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The P2Y$_{12}$ Antagonists, 2MeSAMP and Cangrelor, Inhibit Platelet Activation through P2Y$_{12}$/G$_i$-Dependent Mechanism

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Abstract

Background: ADP is an important physiological agonist that induces integrin activation and platelet aggregation through its receptors P2Y$_1$ (Gα_q-coupled) and P2Y$_{12}$ (Gα_i-coupled). P2Y$_{12}$ plays a critical role in platelet activation and thrombosis. Adenosine-based P2Y$_{12}$ antagonists, 2-methylthioadenosine 5'-monophosphate triethylammonium salt hydrate (2MeSAMP) and Cangrelor (AR-C69931MX) have been widely used to demonstrate the role of P2Y$_{12}$ in platelet function. Cangrelor is being evaluated in clinical trials of thrombotic diseases. However, a recent study reported that both 2MeSAMP and Cangrelor raise intra-platelet cAMP levels and inhibit platelet aggregation through a P2Y$_{12}$-independent mechanism.

Methodology/Principal Findings: The present work, using P2Y$_{12}$ deficient mice, sought to clarify previous conflicting reports and to elucidate the mechanisms by which 2MeSAMP and Cangrelor inhibit platelet activation and thrombosis. 2MeSAMP and Cangrelor inhibited aggregation and ATP release of wild-type but not P2Y$_{12}$ deficient platelets. 2MeSAMP and Cangrelor neither raised intracellular cAMP concentrations nor induced phosphorylation of vasodilator-stimulated phosphoprotein (VASP) in washed human or mouse platelets. Furthermore, unlike the activators (PGI$_2$ and forskolin) of the cAMP pathway, 2MeSAMP and Cangrelor failed to inhibit Ca^{2+} mobilization, Akt phosphorylation, and Rap1b activation in P2Y$_{12}$ deficient platelets. Importantly, while injection of Cangrelor inhibited thrombus formation in a FeCl$_3$-induced thrombosis model in wild-type mice, it failed to affect thrombus formation in P2Y$_{12}$ deficient mice.

Conclusions: These data together demonstrate that 2MeSAMP and Cangrelor inhibit platelet function through the P2Y$_{12}$-dependent mechanism both *in vitro* and *in vivo*.

Introduction

Platelets play a key role in hemostasis. Platelet activation includes a series of positive feedback loops that rapidly amplify activation signals to enable robust platelet recruitment and stabilization of thrombi at the sites of vascular injury. Two important mechanisms for amplification are the release of granule cargo (mainly ADP) and synthesis of TXA$_2$ from cyclooxygenase 1 (COX1) signaling. Granule cargo release is required for full platelet responses induced by weak agonists or low concentrations of strong agonists. One of the important substances secreted from dense granules is ADP, which induces integrin activation and platelet aggregation through its receptors, P2Y$_1$ and P2Y$_{12}$ [1]. P2Y$_1$ couples to Gα_q (G$_q$) that transmits cellular signals mainly through its interaction and stimulation of phospholipase Cβ (PLCβ) [2]. Activation of PLCβ results in generation of inositol

trisphosphate (IP3) and diacyl glycerol (DAG) that elicit calcium release and protein kinase C (PKC) activation, respectively [3]. P2Y$_{12}$ couples to Gα_i (G$_i$) family members [4,5], primarily by coupling to G$_{i2}$ [6,7]. Activation of G$_i$ downstream from P2Y$_{12}$ inhibits cAMP accumulation and induces activation of the small GTPase Rap1b in a phosphoinositide 3-kinase (PI3K)-dependent manner.

Mice lacking P2Y$_{12}$ exhibit highly prolonged bleeding times and impaired thrombus formation [5,8]. P2Y$_{12}$ deficient patients have increased bleeding times and abnormal platelet aggregation and secretion, probably due to abnormal cAMP regulation [9,10]. Antiplatelet therapies targeting P2Y$_{12}$, such as clopidogrel, ticagrelor, and prasugrel, have proven benefit in preventing or treating acute arterial thrombosis, and novel P2Y$_{12}$ inhibitors are still in development [11,12,13,14]. P2Y$_{12}$ is required for ADP-

induced platelet activation, and also contributes to platelet activation induced by other platelet agonists, such as thrombin and thromboxane A$_2$ (TXA$_2$) [8,15]. Evidence supporting this notion comes not only from patients and mice lacking functional P2Y$_{12}$, but also from the use of the adenosine-based P2Y$_{12}$ antagonists, 2MeSAMP and Cangrelor [8,10,16,17,18]. However, a recent study reported that both 2MeSAMP and Cangrelor significantly increase intra-platelet cAMP levels in a P2Y$_{12}$/G$_i$-independent manner [19]. Thus, these findings raise a question not only about the specificity of these P2Y$_{12}$ antagonists, but also about the role of P2Y$_{12}$ in platelet activation.

Our data from studies using human platelets and P2Y$_{12}$ deficient mice demonstrate that both 2MeSAMP and Cangrelor do not significantly raise cAMP levels, nor induce vasodilator-stimulated phosphoprotein (VASP) phosphorylation in platelets. Furthermore, 2MeSAMP and Cangrelor inhibit platelet aggregation and in vivo thrombosis only in wild-type mice, but not in P2Y$_{12}$ deficient mice. Taken together, the results therefore indicate that these adenosine-based P2Y$_{12}$ antagonists inhibit platelet function through the P2Y$_{12}$-dependent mechanism.

Materials and Methods

Materials

α-Thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). PAR 4 peptide AYPGKF was custom-synthesized at Biomatik USA, LLC (Wilmington, DE). FeCl$_3$, ADP, and 2MeSAMP were from Sigma. Luciferase/luciferin reagent was from Chrono-log (Havertown, PA). Forskolin was purchased from Calbiochem (San Diego, CA). Fura-2/AM and Pluronic F-127 were from Invitrogen (Carlsbad, CA, USA). RalGDS-RBD fused to GST was a generous gift from Dr. Johannes L. Bos, University Medical Center, Utrecht, the Netherlands. Mouse monoclonal antibodies against VASP phosphorylated at residues serine 157 or serine 239 were purchased from Santa Cruz Biotechnology Inc. Rabbit monoclonal antibodies against the phosphorylated Ser[473] or Thr[308] residues of Akt were from Cell Signaling Technology (Beverly, MA). cAMP ELISA kit was from Amersham Biosciences.

Animals

P2Y$_{12}$ deficient mice were generated as described previously [5]. Littermate wild-type mice from heterozygous breeding were used as controls. All animal procedures were conducted in accordance with appropriate regulatory standards approved by the animal research committee at University of Kentucky, following institutional guidelines for the proper and humane use of animals in research.

Preparation of Platelets

Washed mouse platelets were prepared as described previously [8]. Platelets were resuspended in modified Tyrode's buffer (12 mM NaHCO$_3$, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, 2 mM MgCl$_2$, 0.42 mM NaH$_2$PO$_4$, 10 mM HEPES, pH 7.4) at 3×10^8/ml, and incubated for 1 h at 22°C before use. Washed human platelets were prepared as described previously [8], and resuspended in modified Tyrode's buffer. All participants were provided with written informed consent, and the study was approved by the research ethics boards at University of Kentucky.

Platelet Aggregation and Secretion

Platelet aggregation at 37°C was measured by detecting changes in light transmission using a turbidometric platelet aggregometer (Chrono-Log) with stirring (1000 rpm). Platelet secretion was determined by measuring the release of ATP using luciferin/luciferase reagent. Luciferin/luciferase reagent (12 μl) was added to 238 μl of a washed platelet suspension 1 min before stimulation.

Western Blot Analysis of Akt and VASP Phosphorylation in Platelets

Washed platelets (3×10^8/ml) were preincubated with Cangrelor (1 μM), 2MeSAMP (10 μM), or forskolin (10 μM) for 5 min, and then stimulated with thrombin or AYPGKF in a platelet aggregometer at 37°C for 5 min and then solubilized in SDS-PAGE sample buffer. Platelet lysates were analyzed by SDS-PAGE on 4–15% gradient gels and immunoblotted using rabbit monoclonal antibodies specific for the phosphorylated Akt residues Ser[473] or Thr[308] [20]. To detect VASP phosphorylation, washed platelets were incubated with Cangrelor, 2MeSAMP, or forskolin at 37°C for 5 min. VASP phosphorylation was analyzed by Western blot as described previously [8].

Determination of Intracellular cAMP Levels

Washed platelets (3×10^8/ml) from healthy donors, P2Y$_{12}$ deficient or wild-type mice were resuspended in Tyrode's solution and incubated with 2MeSAMP, Cangrelor, or forskolin for 5 min at 37°C. The reaction was stopped by addition of an equal volume of ice-cold 12% (wt/vol) trichloroacetic acid. Samples were mixed and centrifuged at 2000 g for 15 minutes at 4°C. Each supernatant fraction was washed with 5 volumes of water-saturated diethyl ether 4 times and then lyophilized. cAMP levels were measured using a cAMP enzyme immunoassay kit [8].

To measure intra-platelet cAMP concentrations by mass spectrometry, cAMP was extracted from platelets (3×10^8) by using a mixture of 3:2 ice-cold acetonitrile and water. 2′ Deoxyadenosine 3′, 5′-cyclic monophosphate (2-dAcAMP) from Sigma Aldrich was used as an internal standard. The supernatant fraction containing cAMP was evaporated to dryness under N2 after centrifugation at 20,000 g for 30 min at 4°C, and reconstituted with 1:1 acetonitrile and water. cAMP was quantitated by HPLC- electrospray ionization (ESI) tandem mass spectrometry using an AB Sciex (Foster City, CA) 4000 Q-Trap hybrid linear ion trap triple-quadrupole mass spectrometer equipped with a Turbo V electrospray ion source. cAMP and 2-dAcAMP were analyzed on a Kinetex PFP, 100×4.6 mm, 2.6 u column from Phenomenex with 0.1% formic acid in water and 0.1% formic acid in acetonitrile as solvents at a flow rate of 0.5 mL/min. The mass spectrometer was operated in the positive ESI mode monitoring the following MRM transitions: 314.13/135.9 and 314.13/118.9 for 2-dAcAMP; 330.21/136 and 330.21/118.9 for cAMP. Recovery was calculated using the internal standard and cAMP levels were determined using an off line calibration and normalized to platelet counts [21].

Rap1b Activation

Washed platelets (3×10^8/ml) from P2Y$_{12}$ deficient mice were pre-incubated with 2MeSAMP (10 μM), Cangrelor (1 μM), or forskolin (10 μM) for 5 min, and then stimulated with thrombin (0.25 U/ml) or AYPGKF (500 μM) for an additional 5 min at 37°C with stirring. Rap1b activation was measured as described previously [22].

Ca^{2+} Mobilization

Intra-platelet Ca^{2+} was measured using Fura-2/AM as described previously [22]. Labeled platelets were preincubated with Cangrelor (1 μM), 2MeSAMP (10 μM), forskolin (10 μM), or PGI$_2$ (1 μM).

In vivo Thrombosis

An *in vivo* thrombosis model was performed as described previously [23]. 20% of FeCl$_3$ was applied to a filter paper disc that was immediately placed on top of the artery for 3 minutes. Cangrelor (4 µg per mouse) or saline was injected into the fundus oculi of the mice 5 min prior to the initiation of carotid artery injury.

Statistics

Statistical significance was determined using a Student *t* test. Bar charts show mean ± standard deviation (SD). Fisher exact test was used for analysis of the *in vivo* thrombosis model. A *p*-value of less than 0.05 was considered significant.

Results

2MeSAMP and Cangrelor Inhibit Platelet Activation through P2Y$_{12}$

In order to determine whether inhibition of platelet activation by 2MeSAMP and Cangrelor is mediated specifically through P2Y$_{12}$, the effects of 2MeSAMP and Cangrelor on platelet aggregation and secretion of ATP were examined. cAMP-dependent protein kinase (PKA) is a strong inhibitory signaling pathway of platelet activation. As expected, the PKA pathway activators, forskolin and PGI$_2$, respectively inhibited aggregation and ATP secretion in both wild-type (Fig. 1A) and P2Y$_{12}$ deficient platelets (Fig. 1B) in response to the thrombin receptor PAR4 activator, the peptide AYPGKF. In contrast, 2MeSAMP and Cangrelor inhibited aggregation and ATP release in wild-type platelets elicited only by low concentrations of AYPGKF (Fig. 1C). Similar to 2MeSAMP and Cangrelor treatment, aggregation and secretion were reduced in P2Y$_{12}$ deficient platelets in response to low-dose AYPGKF (Fig. 1C), but not to high-dose AYPGKF (Fig. 1D). Unlike forskolin and PGI$_2$, 2MeSAMP and Cangrelor did not affect aggregation and secretion of P2Y$_{12}$ deficient platelets (Fig. 1C and 1D). These results not only demonstrate a role of the G$_i$ pathway activated by ADP through P2Y$_{12}$ in platelet activation in response to low-dose AYPGKF, but also indicate that the effects of 2MeSAMP and Cangrelor on platelet aggregation and secretion are P2Y$_{12}$-dependent. ADP failed to stimulate aggregation and secretion of P2Y$_{12}$ deficient platelets and 2MeSAMP- or Cangrelor-treated platelets (data not shown).

2MeSAMP and Cangrelor Failed to Stimulate cAMP Production in Platelets

In contrast to a previous report showing that 2MeSAMP and Cangrelor increase intra-platelet cAMP concentrations through a P2Y$_{12}$-independent mechanism [19], we found that Cangrelor, up to 1 µM, failed to increase intracellular cAMP levels in human or mouse platelets (Fig. 2A and 2B). As a positive control, forskolin markedly increased intra-platelet cAMP concentrations (Fig. 2A and 2B). While 2MeSAMP at concentrations at or below 10 µM did not increase cAMP levels in human platelets, it apparently increased intra-platelet cAMP levels at a higher concentration (50 µM) (Fig. 2A). Surprisingly, 2MeSAMP at 50 µM 'enhanced' cAMP concentrations in Tyrode's solution in the absence of platelets. These results suggest that 2MeSAMP may cross-react with the cAMP ELISA assay, thereby causing false positive results. 2MeSAMP at concentrations at or below 10 µM did not increase cAMP levels in platelets from wild-type or P2Y$_{12}$ deficient mice (Fig. 2B). Due to the fact that 2MeSAMP at high concentrations (>50 µM) interferes with the cAMP ELISA assay, we did not test high concentrations of 2MeSAMP in mouse platelets.

To further determine whether 2MeSAMP and Cangrelor can stimulate cAMP production in platelets, we developed an HPLC electrospray ionization tandem mass spectrometry method to measure cAMP concentrations. 2MeSAMP (50 µM) or Cangrelor (1 µM) failed to enhance cAMP levels in mouse platelets or Tyrode's solution, as measured by this mass spectrometry-based assay (Fig. 2C). Using this assay we observed that forskolin stimulated cAMP production in platelets but not in solution in the absence of platelets.

2MeSAMP and Cangrelor Failed to Stimulate VASP Phosphorylation

VASP is a well-known substrate for PKA [8,24,25]. Stimulation of platelets with forskolin induced VASP phosphorylation in human platelets (Fig. 3A). Thus, if 2MeSAMP and Cangrelor increase intracellular cAMP levels in platelets, they should also be able to induce VASP phosphorylation. However, neither 2MeSAMP nor Cangrelor, even at high concentrations, induced VASP phosphorylation in human platelets (Fig. 3A). 2MeSAMP and Cangrelor, respectively failed to induce VASP phosphorylation in both wild-type and P2Y$_{12}$ deficient mouse platelets (Fig. 3B and 3C). These results indicate that 2MeSAMP and Cangrelor are unable to activate the cAMP pathway through a P2Y$_{12}$-independent mechanism in platelets.

Forskolin and PGI$_2$, but not 2MeSAMP and Cangrelor, Inhibited AYPGKR-elicited Ca^{2+} Mobilization

PGE$_1$, by activating G$_s$-coupled receptors and increasing the generation of the intracellular cAMP, inhibited platelet activation in both wild-type and VASP deficient mice [26], demonstrating that cAMP/PKA signaling inhibits platelet activation through a VASP-independent mechanism. Stimulation of platelets with thrombin or TXA$_2$ induces Ca^{2+} elevation through G$_q$-dependent activation of PLC β. To determine whether or not PKA inhibition of platelet activation involves the PLC β/Ca^{2+} signaling, the effect of PGI$_2$ and forskolin on AYPGKF-elicited Ca^{2+} elevation was examined. AYPGKF-induced Ca^{2+} mobilization was abolished by PGI$_2$ and forskolin, respectively (Fig. 4). In contrast, 2MeSAMP and Cangrelor did not affect AYPGKF-elicited Ca^{2+} elevation in P2Y$_{12}$ deficient platelets, supporting and extending our previous observation that, unlike PGI$_2$ and forskolin, 2MeSAMP and Cangrelor cannot activate the cAMP pathway through a P2Y$_{12}$-independent mechanism in platelets.

Forskolin, but not 2MeSAMP and Cangrelor, Inhibited Akt Phosphorylation in P2Y$_{12}$ Deficient Platelets

Akt phosphorylation in response to thrombin receptor stimulation involves both P2Y$_{12}$-dependent and -independent mechanisms [20]. The effect of 2MeSAMP and Cangrelor on AYPGKF-induced Akt phosphorylation appears to be specifically mediated through the P2Y$_{12}$-dependent pathway, because although 2MeSAMP and Cangrelor reduced AYPGKF-induced Akt phosphorylation in wild-type platelets (data not shown), they failed to inhibit Akt phosphorylation in P2Y$_{12}$ deficient platelets in response to thrombin (Fig. 5A) or AYPGKF (Fig. 5B). In contrast, forskolin abolished Akt phosphorylation in P2Y$_{12}$ deficient platelets elicited by thrombin (Fig. 5A) or AYPGKF (Fig. 5B).

Forskolin, but not 2MeSAMP and Cangrelor, Inhibited AYPGKF-elicited Rap1b Activation in P2Y$_{12}$ Deficient Platelets

Rap1b plays important roles in integrin inside-out signaling, and platelet aggregation [22,27]. P2Y$_{12}$/G$_i$ signaling is critical for
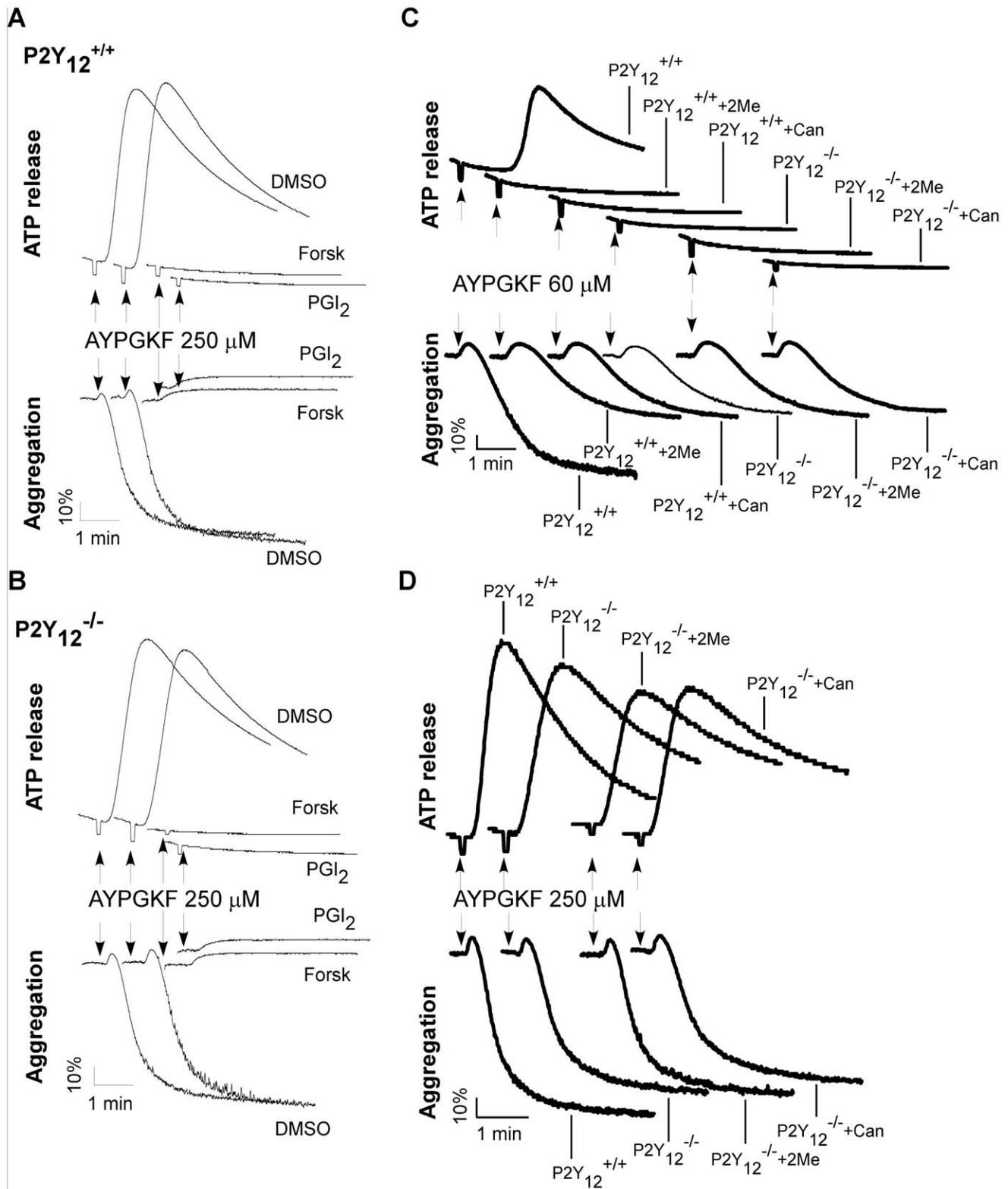
Figure 1. 2MeSAMP and Cangrelor inhibit platelet aggregation and secretion through P2Y$_{12}$-dependent mechanism. (A-B) Washed platelets from wild-type mice (A) or P2Y$_{12}$ deficient mice (B) were pre-incubated with PGI$_2$ (1 µM) or forskolin (10 µM) at 37°C for 5 min, and added with AYPGKF 250 µM to induce ATP release and aggregation. (C–D) Washed platelets (3×10^8/ml) from P2Y$_{12}$ deficient mice and littermate wild-type controls were pre-incubated with Cangrelor (1 µM) (Can) or 2MeSAMP (10 µM) (2Me) at 37°C for 5 min, and added with AYPGKF 60 µM (C) or 250 µM (D) to induce ATP release and aggregation. Data shown are representative of three independent experiments.
doi:10.1371/journal.pone.0051037.g001

A Human platelets

cAMP(pmols/10^8 platelets)

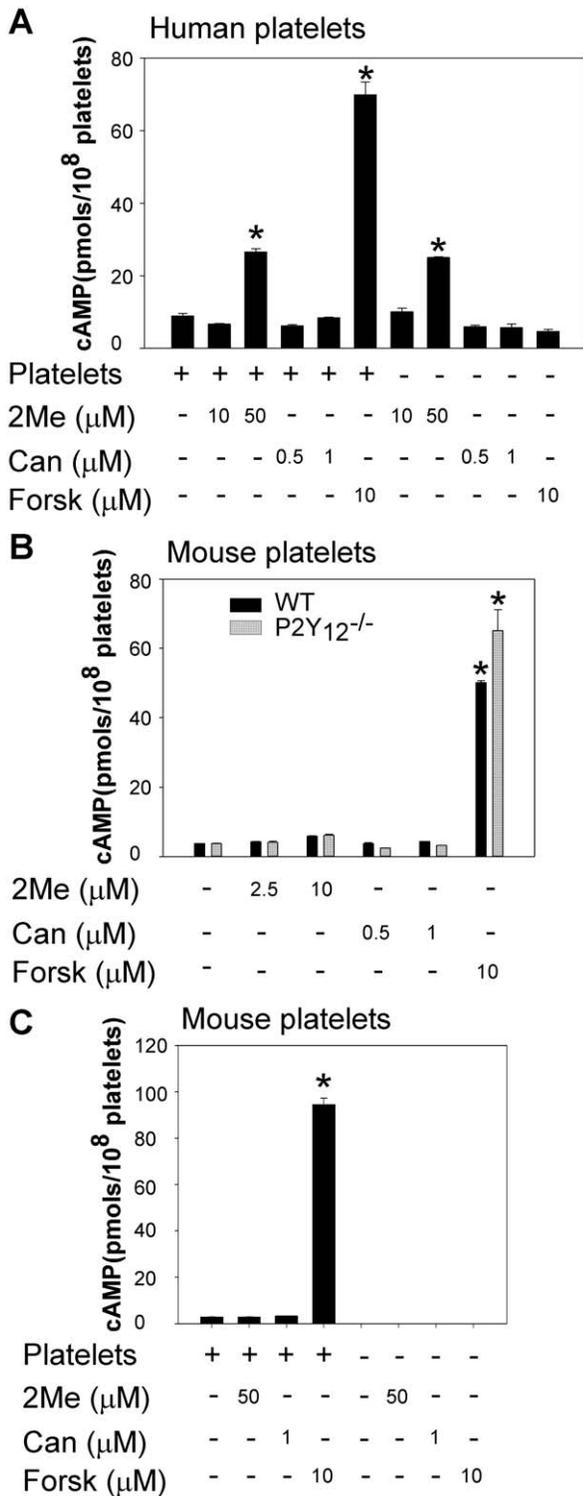| Platelets | + | + | + | + | + | + | − | − | − | − | − |
2Me (μM)	−	10	50	−	−	−	10	50	−	−	−
Can (μM)	−	−	−	0.5	1	−	−	−	0.5	1	−
Forsk (μM)	−	−	−	−	−	10	−	−	−	−	10

B Mouse platelets

■ WT   ▨ P2Y$_{12}^{-/-}$

cAMP(pmols/10^8 platelets)

2Me (μM)	−	2.5	10	−	−	−
Can (μM)	−	−	−	0.5	1	−
Forsk (μM)	−	−	−	−	−	10

C Mouse platelets

cAMP(pmols/10^8 platelets)

Platelets	+	+	+	+	−	−	−	−
2Me (μM)	−	50	−	−	−	50	−	−
Can (μM)	−	−	1	−	−	−	1	−
Forsk (μM)	−	−	−	10	−	−	−	10

Figure 2. 2MeSAMP and Cangrelor failed to stimulate cAMP production. (A) Washed human platelets were incubated with 2MeSAMP (2Me), Cangrelor (Can), or forskolin (Forsk) at 37°C for 5 min. The reactions were stopped by the addition of an equal volume of 12% (w/v) trichloroacetic acid. cAMP concentrations were determined by using a cAMP immunoassay kit. Statistical differences were examined by Student t test. Data are mean ± SD. *$P<0.005$ versus platelets in the absence of antagonists and forskolin. (B) Washed platelets from P2Y$_{12}$ deficient mice or wild-type controls were pre-incubated with 2MeSAMP, Cangrelor, or forskolin for 5 min. The

reactions were stopped by the addition of an equal volume of 12% (w/v) trichloroacetic acid. cAMP concentrations were determined by using a cAMP immunoassay kit. Statistical differences were examined by Student t test. Data are mean ± SD. *$P<0.001$ versus platelets of same genotype in the absence of antagonists and forskolin. (C) Washed platelets from wild-type mice were pre-incubated with 2MeSAMP, Cangrelor, or forskolin for 5 min. cAMP concentrations were determined by HPLC electrospray ionization tandem mass spectrometry as described under *Experimental Procedures*. Statistical differences were examined by Student t test. Data are mean ± SD. *$P<0.001$ versus platelets in the absence of antagonists and forskolin.
doi:10.1371/journal.pone.0051037.g002

Rap1b activation in response to ADP and other agonists [28,29,30,31]. However, we have recently shown that the thrombin mimetic PAR4 peptide AYPGKF and collagen at high concentrations elicited Rap1b activation in P2Y$_{12}$ deficient platelets [22]. AYPGKF-induced, P2Y$_{12}$-independent, activation of Rap1b requires Ca^{2+}. Thus, if forskolin inhibits AYPGKF-induced Ca^{2+} elevation, it should also inhibit agonist-induced Rap1b activation in the P2Y$_{12}$ deficient platelets. Indeed, forskolin inhibited thrombin- or AYPGKF-induced Rap1b activation in P2Y$_{12}$ deficient platelets (Fig. 5C and 5D). In contrast, 2MeSAMP and Cangrelor did not affect thrombin- or AYPGKF-induced Rap1b activation in P2Y$_{12}$ deficient platelets (Fig. 5C and 5D). These data demonstrate that 2MeSAMP and Cangrelor inhibited agonist-induced Rap1b activation in a P2Y$_{12}$-specific manner.

Cangrelor Inhibited Thrombus Formation in Wild-type but not P2Y$_{12}$ Deficient Mice

All the above data indicate that the *in vitro* effects of 2MeSAMP and Cangrelor on platelets are mediated specifically through P2Y$_{12}$. Unlike clopidogrel (Plavix), which is a pro-drug, Cangrelor is an active drug that does not require metabolic conversion for activity. Although the CHAMPION clinical trials did not show clinical benefit of cangrelor beyond that of clopidogrel for percuataneous coronary interventions [32], the BRIDGE study of short-term use of Cangrelor prior to surgery recently concluded with promising results [33]. Thus, it is important to demonstrate whether or not inhibition of thrombosis *in vivo* by Cangrelor is specifically mediated through P2Y$_{12}$. The role of P2Y$_{12}$ in *in vivo* thrombus formation was evaluated using a FeCl$_3$-induced carotid artery thrombosis model. No occlusive thrombi formed in P2Y$_{12}$ deficient mice when low-concentrations of FeCl$_3$ ($\leq$10%) were applied (data not shown). However, when 20% FeCl$_3$ was used to cause vessel injury, P2Y$_{12}$ deficient mice were able to initiate thrombus formation (Fig. 6C). Unlike wild-type mice, in which occlusive thrombi were formed within 10 minutes (Fig. 6A and 6E), thrombi were repeatedly formed and washed away in 60% (9 mice out of 15) of the P2Y$_{12}$ deficient mice examined (Fig. 6C and 6E). Injection of Cangrelor into wild-type mice inhibited stable thrombus formation in a manner similar to that observed in P2Y$_{12}$ deficient mice (Fig. 6B). In contrast, injection of Cangrelor failed to affect stable thrombus formation in P2Y$_{12}$ deficient mice (Fig. 6D and 6E). These results demonstrate that Cangrelor mediated inhibition of thrombus formation *in vivo* is P2Y$_{12}$ dependent.

Discussion

The purpose of this study was to re-examine the specificity of the P2Y$_{12}$ antagonists, Cangrelor and 2MeSAMP in inhibiting platelet functions. This study was initiated to test the assertion that Cangrelor and 2MeSAMP do not inhibit platelet activation solely through a P2Y$_{12}$-dependent manner. Additionally, we discovered that inhibition of platelet activation by the cAMP/PKA pathway

A Human platelets

P-VASP (Ser239) →

β-actin →

2Me (μM)	-	-	10	50	-	-
Can (μM)	-	-	-	-	0.5	1
Forsk (μM)	-	10	-	-	-	-

P2Y$_{12}$ $^{+/+}$

B

P-VASP → (Ser157)

P-VASP → (Ser239)

β-actin →

DMSO	-	+	-	-	-
Forsk	-	-	+	-	-
2Me	-	-	-	+	-
Can	-	-	-	-	+

P2Y$_{12}$ $^{-/-}$

C

P-VASP → (Ser157)

P-VASP → (Ser239)

β-actin →

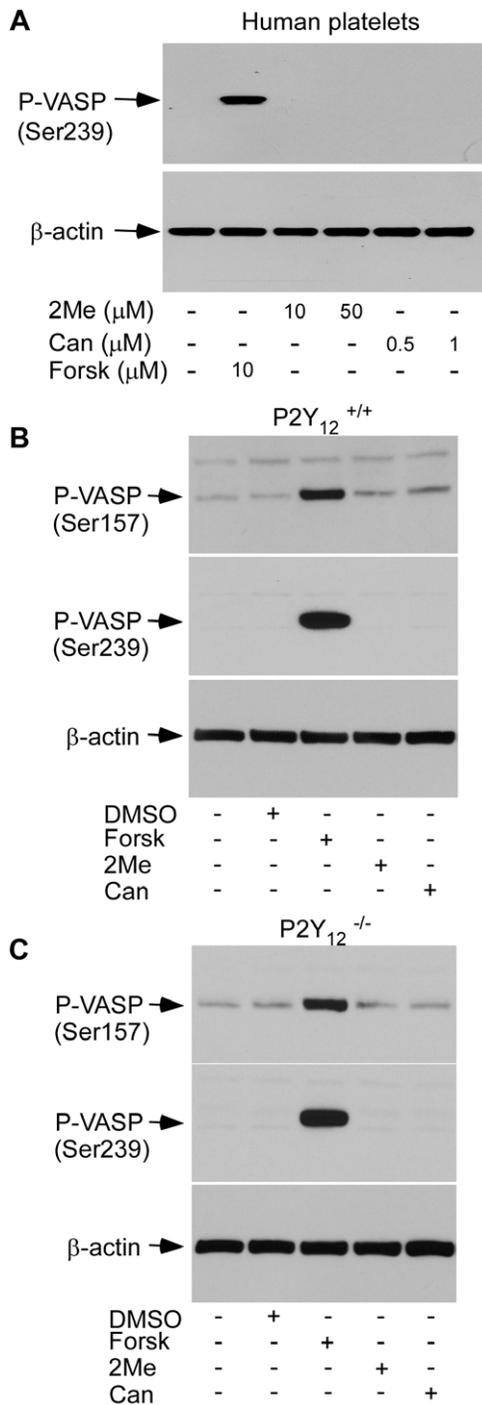| DMSO | - | + | - | - | - |
Forsk	-	-	+	-	-
2Me	-	-	-	+	-
Can	-	-	-	-	+

Figure 3. 2MeSAMP and Cangrelor failed to stimulate VASP phosphorylation in platelets. (A) Washed human platelets were incubated with 2MeSAMP (2Me), Cangrelor (Can), or forskolin (Forsk) at 37°C for 5 min. The reactions were stopped by adding equal volume of 2×SDS sample buffer. Phosphorylation of VASP was detected by Western blotting with mouse monoclonal antibodies specifically recognizing the phosphorylated VASP residues Ser[239]. (B–C) Washed platelets from wild-type (B) or P2Y$_{12}$ deficient mice (C) were pre-incubated with DMSO, forskolin (10 μM), 2MeSAMP (10 μM), or Cangrelor (1 μM) for 5 min. Reactions were stopped by adding equal volume of 2×SDS sample buffer. Phosphorylation of VASP was detected by Western blotting with mouse monoclonal antibodies specifically recognizing the phosphorylated VASP residues Ser[157] or Ser[239].
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involves multiple signaling pathways including PLCβ, Akt, and Rap1b.

Cangrelor and 2MeSAMP have been extensively employed to identify the role of P2Y$_{12}$ in platelet activation. However, a recent study reported that both Cangrelor and 2MeSAMP inhibit platelet activation by increasing intra-platelet cAMP concentrations through a P2Y$_{12}$-independent mechanism [19]. If their data are correct, any conclusions on the role of P2Y$_{12}$ in platelet activation drawn from experiments using these two antagonists need to be re-evaluated. More importantly, Cangrelor is being evaluated in clinical trials of thrombotic diseases with promising results [33]. Therefore, it is necessary to understand the detail mechanisms by which Cangrelor inhibits platelet activation. The key evidence showing that Cangrelor and 2MeSAMP inhibit platelet activation via a P2Y$_{12}$-independent mechanism is that Cangrelor and 2MeSAMP dose-dependently increased intra-platelet cAMP levels in the absence of agonists. In that study, incubation of platelets with 0.05 μM of Cangrelor or 5 μM of 2MeSAMP dramatically increased cAMP concentrations. In contrast, our data indicate that Cangrelor (≤1 μM) and 2MeSAMP (≤10 μM) failed to stimulate cAMP production in both human and mouse platelets. We show that 1 μM of Cangrelor and 10 μM of 2MeSAMP are sufficient to inhibit P2Y$_{12}$ function in isolated platelets (Fig. 1), which are the levels of those antagonists used to demonstrate the role of P2Y$_{12}$ in most platelet studies [8,18,29,30,34,35,36]. The reason for the discrepancy between our study and the previous report are unknown. However, we found that 2MeSAMP at high concentrations (≥50 μM) apparently increases cAMP concentrations in platelets. But because these effects were observed when this compound was added to platelet-free Tyrode's solution, we suspect that at high concentrations 2MeSAMP may cross-react with the antibody used for measurement of cAMP by ELISA and thereby produce a false positive result. To address this issue, we developed tandem mass spectrometry method to measure cAMP concentrations. In this highly specific and sensitive assay, neither 2MeSAMP (50 μM) nor Cangrelor (1 μM) enhanced cAMP concentrations in platelets or in solution. One difference we noticed between the previous study [19] and this study in measuring intra-platelet cAMP is that platelet-rich plasma was used in the previous study but we used washed platelets. It is not clear whether plasma affects cAMP measurement. Agents that elevate cAMP are strong inhibitors of platelet activation [37]. In agreement with the results that Cangrelor and 2MeSAMP could not induce cAMP production in platelets, they failed to inhibit platelet secretion and aggregation in P2Y$_{12}$ deficient platelets. In contrast, PGI$_2$ and forskolin, respectively abolished platelet activation in both wild-type and P2Y$_{12}$ deficient mice.

Further evidence supporting the conclusion that Cangrelor and 2MeSAMP are unable to induce P2Y$_{12}$-independent activation of the cAMP-PKA pathway in platelets was provided by our study of VASP phosphorylation. VASP is a well-established substrate for PKA. Thus, if Cangrelor and 2MeSAMP increase intra-platelet cAMP concentrations to a level similar as PGI$_2$, they should be able to induce VASP phosphorylation. However, unlike PGI$_2$ or forskolin, both Cangrelor and 2MeSAMP, even at high concentrations, failed to stimulate VASP phosphorylation in intact platelets.

Although it is well established that cAMP/PKA signaling inhibits platelet activation, the mechanisms by which cAMP/PKA inhibits platelet activation are not fully understood. In this regard, PKA can phosphorylate glycoprotein (GP) Ibβ, resulting in inhibition of GPIb-IX-V-stimulated platelet activation [38]. PKA has been shown to phosphorylate PLCβ and inhibit PLCβ signaling in COS 7 cells [39]. Ca^{2+} mobilization from the PLCβ
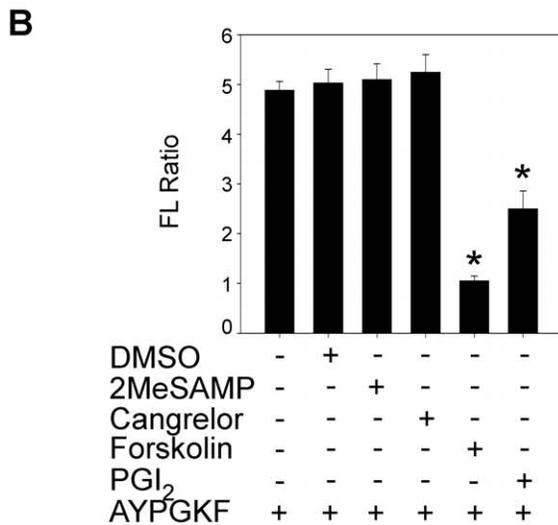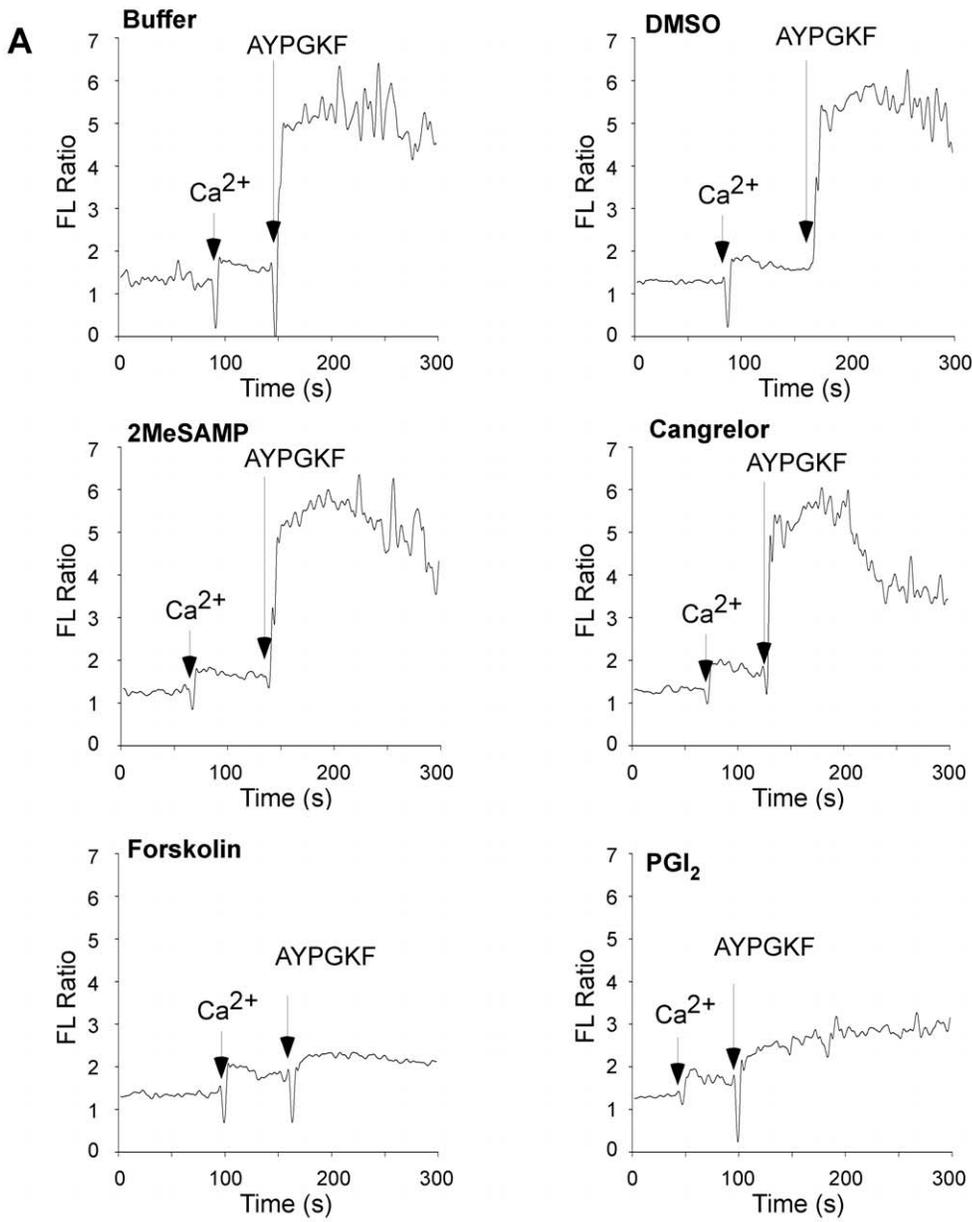
Figure 4. The PKA activators, but not 2MeSAMP and Cangrelor, inhibited Ca^{2+} mobilization in P2Y$_{12}$ deficient platelets. Washed platelets from P2Y$_{12}$ deficient mice were labeled with 12.5 μM Fura-2/AM/0.2% Pluronic F-127 and resuspended in Tyrode's solution at 3×10^8/ml. Platelets were preincubated with Cangrelor (1 μM), 2MeSAMP (10 μM), forskolin (10 μM), or PGI$_2$ (1 μM), and then stimulated with AYPGKF (500 μM). Changes in the intracellular Ca^{2+} levels were measured every 2 s and expressed as a ratio of fluorescence (FL) detected at 509 nm emission with an excitation wavelength of 340 nm and 380 nm (A). Summarized data from three experiments are shown (B). Statistical differences were examined by Student t test. Data are mean $\pm$ SD. *$P<0.001$ versus DMSO.
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signaling is a key event in platelet activation induced by GPCR agonists such thrombin and TXA$_2$. Our data indicate that PKA-dependent inhibition of platelet activation involves the PLCβ pathway, because pre-treatment of platelets with forskolin or PGI$_2$ abolished AYPGKF-elicited Ca^{2+} elevation (Fig. 4). Thus, if Cangrelor and 2MeSAMP inhibits platelet activation by increasing cAMP concentrations through P2Y$_{12}$-independent mechanisms, they should be able to inhibit AYPGKF-elicited Ca^{2+} elevation. However, neither Cangrelor nor 2MeSAMP had an effect on AYPGKF-stimulated Ca^{2+} mobilization in P2Y$_{12}$ deficient platelets.

Forskolin and PGI$_2$, respectively inhibited multiple signaling pathways that are known to play important roles in mediating platelet secretion and aggregation, such as Akt phosphorylation [40] and Rap1b activation [27]. In contrast, Cangrelor and 2MeSAMP had no effect on AYPGKF-stimulated phosphorylation of Akt and Rap1b activation in P2Y$_{12}$ deficient platelets. These data demonstrate that the inhibitory effect of Cangrelor and 2MeSAMP on platelet activation is P2Y$_{12}$-dependent and does not involve the P2Y$_{12}$-independent activation of the cAMP/PKA pathway.

P2Y$_{12}$ is not only important for agonist-induced platelet activation *in vitro*, but also contributes to thrombosis *in vivo*
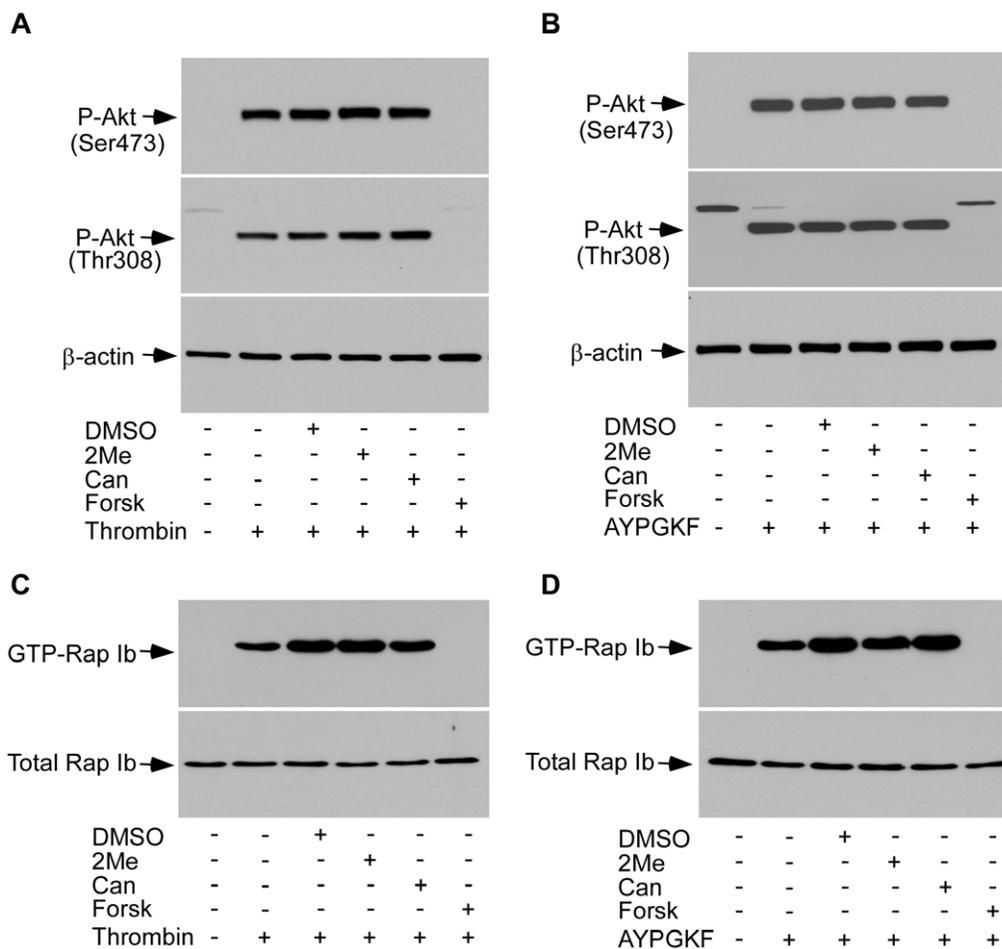

Figure 5. The PKA activators, but not 2MeSAMP and Cangrelor, inhibited Akt phosphorylation and Rap1b activation in P2Y$_{12}$ deficient platelets. (A-B) Washed platelets from P2Y$_{12}$ deficient mice were pre-incubated with 2MeSAMP (10 μM) (2Me), Cangrelor (1 μM) (Can), or forskolin (10 μM) (Forsk) at 37°C for 5 min, and then stimulated with thrombin (0.25 U/ml) (A) or AYPGKF (500 μM) (B). Akt phosphorylation was detected by Western blotting with a rabbit monoclonal antibody specifically recognizing the phosphorylated Akt residue Ser^{473} or Thr^{308}. A mouse monoclonal antibody against β-actin (Sigma-Aldrich) was used to verify equal loading. (C–D) Washed platelets from P2Y$_{12}$ deficient mice were pre-incubated with 2MeSAMP (10 μM) (2Me), Cangrelor (1 μM) (Can), or forskolin (10 μM) (Forsk) at 37°C for 5 min, and then stimulated with thrombin (0.25 U/ml) (C) or AYPGKF (500 μM) (D). GTP-bound Rap1 was precipitated with GST-RalGDS RBD bound to glutathione-agarose beads and detected by Western blot.
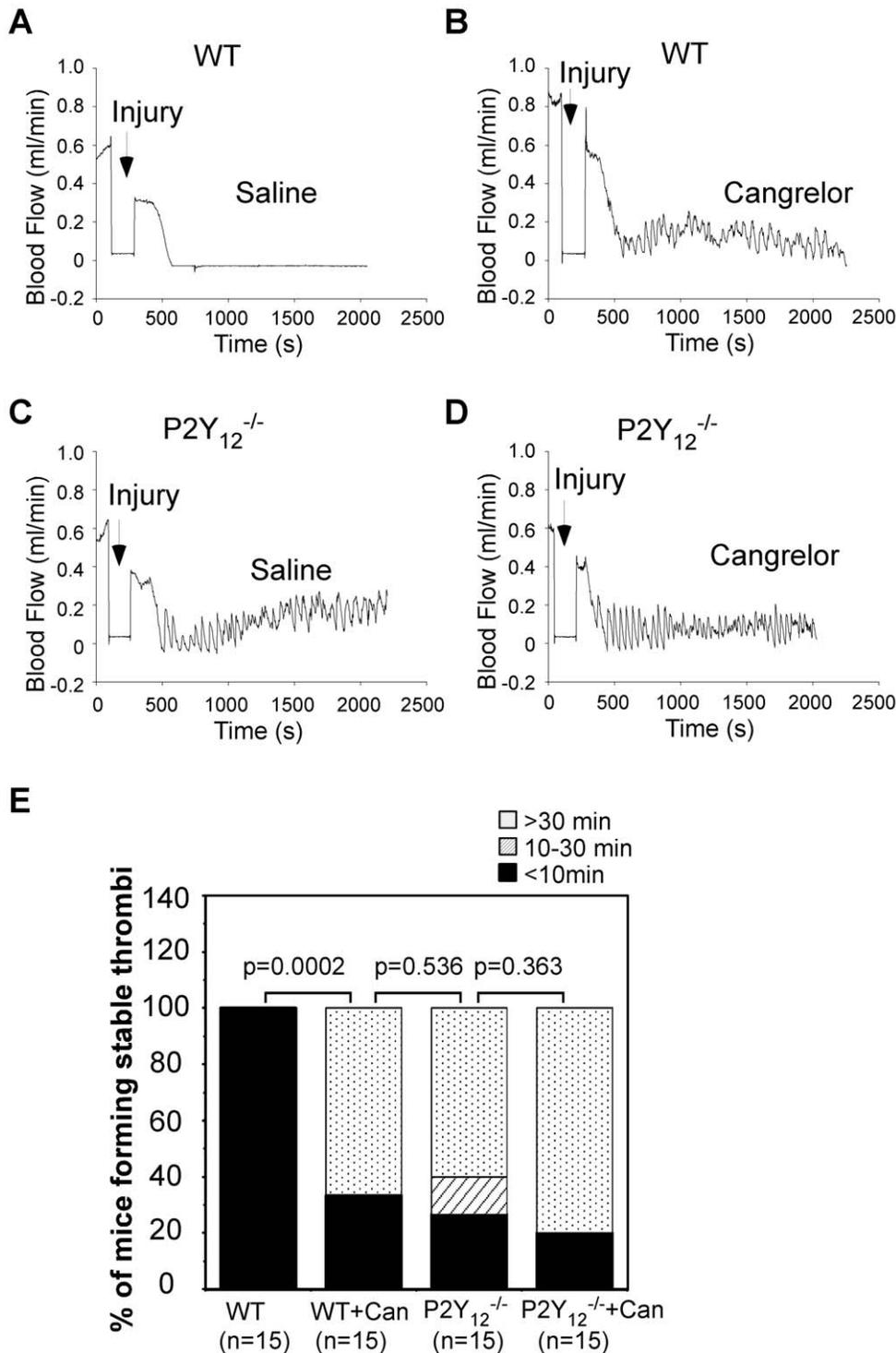doi:10.1371/journal.pone.0051037.g005

Figure 6. Cangrelor inhibited thrombus formation in wild-type but not P2Y$_{12}$ deficient mice. (A–D) FeCl$_3$-induced carotid artery injury was performed and time to occlusive thrombus formation recorded as described under *Experimental Procedures*. Wild-type (A and B) or P2Y$_{12}$ deficient mice (C and D) were injected with saline (A and C) or Cangrelor (B and D) 5 min prior to the carotid artery injury. (E) Mice were calculated by time length of forming occlusive thrombi (<10 min, 10–30 min, >30 min). Statistical differences were examined by Fisher exact test.
doi:10.1371/journal.pone.0051037.g006

[8,41]. Using a FeCl$_3$-induced carotid artery thrombosis model, we found that the initial thrombus formation is not impaired in P2Y$_{12}$ deficient mice. However, most P2Y$_{12}$ deficient mice cannot form stable thrombi under our experimental conditions. Likewise,

injection of Cangrelor into wild-type mice inhibits stable thrombus formation. Accordingly, injection of Cangrelor has no effect on thrombus formation in P2Y$_{12}$ deficient mice. Initial thrombus formation caused by vascular injury is mediated by von Will-

ebrand factor bound to subendothelial matrix. Stable thrombus formation requires integrin activation, a process in which P2Y$_{12}$ plays an important role. Our results demonstrate that Cangrelor inhibition of thrombus formation *in vivo* is also P2Y$_{12}$ dependent.

Acknowledgments

Author Contributions

Conceived and designed the experiments: BX GZ SSS ZL. Performed the experiments: BX GZ HR MS AJM ZL. Analyzed the data: BX GZ HR AJM TKG SSS ZL.

References

1. Shankar H, Kahner BN, Prabhakar J, Lakhani P, Kim S, et al. (2006) G-protein-gated inwardly rectifying potassium channels regulate ADP-induced cPLA2 activity in platelets through Src family kinases. Blood 108: 3027–3034.
2. Jin J, Daniel JL, Kunapuli SP (1998) Molecular basis for ADP-induced platelet activation. II. The P2Y1 receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. J Biol Chem 273: 2030–2034.
3. Berridge MJ (1993) Inositol trisphosphate and calcium signalling. Nature 361: 315–325.
4. Hollopeter G, Jantzen HM, Vincent D, Li G, England L, et al. (2001) Identification of the platelet ADP receptor targeted by antithrombotic drugs. Nature 409: 202–207.
5. Foster CJ, Prosser DM, Agans JM, Zhai Y, Smith MD, et al. (2001) Molecular identification and characterization of the platelet ADP receptor targeted by thienopyridine antithrombotic drugs. J Clin Invest 107: 1591–1598.
6. Ohlmann P, Laugwitz KL, Nurnberg B, Spicher K, Schultz G, et al. (1995) The human platelet ADP receptor activates Gi2 proteins. Biochem J 312 (Pt 3): 775–779.
7. Jantzen HM, Milstone DS, Gousset L, Conley PB, Mortensen RM (2001) Impaired activation of murine platelets lacking G alpha(i2). J Clin Invest 108: 477–483.
8. Andre P, Delaney SM, LaRocca T, Vincent D, DeGuzman F, et al. (2003) P2Y12 regulates platelet adhesion/activation, thrombus growth, and thrombus stability in injured arteries. J Clin Invest 112: 398–406.
9. Cattaneo M, Lecchi A, Randi AM, McGregor JL, Mannucci PM (1992) Identification of a new congenital defect of platelet function characterized by severe impairment of platelet responses to adenosine diphosphate. Blood 80: 2787–2796.
10. Dorsam RT, Kunapuli SP (2004) Central role of the P2Y12 receptor in platelet activation. J Clin Invest 113: 340–345.
11. Committee CS (1996) A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). CAPRIE Steering Committee. Lancet 348: 1329–1339.
12. Steg PG, James S, Harrington RA, Ardissino D, Becker RC, et al. (2010) Ticagrelor versus clopidogrel in patients with ST-elevation acute coronary syndromes intended for reperfusion with primary percutaneous coronary intervention: A Platelet Inhibition and Patient Outcomes (PLATO) trial subgroup analysis. Circulation 122: 2131–2141.
13. Smyth SS, Woulfe DS, Weitz JI, Gachet C, Conley PB, et al. (2009) G-protein-coupled receptors as signaling targets for antiplatelet therapy. Arterioscler Thromb Vasc Biol 29: 449–457.
14. Damman P, Woudstra P, Kuijt WJ, de Winter RJ, James SK (2012) P2Y12 platelet inhibition in clinical practice. J Thromb Thrombolysis 33: 143–153.
15. Paul BZ, Jin J, Kunapuli SP (1999) Molecular mechanism of thromboxane A(2)-induced platelet aggregation. Essential role for p2t(ac) and alpha(2a) receptors. J Biol Chem 274: 29108–29114.
16. Kunapuli SP, Ding Z, Dorsam RT, Kim S, Murugappan S, et al. (2003) ADP receptors–targets for developing antithrombotic agents. Curr Pharm Des 9: 2303–2316.
17. Kim S, Foster C, Lecchi A, Quinton TM, Prosser DM, et al. (2002) Protease-activated receptors 1 and 4 do not stimulate G(i) signaling pathways in the absence of secreted ADP and cause human platelet aggregation independently of G(i) signaling. Blood 99: 3629–3636.
18. Storey RF, Newby LJ, Heptinstall S (2001) Effects of P2Y(1) and P2Y(12) receptor antagonists on platelet aggregation induced by different agonists in human whole blood. Platelets 12: 443–447.
19. Srinivasan S, Mir F, Huang JS, Khasawneh FT, Lam SC, et al. (2009) The P2Y12 antagonists, 2-methylthioadenosine 5′-monophosphate triethylammonium salt and cangrelor (ARC69931MX), can inhibit human platelet aggregation through a Gi-independent increase in cAMP levels. J Biol Chem 284: 16108–16117.
20. Xiang B, Zhang G, Liu J, Morris AJ, Smyth SS, et al. (2010) A G(i) -independent mechanism mediating Akt phosphorylation in platelets. J Thromb Haemost 8: 2032–2041.
21. Au JL, Su MH, Wientjes MG (1989) Extraction of intracellular nucleosides and nucleotides with acetonitrile. Clin Chem 35: 48–51.
22. Zhang G, Xiang B, Ye S, Chrzanowska-Wodnicka M, Morris AJ, et al. (2011) Distinct Roles for Rap1b Protein in Platelet Secretion and Integrin alphaIIbbeta3 Outside-in Signaling. J Biol Chem 286: 39466–39477.
23. Zhang G, Xiang B, Dong A, Skoda RC, Daugherty A, et al. (2011) Biphasic roles for soluble guanylyl cyclase (sGC) in platelet activation. Blood 118: 3670–3679.
24. Eigenthaler M, Nolte C, Halbrugge M, Walter U (1992) Concentration and regulation of cyclic nucleotides, cyclic-nucleotide-dependent protein kinases and one of their major substrates in human platelets. Estimating the rate of cAMP-regulated and cGMP-regulated protein phosphorylation in intact cells. Eur J Biochem 205: 471–481.
25. Butt E, Abel K, Krieger M, Palm D, Hoppe V, et al. (1994) cAMP- and cGMP-dependent protein kinase phosphorylation sites of the focal adhesion vasodilator-stimulated phosphoprotein (VASP) in vitro and in intact human platelets. J Biol Chem 269: 14509–14517.
26. Hauser W, Knobeloch KP, Eigenthaler M, Gambaryan S, Krenn V, et al. (1999) Megakaryocyte hyperplasia and enhanced agonist-induced platelet activation in vasodilator-stimulated phosphoprotein knockout mice. Proc Natl Acad Sci U S A 96: 8120–8125.
27. Chrzanowska-Wodnicka M, Smyth SS, Schoenwaelder SM, Fischer TH, White GC, 2nd (2005) Rap1b is required for normal platelet function and hemostasis in mice. J Clin Invest 115: 680–687.
28. Cifuni SM, Wagner DD, Bergmeier W (2008) CalDAG-GEFI and protein kinase C represent alternative pathways leading to activation of integrin alphaIIbbeta3 in platelets. Blood 112: 1696–1703.
29. Woulfe D, Jiang H, Mortensen R, Yang J, Brass LF (2002) Activation of Rap1B by G(i) family members in platelets. J Biol Chem 277: 23382–23390.
30. Lova P, Paganini S, Sinigaglia F, Balduini C, Torti M (2002) A Gi-dependent pathway is required for activation of the small GTPase Rap1B in human platelets. J Biol Chem 277: 12009–12015.
31. Larson MK, Chen H, Kahn ML, Taylor AM, Fabre JE, et al. (2003) Identification of P2Y12-dependent and -independent mechanisms of glycoprotein VI-mediated Rap1 activation in platelets. Blood 101: 1409–1415.
32. Faxon DP (2010) Antiplatelet therapy: cangrelor for ACS-lessons from the CHAMPION trials. Nat Rev Cardiol 7: 124–125.
33. Angiolillo DJ, Firstenberg MS, Price MJ, Tummala PE, Hutyra M, et al. (2012) Bridging antiplatelet therapy with cangrelor in patients undergoing cardiac surgery: a randomized controlled trial. JAMA 307: 265–274.
34. Kim S, Mangin P, Dangelmaier C, Lillian R, Jackson SP, et al. (2009) The role of PI 3-K{beta} in glycoprotein VI-mediated akt activation in platelets. J Biol Chem 284: 33763–33772.
35. Lova P, Campus F, Lombardi R, Cattaneo M, Sinigaglia F, et al. (2004) Contribution of protease-activated receptors 1 and 4 and glycoprotein Ib-IX-V in the G(i)-independent activation of platelet Rap1B by thrombin. J Biol Chem 279: 25299–25306.
36. Maloney SF, Brass LF, Diamond SL (2010) P2Y12 or P2Y1 inhibitors reduce platelet deposition in a microfluidic model of thrombosis while apyrase lacks efficacy under flow conditions. Integr Biol (Camb) 2: 183–192.
37. Johnston-Cox HA, Yang D, Ravid K (2011) Physiological implications of adenosine receptor-mediated platelet aggregation. J Cell Physiol 226: 46–51.
38. Bodnar RJ, Xi X, Li Z, Berndt MC, Du X (2002) Regulation of glycoprotein Ib-IX-von Willebrand factor interaction by cAMP-dependent protein kinase-mediated phosphorylation at Ser 166 of glycoprotein Ib(beta). J Biol Chem 277: 47080–47087.
39. Liu M, Simon MI (1996) Regulation by cAMP-dependent protein kinease of a G-protein-mediated phospholipase C. Nature 382: 83–87.
40. Woulfe DS (2010) Akt signaling in platelets and thrombosis. Expert Rev Hematol 3: 81–91.
41. Liu J, Joglekar M, Ware J, Fitzgerald ME, Lowell CA, et al. (2008) Evaluation of the physiological significance of botrocetin/von Willebrand factor in vitro signaling. J Thromb Haemost 6: 1915–1922.