Disturbances of blood flow play an important role in promoting platelet activation and arterial thrombus formation in stenosed, injured, atherosclerotic arteries. To date, glycoprotein Ib (GPIb) has been considered the primary platelet mechanosensory receptor, responding to increased shear with enhanced adhesive and signaling function. We demonstrate here that von Willebrand factor-GPIb interaction is inefficient at inducing platelet activation even when platelets are exposed to very high wall shear stresses (60 dyn/cm²). Rapid platelet activation under flow was only observed under experimental conditions in which transiently adherent platelets were exposed to sudden accelerations in blood flow. Platelet responsiveness to temporal shear gradients was integrin αIIbβ3-dependent and occurred only on a von Willebrand factor substrate, as platelets forming integrin αIIbβ3 adhesive contacts with immobilized fibrinogen were unresponsive to sudden increases in shear. The calcium response induced by temporal shear gradients was distinct from previously identified integrin αIIbβ3 calcium responses in terms of its transient nature, its requirement for platelet co-stimulation by the P2Y₃ purinergic ADP receptor, and its dependence on the influx of extracellular calcium. Our studies demonstrate a key role for temporal shear gradients in promoting platelet activation. Moreover, they define for the first time the involvement of integrin αIIbβ3 mechanotransduction in regulating platelet activation.

The transduction of biomechanical stimuli into biochemical signals is fundamental to the regulation of a wide range of physiological processes, including sensory perception, blood pressure regulation, bone remodeling, and maintenance of muscle mass (1–3). Mechanotransduction mechanisms are particularly relevant to the vasculature, where hemodynamic forces generated as a result of blood flow modulate the phenotypic characteristics of vascular (endothelial and smooth muscle cells) and circulating blood cells (platelets and leukocytes). Shear effects on vascular cells are mediated in part by transmembrane extracellular matrix receptors, principally involving integrins. Various members of the β₁, β₂, and β₃ families of integrin receptors have been demonstrated to transduce mechanical signals that are critically linked to the development of atherosclerotic lesions (4, 5).

The importance of integrin mechanotransduction in regulating the adhesive function of circulating blood cells has been less clearly defined, although it is important given the key role played by platelets and leukocytes in the atherothrombotic process. In general, rapid transduction of biomechanical stimuli is typically mediated through mechanically gated ion channels (1) and, in the case of platelets, such membrane channels may be linked to the shear-regulated binding of von Willebrand factor (vWf) to its surface receptor, glycoprotein (GP) Ib/V/IX. Mechanotransduction mechanisms operating downstream of integrins are typically linked to slower adaptive responses such as cytoskeletal remodeling and gene transcription (1); however, the role of integrin mechanotransduction in regulating functional processes in rapidly responding cells such as platelets remains largely unknown.

Insight into the mechanisms by which shear forces regulate platelet function requires consideration of the hemodynamic conditions operating at sites of atherothrombosis. For example, shear forces change significantly at sites of arterial stenosis, increasing dramatically at the apex of the stenosis and decreasing rapidly in the post-stenotic recirculation region (6). Flow patterns in the post-stenotic region can change from unidirectional laminar flow to disturbed flow patterns involving eddy formation, flow reversal, and shear gradients. Significantly, a major role for rapid accelerations of blood flow in promoting platelet deposition and thrombus formation has been demonstrated in vivo (7); furthermore, propagation of thrombi has also been established to occur downstream of the initial shear flux in the post-stenotic region (7). Although such disturbances of blood flow patterns have long been identified as powerful atherogenic stimuli and have been demonstrated to be important in regulating endothelial cell function (8, 9), the role of such flow changes in regulating platelet function remains ill-defined.

In this study we demonstrate a key role for shear gradients in inducing platelet activation. Temporal shear gradients stimulate platelet activation through a signaling mechanism involving the platelet P2Y₁ purinergic receptor and integrin αIIbβ3. We demonstrate that cooperative signaling by these receptors is necessary for efficient shear activation of platelets. Moreover, we demonstrate that this mechanosensory signaling mechanism is operational over a narrow temporal shear range. Overall, our studies define for the first time an important role for P2Y receptors in integrin mechanotransduction.
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(10). The anti-\(\alpha_{IIb}\beta_{3}\) chimeric Fab fragment of the monoclonal antibody 7E3 (c7E3 Fab-abxicabimab) was from Eli Lilly and Centocor (Leiden, The Netherlands). All other reagents were obtained from sources described previously (10–13).

Platelet Preparation—Washed human platelets were prepared as described previously (10, 14) with some minor modifications. Whole blood was collected into acid citrate dextrose (ACD) anticoagulant at a ratio of 6:1 (blood/ACD), to which mixture 20 units/ml Clexane was added before being allowed to incubate at 37 °C for 15 min. Separation of platelet-rich plasma and red blood cells was achieved by centrifugation at 300 × g for 16 min. In all of the subsequent steps involving centrifugation, platelets were allowed to rest at 37 °C for 10 min prior to continuation of the next step in the washing procedure. Platelets were isolated from plasma components by centrifugation at 1,700 × g for 7 min, followed by resuspension in an equal volume of platelet washing buffer (4.3 mM K$_2$HPO$_4$, 4.3 mM Na$_2$HPO$_4$, 24.3 mM NaH$_2$PO$_4$, 113 mM NaCl, 5.5 mM d-glucose, and 10 mM theophylline, pH 6.5). Platelets were washed by centrifugation at 1,500 × g for 7 min, followed by a final resuspension at a concentration of 3 × 10$^9$/ml in modified Tyrode’s buffer (10 mM Heps, 12 mM NaHCO$_3$, 137 mM NaCl, 2.7 mM KCl, and 5 mM glucose, pH 7.3) containing bovine serum albumin (5 mg/ml), calcium (1 mM), and apyrase (0.02 units/ml) (ADPase activity).

In Vitro Flow Studies—Flow assays were performed as described previously (10). Briefly, glass microcapillary slides (Lomb Scientific, New South Wales, Australia) were coated with vWf (100 µg/ml) or fibronectin (100 µg/ml) for 2 h at room temperature and then blocked with 10% heat-inactivated human serum. Washed platelets (5 × 10$^9$/ml) in Tyrode’s buffer with 1 mM Ca$^{2+}$ were reconstituted with washed red blood cells (50% v/v) and perfused through the matrix-coated microcapillary tubes. To ascertain at what shear rates of 3.6, 10.8, or 60 dyn/cm$^2$ for 2 min at 37 °C. Platelet-matrix interactions were monitored for 100 frames (0.586 frames/sec) using confocal fluorescence microscopy (TCS-SP, Leica). Stationary adhesion was defined as platelet movement of less than a single cell diameter over a 30-s observation period.

For temporal shear gradient experiments, washed platelets (1.5 × 10$^9$/ml) were allowed to transiently engage the vWf surface prior to the application of a shear gradient (0.9, 3.6, 10.8, or 60 dyn/cm$^2$). A pre-shear time period fixed at 5 min was employed to allow platelet-vWf matrix interactions to occur. During this period, platelets in suspension settle under gravity onto the vWf surface. Although the pre-shear time window was fixed, the platelet interaction times were variable and depended on the time of initial engagement of the surface over the 5-min period. Platelet contact on the matrix was defined when platelets remained in focus on the surface of the microcapillary tube. The mean platelet interaction time reflects the amount of time that has elapsed following initial contact of a single platelet with the matrix up until the point of application. The mean interaction time of platelets with the vWf surface prior to the application of shear was 15 ± 3 s. These findings highlight the fact that although a 5-min pre-adhesion step was utilized in all studies, the vast majority of platelets only interacted with the matrix for a short period of time before the application of shear.

Platelet计算器 were generated according to published methods (15, 16). Briefly, incremental flow rate increases were produced by a programmable Harvard PHD syringe driver operating in-line with a glass microcapillary.

Analysis of Cytosolic Calcium Flux under Static and Flow Conditions—The changes in cytosolic calcium levels were monitored according to published methods (10, 12). Briefly, washed platelets (1 × 10$^9$/ml) were loaded with Oregon Green 488 1,2-bis(2-aminoethoxy)ethane-$N,N,N',N''$-tetraacetic acid tetraacetoxymethylester (1 µM), and FuraRed/AM (1.25 µM) for 30 min at 37 °C. Dye-loaded platelets (1 × 10$^9$/ml) were then either allowed to adhere to vWf under static conditions or reconstituted with red blood cells (50% v/v) prior to perfusion through vWf-fibronectin, or vWf/fibronectin-coated microcapillary tubes. Transient changes in cytosolic calcium flux, sequential confocal images of adherent platelets were captured at a scan rate of 0.586 frames/sec for 37.5 s at the indicated time points. Real time platelet calcium flux was calculated from ratiometric fluorescence measurements and converted to intracellular calcium concentrations as described previously (10, 12).

Statistical Analysis—Statistical significance of results was determined using one-way analysis of variance, and p values are indicated where appropriate (*, p < 0.05 and **, p < 0.01; Figs. 2–7). All bars (Figs. 1–7) represent mean ± S.E. unless otherwise stated.

FIG. 1. High shear forces per se do not enhance the signaling function of GPIb. Calcium dye-loaded, washed platelets reconstituted with packed red blood cells (50% v/v) were perfused through vWf-coated (100 µg/ml) microcapillary tubes under steady-state flow conditions (wall shear stresses of 3.6, 10.8, or 60 dyn/cm$^2$). A, the proportion of platelets exhibiting sustained calcium signaling was analyzed and expressed as a percentage of totally adherent platelets (translocating with stable adhesion). B, the proportion of platelets forming stationary adhesion contacts was quantified and expressed as a percentage of totally adherent platelets (translocating with stable adhesion). C, calcium dye-loaded platelets pretreated with the integrin $\alpha_{IIb}\beta_{3}$ receptor antagonist c7E3 Fab were perfused through vWf-coated microcapillary tubes in the presence of ristocetin (1 mg/ml) at wall shear stresses of 3.6, 10.8, or 60 dyn/cm$^2$. The percentage of platelets undergoing a calcium response was quantified and expressed as a percentage of totally adherent platelets.

To gain insight into the mechanisms by which shear forces stimulate platelet activation, we employed an in vitro flow-based adhesion assay that enables simultaneous analysis of platelet adhesion and activation (by monitoring cytosolic calcium flux) on a von Willebrand factor substrate (10). With this assay, changes in cytosolic calcium flux correlate closely with platelet activation and the development of stationary adhesion contacts (12). Consistent with previous findings (18, 19), exposure of platelets in suspension to progressive increases in shear resulted in a corresponding increase in platelet aggregation (data not shown). Similarly, increasing shear induced a corresponding increase in the number of platelets tethering to von Willebrand factor (20) (data not shown). However, there was no positive relationship between increasing shear and the subsequent cytosolic calcium flux and concomitant stationary adhesion formation (Fig. 1 A and B). In fact, increases in steady-state shear reduced the proportion of platelets forming firm adhesion contacts, resulting in an increased number of rolling cells (Fig. 1 D).

It is generally assumed that GPIb is the principal mechano-receptor on the surface of platelets, responding to increases in shear with enhanced adhesive and signaling function (21, 22). However, analysis of the effects of steady-state increases in blood flow on GPIb-dependent calcium flux revealed no significant shear-dependent increase in GPIb signaling (data not shown). Even when platelets were artificially anchored to the matrix through vWf-GPIb adhesive bonds by perfusing platelets in the presence of ristocetin (1 mg/ml), no shear-dependent increase in cytosolic calcium flux was observed (Fig. 1 C).

In control studies we confirmed that this lack of increase in cal-
Fig. 2. Temporal shear gradients induce platelet activation. A, population analysis examining the effect of steady-state (60 dyn/cm²) or temporal shear gradients (60 dyn/cm²/s) on Ca²⁺ flux on vWF adherent platelets. Calcium dye-loaded, washed platelets were either reconstituted with red blood cells (50%) and flowed over vWF-coated capillary slides at 60 dyn/cm² or allowed to transiently engage the vWF surface under static conditions for 5 min (Static) and expressed to a shear gradient (ΔShear) (60 dyn/cm²/s). B, single platelet Δ[Ca²⁺], profiles (---) and concomitant displacement (µm) versus time (s) (-----) graphs demonstrating platelet adhesive behavior at the surface of immobilized vWF. The arrow (↓) indicates the point of application of rapid shear gradient. i. Trans-Sustained, platelet undergoes an initial transient calcium spike followed by the development of an oscillatory calcium response and firm platelet adhesion; ii. Transient, platelet undergoing a single Δ[Ca²⁺] spike that coincides with the development of a transient stationary adhesion contact; iii. No Response, platelet displaying minimal Δ[Ca²⁺] and rapid translocation on the vWF surface following application of shear. For PAC-1 immunofluorescence studies, dye-loaded platelets were allowed to adhere to vWF and exposed to a rapid shear gradient (60 dyn/cm²/s) in the presence of PAC-1 monoclonal antibody (1 µg/ml). Adherent platelets were subsequently fixed, labeled with a fluorescein isothiocyanate-conjugated secondary antibody, and visualized using fluorescence (PAC-1) and differential interference contrast (DIC) microscopy.
shear gradient of 10.8 dyn/cm²/s (Fig. 3A). This increase in the percentage of platelets undergoing a trans-sustained response with increasing shear gradients was not an artifact of enrichment for preactivated platelets, as increased shear differentials actually increased the number of platelets adhering to the matrix (data not shown). These findings suggest that increased tensile force on adhesive bonds activates mechanosensory mechanisms in platelets.

To determine whether platelet responsiveness to rapid shear changes was dependent on the rate of the applied shear gradient, transient adherent platelets were exposed to various ramped increases in fluid flow. As shown in Fig. 3B, a reduction in the rate of shear stress application lead to a corresponding decrease in the efficiency of platelet activation, which was particularly marked when the rate was increased from 6 to 3 dyn/cm²/s. Altering the slope of the shear gradient had no significant effect on the magnitude or pattern of the calcium flux in responding cells (data not shown) but primarily influenced the proportion of platelets exhibiting a cytosolic calcium response. Platelet activation induced by temporal shear gradients required ligand engagement of integrin αIIbβ3, as it was completely eliminated by pretreating platelets with integrin αIIbβ3 antagonists (c7E3 Fab or Aggrastat) (Fig. 4A). Significantly, rolling platelets never exhibited a significant change in calcium flux when exposed to rapid increases in shear (data not shown), suggesting that mechanotransduction primarily occurs through sustained integrin αIIbβ3-dependent adhesive bonds.

To examine whether the platelet activating effects of temporal shear gradients was vWF-specific or occurred on other physiologically relevant matrices, flow-based adhesion studies were performed on a fibrinogen matrix. In contrast to vWF, there was no calcium flux induced by shear gradients on this substrate, with the majority of platelets detaching to bulk flow (Fig. 4B). These studies demonstrate that vWF-integrin αIIbβ3 adhesive bonds sense and respond to rapid shear changes. Moreover, they demonstrate that the mechanosensory function of this receptor operates over a narrow temporal shear range.

To examine the relationship between the initial rapid acceleration in shear relative to sustained steady-state shear in promoting platelet activation on vWF, platelets were exposed to a short (1-s) pulse of flow (0 to 60 dyn/cm²). As demonstrated in Fig. 5, A and B, exposure of platelets to short shear pulses was associated with the induction of transient calcium response only, with <2% of cells exhibiting a subsequent sustained calcium response. As a consequence, the majority of platelets responding to short duration shear impulses subsequently detached from the matrix surface, suggesting that the induction of a sustained calcium response required the maintenance of steady-state flow. Consistent with this possibility, when platelets experiencing high wall shear rates were suddenly exposed to rapid decelerations in blood flow, >95% of cells subsequently detached from the matrix (data not shown). Examination of the shear threshold required to sustain platelet adhesion to the vWF surface revealed a progressive increase in the number of platelets remaining anchored to the vWF substrate as a function of steady-state shear, with maximal platelet adhesion and calcium flux at 10.8 dyn/cm². Furthermore, conversion of the transient integrin αIIbβ3-dependent calcium signal to a sustained calcium response was dependent on the maintenance of a minimal shear threshold of 3.6 dyn/cm² (Fig. 5C). These studies demonstrate that platelets can respond to shear conditions by exhibiting two distinct calcium responses, a transient calcium response linked to sudden accelerations in blood flow and a sustained calcium response dependent on the maintenance of steady-state shear.

Studies in nucleated cells have demonstrated that integrin responsiveness to biomechanical stimuli requires receptor occupancy and potentially post-ligand binding events such as the development of focal adhesions (4). To examine whether the shear responsiveness of transiently adherent platelets was related to the time of platelet pre-adhesion on the vWF surface (platelet-matrix interaction time), we compared the proportion of shear-responsive platelets as a function of pre-adhesion time. These studies revealed that there was no relationship between the time of platelet interaction with the matrix and their subsequent responsiveness to rapid shear changes, such that platelets interacting with vWF for a few seconds were equally as responsive to shear as platelets interacting for several minutes (data not shown). Furthermore, analysis of cytosolic calcium levels prior to the application of shear revealed that there was minimal integrin αIIbβ3-dependent calcium flux, indicating that newly formed integrin αIIbβ3 adhesive bonds respond rapidly to biomechanical stimuli.

Rapid transduction of biomechanical signals is typically linked to mechanically gated ion channels, whereas mechanically regulated second messenger generation is primarily linked to slower adaptive cellular responses. Gadolinium is a well defined inhibitor of mechanically gated ion channels (23); however, it had no effect on calcium flux induced by temporal shear gradients even when used at concentrations as high as 0.25–1 mM (Fig. 6). In contrast, pretreating platelets with signaling inhibitors against the IP₃ receptor (2-aminoethoxydi-
phenyl borate) or phospholipase C (U73122) completely eliminated shear gradient-induced calcium flux (Fig. 6) and prevented integrin αIIbβ3 activation and stable platelet adhesion on a vWf matrix (data not shown). Inhibition of Rho kinase and mitogen-activated protein kinase, two enzymes that have a well-defined role in integrin mechanotransduction (4), had no inhibitory effect on shear gradient-induced calcium flux and platelet adhesion, whereas inhibitors of Src family kinases (4-amino-5-(4-chlorophenyl)-7-(t-butylypyrazolo][3,4-d]pyrimidine) or phosphatidylinositol 3-kinase (LY294002 or wortman- 
nin) completely eliminated these responses (Fig. 6). These studies demonstrate a key role for mechanically regulated second messenger generation in enabling efficient platelet activation in response to rapid changes in shear.

ADP release from platelet-dense granules plays an important role in potentiating platelet activation in response to all physiological stimuli through activation of the P2Y1 and P2Y12 purinergic receptors. To investigate the role of these receptors in promoting shear gradient-induced transient calcium flux, platelets were pretreated with specific antagonists against P2Y1 (A3P5PS) and P2Y12 (ARC-C69931MX) (24, 25). As demonstrated in Fig. 7A, blocking the P2Y1 receptor completely eliminated the transient calcium flux induced by temporal shear gradients, whereas the P2Y12 antagonist had no effect. Interestingly, preventing transmembrane calcium influx also completely eliminated the shear gradient-induced calcium flux (Fig. 7A). Notably, a small percentage of platelets were still able to form firm adhesion contacts with the vWf substrate and undergo a sustained oscillatory calcium response independent of shear gradients, ADP release, and influx of extracellular calcium (Fig. 7B). In control studies, we demonstrated that inhibition of thromboxane A2 generation with aspirin had no effect on platelet activation by temporal shear gradients (data not shown), confirming a specific role for ADP in this process.

**DISCUSSION**

In this study we have defined a new mechanism responsible for mediating the rapid transduction of biomechanical signals in adherent cells. This mechanism involves cooperative signaling between the P2Y1 purinergic receptor and integrin αIIbβ3, leading to a rapid, transient increase in cytosolic calcium. We have demonstrated that this shear-induced calcium signal is distinct from previously identified integrin αIIbβ3-regulated calcium signals in terms of its transient nature, its dependence on extracellular calcium influx, and its requirement for a soluble co-stimulus. Moreover, we have demonstrated that the
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FIG. 6. Platelet activation in response to temporal shear gradients is dependent on second messenger generation. Population analysis demonstrating the effects of the mechanically gated ion channel inhibitor (gadolinium) or a variety of signaling inhibitors (2-aminooxydiphenyl borate (APB-2) against the IP3 receptor, U73122 against phospholipase C, PD98059 and SB 203580 against mitogen-activated protein kinase, Y27632 against Rho kinase, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) against Src, and LY294002 and wortmannin against phosphatidylinositol 3-kinase) on shear gradient (d/shear)-induced calcium flux. Calcium dye-loaded, washed platelets were incubated with the inhibitors for 10 min and allowed to transiently engage the vWF surface prior to the application of a shear gradient (60 dyn/cm²/s). The results represent the mean percentage of adherent platelets ± S.E. undergoing a calcium response in response to a shear gradient from 0 to 60 dyn/cm²/s (n = 3).

Flow conditions regulating this calcium signal are distinct from those regulating the onset of sustained integrin αIIbβ3 calcium responses in that the former are specifically induced by shear gradients, whereas the latter are regulated by changes in the magnitude of steady-state shear. Overall, these studies define a unique biomechanical transduction mechanism responsible for the induction of rapid shear activation of platelets.

An important finding in this study is the ability of rapid shear gradients to stimulate platelet activation. Measurements of hydrodynamic shear in stenosed arterial segments in vivo have demonstrated that narrowing of the vessel lumen leads to perturbation of the rheological profile, leading to significant flow acceleration (leading to temporal shear gradients) (6). Furthermore, measurements and observations of the shear conditions in vascular regions exhibiting non-Newtonian flow characteristics have demonstrated that wall shear stress fluctuates in magnitude and changes direction as a result of the cardiac cycle (9, 26). From these studies, shear stresses have been estimated to vary from negative values through zero values at the edges of flow separation regions and up to values of 40–60 dyn/cm². The shear gradients employed in this study are therefore well within this pathophysiological range.

The experimental approach utilized in the present study does not attempt to mimic the complex rheological changes that are relevant to arterial thrombus formation in vivo. Rather, we have attempted to examine the significance of steady-state shear changes and shear gradients on the platelet adhesion/activation process on a purified vWF matrix using a Newtonian or laminar shear system. By necessity, a pre-shear adhesion step was essential to enable formation of vWF-integrin αIIbβ3 adhesion bonds without significant platelet activation. These experimental conditions were necessary to demonstrate platelet activation by temporal shear gradients, as such effects were never observed in translocating platelets. Clearly, insight into the pathophysiological significance of our findings requires the development of more complex experimental systems that more closely approximate the blood flow environment in vivo. The simplified model presented in the present study delineates, for the first time, a key role for shear flux in modulating the adhesive and signaling functions of vWF-integrin αIIbβ3 adhesive interactions.

An unexpected finding from this study was the demonstration that integrin αIIbβ3 rather than GPIbα was the major mechanosensory receptor mediating platelet activation in response to temporal shear gradients. Previous studies have reported a GPIb-dependent calcium release mechanism that coincided with a period of transient stationary adhesion during surface translocation (27). Given the correlation between transient arrest and the GPIb-dependent calcium flux, the authors proposed a mechano-transduction model for GPIb-dependent platelet activation. Similarly, we have reported previously that GPIb-dependent calcium flux is only observed in platelets forming stationary albeit transient adhesion contacts under flow (21). These studies have suggested that an initial GPIb-dependent calcium flux triggers a low level of integrin αIIbβ3 activation that promotes stable platelet adhesion and the onset of a sustained cytosolic calcium signal (12, 27). However, neither of these previous studies examined the effect of shear gradients on integrin αIIbβ3-dependent calcium signaling. Furthermore, we also demonstrated that a small percentage of platelets were able to form firm adhesion contacts with the vWF substrate and undergo an αIIbβ3-dependent, sustained oscillatory calcium response independent of shear gradients and released ADP. Recent studies have demonstrated that blocking P2Y1 prevents stationary platelet adhesion and aggregation on immobilized vWF and completely abolishes αIIbβ3-dependent sustained oscillatory calcium response (28). We have reported previously that platelets can form firm adhesion contacts with a vWF matrix under static conditions independent of ADP, albeit less efficiently than in the absence of ADP receptor antagonists (10). The most likely explanation for these findings is that a small percentage of platelets exhibit a GPIbα signal that promotes a sufficient level of integrin αIIbβ3 activation to form stable adhesive interactions independent of ADP.

Our studies have demonstrated that platelet responsiveness to rapid accelerations in shear requires the cooperative signaling function of P2Y1 and integrin αIIbβ3. Growing evidence suggests that cooperative signaling mechanisms operating between multiple surface receptors are important for both rapid and delayed mechanosensory functional responses (29). In the case of integrins there is evidence that functional association with other surface receptors, including G protein-coupled receptors, integrin-associated proteins, and mechanically gated ion channels is important for efficient mechanotransduction (4, 30, 31). Although our studies do not support an important role for mechanically gated ion channels in integrin αIIbβ3 mechanotransduction, they demonstrate a cooperative signaling function for the P2Y1 purinergic receptor in this process. ADP binding to P2Y1 stimulates internal calcium release, whereas vWF ligation of integrin αIIbβ3 induces both calcium mobilization and transmembrane calcium influx. In this context, it was surprising that the calcium response induced by temporal shear gradients was completely eliminated by chelators of extracellular calcium with no apparent contribution from P2Y1 or integrin αIIbβ3-regulated internal calcium stores in this process. This finding, combined with the fact that rapid mechanosensitive functional responses are typically linked to the activation of mechanically gated ion channels, raised the possibility that shear-sensitive membrane channels rather than second messenger generation was the principal mechanism responsible for this calcium signal. However, in this study the use of inhibitors of mechanically gated ion channels and well-defined signaling enzymes required for integrin αIIbβ3-dependent calcium flux revealed that this hypothesis may not be the case.
In platelets, calcium influx mechanisms have been principally linked to store-operated calcium channels (32) and, to date, there is no definitive evidence that voltage-gated or mechanically gated ion channels exist on the platelet surface. Our inability to detect second messenger-dependent internal calcium release in response to temporal shear gradients was surprising but presumably reflects technical limitations of our calcium-imaging assay, which may not detect subtle changes in near-membrane calcium flux. Alternatively, our findings may highlight the IP3/IP3 receptor dependence of the store-operated calcium influx pathway independent of internal calcium release. Consistent with this is the conformational coupling model of calcium influx (17) in which calcium influx is dependent on the formation of a macromolecular complex (signalosome) involving IP3, the IP3 receptor, and phospholipase C along with capacitative calcium influx channels. Resolution of this issue will require the development of high sensitivity calcium imaging systems that can detect subtle changes in near-membrane calcium flux.

Our current working model for the temporal shear gradient-induced calcium response suggests an important role for the P2Y1 receptor in stimulating rapid changes in integrin \( \alpha_{IIb}\beta_3 \) affinity through inside-out signaling events. It is possible that this signal potentiates initial GPIb-dependent signals necessary for sustained integrin \( \alpha_{IIb}\beta_3 \) activation. The subsequent increased affinity/avidity interaction between integrin \( \alpha_{IIb}\beta_3 \) and vWF initiates outside-in signals that stimulate an elementary cytosolic calcium response necessary for transmembrane calcium influx. This initial transient calcium response is likely to feed back on the integrin \( \alpha_{IIb}\beta_3 \) itself, enhancing platelet adhesion and increasing the probability of platelets maintaining a sustained calcium response. A key issue that will require future investigation is determining whether both GPIb and integrin \( \alpha_{IIb}\beta_3 \) are required to act as mechanosensors for temporal shear gradient-induced platelet activation, a possibility given that temporal shear effects are only observed on a vWF substrate. It also remains to be established how shear effects

**FIG. 7.** Role of ADP in potentiating platelet activation in response to temporal shear gradients. Calcium dye-loaded platelets were treated with either the P2Y1 and P2Y12 receptor antagonists A3P5PS (250 \( \mu \)M) and AR-C69931MX (10 \( \mu \)M) or 1 mM EGTA and 1 mM \( \text{Mg}^{2+} \) for 10 min prior to the assay and then allowed to transiently engage the vWF surface prior to the application of a shear stress gradient of 60 dyn/cm²/s. The arrow (↓) shows the point of shear gradient application. A, population analysis demonstrating the effects of the P2Y1 and P2Y12 receptor antagonists A3P5PS and AR-C69931MX, respectively, or 1 mM EGTA/Mg²⁺ on shear gradient-induced calcium flux. Results represent the percentage of platelets undergoing a calcium response in response to a shear gradient between 0 and 60 dyn/cm²/s (n = 3). B, analysis of the platelets that form firm adhesion contacts with the vWF substrate and undergo a sustained integrin \( \alpha_{IIb}\beta_3 \)-dependent oscillatory calcium response after a period of time independent of the shear gradient. Effect of A3P5PS, AR-C69931MX, and EGTA/Mg²⁺ on sustained calcium signaling in vWF-adherent platelets following exposure to temporal shear gradients (60 dyn/cm²/s). C, effect of A3P5PS, AR-C69931MX, and EGTA/Mg²⁺ on sustained calcium signaling in vWF-adherent platelets following exposure to temporal shear gradients (60 dyn/cm²/s). The calcium profiles are of selected single platelet recordings to highlight the distinct nature of the transient and sustained calcium responses.
on integrin αIIbβ3 adhesive bonds can stimulate such rapid platelet activation, given that the process requires ADP release, subsequent integrin αIIbβ3 responsiveness to P2Y1 signals, outside-in second messenger generation, and the activation of transmembrane calcium channels, all within a 1-s time frame. Unraveling such mechanisms will be important, not only for a more complete understanding of how shear affects platelet function but also to identify new approaches to modulating shear activation of platelets.

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