ATP Augments von Willebrand Factor-dependent Shear-induced Platelet Aggregation through Ca\(^{2+}\)-Calmodulin and Myosin Light Chain Kinase Activation*

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Shear stress triggers von Willebrand factor (VWF) binding to platelet glycoprotein Ib\(\alpha\) and subsequent integrin \(\alpha_{\text{IIb}}\beta_{\text{IIIa}}\)-dependent platelet aggregation. Concomitantly, nucleotides are released from platelet-dense granules, and ADP is known to contribute to shear-induced platelet aggregation (SIPA). We found that the impaired SIPA of platelets from a Hermansky-Pudlak patient lacking dense granules was restored by that the impaired SIPA of platelets from a Hermansky-Pudlak patient. Nucleotides are released from platelet-dense granules and, as well as by ADP, confirming that in addition to ADP (via \(P_{2Y}\)) and \(P_{2Y_{12}}\), ATP (via \(P_{2X}\)) also contributes to SIPA. Likewise, SIPA of apyrase-treated platelets was restored upon \(P_{2X}\) activation with \(1\)-, \(\beta\)-, \(\gamma\)-methylene ATP, which promoted granule centralization within platelets and stimulated P-selectin expression, which is a marker of \(\alpha\)-granule release. In addition, during SIPA, platelet degranulation required both extracellular Ca\(^{2+}\) and VWF-glycoprotein Ib\(\alpha\) interactions without involving \(\alpha_{\text{IIb}}\beta_{\text{IIIa}}\). Neither platelet release nor SIPA was affected by protein kinase C inactivation, even though protein kinase C blockade inhibits platelet responses to collagen and thrombin in stirring conditions. In contrast, inhibiting myosin light chain (MLC) kinase with ML-7 reduced platelet release and SIPA by 30%. Accordingly, the potentiating effect of \(P_{2X}\) stimulation on the aggregation of apyrase-treated platelets coincided with intensified phosphorylation of MLC and was abrogated by ML-7. SIPA-induced MLC phosphorylation occurred exclusively through released nucleotides and selective antagonism of \(P_{2X}\), with MRS2159-reduced SIPA, ATP release, and potentially inhibited MLC phosphorylation. We conclude that the \(P_{2X}\) ion channel induces MLC-mediated cytoskeletal rearrangements, thus contributing to SIPA and degranulation during VWF-triggered platelet activation.

Blood platelets are constantly subjected to hemodynamic forces imposed by the blood flow, including fluid shear stress.

High shear stress is generated at sites of arterial injury where laminar blood flow is forced through a stenosis (1, 2). Shear-stress-triggered platelet activation and subsequent aggregation, termed SIPA (3), may thus contribute to the pathogenesis of vascular diseases. In addition, platelets from patients with acute myocardial infarction (4) or cerebral ischemia (5) display enhanced SIPA in vitro.

High shear stress is required for the interaction between von Willebrand factor (VWF) and platelet glycoprotein Ib\(\alpha\) (GP Ib\(\alpha\)) (for review, see Ref. 6), but the effects of shear forces on GP Ib\(\alpha\) signaling are only just beginning to be defined. Downstream effectors that have been implicated in the VWF-dependent activation of \(\alpha_{\text{IIb}}\beta_{\text{IIIa}}\) include phosphatidylinositol 3-kinase (PI3K) (7), protein kinase C (PKC) (8), Syk, and Src, as well as co-associated immunoreceptor tyrosine-activated motif-containing transmembrane proteins and adapter proteins (2). A recent study of platelet adhesion to dimeric VWF A1 domain, which recognizes only GP Ib\(\alpha\), showed that GP Ib\(\alpha\) itself can signal to activate \(\alpha_{\text{IIb}}\beta_{\text{IIIa}}\) through sequential actions of Src kinases, Ca\(^{2+}\)-oscillations, and PI3K/PKC (9). It has also been proposed that GP Ib\(\alpha\) signals directly as a consequence of its association with structural cytoskeletal proteins (10), among which GP Ib\(\alpha\)-bound filamin A, filamentous actin cross-linked by \(\alpha\)-actinin, vinculin, and talin would directly link the cytoplasmic domain of GP Ib\(\alpha\) with the \(\beta_{\text{IIIa}}\) tail of \(\alpha_{\text{IIb}}\beta_{\text{IIIa}}\) (11).

Studies examining SIPA have suggested that VWF primarily stimulates platelet activation through an indirect pathway linked to ADP release (12, 13). These studies have also shown that platelet activation initiated by VWF-GP Ib\(\alpha\) interaction requires a transmembrane Ca\(^{2+}\) influx independent of released ADP and VWF binding to \(\alpha_{\text{IIb}}\beta_{\text{IIIa}}\) (14, 15), which promotes dense granule secretion of ADP and activates integrin \(\alpha_{\text{IIb}}\beta_{\text{IIIa}}\) through engagement of the P2 receptors for ADP. A role for the platelet ADP receptors \(P_{2Y_{1}}\) and \(P_{2Y_{12}}\) (reviewed in Ref. 16) in SIPA has subsequently been confirmed (17, 18). Reséndiz et al. (19) recently reported that the selective \(P_{2Y_{12}}\) antagonist AR-C69931MX inhibits the shear-induced aggregation of washed platelets and showed that \(P_{2Y_{12}}\) mediates shear-induced PI3K activation, a process coupled to phosphorylation of PI3K-associated Syk tyrosine kinase.

In blood vessels, high shear stress causes the release of high levels of nucleotides, including ATP and ADP, into the extracellular environment; these nucleotides mainly originate from endothelial cells (20) and platelet-dense granules (21). Although the
shear-induced ATP release from endothelial cells probably involves vesicular exocytosis (22), the molecular mechanisms of such a release are largely unknown. In platelets, the time course of this ATP release appeared to parallel the association of myosin with the actin cytoskeleton, suggesting that these two processes are related (21). In addition, Ca\(^{2+}\)- influx-dependent phosphorylation of myosin light chain, preceding the myosin-actin interactions, has been proposed to be an initial step during SIPA (21).

ATP is the physiological agonist at P2X\(_{1}\), but its role in platelet activation is only starting to be unraveled. In the aggregometer, the selective P2X\(_{1}\) agonists \(\alpha,\beta\)-meATP and \(\alpha,\beta\)-meATP evoke a transient Ca\(^{2+}\) influx accompanied by rapid and reversible platelet shape change and myosin light chain (MLC) phosphorylation, provided that measures are taken to avoid desensitization of P2X\(_{1}\) by ATP spontaneously released during platelet preparation (23–25). Recent studies, using P2X\(_{1}\) knock-out mice (26) or transgenic mice overexpressing this ion channel selectively in the megakaryocytic cell lineage (27), have reported a prominent role for P2X\(_{1}\) in platelet aggregation and thrombus formation in shear stress conditions. Notably, P2X\(_{1}\) overexpressing platelets displayed potent SIPA in conditions where wild-type platelets hardly aggregated (27). In the present study, we have investigated the role of the P2X\(_{1}\)-mediated Ca\(^{2+}\) influx and the associated downstream signaling pathways in VWF-dependent shear-induced granule release and aggregation of washed human platelets.

**EXPERIMENTAL PROCEDURES**

**Materials**—ADP, \(\alpha,\beta\)-meATP, the P2X\(_{1}\) antagonist MR2159, the P2Y\(_{1}\) antagonist MR2179, and the ecto-5nucleotidase apyrase (grade 1) were purchased from Sigma. AR-C69091XM was a gift from AstraZeneca R&D, Charnwood, UK. The P2X\(_{1}\) antagonist MR2159 at the concentration used in this study was verified to selectively inhibit a P2X\(_{1}\)-mediated platelet shape change without affecting platelet shape change and aggregation induced by ADP. The calmodulin inhibitor W-7, the MLCK kinase inhibitor ML-7, the Rho kinase inhibitor Y-27632, and the PKC inhibitors GF109203X, Go6976, and Go6983 were purchased from Calbiochem. Human VWF was purified from plasma cryoprecipitate by gel filtration on a Sepharose 4B-CL column. Tirofiban (Aggrastat) was a gift from Bristol-Myers Squibb. Human VWF was purified by high pressure liquid chromatography on an Adsorbosphere H5 C18, 7 \(\mu\)m, 250 × 4.6-mm column (Alltech) as described by Oury et al. (24).

**Preparation of Washed Human Platelets**—Washed human platelets (2.5–3.5 × 10\(^{10}\) platelets/\(\mu\)l) were prepared as described previously (29). Apyrase (0.5 units/ml) was added to the blood sample and platelet resuspension buffer when indicated. Platelet preparations were free of red blood cells, as validated via microscopy and blood cell count (CellDyn 1300, Abbott, Abbott Park, IL).

**Shear-induced Platelet Aggregation, ATP Secretion, and P-selectin Expression**—Shear-induced aggregations of washed human platelets were performed in an annular ring-shaped viscometer generating laminar flow (Ravenfield viscometer; Heywood, Lancashire, UK) at 37 °C in the presence of 2 mM CaCl\(_2\) and 10 \(\mu\)g/ml human soluble VWF unless otherwise indicated. A shear rate of 9000 s\(^{-1}\) was used, which corresponds to a shear stress of 80 dynes/cm\(^2\). The threshold shear rate causing platelet aggregation ranged between 3000 s\(^{-1}\) and 5000 s\(^{-1}\). At defined shear rates, platelet samples were collected and fixed in 1% paraformaldehyde; the percentage of platelet aggregation was calculated by comparing single platelet counts before and after shearing. Shear-induced P-selectin surface translocation on tirofiban-treated washed platelets was detected by flow cytometry with the phycoerythrin-conjugated anti-P-selectin CD62P antibody (BD Biosciences). For ATP secretion assays, the reactions were stopped with an ice-cold HEPES buffer (20 nm HEPES, pH 7.4, 150 mM NaCl, 5 mM EDTA). After immediate centrifugation at 4 °C, ATP was measured in the supernatants by the addition of a luciferase/luciferin reagent (Chrono-Lume, Vilvoorde, Belgium). At least five independent experiments were performed on platelets from different individuals. The data are represented as the mean ± S.D. Statistical analysis of the data was done using nonpaired Student’s t tests.

**Immunoblotting Analyses**—Platelets (0.3 ml platelet suspensions) were lysed in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% bromphenol blue). Sample aliquots were loaded on SDS-PAGE (12.5%) and subjected to Western blotting. Thr-18/Ser-19 MLC phosphorylation was detected with the anti-phospho-MLC polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) according to the instructions of the manufacturers.

**Electron Microscopy**—Washed platelets were immediately fixed overnight at 4 °C in 2.5% (w/v) glutaraldehyde, 0.1 mM phosphate buffer, pH 7.2. After centrifugation at 800 × g for 10 min, a condensed pellet of washed platelets was formed. After fixation in 1% OsO\(_4\) (w/v), 0.1 mM phosphate buffer, pH 7.2, and dehydration in a graded series of ethanol, the pellets were embedded in epoxy resin. Ultrathin sections were cut and stained with uranyl acetate and lead citrate before examination with a Zeiss EM 10 electron microscope (Oberkochen, Germany).

**RESULTS**

**VWF-GP Ibα Interactions and Extracellular Ca\(^{2+}\) in SIPA**—When suspensions of washed human platelets were subjected to high shear stress (80 dynes/cm\(^2\); 9000 s\(^{-1}\) shear rate) in the presence of soluble human VWF in an annular ring-shaped viscometer, aggregation rapidly occurred, reaching a plateau after 3 min (Fig. 1A). In contrast, a shear rate of 1000 s\(^{-1}\) did not cause platelet aggregation (Fig. 1A). The inclusion of the VWF A1 domain-blocking antibody Ab4W-2, neutralizing anti-GP Ibα antibody G19H10, or the \(\alpha_{\text{IIb}}\beta_{\text{3}}\) antagonist tirofiban (Fig. 1B) confirmed the findings by others (30) that SIPA requires VWF interactions with GP Ibα and the activation of \(\alpha_{\text{IIb}}\beta_{\text{3}}\).

Platelet aggregation was accompanied by gradually increasing shear stress-dependent ATP release (Fig. 1A). ATP release was initiated as early as 10 s after exposure to shear stress. When measured at the maximal aggregation time point, this release clearly relied on VWF-GP Ibα signaling, as it was at least partly inhibited by Ab4W-2 and G19H10 (Fig. 1B). Omitting the addition of extracellular Ca\(^{2+}\) also reduced the amount of released ATP, although only partly (Fig. 1B). The combined absence of VWF and extracellular Ca\(^{2+}\) almost abolished platelet degranulation (Fig. 1B). In contrast, \(\alpha_{\text{IIb}}\beta_{3}\) antagonism had no effect on the degree of platelet degranulation (Fig. 1B). Therefore, both VWF-GP Ibα signaling and Ca\(^{2+}\) contribute to platelet release under shear stress, a process that is independent of \(\alpha_{\text{IIb}}\beta_{3}\) outside-in signals.

**MLC Kinase (but Not PKCs) Controls the SIPA-induced Platelet Degranulation**—Platelet granule release that is induced by most agonists requires an increase of intracellular Ca\(^{2+}\) and PKC activation (reviewed in Ref. 31). Whether or not PKC plays a role in SIPA is controversial (8, 32). In our experimental conditions, the nonselective PKC inhibitor GF109203X, which blocks both conventional (\(\alpha,\beta\), \(\beta\), \(\gamma\)) and novel (\(\delta,\eta,\xi\)) PKC isoforms, failed to inhibit either platelet aggregation or release produced by shear stress (Fig. 2A), although it prevented such responses to collagen (2 \(\mu\)g/ml) or to the PAR-1-activating peptide TRAP\(_{6}\) (1 \(\mu\)M) under stirring conditions (Fig. 2B). Similarly, the PKC a/b-specific inhibitor Go6976 did not affect SIPA and associated platelet release (Fig. 2A), although shear stress-independent collagen-induced release and aggregation were fully blocked (Fig. 2B). In agreement with previous reports that PKC a/b isoforms do not play a major role downstream of PAR-1 (33), Go6976 had a minimal inhibitory effect on TRAP\(_{6}\)- induced granule release (Fig. 2B). Finally, to determine whether other PKC isoforms could be involved in platelet release accompanying SIPA, we used Go6983, which blocks the platelet-expressed atypical PKC \(\xi\) isoform in addition to PKC \(\alpha,\beta,\delta,\gamma,\) and \(\delta\). This inhibitor had no effect on shear-induced
platelet release or aggregation, although it diminished TRAP$_1$-induced ATP release by about 50% without affecting platelet aggregation (Fig. 2B). These results indicate that shear-induced platelet dense granule release and aggregation are dependent on signaling pathways distinct from those activated downstream of the collagen receptor GPVI or PAR-1. They also suggest that the known conventional, novel, and atypical PKC isoforms do not play a major role in platelet degranulation during SIPA.

In addition to PKC, GPVI-mediated platelet dense granule release also depends on Ca$^{2+}$-sensitive MLC kinase activation (25). Inhibiting this kinase with ML-7 abolished both the ATP release and platelet aggregation induced by collagen (2 μg/ml) (Fig. 2B). As shown in Fig. 2A, ML-7 reduced the shear-induced ATP release and platelet aggregation by about 30%, indicating a role for MLC kinase in SIPA and associated platelet degranulation.

**Secreted ADP and ATP in VWF-dependent Shear-induced Platelet Release and Aggregation**—Under stirring conditions in an aggregometer, MLC kinase is activated downstream of the P2X$_1$-mediated Ca$^{2+}$ influx, playing an essential role during cytoskeletal rearrangements evoked by selective agonists (αβ$\gamma$-meATP and i-β,γ$\gamma$-meATP) of this ion channel (25). The activation of MLC kinase can also result from phospholipase C-dependent Ca$^{2+}$ mobilization from internal stores downstream of the G$_q$-protein-coupled P2Y$_1$ receptor or of GPVI (34, 35). In agreement with earlier reports (17–19), the selective antagonists of P2Y$_1$ (MRS2179, 10 μM) and P2Y$_12$ (AR-C69931MX, 1 μM) inhibited SIPA by more than 50% (Fig. 3). In previous studies of platelet aggregation performed under stirring conditions in an aggrego-
P2X1 Signaling in SIPA

Fig. 2. MLC kinase versus PKC in SIPA and platelet ATP release. A, aggregation of washed human platelets was induced for 3 min at a shear rate of 9000 s⁻¹ following preincubation with the protein kinase C inhibitors GF109203X (+ GF, 10 μM, 5 min), Go6976 (1 μM, 5 min), or Go6983 (1 μM, 5 min) or the MLC kinase antagonist ML-7 (10 μM, 10 min). The effect of these inhibitors on ATP secretion measured after 3 min of shearing is also shown. *, p < 0.05. B, aggregation of washed human platelets produced in an aggregometer by collagen (2 μg/ml collagen) or TRAP₁₋₄ (1 μM SFFLRN) in the absence (control) or presence (+) of the same inhibitors, as indicated. The corresponding concentrations (μM) of released ATP are shown in brackets.

Fig. 3. P2X₁, P2Y₁, and P2Y₁₂ in SIPA and ATP release. A, aggregation of washed platelets was induced for 3 min at a shear rate of 9000 s⁻¹ following a 1-min preincubation with the selective P2 receptor antagonists for P2Y₁₂ (AR-C69931MX, 1 μM), P2Y₁ (MRS2179, 10 μM), and P2X₁ (MRS2159, 10 μM) or with combined MRS2179 + MRS2159. *, p < 0.001; #, p = 0.04 versus control. B, ATP secretion measured after 3 min of shearing in the presence or absence of the P2 receptor antagonists. *, p < 0.05; NS, not significant.

P2X₁ signaling in SIPA: A new target for platelet inhibition.

The results indicate that P2X₁ antagonists inhibit aggregation, release, and secretion in SIPA, suggesting a novel role for P2X₁ in platelet activation. The study also highlights the importance of understanding the interplay between different P2 receptors and nucleotide signaling in platelet function.

Further experiments are needed to elucidate the precise mechanisms by which P2X₁ antagonists modulate platelet responses and to assess their potential as therapeutic agents for the treatment of thrombotic disorders.
meATP amplification (Fig. 4B). Notably, these inhibitors had no effect on the SIPA of apyrase-treated platelets without additional stimulation by L-\(\gamma\)-meATP (data not shown), indicating that the activation of these pathways exclusively depends on P2X\(_1\) potentiation. The fact that W-7 inhibited the L-\(\beta\)-\(\gamma\)-meATP-driven SIPA to a larger extent than ML-7 may indicate the existence of an additional CaM-dependent pathway activated downstream of P2X\(_1\). Thus, exogenous P2X\(_1\) activation selectively triggers CaM-dependent pathways contributing to SIPA. Moreover, in the presence of the P2Y\(_{12}\) receptor antagonist AR-C69931MX, the L-\(\beta\)-\(\gamma\)-meATP-driven SIPA was reduced to the same level (16.2% vs. 11006 control) in the presence or in the absence of the selective P2Y\(_1\) agonist L-\(\gamma\)-TFA (10 \(\mu\)M) following preincubation with the neutralizing anti-VWF monoclonal antibody AdvW-2 (20 \(\mu\)g/ml), the \(\alpha_{IIb}\beta_3\) antagonist tirofiban (+tir, 1 \(\mu\)g/ml), the CaM antagonist W-7 (100 \(\mu\)M), the MLC kinase antagonist ML-7 (10 \(\mu\)M), or the P2Y\(_{12}\) antagonist AR-C69931MX, as indicated. $, p < 0.004$ versus control; *, $p = 0.001$ versus apyrase – L-\(\beta\)-\(\gamma\)-meATP; **, $p < 0.005$; $\dagger$, $p < 0.01$ versus apyrase + L-\(\beta\)-\(\gamma\)-meATP; $\ddagger$, $p = 0.02$ versus presence of W-7. The presence of apyrase and L-\(\beta\)-\(\gamma\)-meATP are represented by solid black lines under-scoring the graph.

**P2X\(_1\) Signaling in SIPA**

We investigated whether P2X\(_1\), to potentiate platelet aggregation during SIPA was further investigated by electron microscopy of platelet aggregates (Fig. 5). Following 3 min of shearing, the apyrase (0.5 units/ml)-treated platelets formed small aggregates and displayed granule centralization and pseudopodia, but most of the granules were intact (Fig. 5c). On the contrary, in the presence of L-\(\beta\)-\(\gamma\)-meATP, larger platelet aggregates were observed in which more platelets were degranulated; in the platelets still showing intact granules, their centralization appeared more advanced than in the absence of L-\(\beta\)-\(\gamma\)-meATP, indicative of more pronounced cytoskeletal rearrangements (Fig. 5d). Platelets subject to low shear stress (1000 s\(^{-1}\) shear rate) did not show any morphological changes (Fig. 5b) as compared with resting platelets (Fig. 5a). Thus, P2X\(_1\)-driven MLC kinase activation in SIPA contributes both to cytoskeletal rearrangements in platelets and to degranulation.

**Shear-induced MLC Phosphorylation Depends on P2X\(_1\), P2Y\(_1\), and P2Y\(_{12}\) Activation of CaM-regulated MLC kinase and Rho kinase results in Ca\(^{2+}\)-dependent and -independent phosphorylation of MLC (34), which is a key step in platelet activation. Shear stress caused phosphorylation of MLC (detected as early as 20 s and reaching its maximum after 3 min) (Fig. 6A), which was inhibited both by ML-7 and the Rho kinase inhibitor Y-27632 (Fig. 6B). Such phosphorylation was not affected by the \(\alpha_{\text{IIb}}\beta_3\) antagonist tirofiban (Fig. 6B), indicating that it occurred independently of \(\alpha_{\text{IIb}}\beta_3\) outside-in signals. In agreement with a role for Rho kinase in SIPA, platelet aggregation was reduced from 43.9 ± 9.5% to 23.6 ± 8.7% by adding Y-27632 (5 \(\mu\)M) during SIPA (37).

We found that antagonizing P2X\(_1\) (MRS2159), P2Y\(_1\),
apyrase, the addition of L-β,γ-meATP (10 μM) and the MLC kinase inhibitor ML-7 (10 μM) as indicated. #, p = 0.01 versus control; *, p = 0.02 versus absence of L-β,γ-meATP, **, p = 0.04 versus presence of L-β,γ-meATP.

Lower panels (a–d), transmission electron microscopy of SIPA. Apyrase (0.5 unit/ml)-treated washed human platelets were subjected or not (a) to a shear rate of 1000 s⁻¹ (b) or 9000 s⁻¹ (c) in the absence or presence of L-β,γ-meATP (10 μM) (d) for 3 min before being analyzed by electron microscopy. Scale bars = 1 μm.

(MRS2179), or P2Y₁₂ (AR-C69931MX) inhibited phosphorylation of MLC to a variable degree (Fig. 6B). Phosphorylation was inhibited potently by P2X₁ neutralization. Correspondingly, the addition of 0.5 units/ml apyrase (i.e. abolishing platelet activation via P2Y₁ and P2X₁) almost abolished MLC phosphorylation (Fig. 6C); moreover, shearing for 20 s resulted in a phosphorylation state below that of resting platelets (Fig. 6C). The addition of 5 units/ml apyrase abrogated the overall response (Fig. 6D), which could not be restored by doubling the concentration of exogenous VWF (20 μg/ml) (Fig. 6D), even though this led to an ~20% increase of aggregation (not shown). Thus, VWF does not trigger MLC phosphorylation without involving secreted nucleotides.

Fig. 6C also shows that, in the presence of 0.5 units/ml apyrase, the addition of L-β,γ-meATP prior to shear potently enhanced MLC phosphorylation induced after 20 and 60 s; such L-β,γ-meATP-elicted phosphorylation was quickly reversible as it was no longer detected after 3 min. Therefore, the ability of L-β,γ-meATP to cause MLC phosphorylation coincides with its ability to potentiate the shear-induced aggregation of apyrase-treated platelets.

FIG. 5. P2X₁ activation enhances platelet degranulation. Upper panel, flow cytometry analyses of P-selectin surface expression on tirofiban- and apyrase (0.5 units/ml)-treated washed platelets exposed to VWF at a shear rate of 9000 s⁻¹ for 5 min or not (control) in the presence or absence of L-β,γ-meATP (10 μM) and the MLC kinase inhibitor ML-7 (10 μM) as indicated. #, p = 0.01 versus control; *, p = 0.02 versus absence of L-β,γ-meATP, **, p = 0.04 versus presence of L-β,γ-meATP. Lower panels (a–d), transmission electron microscopy of SIPA. Apyrase (0.5 unit/ml)-treated washed human platelets were subjected or not (a) to a shear rate of 1000 s⁻¹ (b) or 9000 s⁻¹ (c) in the absence or presence of L-β,γ-meATP (10 μM) (d) for 3 min before being analyzed by electron microscopy. Scale bars = 1 μm.

DISCUSSION

In the present study, we describe a role for the P2X₁-mediated CaM and MLC kinase activation in VWF-dependent SIPA through secreted ATP. This pathway contributes to the extracellular Ca²⁺-dependent platelet degranulation induced by shear stress and acts in conjunction with VWF-GPIbα signaling. As schematically represented in Fig. 7, platelet activation initiated by the VWF-GPIbα interaction required a transmembrane Ca²⁺ influx independent of both ADP and VWF binding to α₃β₁(14, 15). Our observation that the P2X₁ receptor antagonist MRS2159 only partly inhibits platelet degranulation and aggregation (to a maximum of 60%), whereas omitting extracellular Ca²⁺ or neutralizing VWF abolishes it, suggests that the VWF/GPIb-associated transmembrane Ca²⁺ influx only partly relies on P2X₁-mediated Ca²⁺ influx.

In contrast to the effects on aggregation, the removal of VWF or antagonizing its binding to GPIbα only partially reduced the shear-induced platelet ATP release unless extracellular Ca²⁺ was omitted at the same time. The antagonist of α₃β₁(tirofiban, had no effect on shear-induced platelet release, excluding contributions by VWF-α₃β₁ outside-in signaling to secretion regulation. Antagonizing P2X₁ with MRS2159 diminished the ATP release, which was also seen with the antagonism of P2Y₁ and P2Y₁₂, indicating a role for all of these receptors in the amplification of the VWF/GPIbα-triggered platelet degranulation (Fig. 7).

The ATP-gated P2X₁ ion channel only operates in the presence of physiological extracellular Ca²⁺ concentrations (23, 24). Ca²⁺ is a key element in granule secretion (reviewed in Ref. 31). The proteins that mediate the effects of Ca²⁺ in secretion fall into two categories: the EF hand proteins and the Ca²⁺/phospholipid-binding proteins. EF hand proteins found in platelets include calmodulin and calcyolin. Calmodulin binds to platelet α-granules. Ca²⁺/calmodulin-dependent phosphorylation of MLC ki-
P2X<sub>1</sub> activation can restore the severely defective SIPA of platelets lacking dense granules (Hermansky-Pudlak patient) corroborates a role for P2X<sub>1</sub>-mediated cytoskeletal rearrangements in facilitating α-granule release.

Thus, our findings suggest that P2X<sub>1</sub> participates in the extracellular Ca<sup>2+</sup>-dependent shear-induced platelet granule release through CaM and MLC kinase-triggered MLC phosphorylation and concurrent cytoskeletal rearrangements, facilitating SIPA and leading to granule centralization as an onset to degranulation (Fig. 7). Because P2Y<sub>12</sub> antagonism also inhibited shear-induced MLC phosphorylation (although weakly), a role for Ca<sup>2+</sup> mobilized from internal stores in MLC kinase activation is possible (34, 35). Rapid P2X<sub>1</sub> and slower P2Y<sub>1</sub> signals (activated by co-released ATP and ADP, respectively) converge to a common effector (MLC kinase) contributing to cytoskeleton remodeling and platelet release (Fig. 7). Accordingly, combined P2X<sub>1</sub> and P2Y<sub>1</sub> antagonism leads to the same inhibition of SIPA at 3 min as P2Y<sub>1</sub> antagonism only.

The study by Schoenwaelder et al. (37) defines an important role for RhoA and Rho kinase in SIPA but not in platelet release. The inhibition of shear-induced MLC phosphorylation by selective P2Y<sub>1</sub> or P2Y<sub>12</sub> antagonism suggests that ADP activates Rho kinase through G<sub>i</sub> (40) and G<sub>q</sub> (41) also under shear stress (Fig. 7). Further investigations are required to determine the function of these pathways during SIPA. The failure of Rho kinase inhibitors to affect platelet release (Ref. 37 and the present study) may be explained by the existence of two pools of myosin present in different cell compartments (42) and emphasizes the role of MLC kinase activation in nucleotide-mediated granule release. In agreement with findings by Reséndez et al. (19), we further found that P2Y<sub>12</sub> antagonism P13K activation during SIPA. The resulting Akt (protein kinase B) phosphorylation was inhibited by P2Y<sub>12</sub> neutralization. P13K inhibition partially inhibited SIPA but had no effect on ATP release (not shown); i.e. P2Y<sub>12</sub>-recruited P13K activates platelets via a separate pathway (Fig. 7) potentially by regulating the function of α<sub>IIb</sub>β<sub>3</sub> via Akt and the integrin-linked kinase ILK (43).

Platelet granule release induced by most platelet agonists depends on PKC activation (reviewed in Ref. 31). However, none of the PKC inhibitors used in this study affected the shear-induced platelet release or aggregation. The nonsselective PKC inhibitor GF109203X (which blocks both diacylglycerol- and Ca<sup>2+</sup>-sensitive conventional PKC isoforms and the diacylglycerol-sensitive Ca<sup>2+</sup>-insensitive novel isoforms), the more potent PKC α/β-specific inhibitor Go6976, and Go6983 (which inhibits the atypical isoform in addition to α, β, γ, and δ) were all without effect, even though they inhibited platelet release in response to collagen or TRAP1<sub>1-6</sub>. This finding is consistent with a previous study in which the nonsselective protein kinase inhibitor staurosporine failed to inhibit SIPA (32). This is in contrast to the study by Kroll et al. (8) showing inhibition of the full aggregation response by the staurosporine analogue Ro 31-7549. The effect of this inhibitor on platelet release was not reported in their study. Ro 31-7549, similar to Ro 31-8220 and GF109203X, belongs to a category of nonsselective PKC inhibitors showing similar potency in inhibiting collagen-, thromboxane A<sub>2</sub> analogue-, and thrombin-induced platelet release and aggregation (29, 33, 44). These authors show that SIPA is associated with the phosphorylation of pleckstrin, a PKC substrate that occurs in the absence of diacylglycerol or hydrolysis of phosphatidylinositol 4,5-bisphosphate in a Ca<sup>2+</sup>-dependent manner. It has to be noticed that EGTA, a chelator of extracellular Ca<sup>2+</sup>, had minimal effect on such phosphorylation, excluding a role for transmembrane Ca<sup>2+</sup> influx. They conclude that a diacylglycerol-independent pathway of PKC activation
contributes to SIPA. Thus, although involvement of an unknown PKC isoform in shear-induced platelet granule release cannot be ruled out, our data clearly distinguished this process from the GPVI- and PAR-1-mediated granule release (33). Finally, the study by Nakai et al. (21) describes the shear-induced cytoskeleton translocation of actin, actin-binding protein, and myosin as a rapid and transient movement of MLC kinase wherein the amount of cytoskeletal PKC did not change, unlike what is seen with thrombin. This observation further suggests distinct roles of PKC (if any) and MLC kinase during platelet responses to shear stress. The transient association of MLC kinase with the cytoskeleton may coincide with the quickly reversible MLC phosphorylation produced via P2X activation.

In conclusion, P2X contributes to SIPA through its ability to rapidly elicit Ca2+-influx-triggered signals leading to prompt CaM-dependent signaling, platelet activation, and platelet granule release. Further studies are required to elucidate the mechanisms of VWF/GPIIb-dependent platelet granule release. It also remains to be determined whether the presently identified P2X/CaM/MLC kinase pathway plays a role during platelet activation processes accompanying platelet recruitment and adhesion on immobilized VWF and, most importantly, during thrombosis.

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