

P2Y₁₂ receptor modulation of ADP-evoked intracellular Ca²⁺ signalling in THP-1 human monocytic cells

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ABSTRACT AND KEYWORDS

Background and purpose

The G_i-coupled, ADP-activated P2Y₁₂ receptor is well characterised as playing a key role in platelet activation via crosstalk with P2Y₁ in ADP-evoked intracellular Ca²⁺ response. There is limited knowledge on the role of P2Y₁₂ in ADP-evoked Ca²⁺ responses in other blood cells. Here we investigate the role of P2Y₁₂ receptor activation in modulation of ADP-evoked Ca²⁺ responses in human THP-1 monocytic cells.

Experimental approach

A combination of intracellular Ca²⁺ measurements, RT-PCR, immunocytochemistry, leukocyte isolation and siRNA-mediated gene knockdown were used to identify the role of P2Y₁₂ receptor activation.

Key results

ADP-evoked intracellular Ca²⁺ responses (EC₅₀ 2.7 μM) in THP-1 cells were abolished by inhibition of phospholipase C (U73122) or sarco/endoplasmic reticulum Ca²⁺-ATPase (thapsigargin). Loss of ADP-evoked Ca²⁺ responses following treatment with MRS2578 (IC₅₀ 200 nM) revealed a major role for P2Y₆ in mediating ADP-evoked Ca²⁺ responses. ADP-evoked responses were attenuated either with pertussis toxin treatment, or P2Y₁₂ inhibition with two chemically distinct antagonists (ticagrelor, IC₅₀ 5.3 μM; PSB-0739, IC₅₀ 5.6 μM). ADP-evoked responses were suppressed following siRNA-mediated P2Y₁₂ gene knockdown. The inhibitory effects of P2Y₁₂ antagonists were fully reversed following adenylate cyclase inhibition (SQ22536). P2Y₁₂ receptor expression was confirmed in freshly isolated human CD14⁺ monocytes.

Conclusion and implications

Taken together, these data suggest that P2Y₁₂ activation positively regulates P2Y₆-mediated intracellular Ca²⁺ signalling through suppression of adenylate cyclase activity in human monocytic cells.

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Abbreviations: adenosine 5'-diphosphate (ADP); phospholipase C (PLC); inositol 1,4,5-triphosphate (IP₃); phosphatidylinositol 4,5-bisphosphate (PIP₂), cyclic adenosine monophosphate (cAMP); phosphatidylinositol-3-kinase (PI3K); uridine diphosphate (UDP); protein kinase A (PKA).

Keywords: Purinergic, ADP, monocyte, P2Y receptor, calcium signalling

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 P2Y₁, P2Y₆, P2Y₁₂ and P2Y₁₃, which are members of the G protein-coupled P2Y receptor family. Activation of G_q-coupled P2Y₁ and P2Y₆ causes downstream IP₃ generation via PLC-mediated breakdown of PIP₂, and subsequent calcium ion (Ca²⁺) release through endoplasmic reticulum IP₃ receptors (Erb and Weisman, 2012). Conversely, P2Y₁₂ and P2Y₁₃ 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 P2Y₁ and P2Y₁₂ receptors, and the functionality of both receptors is required for normal platelet aggregation (Jin and Kunapuli, 1998). The emergence of P2Y₁₂ 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 Ca²⁺ in response to ADP is deemed responsible for the ADP-mediated activation of platelets (Dorsam and Kunapuli, 2004). Although P2Y₁₂ is G_i-coupled and should therefore not cause intracellular Ca²⁺ mobilisation, outcomes from a previous study suggest that P2Y₁₂ potentiates the G_q P2Y₁-mediated Ca²⁺ response to ADP in a cross-talk mechanism (Hardy et al., 2004). Hardy et al. (2004) propose that P2Y₁₂ mediates inhibition of adenylate cyclase and activation of PI3K, which collectively positively modulates the Ca²⁺ signal induced by ADP. The same P2Y₁-P2Y₁₂ 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 P2Y₁₂ 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 P2Y₁₂ in early atherogenesis, rather than platelet P2Y₁₂. In addition to cardiovascular disease, P2Y₁₂ has potentially been implicated in immune responses to ADP in macrophages (Zhang et al., 2016) and dendritic cells (Ben Addi et al., 2010) in functions such as antigen uptake and chemotaxis. Therefore, non-platelet roles for P2Y₁₂ 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 apply the THP-1 monocytic cell line as an experimental model to investigate the expression of P2Y₁₂ and the contribution of the receptor in ADP-evoked Ca²⁺ responses, exploring the signal transduction mechanisms involved. As there have been no publications reporting a role for P2Y₁₂ in monocytes, this investigation reveals a new role for P2Y₁₂ in non-platelet Ca²⁺ responses, and contributes to our understanding of how monocytes function in health and disease.

MATERIALS AND METHODS

Chemicals and reagents

Unless otherwise stated, all chemicals and reagents were purchased from Sigma-Aldrich, with the exception of *pertussis toxin* and SQ22536 (Tocris). All chemicals (agonists and antagonists) were diluted using physiological saline (SBS buffer) (containing (mM): NaCl, 130; KCl, 5; MgCl₂, 1.2; CaCl₂, 1.5; D-glucose, 8; HEPES, 10; pH 7.4) with the exception of CCL2 (SBS buffer containing 1%(w/v) bovine serum albumin), MRS2578, SQ22536 and ticagrelor (SBS buffer containing 1% (v/v) DMSO).

Isolation of CD14⁺ human monocytes

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

Intracellular Ca²⁺ measurements & drug treatments

1 x 10⁶ THP-1 cells/mL were loaded for 1 h with 2 μM fura-2 AM in SBS buffer plus 0.01% (w/v) pluronic acid at 37°C. Cells were then pelleted and washed using SBS and plated at a density of 2x10⁵ cells/well. The plated cells were allowed to settle for 1 hour at 37°C, during which stage antagonists were added 30 minutes into the hour, unless otherwise stated. Measurements were taken at 37°C using the FlexStation 3 instrument (Molecular Devices) measuring fura-2 fluorescence (340 nm excitation when Ca²⁺-bound; 380 nm excitation when unbound; 510 nm emission) at intervals of 2 seconds giving. For all Ca²⁺ experiments, the signal reported is 'F ratio', the ratio between Fura-2 emission at 510 when excited at 340 and 380nm. For hexokinase-treated of ADP stocks, 1mM ADP in SBS solution was treated for 1 hour at 37°C with 3 U/mL hexokinase from *Saccharomyces cerevisiae* (Sigma) prior to agonist application.

Cell culture

Cells from the THP-1 cell line were cultured at 37°C, 5% CO₂ in RPMI 1640 medium containing 2 mM L-glutamine and supplemented with 10% (v/v) foetal bovine serum (FBS), 50 IU/mL penicillin and 50 μg/mL streptomycin. Cells were maintained at a density between 1 x 10⁵ and 1 x 10⁶ cells/mL.

siRNA-mediated gene knockdown

THP-1 cells (2 x 10⁵ 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.) using the manufacturer's protocol in 96-well format.

RNA extraction & RT-PCR

Total RNA was extracted from THP-1 cells and CD14⁺ monocytes using Tri reagent (Sigma Aldrich) with a subsequent DNase I treatment (Ambion). Complementary DNA was synthesised from 1 μg of total RNA using Superscript II reverse transcriptase (Invitrogen). PCR was performed using a Taq polymerase readymix (Sigma Aldrich) using primer pairs designed using the following sequences (accession numbers): *P2RY1* - GTTCAATTTGGCTCTGGCCG (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 phosphate-buffered saline (PBS) followed by fixative with 4%(w/v) paraformaldehyde. Cells were permeabilised with 0.25%(v/v) triton X-100 for 10 minutes followed by blocking with 1%(w/v) bovine serum albumin (BSA) for 30 minutes at room temperature. Primary and secondary antibodies were diluted in PBS containing 1%(w/v) BSA and incubated with cells overnight at 4°C and for 1 hour at room temperature, respectively. Cells were mounted in Vectashield containing nucleus counterstain (4',6-diamidino-2-phenylindole; DAPI). Goat polyclonal anti-P2Y₁₂ (Santa Cruz Biotechnology Inc) was used with Alexa 488-conjugated rabbit anti-goat (Abcam). Rabbit polyclonal anti-P2Y₁, anti-P2Y₆ and anti-P2Y₁₃ (Alomone) were used with Alexa 488-conjugated goat anti-rabbit (Invitrogen). Cell imaging was performed using a laser-scanning Zeiss LSM510 Meta confocal microscope.

Transmigration assays

Transwell migration assays were performed as previously described (Sivaramakrishnan et al., 2012; Campwala et al., 2014). Briefly, assays were performed in 24-well plates using polyethylene terephthalate (PET) membrane transwell inserts with 3 µm pores. 1x10⁶ THP-1 cells 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 hours at 37°C, and migrated cells counted on the underside of the transwell support using crystal violet staining. Chemotactic index was calculated as the ratio of cells that migrated to ADP over vehicle control.

Data analysis

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 ± standard error of the mean (error bars). Statistical significance was determined using paired Student t-tests. Each point in the dose-response plots represents the average of the peak Ca²⁺ response. A confidence interval of 5% (*P*<0.05) is used throughout for statement of significance.

Dose-response relationships were fitted using the equation of the Hill equation:

$$\text{response (y)} = \frac{(\text{max} - \text{min})}{1 + 10^{(\text{Log EC}_{50} - x)^{\text{HillSlope}}}}$$

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.*, 2017).

RESULTS

ADP-evoked intracellular Ca²⁺ responses are mediated by P2Y₆ receptor activation and modulated by G_i-dependent signalling

ADP evoked concentration-dependent increases in intracellular Ca²⁺ with a half-maximal concentration of 2.7±0.3 μM (N=5) (**Figures 1A and 1B**). Responses to maximal ADP concentrations were abolished following either phospholipase C inhibitor treatment (U73122) (**Figure 1C**) or calcium depletion of the ER store (thapsigargin) (**Figure 1D**). 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 Ca²⁺ responses ADP stocks were treated with hexokinase. Ca²⁺ response evoked by treated ADP were similar to untreated ADP, with a half-maximal concentration of 2.2 ±0.5 μM (N=5) (*P*>0.05 versus untreated ADP).

P2Y₆ antagonism by MRS2578 inhibited the ADP response almost completely with a half-maximal concentration of 200±20 nM (N=3) (**Figures 1E and 1F**). MRS2578 inhibited the ADP response in a non-competitive fashion (**Figure 1G**), consistent with the reported mode of antagonism at P2Y₆ (Mamedova et al., 2004) and with previous findings (Sivaramakrishnan et al., 2012; Campwala et al., 2014). Selective inhibition of P2Y₁ with MRS2500 (tested up to 10 μM) (**Figure 1H**) or P2Y₁₃ with MRS2211 (tested up to 10 μM) (**Figure 1I**) had no significant effect of ADP evoked Ca²⁺ responses. In addition to a dependency of phospholipase C activity and release of ER Ca²⁺ stores, we investigated the effect of G_i-dependent signalling by using *Bordetella pertussis* toxin (PTx). PTx treatment (5 nM, 3 hours) caused a significant attenuation of ADP-evoked intracellular Ca²⁺ response (**Figure 2A**), significantly suppressing the maximal response by approximately 30% and the half-maximal concentration for ADP (EC₅₀ 1.9±0.3 μM vs 2.9±0.2 μM with PTx; *p*<0.05, N=5) (**Figures 2B**). In control experiments, CCL2-evoked Ca²⁺ responses were abolished by PTx treatment (**Figure 2C**), in agreement with our previous observations. Together these findings suggest that ADP-evoked Ca²⁺ responses are mediated via P2Y₆ activation and release of ER Ca²⁺, and that G_i-dependent signalling either constitutively or following ADP activation, positively modulates the ADP-evoked response.

We next determined the expression of ADP receptors in THP-1 cells in an effort to probe further the molecular basis of ADP-evoked Ca²⁺ responses and identify receptors that may modulate them. RT-PCR analysis of P2Y receptors activated by ADP (Erb and Weisman, 2012; Communi et al., 1996) in THP-1 cells revealed expression of P2Y₁, P2Y₆, P2Y₁₂ and P2Y₁₃ (**Figure 3A**). P2Y₁₂ receptor mRNA expression was confirmed in freshly isolated CD14⁺ monocytes (**Figure 3B**). P2Y₁, P2Y₆, P2Y₁₂ and P2Y₁₃ protein expression was confirmed by immunocytochemistry and confocal microscopy (**Figure 3C**).

Effect of P2Y₁₂ antagonism and knockdown on ADP-evoked Ca²⁺ responses in THP-1 cells

Of the ADP receptors expressed by THP-1 monocytes, P2Y₁₂ is a known G_i-coupled receptor. The role of P2Y₁₂ activation during ADP challenge was investigated using PSB-0739, a high affinity competitive antagonist (Baqi et al., 2009), and ticagrelor, a P2Y₁₂ antagonist used clinically as an anti-thrombotic agent (Husted et al., 2006). Ticagrelor inhibited ADP-evoked Ca²⁺ responses in THP-1 cells in a concentration-dependent fashion (**Figure 4A**). Ticagrelor inhibited ADP-evoked Ca²⁺ responses with a half-maximal concentration of 4.7±1.8 μM (N=5) and a non-competitive mode of action (**Figures 4B and 4C**). This is consistent with the observations of van Giezen et al (2009) where ticagrelor binds to P2Y₁₂ 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 Ca²⁺ responses with a half-maximal concentration of 5.4±1.8 μM (N=5) (**Figures 4D and 4E**) 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 μ M 6-S-[(4-nitrophenyl)methyl]-6-thioinosine (NBMPR), but observed no effect on ADP-evoked Ca²⁺ responses (data not shown). Next, we undertook a gene knockdown strategy to support our observations with P2Y₁₂ receptor antagonists. siRNA targeting of P2Y₁₂ or control gene GADPH achieved approximately 40% mRNA knockdown versus scrambled siRNA control cells. ADP-evoked Ca²⁺ responses were attenuated by approximately 30% in cells with P2Y₁₂ knockdown versus either GADPH knockdown or scrambled control cells (**Figures 4G and 4H**). To investigate the biological relevance of P2Y₁₂ receptor inhibition in THP-1s, we assayed transmigration in response to ADP. In these experiments, ADP stimulated significant THP-1 transmigration over a 2 hour period over vehicle (**Figure 4I**). Addition of either ticagrelor or PSB-0739 attenuated cell migration to ADP (**Figure 4I**). Together these data reveal that P2Y₁₂ receptor activity is functional important for the generation of ADP evoked intracellular Ca²⁺ responses and migration in THP-1 cells.

Adenylate cyclase inhibition reverses antagonistic effect of ticagrelor

We hypothesised that the molecular mechanism underlying the positive contribution of P2Y₁₂ receptor to ADP-evoked Ca²⁺ responses could be due to a number of possibilities. Firstly, that P2Y₁₂ is solely G_i-coupled and its activity positively regulates P2Y₆-mediated Ca²⁺ signalling through classical suppression of adenylate cyclase, or secondly that P2Y₁₂ can directly elicit a Ca²⁺ response, either through promiscuous G_q coupling or via G_i-coupling that directly elicits Ca²⁺ signalling through activation of atypical phospholipase C, a 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 μ M SQ22536 had no effect on ADP-evoked Ca²⁺ responses (**Figure 5A**). However, SQ22536 could reverse the effect of ticagrelor, restoring the ADP concentration-response relationship and maximum response ($F_{max} = 3.32 \pm 0.26$ for ADP + 5 μ M ticagrelor + 300 μ M SQ22536; $F_{max} = 2.64 \pm 0.21$, ADP + 5 μ M ticagrelor; $F_{max} = 1.06 \pm 0.10$; $N=5$. The F_{max} for ADP + 5 μ M ticagrelor vs. ADP + vehicle control is significantly different to $P<0.05$) (**Figures 5B and 5C**).

DISCUSSION

Demonstrated in this study are novel findings identifying a role for P2Y₁₂ in regulating intracellular Ca²⁺ signalling in non-platelet cells. We have utilised THP-1 cells, a model used extensively to investigate human monocyte function, to demonstrate a functional role for P2Y₁₂. In addition, we identify P2Y₁₂ expression in human CD14⁺ monocytes. There have been no published reports on the expression of P2Y₁₂ in monocytes thus far, however the involvement of P2Y₁₂ function in macrophage was investigated by Kronlage et al (2010). Stimulation of P2Y₁₂ in macrophage was found to induce cell spreading with formation of lamellipodia, and inhibition of multiple purinergic receptors, including P2Y₁₂, attenuated chemotaxis (Kronlage et al., 2010). The signal transduction mechanisms downstream of P2Y₁₂ were not investigated in this study. P2Y₁₂ receptor expression has also been reported in glial cells, smooth muscle and endothelium (Cattaneo, 2007).

In this study, we demonstrate that ADP evokes intracellular Ca²⁺ signalling via P2Y₆ receptor activation. P2Y₆ is often considered as the metabotropic receptor for UDP. Though UDP is a more potent agonist at the human P2Y₆ receptor by several orders of magnitude, ADP is a full agonist with a half-maximal concentration for inositol 1,4,5-triphosphate 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 tumour necrosis factor alpha (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 *E. coli*-infected mice, and from macrophages exposed to lipopolysaccharide (LPS), protected mice from *E. coli*-induced peritonitis via macrophage recruitment (Zhang et al., 2016). 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., 2016). Inhibition of downstream P2Y₁₂ signalling, or macrophage P2Y₁₂ deficiency, blocked immune responses to ADP, in turn allowing more bacteria to persist in infected mice (Zhang et al., 2016). This investigation by Zhang et al. revealed that upon sensing danger, macrophages release ADP, which binds to receptors such as P2Y₁₂, mediating actions such as chemokine release and consequent recruitment of immune cells. Such findings may implicate ADP and P2Y₁₂ 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 P2Y₁₂ inhibitor AR-C69931MX partially blocked the calcium response to 10 μM ADP, which was also shown to be completely abolished by selective P2Y₁ inhibition. This suggested that P2Y₁₂ is able to positively modulate the P2Y₁-mediated calcium response to ADP in platelets. Subsequently, Hardy et al. identified that the PI3K inhibitor LY294002 (10 μM) partially inhibited the P2Y₁₂-dependent part of the calcium response to ADP. Moreover, the adenylate cyclase inhibitor SQ22536 partially restored ADP-evoked calcium responses in the presence of the P2Y₁₂ inhibitor AR-C69931MX (Hardy et al., 2004). Taken together, these findings by Hardy et al. indicated in platelets that P2Y₁₂ regulates P2Y₁-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 P2Y₁₂ and P2Y₆ activation, but not dependent on P2Y₁ or P2Y₁₃. Therefore, the basic principle identified reported by Hardy et al. is supported here for THP-1 monocytic cells, only with P2Y₆ acting as the equivalent of P2Y₁.

Of interest is the observation that adenylate cyclase inhibition with SQ22536 on ADP-evoked Ca²⁺ signals in the absence of P2Y₁₂ antagonism. This data suggests adenylate cyclase activity does not exert a suppressive effect when P2Y₆ and P2Y₁₂ 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 P2Y₁₂, and a G_s-dependent pathway mediated by another ADP receptor or possible adenosine receptor. A predominance of G_s signaling could be revealed following P2Y₁₂ inhibition, and increased adenylate cyclase activity suppresses ADP-evoked Ca²⁺ signaling. Previous reports (Communi et al., 1997) have highlighted promiscuity for G_s and G_q coupling by P2Y₁₁ which is expressed by THP-1 cells (unpublished data). However, P2Y₁₁ is ATP activated and not activated by ADP at concentrations used in this study (Communi et al., 1997).

How might P2Y₁₂ regulate P2Y₆-dependent signaling via adenylate cyclase? Firstly, this is unlikely to involve PKA-dependent phosphorylation of the P2Y₆ receptor or receptor desensitization. Compared to other P2Y receptors, such as P2Y₄, P2Y₆ display limited reduction in cell surface receptor number even in the presence of maximal agonist concentrations (Brinson & Harden, 2000). There are no consensus PKA phosphorylation sites in the cytoplasmic loops of P2Y₆, though this cannot discount PKA phosphorylation of an auxiliary protein that positively regulates P2Y₆ activity.

There is currently much interest in the biological effects of P2Y₁₂ antagonists beyond platelets (Nylander & Schultz, 2016). Although further work is required to investigate the role

of P2Y₁₂ in freshly isolated human monocytes, this work suggests that physiological and pharmacological modulation of P2Y₁₂ can influence ADP-evoked intracellular Ca²⁺ signalling in THP-1 cells and perhaps monocytes, which will likely influence key monocyte functionality such as migration, adhesion and cytokine production.

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Competing interests: None

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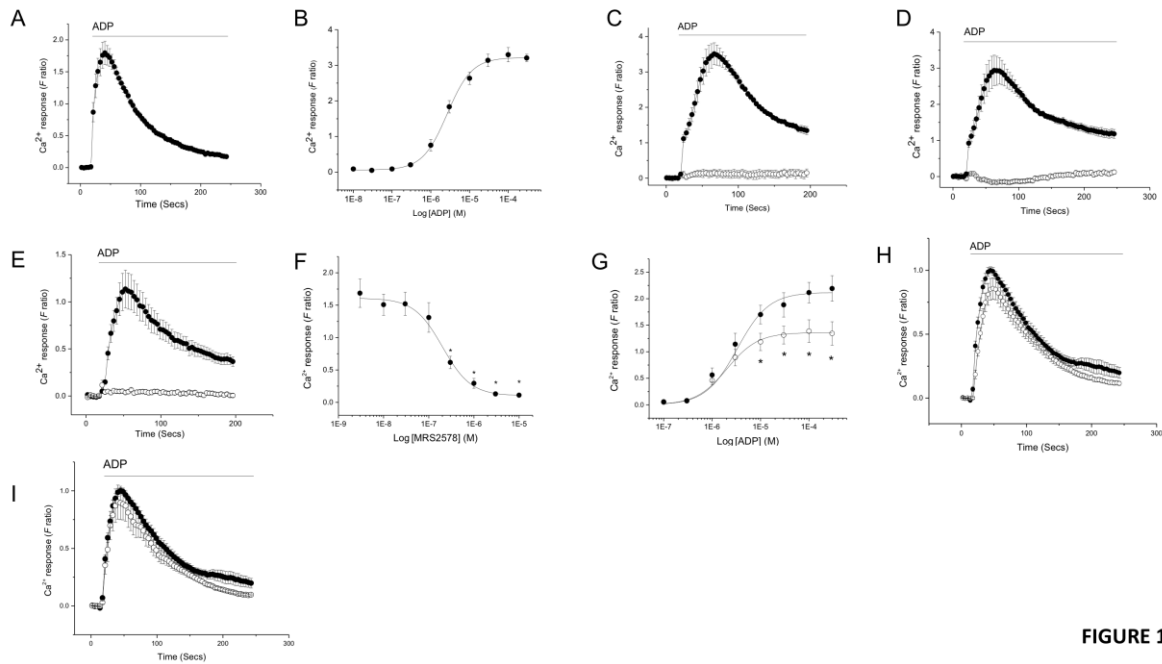


FIGURE 1

Figure 1 P2Y₆ mediates ADP-evoked intracellular Ca²⁺ responses in THP-1 cells. (A) Averaged (N=5) intracellular Ca²⁺ response evoked by ADP (30 μM). (B) Concentration-dependency of ADP-evoked responses (EC₅₀ 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 P2Y₆ antagonist MRS2578 on intracellular Ca²⁺ 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 Ca²⁺ responses evoked by 3 μM ADP in the presence of vehicle (*closed circles*) or in the presence of P2Y₁ antagonist 1 μM MRS2500 (H; *open circles*) or P2Y₁₃ antagonist 10 μM MRS2211 (I; *open circles*). For all experiments, F ratio is the ratiometric measurement of intracellular Ca²⁺ using fura-2. * p < 0.05

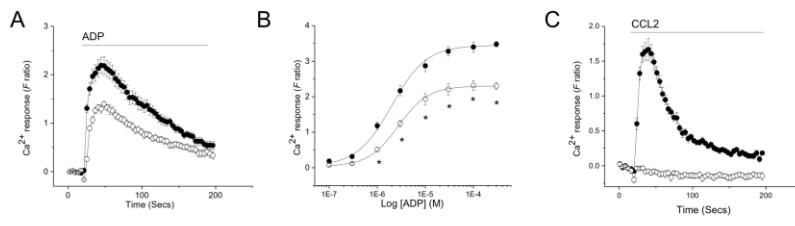


FIGURE 2

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 ($3\mu M$) in the absence (*closed circles*) and presence (*open circles*) of 5nM pertussis toxin (3 hr pre-incubation). (B) ADP concentration-response relationship in absence (*closed circles*) and presence (*open circles*) of 5nM pertussis toxin ($N=5$). (C) Positive control showing abolition of control CCL2-evoked response (*closed circle*) by 5nM pertussis toxin (*open circles*) ($N=5$). Responses evoked by 50ng/mL CCL2. For all experiments, F ratio is the ratiometric measurement of intracellular Ca^{2+} using fura-2. * $p<0.05$.

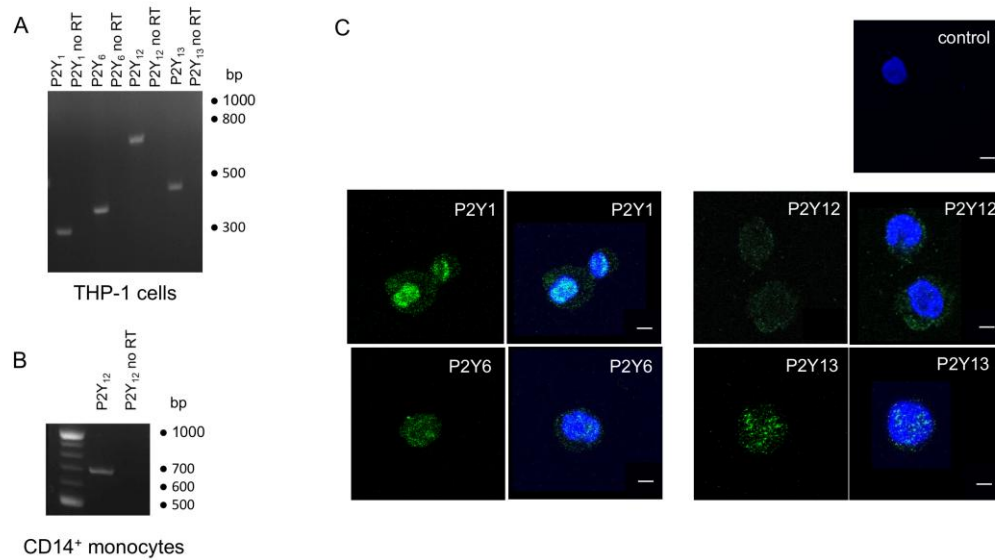


FIGURE 3

Figure 3 Expression of ADP-activated P2Y receptors in THP-1 cells. (A) RT-PCR analysis of P2Y₁ (326 bp), P2Y₆ (391 bp), P2Y₁₂ (698 bp) and P2Y₁₃ (461 bp) expression in THP-1 monocytes. RT-PCR analysis of P2Y₁₂ (698) expression in freshly isolated CD14⁺ monocytes from human peripheral blood. For (A) and (B): predicted PCR amplicon 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*) visualised by using a AF488-conjugated secondary antibody (*lefthand panels*). *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 5µm. Experiments are representative of at least 3 independent experiments.

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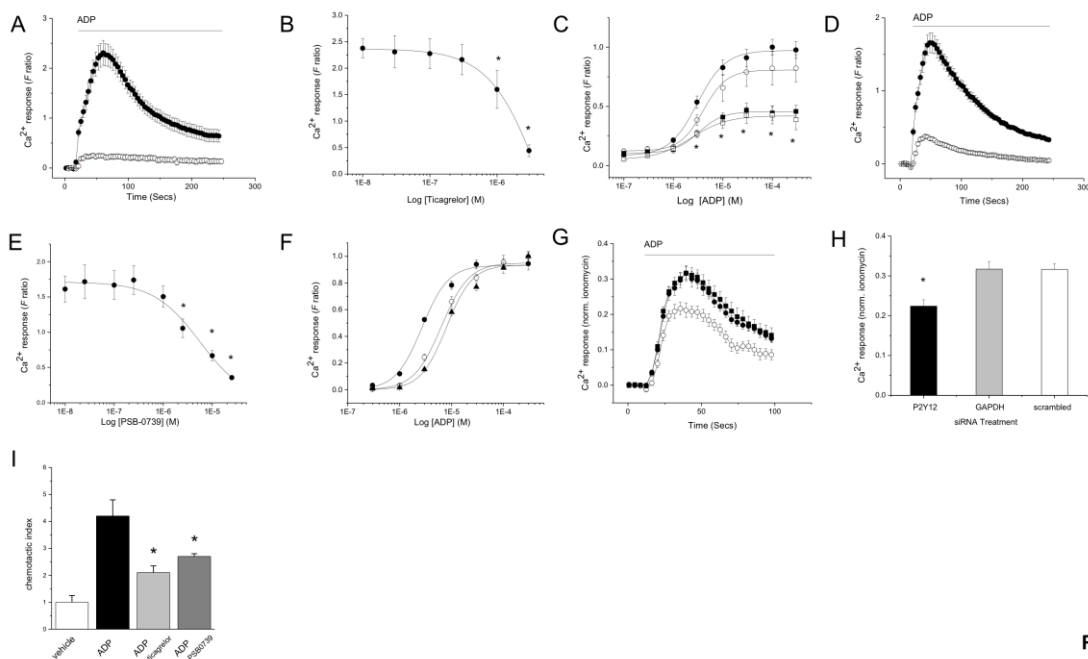


FIGURE 4

Figure 4 Effect of P2Y₁₂ receptor antagonism and gene knockdown on ADP-evoked intracellular Ca²⁺ responses in THP-1 cells. (A) Averaged ($N=5$) ADP-evoked Ca²⁺ response in the absence (*closed circles*) and presence (*open circles*) of 3 μ M ticagrelor. (B) Ticagrelor concentration-inhibition curve (IC_{50} $4.7 \pm 1.8 \mu$ M; $N=5$) for responses evoked by 3 μ M 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 μ M ticagrelor (*open squares*). (D) Averaged ($N=5$) ADP-evoked Ca²⁺ response in the absence (*closed circles*) and presence (*open circles*) of 25 μ M PSB-0739. (E) PSB-0739 concentration-inhibition curve (IC_{50} $5.4 \pm 1.8 \mu$ M; $N=5$) for responses evoked by 3 μ M ADP. (F) ADP concentration response curve ($N=4$) in the presence of vehicle (*closed circles*) or 5 (*open circles*) and 10 μ M PSB-0739 (*closed triangles*). (G) Effect of siRNA-mediated silencing of P2Y₁₂ on ADP-evoked Ca²⁺ responses. Averaged ($N=5$) Ca²⁺ responses evoked by 3 μ M ADP in THP-1 cells following siRNA-mediated mRNA knockdown of P2Y₁₂ (*open circles*) or GAPDH (*closed squares*) compared to cells transfected with scrambled siRNA (*closed circles*) ($N=5$). (H) Bar chart showing effect of different siRNA treatment on peak Ca²⁺ responses evoked by 3 μ M ADP ($N=5$). Responses in panels G and H are normalised to the magnitude of Ca²⁺ response elicited by 100 μ M ionomycin to control for cell number. For all experiments, F ratio is the ratiometric measurement of intracellular Ca²⁺ using fura-2. * $p < 0.05$ (I) P2Y₁₂ antagonists attenuate THP-1 transwell migration towards ADP. Chemotactic indexes comparing cell movement over 2 hours in control conditions (*vehicle*), 3 μ M ADP alone or in the presence of either ticagrelor (3 μ M) or PSB-0739 (25 μ M) (* $p < 0.05$ versus ADP alone; $N=5$).

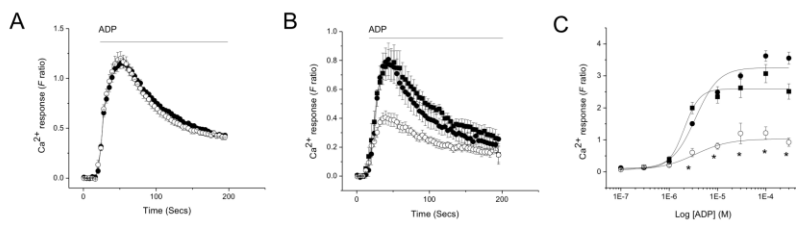


FIGURE 5

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

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