Micromolar Ca$^{2+}$ Concentrations Are Essential for Mg$^{2+}$-dependent Binding of Collagen by the Integrin $\alpha_2\beta_1$ in Human Platelets*

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Integrin receptor $\alpha_2\beta_1$ requires micromolar Ca$^{2+}$ to bind to collagen and to the peptide GPC(GPP)$_2$GFOGER. The I-domain contains hydroxyproline, which represents the minimum recognition sequence for the collagen $\alpha_2$ I-domain (Knight, C. G., Morton, L. F., Peachey, A. R., Tuckwell, D. S., Farndale, R. W., and Barnes, M. J. (2000) J. Biol. Chem. 275, 35–40). Platelet adhesion to these ligands is completely dependent on $\alpha_2\beta_1$ in the presence of 2 mM Mg$^{2+}$. However, we show here that this interaction was abolished in the presence of 25 μM EGTA. Adhesion of Glanzmann’s thrombasthenic platelets, which lack the fibrinogen receptor $\alpha_{IIb}\beta_3$, was also inhibited by micromolar EGTA. Mg$^{2+}$-dependent adhesion of platelets was restored by the addition of 10 μM Ca$^{2+}$, but millimolar Ca$^{2+}$ was inhibitory. Binding of isolated $\alpha_2\beta_1$ to GFOGER-GPP was 70% inhibited by 50 μM EGTA but, as with intact platelets, was fully restored by the addition of micromolar Ca$^{2+}$. 2 mM Ca$^{2+}$ did not inhibit binding of isolated $\alpha_2\beta_1$ to collagen or to GFOGER-GPP. Binding of recombinant $\alpha_2$ I-domain was not inhibited by EGTA, nor did millimolar Ca$^{2+}$ inhibit binding. Our data suggest that high affinity Ca$^{2+}$ binding to $\alpha_2\beta_1$, outside the I-domain, is essential for adhesion to collagen. This is the first demonstration of a Ca$^{2+}$ requirement in $\alpha_2\beta_1$ function.

The widely expressed integrin receptor family mediates many cell-cell and cell-matrix interactions. Each receptor comprises a heterodimeric, non-covalent complex of an $\alpha$ and a $\beta$ subunit (1). The N-terminal region of the $\alpha$ subunits has been modeled as a seven-bladed $\beta$-propeller (2), containing EF-hand-like cation-binding motifs (3). Nine $\alpha$ subunits, including $\alpha_1$ and $\alpha_2$, contain an inserted domain (I-domain) of about 200 amino acids, homologous to the von Willebrand Factor A-domain, located between blades 2 and 3 of the proposed $\beta$-propeller structure (4). The isolated I-domain of $\alpha_2$ is capable of ligand binding (4–6). The I-domain within $\alpha_2\beta_1$ is crucial for collagen binding of the entire integrin. I-domain crystal structures reveal the presence of a single metal ion-dependent adhesion site (MIDAS) (7–9), which is thought to be occupied by Mg$^{2+}$ in vivo.

The platelet integrin $\alpha_2\beta_1$ is a collagen receptor that plays an important role in hemostasis. Injury to the endothelium of a blood vessel results in the exposure of collagen fibers to circulating platelets, resulting in their adhesion and activation, leading to platelet aggregation and clot formation (10, 11). Integrin $\alpha_2\beta_1$ is essential for the recognition of collagen by platelets under flow conditions (10, 12) and platelets lacking functional integrin $\alpha_2\beta_1$ do not respond to stimulation by collagen (13), resulting in bleeding disorders. The sequence GFOGER, within a triple-helical structure, was recently identified as the minimum binding motif within collagen I for $\alpha_1$ and $\alpha_2$ I-domains (14) and has been co-crystallized with the $\alpha_2$ I-domain, verifying its interaction with the $\alpha_2$ MIDAS (15). The availability of this peptide allows the properties of $\alpha_2\beta_1$ to be resolved from those of other, non-integrin, platelet receptors for collagen (11, 16).

The affinity of some integrins depends upon Ca$^{2+}$ (17–20). Micromolar Ca$^{2+}$ is required to stabilize the 2$\beta$ heterodimeric structure of the platelet fibrinogen receptor, $\alpha_{IIb}\beta_3$ (21), which is necessary for ligand binding (22). In addition, fibrinogen binding to $\alpha_{IIb}\beta_3$ is supported by millimolar levels of either Ca$^{2+}$ or Mg$^{2+}$ (23). In marked contrast, micromolar Ca$^{2+}$ inhibits Mg$^{2+}$-dependent ligand binding of $\alpha_2\beta_1$ (24, 25). Together, this evidence suggests that Ca$^{2+}$ has a crucial role in integrin function.

Against this background, we examined the effects of the Ca$^{2+}$ chelator, EGTA, on adhesion of platelets, of $\alpha_2\beta_1$, and of recombinant $\alpha_2$ I-domain to immobilized type I collagen and to the peptide GFOGER-GPP. We demonstrate a requirement for micromolar Ca$^{2+}$ in $\alpha_2\beta_1$-mediated platelet adhesion and the binding of isolated $\alpha_2\beta_1$ to these substrates. Both the stimulatory and inhibitory Ca$^{2+}$-binding sites appear to lie outside the I-domain.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human platelets were from fresh whole blood, provided by the National Blood Service, Long Road, Cambridge, UK. Platelets lacking $\alpha_{IIb}\beta_3$ were prepared from whole blood, kindly provided by Dr. M. Makris (Royal Hallamshire Hospital, Sheffield, UK), from two type I Glanzmann’s patients. Monomeric type I collagen for use in solid phase adhesion assays was purified from bovine skin, following limited pepsin digestion, as described previously (26, 27). The anti-human integrin $\alpha_2$ (subunit) monoclonal antibody 6F1 (28) was a generous gift from Dr. B. S. Coller (Mount Sinai Hospital, New York, NY). GR144053F was a gift from Glaxo Wellcome (Stevenage, Hertfordshire, U.K.).

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§ The abbreviations used are: MIDAS, metal ion-dependent adhesion site; BSA, bovine serum albumin; CRP, collagen-related peptide; GST, glutathione S-transferase.

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UK. Chemicals were from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise stated. Recombinant α2β1 domain, as a gluthathione S-transferase (GST) fusion protein, was produced (5, 29) and used in solid phase binding assays as described previously (5, 29–31). Peptide GFO(GP)GPP, GFOER(GP), GPC (denoted GFOGER-GPP) and collagen-related peptides were synthesized as described previously (14, 30, 31). The central GFOGER sequence is the minimum recognition sequence for the α2β1 domain (14) and the flanking GPP sequences stabilize the triple-helical conformation, which is essential for recognition (14, 26). Integrin α2β1 was purified from solubilized membranes of human platelets by affinity chromatography on collagen-Sepharose (32, 33), biotinylated using an Amersham Pharmacia Biotech ECL biotinylation module, according to the manufacturer’s instructions. Purity of the preparation was assessed by separation on SDS-polyacrylamide gel electrophoresis, followed by staining with Gelcode blue stain reagent (Pierce and Warriner, Chester, UK) and image analysis with Leica Q500 (34).

Static Platelet Adhesion Assay—96-well plates (Immulon 2, Dynex Technologies, Ashford, Middlesex, UK) were coated with 100 μl per well of monomeric type I collagen or peptides GFOGER-GPP or CRP at 10 μg/ml in 0.1 M acetic acid for 1 h at 20 °C. Platelet-rich plasma was prepared from fresh whole blood after 2 spins for 1 min at 1200 × g. 10% (v/v) of ACD buffer (39 mM citric acid, 75 mM tri-sodium citrate-2H2O, 135 mM D-glucose, pH 4.5) and prostaglandin E1 (100 ng/ml final concentration; a gift from Dr. Geoffrey Cook) were synthesized as described previously (14, 30, 31). The central GFOGER sequence is the minimum recognition sequence for the α2β1 domain (14) and the flanking GPP sequences stabilize the triple-helical conformation, which is essential for recognition (14, 26). Integrin α2β1 was purified from solubilized membranes of human platelets by affinity chromatography on collagen-Sepharose (32, 33), biotinylated using an Amersham Pharmacia Biotech ECL biotinylation module, according to the manufacturer’s instructions. Purity of the preparation was assessed by separation on SDS-polyacrylamide gel electrophoresis, followed by staining with Gelcode blue stain reagent (Pierce and Warriner, Chester, UK) and image analysis with Leica Q500 (34).

FIG. 1. Platelet number is directly proportional to A405 nm. Platelets were loaded into 96-well plates and lysed for 1 h, and the absorbance of the p-nitrophenol product was read at 405 nm, as described under “Experimental Procedures.” Data are from a single experiment, representative of five identical experiments using platelets from different donors and expressed as the mean of triplicate readings ± S.D. Where error bars are absent, they were too small to be reproduced.

FIG. 2. EGTA inhibits platelet adhesion to collagen and to GFOGER-GPP. Platelets were preincubated with EGTA for 20 min in the presence of 2 mM MgCl2 and allowed to adhere to wells coated with monomeric bovine type I collagen or GFOGER-GPP as described under “Experimental Procedures.” Data are from a single experiment, representative of three identical experiments using platelets from different donors and expressed as the mean of triplicate readings ± S.D., scaled to an A405 = 1 for adhesion to collagen in the presence of 2 mM Mg2+ alone. Where error bars are absent, they were too small to be reproduced.

RESULTS AND DISCUSSION

Platelet Adhesion via Integrin α2β1, Is Calcium-dependent—We first demonstrate the linearity of the assay used to measure platelet adhesion (Fig. 1) and are in agreement with Bellavite and coworkers (35) that the relationship between absorbance at 405 nm and platelet number is linear, in our conditions up to A405 = 3.0. Platelet adhesion to monomeric bovine type I collagen and to the peptide GFOGER-GPP requires the presence of Mg2+ (14, 24, 25), although other divalent cations such as Co2+ and Mn2+, but not Ca2+, can replace Mg2+ in α2β1 binding (24) and α2β1 I-domain binding.3 Previous work (5, 24, 25) suggests that Ca2+ inhibits Mg2+-dependent adhesion. However, we found that micromolar concentrations of EGTA, a

3 L. F. Morton (this laboratory), unpublished observations.
Ca\(^{2+}\) chelator, blocked platelet adhesion to collagen and to GFOGER-GPP in the presence of 2 mM Mg\(^{2+}\) (Fig. 2). Platelet adhesion to collagen-related peptide (CRP), which is mediated by the receptor glycoprotein VI in a cation-independent manner (37), was unaffected by EGTA (data not shown).

When platelets from normal donors were preincubated with an RGD mimic, GR144053F, at the previously established maximal level for blockade of \(\alpha_{\text{IIb}}\beta_3\)-dependent adhesion to either monomeric collagen I or to GFOGER-GPP was inhibited (Fig. 3). One explanation for the involvement of \(\alpha_{\text{IIb}}\beta_3\) might be that platelet microaggregates form on the initial layer of adherent platelets. Alternatively, the adhesion might involve indirect binding of platelets to substrate in an \(\alpha_{\text{IIb}}\beta_3\)-dependent manner, as has been proposed previously (26, 28). We and others (14, 26, 28) have shown that this component of adhesion is secondary to the initial \(\alpha_{\text{IIb}}\beta_3\)-dependent adhesive process, being entirely inhibited by 6F1 (Fig. 3).

If the observed effects of EGTA were solely due to inhibition of \(\alpha_{\text{IIb}}\beta_3\), the degree of inhibition of adhesion induced by GR144053F would be the same as for EGTA alone and no additional inhibition of adhesion would occur in the presence of both of these substances. However, the left-hand side of Fig. 3 demonstrates that this is not the case; with normal platelets, 2 \(\mu\)M GR144053F resulted in \(~50\%\) inhibition of adhesion to collagen I and \(~70\%\) inhibition of adhesion to GFOGER-GPP, whereas 25 \(\mu\)M EGTA reduced adhesion to about a quarter of these values. The effect of EGTA was greater than that of GR144053F for adhesion to both collagen and to GFOGER-GPP (\(p < 0.001\), analysis of variance). Therefore, blockade of normal platelet adhesion by EGTA cannot be attributed solely to inhibition of \(\alpha_{\text{IIb}}\beta_3\) but must include inhibition of binding through \(\alpha_{\text{IIb}}\beta_1\). We do not understand why GR144053F differentially inhibits platelet adhesion to collagen I and GFOGER-GPP.

Preincubation with a monoclonal antibody specific for the integrin \(\alpha_\text{IIb}\) subunit, 6F1, completely abrogated platelet adhesion to either collagen or to GFOGER-GPP (Fig. 3), thus demonstrating the absolute requirement of this receptor for adhesion to these ligands. In control experiments (Fig. 3), 6F1 did not block adhesion of platelets to CRP (37). These observations suggest that the inhibition of normal platelet adhesion by EGTA in excess of that caused by GR144053F is due to direct action on \(\alpha_{\text{IIb}}\beta_1\).

To confirm the independence of the effect of EGTA from \(\alpha_{\text{IIb}}\beta_3\), we examined adhesion using platelets lacking the \(\alpha_{\text{IIb}}\beta_3\) receptor, from two Type I Glanzmann’s patients (Fig. 3, right-hand side). Adhesion of these platelets to collagen I and to the \(\alpha_{\text{IIb}}\beta_3\)-specific GFOGER-GPP was 80% inhibited by micromolar EGTA (Fig. 3) but was completely insensitive to GR144053F.

\(^4\) E. M. Wijnen (this laboratory), unpublished observations.

\(^5\) Determined by flow cytometry (M. Makris, unpublished observation).
Ca\(^{2+}\) Requirement for \(\alpha_2\beta_1\) Adhesion

Fig. 5. \(\alpha_2\beta_1\) preparation is at least 90% pure. 9 \(\mu\)g of \(\alpha_2\beta_1\) preparation was separated by SDS-polyacrylamide gel electrophoresis on a 6% gel.

validating the specificity of GR144053F for \(\alpha_{Ib}\beta_3\). The data in
Fig. 3 show that the EGTA-induced inhibition of platelet adhesion occurs in the absence of \(\alpha_{Ib}\beta_3\), supporting the concept that the inhibition occurs at the level of \(\alpha_2\beta_1\).

Although EGTA chelates Ca\(^{2+}\), its inhibitory effect could be due to the removal of other ions such as Zn\(^{2+}\) or Co\(^{2+}\), possibly present at trace levels in the medium, which might be essential for ligand binding. However, addition of micromolar Ca\(^{2+}\) to EGTA-inhibited platelets restored adhesive function, confirming that Ca\(^{2+}\) is sufficient to support adhesion in the presence of Mg\(^{2+}\) (Fig. 4). However, platelet adhesion to collagen I and to GFOGER-GPP was inhibited in the presence of millimolar Ca\(^{2+}\), in agreement with the work of others (24, 25). Over several different experiments, rescue of platelet adhesion occurred in the estimated free Ca\(^{2+}\) concentration ranges (36): 58–323 nM (collagen I) and 20–323 nM (GFOGER-GPP), where 50\% maximal adhesion occurred at a free Ca\(^{2+}\) concentration of 88 ± 28 or 110 ± 57 nM for collagen I and GFOGER-GPP, respectively (the latter values are given as mean ± S.E. of five determinations).

It is not surprising that the amount of Ca\(^{2+}\) needed to restore EGTA-inhibited platelet adhesion varied between experiments. The platelets obtained from individual donors are likely to vary in expression of \(\alpha_2\beta_1\), that they may secrete their granule load of Ca\(^{2+}\) in the presence of Mg\(^{2+}\) (mean ± S.D., scaled to an A\(_{500}\) = 1 for adhesion to collagen in the presence of 2 mM Mg\(^{2+}\) alone. Where error bars are absent, they were too small to be reproduced.

been speculated that integrin \(\beta\) subunits may contain I-domain-like elements (7, 38), and it is possible that \(\beta_1\) adheres to sites other than GFOGER within collagen. However, use of monoclonal antibodies directed against the \(\beta_1\) subunit did not result in significant blockade of binding (data not shown). Others have found that recombinant constructs, including \(\alpha_2\) sequence up to the end of the first EF-hand as well as the I-domain show enhanced capacity to bind collagen (4), although the mechanism is unclear. These regions of \(\alpha_2\) may either increase the affinity of the MIDAS or bind to collagen at a site distinct from GFOGER. However, it is clear that adhesion of isolated \(\alpha_2\beta_1\) to the \(\alpha_2\) I-domain-specific peptide, GFOGER-GPP, is highly sensitive to EGTA, suggesting that Ca\(^{2+}\) has a role in affinity regulation of the I-domain.

Ligand Binding to Isolated \(\alpha_2\beta_1\) Shows Partial Ca\(^{2+}\) Dependence—The purity of the \(\alpha_2\beta_1\) preparation used in these assays was judged to be ~90% by densitometric analysis of the polyacrylamide gel shown in Fig. 5. As with platelets, binding of purified \(\alpha_2\beta_1\) to collagen and to GFOGER-containing peptides is completely abolished by 6F1 (14, 30) and is also inhibited by increasing concentration of EGTA (Fig. 6A). At 2 mM EGTA, there is very little further inhibition of adhesion (data not shown). Eleven repeat experiments found that in the presence of 50 \(\mu\)M EGTA, \(\alpha_2\beta_1\) binding to GFOGER-GPP is reduced to 30 ± 3% but adhesion to collagen is only reduced to 66 ± 4% (mean ± S.E.), perhaps because \(\alpha_2\beta_1\) binds to collagen sequences other than GFOGER in a Ca\(^{2+}\)-independent manner. It is possible that, when removed from its proper membrane context, the unconstrained integrin displays novel collagen binding activity in regions other than its I-domain. It has also

FIG. 6. EGTA inhibits \(\alpha_2\beta_1\) adhesion to collagen and to GFOGER-GPP, but adhesion of \(\alpha_2\) I-domain to these ligands is insensitive to EGTA or Ca\(^{2+}\). A, biotinylated \(\alpha_2\beta_1\) was preincubated with EGTA for 15 min, before measuring adhesion. B, recombinant GST-\(\alpha_2\) I-domain fusion protein was preincubated with EGTA or CaCl\(_2\) for 15 min before measuring adhesion. All adhesions were performed in the presence of 2 mM MgCl\(_2\). Data are from a single experiment, representative of three identical experiments and expressed as the mean of triplicate readings ± S.D., scaled to an A\(_{500}\) = 1 for adhesion to collagen in the presence of 2 mM Mg\(^{2+}\) alone. Where error bars are absent, they were too small to be reproduced.
sitivity to EGTA suggests that the activatory Ca\(^{2+}\) binding site is not found within the I-domain and must lie elsewhere within \(\alpha_\beta_1\). Also, the well-documented inhibitory effect of Ca\(^{2+}\) on \(\alpha_\beta_1\)-mediated adhesion (24, 25) cannot be due to direct competition for the Mg\(^{2+}\) ion bound at the MIDAS site within the I-domain, which is in agreement with others (4).

Ca\(^{2+}\) Restores EGTA-inhibited \(\alpha_\beta_1\) Binding.—The EGTA-induced inhibition of isolated \(\alpha_\beta_1\)-ligated platelets can be restored by the addition of Ca\(^{2+}\) (Fig. 7). A 60% reduction in \(\alpha_\beta_1\) binding to GFOGER-GPP was observed in the presence of 50 \(\mu\)M EGTA, which could subsequently be restored by addition of Ca\(^{2+}\). A similar pattern was observed for binding to collagen. Recovery of adhesion corresponds to estimated free Ca\(^{2+}\) concentrations in the range of 2–50 \(\mu\)M (36). The rescue of adhesion observed here is similar to that observed with whole platelets, but a striking difference is that 2 \(\mu\)M Ca\(^{2+}\) does not significantly inhibit adhesion of integrin \(\alpha_\beta_1\) to its ligands. In fact, the presence of 10 \(\mu\)M Ca\(^{2+}\) results in only partial reduction of adhesion to either collagen I or to GFOGER-GPP (Fig. 7), suggesting that the inhibitory Ca\(^{2+}\)-binding site is either not present or non-functional in the isolated integrin. Alternatively, millimolar Ca\(^{2+}\) might bind other cell surface proteins, which then regulate \(\alpha_\beta_1\) ligand-binding affinity.

In conclusion, we have demonstrated an essential requirement for the activatory Ca\(^{2+}\) binding site in Mg\(^{2+}\)-dependent \(\alpha_\beta_1\)-mediated platelet adhesion. Adhesion of purified \(\alpha_\beta_1\) to GFOGER-GPP was significantly Ca\(^{2+}\)-dependent, whereas, adhesion to collagen had a large Ca\(^{2+}\)-independent component. The stimulatory Ca\(^{2+}\) binding site(s) is not situated within the I-domain and, in agreement with others, Ca\(^{2+}\)-mediated inhibition of \(\alpha_\beta_1\) adhesion does not act by competition with the Mg\(^{2+}\) ion at the MIDAS (4). These findings contribute to the understanding of the role of cations in the regulation of \(\alpha_\beta_1\) function.

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