Platelet secretion (exocytosis) is critical in amplifying platelet activation, in stabilizing thrombi, and in arteriosclerosis and vascular remodeling. The signaling mechanisms leading to secretion have not been well defined. We have shown previously that cGMP-dependent protein kinase (PKG) plays a stimulatory role in platelet activation via the glycoprotein Ib-IX pathway. Here we show that PKG also plays an important stimulatory role in mediating aggregation-dependent platelet secretion and secretion-dependent second wave platelet aggregation, particularly those induced via Gα-coupled agonist receptors, the thromboxane A2 (TXA2) receptor, and protease-activated receptors (PARs). PKG I knock-out mouse platelets and PKG inhibitor-treated human platelets showed diminished aggregation-dependent secretion, and also showed a diminished secondary wave of platelet aggregation induced by a TXA2 analog and thrombin receptor-activating peptides that were rescued by the granule content ADP. Low dose collagen-induced platelet secretion and aggregation were also reduced by PKG inhibitors. Furthermore PKG I knock-out and PKG inhibitors significantly attenuated activation of the Gq pathway that is mediated by secreted ADP. These data unveil a novel PKG-dependent platelet secretion pathway and a mechanism by which PKG promotes platelet activation.

The secretion of granule contents (exocytosis) is an important cellular function shared by blood platelets, leukocytes, neurons, endocrine glands, and many other cell types. In platelets, the secretion of granules plays critical roles in amplifying platelet activation, recruitment of platelets into aggregates, and formation and stabilization of thrombi at the site of vascular injury (1). The mechanisms of granule secretion have not been totally clear but are known to require molecules that are shared by different secretory cells. These include soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs),1 which mediate fusion between granules (vesicles) and plasma membranes and cargo release (1–3). There are many other similarities between different cell types in the secretion of granule contents. For example, platelet-dense granules uptake, store, and secrete the neurotransmitter serotonin in a way similar to certain neuron cell presynaptic vesicles (1). Secretion of granules is a regulated process in platelets and many other secretory cells and is known to involve common signaling mechanisms such as the elevation of intracellular calcium levels and phosphorylation of SNARE proteins (1). Platelet secretion can be induced by soluble agonists such as thromboxane A2 (TXA2) and thrombin that binds to G-protein-coupled heptahelical receptors (4–8) or by adhesion proteins such as collagen and von Willebrand factor (9–11). So called “weak agonists,” or low concentrations of agonists, induce activation of integrins and platelet aggregation and subsequently require integrin outside-in signaling to induce aggregation-dependent platelet secretion. However, “strong agonists” such as collagen can induce aggregation-independent granule secretion via pathways that do not require integrin outside-in signaling. TXA2 induces a unique two-wave platelet secretion: a small peak of aggregation-independent platelet secretion, which precedes the first wave of platelet aggregation, and a large peak of aggregation-dependent second wave platelet secretion, which induces the second wave of platelet aggregation (12). Thus, TXA2 is a useful tool in differentiating signaling mechanisms associated with aggregation-dependent or -independent platelet secretion.

cGMP is an important secondary messenger synthesized by guanylyl cyclases (13, 14). Elevation of cGMP activates cGMP-dependent protein kinase (protein kinase G, PKG), which plays key roles in regulating physiological functions including vessel dilation, neuron function, and platelet activation (13, 15). The roles of the cGMP-PKG pathway in platelet activation have been controversial (13, 16–18). Although it has been believed for many years that the cGMP-PKG pathway inhibits platelet function (13), we have shown recently that the cGMP-PKG pathway in fact plays a biphasic role in platelet activation (19). An early stimulatory role of cGMP is important in the adhesion receptor, the glycoprotein Ib-IX (GPIb-IX), dependent platelet activation (19). A late inhibitory role of cGMP requires high concentrations of cGMP and is predominantly dependent upon cGMP-mediated activation of cAMP-dependent protein kinase (PKA) in humans (19–21). However, the role of PKG in platelet activation remains apparently controversial because PKG knockout, although showing reduced GPIb-IX-dependent platelet activation, did not affect platelet aggregation induced by a

1 The abbreviations used are: SNARE, N-ethylmaleimide-sensitive factor attachment protein receptor; TXA2, thromboxane A2; PKG, protein kinase G/cGMP-dependent protein kinase; GPIb-IX, glycoprotein Ib-IX; PTPX, pertussis toxin; Rp-pCPT-cGMPS, Rp-isomer-2′-O-(etheno-8-bromo-β-phenyl-1-guanosine 3′,5′-cyclic monophosphorothioate; Rp-Br-PET-cGMPS, Rp-isomer-2′-O-etheno-8-bromo-β-phenyl-1-guanosine 3′,5′-cyclic monophosphorothioate; RP, platelet-rich plasma; TRAP, thrombin receptor-activating peptide; PAR, protease-activated receptor.
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Materials—The TXA2 analog U46619, pertussis toxin (PTX), and PKG inhibitors Rp-isomer-8-(4-chlorophenylthio)-guanosine 3’5’-cyclic monophosphorothioate (Rp-pCPT-cGMPS), Rp-isomer-N’-etheno-8-bromo-β-phenyl-1-guanosine 3’5’-cyclic monophosphorothioate (Rp-Br-PET-cGMPS), and KT5823 were purchased from Calbiochem (San Diego, CA). ADP, apyrase (grade III), and forskolin were from Sigma. Collagen and luciferase/luciferin reagent were from Chrono-log (Havertown, PA). Indo-1 was purchased from Molecular Probes (Eugene, OR).

Platelet Aggregation and Secretion—Fresh blood from healthy volunteers was anticoagulated with 1/7 volume of ACD as anticoagulant as described previously (25). Male and female mice (6–8 weeks) were used as described previously (12). For each experiment, blood was pooled from 5–6 mice of each genotype. The platelets were then washed twice with PBS-W (W): 137 mM NaCl, 2.7 mM KCl, 1.2 mM MgCl2, 81 mM Na2HPO4, 0.45 mM KH2PO4, 0.8 mg/ml glucose, pH 7.3. Washed platelets from healthy human donors were resuspended in Tyrode’s buffer and preincubated with or without apyrase, PKG inhibitors Rp-pCPT-cGMPS, RP-Br-PET-cGMPS, or KT5823 (250 μM) were then added to the platelets. After incubation for 5 min in a platelet aggregometer at 37 °C with stirring (1000 rpm). To examine the effects of PKG inhibitors, PRP or washed platelets were preincubated with KT5823 (5 μM) or Rp-pCPT-cGMPS (250 μM) at 37 °C for 5 min. PRP was also preincubated with buffer (Control) or AP (apyrase, vehicle for KT5823). Platelet aggregation was induced by adding U46619 (C) or ADP (D).

Calcium Mobilization—Calcium mobilization was measured in Indo-1/AM-loaded cells. Briefly, washed platelets were resuspended in PBS-W at 1 x 10^7/ml and incubated with 5 μM Indo-1/AM at 37 °C for 45 min. After washing with PBS-W once more, platelets were resuspended to 2 x 10^8/ml in modified Tyrode’s solution. Continuous fluorescent measurements of calcium-bound and free Indo-1/AM were made using a PTI spectrophotometer, detecting at 405 and 485 nm, respectively, with an excitation wavelength of 340 nm. The intracellular Ca^2+ level was expressed as relative fluorescence, calculated based on the ratio of Indo-1 fluorescence at 405 and 485 nm and standardized for Indo-1 loading and cell responsiveness.

RESULTS

PKG Plays a Stimulatory Role in Platelet Aggregation—Induced by Several G-protein-coupled Receptor Agonsists—Platelets can be activated via several different types of signaling pathways. G-protein-coupled signaling pathways play a major role in platelet activation induced by soluble platelet agonists such as thrombin, TXA2, and ADP (6–8, 26, 27). The glycoprotein VI/Fc receptor γ chain pathway mediates collagen-induced platelet activation (28–30). The GPlb-IX-dependent activation pathway mediates von Willebrand factor-induced platelet activation (11, 31–33) and is also important in low dose thrombin-induced platelet activation (34–37). We reported recently that PKG plays a stimulatory role in GPlb-IX-dependent integrin activation (19, 20). To investigate whether and how PKG plays roles in platelet activation via various pathways, we examined the platelet aggregation response of PKG I knock-out mouse or PKG inhibitor-treated human platelets to GPlb-IX-independent platelet agonists. These include U46619 (a stable thromboxane A2 analog) and ADP (Fig. 1). PKG I knockout (Fig. 1A) or PKG inhibitors Rp-pCPT-cGMPS, KT5823 (Fig. 1C), or Rp-Br-PET-cGMPS (see below) significantly reduced platelet aggregation induced by U46619 but had no significant effect on platelet aggregation induced by ADP (Fig. 1D). Thus, PKG plays an important role in promoting platelet aggregation in-
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human platelet aggregation as we have shown previously (19), they only partially inhibited human platelet aggregation induced by PAR1 or PAR4 TRAPs (Fig. 2), suggesting that PKG plays distinct roles in GPIb-IX-dependent platelet response and PAR-induced platelet responses.

PKG Mediates Secretion of Dense Granules—The partial inhibitory effects of PKG inhibitors on U46619 and TRAP-induced platelet aggregation resemble the pattern of platelet aggregation in the absence of aggregation-dependent platelet secretion. Thus, we further investigated the roles of PKG in platelet dense granule secretion using real time luciferin-luciferase assay. As we have shown recently (12), U46619 sequentially induced the first wave of dense granule secretion, the first wave of platelet aggregation, and then the second wave of aggregation-dependent secretion followed by the second wave of platelet aggregation (Fig. 2). The second wave of dense granule secretion and the subsequent second wave of platelet aggregation were abolished in PKG I knock-out mouse platelets (Fig. 2A) and in PKG inhibitor-treated human platelets (Fig. 2B). Thrombin or TRAPs induce both aggregation-dependent and aggregation-independent secretion (not shown). At low concentrations, the aggregation-dependent secretion is predominant. Low dose TRAP1- or TRAP4-induced platelet secretion was also significantly inhibited by PKG inhibitors (Fig. 2, C and D). Thus, PKG plays an important role in mediating a common aggregation-dependent secretion pathway induced by several G-protein-coupled receptors.

The Roles of PKG in Collagen-induced Platelet Secretion of Dense Granules—Collagen can induce significant platelet secretion in an aggregation-independent manner, and platelet aggregation induced by a relatively high dose of collagen is not significantly affected by knockout of PKG I (22). However, these findings do not exclude the possibility that aggregation-dependent secretion can still occur. Indeed, platelet secretion induced by a low dose thrombin (0.5 μg/ml) is mostly aggregation-dependent because the integrin inhibitor RGDS almost completely inhibited ATP secretion induced by collagen at this low concentration (Fig. 3A). Thus, to investigate whether the role of

**Fig. 2.** Effects of PKG knockout and PKG inhibitors on platelet secretion and aggregation induced by G-protein-coupled agonists. A, washed wild type (WT) or PKG I knock-out (KO) mouse platelets were stimulated with U46619 and simultaneously recorded for secretion of ATP and aggregation in a lumaggregometer. B, C, and D, washed human platelets were preincubated with buffer (Control) or PKG inhibitors Rp-pCPT-cGMPS (250 μM) or Rp-Br-PET-cGMPS (250 μM) at 37°C for 5 min. Platelets were then stimulated with U46619 (B), PAR1 TRAP SFLRN (C), or PAR4 TRAP AVPGKF (D). Secretion and aggregation were concomitantly recorded. In all of the above experiments, luciferin/luciferase reagent was added to the platelets 1 min prior to adding agonists.

**Fig. 3.** Effects of PKG inhibitors on platelet secretion and aggregation induced by collagen. A and B, washed human platelets were preincubated with or without PKG inhibitors Rp-pCPT-cGMPS or Rp-Br-PET-cGMPS (250 μM) for 5 min at 37°C and then stimulated with collagen at 0.5 μg/ml (A) or 5 μg/ml (B). To examine the aggregation dependence of platelet secretion, platelets were also incubated with RGDS at 37°C for 5 min and then stimulated with collagen. Platelet aggregation and secretion of ATP were recorded as described under “Experimental Procedures.”
PKG is common in aggregation-dependent platelet secretion induced by agonists other than the G-protein-coupled receptors, we examined the effect of PKG inhibitors on collagen-induced platelet secretion of dense granules. Both Rp-cPT-cGMPS and Rp-Br-PET-cGMPS inhibited platelet secretion and aggregation induced by 0.5 μg/ml collagen in a manner similar to RGDS, suggesting that PKG is important for platelet secretion and aggregation induced by this low concentration of collagen. Consistent with previous reports, platelet aggregation induced by a higher concentration of collagen (5 μg/ml) was not significantly affected by PKG inhibitors. However, platelet secretion in PKG inhibitor-treated platelets was partially but significantly reduced even at the higher collagen concentration (Fig. 3B). These results indicate that PKG is important in platelet secretion induced by collagen.

**Inhibition of ADP Secretion Is Responsible for Decreased Platelet Aggregation in PKG Inhibitor-treated Platelets**—A major substance secreted from dense granules is ADP, which induces integrin activation and thus promotes platelet aggregation. As shown in Fig. 1, ADP-induced platelet aggregation was not significantly affected by PKG I knockout or PKG inhibitors, suggesting that ADP-induced integrin activation and platelet aggregation do not require PKG signaling. Therefore, we hypothesized that the inhibitory effects of PKG I knockout or PKG inhibitors on TXA2- or TRAP-induced platelet aggregation result from inhibition of ADP secretion. To investigate this hypothesis, we examined whether restoration of low concentrations of ADP can reverse the inhibitory effects of PKG inhibitors during U46619-induced platelet aggregation. Fig. 4 shows that PKG inhibitors diminished secretion and second wave platelet aggregation. Addition of a low concentration of ADP (0.5 μM), although insufficient by itself to induce platelet aggregation (not shown), restored the second wave of aggregation induced by U46619 in PKG inhibitor-treated platelets. These results suggest that the inhibitory effects of PKG inhibitors on the second wave of platelet aggregation mainly result from inhibition of secretion of ADP from dense granules.

**PKG-dependent Secretion of ADP Promotes Platelet Aggregation via the G_i Signaling Pathway**—The above data show that a major role for PKG in platelet aggregation is to mediate signal transduction leading to secretion of dense granule ADP. ADP binds to two different receptors on platelets, the G_i-coupled P2Y12 receptor and the G_q-coupled P2Y1 receptor. G_i is also the major G-protein mediating signaling of receptors for TXA2 and TRAPs. The role of ADP in potentiating platelet aggregation induced by TXA2 or low dose TRAP only requires the G_i-coupled P2Y12 pathway because it is inhibited by P2Y12 antagonists but not P2Y1 antagonists (12, 43). Therefore, we reason that low concentrations of G-protein-coupled agonists other than ADP may require secreted ADP to enhance the G_i signaling pathway and thus allow full scale platelet aggregation and that PKG inhibition reduces secretion of ADP and thus inhibits the ADP-dependent G_i-coupled pathway. To test this hypothesis, we examined the effects of PKG inhibitors or PKG I knockout on TXA2-induced inhibition of adenyl cyclase function. To increase the sensitivity of the assay, we used forskolin (a standard reagent for elevating cAMP levels) to elevate cAMP and examined the effects of agonists and PKG inhibitors. Fig. 5 shows that U46619 caused inhibition of forskolin-stimulated adenyl cyclase (Fig. 5A and B), which was completely reversed by PTX (Fig. 5A), an inhibitor of the G_i pathway, indicating a

![Figure 4](image1.png)

**Fig. 4. Restoration of the PKG-dependent second wave of platelet aggregation by exogenous ADP.** Washed human platelets in modified Tyrode’s buffer (3 × 10⁷/ml) were preincubated with buffer (Control) or Rp-cPT-cGMPS (250 μM) and then stimulated with U46619 (500 nM). Rp-cPT-cGMPS-treated platelets were also stimulated with U46619 followed by 0.5 μM ADP.

![Figure 5](image2.png)

**Fig. 5. Roles of PKG in stimulating the ADP-dependent G_i pathway.** A and B, washed human platelets (1 × 10⁷/ml) were preincubated at 37°C for 5 min with or without (A) PTX (0.5 mM), (B) Rp-cPT-cGMPS (250 μM), or apyrase (1 units/ml). Platelets were further treated for 5 min with forskolin (10 μM) in the presence (U46619) or absence (Control) of stimulation by U46619 (1 μM). The reactions were stopped by the addition of the same volume of 12% (w/v) trichloroacetic acid. cAMP concentrations were determined by using a cAMP immunoassay kit. Results are expressed as means ± S.D. (n = 3). C, wild type (PKG⁺/⁺) or PKG⁻/⁻ mouse platelets were stimulated with or without U46619 or with ADP in the presence of forskolin. Levels of cAMP were then determined as described in A.
requirement for G\textsubscript{i} signaling. Addition of an ADP scavenger, apyrase, also reversed U46619-induced inhibition of adenyl cyclase (Fig. 5B), suggesting that U46619-induced activation of the G\textsubscript{i} pathway is dependent on secreted ADP. U46619-induced inhibition of adenyl cyclase was completely reversed by the PKG inhibitor Rp-pCPT-cGMPS (Fig. 5B) and in PKG I knock-out platelets (Fig. 5C). In contrast, ADP-induced inhibition of adenyl cyclase was not affected by PKG knockout. These data indicate that PKG mediates signals leading to ADP secretion that plays important roles in activating the G\textsubscript{i} pathway and in inducing second wave platelet aggregation.

The Role of PKG in Platelet Secretion of \(\alpha\)-Granules—The above results indicate that PKG plays an important role in aggregation-dependent secretion of platelet dense granules. To determine whether PKG also plays a role in platelet secretion of \(\alpha\)-granules, we examined the effect of PKG inhibitors on surface expression of P-selectin induced by platelet agonists. Expression of P-selectin was examined by flow cytometry using the monoclonal anti-human P-selectin antibody SZ51. Fig. 6 shows that both Rp-pCPT-cGMPS and Rp-Br-PET-cGMPS significantly inhibited P-selectin expression induced by U46619. P-selectin expression induced by TRAPs was also partially but significantly inhibited by PKG inhibitors, although to a lesser degree. Thus, PKG also plays an important role in platelet secretion from \(\alpha\)-granules.

Effects of PKG Inhibitors and PKG Knockout on Calcium Mobilization—A common signaling event upstream from secretion is the elevation of intracellular calcium levels. To investigate the relationship between PKG and calcium elevation, we examined the effects of PKG inhibitors and PKG deficiency on U46619-induced intracellular calcium mobilization. As shown in Fig. 7A, U46619-induced calcium mobilization in PKG I knock-out mouse platelets was identical to that of wild type platelets. Similarly, U46619-induced calcium mobilization was not affected by the PKG inhibitor Rp-pCPT-cGMPS in human platelets (Fig. 7B). These results suggest that PKG is not required for agonist-induced calcium mobilization. Thus, PKG is likely to be involved in signaling events either downstream from or parallel to calcium mobilization in platelet secretion signaling.

**DISCUSSION**

In this study, we show that PKG plays an important role in mediating aggregation-dependent platelet secretion of dense granules and \(\alpha\)-granules. We also show that by inducing secretion of ADP, PKG plays a general stimulatory role in promoting platelet activation and stabilizing platelet aggregation. These findings suggest a novel signaling mechanism mediating platelet granule secretion and provide a new mechanism for the stimulatory roles of the cGMP-PKG pathway in platelet activation. Because regulated secretion is important not only in platelet function but also in leukocytes, neurons, and other cell types, identification of a novel PKG-dependent signaling pathway that stimulates exocytosis is of general significance to the understanding of the regulatory mechanisms of degranulation, exocytosis, and vesicle trafficking.

Although it has been believed for the past 30 years that the cGMP-PKG pathway inhibits platelet activation, we have shown recently that cGMP promotes GPIb-IX-dependent integrin activation and platelet aggregation. GPIb-IX is a unique receptor that, unlike thromboxane A\textsubscript{2} or collagen receptors, does not appear to require secretion of granules to induce activation of integrin. GPIb-IX-mediated integrin activation can be reconstituted in the cultured Chinese hamster ovary cells that do not have platelet-specific granules (19, 20, 33, 44), although it is significantly amplified by ADP secr-
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endothelial nitric oxide synthase can be activated in endothelial secretion. In this respect, it is interesting to note that activation of the PKG pathway by an integrin-independent elevation (although at lower levels) without requiring integrin shown). Also, most platelet agonists can directly cause calcium tides that inhibit integrin-ligand interaction do not signifi-
cantly inhibit the cGMP elevation induced by this agonist (not

dentified secretion signaling molecules and mechanisms (46). Our finding that PKG is important in aggregation-depend-
ent secretion explains why there have been apparent agonist-specific differences in the effects of PKG inhibition on platelets. PKG knockout or inhibitors have no significant effect on platelet aggregation induced by high concentrations of colla-
gen (Fig. 3) or thrombin (data not shown). These are so called strong agonists that can induce platelet secretion via the aggregation-independent secretion pathway. Although PKG-mediated aggregation-dependent secretion may also occur in platelet responses to these agonists (Fig. 3), levels of aggrega-
tion-independent secretion are already sufficient to mediate full scale platelet aggregation. Thus, PKG deficiency would have no significant effect on platelet aggregation induced by these agonists. On the other hand, PKG knockout or inhibitors significantly inhibited the second wave of platelet aggregation induced by low concentrations of TXA2, throm-
bin, TRAPs, and collagen, which requires aggregation-de-
pendent platelet secretion of granules by PKG inhibitors or PKG I knockout has no significant effect on ADP-induced platelet aggregation. Therefore, we have not only identified a novel secretion signaling pathway but also provided significant new insights into the complex interaction between secretion and aggregation and between differ-
et platelet activation pathways.

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