PLATELETS ACTIVATED by adenosine diphosphate (ADP) and other physiologic stimuli express specific and saturable receptors for fibronogen.1 Binding of fibronogen requires divalent cations and is correlated with the onset of platelet aggregation.2-4 Growing evidence suggests that divalent cation-dependent complexes of two membrane glycoproteins (GP), Iib and IIIa, play an essential role in fibronogen receptor expression and therefore in the aggregation process.5-7 Part of this evidence has accumulated from studies with monoclonal antibodies such as AP-28 and Tab,9 which react with complex-dependent epitopes on GP IIb-IIIa and inhibit both fibronogen binding to stimulated platelets and platelet aggregation. Other such antibodies have recently been catalogued.10

That GP Iib and GP IIIa may form complexes in the presence of $\text{Ca}^{2+}$ was first shown in studies in which Triton X-100 extracts of platelets or isolated membranes were analyzed by crossed immunoelectrophoresis (CIE). A major immunoprecipitate was observed that, after divalent cation chelation with EDTA, was replaced by two new precipitates, one containing GP Iib and the other, GP IIIa.5-12 Other studies on the extracted glycoproteins showed the complex to be a heterodimer13,14 and that the complexes can bind $4^{5}\text{Ca}^{2+}$,15-17 possibly through GP IIb.16 Indeed, they may represent the major calcium-binding site on the platelet surface.17,18 and participate in the transport of this cation across the plasma membrane.19 Binding studies performed with the monoclonal antibodies AP-29 and T109 have confirmed the presence of the heterodimer in the membrane of inactivated platelets.

The results of studies by Zucker and Gran19,20 showed that platelets incubated at 37 °C for short periods caused an irreversible loss of their aggregation response to adenosine diphosphate and markedly diminished their capacity to bind fibronogen. AP-2 is a monoclonal antibody that reacts with a determinant specific to the glycoprotein (GP) Iib–IIla complex. We now report that in a direct binding assay, the number of sites for AP-2 on platelets incubated with EDTA at 37 °C fell to ~30% of those present on control platelets. This effect of EDTA was not observed at room temperature. Analysis of the treated platelets by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed normal amounts of GP Iib and GP IIIa. However, studies using crossed immunoelectrophoresis with $^{125}\text{I}-\text{AP-2}$, $^{125}\text{I}-\text{Tab}$ (anti-GP Iib), or $^{125}\text{I}-\text{AP-3}$ (anti-GP IIIa) in intermediate gels showed that at 37 °C, EDTA was inducing an irreversible change in GP Iib–IIla complexes. A reduction in size and probable dissociation of the GP Iib–IIIa precipitate was accompanied by the appearance of precipitates having the characteristics of those given by free GP Iib and free GP IIIa and the location of a new major cathodal precipitate, which bound Tab and AP-3 but not AP-2. Membrane modifications associated with the loss of antigenic determinants on GP Iib–IIIa may explain EDTA-induced loss of platelet aggregability at 37 °C.

MATERIALS AND METHODS
The following materials were obtained commercially: prostaglandin E1 (PGE1), grade 1 arbovine, bovine serum albumin (BSA, fraction V), N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), ADP, phenylmethylsulfonyl fluoride (PMSF), and synthetic hemisulfate leupeptin were from Sigma Chemical Co (St Louis); ethylenediamine tetraacetic acid disodium salt (EDTA) from Prolabo (Paris); purified human fibronogen from Kabi Diagnostics (Stockholm); $^{125}\text{I}-\text{fibronogen}$ was obtained from the Service des Radioisotopes of the Centre National de Transfusion Sanguine (Paris); Na$^{109}$i from the Centre de l’Energie Atomique (Orsay, France); agarose HSA type from Litex (Glostrup, Denmark); Triton X-100 from BDH Chemicals Ltd (Poole, England); glycin, tris (hydroxymethyl)aminomethane (Tris), sodium dodecyl sulfate (SDS), acrylamide, bisacrylamide, ammonium persulfate, 2-mercaptoethanol, TEMED, and Coomassie blue R-250 (CB-R) from Bio-Rad (Richmond, Calif.). All other chemicals were of reagent grade. The monoclonal antibodies Tab22 and AP-319 were kindly provided as purified IgG by Dr R. McEver (The University of Texas Health Science Center, San Antonio, Tex) and Dr P.J. Newman (The Blood Center of Southeastern Wisconsin, Milwaukee), respectively.

Platelet isolation. Blood (6 vol) was obtained from adult human volunteers and anticoagulated with acid-citrate-dextrose anticoagulant (ACD; NIH formula A) (1 vol).24 Platelet-rich plasma prepared by centrifugation at 20 °C for 15 minutes at 120 g was acidified to pH 6.5 by addition of a one tenth vol ACD-A. PGE1 (100 nmol/L) and aspartate (25 μg/mL) were also added. Washed platelets were prepared by the method of Patscheke25 as modified by Legrand et al.26 In the standard procedure, the platelets were washed in 36 mmol/L citric acid, 5 mmol/L glucose, 5 mmol/L KCl, 2 mmol/L CaCl2, 1 mmol/L MgCl2, 103 mmol/L NaCl, 3.5 mg/mL BSA, 100 nmol/L PGE1, 25 μg/mL aspartate, pH 6.5 (washing buffer). Unless otherwise stated, the platelets were finally resuspended in a modified fibrinogen in the presence of ADP and exhibited a reduced capacity to bind $^{45}\text{Ca}^{2+}$.4,17 Recent advances in our knowledge of fibronogen receptor expression suggest that EDTA may be acting on the GP Iib–IIIa heterodimers. To investigate this possibility, we have examined the effect of incubating platelets with EDTA at 37 °C, studying in parallel (a) platelet aggregation and fibronogen binding, (b) AP-2 binding, and (c) GP Iib–IIIa stability. Results show that at 37 °C and alkaline pH, EDTA directly influences the structure of the Iib–IIIa heterodimers in the platelet membrane.

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Tyrode’s solution consisting of 137 mmol/L NaCl, 2 mmol/L KCl, 12 mmol/L NaHCO₃, 0.3 mmol/L NaH₂PO₄, 2 mmol/L CaCl₂, 1 mmol/L MgCl₂, 5.5 mmol/L glucose, 5 mmol/L HEPES, pH 7.4 (HBMT).

Incubation with EDTA. Twice-washed platelets were suspended at 10⁴/mL in HBMT or the same buffer lacking calcium and magnesium but containing 2 mmol/L EDTA (HBMT-EDTA). In some experiments, the concentration of EDTA was varied as detailed in the text. Both buffers also contained 100 nmol/L PGE₁ and 25 µg/mL apyrase. The platelet suspensions were incubated at 20 °C or 37 °C for periods of up to 15 minutes in a thermostatically controlled water bath. Control incubations were performed in HBMT alone. After the incubation, the platelet suspensions were diluted with 5 vol washing buffer and the platelets were resuspended at room temperature in HBMT containing 3.5 mg/mL BSA but lacking apyrase and PGE₁. To standardize the experimental conditions, all subsequent studies were started 30 minutes after the final platelet resuspension. Such platelets are subsequently referred to as “pretreated” platelets.

Platelet aggregation. Normal or pretreated washed platelets in HBMT were adjusted to 3 x 10⁹ platelets per milliliter. Platelet aggregation was studied photometrically using a Labintec aggregometer (Central Labo, Paris). The platelet suspension (0.36 mL) was stirred (1,100 rpm) at 37 °C in the presence of 0.2 mg/mL fibrinogen and 5 or 10 µmol/L ADP. Changes in light transmission were continuously recorded and compared with the maximal change observed with HBMT buffer alone.

Fibrinogen binding assay. The binding of purified human ¹²⁵I-fibrinogen to ADP-stimulated control and EDTA-pretreated platelets was measured according to the procedure of Legrand et al. The characteristics of the ¹²⁵I-fibrinogen preparation have already been reported. Briefly, the assay was performed as follows: 0.4-mL vol of a washed platelet suspension in HBMT (5 to 8 x 10⁹ platelets per milliliter) were mixed with 80 µL of HBMT and 40 µL of a 12 µmol/L solution of ¹²⁵I-fibrinogen in HBMT to give a final concentration of 1 µmol/L, at which steady-state binding of fibrinogen to its platelet receptor reaches saturation under our experimental conditions. Binding was initiated three minutes later by adding 30 µL of a 200-µmol/L solution of ADP dissolved in HBMT to give a final concentration of 10 µmol/L, and continued for 30 minutes at room temperature, before trichloroacetic acid 0.15 mL were layered onto 0.5 mL of an inert oil mixture (d = 1.014). The samples were centrifuged for three minutes at 12,000 g in an Eppendorf model 5414 centrifuge (Brinkman Instruments, Inc, Westbury, NY). Supernatants and pellets were fractionated and their associated ¹²⁵I-radioactivity was measured in a Gamma 7000 counter (Beckman Instruments, Inc, Fullerton, Calif). Nonspecific binding of ¹²⁵I-fibrinogen was defined as that observed in the presence of a 20-fold excess of unlabeled fibrinogen initially added in a 80-µL vol instead of the buffer alone (see above). It represented approximately 35% of the total binding, from which it was subtracted to give the specific binding.

AP-2 binding assay. AP-2 was isolated from ascites fluid and the monoclonal IgG labeled with ¹²⁵I using the chloramine T method. The assay for the direct binding of ¹²⁵I-labeled antibody to washed platelets was adapted from that previously used by us. Briefly, normal or pretreated washed platelet suspensions were first adjusted to 10⁷ platelets per milliliter with HBMT. In the standard assay, aliquots (0.3 mL) were then mixed with near-saturating concentrations of ¹²⁵I-AP-2 (≥ 3 µg/mL). For Scatchard analysis (see ref. 8), aliquots were mixed with increasing amounts of ¹²⁵I-AP-2 (0.5 to 5.0 µg/mL) as detailed in the text. After a 30-minute incubation at room temperature, 0.14-mL samples were layered in duplicate over 0.5 mL 20% (vol/vol) sucrose in HBMT. Platelets were pelleted by centrifugation at 12,000 g for two minutes in an Eppendorf centrifuge. Each supernatant was aspirated and the radioactivity in both supernatants and pellets measured in a Beckmann 7000 gamma counter. Nonspecific binding was measured in the presence of a 50-fold excess of unlabeled AP-2. As this was found to be negligible (≤3% of total binding), the data is presented as total binding.

Crossed immunoelectrophoresis. Pretreated platelets resuspended in HBMT were diluted with 5 vol washing buffer and the platelets sedimented. The pellets were resuspended in 0.1 mol/L glycine, 0.038 mol/L Tris, pH 8.7 (Tris-glycine) prechilled to 4 °C. Platelet solubilization was performed at 4 °C by incubation for 30 minutes with 1% (vol/vol) Triton X-100. In selected experiments, the solubilization step was performed in the additional presence of 500 µg/mL leupeptin and 1 mmol/L PMSF. The Triton X-100 insoluble material was removed by ultracentrifugation and the supernatants stored at −80 °C until use. CIE was performed according to the procedures detailed by Kunicki et al and Pidard et al. Briefly, first-dimension electrophoresis was performed at 10 V/cm for one hour in 1% (wt/vol) agarose containing 0.5% (vol/vol) Triton X-100 in Tris-glycine. Second-dimension electrophoresis was performed at 2 V/cm for 18 hours into a bifasic gel system consisting of (a) an intermediate gel containing 1% agarose, 0.5% Triton X-100 and, on occasion, trace amounts of ¹²⁵I-AP-2, ¹²⁵I-Tab or ¹²⁵I-AP-3 and (b) an upper gel containing precipitating concentrations of a polyspecific rabbit antihuman platelet antibody preparation. Further experimental details are given in the figure legends. Immunoprecipitates were located by Coomassie blue R250 (CB-R) staining, and those containing ¹²⁵I, by autoradiography.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Pretreated platelets in HBMT were pelleted and resuspended at 2 x 10⁹/mL in 10 mmol/L Tris-HCl, 150 mmol/L NaCl, 3 mmol/L EDTA, pH 7.0, and solubilized by heating at 100 °C for five minutes after the addition of a one fifth vol of 12% (wt/vol) SDS containing 30 mmol/L N-ethylmaleimide. Aliquots of Triton X-100-soluble platelet extracts were diluted with an equal vol of 10 mmol/L Tris-HCl, 150 mmol/L NaCl, 3 mmol/L EDTA, pH 7.0, before being treated similarly. Triton X-100-insoluble material was resuspended in the same buffer before SDS solubilization. Samples were prepared with (reduced) or without (nonreduced) disulfide reduction, and electrophoresis was performed using 7% or 6% acrylamide rod gels, or 7% to 12% gradient acrylamide slab gels, according to the procedures detailed by Norden et al. Glycoproteins were located by periodic-acid Schiff (PAS) staining, and proteins, by CB-R staining. Densitometric scanning of PAS-stained rod gels was performed using a Vernon Integrating Photometer (Vernon Integrating Photometers Ltd, Paris). In some experiments, membrane glycoproteins were labeled with ¹²⁵I using the lactoperoxidase-catalyzed procedure detailed by Norden et al before the platelets were exposed to EDTA. ¹²⁵I-labeled polypeptides were revealed by autoradiography of dried polyacrylamide slab gels as previously described by us.

RESULTS

Experimental conditions. The aim of this study was to examine the effect of controlled incubation with EDTA on the platelet aggregation response and on the GP Ibα-IIIa complex. All platelet washing steps were therefore performed in the presence of divalent cations but at pH 6.5, to prevent the formation of platelet aggregates during centrifugation. Incubations at 37 °C were performed at pH 7.4 in the presence of (a) apyrase to prevent platelets becoming refractory to released ADP and (b) PGE₁, to help maintain platelet integrity and inhibit granule release.
**Platelet aggregation.** Washed human platelets that had been incubated for 15 minutes at 37 °C with 2 mmol/L EDTA failed to aggregate when resuspended in HBMT containing divalent cations and stimulated by ADP in the presence of added fibrinogen. A typical experiment is shown in Fig 1. Platelets that were incubated at 37 °C under control conditions in the presence of 2 mmol/L CaCl2 and 1 mmol/L MgCl2 were normally aggregated. Incubation of the platelets at 37 °C with equimolar amounts of Ca2+ and EDTA (CaEDTA-treated platelets) did not affect platelet aggregability. This suggested that the primary effect of EDTA was to chelate membrane-bound calcium. The phenomenon was time and temperature dependent. Incubation of platelets with 2 mmol/L EDTA for 15 minutes at 20 °C did not result in a loss of aggregability. Incubation of platelets with 2 mmol/L EDTA for shorter periods at 37 °C resulted in partial inhibition of the aggregation response (not shown). We also consistently observed a reduced “swirling” phenomenon in the final suspensions of unstimulated EDTA-treated platelets when compared with the control preparations. This may indicate a loss of their discoid shape during exposure to EDTA at 37 °C, a phenomenon previously described for EDTA-treated platelets by White.30 In agreement with this conclusion, aggregation studies performed at lower concentrations of EDTA-treated platelets (1 × 10⁹/mL) did not show the typical decrease in light transmission that normally follows the addition of ADP to stirred platelet suspensions and that reflects the change from disk-shaped to spherical platelets with pseudopods (not shown).

**Fibrinogen binding.** Control and EDTA-treated platelets were next compared as to their ability to bind ¹²⁵I-fibrinogen when activated with 10 μmol/L ADP in HBMT containing divalent cations. In experiments performed using platelets from four donors, the specific ADP-induced binding of fibrinogen to platelets preincubated at 37 °C under control conditions was 372 ± 130 ng fibrinogen per 10⁹ platelets. In contrast, platelets of the same donors preincubated with 2 mmol/L EDTA for 15 minutes at 37 °C bound only 162 ± 90 ng fibrinogen per 10⁹ platelets (mean ± SD). Thus fibrinogen binding was reduced to approximately 43% of its normal value.

**Monoclonal antibody binding.** Platelets prepared for aggregation studies were also tested for their capacity to bind the monoclonal antibody AP-2. Using near-saturating concentrations of ¹²⁵I-AP-2, we found that platelets preincubated at 37 °C for 15 minutes with 2 mmol/L EDTA bound only 29.7% of the amount of antibody that bound to platelets incubated at 37 °C under control conditions (Table 1). No such reduction was observed when platelets were treated with CaEDTA at 37 °C or with EDTA at 20 °C. Increasing the concentration of EDTA (10 mmol/L) or the duration of the incubation at 37 °C caused only a small additional fall in the number of AP-2 binding sites. For example, approximately 20% of the binding sites remained on platelets pretreated with 2 mmol/L EDTA for 45 minutes at 37 °C (data not shown).

Scatchard analysis after the incubation of washed platelets with increasing concentrations of ¹²⁵I-AP-2 (see Materials and Methods) showed that the platelets bound 39,388 ± 5,832 IgG molecules per platelet with an affinity Kd = 0.81 ± 0.21 mmol/L (mean ± SD, n = 9). These values were similar to those previously reported by us.4 Preincubation of the washed platelets at 37 °C for 15 minutes under the control conditions did not significantly change the platelet capacity to bind AP-2 (Table 1). In a single experiment, platelets that had been treated with 2 mmol/L EDTA for 15 minutes at 37 °C were also incubated with varying concentrations of ¹²⁵I-AP-2. Here, Scatchard analysis showed that the number of AP-2 molecules that had bound had fallen to 17,950 per platelet with an affinity Kd = 1.02 mmol/L. The latter value was unchanged from that obtained for untreated platelets, thus EDTA was primarily affecting the number of AP-2 receptors.

**Crossed immunoelectrophoresis.** Platelets prepared for aggregation studies were also solubilized with 1% Triton X-100 and the soluble extracts analyzed by CIE. Initial experiments established that the pattern of immunoprecipitates obtained for platelets incubated at 37 °C under the control conditions was identical to that obtained for washed platelets. These experiments established that the pattern of immunoprecipitates obtained for platelets incubated at 37 °C under the control conditions was identical to that obtained for washed platelets.

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### Table 1. ¹²⁵I-AP-2 Binding to Pretreated Washed Platelets

<table>
<thead>
<tr>
<th>Conditions of Incubation (15 min)</th>
<th>Amount of bound ¹²⁵I-AP-2 (ng IgG/10⁸ platelets)</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mmol/L Ca⁺², 1 mmol/L Mg²⁺, 20 °C</td>
<td>1,044 ± 126 (n = 3)</td>
<td>100.0</td>
</tr>
<tr>
<td>2 mmol/L Ca⁺², 1 mmol/L Mg²⁺, 37 °C</td>
<td>1,176 (n = 1)</td>
<td>112.6</td>
</tr>
<tr>
<td>2 mmol/L Ca⁺², 2 mmol/L Mg²⁺, EDTA: 37 °C</td>
<td>1,130 (n = 2)</td>
<td>108.2</td>
</tr>
<tr>
<td>2 mmol/L EDTA, 20 °C</td>
<td>1,082 (n = 1)</td>
<td>103.6</td>
</tr>
<tr>
<td>0.5 mmol/L EDTA, 37 °C</td>
<td>330 (n = 2)</td>
<td>31.6</td>
</tr>
<tr>
<td>2.0 mmol/L EDTA, 37 °C</td>
<td>310 ± 97 (n = 4)</td>
<td>29.7</td>
</tr>
<tr>
<td>10.0 mmol/L EDTA, 37 °C</td>
<td>286 (n = 1)</td>
<td>27.4</td>
</tr>
</tbody>
</table>

The treated platelets were sedimented and resuspended at 1 × 10⁸ platelets per milliliter in HBMT for the ¹²⁵I-AP-2 binding assay performed using ≥3 μg/mL AP-2 (see Materials and Methods). Results are expressed as the mean ± SD (where calculated) and as the percentage of the control incubation at 20 °C arbitrarily designated as 100%.

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platelets prepared without pretreatment. In contrast, as shown in Fig 2, analysis of platelets that had been incubated for 15 minutes with 2 mM EDTA at 37°C revealed a marked reduction in the size of the immunoprecipitate given by the GP IIb-IIIa complex and the presence of a new, weakly stained precipitate in a more cathodal position (see next section). The use of small amounts of 125I-AP-2 in the intermediate gel confirmed that the former precipitate contained GP IIb-IIIa but that no new AP-2 binding immunoprecipitates were present after the EDTA treatment. The area occupied by the GP IIb-IIIa precipitate on each plate was measured by planimetry and expressed relative to that of another major membrane antigen previously identified as GP IIb.5 The size and form of the GP IIb containing immunoprecipitate was unchanged after platelet incubation with EDTA, and it therefore served as an internal standard. Table 2 shows that for three experiments, the apparent GP IIb-IIIa content of EDTA-treated platelets was about 39% of that present in platelets incubated under the control conditions. No such reduction was seen for platelets treated with EDTA at 20°C or CaEDTA at 37°C. A number of other immunoprecipitates located by CB-R staining are also identified in Fig 2. Neither GP Ib, highly sensitive to degradation by proteolytic enzymes,3 nor α-granule proteins, such as fibrinogen, von Willebrand factor, and thrombospondin, appeared to have been modified during the platelet incubation at 37°C. However, as an extra precaution against proteolysis, both

Fig 2. Reduced size of the GP IIb-IIIa immunoprecipitate on analysis of platelets incubated with EDTA at 37°C. Washed human platelets were incubated for 15 minutes (A, C) + 2 mM/L Ca²⁺, 1 mM/L Mg²⁺ at 37°C, and (B, D) + 2 mM/L EDTA at 37°C. After sedimentation and resuspension in HBMT for 30 minutes, aliquots were prepared for CIE analysis (see Materials and Methods). Seventy micrograms of platelet protein solubilized in 1% Triton X-100 was electrophoresed from left to right in the first dimension. Electrophoresis in the second dimension was through an intermediate gel containing 3.5 x 10⁶ cpm 125I-AP-2. The upper gel contained 0.75 mg/cm² of polyclonal rabbit antihuman platelet antibody. In (A) and (B), immunoprecipitates were located by CB-R staining, and in (C) and (D), GP IIb-IIIa complexes that had bound AP-2 were detected by autoradiography. Note on (B) the appearance of a new precipitate, indicated by the heavy arrow. Alb, albumin; Fib, fibrinogen; TSP, thrombospondin.
Table 2. Quantitative Analysis of the GP IIb-IIIa Precipitate Obtained After CIE of Pretreated Washed Platelets

<table>
<thead>
<tr>
<th>Conditions of Incubation</th>
<th>Comparative Size of GP IIb-IIIa Precipitate</th>
<th>Ratio of GP IIb-IIIa to GP IIb</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mmol/L Ca(^{2+}), 1 mmol/L Mg(^{2+}); 20 °C</td>
<td>2.92 ± 0.17 (n = 3)</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>2 mmol/L Ca(^{2+}); 1 mmol/L Mg(^{2+}); 37 °C</td>
<td>3.14 (n = 1)</td>
<td>107.5</td>
<td></td>
</tr>
<tr>
<td>2 mmol/L Ca(^{2+}), 2 mmol/L EDTA; 37 °C</td>
<td>2.77 (n = 2)</td>
<td>94.9</td>
<td></td>
</tr>
<tr>
<td>2 mmol/L EDTA; 20 °C</td>
<td>3.68 (n = 1)</td>
<td>126.0</td>
<td></td>
</tr>
<tr>
<td>0.5 mmol/L EDTA; 37 °C</td>
<td>1.05 (n = 1)</td>
<td>36.0</td>
<td></td>
</tr>
<tr>
<td>2.0 mmol/L EDTA; 37 °C</td>
<td>1.13 ± 0.01 (n = 4)</td>
<td>38.7</td>
<td></td>
</tr>
<tr>
<td>10.0 mmol/L EDTA; 37 °C</td>
<td>0.88 (n = 1)</td>
<td>30.1</td>
<td></td>
</tr>
</tbody>
</table>

Tab confirmed that only GP IIb-IIIa complexes were present in Triton X-100 extracts of platelets preincubated at 37 °C under control conditions (Fig 3) or with EDTA at room temperature (not shown). In contrast, when extracts of platelets that had been treated with EDTA at 37 °C were analyzed, two, or sometimes three, immunoprecipitates were labeled with the antibody. The first of these corresponded to that given by residual GP IIb-IIIa complexes and was identical to that which bound AP-2 (see Fig 2). The second was the new cathodal immunoprecipitate previously noted to be present on CB-R-stained gels and that clearly shows a line of antigenic identity to GP IIb-IIIa (Fig 3B) but which did not bind AP-2. This was a surprise, for previous studies had shown dissociated GP IIb to be present exclusively in an immunoprecipitate located directly above that containing residual GP IIb-IIIa. Such an immunoprecipitate was occasionally observed (open arrow in Fig 3B). However, its presence was often difficult to confirm due to the intense labeling of the residual GP IIb-IIIa precipitate by \(^{125}\)I-Tab. Figure 3 also shows CIE studies performed with \(^{125}\)I-Tab in the intermediate gel when Triton X-100 extracts of the EDTA-treated platelets were made 5 mmol/L with EDTA and further incubated for 15 minutes at room temperature just before the CIE. These are conditions known to induce a dissociation of Triton X-100-solubilized GP IIb-IIIa heterodimers. The precipitate containing free GP IIb was now increased in size (Fig 3C), while that given by the residual GP IIb-IIIa complexes was decreased. By continuing the second incubation at room temperature for one hour, a total dissociation of residual GP IIb-IIIa was obtained (not shown). However, no modification in either the location, the shape, or the \(^{125}\)I-Tab labeling intensity of the new cathodal precipitate was observed under these conditions.

\(^{125}\)I-AP-3 also labeled three immunoprecipitates during CIE of extracts of platelets incubated with EDTA at 37 °C (Fig 3D). The major arc corresponded to that given by residual GP IIb-IIIa complexes. Of the two cathodal arcs reacting with \(^{125}\)I-AP-3, one was identified as the new GP IIb-IIIa-related precipitate also labeled by \(^{125}\)I-Tab. This
conclusion was based on its size, shape, and line of partial antigenic identity with the IIb-IIIa precipitate (compare Fig 3B and D). The third component (open arrow in Fig 3D) exhibited the features previously reported for free GP IIIa on CIE analysis, ie, a slower electrophoretic mobility than the GP IIb-IIIa heterodimer, leading to a more cathodal precipitate and an absence of reactivity with 125I-Tab and 125I-AP-2.

The presence of both GP IIb and GP IIIa within the new cathodal immunoprecipitate was confirmed by SDS-PAGE analysis after excision and SDS solubilization of this particular precipitation line from unstained agarose gels, according to the procedure previously detailed by Kunicki et al. The study was performed with Triton X-100 extracts of platelets whose surface proteins had been labeled by 125I by the lactoperoxidase-catalyzed procedure before their incubation with EDTA at 37 °C. Autoradiograms of dried polyacrylamide gels revealed the presence of two 125I-labeled components in this new immunoprecipitate, with nonreduced and reduced molecular weights identical to those of GP IIb and GP IIIa (not shown).

DISCUSSION

It is well known that divalent cations are essential cofactors of platelet aggregation. Zucker and Grant highlighted a special case when they described how pretreatment of platelet-rich plasma with EDTA at 37 °C resulted in an irreversible loss in the platelet aggregation response to ADP. This effect of EDTA was temperature dependent, occurring at 37 °C but not at 20 °C, and was only observed at alkaline pH (pH > 7.2). Aggregation induced by epinephrine and the ionophore A 23187 was also inhibited, and although the treated platelets were aggregated by thrombin, the velocity of aggregation was decreased. The subsequent observations that washed platelets incubated with EDTA at 37 °C (a) had a reduced capacity to bind 45Ca2+ and (b) bound little or no fibrinogen in the presence of Ca2+ suggested that EDTA was influencing a crucial step at a late stage of the aggregation mechanism.

In confirming the temperature-dependent inhibitory effect of EDTA on the platelet aggregation response, we have shown that it is accompanied by structural changes in the GP IIb-IIIa complex. One manifestation of these changes was an irreversible fall in the number of AP-2 binding sites expressed by platelets treated with EDTA at 37 °C and alkaline pH. Significantly, this fall was of similar magnitude to the loss in the ability of the platelets to bind fibrinogen after ADP stimulation. The binding of AP-2 to GP IIb-IIIa complexes of normal platelets is followed by the inhibition of both platelet aggregation and fibrinogen binding after platelet activation. As increasing evidence suggests that GP IIb-IIIa complexes constitute the fibrinogen receptor on the platelet surface, it is probable that the reduced binding of both AP-2 and fibrinogen after incubation of platelets with EDTA at 37 °C are related events.

Our results with AP-2 may be compared with those reported by Coller et al using the monoclonal antibody 1OE5, and those of Brass et al who used another antibody, A2A9. Both 1OE5 and A2A9 appear to react with complex-dependent determinants on GP IIb–IIIa. Coller et al observed a 51% reduction in the amount of 1OE5 that bound to platelets after citrated platelet-rich plasma had been incubated with EDTA at pH 8.3 for 15 minutes at 37 °C. In contrast, Brass et al reported a complete loss in the binding of A2A9 to gel-filtered platelets incubated with EDTA for one hour at 37 °C and pH 7.4. However, the binding of AP-2, 1OE5, or A2A9 was not affected by EDTA when the temperature and pH of the incubation medium were kept within the ranges of 20 to 25 °C and pH 7.4 to 7.8, respectively. Nonetheless, under these conditions, the binding of a newly described monoclonal antibody, 7E3, was decreased by more than 40%. Finally, the binding of another anti-GP IIb–IIIa monoclonal antibody, T10, was reduced after the incubation of gel-filtered platelets for 30 minutes at room temperature and at pH 8.5. Subtle differences in the specificity of the different antibodies for their determinants on GP IIb–IIIa probably account for the variations, which may also reflect progressive changes in the conformation or organization of the complexes during platelet incubation with EDTA at different pH and temperatures.

CIE experiments confirmed a direct temperature-dependent effect of EDTA on GP IIb–IIIa complexes in the platelet membrane. This was first observed as a reduction in the size of the immunoprecipitate containing GP IIb–IIIa. The good correlation between the extent of the EDTA-induced loss of AP-2 binding sites on intact platelets at 37 °C and the reduction in size of the GP IIb–IIIa precipitate strongly suggests that the changes observed after CIE did not arise subsequent to the platelet solubilization step, a view also supported by the fact that solubilization with Triton X-100 was performed under conditions that might stabilize preexisting IIb–IIIa heterodimers, ie, at low temperature and in the presence of Ca2+ released from disrupted organelles.

The labeling by both Tab and AP-3 of a second major immunoprecipitate in a more cathodal position to GP IIb–IIIa during CIE of Triton X-100–soluble extracts of platelets that had been preincubated with EDTA at 37 °C was unexpected. These monoclonal antibodies react with EDTA-resistant determinants on GP IIb and GP IIIa, respectively. The results of studies performed after the incubation of Triton X-100–soluble platelet extracts with EDTA at 4 °C or 20 °C have shown that free GP IIb migrated at a rate similar to that of the GP IIb–IIIa complexes in the first-dimension electrophoresis, while dissociated GP IIIa gave a more cathodal immunoprecipitate. Furthermore, any decrease in the precipitate given by GP IIb–IIIa due to a dissociation of the complex was directly compensated for by an increase in the arcs given by free GP IIb and free GP IIIa. However, although small amounts of free GP IIb and free GP IIIa were visualized on CIE analysis of platelets pretreated with EDTA at 37 °C, the bulk of GP IIb and GP IIIa not present in the heterodimer precipitate was located within the new cathodal arc. Re-addition of EDTA to the Triton X-100–soluble extracts prepared from platelets pretreated with EDTA at 37 °C resulted in dissociation of residual GP IIb–IIIa complexes and the increased appear-
ance of 125I-Tab- or 125I-AP-3-labeled arcs in the position of free GP IIb and free GP IIIa (data not presented). In contrast, the antigens within the new major cathodal immuno-precipitate were not sensitive to further EDTA treatment. Thus it appears that this precipitate contains GP IIb and GP IIIa reorganized into a new common structure that specifically forms under conditions of low calcium concentration and elevated temperature and that lacks the IIb–IIIa heterodimer-specific epitopes recognized by antibodies such as AP-2. The fact that the glycoproteins within this precipitate have a more cathodal migration is suggestive of polymer formation. That this may be so is also suggested by the parallel studies of Shattil et al and Fitzgerald and Phillips. These authors have described populations of GP IIb–IIIa complexes solubilized from platelets treated with EDTA at 37 °C that showed electrophoretic properties in polyacrylamide gels under non-denaturing conditions or sedimentation profiles after centrifugation across sucrose density gradients that were indicative of the presence of high-molecular-weight aggregates.

The temperature dependence of the EDTA effect is striking. As temperature has only a limited effect on the chelating capacity of EDTA (see ref. 19), it appears that another explanation is required. One possibility is that some of the Ca²⁺ binding sites located on GP IIb or GP IIIa are only accessible to EDTA at 37 °C, perhaps due to temperature-dependent membrane fluidity changes. This may then lead to dissociation of GP IIb–IIIa complexes, followed by the recombination of the monomeric glycoproteins into high-molecular-weight aggregates lacking the structural specificities of the heterodimers. Alternative effects of EDTA also cannot be excluded. These could involve the undetected solubilization of loosely bound peripheral membrane proteins important for GP IIb–IIIa organization or stimulation of membrane enzymatic activities leading to degradation or cross-linking of GP IIb and GP IIIa. However, the observed lack of inhibition by leupeptin and the normal migration of GP IIb and GP IIIa on SDS-PAGE would seem to rule out both an effect of the major platelet Ca²⁺-dependant protease and a covalent bonding of separated polypeptide chains.

In conclusion, our studies point to a temperature-dependent effect of EDTA on GP IIb–IIIa complexes, which may explain the loss of aggregability of the treated platelets through their inability to bind fibrinogen after platelet activation. As such, our results differ from those recently presented by Zucker et al, who also examined the behavior of GP IIb and GP IIIa in Triton X-100-soluble extracts of human platelets incubated with EDTA at 37 °C. These authors showed the presence of GP IIb–IIIa complexes in Triton X-100 extracts of membranes prepared from the EDTA-treated platelets but did not quantitate the levels of GP IIb–IIIa present or examine the intact platelets in antibody binding studies. The effect of EDTA on GP IIb–IIIa appears to be subtle. However, the changes appear to concern functionally important regions of the complex, and thus EDTA treatment may represent a useful approach to elucidating the role of this complex in the responses of platelets to activation.

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