Role of $P2Y_{12}$ Inhibition in PAR1 Stimulated Platelet Dense Granule Release

by
Nicole Laschober

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Bioengineering
(Honors Scholar)

Presented May 3, 2019
Commencement June 2019

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Abstract approved:_____

Owen McCarty

Platelet activation uses a complex set of pathways to respond to vascular injury during hemostasis. However, thrombosis relies on the same set of pathways. Antiplatelet therapies used to prevent thrombotic events often target the $P2Y_{12}$ platelet receptor for inhibition but can result in bleeding diathesis or in abnormally high ADP levels in the blood. Thus, an ATP secretion assay was used to measure dense granule release in order to elucidate pathways involved in platelet activation and degranulation. $P2Y_{12}$ inhibition with PAR1 activation resulted in enhanced dense granule release, but PAR4 and GPVI activation did not have any effect. PLC_{β} and PKC δ inhibition and calcium chelation reduced dense granule release, while PI3K, PAR1, $P2Y_1$, and GP IIb/IIIa inhibition did not have an impact. It is possible that there is an ADP-mediated response mechanism that releases dense granules in a secondary wave if $P2Y_{12}$ is inhibited, which should be further examined.

Key Words: platelets, ADP, dense granules, $P2Y_{12}$

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Nicole Laschober, Author

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Introduction

Problem definition

The platelet receptor $P2Y_{12}$ is a common target for antithrombotic drugs due to its specificity to platelets and role in activation and clotting. However, many of these drugs allow excessive bleeding, and findings show that certain platelet antagonists initiate unwanted dense granule release when $P2Y_{12}$ is inhibited which reverses the desired effect of antiplatelet therapy [1].

Statement of Purpose

This thesis will focus on understanding the role of $P2Y_{12}$ inhibition in dense granule release with protease-activated receptor (PAR) activation, and elucidating other pathways involved in degranulation.

Background

Platelets respond to vascular injury through a signaling cascade resulting in activation and aggregation to potentiate blood clotting, a protective mechanism to prevent blood loss. Surface receptors respond to a variety of stimuli, initiating activation and producing stimuli-dependent responses through two main mechanisms: a tissue factor (TF) pathway and a contact pathway [2]. The TF, or extrinsic, pathway is the primary method for the normal platelet function of hemostasis, resulting from platelet contact with the TF protein expressed on cell surfaces and other subendothelial proteins exposed by the vessel injury. The contact, or intrinsic, pathway results in thrombosis rather than hemostasis, as it doesn't involve TF contact but artificial surface contact. A series of zymogens and circulating protein cofactors are involved in both clotting cascades, and the two pathways merge at the formation of fXa, which initiates thrombin production; thrombin

binds to the PAR1 and PAR4 receptors, and along with other external stimuli, ultimately contributes to the activation of platelets. Adenosine diphosphate (ADP) is one of these key agonists, released from red blood cells and endothelial cells in response to vascular injury or shear stress [3].

The platelet surface overall is made of glycoproteins (GP) with many receptors designed for adhesion and aggregation [4]. GPIb-IX-V initially interacts with the subendothelial proteins, resulting in tethering to von Willebrand factor (vWF) and instigation of the platelet plug. Additional subendothelial proteins that interact with platelet receptors include vitronectin and collagen. The α2β1 and GPVI receptors strengthen platelet adhesion to the subendothelial matrix through collagen binding, and thrombin cleaves platelet fibrinogen to form fibrin, which also strengthens the growing platelet clot [5]. The binding affinity of integrin αIIbβ3, the fibrinogen receptor on platelet surfaces, increases during activation and mediates platelet-platelet interaction via crosslinking [4, 5]. These changes help the clot resist shear stress from blood flow and create a stable plug to prevent blood loss.

These protein interactions result in platelet activation and also degranulation, which further enhances coagulation through continued platelet recruitment and activation [6]. Alpha granules, dense granules, and lysosomes contain a variety of proteins and other molecules; specifically, dense granules contain the small molecules serotonin, calcium, pyrophosphate, adenosine triphosphate (ATP), and ADP, while alpha granules contain fibrinogen, factor V, and P-selectin, among other things [5, 7]. ADP secreted from dense granules, in addition to circulating ADP, acts as a platelet agonist at the $P2Y_1$ and $P2Y_{12}$ receptors. Dense granule derived ADP creates an

activation loop, useful for sustaining activation and quickly recruiting and activating other platelets during clot formation, as shown in **Figure 1**.

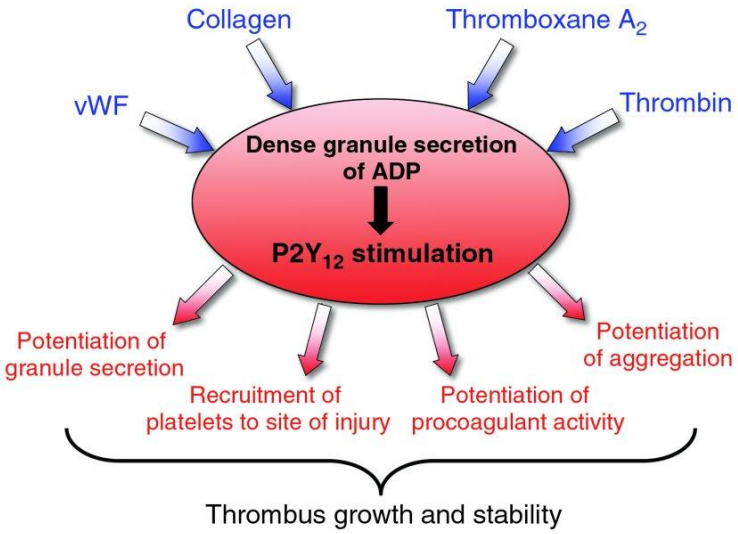


Figure 1. Activation of platelets with a variety of agonists initiates degranulation, which has substantial impact on clot formation and platelet function. The $P2Y_{12}$ receptor is subsequently activated through the contents of dense granules and potentiates clot formation. Figure adapted from [5].

The P2Y receptors are metabotropic and G-protein coupled. $P2Y_1$ is linked to the protein subunit $G\alpha_q$, which has been shown to decrease secretion of dense granules when deficient in humans and mice [8]. The phospholipase C (PLC) pathway downstream of $P2Y_1$ is also integral to platelet aggregation, through calcium production and inhibition of adenylyl cyclase [9]. Protein kinase C (PKC), which is further downstream, is phosphorylated with receptor activation; when phosphorylated, it negatively regulates the kinase activity of the glycogen synthase kinase 3 β (GSK3β), which is a negative regulator of platelet activity. Thus, PKC phosphorylation also contributes to platelet activation through a reduction of GSK3β's inhibitory activity [10, 11]. PKC activation is facilitated by calcium release, resulting in additional platelet activation and degranulation; thus, the mobilization of calcium and subsequent PKC activation are necessary for

dense granule release [12]. However, inhibition of PKC interestingly enhances calcium release with PAR1 activation [13]. Additionally, studies have shown that PKC is inhibitory at low ADP concentrations, but is activating at high ADP concentrations [14]. This shows the necessity for further studies on the role of PKC in dense granule release [6]. Furthermore, the subunit PKC δ has been shown to be important for agonist-induced degranulation and is also downstream of the PAR receptors and GPVI [8].

The $P2Y_{12}$ receptor is coupled with the protein subunit $G\alpha_i$, which causes cyclic AMP and adenylyl cyclase inhibition when stimulated. $P2Y_{12}$ also associates with the $G\beta\gamma_i$ subunit, which activates the PI3 kinase (PI3K) and AKT; AKT causes phosphorylation of GSK3β, which results in negative regulation except when reduced by PKC as previously described [1]. **Figure 2** shows some of these pathways and associated agonists which potentiate activation and hemostasis.

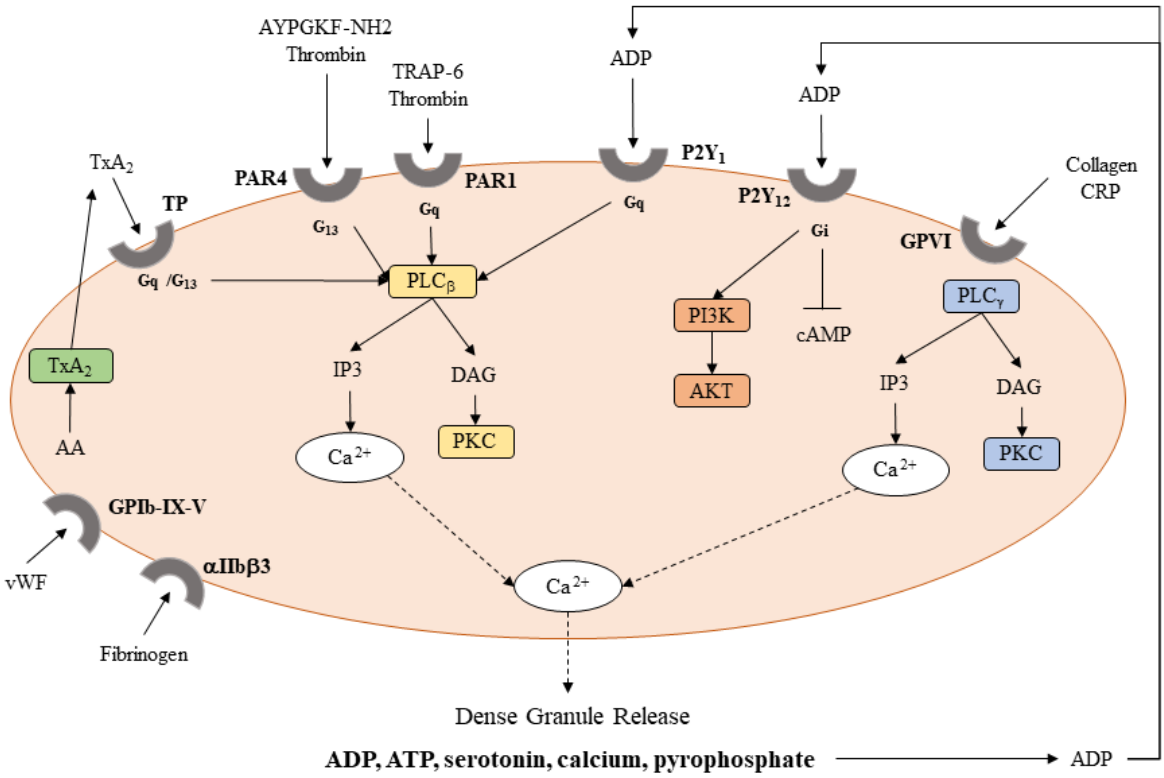


Figure 2. Platelet activation involves a variety of agonists, including thrombin and ADP. Stimulation of each receptor causes downstream effects resulting in degranulation and an activation loop that advances clot growth and stabilization.

Stimulation of the P2Y receptors, along with other key receptors such as PAR1 and PAR4, causes a signal relay mediated downstream to potentiate hemostasis; however, thrombosis relies on the same set of pathways. Understanding the pathways which mediate platelet activation essential for hemostasis is key in designing therapies that target dispensable pathways that trigger deleterious platelet activation in diseased blood vessels prone to thrombosis. The $P2Y_{12}$ receptor is a useful target for antithrombotic drugs such as ticlopidine and clopidogrel due to the importance of ADP in the activation and coagulation process and the receptor's specificity to platelets [15, 16]. For example, patients with dysfunctional $P2Y_{12}$ receptors or an ADP deficiency exhibit a bleeding diathesis; $P2Y_{12}$ inhibitors are thus effective antithrombotic agents [9, 17]. Other receptors are also

targeted by antithrombotic drugs, such as GP IIb/IIIa by Integrilin (eptifibatide). Thus, anti-platelet therapy that targets receptors like $P2Y_{12}$ is often used for patients who suffer from thrombotic events such as stroke in order to prevent platelet activation and clotting in diseased vessels. However, because hemostasis and thrombosis have shared activation pathways, bleeding is a frequent problem for patients undergoing this therapy. With inhibition of key platelet receptors, a small cut could result in large loss of blood. Conversely, high levels of ADP have been detected in some serum from select patients undergoing anti-$P2Y_{12}$ therapy, which has the opposite from intended effect and could lead to increased risk of thrombosis and stroke [1]. By studying the pathways involved in dense granule release, the relationship between ADP and receptor inhibition can be better understood in order to improve the safety and efficacy of anti-platelet therapy.

Materials and Methods

Reagents

The buffer HEPES-Tyrode (HT) used as a solvent and assay control is made up of 129 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, and 1 mM $MgCl_2$; used at pH 7.3. Dimethyl sulfoxide (DMSO) was used as a vehicle at 0.01%. Chronolume (Chrono-Log Corporation, Havertown, PA, USA) was used as an ATP detection agent.

Inhibitors used include MRS2395 (2,2-dimethyl-propionic acid 3-[2-chloro-6-methylaminopurin-9-yl]-2-[2,2-dimethyl-propionyloxymethyl]-propyl ester), a $P2Y_{12}$ inhibitor; RWJ56110, a PAR1 inhibitor; MRS2179, a $P2Y_1$ inhibitor; wortmannin, a PI3K inhibitor; Integrilin (eptifibatide), a GP IIb/IIIa inhibitor; Go6976, a PKC α/β inhibitor; Rottlerin, a PKC δ inhibitor; Ro 31-8220, a pan-PKC inhibitor; BAPTA, a calcium chelator; and U73122, a PLC_β inhibitor. U73343, an inactive analogue of U73122, was used as a negative control. MRS2395 and wortmannin were obtained from SigmaAldrich (St Louis, MO, USA). RWJ56110 and Integrilin were obtained from sources previously described [1]. MRS2179, Go6976, Rottlerin, Ro 31-8220, BAPTA, U73122, and U73343 were obtained from Tocris (Bristol, UK).

Platelet agonists include adenosine diphosphate (ADP), a P2Y agonist; thrombin, a PAR1 and PAR4 agonist; thrombin receptor-activating peptide (TRAP-6, sequence SFLLRN), a PAR1 agonist; AYPGKF-NH_2, a PAR4 agonist; and CRP, a GPVI agonist. Thrombin and ADP were obtained from SigmaAldrich (St Louis, MO, USA). AYPGKF-NH2 was obtained from Abgent (San Diego, CA, USA). TRAP-6 was obtained from Tocris (Bristol, UK). CRP was obtained from R. Farndale (Cambridge University, Cambridge, UK).

Isolation of Human Washed Platelets (WP)

Fresh adult blood was drawn from healthy donors by venipuncture into 3.8% sodium citrate (9:1, v:v) as described [18, 19]. Anticoagulated blood was centrifuged at 200 g for 20 min, allowing extraction of platelet-rich plasma which was further centrifuged at 1000 g for 10 min in the presence of prostacyclin (0.1 μg/mL). A platelet pellet was obtained and resuspended in HT buffer, then washed via centrifugation at 1000 g for 10 min in HT buffer in the presence of prostacyclin (0.1 μg/mL). After washing, purified platelets were resuspended in HT buffer to the necessary concentration.

ATP Secretion Assays

WP were resuspended to 2×10^8 mL^{-1} as described, and 70 μL were added to 10 μL selected inhibitor or vehicle control DMSO (0.01%) in triplicate in a white Corning Costar flat bottom 96-well plate as described [1]. Following a 15-minute orbital shake incubation at 37°C, 10 μL selected agonists or HT buffer control were added to each well and incubated with orbital shaking at room temperature for either 30 seconds or 15 minutes to activate the platelets. After brief incubation, 10 μL Chronolume was added to each well to detect ATP released from dense granules and exude luminescence, as measured by an Infinite® M200 spectrophotometer (TECAN, Mannerdorf, Switzerland). Each condition was triplicated on a well plate and total assays were also triplicated using three unique platelet donors. Results were analyzed using Student's paired *t* test to compare treatments.

Results

PAR1 stimulation enhances dense granule release when $P2Y_{12}$ is inhibited

To understand the role of the $P2Y_{12}$ receptor in dense granule release for hemostatic amplification, low (10 μM) and high (50 μM) concentrations of MRS2395 were incubated with WP before activation by TRAP-6 or ADP. Luminescence, and thus granule release, increased from low to high MRS2395 and low (10 μM) to high (30 μM) TRAP-6 as shown in **Figure 3A**. Thus, stronger $P2Y_{12}$ inhibition caused higher dense granule release with PAR1 platelet activation. This trend remains even when activation time, the period of orbital shaking after addition of the agonist, is increased from 30 seconds to 15 minutes. Interestingly, the addition of both low (10 μM) or high (30 μM) ADP in combination with low concentration TRAP-6 eliminated any significant increase in dense granule release (**Figure 3B**), showing that the previous trend may be ADP mediated.

Thrombin was also tested during $P2Y_{12}$ inhibition but showed no significant difference in luminescence between high and low agonist or inhibitor concentration or activation time. It is possible that the potency of thrombin caused all dense granules to be released upon initial activation. CRP and AYPGKF-NH_2 were also tested, however, WP activated with both high and low concentrations of these agonists also did not show differences in dense granule release as compared to the vehicle (**Figure 3C**).

Low concentration MRS2395, TRAP-6, and ADP and 30 second incubation time were selected for additional experiments due to their best exhibition of the observed trend.

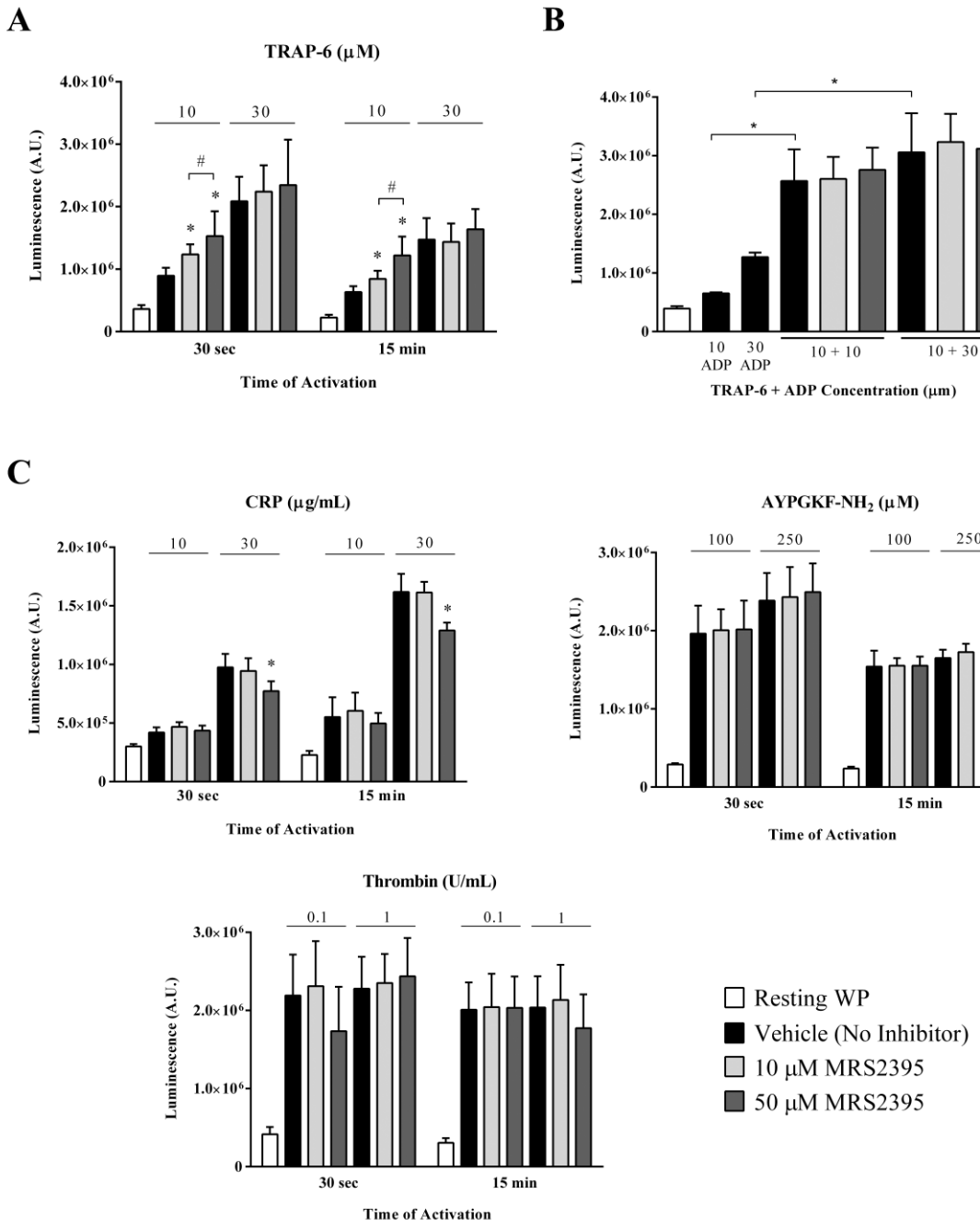


Figure 3. Effect of $P2Y_{12}$ inhibition on platelet dense granule release. **(A)** Dense granule release is enhanced with increased inhibition by MRS2395. **(B)** Activation with ADP does not impact dense granule release when used with TRAP-6. **(C)** Activation with CRP, AYPGKF-NH_2, and thrombin does not impact dense granule release. The significant decrease with CRP activation in a few conditions may be due to donor differences. * indicates statistical significance as compared to the vehicle of the same condition. # indicates statistical significance between conditions. ($p<0.05$)

Downstream inhibition prevents dense granule release enhancement with PAR1 stimulation and $P2Y_{12}$ inhibition

Downstream pathways of the both the $P2Y_{12}$ receptor and PAR1 were also tested for effects on dense granule release when activated with TRAP-6. Firstly, the PLC subunit β was inhibited using U73122 both alone and in combination with MRS2395, as shown in **Figure 4A**. The presence of U73122 in both conditions caused significant decrease in dense granule release as compared to the vehicle and to MRS2395 alone, when activated with TRAP-6, showing that TRAP-6 cannot successfully activate platelets to dense granule release when PLC_{β} is inhibited. The inactive analogue of U73122, U73343, was also tested alone and in combination with low concentration MRS2395; there was no difference between these conditions and either the vehicle or MRS2395 alone as expected.

Next, the calcium chelator BAPTA was tested; calcium, which plays a key role in denes granule release, is released further downstream from PLC_{β}. Used alone and in combination with MRS2395, there was also a significant decrease in dense granule release in this condition as shown in **Figure 4B**. As compared to the similar levels of luminescence measured between the two U73122 conditions, there was instead decreased dense granule release from the already decreased BAPTA alone when MRS2395 was added.

Ro 31-8220, the pan-PKC inhibitor, and Rottlerin, the PKC δ inhibitor, also showed an analogous significant trend to the U73122 dense granule reduction when used alone and in combination with MRS2395. Thus, pan-PKC inhibition confirms the trend shown with PKC δ as expected. The PKC δ-inhibited condition also shows a slight enhancement in release when jointly inhibited with

MRS2395. However, the PKC α/β inhibitor Go6976 had no significant change in dense granule release when compared to either the vehicle or the MRS2395 condition (**Figure 4C**). PI3K is downstream of $P2Y_{12}$ but not PAR1; when inhibited with wortmannin, alone and in combination with low concentration MRS2395, there was also no change in dense granule levels (**Figure 4D**).

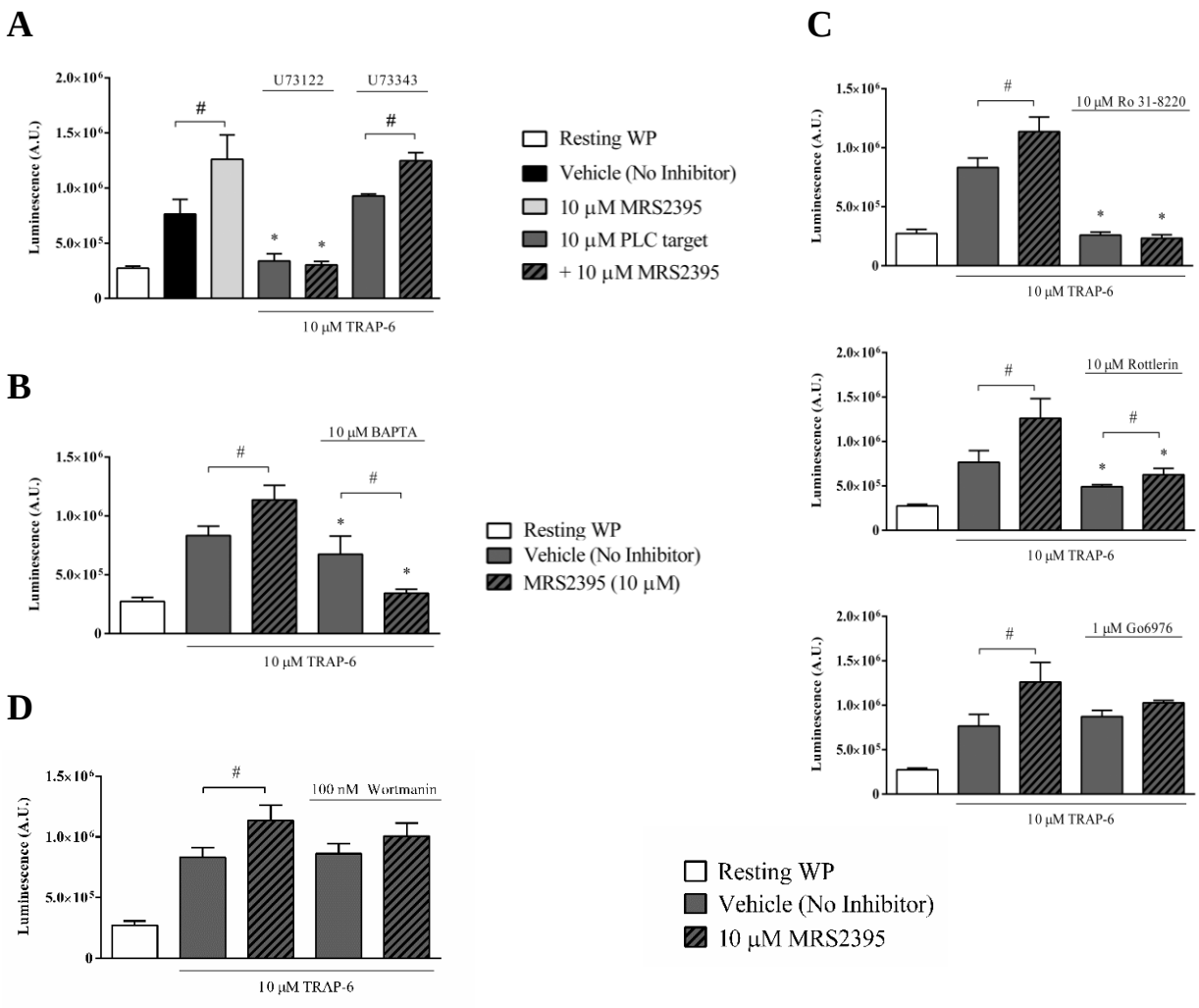


Figure 4(A) PLCβ inhibition with U73122 blocks dense granule release. The inactive analogue shows no difference from controls, as expected. **(B)** Calcium chelation with BAPTA increasingly blocks dense granule release when used in combination with MRS2395. **(C)** PCK inhibitors are compared. PKC δ has decreased release overall but enhanced release with $P2Y_{12}$ inhibition, pan-PKC has decreased degranulation, but PKC α/β inhibition has no impact on dense granule release. **(D)** PI3K inhibition has no impact on dense granule release. * indicates statistical significance as compared to the vehicle of the same condition. # indicates statistical significance between conditions. ($p<0.05$).

PAR1 inhibition prevents dense granule release when stimulated with TRAP-6 and ADP together

To further elucidate the pathway responsible for dense granule release, RWJ56110 was used to inhibit PAR1 in platelets activated by both TRAP-6 and ADP, used alone and in combination. Results in **Figure 5** show that dense granule release is significantly reduced by PAR1 inhibition when activated with TRAP-6 or TRAP-6 and ADP together, but not ADP alone. Both of the inhibited TRAP-6 conditions are comparable to the inhibited and uninhibited ADP alone conditions, showing that although ADP activation has less strength than TRAP-6, it can still reach full potency with only one of two ADP-receptive binding sites.

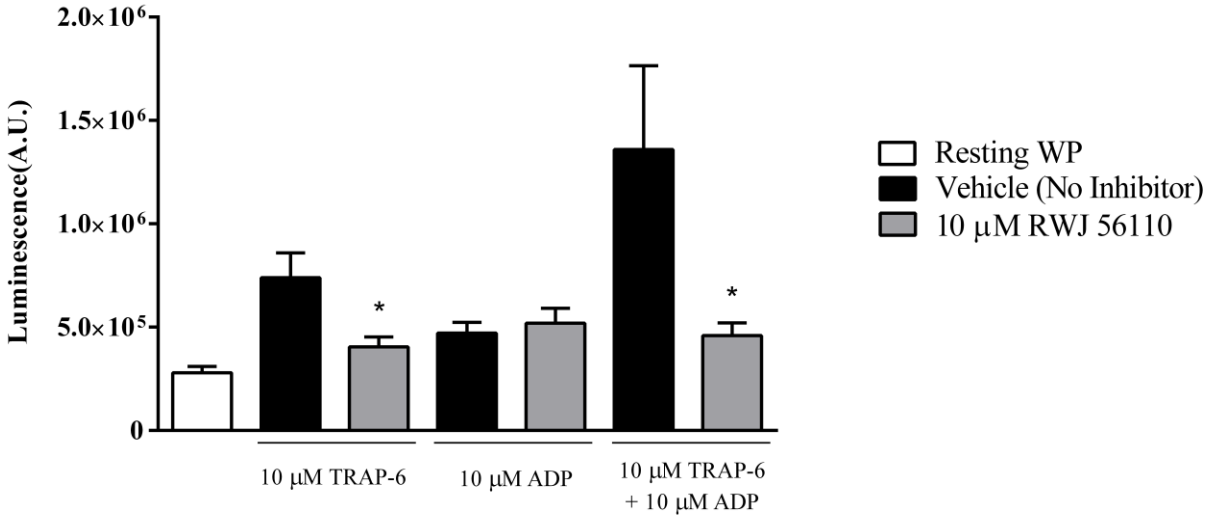


Figure 5. Combined TRAP-6 and ADP stimulation with PAR1 inhibition. TRAP-6 activation without inhibition initiates stronger degranulation than ADP, but ADP still reaches complete activation with PAR1 inhibition. * indicates statistical significance as compared to the vehicle of the same condition. ($p<0.05$)

$P2Y_1$ inhibition has no effect on dense granule release when used alone or in combination with $P2Y_{12}$ or GP IIb/IIIa inhibition

Similar to $P2Y_{12}$ inhibition conditions, the $P2Y_1$ receptor was blocked using MRS2179 at low (10 μM) and high (50 μM) concentrations. TRAP-6, AYPGKF-NH_2, and CRP stimulation caused no

change in dense granule release from their respective vehicle conditions at both low and high concentrations (**Figure 6A-C**). Activation with ADP had a significant decrease for high ADP and low MRS2179, but as the trend did not apply to any of the other concentration conditions, this may have been due to donor differences (**Figure 6D**).

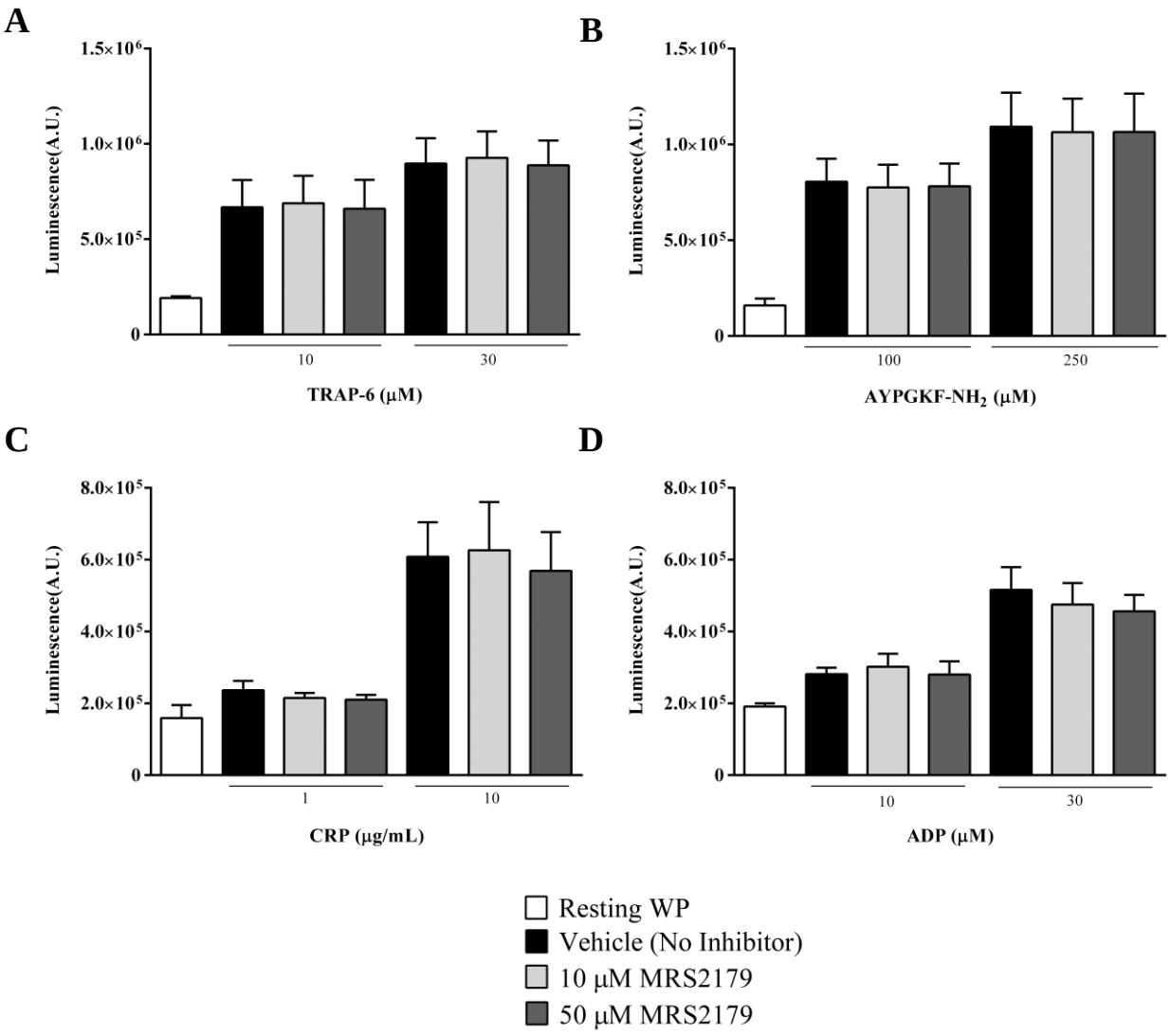
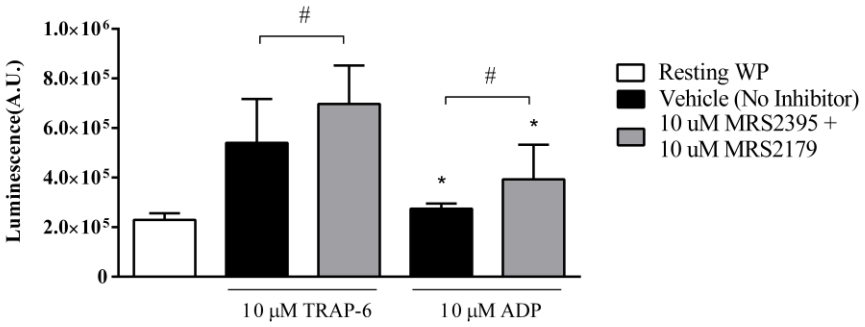


Figure 6. Effects of $P2Y_1$ inhibition on dense granule release with activation by **(A)** TRAP-6, **(B)** AYPGKF-NH_2, **(C)** CRP, and **(D)** ADP. Dense granule release is not significantly impacted by inhibition in any of these conditions. ($p<0.05$)

MRS2179 was also used synergistically with MRS2395 to block both P2Y receptors with TRAP-6 and ADP activation. While there was no change in dense granule release between the vehicle and inhibited conditions when using ADP, there was a significant increase in when synergistically inhibited and activated with TRAP-6, as shown in **Figure 7A**. This may be due to the combination of MRS2395 and TRAP-6 alone and independent of the $P2Y_1$ inhibition. Additionally, the GP IIb/IIIa inhibitor Integrilin was used in combination with each of the P2Y inhibitors (**Figure 7B**); these inhibition combinations did not have any impact on dense granule release when compared to their respective vehicle conditions.

A



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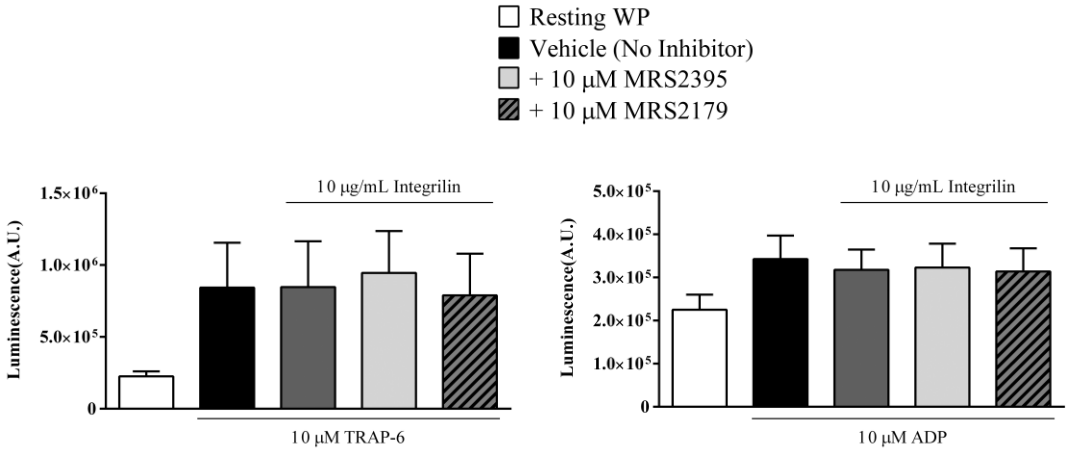

Figure 7(A) Synergistic P2Y inhibition results in differences in dense granule release when activated with TRAP-6 or ADP. **(B)** GP IIb/IIIa inhibition has no impact on dense granule release when used alone or in combination with P2Y inhibition. * indicates statistical significance as compared to the vehicle of the same condition. # indicates statistical significance between conditions ($p<0.05$).

Discussion

These experiments examined the receptors and pathways necessary for dense granule release in platelets. Significantly, inhibition of the $P2Y_{12}$ receptor by MRS2395 initiates an increase in dense granule release when platelets are activated at the PAR1 receptor by TRAP-6, but not ADP and TRAP-6 together. Higher levels of inhibition would be expected to cause lower levels of dense granule release, due to the important role that the $P2Y_{12}$ receptor plays in activation amplification with ADP, so this observation with the opposite trend may explain the high levels of ADP found in the serum of patients undergoing antiplatelet therapy. This may indicate that platelets have a feedback mechanism or sensing system for ADP that causes more dense granules to be released when the expected amplification loop is broken. Thus, when ADP contained in the granules cannot bind after secretion due to $P2Y_{12}$ inhibition, the platelets may continue releasing dense granules in an effort to perform the normal amplification. This trend is absent with PAR4 and GPVI activation, when platelets are stimulated with ADP, CRP, thrombin, or APYGKF-NH_2, which may indicate a distinct role the PAR1 receptor plays in platelet activation. While thrombin may have been expected to show a trend similar to TRAP-6, it is possible that the higher strength of activation by thrombin due to targeting both PAR receptors causes complete release of all stored dense granules at initial activation, not depending on the amplification loop to ensure complete activation. This is also demonstrated by the luminescence of thrombin reaching a value comparable or higher than to the other agonist conditions.

Additionally, internal activation pathways necessary for dense granule release were examined. Inhibition of PKC, PLC_{β}, and the PKC isoform δ resulted in decreased dense granule release compared to uninhibited platelets when activated with TRAP-6, showing that the PAR1 pathway

is dependent on downstream activation of these molecules for degranulation. The enhancement of dense granule release in PKC δ- and $P2Y_{12}$-inhibited platelets is in agreement with the finding that PKC δ plays a key role in PAR1-simulated platelet dense granule release [8].

On the other hand, PKC α/β and PI3K did not have significant change in dense granule release as compared to their vehicle conditions, showing that they are not necessary for degranulation when PAR1-activated. Furthermore, testing demonstrated that calcium chelation partially inhibits the ability of platelets to release dense granules; intracellular calcium plays a role in PKC isoform activation, and the further blocking of dense granule release by the addition of MRS2395 to BAPTA shows that the ability of MRS2395 to inhibit $P2Y_{12}$ decreases with the absence of calcium [20]. This corroborates the finding that intracellular calcium enhances the efficacy of MRS2395 [20]. Additionally, MRS2395 has been shown to significantly increase intracellular calcium with TRAP-6 activation, which could help explain the enhancement effects of $P2Y_{12}$ inhibition on dense granule release [1].

Future Work

These experiments have elucidated a greater understanding of the pathways and molecules that mediate platelet dense granule release. The possible feedback mechanism mediated by ADP in platelets that are $P2Y_{12}$-inhibited and PAR1-activated should be further examined to continue to improve antiplatelet therapies using $P2Y_{12}$ inhibition, in order to prevent adverse effects such as increased ADP due to dense granule release. For this task, preventing platelet feedback in the activation loop may be crucial to reducing the response-mediated secondary degranulation.

Additionally, understanding the pathways and interactions of downstream activation in platelets is crucial to further development of safe platelet therapeutics to prevent thrombotic events and decrease risk of bleeding. Specifically, targets such as calcium, PLC_{β}, and PKC δ or pan-PKC may be available as novel or underused molecules of inhibition that are necessary for platelet activation. Although less specific to platelets as compared to $P2Y_{12}$, combination therapy with $P2Y_{12}$ inhibition such as BAPTA and MRS2395 may have positive results. As calcium is a key player in platelet activation and interacts with other inhibition pathways, calcium patterns within platelets would also be beneficial to study further.

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