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**BIOCHEMICAL AND MOLECULAR CHARACTERISATION OF
THE PROPOSED P2Y₁-P2Y₁₂ RECEPTOR HETERODIMER IN
TSA201 CELLS**

by

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A thesis submitted in partial fulfilment for the degree of Master of Research in
Pharmacology

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List of Abbreviations

AC	Adenylyl cyclase
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
APS	Ammonium persulfate
AR	Adrenoreceptor
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
BRET	Bioluminescent resonant energy transfer
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CFA	Complete Freund's adjuvant
CFP	Cyan fluorescent protein
CNS	Central nervous system
Co-IP	Co-immunoprecipitation
DMEM	Dulbeco's Modified Eagles medium
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglia
DTT	Dithiothreitol
EBI	European Bioinformatics Institute
ECACC	European Collection of Authenticated Cell Cultures
ECL	Enhanced chemiluminescence
ESSFT	Extra sum of squares F Test
FIIP	Formalin induced inflammatory pain
FP	Fluorescent protein
FRET	Försters resonant energy transfer
GABA	γ -aminobutyric acid
GABA	γ -aminobutyric acid
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
GTP	Guanosine triphosphate
HA	Haemagglutinin
HEK-293	Human embryonic kidney cells

HRP	Horseradish peroxidase
IASP	International Association for the Study of Pain
IP	Immunoprecipitation
IP ₃	Inositol 1,4,5-trisphosphate
LDS	Lithium dodecyl sulphate
mRNA	Messenger ribonucleic acid
MUSCLE	MULTiple Sequence Comparison by Log- Expectation
PCR	Polymerase chain reaction
PEI	Polyethylenimine
PI3-K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP ₂	Phosphatidylinositol 4,5-bisphosphate
PLC	Phospholipase C
PTX	Pertussis toxin
PVDF	Polyvinylidene difluoride
RET	Resonant energy transfer
RNA	Ribonucleic acid
RT	Reverse transcriptase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
siRNA	Small interfering RNA
STR	Short tandem repeats
SV40	Simian virus 40
TBE	Tris-Borate-EDTA
TEMED	Tetramethylethylenediamine
TRPV	Transient receptor potential cation channel subfamily V
tsA201	Temperature sensitive antigen 201
UDP	Uridine diphosphate
UTP	Uridine triphosphate
WT	Wild type
YFP	Yellow fluorescent protein

Abstract

The P2Y receptor family represents a wide-ranging set of G-protein-coupled receptors that respond to purinergic ligands, and are involved in many physiological processes. From signalling assays in tsA201 cells, the Kennedy lab has previously proposed the formation of a constitutive heterodimer between co-expressed human P2Y₁ and P2Y₁₂ receptors; this could represent a target for the control of pain sensing neurones. Therefore, it was aimed in this project to characterise if the receptors physically interacted using co-immunoprecipitation, and also to investigate the endogenous expression of other potentially interacting hP2Y receptors in the tsA201 cell line.

In this study, tsA201 cells were determined to express mRNA for hP2Y_{1, 2, 4, 6, 12, 14} receptors using reverse transcriptase PCR and confirmatory Sanger sequencing. Furthermore, the cells demonstrated Ca²⁺ responses to all of the natural hP2Y receptor agonists tested, the order of potency being ADP > ATP > UTP > UDP. When co-expressing hP2Y₁ and hP2Y₁₂ receptors oppositely tagged with a HA or fluorescent protein (FP), immunoprecipitating for the HA tag and blotting for the FP showed a visible high molecular weight protein complex of ~130 to >250 KDa for either combination of receptors. This implied that the FP tagged receptor had co-immunoprecipitated with the other HA tagged receptor as this protein was also visible when blotting for the HA tag, signifying the presence of both tagged receptors in the complex.

These results were overall suggestive of functional hP2Y_{1, 2, 12} receptor expression in the tsA201 cell line, and possibly also hP2Y_{4, 6, 14} receptors. The high molecular weight protein complex detected could represent a constitutive hP2Y₁-hP2Y₁₂ receptor heterodimer as proposed. Whilst it was not possible to specifically either confirm or refute the physical formation of the heterodimer with the data acquired, it was potentially supportive, and furthermore suggested other future paths of investigation.

Chapter 1: Introduction

Over 20% of the population in Europe have been reported to suffer from chronic pain (van Hecke *et al.*, 2013). Unfortunately, current treatments are typically of poor efficacy and are associated with side effects, mainly due to an inadequate understanding of the underlying mechanisms; this highlights the need for research into fresh methods by which pain may be managed (Labianca *et al.*, 2012; van Hecke *et al.*, 2013). In recent years the Kennedy lab group at the University of Strathclyde has been characterising a potential novel target for modulating pain sensing neurones activity: in 2010 it was postulated that P2Y₁ and P2Y₁₂ purinergic receptors may form a G protein-coupled receptor (GPCR) dimer complex (Shrestha *et al.*, 2010). This followed observations in temperature sensitive antigen 201 (tsA201) cells, which suggested that when recombinant receptors were transiently co-expressed and targeted by selective ligands they were able to inhibit neuronal currents in co-expressed two pore potassium channels with novel pharmacological effects; these channels have a well-defined role in pain sensing. Later Ca²⁺ signalling assays conducted in the same cell line corresponded with the electrophysiology data. It is therefore in this project intended to biochemically characterise the mechanisms by which these two receptors interact, and investigate other potentially interacting factors which may have been responsible for the signalling effects observed.

In this introduction the following areas will be covered: firstly, P2 purinergic receptor signalling will be examined, with the focus being on P2Y receptors. Subsequently, the roles of P2Y, and briefly P2X, receptors in pain will be covered; additional contextual information on the neurobiology and pathophysiology of pain will also be presented. The concept of GPCR dimerisation will subsequently be reviewed, including research methods, functional consequences and known/proposed P2Y receptor dimers. Lastly the aims of this project will be defined.

1.1.0 P2 Purinergic Receptor Signalling

Although traditionally considered only as a product of intracellular energy transfer in the Krebs cycle and as source of a phosphate group for kinases, in the past 40 years it has since been recognised that adenosine triphosphate (ATP) and other purine molecules such as adenosine form part of a wide ranging extracellular signalling system. Initially identified as an inhibitory neurotransmitter from non-adrenergic, non-cholinergic nerves in the gut, later experiments revealed that ATP was a co-transmitter in many neurones, which was highly controversial at the time as it violated the so called "Dale's principle" of each neurone only releasing a single neurotransmitter (Burnstock, 2009; Burnstock, 2013b). With further

research it was recognised that purinergic signalling receptors could be separated into two families based on their agonist selectivity, with P1 receptors (also termed as adenosine receptors) being selective to adenosine, and P2 receptors to ATP and adenosine diphosphate (ADP). P2 receptors were later split into P2X and P2Y receptors based on their pharmacology (Burnstock and Kennedy, 1985), and starting in the early 1990's the receptors began to be cloned, confirming the differences between the two branches (Abbracchio and Burnstock, 1994) and leading to the discovery of many additional subtypes in each (reviewed in Weisman *et al.*, 2006). Various drugs targeting aspects of purinergic signalling are now in clinical use, and more continue to be developed.

1.1.1 P2X Receptors

P2X receptors represent a diverse family of ionotropic receptors widely expressed throughout various systems of the body. Seven subunits have been presently characterised, with all responding strongly to ATP, but not ADP, uridine-triphosphate (UTP), uridine-diphosphate (UDP) or UDP-glucose. P2X subunits can form homomeric or heteromeric receptors (Khakh *et al.*, 2001; Kennedy *et al.*, 2013) and have been associated with a wide variety of roles, including amongst others cardiovascular disease (Kennedy *et al.*, 2013), cancer, kidney disease and osteoporosis (Burnstock, 2013). They have additionally been identified as having important roles in pain, as briefly surmised later on in section 1.2.5.

1.1.2 P2Y Receptors

P2Y receptors represent a family of G protein-coupled receptors (GPCRs) that respond preferentially to ATP, ADP, UTP, UDP or UDP-glucose depending on the receptor subtype. P2Y receptors follow the standard GPCR structure of 7 transmembrane domains and belong to the Class A "rhodopsin like" family of GPCRs, which is the largest class (Abbracchio *et al.*, 2006). These bind ligands on the transmembrane helices or extracellular loops, and represent the target of purines, many neuropeptides and most amine neurotransmitters. This contrasts with the smaller Class B ("glucagon/secretin") and Class C ("metabotropic glutamate") families of GPCRs, which bind ligands on their extracellular tails and are respectively receptors for peptide hormones and ligands such glutamate (Rang *et al.*, 2011).

Currently eight P2Y subtypes have been characterised in humans (surmised in table 1), coupled to heterotrimeric G proteins. P2Y₁, P2Y₂, P2Y₄, P2Y₆ and P2Y₁₁ receptors mainly couple to G_{q/11} α subunits, whilst P2Y₁₂, P2Y₁₃ and P2Y₁₄ receptors couple to G_{i/o}; the P2Y₁₁

receptor additionally couples to G_s (Abbracchio *et al.*, 2006). Under certain conditions the receptors may also couple with other α subunits: the $P2Y_{13}$ receptor for instance will also couple to G_s when stimulated by high concentrations of ADP (Marteau *et al.*, 2003). The sequence gaps in nomenclature represent receptors which were initially classified as $P2Y$ receptors when first cloned, but are now classified as other receptor types.

Table 1: Properties of human $P2Y$ receptors

Receptor	Natural Agonist	G protein - Main Effector(s)
$P2Y_1$	ADP	G_q - $PLC\beta$, Rac, Rho activation
$P2Y_2$	ATP/UTP	G_q - $PLC\beta$ activation G_o - $PLC\beta$, Rac activation G_{12} - Rho activation
$P2Y_4$	UTP	G_q - $PLC\beta$ activation G_o - $PLC\beta$ activation
$P2Y_6$	UDP	G_q - $PLC\beta$ activation $G_{12/13}$ - Rho activation
$P2Y_{11}$	ATP	G_q - $PLC\beta$ activation G_s - AC activation
$P2Y_{12}$	ADP	$G_{i/o}$ - AC inhibition $PLC\beta$, RhoA activation
$P2Y_{13}$	ADP	$G_{i/o}$ - AC inhibition $PLC\beta$, RhoA activation
$P2Y_{14}$	UDP/UDP-glucose	$G_{i/o}$ - AC inhibition $PLC\beta$ activation

Summary of the natural agonists and main G protein effects in human $P2Y$ receptors (Adapted from Erb and Weisman, 2012).

G Protein Cycle and Targets:

Binding of agonists to the receptor initiates the G protein cycle. On the α subunit the exchange of Guanosine diphosphate (GDP) for intracellular guanosine triphosphate (GTP) is promoted, with the α subunit then disassociating from the $\beta\gamma$ complex and interacting with its target protein. GTPase activity on the α subunit increases following target interaction, hydrolysing the GTP to GDP; the α subunit then re-joins with the $\beta\gamma$ complex and the cycle is ready to repeat (Rang *et al.*, 2011). With $G_{q/11}$ coupled α subunits phospholipase C- β (PLC β) is activated, cleaving plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP₂) to inositol 1,4,5-trisphosphate (IP₃); intracellular Ca²⁺ release is then triggered following the action of IP₃ on endoplasmic reticulum IP₃ receptors. PIP₂ cleavage additionally forms diacylglycerol, which consequently activates protein kinase C. Subunits coupled to $G_{i/o}$ and G_s however are respectively associated with the inhibition and stimulation of adenylyl cyclase (AC) activity, leading to a corresponding decrease/increase in cyclic adenosine monophosphate (cAMP) formation and hence protein kinase A activity. The released $\beta\gamma$ complex can also modulate its own targets, including ion channels such as N-type Ca²⁺ channels (Simon *et al.*, 2002), and PLC β to invoke IP₃ based signalling in $G_{i/o}$ coupled receptors (Murthy and Makhlouf, 1998). Along with these typical second messenger actions, P2Y receptors are able to mediate cellular activities through other pathways (reviewed in Erb and Weisman, 2012). For example $G_{q/11}$ subunits additionally bind to and stimulate the p63RhoGEF guanine nucleotide exchange factor, which can then act on downstream Rho targets (Lutz *et al.*, 2005).

1.1.3 Human P2Y₁ and P2Y₁₂ Receptor Tissue Distribution

P2Y receptors are widely expressed in the human body, with P2Y₁₂ receptors mainly expressed on platelets, where in concert with P2Y₁ receptors they are involved in platelet activation and aggregation, resulting in the eventual cessation of bleeding (O'Connor *et al.*, 2011). hP2Y₁₂ receptors are also present on other tissues, including endothelial, epithelial and dendritic cells, vascular smooth muscle and leukocytes, where amongst other functions they have been identified as having roles in inflammation and vasoconstriction (Gachet, 2012; Shirashaki *et al.*, 2013; Nylander and Schulz, 2016). Additional expression is shown on brain glial cells and on neurons (Hollopeter *et al.*, 2000, Satoh *et al.*, 2016), where they are proposed to have important roles in pain as described later in Section 1.2.5.

In contrast, the expression of hP2Y₁ receptors is substantially wider compared to hP2Y₁₂ receptors, with mRNA highly expressed in ovary, intestine, prostate, placenta and brain

tissue, lesser expressed in heart, lung and placental tissue and negligibly expressed in kidney, liver, bone and cartilage tissue (Janssens *et al.*, 1996; Moore *et al.*, 2001); the expression pattern for certain tissues widely varies between studies. hP2Y₁ receptors have been located in all regions of the brain (Moore *et al.*, 2001) and are widely expressed on neurons (Moore *et al.*, 2000); they have likewise been proposed to have key roles in pain as detailed later on.

This wide distribution highlights one of the potential difficulties of targeting P2Y receptors clinically, due to the likelihood of off-target effects; targeting the neuronal P2Y₁ or P2Y₁₂ receptors for instance for pain control purposes could also lead to abnormalities in platelet or immune cell function. Interestingly a proportion of the clinical effects of anti-thrombolytic platelet P2Y₁₂ receptor antagonists may result from their additional actions on endothelial and vascular smooth muscle cells (Nylander and Schulz, 2016).

1.1.4 Clinical P2Y Receptor Ligands in Current Use

Despite the ubiquitous expression of P2Y receptors and the many potential drug targets, this has unfortunately so far largely not translated into clinically used drugs for the majority of subtypes; mainly this is due to the difficulties of developing sufficiently selective ligands (Jacobson *et al.*, 2009). Currently out of the few drugs in human use, anti-thrombolytic platelet P2Y₁₂ receptor targeting antagonists represent the largest class. The most common of these is the pro-drug clopidogrel (Plavix), but as this relies on efficient enzymatic conversion to its active metabolite it is less effective in some patients expressing enzyme polymorphisms, and newer directly active competitive P2Y₁₂ receptor antagonists such as cangrelor (also known by its developmental name ARC-69931MX and the trade name Kengrexal) and ticagrelor (Brilinta) have since been developed (O'Connor *et al.*, 2011). Cangrelor is highly selective for the P2Y₁₂ receptor, and coupled with its potent inhibitory properties – a reported pA₂ of 9.2 (Horváth *et al.*, 2014) - it is also commonly used to test for the presence of functional P2Y₁₂ receptor expression (Suzuki *et al.*, 2011).

1.1.5 Cell Lines Used for *In-vitro* P2Y Receptor Studies

Human embryonic kidney cells (HEK-293) – plus derivatives such as the Simian virus 40 (SV40) transformed tsA201 and HEK-293T lines -, and the human astrocytoma 1321N1 cell lines are some of the more commonly used lines in P2Y receptor research. The former is for its ability to transiently express recombinant receptors with high efficiency (Thomas and

Smart, 2005), and the latter for the lack of any known endogenous P2Y receptor expression (Parr *et al.*, 1994; Suzuki *et al.*, 2011). However, despite the popularity of HEK-293 type cell lines for studying transfected P2Y receptors, there have been few studies looking at the overall endogenous mRNA expression of the P2Y or other purinergic receptors; even fewer have studied functional expression. This is particularly important considering that HEK-293 cells are known to endogenously express P2Y receptor subtypes (Choi *et al.*, 2008; Suzuki *et al.*, 2011), and it is correspondingly difficult to characterise if any observed signalling or biochemical effects were induced or modulated through the endogenous receptors presence; this has previously been responsible for misleading and unclear data (Abbracchio *et al.*, 2005). 1321N1 cells are therefore more appropriate for P2Y research due to their lack of endogenous expression, but their use is limited by the cells being much more difficult to efficiently transiently transfect (Marucci *et al.*, 2011) compared to HEK-293 cell lines.

1.2.0 The Role of the Purinergic System in Pain

The role of the purinergic system in pain was first described when it was noted that ATP invoked a painful response in blister tests (Collier *et al.*, 1966). P2Y receptors have been implicated in the pathogenesis and maintenance of both acute and chronic pain; the different types of pain will be discussed first to place later information in context. Whilst glial cell P2Y receptors are involved in pain, the better studied peripheral receptors will be mainly concentrated on later in this section; P2X receptors roles in pain will also be briefly discussed at the end.

1.2.1 What is Pain?

Pain is defined by the International Association for the Study of Pain (IASP) (2012) as “*An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage.*” It can be broadly separated into two categories, acute and chronic. Acute pain is generally defined as a response to tissue damage that provides a protective function and lasts for a short time, arbitrarily set by most sources as up to 30 days (Thienhaus and Cole, 2002); it can typically be described as being nociceptive or inflammatory. Pain which continues after this is described as chronic, and is accompanied by changes in the central nervous system (CNS); chronic pain can be a result of long term inflammatory pain or from neuropathic pain, which is of a distinct cause (Voscopoulos and Lema, 2010).

1.2.2 Nociceptive pain

Nociceptive pain is defined by the IASP (2012) as “*Pain that arises from actual or threatened damage to non-neural tissue and is due to the activation of nociceptors.*” This distinguishes from the definition of neuropathic pain, and describes pain experienced with a conventionally functioning somatosensory nervous system.

Pain transduction begins at the nociceptors, peripheral specialised neuronal fibres that respond to the presence of various noxious stimuli, including heat, mechanical pressure and chemicals (see Basbaum *et al.*, 2009 and Dubin and Patapoutian, 2010 for reviews); these are located throughout the somatic (e.g skin, muscles) and visceral tissues to mediate pain sensing (Kruger *et al.*, 2003). Upon stimulation, reaching their activation threshold and depolarising, nociceptors transmit an action potential along their axon to higher brain centres. There are several types of nociceptive fibres, which differ in both their conductive speed and structure: of the two types associated with pain signalling, medium diameter myelinated A δ fibres mediate initial sharp, well localised, “fast” pain, and have a lower activation threshold. These contrast with the small diameter unmyelinated C fibres which mediate dull, “slow”, un-localised pain, additionally having a higher activation threshold. (Basbaum *et al.*, 2009; Gereau and Cavallone, 2013; Steeds, 2013).

Nociceptive fibres synapse in the dorsal horn of the spinal column, and the signals are then transmitted along the “pain pathways” to the brain for processing (Gereau and Cavallone, 2013; Steeds, 2013). The ascending pain pathways transmit nociceptive signals to higher brain centres, the main two pathways being the spinothalamic and the spinoreticulothalamic tracts; the former relays the strength and site of painful stimuli, whilst the latter induces psychological effects of a painful experience (Almeida *et al.*, 2000; Basbaum *et al.*, 2009).

The descending pathways however are associated with the modulation of pain transmission, with the fibres utilising 5-HT and noradrenaline at synapses, and enkephalins released from spinal cord interneurons acting at pre-synaptic μ -opioid receptors to inhibit neurotransmitter release from nociceptive fibres (Ossipov *et al.*, 2010; Steeds, 2013; Gereau and Cavallone, 2013).

1.2.3 Inflammatory Pain: the Development of Peripheral and Central Sensitisation

The concept of neuroplasticity – where the nervous system adapts to new conditions – is a key factor in the development of pain (Puretić and Demarin, 2012). Tissue damage induces inflammatory pain, caused by the release of inflammatory mediators and cytokines from surrounding cells, including bradykinin, prostaglandins and tumour necrosis factor- α ; these interact with voltage and ligand gated channels on chemical sensitive C fibres, causing fibre stimulation and nociceptive transmission (Voskopoulos and Lema, 2010) (Figure 1). The nociceptor also induces the release of mediators such as substance P for stimulation and sensitisation of itself – neurogenic inflammation (Richardson and Vasco, 2002). These factors induce peripheral sensitisation: the local nociceptive fibres become more sensitive through a lowering of the activation threshold so that normally non-painful stimuli induce pain (allodynia), and there are further increased responses to painful stimuli (hyperalgaesia). In acute pain this temporary sensitisation usefully deters further damage to tissues, and returns to normal following tissue healing (Bennet, 2000; Ringkamp *et al.*, 2013; Cohen and Mao, 2014).

Continued inflammatory pain in conditions such as rheumatoid arthritis typically leads to a state of chronic pain where the inflammation and sensitisation no longer serve any useful protective purpose. Prolonged peripheral sensitisation induces central sensitisation, causing the CNS to be in a state of hyper-excitability and enhancing pain processing because of neuronal remodelling (Puretić and Demarin, 2012). Beyond the site of the tissue damage large diameter A β fibres that mediate touch sensing may have their normally non-painful signals processed as pain (secondary allodynia), and changes in the descending pain pathways can inhibit anti-nociceptive modulation (secondary hyperalgaesia) (Devor, 2009; Ringkamp *et al.*, 2013; Cohen and Mao, 2014).

Figure 1: Interaction of inflammatory mediators on peripheral nociceptor surface receptors.

Tissue damage leads to the release of inflammatory mediators – the so called “inflammatory soup” - from surrounding cells and the nociceptor itself; these interact with surface receptors on the nociceptor, leading to nociceptive signalling and eventual peripheral sensitisation.

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1.2.4 Neuropathic Pain

Neuropathic pain is described by the IASP Taxonomy (2012) as “*Pain caused by a lesion or disease of the somatosensory nervous system.*” As such neuropathic pain differs from nociceptive pain as it is mediated by direct damage to the neuronal fibres of the somatosensory system rather than nociceptor stimulation. Neuropathic pain is frequently described as an unremitting, “shooting”, burning sensation that does not respond well to classical analgesics, and is associated with allodynia and hypersensitivity from abnormal action potential generation in the nociceptive pathways (Costigan *et al.*, 2009; Voscopoulos and Lema, 2010; Cohen and Mao, 2014). Nerve injury induces Wallerian degeneration, resulting in production of algescic mediators and cytokines that act on the nociceptive fibre, and induce hypersensitivity in nearby uninjured fibres (Üçeyler and Sommer, 2006). Many of the mechanisms resulting in chronic neuropathic pain are similar to those in nociceptive pain: analogous neuronal remodelling mediating central sensitisation occurs, with the descending pathways losing their inhibitory functions and the changes being mediated by similar mechanisms (Costigan *et al.*, 2009; Cohen and Mao, 2014).

1.2.5 P2 Receptors and Pain

Tissue injury results in the release of ATP and other purines that can act as mediators at P2 receptors expressed on sensory dorsal root ganglia (DRG), thereby influencing signalling through nociceptive fibres. With the possible exception of P2Y₁₁ all P2Y receptor subtypes have been located on sensory DRG (Burnstock, 2013a; Magni and Ceruti, 2013), and there is extensive data on the role of P2Y receptors in pain from animal models.

P2Y₁ receptors appear to mediate heat sensitive nociception via interaction with transient receptor potential cation channel subfamily V member 1 (TRPV₁) receptors (Mallin and Moliver, 2010); low level P2Y₁ receptor stimulation with the selective agonist MRS2365 has been associated with an increase in thermal pain threshold, but has no effect in higher doses (Andó *et al.*, 2010). Inhibition or knockout of P2Y₁ inhibits the inducement of peripheral inflammatory sensitisation by complete Freund's adjuvant (CFA) (Mallin and Moliver, 2010), and additionally in formalin induced inflammatory pain (FIIP) P2Y₁ receptors are similarly pro-nociceptive (Barragán-Iglesias *et al.*, 2015). P2Y₂ and/or P2Y₄ receptor activation with UTP raises the pain threshold in the hot plate test (Andó *et al.*, 2010), and P2Y₂ receptors appear to also be involved with TRPV₁ and peripheral sensitisation (Moriyama *et al.*, 2003);

chronic inflammatory pain has been further reported to upregulate P2Y₂ receptor expression (Zhu *et al.*, 2015).

The roles of P2Y₄ and P2Y₆ receptors in DRG are less clear, which is mainly due to a lack of sufficiently selective ligands. UTP or UDP administration is associated with an increase in pain threshold in the hotplate test (Andó *et al.*, 2010), and both the receptors have been suggested to have an inhibitory role in spinal pain transmission (Okada *et al.*, 2002). Recently though a study in rats by Barragán-Iglesias *et al.* (2015) using FIIP has suggested the involvement and upregulation of peripheral P2Y₆ receptors in the development of chronic inflammatory pain; in the same study P2Y₁₁ mRNA was located in rat DRG for the first time, and this was analogously upregulated. Activation of either P2Y₆ or P2Y₁₁ receptors before injection of formalin led to increased pain responses as quantified by flinching behaviour, and these were correspondingly reduced by the presence of antagonists prior to formalin injection.

P2Y₁₂, ₁₃, ₁₄ receptors have been suggested to have an inhibitory effect on nociceptive and inflammatory pain signalling; stimulation of any of these receptors has been associated with anti-nociceptive effects in CFA induced inflammatory pain when P2Y₁ receptors are antagonised or knocked out (Mallin and Moliver, 2010). In contrast to this report though P2Y₁₂ expression levels have also been seen to be up-regulated during hyperalgesia (Horváth *et al.*, 2014), and selective P2Y₁₂ antagonists to be anti-nociceptive in all pain types in rodent models, with receptor knockout mice displaying effects similar to pharmacological inhibition (Andó *et al.*, 2010; Horváth *et al.*, 2014)

The effect of P2Y receptors on neuropathic pain has revealed some interesting potential drug targets. The P2Y₁ selective agonist MRS2365 has been associated with the reversal of mechanical allodynia following surgery in neuropathic pain models; UTP acting at the P2Y₂ and/or P2Y₄ receptors was also anti-nociceptive, but to a much lesser extent (Andó *et al.*, 2010). Selective P2Y₁₂ antagonism in particular has been associated with profound anti-allodynic effects and inhibition of neuropathic pain development in several studies (Tozaki-Saitoh *et al.*, 2008; Andó *et al.*, 2010; Horváth *et al.*, 2014). It has been demonstrated that a time dependence of peripheral and central P2Y₁₂ mRNA expression appears to exist, which correlates with the development of central sensitisation in rodent models for both inflammatory and neuropathic pain; this can be prevented by selective P2Y₁₂ receptor antagonists or receptor knockout (Tozaki-Saitoh *et al.*, 2008; Horváth *et al.*, 2014).

Amongst their many functions P2X receptors have also been identified as having roles in pain: all sensory dorsal root ganglia neurones have been found to express P2X receptors (Burnstock, 2013a). P2X₃, P2X₄ and P2X₇ receptors are especially implicated in the development and/or maintenance of chronic pain, which is abolished by selective receptor knockout or pharmacological inhibition (Hughes *et al.*, 2007; Fabbretti, 2013; Tsuda *et al.*, 2013).

Despite all this extensive research in animal models, there is however very little data regarding the role of the P2 receptors, or the effects of ligands targeting them, in any form of human pain (Burnstock and Sawynok, 2010). Whilst P2Y₁₂ receptor antagonists have been clinically used as anti-thrombolytic agents for several decades now, despite an extensive search of the literature it has only been possible to find minimal even anecdotal evidence of these drugs affecting pain perception in humans; this could be related to the doses used in clinical practice being lower than in animal model pain studies.

In a small, short duration, double blind, placebo controlled study with sickle cell anaemia patients, the platelet P2Y₁₂ receptor antagonist prasugrel was associated with a lower rate and intensity of sickle cell related pain as quantified by patient diaries, but this did not reach the level of statistical significance (Wun *et al.*, 2013). Subsequently a larger, multinational study of longer duration likewise did not find a significant effect, and the differences between the groups were not as pronounced as in the earlier study (Heeney *et al.*, 2015). Whilst the thienopyridine class P2Y₁₂ receptor antagonists clopidogrel and ticlopidine have been associated with a development or increase in joint pain and polyarthrititis as a side effect in some patients (Garg *et al.*, 2000; Dakik *et al.*, 2002; Agrawal *et al.*, 2013), this seems to be a class specific effect as it is typically relieved by switching affected patients to other P2Y₁₂ antagonists such as prasugrel (Coulter and Montandon, 2012; Agrawal *et al.*, 2013). Because of the overall limited data available, it must be taken with caution therefore as to the extent that any animal model studies may be ultimately translatable to humans.

1.3.0 G Protein-Coupled Receptor Dimerisation

1.3.1 The Concept of Dimerisation

Traditionally GPCRs were considered to be monomeric entities that coupled with G proteins on a 1:1 stoichiometric basis to cause physiological effects. However, whilst this model is indeed possible (Chabre and le Maire, 2005), it is now well established this simplistic view

may not be appropriate in a number of cases, and that multiple GPCRs can interact with one another in various compartments to form dimeric complexes, which may be linked to one or several G proteins if expressed on the cell surface (Ferré *et al.*, 2009; Kamal *et al.*, 2011; Ferré *et al.*, 2014) as illustrated in Figure 2. These may have different functional properties compared to their singular receptors, and have been long proposed as lucrative potential new drug targets, although this has not come to fruition yet (Bortolato *et al.*, 2009). The alternate terms “oligomer” or “multimer” may be used in other literature, but in this thesis the term “dimer” will be used as it is seen as the smallest multimeric unit.

Dimers can be receptors both of the same type interacting together – “homodimers” – or of entirely different types, so called “heterodimers” (Ferré *et al.*, 2009); indeed, two receptors does not seem to be the limit either, with evidence of higher order dimers – “multimers” - appearing for numerous receptor types (Vischer *et al.*, 2011). In some cases, such as most Class C GPCRs, it has been conclusively demonstrated that dimerisation is essential for normal receptor expression and function (Pin *et al.*, 2007).

Figure 2: Possible stoichiometric models of G protein coupling to receptors

a) 1:1, the simplest possible model, a monomeric receptor coupled to a heterotrimeric G protein b) 2:2, two G proteins, each coupled to a protomer within a dimer complex c) 2:1, a single G protein-coupled to both protomers of a dimer complex (Figure reproduced from Kamal *et al.*, 2011 under an Open Access Licence).

1.3.2 A Brief History of Dimerisation and Research Techniques

The first indications of GPCR dimerisation came indirectly in the mid 1970's with pharmacological binding assays that appeared to show negative or positive cooperativity, suggesting the presence of multiple binding sites (Limbird and Lefkowitz, 1976; Maggi *et al.*, 1980). Beginning in 1982 target size analysis experiments using radiation inactivation indicated that functional receptors tended to have a molecular weight twice or more the size of singular receptors measured using biochemical techniques, leading to suggestions that

the functional receptors were formed of two or further subunits (Fraser and Venter, 1982; Ng *et al.*, 2013). Using target size analysis several possible homodimers were identified, including for β 2 adrenoceptors (AR) (Fraser and Venter, 1982), α 1AR (Venter *et al.*, 1984) and opioid receptors (McLawhon *et al.*, 1983), but the technique was limited as it could not differentiate between receptor-receptor and other receptor-protein interactions (Rios *et al.*, 2001).

Although this approach, and others such as trans-complementation where truncated mutated receptors of differing types displayed restored signalling and function when co-expressed (Maggio *et al.*, 1993), provided a large pool of evidence supporting the concept of dimerisation, there was no direct proof. The development of differential epitope tagging of recombinant receptors combined with co-immunoprecipitation and Western blotting was a major step as it was able to detect direct receptor-receptor interactions. With the first application of this technique β 2AR homodimerisation was demonstrated by Hebert *et al.* (1996), providing the first direct physical evidence of dimerisation; it was shortly expanded to probing the heterodimerisation of δ - κ opioid receptors (Jordan and Devi, 1999) and has since become the most commonly used biochemical method (Szidonya *et al.*, 2008). It does however have drawbacks such as that it requires a high level of receptor overexpression, which may artificially induce dimer formation (Szidonya *et al.*, 2008); furthermore, incomplete solubilisation may cause formation of non-specific aggregates (Salim *et al.*, 2002).

Biochemical techniques have a disadvantage in that they are unable to be used with living cells. More recently biophysical resonant energy transfer (RET) techniques such as bioluminescent RET (BRET) and Försters RET (FRET) have become popular, both for their ease of use and for their ability to quantify dimer formation in living cells (Ng *et al.*, 2013). RET (Figure 3) uses energy transfer from an excited fluorescent/luminescent “donor” molecule – for example cyan fluorescent protein [CFP] - expressed recombinantly on one receptor to an “acceptor” – in the case of CFP, typically yellow fluorescent protein (YFP) - on another receptor if in close enough proximity - approximately 50 Å for BRET (Angers *et al.*, 2000) –, producing detectable fluorescence (Goddard and Watts, 2012). The RET approach offers the some of the best resolution strategies for studying dimers (Goddard and Watts, 2012; Ng *et al.*, 2013) and has been used to suggest the development of many different dimers. However, RET is limited as it functions on close rather than physical association, so it is important that protocols are followed and controls taken to avoid mis-interpretation (Marullo and Bouvier, 2007). Furthermore, a major additional drawback is that it can typically only be used in a tagged, recombinant overexpression system, and as so RET is usually unable to be used to detect native dimers (Persani *et al.*, 2007).

It has additionally become apparent that higher order “multimers” (also termed oligomers) may also form between GPCR dimers. Although potentially eluded to by early methods it wasn't until the development of modern co-immunoprecipitation and RET techniques that the concept was able to gain acceptance, but even these are unable to easily provide data on the order of a dimer. Amongst other studies, using three unique tags Park and Wells (2004) were able to co-immunoprecipitate muscarinic M₂ receptors of at least trimeric order. Some of the most compelling potential evidence for multimers has stemmed from the publication of GPCR structures using atomic force microscopy, with the rhodopsin structure appearing to consist of paracrystalline arrays of receptors (Fotiadis *et al.*, 2004). However, the structure has been criticised on methodological grounds (Chabre and le Maire, 2005), and it has been pointed out that interpretation of atomic force microscopy images is somewhat subjective (Gurevich and Gurevich, 2008). Both the potential functional consequences and the existence of multimers *in-vivo* are still largely unclear.

Figure 3: The mechanism behind the principle of resonant energy transfer methods

A fluorescent donor molecule - cyan fluorescent protein in this case - on one receptor is excited by a background fluorescent source, and if it is within close enough proximity then the partner acceptor molecule – yellow fluorescent protein in this case – will subsequently emit detectable fluorescence of a known wavelength from energy transfer. Although this example shows Försters resonant energy transfer (FRET), the principle is similar for other RET technologies

Figure reproduced from Methods, 27(4), Overton MC and Blumer KJ., “Use of fluorescence resonance energy transfer to analyze oligomerization of G-protein-coupled receptors expressed in yeast”, 2002 with permission of Elsevier.

1.3.3 The Dynamic Nature of Dimerisation

One point that has become clear is that GPCR dimerisation is typically dynamic, and as well as existing as dimers in a basal state the order of a GPCR may change in response to various factors. Using a biophysical single molecule technique for instance it has demonstrated that β_1 AR, β_2 AR, and γ -aminobutyric acid B (GABA_B) receptors each move between monomeric, dimeric or multimeric states based on a number of factors including agonist stimulation and receptor density (Calebiro *et al.*, 2012). Additionally, a large proportion of hP2Y₁ receptors transfected into the HEK-293 cell line have been seen to exist as homodimers, but upon agonist stimulation the proportion of dimers to monomers further increases (Choi *et al.*, 2008)

1.3.4 Functional Examples of GPCR dimers

Although by now it is commonly accepted that GPCRs form dimers, perhaps the most important information to date has come from cases where it has been found to affect GPCR function; some prominent examples will now be discussed.

Receptor expression:

It has been found that for cellular expression it is necessary for some GPCRs to first dimerise. The most well-known example is likely the GABA_B receptor, which after being initially cloned was found to have substantially lower affinity for agonists than native receptors (Kaupmann *et al.*, 1997), as it was unable to be expressed on the cell surface (Couve *et al.*, 1998). Shortly another GABA_B form was identified, $\text{GABA}_{B(2)}$, and it was found to be vital for this to be co-expressed with the original cloned receptor ($\text{GABA}_{B(1)}$) in order for a functional GABA_B receptor to be expressed, leading to the conclusion that GABA_B receptors are inherently heterodimers of these two subunits (Jones *et al.*, 1998; Kaupmann *et al.*, 1998; White *et al.*, 1998). It has since been identified that in the heterodimer complex the $\text{GABA}_{B(1)}$ subunit expresses the orthosteric ligand binding site, whilst the $\text{GABA}_{B(2)}$ subunit couples with the G protein to mediate the signalling effects (Margeta-Mitrovic *et al.*, 2001; Bettler *et al.*, 2004). However there is evidence which challenges this essential heterodimerisation theory, as there is partial overlap or separation of function in certain cases: $\text{GABA}_{B(2)}$ has appeared to independently couple to AC despite being lacking a ligand binding site (Martin *et al.*, 1999), and $\text{GABA}_{B(1)}$ alone has been reported to inhibit TRPV₁

sensitisation (Hanack *et al.*, 2015). This may result from alternative isoforms of GABA_{B(1)} (Calver *et al.* 2000; Jacobson *et al.*, 2006 Pinard *et al.*, 2010).

Since this seminal discovery of GABA_B's essential heterodimerisation it has become apparent that most Class C GPCRs function likewise, but some have been found to not be heteromers and may act through “cross talk” mechanisms that do not involve physical association (Prezeau *et al.*, 2010).

Pharmacology:

For some dimer pairs it has been established that the fundamental pharmacology can alter upon dimerising; there have long been indirect indications of individual receptor types seeming to alter their binding affinity for specific ligands when other receptor types were co-expressed (Maggi *et al.*, 1980). The opioid receptor family is probably the best characterised example in this area, with the first direct indications coming from Jordan and Devi (1999). Potential heterodimerisation between transfected δ -opioid (δ -OR) and κ -opioid (κ -OR) receptors was established using co-immunoprecipitation, and furthermore as summarised in table 2 δ - κ opioid dimers appeared to show practically no binding affinity for κ or δ selective agonists, but incubation with specific ligands to one receptor type appeared to synergistically increase the binding affinity to agonists for the other receptor; δ - κ opioid dimerisation has subsequently been supported by later BRET analysis (Ramsay *et al.*, 2002). Potential μ - δ opioid dimers have also been found using co-immunoprecipitation (George *et al.*, 2000; Gomes *et al.*, 2000) and BRET (Gomes *et al.*, 2004), and are reported to have similar ligand binding characteristics to δ - κ opioid dimers (Gomes *et al.*, 2004; Gomes *et al.*, 2011).

Although Jordan and Devi (1999) were unable to co-immunoprecipitate μ - κ opioid dimers, later studies have indicated heterodimers through both co-immunoprecipitation and BRET (Wang *et al.*, 2005; Chakrabarti *et al.*, 2010). μ - κ opioid dimers in contrast seem to retain their affinity for κ selective agonists, whilst maintaining the reduced μ -receptor binding affinity; binding of κ selective agonists seems to increase μ -receptor binding affinity, but no opposite effect has been observed (Wang *et al.*, 2005)

Table 2: Ligand binding affinities of the δ - κ opioid dimer and its constituent GPCRs.

Ligand	K _i (nM)		
Agonists	κ -OR	δ -OR	κ -OR/ δ -OR
U69593 (κ -OR selective)	14.4 $\pm$ 0.2	> 1000	> 1000
DPDPE (δ -OR selective)	> 1000	21.8 $\pm$ 2.1	> 1000
Antagonists			
Norbinaltorphimine (mainly κ -OR selective)	4.9 $\pm$ 0.3	31.8 $\pm$ 1.2	126 $\pm$ 9.6
TIPP (δ -OR selective)	> 1000	0.28 $\pm$ 0.6	> 1000
Combinations of ligands			
U69593 (+ 10 μ M DPDPE)	14.4 $\pm$ 0.4	-	9.2 $\pm$ 1.4
DPDPE (+ 10 μ M U69593)	-	24.8 $\pm$ 1.6	20.0 $\pm$ 1.3

The effect of δ - κ dimerisation on ligand binding affinity with a competition binding assay using the non-selective opioid receptor 3 [H]-diprenorphine. $\pm$ standard error of the mean. Adapted from Jordan and Devi (1999).

Signalling:

It has become apparent that dimerisation will sometimes alter the signal transduction mechanisms used by the constituent GPCRs. A prominent example is the dopamine D₁-D₂ heterodimer; D₁ and D₂ receptors are known to couple to G_s and G_{i/o} subunits respectively, and act through the second messenger AC system (Beaulieu and Gainetdinov, 2011). However, over the years reports were published where it appeared that a D₁ like receptor was able to stimulate intra-cellular Ca²⁺ release in striatal brain tissue (Undie *et al.*, 1994; Wang *et al.*, 1995; Pacheto and Jope, 1997; Jin *et al.*, 2003; Tang and Bezprozvanny, 2004). This effect was insensitive to pertussis toxin (PTX) – a potent inhibitor of AC - (Wang *et al.*, 1995) and was blocked by a G_{q/11} monoclonal antibody (Pacheto and Jope, 1997), which suggested G_{q/11} coupling. This release of intra-cellular Ca²⁺ was later found to only occur when D₁ and D₂ receptors were co-expressed together, and this could be blocked by PLC inhibitors, suggesting the possible formation of a G_{q/11} coupled dimer (Lee *et al.*, 2004; Rashid *et al.*, 2007). Co-immunoprecipitation (Lee *et al.*, 2004) and RET (So *et al.*, 2005) studies indicated formation of a D₁-D₂ heterodimer, which was later located in native striatal tissue (Lee *et al.*, 2004; Rashid *et al.*, 2007).

1.3.5 Criticism of Dimerisation

Whilst the concept of dimerisation is now well established, and numerous studies on an array of GPCRs appear to support the notion, it has been questioned as to how much of this “evidence” can be seen as biologically relevant (Chabre and le Maire, 2005; Pin *et al.*, 2007; Ferré *et al.*, 2009; Teitler and Klein, 2012; Ferré *et al.*, 2014). As previously stated all of the methods used to investigate *in-vitro* have their inherent advantages and disadvantages, but this has created a pool of disjointed and contradictory data which can have multiple interpretations (Gurevich and Gurevich, 2008; Palczewski, 2010; Vischer *et al.*, 2011). The evidence for class A and B GPCRs forming dimers is much less clear cut than with class C GPCRs (Ferré *et al.*, 2014), especially as only a small proportion of reported dimers have been characterised in native cells, even fewer *in-vivo*, and only a select number meet the guidelines reached by the International Union of Basic and Clinical Pharmacology (Pin *et al.*, 2007). GPCR dimerisation remains a controversial area, and a detailed assessment of the evidence is beyond the scope of this review.

1.3.6 P2Y Receptor Dimers

It has been reported that P2Y receptors appear to be able to form both homodimers and heterodimers. As previously detailed in section 1.3.3 P2Y₁ receptors appear to constitutively form homodimers (Choi *et al.*, 2008); P2Y₄ receptor homodimerisation has been reported by d'Ambrosi *et al.* (2006) using co-immunoprecipitation of tagged transfected receptors; later work by the same group has found that native P2Y₄ receptors appear to exist predominantly as homodimers and homo-multimers (d'Ambrosi *et al.*, 2007) using blue native and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). In the same study it was also found that not only does P2Y₆ seem also able to form homodimers, but that it can additionally form heterodimers with P2Y₄ receptors in transfected and endogenous cells; whilst the native P2Y₄ receptors preferentially consisted as homodimers and homo-oligomers, P2Y₆ receptors in contrast formed homodimers in response to agonist stimulation. P2Y₁₂ receptors have been observed as predominantly existing as homo-oligomers; interestingly the active metabolite of the antithrombotic agent clopidogrel appears to interfere in this homodimerisation, which may represent part of its mechanism of action (Savi *et al.*, 2006). The P2Y₁₂ receptors crystal structure when in complex with an antagonist molecule has further suggested dimeric interactions between P2Y₁₂ receptors via cholesterol molecules (Zhang *et al.*, 2015).

Both P2Y₁ and P2Y₂ receptors have been reported to form heterodimers with adenosine A₁ receptors. The P2Y₁-A₁ dimer has complex effects on the pharmacology and signalling properties, including activation of both G_i and G_q subunits, and a substantial reduction in binding affinity for A₁ ligands, but a comparative increase for P2Y₁ ligands (Yoshioka *et al.*, 2001). P2Y₂-A₁ receptor dimerisation on the other hand didn't appear to have any effect on ligand binding affinity, but simultaneous stimulation with agonists for both GPCRs enhanced G_q signalling and inhibited G_i based signalling (Suzuki *et al.*, 2006).

P2Y₁ and P2Y₁₁ receptors have been reported to form heterodimers using both co-immunoprecipitation and FRET. Interestingly this dimerisation not only appeared to enable the agonist induced internalisation of the P2Y₁₁ receptor, something that its monomer is not capable of, but it was also able to alter the ligand selectivity of the receptor (Ecke *et al.*, 2008). Work in the Kennedy lab group has researched possible dimerisation between P2Y₁ and P2Y₁₂ receptors in tsA201 cells. The two co-expressed receptors appeared to interact to modulate the signalling activity of co-transfected two pore potassium channels, showing the pharmacological profile of both receptors, but the signalling properties of P2Y₁ (Shrestha *et al.*, 2010), and the formation of a heterodimer being proposed as the mechanism. Later Ca²⁺ signalling based pharmacological assays were able to reproduce this data (Naughton *et al.*, 2014).

1.4.0 Hypothesis and Aims

It is hypothesised that P2Y₁ and P2Y₁₂ receptors will form a GPCR dimer complex upon co-expression in the tsA201 cell line, which will be detectable by co-immunoprecipitation. Therefore, it is aimed in this project to characterise the mechanisms by which the two receptors interact, in addition to evaluating other potentially interacting factors which may have had a role in influencing the signalling data seen previously.

It is firstly intended to characterise the expression of endogenous human P2Y receptor subtypes in the tsA201 cell line by reverse transcriptase polymerase chain reaction and functional assays. Recombinant haemagglutinin (HA) or fluorescent protein (FP) tagged human P2Y₁ and P2Y₁₂ receptors will next be transfected into the tsA201 cell line, and Ca²⁺ flux imaging utilised to verify that the tagged receptors are functionally expressed and operate similarly. Whether the proposed P2Y₁-P2Y₁₂ receptor dimer forms physically will then be lastly investigated, using co-immunoprecipitation with subsequent Western blotting against the receptor tags.

Chapter 2: Materials and Methods

2.1.0 Cell Culture

All cell culture was carried out under aseptic conditions in a class II biological safety hood.

2.1.1 Culture of tsA201 Cells

tsA201 cells (European Collection of Authenticated Cell Cultures [ECACC] 96121229) were thawed from frozen stocks and maintained at 37°C in a 5% CO₂ 95% O₂ humidified atmosphere in Dulbecco's Modified Eagles medium (DMEM) augmented with 10% foetal bovine serum, 1% non-essential amino acids and 1% penicillin (10000 U/ml)/streptomycin (10000 U/ml) - referred to herein as "complete" media. All media components were sourced from Thermo Fisher Scientific.

The cells were grown in Corning T25 or T75 flasks and when at 70 – 90% confluence were washed twice with serum-free DMEM prior to being incubated with trypsin for 5 minutes at 37°C to detach. The cells were then brought up to the flasks working volume with complete media to inactivate the trypsin, and subsequently split and re-plated at between 1:2 and 1:10 in a new flask. Later on in the project the initial washes were omitted and versene solution was used in place of the trypsin, with the cells being placed in the incubator for 2 minutes to detach; the versene was subsequently removed by centrifugation after addition of complete media. The remaining cells were discarded or used for other experiments detailed further on; the media was changed every 2 days and the cells were utilised between passages 11 and 37.

2.1.2 Preparation of Coverslips of Cells for LS-50B Ca²⁺ Flux Imaging

tsA201 cells from splitting were gently pipetted onto 13 mm diameter poly-L-lysine (1 mg/ml) coated glass coverslips in a 35mm sterile petri dish at a dilution suitable for a ≥80% confluent monolayer to have formed in 24 hours. The dishes were then placed in the incubator for two hours for the cells to adhere to the coverslips, and for wild type only cells the petri dish was filled with complete media 2 hours later for use in 24-48 hours.

2.1.3 Transient Transfection of Coverslip-plated Cells with Lipofectamine-2000

For experiments where Ca^{2+} flux was recorded, Lipofectamine-2000 (Thermo Fisher Scientific) was used to transiently insert cDNA sequences expressed in plasmids (HA-hP2Y₁, HA-hP2Y₁₂, hP2Y₁-YFP and hP2Y₁₂-CFP receptor containing plasmids were used) into the coverslip-plated cells. After allowing the cells 2 hours to adhere to the coverslips, the dishes had 2 ml of serum-free DMEM added and were allowed to incubate for 15 minutes; 1 µg of the respective plasmid was used in a cDNA:Lipofectamine-2000 mix with a ratio of 1 µg:1.5 µl, prepared in serum-free DMEM. This was left to incubate in the hood for 5 minutes before being slowly added to the dish of coverslips, which was gently rocked to mix; the dishes were then placed back in the incubator for 24 hours before the media (and plasmid) was aspirated off and replaced with complete media. The coverslip-plated cells were then used for Ca^{2+} flux imaging 24-48 hours later.

2.1.4 Transient Transfection of 60 mm Dishes of Cells with Linear Polyethylenimine (PEI)

For biochemical experiments, PEI (linear, MW-2500 from Polysciences Inc) was used for transient transfection of plasmids (containing HA-hP2Y₁, HA-hP2Y₁₂, hP2Y₁-YFP or hP2Y₁₂-CFP receptors or green fluorescent protein [GFP]) due to the prohibitive cost of using Lipofectamine-2000 for the quantity required. 60 mm dishes were seeded with cells on a 1:4 ratio of cells to complete media and later transfected when overall confluence was between 50% and 80%. 2.5 µg of each plasmid to be inserted (2.5 µg or 5.0 µg total cDNA content) and 15 µg PEI were separately diluted in serum-free DMEM and after 5 minutes combined together by adding the PEI to the cDNA and leaving for at least 15 minutes at room temperature before adding to the dishes in serum-free DMEM drop by drop. The dishes were placed back in the incubator for 24 hours, with the media then replaced with complete media before the cells were harvested for immunoprecipitation experiments after a further 24 hours.

2.2.0 Assessment of Receptor Function by Ca²⁺ Flux Imaging

2.2.1 HEPES-Krebbs Buffer

HEPES-Krebbs buffer (122 mM NaCl, 5 mM KCl, 10 mM HEPES, 0.5 mM KH₂PO₄, 0.5 mM NaH₂PO₄, 1 mM MgCl₂, 11 mM glucose, 1.8 mM CaCl₂) was prepared immediately before each experiment and titrated to pH 7.30 with NaOH.

2.2.2 Cal-520 AM Fluorescent Dye Preparation

Cal-520 AM fluorescent dye (Abcam) was prepared from a frozen 1 mM in DMSO stock to a final 5 µM concentration in HEPES-Krebbs buffer plus 0.05% F-127 Pluoronic acid (Thermo Fisher Scientific). The dye was added to the cells (for coverslips 1.5 ml of solution per dish and for 96 well plates 100 µl per well) and incubated at 37°C for 1 hour; 96 well plates were also given an additional 30 minutes of incubation at room temperature as recommended in the dyes product literature. Coverslips were washed twice with buffer prior to use whilst 96 well plates were aspirated and the dye replaced with 90 µl of buffer in each well; all assays were ran at room temperature as recommended.

2.2.3 Ca²⁺ Flux Imaging of Transfected Tagged Receptor Constructs with the Perkin-Elmer LS-50B Luminance-spectrophotometer

In order to test whether the introduction of HA or FP tags interfered with normal receptor functioning and they could be functionally expressed, Ca²⁺ flux assays were used to determine if the receptors responded as expected to pharmacological ligands when transfected into coverslip-plated tsA201 cells. The coverslip being assayed was placed in a cuvette in the path of the LS-50B luminance/spectrophotometer (Perkin-Elmer) excitation beam and photodetector. HEPES-Krebbs buffer was continuously perfused into the cuvette at 4 ml/min by gravity and aspirated out by suction to maintain a constant flow of new buffer. Recordings were each made at a 10 Hz sampling frequency with 490±10 nm excitation and 525±10 nm emission settings using Perkin-Elmer FL Winlab V4.00.02 software.

After recording 30 seconds of baseline, ADP (Sigma-Aldrich) was added by changing the perfusion buffer over to a separate source containing the necessary concentration of ligand

made up in buffer. This was allowed to perfuse until the Ca^{2+} response had peaked and the source was then switched back to buffer only; each recording was a total of 5 minutes long with a subsequent 5 minute washout period before the next ADP addition (10 minutes for 1 μM ADP and above). Each coverslip was initially subjected to two additions of 10 μM ADP before proceeding to further experimentations, and after data capture the files were imported into GraphPad Prism 7 (GraphPad Inc), with every 20 time points averaged and response calculated by manually subtracting the baseline from the peak response.

For wild type (WT) and hP2Y_1 receptor transfected tsA201 cells ADP concentration-response curves were produced, with all responses being normalised as a percentage of that of the final 10 μM ADP concentration. These were fitted with the "log(agonist) vs. response -- variable slope" equation in GraphPad Prism 7 to calculate the $\log\text{EC}_{50}$ - which is expressed in this thesis as the negative logarithmic form (pEC_{50}) -, Hill slope and E_{max} ; the bottom parameter was constrained to 0 whilst the other parameters were fitted automatically;

hP2Y_{12} receptor transfected cells however were tested by studying their response to the hP2Y_{12} receptor antagonist cangrelor using a protocol developed previously in the lab group (see Naughton, 2014). 3 or 4 repeated additions of 300 nM ADP were made at 10 minute intervals to determine if the response was reproducible, and the coverslip then perfused with 1 μM of cangrelor for 5 minutes to equilibrate. The Ca^{2+} response of 300 nM ADP diluted in 1 μM cangrelor was then recorded, and after 5 minutes of washout to remove the antagonist the response to 300 nM ADP alone was tested again; all responses were expressed as a percentage of the second initial 10 μM ADP response.

2.2.4 Ca^{2+} Flux Imaging with the Molecular Devices FlexStation 3 Platereader

tsA201 cells were split into clear bottomed, black walled 96 well plates (Corning) at a low density and left to grow until uniformly confluent. Two hours before experimentations the complete media was replaced with serum-free DMEM, and the cells subsequently incubated with 5 μM Cal-520 AM as described in section 2.2.2; afterwards the wells were aspirated and filled with 90 μl of HEPES-Krebbs buffer, with the plate then loaded in the reading chamber of a Molecular Devices FlexStation 3 robotic platereader. The automated micro fluidics and dispensing system was loaded with the natural hP2Y ligands at 10x the concentrations (to account for serial dilution when added to the 90 μl buffer already in each well of cells) being tested along with a buffer only control well for each lane. ADP, ATP, UDP and UTP (all from Sigma-Aldrich) were prepared in buffer with the platereader programmed to apply the loaded

concentrations of each to 3 adjacent lanes of the plate for triplicate reads. Upon running the Ca^{2+} assay the software (SoftMax Pro 7) was set with the excitation and emission frequencies as 490 nm and 525 nm, to report averaged reads every 3 seconds, apply 10 μl of respective agonist to the wells in the lane 17 seconds into each read and finish recording after 300 seconds; the photomultiplier tube sensitivity was set as “high”. After the assay had finished the data was automatically transformed in SoftMax Pro to present as maximum – minimum and analysed in GraphPad Prism 7, where the data was normalised as a percentage of the response to 10 μM of agonist and the “log(agonist) vs. response -- variable slope” equation fitted. The bottom parameter was constrained to the average percent response to buffer only for the three lanes of each agonist, whilst all other equation parameters were fitted automatically up to the maximal response.

2.3.0 Molecular Biology Techniques

All molecular biology related work was conducted using RNase/DNase free consumables and filtered aerosol resistant pipette tips to avoid degradation or cross contamination of samples.

2.3.1 RNA Extraction

After growing to at least 70% confluence tsA201 cells were passaged with trypsin as previously detailed, centrifuged at 1000 RPM for 5 minutes, the pellet re-suspended in a known volume of complete media and a cell count performed using a haemocytometer to calculate the cell density and total count. After adjusting to approximately 3×10^6 cells in total the cells were then spun down at 1000 RPM for 5 minutes, the supernatants removed and the pellet lysed by addition of a mix of 350 μl Bioline Buffer RLY and 3.5 μl β -mercaptoethanol. The pellet was then vigorously mixed by vortexing until homogenous and RNA extraction proceeded to or the lysate frozen at -80°C for later extraction.

Because of the thick nature of the lysates they were passed through a Qiagen Qias shredder at full centrifuge speed for 2 minutes to homogenise; the standard Bioline Isolate II RNA Mini kit protocol for cultured cells was subsequently used for extraction, including the on-column DNase I step; the RNA was eluted with 60 μl of RNase free water. RNA concentration and purity were then assessed through a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific).

Because of issues associated with genomic DNA contamination of the eluted RNA, a post DNase I reaction was further performed on the RNA using NEB DNase I enzyme (New England Biolabs). NEB DNase buffer was added to the RNA to a final concentration of 10% (v/v) along with 2 U of DNase I enzyme and the total volume brought up to 100 µl with RNase free water. This was then incubated at 37°C for 10 minutes in a Perkin-Elmer DNA Thermal Cycler 480 and the DNase I was subsequently inactivated by incubation at 75°C for 10 minutes; afterward cleanup of the RNA was performed using the Bioline Isolate II RNA mini kit again, following the “RNA cleanup” protocol detailed in the product literature. Concentration and purity were lastly assessed again with the Nanodrop 2000c spectrophotometer: concentrations of ≥ 150 ng/µl, and respective $A_{260/280}$ and $A_{260/230}$ ratios of 2.00 to 2.10 and ≥ 2.00 were taken as appropriate for further use.

2.3.2 cDNA Synthesis

The extracted RNA was subsequently used to prepare cDNA (RT+) with Quanta Biosciences qScript cDNA Supermix. In RNase free water supermix and RNA were combined on a 1 µl/0.25 µg ratio and brought up to a total volume of 5x that of the supermix; no reverse transcriptase (RT-) controls were further prepared identically except for the replacement of the supermix with water. The reactions were placed on a Perkin-Elmer DNA Thermal Cycler 480 programmed to 25°C degrees for 5 minutes, 42°C for 30 minutes and finally 85°C for 5 minutes to inactivate. The cDNA and RT- controls were then stored at -20°C until use.

For one application, 50 U of NEB RNase I_f was applied to half the volumes of newly synthesised RT+ and RT- templates to remove present RNA, with the samples incubated at 37°C for 30 minutes; the remaining volumes of the templates were not treated with the enzyme, but were still subjected to the incubation.

2.3.3 Reverse Transcriptase Polymerase Chain Reaction

The produced cDNA was subsequently amplified by polymerase chain reaction. Primers designed against all the hP2Y receptor subtypes were utilised as detailed in Table 3; these would therefore amplify the product sequences listed in Table 4.

Table 3: Primer sequences used for the RT-PCR

Receptor Gene	Forward (sense) primer	Reverse (anti-sense) primer	Expected Product Size (bp)
hP2Y1	CTTGGCGGGAGATACTTTCAG	CACAGGCTTGTATCTCCATTCTG	147
hP2Y2	CCTTTACTGCAGCATCCTCTTC	CAGCACCAACACCCACAC	139
hP2Y4	GTGAGGGAACCCAATAGTGATAC	AAAGAGTCGGCAACTGAAGG	147
hP2Y6	ACATCACCAAGACAGCCTAC	CTGGGTGAAGTAGAAGAGGATG	149
hP2Y11	GGAAGTGGGTAGCAGACAC	TGGAACCCACTGAGTTTGTC	122
hP2Y12	ATGCCTGGATCCGTTTCATC	TCATTGGGTCACCACCATC	141
hP2Y13	ATGACTGCCGCCATAAGAAG	TGTACTATCCGAGTGTCTCTGG	131
hP2Y14	TTTACGTGCCCAGCTCTAAG	CTGCACACAAACACGTTTCAG	142

Table 4: Sequences amplified by the RT-PCR primers

Receptor Gene	Amplified Sequence
hP2Y ₁	CGGCTGTCTACATCTTGGTATTCATCATCGGCTTCCTGGGCAACAGCGTGGCCATCTGGATGTT CGTCTTCCACATGAAGCCCTGGAGCGGCATCTCCGTGTACATGTTCAATTGGCTCTGGCCGAC TTCTTGTACGTGCTGACTCTG
hP2Y ₂	CCTTTACTGCAGCATCCTCTTCCTCACCTGCATCAGCGTGACCGGTGTCTGGGCGTCTTACGA CCTCTGCGCTCCCTGCGCTGGGGCCGGGCCCGCTACGCTCGCCGGGTGGCCGGGGCCG
hP2Y ₄	GTGAGGGAACCCAATAGTGATACCTGGTAAGGTGCTTCTTCCTCTTTTCCAGGCTCTGGAGAGA AGCCCTCACCTGAGGGTTGCAGGGAGGCAGGGATATCATGGGTGACCTCATCTCTCATAACCC CTTCAGTTGCCGACTCTTT
hP2Y ₆	ACATCACCAAGACAGCCTACCTGGCAGTGCGCTCGACGCCGGGCGTCCCCTGCACTGTATTGGA GGCCTTTCAGCGGCCTACAAAGGCACGCGGCCGTTTGCCAGTGCCAACAGCGTGTGGACCCC ATCCTCTTCTACTTCACCCAG
hP2Y ₁₁	GGAAGTGGGTAGCAGACACAGGCTGAGGATCGGCACGGGAGCATGGCAGCCAACGTCTCGGGTG CCAAGTCTGCCCCTGCCAAGTCTTGGCAGCTGCCGACGACAACTCAGTGGGTTCCA
hP2Y ₁₂	ATGCCTGGATCCGTTTCATCTATTTTTTCCCTTTGCAAGTCCTTCAGAAATTCCTTGATAAGTATG CTGAAGTGCCCCAATTCTGCAACATCTCTGTCCAGGACAATAGGAAAAAAGAACAGGATGGTG GTGACCCAAATGA
hP2Y ₁₃	ATGACTGCCGCCATAAGAAGACAGAGAGAACTGAGTATCCTCCCAAAGGTGACACTGGAAGCAA TGAACACCACAGTGATGCAAGGCTTCAACAGATCTGAGCGGTGCCCCAGAGACACTCGGATAGT ACA
hP2Y ₁₄	TTTACGTGCCAGCTCTAAGAGTTTCATCATCTATCTCAAGAACATTGTTATTGCTGACTTTGT GATGAGCCTGACTTTTCCTTTCAAGATCCTTGGTGACTCAGGCCTTGGTCCCTGGCAGCTGAAC GTGTTTGTGTGCAG

The primers were designed to amplify the above sequences for each receptor when used in RT-PCR; these sequences were also compared with the Sanger sequenced gel bands (see section 2.3.5).

After preliminary experiments with several polymerases, Promega GoTaq Green Hotstart PCR Mastermix polymerase was used for the amplification. The components were combined in 0.2 ml thin wall PCR tubes on a ratio of 12.5 volumes of polymerase per 1 volume of template, with 0.4 μ M of each of the respective forward and reverse primers and brought up to a total volume twice that of the polymerase with RNase free water; this was individually adjusted however when needed for specific targets. The templates used were cDNA (RT+), in addition to RT- and water controls: these were to respectively amplify the target nucleotide sequence if present on the cDNA, identify any contaminants in the RNA and identify contamination or off-target effects produced exclusively by the reaction components. The combined components were then subsequently placed in a Primus 96 Plus thermal cycler (MWG Biotech AG) programmed for 2 minutes of initial denaturation at 95°C, and then 35 cycles of denaturation at 95°C for 45 seconds, 30 seconds of annealing at 55°C and 30 seconds of extension at 72°C. After the final cycle there was an additional final extension at 72°C for 5 minutes before the reactions were held at 8°C. Afterwards the PCR reactions were stored at -20°C until use or agarose gel electrophoresis immediately proceeded to.

Positive control reactions were further prepared as above, using as the template either 1 ng of plasmid containing the receptor cDNA sequence or cDNA isolated from cells endogenously expressing the required receptor mRNA. Due to challenges encountered with the plasmids potentially contaminating reaction components, all PCR reactions were eventually made up in a class II hood which had been exposed to short wavelength UVc light for 20 minutes to remove any DNA aerosols, and the positive controls were not opened in the presence of other reaction component stocks. The positive control PCR reactions were prepared separately using different pipettes to avoid contamination of the source templates and other reagents.

The following positive controls were used for each receptor subtype gene (Table 5):

Table 5: RT-PCR hP2Y receptor positive control templates

Receptor	Control
Gene	
hP2Y ₁	hP2Y ₁ -YFP plasmid
hP2Y ₂	hP2Y ₂ -HA plasmid
hP2Y ₄	1321N1-hP2Y ₄ receptor cells mRNA
hP2Y ₆	Hep-G2 cells mRNA
hP2Y ₁₁	hP2Y ₁₁ plasmid
hP2Y ₁₂	hP2Y ₁₂ -CFP plasmid
hP2Y ₁₃	hP2Y ₁₃ plasmid
hP2Y ₁₄	hP2Y ₁₄ -HA plasmid

2.3.4 Agarose Gel Electrophoresis

Agarose gels were prepared by dissolving molecular grade agarose (Bioline) in 1x Tris-Borate-EDTA (TBE) buffer (UltraPure 10x TBE buffer, Thermo Fisher Scientific) to the percentage required: typically 2% (w/v) gels were prepared, but for some applications 1% (w/v) gels were also used. This mixture was weighed, then boiled in a 900W microwave until the agarose had dissolved and distilled water added to make up any volume lost through convection. After allowing to cool for a few minutes, ethidium bromide (Sigma-Aldrich) was added to the dissolved agarose to a concentration of 0.4 µg/ml and the solution gently poured into the gel tank (Bioscience Services Mini Q) to a thickness of 6 mm with a 16 well (5 mm x 5 mm x 1 mm per tooth) comb then carefully added; the gel was allowed to set at room temperature for at least 30 minutes. Running buffer was prepared by addition of ethidium bromide to 1x TBE buffer to a final concentration of 0.625 µg/ml, this was added to the tank with the gel submerged to a final depth of 6 mm after the comb was removed. 15 µl of sample was loaded per well in addition to one or two lanes with 5 µl of DNA size ladder (Bioline Hyperladder 1 kb or 100 bp); the power supply electrodes were attached to the tank and the electrophoresis ran at 4.0 - 4.5 V/cm until the yellow tracking dye had ran off the bottom. The gel was then removed from the tank to an enclosed UV transilluminator where it was photographed with a Pulnix TM-300 (JAI) imaging system and Syngene Genesnap software. Adjustments to global image brightness, contrast and density were made in Adobe Photoshop Elements 8.0.

2.3.5 Gel Band Purification and Sanger Sequencing

In order to confirm the identity of the expressed bands on the agarose gels, the bands were excised, purified and sequenced by the Sanger method (Sanger *et al.*, 1977). Sufficient volumes of PCR reactions were prepared to load 3 adjacent wells of a gel with 15 µl each of the product; initially 1% agarose gels were used, but shortly 2% gels were used so to maximise the separation between bands. Following gel electrophoresis the gels were visualised on a UV transilluminator, and a scalpel used to carefully excise the bands of interest and remove as much exogenous agarose as possible. The excised DNA in the bands was then purified, initially using a Sigma GenElute Gel Extraction Kit as per the manufacturer's instructions and eluting with 25 µl of RNase free water. Because of poor yields that frequently failed to sequence, later purifications were made using an Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare) with the protocol recommended in the product literature; the DNA was eluted with 40 µl of RNase free water and then passed back through the spin column membrane to concentrate. After concentration measurement on the Nanodrop 2000c spectrophotometer the purified DNA was sent to GATC Biotech - along with samples of the respective forward and reverse primers – for Sanger sequencing using their "SUPREMERun" service.

The received sequences were subsequently viewed in FinchTV V1.4.0 (Geospiza Inc, available at <http://www.geospiza.com/ftvdlinfo.html>), with poor quality bases at the beginning/end of each read being removed before exporting the sequence in FASTA format (with reverse reads converted to the reverse complement). Subsequently the NCBI nucleotide Mega "Basic Local Alignment Search Tool" (BLAST) was used to search for highly similar sequences. The Human Genomic and Transcript databases were searched and the default options used except for disabling low-complexity regions filtering.

The forward and reverse read sequences were afterwards aligned with each other and the expected PCR product sequence using the European Bioinformatics Institute (EBI) Multiple Sequence Comparison by Log-Expectation (MUSCLE) (available at <http://www.ebi.ac.uk/Tools/msa/muscle/>). These were exported in the GCG MSF format for processing with Boxshade Server (available at http://www.ch.embnet.org/software/BOX_form.html) to highlight similarity, selecting the "RTF new" format and setting "fraction of sequences" as 0.05.

2.3.6 Verification of the tsA201 Cell Line by Short Tandem Repeats

A short tandem repeats (STR) test was performed on the cells being maintained, in order to confirm the identity of the tsA201 cells prior to undertaking future experimentations. 90% confluent cells in a T75 were detached with trypsin and collected as previously described; after a cell count and centrifuging down at 5000 RPM for 5 minutes the resulting pellet was re-suspended in DMEM to a volume equal to 1000 cells/ μ l and 100 μ l of cells then applied to the collection card supplied by Public Health England for the test. After allowing to air dry for 2 hours the card was sent to Public Health England for STR cell line validation and the resulting profile compared with that deposited in the European Collection of Authenticated Cell Cultures.

2.4.0 Co-immunoprecipitation and Western Blotting

2.4.1 Immunoprecipitation

After 48 hours of growth and reaching confluence, transfected 60 mm dishes of tsA201 cells (expressing either singular or co-expressed transfected receptors) were washed with ice cold phosphate buffered saline (PBS), placed on ice and then lysed by addition of lysis buffer (50 mM Tris-HCl pH 7.4, 1% Triton-X-100 plus Roche Mini Protease Inhibitor cocktail 1 tablet/10 ml added on the day of the experiment). The cells were scraped down and passed several times through a 21G or 23G syringe to break up clumps before being transferred to 1.5 ml tubes. The lysates were left to rotate end over end at 40 RPM in a 4°C atmosphere for at least 60 minutes, then spun down at 10000 RPM for 10 minutes in a 4°C atmosphere to pellet any cellular debris that had not lysed. The supernatants – crude whole cell lysates - were removed to new tubes and 50 μ l of each was subsequently placed in another set of tubes along with 50 μ l of 2x NuPage- lithium dodecyl sulphate (LDS) sample buffer (Thermo Fisher Scientific) plus 50 mM dithiothreitol (DTT); these were then boiled at 95°C for 2 minutes to denature, providing the “input” samples.

The lysates were pre-cleared with Calbiochem Protein G Plus/Protein A Agarose Suspension (Merck Millipore). 20 μ l of beads were placed in new tubes and washed twice with lysis buffer before the supernatants were removed to leave the washed pellets. 300 μ l of each whole cell lysate was added to a tube of beads and left to rotate end over end in a 4°C

atmosphere for at least 60 minutes before being pelleted by centrifugation and the pre-cleared supernatants transferred into new tubes.

20 µl per sample of the respective agarose immunoprecipitation beads – anti-HA-agarose produced in mouse (Sigma-Aldrich) for HA immunoprecipitation and GFP-Trap (ChromoTek) for GFP – were washed twice with lysis buffer to remove any residual storage buffer, the supernatants were then removed and 300 µl of each pre-cleared lysate added. These were left to rotate overnight at 40 RPM in a 4°C atmosphere, the next day the bead-lysate complexes were washed 3 times with lysis buffer to remove any un-bound protein before being pelleted by centrifugation and the supernatants removed. For the GFP immunoprecipitations only, 30 µl was removed from each to separate tubes and 30 µl 2x NuPage-LDS sample buffer plus 50 mM DTT added to elute the immunoprecipitated (IP) complexes; these samples were then boiled at 95°C for 2 minutes to denature. For HA immunoprecipitations the HA tagged proteins were eluted with 30-40 µl of 200 µg/ml HA-Peptide (Sigma-Aldrich), which was added to the washed bead-lysate complexes, gently vortexed and then left at room temperature for 20 minutes, lightly vortexing every 3 minutes. The tubes were then centrifuged at 5000 RPM for 5 minutes and the supernatants – IP samples – were carefully transferred to new tubes. Both the IP samples and the leftover beads – HA-peptide controls - then had 2x NuPage-LDS sample buffer plus 50 mM DTT added at the same volume as the HA-peptide and were subsequently boiled at 95°C for 2 minutes to denature.

2.4.2 SDS-PAGE and Western Blotting

The Biorad Mini-Protean Tetra system was used for Western blotting. 15-20 µl of sample per lane were separated by 8.5% resolving gels (30% [w/v] acrylamide, 0.8% [w/v] bis-acrylamide in 380 mM Tris-HCL pH 8.8, 0.10% [w/v] sodium dodecyl sulphate [SDS]). 0.02% (w/v) ammonium persulfate (APS) and 0.02% (v/v) tetramethylethylenediamine (TEMED) were added to these components to polymerise the gel, which was quickly poured between pre-assembled 1.0 mm (medium) glass plates (Biorad) with a thin layer of 100% isopropanol on top to ensure level setting and give an indication of when the gel had set. After setting the isopropanol was washed out with distilled water and exogenous liquid gently drawn out with filter paper; the 4% stacking gel (30% [w/v] acrylamide, 0.8% [w/v] bis-acrylamide diluted in 123 mM tris-HCL pH 6.6, 0.01% [w/v] SDS, 0.1% [w/v] APS, 0.07% [v/v] TEMED) was quickly poured on top of the resolving gel and a 1.0 mm 10 or 15 well comb (Biorad) inserted gently between the plates and into the stacking gel. After the stacking gel had set the plates

were transferred to Biorad Mini-Protean Tetra electrophoresis holders, placed in the electrophoresis chamber and the comb removed; the tank was then filled with running buffer (25 mM Tris, 192 mM Glycine, 3.5 mM SDS) up to the respective level for the number of gels. The samples were loaded with a Hamilton micro-syringe along with a lane of pre-stained protein standards ladder (Thermo Fisher Scientific); the chamber between the plates was topped up with running buffer before running the electrophoresis at 115 V until the bromophenol blue tracking dye had ran off the bottom of the gel.

The gels were subsequently transferred to polyvinylidene difluoride (PVDF) membranes with the Biorad Mini Trans-blot system. The membranes were activated by soaking in 100% methanol until translucent, followed by ice cold transfer buffer (25 mM tris, 192 mM glycine, 20% methanol [v/v]). All components for the transfer were soaked in ice cold transfer buffer and each transfer cassette arranged with the bottom side of the cassette down followed by 3 mm sponge, 1 mm filter paper, the gel, the activated membrane, 1 mm filter paper and 1 mm sponge. The cassette was closed and inserted into the Biorad Mini Tran-blot Cell transfer chamber along with a cooling bath of frozen transfer buffer and the chamber filled to the level of the cassette clip with transfer buffer. The power supply was set to run at 300 mA for 105 minutes before the cassette was removed, the membrane extracted and put to block in 2% (w/v) bovine serum albumin (BSA) (diluted in 0.03% NATT buffer: 300 mM NaCl, 40 mM tris, 0.03% [v/v] Tween-20, pH7.4) on an orbital shaker at room temperature for at least 2 hours. The primary antibody – in 0.2% (w/v) BSA diluted in 0.03% NATT buffer - was then applied either by heat sealing the membrane in a plastic liner with the antibody or by adding the antibody directly to the membranes container. Preferentially the antibody was left to bind overnight (>12 hours) at room temperature on an orbital shaker, but in some cases where this was not possible the primary antibody was left to bind for at least 2 hours. The primary antibodies that were used are detailed in Table 6:

Table 6: Primary antibodies used for Western blotting

Antibody	Supplier	Species	Dilution
Anti-αGFP	Chromobio	Rat	1:1000
Anti-HA	BioLegend	Mouse	1:5000/1:10000
Anti-β-tubulin	Sigma-Aldrich	Mouse	1:3333

After the primary antibody had been applied for sufficiently long the membranes were washed in 0.03% NATT buffer 4 times at 15 minute intervals; horseradish peroxidase (HRP) conjugated secondary antibody for the respective species of the primary antibody was applied at 1:3333 dilution in 0.2% (w/v) BSA for at least an hour and half at room

temperature on an orbital shaker before another 4 washes were undertaken. Enhanced chemiluminescence (ECL) was then used for detection: ECL-1 (100 mM Tris-HCL pH 8.5, 2.5 mM luminol, 1.15 mM coumaric acid) and ECL-2 (100 mM Tris-HCL pH 8.5, 0.064% [v/v] H₂O₂) were applied to the membrane on a 1:1 basis to activate the conjugated HRP. The membranes were then placed in an exposure cassette beneath plastic wrap, and under safelight conditions exposed on UltraCruz autoradiography film (Santa Cruz Biotechnology), which was processed in a Kodak M-35M X-OMAT processor. The film was then scanned on a HP Deskjet 2540 and adjustments to the images global brightness, contrast and density were made in Adobe Photoshop Elements 8.0.

2.4.3 PVDF Membrane Stripping

In order to re-probe the PVDF membranes they were stripped of any bound antibodies using Restore Western Blot Stripping Buffer (Thermo Fisher Scientific) at room temperature for 15 minutes on an orbital shaker. Unfortunately, this did not sufficiently remove higher affinity antibodies such as anti-HA or anti- β -tubulin and extending the incubation time did not improve removal; using it at 37°C as recommended in the product literature was associated with both the antibodies and proteins being removed from the membrane. It was therefore necessary in some cases to re-run samples on separate membrane or probe with antibodies in a strict order.

2.5.0 Statistical Analysis

All statistical analysis was undertaken in GraphPad Prism 7. The fitted parameters of different concentration-response curves were statistically compared with non-linear regression using the “compare” function with the extra sum of squares F test (ESSFT); p values of ≤ 0.05 were taken as statistically significant and the Holm-Sidak correction used to account for multiple comparisons.

The transfected hP2Y₁₂ receptors responses were evaluated by first analysing the time-course of the 3 or 4 repeated 300 nM ADP control responses with linear regression to determine if the repeated stimulation caused any overall changes in response by the cells. The repeated 300 nM ADP responses were then averaged, and two-way analysis of variance (ANOVA) used to compare these responses to those of 300 nM ADP both during and after cangrelor being present. The responses factor was set as repeated measures and the differently tagged plasmids as the second factor; when there was an overall significant

difference for the factor and no significant interaction between the two factors multiple comparisons were made with a *post-hoc* Tukey test; p values of ≤ 0.05 were taken as statistically significant.

Chapter 3: Results

3.1.0 Cell Line Verification by Short Tandem Repeats

After receiving the cell line verification by short tandem repeats (STR) results, the received values for each genetic loci were compared to those listed for the tsA201 cell line by the European Collection of Authenticated Cell Cultures, and are both detailed in Table 7. The loci values were additionally checked against the German Collection of Microorganisms and Cell Cultures Profile Database (available at <https://www.dsmz.de/services/services-human-and-animal-cell-lines/online-str-analysis.html>) to identify if they could also correspond to other cell lines. Although there was a complete match with that of HEK-293 derived Hkb20 cells (score 1.00), the tsA201 line was unfortunately not listed in the database. All of the cell lines with a score of >0.80 were HEK-293 derivatives, which is reflective of their high degree of similarity, but regrettably no reference profile for the tsA201 cell line could be found in other databases using the full 18 loci received as the cells have not been commonly deposited in cell banks. Based on the fact that the cells were behaving functionally identically to those in previous experimentations in the lab group using the cell line, it was decided to proceed based on them being tsA201 cells.

Table 7: The received and the reference European Collection of Authenticated Cell Cultures Short Tandem Repeats profiles for the tsA201 cells.

Loci	Received Profile	ECACC Reference Profile
Amelogenin	X,X	X,X
vWA	16,19	16,19
D16S539	9,13	9,13
CSF1PO	11,12	11,12
TPOX	11,11	11,11
TH01	7,9.3	7,9.3
D5S818	8,9	8,9
D13S317	12,12	12,12
D7S820	11,11	11,11
FGA	23,23	
D3S1358	15,17	
D2S1338	19,19	
Penta D	9,10	
Penta E	7,15	
D8S1179	12,14	
D21S11	28,30.2	
D18S51	17,18	
D19S433	18,18	

DNA from the tsA201 cells being used was screened for various short tandem repeats markers by Public Health England for cell line verification, and the values received are listed as well as those deposited in the European Collection of Authenticated Cell Cultures for the cell line.

3.2.0 Endogenous hP2Y Receptor Expression in the tsA201 Cell Line

3.2.1 Reverse Transcriptase PCR Revealed the Expression of mRNA for Multiple hP2Y Receptor Subtypes

The aim of these experiments was to determine the endogenous mRNA expression of the various hP2Y receptor subtypes in the tsA201 cell line with RT-PCR. As is illustrated in Figures 4A-D, following gel electrophoresis of the PCR reactions strong bands were produced in the RT+ lanes for hP2Y₁, hP2Y₁₂ and hP2Y₁₄; there were also weaker bands expressed in the RT+ lanes for hP2Y₂, hP2Y₄ and hP2Y₆ receptors, but neither hP2Y₁₁ nor hP2Y₁₃ receptors expressed RT+ bands. All of the RT+ bands shown were of the expected sizes and corresponded with that of the positive controls (the hP2Y₁₁ and hP2Y₁₃ receptors positive controls however repeatedly failed to show products); they are representative of at least 3 individual experiments, although the positive control bands are only representative of at least a single experiment in some cases as the plasmid templates did not become available until later on in the research. To specifically confirm the RT+ bands identities, the reactions were repeated with the gel bands purified and then Sanger sequenced (see section 3.2.2).

However, in the case of the hP2Y₁, ₂, ₁₂ receptors (and very inconsistently the hP2Y₄, ₁₄ receptors) there were frequently also weaker bands present in the RT- control lanes. Generally the staining intensity was much weaker compared to the RT+ bands or positive controls, but regularly the hP2Y₁₂ receptor RT+ was only very slightly more intense than the RT- (Figure 4D). The RT-band effect was inconsistent and varied greatly between each PCR reaction: batch to batch variation in the synthesised cDNA and RT- appeared to be at least partially responsible for this. To ascertain the RT- bands identity to determine if they were genomic DNA contamination they were sequenced (see section 3.2.3); using a different DNase I enzyme (Precision DNase Kit, Primer Design UK) did not appear to remove or further reduce the bands (data not shown).

Figure 4: Representative RT-PCR agarose gels of tsA201 hP2Y receptors

Cultured tsA201 cells RNA was extracted, treated with DNase I enzyme and cDNA then synthesised before amplifying with PCR using gene specific primers (see section 2.3.0) as indicated for (A) hP2Y1, 2 (B) 4 (C) 6, 11 and (D) 12, 13, 14 receptors and running the products on 2% (w/v) agarose gels along with 5 µl DNA size ladder (outermost lanes). Water (W) and RT- control PCR products were additionally prepared and loaded for each receptor type, along with the RT+ product and a positive control reaction using an appropriate plasmid (+) or RT+ and RT- from an appropriate cell line (HepG2 or 1321N1-hP2Y4 cells) to act as a positive control. The RT+ bands are representative of at least 3 independent experiments, whilst the positive control bands are representative of at least 1 independent experiment.

Although it is accepted to be seen as generally unlikely, it was also tested as to whether the polymerase itself could exhibit reverse transcriptase activity (Figure 5) and hence produce amplicons in the RT- controls. For this RNase I_f treated cDNA and RT- were utilised in the standard PCR reaction protocol detailed in section 2.3.3, with hP2Y₁ and hP2Y₂ receptors as the test subjects due to them both exhibiting consistent RT- bands and the hP2Y₂ receptor being typically weaker staining overall. For both replicate PCR reactions where the RNase treated templates were used, whilst the overall intensity of the RNase treated RT- was substantially reduced for the hP2Y₁ receptor, the RT+ intensity was relatively unaffected. Unfortunately, the RNase appeared to have little to no effect on the hP2Y₂ receptor RT- band density compared to un-treated RT-, and also induced a bright primer-dimer in the treated RT-. In light of these results it was decided that RNase I_f treated cDNA or RT- controls would not be used in future PCR reactions, although it did raise questions as to why the hP2Y₁ receptor RT- was reduced in intensity by the enzyme.

Figure 5: The effect of RNase I_f treatment of RT- and RT+ template on hP2Y₁ and hP2Y₂ receptor RT- and RT+ staining intensity.

Half the volumes of newly synthesised tsA201 RT- and RT+ templates were treated with 50 U of NEB RNase I_f (RNase+) and used in PCR reactions with primers for amplifying hP2Y₁ or hP2Y₂ receptor sequences, in addition a second set of reactions was prepared using the remaining volumes of templates which had not been RNase I_f treated (RNase-). The reactions were then run out on 2% (w/v) agarose gels with ethidium bromide staining. This figure is representative of two sets of PCR reactions and agarose gels from the same RT+ and RT- sources.

Furthermore, signals were inconsistently present in the water controls for hP2Y₁ and hP2Y₁₂ receptors and more rarely the hP2Y₂ receptor, with the strength varying from reaction to reaction; these signals would appear with no clear pattern and when using either new or old reaction components. Switching polymerase to GoTaq Green Hotstart Mastermix from the Bioline Mytaq Mix that was first trialled appeared to remove the bands, but they started to appear again after using the GoTaq for a few weeks, suggesting contamination of the reaction mix in some form. Because the bands were of approximately the same sizes as the expected products, steps were taken to exclude aerosol genomic contamination by the plasmid positive controls or previous PCR products as a cause. Toward the end of the project the PCR reactions began to be made up in a Class II hood which had been UVC irradiated for at least 20 minutes to degrade any present DNA and prevent airborne contamination from the outside atmosphere. New, sealed reagents were used which were not opened except in the hood when none of the templates were present. Whilst this appeared to remove the bands in the majority of cases and also reduced the RT- bands, even these measures did not prevent the water amplicons from appearing in some PCR reactions; the exact cause was never entirely satisfactorily determined.

The hP2Y₁₁ and hP2Y₁₃ receptor PCR reactions persistently did not produce any visible products of the expected size, including with the positive controls. To try to determine the reason, PCR reactions against the two receptors were performed, but with the primer concentrations increased to 1 μ M and 10 ng of each control plasmid used. As a further control a reaction with HepG2 cells cDNA as the template was also included for each as the cell line is reported to express mRNA for both receptors – although the presence of hP2Y₁₃ receptor mRNA appears to differ between reports (Jacquet *et al.*, 2013; Goree *et al.*, 2014). Unfortunately, these changes failed to produce any products of the expected size and those present were substantially larger (Figure 6), suggesting that either the primers were inappropriate, the plasmids were unsuitable as a positive control and/or the HepG2 cDNA did not express the required mRNA.

Figure 6: RT-PCR of hP2Y₁₁ and hP2Y₁₃ receptor sequences with increased primers concentrations and plasmid template amounts, plus HepG2 cDNA positive controls.

The hP2Y₁₁ and ₁₃ receptors PCR reactions did not appear to indicate formation of any products of the expected size in the RT+ or plasmid positive controls (+) lanes using the standard protocol, so they were repeated with the primers raised to 1 μ M each and the plasmid amounts increased to 10 ng. Furthermore, a HepG2 RT+ and RT- template reaction was made for each to act as an alternate positive control. These were run out on a 2% (w/v) agarose gel with ethidium bromide staining and 5 μ l of DNA size ladder (outermost lanes). This is representative of a single experiment.

3.2.2 The RT+ Gel Bands Sequences Corresponded to the Expected Targets

The sequenced hP2Y₁ RT+ bands (Figure 7A) corresponded to that of hP2Y₁ receptor mRNA (accession NM_002563.4) with 100% nucleotide sequence identity in both the forward and reverse reads; when aligned the reads corresponded with each other and the expected PCR product with no differences. The hP2Y₂ receptors RT+ bands sequence (Figure 7B) matched with that of all 3 transcript variants of hP2Y₂ receptor mRNA (accession numbers NM_176072.2, NM_176071.2 and NM_002564.3) with 99% sequence identity in the forward read and 100% in the reverse. Alignment of the forward and reverse reads for the hP2Y₂ receptor RT+ band sequences revealed them to correspond to each other except for a single base change from C to A in the forward read at position 27, which would have been responsible for the lower sequence identity in the read. This is not recorded in the GPCR Natural Variants Database (available at <http://nava.liacs.nl/index.html>) as a known variant of the hP2Y₂ receptor, so it was likely simply a misread base. These reads both aligned precisely with the expected hP2Y₂ receptor PCR product sequence, apart from the aforementioned base change.

However, the reverse hP2Y₂ receptor RT+ read also produced a 65% sequence identity match with 8 of the transcript variants of hP2Y₆ receptor mRNA, although this was only over a small section toward the start of the sequence. When the full length sequences of hP2Y₂ and hP2Y₆ receptors mRNA were aligned with each other in NCBI nucleotide BLAST, despite having 79% sequence identity this only covered 6% of the sequences total lengths (data not shown), and was therefore determined to be the reason for this match.

The sequenced hP2Y₄ receptor RT+ bands (Figure 7C) corresponded to hP2Y₄ receptor mRNA (accession number NM_002565.3) with 100% sequence identity for both the forward and reverse reads. Additionally, sequencing of the purified hP2Y₆ receptor RT+ bands (Figure 7D) revealed a sequence which corresponded to 8 transcript variants (accession numbers NM_176797.2, NM_176798.2, NM_176796.2, NM_001277204.1, NM_001277205.1, NM_001277206.1, NM_001277207.1, NM_001277208.1) of hP2Y₆ receptor mRNA, with 100% sequence identity for all in both the forward and reverse reads. For both the hP2Y₄ and hP2Y₆ receptor RT+ bands their respective forward and reverse reads aligned with each other with no missing or changed bases, and furthermore aligned with their expected PCR product sequences.

The purified hP2Y₁₂ receptor RT+ bands sequence (Figure 7E) matched with that of both hP2Y₁₂ receptor mRNA transcript variants (accession numbers NM_176876.2 and NM_022788.4) with 100% sequence identity in the forward read, and 99% for the reverse read due to a single missing base. Additionally, the sequenced hP2Y₁₄ receptor RT+ bands (Figure 7F) corresponded to the sequences for both known transcript variants of hP2Y₁₄ receptor mRNA (Accession numbers NM_001081455.1 and NM_014879.3) with 100% sequence identity in both the forward and reverse reads. For both of the receptors the respective forward and reverse reads were complementary, and further they matched with their expected PCR products with no differences apart for the missing base in the hP2Y₁₂ receptor reverse read.

A

hP2Y1 RT+ Reverse

1 TCTTGCGGGGAGATACTTTTCAGAAGGAGACTCTCCCGAGCCACAAGGAAAGCTTCTAGAA

P2Y1 RT+ Forward

1 -----CTTCTAGAA

hP2Y1 PCR Product

1 -CTTGCGGGGAGATACTTTTCAGAAGGAGACTCTCCCGAGCCACAAGGAAAGCTTCTAGAA

hP2Y1 RT+ Reverse

61 GAAGTGAGGCAAATTTGCAATCCAAGAGTGAAGACATG-----

hP2Y1 RT+ Forward

10 GAAGTGAGGCAAATTTGCAATCCAAGAGTGAAGACATGACCCCTCAATATTTTACCTGAGT

hP2Y1 PCR Product

60 GAAGTGAGGCAAATTTGCAATCCAAGAGTGAAGACATGACCCCTCAATATTTTACCTGAGT

hP2Y1 RT+ Reverse

hP2Y1 RT+ Forward

70 TCAAGCAGAATGGAGATACAAGCCTGTGA

hP2Y1 PCR Product

120 TCAAGCAGAATGGAGATACAAGCCTGTG-

B

hP2Y2 RT+ Forward

1 -----CTGGGCGTCT

hP2Y2 RT+ Reverse

1 TCCTTTACTGCAGCATCCTCTTCCTCACCTGCATCAGCGTGCACCGGTGTCTGGGCGTCT

hP2Y2 PCR Product

1 -CCTTTACTGCAGCATCCTCTTCCTCACCTGCATCAGCGTGCACCGGTGTCTGGGCGTCT

hP2Y2 RT+ Forward

11 TACGACCTCTGCGCTCCA TGGCGTGGGGCCGGGCCCGCTACGCTCGCCGGGTGGCCGGGG

hP2Y2 RT+ Reverse

61 TACGACCTCTGCGCTCCCTGCGC-----

hP2Y2 PCR Product

60 TACGACCTCTGCGCTCCCTGCGCTGGGGCCGGGCCCGCTACGCTCGCCGGGTGGCCGGGG

hP2Y2 RT+ Forward

71 CCGTGTGGGTGTTGGTGCTGA

hP2Y2 RT+ Reverse

hP2Y2 PCR Product

120 CCG-----

C

hP2Y4 RT+ Reverse

1 GTGAGGGAACCCAATAGTGATACCTGGTAAGGTGCTTCTTCCTCTTTTCCAGGCTCTGGA

hP2Y4 RT+ Forward

1 -----GGCTCTGGA

hP2Y4 PCR Product

1 GTGAGGGAACCCAATAGTGATACCTGGTAAGGTGCTTCTTCCTCTTTTCCAGGCTCTGGA

hP2Y4 RT+ Reverse

61 GAGAAGCCCTCACCCCTGAGGGTTGCAGGGAGGCA-----

hP2Y4 RT+ Forward

10 GAGAAGCCCTCACCCCTGAGGGTTGCAGGGAGGCAGGGATATCATGGGTGACCTCATCTCT

hP2Y4 PCR Product

61 GAGAAGCCCTCACCCCTGAGGGTTGCAGGGAGGCAGGGATATCATGGGTGACCTCATCTCT

hP2Y4 RT+ Reverse

hP2Y4 RT+ Forward

70 CATAACCCCTTCAGTTGCCGACTCTTT

hP2Y4 PCR Product

121 CATAACCCCTTCAGTTGCCGACTCTTT

D

hP2Y6 RT+ Forward

1 -----TGCACTGTAT

hP2Y6 RT+ Reverse

1 ACATACCCAAGACAGCCTACCTGGCAGTGCGCTCGACGCCGGGCGTCCCCTGCACTGTAT

hP2Y6 PCR Product

1 ACATACCCAAGACAGCCTACCTGGCAGTGCGCTCGACGCCGGGCGTCCCCTGCACTGTAT

hP2Y6 RT+ Forward

11 TGGAGGCCTTTGCAGCGGCCCTACAAAGGCACGCGGCCGTTTGCCAGTGCCAACAGCGTGC

hP2Y6 RT+ Reverse

61 TGGAGGCCTTTGCAGCGGCCCTACAAAGGCACGCGGC-----

hP2Y6 PCR Product

61 TGGAGGCCTTTGCAGCGGCCCTACAAAGGCACGCGGCCGTTTGCCAGTGCCAACAGCGTGC

hP2Y6 RT+ Forward

71 TGGACCCCATCCTCTTCTACTTCACCCAGA

hP2Y6 RT+ Reverse

hP2Y6 PCR Product

121 TGGACCCCATCCTCTTCTACTTCACCCAG-

E

```
hP2Y12 RT+ Reverse
1 ATGCCTGGATCCGTTTCATCTATTTTTTCCTTTGCAAGTCCTTCAGAAATTCCTTGATAAG
hP2Y12 RT+ Forward
1 -----TTCCTTGATAAG
hP2Y12 PCR Product
1 ATGCCTGGATCCGTTTCATCTATTTTTTCCTTTGCAAGTCCTTCAGAAATTCCTTGATAAG

hP2Y12 RT+ Reverse
61 TATGCTGAAGTGCCCCAATTCTGCAACATCTCT-TCCCA-----
hP2Y12 RT+ Forward
13 TATGCTGAAGTGCCCCAATTCTGCAACATCTCTGTCCCAGGACAATAGGAAAAAAGAACA
hP2Y12 PCR Product
61 TATGCTGAAGTGCCCCAATTCTGCAACATCTCTGTCCCAGGACAATAGGAAAAAAGAACA

hP2Y12 RT+ Reverse
-----
hP2Y12 RT+ Forward
73 GGATGGTGGTGACCCAAATGA
hP2Y12 PCR Product
121 GGATGGTGGTGACCCAAATGA-
```

F

```
hP2Y14 RT+ Reverse
1 TTTACGTGCCCAGCTCTAAGAGTTTCATCATCTATCTCAAGAACATTGTTATTGCTGAC
hP2Y14 RT+ Forward
1 -----GTTATTGCTGAC
hP2Y14 PCR Product
1 -TTTACGTGCCCAGCTCTAAGAGTTTCATCATCTATCTCAAGAACATTGTTATTGCTGAC

hP2Y14 RT+ Reverse
61 TTTGTGATGAGCCTGAC-----
hP2Y14 RT+ Forward
13 TTTGTGATGAGCCTGACTTTTCCTTTCAAGATCCTTGGTGACTCAGGCCTTGGTCCCTGG
hP2Y14 PCR Product
60 TTTGTGATGAGCCTGACTTTTCCTTTCAAGATCCTTGGTGACTCAGGCCTTGGTCCCTGG

hP2Y14 RT+ Reverse
-----
hP2Y14 RT+ Forward
73 CAGCTGAACGTGTTTGTGTGCAGA
hP2Y14 PCR Product
120 CAGCTGAACGTGTTTGTGTGCAG-
```

Figure 7: Alignments of the forward and reverse reads for the Sanger sequenced RT+ bands with the expected PCR products nucleotide sequences.

To confirm the identity of the expressed RT+ gel bands they were excised, purified and Sanger sequenced by GATC Biotech. The forward and reverse read sequences were then aligned with one another and the expected PCR product sequences with MUSCLE and processed with Boxshade Server to highlight similarity between the sequences. (A)hP2Y₁ (B)hP2Y₂ (C)hP2Y₄ (D)hP2Y₆ (E)hP2Y₁₂ (F)hP2Y₁₄ receptors .

3.2.3 The RT- Gel Bands Sequences Matched with those of the Expected PCR Products

The sequenced hP2Y₁ receptor RT- bands (Figure 8A) matched with that of hP2Y₁ receptor mRNA (accession NM_002563.4) with 96% sequence identity in the forward read and 98% in the reverse read; in both cases this was due to several missing bases. Alignment of the sequences with each other and the expected PCR product was generally complementary aside from several missing and changed bases.

Sequencing the hP2Y₂ receptor RT- bands (Figure 8B) revealed both reads to correspond to all three transcript variants (accession numbers NM_176072.2, NM_176071.2 and NM_002564.3) of hP2Y₂ receptor mRNA with 100% sequence identity. Likewise, the hP2Y₁₂ receptor RT- band sequence (Figure 8C) matched with both transcript variants of hP2Y₁₂ receptor mRNA (accession numbers NM_176876.2 and NM_022788.4 with 100% sequence identity for both read directions. For both receptors the forward and reverse read sequences aligned and matched with their expected PCR product with no differences.

As with the RT+ product, the reverse read of the hP2Y₂ receptor RT- product also matched with 8 transcript variants of hP2Y₆ receptor mRNA (see section 3.2.2), although with 85% sequence identity compared to 65% with the RT+. The hP2Y₄ and hP2Y₁₄ receptors RT- bands were not sequenced due to their typically extreme faintness and very inconsistent appearance.

A

```
hP2Y1 RT- Forward
1 -----TTTCTAGG
hP2Y1 RT- Reverse
1 TCTTGCGGGAGATACTTTCAGAAGGAGACTCTCCCGAGCCACAACCGAAAGCTTCTA-G
hP2Y1 PCR Product
1 -CTTGCGGGAGATACTTTCAGAAGGAGACTCTCCCGAGCCACAA-GGAAAGCTTCTA-G

hP2Y1 RT- Forward
9 AAGAAGTTGAGGCAAATTTGCAATCCAAGAGTGAAGACATGACCAATCAATATTTTACCT
hP2Y1 RT- Reverse
60 AAGAAG-TGAGGCAAATTTGCAATCCAAGAGTGAAGACATGACC-CTC-----
hP2Y1 PCR Product
58 AAGAAG-TGAGGCAAATTTGCAATCCAAGAGTGAAGACATGACC-CTCAATATTTTACCT

hP2Y1 RT- Forward
69 GAGTTCAAGCAGAATGGAGATACAAGCCTGTGATGA
hP2Y1 RT- Reverse
-----
hP2Y1 PCR Product
116 GAGTTCAAGCAGAATGGAGATACAAGCCTGTG----
```

B

```
hP2Y2 RT- Forward
1 -----CGTCT
hP2Y2 RT- Reverse
1 TCCTTTACTGCAGCATCCTCTTCCTCACCTGCATCAGCGTGCACCGGTGTCTGGGCGTCT
hP2Y2 PCR Product
1 -CCTTTACTGCAGCATCCTCTTCCTCACCTGCATCAGCGTGCACCGGTGTCTGGGCGTCT

hP2Y2 RT- Forward
6 TACGACCTCTGCGCTCCCTGCGCTGGGGCCGGGCCCGCTACGCTCGCCGGGTGGCCGGGG
hP2Y2 RT- Reverse
61 TACGACCTCTGCGCTCCCTGCG-----
hP2Y2 PCR Product
60 TACGACCTCTGCGCTCCCTGCGCTGGGGCCGGGCCCGCTACGCTCGCCGGGTGGCCGGGG

hP2Y2 RT- Forward
66 CCGTGTGGGTGTTGGTGCTGA
hP2Y2 RT- Reverse
-----
hP2Y2 PCR Product
120 CCG-----
```


C

```
hP2Y12 RT- Forward
1 -----CCTTGATAAG
hP2Y12 RT- Reverse
1 ATGCCTGGATCCGTTTCATCTATTTTTCCTTTGCAAGTCCTTCAGAAATTCCTTGATAAG
hP2Y12 PCR Product
1 ATGCCTGGATCCGTTTCATCTATTTTTCCTTTGCAAGTCCTTCAGAAATTCCTTGATAAG

hP2Y12 RT- Forward
11 TATGCTGAAGTGCCCCAATTCTGCAACATCTCTGTCCCAGGACAATAGGAAAAAAGAACA
hP2Y12 RT- Reverse
61 TATGCTGAAGTGCCCCAATTCTGCAACATCT-----
hP2Y12 PCR Product
61 TATGCTGAAGTGCCCCAATTCTGCAACATCTCTGTCCCAGGACAATAGGAAAAAAGAACA

hP2Y12 RT- Forward
71 GGATGGTGGTGACCCAAATGAA
hP2Y12 RT- Reverse
-----
hP2Y12 PCR Product
121 GGATGGTGGTGACCCAAATGA-
```

Figure 8: Alignments of the forward and reverse reads for the Sanger sequenced RT- bands with the primers resulting PCR products sequences.

To ascertain the identity of the expressed RT- gel bands they were excised, purified and Sanger sequenced by GATC Biotech. The forward and reverse read sequences were then aligned with one another and the sequence of the PCR product amplified by the primers using MUSCLE, and processed with Boxshade Server to highlight similarity between the sequences. (A)hP2Y₁ (B)hP2Y₂ (C)hP2Y₁₂ receptors.

3.2.4 FlexStation 3 Ca²⁺ Flux Imaging of Wild Type tsA201 Cells with Natural hP2Y Agonists

Whilst the previous section reports the expression of mRNA for the hP2Y receptor subtypes in the tsA201 cell line, the next experiment examined functional expression of the receptors using Ca²⁺ flux as a marker. Of the 4 natural hP2Y agonists (ADP, ATP, UDP, UTP) tested on the same 96 well plate of wild type tsA201 cells (Figures 9A-E), all evoked a concentration-dependent rise in fluorescence signal, indicating the release of Ca²⁺.

ADP (Figure 9A) was associated with an initial response at 100 nM and then a steady increase until starting to plateau after 10 μ M and peaking with 100 μ M ADP. The pEC₅₀ was calculated as 6.32 ($\pm$ 0.04 SEM) with the fitted Hill slope as 1.22 ($\pm$ 0.10 SEM) and the E_{max} as 105.90% ($\pm$ 1.80 SEM) of the response to 10 μ M ADP

ATP (Figure 9B) had no appreciable effect up until 1 μ M where there was a sharp increase in response. The response subsequently peaked at 100% at 10 μ M and afterwards there was a steady decrease with increasing ATP concentrations. The calculated pEC₅₀ was 6.04 ($\pm$ 0.01 SEM) with a Hill slope of 1.94 ($\pm$ 0.33 SEM) and an E_{max} of 100.90% ($\pm$ 1.24 SEM) of the response to 10 μ M ATP.

UDP (Figure 9C) did not cause any response until 100 μ M where there was a small increase in Ca²⁺ signal. There was then a much more rapid rise with 1 mM UDP, but this was not sustained and the response fell at the top 10 mM concentration. Unfortunately, there were insufficient points in order to effectively fit a response curve.

UTP (Figure 9D) had no visible effect up until 1 μ M, where there was a then a sharp rise in response before beginning to plateau at 100 μ M, peaking at 1 mM and falling at 10 mM. There was a pEC₅₀ of 4.93 ($\pm$ 0.10 SEM) with a Hill slope of 0.97 ($\pm$ 0.19 SEM) and an E_{max} of 99.94% ($\pm$ 4.77 SEM) of the response to 1 mM UTP.

When the averaged raw data for the 96 well plate was plotted as a percentage of the average response to that of 1 mM UTP (Figure 9E), the largest fitted E_{max} was that of UTP with 100.10% ($\pm$ 2.29 SEM) followed by ADP at 82.44% ($\pm$ 1.40 SEM), ATP with 74.71% ($\pm$ 0.63 SEM) and lastly UDP at approximately 29.54%.

Figure 9: The effects of natural P2Y receptor agonists on Ca^{2+} flux signal in wild type tsA201 cells.

Wild type tsA201 cells grown in a 96 well plate and incubated with 5 μM Cal-520 AM fluorescent dye for 1.5 hours were stimulated with various concentrations of (A) ADP, a single read (B) ATP, note the -6 point only is a duplicate read (C) UDP and (D) UTP, using the automated dispensing system of a Molecular Devices FlexStation 3 robotic platereader, for triplicate reads except where noted and as further detailed in the text. Responses are expressed as a percentage of the response to 10 μM of the agonist, except for UTP and UDP where they are a percentage of the response to 1 mM of each due to the small or absent responses to 10 μM ; the data was then fitted with the Hill equation in GraphPad Prism 7 with the bottom constrained to the average response to buffer only. (A) additionally superimposes the average response curve ($n=4$) to ADP produced by the LS-50B luminance/spectrophotometer as a comparison. (E) Plots the signals on the 96 well plate on the same graph, expressed as a percentage of the average response to 1 mM UTP so allow comparison of the fitted E_{max} values. All error bars in this figure indicate SEM.

3.3.0 Confirmation of Functional Tagged Receptor Expression using Ca^{2+} Flux Bioassay

The experiments detailed so far characterised the native expression of hP2Y receptor subtypes in the tsA201 cell line, the other aim of this project however was to biochemically study the interactions between co-expressed recombinant hP2Y₁ and hP2Y₁₂ receptors. Previous experiments in the Kennedy lab group used hP2Y₁ and hP2Y₁₂ receptors tagged with both a HA and an FP tag, which would be unsuitable for use in co-immunoprecipitation experiments. Therefore, prior to the start of the project a new set of recombinant receptors expressing a single HA (N-terminus) or C-terminus CFP/YFP tag (HA-hP2Y₁, HA-hP2Y₁₂, hP2Y₁-YFP, hP2Y₁₂-CFP receptors) were designed, and in the next set of experiments it was tested as to whether they could be functionally expressed in the tsA201 cell line using Ca^{2+} flux as a bioassay.

3.3.1 Ca^{2+} Imaging of Wild Type and Tagged hP2Y₁ Receptor Transfected tsA201 Cells Demonstrated Functional Expression of the Constructs

When WT tsA201 cells Ca^{2+} responses were imaged with the LS-50B luminance/spectrophotometer – see Figure 10A for a representative trace - there was no response to ADP until 100 nM. The average responses rose rapidly with increasing ADP concentrations before starting to plateau at 3 μM , immediately before the final 10 μM concentration (Figure 10D). A pEC₅₀ of 6.49 (± 0.04 SEM) was fitted, with a Hill slope of 1.35 (± 0.14 SEM) and an E_{max} of 98.77% (± 2.85 SEM).

Comparison of the FlexStation 3 robotic platereader generated ADP concentration-response curve with the averaged WT tsA201 ADP concentration response curve (n=4) produced by the LS-50B luminance/spectrophotometer (see Figure 9A) using the extra sum of squares F test (ESSFT) did not find any statistically significant differences in the fitted pEC₅₀, Hill slope or E_{max} parameters, and therefore confirmed the two methods as being comparable.

When 1 μg of either hP2Y₁-YFP or HA-hP2Y₁ receptor cDNA was transfected into the cell line and the Ca^{2+} response measured with the LS-50B – see Figures 10B and 10C for respective representative traces - there was a shift to the left in the concentration-response curve (Figure 10D) compared to the WT cells. The initial 300 pM of ADP produced a small response for each, contrasting with there being no response at the initial 10 nM

concentration in wild type cells. Compared to the WT only cells, the response curves were shallower for both plasmids, but began to plateau out at similar concentrations.

The fitted pEC_{50} values for the HA-hP2Y₁ and hP2Y₁-YFP receptor plasmids were 7.13 (± 0.17 SEM) and 6.96 (± 0.11 SEM) respectively, which were not significantly different from one another using the ESSFT, although only the HA-hP2Y₁ pEC_{50} was statistically significant from that of the WT cells ($p \leq 0.05$). The Hill slopes were respectively 0.40 (± 0.04 SEM) and 0.49 (± 0.04 SEM), which were not significantly different, but both were significant from the Hill slope of the WT cells ($p \leq 0.001$). The plasmids fitted E_{max} values were not significantly altered from one another, but only the HA-hP2Y₁ receptors E_{max} of 114.90% (± 6.25 SEM) of the response to 10 μ M ADP was statistically significant from the wild type cells ($p \leq 0.05$). This overall suggests that both of the tagged hP2Y₁ receptors were functionally expressed in the tsA201 cells following transfection.

Figure 10: The effect of ADP stimulation on intracellular Ca^{2+} signalling in wild type tsA201 cells and when transfected with hP2Y₁-YFP or HA-hP2Y₁ receptor cDNA.

The Ca^{2+} flux of coverslips of tsA201 cells was assayed on an LS-50B luminance/spectrophotometer using Cal-520 AM fluorescent Ca^{2+} dye. Representative Ca^{2+} traces of un-transfected wild type (WT) cells (A) and cells expressing hP2Y₁-YFP receptors (B) or HA-hP2Y₁ receptors (C) are shown. Cells were subjected to different concentrations of ADP stimulation (at the arrow point) until the response peaked; these are representative of at least 3 independent experiments each. (D) Concentration-response curves were generated and graphically present the averaged responses to the concentrations of ADP on the cells ($n=3$ for hP2Y₁-YFP receptors, $n=4$ for WT and $n=5$ for hP2Y₁-HA receptors) and fitted with the Hill equation in GraphPad Prism 7. Error bars indicate SEM.

3.3.2 Ca²⁺ Imaging Established that the Tagged hP2Y₁₂ Receptors could be Functionally Expressed in the tsA201 Cell Line

hP2Y₁₂ receptors couple to the G_{i/o} subunit and therefore when activated do not directly mediate any increase in Ca²⁺ flux (Abbracchio et al., 2006). Consistent with this the ADP evoked release of intracellular Ca²⁺ is not inhibited by the selective hP2Y₁₂ receptor antagonist cangrelor in wild type tsA201 cells, but is abolished by the selective hP2Y₁ receptor antagonist MRS2179. When recombinant hP2Y₁₂ receptors are overexpressed in the tsA201 cell line though, the Ca²⁺ response becomes sensitive to cangrelor and is also abolished by MRS2179 (Naughton et al., 2014); this data forms the core of the hypothesis by Naughton (2014) that hP2Y₁ and hP2Y₁₂ receptors can form a functional heterodimer. Consequently, in the next set of experiments it was aimed to determine the functional expression of tagged hP2Y₁₂ receptors transfected into the tsA201 cell line, using the inhibition by cangrelor of ADP-induced intracellular Ca²⁺ release as a bioassay.

For either of the hP2Y₁₂-CFP or HA-hP2Y₁₂ receptor plasmids transfected (1 µg) into the tsA201 cell line, the repeated 3 or 4 additions of 300 nM ADP did not cause any visible appreciable change in response and linear regression analysis did not find any slopes statistically significant from zero (data not shown); see figures 11A and 11C for representative hP2Y₁₂-CFP and HA-hP2Y₁₂ receptor traces of the repeated ADP additions.

As further illustrated in Figures 11B, D and E, 1 µM cangrelor by itself caused no change in fluorescence signal, but the average responses to 300 nM ADP were reduced by 68.0% (±6.6 SEM, n=5) and 67.8% (±5.3 SEM, n=5) in the presence of 1 µM cangrelor for the hP2Y₁₂-CFP and HA-hP2Y₁₂ receptor plasmids correspondingly. Following 5 minutes of washout to remove the cangrelor, the mean responses of hP2Y₁₂-CFP and HA-hP2Y₁₂ receptors to 300 nM ADP were not significantly different from before the addition of cangrelor, but were significantly greater than in its presence (p≤0.0001 and p≤0.001 respectively). The plasmids showed no overall statistically significant differences in response between each other. As the Ca²⁺ responses were suppressed by the presence of the selective P2Y₁₂ antagonist cangrelor, it suggested that the tagged hP2Y₁₂ receptors had been functionally expressed in the cell line.

Figure 11: The effect of ADP stimulation on intracellular Ca^{2+} after transfecting hP2Y₁₂-CFP or HA-hP2Y₁₂ receptor cDNA into the tsA201 cell line.

1 μg of hP2Y₁₂-CFP or hP2Y₁₂-HA containing plasmid was transfected into tsA201 cells on coverslips and imaged on an LS-50B luminance/spectrophotometer using Cal-520 AM fluorescent Ca^{2+} dye. Representative Ca^{2+} traces are shown for (A) hP2Y₁₂-CFP and (C) HA-hP2Y₁₂ transfected cells when subjected to repeated 300 nM ADP control stimulations (at the arrow point). (B) hP2Y₁₂-CFP and (D) HA-hP2Y₁₂ show the traces for the last 300 nM ADP control stimulation, and subsequently those for the response to the 1 μM of the P2Y₁₂ receptor antagonist cangrelor alone, the response to 300 nM ADP in the presence of 1 μM cangrelor and the response to 300 nM ADP after 5 minutes of washout. These two sets of traces are each representative of 5 independent experiments.

(E) Graphically presents the averaged responses from the Ca^{2+} traces for each plasmid (n=5 each) as a percentage of the initial 10 μM ADP concentration, error bars represent SEM; the plasmids were not overall statistically different from one another. *** = $p \leq 0.001$ **** = $p \leq 0.0001$ ns = not significant.

3.4.0 Co-immunoprecipitation and Western Blotting

The previous set of experiments confirmed that HA or FP tagged hP2Y₁ and hP2Y₁₂ receptors were functionally expressed when transfected into the tsA201 cells. The final set of experiments therefore aimed to investigate whether they would co-immunoprecipitate when expressed together, after first determining whether the singular HA or FP tagged receptors themselves could be immunoprecipitated.

3.4.1 GFP-Immunoprecipitation and Blotting Showed that Individual FP Tagged Receptors could be Immunoprecipitated

Following transfection of tsA201 cells with 2.5 µg of GFP, P2Y₁-YFP or P2Y₁₂-CFP and immunoprecipitating for GFP, probing with anti-GFP revealed no signal in the no-cDNA untransfected control lanes for either the inputs or for the IP samples (Figure 12A). In all transfected lanes there was prominent signal at ~27KDa visible in both the inputs and IP samples, corresponding to the GFP; this was substantially stronger in the IP lanes. The hP2Y₁-YFP receptor IP sample presented as a “smear” signal from ~40 to >250 KDa (based on the pre-stained protein standards), with the band at ~70 KDa likely representing the hP2Y₁-YFP receptor (predicted molecular weight 69.0 KDa), and other bands post-translationally modified forms of the receptor such as homodimers or those undergone glycosylation. The hP2Y₁₂-CFP receptor IP sample presented as clearly defined bands with the band at ~65 kDa probably representing the CFP tagged hP2Y₁₂ receptor (predicted molecular weight 66.3 KDa) and the other bands post-translational modifications of the receptor as with the hP2Y₁₂-YFP. These signals typically did not show clearly in the input samples, and by being visible in the IP samples overall suggested that the FP tagged complexes had been successfully immunoprecipitated from the other proteins present in the inputs and exposed the epitope.

3.4.2 HA-Immunoprecipitation and Blotting Demonstrated Successful Immunoprecipitation of Individually Expressed HA Tagged Receptors

After transfecting tsA201 cells with 2.5 µg of HA tagged hP2Y₁ or P2Y₁₂ receptor and immunoprecipitating for the HA tag, probing with anti-HA showed no signal in the untransfected no-cDNA control lanes for the inputs or IP samples (Figure 12B); although prominent bands were visible in the HA-peptide control lanes these corresponded to the light

and heavy IgG chains of the beads. The HA-hP2Y₁ receptor IP lane presented as a “smear” ranging from ~30 to 250 KDa (based on the pre-stained protein standards), with the band at ~40 KDa probably representing the HA-hP2Y₁ receptor (predicted molecular weight 43.2 KDa) and the other bands post-translational modifications of the receptor; the signal strength was substantially stronger in the IP sample compared to the input. The HA-hP2Y₁₂ IP lane also presented as a “smear”, but from ~36 to 55 KDa with the band at ~40 KDa likely representing the HA-hP2Y₁₂ receptor (predicted molecular weight 40.5 KDa); these were barely detectable in the input sample. The presence of “smear” signals in the HA-peptide controls similar to those of the IP samples suggested that the some of the bound proteins had not been competed off the beads by the peptide, however using either 6 µg or 16 µg of HA-peptide did not appear to alter the result (data not shown) appreciably so 8 µg was used to elute in future experimentations. As a whole the HA tagged complexes appeared to have been successfully immunoprecipitated.

Figure 12: Immunoprecipitation of singular FP tagged and HA tagged hP2Y₁ and hP2Y₁₂ receptors.

tsA201 cells were transfected with (A) fluorescent protein (FP) tagged constructs of hP2Y₁ or hP2Y₁₂ receptors and (B) HA tagged hP2Y₁ or hP2Y₁₂ receptors, along with a control lane containing no transfected cDNA. The cells were lysed and denatured to provide crude whole cell lysates as input samples, then immunoprecipitated for GFP for the FP tagged receptors and HA for the HA tagged receptors; the HA samples have a further set of "HA-peptide" lanes representing immunoprecipitated proteins not competed off of the beads. The respective protein tag was then blotted for. The HA immunoprecipitation is representative of 2 independent experiments whilst the GFP IP represents a single experiment.

3.4.3 HA-Immunoprecipitation of Differentially Tagged Co-expressed hP2Y₁ and hP2Y₁₂ Receptors and Blotting with Anti-GFP Demonstrated a High Molecular Weight Protein Complex

Based on the results of the individual immunoprecipitations it was decided to immunoprecipitate for the HA tag as it gave a clearer signal, and then determine if the FP tag was co-immunoprecipitated. Samples were prepared with 2.5 µg each of a HA-hP2Y₁ or HA-hP2Y₁₂ receptor and the correspondingly opposite hP2Y₁₂-CFP or hP2Y₁-YFP receptor co-transfected together. Samples were also prepared of each HA tagged receptor co-transfected with 2.5 µg GFP to detect any non-specific precipitation of the GFP. The samples were immunoprecipitated for the HA tag and then blotted for the FP tag to determine if the FP tagged receptor were precipitated along with the HA tagged receptor (Figure 13A).

No signals presented in the un-transfected no-cDNA control lane for the inputs or IP samples, and likewise no signals were visible in any of the IP lanes transfected with a HA tagged receptor and GFP. This suggested that the GFP alone did not immunoprecipitate with the receptors, but that it had successfully transfected as evidenced by the band at ~27 KDa in the input lanes. In both of the co-transfected receptor IP lanes a high molecular weight complex of ~130 to >250 KDa (based on the pre-stained protein standards) was detected, with the signal strength in the HA-hP2Y₁₂ + hP2Y₁-YFP receptors IP lane being stronger and additionally visible in the input lane. After stripping and blotting with anti-HA this higher weight complex was again visible (Figure 13B), although more faintly, as were bands at ~45 KDa and ~40 KDa in the respective lanes, which likely corresponded to the monomeric HA tagged hP2Y₁ and hP2Y₁₂ receptors. Interestingly co-expression of the two receptors appeared to reduce the signal strength compared to the singular HA tagged receptors co-expressed with GFP.

After re-running the samples (due to stripping difficulties where the higher affinity anti-HA could not be removed - detailed in section 2.4.3) and blotting with anti-β-tubulin there was a strong band expressed across all of the input lanes at 55 KDa, which did not present in the IP lanes (Figure 13C). The membrane was stripped and anti-HA blotted with again (using a lower 1:10000 concentration due the intense staining at 1:5000) to determine if the membrane was representative of the first; identical results presented aside from the higher molecular weight complexes being slightly more visible (Figure 13D). This is unfortunately only representative of a single co-IP experiment however due to insufficient time being available for further repeats.

Figure 13: HA co-IP of co-expressed tagged hP2Y₁ and hP2Y₁₂ receptors in tsA201 cells

tsA201 cells were co-transfected with a HA tagged form of hP2Y₁ or hP2Y₁₂ and an FP tagged construct of the opposing receptor; other samples had no cDNA transfected (control) or had HA tagged hP2Y₁ or hP2Y₁₂ receptor cDNA plus separate GFP cDNA transfected. These were lysed and denatured to provide crude "input" samples before being immunoprecipitated for the HA tag to purify. The FP tag was then blotted for with anti-GFP to detect any physical association of the HA tagged and FP tagged receptors following the immunoprecipitation (**A**). After stripping the membrane was re-probed with anti-HA to confirm that the HA tagged receptors were present in the IP samples (**B**). The samples were re-run on a separate gel and the membrane then blotted for the loading control anti- β -tubulin to confirm that protein was present (**C**). Following stripping this second membrane was re-probed with anti-HA to ensure that the results between the two membranes were complementary – note that the β -tubulin staining remains as it did not strip fully (**D**). This panel is representative of a single experiment.

Chapter 4: Discussion and Conclusions

4.1.0 Endogenous hP2Y Receptor Expression in the tsA201 cell line

As the overall expression of hP2Y receptor subtypes present in the tsA201 cell line is relatively unknown, qualitative RT-PCR and Sanger sequencing were used to characterise individual receptor mRNA expression: this demonstrated the presence of mRNA for hP2Y₁, ₂, ₄, ₆, ₁₂, ₁₄ receptors. Ca²⁺ flux imaging was also used to try to ascertain functional hP2Y receptor expression, which demonstrated concentration dependent responses to all 4 of the natural P2Y agonists tested, these being ADP, ATP, UDP and UTP.

The tsA201 cell line is an SV40 transformed derivative of the HEK-293 cell line stably expressing the large-T antigen to improve plasmid copy number and protein expression (Mellon *et al.*, 1981). Previous whole genome sequencing of the HEK-293 cell line and related derivatives (including the similarly SV40 transformed HEK-293T line) has revealed them to be typically complementary in expression of most genes, including expressing mRNA sequences for all of the hP2Y receptors (Lin *et al.*, 2014), but many individual studies have found differing results using complementary techniques as will be further detailed.

The presence of P2Y₁ mRNA in this study is consistent with previous reports in HEK-293 cells and derived cell lines (Schachter *et al.*, 1997; Moore *et al.*, 2001; Fischer *et al.*, 2003; Wirkner *et al.*, 2004; Atwood *et al.*, 2011). Functional hP2Y₁ receptor expression in the tsA201 cell line is strongly suggested from the concentration dependent increases in Ca²⁺ signal upon ADP stimulation in this project, and from past work in the Kennedy lab where the responses to ADP continued in the presence of the antagonist cangrelor, which inhibits ADP sensitive hP2Y₁₂, ₁₃ receptors, but were abolished by the selective P2Y₁ receptor antagonist MRS2179 (Naughton *et al.*, 2014). This has been observed in other HEK-293 derived cell lines (Suzuki *et al.*, 2011), with similar pEC₅₀ values for ADP also reported (Mundell and Benovic, 2000; Werry *et al.*, 2002). In addition, Western blotting and immunocytochemical staining have demonstrated hP2Y₁ protein expression in HEK-293 cell lines (Fischer *et al.*, 2003; Wirkner *et al.*, 2004).

As ADP also acts on the hP2Y₁₂ and hP2Y₁₃ receptors there is the possibility of expression of these in the tsA201 cell line: this is supported by the presence of hP2Y₁₂ receptor mRNA in this project, but PCR products for hP2Y₁₃ receptor mRNA were absent. Although both receptors are G_{i/o} linked and not traditionally associated with signalling through Ca²⁺, hP2Y₁₂ receptors are known to modulate the Ca²⁺ response induced through the hP2Y₁ receptor (Hardy *et al.*, 2004; Suzuki *et al.*, 2011). Data from the Kennedy lab group has previously

suggested the presence of a low level of functional hP2Y₁₂ receptors in tsA201 cells, based on how cangrelor can subtly alter the Ca²⁺ response induced through the hP2Y₁ receptor (Naughton, 2014). Analogous conclusions were drawn by Suzuki *et al.* (2011) in similarly SV40 transformed HEK-293T cells, although cangrelor caused a decrease in Ca²⁺ signal compared to an increase as described by Naughton (2014), which may be related to the increased concentration used. Inbe *et al.* (2004) also found hP2Y₁₂ receptor mRNA in HEK-293 cells, and contrastingly hP2Y₁₃ receptor mRNA as well, which is consistent with the whole genome sequencing of HEK-293 cell lines by Lin *et al.* (2014), where hP2Y_{12, 13} receptor mRNA was present in all those sequenced, although at varying expression levels.

The responses to ATP and UTP in the tsA201 cells in this project are overall likely indicative of hP2Y₂ and/or hP2Y₄ expression: both of these stained faintly on agarose gels after RT-PCR and their identities were confirmed by Sanger sequencing. Whilst ATP also acts as a full agonist on the G_q coupled hP2Y₁₁ receptors, no PCR products were observed in difference to reports in other HEK-293 derived cell lines (Moore *et al.*, 2001; Wirkner *et al.* 2004). Despite both hP2Y₂ and hP2Y₄ receptors responding to UTP, because ATP acts as an antagonist at the hP2Y₄ receptor (Kennedy *et al.*, 2000) any ATP induced Ca²⁺ responses in the tsA201 cells are likely from hP2Y₂ receptor binding due to the lack of hP2Y₁₁ receptor mRNA, although ATP additionally acts as a partial agonist at the hP2Y₁ receptor as detailed later. The reduced potencies of ATP and UTP compared to ADP suggests that any hP2Y_{2, 4} receptors are expressed at a lower level compared to ADP sensitive hP2Y₁ receptors.

Dickson *et al.* (2013) have demonstrated UTP sensitivity and a decreased response to UTP in the presence of 100 µM suramin in the tsA201 cell line, along with hP2Y₂ protein expression using Western blotting; this was reduced by hP2Y₂ receptor small interfering RNA (siRNA) knockdown. Whilst they also proposed that hP2Y₁₁ was absent based on the inability of UTP to raise cAMP, UTP has since been found to not act at the hP2Y₁₁ receptor (Morrow *et al.*, 2014). Mundell and Benovic (2000) have also reported 100 µM of ATP or UTP to analogously raise inositol phosphate accumulation in HEK-293 cells, which suggests hP2Y₂ and/or hP2Y₄ receptor expression. In other HEK-293 derived cell lines there is much conflicting evidence both suggesting and contesting hP2Y₂ receptor expression (Moore *et al.*, 2001; Schäfer *et al.*, 2003; Wirker *et al.*, 2004).

There is the possibility that the ATP induced Ca²⁺ responses observed were due to ATP acting exclusively as a partial agonist at the hP2Y₁ receptor in the tsA201 cells (Palmer *et al.*, 1998; Waldo and Harden, 2004). This is seen as unlikely though because the fitted E_{max} parameters of the ADP and ATP concentration response curves from the FlexStation 3 were

very similar, rather than ATP showing the clear sub-maximal response associated with partial agonism. The Hill slope of above unity for ATP could further suggest it acting on multiple targets – it is accepted though that the Hill slope and $E_{\max}$ obtained for the ATP are likely somewhat inaccurate due to there only being a single point in the middle of the curve and few points overall from 0 – 100%. The additional partial agonist action of ATP at the hP2Y₁ receptor may also perhaps explain the greater pEC₅₀ of ATP compared to UTP as the two are usually seen as equal in potency at the hP2Y₂ receptor (Velázquez *et al.*, 2000). Contrastingly Mundell and Benovic (2000) have reported that in WT HEK-293 cells ADP, ATP and UTP each raised inositol phosphate accumulation by similar levels; although this may be reflective of the different signalling assay used, Ca²⁺ concentration-response curves for ADP and ATP also revealed them to have very similar EC₅₀ values.

Despite the responses to UTP and the hP2Y₄ receptor mRNA expression in this project, there is unfortunately little prior evidence to directly support or refute hP2Y₄ receptor functional expression specifically in the tsA201 cell line. Similar results have been published for hP2Y₄ mRNA expression in the parent HEK-293 cell line, with Moore *et al.* (2001) showing hP2Y₄ as having the second highest mRNA expression level of the P2Y receptors characterised and Wirker *et al.* (2001) finding mRNA, plus also a small number of hP2Y₄ receptors with immunocytochemistry. The UTP concentration-response curve demonstrated a higher $E_{\max}$ compared to ATP when plotted on the same graph, which could indicate both UTP sensitive hP2Y₂ and hP2Y₄ receptors being activated and inducing greater Ca²⁺ release. Based on the past literature and the findings of this project, it is therefore likely that tsA201 cells express functional hP2Y₂ receptors, but it is unclear as to functional hP2Y₄ receptor expression without further study.

hP2Y₆ and hP2Y₁₄ receptor mRNA were detected in the tsA201 cell line in this project. This corresponds with the previous whole genome sequencing by Lin *et al.*, (2014) in the HEK-293 cell line and derivatives; other studies have found hP2Y₆ receptor (Wirker *et al.*, 2001) or hP2Y₁₄ receptor (Moore *et al.*, 2003) mRNA, but Schäfer *et al.* (2003) and Moore *et al.* (2001) did not find evidence of hP2Y₆ receptor mRNA (hP2Y₁₄ receptor mRNA was not searched for in these studies). As a small response was found to high (≥ 1 mM) concentrations of UDP in this project it suggests a low level of functional expression of either or both types of receptor as they uniquely respond to UDP out of the hP2Y receptors. It is unfortunately not possible to differentiate between the two though with the present data due to the lack of any insights on the response to UDP-glucose, which only acts on the hP2Y₁₄ receptor.

Despite initial reports suggesting a competitive antagonist action of UDP on the hP2Y₁₄ receptor (Fricks *et al.*, 2008), later assays have confirmed its action as an agonist at the hP2Y₁₄ receptor (Carter *et al.*, 2009). Previous Ca²⁺ signalling assays in tsA201 cells in the Kennedy lab group have suggested a small response to UDP-glucose from 100 nM to 100 μ M, but similarly to this project no response to UDP at 10 μ M (Naughton, 2014). In contrast, other studies with WT HEK-293 derived cell lines have reported no Ca²⁺ response to UDP or UDP-glucose (Werry *et al.*, 2002), except when co-expressing chimeric G α subunits (Moore *et al.*, 2003). This is because hP2Y₁₄ does not couple to the G α subunit, so it is unclear why Naughton (2014) found a response; there is evidence that in other cell lines the P2Y₁₄ G $\alpha_{i/o}$ $\beta\gamma$ subunit may act on PLC β and induce Ca²⁺ signalling, or that the hP2Y₁₄ receptor may mediate Ca²⁺ signalling through the hP2Y₆ receptor, similar to the hP2Y₁-hP2Y₁₂ relationship (Bianco *et al.*, 2005; Wypych and Barańska, 2013). These signalling effects have not been characterised in HEK-293 type cell lines though, and additionally both UDP and UDP-glucose have been identified as failing to inhibit forskollin mediated cAMP accumulation in wild type HEK-293 cells (Carter *et al.*, 2009), which suggests the absence of hP2Y₁₄ receptors.

Without further data using UDP-glucose, cAMP inhibition assays and selective hP2Y₆ ligands, any conclusions on the presence of functional hP2Y₆ or hP2Y₁₄ receptors in the tsA201 cell line are at best speculation. However, based on the available data hP2Y₆ receptors are more likely due to them being well established as coupling to the G α subunit and signalling through Ca²⁺, unlike the hP2Y₁₄ receptor.

It is unclear as to the expression of hP2Y₁₁ or hP2Y₁₃ receptor mRNA in this project as RT-PCR showed no RT+ products of the expected size, but it also unfortunately appeared to fail to amplify any of the positive controls correctly. Raising the primers concentrations and plasmid amounts only gave products which were substantially larger than expected, and likely represent mis-primes or the plasmid vector itself as the cyclor extension time was insufficient to generate products of the size generated (Tate, personal communication, 2016). The reason for this became apparent when the plasmid sequences were later available: the primers could not bind due to small numbers of nucleotide base changes in the plasmids encoded receptor sequences. This information unfortunately came too late for other positive controls to be sourced; HepG2 cDNA was evaluated as an alternative, but gave no products either, and it is therefore possible that it did not express mRNA for either receptor despite the previous reports.

The products formed in the RT- controls in this study are likely reflective of a low concentration of contaminating genomic DNA based on their sequences matching the expected PCR products. This is despite the multiple rounds of DNase I treatment, but Schachter *et al.* (1997) have reported experiencing a similar issue with hP2Y₁ and hP2Y₂ receptor RT-PCR. It is interesting that RNase I_f treatment of the templates caused large reductions in hP2Y₁ receptor RT- band staining, but not with the hP2Y₂ receptor RT-, and it did not affect the RT+ for either, which could suggest an off-target reverse transcriptase action of the polymerase on the RNA present in the RT-. This is seen as unlikely with a Taq based PCR polymerase mastermix like used (Shi *et al.*, 2015), especially as reverse transcriptase activity is Mn²⁺ dependent compared to the Mg²⁺ required for PCR polymerase activity (Mülhardt and Beese, 2007). Alternatively, the products could represent pseudo-genes for the receptors, but no pseudo-genes are currently known to exist in the hP2Y receptor family, except for possibly the hP2Y₆ receptor (Maier *et al.*, 1997).

Similarly, the reasons for the amplicons forming in the water controls are unclear, but contamination of the reagents by airborne plasmid DNA or previous PCR products looks to be the most obvious cause, and these likely additionally contributed to the RT- bands. This theory is supported by how setting up the PCR reactions in a UV exposed laminar flow cabinet appeared to eliminate the amplicons on nearly all occasions, and also reduced or removed the RT- bands in the majority of cases.

It is accepted that the functional data presented here is limited in that selective pharmacological ligands were not used, so any conclusions on most individual receptors presence are more speculative than explicit. Additionally, another especially valid point is that as briefly mentioned the P2Y₁₂ like G_{i/o} coupled receptors are traditionally not characterised as signalling, at least directly, through intracellular Ca²⁺.

4.2.0 Tagged Receptor Construct Cellular Expression

In order to determine if the HA or FP tagged hP2Y₁ and hP2Y₁₂ receptor constructs were functionally expressed in the tsA201 cell line following transfection, Ca²⁺ flux assays were used to determine the responses to ligands. ADP stimulated a response in wild type tsA201 cells with a similar potency to past studies with the cell line in the lab group (Naughton *et al.*, 2014) and also other HEK-293 type cell lines (Mundell and Benovic, 2000; Werry *et al.*, 2002; Jones *et al.*, 2014). Transfection of 1 µg of either HA-hP2Y₁ or hP2Y₁-YFP receptor cDNA was associated with a leftward shift in the fitted concentration response curve, a

corresponding increase in the pEC_{50} and a slightly raised E_{max} . This is consistent with receptor overexpression and there being an increased number of binding sites present (Chen *et al.*, 2000).

The transfection also significantly reduced the Hill slope for both of the tagged hP2Y₁ receptor constructs compared to the WT cells, which was likely a result of the receptor overexpression moving a greater proportion of receptors to a higher affinity, activated state and thereby raising basal activity levels (Chen *et al.*, 2000; Kenakin, 2014). Another possibility is that this reflects the heterogeneous mixed population of the overexpressed transfected receptors and the endogenous receptors present, so the balance of the P2Y receptors expressed had been un-naturally shifted, which thereby induced differing combinations of receptor conformational or dimeric states that showed varying affinities to ADP (Colquhoun, 1998).

To test for expression of the tagged hP2Y₁₂ receptor constructs, the cells were transfected with 1 μ g of HA-hP2Y₁₂ or hP2Y₁₂-CFP cDNA. The cells were repeatedly stimulated with 300 nM of ADP (this being close to the EC_{50} of WT tsA201 cells), which demonstrated reproducible responses for both plasmids; the response was then measured again when in the presence of 1 μ M of the hP2Y₁₂ receptor antagonist cangrelor and again after washout. In WT tsA201 cells cangrelor has been found in previous work in the lab group to have no inhibitory effect on Ca²⁺ signals (Naughton *et al.*, 2014) due to its target hP2Y₁₂ receptors not being G_q coupled, but in this project when either of the hP2Y₁₂ receptor plasmids were expressed in the cell line the cangrelor significantly suppressed the Ca²⁺ response and the prior response to 300 nM ADP was restored after washout. This corresponds with the previous studies in the lab group, and suggests that hP2Y₁₂ receptors had been functionally expressed as the ADP induced Ca²⁺ signals were sensitive to cangrelor, which only directly acts competitively on hP2Y₁₂ receptors and has no action on the endogenous hP2Y₁ receptors inducing the Ca²⁺ signal (Suzuki *et al.*, 2011). It is fully accepted however that this was not the most appropriate method to determine hP2Y₁₂ receptor expression as they do not directly couple to Ca²⁺ signalling, and cAMP inhibition assays would have been more suitable.

Overall this suggested that the HA or FP tagged forms of the hP2Y₁ and hP2Y₁₂ receptors could be functionally expressed in the tsA201 cell line. Therefore, they could be used in co-immunoprecipitation studies to try to detect physical interactions between the two receptors.

4.3.0 Interactions between the hP2Y₁ and hP2Y₁₂ receptors

To try to determine if the hP2Y₁ and hP2Y₁₂ receptors physically interacted to form a functional constitutive heterodimer as per the theory proposed by Shrestha *et al.* (2010), co-immunoprecipitation experiments were performed. In the first set of experiments it was confirmed that when transfected in individually both the HA and FP tagged forms of the receptors could be successfully immunoprecipitated from the initial whole cell lysate “inputs” using the respective antibody conjugated agarose beads. The “smear” staining and multiple bands present on the Western blots likely represent various post-translationally modified forms of the receptors, for example by glycosylation (Yoshioka *et al.*, 2001; Zhong *et al.*, 2004) and homodimer formation. Both hP2Y₁ (Choi *et al.*, 2008) and hP2Y₁₂ receptors are known to form homodimers, with the heavier bands visible in the hP2Y₁₂ receptor anti-GFP IP corresponding with the report by Savi *et al.* (2006) of dimeric and oligomeric hP2Y₁₂ receptor complexes. These were not as prominent in the anti-HA IP, which may be related to the resolving gel percentage being inappropriate for the size of the protein, and/or the concentration of DTT used, which P2Y₁₂ receptor homo-oligomers are sensitive to (Savi *et al.*, 2006).

As the individual receptors could be immunoprecipitated, it was then moved on to the main aim of whether co-expressed receptors could be HA co-immunoprecipitated: one HA tagged hP2Y₁ or hP2Y₁₂ receptor was co-expressed with an FP tagged version of the opposing receptor. These were in addition to no-cDNA and HA tagged receptor plus GFP control samples to detect any non-specific signals or protein aggregation.

On the one co-IP experiment performed, the formation of a detectable higher molecular weight protein of ~130 to >250 KDa (based on pre-stained protein standards) and expressing an FP tag was visible in samples expressing HA-hP2Y₁+hP2Y₁₂-CFP or HA-hP2Y₁₂+hP2Y₁-YFP receptors when immunoprecipitating for the HA tag and blotting for the FP tag. This suggested a physical association between the two receptors as the FP tagged receptor had been immunoprecipitated with the HA tagged receptor rather than being purified out. With the tools used though it is difficult to know with any certainty what the complex was comprised of due to the likelihood of both post-translational modifications of the receptors and from dimers forming between other receptors or proteins present (discussed further later on).

The no-cDNA, HA-hP2Y₁ receptor co-expressed with GFP and HA-hP2Y₁₂ receptor co-expressed with GFP controls did not show signals, implying that this was not due to protein aggregation during cell lysis, however protein was present in all the inputs before the IP as demonstrated by the strong anti- β -tubulin staining at the expected ~55 KDa. This heavier FP tag expressing protein complex was furthermore visible when blotting for the HA tagged receptor, which confirmed the presence of both tagged receptors in the complex. These results potentially could suggest the formation of a constitutive heterodimer complex between the hP2Y₁ and hP2Y₁₂ receptors, but based on the tools used, and as it is only from a single experiment, it is not possible to currently either support or refute this hypothesis; there is past literature (detailed below) both for and against the formation of a specific heterodimer. It is also important to remember the dynamic nature of dimerisation as this experiment only represents the cells at a single point in time, and any heterodimer may form or dissociate in response to other factors such as receptor density or agonist stimulation.

Evidence for the formation of a constitutive heterodimer between hP2Y₁ and hP2Y₁₂ receptors has been previously presented by Schicker *et al.* (2009) based on FRET studies in the tsA201 cell line where co-transfection of the two receptors was associated with a significant FRET signal. However, this was also the result when most other hP2Y receptors were transfected in, raising doubt as to whether this could have been a non-specific effect of the FRET study - even though the authors took great care with positive and negative controls -, or if purinergic receptors will promiscuously dimerise with other receptors to exert required physiological signalling pathways as the authors proposed. Indeed, not only P2Y receptors, but also various co-expressed adenosine receptor subtypes were associated with an increase in FRET signal, including adenosine A₁ or A_{2A} receptors when co-expressed with hP2Y₁ or hP2Y₁₂ receptors. A₁-P2Y₁ heterodimers have already been characterised previously (Yoshioka *et al.*, 2001; Yoshioka *et al.*, 2002), but for both the hP2Y₁ and hP2Y₁₂ receptors physical interaction was also confirmed with A_{2A} receptors by co-IP in the Schicker *et al.* study. It is known that HEK-293T cells (Suzuki *et al.*, 2011) and the parent HEK-293 cell line (Inbe *et al.*, 2004) endogenously express A₁ and A_{2A} receptors, so there could a third or potentially even greater number of partners involved in any dimerisation or crosstalk between hP2Y₁ and hP2Y₁₂ receptors in tsA201 cells. This may potentially explain the high molecular weight of the co-immunoprecipitated complex in this project, especially as a possible multimeric P2Y₁-P2Y₁₂-A_{2A} receptor complex has already been described by Nakata *et al.* (2010).

The other possibility for the proposed dimerisation effects previously observed in the signalling assays is that they could be from receptor cross talk without any physical

interaction. The $G_{i/o}$ linked hP2Y₁₂ receptor is unable to directly induce Ca²⁺ release itself, but there is extensive research in both platelets (Hardy *et al.*, 2004) and C6 glioma cells (Barańska *et al.*, 2004; Suplat *et al.* 2007; Wypych and Barańska, 2013) which indicates that hP2Y₁ and hP2Y₁₂ receptors have a synergistic relationship to regulate agonist induced Ca²⁺ release, similar to as described in the tsA201 cells by Naughton *et al.*, 2014. The signalling appears to be triggered and negatively modulated through the hP2Y₁ receptor and positively modulated through the activated hP2Y₁₂ receptors inhibitory actions on cAMP as the effect is PTX-sensitive, again similar to as characterised in the previous tsA201 cell signalling studies. The platelet and C6 glioma studies mentioned have further indicated that the link between the two receptors which enables hP2Y₁₂ to modulate Ca²⁺ release appears be the activation of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3-K), the activity of which is up-regulated by the hP2Y₁₂ receptor mediated inhibition of cAMP, but is down-regulated by hP2Y₁ receptor activation. This data however has not been characterised yet in any HEK-293 type cells so it is unknown if it may apply to the signalling effects seen in tsA201 cells. The overall potential signalling pathways and heterodimer formations in the tsA201 cell line in this discussion section are visually presented in Figure 14.

It is interesting to note that for the co-IP experiment performed in this project, co-expression of both hP2Y₁ and hP2Y₁₂ receptors appeared to cause a decrease in expression on the Western blot compared to the singular receptors co-expressed with GFP controls; this may suggest that the hP2Y₁ and hP2Y₁₂ receptors have a dynamic effect on each other's expression levels. Whilst siRNA knockdown of hP2Y₁ receptors did not appear to have a subsequent effect on hP2Y₁₂ mRNA expression in C6 glioma cells in one study (Wypych and Pomorski, 2012), factors such as the presence or absence of serum in the culture media have been found to alter the molecular and surface expression of the two receptors and promote the modulating pathway of one receptor over the other (Wypych and Barańska, 2013). The other possibility is that this reduced expression was simply from a non-specific decrease in transfection efficiency induced by co-expression of the additional GPCR, but there was insufficient time to test this hypothesis using an unrelated receptor.

Figure 14: Potential hP2Y receptor mediated signalling pathways and heterodimers in the tsA201 cell line

Please note that only the major signalling pathways and potential heterodimers are represented here for simplicity and also because of a lack of data in past literature or in the authors' experimental support or refute their presence in the tsA201 cell line or the parent HEK-293 line. Only full agonists of the P2Y receptors are shown here and all receptors are the human form.

Abbreviations: AC, adenylyl cyclase; ADP, adenosine diphosphate; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; PLC, phospholipase C; PI3-K, phosphatidylinositol 3-kinase; UDP, uridine diphosphate; UDP-G, uridine diphosphate glucose; UTP, uridine triphosphate.

One particularly pertinent point though is that the signalling phenomenon proposed to indicate formation of a hP2Y₁-hP2Y₁₂ receptor heterodimer by the Shrestha *et al.* (2010) and Naughton *et al.* (2014) studies has only ever been characterised in tsA201 cells. Therefore, the signalling effects could be from the influence of any of the many endogenously expressed purinergic family receptors, or indeed simply be a cell line specific effect. It is therefore proposed that signalling, co-localisation and co-IP studies need to be characterised in another cell line to exclude this possibility, whether or not the two receptors ultimately are confirmed to co-immunoprecipitate in tsA201 cells.

This itself however leads on to another important question: even if this dimer complex can be co-immunoprecipitated in an overexpression system, does it exist in native cells, or moreover in an *in-vivo* system and if so what is its functional purpose? Although P2Y₁ and P2Y₁₂ receptors are both known to have roles in pain in animal models, there is a present deficiency in information on their roles in human pain. The mixed pro/anti nociceptive effects of stimulating/inhibiting ligands at the individual P2Y₁ and P2Y₁₂ receptors further makes it uncertain as to the form of any ligand that could target the dimer – would it be an agonist or antagonist?. It is unclear therefore as to whether a hP2Y₁-hP2Y₁₂ receptor heterodimer could even be a viable target for the modulation of pain sensing neurones, as postulated from the results of the Shrestha *et al.* (2010) study.

4.4.0 Conclusions

To conclude, in this project it was aimed to investigate endogenous hP2Y receptor expression in the tsA201 cell line, confirm that the new tagged receptor constructs could be functionally expressed in the cell line and examine whether the tagged hP2Y₁ and hP2Y₁₂ receptors physically interacted and could be co-immunoprecipitated. Molecular expression of hP2Y_{1, 2, 4, 6, 12, 14} receptor mRNA was indicated by RT-PCR and Sanger sequencing. Furthermore, functional Ca²⁺ flux assays indicated responses to ADP, ATP, UDP and UTP, which based on previous literature suggests functional expression of hP2Y_{1, 2, 12} receptors and possibly hP2Y_{4, 6, 14} receptors, but additional studies need to be completed to clarify this. The HA or FP tagged receptor constructs were successfully functionally expressed in the cell line, and when the hP2Y₁ and hP2Y₁₂ receptors were co-expressed together it was possible to co-immunoprecipitate a higher molecular weight complex bearing the opposing tag to that immunoprecipitated, therefore implying the formation of a possible constitutive heterodimer complex. Because of other potentially interacting receptors, and as this co-IP data is based

on a single experiment, it is not possible with the current information to confirm or refute the formation of specific hP2Y₁-hP2Y₁₂ heterodimers in the tsA201 cell line.

4.5.0 Potential Future Work

The data presented in this study is suggestive of several hP2Y receptor subtypes being expressed in the tsA201 cell line. P2Y receptor antibodies have been associated with a high degree of cross-reactivity (Tung *et al.*, 2004), but various studies discussed here have been able to successfully use antibody mediated techniques. It is suggested therefore that this would be a viable future direction in which to ascertain endogenous tsA201 P2Y receptor expression, along with using selective ligands, cAMP assays and quantitative RNA sequencing of the cell line to give an overall, detailed insight into the receptors expressions.

The co-IP data in the tsA201 cell line needs to be repeated in order to confirm any reproducibility, along with investigating the effect of factors such as agonist stimulation on any potential dimer formation; co-localisation and RET studies could be used to further investigate these in live cells and to confirm if the receptors co-localise. As discussed there is a strong probability of interactions with other endogenous receptors in the tsA201 cell line, so the signalling and co-IP data ideally needs to additionally be characterised in another cell line to exclude this possibility; selective gene knockdown of endogenous receptors in the tsA201 cells could also be used to determine whether the presence/absence of any specific receptor type alters the results. Utilising stably transfected 1321N1 cells, which lack endogenous P2Y receptors, could represent one option as it removes the presence of most other potentially influencing receptors, and furthermore bypasses the possibility of experimental variances induced through transient transfection. As PI3-K has previously been reported to be the linking mechanism through which hP2Y₁ and hP2Y₁₂ receptors can both modulate Ca²⁺ release in other cell types, its activity should be additionally assessed to determine if this is simply a subset of the crosstalk already characterised in other cells, or a unique mechanism. Depending on the results of these studies, potential longer term work could involve trying to investigate the effects in native receptors, in *ex-vivo* tissue or *in-vivo*.

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