AN IMPORTANT STIMULATORY ROLE FOR THE cGMP-DEPENDENT PROTEIN KINASE II IN PLATELET ACTIVATION, IN VIVO THROMBOSIS AND HAEMOSTASIS

Running title: The role of PKG II in platelet activation

Zhenyu Li[1*#], Ying Liang,[1*] Can Wang[1], Guoying Zhang[1#], Jens Schlossmann[2], Melanie Baron[2], Matthias Schiemann[4], Sven Fraterman[3], Matthias Wilm[3], Franz Hofmann[2], Robert Feil[5], and Xiaoping Du[1]

From [1]Department of Pharmacology, College of Medicine, University of Illinois, 835 South Wolcott Ave, Chicago, IL 60612, USA, [2]Institute für Pharmakologie und Toxikologie der Technischen Universität München, Biedersteiner Straße 29, 80802 München, Germany, [3]Protein and Peptide Group, EMBL, Meyerhofstraße 1, 69117 Heidelberg, Germany, [4]Institute für Mikrobiologie, Immunologie und Hygiene der Technischen Universität München, Trogerstraße 9, 81675 München, Germany, [5]Interfakultäres Institut für Biochemie, University of Tübingen, Auf der Morgenstelle 34, 72076 Tübingen, Germany

Address correspondence to: Xiaoping Du, Department of Pharmacology, College of Medicine, University of Illinois at Chicago, 835 South Wolcott Ave., Chicago, IL 60612. Tel. 312-355 0237; Fax: 312-996-1225; E-mail: xdu@uic.edu.

#Current Addresses: Z.L. and G.Z.: Department of Medicine, Division of Cardiology, University of Kentucky Lexington, KY. J.S.: Department of Pharmacology and Toxicology, Institute of Pharmacy, University of Regensburg, Universitätsstr. 31, D-93040 Regensburg, Germany. M.W.: Bioanalytics UCD Conway Institute of Biomolecular and Biomedical Research, University College Dublin (UCD),Dublin, Ireland.

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Bullet point summary:

What is already known：

- cGMP plays biphasic roles in platelet activation, in which PKG I plays an important role.

What this study adds：

- PKG II is expressed in platelets and important in stimulating collagen-induced platelet activation.
- PKG II is important in thrombosis and haemostasis *in vivo*.

Clinical significance：

- Specific PKG II inhibition may selectively inhibit thrombosis without perturbing beneficial inhibitory effects of cGMP,

Abstract

Background and Purpose: The intracellular second messenger cGMP mediates signals by activating two types of cGMP-dependent protein kinases (PKG), PKG I and PKG II, differentially expressed in different cells. In platelets, cGMP mediates biphasic signals that stimulate and inhibit platelet activation, and the downstream signaling of cGMP is mediated by PKG I, the only PKG known to be expressed in platelets. However, functional defects of PKG I knockout platelets did not fully explain the roles of cGMP and the effect of PKG inhibitors on platelet activation.

Experimental Approach: To determine if PKG II is present in platelets and plays a role in platelet activation, we performed RT-PCR and isolation of PKG II protein using cGMP-conjugated beads. We further determined platelet aggregation and ATP release *in vitro*, and $FeCl_3$-injured carotid artery thrombosis as well as tail bleeding time *in vivo*.

Key Results: PKG II is expressed in platelets and plays an important role in selectively stimulating platelet activation but not in the negative regulatory role of cGMP. Collagen-induced platelet aggregation and ATP secretion were reduced in PKG II-deficient mice but not PKG I-deficient mice. In contrast, low-dose thrombin-induced platelet activation depended on PKG I but not PKG II. Tail bleeding time and $FeCl_3$-induced artery thrombus formation were significantly prolonged in PKG II knockout mice.

Conclusion and Implication: PKG II-mediated cGMP signals are important in platelet activation, thrombosis and haemostasis *in vitro* and *in vivo*.

Keywords: platelet, cGMP, kinase, platelet aggregation, thrombosis.

Platelet activation plays a critical role in the development of thrombotic diseases such as heart attack and stroke (Ruggeri, 2002). An important characteristic of platelet activation is the positive feedback loops that can dramatically amplify initial activation signals(Estevez & Du, 2017). Thus, exposure of platelets to minor vascular injury and low concentrations of agonists can result in full scale platelet aggregation and effectively plug the wound. On the other hand, platelet activation also requires self-control and negative feedback loops, so that normal hemostatic response to vascular injury will not uncontrollably develop into occlusive thrombosis and ischemia. The intracellular secondary messenger cGMP has been shown to be involved both the stimulatory amplification of platelet activation and inhibitory regulation of platelet activation (Hofmann, 2005; Li, Ajdic, Eigenthaler & Du, 2003; Li et al., 2003; Wen, 2017). Elevation of cGMP by platelet agonists such as collagen and thrombin initially induces secretion of platelet granule contents such as ADP that amplify platelet response (Li, Zhang, Marjanovic, Ruan & Du, 2004). High concentrations of cGMP may later inhibit platelet aggregation and thrombus formation. In this respect, a recent study suggests that shear-enhanced, NO-dependent cGMP generation in activated platelets may play a role in the auto-regulatory brake of thrombosis(Wen et al., 2018).

It is believed that the signaling of cGMP in platelets is mainly mediated by PKG (Hofmann, 2005). There are two mammalian PKGs, PKG I and PKG II, which are encoded by separate genes. They have homologous structural features; an N-terminal dimerization domain, a central cGMP-binding domain and a C-terminal ATP-binding/catalytic domain. Only PKG I (mainly the PKG Iβ

isoform) is known to be expressed in platelets (Geiselhoringer, Gaisa, Hofmann & Schlossmann, 2004; Jang, Azzam, Dickinson, Davidson & Haslam, 2002). PKG I knockout results in inhibition of either the stimulatory effect (Li et al., 2003; Li, Zhang, Marjanovic, Ruan & Du, 2004) or the inhibitory effect of cGMP in platelets (Massberg et al., 1999). However, there are apparent discrepancies in the functional effects of PKG I knockout mice and PKG inhibitors. PKG inhibitors are more potent in inhibiting platelet activation than PKG I knockout (Li et al., 2003), and can still be effective in inhibiting platelet activation under experimental conditions when PKG I knockout did not inhibit platelet activation (Marshall et al., 2004). To understand the mechanism of this "non-specificity" of PKG inhibitors, we have explored whether there is another downstream molecule that mediates the effect of cGMP on platelet activation. In the present study, we show that PKG II, which was previously believed to be absent from the cardiovascular system, is in fact expressed in platelets, and plays an important role in stimulating platelet secretion and aggregation. Significantly, the role of PKG II in platelet activation is distinct from that of PKG I, and does not appear to be involved in the inhibitory phase of cGMP. Furthermore, our data indicate that PKG II plays an important role in thrombus formation and haemostasis *in vivo*.

METHODS

Mice— The generation of a PKG I and PKG II null (−) allele by homologous recombination has been described previously (Pfeifer, Aszodi, Seidler, Ruth, Hofmann & Fassler, 1996; Wegener et al., 2002). PKG II knockout mice were back-crossed to C57BL/6 background. PKG II knockout and wild type mice obtained from heterozygotes breeding were used for the experiments. All animal procedures were performed in accordance with and approved by Animal Care Committee of University of Illinois at Chicago. Experiments strictly adhered to ARRIVE guidelines.

Materials—Luciferin/luciferase reagent and collagen were purchased from Chronolog, Havertown, PA. Human α-thrombin was from Enzyme Research Laboratories, South Bend, IN. Polyclonal anti-PKG II antibody was from Santa Cruz. Anti-PKG I antibody and PKG inhibitor, Rp-Br-PET-cGMPS were purchased from Calbiochem. Antibody specifically recognizing Ser^{157}-phosphorylated VASP, 5C6, was from Santa Cruz. A monoclonal antibody against β-actin (AC74) was from Sigma.

Preparation of mouse washed platelets— Blood was collected from abdominal aorta of the isofluorane-anesthetized mice (8-12 week old) using 1/7 volume of ACD (85 mM trisodium citrate, 83 mM dextrose, and 21 mM citric acid) as anticoagulant. Blood from 5 to 6 mice of either genotype was pooled and platelets were isolated by differential centrifugation as previously described (Li, Zhang, Le Breton, Gao, Malik & Du, 2003). Platelets were washed two times with CGS buffer (0.12 M sodium chloride, 0.0129 M trisodium citrate, and 0.03 M D-glucose, pH 6.5), resuspended in modified Tyrode's buffer (Du, Plow, Frelinger, O'Toole, Loftus & Ginsberg, 1991) and allowed to rest for at least 1 hour at room temperature before use.

Platelet aggregation and secretion—Platelet aggregation was measured in a turbidometric platelet aggregometer (Chronolog) at 37 °C with stirring (1000 rpm). To investigate the effects of a cGMP analog pCPT-cGMP or the PKG inhibitor Rp-Br-PET-cGMPS on platelet aggregation and secretion, platelets were preincubated with pCPT-cGMP (100 μM) or Rp-Br-PET-cGMPS (200 μM) for 5 minutes at 37 °C, collagen or thrombin was then added to induce platelet aggregation. Platelet secretion was monitored in parallel with platelet aggregation as ATP release in a platelet lumiaggregometer (Chronolog) with the addition of luciferin-luciferase reagent to platelet suspension. Quantification was performed using ATP standards. Experiments were repeated at least three times. Statistical significance was examined using a paired *t* test and data from three or more experiments.

PKG II mRNA detection in platelets—RNA was isolated from human or mouse platelets with the total RNA extraction kit from Promega, Madison, WI. Total RNA was reverse-transcribed using Thermoscript RT-PCR (Invitrogen, Carlsbad, CA), following the instructions of the manufacturer. One tenth of each reaction was used as a cDNA template for the PCR reaction. Primers were designed to be complementary with both mouse and human sequence. PKG II cDNA of human platelets was amplified with a forward primer 5'-GAATTCTAGTGTATGAGCTC-3', and a reverse primer 5'-TCAGAAGTCTTTATCCCA-3', amplifying the C-terminal fragment of PKG II cDNA. PKG II cDNA of mouse platelets was amplified with a forward primer 5'-TGCCCTGAATAAGAACCAGT-3', and a reverse primer 5'-GTTCTGGAAAACCTCTCTAT-3', amplifying a fragment of PKG II cDNA near N-terminal. PCR products (expected size 380 bp for human, 310 bp for mouse) were separated on a 2% agarose gels containing ethidium-bromide and visualized with a UV transluminator.

Platelet PKG II levels—Washed platelets in Tyrode buffer (1 x 10^9/ml) were solubilized by adding equal volume of 2% Triton X-100, 100 mM Tris, 10 mM EGTA, 0.15 M NaCl, 2 mM phenylmethylsulfonyl fluoride, and 0.2 mM E64, pH 7.4. The lysates were incubated with cGMP-agarose beads, and bound proteins were analyzed by SDS-PAGE and immunoblotting with a polyclonal anti-PKG II antibody (Santa Cruz Biotechnology Inc).

Mass spectrometry—Coomassie stained protein bands were excised from the gel and subjected to in-gel reduction, alkylation, and trysin digestion. The peptide mixtures were then analyzed by nanoscale LC-MS-MS using an Eksigent 1D-plus nanoflow system (Eksigent Technologies, Dublin, USA) connected to a QSTAR Pulsar i quadrupole time-of-flight mass spectrometer (AB/MDS Sciex, Toronto, Canada) equipped with a nanoelectrospray ion source. Linear gradient elution was from 100% buffer A (H_2O-acetic acid, 100:0.5, v/v) to 50% buffer B (H_2O-acetonitrile-acetic acid, 20:80:0.5, v/v) with a flow rate of 200 nl/min in 60 min. Protein identification was performed with the Mascot software package (Matrix Science, London, UK).

Western blot analysis of VASP phosphorylation—Washed platelets from wild type or PKG II knockout mice were resuspended in Tyrode's solution (3 x 10^8/ml) and incubated with Br-cGMP (100 μM), pCPT-cGMP (100 μM), or SNP (100 μM) in the aggregometer at 37 °C for 5 min and solubilized in SDS-PAGE sample buffer. Platelet lysates were analyzed by SDS-PAGE on 4% to 15% gradient gel and electrotransfered to polyvinylidenefluoride membranes. Phosphorylation of VASP at Ser^{157} was detected as described previously (Hofmann, 2005; Li, Ajdic, Eigenthaler & Du, 2003; Li et al., 2003) by immunoblotting with a monoclonal antibody specifically recognizing Ser^{157}-phosphorylated VASP, 5C6.

Bleeding time—8-10 week-old mice were anesthetized with intraperitoneal injection of pentobarbital. The distal portion of the tail (5 mm) was amputated with a scalpel, and the tail was immersed in 0.15 M NaCl at 37 °C as previously described (Li et al., 2003). Time to stable cessation of the bleeding was defined as the time where no re-bleeding for longer than 60 s was recorded. Statistical analysis was performed using the Mann-Whitney test.

In vivo thrombosis—The *in vivo* thrombosis model was performed as described previously (Marjanovic, Li, Stojanovic & Du, 2005). Briefly, 8–10-Week-old mice were anesthetized with intraperitoneal injection of pentobarbital. The left carotid artery was isolated from surrounding tissues (Day, Reeve, Myers & Fay, 2004). MA-0.5PSB nanoprobe (Transonic Systems, Ithaca, NY) was hooked to the artery, and blood flow was monitored with a TS420 flowmeter (Transonic Systems). After stabilization, 1.2 μl of 5% $FeCl_3$ was applied to a filter paper disc (1-mm diameter) that was immediately placed on top of the artery for 3 min. After removing the filter paper, blood flow was monitored continuously until 5 min after occlusion. Time to occlusion was calculated as a difference in time between the removal of the filter paper and stable occlusion (no blood flow

for 2 min). Statistical analysis was performed using the Mann-Whitney test for the evaluation of differences in median occlusion time.

Data and statistical analysis—Presented data and statistical analysis comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis et al., 2018). For parametric data, statistical significance was analyzed using Student's t-test (2 groups) using Graphpad software. For nonparametric data, statistical significance was determined using Mann-Whitney test. $P<0.05$ was considered as significant difference.

RESULTS

Expression of PKG II in platelets—We have previously shown that PKG inhibitors significantly diminished platelet secretion and aggregation induced by low concentrations of collagen (Li, Zhang, Marjanovic, Ruan & Du, 2004). To study whether PKG I is important in collagen-induced platelet activation, washed platelets from PKG I-deficient mice and wild type littermates were stimulated by collagen. Interestingly, platelet secretion and aggregation induced by low concentrations of collagen were not affected in PKG I-deficient platelets (Fig. 1A). To determine whether PKG II is present in platelets and mediates collagen-induced platelet activation, we performed RT-PCR using primers specific for a 3'-terminal fragment of PKG II mRNA purified from human platelets as template. Indeed, we detected the presence of PKG II mRNA in platelets (Fig. 1B). We further cloned the complete cDNA of PKG II by RT-PCR and verified the correct sequence. Furthermore, PKG II mRNA was detected in wild type mouse platelets, but not PKG II-deficient platelets (Fig. 1C). Importantly, cGMP-conjugated agarose beads precipitated PKG II protein from platelet lysates as detected by western blot using an antibody against PKG II (Fig. 1D). To verify that the detected PKG II was not from contamination of other blood cells, we performed a flow cytometric analysis of isolated platelets using a monoclonal antibody against a platelet-specific integral membrane glycoprotein, GPIX. The results showed that 99.8% of the isolated cells were positive for GPIX. The identity of PKG II protein in platelets was further verified by mass spectrometric sequencing of the Coomassie blue-stained protein immunoprecipiated by an anti-PKG II antibody followed by trypsin digestion. To estimate the expression level of PKGs in human platelets, the amount of PKG I and II in 50 μg total protein of human platelet lysate was compared to 50 ng of purified PKG I and PKG II protein, respectively, by western blot. Preliminary estimation revealed the levels of ~5 ng of PKG II and ~15 ng of PKG I in 50 μg of the platelet lysate, respectively (Fig. 1E, 1F).

The role of PKG II in stimulating platelet secretion and aggregation—To determine the role of PKG II in platelet activation, washed platelets from wild type and PKG II knockout (PKG $II^{-/-}$) mice were exposed to platelet agonists. Platelet aggregation and ATP release induced by low concentrations of collagen were significantly reduced in PKG II-deficient mice (Fig. 2A and 2B). Similarly, treatment of wild type mouse platelets with a selective PKG inhibitor, Rp-Br-PET-cGMPS, inhibited platelet aggregation and secretion induced by low doses of collagen, which is consistent with previous data using Rp-pCPT-cGMPS (Li, Zhang, Marjanovic, Ruan & Du, 2004). In contrast, treatment of PKG II knockout platelets with Rp-Br-PET-cGMPS had no further effect on collagen-induced platelet aggregation and secretion (Fig. 3A and 3B). These data indicated that PKG II is an important downstream mediator of cGMP signalling in collagen-induced platelet activation. Interestingly, under identical experimental conditions, platelet aggregation and secretion induced by a low concentration of thrombin (0.02 U/ml) did not appear to be inhibited in PKG $II^{-/-}$ platelets (Fig. 4A, 4B), but were clearly inhibited by the PKG inhibitors Rp-Br-PET-cGMPS and Rp-pCPT-cGMPS, in both wild type and PKG II-deficient platelets (Fig. 4C, 4D).

These findings indicated that PKG I but not PKG II plays a major role in stimulating low dose thrombin-induced platelet aggregation and secretion, which is consistent with our previous data (Li et al., 2003; Li, Zhang, Marjanovic, Ruan & Du, 2004). Thus, PKG I and PKG II may play distinct roles in different platelet activation pathways: PKG I is important mainly in thrombin pathway but PKG II is mainly important in collagen pathway. However, our results do not exclude the possibility that PKG II may partially compensate or be masked by the role of PKG I.

PKG II is not involved in cGMP-mediated platelet inhibition—The effects of cGMP on platelets are biphasic. It is known that preincubation of platelets with high concentrations of cGMP analogs inhibits platelet function via a PKG I-mediated pathway (Massberg et al., 1999). To investigate whether PKG II is also involved in mediating the inhibitory effect of high concentrations of cGMP, platelets from wild type or PKG II knockout mice were preincubated with pCPT-cGMP (100 μM) for 5 minutes, and then exposed to agonists. Platelet aggregation and secretion induced by high-dose collagen (Fig. 5A) or thrombin (Fig. 5B) were inhibited by the preincubation of platelets with pCPT-cGMP in both wild type and PKG II-deficient platelets, suggesting that PKG II is not required in cGMP-mediated platelet inhibition. Accordingly, phosphorylation of vasodilator-stimulated phosphoprotein (VASP) induced by cGMP analogs or the NO-releasing agent SNP was not affected by PKG II knockout (Fig, 5C**)**. This is in contrast to the known inhibitory effect of PKG I knockout and PKG inhibitors on VASP phosphorylation induced by cGMP analogs or NO donors in mice (Li, Ajdic, Eigenthaler & Du, 2003; Massberg et al., 1999).

The role of PKG II in thrombosis and haemostasis in vivo—We next investigated whether PKG II deficiency affected *in vivo* thrombus formation using the $FeCl_3$-injured carotid artery thrombosis model. The time to the formation of stable occlusive thrombus in PKG $II^{-/-}$ mice (median, 420.0 seconds, n=15) was significantly prolonged compared to wild type mice (median, 321.0 seconds, n=15) (p=0.031) (Fig. 6A). Tail-bleeding time analysis indicated a remarkable bleeding diathesis in PKG $II^{-/-}$ mice. The median bleeding time was 73.50 seconds (n=28) in wild type mice. In contrast, the median bleeding time of PKG II knockout mice was 454.50 seconds (n=28, p=0.0062) (Fig. 6B). Thus, PKG II plays an important role in thrombosis and haemostasis *in vivo*.

DISCUSSION

Our data reveal that PKG II is expressed in platelets. More importantly, we present the novel finding that PKG II plays an important stimulatory role in collagen-induced platelet activation pathways *in vitro*, and in thrombosis and haemostasis *in vivo*. PKG II is known to be expressed in epithelial cells of intestinal mucosa, kidney, and lung tissues, and in neurons, but not in the cardiovascular system (Hofmann, 2005; Hofmann, Feil, Kleppisch & Schlossmann, 2006; Jarchau et al., 1994; Marshall et al., 2004; Uhler, 1993). Thus, our findings suggest a new downstream effector of the NO-cGMP pathway in platelets and support the emerging new concept that cGMP is a signaling mediator that plays an important stimulatory role in platelet activation and *in vivo* thrombosis.

The role of cGMP and PKG in platelets has been controversial. Early studies in 1970s suggested a stimulatory role for cGMP in platelets (Chiang, Beachey & Kang, 1975; Chiang, Dixit & Kang, 1976). Since the discovery that NO and demonstration of the inhibitory effect of NO and cGMP analogs on platelet activation, however, it became a prevalent believe that the cGMP/PKG pathway is inhibitory to platelet activation (Haslam, Dickinson & Jang, 1999). We demonstrated that cGMP and PKG in fact play biphasic roles in platelet activation. While high concentrations of NO donors and cGMP analogs inhibit platelet activation via the PKG I- and PKA-dependent signaling pathways (Butt et al., 1992; Haslam, Dickinson & Jang, 1999; Li, Ajdic, Eigenthaler &

Du, 2003; Massberg et al., 1999; Mellion, Ignarro, Ohlstein, Pontecorvo, Hyman & Kadowitz, 1981; Saxon & Kattlove, 1976), low concentrations of NO and cGMP stimulate platelet secretion and aggregation (Li, Ajdic, Eigenthaler & Du, 2003; Li et al., 2003; Li, Zhang, Marjanovic, Ruan & Du, 2004). Whereas initially disputed by some studies (Gambaryan et al., 2004; Marshall et al., 2004), a stimulatory role of the cGMP-PKG pathway in platelets was further verified and developed by several independent groups(Vogel et al., 2015; Weng et al., 2010; Zhang et al., 2015) and by a series of our follow up studies (Li, Zhang, Feil, Han & Du, 2006; Li et al., 2010; Marjanovic, Li, Stojanovic & Du, 2005; Marjanovic, Stojanovic, Brovkovych, Skidgel & Du, 2008; Stojanovic et al., 2006; Yin, Liu, Li, Berndt, Lowell & Du, 2008; Yin, Stojanovic, Hay & Du, 2008; Zhang et al., 2009; Zhang et al., 2011). Moreover, we demonstrated that the PI3K-Akt-NOS-NO-sGC-PKG-MAPK pathway mediates the stimulatory role of cGMP (Chen, Ruggeri & Du, 2018; Li, Zhang, Feil, Han & Du, 2006; Li et al., 2010; Marjanovic, Li, Stojanovic & Du, 2005; Marjanovic, Stojanovic, Brovkovych, Skidgel & Du, 2008; Stojanovic et al., 2006; Yin, Liu, Li, Berndt, Lowell & Du, 2008; Yin, Stojanovic, Hay & Du, 2008; Zhang et al., 2009; Zhang et al., 2011). However, the controversy remains with regard to why PKG inhibitors inhibited platelet activation even under certain experimental conditions where PKG I knockout showed no significant effect (Marshall et al., 2004), especially in collagen-induced platelet activation (Fig. 1) (Massberg et al., 1999). Here we provide new evidence that the stimulatory roles of cGMP in platelet activation can be mediated by PKG II in addition to PKG I. We further show that PKG I and PKG II play distinct roles in that PKG I is primarily important in thrombin-induced pathway (Li et al., 2003) and PKG II is mainly important in collagen-induced activation pathway. Our findings explain at least some of the apparent discrepancies in previous studies. More importantly, we identify PKG II as a new downstream effector of the NO-cGMP signaling pathway that stimulates platelet secretion and platelet aggregation.

In accordance with a stimulatory role of PKG II in platelet activation *in vitro*, PKG II-deficient mice show impaired thrombosis and haemostasis. PKG II knockout mice showed significantly prolonged tail bleeding time, and a prolonged occlusion time in the $FeCl_3$-induced carotid artery thrombosis experiment (Fig. 6). Previously, depending on tissue specificity, PKG I knockout mice reportedly ranged from having a relatively mild prolongation of bleeding time (Li et al., 2003) to no significant effect (Wen et al., 2018), possibly reflecting the biphasic nature of the role of PKG I in platelets in contrast to PKG II. The defects of PKG II knockout mice are similar to platelet-specific sGC knockout mice, which also showed prolonged bleeding time and carotid artery thrombosis (Zhang et al., 2011). Whole body knockout of sGC, interestingly, showed shortened bleeding time (Dangel, Mergia, Karlisch, Groneberg, Koesling & Friebe, 2010; Thoonen et al., 2015), in contrast to platelet-specific sGC knockout as well as PKG I and PKG II knockouts, supporting a critical role of sGC in vessel relaxation/constriction, which is also important in haemostasis. Furthermore, defective thrombus formation and/or prolonged bleeding time were observed in mice that lack the key enzymes leading to activation of the cGMP pathway in platelets, including Akt (Chen, De, Damron, Chen, Hay & Byzova, 2004; Stojanovic et al., 2006; Woulfe, Jiang, Morgans, Monks, Birnbaum & Brass, 2004) or NO synthases (Iafrati et al., 2005; Marjanovic, Li, Stojanovic & Du, 2005; Marjanovic, Stojanovic, Brovkovych, Skidgel & Du, 2008), supporting a role for the cGMP pathway in platelet activation, thrombosis and haemostasis.

While PKG II is important in the stimulatory phase of the cGMP effect, unlike PKG I, PKG II is not involved in the inhibitory effect of high concentrations of cGMP on platelet activation. Platelet inhibition by high concentrations of cGMP is not affected by PKG II deficiency. These results are consistent with previous findings that platelet inhibition induced by high concentrations of cGMP involves cGMP-induced activation of cAMP-dependent protein kinase (Jang, Azzam, Dickinson, Davidson & Haslam, 2002; Li, Ajdic, Eigenthaler & Du, 2003), PKG I (Massberg et

al., 1999) and a downstream effector of PKG I, IRAG (Antl et al., 2006). Thus, the two different PKG isoforms have clearly distinct regulatory roles in platelets. PKG I plays biphasic roles in mediating low-dose thrombin-induced platelet activation as well as NO- and cGMP-dependent platelet inhibition, while PKG II only plays an important stimulatory role in low-dose collagen-induced platelet activation. Thus, potential drugs specifically targeting PKG II may seletively inhibit thrombosis without perturbing the beneficial effect of NO-cGMP in auto-regulating platelet activation and thrombus size.

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FIGURE LEGENDS

Figure 1. Expression of PKG II in platelets. (A) Platelet aggregation traces showing similar responses of PKG $I^{-/-}$ or PKG $I^{+/+}$ mouse platelets in modified Tyrode's buffer to 0.5 µg/ml collagen. (B**)** RT-PCR amplification of a C-terminal fragment of PKG II using human platelets mRNA as a template. (C) RT-PCR amplification of a N-terminal fragment of PKG II using mRNA from wild type or PKG $II^{-/-}$ platelets as templates. (D) Human platelet lysates were incubated with cGMP-agarose beads,and the bead-bound proteins were analyzed by SDS-polyacrylamide gel

electrophoresis. PKG II and PKG I were detected by western blot with polyclonal anti-PKG II (Santa Cruz) and PKG I antibodies. (E) and (F) Estimation of relative PKG II and I concentration in platelets (Plts). 50 μg of platelet extract proteins were separated on a SDS gel and were blotted together with either 50 ng pure PKG II (E) or PKG I (F) protein.

Figure 2. Effects of PKG II knockout on collagen-induced platelet secretion and aggregation. (A) Washed wild type (PKG $II^{+/+}$) or PKG $II^{-/-}$ mouse platelets were stimulated with various concentrations of collagen and simultaneously recorded for ATP secretion and aggregation. (B) Statistical data of maximal platelet aggregation and ATP release induced by 0.6 μg/ml collagen from 5 experiments (Student t-test, **p<0.01).

Figure 3. Effects of PKG inhibitors on collagen-induced platelet aggregation and secretion in PKG $II^{+/+}$ and PKG $II^{-/-}$ mice. Washed platelets ($3x10^8$/ml) from PKG $II^{+/+}$ or PKG $II^{-/-}$ mice were preincubated with a PKG inhibitor, Rp-Br-PET- cGMPS (200 μM), at 37°C for 5 min, collagen 0.6 μg/ml (A) or 0.75 μg/ml (B) was added to induce platelet aggregation and ATP release.

Figure 4. Effects of PKG II knockout on thrombin-induced platelet secretion and aggregation. (A and B) Washed wild type (PKG $II^{+/+}$) (A) or PKG $II^{-/-}$ (B) mouse platelets were preincubated with or without PKG inhibitors Rp-Br-PET- cGMPS (200 μM) or Rp-pCPT-cGMPS (200 μM), at 37°C for 5 min, and then stimulated with thrombin (0.02 U/ml). (C) Quantification and statistical analysis of the effect of Rp-Br-PET-cGMPS on platelet aggregation (n=7) and platelet secretion of ATP (n=5). (D) Quantification and statistical analysis of the effect of Rp-pCPT-cGMPS on platelet aggregation (n=9) and platelet secretion of ATP (n=8). **p<0.01, ****p<0.0001, t-test.

Figure 5. The effects of PKG II deficiency on cGMP-induced platelet inhibition and VASP phosphorylation. Washed platelets ($3x10^8$/ml) from PKG $II^{+/+}$ or PKG $II^{-/-}$ mice were preincubated with a cGMP analog, pCPT-cGMP (100μM), at 37°C for 5 min, collagen (2 μg/ml) (A) or thrombin (0.02 U/ml) (B) was added to induce platelet aggregation and ATP release. (C) Washed platelets (3×10^8/ml) from wild type or PKG $II^{-/-}$ mice were incubated with Br-cGMP, pCPT-cGMP, or SNP (100 μM) in the aggregometer at 37°C for 5 min and solubilized in SDS-PAGE sample buffer. Phosphorylation of VASP at Ser^{157} was detected by immunoblotting with a monoclonal antibody specifically recognizing Ser^{157}-phosphorylated VASP, 5C6.

Figure 6. The effects of PKG II deficiency on in vivo thrombosis and the tail bleeding time. (A) $FeCl_3$-induced carotid artery injury was performed and time to occlusive thrombosis recorded as described under *Experimental Procedures*. The occlusion time of each mouse is shown as circles. The bars represent the median occlusion time, (420.0 s for PKG $II^{-/-}$ (n=15), and 321.0 s for PKG $II^{+/+}$ (n=15), p=0.031, Mann-Whitney test). (B) Bleeding time tests were performed blind to genotype in littermate mice (8-10 weeks old) generated from mating PKG $II^{+/-}$ mice. The solid triangles represent the bleeding time of a single mouse. The bars represent the median bleeding time of the group. The difference between PKG $II^{+/+}$ (n=28) and PKG $II^{-/-}$ (n=28) mice is statistically significant (p=0.0062, Mann-Whitney test).

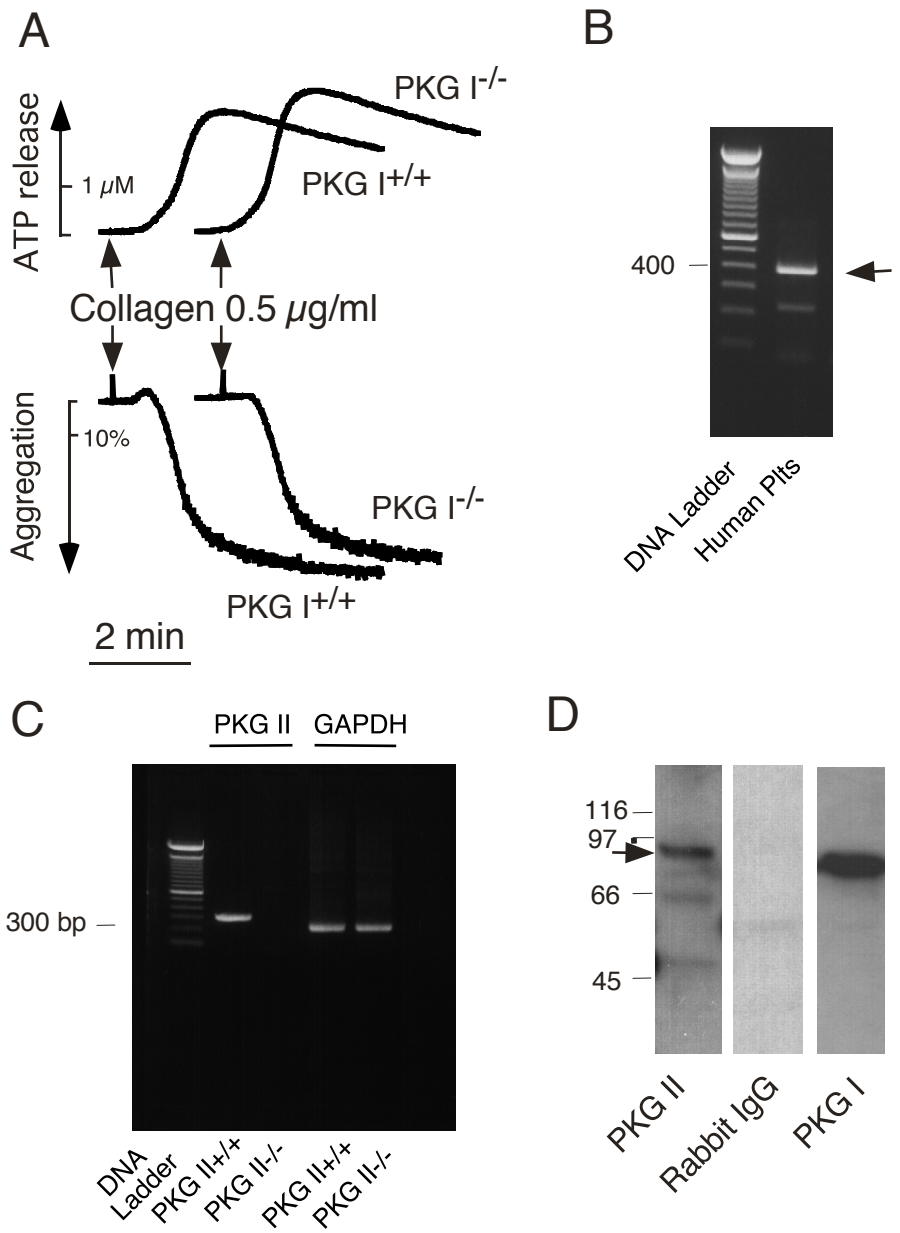
A
ATP release
1 μM
PKG I-/-
PKG I+/+
Collagen 0.5 μg/ml
Aggregation
10%
PKG I-/-
PKG I+/+
2 min
B
400
DNA Ladder
Human Plts
C
PKG II
GAPDH
300 bp
DNA Ladder
PKG II+/+
PKG II-/-
PKG II+/+
PKG II-/-
D
116
97
66
45
PKG II
Rabbit IgG
PKG I
E
PKG II
Plts
PKG I
PKG II
F
PKG I
Plts
PKG I
PKG II

Fig. 1

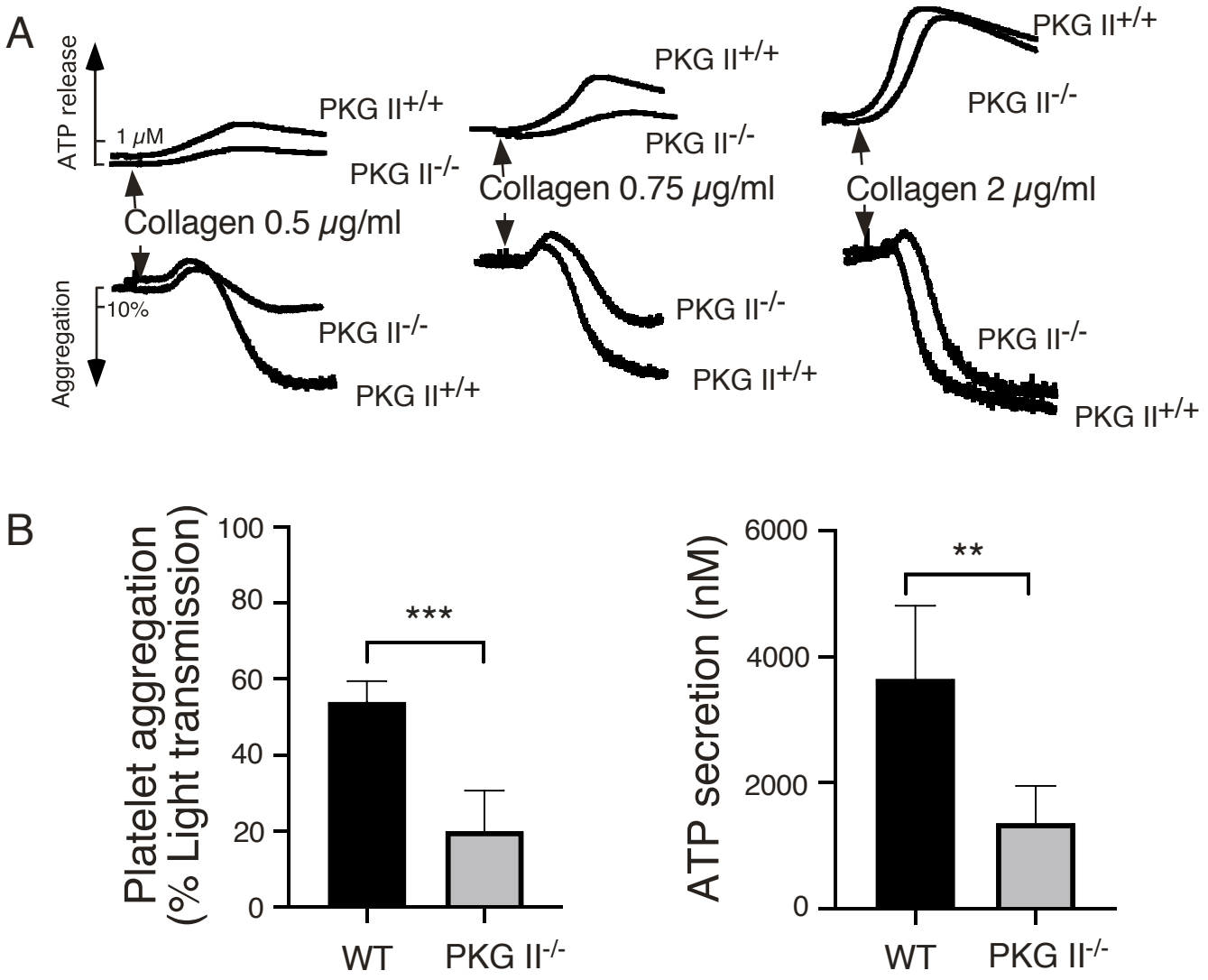
A
ATP release
1 μM
PKG II+/+
PKG II-/-
Collagen 0.5 μg/ml
Aggregation
10%
PKG II-/-
PKG II+/+
PKG II+/+
PKG II-/-
Collagen 0.75 μg/ml
PKG II-/-
PKG II+/+
PKG II+/+
PKG II-/-
Collagen 2 μg/ml
PKG II-/-
PKG II+/+
B
Platelet aggregation (% Light transmission)
100
80
60
40
20
0
***
WT
PKG II-/-
ATP secretion (nM)
6000
4000
2000
0
**
WT
PKG II-/-

Fig. 2

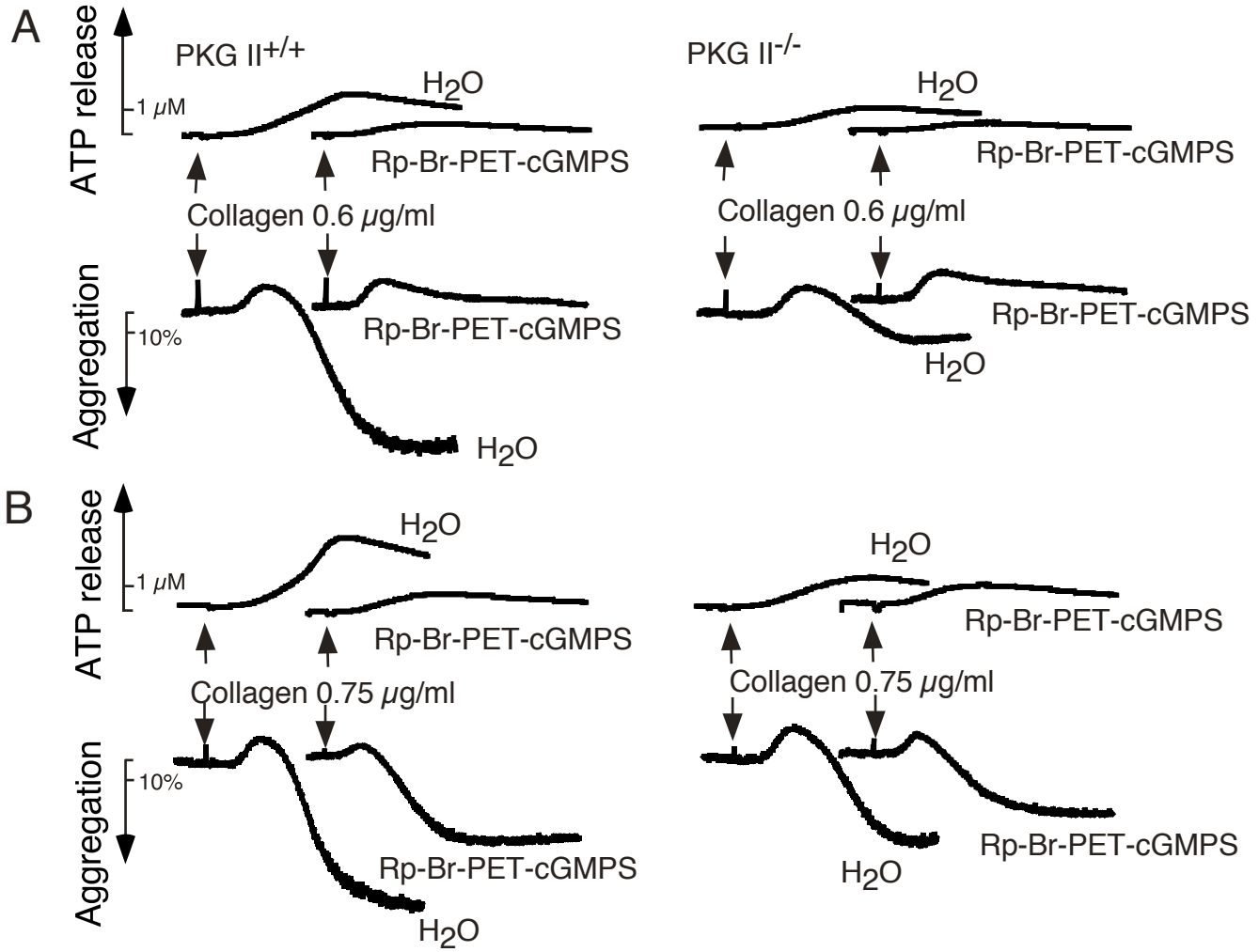
A
ATP release
1 μM
PKG II+/+
H2O
Rp-Br-PET-cGMPS
Collagen 0.6 μg/ml
Aggregation
10%
Rp-Br-PET-cGMPS
H2O
PKG II-/-
H2O
Rp-Br-PET-cGMPS
Collagen 0.6 μg/ml
Rp-Br-PET-cGMPS
H2O
B
ATP release
1 μM
H2O
Rp-Br-PET-cGMPS
Collagen 0.75 μg/ml
Aggregation
10%
Rp-Br-PET-cGMPS
H2O
H2O
Rp-Br-PET-cGMPS
Collagen 0.75 μg/ml
Rp-Br-PET-cGMPS
H2O

Fig. 3

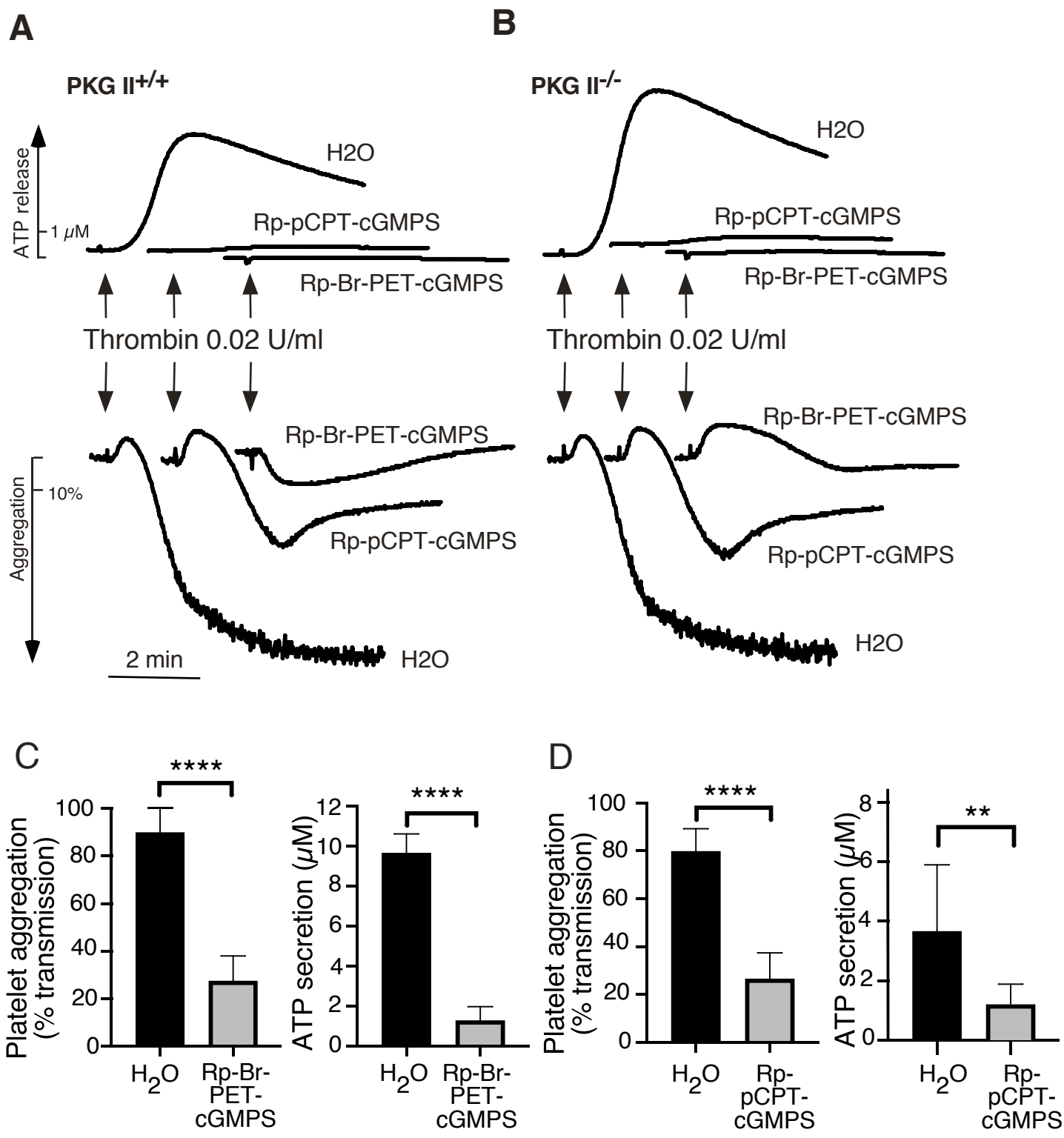
A
B
PKG II+/+
PKG II-/-
ATP release
1 μM
H2O
Rp-pCPT-cGMPS
Rp-Br-PET-cGMPS
Thrombin 0.02 U/ml
Aggregation
10%
2 min
C
D
Platelet aggregation (% transmission)
ATP secretion (μM)
****
**
H2O
Rp-Br-PET-cGMPS
Rp-pCPT-cGMPS

Fig. 4

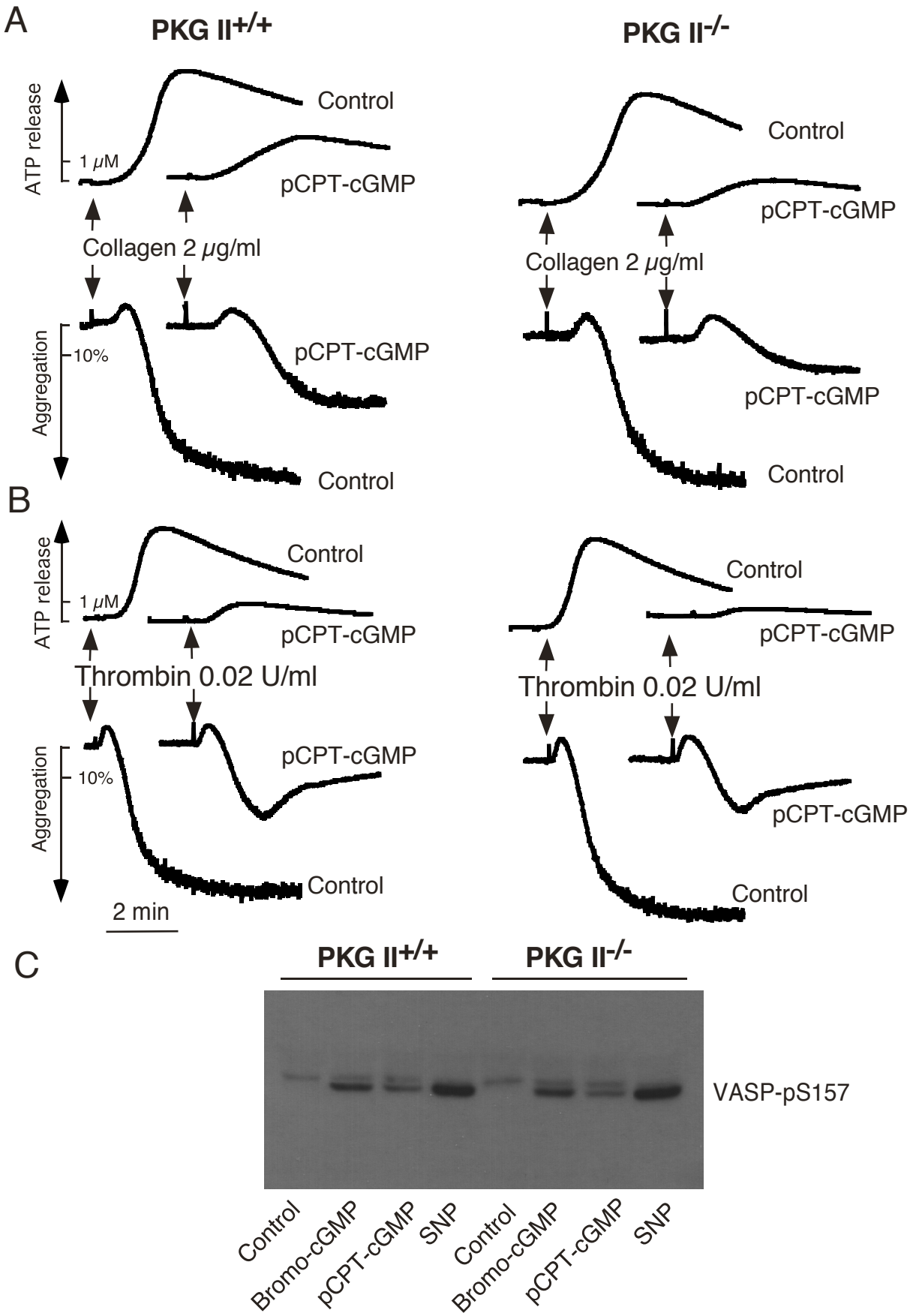
A
PKG II+/+
PKG II-/-
ATP release
1 μM
Control
pCPT-cGMP
Collagen 2 μg/ml
Aggregation
10%
pCPT-cGMP
Control
B
Thrombin 0.02 U/ml
2 min
C
VASP-pS157
Control
Bromo-cGMP
pCPT-cGMP
SNP

Fig. 5

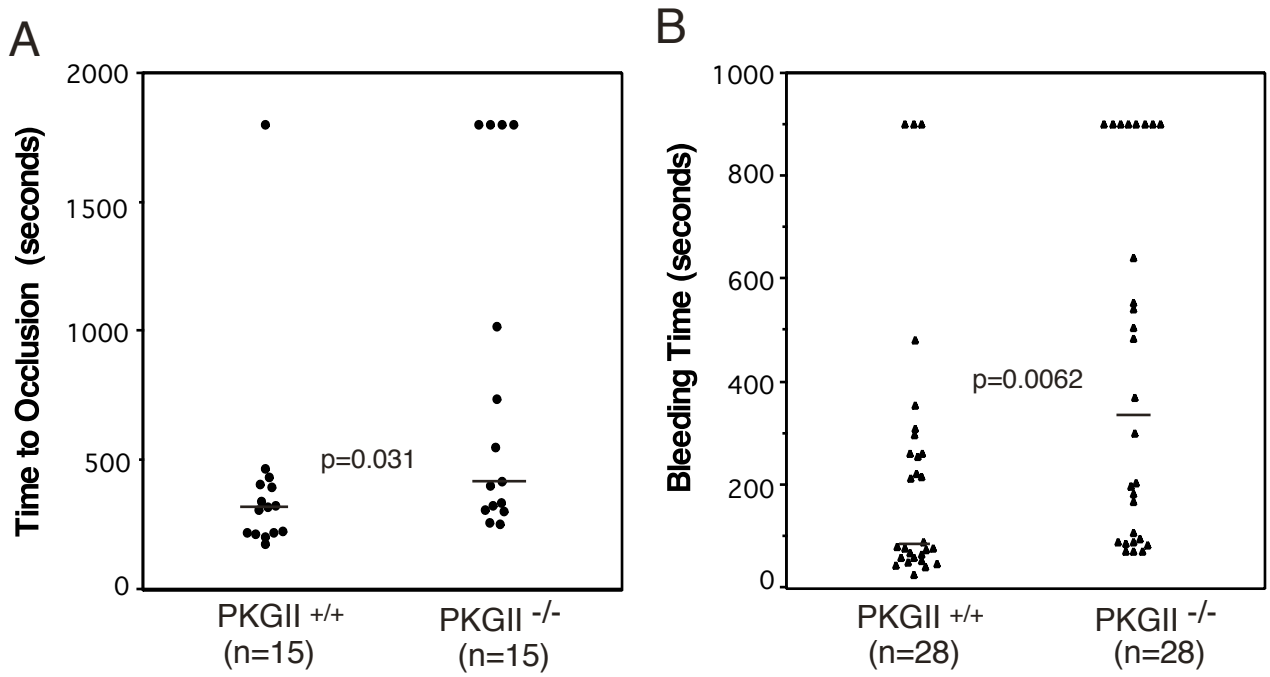
A
Time to Occlusion (seconds)
2000
1500
1000
500
0
p=0.031
PKGII +/+
(n=15)
PKGII -/-
(n=15)
B
Bleeding Time (seconds)
1000
800
600
400
200
0
p=0.0062
PKGII +/+
(n=28)
PKGII -/-
(n=28)

Fig. 6