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A Stimulatory Role for cGMP-Dependent Protein Kinase in Platelet Activation

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Summary

It is currently accepted that cGMP-dependent protein kinase (PKG) inhibits platelet activation. Here, we show that PKG plays an important stimulatory role in platelet activation. Expression of recombinant PKG in a reconstituted cell model enhanced von Willebrand factor (vWF)-induced activation of the platelet integrin $\alpha_{IIb}\beta_3$. PKG knockout mice showed impaired platelet responses to vWF or low doses of thrombin and prolonged bleeding time. Human platelet aggregation induced by vWF or low-dose thrombin was inhibited by PKG inhibitors but enhanced by cGMP. Furthermore, a cGMP-enhancing agent, sildenafil, promoted vWF- or thrombin-induced platelet aggregation. The cGMP-stimulated platelet responses are biphasic, consisting of an initial transient stimulatory response that promotes platelet aggregation and a subsequent inhibitory response that limits the size of thrombi.

Platelet adhesion and activation are critical to the development of thrombotic diseases such as heart attack and stroke (Ginsberg et al., 1992; Shattil et al., 1998). Under flow conditions seen in stenotic arteries, the initial platelet adhesion and activation require the interaction between subendothelial bound von Willebrand factor (vWF) and an important platelet adhesion receptor, the glycoprotein Ib-IX complex (GPIb-IX) (Du and Ginsberg, 1999; Sakariassen et al., 1979; Savage et al., 1996; Weiss et al., 1978). GPIb-IX interaction with vWF initiates platelet adhesion and triggers activation of the platelet integrin $\alpha_{IIb}\beta_3$, leading to integrin-dependent stable platelet adhesion, spreading and aggregation (De Marco et al., 1985; Gralnick et al., 1985; Gu et al., 1999; Savage et al., 1996, 1992). The importance of GPIb-IX and vWF in thrombosis and hemostasis is manifested in the inherited diseases Bernard-Soulier syndrome and von Willebrand disease in which a genetic deficiency in GPIb-IX or vWF, respectively, causes defects in platelet adhesion and activation and results in bleeding disorders (Lopez et al., 1998). GPIb-IX is also a receptor for the potent platelet agonist thrombin and synergizes with protease-activated receptors (PAR) in thrombin-induced platelet activation (Greco et al., 1996; Jamieson and Okumura, 1978; Ramakrishnan et al., 2001; Yamamoto et al., 1985).

Cyclic guanosine monophosphate (cGMP) is an important secondary messenger synthesized by guanylyl cyclases. Elevation of intracellular cGMP activates cGMP-dependent protein kinase (PKG) (Haslam et al., 1999). PKG regulates the functions of several intracellular molecules and pathways including the vasodilator-stimulated phosphoprotein (VASP) (Halbrugge et al., 1990) and extracellular stimuli-responsive kinase (ERK) pathway (Hood and Granger, 1998; Li et al., 2001). One important finding in cGMP signaling is that cGMP mediates nitric oxide (NO)-induced vascular smooth muscle relaxation (Furchgott and Vanhoutte, 1989). Based on this mechanism, a specific cGMP-enhancing agent, sildenafil (VIAGRA), has been developed and used to treat erectile dysfunction. Sildenafil selectively inhibits phosphodiesterase 5 (PDE5) that hydrolyzes cGMP and thus enhances intracellular cGMP levels (Corbin and Francis, 1999). The role of the cGMP-PKG pathway in platelet activation was controversial since increases in platelet cGMP levels were observed in response to both platelet agonists (thrombin, ADP, or collagen) and inhibitors (NO donors such as sodium nitroprusside) (for review, see Haslam et al., 1999). It is currently accepted that PKG inhibits platelet activation (Haslam et al., 1999). This concept is supported by the finding that preincubation of cGMP analogs with platelets inhibits platelet activation (Massberg et al., 1999; Mellion et al., 1981). Consistent with this concept, nitric oxide (NO) donors that inhibit platelet activation enhance intracellular cGMP (Haslam et al., 1999). This concept, however, does not explain why physiological platelet activators elevate platelet cGMP levels. Also, sildenafil enhances intracellular cGMP, but does not significantly inhibit platelet activation (Wallis et al., 1999). On the other hand, a small number of patients taking sildenafil have developed unexplained thrombotic conditions including heart attack (Arora et al., 1999; Feenstra et al., 1998; Mitka, 2000; Porter et al., 1999).

In this study, we present the novel finding that the cGMP-PKG pathway plays an important stimulatory role in GPIb-IX-mediated platelet activation and propose a new concept that cGMP-induced platelet responses are biphasic, consisting of an early stimulatory response that promotes platelet activation followed by a delayed platelet inhibition that serves to limit the size of platelet aggregates. Our findings explain previous controversies and provide revisions to the current concept of cGMP signaling in platelet activation.

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Results

PKG Promotes GPIb-IX-Mediated Integrin Activation in a Reconstituted Integrin Activation Model

We have recently shown that activation of the platelet integrin $\alpha_{IIb}\beta_3$ can be reconstituted in Chinese hamster ovary (CHO) cells expressing both recombinant human GPIb-IX and integrin $\alpha_{IIb}\beta_3$ (123 cells) (Gu et al., 1999). In this model, binding of vWF to GPIb-IX triggers activation of integrin $\alpha_{IIb}\beta_3$, allowing specific binding of fibrinogen (a physiological ligand of integrin $\alpha_{IIb}\beta_3$). We found in preliminary studies that a PKG inhibitor, KT5823, inhibited GPIb-IX-mediated integrin activation in this system (data not shown). To specifically identify the roles of PKG in GPIb-IX-mediated integrin activation, cDNAs encoding human PKG Iα or PKG Iβ were transfected into 123 cells. Results obtained with PKG Iα (Figure 1) and PKG Iβ (data not shown) were similar. Immunoblotting and PKG activity assays confirmed that human PKG I was expressed (Figure 1A). Three different clones each of PKG Iα- or Iβ-expressing cells were examined for vWF-induced integrin activation, as indicated by specific binding of soluble fibrinogen. We found that a low concentration of vWF (12 μg/ml) induced a slight increase in fibrinogen binding to vector-transfected 123 cells. In contrast, all tested 123 cell lines expressing human PKG showed a significantly enhanced fibrinogen binding (Figures 1B and 1C). More importantly, vWF-induced fibrinogen binding was dramatically increased in PKG-transfected cells in the presence of a membrane-permeable PKG activator, 8-bromo-cGMP (Figures 1B and 1C). Thus, recombinant human PKG stimulates GPIb-IX-mediated integrin activation in the reconstituted CHO cell model.

GPIb-IX-mediated integrin activation is important in stable platelet adhesion to immobilized vWF. Thus, we examined if PKG could augment this process. CHO cells expressing comparable levels of recombinant human GPIb-IX and/or integrin $\alpha_{IIb}\beta_3$ (Gu et al., 1999) were perfused into vWF-coated capillary tubes. GPIb-IX-expressing cells (1b9 cells) displayed transient adhesion and rolling on the vWF surface but failed to stably adhere (Figure 2A). Cells expressing integrin $\alpha_{IIb}\beta_3$ alone (2b3a cells) also showed minimal adhesion to vWF. 123 cells that express both GPIb-IX and integrin $\alpha_{IIb}\beta_3$ adhered to vWF as expected (Figure 2A). Remarkably, PKG-transfected 123 cells showed an enhanced stable cell adhesion compared to 123 cells (Figure 2A). Stable adhesion of PKG-expressing cells was inhibited by RGDS peptide (an integrin inhibitor), but not by the same concentration of control RGES peptide, indicating that PKG-stimulated stable adhesion is integrin dependent (Figure 2B). Furthermore, integrin-dependent adhesion of PKG-expressing 123 cells to vWF was enhanced by a specific cGMP-enhancing drug, sildenafil, or by 8-bromo-cGMP (data not shown). These data demonstrate that PKG promotes GPIb-IX- and integrin-dependent cell adhesion to vWF.

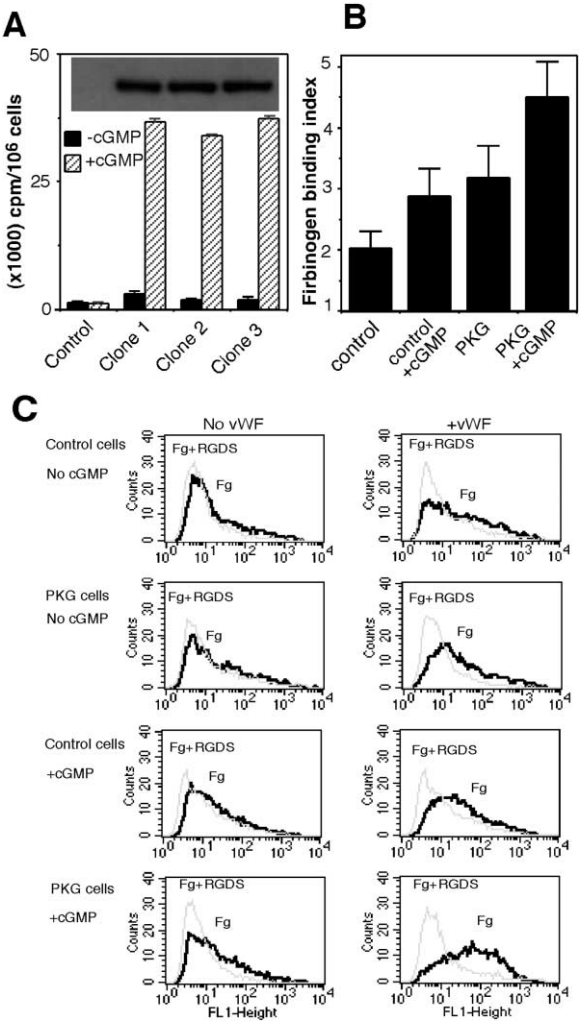

Figure 1. Expression of Recombinant PKG Promotes GPIb-IX-Mediated Integrin Activation

(A) CHO cells expressing GPIb-IX and integrin $\alpha_{IIb}\beta_3$ (123 cells) were transfected with cDNA encoding PKGIα or vector. Expression of PKG Iα in 123 cells was detected by immunoblotting with an anti-human PKG I antibody (Insert), and recombinant PKG activity was determined in the absence or presence of 20 μM 8-bromo-cGMP as previously described (Colbran et al., 1992). Results are expressed as mean $\pm$ SD (n = 3).

(B and C) The reconstitution of GPIb-IX-mediated integrin activation in CHO cells has been described previously (Gu et al., 1999). PKG- or vector-transfected 123 cells were incubated with Oregon green-labeled fibrinogen (Fg) (30 μg/ml) and 1 mg/ml ristocetin (no cGMP) for 30 min with (+vWF) or without (no vWF) the addition of 12 μg/ml of vWF. These cells were also incubated with Oregon green-labeled Fg, ristocetin, and 0.1 mM 8-bromo-cGMP (+cGMP) with or without the addition of vWF. Nonspecific binding was estimated by adding RGDS (Fg + RGDS). Cells were analyzed by flow cytometry. Quantitative results from three experiments are expressed as fibrinogen binding index (total bound fluorescence [Fg]/nonspecifically bound fluorescence [Fg + RGDS]) and shown in (B) (mean $\pm$ SD). Results from a representative experiment are shown in (C).

GPIb-IX- and Integrin-Dependent Platelet Spreading on vWF Is Inhibited in PKG Knockout Mice

A physiological role for GPIb-IX-mediated integrin activation is to mediate platelet spreading on immobilized vWF. Thus, if PKG plays a role in GPIb-IX-mediated integrin activation, deletion of the PKG gene should result in decreased platelet spreading on vWF. To specifically determine the physiological role of PKG in GPIb-IX-mediated integrin activation, platelets from wild-type

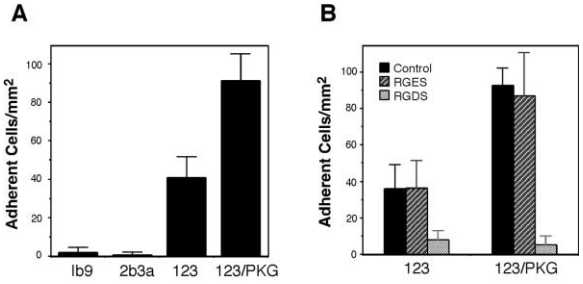

Figure 2. PKG Enhances GPIb-IX and Integrin-Dependent Cell Adhesion under Flow

(A) Stable adhesion of CHO cells expressing recombinant GPIb-IX alone (1b9); integrin $\alpha_{IIb}\beta_3$ alone (2b3a); both GPIb-IX and integrin $\alpha_{IIb}\beta_3$ (123); or expressing GPIb-IX, integrin $\alpha_{IIb}\beta_3$, and PKG Iα (123PKG) under flow was performed as described in Experimental Procedures. Adherent cells at 15 randomly selected locations in a vWF-coated capillary tube were counted. A significantly increased stable adhesion of 123PKG cells compared to 123 cells was observed ($p < 0.001$).

(B). Vector transfected 123 cells or 123PKG cells were perfused into vWF-coated capillary tubes either alone or in the presence of an integrin inhibitor RGDS peptide (4 mM) (+RGDS), or a control peptide, RGES (4 mM) (+RGES). Stable adhesion of 123PKG cells was significantly higher than vector-transfected 123 cells ($p < 0.001$) and was inhibited by RGDS peptide ($p < 0.001$), but not RGES peptide ($p = 0.713$).

or PKG knockout mice (Massberg et al., 1999; Pfeifer et al., 1998; Wegener et al., 2002) were allowed to adhere and spread on immobilized vWF stained with phalloidin and examined by fluorescence microscopy. As expected, wild-type mouse platelets fully spread on vWF (Figure 3A; WT). Spreading of wild-type platelets was integrin dependent since it was blocked by the integrin inhibitor, RGDS (Figure 3A, RGDS). In contrast to wild-type mice, platelets from PKG knockout mice spread poorly on vWF, in a manner similar to RGDS-treated platelets (Figure 3A, KO). Consistent with the results obtained with PKG knockout platelets, treatment of wild-type platelets with the PKG inhibitor, Rp-pCPT-cGMP, also inhibited platelet spreading on vWF (Figure 3A, WT + Rp-pCPT-cGMP). These results indicate that PKG plays an important role in GPIb-IX-mediated platelet activation and integrin-dependent platelet spreading on vWF. These results also indicate that the PKG inhibitor Rp-pCPT-cGMP specifically inhibited PKG function in platelets, equivalent to the results obtained with PKG knockout mice.

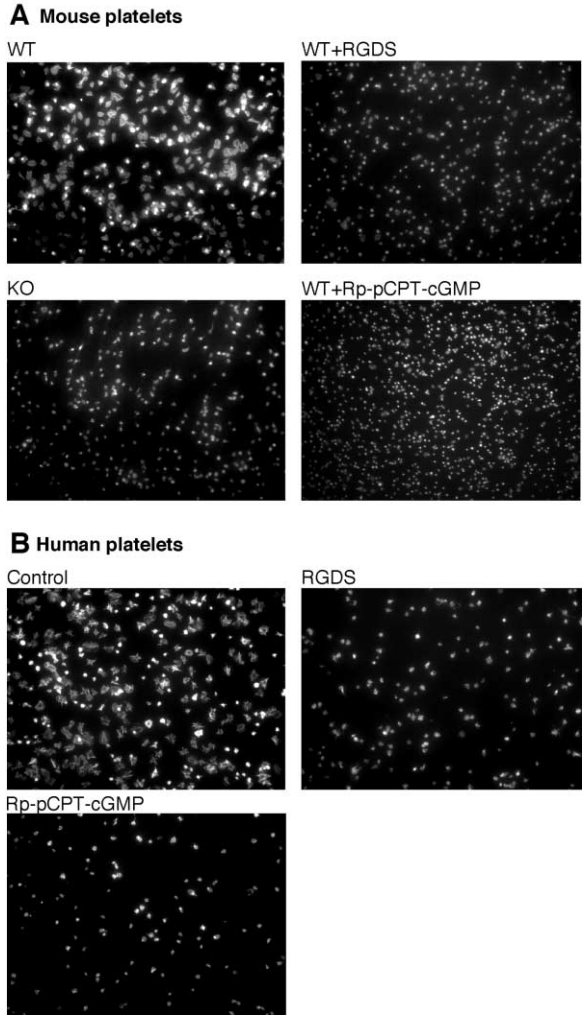

Figure 3. Inhibition of Platelet Spreading on vWF in PKG Knockout Mice and in PKG Inhibitor-Treated Mouse and Human Platelets

(A) Washed platelets from wild-type mice (WT) or homozygous PKG knockout mice (KO) were allowed to adhere and spread on immobilized vWF at 37°C for 1 hr as described in Experimental Procedures. Wild-type mouse platelets were also allowed to adhere to immobilized vWF in the presence of integrin inhibitor RGDS peptide (RGDS) (4 mM) or PKG inhibitor Rp-pCPT-cGMP (0.25 mM).

(B) Washed human platelets were treated without (control) or with integrin inhibitor RGDS or PKG inhibitor Rp-pCPT-cGMP and allowed to adhere and spread on immobilized vWF. Adherent platelets were stained with Rhodamine-labeled phalloidin and examined under a Nikon inverted fluorescence microscope (60× objective lens).

PKG Promotes vWF-Induced Human Platelet Activation

The above studies establish that PKG promotes GPIb-IX-dependent integrin activation in a reconstituted integrin activation model in CHO cells and in mouse platelets. It has recently been shown that vWF induces much more robust GPIb-IX-dependent platelet activation in humans than in mice (Canobbio et al., 2001). Thus, we examined whether PKG is important in GPIb-IX-dependent integrin activation in human platelets. Figure 3B shows that spreading of human platelets on vWF was integrin dependent because it was inhibited by RGDS. Spreading of human platelets on vWF was also inhibited by the PKG inhibitor, Rp-pCPT-cGMP (Figure 3B), indicating that PKG is important in integrin-dependent human platelet spreading on vWF.

In humans, vWF binding to GPIb-IX not only induces integrin-dependent platelet spreading on vWF, but also induces fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ and integrin-dependent platelet aggregation. We therefore examined the effects of PKG inhibitors or stimulators on vWF-induced fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ and platelet aggregation. GPIb-IX on platelets does not normally bind soluble vWF, but it does bind to subendothelial-bound vWF at sites of vascular injury. In in vitro studies,

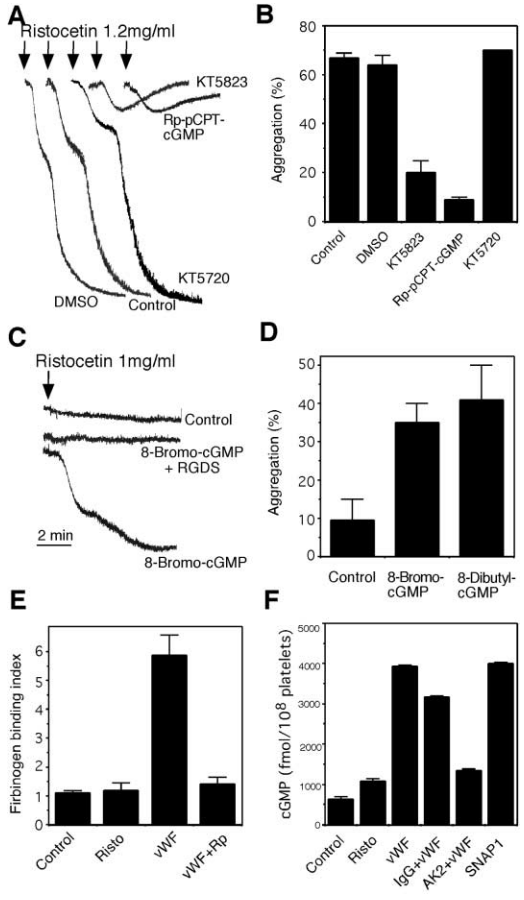

Figure 4. Effects of PKG Inhibitors and Activators on Integrin-Dependent Human Platelet Aggregation Induced by vWF-GPIb-IX Interaction

(A) Platelet rich-plasma (PRP) from a healthy human donor was preincubated at 37°C for 5 min with PKG inhibitors KT5823 (2 μM) or Rp-pCPT-cGMP (0.25 mM). PRP was also incubated with DMSO (vehicle for KT5823), the PKA inhibitor KT5720 (2 μM), or buffer (control). The vWF modulator, ristocetin, was then added to induce vWF-GPIb-IX interaction. Ristocetin-induced platelet aggregation was recorded using a platelet aggregometer.

(B) Quantitative data (mean ± SD) from three experiments as described in (A).

(C) A subthreshold concentration of ristocetin was added to PRP immediately followed by addition of 8-bromo-cGMP or buffer (control) to induce platelet aggregation. PRP was also preincubated with the integrin inhibitor RGDS before adding ristocetin and 8-bromo-cGMP (8-bromo-cGMP + RGDS).

(D) A subthreshold concentration of ristocetin (1 mg/ml) was added to PRP immediately followed by addition of buffer (control), 8-bromo-cGMP (100 μM,) or 8-dibutyl-cGMP (100 μM) to induce platelet aggregation. The data represent the mean ± SD of percentage of aggregation (light transmission) from three experiments.

(E) Washed platelets were preincubated without or with 0.25 mM PKG inhibitor, Rp-pCPT-cGMP (vWF + Rp), at 22°C for 10 min. The platelets were then allowed to bind Oregon green 488-labeled fibrinogen (30 μg/ml) in the absence (control) or presence of 20 μg/ml vWF and 1 mg/ml ristocetin (vWF) or in the presence of ristocetin alone as an additional control. Fibrinogen binding was examined by flow cytometry. Nonspecific binding of fibrinogen was estimated by adding a saturating concentration of integrin inhibitor, RGDS peptide (1 mM). The fibrinogen binding index is the ratio between mean fluorescence intensity in the absence of RGDS and the mean fluorescence intensity in the presence of RGDS peptide. The fibrinogen binding index is one when there is no specific fibrinogen binding.

the vWF modulators ristocetin or botrocetin mimic the effects of subendothelial matrix to induce vWF binding to human GPIb-IX. At appropriate concentrations, ristocetin-induced vWF binding to GPIb-IX causes reversible agglutination of platelets and activation of integrin $\alpha_{IIb}\beta_3$, leading to a second wave of platelet aggregation (Figures 4A and 4B). We found that the inhibitors of PKG, Rp-pCPT-cGMP and KT5823, diminished the integrin-dependent second wave of platelet aggregation (Figures 4A and 4B) and fibrinogen binding to platelets (Figure 4E) induced by vWF and ristocetin. Furthermore, 8-bromo-cGMP or 8-dibutyl-cGMP, when added to platelets together with a subthreshold concentration of ristocetin, induced integrin-dependent platelet aggregation (Figures 4C and 4D). Thus, we conclude that PKG plays an important stimulatory role in GPIb-IX-dependent human platelet activation.

vWF Induces an Increase in Platelet cGMP Levels

PKG activity is regulated by cGMP levels. Thus, if PKG is activated downstream of GPIb-IX, ligand binding to GPIb-IX would be expected to stimulate an increase in intracellular cGMP levels. Indeed, vWF induced a dramatic increase in platelet cGMP. The vWF-induced increase in cGMP levels was inhibited by a blocking anti-GPIbα monoclonal antibody, AK2, but not control IgG (Figure 4F). The cGMP levels in vWF-stimulated platelets were comparable to those in platelets stimulated with glyco-SNAP1 (a compound that releases nitric oxide and thus activates PKG) (Figure 4F). Thus, ligand binding to GPIb-IX increases the intracellular cGMP level to an extent that is capable of activating PKG.

The Role of PKG in Low-Dose Thrombin-Induced Platelet Aggregation

In addition to binding vWF, GPIb-IX also facilitates low-dose thrombin-induced platelet aggregation (Greco et al., 1996; Jamieson and Okumura, 1978; Yamamoto et al., 1985). To study the role of PKG in low-dose thrombin-induced platelet activation, 0.05 units/ml of α-thrombin was used to induce human platelet aggregation, which was integrin- and GPIb-IX-dependent as indicated by the inhibitory effects of RGDS and a monoclonal antibody specific for the thrombin binding site of GPIbα (Ruan et al., 1987; Ward et al., 1996) (Figure 5A). Platelet aggregation was inhibited by the PKG inhibitors KT5823 (2 μM) and Rp-pCPT-cGMP (Figures 5A and 5B). On the other hand, the membrane-permeable cGMP analog, 8-bromo-cGMP, induced platelet aggregation when added to platelets together with a subthreshold concentration (0.02 units/ml) of thrombin (Figure 5C).

To further examine the roles of PKG in low-dose

(F) Washed platelets (3×10^8/ml) were preincubated at 37°C for 10 min with buffer, control IgG, or a blocking monoclonal antibody against GPIbα, AK2. The platelets were then further incubated in a platelet aggregometer for 5 min after addition of buffer (control), 1.0 mg/ml ristocetin only (Risto), ristocetin plus 15 μg/ml vWF (vWF), or glyco-SNAP1 (100 μM). The reaction was stopped by addition of 400 μl of ice-cold 12% (w/v) trichloroacetic acid. cGMP concentrations were determined using a cGMP enzyme immunoassay kit. Results are expressed as mean ± SD (n = 3).

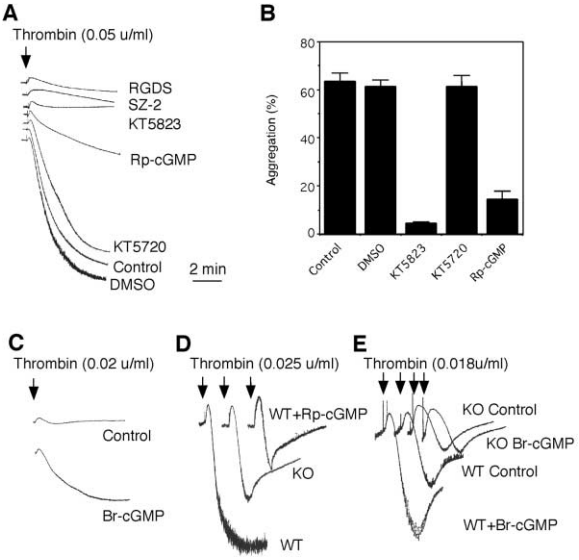

Figure 5. Effects of PKG Inhibitors and Stimulators and Effect of the PKG Knockout on Thrombin-Induced Platelet Aggregation

(A) Washed platelets were preincubated with 40 μg/ml anti-GPIbα monoclonal antibody SZ2, integrin inhibitor RGDS, or PKG inhibitors KT5823 (2 μM) or Rp-pCPT-cGMP (0.25 mM) for 5 min. The platelets were also preincubated with DMSO (vehicle for KT5823), KT5720, or buffer (control) as controls. α-thrombin (0.05 units/ml) was then added to induce platelet aggregation. Nonspecific IgG and RGES peptide were also examined as additional controls but had no effect (data not shown).

(B) Experiments described in (A) were repeated three times with the results quantified as percentage of light transmission (mean ± SD).

(C) A subthreshold dose of thrombin was added to human platelets followed immediately by addition of the PKG activator, 8-bromo-cGMP, or buffer (control). Shown in the figure are representative results of at least three experiments.

(D) Washed platelets from wild-type (WT) or $PKG^{-/-}$ mice (KO) in modified Tyrode's solution (Du et al., 1991) were stimulated with 0.025 u/ml of α-thrombin in a turbidometric platelet aggregomoter. Wild-type platelets were also preincubated with the PKG inhibitor Rp-pCPT-cGMP (0.25 mM) for 5 min and then exposed to 0.025/ml α-thrombin.

(E) A subthreshold dose of thrombin was added to wild-type (WT) or $PKG^{-/-}$ (KO) mouse platelets followed immediately by addition of the PKG activator 8-bromo-cGMP (10 μM) or buffer (control).

thrombin-induced platelet activation, a low dose of α-thrombin was added to platelets from wild-type or PKG knockout mice to induce aggregation. We found that platelet aggregation was markedly reduced in PKG knockout mice compared to wild-type mice (Figure 5D). Wild-type platelets treated with the PKG inhibitor Rp-pCPT-cGMP also showed reduced platelet aggregation similar to PKG knockout mice (Figure 5D). As previously reported (Massberg et al., 1999), collagen-induced platelet aggregation was normal in PKG knockout mice or PKG inhibitor-treated wild-type mice (data not shown), suggesting that PKG promotes platelet aggregation in an agonist-selective manner. Furthermore, addition of a membrane-permeable cGMP analog, 8-bromo-cGMP, enhanced platelet aggregation induced by a subthreshold concentration of thrombin in wild-type platelets, but not in PKG knockout platelets (Figure 5E). These data indicate that PKG promotes platelet activation induced by low doses of thrombin.

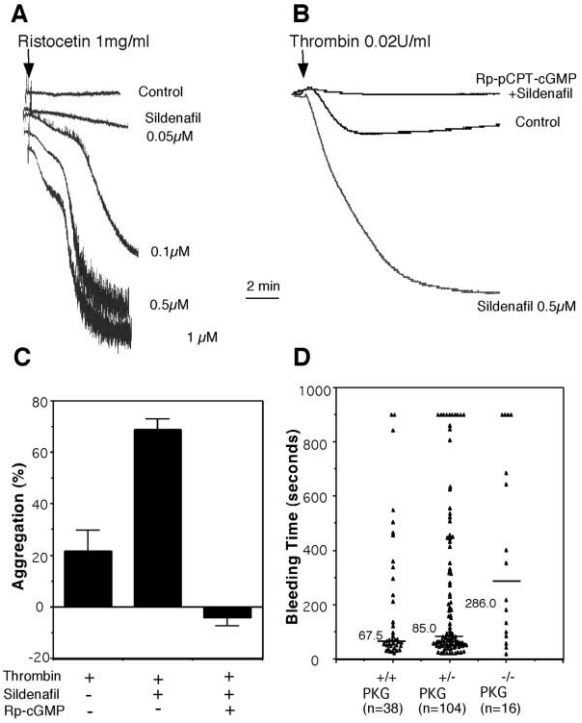

Figure 6. Effects of Sildenafil on Human Platelet Aggregation and Effect of the PKG Knockout on In Vivo Bleeding Time in Mice

(A) Increasing concentrations of sildenafil or control 0.15 M NaCl solution were added to human PRP followed by a subthreshold concentration of ristocetin. Platelet aggregation was recorded using a platelet aggregometer.

(B) Washed human platelets were preincubated in the absence or presence of a PKG inhibitor, Rp-pCPT-cGMP. Sildenafil or control buffer was then added to the platelets followed by addition of subthreshold doses of thrombin to induce platelet aggregation.

(C) Experiments described in (B) were repeated three times with the results quantified as percentage of light transmission.

(D) Bleeding time tests were performed blind to genotype in littermate mice (5–6 weeks old) generated from mating $PKG^{+/-}$ mice, using methods described under Experimental Procedures. Genotypes of the tested mice were subsequently determined by PCR analysis. The solid triangles represent the bleeding time of a single mouse. The bar represents the median bleeding time of the group. Note that although the variance of bleeding time was broad in both the control and $PKG^{-/-}$ groups (reflecting that multiple factors may influence the outcome of this in vivo test), the difference between $PKG^{+/+}$ and $PKG^{-/-}$ mice is statistically significant ($p = 0.017$).

The cGMP-Enhancing Drug Sildenafil Promotes Platelet Activation In Vitro

The cGMP-enhancing drug sildenafil increases intracellular cGMP and activates PKG. Thus, if PKG stimulates platelet activation, sildenafil might also be stimulatory. Indeed, addition of sildenafil to platelets together with subthreshold concentrations of either ristocetin (Figure 6A) or thrombin (Figures 6B and 6C) induced platelet aggregation. The stimulatory role of sildenafil was PKG dependent since it was inhibited by preincubation of platelets with the PKG inhibitor Rp-pCPT-cGMP. Since sildenafil functions by specifically increasing endogenous cGMP, this result further excludes the possibility of nonspecific effects of exogenous cGMP analogs and supports the conclusion that the cGMP-PKG pathway

promotes platelet activation. This result also provides novel in vitro evidence that sildenafil may potentiate platelet activation.

Prolonged Bleeding Time in PKG Knockout Mice

The above ex vivo data show that PKG promotes platelet activation. To investigate if the stimulatory role of PKG is important for hemostasis in vivo, tail bleeding time tests were performed blind to genotype in 5- to 6-week-old avertin-anesthetized littermates generated from heterozygous breeding of $PKG^{+/-}$ mice. Genotypes of these mice were subsequently determined by PCR analyses, using DNA extracted from the amputated tail after the bleeding time tests. Among the 158 mice tested, 38 were $PKG^{+/+}$ mice (wild-type) with a median bleeding time of 67.5 s, and 104 were $PKG^{+/-}$ mice with a median bleeding time of 85.0 s. In contrast, the median bleeding time of 16 $PKG^{-/-}$ mice (286.0 s) was significantly prolonged ($p = 0.017$) (Figure 6D). Thus, PKG plays an important role in supporting hemostasis in vivo.

cGMP Induces Biphasic Platelet Responses

The stimulatory role of the cGMP pathway in GPIb-IX-dependent platelet activation appears to contradict the concept that cGMP inhibits platelet activation. However, we noted that there is a major difference in the timing of cGMP elevation between our experiments and those in previous studies. We therefore examined the relationship between the timing of cGMP elevation and its effects on platelets. Addition of cGMP analogs (0.05–0.5 mM) simultaneously with platelet agonists (ristocetin [Figure 4C] or thrombin [Figure 5B]) had no inhibitory effects but promoted platelet aggregation (also see Figure 7A). However, when platelets were preincubated with cGMP for increasing lengths of time, the stimulatory effect was diminished, and cGMP became increasingly inhibitory (Figure 7A). With increasing cGMP concentrations, the inhibitory phase appeared progressively earlier. Only at unusually high 8-pCPT-cGMP concentrations (>1 mM) were the inhibitory effects seen without preincubation (data not shown). Thus, cGMP exerts a time- and concentration-dependent biphasic effect on platelet activation. Consistent with the delayed inhibitory effects of cGMP on platelet aggregation, we also observed that the cGMP-induced phosphorylation of VASP (a protein important in the inhibitory effects of cGMP [Aszodi et al., 1999; Hauser et al., 1999]) is also delayed with significant phosphorylation occurring only after a 10 min incubation with cGMP (Figure 7B). In contrast to VASP phosphorylation, a different cGMP-induced intracellular event, the cGMP-induced phosphorylation of ERK (a protein kinase important in stimulating platelet activation) (Li et al., 2001), occurred rapidly, reaching a maximum within 1 min of cGMP incubation. It was, however, rapidly dephosphorylated with increasing length of cGMP incubation (Figure 7B). These data indicate that the apparently paradoxical effects of cGMP in platelets are in fact biphasic effects: an early stimulatory effect and a delayed inhibitory effect.

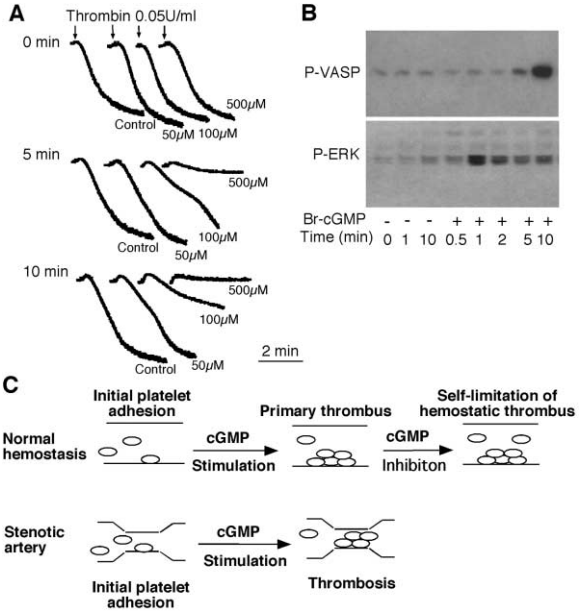

Figure 7. Biphasic Roles of cGMP in Platelet Activation

(A) Washed platelets were preincubated without (control) or with 8-pCPT-cGMP (50, 100, and 500 μM) for 0, 5, and 10 min and then exposed to thrombin to examine the inhibitory effects of cGMP on platelet aggregation. At the zero time point, cGMP had no inhibitory effect but promoted platelet aggregation induced by subthreshold concentrations of thrombin (cf. Figure 5). After prolonged incubation, cGMP became inhibitory.

(B) Washed platelets (1×10^9/ml) were incubated with 8-bromo-cGMP for increasing lengths of time and then solublized directly into SDS-PAGE sample buffer. Phosphorylation of VASP was detected by immunoblotting with the monoclonal antibody 16C2, which specifically recognizes phosphorylated Ser^{239} in VASP (Smolenski et al., 1998). Phosphorylation of ERK MAP kinase was detected by immunoblotting, using a rabbit antibody specific for the phosphorylated Thr^{202}/Tyr^{204} site of ERK. Data shown are representative of three independent experiments.

(C) Upon vascular injury, platelets adhere to the subendothelial matrix via GPIb-IX interaction with vWF and are exposed to soluble agonists such as thrombin. This induces cGMP elevation, which synergizes with G protein-coupled signaling pathways, leading to platelet activation and formation of a hemostatic thrombus. Continued cGMP elevation in aggregated platelets induces a second phase of inhibitory cGMP signaling, resulting in inhibition of further recruitment of platelets. This prevents the overgrowth of hemostatic thrombus (Freedman et al., 1997), thus reducing the probability of thrombosis during normal hemostasis. However, in patients at thrombotic risk, the initial phase of cGMP-promoted thrombus formation may be sufficient to cause thrombosis.

Discussion

Two novel concepts are derived from the data presented in this paper: first, that the cGMP-PKG pathway plays an important stimulatory role in GPIb-IX-dependent platelet activation induced by vWF and thrombin; and second, that platelet responses to cGMP are biphasic, consisting of an early stimulatory response that promotes thrombus formation and a late inhibitory response that limits the size of platelet aggregates. A direct pharmacological implication derived from these concepts and data is that cGMP-enhancing drugs, such as sildenafil, may promote platelet activation in the presence of subthreshold concentrations of the platelet agonists, thrombin, and vWF.

The conclusion that GPIb-IX mediates platelet activation via the PKG pathway is based on data derived from

experiments using platelets from PKG knockout mice, pharmacological studies in human platelets, and studies using recombinant PKG expression in a reconstituted integrin activation model. The results obtained using recombinant DNA techniques and PKG knockout mice exclude the possibility of nonspecific effects of pharmacological reagents, and studies in human platelets indicate that the results obtained in the CHO cell model and knockout mouse model appropriately reflect the events that occur in human platelets. Furthermore, the effects of PKG inhibitors and activators as well as cGMP-enhancing agents with different mechanisms of action are consistent with each other and with the experiments using recombinant DNA technologies, making it unlikely that potential nonspecific effects of one drug could influence the outcome of our studies. GPIb-IX, as a receptor for vWF, plays critical roles in platelet adhesion and activation. Although it is known that vWF binding to GPIb-IX activates platelets, the signaling pathways remain poorly defined. Thus, our finding of a PKG-dependent platelet activation pathway is significant not only because it provides convincing evidence of a stimulatory role for PKG in platelet activation, but also because it defines a novel signaling mechanism of platelet adhesion and activation.

An important platelet agonist, thrombin, requires PARs to induce platelet activation. However, at low thrombin concentrations, PARs are not sufficient, and a different thrombin receptor, GPIb-IX, is also required. At present, it is not clear how GPIb-IX is involved in thrombin-induced platelet activation. One hypothesis is that GPIb-IX functions as a thrombin-presenting receptor that enhances the interaction between PARs and thrombin (Coughlin, 2000). Alternatively, it is hypothesized that GPIb-IX may transmit signals that synergize with PARs in promoting platelet activation (Greco et al., 1996). Our data suggest a possible PKG-dependent signaling mechanism for the synergism between GPIb-IX and PARs. It is important to note that the stimulatory role of cGMP manifests only when platelets are exposed simultaneously to the agonists, α-thrombin, or vWF. Elevation of cGMP alone is not sufficient to activate platelets (data not shown). Thus, it appears that the cGMP-PKG pathway is not the only pathway required for GPIb-IX-mediated integrin activation. In this respect, it has been shown that vWF-induced platelet activation involves the coordination of different signaling pathways and requires the Fcγ receptor IIa/Fc receptor γ-chain and Syk signaling pathway (Canobbio et al., 2001; Sullam et al., 1998; Wu et al., 2001).

Although a stimulatory role for the cGMP-PKG pathway in platelet activation apparently contradicts previous observations of inhibitory effects of cGMP analogs or NO donors on platelet activation, this contradiction is well explained by our finding that cGMP induces biphasic platelet responses. That is, the early stimulatory response to cGMP is followed by an inhibitory response (Figure 7). Consistent with our finding, inhibitory effects of cGMP reported in previous publications were observed only after prolonged preincubation of cGMP analogs with platelets. The lower the cGMP concentration, the longer the required preincubation time (Massberg et al., 1999; Mellion et al., 1981). Also, cGMP-induced platelet inhibition requires a longer period of preincubation than for high concentrations of NO donors (Azula et al., 1996; Maurice and Haslam, 1990), suggesting that the inhibitory effects of NO donors may involve either a higher peak of cGMP elevation or other pathways in addition to cGMP (Azula et al., 1996; Maurice and Haslam, 1990; Yan and Smith, 2000). Interestingly, it was reported that cGMP elevation induced by endogenous platelet NO had no inhibitory effect on primary platelet activation but, after a delay, inhibited recruitment of subsequently added platelets to already formed aggregates (Freedman et al., 1997). It was also shown that while platelet adhesion and thrombus formation occurred normally at a site of ischemia/reperfusion injury (involving multiple platelet agonists) in wild-type mice, more platelets appeared to be recruited to the site of ischemia/reperfusion injury in PKG-deficient mice (Massberg et al., 1999), Thus, the biphasic response of platelets to cGMP elevation may be of physiological significance because this response would not only mediate rapid activation of platelets upon vascular injury and formation of a primary hemostatic thrombus, but would also serve to inhibit the overgrowth of thrombus to prevent the occlusion of blood vessels during normal hemostasis (Figure 7C). The mechanisms for the biphasic effect of cGMP remain to be investigated. Our data suggest that cGMP induces an early activation of the ERK pathway that is important in platelet activation (Li et al., 2001), followed by a delayed VASP phosphorylation, which is involved in platelet inhibition (Figure 7B). We have also found that cAMP-dependent protein kinase is important in cGMP-induced VASP phosphorylation and platelet inhibition (Z. Li et al., submitted). Thus, one of the possible mechanisms for the transition from stimulatory to inhibitory responses may involve the crosstalk between the cGMP and cAMP pathways.

Our finding of a stimulatory role of the cGMP-PKG pathway in platelet activation is important not only to the understanding of platelet physiology, but also to the pharmacology and therapeutic applications of cGMP-enhancing drugs such as sildenafil. Our data suggest that although sildenafil by itself is not sufficient to cause platelet aggregation, this drug enhances platelet activation in the presence of subthreshold concentrations of thrombin or vWF, agonists that are likely to be present at sites of vascular injury or atherosclerotic lesions. Furthermore, the half maximal effective concentration of sildenafil that promotes vWF-induced platelet activation is $\sim$0.1 μM. In comparison, the mean peak plasma concentration of sildenafil in healthy males after oral administration of a 100 mg tablet is $\sim$0.66 μM (http://www.pfizer.com/hml/pi's/viagrapi.pdf). Thus, sildenafil may possibly potentiate platelet activation in patients with preexisting thrombotic conditions. Further clinical studies may help to determine if this effect of sildenafil could explain the thrombotic complications in a small number of patients taking sildenafil.

Experimental Procedures

Effects of Recombinant PKG Expression on GPIb-IX-Mediated Integrin Activation

Human PKG Iβ cDNA was cloned by RT-PCR, using human platelet mRNA as the template. The sequence of the PKG Iβ cDNA matches the published sequence (Sandberg et al., 1989). Cloning of human

PKG Iα cDNA was described previously (Browning et al., 2000). PKG Iα or Iβ in *pCDNA3.1/Zeo+* vector was transfected into 123 cells using Lipofectamine plus (BRL). Expression of PKG was assessed by immunoblotting with an anti-human PKG I antibody (Calbiochem). The kinase activity of the expressed enzymes was measured in cell homogenates using BPDEtide (Calbiochem) as substrate as detailed previously (Colbran et al., 1992). vWF-induced activation of integrin $\alpha_{IIb}\beta_3$ was examined by flow cytometric analysis of Oregon green-labeled fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ as described previously (Gu et al., 1999).

Transfected CHO Cell Adhesion to vWF

Purified human vWF (50 μg/ml in 0.1 M $NaHCO_3$ [pH 8.3]) was added to glass microcapillary tubes (inner diameter of 1.17 mm and a length of 7.5 cm) (Warner Instrument Corp) and incubated at 22°C for 3 hr. The vWF-coated microcapillary tubes were then incubated with 5% nonfat dry milk in phosphate-buffered saline solution (pH 7.4) (PBS) to block nonspecific binding. CHO cells expressing human platelet receptors were resuspended in modified Tyrode's buffer (pH 7.4) containing 1 mg/ml of bovine serum albumin (BSA) (Du et al., 1991) and at a final cell count of 5×10^6/ml. The cell suspension was aspirated through the tube by a syringe pump (Harvard Apparatus, Inc.) at a flow rate of 1.42 ml/min (150/s) for 1.2 min. The tube was then washed with modified Tyrode's buffer for an additional 5 min at the same flow rates to remove transiently adherent cells. Cell interaction with immobilized vWF was observed in real time under a Zeiss inverted microscope equipped with a CCD camera and was recorded using a video cassette recorder. The number of stably adherent cells on immobilized vWF was counted on images obtained at 15 randomly selected positions in a vWF-coated tube.

PKG Knockout Mice and Mouse Platelet Preparation

The generation of a PKG null (−) allele by homologous recombination has been described previously (Wegener et al., 2002). Male and female mice (6–8 weeks) were anesthetized by intraperitoneal injection of pentobarbital. Whole blood from homozygous PKG knockout mice or wild-type mice was collected from the inferior vena cava using 1/7 volume of ACD (2.5% trisodium citrate, 2% dextrose, and 1.5% citric acid) as anticoagulant. For each experiment, platelets were pooled from five to six mice of each genotype. The platelets were then washed twice with CGS (0.12 M sodium chloride, 0.0129 M trisodium citrate, 0.03 M D-glucose, [pH 6.5]), resuspended in Tyrode's buffer at 2×10^8/ml, and incubated at room temperature for 1 hr before use.

Human Platelet Preparation and Aggregation Assay

Fresh blood from healthy volunteers was anticoagulated with 1/7 volume of ACD. Platelets were washed with CGS, resuspended in modified Tyrode's solution, and allowed to incubate at 22°C for 1–2 hr as previously described (Du et al., 1991). In experiments using platelet-rich plasma (PRP), 1/10 volume of 3.8% trisodium citrate was used as anticoagulant. Platelet aggregation was measured using a turbidometric platelet aggregometer (Chrono-Log) at 37°C. vWF-dependent platelet aggregation was induced by addition of ristocetin or botrocetin to PRP. Washed platelets were used for platelet aggregation induced by α-thrombin (Enzyme Research Laboratories). To examine the effects of PKG, various concentrations of the membrane-permeable cGMP analogs 8-bromo-cGMP, 8-pCPT-cGMP, or 8-dibutyl-cGMP (Calbiochem), or sildenafil citrate (Pfizer) were added simultaneously with subthreshold concentrations of agonists. The PKG inhibitors KT5823 (2 μM) and Rp-pCPT-cGMP (0.25 mM) (Calbiochem) were preincubated with platelets at 37°C for 5 min before addition of agonists.

Platelet Spreading on vWF

Washed mouse or human platelets were allowed to adhere and spread on 20 μg/ml vWF-coated glass chamber slides (Nunc) at 37°C for 1 hr in the presence of a low concentration of the vWF modulator, botrocetin (0.5 μg/ml), which enhances vWF-GPIb-IX interaction. After three washes, platelets were fixed by adding 4% paraformaldehyde in PBS, permeabilized by adding 0.1 M Tris, 0.01 M EGTA, 0.15 M NaCl, 5 mM $MgCl_2$ [pH 7.4], containing 0.1% Triton X-100, 0.5 mM leupeptin, 1 mM PMSF, and 0.1 mM E64, and then incubated with 2 μg/ml of rhodamine-labeled phalloidin at 22°C for 30 min. After additional washes, the slides were mounted and examined under a Nikon fluorescence microscope.

Measurement of cGMP Levels

Washed platelets (3×10^8/ml) in 400 μl Tyrode's buffer were stirred at 37°C after addition of control buffer, 1 mg/ml ristocetin alone, 15 μg/ml vWF, and 1 mg/ml ristocetin, or 100 μM glyco-SNAP1 (Calbiochem). The reaction was stopped by addition of 400 μl of ice-cold 12% (w/v) trichloroacetic acid. Samples were mixed and centrifuged at $2000 \times g$ for 15 min at 4°C. The supernatant was removed and washed four times with 5 volumes of water-saturated diethyl ether and then lyophilized. cGMP levels were measured using a cGMP enzyme immunoassay kit from Amersham-Pharmacia Biotech.

Bleeding Time Analysis in PKG Knockout Mice

Mice were anesthetized with 2.5% (10 ml/kg) Avertin (100% [w/v] tribromoethyl alcohol in tertiary amyl alcohol) in PBS. Tails were amputated 0.5 cm from the tip and immediately immersed in 0.15M sodium chloride at 37°C. Bleeding time was followed visually and time to cessation was recorded. If bleeding did not naturally stop after 900 s, the wound was pressured to prevent death from hemorrhage. The amputated segments of tails were saved for subsequent DNA preparation and PCR analysis of genotype as described previously (Wegener et al., 2002).

Analysis of cGMP-Induced VASP and ERK Phosphorylation in Platelets

Washed human platelets (1×10^9/ml) were incubated with 8-bromo-cGMP for increasing lengths of time at 37°C. The platelets were then solubilized in SDS-PAGE sample buffer, analyzed by SDS-PAGE on 4%–15% gradient gels, and electrotransfered to PVDF membranes. The membranes were immunoblotted with a monoclonal antibody, 16C2, specific for the phosphorylated Ser^{239} site of VASP (0.2 μg/ml) (Smolenski et al., 1998) or a rabbit antibody specific for the phosphorylated form of ERK (New England Biolabs) as previously described (Li et al., 2001).

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