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FIGURE CAPTIONS

Fig. 1 ADP (3 μM)-induced platelets (final count; $2.0 \times 10^5/\mu\text{L}$) aggregation in the presence of latex beads samples (a) non-coated latex beads, (b) H12-latex beads, (c) and (d) H12/rGPIIb α -latex beads). The final concentration of beads was $2.0 \times 10^3/\mu\text{L}$. Number of H12 molecules conjugated to the one latex bead was (a) 0, and (b)-(d) 2.0×10^5 . Number of rGPIIb α molecules conjugated to one latex bead was (a) 0, (b) 0, (c) 1.7×10^3 , and (d) 5.0×10^3 . Arrow shows addition of ADP.

Fig. 2 Average surface coverage of (a) latex beads samples or (b) thrombocytopenia-imitation blood (platelet count; $2.0 \times 10^4/\mu\text{L}$) mixed with sample latex beads and passed over the

surface; control latex beads (\bullet ; $1.0 \times 10^5 / \mu\text{L}$), H12-latex beads (\diamond ; $1.0 \times 10^5 / \mu\text{L}$), rGPIb α -latex beads (\triangle ; $1.0 \times 10^5 / \mu\text{L}$), a mixture of H12-latex beads ($5.0 \times 10^4 / \mu\text{L}$) and rGPIb α -latex beads ($5.0 \times 10^4 / \mu\text{L}$) (\circ), and calculation value of $((\diamond+\triangle)/2)$ at various shear rates (\square). The Average surface coverage was calculated after blood was circulated for 180 s.

All measurements were performed in triplicate.

Fig. 3 Average surface coverage of H12-latex beads in thrombocytopenia-imitation blood containing (\circ) H12-latex beads ($5.0 \times 10^4 / \mu\text{L}$) and rGPIb α -latex beads ($5.0 \times 10^4 / \mu\text{L}$), (\triangle) H12-latex beads ($1.0 \times 10^5 / \mu\text{L}$), and (\square) control latex beads ($1.0 \times 10^5 / \mu\text{L}$) at a shear rate of 1600 s^{-1} .

Fig. 4 (a) SEM images of platelet thrombus formed on the collagen surface in the presence of H12-latex beads and rGPIb α -latex beads at a shear rate of 1600 s^{-1} . **(b)** Number of H12-latex beads (opened bar) or rGPIb α -latex beads (filled bar) on the thrombus was estimated using scanning electron microscope after blood was passed over the collagen surface (the number of thrombus: N=120). Error bars represented standard deviation.

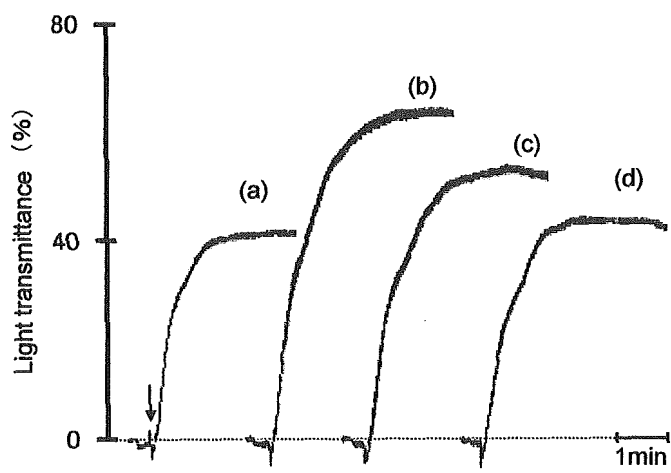


Fig. 1 Okamura et al.

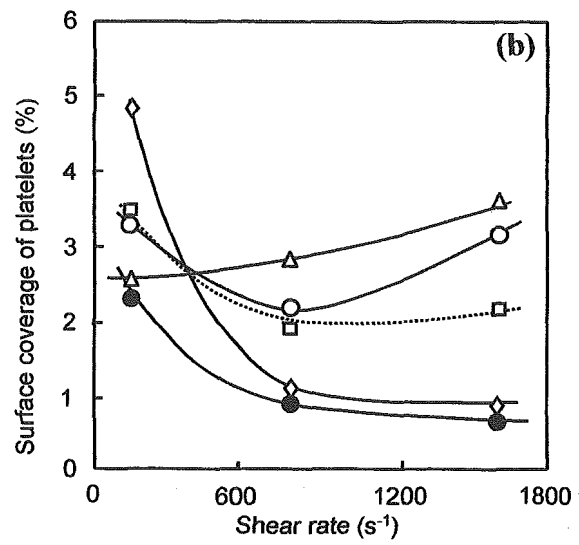
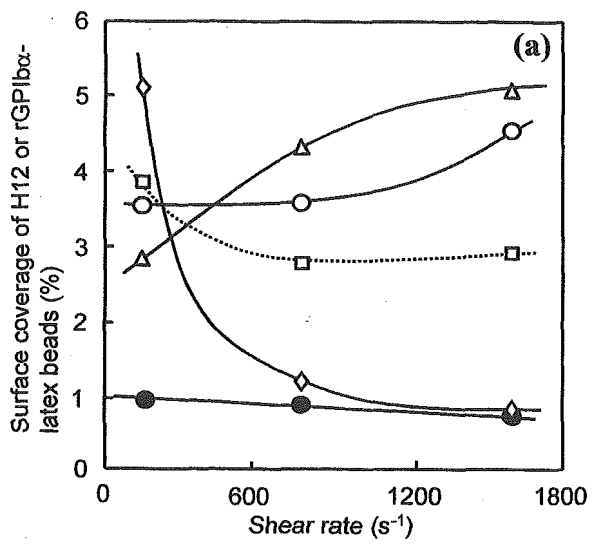


Fig. 2 Okamura et al.

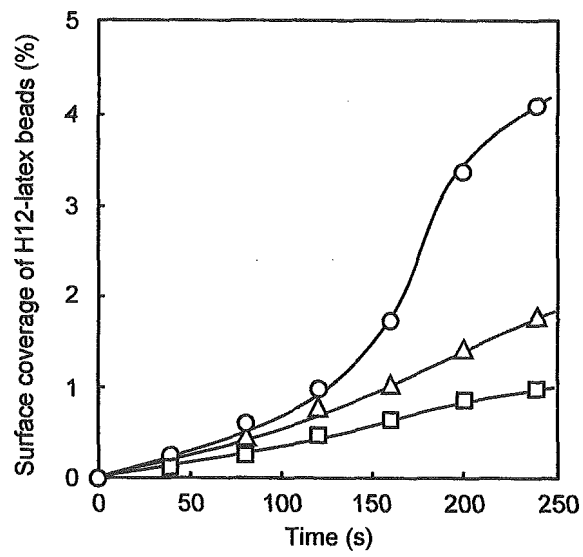


Fig. 3 Okamura et al.

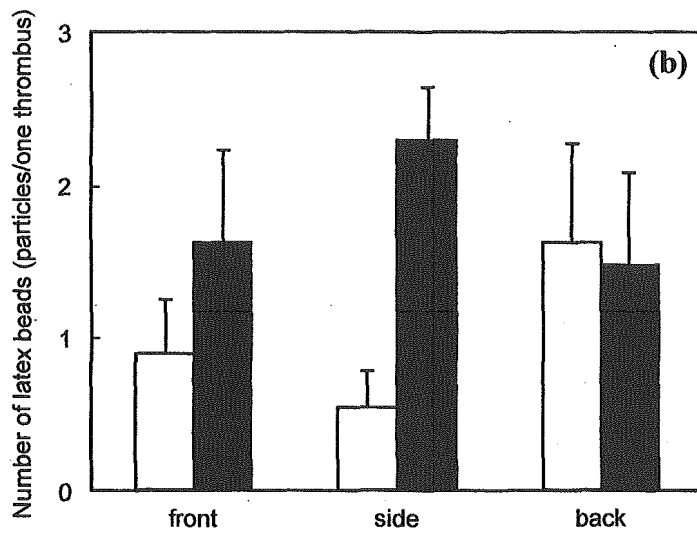
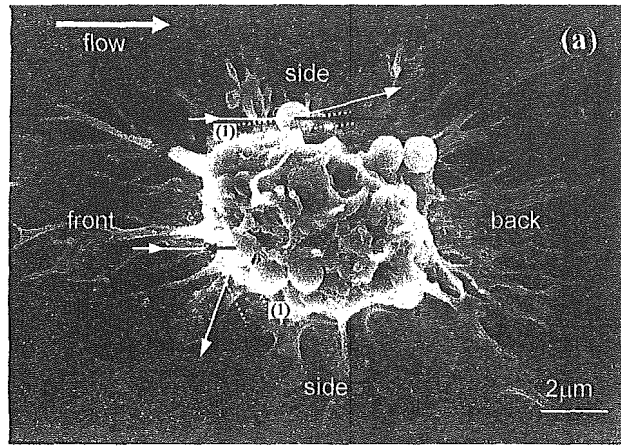


Fig. 4 Okamura et al.

PRECLINICAL STUDY

Dependence of Platelet Thrombus Stability on Sustained Glycoprotein IIb/IIIa Activation Through Adenosine 5'-Diphosphate Receptor Stimulation and Cyclic Calcium Signaling

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Kanagawa, Japan; and La Jolla, California

OBJECTIVES	We sought to evaluate the mechanisms that support the stability of platelet aggregates on a thrombogenic surface exposed to flowing blood.
BACKGROUND	Activation of the membrane glycoprotein (GP) IIb/IIIa—mediated in part through the P2Y ₁ and P2Y ₁₂ adenosine 5'-diphosphate (ADP) receptors—is necessary for platelet aggregation. Platelets in growing thrombi exhibit cyclic calcium signal, suggesting that sustained activation may be required for thrombus stability.
METHODS	Blood was perfused over type I collagen fibrils at the wall shear rate of 1,500 s ⁻¹ . Three-dimensional visualization of platelet thrombi was obtained in real time with confocal microscopy. The intracytoplasmic Ca ²⁺ concentration ([Ca ²⁺] _i) was measured in fluo-3AM-loaded platelets.
RESULTS	The height of platelet thrombi in control blood was 13.5 ± 3.3 μm after 6 min, and increased to 16.3 ± 4.5 μm (n = 8) after an additional 6 min. In contrast, the height was reduced to 5.4 ± 2.2 and 3.3 ± 1.3 μm, respectively (p < 0.01, n = 8), when the blood used in the second 6-min perfusion contained a P2Y ₁ (MRS2179) or P2Y ₁₂ (AR-C69931MX) inhibitor. The [Ca ²⁺] _i of platelets within forming thrombi oscillated between 212 ± 38 nmol/l and 924 ± 458 nmol/l, with cycles lasting 4.2 ± 2.8 s that were inhibited completely by AR-C69931MX and partially by MRS2179. Accordingly, thrombi became unstable upon perfusion of blood containing the Ca ²⁺ channel blocker, lanthanum chloride. Flow cytometric studies demonstrated that AR-C69931MX, MRS2179, and lanthanum chloride reduced monoclonal antibody PAC-1 binding to platelets, indicating a decrease of membrane-expressed activated GP IIb/IIIa.
CONCLUSIONS	Continuous P2Y ₁ and P2Y ₁₂ stimulation resulting in cyclic [Ca ²⁺] _i oscillations is required for maintaining the activation of GP IIb/IIIa needed for thrombus stability in flowing blood. (J Am Coll Cardiol 2006;47:155–62) © 2006 by the American College of Cardiology Foundation

Arterial thrombosis may initiate after the rupture of an unstable atherosclerotic plaque, and it involves multiple platelet adhesion and agonist receptors (1) as well as activation of clotting with fibrin deposition (2,3). Two adenosine 5'-diphosphate (ADP) receptors, P2Y₁ and P2Y₁₂, mediate platelet stimulation induced not only by exogenous ADP (4–6), but also by shear forces or interactions with extracellular matrixes that cause ADP release from storage granules (7–9). In particular, P2Y₁₂ concurs to the stability of platelet aggregates (10) and may exert a

similar effect in developing arterial thrombi (11). In experimental studies, platelets that adhere and aggregate onto immobilized von Willebrand factor (VWF) exhibit cyclic oscillations in intracytoplasmic Ca²⁺ concentration ([Ca²⁺]_i) (12). These have been shown to depend on the concurrent function of P2Y₁₂ and glycoprotein (GP) IIb/IIIa (integrin IIb₃), and are linked to the recruitment and activation of flowing platelets into growing thrombi (12). Such findings, and the results obtained with a thrombosis model in P2Y₁₂-deficient mice (13), suggest that continuing stimulation of ADP receptors may be required to initiate as well as propagate thrombus growth on damaged vascular surfaces. Experiments with selective inhibitors have confirmed the importance of P2Y₁₂ in this regard (12), while P2Y₁ may be key for the initial activation of ADP-stimulated platelets (14) but have no role in thrombus propagation. In the present study, we have used a fibrillar type I collagen surface exposed to flowing blood and specific antagonists of the ADP receptors and GP IIb/IIIa to evaluate how aggregating platelets are incorporated irreversibly into a thrombus. Our findings may contribute to

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Abbreviations and Acronyms

ADP	adenosine diphosphate
[Ca ²⁺] _i	intracytoplasmic Ca ²⁺ concentration
FITC	fluorescein isothiocyanate
GP	glycoprotein
PPP	platelet-poor plasma
PRP	platelet-rich plasma
VWF	von Willebrand factor

clarifying the mechanism of action of antithrombotic agents.

METHODS

Blood samples. Venous blood was obtained from medication-free volunteers (6 men, 2 women; age 28 to 43 years) with their informed consent, and transferred into plastic tubes containing 1/10 volume of the thrombin inhibitor Argatroban (Mitsubishi Kagaku, Tokyo, Japan) to yield a final concentration of 100 mol/l (15,16), which does not decrease the plasma divalent cation concentration. Platelet-rich plasma (PRP) was separated by centrifugation at 100 g for 15 min and platelet-poor plasma (PPP) by further centrifugation at 800 g for 10 min. The platelet count in PRP was adjusted to 3 × 10⁵/l.

Reagents. AR-C69931MX (17) was from AstraZeneca (Loughborough, Leicestershire, United Kingdom). MRS2179 (18) was obtained from Dr. Savi (Sanofi-Synthelabo Recherche, Toulouse, France). Lanthanum chloride (19), acid insoluble fibrillar collagen type I from bovine Achilles tendon,

mepacrine (quinacrine hydrochloride), acetyl salicylic acid, ADP, and epinephrine were from Sigma Chemical Co. (St. Louis, Missouri). Tirofiban (Aggrastate) was from Merck & Co. (Allentown, Pennsylvania). Fluo-3 acetoxymethyl ester (Fluo-3AM) was from Molecular Probe (Eugene, Oregon). **Measurement of thrombus volume.** Platelets were rendered fluorescent by adding 10 mol/l mepacrine (16,20) or 1 g/ml of the fluorescein isothiocyanate (FITC)-labeled Fab fragment of the anti-GP IIb/IIIa monoclonal antibody, YM337 (Yamanouchi Pharmaceutical Co. Ltd., Tokyo, Japan). Thrombi of similar size were obtained in either case (not shown). A rectangular flow chamber with type I collagen fibrils coated on the glass bottom (15,16,20) was assembled onto the stage of an inverted epifluorescence microscope (Leica, Germany). Blood was aspirated through the chamber with a syringe pump (Harvard Apparatus, Holliston, Massachusetts) at a constant flow rate to yield a wall shear rate of 1,500 s⁻¹. Images were digitized on-line with a color CCD video camera (L-600, Leica, Germany). Thrombus growth was evaluated in two dimensions by measuring the surface area covered by platelets (16) and in three dimensions by confocal microscopy (Fig. 1) as previously reported (15). The effect of inhibitors of platelet function on the stability of platelet thrombi was tested in two-stage experiments, consisting in the perfusion of untreated blood for 6 min followed by blood containing or not a test substance for an additional 6 min.

Measurement of [Ca²⁺]_i. Platelets in PRP were incubated for 30 min at 37°C with fluo-3AM (8 mol/l), then mixed with erythrocytes separated from the same blood and

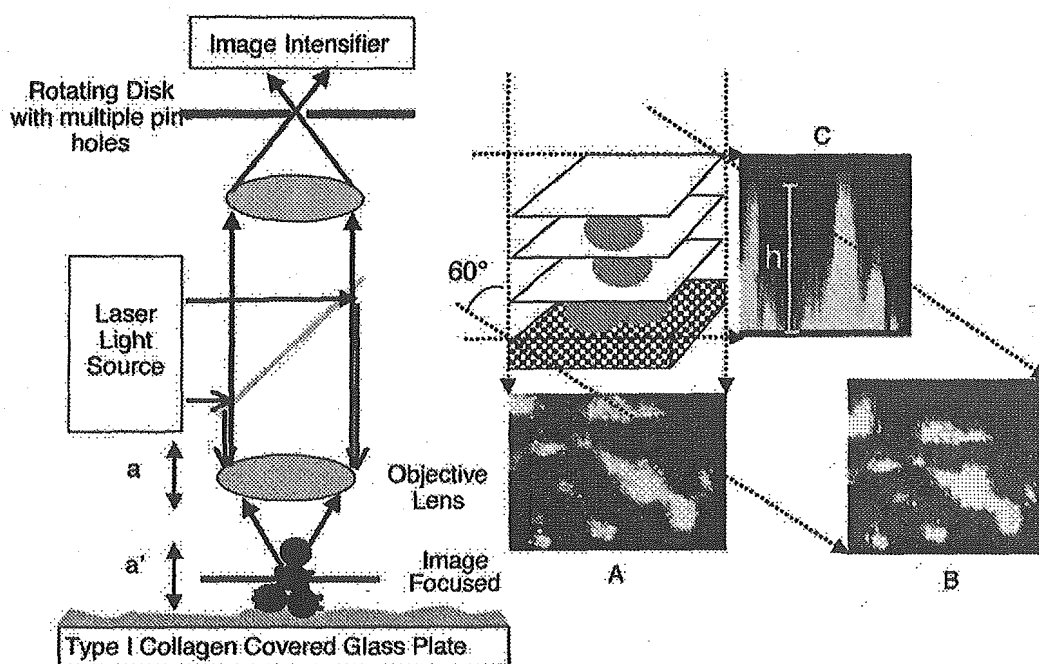


Figure 1. Three-dimensional projection imaging of platelet thrombi. A piezo-electric motor (a) moved the objective lens at a constant speed of 0.4 mol/l/s to provide scanning images of the platelet thrombi (a'). The sum of the confocal images in a bottom to top stack (z-axis) was projected on planes at 10° intervals relative to the x axis to obtain the three-dimensional projection images shown on the right, including projections at 0 degrees (top view; A), 60° (B), and 90° (front view; C). The maximum height (h) of the platelet thrombi was calculated from the front view projection, as shown in C.

washed three times by centrifugation and resuspension in a buffer composed of 10 mmol/l HEPES, 140 mmol/l NaCl, pH 7.4 (HEPES buffer). The washed cells were resuspended in homologous PPP containing Argatroban (100 mol/l) at a 40% hematocrit. Unlike previously suggested (21) but in agreement with recent reports (22), we did not use probenecid to prevent the leakage of fluo-3 because of its effects on platelet function (23). The $[Ca^{2+}]_i$ of 10 randomly selected platelets incorporated at different positions within a thrombus was measured using confocal microscopy. Variations in the fluorescence intensity of fluo-3AM were converted into $[Ca^{2+}]_i$ using the equation:

$$Ca^{2+}_i = Kd \cdot F / (F_{max} - F)$$

where Kd (495 nmol/l) is the dissociation constant of fluo-3AM for the interaction with Ca^{2+} (21); F is the measured fluorescent intensity of single platelets; F_{max} is the fluorescence intensity of single platelets treated with the Ca^{2+} ionophore A23187 (10 mol/l; Sigma) in the presence of 2 mmol/l Ca^{2+} ; and F_{min} is the fluorescent intensity of unstimulated single platelets.

Flow cytometry. The platelet binding of FITC-conjugated PAC-1, a monoclonal antibody that selectively interacts with activated GP IIb/IIIa, was measured by flow cytometry (FACScan, Becton-Dickinson, San Jose, California). Platelets in PRP were activated with the combination of ADP and epinephrine (25 mol/l each) or with the thrombin receptor activation peptide (1 mmol/l). Then, FITC-conjugated PAC-1 was added at a final concentration of 2.77 g/ml, followed by HEPES buffer containing or not AR-C69931MX (100 nmol/l), MRS2179 (100 mol/l), or lanthanum chloride (1 mmol/l). PAC-1 binding was measured 5, 10, 30, 45, and 60 min after addition of the last

solution. All these experiments were performed under static conditions. The median fluorescence of 10,000 single platelets was calculated using the CellQuest software (Becton Dickinson Biosciences).

Statistical analysis. All numerical data are expressed as mean values \pm SD unless otherwise specified. The effect of various concentrations of AR-C69931MX and MRS2179 on the surface coverage by platelets was tested by one-way analysis of variance. Differences between two groups of data were compared by Newman-Keuls test. A p value of ≤ 0.05 was considered to denote statistical significance.

RESULTS

P2Y₁ and P2Y₁₂ antagonists inhibit platelet thrombus growth. In agreement with previous results (7,8), ADP receptor antagonists inhibited thrombus growth on type I collagen fibrils exposed to blood flowing with a wall shear rate of 1,500 s⁻¹. Platelet surface coverage decreased from 38.0 \pm 6.6% to 13.6 \pm 3.9% after blocking P2Y₁₂ with 100 nmol/l AR-C69931MX, and to 19.4 \pm 5.4% after blocking P2Y₁ with 100 mol/l MRS2179 (p ≤ 0.01). Both antagonists also inhibited thrombus volume (Fig. 2). Untreated blood perfused for 6 min formed multilayered thrombi with a height of 13.2 \pm 2.3 μ m (n = 8), which was reduced to a single layer of platelets with a height of 3.2 \pm 1.1 μ m by 100 nmol/l AR-C69931MX (n = 8). With blood containing 100 mol/l MRS2974, the thrombus height was 6.1 \pm 3.5 μ m (n = 8), less than with untreated blood (p ≤ 0.01) but more than with the P2Y₁₂ antagonist (p ≤ 0.01).

Inhibitors of P2Y₁, P2Y₁₂, and Ca²⁺ channels reduce platelet thrombus size. Blood perfused over collagen type I fibrils for 6 min at the wall shear rate of 1,500 s⁻¹ formed

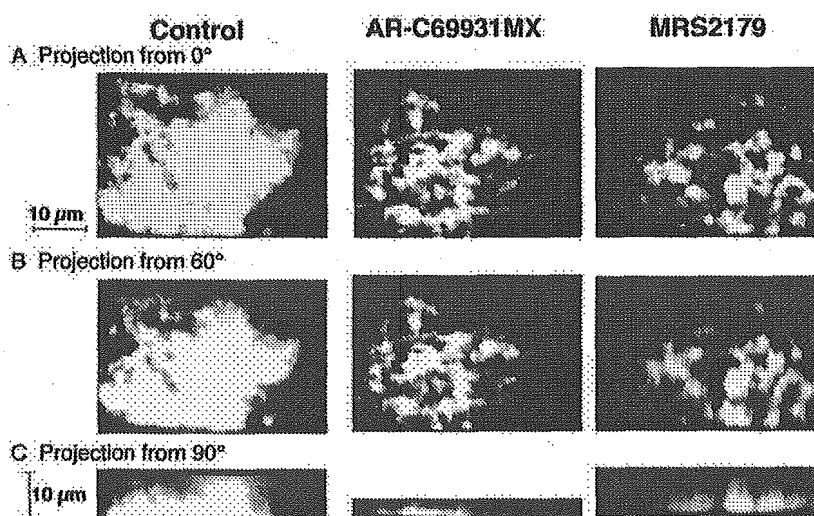


Figure 2. Three-dimensional projection images of thrombi formed in the presence or absence of adenosine diphosphate receptor antagonists. Blood with fluoresceinated platelets was perfused over immobilized collagen type I fibrils for 6 min at the wall shear rate of 1,500 s⁻¹ in the absence (Control) or presence of MRS2179 (100 mol/l) or AR-C69931MX (100 nmol/l), as indicated. The platelet thrombi were scanned in the z-axis by confocal microscopy, and the resulting images were projected on planes rotated around the x-axis at 10° intervals (please see the online version of this article for supplemental videos). The figure shows projection images from the top (A), 60° (B), and the front (C). These images are representative of the results obtained in eight separate experiments.

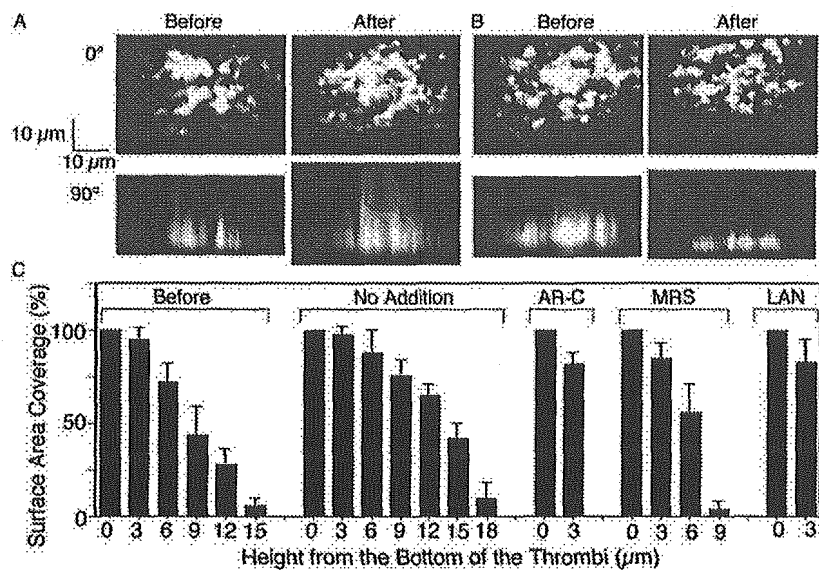


Figure 3. Reduction in the size of platelet thrombi exposed to blood containing different antagonists of platelet function. These experiments were performed as described in the caption of Figure 2, with the difference that the surface was exposed to two subsequent aliquots of blood each perfused for 6 min. (A) Representative images (0-degree and 90° projections) of platelet thrombi after perfusion of the first (before) or second (after) control blood aliquot (please see the online version of this article for supplemental videos). (B) Representative images of platelet thrombi after perfusion of the first control blood aliquot (before) or a second blood aliquot containing the P2Y₁₂ inhibitor AR-C69931MX (100 nmol/l) (please see the online version of this article for supplemental videos). (C) Cross-sectional area occupied by fluorescent platelets in horizontal planes passing through the thrombi at the indicated distance from the collagen surface, calculated as percentage of the area in the plane closest to collagen surface. The bars labeled “Before” show the area of thrombi formed after perfusion of untreated blood for 6 min. The bars labeled “No Addition,” AR-C, MRS, and LAN show the area of thrombi remaining on the surface after an additional 6 min perfusion of untreated blood, or blood treated with the P2Y₁₂ inhibitor AR-C69931MX (100 nmol/l), or the P2Y₁ inhibitor MRS2179 (100 mol/l), or the putative Ca²⁺ channel inhibitor lanthanum chloride (LAN) (1 mmol/l), respectively. Mean and SEM of eight experiments are shown.

platelet thrombi with a height of 13.5 ± 3.3 μ m (n = 8; Fig. 3), increasing to 16.3 ± 4.5 μ m after an additional 6 min. In contrast, when blood perfused in the second 6-min period contained 100 nmol/l AR-C69931MX, thrombus size progressively decreased until a single layer of adherent

platelets with a height of 3.3 ± 1.3 μ m remained. A similar effect, resulting in a 3.4 ± 1.2 μ m high single layer of adherent platelets, was observed with the Ca²⁺ channel blocker, lanthanum chloride (1 mmol/l), whereas 100 mol/l MRS2974 reduced thrombus height to 5.4 ± 2.2

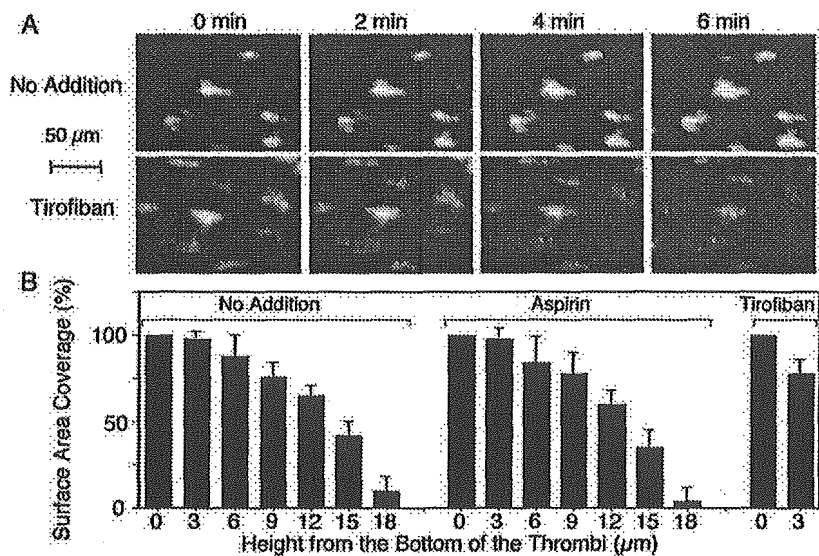


Figure 4. Changes in the two-dimensional and three-dimensional structure of platelet thrombi exposed to blood containing antiplatelet agents. These experiments were performed essentially as described in the caption of Figure 3. (A) Representative two-dimensional fluorescence microscopy images of platelet thrombi immediately after perfusion of the first control blood aliquot (0 min) or at different times after beginning the second perfusion with either untreated blood (No Addition) or blood containing tirofiban (0.5 mol/l), as indicated (please see the online version of this article for supplemental videos). (B) Cross-sectional area of thrombi at the indicated distances from the collagen surface after perfusion of untreated blood for 6 min (No Addition), or after perfusion for an additional 6 min of blood containing aspirin (100 mol/l) or tirofiban (0.5 mol/l), as indicated. See Figure 3C for additional details.

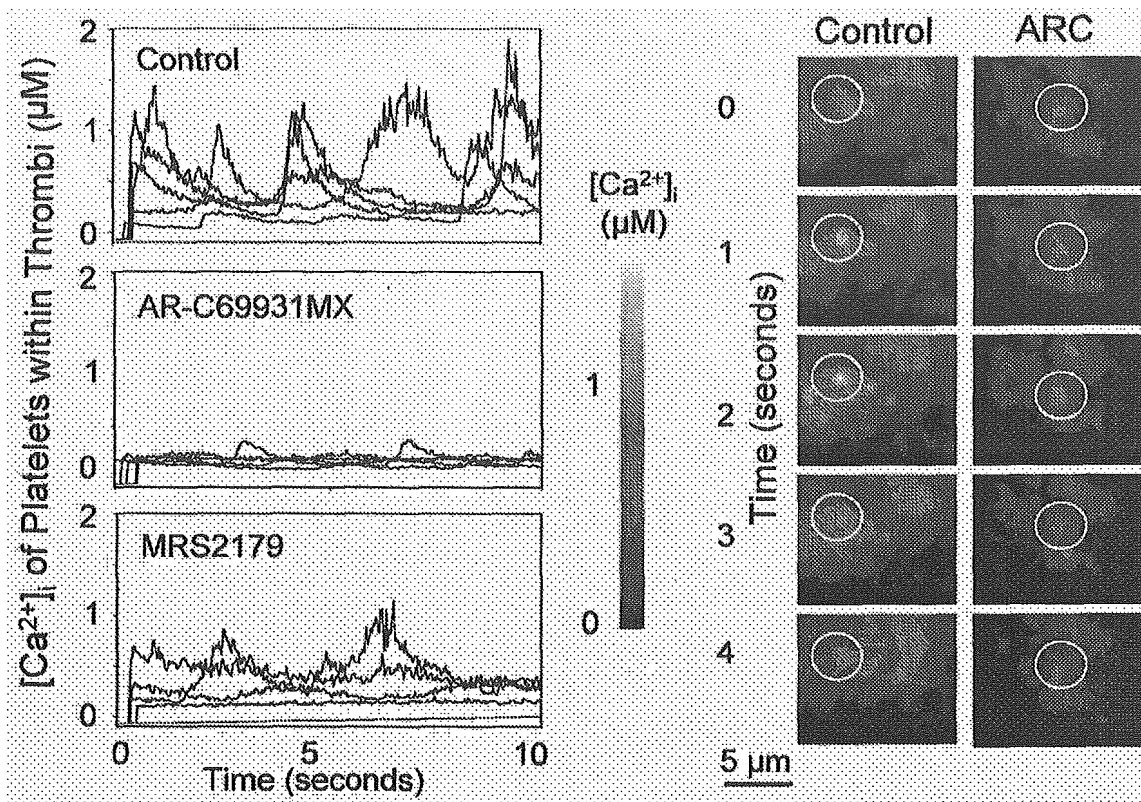


Figure 5. Changes in the intracytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) of platelet thrombi caused by adenosine diphosphate receptor antagonists. These experiments were conducted as described in the caption of Figure 3, except that blood was replaced with a cell suspension containing fluo-3AM-loaded platelets, washed erythrocytes, and homologous platelet-poor plasma with the specific thrombin inhibitor argatroban (100 $\mu\text{mol/l}$) as the anticoagulant. The cell suspension was perfused over type I collagen fibrils at the shear rate of $1,500\text{ s}^{-1}$ for 4 min to form platelet thrombi. Then, the same cell suspension without or with the addition of the $P2Y_{12}$ antagonist AR-C69931MX (100 nmol/l), or the $P2Y_1$ antagonist MRS2179 (100 $\mu\text{mol/l}$), was perfused for an additional 4 min. The $[Ca^{2+}]_i$ of platelets incorporated into thrombi was measured during the second perfusion. (Left panels) Intracytoplasmic Ca^{2+} concentration of five randomly selected platelets recorded for 10 s beginning 2 min after the start of the second perfusion. (Right panels) Images reflecting the concentration of Ca^{2+} ions in platelets within thrombi formed during perfusion of untreated blood (Control) or blood containing 100 nmol/l AR-C69931MX (ARC).

m. The latter value was less than with untreated blood but more than with the anti- $P2Y_{12}$ antagonist (n 8; p 0.01 for both comparisons; Fig. 3). Addition of 0.5 $\mu\text{mol/l}$ tirofiban, a GP IIb/IIIa antagonist (15), to the blood used in the second perfusion also reduced thrombi to a single layer of adherent platelets with a height of $3.1 \pm 1.1\ \mu\text{m}$ (n 8; Fig. 4), whereas 100 $\mu\text{mol/l}$ aspirin allowed continued growth to a height of $15.8 \pm 5.5\ \mu\text{m}$ (n 8; Fig. 4).

Platelets within thrombi exhibited cyclic $[Ca^{2+}]_i$ oscillations (Fig. 5). The lowest average $[Ca^{2+}]_i$ was 212 ± 38 (SEM) nmol/l and the highest $924 \pm 458\ \text{nmol/l}$, with a cycle length from peak to peak of $4.2 \pm 2.8\ \text{s}$ (Fig. 5). These $[Ca^{2+}]_i$ variations appeared to depend on specific ion channels because they were blocked by the Ca^{2+} channel antagonist lanthanum chloride (not shown). Addition of the $P2Y_{12}$ antagonist to the perfused blood rapidly decreased the platelet $[Ca^{2+}]_i$ within formed thrombi; after 2 min the value ranged between $182 \pm 22\ \text{nmol/l}$ and $244 \pm 96\ \text{nmol/l}$ without detectable cycles in most platelets (Fig. 5). After addition of the $P2Y_1$ antagonist, the low and high $[Ca^{2+}]_i$ values ranged between $192 \pm 34\ \text{nmol/l}$ and $558 \pm 211\ \text{nmol/l}$, respectively, and

some but not all platelets showed measurable cycles of $5.8 \pm 2.5\ \text{s}$ (Fig. 5). Neither tirofiban, in spite of the pronounced effect on platelet thrombus size, nor aspirin had any influence on the cyclic $[Ca^{2+}]_i$ of aggregated platelets (not shown).

Inhibition of $P2Y_1$ and $P2Y_{12}$, and blockade of Ca^{2+} entry reduce activated GP IIb/IIIa on platelets. Fluorescein isothiocyanate-conjugated PAC-1 binding to platelets, measured as the median fluorescence intensity of 10,000 platelets, increased when platelets were activated by the combination of ADP and epinephrine or the thrombin receptor activating peptide (the results were similar and only the former are shown). Bound PAC-1 slowly but significantly decreased in time when no additional exogenous agonist was added after the initial activation (Fig. 6). The reduction was more marked after adding the $P2Y_{12}$ inhibitor (Fig. 6), suggesting that continuous stimulation is necessary to maintain the active state of GP IIb/IIIa. A similar effect was observed with the $P2Y_1$ inhibitor or the Ca^{2+} channel blocker (Fig. 7), but not with aspirin (data not shown).

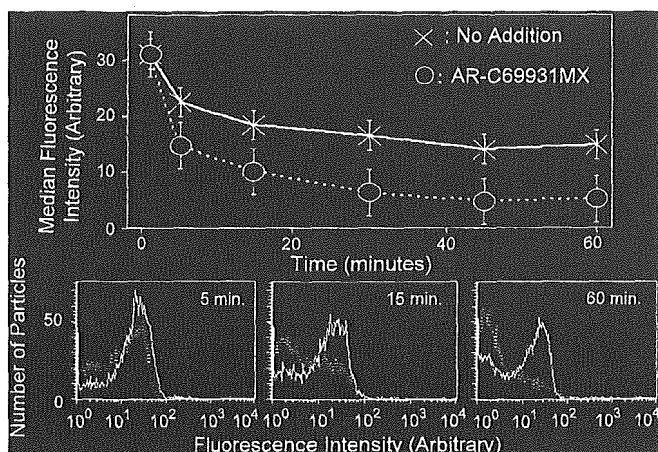


Figure 6. Changes in PAC-1 binding to activated platelets caused by adenosine diphosphate (ADP) receptor antagonists. To activate platelets, 25 μ l of HEPES buffer containing ADP and epinephrine (400 μ mol/l each) was mixed with 375 μ l of platelet-rich plasma and incubated for 20 min. Fifty μ l of fluorescein isothiocyanate-conjugated PAC-1 (25 μ g/ml) was then added, and the fluorescence of individual platelets was measured 5, 15, 30, 45, and 60 min after the addition of 50 μ l of HEPES buffer containing or not the P2Y₁₂ antagonist AR-C69931MX (100 nmol/l). (Upper panel) Mean and SEM of the median fluorescence of 10,000 platelets in eight experiments. (Lower panels) Representative flow cytometric results at selected time points in one experiment without (solid lines) or with (dotted lines) the addition of AR-C69931MX.

DISCUSSION

Our findings show that platelet thrombi growing on collagen may disperse several minutes after the initial aggregation when exposed to blood containing an ADP receptor antagonist or a putative Ca²⁺ channel blocker or a GP IIb/IIIa inhibitor. Thrombus stability, therefore, may depend on sustained ligand binding to activated GP IIb/IIIa, which in turn may be mediated by [Ca²⁺]_i elevations induced by the continuous stimulation of specific signaling pathways (Fig. 8). Our findings add to the concept of intercellular calcium communication, suggesting that it operates bidirectionally from platelets at the growing edge of a thrombus not only to activate newly recruited platelets (12) but also to ensure stability throughout the entire aggregate. Such a conclusion is based on the observation that perfusion of blood containing ADP receptor antagonists reduced the cyclic [Ca²⁺]_i increases within platelet thrombi even several minutes after the initial adhesive contacts had been established. Our experiments, however, could not define whether platelets at the center of thrombi differed in their Ca²⁺ responses from those at the outer edges. It has been proposed that cooperation between P2Y₁₂-mediated activation and GP IIb/IIIa engagement by ligand promotes the activation of newly recruited platelets in a thrombus (12). We observed, however, that blocking GP IIb/IIIa on the perfused platelets had no effect on the [Ca²⁺]_i oscillations within the thrombus even though it caused progressive disaggregation. Thus, the effect of GP IIb/IIIa inhibition on Ca²⁺ signals may reflect the need of maintaining newly recruited platelets in contact with adherent ones until activation is induced. In the case of platelets

already aggregated into a thrombus, the replacement of an adhesive ligand by a GP IIb/IIIa antagonist may result in the detachment of platelets at the edge without effects on the activation of those still in the thrombus.

We propose that cyclic [Ca²⁺]_i increases within platelet thrombi reflect a mechanism that maintains GP IIb/IIIa activation and, thus, thrombus stability. The experiments with lanthanum, a putative Ca²⁺ channel blocker, support such a concept, but in this study we could not identify the ion channel involved or the signaling pathway regulating its function. Lanthanum, therefore, could have other effects, such as altering the ion binding sites of adhesive receptors and/or ligands (24) resulting in thrombus dissolution independently of Ca²⁺ channel blocking. Moreover, the cyclic [Ca²⁺]_i increases of aggregated platelets within thrombi could be a consequence, not the cause of, maintaining GP IIb/IIIa activation. In the alternative, the observation that [Ca²⁺]_i oscillations are linked to ADP receptor function may suggest an involvement of store-dependent Ca²⁺ entry (25–27) mediated by P2Y₁₂ stimulation. From a technical viewpoint, changes in platelet volume could have affected the results obtained with fluo-3AM, a single-wave Ca²⁺ indicator, thus influencing our conclusions. It should be noted that we have attempted to minimize such possible effects by using ultra-fast confocal microscopy, and no cyclic platelet volume changes have been reported during thrombus growth. In the end, sustained platelet activation necessary for thrombus stability may involve various signaling molecules, including ephrin/eph kinases (28), regulated by

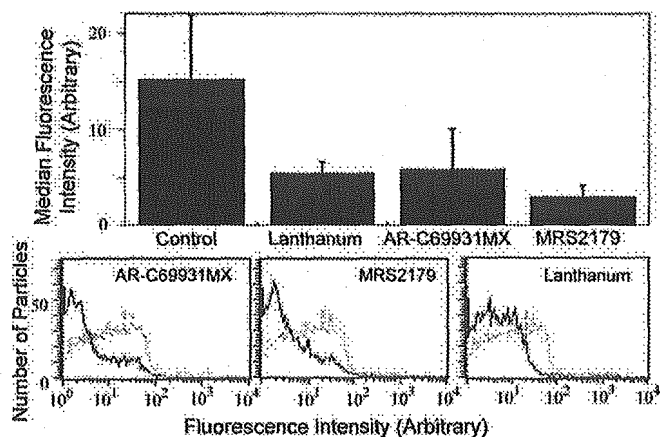


Figure 7. Effect of different platelet inhibitors on PAC-1 binding to activated platelets. These experiments were performed as described in the caption for Figure 6, with the only difference that the P2Y₁ inhibitor, MRS2179, or the putative Ca²⁺ channel blocker, lanthanum chloride, was also added to platelets after activation by adenosine diphosphate and epinephrine. The upper panel shows the mean and SEM of the median fluorescence of 10,000 platelets measured 30 min after addition of AR-C69931MX (final concentration: 100 nmol/l), MRS2179 (final concentration: 100 μ mol/l), or lanthanum chloride (final concentration: 1 mmol/l) as compared to control in which only buffer was added (n = 8). The lower panels show the actual flow cytometric results of one representative experiment. Solid lines represent the results in the presence of the inhibitor shown in each panel, while dotted lines represent the results in the absence of inhibition.

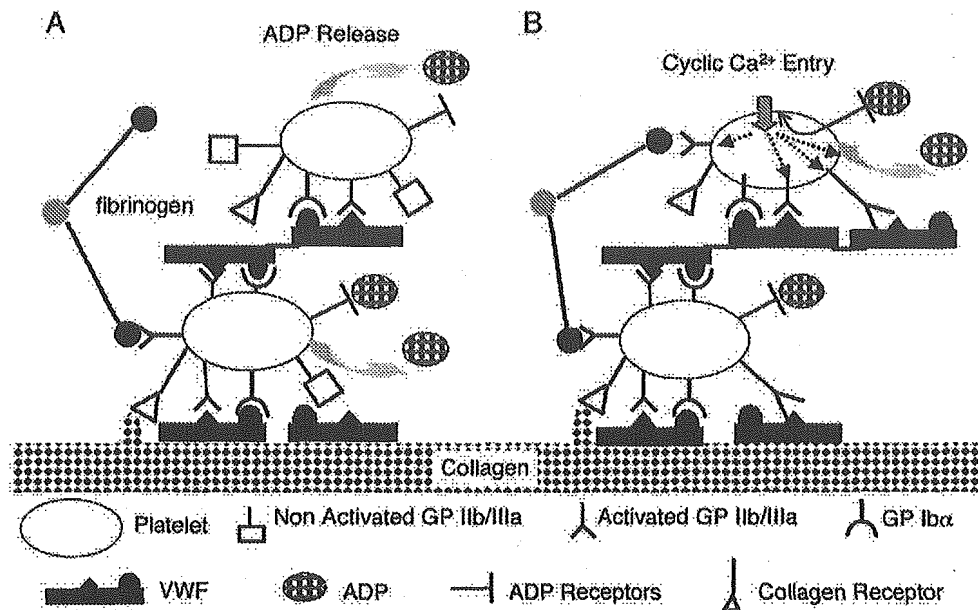


Figure 8. Schematic representation of the mechanism that stabilizes platelets at the edge of a growing thrombus exposed to elevated shear rates. (A) Circulating platelets adhere and become activated onto collagen through multiple adhesive interactions, initiated by glycoprotein (GP) Ib binding to von Willebrand factor (VWF) under high shear rate conditions. Full activation depends on released adenosine diphosphate (ADP) and leads to the binding of soluble adhesive ligands such as VWF and fibrinogen. These form the new substrate for the recruitment of circulating platelets, again initiated under high shear rate conditions by GP Ib-VWF binding. Adhesive interactions and soluble agonists present in the environment of the growing thrombus lead to activation of the newly recruited platelets and further ADP release. (B) Cyclic Ca²⁺ signaling induced by released ADP and mediated by P2Y₁ and P2Y₁₂ maintains GP IIb/IIIa activation necessary for the sustained binding of adhesive molecules and stability of the aggregate. The first layer of platelets interacting with the collagen surface may not require sustained ADP stimulation for stable adhesion.

platelet-platelet contacts at the upper edge of the growing thrombus.

Although there is agreement that P2Y₁₂ is involved in the calcium signaling that sustains activation, conclusions on the role of P2Y₁ are not univocal, perhaps because previous studies were focused on the activation of newly recruited platelets (12) while we analyzed the activation of platelets already incorporated within thrombi. The two processes may be distinct, or the discrepant results may be caused by methodological differences. For example, continuous rather than periodic monitoring of [Ca²⁺]_i cycles may better reveal the relatively weak effect of blocking P2Y₁. Moreover, others have reported that only the combined blockade of P2Y₁ and P2Y₁₂ can inhibit platelet thrombus formation on the surface of collagen (7), whereas we have shown previously (8) and confirm here that inhibition of either receptor is almost equally effective in doing so. This discrepancy may result from differences in experimental observation times, because we measured thrombus volume after several minutes when the stability may be more influenced by interplatelet communication and less by the effects of the platelet-collagen interactions at the base of the thrombus.

Perhaps the main limitation of an *ex vivo* blood perfusion model is the need to use an anticoagulant to preserve blood fluidity, which in turn prevents the generation of thrombin and, consequently, of fibrin, both likely to have a role in providing thrombus stability (3,29). Nonetheless, ADP-induced signaling pathways may contribute to the overall effects of other agonists, including thrombin, that lead to

nucleotide release from storage granules after primary platelet stimulation. Moreover, the mechanism highlighted here may provide initial stability to aggregated platelets exposed to flowing blood and, through the procoagulant function of activated platelets (30), contribute to fibrin formation. Our results, therefore, provide new insights into the mechanism of action of antiplatelet agents used in clinical practice and currently targeted against the P2Y₁₂ receptor. In situations such as acute coronary syndromes or coronary interventions with elevated thrombotic risk, P2Y₁₂ inhibitors may be used in combination with anticoagulants such as heparin, suggesting that an experimental model in which thrombin activity and fibrin formation are inhibited may be representative of at least some clinical conditions.

Conclusions. Our studies, although directly relevant only for thrombus formation on collagen fibrils under artificial blood flow conditions, suggest a possible role for distinct ADP receptors and their operating calcium signals in sustaining the long-term GP IIb/IIIa activation required to maintain platelet aggregate stability before the occurrence of fibrin generation. Such a mechanism may provide a more comprehensive understanding of the pharmacological effects of anti-P2Y₁₂ drugs, and suggest novel strategies to achieve the dispersion of platelet thrombi after they are formed.

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APPENDIX

For the supplemental videos, please see the online version of this article.