HEMOSTASIS, THROMBOSIS, AND VASCULAR BIOLOGY

Collagen Mediates Changes in Intracellular Calcium in Primary Mouse Megakaryocytes Through syk-Dependent and -Independent Pathways

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We have characterized changes in [Ca^{2+}]_i in primary mouse megakaryocytes in response to fibrillar collagen and in response to cross-linking of the collagen receptor, the integrin αβ1. The response to collagen was markedly different from that seen to a triple helical collagen-related peptide (CRP), which signals via the tyrosine kinases p59^fyn and p72^syk. This peptide binds to the collagen receptor glycoprotein VI (GPVI), but not to the integrin αβ1. Collagen elicited a sustained increase in [Ca^{2+}]_i composed primarily of influx of extracellular Ca^{2+} with some Ca^{2+} release from internal stores. In contrast to CRP, this response was only partially (−30%) inhibited by the src-family kinase inhibitor PP1 (10 μmol/L) or by microinjection of the tandem SH2 domains of p72^syk. Collagen also caused an increase in [Ca^{2+}]_i in megakaryocytes deficient in either p59^fyn or p72^syk, although the response was reduced by approximately 40% in both cases: Cross-linking of the α2 integrin increased [Ca^{2+}]_i in these cells exclusively via Ca^{2+} influx. This response was reduced by approximately 50% after PP1 pretreatment, but was significantly increased in fyn-deficient megakaryocytes. Collagen therefore increases [Ca^{2+}]_i in mouse megakaryocytes via multiple receptors, including GPVI, which causes Ca^{2+} mobilization, and αβ1, which stimulates a substantial influx of extracellular Ca^{2+}.

COLLAGENS ARE IMPORTANT and widespread extracellular matrix proteins. In blood vessels, type I and type III collagen form the subendothelial matrix, where they play an important role in the maintenance of vascular integrity. After damage to the blood vessel lining, platelets adhere to these collagens and are subsequently activated, leading to the formation of a hemostatic plug. Platelets express several receptors for collagen, including the integrin αβ1, glycoprotein IV (GPIV), and glycoprotein VI (GPVI), and other proteins of 65 kD and 85-90 kD and GPIIb/IIIa. These receptors appear to be variously responsible for adhesion to and activation of platelets by collagen. A two-step, two-site model of collagen action has been proposed involving initial adhesion to αβ1, followed by subsequent binding to an activating receptor, which mounting evidence suggests is GPVI. The signaling pathway that is thought to be activated downstream of GPVI in human platelets involves its association with the Fc receptor γ chain (FcR γ chain), which is better known as a receptor for IgG and IgE. After stimulation of platelets by collagen or a collagen-related peptide (CRP), the FcR γ chain is phosphorylated on tyrosine residues, probably within a conserved cytoplasmic domain known as the immune receptor tyrosine-based activation motif or ITAM. ITAM phosphorylation allows subsequent binding and activation of the tyrosine kinase p72^syk via its SH2 domains, leading to activation of phospholipase Cγ2 (PLCγ2), an increase in intracellular calcium [Ca^{2+}], and activation of protein kinase C. This pathway is essential for platelet secretion and aggregation by collagen, because platelets from mice deficient in either p72^syk or the FcR γ chain are not activated when stimulated with fibrillar collagen.

Less is known of the signaling capacity of other platelet collagen receptors. The integrin αβ1 is essential for efficient platelet adhesion to collagen, particularly under conditions of high shear. However, its role in intracellular signaling is less clear. Keely and Parise have shown that αβ1 is necessary for full collagen-induced activation and phosphorylation of p72^syk and PLCγ2, whereas Kamiguti et al have also suggested a role for αβ1 in p72^syk phosphorylation. In pancreatic acinar cells, αβ1 has been linked to increases in [Ca^{2+}], and a number of other reports have shown calcium elevation after cross-linking of other integrins such as αβ1. Platelet GPIV is known to bind collagen and is associated with the src-family kinases p59^fyn, p53/56^fyn, and p62^fyn, suggesting that it may be capable of transducing intracellular signals.

Less is known regarding the signaling pathways activated by collagen in other cell types and in particular the platelet precursor cell, the megakaryocyte. Megakaryocytes are large polyploid bone marrow cells that mature in the bone marrow, before releasing platelets into the bloodstream. At full maturity, they express many of the cell surface proteins that are expressed on platelets, including the fibrinogen receptor GPIIb/IIIa (αβ3), the receptor for von Willebrand’s factor, GPIb, and αβ1. They also respond to classic platelet agonists such as thrombin and ADP and to CRP. CRP consists of a glycine-proline-hydroxyproline repeat sequence, cross-linked via cysteine residues in its C- and N-terminals, and binds to GPVI, but not αβ1. In megakaryocytes, it elicits an increase in [Ca^{2+}]_i in these cells through a tyrosine kinase-dependent pathway that is thought to be the same as that found in platelets, with crucial roles for p72^syk and the src-family kinase p59^fyn.

The presence of other collagen receptors prompted us to investigate the nature of the response in megakaryocytes to collagen itself and, after cross-linking of one of its receptors, the αβ1 integrin. This showed differences in the signaling pathways activated by collagen in megakaryocytes and platelets. Collagen can activate at least two pathways in megakaryocytes leading to the mobilization of calcium from intracellular stores.

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Submitted August 27, 1998; accepted February 1, 1999.

Supported by The Wellcome Trust and the British Heart Foundation.

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0006-4971/99/9311-0034$3.00/0
and the influx of extracellular calcium. The evidence suggests a role for both GPV1 and the integrin $\alpha_\text{IIb}\beta_3$ in the response to collagen and may indicate a difference in the role of collagen signaling in the two cell types.

**MATERIALS AND METHODS**

**Materials.** Collagen fibers (predominantly type I) derived from equine Achilles tendon were obtained from Nycomed (Manchester, Germany). CRP [GCP(P')$_3$G-P]$_9$GCP(G, single letter amino acid code; P' = hydroxyproline) was provided by Dr M. Barnes (Strangeways Laboratories, Cambridge, UK) and cross-linked as described. Armenian hamster anti-mouse CD49b (integrin $\alpha_\text{IIb}$ subunit) and mouse antihamster IgG known to recognize the anti-$\alpha_\text{IIb}$ monoclonal antibody (MoAb) were obtained from Pharmingen (Cambridge Bioscience, Cambridge, UK). PP1 was provided by Dr J. Hanke (Pfizer, Groton, CT). Bovine thrombin, apyrase, staurosporine, and poly-L-lysine (70,000 molecular weight [MW]) were from Sigma (Poole, Dorset, UK). FURA-2 and calibration standards were from Molecular Probes (Eugene, OR). A recombinant protein containing glutathione-S-transferase (GST) fusion protein linked to the tandem SH2 domains of p72^syk was expressed in Echerichia coli and purified as previously described. GST was then removed by thrombin cleavage, and thrombin was removed by passage over Hi-Trap heparin columns (Pharmacia, Uppsala, Sweden). All other reagents were from previously reported sources.

**Protein-deficient mice.** BALB/c radiation chimeric mice reconstituted with syk-deficient fetal liver were generated as previously described. Fyn-deficient mice on a C57BL/6 background were obtained from Jackson Laboratories (Bar Harbor, ME).

**Preparation of mouse megakaryocytes.** Mouse megakaryocytes were prepared as previously described. Briefly, 4- to 8-week-old BALB/c or protein-deficient mice were killed by cervical dislocation and the femurs were removed. Bone marrow was flushed out gently in calcium/magnesium-free Hanks' buffer containing 0.4% wt/vol bovine serum albumin (BSA) and 0.2 U/mL apyrase using a 23-gauge needle. Cells were collected by centrifugation (200 $g$ for 8 minutes) in the presence of 1 µg/mL prostacyclin. For experimentation, cells were resuspended in a modified Hanks' buffer, containing low calcium (200 µmol/L) that prolonged cell survival (143 µmol/L NaCl, 5.6 µmol/L KCl, 2 µmol/L MgCl$_2$, 10 µmol/L HEPES, 10 µmol/L glucose, 0.2 µmol/L CaCl$_2$, and 0.4% BSA, pH 7.2). Cells were maintained at 37°C in this buffer until experimentation.

**Single-cell microinjection and calcium imaging.** Cell suspension (50 µL) was plated onto poly-L-lysine (70,000 MW)-coated coverslips and allowed to settle for 3 minutes. Excess suspension was removed and modified Hanks' buffer was added as bathing medium. Unless stated otherwise, experiments were performed with [Ca$_{2+}$]$_e$ = 200 µmol/L and at room temperature (22°C ± 2°C). Cells were viewed on an inverted Axiovert 110 microscope (Zeiss, Herts, UK), and stage III/IV megakaryocytes were identified on the basis of size and morphology. We have previously confirmed their identity as megakaryocytes through specific staining with the 1C2 Ab, which is specific to mouse megakaryocytes.

**Characterization of collagen-mediated changes in [Ca$_{2+}$]$_i$.** The addition of fibrillar type I collagen (100 µg/mL) to murine megakaryocytes in the presence of 200 µmol/L extracellular calcium ([Ca$_{2+}$]$_o$) caused a steady increase in [Ca$_{2+}$]$_i$, from a basal of 66 ± 7 nmol/L to a peak of 217 ± 19 nmol/L after a delay of approximately 30 to 60 seconds, leading to a sustained plateau (mean ± SEM, $n = 10$; increase over basal = 151 ± 26 nmol/L; Figs 1A and 2A). This plateau generally persisted for at least 10 minutes after the addition of collagen, decreasing slightly in this time. A similar profile of changes in [Ca$_{2+}$]$_i$ were seen in megakaryocytes isolated from human bone marrow (unpublished observations). The increase in [Ca$_{2+}$]$_i$ seen after collagen stimulation was highly dependent on the level of [Ca$_{2+}$]$_o$, suggesting a prominent role for calcium influx in this response (Fig 1A). The addition of 10 mmol/L EGTA caused a small decrease in basal [Ca$_{2+}$]$_i$ (57 ± 21 nmol/L, $n = 6$). Collagen elicited a small but significant increase in [Ca$_{2+}$]$_i$ in the presence of EGTA, with a mean peak [Ca$_{2+}$]$_i$ value of 100 ± 24 nmol/L ($n = 6$), giving an increase over basal of 44 ± 12 nmol/L ($P < .01$ v basal). This indicates that collagen is capable of mobilizing calcium from intracellular stores in mouse megakaryocytes. The peak calcium level in the presence of 10 mmol/L EGTA was significantly reduced compared with that seen in 200 µmol/L [Ca$_{2+}$]$_o$ ($P < .05$), indicating that a significant component of the response was due to influx of [Ca$_{2+}$]$_o$. This was further demonstrated by increasing the [Ca$_{2+}$]$_o$ to 1.2 mmol/L, which caused a further elevation in the response to collagen, with increases of greater than 500 nmol/L above basal seen in all cells tested (peak [Ca$_{2+}$]$_i$ = 695 ± 150 nmol/L, $n = 6$; $P < .01$ v peak value in 200 µmol/L [Ca$_{2+}$]$_o$; Fig 1A). The addition of 1.2 mmol/L [Ca$_{2+}$]$_o$ caused no significant increase in the resting level of [Ca$_{2+}$]$_i$ in these cells (108 ± 37 nmol/L, $n = 6$).

**RESULTS**

Characterization of collagen-mediated changes in [Ca$_{2+}$]$_i$.
Collagen-mediated increase in intracellular calcium ([Ca^{2+}]_i) in megakaryocytes from wild-type and syk^{-/-} mice. Megakaryocytes from either (A) wild-type Balb/c mice or (B) radiation chimeric Balb/c mice repopulated with syk^{-/-} fetal liver were isolated from bone marrow as described. After plating onto poly-L-lysine-coated coverslips, stage III/IV megakaryocytes were identified and injected with FURA-2 and then stimulated with collagen (100 µg/mL) as indicated. [Ca^{2+}]_i levels were adjusted 2 minutes before the addition of agonist. FURA-2 fluorescence was measured as described and [Ca^{2+}]_i was determined from a precalculated calibration curve. Representative traces in (A) and (B) are each taken from cells from a single animal, but a representative of at least 6 (A) or 3 (B) cells from a minimum of three mice.

The role of syk- and src-family kinases in the collagen-mediated changes in [Ca^{2+}]_i. Initial results suggested that, in megakaryocytes, in addition to GPVI, collagen activates other cell membrane receptors. Further evidence for this was obtained from work investigating the role of syk- and src-family kinases in this change in [Ca^{2+}]_i. Initially, we investigated the role of p72^syk in the Ca^{2+} response to collagen in these cells (Fig 2 and Table 1). Our previous work with CRP demonstrated that the pathway by which this agonist increased [Ca^{2+}]_i, in mouse megakaryocytes was entirely dependent on the tyrosine kinase p72^syk.22 This was shown partly by microinjection of a recombinant protein composed of the tandem SH2 domains of p72^syk. The inhibition is believed to be through the binding of the recombinant protein to the phosphorylated ITAM motif on the FcRγ chain, which then prevents further signal propagation via this pathway. Injection of this protein therefore allows us to investigate the role of the GPV/FcRγ chain pathway in the collagen response, in addition to other syk-SH2-dependent signaling pathways. In agreement with our previous study, microinjection of the SH2 domains of p72^syk protein did not significantly affect the resting calcium levels in the cells (56 ± 7 nmol/L, n = 10; Table 1). However, the addition of collagen in the presence of this protein caused a large increase in [Ca^{2+}]_i, levels, although the peak value of 188 ± 11 nmol/L gave a mean increase above basal that was significantly reduced compared with control cells (133 ± 11 nmol/L, n = 10; P < .05; Table 1 and Fig 2C). To further investigate the role of p72^syk in collagen-mediated calcium increases, radiation chimeric mice, repopulated with syk^{-/-} fetal liver, were produced as previously described.23 Syk^{-/-} megakaryocytes isolated from these animals had a similar basal [Ca^{2+}]_i, to control cells (73 ± 6 nmol/L, n = 6). Collagen caused a significant increase in [Ca^{2+}]_i, in syk^{-/-} cells, with a peak value of 179 ± 10 nmol/L and an increase above basal levels of 114 ± 10 nmol/L (P < .01 vs basal). This is approximately 40% lower than in control cells (P < .05; Table 1 and Fig 2D). The increase in [Ca^{2+}]_i, mediated by collagen in the syk^{-/-} megakaryocytes was, like the response in control cells, highly dependent on the concentration of [Ca^{2+}]_i, indicating that collagen could initiate calcium influx independently of p72^syk (Fig 1B). Interestingly, there was a small but significant increase in [Ca^{2+}]_i, in syk^{-/-} megakaryocytes (P < .05) in the presence of 10 nmol/L EGTA, suggesting the existence of a minor syk-independent pathway for collagen-mediated mobilization of [Ca^{2+}]_i, from intracellular stores.

We then investigated the role of Src-family kinases in the collagen-mediated increases in [Ca^{2+}]_i, because the response to CRP in mouse megakaryocytes is mediated predominantly via the src-family kinase p59^{fyn}. Pretreatment of mouse megakaryocytes with the Src-family kinase inhibitor PP1 26 (10 µmol/L) affected neither the resting [Ca^{2+}]_i, (67 ± 13 nmol/L, n = 9) nor the peak in [Ca^{2+}]_i, after collagen stimulation (201 ± 18 nmol/L), although the increase above resting levels was significantly reduced (136 ± 8 nmol/L, n = 9; P < .05; Table 1 and Fig 2E). However, interestingly, the duration of the collagen response was reduced in the presence of PP1, suggesting a role for a Src-family kinase in the maintenance of calcium influx. Because PP1 may not be specific to p59^{fyn} at this concentration, we also studied the collagen-mediated change in [Ca^{2+}]_i, in megakaryocytes from fyn-deficient mice. In these cells, collagen elicited a smaller increase in [Ca^{2+}]_i, to that seen in control cells, but this failed to reach significance (peak [Ca^{2+}]_i = 183 ± 26 nmol/L, rise above basal = 106 ± 27 nmol/L, n = 4; Fig 2F).
Fig 2. The role of Syk- and Src-family kinases in collagen- and CRP-mediated calcium increases in mouse megakaryocytes. Single mouse megakaryocytes from Balb/c bone marrow were microinjected with FURA-2, and the changes in \([\text{Ca}^{2+}]_i\) were determined in response to (A) 100 \(\mu\)g/mL collagen or (B) 2 \(\mu\)g/mL CRP. The response to collagen (100 \(\mu\)g/mL) was then determined (C) after comicroinjection of a recombinant fusion protein containing the tandem SH2 domains of syk; (D) in syk-deficient megakaryocytes; (E) in the presence of the Src-family kinase inhibitor, PP1 (10 \(\mu\)mol/L for 3 minutes); or (F) in fyn-deficient megakaryocytes. Each trace is representative of 4 to 10 cells from a minimum of three different mice. In each case, \([\text{Ca}^{2+}]_i\) was fixed at 200 \(\mu\)mol/L.
Collagen Signaling in Megakaryocytes

### Table 1. Collagen-Mediated Changes in [Ca^{2+}] in Mouse Megakaryocytes in the Presence of Tyrosine Kinase Inhibitors and in Kinase-Deficient Megakaryocytes

<table>
<thead>
<tr>
<th></th>
<th>Peak [Ca^{2+}] Value (nmol/L; mean ± SEM)</th>
<th>Increase in [Ca^{2+}] (nmol/L; mean ± SEM)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resting (100 µg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>66 ± 7</td>
<td>161 ± 17</td>
<td>10</td>
</tr>
<tr>
<td>Syk SH2 domains</td>
<td>56 ± 7</td>
<td>153 ± 11</td>
<td>10</td>
</tr>
<tr>
<td>Syk-deficient</td>
<td>73 ± 6</td>
<td>114 ± 12</td>
<td>6</td>
</tr>
<tr>
<td>10 µmol/L PP1</td>
<td>67 ± 13</td>
<td>136 ± 8</td>
<td>9</td>
</tr>
<tr>
<td>Fyn-deficient</td>
<td>77 ± 13</td>
<td>106 ± 27</td>
<td>4</td>
</tr>
<tr>
<td>3 µmol/L Staurosporine</td>
<td>74 ± 19</td>
<td>155 ± 37</td>
<td>8</td>
</tr>
</tbody>
</table>

The table indicates the resting and peak [Ca^{2+}]. For each group, the difference in peak and resting calcium was calculated. The increase in [Ca^{2+}] for each group was calculated as the difference between the peak calcium and the resting calcium.

To test the overall kinase-dependency of the collagen response, we used the general kinase inhibitor, staurosporine, which is known to prevent CRP-mediated calcium changes in these cells and also functional responses to collagen in human platelets. Preincubation of mouse megakaryocytes with staurosporine (3 µmol/L for 5 minutes) did not significantly affect either the resting level of [Ca^{2+}], (74 ± 19 nmol/L, n = 8) or the change in [Ca^{2+}], seen after the addition of 100 µg/ml collagen (peak [Ca^{2+}] = 224 ± 33 nmol/L; increase above basal = 155 ± 37 nmol/L; data not shown). This result strongly suggests that collagen can increase [Ca^{2+}], independently of tyrosine phosphorylation in these cells.

The role of α2β1 in collagen-mediated changes in [Ca^{2+}], in mouse megakaryocytes. Taken together, these results show that the collagen-mediated increase in [Ca^{2+}], in mouse megakaryocytes is partially mediated by the syk-dependent pathway previously described for CRP in these cells. This implies the existence of a second pathway of calcium elevation. Of the proposed receptors for collagen other than GPVI, the integrin α2β1 is the best characterized, and its presence on megakaryocytes has been described. Further, we investigated whether this integrin was capable of causing a change in [Ca^{2+}], in mouse megakaryocytes using a previously characterized MoAb to the α2 integrin subunit, which is known to block the adhesion of T cells to collagen gels. This was used in preference to anti-β1 subunit antibodies to avoid coincident cross-linking of, for instance, α2β1 or α3β1 integrins.

Mouse megakaryocytes were coated with the antismouse α2 MoAb (10 µg/mL) for 5 minutes and the excess was removed. A suitable cell was microinjected with FURA-2 and the primary antibody was cross-linked by the addition of the antihamster IgG secondary antibody (20 µg/mL). Cross-linking of α2 subunits caused a small but significant increase in [Ca^{2+}], in the presence of 200 µmol/L [Ca^{2+}], after a delay of 20 to 30 seconds (increase above basal = 95 ± 8 nmol/L, n = 8; Fig 3C). The response was smaller but similar in profile to that seen with collagen, consisting of an initial slow increase in [Ca^{2+}], followed by a prolonged plateau. The addition of primary antibody (10 µg/mL) or secondary antibody (20 µg/mL) alone to the incubation medium caused no significant change in cytosolic calcium levels (Fig 3A and B). The response seen after cross-linking with the anti-α2 Ab was dependent on [Ca^{2+}].

With [Ca^{2+}], at 1.2 mmol/L, cross-linking caused a significantly larger and prolonged response than in 200 µmol/L calcium (increase above basal = 279 ± 44 nmol/L, n = 11; P < .05; Fig 3D). In the presence of 10 mmol/L EGTA, clustering of α2 subunits did not cause any significant increase in [Ca^{2+}]. This suggests that in [Ca^{2+}], caused by cross-linking of the α2 subunit appear, therefore, to be mediated entirely by influx of [Ca^{2+}].

Because the influx of [Ca^{2+}], after collagen activation was partially sensitive to PP1 treatment, we investigated the role of src-family kinases in the response to cross-linking of the integrin α2β1 subunit. Preincubation with the src-family kinase inhibitor PP1 before the addition of the cross-linking antibody caused an approximate 50% decrease in the increase above resting [Ca^{2+}], with [Ca^{2+}], at 1.2 mmol/L (137 ± 31 nmol/L, n = 12). This was also the case when [Ca^{2+}], was at 200 µmol/L (Fig 3E).

DISCUSSION

The data presented in this study provide, to our knowledge, the first description of the changes in [Ca^{2+}], mediated by collagen in primary megakaryocytes. The study also provides evidence for the activation of at least two collagen receptors in these cells, which can influence [Ca^{2+}], via multiple pathways. We have delineated roles for (1) a collagen-stimulated mobilization of calcium from intracellular stores, dependent on p72^syk^ and probably mediated by GPVI; and (2) a collagen-mediated influx of extracellular calcium, which is p72^syk^- and p59^fyn^- independent and may be mediated by α2β1. These results demonstrate the complex nature of collagen signaling in these cells and highlight an important difference between signaling by this agonist in megakaryocytes and in platelets, in which the increase in [Ca^{2+}], mediated by collagen is mediated entirely by a tyrosine kinase-dependent pathway. We believe that this difference may reflect the differing physiological roles of the response to collagen in the two cell types.

Our previous studies using the synthetic collagen agonist, CRP, have demonstrated that a calcium-mobilizing pathway similar to that activated by GPVI/FcR γ chain in platelets exists in mouse megakaryocytes. The tyrosine kinases p59^fyn^ and p72^syk^ play crucial roles in this pathway, which is presumed to culminate in the activation of PLCγ2 and the generation of the calcium-mobilizing messenger, inositol 1,4,5-trisphosphate [Ins(1,4,5)P3]. The response to CRP is virtually abolished in mice megakaryocytes deficient in either p72^syk^ or p59^fyn^. This response consists almost entirely of calcium release from intracellular stores, because it is not significantly altered in the
presence of EGTA. In contrast to CRP, the collagen response was highly dependent on the level of extracellular calcium, indicating that a large component of the response is mediated by calcium influx. This suggests that the p59\textsuperscript{fyn}/p72\textsuperscript{syk} pathway was not the only pathway leading to an increase in [Ca\textsuperscript{2+}]i. Moreover, whereas collagen undoubtedly releases stored calcium in these cells, because there was still a response in the presence of EGTA, at physiologically relevant concentrations of...
[Ca\textsuperscript{2+}]\textsubscript{i}, influx comprises the greater part of the collagen response.

Evidence for the separation of the mechanisms underlying entry and mobilization of calcium by collagen came from the use of tyrosine kinase inhibitors and protein-deficient megakaryocytes. Collagen caused a significant increase in [Ca\textsuperscript{2+}]\textsubscript{i} in the presence of the general kinase inhibitor staurosporine, in the presence of the src-family kinase inhibitor PP1, and after microinjection of a protein containing the tandem SH2 domains of p72\textsuperscript{src}. Each of these treatments is known to block the response to CRP in these cells.\textsuperscript{22} Staurosporine and PP1 have also been shown to block the calcium mobilizing responses of collagen and CRP in human platelets.\textsuperscript{28,29} After microinjection of the tandem SH2 domains of p72\textsuperscript{src}, there was approximately 30% reduction in the peak response to collagen. This protein is thought to act by binding to phosphorylated FcR \( \gamma \) chain ITAM and preventing downstream signaling. These results suggest that the FcR \( \gamma \) chain pathway is responsible for only part of the collagen response. This was further supported by the persistence of a collagen response in syk-deficient megakaryocytes, whereas CRP-mediated calcium mobilization is absent. Importantly, collagen could also mediate substantial calcium influx in syk-deficient megakaryocytes, showing that calcium influx can be mediated independently of this kinase. The maintenance of calcium influx stimulated by collagen was partly affected by PP1, reflecting a role for a Src-family kinase in this response. A role for this kinase family in calcium influx has been demonstrated in HEL cells,\textsuperscript{30} whereas in platelets the presence of a tyrosine kinase-dependent route of calcium influx has been described.\textsuperscript{31}

Because a component of the collagen response is dependent on the tyrosine kinase p72\textsuperscript{src} and is affected in duration by PP1, it is noteworthy that the general kinase inhibitor, staurosporine, does not have the same effect. It is possible that collagen influences the calcium levels in megakaryocytes through both positive and negative kinase-dependent mechanisms. One could then envisage a situation in which a general kinase inhibitor such as staurosporine would affect both stimulatory and inhibitory pathways. For example, staurosporine inhibits serine/threonine kinases such as protein kinase C (PKC), which can directly affect capacitative calcium influx, or calcium extrusion mechanisms.\textsuperscript{32-34}

Cross-linking of the integrin \( \alpha_\text{IIb}\beta_1 \) in mouse megakaryocytes demonstrated that clustering of the integrin \( \alpha_\text{IIb}\beta_1 \) could also stimulate calcium influx. The integrins are a family of heterodimeric cell surface molecules that interact with a variety of extracellular matrix components such as collagens, fibronectin, and vitronectin.\textsuperscript{35} The integrin \( \alpha_\text{IIb}\beta_1 \) is an important receptor for collagen that is present on platelets, megakaryocytes, and a variety of other cell types. Whereas the signaling functions of \( \alpha_\text{IIb}\beta_1 \) are not well defined in platelets, there is evidence that binding of collagen to this receptor is necessary for full activation of p72\textsuperscript{src} and PLC\( \gamma \).\textsuperscript{13,14} and may mediate collagen activation of p60\textsuperscript{src}.\textsuperscript{36} In pancreatic acinar cells, the increase in [Ca\textsuperscript{2+}]\textsubscript{i}, caused by collagen appears to be mediated predominantly by \( \alpha_\text{IIb}\beta_1 \).\textsuperscript{15,37} The calcium signaling capacity of integrins in other cells has also been demonstrated, with contributions from tyrosine kinase-dependent and -independent pathways.\textsuperscript{16} For example, both soluble fibronectin and vitronectin mediate an increase in [Ca\textsuperscript{2+}]\textsubscript{i} in human endothelial cells,\textsuperscript{38} whereas the \( \alpha_\text{IIb}\beta_1 \) integrin in rat osteoclasts is responsible for calcium increases to vitronectin in this cell line.\textsuperscript{39} Integrins also regulate or induce protein tyrosine phosphorylation in many cell types.\textsuperscript{40} This seems to be predominantly linked to the formation of focal adhesions, but can lead to phosphorylation or activation of a variety of other proteins, including ZAP-70, PLC\( \gamma \)1, and Src-family kinases.\textsuperscript{37,40,41}

Cross-linking of the integrin \( \alpha_\text{IIb}\beta_1 \) subunit caused an increase in [Ca\textsuperscript{2+}]\textsubscript{i} in mouse megakaryocytes. This response appears to be mediated exclusively by the influx of extracellular calcium and is maintained at a lower level in the presence of PP1 but potentiated in fyn-deficient cells. It is therefore possible that differing src-family kinases play stimulatory and inhibitory roles in \( \alpha_\text{IIb}\beta_1 \) signaling in megakaryocytes. The pathway mediating the component of the response to \( \alpha_\text{IIb}\beta_1 \) cross-linking that is independent of src-family kinases is unknown, although there is some evidence to suggest that integrins can directly activate calcium influx channels independently of kinases.\textsuperscript{16}

A major finding of this study is the difference in calcium signaling pathways activated by collagen in megakaryocytes compared with those activated in platelets. In platelets, collagen mediates aggregation and dense granule release via a tyrosine kinase-dependent pathway, which can be inhibited by staurosporine and PP1.\textsuperscript{10,21,28} This is the predominant pathway in these cells, because mice platelets lacking p72\textsuperscript{src} or the FcR \( \gamma \) chain fail to undergo aggregation or secretion in response to collagen.\textsuperscript{11} However, in megakaryocytes, it appears that calcium influx, probably mediated by \( \alpha_\text{IIb}\beta_1 \), is the predominant pathway. This may reflect differences in the level of expression or the signaling pathways coupled to the integrin \( \alpha_\text{IIb}\beta_1 \) between megakaryocytes and platelets. This is likely to reflect important differences in the physiological role of collagen in the two cell types. For example, whereas collagen, CRP, and thrombin induce similar responses in the platelet, there are notable differences in megakaryocyte morphology after stimulation by the three agonists. We have sometimes observed a shape change or blebbing response of megakaryocytes in response to CRP (30% of cells) or thrombin (60% of cells; unpublished observations), which others have speculated is related to proplatelet formation.\textsuperscript{42} However, the addition of collagen did not cause blebbing, suggesting that the adhesion molecule overrides the response to cross-linking of GPVI, thereby protecting against premature proplatelet shedding. Collagen may also perform other functions in the megakaryocyte that have little significance to platelet function. We speculate, for example, that collagen may play a role in the migration and subsequent adhesion of mature megakaryocytes to the blood vessels, leading to protrusion into the circulation.\textsuperscript{19} It is notable that integrin-mediated Ca\textsuperscript{2+} fluxes have been shown to regulate adhesion and migration in other cells.\textsuperscript{39,43,44}

In conclusion, we have demonstrated the complex nature of the collagen-induced changes in [Ca\textsuperscript{2+}]\textsubscript{i}, in primary megakaryocytes. Collagen causes both calcium mobilization from intracellular stores and the influx of calcium from the extracellular solution. Although multiple pathways are involved in this response, mobilization from intracellular stores is mediated predominantly via a syk-dependent pathway, which may be via the GPVI pathway previously described in these cells. In
contrast, calcium influx occurs via a predominantly syk-independent pathway, which is at least partly mediated by $\alpha_2\beta_1$.

ACKNOWLEDGMENT

The authors thank Drs M. Barnes and R. Farndale for the CRP. J. Hanke for the gift of PP1, and Dr Che-Leung Law for the Syk fusion construct.

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