British Journal of Pharmacology (2002) 137, 107–117
www.nature.com/bjp

Distinct roles of GPVI and integrin $\alpha_2\beta_1$ in platelet shape change and aggregation induced by different collagens

*,[1]Gavin E. Jarvis, [1]Ben T. Atkinson, [1]Daniel C. Snell & [1]Steve P. Watson

[1]Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3QT

1 Various platelet membrane glycoproteins have been proposed as receptors for collagen, in some cases as receptors for specific collagen types. In this study we have compared the ability of a range of collagen types to activate platelets.

2 Bovine collagen types I–V, native equine tendon collagen fibrils and collagen-related peptide (CRP) all induced platelet aggregation and shape change.

3 Responses were abolished in FcRγ chain-deficient platelets, which also lack GPVI, indicating a critical dependence on the GPVI/FcRγ chain complex.

4 Responses to all collagens were unaffected in CD36-deficient platelets.

5 A monoclonal antibody (6F1) which binds to the α_2 integrin subunit of human platelets had a minimal effect on the rate and extent of aggregation induced by the collagens; however, it delayed the onset of aggregation following addition of all collagens. For shape change, 6F1 abolished the response induced by collagen types I and IV, substantially attenuated that to collagen types II, III and V, but only partially inhibited Horm collagen.

6 Simultaneous blockade of the $P2Y_1$ and $P2Y_{12}$ receptors, and inhibition of cyclo-oxygenase demonstrated that CRP can activate platelets independently of ADP and TxA_2; however, responses to the collagens were dependent on these mediators.

7 This study confirms the importance of the GPVI/FcRγ chain complex in platelet responses induced by a range of collagen agonists, while providing no evidence for collagen type-specific receptors. It also provides evidence for a modulatory role of $\alpha_2\beta_1$, the significance of which depends on the collagen preparation.

British Journal of Pharmacology (2002) **137,** 107–117. doi:10.1038/sj.bjp.0704834

Keywords: Collagen types; platelet activation; collagen receptors; CD36; GPVI; $\alpha_2\beta_1$

Abbreviations: CRP, collagen-related peptide; GP, glycoprotein; GPO, glycine-proline-hydroxyproline; PLCγ2, phospholipase Cγ2; TxA_2, Thromboxane A_2

Introduction

In the circulation, blood platelets come into contact with the non-thrombogenic endothelial lining of the vasculature. Disruption of this lining as a consequence of tissue damage or loss of endothelial cells results in the exposure of platelets to collagen leading to their adhesion and activation. The manner in which the interaction of platelets with collagen leads to platelet activation has been the subject of much research in recent years, resulting in the identification of two collagen receptors on the platelet surface, glycoprotein (GP) VI and the integrin $\alpha_2\beta_1$.

GPVI is a signalling receptor whose function is critically dependent on the presence of the associated FcRγ chain (Poole *et al*., 1997). Engagement of GPVI results in the activation of a tyrosine phosphorylation cascade which includes the Src kinases Fyn and Lyn (Quek *et al*., 2000), the adapters LAT (Gibbins *et al*., 1998) and SLP-76 (Gross *et al*., 1999), and activation of phospholipase Cγ2 (PLCγ2) (Asselin *et al*., 1997). Activation of PLCγ2 causes mobilization of intracellular calcium and platelet activation. An amino acid motif which comprises approximately 10% of the triple helix of collagen molecules, glycine–proline–hydroxyproline (GPO), binds to GPVI (Knight *et al*., 1999) and a synthetic collagen-like peptide consisting of repeat GPO sequences (collagen-related peptide, CRP) is a potent GPVI agonist (Morton *et al*., 1995). CRP is thought to activate GPVI by clustering the receptor with its multiple GPO motifs. An antibody to murine GPVI, JAQ1, is also able to potently activate platelets in a similar manner following cross-linking (Nieswandt *et al*., 2000).

The integrin α_2 subunit (also known as GPIa) was proposed as a collagen receptor following the identification of a patient with a haemorrhagic disorder whose platelets failed to respond to collagen, and which had 15–25% of normal levels of GPIa on their surface (Nieuwenhuis *et al*., 1985). However, it has been suggested that additional abnormalities were present which were important in the aetiology of this case (Coller *et al*., 1989). Further patients in which dysfunctional $\alpha_2\beta_1$ has been associated with a selective deficit in platelet collagen responsiveness have been identified, including one who lacked intact thrombospondin and α_2 integrin (Kehrel *et al*., 1988), one with auto-antibodies to α_2 (Deckmyn *et al*., 1990), and one with a myeloproliferative disorder (Handa *et al*., 1995).

Collagen interacts with the I-domain of $\alpha_2\beta_1$ integrin and affinity for this binding site can be conferred by specific

*Author for correspondence;
E-mail: gavin.jarvis@pharmacology.oxford.ac.uk

amino acid sequences including GFOGER (Emsley *et al*., 2000; Knight *et al*., 2000). However, the physical properties of collagen (quaternary structure) also influence its interaction with platelets, with native fibres, enzymatically-digested fibrils and re-constituted collagen fibrils showing differential dependence on $\alpha_2\beta_1$ for adhesion (Savage *et al*., 1999). It has been demonstrated that, in a manner analogous to $\alpha_{IIb}\beta_3$ integrin for fibrinogen, $\alpha_2\beta_1$ exists in the quiescent platelet in a conformation with low affinity for collagen and that activation of the platelet can convert the integrin into a high affinity state (Jung & Moroi, 1998). Platelets adhere to a range of collagen types (Sixma *et al*., 1995) in a manner that is dependent upon $\alpha_2\beta_1$ (Saelman *et al*., 1994) suggesting that this integrin may contribute to the firm interaction between platelets and sub-endothelial collagen. A role for $\alpha_2\beta_1$ in generating intracellular signals in platelets is more controversial. Although limited evidence exists to support such a role (Ivaska *et al*., 1999), it is still widely considered that the primary role of $\alpha_2\beta_1$ is to support adhesion of platelets to collagen (Clemetson & Clemetson, 2001).

A two-step, two-site model has been proposed in which $\alpha_2\beta_1$ integrin is primarily responsible for the firm adhesion of platelets to collagen and GPVI mediates the activation of the platelets following this adhesion (Barnes *et al*., 1998; Santoro *et al*., 1991). However, evidence exists suggesting that this model is not an adequate description of the platelet–collagen interaction (Watson *et al*., 2000). For example, it is clear that other receptor complexes play a critical role in the interaction of platelets with collagen, principally the GPIb-IX-V complex, which mediates the initial tethering of platelets to collagen *via* von Willebrand factor under conditions of high shear (Cauwenberghs *et al*., 2000). Other proteins have also been proposed as putative receptors for collagen including CD36 (Tandon *et al*., 1989), GPV (Moog *et al*., 2001), a 65 kD non-integrin receptor for type I collagen (Chiang *et al*., 1997) and a putative type III collagen-binding protein (Monnet & Fauvel-Lafeve, 2000).

Uncertainty over the role of putative platelet collagen receptors may be partly due to the use of collagens of different types, species and preparations. Collagens share common features, including the characteristic triple helical structure which forms as a result of the repeat amino acid sequence glycine-X-Y. However, the existence of distinct collagen types and preparations also affords a potential means for dissecting out the contribution to platelet activation made by different surface receptors. The classical fibril forming collagens, types I, II and III, all have platelet aggregating activity (Balleisen *et al*., 1975; Barnes *et al*., 1976; Trelstad & Carvalho, 1979). Type IV (from basement membrane) and V (formerly type 'A-B') collagens have been investigated less extensively with some investigators concluding that they do not elicit platelet activation (Trelstad & Carvalho, 1979) and others showing that under appropriate conditions they do (Barnes *et al*., 1980; Chiang *et al*., 1980). As already mentioned, it has been suggested that there are type I (Chiang *et al*., 1997) and type III (Monnet & Fauvel-Lafeve, 2000) collagen specific receptors, but also that CD36 (also known as GPIIIb or GPIV) is a specific receptor for type V collagen (Kehrel *et al*., 1993). Since particular amino acid motifs are known to bind specifically to certain receptors (GPO binds GPVI (Knight *et al*., 1999), and GER confers binding affinity for integrins $\alpha_2\beta_1$ and $\alpha_1\beta_1$ (Xu *et al*., 2000)), it is possible that collagen interacts with these and other receptors *via* as yet unidentified motifs, which may be restricted to particular collagen types.

The aim of this study was to investigate the response of human and murine platelets to a range of collagen agonists in order to characterize the role of GPVI, $\alpha_2\beta_1$ and CD36 in the platelet–collagen interaction, and also to potentially identify collagen type- and preparation-specific receptors. No systematic survey has been conducted to characterize the activity of a broad range of collagen agonists on platelets devoid of GPVI/FcRγ chain or CD36. The ability of anti-α_2 blocking antibodies to inhibit adhesion of platelets to collagen types I–VIII has previously been demonstrated (Saelman *et al*., 1994) although the role of the integrin $\alpha_2\beta_1$ in aggregation and shape change to a wide range of collagen agonists has been less extensively investigated. We used reconstituted bovine collagen types I–V, the commonly used native type I fibrils from equine tendon (Horm collagen) and the synthetic CRP, and examined their ability to induce shape change and aggregation. We investigated the role of $\alpha_2\beta_1$ with the anti-α_2 antibody 6F1 (Coller *et al*., 1989) and that of GPVI and CD36 with platelets from genetically-modified mice (Febbraio *et al*., 1999; Park *et al*., 1998). In addition, we investigated the ability of the collagen agonists to activate platelets in the absence of the positive feedback pathways of ADP and thromboxane A_2 (TxA_2).

Methods

Materials

Bovine collagen types I–V were obtained from Koken Co. Ltd (Tokyo, Japan); Horm collagen (Nycomed, Munich, Germany) is a preparation of native collagen fibrils prepared from equine tendon; CRP [YGKO$(GPO)_{10}$GKOG] was synthesized by Tana Laboratories (Texas, U.S.A.); anti-α_2 monoclonal antibody 6F1 was kindly donated by Prof Barry Coller (Rockefeller University, New York, U.S.A.); anti-CD36 monoclonal antibody 10.5 was kindly donated by Dr John McGregor (INSERM U331, Lyon, France); heparin sodium (Monoparin® 25,000 u ml^{-1}) was obtained from CP Pharmaceuticals (Wrexham, U.K.). AR-C67085 was supplied by AstraZeneca R&D Charnwood, Loughborough, U.K. Lotrafiban was supplied by Glaxo-SmithKline, King of Prussia, U.S.A. Other reagents were obtained from Sigma (Poole, Dorset, U.K.) or BDH (Poole, Dorset, U.K.).

Preparation of collagens

Pepsin-digested bovine collagens were supplied in sterile solution, pH 3.0 at 3 mg ml^{-1}. Insoluble (reconstituted, fibrillar) preparations of each type of collagen were prepared as previously described (Jung & Moroi, 1998). Each collagen was dialysed overnight at 4°C against a HEPES–Tyrode's solution (mM): NaCl 136, KCl 2.7 NaH_2PO_4 0.42, $NaHCO_3$ 12, glucose 5.55, HEPES 5, pH 7.4. The collagen was incubated for 1 h at 37°C, during which time the collagen polymerized and formed a gel. The gel was pelleted by

centrifugation at $8000 \times g$ for 15 min and re-suspended in a small volume of the HEPES–Tyrodes buffer. The collagen was homogenized with a homogenizer to give a fine suspension of macromolecular collagen. Prior to each use, the polymerized collagens were vigorously vortexed, although the preparations remained dispersed in suspension indefinitely. These collagens have been used previously by other investigators (Jung & Moroi, 1998; Kawamoto & Kaibara, 1992). The purity of the collagens as determined using electrophoresis by the manufacturing company was as follows: type I–94%; type II–100%; type III–88%; type IV–83%; type V–81%. In the case of type I the only identifiable contaminant was collagen type III, and for collagen types III, IV and V, was collagen type I. Although the levels of contaminating type I collagen were not insignificant, our results clearly showed that platelet activation was caused by the main constituent of each preparation. For example, the preparation of type V collagen was clearly more potent than the preparation made from its major contaminant, type I collagen, indicating that the type V collagen was the major active ingredient. The concentration of collagen in suspension was determined using a hydroxyproline assay (Bergman & Loxley, 1963). The proportion of hydroxyproline in each collagen was determined from the supplied soluble collagen and this was used to calculate the final concentrations of polymerized collagen after the dialysis and cross-linking.

Preparation of cross-linked CRP

CRP peptide was dissolved in ice-cold phosphate buffered saline at 2 mg ml^{-1} and then cross-linked by adding 0.25% glutaraldehyde for 3 h at 4°C under stirring condition. The solution was then dialyzed three times in 1 l of ice-cold phosphate buffered saline using Pierce 10,000 molecular weight cut-off dialysis cassettes (Perbio, Tattenhall, U.K.).

Preparation of heparinized platelet rich plasma

Blood was collected from healthy human donors into syringes containing 100 u ml^{-1} heparin sodium, to give a final concentration of heparin of 10 u ml^{-1}. The blood was centrifuged at $240 \times g$ and the platelet rich plasma (PRP) pipetted off. A small sample of blood was centrifuged at 13,000 r.p.m. in a micro-centrifuge to produce platelet poor plasma (PPP). We chose to investigate platelet responses primarily in heparinized human PRP, since this preserves many of the physiological conditions under which platelets normally function, including divalent cation concentrations and the presence of plasma proteins.

Preparation of human washed platelets

Blood was collected from healthy human donors into syringes containing 0.11 M sodium citrate to give a final concentration of 11 mM citrate. The blood was centrifuged at $240 \times g$ for 15 min and the PRP removed. Prostacyclin (800 nM) was added to the PRP which was then centrifuged at $125 \times g$ to sediment out any residual red blood cells. The red cell free PRP was removed and centrifuged at $640 \times g$ for 15 min to pellet the platelets. The PPP was poured off and the pellet re-suspended in 10 ml of the following modified Tyrode's solution (mM): NaCl 137, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, KCl 2.7, $MgCl_2$ 1.1, glucose 5.6, pH 7.4, 37°C, containing 800 nM prostacyclin. The re-suspended platelets were centrifuged at $640 \times g$, the supernatant discarded, and the platelets re-suspended in 10 ml of the modified Tyrode's solution. The platelet count was adjusted to 200×10^6 ml^{-1} and the platelets allowed to rest for at least 1 h prior to use in a sealed syringe at room temperature. Prior to activation of the platelets, 1 mM $CaCl_2$ was added to the suspension.

Genetically modified mice

CD36 deficient mice (Febbraio *et al*., 1999) were provided by Dr Maria Febbraio of Cornell University, U.S.A. FcRγ chain deficient mice (Park *et al*., 1998) were provided by Dr Takashi Saito of the Chiba University Graduate School, Japan. Both strains were on a C57/B6 background, and age, sex and strain matching controls were used alongside these mice for the experiments described.

Preparation of murine washed platelets

Approximately 1 ml of blood was collected from each mouse from the hepatic portal vein under terminal CO_2 narcosis into acid–citrate–dextrose anticoagulant. The blood was centrifuged for 7 min at $200 \times g$ and the PRP removed. 200 μl of modified Tyrode's (mM): NaCl 134, KCl 2.9, Na_2HPO_4 0.34, $NaHCO_3$ 12, HEPES 20, $MgCl_2$ 1, pH 6.6, were added and the blood centrifuged in the same way. The procedure was carried out three times, on each occasion, the supernatant being collected and pooled. Prostacyclin (800 nM) was then added to the PRP which was centrifuged for 7 min at $1000 \times g$ to pellet the platelets. The supernatant was discarded and the platelets re-suspended in modified Tyrode's at pH 7.4. In order to minimize the number of mice used the platelet count was adjusted to 150×10^6 ml^{-1}, lower than that for human washed platelets. The platelets were allowed to rest in a sealed tube at room temperature for 1 h prior to use.

Measurement of platelet aggregation

Platelet aggregation was measured using a turbidimetric method (Born & Cross, 1963) with a BioData PAP-4 aggregometer (Alpha Laboratories, Eastleigh, U.K.). The assay was performed at 37°C with a sample stir speed of 1000 r.p.m. in heparinized human PRP, or in washed murine platelets. A value for the maximal rate and extent of aggregation was produced automatically by the PAP-4 aggregometer. The time to the onset of the aggregation response was estimated visually from the trace recordings as the period of time from the addition of the agonist to the first clear indication of a reduction in the optical density of the platelet suspension.

Measurement of platelet shape change

Shape change measurements were carried out using a Chronolog 490-2D aggregometer (Labmedics, Manchester, UK). Heparinized human PRP was used in the presence of 10 μM of the $\alpha_{IIb}\beta_3$ antagonist lotrafiban to prevent

aggregation, which frequently obscures the shape change response. The maximum rate of and extent of shape change were determined manually from the trace recording. The time to the onset of response was determined as the period of time from the addition of the agonist to the initial increase in the optical density of the platelet suspension.

Scanning electron microscopy

Scanning electron microscopy was carried out as previously described (Gear, 1981). Basal or stimulated washed human platelets (300 μl, at a concentration of 200×10^6 platelet ml^{-1}) were mixed with an equal volume of 4% glutaraldehyde in 0.15 M NaCl, in phosphate buffer pH 7.4 (pre-warmed to 37°C). Platelets were fixed 4 min after addition of the agonist. The platelets were then collected with gentle suction onto 0.6 μm pore size polycarbonate filters (Whatman) that had been pre-rinsed with 2% glutaraldehyde in 0.15 M NaCl, pH 7.4. Filters were transferred to vials and rinsed once with 0.15 M NaCl, pH 7.4 and twice with distilled water for removal of glutaraldehyde. Dehydration of filters was accomplished by washing with 10%, 25%, 50%, 75%, 95% and 100% ethanol. The filters were subjected to critical point drying (on a Polaron CPD7501 critical point drier) coated with gold (using a Nonotech SemPrep 2 sputter coater) and analysed on a Philips 515 scanner.

Data analysis

Concentration–response curve data were fitted to a three parameter logistic equation (de Lean *et al*., 1978) of the form:

$$R = \frac{(Max)}{\left(1 + \left(\frac{(10^{-pA_{50}})}{A}\right)^{n_H}\right)} \quad (1)$$

Where:

R = response (dependent variable)
A = concentration of agonist (independent variable)
... and ...
Max = response when $A = \infty$
pA_{50} = $-\log_{10}$ of concentration of A that gives a response equal to $(Max)/2$
n_H = Hill coefficient

The agonist location parameter (pA_{50}) was estimated as the negative logarithm as the error associated with this parameter is logarithmically distributed (de Lean *et al*., 1982; Hancock *et al*., 1988). Comparison of different data sets was conducted using an extra sum of squares analysis (Bates & Watts, 1988). Separate data sets were fitted to the model independently and a residual sum of squares calculated. The same data was then re-fitted with each of the three parameters individually constrained across the data sets and a new residual sum of square value obtained for each constrained parameter. The difference between these two values allowed a statistical assessment of whether any particular parameter was significantly different between the two data sets. Data were analysed using Microsoft® Excel 2000. P values of <0.05 were considered significant. Other data was analysed using Student's t-test. Results are presented as the mean ± s.e.mean.

Results

All collagen agonists induce aggregation and shape change of human platelets

All of the agonists induced aggregation and shape change of human platelets. Figure 1 shows concentration–response curves and Figure 2 shows example traces for each of the agonists for shape change and aggregation. For the rate of aggregation the more potent agonists were CRP, Horm collagen and the bovine types II and V which had A_{50} values in the range of 0.4–0.7 μg ml^{-1}. The less potent agonists were bovine types I, III and IV which had A_{50} values in the range of 1–6 μg ml^{-1}. The A_{50} values for the extent of aggregation were similar to those for the rate of aggregation (data not shown). Figure 1a illustrates that the range of concentrations over which the agonists induce shape change is higher than for aggregation with all A_{50} values lying in the range of 1–12 μg ml^{-1}. The A_{50} values for extent of shape change were lower than for rate, lying between 0.2 and 2 μg ml^{-1} (data not shown). The delay from addition of the agonists to the onset of the response (lag phase) is shown in Figure 3. At maximal concentrations of agonists, the lag phase for aggregation was between 30–60 s, whereas for shape change it was 10–30 s.

The role of FcRγ chain and CD36 in collagen-induced aggregation of murine platelets

The role of the GPVI/FcRγ chain complex and CD36 were investigated using murine platelets deficient in these proteins (Figure 4). Washed platelets were prepared from control mice ($n=12$) and strain matched mice deficient in the FcRγ chain ($n=11$) and CD36 ($n=11$). Control platelets aggregated in response to all the collagens and CRP; however, none of the collagens, nor CRP, induced aggregation of the FcRγ chain-deficient platelets (Figure 4b). Furthermore, a clear shape change response was observed on the aggregation traces of the control platelets but was completely absent in the FcRγ chain-deficient platelets. FcRγ chain-deficient platelets aggregated normally in response to thrombin (data not shown).

Aggregation responses induced by the collagens and CRP were measured in the CD36-deficient platelets (Figure 4a). No differences in the lag phase, the rate or extent of aggregation were observed between the control and the knock-out mice. Furthermore, studies performed in human platelets showed that cross-linking of a monoclonal antibody to CD36 (10.5, 4 μg ml^{-1}) in the presence of F(ab)$_2$ fragments of IV.3 to block the FcγRIIa receptor was unable to induce platelet aggregation or tyrosine phosphorylation (data not shown).

Effect of 6F1 on aggregation of human platelets

The effect of the anti-α_2 antibody 6F1 (10 μg ml^{-1}) on the rate of aggregation induced by the collagen agonists is shown in Figure 5. There was a small effect on each agonist with the exception of CRP which appeared to be completely unaffected. This effect was manifest as a small reduction in the maximum response which was statistically significant for collagen types I ($P=0.021$), II ($P=0.007$) and IV ($P=0.006$), but not for collagen types III ($P=0.075$), V ($P=0.218$) or

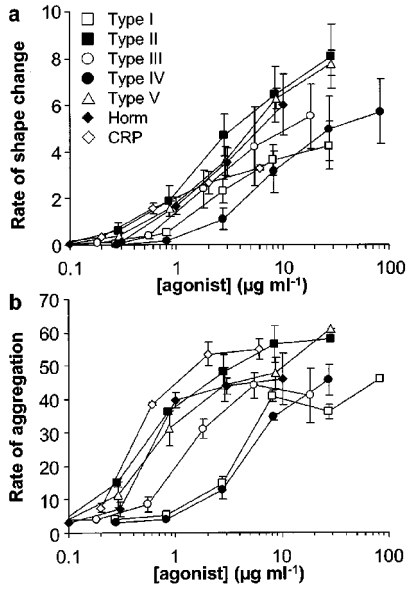

Figure 1 Concentration-response curves for (a) the rate of shape change and (b) the rate of aggregation induced by bovine collagen types I–V, Horm collagen and CRP. The results are indicated in arbitrary values as the mean ± s.e.mean. Data was obtained in human heparinized PRP from three separate experiments.

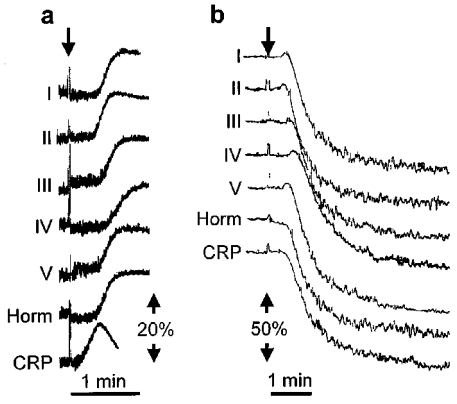

Figure 2 Example traces showing (a) shape change and (b) aggregation responses of platelets induced by bovine collagen types I–V, Horm collagen and CRP. Concentrations of the agonists were: type I, 10 μg ml^{-1}; type II, 3 μg ml^{-1}; type III, 5 μg ml^{-1}; type IV, 10 μg ml^{-1}; type V, 3 μg ml^{-1}; Horm, 3 μg ml^{-1}; CRP, 2 μg ml^{-1}. The shape change traces are all from the same, single experiment and were measured using a Chronolog 490-2D aggregometer in heparinized PRP in the presence of 10 μM lotrafiban. The aggregation traces are from another separate, single experiment and were measured using a PAP-4 aggregometer in heparinized PRP. The scale indicates the percentage change in optical density where the difference between platelet rich plasma and platelet poor plasma is equivalent to 100%.

Horm collagen ($P=0.266$). In no case was the pA_{50} or Hill coefficient for the rate of aggregation significantly altered in the presence of 6F1.

The clearest effect of 6F1 on platelet aggregation was the prolongation of the lag phase (Figures 3b and 5b). This was observed with all collagens at all concentrations, although it was most marked for collagen types I and IV. There was no effect on CRP, and the small increases in the lag phase to

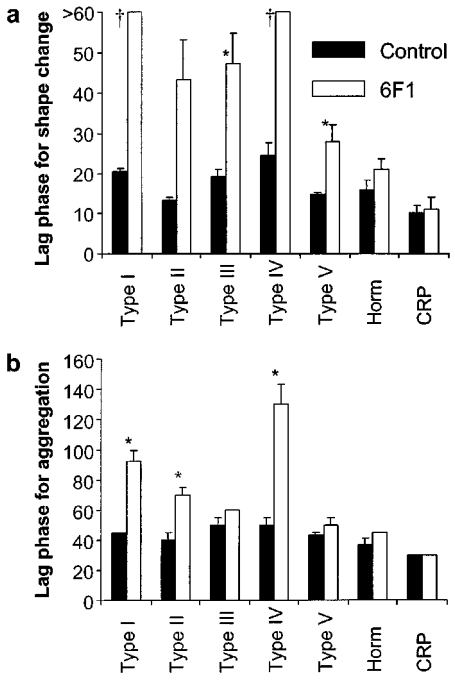

Figure 3 The effect of the anti-α_2 monoclonal antibody 6F1 (10 μg ml^{-1}) on the time to the onset (lag phase) of (a) shape change and (b) aggregation following the addition of bovine collagen types I–V, Horm collagen and CRP. The data shown represent the lag phases for maximal concentrations of the agonists. These are shorter for shape change since it occurs before the onset of aggregation. Results are measured in seconds and are the mean ± s.e.mean. †The shape change response induced by collagen types I and IV was abolished in the presence of 6F1, and hence a lag time could not be measured. An arbitrary time of 60 s was assigned in those cases where the response was completely abolished or where the onset of the response was after this time. Statistical analysis was performed using Student's *t*-test. *$P<0.05$. Data was obtained in human heparinized PRP from three separate experiments.

collagen types III, V and Horm collagen were not statistically significant. There was, however, a statistically significant increase in the lag phase to collagen types I ($P=0.002$), II ($P=0.013$) and IV ($P=0.005$).

Effect of 6F1 on shape change of human platelets

Shape change measurements were made in an identical manner to aggregation, except that the $\alpha_{IIb}\beta_3$ integrin antagonist lotrafiban (10 μM) was present in order to prevent aggregation and thereby accentuate the shape change response.

In contrast to the effect on aggregation, 6F1 had a profound effect on the shape change response induced by the collagens (Figure 6). Shape change induced by collagen types I and IV was abolished, whereas that induced by collagen types II, III and V was substantially attenuated. The response to Horm collagen was also inhibited but not to the same extent as the reconstituted bovine collagens. As expected, the response to CRP was completely unaffected.

The lag phase of the shape change response following addition of the collagens was prolonged in the presence of 6F1 (Figure 3a). This prolongation was statistically significant for collagen types III ($P=0.022$) and V ($P=0.034$) at maximal concentrations of these agonists. It was not possible

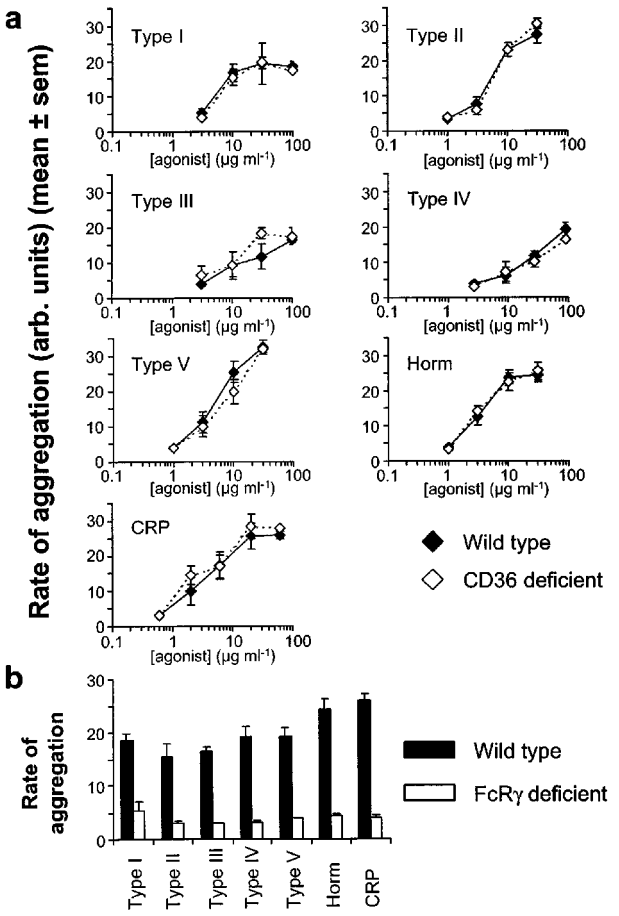

Figure 4 Responses induced by bovine collagen types I–V, Horm collagen and CRP in murine platelets deficient in CD36 and FcRγ chain. (a) Concentration-response curves show the rate of aggregation of control and CD36-deficient murine washed platelets in response to bovine collagen types I–V, Horm collagen and CRP. No effect of the CD36 deficiency was observed. The experiment was conducted on three separate occasions on a total of 12 control and 11 CD36-deficient mice. (b) Rate of aggregation induced in control and FcRγ chain-deficient platelets by bovine collagen types I–V, Horm collagen and CRP. Concentrations of the agonists were: type I, 100 μg ml^{-1}; type II, 100 μg ml^{-1}; type III, 100 μg ml^{-1}; type IV, 90 μg ml^{-1}; type V, 100 μg ml^{-1}; Horm, 30 μg ml^{-1}; CRP, 60 μg ml^{-1}. The experiment was conducted on three separate occasions on a total of 12 control and 11 FcRγ chain-deficient mice. The reduction in the response in the FcRγ chain-deficient platelets is significant in all cases.

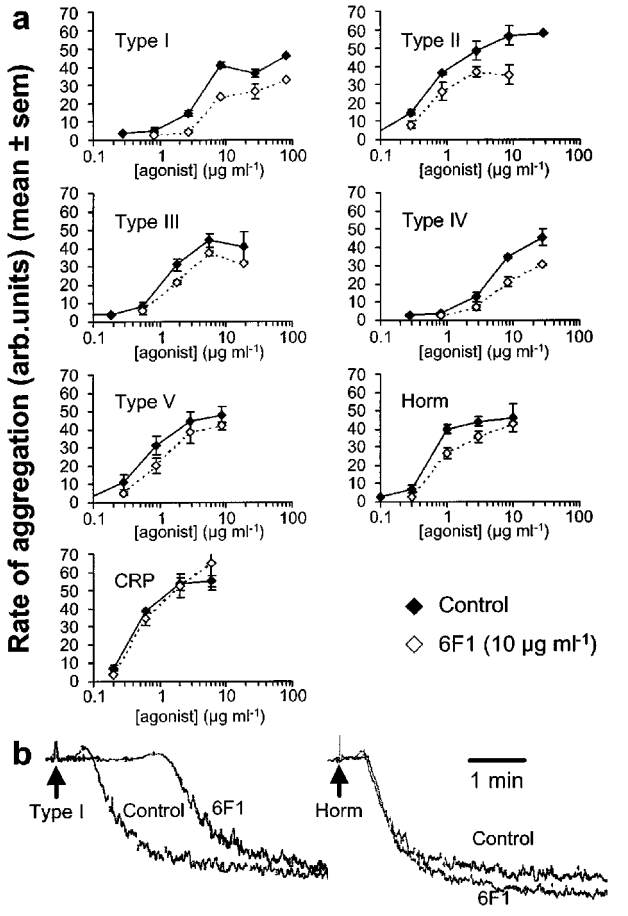

Figure 5 The effect of the anti-α_2 monoclonal antibody 6F1 on aggregation induced by bovine collagen types I–V, Horm collagen and CRP. (a) Concentration-response curves show the rate of aggregation in the absence or presence of 10 μg ml^{-1} 6F1. Data were obtained in human heparinized PRP from three separate experiments and are shown as mean$\pm$s.e.mean. (b) Traces illustrating the effect of 6F1 (10 μg ml^{-1}) on collagen-induced aggregation in heparinized human PRP. Aggregation was induced by bovine type I collagen (30 μg ml^{-1}) or Horm collagen (10 μg ml^{-1}). These traces illustrate the increase in the lag phase for bovine collagen type I caused by 6F1. Traces shown are representative of three separate sets of experiments.

to measure a lag phase for collagen types I and IV since the response was abolished. Although the increases in the lag phase for type II and Horm collagens were evident at all concentrations, the differences were not statistically significant. CRP was completely unaffected in the presence of 6F1.

Scanning electron microscopy

In order to confirm that the shape change trace recordings reflected the morphological changes in the platelets, scanning electron microscopy was carried out on platelets activated with either 3 μg ml^{-1} of Horm collagen or 4 μg ml^{-1} of bovine type I collagen, both in the absence and presence of 6F1 (10 μg ml^{-1}). These experiments were performed in washed human platelets rather than PRP owing to the difficulties of fixing platelets in plasma. Trace recordings revealed the same pattern of shape change response in the washed platelet preparation as in the heparinized PRP (data not shown). The electron micrographs are shown in Figure 7. In the absence of the antibody, shape change induced by both collagens is clearly evident. In the presence of 6F1, shape change is still observed in those platelets activated by the Horm collagen, whereas for those platelets to which the bovine type I collagen had been added, there was no shape change response. Most platelets showed little or no signs of activation, and retained the smooth discoid form characteristic of a quiescent platelet.

Platelet activation in the absence of ADP- and TxA_2-mediated activation

Human platelets were pre-incubated with indomethacin (10 μM) to prevent TxA_2 production, and AR-C67085

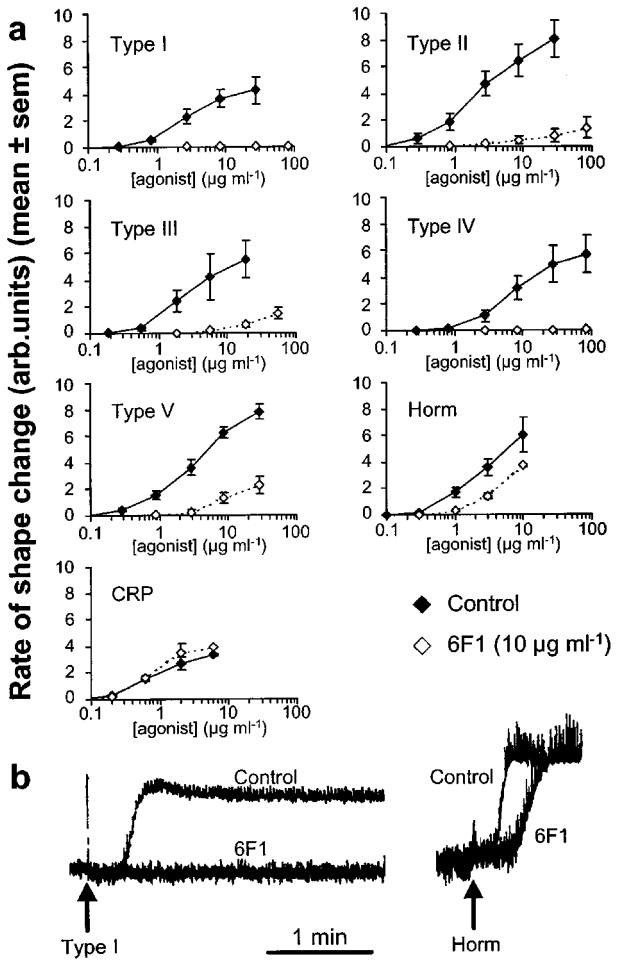

Figure 6 The effect of the anti-α_2 monoclonal antibody 6F1 on shape change induced by bovine collagen types I–V, Horm collagen and CRP. (a) Concentration-response curves show the rate of shape change in the absence or presence of 10 μg ml^{-1} 6F1. Data were obtained in human heparinized PRP from three separate experiments. (b) Traces illustrating the effect of 6F1 (10 μg ml^{-1}) on collagen-induced shape change in heparinized human PRP. Control responses were induced by 10 μg ml^{-1} bovine type I collagen and 10 μg ml^{-1} Horm collagen and in the presence of 6F1 by 30 μg ml^{-1} type I collagen and 10 μg ml^{-1} Horm collagen. The traces illustrate the inhibitory effect of 6F1 on the shape change response induced by type I collagen, and the small effect on that induced by Horm. Traces shown are representative of three separate sets of experiments.

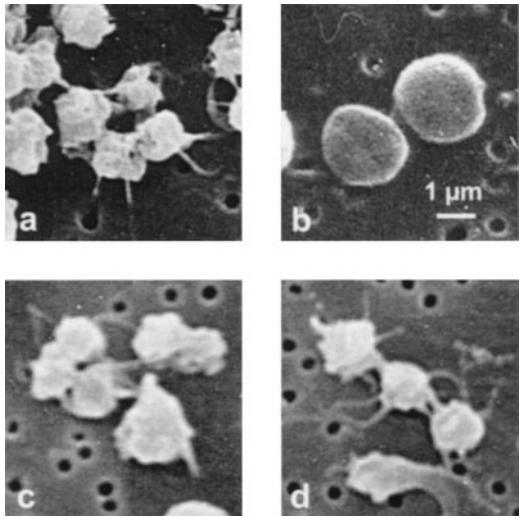

Figure 7 Scanning electron micrographs of platelets activated with bovine type I collagen or Horm collagen in the absence or presence of the anti-α_2 monoclonal antibody 6F1 (10 μg ml^{-1}). Human washed platelets were activated with 4 μg ml^{-1} bovine type I collagen (a, b) or 3 μg ml^{-1} Horm collagen (c, d) in the absence (a and c) or presence (b and d) of 10 μg ml^{-1} of the anti-α_2 antibody 6F1. The platelets were fixed with glutaraldehyde and analysed with a scanning electron microscope. Panels a, c, d illustrate the characteristic morphology of shape changed platelets, whereas panel b shows typical smooth discoid non-activated platelets. These pictures reflect the shape change trace data which also indicate that 6F1 inhibited shape change induced by bovine type I, but not Horm collagen.

(1 μM) and A3P5P (1 mM) to prevent activation by ADP of the $P2Y_{12}$ and $P2Y_1$ receptors respectively. Under these conditions, the aggregation response to high concentrations of all five bovine collagens (30 μg ml^{-1}) and Horm collagen (10 μg ml^{-1}) were substantially inhibited or blocked (Figure 8). The response to a modest concentration of CRP (2 μg ml^{-1}), however, was only inhibited by approximately 50%, whilst that to high concentrations of CRP was minimally affected (data not shown). Similar results were observed for shape change.

Discussion

The purpose of the study was to use a range of collagen agonists in order to identify potential collagen type- and preparation-specific receptors. We investigated their ability to induce shape change and aggregation in normal platelets, in platelets deficient in the activatory collagen receptor complex GPVI/FcRγ chain, and in platelets in which the adhesion receptor $\alpha_2\beta_1$ integrin had been blocked. We also investigated the ability of the collagen agonists to directly activate platelets in the absence of the secondary mediators ADP and TxA_2. In addition, we specifically examined the suggestion that CD36 is a collagen receptor using platelets deficient in this protein. Our results confirm the central importance of the GPVI/FcRγ chain complex in these collagen-induced responses, and also the modulatory role of $\alpha_2\beta_1$ integrin. However, we were unable to demonstrate a role for CD36 or the presence of any collagen type-specific receptors.

Although the concept of different collagen types acting upon different receptors is attractive, the comparison of different collagens in hindered by various confounding factors. Collagen needs to assume a suitable tertiary and quaternary structure before it is able to activate platelets (Morton *et al*., 1995). The size, diameter and morphology of collagen fibers have a significant impact upon their ability to activate platelets. Furthermore, collagen is a multimeric ligand which binds to different and multiple copies of receptors, making it difficult to clearly identify the role of any particular receptor. For these reasons we chose to investigate the effects of a range of collagen agonists under circumstances in which the two main collagen receptors, GPVI and $\alpha_2\beta_1$, were blocked.

The importance of the GPVI/FcRγ chain complex in collagen-induced responses is underlined by these data. We

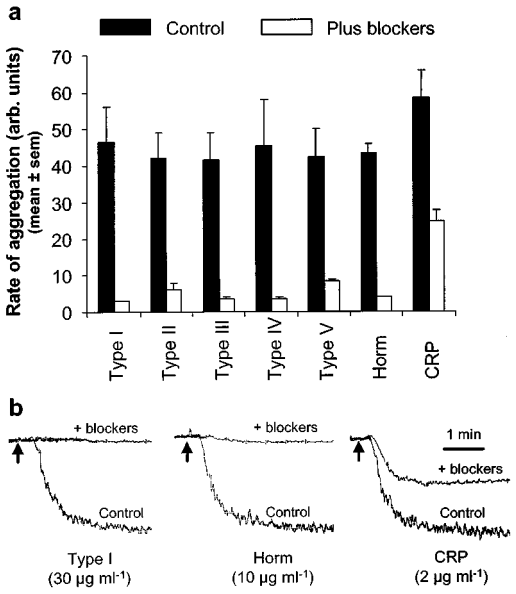

Figure 8 The role of the secondary mediators ADP and TxA_2 in collagen-induced aggregation. The effect of the combination of indomethacin (10 μM), A3P5P (1 mM), and AR-C67085 (1 μM) on aggregation induced by bovine collagen types I–V, Horm collagen and CRP was investigated. (a) Bar chart showing the inhibitory effect on the following concentrations of the agonists: types I–V, 30 μg ml^{-1}; Horm, 10 μg ml^{-1}; CRP 2 μg ml^{-1}. Data were obtained in human heparinized PRP from two separate experiments. (b) Traces illustrating the inhibitory effect of the combination of blockers on type I and Horm collagens, and CRP.

have previously shown that Horm collagen-induced release of 5-hydroxytryptamine was abolished in FcRγ chain-deficient platelets (Poole *et al*., 1997). These data now extend that finding to platelet shape change and aggregation induced by a range of different collagen types. Collagen-induced responses are therefore dependent on signalling through an FcRγ chain-dependent pathway, whether mediated by GPVI or another FcRγ chain-associated receptor. Alternatively, since the presence of the FcRγ chain is essential for the expression of GPVI (which is absent in the knock-out mice) it may be that binding of collagen to GPVI is essential and that a signalling mechanism other than *via* the FcRγ chain contributes to the collagen response. The reported observation that platelets from a patient lacking GPVI did not aggregate in response to human collagen types I and III and bovine collagen types I, III, IV and V (Moroi *et al*., 1989) is consistent with our results and supports the view that GPVI/FcRγ chain complex is of critical importance in collagen-induced platelet activation.

The observation that none of the collagens induce shape change or aggregation in the absence of the FcRγ chain does not support the view that there are collagen type-specific receptors as claimed by previous investigators (Chiang *et al*., 1997; Kehrel *et al*., 1993; Monnet & Fauvel-Lafeve, 2000). This does not definitively exclude the possibility that such receptors exist, but suggests that they are incapable of eliciting a functional response in the absence of the GPVI/FcRγ chain complex. Although in the absence of the FcRγ chain there was no observable shape change or aggregation, we have previously reported a small degree of collagen-induced tyrosine phosphorylation in these murine platelets (Poole *et al*., 1997). The lack of any direct evidence to support a role for $\alpha_2\beta_1$ in mediating tyrosine phosphorylation (Hers *et al*., 2000) suggests that another collagen receptor may be involved in this response.

Since CD36 has been proposed as a primary receptor for collagen (Tandon *et al*., 1989) and is also known to be associated with Src kinases (Huang *et al*., 1991), we investigated its potential role in collagen-induced platelet activation using CD36-deficient mice. However, no role for CD36 in collagen-induced platelet activation could be inferred from the full concentration-response relationships observed in these mice. Furthermore, studies in human washed platelets showed that cross-linking an anti-CD36 antibody (in the presence $F(ab)_2$ fragments of the monoclonal antibody IV.3 to block the FcγRIIa receptor) was unable to induce either tyrosine phosphorylation or a functional platelet response (data not shown). These data are consistent with those of other investigators who have shown that the absence of CD36 on human platelets has little effect on platelet aggregation induced by type I collagen (Daniel *et al*., 1994; McKeown *et al*., 1994; Yamamoto *et al*., 1992), and extend these observations to responses induced by collagen types II–V. The lack of any effect on collagen type V in particular argues against the proposition that CD36 is a collagen type V specific receptor (Kehrel *et al*., 1993). The consistent lack of any identifiable role for CD36 suggests that it is not an important mediator of the activation of platelets by collagen.

The role of integrin $\alpha_2\beta_1$ was investigated using the monoclonal antibody 6F1 which binds to the α_2 subunit. This antibody has been widely used to investigate the interaction of collagen with platelet $\alpha_2\beta_1$ (Monnet *et al*., 2000; Moroi *et al*., 2000; Siljander & Lassila, 1999) and is able to prevent adhesion of platelets to immobilized collagen (Saelman *et al*., 1994) in a manner similar to other anti-$\alpha_2\beta_1$ antibodies including Gi9 (Moroi *et al*., 2000) and 176D7 (Verkleij *et al*., 1998). As anticipated, the anti-α_2 antibody 6F1 had no effect on the response of platelets induced by the GPVI specific ligand CRP. 6F1 did, however, interfere with the responses to the collagens, indicating that interaction of the collagens with $\alpha_2\beta_1$ plays a role in the activation of platelets. However, the role of the integrin appears to vary depending on the preparation of collagen used, as previously shown by other investigators (Monnet *et al*., 2000). Of the collagens, the Horm preparation was least affected by 6F1. This may reflect the highly cross-linked nature of these native fibrils (Fauvel *et al*., 1985). There was little effect on Horm-induced aggregation, although there was a partial inhibition of the shape change response. By contrast, all of the other collagens were more affected by 6F1. Although there was no substantial change in the rate (or extent, data not shown) of aggregation, there was an increase in the lag phase for this response, an effect which was most marked for collagen types I and IV. This relative ineffectiveness of 6F1 in inhibiting collagen-induced aggregation has been previously noted for type I collagen (Morton *et al*., 1995). However, 6F1 was more effective at inhibiting platelet shape change; in particular, it abolished this response induced by collagen types I and IV, and substantially inhibited the response to the other bovine collagens. Our results are consistent with those recently obtained in β_1-deficient murine platelets in which an increased delay in the response to collagen was also observed;

furthermore, the effects of β_1 ablation were less evident using Horm collagen (Nieswandt *et al*., 2001b). Given the dependence of all the collagens on the presence of the FcRγ chain, the difference in sensitivity to 6F1 may reflect a greater relative ability of the Horm collagen to interact with GPVI over $\alpha_2\beta_1$ compared to the other collagens. This may arise as a result of differences in the fibrillar structure of the collagens (Savage *et al*., 1999), but could also be due to different primary structural determinants.

The role played by $\alpha_2\beta_1$ in the collagen-induced platelet activation remains unclear. The interaction of collagen with $\alpha_2\beta_1$ could generate a signal contributing to platelet activation. However, the binding of collagen to $\alpha_2\beta_1$ could simply enhance the interaction of the collagen molecule with GPVI thereby facilitating the activation of this receptor. The effects of 6F1 reported here could be manifest as a consequence of either of these possibilities. Both of these possibilities require that collagen is able to bind to $\alpha_2\beta_1$ relatively quickly. $\alpha_2\beta_1$ exists in different affinity states for collagen (Emsley *et al*., 2000), and it has been suggested that an inside-out signal is required to convert low affinity $\alpha_2\beta_1$ to a high affinity collagen binding conformation in a manner analogous to $\alpha_{IIb}\beta_3$ and fibrinogen binding (Jung & Moroi, 1998). Such a signal may arise from a weak interaction with GPVI, or alternatively *via* binding of collagen to another receptor. Alternatively, the differing affinity states of the integrin may exist in a dynamic equilibrium, regulated by the underlying activation status of the platelet. The stable binding of macromolecular collagen to a small proportion of $\alpha_2\beta_1$ in a high affinity state for collagen could result in a shift in the proportion of the remaining low affinity sites to a high affinity status thus enabling further binding. It could also facilitate sufficient activation *via* GPVI or other receptors to further activate $\alpha_2\beta_1$ *via* inside-out signalling.

It is noteworthy that while 6F1 was able to abolish the shape change response induced by collagen types I and IV, it only delayed and did not completely abolish aggregation induced by the same collagens. The most significant difference between the measurement of shape change and aggregation in these studies was the presence of the $\alpha_{IIb}\beta_3$ antagonist lotrafiban which was employed to prevent aggregation and hence to accentuate the shape change response. Since $\alpha_{IIb}\beta_3$ can generate intracellular signals on activation of platelets (Phillips *et al*., 2001), it is possible that by preventing aggregation with the antagonist lotrafiban, $\alpha_{IIb}\beta_3$-mediated signalling may have been inhibited, resulting in an apparent augmentation of the inhibitory effect of 6F1 in the shape change experiments. Further studies are ongoing to address the role of $\alpha_{IIb}\beta_3$ in collagen-induced platelet activation.

It has been suggested that the response to collagen arises as a result of a synergistic interaction between a low level of activation of GPVI and Gi-coupling agonists such as ADP (Nieswandt *et al*., 2001a). However, the nature of the initial signal that is responsible for the release of these mediators remains unclear. The ability of the ADP and TxA_2 blockers to abolish the responses to collagen suggests that under these conditions collagen may be unable to generate signals that can directly activate $\alpha_{IIb}\beta_3$ or induce actin polymerization, but that it is able to induce release of these secondary mediators. In contrast, the responses to CRP in the presence of ADP and TxA_2 blockers indicate that GPVI is able to directly induce aggregation when activated sufficiently strongly. These data suggest that collagen activates GPVI weakly, and it therefore remains unclear whether collagen induces the initial release by signalling solely *via* GPVI or whether some additional signalling pathway is involved (Atkinson *et al*., 2001).

In conclusion, we have confirmed the ability of collagen types I–V to induce shape change and aggregation of platelets through interactions with GPVI and $\alpha_2\beta_1$ and in a manner which is substantially dependent on the release of ADP and TxA_2. However, we have been unable to demonstrate the presence of collagen type-specific receptors capable of inducing shape change or aggregation in the absence of the GPVI/FcRγ chain complex. Furthermore, data from genetically-modified mice provide no evidence for a role of CD36 in collagen-induced responses. Although blockade of $\alpha_2\beta_1$ does not abolish responses induced by collagen, it can clearly facilitate a more brisk and rapid activation which could be of great importance under flow conditions, and may play a significant role in initiating thrombus formation *in vivo*. The ability of CRP to induce shape change and aggregation when ADP and TxA_2 are inhibited, in contrast to the collagens, suggests that collagen activates GPVI more weakly than CRP, raising the possibility that collagen acts *via* a further uncharacterized receptor. These data suggest that the primary activatory role of collagen is to induce the release of secondary platelet activators and that both GPVI and $\alpha_2\beta_1$ play important but distinct roles in this process.

This work was supported by the British Heart Foundation. S.P. Watson is a British Heart Foundation Senior Research Fellow. B.T. Atkinson is a holder of a British Heart Foundation Research Studentship. We would like to thank Prof Barry Coller (Mount Sinai School of Medicine, New York, U.S.A.) for supplying the 6F1 monoclonal antibody, Dr John McGregor (INSERM U331, Lyon, France) for supplying the 10.5 monoclonal antibody, Takashi Saito (Chiba University Graduate School, Japan) for supplying the FcRγ chain deficient mice, Dr Maria Febbraio (Cornell University, U.S.A.) for supplying the CD36 deficient mice, AstraZeneca R&D Charnwood for supplying AR-C67085, and GlaxoSmithKline for supplying lotrafiban.

References

ASSELIN, J., GIBBINS, J.M., ACHISON, M., LEE, Y.H., MORTON, L.F., FARNDALE, R.W., BARNES, M.J. & WATSON, S.P. (1997). A collagen-like peptide stimulates tyrosine phosphorylation of syk and phospholipase C gamma2 in platelets independent of the integrin alpha2beta1. *Blood,* **89,** 1235–1242.

ATKINSON, B.T., STAFFORD, M.J., PEARS, C.J. & WATSON, S.P. (2001). Signalling events underlying platelet aggregation induced by the glycoprotein VI agonist convulxin. *Eur. J. Biochem.,* **268,** 5242–5248.

BALLEISEN, L., GAY, S., MARX, R. & KUHN, K. (1975). Comparative investigation on the influence of human and bovine collagen types I, II and III on the aggregation of human platelets. *Klin. Wochenschr.,* **53,** 903–905.

BARNES, M.J., BAILEY, A.J., GORDON, J.L. & MACINTYRE, D.E. (1980). Platelet aggregation by basement membrane-associated collagens. *Thromb. Res.,* **18,** 375–388.

BARNES, M.J., GORDON, J.L. & MACINTYRE, D.E. (1976). Platelet-aggregating activity of type I and type III collagens from human aorta and chicken skin. *Biochem. J.,* **160,** 647 – 651.

BARNES, M.J., KNIGHT, C.G. & FARNDALE, R.W. (1998). The collagen-platelet interaction. *Curr. Opin. Hematol.,* **5,** 314 – 320.

BATES, D.M. & WATTS, D.G. (1988). *Nonlinear regression analysis and its applications*. New York: Wiley.

BERGMAN, I. & LOXLEY, R. (1963). Two improved and simplified methods for the spectrophotometric determination of hydroxyproline. *Anal. Chem.,* **35,** 1961 – 1965.

BORN, G.V.R. & CROSS, M.J. (1963). The aggregation of blood platelets. *J. Physiol.,* **168,** 178 – 195.

CAUWENBERGHS, N., VANHOORELBEKE, K., VAUTERIN, S. & DECKMYN, H. (2000). Structural determinants within platelet glycoprotein Ibalpha involved in its binding to von Willebrand factor. *Platelets,* **11,** 373 – 378.

CHIANG, T.M., MAINARDI, C.L., SEYER, J.M. & KANG, A.H. (1980). Collagen-platelet interaction. Type V(A-B) collagen induced platelet aggregation. *J. Lab. Clin. Med.,* **95,** 99 – 107.

CHIANG, T.M., RINALDY, A. & KANG, A.H. (1997). Cloning, characterization, and functional studies of a nonintegrin platelet receptor for type I collagen. *J. Clin. Invest.,* **100,** 514 – 521.

CLEMETSON, K.J. & CLEMETSON, J.M. (2001). Platelet collagen receptors. *Thromb. Haemost.,* **86,** 189 – 197.

COLLER, B.S., BEER, J.H., SCUDDER, L.E. & STEINBERG, M.H. (1989). Collagen-platelet interactions: evidence for a direct interaction of collagen with platelet GPIa/IIa and an indirect interaction with platelet GPIIb/IIIa mediated by adhesive proteins. *Blood,* **74,** 182 – 192.

DANIEL, J.L., DANGELMAIER, C., STROUSE, R. & SMITH, J.B. (1994). Collagen induces normal signal transduction in platelets deficient in CD36 (platelet glycoprotein IV). *Thromb. Haemost.,* **71,** 353 – 356.

DE LEAN, A., HANCOCK, A.A. & LEFKOWITZ, R.J. (1982). Validation and statistical analysis of a computer modeling method for quantitative analysis of radioligand binding data for mixtures of pharmacological receptor subtypes. *Mol. Pharmacol.,* **21,** 5 – 16.

DE LEAN, A., MUNSON, P.J. & RODBARD, D. (1978). Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am. J. Physiol.,* **235,** E97 – E102.

DECKMYN, H., CHEW, S.L. & VERMYLEN, J. (1990). Lack of platelet response to collagen associated with an autoantibody against glycoprotein Ia: a novel cause of acquired qualitative platelet dysfunction. *Thromb. Haemost.,* **64,** 74 – 79.

EMSLEY, J., KNIGHT, C.G., FARNDALE, R.W., BARNES, M.J. & LIDDINGTON, R.C. (2000). Structural basis of collagen recognition by integrin alpha2beta1. *Cell,* **101,** 47 – 56.

FAUVEL, F., KARNIGUIAN, A., LEGER, D., PIGNAUD, G. & LEGRAND, Y.J. (1985). Collagen induced platelet aggregation: a comparison between several commercially available collagens. *Thromb. Res.,* **38,** 707 – 711.

FEBBRAIO, M., ABUMRAD, N.A., HAJJAR, D.P., SHARMA, K., CHENG, W., PEARCE, S.F. & SILVERSTEIN, R.L. (1999). A null mutation in murine CD36 reveals an important role in fatty acid and lipoprotein metabolism. *J. Biol. Chem.,* **274,** 19055 – 19062.

GEAR, A.R. (1981). Preaggregation reactions of platelets. *Blood,* **58,** 477 – 490.

GIBBINS, J.M., BRIDDON, S., SHUTES, A., VAN VUGT, M.J., VAN DE WINKEL, J.G., SAITO, T. & WATSON, S.P. (1998). The p85 subunit of phosphatidylinositol 3-kinase associates with the Fc receptor gamma-chain and linker for activitor of T cells (LAT) in platelets stimulated by collagen and convulxin. *J. Biol. Chem.,* **273,** 34437 – 34443.

GROSS, B.S., LEE, J.R., CLEMENTS, J.L., TURNER, M., TYBULEWICZ, V.L., FINDELL, P.R., KORETZKY, G.A. & WATSON, S.P. (1999). Tyrosine phosphorylation of SLP-76 is downstream of Syk following stimulation of the collagen receptor in platelets. *J. Biol. Chem.,* **274,** 5963 – 5971.

HANCOCK, A.A., BUSH, E.N., STANISIC, D., KYNCL, J.J. & LIN, C.T. (1988). Data normalization before statistical analysis: keeping the horse before the cart. *Trends Pharmacol. Sci.,* **9,** 29 – 32.

HANDA, M., WATANABE, K., KAWAI, Y., KAMATA, T., KOYAMA, T., NAGAI, H. & IKEDA, Y. (1995). Platelet unresponsiveness to collagen: involvement of glycoprotein Ia-IIa (alpha 2 beta 1 integrin) deficiency associated with a myeloproliferative disorder. *Thromb. Haemost.,* **73,** 521 – 528.

HERS, I., BERLANGA, O., TIEKSTRA, M.J., KAMIGUTI, A.S., THEAKSTON, R.D. & WATSON, S.P. (2000). Evidence against a direct role of the integrin alpha2beta1 in collagen-induced tyrosine phosphorylation in human platelets. *Eur. J. Biochem.,* **267,** 2088 – 2097.

HUANG, M.M., BOLEN, J.B., BARNWELL, J.W., SHATTIL, S.J. & BRUGGE, J.S. (1991). Membrane glycoprotein IV (CD36) is physically associated with the Fyn, Lyn, and Yes protein-tyrosine kinases in human platelets. *Proc. Natl. Acad. Sci. U.S.A.,* **88,** 7844 – 7848.

IVASKA, J., REUNANEN, H., WESTERMARCK, J., KOIVISTO, L., KAHARI, V.M. & HEINO, J. (1999). Integrin alpha2beta1 mediates isoform-specific activation of p38 and upregulation of collagen gene transcription by a mechanism involving the alpha2 cytoplasmic tail. *J. Cell Biol.,* **147,** 401 – 416.

JUNG, S.M. & MOROI, M. (1998). Platelets interact with soluble and insoluble collagens through characteristically different reactions. *J. Biol. Chem.,* **273,** 14827 – 14837.

KAWAMOTO, Y. & KAIBARA, M. (1992). Reconstituted collagen is not capable of activating factor XII but causes intrinsic coagulation by activating platelets. *Blood Coagul. Fibrinolysis,* **3,** 371 – 379.

KEHREL, B., BALLEISEN, L., KOKOTT, R., MESTERS, R., STENZINGER, W., CLEMETSON, K.J. & VAN DE LOO, J. (1988). Deficiency of intact thrombospondin and membrane glycoprotein Ia in platelets with defective collagen-induced aggregation and spontaneous loss of disorder. *Blood,* **71,** 1074 – 1078.

KEHREL, B., KRONENBERG, A., RAUTERBERG, J., NIESING-BRESCH, D., NIEHUES, U., KARDOEUS, J., SCHWIPPERT, B., TSCHOPE, D., VAN DE LOO, J. & CLEMETSON, K.J. (1993). Platelets deficient in glycoprotein IIIb aggregate normally to collagens type I and III but not to collagen type V. *Blood,* **82,** 3364 – 3370.

KNIGHT, C.G., MORTON, L.F., ONLEY, D.J., PEACHEY, A.R., ICHINOHE, T., OKUMA, M., FARNDALE, R.W. & BARNES, M.J. (1999). Collagen-platelet interaction: Gly-Pro-Hyp is uniquely specific for platelet Gp VI and mediates platelet activation by collagen. *Cardiovasc. Res.,* **41,** 450 – 457.

KNIGHT, C.G., MORTON, L.F., PEACHEY, A.R., TUCKWELL, D.S., FARNDALE, R.W. & BARNES, M.J. (2000). The collagen-binding A-domains of integrins alpha(1)beta(1) and alpha(2)beta(1) recognize the same specific amino acid sequence, GFOGER, in native (triple-helical) collagens, *J. Biol. Chem.,* **275,** 35 – 40.

MCKEOWN, L., VAIL, M., WILLIAMS, S., KRAMER, W., HANSMANN, K. & GRALNICK, H. (1994). Platelet adhesion to collagen in individuals lacking glycoprotein IV. *Blood,* **83,** 2866 – 2871.

MONNET, E. & FAUVEL-LAFEVE, F. (2000). A new platelet receptor specific to type III collagen. Type III collagen-binding protein. *J. Biol. Chem.,* **275,** 10912 – 10917.

MONNET, E., SIZARET, P., ARBEILLE, B. & FAUVEL-LAFEVE, F. (2000). Different role of platelet glycoprotein GP Ia/IIa in platelet contact and activation induced by type I and type III collagens. *Thromb. Res.,* **98,** 423 – 433.

MOOG, S., MANGIN, P., LENAIN, N., STRASSEL, C., RAVANAT, C., SCHUHLER, S., FREUND, M., SANTER, M., KAHN, M., NIESWANDT, B., GACHET, C., CAZENAVE, J.P. & LANZA, F. (2001). Platelet glycoprotein V binds to collagen and participates in platelet adhesion and aggregation. *Blood,* **98,** 1038 – 1046.

MOROI, M., JUNG, S.M., OKUMA, M. & SHINMYOZU, K. (1989). A patient with platelets deficient in glycoprotein VI that lack both collagen-induced aggregation and adhesion. *J. Clin. Invest.,* **84,** 1440 – 1445.

MOROI, M., ONITSUKA, I., IMAIZUMI, T. & JUNG, S.M. (2000). Involvement of activated integrin alpha2beta1 in the firm adhesion of platelets onto a surface of immobilized collagen under flow conditions. *Thromb. Haemost.,* **83,** 769 – 776.

MORTON, L.F., HARGREAVES, P.G., FARNDALE, R.W., YOUNG, R.D. & BARNES, M.J. (1995). Integrin alpha 2 beta 1-independent activation of platelets by simple collagen-like peptides: collagen tertiary (triple-helical) and quaternary (polymeric) structures are sufficient alone for alpha 2 beta 1-independent platelet reactivity. *Biochem. J.,* **306,** 337 – 344.

NIESWANDT, B., BERGMEIER, W., ECKLY, A., SCHULTE, V., OHLMANN, P., CAZENAVE, J.P., ZIRNGIBL, H., OFFERMANNS, S. & GACHET, C. (2001a). Evidence for cross-talk between glycoprotein VI and Gi-coupled receptors during collagen-induced platelet aggregation. *Blood,* **97,** 3829 – 3835.

NIESWANDT, B., BERGMEIER, W., SCHULTE, V., RACKEBRANDT, K., GESSNER, J.E. & ZIRNGIBL, H. (2000). Expression and function of the mouse collagen receptor glycoprotein VI is strictly dependent on its association with the FcRgamma chain. *J. Biol. Chem.,* **275,** 23998 – 24002.

NIESWANDT, B., BRAKEBUSCH, C., BERGMEIER, W., SCHULTE, V., BOUVARD, D., MOKHTARI-NEJAD, R., LINDHOUT, T., HEEMSKERK, J.W., ZIRNGIBL, H. & FASSLER, R. (2001b). Glycoprotein VI but not alpha2beta1 integrin is essential for platelet interaction with collagen. *EMBO J.,* **20,** 2120 – 2130.

NIEUWENHUIS, H.K., AKKERMAN, J.W., HOUDIJK, W.P. & SIXMA, J.J. (1985). Human blood platelets showing no response to collagen fail to express surface glycoprotein Ia. *Nature,* **318,** 470 – 472.

PARK, S.Y., UEDA, S., OHNO, H., HAMANO, Y., TANAKA, M., SHIRATORI, T., YAMAZAKI, T., ARASE, H., ARASE, N., KARASAWA, A., SATO, S., LEDERMANN, B., KONDO, Y., OKUMURA, K., RA, C. & SAITO, T. (1998). Resistance of Fc receptor-deficient mice to fatal glomerulonephritis. *J. Clin. Invest.,* **102,** 1229 – 1238.

PHILLIPS, D.R., PRASAD, K.S., MANGANELLO, J., BAO, M. & NANNIZZI-ALAIMO, L. (2001). Integrin tyrosine phosphorylation in platelet signaling. *Curr. Opin. Cell Biol.,* **13,** 546 – 554.

POOLE, A., GIBBINS, J.M., TURNER, M., VAN VUGT, M.J., VAN DE WINKEL, J.G., SAITO, T., TYBULEWICZ, V.L. & WATSON, S.P. (1997). The Fc receptor gamma-chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen. *EMBO J.,* **16,** 2333 – 2341.

QUEK, L.S., PASQUET, J.M., HERS, I., CORNALL, R., KNIGHT, G., BARNES, M., HIBBS, M.L., DUNN, A.R., LOWELL, C.A. & WATSON, S.P. (2000). Fyn and Lyn phosphorylate the Fc receptor gamma chain downstream of glycoprotein VI in murine platelets, and Lyn regulates a novel feedback pathway. *Blood,* **96,** 4246 – 4253.

SAELMAN, E.U., NIEUWENHUIS, H.K., HESE, K.M., DE GROOT, P.G., HEIJNEN, H.F., SAGE, E.H., WILLIAMS, S., MCKEOWN, L., GRALNICK, H.R. & SIXMA, J.J. (1994). Platelet adhesion to collagen types I through VIII under conditions of stasis and flow is mediated by GPIa/IIa (alpha 2 beta 1-integrin). *Blood,* **83,** 1244 – 1250.

SANTORO, S.A., WALSH, J.J., STAATZ, W.D. & BARANSKI, K.J. (1991). Distinct determinants on collagen support alpha 2 beta 1 integrin-mediated platelet adhesion and platelet activation. *Cell Regul.,* **2,** 905 – 913.

SAVAGE, B., GINSBERG, M.H. & RUGGERI, Z.M. (1999). Influence of fibrillar collagen structure on the mechanisms of platelet thrombus formation under flow. *Blood,* **94,** 2704 – 2715.

SILJANDER, P. & LASSILA, R. (1999). Studies of adhesion-dependent platelet activation: distinct roles for different participating receptors can be dissociated by proteolysis of collagen. *Arterioscler. Thromb. Vasc. Biol.,* **19,** 3033 – 3043.

SIXMA, J.J., VAN ZANTEN, G.H., SAELMAN, E.U., VERKLEIJ, M., LANKHOF, H., NIEUWENHUIS, H.K. & DE GROOT, P.G. (1995). Platelet adhesion to collagen. *Thromb. Haemost.,* **74,** 454 – 459.

TANDON, N.N., KRALISZ, U. & JAMIESON, G.A. (1989). Identification of glycoprotein IV (CD36) as a primary receptor for platelet-collagen adhesion. *J. Biol. Chem.,* **264,** 7576 – 7583.

TRELSTAD, R.L. & CARVALHO, A.C. (1979). Type IV and type 'A-B' collagens do not elicit platelet aggregation or the serotonin release reaction. *J. Lab. Clin. Med.,* **93,** 499 – 505.

VERKLEIJ, M.W., MORTON, L.F., KNIGHT, C.G., DE GROOT, P.G., BARNES, M.J. & SIXMA, J.J. (1998). Simple collagen-like peptides support platelet adhesion under static but not under flow conditions: interaction via alpha2 beta1 and von Willebrand factor with specific sequences in native collagen is a requirement to resist shear forces. *Blood,* **91,** 3808 – 3816.

WATSON, S., BERLANGA, O., BEST, D. & FRAMPTON, J. (2000). Update on collagen receptor interactions in platelets: is the two-state model still valid? *Platelets,* **11,** 252 – 258.

XU, Y., GURUSIDDAPPA, S., RICH, R.L., OWENS, R.T., KEENE, D.R., MAYNE, R., HOOK, A. & HOOK, M. (2000). Multiple binding sites in collagen type I for the integrins alpha1beta1 and alpha2beta1. *J. Biol. Chem.,* **275,** 38981 – 38989.

YAMAMOTO, N., AKAMATSU, N., YAMAZAKI, H. & TANOUE, K. (1992). Normal aggregations of glycoprotein IV (CD36)-deficient platelets from seven healthy Japanese donors. *Br. J. Haematol.,* **81,** 86 – 92.

(Received April 22, 2002
Revised May 31, 2002
Accepted June 10, 2002)