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Potential of TRAP-6-induced platelet dense granule release by blockade of P2Y₁₂ signaling with MRS2395

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Abstract

The release of ADP from platelet dense granules and its binding to platelet P2Y₁₂ receptors is key to amplifying the initial haemostatic response and propagating thrombus formation. P2Y₁₂ has thus emerged as a therapeutic target to safely and effectively prevent secondary thrombotic events in patients with acute coronary syndrome or a history of myocardial infarction. Pharmacological inhibition of P2Y₁₂ receptors represents a useful approach to better understand the signaling mediated by these receptors and to elucidate the role of these receptors in a multitude of platelet hemostatic and thrombotic responses. The present work examined and compared the effects of four different P2Y₁₂ inhibitors (MRS2395, ticagrelor, PSB 0739 and AR-C 66096) on platelet function in a series of *in vitro* studies of platelet dense granule secretion and trafficking, calcium generation, and protein phosphorylation. Our results show that in platelets activated with the PAR-1 agonist TRAP-6 (thrombin receptor-activating peptide), inhibition of P2Y₁₂ with the antagonist MRS2395, but not ticagrelor, PSB 0739 or AR-C 66096, potentiated human platelet dense granule trafficking to the plasma membrane and release into the extracellular space, cytosolic Ca²⁺ influx and phosphorylation of GSK3β-Ser9 through a PKC-dependent pathway. These results suggest that inhibition of P2Y₁₂ with MRS2395 may act in concert with PAR-1 signaling and result in the aberrant release of ADP by platelet dense granules, thus reducing or counteracting the anticipated anti-platelet efficacy of this inhibitor.

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Authorship

A.M. designed the project, performed research, analyzed data and wrote the manuscript. N.B.L. performed research and analyzed data. A.T.P.N. and J.P. performed experiments. R.A.R. wrote the manuscript. C.D.W. and J.E.A. supervised the research and edited the manuscript. O.J.M. supervised research and wrote the manuscript.

Disclosures

The authors have no conflicts of interest to declare.

Keywords

platelets; ADP; dense granules; P2Y₁₂

Introduction

Platelets play a central role in hemostasis, becoming activated at sites of vessel damage and aggregating to form a plug and restore blood patency. This activation regime is potentiated by adenosine di-phosphate (ADP), which is released from red blood cells and endothelial cells upon vessel damage or shear stress and is further released by activated platelets from platelet dense granules.[1] ADP binds platelet surface receptors, triggering an intracellular signaling cascade that causes release of intracellular calcium stores, secretion of granule contents, shape change, and platelet-platelet aggregation.

Three purinergic adenosine (P2) receptors are present on platelets: the ATP-binding ion channel P2X₁ and two ADP-binding G-protein coupled receptors (GPCRs), P2Y₁ and P2Y₁₂. The P2Y₁ receptor activates the G-protein subunit G_{αq}, mobilizing intracellular calcium stores to facilitate platelet shape change. The P2Y₁₂ receptor activates G_{αi} to inhibit adenylyl cyclase and cyclic AMP (cAMP) production, causing reduced phosphorylation of vasodilator-stimulated phosphoprotein (VASP) and subsequent platelet actin polymerization and shape change. Moreover, the P2Y₁₂ receptor activates the Gβγ_i subunit to activate the kinases PI3 kinase (PI3K) and AKT.[2] AKT phosphorylates glycogen synthase kinase 3β (GSK3β), a serine-threonine kinase that is constitutively active in platelets as a negative regulator of platelet activity.[3] In addition, protein kinase C (PKC) has also been shown to phosphorylate and negatively regulate the kinase activity of GSK3β.[22] Ultimately, phosphorylation of GSK3β on Ser9 suppresses its inhibitory activity, thereby facilitating platelet activation. Activation of platelets is also potentiated by agonists to other platelet GPCRs, such as the thromboxane receptor (TP) and the thrombin protease activated receptors (PAR-1 and PAR-4), indicating common G-protein signaling pathways between these receptors. One common effector is membrane-bound phospholipase C β (PLCβ), which is activated by G_q to facilitate hydrolysis of membrane inositol phospholipid into inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG). IP₃ moves into the cytosol to trigger calcium release from the platelet dense tubular system, and the released calcium enables recruitment of PKC to the membrane, where it binds DAG and is phosphorylated.[4] Activation of PKC facilitates platelet dense granule release, including the release of ADP to further activate platelets. Intriguingly, inhibition of PKC enhances PAR-1-dependent calcium release, suggesting PKC may play an inhibitory role in calcium signaling, despite its canonical stimulatory role in dense granule secretion.[5] Additional studies reveal an inhibitory role of PKC under low concentrations of ADP but an activating role at high concentrations.[6] A better understanding is needed regarding the mechanisms of PKC-mediated dense granule secretion downstream of GPCRs such as PAR-1 and P2Y₁₂. [7]

Platelet activation, while critical to hemostasis, is also a key factor in arterial thrombosis. The P2Y₁₂ receptor is largely specific to platelets, making it a target for antithrombotic drugs.[8] Several classes of P2Y₁₂ inhibitors have been developed and are clinically in use

to prevent secondary thrombotic events in patients with acute coronary syndrome or recent percutaneous coronary intervention.[9] P2Y₁₂ inhibitors were initially developed from thienopyridines and include clopidogrel, ticlopidine, and prasugrel, pro-drugs that are metabolized in the liver and act by binding irreversibly to P2Y₁₂. Newer generation P2Y₁₂ inhibitors such as ticagrelor and cangrelor are direct-acting, reversible ADP analogues with a more rapid onset and offset than the thienopyridines and no need for metabolism in the liver, properties which may improve safety and efficacy.[9, 10]

Pharmacological inhibition of P2Y₁₂ continues to be crucial to better understand the signaling mediated by this receptor and to elucidate its role in a multitude of platelet hemostatic and thrombotic responses. Our findings suggest the possibility that select P2Y₁₂ inhibitors, including MRS2395, may act in concert with PAR-1 signaling to potentiate platelet dense granule release of ADP, thus reducing or counteracting the anticipated anti-platelet efficacy of these inhibitors. These findings are important and should alert against the development of P2Y₁₂ inhibitors structurally related to MRS2395 for therapeutic applications.

Materials and Methods

Reagents.

MRS2395 [2,2-Dimethyl-propionic acid 3-(2-chloro-6-methylaminopurin-9-yl)-2-(2,2-dimethyl-propionyloxymethyl)-propyl ester], adenosine diphosphate (ADP), wortmannin, thrombin and all other chemicals and reagents were purchased from Sigma-Aldrich (St Louis, MO, USA) or previously mentioned sources unless specified otherwise.[12, 13] TRAP-6 (SFLLRN), U73122, U73343, Ro 31-8220, Rottlerin, Go6976, MRS2179, BAPTA, PSB 0739 and AR-C 66096 were obtained from Tocris (Bristol, UK). Ticagrelor was purchased from Oxchem Corporation (Wood Dale, IL, USA). AYPGKF-NH₂ was obtained from Abgent (San Diego, CA, USA). Oregon Green® 488 BAPTA-1 AM (OG488 BAPTA-1 AM) was from Thermo Fisher Scientific (Carlsbad, CA, USA). PAC-1-FITC and anti-CD62P-APC were obtained from BD Bioscience (San Jose, CA, USA). Anti-AKT, phospho (p)AKT-Ser473, pGSK3β-Ser9 and pPKC-Ser substrates were from Cell Signaling Technology (Danvers, MA, USA).

Isolation of human washed platelets.

Platelets were isolated from human venous blood drawn from healthy volunteers by venipuncture into sodium citrate (1:9; v/v), in accordance with an Institutional Review Board-approved protocol at Oregon Health & Science University, as previously described. [12, 13] Briefly, anticoagulated blood was centrifuged (200×g, 20 min) to obtain platelet-rich plasma (PRP). PRP was centrifuged (1000×g, 10 min) in the presence of prostacyclin (0.1 µg mL⁻¹) to obtain a platelet pellet. The platelet pellet was resuspended in modified HEPES/Tyrode buffer (129 mM NaCl, 0.34 mM Na₂HPO₄, 2.9 mM KCl, 12 mM NaHCO₃, 20 mM HEPES, 5 mM glucose, 1 mM MgCl₂; pH 7.3) and washed once via centrifugation at 1000×g for 10 min in modified HEPES/Tyrode buffer in the presence of prostacyclin (0.1 µg mL⁻¹). Purified platelets were resuspended in modified HEPES/Tyrode buffer at the indicated concentrations.

Platelet aggregation.

Aggregation studies were performed using 250 μ L of PRP pretreated with inhibitors for 15 min. Platelet aggregation was initiated by ADP (3 μ M), and changes in light transmission were monitored for 5 minutes using a PAP-4 aggregometer, as previously described.[13]

Platelet dense granule secretion assay.

The release of ATP stored in platelet dense granules was measured as light output generated following an ATP-luciferin-luciferase reaction. The assay was carried out as previously described.[14, 15] Briefly, human washed platelets (2×10^8 /mL; 70 μ L) were incubated in a white Corning Costar flat bottom 96-well plate in the presence of select inhibitors or vehicle control (10 μ L) for 15 minutes at 37°C under an orbital shake. Platelet agonists (10 μ L) were then added and allowed to activate platelets for 30 seconds at 37°C on an orbital shaker. Finally, 10 μ L of the detection reagent Chronolume (Chrono-Log Corporation, Havertown, PA, USA) was added to the wells and the corresponding sample luminescence was detected with an Infinite® M200 spectrophotometer (TECAN, Switzerland). The assay was performed in duplicate for at least three independent experiments.

Imaging of dense granule intracellular localization and reorganization by Superresolution Structured Illumination Microscopy (SR-SIM).

For imaging experiments, 12mm #1.5 glass coverslips (Fisher Scientific) were coated with poly-L-lysine (Sigma). Inhibitors (MRS2395, 10 μ M; ticagrelor, 20 ng/mL; PSB 0739, 10 μ M; AR-C 66096, 10 μ M) were added to platelets in solution (4×10^7 /mL) for 15 min prior to activation with TRAP-6 (10 μ M) for 30 sec at 37°C, followed by fixation with 4% paraformaldehyde and seeding onto proteins at room temperature for 1 hr. Adherent platelets were permeabilized with a blocking solution (1% BSA + 0.01% SDS in PBS). Platelets were then stained with CD63 (MX49.129.5; Santa Cruz Biotechnology) and MRP4/ABCC4 (D1Z3W; Cell Signaling Technology) overnight at 4°C at a 1:100 dilution in the blocking solution. Alexa Fluor secondary antibodies (1:500; Life Technology) were added in the blocking solution for 2 hrs. Coverslips were mounted with Fluoromount G (SouthernBiotech) onto glass slides. Platelets were imaged using SR-SIM with a Zeiss 100 $\times$ oil immersion 1.46 NA alpha plan-apochromat lens on a Zeiss Elyra PS.1 microscope.

Intracellular calcium measurements.

The increase in intracellular calcium concentration [Ca^{2+}] in response to TRAP-6 (10 μ M) or ADP (10 μ M) was measured using OG488-BAPTA-1 AM-loaded platelets. In brief, human washed platelets (2×10^8 /mL) were incubated with 10 μ M OG488-BAPTA-1 AM for 30 min at 37°C. Dye-loaded platelets were then washed as described above to remove excess of dye. Subsequently, washed platelets (2×10^8 /mL; 80 μ L) were incubated in a black Corning Costar clear flat bottom 96-well plate in the presence of select inhibitors or vehicle control (10 μ L) for 15 min at 37°C on an orbital shaker. Platelet agonists (10 μ L) were then added, and fluorescence was measured continuously for 1 min at 37°C using an excitation wavelength of 494 nm and emission wavelength of 523 nm on an Infinite® M200 spectrophotometer. The assay was performed in duplicate for at least three independent experiments.

Western blotting.

For Western blot experiments, human washed platelets (300 μ L, 5×10^8 /mL in modified HEPES/Tyrode buffer) were incubated with vehicle control or select inhibitors for 15 min at 37°C. TRAP-6 (10 μ M) was added and allowed to activate platelets for 30 sec at 37°C with stirring at 300 rpm. Subsequently, platelets were lysed in RIPA buffer (1X, Pierce, Thermo Scientific, Rockford, USA) supplemented with a protease and phosphatase inhibitor cocktail (1X, Pierce). Lysates were denatured in an equal volume of Laemmli sample buffer (Bio-Rad) with 0.5 M dithiothreitol (100°C, 5 min), separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and immunoblotted with indicated antibodies and HRP-conjugated secondary antibodies, as previously described.[16] Protein was detected using ECL (Amersham Biosciences).

Flow cytometry analysis.

Flow cytometry experiments were carried out as previously described.[16] Briefly, washed human platelets (2×10^8 /mL) were preincubated with vehicle (0.01% DMSO) or MRS2395 (10 μ M) for 15 min at 37°C and then stimulated with ADP (10 μ M final concentration) for 15 min in the presence of FITC-conjugated anti-human PAC-1 or APC-conjugated anti-human CD62P antibodies (BD Biosciences, USA). Samples were then fixed by 1% paraformaldehyde for 10 min and diluted in HEPES/Tyrode buffer, followed by flow cytometric analysis on a FACSCantoII system (Becton Dickinson, USA). Data are expressed as percentage of the maximal response obtained in the response to ADP.

Statistical Analysis.

Data were analyzed using GraphPad PRISM 4.0 software (San Diego, CA, USA). To determine statistical significance, Student's paired *t* test was used for comparison between treatments. Results are expressed as the mean $\pm$ standard error of the mean (SEM). Differences were considered significant at *p* values less than 0.05. For aggregation studies, percentage maximum aggregation was used for data analysis; the IC₅₀ were determined by a variable slope model of nonlinear regression analysis.

Results

Inhibition of P2Y₁₂ by MRS2395 enhances TRAP-6-induced platelet dense granule release.

The G_i-coupled ADP receptor P2Y₁₂ is key to the amplification signal necessary to sustain platelet aggregation induced by ADP and other platelet agonists.[2] However, whether P2Y₁₂ is involved in the release of its autocrine ligand remains ill-defined. The primary objective of this study was to determine the effect of P2Y₁₂ inhibition on the release of platelet dense granules, which store ADP. For this purpose, we used the P2Y₁₂ inhibitor MRS2395.[17] The initial experiments aimed to define the effect of increasing doses of MRS2395 (1–100 μ M) on platelet aggregation induced by ADP. As seen in Figure 1A & B, MRS2395 inhibited platelet aggregation in a concentration-dependent manner with an IC₅₀ of 7 μ M following human PRP exposure to 3 μ M ADP. To determine whether ligation of P2Y₁₂ by ADP stimulates ADP release, we next investigated the effect of MRS2395 on the release of dense granules in response to specific platelet agonists using a luciferin-luciferase

assay.[17] MRS2395 (10 and 50 μ M) by itself did not have an effect on platelet dense granule release (Figure 1C). As shown in Figure 1D, perhaps surprisingly, MRS2395 did not directly affect the ability of exogenous ADP to induce platelet dense granule release. Similarly, MRS2395 did not synergize with the P2Y₁ inhibitor MRS2179 (20 μ M) or with the COX-1 inhibitor indomethacin (10 μ M) in antagonizing ADP-induced dense granule release (Supplemental Figure 1). Moreover, MRS2395 had no effect on platelet dense granule release in response to thrombin or AYPGKF-NH₂ (PAR-4 agonist). Intriguingly, MRS2395 significantly enhanced platelet dense granule release following activation of the G_q-coupled receptor PAR-1 with 10 μ M of the PAR-1 selective peptide TRAP-6 (Figure 1H); this effect was lost when platelets were stimulated with 30 μ M TRAP-6 or with 10 μ M TRAP-6 in combination with 10 μ M ADP (Figure 1H & I). In contrast, MRS2395 (50 μ M) inhibited platelet dense granule release in response to the GPVI agonist CRP (10 μ g/mL; Figure 1E). Moreover, the low dose of 10 μ M MRS2395 retained the ability to inhibit dense granule release in response to the GPVI agonist CRP when used in combination with 10 μ M TRAP-6 (Figure 1I).

Taken together, these data suggest that targeted inhibition of the P2Y₁₂ receptor with MRS2395 results in differential regulation of dense granule release dependent on the platelet agonist, with a surprising increase in ADP release in response to PAR-1 agonist TRAP-6. In agreement with previous studies,[17, 18] inhibition of the P2Y₁₂ receptor with MRS2395 (10 μ M) significantly reduced ADP-induced α (alpha)-granule secretion and α IIb β 3 activation as detected by flow cytometry analysis of P-selectin expression (CD62P) and PAC-1 binding, respectively (Supplemental Figure 2).

Inhibition of P2Y₁₂ with MRS2395 enhances the localization of dense granules at the plasma membrane after platelet activation with TRAP-6.

We next studied whether autocrine P2Y₁₂ signaling regulates dense granule trafficking in platelets following platelet activation with TRAP-6. Platelets were stained and imaged using SR-SIM to study the spatial localization of the dense granule marker CD63 and the ATP-binding cassette transporter multidrug resistance protein 4 (MRP-4), which is required for pumping nucleotides stored in dense granules, such as ATP and ADP, into the extracellular space.[19]

As shown in Figure 2, MRP-4 (green) was expressed and distributed in a punctate pattern throughout the intracellular space and plasma membrane of resting platelets. In contrast, CD63 was primarily detected in the intracellular compartment of resting platelets (Figure 2). Treatment of resting platelets with MRS2395 alone did not affect the distribution of MRP-4 or CD63. After activation with TRAP-6, both MRP-4 and CD63 redistributed to the periphery of the platelets. Importantly, treatment of platelets with MRS2395 prior activation with TRAP-6 resulted in an increase in the accumulation of CD63 at the plasma membrane, suggesting that MRS2395 facilitates the coordination of dense granule release under TRAP-6 stimulation.

MRS2395-dependent potentiation of TRAP-6-induced dense granule secretion is PI3K-independent and PLC-dependent.

We next sought to study the downstream signaling pathways by which MRS2395 potentiates dense granule secretion in platelets following activation with TRAP-6. To this end, we used pharmacological inhibitors of PI-3K and PLC, molecules that are involved in the crosstalk between the PAR-1 and P2Y₁₂ receptors. Platelets were treated with the PI-3K inhibitor wortmannin (100 nM), the PLC inhibitor U73122 (10 μ M) or its inactive analogue U73343 (10 μ M) in presence or absence of the P2Y₁₂ blocker MRS2395 (10 μ M) prior to activation with TRAP-6 (10 μ M). As shown in Figure 3A, wortmannin alone had no effect on platelet dense granule release induced by TRAP-6. Moreover, the ability of MRS2395 to enhance platelet dense granule release in response to TRAP-6 was unaffected by platelet treatment with wortmannin, indicating that MRS2395 acts in a PI3K-independent manner. In contrast, pre-treatment of platelets with the PLC inhibitor U73122 alone abrogated dense granule release incited by TRAP-6, whilst the inactive analog U73343 had no effect on this response (Figure 3B). Importantly, the inhibitory effect of U73122 on TRAP-6-induced dense granule release persisted in the presence of MRS2395 (Figure 3B).

MRS2395-dependent potentiation of TRAP-6-induced dense granule secretion is PKC and calcium-dependent.

The activation of PKC and the mobilization of intracellular calcium are required events for platelet dense granule secretion.[20] Thus, we next sought to determine whether PKC and calcium were involved in the ability of MRS2395 to enhance platelet dense granule release in response to TRAP-6. To this end, we used the pan-PKC inhibitor Ro 31-8220 (10 μ M) and the calcium chelator BAPTA (10 μ M). As shown in Figure 4A, pre-treatment of platelets with Ro 31-8220 alone abrogated dense granule release by TRAP-6. In addition, the inhibitory effect of Ro 31-8220 on TRAP-6-induced dense granule release persisted in the presence of MRS2395. Importantly, TRAP-6-induced dense granule release was unaffected by calcium chelation with BAPTA. Rather, an inhibitory effect of BAPTA on dense granule secretion was only observed when TRAP-6 was used in combination with MRS2395 (Figure 4A).

There is increasing evidence that members of the PKC superfamily can exert opposing regulatory roles in a fashion unique to the modes of platelet activation or inhibition.[21] Therefore, we next investigated the ability of Rottlerin (10 μ M) and Go6976 (1 μ M), which are reported to be selective for the PKC isoforms δ and α/β , respectively, to inhibit the potentiation of TRAP-6-induced platelet dense granule release by blockade of P2Y₁₂ with MRS2395. As demonstrated in Figure 4B, Rottlerin alone significantly reduced dense granule release induced by TRAP-6. Moreover, the inhibitory effect of Rottlerin on TRAP-6-induced dense granule release persisted in the presence of MRS2395. Interestingly, TRAP-6-triggered dense granule release was unaffected by Go6976. In contrast, the potentiating effect of MRS2395 on TRAP-6-induced dense granule secretion was partially reduced, but not eliminated, by Go6976 (Figure 4B).

We next addressed whether blockade of P2Y₁₂ with MRS2395 had an effect on cytosolic Ca²⁺ influx upon platelet activation with TRAP-6 or ADP. Treatment of resting platelets

with MRS2395 alone did not affect the baseline concentration of cytosolic Ca^{2+} (Figure 4C). The PAR-1 agonist TRAP-6 caused a rapid increase in cytosolic Ca^{2+} compared to resting platelets, and this response was enhanced in presence of MRS2395 (Figure 4C). Platelet activation with exogenous ADP alone induced an increase in intracellular Ca^{2+} , a response that was reduced by MRS2395 (Figure 4C). As expected, addition of the calcium chelator BAPTA decreased platelet intracellular Ca^{2+} concentrations regardless of the agonist used (Figure 4C).

Glycogen Synthase Kinase 3 β (GSK3 β) plays a role in the ability of MRS2395 to potentiate platelet activation downstream of the PAR-1 receptor.

Previous studies have shown that GSK3 β functions as endogenous negative regulator of platelet activity and that phosphorylation of GSK3 β at serine 9 (Ser⁹) by PKC α or AKT abrogates the inhibitory function of GSK3 β on platelet dense granule secretion downstream of either PAR-1 or P2Y₁₂. [3, 22] Therefore, we next sought to determine whether the unexpected effect of the P2Y₁₂ antagonist MRS2395 (10 μM) on TRAP-6-induced platelet dense granule secretion was due to changes in phosphorylation of serine residues of GSK3 β , AKT and PKC substrates, the latter indicative of PKC activity. To this end, washed platelets were pretreated with the P2Y₁₂ inhibitor MRS2395 or vehicle alone (0.01% DMSO) for 15 min prior to being stimulated with TRAP-6 (10 μM , 30 sec), lysed and immunostained for pGSK3 β -Ser9, pAKT-Ser473 and pPKC-Ser substrates. As demonstrated in Figure 4D, lane a, the MRS2395 compound alone induced a very weak increase in phosphorylation of GSK3 β -Ser9 and PKC-Ser substrates in resting platelets, whilst having no effect on AKT-Ser473 phosphorylation. As expected, GSK3 β -Ser9 was phosphorylated following 30 sec of activation with the PAR-1 agonist TRAP-6; along these lines, a dramatic increase in phosphorylation of PKC-Ser substrates and of AKT-Ser473 was observed following platelet activation with TRAP-6 (Figure 4D, lane b). Interestingly, GSK3 β -Ser9 phosphorylation was enhanced when platelets were treated with the MRS2395 compound prior to stimulation with TRAP-6; this response correlated with an increase in the phosphorylation of the PKC-Ser substrates. In contrast, treatment with MRS2395 was associated with a significant decrease in AKT-Ser473 phosphorylation in platelets activated with TRAP-6 (Figure 4D, lane b).

As we observed a potential role for PKC and Ca^{2+} in the ability of MRS2395 to potentiate TRAP-6-induced platelet dense granule secretion, we next studied whether inhibition of PKC with Ro 31-8220 or chelation of calcium with BAPTA blocked the effect of MRS2395 on TRAP-6-induced serine phosphorylation of GSK3 β , PKC substrates and AKT. To this end, we treated washed platelets with the pan-PKC inhibitor Ro 31-8220 or the calcium chelator BAPTA alone or in combination with MRS2395 prior to stimulation with TRAP-6 for 30 sec. The PKC inhibitor Ro-31-8220 used alone or in combination with MRS2395 abrogated GSK3 β -Ser9 phosphorylation and largely inhibited PKC-Ser substrate phosphorylation in TRAP-6-activated platelets (Figure 4D, lane c). In addition, the phosphorylation of AKT-Ser473 was partially reduced by Ro 31-8220 alone as compared to vehicle control; this effect was lost in the presence of MRS2395 (Figure 4D, lane c). BAPTA alone had no significant effect on phosphorylation of GSK3 β -Ser9 following platelet activation with TRAP-6 as compared to vehicle control, whereas we observed a reduced

PKC activity and phosphorylation of AKT-Ser473 (Figure 4D, lane d vs. b). In contrast, TRAP-6-mediated phosphorylation of GSK3 β -Ser9 was blocked when platelets were treated with a cocktail of BAPTA and MRS2395; this observation was associated with a decrease in AKT-Ser473 phosphorylation as compared to platelets activated with TRAP-6 in absence of MRS2395.

In light of the fact that inhibition of the PI-3K pathway had no effect on the ability of MRS2395 to potentiate TRAP-6-induced dense granule release, we did not expect to see an effect of the PI-3K inhibitor wortmannin on the ability of MRS2395 to potentiate the phosphorylation of GSK3 β -Ser9 or PKC-Ser substrates in response to TRAP-6. As shown in Figure 5, lane e, inhibition of PI-3K reduced but did not abolish AKT-Ser473 phosphorylation in response to TRAP-6; addition of MRS2395 in combination with wortmannin rescued this response. In good agreement with our previous results, the ability of MRS2395 to enhance GSK3 β -Ser9 phosphorylation in TRAP-6-activated platelets persisted in the presence of wortmannin (Figure 4D, lane e).

Finally, to determine the potential involvement of tyrosine kinase signaling in the ability of MRS2395 to enhance TRAP-6-induced platelet activation, we probed platelet lysates with the monoclonal antibody 4G10 that recognizes phosphotyrosine. Platelet activation with TRAP-6 for 30 sec resulted in a mild increase in protein tyrosine phosphorylation, while treatment with MRS2395 had no significant effect on this response, indicating that tyrosine kinase signaling is not involved in the ability of MRS2395 to enhance TRAP-6-induced platelet activation (Supplemental Figure 3).

Effect of the P2Y₁₂ antagonists ticagrelor, PSB 0739 and AR-C 66096 on platelet dense granule release and trafficking.

We next evaluated whether the functional effects of MRS2395 on TRAP-6-induced platelet activation were observed with ticagrelor, a clinically approved P2Y₁₂ receptor blocker. Ticagrelor inhibited platelet aggregation in a concentration-dependent manner with an IC₅₀ of 19.9 ng/mL following human PRP exposure to 3 μ M ADP (Figure 5A & B). Ticagrelor (20 ng/mL) by itself did not have an effect on platelet dense granule release (Figure 5C). Similarly to MRS2395 (Figure 1D), ticagrelor failed to inhibit the release of platelet dense granule triggered by exogenous ADP (10 μ M) (Figure 5D). However, ticagrelor synergized with the P2Y₁ inhibitor MRS2179, but not with the COX-1 inhibitor indomethacin, in antagonizing ADP-induced dense granule release (Supplemental Figure 1). As shown in Figure 5E & G, contrary to the action of the MRS2395 compound (Figure 1H), ticagrelor, at a concentration as low as 20 ng/mL, strongly inhibited platelet dense granule release induced by TRAP-6 (10 μ M) or by the GPVI agonist CRP (10 μ g/mL). Moreover, ticagrelor retained the ability to inhibit platelet dense granule release in response to TRAP-6 (10 μ M) when used in combination with 10 μ M ADP or 10 μ g/mL CRP (Figure 5H). In contrast, ticagrelor had no effect on platelet dense granule release in response to AYPGKF-NH₂ (250 μ M; Figure 5F). To assess whether the results showing platelet dense granule inhibition were related to ticagrelor specifically or to P2Y₁₂ inhibitors in general, we investigated the effect of two additional P2Y₁₂ antagonists, PSB 0739 and AR-C 66096, on the release of dense granules in response to a range of agonists in human platelets. As demonstrated in Figure

6A, neither PSB 0739 nor AR-C 66096 by themselves had an effect on platelet dense granule release. Contrary to MRS2395 and ticagrelor, both PSB 0739 and AR-C 66096 were able to reduce, but to different extents, ADP-induced platelet dense granule release (Figure 6B). Similarly to ticagrelor, PSB 0739 and AR-C 66096 strongly inhibited platelet dense granule release induced by TRAP-6 (10 μ M) or by the GPVI agonist CRP (10 μ g/mL) (Figure 6C & E). Importantly, PSB 0739, but not AR-C 66096, significantly decreased platelet dense granule release in response to the PAR-4 agonist AYPGKF-NH₂ (250 μ M; Figure 6D).

The inhibitory activity of ticagrelor, PSB 0739 and AR-C 66096 on TRAP-6-induced platelet dense granule release was also determined by visualization of CD63 and MRP-4 localization via SR-SIM. In contrast to MRS2395 treatment (Figure 2), inhibition of P2Y₁₂ with ticagrelor or AR-C 66096 prevented the relocalization of CD63 and MRP-4 to the periphery of the platelets upon activation with TRAP-6 (Figure 7). Surprisingly, CD63 and MRP-4 displayed a polarized distribution in platelets pretreated with PSB 0739 prior activation with TRAP-6 (Figure 7).

Effect of ticagrelor, PSB 0739 and AR-C 66096 on Ca²⁺ mobilization and GSK β phosphorylation of platelets activated with TRAP-6.

We next addressed whether blockade of P2Y₁₂ with ticagrelor, PSB 0739 or AR-C 66096 had an effect on cytosolic Ca²⁺ influx upon platelet activation with TRAP-6 or ADP. In platelets treated with any of the three P2Y₁₂ antagonists tested, the Ca²⁺ signal induced by TRAP-6 and ADP was reduced when compared to vehicle control (Figure 8A).

To determine whether the inhibition of TRAP-6-induced dense granule release occurred as a result of impaired GSK β signaling, we investigated the effect of ticagrelor, PSB 0739 and AR-C 66096 on phosphorylation of GSK β -Ser9, AKT-Ser473 and PKC-Ser substrates. Contrary to the phosphorylation profile observed in presence of MRS2395 (Figure 4D), GSK β -Ser9 phosphorylation was dramatically reduced when platelets were treated with ticagrelor, PSB 0739 or AR-C 66096 prior to stimulation with TRAP-6; this response was corroborated by the observation that all the three P2Y₁₂ antagonists significantly inhibited, but to different extents, AKT-Ser473 phosphorylation in response to TRAP-6 (Figure 8B). Similarly to MRS2395, targeted inhibition of P2Y₁₂ with ticagrelor slightly increased the phosphorylation of PKC-Ser substrates in TRAP-6-activated platelets (Figure 8B). In contrast, inhibition of P2Y₁₂ with AR-C 66096 decreased the phosphorylation of PKC-Ser substrates in TRAP-6 activated platelets, while PSB 0739 had no effect on it (Figure 8B).

Discussion

In this study, we investigated whether the inhibition of the platelet ADP receptor P2Y₁₂ acts in concert with select platelet activation pathways to promote platelet dense granule release. We have demonstrated that TRAP-6-induced dense granule release, dense granule trafficking to the plasma membrane and cytosolic Ca²⁺ influx are potentiated by targeting P2Y₁₂ with MRS2395 yet are inhibited by targeting P2Y₁₂ with ticagrelor. We show that the ability of MRS2395 to promote TRAP-6 induced platelet dense granule release is PLC-, PKC- and Ca²⁺-dependent yet independent of PI-3K. Biochemical analysis of platelet lysates revealed

that the ability of MRS2395 to potentiate TRAP-6-induced platelet dense granule release correlated with an increase in phosphorylation of GSK3 β on Ser9.

Upon platelet activation, ADP stored in platelet dense granules is released and binds P2Y₁₂ receptors to amplify the initial haemostatic response and to propagate thrombus formation. [1,2] P2Y₁₂ has thus emerged as a therapeutic target to safely and effectively prevent cardiovascular events including heart attack and stroke. Two different classes of P2Y₁₂ inhibitors have been developed: adenosine-based derivatives (*i.e.* ticagrelor, MRS2395) and thienopyridine derivatives (*i.e.* clopidogrel, ticlopidine). [9,10] Most of these P2Y₁₂ antagonists have been designed to act against P2Y₁₂-G_i-mediated signaling, thus enhancing the generation of the second messenger cyclic AMP (cAMP) by reducing the inhibition of adenylate cyclase promoted by ADP. However, several studies have questioned the specificity of a number of P2Y₁₂ antagonists, and additional P2Y₁₂-independent mechanisms of action have been identified that may impact the effectiveness and outcome of anti-P2Y₁₂ therapy. For example, studies have suggested that ticagrelor inhibits platelet function not only by antagonizing P2Y₁₂ but also by promoting the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) and by increasing the levels of extracellular adenosine via blockade of the ENT1 adenosine transporter on red blood cells and platelets. [11,24] These observations have raised the question of whether the release of ADP, which is substrate for adenosine generation, is relevant to the effect of P2Y₁₂ antagonists. While the role of P2Y₁₂ in amplification of the signaling cascade that sustains platelet activation is well known, to date there has been no characterization of whether targeted inhibition of the P2Y₁₂ receptor feeds back to invoke platelet ADP release. In the current study, we employed four different P2Y₁₂ inhibitors, the ADP acyclic analogue MRS2395, the ATP analog AR-C 66096 and the non-nucleotide antagonist PSB 0739, all three widely used for research purposes, and the clinically available drug ticagrelor, to study whether targeted inhibition of P2Y₁₂ signaling regulates platelet dense granule release of ADP.

Surprisingly, functional experiments revealed that MRS2395 specifically potentiated platelet dense granule release in response to activation of PAR-1 with TRAP-6, while it had no effect when platelets were stimulated with ADP, thrombin or the PAR-4 ligand AYPGKF-NH₂. In contrast, ticagrelor, PSB 0739 and AR-C 66096 significantly inhibited platelet dense granule release in response to TRAP-6 and CRP. Moreover, similar to MRS2395, ticagrelor failed to inhibit ADP-elicited platelet dense granule release, while both PSB 0739 and AR-C 66096 significantly reduced platelet secretory response to ADP but to different extents. Interestingly, PSB 0739 was the only P2Y₁₂ antagonist able to significantly decrease dense granule release upon platelet activation with the PAR-4 agonist AYPGKF-NH₂.

There are a number of mechanisms through which MRS2395 may enhance platelet dense granule release in response to TRAP-6. The activation of the G_q cascade by engagement of the PAR-1 receptor leads to the recruitment and activation of PLC β . Activated PLC β cleaves phosphatidylinositol 4,5-trisphosphate into inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). Production of diacylglycerol and the increase in intracellular calcium synergize to activate the serine-threonine kinase PKC; the latter then phosphorylates downstream molecular targets, including GSK3 β , which, in resting platelets, functions as a negative regulator of dense granule secretion. In the present study, we found evidence for

increased PKC activation in platelets treated with MRS2395 as detected by the increased phosphorylation profile of serine residues of PKC substrates downstream of PAR-1 signaling. Moreover, inhibition of PLC or PKC with the broad spectrum antagonists U73122 and Ro 31-8220, respectively, blocked both the ability of TRAP-6 alone or in concert with MRS2395 to induce platelet dense granule release. Interestingly, treatment of platelets with MRS2395 also enhanced PAR-1 mediated GSK3 β -Ser9 phosphorylation, possibly as a result of increased PKC activity, as the phosphorylation status of GSK3 β -Ser9 dramatically declined in presence of the PKC inhibitor Ro 31-8220. GSK3 β can also be phosphorylated by AKT following activation of PI-3K signaling, which is triggered in response to several platelet agonists, including TRAP-6 and ADP.[22] Consistent with our previous results, we demonstrate that the ability of MRS2395 to potentiate TRAP-6-induced dense granule release and GSK3 β phosphorylation was not affected by the PI-3K inhibitor wortmannin, ruling out a role for AKT and supporting the hypothesis that other kinases, such as PKC, may be involved. These findings are also supported by a study by Moore and colleagues showing that TRAP-6-mediated early phosphorylation of GSK3 β -Ser9 is partly AKT independent and more likely to be PKC dependent.[22] In contrast, our results show that treatment of platelets with ticagrelor, PSB 0739 or AR-C 66096 ablated PAR-1 mediated GSK3 β phosphorylation, possibly as a result of decreased AKT activity, in line with our observation that all of the three P2Y₁₂ inhibitors also blocked the phosphorylation of AKT at the serine residue 473. Interestingly, ticagrelor and AR-C 66096 were the most effective at reducing AKT phosphorylation, while PSB 0739 and MRS2395 displayed a similar inhibitory activity against p-AKT_{Ser473}.

PKC is a promiscuous enzyme functioning not only as a positive regulator of platelet activation but also as an endogenous intracellular inhibitor downstream of PAR-1 and P2Y₁₂ receptor-mediated signaling.[5,6] The PKC family is divided into 3 groups based on the cofactors involved in their activation: the diacylglycerol (DAG) and calcium sensitive conventional isoforms (cPKC; i.e., α and β), the DAG-sensitive and calcium-insensitive novel isoforms (nPKC; i.e., δ and θ), and the phosphatidyl inositide triphosphate sensitive atypical isoforms (aPKC; i.e., ζ and γ).[4] Through the use of PKC isoform-specific inhibitors, we demonstrate that PKC α/β might specifically regulate the potentiating effect of MRS2395 downstream of PAR-1, as the PKC α/β inhibitor Go6976 significantly reduced the ability of MRS2395 to potentiate PAR-1 mediated dense granule secretion whilst having no effect on TRAP-6-induced platelet dense granule secretion in the absence of MRS2395. Previous findings by the Kunapuli group demonstrated that PKC δ plays a key positive role in PAR-1-mediated dense granule secretion.[23] Our results are in agreement with these findings, as we observe that Rottlerin abrogated PAR-1 mediated dense granule release; moreover, this effect persisted in presence of the MRS2395 compound. It is well known that a rise in the cytosolic Ca²⁺ concentration is key to the activation of cPKC isoforms.[4] Indeed, we found that MRS2395 significantly increased the intracellular influx of Ca²⁺ when platelets were activated with TRAP-6. Importantly, treatment of platelets with the calcium chelator BAPTA specifically ablated the potentiating effect of MRS2395 on platelet dense granule release in response to TRAP-6, confirming that a rise in intracellular Ca²⁺ is key to the enhancing effect of MRS2395 on platelet secretion. In agreement with these

findings, the increase in phosphorylation of platelet GSK3 β -Ser9 induced by MRS2395 downstream of PAR-1 activation dramatically declined in the presence of BAPTA.

All together, our data suggest a potential deleterious mechanism of action of the P2Y₁₂ inhibitor MRS2395 on platelet function, an effect that may or may not be conserved by other P2Y₁₂ inhibitors. Thus, the regulation of platelet dense granule secretion as a result of targeted inhibition of P2Y₁₂ with MRS2395 should be taken into account when interpreting experimental results based on use of this reagent. Our results suggest that MRS2395 might enhance the sensitivity of platelets to secondary ADP-P2Y₁₂ signaling downstream of the G_q-bound receptor PAR-1 while having an inhibitory effect in response to activation of receptors that signal through an immunoreceptor tyrosine-based activation motif (ITAM) such as the collagen receptor GPVI. It could be speculated that platelets, similar to neurons, are plastic and thus able to strengthen or weaken their response to changes in their microenvironment. In line with this idea, it is possible that exogenous signals, agonists or inhibitors that modulate platelet homeostasis away from an equilibrium state may also trigger compensatory platelet responses.

In conclusion, we show for the first time that targeted inhibition of the P2Y₁₂ receptor results in differential regulation of dense granule release, dependent on the platelet agonist and the P2Y₁₂ antagonist used. Given that the release of ADP and its subsequent positive feedback signaling through P2Y₁₂ plays a crucial role in platelet activation, a better understanding of the regulation of P2Y₁₂ receptor sensitivity to different platelet agonists and antagonists will be of considerable importance for the design of improved therapeutics in the treatment of thrombotic disease and prevention of unwanted bleeding.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Effect of the P2Y₁₂ inhibitor MRS2395 on aggregation and dense granule secretion of human washed platelets. A-B.

PRP was incubated with increasing doses (1–100 μ M) of MRS2395 at 37°C for 15 minutes prior activation with 3 μ M ADP for 5 minutes. Aggregation traces (**A**) and corresponding dose-response bar graph (**B**) are representative of three independent experiments. Data are mean $\pm$ SEM. #: $p < 0.05$ for comparison of MRS2395-treated platelets vs. vehicle. **C-I.** Human washed human platelets (2×10^8 /mL) were incubated with vehicle (0.01% DMSO) or MRS2395 (10 or 50 μ M) prior to incubation with platelet buffer (unstimulated, **C**) or with

ADP (10 or 30 μ M, **D**), CRP (1 or 10 μ g/mL, **E**), thrombin (1 or 10 U/mL, **F**), AYPGKF-NH₂ (100 or 250 μ M, **G**), TRAP-6 alone (10 or 30 μ M, **H**) or a cocktail of TRAP-6 (10 μ M, **I**) with either ADP (10 μ M) or CRP (10 μ g/mL). Platelet dense granule secretion was measured as function of ATP release using luminometry. Values are mean $\pm$ SEM of raw luminescence ($\times 10^6$) of at least 3 independent experiments. #: $p < 0.05$ for comparison of MRS2395-treated platelets vs. vehicle. AU: Arbitrary units.

Fig. 2. Effect of MRS2395 on the localization and distribution of CD63 and MRP-4 in human washed platelets activated with TRAP-6.

Human washed human platelets ($4 \times 10^7/\text{mL}$) were incubated with vehicle (0.01% DMSO) or MRS2395 (10 μM) prior to activation with TRAP-6 for 30 sec. Replicate samples of washed human platelets were then placed on glass coverslips coated with poly-lysine prior to fixation, blocking and staining for MRP-4 (green) and CD63 (red) and visualization by SR-SIM (scale bar = 2 μm).

Fig. 3. Effect of wortmannin and U73122 on TRAP-6-induced dense granule release of MRS2395-treated platelets. A-B.

Washed human platelets ($2 \times 10^8/\text{mL}$) were incubated with vehicle (0.01% DMSO) or MRS2395 (10 μM) in combination with wortmannin (100 nM, **A**), U73122 (10 μM , **B**) or U73343 (10 μM , **B**) prior to activation with 10 μM TRAP-6 for 30 sec. Platelet dense granule secretion was measured as function of ATP release using luminometry. Values are mean $\pm$ SEM of raw luminescence ($\times 10^6$) of at least 3 independent experiments. #: $p < 0.05$ for comparison of single drug-treated platelets vs. vehicle. *: $p < 0.05$ for comparison of drug cocktail-treated platelets vs. vehicle in presence of MRS2395. AU: Arbitrary units.

Fig. 4. Role for PKC, Ca^{2+} and GSK3 β -Ser9 in TRAP-6-induced activation of MRS2395-treated platelets. A-B.

Washed human platelets ($2 \times 10^8/\text{mL}$) were incubated with vehicle (0.01% DMSO) or MRS2395 (10 μM) in combination with Ro 31-8220 (10 μM , **A**), BAPTA (10 μM , **A**), Rottlerin (10 μM , **B**) or Go6976 (1 μM , **B**) prior to activation with 10 μM TRAP-6 for 30 sec. The effect of the inhibitors alone on TRAP-6-induced platelet dense granule secretion was also tested. Platelet dense granule secretion was measured as function of ATP release using luminometry. Values are mean $\pm$ SEM of raw luminescence ($\times 10^6$) of at least 3 independent experiments. #: $p < 0.05$ for comparison of single drug-treated platelets vs vehicle. *: $p < 0.05$ for comparison of drug cocktail-treated platelets vs. vehicle in presence of MRS2395. AU: Arbitrary units. **C.** OG-488 BAPTA-AM loaded washed platelets ($2 \times 10^8/\text{mL}$) were treated with vehicle (0.01% DMSO), MRS2395 (10 μM) or BAPTA (10 μM) prior to activation with TRAP-6 (10 μM , left panel) or ADP (10 μM , right panel). The effect of MRS2395 (10 μM) in combination with BAPTA (10 μM) was also tested. Values

are mean $\pm$ SEM of raw fluorescence of 3 independent experiments. Fluorescence was measured for 60 sec. **D.** Washed human platelets ($5 \times 10^8/\text{mL}$) were incubated with vehicle (0.01% DMSO) or MRS2395 (10 μM) alone or in combination with Ro 31-8220 (10 μM), BAPTA (10 μM) or wortmannin (100 nM) prior to activation with TRAP-6 for 30 sec. The effect of the inhibitors alone on TRAP-6-induced platelet activation was also tested. Treated platelets were subsequently lysed and blotted for phosphorylated GSK3 β -Ser9, phosphorylated PKC-Ser substrates and phosphorylated AKT-Ser473. Total AKT and tubulin served as loading control. Blots are representative of 3 experiments.

Fig. 5. Effect of the P2Y₁₂ inhibitor ticagrelor on aggregation and dense granule secretion of human washed platelets. A-B.

PRP was incubated with increasing doses (1–200 ng/mL) of ticagrelor at 37°C for 15 minutes prior to activation with 3 μ M ADP for 5 minutes. Aggregation traces (A) and corresponding dose-response bar graph (B) are representative of three independent experiments. Data are mean $\pm$ SEM. #: $p < 0.05$ for comparison of ticagrelor-treated platelets vs. vehicle. **C-H.** Human washed human platelets (2×10^8 /mL) were incubated with vehicle (0.01% DMSO) or ticagrelor (20 ng/mL) prior to incubation with platelet buffer (unstimulated, C) or with ADP (10 μ M, D), TRAP-6 (10 μ M, E), AYPGKF-NH₂ (250 μ M, F) CRP (10 μ g/mL, G), or a cocktail of TRAP-6 (10 μ M, H) with either ADP (10 μ M) or CRP (10 μ g/mL). Platelet dense granule secretion was measured as function of ATP release using luminometry. Values are mean $\pm$ SEM of raw luminescence ($\times 10^6$) of at least 3 independent experiments. #: $p < 0.05$ for comparison of ticagrelor-treated platelets vs. vehicle. AU: Arbitrary units.

Fig. 6. Effect of the P2Y₁₂ inhibitors PSB 0739 and AR-C 66096 on dense granule secretion of human washed platelets. A-E.

Human washed human platelets ($2 \times 10^8/\text{mL}$) were incubated with vehicle (0.01% DMSO), PSB 0739 (10 μM) or AR-C 66096 (10 μM) prior to incubation with platelet buffer (unstimulated, **A**) or with ADP (10 μM , **B**), TRAP-6 (10 μM , **C**), AYPGKF-NH₂ (250 μM , **D**) or CRP (10 $\mu\text{g/mL}$, **E**). Platelet dense granule secretion was measured as a function of ATP release using luminometry. Values are mean $\pm$ SEM of raw luminescence ($\times 10^6$) of at least 3 independent experiments. #: $p < 0.05$ for comparison of PSB 0739 or AR-C 66096-treated platelets vs. vehicle. AU: Arbitrary units.

Fig. 7. Effect of ticagrelor, PSB 0739 and AR-C 66096 on the localization and distribution of CD63 and MRP-4 in human washed platelets activated with TRAP-6.

Human washed human platelets ($4 \times 10^7/\text{mL}$) were incubated with vehicle (0.01% DMSO) or the indicated P2Y₁₂ antagonist prior to activation with TRAP-6 for 30 sec. Replicate samples of washed human platelets were then placed on glass coverslips coated with poly-lysine prior to fixation, blocking and staining for MRP-4 (green) and CD63 (red) and visualization by SR-SIM (scale bar = 2 μm).

Fig. 8. Effect of ticagrelor, PSB 0739 and AR-C 66096 on cytosolic Ca^{2+} influx and phosphorylation of GSK3 β -Ser9, PKC-Ser substrates and AKT-Ser473 of TRAP-6 activated platelets.

A. OG-488 BAPTA-AM loaded washed platelets ($2 \times 10^8/\text{mL}$) were treated with vehicle (0.01% DMSO), ticagrelor (20 ng/mL), PSB 0739 (10 μM) or AR-C 66096 (10 μM) prior to activation with TRAP-6 (10 μM , left panel) or ADP (10 μM , right panel). Values are mean $\pm$ SEM of raw fluorescence of 3 independent experiments. Fluorescence was measured for 60 sec. **B.** Washed human platelets ($5 \times 10^8/\text{mL}$) were incubated with vehicle (0.01% DMSO), ticagrelor (20 ng/mL), PSB 0739 (10 μM) or AR-C 66096 (10 μM) prior to activation with TRAP-6 for 30 sec. Treated platelets were subsequently lysed and blotted for phosphorylated GSK3 β -Ser9, phosphorylated PKC-Ser substrates and phosphorylated AKT-Ser473. Tubulin served as loading control. Blots are representative of 3 experiments.