PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **179,** 232–239 (1985)

pH and Magnesium Alter ^{45}Calcium Binding to Platelets at Sites Other than Glycoproteins I or IIb/IIIa[1] (42092)

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Abstract. Calcium is a cofactor of human platelet aggregation. Moreover a direct correlation between the ability of platelets to bind this divalent cation and to aggregate has been demonstrated. Since magnesium can substitute for calcium in supporting aggregation, especially in the presence of low calcium concentrations, and platelet aggregation is inhibited at low pH, the present study was designed to examine the effects of magnesium and low pH on ^{45}calcium binding to human platelets, and to determine whether such effects might be associated with calcium binding to glycoproteins I (GPI) or IIb/IIIa (GPIIb/IIIa), the putative fibrinogen receptor. ^{45}Calcium binding to aspirin-treated platelets that had been depleted of surface-associated calcium by brief exposure to EDTA was evaluated. Magnesium (5–10 m*M*) or a change in hydrogen ion concentration to decrease the pH from 7.5 to 6.0 was found to inhibit the binding of ^{45}calcium to platelets from healthy donors by 34 ± 6 and 32 ± 8% (mean ± SD, n = 13), respectively. Similar results were obtained with platelets incubated with chymotrypsin to selectively remove GPI or platelets from a patient with the Bernard Soulier Syndrome, congenitally deficient in GPI. In contrast, calcium binding to platelets from two patients with thrombasthenia, lacking GPIIb/IIIa, was reduced 49 ± 6% and 42 ± 8% (n = 4) by magnesium and hydrogen ions, respectively. This apparently increased inhibition was attributed to the combined effects of an overall decrease (~50%) in calcium binding to thrombasthenic platelets compared with that in control platelets, and a similar absolute reduction in calcium binding in the presence of magnesium and/or hydrogen ions. No additional inhibition of ^{45}calcium binding was noted in the presence of magnesium and at low pH, indicating that magnesium and hydrogen ions may affect the same platelet membrane binding sites. The data suggest that although modulation of platelet aggregation by magnesium and pH is accompanied by changes in platelet-associated calcium, calcium binding to the three major platelet membrane glycoproteins, GPI, IIb, and IIIa is unaffected. © 1985 Society for Experimental Biology and Medicine.

Much attention has recently focused on defining the role of extracellular calcium in human platelet aggregation. Extracellular calcium is required for fibrinogen binding to activated platelets (1, 2), and some of this calcium may be associated with GPIIb and IIIa since these glycoproteins form a calcium-dependent complex (3, 4), and platelets from patients with thrombasthenia bind less calcium than platelets from healthy donors (5, 6). Because most if not all platelet membrane-associated calcium binding sites appear to be saturated in a plasma milieu or other divalent cation-rich media (5), chelating agents (EGTA) (7, 8), ion exchange resins (9), or gel-filtration techniques (5, 10) are commonly used to remove the bulk of this extracellular calcium before calcium binding is evaluated. Under these conditions, ^{45}calcium binding studies suggest that there are two classes of saturable, high affinity, platelet membrane calcium binding sites (10), both of which may be associated with GPIIb and/or IIIa (11), and a third class of binding sites that is unsaturable.

Since both fibrinogen binding (2) and platelet aggregation (12) can be supported by either extracellular calcium or magnesium and are inhibited at low pH (13), the present study was designed to evaluate such modulations of platelet function in terms of their effect on platelet membrane-associated calcium, particularly calcium associated with GPIIb/IIIa. Platelet-rich plasma was briefly exposed to EDTA, and the platelets were washed to remove the bulk of extracellular and membrane-associated calcium. As reported by others, the resulting loss in platelet

[1] Supported by National Institute of Health: NHLBI Grant HL-28183.

0037-9727/85 $1.50
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aggregability was rapidly reversed by adding either calcium or magnesium (14), and was pH dependent (15).

Materials and Methods. *Platelet preparation.* Blood was collected into 0.1 vol 3.2% sodium citrate and 0.05 vol 1 m*M* acetylsalicylic acid (Merck and Co., West Point, Pa.). Platelet-rich plasma was prepared (5), incubated for 20 min at 22°C with 10 m*M* EDTA, and centrifuged (1000*g*, 20 min). The platelets were washed once with 0.02 *M* phosphate-buffered 0.15 *M* NaCl (PBS), pH 7.5, containing 5 m*M* EDTA, and resuspended in either divalent cation-free 0.01 *M* Hepes (*N*-2-hydroxyethyl-piperazine-*N*-2-ethane sulfonic acid)-buffered modified Tyrode's solution, pH 7.5 (HBMT) (5), HBMT containing 2–10 m*M* $MgCl_2$, or HBMT at pH 6.0. As described previously (5), this procedure resulted in the removal of most of the platelet membrane-associated calcium, and consequently an inability of platelets to aggregate. The latter was restored immediately by adding at least 30 μM calcium. ADP (10 μM)-induced platelet aggregation was monitored at room temperature in a dual channel aggregometer (Chronolog Corp., Havertown, Pa.) with baselines set at 10% light transmission using platelet samples and 90% light transmission using the appropriate buffer blank.

^{45}Calcium binding. Experiments were performed at 22°C using $^{45}CaCl_2$ (Amersham Corp., Arlington Heights, Ill.) mixed with unlabeled 0.1 *M* $CaCl_2$ and diluted with 0.15 *M* NaCl to a final concentration of 5 m*M* (250 μCi/ml). As the purpose of this study was to correlate calcium binding to platelets depleted of surface-associated calcium with platelet aggregability, platelets in HBMT were incubated with increasing concentrations of ^{45}calcium (25–1000 μM) for only 2 min. One-half of the suspension was then centrifuged through silicone oil to determine total calcium binding (5). The remainder was incubated with 5 m*M* EDTA for 5 min and centrifuged through silicone oil to assess calcium uptake (5). The difference between the amount of calcium bound to platelets before and after this brief exposure to EDTA was defined as surface-associated calcium (5). ^{45}Calcium binding was analyzed as recommended by Klotz (16) and according to the method of Scatchard (17). Free calcium concentrations were measured directly using a Nova 2 ionized calcium analyzer (Nova Biomedical Corp., Newton, Mass.).

To examine the effects of magnesium and pH on the reassociation of calcium with membrane calcium-depleted platelets, similar studies were performed with platelets in HBMT containing 2–10 m*M* $MgCl_2$ and/or HBMT at pH 6.0.

Effects of magnesium and pH on the rebinding of ^{45}calcium to platelets lacking GPI or GPIIb/IIIa. ^{45}Calcium binding studies were performed as described above using platelets from two patients with Glanzmann's thrombasthenia (N.L., Ma. Mo.), lacking platelet membrane GPIIb/IIIa, and from one patient with the Bernard Soulier Syndrome (T.H.) lacking GPI. These platelets were studied on two separate occasions courtesy of Dr. Margaret M. Johnson, Wilmington, Delaware. Quantitation of platelet membrane GPI or GPIIb/IIIa was performed previously using monoclonal antibodies (18, 19).

Platelets from healthy volunteers lacking GPI as a result of chymotrypsin treatment were also studied. These platelets were prepared as described elsewhere (20). Briefly, platelets were separated from plasma by centrifugation (280*g*, 12–15 min), and resuspended in calcium-poor HBMT (21) containing 1 m*M* $MgCl_2$. Platelets were incubated with 0.1 μM PGE_1 (Sigma Chemical Co., St. Louis, Mo.; stored as a 2.8 m*M* stock solution in ethanol at −10°C) and 500 μg/ml chymotrypsin (α-chymotrypsin, 51 U/mg, Worthington Biochemical Co., Freehold, N.J., Lot CDAG 35A634). After 5 min, a 10-fold molar excess of PMSF (phenylmethylsulfonylfluoride, Sigma Chemical Co.) was added, and platelets were incubated with 10 m*M* EDTA for an additional 20 min to deplete them of surface-associated calcium. They were centrifuged, washed once with 5 m*M* EDTA-containing PBS, and resuspended in HBMT. These platelets lost 94 ± 5% (SD, n = 10) of their surface GPI as determined by their inability to bind a monoclonal antibody specific for this glycoprotein (6D1) (18).

Electrophoretic mobility of platelets depleted of surface-associated calcium before and after reexposure to calcium in the presence and absence of magnesium or low pH. The

electrophoretic mobility of platelets was determined as described by Coller (22), using a 1-mm radius cylindrical cell apparatus (Mark I, Rank Bros., Bottisham, Cambs, U.K.) submerged in a 25°C water bath. The electrophoretic mobility of washed erythrocytes in buffer (0.15 *M* NaCl-0.0001 *M* $NaHCO_3$, pH 7.2) was used as an internal control. Values of -1.09 ± 0.06 μm/sec/V/cm (mean ± SD, $n = 3$) were obtained which are in agreement with those reported by others (22, 23).

Results. *^{45}Calcium binding to platelets from healthy volunteers: Effect of magnesium and pH.* The amount of calcium bound to platelets depleted of membrane-associated calcium increased with increasing calcium concentrations in the buffer medium. Greater than 95% of calcium bound to platelets after 2 min was accessible to removal by EDTA, suggesting that it was membrane-bound and had not been internalized (5). As reported by others (10, 11), platelets depleted of loosely associated surface calcium following brief calcium deprivation rebound ^{45}calcium at pH 7.5 in an apparently biphasic manner, suggesting the presence of at least two membrane-associated calcium binding compartments. The first approached saturation at 200 μ*M* free ^{45}calcium, while the second failed to saturate even at 1 m*M* free ^{45}calcium (Fig. 1). Restoration of platelet aggregation in response to ADP (10 μ*M*) paralleled calcium binding to the saturable compartment (Fig. 2). To further characterize this compartment, Scatchard plots were constructed (Fig. 1, insert) and resolved into two compartments according to the methods of Rosenthal (24) and Feldman (25). This analysis suggested that calcium bound to approximately 360,000 ± 100,000 (SD, $n = 4$) saturable platelet membrane-associated binding sites with a $K_{d,\mathrm{app}}$ of $3.0 \pm 1.5 \times 10^{-5}$ *M*.

In the presence of 10 m*M* $MgCl_2$ or at pH 6.0, calcium binding was reduced 34 ± 6 and 32 ± 8% (SD, $n = 9$), respectively, over a range of calcium concentrations (25–1000 μ*M*) (Table I). These data indicate that both the saturable and nonsaturable components were equally sensitive to magnesium and pH. No additive inhibition was noted at pH 6.0 and in the presence of $MgCl_2$, suggesting that magnesium and hydrogen ions competed for the same calcium binding sites. Both magnesium and hydrogen ions rapidly displaced

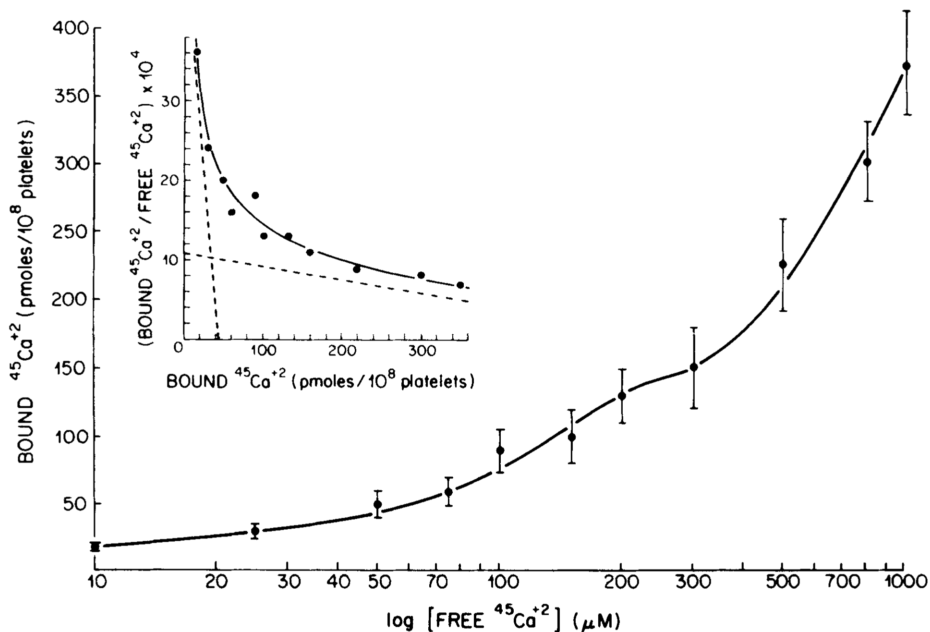

FIG. 1. ^{45}Calcium binding to platelets depleted of surface-associated calcium by brief incubation with EDTA. Scatchard analysis of the data is shown in the insert. Error bars indicate SD of four experiments.

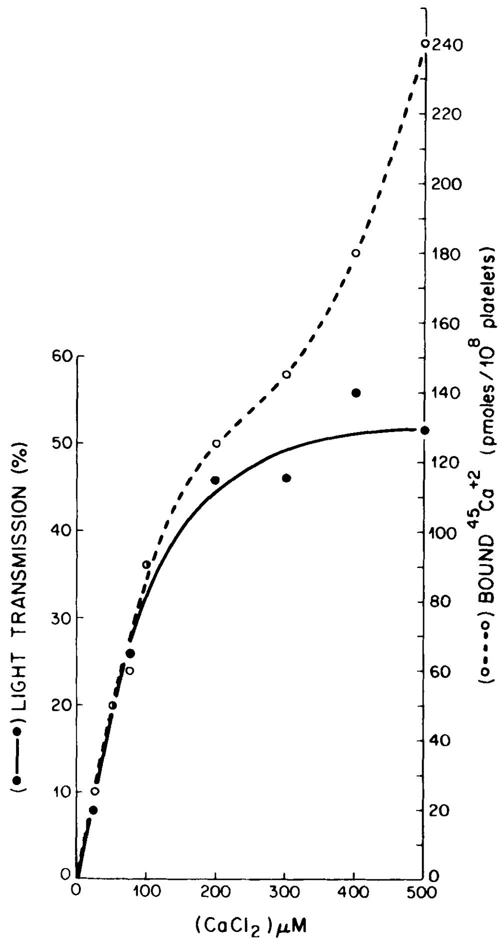

FIG. 2. Correlation between ^{45}calcium binding (---) and platelet aggregation (—) at $CaCl_2$ concentrations between 25 and 500 μM. Platelets were aggregated with 10 μM ADP in the presence of 0.2 mg/ml purified fibrinogen.

surface bound ^{45}calcium as illustrated in Fig. 3.

Effect of magnesium and pH on calcium binding to platelets lacking GPI or GPIIb/GPIIIa. To assess whether the magnesium or pH-dependent decrease in calcium binding was associated with changes in calcium binding to one of the three major platelet membrane glycoproteins, particularly the fibrinogen receptor, experiments were performed using (a) platelets from two patients with thrombasthenia that lack GPIIb and IIIa, (b) platelets from one patient with the Bernard Soulier Syndrome lacking GPI, and (c) chymotrypsin-treated platelets also lacking GPI. Table II depicts results of studies measuring calcium binding at 50, 100, 200, and 1000 μM free calcium. All platelets except those from the patients with thrombasthenia bound 28–32% less ^{45}calcium in the presence of magnesium and/or at pH 6.0. ^{45}Calcium binding to thrombasthenic platelets was reduced 49 ± 6 and 42 ± 8% by magnesium and hydrogen ions, respectively. This in-

TABLE I. EFFECT OF MAGNESIUM AND LOW pH ON ^{45}CALCIUM BINDING TO PLATELETS FROM NORMAL VOLUNTEERS

	^{45}Calcium bound[a] to platelets suspended in		
$^{45}CaCl_2$ (μM)	HBMT (mean ± SD)[c]	HBMT + Mg[b] (mean ± SD)[c]	HBMT, pH 6.0 (mean ± SD)[c]
25	29 ± 6	20 ± 4	20 ± 4
50	38 ± 7	23 ± 6	27 ± 5
75	50 ± 8	33 ± 8	N.D.[d]
100	56 ± 6	40 ± 8	43 ± 6
150	69 ± 19	45 ± 4	52 ± 3
200	96 ± 30	56 ± 15	N.D.
300	104 ± 15	76 ± 16	74 ± 12
500	152 ± 16	93 ± 18	N.D.
1000	281 ± 95	207 ± 71	203 ± 64

[a] pmoles per 10^8 platelets.
[b] 10 mM $MgCl_2$.
[c] n = 13.
[d] N.D. = not done.

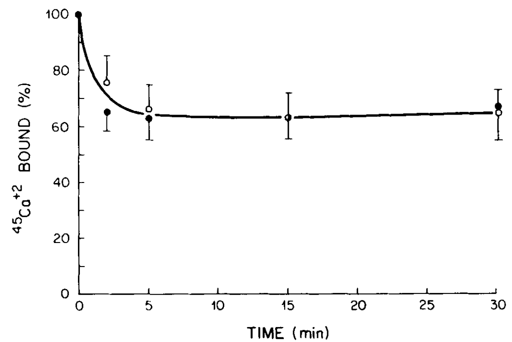

FIG. 3. Displacement of platelet-bound ^{45}calcium by 20 mM $MgCl_2$ (O) or a decrease in pH to pH 6.0 (●). The initial ^{45}calcium binding occurred in Mg^{+2}-free HBMT, pH 7.5, containing $^{45}CaCl_2$ (150 μM). After 2 min, either $MgCl_2$ (20 mM) was added to the platelet suspension, or the pH was lowered to pH 6.0 by gradually adding HCl (0.1 N) diluted in 0.15 M NaCl. Error bars indicate the SD, n = 5.

TABLE II. ^{45}CALCIUM BINDING TO NORMAL PLATELETS, CHYMOTRYPSIN-TREATED PLATELETS, AND PLATELETS FROM PATIENTS WITH THROMBASTHENIA AND THE BERNARD SOULIER SYNDROME

		^{45}Calcium bound[a] to platelets in			
$^{45}CaCl_2$ (μM)	Platelets	HBMT (mean ± SD)[c]	HBMT + Mg[b] (mean ± SD)[c]	HBMT, pH 6.0 (mean ± SD)[c]	HBMT + Mg, pH 6.0 (mean ± SD)[c]
50	Normal	38 ± 7	27 ± 3	26 ± 5	27 ± 4
	Thrombasthenic	14 ± 3	7 ± 1	6 ± 2	7 ± 2
	Bernard Soulier	92 ± 1	66 ± 7	63 ± 9	69 ± 11
	Chymotrypsin	38 ± 4	26 ± 5	29 ± 4	27 ± 2
100	Normal	63 ± 10	43 ± 8	45 ± 6	40 ± 5
	Thrombasthenic	35 ± 7	17 ± 6	14 ± 6	13 ± 8
	Bernard Soulier	169 ± 14	118 ± 12	109 ± 14	115 ± 9
	Chymotrypsin	68 ± 7	47 ± 6	46 ± 5	49 ± 8
200	Normal	107 ± 12	76 ± 10	77 ± 10	76 ± 12
	Thrombasthenic	56 ± 8	26 ± 7	25 ± 6	29 ± 10
	Bernard Soulier	221 ± 34	153 ± 35	159 ± 21	155 ± 25
	Chymotrypsin	124 ± 20	86 ± 17	87 ± 16	86 ± 19
1000	Normal	213 ± 31	154 ± 26	152 ± 24	155 ± 15
	Thrombasthenic	104 ± 23	53 ± 12	40 ± 15	64 ± 17
	Bernard Soulier	596 ± 47	424 ± 36	429 ± 38	422 ± 37
	Chymotrypsin	238 ± 26	171 ± 16	175 ± 20	176 ± 19

[a] pmoles/10^8 platelets.
[b] Platelets in HBMT containing 10 mM $MgCl_2$.
[c] For normal platelets and chymotrypsin-treated platelets n = 13, for platelets from patients with thrombasthenia n = 4, for platelets from patients with the Bernard Soulier Syndrome n = 2.

creased inhibition was attributed to the overall decrease (approximately 50%) in ^{45}calcium binding to thrombasthenic platelets observed under control conditions rather than an increased effect of magnesium or hydrogen ions. Platelets from the patient with Bernard Soulier Syndrome which are larger than normal bound two- to threefold more ^{45}calcium in HBMT at pH 7.5 than control platelets, while chymotrypsin-treated platelets, which also lack GPI but are of normal size, bound the same amount of calcium.

Effects of changes in platelet membrane-associated calcium on electrophoretic mobility. Calcium deprivation as performed in this study had no measurable effect on platelet electrophoretic mobility. Values of −0.88 ± 0.02 and −0.89 ± 0.01 μm/sec/V/cm were obtained for platelets depleted of surface-associated calcium before and after recalcification (1 mM $CaCl_2$), respectively. Recalcification in the presence of magnesium (10 mM) or at pH 6.0 also failed to change the electrophoretic mobility measurements (−0.87 ± 0.03 and −0.89 ± 0.02 μm/sec/V/cm, respectively).

Discussion. Platelet aggregation requires the presence of certain cofactors, most notably fibrinogen and the divalent cations calcium and/or magnesium. While the interaction between platelets and fibrinogen via the GPIIb/IIIa complex has been extensively characterized (4, 26–29) and correlates directly with the ability of platelets to aggregate (1), the regulation of platelet function by extracellular divalent cations is less clear. It is known, however, that platelet aggregation in citrated plasma or plasma anticoagulated with heparin is completely inhibited by EDTA and less effectively by EGTA (12). Platelet aggregation under such conditions is restored by addition of calcium and/or magnesium (30). These observations as well as more recent studies (2) characterizing the divalent cation dependence of platelet–fibrinogen interactions have led to the conclusion that magnesium can support primary platelet aggregation and fibrinogen binding in the absence of sufficient calcium.

The interaction between calcium and platelets is complex. At least three apparently independent classes of membrane-associated

binding sites have been described (10): two saturable high affinity binding sites and a lower affinity, nonsaturable site. Studies are complicated not only by the need to first deplete platelets of membrane-associated calcium, but also by the fact that calcium binding is accompanied by a simultaneous uptake of calcium into an intracellular compartment, most likely the dense tubular system (5, 10).

In order to characterize more fully those platelet–calcium interactions that modulate aggregation, this study evaluated calcium binding to platelets in the presence of magnesium and at a low pH, conditions known to respectively support and inhibit platelet aggregation (13). Platelets were depleted of loosely associated surface calcium by brief exposure to EDTA at 22°C. ^{45}Calcium was added subsequently. Its association with platelets was quantified after 2 min because platelet aggregation could be restored immediately upon adding $CaCl_2$, and minimal calcium uptake was noted under these conditions.

This revealed the presence of new low affinity calcium binding sites on the platelet membrane that were sensitive to the presence of magnesium and low pH, and whose binding of calcium paralleled the restoration of platelet aggregation following platelet exposure to EDTA. Compared to studies reported by Brass and Shattil (10, 11) which examined the association of calcium with platelets at equilibrium when a steady state had been reached between calcium binding and calcium uptake, these binding sites expressed a K_d at least 100-fold lower than that reported by the above authors for high affinity platelet membrane calcium binding sites.

Although calcium binding to fibrinogen and its contribution to the restoration of platelet aggregation cannot be discounted on the basis of this study, it was considered of minor importance. For example, previous studies have shown that prolonged calcium deprivation (>30 min) is necessary before fibrinogen loses its clottability (31, 32). Since platelet aggregation was initiated immediately after adding fibrinogen to platelet suspensions containing various amounts of calcium in the present study, it is doubtful that the observed loss in platelet aggregability resulted from decreased fibrinogen function rather than insufficient platelet membrane-associated calcium. Moreover, calcium binding to platelets depleted of membrane-associated calcium does not increase when platelets are stimulated with ADP and bind fibrinogen (5), and the calcium binding sites on the γ chain of fibrinogen are not directly involved in platelet–fibrinogen interactions (33).

Magnesium and hydrogen ions which modulate the ability of platelets to aggregate were noted to decrease calcium binding (30%) to both the saturable and nonsaturable calcium binding sites. Since no additive inhibition of calcium binding was observed in the presence of both magnesium and increased hydrogen ion concentrations, these ions appear to interact with the same platelet membrane binding sites. The complexity of platelet–calcium interactions, however, makes interpretation of double-reciprocal plots (34) of calcium binding to platelets at different magnesium and hydrogen ion concentrations difficult. Curved rather than straight lines are generated and do not easily permit the distinction between direct competitive effects between calcium, magnesium, and hydrogen ions and allosteric effects.

The observation that magnesium and hydrogen ions only partially inhibit calcium binding to the saturable and nonsaturable binding sites may suggest that the latter are composed of heterogeneous rather than homogeneous receptors. Since the percentage inhibition of calcium binding remained relatively constant over a wide range of calcium concentrations, it is unlikely that the observed inhibition of calcium binding was due primarily to an inhibition of calcium binding to the nonsaturable platelet membrane compartment, which can interfere with the characterization of the high affinity, saturable compartment because of its comparatively large number of binding sites.

To date, the only platelet membrane calcium binding sites identified are GPIIb and IIIa (5, 6, 11). Lack of these glycoproteins (5, 11) or disruption of the complex (9) results in decreased calcium binding, diminished fibrinogen binding (1), and failure of platelets to aggregate (15). It has been suggested that GPIIb and IIIa bind calcium with both high and low affinity (11). This is based

on studies of calcium binding to platelets from patients with thrombasthenia, illustrating a decrease in the number of both high and low affinity sites with no change in receptor affinities (10).

The high affinity binding sites have been proposed to regulate GPIIb/IIIa complex formation (27). They are apparently specific for calcium (3), saturate at 10^{-6} M calcium, and are generally absent on platelets from patients with thrombasthenia (10, 11). In comparison, lower affinity sites appear to be specific for both calcium and magnesium, saturate at 10^{-3} M divalent cations (35), and are only partially absent from thrombasthenic platelets (11). These sites resemble the saturable platelet membrane calcium binding component described in the present study. They are specific for either calcium or magnesium, and their binding of calcium is only partially inhibited at pH 6.0 and/or in the presence of excess magnesium. Because the interactions between calcium and magnesium or hydrogen ions were quantitatively similar if platelets from healthy volunteers or patients with thrombasthenia were examined, it appears that calcium binding sites that modulate platelet aggregation under conditions chosen in the present study, constitute lower affinity calcium binding sites that do not represent GPIIb and IIIa. Furthermore, the effect of magnesium and pH on calcium binding to platelets deficient in GPI supports previous studies by Gogstad *et al.* (36) demonstrating that GPIb fails to bind ^{45}calcium following the separation of platelet membrane constituents by crossed immunoelectrophoresis.

Since changes in calcium binding brought about by the presence of magnesium or low pH in this study were found to have no measurable effect on calcium binding to the two glycoproteins occupying a central role in platelet function, it was postulated that these ions might alter platelet surface charge instead, and thus modulate the aggregation response (23). The electrophoretic mobility of platelets depleted of surface-associated calcium, however, was unchanged before or after rebinding calcium in the presence or absence of magnesium or at low pH.

In conclusion, data from the present study suggest that whereas modulation of platelet aggregation by magnesium and low pH is associated with changes in platelet membrane-associated calcium, calcium binding to the three major platelet membrane glycoproteins GPI, IIb, and IIIa is unaffected. Thus, calcium binding to GPIIb and IIIa alone is not sufficient for fibrinogen binding or platelet aggregation, and other divalent cation binding sites may serve equally important regulatory functions.

The author thanks Jean Ann Wainer for expert technical assistance, Dr. Margaret M. Johnson, Wilmington, Delaware, for facilitating the study of platelets from patients with thrombasthenia and the Bernard Soulier Syndrome, and Dr. Barry Coller for advice on studies of platelet electrophoretic mobility and use of the electrophoretic mobility apparatus.

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Received October 25, 1984. P.S.E.B.M. 1985, Vol. 179.
Accepted March 1, 1985.