Thrombin-induced Exposure and Prostacyclin Inhibition of the Receptor for Factor VIII/von Willebrand Factor on Human Platelets

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ABSTRACT The receptor for Factor VIII/von Willebrand factor (F. $VIII_{vWF}$) is not readily available on circulating platelets. We have found that the stimulation of platelets with traces of thrombin at concentrations that are generated physiologically (0.008 U–0.05 U/ml) induced concentration-dependent binding of ^{125}I-labeled F. $VIII_{vWF}$ in a steady-state system. The binding induced by thrombin was specific because it was inhibited by a 100-fold molar excess of unlabeled F. $VIII_{vWF}$ factor, by rabbit monospecific antibody against Factor VIII, and was not inhibited by an excess of fibrinogen or fibronectin. Binding induced by thrombin required metabolically active platelets, in contrast to a system with ristocetin that also prompted binding to glutaraldehyde-treated platelets. The thrombin effects on binding of ^{125}I-F. $VIII_{vWF}$ was not observed when platelets were washed with EDTA-containing buffers; EDTA and EGTA both inhibited thrombin-induced binding. Platelet membrane glycoproteins were required because enzymatic stripping of them from the platelet surface with chymotrypsin reduced binding 2.5–5.0-fold. Prostacyclin, in the concentration range of 1 to 50 nM, had two distinct effects on the receptor for F. $VIII_{vWF}$: (*a*) it prevented exposure of this receptor when added 10 min before thrombin, and (*b*) it reversed the binding of ^{125}I-F. $VIII_{vWF}$ to the platelet receptor when added 30 min after thrombin and the ligand, i.e., when binding was at equilibrium. The dual effect of prostacyclin on the receptor for F. $VIII_{vWF}$ was reproduced by dibutyryl cyclic AMP.

INTRODUCTION

Formation of a platelet plug at the site of vascular injury is of fundamental importance in normal hemostasis. Plasma Factor VIII/von Willebrand factor (F. $VIII_{vWF}$)[1] is required for the interaction of platelets with an injured vessel wall (1). Individuals lacking this glycoprotein demonstrate a bleeding tendency, owing to their inability to form a functionally competent hemostatic plug (2). However, the exact in vivo mechanism(s) of the interaction between platelets and F. $VIII_{vWF}$ is not known.

Under normal physiologic conditions, plasma F. $VIII_{vWF}$ is loosely associated with platelets. After their removal from the plasma atmosphere, such platelets do not adhere to the subendothelial space in a rabbit aorta, and they do not aggregate in the presence of the antibiotic ristocetin (3, 4). Aggregation or agglutination of platelets in the presence of ristocetin requires F. $VIII_{vWF}$, and this factor becomes tightly "associated" with human platelets (5–8). It has been recently reported that ristocetin induces binding of 31,000 molecules of F. $VIII_{vWF}$ (M_r 1.1×10^6) per one platelet (9). This ristocetin-induced binding of F. $VIII_{vWF}$ is of relatively high affinity and demonstrates a functional correlation with platelet aggregation (10). Whereas ristocetin is a useful laboratory tool to study and diagnose abnormalities in the interaction of von Willebrand factor with human platelets (4), we undertook a search for a physiologic mechanism(s) that can be operative in vivo to induce and regulate the interaction of F. $VIII_{vWF}$ with human platelets.

Our results indicate that thrombin at concentrations that are generated physiologically (11) allows F.

VIII$_{VWF}$ to bind to platelets. Because there is evidence for thrombin activity and for formation of fibrin appearing very promptly at the site of a vascular wound (1, 11, 12), the ability of thrombin to induce binding of F. VIII$_{VWF}$ can represent one of the important mechanisms governing formation and/or growth of a hemostatic plug. On the other hand, prostacyclin (PGI$_2$), a known inhibitor of platelet aggregation produced by endothelial cells (13, 14) lining the vessel in the vicinity of a vascular injury, is a likely agent to be involved in prevention of F. VIII$_{VWF}$-mediated interaction of platelets with the vessel wall. Indeed, prostacyclin has proven to be not only inhibitory toward exposure of the receptor for F. VIII$_{VWF}$ by thrombin, but even more striking, it was also able to reverse the binding of already attached F. VIII$_{VWF}$.

METHODS

Materials. Human F. VIII concentrate was obtained from American National Red Cross. Ristocetin, human thrombin (>90% homogeneity, 3,000 National Institutes of Health [(NIH) Units/mg], EDTA, EGTA, polyethelyne glycol (mol wt 4,000), and diisopropylfluorophosphate were purchased from Sigma Chemical Co., St. Louis, MO. Bovine thrombin was purchased from Parke, Davis & Co., Detroit, MI. Purified human α-thrombin (sp act 2,730 U/mg) was obtained through the courtesy of Dr. John Fenton III (New York Department of Public Health, Albany, NY) and Dr. Leon Cunningham (Vanderbilt University, Nashville, TN). Human fibrinogen was purchased from Kabi, Stockholm, Sweden. Rabbit antihuman fibrinogen immunoglobulin G (IgG) was purchased from N. L. Cappel Laboratories, Inc., Cochranville, PA, rabbit antihuman F. VIII and dibutyryl cyclic AMP (dcAMP) from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA. Bio-Gel A15m was purchased from Bio-Rad Laboratories, Richmond, CA. Sepharose 4B and 2B were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Both ^{125}I- and [^{3}H]serotonin were purchased from Amersham Corp., Arlington Hts., IL. Imipramine was obtained from Ciba-Geigy Corp., Summit, NJ. Alpha chymotrypsin (49.2 U/mg) was purchased from Worthington Biochemical Corp., Freehold, NJ. PGI$_2$ and 6-keto prostaglandin F$_{1\alpha}$ (6 keto PGF$_{1\alpha}$) were obtained through the courtesy of Dr. John Pike, Upjohn Co., Kalamazoo, MI.

Preparation of albumin gradient gel-filtered platelets (AGFP). Blood was drawn from normal healthy donors, who had not taken any medication in the previous 2 wk, into syringes containing 0.11 M citrate buffer, pH 5.0, to have a final blood buffer ratio of 9:1. It was centrifuged at 160 g for 15 min. The platelet-rich plasma was collected, layered on the top of a stepwise albumin gradient, and after centrifugation, the layer of platelets was filtered through a Sepharose 2B column equilibrated with Hepes buffer, pH 7.35, containing 0.1% dextrose and 0.35% albumin (15). Fractions from the first peak were collected, and platelets were counted in a Coulter model ZBI particle counter (Coulter Electronics Inc., Hialeah, FL). AGFP were used in experiments unless otherwise stated.

Preparation of glutaraldehyde-fixed platelets. Platelets obtained by the stepwise albumin gradient-gel filtration method were fixed with an equal volume of 0.1% glutaraldehyde containing 0.14 M NaCl, 5 mM EDTA, 5.5 mM glu-

cose, and 0.01 M Tris-HCl, pH 7.4, for 2 min at room temperature (16). Fixed platelets were centrifuged, washed three times in the above buffer from which glucose was omitted, and used for the experiment the next day.

Preparation of washed platelets. The procedure of Kao et al. (9) was followed. Briefly, platelet-rich plasma was obtained as above and centrifuged at 6,000 g for 10 min at room temperature. The platelet pellet was washed five times by resuspending it in 16 ml of 0.025 M Tris-HCl, 0.15 M NaCl, 0.01 M EDTA, pH 7.4, and centrifuging at 6,000 g for 10 min at room temperature. After the last wash, the platelets were resuspended in 0.025 M Tris-HCl, 0.15 M NaCl, 0.1% bovine serum albumin, pH 7.4.

Chymotrypsin treatment of platelets. Albumin gradient gel-filtered platelets were treated with chymotrypsin (300 µg/ml) for 30 min and then the reaction was stopped with 0.5 mM toluenesulfonyl fluoride. Untreated platelets were processed in a similar way but without addition of chymotrypsin.

Preparation of [^{3}H]serotonin-labeled platelets (AGFP) was done as described (17). The final count of platelets was adjusted to 1.0×10^8 platelets in each sample, and the release reaction was performed at room temperature without stirring. Samples were taken for total radioactivity counts, the incubation mixture centrifuged at 8,000 g for 2 min, and the supernatant assayed for released radioactivity. Imipramine, at a concentration of 0.15 µg/ml, was used in all release experiments.

Purification of F. VIII$_{VWF}$. The general procedure of Sodetz et al. (18) was followed with two added modifications. Briefly, human F. VIII concentrate (807–915 NIH units) was precipitated with polyethylene glycol. The precipitate was washed and dissolved in 0.05 M Tris-HCl, 0.15 M NaCl, 1 mM diisopropylfluorophosphate, and 0.02% NaN$_3$, pH 7.4, and applied to Bio-Gel A15 m packed in a 2.5×100-cm siliconized glass column. The column was eluted with a solution of 0.05 M Tris-HCl, 0.15 M NaCl, and 0.02% NaN$_3$, pH 7.4. The void volume fractions containing F. VIII procoagulant activity were pooled and precipitated at 4°C by the addition of solid ammonium sulfate until 35% saturation was attained. The precipitate was dissolved in 0.05 M phosphate, 0.15 M NaCl, pH 8.0, and dialyzed at 4°C against the same buffer. To remove traces of fibrinogen, dialyzed, purified material was applied to an antihuman fibrinogen IgG-Sepharose 4B affinity column prepared as described below. The column was eluted at room temperature with 0.05 M phosphate, 0.15 M NaCl, (pH 8.0), 0.01 M glycine-HCl (pH 2.2), and 6 M urea, 0.05 M phosphate, (pH 6.0). Fractions eluted with 0.05 M phosphate, 0.15 M NaCl, pH 8.0, were combined and precipitated by the addition of solid ammonium sulfate until 35% saturation was attained. The precipitate was collected by centrifugation at 12,000 g for 10 min, dissolved in the same buffer, and dialyzed. In some experiments, this material was subjected to an additional step of purification to remove fibronectin, using gelatin-Sepharose 4B affinity chromatography as described below (19).

Labeling of F. VIII$_{VWF}$. Purified F. VIII$_{VWF}$ was labeled with ^{125}I, using the iodine monochloride technique of McFarlane (20). The specific radioactivity of ^{125}I-F. VIII$_{VWF}$ was on average 5.4×10^8 cpm/mg, and 95% radioactivity was precipitated with 10% TCA. The activity of F. VIII$_{VWF}$ was assayed for the ristocetin-cofactor activity before and after radioiodination using platelets separated by a stepwise albumin gradient-gel filtration method. In a twofold dilution method, both iodinated F. VIII$_{VWF}$ and unlabeled protein after affinity chromatography with antifibrinogen IgG had 74 units of ristocetin cofactor activity per milligram

protein. Before affinity chromatography, the ristocetin-cofactor activity was, on the average, 56 U/mg protein.

Binding of ^{125}I-F. VIII$_{VWF}$ to platelets. All binding assays were performed in 0.5 ml reaction mixture containing from 5×10^7 to 1×10^8 platelets. Platelets were preincubated with thrombin or ristocetin for 3 to 5 min, and then ^{125}I-F. VIII$_{VWF}$ was added to each sample. When thrombin was used, hirudin in concentration of 0.2 U was added for 2 min to neutralize unbound thrombin. The sample was incubated at room temperature in polypropylene tubes (12×75 mm) for an additional 50 min without agitation. After incubation, the mixture was applied to the top of a 9:1 (vol/vol) mixture of dibutylphthalate and Apiezon oil C (James G. Biddle Co., Plymouth Meeting, PA), and the platelets were separated from unbound ^{125}I-F. VIII$_{VWF}$ by centrifugation for about 2 min at 8,000 g using a microfuge (21). The supernatant was sampled for free radioactivity, and bound radioactivity was measured in the platelet pellet cut off together with the tip of the tube after rapid freezing of its contents in a mixture of dry ice and acetone. Their radioactivities were determined in a Searle Gamma Counter (Searle Radiographics Inc., Des Plaines, IL). Specific binding was determined by subtracting from the observed amount of bound ^{125}I-F. VIII$_{VWF}$ that amount which bound in a duplicate incubation mixture containing 100-fold M excess of unlabeled F. VIII$_{VWF}$. The amount of ^{125}I-F. VIII$_{VWF}$ bound in the presence of an excess of unlabeled ligand ranged from 10 to 22% of the observed ^{125}I-F. VIII$_{VWF}$ bound to platelets stimulated with thrombin. The amount of ^{125}I-F. VIII$_{VWF}$ associated with unstimulated control platelets was very similar in the absence and presence of an excess of unlabeled F. VIII$_{VWF}$.

Preparation of fibrinogen-Sepharose 4B column. Human fibrinogen was dissolved in distilled water to a final concentration of 0.3% and then dialyzed against 0.15 M NaCl. A saturated solution of ammonium sulfate was added to the solution with stirring to give 16% saturation. The fibrin-like material was collected on a glass rod, redissolved in 0.15 M NaCl, and then precipitated once more by the addition of ammonium sulfate until 16% saturation was attained (22). The final product of fibrinogen was coupled to Sepharose 4B (23).

Preparation of monospecific antihuman fibrinogen IgG-Sepharose 4B column. Rabbit antihuman fibrinogen IgG was dissolved in 0.015 M Tris-HCl, 0.14 M NaCl, pH 7.5, and then dialyzed against the same buffer. After dialysis, the solution was applied to a fibrinogen-Sepharose 4B affinity column and eluted in sequence with 0.15 M Tris-HCl, 0.14 M NaCl, pH 7.5; 0.1 M carbonate, pH 9.7; 1 M NaCl, 0.015 M Tris-HCl, pH 7.5; 0.1 M glycine-HCl, pH 2.2; and 4 M urea, 0.05 M phosphate, pH 6.0 (23). The eluates with 0.1 M glycine-HCl, pH 2.2; and with 4 M urea, 0.05 M phosphate, pH 6.0, were collected and concentrated, respectively. After checking the purity and specificity of antihuman fibrinogen IgG, the material was coupled to Sepharose 4B (23).

Preparation of fibronectin. Gelatin-Sepharose 4B was used to isolate fibronectin and to separate fibronectin from purified F. VIII$_{VWF}$. The sample was applied to gelatin-Sepharose 4B with 0.05 M phosphate, 0.15 M NaCl, pH 7.2, and fibronectin was eluted with 8 M urea, 0.05 M Tris-HCl, pH 7.5 (19).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was performed according to the procedure of Weber and Osborn (24). Samples were reduced in the presence of 1% dithiothreitol at 37°C

for 30 min. The percentages of acrylamide used for electrophoresis were either 5 or 7.5%.
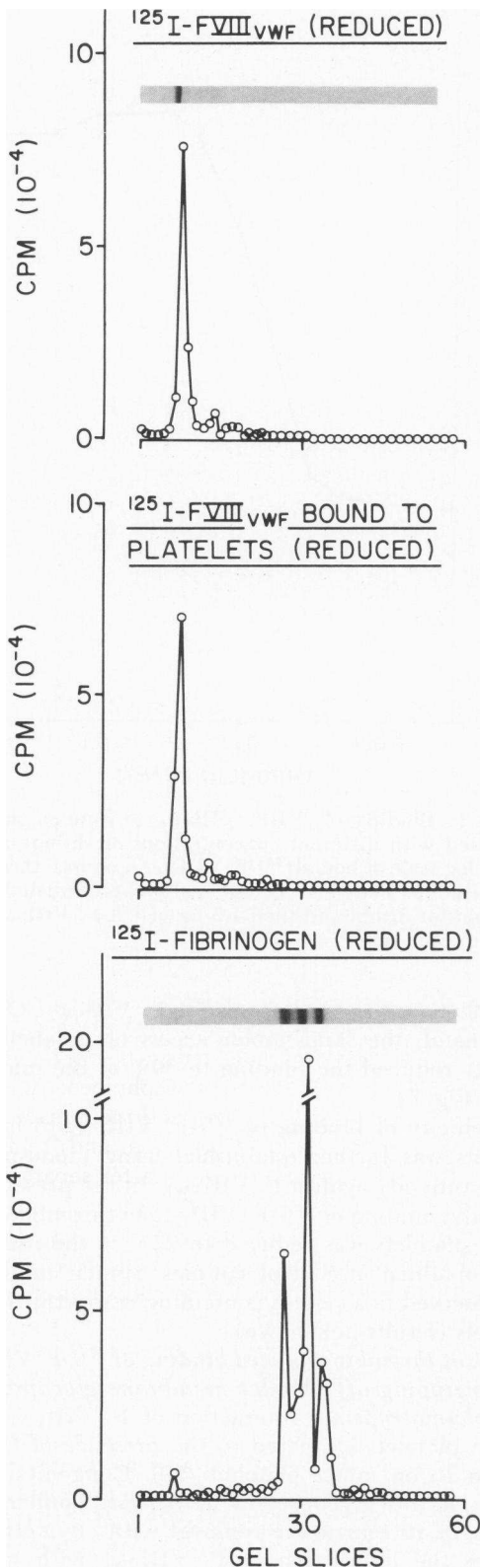
Other assays. Protein concentrations in the samples were measured by the method of Lowry (25). Fibrinogen content of purified F. VIII$_{VWF}$ was checked with the staphylococcal clumping test which can detect 0.5 μg fibrinogen/ml (26) and by radioimmunoassay detecting at least 10 ng of fibrinogen per milliliter. Immunodiffusion reactions were performed by the double diffusion method of Ouchterlony (27) using the diffusion plates prepared with 1.2% agar in veronal buffer, pH 8.5, $I = 0.5$.

RESULTS

We have used purified ^{125}I-F. VIII$_{VWF}$ for quantitative binding studies. F. VIII$_{VWF}$ isolated from human Factor VIII concentrate by a standard procedure (18), was purified further by affinity chromatography to assure removal of fibrinogen and fibronectin, which contaminate Factor VIII preparations (28). Such purified F. VIII$_{VWF}$ gave a single band on SDS-PAGE under reducing conditions. Electrophoresis of iodinated F. VIII$_{VWF}$ produced a single peak of radioactivity that was parallel with the protein band (Fig. 1, top panel). Whereas fibrinogen was present in concentrations of 22 μg/1 mg of preparations of F. VIII$_{VWF}$ before immunoaffinity chromatography, it was not detected thereafter using immunodiffusion or the staphylococcal clumping test with a limit of detectability 0.5 μg fibrinogen/ml (26). In an even more sensitive radioimmunoassay procedure, the fibrinogen content was <60 ng/1 mg of F. VIII$_{VWF}$, i.e., <0.00006% of F. VIII$_{VWF}$ weight.

Human platelets used in these experiments were essentially free of plasma F. VIII$_{VWF}$, as judged by a complete lack of responsiveness to ristocetin. They were also separated from plasma fibrinogen, as verified by the lack of a response to ADP and by the absence of detectable plasma fibrinogen in the supernatant of sedimented platelets, using the staphylococcal clumping test. These platelets maintained their reactivity to thrombin in terms of aggregation and release of [^{3}H]serotonin for at least 120 min after separation.

Binding of F. VIII$_{VWF}$ to human platelets. Binding of ^{125}I-F. VIII$_{VWF}$ was observed when platelets were treated with thrombin in a concentration of 0.05 U/ml. At this concentration, the equilibrium of binding occurred within 30 min (Fig. 2). Because we were testing lower thrombin concentrations as well, 60-min incubations were usually performed. In subsequent experiments, different concentrations of thrombin, ranging from 0.001 U/ml to 1 U/ml, were tested. The extent of ^{125}I-F. VIII$_{VWF}$ binding was proportional to the thrombin concentration added to the platelets before ^{125}I-F. VIII$_{VWF}$ (Fig. 3). Binding of F. VIII$_{VWF}$ to platelets was significantly induced with as little throm-

bin as 0.008 U/ml, reaching a maximum at thrombin concentrations of 0.01 U/ml–0.05 U/ml. Above this range, thrombin caused some decrease in binding of ^{125}I-F. VIII$_{VWF}$. Therefore, in most experiments presented below, we used thrombin at a concentration of 0.05 U/ml. Bovine thrombin and two different human α-thrombin preparations (one purified and provided by Dr. John Fenton III) produced a similar effect when tested with the same platelet preparation in terms of binding results.

Thrombin was required for its action on platelets and not on ^{125}I-F. VIII$_{VWF}$, because preincubation of ^{125}I-F. VIII$_{VWF}$ with thrombin, followed by inactivation of free thrombin with hirudin, did not result in increased binding of ^{125}I-F. VIII$_{VWF}$ to untreated platelets. Furthermore, ^{125}I-F. VIII$_{VWF}$ bound to platelets was analyzed in SDS-PAGE under reducing conditions. The radiolabeled protein pattern remained the same as ^{125}I-F. VIII$_{VWF}$ analyzed before binding to platelets (Fig. 1, middle panel). The platelet-associated ^{125}I pattern did not exhibit radioactivity associated with ^{125}I-fibrinogen (comparative control in Fig. 1, lower panel).

The concentration of platelets was an important factor in binding of ^{125}I-F. VIII$_{VWF}$. At a low platelet concentration (5×10^6 cells), binding measured after a 60-min incubation gave variable results, probably owing to poor recovery of platelets in the pellet. With 10 times more platelets (5×10^7 cells), binding of ^{125}I-F. VIII$_{VWF}$ could be readily measured, and at 1.0×10^8 platelets/ml (a concentration within the physiologic range), binding was optimal.

FIGURE 1 SDS polyacrylamide (5%) gel electrophoresis of purified ^{125}I-F. VIII$_{VWF}$. ^{125}I-F. VIII$_{VWF}$ was dissolved in 1% SDS with 1% dithiothreitol and was subject to disc gel electrophoresis as described in Methods. The gels were then stained with 0.1% Coomassie Brilliant Blue and cut into 1.5-mm slices, which were counted for ^{125}I. Recovery of the radioactivity applied to the gels was >90%. Upper panel: gel that contained only ^{125}I-F. VIII$_{VWF}$. Middle panel: gel that contained ^{125}I-labeled material bound to thrombin-stimulated platelets. Suspension of platelets (1×10^8 cells) were treated with thrombin (0.05 U) and incubated with ^{125}I-F. VIII$_{VWF}$ (8.2 μg) at 22°C for 60 min. After sedimentation of the platelets through an oil mixture (Methods), the pelleted platelets were dissolved in 3% SDS and 4 M urea by heating at 100°C for 5 min, and a portion of solubilized platelets was subjected to disc gel electrophoresis under reducing conditions as described in Methods. Distribution of ^{125}I associated with thrombin-stimulated platelets corresponds to that of ^{125}I-F. VIII$_{VWF}$ in the upper panel and does not correspond to the pattern of radioactivity distribution characteristic of ^{125}I-fibrinogen run as comparative control (lower panel). Lower panel: gel that contained only purified ^{125}I human fibrinogen labeled by the iodine monochloride method (20).

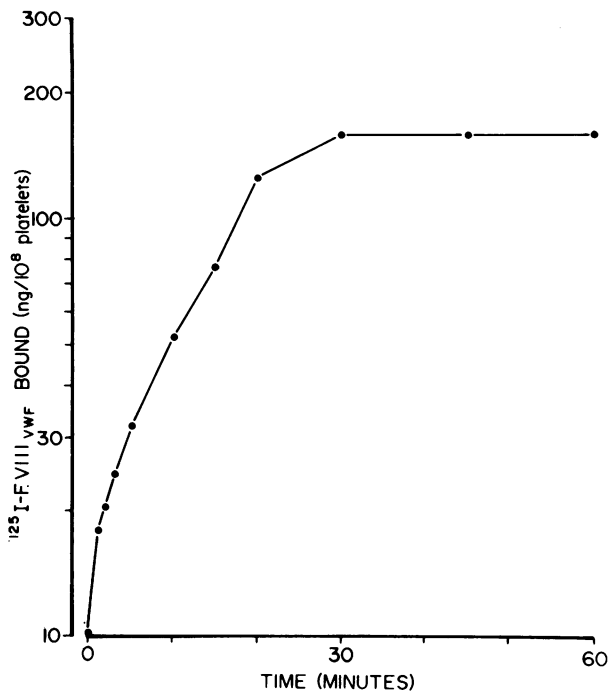FIGURE 2 Time-course of ^{125}I-F. VIII$_{VWF}$ binding to human platelets induced by thrombin (0.05 U/ml). Ordinate: log scale of bound ^{125}I-F. VIII$_{VWF}$; abscissa: time in minutes. Platelets (1 × 10^8 cells) were treated with thrombin for 3 min and then ^{125}I-F. VIII$_{VWF}$ (0.8 μg) was added. At specified time intervals, platelets were rapidly centrifuged (8,000 g for 2 min) through an oil mixture to separate platelet-bound ligand from unbound material.
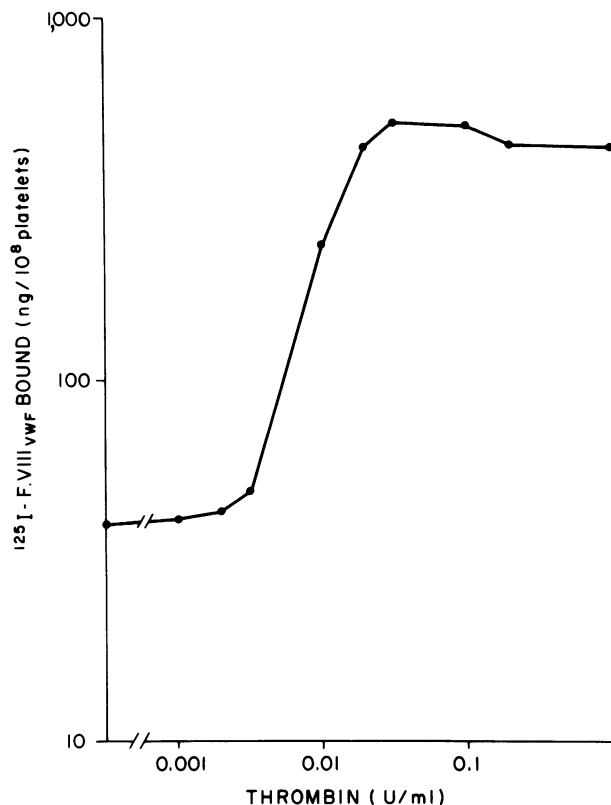
FIGURE 3 Binding of ^{125}I-F. VIII$_{VWF}$ to human platelets stimulated with different concentrations of thrombin. Ordinate: log scale of bound ^{125}I-F. VIII$_{VWF}$; abscissa: thrombin concentration. Platelets (1 × 10^8 cells) were treated with thrombin for 3 min and then 1.2 μg of ^{125}I-F. VIII$_{VWF}$ was added to each tube.

Binding of ^{125}*I-F. VIII$_{VWF}$ to metabolically intact platelets as compared with glutaraldehyde-fixed platelets.* In these experiments, we compared the effect of thrombin on binding of F. VIII$_{VWF}$ to metabolically intact platelets with that of glutaraldehyde-fixed platelets. Only intact platelets showed responsiveness to thrombin in terms of induction of binding of ^{125}I-F. VIII$_{VWF}$ (Fig. 4). In contrast to thrombin, ristocetin induced binding to both metabolically intact platelets and glutaraldehyde-treated platelets. The binding shown in Fig. 4 represents subsaturating conditions because the percentage of total added ^{125}I-F. VIII$_{VWF}$ that was bound increased upon addition of increasing concentrations of ^{125}I-F. VIII$_{VWF}$.

Specificity of thrombin-induced binding. To establish the specificity of binding of ^{125}I-F. VIII$_{VWF}$ to thrombin-treated platelets, the effect of a molar excess of unlabeled F. VIII$_{VWF}$, fibrinogen, or fibronectin was examined. Although we did not detect these two proteins in our purified preparations of F. VIII$_{VWF}$ (see above), we checked whether the binding of ^{125}I-F. VIII$_{VWF}$ is influenced by these two plasma proteins. A 100-fold molar excess of fibrinogen or fibronectin did

not influence the binding of ^{125}I-F. VIII$_{VWF}$. On the other hand, the same molar excess of unlabeled F. VIII$_{VWF}$ reduced the binding to 20% of the maximal value (Fig. 5).

Specificity of binding of ^{125}I-F. VIII$_{VWF}$ to human platelets was further established using monospecific rabbit antibody against F. VIII$_{VWF}$. In the presence of antibody, binding of ^{125}I-F. VIII$_{VWF}$ to thrombin-stimulated platelets was reduced to 22% of the maximal value obtained in control samples. Similar inhibition was observed in a system containing ristocetin-treated platelets (results not shown).

Lack of thrombin-induced binding of ^{125}*I-F. VIII$_{VWF}$ after stripping off platelet membrane glycoproteins with chymotrypsin.* Interaction of F. VIII$_{VWF}$ with human platelets is related to the presence of Glycoprotein Ib on intact platelets (29). Congenital deficiency of this glycoprotein in Bernard-Soulier Syndrome, or its enzymatic removal with chymotrypsin reduces the interaction of F. VIII$_{VWF}$ with human platelets (30). Treatment of platelets with chymo-
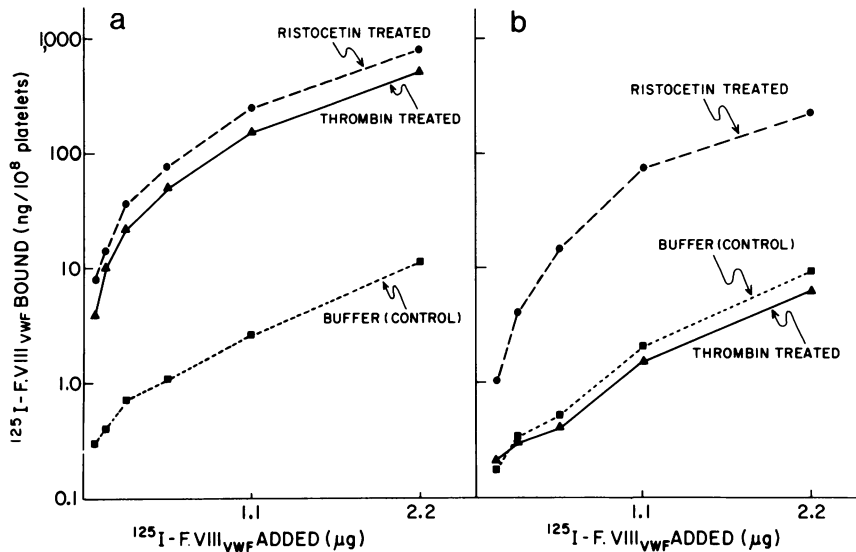
FIGURE 4 (a) Intact platelets, (b) glutaraldehyde-fixed platelets. Comparison of binding of ^{125}I-F. VIII$_{VWF}$ to intact platelets and glutaraldehyde-fixed platelets. Ordinates: log scale of bound ^{125}I-F. VIII$_{VWF}$; abscissa: total ^{125}I-F. VIII$_{VWF}$ added to platelets. Platelets (1×10^8 cells) treated with thrombin (0.05 U/ml) or ristocetin (1 mg/ml) were incubated with different concentrations of ^{125}I-F. VIII$_{VWF}$ for 60 min at room temperature without stirring.

trypsin (300 µg/m) reduced significantly the level of glycoprotein I complex as determined in SDS-PAGE of the solubilized platelet pellet obtained after chy-

FIGURE 5 Inhibition of binding of ^{125}I-F. VIII$_{VWF}$ to human platelets by unlabeled F. VIII$_{VWF}$ as compared with fibrinogen and fibronectin. Platelets were treated with thrombin (0.05 U/ml) followed by hirudin (0.2 U/ml). Then unlabeled ligands at different concentrations were added with a constant amount of ^{125}I-F. VIII$_{VWF}$ (0.22 µg). Molar concentrations were based on F. VIII$_{VWF}$ subunit ($M_r = 240,000$). The 100% binding was 97 ng ^{125}I-F. VIII$_{VWF}$/10^8 platelets as determined in the absence of unlabeled ligand.

motrypsin treatment. Likewise, binding of ^{125}I-F. VIII$_{VWF}$ to these platelets stimulated with thrombin or with ristocetin was reduced to negligible levels (results not shown).

Effect of chelators on thrombin-induced binding of ^{125}I-F. VIII$_{VWF}$ to human platelets. Previous reports indicate that ristocetin-induced binding of F. VIII$_{VWF}$ to human platelets was not inhibited by EDTA (9). Platelets used in those experiments were prepared by repeated centrifugation and washing using EDTA-containing Tris buffer. To compare our system based on thrombin-stimulated binding with that based on ristocetin-induced binding, we examined the effect of the different methods of preparing platelets on binding of F. VIII$_{VWF}$ stimulated by thrombin and by ristocetin. In method 1, which was based on stepwise albumin gradient and gel filtration (15) both thrombin and ristocetin induced the binding of ^{125}I-F. VIII$_{VWF}$ to platelets (Fig. 6). In method 2, based on the washing of platelets with EDTA-containing Tris buffer (9), the binding of ^{125}I-F. VIII$_{VWF}$ to platelets was induced by ristocetin, which gave almost identical concentration-dependent curves as with platelets prepared by method 1. However, thrombin treatment of platelets prepared by method 2 did not induce binding of ^{125}I-F. VIII$_{VWF}$ to platelets. Addition of CaCl$_2$ (2 mM) to the incubation mixture restored the binding reactivity of thrombin-treated platelets prepared by method 2. Because of the lack of thrombin-induced binding to platelets prepared by washing with buffer containing EDTA, the effect of chelators was examined. Both
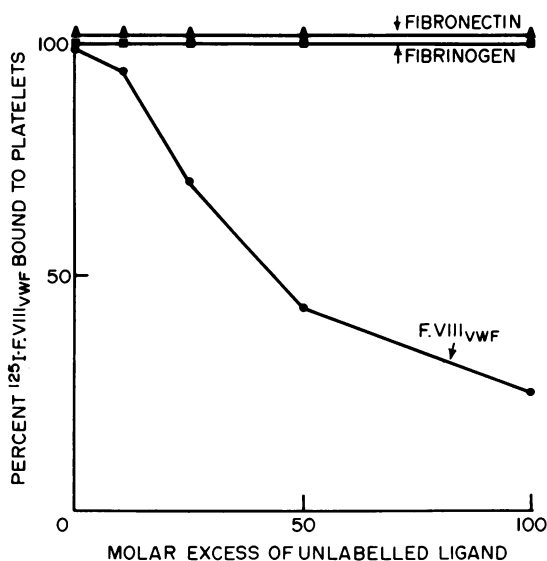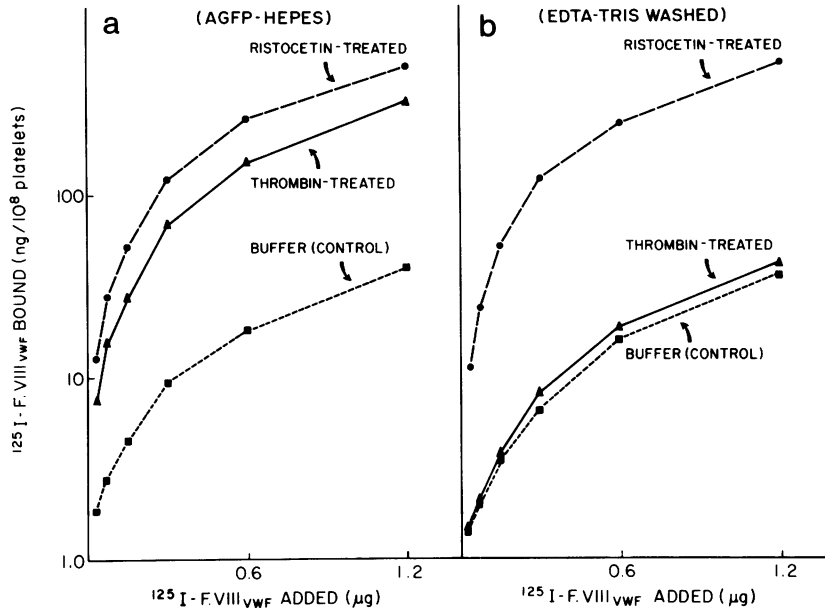
FIGURE 6 (a) Platelets prepared by method 1, (b) platelets prepared by method 2. Comparison of binding of ^{125}I-F. VIII$_{VWF}$ to human platelets prepared by method 1 (albumin gradient-gel filtration) and method 2 (EDTA-Tris buffer washing). Ordinate: log scale of bound ^{125}I-F. VIII$_{VWF}$; abscissa: total ^{125}I-F. VIII$_{VWF}$ added to platelets. Platelets were treated with thrombin (0.05 U/ml) or ristocetin (1 mg/ml) and binding of different concentrations ^{125}I-F. VIII$_{VWF}$ was determined.

EDTA (2 mM) and EGTA (2 mM) added to platelets prepared by method 1 inhibited thrombin-induced binding of ^{125}I-F. VIII$_{VWF}$ by 92 and 90%, respectively. Inhibition was observed when chelators were added 5 min before thrombin or 8 min after thrombin. These chelators did not inhibit thrombin-induced release of [³H]serotonin from platelets prepared by method 1, thus indicating that they are affecting receptor interaction with F. VIII$_{VWF}$ rather than thrombin binding and subsequent activation of platelets.

Prostacyclin inhibition of binding of ^{125}I-F. VIII$_{VWF}$ to thrombin-activated platelets. In these experiments, we examined whether prostacyclin inhibits the process of F. VIII$_{VWF}$ receptor exposure by thrombin and ristocetin. Prostacyclin inhibited, in a concentration-dependent manner, binding of ^{125}I-F. VIII$_{VWF}$ to human platelets induced by low thrombin concentration (0.01 U/ml) (Fig. 7). On the other hand, PGI$_2$ was not inhibitory toward binding of ^{125}I-F. VIII$_{VWF}$ to human platelets treated with ristocetin. Although PGI$_2$ was inhibitory at the concentration range 1–10 nM, the inhibition by 6 keto PGF$_{1\alpha}$ was not observed at 1 nM and only 24% binding was inhibited at 10 nM. This effect of 6 keto PGF$_{1\alpha}$ represented ~2% of the inhibitory potency of PGI$_2$.

The effects of time of addition of PGI$_2$ on inhibition of binding of ^{125}I-F. VIII$_{VWF}$ to human plate-

lets. To distinguish between the two possible modes of action of prostacyclin on the interaction of F. VIII$_{VWF}$ with human platelets, i.e., the effect of PGI$_2$ on the process of receptor induction by thrombin, and the effect of PGI$_2$ on the already formed ligand-receptor complex, the following experiments were conducted. PGI$_2$ was added to platelets at different stages of thrombin-platelet and platelet-^{125}I-F. VIII$_{VWF}$ interactions.

In the first experiments, PGI$_2$ was added to human platelets 3 min before addition of thrombin, which was followed 8 min later by addition of ^{125}I-F. VIII$_{VWF}$. A concentration-dependent inhibition of ^{125}I-F. VIII$_{VWF}$ binding by PGI$_2$ was observed as shown in Fig. 7. Addition of PGI$_2$ 10 min before thrombin (0.01 U/ml) did not change the inhibitory effect of this prostaglandin. A more striking effect of the prostacyclin action was noted when prostacyclin was added following thrombin and ^{125}I-F. VIII$_{VWF}$. Whether PGI$_2$ was added 3, 10, or even 30 min after, the effect was the same, namely inhibition of binding of ^{125}I-F. VIII$_{VWF}$ by the same range of prostacyclin concentrations.

The effect of PGI$_2$ added to platelets after ^{125}I-F. VIII$_{VWF}$ was investigated in more detail by following the time-course of binding (Fig. 8). When binding reached equilibrium at 30 min after addition of ^{125}I-F. VIII$_{VWF}$, prostacyclin (a concentration of 50 nM),
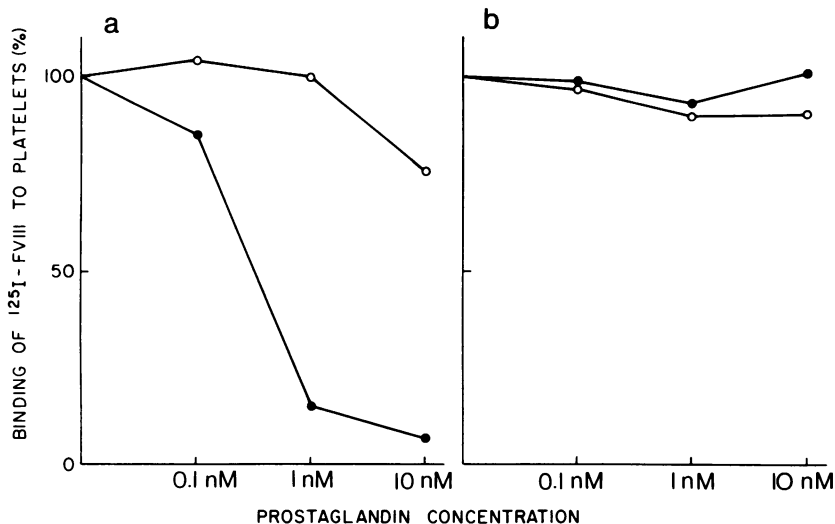
FIGURE 7 (a) Thrombin-treated platelets, (b) ristocetin-treated platelets. The effect of prostacyclin (solid circles) and 6 keto $PGF_{1\alpha}$ (open circles) on binding of ^{125}I-F. $VIII_{VWF}$ to human platelets. Platelets (1×10^8 cells) were treated with different concentrations of prostaglandins for 3 min, followed by addition of thrombin (0.01 U/ml) or ristocetin (1 mg/ml). After 3 min, free thrombin was inactivated by hirudin (0.04 U/ml) and then 0.34 μg of ^{125}I-F. $VIII_{VWF}$ was added to each tube. Binding was determined after 60 min incubation at room temperature without stirring. Binding of ^{125}I-F. $VIII_{VWF}$ to platelets pretreated with Tris buffer, pH 9.37 (diluent for PGI_2 and 6 keto $PGF_{1\alpha}$), was accepted as 100%.
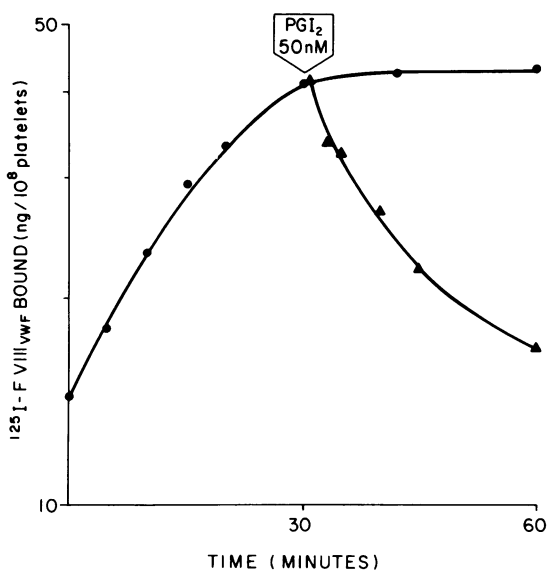

FIGURE 8 Reversal of binding of ^{125}I-F. $VIII_{VWF}$ to human platelets by prostacyclin. Ordinate: log scale of bound ^{125}I-F. $VIII_{VWF}$; abscissa: time in minutes. Platelets (1×10^8 cells) were treated with thrombin (0.01 U/ml) for 3 min followed by hirudin (0.02 U/ml) for 2 min and then ^{125}I-F. $VIII_{VWF}$ (0.38 μg) was added. The time-course of binding was determined. The solid circles represent binding to platelets treated with thrombin and Tris buffer, pH 9.37; solid triangles show the reversal of binding following addition of 50 nM PGI_2 to a parallel portion of the reaction mixture.

was added to one of two sets of tubes containing the incubation mixture. Tris buffer, pH 9.37, was added to the other set, which served as a positive control for binding. After addition of PGI_2 there was a gradual dissociation of bound ^{125}I-F. $VIII_{VWF}$, indicating that prostacyclin is able to reverse binding of ^{125}I-F. $VIII_{VWF}$, which had already attained equilibrium.

Inhibitory effect of PGI_2 on binding of ^{125}I-F. $VIII_{VWF}$ is mimicked by dcAMP. PGI_2 is the most potent activator of platelet adenylate cyclase known to date (31, 32). Therefore, its inhibitory effect on the binding of ^{125}I-F. $VIII_{VWF}$ can be mediated by cAMP, which is generated as a result of PGI_2 action. To examine this possibility, we performed a series of experiments in which PGI_2 was substituted with dcAMP. We have observed a concentration-dependent inhibition of binding by dcAMP (Fig. 9). Again, like PGI_2, dcAMP was added at different stages in regard to the binding process. Addition of dcAMP 5 min before thrombin, i.e., 10 min before ^{125}I-F. $VIII_{VWF}$ (curve A), 5 min after thrombin, i.e., simultaneously with ^{125}I-F. $VIII_{VWF}$ (curve B), 8 min after thrombin (curve C), or 35 min after thrombin, i.e., 30 min after ^{125}I-F. $VIII_{VWF}$ (curve D), resulted in concentration-dependent inhibition of binding. Although the effect of dcAMP added 30 min after ^{125}I-F. $VIII_{VWF}$ was less pronounced, it still caused a 50% reversal of binding at a concentration of 0.3 mM of dcAMP.
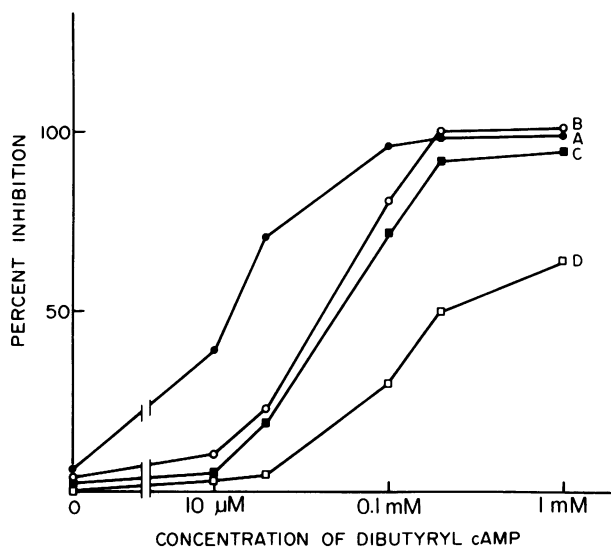
FIGURE 9 Inhibition of binding of ^{125}I-F. VIII$_{VWF}$ to human platelets by dcAMP added to platelets at different times in reference to the addition of thrombin (0.01 U/ml). In each instance, platelets were treated with thrombin and with hirudin before the addition of ^{125}I-F. VIII$_{VWF}$ (0.4 μg). A, dcAMP 5 min before thrombin. B, dcAMP 5 min after thrombin. C, dcAMP 8 min after thrombin. D, dcAMP 30 min after ^{125}I-F. VIII$_{VWF}$ (35 min after thrombin).

DISCUSSION

Our results indicate that in intact platelets thrombin induces binding of F. VIII$_{VWF}$ to a specific membrane receptor. The specificity of the interaction between F. VIII$_{VWF}$ and human platelets is based on the following evidence. By following the additional step of immunoaffinity chromatography with monospecific anti-human fibrinogen IgG, we obtained F. VIII$_{VWF}$ preparations essentially devoid of another plasma protein, fibrinogen. Fibrinogen interacts with a distinct receptor on platelets stimulated with thrombin (33). Furthermore, thrombin-induced binding of ^{125}I-F. VIII$_{VWF}$ was specific because it was not inhibited by 100-fold molar excess of either fibrinogen or fibronectin, but was inhibited by an excess of unlabeled, purified F. VIII$_{VWF}$ and by monospecific rabbit antibody against human F. VIII. Binding of F. VIII$_{VWF}$ to human platelets in our system was induced by a very low concentration of thrombin (0.1–0.5 nM), which can be generated physiologically (11). Such low concentrations of thrombin exert a direct effect on platelets to induce the exposure of specific receptors for F. VIII$_{VWF}$, rather than to induce changes in F. VIII$_{VWF}$ promoting its subsequent interaction with intact platelets. Preincubation of thrombin with ^{125}I-F. VIII$_{VWF}$ remained without a measurable effect on the interaction of this complex with unstimulated platelets.

Comparison of the binding of F. VIII$_{VWF}$ to platelets stimulated with thrombin with that of platelets treated with ristocetin has demonstrated several hitherto unrecognized aspects of this interaction. Although the extent of binding induced by thrombin at low concentrations (0.5 nM or 0.05 U/ml) is similar to that of ristocetin, the process of exposure of the F. VIII$_{VWF}$ receptor by thrombin and ristocetin is different. Induction of F. VIII$_{VWF}$ binding by thrombin requires metabolically intact platelets and is sensitive to EDTA and EGTA. The thrombin-induced receptor is inhibited by prostacyclin. Ristocetin-induced binding does not require metabolically active platelets, is not sensitive to chelators (9, 10), and is not sensitive to inhibition by prostacyclin. Thus, it appears that the receptor for F. VIII$_{VWF}$ can be induced in human platelets through two different mechanisms.

Binding of F. VIII$_{VWF}$ to its platelet receptor, induced by thrombin, represents one of a few examples of receptor induction by this proteolytic enzyme. Previously, it was demonstrated that thrombin induces binding of Factor Xa to human platelets through mobilization of platelet Factor V (34). Also, thrombin caused a marked increase in, and redistribution of, the platelet receptor for lentil phytohemagglutinin (35). Recently, we have shown that thrombin induces binding of fibrinogen to the human platelet receptor (33). It appears that thrombin interacts with membrane mechanism(s) in human platelets, which results in exposure of membrane receptors for a number of different ligands. It was established that in the case of Factor Xa binding, this thrombin-dependent mechanism required the release reaction (34). The effect of thrombin does not appear to involve stimulation of de novo receptor formation because the protein synthesis in platelets is very limited (36). Furthermore, the receptor for F. VIII$_{VWF}$ appears to be present in platelets because membranes prepared from disrupted platelets are able to bind plasma F. VIII$_{VWF}$ (37).

The thrombin-sensitive membrane component(s) responsible for exposure of the receptor for F. VIII$_{VWF}$ is apparently of restricted sensitivity because other proteolytic enzymes, such as cymotrypsin, not only do not induce binding of F. VIII$_{VWF}$ to platelets (in striking contrast to fibrinogen binding, e.g., ref. 38), but also make them refractory to subsequent receptor exposure by thrombin or ristocetin. The platelet membrane glycoprotein of M_r 68,000–85,000 (Glycoprotein V), has been proposed as the thrombin substrate in intact platelets (39, 40). Whether this glycoprotein is linked to Glycoprotein Ib or to some other mechanism(s) controlling the availability of F. VIII$_{VWF}$ receptors remains to be examined.

Calcium is required for the binding of F. VIII$_{VWF}$ to platelets because EDTA used while platelets are washed prevents binding, and EDTA added to platelets before thrombin, or even 8 min after thrombin,

inhibits binding of F. VIII$_{VWF}$. This is in all likelihood the main reason why platelets washed with an EDTA-containing buffer (9), which we used in method 2 of platelet separation, did not bind F. VIII$_{VWF}$ in response to thrombin. It is possible that EDTA removes from the platelet membrane the surface-bound calcium essential for formation of a complex between F. VIII$_{VWF}$ and its receptor.

The mechanism of inhibition of binding of ^{125}I-F. VIII$_{VWF}$ to human platelets by PGI$_2$ seems to be dependent on cAMP, because dcAMP can cause a similar inhibition of binding. Therefore, this mechanism can be related to several platelet reactions that are influenced by cAMP, such as phosphorylation of platelet proteins (41) and inhibition of calcium flux (42). The receptor for F. VIII$_{VWF}$ is induced by thrombin, which is known to inhibit adenylate cyclase in platelets (43). The inhibition of adenylate cyclase by thrombin appears to be reversible because PGI$_2$ is able to obviate this effect of thrombin. The availability of divalent cations is a prerequisite for F. VIII$_{VWF}$ binding to its receptor on thrombin-stimulated platelets. The known inhibitory effect of cAMP on calcium mobilization (42) may thus contribute to inhibition of thrombin-induced binding of F. VIII$_{VWF}$ by prostacyclin. The dissociation of bound F. VIII$_{VWF}$ from its receptor by PGI$_2$ indicates that this ligand is not internalized by platelets, at least during the first 30 min of interaction.

Dual regulation of the platelet receptor for F. VIII$_{VWF}$ by PGI$_2$, as described herein, provides the mechanism that may explain the known inhibitory effect of prostacyclin on formation of platelet aggregates, due to platelet spreading, on the subendothelial layer of the aorta and the inhibition of platelet thrombus formation (44, 45) because both phenomena require F. VIII$_{VWF}$ (46). The inhibitory effect of PGI$_2$ is not limited to the receptor for F. VIII$_{VWF}$ on human platelets. The receptor for fibrinogen (33) and the receptor for the Fc fragment of IgG are also regulated by PGI$_2$ (47).

Our results provide evidence that the proteolytic enzyme thrombin induces exposure of a specific receptor for VIII$_{VWF}$ in human platelets and that prostacyclin can prevent this process. Analysis of thrombin-induced membrane changes governing platelet receptor exposure for F. VIII$_{VWF}$ as presented in this study can facilitate a search for the initial signal for platelet binding of F. VIII$_{VWF}$ in vivo.

ACKNOWLEDGMENTS

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