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Calcium binding to human platelet integrin GPIIb/IIIa and to its constituent glycoproteins

Effects of lipids and temperature

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Platelet plasma membrane glycoproteins IIb (GPIIb) and IIIa (GPIIIa) form a Ca^{2+}-dependent heterodimer, GPIIb/IIIa, which serves as the receptor for fibrinogen and other adhesive proteins at the surface of activated platelets. Using equilibrium dialysis measurements, it was established that both GPIIb and GPIIIa in solution have low-affinity Ca^{2+}-binding sites (K_d 0.2–0.3 mM), five in GPIIb and two in GPIIIa, and it was confirmed that only the α-chain of GPIIb (GPIIbα) binds Ca^{2+}. Furthermore, Ca^{2+} binding was found with two CNBr fragments of GPIIb, GPIIbα-(1–285) and GPIIbα-(314–489), which carry three out of the four putative Ca^{2+}-binding sites. GPIIb/IIIa in solution has a single high-affinity Ca^{2+}-binding site (K_{d_1} 80 ± 30 nM at 21 °C), whose degree of saturation regulates the state of association of GPIIb and GPIIIa in the GPIIb/IIIa heterodimer at room temperature, and 3–4 medium-affinity Ca^{2+}-binding sites (K_{d_2} 40 ± 15 μM at 21 °C). When GPIIb/IIIa was incorporated into liposomes, K_{d_1} decreased by an order of magnitude (9 ± 3 nM at 21 °C) and reached the dissociation constant estimated for the high-affinity Ca^{2+}-binding sites at the platelet surface [Brass & Shattil (1982) J. Biol. Chem. **257**, 1400–1405], whereas K_{d_2} remained unchanged. The high-affinity Ca^{2+}-binding site of GPIIb/IIIa in solution at 4 °C has almost the same affinity (K_{d_1} 65 ± 20 nM) as at 21 °C; however, at 37 °C, either its affinity decreases enough so as to become experimentally indistinguishable from the medium-affinity Ca^{2+}-binding sites determined at this temperature (number of binding sites 3.9 ± 1.2 mol of Ca^{2+}/mol of GP, K_d 25 ± 11 μM), or vanishes altogether. Studies on Ca^{2+}-dependent dissociation of GPIIb/IIIa at 37 °C in solution seem to support the former interpretation. Further work will be necessary to decide whether the dissociation of GPIIb/IIIa in the platelet membrane at 37 °C is regulated by the degree of saturation of the high-affinity Ca^{2+}-binding site, as occurs in solution. It is suggested that the high-affinity Ca^{2+}-binding site could be related to the putative GPIIIa-binding region in GPIIb (residues 558–747 of the α chain).

INTRODUCTION

It is well established that extracellular Ca^{2+} is required for physiological platelet aggregation (Born & Gross, 1964; Bennett & Vilaire, 1979; Marguerie *et al.*, 1979), that platelet glycoproteins IIb (GPIIb) and IIIa (GPIIIa) form a Ca^{2+}-dependent complex in solution (Kunicki *et al.*, 1981; Jennings & Phillips, 1982; Brass *et al.*, 1985) and in the platelet membrane (Fujimura & Phillips, 1983*a*; Brass *et al.*, 1985; Fitzgerald & Phillips, 1985), and that there are several classes of rapidly exchangeable Ca^{2+}-binding sites at the surface of intact platelets (Brass & Shattil, 1982; Peerschke, 1985; Johnston & Heptinstall, 1988). Studies on Ca^{2+} transport across the platelet plasma membranes of normal and thrombasthenic platelets suggested that GPIIb/IIIa is necessary for platelet Ca^{2+} homeostasis (Brass, 1985). This has been supported by Ca^{2+}-influx measurements both during platelet activation (Yamaguchi *et al.*, 1987) and in GPIIb/IIIa-carrying liposomes (Rybak *et al.*, 1988).

From the differences between the Ca^{2+} bound to the surface of normal and thrombasthenic platelets, and assuming that these differences are due only to the GPIIb/IIIa deficiency in thrombasthenic platelets, Brass & Shattil (1984) estimated that each GPIIb/IIIa complex has two high-affinity (K_{d_1} 9 ± 2 nM) and six medium-affinity (K_{d_2} 0.4 ± 0.1 μM) Ca^{2+}-binding sites. There are discrepancies between results from several laboratories on the Ca^{2+}-binding ability of isolated GPIIb and GPIIIa (Fujimura & Phillips, 1983*b*; Gogstad *et al.*, 1983; Karpatkin *et al.*, 1986). Equally, there appear to be severe discrepancies in the literature with regard to the Ca^{2+} concentration required to maintain the association of GPIIb with GPIIIa in the heterodimer GPIIb/IIIa, both in solution and in the natural membrane (Fujimura & Phillips, 1983*a*; Brass *et al.*, 1985; Fitzgerald & Phillips, 1985; Steiner *et al.*, 1989; Rivas, 1989).

In the present work we have carried out direct equilibrium measurements of Ca^{2+} binding to pure GPIIb, GPIIIa and GPIIb/IIIa in Triton X-100 solutions and to GPIIb/IIIa reconstituted in liposomes. We have studied the effects of temperature and lipids on the high-affinity Ca^{2+}-binding site determined in GPIIb/IIIa and confirmed the presence of some of the four Ca^{2+}-binding sites predicted for the α chain of GPIIb (GPIIbα) from its cDNA-derived amino acid sequence (Poncz *et al.*, 1987). These findings are discussed in relation to the actual discrepancies pointed out above.

MATERIALS AND METHODS

Materials

Water (containing 2–4 μM-Ca^{2+}) from a Milli Q purification

Abbreviations used: GPIIb and GPIIIa, glycoproteins IIb and IIIa respectively; GPIIb/IIIa, heterodimer of GPIIb and GPIIIa, GPIIbα and GPIIbβ, the α and β chains of GPIIb, after reduction of the single disulphide bond joining them and further carboxymethylation; *N*, number of binding sites (mol of Ca^{2+} bound/mol of glycoprotein).

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system (Millipore, Bedford, MA, U.S.A.) was used. ^{45}Ca (830 μCi/μmol) was from Amersham. The rest of the chemicals and biochemicals were of analytical or chromatography grade. Chromatographic columns and buffers, as well as the preparation of platelets and platelet plasma membranes, and the isolation of GPIIb, GPIIIa, GPIIbα, GPIIbβ and the CNBr fragments of GPIIb, were as previously described (Eirin *et al.*, 1986; Calvete & González-Rodríguez, 1986; Calvete *et al.*, 1989). The GPIIb/IIIa heterodimer was isolated as described elsewhere (Rivas, 1989; G. A. Rivas, J. J. Calvete & J. González-Rodriguez, unpublished work). Briefly, after differential extraction of platelet plasma membranes with Triton X-100 as described previously (Eirin *et al.*, 1986), the 4% Triton supernatant was subjected to ion-exchange chromatography on a DEAE-Sephacel column (5 cm × 20 cm) equilibrated in 50 mM-Tris/HCl/25 mM-NaCl/0.1 mM-$CaCl_2$/0.5% Triton X-100, pH 7.0. The retained fraction that was eluted with 0.5 M-NaCl in the same buffer contained 2% of the total phospholipids, 60% of the total protein and 80–90% of the GPIIb/IIIa loaded on to the column, providing a 4-fold enrichment in GPIIb/IIIa with respect to the original membrane. This fraction was directly loaded on to a Sephacryl S 300 column (5 cm × 140 cm) equilibrated in 50 mM-Tris/HCl/0.1 mM-$CaCl_2$/0.025% NaN_3/0.2% Triton X-100, pH 7.4. The second band eluted from this column was the GPIIb/IIIa fraction, which contained no detectable phosphorus (less than 1 μg/mg of protein) and 85% of the GPIIb/IIIa originally loaded on to the column. This fraction, after being concentrated on a DEAE-Sephacel column, was rechromatographed on the same Sephacryl S 300 column, from whose main fraction pure GPIIb/IIIa was obtained with a 47±8% yield with respect to the GPIIb/IIIa content of the starting platelet membrane. All of these isolation steps were performed at 4 °C.

Analytical procedures

Protein assay was done according to Markwell *et al.* (1978). Amino acid and amino sugar analyses were performed after sample hydrolysis at 110 °C in 6 M-HCl for 24 h and in 4 M-HCl for 4 h in a Biotronik amino acid analyser. SDS/PAGE was carried out according to Laemmli (1970). Electroblotting was carried out after gel electrophoresis by transferring the protein bands to nitrocellulose membranes by a standard procedure (Towbin *et al.*, 1979). Binding of Ca^{2+} to the transferred bands and ^{45}Ca autoradiography was effected according to the method of Maruyama *et al.* (1984).

Incorporation of GPIIb/IIIa into liposomes

GPIIb/IIIa (2 mg/ml) in 50 mM-Tris/HCl/0.1 mM-$CaCl_2$/0.025% (w/v) NaN_3/1% (w/v) Triton X-100 buffer, pH 7.4, was mixed with egg-yolk lecithin (Sigma) at a 1:6 (w/w) protein/lecithin ratio, and left at room temperature for 1 h under gentle agitation. Then the mixture was diluted with the same buffer but without detergent (liposome buffer) to bring the Triton X-100 to below its critical micellar concentration (0.016%). The diluted mixture was concentrated in a membrane concentration device (Amicon) and subjected to several dilution/concentration cycles to eliminate the detergent. The GPIIb/IIIa/lecithin multilayers formed in the final 2 ml sample were pelleted at 90000 rev./min in an Airfuge (Beckman). The pellet was resuspended in 1 ml of liposome buffer and subjected to four cycles (30 s each) of sonication at 4 °C under an N_2 atmosphere using a Soniprep 150 (MSE). The sonicated vesicles were subjected to size-exclusion chromatography on a Sephacryl S 1000 column (1.5 cm × 140 cm) equilibrated in a liposome buffer. The column had previously been presaturated with egg-yolk lecithin as described before (Nozaki *et al.*, 1982) and calibrated using latex spheres of 109±2.7 and 305±8.4 nm average diameter (Sigma). That the interior of these liposomes (150±30 nm in diameter) becomes equilibrated with the external Ca^{2+} was determined by fluorescence using Quin 2 (10 μM)-loaded liposomes.

Ca^{2+}-binding assay

Equilibrium dialysis and regulation by EGTA of the free Ca^{2+} concentration were used to obtain the Ca^{2+}-binding isotherms of GPIIb, GPIIbα, GPIIbβ, GPIIIa and GPIIb/IIIa in 50 mM-Tris/HCl/0.1% Triton X-100, pH 7.4 (equilibrium buffer), and of GPIIb/IIIa incorporated into liposomes of egg-yolk lecithin in liposome buffer. Before use, glassware, plasticware and dialysis tubing were treated according to Marguerie *et al.* (1977) and Potter *et al.* (1983). Total Ca^{2+} concentrations were determined by atomic absorption spectrometry (Perkin–Elmer, model 303), using Ca^{2+} standard solutions, either prepared according to Forstner & Manner (1971) or obtained from Merck. ^{45}Ca was measured in a scintillation counter (LKB Rackbeta, model 1217) using a ^{45}Ca program. Below 10 μM, the required free Ca^{2+} concentration was adjusted by addition of EGTA, as calculated according to Fabiato (1981) to account for the influence of pH, temperature and ionic strength on the equilibrium constants used. Demineralization of the glycoproteins (3–5 mg/ml) in equilibrium buffer was done overnight at 4 °C by dialysis against the same buffer with enough EGTA to reduce free Ca^{2+} below 1 nM (Marguerie *et al.*, 1977), except for GPIIb/IIIa, which was dialysed at 22 °C for 1 h in order to avoid irreversible dissociation of the heterodimer (Rivas, 1989). By this procedure Ca contents below 0.2 mol/mol of glycoprotein were always achieved, as determined by atomic absorption spectrometry.

Portions of 250 μl of the glycoprotein solution (3.5 mg/ml) in equilibrium buffer were dialysed at 22 °C overnight against 200 ml of equilibrium buffer enriched with ^{45}Ca and containing between 1 nM and 1 mM free Ca^{2+}, calculated as indicated above and taking into consideration the ^{45}Ca, the contaminating Ca^{2+} and the added Ca^{2+} in the buffer, and the added EGTA. Once the equilibrium was reached, two 100 μl samples of the solution inside and outside the dialysis bags were taken for ^{45}Ca measurements. The rest of the solution inside was used for glycoprotein determination and electrophoretic analysis. The Ca^{2+} bound to the glycoprotein was calculated according to Potter *et al.* (1983), and the data were fitted to the expression $B = \Sigma(N_i \cdot k_i \cdot [Ca^{2+}]/1 + k_i \cdot [Ca^{2+}])$ by a Marquardt non-linear least-squares procedure, where B is the mol of Ca^{2+} bound/mol of glycoprotein, N_i is the number of Ca^{2+}-binding sites of class i and affinity constant k_i, and $[Ca^{2+}]$ is the free Ca^{2+} concentration.

RESULTS

Ca^{2+}-binding isotherm of GPIIb and its subunits

Fig. 1 shows the Ca^{2+}-binding isotherm (21 °C) of pure GPIIb in equilibrium buffer measured by equilibrium dialysis (see the Materials and methods section) in the range 1 nM–0.5 mM-Ca^{2+}. The continuous line is the best fit to the experimental data (average of 12 experiments) and corresponds to a single class of low-affinity binding sites (N 5.4±0.9 mol of Ca^{2+}/mol of GPIIb; K_d 0.17±0.03 mM). Enhancement of the ionic strength of the buffer by addition of NaCl up to 250 mM (final concentration) did not significantly change these results. When the Ca^{2+}-binding isotherms for the isolated α and β chains of GPIIb were obtained (results not shown), it was found that GPIIbα bound Ca^{2+} in the same manner as did GPIIb (N 4.9±0.6 mol/mol; K_d 0.30±0.05 mM), whereas GPIIbβ did not bind Ca^{2+} in the range of Ca^{2+} concentrations studied. These results agree with those obtained by ^{45}Ca autoradiography after separation of the

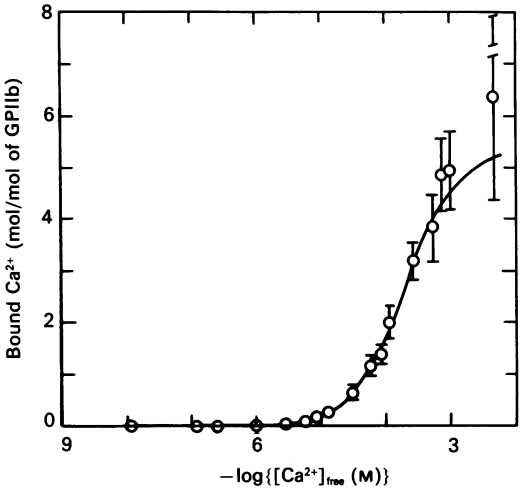

Fig. 1. Isotherm of Ca^{2+} binding to GPIIb in solution

Ca^{2+}-binding was measured at 21±1 °C as a function of the free Ca^{2+} concentration by equilibrium dialysis using ^{45}Ca, as described in the Materials and methods section. The continuous line represents the best fit of the experimental data (averages of 12 experiments) to the binding equation given in the Materials and methods section.

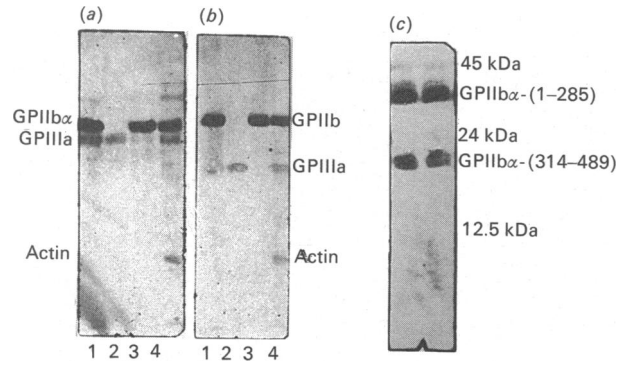

Fig. 2. Solid-phase Ca^{2+}-binding to GPIIb, GPIIIa and the CNBr cleavage fragments of GPIIb

Reduced (*a*) and non-reduced (*b*) GPIIb and GPIIIa, and the non-reduced CNBr fragments of GPIIb (*c*) were first separated by SDS/PAGE, then electroblotted on to nitrocellulose membranes and incubated with 1–10 μM-Ca^{2+} followed by ^{45}Ca autoradiography (see the Materials and methods section). Polyacrylamide gels of 7–12% (*a* and *b*) and 15% (*c*) were used. (*a*) and (*b*): lane 1, GPIIb/IIIa heterodimer; lane 2, GPIIIa; lane 3, GPIIb; lane 4, platelet plasma membrane. The preparation and the chemical and immunochemical identification of those CNBr fragments [GPIIbα-(1–285) and GPIIbα-(314–489)] of GPIIb showing Ca^{2+} binding was done as described previously (Calvete *et al.*, 1989). Molecular mass markers are given in kDa.

two subunits by SDS/PAGE, electroblotting on to nitrocellulose membranes and incubation with different Ca^{2+} concentrations within the range 1–100 μM (Fig. 2*a*).

Ca^{2+}-binding isotherm of GPIIIa

Fig. 3 displays the Ca^{2+}-binding isotherm (21 °C) of isolated GPIIIa in equilibrium buffer. The continuous line is the best fit to the experimental data (average of 8 experiments) and

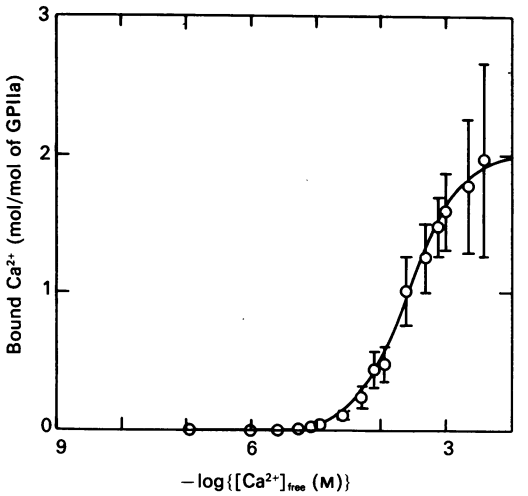

Fig. 3. Isotherm of Ca^{2+} binding to GPIIIa in solution

Ca^{2+}-binding measurements at 21±1 °C and analysis of the experimental data (averages of 8 experiments) were carried out as described in the legend to Fig. 1.

corresponds to a single class of low-affinity binding sites (N 2.0±0.4 mol/mol; K_d 0.30±0.07 mM). As observed above for GPIIb, these results did not change appreciably on increasing the ionic strength of the buffer. Because GPIIIa has a great tendency to autoaggregate (Rivas *et al.*, 1991), which is lower at pH 9.0, Ca^{2+}-binding experiments were also done at this pH; however, no significant changes were observed in the binding data. These results are in good agreement with those obtained by ^{45}Ca autoradiography after SDS/PAGE, electroblotting and incubation with different Ca^{2+} concentrations (Fig. 2*b*).

Ca^{2+}-binding isotherms of GPIIb/IIIa in solution

Fig. 4(*b*) gives the Ca^{2+}-binding isotherm (average of 10 experiments) of the isolated GPIIb/IIIa complex in equilibrium buffer measured at 21 °C in the range 1 nM–1 mM-Ca^{2+}. The best fit to the experimental points between 1 nM- and 0.5 mM-Ca^{2+} (continuous line) corresponds to two classes of binding sites: a single high-affinity site (N_1 0.9±0.2 mol/mol; K_{d_1} 80±30 nM); and between three and four medium-affinity sites (N_2 3.4±0.6 mol/mol; K_{d_2} 40±15 μM). If we include the experimental data up to 1 mM-Ca^{2+} (broken line), a third class of non-saturable low-affinity sites appears, without any changes in the binding parameters of the other two classes of sites. The experimental accuracy in this region did not allow us to obtain reliable binding data for this third class of binding sites.

Ca^{2+}-binding isotherm of GPIIb/IIIa incorporated into liposomes

When GPIIb/IIIa was incorporated into single-wall egg-yolk lecithin liposomes, at a protein/lipid ratio of 1:6 (w/w) and subjected to ^{45}Ca equilibrium dialysis measurements in the range 1 nM–1 mM-Ca^{2+}, the binding isotherm at 21±1 °C (average of 6 experiments) was obtained (results not shown). The best fit to the experimental data corresponded to two classes of binding sites: a single high-affinity site (N_1 0.8±0.2 mol/mol; K_{d_1} 9.0±2.8 nM) with an affinity one order of magnitude higher than that for the same site in the complex in solution (see above); and 3–4 medium-affinity sites (N_2 3.6±0.9 mol/mol; K_{d_2} 40±2 μM), as in the complex in solution. Again, because of the poor accuracy of the experimental results in the millimolar range, no attempt was made to fit the experimental data in this region, although a class of non-saturable low-affinity sites was apparent.

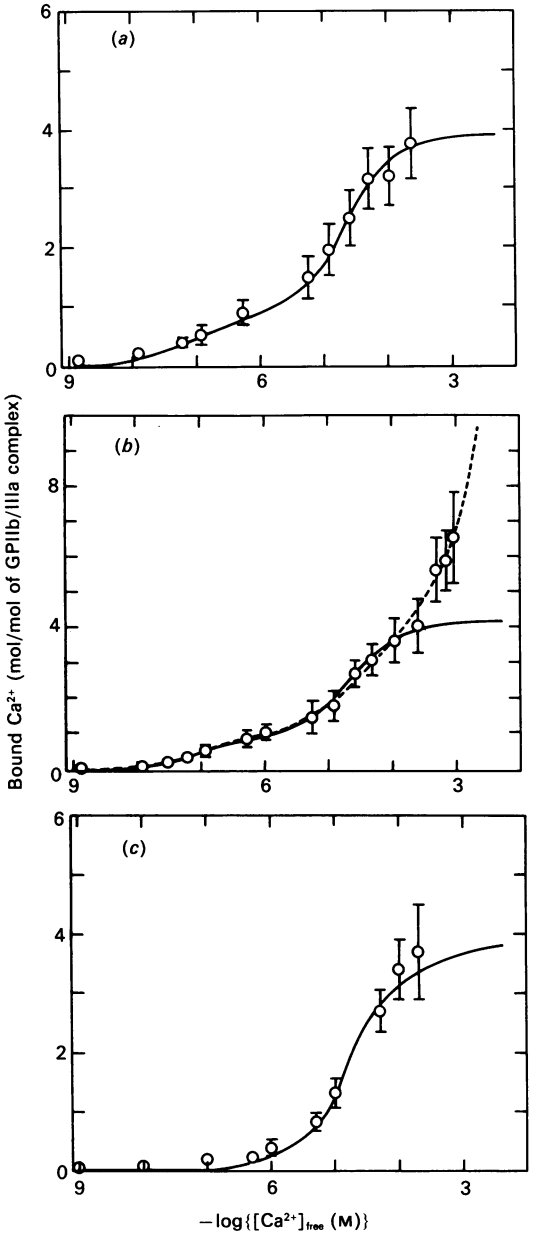

Fig. 4. Isotherms of Ca^{2+} binding to the GPIIb/IIIa heterodimer in solution at various temperatures

Ca^{2+}-binding measurements were performed at 4 ± 1 °C (*a*, averages of 4 experiments), 21 ± 1 °C (*b*, averages of 10 experiments) and 37 ± 1 °C (*c*, averages of 4 experiments). Experimental data analysis was as in the legend to Fig. 1. The broken line in (*b*) is the best fit obtained when the experimental data up to 1 mM-Ca^{2+} is included.

Effect of temperature on the high-affinity Ca^{2+}-binding site of GPIIb/IIIa in solution

The Ca^{2+}-binding isotherm of GPIIb/IIIa in solution at 4 °C is shown in Fig. 4(*a*). The best fit to the experimental data corresponds to a single high-affinity site with an affinity (K_{d_1} 65 ± 20 nM) almost the same as that found above for this site at 21 °C. At 37 °C either the high-affinity site was lost or its affinity decreased so much as to become experimentally indistinguishable from the medium-affinity Ca^{2+}-binding sites determined at 37 °C (N 3.9 ± 1.2 mol/mol, K_d 25 ± 11 μM) (Fig. 4*c*).

Localization of the Ca^{2+}-binding sites predicted in GPIIb

When isolated GPIIb is cleaved with CNBr and the cleavage products are separated by SDS/PAGE, three main CNBr products are obtained (Calvette *et al.*, 1989): the 35 kDa fragment, which corresponds to the GPIIbα-(1–285) peptide sequence; the 20 kDa fragment, identified with the GPIIbα-(704–856) and GPIIbβ_2-(1–32) peptide stretch; and the 18 kDa fragment, which corresponds to the GPIIbα-(314–489) peptide sequence. Electroblotting these fragments into nitrocellulose membranes and incubation with $^{45}Ca^{2+}$ in the range 1–10 μM, followed by ^{45}Ca autoradiography, showed Ca^{2+} binding only to the fragments containing the GPIIbα-(1–285) and -(314–489) peptide stretches (Fig. 2*c*).

DISCUSSION

The cDNA-derived amino acid sequences for GPIIb (Poncz *et al.*, 1987) and GPIIIa (Fitzgerald *et al.*, 1987) predict four Ca^{2+}-binding sites for the α chain of GPIIb [α-(243–254), α-(297–308), α-(365–376) and α-(426–437)] and none in GPIIIa. Here we have established that both GPIIb and GPIIIa in solution have low-affinity Ca^{2+}-binding sites (K_d 0.2–0.3 mM), five in GPIIb and two in GPIIIa, and we have confirmed that only the α chain of GPIIb binds Ca^{2+}. In addition, we have found Ca^{2+} binding to fragments of CNBr cleavage of GPIIb, i.e. GPIIbα-(1–285) and GPIIbα-(314–489) (Calvete *et al.*, 1989), which carry three out of the four putative Ca^{2+}-binding sites [α-(243–254), α-(365–376) and α-(426–437)]. As happens in other Ca^{2+}-binding proteins (McLennan *et al.*, 1985; Stuart *et al.*, 1986), there are no apparent structural bases (Ca^{2+}-binding β turns) for predicting one out of the five binding sites determined here in GPIIbα or for predicting the two binding sites determined in GPIIIa. Direct demonstration of Ca^{2+} binding to GPIIb and GPIIIa supports previous findings and suggestions that the conformation of both glycoproteins and their individual contributions to fibrinogen binding to the GPIIb/IIIa complex are Ca^{2+}-dependent (Fujimura & Phillips, 1983*b*; Yamamoto *et al.*, 1989).

GPIIb/IIIa in solution at 21 °C has a single high-affinity Ca^{2+}-binding site, as determined by equilibrium dialysis, whose K_d (80 ± 30 nM) is five times lower than the Ca^{2+} concentration (400 nM) at which half-maximal binding of a GPIIb/IIIa-specific monoclonal antibody to GPIIb/IIIa occurs at 25 °C (Brass *et al.*, 1985), and very close to the Ca^{2+} concentration (100–200 nM) required for half-maximal dissociation of GPIIb/IIIa, as assessed in the 21–25 °C range by density-gradient and analytical centrifugation (Steiner *et al.*, 1989; G. A. Rivas, R. Usobiaga & J. González-Rodríguez, unpublished work). This seems to demonstrate that the stability of the GPIIb/IIIa heterodimer in solution at room temperature is regulated by the degree of saturation of its high-affinity Ca^{2+}-binding site, i.e. in the nanomolar range. At 4 °C, K_{d_1} (65 ± 20 nM) does not appreciably change and GPIIb/IIIa remains undissociated even at 0.1 nM, as assessed by analytical ultracentrifugation (G. A. Rivas, R. Usobiaga & J. González-Rodríguez, unpublished work). This shows that the stability of the complex at lower temperatures is Ca^{2+}-independent within this range of Ca^{2+} concentrations. It also explains the inability of the chelating agents to dissociate GPIIb/IIIa in solution at 4 °C (Rosa *et al.*, 1984) and why Fitzgerald & Phillips (1985) could assess the GPIIb/IIIa heterodimer in intact human platelets after lysis and solubilization at 4 °C in Triton X-100 buffers containing EDTA.

At 37 °C in solution, the accuracy of the binding data does not permit us to decide whether the high-affinity Ca^{2+}-binding site becomes structurally lost, or whether its affinity decreases to the point of becoming experimentally indistinguishable from the medium-affinity sites determined in these conditions (N 3.9 ± 1.2 mol/mol; K_d 25 ± 11 μM). The latter interpretation seems to be supported by the Ca^{2+} concentration required for

half-maximal dissociation of the GPIIb/IIIa heterodimer in solution, somewhere between 10 μM-Ca^{2+} [as assessed by the susceptibility of GPIIb/IIIa to thrombin attack and by sucrose-density gradient ultracentrifugation at 37 °C (Fujimura & Phillips, 1983*a*)], and 5.3 $\pm$ 0.4 μM-Ca^{2+} [as assessed by analytical ultracentrifugation at 35 °C (G. A. Rivas, P. Usobiaga & J. González-Rodríguez)]; that is, a Ca^{2+} concentration between 2.5 and 5 times lower than the K_d determined for the medium-affinity binding sites. If this interpretation is correct it would imply that the high-affinity Ca^{2+}-binding site still regulates the stability of the GPIIb/IIIa heterodimer in solution at 37 °C.

When GPIIb/IIIa is incorporated into liposomes, the K_d of the high-affinity Ca^{2+}-binding site reaches the affinity estimated by Brass & Shattil (1984) for the high-affinity Ca^{2+}-binding sites of GPIIb/IIIa in intact platelets (K_{d_1} 9 $\pm$ 2 nM). This suggests a stabilization of the GPIIb/IIIa structure by incorporation into the lipid bilayer, with a subsequent decrease in the dissociation constant of the high-affinity Ca^{2+}-binding site, compared with that of the GPIIb/IIIa structure in solution. Phospholipid-dependent Ca^{2+} binding has been observed previously in other proteins, such as calpactin (Glenney, 1986) and protein kinase C (Bazzi & Nelsestuen, 1990).

Several laboratories have reported that with some antibodies and/or under certain circumstances, the number of surface GPIIb/IIIa molecules per platelet is about (7–10) $\times$ 10^4 (Woods *et al.*, 1986; Calvete *et al.*, 1986; Niiya *et al.*, 1987), i.e. twice the value most often found in the literature for resting platelets (Ruggeri & Hodson, 1989). The most likely interpretation seems to be that the newly exposed GPIIb/IIIa molecules could occur as a result of the opening of the surface-connected canalicular system, which is accessible to some, but not to other, extracellular proteins. If this were so, Ca^{2+} would have access to a pool of GPIIb/IIIa which is inaccessible to the majority of the antibodies in the resting platelets, and so the actual number of surface GPIIb/IIIa molecules per platelet would rise to (8–9) $\times$ 10^4. Had Brass & Shattil (1984) used this value, they would have estimated a single high-affinity and three medium-affinity Ca^{2+}-binding sites per GPIIb/IIIa molecule, in agreement with our findings.

So far, we have discussed the agreements between our data and previous results from other laboratories. Now we will deal with the main discrepancies that still remain. First, the dissociation constant of the medium-affinity Ca^{2+}-binding sites of GPIIb/IIIa (K_{d_2} 0.4 $\pm$ 0.1 μM) estimated by Brass & Shattil (1984) is two orders of magnitude lower than that determined here for GPIIb/IIIa incorporated in liposomes (40 $\pm$ 20 μM). Further studies will be required to determine whether this large difference is due to structural differences between GPIIb/IIIa in the platelet membrane and GPIIb/IIIa in liposomes of egg-yolk lecithin, or whether it is due to limitations in the previous estimations. Secondly, the apparent discrepancies in the Ca^{2+} concentration required for half-maximal dissociation of GPIIb/IIIa in the natural membrane at 37 °C seem to depend on the criteria used to assess this dissociation. Thus, whereas a value of 1–2 μM-Ca^{2+} was found when using density-gradient centrifugation (Fitzgerald & Phillips, 1985), one of 0.4 μM-Ca^{2+} was obtained when the binding of a GPIIb/IIIa-specific monoclonal antibody was used (Brass *et al.*, 1985), and a value of only 10 nM-Ca^{2+} was found when using the susceptibility to thrombin attack as a measure (Fujimura & Phillips, 1983*a*). Further work will be necessary to decide whether the dissociation of GPIIb/IIIa in the natural membrane at 37 °C is regulated by the degree of saturation of the high-affinity Ca^{2+}-binding site, as happens in solution.

Finally, there are some guiding clues on the location of the high- and medium-affinity Ca^{2+}-binding sites in GPIIb/IIIa. Thus Lam *et al.* (1989) found that the α chain of GPIIb contains sufficient information for the formation of a complex with GPIIIa and to support the binding of Arg-Gly-Asp peptides to GPIIb/IIIa. More specifically, Calvete *et al.* (1991) have suggested that the region of GPIIbα between residues 558 and 747 may carry part of the surface of interaction of GPIIb with GPIIIa in the GPIIb/IIIa heterodimer, which would imply that at least the high-affinity Ca^{2+}-binding site of GPIIb/IIIa could be related to this region of GPIIbα, where none of the four predicted Ca^{2+}-binding sites are located.

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