Distinct Spatio-Temporal Ca++ Signaling Elicited by Integrin α2β1 and Glycoprotein VI under Flow

Condensed Title: Platelet Collagen Receptors and Calcium Signaling

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Abbreviations used: ECM, extracellular matrix; GP, glycoprotein; VWF, von Willebrand factor; FcRγ, Fc receptor γ subunit; ITAM, immune-receptor tyrosine-based activation motif; PLC, phospholipase C; [Ca++]i, intracellular Ca++ concentration; ACD, citric acid/citrate/dextrose; PRP, platelet-rich plasma; AM, acetoxymethyl ester; PBS, phosphate-buffered saline; PI 3-K, phosphatidylinositol 3-kinase; EGTA, ethyleneglycoltetraacetic acid; 8-BR, 8-Br-guanosine; ASA, acetyl salicylic acid; FITC, fluorescein isothiocyanate; MFI, mean fluorescence intensity; CI, confidence intervals of a mean.

Key Words: Platelets, collagen, signaling, adhesion, aggregation
ABSTRACT

We studied how integrin α2β1 and glycoprotein (GP) VI contribute to collagen-induced platelet activation under flow conditions by evaluating stable adhesion and intracellular Ca++ concentration ([Ca++]i) of FLUO 3-AM-labeled platelets perfused over acid-soluble type I or microfibrillar type VI collagen. Adhering platelets displayed two kinds of [Ca++]i oscillations. Rapid α-type peaks were unaffected by the membrane-impermeable Ca++ chelator, EGTA, but abolished by membrane-permeable BAPTA-AM. Longer lasting γ-like peaks were always preceded by at least one α-like peak and abolished by intracellular or extracellular Ca++ chelation. Inhibition of phosphatidylinositol 3-kinase or phospholipase C and modulation of cyclic nucleotides, but not blockage of ADP receptors, prevented both Ca++ responses. Human or mouse platelets lacking GPVI function exhibited α-like but not γ-type Ca++ peaks, whereas those lacking α2β1 showed markedly reduced to absent α-type and no γ-like Ca++ peaks. Specific α2β1 ligation induced α-like but not γ-like peaks. Thus, α2β1 may generate Ca++ signals that are reinforced by GPVI and required for subsequent longer lasting Ca++ oscillation mediated by GPVI through transmembrane ion flux. Our results delineate a GPVI-independent signaling role of α2β1 in response to collagen stimulation.
INTRODUCTION

Platelet interaction with exposed extracellular matrix (ECM) at sites of vascular injury is a crucial step in hemostasis and thrombosis. Collagens in ECM mediate both platelet adhesion and activation through direct and indirect mechanisms influenced by fluid dynamic conditions. Above a threshold shear rate, the initial interaction between circulating platelets and the vessel wall is mediated by the binding of glycoprotein (GP) Ib to von Willebrand factor (VWF) immobilized onto collagen fibrils. The GPIb-VWF interaction promotes the initial tethering, but subsequent firm platelet adhesion is also supported by two collagen receptors, GPVI and the integrin α2β1, whose individual roles in collagen binding and platelet activation have been extensively studied in recent years. In vivo and ex vivo experiments have suggested that GPVI may be the principal receptor responsible for collagen-induced platelet activation. The signaling pathway elicited by the engagement of GPVI is strictly dependent on the Fc receptor γ subunit (FcRγ), which contains an immune-receptor tyrosine-based activation motif (ITAM) and forms a non-covalent membrane-expressed complex with GPVI.

The contribution of α2β1 to collagen-induced platelet activation and thrombus formation has been more controversial, but several observations suggest that it may have an important role. Patients with defective α2β1 manifest a mild bleeding tendency, and variations in the expression of this receptor correlate with a predisposition to thrombotic events. In mice, α2β1 deficiency results in impaired platelet adhesion to collagen and delayed thrombus formation - although this conclusion may be influenced by the type of thrombosis model used - and strain-related differences in its expression are associated with variable response to collagen. It is through that, like other integrins, α2β1 requires activation resulting from inside-out signaling as well as divalent cations to engage its ligands with high affinity, but while this may be a requisite...
for subsequent outside-in signaling, it may not be necessary for initial platelet-collagen contact. Thus, even in a low affinity state, \( \alpha_2\beta_1 \) may mediate platelet adhesion to collagen preceding GPVI-induced activation.\(^{12}\) It is also apparent that \( \alpha_2\beta_1 \) engagement generates tyrosine kinase-based intracellular signals, which underlie platelet spreading,\(^{13}\) through a pathway sharing many features with that elicited by GPVI.\(^{12}\) Of note, native collagen is an insoluble matrix protein, and the preparations used in \textit{ex vivo} experiments undergo manipulations that may variably influence the interaction with platelet receptors. For example, \( \alpha_2\beta_1 \) is required for normal platelet adhesion to pepsin-treated acid soluble collagen but not to acid-insoluble fibrils.\(^{14}\) Thus, the use of different collagen preparations may explain some of the discrepancies found in the literature with respect to the relative functions of the platelet collagen receptors.

Here, we have used acid soluble type I collagen and collagen type VI tetramers to study \( \alpha_2\beta_1 \) and GPVI function under flow conditions. The former collagen type was used to highlight the potential functions of \( \alpha_2\beta_1 \),\(^{14}\) the latter because collagen type VI, which forms mixed fibrils with the fibrillar collagens type I and III in ECM,\(^{15}\) is likely to be readily exposed to flowing blood at sites of vascular injury and, thus, of physiopathological significance.\(^{16}\) We found that engagement of \( \alpha_2\beta_1 \) under flow conditions induces the appearance of transient variations in intracellular Ca\(^{++}\) concentration ([Ca\(^{++}\)]\(_{i}\)) resulting from store release, and is a requisite for subsequent GPVI-mediated Ca\(^{++}\) signals induced by both collagen types. The sequential function of the two receptors underlines a potential synergy in thrombus formation responsive to the collagen composition of the vascular lesion and local fluid dynamic conditions.
MATERIALS AND METHODS

Preparation of blood samples. Venous blood from medication-free consenting volunteers -- in accordance with the Declaration of Helsinki, under protocols approved by the Ethics Committee of Centro Di Riferimento Oncologico and the Institutional Review Board of The Scripps Research Institute -- was mixed with 1/6 final volume of citric acid/citrate/dextrose (ACD), pH 4.5. The procedures to obtain platelet-rich plasma (PRP), load platelets with the fluorescent calcium probe FLUO 3-acetoxymethyl ester-AM (FLUO 3-AM; Molecular Probes, Eugene, OR; 8 μM), and prepare a washed erythrocyte suspension have been described previously in detail. In selected experiments, platelets were loaded simultaneously with FLUO 3-AM and BAPTA-acetoxymethyl ester-AM (Molecular Probes; 80 μM). PRP containing 2-8 x10^8 loaded platelets/ml was mixed with washed erythrocytes, to obtain a suspension with hematocrit of 42-45%, and apyrase (grade III; 142 ATPase U/mg of protein; Sigma) was added at the final concentration of 5 ATPase U/ml. The mixture was centrifuged at 1000 g for 15 min, the supernatant was discarded and the cell pellet was suspended in Hepes-Tyrode buffer, pH 7.4, containing 2 mM each Ca++ and Mg++ and 1.75 mM Probenecid (Sigma, St. Louis, MO) to prevent FLUO 3-AM leakage from platelets. These procedures did not alter platelet function as evidenced by three measurements that were comparable to those of unlabeled platelets: agonist-induced aggregation, P-selectin expression and binding of IAC-1, a monoclonal antibody that recognizes an activation-dependent epitope on the integrin (kindly provided by Dr. Gerlinde Van de Walle, Leuven, Belgium).

Mouse platelet preparation. All mice experiments were approved by the Institutional Animal Care and Use Committees of Centro Di Riferimento Oncologico and of The Scripps Research Institute. Blood of GPVI null, α2 null or wild type C57BL/6 mice was collected
from the retro-orbital venous plexus using heparin-coated- glass capillary tubes or by cardiac
puncture, and mixed with 1/8th volume of ACD. Mouse PRP was obtained by centrifugation at
100 g for 3 min at 22-25 °C, mixed with an equal volume of Hepes-Tyrode buffer, pH 6.5,
containing 5 U/ml apyrase, and centrifuged at 180 g for 12 min at 22-25°C. The procedure was
repeated once, and platelets resuspended at 5x10⁸/ml in Hepes-Tyrode buffer, pH 7.4, containing
0.1% bovine serum albumin (BSA), 1.75 mM probenecid, 0.01% pluronic acid, were loaded with
16 μM FLUO 3-AM for 40 minutes at 37 °C. One ml of these labeled platelets was mixed with 5
ml of human erythrocyte suspension (50% hematocrit) and 4 ml of Hepes-Tyrode buffer, pH 6.5,
containing 5 U/ml apyrase. The mixture was centrifuged at 1000 g for 10 min, the supernatant
was discarded and the cell pellet was suspended in Hepes-Tyrode buffer, pH 7.4, containing 2
mM each Ca²⁺ and Mg²⁺ and 1.75 mM Probenecid in a proportion such that the hematocrit was
42-45% and immediately used for perfusion experiments.

**Perfusion experiments.** Acid-soluble type I collagen from human placenta (Sigma) was
dissolved in 0.1 M acetic acid at 2.5 mg/ml. Collagen type VI, purified from human placenta as
previously reported,¹⁶ was diluted in phosphate-buffered saline (PBS; 20 mM Na₂HPO₄, 20 mM
NaH₂PO₄, 2.7 mM KCl, 0.15 M NaCl, pH 7.4) at 200 μg/ml. One-hundred μl of collagen
solution was used to coat glass coverslips for 60 min at 22-25 °C.¹⁴ The GFOGER peptide
(GPC[GPP]₃GFOGER[GPP]₅GPC; single-letter amino acid notation, where O = hydroxyproline)
was synthesized by SYNPEP Laboratories (Dublin, CA, USA) and cross-linked using N-
succinimidyl 3-[2-pyridyldithio]propionate (SPDP).²²,²³ A 50 μg/ml solution of cross-linked
GFOGER peptide was incubated on a coverslip overnight at 4°C. After coating, coverslips were
washed with PBS and kept in a moist environment until assembled in a modified Hele-Shaw
flow chamber.³,²⁴ In the case of the GFOGER peptide, coated coverslips were treated with 1%
fatty acid–free purified BSA in PBS for 2 hours at room temperature and washed twice with Hepes-Tyrode buffer before use. The flow chamber was positioned on the stage of an inverted microscope equipped with epifluorescent illumination (Diaphot-TMD, Nikon Instech, Kanagawa, Japan), an intensified CCD videocamera (C-2400-87, Hamamatsu Photonics, Shizuoka, Japan) and appropriate filters. The total area of an optical field corresponded to approximately 0.007 mm². Blood cells were aspirated through the chamber with a syringe pump (Harvard Apparatus, Boston, MA) at a flow rate calculated to obtain the desired wall shear rate at the inlet. Thrombus volume was measured as reported,3,25 using blood containing 40 U/ml heparin and 10 μM mepacrine to render platelets fluorescent. Stacks of confocal sections through the height of forming thrombi were obtained at selected time points using a Zeiss (Thornwood, NY) LSM 450 inverted confocal microscope. Image analysis for volume calculation was performed with Metamorph (Universal Imaging Co., West Chester, PA).

When indicated, various reagents were added to the blood cell suspension before perfusion, including PP2, an inhibitor of Src family kinases, or PP3, its non-inhibitory analog (Calbiochem-Nova Biochem, GMBH, Bad Soden, Germany); PD173952, a different Src family kinase inhibitor (a gift from Pfizer Global Research and Development, Sandwich, United Kingdom); wortmannin or LY294002, two phosphatidylinositol 3-kinase (PI 3-K) inhibitors (Sigma); U73122, a phospholipase C (PLC) inhibitor, or U73343, its inactive analog (Calbiochem-Nova Biochem); MRS 2216, a P2Y₁ inhibitor (kindly provided by Dr. KA Jacobson, National Institutes of Health, Bethesda, MD);26 AR-C69931MX, a P2Y₁₂ inhibitor (Astra Zeneca Pharmaceutical LP, Wilmington, DE);27,28 ethyleneglycoltetracetic acid (EGTA; Sigma); dibutyryl adenosine 3',5'-cyclic monophosphate (sodium salt), a cAMP analog, or 8-bromoguanosine 3',5'-cyclic monophosphate (sodium salt), a cGMP analog, (Sigma); acetyl salicylic acid (ASA), an inhibitor
of cyclooxygenase-1 and thromboxane A2 generation, prepared from powdered lysin-acetylsalicylate (Sanofi-Sythelabo S.p.A., Milano, Italy) as a 10 mM stock solution in 0.9% NaCl. Monoclonal antibodies included LJ-CP8, specific for the integrin αIIbβ3 (GP IIb-IIIa complex) and blocking both VWF and fibrinogen binding;29 Fab 9O12.2, directed against the extracellular portion of human GPVI;30 R2-7E4, specific for the α2 integrin subunit and blocking α2β1 function;14 and TS2/16, specific for the β1 integrin subunit.31 Experiments were recorded in real time at the rate of 25 frames/s, which resulted in a time resolution of 0.08 s. Selected video sequences were digitized in real time using a Matrox-Digisuite board (Matrox Graphics Inc., Dorval, Quebec, Canada).

**Measurement of Ca++ mobilization in adhering platelets.** Images were analyzed with a custom-made software (Amplimedical, Casti Imaging Division, Venice, Italy) that tracks single platelet area, position of the corresponding centroid and variations of pixel light intensity. Fluorescence intensity variations were converted into [Ca++]i using the equation:

\[
[Ca^{++}]_i = K_d \frac{F_{min}}{F_{max}} - F
\]

where \(K_d\) is the dissociation constant of the interaction between FLUO 3-AM and Ca++ (864 nM at 37 °C);\(^{18}\) \(F\) is the measured fluorescence intensity of a single platelet; \(F_{max}\) is the fluorescence intensity of a single platelet treated with the Ca++ ionophore A23187 (10 μM; Sigma) in the presence of 2 mM CaCl₂; and \(F_{min}\) is the fluorescence intensity of an unstimulated single platelet. The [Ca++]i of the resting state was calculated in single platelets in which the fluorescence intensity in each of at least 10 consecutive frames was within 15% of the value in the first frame and <200 nM. A Ca++ oscillation was recorded (i.e. a platelet was activated) when the following conditions were met: a change of [Ca++]i was more than 3 SD above the resting state value in at least 3 consecutive frames, the magnitude was at least 200 nM and there was an identifiable
peak. A ([Ca++]i) variation was defined as an $\alpha$-like peak when the duration was $2.2 \pm 0.36$ s, and a $\gamma$-like peak when the duration was greater than 5 s. The peak Ca++ concentration was typically greater in the latter than the former, but this parameter was not a discriminator between the two. Platelets were identified as firmly adherent when present in the first through last frames analyzed ($t_x - t_0$), typically a time interval of 30 s, with centroid movements of less than one platelet diameter (2 $\mu$m).

**Flow Cytometric Analysis.** Platelet membrane $\alpha2\beta1$ density was determined by measuring binding of the monoclonal antibody R2-7E4. For comparison, the binding of LJ-CP8, was measured in parallel. $3 \times 10^6$ platelets were incubated with the selected monoclonal antibodies at saturating concentration (75 $\mu$g/ml) for 30 minute at room temperature; polyclonal fluorescein isothiocynate (FITC)-conjugated rabbit anti-mouse F(ab)$_2$ (DAKO, Glostrup, Denmark) was added in a 1:30 v/v ratio and incubated for an additional 15 min. The samples were analyzed by acquiring a total of 20,000 events on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with CellQuest Pro Software. For analysis, a gate was set around the platelet population as defined by forward and side scatter characteristic and the mean fluorescence intensity (MFI) was recorded.

**Statistical Analysis.** Experiments were performed at least 3 times and data are shown as the mean $\pm$ 95% confidence intervals (CI) or standard error of the mean (SEM). Statistical significance was evaluated with the two-tailed Student’s $t$-test (degrees of freedom $\geq 10$) using Sigma Plot Version 9.0 software (Systat Software Inc, London, UK).
RESULTS

Two types of \([\text{Ca}^{++}]_i\) elevations mediated by different receptors in platelets interacting with acid-soluble type I collagen under flow. Flowing platelets interacting with immobilized acid-soluble type I collagen exhibited two types of \([\text{Ca}^{++}]_i\) oscillations that differed with respect to ion concentration and duration (Fig. 1A). One type, which occurred before the other, was characterized by rapid increase to 1-2 \(\mu\text{M} [\text{Ca}^{++}]_i\) lasting for a few seconds and recalled the \(\alpha\) peaks seen during GPIb\(\alpha\)-mediated platelet rolling onto VWF.\(^{32}\) These \([\text{Ca}^{++}]_i\) peaks were thus defined as \(\alpha\)-like. The second type reached the same or higher \([\text{Ca}^{++}]_i\) levels but lasted for several seconds, recalling the \(\gamma\) peaks seen during \(\alpha\)IIb\(\beta\)3-mediated stable platelet adhesion to VWF.\(^{32}\) They were thus identified as \(\gamma\)-like peaks. In three experiments, we found that 42.3 ± 6.9% (mean ± 95% CI) of the platelets tethering to acid-soluble type I collagen exhibited \(\alpha\)-like and 33.8 ± 5.5% exhibited \(\gamma\)-like \([\text{Ca}^{++}]_i\) peaks, the latter being ~80% of the former. A function-blocking anti-\(\alpha\)IIb\(\beta\)3 monoclonal antibody (LJ-CP8) had no effects on these \(\text{Ca}^{++}\) signals (Fig. 1B, E, F; Table 1), while an anti-\(\alpha\)2\(\beta\)1 monoclonal antibody (R2-7E4) fully inhibited platelet tethering and adhesion to collagen (Fig. 1G) and completely blocked all \([\text{Ca}^{++}]_i\) oscillations (Fig. 1C, E). An anti-GPVI monoclonal antibody (9O12.2), on the other hand, inhibited the initial platelet tethering to collagen (Fig. 1G), the appearance of \(\alpha\)-like \([\text{Ca}^{++}]_i\) peaks (Fig. 1D, E; Table 1) and their frequency per platelet (Fig. 1F) only partially, albeit significantly, while markedly decreasing the number of firmly adherent platelets (Table 1) and completely preventing the appearance of \(\gamma\)-like \([\text{Ca}^{++}]_i\) oscillations (Fig. 1D, E; Table 1).

Effect of inhibiting selected signaling pathways on \([\text{Ca}^{++}]_i\) elevations in platelets interacting with immobilized type I acid-soluble collagen under flow. Two different PI 3-K inhibitors, wortmannin and LY 294002, equally abolished all \(\text{Ca}^{++}\) oscillations and caused a
significant, albeit partial, reduction of firm platelet adhesion to acid-soluble type I collagen (Table 1). The latter effect was more pronounced with the PLC inhibitor, U73122, which also blocked all Ca^{++} signals (Table 1). The structurally related but inactive analog, U73343, was without effect (not shown). PG E_{1}, an inhibitor of platelet activation acting at different levels, had an effect similar to that of the PLC inhibitor (Table 1). Blocking tromboxane A2 synthesis with ASA resulted in a modest, albeit significant reduction in the percentage of platelets exhibiting \( \alpha \)-like peaks, but without consequence on the number of platelets exhibiting \( \gamma \)-like peaks or establishing firm adhesion (Table 1). Of note, concurrent blockage of the two platelet ADP receptors, P2Y_{1} with MRS 2216 and P2Y_{12} with AR-C69931, had no influence on \([Ca^{++}]_{i}\) oscillations or platelet adhesion (Table 1).

Inhibition of Src family kinases with PP2 resulted in the complete dose-dependent inhibition of both \( \alpha \)-like and \( \gamma \)-like \([Ca^{++}]_{i}\) oscillations in platelets interacting with acid-soluble type I collagen under flow, however \( \gamma \)-like peaks were abolished at a concentration of inhibitor that only reduced the frequency of \( \alpha \)-like peaks by 50\% (Fig. 2A). A comparable, complete abrogation of \( \alpha \)-like peaks required a 5-fold higher concentration of inhibitor (Fig. 2A). A high concentration of PP2 (50 \( \mu \)M) also significantly decreased the percentage of firmly adherent platelets (Table 1). PD173952, a structurally unrelated Src kinase inhibitor, at the concentration of 25 \( \mu \)M demonstrated an inhibitory activity similar to that of PP2 (not shown), while PP3, a non-inhibitory PP2 analog, was without effect (Table 1). The cGMP stable analog, 8Br-cGMP, also inhibited dose-dependently both types of \([Ca^{++}]_{i}\) oscillations, and the inhibitor concentration required to abolish \( \alpha \)-like peaks was approximately 6-fold higher that that required to abolish \( \gamma \)-like peaks (Fig. 2B). At the high concentration of 80 \( \mu \)M, 8Br-cGMP markedly reduced the
number of firmly adherent platelets (Table 1). The stable cAMP analog, dibutyryl-cAMP, at the concentration of 100 μM abolished both α-like and γ-like peaks (not shown).

\[\text{[Ca}^{++}\text{]}_i \text{ elevations induced by platelet adhesion to acid-soluble type I collagen differ} \]
\[\text{entially depend on release from intracellular stores and transmembrane ion flux.}\]
Platelet adhering to collagen in the presence of 5 mM EGTA, a membrane-impermeable Ca\text{++} chelator, showed normal α-like but absence of γ-like [Ca\text{++}]_i oscillations (Fig. 3), indicating that the latter but not the former depend on transmembrane ion flux. Of note, EGTA had no significant effect on the number of firmly adherent platelets (Table 1). In contrast, all [Ca\text{++}]_i oscillations were obliterated by the membrane-permeable chelator, BAPTA-AM, whether in the absence or presence of EGTA (Fig. 3), and the number of firmly adherent platelets was significantly reduced (Table 1). Thus, α-like [Ca\text{++}]_i peaks are a consequence of release from intracellular stores, which is also a requisite for the subsequent appearance of γ-like Ca\text{++} signals.

\[\text{[Ca}^{++}\text{]}_i \text{ elevations induced by platelet adhesion to an immobilized anti-}\alpha_2\beta_1\text{antibody or the GFOGER peptide.}\]
Platelets perfused over immobilized TS2/16, an anti-β1 integrin subunit antibody, exhibited frequent α-like [Ca\text{++}]_i oscillations upon adhesion that reached higher levels than seen on collagen, but lacked the sustained duration of typical γ-like peaks (Fig. 4A). Platelet incubation with the anti-GPVI monoclonal antibody, 9O12.2, prior to perfusion had no effect on the appearance of the α-like [Ca\text{++}]_i peaks, which were completely inhibited by the Src family kinase inhibitor, PP2, in a dose-dependent manner (Fig. 4B). Addition of EGTA had no effect on these [Ca\text{++}]_i oscillations (not shown). Platelets perfused over the triple-helical peptide, GFOGER, which interacts specifically with α2β1, exhibited α-like [Ca\text{++}]_i oscillations similar to those seen on antibody TS2/16 (Fig. 4C). These α-like [Ca\text{++}]_i peaks were inhibited by treating the platelets with the anti-α2β1 antibody, R2-7E4, or with PP2 (not shown).
[Ca++]i elevations and thrombus formation in WT, GPVI-/- and α2-/- mouse platelets interacting with acid-soluble type I collagen. The perfusion of WT mouse platelets over acid-soluble type I collagen resulted in adhesion and appearance of the same two types of [Ca++]i oscillations seen in human platelets (Fig. 5A, C). In contrast, the platelets of GPVI-/- mice exhibited only α-like [Ca++]i peaks with complete absence of γ-like peaks (Fig. 5B), while those of α2-/- mice showed no [Ca++]i oscillations (Fig. 5D, E) while contacting the collagen surface only transiently without achieving firm adhesion (Fig. 5F). In six distinct experiments, we found that 61.7 ± 12% of WT mouse platelets tethering to acid-soluble type I collagen exhibited α-like and 21 ± 7.6% exhibited γ-like [Ca++]i peaks (Fig. 5E). Fewer GPVI-/- platelets (39.8 ± 6.5%) exhibited α-like [Ca++]i peaks with absence of γ-like peaks (Fig. 5E), and fewer reached firm adhesion (Fig. 5F). Thus, the results obtained with GPVI-/- or α2-/- mouse platelets were consistent with those of human platelets treated with the anti-GPVI antibody, 9O12.2, or the anti-α2β1 antibody, R2-7E4, respectively. Thrombus formation in GPVI-/- or α2-/- blood was completely abolished (Fig. 5G).

[Ca++]i elevations in platelets adhering to type VI collagen. Human platelets perfused over purified collagen type VI established firm adhesion essentially in the same proportion (74%) as on acid-soluble collagen type I (75 ± 11%; see Table 1), and exhibited the same two types of [Ca++]i oscillations (Fig. 6A). Treating platelets with an anti-αIIbβ3 antibody significantly decreased the number of firmly adherent platelets while having no effect on [Ca++]i oscillations (Fig. 6B,C). Thus, to avoid any confounding effect caused by variable αIIbβ3 function in different samples, platelets perfused over collagen type VI were always treated with the anti-αIIbβ3 antibody. When, in addition to the latter, platelets were treated with an anti-α2β1 or anti-GPVI antibody, not only was firm adhesion reduced more markedly but the occurrence of α-like
[Ca++]i peaks was significantly reduced (the effect was more pronounced for the anti-α2β1 antibody) and γ-like [Ca++]i peaks were essentially obliterated (Fig. 6B,C). Concurrent platelet treatment with the anti-α2β1 and anti-GPVI antibodies, in addition to the anti-αIIbβ3 antibody, completely abolished both types of [Ca++]i oscillations (Fig. 6C).

Effect of α2β1 density on platelet adhesion and [Ca++]i elevations. The levels of α2β1 surface expression on the platelet membrane have been reported to vary in normal individuals by up to ten-fold, suggesting that there might be corresponding variations in the extent of collagen-induced Ca++ signaling and platelet adhesion. To evaluate this possibility, platelets from individuals with either low or high α2β1 membrane density (Fig. 7A), but comparable levels of αIIbβ3 (not shown), were perfused over acid-soluble type I collagen. In either case there was a time-dependent platelet accrual on the surface, but the rate at which this occurred was faster and the extent greater for the high α2β1 density platelets (Fig. 7B). Of note, the latter exhibited higher α-like as well as higher and longer lasting γ-like [Ca++]i peaks than seen with the low α2β1 density platelets (Fig. 7C, D). Treatment with the anti-GPVI antibody prior to perfusion abolished the occurrence of γ-like peaks and essentially eliminated the difference between high or low α2β1 density platelets, both of which showed only α-like [Ca++]i oscillations upon interaction with collagen (Fig. 7E, F).
DISCUSSION

We identified two distinct types of \([\text{Ca}^{++}]_i\) elevations in platelets that depend on collagen binding by two different receptors, \(\alpha2\beta1\) and GPVI, under flow conditions. Our results - concordant in antibody-treated human platelets or mouse platelets with receptor ablation - link short lasting \(\alpha\)-type and long lasting \(\gamma\)-type \(\text{Ca}^{++}\) peaks to \(\alpha2\beta1\) and GPVI ligation, respectively. Such \(\text{Ca}^{++}\) peaks are not independent of one another, in agreement with the concept of a collagen-induced reciprocal signaling by integrin and non-integrin receptors.\(^{31}\) Evidence supporting this conclusion derives from experiments performed with acid-soluble collagen type I and fibrillar collagen type VI. In either case, lack of GPVI function has a significant albeit partial effect on the appearance of \(\alpha\)-type while obliterating \(\gamma\)-type \(\text{Ca}^{++}\) peaks; lack of \(\alpha2\beta1\) function markedly reduces or abolishes \(\alpha\)-type and concurrently obliterates \(\gamma\)-type peaks. Thus, \(\alpha\)-type \([\text{Ca}^{++}]_i\) elevations that require release from intracellular stores, but not \(\gamma\)-type that depend on transmembrane ion flux, can occur in the absence of GPVI, and \(\gamma\)-type can only appear after \(\alpha\)-type peaks. This is compatible with the concept that depletion of intracellular stores activates a pathway leading to the opening of plasma membrane channels for regulated \(\text{Ca}^{++}\) entry.\(^{34}\) The coupling of \(\alpha2\beta1\) ligation to intracellular \(\text{Ca}^{++}\) oscillations in the absence of GPVI signaling is confirmed by the onset of \(\alpha\)-type but not \(\gamma\)-type \(\text{Ca}^{++}\) peaks upon platelet interaction with an \(\alpha2\beta1\)-specific peptide. Because \(\alpha\)-type \(\text{Ca}^{++}\) transients predominantly mediated by \(\alpha2\beta1\) lead to more sustained \(\text{Ca}^{++}\) elevations associated with the up-regulation of platelet adhesion and aggregation\(^{32}\), our studies highlight a reason why \(\alpha2\beta1\) may be necessary for full platelet activation. The importance of such a function may vary with the type of collagen involved.

It is apparent from our findings that initial \(\alpha\)-type and subsequent sustained \(\gamma\)-type \(\text{Ca}^{++}\) transients are neither sufficient nor a requisite, respectively, for firm platelet adhesion to
collagen. In fact, treating human platelets with EGTA abolishes γ-type peaks without significant consequences on platelet adhesion, and blocking GPVI function only modestly inhibits α-type peaks while markedly decreasing platelet adhesion. Moreover, lack of Ca++ signaling is still compatible with firm platelet adhesion to collagen, albeit only at ~50% of normal, as seen after intracellular Ca++ chelation or PI 3-K blockade. Thus, sufficient activation-inducing signals can occur in platelets to support adhesion against shear stress (which requires platelet spreading) without changes in intracytoplasmic Ca++ levels. Of note, our findings indicate that PI 3-K as well PLC activity lie upstream of Ca++ release from cytoplasmic organelles induced by the two collagen receptors, as both are required to achieve Ca++ elevations; in contrast, only PLC is absolutely required to establish firm platelet adhesion to collagen, with PI 3-K contributing to maximal efficiency. Altogether, our studies indicate that distinct changes in [Ca++]i, detectable in real time and under flow conditions, are markers that differentiate the signaling and adhesive functions of GPVI and α2β1 in spite of their proposed convergence on the modulation of a similar set of cytoplasmic proteins during platelet activation.

Models have been proposed to explain how two receptors may synergize in mediating collagen responses. In a two-step process, α2β1 is thought to initiate the interaction with the substrate, but outside-in signaling required for platelet activation has been considered so far to involve predominantly the GPVI-FcRγ complex. Our evidence supports the existence of a functional synergy between α2β1 and GPVI, but not simply to the effect that GPVI provides primary signals subsequently amplified by α2β1. Not only did we find that α2β1 can signal independently of GPVI or αIIbβ3 (GPIIb-IIIa), as clearly confirmed by the induction of α-type Ca++ peaks upon selective α2β1 ligation by the GFOGER peptide, but also that GPVI functions are facilitated by preceding α-type Ca++ elevations dependent on α2β1 engagement. Such
conclusions do not exclude the possibility that α2β1 ligand-binding affinity is further enhanced following GPVI interaction with collagen and/or αIIbβ3 activation. With the resolution afforded by our methodology, the functional activities of α2β1 and GPVI appear to be at least concurrent, a concept supported by the finding that variations in α2β1 platelet surface expression influence the extent of GPVI-dependent Ca++ signaling. That our findings were not biased by uncontrolled modification of the integrin before exposure to collagen was indicated by the lack of binding of antibody IAC 1, a selective marker of active α2β1 conformation.36

Not unexpectedly, we found that cytoplasmic levels of cGMP and cAMP regulate collagen-induced platelet activation, as the same result was previously observed for the two corresponding sequential Ca++ signals elicited by GPIbα interaction with VWF under high flow conditions.17,32 Src kinase inhibitors also display comparable effects on collagen- and VWF-induced activation,17 and given the greater inhibitor concentration required to block α and α-like as opposed to γ and γ-like peaks, it appears that the role of Src family kinases varies in sequential steps of activation. In contrast, the role of PLC in collagen- as compared to VWF-induced Ca++ signaling may differ, as in the latter case PLC inhibition caused only partial decrease of initial α peaks but complete abrogation of subsequent γ peaks,40 while both collagen-induced α-like and γ-like peaks were abolished. The contributions of PI 3-K to platelet activation mediated by collagen and its receptors as compared to GPIbα and VWF-A1 also appear to differ, since in the latter case PI 3-K inhibitors had no effect on α peaks but prevented the appearance of sustained γ peaks,17,39,40 while both corresponding collagen-induced Ca++ signals were abolished. Thus, Ca++ release from intracellular stores may follow distinct pathways in platelets and involve a variable interplay of PLC- and PI 3-K-dependent mechanisms depending on the initiating adhesive event. This may favor a synergy of VWF bound to collagen fibrils in initiating the activation of...
platelets tethered at sites of vascular injury under high flow conditions. Of note, marking another
difference with the process of activation induced by interaction with VWF-GPIb, blockage of
both platelet ADP receptors had no effect on Ca\textsuperscript++ signals following interaction with collagen.
This signifies that secreted ADP, and possibly thromboxane A2, intervene at a late stage in
collagen-induced aggregation. In this context, it is difficult to ascertain at present whether the
small, albeit significant, effect of aspirin on initial collagen-induced α-like Ca\textsuperscript++ peak intensity is
relevant with respect to the process of thrombus formation.

In considering the meaning of experimental results obtained with single collagen
preparations extracted from tissues, it should be considered that native collagen is an insoluble
protein whose thrombogenic activity is likely influenced by multiple interactions with other
matrix components in a complex supramolecular assembly. Moreover, evidence is emerging
that the function of collagen and/or collagen receptors on platelets may be subjected to the
modifying effects of still unknown gene products. Here, to highlight the role of α2β1, we have
used acid soluble collagen type I, which does not exist as such in vivo but is composed of an
assembly of fibrils in a helical configuration that may mimic the properties of the spiraled
collagens identified in normal and pathological tissues. To extend the significance of our
findings we have also used collagen type VI, which platelets contact directly when the
subendothelial matrix is exposed or, associated with collagen type I and III, when lesions reach
deeper layers of the vessel wall. In both instances we found evidence that α2β1 contributes to
adhesion and aggregation, but results might be different in the context of platelet interactions
with other collagen types and/or with native collagens in tissues. These considerations
notwithstanding, our studies on the signaling role of α2β1 contribute to a plausible mechanistic
explanation for the increased lag phase and reduced extent of collagen-induced platelet
aggregation resulting from functional deficiencies of the receptor, whether caused by inhibitory monoclonal antibodies\textsuperscript{12} or low levels of the protein.\textsuperscript{11,43} By the same token, akin to the phenotype resulting from increased cytoplasmic Ca\textsuperscript{+}\textsuperscript{+} levels in mice over-expressing platelet P2X1,\textsuperscript{44} a more rapid and robust $\alpha_2\beta_1$-mediated Ca\textsuperscript{+}\textsuperscript{+} release from intracellular store may contribute to explaining the thrombotic tendency observed in patients bearing the $\alpha_2$ 807T polymorphism linked to expression of the receptor.\textsuperscript{8,33}

**Aknowledgements**

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Statement on authors’ contributions: M.Mazzucato designed experiments, analyzed data and contributed to writing the manuscript; M.R.C. performed experiments, analyzed data and contributed to writing the manuscript; M.B. performed experiments and analyzed data; M.J.-P. provided essential reagents; M.Mongiat performed experiments; P.M. performed experiments; T.J.K. provided essential reagents, Z.M.R. contributed to designing experiments, provided essential reagents, evaluated results and wrote the manuscript; L.D.M. supervised research, designed experiments, evaluated results and wrote the manuscript. There are no conflicts of interest to declare.
References


Table 1. Effect of different antibodies and inhibitors on platelet adhesion and $[Ca^{++}]_{i}$ elevations in platelets interacting with immobilized acid-soluble type I collagen under flow.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Concentration</th>
<th>Firmly Adherent Platelets (%)</th>
<th>$[Ca^{++}]_{i}$ Elevations (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$\alpha$-like</td>
</tr>
<tr>
<td>Anti-GP VI</td>
<td>50 µg/ml</td>
<td>14 ± 6**</td>
<td>79 ± 11*</td>
</tr>
<tr>
<td>Anti-$\alpha$IIbβ3</td>
<td>100 µg/ml</td>
<td>88 ± 15</td>
<td>101 ± 22</td>
</tr>
<tr>
<td>Wortmannin (PI 3-K inhibitor)</td>
<td>100 nM</td>
<td>50 ± 6*</td>
<td>0</td>
</tr>
<tr>
<td>LY294002 (PI 3-K inhibitor)</td>
<td>50 µM</td>
<td>52 ± 11*</td>
<td>0</td>
</tr>
<tr>
<td>U73122 (phospholipase C inhibitor)</td>
<td>21 µM</td>
<td>10 ± 3**</td>
<td>0</td>
</tr>
<tr>
<td>PG E1 (increases cAMP)</td>
<td>0.28 µM</td>
<td>10 ± 2**</td>
<td>0</td>
</tr>
<tr>
<td>ASA (cyclooxygenase-1 inhibitor)</td>
<td>400 µM</td>
<td>81 ± 10</td>
<td>81 ± 6*</td>
</tr>
<tr>
<td>MRS 2216+AR-C69931 (ADP receptors inhibitors)</td>
<td>12.5±5 µM</td>
<td>82 ± 9</td>
<td>102 ± 16</td>
</tr>
<tr>
<td>PP2 (Src-family kinases inhibitor)</td>
<td>50 µM</td>
<td>14 ± 5**</td>
<td>0</td>
</tr>
<tr>
<td>PP3 (Non-inhibitory PP2 analog)</td>
<td>50 µM</td>
<td>78 ± 12</td>
<td>91 ± 5</td>
</tr>
<tr>
<td>8-Br-cGMP (cGMP analog)</td>
<td>80 µM</td>
<td>14 ± 5**</td>
<td>0</td>
</tr>
<tr>
<td>EGTA (Non membrane-permeable Ca^{++} chelator)</td>
<td>10 mM</td>
<td>85 ± 14</td>
<td>110 ± 18</td>
</tr>
<tr>
<td>BAPTA-AM (Membrane-permeable Ca^{++} chelator)</td>
<td>80 µM</td>
<td>43 ± 16*</td>
<td>0</td>
</tr>
</tbody>
</table>

Blood cell suspensions were prepared as described in the legend to Fig. 1 and perfused over immobilized acid-soluble type I collagen at the wall shear rate of 250 s⁻¹ without or with prior incubation for 10 min at 37 °C with the indicated reagents. The frequency of platelets exhibiting different types of $[Ca^{++}]_{i}$ peaks was calculated as a fraction of the total number of platelets analyzed in the field of view during a 3-minute observation period and normalized to the corresponding value measured in untreated (control) platelets. Firmly adherent platelets were defined as those whose centroid was displaced by less than 1 cell diameter over 30 s, and are reported here as percentage of all the platelets present in the field of view during the same observation period. All measurements were performed after perfusion for at least 120 s. In control experiments, 75±11% of platelets analyzed established firm adhesion to collagen, 42±7% exhibited $\alpha$-like $Ca^{++}$ peaks, and 34±6% exhibited $\gamma$-like peaks. All results are the mean ± 95% confidence intervals of measurements obtained analyzing 180-250 platelets in each of at least 3 separate experiments. *P <0.05; **P <0.01.
FIGURE LEGENDS

Figure 1. Distinct [Ca++]i elevations in platelets interacting with acid-soluble type I collagen under flow. Washed human platelets (2x10^7/ml) loaded with 8 μM FLUO 3-AM were suspended with erythrocytes (40% hematocrit) in Heps-Tyrode buffer containing 2 mM Ca++ and Mg++ and perfused over collagen for 10 min at the shear rate of 250 s⁻¹. The low platelet count was required for accurate image analysis. A. After perfusion for 120 s, [Ca++]i in surface interacting platelets was monitored for 90 s. Both short (α-like) and long lasting (γ-like) peaks were observed. B. An anti-αIIbβ3 antibody (LJ-CP8, 100 μg/ml) had no effect on Ca++ signals. C. Platelets treated with an anti-α2β1 antibody (R2-7E4, 100 μg/ml) exhibited no [Ca++]i oscillations and only transient contacts with the surface. D. An anti-GPVI antibody (Fab 9O12.2, 50 μg/ml) had no apparent effect on α-like but abolished γ-like [Ca++]i oscillations. E. The number of activated platelets exhibiting at least one α-like (black bars) or γ-like (grey bars) [Ca++]i elevation over a 3 min period was measured as a fraction of the total number of surface interacting platelets, in the absence (control) or presence of different antibodies, as indicated. F. The number of α-like [Ca++]i elevations in each activated platelet was measured over a 3 min period, in the absence (control) or presence of different monoclonal antibodies, as indicated. At least 250 control or antibody-treated platelets were evaluated. G. Number of control and antibody-treated platelets (including transient and stable adhesion) present in each optical field measured every 10 s for 200 s. The results shown in B-D represent the mean ± 95% CI of the values measured in at least 8 experiments. Asterisks indicate a significant difference from the corresponding control (*P <0.05; **P <0.01; *** P <0.001).

Figure 2. Effect of Src kinase inhibition and cGMP modulation on [Ca++]i elevations in platelets interacting with acid-soluble type I collagen under flow. Blood cell suspensions (see
legend to Fig. 1 for details) were perfused at the shear rate of 250 s\(^{-1}\) over immobilized collagen, without or with prior incubation for 10 min at 37 °C with varying concentrations of (A) the Src kinase inhibitor, PP2, or (B) the cGMP analogue, 8-Bromo-cyclic guanosine monophosphate (8-Br-cGMP). Platelets in which at least one \([\text{Ca}^{++}]_i\) elevation occurred were considered activated and those exhibiting \(\alpha\)-like or \(\gamma\)-like \([\text{Ca}^{++}]_i\) peaks was enumerated. Results are shown relative to the values observed in untreated blood cell suspensions (control). Data are the mean ± 95% CI of 3 (panel A) or 4 (panel B) different experiments.

**Figure 3. Effect of chelating agents on \([\text{Ca}^{++}]_i\) elevations in platelets interacting with acid-soluble type I collagen under flow.** Blood cell suspensions (see legend to Fig. 1 for details) were perfused over collagen at the shear rate of 250 s\(^{-1}\) without or with prior treatment with a \([\text{Ca}^{++}]_i\) chelator, either the membrane impermeable EGTA (5 mM), the membrane permeable BAPTA-AM (80 \(\mu\text{M}\)), or the two combined. Results are expressed as described in the legend to Fig. 2 (n=3).

**Figure 4. \([\text{Ca}^{++}]_i\) elevations in platelets interacting with an immobilized anti-integrin \(\beta1\) monoclonal antibody or with the \(\alpha_2\beta_1\)-specific triple-helical GFOGER peptide.** Blood cell suspensions (see legend to Fig. 1 for details) were perfused at the shear rate of 250 s\(^{-1}\) over the anti-\(\beta1\) antibody TS2/16 (A, B) or the peptide GFOGER (C). A. Typical \(\alpha\)-like (short lasting) \([\text{Ca}^{++}]_i\) peaks were identified in platelets interacting with TS2/16. B. Blood cell suspensions were perfused over TS2/16 without or with prior treatment for 10 min at 37°C with the anti-GPVI monoclonal antibody, Fab 9O12.2 (50 \(\mu\text{g/ml}\)), or the Src kinase inhibitor, PP2, at the indicated concentrations. Results are expressed as described in the legend to Fig. 2 (n=3); note the absence of \(\gamma\)-like peaks under these conditions. C. Typical \(\alpha\)-like peaks were seen in platelets (5x10\(^7\)/ml) interacting with the GFOGER peptide (coating solution concentration: 50 \(\mu\text{g/ml}\)).
Figure 5. [Ca++]i elevations and thrombus formation in mouse platelets adhering to acid-soluble type I collagen under flow. Mice were anesthetized and blood was drawn from the retro-orbital plexus, using heparin-coated capillary tubes, or by cardiac puncture. Platelets were washed, loaded with FLUO 3-AM (16 μM), suspended (5x10^7/ml) with washed human erythrocytes (40% hematocrit) in Hepes-Tyrode buffer, pH 7.4, containing 2 mM Ca++ and Mg++ and 1.75 mM Probenecid, and perfused over immobilized acid-soluble type I collagen at the shear rate of 400 (A-F) or 1,500 s⁻¹ (G). A, C. Platelets from WT mice exhibited α-like (short lasting) and γ-like (long lasting) [Ca++]i peaks. B. Platelets from GPVI-/- mice exhibited only α-like peaks. D. Platelets from α2-/- mice exhibited no [Ca++]i oscillations and only transient contact with the surface. E. Number of platelets exhibiting α-like and γ-like [Ca++]i elevations (activated platelets) calculated relative to total adhering platelets. F. Percentage of firmly adherent platelets calculated as described in Materials and Methods. Data in B-C are the mean ± 95% CI of 3 different experiments. G. Volume of thrombi over a 0.38 mm² collagen surface after perfusion for 3 min at 37 °C of blood containing 40 U/ml heparin and 10 μM mepacrine. Data are the mean ± SEM of measurements in 5 different positions of the chamber. The three images show the thrombi formed by fluorescent platelets at the end of perfusion; only single platelets are seen in the case of GPVI-/- and α2-/- mice. Asterisks indicate a significant difference from the corresponding control (WT) (*P <0.05; **P <0.01; ***P <0.001).

Figure 6. [Ca++]i elevations in platelets adhering to collagen type VI under flow. Human blood cell suspensions (see legend to Fig. 1 for details) were perfused at the shear rate of 250 s⁻¹ over immobilized collagen type VI (coating solution 350 μg/ml) without or with prior incubation with the indicated antibodies (see legend to Fig. 1). A. Typical α-like (short lasting) and γ-like (long lasting) [Ca++]i peaks were observed. B. Firmly adherent platelets, calculated as described
in Materials and Methods. C. Activated platelets, calculated as described in the legend to Fig. 2. Data are the mean ± 95% CI of at least 8 different experiments. Asterisks indicate a significant difference from platelets treated with the anti-αIIbβ3 antibody (*P <0.05; **P <0.01; ***P <0.001).

**Figure 7. Effect of α2β1 density on platelet adhesion and [Ca++]i elevations.** A. The density of α2β1 on the platelet membrane was determined in two normal donors by flow cytometry using the monoclonal antibody R2-7E4. The difference in MFI was significant (P <0.001). B. Washed blood cell suspensions, derived from donors whose platelets were known to have high or low α2β1 density, were prepared as described in the legend to Fig. 1 and perfused over immobilized acid-soluble collagen type I at the shear rate of 250 s⁻¹. The time-course of the accumulation of firmly adherent platelets, measured every 10 s for 200 s, is shown. Data are the mean ± 95% CI of 3 different experiments. The two curves are significantly different (P<0.01). C, D. Typical α-like (short lasting) and γ-like (long lasting) [Ca++]i peaks were identified with both types of platelets. E, F. Note that, in either case, γ-like peaks were essentially abolished after platelet incubation with the anti-GPVI antibody, Fab 9O12.2 (50 μg/ml), while α-like peaks could still be observed.
Figure 1
Figure 2
Figure 3

Activated Platelets (% of Control)

EGTA
BAPTA-AM
EGTA + BAPTA-AM

α-like
γ-like
Figure 4
**Figure 5**

A - WT
B - GPVI−/−
C - WT
D - α2−/−

[Graphs showing calcium concentration over time for different genotypes: WT, GPVI−/−, α2−/−, α-like, γ-like.]

E - Activated Platelets (%)
F - Firmly Adherent Platelets (%)

G - Thrombus Volume (mm³ x 10^-3)

Bar graphs showing activated platelets and thrombus volume for WT, GPVI−/−, and α2−/− genotypes.
Figure 6

(A) [Ca^{++}]_i (μM) over time (s).

(B) Firmly Adherent Platelets (% of Control).

(C) Activated Platelets (% of Control).

Legend:
- Anti-α\text{IIbβ3}:
  - +: +
  - ++: ++
  - +++: +++
- Anti-α\text{2β1}:
  - -:
  - +:
- Anti-GPVI:
  - -:
  - +:
  - ++:

* Significant difference compared to control.
** Highly significant difference compared to control.
**Figure 7**

**Panel A**
- **High α2β1 density**
  - [Graph showing fluorescence intensity versus log fluorescence intensity with two peaks labeled as 'Low' and 'High'.]

**Panel B**
- **Low α2β1 density**
  - [Graph showing adherent platelets (N) versus time (s) with error bars indicating variability.]

**Panel C**
- **High α2β1 density**
  - [Graph showing [Ca^2+]] versus time (s) with labels for 'α-like', 'γ-like', and 'Control'.]

**Panel D**
- **Low α2β1 density**
  - [Graph showing [Ca^2+]] versus time (s) with labels for 'α-like', 'γ-like', and 'Control'.]

**Panel E**
- [Graph showing [Ca^2+] with a peak labeled as 'α-like + Anti-GPVI'.]

**Panel F**
- [Graph showing [Ca^2+] with a peak labeled as 'α-like + Anti-GPVI'.]
Distinct spatio-temporal Ca++ signaling elicited by integrin α2β1 and glycoprotein VI under flow

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