Identification and Function of the High Affinity Binding Sites for Ca^{2+} on the Surface of Platelets

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Abstract. Extracellular Ca^{2+} is required for platelet aggregation and secretion in response to ADP or epinephrine. Recently, we reported that the platelet surface contains two classes of high affinity binding sites for extracellular Ca^{2+}. To identify these sites and clarify their role in platelet function, we have now (*a*) studied platelets congenitally deficient in surface membrane glycoproteins and (*b*) examined the effect of removing surface-bound Ca^{2+} on platelet responses to ADP and epinephrine. Unstimulated normal platelets contained 86,000 Ca^{2+}-binding sites/platelet with a dissociation constant (K_d) of 9 nM and 389,000 sites with a K_d of 400 nM. In contrast, thrombasthenic platelets, which lack glycoproteins IIb and IIIa, exhibited a 92% reduction in the number of higher affinity Ca^{2+}-binding sites and a 63% reduction in the number of lower affinity sites. Bernard-Soulier platelets, which lack glycoprotein Ib, were not deficient in Ca^{2+}-binding sites. After stimulation with ADP, both normal and thrombasthenic platelets developed ~138,000 new Ca^{2+}-binding sites/platelet (K_d = 400 nM), while the larger Bernard-Soulier platelets developed 216,000 new sites. These data suggest that IIb and IIIa represent the major Ca^{2+}-binding glycoproteins on unstimulated platelets, while neither these glycoproteins nor Ib represent the new Ca^{2+}-binding sites on stimulated platelets.

Removal of Ca^{2+} from the platelet surface inhibited platelet function. Despite the presence of 1 mM Mg^{2+}, ADP- and epinephrine-induced aggregation and [^{14}C]serotonin release were markedly decreased at free Ca^{2+} concentrations < 7 nM, a value similar to the K_d of the higher affinity Ca^{2+}-binding sites. Moreover, gadolinium, a lanthanide that competed for these Ca^{2+}-binding sites, also inhibited aggregation and serotonin release. These studies demonstrate, therefore, that the binding of extracellular Ca^{2+} to glycoproteins IIb/IIIa on unstimulated platelets or to additional membrane proteins on stimulated platelets is necessary for maximal platelet responses to ADP and epinephrine. Thus, the requirement for extracellular Ca^{2+} during platelet activation by these agonists may actually represent a requirement for surface-bound Ca^{2+}.

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Introduction

Extracellular Ca^{2+} is required for several of the reactions that take place on the platelet surface when platelets are activated by agonists, including the binding of fibrinogen to membrane glycoproteins IIb and IIIa (1, 2). Since extracellular Ca^{2+} exists in rapid equilibrium with Ca^{2+} bound to the platelet surface (3), at least part of the requirement for extracellular Ca^{2+} may reflect Ca^{2+} bound to the platelet membrane at sites involved in the surface reactions. Previously, we demonstrated that there are a minimum of two classes of high affinity, saturable binding sites for Ca^{2+} on the surface of intact platelets (3). Stimulation by ADP or epinephrine produced additional Ca^{2+}-binding sites, even in the absence of platelet aggregation and secretion.

The high affinity (dissociation constant [Kd] < 10^{-6} M) of all of these binding sites for Ca^{2+} suggested that they may be located on membrane proteins or glycoproteins rather than membrane phospholipids (4). Accordingly, we performed the present studies (*a*) to identify the specific membrane proteins that represent the high affinity Ca^{2+}-binding sites on unstimulated platelets; (*b*) to determine whether these same membrane proteins are involved in ADP-stimulated Ca^{2+} binding; and (*c*) to

examine the effect on platelet aggregation and secretion of selectively removing Ca^{2+} from its binding sites on the platelet surface. The results suggest that the requirement for extracellular Ca^{2+} during ADP- and epinephrine-induced platelet aggregation and secretion reflects a requirement for Ca^{2+} bound to specific membrane proteins.

Methods

Preparation of gel-filtered platelets. Platelet-rich plasma from normal and thrombasthenic donors was obtained by differential centrifugation of blood anticoagulated with 13 mM sodium citrate and incubated for 30 min at room temperature with 1 mM aspirin to inhibit platelet cyclooxygenase (3). Platelet-rich plasma from donors with the Bernard-Soulier syndrome was collected after the erythrocytes had settled by gravity for 3 h in an air-tight plastic container. Platelets were isolated from plasma by gel filtration on Sepharose 2B (Pharmacia Fine Chemicals, Piscataway, NJ) using an elution buffer that contained 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 1 mg/ml bovine serum albumin (Sigma Chemical Co., St. Louis, MO), and 4 mM Hepes, pH 7.40 (3, 5). This buffer was prepared with water that had been depleted of calcium by passage through Chelex 100 ion exchange resin (Bio-Rad Laboratories, Richmond, CA). The final total Ca^{2+} content of the buffer measured by atomic absorption spectroscopy was 11 μM. This value was not affected by the presence of gel-filtered platelets, and it was included in the calculation of the Ca^{2+} concentration of all buffer solutions used in this study. The final free Ca^{2+} concentration measured by Ca^{2+} electrode was 5 μM. Normal platelets prepared in this manner aggregate in response to ADP when stirred with added fibrinogen, but do not undergo the release reaction. In the absence of stirring and fibrinogen, aggregation does not occur (3).

Platelets from three donors with Glanzmann's thrombasthenia (L.M., M.M., and N.L.) and one of the two donors with the Bernard-Soulier syndrome (A.J., previously A.H.) were obtained through the courtesy of Dr. Margaret Johnson of the Wilmington Medical Center, Wilmington, DE. Platelets from the second donor with the Bernard-Soulier syndrome (R.O.) were obtained with the assistance of Dr. Scott Murphy, Thomas Jefferson University, Philadelphia, PA. All five patients have classical findings and histories for their respective diseases and have been described previously by multiple investigators (1, 6–11). Platelets from all three of the thrombasthenic donors contained <7% of the normal amount of glycoproteins IIb and IIIa (measured antigenically with an anti-IIb-IIIa monoclonal antibody) and do not bind fibrinogen (1, 6).

Measurement of surface-bound Ca^{2+} on unstimulated platelets. Ca^{2+} binding to the surface of unstimulated platelets was measured as previously described (3). Briefly, $^{45}CaCl_2$ (1–1,500 μM) and EGTA (500 μM) were added to gel-filtered platelets to obtain buffered extracellular free Ca^{2+} concentrations that ranged from 10^{-9} M to 10^{-3} M and platelet concentrations that ranged from $1.5–3.0 \times 10^8$/ml (3, 12). After 10 min at room temperature, 0.4-ml aliquots of the platelet suspension were layered on top of silicone oil and the platelets sedimented by centrifugation for 2 min at 12,000 *g* in a microcentrifuge. The amount of $^{45}Ca^{2+}$ in the platelet pellet was measured by scintillation counting and corrected for trapped extracellular space using [^{3}H]sorbitol (New England Nuclear, Boston, MA) (3). Surface-bound $^{45}Ca^{2+}$ was defined as the difference between this value for platelet-associated $^{45}Ca^{2+}$ and the value obtained when 3.5 mM EGTA was added to the platelet suspension immediately before sedimenting the platelets. The number and affinity of the Ca^{2+}-binding sites measured in this way were calculated by nonlinear regression analysis using the general equation for ligand binding to one or more noninteracting classes of binding sites (3, 13).

Measurement of surface-bound Ca^{2+} on ADP-stimulated platelets. Measurements of surface-bound Ca^{2+} on platelets stimulated by ADP were made over a more restricted range of free Ca^{2+} concentrations (3×10^{-8} to 1×10^{-5} M) using 50 μM EGTA to buffer the free Ca^{2+} concentration. Otherwise the procedure was the same as used in the absence of ADP. Over this limited range of Ca^{2+} concentrations, binding occurred predominantly at a single class of sites, and the data were analyzed by the method of Scatchard (14).

Free Ca^{2+} concentration. Free Ca^{2+} concentrations > 1 μM were measured with a Ca^{2+}-specific electrode (Orion Research, Inc., Cambridge, MA). Free Ca^{2+} concentrations < 1 μM were calculated by the method of Portzehl (12) using published association constants for EGTA that were corrected for pH (15). In addition, the apparent association constant for Ca^{2+}-EGTA was confirmed directly in the platelet incubation buffer (minus Mg^{2+} and albumin) using the method of Bers (16).

Platelet aggregation and serotonin release. Platelets in plasma were labeled with [^{14}C]serotonin (3). Following gel filtration, imipramine (1 μM) and fibrinogen (0.1 mg/ml) were added and platelet aggregation and [^{14}C]serotonin release in response to ADP and epinephrine were measured at various free Ca^{2+} concentrations (3). In some of the studies, platelet aggregation and [^{14}C]serotonin release were measured in the presence of 10–100 μM $GdCl_3$ (ICN Pharmaceuticals, Plainview, NY). In this case, the pH of the gel filtration buffer was decreased to 6.9 to avoid formation of insoluble Gd^{3+} salts (17). The cyclic AMP content of platelets incubated with 100 μM $GdCl_3$ for 15 min at room temperature was measured as described previously (18).

Results

Ca^{2+} binding to unstimulated thrombasthenic platelets. Surface-bound Ca^{2+} was measured at free Ca^{2+} concentrations ranging from 10^{-9} M to 10^{-3} M using aspirin-treated, gel-filtered platelets. The results obtained with platelets from seven normal donors are summarized in Table I and are comparable to those reported previously (3). Normal platelets had two classes of saturable binding sites for Ca^{2+} with an apparent K_d of 9 and 400 nM, respectively. Nonsaturable Ca^{2+} binding averaged 0.05% of the free Ca^{2+} concentration.

Thrombasthenic platelets, which are deficient in glycoproteins IIb and IIIa, demonstrated abnormal Ca^{2+} binding. For purposes of comparison, the combined data from the three thrombasthenic donors and the normal donors are shown in Fig. 1. The analysis of each individual thrombasthenic donor is shown in Table I. Although the thrombasthenic platelets also contained two classes of saturable Ca^{2+}-binding sites with apparent dissociation constants similar to normal platelets, the amount of Ca^{2+} bound by each class of sites was markedly reduced. Ca^{2+} binding to the higher affinity sites was reduced by an average of 92%, while binding to the lower affinity sites was reduced by an average of 63%. These data suggest, but do not prove, that the majority of both classes of saturable binding sites for Ca^{2+} on unstimulated normal platelets is associated with glycoproteins IIb and IIIa.

Ca^{2+} binding to ADP-stimulated thrombasthenic platelets. Studies of ADP-stimulated platelets required a modification of

Table I. Ca^{2+}-binding Sites on Unstimulated Thrombasthenic Platelets

Platelets	K_d	Sites/platelet	K_d	Sites/platelet
	nM		*nM*	
Normal	9±2	86,000±11,000	400±100	389,000±35,000
Thrombasthenic	16	5,000	100	113,000
	1	15,000	900	141,000
	5	2,000	200	177,000

Platelets were isolated from plasma by gel filtration in buffer containing 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 1 mg/ml bovine serum albumin and 4 mM Hepes, pH 7.40. $^{45}CaCl_2$ and 500 μM EGTA were added and the platelets were incubated for 10 min at room temperature. The final total calcium concentration (including residual calcium in the gel-filtration buffer) ranged from 10^{-5} to 1.5×10^{-3} M, giving free Ca^{2+} concentrations from 10^{-9} to 10^{-3} M (Methods). The amount of surface-bound $^{45}Ca^{2+}$ was measured after sedimenting the platelets through silicone oil and was analyzed for each individual donor by nonlinear regression (3, 13). The results shown for the normal platelets are the mean±SEM for seven donors.

the methods used to study unstimulated platelets. Platelets incubated at 37°C with EGTA or EDTA irreversibly lose their ability to aggregate in response to ADP in a time- and concentration-dependent manner (19, 20). To avoid this potential problem, we first examined the ability of ADP to stimulate platelet aggregation after normal platelets had been incubated for 15 min at room temperature at various concentrations of EGTA and Ca^{2+}. When the Ca^{2+} concentration was buffered with 50 μM EGTA, ADP-induced platelet aggregation was fully maintained at free Ca^{2+} concentrations at least as low as 30 nM. Therefore, Ca^{2+} binding to ADP-stimulated platelets was studied between 30 nM and 10 μM extracellular free Ca^{2+}. The upper limit of 10 μM was chosen to minimize nonsaturable Ca^{2+} binding (Fig. 1). Over this range of free Ca^{2+} concentrations, surface-bound Ca^{2+} reflects binding of extracellular Ca^{2+} predominantly to the lower affinity class of binding sites (K_d = 400 nM). This permitted the data on a Scatchard graph to be analyzed by linear regression (Fig. 2). Normal platelets stimulated by ADP developed an average of 138,000±23,000 new Ca^{2+}-binding sites with no change in the apparent K_d (Figs. 2 and 3). This increase in Ca^{2+}-binding sites after ADP is in good agreement with the value of 140,000 sites/platelet found in our previous study using a platelet incubation system not buffered with EGTA (3). The absence of a change in the apparent K_d after ADP suggests that the new Ca^{2+}-binding sites also have a K_d of ~400 nM. In contrast to the marked deficiency of Ca^{2+}-binding sites on unstimulated thrombasthenic platelets, thrombasthenic platelets stimulated with ADP developed a normal number of new Ca^{2+}-binding sites (Fig. 3). This suggests that the ADP-induced Ca^{2+}-binding sites are not associated with glycoprotein IIb or IIIa.

Ca^{2+} binding to Bernard-Soulier platelets. To determine whether the ADP-stimulated Ca^{2+}-binding sites are associated with glycoprotein Ib, Ca^{2+} binding was measured using Bernard-Soulier platelets, which are deficient in this glycoprotein (2). Unstimulated Bernard-Soulier platelets from two donors contained twice the number of higher and lower affinity Ca^{2+}-binding sites found on unstimulated normal platelets. This may be related to the larger size and increased membrane surface area of these platelets. After stimulation by ADP, Bernard-Soulier platelets

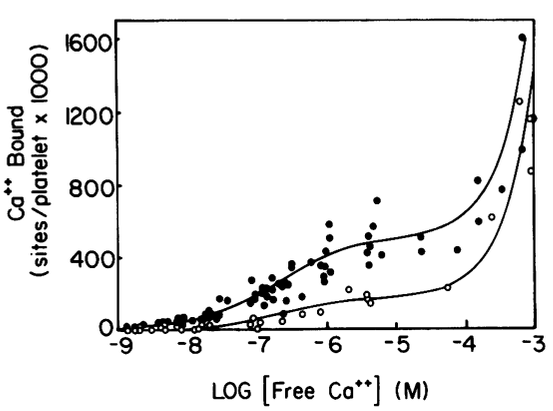

Figure 1. Ca^{2+}-binding sites on unstimulated thrombasthenic platelets. Gel-filtered platelets were prepared from three donors with thrombasthenia (open circles) and seven normal donors (filled circles). Surface-bound Ca^{2+} was measured at free Ca^{2+} concentrations from 1 nM to 1 mM. Platelets from all donors were studied across this entire range of free Ca^{2+} concentrations. Each data point represents the mean of triplicate determinations. The solid lines were generated by nonlinear regression analysis of the grouped data.

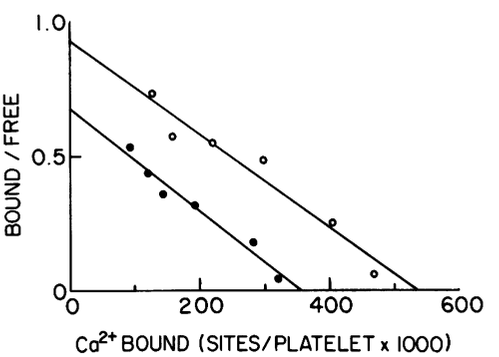

Figure 2. The effect of ADP on Ca^{2+} binding to normal platelets. $CaCl_2$ and EGTA (50 μM) were added to gel-filtered platelets to obtain free Ca^{2+} concentrations from 30 nM to 10 μM. At the same time, ADP (10 μM) was added to half of the platelets. 10 min later, the amount of surface-bound Ca^{2+} on platelets stimulated by ADP (open circles) or on control platelets (filled circles) was measured. The results obtained are displayed as a Scatchard plot. This figure illustrates the data obtained on platelets from a single normal donor, but it is representative of the data from all the donors studied. ADP increased the number of Ca^{2+}-binding sites on these normal platelets from 354,000 sites/platelet (K_d = 221 nM) to 534,000 sites/platelet (K_d = 243 nM).

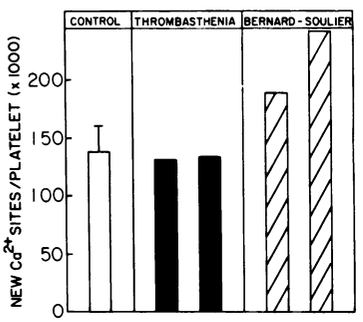

Figure 3. ADP-induced Ca^{2+}-binding sites on normal, thrombasthenic, and Bernard-Soulier platelets. Surface-bound Ca^{2+} was measured and analyzed as shown in Fig. 2. The values shown are the new Ca^{2+}-binding sites stimulated by ADP and represent the difference between the number of Ca^{2+}-binding sites measured in the presence and absence of ADP. The results for the normal platelets are mean ±SEM for eight donors. The values shown for the thrombasthenic and Bernard-Soulier platelets are the results from each individual donor.

developed an average of 216,000 new lower affinity Ca^{2+}-binding sites/platelet, an increase that was also greater than normal (Fig. 3). Since Bernard-Soulier platelets are deficient in glycoprotein Ib, these data suggest that the new Ca^{2+}-binding sites stimulated by ADP are not associated with this glycoprotein.

Effect of Ca^{2+} binding on platelet aggregation and serotonin release. Both classes of Ca^{2+}-binding sites on unstimulated platelets exhibit apparent K_d that are several orders of magnitude lower than the 1 mM free Ca^{2+} concentration of plasma. Presumably, therefore, these sites are normally fully occupied. To determine whether surface-bound Ca^{2+} is necessary for normal platelet function, two separate approaches were taken. First, platelet aggregation and [^{14}C]serotonin release in response to ADP and epinephrine were measured at low free Ca^{2+} concentrations but in the presence of 1 mM Mg^{2+}. In contrast to the Ca^{2+}-binding studies, the platelets were not preincubated with aspirin and they were stirred in the presence of fibrinogen so that platelet aggregation and serotonin release could take place. At 0.8 nM free Ca^{2+}, 10 μM ADP stimulated only partial aggregation and <5% [^{14}C]serotonin release (Fig. 4). This reduction of aggregation and serotonin release was not due to a direct effect of the EGTA or of the low Ca^{2+} concentration on fibrinogen, because fibrinogen incubated at 0.8 nM free Ca^{2+} for up to 2 h was still able subsequently to support aggregation. The extent of ADP-induced platelet aggregation and [^{14}C]serotonin release increased with increasing Ca^{2+}, and was maximal at 7 nM free Ca^{2+} (Fig. 4). Similar results were obtained when epinephrine was used as the agonist. The reductions of ADP or epinephrine-induced aggregation and [^{14}C]serotonin release at Ca^{2+} concentrations < 7 nM were reversed by adding Ca^{2+} back to the platelet mixture. These experiments suggest that the higher affinity Ca^{2+}-binding sites (K_d = 9 nM) on the platelet surface must be at least partially occupied by Ca^{2+} for maximal platelet aggregation and secretion to occur in response to ADP and epinephrine. Mg^{2+}, which was present in these experiments, cannot substitute for Ca^{2+} in this regard but it can support partial aggregation.

The second approach used to assess the functional role of surface-bound Ca^{2+} took advantage of the binding properties of the lanthanide, Gd^{3+}. Gd^{3+} exhibits a high affinity for Ca^{2+}-binding sites on cell membranes (17). In preliminary studies we incubated gel-filtered platelets with $^{153}GdCl_3$ for up to 1 h. We found that the volume of distribution of $^{153}Gd^{3+}$ centrifuged through silicone oil with platelets was the same as the volume of the trapped extracellular space measured with [^{3}H]sorbitol. This suggests that, as with other cells (17), Gd^{3+} is unable to enter the platelet. Thus, we used Gd^{3+} to displace surface-bound $^{45}Ca^{2+}$ selectively. At 5 μM free Ca^{2+} and 1 mM Mg^{2+}, $GdCl_3$ caused a dose-dependent decrease in both surface-bound Ca^{2+} and ADP-induced platelet [^{14}C]serotonin release (Fig. 5). Gd^{3+} also decreased platelet aggregation, but to a lesser extent (Fig. 5). These inhibitory effects of Gd^{3+} were reversed by the addition of excess Ca^{2+}, but not Mg^{2+}, to the incubation mixture. Another lanthanide, La^{3+}, has been reported to inhibit platelet function by increasing the levels of platelet cyclic AMP (21). However, in two experiments, 100 μM $GdCl_3$ had no effect on platelet cyclic AMP content (control = 0.70 and 0.81 pmol cyclic AMP/10^8 platelets; $GdCl_3$ = 0.77 and 0.91 pmol).

Discussion

The plasma membrane of human platelets contains high affinity Ca^{2+}-binding sites that can be characterized by equilibrium binding studies performed with intact platelets at submicromolar extracellular free Ca^{2+} concentrations. Approaching in this manner, we previously found that normal platelets have at least

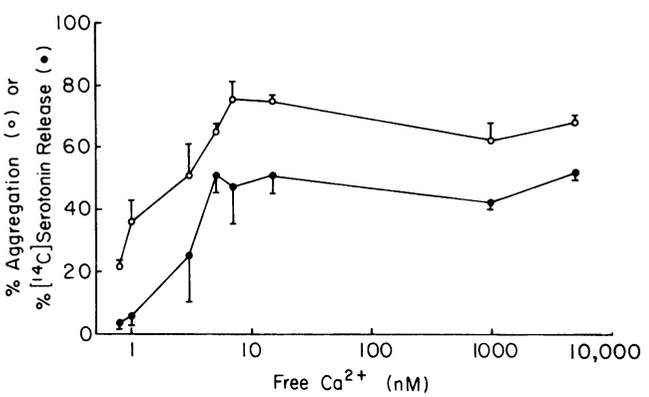

Figure 4. The effect of the extracellular free Ca^{2+} concentration on platelet responses to ADP. Aggregation and [^{14}C]serotonin release in response to 10 μM ADP were measured at 37°C in the presence of 0.1 mg/ml fibrinogen and 1 mM $MgCl_2$. The ADP was added immediately after addition of 500 μM EGTA and sufficient $CaCl_2$ to give the initial free Ca^{2+} concentrations indicated. The results shown are the percentage of platelet aggregation (open circles) and [^{14}C]serotonin release (filled circles) measured 3 min after the addition of ADP and represent the mean±SEM of three experiments. Percent aggregation refers to the percent change in light transmittance through the platelet suspensions with unstimulated platelet suspensions set at 0% and incubation buffer without platelets set at 100%.

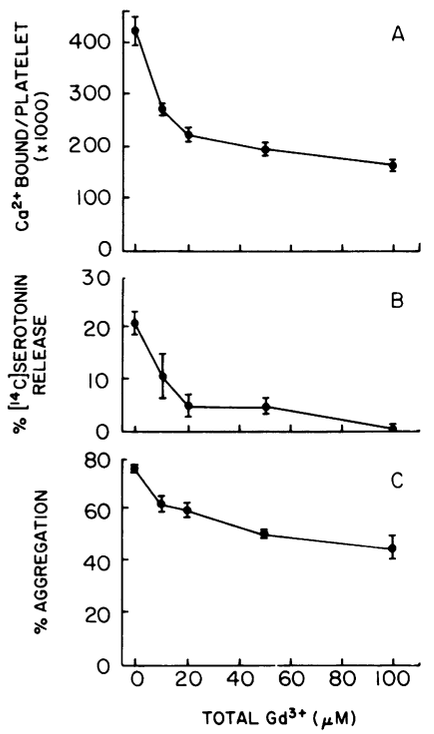

Figure 5. The effect of Gd^{3+} on surface-bound Ca^{2+}, platelet aggregation and [^{14}C]serotonin release. Gel-filtered platelets were prepared at pH 6.9. (*A*) Aspirin-treated platelets were incubated with $^{45}CaCl_2$ (5 μM free Ca^{2+}) and 10–100 μM $GdCl_3$. After 10 min, surface-bound Ca^{2+} was measured. Using platelets not preincubated with aspirin, platelet [^{14}C]serotonin release (*B*), and aggregation (*C*) in response to 50 μM ADP were measured. Data represent the mean ±SEM of three experiments.

two classes of saturable Ca^{2+}-binding sites and develop additional Ca^{2+}-binding sites after stimulation with ADP or epinephrine (3). In these studies we have used abnormal platelets with defined deficiencies of plasma membrane glycoproteins to help localize the saturable Ca^{2+}-binding sites. We have also explored the role of these sites in platelet function by observing the effect of removing or displacing Ca^{2+} from the platelet surface on platelet aggregation and secretion.

The studies performed with abnormal platelets show that unstimulated thrombasthenic platelets, which are deficient in the membrane glycoproteins IIb and IIIa, had markedly fewer high affinity Ca^{2+}-binding sites than normal. These data may explain the observations of Peerschke et al. (22), who found that thrombasthenic platelets have a reduced uptake of extracellular $^{45}Ca^{2+}$. However, their studies did not specifically quantitate surface-bound Ca^{2+}. Despite the deficiency of Ca^{2+}-binding sites on unstimulated thrombasthenic platelets, ADP-stimulated thrombasthenic platelets had normal numbers of new Ca^{2+}-binding sites. Similarly, Bernard-Soulier platelets, which are deficient in membrane glycoprotein Ib, demonstrated no deficiency of ADP-stimulated Ca^{2+}-binding sites. Taken together, these studies suggest that most of the saturable binding sites for Ca^{2+} on unstimulated normal platelets are associated with glycoproteins IIb and IIIa. Confirmation of this suggestion must await direct Ca^{2+}-binding studies with isolated platelet membrane glycoproteins. In contrast to the unstimulated platelet, glycoproteins IIb and IIIa, as well as glycoprotein Ib, can be excluded as the location for the new Ca^{2+}-binding sites that appear on the surface of platelets stimulated with ADP.

These conclusions are dependent upon adequate definition of the membrane abnormalities present in thrombasthenic and Bernard-Soulier platelets. It is generally accepted that a deficiency in glycoproteins IIb and IIIa is the major change in thrombasthenic platelet membranes; however, other as yet poorly characterized changes in membrane glycoproteins have been detected by two-dimensional gel electrophoresis (23). Nonetheless, the identification of glycoproteins IIb and IIIa as a major binding site for extracellular Ca^{2+} is supported by other observations. When glycoproteins IIb and IIIa are isolated from platelet membranes, they form a Ca^{2+}-dependent complex (24, 25). In stimulated platelets, this complex appears to mediate fibrinogen binding, a process that also requires divalent cations (1). In contrast, glycoprotein Ib, which binds von Willebrand factor in the presence of ristocetin, does not appear to bind extracellular Ca^{2+} and has no known structural or functional requirement for Ca^{2+} (2). Similarly, glycoprotein V, which may also be decreased in Bernard-Soulier platelets, appears to be involved in thrombin-induced platelet activation, a process that does not require extracellular Ca^{2+} (2, 26).

Since there are ~44,000 glycoprotein IIb-IIIa complexes/platelet (1, 6), the number of Ca^{2+} ions bound to each complex may be estimated using the data in Table I. Normal platelets have an average of 86,000 higher affinity and 389,000 lower affinity Ca^{2+}-binding sites. Thrombasthenic platelets have an average of 7,000 higher affinity and 144,000 lower affinity sites/platelet. Assuming that the differences between the normal platelets and the thrombasthenic platelets are due exclusively to the absence of glycoproteins IIb and IIIa, normal platelets contain approximately two Ca^{2+}-binding sites per IIb-IIIa complex with an average K_d of 9 nM and six Ca^{2+}-binding sites per IIb-IIIa complex with a K_d of 400 nM. Recently, Fujimura and Phillips (24), using isolated IIb and IIIa, demonstrated that the Ca^{2+} concentration required for formation of the glycoprotein IIb-IIIa complex is ~1 μM. This Ca^{2+} concentration is similar to the K_d of the lower affinity Ca^{2+}-binding sites that we observed on intact platelets. However, the relationship between the Ca^{2+} required for formation of the IIb/IIIa complex within the platelet membrane and the Ca^{2+} that we have found to be bound to glycoproteins IIb and IIIa using intact platelets remains to be explored.

Using two separate approaches, we found that removing Ca^{2+} from the platelet surface decreased the ability of platelets to aggregate and release serotonin in response to ADP and epinephrine. Thus, despite the presence of 1 mM free Mg^{2+}, platelet

aggregation and [^{14}C]serotonin release were markedly decreased at free Ca^{2+} concentrations < 7 nM. This free Ca^{2+} concentration is similar to the apparent K_d of the higher affinity Ca^{2+}-binding sites on the platelet surface and suggests that these sites must be at least partially occupied for normal platelet function. Furthermore, the lanthanide, Gd^{3+}, was able to displace Ca^{2+} from its specific binding sites on the platelet surface, resulting in an inhibition of platelet aggregation and serotonin release.

The fact that 1 mM Mg^{2+} alone was unable to support full aggregation and serotonin release but could do so if at least 7 nM free Ca^{2+} was simultaneously present suggests that divalent cation binding sites on platelets are heterogeneous (20, 27). Although not specifically addressed by the present studies, the data are consistent with the presence of divalent cation binding sites on platelets distinct from the high affinity binding sites specific for Ca^{2+}. These sites would be capable of binding either Ca^{2+} or Mg^{2+} at millimolar concentrations of these cations. Occupation of such sites by either Ca^{2+} or Mg^{2+} may be required for partial platelet aggregation. In contrast, the high affinity binding sites defined in the present study are specific for Ca^{2+}, and occupancy of these by Ca^{2+} appears to be required for both serotonin release and maximal platelet aggregation in response to ADP or epinephrine.

How might the binding of extracellular Ca^{2+} to specific high affinity sites on glycoproteins IIb, IIIa or other membrane proteins be involved in platelet activation? On the one hand, the high affinity binding of extracellular Ca^{2+} to IIb or IIIa may be a prerequisite for these glycoproteins to bind fibrinogen maximally and therefore support maximal aggregation (1). On the other hand, the binding of extracellular Ca^{2+} may also influence the platelet secretory response directly, independent of its effect on platelet aggregation. For example, a number of surface membrane proteins believed to be involved in receptor-mediated platelet activation are Ca^{2+} dependent (28, 29). However, further studies will be necessary to clarify the topography of these membrane proteins and determine whether they are capable of binding extracellular Ca^{2+} during ADP- or epinephrine-induced platelet activation.

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