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Loss of $P2Y_1$ receptor desensitization does not impact hemostasis or thrombosis despite increased platelet reactivity *in vitro*

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Abstract

Background—Hemostatic plug formation at sites of vascular injury is strongly dependent on rapid platelet activation and integrin-mediated adhesion and aggregation. However, to prevent thrombotic complications, platelet aggregate formation must be a self-limiting process. The second wave mediator ADP activates platelets via Gq-coupled $P2Y_1$ and Gi-coupled $P2Y_{12}$ receptors. Following ADP exposure, the $P2Y_1$ receptor undergoes rapid phosphorylation-induced desensitization, a negative feedback mechanism thought to be critical for limiting thrombus growth.

Methods—Here, we analyzed a novel knock-in mouse strain expressing a $P2Y_1$ receptor variant that cannot be phosphorylated beyond residue 340 ($P2Y_1^{340\text{-}0P}$), thereby preventing desensitization of the receptor.

Results—$P2Y_1^{340\text{-}0P}$ mice followed a Mendelian inheritance pattern and peripheral platelet counts were comparable between $P2Y_1^{340\text{-}0P/340\text{-}0P}$ and control mice. *In vitro*, $P2Y_1^{340\text{-}0P/340\text{-}0P}$ platelets were hyperreactive to ADP, showed a robust activation response to the $P2Y_1$ receptor-selective agonist, MRS2365, and did not desensitize in response to repeated ADP challenge.

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Author Contributions

D.S.P., T.N.B., W.B., T.K.H. and R.A.N. designed the study; D.S.P. and W.J.S. performed most of the experiments, analyzed and interpreted the data; T.N.B. performed the initial desensitization studies; E.G.K. performed and analyzed the microfluidics studies; T.K. performed the $FeCl_3$ thrombosis assay, J.M.N.M. performed the IVC stenosis model, K.O.P. was responsible for mouse colony maintenance and genotyping; S.M. assisted with experiments and performed some of the initial *in vitro* experiments; N.M. edited the manuscript; D.S.P., W.B. and R.A.N. wrote the manuscript.

Conflict of Interest

All authors have no conflicts of interest to declare.

We observed increased calcium mobilization, protein kinase C substrate phosphorylation, alpha granule release, activation of the small GTPase Rap1, and integrin inside-out activation/aggregation. This hyperreactivity, however, did not lead to increased platelet adhesion or excessive plug formation under physiological shear conditions.

Conclusion—Our studies demonstrate that receptor phosphorylation at the C-terminus is critical for $P2Y_1$ desensitization in platelets and that impaired desensitization leads to increased $P2Y_1$ receptor signaling *in vitro*. Surprisingly, desensitization of the $P2Y_1$ receptor is not required for limiting platelet adhesion/aggregation at sites of vascular injury, likely because ADP is degraded quickly or washed away in the bloodstream

Keywords

platelets; signaling; P2Y1 receptor; thrombosis; hemostasis

Introduction

Platelets play an essential role in hemostasis by preventing the loss of blood following vessel injury. Under homeostatic conditions, platelets circulate in the bloodstream in a non-adhesive state, but they interact with the subendothelial matrix at sites of vascular injury and, following initiation of a complex network of signaling pathways, form a platelet plug [1,2]. The first step in the adherence of platelets to sites of injury is the interaction of platelets with von Willebrand factor (vWF), mediated by the GPIb-V-IX complex, and collagen, which is mediated by GPVI and integrin α2β1. The coagulation protease thrombin further promotes human platelet activation through the G protein-coupled receptors (GPCRs) PAR1 and PAR4. Secondary mediators released from activated platelets, including ADP and serotonin released from dense granules and thromboxane A2 (TxA2) produced by cyclooxygenase 1, are critical for sustaining platelet activation and thrombus growth. These soluble agonists engage GPCRs and initiate an inside-out signaling cascade that results in the activation of the most abundant platelet integrin, αIIbβ3. Activated αIIbβ3 mediates clot formation by binding to the RGD and AGD motifs on fibrinogen and other ligands, resulting in the formation of a stable platelet plug.

ADP released from dense granules activates two distinct P2Y receptors expressed on platelets: $P2Y_1$ and $P2Y_{12}$ [3]. The $P2Y_1$ receptor, which couples to the Gq pathway, initiates platelet aggregation by promoting shape change and generating second messengers via phospholipase C-β. The $P2Y_{12}$ receptor is the primary Gi-coupled receptor on platelets, which modulates adenylyl cyclase and PI3 kinase activity. Both $P2Y_1$ and $P2Y_{12}$ receptor signaling pathways converge on the small GTPase Rap1 to ultimately trigger inside-out activation of platelet integrins [4]. $P2Y_1$ receptor-promoted Ca^{2+} signaling results in activation of the Ca^{2+}-sensitive Rap1 guanine nucleotide exchange factor (GEF), CalDAG-GEFI, leading to rapid but reversible integrin activation [5]. $P2Y_{12}$ receptor-promoted PI3K signaling leads to the inhibition of the Rap1 GTPase-activating protein (GAP), Rasa3, which is required for sustained Rap1 and integrin activation [6]. In ADP-activated platelets, simultaneous engagement of these pathways is required for integrin-mediated aggregation to occur.

An important property of ADP-promoted aggregation of platelets is the propensity of the $P2Y_1$ receptor to undergo rapid and profound desensitization. $P2Y_1$ receptor desensitization was first recognized in mice lacking CD39, an ATP diphosphohydrolase expressed on endothelial cells that degrades ATP and ADP. Interestingly, mice deficient in CD39, instead of displaying the expected hyper-aggregatory phenotype due to the lack of ATP/ADP catabolism, showed a complete absence of platelet aggregation in response to ADP [7]. This aggregation defect in mutant platelets was shown to be due to loss of $P2Y_1$ receptor signaling, as simultaneous exposure to ADP and serotonin, which activates the Gq-coupled 5-HT_{2a} receptor, resulted in full platelet aggregation. Exposure to ADP and epinephrine, which activates the α2-adrenergic receptor that acts as a surrogate for the $P2Y_{12}$ receptor, had no effect. These data suggested that the higher levels of ADP in the blood of CD39-null mice promoted desensitization of the $P2Y_1$ receptor, resulting in a loss of ADP-promoted aggregation. Differential regulation of platelet P2Y receptors was confirmed shortly thereafter [8], and kinetic studies demonstrated that the $P2Y_1$ receptor on human platelets desensitized within seconds following addition of a $P2Y_1$ receptor-selective agonist, MRS2365 [9].

The realization that $P2Y_1$ receptors were rapidly desensitized in platelets stimulated substantial interest in understanding the molecular mechanisms involved in this process. Studies by Mundell et al. confirmed the rapid desensitization of the $P2Y_1$ receptor and reported that phosphorylation of the receptor by PKC isozymes was important for receptor desensitization and internalization in cell lines [10]. A role for PKC signaling in $P2Y_1$ regulation in platelets was also suggested; however, these studies did not investigate the near-immediate desensitization events characteristic for $P2Y_1$. Other labs also relied on measuring receptor internalization in cultured cells as a proxy for desensitization. As shown for other GPCRs, regulation occurs by rapid phosphorylation of intracellular Ser and Thr residues, followed by binding of arrestins and a slower internalization of the receptor [11]. Reiner *et al.* showed that mutation of Ser352 and Thr358 decreased phosphorylation by ~70% and markedly inhibited internalization of a $P2Y_1$-YFP construct [12]. Our lab reported that mutation of Ser352 and Ser354 completely ablated HA-$P2Y_1$ receptor phosphorylation and also markedly reduced receptor internalization [13]. For both studies, pan inhibitors of PKC isozymes or an inhibitor of CaM Kinase II had no effect on internalization. Taken together, these studies predicted that removal of phosphorylation sites in the C-terminus of the $P2Y_1$ receptor would result in a receptor unable to either desensitize or internalize in response to agonist.

Therefore, to examine the physiological importance of $P2Y_1$ receptor desensitization in platelet-mediated hemostasis, we generated a mouse line harboring a $P2Y_1$ receptor allele lacking all phosphorylation sites beyond position 339 in its C-terminal tail (termed $P2Y_1$-340-0P) to prevent desensitization in response to agonist stimulation. We chose to knock in the $P2Y_1$-340-0P construct due to the identification of different phosphorylation sites reported by Qi *et al.* and Reiner *et al.* [12,13], the various kinases predicted to phosphorylate the receptor and the possibility that platelets may have evolved unique mechanisms to rapidly phosphorylate and desensitize the $P2Y_1$ receptor. Homozygous $P2Y_1^{340\text{-}0P/340\text{-}0P}$ mice were born at Mendelian ratios and their platelet count and platelet size were normal. Compared to those from control mice, platelets from $P2Y_1^{340\text{-}0P/340\text{-}0P}$

mice demonstrated marked hyperreactivity to ADP and the $P2Y_1$ receptor-selective agonist, MRS2365, and they were able to respond to repeated challenges with ADP without loss of responsiveness, indicating a loss of $P2Y_1$ receptor desensitization. Unexpectedly, adult $P2Y_1^{340\text{-}0P/340\text{-}0P}$ mice showed no obvious phenotype *in vivo* compared to their $P2Y_1^{+/+}$ littermates, including clotting time following laser-induced injury and $FeCl_3$-induced injury models. These results suggest that preventing agonist-promoted desensitization of the $P2Y_1$ receptor by removing intracellular phosphorylation sites results in hyperresponsive platelets *in vitro*, but no detectable change in hemostatic or thrombotic plug formation *in vivo*.

Methods

Reagents

Low molecular weight enoxaparin sodium (Lovenox) was purchased from Sanofi-Aventis (Bridgewater, NJ), heparin-coated capillaries were obtained from Thermo Fisher Scientific (Waltham, MA), and apyrase was acquired from Sigma-Aldrich (St. Louis, MO). MRS2365 was purchased from Tocris Biosciences (Bristol, UK) and ADP was purchased from Sigma-Aldrich. Fibrillar collagen type I was purchased from Chrono-Log Corporation (Havertown, PA). PAR4-activating peptide (Par4p) was purchased from GL Biochem Inc. (Boston, MA). Convulxin was purchased from Kenneth Clemetson, Theodor Kocher Institute (Bern, Switzerland). Anti-GPIX (clone Xia.B4) and JON/A antibodies were purchased from Emfret Analytics (Eibelstadt, Germany). The JON/A antibody, which recognizes only the activated form of murine αIIbβ3, was labeled with phycoerythrin as described previously [14]. Anti P-selectin antibody (clone RB40.34) was obtained from BD Biosciences (San Jose, CA).

Mouse studies

All animal procedures were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. Mice were housed in micro-washed cages under a 12-hour light/dark cycle and provided food and water *ad libitum.*

Control and $P2Y_1^{340\text{-}0P}$ mutant mice were generated using the CRISPR-Cas9 system. Cas9 guide RNAs were developed to target the $P2Y_1$ start codon for insertion of an HA tag, and the $P2Y_1$ C-terminus to facilitate insertion of the $P2Y_1$-340/0P mutations. Candidate guide RNAs were cloned into a T7 promoter vector, followed by T7 *in vitro* transcription and RNeasy (Qiagen) spin column purification, with elution in microinjection buffer (5 mM Tris-HCl pH 7.5, 0.1 mM EDTA). Guide RNAs were tested for activity in an *in vitro* cleavage assay with recombinant Cas9 protein and PCR-amplified target site, followed by agarose gel electrophoresis. The protospacer sequences of guide RNAs chosen for targeting of the $P2Y_1$ locus were g61T (5'-GGAAAGAAGAGGATGACCG-3') and g65T (5'-TGGAGACACGAGTTTGTGA-3'). The guide RNAs included an additional heterologous guanine (G) at the 5' end to allow T7 in vitro transcription. The donor plasmid had a 1563 bp 5' homology arm and a 1587 bp 3' homology arm flanking the HA-$P2Y_1$-340-0P coding sequence. For generating the HA-tagged wild-type $P2Y_1$ receptor knock-in, a similar donor was produced with the same HA tag at the 5' end of the wild-type $P2Y_1$ receptor coding sequence. The donor vectors were prepared by Qiagen High Speed Maxiprep protocol and resuspended in microinjection buffer. Recombinant Cas9 protein was

expressed in *E. coli* and purified by the UNC Protein Expression and Purification Core Facility.

Targeting of C57BL/6 ES cells and embryo microinjection were performed by Dr. Dale Cowley at the Animal Models Core at the University of North Carolina at Chapel Hill. For genotyping, forward primers were designed to anneal in the wild type ($P2Y_1$-Fwd 5'-ACACGAGTTTGTGAAGGCA-3') or $P2Y_1^{340\text{-}0P}$ (340-0P-Fwd 5'-GCTGCCAGGAGGGCTGA-3') C-terminal coding sequence. A common downstream reverse primer ($P2Y_1$-Rev 5'-GACTCACGACTTTCTTCTTTGGAG-3') was used for both reactions (Supplemental Figure 1B). Amplified PCR products were run on a 2.5% agarose gel to confirm the generation of homozygous control and mutant mice. $P2Y_1^{-/-}$ mice were obtained from Jackson Labs and their genotype was confirmed using the provided primer sequences and protocol (Jackson Labs, Bar Harbor, ME).

Whole blood aggregation

For human aggregation studies, blood was collected from volunteers with approval of the Institutional Review Board of the University of North Carolina at Chapel Hill. Whole blood (~30 mL) was collected by gravity into tubes containing 3 mL heparin sulfate (100 U/mL) from healthy adult human subjects who had not taken NSAIDs or antiplatelet medications within 72 h of collection. For mouse aggregation studies, blood was drawn from isoflurane-anesthetized mice by retro-orbital capillary insertion and collected in a microcentrifuge tube containing 30 U/mL of heparin. The blood-heparin mixture was gently inverted and placed at 37°C until testing. Prior to each assay, 500 μL of whole human blood or 300 μL of whole mouse blood was combined with sterile 0.9% saline to a final volume of 1 mL, placed in an impedance cuvette (Chrono-Log, Havertown, PA), and stirred at 37°C for approximately 5 minutes. Baseline impedance was recorded for 1 minute, at which time the indicated drugs were added and data were recorded for 4 minutes, and the rate of loss of impedance was determined.

Washed human platelets

Human blood was collected as indicated above, supplemented with 0.01 U/mL of apyrase, and centrifuged at 1,200 x G for 15 minutes at room temperature to obtain platelet-rich plasma (PRP). PRP was diluted 1:1 in Tyrode's buffer and centrifuged at 100 x G for 10 minutes to pellet contaminating red blood cells and white blood cells. PRP was treated with 1 μg/mL of prostaglandin I_2 (Cayman Chemical, Ann Arbor, MI) and centrifuged at 700 x *g* for 5 min, after which the resulting platelet pellet was resuspended in Tyrode's buffer (137 mM NaCl, 0.3 mM Na_2HPO_4, 2 mM KCl, 12 mM $NaHCO_3$, 5 mM HEPES, 5 mM glucose, 0.35% BSA, pH 7.3) and allowed to rest at 37°C for 45 minutes before experimental manipulation.

Platelet-rich plasma and washed mouse platelets

Blood was collected from anesthetized mice as described above and supplemented with 0.01 U/mL apyrase. Platelet-rich plasma was obtained by centrifuging the blood at 130 x *g* for 4 minutes, transferring the plasma layer and the top of the RBC layer to a new tube, spinning these samples at 100 x *g* for 5 minutes and collecting the PRP layer. For washed platelet

experiments, PRP was treated with 1 μg/mL of prostaglandin I_2 and centrifuged at 700 x *g* for 5 min, after which the resulting platelet pellet was resuspended in 1 mL of Tyrode's buffer.

Light Transmission Aggregometry

Light transmission was measured using PRP with the platelet concentration diluted to 3.0 × 10^8 platelets/mL with Tyrode's buffer (PRP:Tyrode's ~ 1:1). Ca^{2+} was added to a final concentration of 1 mM, and aggregation was performed at 37°C under stirring conditions using a Chrono-log 4-channel optical aggregation system (Chrono-log, Havertown, PA).

Immunoblot analysis

Platelet proteins were solubilized in RIPA buffer (50 mM Tris, 200 mM NaCl, 2.5 mM $MgCl_2$, 1% NP-40, 10% glycerol, supplemented with proteases inhibitors) and separated by SDS-polyacrylamide gel electrophoresis using 10% acrylamide gels (Bio-Rad, Hercules, CA), then transferred to polyvinylidene fluoride membranes (Millipore, Burlington, MA) as previously described [15]. Rap1 protein from platelet lysates before and after pulldown of Rap1-GTP with RALGDS-RBD conjugated beads (Millipore) for 45 minutes was detected using anti-Rap1a/b antibody (rabbit polyclonal, Cell Signaling, Danvers, MA). PKC activation was assessed with an anti-Phospho-(Ser) PKC substrate antibody (rabbit polyclonal, Cell Signaling). Immunoblots were developed using an Odyssey Infrared Imaging System (Li-Cor Biosystems, Lincoln, NE).

Flow Cytometry

Quantification of platelet surface adhesion receptors—Diluted samples of whole blood were incubated for 15 min with 10 μg/mL of fluorophore-conjugated antibodies to the indicated platelet surface receptors and immediately analyzed by flow cytometry. Antibodies targeting GPIX (clone Xia.B4), GPIbα (Sam.G4), and GPVI (Jaq1) were purchased from Emfret Analytics, whereas those targeting integrin αIIbβ3 (MWreg-30) and control IgG were purchased BD Biosciences (San Jose, CA).

αIIbβ3 activation and α-granule secretion—Whole blood was collected from anesthetized mice as previously described and diluted in modified Tyrode's buffer containing 1 mM $CaCl_2$. Cell suspensions were then stimulated with ADP, MRS2365, Par4p or convulxin for 10 minutes at room temperature in the presence of 2 μg/mL PE-conjugated JON/A antibody (which detects activated αIIbβ3), 2.5 μg/mL of Alexa Fluor 488-conjugated anti-P-selectin antibody, and 2.5 μg/mL of Alexa Flour 647-conjugated anti-GPIX antibody. Samples were diluted with sterile-filtered PBS to stop the reaction and analyzed immediately by flow cytometry on a BD C6 Plus flow cytometer (BD Biosciences).

Measurement of Calcium Mobilization

Washed platelets in Tyrode's buffer were diluted to 1 x 10^7 platelets/mL and loaded with 5 μM Fluo-4 and 5 μM Fura Red (Thermo Fisher Scientific, Waltham, MA) for 30 minutes at 37° C in the dark. Afterward, the samples were diluted to 1 x 10^6 platelets/mL in Tyrode's buffer and activated with the indicated concentrations of agonists in the presence of 1 mM

Ca^{2+}, while being continuously sampled by a BD C6 Plus flow cytometer. Kinetic calcium mobilization was analyzed in Flow Jo (Version 10) by plotting the ratio of Fluo4 to Fura Red mean fluorescence intensities (MFI) over time.

Microfluidic thrombosis assay

Ex vivo microfluidics studies were performed as previously described [16]. Briefly, heparinized mouse blood was incubated with anti-GPIX-647 to label platelets and perfused at venous (400 s^{-1}) or arterial (1600 s^{-1}) shear rate through a microfluidic chamber coated with an immobilized strip of fibrillar type 1 equine collagen (100 μg/mL; Chrono-Log) and visualized on Nikon TE300 microscope equipped with a Nikon 20x Plan-Fluor objective and an Orca Flash 4.0 camera (Hamamatsu, Japan). Platelet adhesion was quantified using NIS Elements 4.1.3 software (Nikon) to determine the sum intensity (sum intensity = platelet area x platelet intensity) of a region of interest defined as the middle two-thirds of the collagen strip.

Ferric chloride-induced carotid artery thrombosis

Adult male mice, 12-16 weeks old, were anesthetized with vaporized isoflurane. A 1 mm x 2 mm filter paper soaked in 2.5% ferric chloride was applied to the top of the carotid artery for 5 minutes, followed by brief rinsing with saline before ultrasonic probe placement. Blood flow velocity was measured for 30 minutes with a microvascular ultrasonic flow probe (MA0.5PSB, Transonic Systems Inc, Ithaca, NY). Time to occlusion was defined as the cessation of blood flow (0 mL/minute) for ≥2 minutes.

Inferior vena cava stenosis

Venous thrombosis was induced in the inferior vena cava (IVC) using a partial flow restriction (stenosis) procedure as previously described [17]. Briefly, 12-16-week-old male mice were anesthetized by isoflurane prior to laparotomy to ligate the IVC using a 30-gauge blunt needle spacer and cauterization of side branches below the left renal branch. The abdominal wall was closed with a simple continuous suture. Mice were administered 0.5 mL of warm sterile saline and allowed to recover in a warmed cage. Buprenorphine (0.1 mg/kg) was given for pain management immediately prior to surgery and every 12 h until sacrifice and harvest of thrombi at 48 h post-surgery.

Saphenous vein laser injury

Mice (12-16 weeks of age) were anesthetized with ketamine/xylazine (100 mg/kg and 10 mg/kg, respectively) and injected with Alexa Fluor 488-conjugated GPIX antibody (2.5 μg) to label circulating platelets. The saphenous vein was exposed and injured once as previously described [18]. Platelet accumulation at the site of laser injury was assessed by intravital microscopy using a Zeiss Examiner Z1 microscope (Zeiss, Oberkochen, Germany) equipped with a Hamamatsu Orca Flash 4.0 camera (Hamamatsu, Japan). Image acquisition and fluorescence quantification was done with Slidebook 6.0 (Intelligent Imaging Innovations, Denver, CO).

Peripheral blood cell counts

Whole blood from the retro-orbital plexus of mice was collected into EDTA-containing tubes. Complete blood counts were determined using an Element HT5 analyzer (Heska, Loveland, CO, USA).

Statistical analysis

Data are presented as the mean ± standard error of the mean unless otherwise indicated. Students' unpaired t-test was used to determine significance between 2 groups. Two-way analysis of variance (ANOVA) was used to compare multiple groups. A *p*-value less than 0.05 was defined as statistically significant. All statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

Results

The $P2Y_1$ receptor undergoes rapid desensitization in mouse platelets

The human $P2Y_1$ receptor ($hP2Y_1$) has been shown to undergo a rapid and nearly complete desensitization in platelets following preincubation with the $P2Y_1$-selective agonist, MRS2365 [9]. To determine whether the mouse $P2Y_1$ receptor ($mP2Y_1$) desensitizes in a similar manner, platelets from wild-type BL6 mice or from human volunteers were pretreated with 3 μM MRS2365 for various times, followed by a challenge with 10 μM ADP. As shown in Figure 1A, both human and mouse platelets demonstrated a rapidly diminished capacity to aggregate to 10 μM ADP following increasing times of MRS2365 incubation, with platelets from both species showing a 50% loss of aggregation within ~18-22 s. To investigate whether other Gq-coupled receptors in platelets show agonist-promoted desensitization, we stimulated Gq signaling in mouse platelets with either MRS2365 ($P2Y_1$ receptor) or serotonin ($5\text{-}HT_{2a}$ receptor), followed by addition of ADP or epinephrine, which mimics $P2Y_{12}$/Gi-signaling. As shown in Figure 1B, while the $P2Y_1$ receptor desensitized rapidly to MRS2365, the $5\text{-}HT_{2a}$ receptor remained activated and capable of promoting platelet aggregation for up to 120 s before epinephrine addition. Moreover, following MRS2365-induced desensitization, platelets aggregated in response to serotonin and epinephrine to the same level as those without MRS2365 preincubation (Figure 1C). These data indicate that rapid desensitization of the $P2Y_1$ receptor is not due to desensitization of Gq signaling pathways in platelets.

Generation of mice containing a non-desensitizing $P2Y_1$ receptor

Previous structure-function studies on the $P2Y_1$ receptor by our lab and others demonstrated an important role for phosphorylation of C-terminal serine and threonine residues as regulators of receptor desensitization and internalization [12,13,19,20], but the specific kinase involved was not convincingly identified. To confirm the importance of phosphorylation of these residues for $P2Y_1$ receptor desensitization in a physiological context, we generated a knock-in mouse line in which all Ser and Thr residues within the C-terminal tail ($P2Y_1$-340-0P) were mutated to Ala residues (Supplemental Figure 1A). These mutations prevent phosphorylation while retaining the full-length C-terminal tail of the receptor, which may be involved in other cellular regulation pathways [21]. Using a

CRISPR/Cas9 targeting system and specific guide RNAs, we successfully generated founder mice containing a single allele encoding either the HA-tagged mP2Y$_1$ receptor or the HA-tagged mP2Y$_1$-340-0P receptor (details in Materials and Methods and Supplemental Info).

Breeding of P2Y$_1^{340\text{-}0P/WT}$ mice to generate homozygous P2Y$_1^{340\text{-}0P/340\text{-}0P}$ mice followed Mendelian genetics (data not shown). P2Y$_1^{340\text{-}0P/340\text{-}0P}$ mice exhibited normal T cell and red blood cell counts (Supplemental Table 1) and the peripheral platelet count and platelet size were comparable to controls (Supplemental Figure 1C,D). However, a significant elevation in the peripheral monocyte and neutrophil counts were observed in P2Y$_1^{340\text{-}0P/340\text{-}0P}$ mice (Supplemental Table 1), suggesting that P2Y$_1$ receptor desensitization is important for myeloid cell homeostasis but not necessary for maintaining platelets in a quiescent state and preventing premature platelet clearance in adult mice. No difference in the presence of the major adhesion surface receptors on platelets was observed by flow cytometric quantification (Supplemental Figure 1E). Despite repeated attempts, we were unable to quantify P2Y$_1$ receptors in either P2Y$_1^{+/+}$ or P2Y$_1^{340\text{-}0P/340\text{-}0P}$ platelets by flow cytometry or Western blotting using commercially available antibodies to the P2Y$_1$ receptor or to the HA tag. This is likely due to the very low abundance of P2Y$_1$ receptors in platelets [22–24]. Radioligands such as ^{3}H-2MeSADP or [^{32}P]MRS2500, which have been used in the past to determine P2Y$_1$ receptor levels in platelets, are not currently commercially available.

Platelets from P2Y$_1^{340\text{-}0P/340\text{-}0P}$ mice are hyper-responsive to ADP

To examine the ramifications of removing Ser and Thr phosphorylation sites from the C-terminal tail of the P2Y$_1$ receptor on platelet activation, we first studied the signaling and aggregation responses of platelets from both P2Y$_1^{340\text{-}0P/340\text{-}0P}$ and P2Y$_1^{+/+}$ mice in response to ADP. Platelets in platelet-rich plasma (PRP) obtained from P2Y$_1^{340\text{-}0P/340\text{-}0P}$ mice aggregated more robustly to ADP stimulation at all doses tested (Figure 2A,B). Additionally, aggregation was more sustained in P2Y$_1^{340\text{-}0P/340\text{-}0P}$ platelets when compared to that in control platelets (Figure 2A). P2Y$_1^{340\text{-}0P/340\text{-}0P}$ platelets showed significantly increased integrin activation (Figure 2C) at ADP concentrations ≥ 1 μM and α-granule secretion (Figure 2D) at the highest concentration of ADP (10 μM). Taken together, these results indicate that platelets expressing a non-desensitizing P2Y$_1$ receptor respond more robustly to and at lower concentrations of ADP than platelets expressing the wild-type receptor.

To assess whether the removal of C-terminal phosphorylation sites in the P2Y$_1$-340-0P receptor disrupted desensitization, platelets from either P2Y$_1^{+/+}$ or P2Y$_1^{340\text{-}0P/340\text{-}0P}$ mice were challenged with repeated doses of ADP in the presence of apyrase to degrade the ADP in between challenges (Figure 2E,F). Platelets from P2Y$_1^{340\text{-}0P/340\text{-}0P}$ mice aggregated to the same extent for eight successive additions of ADP, whereas platelets from P2Y$_1^{+/+}$ mice showed progressive loss of aggregation to successive additions of ADP, with no capacity to aggregate after the last addition of ADP. These data are consistent with the rapid and profound desensitization of the wild-type P2Y$_1$ receptor in control platelets, whereas the P2Y$_1$-340-0P receptor appears to be completely refractory to desensitization.

The $P2Y_1$ receptor couples to Gq and PLC-β, resulting in the IP_3-mediated release of Ca^{2+} from the endoplasmic reticulum. Consistent with the aggregation data, ADP promoted a more sustained mobilization of Ca^{2+} in platelets from $P2Y_1^{340\text{-}0P/340\text{-}0P}$ mice compared to that in platelets from $P2Y_1^{+/+}$ mice (Figure 3Ai,3Aiii) without affecting peak Ca^{2+} levels (Figure 3Aii). $P2Y_1^{340\text{-}0P/340\text{-}0P}$ platelets also exhibited increased PKC substrate phosphorylation (Figure 3B,C) and significantly increased P-selectin surface expression following ADP stimulation (Figure 2D), albeit only at the highest concentration of ADP. Furthermore, Rap1-GTP levels were similar in $P2Y_1^{340\text{-}0P/340\text{-}0P}$ platelets and control platelets 1 min after addition of ADP (Figure 3D,E), but were significantly higher when examined at 6 min of stimulation, consistent with the sustained activity of the non-desensitizing receptor relative to wild-type receptor.

Selective stimulation of $P2Y_1$ signaling causes aggregation of $P2Y_1^{340\text{-}0P/340\text{-}0P}$ platelets

ADP-induced aggregation requires the simultaneous activation of both $P2Y_1$ and $P2Y_{12}$ receptors. The $P2Y_1$ receptor-selective agonist, MRS2365, causes shape change (a $P2Y_1$ receptor-specific response), but not aggregation, in wild-type platelets [9]. Consistent with this, MRS2365 promoted shape change but very limited aggregation in $P2Y_1^{+/+}$ platelets (Figure 4A). In marked contrast, addition of MRS2365 to platelets from $P2Y_1^{340\text{-}0P/340\text{-}0P}$ mice promoted a rapid and sustained aggregation similar to that of platelets stimulated with ADP (Figure 2A). MRS2365 induced a highly potent aggregation response in $P2Y_1^{340\text{-}0P/340\text{-}0P}$ platelets, with an EC_{50} of ~10 nM (Figure 4B). Consistent with the aggregation data, JON/A-PE binding (Figure 4C), but not P-selectin surface expression, was significantly increased in $P2Y_1^{340\text{-}0P/340\text{-}0P}$ platelets following MRS2365 stimulation. Ca^{2+} mobilization (Supplemental Figure 2A), PKC substrate phosphorylation (Supplemental Figure 2B,C), and Rap1 activation (Figure 4E,F) were also significantly elevated in MRS2365-stimulated $P2Y_1^{340\text{-}0P/340\text{-}0P}$ platelets when compared to control platelets.

$P2Y_1^{340\text{-}0P/340\text{-}0P}$ platelets show enhanced aggregation response to collagen, but not thrombin, under static conditions

We next examined the response of platelets to stimulation via the PAR4 and GPVI agonist receptors. In response to the thrombin mimetic, PAR4-activating peptide (Par4p), aggregation (Figure 5), JON/A-PE binding (Supplemental Figure 3A), and P-selectin expression (Supplemental Figure 3B) of $P2Y_1^{340\text{-}0P/340\text{-}0P}$ platelets were not significantly different to those of control platelets. Convulxin-induced JON/A-PE binding (Supplemental Figure 3C) or P-selectin expression (Supplemental Figure 3D) also were not strongly increased in $P2Y_1^{340\text{-}0P/340\text{-}0P}$ compared to control platelets. In contrast, a leftward shift in the concentration-response relationship and more sustained aggregation to collagen were observed for $P2Y_1^{340\text{-}0P/340\text{-}0P}$ platelets compared to control platelets (Figure 5C,D).

Normal adhesion response of $P2Y_1^{340\text{-}0P/340\text{-}0P}$ platelets under flow conditions *in vitro* and *in vivo*

We next assessed whether $P2Y_1^{340\text{-}0P/340\text{-}0P}$ platelets showed hyper-reactivity and increased adhesiveness under flow conditions both *in vitro* and *in vivo*. To mimic vascular shear stress *in vitro*, we examined platelet adhesion to collagen under venous or arterial shear stress conditions in a microfluidics chamber. Surprisingly, platelet adhesion to and activation by

collagen under flow conditions was not significantly different between $P2Y_1^{340\text{-}0P/340\text{-}0P}$ platelets and $P2Y_1^{+/+}$ samples (Figure 6A, Supplemental video 1,2). *In vivo*, carotid artery occlusion times were not significantly different between $P2Y_1^{340\text{-}0P/340\text{-}0P}$ and $P2Y_1^{+/+}$ mice challenged by $FeCl_3$ application (Figure 6B). The occurrence or weight of venous thrombi, as assessed by the IVC stenosis model, also were not different between $P2Y_1^{340\text{-}0P/340\text{-}0P}$ and control mice (Figure 6C). Furthermore, we did not observe a reduction in bleeding times (Figure 6D) or a significant increase in platelet adhesion (Figure 6E) in $P2Y_1^{340\text{-}0P/340\text{-}0P}$ mice following laser-induced injury to the saphenous vein.

Conclusion

The Gq-coupled $P2Y_1$ receptor has been shown to undergo rapid and profound agonist-promoted desensitization in platelets, but the physiological relevance for platelet count and vascular hemostasis is unclear. In this study, we describe the generation and analysis of mice expressing a $P2Y_1$ receptor variant that does not desensitize ($P2Y_1$-340-0P). Mice homozygously expressing the $P2Y_1$-340-0P receptor were viable and exhibited normal peripheral platelet counts. $P2Y_1^{340\text{-}0P/340\text{-}0P}$ platelets were hyperresponsive to ADP, the $P2Y_1$ receptor-selective agonist, MRS2365 and collagen. Despite the hyper-reactivity observed *in vitro*, no significant difference was detected in thrombus formation under flow conditions, both *in vivo* and *ex vivo*. Together, these studies suggest that in mice, $P2Y_1$ receptor desensitization limits platelet responses to ADP, but it does not play a major role in preventing premature platelet activation/clearance or in regulating the size of a hemostatic plug or a pathological thrombus.

The $P2Y_1$ receptor is among the least abundant agonist receptors on the platelet surface [22–24]. Its low copy number and rapid desensitization are thought to account for the weak and very transient signaling response elicited by $P2Y_1$ receptor agonists. Our studies provide the first genetic proof that $P2Y_1$ receptor desensitization is indeed critical to limit platelet reactivity to ADP. Impaired $P2Y_1$ receptor desensitization led to prolonged Ca^{2+} mobilization (albeit with similar peak levels) and significantly increased integrin activation and granule secretion *in vitro*. One of the limitations of our studies is that we were not able to biochemically determine whether, in addition to impaired desensitization, $P2Y_1^{340\text{-}0P/340\text{-}0P}$ platelets exhibited receptor expression levels similar to those of $P2Y_1^{+/+}$ control platelets. However, our Ca^{2+} mobilization studies showed similar peak Ca^{2+} levels for $P2Y_1^{340\text{-}0P/340\text{-}0P}$ and $P2Y_1^{+/+}$ control platelets following ADP activation, whereas Ca^{2+} transients were prolonged in $P2Y_1^{340\text{-}0P/340\text{-}0P}$ platelets. Interestingly, Hechler *et al.* demonstrated slightly shortened bleeding times and a mild prothrombotic phenotype for transgenic mice overexpressing the $P2Y_1$ receptor (~300 copies compared to ~150 copies in control platelets) [22]. In platelets from these mice, the increase in receptor expression correlated with an increase in Ca^{2+} mobilization, while only a small effect on the duration of the calcium signal was observed. Together, these studies suggest that (1) $P2Y_1$ receptors are likely expressed at similar levels on circulating WT and $P2Y_1^{340\text{-}0P/340\text{-}0P}$ platelets, and (2) that the number of $P2Y_1$ receptors expressed on the platelet surface, but not the capacity of the receptor to desensitize, is critical for the hemostatic function of platelets. A likely explanation for this finding is that ADP in the blood stream is quickly catabolized by nucleotidases and/or that it is washed away from adherent platelets under

shear conditions. Consistent with this conclusion, we observed normal adhesive behavior for $P2Y_1^{340\text{-}0P/340\text{-}0P}$ platelets under flow conditions *in vitro* and *in vivo*.

The $P2Y_1$ receptor is not the only GPCR on the platelet surface that rapidly desensitizes following agonist stimulation. Studies on the Gq-coupled Thromboxane A2 (TP) receptor showed that it also undergoes rapid agonist-induced desensitization and that activation of either $P2Y_1$ or TP receptors heterologously desensitizes the response of the other receptor [25]. Mechanistic information is missing on how TP desensitization is regulated. Rapid desensitization was also shown for the thrombin receptor, PAR1 [26]. Mechanistic studies on PAR1 desensitization demonstrated that its C-terminal tail is important for receptor desensitization, but not for its internalization [27]. In fibroblasts expressing exogenous PAR1 protein, conversion of multiple Ser residues to Ala at the central region of the tail markedly impaired receptor desensitization, but not internalization of the receptor [27]. Information on whether the same mechanism(s) regulates desensitization in platelets is not available. Thus, our studies are the first to provide definitive evidence that Ser and Thr residues in the C-terminal tail are essential for the desensitization of one of the critical platelet GPCRs, $P2Y_1$.

Early studies of receptor desensitization in platelets suggested that PKC isozymes, activated by downstream $P2Y_1$ receptor signaling pathways, play a major role in shutting off $P2Y_1$ receptor signaling [10]. However, later studies showed that pan-inhibitors of PKC enzymes had no effect in receptor internalization in cell lines [12,13]. We were not able to directly study $P2Y_1$ phosphorylation in platelets due to its low expression level. However, our studies also do not support a role for PKC isozymes in the phosphorylation and desensitization of the $P2Y_1$ receptor, as we observed very little PKC activation in mouse platelets stimulated with ADP. Recently, Zhao *et al.* reported that deletion of G protein-coupled receptor kinase 2 (GRK2) in platelets affects $P2Y_1$ receptor signaling [28]. GRKs phosphorylate GPCRs and induce the binding of arrestins, thereby inducing receptor desensitization [29] and endocytosis. GRK2-deficient platelets exhibited prolonged ADP-promoted Ca^{2+} mobilization, did not desensitize following repeated additions of ADP, and were hyperreactive towards MRS2365, similar to what we describe for $P2Y_1^{340\text{-}0P/340\text{-}0P}$ platelets. However, deletion of GRK2 in platelets also resulted in enhanced $P2Y_{12}$ signaling and a $P2Y_{12}$-dependent increase in platelet accumulation following laser-induced injury of the cremaster muscle arterioles and in faster $FeCl_3$-induced carotid artery occlusion, consistent with a role for GRK2 in desensitization of both $P2Y_1$ and $P2Y_{12}$ receptors.

Considering the high sequence identity (94%) and similar desensitization kinetics between the murine and human receptors, it is likely that the $P2Y_1$ receptor is regulated in a similar fashion in humans. Genome-wide association studies identified polymorphisms in the $P2Y_1$ receptor that are associated with altered platelet activation response to ADP [30] and with on-aspirin platelet reactivity [31,32]. To the best of our knowledge, none of these polymorphisms affect the amino acid sequence of the C-terminus of the receptor and changes to the duration of the activation signal were not reported. Identifying functionally relevant polymorphisms in the C-terminus of $P2Y_1$ receptor would be unlikely, however, as it is enriched in Ser and Thr residues, which are thought to contribute to receptor

desensitization [12,13]. Thus, it is likely that more than one phosphorylation site would have to be impacted to see functional changes in $P2Y_1$ receptor signaling.

In summary, we provide the first definitive proof that phosphorylation of Ser and Thr residues within the C-terminal tail of the $P2Y_1$ receptor is crucial for its desensitization in platelets. Disabling this desensitization mechanism led to platelet hyperreactivity under static conditions, while platelet adhesion and thrombus formation under flow conditions were not affected.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Essentials

- Platelet $P2Y_1$ receptors undergo rapid phosphorylation-induced desensitization.
- Mice expressing a non-desensitizing $P2Y_1$ ($P2Y_1$-340-0P) are viable and have normal platelet counts.
- Platelets expressing $P2Y_1$-340-0P are hyperreactive to ADP and collagen stimulation *in vitro.*
- Hemostatic and thrombotic plug formation are unaffected in $P2Y_1$-340-0P mice.

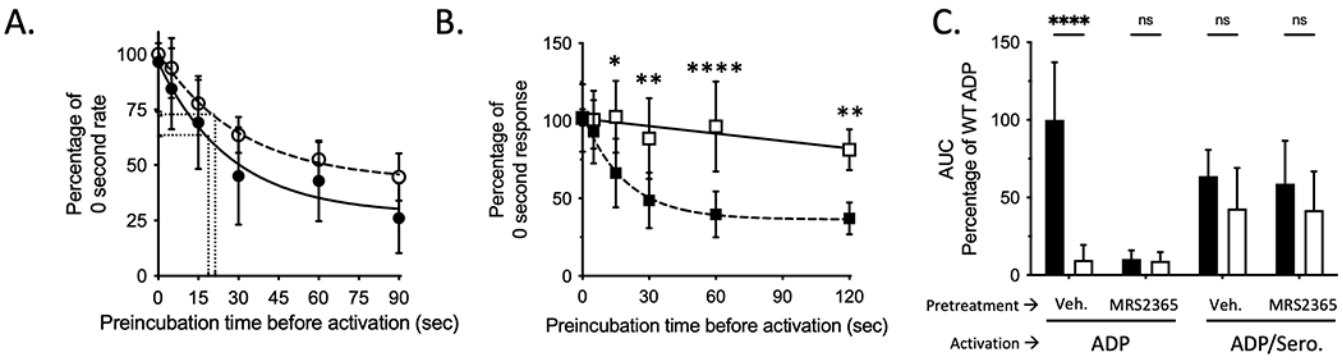

Figure 1. Desensitization of the P2Y$_1$ receptor in mouse and human platelets.

A) Washed platelets from either mouse (open circles) or human (closed circles) blood were pretreated with 3 μM MRS2365 for the indicated times prior to stimulation of 10 μM ADP. The data are presented as the mean ± SD of the percentage of the rate of aggregation (measured by impedance) at 0 seconds of pretreatment. Dotted lines indicate 50% decrease in relative response; $t_{1/2}$ (mouse) = 21.25 s; $t_{1/2}$ (human) = 18.72 s; n = 12 for human; n = 9 for mouse. **B)** Human whole blood was pretreated with either 3 μM MRS2365 (closed squares; P2Y$_1$-specific agonist) or 10 μM serotonin (open squares; 5-HT$_{2A}$-specific agonist) for the indicated times prior to the addition of 10 μM ADP or 5 μM epinephrine, respectively, and platelet aggregation was measured by impedance. The data are presented as the mean ± SD of the percentage of the maximum aggregation at 0 seconds of pretreatment. *p<0.05; **p<0.01; ****p<0.0001. n= 6. **C)** Desensitization of the P2Y$_1$ receptor was quantified in whole blood from wild-type (black bars) or P2Y$_1^{-/-}$ mice (open bars). Blood samples were pretreated with vehicle or 3 μM MRS2365 for 90s prior to addition of 10 μM ADP alone or simultaneous addition of 10 μM ADP and 10 μM serotonin. Data are presented as the mean ± SD of the aggregation AUC as a percentage of wild-type, vehicle stimulated with ADP (measured by impedance). ****p<0.0001. n = 6 for WT; n = 7 for P2Y$_1^{-/-}$.

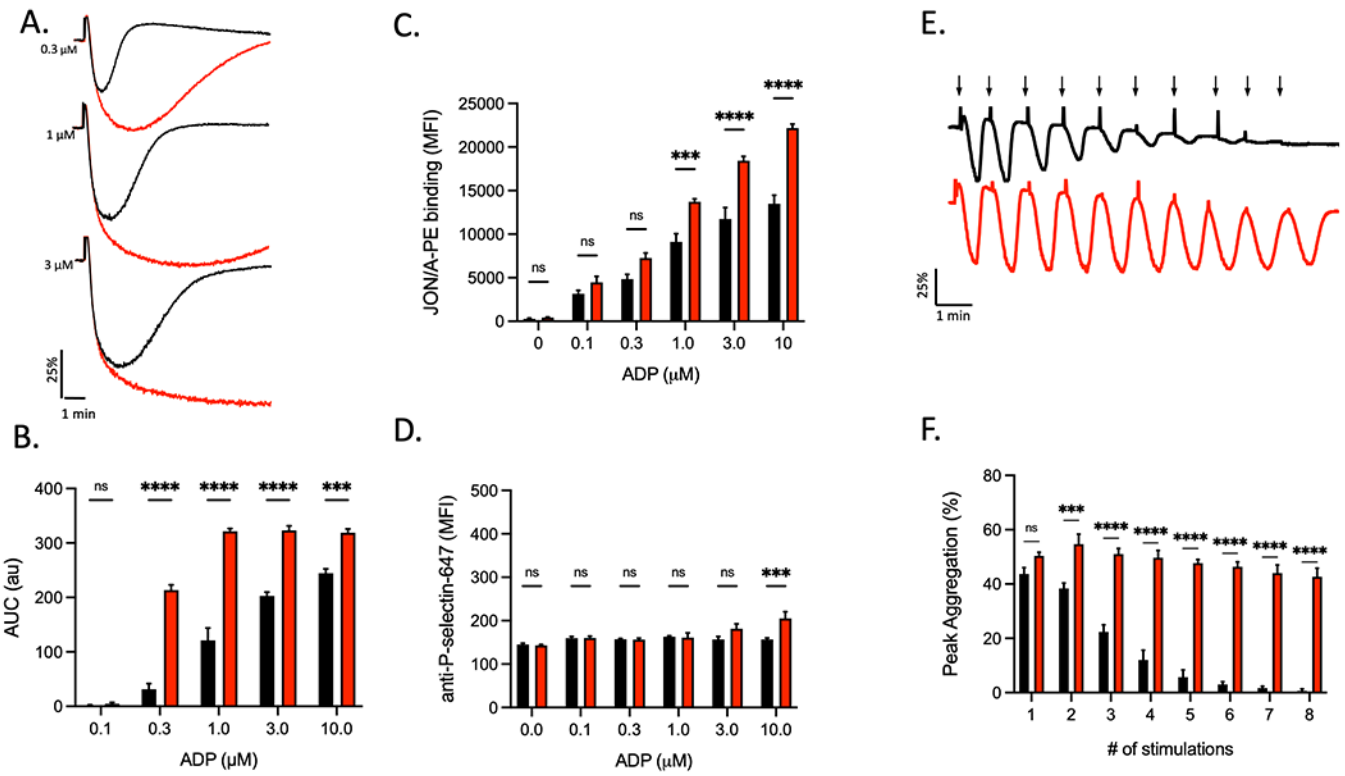

Figure 2. Platelets from $P2Y_1^{340\text{-}0P/340\text{-}0P}$ mice are hyper-responsive to ADP and protected from desensitization.

A,B) Platelets in PRP from $P2Y_1^{+/+}$ (black lines; black bars) and $P2Y_1^{340\text{-}0P/340\text{-}0P}$ (red lines; red bars) mice were stimulated with the indicated doses of ADP and analyzed by standard aggregometry. A) Representative aggregometry traces; B) Area under the curve (AUC) quantification measured from t=0 to t=6 min (n=3 per group). **C,D)** Integrin activation and α-granule secretion in diluted whole blood from $P2Y_1^{+/+}$ (black bars) and $P2Y_1^{340\text{-}0P/340\text{-}0P}$ (red bars) mice stimulated with the indicated doses of ADP and analyzed by flow cytometry. C) MFI for JON/A-PE staining (activated αIIbβ3, n=5/group); D) MFI for anti P-selectin-647 (α-granule marker, n=4/group). **E,F)** $P2Y_1$ receptor desensitization following repeated stimulation with ADP. E) Representative aggregation traces of PRP prepared from $P2Y_1^{+/+}$ (black lines) and $P2Y_1^{340\text{-}0P/340\text{-}0P}$ (red lines) mice pretreated with 2 U/mL of apyrase. Arrows indicate repeated stimulation of PRP with 200 μM of ADP. F) Quantification of peak aggregation ($P2Y_1^{+/+}$ - black bars; $P2Y_1^{340\text{-}0P/340\text{-}0P}$ - red bars) (n=3 per group). Statistical significance was determined by 2-way ANOVA (***p<0.001, ****p<0.0001). All data are expressed as the mean ± SEM.

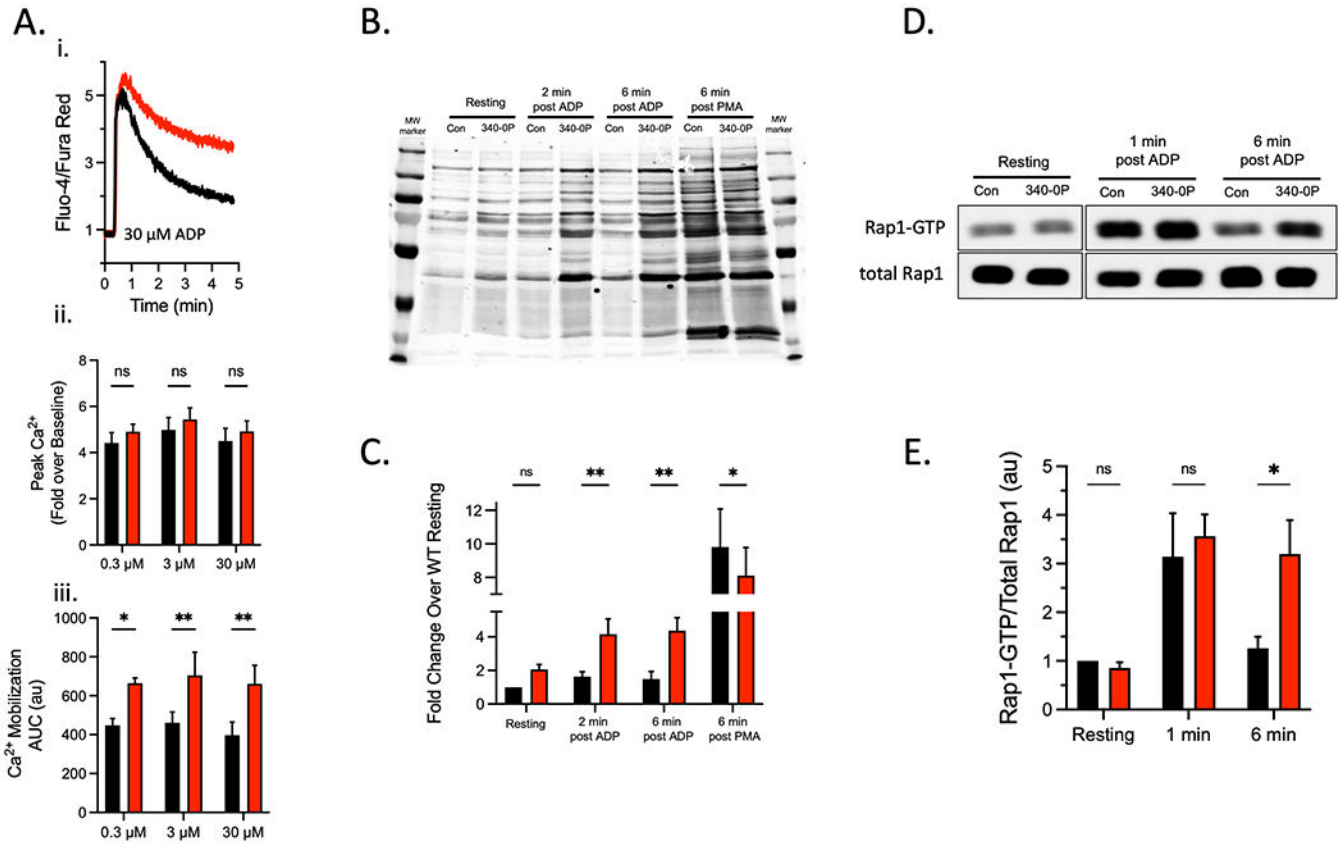

Figure 3. Platelets from P2Y$_1^{340\text{-}0P/340\text{-}0P}$ mice exhibit prolonged ADP-promoted Ca^{2+} mobilization, PKC activation and Rap1-GTP loading.

A) Washed platelets from P2Y$_1^{+/+}$ (black line; black bars) or P2Y$_1^{340\text{-}0P/340\text{-}0P}$ (red line; red bars) mice were loaded with Fluo-4 and Fura-red, stimulated with ADP at indicated concentrations and analyzed by flow cytometry. i) cytosolic Ca^{2+} mobilization (representative traces of 3 independent experiments). ii) quantification of peak Ca^{2+} levels and iii) AUC of cytosolic Ca^{2+} measured from t=0 to t=5 min**. B,C)** Washed platelets from P2Y$_1^{+/+}$ and P2Y$_1^{340\text{-}0P/340\text{-}0P}$ mice were stimulated with either 1 µM ADP or 500 nM Phorbol-12-myristate-13-acetate (PMA; direct activator of PKC) for the indicated times before lysis and immunoblotting for PKC substrate phosphorylation. B) Representative blot; C) Quantification (n=3 per group). **D,E)** Washed platelets from P2Y$_1^{+/+}$ and P2Y$_1^{340\text{-}0P/340\text{-}0P}$ mice were stimulated with 1 µM ADP for indicated time prior to lysis and pulldown of activated Rap1-GTP with RAL-GDS beads and immunoblotting for Rap1. D) Representative blot; E) Quantification of Rap1-GTP/Total Rap1 (n=3 per group). Statistical significance was determined by 2-way ANOVA (*p<0.05, **p<0.01). All data are expressed as the mean ± SEM.

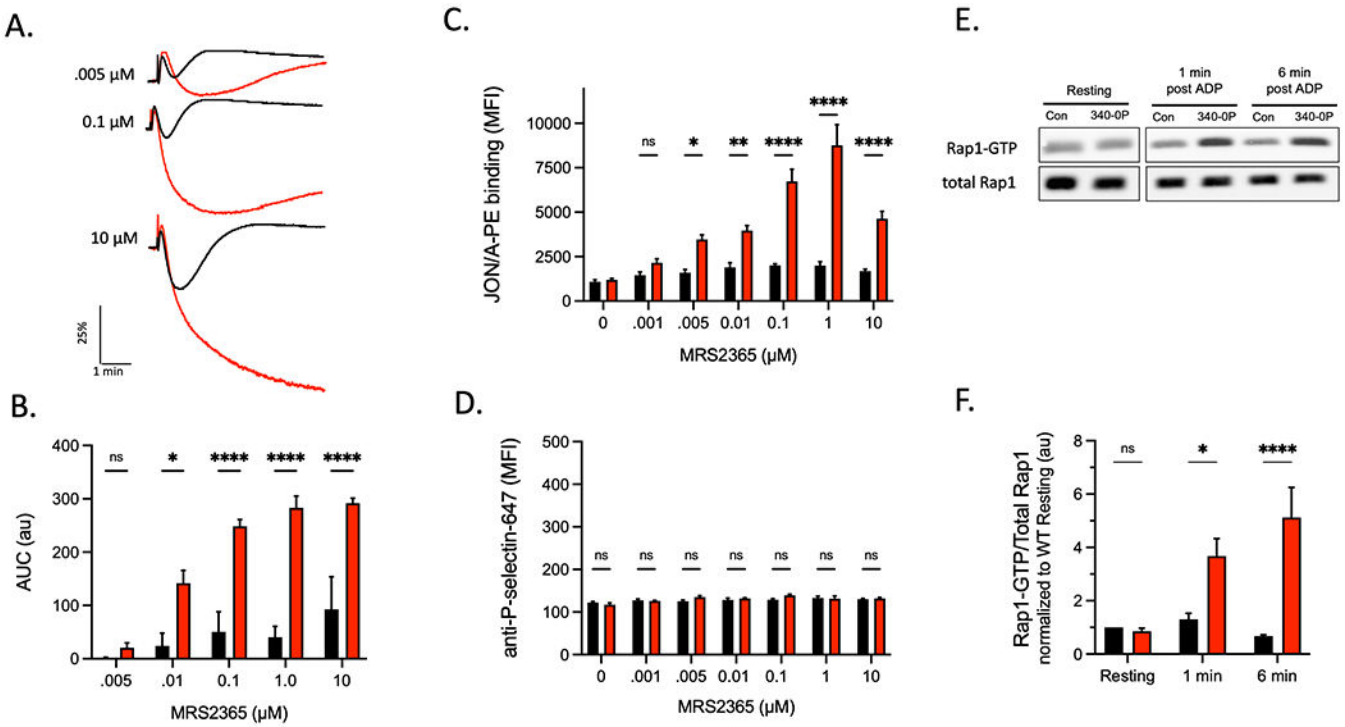

Figure 4. Enhanced activation response of $P2Y_1^{340\text{-}0P/340\text{-}0P}$ platelets to the $P2Y_1$-selective agonist, MRS2365.

A,B) Aggregometry analysis of PRP from $P2Y_1^{+/+}$ (black lines; black bars) and $P2Y_1^{340\text{-}0P/340\text{-}0P}$ (red lines; red bars) mice stimulated with indicated doses of MRS2365. A) Representative traces; B) AUC quantification measured from t=0 to t=6 min (n=3-4 per group). **C,D)** Integrin activation and α-granule secretion in diluted whole blood from $P2Y_1^{+/+}$ (black bars) or $P2Y_1^{340\text{-}0P/340\text{-}0P}$ (red bars) mice stimulated with the indicated doses of MRS2365 and analyzed by flow cytometry. MFI for JON/A-PE staining (activated αIIbβ3, n=5/group); D) MFI for anti P-selectin-647 (α-granule marker, n=5/group). **E,F)** Washed platelets from $P2Y_1^{+/+}$ and $P2Y_1^{340\text{-}0P/340\text{-}0P}$ mice were stimulated with MRS2365 for indicated time prior to lysis and pulldown of activated Rap1-GTP with RAL-GDS beads and immunoblotting for Rap1. D) Representative blot; E) Quantification of the ratio of Rap1-GTP to total Rap1 (n=3 per group). Statistical significance was determined by 2-way ANOVA (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). All data are expressed as the mean ± SEM.

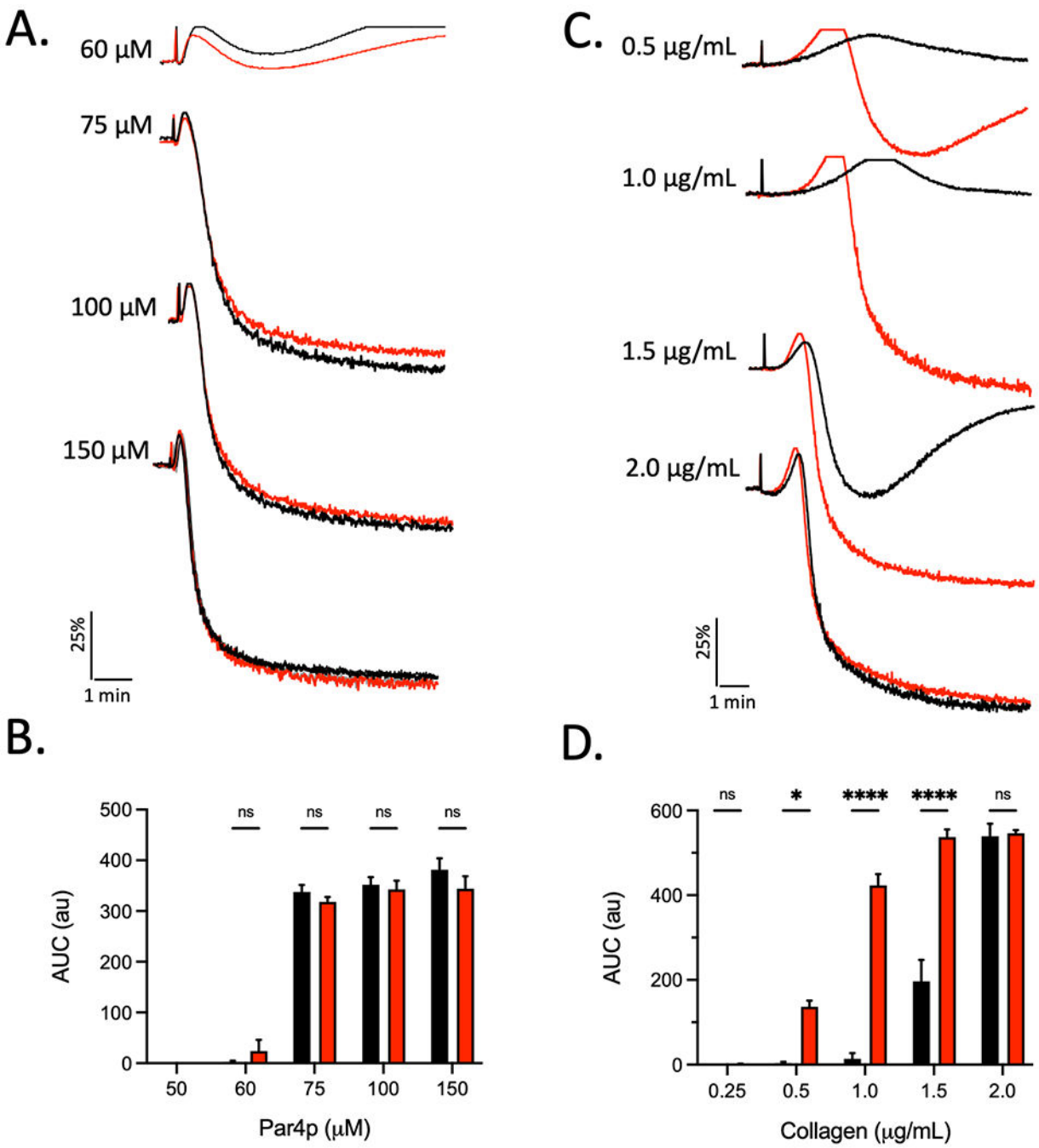

Figure 5. $P2Y_1^{340\text{-}0P/340\text{-}0P}$ platelets are hyper-responsive to collagen but not Par4p.

A,B) Aggregometry analysis of PRP from $P2Y_1^{+/+}$ (black lines; black bars) and $P2Y_1^{340\text{-}0P/340\text{-}0P}$ (red lines; red bars) mice stimulated with indicated doses of PAR4p. A) Representative traces; B) AUC quantification measured from t=0 to t=6 min (n=3-4 per group). **C,D)** Aggregometry analysis of PRP from $P2Y_1^{+/+}$ (black lines; black bars) and $P2Y_1^{340\text{-}0P/340\text{-}0P}$ (red lines; red bars) mice stimulated with indicated doses of collagen. C) Representative traces; D) AUC quantification measured from t=0 to t=6 min (n=3-4 per

group). Statistical significance was determined by 2-way ANOVA (*$p<0.05$, ****$p<0.0001$). All data are expressed as the mean ± SEM.

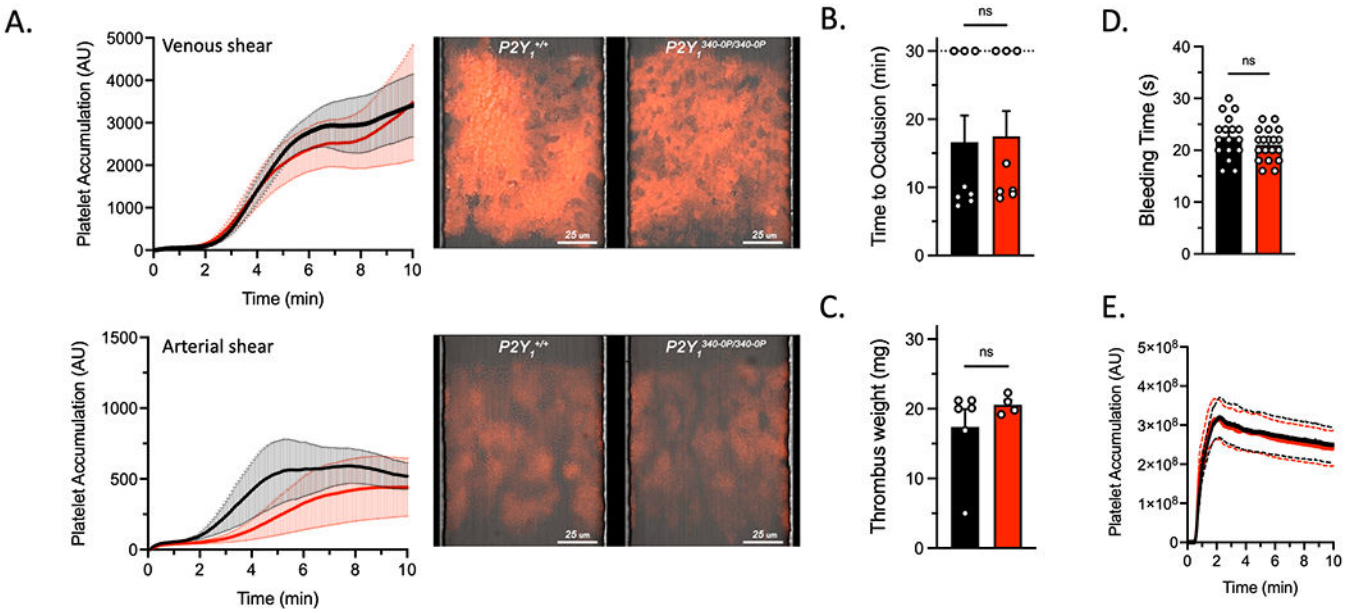

Figure 6. Normal adhesive function of $P2Y_1^{340\text{-}0P/340\text{-}0P}$ platelets under flow conditions *in vitro* and *in vivo*.

A) Microfluidics chamber studies. Whole blood from $P2Y_1^{+/+}$ (black lines) and $P2Y_1^{340\text{-}0P/340\text{-}0P}$ (red lines) mice was perfused over immobilized collagen at venous ($400s^{-1}$) or arterial ($1{,}600s^{-1}$) shear rates and platelet adhesion was quantified (n=4-5 per group). Representative micrographs at t = 10 minutes are shown, platelets in red. Scale bar = 25 μm **B)** Arterial thrombosis. $P2Y_1^{+/+}$ (black bars) and $P2Y_1^{340\text{-}0P/340\text{-}0P}$ (red bars) mice were subjected to the $FeCl_3$-induced carotid artery injury model (2.5% w/v for 5 min) while monitoring blood flow using an ultrasound probe to determine the time to vessel occlusion (n=8/group). Dotted line indicates the end of observation period. **C)** Venous thrombosis. Thrombus weights from $P2Y_1^{+/+}$ (black bars) and $P2Y_1^{340\text{-}0P/340\text{-}0P}$ (red bars) mice subjected to IVC stenosis model (n=4-6/group). **D,E)** Hemostasis. D) $P2Y_1^{+/+}$ (black bars) and $P2Y_1^{340\text{-}0P/340\text{-}0P}$ (red bars) mice were subjected to the saphenous vein laser injury model of hemostasis to quantify bleeding times following injury (5-7 injuries per mouse, 3 mice/group). E) Quantification of platelet accumulation at the site of injury for 10 minutes after laser ablation of endothelium ($P2Y_1^{+/+}$, black lines; $P2Y_1^{340\text{-}0P/340\text{-}0P}$ red lines) (5-7 injuries per mouse, 3 mice/group). Statistical significance was determined by unpaired Students' T-test for B-D. All data are expressed as the mean ± SEM.