Shear-dependent suppression of platelet thrombus formation by phosphodiesterase 3 inhibition requires low levels of concomitant Gs-coupled receptor stimulation

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Summary
Phosphodiesterase (PDE)3 inhibitors exert potent antiplatelet effects through maintaining elevated intracellular cyclic adenosine monophosphate levels, but do not prolong bleeding time. To resolve this discrepancy, we hypothesised that PDE3 inhibitors effectively suppress shear-induced platelet thrombus formation initiated by the interaction of the platelet receptor GPIb/V/IX with its ligand, von Willebrand factor (VWF), since arterial thrombosis is more dependent on shear stress as compared with haemostatic plug formation. To test this hypothesis, we compared the in vitro effects of K-134 (a PDE3 inhibitor), tirofiban (a GPIb/IIa inhibitor) and acetylsalicylic acid (ASA) on ristocetin-induced platelet aggregation and platelet thrombus formation on VWF or collagen surfaces under flow conditions. K-134 inhibited GPIb/IIa-dependent platelet aggregation to the same extent as tirofiban and more potently than ASA. Likewise, K-134 and tirofiban effectively inhibited stable platelet thrombus formation (platelet firm adhesion and subsequent aggregation) on the VWF or collagen surface under high shear, but ASA only inhibited aggregation. Notably, inhibition by K-134 became evident only when a low concentration of PGE1 was present. These inhibitors did not block shear-induced initial platelet contact with VWF via GPIb/V/IX. In contrast, under low shear, the inhibitory effects of K-134 on platelet aggregation on the collagen surface were lower than tirofiban or ASA. The observed shear-dependent suppression of platelet thrombus formation by PDE3 inhibitor in the presence of low levels of adenylate cyclase stimulator may contribute to high therapeutic benefit with low risk of bleeding.

Keywords
Phosphodiesterase 3 inhibitor, GPIb/V/IX, VWF, shear-induced platelet thrombus formation, PGE1

Introduction
In platelets, cyclic adenosine monophosphate (cAMP) is a versatile negative regulator of key signalling pathways including Ca2+ mobilisation and integrin αIIbβ3 (glycoprotein (GP)IIb/IIIa) activation, virtually through serine/threonine phosphorylation by the cAMP-dependent protein kinase (PK)A. The cAMP is synthesised from adenosine triphosphate (ATP) by adenylate cyclase (AC), activated by Gs-coupled receptor stimulation with endogenous agonists such as prostaglandin (PG)I2 (also known as prostacyclin) or adenosine, and is degraded to 5’-AMP by cyclic guanosine monophosphate (cGMP)-inhibited cAMP phosphodiesterase (PDE)3. As a result, cAMP concentrations in platelets are regulated by the activity balance between AC and PDE3 (1). In fact, a prominent functional synergy exists in vitro and ex vivo between AC stimulation (e.g. by the PGI2 analogue PGE1) and PDE3 inhibition (e.g. by the PDE3 inhibitor cilostazol) to suppress platelet activation (1).

Cilostazol is the only PDE3 inhibitor to date approved for clinical use to manage intermittent claudication in patients with peripheral arterial disease (PAD) (2), and has been shown to inhibit agonist-induced human platelet aggregation ex vivo as effectively as the cyclooxygenase inhibitor acetylsalicylic acid (ASA) and the P2Y12 inhibitor clopidogrel (3). Although use of ASA or clopidogrel is complicated by an increased risk of bleeding, cilostazol does not prolong human bleeding time (3, 4), and the risk of haemorrhage associated with treatment is quite low (5, 6). To clarify this discrepancy, we hypothesised that PDE3 inhibitors suppress platelet thrombus formation in a shear-dependent manner, since pathological thrombus at injured arterioles or stenosed arteries is more dependent on high shear stress than physiological haemostatic plug formation.

Thrombus formation on von Willebrand factor (VWF) and collagen surfaces under high shear requires association of platelet GPIb/V/IX with the A1 domain of VWF (7, 8), and GPIb/V/IX engagement itself activates GPIb/IIa independently of other receptors (9). Conversely, under low shear, direct platelet binding to collagen via GPVI, and platelet-to-platelet crosslinking via GPIb/IIa and fibrinogen are functionally significant in thrombus formation on collagen (10, 11), whereas GPIb/V/IX is not necessarily required (8).
Therefore, to test the aforementioned hypothesis, we explored the effect of PDE3 inhibition both on platelet aggregation induced by ristocetin, a non-physiological inducer of VWF binding to GPIb and on platelet thrombus formation on a VWF or collagen surface under flow conditions at wall shear rates of 150 s⁻¹ (low shear) or 1,500 s⁻¹ (high shear) by utilising parallel plate flow chambers in vitro. Cilostazol is not appropriate for in vitro experiments to deduce its clinical effect because its active metabolites also play major roles in its pharmacological effects on human (12). Cilostazol neither increases cAMP levels of human platelets nor inhibits shear stress-induced platelet aggregation at therapeutic concentration in the absence of Gs stimulator in vitro (13). Moreover, the specificity of cilostazol is less selective for PDE3 isozyme (14). Hence, to specifically evaluate the effect of PDE3 inhibition on platelet function in vitro, we used a more potent and selective PDE3 inhibitor, K-134 (6-(3-(3-cyclopropyl-3-(2-hydroxycyclohexyl)ureido)propoxy)-2(1H)-quinolinone, also known as OPC-33509), which is a cilostazol analogue but is not a pro-drug (14, 15). While IC₅₀ of cilostazol towards PDE2, PDE3A and PDE5 are 45.2, 0.20 and 4.4 μM, respectively, those of K-134 are >300, 0.10 and 12.1 μM, respectively (14). Here, we demonstrated that the K-134 effectively suppressed in vitro platelet thrombus formation under flow conditions in a more shear-dependent manner than ASA or the GPIIb/IIIa inhibitor tirofiban, and the effect of K-134 was apparent only in the presence of a low concentration of the AC stimulator PGE1. We propose that our data indicate a mechanism whereby PDE3 inhibition exhibits efficient antiplatelet effects on arterial thrombosis with a minimal impact on primary haemostasis.

Materials and methods

Antiplatelet reagents

The PDE3 inhibitors K-134 and cilostazol were obtained from Kowa (Tokyo, Japan). The GPIIb/IIIa inhibitor tirofiban was purchased from Toronto Research Chemicals (Toronto, Canada). The cyclooxygenase inhibitor ASA, Arg-Gly-Asp-Ser (RGDS) peptides (inhibitors of integrins with RGD binding sites), and forskolin (an AC activator) were from Sigma-Aldrich (St Louis, MO, USA). These reagents were dissolved in dimethyl sulfoxide (DMSO).

PGE1 was purchased from Cayman Chemical (Ann Arbor, MI, USA) and dissolved in ethanol (EtOH). An inhibitory mouse monoclonal antibody against human GPIbα, GUR83–35, was obtained from Takara Bio (Shiga, Japan), and the isotype-matched control mouse IgG1 was purchased from SIGMA-Aldrich. The selection of K-134 concentrations is based on the phase-I study and non-clinical study, in which K-134 treatment with a maximal serum concentration of 1–5 μM showed antiplatelet effects on human ex vivo and beneficial effects in a rat model of thrombosis or ischaemia (manuscript in preparation).

Blood sampling

After obtaining informed consent according to the Declaration of Helsinki, blood was collected from the antecubital vein of healthy, medication-free volunteers through a 21-gauge needle and was anticoagulated with D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone dihydrochloride (PPACK; Calbiochem, San Diego, CA, USA) (final concentration (fc), 40 μM) or a 10% volume of 3.8% (w/v) sodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation (100 g, 15 minutes [min], 22°C) of blood, and the platelet count was adjusted to 2.0 ×10⁹/μl with platelet-poor plasma prepared by centrifugation (2,200 g, 10 min, 22°C) for agonist-induced platelet aggregation assay. The platelet concentration was determined using an automated haematology analyzer (K-4500; Sysmex, Kobe, Japan).

Agonist-induced platelet aggregation

PPACK-anticoagulated PRP was incubated with antiplatelet agents with or without 6 nM PGE1 at 37°C for 2 min and stimulated with adenosine diphosphate (ADP) (fc, 10 μM; MC Medical, Tokyo, Japan) or collagen (fc, 1.75–2.75 μg/ml; Chronolog, Havertown, PA, USA). ADP- or collagen-induced platelet aggregation was quantified by measuring maximum aggregation rate (MAR; percent of maximal light transmittance) within 5 min after addition of trigger using an aggregometer (Hema Tracer T-638; Nico Bioscience, Tokyo, Japan) (n=3). All volunteers’ PRP samples showed similar dose-response curves for ADP (EC₅₀: about 5 μM, MAR induced by 10 μM ADP: 55–65%). The concentration of collagen was adjusted to give the EC₅₀. Ristocetin-induced platelet aggregation (RIPA) was performed by adding ristocetin solution (fc, 1.5 mg/ml; Sigma-Aldrich) to citrated PRP after incubation with antiplatelet agents at 37°C for 5 min. The effects of antiplatelet agents on RIPA were evaluated by aggregation rates at 10 min after addition of trigger. All volunteers’ PRP samples (n=4) showed similar dose-response curves for ristocetin (EC₅₀: about 1.2 mg/ml, aggregation rate at 10 min: > 90%).

Preparation of thrombogenic substrate-coated surfaces

Human VWF (10 μg/ml; purified from plasma as previously described [16]) and type I collagen derived from porcine tendon (30 μg/ml; Cellmatrix Type I-A, Nitta Gelatin, Osaka, Japan) were prepared in Dulbecco’s phosphate-buffered saline (PBS). Glass coverslips (No. 5, diameter 24 mm, thickness 0.5 mm; Matsunami Glass, Osaka, Japan) were immersed in VWF (22°C, 2.5 h) or collagen (4°C, 12 h) solution, carefully rinsed with PBS, and then blocked with bovine serum albumin (20 mg/ml; Sigma-Aldrich) in PBS (22°C, ≥ 2 h). After additional rinsing with PBS, coverslips were assembled into a parallel plate flow chamber just before perfusion experiments.

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**Perfusion experiments**

PPACK-anticoagulated whole blood (5 ml) was incubated with a fluorescent marker (DiOC₆; Molecular Probes, Eugene, OR, USA) and antiplatelet agents at 37°C for 5 min, then aspirated using a roller pump (Minipulse 3; Gilson, Villiers Le Bel, France) and perfused over thrombogenic substrate-coated glass coverslips at a wall shear rate of 150 or 1,500 s⁻¹ in a recirculating chamber (circulation cycle, 180 s or 18 s, respectively) mounted on an inverted epifluorescence microscope (Eclipse TE300; Nikon, Tokyo, Japan) equipped with a charge-coupled device camera (C2400–80V; Hamamatsu Photonics, Hamamatsu, Japan). These experiments were performed at 37°C and recorded on S-VHS videotape using a video cassette recorder (BR-S662; Victor, Tokyo, Japan), and digital images were captured using an Argus-50 image processor (Hamamatsu Photonics) with fixed contrast and brightness. The percentage of total area covered with platelets (designated as surface coverage) and the mean size of surface-bound platelet aggregates (designated as average platelet aggregate size) were calculated using the Argus-50 software. Surface coverage reflects platelet adhesion to the thrombogenic substrate-coated surface (2-dimensional thrombus growth), while average platelet aggregate size represents the degree of platelet aggregation (3-dimensional thrombus growth) on the surface (17, 18). We chose threshold values (grayness level from 0–255, where 0 is black) of 50 for surface coverage and 200 for average platelet aggregate size, to eliminate background fluorescence and fluorescence of single platelets that do not aggregate, respectively.

**Measurement of cAMP and cGMP**

Citrated PRP was pre-incubated with K-134 with or without 6 nM PGE1 at 37°C for 5 min, and stimulated with ristocetin (fc, 1.5 mg/ml) for 10 min under stirring. Platelet intracellular cAMP and cGMP levels were analysed using an Amersham cAMP/cGMP Biotrak Enzymeimmunoassay system (GE Healthcare, Buckinghamshire, UK) and were presented as a concentration of cAMP or cGMP pmol/10⁸ platelets.

**Statistical analysis**

Statistical analyses were performed using SAS Preclinical Package version 5.0 software (SAS Institute Japan, Tokyo, Japan).

**Results**

**Effects of PDE3 inhibitors on RIPA**

First, we compared the inhibitory effects of the PDE3 inhibitors cilostazol and K-134 with other antiplatelet agents on RIPA in vitro.
In effect, 3 μM K-134 and 30 μM cilostazol significantly (p<0.001) inhibited RIPA (evaluated at 10 min after addition of trigger) to the same extent as 0.1 μM tirofiban and 1 μM PGE1, and more potently than 1,000 μM ASA (Fig. 1A, B). To explore the effects of PDE3 inhibitor on GPIIb/IIIa-independent platelet agglutination mediated through direct interaction between VWF and GPIb/VIIX, PRP was incubated with an integrin-antagonist peptide, RGDS, before ristocetin stimulation. Under such conditions, K-134 caused no change in agglutination extent and the same was true with PGE1 (not shown) and forskolin, which also increases...

Table 1: Effects of PDE3 inhibitors on ristocetin-, ADP- or collagen-induced platelet aggregation. Calculated half-maximal inhibitory concentration (IC50) values are expressed as the mean from 3–4 human volunteers. Values in parentheses indicate 95% confidence intervals.

<table>
<thead>
<tr>
<th>IC50 (μM)</th>
<th>Ristocetin</th>
<th>Ristocetin (+PGE1)</th>
<th>ADP</th>
<th>ADP (+PGE1)</th>
<th>Collagen</th>
<th>Collagen (+PGE1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-134</td>
<td>2.2 (2.0–2.4)</td>
<td>0.77 (0.47–1.1)</td>
<td>6.1</td>
<td>0.95 (0.76–1.2)</td>
<td>0.74 (0.55–1.0)</td>
<td>0.24 (0.18–0.33)</td>
</tr>
<tr>
<td>Cilostazol</td>
<td>22 (18–25)</td>
<td>8.3 (4.8–12)</td>
<td>32  (24–42)</td>
<td>7.3 (6.5–8.2)</td>
<td>6.4 (5.5–7.5)</td>
<td>1.6 (1.4–1.8)</td>
</tr>
<tr>
<td>Tirofiban</td>
<td>0.045 (0.011–0.072)</td>
<td>0.061 (0.049–0.072)</td>
<td>0.028 (0.018–0.042)</td>
<td>0.027 (0.021–0.034)</td>
<td>0.022 (0.019–0.026)</td>
<td>0.016 (0.012–0.022)</td>
</tr>
<tr>
<td>ASA</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>59 (39–91)</td>
<td>55 (40–73)</td>
</tr>
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Figure 2: Effects on thrombus formation on a VWF surface under flow. PACK-anticoagulated blood was incubated with DIOC6 for platelet visualisation and DMSO (control) or each antplatelet agent, and perfused over VWF-immobilised glass coverslips in a parallel plate flow chamber at a wall shear rate of 150 s⁻¹ (A) or 1,500 s⁻¹ (A–D) in the absence (A, B) or presence (C, D) of 6 nM PGE1. To confirm the GPIbα-dependency of platelet binding to VWF, 15 μg/ml GUR83–35 was used as a GPIbα blocking antibody. The effect of each inhibitor on platelet initial accumulation via GPIbα and on subsequent platelet aggregation was evaluated by measuring surface coverage at 0–120 s after initiation of perfusion. A, C) Representative images, corresponding to a 220 × 220 μm area at 20 or 120 s after initiation of perfusion. ***p<0.001 vs. control (Tukey’s test was performed at 120 s). Values are mean ± SD (n=6 for control and n=3 for others).
tracellular cAMP levels via direct AC activation (Fig. 1C). It was found that GUR83–35, a monoclonal antibody against GPIIbα, completely suppressed the agglutination phenomenon (not shown). These results indicate that an intracellular CAMP level heightened by PDE3 inhibition or AC activation is a crucial downregulator for platelet aggregation via VWF-GPIb/V/IX-mediated signals, but not for VWF-GPIb/V/IX interaction itself that is independent of GPIIb/IIIa.

Next, we examined the synergistic effects of concomitant stimulation of AC on the observed PDE3 inhibition of RIPA. Inducible Gs-coupled receptor stimulation by vascular wall-derived PGII or adenosine is thought to be pathologically relevant in the circulation to defend against thrombosis (19, 20). Therefore, to mimic the in vivo situation in vitro, we conducted aggregation study in the presence of a very low concentration (6 nM) of PGE1. Our preliminary experiments demonstrated that 6 nM PGE1 was close to the optimal concentration that could elevate CAMP levels in the presence of PDE3 inhibitor, but did not affect agonist-induced platelet aggregation in the absence of PDE3 inhibitor (not shown). Indeed, the inhibitory effects of PDE3 inhibitors on not only ADP- or collagen- but also ristocetin-induced platelet aggregation were readily augmented in the presence of 6 nM PGE1 (Table 1). In contrast, the effects of tirofiban and ASA were unaffected by PGE1. Taken together, these results indicate that similar to other signals stimulated by ADP and collagen under stirring conditions, the GPIIb/V/IX signal elicited by VWF binding is a CAMP-sensitive pathway.

**PDE3 inhibition prevents stable platelet thrombus formation on a VWF surface under flow**

To further clarify the effect of PDE3 inhibition on VWF-GPIb/V/IX-mediated platelet activation under physiological conditions, PPACK-anticoagulated blood was perfused over VWF-coated glass coverslips. Under arterial flow conditions at a high wall shear rate of 1,500 s⁻¹, platelets exhibited rapid and progressive attachment onto the VWF surface with a peak surface coverage of ~70% at 20 s after perfusion (Fig. 2A, B). Platelets then started to detach over time (possibly due to the unstable platelet adherence induced by recirculating small platelet aggregates), and stable thrombus formation (surface coverage ~30%) was finally constructed by 120 s. In contrast, at a venous wall shear rate of 150 s⁻¹, platelet thrombus tended to grow slowly on the VWF surface with surface coverage of about 10% even at 120 s (Fig. 2A, B), and was not completely abolished by the previous addition of GUR83–35 (not shown). The effect of K-134 on platelet thrombus formation was studied under arterial flow conditions (1,500 s⁻¹). Unexpectedly, K-134 at a therapeutic concentration of 3 μM did not affect the whole profile of thrombus formation, while tirofiban showed an apparent inhibitory effect on the second phase of thrombus formation (at 20–120 s). Although tirofiban did not affect the first phase of platelet contact at 0–20 s, GUR83–35 completely blocked this platelet interaction (Fig. 2A, B) but the isotype-matched control mouse IgG1 did not show any effect (not shown), indicating that the first phase of the interaction involves GPIIb/IIIa-independent initial contact of platelets onto VWF via GPIb/V/IX (Fig. 2A, B). Prominently enough, although 6 nM PGE1 itself had no inhibitory effect on platelet thrombus formation at such low concentration, K-134 showed a potent inhibitory effect on the second phase of platelet interaction in the presence of 6 nM PGE1 (Fig. 2C, D). The extent of inhibition by K-134 resembled that by tirofiban and no synergic effect from both inhibitors was observed. In contrast, ASA showed no inhibitory effect under these experimental conditions. These results of perfusion experiments thus indicate that under physiological flow conditions, antplatelet effects by PDE3 inhibition are mediated by elevated CAMP to efficiently block the initial signalling of the VWF-GPIb/V/IX pathway and subsequent autocrine activation signals, and the inhibitory effect is only significant in the presence of concomitant low level stimulation of AC.

**PDE3 inhibition suppresses platelet thrombus formation on a collagen surface in a shear-dependent manner**

To evaluate the effect of PDE3 inhibition on platelet thrombus formation mediated simultaneously by both collagen and VWF, we performed perfusion experiments over glass coverslips coated with type I collagen fibrils, to which plasma-derived VWF was expected to adsorb via the A3 domain. Indeed, we observed that platelet thrombus formation on the collagen surface under high shear (1,500 s⁻¹) was completely blocked by anti-GPIbα antibody...
Figure 4: Effects on thrombus formation on a collagen surface under high shear. Blood treated as described in the legend for Figure 2 was perfused over collagen-immobilised glass coverslips at a wall shear rate of 1,500 s\(^{-1}\). A) Representative images corresponding to a 340 x 340 μm area, captured at 270 s after initiation of perfusion. Effects on platelet adhesion and aggregation were evaluated by measuring surface coverage (B) and average platelet aggregate size (C), respectively. **p<0.01, ***p<0.001 vs. control, †p<0.05, ‡p<0.001 vs. ASA with PGE1 group (Tukey’s test). Values are mean ± SD (n=8 for control, n=4 for others).

GUR83–35, while the antibody was without effect on thrombus formation under low shear (150 s\(^{-1}\)) (Fig. 3). Consistent with previous findings (8, 11), these results indicate that platelet adhesion and subsequent aggregation onto the collagen surface

Figure 5: Effects on thrombus formation on a collagen surface under low shear. Blood treated as described in the legend for Figure 2 was perfused over collagen-immobilised glass coverslips at a wall shear rate of 150 s\(^{-1}\). A) Figure shows representative images, corresponding to a 340 x 340 μm area, captured at 270 s after initiation of perfusion. Effects on platelet adhesion and aggregation were evaluated by measuring surface coverage (B) and average platelet aggregate size (C), respectively. **p<0.01, ***p<0.001 vs. control, †p<0.05 vs. 3 μM K-134 with PGE1 group (Tukey’s test). Values are mean ± SD (n=4).
under flow conditions is mediated through VWF-GPIb/V/IX engagement in a shear rate-dependent manner. We then tested the effects of antiplatelet agents on platelet thrombus formation on the collagen surface under flow conditions. Under high shear, K-134 at least at a therapeutic concentration (\(\sim 3 \mu M\)) was found to be inhibitory only when a low concentration of PGE1 (6 nM) was present (\(*/L50480\) Fig. 4A). In fact, K-134 readily suppressed irreversible platelet adhesion (surface coverage at 270 s) and stable thrombus formation by platelet aggregation (average platelet aggregate size at 270 s) on the collagen surface in a dose-dependent manner in the presence of PGE1 (\(*/L50480\) Fig. 4B, C). Conversely, tirofiban showed potent inhibitory effects on both parameters regardless of the presence or absence of PGE1, whereas ASA was without effects on surface coverage, but effectively (with a weaker effect than K-134 or tirofiban) inhibited platelet aggregate size. In contrast, under low shear were significantly weaker than those of 0.1 \(\mu M\) tirofiban or 100 \(\mu M\) ASA (\(p<0.05\)).

Taken together, these results indicate that shear-dependent platelet thrombus formation \textit{in vitro} initiated by GPIb/V/IX signalling is sensitive to cAMP-mediated regulatory pathways elicited by PDE3 inhibition in the presence of a low concentration of AC stimulator PGE1.

**PDE3 inhibition increases platelet intracellular cAMP levels but not cGMP**

To ascertain if the inhibitory effect of K-134 are mediated through cAMP or an alternative mechanism, we measured the intracellular cAMP and cGMP levels of platelets treated with K-134. Indeed, K-134 dose-dependently increased platelet cAMP levels but not cGMP, and the effect was potentiated by a low concentration of PGE1 (\(*/L50480\) Fig. 6A, B). Furthermore, the cAMP-elevating effect of K-134 was also maintained in platelets activated by ristocetin under stirring conditions, while K-134 had no effect on cGMP levels (\(*/L50480\) Fig. 6C, D).
Discussion

The GPIb/V/IX engagement with VWF immobilised on the subendothelial collagen surface under arterial flow conditions not only mediates platelet recruitment, but also initiates cellular activation leading to integrin-dependent firm platelet adhesion and aggregation at the site of vascular injury (8, 11). In fact, our findings (Figs. 1, 2) indicated that the observed suppression of VWF-mediated platelet thrombus formation by PDE3 inhibitors was due to efficient blocking of VWF-induced GPIb/V/IX signalling by elevated cAMP, but was not attributable to the down-regulation of GPIb/V/IX binding affinity with the ligand led by PKA-induced phosphorylation of the GPIbα subunit intracellular domain, which is contrary to previously reported study (21). Consistent with our finding, Mazzucato et al. (18, 22) reported that the magnitude of platelet translocation velocity on immobilised VWF in the presence of cAMP elevating reagents appeared to be the same as that obtained by GPIb/IIa block in similar perfusion experiments, while raising cAMP levels achieved complete inhibition of intracellular Ca^{2+} elevation and GPIb/IIa-mediated stable adhesion and aggregation.

PDE3 inhibitor reduced stable platelet thrombus formation on the collagen surface under high shear in the presence of PGE1 more potently than ASA, but had much lower inhibitory effects under low shear than tirofiban or ASA (Figs. 4, 5). There is a possible simple explanation as to why PDE3 inhibitor interfered with thrombus formation on the collagen surface in a more shear-dependent manner than tirofiban and ASA; while the secretion-independent signal mediated by VWF bound onto collagen is very sensitive to cAMP, that by collagen through GP VI is resistant to cAMP. In fact, PGE1, but not ADP receptor antagonists and ASA, could obliterate VWF-GPIb/V/IX-mediated initial intracytoplasmic Ca^{2+} oscillation (18). Moreover, cAMP analog strongly blocked GPIb/IIa activation induced by the dimeric VWF A1 domain (through GPIb/V/IX) in the presence of inhibitors of autocrine signalling through ADP and thromboxane A2 (TXA2) receptors (9). In addition, Shaun P. Jackson's group reported that platelet adhesion to VWF under high shear (at 600 and 1,800 s^{-1}) was not dependent on either ADP or TXA2 (23). A primary collagen receptor of platelets is GPVI, which activates c-Src and Syk kinases leading to full cellular activation. In fact, tyrosine phosphorylation of these kinases induced by an anti-GPVI antibody or collagen was not abrogated with PGI2 treatment (24). Also, marked elevation of cAMP by forskolin reportedly did not inhibit collagen-induced secretion-independent signalling events including protein-tyrosine phosphorylation, polyphosphoinositide liberation and granular secretion (25). In contrast, GP IV-mediated platelet aggregation (which is secretion-dependent) was completely inhibited by cAMP-elevating agents (24). Thus our findings were consistent with the fact that stable platelet thrombus formation on collagen under high shear is dependent on GPIb/V/IX signalling triggered by VWF and that under low shear that is mediated by GP VI signalling triggered by collagen itself (8, 10, 23).

Gs stimulation with PGE1 or adenosine and PDE3 inhibition synergistically accumulate cAMP to suppress platelet activation (1, 20). In fact, our results indicated that the observed antiplatelet effects of K-134 are most likely to be due to cAMP rather than to cGMP elevation (Fig. 6). Although K-134 alone potently inhibited VWF-dependent platelet aggregation induced by ristocetin (Fig. 1A, B), the inhibitory effect was readily enhanced by a low level of Gs stimulation with 6 nM PGE1 (Table 1). Notably enough, however, an absolute requirement of PGE1 was observed with K-134 at the therapeutic concentrations needed to inhibit VWF-dependent platelet thrombus formation under flow conditions (Figs. 2, 4). This discrepancy may be due to difference in threshold level of cAMP required to block intracellular signalling; one is ADP- and TXA2-dependent GPIb/IIa activation pathway downstream of VWF-GPIb/V/IX interaction induced by ristocetin (aggregometer studies) and the other is secretion-independent integrin activation pathway induced by shear (flow chamber studies) ([23, 26] and Figs. 1, 2, 6). Thus, our in vitro findings obtained under physiological flow conditions raised the notion that VWF-induced platelet thrombus formation may be efficiently blocked by PDE3 inhibitor under in vivo conditions where the endogenous Gs stimulators (PGI2 and adenosine) are up-regulated. As a matter of fact, considerable amounts of PGI2 were reportedly produced locally in response to mural platelet thrombus formation on de-endothelialised arterial wall (19). In addition, adenosine was released in hypoxic tissues during ischaemia and exerted ischaemic preconditioning effects (27, 28). Since both of these substances have very short biological lives in vitro, the stable PGI2 analogue, PGE1 may instead mimic the in vivo supporting effects on PDE3 inhibitor-driven suppression of VWF-dependent platelet thrombus formation under arterial shear conditions. Meanwhile, we speculated that there is no feasibility of clinical use of combined PDE3 inhibitor and Gs stimulator, since long-term administration of Gs stimulator leads to decreased sensitivity of human platelets to the drug (29).
Unlike tirotrobin and ASA, PDE3 inhibitor does not prolong human bleeding time, even under repeated dosing, but exhibits significant inhibitory effects on agonist-induced platelet aggregation ex vivo (3, 4). Considering the observed high shear stress and Gs stimulation-dependent antiplatelet effects of PDE3 inhibitor in vitro (Figs. 4, 5), it may exert in vivo antiplatelet activity in a shear-dependent manner when concomitant stimulation of Gs-coupled receptors is induced at the site of arterial thrombosis and ischaemia. In contrast, tirotrobin and ASA inhibit platelet aggregation regardless of shear and Gs stimulation, and thus may affect physiological haemostatic plug formation. To further understand the mechanism of their low risk of bleeding, we need to explore how the local concentrations of endogenous Gs stimulators are regulated at sites of vascular perturbation.

Care should be taken when the results of our in vitro perfusion experiments at a high shear rate of 1,500 s⁻¹ are interpreted into the pathological conditions in vivo, since the regions of arterial stenosis are exposed to much higher shear rates ranging from 1,000 up to 10,000 s⁻¹ (11). Nonetheless, considering the rebound phenomenon of Gs-stimulator (29), PDE3 may be a better drug-target to elevate platelet cAMP levels for the treatment of PAD (2) or secondary Prevention of cerebral infarction (6) that needs long-term drug administration.

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Conflict of Interest

The study was supported in part by a research grant from Kowa Company, Ltd. (Tokyo, Japan). H.Y. was an employee of Kowa Company, Ltd., Japan.

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