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Diverse signaling of the platelet P2Y₁ receptor leads to a dichotomy in platelet function.

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Abstract

Platelet P2Y₁ receptor signaling via RhoGTPases is necessary for platelet-dependent leukocyte recruitment, where no platelet aggregation is observed. We investigated signaling cascades involved in distinct P2Y₁-dependent platelet activities *in vitro*, using specific inhibitors for phospholipase C (PLC) (U73122, to inhibit the canonical pathway), and RhoGTPases: Rac1 (NSC23766) and RhoA (ROCK inhibitor GSK429286). Human platelet rich plasma (for platelet aggregation) or isolated washed platelets (for chemotaxis assays) was treated with U73122, GSK429286 or NSC23766 prior to stimulation with adenosine diphosphate (ADP) or the P2Y₁ specific agonist MRS2365. Aggregation, chemotaxis (towards f-MLP), or platelet-induced human neutrophil chemotaxis (PINC) towards macrophage derived chemokine (MDC) was assessed. Molecular docking of ADP and MRS2365 to P2Y₁ was analyzed using AutoDock Smina followed by GOLD molecular docking in the Accelrys Discovery Studio software.

Inhibition of PLC, but not Rac1 or RhoA, suppressed platelet aggregation induced by ADP and MRS2365. In contrast, platelet chemotaxis and PINC, were significantly attenuated by inhibition of platelet Rac1 or RhoA, but not PLC. MRS2365, compared to ADP had a less pronounced effect on P2Y₁-induced aggregation, but a similar efficacy to stimulate platelet chemotaxis and PINC, which might be explained by differences in molecular interaction of ADP compared to MRS2365 with the P2Y₁ receptor. Platelet P2Y₁ receptor activation during inflammation signals through alternate pathways involving Rho GTPases in contrast to canonical P2Y₁ receptor induced PLC signaling. This might be explained by selective molecular interactions of ligands within the orthosteric site of the P2Y₁ receptor.

Keywords

Platelets; P2Y₁ receptor; PLC; RhoA; Rac1; inflammation.

1. Introduction

It is now understood that platelet activation and recruitment to sites of inflammation is a requisite part of host defence against pathogens (McMorran et al., 2009; Yeaman 2014; Page and Pitchford 2013, Amison et al, 2017a). Conversely, inappropriate platelet activation occurs in a range of inflammatory and auto-immune disorders, and in animal models evidence for an absolute requirement of platelets in the recruitment of leukocytes to various inflamed organs continues to grow; for example in allergic and non-allergic inflammation (Kornerup et al., 2010; Lellouch Tubiana et al., 1988; Coyle et al., 1990; Pitchford et al., 2003; 2005, Amison et al, 2017b), atherosclerosis and damaged arterial surfaces (Bienvenu et al., 2001; Theoret et al., 2001), rheumatoid arthritis (Schmitt-Sody et al., 2005; Boilard et al., 2010), adult respiratory distress syndrome (Zarbock et al., 2006; Grommes et al., 2012; Looney et al., 2009), acute pancreatitis (Abdulla et al., 2011), glomerulonephritis (Devi et al., 2010), and experimental autoimmune encephalitis (Langer et al., 2012). This pro-inflammatory role of platelets is distinct from their function in haemostasis since we have identified drugs that can inhibit platelet dependent leukocyte recruitment without influencing platelet aggregation. We were the first to identify a divergent, or 'biased', signalling event centred on the platelet purinergic P2Y₁ receptor that controls leukocyte recruitment during inflammation, but that does not affect haemostasis (Amison et al., 2015; 2017b). This divergence in platelet function supports the hypothesis: 'Dichotomy in platelet activation', whereby platelet activation is considered distinct following activation with inflammatory stimuli versus aggregatory stimuli (Page 1988).

The role of purinergic receptor activation in the regulation of leukocyte activation, adhesion molecule expression and motility has received considerable attention (Chen et al., 2006; Ferrari et al., 2016). Platelets express 4 purinergic receptor sub types on their surface (P2Y₁, P2Y₁₂, P2Y₁₄, P2X₁), which control distinct aspects of platelet function (Jacobson et al., 2011). The purinergic

nucleotide, adenosine-diphosphate (ADP), can activate platelets by signalling through the purinergic G Protein coupled receptor, P2Y₁. The P2Y₁ receptor is coupled to G_{αq}, which results in Ca²⁺ mobilisation and Protein Kinase C (PKC) activation following activation of phospholipase C (PLC), initiating platelet shape change, granule release, thromboxane (TXA₂) production and initial aggregation (Gachet 2006; Leon et al., 2003). The activation of P2Y₁ receptors on platelets also triggers an increase in surface P-selectin expression (Leon et al., 2003; Storey et al., 2002), suggesting that P2Y₁ receptor activation of platelets might also be involved in P-selectin-dependent inflammatory responses (Page and Pitchford 2013; Pitchford et al., 2003; 2005; Kornerup et al., 2010). It is therefore likely that, as with leukocytes, purinergic receptor signalling is involved in platelet activation during inflammation. We have therefore investigated whether platelet purinergic receptors become activated in response to immune and inflammatory stimuli, and the downstream signalling events resulting from such activation (e.g. Rho-GTPases). Rho-GTPases (for example RhoA, Rac1, cdc42) are important for the adhesion, activation and motility of leukocytes, but are also found in platelets (Aslan and McCarty 2013). Our recent data implicates purinergic nucleotide signalling, with Rho-GTPases downstream of P2Y₁, receptor activation controlling pulmonary leukocyte recruitment during allergic airways inflammation (Amison et al., 2015). The formation of platelet-leukocyte complexes *in vivo*, and platelet-P-selectin dependent neutrophil migration *in vitro* were dependent on activation of platelet P2Y₁ receptors via RhoA signalling (Amison et al., 2015). Furthermore, we recently investigated the role of another small GTPase, Rac1, in the platelet activation associated with both allergic and LPS-induced airway inflammation. Thus, we have demonstrated that direct activators of Rac1 (guanine nucleotide exchange factors Rac-GEFs) in platelets are also required for leukocyte recruitment (Pan et al., 2015). Furthermore, others have shown that Rac1 signalling in platelets can be induced by ADP activation of P2Y₁ receptors, although this has not been tested in an inflammatory setting (Eckly et al., 2001). Since RhoA activity is insufficient for full platelet aggregation in response to ADP or other primary agonists (Soulet et al., 2005; Pearce et al., 2007), and Rac1 activation by specific Rac-GEFs appear to play minor roles, if

any, in platelet aggregation (Qian et al., 2012; Aslan et al., 2011; Pleines et al., 2012), it is possible that Rho-GTPases link P2Y₁ receptors selectively to platelet functions involved in inflammatory processes, but are largely redundant in regulating ADP-induced platelet aggregation. Furthermore, it is not known what effect platelet activation induced by P2Y₁ receptor stimulation of the canonical PLC signalling pathway has on platelet functions associated with inflammation and host defence. The aim of the present experiments discussed here was to evaluate the contributions of PLC compared to Rac1 and RhoA signalling to P2Y₁ receptor induced platelet aggregation, platelet motility, and platelet-induced neutrophil chemotaxis (PINC), using *in vitro* assays of platelet functions involved in haemostasis and host defence.

2. Methods and Methods

2.1. Materials and Reagents

To assess the role of RhoGTPases and PLC on different aspects of platelet function, specific protein inhibitors were obtained as described. The PLC specific inhibitor U73122 was purchased from Bio-Techne and was prepared at 0.01, 0.1, 1 and 10 µmol/l (in 0.1% dimethyl sulfoxide, DMSO). U46619, the synthetic stable analogue of PGH₂ which is a thromboxane A₂ (TXA₂) receptor (TP) agonist was purchased from Tocris and was pre-dissolved in methyl acetate and then diluted in saline to 0.001, 0.01, 0.1 and 1.0 µmol/l. The specific Rac1 inhibitor NSC23766 was purchased from Bio-Techne and was prepared at 0.1, 1 and 10 µmol/l (in saline). The Rho-associated Kinase (ROCK) inhibitor GSK429286 was purchased from Bio-Techne and was prepared at 1, 10 and 100 µmol/l (in 0.1% DMSO). The P2Y₁ receptor antagonist MRS2500, and the P2Y₁₂ receptor antagonist AR-C66096 were purchased from Bio-Techne and prepared to 1 µmol/l in saline. The P2Y₁ receptor specific agonist MRS2365 was purchased from Bio-Techne and prepared to 1 µmol/l in saline. ADP was purchased from Sigma Aldrich and prepared to 0.1 and 10 µmol/l in saline. Apyrase was purchased from Sigma Aldrich (#A6535) and prepared to 1U/ml in saline. The P2Y₁₂ specific antagonist AR-C66096 was

purchased from Bio-Techne and prepared to 1 μ mol/l in saline. Aspirin was purchased from Sigma (A2093), and was prepared at 0.1% DMSO. Platelets were treated with 1mM aspirin for 30 mins at 37degrees prior to stimulation with agonists.

2.2. Isolation of Human cells

Platelets and Neutrophils were isolated from human peripheral venous blood obtained from healthy volunteers who had not taken NSAIDs or other anti-inflammatory drugs in the previous seven days, and were not prescribed anti-platelet drugs. For all studies blood was collected in accordance with local ethical approval from King's College London and adhered to regulations outlined by the Human Tissue Act 2004.

2.2.1 Human platelet isolation for aggregation studies.

Citrated (1:9) whole venous blood (20ml) was collected from healthy volunteers as described above. Blood was centrifuged at 120 g for 20 mins at room temperature to isolate platelet rich plasma (PRP). PRP was collected and incubated with drugs for 10 mins at room temperature as explained in section 2.3 below.

2.2.2 Platelet Isolation (used for both platelet chemotaxis and PINC).

Citrated (1:9) whole venous blood was collected from healthy volunteers as described above. Blood was centrifuged at 120 g for 20 mins at room temperature to isolate PRP. PRP was then treated with 2.5 μ M PGE₁ and centrifuged at 800 g for 10 mins at room temperature to isolate the platelet pellet. The supernatant was removed and the platelet pellet re-suspended in RPMI 1680 cell media and incubated with drugs as detailed in sections 2.4 and 2.5 below. Platelets used for the PINC assay underwent a further washing step, because in this assay pre-incubation with drugs was required. It was subsequently necessary to remove the drugs from the media before platelets were added to neutrophils (Section 2.5). 2.5 μ M PGE₁ was then added to the platelet suspension and again

centrifuged at 800 g for a further 10 mins at room temperature. Upon final centrifugation, the supernatant was aspirated off and the pellet re-suspended in 1ml RPMI. Platelets were quantified using a haemocytometer and diluted to a working concentration of 1×10^8 platelets/ml in RPMI containing 2mM Ca^{2+} . Platelets were left for 1h prior to experimentation to ensure the effects of PGE_1 had expired.

2.2.3. Human neutrophil isolation

Whole human peripheral venous blood was added to Histopaque 10771 containing leucosep tubes to isolate the individual cell layers through optimised density gradient centrifugation as per the manufacturer's instructions. All layers were removed except for the pelleted neutrophil layer and erythrocytes below. This layer was then mixed with Solution A (40ml Voluven, 8ml PBS, 2ml Acid-Citrate-Dextrose) and centrifuged. The resulting top layer was collected and treated with ultrapure water to remove any remaining erythrocytes. This was centrifuged and the remaining pellet re-suspended in RPMI 1640 media and quantified on an improved Neubauer haemocytometer in Turk's nuclear stain using a Zeiss Axioskop microscope with a x20 objective. Neutrophils were then diluted to a working concentration of 5×10^6 neutrophils/ml in RPMI 1640. The purity of the neutrophil suspension using this technique is >95%.

2.3. Platelet Aggregation.

An *in vitro* assay of platelet aggregation was used to elucidate the importance of each of the downstream Rho GTPases and PLC in this biological process. PRP was isolated from citrated peripheral venous blood and incubated with vehicle (saline) controls and increasing concentrations of U73122 (PLC), NSC23766 (Rac1) or GSK429286 (ROCK) for 10 mins at room temperature. In some experiments platelets were incubated with either vehicle or 1u/ml apyrase prior to drug treatment. In other experiments, platelets were treated with aspirin (1mmol/l) for 30 mins at 37degrees prior to stimulation with agonists. After treatment, platelets in the presence of drug were stimulated with

10 μ mol/l ADP or 1 μ mol/l of the P2Y₁ receptor specific agonist MRS2365 and aggregation measured over 10 mins via light transmission aggregometry in a shaking plate reader (SpectraMax 340PC, Molecular Devices) at 595nm at 37°C.

2.4. Platelet chemotaxis.

An *in vitro* assay of platelet chemotaxis was used to elucidate the importance of each of the downstream signalling proteins on this inflammatory function of platelets. 24 well cell culture plates were blocked with RPMI 1640 medium plus 1% BSA for 1 h before blocking buffer was removed, and 30nmol/l f-MLP added to each well. Washed platelets (1x10⁸ platelets/ml) were isolated from citrated human peripheral venous blood and then incubated with vehicle controls or increasing concentrations of U73122 (PLC), NSC23766 (Rac1) or GSK429286 (ROCK) for 10 mins at room temperature. In some experiments platelets were incubated with either vehicle or 1u/ml apyrase before drug treatment. In other experiments, platelets were treated with aspirin (1mmol/l) for 30 mins at 37degrees prior to stimulation with agonists. Platelets in the presence of drug were then pre-incubated for 5 mins with either 100nmol/l ADP, below the threshold concentration for aggregation, or 1 μ mol/l of the P2Y₁ receptor agonist MRS2365, or the TP receptor agonist U46619 (1, 10, 100, 1000nmol/l). Platelet chemotaxis was then measured over 90 mins in a transwell setup using inserts with membranes consisting of 3 μ m pores (Becton Dickinson). After 90 mins, the transwell inserts were removed and the media from the bottom chamber mixed 1:1 with stromatol, and platelet numbers quantified on an Improved Neubauer haemocytometer using a Zeiss Axioskop microscope with a x40 objective. Data are represented as a chemotactic index, which is the ratio of platelet migration in the positive control (30nM f-MLP) in the presence or absence of inhibitors divided by the basal platelet movement to vehicle control (no chemokine).

2.5. Platelet-induced neutrophil chemotaxis (PINC) assay

An *in vitro* assay of PINC was used to elucidate the importance of each of the proteins downstream of the P2Y₁ receptor on subsequent neutrophil activation. Chemotaxis plates (3µm pore size, Neuroprobe) were blocked with Tyrode's medium plus 1% BSA for 1h before blocking buffer was removed and 100nmol/l macrophage-derived chemokine (MDC) was added to each well. Washed platelets (1x10⁸ platelets/ml) were isolated from citrated human peripheral venous blood and incubated with vehicle controls or increasing concentrations of U73122 (PLC), NSC23766 (Rac1) or GSK429286 (ROCK) at room temperature for 10 mins. In some experiments platelets were incubated with 1u/ml apyrase prior to incubation with drugs. In other experiments, platelets were treated with aspirin (1mmol/l) for 30 mins at 37degrees prior to stimulation with agonists. Platelets in the presence of drug were subsequently incubated with either 100nmol/l ADP below the threshold concentration for aggregation, or 1µmol/l MRS2365, or the TP receptor agonist U46619 (1, 10, 100, 1000nmol/l) in later experiments for 5 mins at 37°C and then washed. Following stimulation, platelets were mixed at a 1:1 volumetric ratio with neutrophils (5x10⁶ neutrophils/ml) isolated as described above and placed on the top membrane of the chemotaxis plates. Chemotaxis was then allowed to occur over 90 mins at 37°C, at which point the top membrane was removed and the media in the bottom chamber collected and mixed 1:1 with Turk's nuclear stain, and neutrophils quantified on an improved Neubauer haemocytometer using a Zeiss Axioskop microscope with a x20 objective. Data are represented as a chemotactic index, which is the ratio of neutrophil migration (with platelets) in the positive control (100nM MDC) in the presence or absence of inhibitors divided by the basal neutrophil movement (with platelets) to vehicle control (no chemokine).

2.6. Molecular docking of ligands with the P2Y₁ receptor.

Molecular docking was performed to generate several distinct binding orientations and binding affinity for each binding mode. Subsequently, the lowest binding free energy was considered as the most favourable binding mode for the system. AutoDock Smina (Koes et al., 2013; Trott and Olson

2009), which uses the AutoDock Vina scoring function by default, was used for the blind molecular docking of the ligands to the P2Y₁ structure (protein databank PDB ID: 4XNW, 4XNV) for finding the best binding site by exploring all probable binding cavities of the proteins. Smina was performed with default settings, which samples nine ligand conformations using the Vina docking routine of stochastic sampling. Then, Genetic Optimization for Ligand Docking (GOLD) molecular docking was applied for the docking of ADP and MRS2365 to the Smina-located best binding site of the P2Y₁ receptor for performing flexible molecular docking as described elsewhere (Jones et al., 1995; 1997). Based on the fitness function scores and ligand binding positions, the best-docked poses for the ligands were selected. The GOLD molecular docking procedure was performed by applying the GOLD protocol in the Accelrys Discovery Studio software (Jones et al., 1997). The Genetic algorithm (GA) was used in GOLD ligand docking software to examine thoroughly the ligand conformational flexibility along with the partial flexibility of the protein (Nissink et al., 2002). The maximum number of runs was set to 20 for the ligand and the default parameters selected were 100 population size, 5 for the number of islands, 100,000 for the number of operations and 2 for the niche size. Default cut-off values of 2.5Å (dH-X) for hydrogen bonds and 4.0Å for the van-der-Waals distance were applied. When the top solutions attained the Root Mean Squared Deviation (RMSD) values within 1.5 Å, the Genetic Algorithm (GA) docking was terminated.

2.7. Statistical analysis

Data from *in vitro* platelet aggregation studies are displayed as means $\pm$ standard error of mean (S.E.M), whereas data from *in vitro* chemotaxis studies are displayed as a chemotactic index because basal migration differs with each platelet donor. Exact group sizes are shown in the individual figure legends, and are ≥ 5 to achieve necessary statistical power. The observer was blinded to treatment groups for quantification of microscopy counts for the chemotaxis assays. Data were analysed by means of 1-way ANOVA using GraphPad Prism, followed by the Dunnett multiple comparison post-test. A *P* value of less than 0.05 was considered significant.

3. Results

3.1 *Rho GTPases, compared to PLC are not involved with platelet aggregation stimulated by ADP.*

We have investigated whether signalling via RhoA and Rac1 could elicit platelet aggregation in a similar manner to PLC *in vitro*. Platelets were stimulated with 10 μ mol/l ADP, which induced a significant aggregatory response ($P < 0.05$ **Fig. 1A-C**). As previously reported elsewhere, pre-incubation of platelets with a PLC inhibitor (U73122) caused a concentration-dependent suppression of platelet aggregation (U73122: 1 μ mol/l, $P < 0.05$; 10 μ mol/l, $P < 0.05$ **Fig. 1A**). In contrast, pre-incubation of platelets with increasing concentrations of a Rac1 inhibitor (NSC23766, 0.1-10 μ mol/l) or a ROCK inhibitor (GSK429286, 1-100 μ mol/l) did not suppress platelet aggregation stimulated by ADP (**Fig. 1B & 1C**).

3.2 *Platelet Rho GTPases, but not PLC are necessary for platelet motility and PINC.*

Platelet-induced neutrophil chemotaxis (PINC) towards MDC requires the addition of ADP (or the P2Y₁ receptor specific agonist MRS2365. **Supplementary Figure 1A**). Neutrophil chemotaxis towards MDC does not occur in the absence of platelets, even in the presence of ADP or MRS2365 (**Supplementary Figure 1A**). Previously, using an *in vitro* assay of PINC, we reported that the mechanism was platelet P2Y₁ dependent, and not dependent on P2Y₁₂ receptor stimulation, which is the other purinergic receptor expressed on platelets that can be activated by ADP (Amison et al., 2015). This assay has therefore been used to predict the mechanisms of platelet activation involved in platelet-dependent leukocyte recruitment in several animal models of inflammation using different stimuli (Amison et al., 2015; 2017b). Using this assay, and separately a platelet chemotaxis assay, we investigated whether these P2Y₁-dependent actions required signalling via PLC, or Rho-GTPases other than RhoA. The addition of platelets led to a significant increase in PMN chemotaxis towards the chemokine MDC (**Fig. 2A-2C**). However, inhibition of platelet PLC activity with U73122 was unable to suppress this platelet-mediated effect at concentrations that led to complete

suppression of platelet aggregation (**Fig. 2A**). In contrast, inhibition of platelet Rac1 (NSC23766) suppressed platelet-dependent PMN chemotaxis towards MDC at 1 μ mol/l ($P<0.05$), and 10 μ mol/l ($P<0.05$) to basal levels (**Fig. 2B**). Similarly, inhibition of platelet RhoA activity with the ROCK inhibitor (GSK429286) also suppressed platelet-dependent PMN chemotaxis towards MDC at 1 μ mol/l ($P<0.05$), 10 μ mol/l ($P<0.05$) and 100 μ mol/l ($P<0.05$) to basal levels (**Fig. 2C**).

Next, we used another assay of platelet function applicable to platelet activation during inflammation. We and others have recorded platelet migration into inflamed tissue, and the ability of platelets to undergo chemotaxis *in vitro* (Pitchford et al., 2008; Czapiga et al., 2005; Kraemer et al., 2012). We have used a transwell chemotaxis assay to investigate platelet motility towards fMLP and the possible involvement of PLC or RhoGTPases in this activity. Platelet chemotaxis towards fMLP requires the presence of ADP, or the P2Y₁ receptor specific agonist MRS2365 (**Supplementary Figure 1B**). Platelet chemotaxis towards fMLP in the presence of 100nM ADP or 1 μ M MRS2365 was inhibited by the P2Y₁ antagonist MRS2500, but not the P2Y₁₂ antagonist AR-C66096 (**Supplementary Figure 2**). This shows that platelet chemotaxis towards fMLP is dependent on platelet P2Y₁ receptor stimulation by either ADP (**Supplementary Figure 2A**), or the synthetic P2Y₁ agonist MRS2365 (**Supplementary Figure 2B**). Similar to the PINC assay, inhibition of platelet PLC activity was not able to suppress platelet motility in response to fMLP, even at concentrations of U73122 that completely suppressed platelet aggregation (**Figure 2D**). However, the Rac1 inhibitor (NSC23766) and ROCK inhibitor (GSK429286) each caused a significant concentration-dependent inhibition of platelet motility (**Fig. 2E & F**).

3.3. ADP-dependent platelet motility and PINC occurs independently of TXA₂ production and activation of platelets.

ADP stimulation of platelets leads to the synthesis and release of TXA₂ which acts on TP receptors and these have the potential to mobilize several families of G-protein signalling molecules that are capable of stimulating RhoA (and Rac1) activity (Woodward et al., 2011). However, aspirin treated

platelets did not inhibit the ability of platelets to stimulate neutrophil chemotaxis towards MDC (**Fig. 3A**), or platelet motility towards fMLP (**Fig. 3B**). Furthermore, direct stimulation of platelets with the TXA₂ analogue U46619 did not induce activity in these assays, compared to platelet incubation with ADP, suggesting TP receptor stimulation was not involved in ADP-induced RhoA or Rac1 activity on platelet function (**Fig. 3C & 3D**), at concentrations of U46619 used to induce full platelet aggregation (**Fig. 3E**). Furthermore, aspirin (1mmol/l) significantly inhibited ADP induced platelet aggregation (**Fig. 3E**), at a concentration that had no effect on platelet chemotaxis or PINC as mentioned above (**Fig. 3A & 3B**).

3.4. Platelet aggregation induced via P2Y₁ receptor activation is dependent on PLC, but not Rac1 or RhoA.

To investigate whether the activity of the various signalling molecules in the functional assays was downstream of P2Y₁ receptor activation, we replaced the ADP stimulation of platelets with a P2Y₁ receptor specific agonist MRS2365 (Bourdon et al., 2006). Increasing concentrations of MRS2365 (0.01µmol/l-10.0 µmol/l) revealed maximal aggregation at 1µmol/l (**Fig. 4A**). Pre-incubation of platelets with a PLC inhibitor (U73122, 10µmol/l), caused significant suppression of platelet aggregation (P<0.05, **Fig. 4B**).

The addition of apyrase was used in some groups to quench endogenously released ADP that could then act on P2Y₁₂ receptors, and therefore to confirm that these signalling pathway inhibitors were directly suppressing activity downstream of P2Y₁ receptor stimulation. Compared to solely P2Y₁ receptor mediated platelet aggregation under these conditions, platelet aggregation induced by ADP was more extensive, as it presumably culminated in both P2Y₁ and P2Y₁₂ receptor stimulation (**Fig. 4**). The effect of apyrase on MRS2365-induced aggregation was less pronounced (MRS2365+ vehicle: 31.8 ±5.0% vs MRS2365 +apyrase 23.6 ±3.9%), suggesting the ability of MRS2365 to induce further

release of ADP that might then act on the P2Y₁₂ receptor was limited. Nevertheless, P2Y₁ receptor-dependent platelet aggregation was suppressed by PLC inhibition as has previously been extensively reported. However, neither Rac1 (**Fig. 4C**) nor ROCK inhibition (**Fig. 4D**) could suppress MRS2365 induced platelet aggregation, at concentrations that caused significant inhibition of platelet-induced PMN motility and platelet chemotaxis. We next examined whether suppression of these activities via inhibition of these signalling pathways were also platelet P2Y₁ receptor dependent.

3.5. Platelet-dependent PMN chemotaxis and platelet motility stimulated by P2Y₁ receptor activation is dependent on Rac1, and RhoA, but not PLC

We have previously reported that platelet-induced PMN motility could be suppressed by antagonism of P2Y₁, but not P2Y₁₂ receptors, and that this correlated with suppression of platelet RhoA activity. To exclude the possibility that P2Y₁ receptor stimulation by ADP might also lead to P2Y₁₂ receptor activation in these assays, we investigated platelet activity in the PINC assay, and separately, platelet chemotaxis under conditions with apyrase pre-treatment. Furthermore, we investigated the unexplored relationship of other RhoGTPase signalling following activation of P2Y₁ receptors on platelets. As expected, PLC inhibition had no effect on platelet-induced neutrophil motility (**Fig. 5A**) or platelet chemotaxis (**Fig. 5D**) when platelets were pre-incubated with a P2Y₁ receptor specific agonist (MRS2365). We next explored whether Rho GTPase signalling events for PINC, or platelet chemotaxis, were also specifically through P2Y₁ receptors by stimulating with MRS2365, whilst simultaneously quenching any effect of autologous ADP with the presence of apyrase. Thus, PINC in response to MDC (**Fig. 5A-C**) and platelet chemotaxis in response to fMLP (**Fig. 5D-F**), could be induced by stimulation of platelet P2Y₁ receptors. This was significantly suppressed by inhibition of Rac1 ($P < 0.05$, **Fig. 5B**; $P < 0.05$, **Fig. 5E**), and RhoA ($P < 0.05$, **Fig. 5C**; $P < 0.05$, **Fig. 5F**). Apyrase, which does not breakdown the specific P2Y₁ receptor agonist (MRS2365), had no effect on the ability to stimulate PINC or platelet chemotaxis, nor was this response reliant on the release of ADP from platelet dense granules (**Fig. 5**). However, PINC and platelet chemotaxis could be suppressed under

these conditions to basal levels via inhibition of Rac1 ($P < 0.05$, **Fig. 5B**; $P < 0.05$, **Fig. 5E**); or RhoA ($P < 0.05$, **Fig. 5C**; $P < 0.01$, **Fig. 5F**).

3.6. Comparison between ADP and MRS2365 responses and their molecular docking with the P2Y₁ receptor.

Platelet aggregation induced by MRS2365 was noticeably different compared to ADP (**Fig. 4**), whilst MRS2365 stimulation of platelets in the PINC assay, and platelet chemotaxis (**Fig. 5**), were similar or greater than platelet stimulation in response to ADP. Whilst we have reported the dependency for P2Y₁ receptor rather than the P2Y₁₂ receptor in these *in vitro* assays, and it is accepted that full platelet-induced aggregation by ADP involves both P2Y₁ and P2Y₁₂ receptors (**Fig. 6A & B**), the early phase (60 seconds) of platelet aggregation induced by ADP and the P2Y₁ receptor agonist MRS2365 was not inhibited by a selective P2Y₁₂ receptor antagonist (AR-C66096) (**Fig. 6A inset, & 6B**). Furthermore, MRS2365 induced aggregation of platelets was significantly inhibited by AR-C66096 at 10 mins (**Fig. 6B**), suggesting that MRS2365 had the ability to induce further ADP release from dense granules to account for a P2Y₁₂ receptor dependent component of platelet aggregation. Thus, despite the initial dependency of ADP-induced platelet aggregation via P2Y₁ receptor stimulation being similar in magnitude for both MRS2365 and ADP, the eventual scale of platelet aggregation was markedly different. In contrast, granule release of ADP did not have an effect on MRS2365-induced platelet chemotaxis or PINC (**Fig. 5**). As well as this function not being dependent on P2Y₁₂ receptor stimulation as a consequence of initial P2Y₁ receptor stimulation, we assumed the whole P2Y₁ receptor response in the motility assays was due to MRS2365 having 'full' agonistic properties similar to ADP in these RhoA and Rac1 dependent assays (rather an additive combination of MRS2365 induced ADP stimulation). We next investigated why MRS2365 and ADP might have comparatively different efficacies on the P2Y₁ receptor dependent function of ADP-induced platelet aggregation, in contrast to platelet chemotaxis and PINC based on their molecular docking with the P2Y₁ receptor. Whilst both ADP and MRS2365 bind to the orthosteric site of P2Y₁ receptors,

molecular docking showed that ADP bound to the ligand binding site with a ChemScore of 11.534 and a binding affinity of -15.89 Kcal/mole, while MRS2365 provided a lower ChemScore of 9.32 and a binding affinity of -14.45 Kcal/mole. Although both ligands occupied the ligand binding site, there were considerable differences in the interactions of the ligands with the key residues within the binding site. Both ADP and MRS2365 interacted with Arg195, Arg287, Thr205 and Arg310 (**Fig. 6C & E**). However, ADP formed hydrogen bonds with Thr206, Tyr303 and Gln50 (**Fig. 6D**), whereas electrostatic interaction was observed between MRS2365 and Arg128, whilst also forming hydrogen bonds with Lys41 (**Fig. 6F**). The differences in the interaction within the binding site can be partly explained by the recent observation of the molecular interactions of a structurally similar antagonist, MRS2500, with the P2Y₁ receptor (Yuan et al., 2016). These differences can potentially explain the differences in responses that were observed in the present studies for ADP and MRS2365.

4. Discussion

Platelets are able to carry out a range of functions that are now recognised as being important in both haemostasis and host defence against pathogens. Yet, whilst these biological processes are sometimes intertwined, the control of these cellular activities are distinct. We show here that the activation of platelets by ADP via activation of P2Y₁ receptors is necessary for platelet functions applicable to haemostasis (aggregation) and also host defence (platelet motility, and platelet induced neutrophil chemotaxis).

Whilst *in vitro* assays of platelet aggregation have been widely used to interpret pathways of platelet activation, *in vitro* assays that represent different platelet functions that occur *in vivo* can be used to elucidate non-classical platelet activation pathways pertinent to inflammation and host defence. For

example, two activities of platelets that are distinct from aggregation are the ability of platelets to migrate extravascularly in response to inflammatory stimuli (Pitchford et al., 2008; Kraemer et al., 2010), and the platelet P-selectin dependent intravascular crawling of leukocytes (neutrophils) as a requisite step of the leukocyte adhesion cascade (Sreeramkumar et al., 2014; Zuchtriegel et al., 2016). We have therefore adapted two *in vitro* assays that have been described elsewhere to measure these non-haemostatic functions of platelets. Thus, platelet motility has been reported with the use of transwell chemotaxis plates where platelet chemotaxis towards different chemotactic substances (fMLP, SDF-1 α) requires polarization of platelet membranes with actin polymerization and P-selectin expression to allow isolated focal platelet adhesion to vinculin and subsequent motility (Kraemer et al., 2010; Czapiga et al., 2005). We used fMLP as a platelet chemoattractant here because it has been reported to induce robust platelet chemotaxis and is pertinent to host defence being a constituent of bacterial cell walls, but has not been described to induce platelet aggregation (Czapiga et al., 2005). We have also investigated PINC, akin to *in vivo* neutrophil intravascular crawling, that has previously been optimized *in vitro* to investigate interactions between platelets and neutrophils at the single cell level under precise conditions that are platelet P-selectin dependent (Bengtsson et al., 1999; Kornerup et al., 2010; Amison et al., 2015), and using microfluidic assays (Frydman et al., 2017; Rainger et al., 1997). Here, we used MDC as the chemotactic substance, because it has been shown to activate platelets via the activation of CCR4 receptors, and is highly expressed during LPS induced neutrophilic inflammation with no associated thrombosis or changes to haemostasis (Kornerup et al., 2010; Amison et al., 2017b; Clemetson et al., 2000).

The apparent contradiction that activation of platelets by P2Y₁ receptors is implicated in the diverse platelet functions of platelet aggregation, motility, and neutrophil crawling, might be explained by our results presented here, whereby platelet activation by P2Y₁ receptor stimulation pertinent to

inflammation and host defence is involved in signalling via a non-canonical pathway(s) that does not involve PLC activity that leads to platelet aggregation. Indeed, as reported previously, the PLC inhibitor U73122 inhibited platelet aggregation at concentrations (1-10 μ M) achieved elsewhere (Bleasdale et al., 1990). Recent reports suggest U73122 also affects other signalling pathways, for example TRPM4 channels (Leitner et al., 2016), and inhibition of 5-lipoxygenase (Hornig et al., 2012). However, despite these recognised off-target effects of the U73122, our data make it apparent that only PLC-dependent platelet aggregation was inhibited by U73122, and not P1NC or platelet motility.

We show that platelet P2Y₁ receptor activation influences the signalling of Rho GTPases, specifically platelet RhoA and Rac1, in assays that involve co-stimulation with the inflammatory mediators, fMLP and MDC. We have previously reported a requirement for platelet P2Y₁ activation via RhoA signalling for leukocyte recruitment in a murine model of allergic inflammation (Amison et al., 2015). Here we report a requirement for platelet RhoA activity in mediating P2Y₁ receptor dependent platelet chemotaxis towards fMLP using the ROCK inhibitor GSK429286. We have also shown that platelet Rac1 activity is necessary for leukocyte recruitment in murine models of allergic and non-allergic inflammation that are dependent on P2Y₁ receptor activation (Pan et al., 2015; Amison et al., 2017b). Furthermore, we also report that the Rac1 inhibitor NSC23766 (1-10 μ M) suppressed two P2Y₁ receptor dependent functions, namely P1NC towards MDC, and platelet chemotaxis towards fMLP, but not ADP, or MRS2365 induced platelet aggregation. Thus, the effects of NSC23766 occurred at least 10 fold below off-target effects reported for NSC23766 on platelet function (Dutting et al., 2015).

It is interesting to note that the ROCK inhibitor (GSK429286) was less effective in inhibiting platelet chemotaxis compared to P1NC, whereas the Rac1 inhibitor (NSC23766) acted with similar potency in both assays, suggesting that it is plausible therefore that Rac1 activity is more important for

chemotaxis than RhoA. The potency of RhoA is more identifiable in the PINC, that we have previously reported to require a P-selectin cell-cell adhesion step (Amison et al., 2015). Whilst Rac1 has been reported to be essential for cell migration, and that RhoA signalling inhibits cell migration (Machacek et al., 2009), cell adhesion and cell polarization are both necessary for cell transit that require a sequential and repeated organisation such that Rho and Rac GTPases act in a complementary relationship (rather than in a strictly opposing manner) (Lawson and Burridge 2014).

Whilst the 'canonical' pathway of ADP-induced platelet activation via P2Y₁ receptor stimulation leading to aggregation occurs via PLC activity, we show here for the first time that inhibition of platelet PLC had no effect on platelet functions *in vitro* of relevance to inflammation and host defence. Furthermore, we have demonstrated that platelet Rho GTPase activity, stimulated by ADP-induced activation of platelet P2Y₁ receptors was not necessary for full aggregation responses to ADP, as reported elsewhere (Pleines et al, 2012; Soulet et al., 2005; Eckly et al., 2001; Aslan and McCarty 2013). We have also shown that it is possible for distinct molecular docking arrangements at the orthosteric site of P2Y₁ receptors, and in making comparisons between the efficacies of MRS2365 and ADP on different P2Y₁-dependent activities, suggests that the differences in the molecular interaction of these P2Y₁ receptor agonists with the receptor may affect these selective signalling events. Furthermore, by incubating platelets with aspirin, we show that the effect of ADP on platelet chemotaxis, and PINC is not dependent on ADP induced TXA₂ production and potential activation of TP receptors to signal via RhoA or Rac1 (Woodward et al., 2011). In agreement with this, the stable TXA₂ analogue U46619 was unable to stimulate platelet chemotaxis or PINC.

The scenario of P2Y₁ receptor signalling modulating platelet activation during inflammation may therefore, be distinct from the signalling involved in haemostasis and is suggestive of biased agonism. It has been hypothesized that P2Y receptors have this potential, as has been reported for other GPCRs (Jacobson et al., 2011; Kenakin et al., 2012), since biased agonism with several P2Y₁

receptor agonists (2MeSADP, MRS2365, and Ap4A) with distinct chemical structures had qualitatively different functional outcomes in experiments using P2Y₁ receptor expressing cell lines (Gao and Jacobson 2017). Indeed, independent ERK1/2 signalling mediated via β -arrestin2 and Gq proteins also suggest that distinct 'active' P2Y₁ receptor conformations are coupled to each effector (Wisler et al., 2014; Edelstein and Changeux 2016). There are several potential explanations for the results we have obtained above revealing biased signalling via RhoGTPases: 1. The activity of minor receptor signalling pathways appear 'silent' until the required function of the platelet (e.g. during inflammation) is influenced by the inflammatory milieu; 2. A direct cross-talk, or receptor heterodimerization may occur between the P2Y₁ receptor and other receptors involved in platelet functions involved in host defence, which might induce a biased signalling pathway, since P2Y₁ receptor has been shown to heterodimerize with other GPCRS (Yoshioka et al., 2001); 3. Biased signalling is dependent on the particular agonist used (Gao and Jacobson 2017). It is interesting to note that there is significant potential for ADP activation of platelets to directly stimulate non-canonical signalling pathways (Beck et al., 2017), and the inability of the TP receptor, itself acting via G protein Gq to stimulate platelet chemotaxis and PINC might be due to any one of these three (or more) reasons, whilst purinergic receptors have been shown to modulate chemotaxis of leukocytes, presumably because purinergic receptors contribute to cell polarization (Ferrari et al., 2016).

In the assays described here, ADP and the selective P2Y₁ agonist MRS2365 (Bourdon et al., 2006), were used to stimulate P2Y₁ receptors, and MRS2365 revealed a strong ability to induce platelet chemotaxis and PINC in comparison to ADP. Whilst these agonists interact with the orthosteric site of the P2Y₁ receptor in different modes, it is speculative to assume this might provide biased agonist and signalling properties. However, the circumstances of platelet activation via P2Y₁ receptors *in vivo* may be more complex. ADP is only one of several endogenous agonists for P2Y₁ receptors and it is not yet known whether these agonists would reveal a preferential downstream signalling pathway

during inflammation (e.g. Rho family GTPase signalling), or indeed if they are relevant to platelet activation (Jacobson et al., 2011a; Kenakin et al., 2012; Patel et al., 2001; Gustafsson et al 2011; Zhang et al., 2015).

In conclusion, we have demonstrated that platelet activation induced by ADP stimulation of P2Y₁ receptors can elicit distinct signalling pathways leading to different physiological functions of platelets, PLC activation leading to aggregation, but not motility or interactions with neutrophils; and Rho-GTPase activation leading to motility and interactions with neutrophils, but not aggregation. This is suggestive of functional selectivity or a 'biased agonism' at P2Y₁ receptors.

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6. Author contributions

RA, KR, CP, SP were involved with the conception, hypotheses delineation, and design of the study. RA, SP, SJ, KR were involved with the acquisition of the data, analysis and interpretation of the data. SP wrote the article. All authors were involved with manuscript revision prior to submission. There are no conflicts of interest to declare.

Figure Legends**Figure 1. PLC inhibition but not RhoGTPase inhibition suppresses platelet aggregation towards**

ADP. Platelet rich plasma (PRP) was harvested from the peripheral blood of healthy donors and were incubated for 10 mins with inhibitors of PLC (U73122, **A**), Rac1 (NSC23766, **B**), or ROCK (GSK429286, **C**). Platelet aggregation towards 10 μ mol/LM ADP was then recorded over a 10 minute period. Traces are displayed for the whole period, and percentage aggregation portrayed in each histogram. Data = means $\pm$ S.E.M. n= 6 per group. *P<0.05 vs resting PRP; #P<0.05 vs ADP stimulated platelets.

Figure 2. Inhibition of activity of RhoGTPases but not PLC activity suppresses platelet-induced

neutrophil chemotaxis and platelet motility induced by ADP. Platelet rich plasma (PRP) was harvested from the peripheral blood of healthy donors, washed and subsequent platelets incubated for 10 mins with inhibitors of PLC (U73122, **A & D**), Rac1 (NSC23766, **B & E**), or ROCK (GSK429286, **C & F**). Platelets were washed and then stimulated with 100nmol/L ADP before mixture with PMNs (purified from peripheral blood). PMN chemotaxis towards MDC (100nmol/L) was analysed as a chemotactic index (**A-C**). Or, platelets were washed and then stimulated with 100nmol/L ADP before chemotaxis towards fMLP (30nmol/L) was analysed as a chemotactic index (**D-F**). Data: means $\pm$ S.E.M. n= 5 per group (B), or 6 per group (A, C-F). A-C: *P< 0.05 vs column 3, or #P< 0.05 vs column 4; and D-F: *P< 0.05 vs negative control; or #P< 0.05 vs fMLP in the absence of inhibitors.

Figure 3. Inhibition of TXA₂ synthesis and release, or activation of platelets by TXA₂ analogue

(U46619) did not affect platelet-induced neutrophil chemotaxis and platelet motility. Platelet rich plasma (PRP) was harvested from the peripheral blood of healthy donors, washed and subsequent platelets incubated for 30 mins with aspirin (1mmol/L) before stimulation with 100nmol/L ADP (**A,B**), or U46619 (1-100nmol/L. **C,D**) and platelet chemotaxis towards fMLP (30nmol/L) (**A, C**); or platelets washed again then mixed with PMNs (purified from peripheral blood) and PMN chemotaxis towards

MDC (100nmol/L. **B,D**) were analysed as a chemotactic index. In other experiments, platelet aggregation towards U46619 (1 μ mol/L), ADP (10 μ mol/L) or MRS2365 (1 μ mol/L) was measured after incubation for 30 mins with aspirin (1mmol/L) or vehicle (**E**). Data: means $\pm$ S.E.M. n= 5 per group (D), or 6 per group (A-C, E). A-E: *P<0.05 and ***P< 0.001 vs column 1, or E ###P< 0.001 vs stimulation with ADP in the absence of aspirin.

Figure 4. Inhibition of PLC activity, but not RhoGTPase activity, suppresses P2Y₁ mediated platelet aggregation. Platelet rich plasma (PRP) was harvested from the peripheral blood of healthy donors and were stimulated with increasing concentrations of MRS2365 **A**) or incubated for 10 mins with inhibitors of PLC (U73122, **B**), Rac1 (NSC23766, **C**), or ROCK (GSK429286, **D**). Platelet aggregation towards 10 μ mol/L ADP or 1 μ mol/L MRS2365 (P2Y₁ agonist) was then recorded over a 10 minute period. In annotated groups, platelets were incubated with apyrase (1U/ml). Traces are displayed for the whole period, and percentage aggregation portrayed in each histogram. Data = means $\pm$ S.E.M. n= 5 per group (A), or 6 per group (B-D). *P<0.05 vs resting PRP; #P<0.05 where indicated.

Figure 5. Inhibition of activity of RhoGTPases, but not PLC activity, suppresses P2Y₁-dependent platelet-induced neutrophil chemotaxis or platelet motility. Platelet rich plasma (PRP) was harvested from the peripheral blood of healthy donors, washed and subsequent platelets incubated for 10 mins with inhibitors of PLC (U73122, **A & D**), Rac1 (NSC23766, **B & E**), or ROCK (GSK429286, **C & F**). Platelets were washed and then stimulated with 100nmol/L ADP or 1 μ mol/L MRS2365 (P2Y₁ agonist) and in annotated groups, platelets were incubated with apyrase (1U/ml); before mixture with PMNs (purified from peripheral blood), and the chemotaxis of these PMNs towards MDC (100nmol/L) (A-C), or platelet chemotaxis towards fMLP (30nmol/L) (D-F). Chemotaxis was analysed as a chemotactic index. Data: means $\pm$ S.E.M. n= 5 per group (A, C, E, F), or 6 per group (B, D). A-C:

*P<0.05 vs column 3; or #P< 0.05 where indicated. D-F: *P< 0.05 vs negative control; or #P< 0.05 vs fMLP in the absence of inhibitors.

Figure 6. Comparison of ADP and MRS2365 activity on platelet aggregation, and their molecular docking characteristics with the P2Y₁ receptor. Platelet rich plasma (PRP) was harvested from the peripheral blood of healthy donors, washed and incubated for 10 mins with AR-C66096 (1μmol/L) before stimulation with ADP or MRS2365 and aggregation recorded for 10 mins (**A**. Inset: response up to 60 seconds), and percentage aggregation measured at 60 seconds and 10 mins (**B**). Three-dimensional model of ADP in the ligand binding site of P2Y₁ (**C**), Two-dimensional model showing the interactions of ADP with the key residues within the binding site (**D**). Three-dimensional model of MRS2365 in the ligand binding site of P2Y₁ (**E**), Two-dimensional model showing the interactions of MRS2365 with the key residues within the binding site (**F**). Data: means ± S.E.M. n=5 per group. *P< 0.05 vs no stimulation (white bar); #P< 0.05 vs MRS2365 or ADP in absence of AR-C66096; ^P<0.05 as indicated.

Supplementary Figure 1. Effect of exogenously applied ADP or MRS2365 on platelet-induced neutrophil chemotaxis, and platelet motility. Platelet rich plasma (PRP) was harvested from the peripheral blood of healthy donors, washed and subsequent platelets were stimulated with 100nmol/L ADP or 1μmol/L MRS2365 (P2Y₁ agonist); before mixture with PMNs (purified from peripheral blood), and the chemotaxis of these PMNs towards MDC (100nmol/L) (**A**), or platelet chemotaxis towards fMLP (30nmol/L) (**B**). Chemotaxis was analysed as a chemotactic index. Data: means ± S.E.M. n= 5 per group. (**A**): ***P<0.001 vs as indicated (**B**): **P<0.01, ***P<0.001 vs groups without fMLP, or ##P< 0.01 vs PBS+fMLP group (column 2).

Supplementary Figure 2. Effect of P2Y₁ and P2Y₁₂ receptor antagonists on platelet motility towards

fMLP. Platelet rich plasma (PRP) was harvested from the peripheral blood of healthy donors, washed and subsequent platelets were stimulated with (A) 100nmol/L ADP or (B) 1μmol/L MRS2365 (P2Y₁ agonist); in the presence or absence of P2Y₁ antagonist MRS2500 (1μM) or P2Y₁₂ antagonist AR-C66096 (11μM) before chemotaxis towards fMLP (30nmol/L). Chemotaxis was analysed as a chemotactic index. Data: means ± S.E.M. n= 5 per group. *P<0.05, ***P<0.001 vs column 1: #P<0.01, ##P<0.01 vs column 2.

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Figure 1

A PLC inhibitor

B Rac1 inhibitor

C ROCK inhibitor

Figure 2

Figure 3

Figure 4

A

B PLC inhibitor

C Rac1 inhibitor

D ROCK inhibitor

Figure 5

A PLC inhibitor

B Rac1 inhibitor

C ROCK inhibitor

D PLC inhibitor

E Rac1 inhibitor

F ROCK inhibitor

Figure 6