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RESEARCH PAPER

P2Y$_{12}$ receptor modulation of ADP-evoked intracellular Ca^{2+} signalling in THP-1 human monocytic cells

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BACKGROUND AND PURPOSE

The G$_i$-coupled, ADP-activated P2Y$_{12}$ receptor is well characterized as playing a key role in platelet activation *via* crosstalk with the P2Y$_1$ receptor in ADP-evoked intracellular Ca^{2+} responses. However, there is limited knowledge on the role of P2Y$_{12}$ receptors in ADP-evoked Ca^{2+} responses in other blood cells. Here, we investigated the role of P2Y$_{12}$ receptor activation in the modulation of ADP-evoked Ca^{2+} responses in human THP-1 monocytic cells.

EXPERIMENTAL APPROACH

A combination of intracellular Ca^{2+} measurements, RT-PCR, immunocytochemistry, leukocyte isolation and siRNA-mediated gene knockdown were used to identify the role of P2Y$_{12}$ receptor activation.

KEY RESULTS

ADP-evoked intracellular Ca^{2+} responses (EC$_{50}$ 2.7 μM) in THP-1 cells were abolished by inhibition of PLC (U73122) or sarco/endoplasmic reticulum Ca^{2+}-ATPase (thapsigargin). Loss of ADP-evoked Ca^{2+} responses following treatment with MRS2578 (IC$_{50}$ 200 nM) revealed a major role for P2Y$_6$ receptors in mediating ADP-evoked Ca^{2+} responses. ADP-evoked responses were attenuated either with pertussis toxin treatment, or P2Y$_{12}$ receptor inhibition with two chemically distinct antagonists (ticagrelor, IC$_{50}$ 5.3 μM; PSB-0739, IC$_{50}$ 5.6 μM). ADP-evoked responses were suppressed following siRNA-mediated P2Y$_{12}$ gene knockdown. The inhibitory effects of P2Y$_{12}$ antagonists were fully reversed following adenylate cyclase inhibition (SQ22536). P2Y$_{12}$ receptor expression was confirmed in freshly isolated human CD14$^+$ monocytes.

CONCLUSIONS AND IMPLICATIONS

Taken together, these data suggest that P2Y$_{12}$ receptor activation positively regulates P2Y$_6$ receptor-mediated intracellular Ca^{2+} signalling through suppression of adenylate cyclase activity in human monocytic cells.

Abbreviations

IP$_3$, inositol 1,4,5-triphosphate; PIP$_2$, phosphatidylinositol 4,5-bisphosphate; UDP, uridine diphosphate

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Introduction

The adenine nucleotide **ADP** serves as an important signalling molecule that is implicated in processes such as platelet aggregation (Dorsam and Kunapuli, 2004) and immune modulation (Ben Addi *et al.*, 2010). ADP binds to **$P2Y_1$**, **$P2Y_6$**, **$P2Y_{12}$** and **$P2Y_{13}$** receptors, which are members of the G protein-coupled P2Y receptor family. Activation of G_q-coupled $P2Y_1$ and $P2Y_6$ receptors causes downstream inositol 1,4,5-triphosphate (IP_3) generation *via* PLC-mediated breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) and subsequent calcium ion (Ca^{2+}) release through endoplasmic reticulum IP_3 receptors (Erb and Weisman, 2012). Conversely, $P2Y_{12}$ and $P2Y_{13}$ receptors are G_i-coupled and hence inhibit production of cAMP and stimulate PI3K when activated (Erb and Weisman, 2012).

Platelet aggregation in response to ADP is one of the earliest examples of extracellular nucleotide signalling. It is firmly established that ADP activates platelets *via* the $P2Y_1$ and $P2Y_{12}$ receptors, and the functionality of both receptors is required for normal platelet aggregation (Jin and Kunapuli, 1998). The emergence of the $P2Y_{12}$ receptor as a vital component in ADP-evoked platelet activation has led to the development of anti-platelet drugs targeting this receptor, such as **ticagrelor** and **clopidogrel**. The rise in cytosolic Ca^{2+} in response to ADP is deemed responsible for the ADP-mediated activation of platelets (Dorsam and Kunapuli, 2004). Although the $P2Y_{12}$ receptor is G_i-coupled and should therefore not cause intracellular Ca^{2+} mobilization, outcomes from a previous study suggest that the $P2Y_{12}$ receptor potentiates the G_q $P2Y_1$ receptor-mediated Ca^{2+} response to ADP in a crosstalk mechanism (Hardy *et al.*, 2004). Hardy *et al.* (2004) proposed that $P2Y_{12}$ receptors mediate inhibition of **adenylate cyclase** and activation of PI3K, which collectively positively modulates the Ca^{2+} signal induced by ADP. The same $P2Y_1$-$P2Y_{12}$ crosstalk mechanism described in platelets by Hardy *et al.* (2004) was also suggested to be present in glioma C6 cells (Suplat *et al.*, 2007).

The role of $P2Y_{12}$ receptors in cells other than platelets is not well described, although recent evidence is promising. Findings by West *et al.* (2014) indicate a role for vessel wall $P2Y_{12}$ receptors in early atherogenesis, rather than platelet $P2Y_{12}$ receptors. In addition to cardiovascular disease, the $P2Y_{12}$ receptors has potentially been implicated in immune responses to ADP in macrophages (Zhang *et al.*, 2018) and dendritic cells (Ben Addi *et al.*, 2010) in functions such as antigen uptake and chemotaxis. Therefore, non-platelet roles for $P2Y_{12}$ receptors have been suggested and should be further investigated.

Monocytes are essential immune cells that, together with their progeny, facilitate innate immune defence *via* phagocytosis and cytokine production, but also activate the adaptive immune system through antigen uptake and presentation (Ziegler-Heitbrock, 2006). In this study, we applied the THP-1 monocytic cell line as an experimental model to investigate the expression of $P2Y_{12}$ receptors and the contribution of the receptor in ADP-evoked Ca^{2+} responses, exploring the signal transduction mechanisms involved. As there have been no publications reporting a role for $P2Y_{12}$ receptors in monocytes, this investigation reveals a new role for $P2Y_{12}$ receptors in non-platelet Ca^{2+} responses and contributes to our understanding of how monocytes function in health and disease.

Methods

Isolation of $CD14^+$ human monocytes

Peripheral blood mononucleated cells (PBMCs) were isolated from the blood of human volunteers using Histopaque-1077 (Sigma-Aldrich, Haverhill, UK). CD14+ monocytes were magnetically labelled from a PBMC suspension using MACS CD14 MicroBeads (Miltenyi Biotec, Germany) and positively selected for *via* the MACS Cell Separation Column (Miltenyi Biotec, Germany) together with the QuadroMACS Separator (Miltenyi Biotec, Germany).

Intracellular Ca^{2+} measurements and drug treatments

THP-1 cells, $1 \times 10^6 \cdot mL^{-1}$ were loaded for 1 h with 2 μM fura-2 AM in SBS buffer plus 0.01% ($w \cdot v^{-1}$) pluronic acid at 37°C. Cells were then pelleted and washed using SBS and plated at a density of 2×10^5 cells per well. The plated cells were allowed to settle for 1 h at 37°C, during which stage antagonists were added 30 min into the hour, unless otherwise stated. Measurements were taken at 37°C using the FlexStation 3 instrument (Molecular Devices, Wokingham, UK) measuring fura-2 fluorescence (340 nm excitation when Ca^{2+}-bound; 380 nm excitation when unbound; 510 nm emission) at intervals of 2 s giving. For all Ca^{2+} experiments, the signal reported is 'F ratio', the ratio between Fura-2 emission at 510 when excited at 340 and 380 nm. For **hexokinase**-treated ADP stocks, 1 mM ADP in SBS solution was treated for 1 h at 37°C with 3 $U \cdot mL^{-1}$ hexokinase from *Saccharomyces cerevisiae* (Sigma, Haverhill, UK) prior to agonist application.

Cell culture

Cells from the THP-1 cell line were cultured at 37°C, 5% CO_2 in RPMI 1640 medium containing 2 mM L-glutamine and supplemented with 10% ($v \cdot v^{-1}$) FBS, 50 $IU \cdot mL^{-1}$ penicillin and 50 $\mu g \cdot mL^{-1}$ streptomycin. Cells were maintained at a density between 1×10^5 and 1×10^6 cells $\cdot mL^{-1}$.

siRNA-mediated gene knockdown

THP-1 cells (2×10^5 final amount) were incubated overnight in complete RPMI (10% FBS) without antibiotic before cells were transfected using Dharmacon siRNA (25 nM final concentration) *via* DharmaFECT 2 transfection reagent (obtained from Dharmacon Research, Inc., Cambridge, UK) using the manufacturer's protocol in 96-well format.

RNA extraction and RT-PCR

Total RNA was extracted from THP-1 cells and $CD14^+$ monocytes using Tri reagent (Sigma Aldrich, Haverhill, UK) with a subsequent DNase I treatment (Ambion). Complementary DNA was synthesized from 1 μg of total RNA using Superscript II reverse transcriptase (Invitrogen, Waltham, USA). PCR was performed using a Taq polymerase readymix (Sigma Aldrich, Haverhill, UK) using primer pairs designed using the following sequences (accession numbers): *P2RY1* - GTTCAA

TTTGGCTCTGGCCG (5′-3′), TTTTGTTTTTGCGGACCCCG (3′-5′) (NM_002563); *P2RY6* - GCTCTCACTGTCATCGGCTT (5′-3′), TCTGCCATTTGGCTGTGAGT (3′-5′) (NM_176798); *P2RY12* - ACTGGGAACAGGACCACTGA (5′-3′), CAGAATTGGGGCACTTCAGC (3′-5′) (NM_022788); *P2RY13* - TTCCCAGCCCTCTACACAGT (5′-3′), GGCCCCTTTAAGGAAGCACA (3′-5′) (NM_176894).

Immunocytochemistry

THP-1 cells adhered to glass coverslips were washed twice in PBS followed by fixative with 4% (wv^{-1}) paraformaldehyde. Cells were permeabilized with 0.25% (vv^{-1}) triton X-100 for 10 min followed by blocking with 1% (wv^{-1}) BSA for 30 min at room temperature. Primary and secondary antibodies were diluted in PBS containing 1% (wv^{-1}) BSA and incubated with cells overnight a 4°C and for 1 h at room temperature respectively. Cells were mounted in Vectashield containing nucleus counterstain (DAPI). Goat polyclonal anti-$P2Y_{12}$ (Santa Cruz Biotechnology, Texas, USA) was used with Alexa 488-conjugated rabbit anti-goat (Abcam, Cambridge, UK). Rabbit polyclonal anti-$P2Y_1$, anti-$P2Y_6$ and anti-$P2Y_{13}$ (Alomone, Jerusalem, Israel) were used with Alexa 488-conjugated goat anti-rabbit (Invitrogen, Waltham, USA). Cell imaging was performed using a laser-scanning Zeiss LSM510 Meta confocal microscope.

Transmigration assays

Transwell migration assay was performed as previously described (Sivaramakrishnan *et al.*, 2012; Campwala *et al.*, 2014). Briefly, assays were performed in 24-well plates using polyethylene terephthalate membrane transwell inserts with 3 μm pores. THP-1 cells, 1×10^6 in RPMI (no serum) with vehicle or drug treatment were added to the upper chamber, and 3 μM ADP or vehicle added to the lower chamber. Assays were performed for 2 h at 37°C, and migrated cells counted on the underside of the transwell support using crystal violet staining. Chemotaxic index was calculated as the ratio of cells that migrated to ADP over vehicle control.

Data analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). Data analysis was performed using Origin Pro 9.0 software (Origin Lab, USA). Dose–response curves were fitted assuming a Hill coefficient of 1, with the Hill equation used to determine the degree of ligand–receptor cooperation. Figure data points represent mean values ± SEM (error bars). Statistical significance was determined using Student's paired *t*-tests. Each point in the dose–response plots represents the average of the peak Ca^{2+} response. A confidence interval of 5% ($P < 0.05$) is used throughout for statement of significance.

Dose–response relationships were fitted using the Hill equation:

$$response\ (y) = \frac{(\max - \min)}{1 + 10^{(\mathrm{Log\ EC50} - X)^{nH}}}$$

where n_H represents the Hill slope.

Chemicals and reagents

Unless otherwise stated, all chemicals and reagents were purchased from Sigma-Aldrich (Haverhill, UK), with the exception of *pertussis toxin* and SQ22536 (Tocris, Bristol, UK). All chemicals (agonists and antagonists) were diluted using physiological saline (SBS buffer) (containing (mM): NaCl, 130; KCl, 5; $MgCl_2$, 1.2; $CaCl_2$, 1.5; D-glucose, 8; HEPES, 10; pH 7.4) with the exception of **CCL2** [SBS buffer containing 1% (wv^{-1}) BSA], **MRS2578**, SQ22536 and ticagrelor [SBS buffer containing 1% (vv^{-1}) DMSO].

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a,b).

Results

ADP-evoked intracellular Ca^{2+} responses are mediated by $P2Y_6$ receptor activation and modulated by G_i-dependent signalling

ADP evoked concentration-dependent increases in intracellular Ca^{2+} with an EC_{50} of 2.7 ± 0.3 μM (n = 5) (Figure 1A, B). Responses to maximal ADP concentrations were abolished following either PLC inhibitor treatment (**U73122**) (Figure 1C) or calcium depletion of the ER store (**thapsigargin**) (Figure 1 D). A study by Mahaut-Smith *et al.* (2000) identified that commercially available ADP contains some ATP. To exclude any contribution of trace ATP to ADP-evoked Ca^{2+} responses, ADP stocks were treated with hexokinase. The Ca^{2+} responses evoked by treated ADP were similar to untreated ADP, with an EC_{50} of 2.2 ± 0.5 μM (n = 5) ($P > 0.05$ vs. untreated ADP) (See Supporting Information Figure S1).

$P2Y_6$ antagonism by MRS2578 inhibited the ADP response almost completely with an EC_{50} of 200 ± 20 nM (n = 3) (Figure 1E, F**)**. MRS2578 inhibited the ADP response in a non-competitive fashion (Figure 1G), consistent with the reported mode of antagonism at $P2Y_6$ receptors (Mamedova *et al.*, 2004) and with previous findings (Sivaramakrishnan *et al.*, 2012; Campwala *et al.*, 2014). Selective inhibition of $P2Y_1$ receptors with **MRS2500** (tested up to 10 μM) (Figure 1H) or $P2Y_{13}$ receptors with **MRS2211** (tested up to 10 μM) (Figure 1I) had no significant effect on ADP- evoked Ca^{2+} responses. In addition to a dependence on PLC activity and release of ER Ca^{2+} stores, we investigated the effect of G_i-dependent signalling by using *Bordetella pertussis* toxin (PTx). PTx treatment (5 nM, 3 h) caused a significant attenuation of ADP-evoked intracellular Ca^{2+} responses (Figure 2A), significantly suppressing the maximal response by approximately 30% and the EC_{50} for ADP (EC_{50} 1.9 ± 0.3 μM vs. 2.9 ± 0.2 μM with PTx; $P < 0.05$, n = 5) (Figure 2B). In control experiments, CCL2-evoked Ca^{2+} responses were abolished by PTx treatment (Figure 2C), in agreement with our previous observations. Together, these findings suggest that ADP-evoked Ca^{2+} responses are mediated *via* $P2Y_6$ receptor activation and release of ER Ca^{2+} and that G_i-dependent signalling either constitutively or following ADP activation, positively modulates the ADP-evoked response.

Figure 1

$P2Y_6$ receptor mediates ADP-evoked intracellular Ca^{2+} responses in THP-1 cells. (A) Averaged ($n = 5$) intracellular Ca^{2+} response evoked by ADP (30 μM). (B) Concentration-dependency of ADP-evoked responses (EC_{50} 2.7 ± 0.3 μM; $n = 5$). Abolition of responses evoked by 30 μM ADP in control conditions (closed circles) or following pre-incubation (open circles) with 5 μM U73122 (C) or 1 μM thapsigargin (D); $n = 5$ for both. (E) ADP concentration-response curve in the absence (closed circles) and presence (open circles) of 300 nM MRS2578 ($n = 5$). (F) Concentration-inhibition curve for $P2Y_6$ antagonist MRS2578 on intracellular Ca^{2+} response evoked by ADP (3 μM; $n = 5$). (G) ADP concentration-response curve in the absence (closed circles) and presence (open circles) of 300 nM MRS2578 ($n = 5$). (H and I) Averaged ($n = 5$) intracellular Ca^{2+} responses evoked by 3 μM ADP in the presence of vehicle (closed circles) or in the presence of $P2Y_1$ antagonist 1 μM MRS2500 (H; open circles) or $P2Y_{13}$ antagonist 10 μM MRS2211 (I; open circles). For all experiments, *F* ratio is the ratiometric measurement of intracellular Ca^{2+} using fura-2. $^*P < 0.05$.

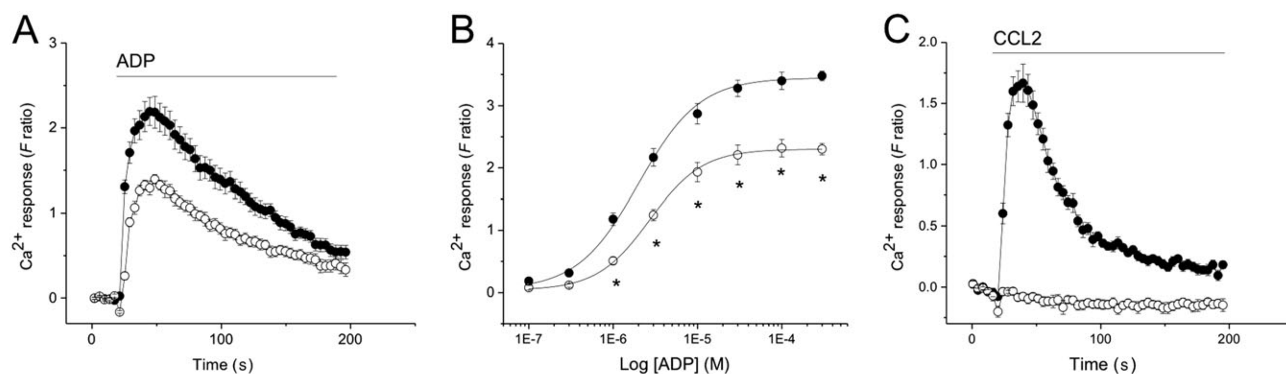

Figure 2

Pertussis toxin attenuates ADP-evoked intracellular Ca^{2+} responses in THP-1 cells. (A) Averaged ($n = 5$) intracellular Ca^{2+} response evoked by ADP (3 μM) in the absence (closed circles) and presence (open circles) of 5 nM pertussis toxin (3 h pre-incubation). (B) ADP concentration–response relationship in absence and presence of 5 nM pertussis toxin ($n = 5$). (C) Positive control showing abolition of control CCL2-evoked response by 5 nM pertussis toxin ($n = 5$). Responses evoked by 50 ng·mL^{-1} CCL2. For all experiments, *F* ratio is the ratiometric measurement of intracellular Ca^{2+} using fura-2. $^*P < 0.05$.

We next determined the expression of ADP receptors in THP-1 cells in an effort to probe further the molecular basis of ADP-evoked Ca^{2+} responses and identify receptors that may modulate them. RT-PCR analysis of P2Y receptors activated by ADP (Communi *et al.*, 1996; Erb and Weisman, 2012) in THP-1 cells revealed the expression of $P2Y_1$, $P2Y_6$, $P2Y_{12}$ and $P2Y_{13}$ receptors (Figure 3A). $P2Y_{12}$ receptor mRNA expression was confirmed in freshly isolated $CD14^+$ monocytes (Figure 3B). $P2Y_1$, $P2Y_6$, $P2Y_{12}$ and $P2Y_{13}$ receptor protein expression was confirmed by immunocytochemistry and confocal microscopy (Figure 3C).

Effect of $P2Y_{12}$ receptor antagonism and knockdown on ADP-evoked Ca^{2+} responses in THP-1 cells

Of the ADP receptors expressed by THP-1 monocytes, the $P2Y_{12}$ receptor is a known G_i-coupled receptor. The role of $P2Y_{12}$ receptor activation during ADP challenge was investigated using **PSB-0739**, a high affinity competitive antagonist (Baqi *et al.*, 2009), and ticagrelor, a $P2Y_{12}$ antagonist used clinically as an anti-thrombotic agent (Husted *et al.*, 2006). Ticagrelor inhibited ADP-evoked Ca^{2+} responses in THP-1 cells in a concentration-dependent fashion (Figure 4A). Ticagrelor inhibited ADP-evoked Ca^{2+} responses with an EC_{50} of 4.7 ± 1.8 μM (*n* = 5) and a non-competitive mode of action (Figure 4B, C). This is consistent with the observations of van Giezen *et al.* (2009) where ticagrelor binds to $P2Y_{12}$ at a site distinct from that of ADP, producing a non-competitive inhibition of ADP-induced aggregation in human platelets. PSB-0739 inhibited ADP-evoked Ca^{2+} responses with an EC_{50} of 5.4 ± 1.8 μM (*n* = 5) (Figure 4D, E) and caused a rightward parallel shift in the ADP concentration–response curve (Figure 4F). Inhibition of the ADP response was approximately 80% at the highest concentrations of PSB-0739 tested. Several studies have suggested that ticagrelor produces cardiovascular benefit through a pleiotropic inhibition of the equilibrative nucleoside transporter 1 (ENT-1) (Aungraheeta *et al.*, 2016), and a consequent elevation in extracellular adenosine. To rule out this mechanism of action in THP-1 cells, we inhibited ENT-1 using 3 μM 6-*S*-[(4-nitrophenyl)methyl]-6-thioinosine (**NBMPR**), but observed no effect on ADP-evoked Ca^{2+} responses (data not shown). Next, we undertook a gene knockdown strategy to support our observations with $P2Y_{12}$ receptor antagonists. siRNA targeting of $P2Y_{12}$ receptors or control gene GADPH achieved approximately 40% mRNA knockdown versus scrambled siRNA control cells. ADP-evoked

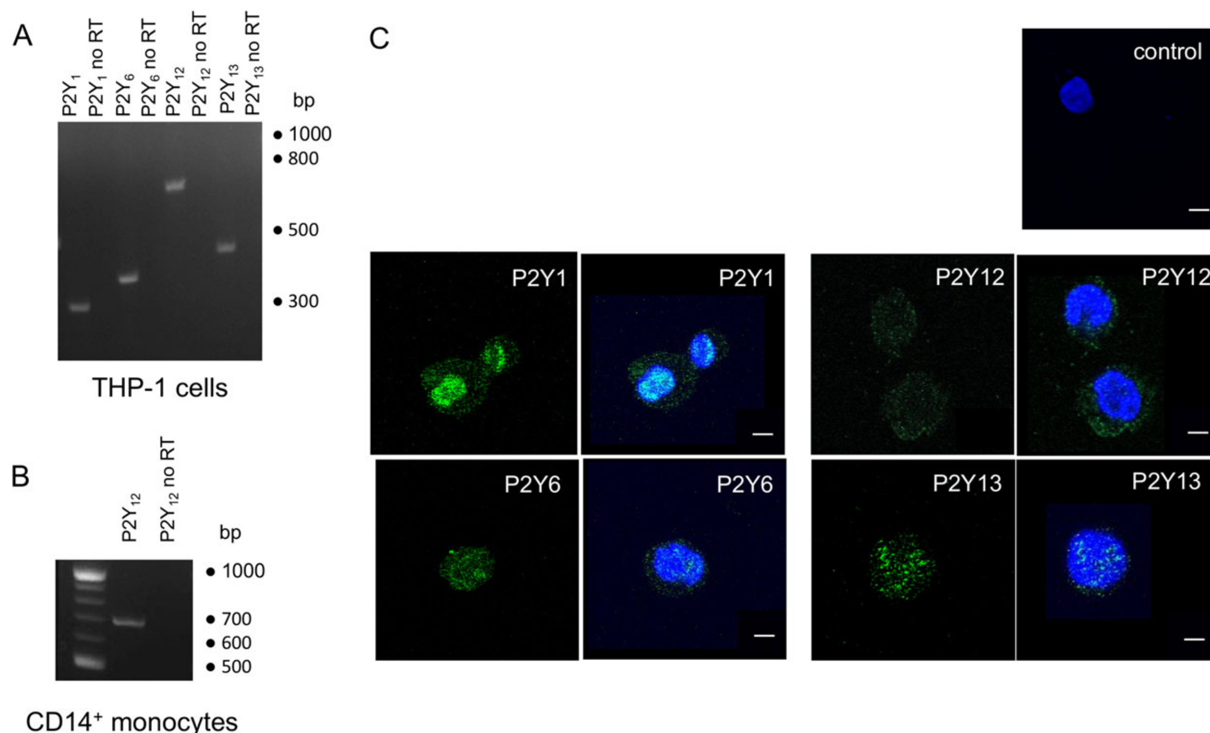

Figure 3

Expression of ADP-activated P2Y receptors in THP-1 cells. (A) RT-PCR analysis of $P2Y_1$ (326 bp), $P2Y_6$ (391 bp), $P2Y_{12}$ (698 bp) and $P2Y_{13}$ (461 bp) receptor expression in THP-1 monocytes. RT-PCR analysis of $P2Y_{12}$ receptors (698) expression in freshly isolated $CD14^+$ monocytes from human peripheral blood. For (A) and (B): predicted PCR ampilicon size given in parentheses; no RT (no reverse transcriptase) denotes negative control experiments for genomic DNA contamination. (C) Representative confocal microscopy images showing P2Y primary antibody immunoreactivity in fixed THP-1 cells. Cells are labelled with polyclonal antibodies against P2Y receptor subunits and fluorescence (green) visualized by using a AF488-conjugated secondary antibody (lefthand panels). The "control" is representative of an experiment where primary antibodies have been omitted. Cells are counterstained with diamidino-2-phenylindole to identify nuclei (blue; in overlay in righthand panel). Scale bar is 5 μm. Experiments are representative of at least three independent experiments.

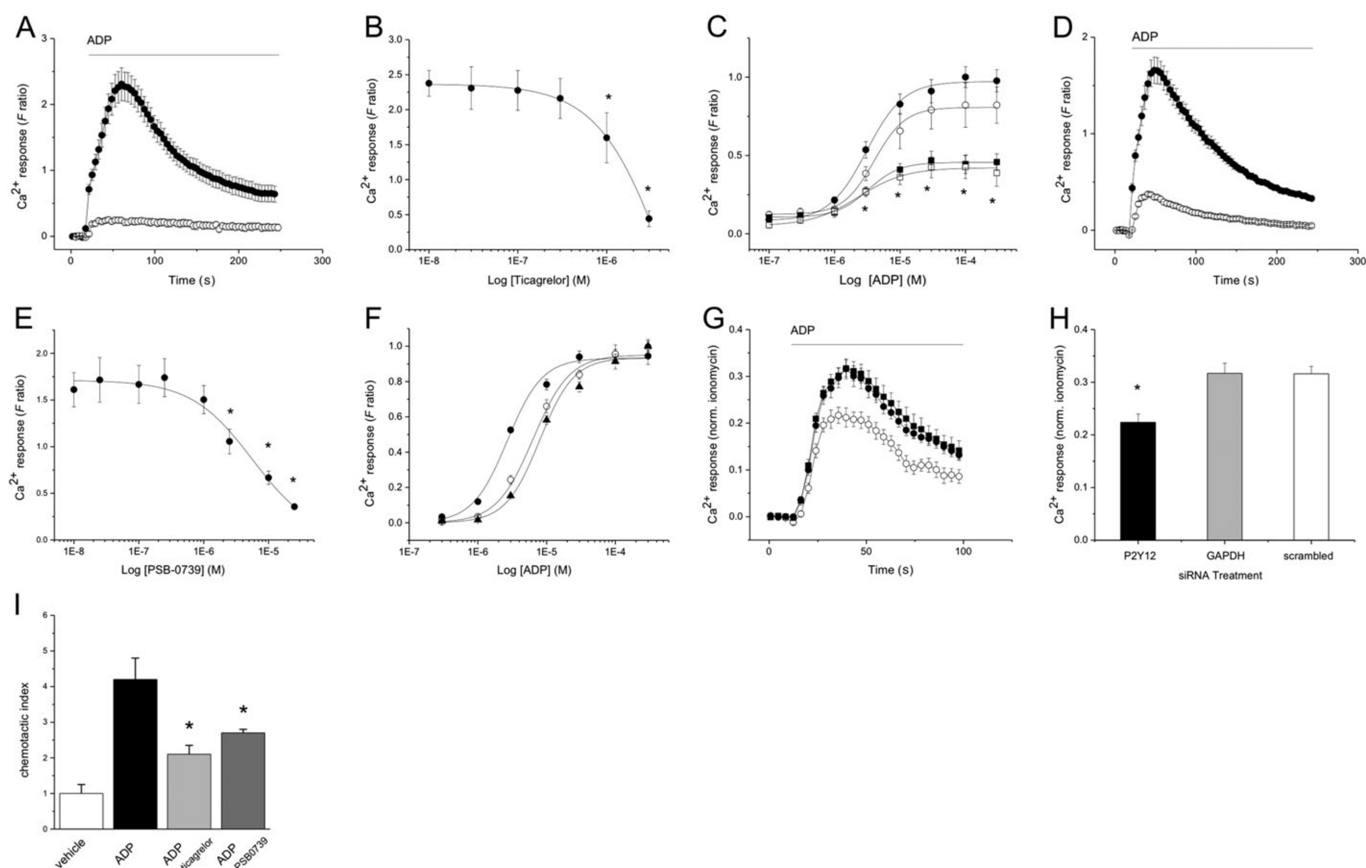

Figure 4

Effect of $P2Y_{12}$ receptor antagonism and gene knockdown on ADP-evoked intracellular Ca^{2+} responses in THP-1 cells. (A) Averaged (*n* = 5) ADP-evoked Ca^{2+} response in the absence (closed circles) and presence (open circles) of 3 μM ticagrelor. (B) Ticagrelor concentration-inhibition curve (IC_{50} 4.7 ± 1.8 μM; *n* = 5) for responses evoked by 3 μM ADP. (C) ADP concentration–response curve (*n* = 5) in the presence of vehicle (closed circles) or 0.1 (open circles), 1 (closed squares) and 5 μM ticagrelor (open squares). (D) Averaged (*n* = 5) ADP-evoked Ca^{2+} response in the absence (closed circles) and presence (open circles) of 25 μM PSB-0739. (E) PSB-0739 concentration-inhibition curve (IC_{50} 5.4 ± 1.8 μM; *n* = 5) for responses evoked by 3 μM ADP. (F) ADP concentration response curve (*n* = 4) in the presence of vehicle (closed circles) or 5 (open circles) and 10 μM PSB-0739 (triangles). (G) Effect of siRNA-mediated silencing of $P2Y_{12}$ receptors on ADP-evoked Ca^{2+} responses. Averaged (*n* = 5) Ca^{2+} responses evoked by 3 μM ADP in THP-1 cells following siRNA-mediated mRNA knockdown of $P2Y_{12}$ (open circles) or GAPDH (closed squares) compared with cells transfected with scrambled siRNA (closed circles) (*n* = 5). (H) Bar chart showing effect of different siRNA treatment on peak Ca^{2+} responses evoked by 3 μM ADP (*n* = 5). Responses in panels G and H are normalized to the magnitude of Ca^{2+} response elicited by 100 μM ionomycin to control for cell number. For all experiments, *F* ratio is the ratiometric measurement of intracellular Ca^{2+} using fura-2. *$P < 0.05$. (I) $P2Y_{12}$ antagonists attenuate THP-1 transwell migration towards ADP. Chemotactic indexes comparing cell movement over 2 h in control conditions (vehicle), 3 μM ADP alone or in the presence of either ticagrelor (3 μM) or PSB-0739 (25 μM) (*$P < 0.05$ vs. ADP alone; *n* = 5).

Ca^{2+} responses were attenuated by approximately 30% in cells with $P2Y_{12}$ knockdown versus either GADPH knockdown or scrambled control cells (Figure 4G, H). To investigate the biological relevance of $P2Y_{12}$ receptor inhibition in THP-1 cells, we assayed transmigration in response to ADP. In these experiments, ADP stimulated significant THP-1 cell transmigration over a 2 h period compared to vehicle (Figure 4I). The addition of either ticagrelor or PSB-0739 attenuated cell migration to ADP (Figure 4I). Together, these data reveal that $P2Y_{12}$ receptor activity is functionally important for the generation of ADP-evoked intracellular Ca^{2+} responses and migration in THP-1 cells.

Adenylate cyclase inhibition reverses antagonistic effect of ticagrelor

We hypothesized that the molecular mechanism underlying the positive contribution of the $P2Y_{12}$ receptor to ADP-evoked Ca^{2+} responses could be due to a number of possibilities. Firstly, that the $P2Y_{12}$ receptor is solely G_i-coupled and its activity positively regulates $P2Y_6$ receptor-mediated Ca^{2+} signalling through classical suppression of adenylate cyclase, or secondly, that the $P2Y_{12}$ receptor can directly elicit a Ca^{2+} response, either through promiscuous G_q-coupling or *via* G_i-coupling that directly elicits Ca^{2+} signalling through activation of atypical PLC, for signalling on the CCL2-**CCR2** axis (Myers *et al.*, 1995). To explore this further, we investigated

the requirement of adenylate cyclase activity for mediating the inhibitory action of ticagrelor. Adenylate cyclase inhibition with 300 μM SQ22536 had no effect on ADP-evoked Ca^{2+} responses (Figure 5A). However, SQ22536 could reverse the effect of ticagrelor, restoring the ADP concentration–response relationship and maximum response (F_{max} = 3.32 ± 0.26 for ADP + 5 μM ticagrelor + 300 μM SQ22536; F_{max} = 2.64 ± 0.21, ADP + 5 μM ticagrelor; F_{max} = 1.06 ± 0.10; n = 5. The F_{max} for ADP + 5 μM ticagrelor vs. ADP + vehicle control is significantly different, $P < 0.05$) (Figure 5B, C).

Discussion

In this study, we demonstrated novel findings identifying a role for $P2Y_{12}$ receptors in regulating intracellular Ca^{2+} signalling in non-platelet cells. We utilized THP-1 cells, a model used extensively to investigate human monocyte function, to demonstrate a functional role for the $P2Y_{12}$ receptor. In addition, we identified $P2Y_{12}$ receptor expression in human $CD14^+$ monocytes. There have been no published reports on the expression of $P2Y_{12}$ receptors in monocytes thus far; however, the involvement of $P2Y_{12}$ receptors in the function of macrophages was investigated by Kronlage *et al.* (2010). Stimulation of $P2Y_{12}$ receptors in macrophages was found to induce cell spreading with formation of lamellipodia and inhibition of multiple purine receptors, including $P2Y_{12}$, attenuated chemotaxis (Kronlage *et al.*, 2010). The signal transduction mechanisms downstream of the $P2Y_{12}$ receptor were not investigated in this study. $P2Y_{12}$ receptor expression has also been reported in glial cells, smooth muscle and endothelium (Cattaneo, 2007).

In this study, we demonstrated that ADP evokes intracellular Ca^{2+} signalling *via* $P2Y_6$ receptor activation. The $P2Y_6$ receptor is often considered as the metabotropic receptor for uridine diphosphate (UDP). Although UDP is a more potent agonist at the human $P2Y_6$ receptor by several orders of magnitude, ADP is a full agonist with a half-maximal concentration for IP_3 production in the micromolar range (Communi *et al.*, 1996). ADP is an important signalling cue in monocyte/macrophage biology. For example, in the same cell-line used in this investigation, THP-1, ADP stimulation caused release of the cytokine TNF-α (Mattana *et al.*, 2002). TNF-α release causes an inflammatory innate immune response including immune cell recruitment. In addition, ADP has been shown in this investigation to induce calcium signals in THP-1 cells, and calcium signalling in circulating monocytes has in fact been suggested to result in the development of mature dendritic cells (Czerniecki *et al.*, 1997). Such examples indicate that ADP contributes to monocyte function, with specific reference to immune responses and differentiation. ADP released from *Escherichia coli*-infected mice, and from macrophages exposed to LPS, protected mice from *E. coli*-induced peritonitis *via* macrophage recruitment (Zhang *et al.*, 2018). Additionally, ADP caused production of CCL2, a crucial chemokine in immune cell recruitment, which accordingly attracted more macrophages in a transwell assay (Zhang *et al.*, 2018). Inhibition of downstream $P2Y_{12}$ receptor signalling, or macrophage $P2Y_{12}$ receptor deficiency, blocked immune responses to ADP, in turn allowing more bacteria to persist in infected mice (Zhang *et al.*, 2018). This investigation by Zhang *et al.* revealed that upon sensing danger, macrophages release ADP, which binds to receptors such as $P2Y_{12}$, mediating actions such as chemokine release and consequent recruitment of immune cells. Such findings may implicate ADP and $P2Y_{12}$ receptors as being crucial in forming a comprehensive immune response to infectious disease.

It was discovered in platelets by Hardy *et al.* (2004) that the selective pharmacological $P2Y_{12}$ receptor inhibitor AR-C69931MX partially blocked the calcium response to 10 μM ADP, which was also shown to be completely abolished by selective inhibition the $P2Y_1$ receptor. This suggested that the $P2Y_{12}$ receptor is able to positively modulate the $P2Y_1$ receptor-mediated calcium response to ADP in platelets. Subsequently, Hardy *et al.* identified that the PI3K inhibitor LY294002 (10 μM) partially inhibited the $P2Y_{12}$ receptor-dependent part of the calcium response to ADP. Moreover,

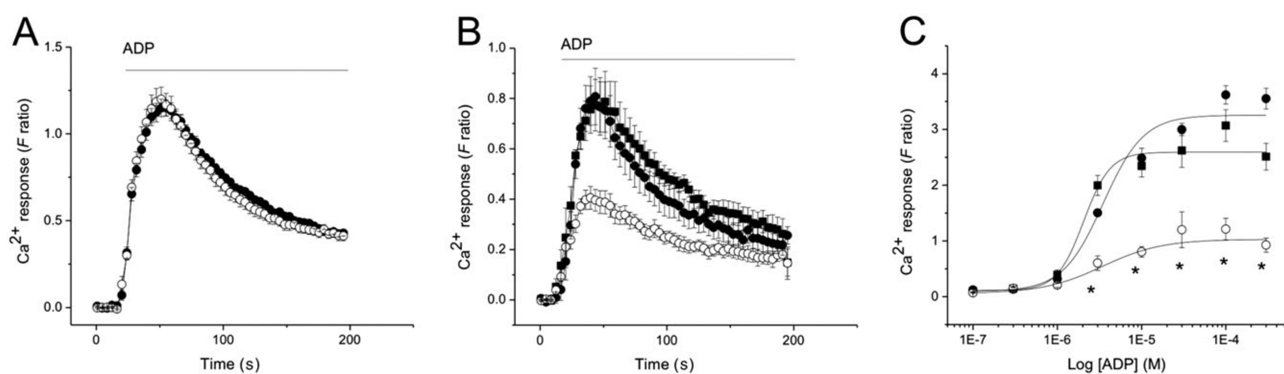

Figure 5

Inhibition of adenylate cyclase reverses antagonistic action of ticagrelor on ADP-evoked Ca^{2+} responses in THP-1 cells. (A) Averaged (n = 5) 3 μM ADP-evoked Ca^{2+} in the presence of vehicle (closed circles) or following adenylate cyclase inhibition with 300 μM SQ22536. (B) Averaged (n = 5) 3 μM ADP-evoked Ca^{2+} in the presence of vehicle, 5 μM ticagrelor (open circles) or 5 μM ticagrelor plus 300 μM SQ22536 (closed squares). (C) ADP concentration–response curve (n = 5) in the presence of vehicle (closed circles), 5 μM ticagrelor (open circles) or 5 μM ticagrelor plus 300 μM SQ22536 (closed squares). For all experiments, F ratio is the ratiometric measurement of intracellular Ca^{2+} using fura-2. *$P < 0.05$.

the adenylate cyclase inhibitor SQ22536 partially restored ADP-evoked calcium responses in the presence of the $P2Y_{12}$ receptor inhibitor AR-C69931MX (Hardy *et al.*, 2004). Taken together, these findings by Hardy *et al.* indicated that in platelets the $P2Y_{12}$ receptor regulates $P2Y_1$ receptor-mediated calcium responses to ADP through activation of PI3K and inhibition of adenylate cyclase. The findings in this investigation suggest that, of the ADP-activated P2Y receptors, ADP-induced calcium responses in THP-1 cells are dependent on $P2Y_{12}$ and $P2Y_6$ receptor activation, but not dependent on $P2Y_1$ or $P2Y_{13}$ receptors. Therefore, the basic principle identified and reported by Hardy *et al.* is supported here for THP-1 monocytic cells, only with the $P2Y_6$ receptor acting as the equivalent of the $P2Y_1$ receptor.

An inhibitory action of SQ22536 on ADP-evoked Ca^{2+} signals is not observed in the absence of $P2Y_{12}$ antagonism. These data suggest adenylate cyclase activity does not exert a suppressive effect when $P2Y_6$ and $P2Y_{12}$ receptors are concurrently activated by ADP. There are mechanistic explanations that could explain this observation. For example, there may be no net change in adenylate cyclase activity upon ADP challenge due to co-activation of a G_i-dependent pathway, mediated by $P2Y_{12}$ receptors and a G_s-dependent pathway mediated by another ADP receptor or possible adenosine receptor. A predominance of G_s signalling could be revealed following $P2Y_{12}$ receptor inhibition and increased adenylate cyclase activity suppresses ADP-evoked Ca^{2+} signalling. Previous reports (Communi *et al.*, 1997) have highlighted promiscuity for G_s- and G_q-coupling by $P2Y_{11}$ receptors that is expressed by THP-1 cells (unpublished data). However, the $P2Y_{11}$ receptor is activated by ATP not by ADP at concentrations used in this study (Communi *et al.*, 1997).

How might the $P2Y_{12}$ receptor regulate $P2Y_6$ receptor-dependent signalling *via* adenylate cyclase? Firstly, this is unlikely to involve PKA-dependent phosphorylation of the $P2Y_6$ receptor or receptor desensitization. Compared with other P2Y receptors, such as $P2Y_4$, the $P2Y_6$ receptor displays limited reduction in cell surface number even in the presence of maximal agonist concentrations (Brinson and Harden, 2000). There are no consensus PKA phosphorylation sites in the cytoplasmic loops of $P2Y_6$, receptors, although this cannot discount the possibility of PKA phosphorylation of an auxiliary protein that positively regulates $P2Y_6$ receptor activity.

There is currently much interest in the biological effects of $P2Y_{12}$ antagonists beyond platelets (Nylander and Schulz, 2016). Although further work is required to investigate the role of the $P2Y_{12}$ receptor in freshly isolated human monocytes, this work suggests that physiological and pharmacological modulation of $P2Y_{12}$ receptors can influence ADP-evoked intracellular Ca^{2+} signalling in THP-1 cells and perhaps monocytes, which will likely influence key monocyte functions such as migration, adhesion and cytokine production.

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Author contributions

J.J.M. and J.A.L. carried out the experiments and analysed the data. J.J.M., J.A.L. and S.J.F. designed the experiments and co-wrote the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article.

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Figure S1 Ca^{2+} response in THP-1 cells evoked by hexokinase-treated ADP. (A) Average (*N* = 5) intracellular Ca^{2+} response evoked by ADP (20 μM). Concentration-response curve of ADP-evoked responses (EC_{50} 2.2±0.5 μM; *N* = 5).