P2Y$_{12}$-dependent modulation of ADP-evoked calcium responses in human monocytes

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Abstract

The G_i-coupled, ADP-activated $P2Y_{12}$ receptor is well-characterised as playing a key role in platelet activation via crosstalk with $P2Y_1$. A crucial aspect of $P2Y_{12}$-$P2Y_1$ crosstalk in platelets involves ADP-induced intracellular calcium (Ca^{2+}) mobilisation, however there is limited knowledge on the role of $P2Y_{12}$ in ADP-evoked Ca^{2+} responses in other blood cells. Here, we investigate the expression of $P2Y_{12}$ in human monocytes and the contribution of $P2Y_{12}$ in THP-1 ADP-evoked Ca^{2+} responses.

RT-PCR analysis showed that all ADP-binding P2Y receptors were expressed in THP-1 monocytes at the mRNA level, with $P2Y_{12}$ expressed in $CD14^+$ primary monocytes. $P2Y_{12}$ protein was found to be expressed in THP-1 cells, using immunocytochemistry. ADP-evoked Ca^{2+} responses in fura-2-loaded THP-1 cells were completely abolished by a sarco/endoplasmic reticulum Ca^{2+}-ATPase inhibitor, and by a phospholipase C inhibitor, indicating that these Ca^{2+} responses are mediated through GPCRs. Ca^{2+} responses induced by ADP were significantly reduced by the $P2Y_{12}$ inhibitors ticagrelor and PSB-0739 and by the $P2Y_6$ antagonist MRS2578, but not by $P2Y_1$ or $P2Y_{13}$ inhibitors. Using $P2Y_6$-overexpressing astrocytomas, ticagrelor was found to be selective for $P2Y_{12}$ over $P2Y_6$ at the concentrations used here, although PSB-0739 was not. ADP-induced Ca^{2+} responses were significantly decreased in THP-1 cells with siRNA-mediated $P2Y_{12}$ knockdown. Pertussis toxin-mediated G_i inhibition caused a significant reduction in ADP-induced Ca^{2+} responses, as did the PI3K inhibitor LY294002. Use of the adenylate cyclase inhibitor SQ22536 had no effect on sole ADP-evoked Ca^{2+} responses, but caused significant recovery of Ca^{2+} responses inhibited by $P2Y_{12}$ antagonists.

The results from this study suggest that Ca^{2+} responses evoked by ADP in THP-1 cells are dependent on both $P2Y_{12}$ and $P2Y_6$ activation, with $P2Y_{12}$ positively modulating G_q-coupled $P2Y_6$-mediated Ca^{2+} responses via adenylate cyclase inhibition and PI3K activation. Overall, this investigation reveals a new role for $P2Y_{12}$ in non-platelet Ca^{2+} responses, and contributes to our understanding of how monocytes function in health and disease.

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List of Abbreviations

The following lists abbreviations commonly used in this thesis:

AC – Adenylate cyclase

ADP – Adenosine diphosphate

Ca^{2+} - Calcium ions

cAMP – Cyclic adenosine monophosphate

CCL2 - CC-chemokine ligand 2

CD14 – Cluster of differentiation 14

DAG – Diacylglycerol

EC_{50} – Half-maximal effective concentration

E_{max} – Maximal effect of agonist

ENT - Equilibrative nucleotide transporter-1

ER – Endoplasmic reticulum

IC_{50} – Half-maximal inhibitory concentration

IP_3 - Inositol 1,4,5-trisphosphate

mRNA – Messenger ribonucleic acid

PI3K - Phosphoinositide 3-kinase

PLCβ – Phospholipase C beta

PLC γ - Phospholipase C gamma

PMCA - Plasma membrane Ca^{2+} ATPase

SEM – Standard error of the mean

SERCA - Sarcoendoplasmic reticular Ca^{2+} ATPase

siRNA – Small interfering ribonucleic acid

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Chapter 1 – Introduction

1.1 - Monocytes

1.1.1 - Monocytes – Background

Derivation, development & subtypes

Known as one of the 'professional' phagocytes, monocytes are a type of leukocyte that exists in all vertebrates and amount to 10% of the nucleated cells in human blood (van Furth and Sluiter, 1986). Monocytes originate from primary lymphoid organs that include the bone marrow and the foetal liver, where they derive from the common myeloid progenitor cell (Ginhoux and Jung, 2014). Monocytes undergo apoptosis in the absence of appropriate stimuli, with pro-inflammatory cytokines such as IL-1β and TNF-α proving fundamental in preventing monocyte apoptosis (Mangan and Wahl, 1991). This indicates that monocyte viability is modulated by the presence of inflammatory markers (Mangan and Wahl, 1991).

There are several subsets of human monocyte population, which are essentially based on expression of CD14 and CD16 molecules. Some of the main sub-populations include: the classical subset (high CD14 expression, no CD16 expression), the non-classical subset (low CD14 expression, high CD16 expression) and the intermediate subset (high CD14 expression, low CD16 expression) (Wong et al., 2012). The subpopulations of monocytes have been shown to give rise to varying physiological effects, including differential cytokine production (Frankenburger et al., 1996) and antigen processing (Wong et al., 2011) between the different monocyte subtypes.

Morphology & physiological function

Monocytes are spherical in shape and feature distinctive bean-shaped and single-lobed nuclei, and are the largest of all leukocytes. The cytoplasm contains dispersed granules, and many mitochondria, microfilaments and microtubules (Kaushansky and Williams, 2010).

Monocytes constitutively circulate in the bloodstream and play a key role in immune surveillance whereby they migrate from the bloodstream to sites of infection and inflammation, where they are capable of differentiating into macrophages and dendritic cells (Serbina et al., 2008), thus forming an essential role in the immune system. In response to injury or infection, monocytes and their progeny facilitate innate immune defence via phagocytosis and cytokine production, but they can also activate the adaptive immune system through antigen uptake and presentation, providing a comprehensive immune response. It has long been thought that monocytes also continually replenish tissue-based dendritic cell and macrophage populations in the steady state, but novel experimental methods, including Cre–*loxP*-based fate-mapping systems, for example (Yona et al., 2013), have demonstrated that most tissue macrophage populations do not derive from monocytes (Hashimoto et al., 2013).

CC-chemokine ligand 2 (CCL2) is a chemokine that binds to the CCR2 receptor and is released from cells activated during innate immune responses to a variety of microbial molecules (Tsou et al., 2007), and indeed a number of different infections cause an increased circulating CCL2 level. CCL2 is fundamental to the recruitment of monocytes to sites of infection, and furthermore, murine models have shown that CCL2- and/or CCR2-deficient mice have reduced monocyte recruitment in response to infections with *Listeria monocytogenes* (Kurihara et al., 1997), *Toxoplasma gondii* (Dunay et al., 2008) and several fungal (Osterholzer et al., 2009) and viral (Dawson et al., 2000. Lim et al., 2010) infections.

1.1.2 - Monocytes – Roles in human disease

Atherosclerosis

In addition to their beneficial and crucial inflammatory role for the innate immune system, monocytes also heavily contribute to the development of atherosclerosis, an inflammatory disease which is the leading cause of cardiovascular disease. **Figure A** illustrates the role of monocytes in atherosclerosis together with the description in this section. Monocytes are directly involved in the early stages of atherosclerosis development through recruitment to the arterial endothelium via CCL2-mediated chemotaxis, whereby they undergo endothelial adhesion, 'rolling', and subsequent migration **(Figure A – 1)** into the intima (Galkina and Ley, 2009). Mice lacking the CCL2

protein, or its receptor CCR2, developed much smaller atherosclerotic lesions (Gu et al., 1998. Boring et al., 1998), emphasising the crucial role of the CCL2/CCR2 interaction in
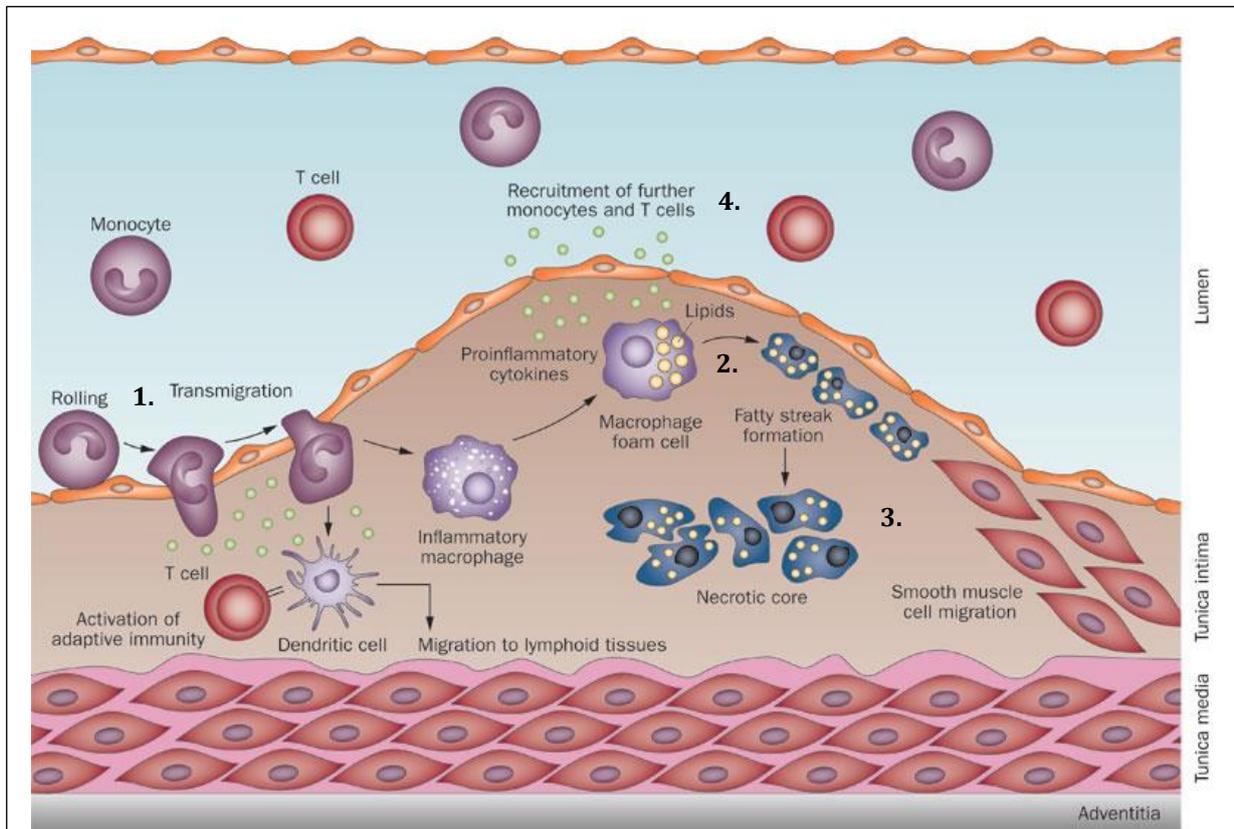
Figure A – Monocytes in the initiation and development of atherosclerosis

The following provides a brief summary of the contribution of monocytes and their derivatives in atherosclerosis, with more detail provided in-text. Endothelial cells are activated upon initial injury, mediating the rolling and transmigration of recruited monocytes (**1**), which then differentiate into macrophages or dendritic cells. Macrophages consume lipoproteins (**2**), leading to fatty streak formation, with such macrophages enduring apoptosis, thus contributing to the destabilising necrotic core (**3**). Further progression of the plaque is facilitated through further recruitment of monocytes via inflammatory cytokine release (**4**). The schematic diagram was taken from Heine et al., (2012) and annotated.

monocyte recruitment in atherosclerosis.

In the intima, monocytes differentiate into macrophages, which accrue oxidised low-density lipoprotein (oxLDL) and other lipids through their scavenger receptors (**Figure A – 2**), subsequently forming an early-stage plaque (Galkina and Ley, 2009).

Apolipoprotein E (apoE) is a glycoprotein that is essential in lipid transport and metabolism, and apoE-deficient mice provide a useful model for studying atherosclerosis as such mice have impaired plasma lipid clearance and subsequently rapidly develop atherosclerotic lesions (Kolovou et al., 2008). Studies have shown that apoE-deficient, hypercholesteraemic mice fed on a high-fat diet exhibited a dramatic increase in inflammatory monocytes, which were shown to adhere to an activated endothelium, infiltrate lesions, and become lesional macrophages (Swirski et al., 2007). Statin-induced cholesterol reduction caused a decrease in the number of inflammatory monocytes and consequently a reduction in lesion formation, therefore highlighting the level of monocyte recruitment during atherosclerosis (Swirski et al., 2007).

Macrophages containing oxLDL become 'foam cells' and undergo apoptosis and necrosis, resulting in the progression of the atherosclerotic plaque, with accumulation of necrotic tissue, deposition of collagen and migration of smooth muscle also following (**Figure A – 3**) (Pamukcu et al., 2010). An inflammatory process is then established, with activated monocytes containing a higher number of toll-like receptors and releasing an excessive amount of inflammatory cytokines. This causes further transendothelial migration of monocytes to the plaque environment (**Figure A – 4**), consequently giving rise to plaque growth and destabilisation (Jaipersad et al., 2014).

The instability of an atherosclerotic plaque is a high contributor to the chance of a thrombotic event, with approximately 75% of acute coronary events caused by the volatility of the responsible plaque (Jaipersad et al., 2014). When a plaque becomes structurally unstable, it is more likely to rupture, exposing and releasing lipids and tissue factor, which in turn activate the coagulation cascade, causing thrombus formation and potentially resulting in a myocardial infarction.

The excessive release of inflammatory mediators by monocytes, such as IL-1β, IL-6 and TNF-α, contributes to the instability of an atheromatous plaque, not only by further recruitment of inflammatory cells, but also by contributing to neovascularisation (Shantsila and Lip, 2009). Activated monocytes produce matrix metalloproteinases (MMPs) (Shantsila and Lip, 2009), which allow vessel growth in neovascularisation, which is associated with plaque rupture (Kumamoto, 1995).

As described here, monocytes greatly participate in the pathology of atherosclerosis. From their initial recruitment, adhesion and migration across the endothelium, to differentiating into macrophages/foam cells, to contributing to plaque instability and therefore rupture, monocytes are involved in many stages of atherosclerosis. Further studies into how monocytes elicit such functions would undoubtedly be of great value due to their involvement in the pathological process of a leading cause of cardiovascular disease.

Liver fibrosis

Liver fibrosis occurs when type-I collagen is deposited to the extracellular space upon damage caused by, for example, chronic alcoholism or hepatitis B infection, which affects the functionality of the liver (Heymann, et al., 2009). It has been indicated in a mouse model of liver fibrosis that inflammatory monocytes contribute to the fibrosis progression by infiltrating the liver and activating collagen-producing cells, hepatic stellate cells, by releasing cytokines such as TGF-β (Karlmark et al., 2009). CCR2-deficient mice exhibited reduced fibrosis compared to the control, which is suggested to be due to a decrease in inflammatory monocyte migration into the liver (Karlmark et al., 2009). Additionally, CX_3CR1-knockout mice demonstrated a more severe fibrosis phenotype owing to higher numbers of infiltrating inflammatory monocytes, indicating that the fractalkine receptor CX_3CR1 is protective in this case through the control of intrahepatic monocyte survival and differentiation (Karlmark et al., 2010). It is therefore evident that invading inflammatory monocytes contribute to the development of liver fibrosis. However, differentiation of infiltrating monocytes to macrophages in the liver leads to production of matrix metalloproteinases that contribute to a regression of fibrosis through breakdown of type-I collagen (Heymann, et al., 2009). Thus, monocytes cause progression of liver fibrosis during the early phases of the disease, but subsequently promote regression at later stages.

Cancer progression

Recent findings indicate a role for monocytes in carcinogenesis and metastasis of cancerous tumours. Myeloid-derived suppressor cells (MDSCs) are indicated to contribute to cancer progression through the promotion of angiogenesis and metastasis, but also via inhibition of anti-tumour immune activity (Monu and Frey, 2012). It is suggested that MDSCs derived from monocytes release IL-10, consequently reducing the anti-tumour functions of T-cells (Karlmark et al., 2012).

Moreover, a chemical-induced model of carcinogenesis revealed that histamine deficiency promotes carcinogenesis, which is coupled with infiltration of inflammatory monocytes (Yang et al., 2010). Inflammatory monocytes have been shown to infiltrate mammary tumours in mice, produce vascular endothelial growth factor, and subsequently promote metastasis of the tumour to the lung (Qian et al., 2011). Inhibition of CCL2 signalling, a major monocyte chemoattractant, blocked the recruitment of inflammatory monocytes and reduced metastasis, prolonging the survival of the mice (Qian et al., 2011). These findings, which include studies *in vivo*, indicate that monocytes have a major part to play in different stages of tumour progression.

It is evident that monocytes are crucial cells in human health and disease, and increasing the understanding of the underlying mechanisms to their functions in such situations would be strongly beneficial.

1.2 - Calcium signalling

1.2.1 - Calcium signalling – Basic principles and mechanism

Cellular signalling consists of information transmission, occurring through protein-protein interactions or via diffusible factors known as second messengers (Berridge, 2014). Calcium ions (Ca^{2+}) are a type of second messenger, and Ca^{2+} signalling is key to human physiology, required for fertilisation at the beginning of life and cellular apoptosis at the end, controlling many cellular processes in between. The basic mechanism for calcium signalling comprises a stimulus triggering a transient increase in cytoplasmic Ca^{2+} concentration, known as a calcium 'response', or 'signal'. In order for calcium responses to occur, at rest the intracellular Ca^{2+} concentration is maintained at a concentration 20,000-100,000-fold lower than the extracellular Ca^{2+} concentration

(Clapham, 2007). **Figure B** illustrates and describes the mechanisms behind intracellular homeostasis and release.
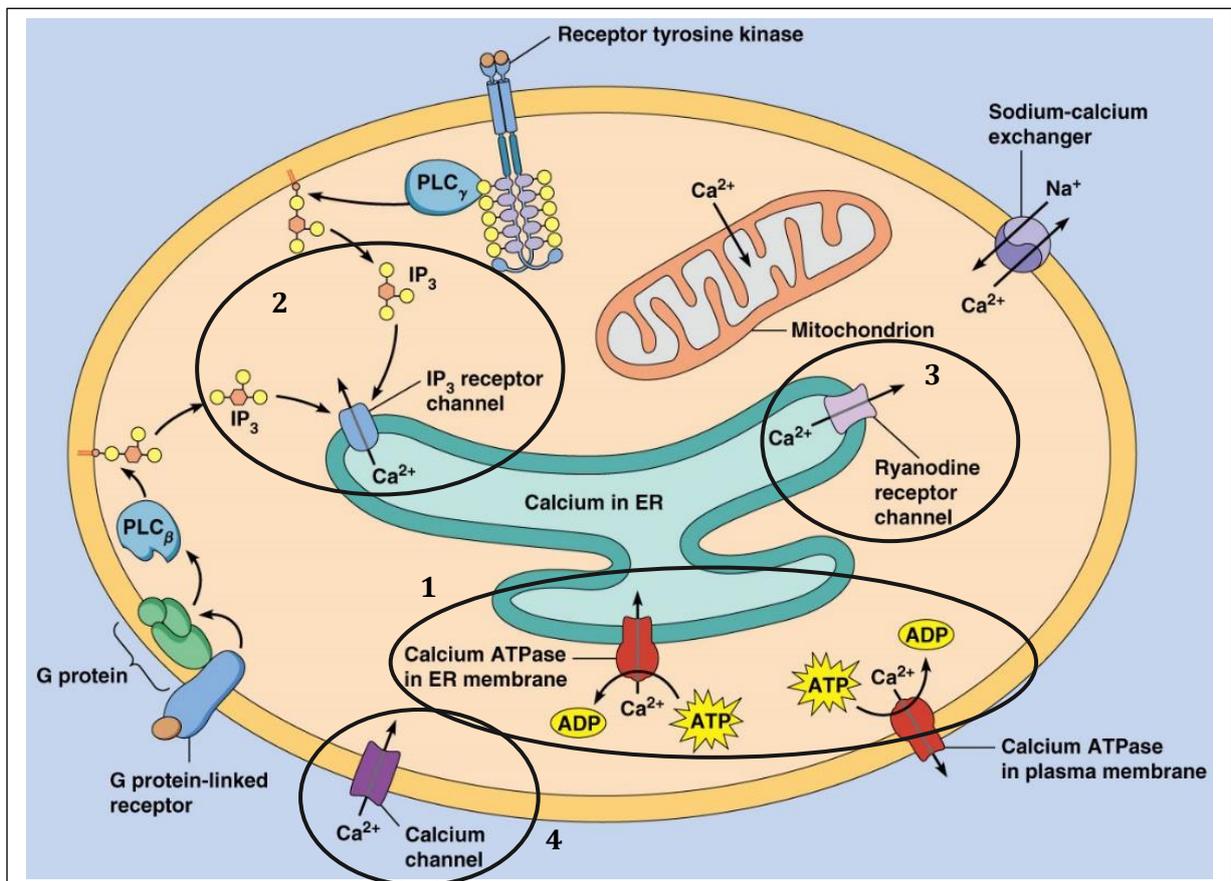
Figure B – The core of intracellular calcium homeostasis and release.

The physiological intracellular concentration of calcium is around 100 nM, which is maintained by Ca^{2+} buffers and also by sarcoendoplasmic reticular Ca^{2+} ATPase (SERCA) and plasma membrane Ca^{2+} ATPase (PMCA) pumps (**1**), which actively transport Ca^{2+} into the endoplasmic reticulum (ER) and out of the cell through the cell membrane, respectively (Clapham, 2007). Some cytosolic Ca^{2+} is also transported into the mitochondria.

2. Ca^{2+} can be released from the ER via inositol 1,4,5-trisphosphate (IP_3) and ryanodine (**3**) receptors. IP_3 production occurs upon extracellular stimuli-dependent activation of G-protein-coupled receptors and receptor tyrosine kinases, which then causes release of Ca^{2+} stored in the ER via the IP_3 receptor (Berridge, 2014). Ryanodine receptors mediate calcium release from the ER following an increase in cytoplasmic Ca^{2+} concentration, in a calcium-induced calcium release manner, subsequently allowing amplification of previous intracellular calcium release. Calcium responses can also occur from influx of extracellular Ca^{2+} through membrane ion channels (**4**), which open upon activation by a stimulus. The schematic diagram was taken from Hardin et al., (2012) and annotated.

A combination of Ca^{2+}-ATPase pumps, Na^+/Ca^{2+} exchangers and Na^+/Ca^{2+}-K^+ exchangers help to maintain the intracellular Ca^{2+} concentration at around 100 nM. Ca^{2+}-ATPase pumps function in an 'uphill' manner, with SERCA transferring two Ca^{2+} ions into the ER per ATP molecule hydrolysed, and the PMCA removing one Ca^{2+} from the cell per ATP molecule (Figure B). Conversely, Na^+/Ca^{2+} and Na^+/Ca^{2+}-K^+ exchangers take advantage of inward Na^+ currents to send Ca^{2+} out of the cell. The Na^+/Ca^{2+} exchanger transports one Ca^{2+} ion for every three Na^+ ions while the Na^+/Ca^{2+}-K^+ exchanger co-transports one Ca^{2+} and one K^+ for every four Na^+ ions (Clapham, 2007).

Calcium buffer proteins are another key contributor in regulating cytosolic Ca^{2+} concentration, which include the parvalbumins, calbindins and the calretinin protein (Schwaller, 2010). Calcium buffers contain evolutionarily conserved structural aspects to allow Ca^{2+}-binding to occur with high affinity. Specifically, the EF-hand motif is a feature in all the calcium buffer proteins. The resting intracellular Ca^{2+} concentration is typically 20-100 nM, whereas the dissociation constants of calcium buffers is higher, at around 0.2-1.5 μM. Owing to this difference, calcium buffers exist in a free state in basal conditions, and are thus primed to bind Ca^{2+} upon cytosolic influx of Ca^{2+}, helping to spatiotemporally modulate calcium signals and restore the resting Ca^{2+} after the signal has passed (Schwaller, 2010). Together with the calcium-binding pumps and exchangers, calcium buffers maintain an intracellular environment viable for effective, but regulated calcium signalling.

As introduced in figure B, there are various means by which calcium influx into the cytosol occurs. The phospholipase C pathway is arguably the most common method of intracellular calcium release, and occurs initially through activation of cell surface receptors such as G protein-coupled receptors. This causes phospholipase C to hydrolyse PIP2 into IP_3 and DAG, with IP_3 triggering calcium release from the ER via the IP_3 receptor.

There are several different types of calcium ion channels that allow direct calcium entry into the cell (figure B-4), that differ in the type of stimulus that activates them. For instance, there are channels that allow influx of calcium upon direct binding of agonists such as ATP to the extracellular domains of the respective ion channel, thus also acting as a receptor. Additionally, in excitable cells such as neurons and muscle cells,

membrane depolarisation causes activation of voltage-gated calcium channels, permitting calcium entry. Entry of extracellular calcium can also occur from second messengers such as arachidonic acid acting at second messenger-operated channels on the cytoplasmic side of the membrane (Berridge, 2014).

Upon activation of the IP_3 receptor and calcium release from the ER, the calcium sensor STIM1 senses the decrease in ER Ca^{2+} concentration and activates ORAI1, a Ca^{2+} release-activated Ca^{2+} channel, allowing store-operated Ca^{2+} entry (Fahrner et al., 2013). The purpose of store-operated calcium entry was initially thought to be to replenish depleted calcium stores. However, now it has been indicated that the main function may be to activate specific signalling pathways by offering Ca^{2+} to intraceullar recipients localised to confined sites close to areas of Ca^{2+} entry (Putney, 2011). In fact, store-operated calcium release may be the main signalling pathway for T cell activation and mast cell degranulation (Fahrner et al., 2013).

The endoplasmic reticulum is the most characterised intracellular store capable of releasing calcium into the cytoplasm, as described in figure B. However, the ER is not the only recognised store of calcium ions, as acidic organelles such as lysosomes (Churchill et al., 2002), vacuoles (Patel and Docampo, 2010) and acidocalcisomes (Luo et al., 2004) have also been shown to store calcium. The calcium-mobilising second messenger nicotinic acid adenine dinucleotide phosphate (NAADP) has been suggested to cause calcium release from lysosomes (Churchill et al., 2002), although there is little evidence for NAADP-induced lysosomal calcium release in leukocytes.

1.2.3 - Calcium signalling - Versatility of Ca^{2+} signalling and functions
Calcium responses are temporary but the length of time the transients can persist varies widely, which allows many different cellular processes to be mediated through calcium signals. For instance, calcium transients responsible for exocytosis last microseconds, but responses regulating fertilisation occur for hours (Berridge, 2014). Moreover, transient calcium signals can be generated as singular 'on demand' pulses upon intermittent stimulation, like in neurotransmitter release. Alternatively, they can occur as oscillations of brief calcium spikes during continuous cellular stimulation, with the

frequency of oscillations dependent on the level of stimulation, as is the case in cell proliferation (Berridge, 2014).

In addition to the varied temporal aspects of calcium signalling, there are spatial ones too, demonstrating the incredible versatility of calcium signalling. In a similar fashion to how calcium signals can occur over a wide time domain, a vast spectrum of locality to calcium responses exists. In muscle contraction calcium elevation occurs globally, whereas neurotransmitter release is mediated through extremely local, specific delivery of Ca^{2+} to the synaptic vesicle (Berridge, 2014). Regenerating waves of Ca^{2+} can also occur in some cells, such as pancreatic acinar cells, where calcium signals arise in precise, fixed points and are propagated along the cytoplasm through the positive feedback of calcium-induced calcium release (Dove, 2002).

Once calcium is released into the cytoplasm, various functions can be mediated through Ca^{2+} sensors, which transmit information via Ca^{2+} effectors. Ca^{2+} sensors include calmodulin, troponin C and DAG kinase α, whereas phosphorylase kinase, calcineurin and Ca^{2+}/calmodulin-dependent protein kinases are examples of the many Ca^{2+} effectors (Berridge, 2014). The physiological functions calcium signalling is involved in include synaptic transmission, cell proliferation, cellular motility, muscle contraction among many more. With particular reference to monocytes, calcium responses have been shown to be crucial to their chemotactic (Badolato et al., 1995) and phagocytic (Hishikawa, 1991) functions as well as their ability to respond to pro-inflammatory molecules such as chemokines (Gouwy et al., 2008). Improving the understanding of calcium signalling in monocytes would subsequently be extremely beneficial.

1.3 - Purinergic Signalling

1.3.1 - Purinergic Signalling – Background

General Concept & Roles in the Human Body

The incredibly important and rapidly growing field of purinergic signalling revolves around the function of purine nucleotides and nucleosides which, aside from their obvious critical role in DNA, and as an energy source, also have the capability to act as signalling molecules. Purine nucleosides such as adenosine, and nucleotides such as ATP and ADP bind to their respective purinergic receptors on the surfaces of cells, and cause a downstream cellular change. In the human body, purinergic signalling mediates the function of many different processes, in a wide range of organ systems.

For instance, in the immune system, it is well characterised that large increases of extracellular ATP acts as a 'danger signal', due to ATP release from dying cells (Trautmann, 2009). Furthermore, ATP and adenosine are heavily implicated in more complex immunomodulation. For example, adenosine reduces peripheral blood mononucleated cell (PBMC) release of pro-inflammatory cytokines like TNF-α (Bouma et al., 1994), but increases the release of anti-inflammatory mediators such as IL-10 too (Le Moine et al., 1996). Moreover, ATP acts as an incredibly potent stimulus in $P2X_7$-mediated, NALP3-dependent inflammasome activation (Sutterwala et al., 2006). These are just a few examples of the ever-growing findings for purinergic signalling in the immune system.

Purinergic signalling is also heavily involved in the nervous system, where it is well recognised that the purinergic nucleotide ATP is a co-transmitter, or a sole transmitter, in most nerves in the central and peripheral nervous systems (Burnstock, 2007). Furthermore, among many other neuronal functions, purinergic signalling is involved in neuronal-glial and glial-glial communication, which is vital in the processing of information in the brain (Fields and Burnstock, 2006).

In addition to the above, purinergic signalling is also implicated in many other physiological systems in the human body, including the digestive, skeletal, and respiratory systems. However, purine nucleotides and nucleosides also contribute to pathological conditions, such as Alzheimer's disease (Puertoet al.,2013), cancer (Di Virgilio, 2012) and cardiovascular disease (Ralevic and Burnstock, 2003).

Early History

The story behind purinergic signalling first began in 1776, when uric acid, a metabolite of purines, was isolated from bladder stones by Carl Wilhelm Scheele (Scheele, 1776). However, it was not until the late nineteenth century that several fundamental purines themselves, including adenine, were discovered (Jones, 1953). Upon solving the structure of caffeine and other associated compounds, Emil Fischer initially coined the term 'purines' in the early twentieth century (Fischer, 1907). As for purine nucleotides, adenosine 5'-triphosphate (ATP) was discovered in 1929 by the independent efforts of Cyrus Hartwell Fiske and Yellagaprada SubbaRow in the USA and Karl Lohmann in Germany (Fiske and SubbaRow 1929; Lohmann 1929).

Experimental study into the biological effects of purines subsequently ensued, including early studies into their effects on the nervous system in the 1940s and 1950s, and studies into the more peripheral effects of purines in the 1960s and 1970s (Burnstock and Verkhratsky, 2012). A significant moment came in 1978 when Geoffrey Burnstock suggested the very first classification of the receptors for purines, with the sub-types denoted, as they still are today, as P1 and P2 purinergic receptors (Burnstock, 1978). Further details about purinergic receptor sub-classification are described below.

1.3.2 - Purinergic Signalling – Receptor subtypes

There are two main classes of purinergic receptor, named P1 and P2, with both classes also possessing further classification into distinct subtypes. Adenosine is the agonist of the P1 receptors, with the four types of P1 receptor present in humans being A_1, A_{2A}, A_{2B}, and A_3. All adenosine receptors are G-protein coupled receptors (GPCRs), with the A_{2A}, A_{2B} receptors coupling to G_s proteins, which cause activation of adenylate cyclase (AC) and consequently an increase in cytosolic cyclic adenosine monophosphate (cAMP). Conversely, A_1 and A_3 interact with $G_{i/o}$ proteins, which inhibit AC and cause subsequent decrease in cytosolic cAMP concentration (Fredholm et al., 2011). A_{2B} can also couple to G_q, inducing IP_3 generation and intracellular calcium release (Haskó et al., 2009). All of the adenosine receptors possess 7 transmembrane domains and all have different, although sometimes overlapping, functions. For example, activation of the A_1 receptor inhibits neurotransmitter release from nerve cells, but as with A_{2A}, A_1 also

plays a role in the heart, with A_1 causing a reduction in heart rate, and A_{2A} promoting coronary vasodilation (Gao and Jacobson, 2011).

There are two main sub-types of P2 receptors, namely the ionotropic P2X and metabotropic P2Y receptors. P2X receptors are ligand-gated ion channels which are opened upon binding of ATP (North, 2002). Seven distinct ATP subtypes are known to exist in humans, known as $P2X_1$ through $P2X_7$, with all of them able to bind ATP. P2Y receptors are G protein-coupled receptors, which bind to, and hence are activated by, a variety of nucleotides, depending on the specific P2Y receptor subtype. The P2Y receptors that have so far been identified in humans are detailed in the following table.

P2Y Subtype	Known ligand(s)	G-protein coupling & signal transduction
$P2Y_1$	ADP	G_q – PLCβ, Rac, Rho activation
$P2Y_2$	ATP, UTP	G_q – PLCβ activation
		G_o – PLCβ, Rac activation
		G_{12} – Rho activation
$P2Y_4$	UTP	G_q – PLCβ activation
		G_o – PLCβ activation
$P2Y_6$	UDP, ADP	G_q – PLCβ activation
		$G_{12/13}$ – Rho activation
$P2Y_{11}$	ATP	G_q – PLCβ activation
		G_s – AC activation
$P2Y_{12}$	ADP	$G_{i/o}$ – AC inhibition
$P2Y_{13}$	ADP	$G_{i/o}$ – AC inhibition
$P2Y_{14}$	UDP-glucose	$G_{i/o}$ – AC inhibition

Data derived from (Erb and Weisman, 2012).

As with the adenosine P1 receptors, the P2X and P2Y receptors have a multitude of functions in health and disease, but the focus here is the ADP receptor $P2Y_{12}$.

1.4 - P2Y$_{12}$ Receptor

1.4.1 - P2Y$_{12}$ Receptor – Background & Function

In 2001, an elusive ADP receptor in platelets that could inhibit the activity of adenylate cyclase was cloned by three independent groups (Hollopeter et al., 2001. Zhang et al., 2000. Takasaki et al., 2001), and named as P2Y$_{12}$ by Hollopeter et al., (2001). This receptor, which is situated in the plasma membrane lipid rafts and predominantly takes the form as a homo-oligomer (Savi et al., 2006), is commonly expressed in platelets, glial cells, endothelial cells and smooth muscle cells (Cattaneo, 2006). ADP and its analogues, such as 2-methylthio-ADP, cause activation of P2Y$_{12}$, whereas ATP and its analogues inhibit the activity of the receptor (Kauffenstein et al., 2004). Once stimulated, P2Y$_{12}$ causes inhibition of adenylate cyclase and subsequent reduction in cAMP levels through activation of the G$_{\alpha i}$ subunit of the G protein that the receptor is coupled to (Quinton et al., 2005).

The most established role of P2Y$_{12}$ is arguably the activation, and aggregation, of platelets. Following interaction of platelets with a damaged endothelium, granule contents are secreted, which overall comprise over 300 active substances (Golebiewska and Poole, 2015). There are different types of intracellular granule, such as alpha and dense, each comprising different contents (Golebiewska and Poole, 2015). The released contents of dense granules include ADP, which elicits its effect though the P2Y$_1$ and P2Y$_{12}$ receptors on platelets, with P2Y$_{12}$ forming a crucial role in platelet function. For example, P2Y$_{12}$ has been found to potentiate dense granule secretion (Dangelmaier et al., 2001), activate the fibrinogen receptor (Jin and Kunapuli, 1998), and cause thrombus formation (van Gestel, 2003). Furthermore, although P2Y$_{12}$ has not been found to play a direct role in ADP-evoked platelet morphology change or intracellular calcium release, the G$_i$ signalling activated by P2Y$_{12}$ can ultimately induce platelet aggregation upon simultaneous G$_q$/G$_{12/13}$ pathway activation (Jin and Kunapuli, 1998), or by itself with high ADP concentrations (Ohlmann et al., 2000). The P2Y$_{12}$ signalling pathway also potentiates procoagulant activity (Kunapuli et al., 2003), α-granule release (Zhao et al., 2001), and P-selectin expression (Storey et al., 2002) in platelets. To summarise, P2Y$_{12}$ activation plays a vital role in haemostasis by stimulating granule secretion, platelet aggregation, procoagulant activity, and recruitment of platelets to injury sites.

1.4.2 - P2Y$_{12}$ Receptor – Roles in human disease

P2Y$_{12}$ has a vital role in haemostasis, as previously described, with those having a defect in P2Y$_{12}$ suffering bleeding diathesis, as characterised by excessive post-surgical and post-traumatic blood loss (Cattaneo, 2010). However, along with its haemostatic role in health, P2Y$_{12}$ is implicated in thrombus formation in atherosclerosis. Upon rupture of a vulnerable plaque, lipids, collagen and smooth muscle cells are exposed, causing activation of platelets and the coagulation cascade, resulting in the formation of a potentially occluding thrombus (Bentzon et al., 2014). Attempting to avoid the formation of a potentially life-threatening coronary thrombus using antiplatelet therapies is therefore of great use. Research from as early as the 1980s, even before P2Y$_{12}$ was cloned, showed that inhibition of P2Y$_{12}$ significantly reduced the risk of vascular thrombotic events (Cattaneo, 2015). One of the initial P2Y$_{12}$-targeted thienopyridine antiplatelet drugs, ticlopidine, proved effective in inhibiting platelet function and reducing vascular thrombotic events (Cattaneo, 2010). Ticlopidine is rarely used now, however, due to its toxicity. Other new generation anti-P2Y$_{12}$ drugs such as ticagrelor and prasugrel are now commonly used antiplatelet therapies that have proved more effective in preventing major cardiovascular events in those with acute coronary syndromes, with ticagrelor in particular being associated with a significant reduction in mortality (Cattaneo, 2015).

The role for platelet P2Y$_{12}$ in atherothrombosis is clear, however this receptor has potential atherosclerotic functions in other cells. Notably, findings by West et al. (2014) suggest a role for vessel wall, rather than platelet, P2Y$_{12}$ in early atherogenesis. Compared to the ApoE-knockout control, ApoE-deficient mice lacking in vessel wall P2Y$_{12}$ exhibited significantly reduced atheroma, which was conversely not the case in ApoE-knockout mice deficient in platelet P2Y$_{12}$. Although, this study did not state which specific cell types in the vessel wall express P2Y$_{12}$. Furthermore, mice given doses of ticagrelor or clopidogrel lasting four weeks did not show any significant reduction in atheroma formation compared to the control. This study used bone marrow transplants generating chimeric mice in an attempt to establish the role of platelet or vessel wall P2Y$_{12}$. However, the study did not investigate the effect of P2Y$_{12}$ deficiency in other bone-marrow derived cells, such as monocytes. Therefore, although an atherogenic role

for $P2Y_{12}$ outside platelets has been suggested, these findings are limited, and so investigating the expression and function of $P2Y_{12}$ in human monocytes would be very useful. In addition to cardiovascular disease, $P2Y_{12}$ 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 $P2Y_{12}$ have been suggested and should be further investigated. This project aims to establish the expression of $P2Y_{12}$ in monocytes and to gain insight into the role of the receptor in ADP-evoked calcium responses in THP-1 cells.

Chapter 2 – Materials & Methods

2.1 - Chemicals, reagents and dilutions

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 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% bovine serum albumin), MRS2578, LY294002, SQ22536 and ticagrelor (SBS buffer containing 1% DMSO).

2.2 – THP-1 Cell culture & CD14+ cells

Cells from the THP-1 cell line, which is a human monocytic cell line cultured from the blood of an acute monocytic leukaemia patient (Tsuchiya et al., 1980), were cultured at 37°C and 5% CO_2 in RPMI 1640 medium containing 2 mM L-glutamine (ThermoFisher) and supplemented with 10% (v/v) foetal bovine serum (FBS), 50 IU/ml penicillin and 50 μg/ml streptomycin (ThermoFisher). Cells were maintained at a density between 1×10^5 and 1×10^6 cells/ml.

CD14+ monocyte RNA was kindly donated by Dr. Priscilla Day, a colleague, who extracted the RNA from CD14+ cells obtained from PBMCs isolated from the blood of human volunteers.

2.3 - Measurement of intracellular calcium

1×10^6 THP-1 cells/ml were loaded with 2 μM Fura-2 in SBS buffer plus 0.01% (w/v) pluronic acid to ensure that fura-2 is internalised into the cell and can therefore sufficiently bind to intracellular Ca^{2+}. The loading time was 1 hour at 37°C with gentle shaking of the cells every 15 minutes. After loading, the cells were pelleted and washed with 20 ml excess SBS buffer before being plated in a clear-bottomed 96-well plate at a density of 2×10^5 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. The FlexStation® 3 instrument (Molecular Devices) adds a 50 μl volume of prepared agonist to the cells and subsequently measures fura-2 fluorescence (340 nm excitation when Ca^{2+}-bound; 380 nm excitation when unbound; 510 nm emission) at intervals of 4 seconds at 37°C giving representative traces of F ratio (ratio between 340-

380nm excitation) vs. time. The operation of the FlexStation instrument was managed via Softmax Pro software, which also allowed raw data to be collected. SoftMax Pro subtracted the detected resting Ca^{2+} level prior to challenging with agonists, giving representative traces of F ratio vs. time starting at 0.

2.4 - Immunocytochemistry

Cover slips were inserted into 6-well clear-bottomed plates and were coated with polylysine overnight to improve adherence of the THP-1 cells which were then seeded on the slides and incubated for 2 hours. The cells were then washed twice with phosphate-buffered saline (PBS), fixed with 4% (w/v) paraformaldehyde in PBS (pH 7.4), washed twice with PBS and then permeabilised by incubating with 0.25 % (v/v) Triton X-100 (ThermoFisher) in PBS for 10 minutes. After washing with PBS three times for 5 minutes, the cells were blocked by incubating them with 1% (w/v) BSA in PBS for 30 minutes at room temperature. The primary antibody (Goat polyclonal IgG, Santa Cruz Biotechnology no. # sc-27152) was diluted to an antibody/cells ratio of 1/200 in 1% (w/v) BSA and added to the cells which were then incubated overnight at 4 °C. The secondary antibody (Rabbit anti-Goat polyclonal IgG conjugated to Alexa Fluor® 488, Abcam no. #ab150141) was diluted to an antibody/cells ratio of 1/1000 in 1% (w/v) BSA and added to the cells which were then incubated in the dark for 60 minutes at room temperature. VECTASHIELD®, containing DAPI (Vector Laboratories), was added to microscope slides and, after washing with PBS three times for 5 minutes, the cover slips, containing the cells, were placed cells-side down onto the VECTASHIELD®. The edges of the cover slip were sealed with nail polish to ensure an air-tight environment for the cells, and the microscope slides were kept at 4 ºC in the dark.

2.5 - RNA extraction

1×10^6 cells were centrifuged at 2000 rpm for 7 minutes, and the supernatant was removed. The pellet was lysed with 1 ml Tri reagent and vortexed until no cellular debris was present. The cell lysate was allowed to stand for 5 minutes at room temperature before 0.1 ml 1-bromo-3-chloropropane was added to the lysate, which was then vortexed and allowed to stand for 15 minutes at room temperature. The mixture was then centrifuged at 14,800 x g for 15 minutes at 4 °C, which caused separation of the resulting suspension into three phases, with the top aqueous phase

contains the RNA. The aqueous phase was transferred to a new 1.5 ml Eppendorf tube, along with 0.5 ml 2-propanol, and the mixture was vortexed for 15 seconds. The mixture was allowed to stand at room temperature for 10 minutes before being centrifuged at 14,800 x g for 10 minutes at 4 °C. The resulting pellet contains the RNA, therefore the supernatant was carefully removed, and the pellet was vortexed for 15 seconds with 1 ml 75% pre-chilled ethanol to wash the pellet. The mixture was centrifuged at 14,800 x g for 10 minutes and the supernatant was removed before allowing the pellet to be air-dried for 15 minutes in a sterile hood. A 40 μl amount of RNase-free water was added, and the mixture gently resuspended before heating for 5 minutes at 65 ºC. The resulting RNA quality and quantity was determined using a Nanodrop spectrophotometer.

2.6 - Reverse-Transcription Polymerase Chain Reaction

2.6.1 - Reverse transcription

RNA was primed by adding 200 ng of random hexamers (Invitrogen) to 1 μg RNA, with RNase-free water added to make the solution up to a total volume of 11 μl, which was then incubated for 10 minutes at 70 ºC. A master-mix was made, containing 4 μl 5x first strand buffer (Invitrogen), 2 μl 0.1M DTT (Invitrogen), 1 μl Superscript II reverse transcriptase (200U) (Invitrogen) (substituted with 1 μl RNase-free water for no-RT control), 0.5 μl dNTPs (10 μM) (Promega), 0.75 μl RNasin® ribonuclease inhibitor (30U) (Promega) and 0.75 μl RNase-free water, which was added to the randomly primed RNA and incubated for 1 hour at 42 ºC and then for 10 minutes at 70 ºC. The resulting cDNA was then stored at -20 ºC.

2.6.2 - Polymerase chain reaction

PCR was performed according to the procedure listed in the Sigma-Aldrich 'ReadyMix™ Taq PCR Reaction Mix with $MgCl_2$' product technical bulletin, which was implemented using the conditions outlined below. Separate template-positive and non-template tubes were prepared for each PCR reaction. Each template-positive PCR tube consisted of 12.5 μl ReadyMix™ Taq PCR Reagent Mix, 0.5 μl forward primer, 0.5 μl reverse primer, 2 μl template cDNA and the total volume was made up to 25 μl using PCR water. Each non-template tube contained the same amounts of reagents as above, with the exception of 2 μl template cDNA, which was substituted with 2 μl PCR water. The tubes for each reaction

were flicked to ensure mixing, and centrifuged briefly to allow sedimentation before being inserted into an 'Eppendorf Mastercycler EP S' thermal cycler. The samples were heated to 94 ºC for 4 minutes to enable activation of the enzymes included in the ReadyMix™ Taq PCR Reagent Mix. The samples were then subjected to 35 cycles of the following thermal cycling conditions; the cDNA templates were denatured at 94 ºC for 1 minute, annealing of primers at 55 ºC for 2 minutes, and extension of product at 72 ºC for 3 minutes. The PCR products were then kept in the cycler at 4 ºC until they could be removed 1-2 hours later.

Primer details

Gene	Accession no.	Sequence	Size (bp)
P2RY1	NM_002563	GTTCAATTTGGCTCTGGCCG (5'-3') TTTTGTTTTTGCGGACCCCG (3'-5')	326
P2RY6	NM_176798	GCTCTCACTGTCATCGGCTT (5'-3') TCTGCCATTTGGCTGTGAGT (3'-5')	391
P2RY12	NM_022788	ACTGGGAACAGGACCACTGA (5'-3') CAGAATTGGGGCACTTCAGC (3'-5')	698
P2RY13	NM_176894	TTCCCAGCCCTCTACACAGT (5'-3') GGCCCCTTTAAGGAAGCACA (3'-5')	461

2.7 - Quantitative Real-time Polymerase Chain Reaction

The following was added into each well of a 96-well qPCR plate; 10 µl TaqMan® Gene Expression Master Mix (ThermoFisher), 1 µl Applied Biosystems® Custom TaqMan® MGB Probes (P2Y$_{12}$, GAPDH, B2M) and 4 µl molecular grade water. Followed by 5 µl of 2 ng/µl cDNA into each well. The plate was then sealed and centrifuged at 1000 rpm for 1 minute before being subjected to the default run method included in the Applied Biosystems® 7500 Fast Real-Time PCR System, in which the plate was analysed. Relative quantifications of mRNA were calculated using the $\Delta\Delta C_T$ method. The geomean of GAPDH and B2M expression was used for a housekeeping control.

2.8 - Agarose Gel Electrophoresis

A 2% (w/v) Agarose gel was made using Agarose powder and 1 X TAE buffer, with 3 µl ethidium bromide added. A 5 µl amount of loading dye was added to the samples intended to be run and also to the DNA ladder. The DNA ladder and the samples were added to the wells (30 µl) and electrophoresis was performed at 90V.

2.9 - Human P2Y6-overexpressing 1321N1 cells

Astrocytomas from the 1321N1 cell line overexpressing human $P2Y_6$ were donated as a gift from Jean-Marie Boeynaems (The Université libre de Bruxelles). The transfection, as conducted by Jean-Marie Boeynaems, was achieved using a recombinant pcDNA3 plasmid encoding human $P2Y_6$, as described in Communi et al. (1996). The cells were cultured under G418 selection (0.4 mg/ml) at 37°C and 5% CO_2 in DMEM medium supplemented with 10% (v/v) FBS, 50 IU/ml penicillin and 50 µg/ml streptomycin. For calcium experiments 2.5×10^4 cells/well were plated overnight in clear-bottomed black 96-well plates and measured for intracellular calcium the next day using the method described previously.

2.10 - P2Y$_{12}$-knockdown THP-1 cells

THP-1 cells were incubated overnight in complete RPMI (+ 10% FBS) without antibiotic before cells were transfected using siRNA and DharmaFECT 2 transfection reagent (Dharmacon Research) using the following protocol in 96-well format. Two tubes denoted as tube 1 and tube 2 were prepared using the following method (quantities are **per well**); tube 1 - 0.5 µl of 5 µM siRNA was gently mixed with 9.5 µl Optimem (ThermoFisher), tube 2 - 0.5 µl of DharmaFECT 2 was gently mixed with 9.5 µl Optimem. Tubes 1 & 2 were incubated for 5 mins at room temperature before adding the contents of tube 1 to tube 2 to form a transfection medium, and then incubating at room temperature for a further 20 mins. THP-1 cells were then counted, centrifuged and the resulting pellet resuspended with complete RPMI. Cells were prepared for a final amount of 1×10^5 cells/well, with a volume of 80 µl of the cell suspension added to the aforementioned transfection medium. The cells, combined with the transfection medium, were then plated in a 96-well plate and incubated at 37°C, 5% CO_2 for 48 hrs before proceeding with specified experiments.

Three different siRNA treatments were prepared per experiment; $P2Y_{12}$-targeted (ThermoFisher #AM16708), GAPDH-targeted (ThermoFisher #AM4605), or negative control siRNA (Ambion® Silencer® Negative Control #1 - ThermoFisher #AM4611), and the final siRNA concentration in each experiment was 25 nM.

2.11 - Statistical analysis

Data analysis was performed using Origin Pro 9.0 software (Origin Lab, USA). Dose–response curves were fitted by Origin, which used the Hill equation below 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 mean of the peaks from the respective Flexstation f-ratio outputs.

As obtained from Origin software, the following shows the equation of the Hill curve used when plotting dose-response curves, where k represents the Michaelis constant and n represents the Hill coefficient:

$$y = START + (END - START)\frac{x^n}{k^n + x^n}$$

2.12 - Pharmacological inhibitors used

Name	Function	Reported IC_{50}	IC_{50} Reference
U73122	PLC inhibitor	1.5-1.8 μM (rodent osteoblastic cells)	Tatrai et al., 1994
Thapsigargin	SERCA inhibitor	30 nM (rat liver microsomes)	Thastrup et al., 1990
Ticagrelor	$P2Y_{12}$ antagonist	0.24 μM (platelet aggregation)	FDA, 2009
PSB-0739	Selective $P2Y_{12}$ antagonist	K_i – 25 nM	(Baqi et al., 2009)
MRS 2578	Selective $P2Y_6$ antagonist	37 nM ± 16 (1321N1 astrocytomas)	Mamedova et al., 2004
MRS 2500	Selective $P2Y_1$ antagonist	0.95 nM (platelet aggregation)	Cattaneo et al., 2004
MRS 2211	Selective $P2Y_{13}$ antagonist	pIC_{50} – 5.97 (1321N1 astrocytomas)	Kim et al., 2005
NBMPR	ENT-1 inhibitor	3.6 nM ± 1.4 (*Xenopus oocytes*)	Yao et al., 1997
LY294002	PI3K inhibitor	1.4 μM	Viahos et al., 1994
Pertussis Toxin	Inhibits coupling to G_i protein	~16 ng/ml (2hr incubation) (Rat adenohypophyseal cells)	Musset et al., 1990
SQ22536	Adenylate cyclase inhibitor	21 μM (Human blood platelets)	Harris et al., 1979

Chapter 3 – Results

3.1 - Establishing ADP-evoked calcium responses in THP-1 monocytes

3.1.1 - Introduction & Aim

Calcium responses in human cells can occur through different methods. The mechanism investigated is the phospholipase C pathway, which is described as follows. A certain type of cell surface receptor, called G protein-coupled receptors, are activated by external stimuli, such as extracellular nucleotides, causing activation of PLC. PIP2 is then hydrolysed by PLC into IP_3 and DAG. IP_3 subsequently causes release of calcium ions from the endoplasmic reticulum through IP_3 receptors, giving rise to a sharp increase in cytosolic calcium ion concentration.

Before investigating the function of $P2Y_{12}$ in monocytes, it would be useful to establish ADP-evoked calcium responses, and attempt to confirm whether ADP causes calcium responses via the classical signalling transduction pathway described above. In order to do this, calcium responses were measured in THP-1 cells when stimulated with ADP. Moreover, the effect of selective inhibitors for some proteins involved in the signalling pathway described above on ADP-evoked calcium responses was also determined. The half-maximal effective concentration (EC_{50}) for ADP-evoked maximal platelet aggregation in Ossabaw swines was reported as being 3.64 µM ± 2.2 µM (Kreutz et al., 2011), thus influencing the choice of ADP concentrations used in figure 1.

Figure 1 – ADP evokes calcium responses in THP-1 cells in a dose-dependent manner

Fura-2-loaded THP-1 monocytes were challenged with either 3 µM ADP (A), or increasing concentrations of ADP (B). Shown is a representative time-resolved trace (A), and a dose response curve of the peaks of the calcium responses (B), represented in the form of F Ratio (340/380), with the data points representing mean ± SEM. ADP EC_{50} = 2.66 µM ± 0.25. N=3

The findings shown in **figure 1** suggest that ADP does induce calcium responses in THP-1 cells, with an observed EC50 of 2.66 µM ± 0.25.
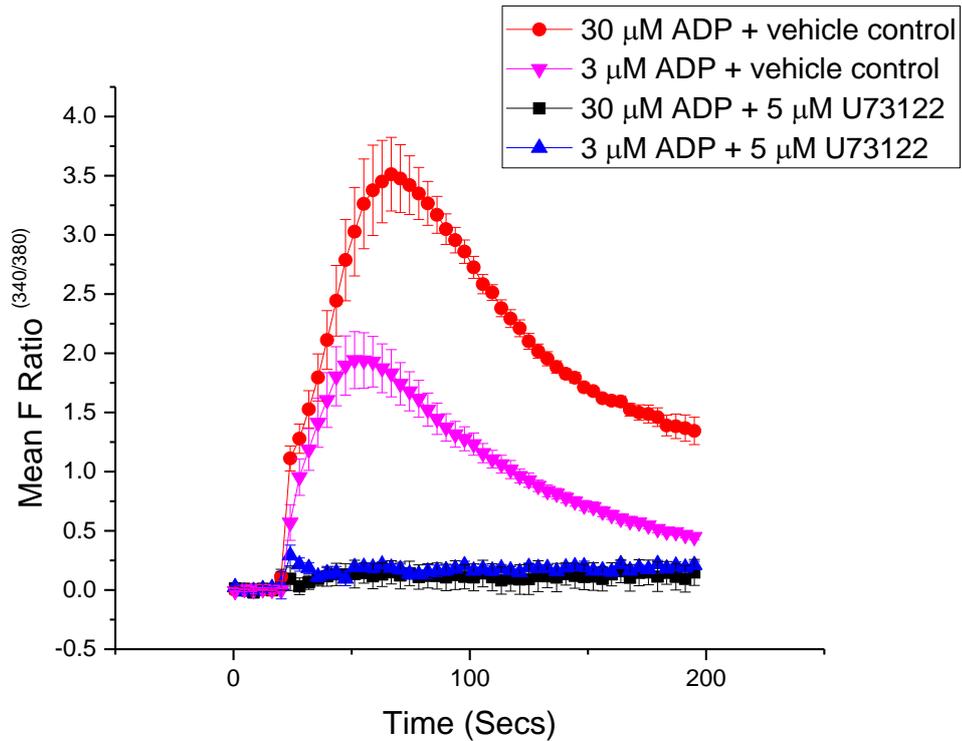
Figure 2 – The Phospholipase C inhibitor U73122 abolishes ADP-evoked calcium responses in THP-1 monocytes

Fura-2-loaded THP-1 monocytes were pre-incubated for 30 mins with 5 µM U73122 or a vehicle control before being challenged with 30 or 3 µM ADP. Shown are the representative traces of the calcium responses, represented in the form of F Ratio (340/380), with the data points representing mean ± SEM. N=3.

To test whether calcium responses evoked by ADP are mediated through activation of PLC, the effect of a selective PLC inhibitor U73122 (5 μM) on ADP-evoked calcium responses was determined in THP-1 cells, with the results shown in **figure 2**. U73122 totally abolished calcium responses induced by maximal (30 μM), and approximate EC50 (3 μM) ADP concentrations, indicating that ADP-induced calcium responses are dependent on PLC activation. As G_q-coupled receptor activation causes PLC stimulation, this indicates that the calcium responses evoked by ADP are primarily mediated through a G_q-coupled receptor.

Another study showed that U73122 inhibited endothelin-1- and parathyroid hormone-evoked intracellular calcium signals in rodent osteoblastic cells with an IC_{50} of 1.5-1.8 μM (Tatrai et al., 1994).
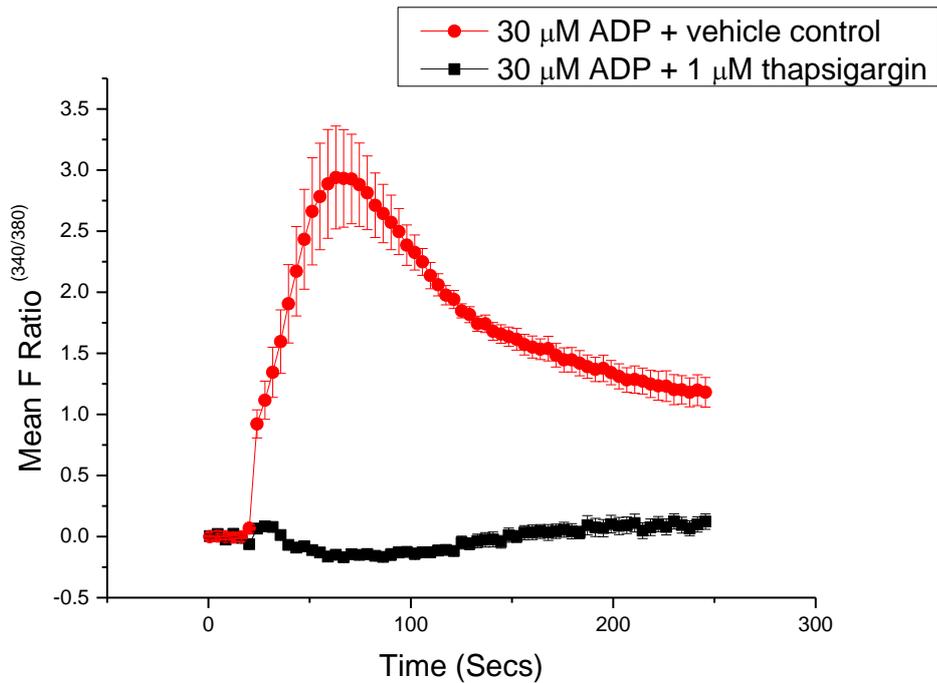
Figure 3 – The SERCA inhibitor thapsigargin abolishes ADP-evoked calcium responses in THP-1 monocytes

Fura-2-loaded THP-1 monocytes were pre-incubated for 30 mins with 1 µM thapsigargin or a vehicle control before being challenged with 30 µM ADP. Shown are the representative traces of the calcium responses, represented in the form of F Ratio (340/380), with the data points representing mean ± SEM. N=3.

Classical G_q protein-coupled receptor-mediated calcium responses occur through calcium release from the endoplasmic reticulum (ER), and the sarco-endoplasmic reticulum. The Ca^{2+}-ATPase (SERCA) calcium ion pump is responsible for transferring calcium ions into the ER and therefore maintaining the calcium store. Inhibition of SERCA would deplete the ER calcium store, and the effect of the SERCA inhibitor thapsigargin on ADP-evoked calcium responses was investigated in THP-1 cells.

Figure 3 shows that thapsigargin eliminates calcium responses evoked by a maximal concentration of ADP, indicating that ADP-induced calcium responses are dependent upon the ER store of calcium ions. In summary, the results from figures 2 and 3 suggest that calcium responses evoked by ADP in THP-1 cells are mediated by the signal transduction pathway downstream of G_q protein-coupled receptor activation, which was the expected outcome. A previous study demonstrated that thapsigargin inhibits the Ca^{2+}-ATPase activity of rat liver microsomes with an IC_{50} value of 30 nM (Thastrup et al., 1990), supporting the choice of thapsigargin concentration in figure 3 as a maximal concentration. Furthermore, thapsigargin-mediated inhibition of the endoplasmic reticulum isoform of the Ca^{2+}-ATPase in rat liver microsomes was shown to be selective, as thapsigargin had little effect on plasma membrane Ca^{2+}-ATPases in hepatocytes or erythrocytes, or on the sarcoplasmic reticulum of cardiac or skeletal muscle cells (Thastrup et al., 1990).

3.2 - Characterising the expression of P2Y$_{12}$ in human monocytes

3.2.1 - Introduction & Aim

It has been shown in previous studies that the P2Y$_{12}$ receptor is expressed in platelets, microglia, endothelium and smooth muscle cells (Cattaneo, 2006), however there are no publications to date that have characterised the expression of P2Y$_{12}$ in monocytes. In order to investigate the involvement of P2Y$_{12}$ in ADP-evoked calcium responses in monocytes, it was necessary to confirm the gene and protein expression of P2Y$_{12}$ in these cells. P2Y$_{12}$ gene expression in both THP-1 cell line monocytes and CD14+ primary monocytes was determined to ensure that P2Y$_{12}$ expression is not a cell-line specific artefact. The aim here was also to establish the gene expression of other P2Y receptors activated by ADP to highlight the other possible candidate receptors involved in mediating the ADP-evoked calcium response in monocytes. This was a useful step which helped to assess what ADP-activated P2Y receptors are present in monocytes before pharmacologically investigating the specific potential receptors involved in mediating ADP-induced calcium responses. RT-PCR was used to determine gene expression, with immunocytochemistry used to establish the protein expression.

Figure 4 – The P2Y$_{12}$ gene is expressed at the mRNA level in THP-1 and CD14+ monocytes

PCR was conducted as stated in materials and methods using cDNA from THP-1 (**A**) and primary CD14+ (**B**) monocytes, with the products separated using agarose gel electrophoresis. Expected band sizes for ADP P2Y receptors (bp) - P2Y$_1$: 326, P2Y$_6$: 391, P2Y$_{12}$: 698, P2Y$_{13}$: 461. 'no RT' indicates negative control products from PCR reactions in the absence of reverse transcriptase. N=1.

The results in **figure 4** show that the ADP-responsive P2Y receptors P2Y$_1$, P2Y$_6$, P2Y$_{12}$ and P2Y$_{13}$ are expressed at the mRNA level in THP-1 cells. Therefore, all such receptors were considered when proceeding with investigating the P2Y receptors responsible for ADP-evoked calcium responses in THP-1 cells. P2Y$_{12}$ mRNA was shown also to be expressed in primary CD14+ monocytes to indicate that P2Y$_{12}$ epression in THP-1 monocytes is not a cell-line specific artefact.
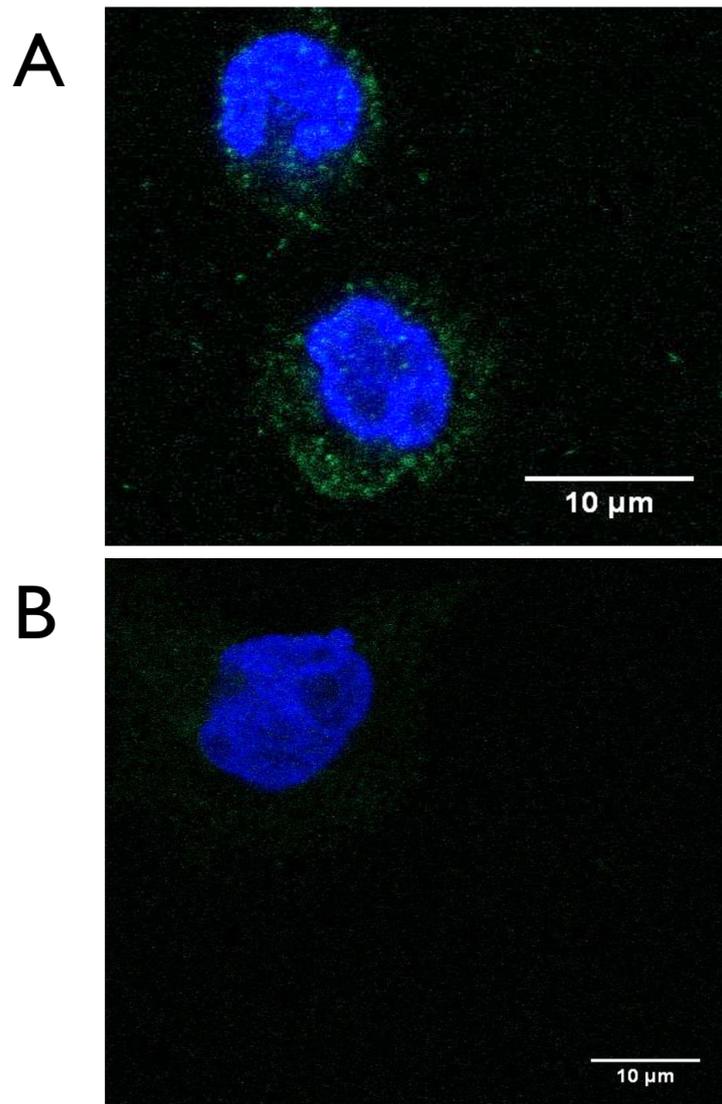
Figure 5 – P2Y$_{12}$ protein is expressed in THP-1 cells

Immunocytochemistry was performed as stated in materials and methods to generate THP-1 monocytes stained for P2Y$_{12}$ (intracellular epitope) using a fluorescent-green AF488-conjugated secondary antibody. The images taken from confocal microscopy above show (**A**) cells with both primary and secondary antibodies present and (**B**) the negative control with only the secondary antibody present. Cells were counter-stained with DAPI (blue). The two images have been equally exposed. N=1.

An antibody directed against an intracellular $P2Y_{12}$ epitope was used during the immunofluorescent staining of $P2Y_{12}$, and the confocal images of the cells show in **figure 5** that $P2Y_{12}$ protein is expressed in THP-1 monocytes. This correlates with the results from figure 4 to confirm that THP-1 $P2Y_{12}$ mRNA is translated into protein in the cell.

3.3 - Pharmacological dissection of ADP-activated P2Y receptors in THP-1 cells
3.3.1 - Introduction & Aim
The G protein-coupled P2Y receptors $P2Y_1$, $P2Y_6$, $P2Y_{12}$ and $P2Y_{13}$ all have the capability to be activated by ADP and may theoretically contribute to the ADP-induced calcium response. Calcium responses triggered through the phospholipase C pathway can occur upon the activation of G_q-coupled receptors, which causes PLC-mediated breakdown of PIP2 into IP_3 and DAG and subsequent calcium ion (Ca^{2+}) release through endoplasmic reticulum IP_3 receptors. $P2Y_1$ and $P2Y_6$ are G_q-coupled and therefore can evoke such calcium responses when activated by ADP. $P2Y_{12}$ is G_i coupled, and G_i-coupled P2Y receptors have not been shown to directly cause cytosolic Ca^{2+} release, but cause inhibition of adenylate cyclase instead. Therefore, it may seem unlikely that $P2Y_{12}$ would contribute to ADP-evoked calcium responses. However, there have been numerous studies in platelets demonstrating that $P2Y_{12}$ modulates the calcium response mediated by $P2Y_1$ activation, indicating that it would be worthwhile to investigate whether $P2Y_{12}$ is involved in ADP-evoked calcium responses in THP-1 cells.

The objective here was to determine the effect of using pharmacological inhibitors for $P2Y_1$, $P2Y_6$, $P2Y_{12}$ or $P2Y_{13}$ to indicate which of these ADP receptors have an involvement in causing calcium responses to ADP in THP-1 cells. The focus here was on the involvement of $P2Y_{12}$, however it was important that all aforementioned ADP-activated P2Y receptors were considered, particularly as they were all found to be expressed in THP-1 monocytes.

Figure 6 – Ticagrelor attenuates ADP-evoked calcium responses in a dose-dependent manner

Fura-2-loaded THP-1 monocytes were pre-incubated for 30 mins with 3 µM ticagrelor (A), or increasing concentrations of ticagrelor (B) before being challenged with 3 µM ADP. Shown are representative time-resolved traces (A), and an inhibitory dose response curve of the peaks of the calcium responses (B), represented in the form of F Ratio (340/380), with the data points representing mean ± SEM. Ticagrelor IC_{50} = 1.29 µM (calculated manually due to plotting issues with Origin). N=3. * denotes $P<0.005$ vs '3 µM ADP + vehicle control'

Ticagrelor is a clinically-administered P2Y$_{12}$ antagonist, and **Figure 6** illustrates the effect of using ticagrelor to inhibit the calcium responses induced by an approximate EC$_{50}$ ADP concentration of 3 µM in THP-1 cells. There is a clear concentration-dependent effect of ticagrelor on ADP-evoked calcium responses indicated by the inhibitory dose-response curve generated.

The FDA reported in 2009 that ticagrelor inhibited 6.5 µM ADP-induced platelet aggregation in human blood in a dose dependent manner, yielding a half-maximal inhibitory concentration (IC$_{50}$) of 0.24 µM. This helped determine the concentration range of ticagrelor to use for figure 6.

Figure 7 – Ticagrelor inhibits ADP-evoked calcium responses in a non-competitive manner

Fura-2-loaded THP-1 monocytes were pre-incubated for 30 mins with 0.1, 1 or 5 μM ticagrelor or vehicle control before being challenged with 30 μM (A), or increasing concentrations of ADP (B). Shown are representative time-resolved traces (A) and the dose response curves of the peaks of the calcium responses (B), represented in the form of F Ratio (340/380), with the data points representing mean ± SEM. 'ADP + vehicle control' $E_{max} = 0.7$, 'ADP + 0.1 μM ticagrelor' $E_{max} = 0.58$, 'ADP + 1 μM ticagrelor' $E_{max} = 0.33$, 'ADP + 5 μM ticagrelor' $E_{max} = 0.30$. The data was normalised to the highest value generated from all repeats across the treatments. N=3. * denotes $P < 0.05$ vs 'ADP + vehicle control' of E_{max} value, and between peaks in (A), as determined by a paired student's t-test.

The effect of 3 increasing Ticagrelor concentrations on calcium responses induced by ADP is illustrated in **figure 7**. Ticagrelor concentrations of 1 and 5 μM caused a significant reduction in the maximal effect of the agonist (E_{max}), with 5 μM ticagrelor representing an approximate IC_{50} concentration. This reduction in E_{max} demonstrates that ticagrelor acts as a non-competitive inhibitor for ADP-evoked calcium responses in THP-1 cells. This is consistent with the findings observed by van Giezen et al. (2009), who report that ticagrelor binds to $P2Y_{12}$ independently from ADP, and non-competitively inhibits ADP-induced aggregation of human washed platelets.
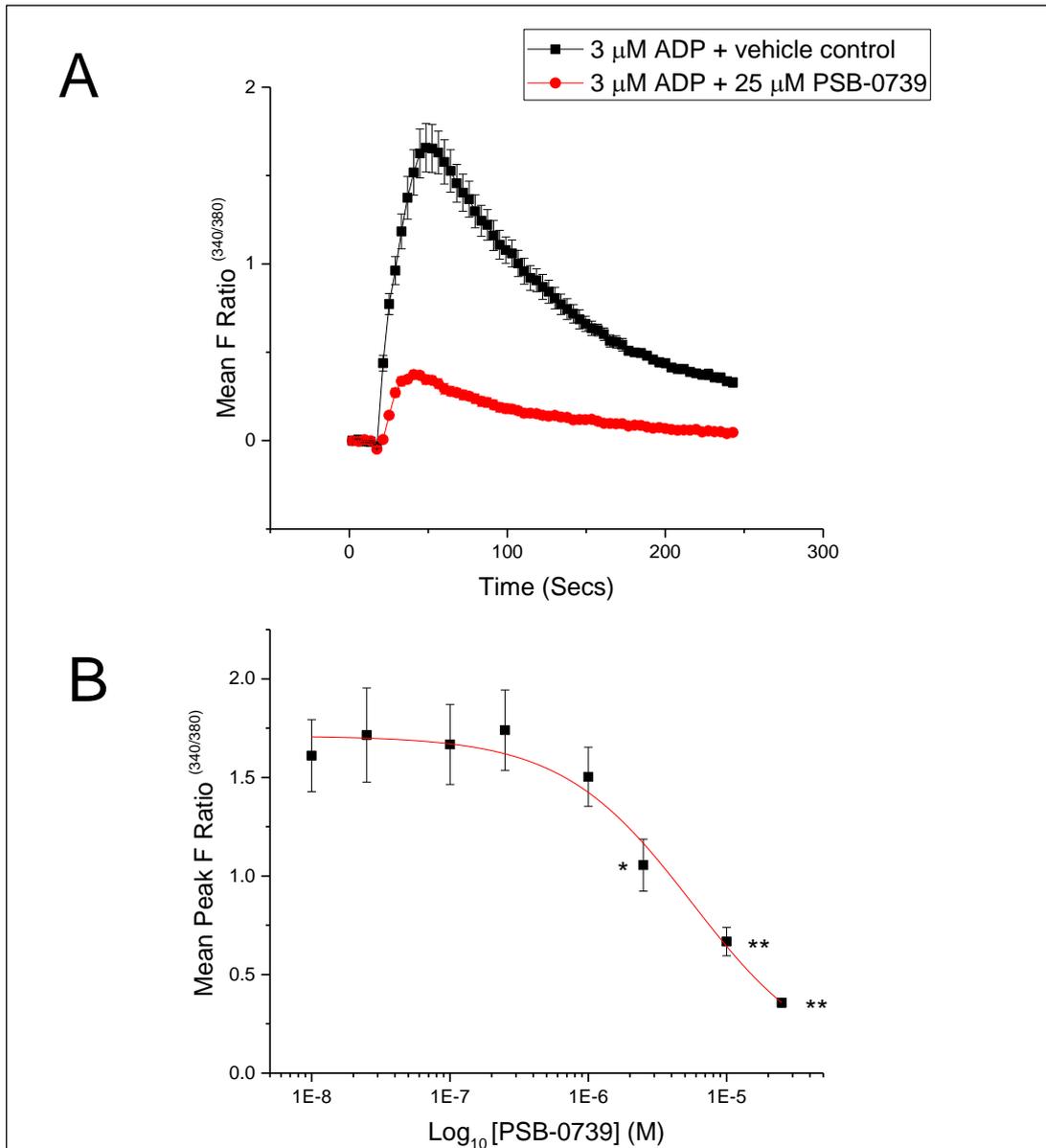
Figure 8 – PSB-0739 reduces ADP-evoked calcium responses in a dose-dependent manner in THP-1 cells

Fura-2-loaded THP-1 monocytes were pre-incubated for 30 mins with 25 μM PSB-0739 (A) or increasing concentrations of PSB-0739 (B) before being challenged with 3 μM ADP. Shown are representative time-resolved traces (A) and an inhibitory dose response curve of the peaks of the calcium responses (B), represented in the form of F Ratio (340/380), with the data points representing mean ± SEM. PSB-0739 IC_{50} = 5.61 μM ± 2.1. N=3. * denotes P<0.05 and ** denotes P<0.01 vs '3 μM ADP + vehicle control'.

To determine whether P2Y$_{12}$ inhibitors other than ticagrelor could have similar effects to the findings presented in figures 6 and 7, the impact of PSB-0739, an alternative selective P2Y$_{12}$ antagonist, on 3 μM ADP-evoked calcium responses was assessed, the data are presented in **figure 8**. As seen from the evident inhibitory dose-response curve shown, PSB-0739 caused a concentration-dependent effect on ADP-induced calcium responses in THP-1 cells. PSB-0739 was found here to have an IC$_{50}$ of 5.6 μM ± 2.1 for ADP-evoked calcium responses, which is a similar IC$_{50}$ to that observed for ticagrelor from figure 4. There are no relevant published IC$_{50}$ values to compare to, however PSB-0739 has a reported K$_i$ of 25 nM (Baqi et al., 2009), which would suggest it is highly potent, however K$_i$, which measures inhibitor-receptor affinity, does not always correlate with efficacy.
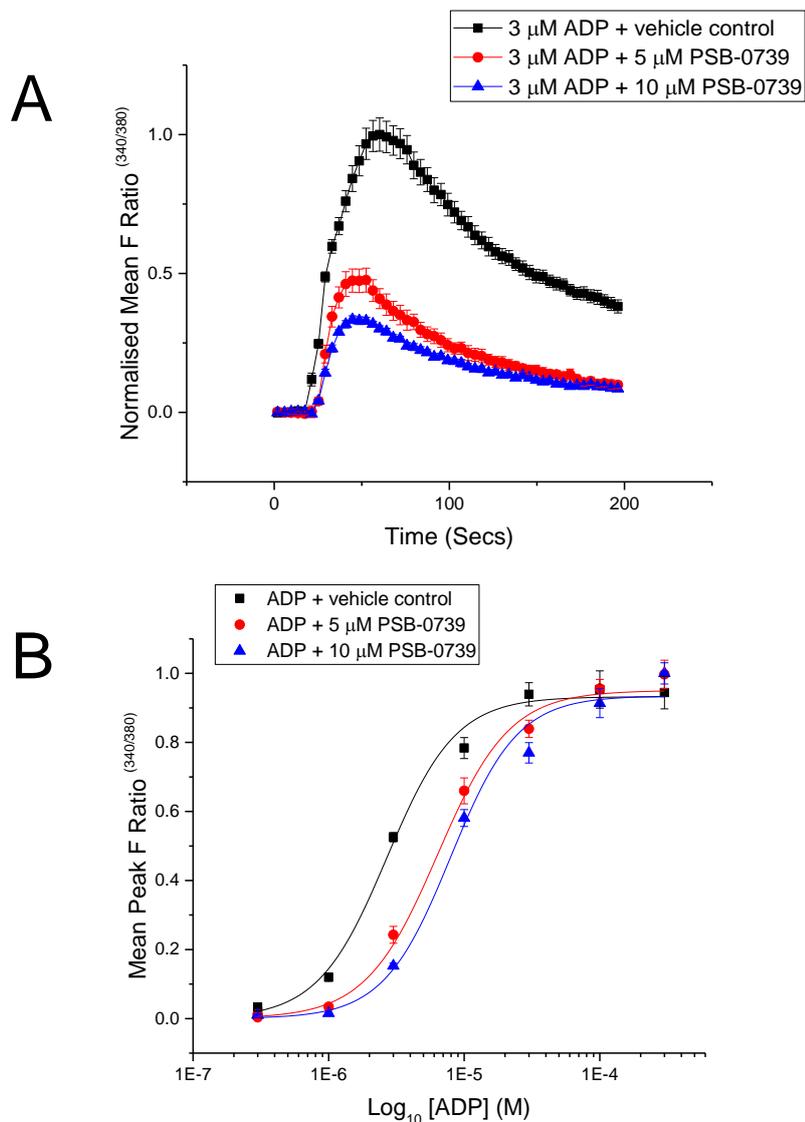
Figure 9 – PSB-0739 inhibits ADP-evoked calcium responses in a competitive manner in THP-1 cells

Fura-2-loaded THP-1 monocytes were pre-incubated for 30 mins with 5 or 10 μM PSB-0739 or a vehicle control before being challenged with 3 μM (A) ADP or increasing concentrations of ADP (B). Shown are the representative time-resolved traces (A) and the dose response curves of the peaks of the calcium responses (B), represented in the form of F Ratio (340/380), with the data points representing mean ± SEM. 'ADP + vehicle control' EC_{50} = 2.68 μM ± 0.26, 'ADP + 5 μM PSB-0739' EC_{50} = 6.36 μM ± 0.74, 'ADP + 10 μM PSB-0739' EC_{50} = 7.93 μM ± 1.47. The data was normalised to the highest value generated from all repeats across the treatments. N=3. EC_{50} values in (B) between effect of 10 μM PSB-0739 vs. vehicle control are significantly different to P<0.05.

Figure 9 shows the effect of pre-incubating two different PSB-0739 concentrations on subsequent calcium responses induced by ADP. PSB-0739 caused a dose-dependent rightward shift in the ADP-evoked dose-response curve, and a dose-dependent increase in EC_{50} value, indicating that PSB-0739 acts competitively in inhibiting ADP-evoked calcium responses. Furthermore, 10 μM caused a statistically significant increase in EC_{50}. It is well established in literature that PSB-0739 acts as a competitive antagonist, as found by Hoffmann et al. (2009) for example, who found that PSB-0739 caused a rightward shift in the inhibitory dose-response curve caused by 2-methylthio-ADP-induced reduction of forskolin-evoked cAMP production in Chinese hamster ovary cells.

Overall the results shown in figures 6-9 suggest that ADP-evoked calcium responses in THP-1 cells are dependent on $P2Y_{12}$ activation due to the fact that the $P2Y_{12}$ inhibitors Ticagrelor and PSB-0739 cause significant decrease in ADP-evoked calcium responses.

Figure 10 – The P2Y$_6$ inhibitor MRS 2578 reduces ADP-evoked calcium responses in a dose-dependent manner

Fura-2-loaded THP-1 monocytes were pre-incubated for 30 mins with a vehicle control or 10 μM MRS 2578 (A), or increasing concentrations of MRS 2578 (B), before being challenged with 3 μM ADP. Shown are representative time-resolved traces (A) and an inhibitory dose response curve of the peaks of the calcium responses (B), represented in the form of F Ratio (340/380), with the data points representing mean ± SEM. MRS 2578 IC$_{50}$ = 0.2 μM ± 0.029. * denotes $P<0.05$ and ** denotes $P<0.01$ vs '3 μM ADP + vehicle control'.

P2Y$_6$ is primarily a UDP receptor but it can also be activated by its partial agonist ADP (Communi et al., 1996), and with the purpose of determining the role that ADP receptors other than P2Y$_{12}$ play in ADP-induced calcium responses, the effect of a P2Y$_6$ inhibitor on ADP-evoked calcium responses was established. In a similar fashion to the P2Y$_{12}$ inhibitors, the P2Y$_6$ inhibitor MRS 2578 caused a dose-dependent reduction in calcium responses evoked by an approximate EC$_{50}$ concentration of ADP, as demonstrated in **figure 10**.

MRS 2578 has been reported elsewhere to have a concentration-dependent inhibitory effect on phospholipase C (PLC) activity induced by P2Y$_6$ activation with an IC$_{50}$ of 37 nM ± 16 in human P2Y$_6$-transfected 1321N1 human astrocytomas (Mamedova et al., 2004). This served as a useful guide in determining the concentration range of MRS 2578 to be used here.
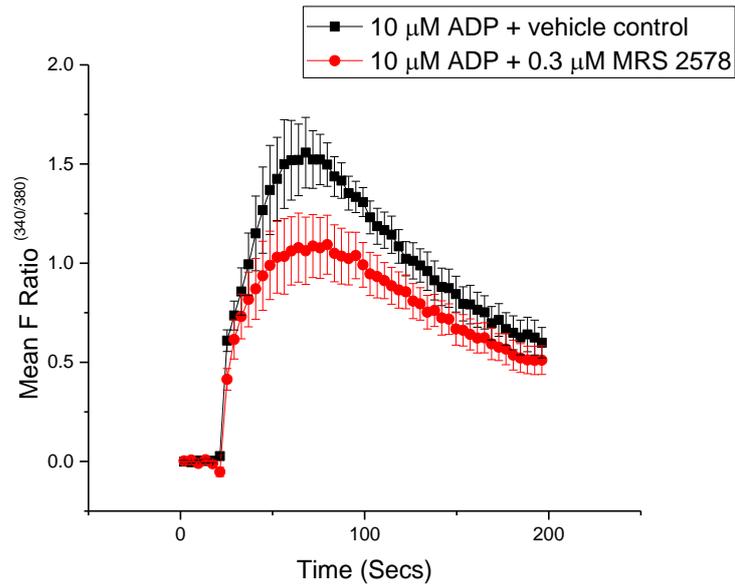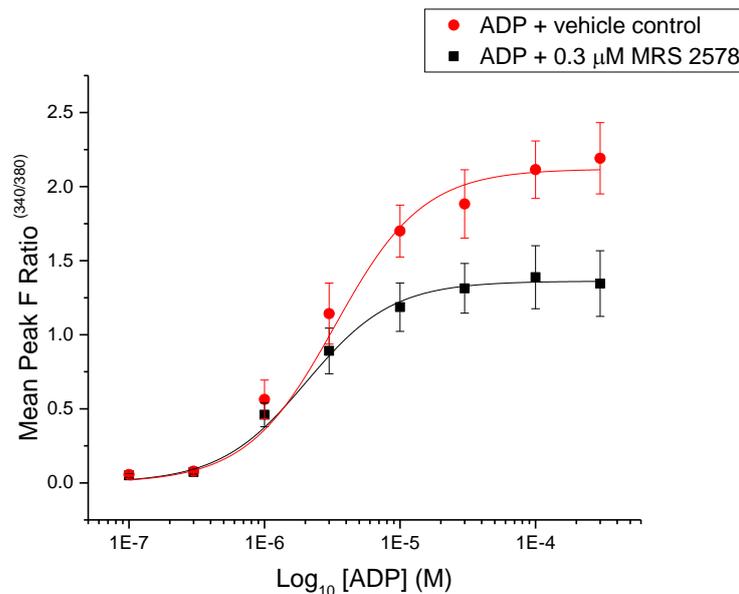
Figure 11 – MRS 2578 inhibits ADP-evoked calcium responses in a non-competitive manner

Fura-2-loaded THP-1 monocytes were pre-incubated for 30 mins with 0.3 μM MRS 2578 or a vehicle control before being challenged with 10 μM ADP (A) or increasing concentrations of ADP (B). Shown are representative time-resolved traces (A), and dose-response curves of the peaks of the calcium responses (B), represented in the form of F Ratio (340/380), with the data points representing mean ± SEM. ADP + 'vehicle control' E_{max} = 2.12, 'ADP + 0.3 μM MRS 2578' E_{max} = 1.36. N=3. E_{max} values in (B) between effect of 0.3 μM MRS 2578 vs. vehicle control are significantly different to $P < 0.005$.

The effect of an approximate IC_{50} concentration of MRS 2578, as determined from figure 10, on ADP-evoked calcium responses is shown in **figure 11**. At a concentration of 0.3 μM, MRS 2578 caused a significant reduction in the E_{max} of calcium responses generated by ADP, indicating that MRS 2578 is acting as a non-competitive inhibitor for ADP-evoked calcium responses. A similar effect was found elsewhere by Mamedova et al. (2004) where 10 μM MRS 2578 was found to non-competitively inhibit UDP-induced accumulation of inositol phosphate in human $P2Y_6$-transfected 1321N1 human astrocytoma cells.
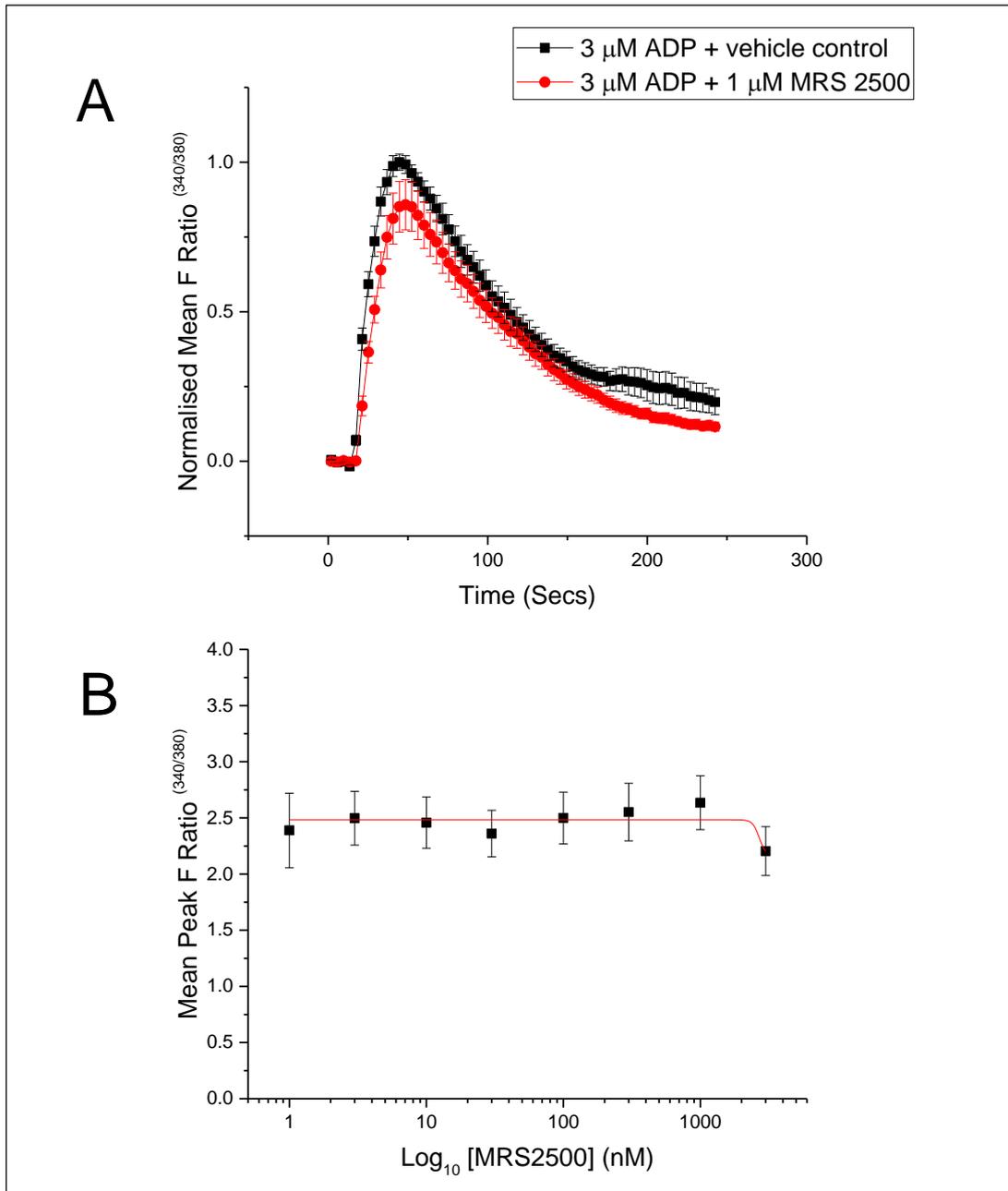
Figure 12 – MRS 2500 had no significant effect on ADP-evoked calcium responses

Fura-2-loaded THP-1 monocytes were pre-incubated for 30 mins with a vehicle control or 1 μM MRS 2500 (A), or increasing concentrations of MRS 2500 (B), before being challenged with 3 μM ADP. Shown are representative time-resolved traces (A) and the dose-response plot of the peaks of the calcium responses (B), represented in the form of F Ratio (340/380), with the data points representing mean ± SEM. N=3.

P2Y$_1$ is another P2Y receptor that is activated by ADP, and **figure 12** shows the effect of using MRS 2500, a selective P2Y$_1$ antagonist, on ADP-evoked calcium responses in THP-1 monocytes. Based on a reported IC$_{50}$ value for MRS 2500 of 0.95 nM for the inhibition of ADP-induced human platelet aggregation (Cattaneo et al., 2004), a concentration range of 1 – 3000 nM was used. None of the concentrations of MRS 2500 used had any notable effect on calcium responses induced by an approximate EC$_{50}$ concentration of ADP.

Figure 13 – MRS 2211 only reduced ADP-evoked calcium responses at a supra-maximal concentration

Fura-2-loaded THP-1 monocytes were pre-incubated for 30 mins with a vehicle control or 30 μM MRS 2211 (A), or increasing concentrations of MRS 2211 (B), before being challenged with 3 μM ADP. Shown are representative time-resolved traces (A) and a dose-response plot of the peaks of the calcium responses (B), represented in the form of F Ratio (340/380), with the data points representing mean ± SEM. N=3. * $P < 0.05$ vs. 3 μM ADP + vehicle control.

P2Y$_{13}$, like P2Y$_{12}$, is a G$_i$-coupled ADP receptor, and so establishing the role of this receptor in ADP-evoked calcium responses would be useful. However, from a concentration range of MRS 2211, a P2Y$_{13}$ antagonist, only the top concentration of 30 μM caused significant inhibition of ADP-evoked calcium responses (**figure 13**). Since it has been found in other studies that MRS 2211 was observed to have a pIC$_{50}$ of 5.97 when inhibiting 100 nM ADP-induced IP$_3$ production in 1321N1 cells (Kim et al., 2005), the inhibitory effect of 30 μM MRS 2211 on ADP-evoked calcium responses in THP-1 cells is likely non-specific. This suggests that MRS 2211 does not affect ADP-evoked calcium responses in THP-1 cells.

In summary of the findings from figures 10 – 13, the inhibitor for P2Y$_6$, but not the inhibitors for P2Y$_1$ and P2Y$_{13}$, caused a decrease in ADP-evoked calcium responses. Therefore, overall the results in this chapter suggest that calcium responses induced by ADP are dependent on activation of P2Y$_{12}$ and P2Y$_6$. However, the selectivity of the antagonists used must be considered, and the selectivity of the P2Y$_{12}$ inhibitors ticagrelor and PSB-0739 is studied in the next section.

3.4 - Investigating the pharmacological selectivity and characteristics of ticagrelor and PSB-0739

3.4.1 – Introduction & Aim

Both of the $P2Y_{12}$ antagonists used in this investigation, ticagrelor and PSB-0739, are reported to be selective for binding to $P2Y_{12}$ over other receptors. Ticagrelor was shown to reduce thrombus formation in $P2Y_{12}$-positive mice in a murine laser-injury model to levels found in $P2Y_{12}$-negative mice, but no reduction in thrombus formation occurred in ticagrelor-treated $P2Y_{12}$-deleted mice (Patil et al., 2007). PSB-0739 (30 nM and 1 µM) was shown in CHO Flp-In cells stably expressing recombinant human $P2Y_{13}$ not to block the effect of 2-methylthio-ADP in inhibiting forskolin-induced increases in cellular cAMP. Furthermore, 1 µM PSB-0739 caused minor, or no effect on the stimulation of the recombinant human $P2Y_1$, $P2Y_{11}$ or $P2Y_{14}$ receptors stably expressed in 1321N1 cells (Hoffmann et al., 2009). Although there is previous evidence for selectivity of ticagrelor and PSB-0739 in certain experiments, it was valuable to investigate their selectivity at $P2Y_{12}$ in the type of experiments performed in this study, calcium experiments, in THP-1 cells. Experiments involving $P2Y_{12}$ and $P2Y_6$ antagonists indicated that ADP-evoked calcium responses are dependent on activation of $P2Y_{12}$ and $P2Y_6$, so it was important to establish whether the two $P2Y_{12}$ antagonists used were in fact selective for $P2Y_{12}$ over $P2Y_6$.

In addition to $P2Y_{12}$ inhibition, ticagrelor has been reported to also cause inhibition of the equilibrative nucleotide transporter-1 (ENT-1), preventing adenosine uptake into the cell and therefore resulting in extracellular accumulation of adenosine. It was observed here that concentrations of ticagrelor greater than 5 µM caused an increase in resting baseline Ca^{2+}, which may be due to adenosine accumulation. Therefore, another aim in this section was to determine whether high concentrations of ticagrelor caused increasing baseline Ca^{2+} concentration due to an adenosine-mediated effect, or because of a different effect.

Figure 14 – Ticagrelor has no significant effect on ADP-evoked calcium responses in hP2Y$_6$-overexpressing 1321N1 cells

Fura-2-loaded hP2Y6-overexpressing 1321N1 astrocytomas were pre-incubated with a vehicle control or 3 μM ticagrelor (A), or increasing concentrations of ticagrelor (B), before being challenged with 3 μM ADP. Shown are representative time-resolved traces (A) and an inhibitory dose response plot of the peaks of the calcium responses (B), represented in the form of F Ratio (340/380), with the data points representing mean ± SEM. N=3.

Human P2Y$_6$-overexpressing 1321N1 astrocytomas were used to help determine whether the P2Y$_{12}$ antagonists used in this study had any affect at the P2Y$_6$ receptor, therefore giving insight into how selective the antagonists are. ADP-induced calcium responses in these cells were not significantly affected when pre-incubated with ticagrelor, as seen from **figure 14**. In regular THP-1 cells ticagrelor caused a dose-dependent effect on ADP-evoked calcium responses, but this was not evident in P2Y$_6$-overexpressing 1321N1 cells.

Figure 15 – Ticagrelor causes partial reduction of an ADP-evoked dose-response curve in hP2Y$_6$-overexpressing 1321N1 cells, but causes no significant E$_{max}$ inhibition

Fura-2-loaded hP2Y6-overexpressing 1321N1 astrocytomas were pre-incubated for 30 mins with 5 μM ticagrelor or vehicle control before being challenged with 30 μM ADP (A), or increasing concentrations of ADP (B). Shown are representative time-resolved traces (A) and dose response curves of the peaks of the calcium responses (B), represented in the form of F Ratio (340/380), with the data points representing mean ± SEM. N=3. * denotes P<0.05, as determined by a paired student's t-test.

The IC_{50} concentration of ticagrelor for inhibiting ADP-evoked calcium responses in THP-1 cells, 5 µM, was commonly used in this investigation in several experiments. Therefore, it was useful to know the effect of this ticagrelor concentration on ADP-evoked calcium responses in $P2Y_6$-overexpressing 1321N1 cells, this is shown in **figure 15**. Ticagrelor at 5 µM did cause significant decreases in calcium responses evoked by 1 µM and 0.3 µM ADP, and a reduction in the sustained phase of the calcium response to 30 µM ADP, which is also observed in standard THP-1 cells. Ticagrelor however had no significant effect on the E_{max} of the dose-response curve. Consequently, unlike what was found in THP-1 cells, ticagrelor does not cause non-competitive inhibition of ADP-evoked calcium responses in $P2Y_6$-overexpressing 1321N1 astrocytomas, which is the reported mode of action for this antagonist. This reinforces the findings from figure 14, and taken together these results indicate that ticagrelor is selective at $P2Y_{12}$ over $P2Y_6$.
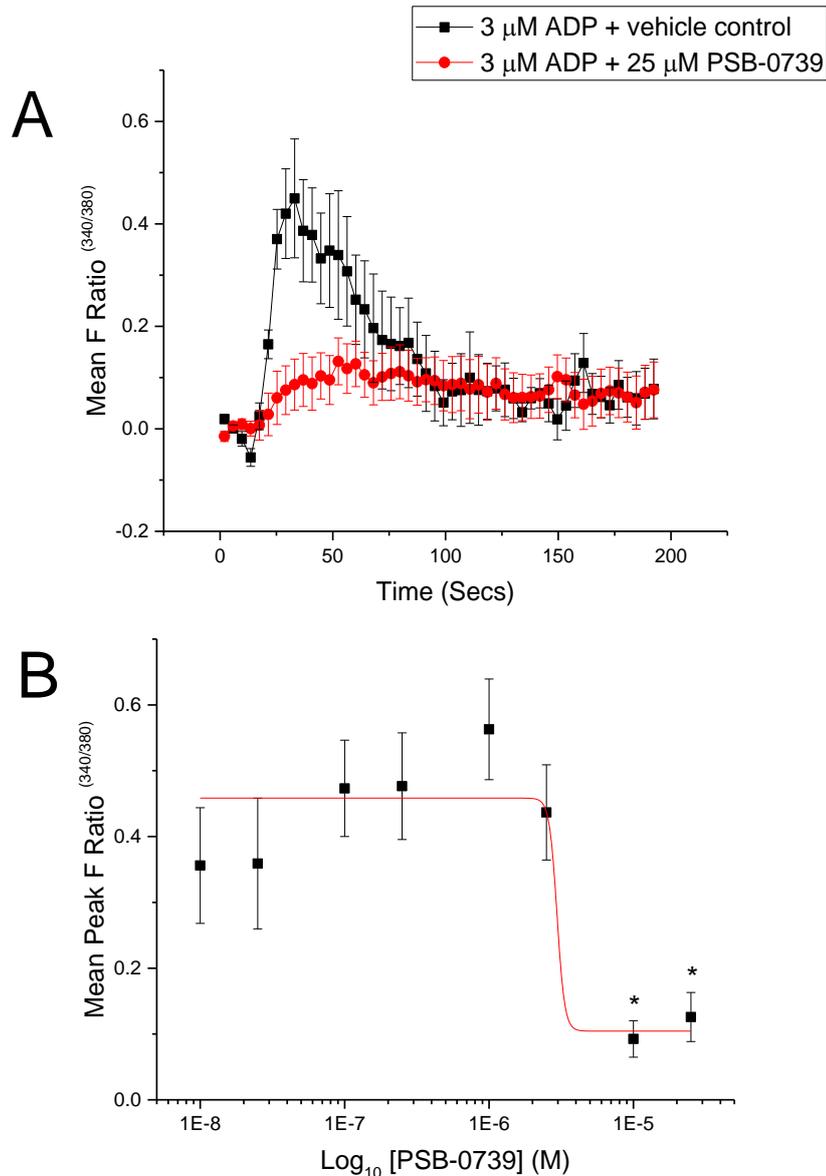
Figure 16 – PSB-0739 reduces ADP-evoked calcium responses in a dose-dependent manner in hP2Y$_6$-overexpressing 1321N1 cells

Fura-2-loaded hP2Y6-overexpressing 1321N1 astrocytomas were pre-incubated for 30 mins with a vehicle control or 25 μM PSB-0739 (A), or increasing concentrations of PSB-0739 (B), before being challenged with 3 μM ADP. Shown are representative time-resolved traces (A) and an inhibitory dose response curve of the peaks of the calcium responses (B), represented in the form of F Ratio (340/380), with the data points representing mean ± SEM. PSB-0739 IC$_{50}$ = 2.96 μM ± 0.4. N=3. * P<0.05 vs. 3 μM ADP + vehicle control.

P2Y$_6$-overexpressing 1321N1 cells were also used to determine the selectivity for the P2Y$_{12}$ antagonist PSB-0739 at P2Y$_{12}$ over P2Y$_6$. When the effect of PSB-0739 on 3 μM ADP-evoked calcium responses was investigated, 10 and 25 μM PSB-0739 caused significant reduction in ADP-induced calcium responses, as presented in **figure 16**. Although there was no progressive inhibitory dose-response trend as seen in THP-1 cells (**figure 8**), an IC$_{50}$ value of 2.96 μM was still obtained for P2Y$_6$-overexpressing 1321N1 cells, which is very similar to that obtained in THP-1 cells. Therefore, the results from figure 14 suggest that PSB-0739 does cause reduction of ADP-evoked calcium responses in a dose-dependent manner in P2Y$_6$-overexpressing 1321N1 astrocytoma cells.
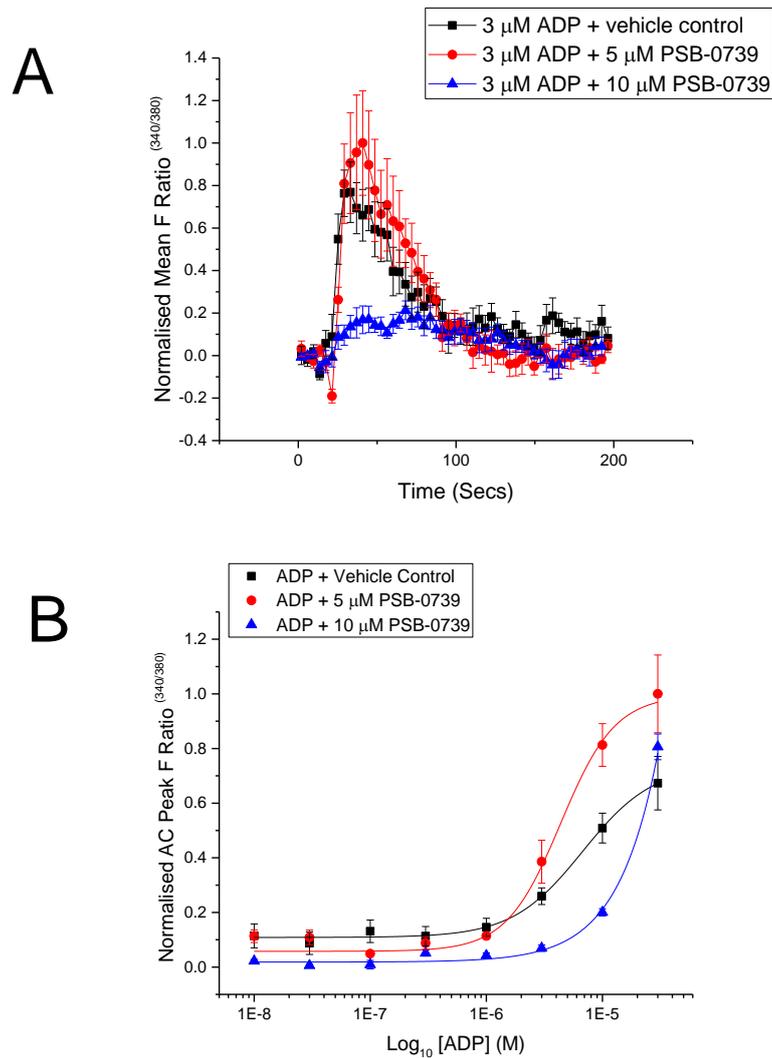
Figure 17 – 10 μM PSB-0739 caused a competitive inhibition of ADP-evoked calcium responses in hP2Y6-overexpressing 1321N1 cells

Fura-2-loaded hP2Y6-overexpressing 1321N1 astrocytomas were pre-incubated for 30 mins with 5 or 10 μM PSB-0739 or a vehicle control before being challenged with 3 μM ADP (A), or increasing concentrations of ADP. Shown are representative time-resolved traces (A) and dose response curves of the peaks of the calcium responses (B), represented in the form of F Ratio (340/380), with the data points representing mean ± SEM. The data was normalised to the highest value generated from all repeats across the treatments. N=3. 'ADP + vehicle control' EC_{50} = 4.02 μM. 'ADP + 5 μM PSB-0739' EC_{50} = 4.37 μM. 'ADP + 10 μM PSB-0739' EC_{50} = 21.44 μM. EC_{50} values in (B) between the effect of 10 μM PSB-0739 vs. vehicle control are significantly different to $P<0.05$.

Figure 17 shows that 10 μM, but not 5 μM, PSB-0739 caused a significant increase in the EC_{50} obtained for peak calcium responses evoked by a concentration range of ADP in $P2Y_6$-overexpressing 1321N1 cells. Surprisingly, 5 μM PSB-0739 significantly potentiated calcium responses induced by 10 μM and 30 μM, however as PSB-0739 has been widely reported as a competitive antagonist, comparing the overall EC_{50} is a more reliable measure of effectiveness. The results from this figure and figure 16 are comparable with those from when the effect of PSB-0739 on ADP-evoked calcium responses was determined in THP-1 cells (figures 8 & 9). Overall, it can be concluded that PSB-0739 causes inhibition of ADP-evoked calcium responses in both THP-1 cells and $P2Y_6$-overexpressing 1321N1 cells, indicating that PSB-0739 is not selective at $P2Y_{12}$ over $P2Y_6$.
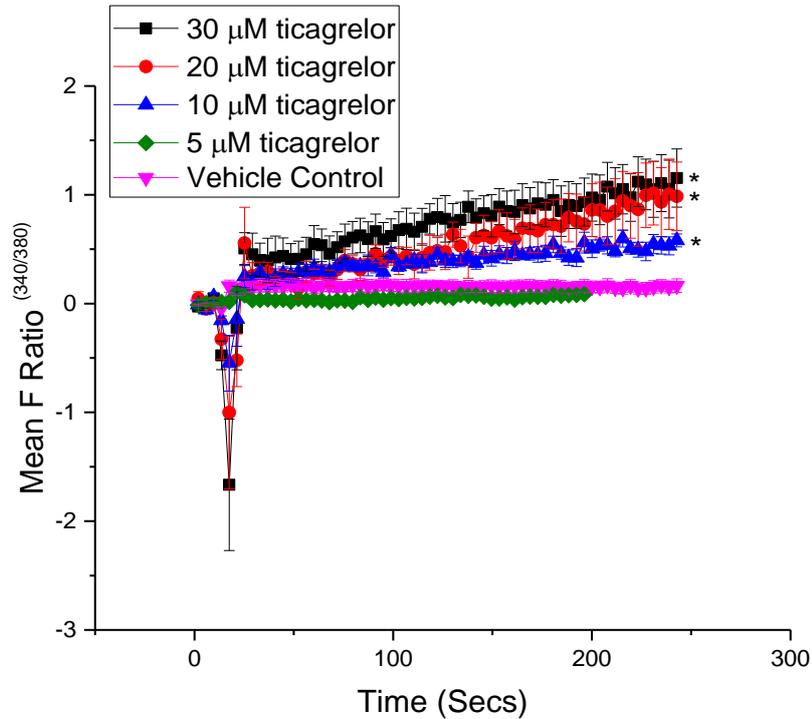
Figure 18 – Ticagrelor increases cytosolic Ca^{2+} in a dose-dependent manner

Fura-2-loaded THP-1 monocytes were challenged with a vehicle control or increasing concentrations of ticagrelor. Shown are the representative traces of the calcium responses, represented in the form of F Ratio (340/380), with the data points representing mean ± SEM. * denotes $P<0.05$, as determined by a paired student's t-test of endpoint values when compared to vehicle control. N=3.

It was observed that ticagrelor concentrations at 10 µM or higher caused an increase in the resting Ca^{2+} concentration before applying the nucleotide agonist, as seen by the 'loading' value from the SoftMax Pro software. Therefore, experiments using ticagrelor at concentrations of 10 µM or above were performed (**Figure 18**), which showed that ticagrelor, at these concentrations, does indeed cause an elevation of baseline calcium in a dose-dependent manner. This elevation of cytosolic calcium may be contributing to the inhibitory effect of ticagrelor. The effect of 10 µM ticagrelor on baseline Ca^{2+} is investigated further, however 5 µM ticagrelor was the concentration that was most commonly used as an inhibitory concentration in this study, and this concentration was shown not to cause an increase in cytosolic Ca^{2+}.

Figure 19 – The ENT-1 inhibitor NBMPR does not increase cytosolic Ca²⁺
Fura-2-loaded THP-1 monocytes were challenged with 10 or 3 μM NBMPR. Shown are the representative traces of the calcium responses, represented in the form of F Ratio (340/380), with the data points representing mean ± SEM. N=3.
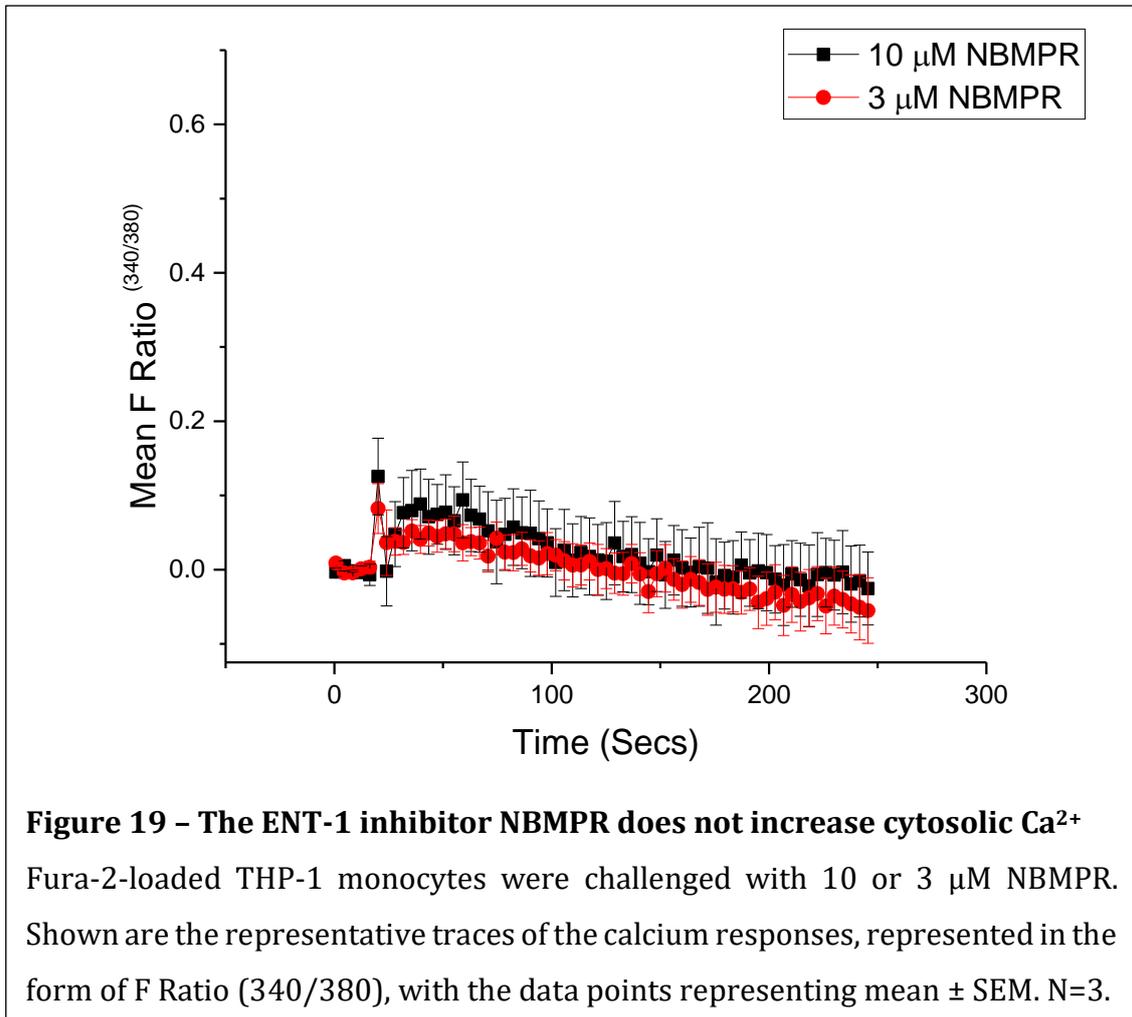
A potential explanation for ticagrelor-mediated Ca^{2+} elevation could be given by the fact that ticagrelor has previously been found to cause inhibition of the ENT-1 transporter protein. Therefore, ticagrelor may prevent uptake of adenosine back into the cell, causing activation of adenosine receptors and subsequent elevation of cytosolic Ca^{2+}. In an attempt to replicate this potential mechanism, the effect of an ENT-1 inhibitor nitrobenzylmercaptopurineriboside (NBMPR) was determined, as demonstrated in **figure 19**. It has been shown in another study that NBMPR caused inhibition of uridine influx in *Xenopus* oocytes expressing human ENT-1 with an IC_{50} of 3.6 nM ± 1.4 (Yao et al., 1997), which was used as a guide here to decide on a maximal NBMPR concentration. NBMPR does not raise the baseline calcium level in a similar fashion to ticagrelor, indicating that ticagrelor-induced cytosolic Ca^{2+} elevation is not due to inhibition of ENT-1.
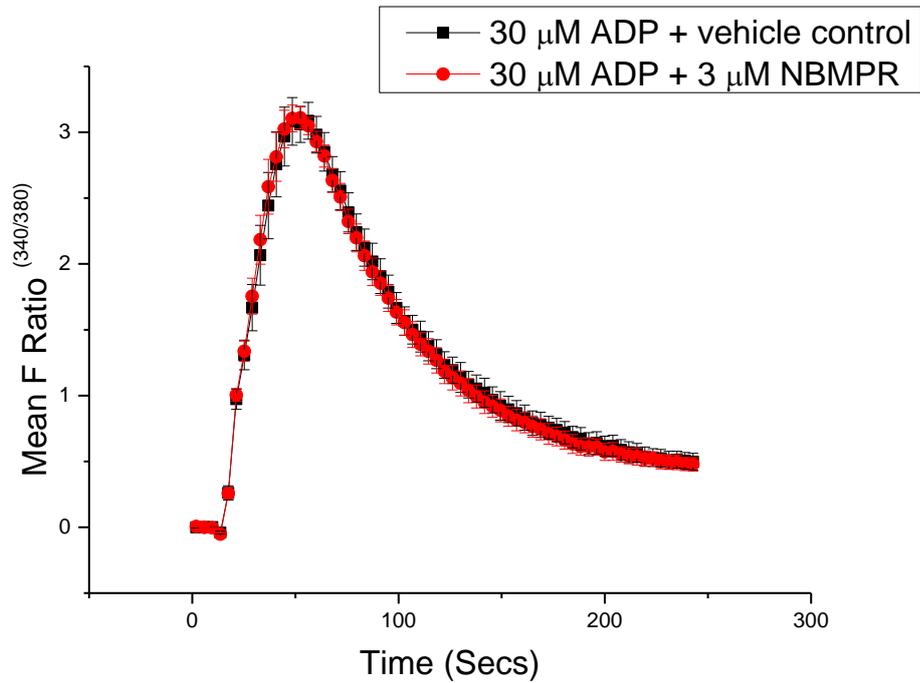
Figure 20 – The ENT-1 inhibitor NBMPR has no effect on ADP evoked calcium responses

Fura-2-loaded THP-1 monocytes were pre-incubated for 30 mins with 3 μM NBMPR or a vehicle control before being challenged with 30 μM ADP. Shown are the representative traces of the calcium responses, represented in the form of F Ratio (340/380), with the data points representing mean ± SEM.

Figure 20 shows that 3 μM NBMPR had no effect on calcium responses evoked by 30 μM ADP, suggesting that inhibition of ENT-1 has no effect on ADP-evoked calcium responses. Overall, the results from figures 19 and 20 indicate that elevation of baseline calcium caused by ticagrelor at 10 μM or above is not due to ENT-1 inhibition. It was observed through light microscopy that 30-minute pre-incubation with 10 μM ticagrelor or above caused a decrease in the number of visible THP-1 cells, when compared to cells preincubated with 5 μM ticagrelor or lower. This indicates that ticagrelor causes cell death at higher concentrations of 10 μM or above. Further study is required into the other effects of ticagrelor observed here at higher concentrations. In this study, however, as 5 μM ticagrelor is the maximum concentration that has been used, effects of ticagrelor at 10 μM or above has no impact on the results found here using the drug.

3.5 - Using siRNA in THP-1 monocytes to determine the effect of P2Y$_{12}$ knockdown on ADP-evoked calcium responses

3.5.1 - Introduction & Aim

Findings from previous experiments in this investigation have indicated that ADP-evoked calcium responses in THP-1 cells are dependent on activation of P2Y$_{12}$. However, these conclusions were entirely drawn from experiments looking at the effect of pharmacological inhibitors on ADP-evoked calcium responses. Although experiments were conducted in this study to investigate the selectivity of the P2Y$_{12}$ inhibitors used, there is still the possibility that the antagonists used here are not selective. Therefore, a more certain method for inhibiting P2Y$_{12}$ in ADP-evoked calcium responses in THP-1 cells would strengthen the suggested conclusions here. Small-interfering RNA (siRNA) are commonly used for transient gene-silencing due to their role in causing post-transcriptional degradation of targeted mRNA.

P2Y$_{12}$-targeted siRNA is used here in an attempt to cause a transient knockdown of P2Y$_{12}$ in THP-1 monocytes, hence providing a more reliable experiment for a reduction in P2Y$_{12}$ activity than simply assessing the effect of pharmacological P2Y$_{12}$ inhibitors. This helps to provide stronger conclusions about the role of P2Y$_{12}$ in mediating ADP-evoked calcium responses in THP-1 monocytes.
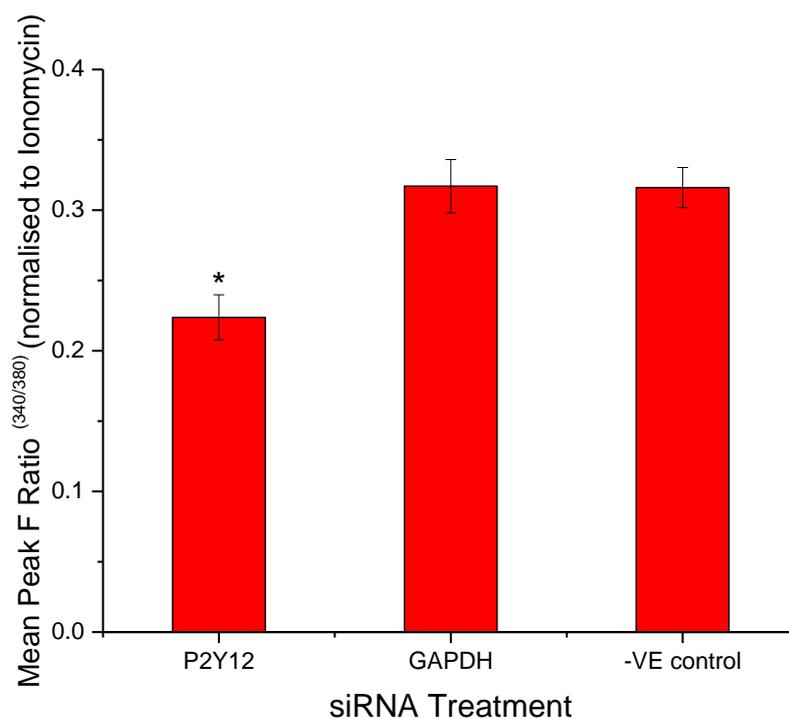
Figure 21 – P2Y$_{12}$-targeted siRNA significantly decreased ADP-evoked calcium responses in THP-1 cells.

THP-1 monocytes were transfected with 25 nM P2Y$_{12}$-targeted, GAPDH-targeted (positive control), or negative-control siRNA using DharmaFECT 2 transfection reagent for 48 hrs as outlined in Materials & Methods. 2×10^5 cells/well were then loaded before being challenged with 3 µM ADP, or 1 µM ionomycin. Ionomycin responses were used to normalise for any differences in cell number. Shown is a bar chart representing the peak size of the calcium responses, represented in the form of F Ratio (340/380), with the data points representing mean ± SEM. N=3. * P<0.05.

ADP-evoked calcium responses were measured in THP-1 cells transfected with siRNA targeted to P2Y$_{12}$, and were compared to those in THP-1 cells transfected with GAPDH-targeted siRNA or negative control siRNA. As **figure 21** shows, THP-1 cells transfected with P2Y$_{12}$-targeted siRNA exhibited significantly smaller calcium responses to ADP-induced calcium responses, compared to the controls. These results support the previous findings in this study that suggest that P2Y$_{12}$ is involved in mediating ADP-evoked calcium responses in THP-1 cells.
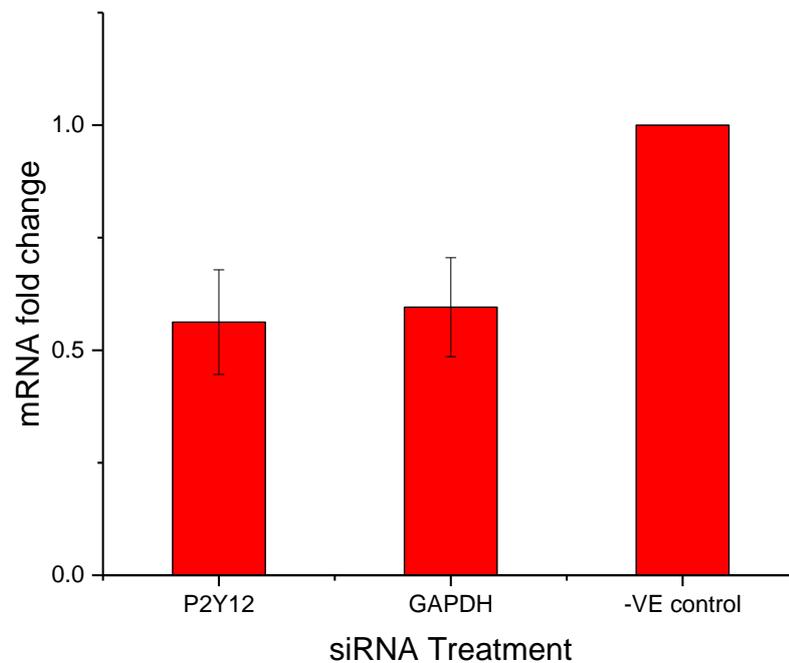
Figure 22 – P2Y$_{12}$-targeted and GAPDH-targeted siRNA caused knockdown of over 40% in the respective mRNA.

THP-1 monocytes were transfected with 25 nM P2Y$_{12}$-targeted, GAPDH-targeted (positive control), or negative-control siRNA using DharmaFECT 2 transfection reagent for 48 hrs with the method outlined in Materials & Methods. The same respective populations of cells used for figure 21 were used here. mRNA expression was determined using Taqman probes with the qRT-PCR method described in Materials and Methods. Shown is a bar chart representing the relative quantifications of mRNA, as calculated using the $\Delta\Delta C_T$ method, with the data points representing mean ± SEM. N=3. The mean of B2M and GAPDH mRNA was used as housekeeping genes during the $\Delta\Delta C_T$ method.

Although the results in figure 21 show that $P2Y_{12}$-targeted siRNA causes a reduction in ADP-evoked calcium responses, it was also necessary to measure the gene expression of $P2Y_{12}$ in the same populations of cells as those used for the calcium experiments. This is important as it would show whether the reduction in calcium responses in THP-1 cells treated with $P2Y_{12}$-targeted siRNA correlates with a reduction in gene expression, which should be the case. **Figure 22** demonstrates that, compared to the scrambled negative control, there was over a 40% reduction in both $P2Y_{12}$ and GAPDH mRNA in THP-1 cells treated with siRNA targeted to $P2Y_{12}$ and GAPDH respectively.

The results in this chapter suggest that molecular knockdown of the $P2Y_{12}$ receptor causes a reduction in calcium responses evoked by ADP. This strongly indicates that $P2Y_{12}$ is involved in mediating ADP-evoked calcium responses in THP-1 cells, which supports the similar conclusions made previously in this investigation.

3.6 - Discovery of a potential signalling transduction mechanism behind ADP-evoked calcium responses in THP-1 monocytes

3.6.1 - Introduction & Aim

The results shown thus far indicate that ADP-evoked calcium responses in THP-1 monocytes are dependent on activation of both $P2Y_{12}$ and $P2Y_6$, indicating that $P2Y_{12}$ contributes to calcium responses evoked by ADP in these cells.

It is important to attempt to understand the signalling mechanism behind the involvement of $P2Y_{12}$ in ADP-induced calcium responses, and whether there is a cross-talk mechanism behind $P2Y_{12}$ and $P2Y_6$ in mediating the calcium responses evoked by ADP in THP-1 monocytes.

ADP activates P2Y purinergic receptors, such receptors being G protein-coupled receptors (GPCRs). The downstream signalling pathway following GPCR activation depends on the type of G protein in question. For instance, $P2Y_6$ is coupled to a G_q protein, which causes hydrolysation of phosphatidylinositol 4,5-bisphosphate (PIP2) into diacyl glycerol (DAG) and inositol trisphosphate (IP_3) through activation of phospholipase C (PLC). Subsequent IP_3 generation causes release of calcium ions from the endoplasmic reticulum (ER). Conversely, the effects of G_i, the G protein coupled to $P2Y_{12}$, include inhibition of adenylate cyclase, resulting in decreased cytosolic cAMP concentration, and activation of phosphoinositide 3-kinase (PI3K). Subsequently, by using selective antagonists for some of the proteins involved in these transduction pathways, valuable insight would be gained into the nature of ADP-evoked calcium responses described previously.

Since $P2Y_{12}$ is G_i-coupled, and therefore unlikely to cause calcium mobilisation, the receptor may be modulating the calcium response evoked by a G_q-coupled P2Y receptor, for example $P2Y_6$. A cross-talk mechanism has been reported between G_q-coupled $P2Y_1$ and G_i-coupled $P2Y_{12}$ in platelets whereby $P2Y_{12}$ potentiates the $P2Y_1$-mediated ADP-evoked calcium response through inhibition of adenylate cyclase and activation of PI3K (Hardy et al., 2004). Since in THP-1 cells ADP-induced calcium responses have been shown to be dependent on $P2Y_6$ activation as well as $P2Y_{12}$, a similar mechanism to the $P2Y_1$-$P2Y_{12}$ cross-talk in platelets, but for $P2Y_6$ and $P2Y_{12}$ in THP-1 monocytes.

Overall, the experiments in this section attempted to determine the signalling transduction mechanism behind ADP-evoked calcium responses in THP-1 monocytes and investigate any potential crosstalk mechanism between $P2Y_{12}$ and $P2Y_6$. This is achieved by assessing the effect of selective inhibitors for certain proteins implicated in different stages of the relevant signalling transduction pathways on ADP-evoked calcium responses.
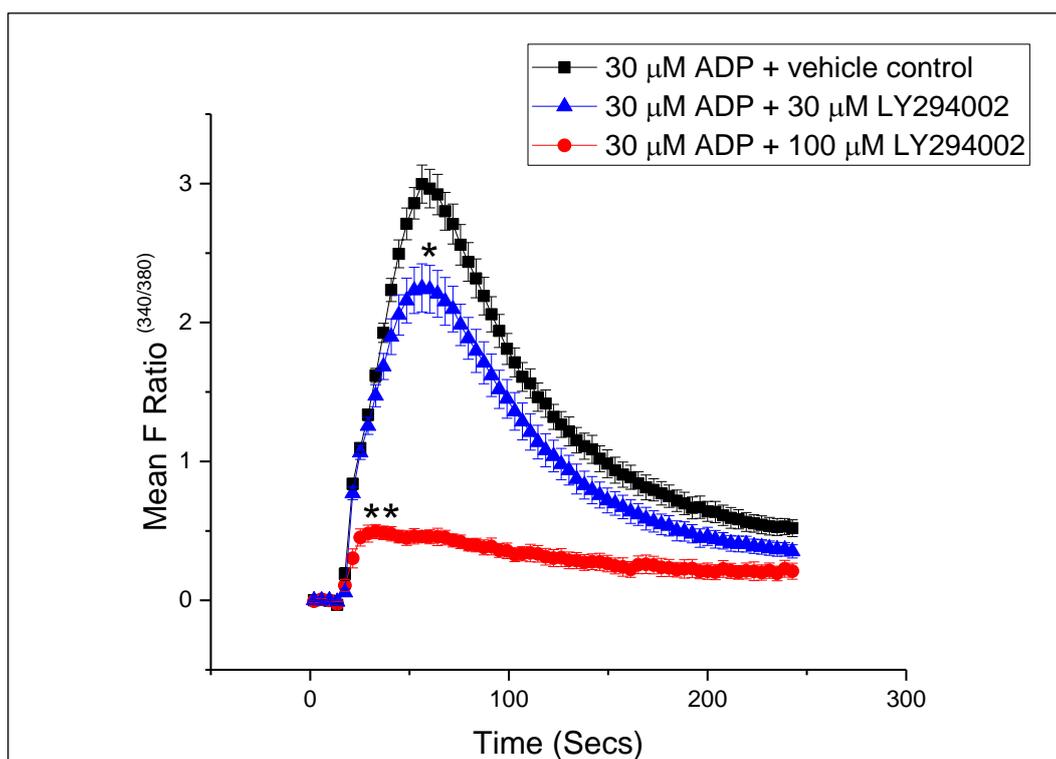
Figure 23 – ADP-evoked calcium responses are sensitive to the PI3K inhibitor LY294002

Fura-2-loaded THP-1 monocytes were pre-incubated for 30 mins with 30 or 100 µM LY294002 or a vehicle control before being challenged with 30 µM ADP. Shown are the representative traces of the calcium responses, represented in the form of F Ratio (340/380), with the data points representing mean ± SEM. N=3. * denotes $P<0.005$ and ** denotes $P<0.001$ of peak values, as determined by a paired student's t-test.

Figure 23 shows the effect of a PI3K inhibitor on ADP-evoked calcium responses. Calcium responses stimulated by a maximal concentration of ADP were significantly inhibited by the PI3K inhibitor LY294002, suggesting that ADP-evoked calcium responses are dependent upon PI3K activity and therefore dependent upon activation of a G_i-coupled receptor, due to G_i activation causing stimulation of PI3K.

LY294002 has been shown, with an IC_{50} value of 1.4 μM, to negate PI3K activity, without causing inhibition of other protein or lipid kinases such as PI4K, PKC or MAP-kinase (Viahos et al., 1994), indicating that LY294002 is selective for PI3K. Conversely, PI4K inhibition, together with PI3K inhibition, was however observed in platelets treated with LY294002 (Rosado and Sage, 2000). However, the experiments in the study by Viahos et al. involved experiments in several different cell types, rather than platelets.

A previous study showed that 50 μM LY294002 caused significant decrease in carbachol- or caffeine-evoked calcium transients in bovine aortic smooth muscle cells (Ethier and Madison, 2002), and these findings were used as an indicator for choosing a sensible LY294002 concentration for causing inhibition of PI3K.

Figure 24 – The G_i protein blocker pertussis toxin (PTx) abolishes CCL2-evoked calcium responses in THP-1 monocytes

Fura-2-loaded THP-1 monocytes were pre-incubated for 3 hours with 5 ng/μl pertussis toxin or a vehicle control before being challenged with 50 ng/ml CCL2. Shown are the representative traces of the calcium responses, represented in the form of F Ratio (340/380), with the data points representing mean ± SEM. N=3.

The involvement of G_i protein activity in mediating ADP-stimulated calcium responses was further investigated by establishing the effect of the G_i inhibitor pertussis toxin. It is well established that CCR2, the receptor for CCL2, is G_i-coupled and can also elicit calcium responses when stimulated by CCL2. As a positive control for pertussis toxin fully functioning as a G_i protein blocker, the effect of pertussis toxin on CCL2-evoked calcium responses was determined, as shown in **figure 24**. Pertussis toxin, at 5 ng/μl, completely negated the CCL2-evoked calcium response, indicating that pertussis toxin is having a maximum effect at this concentration.
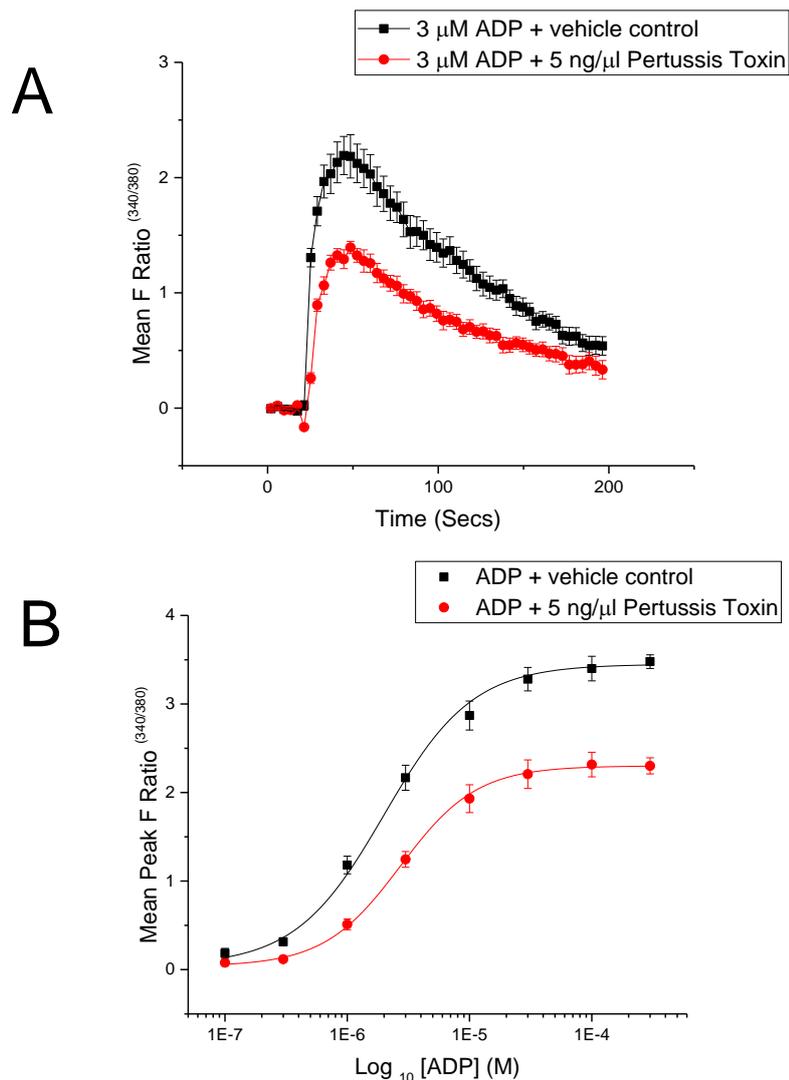
Figure 25 – The G_i protein blocker pertussis toxin (PTx) causes significant inhibition of an ADP-evoked dose-response

Fura-2-loaded THP-1 monocytes were pre-incubated for 3 hours with 5 ng/µl PTx or a vehicle control before being challenged with 3 µM ADP (A) or increasing concentrations of ADP (B). Shown are representative time-resolved traces (A), and dose-response curves of the peaks of the calcium responses (B), represented in the form of F Ratio (340/380), with the data points representing mean ± SEM. 'ADP + vehicle control' EC_{50} = 1.87 µM ± 0.33 E_{max} = 3.45 ± 0.07. 'ADP + 5 ng/µl' EC_{50} = 2.78 µM ± 0.21 E_{max} = 2.3 ± 0.04. N=3. The respective EC_{50} and E_{max} of the different conditions in (B) are significantly different to P<0.05.

In **figure 25**, the effect of the G_i inhibitor pertussis toxin on calcium responses induced by ADP was assessed. Pertussis toxin caused significant increase of the EC_{50}, and significant decrease in the E_{max} of an ADP-evoked dose-response curve. The concentration used here was equal to that used in figure 24, which demonstrated a positive control. These results indicate that calcium responses induced by ADP are dependent upon activation of G_i.

In summary of the outcomes from figures 23 – 25, it is indicated that ADP-stimulated calcium responses in THP-1 monocytes are sensitive to pertussis toxin and the PI3K inhibitor LY294002, implying that activation of a G_i-coupled receptor is also involved in mediating the calcium response. Therefore, both G_q-, and G_i-coupled receptors are suggested to be involved in mediating the ADP-induced calcium response, potentially implicating a crosstalk mechanism between $P2Y_{12}$ and $P2Y_6$ in eliciting the calcium response.
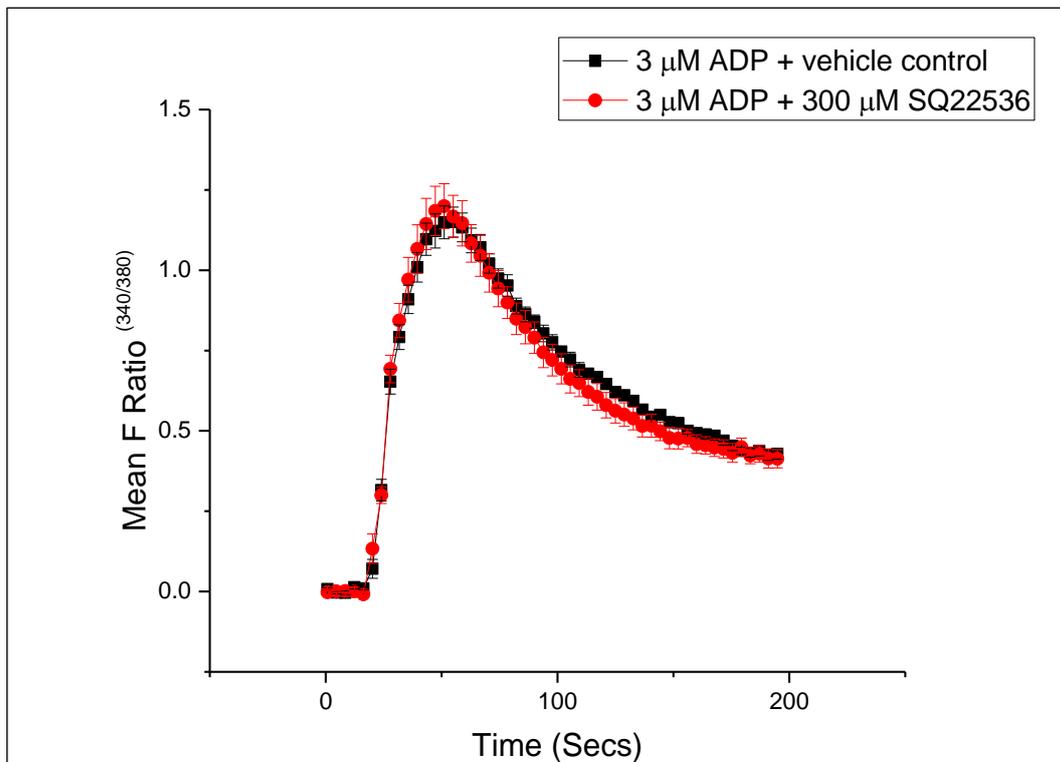
Figure 26 – The adenylate cyclase inhibitor SQ22536 has no effect on calcium responses induced by ADP alone.

Fura-2-loaded THP-1 monocytes were pre-incubated for 30 mins with 300 μM SQ22536 or a vehicle control before being challenged with 3 μM ADP. Shown are the representative traces of the calcium responses, represented in the form of F Ratio (340/380), with the data points representing mean ± SEM. N=3.

In an attempt to investigate a potential crosstalk transduction mechanism for ADP-evoked calcium responses involving adenylate cyclase (AC) inhibition, the pharmacological AC inhibitor SQ22536 was used. Before specifically looking at the involvement of $P2Y_{12}$ in this hypothetical mechanism, it was important to test the effect of SQ22536 on ADP-evoked calcium responses alone as a negative control. **Figure 26** shows that SQ22536 had no effect on ADP-stimulated calcium responses alone in THP-1 cells at 300 μM.
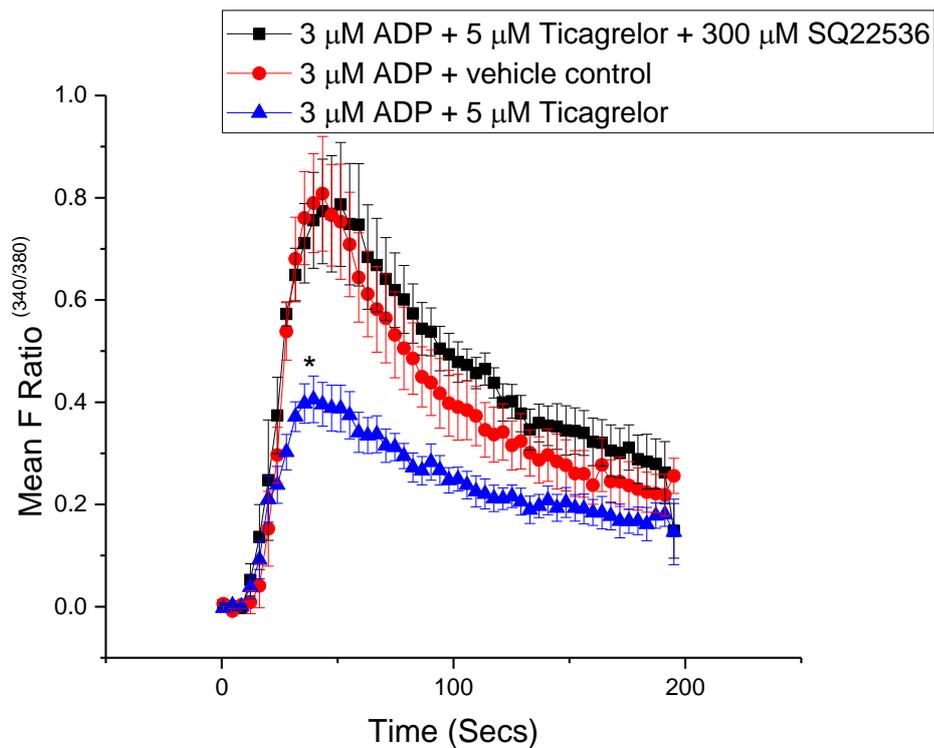
Figure 27 – The adenylate cyclase inhibitor SQ22536 restores ADP-evoked calcium responses inhibited by ticagrelor

Fura-2-loaded THP-1 monocytes were pre-incubated for 30 mins with 5 µM ticagrelor with or without 300 µM SQ22536 or a vehicle control before being challenged with 3 µM ADP. Shown are the representative traces of the calcium responses, represented in the form of F Ratio (340/380), with the data points representing mean ± SEM. N=3. * indicates significance between the peak vs '3 µM ADP + vehicle control, P<0.05.

To determine whether $P2Y_{12}$ could be involved in ADP-induced calcium responses through AC inhibition, the effect of SQ22536 on ADP-evoked calcium responses inhibited by either ticagrelor or PSB-0739 was established. **Figure 27** illustrates that ADP-evoked calcium responses inhibited by ticagrelor are significantly increased when in the presence of SQ22536, compared to when SQ22536 is not present. This suggests that $P2Y_{12}$ may be involved in mediating ADP-induced calcium responses through inhibition of adenylate cyclase. It was determined in human blood platelets that a 3-6-fold increase in adenylate cyclase activity by prostaglandin E1 was inhibited in a dose-dependent manner by SQ22536, with an IC_{50} value of 21 µM (Harris et al., 1979), with this value serving as an aid in determining a suitable SQ22536 concentration for this experiment.
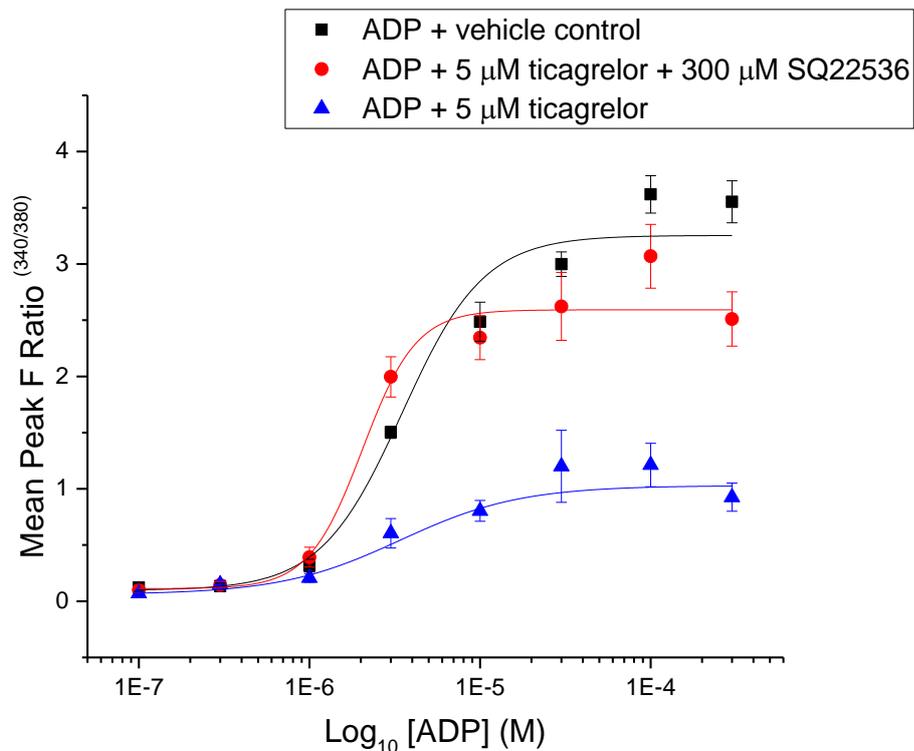
Figure 28 – The adenylate cyclase inhibitor SQ22536 significantly increases the E_{max} of an ADP-evoked dose response curve inhibited by ticagrelor

Fura-2-loaded THP-1 monocytes were pre-incubated for 30 mins with 5 µM ticagrelor with or without 300 µM SQ22536 or a vehicle control before being challenged with increasing concentrations of ADP. Shown are the dose response curves of the peaks of the calcium responses, represented in the form of F Ratio (340/380), with the data points representing mean ± SEM. 'ADP + vehicle control' E_{max} = 3.25 ± 0.18, 'ADP + 5 µM ticagrelor + 300 µM SQ22536' E_{max} = 2.59 ± 0.14, 'ADP + 5 µM ticagrelor' E_{max} = 1.03 ± 0.12. N=3. The E_{max} of 'ADP + 5 µM ticagrelor' vs. 'ADP + vehicle control' is significantly different to $P<0.05$.

To reinforce the results from figure 27, it was assessed whether SQ22536 would cause any recovery of the ticagrelor-induced reduction in E_{max} of an ADP-evoked dose response curve. The AC inhibitor SQ22536 significantly increased the E_{max} value of an ADP-induced dose-response curve inhibited by the presence of ticagrelor in THP-1 cells, compared to just ticagrelor pre-treatment alone. As previously described, the reported mechanism of inhibition of ticagrelor is non-competitive, which is shown again here by the decrease in E_{max}. Since SQ22536 lessened the ticagrelor-mediated non-competitive inhibition of ADP-evoked calcium responses, these results further suggest that $P2Y_{12}$ may be contributing to ADP-stimulated calcium responses via inhibition of adenylate cyclase.
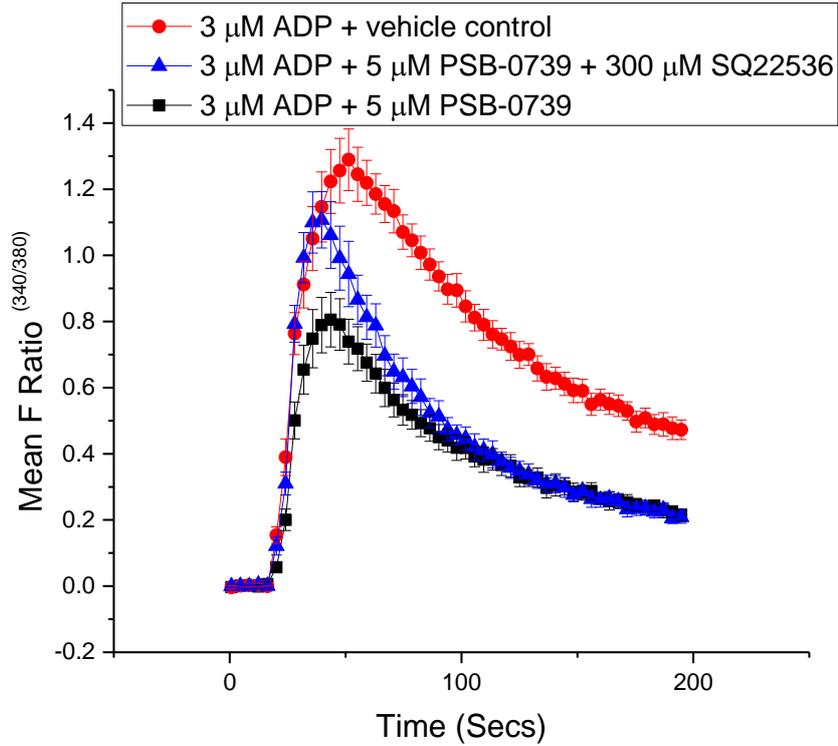
Figure 29 – The adenylate cyclase inhibitor SQ22536 significantly increases ADP-evoked calcium responses inhibited by PSB-0739
Fura-2-loaded THP-1 monocytes were pre-incubated for 30 mins with 5 µM PSB-0739 with or without 300 µM SQ22536 or a vehicle control before being challenged with 3 µM ADP. Shown are the representative traces of the calcium responses, represented in the form of F Ratio (340/380), with the data points representing mean ± SEM. N=3. The difference between the peak of '3 µM ADP + 5 µM PSB-0739' vs. '3 µM ADP + vehicle control' is significant to $P<0.05$.

The experiment conducted for figure 27 was repeated, only using PSB-0739 instead of ticagrelor as a $P2Y_{12}$ inhibitor, with the results shown in **figure 29**. PSB-0739 caused a significant decrease in calcium responses induced by an approximate EC50 concentration of ADP (3 µM), however SQ22536 caused significant increase of ADP-evoked calcium responses inhibited by PSB-0739. These results support the findings from figures 27 and 28, which suggest that ADP-induced calcium responses are dependent on $P2Y_{12}$ activation because of $P2Y_{12}$-mediated adenylate cyclase inhibition. The increase in ADP-induced calcium responses by SQ22536 observed in figures 27-29 is indicated to be due to the presence of the respective $P2Y_{12}$ inhibitor and not simply SQ22536 having an effect itself, as the negative control in figure 26 showed no effect of SQ22536 on ADP-evoked calcium responses alone.

Overall, the results in this chapter indicate that calcium responses evoked by ADP in THP-1 monocytes are dependent upon both G_{q}- and G_{i}-coupled receptor activation, with a potential crosstalk mechanism suggested whereby $P2Y_{12}$ positively modulates a primarily $P2Y_{6}$-mediated calcium response to ADP through inhibition of adenylate cyclase, together with activation of PI3K.

Chapter 4 - Discussion

4.1 - The physiological significance of identifying a role for P2Y$_{12}$ in ADP-evoked calcium responses in monocytes

Demonstrated in this study are novel findings identifying P2Y$_{12}$ as being involved in the calcium response to ADP in THP-1 monocytes. Furthermore, evidence is given for the expression of P2Y$_{12}$ in cell-line, and primary monocytes, when it had been previously established that P2Y$_{12}$ expression is mainly attributed to platelets, glial cells, smooth muscle cells and endothelial cells (Cattaneo, 2006).

There have been no published reports on the role of P2Y$_{12}$ in monocyte function thus far, however the involvement of P2Y$_{12}$ in macrophage chemotaxis was investigated by Kronlage et al., (2010). Stimulation of P2Y$_{12}$ in macrophages was found to induce cell spreading with formation of lamellipodia, and inhibition of multiple purinergic receptors, including P2Y$_{12}$, blocked macrophage chemotaxis (Kronlage et al., 2010). It was indicated from these findings that collaborative autocrine signalling of purinergic receptors, such as P2Y$_{12}$ and P2Y$_2$, ensure directional motility of macrophages upon exposure to chemotactic gradients (Kronlage et al., 2010).

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 P2Y$_{12}$ signalling, or macrophage P2Y$_{12}$ 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 P2Y$_{12}$, mediating actions such as chemokine release and consequent recruitment of immune cells.
Such findings may implicate ADP and P2Y$_{12}$ as being crucial in forming a comprehensive immune response to infectious disease.

P2Y$_{12}$ was shown to be expressed in dendritic cells also, and calcium responses to ADP in these cells was blocked by the P2Y$_{12}$ antagonist AR-C69931MX (Ben Addi et al., 2010). Subsequently, ADP-evoked calcium responses were entirely abolished in P2Y$_{12}$-deficient dendritic cells (Ben Addi et al., 2010). Moreover, results presented by Ben Addi et al. (2010) suggested that P2Y$_{12}$ activation increased antigen uptake and subsequent activation of T cells. Hence, this study indicated that ADP-induced calcium responses in dendritic cells are dependent on P2Y$_{12}$, and that this receptor is involved in immune cellular functions. Since macrophages and dendritic cells are derived from monocytes, the findings presented here and in other studies indicate that P2Y$_{12}$ may also play a role in the physiological role of monocytes and therefore it would be useful for a functional investigation of P2Y$_{12}$ in monocytes to take place.

There is evidence to suggest that ADP contributes to the function of monocytes. 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.

In summary of previous studies, P2Y$_{12}$ has been indicated in similar cell types to monocytes to have roles in mediating ADP-evoked calcium responses and also in physiological ADP-induced immune functions such as chemotaxis and antigen uptake. Although the role of P2Y$_{12}$ has not been specifically studied in monocytes themselves, it has been suggested that ADP and calcium signalling modulates functional responses in such cells. Taken together with aforementioned reports, the current investigation, which has indicated that P2Y$_{12}$ positively modulates THP-1 ADP-evoked calcium responses, therefore implies that P2Y$_{12}$ may have a functional role in monocyte physiology.

Furthermore, monocytes are implicated in crucial early stages of atherosclerosis, in which they are recruited to the artery endothelium and subsequently migrate into the intima, where they differentiate into macrophages (Galkina and Ley, 2009). The presence of P2Y$_{12}$ may be contributing to such pathogenic processes of monocytes in atherosclerosis, particularly when one considers that vessel wall P2Y$_{12}$ has been indicated to contribute to early atherogenesis (West et al., 2014). Also, recent reports have shown that mice supplemented with ticagrelor developed more stable atherosclerotic plaques, as characterised by a reduced necrotic core and a thickening of the fibrous cap (Preusch et al., 2016). Moreover, in vitro experiments revealed that ticagrelor inhibits oxidised LDL uptake and reduces apoptosis in serum-starved macrophages from the RAW 264.7 cell-line (Preusch et al., 2016), indicating that ticagrelor may promote plaque stability through macrophage P2Y$_{12}$ interaction. The results from studies by West et al. (2014) and Preusch et al. (2016) imply that P2Y$_{12}$ may have platelet-independent roles in vascular disease which need further study. This is made more relevant now since P2Y$_{12}$ has been shown here to be involved in monocyte responses to ADP, with monocytes having highly important atherogenic functions.

4.2 - A potential crosstalk mechanism between P2Y$_{12}$ and P2Y$_6$ in mediating ADP-evoked calcium responses in human monocytes

In using a combination of pharmacological inhibitors and siRNA-mediated P2Y$_{12}$ knockdown, the findings in this project have strongly suggested that ADP-evoked calcium responses in THP-1 monocytes are dependent on P2Y$_{12}$ activation. P2Y$_{12}$ has been shown to be G$_i$-coupled (Hollopeter et al., 2001), but there have been no reported findings thus far to indicate that G$_i$-coupled purinergic P2Y receptors such as P2Y$_{12}$ directly elicit calcium responses when activated by extracellular stimuli. This is owing to the fact that activation of G$_i$ does not classically cause IP$_3$ production and therefore does not lead to release of Ca^{2+} from the ER. Therefore, how P2Y$_{12}$ may be contributing to the stimulation of the calcium response was also investigated.

Activation of platelets by ADP occurs through activation of P2Y$_1$ and P2Y$_{12}$, with P2Y$_1$ stimulation giving rise to G$_q$-mediated activation of PLC and a subsequent calcium response (Jin et al., 1998). Knockdown or pharmacological inhibition of P2Y$_1$ has been shown in previous studies to abolish ADP-evoked calcium responses in platelets, and for

this reason it is accepted that $P2Y_1$ is categorically required for ADP-induced calcium responses in these cells (Jin et al., 1998). However, it has also been demonstrated that $P2Y_1$ is not solely responsible for eliciting the calcium response to ADP, with some studies showing $P2Y_{12}$ to be implicated in modulating the response also (Fox et al., 2004).

It was discovered in platelets by Hardy et al. (2004) that the selective pharmacological $P2Y_{12}$ inhibitor AR-C69931MX partially blocked the calcium response to 10 μM ADP, which was also shown to be completely abolished by selective $P2Y_1$ inhibition. This suggested that $P2Y_{12}$ is able to positively modulate the $P2Y_1$-mediated calcium response to ADP in platelets. Subsequently, Hardy et al. identified that the PI3K inhibitor LY294002 (10 μM) partially inhibited the $P2Y_{12}$-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 $P2Y_{12}$ inhibitor AR-C69931MX (Hardy et al., 2004). Taken together, these findings by Hardy et al. indicated in platelets that $P2Y_{12}$ regulates $P2Y_1$-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 $P2Y_6$ activation, but not dependent on $P2Y_1$ or $P2Y_{13}$. Subsequently it would be logical that $P2Y_{12}$ may be potentiating the $P2Y_6$ G_q-mediated calcium response, without stimulation of both receptors being required. In further investigation of this potential mechanism, it was indicated in THP-1 cells that $P2Y_{12}$ may modulate the $P2Y_6$-mediated response through PI3K activation and inhibition of adenylate cyclase. The basic principle identified by Hardy et al. (2004) is supported here, only with $P2Y_6$ acting as the equivalent of $P2Y_1$. A proposed signalling transduction mechanism for how $P2Y_{12}$ supports the $P2Y_6$-mediated calcium response to ADP in THP-1 cells is illustrated and explained in **figure 30**. An interesting observation from this study was that $P2Y_1$, although expressed at the gene level in THP-1 cells, seemingly was not involved in ADP-evoked calcium responses in THP-1 cells. This may be explained by the fact that mRNA expression does not always correlate with respective protein levels, so $P2Y_1$ protein expression would need to be determined in THP-1 cells to further investigate this finding.
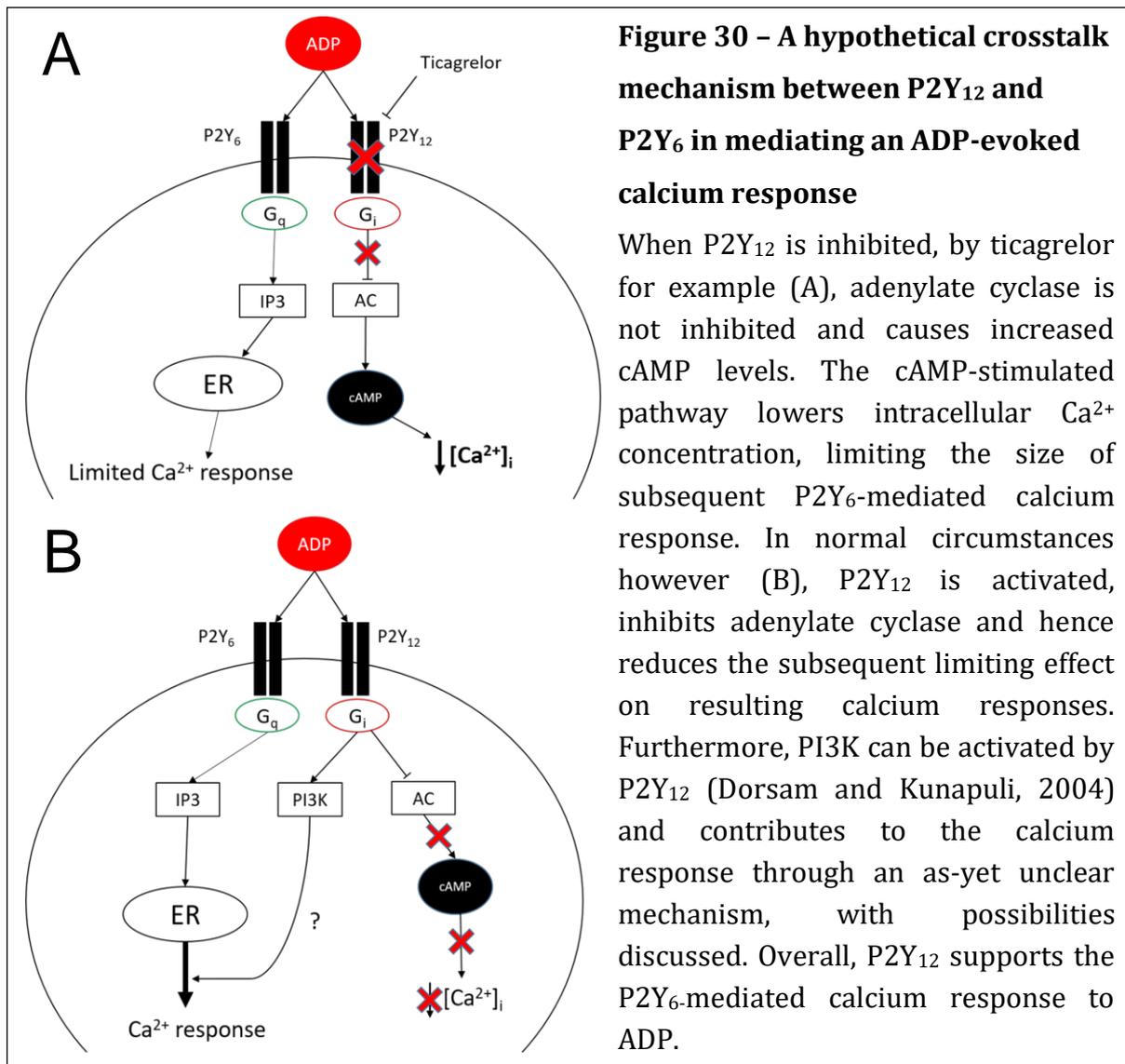
Figure 30 – A hypothetical crosstalk mechanism between P2Y$_{12}$ and P2Y$_6$ in mediating an ADP-evoked calcium response

When P2Y$_{12}$ is inhibited, by ticagrelor for example (A), adenylate cyclase is not inhibited and causes increased cAMP levels. The cAMP-stimulated pathway lowers intracellular Ca^{2+} concentration, limiting the size of subsequent P2Y$_6$-mediated calcium response. In normal circumstances however (B), P2Y$_{12}$ is activated, inhibits adenylate cyclase and hence reduces the subsequent limiting effect on resulting calcium responses. Furthermore, PI3K can be activated by P2Y$_{12}$ (Dorsam and Kunapuli, 2004) and contributes to the calcium response through an as-yet unclear mechanism, with possibilities discussed. Overall, P2Y$_{12}$ supports the P2Y$_6$-mediated calcium response to ADP.

The cAMP pathway may decrease the concentration of Ca^{2+} by stimulating the plasma membrane calcium ATP-ase (PMCA) channel to remove Ca^{2+} from the cell (Suplat et al., 2007), therefore limiting the magnitude of calcium responses.

The exact mechanism as to how PI3K may contribute to the P2Y$_{12}$-dependent potentiation of ADP-evoked calcium responses would still need to be investigated. One possibility could be that receptor-associated PI3K causes generation of PIP3, recruiting PLC γ and subsequently generating IP$_3$, which releases calcium ions (Rameh et al., 1998). An important point to consider is that the respective adenylate cyclase and PI3K pathways outlined above are suggested to be separate and are therefore not dependent on each other, but happen to both be activated by P2Y$_{12}$ stimulation.

The findings demonstrated here are not the only example of potential purinergic receptor crosstalk. Indeed, glioma C6 cells are indicated to exhibit the same $P2Y_{12}$-$P2Y_1$ crosstalk mechanism as that observed in platelets whereby $P2Y_{12}$ positively modulates $P2Y_1$-mediated calcium responses to ADP via adenylate cyclase inhibition and PI3K activation (Suplat et al., 2007). Intriguingly, Seo et al (2008). also reported the possibility of $P2Y_1$-$P2Y_{12}$ crosstalk in rat microglia interleukin-10 (IL-10) production, but with $P2Y_{12}$ negatively regulating $P2Y_1$ activity in this case. This was concluded on account of a $P2Y_{12}$ antagonist (2meSAMP) or a G_i protein blocker (pertussis toxin) causing upregulation of IL-10 production following stimulation with an ADP analogue (Seo et al., 2008).

It was identified in mice that simultaneous inhibition of multiple purinergic receptors, including $P2Y_{12}$ and $P2Y_2$, blocked chemotaxis of macrophages (Kronlage et al., 2010). However, mice with either $P2Y_{12}$ or $P2Y_2$ deficiency exhibited efficient chemotaxis (Kronlage et al., 2010), indicating that a combined parallel effort of purinergic receptors, including $P2Y_{12}$ and $P2Y_2$, may be required for proper chemotaxis of macrophages.

Overall, the results from this present investigation support the previous ones described here which suggest that $P2Y_{12}$, although G_i coupled, is involved in mediating cellular responses to ADP through crosstalk with another P2Y receptor. The cellular responses are not only at the level of calcium signalling, but are also, in some cases, functional responses such as cytokine production and chemotaxis, in several different cell types too. Subsequently, such growing evidence theoretically implicating a G_i-coupled receptor as playing a role in calcium responses means that it would be unwise in any future studies investigating G protein-coupled receptors to rule out G_i-coupled receptors as being involved in calcium responses. This notion is supported by reports that suggest PI3K activation could itself induce release of calcium ions through consequent PIP3 production, PLC γ recruitment and IP_3 generation (Rameh et al., 1998. Bae et al., 1998), with G_i-coupled receptors indeed causing activation of PI3K (Dorsam and Kunapuli, 2004).

If the theory presented here regarding $P2Y_{12}$-$P2Y_6$ crosstalk in monocytes holds true, it would cause further complexity into any current or future theories for the role of $P2Y_6$ in monocytes. For instance, Campwala et al (2014). indicated that $P2Y_6$ activation potentiates CCL2-evoked calcium signalling in monocytes, where it was found that pharmacological $P2Y_6$ inhibition or stable $P2Y_6$ knockdown attenuated chemotaxis and calcium responses to CCL2 in THP-1 cells. As stated however, $P2Y_{12}$ may positively modulate $P2Y_6$-mediated calcium responses to ADP, so $P2Y_{12}$ could have contribution in the functional monocyte response to CCL2. This is significant as CCL2 (or MCP-1) is a chemoattractant cytokine which has a key role in recruiting monocytes to inflammatory sites upon injury or infection but is also implicated in inflammatory conditions such as atherosclerosis (Deshmane et al., 2009).

It is stipulated here that upon $P2Y_{12}$ inhibition the downstream signalling pathway stimulated by cyclic AMP (cAMP) causes an inhibitory effect on subsequent calcium responses to ADP via activation of plasma membrane calcium efflux pumps. Interestingly, a previous study suggested that not only does $P2Y_{12}$ deficiency reduce macrophage immune responses to *E. coli* infection in mice, but cAMP analogues such as 8-bromo-cAMP also reduced ADP-induced CCL2 production and protection against bacterial infection (Zhang et al., 2016). Although the simultaneous effect of both $P2Y_{12}$ deficiency and addition of cAMP was not investigated by Zhang et al. (2016), which would be a more definitive measure, the results show that there is a possibility that the mechanism proposed in this investigation may run true in macrophages. This notion should be investigated further as the crosstalk mechanism involving $P2Y_{12}$, $P2Y_6$ and cAMP hypothesised here may have significance in the function of immune responses.

4.3 - Off-target effects of pharmacological $P2Y_{12}$ inhibitors in THP-1 monocytes

Ticagrelor is a clinically-administered reversible $P2Y_{12}$ inhibitor that is used to treat patients with acute coronary syndromes by acting as a platelet aggregation inhibitor (Wallentin et al., 2009). It is widely accepted that in addition to $P2Y_{12}$ inhibition, ticagrelor can also cause inhibition of the type 1 equilibrative nucleoside transporter (ENT-1) that allows intracellular transport of extracellular adenosine (Cattaneo et al., 2014). Inhibition of ENT-1 by ticagrelor causes extracellular accumulation of adenosine,

which has been suggested to contribute to the inhibition of platelet function by ticagrelor through activation of adenosine receptors (Aungraheeta et al., 2016).

In this study, it was observed that ticagrelor caused a gradual increase in calcium ion concentration when added as an agonist to THP-1 cells at concentrations of 10 μM or above. It was thought that this observation could be owing to the reported effect of ticagrelor causing accumulation of extracellular adenosine due to ENT-1 inhibition. However, upon investigation, further results indicated that use of an ENT-1 inhibitor did not cause a similar effect seen with ticagrelor treatment. This suggested that ENT-1 inhibition was not responsible for the effect of high concentrations of ticagrelor on calcium in THP-1 cells. Furthermore, when looking at THP-1 cells after pre-treatment with ticagrelor at 10 μM or higher, smaller numbers of cells were identified compared to cells pre-treated with 5 μM or below. This suggested that ticagrelor may be causing cell death at higher concentrations in THP-1 cells, which is likely to be the reason behind the gradual increase in calcium ions upon treatment with higher ticagrelor concentrations. There are no previous reports of ticagrelor having cytotoxic effects at such concentrations, and these novel findings should be explored further due to the clinical role ticagrelor plays in medicine.

Recent experiments investigating the effect of ticagrelor on endothelial calcium signalling found that ticagrelor (2-10 μM) caused an increase in intracellular calcium, which is suggested to have been from influx of calcium ions from the extracellular space, rather than intracellular calcium release from the ER (Gündüz et al., 2016). This is another possibility behind the increased intracellular calcium concentration induced by high ticagrelor concentrations, but would need further study as the nature of the suggested ticagrelor-evoked calcium influx effect presented by Gündüz et al. (2016) is unclear.

It may be the case that ticagrelor has off-target or cytotoxic effects at certain concentrations, however ticagrelor had no effect on intracellular calcium at the concentrations used to investigate the role of $P2Y_{12}$ in calcium responses to ADP. Therefore, such potential alternative effects of ticagrelor do not impact on the conclusions made here about $P2Y_{12}$. Furthermore, ticagrelor was shown to be selective

for $P2Y_{12}$ over $P2Y_6$ at the concentrations used, reinforcing its use as a suitable pharmacological tool here.

Previously it has been stated that $P2Y_{12}$ is primarily expressed in platelets, glial cells, endothelial cells and smooth muscle cells (Cattaneo, 2006), but results demonstrated here indicating that the receptor is expressed in human monocytes broaden the scope regarding the expression of $P2Y_{12}$ in the human body. As previously discussed in this chapter, there are growing reports identifying potential functional roles for $P2Y_{12}$ in leukocytes, such as macrophages and dendritic cells. Together with this investigation, these findings may question the effects clinically-used antiplatelet drugs, such as the $P2Y_{12}$ inhibitor ticagrelor, may be having on cells other than platelets when administered to patients. This is supported by a study involving patients receiving antiplatelet therapy, specifically the $P2Y_{12}$ inhibitor clopidogrel, where it was demonstrated that clopidogrel reduced leukocyte activation, with the leukocytes also found to express $P2Y_{12}$ (Diehl et al., 2009). This suggests that clopidogrel can act directly on leukocytes when administered, and not only on platelets. Hence, it would be wise to investigate any effects ticagrelor treatment has on leukocytes such as monocytes, dendritic cells and macrophages, as such clinically-used $P2Y_{12}$ inhibitors may be having negative impacts on immune function.

PSB-0739 is a pharmacological $P2Y_{12}$ inhibitor that has been reported to be highly potent, and selective at $P2Y_{12}$ over $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{13}$, $P2Y_{14}$, $P2X_2$, and $P2X_7$ (Baqi et al., 2009). On the contrary to ticagrelor however, PSB-0739 was not found to be selective for $P2Y_{12}$ over $P2Y_6$ in this study, indicating that the effects of this antagonist on ADP-evoked calcium responses are not due to inhibition of $P2Y_{12}$. Hence, conclusions about the role of $P2Y_{12}$ in monocytes cannot be drawn from experiments involving PSB-0739, and it would be wise for future studies to take care drawing conclusions from experiments using PSB-0739 to study $P2Y_{12}$. Nevertheless, in this investigation, experiments involving siRNA-mediated $P2Y_{12}$ knockdown support the results from experiments investigating the effect of ticagrelor on calcium responses induced by ADP, signifying that overall there is strong evidence for $P2Y_{12}$ involvement in ADP-evoked calcium responses.

4.4 - Conclusions

The findings presented here suggesting that $P2Y_{12}$ positively modulates ADP-evoked calcium responses in THP-1 monocytes broadens current knowledge in monocyte biology, subsequently having significance in expanding the current understanding the physiological function of monocytes in health and disease. Furthermore, an intricate crosstalk mechanism underpinning the involvement of $P2Y_{12}$ in ADP-evoked calcium responses has been indicated, drawing parallels with similar observations in platelets and other cell types. Consequently, this advances current knowledge in downstream signal transduction of G protein-coupled receptor activation and may have implications in the function of the immune system. Investigations into the use of the pharmacological $P2Y_{12}$ inhibitors used have implied that PSB-0739 may not be specific for $P2Y_{12}$, and have also shed further light on alternative effects of ticagrelor other than $P2Y_{12}$ inhibition. Finally, it is suggested that the platelet-independent effects of ticagrelor must be considered following the results shown here and in other studies that demonstrate $P2Y_{12}$ expression and potential functional significance in cells other than platelets.

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