GPIb-dependent platelet activation is dependent on Src kinases but not MAP kinase or cGMP-dependent kinase

Stuart J. Marshall, Yotis A. Senis, Jocelyn M. Auger, Robert Feil, Franz Hofmann, Gary Salmon, J. Thomas Peterson, Frank Burslem, and Steve P. Watson

Glycoprotein Ib-IX-V (GPIb-IX-V) mediates platelet tethering to von Willebrand factor (VWF), recruiting platelets into the thrombus, and activates integrin αIIbβ3 through a pathway that is dependent on Src kinases. In addition, recent reports indicate that activation of αIIbβ3 by VWF is dependent on protein kinase G (PKG) and mitogen-activated protein (MAP) kinases. The present study compares the importance of these signaling pathways in the activation of αIIbβ3 by GPIb-IX-V. In contrast to a recent report, VWF did not promote an increase in cyclic guanosine monophosphate (cGMP), while agents that elevate cGMP, such as the nitrous oxide (NO) donor glyco–SNAP-1 (N-(β-D-glucopyranosyl)-N^2-acetyl-S-nitroso-D,L-penicillaminamide) or the type 5 phosphodiesterase inhibitor, sildenafil, inhibited rather than promoted activation of αIIbβ3 by GPIb-IX-V and blocked aggregate formation on collagen at an intermediate rate of shear (800 s^{-1}). Additionally, sildenafil increased blood flow in a rabbit model of thrombus formation in vivo. A novel inhibitor of the MAP kinase pathway, which is active in plasma, PD184161, had no effect on aggregate formation on collagen under flow conditions, whereas a novel inhibitor of Src kinases, which is also active in plasma, PD173952, blocked this response. These results demonstrate a critical role for Src kinases but not MAP kinases in VWF-dependent platelet activation and demonstrate an inhibitory role for cGMP-elevating agents in regulating this process. (Blood. 2004;103:2601-2609)

Introduction

Glycoprotein Ib-IX-V (GPIb-IX-V), the receptor for von Willebrand factor (VWF), plays a critical role in thrombus formation in damaged blood vessels under high shear.[1-4] A fast on-rate of association between VWF and GPIb-IX-V allows the adhesion protein to tether (or capture) rapidly flowing platelets into the developing thrombus. A fast off-rate of dissociation, however, means that platelets rapidly detach from VWF, unless integrins such as αIIbβ3 or α2β1 are activated by intracellular signals, thereby enabling them to bind their ligands and mediate stable adhesion and thrombus growth. Several surface receptors are able to mediate integrin activation, including the collagen receptor GPVI, the G protein–coupled receptors for adenosine diphosphate (ADP) and thromboxanes, $P2Y_1$, $P2Y_{12}$, and thromboxane prostanoid (TP) receptors.[5] Significantly, these receptors act in synergy to mediate integrin activation and thrombus growth.[6-9]

It is recognized that GPIb-IX-V is also able to stimulate activation of αIIbβ3. However, the GPIb-IX-V complex generates a much weaker signal than many of the other agonists involved in thrombus formation, including collagen and ADP, thereby questioning the significance of this event. Indeed, the extent of activation is heavily dependent on experimental conditions, with activation being more readily seen in plasma than in washed platelets for reasons that remain unclear.[10-15] It is also difficult to ascertain the significance of activation of αIIbβ3 by GPIb-IX-V because of its role in platelet tethering. This is further hampered by the fact that VWF is a ligand for integrin αIIbβ3, which generates a more powerful intracellular signal,[11,16-19] and because the interaction between VWF and GPIb-IX-V takes place only in suspension under physiologic conditions at high shear. The latter can be overcome using modulators, such as the antibiotic ristocetin or the snake venom toxin, botrocetin, which cause a conformational change in VWF that enables it to bind to GPIbα, although this is unlikely to be a true representation of events at high shear or following adhesion to VWF.

There is increasing evidence for a role of Src kinases in signaling by GPIb-IX-V. Thus, activation of αIIbβ3, formation of filopodia, and elevation of intracellular Ca^{2+} by GPIb-IX-V are blocked by the Src family kinase inhibitors PP1 and PP2.[10,18,20] More recently, Manguin et al have shown a critical role for phospholipase C γ2 (PLCγ2) in signaling by GPIb-IX-V downstream of Src kinases, consistent with earlier reports of tyrosine phosphorylation of the phospholipase by GPIb-IX-V.[10,20,21] These observations extend earlier studies that demonstrated tyrosine phosphorylation of spleen tyrosine kinase (Syk) and the adapter

From the Department of Pharmacology, University of Oxford, Oxford United Kingdom; Division of Medical Sciences, The Medical School, University of Birmingham, Edgbaston, Birmingham, United Kingdom; Institut für Pharmakologie und Toxikologie, Technische Universität (TU) München, München, Germany; Pfizer Global Research and Development, Sandwich, Kent, United Kingdom; and Cardiovascular Pharmacology, Pfizer Global Research and Development, Ann Arbor, MI.

Submitted October 7, 2003; accepted November 19, 2003. Prepublished online as *Blood* First Edition Paper, December 18, 2003; DOI 10.1182/blood-2003-09-3319.

Supported by the Biotechnology and Biological Services Research Council (BBSRC), the British Heart Foundation (BHF), and Pfizer Global Research and Development. S.P.W. holds a BHF Professorship. S.J.M. held a BBSRC "CASE" Award. J.M.A. holds a BHF Studentship.

An Inside *Blood* analysis of this article appears in the front of this issue.

Reprints: Yotis Senis, Division of Medical Sciences, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom; e-mail: y.senis@bham.ac.uk.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

SLP-76 (Src homology 2 [SH2] domain-containing leukocyte protein of 76 kDa) in signaling by GPIb-IX-V.[22,23] Thus, it is becoming increasingly clear that the signaling cascade used by GPIb-IX-V has many similarities to those of the collagen receptor GPVI and the integrin αIIbβ3, namely activation of PLCγ2 downstream of Src kinases via a pathway that involves the tyrosine kinase Syk and SLP-76.

Recently, Du and colleagues[24,25] have proposed a distinct mechanism of αIIbβ3 activation by GPIb-IX-V that is mediated through activation of cyclic guanosine monophosphate (cGMP)–dependent protein kinase, protein kinase G (PKG), and the p42/44 mitogen-activated protein (MAP) kinase pathway. Drawing on studies on cell lines, pharmacologic inhibitors, and PKG "knock-out" mice, Li et al[24] demonstrated that activation of αIIbβ3 by GPIb-IX-V and submaximal concentrations of thrombin, which also binds to the glycoprotein receptor, is strictly dependent on activation of PKG. Further, PKG was shown to lie upstream of p42/44 MAP kinases, with the latter pathway being essential for activation of αIIbβ3 by VWF-ristocetin and thrombin.[25] This conclusion was based on the observation that 2 structurally distinct inhibitors of the MAP kinase pathway, PD98059 and U0126, inhibit activation of αIIbβ3 by VWF-ristocetin and thrombin.

The present study was undertaken to compare the relative contributions of Src kinase and PKG/MAP kinase pathways in the activation of αIIbβ3 by GPIb-IX-V and thrombin. The results confirm a critical role for Src kinases in these pathways, but fail to confirm a role for MAP kinase, whereas PKG is inhibitory.

Materials and methods

Materials

Ristocetin and thrombin were from Sigma Chemical (Poole, United Kingdom). VWF (Haemate P) was from Behringwerke (Marburg, Germany). Lotrafiban was a gift from GlaxoSmithKline (Piscataway, NJ). PD173952, PD184161, and sildenafil were gifts from Pfizer Global Research and Development (Ann Arbor, MI; Sandwich, United Kingdom). Glyco–SNAP-1 (N-(β-D-glucopyranosyl)-N^2-acetyl-S-nitroso-D,L-penicillaminamide), KT5823, Rp-8-Br-PET-cGMPS (guanosine 3′,5′-cyclic monophosphorothioate, 8-(4-chlorophenylthio), Rp isomer), and Rp-8-pCPT-cGMPS (guanosine 3′,5′-cyclic monophosphorothioate, β-phenyl-1, N^2-etheno-8-bromo, Rp isomer) were from Calbiochem (Nottingham, United Kingdom). PP2 was purchased from Calbiochem-Novabiochem (Nottingham, United Kingdom). [^{32}P]Orthophosphoric acid was from Perkin Elmer (Boston, MA). The Ser^{239} vasodilator-stimulated phosphoprotein (VASP) phosphospecific antibody (Ab) 16C2 was from Upstate (Milton Keynes, United Kingdom). The phosphospecific p42/44 MAP kinase Ab and MAP kinase Ab were from Santa Cruz Biotechnology (Santa Cruz, CA). The cGMP direct Biotrak kit was from Amersham Biosciences (Bucks, United Kingdom). Other reagents were from previously described sources.[10,27]

Preparation of human platelet-rich plasma and washed platelets

Platelet-rich plasma (PRP) was prepared from whole blood taken from drug-free donors into sodium citrate 3.8% (wt/vol). The ratio of anticoagulant to whole blood was 1:9. PRP was obtained by centrifugation at 200*g* for 20 minutes. To prepare washed platelets, whole blood was anticoagulated with acid-citrate-dextrose (ACD; 80 mM citric acid, 120 mM sodium citrate, 110 mM glucose). The ratio of anticoagulant to whole blood was 1:7. PRP was obtained by centrifugation at 200*g* for 20 minutes. Platelets were separated from PRP by centrifugation at 1000*g* for 10 minutes in the presence of prostacyclin (1 μM). Platelets were then washed twice in CGS (0.0129 M trisodium citrate, 0.03 M D-glucose, and 0.12 M NaCl; pH 6.5) containing 0.1% bovine serum albumin (BSA) in the presence of prostacyclin (1 μM). The platelet pellet was resuspended to the appropriate concentration in Ca^{2+}-free modified Tyrodes-HEPES buffer (134 mM NaCl, 2.9 mM KCl, 12 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 5 mM glucose, and 1 mM $MgCl_2$, pH 7.3). Optical aggregation studies were carried out using a Chronolog dual channel light aggregometer (Chronolog, Havertown, PA). Platelets (2×10^8/mL) were added to an aggregometer tube at 37°C and stirred at 1200 rpm.

Measurement of cGMP

PRP or washed platelets (3×10^8 platelets/mL) were stirred at 37°C followed by addition of ristocetin/VWF, the NO donors glyco–SNAP-1, and sodium nitroprusside or the phosphodiesterase (PDE) type 5 inhibitor sildenafil. Reactions were stopped by the addition of an equal volume of ice-cold lysis buffer 12% (wt/vol) trichloroacetic acid. Lysates were analyzed for cGMP content using a cGMP direct Biotrak immunoassay kit (Amersham Biosciences) and are presented as the concentration of cGMP nmol/10^7 platelets.

Measurement of [^{32}P]phosphatidic acid

PRP was centrifuged to produce a platelet pellet as described in "Preparation of human platelet-rich plasma and washed platelets" and resuspended in 1 mL of a modified phosphate-free Tyrodes buffer (134 mM NaCl, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, and 1 mM $MgCl_2$; pH 6.5). The platelet suspension was labeled with [^{32}P]orthophosphoric acid (0.5 mCi/mL [18.5 MBq/mL]) for 1 hour at 37°C. Remaining [^{32}P]orthophosphoric acid was removed by washing platelets in 3 mL ACD and 24 mL modified Tyrodes-HEPES buffer followed by centrifugation at 1000*g* for 10 minutes. The pellet was then resuspended at 5×10^8 platelets/mL in platelet-poor plasma. Stimulations were terminated by the addition of 400 μL $CHCl_3$/methanol, 1:1 (vol/vol) and phospholipids were extracted from the sample by centrifugation at 1000*g* for 5 minutes at 4°C in the presence of 200 μL HCl/EDTA (ethylenediaminetetraacetic acid) (42% [vol/vol] 10 N HCl, 58 mM EDTA). Samples were separated by thin layer chromatography. Lipids were detected by autoradiography and analyzed by scintillation counting.

Preparation of mouse platelets

The generation of PKG null (−/−) mice by homologous recombination has been previously described.[26] Male PKG null mice and litter-match controls (wild type; 4-5 weeks old) were anesthetized with ether, and whole blood was isolated by cardiac puncture into 1:10 volume ACD. PRP was prepared from whole blood by centrifugation at 200*g* for 7 minutes. Platelets were washed twice using a modified Tyrode-HEPES buffer and resuspended at 2×10^8 platelets/mL, as described for human platelets. For aggregation experiments platelets were pooled from 16 wild-type and 16 PKG null mice.

Flow studies

Human blood or mouse blood was isolated in sodium heparin (10 IU/mL) as previously described.[27] Blood was perfused through glass microslides, 1×0.1-mm or 2×0.2-mm inner diameter (Camlab, Cambridge, United Kingdom), that had been coated with either 30 or 300 μg/mL type I collagen from equine tendon (Horm; Nycomed, Munich, Germany) before blocking with 2% BSA suspended in phosphate-buffered saline (PBS). A shear rate of 800 s^{-1} with a corresponding flow rate of 0.08 mL/minute in 1×0.1-mm microslides and 0.64 mL/minute in 2×0.2-mm microslides was generated by a syringe pump (Harvard Apparatus, Southnatick, MA). After 2 minutes perfusion with whole blood, Ca^{2+}-free modified Tyrode-HEPES buffer was perfused for 8 minutes through 1×0.1-mm microslides and 3 minutes through 2×0.2-mm microslides at the same shear rate. Platelet thrombi that had formed on the surface of the collagen were visualized with an inverted stage videomicroscope system (DM IRB; Leica, Milton Keynes, United Kingdom). Percent surface coverage was quantified using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). Subsequently, adherent platelets were lysed in ice-cold nonidet P-40 (NP-40) lysis buffer (20 mM Tris [tris(hydroxymethyl)aminomethane], 300

mM NaCl, 2 mM EGTA [ethyleneglycotetraacetic acid], 2 mM EDTA, 2% [vol/vol] NP-40, 1 mM phenylmethylsulphonyl fluoride, 2 mM Na_3VO_4, 10 μg/mL aprotinin, 1 μg/mL pepstatin A, pH 7.3). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels and transferred to polyvinylidene difluoride (PVDF) membranes using a semidry transfer system (Trans-blot SD; BioRad, Hercules, CA). Membranes were blocked by incubation in 10% wt/vol BSA in Tris-buffered saline/Tween 20 (TBS-T) for 1 hour to prevent nonspecific binding of antibodies. Phosphospecific p42/44 MAP kinase antibodies were incubated with the PVDF membranes for 1 hour. Membranes were washed in TBS-T following each incubation with antibodies and then analyzed using an enhanced chemiluminescence (ECL) detection system.

Folts model

The rabbit model of recurrent intravascular thrombus formation has been described in detail elsewhere[28] and represents a modification of the canine model originally described by Folts et al.[29] Briefly, male New Zealand White rabbits (2.0-2.5 kg, Porcellus) where anesthetized with a mixture of Hypnorm (Janssen Animal Health, Beerse, Belgium; containing fentanyl citrate 0.315 mg/mL plus fluanisone 10 mg/mL given 0.5 mL/kg intramuscularly) and Hypnovel (Roche, Welwyn Garden City, United Kingdom; containing midazolam 20 mg/mL given 2 mg/kg intraperitoneally). Anesthesia was maintained during the course of the experiment by an intravenous infusion of 3.5 to 5 mL/hour of a solution of Hypnorm (1:10 [vol/vol]) and Hypnoval (1:20 [vol/vol]) in sterile saline via a catheter placed in the left marginal ear vein, which was also used for drug administration. Through a median incision of the neck, the carotid arteries and trachea were exposed and carefully isolated from the surrounding tissue. Polyethylene catheters were inserted into the trachea to support artificial respiration and also into the abdominal aorta via a femoral artery for continuous blood pressure monitoring. A segment of the exposed left carotid artery was injured by gentle squeezing of the artery between a pair of rubber-covered forceps, and blood flow was subsequently partially restricted (to approximately 60% of baseline) using a rat renal artery Goldblatt clip. Carotid blood flow velocity was measured continuously by a Doppler flow probe positioned proximal to the constrictor. Induction of arterial damage and stenosis produced cyclic fluctuations of arterial blood flow (cyclic flow reductions [CFRs]). In all animals, heparin (250 IU/kg intravenously) was used so as to avoid the contribution of coagulation to thrombus formation and maintenance. In this model, CFRs are primarily due to recurrent cycles of formation and dislodgment of platelet-rich thrombi. CFRs were monitored in all animals for 30 minutes to establish a stable baseline and then in 4 rabbits. Vehicle, 0.01, and 0.03 mg/kg sildenafil were sequentially administered intravenously, and blood flow was monitored for each 20-minute treatment period. Heart rate and arterial blood pressure were monitored continuously throughout the experiment and CFRs were scored for rate and reversibility of flow reductions during each treatment period, using the following qualitative scale: 0 indicates complete reduction of flow without spontaneous reversal; 1, reduction of flow with spontaneous reversal; 2, change in rate of flow reduction with spontaneous reversal; and 3, cessation of CFRs. The effect of treatment on CFR score was evaluated by a 2-sided permutation test.[30] A P value less than .05 defined significant differences between treatment periods.

Analysis of data

All experiments were performed at least 3 times and data are shown as means ± SEM. Statistical analysis was conducted using Student unpaired t test unless stated.

Results

VWF activates PLC and αIIbβ3 through a Src kinase–dependent pathway

Experiments were undertaken to investigate previous reports that GPIb-IX-V stimulates activation of αIIbβ3 through a Src kinase–dependent pathway in plasma. Because many of the currently available inhibitors of Src kinases, including PP1, PP2, and SU6656, have limited efficacy in plasma due to high protein binding, we used a novel inhibitor of this family of kinases that does not have this problem, PD173952.[31] PD173952 (25 μM) markedly inhibited ristocetin-mediated aggregation in plasma (Figure 1Ai) at a concentration that completely inhibited aggregation by collagen, which signals through a Src kinase–dependent pathway (data not shown). The residual increase in light transmission that was observed in the presence of PD173952 is most likely due to agglutination, as a similar increase was seen in the presence of the αIIbβ3 antagonist, lotrafiban (10 μM; Figure 1Ai). A similar set of observations were made in washed platelets using ristocetin and a preparation of VWF isolated from FVIII concentrate, Haemate P (Figure 1Aii), which has been previously reported to stimulate activation of αIIbβ3 in washed platelets.[14] A slightly greater agglutination response was seen in the washed platelets. PD173952 had no effect on aggregation to a submaximal concentration of thrombin in washed platelets (Figure 1Aiii).

Recent reports have indicated that GPIb-IX-V stimulates activation of PLCγ2 downstream of Src kinases.[11] Confirmation that

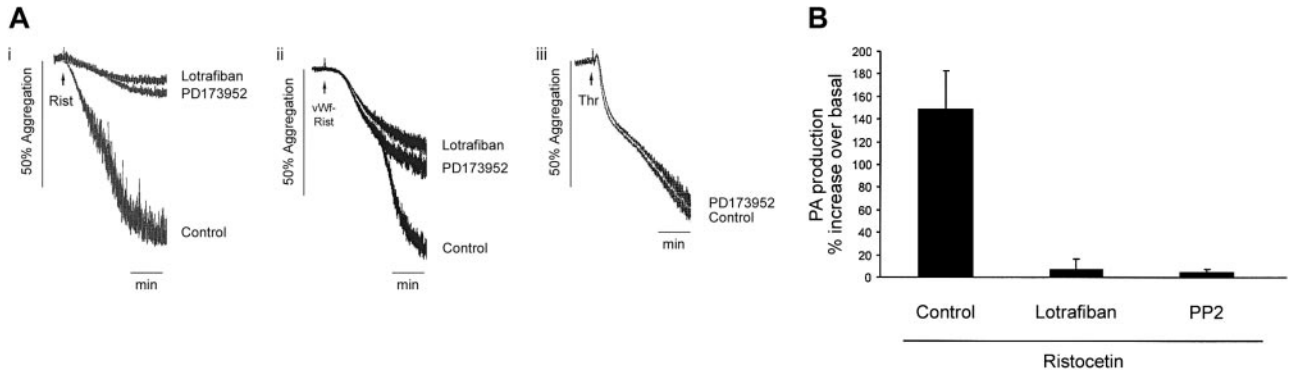

Figure 1. VWF activates PLC and αIIbβ3 through a Src kinase–dependent pathway. (Ai) Human platelet-rich plasma (3×10^8 platelets/mL) and (Aii-iii) human washed platelets (3×10^8 platelets/mL) were preincubated for 5 minutes with vehicle (dimethyl sulfoxide [DMSO] 0.1%), the αIIbβ3 antagonist, lotrafiban (10 μM), or the Src family kinase inhibitor PD173952 (25 μM) followed by stimulation with (i) ristocetin (Rist; 1.5 mg/mL), (ii) VWF-ristocetin (VWF-Rist; 10 μg/mL and 1 mg/mL) or (iii) thrombin (Thr; 0.05 IU/mL). Experiments were conducted in aggregometer cuvettes with stirring at 37°C. Arrows indicate addition of agonist. 100% represents maximal light transmission. (B) Human washed platelets loaded with [^{32}P]orthophosphoric acid (0.5 mCi/mL [18.5 MBq/mL]) were resuspended in plasma (1×10^9 platelets/mL) and preincubated for 5 minutes with vehicle (DMSO 0.1%), the αIIbβ3 antagonist, lotrafiban (10 μM), or the Src kinase inhibitor, PP2 (10 μM), followed by stimulation with ristocetin (1.5 mg/mL) for 5 minutes. Reactions were stopped by addition of an equal volume of chloroform-methanol (1:1, vol/vol). Phospholipids were separated by thin layer chromatography and analyzed by autoradiography. Results are presented as fold increases over basal (mean ± SEM). Results are representative of 3 experiments.

VWF induces activation of PLC was obtained by measurement of [^{32}P]phosphatidic acid (Figure 1B). Phosphatidic acid, a product of metabolism of 1,2-diacylglycerol, has been used in many studies as a marker of activation of PLC.[32] Under these studies, [^{32}P]-labeled phosphatidic acid is derived through the sequential action of PLC and diacylglycerol (DG)–kinase but not by phospholipase D (PLD), as the phosphate in the 1 position of phospholipids are not significantly labeled during short incubations with [^{32}P]orthophosphate. It is important to note that VWF has been reported to activate PLC through engagement of both GPIb-IX-V and αIIbβ3.[11,32,33] Studies with the αIIbβ3 antagonist lotrafiban demonstrated that activation of PLC by VWF was primarily mediated by engagement of the integrin (Figure 1B), consistent with previous reports of the weak nature of the response to GPIb-IX-V. Inhibition of Src kinases also completely inhibited activation of PLC by ristocetin (Figure 1B), but not by thrombin (not shown), as previously reported.[34]

VWF does not stimulate formation of cGMP in platelets

Experiments were undertaken to investigate previous reports that GPIb-IX-V stimulates formation of cGMP in platelets. These studies were performed in plasma using ristocetin and in washed platelets using ristocetin and VWF. The concentrations of VWF and ristocetin that were used in the experiments induced maximal αIIbβ3-dependent aggregation. In neither set of experimental conditions did engagement of GPIb-IX-V lead to a significant increase in cGMP by 5 minutes (Figure 2A). The same result was observed with different concentrations of ristocetin and at shorter times, leading us to conclude that GPIb-IX-V does not elevate

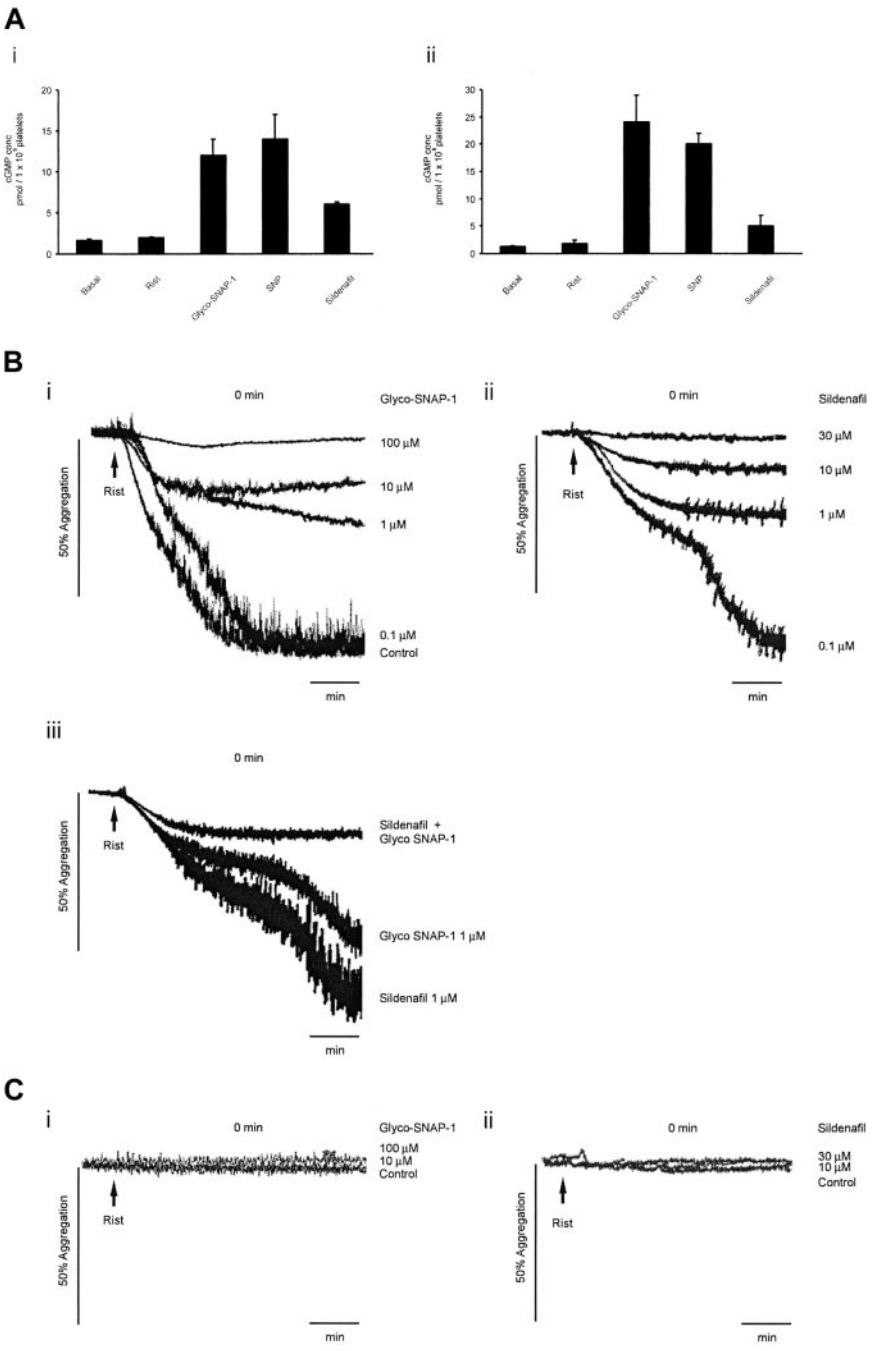

Figure 2. Elevation of cGMP inhibits activation of αIIbβ3 by VWF. (Ai) Human PRP (3×10^8 platelets/mL) or (ii) human washed platelets (3×10^8 platelets/mL) were stimulated with ristocetin alone (Rist; 1.5 mg/mL), ristocetin in the presence of VWF (VWF and rist at 10 μg/mL and 1 mg/mL, respectively), the NO donors glyco–SNAP-1 (100 μM) and sodium nitroprusside (SNP; 100 μM), or the PDE 5 inhibitor sildenafil (30 μM) for 5 minutes. The cGMP content was analyzed using a cGMP direct Biotrak kit (Amersham Biosciences). Results are presented as cGMP concentration/10^8 platelets. Error bars represent SEM. (B) Human PRP (3×10^8 platelets/mL) was stimulated with ristocetin (Rist; 1.5 mg/mL) simultaneously to the addition of the NO donor (i) glyco–SNAP-1 and (ii) the PDE 5 inhibitor sildenafil at the indicated concentrations or (iii) glyco–SNAP-1 (1 μM) and sildenafil (1 μM) alone or in combination. Experiments were conducted in aggregometer cuvettes with stirring at 37°C. Arrows indicate addition of agonist. (C) Human PRP (3×10^8 platelets/mL) was stimulated with threshold concentrations of ristocetin (Rist; 1 mg/mL) simultaneously with the NO donor glyco–SNAP-1 or the PDE 5 inhibitor sildenafil at the indicated concentration. Experiments were conducted in aggregometer cuvettes with stirring at 37°C. Arrows indicate addition of agonist. Results are representative of 5 experiments.

cGMP in platelets (Figure 2A; not shown). In comparison, the NO-elevating agents, sodium nitroprusside (100 μM) and glyco–SNAP-1 (100 μM), stimulated a 5- to 10-fold increase in cGMP (Figure 2A), whereas a smaller increase was observed in response to a supramaximal concentration of the PDE 5 inhibitor, sildenafil (30 μM). The latter result is consistent with the proposal that inhibition of PDE 5 potentiates cGMP formation in platelets, but is unable to induce a maximal increase of the cyclic nucleotide on its own.[35] These results demonstrate that VWF-ristocetin does not lead to formation of cGMP in platelets.

Elevation of cGMP inhibits activation of αIIbβ3 by VWF

Experiments were undertaken to investigate previous reports that agents that increase cGMP potentiate activation of αIIbβ3 by GPIb-IX-V. A key argument in the study by Li et al[24] is that cGMP promotes activation of platelets at early times, whereas inhibition is seen with longer incubations (5 minutes), which is mediated through a protein kinase A–dependent mechanism. Our studies were therefore designed to investigate the response to GPIb-IX-V engagement by ristocetin-VWF and by thrombin following simultaneous addition or after 5 minutes of incubation with cGMP-elevating agents. The experiments with ristocetin were performed in plasma and those for thrombin were performed in washed platelets to avoid fibrin formation. These are the same set of conditions as used by Li et al.[24] Under both sets of conditions, and at both incubation times, however, we observed inhibition of αIIbβ3-dependent platelet activation by ristocetin or thrombin in the presence of glyco–SNAP-1 or sildenafil (Figure 2Bi-ii; not shown). A similar set of results were obtained with sodium nitroprusside (not shown). In addition, we observed a synergistic inhibition of αIIbβ3 activation by ristocetin in the presence of submaximal concentrations of glyco–SNAP-1 and sildenafil (Figure 2Biii). A similar result was seen for thrombin (not shown). Significantly, none of the cGMP-elevating agents were able to potentiate activation of αIIbβ3 by a threshold concentration of ristocetin or thrombin (Figure 2C; not shown). In summary, these studies demonstrate that agents that increase cGMP in platelets inhibit GPIbα- and thrombin-mediated aggregation when given either simultaneously or at longer times.

Inhibitors of protein kinase G do not block phosphorylation and potentiate (rather than inhibit) the effect of cGMP-elevating agents in platelets

A critical line of evidence for a role of cGMP in GPIb-IX-V signaling in the study by Li et al[24] is the inhibitory effect of the PKG-blocking agents, KT5823 and Rp-8-pCPT-cGMPS, on aggregation induced by VWF. Li et al[24] argued that the use of structurally distinct inhibitors of PKG served to minimize the possibility that effects were mediated by non–PKG-dependent mechanisms. However, the ability of KT5823 to block purified PKG has since been questioned,[36] whereas Rp-8-pCPT-cGMPS and the structurally related PKG inhibitor, Rp-8-Br-PET-cGMPS, only work under certain conditions (see the accompanying article by Gambaryan et al, beginning on page 2593).[37]

The evidence presented by Li et al[38] to demonstrate the effectiveness of KT5823 and Rp-8-pCPT-cGMPS in platelets was based on phosphorylation studies. Surprisingly, Li et al[38] demonstrated that KT5823 and Rp-8-pCPT-cGMPS are unable to block phosphorylation of the recognized PKG substrate VASP by VWF-ristocetin or by cGMP-elevating agents. On the other hand, the PKG inhibitors blocked phosphorylation of p42/44 MAP kinases induced by VWF-ristocetin, cGMP-elevating agents, and membrane-permeable cGMP mimetics. These observations led the authors to conclude that VASP had been incorrectly assigned as a PKG substrate in platelets, whereas MAP kinases are regulated downstream of PKG.[38]

We have confirmed the observation that KT5823, Rp-8-pCPT-cGMPS, and Rp-8-Br-PET-cGMPS are unable to block phosphorylation of VASP induced by ristocetin-VWF (Figure 3Ai) or by cGMP-elevating agents (not shown) using the same period of incubation as that of Li et al[38] to block aggregation. In contrast, however, we were unable to confirm the observation that VWF-ristocetin and cGMP-elevating agents, such as glyco–SNAP-1 and sildenafil, are able to induce phosphorylation of p42/44 MAP kinases in platelets (Figure 3Aii). Further, none of the PGK inhibitors are able to block phosphorylation of p42/44 MAP kinase by thrombin or phorbol ester in platelets (Figure 3Aii; data not shown). Taken together, these results demonstrate that cGMP does not lie upstream of the p42/44 MAP kinase pathway in platelets and therefore brings into question the effectiveness of the PKG inhibitors when used under the same experimental conditions as described by Li et al.[38]

Despite these observations, however, Rp-8-pCPT-cGMPS and Rp-8-Br-PET-cGMPS inhibited aggregation induced by ristocetin in plasma and to thrombin in washed platelets (Figure 3B; not shown). In contrast, KT5823 had no effect on the response to either stimulus (data not shown). Further, Rp-8-pCPT-cGMPS (0.5 mM) and Rp-8-Br-PET-cGMPS (0.5 mM) potentiated (rather than inhibited) the ability of the cGMP-elevating agents, glyco–SNAP-1, sildenafil, and sodium nitroprusside, to inhibit aggregation to ristocetin and thrombin (Figure 3B; not shown). These results demonstrate that the PKG inhibitors and cGMP elevators do not have opposing effects as would be expected if their actions were mediated at the level of PKG and thereby indicating that the PKG inhibitors may induce inhibition through a non–PKG-dependent mechanism. This possibility was tested directly through studies on mice that were deficient in PKG. The dose-response curve to thrombin was similar in the wild-type and $PKG^{-/-}$ platelets (not shown). Both Rp-8-pCPT-cGMPS (0.5 mM) and Rp-8-Br-PET-cGMPS (0.5 mM) partially inhibited aggregation to thrombin in wild-type and PKG-deficient platelets, confirming that they are able to block platelet activation through a PKG-independent pathway (Figure 3C). The accompanying manuscript also provides evidence for PKG-independent actions of these 2 inhibitors and other cGMP-based modulators.[36,37]

MAP kinase inhibition does not block activation of αIIbβ3 by VWF

The role of the MAP kinase pathway in mediating aggregation to VWF-ristocetin and thrombin was investigated using a novel inhibitor of mitogen-induced extracellular kinase (MEK) kinases, PD184161 (5-bromo-2-[2-chloro-4-iodo-phenylamino]-N-cyclopropylmethoxy-3,4-difluoro-benzamide), which is biologically effective in plasma ("Inhibition of Src kinases, but not MAP kinases, blocks aggregate formation at high shear"). The structure of PD184161 is presented in Figure 4Ai. Confirmation that PD184161 inhibits activation of the MAP kinase pathway was shown using a phosphospecific antibody to p42/44 MAP kinases (Figure 4Aii). PD184161 causes complete inhibition of p42/44 MAP kinase activation at concentrations as low as 0.1 μM, but had no effect on GPIb-IX-V– or thrombin-mediated aggregation (Figure 4B).

Inhibition of Src kinases, but not MAP kinases, blocks aggregate formation at high shear

The results obtained in this study support a role for Src kinases but neither PKG or MAP kinases in the activation of αIIbβ3 by the GPIb-IX-V complex in the absence of shear. To investigate these observations further, we monitored aggregate formation on collagen in human and mouse platelets at a medium rate of shear,

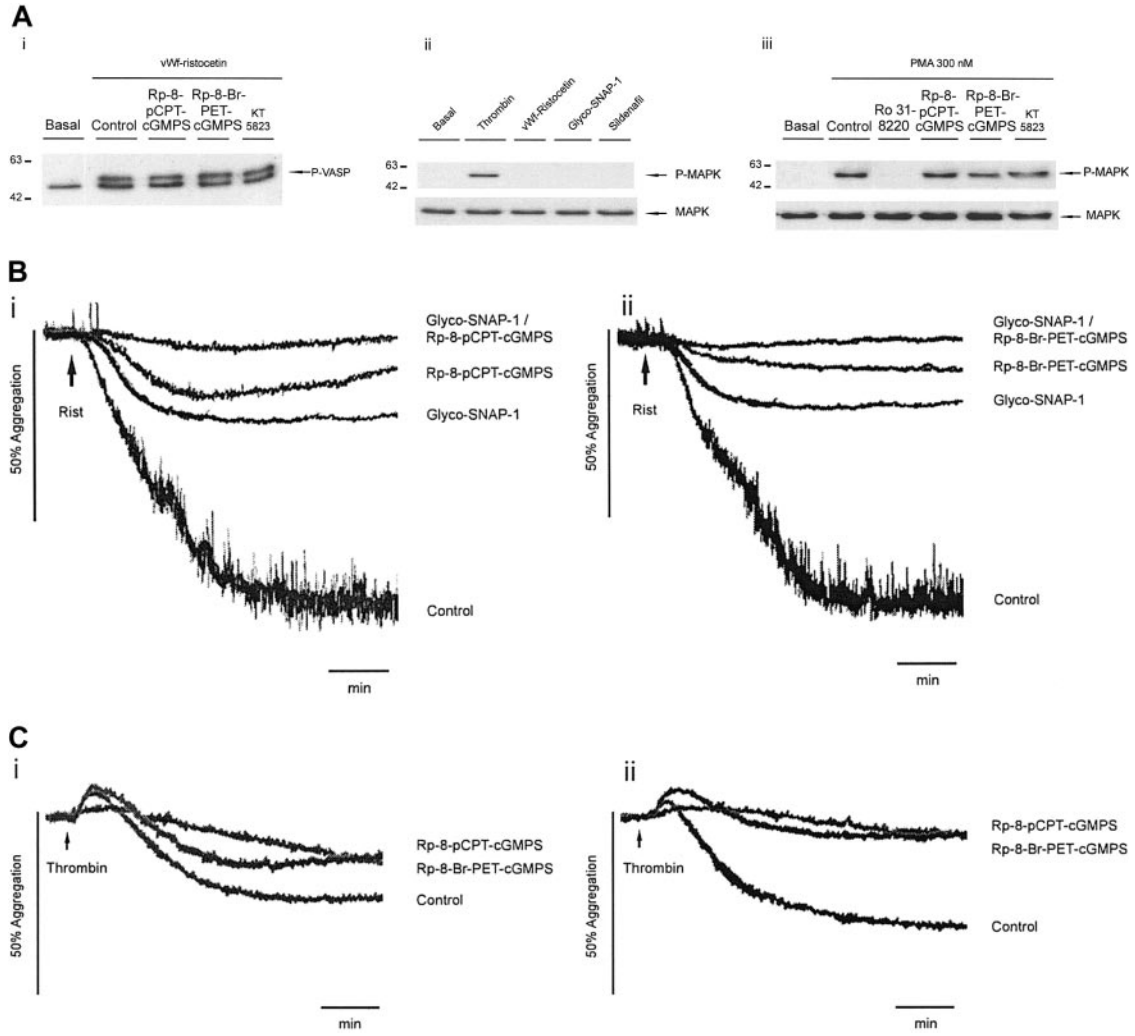

Figure 3. Effect of protein kinase G inhibitors on phosphorylation and aggregation. (A) Human washed platelets (5×10^8/mL), preincubated for 5 minutes with vehicle (DMSO 0.1%), the PKC inhibitor Ro 31-8220 (10 μM), or the PKG inhibitors Rp-8-pCPT-cGMPS (0.5 mM), Rp-8-Br-PET-cGMPS (0.5 mM), and KT5823 (10 μM), were stimulated by VWF and ristocetin (10 μg/mL and 1 mg/mL, respectively), the phorbol ester PMA (300 nM), or the cGMP raising agents glyco–SNAP-1 (10 μM) and sildenafil (30 μM). Reactions were stopped by addition of equal volumes of sample buffer and proteins separated by SDS-PAGE and Western blotted for (i) Ser^{239}-phosphorylated VASP and (ii-iii) phosphorylated p42/44 MAP kinase. Blots were subsequently stripped and reprobed with a pan p42/44 MAP kinase Ab to ensure equal amounts of protein had been loaded. Blots are representative of 4 experiments. (B) Human PRP (3×10^8 platelets/mL) was stimulated with ristocetin (Rist; 1.5 mg/mL) simultaneously with the NO donor glyco–SNAP-1 (1 μM) and the PKG inhibitors Rp-8-pCPT-cGMPS (0.5 mM) and Rp-8-Br-PET-cGMPS (0.5 mM) either on their own or in combination. Experiments were conducted in aggregometer cuvettes with stirring at 37°C. Arrows indicate addition of agonist. Results are representative of 5 experiments. (C) Washed platelets were pooled from (i) 16 wild-type and (ii) 16 PKG null mice and suspended at a concentration of 2×10^8 platelets/mL. Platelets were preincubated for 5 minutes with vehicle (DMSO 0.1%), the PKG inhibitors Rp-8-pCPT-cGMPS (0.5 mM) or Rp-8-Br-PET-cGMPS (0.5 mM), and stimulated by a threshold concentration of thrombin (0.1 IU/mL). Experiments were conducted in aggregometer cuvettes with stirring at 37°C. Arrows indicate addition of agonist.

$800\ s^{-1}$. Similar observations were made in both species (Figure 5; data not shown). Aggregate formation in this system is strictly dependent on the ability of the VWF that is bound to the immobilized collagen to capture circulating platelets via the GPIb-IX-V complex, as demonstrated using the GPIbα-blocking antibody, 6D1 (Figure 5A). Stable adhesion and aggregate formation is also critically dependent on the collagen receptors, GPVI and integrin α2β1, that mediate stable adhesion and generate activation signals. The Src family kinase inhibitor, PD179352 (25 μM), dramatically inhibited platelet adhesion and blocked aggregate formation on collagen, whereas PD184161 (20 μM) and a second inhibitor of the MAP kinase pathway, U0126 (10 μM), had no effect (Figure 5A). Confirmation that PD184161 is able to inhibit activation of p42/44 MAP kinase in whole blood was obtained by Western blotting of the thrombus with phosphospecific antibodies and pan-antibodies to p42/44 MAP kinase, the latter also serving as an index of the amount of protein incorporated into the platelet-rich aggregate (Figure 5B). The p42/44 MAP kinase phosphorylation was markedly inhibited in the presence of PD184161, confirming the activity of PD184161 in whole blood. In contrast, U0126 was unable to block phosphorylation, demonstrating that the ability of U0126 to block ristocetin-induced aggregation in plasma[25] is not mediated by inhibition of p42/44 MAP kinase.

Agents that elevate cGMP inhibit thrombus formation at high shear

Experiments were designed to investigate the effect of cGMP-elevating agents on thrombus formation on collagen in human platelets and in a rabbit model of thrombosis. Thrombus formation on collagen was not altered in the presence of sildenafil (1 μM) or glyco–SNAP-1 (10 μM), whereas the 2 agents in combination had a weak inhibitory effect that was similar to that seen with a higher concentration of sildenafil (Figure 6A). Importantly, under no

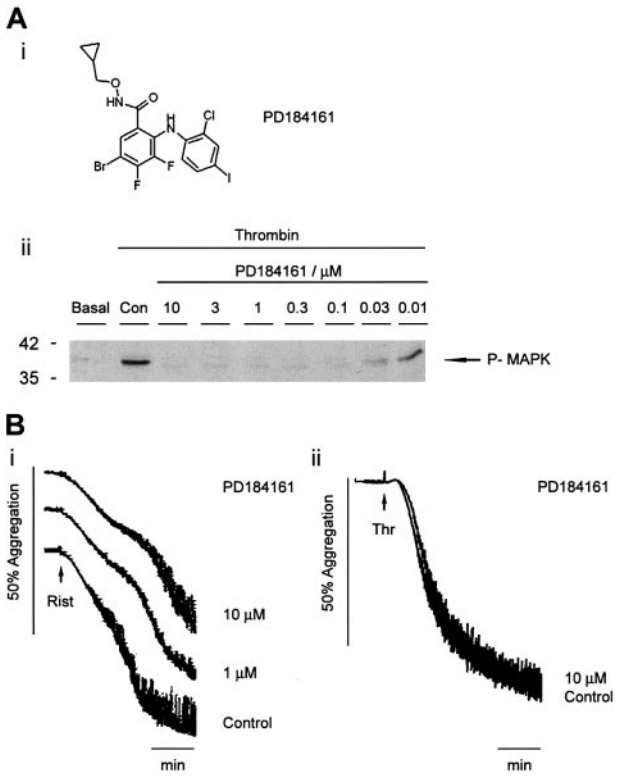

Figure 4. MAP kinase inhibition does not block activation of αIIbβ3 by VWF. (Ai) Structure of the novel MEK inhibitor 5-bromo-2-[2-chloro-4-iodo-phenylamino]-N-cyclopropylmethoxy-3,4-difluoro-benzamide (PD184161). (Aii) Human washed platelets (5×10^8/mL) were preincubated for 5 minutes with vehicle (DMSO 0.1%) or the MEK inhibitor PD184161 at the indicated concentration followed by stimulation with thrombin (0.05 IU/mL). Reactions were stopped by addition of equal volumes of sample buffer, and proteins were separated by SDS-PAGE and Western blotted for phosphorylated p42/44 MAP kinase. Blots are representative of 4 experiments. (Bi) Human platelet-rich plasma (3×10^8 platelets/mL) and (ii) human washed platelets (3×10^8 platelets/mL) were preincubated for 5 minutes with vehicle (DMSO 0.1%) or the MEK inhibitor PD184161 (1-10 μM) followed by stimulation with ristocetin (Rist; 1.5 mg/mL) or thrombin (Thr; 0.05 IU/mL). Experiments were conducted in aggregometer cuvettes with stirring at 37°C. Arrows indicate addition of agonist. Traces are representative of 3 experiments.

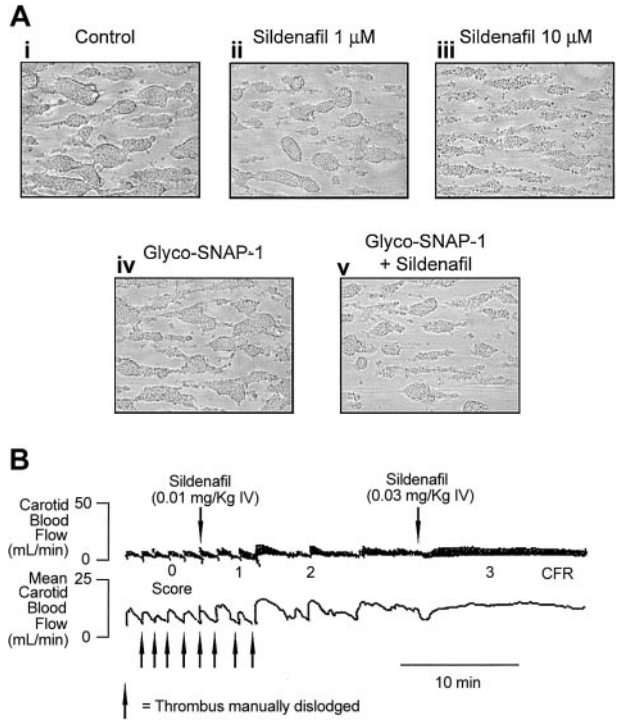

Figure 6. Agents that elevate cGMP inhibit thrombus formation at high shear. (A) Heparinized human blood was incubated with either vehicle DMSO (i; 0.1%), the phosphodiesterase (PDE) 5 inhibitor sildenafil (1 [ii] or 10 [iii] μM), the NO donor glyco–SNAP-1 (iv; 10 μM) or combination of sildenafil (1 μM) and glyco–SNAP-1 (10 μM) (v) for 5 minutes at room temperature prior to being flowed through 2×0.2-mm microslides coated with collagen (300 μg/mL) at 800 s^{-1} for 5 minutes. Excess blood was removed from microslides with modified Ca^{2+}-free Tyrode buffer flowed at 800 s^{-1} for 3 minutes. Thrombi were visualized by light microscope (original magnifications, × 630). Images are representative of 3 experiments. (B) A representative trace for carotid blood flow and mean carotid blood flow (mL/min) from the Folts rabbit model of thrombosis illustrating CFR scores before and after sildenafil (0.01 and 0.03 mg/kg intravenously) administration. CFR scores correspond to the following: 0 indicates complete reduction of flow without spontaneous reversal; 1, reduction of flow with spontaneous reversal; 2, change in rate of flow reduction with spontaneous reversal; and 3, cessation of CFRs.

experimental conditions was potentiation of thrombus formation observed in the presence of cGMP-elevating agents. Consistent with these observations, sildenafil increased the rate of flow in the Folts model of thrombosis (Figure 6B; Table 1). Further, at the doses tested, sildenafil did not alter blood flow in the uninjured vessel, supporting the contention that improved flow in the injured carotid is due to an action on platelet aggregate formation and not a direct vasorelaxant effect. These data suggest that an increase in cGMP inhibits thrombosis formation under high shear conditions both in vitro and in vivo.

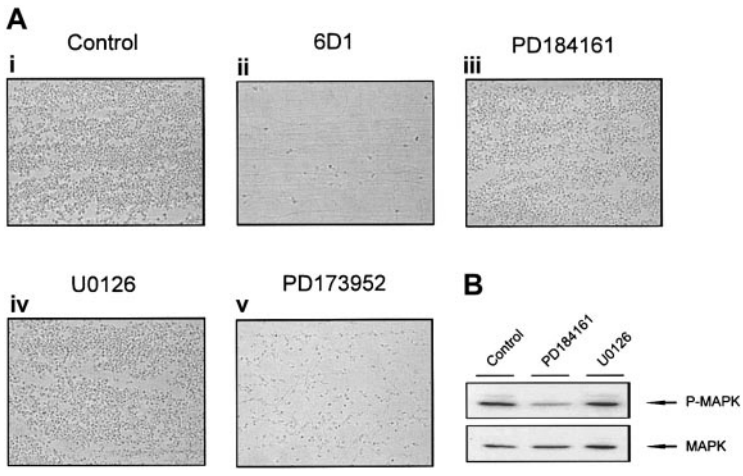

Figure 5. Inhibition of Src kinases, but not MAP kinases, blocks thrombus formation at high shear. (A) Heparinized mouse blood was incubated with either vehicle DMSO (i; 0.1%), the anti-GPIbα monoclonal antibody (mAb) 6D1 (ii; 10 μg/mL), the p42/44 MAP kinase inhibitors PD184161 (iii; 20 μM) and U0126 (iv; 10 μM), or the Src kinase inhibitor PD173952 (v; 25 μM) for 5 minutes at room temperature prior to being flowed through 1×0.1-mm microslides coated with collagen (30 μg/mL) at 800 s^{-1} for 2 minutes. Excess blood was removed from microslides with modified Ca^{2+}-free Tyrode buffer flowed at 800 s^{-1} for 8 minutes. Thrombi were visualized by light microscope (original magnification × 630). (B) Thrombi were lysed with ice-cold lysis buffer containing NP-40 (1% [vol/vol]). Whole cell lysates (WCL) were resolved by SDS-PAGE and immunoblotted for p42/44 MAP kinase with an anti–phospho-p42/44 MAP kinase monoclonal antibody. Membranes were stripped and reprobed with anti-p42/44 MAP kinase polyclonal antibody to determine equal lane loading. Images are representative of 3 experiments performed.

Table 1. Effect of sildenafil on CFR scores in the rabbit model of arterial thrombosis

Sildenafil dose, intravenously	No. of animals achieving CFR scores of 0	No. of animals achieving CFR scores of 1	No. of animals achieving CFR scores of 2	No. of animals achieving CFR scores of 3
Vehicle	3	1	0	0
0.01 mg/kg	0	2	2	0
0.03 mg/kg*	0	1	2	1

The effect of treatment on CFR score was evaluated by a 2-sided permutation test.[30] CFR scores correspond to the following: 0 indicates complete reduction of flow without spontaneous reversal; 1, reduction of flow with spontaneous reversal; 2, change in rate of flow reduction with spontaneous reversal; and 3, cessation of CFRs.

A value of $P < .05$ defined significant differences between treatment periods ($n = 4$).

*$P < .05$, 2-sided permutation test.

Discussion

The aim of this study was to compare the role of Src kinases, PKG, and MAP kinases in the activation of αIIbβ3 by the GPIb-IX-V receptor complex and in thrombus formation. The results demonstrate a critical role for Src kinases, but neither PKG nor MAP kinases, in signaling by GPIb-IX-V. Indeed, the study has generated evidence in support of the more widely accepted view that PKG mediates platelet inhibition by VWF and thrombin.[39-41]

Several lines of evidence argue against a role for PKG in promoting activation of platelets by GPIb-IX-V. These include: (i) the inability of VWF to stimulate formation of cGMP; (ii) the observation that agents that elevate cGMP, such as glyco–SNAP-1, sodium nitroprusside, or sildenafil, inhibit rather than promote αIIbβ3 activation by GPIb-IX-V; (iii) the ability of sildenafil to inhibit platelet aggregation and thrombus formation in vitro and in vivo, respectively; and (iv) the demonstration that the PKG inhibitors, Rp-8-pCPT-cGMPS and Rp-8-Br-PET-cGMPS, potentiate rather than oppose the action of cGMP-elevating agents in inhibiting platelet aggregation. The mechanism underlying the action of Rp-8-pCPT-cGMPS and Rp-8-Br-PET-cGMPS is not known but it does not appear to be related to inhibition of PKG, as they also mediate inhibition in PKC-deficient mice, a conclusion that is also reached in the accompanying manuscript.[37] Rp-8-pCPT-cGMPS and Rp-8-Br-PET-cGMPS do not appear to inhibit the positive feedback effect of ADP, as they inhibit activation by thrombin in the presence of ADP receptor antagonists (not shown).

The present study has provided evidence against a role for the p42/44 MAP kinase pathway in the GPIb-IX-V signaling cascade. This evidence includes the observation that a novel inhibitor of this pathway, which is biologically effective in plasma, PD184161, has minimal effect on the activation of αIIbβ3 by GPIb-IX-V or thrombin and on thrombus formation. Additionally, ristocetin-VWF does not induce activation of p42/44 MAP kinases in platelets. This conclusion is consistent with a number of other studies that report that blockade of p42/44 MAP kinase by PD98059 and U0126 had no effect on thrombin-induced aggregation.[42-44]

An implicit argument in the work of Li et al[24] is that threshold concentrations of thrombin signal through the same pathway as GPIb-IX-V. Although there is no doubt that GPIb-IX-V is a high-affinity binding site for thrombin, there is controversy as to whether it is also a signaling receptor for thrombin. In particular, activation of mouse platelets by thrombin is inhibited completely upon genetic ablation of protease-activated receptor 4, demonstrating that GPIb-IX-V is unable to mediate activation by thrombin in the absence of the G protein–coupled receptor.[45] The role of the high-affinity binding site for thrombin on GPIb-IX-V may therefore be to bring the protease close to its G protein–coupled receptor. This model is consistent with the observation that the Src kinase inhibitor PD173952 is able to block aggregation by VWF-ristocetin, but not to thrombin.

The present study has further emphasized the importance of Src kinases in platelet activation. It is becoming increasingly recognized that Src kinases mediate activation of platelets by the major glycoprotein receptors, including GPVI, the integrins α2β1 and αIIbβ3, and by GPIb-IX-V. Significantly, activation of all 4 of these receptors lie upstream of PLCγ2, thereby identifying Src kinases and the phospholipase as major targets for antithrombotic agents.[11,32,33,46,47] Selective inhibitors of these enzymes may have specific advantages, bearing in mind that glycoprotein receptors mediate the initial events that give rise to activation of platelets at sites of damage to the vasculature.

In conclusion, we have been unable to find support for a critical role of PKG and MAP kinases in the activation of platelets by GPIb-IX-V, but have provided further evidence for a role of Src family kinases in this pathway and in aggregate formation under flow conditions. The present study adds to the growing body of evidence for a critical role of Src kinases in the regulation of platelets by membrane glycoproteins and further emphasizes Src family kinases as important targets for development of novel antithrombotics. The present study has also identified 2 novel tools, PD173952 and PD184161, that are effective in plasma and can be used to establish the roles of Src and MAP kinases, respectively, in thrombus formation in blood.

Acknowledgments

We gratefully acknowledge the excellent support of Susan Waller in the Folts studies. We thank Alan Kraker, Pfizer Global Research and Development (Ann Arbor, MI) for critical discussions throughout the course of this work. The authors also thank Dr Ulrich Walter and colleagues for sharing data[37] and for helpful discussion during the course of this work.

References

1. Jackson SP, Nesbitt WS, Kulkarni S. Signaling events underlying thrombus formation. J Thromb Haemost. 2003;1:1602-1612.
2. Celikel R, McClintock RA, Roberts JR, et al. Modulation of alpha-thrombin function by distinct interactions with platelet glycoprotein Ibalpha. Science. 2003;301:218-221.
3. Savage B, Saldivar E, Ruggeri ZM. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. Cell. 1996; 84:289-297.
4. Ruggeri ZM. Von Willebrand factor, platelets and endothelial cell interactions. J Thromb Haemost. 2003;1:1335-1342.
5. Nieswandt B, Brakebusch C, Bergmeier W, et al. Glycoprotein VI but not alpha2beta1 integrin is essential for platelet interaction with collagen. EMBO J. 2001;20:2120-2130.
6. Atkinson BT, Jarvis GE, Watson SP. Activation of GPVI by collagen is regulated by alpha2beta1

and secondary mediators. J Thromb Haemost. 2003;1:1278-1287.

7. Jin J, Kunapuli SP. Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. Proc Natl Acad Sci U S A. 1998;95:8070-8074.
8. Nieswandt B, Bergmeier W, Eckly A, et al. Evidence for cross-talk between glycoprotein VI and Gi-coupled receptors during collagen-induced platelet aggregation. Blood. 2001;97:3829-3835.
9. Yap CL, Hughan SC, Cranmer SL, et al. Synergistic adhesive interactions and signaling mechanisms operating between platelet glycoprotein Ib/IX and integrin alpha IIbbeta 3: studies in human platelets and transfected Chinese hamster ovary cells. J Biol Chem. 2000;275: 41377-41388.
10. Marshall SJ, Asazuma N, Best D, et al. Glycoprotein IIb-IIIa-dependent aggregation by glycoprotein Ibalpha is reinforced by a Src family kinase inhibitor (PP1)-sensitive signalling pathway. Biochem J. 2002;361:297-305.
11. Mangin P, Yuan Y, Goncalves I, et al. Signaling role for PLCgamma 2 in platelet glycoprotein Ibalpha calcium flux and cytoskeletal reorganization involvement of a pathway distinct from FcR gamma-chain and cgamma RIIA. J Biol Chem. 2003;278:32880-32891.
12. Wu Y, Suzuki-Inoue K, Satoh K, et al. Role of Fc receptor gamma-chain in platelet glycoprotein Ib-mediated signaling. Blood. 2001;97:3836-3845.
13. Kroll MH, Harris TS, Moake JL, et al. von Willebrand factor binding to platelet GpIb initiates signals for platelet activation. J Clin Invest. 1991;88: 1568-1573.
14. Torti M, Bertoni A, Canobbio I, et al. Rap1B and Rap2B translocation to the cytoskeleton by von Willebrand factor involves FcgammaII receptor-mediated protein tyrosine phosphorylation. J Biol Chem. 1999;274:13690-13697.
15. Kulkarni S, Dopheide SM, Yap CL, et al. A revised model of platelet aggregation. J Clin Invest. 2000; 105:783-791.
16. Mazzucato M, Pradella P, Cozzi MR, et al. Sequential cytoplasmic calcium signals in a 2-stage platelet activation process induced by the glycoprotein Ibalpha mechanoreceptor. Blood. 2002; 100:2793-2800.
17. Nesbitt WS, Kulkarni S, Giuliano S, et al. Distinct glycoprotein Ib/V/IX and integrin alpha IIbbeta 3-dependent calcium signals cooperatively regulate platelet adhesion under flow. J Biol Chem. 2002;277:2965-2972.
18. Nesbitt WS, Giuliano S, Kulkarni S, et al. Intercellular calcium communication regulates platelet aggregation and thrombus growth. J Cell Biol. 2003;160:1151-1161.
19. Yap CL, Anderson KE, Hughan SC, et al. Essential role for phosphoinositide 3-kinase in shear-dependent signaling between platelet glycoprotein Ib/V/IX and integrin alpha(IIb)beta(3). Blood. 2002;99:151-158.
20. Ozaki Y, Satoh K, Yatomi Y, et al. Protein tyrosine phosphorylation in human platelets induced by interaction between glycoprotein Ib and von Willebrand factor. Biochim Biophys Acta. 1995;1243: 482-488.
21. Falati S, Edmead CE, Poole AW. Glycoprotein Ib-V-IX, a receptor for von Willebrand factor, couples physically and functionally to the Fc receptor gamma-chain, Fyn, and Lyn to activate human platelets. Blood. 1999;94:1648-1656.
22. Asazuma N, Ozaki Y, Satoh K, et al. Glycoprotein Ib-von Willebrand factor interactions activate tyrosine kinases in human platelets. Blood. 1997; 90:4789-4798.
23. Yanabu M, Ozaki Y, Nomura S, et al. Tyrosine phosphorylation and p72syk activation by an anti-glycoprotein Ib monoclonal antibody. Blood. 1997;89:1590-1598.
24. Li Z, Xi X, Gu M, et al. A stimulatory role for cGMP-dependent protein kinase in platelet activation. Cell. 2003;112:77-86.
25. Li Z, Xi X, Du X. A mitogen-activated protein kinase-dependent signaling pathway in the activation of platelet integrin alpha IIbbeta3. J Biol Chem. 2001;276:42226-42232.
26. Wegener JW, Nawrath H, Wolfsgruber W, et al. cGMP-dependent protein kinase I mediates the negative inotropic effect of cGMP in the murine myocardium. Circ Res. 2002;90:18-20.
27. Best D, Senis YA, Jarvis GE, et al. GPVI levels in platelets: relationship to platelet function at high shear. Blood. 2003;102:2811-2818.
28. Golino P, Ambrosio G, Pascucci I, et al. Experimental carotid stenosis and endothelial injury in the rabbit: an in vivo model to study intravascular platelet aggregation. Thromb Haemost. 1992;67: 302-305.
29. Folts JD, Crowell EB Jr, Rowe GG. Platelet aggregation in partially obstructed vessels and its elimination with aspirin. Circulation. 1976;54:365-370.
30. Edgington ES. Randomization Tests. 3rd ed. New York, NY: Marcel Dekker; 1995.
31. Kraker AJ, Hartl BG, Amar AM, et al. Biochemical and cellular effects of c-Src kinase-selective pyrido[2, 3- d]pyrimidine tyrosine kinase inhibitors. Biochem Pharmacol. 2000;60:885-898.
32. Wonerow P, Pearce AC, Vaux DJ, et al. A critical role for phospholipase Cgamma 2 in alpha IIbeta 3-mediated platelet spreading. J Biol Chem. 2003;278:37520-37529.
33. Goncalves I, Hughan SC, Schoenwaelder SM, et al. Integrin alpha IIbbeta 3-dependent calcium signals regulate platelet-fibrinogen interactions under flow: involvement of PLCgamma 2. J Biol Chem. 2003;278:34812-34822.
34. Briddon SJ, Watson SP. Evidence for the involvement of p59fyn and p53/56lyn in collagen receptor signalling in human platelets. Biochem J. 1999;338:203-209.
35. Wallis RM, Corbin JD, Francis SH, et al. Tissue distribution of phosphodiesterase families and the effects of sildenafil on tissue cyclic nucleotides, platelet function, and the contractile responses of trabeculae carneae and aortic rings in vitro. Am J Cardiol. 1999;83:3C-12C.
36. Bain J, McLauchlan H, Elliott M, et al. The specificities of protein kinase inhibitors: an update. Biochem J. 2003;371:199-204.
37. Gambaryan S, Schwarz UR, Butt E, et al. Potent inhibition of human platelets by cGMP analogs independent of cGMP-dependent protein kinase. Blood. 2004;103:2593-2600.
38. Li Z, Ajdic J, Eigenthaler M, et al. A predominant role for cAMP-dependent protein kinase in the cGMP-induced phosphorylation of vasodilator-stimulated phosphoprotein and platelet inhibition in humans. Blood. 2003;101:4423-4429.
39. Haslam RJ, Dickinson NT, Jang EK. Cyclic nucleotides and phosphodiesterases in platelets. Thromb Haemost. 1999;82:412-423.
40. Jang EK, Azzam JE, Dickinson NT, et al. Roles for both cyclic GMP and cyclic AMP in the inhibition of collagen-induced platelet aggregation by nitroprusside. Br J Haematol. 2002;117:664-675.
41. Aktas B, Honig-Liedl P, Walter U, et al. Inhibition of platelet P2Y12 and alpha2A receptor signaling by cGMP-dependent protein kinase. Biochem Pharmacol. 2002;64:433-439.
42. Borsch-Haubold AG, Pasquet S, Watson SP. Direct inhibition of cyclooxygenase-1 and -2 by the kinase inhibitors SB 203580 and PD 98059: SB 203580 also inhibits thromboxane synthase. J Biol Chem. 1998;273:28766-28772.
43. Song S, Freedman J, Mody M, et al. Porcine von Willebrand factor and thrombin induce the activation of c-Jun amino-terminal kinase (JNK/SAPK) whereas only thrombin induces activation of extracellular signal-related kinase 2 (ERK2) in human platelets. Br J Haematol. 2000;109:851-856.
44. McNicol A, Jackson EC. Inhibition of the MEK/ERK pathway has no effect on agonist-induced aggregation of human platelets. Biochem Pharmacol. 2003;65:1243-1250.
45. Sambrano GR, Weiss EJ, Zheng YW, et al. Role of thrombin signalling in platelets in haemostasis and thrombosis. Nature. 2001;413:74-78.
46. Inoue O, Suzuki-Inoue K, Dean WL, et al. Integrin {alpha}2{beta}1 mediates outside-in regulation of platelet spreading on collagen through activation of Src kinases and PLC{gamma}2. J Cell Biol. 2003;160:769-780.
47. Mangin P, Nonne C, Eckly A, et al. A PLCgamma2-independent platelet collagen aggregation requiring functional association of GPVI and integrin alpha(2)beta(1). FEBS Lett. 2003;542:53-59.