Thrombin Induces a Rapid Redistribution of Glycoprotein Ib-IX Complexes Within the Membrane Systems of Activated Human Platelets

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Previous studies have shown a decreased binding of monoclonal antibodies (MoAbs) to glycoprotein (GP) Ib-IX complexes on thrombin-stimulated platelets, but the reason for this is poorly understood. We have used (1) immunofluorescence procedures and flow cytometry, and (2) immunogold staining and electron microscopy to investigate this phenomenon. Washed platelets were incubated with α-thrombin, adenosine diphosphate, or ionophore A23187 for increasing lengths of time. For α-thrombin, but not the other agonists, flow cytometry confirmed a dose- and time-dependent decrease in the binding of MoAbs specific for GP Iba (AP-1, Bx-1), GP IX (FMC 25), or to the complex itself (SZ 1). Immunogold staining performed using standard transmission or scanning electron microscopy highlighted surface areas devoid of bound antibody. However, a quantitatively normal immunofluorescence was restored if paraformaldehyde-fixed, thrombin-stimulated platelets were permeabilized with Triton X-100 (Sigma Chemical Co, St Louis, MO) before MoAb addition, while immunogold staining was now seen to be concentrated within the interior of the platelet. Glutaraldehyde-fixed samples were then embedded in the resin Lowicryl K4M (Taab Laboratories Equipment Ltd, Aldermaston, England) and immunogold staining performed on thin sections using a monoclonal antibody to glycollacine. An increased presence of GP Ib-IX complexes within surface-connected membrane systems of the thrombin-stimulated platelets was confirmed. Interestingly, GP Ib-IX movement was opposite to the thrombin-induced externalization of internal pools of GP Ib-lla complexes and of the α-granule membrane GP, GMP-140.

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Glycoprotein (GP) Ib is the major sialoglycoprotein at the surface of human platelets. It is composed of a large α-chain (molecular weight [mol wt] 145,000) attached to a small β-chain (mol wt 24,000) through one or more disulfides. The bulk of GP Ib lies exterior to the plasma membrane, where the α-chain may be cleaved by a calcium- and thiol-dependent platelet protease (calpain) with the release of a large, hydrophilic, carbohydrate-rich fragment termed glycollacine. On unstimulated platelets, GP Ibα serves as the receptor for von Willebrand factor (vWF) exposed within the subendothelium of damaged vessels and plays a key role in platelet adhesion. GP Ibα also possesses one or more binding sites for α-thrombin. GP Ibβ has been identified as P24, one of the polypeptides that shows an increased phosphorylation in response to an elevated concentration of adenosine 3′,5′-cyclic monophosphate. Recently, Fox and Berndt reported that phosphorylation of GP Ibβ could play a special role in regulating actin polymerisation. GP Ib is present in the intact platelet membrane as a heterodimer complex with another integral membrane GP, GP IX (mol wt 22,000). GP Ib-IX complexes are absent or quantitatively decreased in platelets of patients with the Bernard-Soulier syndrome, an inherited platelet disorder. A peculiarity of this complex is that it is associated with actin-binding protein and is linked to the membrane cytoskeleton of resting platelets.

Nucleotide sequences corresponding to cDNA inserts that code for the GP Ibα and Ibβ chains have recently been established from messenger RNA isolated from cells of the human erythroleukemic cell line HEL. Computer analysis of the sequences showed that a similar 24-amino acid leucine-rich sequence was common to both subunits of GP Ib; it occurred once in the β chain and seven times in the α chain. The structure of the GP Ibα gene has been elucidated, and a single large coding exon and one intron have been identified. More recently, cDNA coding for GP IX was also analyzed, and again a leucine-rich sequence obtained. This led to the suggestion that GP Ibα, Ibβ, and IX are all members of the same GP family.

Monoclonal antibody (MoAb) binding studies indicate that approximately 20,000 to 25,000 GP Ib-IX molecules are exposed on the surface of normal human platelets. Immunofluorescence procedures and immunogold staining followed by electron microscopy showed the complexes to be distributed over the entire platelet surface. Yet, in 1986, George et al. reported greatly decreased binding of a GP Ib-specific MoAb to thrombin-stimulated platelets. Interestingly, this reduction only appeared to occur when thrombin was the agonist. Michelson and Barnard then reported similar results using several antibodies directed against different epitopes of GP Ib-IX complexes. Furthermore, when platelets were first treated with thrombin and their capacity to bind vWF in the presence of ristocetin evaluated, reduced levels of bound vWF correlated with a diminished capacity of the platelets to agglutinate when stirred. Such a result implies that incubation with thrombin may reduce the ability of platelets to support adhesion to the subendothelium.

We have used (1) immunofluorescence procedures and 1503
flow cytometry, and (2) immunogold staining combined with electron microscopy to investigate possible changes in the organization of GP Ib-IX complexes at the surface of thrombin-activated platelets. We have also examined the effects of platelet stimulation by other agonists such as adenosine diphosphate (ADP) and the ionophore A23187. Our results show that after thrombin stimulation, the bulk of the GP Ib-IX complexes are no longer accessible to MoAbs and are concentrated within surface-connected membrane systems of the platelet. However, accessibility is restored following treatment of paraformaldehyde-fixed platelets with the nonionic detergent Triton X-100. Interestingly, the apparent internal movement of GP Ib-IX complexes contrasts with the previously characterized thrombin-induced externalization of GP IIb-IIIa complexes\textsuperscript{15,23} and GMP-140.\textsuperscript{14,26}

**MATERIALS AND METHODS**

Apyrase, prostaglandin E\(_1\) (PGE\(_1\)), ionophore A23187, ADP, poly-L-lysine, biorunin, Triton X-100, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co (St Louis, MO); glutaraldehyde from Fluka AG (Buchs, Switzerland); Lowicryl K4M and epoxye (Taab 812 resin) from Taab Laboratories Equipment Ltd (Aldermaston, UK); and human \(\alpha\)-thrombin (fibrinindex) from Ortho Diagnostic Systems (Raritan, NJ). Affinity-purified immunoglobulin G (IgG) of goat antiserum to rabbit or mouse IgG coupled to 5 nm gold particles, and silicon enhancement reagents were from Janssen Life Sciences Products (Beerser, Belgium); fluorescein isothiocyanate (FITC)-conjugated affinity purified F(ab\(^\prime\))\(_2\), fragments of a sheep antibody to mouse IgG were from Silenus (Victoria, Australia). \(^{14}\)C\textsuperscript{5} hydroxystriptamine binoxalate (5-HT) (49 mCi/mmol) was purchased from New England Nuclear (Dreieich, FRG).

**Isolation of washed platelets.** Venous blood was taken with informed consent from healthy volunteers and anticoagulated with acid-citrate-dextrose (ACD), National Institutes of Health formula A (1 part anticoagulant to 6 parts blood). Platelet-rich plasma (PRP) was prepared by centrifugation at 120 g for 10 min at room temperature. PGE\(_1\) (100 nmol/mL), 25 \(\mu\)g/mL apyrase, and ACD (1 vol to 9 vol PRP) were added immediately. Platelets were sedimented by centrifugation at 1,200 g for 15 minutes and washed three times as previously described by us.\textsuperscript{27} The washed platelets were resuspended either in modified Tyrode buffer consisting of 137 mmol/L NaCl, 2 mmol/L KCl, 12 mmol/L NaHCO\(_3\), 0.3 mmol/L Na\(_2\)HPO\(_4\), 2 mmol/L CaCl\(_2\), 1 mmol/L MgCl\(_2\), 5.5 mmol/L glucose, 5 mmol/L HEPES, 0.35% bovine serum albumin (BSA), pH 7.4 (Tyrode-Ca\(^{2+}\)), or in the same buffer in which divalent cations were replaced by 5 mmol/L EDTA (Tyrode-EDTA). The platelets were incubated for 20 minutes at 37°C before use.

**Incubation of platelets with \(\alpha\)-thrombin or other agonists.** Human \(\alpha\)-thrombin (0.05 to 0.5 U/mL) was incubated with platelets at 10\(^{9}\) platelets/mL for times ranging from 1 to 10 minutes. Parallel studies were performed with platelets resuspended either in Tyrode-Ca\(^{2+}\) or Tyrode-EDTA. In some experiments, human \(\alpha\)-thrombin inactivated by D-phenylalanine-L-prolyl-L-arginine chloromethyl ketone (PPACK-thrombin; a gift from Dr J. Freyssinet, Strasbourg, France) was used at a concentration of 5 mmol/L (equivalent to \(\alpha\)-thrombin at 0.5 U/mL). Other agonists to be tested were ADP (10 \(\mu\)mol/L) and ionophore A23187 (2 \(\mu\)mol/L, a 1:5,000 dilution of a 10 mmol/L stock solution freshly prepared in DMSO). Controls were performed with buffer alone or with the appropriate concentration of DMSO. All incubations were at 37°C and were performed without stirring after an initial gentle mixing. Incubations were stopped by addition of the appropriate fixative (see below).

**Fixation procedures and Triton X-100 permeabilization.** When platelets were to be analyzed by flow cytometry, they were fixed by addition of an equal volume of 2\% wt/vol paraformaldehyde (PFA) followed by incubation for 30 minutes at room temperature. The Triton X-100 permeabilization step was adapted from the procedures previously described by Woods et al\textsuperscript{29} and Wencel-Drake et al,\textsuperscript{14} and consisted of the addition of 0.5% vol/vol Triton X-100 at the end of the PFA-fixation step. Incubation was then continued for another 30 minutes at room temperature. The cells were then washed and processed as described below. For electron microscopy, unstimulated platelets, or those treated with different concentrations of \(\alpha\)-thrombin or other agonists, were fixed in 1.25\% (vol/vol) glutaraldehyde (diluted in 0.1 mol/L phosphate buffer, pH 7.2) for 10 minutes at 37°C.

**MoAbs.** AP-1, which reacts with the \(\alpha\)-chain of GP I\(_b\),\textsuperscript{11} was kindly given by Dr T. Kunicki (Milwaukee, WI); FMC 25, directed against GP IX,\textsuperscript{28} was supplied by Dr M. Berndt (Sydney, Australia); SZ-1, which recognizes epitopes specific for the GP Ib-IX complex,\textsuperscript{29} was a gift of Dr C. Ruan (Suzhou, China). Some later experiments were performed with Bx-1, a murine MoAb prepared by us in collaboration with the Centre Régional de Transfusion Sanguine of Bordeaux and which binds to the GP I\_b subunit in immunobloting experiments (D. Pintigny, I. Chevalley, G. Vezon, and AT Nureden, unpublished data, February 1990). Other antibodies to be used included S12, specific for the \(\alpha\)-granule membrane glycoprotein, GMP-140,\textsuperscript{29} and kindly provided by Dr R. McEver (Oklahoma City, OK); and AP-2 another gift of Dr T. Kunicki and specific for GP IIb-IIIa complexes.\textsuperscript{29} Isolated IgG of a control murine MoAb prepared against smooth muscle cells and nonreactive with platelets was kindly provided by Dr D. Lamazière (Unité 8 INSERM, Pessac, France).

**Immunofluorescence and Flow Cytometry**

**Sample preparation.** PFA-fixed unstimulated platelets, or those incubated with thrombin, ADP, or ionophore A23187, were washed three times in phosphate-buffered saline, pH 7.2, containing 0.1% BSA (PBS-aleb). Triton X-100 permeabilized cells were treated similarly. They were then resuspended at 10\(^{9}\) platelets/mL and incubated for 1 hour with one of the previously mentioned murine MoAbs, which were used either as ascites fluid or as isolated IgG. Preliminary experiments established minimal saturating concentrations for each antibody. The dilutions were: AP-1 (ascites, 1:2,000); FMC 25 (ascites, 1:1,000); SZ-1 (IgG, 10 \(\mu\)g/mL); Bx-1 (IgG, 23 \(\mu\)g/mL); AP-2 (IgG, 9.2 \(\mu\)g/mL); and S12 (IgG, 2.5 \(\mu\)g/mL). IgG of the control antibody were added in equivalent amounts. After the incubation with the primary antibody, the samples were washed and incubated for 1 hour at room temperature with a 1:30 dilution (vol/vol) of FITC-conjugated F(ab\('\))\(_2\), fragments of sheep antibody to mouse IgG (dilution in PBS-aleb buffer). Platelets were washed three times and resuspended in PBS-aleb at a concentration of 50,000 platelets/\(\mu\)L. On occasion, labeled platelets were visually analyzed using a Nikon Microphot FX fluorescent microscope equipped with epifluorescence and a 50 W mercury lamp (Nippon Kogaku KK, Charenton Le Pont, France).

**Flow cytometry.** A Spectrum III flow cytometer (Ortho) equipped with a 70-\(\mu\)m aperture and a laser argon-ion lamp (emission 488 nm) was used. Samples were first analyzed by forward and wide angled light scatter and the gates set so as to include the majority of the platelets and exclude larger particles, which may be platelet aggregates or other contaminating blood cells. Green fluorescence was measured after passage through a 530-nm long pass
interference filter and the data expressed using a logarithmic scale. A fluorescence histogram was obtained for 10,000 cells.

**Immunocytochemical Studies and Electron Microscopy**

**Immunogold staining performed before embedding.** In the standard procedure, glutaraldehyde-fixed platelets were washed three times and resuspended at 2 × 10^9/mL in PBS-alb. Triton X-100 permeabilized PFA-fixed platelets were treated similarly and without additional glutaraldehyde fixation. All incubations with primary antibodies were performed at saturating concentrations as determined by flow cytometry. The platelets were further washed in 20 mmol/L Tris-HCl, 0.15 mol/L NaCl, pH 8.2, containing 0.1% (wt/vol) BSA. They were then incubated with goat antimouse IgG coupled to 5-nm gold particles for 4 hours at room temperature, followed by overnight incubation at 4°C as previously detailed by us.27

**Transmission electron microscopy.** Platelets were postfixed in 1% (wt/vol) osmic acid, dehydrated with graded alcohols and propylene oxide, and finally embedded in Epon.27 Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and observed in a Philips EM 201 electron microscope (Eindhoven, Holland). Immunostaining on ultrathin sections. In some experiments, immunogold staining was performed on ultrathin sections obtained after embedding. Unstimulated platelets, or platelets incubated with thrombin in Tyrode-Ca²⁺ or Tyrode-EDTA, were fixed in glutaraldehyde as described above. After three washings in PBS-alb they were dehydrated through a series of graded alcohols at -20°C. They were then embedded in Lowicryl K4M at -20°C. Ultrathin sections were mounted on collodium-coated grids. The sections were floated onto a drop of a solution containing the isolated IgG of a rabbit anticytolytic calcium antibody (15 µg/mL) (provided by Dr Clemetson). Characterization of the anticytolytic calcium antibody has been previously reported.28 After 1 hour at room temperature, the sections were rinsed by floating five times on drops of PBS-alb. They were then transferred to a solution containing a 1/70 dilution of goat antirabbit IgG conjugated to 5-nm gold particles. After further washing, the sections were stained with uranyl acetate and lead citrate and examined by transmission electron microscopy.

**Scanning electron microscopy (SEM).** Platelet preparation and immunogold staining were performed as for standard electron microscopy. After the binding of antimouse IgG conjugated to 5-nm gold particles, the platelets were incubated for 8 minutes with silver enhancement reagents as recommended in the manufacturer’s instructions. The platelets were then washed three times in PBS-alb and once with distilled water. A drop of the platelet suspension was allowed to settle onto a poly-L-lysine coated glass coverslip (12 mm in diameter). Coverslips were prepared in advance by incubation for 10 minutes with 1 mg/mL poly-L-lysine (mol wt 290,000). Platelets were dehydrated in graded alcohols and dried by the critical point method. The dried samples were sputter-coated with gold-palladium and observed at 20 kV in a Phillips 505 scanning electron microscope.

**Estimation of the release reaction.** In selected experiments, PRP was incubated with 2 µmol/L (¹⁴C) 5-HT for 30 minutes at 37°C. Stimulation of washed platelets with α-thrombin, ADP, or ionophore A23187 was performed as described above, with the exception that 5 U/mL hirudin was added at the end of the incubations with α-thrombin and before sedimentation of nonfixed samples at 12,000g for 2 minutes in an Eppendorf centrifuge. Measurements of (¹⁴C) 5-HT release into the supernatants were then effected, as previously described by us.27 The results are shown in Table 1. Phase contrast microscopy confirmed that platelet activation by both ADP and ionophore A23187 was accompanied by extensive pseudopod formation.

### Table 1. (¹⁴C) 5-HT Release From Activated Platelets

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Thrombin 0.5 U/mL</th>
<th>Ionophore A23187 2 µmol/L</th>
<th>ADP 10 µmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrode-Ca²⁺</td>
<td>90</td>
<td>40</td>
<td>5</td>
</tr>
<tr>
<td>Tyrode-EDTA</td>
<td>96</td>
<td>65</td>
<td>2</td>
</tr>
</tbody>
</table>

Platelets were preloaded with (¹⁴C) 5-HT in PRP and, after washing, resuspended at (10⁵/mL) in either Tyrode-EDTA or Tyrode-Ca²⁺. Samples were incubated under the standard experimental conditions used throughout this study, at 37°C for 10 minutes in the presence of α-thrombin, ionophore A23187, or ADP before the determination of (¹⁴C) 5-HT release as described in Materials and Methods. A decreased secretory response for ionophore A23187 in buffers containing divalent cations has previously been reported.29

**RESULTS**

**Binding of GP Ib-IX–Specific MoAbs to Activated Platelets as Evaluated by Flow Cytometry**

Figure 1 shows a progressive reduction in the binding of AP-1, an MoAb that recognizes the GP Ib subunit,11 to platelets challenged with α-thrombin for increasing times. The decrease was already evident after 1 minute, was more pronounced after 3 minutes, and was most striking after 10 minutes when the fluorescence profile approached baseline levels (Fig 1, A through D). It may be noticed that the histogram was broader after 10 minutes of stimulation, showing heterogeneity in the extent of the residual expression of GP Ib on the activated platelet surface. Identical results were obtained with Bx-1, a second antibody to GP Ib, and also with FMC 25 and SZ 1, which recognize epitopes on GP IX and GP Ib-IX, respectively5,28 (not illustrated). Thus, these experiments provide support for the original findings of George and Torres24 and Michelson and Barnard,23 and suggest that major changes were occurring in the expression of GP Ib-IX complexes on the surface of thrombin-activated platelets. The above changes were also dependent on the dose of thrombin used, being apparent when platelets were incubated with upwards of 0.05 U α-thrombin/mL (not illustrated), but being maximal with high doses of α-thrombin, such as those used in Fig 1. In contrast, incubation of platelets with equivalent amounts of active-site inhibited PPACK-thrombin did not modify AP-1 binding, showing that an active proteolytic site was required (Fig 1E).

The above results were obtained with platelets suspended in Tyrode-EDTA buffer. When similar experiments were repeated in Tyrode-Ca²⁺, the mean decrease in AP-1 binding was somewhat less marked (Fig 1F), although it should be noted that the extent of (¹⁴C) 5-HT secretion was the same (Table 1). At the same time, the fluorescence profile was broader, and on occasion a double peak was observed, indicating that there was a more heterogeneous response to the α-thrombin with respect to AP-1 binding. Examination of the samples by light microscopy confirmed that platelet aggregation was not a contributory cause of this heterogeneity. Furthermore, no differences were observed in AP-1 binding to unstimulated platelets incubated for 10 minutes at 37°C in Tyrode-EDTA or Tyrode-Ca²⁺ alone (not shown),
suggesting that EDTA itself was not contributing to the changes.

The effects of platelet stimulation with ADP and A23187 were also studied (Fig 1G and H). No detectable modifications of GP Ib expression were noted when platelets resuspended in either buffer were stimulated with ionophore A23187. The mean intensity of fluorescence was slightly lower for platelets incubated with 10 μmol/L ADP in a medium containing EDTA but not in Tyrode-Ca²⁺ (not shown).

One possible explanation for the above findings was that thrombin was inducing a patching or clustering of GP Ib-IX complexes, and that this resulted in a loss of antibody binding sites through steric interference. The distribution of GP Ib-IX complexes on unstimulated and thrombin-activated platelets was therefore examined by fluorescence microscopy. In agreement with the results of others,⁵,¹⁰ unstimulated platelets exhibited a uniform fluorescent "halo", suggestive of a uniform binding of AP-1 to the platelet surface. After thrombin stimulation, the staining was heterogeneous, and although zones of bright intensity were to be seen, the distinguishing feature was the presence of surface areas where little or no fluorescence could be located (results not illustrated).

Evaluation of the Distribution of GP Ib-IX Complexes on the Surface of Unstimulated and Thrombin-Activated Platelets by Immunogold Staining and Electron Microscopy

Initial studies were performed using AP-1 and transmission electron microscopy. Both here, and in the following immunocytochemical studies, results are shown for platelets in Tyrode-Ca²⁺, for incubation of platelets at 37°C in EDTA-containing buffer alone resulted in morphologic changes (see Discussion). Figure 2 shows that on unstimulated platelets, gold particles were regularly distributed over the external surface of the platelet. Staining was quite dense. However, after thrombin treatment, the staining became irregular and large areas devoid of gold particles were apparent. Figure 2b confirms that platelet activation was accompanied by both pseudopod formation and the centralization of granules. Similar results were obtained for FMC 25 and SZ 1 (not illustrated).

To have a more complete view of the platelet surface, these experiments were repeated using SEM. Here, silver enhancement was performed to allow a greater visualization of the small 5-nm gold particles. Figure 3 shows a regular distribution of AP-1, now revealed as large particles on the surface of unstimulated platelets. Staining decreased after thrombin stimulation, with the particles largely confined to central regions of the now spheroid central body of the platelet. Strikingly, there was little staining of the pseudopods. Control experiments performed in the absence of primary antibody showed the surface of both unstimulated and activated platelets to be smooth and no particles or "lumps" were to be seen.
Fig 2. Immunogold staining and transmission electron microscopy show AP-1 bound to the surface of unstimulated and thrombin-stimulated platelets. Washed platelets resuspended at 10^6/mL in Tyrode-Ca\(^2\)+ were incubated at 37°C for 10 minutes (a) in the absence or (b) after the addition of 0.5 U/mL α-thrombin. After fixation with glutaraldehyde, the washed cells were incubated with AP-1 and bound antibody located using goat antimouse IgG coupled to 5-nm gold particles as detailed in Materials and Methods. Note the decreased density of gold beads on the surface of the thrombin-stimulated cells.

Antibody Binding to Paraformaldehyde-Fixed Platelets Permeabilized With Triton X-100

Possible explanations for the above findings include (1) a loss of antigenicity, and therefore of MoAb-binding epitopes, and (2) an altered accessibility resulting from a possible internalization of the complexes. To investigate these possibilities, studies were performed using PFA-fixed platelets permeabilized after incubation with the nonionic detergent, Triton X-100. Antibody binding was first assessed by flow cytometry. Comparison of Fig 4A and B shows that perme-

Fig 3. Immunogold staining combined with SEM gives a global view of the binding of AP-1 to the surface of (a) unstimulated and (b) thrombin-stimulated platelets. Experimental conditions were as detailed in the legend to Fig 2, with the addition that visualization of gold particles was augmented by silver enhancement. Note the decreased staining of the thrombin-activated platelets and the relatively smooth appearance of the pseudopods.
Antibody. In Fig 4C and D the platelets had been stimulated with 0.5 U thrombin for 10 minutes. In accordance with earlier results, AP-1 binding to intact thrombin-stimulated platelets decreased dramatically. However, after permeabilization resulted in a small increase in AP-1 binding to unstimulated platelets. This suggested that an internal pool of GP Ib-IX complexes had been made available to the antibody. In Fig 4C and D the platelets had been stimulated with 0.5 U/mL thrombin for 10 minutes. In agreement with previous studies (see the introduction), the appearance of GMP-140 on the platelet surface (not illustrated) was the feature of unstimulated platelets. After thrombin treatment, a decreased immunogold staining at the platelet surface was accompanied by a marked increase in the labeling of what appeared to be vacuoles and/or surface-connected membrane systems.

Immunogold Staining Performed on Ultrathin Sections of Lowicryl K4M-Embedded Platelets

These studies permitted an evaluation of GP Ib-IX distribution within the platelet in the absence of the Triton X-100 permeabilization step. As preliminary studies showed a poor reactivity of MoAbs with Lowicryl K4M-embedded samples, a rabbit antiglycocalcin antibody was used instead. The results are shown in Fig 6. Unstimulated platelets (Fig 6a), were characterized by a heavy surface labeling and some staining within the open surface canalicular system. We obtained no evidence for the presence of a large cytoplasmic pool of GP Ib. Thrombin stimulation (Fig 6b) was associated with a diminished surface staining and a marked increase in the density of gold particles associated within the surface-connected membrane system. Observation of a large number of platelets showed that the apparent translocation or movement of GP Ib-IX complexes represented a general phenomenon. There was no obvious staining of a-granule membranes.

Distribution of GP IIb-IIIa Complexes and GMP-140 on Thrombin-Stimulated Platelets

The aim of our study was to investigate changes in the organization of GP Ib-IX complexes on thrombin-activated platelets. Nonetheless, it is relevant to define the organization of other major membrane GPs under our experimental conditions. Flow cytometric analysis using MoAbs to GP IIb-IIIa complexes and the a-granule membrane GP, GMP-140, confirmed that incubation of washed platelets with 0.5 U thrombin for 10 minutes at 37°C resulted in an extensive externalization of GP IIb-IIIa complexes together with the appearance of GMP-140 on the platelet surface (not illustrated). Such findings are in agreement with those previously reported by others (see Discussion). Interestingly, the immunocytochemical studies shown in Fig 7 confirm that GP IIb-IIIa complexes were evenly distributed over the surface membrane, including the pseudopods. GMP-140 also was evenly distributed, the lower intensity of staining probably being due to the lower concentration of this GP in platelets.

DISCUSSION

In agreement with previous studies (see the introduction), we observed that platelet stimulation by a-thrombin was followed by reductions in the binding of MoAbs to determinants carried by the GP Ibα chain, GP IX, and the complex itself. Proteolytically active thrombin was required for catalytic-site inactive PPACK-thrombin, which is known to bind to high-, moderate-, and low-affinity thrombin-binding sites on platelets (including those on GP Ibα).3,32 had no effect. Platelet stimulation with ionophore A23187 or with ADP was not accompanied by major modifications in the binding

**Fig 4. Binding of AP-1 to unstimulated and thrombin-stimulated platelets before and after Triton X-100 permeabilization.** Washed platelets (10^6/mL) in Tyrode-EDTA were incubated at 37°C for 10 minutes with (C and D) or without (A and B) 0.5 U/mL a-thrombin. The reaction was stopped by the addition of 1% PFA. After 30 minutes, 0.5% (vol/vol) Triton X-100 was added to half of the samples (B and D). After another 30 minutes, the platelets were washed and antibody binding performed as before. Samples were analyzed by flow cytometry as described in the legend to Fig 1. The broken lines represent samples incubated in the presence of 5 μg/mL control MoAb. The mean fluorescent intensities confirm that Triton X-100 permeabilization almost completely restored the binding of AP-1 to the thrombin-treated platelets.
Fig 5. Immunogold staining and electron microscopy of Triton X-100 permeabilized platelets. Samples of (a) unstimulated and (b) thrombin-stimulated platelets were fixed with PFA and treated with Triton X-100 as described in the legend to Fig 5. After washing, the platelets were incubated with AP-1 and then with goat antimouse IgG coupled to 5-nm gold particles. Samples were dehydrated, embedded, and thin sections examined by transmission electron microscopy. AP-1 was mostly surface-bound on unstimulated platelets (arrowheads), while staining was mostly within the platelet after thrombin stimulation (arrows).

Fig 6. Immunogold staining of thin sections of unstimulated and thrombin-stimulated platelets. Washed platelets resuspended (10^5/mL) in Tyrode-Ca^{2+} were incubated at 37°C for 10 minutes without (a) or with 0.5 U α-thrombin (b) before fixation with glutaraldehyde and embedding in the resin Lowicryl K4M. Thin sections were then incubated with the isolated IgG of a rabbit antiglycopcalicin antibody followed by goat antirabbit IgG bound to 5-nm gold particles, as detailed in Materials and Methods. Bound antibody was then visualized by transmission electron microscopy. Note the high density of immunogold staining within the surface-connected membrane systems of thrombin-activated platelets.
Fig 7. Immunogold staining showing the organization of GP Ib-IIIa complexes and GMP-140 on the surface of thrombin-activated platelets. Washed platelets resuspended at 10⁶/mL in Tyrode-Ca²⁺ were incubated at 37°C for 10 minutes in the presence of 0.5 U/mL α-thrombin. After fixation with glutaraldehyde, the washed cells were incubated with (a) AP-2 (anti-GP Ib-IIIa) or (b) S12 (anti–GMP-140) under saturating conditions as determined by flow cytometry. Bound antibody was detected using goat antimouse IgG coupled to 6-nm gold particles as detailed in Materials and Methods. Note the dense and even staining with AP-2, and the equally homogeneous distribution of S12.

of GP Ib-IX-dependent MoAbs but was followed by pseudopod formation and, in the case of ionophore A23187, by secretion. This suggests that platelet shape change per se is not responsible for the observed changes and that secretion and modifications of GP Ib-IX distribution are not interdependent phenomena.

In our first experiments, MoAb binding was assessed using platelets stimulated in EDTA-containing buffer, conditions previously used by others.²²,²³ However, electron microscopy showed that incubation of platelets at 37°C with EDTA alone resulted in considerable morphologic changes. These included pseudopod formation, the presence of dilated channels within the open surface connecting system, and an increased number of vacuoles. Such effects of incubating platelets with EDTA at 37°C are well known.²⁴ Therefore, experiments were subsequently performed in parallel in Tyrode-EDTA and Tyrode-Ca²⁺. Results showed that EDTA-treatment on its own was not inducing modifications in MoAb binding to GP Ib-IX complexes. However, we cannot rule out that the accentuated loss of AP-1 binding to platelets stimulated in Tyrode-EDTA as compared with Tyrode-Ca²⁺ (see Fig 1) is related to the morphologic changes induced by the EDTA.

When platelets are stimulated with physiologic agonists, a consequence is the induction of conformation changes in GP Ib-IIIa complexes and the expression of fibrinogen-binding determinants.¹³,³⁴ We considered that thrombin may also induce conformational changes in GP Ib-IX complexes and that these lead to an altered antigenicity and a loss of antibody-binding sites. However, changes in conformation should be accompanied by the formation of new antigens, and MoAbs to activation-dependent antigens on GP Ib-IX have not yet been reported in the literature. The virtually complete restoration of antibody binding to thrombin-stimulated platelets after Triton X-100 permeabilization makes it more likely that an altered accessibility accounted for the reducing binding of the MoAbs used in the present study.

Immunofluorescence and permeabilization experiments with antibodies to GP Ib were first performed by Wencel-Drake et al.,¹⁵ who noted that permeabilized, PFA-fixed platelets exhibited an internal staining in addition to the surface halo. The “internal” immunofluorescence was patchy and contrasted with the strong and more homogeneous staining given by antibodies to GP Ib-IIIa complexes. Immunocytochemical studies on frozen thin sections localized the internal pool of GP Ib to internal membrane systems. Thrombin-stimulated platelets were not examined. It is likely that the internal pool of GP Ib detected by these investigators is equivalent to that observed in ultrathin sections of Lowicryl-embedded unstimulated platelets in the present study (Fig 6a). In agreement with Wencel-Drake et al.,¹⁶ both vacuole-like structures and elements of the surface canicular system were labeled. It should be noted that neither we nor Wencel-Drake et al.¹⁵ obtained evidence for the presence of GP Ib-IX complexes in granule membranes.

Woods et al.²⁵ also used nonionic detergent to permeabilize PFA-fixed platelets, and showed that incubation with 0.1% Triton X-100 for 3 minutes was sufficient to allow access of MoAbs to internal pools of GP Ib-IIIa complexes. Our procedure was similar, although we added Triton X-100 to platelets in PFA-containing buffer to ensure a complete fixation of internal antigens exposed to the nonionic detergent. Electron microscopy confirmed that platelets treated in
this way retained a recognizable morphology. Immunogold staining of permeabilized thrombin-stimulated platelets strongly suggested that a majority of the GP Ib-IX complexes were now located in membrane systems that penetrated within the interior of the platelet (Fig 5). This was confirmed by immunogold staining on ultrathin sections of Lowicryl K4M-embedded thrombin-stimulated platelets, where the bulk of the complexes were located in vacuolar-like structures or channels of surface-connected membrane systems.

Binding studies with radiolabeled MoAbs have shown that unstimulated human platelets express on the order of 20,000 to 25,000 copies of GP Ib-IX on their surface.7,17 However, quantitative densitometry after GP staining of platelet proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis led Michelson et al. to conclude that platelets in fact contained upward of 150,000 molecules of GP Ib, with the bulk in an internal pool. We found little evidence for such a large pool either when (1) analyzing the binding of MoAbs to Triton X-100 permeabilized platelets by flow cytometry, or (2) visualizing the binding of a polyclonal antiglycocalicin antibody on thin sections of Lowicryl-embedded platelets. Although acknowledging that our techniques are not quantitative, our results do agree with a previous finding that a large proportion of GP Ib-IX complexes are accessible to neuraminidase when the enzyme is added to platelet suspensions.36

A striking surface change after platelet stimulation with α-thrombin (but not with ADP or ionophore A23187) was the appearance of areas with little or no staining with antibodies to GP Ib-IX. That which did occur was mainly around the central body of the platelet. This was best shown using SEM, a procedure initially applied to the study of thrombin-stimulated platelets by Suzuki et al.21 Our results provided little evidence for the occurrence of a large-scale patching of GP Ib-IX complexes on surface membranes. Such a mechanism has been previously proposed for thrombin-activated platelets by Polley et al. However, these investigators isolated membranes from the platelets before performing immunocytochemical staining, and thus it is possible that changes were induced during this additional experimentation. It is also interesting to note that clustering of GP Ib-IX receptors was not observed when unfixed platelets were incubated at 37°C with an antibody to GP Ib, although the phenomenon was observed after antibody binding to GP Ib-IIIa complexes when patching was followed by endocytosis.19

Clustering of GP Ib-IIIa complexes is a feature on platelets stimulated with ADP in the presence of fibrinogen.28 Here, GP movement appears to be related to receptor occupancy on activated platelets. Recently, Loftus and Albrecht29 reported that occupied GP Ib-IIIa complexes moved from the periphery to the center of platelets spread on formvar grids. Receptor mobility was assessed after the binding of fibrinogen-coated gold beads. Using a similar approach, Escolar et al.30 and Leistikow et al.31 made comparable observations but arrived at different conclusions. Receptor mobility was confirmed, but the fate of many of the fibrinogen-GP Ib-IIIa complexes was shown to be the platelet open surface canalicular system. Experiments were also performed on platelets incubated with thrombin in suspension, when some 5 minutes after platelet activation the bulk of fibrinogen-gold particles were within the open canalicular system.40 One possibility is that we are observing a similar phenomenon. However, there is no evidence thus far that ligand binding to GP Ib-IX complexes is a prerequisite for their movement. An involvement of GP Ib-IIIa complexes in this process can be excluded, for platelets from a patient with Glanzmann’s thrombasthenia and lacking GP Ib-IIIa behaved exactly as normal platelets under our experimental conditions (P.H. and A.T.N., unpublished observation, February 1990). As discussed above, addition of PPACK-thrombin alone is not followed by a decreased binding of MoAbs to GP Ib-IX. However, this does not rule out a dual requirement of both binding and proteolysis. A known substrate for α-thrombin on the platelet surface is GP V.45 The fact that platelets of patients with the inherited disorder, the Bernard-Soulier syndrome, have decreased amounts of GP Ib-IX complexes and of GP V suggests a possible relationship in the organization of these GPs at the platelet surface. Nonetheless, GP V hydrolysis is not essential for thrombin-induced platelet activation.46 Further studies will be required to establish whether GP V hydrolysis is involved in the changes in GP Ib-IX distribution that we have described.

The question of how GP Ib-IX complexes get into the channels remains to be answered. One possibility is by a translocation mechanism. It has been known for some time that the complexes are linked into a distinct membrane skeleton through actin-binding protein.11,12,44-46 Here, the actin-binding protein is linked to short, amorphous actin filaments that require high g forces for their sedimentation. Platelet stimulation by thrombin is accompanied by actin polymerization in the platelet cytoplasm.12 Actin-binding protein provides the link between GP Ib-IX complexes and this cytoskeletal network. It is tempting to speculate that actin filaments are involved in GP Ib-IX movement, a speculation reinforced by the previous finding of George and Torres that cytochalasin E prevented the thrombin-induced decrease in the binding of MoAbs to GP Ib. Incubation of platelets with the ionophore A23187 leads to activation of a neutral Ca2+-activated protease and hydrolysis of actin-binding protein.43 The fact that ionophore A23187 did not induce GP Ib-IX translocation in our study suggests that release of GP Ib-IX complexes from actin-binding protein through calpain activation is not part of the mechanism. Such hydrolysis has been reported with thrombin, but only under conditions where platelet aggregation occurred.44

Another possibility to consider is that the association of the GP Ib-IX complexes with the membrane skeleton of unstimulated platelets anchors the complexes so that they do not redistribute within the new membrane systems that form during thrombin-induced platelet activation. This could explain why pseudopodia, in particular, showed little immunogold staining for GP Ib-IX on thrombin-activated platelets. Under the same experimental conditions, we observed the expression of GMP-140 on the platelet surface and a net increase in the number of GP Ib-IIIa complexes accessible
to the MoAb AP-2. The expression of GMP-140 on the platelet surface is a well-characterized marker of platelet activation.\textsuperscript{22,23,47} Stenberg et al\textsuperscript{26} have previously described how, during secretion, GMP-140 rapidly diffuses into the plasma membrane after the fusion of α-granule membranes with those of the surface-connected canalicular system. These investigators showed how within a matter of minutes of thrombin stimulation, GMP-140 was detected by immuno-gold staining throughout the plasma membrane. It should be emphasized that both in the above study and ours (Fig 7) there was no evidence for GMP-140 clustering. Direct measurement of the binding of radiolabeled MoAbs has shown increases of between 40% and 100% in the number of GP IIb-IIIa complexes exposed on the surface of thrombin-activated platelets.\textsuperscript{22,23,25,26,48} These additional GP IIb-IIIa complexes come from internal pools and, again, appear to be evenly distributed on the surface of thrombin-activated platelets. Thus, there is strong evidence for a movement of membrane GPs out onto the surface during thrombin-induced platelet activation. Perhaps this redistribution is a manifestation of phospholipid flow during secretion and pseudopod formation. What is so intriguing is that the studies of Escolar et al\textsuperscript{49} and Leistikow et al\textsuperscript{41} (discussed above), and now our work on GP Ib-IX complexes, show that movement of subpopulations of membrane GPs may in fact enter channels of the surface-connected canalicular system, presumably at the same time as secretion is occurring.

ACKNOWLEDGMENT

We thank Professor Michel Boisseau for providing laboratory space, and acknowledge the additional technical assistance provided within our laboratory by Annie Pruvost and Annie Paponneau. We especially thank Dr Jackie Larrue (Unité 8 INSERM), Dr J.M. Aran (Unité 229 INSERM), and Dr G. Vezon (Centre Régional de Transfusion Sanguine, Bordeaux, France) for allowing us to perform electron microscopy and flow cytometry in their departments, while A. Guilhaume and Dr A.M. Ferrer provided technical expertise in these studies.

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