Nonreversible Loss of Platelet Aggregability Induced by Calcium Deprivation
By Marjorie B. Zucker and Robert A. Grant

Platelets lose their ability to aggregate when deprived of divalent cations. This usually was studied by incubating human citrated platelet-rich plasma with EDTA or EGTA and then adding enough CaCl₂ to combine with the chelating agent. Incubation for 5–7 min at 37°C caused irreversible loss of the platelets' ability to adhere to glass and to aggregate with ADP, epinephrine, A23187, vasopressin, or serotonin or upon rewarming after chilling and markedly reduced aggregation with collagen or thrombin. Control samples incubated with saline, CaEDTA, or CaEGTA were not inhibited. Untreated platelets washed and incubated in solutions treated with resins that remove divalent cations lost their ability to aggregate in 30 min. More than about 0.26 mM Mg²⁺ partially protected the platelets. Unlike aggregation, ADP-induced shape change, clot retraction caused by thrombin or ADP plus reptilase, and thrombin-induced ¹¹C-serotonin release were not inhibited after incubation. Aggregability was not restored by prolonged incubation with CaCl₂, adding normal plasma, or washing the platelets. Its loss was temperature and pH dependent, occurring in 2 min at 43°C but not in 7 min at 30°C, and at pH 7.8 but much less at pH 7.2. The defect was not associated with an increase in platelet cyclic AMP, a decrease in metabolic ATP, or the presence of free ADP.

Blood platelets in citrated platelet-rich plasma (PRP) respond to stimuli in various ways. When exposed to ADP or thrombin but not to epinephrine they undergo a change in shape from normal circulating discs to spiny spheres. When PRP is stirred with these or certain other substances the platelets aggregate. They secrete the contents of their granules on exposure to collagen, thrombin, or other substances, or during aggregation with ADP or epinephrine in the so-called release reaction.¹² Finally, platelets cause retraction of fibrin clots.³¹ Clots produced by thrombin retract because the thrombin stimulates the platelets while clotting the fibrinogen. Clots produced by reptilase, which does not stimulate platelets, do not retract unless a platelet-active agent such as ADP is also added.³³

The mechanisms of shape change, aggregation, and clot retraction are not well understood. Aggregation requires extracellular fibrinogen and divalent cations and is inhibited by the presence of EDTA, by raising the level of cyclic AMP (cAMP), by treating the platelets with sulfhydryl reagents,¹³ or by depleting metabolic ATP.⁶ Clot retraction, which presumably results from activation of the contractile protein of platelets,¹⁴ is also inhibited by EDTA,⁵ whereas platelet shape change is not.⁷

While studying the effect of chelating agents on platelets, we found that under certain conditions the platelets were unable to aggregate when calcium ions were restored, although they could still support clot retraction and undergo shape change.
change and the release reaction. This paper and the following one report our findings in detail.

MATERIALS AND METHODS

Materials. The following solutions were made in isotonic saline (hereafter termed saline) and kept at -20°C: 1 mM aspirin (Merck), 10 mM ADP (sodium salt, Sigma), 10 mM 5-hydroxytryptamine creatine sulfate (serotonin, Sigma), and 10 U/ml arginine vasopressin (122 U/mg, Sigma). ADP, serotonin, vasopressin, epinephrine (Parke-Davis 1:1000), connective tissue particles, and bovine thrombin (Upjohn) were diluted to ten times the desired final concentration shortly before use and kept cold. Reptilase (Abbott) was dissolved and stored up to several weeks at 4°C. A23187, a gift from Dr. Robert L. Hamill of Lilly Research Laboratories, Indianapolis, Ind., was kept at 4°C as a 20 mM stock solution in dimethylsulfoxide, and the amount added to PRP was no more than 0.4% of the sample volume. The disodium salts of EDTA (Geigy) and EGTA [ethylenebis(oxyethylenenitrilo)tetraacetic acid, Eastman] were prepared as 0.134 M solutions in water, brought to pH 7.4 with 1.0 N NaOH, and diluted further with saline. Human fibrinogen (Grade L, Kabi, Stockholm, Sweden) was dissolved in saline at 10 mg/ml, dialyzed against saline, and stored at -70°C. The chelating resins Biores 70 and Chelex 100 (Biorad) were converted to the sodium form and used in batch procedures.

Modified Tyrode's solution contained 0.2% bovine serum albumin (BSA) (Sigma), 1 mM MgCl₂, and no added CaCl₂. In some experiments saline and Tyrode's solution prepared without CaCl₂ or MgCl₂ were essentially freed of contaminating divalent cations by exposing them twice to Biores 70, then adjusting the pH to 7.8 with 0.1 N NaOH. Thrombin for use with separated platelets was similarly treated with Chelex 100.

Methods. Blood from healthy volunteers was usually drawn into 1/9 vol 0.11 M trisodium citrate plus 1/18 vol 1 mM aspirin. PRP was prepared by centrifugation at 300 g and room temperature and stored for up to 4 hr at 20°C in a capped plastic tube to minimize loss of CO₂.

For most studies 0.4 ml citrated PRP was placed in a glass aggregometer cuvette and warmed to 37°C. Control samples received 100 µl of saline or 60 µl of 0.05 M CaCl₂, 16 µl of neutral EDTA or EGTA (usually 134 mM), and 20 µl of 0.1 N NaOH to return the pH to 7.7 before incubation. Experimental samples received 16 µl of EDTA or EGTA and 10 µl of base. After incubation for the desired period, 60 µl of CaCl₂ and 10 µl more of base were added. The pH fell to about 7.2 before the base was added. This slight temporary fall was unimportant, since the results were the same when the base and chelating agent were added together. Aggregation was tested in a Payton Aggregometer (Payton Associates, Buffalo, N.Y.), usually by adding 0.1 vol of an aggregating agent. It was estimated by measuring the steepest slope of the recorded change in light transmission; similar results were obtained by measuring the maximum change in light transmission. Inhibition represents the decrease in slope, expressed as a percentage of control. Shape change was estimated from the initial decrease in light transmission and confirmed by phase-microscopic observation of samples fixed in 1% formalin. Reagent concentrations are expressed as the final values (see Results).

In some studies, blood was collected in glass tubes containing 1/9 vol 27 mM EDTA, or 27 mM or lower concentrations of EGTA, and 1/18 vol 1 mM aspirin, incubated at 20°C for 5 min, and centrifuged. The PRP, which was at about pH 7.8, was stored at 20°C to prevent loss of aggregability. Inhibition was induced by incubating 0.4 ml of PRP at 37°C for 10 min. Then 60 µl of CaCl₂ was added (50 mM for 27 mM chelating agent, and proportionately less for the lower concentrations of EGTA), and platelet aggregation was tested immediately with ADP, since the samples clotted in about 1.5 min. Control samples were tested after only 1 min incubation at 37°C, prior to addition of CaCl₂ and ADP. Samples were also tested with 60 µl saline instead of CaCl₂. The concentrations of total EDTA, calcium, and magnesium in the PRP were calculated using the hematocrit value to determine the extent of plasma dilution with anticoagulant and aspirin solutions, assuming that the chelating agents do not enter the blood cells and that the calcium and magnesium concentrations in undiluted plasma are 2.5 and 1.0 mM, respectively. The concentrations of ionized magnesium and calcium were then determined as described by Portzehl et al. using binding constants of 1.89 x 10⁶ for CaEDTA and 5 x 10⁵ for MgEGTA at pH 7.8. An additional 40% of the Mg²⁺ was assumed to be bound to protein, since this is the percentage of protein-bound calcium and calcium has the same affinity for albumin. Studies were also carried out with platelets separated from citrated PRP after lowering the pH to 6.5 with 50 µl of 0.11 M citric acid/ml PRP. The PRP was centrifuged at room temperature at 1100 g for 10-15 min and the supernatant discarded. In some experiments, the platelets were suspended in 0.1 vol saline followed immediately by addition of 0.9 vol modified Tyrode's solution. The samples (0.4 ml)
were incubated for different time periods in the cuvettes with 16 μl of 54 mM EDTA or EGTA and 10 μl of 0.1 N NaOH. Then 50 μl of 50 mM CaCl₂, 10 μl of 0.1 N NaOH, 50 μl of fibrinogen (final concentration about 1 mg/ml), and 55 μl of 50 μM ADP were added in rapid succession and aggregation was recorded. The CaCl₂ and all of the base were present during incubation of the control samples.

In experiments in which divalent cations were eliminated without the addition of chelating agents, the platelets were separated from acidified plasma, resuspended in resin-treated saline equal to the original volume of PRP, centrifuged, and resuspended in 0.1 vol treated saline and then in 0.9 vol resin-treated Tyrode’s solution. Control samples were incubated with 0.2 mM CaCl₂ and 1 mM MgCl₂. After incubation, CaCl₂ and MgCl₂ were added to the experimental cation-deficient samples and aggregation was tested using 0.4 ml of platelet suspension, 50 μl of fibrinogen, and 50 μl of 50 μM ADP.

Adhesion of platelets to glass slides was studied with a phase-contrast microscope.

Release was studied with platelets from blood collected in citrate solution, which contained 2.5 μM [³⁵C]-serotonin (57 mCi/mMole, Amersham-Searle, stored at -20°C in 95% ethanol at 8 μCi/ml). Release was induced by adding thrombin to EDTA-treated PRP or, with calcium, to platelets that had been incubated in a divalent cation-free medium. The samples were centrifuged 5 min later, and the radioactivity of the supernatants was determined.

Cyclic AMP was measured by radioimmunoassay (Collaborative Research, Waltham, Mass.) in the laboratory of Dr. Sidney Belman, Dept. of Environmental Medicine, New York University Medical Center. Aspirin-treated citrated PRP (4 ml) was incubated for 5 min with EGTA, CaEGTA, or saline. The samples were centrifuged and the platelet buttons extracted with 1 ml 6% trichloroacetic acid and centrifuged. The supernatants were assayed after removing the acid with water-saturated ether.

Metabolic ATP in platelets was assessed in aspirin-treated citrated PRP incubated for 60 min with 0.4 μM uniformly labeled [¹⁴C]-adenine (286 mCi/mMole; Amersham-Searle). About 70% of the radioactivity was incorporated into the platelets. The PRP was then treated with EDTA-ethanol, and the supernatants were diluted with an equal volume of a mixture of 3 mM ATP, ADP, and inosine monophosphate (IMP). The nucleotides were separated on 0.1-mm cellulose MN 300 polyethyleneimine-impregnated plates (Brinkmann Instrument), using stepwise elution with 1.0 M LiCl for 4 cm and 1.6 M LiCl for another 10 cm. The spots were identified under short-wave ultraviolet radiation, eluted with 0.44 M MgCl₂ 0.13 M Tris pH 9.0 for 60 min at room temperature, and counted in a liquid scintillation spectrometer.

Any ADP that might have been present in citrated PRP during incubation with 5 mM EGTA was destroyed by adding creatine phosphate (CP) and creatine phosphokinase (CPK) as described elsewhere but in final concentrations of 3 mM and 24 U/ml, respectively.

RESULTS

Aggregation and other platelet functions in PRP treated with chelating agents. When citrated PRP was incubated for 5-7 min at 37°C with 5 mM EGTA and the ionized calcium restored with 5 mM CaCl₂, platelet aggregation with 5 μM ADP was usually completely inhibited (Fig. 1A). Even with 50 μM ADP inhibition was essentially complete in 10 min. Similar incubation for less than 1 min, or incubation with CaEGTA for 7 min, caused little or no inhibition of aggregability. Aspirin treatment did not alter the effect of chelating agents; hence aspirin-treated PRP was used in subsequent studies.

The degree of inhibition depended on the concentration of EDTA or EGTA added to citrated PRP: both chelating agents were maximally effective at 5 mM and were nearly as effective at 4 mM (Fig. 2).

The ability of the platelets to aggregate was not restored when EGTA-treated samples were incubated with CaCl₂ for up to 30 min before adding the ADP or when an equal volume of nonincubated citrated plasma was added. To determine if the abnormality was associated with the platelets, the incubated PRP was brought to pH 6.5 and centrifuged and the platelets were resuspended in citrated plasma at pH 7.7. The resuspended platelets from PRP incubated with saline or CaEGTA aggregated well with ADP, whereas the platelets from PRP incubated with EGTA were unresponsive.
Incubation of PRP with EGTA also abolished aggregation induced by 55 \( \mu M \) epinephrine and 80 \( \mu M \) A23187 (a calcium ionophore) (Figs. 1B and 1C) as well as that occurring when chilled PRP was rewarmed.\(^{16}\) Aggregation with 50 mU/ml vasopressin plus 10 mM CaCl\(_2\),\(^{19}\) or with 25 \( \mu M \) serotonin, as well as adhesion of platelets to glass slides, were studied after 7 min incubation with EDTA and were also abolished. Aggregation with 0.5 U/ml thrombin was inhibited only about 70\% after 5 min incubation with EGTA (Fig. 1D), and inhibition was no greater after 30 min incubation than after 10 min. With 1:500 connective tissue in non-aspirin-treated PRP, or 1:100 connective tissue in aspirin-treated PRP, aggregation was 70\%-80\% inhibited after 10 min incubation with EDTA. Primary ristocetin-induced agglutination was not affected after incubating PRP with chelating agents.

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**Fig. 1.** Platelet aggregation induced by (A) ADP, (B) epinephrine, (C) A23187, and (D) thrombin after incubation of citrated PRP at 37\(^\circ\) C for 5 min with 5 mM CaEGTA (a) or EGTA (b).

**Fig. 2.** Inhibition of aggregation induced by 5 \( \mu M \) ADP after incubation of citrated PRP with different concentrations of EGTA and EDTA for 5 min at 37\(^\circ\) C and pH 7.8.
EDTA EFFECT ON PLATELET AGGREGATION

Table 1. Clot Retraction and Serotonin Release in Citrated PRP Incubated With 5 mM EGTA

<table>
<thead>
<tr>
<th>Addition to Citrated PRP</th>
<th>Clot Retraction After Adding CaCl₂ (min)</th>
<th>ADP + Reptilase (min)</th>
<th>¹⁴C Serotonin Released by Thrombin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>None</td>
<td>2+</td>
<td>3+</td>
<td>4+</td>
</tr>
<tr>
<td>EGTA, 0.25 min</td>
<td>2+</td>
<td>3+</td>
<td>4+</td>
</tr>
<tr>
<td>EGTA, 5 min</td>
<td>2+</td>
<td>3+</td>
<td>4+</td>
</tr>
</tbody>
</table>

Incubation of citrated PRP with EGTA or EDTA at 37°C for 5 min failed to alter platelet shape or inhibit the magnitude of the shape change induced by ADP. A23187, thrombin, vasopressin, or serotonin as observed by phase-contrast microscopy of fixed samples or the initial decrease in light transmission (Fig. 1). Incubation also failed to impair the ability of platelets to support clot retraction induced by recalcification plus thrombin or by 10 µM ADP and reptilase or to release ¹⁴C-serotonin when thrombin alone was added (Table 1).

Characteristics of the inhibition of aggregability in PRP. The ability of 5 mM EGTA to impair aggregability depended on both time and temperature of incubation (Fig. 3). Aggregation was always tested at 37°C with 5 µM ADP. It was completely inhibited in samples incubated with EGTA for 2 min at 43°C. Longer periods were necessary for samples incubated at lower temperatures, and no inhibition was noted after 7 min at 30°C. Aggregation was not affected by incubation with CaEGTA at the various temperatures (control samples).

To determine the pH dependence of inhibition, citrated PRP was incubated at different pH values reached by adding 0.15 N HCl or NaOH with or without EGTA or EDTA. The PRP was then raised to pH 7.7 with 0.15 N NaOH or HCl, and aggregation was tested with ADP. Inhibition was maximal at or above pH 8.0 and minimal at or below pH 7.2 (Fig. 4).

When CP and CPK were added to citrated PRP incubated with saline or 5 mM CaEGTA, aggregation in response to 5 µM ADP was diminished but still quite marked. The presence of CP plus CPK did not prevent the usual loss of aggregability after incubation with EGTA, indicating that the loss was not due to refractoriness to ADP that might have accumulated during incubation.

The development of inhibition was also tested in PRP from blood collected with 2.7 mM EDTA or EGTA (4.6-5.3 mM in plasma from blood with a hematocrit of
38%–48%). If the PRP was used soon after preparation, the platelets were disc shaped when warmed to 37°C and aggregated with ADP when CaCl₂ was added. After 10 min incubation of the PRP, aggregation with ADP plus CaCl₂ was abolished, but shape change caused by ADP and clot retraction and ¹⁴C-serotonin release caused by thrombin plus CaCl₂ were not inhibited. If the pH of the PRP was 7.1 during incubation with EDTA and restored to pH 7.8 before testing, aggregation was only partially inhibited.

Blood was also collected in lower concentrations of EGTA, and aggregation was tested with and without added CaCl₂. In PRP with a calculated concentration of less than 4.0 mM EGTA, the Ca²⁺ concentration was about 10⁻⁶ M and Mg²⁺ concentration above about 0.26 mM. These samples showed definite although limited, aggregation with ADP without addition of CaCl₂. When this PRP was incubated for 10 min and then tested with ADP plus CaCl₂, aggregation was not totally abolished, as was true of PRP with higher EGTA and hence lower Mg²⁺ concentrations (Fig. 5).

Aggregation of platelets in modified Tyrode's solution. Platelets suspended in modified Tyrode's solution containing 1 mM MgCl₂ and fibrinogen aggregated with 4 μM ADP without added CaCl₂ or on addition of both CaCl₂ and a chelating agent in equimolar concentrations. Aggregation did not occur when the fibrinogen and CaCl₂ were added after 5 min incubation with 1.73 mM EDTA, and aggregation was considerably reduced after incubation with the same concentration of EGTA (Fig. 6A). The concentration of ionized magnesium was calculated to be
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Fig. 6. Inhibition of aggregation induced by 5 μM ADP after incubation of platelets. (A) Platelets suspended in Tyrode's solution with 1 mM MgCl₂ and no added CaCl₂ and incubated with 2 mM EGTA or EDTA for different periods of time. CaCl₂ (2 mM) and fibrinogen added with ADP. Platelets incubated with 2 mM CaEDTA used as the controls. (B) Platelets incubated in resin-treated Tyrode's solution (RT) with 0.2 mM CaCl₂ and 1.0 mM MgCl₂ added before or after incubation. Platelets incubated in ordinary Tyrode's solution used as controls. Fibrinogen added with ADP.

about 0.5 mM with EGTA and 0.0005 mM with EDTA, whereas the concentration of calcium ions was extremely low with both.

Platelets suspended but not incubated in resin-treated calcium- and magnesium-free Tyrode's solution aggregated with ADP if CaCl₂ and MgCl₂ were added as well as fibrinogen. (No tests were carried out with addition of calcium or magnesium solution alone.) The platelets also aggregated if ADP and fibrinogen were added after 30 min incubation in either untreated modified Tyrode's solution or resin-treated Tyrode's solution containing added divalent cations. However, after incubation in resin-treated Tyrode's solution without divalent cations, the platelets failed to aggregate on addition of the cations, fibrinogen, and ADP (Fig. 6B). Epinephrine-induced aggregation was also abolished after 30 min incubation of platelets in resin-treated Tyrode's solution; aggregation induced by 1 μM A23187 or 0.1 U/ml thrombin was partially inhibited. Clot retraction and ¹⁴C-serotonin release induced by thrombin plus CaCl₂ were the same in samples incubated for 30 min with and without divalent cations.

Other measurements. The amount of cAMP in platelets was unchanged after PRP was treated with a chelating agent; it was 31 pmoles/10⁹ platelets in a saline control, 34 in a CaEGTA control, and 32 in a sample incubated 5 min with EGTA.

Platelets in PRP incubated with EGTA incorporated the same amount of radioactivity into ATP, ADP, and IMP as control platelets; values were about 45%, 6%, and 1%, respectively, of the added radioactivity.
DISCUSSION

Platelets incubated in citrated PRP for 5-7 min with 5 mM EGTA or EDTA at pH 7.8 lost their ability to adhere to glass and to aggregate in response to ADP, epinephrine, A23187, vasopressin, or serotonin or upon rewarming after chilling. Aggregation induced by connective tissue particles or thrombin was much diminished. (The residual response to thrombin may have resulted from platelet adherence to fibrin rather than true aggregation.) In contrast, the platelets' abilities to promote clot retraction and undergo shape change and the release reaction with thrombin were not impaired. This dissociation of aggregation and clot retraction is surprising, since ADP causes retraction of PRP clotted with reptilase as well as aggregation, and most patients with thrombasthenia have impaired clot retraction and absent aggregation.

Aggregability was not restored by prolonged incubation of PRP with CaCl2 or by the addition of normal plasma. Separated platelets retained the defect. The loss of aggregability occurred in the presence of CP and CPK and hence was not due to refactoriness caused by leakage of ADP from the platelets. It was also not associated with elevation of cAMP or reduction of metabolic ATP, both known to inhibit aggregation.

Presumably, the loss of platelet aggregability is due to removal of divalent cations from the platelet surface. A soluble chelating agent was not required, since aggregability was lost, although more slowly, in platelets washed and incubated in solutions from which divalent cations had been removed with resins.

Aggregability was not impaired after incubation of PRP with CaEDTA or CaEGTA; hence chelation of cations with higher affinities for the chelating agents, such as Mn2+ or Zn2+, cannot be responsible. To determine the role of Mg2+, we studied PRP from blood anticoagulated with EGTA or platelets in EGTA-treated Tyrode's solution. Aggregation was only partially inhibited after incubation in the presence of more than about 0.26 mM Mg2+. We were unable to determine from the literature if magnesium can serve as the divalent cation for the aggregation of human platelets. Haslam reported that addition of either magnesium or calcium was effective, but because Ca2+ is ubiquitous it is difficult to draw a firm conclusion from studies made without EGTA. We found that approximately the same concentration of Mg2+ that prevented complete loss of aggregability could support limited but definite aggregation with 5 μM ADP.

The degree of inhibition during incubation depended on the pH and temperature during incubation, decreasing as the pH was reduced from 8.0 to 7.2 and the temperature from 43°C to 23°C. The time and temperature dependence of the development of inhibition cannot be attributed to differences in the concentration of divalent cations during incubation, since the binding constants of EDTA vary only slightly with temperature and binding is virtually instantaneous. Either calcium was removed from the platelets only after a time- and temperature-dependent change in protein or lipid or the rapid removal of cations was succeeded by a slower, temperature-dependent alteration in protein or lipid configuration or an enzymatic step. A difference in the concentration of divalent cations probably also fails to explain the much diminished inhibition noted when EDTA PRP was incubated at pH 7.1 rather than at pH 7.8, since EDTA has a high affinity for calcium and magnesium at both pH values. Presumably, another step in the loss of aggregability takes place better at the higher pH.
Harmful effects of EDTA on platelets have been described by others. Chromium-labeled platelets prepared from EDTA blood disappear more rapidly from the circulation than similar platelets prepared from acid-citrate blood. Chro-
meyer et al. and Haslam and Rosson noted time-dependent inhibitory effects of EDTA and EGTA, respectively, on aggregation. Platelets in EDTA PRP change from discs to spiny spheres during 15 min incubation at 37°C, and they develop dilated surface-connected canaliculi. For unknown reasons, EDTA added to citrated PRP has much less effect on platelet shape. In our studies with citrated PRP, the platelets maintained their disc shape throughout incubation with EDTA, then underwent the usual shape change after ADP was added. These results were confirmed by studies of the platelet size distribution profile.

EDTA also has deleterious effects on plasma proteins. Incubation of EDTA plasma prolongs the thrombin clotting time. Like the defect produced in platelets, the prolongation increases during incubation, reaching its maximum in 10-15 min and becoming more marked as the temperature is increased from 20°C to 37°C. Unlike the platelet defect, however, the thrombin clotting time is promptly re-
ored by adding CaCl₂. Factors V, and VIII lose their activity during incubation of EDTA plasma; it is not restored by adding calcium.

We do not yet know the mechanism causing the loss of platelet aggregability associated with deprivation of divalent cations. Intracellular calcium has been im-
plicated as a second messenger in platelet shape change, aggregation, and release. Our results suggest the presence of a pool of platelet calcium that is im-
portant in aggregation but not shape change or thrombin-induced release. As described in the following paper, calcium loss from this pool is associated with a change in platelet surface charge properties.

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