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The Activation-Induced Decrease in the Platelet Surface Expression of the Glycoprotein Ib-IX Complex Is Reversible

By Alan D. Michelson, Stephen E. Benoit, Michael H. Kroll, Jian-Ming Li, Michael J. Rohrer, Anita S. Kestin, and Marc R. Barnard

Thrombin decreases the platelet surface expression of the glycoprotein (GP) Ib-IX complex. To determine whether this effect is reversible, flow cytometric studies were performed with GPIb-IX–specific monoclonal antibodies. In both whole blood and washed platelet systems, incubation of platelets with thrombin or a combination of adenosine diphosphate and epinephrine resulted in a maximal decrease of the platelet surface expression of GPIb-IX within 5 minutes, after which there was a time-dependent return of the platelet surface GPIb-IX complex, which was maximal by 60 minutes. Exposure of the same platelets to additional exogenous thrombin resulted in a second decrease in platelet surface GPIb-IX, followed by a second reconstitution of platelet surface GPIb-IX. Throughout these experiments there was no measurable release from the platelets of glycocalicin (a proteolytic fragment of GPIb). Experiments in which platelets were preincubated with a biotinylated GPIb-specific MoAb showed that the GPIb molecules that returned to the platelet surface were the same molecules that had been translocated to the intraplatelet pool. The GPIb molecules that returned to the platelet surface were functionally competent to bind von Willebrand factor, as determined by ristocetin-induced platelet agglutination and ristocetin-induced binding of exogenous von Willebrand factor. Inhibitors of protein kinase C and myosin light-chain kinase enhanced the reexpression of platelet surface GPIb. In summary, the activation-induced decrease in the platelet surface expression of the GPIb-IX complex is reversible. Inactivation of protein kinase C and myosin light-chain kinase are important mechanisms in the reexpression of the platelet surface GPIb-IX complex.
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THROMBIN MODULATES the platelet surface expression of receptors for adhesive glycoproteins involved in platelet adhesion and aggregation. Thus, thrombin increases the platelet surface expression of the glycoprotein (GP) IIb-IIIa complex (a receptor for fibrinogen, von Willebrand factor [vWf], fibronectin, and vitronectin).[1-4] In addition, thrombin increases the platelet surface expression of P-selectin (reflecting α-granule secretion)[5] and both 110-kD (LAMP-1) and 53-kD (CD63) lysosomal granule proteins (reflecting lysosomal secretion).[6,7]

In striking contrast, thrombin decreases the platelet surface expression of the GPIb-IX complex (a receptor for vWf).[8-13] This thrombin-induced decrease in the platelet surface expression of the GPIb-IX complex is not the result of proteolysis, as evidenced by: (1) gel electrophoresis,[14] (2) no change in the total platelet content of GPIb,[9,10] (3) no release from the platelets of glycocalicin (a proteolytic fragment of GPIb),[9] and (4) a parallel decrease in the platelet surface binding of a panel of monoclonal antibodies (MoAbs) directed against GPIb, GPIX, and the GPIb-IX complex,[9,12] whereas proteolysis of GPIb only results in a decrease in the binding of GPIb-specific MoAbs.[15] The thrombin-induced decrease in the platelet surface expression of the GPIb-IX complex appears to be the result of a translocation of GPIb-IX to the membranes of the open-surface canalicular system.[11] Adenosine diphosphate (ADP) and the stable thromboxane A_2 analogue, U46619, also decrease the platelet surface expression of the GPIb-IX complex, whereas epinephrine has little effect.[12,16]

In the present study, we examined the hypothesis that the activation-induced decrease in the platelet surface expression of the GPIb-IX complex is reversible.

MATERIALS AND METHODS

Murine MoAbs

GPIb-IX complex-specific. 6D1 (provided by Dr Barry S. Coller, Mount Sinai Medical Center, New York, NY) is directed against the vWf binding site on the α-chain of GPIb.[17,18] TM60 (provided by Dr Naomasa Yamamoto, Tokyo Metropolitan Institute of Medical Science, Japan) is directed against the thrombin and vWf binding sites on the α-chain of GPIb.[19-21] WM23 (provided by Dr Michael C. Berndt, Baker Medical Research Institute, Melbourne, Australia) is directed against the macroglycopeptide portion of the α-chain of GPIb.[22,23] AK1 (provided by Dr Berndt) is directed against the GPIb-IX complex.[24] AK1 only binds to the intact GPIb-IX complex, not to uncomplexed GPIb or GPIX.[24]

GPIIIa-specific. Y2/51 (Dako, Carpinteria, CA) is directed against platelet membrane GPIIIa.[25]

P-selectin–specific. S12 (provided by Dr Rodger P. McEver, University of Oklahoma, Oklahoma City) is directed against P-selectin.[5,26] P-selectin, also referred to as GMP-140,[5] PADGEM protein,[27] and CD62,[28] is a component of the α-granule membrane of resting platelets that is only expressed on the platelet plasma membrane after platelet secretion.[29]

Each antibody was either biotinylated or conjugated with fluorescein isothiocyanate (FITC).[12]

Whole Blood Flow Cytometry

The whole blood flow cytometric method for the analysis of platelet surface glycoproteins has previously been described in detail.[12]

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There are no centrifugation, gel filtration, vortexing, or stirring steps that could artefactually activate platelets. Peripheral blood was drawn from healthy adult volunteers who had not ingested aspirin or other antiplatelet drugs during the previous 10 days, except as indicated in experiments in which the volunteer ingested 700 mg of aspirin 2 hours before phlebotomy. The first 2 mL of blood drawn were discarded and then blood was drawn into a sodium citrate Vacutainer (Becton Dickinson, Rutherford, NJ), which does not result in platelet activation.[30] Within 15 minutes, the anticoagulated blood was diluted 1:2 by volume in modified Tyrode's buffer (137 mmol/L NaCl, 2.8 mmol/L KCl, 1 mmol/L $MgCl_2$, 12 mmol/L $NaHCO_3$, 0.4 mmol/L Na_2HPO_4, 0.35% bovine serum albumin [BSA], 10 mmol/L HEPES, 5.5 mmol/L glucose, pH 7.4) with 2.5 mmol/L (final concentration) of glycyl-L-prolyl-L-arginyl-L-proline (GPRP) (Calbiochem, San Diego, CA) (an inhibitor of fibrin polymerization and platelet aggregation[12]), and incubated at 37°C with an equal volume of platelet agonist or control buffer. The following agonists were studied: purified human α-thrombin (provided by Dr John Fenton II, New York Department of Health, Albany); phorbol myristate acetate (PMA) (Sigma Chemical Co, St Louis, MO); the stable thromboxane A_2 analogue, U46619 (Cayman Chemical, Ann Arbor, MI); ADP (BioData, Horsham, PA); epinephrine (BioData); platelet activating factor (PAF) (Sigma); and vasopressin (Sigma).

Inhibitors were included in some experiments as follows: (1) Hirudin 10 U/mL (Calbiochem) (a specific thrombin inhibitor) was coincubated with ADP and epinephrine; (2) an inhibitor of protein kinase C, either staurosporine 10 μmol/L (Sigma) or 1-O-alkyl-2-O-methylglycerol (AMG)[31] 125 μmol/L (Cayman Chemical), was added 15 minutes after either PMA or U46619; (3) either wortmannin 5 μmol/L (Sigma) (an inhibitor of myosin light-chain kinase[32]) or cytochalasin B 6 μmol/L (Sigma) (an inhibitor of actin polymerization[33]) was added 15 minutes after thrombin; (4) 5-(N-ethyl-N-isopropyl)amiloride (EIA) 20 μmol/L (a specific inhibitor of Na^+/H^+ exchange[34,35]) (obtained from Merck, Sharp, and Dohme, West Point, PA) was incubated with the sample at 37°C for 15 minutes before the addition of thrombin.

All samples were fixed with 1% formaldehyde at various time points after the addition of the agonist. Samples were then diluted and incubated at 22°C for 15 minutes with a saturating concentration of a biotinylated MoAb (directed against either the GPIb-IX complex or P-selectin) and a subsaturating concentration of the FITC-conjugated GPIIIa-specific MoAb, Y2/51. The samples were then incubated at 22°C for 15 minutes with phycoerythrin-streptavidin (Jackson Immunoresearch Laboratories, West Grove, PA). Within 24 hours of antibody tagging, the samples were analyzed in an EPICS Profile flow cytometer (Coulter Cytometry, Hialeah, FL). After identification of platelets by gating on both FITC positivity and their characteristic light scatter, binding of the biotinylated MoAb was determined by analyzing 5,000 individual platelets for phycoerythrin mean fluorescence. To compare results in linear form, data obtained from fluorescence channels in a logarithmic mode were converted to their linear equivalents. As indicated in the figure legends, the binding of the GPIb-specific MoAb before the addition of thrombin was assigned 100 linear U fluorescence. The binding of the P-selectin–specific MoAb 5 minutes after the addition of thrombin 1 U/mL was assigned 100 linear U fluorescence. We[12] and others[36] have shown that this method results in no significant differences in fluorescence intensity between samples analyzed immediately and samples analyzed within 24 hours of antibody tagging.

In experiments to determine whether there was proteolytic loss of GPIb from platelets, a saturating concentration of FITC-conjugated MoAb 6D1 was added 15 minutes before the addition of thrombin 0.5 U/mL and GPRP 2.5 mmol/L. The samples were then fixed at various time points, diluted, incubated (22°C, 15 minutes) with a saturating concentration of a biotinylated MoAb WM23, incubated (22°C, 15 minutes) with phycoerythrin-streptavidin, and analyzed for FITC and phycoerythrin fluorescence by flow cytometry.

Flow Cytometric Analysis of the Platelet Surface GPIb-IX Complex in a Washed Platelet System

The method has been described previously.[15,37] The first 2 mL of blood drawn were discarded and then blood was drawn into a sodium citrate Vacutainer. The anticoagulated blood was centrifuged (150*g*, 15 minutes, 22°C) and the platelet-rich plasma removed and diluted with 3 volumes of citrate-albumin buffer (11 mmol/L glucose, 128 mmol/L sodium chloride, 4.3 mmol/L monobasic sodium phosphate, 7.5 mmol/L dibasic sodium phosphate, 4.8 mmol/L sodium citrate, 2.4 mmol/L citric acid, 0.35% BSA), pH 6.5 with prostaglandin E_1 (PGE_1) 50 ng/mL. Platelets were separated from plasma by centrifugation (1,200*g*, 10 minutes, 22°C) and gentle resuspension in citrate-albumin buffer, pH 6.5 with PGE_1 50 ng/mL. After an additional centrifugation (1,200*g*, 10 minutes, 22°C), the platelets were resuspended in modified Tyrode's buffer, pH 7.4. The washed platelets (75,000/μL) were incubated at 37°C with various concentration of human α-thrombin (or control buffer). At various time points up to 2 hours, aliquots were fixed for 30 minutes at 22°C and diluted 10-fold in modified Tyrode's buffer, pH 7.4. Samples were then incubated with a saturating concentration of an FITC-conjugated GPIb-IX complex-specific MoAb, and analyzed by flow cytometry. In some experiments, 50 μg/mL of cycloheximide (Sigma) (an inhibitor of protein synthesis) was added together with the thrombin. In other experiments, hirudin 10 U/mL was added 5 minutes after the addition of thrombin.

In experiments to determine whether the GPIb molecules that returned to the platelet surface were the same molecules that had been translocated to the intraplatelet pool, washed platelets were prelabeled with a saturating concentration of biotinylated WM23, washed free of excess antibody, and incubated with thrombin 0.1 U/mL, thrombin 0.2 U/mL, or control buffer. The samples were fixed at various time points, incubated with phycoerythrin-streptavidin and a saturating concentration of FITC-conjugated 6D1, and the binding of WM23 and 6D1 assessed by flow cytometry.

Ristocetin-Induced Binding of vWf to Platelets

The ristocetin-induced binding of vWf to platelets was determined by a previously described method.[30] Washed platelets were incubated with various concentrations of human α-thrombin (or control buffer). At various time points up to 2 hours, aliquots were fixed with 1% formaldehyde for 30 minutes at 22°C, diluted 40-fold with modified Tyrode's buffer, pH 7.4, and incubated (22°C, 15 minutes) with pooled platelet-poor plasma from normal donors (as a source of vWf) and ristocetin (BioData) (final concentration 1.4 mg/mL). The mixture was then incubated (22°C, 15 minutes) with 28 μg/mL of either polyclonal FITC-conjugated anti-vWf goat IgG antibody (Atlantic Antibodies, Stillwater, MN) or FITC-conjugated nonspecific goat IgG (Atlantic Antibodies). The sample was then diluted 16-fold in modified Tyrode's buffer, pH 7.4 and analyzed by flow cytometry. The fluorescence of the sample incubated with the nonspecific goat IgG was subtracted from the fluorescence of the sample incubated with the anti-vWf antibody.

Plasma Glycocalicin Assay

Plasma glycocalicin was determined by a competitive inhibition assay, as previously described.[30] Under the conditions described below, saturation of the platelet binding of MoAb 6D1 occurred at 1.7 μg/mL. A subsaturating concentration (1.2 μg/mL) of FITC-conjugated MoAb 6D1 was incubated at 22°C for 20 minutes with either test plasma that had been filtered through a 0.22-μm Acrodisc (Gelman, Ann Arbor, MI) and had the pH buffered to 7.4, or various

concentrations of purified glycocalicin. Samples were then incubated at 22°C for 20 minutes with fixed, washed platelets (final concentration 100,000/μL) and diluted 20-fold in modified Tyrode's buffer, pH 7.4 before the platelet binding of 6D1 was analyzed by flow cytometry. Linear regression analysis was used to generate a standard curve from the purified glycocalicin samples. This standard curve, which was repeated with every experiment, always showed linearity between 0 and 70 nmol/L of glycocalicin. The correlation coefficient (r) was always greater than 0.96. The plasma glycocalicin concentration of unknown plasma samples was derived from the standard curve. All assayed samples were within the linear range of 0 to 70 nmol/L.

Ristocetin-Induced Platelet Agglutination

Platelets (250,000/μL) suspended in autologous plasma were stirred with 1.2 mg/mL ristocetin in a Lumi-Aggregation Module Series 10008 (Payton, Buffalo, NY). Platelet agglutination was detected by change in light transmission, as previously described.[38]

RESULTS

Reversibility of the Thrombin-Induced Decrease in the Platelet Surface Expression of the GPIb-IX Complex

Thrombin has previously been shown to rapidly decrease the platelet surface expression of the GPIb-IX complex.[8-13] To determine whether this effect was reversible, diluted whole blood was incubated with thrombin and aliquots were fixed at various time points for up to 2 hours (Fig 1). The platelet surface expression of the GPIb-IX complex was analyzed by a whole blood flow cytometric assay using MoAb 6D1 (directed against the vWf binding site on GPIbα). Inclusion of the peptide GPRP (an inhibitor of fibrin polymerization and platelet aggregation) allowed us to study the effect of purified human α-thrombin on individual platelets in whole blood.[12] As expected, there was a rapid, concentration-dependent decrease in the platelet surface expression of the GPIb-IX complex, with a maximal decrease at 5 minutes (Fig 1). However, further incubation resulted in a gradual return of the platelet surface expression of the GPIb-IX complex (Fig 1). The return of the platelet surface expression of the GPIb-IX complex was maximal at approximately 60 minutes. Stimulation with lower concentrations of thrombin (0.05 to 0.3 U/mL) resulted in complete reversibility of the platelet surface expression of GPIb within 60 minutes, whereas stimulation with a high concentration of thrombin (2 U/mL) resulted in incomplete reversibility with 20% of the GPIb not returned to the platelet surface (Fig 1). This 20% apparently remained within the platelet because there was no proteolysis of GPIb (see below).

Reversibility of the thrombin-induced decrease in the platelet surface expression of the GPIb-IX complex was also shown in a washed platelet system (Fig 2). Because each platelet is analyzed individually, the flow cytometric method of analyzing platelet surface GPIb is able to detect distinct subpopulations of platelets.[39,40] In Fig 2, with increasing time of incubation, the GPIb-positive population of platelets moved to the left and back to the right as a single peak. These data show that the loss and restoration of the platelet

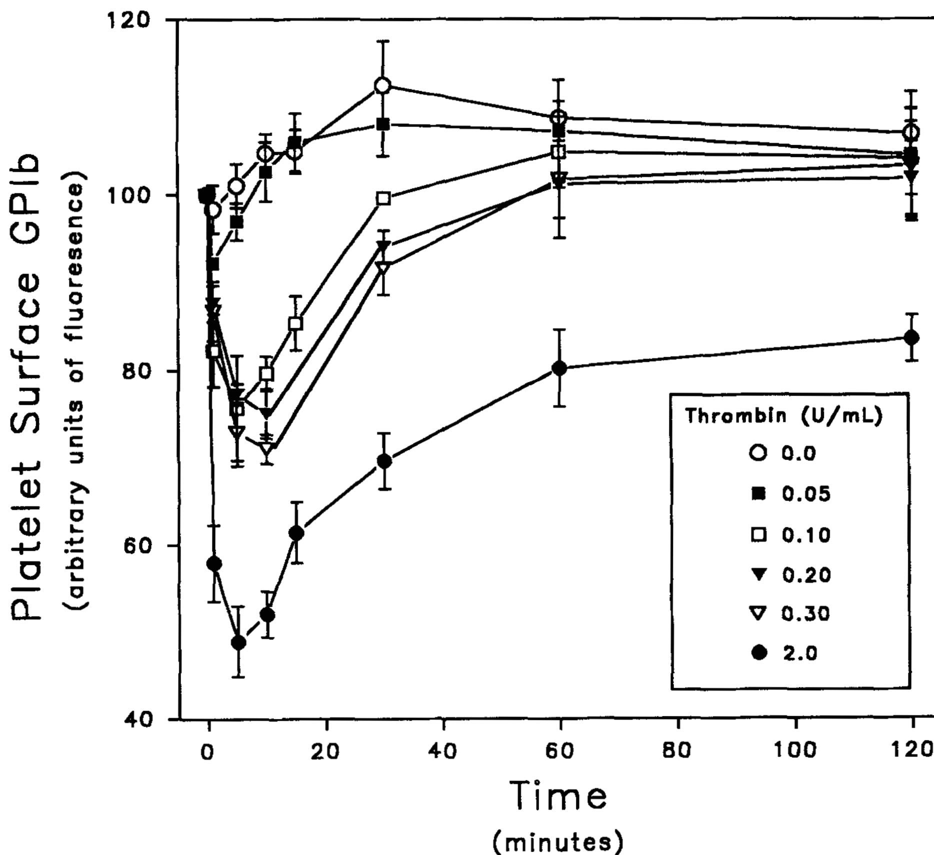

Fig 1. Reversibility of the thrombin-induced decrease in the platelet surface expression of the GPIb-IX complex in whole blood. Human α-thrombin (or control buffer) and GPRP 2.5 mmol/L were added at the 0 time point to whole blood incubated at 37°C. Aliquots were fixed at the indicated time points. The platelet surface binding of the GPIb-specific MoAb 6D1 was analyzed by whole blood flow cytometry, as described in Materials and Methods. The binding of 6D1 before the addition of thrombin was assigned 100 U fluorescence. Data are mean ± SEM, n = 3 to 7 separate experiments.

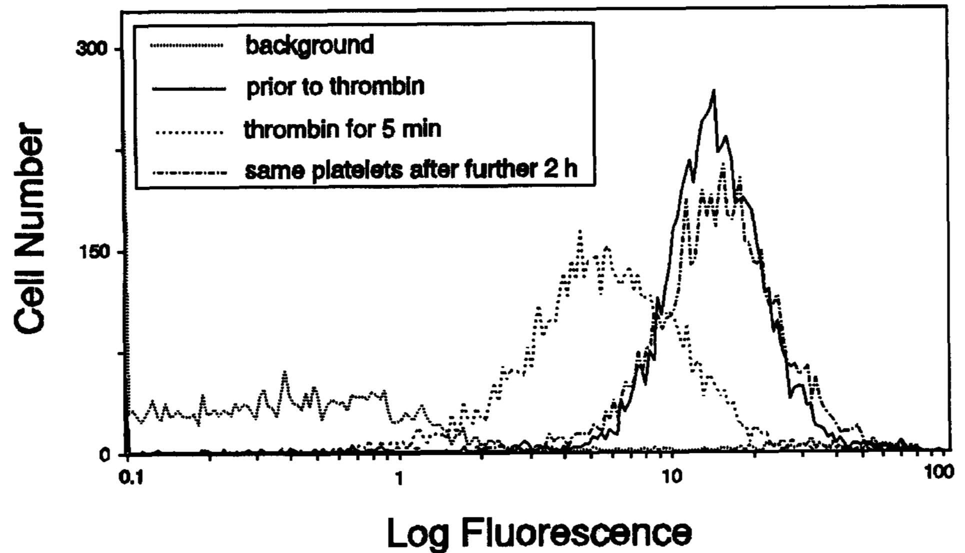

Fig 2. Reversibility of the thrombin-induced decrease in the platelet surface expression of the GPIb-IX complex in a washed platelet system. Washed platelets were incubated at 37°C with thrombin 0.1 U/mL. Aliquots were fixed at the following time points: before the addition of thrombin, after 5 minutes incubation with thrombin, and after a further 2-hour incubation. The platelet surface binding of the GPIb-specific MoAb 6D1 was analyzed by flow cytometry. Background refers to a sample incubated with nonspecific mouse IgG rather than 6D1. The experiment is representative of 8 so performed.

surface expression of the GPIb-IX complex was not confined to a distinct subpopulation of platelets.

In both whole blood and the washed platelet system, a parallel and quantitatively similar reversibility of the thrombin-induced decrease in the platelet surface expression of the GPIb-IX complex was also shown with three other MoAbs that are directed against different epitopes than 6D1: (1) TM60 (directed against the thrombin and vWf binding sites on GPIbα), (2) WM23 (directed against the macroglycopeptide portion of GPIbα), and (3) AK1 (directed against the GPIb-IX complex) (n = 3, data not shown). The fact that AK1 only binds to the intact GPIb-IX complex, not to uncomplexed GPIb or GPIX,[24] shows that the GPIb and GPIX molecules reexpressed on the platelet surface are fully complexed.

Reversibility of the Thrombin-Induced Decrease in the Binding of vWf to the Platelet Surface GPIb-IX Complex

Thrombin has previously been shown to decrease the binding of vWf to the platelet GPIb-IX complex.[10] To determine whether this effect was reversible, washed platelets were incubated with thrombin, aliquots were fixed at various time points up to 2 hours, and the ristocetin-induced binding of vWf to platelets determined by flow cytometry (Fig 3). As expected, there was a rapid decrease in the ristocetin-induced binding of vWf to platelets, with a maximal decrease at 5 minutes. Further incubation resulted in a gradual return in the ability of platelets to bind vWf in response to ristocetin (Fig 3).

The effects of thrombin on vWf binding and on the binding of a GPIb-specific MoAbs (6D1) were directly compared by analyzing aliquots of the same washed platelets (compare the closed circles in Fig 3 to the closed circles in Fig 4B). The data show that almost all the GPIb molecules that returned to the platelet surface were competent to bind vWf.

Reversibility of the thrombin-induced decrease in the binding of vWf to the platelet surface GPIb-IX complex was also shown by ristocetin-induced platelet agglutination (data not shown).

It should be noted that apparent differences in the degree of effect of thrombin between different figures in this study are accounted for by the fact that some of the experiments were performed in whole blood (eg, Fig 1), whereas others were performed in a washed platelet system (eg, Fig 3). The presence of antithrombin III in the plasma portion of whole blood resulted in a more modest effect for a given dose of thrombin, as compared with the washed platelet system in which no antithrombin III was present. In the whole blood system of Fig 1, thrombin 0.2 U/mL resulted in a modest return in the platelet surface expression of GPIb that was equivalent to complete reversibility because the initial decrease in the platelet surface expression of GPIb was also only modest. In contrast, in the washed platelet system of Fig 3, thrombin 0.2 U/mL resulted in a more substantial percent return of the platelet surface expression of GPIb, but this was equivalent to only partial reversibility because the initial decrease in the platelet surface expression of GPIb was much more substantial.

The GPIb Molecules That Returned to the Platelet Surface Were the Same Molecules That Had Been Translocated to the Intraplatelet Pool

The thrombin-induced decrease in the platelet surface expression of the GPIb-IX complex has previously been shown to be on the basis of a redistribution of GPIb-IX complexes to the membranes of the open surface canalicular system.[11] To determine whether the GPIb molecules that returned to the platelet surface were the same molecules that had been translocated to the intraplatelet pool, the following experiment was performed. Washed platelets prelabeled with biotinylated WM23 (a GPIb-specific MoAb) were activated with thrombin, fixed at various time points, diluted 10-fold, incubated with phycoerythrin-streptavidin and FITC-conjugated 6D1 (a GPIb-specific MoAb directed against an epitope distinct from WM23), and the binding of phycoerythrin-streptavidin and 6D1 assessed by flow cytometry (Fig 4). Before the addition of thrombin, the biotinylated WM23 was accessible to the phycoerythrin-streptavidin (Fig 4A, 0 time

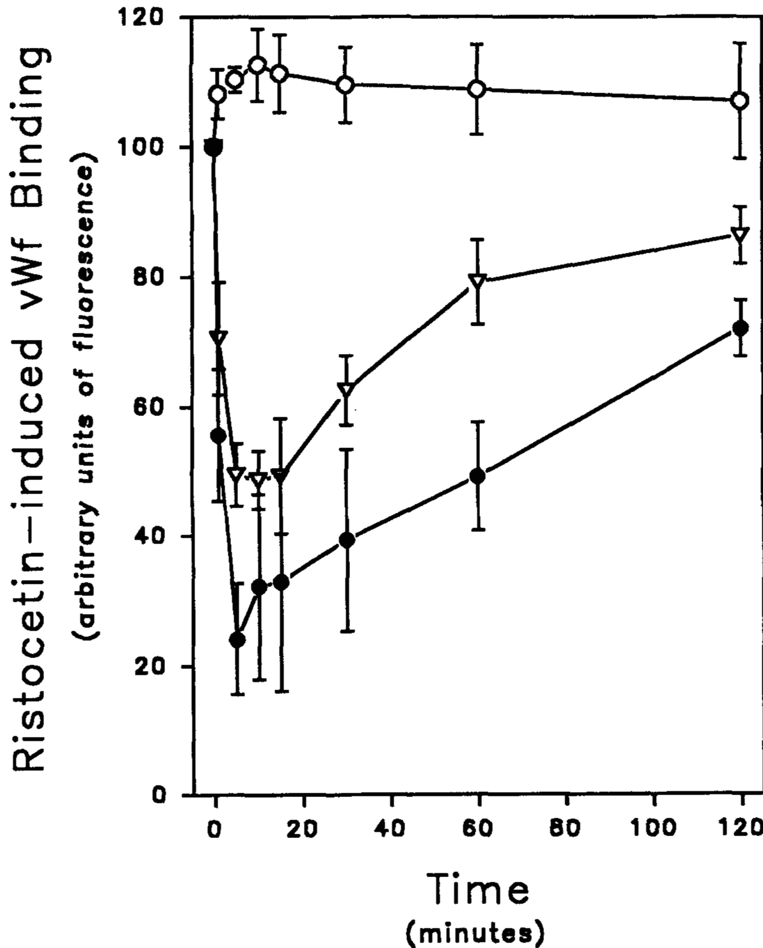

Fig 3. Reversibility of the thrombin-induced decrease in the binding of vWf to the platelet surface GPIb-IX complex. Thrombin (or control buffer) was added at the 0 time point to washed platelets incubated at 37°C. Aliquots were fixed at the indicated time points. After pooled platelet-poor plasma was added as a source of vWf, the ristocetin-induced binding of vWf to platelets was determined by flow cytometry with a polyclonal anti-vWf antibody, as described in Materials and Methods. The ristocetin-induced binding of vWf before the addition of thrombin was assigned 100 U fluorescence. Data are mean ± SEM, n = 3. (○) 0 U/mL, (▽) 0.1 U/mL, and (●) 0.2 U/mL thrombin.

point). After the thrombin-induced redistribution of the GPIb-IX complexes, the biotinylated WM23 was inaccessible to the phycoerythrin-streptavidin (Fig 4A, 5-minute, 10-minute, and 15-minute time points). (The residual approximately 25% phycoerythrin fluorescence was the result of the minority of GPIb molecules that were not translocated to the intraplatelet pool.[11,12] After further incubation, the biotinylated WM23 was again accessible to phycoerythrin-streptavidin (Fig 4A, 60 minute and 120 minute time points). FITC-6D1 binding to the same platelets showed the expected reversible thrombin-induced decrease in the platelet surface expression of the GPIb-IX complex (Fig 4B). Similar results were obtained with thrombin 0.1 U/mL (n = 3, data not shown) and 0.2 U/mL (n = 3, Fig 4).

Exposure of Platelets to Additional Exogenous Thrombin Results in a Repetition of the Reversible Decrease in the Platelet Surface GPIb-IX Complex

We next addressed the question as to whether the same platelets could reversibly decrease their surface expression of the GPIb-IX complex more than once. Figure 5 shows that when platelets were exposed to additional exogenous thrombin 90 minutes after their first exposure to exogenous thrombin, there was a second decrease in platelet surface GPIb-IX, followed by a second reconstitution of the platelet surface expression of GPIb-IX. Successive exposures of the platelets to thrombin resulted in: (1) a smaller decrease in the platelet surface expression of the GPIb-IX complex, although this did not achieve statistical significance (Fig 5: $P > .05$ by Student's *t*-test for the comparison of the time point 5 minutes after addition of the second dose of thrombin versus the time point 5 minutes after addition of the first dose of thrombin); and (2) a significantly smaller recovery of the platelet surface expression of the GPIb-IX complex (Fig 5: $P < .05$ for comparison of the time point 90 minutes after addition of the second dose of thrombin versus the time point 90 minutes after addition of the first dose of thrombin).

Reversibility of the Thrombin-Induced Decrease in the Platelet Surface Expression of the GPIb-IX Complex Is Not Associated With GPIb Proteolysis

In whole blood experiments in which FITC-conjugated 6D1 was added before the addition of thrombin, the expected initial decrease and subsequent return in phycoerythrin fluorescence (reflecting accessibility of biotinylated WM23) was observed. In contrast, FITC fluorescence remained constant, showing that there was no proteolytic loss of GPIb from the platelets during the loss and restoration of the platelet surface expression of GPIb (n = 2, data not shown). This conclusion was supported by the fact that the reversible thrombin-induced

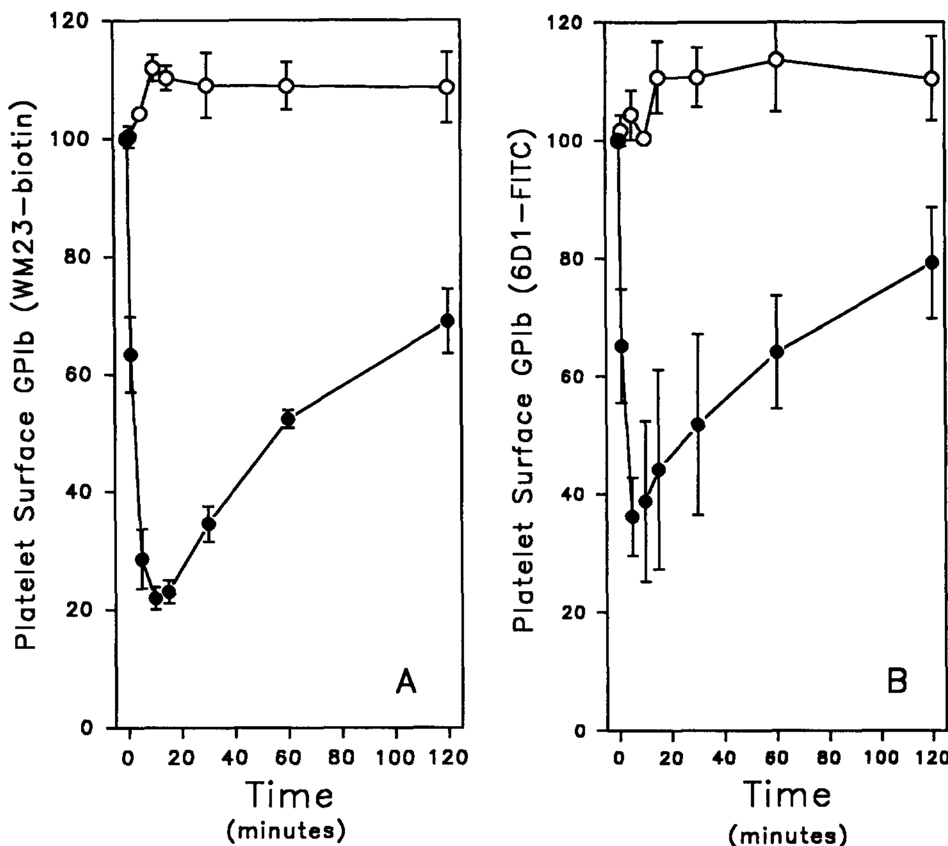

Fig 4. The GPIb molecules that returned to the platelet surface were the same molecules that had been translocated to the intraplatelet pool. Washed platelets were prelabeled with biotinylated WM23 (GPIb-specific MoAb), washed, incubated at 37°C with thrombin 0.2 U/mL (or control buffer) at the 0 time point, fixed at the indicated time points, incubated with phycoerythrin-streptavidin and FITC-conjugated 6D1 (GPIb-specific MoAb directed against an epitope distinct from WM23), and the binding of 6D1 and WM23 simultaneously analyzed by flow cytometry. The binding of 6D1 and WM23 before the addition of thrombin was assigned 100 U fluorescence. The data shown in the closed circles of Fig 4B were obtained from aliquots of the same washed platelet samples used to obtain the data shown in the closed circles of Fig 3. Data are mean ± SEM, n = 3. (○) Control; (●) thrombin.

decrease in the platelet surface expression of the GPIb-IX complex was not associated with any change in the plasma concentration of glycocalicin (n = 3, data not shown).

Reversibility of the Thrombin-Induced Decrease in the Platelet Surface Expression of the GPIb-IX Complex Is Not the Result of New Protein Synthesis

Platelets are capable of synthesis of new proteins, including GPIb.[41] However, new protein synthesis did not account for the reconstitution of the GPIb-IX complex on the platelet surface, because results were unchanged when experiments were performed in the presence of cycloheximide 50 μg/mL, an inhibitor of protein synthesis (data not shown). It has previously been shown that this concentration of cycloheximide can inhibit synthesis of proteins in platelets, as evidenced by inhibition of the incorporation of labeled amino acids into platelet proteins.[42]

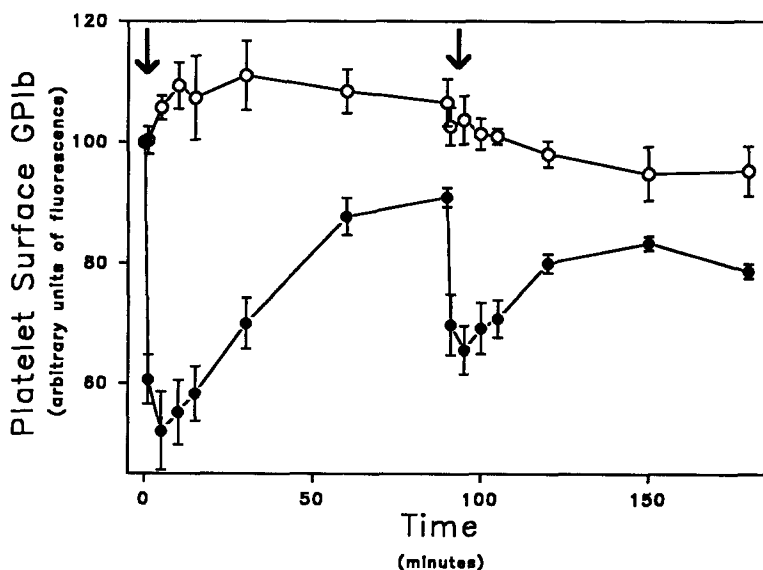

Fig 5. Exposure of platelets to additional exogenous thrombin results in a repetition of the reversible decrease in the platelet surface GPIb-IX complex. Whole blood was incubated at 37°C. Thrombin (1 U/mL) (or control buffer) and GPRP (2.5 mmol/L) were added at the two time points indicated by the arrows. Aliquots were fixed at the indicated time points. The platelet surface binding of the GPIb-specific MoAb 6D1 was analyzed by whole blood flow cytometry. The binding of 6D1 before the addition of any thrombin was assigned 100 U fluorescence. Data are mean ± SEM, n = 3. (○) Control; (●) thrombin.

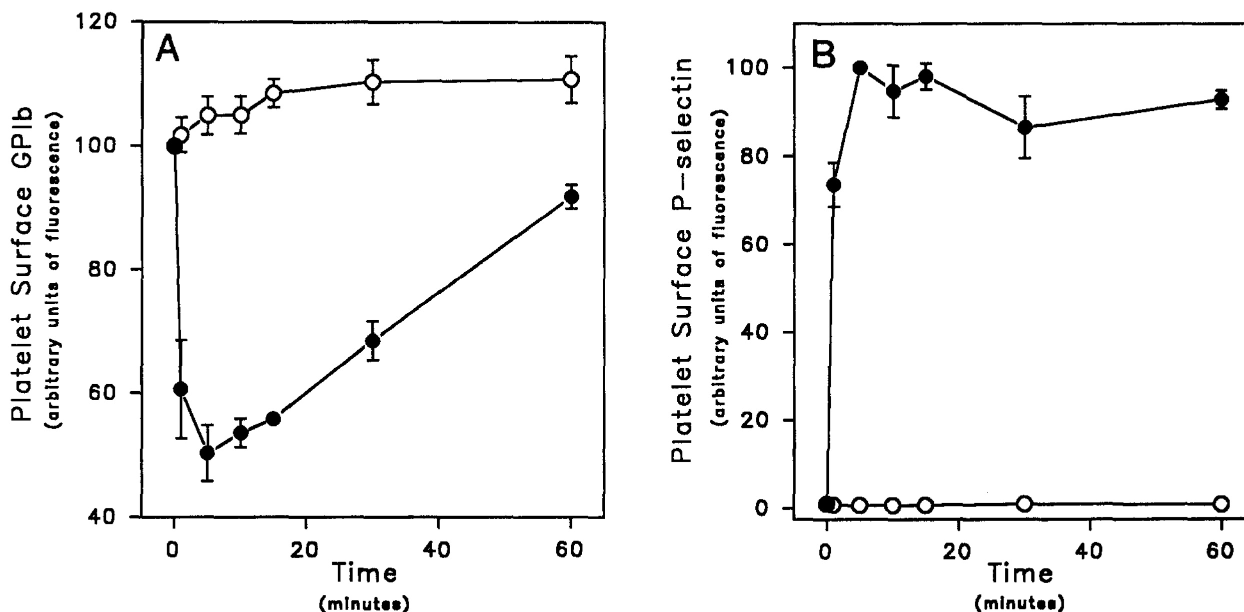

Fig 6. Relative effects of thrombin on the platelet surface expression of the GPIb-IX complex and P-selectin. Thrombin (1 U/mL) (or control buffer) and GPRP (2.5 mmol/L) were added at the 0 time point to whole blood incubated at 37°C. Aliquots were fixed at the indicated time points. The platelet surface binding of the GPIb-specific MoAb 6D1 (A) and the P-selectin-specific MoAb S12 (B) was analyzed by whole blood flow cytometry. In (A), the binding of 6D1 before the addition of thrombin was assigned 100 U of fluorescence. In (B), the binding of S12 5 minutes after the addition of thrombin was assigned 100 U fluorescence. Data from (A) and (B) were obtained from aliquots of the same samples. Data are mean ± SEM, n = 3. (○) No agonist; (●) thrombin.

Restoration of the Platelet Surface Expression of the GPIb-IX Complex Can Occur in a Fully Degranulated Platelet

As expected, the decrease in the platelet surface expression of the GPIb-IX complex induced by thrombin 1 U/mL was associated with translocation of P-selectin to the platelet surface (Fig 6, A and B, 5-minute time point, solid circles). However, the return of the platelet surface expression of the GPIb-IX complex was not associated with any change in the platelet surface expression of P-selectin (Fig 6, A and B, 60-minute time point, solid circles).

Thrombin Is Not Required for the Reexpression of the GPIb-IX Complex on the Platelet Surface

Hirudin inhibits the action of thrombin on platelets and rapidly removes thrombin from its platelet receptors.[43] The addition of 10 U/mL hirudin 5 minutes after the addition of thrombin 0.1 U/mL to washed platelets had no significant effect on the reexpression of the GPIb-IX complex on the platelet surface (data not shown). Thus, the presence of thrombin is not required for the reexpression.

Reversibility of the ADP and Epinephrine-Induced Decrease in the Platelet Surface Expression of the GPIb-IX Complex

ADP and epinephrine synergistically decrease the platelet surface expression of the GPIb-IX complex (Fig 7). Prolonged incubation with ADP or the combination of ADP and epinephrine resulted in a gradual return of the platelet surface expression of the GPIb-IX complex. This reversible decrease in the platelet surface GPIb-IX complex was not the result of trace amounts of thrombin because the results were unchanged by inclusion in the assay of hirudin, a potent inhibitor of thrombin (data not shown).

Augmentation of the ADP-induced decrease in the platelet surface expression of the GPIb-IX complex was also obtained with the combination of ADP 10 μmol/L and PAF 100 nmol/L and the combination of ADP 10 μmol/L and vasopressin 5 IU/mL (data not shown). As observed for the ADP/epinephrine combination, the decreases in the platelet surface GPIb-IX complex induced by ADP/PAF and ADP/vasopressin were reversible.

Biochemical Mechanism of the Reexpression of the GPIb-IX Complex on the Platelet Surface

To determine the mechanism by which the thrombin-induced redistribution of the GPIb-IX complex occurs, intraplatelet signaling pathways were perturbed by various pharmacologic manipulations. Platelets were pretreated in vitro with EIA, a specific inhibitor of Na^+/H^+ exchange,[34,35] or in vivo with oral aspirin 700 mg 2 hours before phlebotomy. Neither EIA nor aspirin had any effect on the thrombin-induced decrease in the platelet surface expression of GPIb or on the subsequent reexpression of GPIb on the platelet surface (n = 3, data not shown). Experiments with the direct protein kinase C activator PMA[44] and the protein kinase C inhibitor staurosporine suggested that protein kinase C may be involved in effecting the decrease in the platelet surface expression of GPIb, and that inhibition of protein kinase C may reverse this process (Fig 8A). To determine if protein kinase C activity generated by a receptor-induced activation

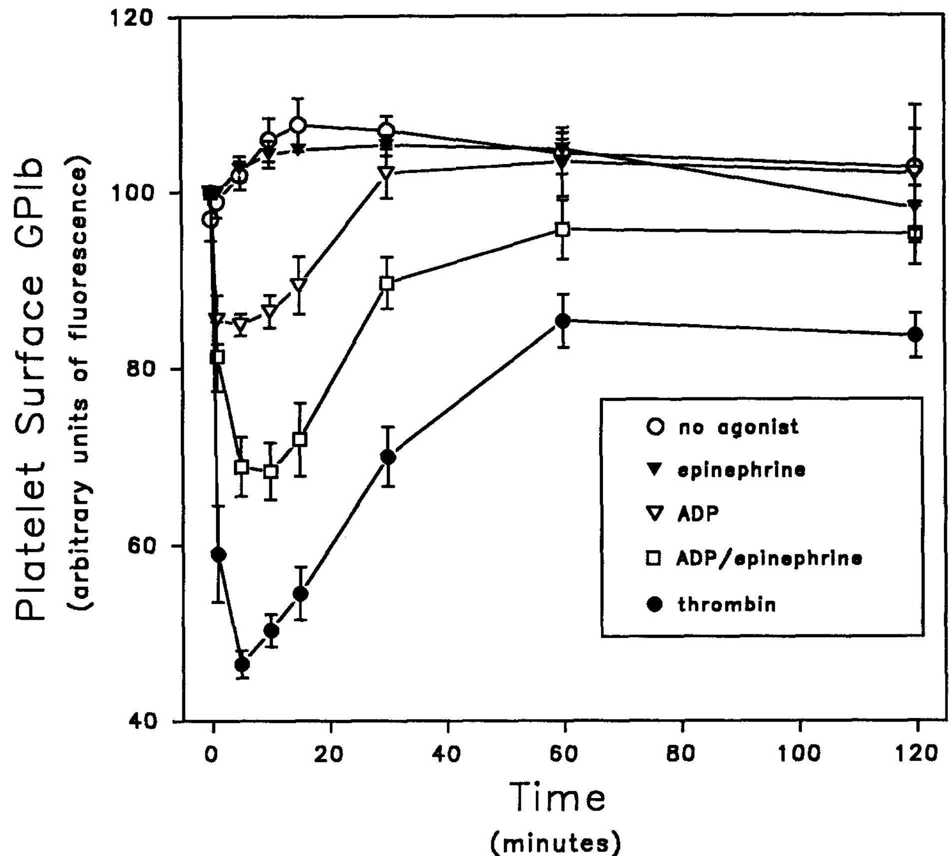

Fig 7. Reversibility of ADP and epinephrine-induced decrease in the platelet surface expression of the GPIb-IX complex. Whole blood was incubated at 37°C with GPRP 2.5 mmol/L and at the 0 time point one of the following agonists was added: epinephrine, 10 μmol/L; ADP, 10 μmol/L; a combination of ADP, 10 μmol/L and epinephrine, 10 μmol/L; thrombin 2 U/mL; or no agonist. Aliquots were fixed at the indicated time points. The platelet surface binding of the GPIb-specific MoAb 6D1 was analyzed by whole blood flow cytometry. The binding of 6D1 before the addition of any agonist was assigned 100 U fluorescence. Data are mean ± SEM, n = 3.

of phospholipase C inhibits the redistribution of GPIb, platelets were stimulated with the synthetic endoperoxide U46619. U46619 resulted in an irreversible decrease in the platelet surface expression of GPIb (Fig 8B). However, addition of a protein kinase C inhibitor (either staurosporine or AMG) 15 minutes after U46619 resulted in reexpression of platelet surface GPIb (Fig 8B).

The fact that the time course of the PMA-induced decrease in the platelet surface expression of GPIb was slower than that of thrombin suggested that additional second messengers are involved when thrombin is the agonist. Addition of the myosin light-chain kinase inhibitor wortmannin 15 minutes after thrombin enhanced the reexpression of platelet surface GPIb (Fig 9). However, cytochalasin B, an inhibitor of actin polymerization, had no effect on the reexpression of platelet surface GPIb (Fig 9).

DISCUSSION

It has previously been shown that thrombin decreases the platelet surface expression of the GPIb-IX complex (a receptor for vWf).[8-13] The following novel conclusions can be drawn from this study: (1) The thrombin-induced decrease in the platelet surface expression of the GPIb-IX complex is reversible, both in a washed platelet system and in whole blood. (2) The loss and restoration of the platelet surface expression of the GPIb-IX complex is not confined to a distinct subpopulation of platelets. (3) The GPIb molecules that returned to the platelet surface are functionally competent to bind vWf. (4) The GPIb molecules that returned to the platelet surface were the same molecules that had been translocated to the intraplatelet pool. (5) Restoration of the platelet surface expression of the GPIb-IX complex can occur in a fully degranulated platelet. (6) Exposure to additional exogenous thrombin results in a repetition of the reversible decrease in the platelet surface GPIb-IX complex. (7) Reversibility of the thrombin-induced decrease in the platelet surface GPIb-IX expression of the GPIb-IX complex is not associated with GPIb proteolysis. (8) The reexpression of the platelet surface GPIb-IX complex is not the result of new protein synthesis by platelets. (9) The combinations of ADP/epinephrine, ADP/PAF, and ADP/vasopressin synergistically decrease the platelet surface expression of the GPIb-IX complex and these decreases are reversible.

In addition, to determine the biochemical mechanism by which the thrombin-induced redistribution of the GPIb-IX complex occurs, intraplatelet signaling pathways were selectively inhibited. In platelets, arachidonic acid is released when phospholipase A_2 cleaves the C-2 acyl group from membrane phospholipids, particularly phosphatidylcholine.[45] This step may be regulated by the platelet Na^+/H^+ exchanger.[46] Released arachidonic acid is then converted to the metabolically active prostaglandin endoperoxides and thromboxane A_2 by the activity of cyclooxygenase (PGG_2/PGH_2 synthase complex). Pretreatment of platelets in vitro with EIA (a specific inhibitor of Na^+/H^+ exchange[34,35]) or in vivo with aspirin had no effect on the thrombin-induced

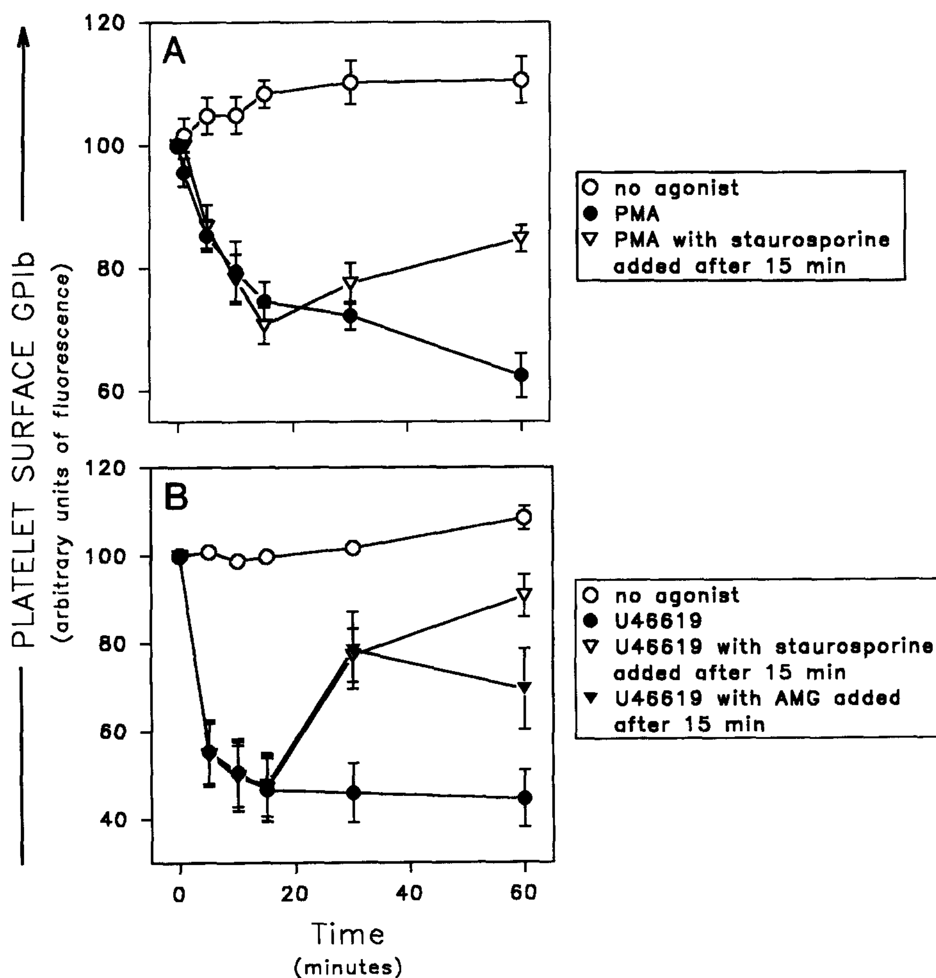

Fig 8. Effect of inhibitors of protein kinase C on the platelet surface expression of the GPIb-IX complex. Either PMA, 10 μmol/L; U46619, 10 μmol/L; or control buffer (no agonist), was added at the 0 time point to whole blood incubated at 37°C. As indicated, an inhibitor of protein kinase C (either staurosporine 10 μmol/L or 1-O-alkyl-2-O-methylglycerol [AMG] 125 μmol/L) was added to some samples at the 15-minute time point. Aliquots were fixed at the indicated time points. The platelet surface binding of the GPIb-specific MoAb 6D1 was analyzed by whole blood flow cytometry. The binding of 6D1 before the addition of any agonist was assigned 100 U fluorescence. Data are mean ± SEM, n = 3.

decrease in the platelet surface expression of GPIb or on the subsequent reexpression of GPIb on the platelet surface. These data show that neither the release and metabolism of arachidonic acid nor signal pathways mediated by phospholipase A_2 and cyclooxygenase are required for the thrombin-induced redistribution of GPIb.

The major signaling pathway involved in platelet activation in response to thrombin is mediated by phospholipase C.[47] Thrombin binding to and cleavage of its receptor causes the dissociation of a heterotrimeric GTP-binding protein. This dissociation is, through unknown mechanisms, coupled to phospholipase C activation, resulting in the generation of the second messengers diacylglycerol and inositol 1,4,5-triphosphate. Diacylglycerol causes the activation of protein kinase C, which promotes platelet secretion and regulates various metabolic processes.[47] Inositol 1,4,5-triphosphate causes the release of stored calcium and the activation of calcium/calmodulin-dependent protein kinases, the best characterized of which is myosin light-chain kinase.[47] Myosin light-chain kinase phosphorylates myosin, which leads to the cytoskeletal assembly required for shape change and secretion.[48] Experiments with the direct protein kinase C activator PMA and the protein kinase C inhibitor staurosporine suggested that protein kinase C may be involved in effecting the decrease in the platelet surface expression of GPIb, and that inhibition of protein kinase C may reverse this process. To corroborate this, the synthetic endoperoxide U46619, together with a protein kinase C inhibitor (staurosporine or AMG), was used. These experiments showed that protein kinase C activity, generated by receptor-induced activation of phospholipase C, inhibits the reexpression of platelet surface GPIb. It was also observed that wortmannin, a myosin light-chain kinase inhibitor,[32] enhanced the reexpression of platelet surface GPIb. However, cytochalasin B, an inhibitor of actin polymerization that blocks the thrombin-induced decrease in the platelet surface expression of GPIb,[10,12] had no effect on the reexpression of platelet surface GPIb. Thus, our data show that both protein kinase C and myosin light-chain kinase, second messengers formed by thrombin-induced platelet phospholipase C activation, inhibit the reexpression of platelet surface GPIb. Inactivation of protein kinase C and myosin light-chain kinase appear to be important mechanisms in the reexpression of platelet surface GPIb. The time frame of the start of the reexpression of platelet surface GPIb (approximately 10 minutes after addition of thrombin) is consistent with the time frame of the decline in thrombin-mediated phosphorylation of substrates of protein kinase C and myosin light-chain kinase (M_r 47,000 and M_r 20,000, respectively).[47]

The use of physiologic agonists (thrombin and ADP) re-

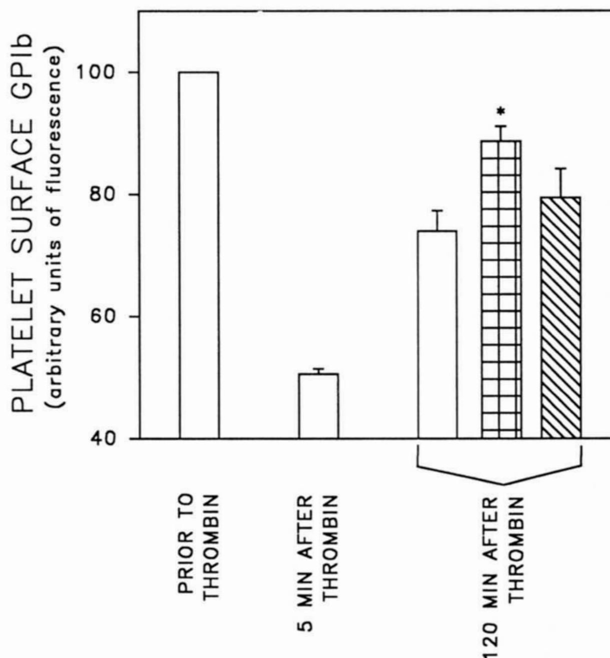

Fig 9. Effect of inhibitors of myosin light-chain kinase and of actin polymerization on the reexpression of the platelet surface GPIb-IX complex. Thrombin 0.5 U/mL with GPRP 2.5 mmol/L was added at the 0 time point to whole blood incubated at 37°C. Either (⊞) wortmannin 5 μmol/L (an inhibitor of myosin light-chain kinase), (▧) cytochalasin B 6 μmol/L (an inhibitor of actin polymerization), or (□) control buffer was added at the 15-minute time point. Aliquots were fixed at the following time points: before the addition of thrombin, 5 minutes after the addition of thrombin, and 120 minutes after the addition of thrombin. The platelet surface binding of the GPIb-specific MoAb 6D1 was analyzed by whole blood flow cytometry. The binding of 6D1 before the addition of thrombin was assigned 100 U fluorescence. Data are mean ± SEM, n = 3. The asterisk indicates $P < .05$ by Student's *t*-test compared with the control sample 120 minutes after the addition of thrombin.

sulted in a reversible decrease in the platelet surface expression of the GPIb-IX complex. The use of nonphysiologic agonists (PMA or U46619) resulted in an irreversible decrease in the platelet surface expression of the GPIb-IX complex. Because PMA causes a direct and prolonged stimulation of protein kinase C,[47] the reexpression of platelet surface GPIb is suppressed. In contrast, the activation of protein kinase C in response to thrombin or ADP is of shorter duration and less magnitude than PMA. We speculate that the duration of protein kinase C activation in response to U46619 is longer than thrombin, perhaps because of the stability of U46619 (as distinct from the instability of native thromboxane A_2).

There are precedents for redistributions of glycoprotein receptor molecules between intraplatelet and platelet surface membrane pools. For example, we have previously reported that after plasmin-mediated proteolysis of platelet surface GPIb, platelets can replenish their surface GPIb pool (within a similar time frame to that reported in this study for thrombin) by recruitment of GPIb molecules from the intraplatelet pool (or from a sequestered surface site).[37] An immunoelectron microscopic study, published after submission of this manuscript for review, reported that the thrombin-induced translocation of platelet surface GPIb to the surface-connected canalicular system is reversible.[49] Furthermore, the GPIIb-IIIa complex is able to actively cycle between intraplatelet and platelet surface pools.[50] Finally, in human cells lines, the tethered ligand thrombin receptor is rapidly internalized after the addition of thrombin.[51] Resensitization of the cells to activation by tethered ligand peptides is associated with the return of receptors to the cell surface.[51]

The pathophysiologic significance of the reversibility of the activation-induced decrease in the platelet surface expression of the GPIb-IX complex remains to be determined. However, previous studies have shown that platelets fully degranulated by thrombin, though they have no granule contents to release, can function normally, including aggregate in response to additional thrombin and circulate in vivo.[52-57] The present study shows that fully degranulated platelets can reexpress the GPIb-IX complex on their surface. Given that the GPIb molecules that return to the platelet surface are functionally competent to bind vWf, they may be able to participate in platelet adhesion. Furthermore, these platelets can be activated again, and reexpress their platelet surface GPIb-IX complex a second time. Thus, consistent with previous studies,[52-57] the present experiments show that a fully degranulated platelet can still be activated, as defined by thrombin-induced modulation of the platelet surface GPIb-IX complex. Taken together, this and previous studies[52-57] suggest that after platelets are activated and degranulated, they may still be able to function. Indeed, it has been proposed that platelets that have been degranulated by thrombin and have taken part in thrombus formation can return to the circulation, where they may contribute to the promotion of hemostasis and/or thrombosis.[58] We are currently examining this hypothesis in vivo in a nonhuman primate model.

Because of its central role in platelet adhesion and its binding sites for vWf and thrombin,[59] the GPIb-IX complex is frequently measured in both in vitro and in vivo studies of platelets. A practical implication of the present data is that in a given in vitro or in vivo experiment, the results of measurements of the platelet surface GPIb-IX complex may vary according to the time point at which samples are analyzed or fixed.

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