The P2Y12 Antagonists, 2MeSAMP and Cangrelor, Inhibit Platelet Activation through P2Y12/Gi-Dependent Mechanism

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Abstract

**Background:** ADP is an important physiological agonist that induces integrin activation and platelet aggregation through its receptors P2Y1 (Gq-coupled) and P2Y12 (Gq-coupled). P2Y12 plays a critical role in platelet activation and thrombosis. Adenosine-based P2Y12 antagonists, 2-methylthioadenosine 5’-monophosphate triethylammonium salt hydrate (2MeSAMP) and Cangrelor (AR-C69931MX) have been widely used to demonstrate the role of P2Y12 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 P2Y12-independent mechanism.

**Methodology/Principal Findings:** The present work, using P2Y12 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 P2Y12 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 (PGI2 and forskolin) of the cAMP pathway, 2MeSAMP and Cangrelor failed to inhibit Ca2+ mobilization, Akt phosphorylation, and Rap1B activation in P2Y12 deficient platelets. Importantly, while injection of Cangrelor inhibited thrombus formation in a FeCl3-induced thrombosis model in wild-type mice, it failed to affect thrombus formation in P2Y12 deficient mice.

**Conclusions:** These data together demonstrate that 2MeSAMP and Cangrelor inhibit platelet activation through the P2Y12-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 TXA2 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, P2Y1 and P2Y12 [1]. P2Y1 couples to Gq1 (Gq) that transmits cellular signals mainly through its interaction and stimulation of phospholipase Cβ (PLCβ) [2]. Activation of PLCβ results in generation of inositol triphosphate (IP3) and diacyl glycerol (DAG) that elicit calcium release and protein kinase C (PKC) activation, respectively [3]. P2Y12 couples to Gαi (Gi) family members [4,5], primarily by coupling to Gαi2 [6,7]. Activation of Gi downstream from P2Y12 inhibits cAMP accumulation and induces activation of the small GTPase Rap1B in a phosphoinositide 3-kinase (PI3K)-dependent manner.

Mice lacking P2Y12 exhibit highly prolonged bleeding times and impaired thrombus formation [5,8]. P2Y12 deficient patients have increased bleeding times and abnormal platelet aggregation and secretion, probably due to abnormal cAMP regulation [9,10]. Antiplatelet therapies targeting P2Y12, such as clopidogrel, ticagrelor, and prasugrel, have proven benefit in preventing or treating acute arterial thrombosis, and novel P2Y12 inhibitors are still in development [11,12,13,14]. P2Y12 is required for ADP-
induced platelet activation, and also contributes to platelet activation induced by other platelet agonists, such as thrombin and thromboxane A2 (TXA2) [6,15]. Evidence supporting this notion comes not only from patients and mice lacking functional P2Y12, but also from the use of the adenosine-based P2Y12 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 P2Y12/G13-independent manner [19]. Thus, these findings raise a question not only about the specificity of these P2Y12 antagonists, but also about the role of P2Y12 in platelet activation.

Our data from studies using human platelets and P2Y12 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 P2Y12 deficient mice. Taken together, the results therefore indicate that these adenosine-based P2Y12 antagonists inhibit platelet function through the P2Y12-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), FeCl3, 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). RaIGDS-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 Ser473 or Thr308 residues of Akt were from Cell Signaling Technology (Beverly, MA). cAMP ELISA kit was from Amersham Biosciences.

Animals

P2Y12 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 NaHCO3, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, 2 mM MgCl2, 0.42 mM Na2HPO4, 10 mM HEPES, pH 7.4) at 3 × 108/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 230 μl of a washed platelet suspension 1 min before stimulation.

Western Blot Analysis of Akt and VASP Phosphorylation in Platelets

Washed platelets (3 × 108/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 Ser473 or Thr308 [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 × 108/ml) from healthy donors, P2Y12 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 four 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 × 108) 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 μm×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: 314.13/118.9 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 × 108/ml) from P2Y12 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].

Ca2+ Mobilization

Intra-platelet Ca2+ was measured using Fura-2/AM as described previously [22]. Labelled platelets were preincubated with Cangrelor (1 μM), 2MeSAMP (10 μM), forskolin (10 μM), or PGI2 (1 μM).
**In vivo Thrombosis**

An *in vivo* thrombosis model was performed as described previously [23]. 20% of FeCl₃ 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 P2Y₁₂**

In order to determine whether inhibition of platelet activation by 2MeSAMP and Cangrelor is mediated specifically through P2Y₁₂, 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 PGI₂, respectively inhibited aggregation and ATP secretion in both wild-type (Fig. 1A) and P2Y₁₂ deficient platelets (Fig. 1B) in response to the thrombin receptor PAR4 activator, the peptide AYPGKF (Fig. 1C). Similar to 2MeSAMP and Cangrelor treatment, aggregation and secretion were reduced in P2Y₁₂ deficient platelets in response to low-dose AYPGKF (Fig. 1C), but not to high-dose AYPGKF (Fig. 1D). Unlike forskolin and PGI₂, 2MeSAMP and Cangrelor did not affect aggregation and secretion of P2Y₁₂ deficient platelets (Fig. 1C and 1D). These results not only demonstrate a role of the Gₛ pathway activated by ADP through P2Y₁₂ 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 P2Y₁₂-dependent. ADP failed to stimulate aggregation and secretion of P2Y₁₂ 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 P2Y₁₂-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 P2Y₁₂ 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 (30 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 [0, 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 P2Y₁₂ deficient mouse platelets (Fig. 3B and 3C). These results indicate that 2MeSAMP and Cangrelor are unable to activate the cAMP pathway through a P2Y₁₂-independent mechanism in platelets.

**Forskolin and PGI₂, but not 2MeSAMP and Cangrelor, Inhibited AYPGKF-elicited Ca²⁺ Mobilization**

PGI₁, by activating Gₛ-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 TXA₂ induces Ca²⁺ elevation through Gₛ-dependent activation of PLC β. To determine whether or not PKA inhibition of platelet activation involves the PLC β/Ca²⁺ signaling, the effect of PGI₂ and forskolin on AYPGKF-elicited Ca²⁺ elevation was examined. AYPGKF-induced Ca²⁺ mobilization was abolished by PGI₂ and forskolin, respectively (Fig. 4). In contrast, 2MeSAMP and Cangrelor did not affect AYPGKF-elicited Ca²⁺ elevation in P2Y₁₂ deficient platelets, supporting and extending our previous observation that, unlike PGI₂ and forskolin, 2MeSAMP and Cangrelor cannot activate the cAMP pathway through a P2Y₁₂-independent mechanism in platelets.

**Forskolin, but not 2MeSAMP and Cangrelor, Inhibited Akt Phosphorylation in P2Y₁₂ Deficient Platelets**

Akt phosphorylation in response to thrombin receptor stimulation involves both P2Y₁₂-dependent and -independent mechanisms [20]. The effect of 2MeSAMP and Cangrelor on AYPGKF-induced Akt phosphorylation appears to be specifically mediated through the P2Y₁₂-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 P2Y₁₂ deficient platelets in response to thrombin (Fig. 5A) or AYPGKF (Fig. 5B). In contrast, forskolin abolished Akt phosphorylation in P2Y₁₂ deficient platelets elicited by thrombin (Fig. 5A) or AYPGKF (Fig. 5B).

**Forskolin, but not 2MeSAMP and Cangrelor, Inhibited AYPGKF-elicited Rap1b Activation in P2Y₁₂ Deficient Platelets**

Rap1b plays important roles in integrin inside-out signaling, and platelet aggregation [22, 27]. P2Y₁₂/Gₛ signaling is critical for...
Figure 1. 2MeSAMP and Cangrelor inhibit platelet aggregation and secretion through P2Y12-dependent mechanism. (A-B) Washed platelets from wild-type mice (A) or P2Y12 deficient mice (B) were pre-incubated with PGI2 (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 × 10⁷/ml) from P2Y12 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.

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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 P2Y12 deficient platelets [22]. AYPGKF-induced, P2Y12-independent, activation of Rap1b requires Ca2+ elevation. Thus, if forskolin inhibits AYPGKF-induced Ca2+ elevation, it should also inhibit agonist-induced Rap1b activation in the P2Y12 deficient platelets. Indeed, forskolin inhibited thrombin- or AYPGKF-induced Rap1b activation in P2Y12 deficient platelets (Fig. 5C and 5D). In contrast, 2MeSAMP and Cangrelor did not affect thrombin- or AYPGKF-induced Rap1b activation in P2Y12 deficient platelets (Fig. 5C and 5D). These data demonstrate that 2MeSAMP and Cangrelor inhibited agonist-induced Rap1b activation in a P2Y12-specific manner.

Cangrelor Inhibited Thrombus Formation in Wild-type but not P2Y12 Deficient Mice

All the above data indicate that the in vitro effects of 2MeSAMP and Cangrelor on platelets are mediated specifically through P2Y12. 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 percutaneous 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 P2Y12. The role of P2Y12 in in vivo thrombus formation was evaluated using a FeCl3-induced carotid artery thrombosis model. No occlusive thrombi formed in P2Y12 deficient mice when low-concentrations of FeCl3 (<10%) were applied (data not shown). However, when 20% FeCl3 was used to cause vessel injury, P2Y12 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 P2Y12 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 P2Y12 deficient mice (Fig. 6B). In contrast, injection of Cangrelor failed to affect stable thrombus formation in P2Y12 deficient mice (Fig. 6D and 6E). These results demonstrate that Cangrelor mediated inhibition of thrombus formation in vivo is P2Y12 dependent.

Discussion

The purpose of this study was to re-examine the specificity of the P2Y12 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 P2Y12-dependent manner. Additionally, we discovered that inhibition of platelet activation by the cAMP/PKA pathway
involves multiple signaling pathways including PLCβ, Akt, and Rap1b.

Cangrelor and 2MeSAMP have been extensively employed to identify the role of P2Y12 in platelet activation. However, a recent study reported that both Cangrelor and 2MeSAMP inhibit platelet activation by increasing intra-platelet cAMP concentrations through a P2Y12-independent mechanism [19]. If their data are correct, any conclusions on the role of P2Y12 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 detailed mechanisms by which Cangrelor inhibits platelet activation. The key evidence showing that Cangrelor and 2MeSAMP inhibit platelet activation via a P2Y12-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 P2Y12 function in isolated platelets (Fig. 1), which are the levels of those antagonists used to demonstrate the role of P2Y12 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 P2Y12 deficient platelets. In contrast, PGI2 and forskolin, respectively abolished platelet activation in both wild-type and P2Y12 deficient mice.

Further evidence supporting the conclusion that Cangrelor and 2MeSAMP are unable to induce P2Y12-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 PGI2, they should be able to induce VASP phosphorylation. However, unlike PGI2 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 glycprotein (GP) Ibβ, resulting in inhibition of GPⅡb-Ⅲa-V-stimulated platelet activation [38]. PKA has been shown to phosphorylate PLCβ and inhibit PLCβ signaling in COS 7 cells [39]. Ca2+ mobilization from the PLCβ

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**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 Ser239. (B–C) Washed platelets from wild-type (B) or P2Y12 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 Ser157 or Ser239. doi:10.1371/journal.pone.0051037.g003
signaling is a key event in platelet activation induced by GPCR agonists such as thrombin and TXA₂. Our data indicate that PKA-dependent inhibition of platelet activation involves the PLCβ pathway, because pre-treatment of platelets with forskolin or PGI₂ abolished AYPGKF-elicited Ca²⁺ elevation (Fig. 4). Thus, if Cangrelor and 2MeSAMP inhibit platelet activation by increasing cAMP concentrations through P2Y₁₂-independent mechanisms, they should be able to inhibit AYPGKF-elicited Ca²⁺ elevation. However, neither Cangrelor nor 2MeSAMP had an effect on AYPGKF-stimulated Ca²⁺ mobilization in P2Y₁₂-deficient platelets.

Forskolin and PGI₂, 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 P2Y₁₂-deficient platelets. These data demonstrate that the inhibitory effect of Cangrelor and 2MeSAMP on platelet activation is P2Y₁₂-dependent and does not involve the P2Y₁₂-independent activation of the cAMP/PKA pathway.

P2Y₁₂ is not only important for agonist-induced platelet activation in vitro, but also contributes to thrombosis in vivo.
Using a FeCl₃-induced carotid artery thrombosis model, we found that the initial thrombus formation is not impaired in P2Y₁₂ deficient mice. However, most P2Y₁₂ 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 P2Y₁₂ deficient mice. Initial thrombus formation caused by vascular injury is mediated by von Will-
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Author Contributions

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

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