Platelet Membrane Alterations Induced by the Local Anesthetic Dibucaine

By Ellinor I.B. Peerschke

Tertiary amine local anesthetics modify a variety of platelet membrane-related functions. The present study explored dibucaine (DB)-induced inhibition of platelet cohesion by examining structural and functional alterations of the human platelet membrane glycoprotein IIb-IIIa complex (GPIIb–IIIa) and platelet Ca²⁺ homeostasis. Complete inhibition of ADP-induced aggregation was achieved five minutes after platelet exposure to 0.1 to 1.0 mmol/L of DB when fibrinogen binding was reduced by 50%. At higher concentrations of DB (~1 mmol/L), ADP-induced fibrinogen binding was completely blocked. Scatchard analysis revealed loss of high-affinity binding sites in addition to reduced but not absent. Fibrinogen binding to chymotrypsin-treated platelets sustained 50% inhibition of fibrinogen binding when incubated with 0.4 to 0.5 mmol/L DB, and kinetic analysis showed that the high-affinity platelet–fibrinogen interactions were reduced but not absent. Fibrinogen binding to chymotrypsin-treated platelets could not be completely inhibited even at high DB concentrations (1 mmol/L). The inhibition of fibrinogen binding to chymotrypsin-treated platelets correlated with changes in binding of a monoclonal antibody (10E5) specific for an epitope on the GPIIb–IIIa complex. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and radioelectrophoresis of DB-treated platelets, however, showed no evidence of a reduction or degradation of GP IIb or IIIa. Platelet incubation with DB (five minutes, 0.1 to 1.0 mmol/L) was also accompanied by: (a) increased platelet membrane-associated Ca²⁺ involving low-affinity binding sites [Kd = 5 x 10⁻⁹ mol/L]; (b) increased Ca²⁺ uptake which correlated with degradation of actin-binding protein (ABP) and digestion of GPIIb as visualized on periodic-acid Schiff (PAS)-stained SDS gels and as inferred from decreased binding of a monoclonal antibody (6D1) directed against this glycoprotein; and (c) enhanced Ca²⁺ exchange. Thus, exposure of platelets to DB results in membrane-related alterations that may contribute to inhibition of platelet cohesion: (a) Decreased fibrinogen receptor exposure by traditional agonists and diminished accessibility of the GPIIb–IIIa complex to extracellular ligands correlate with DB-induced inhibition of platelet aggregation; and (b) increased calcium uptake and exchange across the platelet membrane likely leads to activation of the calcium-dependent protease(s) which was previously shown to correlate with DB-induced inhibition of ristocetin-induced platelet agglutination. © 1986 by Grune & Stratton, Inc.

IN ADDITION to their well-known inhibitory effects on neuronal responses, tertiary amine local anesthetics have been reported to modify a variety of non-neuronal activities including ovum activation, exocytosis, endocytosis, anion transport in erythrocytes, cell spreading and motility, von Willebrand factor (VWF)-mediated platelet agglutination, fibrinogen-mediated platelet aggregation, and the platelet release reaction. They have also been shown to dissolve platelet cytoskeletal components, inhibit protein kinase C, and phospholipase A₂, interfere with platelet adhesion to fibrinogen-coated beads, and, depending on concentration, to potentiate or inhibit ristocetin-induced platelet agglutination. The precise mechanism of action of local anesthetics, however, remains unclear. Some of their effects on cellular activity and reactivity have been attributed to the ability of local anesthetics to perturb membrane phospholipid organization, bilayer fluidity, and membrane protein conformation. Other effects have been ascribed to their ability to displace calcium from various cell membranes and interfere with cellular calcium metabolism. In fact, it has been suggested that local anesthetics affect platelet aggregation by modifying the participation of calcium in this process. The present study was thus designed to investigate further the mechanism of local anesthetic inhibition of platelet cohesion by examining the effects of dibucaine (DB) on the structural and functional integrity of the platelet membrane glycoprotein IIb–IIIa (GPIIb–IIIa) complex, on calcium homeostasis, and on the relationship between platelet calcium homeostasis and degradation of GPIIIa.

MATERIALS AND METHODS

Platelet preparation. Blood was obtained according to the guidelines of the Declaration of Helsinki from healthy volunteers who had not taken aspirin for at least eight days. It was anticoagu-
ter, NY) and by being rocked at 4°C for 30 minutes, and centrifuged at 22°C for five minutes at 12,000 g in a Beckman microfuge (Beckman Instruments Corp, Palo Alto, Calif) to remove remaining debris. Assays were performed in 1% agarose containing a combination of 1% Triton X-100, IgG prepared from the serum of rabbits immunized with whole platelets (Accurate Chemicals, Westbury, NY, Lot 073A) and radiolabeled 1OES antibody (6 × 10⁵ cpm; specific activity 98 cpm/µg). Samples (5 µL) were electrophoresed at 8 mA for 30 minutes and then at 19.5 mA for 18 hours. The gels were sequentially soaked in 0.15 mol/L of NaCl and water, dried on a support medium (Gelbond, FMC Corp, Rockland, Mass) with the aid of an electric dryer, stained with 0.5% Coomasie blue in 45% methanol-10% acetic acid, destained for three to five minutes in the same solution without Coomasie blue, redried, and subjected to radioautography for various lengths of time at −80°C using Kodak XAR-5 film (Eastman Kodak, Rochester, NY) and an intensifying screen (Lightning-Plus, Dupont, Wilmington, Del).

The functional integrity of the GPIIb-IIIa complex was assessed by: (a) comparing the ability of control and DB-treated platelets to bind fibrinogen following stimulation with 10 µmol/L of ADP or treatment with chymotrypsin (Sigma) (500 µg/mL, 15 minutes, 22°C), and (b) evaluating fibrinogen-mediated aggregation of control and DB-treated platelets using a Chronolog (Haverton, PA) dual-channel aggregometer whose baselines were set at 10% light transmission using the unstimulated GFP suspension and 90% light transmission using a buffer blank. All functional studies were performed at room temperature.

LDH release and PF3 availability. Release of lactate dehydrogenase (LDH) from the platelet cytoplasm was measured with a Du Pont ACA (Clinical Systems Division, Du Pont, Wilmington, Del). Platelet factor 3 (PF3) availability was measured by preincubating a mixture of pooled normal plasma at a 1:1 ratio (George King Biomedical Co, Overland Park, Kan) and kaolin (Sigma) for five minutes at 37°C and then adding 0.2 mL of the mixture to 0.2 mL of GFP (150,000/µL). CaCl₂ (0.1 mL of a 0.035 mol/L stock solution) was added 30 seconds later, and clot formation was monitored using a semiautomated impedance device (fibrometer, BBL, Division of Becton Dickinson Co, Cockeysville, Md).

Platelet calcium homeostasis. DB-induced changes in calcium homeostasis were assessed and correlated with degradation of platelet membrane GPIb and actin-binding protein (ABP). The effect of DB in calcium homeostasis was evaluated by characterizing: (a) [⁴⁰Ca]⁺ (Amersham Corp, Arlington Heights, Ill) binding and uptake of platelets in calcium-free and magnesium-free HBMT to which increasing concentrations of Ca²⁺ were added, and (b) Ca²⁺ exchange under steady-state conditions. Scatchard plots of Ca²⁺ binding were constructed with the aid of a Hewlett Packard HP-85 curve-fitting program (Hewlett Packard, Corvallis, Ore) and analyzed according to the methods of Rosenthal and Feldman. Platelet membrane-associated calcium was defined as platelet-associated calcium that was removed following five minutes of incubation with 10 mmol/L of EDTA. Ca²⁺ exchange was quantified by measuring Ca²⁺ influx and efflux as follows: GPP in Ca²⁺-free and Mg²⁺-free HBMT were incubated for 30 minutes at 22°C with 0.2 mmol/L of unlabeled Ca²⁺ and subsequently loaded with tracer amounts of [⁴⁰Ca]⁺ (17.55 mCi/mg; 0.5 to 1.0 × 10⁶ cpm/mL in the presence and absence of DB. Ca²⁺ uptake was assessed at timed intervals up to 120 minutes by centrifugation of platelets through silicone oil. Duplicate samples were subjected to an additional five-minute incubation with 10 mmol/L of EDTA before centrifugation. Following Ca²⁺ uptake, platelet preparations were acidified to pH 6.5 using 0.1 N HCl, centrifuged (1,000 g, 15 minutes, 22°C), and resuspended in buffer containing 1.0 mmol/L of unlabeled Ca²⁺. Ca²⁺ efflux was subsequently monitored at time intervals up to 120 minutes as above, both before and after five-minute incubation with 10 mmol/L of EDTA.

Because the GPIIb-IIIa complex has been implicated in calcium homeostasis, additional studies were performed in which platelets were pretreated with either 10 mmol/L of EDTA or the combination of 10 mmol/L of EDTA and 10 mmol/L of unlabeled CaCl₂ at 37°C and pH 8.1 for 60 minutes. The ability of EDTA-treated platelets with dysfunctional GPIIb-IIIa complexes to take up ⁴⁰Ca in response to exposure to 1.0 mmol/L of DB was monitored after five and 30 minutes and compared with that of control platelets.

Degradation of ABP and platelet membrane GPIb was assessed following platelet incubation with DB in the presence and absence of 10 mmol/L of EDTA. Platelets were lysed in a solution containing one part 3.3% SDS, 6 mmol/L of N-ethylmaleimide, and one part 1% SDS, 12.5 mmol/L Tris (hydroxymethyl) aminomethane chloride, 20% glycerol, and 0.025% bromophenol blue (pH 6.8), heated to 100°C for three minutes, and electrophoresed into 7.5% SDS-polyacrylamide gels. The gels were stained for protein with Coomassie blue or for carbohydrate with PAS. PAS-stained gels were scanned using a Beckman Model CDS 200 gel scanner. DB-induced alterations of platelet membrane GPIb were also monitored by comparing the ability of a radiolabeled monoclonal antibody (6D1) specific for this glycoprotein to bind to control and DB-treated platelets.

RESULTS

Structural and functional integrity of the platelet membrane GPIIb-IIIa complex. DB reduced the binding of a monoclonal antibody (10E5), specific for an epitope on the GPIIb-IIIa complex in a concentration-dependent manner within five minutes of platelet exposure to the anesthetic (Fig 1A). Maximum inhibition (≈50%) occurred at DB concentrations >0.5 mmol/L. The antibody recognized 58,174 ± 14,744 sites on control platelets, but only 23,247 ± 9,877 sites (mean ± SD, N = 16) on platelets treated with 0.5 mmol/L of DB. This inhibition was not reversed when DB was removed by platelet centrifugation and resuspension in fresh buffer: 54% ± 5% (mean ± SD, N = 4) at five minutes and 52% ± 3% at 30 minutes. Platelet incubation with DB for up to 30 minutes, however, had no effect on platelet membrane GPIIIb or GPIIIa as assessed by SDS-PAGE (data not shown). Moreover, when platelets were lysed with Triton X-100 and electrophoresed into 1% agarose gels containing a mixture of unlabeled rabbit anti-whole platelet antiserum and 125I-labeled 10E5, the 10E5 antibody recognized similar quantities of GPIIb-IIIa complexes in control and DB-treated platelet samples (Fig 2). Similar results were obtained when lysed platelets were incubated with the local anesthetic. Furthermore, when 10E5 was bound to platelets before exposure to DB, it did not dissociate on addition of the anesthetic (Table 1).

The effects of DB on platelet aggregation, fibrinogen binding, and 10E5 binding are correlated in Fig 1A. Preincubation (five minutes, 22°C) of platelets with 0.1 to 0.25 mmol/L of DB resulted in complete inhibition of ADP-induced aggregation but in only 50% inhibition of fibrinogen binding. ADP-induced fibrinogen binding was almost completely inhibited at DB concentrations >0.5 mmol/L, as was fibrinogen binding induced by epinephrine, thrombin, and the combination of ADP and epinephrine (Table 2). ADP-
induced fibrinogen binding was similarly inhibited if platelets were first stimulated with 10 μmol/L ADP and then exposed to the anesthetic (Table 3).

In contrast, platelets treated with chymotrypsin were minimally affected by low concentrations of DB (0.1 mmol/L) (Fig 1B). Chymotrypsin-induced platelet aggregation and fibrinogen binding were reduced in parallel following platelet exposure to higher DB concentrations. Neither decreased below ~50% of control levels, however, even at DB concentrations exceeding 0.5 mmol/L.

Scatchard analysis of fibrinogen binding to ADP-stimulated platelets indicated that DB not only decreased total receptor availability, but also specifically obliterated high-affinity fibrinogen binding (Fig 3A). In the presence of DB fibrinogen binding to ADP-treated platelets consistently yielded straight-line Scatchard plots as compared with curvilinear plots obtained with platelets in the absence of DB (Fig 3A), or platelets exposed to DB and stimulated with chymotrypsin (Fig 3B). Platelets stimulated with ADP bound 23,400 ± 6,852 (mean ± SD, N = 5) molecules of fibrinogen [Kd = 1.04 ± 0.6 μmol/L] in the presence of 0.1 mmol/L of DB, as compared with 49,300 ± 10,623 molecules of fibrinogen bound to control platelets in an apparently biphasic manner (initial dissociation constant = 0.10 ± 0.05 μmol/L). Chymotrypsin-treated platelets bound 35,418 ± 11,072 molecules of fibrinogen with an initial high-affinity dissociation constant of 0.09 ± 0.05 μmol/L in the presence of 0.4 mmol/L of DB, and 66,397 ± 12,464 molecules of fibrinogen with an initial high-affinity dissociation constant of 0.08 ± 0.06 μmol/L in the absence of anesthetic.

LDH release and PF3 availability. The effect of DB on PF3 activity and lactate dehydrogenase (LDH) release are shown in Table 4. Increases in PF3 activity were already noted five minutes after platelet exposure to even the lowest DB concentrations (0.1 to 0.25 mmol/L), when virtually no LDH release was detectable, and continued to increase at higher DB concentrations. LDH release required prolonged platelet exposure (30 to 60 minutes) to DB concentrations exceeding 0.5 mmol/L.

Platelet calcium homeostasis. Because platelet incubation with DB has been shown to lead to degradation of both ABP and GPIb by activation of an enzyme(s) that is at least partially sensitive to calcium concentrations, experiments were performed to investigate DB-induced alterations of platelet Ca2+ homeostasis. Although 6D1 binding to platelets was decreased in a concentration-dependent manner five minutes after platelet exposure to DB and continued to decrease during a subsequent 30-minute exposure to the

| Table 1. Effect of 1 mmol/L of DB on the Dissociation of Platelet-Bound 125I-10E5 |
|---------------------|---------------------|
| Time* (min) | 10E5 Binding† (% Relative to T0) |
| 0 | 100 |
| 2 | 98 ± 4 |
| 5 | 102 ± 2 |
| 15 | 101 ± 5 |
| 30 | 99 ± 3 |

*Platelets were incubated with 10 μg/mL 125I-10E5. After 15 minutes 10E5 binding was assessed (T0) and dibucaine (DB) was added. 10E5 binding was quantified at the designated times using aliquots of the platelet suspension.

†Mean ± SD, N = 3
Table 2. Effect of 0.5 mmol/L of DB on Fibrinogen Receptor Exposure by Various Agonists

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Fibrinogen Binding (% of Control)*</th>
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<tr>
<td>ADP (10 μmol/L)</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>Epinephrine (10 μmol/L)</td>
<td>5 ± 7</td>
</tr>
<tr>
<td>Thrombin† (100 μM/mL)</td>
<td>21 ± 10</td>
</tr>
<tr>
<td>ADP (10 μmol/L) and epinephrine (10 μmol/L)</td>
<td>14 ± 4</td>
</tr>
<tr>
<td>Chymotrypsin (500 μg/mL, 15 minutes, 22 °C)</td>
<td>49 ± 15</td>
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*Mean ± SD, N = 5.
†Human thrombin was the gift of Dr John Fenton, New York State Department of Health, Albany.

All platelets were incubated with dibucaine (DB) for 5 minutes before stimulation.

anesthetic, measurable $^{45}\text{Ca}^{2+}$ uptake required either five-minute exposure of platelets to DB concentrations >0.5 mmol/L or 30-minute exposure of platelets to DB concentrations exceeding 0.1 mmol/L. DB-induced Ca$^{2+}$ uptake correlated well with degradation of ABP (Fig 5). In contrast, PAS-stained polyacrylamide gels showed that GPIb degradation required at least a 60-minute exposure of platelets to DB at 0.5 mmol/L or 30-minute exposure to DB concentrations >0.5 mmol/L. Under these conditions, LDH release was also present (Table 4). As reported by others, DB-induced degradation of both ABP and GPIb could be inhibited in the presence of EDTA (10 mmol/L).

The effect of 1 mmol/L of DB on platelet Ca$^{2+}$ influx and efflux under steady-state conditions is shown in Fig 6A and B. Both the rate of Ca$^{2+}$ influx and efflux increased markedly in the presence of 1 mmol/L of DB. Control platelets ($5 \times 10^7$) took up Ca$^{2+}$ at a rate of 0.65 ± 0.20 pmol/min, whereas DB-treated platelets ($5 \times 10^7$) took up Ca$^{2+}$ at an initial rate of 4 ± 1.2 pmol/min. Similar initial Ca$^{2+}$ influx rates were calculated, 0.55 ± 0.18 pmol/5 x 10$^7$ platelets/min for control platelets, and 4.5 ± 1.6 pmol/5 x 10$^7$ platelets per minute for DB-treated platelets, indicating that $^{45}\text{Ca}^{2+}$ measurements were indeed made under equilibrium conditions.

Most DB-induced calcium uptake was mediated by the platelet membrane GPIIb–IIIa complex, as illustrated in Fig 6C. Platelets that were incubated with EDTA for 60 minutes at 37 °C and an alkaline pH to disrupt GPIIb–IIIa complexes were previously reported to take up less Ca$^{2+}$ than control platelets, but amounts similar to those taken up by platelets from patients with thrombasthenia. In this study, EDTA-treated platelets also took up significantly less calcium than did control platelets following exposure to DB.

In addition to calcium uptake, exposure of platelets to DB also altered the fraction of platelet associated $^{45}\text{Ca}^{2+}$ that could be rapidly removed by EDTA and was thus presumed to be located on the surface membrane. Figure 7 depicts the binding of $^{45}\text{Ca}^{2+}$ to platelets before and after five minutes of exposure to 1 mmol/L of DB. Calcium binding was quantified two minutes after addition of increasing concentrations of $^{45}\text{Ca}^{2+}$, since previous studies indicated that most of the $^{45}\text{Ca}^{2+}$ associated with platelets under these conditions was surface bound. As reported earlier, platelets depleted of loosely associated surface Ca$^{2+}$ following brief Ca$^{2+}$ deprivation rebind $^{45}\text{Ca}^{2+}$ in an apparently biphasic manner, suggesting the presence of at least two membrane-associated Ca$^{2+}$-binding compartments. The first approached saturation near 100 μmol/L of free Ca$^{2+}$, whereas the second failed to saturate even at 1 mmol/L of free Ca$^{2+}$. Present data indicate that Ca$^{2+}$ binding to both compartments appears to increase following platelet exposure to DB. Approximately $5 \times 10^7 ± 1.57 \times 10^6$ (mean ± SD, N = 3) molecules of Ca$^{2+}$ bound to the saturable compartment of control platelets with

Table 3. ADP-Induced Fibrinogen Binding

<table>
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<tr>
<th>Platelets</th>
<th>Fibrinogen Binding (% of Control)*</th>
</tr>
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<tr>
<td>Incubated with DB before stimulation</td>
<td>46 ± 16</td>
</tr>
<tr>
<td>Incubated with DB after stimulation</td>
<td>54 ± 15</td>
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Effect of 0.1 mmol/L of dibucaine on fibrinogen receptor exposure and fibrinogen binding to previously exposed receptors. Platelets were stimulated with 10 μmol/L of ADP; fibrinogen binding was assessed in the presence of 1.0 mg/mL $^{125}$I-fibrinogen.

*Mean ± SD, N = 3.
an initial high-affinity dissociation constant of $2.0 \pm 1.0 \times 10^{-3}$ mol/L, as compared with $8 \pm 3 \times 10^5$ sites occupied by $4^2$Ca$^{2+}$ on DB-treated platelets with an initial apparent high-affinity dissociation constant of $5 \pm 2.5 \times 10^{-3}$ mol/L. The difference in high-affinity dissociation constants was statistically significant using the paired Student's $t$ test at $P < .05$.

**DISCUSSION**

The mechanisms by which tertiary amine local anesthetics affect various platelet membrane-related functions, including platelet adhesion to foreign surfaces, platelet agglutination, platelet aggregation, and the release of platelet granule contents, have been of interest because understanding of these mechanisms is likely to enhance present knowledge of the biochemical and biophysical events involved in normal platelet cohesion. Previous studies with other cell systems have shown that the ability of anesthetics to interact with membrane lipids and to displace membrane-bound calcium correlates with anesthetic potency, and that the anesthetics may interact directly with membrane proteins to inhibit their function.

Because local anesthetics modify platelet functions mediated both by the membrane GPIIb-IIIa complex and GPIb, and it has been suggested that the inhibition of platelet function by these agents results from changes in platelet membrane-associated and/or cytosolic calcium, the present study examined platelet membrane alterations accompanying the DB-induced loss of platelet cohesion with emphasis on anesthetic-induced structural and functional alterations of the GPIIb-IIIa complex, and on platelet calcium homeostasis. The data have implications for the cellular control of platelet membrane-associated structures involved in fibrinogen-dependent platelet aggregation, provide direct evidence for DB-induced changes in Ca$^{2+}$ homeostasis, and thus a mechanism whereby the platelet calcium-dependent protease(s) is activated to digest GPIb.

Brief incubation (five minutes) of platelets with low doses of DB (0.1 mmol/L) resulted in complete inhibition of ADP-induced platelet aggregation and a 50% reduction in fibrinogen binding. This reduction in fibrinogen binding was accompanied not only by decreased fibrinogen receptor availability, but also by the absence of high-affinity fibrinogen binding. Recent studies using monoclonal antibodies have shown that both high-affinity and low-affinity platelet fibrinogen interactions are likely mediated by the GPIIb-IIIa complex and suggest that differences in affinity may reflect (a) heterogeneity of the microenvironment surrounding the GPIIb-IIIa complex; (b) the presence of at least two different platelet-binding domains on fibrinogen molecules; and/or (c) interactions among fibrinogen receptors.
resembling negative cooperativity.37 Because high-affinity platelet-fibrinogen interactions appear to be essential for platelet aggregation, and reach a maximum at fibrinogen concentrations supporting maximal platelet aggregation (100 to 200 μg/mL),14 their loss may explain the apparent discrepancy between ADP-induced fibrinogen binding and platelet aggregation observed in the present study. This interpretation is supported by the finding that chymotrypsin-treated platelets incubated with DB also suffered a reduction in total fibrinogen binding, but high-affinity platelet fibrinogen interactions remained intact, albeit reduced, as did platelet aggregation. It is postulated that the DB-induced effect on high-affinity fibrinogen binding to platelets stimulated with ADP results from anesthetic-induced alterations of the GPIIb–IIa microenvironment by direct or indirect modification of platelet membrane constituents that are degraded during fibrinogen receptor exposure by chymotrypsin.

Differences in the extent to which local anesthetics inhibit fibrinogen binding of ADP-treated and chymotrypsin-treated platelets suggest furthermore that certain events contributing to ADP-induced activation transduction may be additionally sensitive to local anesthetics. The mechanism of fibrinogen receptor exposure by chymotrypsin is different from ADP-induced fibrinogen receptor exposure.38 This hypothesis is supported by data from a recent study demonstrating that DB inhibits ADP-induced increases in binding of a monoclonal antibody (7E3) whose rate of binding to platelet GPIIb–IIIa complexes is activation dependent.39 Comparison of the effect of DB on fibrinogen and 10E5 binding to platelets stimulated with ADP and chymotrypsin thus points toward alteration of both GPIIb–IIIa receptor exposure mechanisms (ADP-treated platelets) and receptor accessibility to ligand (chymotrypsin-treated platelets).

In the present study, differences between the DB-induced inhibition of ADP and chymotrypsin-induced fibrinogen binding mimicked those reported when platelets were treated with cytochalasins.40,41 ADP-induced exposure of fibrinogen receptors and maintenance of the receptors in an exposed state were inhibited by cytochalasins, suggesting that they were dependent on platelet cytoskeletal elements.40,41 In contrast, chymotrypsin-treated platelets did not appear to
require cytoskeleton activation for fibrinogen binding. Because local anesthetics are known to cause depolymerization of microfilaments and microtubules and/or to interfere with cytoskeletal assembly, altered GPIIb-IIIa cytoskeletal interactions are likely to be responsible for the different sensitivities of fibrinogen receptors on ADP-treated and chymotrypsin-treated platelets to DB. It is conceivable that the interaction of platelet membrane proteins with membrane-associated cytoskeletal elements alters the exposure of the GPIIb-IIIa complex by modifying its microenvironment and relocating inhibitory membrane constituents that are proteolytically removed by the action of chymotrypsin.

Unlike chymotrypsin-treated platelets exposed to cytochalasins, however, chymotrypsin-treated platelets incubated with the local anesthetic DB lost at least some of their ability to bind fibrinogen. This inhibition correlated remarkably well with the DB-induced inhibition of 10E5 binding, a monoclonal antibody that recognizes an epitope on GPIIb and/or GPIIIa when they form a complex, regardless of platelet activation, and suggested that inhibition of fibrinogen binding is mediated in part by alteration of the GPIIb-IIIa complex by modifying its microenvironment and relocating inhibitory membrane constituents that are proteolytically removed by the action of chymotrypsin.

Exposure of platelets to DB also had a profound effect on platelet Ca\(^{2+}\) homeostasis, affecting Ca\(^{2+}\) binding, Ca\(^{2+}\) exchange, and Ca\(^{2+}\) uptake. Ca\(^{2+}\) binding to both saturable and nonsaturable membrane binding sites was increased and the Ca\(^{2+}\) binding affinity of the saturable sites was decreased. This decreased Ca\(^{2+}\) binding affinity may be due to DB-induced changes in the exposure of platelet membrane proteins and/or phospholipids and may contribute to the observed increase in Ca\(^{2+}\) exchange across the platelet membrane, since Ca\(^{2+}\) binding membrane proteins were previously reported to facilitate plasma membrane Ca\(^{2+}\) transport.

DB enhanced total platelet Ca\(^{2+}\) uptake nearly fourfold. This increase is mediated in large part by the GPIIb-IIIa complex since disruption of the complex by EDTA-treatment at alkaline pH and 37 °C reduces Ca\(^{2+}\) uptake by ~50%. The DB-induced increase in Ca\(^{2+}\) uptake appears to activate the platelet Ca\(^{2+}\)-dependent protease(s), since the accompanying pattern of proteolysis—especially of ABP and GPIb—resembles that described by others for this protease(s). The apparent contradiction between DB-induced increases in Ca\(^{2+}\) uptake and the accompanying decrease in
platelet aggregation suggests that either the observed increase in Ca\textsuperscript{2+} uptake does not result in similar increases in cytosolic-free Ca\textsuperscript{2+} but rather protein or enzyme-bound Ca\textsuperscript{2+} which may be inactive or inhibitory, or that increased levels of cytosolic free Ca\textsuperscript{2+}, while necessary, are not sufficient for platelet activation. Inhibition of a number of proteins and enzymes including ABP, calmodulin, protein C, and phospholipase A, as well as altered fibrinogen receptor availability, likely result in the observed overall inhibition of platelet cohesion.

Although DB-induced increases in Ca\textsuperscript{2+} uptake correlated directly with degradation of ABP, degradation of GPIb required prolonged platelet incubation with the anesthetic and was usually accompanied by LDH release. Exposure of platelets to at least 0.5 mmol/L of DB for 30 minutes resulted in degradation of GPIb as visualized on PAS-stained polyacrylamide gels of lysed platelets. As described previously, the degradation of both GPIb and ABP was incompletely inhibited by EDTA, indicating that DB-induced release of Ca\textsuperscript{2+} from intracellular stores may also be important, and raising questions concerning the location of the Ca\textsuperscript{2+}-dependent protease(s) (cytoplasmic, membrane bound, or released into the extracellular milieu).

More subtle DB-induced alterations of GPIb could be detected using a monoclonal antibody specific for GPIb (6D1). 125I-labeled 6D1 binding to platelets was reduced following even brief exposure of platelets to low concentrations of DB when GPIb remained intact. These early changes in GPIb are consistent with previously reported decreases in VWF binding to DB-treated platelets stimulated with ristocetin. Such early changes in the functional properties of GPIb may result from anesthetic-induced membrane alterations similar to those postulated to inhibit fibrinogen and 10ES binding to the platelet membrane GPIIb–IIIa complex.

In conclusion, the data suggest that exposure of platelets to the tertiary amine local anesthetic DB results in several alterations that contribute to the observed inhibition of platelet cohesion: (a) Although the platelet membrane GPIIb–IIIa complex appears to remain antigenically intact, and certain complex dependent functions are unaltered in the presence of DB, decreased fibrinogen receptor exposure by traditional agonists including ADP, epinephrine, or thrombin and diminished accessibility of the complex to extracellular ligands such as fibrinogen and the monoclonal antibody 10ES correlate with DB-induced inhibition of platelet aggregation; and (b) increased Ca\textsuperscript{2+} uptake and exchange by DB-treated platelets likely contributes to the observed inhibition of platelet aggregation and activation of the Ca\textsuperscript{2+}-dependent protease(s), leading to degradation of ABP and GPIb which was previously shown to correlate with anesthetic-induced inhibition of ristocetin-mediated platelet agglutination.

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