THE UNIVERSITY OF HULL

THE ROLE OF INTRACELLULAR AND EXTRACELLULAR CYSTEINES IN REGULATING HUMAN PROTEINASE-ACTIVATED RECEPTOR-2 ($hPAR_2$) FUNCTION

Being a Thesis Submitted for the Degree of Doctor of Philosophy

In the Department of Cardiovascular Respiratory Medicine, University of Hull

By

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(October 2007)

UNIVERSITY OF HULL
ABSTRACT
DIVISION OF CARDIOVASCULAR & RESPIRATORY STUDIES
POST-GRADUATE MEDICAL INSTITUTE

Doctor of Philosophy

THE ROLE OF INTRACELLULAR AND EXTRACELLULAR CYSTEINES IN REGULATING HUMAN PROTEINASE-ACTIVATED RECEPTOR-2 ($hPAR_2$) FUNCTION

By Andrew Michael Botham

$hPAR_2$, a member of the novel family of proteolytically-activated G-protein coupled receptors termed Proteinase-Activated Receptors (PAR), has recently been implicated in cardiovascular disease. Previous pharmacological studies have found that activation of $hPAR_2$ by mast cell tryptase, (the major PAR_2 activator outside the gastrointestinal tract) can be regulated by receptor *N*-terminal glycosylation. In order to elucidate other post-translational modifications of $hPAR_2$ that can regulate function, we have explored the functional role of three extracellular receptor cysteines (C22, C148, and C226) and one intracellular cysteine (C361).

The putative disulphide bridging site (C148 in ECL1 and C226 in ECL2) of $hPAR_2$ were mutagenically removed both individually and together. Mutagenic removal of C226 resulted in ablation of receptor cell surface expression and intracellular retention of receptor. Mutagenic removal of C148 resulted a receptor successfully expressed at the plasma membrane with only a small reduction in cell surface expression over wild-type. The $hPAR_2$C148A mutant still retained the ability to signal through ERK MAP kinase and internalise post-activation with trypsin but was incapable of agonist mediated Ca^{2+} mobilisation. Removal of C22 resulted in a receptor with similar ERK signalling to wt-$hPAR_2$ but altered agonist-mediated Ca^{2+} mobilisation. $hPAR_2$C22A showed no change in sensitivity toward SLIGKV-NH_2, but a slight decrease in sensitivity towards trypsin, increasing to similar to wt-$hPAR_2$ on pre-treatment with thrombin.

The role of the putative palmitoylation site (C361) in regulating $hPAR_2$ function was explored. We demonstrated, using autoradiography, that C361 is the primary palmitoylation site of $hPAR_2$. $hPAR_2$C361A displayed greater cell surface expression compared to wt-$hPAR_2$. The $hPAR_2$C361A also showed a decreased sensitivity and efficacy (intracellular calcium signalling) towards both trypsin and SLIGKV-NH_2. In stark contrast hPAR2C361A triggered greater and more prolonged ERK phosphorylation compared to that of wt-$hPAR_2$. Inhibitor studies revealed that $hPAR_2$C361A triggered the majority of the ERK signal through Gi, since pertussis toxin completely inhibited this receptors ability to activate ERK. Finally, flow cytometry was utilised to assess the rate, and extent of receptor internalisation following agonist challenge. $hPAR_2$C361A displayed faster internalisation kinetics following trypsin activation, compared to wt-$hPAR_2$, whilst SLIGKV-NH_2 had negligible effect on internalisation for either receptor.

This study has highlighted the importance of post-translational modifications in regulating PAR_2 function. More specifically we have shown that palmitoylation of C361 on $hPAR_2$ plays a pivotal role in regulating the ability of the receptor to signal to Gq and Gi. Thus, we have identified a potential target site within PAR_2 that maybe useful in the design of novel therapeutic agents for cardiovascular disease.

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Acknowledgements

This research took place within the Biomedical Research Lab of the University of Hull. Funding the studentship and research was supplied by the British Heart Foundation.

I would like to thank Dr. S. J. Compton and Prof. A. H. Morice for this opportunity, and everyone within the lab for their help and support during my research. Laura Sadofsky and Rithwik Ramachandran for putting up with me invading their quiet little lab and helping me find my way around. I would also like to thank my parents for their unwavering support.

This thesis is dedicated to Amber. She has supported me physically, financially and emotionally throughout its writing. Our stress is often felt more acutely by the ones we love than ourselves and she has been a mountain of strength for me to draw on.

Publications

Papers

Mosley, M., Pullen, S., **Botham, A**., Gray, A., Napier, C., Mansfield, R. and Holbrook, M. (2006) The molecular cloning and functional expression of the dog CCR5. *Vet Immunol Immunopathol*, **113**, 415-420.

Ramachandran, R., Sadofsky, L. R., Xiao, Y., **Botham, A.M**., Cowan, M., Morice, A. H. and Compton, S. J. (2007) Inflammatory Mediators Modulate Thrombin and Cathepsin-G Signaling in Human Bronchial Fibroblasts by Inducing Expression of Proteinase-Activated Receptor-4. *Am J Physiol-Lung Cell,* 292(3),L788-98

Botham, A.M., Morice, A.H., and Compton, S.J. (In Progress) The palmitoylation site (cysteine 361) within human proteinase-activated receptor2 ($hPAR_2$) regulates receptor agonist sensitivity and cell surface expression. *Manuscript in preparation*

Conference Abstracts

Botham A.M., Morice, A. H., and Compton, S. J. (2005) The putative palmitoylation site (cysteine 361) within human proteinase-activated receptor-2 ($hPAR_2$) regulates cell surface expression, agonist sensitivity and receptor internalisation. *BPS* Journal Abstract.

Botham A.M., Morice, A. H., and Compton, S. J. (2005) The putative palmitoylation site (cysteine 361) within human proteinase-activated receptor-2 ($hPAR_2$) regulates cell surface expression, agonist sensitivity and receptor internalisation. *FASEB* Journal Abstract.

Declaration

I hereby declare that the thesis entitled "The role of intracellular and extracellular cysteines in regulating human proteinase-activated receptor-2 ($hPAR_2$)" has not been submitted for a degree, diploma or any other qualification at any other university. The work presented in this thesis was done soley by the author except where stated.

Andrew Michael Botham

Abbreviations

~	approximately
λ	wavelength
%	per cent
[X]	Concentration of X
βarr1	β-arrestin 1
Abs	Absorbance
amp	Ampicillin
bp(s)	base-pair(s)
BSA	Bovine Serum Albumin
cDNA	cloned DNA
CHO	Chinese Hamster Ovary Cells
CIP	calf-intestine phosphatase
dil^n	dilution
DMF	Dimethyl Formamide
DNA	Deoxyribonulceic Acid
dNTP(s)	deoxynucleotide tri-phosphate(s)
EC_{50}	Concentration of agonist required to elicit 50% of maximal response
EDTA	Ethylenediaminetetraacetic acid
ERK1/2	Extracellular Signal Regulated Kinases1/2 (also p44/42)
FACS	Fluorescence Activated Cell Scan
FCS	Fœtal Calf Serum (heat inactivated)
FITC	Fluorescein
FW	Formula Weight

G418	Geneticin
GPCR	G Protein-Coupled Receptor
GRK	G Protein-coupled receptor Kinases
IC_{50}	Concentration of antagonist required to elicit 50% of maximal inhibition
Inc.	Incubate
IL-*	Interleukin-*
Kb	kilo-base
KCl	Potassium Chloride
KNRK	Kirtsen murine sarcoma virus-transformed Rat Kidney epithelial cells
LB	Luria Bertini
M	Molar
mAb	Monoclonal Antibody
MACS	Magnetic Activated Cell Sorting
MAPK	Mitogen Activated Protein Kinase
MCP-*	Monocyte Chemotactic Protein-*
MCS	Multiple Cloning Site
MEM	Minimal Essential Media
mRNA	Messenger RNA
MW	Molecular Weight
NEB	New England Biosciences
NTP	Nucleotide tri-phosphate
OD	Optical Density
PAR_*	Proteinase Activated Receptor (* - 1,2,3 or 4)
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction

PKC	Protein Kinase C
PLC	PhosphoLipase C
R.T.	Room Temperature
RNA	Ribonulceic Acid
RTase	Reverse Transcriptase
RT-PCR	Reverse Transcription PCR
SD	Standard Deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard Error of the Mean
STI	Soya Bean Trypsin Inhibitor
TAE	Tris acetate/disodium EDTA
temp	Temperature
U.V.	Ultra-violet

Units of Measurement

A	amps
Ci	Curie
Da	Daltons
g	Grams
hr	Hour
l	Litres
M	Molar
min	minutes
mol	moles
^{o}C	Degrees Celsius
secs	Seconds
u/µl	units per µl (enzyme concentration)

V	Volts	

Muliples of Units

k	kilo	10^{3}
c	centi	10^{-2}
m	milli	10^{-3}
μ	micro	10^{-6}
n	nano	10^{-9}

1 GENERAL INTRODUCTION

1.0 GENERAL INTRODUCTION

1.2 G-PROTEIN-COUPLED RECEPTORS (GPCRs)

G-protein coupled receptors (GPCRs) encompass a wide range of proteins and comprise the largest single class of cell-surface receptors encompassing 3% of the genome (Wilson & Bergsma, 2000; Fredriksson et al, 2003). All GPCRs have seven transmembrane domains, an extracellular N terminal domain and an intracellular C terminal domain. GPCRs are coupled to a trimeric signal-transducing G protein complex (Janetopoulos et al, 2001). Binding of an appropriate ligand to the receptor results in activation of the associated G-protein. This consequently activates downstream effector molecules, which result in the generation of an intracellular second messenger. GPCRs can be sub-divided into 6 classes by Kolakowski on the basis of their structural homology, functional similarity, and genetic characteristics (Kolakowski, 1994). Additional methods for the classification of GPCRs exist (Kim et al, 2000) but Kolakowski's method is the most widely used (Gether, 2000). These different families do not share a significant level of sequence similarity but all have the characteristic seven transmembrane (TM) domains. The three main classes present in animals (A-C), and additional smaller families are listed below (pictured as receptor monomers) in reverse order of prevalence.

1.2.1 Family D and E

As well as the main three GPCR families there are three less well characterised families. Family D is unique to fungi and is comprised of fungal pheromone receptors, family E contains cAMP receptors, and finally the Frizzled/Smoothened receptors, adhesion

receptors and insect-specific chemosensory receptors (Kolakowski, 1994; Nat. Rev. Drug. Discov, 2004).

1.2.2 Family C

This family is characterised by a long amino terminus (500-600 amino acids) and a long carboxyl tail. Their ligand binding domain is located at the amino terminus (**fig 1.2a** - pictured in yellow), which is shown by the crystal structure of the metabotropic glutamate receptor to form a disulphide linked dimer (Kunishima et al, 2000; Kubo &

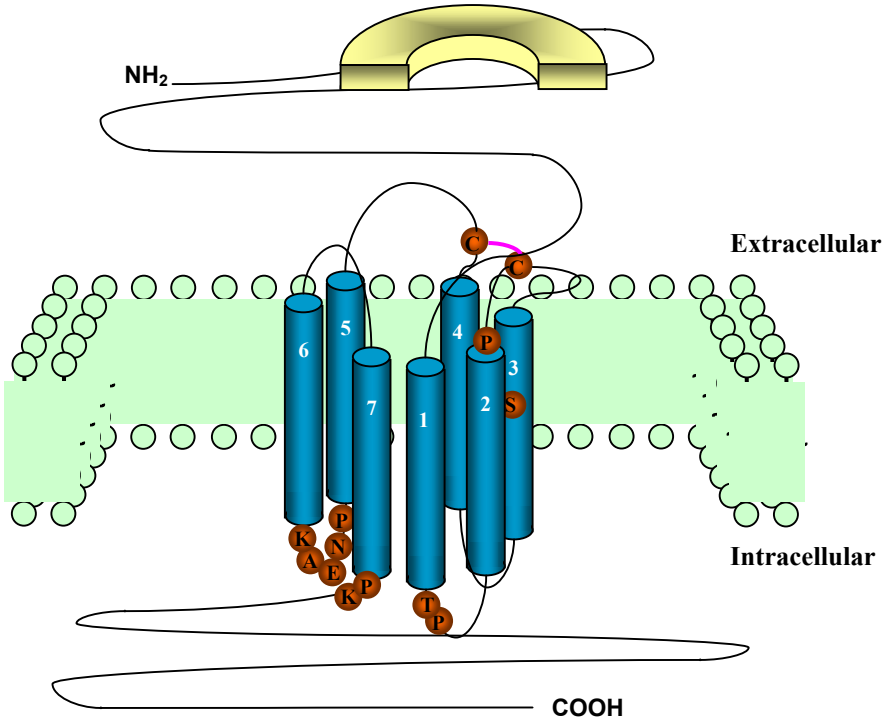


Figure 1.2a – **Representation of Family C (or class III) GPCRs** with transmembrane domains labelled 1-7 N-C terminal, and conserved amino acids found in this GPCR family shown in red. The pink line denotes a disulphide bridge. The yellow arch indicates the glutamate binding domain. Adpated from (Gether, 2000; George et al, 2004).

Tateyama, 2005). This domain is thought to be able to open and close with the agonist bound within. Other than the cysteines in ECL1 and ECL2 forming a putative disulphide bridge, family C do not have any features in common with family A or B but do have a number of their own conserved amino-acids (**fig 1.2a** - shown in red). A feature specific to family C is a short and highly conserved third intracellular loop

(ICL). As with family B the orientation of the TM domains is not known. As well as the metabotropic glutamate receptor this group also includes the Ca^{2+} -sensing and $GABA_B$ γ-aminobutyric acid, type B receptors ($GABA_B$)

1.2.3 Family B – or Secretin-like family

Family B, the parathyroid hormone/calcitonin/secretin receptor family, are characterised by a relatively long N-terminal domain containing several cysteines which form a network of disulphide bridges (potentially within the receptor or with adjacent receptors). This family lacks the palmitoylation site commonly seen in family A and contains conserved amino acids specific to this group (**fig 1.2b** - shown in red). Due to the divergence of the amino-acid sequence it is thought unlikely that the transmembrane domains have a similar orientation to family A (Gether, 2000) and little is known about their actual orientation.

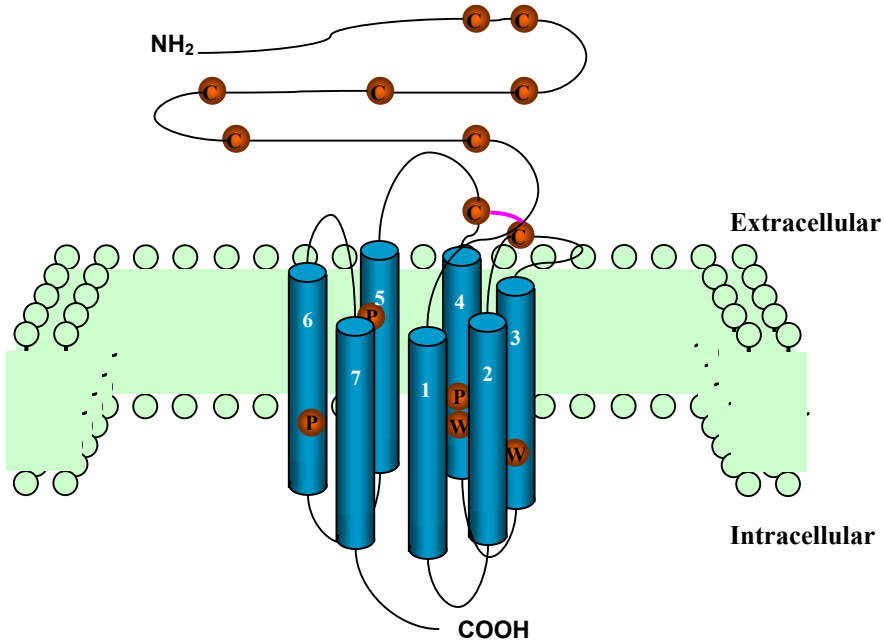


Figure 1.2b – Representation of Family B (or class II)) GPCRs with transmembrane domains labelled 1-7 N-C terminal, and conserved amino acids found in this GPCR family shown in red. The pink line denotes a disulphide bridge. Adpated from (Gether, 2000; George et al, 2004).

1.2.4 Family A – or Rhodopsin-like family.

This is by far the largest subgroup and contains receptors for various neuropeptides, neurotransmitters (such as dopamine and serotonin) and glycoprotein hormones. This receptor family is typified by the rhodopsin and the β2-adrenergic receptor and is characterised by several highly conserved amino acids (**fig 1.2c** - shown in red) and a disulphide bridge that connects the first and second extracellular loops (ECL). Many of these receptors also have potentially palmitoylated cysteine residue(s)

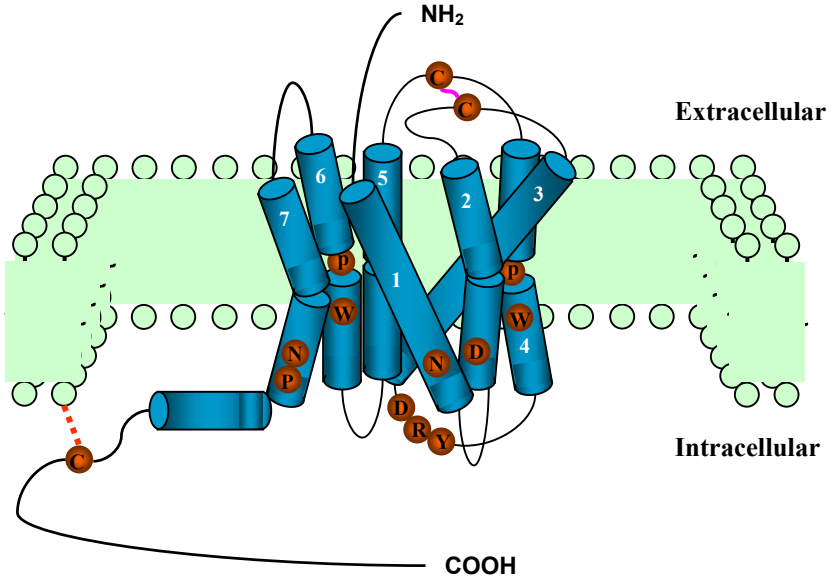


Figure 1.2c – Representation of Family A (or class I)) GPCRs with transmembrane domains labelled 1-7 N-C terminal, and conserved amino acids found in this GPCR family shown in red. The pink line denotes a disulphide bridge. The red dotted line indicates palmitoylation Adpated from (Gether, 2000; George et al, 2004).

(**fig 1.2c** - shown by a dotted red line) in the carboxyl-terminal tail creating an anchor to the membrane and a fourth intracellular loop. The determination of the x-ray crystal structure of rhodopsin (Palczewski et al, 2000) indicates tilting or "kinks" in the transmembrane domains (as shown) caused by the presence of amino-acids such as proline that distort the transmembrane domains helical structure. Family A can be further subdivided into six major subgroups (Kolakowski, 1994; Gether, 2000) as shown in **table 1.2.4a**.:

Table 1.2.4a: Table showing a list of G-protein coupling receptor class A sub-categories	
i. Olfactory, Adenosine, cannabinoid, and melanocortin receptors.	**ii.** Biogenic amine receptors (adrenergic, serotonin, dopamine, muscarinic, histamine)
iii. CCK, endothelin, tachykinin, neuropeptides Y, TRH, nerurotensin, bombesin, and growth hormone secretagogues receptors plus vertebrate opsins .	**iv.** Invertebrate opsins and Bradykinin receptors.
v. Paracrine/autocrine receptors. Chemokine, fMLP, C5A, GnRH, eicosanoid, leukotriene, FSH, LH, TSH, galanin, nucleotide, opioid, oxytocin, vasopressin, somatostatin, and **proteinase-activated receptors** .	**vi.** Melatonin receptors and other non-classified

1.3 PROTEINASE-ACTIVATED RECEPTORS (PAR)

1.3.1 The PAR family members

The proteinase-activated receptors (PARs) are a unique class of GPCR. They are a member of GPCR family A (or rhodopsin-like family) and are much like other receptors in this family. PARs have seven transmembrane domains, three intracellular loops and three extracellular loops, an extracellular N terminus and an intracellular C-terminal tail (see **Fig 1.3.1a**, for a more accurate representation of transmembrane domain orientation see **Fig 1.2c**)(Macfarlane et al, 2001; Hollenberg & Compton, 2002). So far four members of this family have been identified (Vu et al, 1991a; Bohm et al, 1996b; Nystedt et al, 1996; Ishihara et al, 1997; Kahn et al, 1998; Xu et al, 1998), three receptors for thrombin (PAR_1, PAR_3, and PAR_4) and one receptor for trypsin and mast cell tryptase (PAR_2). All four are activated by proteolytic cleavage and several proteases have been found that will activate each receptor.

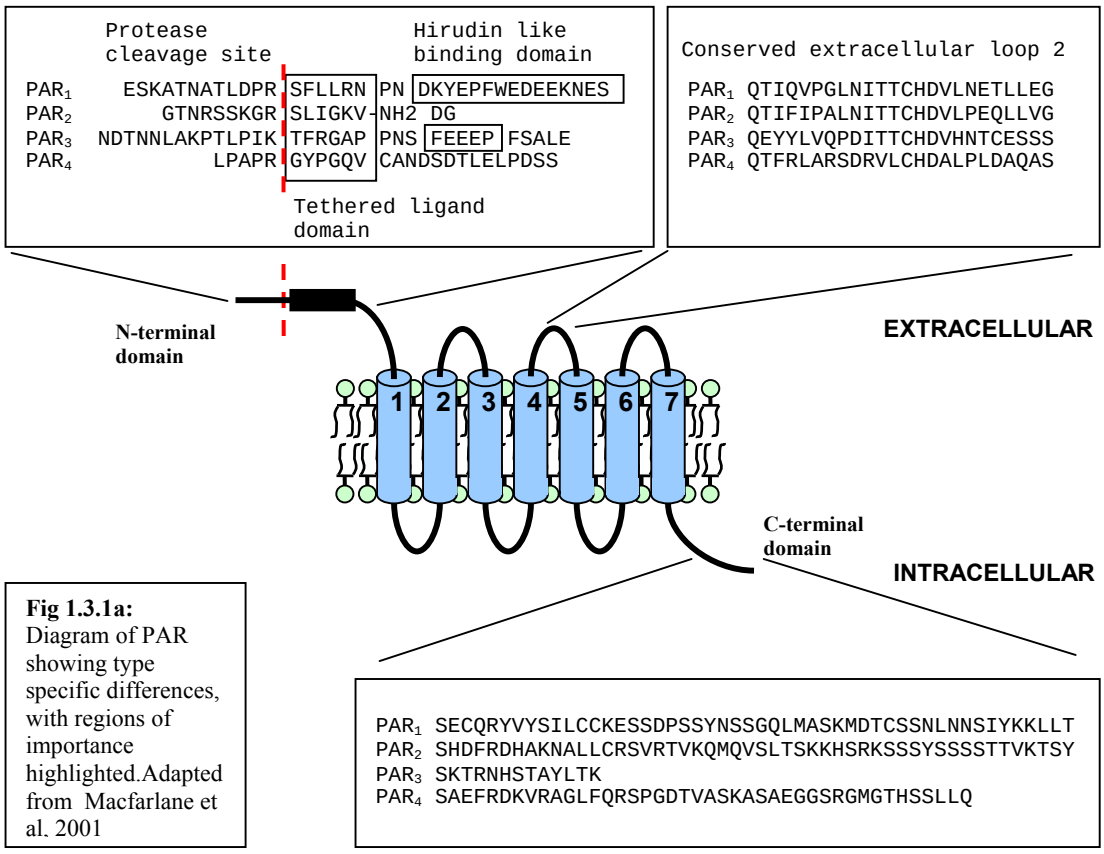


Fig 1.3.1a: Diagram of PAR showing type specific differences, with regions of importance highlighted.Adapted from Macfarlane et al, 2001

1.3.2 Mechanism of Activation

Unlike other GPCRs, PARs have a distinct mode of activation. In the presence of an activating protease cleavage of the N terminus at a specific point exposes a new N terminal region that serves as a "tethered ligand" (TL) domain which subsequently binds to a conserved region in the second extracellular loop of the cleaved receptor, so activating it, and leading to signal transduction (**fig 1.3.2a**). The resulting N-terminal fragment of the receptor has no known function (Macfarlane et al, 2001; Hollenberg & Compton, 2002; Ossovskaya & Bunnett, 2004). It is also possible to activate three of the receptors independent of proteolysis using small peptides, of between five and fourteen amino-acids, analogous in sequence to the tethered ligand (**fig 1.3.2a**) (Vu et al, 1991a; Nystedt et al, 1995a). These PAR activating peptides (or PAR-APs) are available for PAR_1, PAR_2 and PAR_4,(Vu et al, 1991a; Nystedt et al, 1994) and have some cross-reactivity between receptors and species (Blackhart et al, 1996; Hollenberg et al, 1997); as yet no specific activating peptide for PAR_3 has been generated (Ishihara et al, 1997), with the generated sequence activating PAR_1 and PAR_2 instead of PAR_3 (Hansen et al, 2004; Kaufmann et al, 2005).

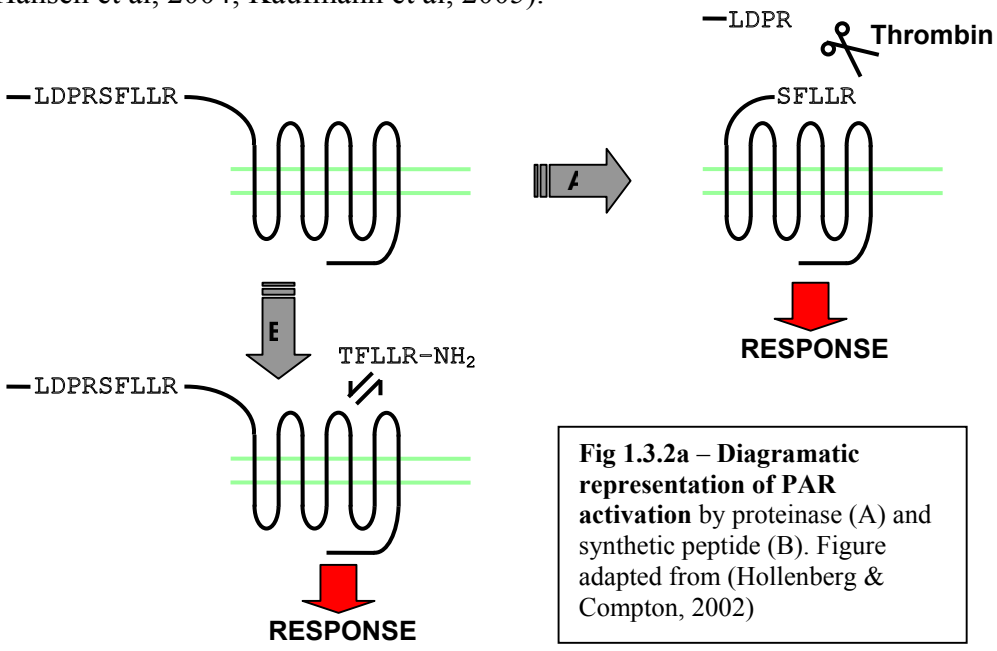


Fig 1.3.2a – Diagramatic representation of PAR activation by proteinase (A) and synthetic peptide (B). Figure adapted from (Hollenberg & Compton, 2002)

1.3.3 PAR signalling pathways

Other than in a research lab environment cells don't exist within isolation and will receive and respond to signals from their surroundings. Even the simplest of bacteria can sense and move towards high concentrations of nutrients. In more complex multicellular organisms survival depends on an elaborate intercellular communication network that control and co-ordinate processes such as cell growth and differentiation. The three largest classes of cell-surface receptor proteins are ion-channel-linked, g-protein-linked, and enzyme-linked receptors. I have outlined below two cell signalling pathways which have been used in this project in order to characterise receptor pharmacology and assess effects of mutations on receptor function.

1.3.3.1 Second Messenger Signalling through Ca2+ mobilisation

Calcium mobilisation is a second messenger signalling system in which calcium ions (Ca^{2+}) act as signal transduction molecules which mediate physiological processes intracellularly. It belongs to a group of pathways that use *phosphatidylinositol* (PI) often referred to as inositol lipid pathways. The signalling cascade (**fig 1.3.3.1a**) can be initiated by receptor tyrosine kinases (RTKs), or by the activation of a GPCR which in turn activates its subsequent G-protein. G-proteins consist of an α, and a $\beta\gamma$ sub-unit which separate on activation following a conversion of GDP-GTP by the α sub-unit. Inositol 1,4,5-tri-phosphate (IP_3) is then liberated from membrane bound phosphatidylinositol 4,5-biphosphate (PIP_2) by phospholipase C subsequently producing another second messenger 1,2-diacylglycerol (DAG). DAG being a lipophilic molecule remains associated with the cell membrane leaving the IP_3 to diffuse into the cytosol. Most intracellular Ca^{2+} ions are located in the mitochondria and endoplasmic reticulum (ER). Upon diffusing into the cytosol IP_3 binds to Ca^{2+}-channel protein on the

surface of the ER. The channel proteins are composed of four identical sub-units, each of which has a cytosolic N-terminal with an IP_3 binding domain and IP_3 binding results in an opening of the channel releasing ER Ca^{2+} stores into the cytosol. A small rise in cytosolic Ca^{2+} induces a variety of cellular responses, but the resulting rise in cytosolic Ca^{2+} concentration is only transient since after it's generation IP_3 is rapidly hydrolyzed to inositol 1,4- bisphosphate, which does not stimulate Ca^{2+} release from the ER (Lodish et al, 1999). The principal function of DAG is to activate a family of protein kinases located within the plasma membrane collectively referred to as protein kinase C (PKC). Whilst inactive PKC is present in the cytosolic compartment and binds to the cytoplasmic leaflet of the plasma membrane following a rise in cytosolic Ca^{2+} ions, leaving it ideally located for activation by DAG (Lodish et al, 1999).

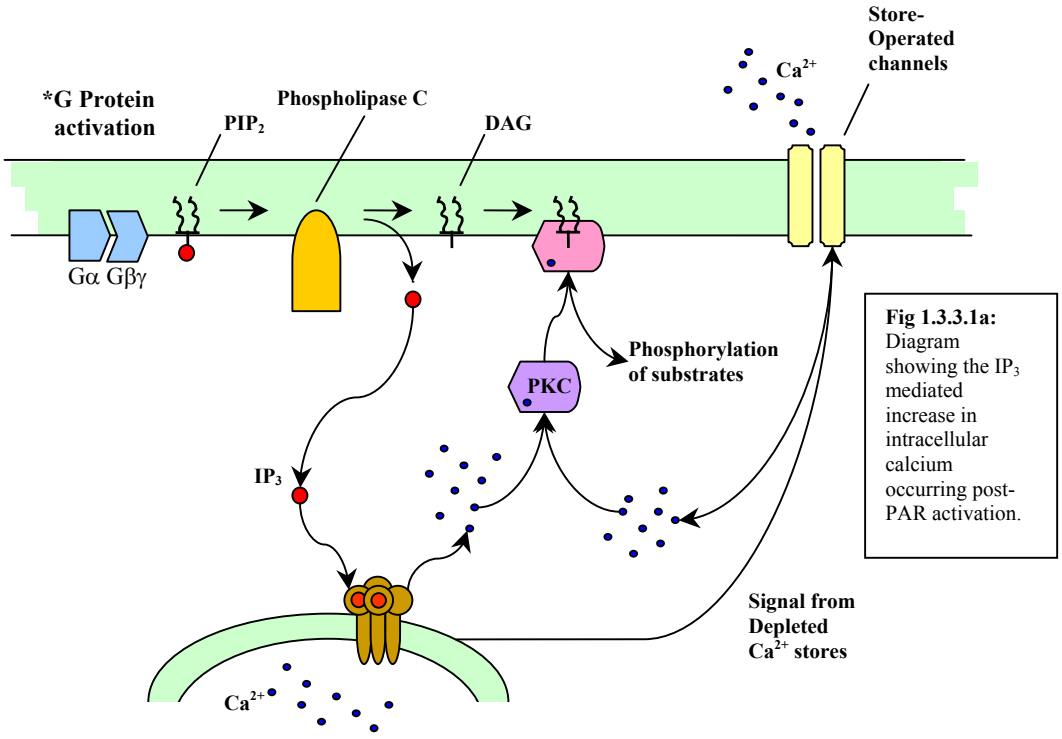


Fig 1.3.3.1a: Diagram showing the IP_3 mediated increase in intracellular calcium occurring post-PAR activation.

1.3.3.2 Mitogen-Activated Protein Kinase (MAPK) Signalling

Mitogen-activated protein kinases (MAP Kinases) are serine/threonine kinases that participate in intracellular signal transduction pathways. MAP kinases are components of protein kinase cascades which, following activation from extracellular stimuli (mitogens), regulate various cellular activities such as gene expression, mitosis, differentiation, and cell survival/apoptosis (Pearson et al, 2001).

The regulation of MAP kinases occurs through phosphorylation cascades, the upstream sequential activation of two (or more) protein kinases leads to the activation of a downstream MAP kinase (**fig 1.3.3.2a**) (Pearson et al, 2001)

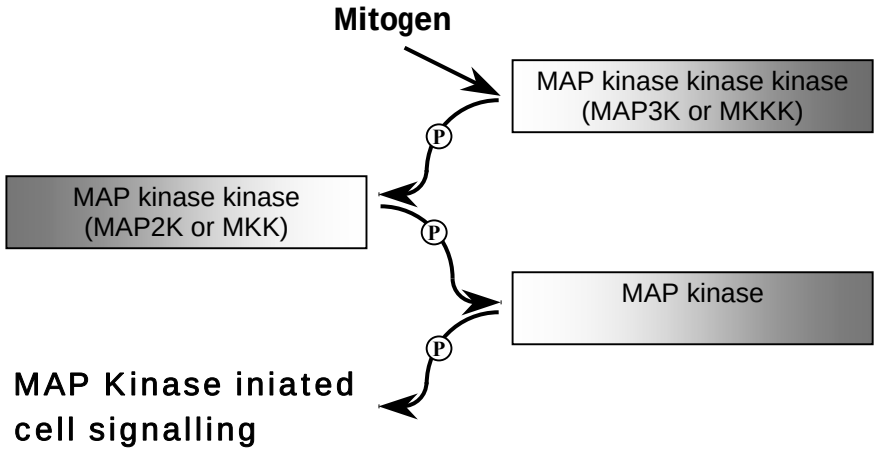


fig 1.3.3.2a – Diagram to demonstrate atypical MAP Kinase phosphorylation cascade. Addition of phosphate (and therefore kinase activation) is indicated by Ⓟ

MAP kinases phosphorylation cascade allows for input at multiple points from other signalling pathways providing a control mechanism to enhance or suppress MAP kinase function (Schonwasser et al, 1998; Corbit et al, 1999). Additionally these cascade intermediates allow for signal amplification by each kinase activating multiple downstream kinases and the successive downstream kinase being more abdundant that its upstream activator.

So far, in mammals, four distinct MAP kinase groups have been identified:-

1. **Extracellular signal-regulated kinases (ERKs)**, these are also known as the classical MAP kinases. ERKs are preferentially activated by growth factors and phorbol ester (a tumour promoter). They primarily regulate cell proliferation and differentiation.

2. c-Jun N-terminal kinases (JNKs), also known as stress-activated protein kinases (SAPK), are activated by stress stimuli such as cytokines, ultraviolet radiation, osmotic shock and heat shock. Their activation can result in cell differentiation and apoptosis.

3. p38 isoforms. Similar to JNK the p38 MAP kinases are responsive to stress stimuli, and signalling through p38 is linked to cell differentiation and apoptosis.

4. ERK5, this is the most recently described MAP kinase and is activated by both growth factors and stress stimuli. Signalling through ERK5 participates in cell proliferation.

1.3.4 TERMINATION OF SIGNALLING IN PARS

Due to the unique way in which PARs are activated the resulting "tethered" ligand remains bound to the receptor and might therefore be expected to produce a continuous signal. The absence of an easily dissociated agonist, present with classical GPCRs, raises the possibility of sustained activation once the receptor has been cleaved by its proteinase. However, this doesn't appear to be the case, the responses observed following PAR activation are short-lived thanks to efficient mechanisms of signal termination (Macfarlane et al, 2001; Hollenberg & Compton, 2002; Trejo, 2003). Termination of signalling in GPCRs classically occurs following an agonist-induced conformational change allowing dissociation from its heterotrimeric G protein (for a historical review see Lefkowitz, 2004. The receptor specific Gα sub-type then signals through its discrete second messenger pathway. Termination of the signalling response is then initiated by members of a protein family called G protein-coupled receptor

kinases (GRKs) (Krupnick & Benovic, 1998; Moore et al, 2007) and/or Protein Kinase C (PKC) depending on determinant phosphorylation sites normally appearing on the C-terminal tail (Krupnick & Benovic, 1998). The GRKs or PKCs bind to, and rapidly phosphorylate the receptor typically within the C-terminal domain (Krupnick & Benovic, 1998). The phosphorylated receptor then attracts β-arrestin binding resulting in uncoupling from the G-protein and blocking of further G protein-initiated signalling through steric hindrance (Lohse et al, 1990; Attramadal et al, 1992).

Since PARs are irreversibly activated the magnitude and duration of the proteinases response is primarily determined by the mechanisms present for signal termination. Studies on PARs (primarily on PAR_1 and to a lesser extent PAR_2) have detailed sequences within the receptor intracellular domains and C-terminal tail, responsible for G protein-coupling, which regulate receptor desensitisation and internalisation, please see specific PAR chapters for information related to each PAR.

1.3.5 PAR DISARMING

In addition to the proteolytic activation of PARs by their respective activating proteinases, other proteinases have been shown to cleave the N-terminus of PARs at sites not resulting in receptor activation. When this cleavage occurs C-terminally of the TL sequence an "amputation" or disarming of the receptor occurs leaving the receptor insensitive to its activating proteinase (**fig 1.3.5a**). This mechanism offers an additional means of receptor desensitisation. Although the receptor can no longer be activated by cleavage with a proteinase, the receptor can still be activated by using the receptor specific activating peptide (Parry et al, 1996).

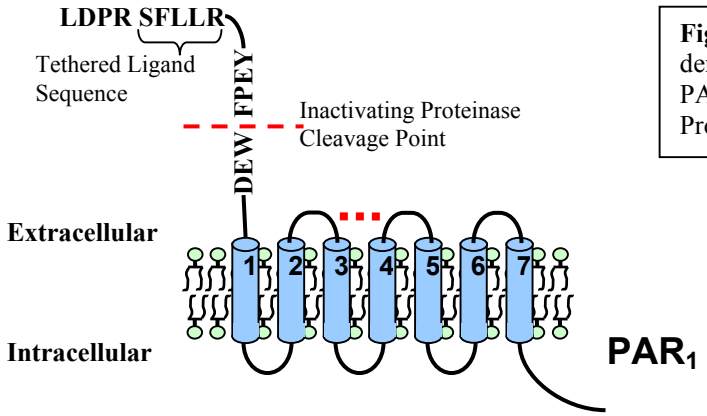


Fig 1.3.5a: Diagram demonstrating disarming of PAR_1 by an inactivating Proteinase.

Disarming or amputation of the tethered ligand of PAR_1 resulting in abolishment of signalling in response to thrombin has been shown following pre-incubation with neutrophil proteinases such as cathepsin G (Molino et al, 1997; Ramachandran et al, 2007), elastase, and proteinase 3 (Renesto et al, 1997). A mast cell proteinase chymase was also shown to desensitise keratinocytes to thrombin induced signalling (Schechter et al, 1998).

A more complicated picture can be seen for disarming with trypsin and plasmin. Trypsin has been reported to leave epithelial cells unresponsive to thrombin (Nakayama et al, 2003; Nakayama et al, 2004). However since trypsin cleaves at three different points in the PAR_1 N-terminus (41-42, and preferentially 70-71 and 82-83) (Nakayama et al, 2004) at low concentrations (< 40 nM) it will disarm PAR_1, whereas higher concentrations will activate the receptor (Kawabata et al, 1999).

Plasmin is observed to activate platelets at only high concentrations (more than 0.2 μM), whereas lower concentrations result in a dose- and time-dependent inhibition of platelet aggregation in response to thrombin (Parry et al, 1996). A later study identified this to be due to a similar mechanism as seen with trypsin, where multiple plasmin cleavage sites exist (Kuliopulos et al, 1999).

Elastase and cathepsin G have both been shown to disarm PAR_2 in both transfected cells and bronchial fibroblasts (Dulon et al, 2003; Ramachandran et al, 2007). The authors showed that the elastase and cathepsin-G treated cells were unresponsive to trypsin but normally responsive to PAR_2-AP, implying proteolytic removal of the tethered ligand (Dulon et al, 2003; Ramachandran et al, 2007). Mast cell tryptase, although an endogenous activator of PAR_2, can also cleave PAR_2 at Lys^{41}-Val^{42} site, which could through amputation of the tethered ligand inactivate PAR_2 (Molino et al, 1997; Ramachandran et al, 2007). However, there is significant evidence in the literature that tryptase preferentially activates PAR_2 (Macfarlane et al, 2001; Hollenberg & Compton, 2002; Hollenberg, 2003; Ossovskaya & Bunnett, 2004). Additionally, cathepsin G has been shown to attenuate thrombin mediated signalling for PAR_3 in transfected cells (Cumashi et al, 2001). The proteolytic disarming of PAR_4 has not been demonstrated.

The fact that a number of different proteinases exist which selectively activate one PAR, whilst selectively disarming another; or that will activate/disarm the same receptor depending on the proteinase concentration adds an interesting and multifaceted control mechanism to PAR related signalling. An example of this is the ability of cathepsin G to simultaneously disarm PAR_1 and activate PAR_4 on human platelets (Sambrano et al, 2000), whereas on cells not expressing PAR_4 such as fibroblasts or endothelial cells simply disarming of PAR_1 would occur (Ossovskaya & Bunnett, 2004; Ramachandran et al, 2007). An example of how this might occur following an inflammatory response is seen with human primary bronchial fibroblasts (HPBF) which upregulate PAR_2 and PAR_4 mRNA expression in response to TNFα and LPS treatment (Ramachandran et al, 2007). Since HPBF usually only express PAR_1, PAR_2 and PAR_3, this allows signalling

through PAR_4 which was not previously possible. Giving the cells the ability to signal with thrombin through both PAR_1 and PAR_4, or signal with cathepsin G through PAR_4 whilst simultaneously disarming PAR_1 and PAR_2 (Ramachandran et al, 2007).
The physiological and pathophysiological processes resultant of proteinase mediated signalling events are therefore a product of that proteinases effect on all the PARs expressed in the tissue environment where the proteinase is present.

1.3.6 RECYCLING OF PARS

Many receptors internalise post-agonist binding, however the eventual fate of the internalised receptor depends on post-endocytic sorting and varies from receptor to receptor (Defea et al, 2000a). Unlike neuropeptide receptors, PARs are not recycled back to the cell membrane following agonist dissociation, rather they depend on the restoration of signalling potential from receptor stores (Trejo & Coughlin, 1999; Defea et al, 2000a). The method of recovery and recycling in the case of PAR receptor signal is dependant on the cell type involved. In the case of megakaryoblastic cells such as platelets, resensitisation of cells post-signalling requires the production of new receptors (Brass et al, 1991). This is not the case in all cells. Most cell types maintain a constant cycling of unactivated receptor (investigated with PAR_1) between the cell surface and intracellular stores (Hein et al, 1994). This "tonic cycling" ensures a pool of uncleaved receptor is present and available in order to rapidly resensitise the cell following a thrombin challenge (Hein et al, 1994). Additionally, approximately one quarter of activated and internalised PAR_1 receptors will then recycle back to the cell surface (Hoxie et al, 1993). These receptors were not self-activating and no longer responded to thrombin challenges, but were capable of being activated by PAR_1-AP (Hoxie et al, 1993). Studies focusing on the recycling of PAR_2 have shown intracellular pools of

receptor within the golgi bodies as well as other superficial vesicles (Bohm et al, 1996a). This is presumably to able a faster restoration of cell surface receptor and therefore resensitisation of response. The C-terminus of PAR_1 appears to be the essential determinant in post-endocytic lysosomal sorting (Trejo et al, 1998). Receptor chimeras produced from domain swapping experiments show PAR_1 when engineered with a substance P cytoplasmic tail recycles readily back to the surface (Trejo et al, 1998), where as a substance P receptor with a PAR_1 cytoplasmic domain is directed to lysosomes (Trejo & Coughlin, 1999). A recent study made a surprising observation that in mouse endothelial fibroblasts (MEF) from β-arrestin knockout mice, expression of β-arrestin 1 alone enhanced restoration of surface PAR_2 after signalling (Kumar et al, 2007). This enhanced resensitisation was sensitive to brefeldin A, a golgi-disrupting drug, suggesting that β-arrestin 1 not only mediates early endocytosis and degradation of PAR_2 but also facilitates PAR_2 trafficking from golgi stores to the plasma membrane following agonist restoration (Kumar et al, 2007).

1.3.7. PAR1

1.3.7.1 The Cloning of PAR_1

PAR_1 was first cloned in 1991 by two independent groups (Rasmussen et al, 1991; Vu et al, 1991a). Originally known as the thrombin receptor, the search for its discovery was instigated by the observation that thrombin mediates cellular responses that are distinct from it's role in the blood clotting cascade (Weksler et al, 1978; Bar-Shavit et al, 1983a; Bar-Shavit et al, 1983b; Babich et al, 1990). A group at the University of California used *Xenopus* oocytes to express mRNA they had isolated from human thrombin responsive megakaryotic HEL and Dami cells. A functional clone was then identified using a dilution cloning approach (Vu et al, 1991a). The other group located

the hamster thrombin receptor within CCL39 hamster lung fibroblasts by amplifying mRNA sequences that showed GPCR transmembrane domain homology (Rasmussen et al, 1991). Located on 5q13 in humans (Bahou et al, 1993b), and 13D2 in the mouse genome (Kahn et al, 1996), the PAR_1 gene encodes a 425aa and 430aa protein respectively, has a 5' untranslated domain (1-224) and a long 3' untranslated region ending in a poly-A tail (Vu et al, 1991a). The receptor was further shown to have characteristics intrinsic to GPCRs i.e. eight hydrophobic domains, seven transmembrane spanning domains and an amino-terminal signal sequence (Vu et al, 1991a). A putative thrombin cleavage site ($LDPR^{41}$↓SFLLRN, where ↓ represents the cleavage site) was shown to be located within the N-terminus. Additionally a high-affinity region for the thrombin's anion-binding exosite was identified, downstream of the thrombin cleavage site (**Fig 1.3a**) (Vu et al, 1991b).

1.3.7.2 Activation of PAR_1

The PAR mechanism of activation was first described for PAR_1 and later extended and investigated for other PARs. The PAR_1 N-terminal domain contains a thrombin recognition and cleavage sequence ($LDPR^{41}$↓SFLLRN) (Vu et al, 1991a) immediately downstream of which is an additional acidic region with high thrombin binding affinity. This region shows remarkable similarity with the leech derived thrombin binding polypeptide hirudin (D^{51}KYEPFWEDEE) (Vu et al, 1991a) which effectively binds the thrombin anion binding exosite, so transiently concentrating thrombin near the receptor activation domain (Vu et al, 1991a). In order to investigate the importance of LDPR as a cleavage recognition sequence, domain swapping was carried out replacing LDPR with DDDK (the cleavage recognition site for the protease enterokinase). The results showed abolition of the thrombin response and activation with enterokinase (Vu et al, 1991b).

The importance of the recognition sequence was further validated using blocking antibodies against the cleavage site, again abolishing thrombin activation (Bahou et al, 1993a; Chen et al, 1994). They also mutated the arginine residue to an alanine which resulted in a receptor that was not responsive to thrombin but still activated with peptide. Removal of the PAR_1 hirudin-like binding domain (residue 51-53) resulted in a 100-fold reduction in thrombin activation efficiency (Vu et al, 1991b). Additionally γ-thrombin, which lacks the anion exosite required for binding to the hirudin-like binding domain is significantly less potent (100-fold) than thrombin in activating PAR_1 (Bouton et al, 1995). Replacing the PAR_1 hirudin-like binding domain with the authentic hirudin thrombin binding domain resulted in activation curves indistinguishable from that of wild-type receptor (Vu et al, 1991b). This site is essential for high affinity binding and potency of thrombin towards PAR_1, reducing the kinetic barrier to thrombin/receptor formation so increasing the potency of thrombin in activating PAR_1. It was therefore proposed that thrombin interacts with PAR_1 through the recognition site LDPR/S and through the anion-binding exosite KYEPFWEDEE.

1.3.7.2.1 Domains Important for Activation

The domain involved in interacting with the tethered ligand (TL) to result in receptor signalling was investigated using domain swapping between *xenopus* PAR_1 and $hPAR_1$ (Gerszten et al, 1994). These showed extracellular loop-2 (ECL2) to play a critical role in conferring TL binding specificity; replacement of the extracellular N-terminus and ECL2 of the *xenopus* receptor with the corresponding domains of human PAR_1 confers specificity to the human tethered ligand (Gerszten et al, 1994; Lerner et al, 1996). Further experiments showed the substitution of just two residues of x*enopus* PAR_1 (Asn^{87} in the N-terminus and Leu^{260} in ECL2) with the analogous residues in human

PAR_1 (Phe^{87} and Glu^{260} respectively) leaves the chimera selective for the human peptide sequence (Gerszten et al, 1994; Lerner et al, 1996). Subsequent studies using mutations in the $hPAR_1$ sequence and PAR_1-AP analogs demonstrated that the Glu^{260} of ECL2 interacts with Arg^5 of the TL SFLLRN (Nanevicz et al, 1995). Interestingly, substitution of eight residues in the $hPAR_1$ ECL2 with their equivalents from the *xenopus* receptor resulted in a constitutively active receptor in the absence of ligand (Nanevicz et al, 1996). This suggests that an alteration in the conformation of the ECL2 of PAR_1 is sufficient to results in signal transduction. The different approach of generating specific monoclonal antibodies targeted against sequences in the N-terminus of PAR_1 provided data identifying Q^{83} to G^{94} as important for binding of the TL (Bahou et al, 1994).

1.3.7.2.2 PAR_1 Activating Proteinases

In oocytes expressing the receptor, thrombin was found to be an extremely potent agonist (~50 pM, (Vu et al, 1991b)). Although trypsin was also found to activate the receptor, it was considerably less effective (Vouret-Craviari et al, 1995). In addition to zymogen activation FVIIa and FXa are capable of signalling through PAR_1 (Camerer et al, 2000; Riewald & Ruf, 2001; Camerer et al, 2002). Although FVIIa signals to *xenopus* oocytes expressing PAR_1, when PAR_1 is co-expressed with tissue factor (TF) FVIIa results in robust signalling (Camerer et al, 2000; Riewald & Ruf, 2001). Similarly, FXa only weakly activates PAR_1, however when allosterically associated in a complex comprising TF-FVIIa-FXa it potently activates PAR_1 (Camerer et al, 2000; Riewald & Ruf, 2001). The TF-FVIIa-FXa complex can cleave and activate PAR_1 in a number of cell types including endothelial cells (Camerer et al, 2000; Riewald & Ruf, 2001). Granzyme A has been capable of cleaving PAR_1 fragments at the activation site, and granzyme A mediated signalling in astrocytes and neurons is ablated with the use of

PAR_1 blocking antibodies (Suidan et al, 1994), thus showing granzyme A to activate PAR_1. The anti-coagulant protein-C (APC) is also capable of activating PAR_1 but only when it is associated with the endothelial protein C receptor (EPCR) which acts as an anchoring protein (Riewald et al, 2002; Riewald & Ruf, 2005). A number of non-mammalian proteases have also proved able to activate PAR_1. The bacterial arginine-specific gingipains-R (RgpB and HRgpA), which are major mediators of periodontitis in humans, can activate PAR_1 in transfected cells (Lourbakos et al, 1998; Lourbakos et al, 2001a; Lourbakos et al, 2001b).

1.3.7.2.3 PAR_1 Peptide Agonists/Antagonists

Activating peptides for PAR_1, known as TRAPs (Thrombin Receptor Activating Peptide), range from 5-14 amino-acids in length corresponding to the post-cleavage amino-terminus. The activating peptide for human PAR_1 was shown to be SFLLRN, but this peptide displayed cross-reactivity with $hPAR_2$ (Blackhart et al, 1996). Substitution of first serine residue with a threonine, similar to the *xenopus* TL, giving the AP TFLLRN-NH_2 made it highly specific to PAR_1 (Hollenberg et al, 1997). Alanine scanning of SFLLRN along with site-directed mutagenesis of the tethered ligand indicates that the critical residues within this domain are Phe^2, Leu^4, and Arg^5. Other substitutions within the sequence can be tolerated (depending on the residue) (Scarborough et al, 1992). Further advances have lead to the production of penta- and tetra-peptides with higher potencies e.g. H-Ala-(pF)Phe-Arg-Cha-hARg-Tyr-NH_2 has an EC_{50} of just 10 nM in inducing platelet aggregation compared to 10 μM for SFLLRN (Feng et al, 1995).

Analogs of PAR_1-APs have been used as templates for the production of possible receptor antagonists (Andrade-Gordon et al, 1999). Several small molecule antagonists

have been produced that are devoid of PAR_1 agonist activity. They bind to PAR_1 and interfere with PAR_1 calcium mobilization and cellular function (platelet aggregation; cell proliferation), and have no effect on PAR_2, PAR_3, or PAR_4. RWJ56110[1] (Andrade-Gordon et al, 1999), RWJ-58259[2] (Andrade-Gordon et al, 2001; Damiano et al, 2003) significantly inhibits PAR_1 signalling and thrombin induced platelet aggregation (Damiano et al, 2003). Preclinical results for RWJ-58259 suggest a potential clinical utility in treating thrombosis and restenosis in humans as well as a useful tool in future PAR research.

1.3.7.3 PAR_1 Signalling

A considerable amount of work has been done to investigate the signalling of PAR_1, considerably more than that of the other three PARs. PAR_1 can couple to multiple heterotrimeric G-proteins. Interactions have been described for Gi, Gq/11 and G12/13 (Macfarlane et al, 2001; Steinhoff et al, 2005) but our knowledge is far from complete.

Two signalling events were identified early on that were associated with G-protein coupling. The first event to be characterised involves interactions with inhibitory G-protein of the G_i class resulting in the inhibition adenyl cyclase and subsequent inhibition of cAMP production in multiple cell types such as osteosarcoma cells (Babich et al, 1990), HEL cells (Brass et al, 1991), fibroblasts (Hung et al, 1992a), vascular smooth muscle (Kanthou et al, 1996) and more recently in astrocytes (Wang et al, 2002b), platelets (Kim et al, 2002), endothelial cells (Vanhauwe et al, 2002) and olfactory sensory neurons (Olianas et al, 2007). The second event to be characterised

[1] (αS)-*N*-([1*S*]-3-amino-1-[((phenylmethyl)amino)propyl]-α-[((1-[(2,6-dichlorophenyl)methyl]-3-[1*H*-indol-6-yl)amino)carbonyl]amino)-3,4-difluoro-benzenepropanamide.
[2] (αS)-*N*-([1*S*]-3-amino-1-[[((phenylmethyl)amino)carbonyl]propyl]-α-[((1-[(2,6-dichlorophenyl)methyl]-3-(1-pyrro lidinylmethyl)-1*H*-indazol-6-yl]amino]carbonyl]amino]-3,4-difluorobenzenepr opanamide)

was the stimulation of phospholipase C (PLC) in catalysing the hydrolysis of polyphosphoinositides to form IP_3, mobilise intracellular calcium, and generate diacylglycerol (DAG) which is the endogenous activator of protein kinase C (**fig 1.3.3.1a**) (Babich et al, 1990; Hung et al, 1992b). Subsequent studies displayed coupling to $G_{q/11}$ with the use of G-protein specific antibodies inhibiting PAR_1 mediated signalling in fibroblasts (Baffy et al, 1994) and through co-immunoprecipitation of $G_{q/11}$ along with PAR_1 (Ogino et al, 1996). Further interactions of PAR_1 with $G_{q/11}$ have been more recently reported in astrocytes (Wang et al, 2002b) and olfactory sensory neurons (Olianas et al, 2007). This combined with further reported signalling via $G_{12/13}$ in thrombin stimulated platelets (Offermanns et al, 1994) and via G_{12} in astrocytoma cells (Aragay et al, 1995; Post et al, 1996) show PAR_1 is capable of initiating an extensive range of downstream signalling pathways including; ERK1/2 MAP kinase via activation of c-Src (Sabri et al, 2000; Vouret-Craviari et al, 2003), JNK and PI-3 kinase (Malarkey et al, 1995).

1.3.7.4 PAR_1 Desensitisation

Desensitisation is an overlapping event involving several processes, including phosphorylation, G protein uncoupling, internalisation (or sequestration), and down-regulation. However, due to their very nature the initial stage of PAR receptor desensitisation involves its activation. The irreversible proteolytic cleavage, which reveals the receptors tethered ligand, removes the possibility of that receptor being activated in the future.

Upon activation of PAR_1 rapid phosphorylation of the C-terminus occurs (Ishii et al, 1994). This phosphorylation is thought to be mediated by GRKs since over expression of GRK3 and GRK5 alongside PAR_1 results in enhanced receptor phosphorylation and

an associated inhibition of calcium signalling (Ishii et al, 1994). Although the involvement of GRKs in the signalling of other PARs remains unknown, additional evidence for GRK3 and 5s importance to PAR_1 signalling can be seen in other studies (Iaccarino et al, 1998; Tiruppathi et al, 2000). Iaccarino *et al* showed GRK3s regulation of thrombin within *in vivo* studies on transgenic mice over-expressing myocardial GRK3, and Tiruppathi *et al* showed GRK5 expression in endothelial cells mediating PAR_1 desensitisation. The next step in desensitisation involves β-arrestins interacting with the newly GRK phosphorylated receptor, so disrupting the PAR/G-protein interaction and terminating receptor signalling. Studies using embryonic fibroblasts from β-arrestin knockout mice highlight the importance of β-arrestin in the termination of PAR_1 signalling, but showed it wasn't important for receptor internalisation (Paing et al, 2002). A more recent study co-expressing PAR_1 with β-arrestin isoforms 1 and 2 in COS7 cells further confirmed β-arrestins regulation of PAR_1 desensitisation, but consequently identified β-arrestin 1 (βarr1) to the preferred isoform involved (Chen et al, 2004). Subsequent co-immunoprecipitation of PAR_1 with βarr1 confirmed this isoform preference. Additionally this study was able to show β-arrestin binding and related signalling inhibition independent of C-terminal phosphorylation using PAR_1 C-terminal mutants (Chen et al, 2004).

1.3.7.5 PAR_1 Internalisation

Internalisation of PAR_1 has been shown to occur through a dynamin- and clathrin-dependant pathway (Hoxie et al, 1993; Paing et al, 2002; Trejo, 2003) confirmed by disruption or inhibition of this pathway affecting receptor endocytosis. Although β-arrestins play a critical role in the internalisation of many GPCRs, internalisation of PAR_1 has previously been shown to internalise via a β-arrestin independent mechanism

(Shapiro et al, 1996; Paing et al, 2002). In fact a later study by the same group displayed the importance of a highly conserved tyrosine based motif site (YXXL). When removed the resulting receptor had greatly impaired agonist-triggered internalisation, and in a mutant where the site was readily exposed within the cytoplasm constitutive agonist-independent activation, internalisation and degradation resulted (Paing et al, 2004). Thus showing phosphorylation, in conjunction with a highly conserved tyrosine-based motif, and not β-arrestin to be the primary regulating factors for PAR_1 internalisation.

1.3.8 PAR_3

1.3.8.1 The Cloning of PAR_3

Knockout gene cloning of the PAR_1 receptor in mice, showing platelets to still respond to thrombin provided evidence to existence of a second thrombin receptor (Connolly et al, 1996). An initial candidate was cloned using the polymerase chain reaction (PCR) with primers from regions shown to be conserved between PAR_1 and PAR_2 (Ishihara et al, 1997). Initially cloned from rat platelet mRNA due to its relative abundance, primers based on this clone were then used to isolate the human form of the receptor (Ishihara et al, 1997). $hPAR_3$ was found to be 374aa in humans and 369aa in mice, and to have retained a 27% amino acid homology to $hPAR_1$ and a 28% amino acid homology with $hPAR_2$. It was shown to have a short c-terminal tail and an N-terminal containing a thrombin cleavage site ($T^{34}LPIK\downarrow TFRGAP^{44}$) with a high affinity thrombin binding domain ($S^{47}FEEFP^{52}$) directly down-stream of the cleavage/activation site.

The PAR_3 gene was located to the same 100-kb spanning gene cluster as both PAR_1 and PAR_2 (5q13 in humans and 13D2 in mouse genome) using genomic analysis and was found to have a similar two exon structure (Kahn et al, 1998; Schmidt et al, 1998).

1.3.8.2 Activation of PAR_3

Initially it was displayed that cells transfected with $hPAR_3$ cDNA produced a calcium signal in response to thrombin (Ishihara et al, 1997). A proline to threonine mutation at position 39 in the N-terminus prevented activation by thrombin showing the cleavage site to be $LPIK^{38}\downarrow T^{39}FRGAP$ (Ishihara et al, 1997). Cleavage results in a N-terminus of TFRGAP, which may interact with the receptor as a tethered ligand (Ishihara et al, 1997). It has since been reported that $hPAR_3$ does not signal through calcium in human endothelial cells (O'Brien et al, 2000). Synthetic peptides produced and based on or designed around the PAR_3 TL sequence fail to activate PAR_3 (Ishihara et al, 1997) but curiously have been revealed to activate PAR_1 and PAR_2 (Hansen et al, 2004; Kaufmann et al, 2005).

1.3.8.3 PAR_3 Signalling.

Cellular studies indicated that phosphoinositide hydrolysis occurs through cleavage at Lys^{38}/Thr^{39} of $hPAR_3$ with thrombin, assisted by the presence of the hirudin-like binding domain $S^{47}FEEFP^{52}$ (see **Fig 1.3a**). Synthetic peptides mirroring the amino acid sequence of the putative tethered ligand of PAR_3 were found to be inactive (Ishihara et al, 1997). The lack of apparent effect of these synthetic peptides maybe for a number of different reasons. Firstly, the conformation of PAR_3 may result in greater ligand specificity than that seen in the other PAR family members. Alternatively the effect of the tethered ligand may be to "release the receptor from inhibition" (i.e. leaving the receptor capable of being activated) rather than activate signalling (Ishihara et al, 1997). The C-terminal region of PAR_3 is considerably shorter than that seen in the other PARs and may result in a difference in signalling and desensitisation, though no studies to date have investigated this. $mPAR_3$ appears to be extremely important in achieving full

thrombin mediated platelet activation in mice. The murine PAR_3 isoform may on its own not be a fully functional receptor and does not signal when expressed on its own (Nakanishi-Matsui et al, 2000). Instead appearing to be a tethering protein for thrombin activation of $mPAR_4$, perhaps allowing thrombin localisation through it's hirudin-like binding domain (Nakanishi-Matsui et al, 2000). Using mouse $mPAR_3$ gene knockouts (Kahn et al, 1998) and by inhibiting binding of thrombin to $mPAR_3$ in mouse platelets (Ishihara et al, 1998) it has been shown to be important in thrombin signalling in mouse platelets at low but not high concentrations of thrombin. However, $mPAR_3$ does not itself mediate transmembrane signalling but rather acts as a co-factor for cleavage and activation of $mPAR_4$ by thrombin (Nakanishi-Matsui et al, 2000). Conflicting reports exist for the ability of $hPAR_3$ to mediate calcium signalling (Ishihara et al, 1997; O'Brien et al, 2000). More recently PAR_3 has been identified as a moderator of signalling in PAR_1, allosterically regulating G-protein association by heterodimerising with PAR_1 (McLaughlin et al, 2007).

1.3.8.4 PAR_3 Desensitisation and Internalisation

As yet the regulatory mechanisms responsible for the termination of signalling in PAR_3 are unknown (Trejo, 2003). The C-terminal tail of PAR_3 is considerably shorter than that of the other PARs (**fig 1.3a**), considering the differences seen between the desensitisation and internalisation mechanisms for PARs 1, 2 and 4 it remains to be determined as to whether the mechanisms for PAR_3 are similar to any of them.

1.3.9 PAR_4

1.3.9.1 The Cloning of PAR_4

In PAR_1 and PAR_3 deficient mice platelets, studies suggested the existence of yet another thrombin receptor (Connolly et al, 1996; Kahn et al, 1998). Using the technique of searching through Expressed Sequence Tag (EST) libraries, two separate laboratories cloned $hPAR_4$ simultaneously (Kahn et al, 1998; Xu et al, 1998). A sequence 36% identical at a.a. level to that of PAR_2's four transmembrane domain was found by Xu *et al*. They then went on to clone the full sequence from a lymphoma Daudi cell line using a DNA probe developed from the EST sequence. Kahn *et al* discovered an 11 amino-acid sequence that was 73% identical to that of PAR_2. The full length clone was produced from mouse embryo DNA using rapid 5' amplification of cDNA ends and the full length human clone isolated using primers based on the mouse sequence. PAR_4 expressed in xenopus oocyte (Kahn et al, 1998), or COS cells (Xu et al, 1998) were shown in be responsive to both trypsin and thrombin. The thrombin concentrations required to activate PAR_4 were found to be higher than that required to activate PAR_1 (Kahn et al, 1998; Xu et al, 1998). The N-terminal region of PAR_4 lacks the highly acidic thrombin-binding site observed in both PAR_1 and PAR_3 which would account for the need for a higher thrombin concentration to activate the receptor.

PCR and somatic cell hybrid mapping methods were used in order to elucidate the gene location of PAR_4 which was mapped to chromosome 19p12 (Xu et al, 1998). Interestingly all other PAR genes are located within 100 bp of each other on chromosome region 5q13 (Bahou et al, 1993b; Nystedt et al, 1995a; Kahn et al, 1996; Schmidt et al, 1998) which suggests differences in the evolutionary develop of PAR_4 compared to the other PARs.

1.3.9.2 Activation of PAR_4

The N-terminal cleavage site of PAR_4 ($R^{47}\downarrow G^{48}YPGQV$) was revealed to be cleaved by both trypsin and thrombin (Xu et al, 1998). Mutation of this cleavage leads an inability of thrombin and trypsin to activate PAR_4 (Xu et al, 1998). By far the most effective agonist of PAR_4 is the neutrophil granular protease cathepsin G (Sambrano et al, 2000), resulting in platelet aggregation, and Ca^{2+} signalling in fibroblasts transfected with PAR_4 (Sambrano et al, 2000). However, PAR_4 is also cleaved and activated by trypsin (Ossovskaya & Bunnett, 2004) and, similar to PAR_1, the gingipains-R, RgpB and HRgpA with a similar potency to thrombin (Lourbakos et al, 2001b). FXa has also been reported to weakly activate PAR_4 (Camerer et al, 2000). Another serine proteinase trypsinogen IV was shown to activate PAR_4 (Cottrell et al, 2004).

Similar to PAR_1 and PAR_2 (see later) PAR_4 was shown to be activated by synthetic hexapeptides derived from its post-cleavage site amino terminus. The peptides GYPGKF (Kahn et al, 1998) and GYPGQV (Xu et al, 1998) which correspond to the TL of $mPAR_4$ and $hPAR_4$ respectively resulted in calcium signalling in transfected cell lines. However this AP is not only less efficient than thrombin at activating PAR_4, but nor is it as efficient as the PAR_1 and PAR_2-APs at activating their respective receptors (Faruqi et al, 2000). Structural activity studies enabled the development of AYPGKF with over 10-fold greater potency than GYPGQV (Faruqi et al, 2000).

1.3.9.3 PAR_4 Signalling

Thrombin cleavage occurs at Arg47/Gly48 in the N-terminal sequence revealing its tethered ligand GYPGQV and it can be activated to a similar efficacy with trypsin (Xu et al, 1998). The activation of PAR_4 has been reported to result in calcium signalling (Kahn et al, 1998; Xu et al, 1998; Camerer et al, 2002) and PAR_4 activation in vascular

smooth muscle stimulated MAPK signalling (Bretschneider et al, 2001). Additionally PAR_4 mediated Src dependant p38 phosphorylation and activation or ERK and PLC has been demonstrated in PAR_1 knockout mice derived cardiomyocytes (Sabri et al, 2003).

1.3.9.4 PAR_4 Desensitisation and Internalisation

Work on the desensitisation and internalisation of PAR_4 is considerably less than that for PAR_1 and PAR_2. However, in a comparison with PAR_1, PAR_4 was shown to desensitise at a significantly slower rate than that of PAR_1 (Shapiro et al, 2000). Receptor post-activation internalisation was also shown to be markedly slower in PAR_4 than PAR_1 (Shapiro et al, 2000). Phosphorylation is an important mediator of PAR_1 desensitisation and internalisation (**1.3.7.4** and **1.3.7.5** respectively), however agonist-mediated phosphorylation was found to be absent in PAR_4 (or at least greatly reduced compared to PAR_1) (Shapiro et al, 2000). Additionally, in a PAR_4 mutant in which the eight C-terminal serines and threonines (see **fig 1.3a**), which are potential phosphorylation sites, were converted to alanines, no change in signalling kinetics or receptor internalisation was seen (Shapiro et al, 2000).

1.3.10 PHYSIOLOGY AND PATHOPHYSIOLOGY OF THE THROMBIN RECEPTORS (PAR_1, PAR_3 AND PAR_4)

1.3.10.1 Tissue distribution of PAR1, PAR3 and PAR4.

The numerous cellular targets for thrombin include not only platelets, endothelial, and smooth muscle cells, but also other cell types such as neutrophils, leukocytes, neurons and glial cells (Hollenberg & Compton, 2002). The primary role of thrombin in revascularisation and the healing of damaged blood vessels (Carney et al, 1992) is therefore facilitated by a multitude of cell types. PAR_1 activating peptide has displayed

effects in a number of different cells types in various systems such as the cardiovascular system (Emilsson et al, 1997; Jiang et al, 1998), the immune system (Naldini et al, 1998; Umarova et al, 2000), the nervous system (Turgeon et al, 1998; Corvera et al, 1999) skeletal muscular system (Mbebi et al, 2001) and many others. This combined with the revelation of a thrombin producing action within brain (Gingrich & Traynelis, 2000), leaving the possibly of extracellular production and action, results in many faceted existence for PAR_1.

Human PAR_3 has been shown to be expressed in numerous tissues including heart, small intestine, bone marrow, airway smooth muscle, vascular endothelium, and astrocytes (Hauck et al, 1999; Bartha et al, 2000). However, no expression of the receptor on human platelets was observed, which differs from that observed with the murine receptor. Mouse PAR_3 is strongly expressed in megakaryocytes, but found at low levels of expression in other tissues such as brain and lungs. There is therefore species specific variation in PAR_3 function (Ishihara et al, 1997). PAR_4 is widely expressed, with the human isoform found in many of the tissue types tested. Northern blot analysis located high levels of human PAR_4 mRNA in lung, pancreas, thyroid, testis, and small intestine. Lower levels of mRNA were present in placenta, skeletal muscle, lymph node, adrenal gland, prostate, uterus, and colon. No expression of $hPAR_4$ was located within brain, kidney, spinal cord, or blood peripheral leukocytes (Xu et al, 1998). PAR_4 mRNA was also detected in human platelets (Xu et al, 1998). PAR_4 has been located in mouse tissues using northern blot analysis in the spleen, trace expression in the heart, lung, skeletal muscle and kidney, with no detectable expression in the brain, liver or testis (Kahn et al, 1998). *In situ* hybridisation of mouse spleen and bone marrow revealed cellular expression of mRNA occurring primarily in platelets and megakaryocytes (Kahn et al, 1998).

1.3.10.2 Platelet aggregation

There have been an abundance of studies showing thrombin to be a major stimulator of platelets resulting in a cascade of effects culminating in platelet aggregation both *in vivo* and *in vitro* (Eidt et al, 1988; Hung et al, 1992b; Kahn et al, 1999).

Many of the processes involved in platelet aggregation are mediated by PAR_1 including stimulating the release of 5-HT (Harmon & Jamieson, 1986), adenosine triphosphate (Detwiler & Feinman, 1973), thromboxane A_2, and other granule contents. Thrombin is equally responsible for the plasma membrane localisation of integrin αIIb/β3, resulting in the adhesion of fibrinogen and von Willebrand factor and platelet aggregation (McGregor et al, 1989; Watts et al, 1989) and the translocation of P-selectin and CD40 ligand to the plasma membrane facilitating platelet binding to endothelial cells (Stenberg et al, 1985; Henn et al, 1998). These actions have further been shown to occur on addition of PAR_1 activating peptide.

Antibodies targeted against the thrombin binding site of PAR_1 results in inhibition of platelet aggregation at low thrombin concentrations (Hung et al, 1992b; Kahn et al, 1999). Interestingly PAR_1 doesn't appear to play a role in mouse platelet aggregation (Derian et al, 1995; Connolly et al, 1996). PAR_1-activating peptides do not activate rodent platelets (Derian et al, 1995; Connolly et al, 1996), additionally platelets in PAR_1 deficient mice responded similarly to wild-type platelets on thrombin challenge (Connolly et al, 1996).

The fact that PAR_1-blocking antibodies inhibited human platelet activation at low concentration of thrombin but not high (Brass et al, 1992; Hung et al, 1992b) suggested that although PAR_1 is important in platelet aggregation it is not the only receptor involved. Combined with the fact that PAR_1 plays no role in mouse platelet aggregation

this lead to the identification of PAR_3 (Ishihara et al, 1997). Similar to PAR_1 in humans, PAR_3 blocking antibodies inhibited mouse platelet activation at low concentrations of thrombin but not high (Ishihara et al, 1998). Additionally the production of PAR_3 knockout mice led to the removal of platelet activation at low concentrations of thrombin, but not high (Kahn et al, 1998). Thus establishing PAR_3 as essential for normal thrombin mouse platelet activation. PAR_3 has no important role in thrombin mediated activation of human platelets (Kahn et al, 1999).

Both humans and rodents benefit from a dual receptor response to thrombin in platelet aggregation (Kahn et al, 1999). In humans low concentrations of thrombin activate platelets through PAR_1 (see 1.4.6.4) and in rodents low concentrations of thrombin activate platelets through PAR_3 (see 1.4.7.4). But both species rely on PAR_4 as a second receptor for platelet aggregation at high thrombin concentrations (Kahn et al, 1999). The implications of having dual thrombin receptors in platelets are uncertain. It is conceivable that response to thrombin over a large concentration range is required or simply the combination of the three receptors mediate signalling through multiple proteinases and ligands.

1.3.10.3 Cardiovascular System

All PARs can be found expressed throughout the cardiovascular system. Expression of PAR_1 (Vu et al, 1991a; McNamara et al, 1993; D'Andrea et al, 1998), PAR_3 (Schmidt et al, 1998; Bretschneider et al, 2003) and PAR_4 (Bretschneider et al, 2001; Fujiwara et al, 2005) can be found on vascular smooth muscle and endothelial cells. Both thrombin and PAR_1 selective agonists result in a relaxation of pre-contracted blood vessels including large volume conducting vessels such as the human pulmonary artery (Hamilton et al, 2001), porcine coronary artery (Hamilton & Cocks, 2000) and rat aorta (Magazine et al,

1996) as well as in a few smaller vessels such as the human and porcine intramyocardial arteries (Hamilton et al, 2002).

The mechanism of prevention of PAR_1-induced vascular relaxation outlined in these studies appears to be removal of the endothelium and thus the secretion of a factor resulting in vascular smooth muscle contraction. The PAR_1 mediated relaxation frequently seen, occurs through the release of nitric oxide (NO), as seen by its suppression of relaxation when inhibiting NO synthase (Magazine et al, 1996; Hamilton & Cocks, 2000; Hamilton et al, 2001; Hamilton et al, 2002). Other PAR_1 mediated NO-independent mechanisms include the endothelial-derived hyperpolarizing factor mediated relaxation seen in porcine coronary arteries (Hamilton & Cocks, 2000). Additional contribution to relaxation is seen as a result of PAR_1 mediated production of cyclooxygenase products in human pulmonary arteries (Hamilton et al, 2001). A more recent study shows endothelium dependant relaxation in internal mammary arteries in response to PAR_1-AP (Ballerio et al, 2007). Activators of PAR_1 can result in the contraction of certain vessels by both endothelium dependant and independent mechanisms (Tay-Uyboco et al, 1995). Additional studies in intact animals have shown intravenous injection of PAR_1-selective AP causes a rapid and sustained hypotension in mice by a NO-independent mechanism (Cheung et al, 1998; Damiano et al, 1999a). Similarly thrombin and PAR_1-AP administration in rats results in reduction in arterial blood pressure that is in part mediated by a NO-dependant mechanism (Sicker et al, 2001). The extravasation of plasma proteins following post-intraplantar injection of thrombin and PAR_1-AP has similarly been studied and shown to cause oedema formation in rat paw (Vergnolle et al, 1999; Andrade-Gordon et al, 2001; de Garavilla et al, 2001).

1.3.10.4 Nervous System

All four PARs have been shown to be expressed throughout the nervous system (Striggow et al, 2001). PAR_1 expression has further been revealed in human astrocytoma cells (Grishina et al, 2005) and peripheral nervous system cells such as rat dorsal root ganglia (Noorbakhsh et al, 2003) in addition to guinea pig myenteric (Corvera et al, 1999) and submucosal neurons (Reed et al, 2003). High densities of PAR_3 have been reported on hippocampus, cortical, amygdale, thalamic, hyperthalamic and striatal neurons along with similar locations for PAR_4 (Striggow et al, 2001), as well all four PARs being expressed on rat astrocytes (Wang et al, 2002a).

The morphology and proliferation of astrocytes has been shown to be affected by both thrombin and PAR_1-AP (Noorbakhsh et al, 2003; Nicole et al, 2005). Thrombin at low concentrations was shown to effect astrocytes by reversing their process extension perhaps resulting in compromising the integrity of the blood brain barrier (Noorbakhsh et al, 2003). Using higher concentrations of thrombin however resulted in astrocyte proliferation (Noorbakhsh et al, 2003). A finding reinforced by a later study showing the astrocyte proliferation disorder, astrogliosis, to be PAR_1 activation mediated (Nicole et al, 2005). The activation of PAR_1 by thrombin in certain neuronal cells have also been shown to have a protective effect against cell death resulting from both hypoglycaemia and oxidative stress (Vaughan et al, 1995). Thrombin and a PAR_1-AP have also been revealed to affect the morphology of tyrosine hydroxylase-positive neurons resulting increased neurite elongation and decreased branching or sprouting (Suidan et al, 1992; Debeir et al, 1998).

An additional role for thrombin as a crucial mediator of CNS injury has been highlighted in a number of studies (Vaughan et al, 1994; Choi et al, 1995; Vaughan et al, 1995; Pike et al, 1996; Akiyama et al, 2000). Brain plaques taken from patients with

Alzheimer's disease have been shown to contain high levels of thrombin (Akiyama et al, 2000). The decrease in protease nexin-1 (a thrombin inhibitor) in an Alzheimer's affected brain further supported the importance of thrombin in this diseases pathology (Vaughan et al, 1994; Choi et al, 1995). However, later *in vitro* studies on cultured neurons displayed the ability of both thrombin and PAR_1-AP to reduce β-amyloid (a putative causative agent in Alzheimer's disease) neurotoxicity (Pike et al, 1996). Whilst in cultured astrocytes although PAR_1 agonists reduced stellation, it attenuates the beta-amyloid-induced increased expression of basic fibroblast growth factor (Pike et al, 1996) suggesting that PAR_1 activation has a dual effect within this disease.

More recently PAR_1 has also been implicated in the neurodegenerative disorders associated with HIV (Boven et al, 2003; Ishida et al, 2006). Brain sections taken from HIV patients with encephalitis showed increased staining for PAR_1, mRNA for PAR_1 was also shown to be upregulated (Boven et al, 2003). It is also been demonstrated that PAR_1 is up-regulated in Parkinson disease effected brain tissue (Ishida et al, 2006), and further provides a thrombin mediated protective pre-conditioning against Parkinson disease related behavioural deficits (Cannon et al, 2006). Pro-generative effects in nerves can be seen in thrombin and PAR_1-AP increasing crushed mouse peripheral nerve regeneration (Balezina et al, 2005). PAR_1 has been localised by confocal microscopy in rat olfactory neurons and thrombin has been shown to result in rapid neurite retraction suggesting a role in neuritogenesis (Olianas et al, 2007).

1.3.10.5 Gastrointestinal system

Of all areas in the body the gut is exposed to the greatest array of proteases under normal physiological conditions and in response to disease making it a prime site for PAR mediated processes. PAR_1 has been located in the endothelial cells within the

lamina propria of the small intestine, as well as in intestinal epithelial cells, smooth muscle cells and within the neurons of the enteric nervous system (Corvera et al, 1999). PAR_1 mRNA has been isolated in the parotid, sublingual and submaxillary glands (Kawabata et al, 2000c) suggesting a role salivation, but administration of PAR_1 agonists *in vivo* cause no such effect (Kawabata et al, 2000c).

In humans PAR_3 mRNA is expressed in the stomach and small intestines, although within which cell type has yet to be characterised (Ishihara et al, 1997). Expression of PAR_4 can be seen to a large extent within the pancreas, small intestine and colon though similar to PAR_3 it has yet to be localised to specific cell types (Kahn et al, 1998; Xu et al, 1998).

Activation of PAR_1 results in stimulation of Cl^- ion secretion from the mucosa of the intestine (Buresi et al, 2001; Buresi et al, 2002) conceivably leading to a two-fold effect. Firstly secretion due to PAR_1 activation could contribute to the diarrhoea that is indicative of inflammatory conditions of the gut (Buresi et al, 2001; Buresi et al, 2002), and secondly the enhanced secretion during inflammation may give a protective outcome by promoting toxin excretion (Buresi et al, 2001; Buresi et al, 2002). Further to this, PAR_1 is seen to be overexpressed in the colon of patients with inflammatory bowel disease (IBD), and administration of PAR_1 agonists to mice resulted in a colonic inflammatory response (Vergnolle, 2005). In addition, intra-colonic administration of PAR_1 antagonists to mice with stimulated IBD reduced the severity of colonic inflammation and mortality (Vergnolle, 2005). Further work showed PAR_1 to have an anti-inflammatory role in colitis, by showing the colitis inhibiting effects of administering PAR_1 agonists and exacerbation of condition in PAR_1 knockout mice (Cenac et al, 2005). The role played by PAR_1 in electrolyte excretion has been examined by applying thrombin and PAR_1-AP to small intestine epithelial cells (Buresi

et al, 2001; Buresi et al, 2002). Both were shown to raise Cl^- levels in a Ca^{2+} dependant manner as well as activation of Src, ERK1/2, transactivation of EGF, phosphorylation of phospholipase A_2, and stimulation of cyclooxgenase 1 and 2 (Buresi et al, 2001; Buresi et al, 2002), which are all capable of inducing Cl^- secretion.

In rodents an additional role is seen in gut motility. Activation of PAR_1 in rats and guinea pigs stimulates contraction of the gastric longitudinal muscles (al-Ani et al, 1995; Saifeddine et al, 1996). Whereas, in mouse gastric fundus the contraction is preceded by an initial relaxation (Cocks et al, 1999b). Contraction of the oesophagus is noted following PAR_1 activation with both low concentrations of thrombin and PAR_1-AP (Kawabata et al, 2000a). Interestingly although low concentrations of thrombin results in oesophageal contraction through a PAR_1 mediated action, higher concentrations of thrombin and PAR_4-APs result in a relaxation of pre-contracted oesophagus (Kawabata et al, 2000a) giving an interesting dual action for thrombin in this system. Further to this administration of selective PAR_1-APs accelerates intestinal transit in mice (Kawabata et al, 2001), thus giving PAR_1 overall effect to promoting movement through the gut.

1.3.10.6 Renal System

Expression of PAR_1 in the kidney was established in the initial cloning study (Rasmussen et al, 1991). Since the expression has been shown to be present in endothelial, mesangial and epithelial cells (Grandaliano et al, 2000) and more recently expression has been shown in primary human renal carcinoma cells (Kaufmann et al, 2002). Expression of $hPAR_3$ has been reported in primary human renal carcinoma cells by both identifying $hPAR_3$ mRNA by RT-PCR, and using confocal and electron-

microscopy (Kaufmann et al, 2002). No work has been done looking into the possible role of PAR_3 in this system.

When treated with thrombin glomerular epithelial and mesangial cells in culture have been shown to proliferate and manufacture prostaglandins, NO, endothelin-1, extracellular matrix components, and chemokines such as MCP1 (monocyte chemotactic protein-1), as well as the serine protease inhibitor PAI-1 (plasminogen activator inhibitor type 1) (Rondeau et al, 1996). A localised excess of PAI-1 seen in several fibrin-related nephropathies may therefore be as a result of thrombin release and could contribute to the severity of renal lesions (Rondeau et al, 1996). PAI-1 presence has also been demonstrated to promote fibrotic tissue development (Eitzman et al, 1996; Hattori et al, 2000). It was observed that in fibrin associated kidney diseases such as crescentic glomerular nephritis and thrombotic microangiopathy there was a reduced cellular PAR_1 expression within the glomerular lesions (Xu et al, 1995). However the study noted that PAR_1 mRNA expression remained the same, suggesting removal of PAR_1 cell surface expression by protease activation and internalisation implicating PAR_1 in the pathogenesis of these diseases (Xu et al, 1995). Later, PAR_1 was definitively shown to play an important role in murine model of crescentic glomerular nephritis (Cunningham et al, 2000). In this *in vivo* study PAR_1 activation was shown to result in enhanced glomerular crescent formation, T cell and macrophage infiltration, fibrin deposition, and elevated serum creatinine, all which are prominent indicators and features of glomerular nephritis (Cunningham et al, 2000). Infusion of hirudin (which strongly inhibits thrombin) in the same model almost completely prevents the development of glomerular fibrin deposition and crescent formation (Cunningham et al, 2000). In the same study, PAR_1 knockout mice showed a lower percentage of crescent formation, less glomerular fibrin deposition and macrophage infiltration as well as less

severe renal failure compared to wild-type (Cunningham et al, 2000). Clearly highlighting the involvement of PAR_1 in the pathogenesis of crescentic glomerular nephritis. PAR_1 has also been shown to play a role in renal haemodynamics (Gui et al, 2003). Application of both thrombin and PAR_1-AP to isolated perfused rat kidneys caused vasoconstriction and an associated significant reduction in renal flow (Gui et al, 2003). The vasoconstrictive effect was ablated with the use of a PKC inhibitor, showing PAR_1-mediated renal vasoconstriction to occur by a PKC dependant mechanism (Gui et al, 2003).

1.3.10.7 Immune System

PAR_1 is expressed widely throughout the immune system. Expression of PAR_1 has been demonstrated on macrophages and monocytes (Colognato et al, 2003) as well as mast cells (D'Andrea et al, 2000). Additionally low-levels of thrombin has been shown to illicit responses from other immune cells such as T cells (Mari et al, 1996) and Jurkat-T leukemic cells (Bar-Shavit et al, 2002) suggesting these cells may express PAR_1. Expression of PAR_3 has been established on eosinophils (Miike et al, 2001) and in conjunction with PAR_1 on Jurkat-T leukemic cells (Bar-Shavit et al, 2002). Expression of PAR_4 has as yet only been described on leukocytes (Vergnolle et al, 2002).
The actions of thrombin within the immune system are primarily concerned with chemotaxis and the release of cytokines from inflammatory cell lines. In monocytes thrombin has been reported to stimulate the release of both IL-8 and IL-6 (Naldini et al, 2000) as well as MCP-1 (Colotta et al, 1994). Additionally, IL-2 release from natural killer cells has been shown to occur under the influence on thrombin (Naldini & Carney, 1996). Thrombin has been shown to result in the liberation of arachidonic acid from human neutrophils (Ramesha & Taylor, 1991), as well as the generation and release of

leukotriene B_4 (LTB_4) in peripheral lymph tissue (Malik et al, 1986). LTB_4 has been shown to act on neutrophils resulting in degranulation (Kannan, 2002). Human eosinophils have shown to express PAR_1, (Bolton et al, 2003) and mRNA for PAR_3 (Miike et al, 2001). Application of PAR_1-AP to eosinophils shape-change (as measured by a significant increase in FSc on a flow-cytometer) (Bolton et al, 2003)

1.3.10.8 Respiratory System

PAR expression is seen throughout the respiratory system and all evidence gathered thus far point towards a pivotal role for PARs within the respiratory system. Expression of PAR_1 has been identified on numerous airway associated cell lines including pulmonary fibroblasts (Trejo et al, 1996; Chambers et al, 1998) and epithelial cells (Asokananthan et al, 2002a), endothelial cells (D'Andrea et al, 1998; Kataoka et al, 2003) as well as smooth muscle cells (Lan et al, 2000; Walker et al, 2005). Expression of PAR_3 within the airway is not as clear cut as PAR_1, but PAR_3 expression has been demonstrated functionally in bovine tracheal smooth muscle cells (Walker et al, 2005). It has demonstrated that PAR_3 mRNA is present in airway smooth muscle cells (Hauck et al, 1999) as well as epithelial cells (Shimizu et al, 2000). Expression of PAR_4 appears to be restricted to just the respiratory epithelial (Shimizu et al, 2000; Asokananthan et al, 2002a), endothelial (Kataoka et al, 2003) and smooth muscle cells (Lan et al, 2000). Additionally all PARs have also been shown on airway epithelial and smooth muscles cells by immunohistochemical staining using anti-receptor antibodies (Knight et al, 2001).

An important function of PAR_1 activation in the lung is the mediation of fibroblast mitogenesis (Blanc-Brude et al, 2005; Walker et al, 2005), though it has also been reported that thrombin and not PAR_1-AP is responsible for the mediation of mitogenesis

(Tran & Stewart, 2003; Walker et al, 2005). The activation of PAR_1 by thrombin has further been demonstrated to stimulate the production of procollagen from fibroblasts (Chambers et al, 1998). This additionally mediates the upregulation of connective tissue growth factor (CTGF) (Chambers et al, 2000), which acts as an autocrine agent promoting fibroblast mitogenesis, and the production of extracellular matrix. Work further supporting the importance of PAR_1 in the pulmonary fibrotic response has been carried out in rats using bleomycin-induced pulmonary fibrosis (Howell et al, 2001; Howell et al, 2002). In this model, PAR_1 expression was greatly increased and the use of a specific thrombin inhibitor resulted in a significant reduction in fibroblast proliferation, procollagen production and in the production of CTGF mRNA (Howell et al, 2001; Howell et al, 2002). Activation of PAR_1 in human airway epithelial cell by Pen C13, an immunodominant mold allergen, was shown to result in IL-8 production, indicating an important role in the pathogenesis of this form of asthma (Chiu et al, 2007).

1.4 PAR_2

1.4.1. Cloning of PAR_2

The emergence of the thrombin receptors' (PAR_1) novel method of activation opened the possibility of other proteinase activated receptors that activate by similar means. In addition, the inability of thrombin to elicit the same response on cells as the PAR_1-AP (SFLLRN) suggested the presence of another receptor (Kinlough-Rathbone *et al*., 1993). Initial southern blotting with genomic DNA was unsuccessful. A 395 aa G-protein coupled receptor (PAR_2) was eventually found in mouse genomic DNA that had 30% similarity to $hPAR_1$ and 28% similarity to $mPAR_1$ (Nystedt *et al*., 1994). The group further reported the cloning on the human PAR_2 ($hPAR_2$) in a subsequent publication (Nystedt et al, 1995a) and was then later cloned by another group (Bohm et al, 1996b).

The extramembranous domains displayed a high level of heterogeneity including a C-terminal that is 29 amino acids shorter than $hPAR_1$ and the absence on hirudin-like binding domain (see **Fig 1.3a**). Now termed PAR_2, when expressed in Xenopus oocytes $mPAR_2$ was shown to be unresponsive to thrombin but responsive to 100-fold lower concentrations of trypsin than $hPAR_1$ (Nystedt *et al*., 1994). The trypsin cleavage site was found to be Arg34 in the N-terminal domain, identical in both the mouse and human isoforms. The proteolytically unmasked TL though was not conserved having a two amino acid variation, for SLIGKV-NH2 for $hPAR_2$ and SLIGRL for $mPAR_2$ (Nystedt et al, 1994). In order to locate the $hPAR_2$, gene fluorescent in-situ hybridisation on chromosome spreads was used and the gene identified in chromosomal region 5q13 (Nystedt et al, 1995a) the same location where PAR_1 had previously been shown to be located (Bahou et al, 1993b).

1.4.2 Activation of PAR_2

The PAR_2 receptor was identified as being a trypsin receptor when revealed to contain a putative trypsin cleavage site $SKGR^{34}\downarrow S^{35}LIG$ (Nystedt et al, 1994). Removal of a N-terminally attached flag (shown by loss of immuno-reactivity to anti-flag antibody) upon activation with trypsin served to confirm receptor cleavage was involved in PAR_2 activation (Bohm et al, 1996a). The trypsin cleavage site was confirmed to be $Arg^{34}\downarrow Ser^{35}$ through analysis of proteinase cleavage products and the fact that mutation of the cleavage site abolished receptor activation by trypsin (Bohm et al, 1996a).

1.4.2.1 Domains Important for Activation

Similar to PAR_1 the ECL2 of PAR_2 was shown to be essential for responsiveness to its TL sequence (Lerner et al, 1996). Domain swapping experiments transferring the ECL2 of PAR_2 into PAR_1 resulted in a receptor with PAR_2-AP specificity (Lerner et al, 1996). The study also showed that ECL3 and the extracellular N-terminus to be important for peptide-mediated receptor activation. Later studies with the use of AP as probes for activation specificity showed specific acidic residues in the ECL2 of PAR_2 to be important for peptide-mediated activation (Al-Ani et al, 1999). Using site-directed mutagenesis to create alanine mutations for the first six residues of the TL this group went on to show that the amino-terminal tethered ligand dipeptide sequence $S^{37}L^{38}$ perform an important function in the activation of PAR_2 (Al-Ani et al, 2004), provided these residues were intact trypsin mediated receptor activation could still occur (Al-Ani et al, 2004).

1.4.2.2 PAR_2 Activating Proteinases

In addition to trypsin, tryptase has been shown to be able to cleave PAR_2 in a number of different tissues, though to a much lower potency than trypsin(Corvera et al, 1997; Mirza et al, 1997; Molino et al, 1997; Schechter et al, 1998; Corvera et al, 1999; Akers et al, 2000; Steinhoff et al, 2000; Berger et al, 2001a; Berger et al, 2001b; Schmidlin et al, 2001; Reed et al, 2003). The efficacy of tryptase as an activator of PAR_2 is dramatically effected by the glycosylation state of the receptor (Compton et al, 2001; Compton et al, 2002; Compton, 2003). When it was first cloned the serine proteinase trypsinogen IV, was also shown to be capable of activating PAR_2 (Cottrell et al, 2004). Similar to PAR_1 coagulation TF, FXa and FVIIa can all activate PAR_2 on endothelial cells (Camerer *et al*., 2000). FVIIa only signals strongly through PAR_2 when PAR_2 is co-expressed with TF (Camerer et al, 2000; Riewald & Ruf, 2001). Activation of PAR_2 by FXa is weak on its own, but when associated in the complex TF-FVIIa-FXa it's capable of potently activating PAR_2 (Camerer et al, 2000; Riewald & Ruf, 2001). Additionally, although TF-FVIIa signals to PAR_2 it does so with much lower efficiency than TF-FVIIa-FXa and observations in PAR knockout suggest FXa signalling to endothelial cells is primarily mediated by PAR_2 (Camerer et al, 2002). Proteinase 3 has been shown to be capable of cleaving PAR_2 N-terminal fragments, and proteinase 3 mediated Ca^{2+} mobilisation is suppressed with PAR_2 desensitisation (Uehara et al, 2002; Uehara et al, 2003). The integral membrane protease, membrane-type serine protease 1 (MT-SP1) has been shown by substrate-specificity analysis to be a potential proteolytic activator of PAR_2 (Takeuchi et al, 2000). Soluble MT-SP1 will specifically activate PAR_2 when expressed on oocytes (Takeuchi et al, 2000). A more recent study has shown MT-SP1 to induce IL-8 and IL-6 upregulation in endothelial cells and monocytes by a PAR_2 mediated pathway (Seitz et al, 2007).

Non-mammalian proteases such as the dust mite allergen proteases Der P3 and Der P9 also cleave PAR_2 at its activation site, and desensitisation experiments suggest that the cytokine release seen in the airway in response to these proteases is at least in part mediated by PAR_2 (Sun et al, 2001). The gingipains-R RgpB can also activate PAR_2 in transfected cells, and will induces IL-6 release in an oral epithelial cell line (Lourbakos et al, 1998; Lourbakos et al, 2001a). Although in transfected cell lines cathepsin G and elastase cleave the PAR_2 N-terminus pre-activation site, disabling the receptor, (Dulon et al, 2003) both have been shown to signal in fibroblast stimulating IL-8 release most likely through PAR_2 (Uehara et al, 2003). Though in a more recent study using human primary lung fibroblasts cathepsin G was again seen to disarm PAR_2 (Ramachandran et al, 2007).

1.4.2.3 PAR_2 Peptide Agonists/Antagonists

In keeping with observations of PAR_1, PAR_2 can be activated using its activating peptide SLIGKV-NH2 in human, and SLIGRL in mouse and rats derived from the TL sequence (Nystedt et al, 1995a; Nystedt et al, 1995b). The $hPAR_2$ peptide is very specific but has a relatively low potency; interestingly the $mPAR_2$ activating peptide is also capable of activating $hPAR_2$ to a higher potency than the native peptide (Blackhart et al, 1996). Analysis and AP analogs have identified residues of particular importance to receptor activation. Alanine scanning of the mouse AP shows Leu^2 and Arg^5 are essential for its biological activity, replacement of Ser^1 or Arg^5 with alanines results in some reduction in potency, whereas alanine substitutions for Gly^4 or Leu^6 have little effect on the AP (Blackhart et al, 1996). Numerous other synthetically derived peptides have been designed for PAR_2 with varying degrees of potency (Maryanoff et al, 2001; Kawabata et al, 2004; McGuire, 2004; Kanke et al, 2005a). By far the most potent is 2-

furoyl-LIGRL-NH_2 which has a 30-fold higher binding affinity for PAR_2 than SLIGRL-NH_2 (Kanke et al, 2005a). No PAR_2 antagonists have been reported to date.

1.4.3 PAR_2 Signalling

It has been widely demonstrated that trypsin and PAR_2-AP stimulate IP_3 production in cells, resulting in calcium signalling in a way that is consistent with coupling to the heterotrimeric G proteins G_q/G_{11} and PLC isoforms (Nystedt et al, 1995a) and possibly G_i (Macfarlane et al, 2001). Whereas coupling to $G_{12/13}$ by PAR_2 has not been reported. Investigation of PAR_2 signalling in human smooth muscle and neuronal cells have demonstrated that activation of PAR_2 results in the subsequent activation of PLC (Berger et al, 2001b) and PKC respectively (Okamoto et al, 2001). Activation of PAR_2 in rat aortic smooth muscle has been reported to consequently activate the MAPK cascade (Belham et al, 1996) and in human keritinocytes PAR_2 attributed activation of both JNK and p38 MAPK (Kanke et al, 2001) was shown to mediate NF-κB DNA binding (Kanke et al, 2001; Buddenkotte et al, 2005). Further to this, NF-κB signalling in keritinocytes has been demonstrated to be dependant on intracellular calcium mobilisation (Macfarlane et al, 2005). Activation of NF-κB via PAR_2 mediated mechanisms has also been shown in epithelial cells (Vliagoftis et al, 2000) and coronary artery smooth muscle cells (Bretschneider et al, 1999).

Activation of the MAPKs ERK1/2 by PAR_2 has been demonstrated in numerous different cell types such as smooth muscles cells (Belham et al, 1996), cardiomyocytes (Sabri et al, 2000) and coronary artery smooth muscles cells through Factor Xa mediated activation of PAR_2 (Koo et al, 2002). It has been further revealed that a 9 aa region within the C-terminal tail of PAR_2 ($A^{355}KNALLCRS^{363}$) is essential for IP_3 formation and downstream calcium signalling as mediated by PLC (Seatter et al, 2004).

The removal of this sequence in a truncated mutant abolishes signalling through G_q whilst leaving the receptor still capable of signalling through JNK, p38 and ERK1/2 MAP kinases (Seatter et al, 2004)

1.4.4 PAR_2 Desensitisation

In contrast to PAR_1, PAR_2 appears to primarily phosphorylated at its C-terminus by Protein Kinase C (PKC) (Bohm et al, 1996a), rather than GRK, making it also the primary mediator of PAR_2 desensitisation. Similar to PAR_1, activation of PAR_2 results in an association with β-arrestin. In the case of PAR_2 through a rapid and transient redistribution of βarr1 to the plasma membrane, when over-expressed in KNRK cells (Dery et al, 1999) though interestingly, unlike PAR_1, βarr1 over-expression had no effect on the magnitude or duration of the Ca^{2+} signalling. Mechanisms of desensitisation in PAR_3 remain un-studied, though PAR_4 desensitisation has been shown to be slower than that of PAR_1 (Shapiro et al, 1996) presumably due to its lack of C-terminal phosphorylation sites.

1.4.5 PAR_2 Internalisation

Like PAR_1, internalisation of PAR_2 has been shown to occur through a dynamin- and clathrin-dependant pathway (Dery et al, 1999; Trejo, 2003) confirmed by disruption or inhibition of this pathway affecting receptor endocytosis. However, contrary to PAR_1, β-arrestin may play an important role in the internalisation of PAR_2. PAR_2 activation results in a rapid and transient redistribution of β-arrestin 1 to the plasma membrane where it co-localises with PAR_2 (Dery et al, 1999). Additionally, co-expression of PAR_2 with a β-arrestin 1 mutant, which constitutively binds clathrin but not the receptor, partially inhibited receptor agonist-mediated endocytosis (Dery et al, 1999;

DeFea et al, 2000b). Results further comfirmed in cells taken from β-arrestin knockout mice where PAR_2 agonist-mediated internalisation was shown to be completely abolished (Stalheim et al, 2005; Kumar et al, 2007). Early internalisation was restored through the expression of β-arrestin 1 and expression of β-arrestin restored prolonged internalisation (Kumar et al, 2007). Interestingly though, over-expression of β-arrestins affected neither the magnitude nor extent of the Ca^{2+} response nor did it enhance PAR_2 internalisation (Dery et al, 1999; DeFea et al, 2000b). Following internalisation activated PAR_2 then remains stably associated with arrestins, as seen by the redistribution of arrestins and PAR_2 together into endocytic vesicles (Dery et al, 1999). The formation of stable arrestin-GPCR complexes is in part regulated by specific serine and threonine rich clusters precisely located in the C-terminal domain (Oakley et al, 2001). Three such domains exist within the PAR_2 C-terminus, and although C-tail truncation mutants deficient in theses regions still desensitise and internalise similar to wild-type receptor they lose the ability to stably associate with β-arrestin on internalisation (Stalheim et al, 2005).

Co-localisation with PAR_2 in these early endosomes was seen with both β-arrestin 1 and 2, however only β-arrestin 1 was found co-localised with PAR_2 in lysosomes (Kumar et al, 2007), suggesting a different fate for β-arrestin 2 associated receptors.

1.4.6 PAR_2 Physiology and Pathophysiology

PAR_2 has been implicated in numerous different physiological systems (**fig 1.4.6a**). The focus of this study is on PAR_2 within the cardiovascular system but a short explanation of PAR_2 in other systems has been included to give a wider picture of PAR_2 importance.

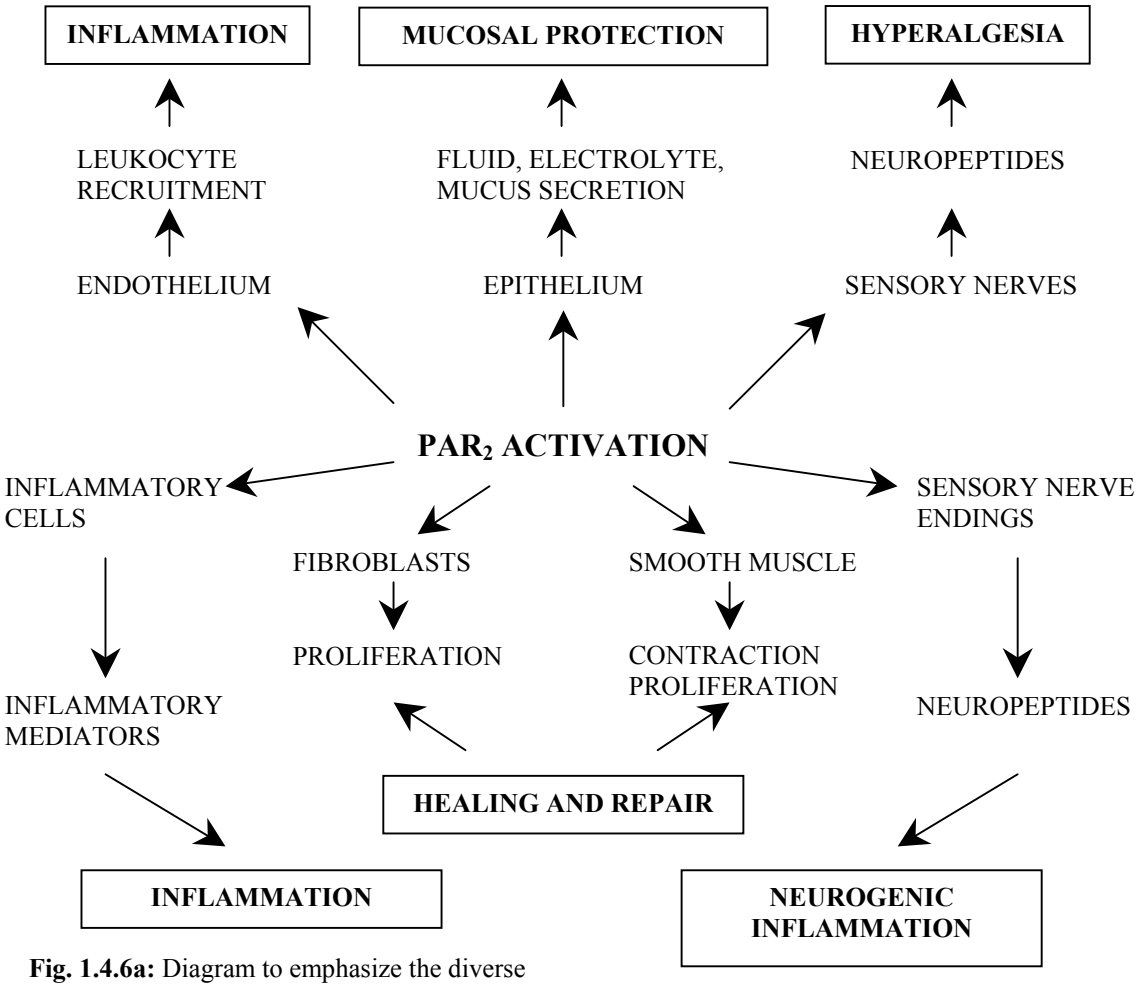


Fig. 1.4.6a: Diagram to emphasize the diverse affects of PAR_2 activation.

1.4.6.1 PAR_2 in the Cardiovascular System

The expression of PAR_2 on both vascular endothelium (Hwa et al, 1996; Mirza et al, 1996) and vascular smooth muscle (D'Andrea et al, 1998; Molino et al, 1998) has suggested a role for the receptor in the control of vascular reactivity. In order to further investigate its proposed role in the cardiovascular system and vasculature there have been numerous studies using PAR_2 agonists. Trypsin and SLIGRL-NH_2 (the murine tethered ligand sequence) have been shown to cause an endothelium dependant relaxation of the rat thoracic aorta (al-Ani et al, 1995). Similarly in porcine coronary arteries SLIGKV-NH2 results in a endothelium dependant relaxation (Hamilton et al, 2002).

The blocking of the PAR_2-AP effect by L-NAME[3] in this study suggests the involvement of nitric oxide release from endothelial cells. Further experiments in animal models have shown PAR_2 agonists to induce a reflex hypertension followed by an endothelial dependant hypotension (Emilsson et al, 1997; Cheung et al, 1998; Cicala et al, 2001). A human *in-vivo* study went on to show PAR_2-AP results in vasodilation caused in a NO and prostaglandin dependant manner (Robin et al, 2003). Up-regulation of PAR_2 cell surface expression in the presence of inflammatory factors, such as tumour necrosis factor-α and interleukin-1β, has been demonstrated *in vitro* using human umbilical vein endothelial cells (Nystedt et al, 1996). Proliferative responses shown to be mediated under the control of PAR_2 occur in endothelial cells (Mirza et al, 1996), as well as coronary artery smooth muscle cells (Bretschneider et al, 1999; Koo et al, 2002). Further implication of PAR_2 in cardiovascular disease is shown by the increased expression seen in rats with endotoxic shock and/or induced arterial restenosis (Cicala et al, 1999; Damiano et al, 1999b). Further to work showing the upregulation of PAR_2 in response to inflammatory stimuli (Ramachandran et al, 2007; Ritchie et al, 2007). Another study showing inflammatory stimuli upregulation of PAR_2 providing a PAR_2 mediated response where it was previously absent demonstrated pre-treatment with TNF_α results in PAR_2-mediated endothelium dependant relaxation in internal mammary arteries (Ballerio et al, 2007).

1.4.6.2 PAR_2 in the Respiratory System

PAR2 is widely expressed in respiratory cells including ciliated and non-ciliated epithelial cells (Knight, 2001; Vliagoftis et al, 2001; Asokananthan et al, 2002a; Ubl et al, 2002), airway smooth muscle cells (Chow et al, 2000; Berger et al, 2001a; Schmidlin

[3] Nα-L-arginine methyl ester, a non-selective inhibitor of nitric oxide synthase.

et al, 2001), endothelial cells (D'Andrea et al, 1998; Schmidlin et al, 2001), and fibroblasts (Akers et al, 2000; Matsushima et al, 2005). A number of PAR_2 agonist proteinases have been identified in the airways and include mast cell tryptase (Molino et al, 1997), trypsin (Cocks et al, 1999a) human airway trypsin (HAT) (Matsushima et al, 2005), proteases derived from house dust mite and fungi allergens (Der P 1, Der P 9) (Sun et al, 2001; Asokananthan et al, 2002b), and the neutrophil proteinases, human leukocyte elastase, cathepsin G and proteinase 3 (Uehara et al, 2003)

The role of PAR_2 in the airway appears varied with some studies demonstrating a protective bronchodilatory role and others illustrating a more destructive bronchoconstrictive effect. In isolated precontracted bronchi from mice, PAR_2 activation resulted in relaxation (Cocks et al, 1999a; Lan et al, 2001). A similar result was obtained from precontracted bronchi from human and guinea pigs (Cocks et al, 1999a). PAR_2 agonists have been shown to cause an epithelium-dependant relaxation in rat trachea and intrapulmonary bronchi (Cocks et al, 1999a; Chow et al, 2000) all of which display a role of PAR_2 in mediating smooth muscle relaxation. However contrary to this, others have reported that PAR_2 activation stimulated contraction of lobar or segmental bronchial rings, mediated in part by direct activation of PAR_2 on smooth muscle (Schmidlin et al, 2001). Further, an *in vivo* study similarly showed administration of PAR_2 agonists intravenously, intratracheally or intranasally caused bronchoconstriction (Ricciardolo et al, 2000; Schmidlin et al, 2002). A possible explanation for this apparent contradiction could be found in one of those studies. Ricciardolo *et al* had found activation of PAR_2 to result in relaxation in the main bronchi and trachea, but a converse contractile response in the smaller intrapulmonary bronchi (Ricciardolo et al, 2000). The analysis of sputum showed an increase in tryptase, an agonist of PAR_2 in mild to moderate asthmatics (Gibson et al, 2000; Louis

et al, 2000) Immunohistochemical staining for PAR_2 in asthmatic and healthy lung sections revealed a significantly greater staining intensity in biopsies from asthmatic patients than in biopsies from healthy patients (Knight et al, 2001). This illustrates an upregulation of PAR_2 in asthmatic disease (Knight et al, 2001). The increased staining intensity is likely to be as a result of inflammation *per se* and localised in the epithelium and not seen in the smooth muscle on biopsy specimens (Knight et al, 2001). Mast cell tryptase, likely acting through PAR_2 results in an increase mast cell infiltration to subepithelial bronchi tissue and hyperresponsiveness (Berger et al, 1999). These results were later confirmed through *in vivo* studies administering PAR_2 agonists resulting in airway hyperresponsiveness and inflammatory cell infiltration (Schmidlin et al, 2002; Ebeling et al, 2005). In an ovalbumin-induced inflammation model eosinophil infiltration in PAR_2 knockout mice was reduced by 73% over wild-type, and increased by 88% in mice overexpressing PAR_2 (Schmidlin et al, 2002)

PAR_2 activation has also been shown to result in upregulation of inflammatory mediators in lung tissue such as IL-6, IL-8 and PGE_2 (Asokananthan et al, 2002a). Allergen stimulated PAR_2 responses in the lung have also been noted (Sun et al, 2001; Kauffman et al, 2006; Chiu et al, 2007). A recent study linking PAR_2 activation to the sensitisation of TRPV1 have showed PAR_2 activation by aerosol administration of PAR_2 agonist to exacerbate TRPV1 dependant cough by activating PKC, prostanoid release and further sensitising TRPV1 (Gatti et al, 2006). All of which lend evidence to a role of PAR_2 in asthma and COPD.

1.4.6.3 PAR_2 in the Gastrointestinal System

Although all PARs are expressed within the gastrointestinal (G.I.) tract (Vergnolle, 2005) studies have mainly focused on PAR_2. High expression of PAR_2 mRNA has been

identified in the small intestine, colon, liver, and pancreas and to a lesser extent in the stomach (Nystedt et al, 1994; Bohm et al, 1996b). PAR_2 expression has been observed on colonic smooth muscles cells (al-Ani et al, 1995), epithelial cells (Bohm et al, 1996a), colonic myocytes and enterocytes (Cenac et al, 2002), neurons (Reed et al, 2003), myofibroblasts (Seymour et al, 2005) and endothelial cells (Vergnolle, 2005). There is a high degree of expression of PAR_2 within the pancreas and gastrointestinal tract where it is activated by pancreatic trypsin, so resulting in the production of cytokines and regulation of G.I. exocrine functioning through a negative feedback loop (Bohm et al, 1996b; Nystedt et al, 1996; Hirota et al, 2006). Additionally PAR_2 activation directly results in the stimulation of amylase release from pancreatic acini (Hirota et al, 2006). Administering PAR-APs systemically in anesthetised rats causes a rapid increase, followed by a short term decrease in the secretion of pancreatic juices before subsequently resulting in a persistant increased secretion (Kawabata et al, 2000c).

PAR_2s direct activation on enterocytes has been shown to result calcium mobilisation, arachidonic acid and prostaglandin release as well as increase chloride secretion (Kong et al, 1997). Increased chloride secretion in response to PAR_2 agonists has also been observed in studies using whole tissue models (Vergnolle et al, 1998; Green et al, 2000). In rat jejunum results show prostaglandin release to be the major mediator of PAR_2 induced chloride secretion and that nerve activation was not involved (Vergnolle et al, 1998). In pig ileum and mouse colon however the PAR_2 induced chloride secretion is dependant on both eicosanoid release and submucosal neurone activation (Green et al, 2000; Cuffe et al, 2002). Increased chloride secretion has been shown to be functionally and clinically relevant in two aspects, so implicating PAR_2 in both. (Kawabata, 2003; Ossovskaya & Bunnett, 2004; Vergnolle, 2005). Firstly chloride

secretion is linked to fluid transport and results in diarrhoea constituting an important clinical relevance in irritable bowel disease or syndrome (Kawabata, 2003; Ossovskaya & Bunnett, 2004; Vergnolle, 2005). Secondly, enhanced fluid transport resulting from increased Cl^- secretion may provide a protective effect by flushing the mucosal surfaces of potentially harmful pathogens (Kawabata, 2003; Ossovskaya & Bunnett, 2004; Vergnolle, 2005).

Administration of PAR_2-APs and trypsin to rat parotid gland slices (Kawabata et al, 2000c) and sublingual glands (Kawabata et al, 2000b) resulting in the secretion of salivary amylase and mucin respectively. *In vivo* investigation shows PAR_2-triggered salivation and amylase secretion to be partially blocked by an inhibitor of NO synthase suggesting a role endogenous NO (Kawabata et al, 2002)

In studies in mouse gastric fundus PAR_2 has been implicated in the mediation of the contractile response (Corvera et al, 1997), however a later study suggests that this may mask an initial relaxation (Cocks et al, 1999b)

1.4.6.4 PAR_2 in the Renal System

The kidney was the first site shown to express PAR_2 in the study for its initial cloning (Nystedt et al, 1995a). Vasodilation of perfused rat kidney as a result of PAR_2 activation has been reported (Trottier et al, 2002). Another study, also using isolated perfused rat kidneys gave supporting results by showing administration of trypsin and PAR_2-AP caused increased renal perfusion indicating a vasodilatory response (Gui et al, 2003). Interestingly this suggests PAR_2 mediates an opposing vasodilatory response to PAR_1 (see **1.3.10.6**). PAR_2 activation by factor Xa has also been implicated in mesengial cell proliferation (Tanaka et al, 2005). This lead to the investigation and confirmation of PAR_2 involvement in a mesangioproliferative glomerulanephritis animal model where

PAR_2 upregulation and factor Xa accumulation occurred (Nomura et al, 2007). Finally, a recent study with PAR_2 knockout mice demonstrated PAR_2 to have a proinflammatory role in cresentic glomerular nephritis (Moussa et al, 2007).

1.4.6.5 PAR_2 in the Nervous System

PAR_2 has been located throughout the central nervous system (Striggow et al, 2001) including astrocytes, neurons, and brain vasculature (D'Andrea et al, 1998; Ubl et al, 1998). PAR_2 has been shown to be expressed on astrocytes both in rodent primary cultures (Ubl et al, 1998; Wang et al, 2003; Bushell et al, 2006; Park et al, 2006) and on acute brain slices (Pompili et al, 2004; Bushell et al, 2006). Expression of PAR_2 has been further demonstrated on brain white matter (Noorbakhsh et al, 2006) and in the microglia (Goldshmidt & Traynelis, 2006; Noorbakhsh et al, 2006). Within the peripheral nervous system PAR_2 expression is also found in guinea pig myenteric (Corvera et al, 1999) and submucosal neurons (Reed et al, 2003), as well as isolated rat dorsal root ganglia (Noorbakhsh et al, 2003). Upregulation of PAR_2 expression has been shown in organotypic hippocampal slice cultures exposed to severe experimental ischaemia (Striggow et al, 2001). PAR_2 upregulation has been further demonstrated following experimental CNS damage (Olejar et al, 2002). *In vivo* upregulation in a model of stroke was later shown following transient occlusion of the middle cerebral artery (Jin et al, 2005). In PAR_2 knock-out mice treated with the same disease model the infarct volume was increased compared to wild-type (Jin et al, 2005) suggesting PAR_2 has a neuroprotective role in this model of ischaemic brain injury.

In models of HIV associated brain dementias PAR_2 expression levels are increased in response to TNFα and IL-1β, with its activation and preventing neural death from the HIV encoding of Tat (a neurotoxic HIV encoded protein) thus showing a

neuroprotective effect (Noorbakhsh et al, 2005). Contrary to this protective effect a neurodegenerative effect has been suggested in other disease states. PAR_2 involvement in multiple sclerosis (MS) is suggested by high levels of mast cells have been noted in the brains and cerebrospinal fluid of (MS) patients (Roznieckí et al, 1995; Noorbakhsh et al, 2003). The implication of PAR_2 involvement in MS was confirmed by more recent work using PAR_2 knockout mice, which showed PAR_2 activation to result in increased neuronal inflammation with ensuing demyelination and axonal injury (Noorbakhsh et al, 2006). In post-mortem samples taken from MS patients PAR_2 expression is increased in CNS white matter and co-localised with markers for astrocytes and macrophages within areas of demyelination (Noorbakhsh et al, 2006). As such whether a neuroprotective or neurodegenerative effect is seen connected with PAR_2 appears to be dependant on what cell type and tissue.

PAR_2 agonists induce pruritus in human skin (Steinhoff et al, 2003) and a prolonged hyperalgesia results from intraplantar injection of low doses of PAR_2 agonists (Vergnolle et al, 2001). Additionally exposure of peripheral tissues to PAR_2 agonists was revealed to activate nociceptors as observed by an associated increase in Fos expression in the superficial laminae of the distal horn in the spinal cord (Hoogerwerf et al, 2001; Vergnolle et al, 2001; Coelho et al, 2002). The superficial laminae of the distal horn in the spinal cord is an area known to show noicecptive inputs and PAR_2s activation of second order neurons in this region further supports the concept of PAR_2 triggering pain perception (Vergnolle et al, 2003). The presence of PAR_2 in dorsal root ganglia (Steinhoff et al, 2000; Hoogerwerf et al, 2001) and the fact that PAR_2 agonists resulted in enhanced capsaicin and KCl-evoked release of calcitonin gene related peptide (Hoogerwerf et al, 2001) gives more evidence for PAR_2 agonist acting directly on primary nerves in induce a nociceptive signal (Vergnolle et al, 2003). More recent

studies show PAR_2 activation sensitising members of the TRPA and TRPV ion channels family and so contribute to hyperalgesia and the sensation of pain (Dai et al, 2007; Grant et al, 2007).

1.4.7 PAR_2 as a Therapeutic Target

A proper determination of the possible therapeutic effects and usefulness of drugs targeted to PAR_2 is perhaps best left until the future development of specific antagonists that can properly access their effects (Kanke et al, 2005b). The discovery of a number of high-affinity PAR_2 activating peptides which resist peptide metabolizing enzymes (Ferrell et al, 2003; Kawabata et al, 2004; McGuire et al, 2004) have enabled specific evaluation of PAR_2 function, but selective antagonists of the PAR_2 receptor have so far been elusive. Structural activity relationship studies on PAR_1-APs lead to the substitution of the N-terminal serine with a neutral hydrophobic N-acyl group (e.g. *trans*-cinnamoyl) creating a range of peptides which were partial agonists or antagonists of PAR_1 (Bernatowicz et al, 1996). Upon application of the same approach to PAR_2-AP, only peptides with agonistic activity were produced e.g. *trans*-cinnamoyl-LIGRLO-NH_2 (Roy et al, 1998). Two of the peptides produced have been described as having some antagonistic activity but only in respect to trypsin-induced PAR_2 activation (Al-Ani et al, 2002) but did not antagonize PAR_2 to activation by PAR_2-AP.

Thus, a better understanding is required of PAR function and mechanism of action in order to uncover novel strategies for the development of therapeutic tools for use in cardiovascular disease. Recently, *N*-linked glycosylation of PAR_2 was demonstrated to have a profound effect on receptor function, regulating mast cell tryptases ability to activate the receptor (Compton et al, 2001). Tryptase itself is a biomarker for human

mast cell activation and is localised in areas of tissue injury, including the outer layers of blood vessels during development of atherosclerotic lesions (Jeziorska et al, 1997). Unlike trypsin no endogenous inhibitors of tryptase activity have been found. Novel therapeutic agents targeted either against PAR_2 glycosylation or drugs that selectively inhibit mast cell tryptase activity, i.e. without affecting other serine protease activity, might therefore be useful in the treatment of cardiovascular diseases that involve vascular inflammation such as atherosclerosis. The classical approach when targeting a GPCR in therapeutic drug design is to focus development of ligands that will act as agonists, antagonists, or allosteric modulators. Although this has proved successful, as a greater understanding is gained of the regulation of GPCRs and more specifically by covalent modifications that may/can relate tissue specific functions, so we gain an alternative group of targets for GPCR drug discovery.

1.5 POST-TRANSLATIONAL MODIFICATIONS OF GPCR

Following translation a protein can undergo a number of modifications termed post-translational modifications. An example of this is proteolytic cleavage, most proteins undergo some proteolytic cleavage in the removal of the initial methionine, but others are synthesized in an inactive form and require cleavage to activate them (Gether, 2000). Those proteins that are destined for secretion from or inclusion in the membrane will contain a signal peptide that requires cleavage within the rough endoplasmic reticulum (RER). However there are a number of different post-translational modifications including glycosylation, phosphorylation, methylation, acylation. Many eukaryotic signalling proteins undergo lipid modifications of intracellular domains such as prenylation, myristoylation, and palmitoylation (Savarese & Fraser, 1992; Lohse, 1993). Many post-translational modification occur at cysteine residues due to the relative reactivity of the cysteines sulfhydryl group (-SH) (Stryer, 2004). These include disulphide bond formation, palmitoylation, and oligomerisation. Glycosylation, phosphorylation, ubiquitination, disulphide bonds, dimerisation and palmitoylation are discussed below.

1.5.1 Glycosylation

Glycosylation is one of four principal post-translational modifications in the synthesis of membrane and secreted protein (Apweiler et al, 1999). The process involves the addition of saccharides to proteins or lipids, and the majority of proteins synthesized in the rough endoplasmic reticulum undergo glycosylation (Kornfeld & Kornfeld, 1985; Wheatley & Hawtin, 1999). Two types of glycosylation occur**:** *N*-linked glycosylation to the amide nitrogen of asparagine side chains and O-linked glycosylation to the

hydroxy oxygen of serine and threonine side chains. Characteristically, GPCR contain at least one site of glycosylation in the *N*-terminal domain, although some exceptions exist (A_2 adenosine receptor lacks *N*-terminal sites but has glycosylation sites in ECL2) (Wheatley & Hawtin, 1999). *N*-glycosylation is extremely common and nearly two-thirds of the sequences stored in SWISS-PROT contain the potential N-glycosylation consensus sequon, NXS/T (where X can be any amino acid but proline) (Apweiler et al, 1999) and are therefore potentially glycosylated. Historically, glycosylation was thought to regulate receptor cell surface expression but a number of studies have found glycosylation to have direct influence on receptor function (Pang et al, 1999; Zhou et al, 2000; Zhang et al, 2001; Compton et al, 2002). The human secretin receptor was shown to have five putative *N*-terminal glycosylation sites (Pang et al, 1999). Individual mutagenesis of two of these sites results in reduced maximal response and increased EC_{50} with agonist (Pang et al, 1999), results confirmed by binding studies showing both mutants had defective binding of secretin. Another group working with the parathyroid hormone (PTH) receptor found four glycosylation sites which when sequentially removed using mutagensis resulted in little change of receptor expression and function, provided at least one site remained glycosylated (Zhou et al, 2000). The fully non-glycosylated mutant displayed reduced cell surface expression and was completely deficient in agonist binding as shown by binding studies (Zhou et al, 2000).

In PAR_2, two glycosylation sites have been identified and studied, one in N-terminal (N30) and one in ECL2 (N222) which were removed using site-directed mutagenesis to produce the mutations N30A, N222Q and N30A/N222Q (Compton et al, 2002). The N30A mutation resulted in a significant increase in sensitivity to tryptase but no other PAR_2 agonist and N222Q resulted no change in sensitivity towards any of the PAR_2 agonist tested (Compton et al, 2002). The N30A/N222Q mutant also had an increased

sensitivity to tryptase, but a loss in sensitivity towards trypsin and SLIGRL-NH_2 likely caused by a loss in cell surface expression (Compton et al, 2002).

1.5.2 Phosphorylation

Phosphorylation is the addition of a phosphate (PO_4) to a protein (via a protein kinase) and is the most prevalent and widely studied of post-translational modification used in signal transduction. Phosphorylation occurs on serine, threonine and tyrosine residues (in that order of frequency)(Pierce et al, 2002) in eukaryotes and is capable of converting what was previously a nonpolar hydrophobic protein to a polar extremely hydrophilic protein. Thus protein phosphorylation is often an important regulatory event. Rapid phosphorylation occurring as a result of agonist stimulation/activation is a post-translational modification that occurs in nearly all GPCRs (Pierce et al, 2002). As such it effects every basic cellular process from metabolism, cell growth and differentiation to immunity, learning and memory (Manning et al, 2002a; Manning et al, 2002b). It is estimated that 30% of the total cellular protein complement are phosphorylated on at least one of their residues (Cohen, 2000).

The mediators of phosphorylation for GPCRs are generally accepted to be members of the GRK family (which specifically phosphorylate serine and threonine residues) or second messenger kinases (Pitcher et al, 1998) e.g. protein kinase A (PKA) and protein kinase C (PKC). PKA and PKC mediated receptor phosphorylation directly uncouples a receptor from it's associated G-protein (Pitcher et al, 1998). PKA-mediated phosphorylation of β2-adrenergic receptor has also been shown to 'switch' receptor coupling from favouring G_s (which results in desensitization) to favouring G_i (Daaka et al, 1997; Zamah et al, 2002). This switch enables stimulation of G_i pathways such as the ERK MAPK pathway (Daaka et al, 1997; Zamah et al, 2002).

GRK mediated phosphorylation is a general mechanism for regulating GPCR activity through the GRK-β-arrestin system (Pierce et al, 2002). GRK will only recognise and phosphorylate the agonist-occupied conformation of the receptor and therefore promote agonist-specific desensitisation. GRK phosphorylation promotes β-arrestin binding to the receptor which sterically inhibits receptor/G-protein interactions.

Several factors contribute to controlling the activity and specificity of kinases towards receptors (Pitcher et al, 1998). Among them are the various mechanisms by which kinases associate with the plasma membrane, so bringing them into closer association with their targets. These include, interaction of prenylated Gβγ subunits with GRK2 and 3; farnesylation of GRK1; and palmitoylation of GRK4 and GRK6 exclusively associating them with membranes (Pitcher et al, 1998).

1.5.3 Ubiquitination

Ubiquitin (Ub), a 76 amino-acid protein, is post-translationally attached to proteins via the co-operative sequential actions of three enzyme components. An activating E1 enzyme, a conjugating E2 and an E3 ligase (Welchman et al, 2005). Ub is covalently attached to proteins and recognize by ub-binding domains (UBD), which are found on proteins of the endocytic sorting machinery. Upon it's activation by E1, Ub is transferred to E2, which with the aid of E3 specifically attaches ubiquitin to a target protein by the ε-amino group of a lysine residue(Hershko et al, 1983). The Ub chain is further lengthened by E2 and E3 to at least four (polyubiquitination) before the protein is sufficiently ubiquitinoylated to be recognised and degraded by the 26S proteosome (Thrower et al, 2000).

Previous studies suggest that Ub is essential for lysosomal sorting in GPCR, but not for internalisation (Marchese & Benovic, 2001; Shenoy et al, 2001). A study focusing on

PAR_2 found that PAR_2 agonists result in the tyrosine phosphorylation of c-Cbl (an E3 ligase) and subsequent mono-ubiquitination of PAR_2 (Jacob et al, 2005). The band size produced on western blot for mono-ubiquitinated PAR_2 suggests multi-mono-ubiquitination (the addition of a single ubiquitin at multiple lysines on the same protein). A PAR_2 mutant lacking in all 14 intracellular lysine residues (termed $PAR_2\Delta14K/R$) was found to be functionally competent with regards to mobilisation of intracellular calcium and to internalise into early endosomes following activation. However, $PAR_2\Delta14K/R$ was not found to continue into late endosomes or lysosomes so was degradation deficient (Jacob et al, 2005). However, ubiquitination is not required for lysosomal trafficking all GPCRs (Tanowitz & Von Zastrow, 2002). Interestingly a more recent study shows Ub to not be essential for agonist-promoted lysosomal degradation for PAR_1, and in fact has a negative controlling effect on PAR_1 constitutive internalisation (Wolfe et al, 2007).

1.5.4 Disulphide Bridging

A disulphide bridge results from the oxidation of the thiol groups in two cysteine residues creating a cysteine bridge. The resulting S-S bond is extremely strong and confers additional stability to a proteins tertiary structure. All of the GPCRs that have previously been investigated, including PARs, possess conserved cysteine residues in the first and second extracellular loops (Strader et al, 1994; Watson & Arkinstall, 1994). In all those studied these cysteines have been demonstrated by biochemical methods (Curtis et al, 1989; Kurtenbach et al, 1990; Boyd et al, 1996) and site-directed mutagenesis (Dixon et al, 1987; Karnik et al, 1988; Dohlman et al, 1990; Karnik & Khorana, 1990; Kosugi et al, 1992; Savarese et al, 1992; Davidson et al, 1994; Gustavsson et al, 1994; Noda et al, 1994; Perlman et al, 1995; Zeng et al, 1999;

Kuwasako et al, 2003; Yang et al, 2007) to form disulphide bridges, and it is a defining feature of class A, B, and C GPCR (Kolakowski, 1994; Gether, 2000; Kim et al, 2000). The stabilising effect of this disulphide bridge is thought to allow proper folding, cell surface expression, and function of the receptor (Gether, 2000). Additionally in those studies where the conserved cysteine residues were replaced with amino acids such as serine, alanine or valine the data produced suggests the extracellular disulphide bond is critical for proper ligand binding and receptor mediated G-protein activation (Karnik et al, 1988; Karnik & Khorana, 1990; Kosugi et al, 1992; Savarese et al, 1992; Davidson et al, 1994; Gustavsson et al, 1994; Perlman et al, 1995; Cook & Eidne, 1997; Yang et al, 2007). However, the impaired receptor function in many of these mutagenesis studies is due to the lack of receptor delivery to the cell surface.

1.5.5 GPCR Dimerisation

The interaction between ligand-activated G-protein coupled receptors and their specific G proteins is mainly described assuming the GPCR exists in a monomer and that a 1:1 stoichiometry between receptor and G-protein occurs (Bulenger et al, 2005). More and more evidence has been coming to light suggesting many if not all GPCRs are capable of forming and functioning within dimers or larger oligomeric complexes, so pointing towards alternative mechanisms of action (Bulenger et al, 2005). The idea that GPCRs could exist as dimers was for many years considered to be controversial and flew in the face of popular opinion (Marshall et al, 1999; Bouvier, 2001; Bulenger et al, 2005). Now however, far from controversial, this concept has progressively become more widely accepted among those studying GPCRs (Bulenger et al, 2005). A significant point in accepting the dimer premise was the finding that the metabotropic $GABA_B$ receptor exists as an obligatory heterodimer (Marshall et al, 1999). This finding,

originally thought to be an exception, was gradually shown in other GPCRs as a result of a large number of studies using resonance energy transfer (RET) methods showing receptors could form both homo and hetero-dimers in living cells (Angers et al, 2002; Eidne et al, 2002; George et al, 2002) and pictures of rhodopsin formed into homodimers in native disc membranes (Fotiadis et al, 2003) finally swayed remaining sceptics. Dimerisation has been shown to have numerous effects on the functioning of GPCRs, affecting signalling, pharmacology, trafficking, internalisation and desensitisation (Angers et al, 2002). In some cases dimerisation has been shown to occur, or be in part aided by, covalent association of N-terminal cysteines in a number of receptors such as the calcium receptor and metabotropic glutamate receptor (Romano et al, 1996; Bai et al, 1998; Goldsmith et al, 1999; Pace et al, 1999; Ray et al, 1999; Romano et al, 2001; Overton & Blumer, 2002). All four PARs have cysteine residues present in the N-terminus, three of which have the residues prior to their activating peptide (PAR_1 – C12, C16, PAR2 – C22, and PAR_3 – C19). Cysteine residues by their nature do not often remain unpaired (Torrecilla & Tobin, 2006) suggesting a possible interaction inter- or intra-molecularly.

1.5.6 Palmitoylation

Palmitoylation is one of the most commonly occurring post-translational modifications occurring in proteins (Bijlmakers & Marsh, 2003). Palmitoylation can be described as a reversible post-translational modification which involves the covalent attachment of a 16-carbon unsaturated fatty acid to cysteine residues on a protein, associating it with the plasma membrane. The reaction involves the nucleophilic attack of the target proteins thiolate ion ($-S^-$) on the ester bond of palmitoyl coenzyme A (Torrecilla & Tobin, 2006).

The result is palmitoylation of the protein by transfer of the fatty acid and the formation of a new thioester bond (see **fig 1.5.6a**).

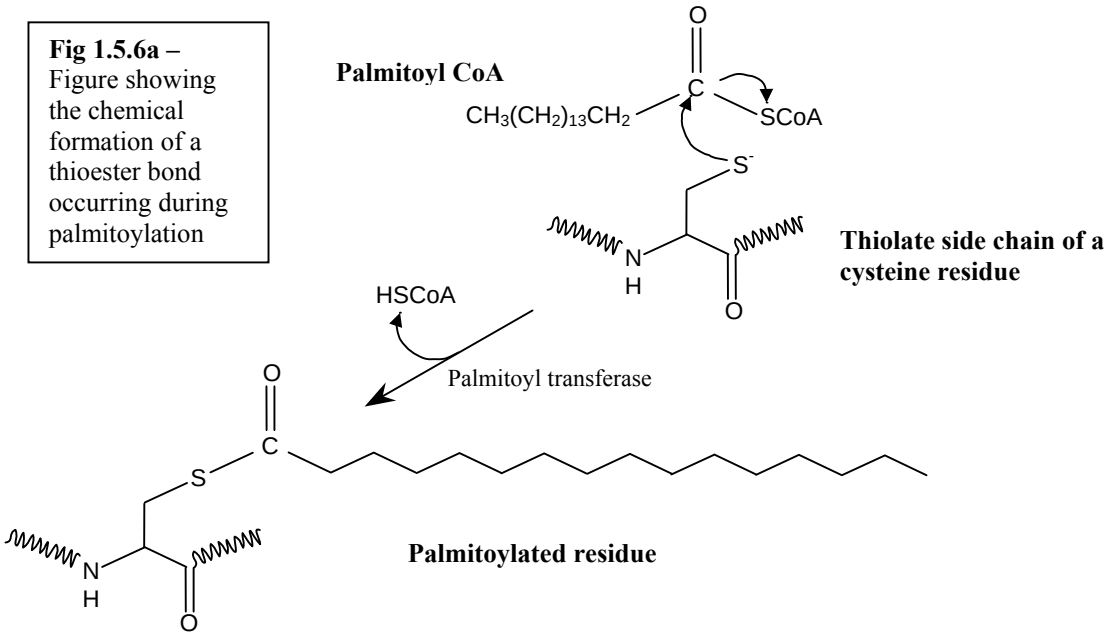


Fig 1.5.6a – Figure showing the chemical formation of a thioester bond occurring during palmitoylation

The covalent thioester linkage enables palmitoylation to be distinguished from other lipid modifications since the thioester-cysteine residue bond is sensitive to treatment with mild-alkali (Schmidt et al, 1979) and is therefore a useful chemical method to differentiate it from the amide or oxygen ester linkages found in other lipid modifications. In order to demonstrate the presence of palmitoylation, a frequently used experimental approach is to chemically dissociate radio-labelled palmitate from the protein using dithiothreitol or hydroxylamine at a neutral pH (Qanbar & Bouvier, 2003). The specific features or motifs required within a protein to target it for palmitoylation are poorly understood, and until recently the existence of specific palmitoylating and depalmitoylating enzymes was a matter of some contention (Magee & Seabra, 2005). However, the discovery of a number of palmitoyl enzyme transferases (PATs), first in yeast and then mammalian cells (Hayashi et al, 2005; Fernandez-Hernando et al, 2006; Hundt et al, 2006) demonstrated palmitoylation to be a protein mediated event (Mitchell et al, 2006). Cysteines situated near either transmembrane domains (TMD) or

membrane associated domains appear to be the preferred sites of palmitoylation, perhaps due to the apparent membrane association of PATs (Lobo et al, 2002; Roth et al, 2002). This is quite clearly not the full story though, as palmitoylation occurs in many cellular locations and not all cysteines located near TMD are palmitoylated, so additional factors must be involved.

During a proteins lifetime it is capable of undergoing numerous cycles of palmitoylation and depalmitoylation, and it is this that has led to the hypothesis that similar to phosphorylation, palmitoylation maybe a cell regulatory mechanism (Mumby, 1997). There are a number of supportive studies for this contention: 1) An increase in palmitate turnover can occur in response to stimuli (James & Olson, 1989) (see **table 1.5.6.1a**), 2) Palmitoylation is dependently regulated in some systems (Melendez & Bizzozero, 1996; Patterson & Skene, 1999; Veit, 2000), 3) Palmitoylation in some proteins is dependant on the cell cycle (Mundy & Warren, 1992) and 4) Palmitoylation can occur in anucleated cells such as erythrocytes (Staufenbiel, 1988) and platelets (Seehafer et al, 1988; Huang, 1989; Sim et al, 2007).

1.5.6.1 Palmitoylation function

The functional implications of palmitoylation initially depend on whether the palmitoylation is constitutive: palmitoylation occurring during or shortly after protein synthesis, or dynamic: regulation of palmitoylation state of a mature GPCR once at the membrane. As such, the function of palmitoylation for a protein could be the independent fulfilment of two separate roles: the processing and targeting of a protein to the correct membrane site, and a signalling function in the final receptor (Escriba et al, 2007). The rapid turnover of palmitoylation supports the idea that is likely to be a regulatory mechanism within the cell, similar to phosphorylation (Mumby, 1997). In fact an increase is palmitate turnover in response to agonist activation has been

demonstrated with a number of GPCRs (see **Table 1.5.6.1a**), such as β2-adrenergic receptor and α2 adrenergic receptor which have activation-dependant palmitoylation/depalmitoylation cycles (Mouillac et al, 1992; Kennedy & Limbird, 1994). However, when assessing the effect of GPCR palmitoylation on G-protein coupling, receptor desensitisation, internalisation and expression there appears to be no general consensus (see **Table 1.5.6.1a**). It should be noted though that caution should be taken when reviewing results from studies in which predictive palmitoylation sites have been removed by site-directed mutagenesis. Firstly, unless palmitoylation of the mutated cysteine has been confirmed, by a method such as [^{3}H]-palmitate incorporation, then there are a number of other covalent modifications occurring on sulfhydryl groups which could equally be responsible for observing signalling irregularities. For example, the radically decreased half-life of the cysteine mutated A1 adenosine receptor (compared to wild-type) (Gao et al, 1999), along with no other apparent effect, may be attributed just as easily to a poorly folded tertiary protein structure being degraded by normal cellular machinery. Interestingly, in some GPCRs the elimination of a specific palmitoylated cysteine seems to exercise a different influence of G-protein coupling depending on which Gα sub-unit is investigated. An example of this is seen with the multiple palmitoylation of the endothelin subtype B receptor on cysteine residues 402, 403 and 405 (C402, C403 and C405) (Okamoto et al, 1997). Loss of all palmitoylation sites results in ablation of all $G\alpha_q$ and $G\alpha_i$ coupling. When palmitoylated C402 alone the receptor is capable of coupling with $G\alpha_q$ but not $G\alpha_i$, with the latter apparently requiring a higher level of palmitoylation for a successful interaction (Okamoto et al, 1997). The group speculated that the observed loss in G-protein coupling and downstream signalling noted in these studies are likely, at least in part, to be affected by enhanced receptor phosphorylation in the absence of palmitoylation. In the case of GPCRs with

C-terminal palmitoylated cysteines the receptor phosphorylation sites are usually within close proximity. Using β-adrenergic as an example, the non-palmitoylated mutant is basally hyper-phosphorylated and doesn't undergo any further phosphorylation upon agonist activation (Moffett et al, 1993). As previously covered in section **1.5.2**, phosphorylation is the first step in receptor desensitisation and initiating receptor uncoupling from its associated G-protein. This was further highlighted in a recent study using knock-in mice that expressed a palmitoylation-deficient form of rhodopsin (Wang et al, 2005). The mutation lead to enhanced receptor phosphorylation resulting in an increased shut-off of photo-transduction following stimulation (Wang et al, 2005). These data then indicate an additional role for palmitoylation in regulating access to receptor phosphorylation sites, and thus inhibiting rapid desensitisation. Additionally, phosphorylation is often related to receptor internalisation, resulting in signal termination and receptor degradation or recycling. As such, with some GPCRs removal of palmitoylation resulted in enhanced phosphorylation, desensitisation and internalisation (see **table 1.5.6.1a**) e.g. 5-HT_{4a} (Ponimaskin et al, 2005) and LH receptor (Munshi et al, 2005), thus providing further evidence of the importance of receptor palmitoylation in GPCR-mediated signal transduction. However, not all receptors studied are uniformly phosphorylated following depalmitoylation, work with a number of GPCRs show their palmitoylation status to have no effect on the ability of the receptor to become phosphorylated (Nakata et al, 1994; Hayashi & Haga, 1997; Blaukat et al, 2001). The M_2-muscarinic receptor has a short C-terminal containing its palmitoylation site, but its phosphorylation sites are found within the ICL3 (Nakata et al, 1994; Hayashi & Haga, 1997). Mutation of the palmitoylation site in this receptor has no affect on phosphorylation (Hayashi & Haga, 1997). The bradykinin B_2 receptor which has C-terminal phosphorylation sites (Blaukat et al, 2001) which might be

expected to be susceptible to the structural implications of palmitoylation, similarly is unaffected by agonist mediated phosphorylation in the palmitoylation deficient mutant (Pizard et al, 2001). As such the apparent relationship between phosphorylation and palmitoylation is not universally applicable.

Table 1.5.6.1a Table showing a list of GPCR previously shown to be palmitoylated along with the effects resulting removal of palmitoylation site by mutagenesis. Abbreviations: 5-HT - 5 hydroxytrptamine, LH-hCG – luteinizing hormone/human chorionic gonadotropin.
X – not investigated, ↑ - increased or enhanced, ↓ - decreased

Receptor	G-protein coupling	palmitate turnover	Phosphor-rylation	β arrestin coupling	Interna-lisation	Flanking sequence	Reference
α2-adrenergic	no change	↑	X	X	X	HDFRRAFKKIL**C**RGDRKRIV	(Kennedy & Limbird, 1994) (Eason et al, 1994)
β2 adrenergic	reduced to G_s	↑	↑	X	X	DFRIAFQELL**C**LRRSSLKAYG	(O'Dowd et al, 1989; Mouillac et al, 1992)
Adenosine A_1	no change	no change	X	X	no change	FFQKHIAKRF**C**K**CC**SIFQQEAPER	(Gao et al, 1999; Ferguson et al, 2002)
Adenosine A_3	X	X	↑	X	↑		(Ferguson et al, 2002)
Bradykinin B_2	no change	X	no change	X	↓		(Soskic et al, 1999; Pizard et al, 2001)
Chemokine CCR5	reduced to G_i	no change	↓	X	↓	FRTAYQQLFR**C**RPASHNAQET	(Fukushima et al, 2001)
Dopamine D_1	no change	↑	X	X	X	RKAFSTLLG**C**YRL**C**PATNNAIETV	(Jensen et al, 1995) (Jin et al, 1997; Jin et al, 1999)
Dopamine D_2	X	X	X	X	X		(Ng et al, 1994)
Endothelin ET_A	reduced to G_i and G_q	X	X	X	X		(Horstmeyer et al, 1996; Doi et al, 1999)
Endothelin ET_B	reduced to G_i and G_q	X	X	X	no change	RFKNCFKSCL**CC**W**C**QSFEEKQSLE	(Okamoto et al, 1997)
$mGlu_4$	X	no change	X	X	X		(Alaluf et al, 1995)
5-$HT1_{(a)}$	reduced to G_i	no change	X	X	X		(Butkerait et al, 1995)
5-$HT1_{(b)}$	X	X	X	X	X		(Ng et al, 1993)
5-$HT4_{(a)}$	no change	↑	↑	X	↑	L**CC**DDERYKRPPILGQTVPCSTTTINGSTHVLRDTVECGGQWESR**C**	(Ponimaskin et al, 2002; Ponimaskin et al, 2005)
LH/hCG	no change	X	↑	↑	↑	DFLLLLSRFG**CC**KRRAELYRRK	(Munshi et al, 2001; Munshi et al, 2005)
Muscarinic M_2	reduced to G_o and G_{12}	X	no change	X	X	KKTFKHLLM**C**HYKNIGATR	(van Koppen & Nathanson, 1991; Hayashi & Haga, 1997)
μ-opioid	X	X	X	X	X		(Chen et al, 1998)
Rhodopsin	no change	X	X	X	X	QFRNCMVTTL**CC**GKNPLGDDEA	(Ovchinnikov Yu et al, 1988; Karnik et al, 1993)
Somatostatin SST_5	reduced	X	↓	X	↓		(Hukovic et al, 1998)
TRH	no change	X	X	X	↓	FRAAFRKL**C**N**C**KQKPTEK	(Nussenzveig et al, 1993)
Vasopressin V_{1A}	no change	↑	↓	X	no change	LLQDCVQSFP**CC**HSMAQKFAKD	(Hawtin et al, 2001)
Vasopressin V_2	no change	↓	no change	↓	↓		(Charest & Bouvier, 2003)

1.5.7 Post-translational Modifications of Cysteines in PARs

Post-translational modifications at intracellular and extracellular cysteine residues on GPCRs have been shown to have a crucial role in receptor function, as discussed above (Morello & Bouvier, 1996; Zeng et al, 1999; Ennion & Evans, 2002).

Thus, an increase in understanding of the presence and effect of these modifications on PAR_2 function may facilitate the development of novel therapeutic tools towards this receptor. However, many post-translational modifications of cysteine residues in the PAR family and their subsequent effects are currently unknown.

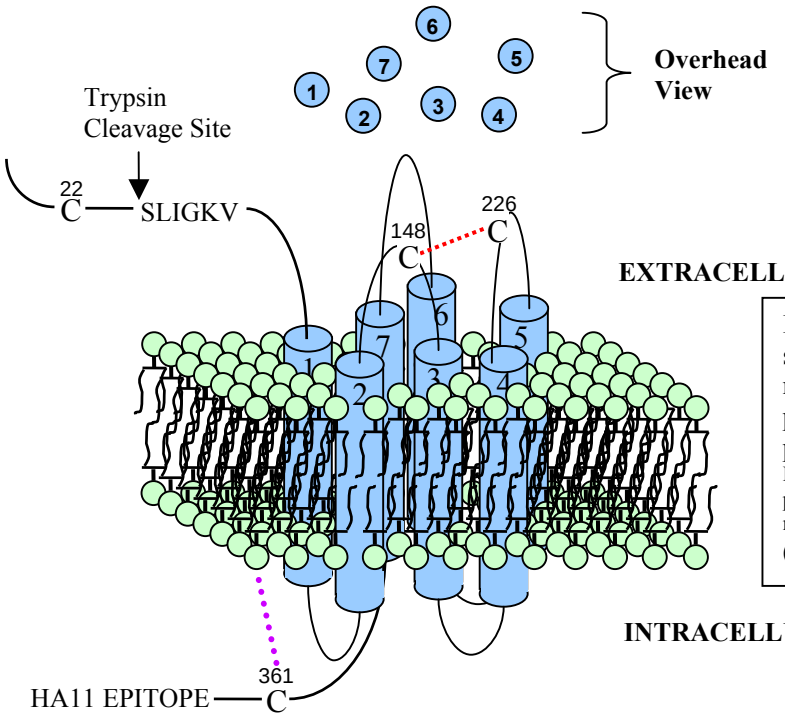


Fig 1.5.7a: Diagram of $hPAR_2$ showing the four cysteine residues of interest and the putative disulphide bridge and palmitoylation site. Helical positions placed according to prediction for Family A GPCR modelled from rhodopsin. (Gether, 2000).

We have identified four cysteine residues of interest (three extracellular C22, C148, and C226, and one intracellular C361) on $hPAR_2$ that are putative sites of post-translational modification (see **Fig. 1.5.7a:** full $hPAR_2$ sequence available in APPENDIX 4) identified by their positions within their respective receptor domains. It is our intention

to investigate the roles of these extracellular and intracellular cysteines in regulating $hPAR_2$ function.

1.5.5.1 Disulphide Bridging in PARs

Interestingly, the existence of this putative disulphide bridge has never been demonstrated in PARs, so the potential importance of these conserved cysteines (C148 and C226 in $hPAR_2$) has yet to be investigated.

1.5.5.2 Dimerisation in PARs

Although there has been a large body of previous work showing dimerisation of GPCRs (especially those of Class A) until recently no work had been carried out displaying that PARs could or do form homo- or hetero dimer. However, a study showing NMR of the structure of the thrombin-cleaved PAR_1 exodomain clearly indicates that PAR_1's mode of binding leaves thrombin still capable of interacting with other macromolecular structures such as PAR_4 (Seeley et al, 2003). This along with the known interaction of PAR_3 as a high affinity thrombin binder for adjacent PAR_4 molecules seen in mouse platelets (Nakanishi-Matsui et al, 2000) spurred on the search to PAR dimers. As such a group recently succeeded in co-immunoprecipitating PAR_1 and PAR_4, as well fluorescenct resonance energy transfer (FRET) studies demonstrating PAR_1 and PAR_4 form a heterodimer (Leger et al, 2006). Further to this a co-factoring of PAR_1 and PAR_4 was shown by acceleration of thrombin cleavage and signalling for PAR_4 when co-expressed with PAR_1. The impact on physiology of this $PAR_{1/4}$ heterodimer is that thrombin is capable of acting as a bivalent functional agonist in human platelets (Leger et al, 2006). An even more recent study has shown PAR_1 and PAR_3 to heterodimerise (McLaughlin et al, 2007). This study displayed that PAR_1 was capable of constitutively

forming both $PAR_{1/1}$ homodimers and $PAR_{1/3}$ heterodimers through using bioluminescent resonance energy transfer (BRET), and that PAR_1's dimerisation affinity was equal in both. The function of a non-signalling PAR_3 in humans has long been unknown, so the finding that the $PAR_{1/3}$ heterodimer signalled in a way distinct from $PAR_{1/1}$ homodimer involving selective coupling to $G_{\alpha 13}$ was made all the more interesting. They were also able to use siRNA to cause PAR knockdown in endothelial cells to demonstrate PAR_3's regulation of PAR_1-mediated increase in endothelial permeability (McLaughlin et al, 2007).

Dimerisation has yet to be demonstrated for PAR_2, however PAR_1 and PAR_2 are very structurally similar and peptides corresponding with the TL of PAR_1 have been shown to activate PAR_2 (Blackhart et al, 1996; Hollenberg et al, 1997), and PAR_1 is suspected to be able to transactivate pre-cleaved PAR_1 receptors (Chen et al, 1994). As such when investigated, the transactivation of PAR_2 was shown to be possible by the TL of an activated PAR_1 receptor (O'Brien et al, 2000), thus enabling the contribution of PAR_2 to the thrombin response. This demonstrates that PAR_1 and PAR_2 are located sufficiently close to one another in the plasma membrane to allow the cleaved N terminus of PAR_1 access to PAR_2.

We have identified a cysteine residue in the N terminus of PAR_2 (C22) which is of unknown purpose; we intend to investigate the affect of removing this cysteine on receptor expression and function.

1.5.5.3 Palmitoylation and PARs

To date, little work has been done on the palmitoylation state of PARs. A recent study by Swift (Swift et al, 2006) on the activation mechanism employed by PAR_1 used a

computer model, based on the 2.8Å x-ray structure of rhodopsin (Palczewski et al, 2000), to predict key structural elements within the receptor, including palmitoylation sites at cysteine 387 and 388 (Swift et al, 2006). In the study C→S site directed mutagenesis of these sites resulted in 4-9 fold shifts in EC_{50} values for thrombin and SFLLRN but had no effect of maximal signal. A single point mutation of C387 to an alanine had no effect on the agonist EC_{50} values suggesting that either palmitoylation is only required at one cysteine, or that mutation C388 is what resulted in the shift in agonist EC_{50} values. An even more recent study investigating palmitoylation in platelets, using pharmacological inhibition or removal of palmitoylation in intact cells and *in vivo*, rather than simple point mutations in single proteins, shows the essential role of palmitoylation in platelet function (Sim et al, 2007). Activation of platelets was shown to cause the translocation of APT1 (acyl-protein thioesterase1: a cellular enzyme responsible for depalmitoylation) from the cytosol to the membrane or cytoskeletal compartments. Thus, demonstrating the responsiveness of cellular palmitoylation machinery to platelet activation. Incubation of permeabilised platelets with APT1, in an effect to reduce platelet protein palmitoylation, lead to inhibition of PAR_1-AP mediated platelet α-granule secretion (Sim et al, 2007). *In vivo*, using cerulenin (which inhibits addition of new palmitate to proteins) to treat platelets prior to infusing them into mice resulted in an inhibition of platelet incorporation into thrombi following laser induced injury (Sim et al, 2007). As such the group suggested that palmitoylation may serve as a novel target for the development of new anti-thrombotic compounds.

Interestingly, although Swift et al used sequence alignment to highlight putative palmitoylation sites in other rhodopsin-like GPCR, including PAR_2, to date no work has been carried out on the palmitoylation state of PAR_2. We have identified a cysteine residue within the C-terminal domain of $hPAR_2$ (C361: **fig 1.5.7a**) that is a putative

palmitoylation site. Due to the wide range of effects connected with palmitoylation within GPCRs it is unclear what role it may play in PAR_2 function.

1.6 WORKING HYPOTHESIS

"Extracellular and intracellular cysteines of $hPAR_2$ undergo modifications that regulate receptor function"

1.7 AIMS

The thesis has 3 broad aims:

❶ Determine whether C22 is a free cysteine and its role in regulating PAR_2 function.

❷ Assess whether C148 and C226 form a disulphide bridge and their role in regulating PAR_2 function.

❸ Determine whether C361 is palmitoylated and its role in regulating PAR_2 function.

2 GENERAL MATERIALS AND METHODS

2.0 GENERAL MATERIALS AND METHODS

2.1 Materials and Reagents

2.1.1 General Laboratory Reagents

De-ionized water was produced using a Purite Select deionizer. HEPEs, Tris, Tris-HCl, NaCl, KCl, $CaCl.6H_2O$, Glucose, Phosphate Buffer Saline (PBS), DMSO, β-mercapethanol, potassium EDTA, Rabbit serum and BSA were all purchased from Sigma-Aldrich Chemicals Group (Dorset, UK).

2.1.2 Cloning Materials

pcDNA3.1 containing $hPAR_2$ C-terminal tagged with a HA11 epitope was supplied by supervisor Dr. S.J. Compton, Respiratory Medicine, University of Hull. PCR primers were designed in house and made by MWG Biotech (Milton Keynes, UK).
XL1-Blue supercompetent cells and QuikChange® Site-Directed Mutagenesis Kit was purchased from Stratagene® Europe (Amsterdam, NL), along with competent *E.Coli* . The rapid DNA ligation kit was purchased from Roche. All restriction enzymes used were purchased from Promega Ltd. (Southampton, UK), along with their specific restriction buffers and Bovine Serum Albumin (BSA). Ampicillin (sodium salt), and ethidium bromide at 10mg/ml were purchased from Sigma-Aldrich Chemicals Group, (Dorset, UK). Accuprime DNA polymerase, Miller's Luria-Bertani (LB) broth base, 1 Kb Plus DNA Ladder, Miller's LB agar powder were all purchased from Invitrogen (Life Technologies Inc. Paisley, UK).

2.1.3 Cell Culture Materials

Alpha-MEM (minimal essential media), heat inactivated foetal calf serum (FCS), Phosphate Buffer Saline (PBS) 10X, Non-enzymatic Cell Dissociation Fluid, and the antibiotics Geneticin (G418), penicillin and streptomycin with L-glutamine were purchased from Invitrogen (Life Technologies Inc. Paisley, UK).

2.1.4 Transfection and Cell Sorting Materials

Chinese Hamster Ovary (CHO) Pro5 parent cells were supplied by ATCC LGC Promochem, (Middlesex, UK). Opti-MEM media with glutamax and Lipofectamine 2000 (1µg/µl) were purchased from Gibco BRL, (Life Technologies Inc. Paisley, UK). B5 rabbit polyclonal anti-human PAR_2 was a kind gift from Professor Morley Hollenberg, University of Calgary, Canada.

2.1.5 FACS Materials

Sam11 mouse anti-human PAR_2 antibody was purchased from Cambridge Bioscience (Cambridge, UK.). FITC-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Sigma-Aldrich Chemicals Group (Dorset, UK).

2.1.6 Ca^{2+} Signalling Materials

Fluo-3, AM *cell permeant* was purchased from Molecular Probes (Invitrogen Inc, Paisley, UK.). Dimethyl Formamide (DMF) and Sulphinpyrazone was purchased from Sigma-Aldrich Chemicals (Dorset, UK.).

2.1.7 Western Blotting Materials

Precision Plus Protein™ Standards were supplied by Bio-Rad. Methanol was supplied in house by the University of Hull Chemistry Department. Acrylamide, Trizma base, glycine, ammonium persulphate and temed were all supplied by Sigma-Aldrich Chemicals (Dorset, UK.). HA11 antibody was produced by Convance and supplied through Cambridge Biosciences (Cambridge, UK). ECL™ Western Blotting System, Hypond P Protein Binding Membrane, Bucks, UK. MPER® mammalian protein extraction reagent was supplied by Pierce (Northumberland, UK) as were Handee® mini-spin columns. Mammalian HA-Tag Immunoprecipitation/Co-immunoprecipitation agarose conjugated antibody was purchased from Vectorlabs (Peterborough, UK).

2.1.8 Confocal Microscopy Materials

Propidium iodide was purchased from Sigma-Aldrich Chemicals Group (Dorest, UK). Coverslips and slides were purchased from Fisher Scientific (Loughborough, UK) and antifade CITI-Fluor AF1 was purchased from CITI-Flour Ltd (London, UK).

2.1.9 Laboratory Equipment

PCR and Mutagenic PCR were carried out on a MWG AG Biotech Primus 96 Plus Thermocycler. DNA was quantified using an Amersham Genequant Pro. Fluorescence Activated Cell Scanning (FACS) was carried out on a Beckton Dickinson FACS Calibur. SDS-page was carried out using Jencons vertical protein gel apparatus purchased from SLS Laboratory Equipment. Gels were analysed/photographed using UVP Laboratory Products Epichem II Darkroom. The confocal microscope use in this work was a Nikon Eclipse (TE2000-E) microscope with a BioRad Radiance

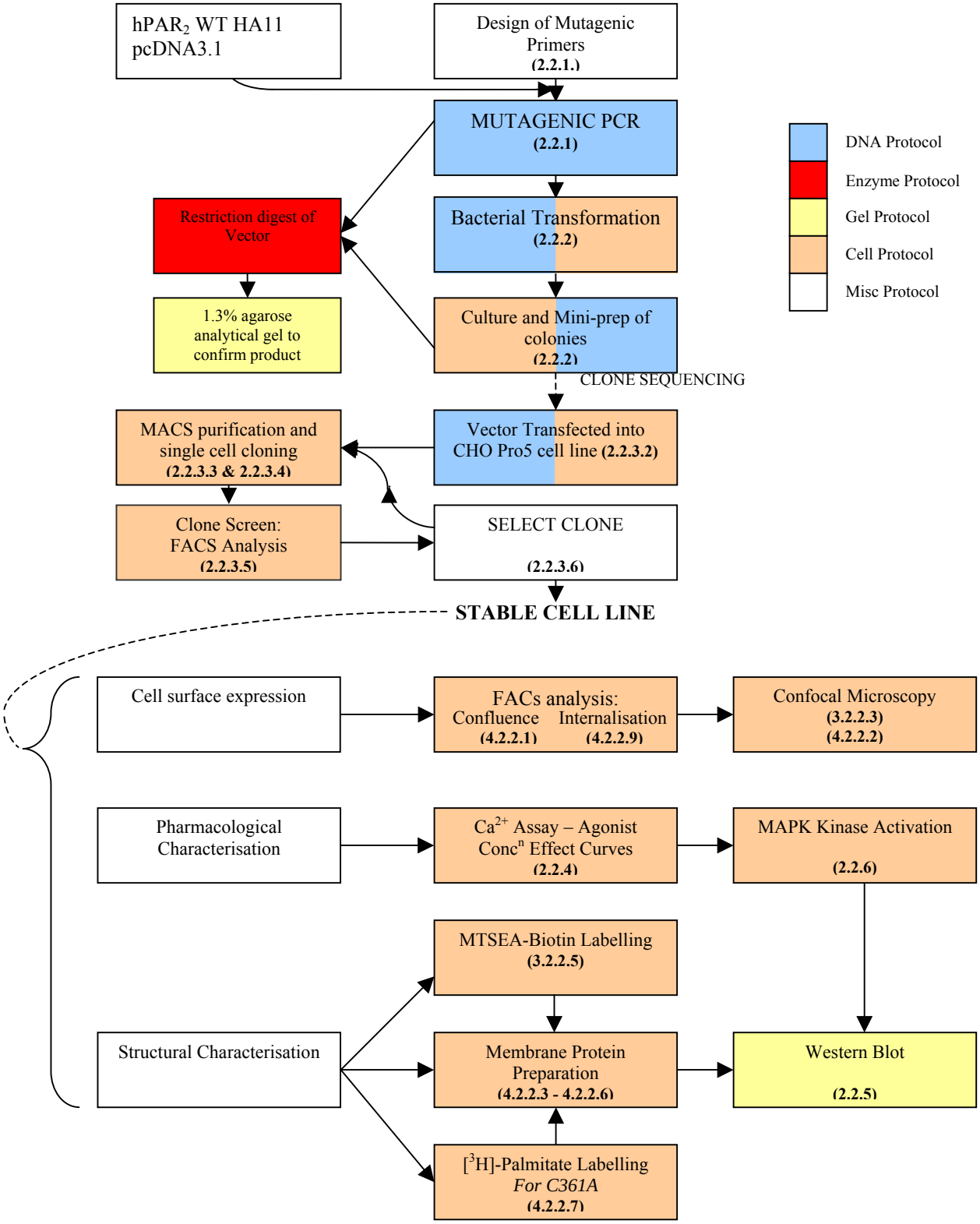
2100 scanning system and lasers. Calcium signalling was carried out on a Photon Technology International (PTI) Fluorimeter.

2.1.10 Calculations and Statisical Analysis

Calculations were carried out using Microsoft Excel XP and graphs were produced using Prism Graphpad Version 4. Statistical tests were carried out depending on the specifics of the datasets to be compared. For a two-dataset comparison of group mean a two-tailed test was used, when this was to compare two different different treatments of of the same cell line, or when this was to compare two different cell lines subjected to the same treatment a paired t-test was adopted. In order to assess a change over a period of time using a specific treatment, or to assess a change over a concentration range for a specific cell line a repeated measures one-way ANOVA table was used. When a comparison of two different time courses or concentration ranges was required a two-way ANOVA table was adopted.

2.2 METHODS

This section will describe the final methods developed to carry out this project. The flow diagram below shows an overview of the research project and how methods used fit together. The boxes are referenced to the particular sub-chapter containing more in-depth information on the protocol.



2.2.1 Mutagenic PCR (MPCR)

The pcDNA 3.1 vector containing wild-type $hPAR_2$ (wt-$hPAR_2$) with a C-terminal HA11 tag was supplied by Dr. S. J. Compton (Respiratory Medicine, University of Hull) from previous work. Specific PCR oligonucleotide primers were designed and custom made for the mutagenic PCR of the wild-type vector (primers for specific mutants can be found in the relevant chapter).

The mutant constructs were prepared as below:

pcDNA3.1 $hPAR_2$.HA11	10 µl	(30 ng)
10X Reaction Buffer	5 µl	
dNTP mix	1 µl	
Pfu Turbo® polymerase	1 µl	(2.5 U/µl)
Mutagenic Primer upstream	1.25 µl	(125 ng)
Mutagenic Primer downstream	1.25 µl	(125 ng)
DEPC dH_2O	31.5 µl	
Total Volume	50 µl	

The mutagenic PCR was performed in a thermocycler as follows:

Step 1 - hotstart - 95°C - 30 secs

Step 2 - denaturation - 95°C - 30 secs

Step 3 - annealing - 55°C - 1 min

Step 4 - extension - 68°C - 7 min (1 min/Kb)

Goto Step 2 for 16 cycles

In order to remove of the parental vector, 1 µl of *Dpn* I restriction enzyme was added to each sample. The samples were then incubated at 37°C for 1 hour.

2.2.2 Bacterial Transformation

The bacterial transformations were carried out as per standard transformation protocol provided by Promega (Edition April 2000, part III).

5 ml falcon tubes were pre-chilled on ice before adding 50 µl of XL1-Blue supercompetent *E.coli* (one tube for each sample to be transformed + one control). The cells were then incubated on ice for 10 mins with 0.5% β-mercaptoethanol, swirling at regular intervals. 1 µl of vector DNA from the mutagenesis PCR was then added to the cells swirled and incubated on ice for 10 mins. An extra transformation using 30 ng of pcDNA3.1(-) was carried out as a control. The cells were then heat shocked at 42^{o}C for 45 secs before returning to ice for a further two mins. 500 µl of LB broth (pre-heated to 37^{o}C) was then added and the cells incubated at 37^{o}C, 200 rpm for 60 mins.

Transformants were then selected via plating 250 µl of transformation onto LB agar plates containing ampicillin (50 µg/ml) and incubating at 37^{o}C overnight along with the transformation control. Colonies were picked at random (5 from each sample plate) and inoculated into 5 ml LB media containing ampicillin (100 µg/ml) and incubated at 37^{o}C, 200 rpm overnight.

Mini-preps were then produced from the overnight cultures using a QIAgen Miniprep Kit as per manufacturers instructions (edition: April 1998, pg 16-19). A small amount of the resulting DNA was digested using Hind III /Not I restriction enzymes in NEB Buffer 2 (as below) and run on a 1.3% agarose gel along side 1 Kb Plus DNA Ladder to assess the presence of insert. The DNA concentration and purity were then assessed using Genequant and an aliquot of sample sent to MWG Biotech for insert sequencing using T7 primers (see **Appendix** for sequence).

2.2.3 Preparation of Receptor Expressing Cell lines

2.2.3.1 Maintainance of CHO Pro5 cells

CHO Pro5 parent cells (un-transfected) were routinely maintained in T75 flasks with 12 ml of growth media produced as below:

Plain Media for Un-transfected Cells

Dulbecco's Alpha-MEM with ribonucleosides	500 ml
10% FCS	(50 ml of 100x solution added)
1 mM penicillin and streptomycin	(5 ml of 100 mM solution added)
2 mM L-glutamine	(5 ml of 200 mM solution added)

Media for both wild-type and mutant human PAR_2 expressing cells was made as plain media and supplemented with 500 mg of Geneticin (G418) to give a final concentration of 1 mg/ml. Cells were seeded into T75 ventilated cap flasks and were maintained at 37^{o}C in a humidified atmosphere containing 5% CO_2.

The Pro5 cells used in this study were passaged at 80-90% confluence (typically every 4 days) in a ratio of 1:10-1:15 (cells:media). Cells were added to fresh media after washing with 5 ml PBS and dissociating the cells from the flask using 5 ml of Cell Dissociation Buffer (non-enzymic).

2.2.3.2 Transfection of Pro5 Cells

The transfection protocol (described below) was carried on CHO Pro5 parent cells at 50-70% confluence in T25 flasks under sterile conditions.

Two 5 ml falcon tubes, both containing 2 ml of OPTI-MEM, were prepared. To one tube 20 µl of Lipofectamine 2000 was added, to the other tube 5 µg of vector DNA was added. Both tubes were then incubated at room temperature (RT) for 15 mins before being combined, vortexed, and then incubated at RT for a further 15 mins. Old growth media was removed from Pro5 un-transfected cells and the cells were washed with 2ml

of OPTI-MEM before aspiration. The DNA/Lipofectamine mix was then added to the flasks and incubated at 37°C, 5% CO_2 for six hours. Three ml of plain media was then added and the flask incubated at 37°C, 5% CO_2 for a further 18 hours. The plain media was then removed and the cells washed with 2 ml PBS before adding 5 ml of fresh media containing G418 selective antibiotic. Cells were the incubated at 37°C, 5% CO_2 for a further 24 hours before being passaged up to a T75 flask to prepare for single cell cloning.

2.2.3.3 Magnetic Activated Cell Sorting (MACS) Purification of Transfected Cells

Running Buffer for MACS Purification

Old media was poured off and the cells washed with 5 ml of PBS (1X) and dissociated using 5 ml of Cell Dissociation Fluid (non-enzymic) before cell number was assessed. The cell suspension was transferred to a 5 ml falcon tube and centrifuged at 500 *g* for 5 min at 4°C. The supernatant was disposed of and the pellet resuspended in running buffer (PBS 1X, EDTA 2 mM, BSA 0.5%) to give a cell concentration of $3x10^7$ cells in 300 µl (100 µl per $1x10^7$ cells). Sam-11 anti-$hPAR_2$ mAb was added to the cell suspensions (1 in 300 ≡ 3 µg/ml) and incubated at 4°C for 30 min swirling gently after 15 min. 1ml of cold-PBS was then added and then centrifuged at 500 *g* for 10 min at 4°C. The supernatant was then disposed of and the pellet resuspended in 80µl of running buffer per $1x10^7$ cells. 20µl of MACS anti-immunoglobulin Microbeads were added per $1x10^7$ cells, mixed well and incubated at 4-8°C for 15 min. 1ml of cold PBS was then added and the sample centrifuged at 500 *g* for 10 min at 4 °C, the supernatant was then disposed of and the pellet resuspended in 500 µl of Running Buffer per $1x10^8$ cells. MidiMACS LS separation columns were prepared by fixing them to the MidiMACS Separation Unit and adding 3 ml of degassed Running Buffer, allowing the buffer to run to waste. The sample was then added to the column and the column was washed 3 x

with 3 ml of Running Buffer and collected as the negative fraction. The column was then removed from the separation unit and the positive fraction eluted with 5 ml of Running Buffer, using the plunger, into a fresh collection tube. The cells were then counted using a haemocytometer to assess cell number.

2.2.3.4 Single Cell Cloning

Following MACS purification, cells were diluted using selective media to give 21 ml of cell suspension with a final cell concentration of 3-5 cells per ml. This suspension was then pipetted into a 96 well plate (200 µl per well) and incubated at 37^{o}C, 5%. After approximately one week, when discreet colonies had formed, 6 clones were selected at random and passaged into T25 flask with 5 ml selective media. When cells reached ~70% confluence they were analysed using Fluorescence Activated Cell Scan (FACS) to assess expression levels (see below).

2.2.3.5 FACS Analysis: Screening for Receptor Expression in Single Cell Clones

The FACS protocol (described below) was completed with Pro5 cells single cell clones following transfection, along with controls of empty-vector transfected Pro5 cells, at 50-70% confluence in T25 flasks under sterile conditions unless stated otherwise.

Old media was poured off to waste and the cells were rinsed with 2 ml of PBS (1x). Cells were then dissociated with 2 ml of cell dissociation fluid and transferred to a pre-chilled 5 ml falcon tube, and centrifuged at 500 *g* for 5 mins at 4^{o}C. The supernatant was disposed of and the pellet resuspended in 300 µl of cold-PBS (1x).

Sam-11 anti-hPAR$_2$ mAb [1 in 300 ≡ 3 µg/ml] was then added and the samples incubated at 4^{o}C for 60 mins with swirling at 15 min intervals. 1 ml of cold-PBS (1x) was then added to each sample before centrifugation at 500 *g* for 5 mins at 4^{o}C. The supernatant was then disposed of, cell pellets were resuspended in 300 µl of cold-PBS

(1x). FITC-conjugated anti-mouse IgG [1 in 100 ≡ 10 µg/ml] was added to each sample and then incubated on ice for 45 mins, swirling at 15 min intervals. 1 ml of cold-PBS (1x) was then added to each sample and then centrifuged at 500 *g* for 5 mins at 4°C. Supernatant was discarded, cell pellets were resuspended in 300 µl of cold-PBS (1x) and then analysed using a FACScan analyser. pcDNA 3.1 empty vector transfected cells were employed as a control.

2.2.3.6 Clone Selection

The clone showing the highest level of expression above control (empty vector transfected cells) in FACS analysis was selected and a second round of MACS purification (**2.2.3.3**) and single cell cloning (**2.2.3.4**) was carried out in order to reduce the chances of a multiple cell population occurring. These second round cells were then analysed again using FACS (**2.2.3.5**).

The clone showing the highest level of expression above control was selected as the expressing clone for all future analysis and passaged up into larger flasks before being frozen down in FCS with 10% DMSO in liquid nitrogen creating cell stocks.

2.2.4 Calcium Signalling

Ca^{2+} Assay Buffer

HEPES 20 mM, NaCl 150 mM, KCl 3 mM, Glucose 10 mM. Once dissolved Sulphinpyrazone 250 µM was added. Buffer was then adjusted to pH 7.4 using 1M NaOH and stored at 4°C and used within one month.

Prior to assay: -

1.5 mM $CaCl.6H_2O$ (540 µl of 280 mM/ 100 ml) was added to the assay buffer.

Fluo-3 dye was reconstituted with 20µl of DMF per tube and mixed using a pipette.

Old media was poured off and the cells rinsed using 5 ml of PBS(1x). Cells were then harvested using 4 ml of cell dissociation fluid (non-enzymic). Cell suspensions were then transferred to a 15 ml falcon tube and centrifuged at 500 *g* for 5 min. The supernatant was disposed to waste and the cell pellet resuspended in 1.5 ml of growth media containing 10 µg/ml of Fluo-3 dye and 3.75 µl of 100 mM sulphinpyrazone and incubated at RT for 30 min at 100 rpm. 4 ml of PBS(1x) was then added and the sample centrifuged at 500 *g* for 5 mins. Supernatant was then disposed of and the cell pellet resuspended in 1.5 ml of calcium assay buffer. The cell suspension was then aliquoted in 100 µl amounts into 15X Ca^{2+} assay cuvettes prepared containing the magnetic flea and 940 µl of Ca^{2+} Assay Buffer.

Cuvette				
1	Ca^{2+} ionophore	2	µM	(4 µl of 1 mM)
2	Agonist	316	xM	(20.8 µl of conc^{n} A)
3	"	100	xM	(6.6 µl of conc^{n} A)
4	"	31.6	xM	(20.8 µl of conc^{n} B)
5	"	10	xM	(6.6 µl of conc^{n} B)
6	"	3.16	xM	(20.8 µl of conc^{n} C)
7	"	1	xM	(6.6 µl of conc^{n} C)
8	Ca^{2+} ionophore	2	µM	(4 µl of 1 mM)
9	Agonist	1	xM	(6.6 µl of conc^{n} C)
10	"	3.16	xM	(20.8 µl of conc^{n} C)
11	"	10	xM	(6.6 µl of conc^{n} B)
12	"	31.6	xM	(20.8 µl of conc^{n} B)
13	"	100	xM	(6.6 µl of conc^{n} A)
14	"	316	xM	(20.8 µl of conc^{n} A)
15	Ca^{2+} ionophore	2	µM	(4 µl of 1 mM)

For Trypsin xM = nM, A = 33 μM, B = 3.3 μM, C = 333 nM.

For $hPAR_2$ AP xM = μM, A = 33 mM, B = 3.3 mM, C = 333 μM.

Responses were calculated for each cuvette by subtracting the baseline fluorescence for the cuvette (prior to agonist addition) from the cuvettes peak fluorescence (post addition of agonist). The average for each agonist concentration was then expressed as a percentage of the A23187 (Ca^{2+} ionophore) [2 μM ≡ 4 μl of 1 mM in 2 ml] mean response (maximum attainable response) and plotted versus agonist concentration, using Prism Graphpad 4, to give the agonist concentration effect curve.

Since Pro5 cell lines have some endogenous PAR_1 expression and trypsin at high concentrations is capable of activating PAR_1 (Vu et al., 1991), a thrombin concentration effect curve was produced (as above) using a concentration range of 0.01 units/ml – 3.16 units/ml at half Log intervals. Curves were produced for cells at both 40% and 70% confluence to match recombinant cell lines.

The protocol was then repeated for SLIGKV-NH2 and trypsin first pre-treating cells with thrombin [3.16 units/ml ≡ 6.32 μl of 1000 units/ml] for 10 min, prior to agonist addition, in order to remove any endogenous PAR_1 activity.

2.2.5 Western Blotting

The western blot protocol (described below) was carried out on membrane protein preparations. They were run along side Bio-Rad Precision Plus Protein™ Standards to allow identification of band size.

10% SDS PAGE - 3.33 ml of acrylamide stock (Acylamide 29.2 g, bis-methylene acrylamide, 0.8 g in 100 ml dH_2O), 4.02 ml of dH_2O, 2.5 ml of 1.5 M Tris-HCL pH 8.8, 100 μl of 10% SDS, 50 μl of 10% APS (freshly made), and 5 μl of TEMED.

Stacking Gel - 1.3 ml of acrylamide stock (Acylamide 29.2 g, bis-methylene acrylamide, 0.8 g in 100 ml dH_2O), 6.1 ml of dH_2O, 2.5 ml of 0.5 M Tris-HCL pH 6.8, 100 µl of 10% SDS, 50 µl of 10% APS (freshly made), and 20 µl of TEMED.

Gel forming apparatus was constructed as per manufacturers instructions. A 10% SDS-PAGE was made (as above) adding TEMED last and quickly mixing before adding to gel former, 5 ml per gel. Propan-2-ol, ~500 µl, was pipetted onto the top of the gel to ensure a flat surface. The gel was then left to set for approx. 30 min at R.T. Once the gel is set the propan-2-ol was poured off and the gel surface washed with dH_2O and dried. The stacking gel was then made (as above) adding TEMED last, mixed and added to the gel former filling it to the top. A 12 sample comb was then insert into gel and the gel left to set for approx. 30 min at R.T. The gel former was placed into the gel tank. The internal gel former reservoir was filled with electrode buffer (25 mM Tris, 200 mM glycine, 1% SDS) to above the sample wells. Then the gel tank was filled upto the max fill mark with electrode buffer.

20 µl of 1 µg/µl samples were prepared with 5 µl of sample buffer and 1 µl of β-mercaptoethanol (for reducing gels only). The samples were then heated to 95^{o}C on a heat block for 1 min. Samples were loaded into sample wells alongside 10 µl of protein standard. The gel was then run at 150-225 V until the dye front had travelled the length of the gel (approx 2 hours).

An appropriate sized piece of protein binding membrane was prepared by soaking in methanol and foam pads and 4 pieces of filter were wet in the blotting buffer (25 mM Tris, 200 mM glycine, 20% methanol). The gel was then carefully removed from the gel plates and the blotting apparatus constructed, submerged in blotting buffer, as below:

Black cassette piece, Pre-soaked sponge pad, 2 pieces of pre-soaked filter paper, SDS-PAGE, Protein blotting membrane (cut to top left), 2 pieces of pre-soaked filter paper, Pre-soaked sponge pad, White cassette piece

The cassette was then clipped in place and transferred quickly (to avoid drying out) into a gel tank containing blotting buffer with the black cassette piece toward the negative pole. The entire blotting apparatus was then placed in ice and run at 25-40 V for a minimum of 90 min, best results were obtained by blotting overnight.

The membrane was then "blocked" for a minimum of 1 hour in 5% W/V non-fat milk powder in PBS (0.1% Tween-20) at room temp on a bench top rocker. The non-fat milk solution was then removed and the membrane washed 4 X 15 min with PBS (0.1% Tween-20). The membrane was then incubated with primary antibody, murine anti-HA11 antibody [1 in 1000 ≡ 1 μg/ml] in PBS (0.1% Tween-20) and 2% non-fat milk at 4^{o}C overnight on a rocker. The antibody was then removed the membrane washed 4 X 15 min with PBS (0.1% Tween-20). The membrane was then incubated with secondary antibody, HRP conjugated anti-mouse antibody [1 in 1000 ≡ 1 μg/ml] in PBS (0.1% Tween-20) and 2% non-fat milk at R.T. for 1 hour. The antibody as then removed the membrane washed 4 X 15 min with PBS (0.1% Tween-20). ECL reagents A and B were mixed together in equal volumes, applied to the protein binding surface and incubated at room temp for 1-15 min. Excess reagent was then removed and the membrane photographed using a gel dock system setup to measure chemiluminescence.

2.2.6 MAPK Kinase signalling

Cells were grown in 6 well plates to ~50% confluence. Cells were then incubated overnight with serum-free media. Cells were then either treated with 20 nM trypsin, 100 μM SLIGKV-NH_2 or remained untreated. At a marked time-point (5, 10, 20, 30, or 60

min) post-agonist addition treatment was halted by removal of media and direct addition of 350 µl of Lamelli's buffer (2% SDS, 10% glycerol, 50Mm Tris-HCl, 5mM EDTA, 0.008% bromophenol blue and 0.5% β-mercaptotheanol). Samples were then stored at -80^{o}C until analysis. Samples were then run on a 10% SDS-PAGE and transferred to a blotting membrane (as previously described **2.2.5**). Membranes were blocked for 60 min in 5% non fat milk in PBS (0.1% Tween 20) before incubating with anti-phosphorylated p44/42 antibody [1 in 1000 ≡ 1µg/ml] in 5% BSA, PBS (0.1% Tween 20) overnight at 4^{o}C. Membranes were then washed in PBS (0.1% Tween 20) 4X 15 min. Membranes were then incubated with donkey anti-rabbit IgG HRP-linked antibody [1 in 1000 ≡ 1µg/ml] for 60 min in PBS (0.1% Tween 20) 2% non-fat milk at R.T. for 60 min. Membranes were again washed in PBS (0.1% Tween 20) 4X 15 min. ECL reagents A and B were mixed together in equal volumes, applied to the protein binding surface and incubated at room temp for 1-15 min. Excess reagent was then removed and the membrane photographed using a UVP Laboratory Products Epichem II Darkroom setup to measure chemiluminescence.

Following visualisation membrane were further washed in PBS (0.1% Tween 20) 4X 15 min to remove any residual ECL. Membrane were then blocked as before with 5% non-fat milk in PBS (0.1% Tween 20) and reprobed with anti-total p44/42 antibody [1 in 1000 ≡ 1µg/ml] in 5% BSA, PBS (0.1% Tween 20) overnight at 4^{o}C. Membranes were then washed, incubated with secondary HRP conjugate and visualised as described above.

3 EXTRACELLULAR CYSTEINES OF $hPAR_2$

3.0 EXTRACELLULAR CYSTEINES OF $hPAR_2$

3.1 Introduction

3.1.1 C148 and C226 of $hPAR_2$

Studying the amino-acid sequence of $hPAR_2$ reveals two cysteine residues within ECL1 and ECL2 (C148 and C226 respectively; see APPENDIX 4 for full sequence). All class A GPCR (the proteinase-activated receptors included) that have been examined possess these conserved cysteine residues. Since the existence of disulphide bridge between these cysteine within ECL1 and ECL2 is one of the defining characteristics of class A GPCR (**1.2.4**) (Kolakowski, 1994; Gether, 2000), and is frequently essential for receptor structural stability and/or function (Gether, 2000) it is our contention that C148 and C226 is a putative site for the formation a dulphide bridge. However, the existence of a disulphide As such is it our intention to investigate whether or not a disulphide bridge is present between C148 and C226 (**fig 1.5.7a**) and what, if any, influence do they have on the function of $hPAR_2$.

3.1.3 C22 of $hPAR_2$

Through examination of the sequence of $hPAR_2$ we have identified a cysteine residue within the extracellular N-terminus which has no aknowldeged function or association. Although $hPAR_2$ has not been shown to form into dimers, dimerisation within GPCRs (Bulenger et al, 2005)and within the PAR family has been demonstrated (Leger et al, 2006). As such one possible function of this cysteine residue is a site for dimerisation. We intend to investigate the affect of removing this cysteine on receptor expression and function.

3.2 MATERIALS AND METHODS

3.2.1 Materials

pcDNA 3.1 $hPAR_2$ wild-type HA11.eYFP was supplied by Dr. S.J. Compton, Division of Cardio-respiratory Studies, Univeristy of Hull. Propidium iodide and formaldehyde was purchased from Sigma, Dorest, UK. CITI-Flour AF1 was purchased from CITI-flour Ltd, Stansted, UK.

3.2.2 Methods

3.2.2.1 Mutagenic Primers

The pcDNA 3.1 vector containing wild-type $hPAR_2$ (wt-$hPAR_2$) with a C-terminal HA11 tag was supplied by Dr. S. J. Compton (Division of Cardio-respiratory Studies, University of Hull) from previous work. Specific PCR oligonucleotide primers were designed and custom made for the mutagenic PCR of the vector to include the following mutations (mutations highlighted on the primers).

$hPAR_2$C22A

5′ CC TCT CTC TCC GCC AGT GGC ACC ATC CAA GG 3′

5′ CC TTG GAT GGT GCC ACT GGC GGA GAG AGA GG 3′

$hPAR_2$C148A

5′ GGG GAA GCT CTT GCT AAT GTG CTT ATT GG 3′

5′ CC AAT AAG CAC ATT AGC AAG AGC TTC CCC 3′

$hPAR_2$C226A

5′ CTG AAC ATC ACG ACC GCT CAT GAT GTT TTG CC 3′

5′ GG CAA AAC ATC ATG AGC GGT CGT GAT GTT CAG 3′

$hPAR_2$C148A/C226A

Generated using two rounds of PCR the first with $hPAR_2$C148A mutagenic primers, the

second with $hPAR_2$C226A mutagenic primers. Mutagenic PCR was carried out as explained in section **2.2.1.** along with subsequent cloning steps.

3.2.2.2 Transient transfection of eYFP tagged $hPAR_2$

The pc DNA3.1 $hPAR_2$ eYFP vector was kindly supplied by Dr. S. J. Compton (Division of Cardio-respiratory Studies, University of Hull) from previous work. The C→A mutations C148A, C226A and C148A/C226A were produced as in **3.2.2.1**. Transfection of vectors in Pro5 cells were then carried out as outlined in **2.2.3.2** except that selective antibiotics were not used. Cells were analysed at between 48 and 72 hours post-transfection to ensure peak transient expression.

3.2.2.3 Confocal Microscopy

Cells were grown on $18mm^2$ glass coverslips and washed with PBS (with Ca^{2+}/Mg^{2+}) before being fixed with 3% formaldehyde for 15 min, and permeabilising with 0.2% v/v Triton X-100 in PBS for 10 min. Cells were then stained using propidium iodide [500 nM] and coverslips washed in PBS prior to being dried and mounted inverted into antifade **CITI-Fluor** AF1 [anti-degradent in glycerol/PBS]. The slides were then analysed on a Nikon Eclipse (TE2000-E) confocal microscope with a BioRad Radiance 2100 scanning system and lasers using argon laser exciting at 488 nM. Emissions for eYFP were captured at 530 nM (+/- 30 nM) and propidium iodide emissions were captured as 600nM and over. An experiment consisted of two pictures being taken from each of triplicate coverslips. A single consensus photo was then selected to be representative of that treatment.

3.2.2.4 Desensitisation of PAR_1

Cells were harvested and prepared for Ca^{2+} signalling analysis as detailed **2.2.4.** Each cuvette was then treated with either 3.16 units/ml of thrombin, 50 nM of TFLLR-NH_2 or 30 units/ml of elastase prior to addition of $hPAR_2$ agoinst. Data analysis was then carried out as detailed in **2.2.4** with thrombin, TFLLR-NH_2, or elastase signal also used as a between cuvette control.

3.2.2.5 MTSEA Biotin immunoblots

Cells were grown in T75 flasks before removing media and washing cell surface with MTSEA Buffer (MTb: 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM $MgCl_2$, 5 mM sodium pyruvate, 5 mM HEPES, pH 7.6). Cells were then incubated with MTSEA-Biotin [0.15 mg/ml] in 3 ml of MTb for 30 min and 37^{o}C. The cell surface was then washed five times with MTb before harvesting cells using 1 mM EDTA. Cells were pelleted by centrifugation at 500 *g* for 5 min. Supernatant was discarded and the cell pellet resuspended in 500 µl of MPER and placed on a rocker for 10 min at RT. Samples were then transferred to ultra-centrifugation tubes and centrifuged at 20000 *g* for 20 min at 4^{o}C. The supernatant from each sample was retained and split in two. Half of each sample was immunoprecipitated using avidin-agarose beads (30 µl) and half immunoprecipitated using anti-HA11 agarose beads (10 µl). The samples were added in minispin columns and the respective agarose beads added. The samples were then incubated overnight with inversion at 4^{o}C. Beads were washed four times with 500 µl of TBS (0.1% Tween-20) pulse centrifuging after each wash and discarding flow-through. Mini-spin columns were then placed into clean collection tubes and 25 µl of 2X SDS loading buffer was added in the column. The sample was then incubated at 95^{o}C for 5 min before pulse centrifugation and collection of elutant. Samples were

retained and stored at -80 ^{o}C until separated by SDS-PAGE and transferred to protein binding membrane as outlined in **2.2.5**.

Protein blots were then blocked using 5% non-fat milk in PBS (0.1% tween-20) for 60 min at RT. The blots were then washed four times for 15 min in PBS (0.1% tween-20) discarding the wash each time. Blots were then labelled with one of two methods. Those produced from anti-HA11 agarose immunoprecipitated samples were incubated with avidin-peroxidase [1 in 500 ≡ 2 μg/ml] in PBS (0.1% tween-20), 2% non-fat milk at 4^{o}C for 120 mins before again washing four times for 15 min in PBS (0.1% tween-20) discarding wash each time**.** Blots were then visualised as outlined in **2.2.5** before reprobing with anti-HA11 antibody [1 in 1000 ≡ 1μg/ml] in PBS (0.1% Tween-20) 2% non-fat milk at 4^{o}C overnight. The blot was again washed four times for 15 min in PBS (0.1% tween-20) discarding wash each time**,** before incubating with goat anti-mouse HRP [1 in 1000 ≡ 1 μg/ml] in PBS (0.1% Tween-20), 2% non-fat milk for 60 mins at RT. The blot was again washed four times for 15 min in PBS (0.1% tween-20) discarding wash each time**,** before visualising as outlined in **2.2.5**.

Those protein blots produced from avidin-agarose immunoprecipitated samples were incubated with anti-HA11 antibody [1 in 1000 ≡ 1μg/ml] in PBS (0.1% Tween-20) 2% non-fat milk at 4^{o}C overnight before washing four times for 15 min in PBS (0.1% tween-20) discarding wash each time**.** The blots were then incubated with goat anti-mouse HRP [1 in 1000 ≡ 1 μg/ml] in PBS (0.1% Tween-20), 2% non-fat milk for 60 mins at RT. The blot was again washed four times for 15 min in PBS (0.1% tween-20) discarding wash each time**,** before visualising as outlined in **2.2.5**. These blots were then reprobed with avidin- peroxidase [1 in 500 ≡ 2 μg/ml] in PBS (0.1% tween-20), 2% non-fat milk at 4oC for 120 mins before again washing four times for 15 min in PBS

(0.1% tween-20) discarding wash each time. The blots when then once again visualised as outlined in **2.2.5**.

3.3 RESULTS

3.3.1 Receptor Cell Surface Expression

Receptor cell surface expression was assessed for wt-$hPAR_2$, $hPAR_2$C22A, $hPAR_2$C148A, $hPAR_2$C226A, and $hPAR_2$C148A/C226A by FACs analysis following staining with anti-$hPAR_2$ mAb Sam-11 (Figure 3.3.1). In each trace the solid purple histogram represents the pcDNA3.1 empty vector control and the green line the receptor transfected cell line. Histogram **A** shows the wt-$hPAR_2$ cell line expression levels to which the mutant receptor expressing cell lines can be compared. Histogram **E** shows $hPAR_2$C22A to be successfully expressed at the cell surface to a similar level as seen with wt-$hPAR_2$ (**A**). $hPAR_2$C148A (**B**) also displays receptor cell surface expression similar to wt-$hPAR_2$ (**A**). However, $hPAR_2$C226A (**C**) and $hPAR_2$C148A/C226A (**D**) showed no discernable cell surface expression after repeated cloning attempts and maintain Sam-11 staining similar to pcDNA3.1 empty vector control.

The bar chart (F) shows $hPAR_2$C22A to have only slightly reduced (90.1% +/-SEM) cell surface expression compared to wild-type, whilst $hPAR_2$C148A displays only ~72% of that of wild-type. Sam-11 staining of $hPAR_2$C226A and $hPAR_2$C148A/C226A was not appreciably increased over control. When analysed using one way ANOVA table 95% CI and Dunnett's Multiple comparison ad-hoc test with wt-$hPAR_2$ as the control column comparison, all cell lines displayed a significant change ($p<0.01$) in Sam-11 cell surface labelling.

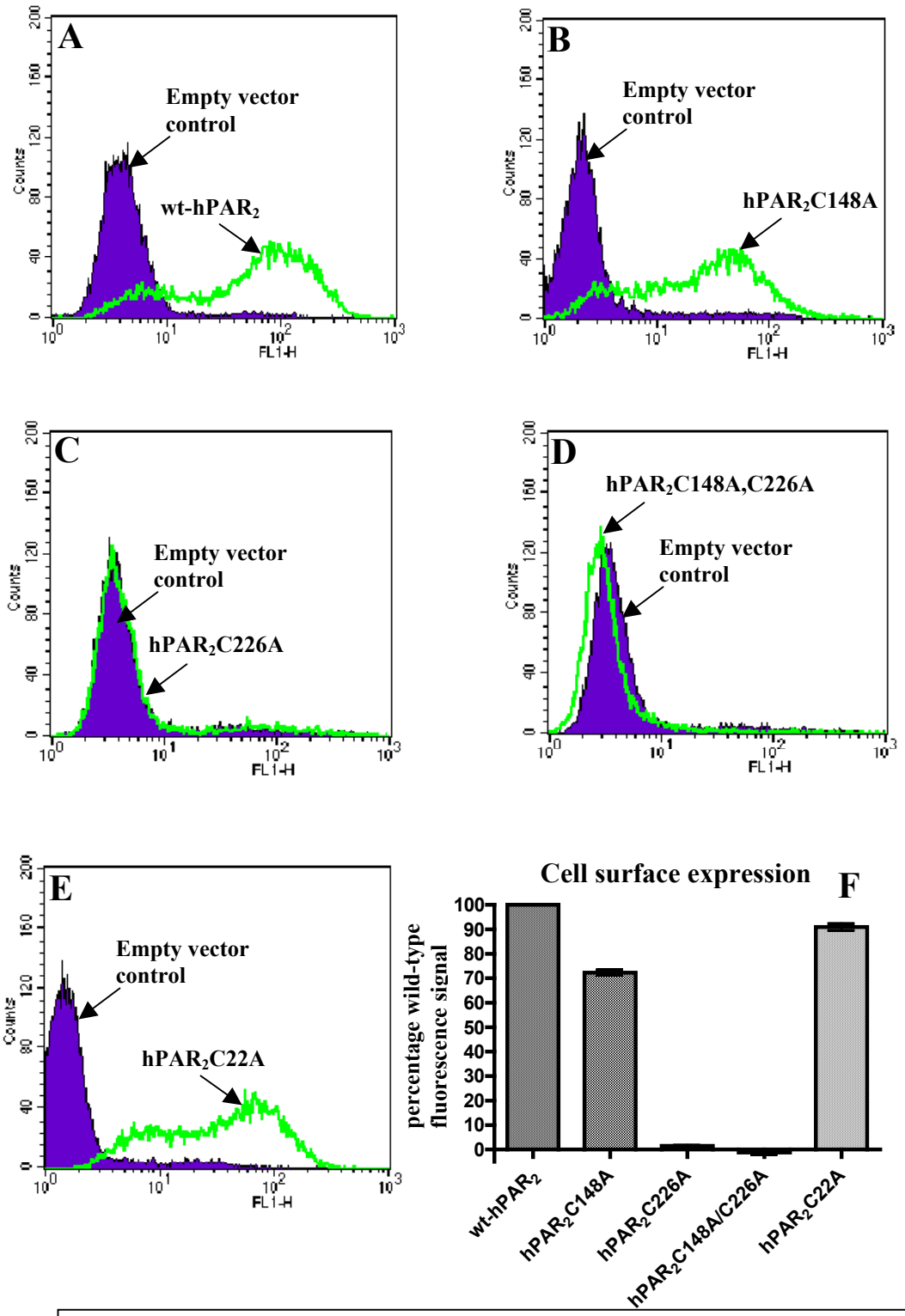


Figure 3.3.1 - Receptor cell surface expression
FACs histograms for:**A** - wt-hPAR$_2$, **B** - hPAR$_2$C148A, **C** - hPAR$_2$C226A, **D** - hPAR$_2$C148A/C226A, **E** - hPAR$_2$C22A. FACS histogram of empty vector transfected controls are shown in solid purple with the receptor transfected cell line shown in green. Histograms shown are representative of multiple experiments. **F** - bar graph showing FACs mean fluorescence expressed as relative receptor expression (compared to wt-hPAR$_2$) of each cell line +/- SEM of 3 separate experiments.

3.3.2 Localisation of receptor expression by confocal microscopy

Repeated cloning attempts of $hPAR_2C226A$ and $hPAR_2C148A/C226A$ failed to produce a cell line displaying receptor cell surface expression (**fig 3.3.1 D and E**). In order to identify the site of receptor expression additional cell lines were produced transiently expressing constructs with C-terminal eYFP tags: $hPAR_2C148A$ eYFP, $hPAR_2C226A$ eYFP, and $hPAR_2C148A/C226A$ eYFP. Transiently transfected cells were permeabilised and co-stained with propidium iodide.

Confocal images of wt-$hPAR_2$ eYFP (**fig 3.3.2 A**) transiently expressed in Pro5 cells showed clearly receptor at the cell membrane, with a small amount appearing punctuate just below the cell membrane. This corroborates the previous FACs trace (**fig 3.3.1 A**) showing staining at the cell membrane for $hPAR_2$. Confocal imaging of $hPAR_2C148A$ eYFP (**B**) shows some eYFP localisation at the cell membrane, confirming the accompanying FACs trace (**fig 3.3.1 B**), however there is also large amount of eYFP signal scattered throughout the cytosol. The confocal pictures for both $hPAR_2C226A$ eYFP (**C**) and $hPAR_2C148A/C226A$ eYFP (**D**) both show no eYFP signal at the cell membrane and a large accumulation of signal surrounding the nucleus, as well as punctate eYFP signal throughout the cytoplasm.

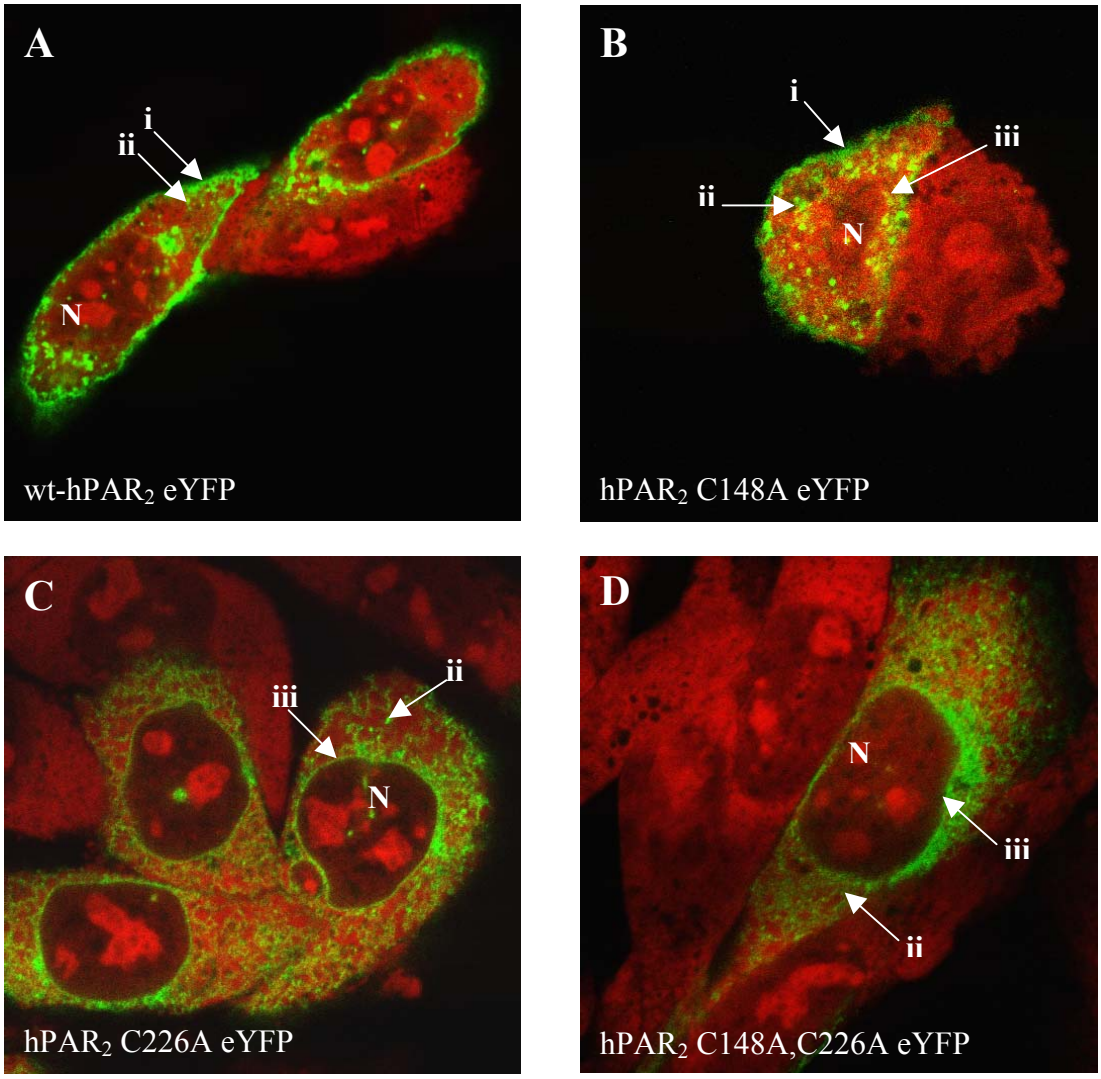


Figure 3.3.2 - Localisation of Receptor Expression by Confocal Microscopy
Confocal images of permeabilised and co-stained with propidium iodide.
eYFP is signal shown in green, propidium iodide signal shown in red.
A - wt-hPAR$_2$ eYFP **B** - hPAR$_2$C148A eYFP
C - hPAR$_2$C226A eYFP **D** - hPAR$_2$C148A/C226A eYFP
Confocal Images shown are representative of two pictures taken from each of three separate slides for each cell line with eYFP signal shown in green and propidium iodide signal shown in red.
i) Cell surface expression, **ii)** internal receptor expression, **iii)** receptor surrounding the nuclear envelope.

3.3.3 Calcium Signalling: Agonist concentration effect curves for hPAR$_2$C148A

In order to assess the effect of the C148A mutation on hPAR$_2$ triggered calcium signalling, agonist concentration effect curves were produced for both the mutant and wild-type receptor. The curves for wt-hPAR$_2$ with both trypsin and SLIGKV-NH2 (**fig 3.3.3(i)**) plateau at ~65% maximum obtainable signal with concentration ranges of 316 pM to 316 nM, and 316 nM to 316 μM for trypsin and SLIGKV-NH2 respectively. The concentration effect curve for hPAR$_2$C148A with trypsin and SLIGKV-NH$_2$ (**fig 3.3.3(ii)**), using a one-sample t test with 95% C.I. $p>0.05$, display no significant deviation from 0 ($p=0.0028$ and $p=0.0035$ respectively) with trypsin reaching a maximum of only 3% at top dose and SLIGKV-NH$_2$ not exceeding 1% over the dose range.

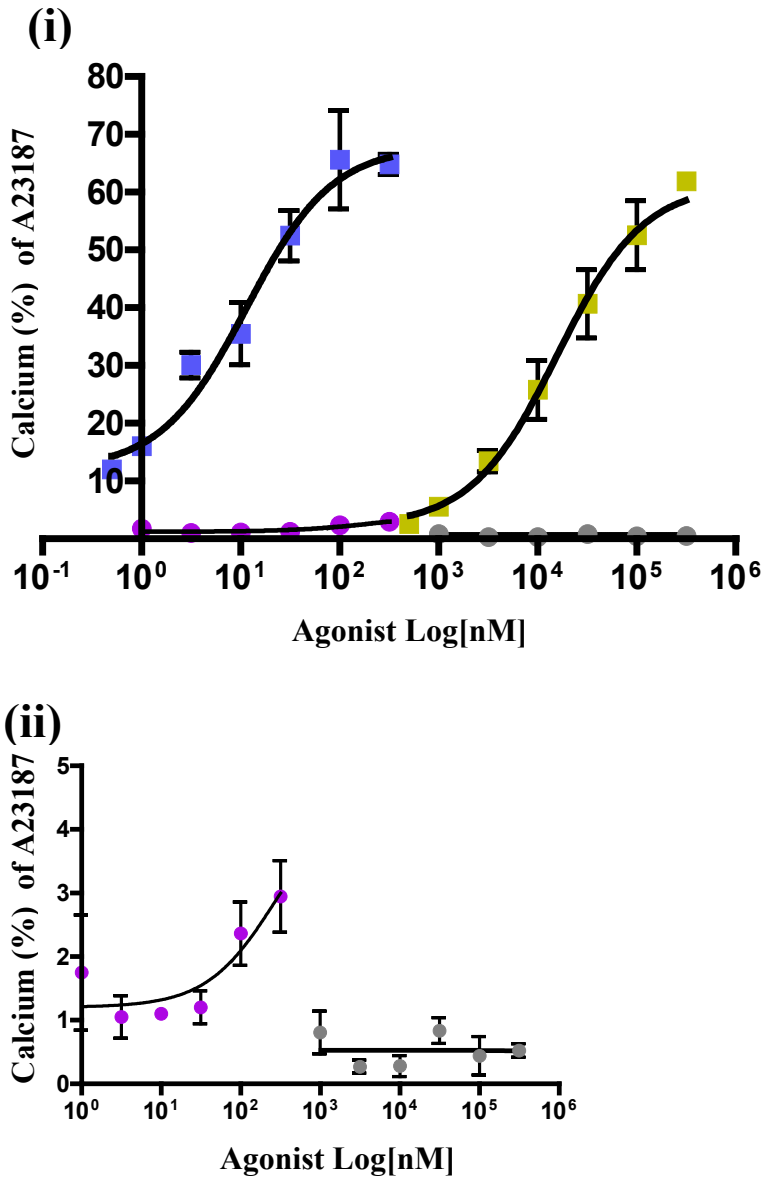


Fig 3.3.3(i)/(ii) - hPAR$_2$C148A agonist stimulated calcium mobilisation
Trypsin and SLIGKV-NH$_2$ concentration effect curves for hPAR$_2$C148A and wt-hPAR$_2$.
(i) data plotted on the same axis to allow comparison, (ii) shows C148A data only on an compressed *y* axis.

wt-hPAR$_2$: with trypsin **hPAR$_2$C148A : with trypsin**
with SLIGKV-NH$_2$ **with SLIGKV-NH$_2$**

Results are expressed as the mean +/- SEM of 3 separate experiments. Each preformed in duplicate.

3.3.4 Calcium Signalling: Agonist concentration effect curves for hPAR$_2$C22A

The effect of the C22A mutation on hPAR$_2$ mediated calcium mobilisation was assessed and concentration effect curves compared to the curves for wild-type receptor produced in **3.3.3** (**fig 3.3.4(i)[A]**). As previously stated curves for wt-hPAR$_2$ with both trypsin and SLIGKV-NH2 (plateau at ~65% maximum obtainable signal with concentration ranges of 316 pM to 316 nM, and 316 nM to 316 μM for trypsin and SLIGKV-NH2 respectively. The concentration effect curve for hPAR$_2$C22A with trypsin plateaued at ~52% maximum obtainable signal within a concentration range of 1 nM to 316 nM, when compared to the trypsin concentration effect curve for wt-hPAR$_2$ using a paired two-tailed t-test there is a significant difference ($P<0.0001$). The concentration effect curve for hPAR$_2$C22A with SLIGKV-NH$_2$ plateaued at ~70% maximum obtainable signal within a concentration range of 1 μM to 316 μM, and unlike the trypsin curve when compared to the SLIGKV-NH$_2$ concentration effect curve in wt-hPAR$_2$, using a paired two-tailed t-test, there was no significent difference ($P=0.1861$). In order to remove endogenous PAR$_1$ activity the concentration effect experiments were repeated with prior treatment of cells with thrombin. The concentration effect curves for wt-hPAR$_2$ and hPAR$_2$C22A cell lines with the PAR$_2$ agonists SLIGKV-NH$_2$ and trypsin following abolition of endogenous PAR$_1$ signal with 3.16 units/ml of thrombin are shown in **Fig 3.3.4(i)[B]**. The curves for wt-hPAR$_2$ with both trypsin and SLIGKV-NH$_2$ plateau at ~55% maximum obtainable signal with concentration ranges of 316 pM to 316 nM, and 316 nM to 316 μM for trypsin and SLIGKV-NH2 respectively. The concentration effect curve for hPAR$_2$C22A with trypsin plateaued at ~68% maximum obtainable signal within a concentration range of 1 nM to 316 nM. For hPAR$_2$C22A with SLIGKV-NH$_2$ the curve plateaued at ~59% maximum obtainable signal with a

concentration range of 1 μM to 316 μM (For EC_{50} and precise maximum curve values see **Table 3.3.4(ii)**.) The thrombin desensitised concentration effect curve for $hPAR_2C22A$ with trypsin yielded a maximum signal slightly larger than seen with wt-$hPAR_2$ (68.15% compared to 55.95%) and similar EC_{50} values as with wt-$hPAR_2$ (5.36 nM compared to 4.00 nM). When compared by two-tailed t-test trypsin concentration effect curves with wt-$hPAR_2$ and $hPAR_2C22A$ show no significant difference (P=0.8458). The thrombin desensitised concentration effect curve for $hPAR_2C22A$ with SLIGKV-NH2 gave a maximum signal approximately the same as that seen with wt-$hPAR_2$ (58.49% compared to 58.70%) and EC_{50} values ~2-fold greater than that seen with wt-$hPAR_2$ (68.82 μM compared to 32.69 μM), however when compared by two-tailed t-test the two groups show a significant difference (P=0.0012) .

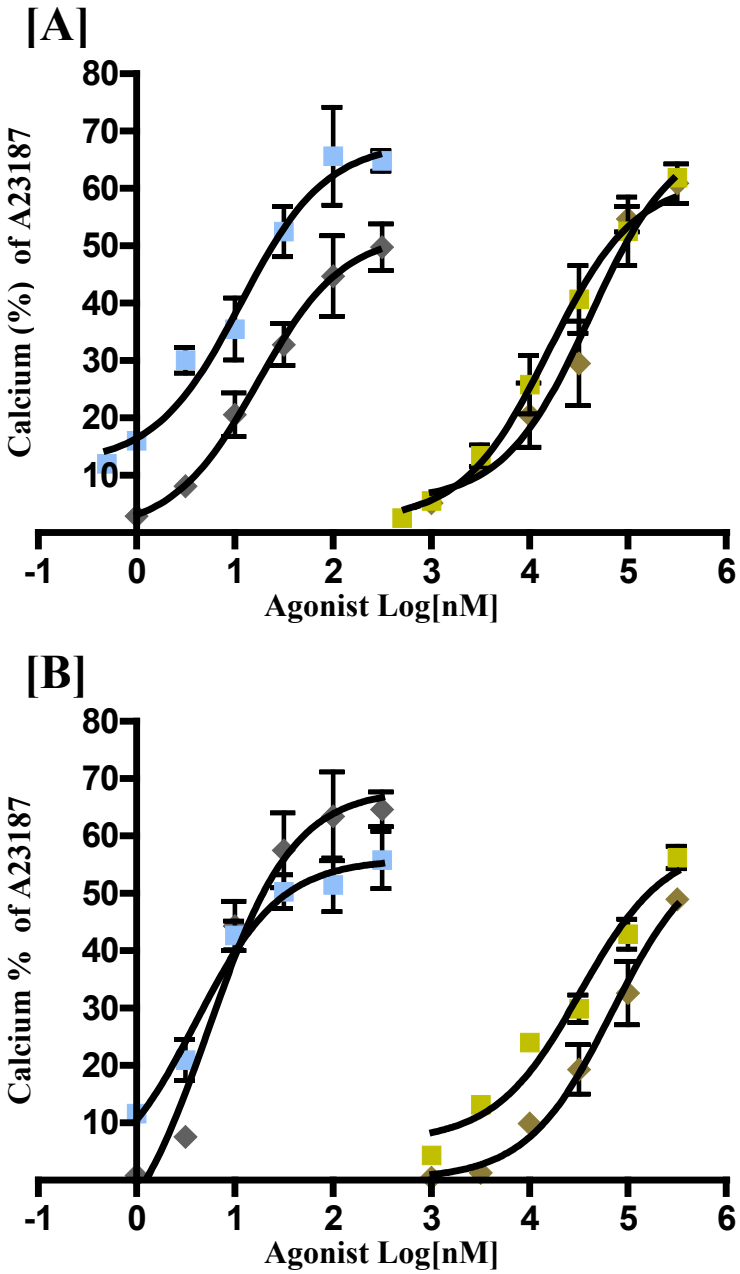


Fig 3.3.4(i) - hPAR$_2$C22A agonist stimulated Ca^{2+} mobilisation
Trypsin and SLIGKV-NH$_2$ concentration effect curves for wt-hPAR$_2$ and hPAR$_2$C22A **[A]** and following PAR$_1$ desensitisation with 3.16 units/ml of thrombin **[B]**.

wt-hPAR$_2$: **with trypsin** **hPAR$_2$C22A :** **with trypsin**
with SLIGKV-NH$_2$ **with SLIGKV-NH$_2$**

Results are expressed as the mean +/- SEM of 4 separate experiments. Each preformed in duplicate.

CURVE		EC_{50} ±SEM	Maximum	EC_{50} fold wt	Maximum fold wt
wt-$hPAR_2$:	Trypsin	11.44 nM **± 1.40**	67.99 %		
	SLIGKV	15.69 µM **± 1.43**	61.43 %		
$hPAR_2$C22A:	Trypsin	17.11 nM **±1.49**	52.20 %	~1.5 ↑	~1.3 ↓
	SLIGKV	40.22 µM **± 1.46**	69.52 %	~2.6 ↑	~1.1 ↑
post cell desensitisation with 3.16 units/ml Thrombin					
wt-$hPAR_2$:	Trypsin	4.00 nM **± 1.53**	55.95 %		
	SLIGKV	32.69 µM **± 1.34**	58.70 %		
$hPAR_2$C22A:	Trypsin	5.36 nM **± 1.46**	68.15 %	~1.3 ↑	~1.2 ↑
	SLIGKV	68.82 µM **± 1.36**	58.49 %	~2.0 ↑	~1.0

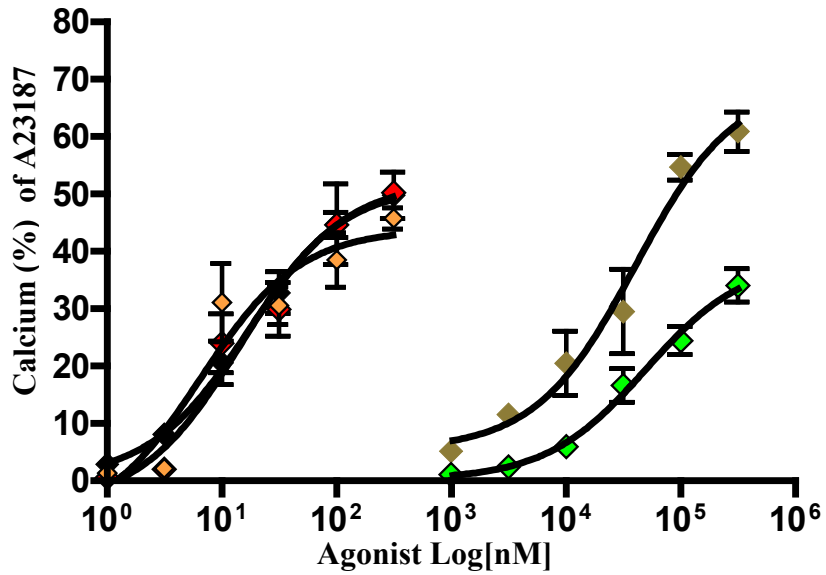
Table 3.3.4(ii) - Table of Curve Fit Data for Fig 3.3.4(i)
Table giving EC_{50} values for wt-$hPAR_2$ and $hPAR_2$C22A agonist concentration effect curves shown in **Fig 3.3.4[A]/[B]**. ↑ indicates a fold increase over wt-$hPAR_2$, and ↓ indicates a fold decrease over wt-$hPAR_2$. EC_{50} values given to 2 decimal places with SEM displayed in **bold**. Slope maximum response values expressed as percentage of A23187 response.

3.3.5 Desensitising PAR_1

To investigate whether the change in agonist activation curves compared to untreated following desensitisation was PAR_1, or thrombin dependant. PAR_1 desensitisation was carried out on $hPAR_2C22A$ using TFLLR-NH_2 instead of thrombin. Assessment of variation was carried out using a repeated measures two-way ANOVA table, where a P value <0.05 is considered significant.

Trypsin concentration effect curves for $hPAR_2C22A$ following pre-treatment with TFLLR-NH_2 lead to a small reduction EC_{50} (~0.8 fold) and a small decrease in curve max (~1.3 fold) but displays no significant difference to $hPAR_2C22A$ with trypsin alone (P=0.7717). After pre-treatment with elastase trypsin concentration effect curves for $hPAR_2C22A$ showed a small reduction in curve maximum (~1.2 fold) and a decrease in EC_{50} (~2.6 fold) to a value similar to that seen following thrombin treatment, but overall showing no significant divergance from $hPAR_2C22A$ with trypsin alone (P=0.7761) . A small increase in the EC_{50} (~1.2 fold) and reduction in curve maximum (~1.8 fold) for SLIGKV-NH_2 was seen following pre-treatment of cells with TFLLR-NH_2 resulting in a significant difference from $hPAR_2C22A$ with SLIGKV-NH_2 alone (P<0.0001).

[A]



[B] CURVE	**EC_{50} ±SEM**	**Maximum**	**EC_{50} fold unt**	**Maximum fold unt**
$hPAR_2C22A$: Trypsin	17.11 nM ±1.49	52.20 %		
SLIGKV	40.22 µM ± 1.46	69.52 %		
$hPAR_2C22A$: Trypsin **Thrombin desensitised**	5.36 nM ± 1.46	68.15 %	~3.2 ↓	~1.3 ↑
SLIGKV	68.82 µM ± 1.36	58.49 %	~1.7 ↑	~1. 2 ↓
$hPAR_2C22A$: Trypsin **TFLLR desensitised**	14.49 nM ± 1.46	51.55 %	~0.8 ↓	~1.3 ↓
SLIGKV	49.58 µM ± 1.36	38.68 %	~1.2 ↑	~1.8 ↓
$hPAR_2C22A$: Trypsin **Elastase desensitised**	6.56 nM ± 1.30	43.89 %	~2.6 ↓	~1.2 ↓

Fig 3.3.5(i) - $hPAR_2C22A$ Ca^{2+} mobilisation - Agonist Concentration Effect Curves pre/post-PAR_1 desensitisation
[A] – Graph showing trypsin and SLIGKV-NH_2 concentration effect curves for $hPAR_2C22A$ and before and after PAR_1 desensitisation with 50 nM TFLLR-NH2 or 10 units/ml elastase. **[B]** – table of curve values for graph **[A]**
$hPAR_2C22A$: ◆ **with trypsin** ◆ **with SLIGKV-NH_2**
◆ **with TFLLR-NH_2 then trypsin** ◇ **with TFLLR-NH_2**
◇ **with elastase then trypsin** **then SLIGKV-NH_2**
Results are expressed as the mean +/- SEM of 4 separate experiments (3 for elastase). Each experiment was preformed in duplicate.

3.3.6 MAPK Signalling: Time course

In an effort to characterise the effect of the C22A and C148A mutation on $hPAR_2$ ERK1/2 (p44/42) MAPK signalling, post agonist activation curves for wt-PAR_2, $hPAR_2$C22A and $hPAR_2$C148A cell lines with PAR_2 agonists (trypsin and SLIGKV-NH_2) were produced (**fig 3.3.6b (i)** and **(ii)** respectively).

The phosphorylated p44/42 blot produced for wt-$hPAR_2$ (fig 3.3.6a(i)), shows an increase in band intensity, over "no treatment", 5 min after agonist addition for both SLIGKV-NH_2 and trypsin. The band intensity then rapidly returns to being equivalent to "no treatment" for SLIGKV-NH_2 treated cells, and more slowly for trypsin treated cells. The phosphorylated p44/42 blot produced for $hPAR_2$C22A and $hPAR_2$C148A (shown in fig 3.3.6a(i)), shows a similar activation trend to wt-$hPAR_2$. When analysed using a one-way ANOVA table it shows significant difference (P=0.3718) between $hPAR_2$C22A, $hPAR_2$C148A and wt-$hPAR_2$. Interestingly the positive control thrombin band was consistently of lower intensity than the equivalent bands in both wt-$hPAR_2$ and $hPAR_2$C148A. Fig 3.3.6b(i)/(ii) show the densitometry values taken from the western blot banding and plotted versus time post-agonist addition. The MAP Kinase activation curve post-SLIGKV-NH_2 addition for both $hPAR_2$C22A and $hPAR_2$C148A remain similar to wt-$hPAR_2$ at all time points post-agonist treatment (fig 3.3.6b(i)). The MAP Kinase activation curve post-trypsin addition for both $hPAR_2$C22A and $hPAR_2$C148A show a slight increase in band intensities compared to wt-$hPAR_2$ at 5 and 10 min post-addition of agonist (fig 3.3.6b(ii)), however when analysed using a one-way ANOVA table it shows a significant difference (P=0.9184) between $hPAR_2$C22A, $hPAR_2$C148A and wt-$hPAR_2$.

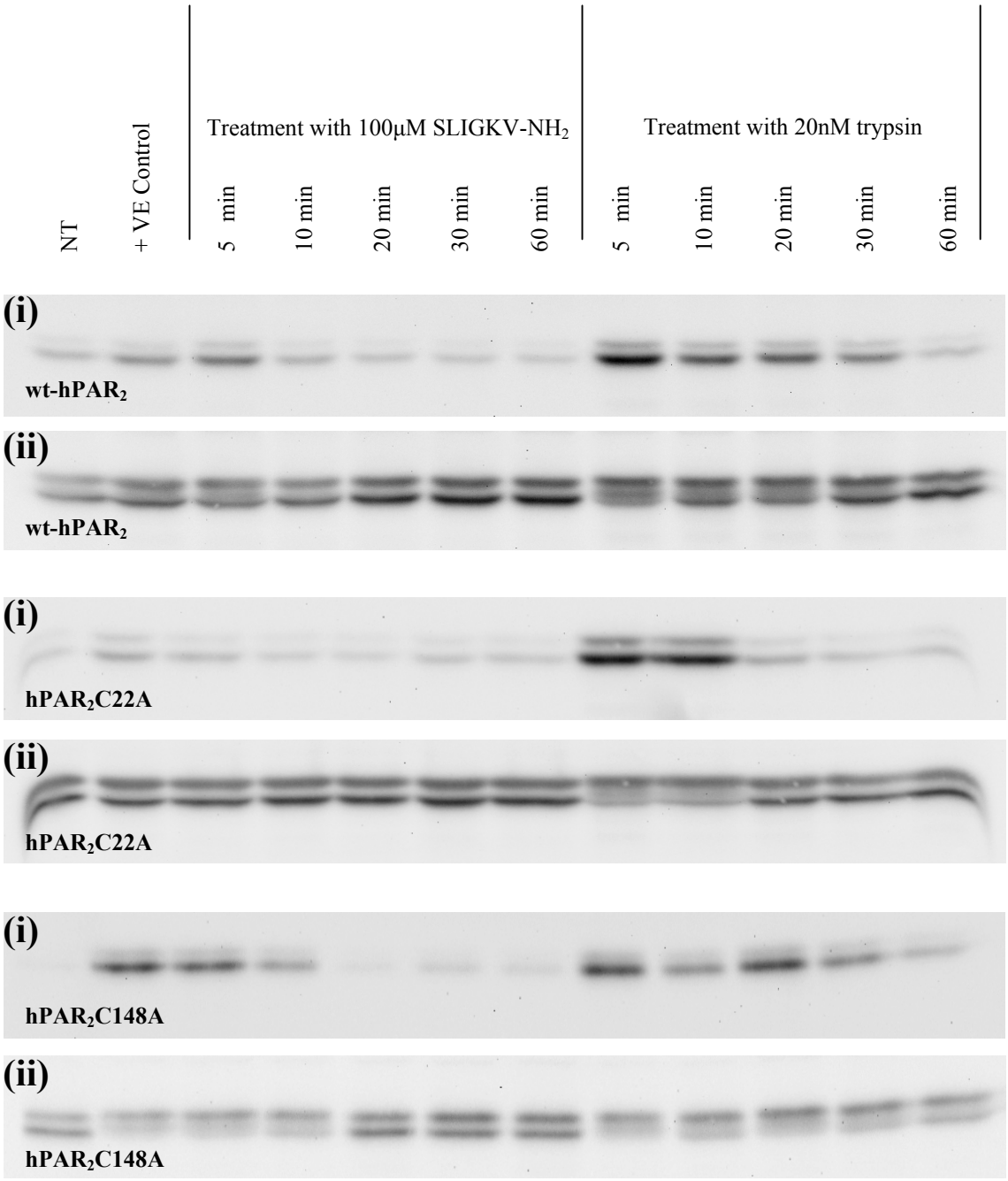


Fig 3.3.6a – Agonist Stimulated MAPK Phosphorylation Blots
Western blots produced for wt-hPAR$_2$, hPAR$_2$C22A, and hPAR$_2$C148A cells treated with agonist and protein harvested at set time points.
NT- no treatment, +VE control – 3.16 units/ml thrombin for 10 min.
(i) – Have been probed with anti-phosphorylated p44/42 antibody.
(ii) – Have been re-probed with anti-total p44/42 antibody.
A 10 min exposure time was used for all blots.
The blots shown are a representation of 3 repeats.

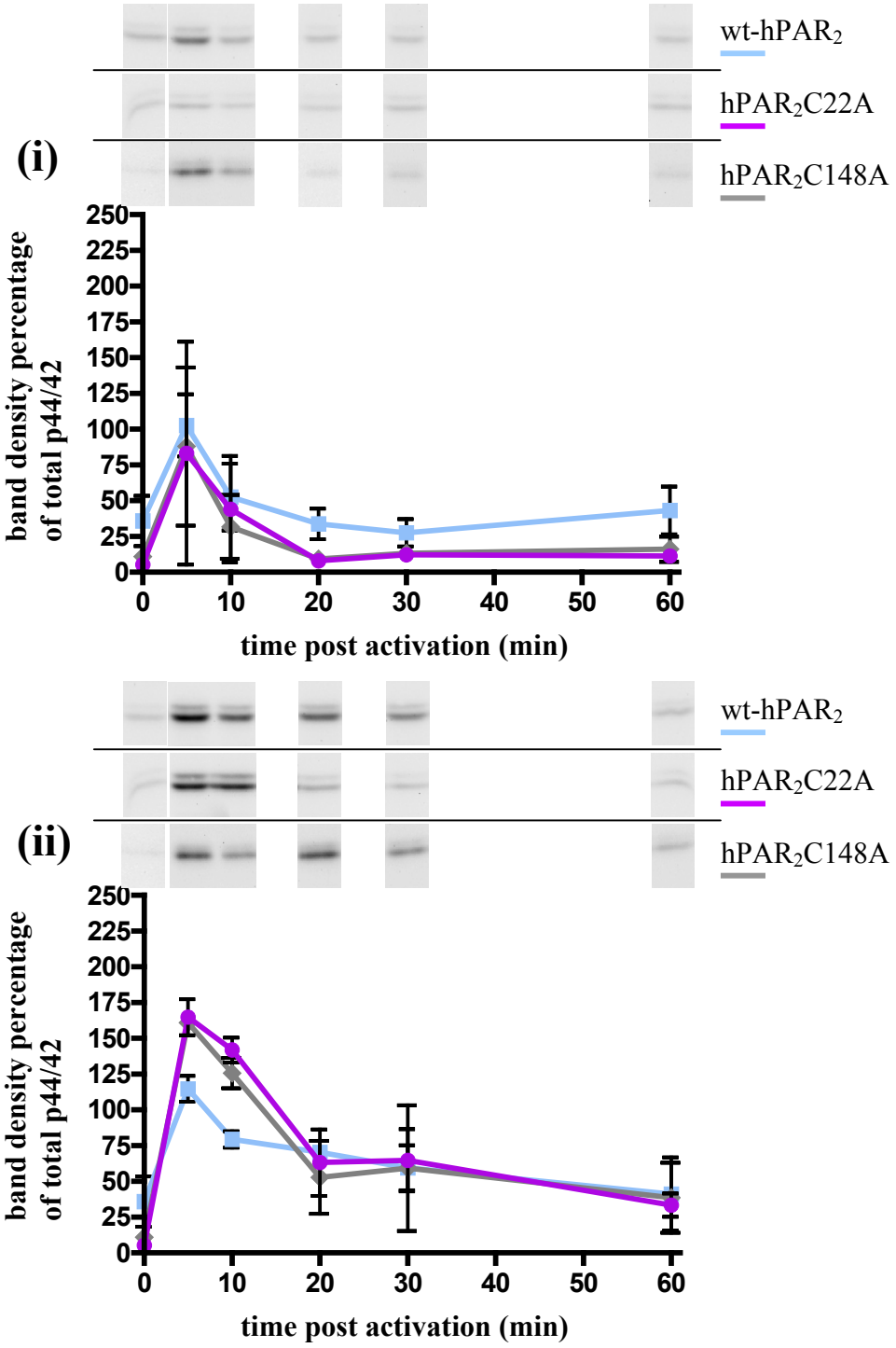


Fig 3.3.6b – Agonist Stimulated MAPK Phosphorylation Curves
Graphs showing densitometric values for bands produced by western blot (see **fig 3.3.6a**) for phosphorylated p44/42 post-SLIGKV-NH_2 treatment **(i)** and post trypsin treatment **(ii)**. Values expressed as a percentage of total p44/42 measured on reprobed blot (see **fig 3.3.6a**).

wt-$hPAR_2$ $hPAR_2$C22A $hPAR_2$C148A

Results are expressed as the mean +/- SEM of 3 separate experiments, while the bands above are representative of the blots.

3.3.7 Post-agonist activation internalisation

Since calcium signalling in response to both trypsin and SLIGKV-NH_2 was abolished in hPAR$_2$C148A we investigated whether or not internalisation of receptor still resulted post-agonist activation. Cells were incubated with agonist for 30 min prior to labelling with anti-PAR$_2$ mAb Sam-11.

Following treatment with trypsin a significant ($P<0.01$) reduction in Sam-11 cell surface staining can be noted in wt-hPAR$_2$ **[B]**, hPAR$_2$C22A **[C]** and hPAR$_2$C148A **[D]**. This is evident in the FACs traces by a shift to the left compared to untreated. By contrast treatment with SLIGKV-NH_2 resulted in no significant change ($P>0.05$) in cell surface staining with Sam-11 compared to untreated, as such the FACs trace very similar to that of untreated cells. No significant change ($P>0.05$) in Sam-11 labelling of empty vector transfected cells is evident following treatment with trypsin with SLIGKV-NH_2 . Fluorescence mean was then expressed as a percentage of untreated cell fluorescence and plotted on a graph **[E]**. The graph shows that cell surface fluorescence following treatment with trypsin drops to ~47% (SEM 3.77%) that of untreated cells in wt-hPAR$_2$. In hPAR$_2$C22A a reduction to ~43% (SEM 5.03%) is seen, similar to that of wild-type. For hPAR$_2$C148A a slightly larger reduction to ~37% (SEM 7.8%) of untreated cell signal is seen. No significant difference ($P=0.4759$) can be seen between each cell line for the post-trypsin treatment Sam-11 staining. Following treatment with SLIGKV-NH_2 no significant difference ($P>0.05$) is seen between cell lines remained around 100% of untreated signal, there is also no significant difference noted between cell lines ($P=0.3819$).

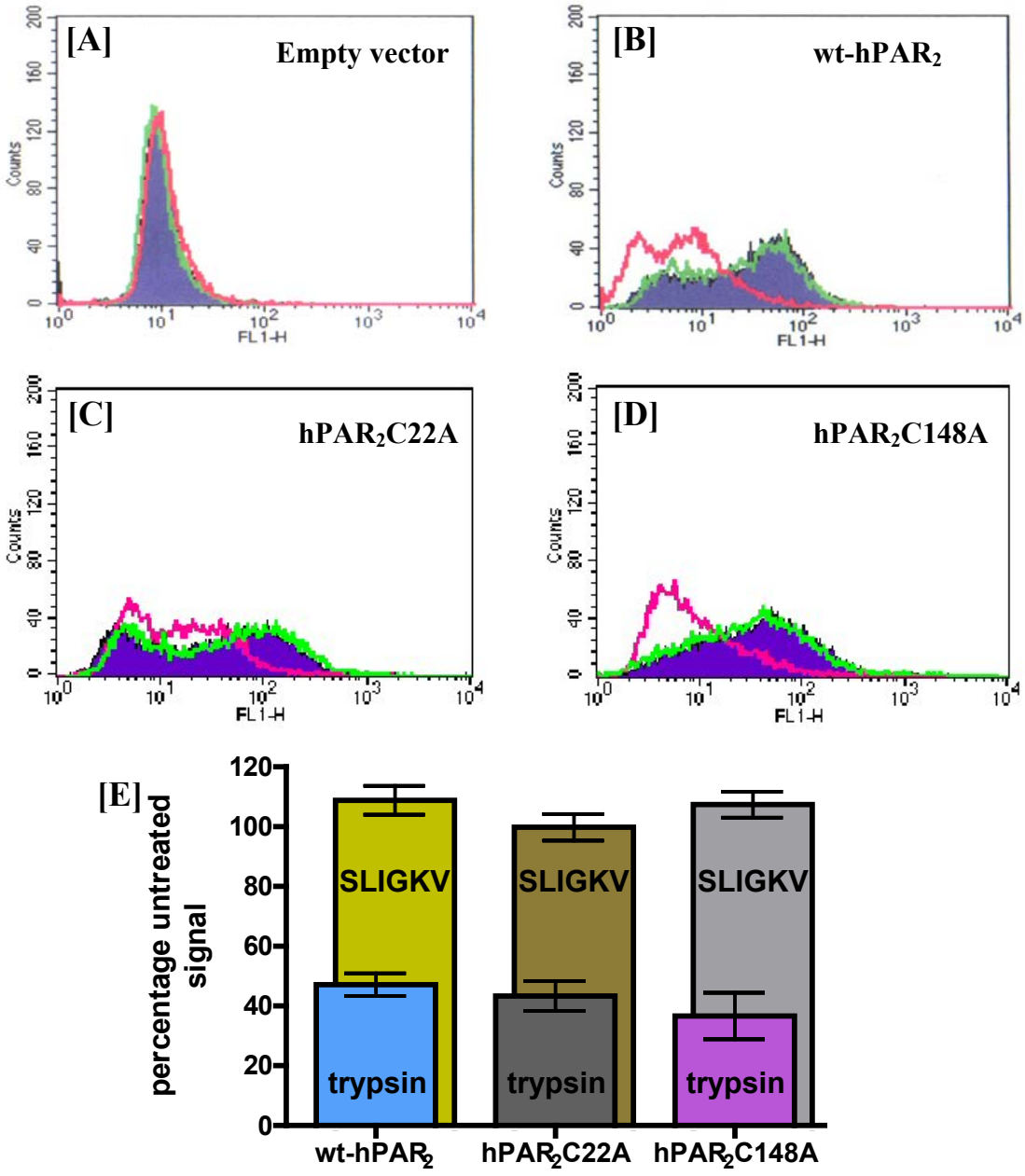


fig 3.3.7 - Post-activation internalisation
FACS histograms of empty vector, wt-hPAR$_2$ hPAR$_2$C22A and hPAR$_2$C148A cells (A, B, C, D respectively) incubated with trypsin (red), SLIGKV-NH$_2$ (green) or untreated (purple infill) for 30 min prior to staining Sam-11 anti-PAR$_2$ antibody. FACS histograms shown are representative of three experiments.
(E) Bar chart showing agonist triggered receptor internalisation for wt-hPAR2, hPAR2C22A and hPAR2C148A. Results are expressed as the mean +/- SEM from three separate experiments.

3.3.8 Internalisation of Endogenous PAR_2

Due to there being no observable reduction in cell surface staining for all cell lines tested following treatment with SLIGKV-NH_2 (**fig 3.3.7**), we investigated whether this could be due to the over-expression of receptor within the cloned cell lines. The internalisation $hPAR_2$ in Human Embryonic Kidney cell (HEK) post-agonist activation was assessed (**fig 3.3.8[A]**). HEK cells incubated with FITC conjugated secondary antibody only were used to ascertain non-specific fluorescence (shown in green).
The FACS histogram shows following treatment with trypsin (shown in blue) there is a reduction in cell surface staining with Sam-11 compared to untreated cells (shown in pink). However, following treatment with SLIGKV-NH_2 (shown in orange) cell surface staining with Sam-11 remains the same as untreated cells. The accompanying graph confirms fluorescence of cells following treatment with trypsin is reduced to ~53% (SEM 11.95%) that of untreated cells which is a significant change ($P<0.01$). When compared to wt-$hPAR_2$ post-trypsin treatment (fig 3.3.7) there shows no significant difference ($P=0.6703$) in Sam11 cell surface staining. Where as following treatment with SLIGKV-NH_2 fluorescence remains at ~ 93% (SEM 6.21%) of untreated cells, which when compared to wt-$hPAR_2$ post-SLIGKV-NH_2 treatment (fig 3.3.7) shows no significant difference ($P=0.8728$).

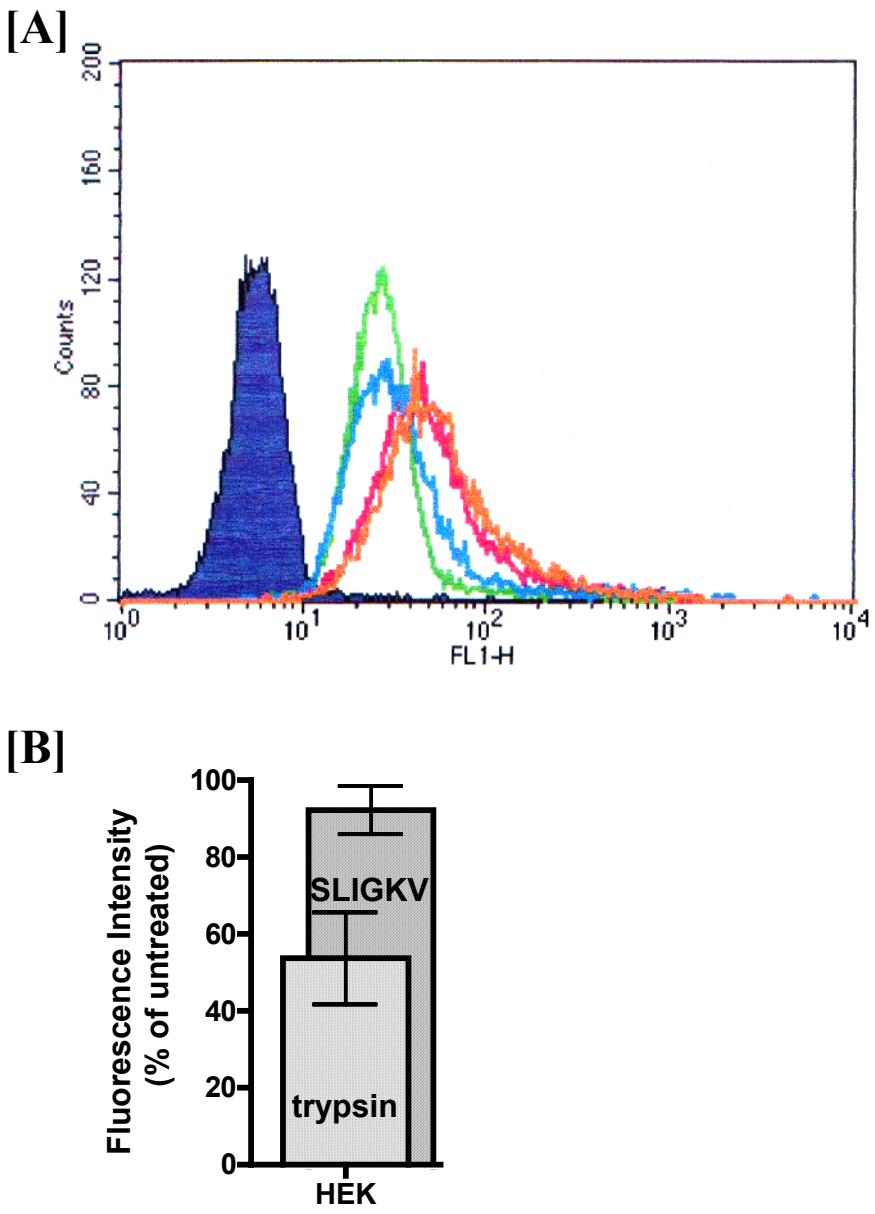


Figure 3.3.8 - Trypsin and SLIGKV-NH_2 Triggered Internalisation of endogenous PAR_2 in HEK Cells.
[A] HEK cells treated with either trypsin (conc), SLIGKV-NH_2 (conc) or not treated (NT) for 30 min prior to staining with Sam-11 anti-PAR_2 antibody and analysis of cell surface expression by FACs. FACs histogram is representative of three experiments. **[B]** Bar chart showing agonist induced internalisation relative to untreated control.

- 1^o antibody only
- 2^o antibody only
- SLIGKV-NH_2
- NT
- Trypsin

Results are expressed as a mean +/- SEM of 3 separate experiments.

3.3.9 Assessment of "free-cysteines"

We investigated whether or not C22 was a "free-cysteine" in $hPAR_2$. A considerable amount of time was spent attempting to get this method to work. Both reducing and no-reducing SDS-PAGEs were used, as well as different concentrations of avidin or anti-HA11-agarose and incubation times. If cells were incubated with MTSEA-biotin for a time period an longer than that used in the final protocol cell death and lysis resulted. Higher concentrations and longer incubation times with avidin-agarose only served to increase the non-specific binding and decreased concentration or incubation resulted in no protein yield. Initial experiments used ultracentrifugation to isolate avidin-agarose labelled receptor from the labelling solution, but this was found to result in loss of agarose so minispin™ columns were used which retained more of the avidin-agarose. wt-$hPAR_2$, $hPAR_2$C22A and empty vector transfected cells were labelled with MTSEA-biotin, immunoprecipitated and analysed by immunoblot. Immunoprecipitation of samples using anti-HA11 agarose and visualisation using avidin HRP (**fig 3.3.9(i) [A1]**) resulted in multiple bands in all lanes ranging from 20 – 120 kDa with empty vector control giving a stronger banding pattern than wt-$hPAR_2$ and $hPAR_2$C22A. No difference in banding is evident between samples or between MTSEA-biotin treated samples and untreated controls. When reprobed with anti-HA11 (**fig 3.3.9(i) [A2]**) additional banding appears in wt-$hPAR_2$ and $hPAR_2$C22A lanes between 40 and 75 kDa with no apparent difference in banding between wt-$hPAR_2$ and $hPAR_2$C22A, or between MTSEA-biotin treated samples and untreated controls. Immunoprecipitation of samples using avidin-agarose and visualisation using anti-HA11 (**fig 3.3.9(i) B1]**) resulted in no detectable banding in any lane. The reprobing of the same immunoblot with avidin-HRP (**fig 3.3.9(i) [B2]**) resulted in banding patterns in all lanes between 75 and 250 kDa. No difference can be seen between empty vector control or wt-$hPAR_2$

and $hPAR_2C22A$, nor is any difference in banding evident between samples or between MTSEA-biotin treated samples and untreated controls. Blots were then repeated using samples that were treated with trypsin prior to MTSEA-biotin labelling (**fig 3.3.9(ii)**) in order to remove the N-terminus pre-cleavage site. The resulting blots showed no difference to those produced without trypsin pre-treatment (**fig 3.3.9(i)**). MTSEA-biotin labelled wt-$hPAR_2$, $hPAR_2C22A$ and empty vector sample were then run alongside $hPAR_2C361A$ and $hPAR_2C148A$ samples as additional controls (**fig 3.3.9(iii)**). Banding resulted between 25 and 130 kDa for all cell lines. Banding intensity for $hPAR_2C361A$ was slightly less than that of other cell lines due to reduced sample loading. No difference in banding pattern can be seen between unlabelled samples (**fig 3.3.9(iii) [A]**) and MTSEA-Biotin labelled samples (**fig 3.3.9(iii) [B]**).

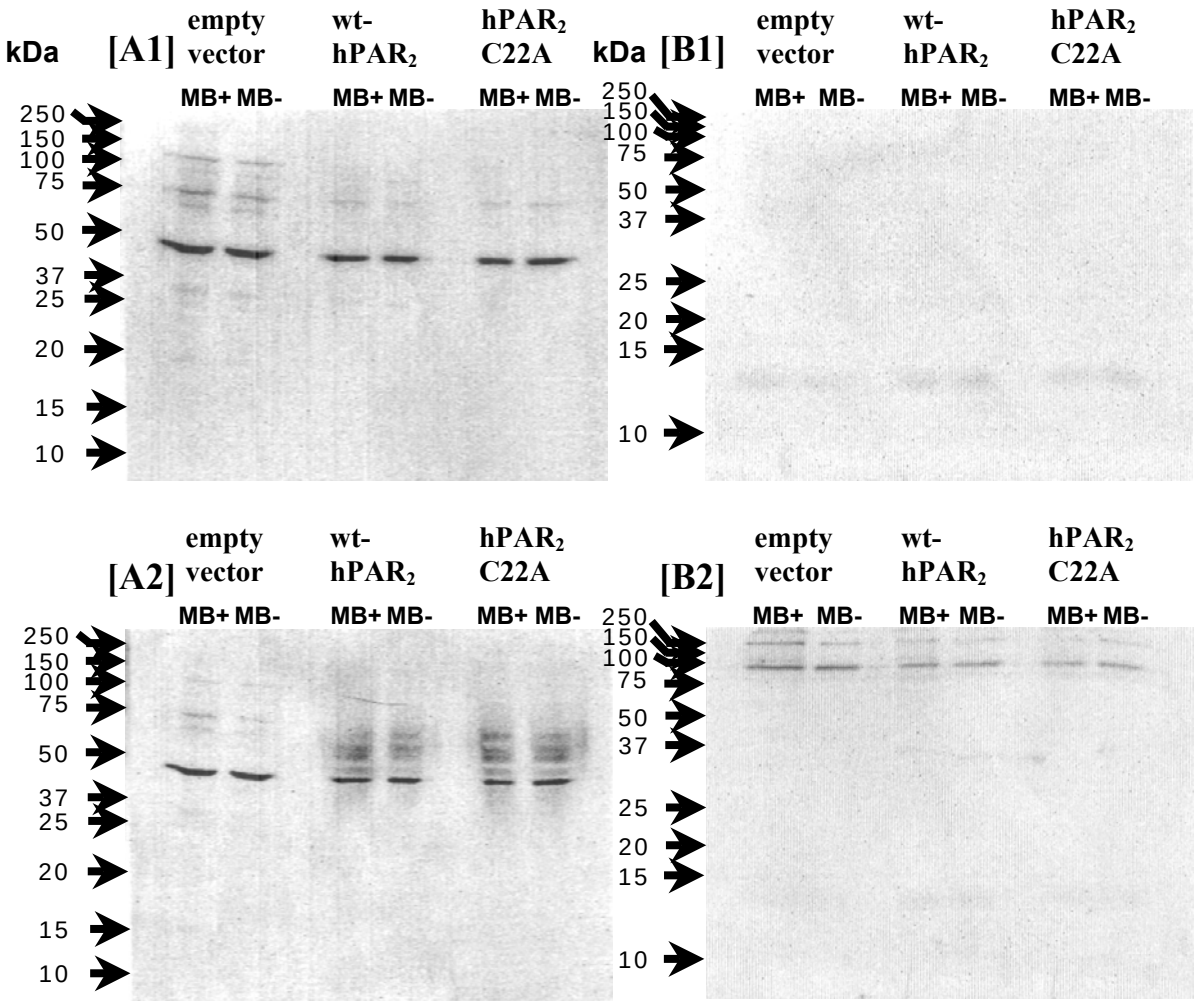


Figure 3.3.9(i) - MTSEA-Biotin immunoblot
wt-hPAR$_2$, hPAR$_2$C22A and empty vector control incubated with (**MB+**) and without MTSEA-Biotin (**MB**-) and immunoprecipitated using anti-HA11 agarose ([A1] and A2]) or avidin-agarose ([B1] and [B2]) and visualised on immunoblot using anti-HA11 ([A1] and [B2]) or avidin [A2] and [B1]. Pictures are representative of 3 repeats. Blots were exposed for 20 min

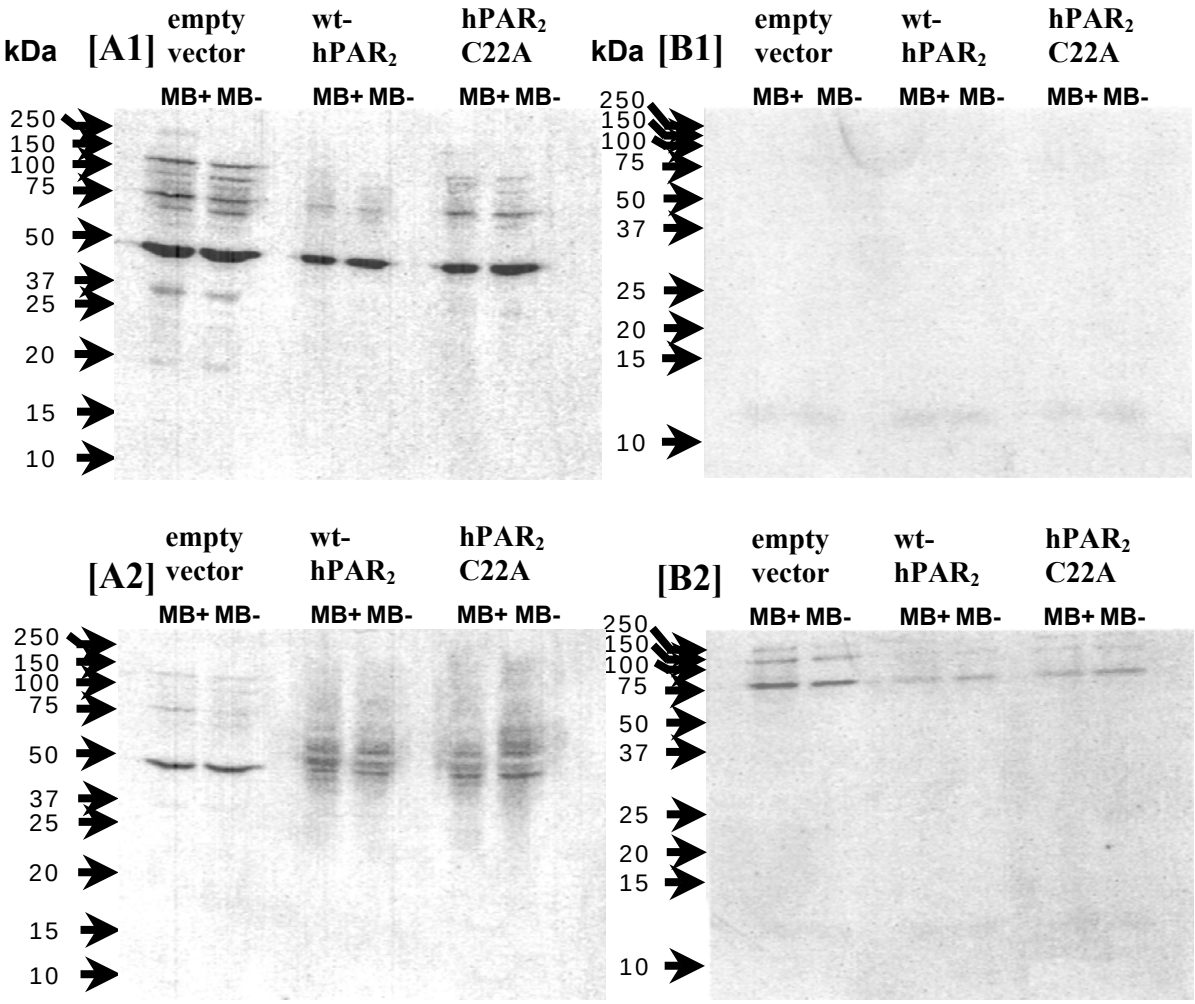


Figure 3.3.9(ii) - MTSEA-Biotin immunoblot post trypsin
wt-hPAR$_2$, hPAR$_2$C22A and empty vector control treated with 20 nM trypsin for 10 min before incubating with (**MB+**) and without MTSEA-Biotin (**MB-**) and immunoprecipitated using anti-HA11 agarose ([A1] and A2]) or avidin-agarose ([B1] and [B2]) and visualised on immunoblot using anti-HA11 ([A1] and [B2]) or avidin [A2] and [B1]. Blots were exposed for 20 min
Pictures are representative of 3 repeats.

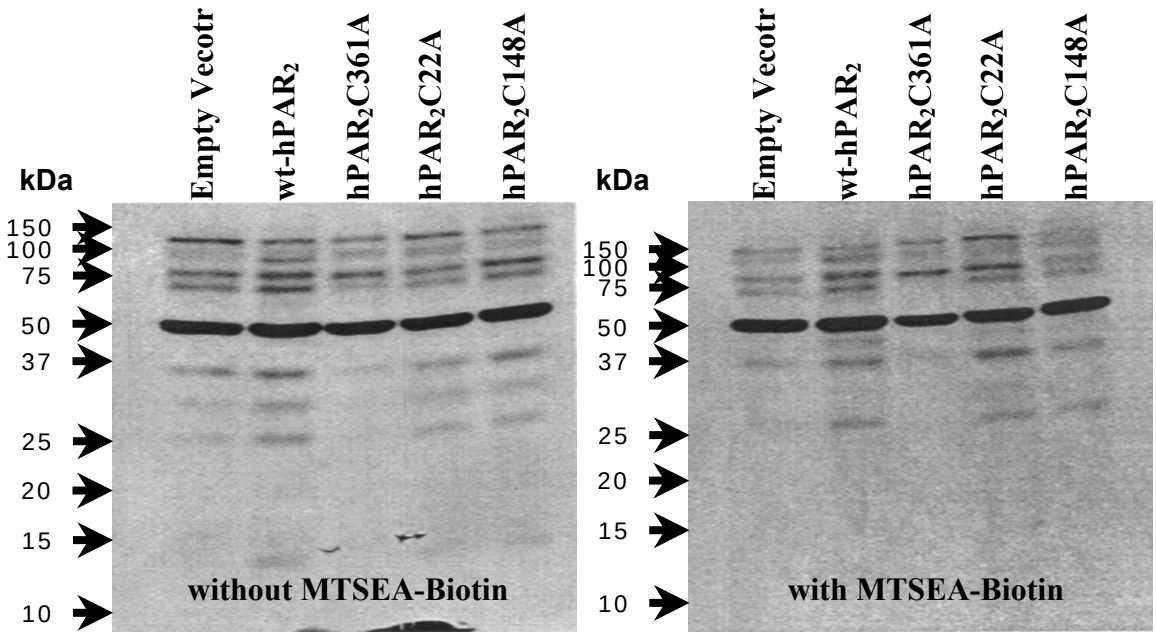


Figure 3.3.9(iii) - MTSEA-Biotin immunoblot cell line controls
empty vector control, wt-hPAR$_2$, hPAR$_2$C361A, hPAR$_2$C22A and hPAR$_2$C148A before incubating with (**[B]**) and without MTSEA-Biotin (**[A]**) and immunoprecipitated using anti-HA11 agarose and visualised on immunoblot using avidin-HRP. Blots were exposed for 20 min.
Pictures are representative of 5 repeats.

3.4 Discussion

This study shows for the first time the importance of C22, C148 and C226 in the expression and function of $hPAR_2$. The removal of C22 resulted in alterations in receptor pharmacology and increased trypsin sensitivity following pre-treatment with thrombin. C226 was found to be essential for successful receptor cell surface expression, whilst mutating C148 to alanine surprisingly resulted in a receptor that failed to couple to calcium, whilst retaining an ability to signal to ERK and undergo agonist mediated internalisation. In conclusion the data suggest that the disulphide bridge of PAR2 is not required for proper cell surface expression, agonist triggered internalisation and ERK signalling.

C226 is essential for receptor cell surface expression, as seen by the ablation of Sam-11 labelling in C226A and C148A/C226A mutants. Confocal imaging of eYFP tagged clones shows a significant retention of $hPAR_2C226A$ and $hPAR_2C148A/C226A$ intracellularly. The expression appears to both ring the nucleus, which is concurrent with retention within the endoplasmic reticulum, and scattered through cytosolic vesicles, presumably for lysosomic degradation. This is similar to results seen in the M_3 Muscarinic receptor where removal of conserved ECL1 and ECL2 causes ablation of receptor cell surface expression but dose not affect receptor folding (Zeng et al, 1999). In the case of rhodopsin removal of this disulphide bridge also results in reduced cell surface expression, though this is thought to be due to improper folding (Karnik et al, 1988; Karnik & Khorana, 1990) something also seen with the β_2-adrenergic receptor (Noda et al, 1994). The C148A mutation on its own still allowed for effective cell surface expression which only was slightly reduced compared to wt-$hPAR_2$. Confocal imaging of $hPAR_2C148A$ eYFP also shows receptor expression ringing the nucleus and

scattered throughout cytosolic vesicles, however receptor is also seen at the plasma membrane showing enough receptor to be properly folded and trafficked to allow cell surface expression. Previous cases where receptor cell surface expression remains intact for disulphide bridging mutants are the gonadotropin-releasing hormone (GnRH) receptor (Cook & Eidne, 1997) and the M_1 muscarinic acetylcholine receptor (Kurtenbach et al, 1990; Savarese et al, 1992). The C22A mutation appears to have no significant effect on receptor cell surface expression compared to wild-type showing it to not be involved in receptor trafficking or to significantly effect receptor structural folding or stabilisation to result in intracellular retention. Removal of the N-terminal cysteine in the GnRH receptor similarly leaves receptor cell surface expression unaffected (Cook & Eidne, 1997). In the GABA receptor cell surface is shown to be reduced on mutation of N-terminal cysteines as they are required to stabilise receptor hetero-dimerisation which is essential for receptor trafficking to the plasma membrane (Jones et al, 1998)

Agonist concentration effect curves for $hPAR_2C148A$ show this mutation to abolish trypsin and SLIGKV-NH_2 mediated Ca^{2+} signalling. A small deviation from baseline is evident at higher trypsin concentrations, though this is likely to be due to endogenous expression of PAR_1 with Pro5 cells. As such, this mutation results in either complete uncoupling of the receptor from its associated G-protein, or a non-functional receptor due to improper folding or disruption of the ligand binding site, or a combination of there factors. Agonist activation in disulphide bridge negative site-directed mutants is often reduced due to either improper formation of the ligand binding site or receptor generally (Kurtenbach et al, 1990; Kosugi et al, 1992; Savarese et al, 1992; Noda et al, 1994; Cook & Eidne, 1997; Zeng et al, 1999). The disulphide bridge in the β2-AR is

thought to stabilise the ligand binding pocket, as such its removal results in reduced ligand sensitivity (Noda et al, 1994). In the case the thyroid stimulating hormone receptor the tertiary structure of the receptor is affected resulting in improper receptor folding in the disulphide bridge absent mutant, so reducing receptor bioactivity (Kosugi et al, 1992). In that rat M_1 muscarinic receptor the disulphide bridge is responsible for proper folding of the receptor and formation of the ligand binding domain, removal of the disulphide bridge leaves the receptor incapable of responding to agonist and binding a [^{3}H]-antagonist (Savarese & Fraser, 1992; Savarese et al, 1992).

The agonist concentration effect curves for hPAR_2C22A suggest this mutation does not significantly effect SLIGKV-NH_2 binding and activation of the receptor. However, the TL appears to have a significantly reduced potency compared to wt-hPAR_2 giving a lower maximal response to trypsin (52.2 % compared to 67.9 %: $P<0.0001$). It may therefore be that this cysteine is required for the efficient binding of trypsin and/or the resulting cleavage of the N-terminus. Interestingly, pre-treatment of hPAR_2C22A with thrombin in an effort to densensitise endogenous PAR_1, resulted in an increased potency and sensitivity to trypsin compared to non-pre-treated hPAR_2C22A, giving a maximal response similar to wt-hPAR_2 and decreasing the EC_{50} value. This may occur as a result of the C→A mutation creating an additional thrombin cleavage site, as removal of the N-terminus at this point may increase trypsin binding efficiency. It may be that if the C→A mutation resulted in inefficient binding and cleavage of the N-terminus by trypsin then its removal at this point could restore trypsin efficiency. Alternatively, the effect may be the result of hetero- or homo-dimerisation present in wt-hPAR_2, occurring at C22, being disrupted in the mutant. Although dimerisation has not been demonstrated in PAR_2, transactivation of PAR_2 by PAR_1 has been previously noted (O'Brien et al, 2000)

and may be made possible by the close association resulting from hetero-dimerisation between the PAR_1 and PAR_2. The agonist concentration effect curves produced using TFLLR-NH_2 to densitise PAR_1 in order to test whether "sensitisation" was PAR_1 dependant or thrombin dependant show no increase in sensitivity to trypsin. This suggests that the sensitisation to trypsin seen following thrombin treatment is not PAR_1 signalling dependant and is, if not thrombin dependant, then PAR_1 protease or TL dependant. Since at high concentrations TFLLR-NH_2 is capable of activating PAR_2 (Hollenberg et al, 1997), the reduction in SLIGKV-NH_2 curves are likely to be due to some interaction by TFLLR-NH_2 with the ligand binding site insufficient to activate PAR_2 but sufficient to block efficient SLIGKV-NH_2 binding. The absence of an increased trypsin sensitivity following the disarming of PAR_1 with elastase suggests that the effect is not as a result of an interaction with PAR_1 N-terminal TL region.

Analysis of ERK1/2 MAP kinase signalling in hPAR_2C22A and hPAR_2C148A show these mutations to have no significant effect on ERK1/2 signalling ($P>0.05$). This shows hPAR_2C148A to be sufficiently correctly folded in order to allow receptor binding and activation by SLIGKV-NH_2 and the trypsin revealed TL. In addition this also shows that MAP kinase signalling for PAR_2 occurs independently of Ca^{2+} signalling, so supporting findings in studies showing β-arrestin regulation of ERK1/2 signalling (Kumar et al, 2007) and activation of PAR_2 promoting β-arrestin dependent actions independently of G-proteins and Ca^{2+} mobilisation (Zoudilova et al, 2007). This was done by siRNA mediated silencing of Gαq/11, inhibition of Gαq/receptor coupling (using GP antagonist 2A peptide), inhibition of signalling intermediates PLCβ (using U73122) and Ca^{2+} (using BAPTA-AM). Interestingly, when using Gαq/11 siRNA and BAPTA-AM a β-arrestin dependant pathway was potentiated, showing not only can β-

arrestin signalling occur independently of G-protein signalling/coupling but in opposition to it (Zoudilova et al, 2007). Thus suggesting the absence of agonist induced Ca^{2+} mobilisation seen in $hPAR_2C148A$ is as a result of either uncoupling or inefficient coupling to its respective G-protein and not as a result of a non-functional/non-ligand binding receptor.

Internalisation of receptor post-agonist activation was investigated for $hPAR_2C148A$ and $hPAR_2C22A$ and compared with wt-$hPAR_2$. All three receptors internalised following trypsin activation. wt-$hPAR_2$ receptor internalised by ~50 % similar to the amount previously seen to internalise post-agonist activation (Stalheim et al, 2005) and $hPAR_2C22A$ internalises to similar extent. $hPAR_2C148A$ has slightly increased internalisation compared to wt-$hPAR_2$. Firstly the fact that the receptor internalises post-trypsin treatment provides further evidence of receptor activation by TL, meaning showing the receptor to be properly folded. Secondly the increased internalisation maybe as a results of β-arrestin related signalling events being potentiated in the absence of Ca^{2+} and signalling through Gαq (Zoudilova et al, 2007). Interestingly, and contrary to previous publications (Dery et al, 1999; DeFea et al, 2000b), none of the receptors internalised post-SLIGKV-NH_2 treatment including wt-$hPAR_2$. Since SLIGKV-NH_2 is a significantly less effective agonist than the TL lack of internalisation post-activation may have been due to over-expression of $hPAR_2$ in the recombinant cell lines used. As such, we further investigated receptor internalisation in a HEK cells endogenously expressing $hPAR_2$ (Yu et al, 1997; Dale et al, 2006) at levels not driven to over-expression. HEK cells too displayed ~50 % receptor internalisation post-trypsin activation again and no significant internalisation of receptor following activation with SLIGKV-NH_2. It may be that under different experimental conditions SLIGKV-NH_2

would be capable of driving receptor internalisation, for example at physiological temperatures receptor endocytosis would occur more readily. In the case of Bohm et al, 1996a; Dery et al, 1999; and Defea et al, 2000, all of which reported internalisation with SLIGKV-NH_2, internalisation experiments were carried out at 37^{o}C in KNRK cells. The internalisation of endogenous PAR_2 was determined with the use of HEK cells in our study, where as Defea et al, 2000 used rat hBRIE cells which may respond differently to SLIGKV-NH_2. Additionally with Dery et al, 1999 the concentration of SLIGKV-NH_2 used was 500 μM, 5 times that used in this study. Since there are a number of different PAR_2-APs available it may also be possible that internalisation would occur following treatment with a different PAR_2-AP, certainly Kumar et al, 2007 used 100 nM 2-furoyl-LIGRL to demonstrate agonist peptide-induced internalisation of receptor.

Interestingly demonstrating SLIGKV-NH_2 to not result in post-activation internalisation of $hPAR_2$ is also contrary to previous studies stating β-arrestin mediated endocytosis to be required for ERK1/2 activation (DeFea et al, 2000b; Kumar et al, 2007; Zoudilova et al, 2007). However, since all of these studies used either β-arrestin knockout, β-arrestin siRNA, and/or a truncated form of β-arrestin incapable of receptor binding it seems likely that β-arrestin receptor binding, which frequently leads in receptor endocytosis, is essential for ERK1/2 activation and not endocytosis per se. The reason for trypsin resulting in $hPAR_2$ internalisation where SLIGKV-NH_2 does not maybe due to a conformational change occurring through the binding of the tethered ligand which does not occur following SLIGKV-NH_2 binding. Alternatively, if $hPAR_2$ exists as a hetero-dimer via covalent association with an amino-acid pre-cleavage site with a complementary site on PAR_1 e.g. C22, trypsin cleavage may be required to dissociate $hPAR_2$ from its dimer allowing endocytosis of the monomer. In this model SLIGKV-NH_2 activated $hPAR_2$ would remain tethered to the cell surface by the other receptor,

this has previously been demonstrated in the δ- opioid receptor which when co-expressed with κ-opioid receptor results in retention of the δ-opioid receptor at the cell surface (Jordan & Devi, 1999). Additionally, co-expression the β2-adrenergic receptor with the κ-opioid receptor also result in the β2-adrenergic receptor being retained at the cell surface post-activation (Jordan et al, 2001). This model may also account for why previous studies have shown internalisation following SLIGKV-NH_2, as if $hPAR_2$ is expressed in a cell line which does not allow for its hetero-dimerisation it may remain as a monomer or form a homodimer. As such on activation a monomer would be free to internalise and in a homodimer both receptors would be activated allowing internalisation.

The use of MTSEA-Biotin in the investigation of extracellular "free-cysteines" showed no extracellular "free-cysteines" in wt-$hPAR_2$ or $hPAR_2$C22A. This suggests that reactive sulfhydryl group of C22A is interacting with something inhibiting the binding of MTSEA. However, since removal of C22 did not result in the production of a free cysteine this suggests that the interaction is not with another cysteine residue located within the receptor. Alternatively, it is possible that C22 is unbound but is in someway masked at the cell surface by normal receptor folding leaving it inaccessable to MTSEA binding as seen with some cysteines in the P2X receptor (Ennion & Evans, 2002), though this seems unlikely given its position within the N-terminal close to the trypsin binding site. Since no positive control was available complete evaluation of this method was not possible. Attempts to use $hPAR_2$C148A as a positive control resulted in negative results possibly due to inter- or intra- receptor interaction with other cysteine residues or the unavailability of C226 to MTSEA due to receptor folding. As such lack

of detection of a free-sulfhydryl group maybe as a result of improper experimental technique.

This study shows for the first time the importance of C226 in the expression of $hPAR_2$ and the importance of C148 in the Gαq coupling and agonist mediated Ca^{2+} mobilization in $hPAR_2$. This study has also identified an interesting role for C22 in affecting the agonist sensitivity of $hPAR_2$.

4 PALMITOYLATION OF $hPAR_2$

4.0 PALMITOYLATION OF hPAR$_2$

4.1 Introduction

4.1.1 Lipid modifications

The modification of many proteins via the covalent attachment of lipid moieties is an essential process to ensure efficient membrane targeting, trafficking and signalling (Escriba et al, 2007). In both eukaryotic and viral systems four major forms of lipid modifications have so far been identified: the co-translational amino (N)-terminal myristoylation of cytosolic proteins; the modification of plasma membrane proteins glycosphosphatidyl inositol (GPI); the carboxy (C)-terminal isoprenylation of cytoplasmic proteins; and the post-translational addition of palmitic acid to integral and peripheral membrane proteins (Casey & Seabra, 1996; Resh, 1999). The addition of palmitic acid to most proteins occurs via the esterification of a free thiol of a cysteine residue (Bijlmakers & Marsh, 2003). As other saturated and unsaturated fatty acids can also be utilised, the term S-acylation (Resh, 1999) or more accurately acyl thioesterification or thioesteracylation (Qanbar & Bouvier, 2003) is the correct terminology. However, it is more frequently referred to as “palmitoylation” and this is the term that will be used here.

4.1.2 Palmitoylation

Palmitoylation is one of four major post-translational lipid modifications and apparently the most common and versatile (Bijlmakers & Marsh, 2003). It involves the acylation of a protein with a 16-carbon saturated fatty acid (palmitic acid) through a thioester or oxyester bond. In palmitoylations most predominant form palmitic acid is covalently attached via a thioester linkage to cysteine residues on the protein (Bijlmakers & Marsh,

2003). In contrast to peripheral proteins which require lipid modifications for tight membrane attachment (Escriba et al, 2007) GPCR are already membrane associated. GPCRs are typically palmitoylated on a cysteine residue in the cytoplasmic tail between 10 to 14 amino acids downstream of the last transmembrane domain (Probst et al, 1992). The first GPCR to be identified as being palmitoylated was rhodopsin (O'Brien & Zatz, 1984) and it was hypothesized that the attached palmitate may incorporate into the plasma membrane and so create an additional intracellular loop. This was later confirmed (Moench et al, 1994) and explains why palmitoylation has such a profound effect on local conformation of this domain, which possibly mediates interactions with specific regulatory proteins. Cysteine residues at similar locations where found in approximately 80% of all GPCRs indicating palmitoylation to be a general characteristic of this receptor type (Escriba et al, 2007). However, the removal of all C-terminal cysteines from the rat μ-opioid receptor had no effect of palmitate incorporation (Chen et al, 1998), suggesting palmitoylation sites exist outside of the C-terminal domain.

Thanks to its relatively unstable thioester linkage palmitoylation is unlike other lipid modifications in that it is readily reversible, and thus has the potential to be regulated (Mumby, 1997). An example of which is seen with β2-adrenergic receptor, which increases its palmitate incorporation upon treatment with agonist (Mouillac et al, 1992). Since a protein may undergo numerous rounds of palmitoylation and depalmitoylation within its lifetime, the dynamic nature of palmitoylation may suggest a regulatory influence on protein function. Gaining a greater understanding of the effects of palmitoylation in respect to the function of specific GPCR may provide a novel target for altering, rather than blocking GPCR-mediated responses. Thus indicating the effects if this modification were to be targeted therapeutically. We have identified a putative

site of palmitoylation with the intracellular C-terminal tail of $hPAR_2$ and intend to investigate its role in receptor function.

4.2 Materials and Methods

4.2.1 Materials

[^{3}H] palmitate, Hyperfilm™ MP x-ray film and Amplify™ was purchased from Amersham Biosciences Ltd (Little Chalfont, Buckinghamshire, U.K.), as was ECL detection reagents and donkey anti-rabbit IgG HRP-linked antibody. µMACS™ HA11 Epitope Tag Protein Isolation Kit was purchased from Miltenyi Biotech, (Bergisch, Germany). Film developer and fixer were purchased from Sigma (Dorset, UK). The MAP kinase antibodies, Phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody and p44/42 MAP kinase antibody were purchased for Cell Signaling Technology, Inc. through New England Biolabs (Herts, UK.). KNRK hPAR2 eYFP cells were kindly supplied by Dr. S.J. Compton, Cardiovascular Respiratory Medicine, University of Hull, from previous work. AF1 antifade was supplied by CITI-flour, Stanstad, UK.). Bisindolylmaleimide I (GF109203X), Phenylarsine oxide and concanavalin A were purchased from Sigma-Aldrich Chemicals Group (Dorest, UK).

4.2.2 Methods

4.2.2.1 FACS Analysis: Expression vs. Confluence

The following protocol was carried out using both PRO5 cells stably expressing $hPAR_2$ wild-type and PRO5 cells stably expressing $hPAR_2$C361A. Controls of un-transfected PRO5 parent cells at ~50% confluence in a T25 were also used to assess baseline. Increasing cell numbers were pipetted into 5X T25 flasks to give the following confluence on the day of analysis: 20%, 30%, 50%, 60% and 70%. Old media was discarded and the cells washed with 2 ml of PBS (1x). Cells were then dissociated with 2 ml of cell dissociation fluid and transferred to a pre-chilled 5 ml falcon tube, and centrifuged at 500 *g* for 5 mins at 4^{o}C. The supernatant was disposed of, and the pellet

resuspended in 300 µl of cold-PBS (1x). Sam11 anti-hPAR$_2$ antibody [1 in 100 ≡ 10 µg/ml] was then added and the samples incubated at 4^{o}C for 60 mins with swirling at 15 min intervals. 1 ml of cold-PBS (1x) was then added to each sample before centrifugation at 500 *g* for 5 mins at 4^{o}C. Supernatant was disposed of, and cell pellets were resuspended in 300 µl of cold-PBS (1x). FITC-conjugated anti-mouse IgG [1 in 300 ≡ 30 µg/ml] was then added to each sample and the samples incubated on ice for 45 mins, swirling at 15 min intervals. 1 ml of cold-PBS (1x) was then added to each sample and the sample centrifuged at 500 *g* for 5 mins at 4^{o}C. Supernatant was disposed of, and cell pellets were resuspended in 300 µl of cold-PBS (1x) before analysis using a Beckton Dickinson FACS Calibur using the un-transfected Pro5 parent cells as the calibrating control.

4.2.2.2 Confocal Microscopy

Cells were grown on 18mm^2 glass coverslips to a confluence of 40-50%, before media was removed and the cells washed with PBS (with Ca^{2+}/Mg^{2+}). Coverslips were then treated with either 100nM trypsin, 100µM SLIGKV-NH_2 or received no treatment for 30 min.

For Sam11 staining coverslips were then washed twice with PBS and incubated with Sam11 anti-PAR$_2$ antibody [1 in 1000 ≡ 1µg/ml] at RT for 60 min. Coverslips were then washed twice with PBS and incubated with FITC conjugated anti-mouse antibody [1 in 1000 ≡ 1µg/ml]. Coverslips were then washed twice with PBS before mounting cell side down onto glass slides. The slides were then analysed on a Nikon Eclipse (TE2000-E) confocal microscope with a BioRad Radiance 2100 scanning system and lasers using the argon laser exciting at 488 nM and detecting fluorescence emissions above 500 nM.

For HA11 staining coverslips were washed twice with PBS (1% BSA) and fixed by treatment with 3% formaldehyde for 15 min, prior to a permeabilising treatment with 0.2% v/v Triton X-100 in PBS for 10 min. Coverslips were then pre-incubated in PBS (1% BSA, 3% Rabbit Serum (RS)) for 15 min prior to addition of anti-HA11 mouse monoclonal antibody [1 in 1000 ≡ 1μg/ml] and incubated at RT for 60 min. Coverslips were then washed twice with PBS (1% BSA) and incubated with FITC conjugated anti-mouse antibody [1 in 1000 ≡ 1μg/ml]. Coverslips were then washed twice with PBS (1% BSA) before mounting cell side down onto glass slides. The slides were then analysed on a confocal microscope using an argon laser exciting at 488 nM and detecting fluorescence emissions above 500 nM. Each experiment consisted of two pictures from each of three coverslip repeats for each treatment and a final consensus photo being chosen.

4.2.2.3 Crude membrane preparation

Initially the production of crude membrane preparations were carried out using a method adapted from Compton et al, 2002. Cells in 75cm^2 flasks were rinsed with PBS and then dH_2O for 30 secs before harvesting in cold membrane buffer (Tris 5 mM, EDTA 0.5 mM, Leupeptin 1 μg/ml, STI 1 μg/ml, Orthovanadate 1 mM, NaF 50 mM. pH 7.5) with the aid of a cell scraper. The cell suspension was then passed several times through a 0.22 gauge needle to disrupt the cell membrane causing cell lysis. The crude lysate was transferred to a 15 ml falcon tube and centrifuged at 500 *g* for 10 min at 4^{o}C to remove the nuclear fraction. The supernatant was then transferred to multiple ultracentrifuge tubes and centrifuged at 20,000 *g* for 45 min at 4^{o}C. Supernatant was then disposed of and the resulting membrane protein pellets resuspended in 500 μl of membrane buffer.

Later the method adopted was to non-enzymically harvest the cells and centrifuge at 500 *g* at 4°C for 5 min to create a cell pellet and the supernatant was discarded. The cell pellet as then resuspended in 500 µl of MPER® mammalian protein extraction reagent. Suspensions were then centrifuged at 15000 *g* for 20 min at 4°C. Supernatants (solubilised membrane protein preparation) were retained and the DNA pellets discarded.

4.2.2.4 Immunoprecipitation of PAR_2 by Profound Method (Pierce)

Initially PAR_2 protein was purified using ProFound™ HA-Tag IP/C-IP kit (Pierce Biotechnology, Inc. Northumberland, UK.). Anti-HA11 agarose (containing10 µg of anti-HA11 antibody) was added to crude membrane preparations and incubated overnight at 4°C with inversion, before transferring to a Handee™ mini-spin column. Samples were then pulse-ultracentrifuged for 10 sec and flow-through was discarded. Samples were then washed three times by adding 500 µl BupH™ (0.05% Tween-20) and pulse-ultracentrifuged for 10 sec before flow-through was discarded. Elution was carried out by transferring the column to a collection tube and adding 20 µl of sample buffer to the centre of the column before incubating at 95°C for 5 min. The column was then pulse-ultracentrifuged for 10 sec and the sample retained and stored at -80°C until used.

4.2.2.5 Immunoprecipitation of PAR_2 by Vector Labs Method

The next means of isolating PAR_2 protein was using the Vector® Fusion-Aid™ HA Kit, (Agarose Anti-HA) (Vector laboratories, Ltd. Peterborough, UK.). This method was carried out using the same protocol as **4.2.2.4** except using Vectorlabs anti-HA11 agarose (containing 10 µg of anti-HA11 antibody).

4.2.2.6 Immunoprecipitation using µMACS™ Protein G microbeads and µMACS™ HA11 Epitope Tag Protein Isolation Kit

Next in order to specifically purify PAR_2 protein we used a primary anti-HA11 antibody (Covance Research Products, Ltd. Harrogate, UK.) and µMACS™ Protein G microbeads and µMACS™ separator column (Miltenyi Biotec Ltd, Surrey, UK.). Crude membrane preparations or lysates (prepared as described in **4.2.2.3**) were incubated overnight with anti-HA11 antibody [2 µg/ml] at 4°C with inversion. 50 µl of Protein G microbeads were then added and incubated at 4°C for 30 min. Samples were then loaded onto membrane buffer pre-rinsed µMACS™ separation columns, already mounted on the µMACS™ magnet, and allowed to run through. The flow-through was discarded and the columns washed four times with 200 µl of membrane buffer (Tris 5 mM, 1% Triton X-100, EDTA 0.5 mM, Leupeptin 1 µg/ml, STI 1 µg/ml, Orthovanadate 1 mM, NaF 50 mM. pH 7.5), discarding the flow-through at the end of each wash. Elution was carried out by applying 20 µl of 1X SDS loading buffer (pre-heated to 95°C) to the column and incubating for 5 min before adding a further 50 µl of 1X SDS loading buffer (pre-heated to 95°C) and collecting the flow-through and storing at -80°C until used.

In order to immunoprecipitate receptor using µMACS™ HA11 Epitope Tag Protein Isolation Kit crude membrane preparation was first incubated with 50 µl of anti-HA11 Tag Microbeads for 30 min at 4°C. Samples were then loaded onto column buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris HCl, pH 8.0) pre-rinsed µMACS™ separation columns, already mounted on the µMACS™ magnet, and allowed to run through. The flow-through was discarded and the columns washed four times with 200 µl of wash buffer 1 (150 mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris HCl, pH 8.0), and then once more with 100 µl of wash buffer 2 (20mM Tris

HCl, pH 7.5) discarding the flow-through after each wash. Elution was carried out by applying 20 µl of 1X Elution buffer (50 mM Tris HCL, 50 mM DTT, 1% SDS, 1mM EDTA, 0.005% bromophenol blue 10% glycerol, pH 6.8)(pre-heated to 95^{o}C) to the column and incubating for 5 min before adding a further 50 µl of 1X SDS loading buffer (pre-heated to 95^{o}C) and collecting the flow-through and storing at -80^{o}C until used.

4.2.2.7 [^{3}H]-Palmitate labelling and receptor visualisation

Pro5 cells expressing wt-hPAR$_2$, hPAR$_2$C361A, and pcDNA3.1 empty vector were seeded in T75 flasks and grown to the required confluence. Cells were then pre-incubated overnight in serum-free medium. Toluene containing the [^{3}H]-palmitic acid was then evaporated overnight under vacuum before the dried [^{3}H]-palmitic acid was dissolved in DMSO. The palmitate labelling media was then made and contained α-MEM containing 1mCi/3ml [^{3}H] palmitate (30-60 Ci/mmol**),** 1% dimethylsulfoxide (DMSO) and 2.5% FCS). Serum-free media was then removed and cells were washed with PBS prior to incubation with 3ml of [^{3}H]-palmitate labelling media for 4h at 37^{o}C. In separate experiments to assess the effect of agonist stimulation on palmitate incorporation wt-hPAR$_2$ cells were treated with either 100 µM SLIGKV-NH$_2$, 20 nM trypsin (trypsin concentration were adjusted to 20 nM for these experiments as high concentrations resulted in the lifting of cells) or no treatment for the final 15 min of labelling. Labelling media was then removed and disposed of. Cells were then washed twice with PBS and 3 ml of 1 mM EDTA in PBS was then added to the cell surface and the cells lifted with a cell scrapper before transferring the resulting cell suspensions to pre-chilled 15 ml falcon tubes. Cells were pelleted by centrifugation at 500 *g* for 5 min at 4^{o}C. Supernatant was discarded and the cell pellet resuspended in 500 µl of MPER®

mammalian protein extraction reagent. Suspensions were then centrifuged at 15000 *g* for 20 min at 4°C. Supernatants (whole cell lysate) were retained and the DNA pellets discarded. A receptor protein preparation was then produced from the crude membrane protein preparation using μMACS™ HA11 Epitope Tag Protein Isolation Kit as per manufacturers protocol. The resulting receptor protein preparation was then split into two halves and frozen at -80°C until use.

Isolated receptor samples were then resolved on SDS-PAGE (as previously described **2.2.5**) under non-reducing conditions. The gel was then fixed for 30 min in gel fixing buffer before treating with Amplify™ fluorographic reagent for 15 min at RT. The gel was then dried under vacuum at 80°C and the resulting dried gel exposed to Hyperfilm™ MP x-ray film, in a radiation cassette, at -80°C for 6 weeks prior to developing.

The remaining half of each sample was analysed by anti-HA11 western blotting (as previously described **2.2.5**).

4.2.2.8 Inhibition of trypsin mediated calcium signal by pertussis toxin

wt-hPAR$_2$ and hPAR$_2$C361A expressing cells were grown in 14X T25 flasks (2 for each concentration of PTX) to the confluences of 50% and 70% respectively. Media was then replaced with serum-free media containing pertussis toxin (PTX) concentrations of 1 – 316 ng/ml, and cells further incubated for 18 hours at 37°C. Cells were then harvested non-enzymically using 1 mM EDTA and centrifuged at 500 *g* for 5 min. Cell pellets were resuspended in 100 μl of growth media, containing sulphinpyrazone [250 μM], and each incubated separately with fluo-3 [50 μM] (prepared as previously stated **2.2.4**) for 30 min at R.T. Excess unabsorbed Fluo-3 was then removed by washing with 1 ml of PBS and centrifuging at 500 *g* for 5 min. Cell pellets were resuspended in 2 ml of

Ca^{2+} assay buffer and transferred fluorimeter cuvettes for analysis. Ca^{2+} signals were recorded in response to addition of 100 nM of trypsin. This trypsin concentration was selected as it is the EC_{90} for trypsin activation of $hPAR_2$. Following trypsin addition 2 μM of A23187 was added in order to measure maximum attainable fluorescence. Each concentration of PTX was measured in duplicate.

NB: 20 nM of trypsin were used during internalisation inhibition experiments as higher concentrations resulted in cell death.

4.2.2.9 FACS Analysis: Receptor Internalisation

The following protocol was carried out using both Pro5 cells stably expressing $hPAR_2$ wild-type at ~40% confluence, and Pro5 cells stably expressing $hPAR_2$ C361A at ~80% confluence in T25 flasks (difference in confluence used to normalise receptor expression levels between cell lines). Controls pcDNA3.1 empty vector transfected cells were grown at ~50% confluence in a T25 and used to assess baseline. 5X T25 flasks for wt-$hPAR_2$ and $hPAR_2$C361A expressing cells were harvested and pooled for each cell line, half the cells were disposed of for the $hPAR_2$ C361A mutant in order to normalise cell number. The cell suspensions were then centrifuged at 500 *g* for 5 mins at 4°C, the supernatant was disposed of and the pellet resuspended in 3.0 ml of cold-PBS(1x) for sample cell lines and 1.2 ml for controls. Cell suspensions were then aliquoted in 300 μl into pre-chilled falcon tubes (10x for samples, 4x for controls). Tubes were then treated with either 100 μM SLIGKV, or 100 nM trypsin, for 0, 10, 20, 30, or 60 min (prior to further treatment tubes were stored on ice and then removed and rolled between the palms of the hands briefly to raise them to RT). Controls were treated for only 0 and 60 mins. Sam-11 cell surface staining was then measured using FACS as in **2.2.3.5**. This protocol was then repeated for time values of 0, 1, 5, and 10 mins. Values for empty

vector control (taken as baseline fluorescence) were subtracted from fluorescence values for each sample and the results expressed as a percentage of the cell lines fluorescence at 0 min minus baseline. These results were plotted on *y* axis versus mins treatment on *x* axis using Prism Graphpad 4 to give a receptor internalisation curve.

4.2.2.10 FACS Analysis: Permeabilised

Cells were grown in T25 flasks (1 per agonist treatment), harvested non-enzymically and pelleted by centrifugation at 500 *g* for 5 mins. Cell pellets were resuspended in 600 µl of PBS (1X) before treatment with trypsin [20 nM], SLIGKV-NH_2 [100 µM] or no treatment for 10 min. Treatment was halted by addition of 2 ml of cold-PBS and transfer to an ice bucket. Samples were then centrifuged at 500 *g* for 5 mins at 4^{o}C, the supernatant was discarded and the cell pellet resuspended in 600 µl of PBS (1X). Samples were split in two and subjected to one of two antibody labelling methods. Half were treated with 300 µl of 3% formaldehyde for 15 min prior to addition of 1 ml of PBS (1X). Cells were then pelleted by centrifugation at 500 *g* for 5 min, the supernatant was discarded and the cell pellet resuspended in 300 µl 0.1% triton X-100 in PBS and incubated at RT for 10 min before addition of 1ml of PBS (1X). Cells were then centrifuged at 500 *g* for 5 min at 4^{o}C and resuspended in 300 µl of PBS (1X) before incubating with anti-HA11 antibody [1 in 300 ≡ 30 ng/ml] for 60 min at 4^{o}C. Cells were then washed with 1 ml of PBS (1X) and centrifuged at 500 *g* for 5 min at 4^{o}C, the supernatant was discarded and the cell pellet resuspended in 300 µl of PBS (1X) before incubating with FITC conjugated anti-mouse IgG antibody [1 in 100 ≡ 100 ng/ml] for 45 min at 4^{o}C. Cells were again washed with 1 ml of PBS (1X) and centrifuged at 500 *g* for 5 min at 4^{o}C, the supernatant was discarded and the cell pellet resuspended in 300 µl of PBS (1X). Supernatant was discarded and cell pellets were resuspended in 300 µl of

cold-PBS (1x) before analysis using a Beckinson Dickinson flow-cytometer. The remaining half were labelled with Sam-11 mAb in a non-permeabilising protocol as outlined in **2.2.3.5**. Values for empty vector control (taken as baseline fluorescence) were subtracted from fluorescence values for each sample and the results expressed as a percentage of the agonist untreated sample minus baseline.

4.2.2.11 Pharmacological Inhibition of Receptor Internalisation Using GF109203X and Phenylarsine Oxide (PAO)

Cells were grown and harvested as in 2.2.3.5 before incubating with GF109203X (100nM - 10 μM, for 30 min (Macfarlane et al, 2005), or PAO (1μM-316μM) for 15 min (Koenig & Edwardson, 1997; Boudin et al, 2000) Cells were then either treated with 20 nM trypsin, 100 μM SLIGKV-NH_2 or no treatment for 10 min. Cells were then labelled and analysed as in **2.2.3.5,** except inhibitors remained present in the buffers throughout. Cells not treated with inhibitor were used as a comparison to assess percentage inhibition of internalisation.

4.2.2.12 Pharmacological Inhibition of Receptor Internalisation with Concanavalin A (Con A) and Hyperosmolar Sucrose

Cells were grown as in **2.2.3.5** the growth media was removed and the cell surface washed with PBS. Cells were then incubated with 250 μg/ml of Con A in serum-free media for 15-20 min at 37^{o}C (Moffett et al, 1993), or allowed to equilibrate in 0.6 M sucrose in PBS (hyperosmolar sucrose) for 30 min (Koenig & Edwardson, 1997; Boudin et al, 2000). Cells were then harvested using a cell scraper and pelleted by centrifugation at 500 *g* for 5 min at 4^{o}C, the supernatant was discarded and the cell pellets resuspended in 300 μl of PBS, for cells treated with Con A, or 0.6 M sucrose for

cells equilibrated in hyperosmolar sucrose. Cells were then either treated with 20 nM trypsin for 10 min or remained untreated. Cells were then labelled and analysed as in **2.2.3.5** with the exception of maintaining sucrose concentration throughout in the hyperosmolar sucrose treated cells. Cells incubated with serum-free media alone for 15-20 min were used as a control for con A, or PBS alone for 30 min as a control for hyperosmolar sucrose, to assess percentage inhibition of internalisation.

4.2.2.13 Inhibition of internalisation by fixing cells

Cells were grown and harvested as in **2.2.3.5** and fixed by incubation in 3% formaldehyde for 10 min either before or after either treatment with 20 nM trypsin for 10 min or no treatment. A wash step of adding 1 ml of PBS (1X) and centrifuging at 500 *g* for 5 min before discarding supernatant and resuspending the cell pellet in 300 µl of either PBS or 3% formaldehyde, was used between steps. Cells were then labelled and analysed as in **2.2.3.5**.

4.3 Results

4.3.1 FACS Analysis: Expression vs. Confluence

As previously observed Pro5 cells display a confluence dependant level of receptor expression (Compton et al, 2002). As such in order to characterise the effect of cell confluence on expression in wt-hPAR$_2$ and hPAR$_2$C361A expressing cells, cells were harvested at different confluence levels and assayed for receptor expression by FACS analysis using the anti-hPAR$_2$ mAb Sam-11. Data obtained from FACS analysis (Figure 4.3.1) showed that receptor expression, for both wt-hPAR$_2$ and hPAR$_2$C361A, was inversely proportional to confluence. The hPAR$_2$C361A cell line maintains a receptor cell surface expression of ~twice that of the wt-hPAR$_2$ at all levels of confluence. Therefore a confluence level of 40% was employed for wt-hPAR$_2$ and a confluence level of 70% was used for hPAR$_2$C361A for subsequent experiments since receptor expression for both cell lines were then matched.

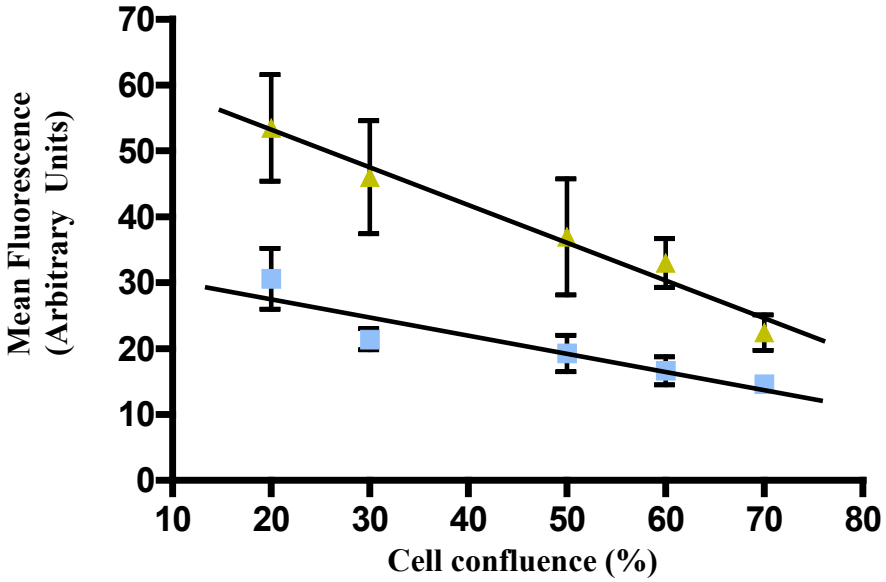


Fig 4.3.1 - Cell confluence versus receptor cell surface expression. Graphs showing the mean fluorescence from FACS analysis plotted versus percentage confluence of cell population used.

wt-hPAR$_2$ **hPAR$_2$C361A**

Results are expressed as the mean +/- SEM of 4 separate experiments.

4.3.2 Localisation of Receptor by Confocal Imaging

To identify the cellular localisation of receptor expression, confocal imaging was carried out (**figure 4.3.2**). When stained with Sam11 anti-PAR_2 antibody (**figure 4.3.2(i)**) empty vector transfected cells (**figure 4.3.2(i) A**) showed no staining. Wt-$hPAR_2$ (**figure 4.3.2(i) B**) and $hPAR_2$C361A (**figure 4.3.2(i) C**) cells showed staining at the cell membrane. However, low levels of fluorescence were seen using this method and a high photomultiplier gain was required to observe any signal. Cells were therefore permeabilised and stained with anti-HA11 antibody (**figure 4.3.2(ii)**). Wt-$hPAR_2$ (**figure 4.3.2(ii) B**) displayed distinct, uniform and robust immunostaining around the cell membrane. Some punctate cytoplasmic staining was also observed. No staining was observed in the empty vector transfected cells (**figure 4.3.2(ii) A**). The staining pattern observed for $hPAR_2$C361A (**figure 4.3.2(ii) C**) was strikingly different to that of wt-$hPAR_2$ (**figure 4.3.2(ii) B**). The ring staining for $hPAR_2$C361A was not uniform and distinct pockets of intense staining were observed at the cell surface. In addition considerable punctate staining was observed in the cytoplasmic region of the cells (**figure 4.3.2(ii) C**). However, the staining for $hPAR_2$C361A appeared brighter than that of wt-PAR_2.

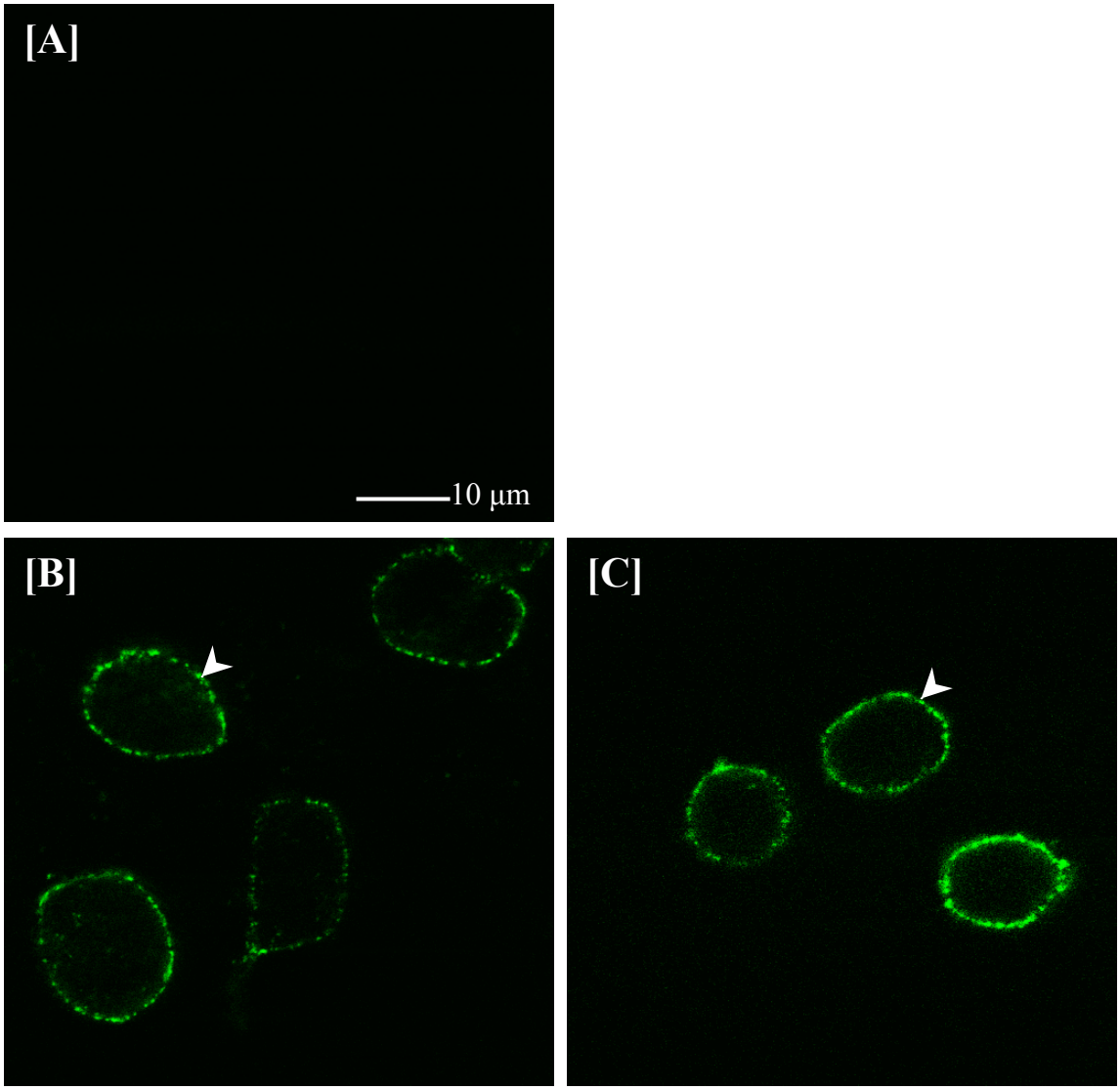


Fig 4.3.2(i) – Confocal Images of wt-hPAR$_2$ and hPAR$_2$C361A using Sam-11
Cells were grown on coverslips and incubated with Sam11 anti-PAR$_2$ primary antibody and anti-mouse FITC conjugated secondary antibody. Coverslips were then mounted and analysed by confocal microscopy.
[A] - pcDNA3.1 empty vector. **[B] -** wt-hPAR$_2$. **[C] -** hPAR$_2$C361A.
Presence of PAR$_2$ at the plasma membrane is indicated with arrowheads➤. Scale bar shown in white on image [A] indicates 10 µM. Images shown are representative of three experiments

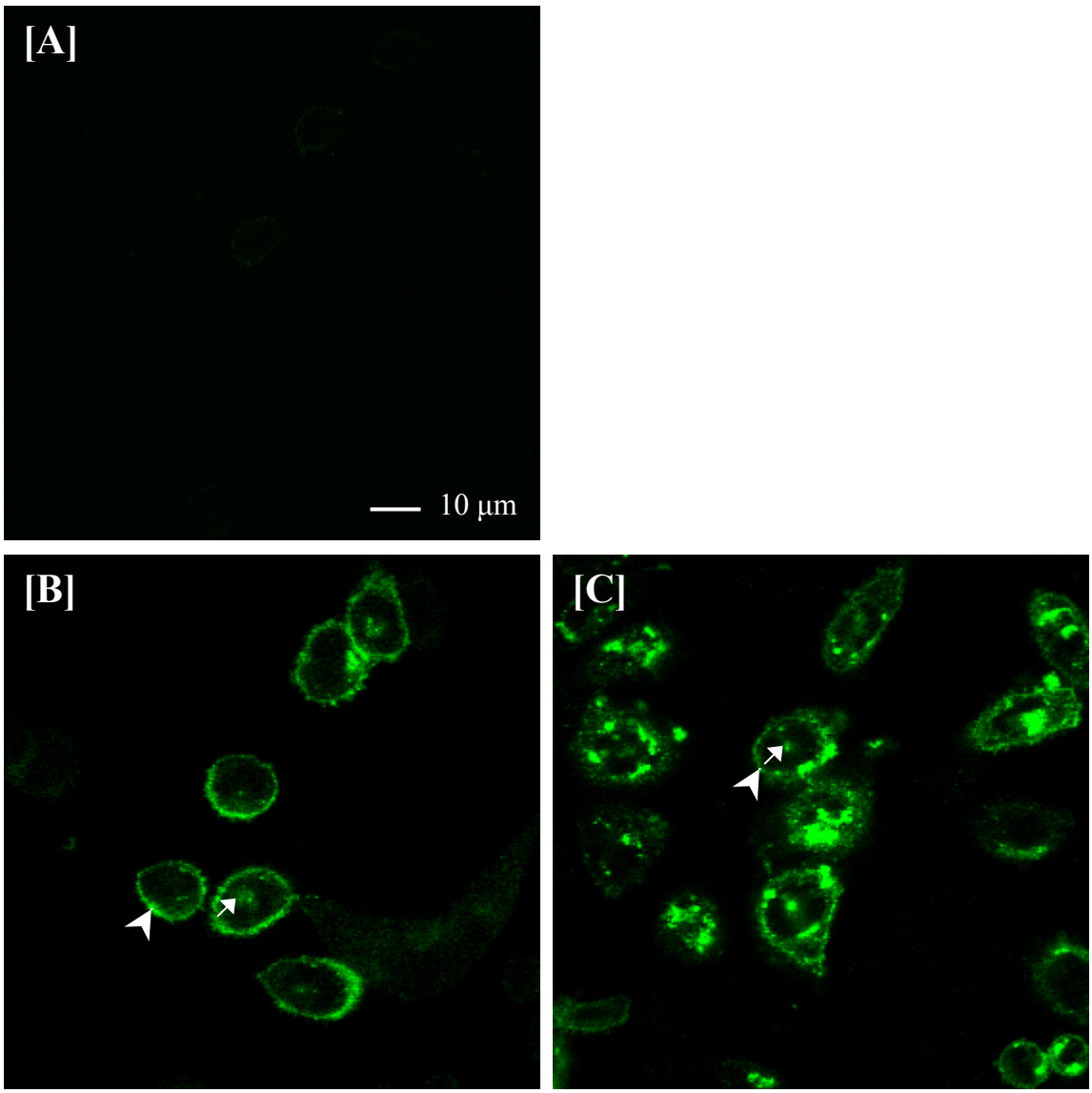


Fig 4.3.2(ii) – Confocal Images of wt-hPAR$_2$ and hPAR$_2$C361A using HA11
Cells were grown on coverslips and fixed and permeabilised prior to incubation with anti-HA11 primary antibody and anti-mouse FITC conjugated secondary antibody. Coverslips were then mounted and analysed by confocal microscopy. **[A] -** pcDNA3.1 empty vector. **[B] -** Wt-hPAR$_2$. **[C] -** hPAR$_2$C361A.
Presence of PAR$_2$ at the plasma membrane is indicated with arrowheads➤, PAR$_2$ contained internally is indicated with arrows↑. Scale bar shown in white on image [A] indicates 10 µM. Images shown are representative of three experiments.

4.3.3 Immunoprecipitation of $hPAR_2$

Figure 4.3.3a shows immunoprecipitated (samples prepared using Vectorlabs anti-HA11 agarose) empty vector (control), wt-$hPAR_2$, and $hPAR_2$C361A (left to right) visualised following immunoblotting using anti-HA11, shown in **Fig 4.3.3a**. Both $hPAR_2$ and $hPAR_2$C361A migrated as multiple bands ranging from 40 to 250 kDa. No visible bands were observed in the empty vector lane. A double band can be observed at ~75 kDa, and single bands at ~95, ~60 and ~45 kDa in wt-$hPAR_2$ and $hPAR_2$C361A but not in the empty vector control. No observable differences were detected between wt-PAR_2 and $hPAR_2$C361A, except that more receptor protein was consistently present in the wt-$hPAR_2$ lane compared to that of the $hPAR_2$C361A lane.

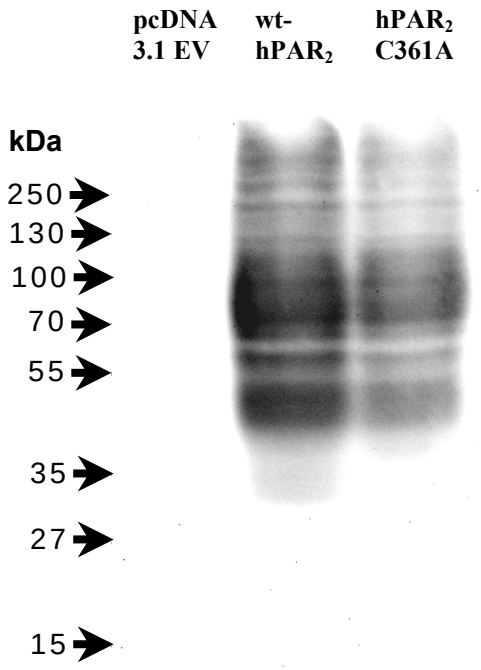


Fig 4.3.3a - Immunoprecipitation of $hPAR_2$
SDS-PAGE and western blot of inmunoprecipitated wt-$hPAR_2$, $hPAR_2$C361A and pcDNA3.1 empty vector cells visualised using anti-HA11 antibody. Blot shown is representative of 5 separate experiments.
Blot was produced using a 20 min exposure time.

4.3.4 [^{3}H]-Palmitate Incorporation

To ascertain whether $hPAR_2$ is palmitoylated on C361, empty vector, wt-$hPAR_2$ and $hPAR_2$C361A cell lines were labelled with [^{3}H]-palmitate prior to immunoprecipitation of HA11 epitope-tagged receptor. Several methods of Immunoprecipitation were attempted (**4.2.2.4** and **4.2.2.5**) with successful results finally being achieved using the μMACs HA11 kit (**4.2.2.6**). Although sufficient receptor concentration was gained with all previous methods as assessed by western blot analysis, the Vectorlabs and Profond anti-HA11 agarose methods and the μMACs protein G method were not sufficiently specific for use in the radioassay as multiple non-specific banding was detected in the empty vector control lanes (data not shown). The μMACs HA11 kit was found to be the most specific at purifying PAR_2 and was then used for the labelling experiments. Autoradiographical and western blot for labelled wt-PAR_2 and $hPAR_2$C361A are shown in **Figure 4.3.4**. The autoradiographic blot seen in **Figure 4.3.4 [A]** shows no detectable radiation signal in the empty vector control lane. In the wt-$hPAR_2$ lane it can be seen that sufficient levels of [^{3}H]-palmitate were incorporated as clear multiple banding patterns were observed. In total four bands can be seen at ~30, 35 ,50, and 75 kDa. In stark contrast $hPAR_2$C361A displayed no detectable [^{3}H]-palmitate incorporation and thus no observable banding patterns. **Figure 4.3.4 [B]** shows multiple banding in all lanes. pcDNA3.1 empty vector control shows three non-specific bands (~25, 95, and 130 kDa) which appear in all three samples. wt-$hPAR_2$ and $hPAR_2$C361A show additional bands at ~30, 40, 50, 55, 60, and 65 kDa. No observable differences were detected in the molecular weights of bands between wt-$hPAR_2$ and $hPAR_2$C361A. No detectable $hPAR_2$ protein was immunoprecipitated from pcDNA 3.1 empty vector control cells.

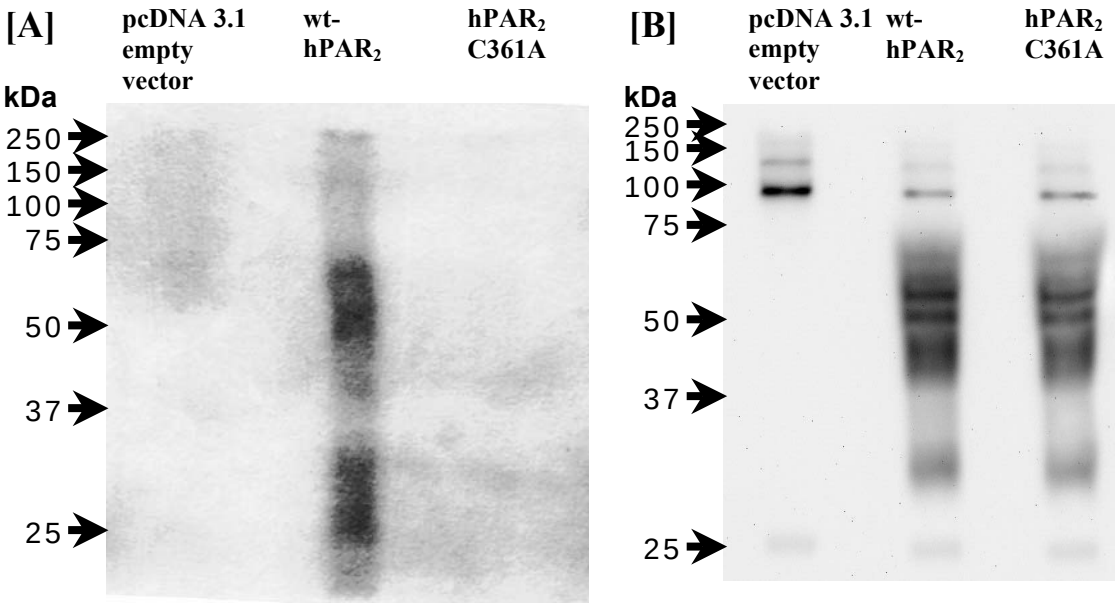


Fig 4.3.4 - [^{3}H]- palmitate incorporation into wt-hPAR$_2$ and hPAR$_2$C361A.
HA11-epitope immunoprecipitated protein preparations from empty vector, wt-hPAR$_2$ and hPAR$_2$C361A cells each labelled with 1 mCi [^{3}H]-palmitate for 4 hours run on a 10% SDS-PAGE. Gel dried under vacuum and exposed to Hyperfilm™ x-ray film at -80^{o}C for 6 weeks **[A]**. A portion of the same samples were analysed on western blot using anti-HA11 antibody to assess receptor loading **[B]** and a 20 min exposure time.
Blots shown are representative of three experiments

4.3.5 The Effect of Agonist Activation on [^{3}H]-Palmitate Incorporation

To ascertain whether incorporation of [^{3}H]-palmitate into $hPAR_2$ is affected by receptor activation we labelled wt-$hPAR_2$ cell lines with [^{3}H]-palmitate before treating with $hPAR_2$ agonists, trypsin and SLIGKV-NH_2 for 10 min. Receptor protein was then immunoprecipitated. Samples were split in two and subsequently run on SDS-PAGE then either exposed to autoradiography (**A**) or subjected to western blotting analysis (**B**).

Figure **4.3.5 [A]** shows an autoradiograph of wt-$hPAR_2$ following either no treatment (nt) or treatment with the $hPAR_2$ agonists SLIGKV-NH_2 and trypsin. All lanes contain multiple bands demonstrating that all samples incorporated sufficient levels of [^{3}H]-palmitate to be easily detectable. All samples produced bands appearing at approximately 20, 22, 25, 65, 140, and 220 kDa. In addition, two bands of approx 90 kDa and 50 kDa are clearly visible in the untreated sample (shown in red parentheses) which aren't present in samples treated with either agonist. The banding in lanes with samples treated with both $hPAR_2$ agonists are less intense than that of the untreated sample, indicating a reduction in [^{3}H]-palmitate incorporation.

Due to technical problems occurring during the protein transfer blot no clearly recognisable banding patterns can be identified in figure **4.3.5 [B]**. However, the blot still demonstrates receptor presence between 50 and 75 kDa for all samples. In addition SLIGKV-NH_2 and trypsin treated samples did not display a reduction in receptor compared to the untreated sample.

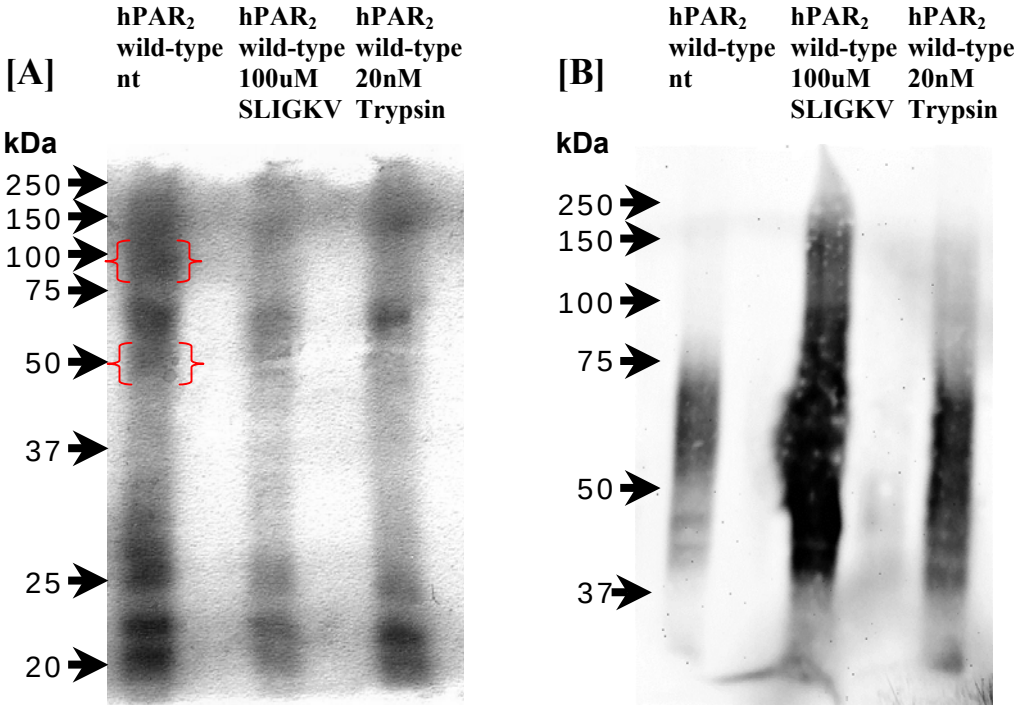


Fig 4.3.5 - [^{3}H] -palmitate incorporation into hPAR$_2$ wild-type following agonist stimulation
HA11-epitope immunoprecipitated protein preparations from wt-hPAR$_2$ cells labelled with 1 mCi [^{3}H]-palmitate for 4 hours and treated with either 100 μM SLIGKV-NH$_2$, 20 nM Trypsin, or no treatment, the run on a 10% SDS-PAGE. Gel dried under vacuum and exposed to Hyperfilm™ MP x-ray film at -80^{o}C for 6 weeks **[A]**. A portion of the same samples were analysed on western blot using anti-HA11 antibody to assess receptor loading **[B]** with a 20 min exposure time.

4.3.6 Calcium Signalling: Agonist Concentration Effect Curves

In an effort to characterise the effect of the C361A mutation on PAR_2 signalling, wt-concentration effect curves for wt-PAR_2 and hPAR_2C361A cell lines with PAR_2 agonists (trypsin and SLIGKV-NH_2) were produced (**Figure 4.3.6**). The concentration effect curves for wt-hPAR_2 and hPAR_2C361A cell lines treated with the PAR_2 agonists SLIGKV-NH_2 and trypsin are shown in **Fig 4.3.6(i)[A]**. The curves for wt-hPAR_2 with both trypsin and SLIGKV-NH_2 plateau at ~65% maximum obtainable signal with concentration ranges of 316 pM to 316 nM, and 316 nM to 316 μM for trypsin and SLIGKV-NH_2 respectively. The concentration effect curve for hPAR_2C361A with trypsin plateaued at ~35% maximum obtainable signal within a concentration range of 1 nM to 316 nM showing a significant difference to wt-hPAR_2 (P=0.0003). For hPAR_2C361A with SLIGKV-NH_2 the curve plateaued at ~20% maximum obtainable signal with a concentration range of 31.6 μM to 1 mM (For EC_{50} and precise maximum curve values see **Table 4.3.6(ii)**) displaying a significant difference to wt-hPAR_2 (P=0.0058).

In order to remove endogenous PAR_1 activity the concentration effect experiments were repeated with prior treatment of cells with thrombin (**3.2.2.4**) (A concentration that was shown to completely desensitise PAR1 in preliminary experiments, data not shown). The concentration effect curves for wt-hPAR_2 and hPAR_2C361A cell lines with the PAR_2 agonists SLIGKV-NH_2 and trypsin following abolition of endogenous PAR_1 signal with 3.16 units/ml of thrombin are shown in **Fig 4.3.6(i)[B]**. The curves for wt-hPAR_2 with both trypsin and SLIGKV-NH2 plateau at ~55% maximum obtainable signal with concentration ranges of 316 pM to 316 nM, and 316 nM to 316 μM for trypsin and SLIGKV-NH_2 respectively. The concentration effect curve for

$hPAR_2C361A$ with trypsin plateaued at ~25% maximum obtainable signal within a concentration range of 1 nM to 316 nM. For $hPAR_2C361A$ with SLIGKV-NH_2 the curve plateaued at ~25% maximum obtainable signal with a concentration range of 31.6 μM to 1 mM (For EC_{50} and precise maximum curve values see **Table 4.3.6(ii)**.) The thrombin desensitised concentration effect curve for $hPAR_2C361A$ with trypsin yielded a maximum signal ~2-fold less than that seen with wt-$hPAR_2$ (27.01% ± 1.781 compared to 55.95% ± 2.513) and EC_{50} values ~6-fold greater than that seen with wt-$hPAR_2$ (22.76 nM ± 1.39 compared to 4.00 nM ± 1.53). The thrombin desensitised concentration effect curve for $hPAR_2C361A$ with SLIGKV-NH_2 gave a maximum signal ~1.9-fold less than that seen with wt-$hPAR_2$ (31.66% ± 5.332 compared to 58.70% ± 3.519) and EC_{50} values ~3-fold greater than that seen with wt-$hPAR_2$ (109.88 μM ± 1.77 compared to 32.69 μM ± 1.34).

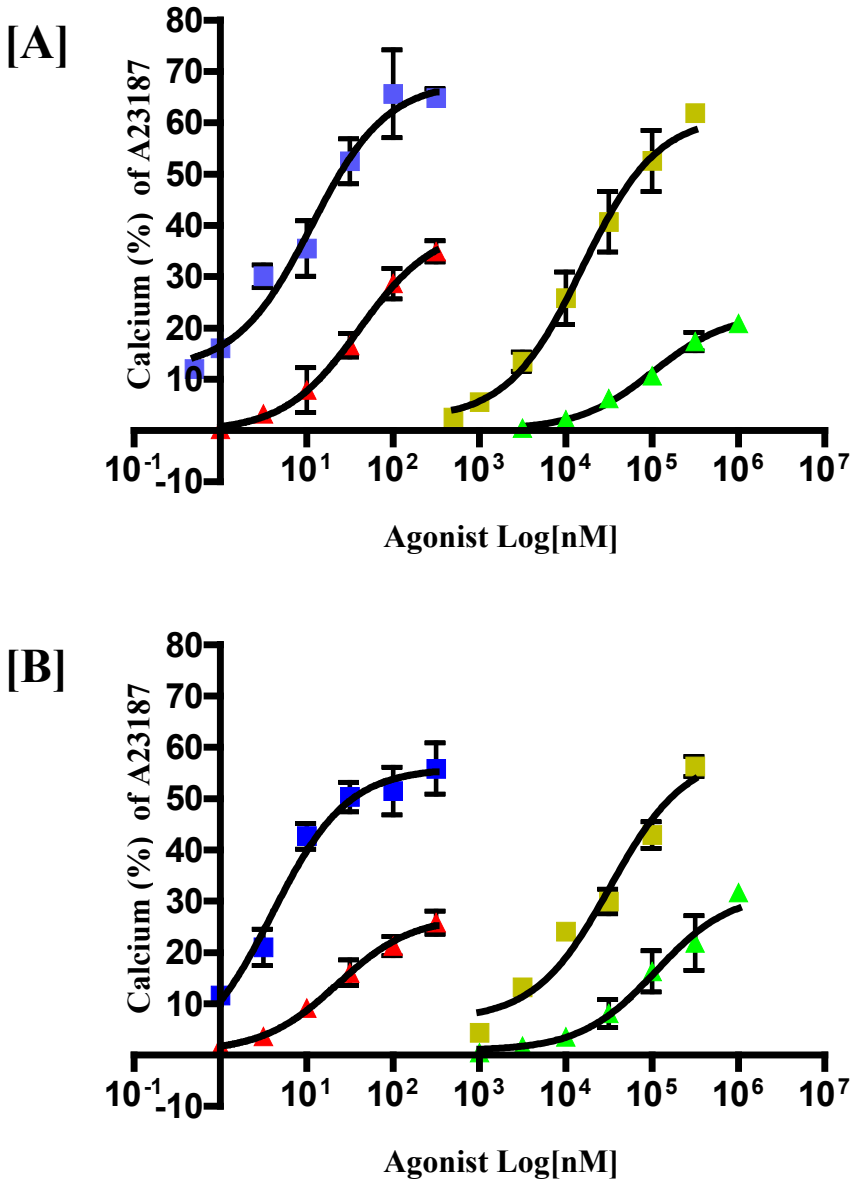


Fig 4.3.6(i) - hPAR$_2$ agonist stimulated Ca^{2+} mobilisation
Concentration effect curves for trypsin and SLIGKV-NH$_2$ stimulated Ca^{2+} mobilisation for wt-hPAR$_2$ and hPAR$_2$C361A **[A]** and following PAR$_1$ desensitisation with 3.16 units/ml of thrombin **[B]**.

wt-hPAR$_2$: **with trypsin** **with SLIGKV** **hPAR$_2$C361A :** **with trypsin** **with SLIGKV**

Results are expressed as the mean +/- SEM of 4 separate experiments. Each preformed in duplicate.

CURVE		EC_{50} ± SEM	Maximum ± SEM	EC_{50} fold wt	Maximum fold wt
wt-$hPAR_2$:	Trypsin	11.44 nM **± 1.40**	67.99 % **± 3.494**		
	SLIGKV	15.69 µM **± 1.43**	61.43 % **± 4.906**		
$hPAR_2$C361A:	Trypsin	40.76 nM **±1.41**	39.65 % **± 3.419**	~3.6 ↑	~1.7 ↓
	SLIGKV	103.45 µM **± 1.34**	22.82 % **± 1.890**	~6.6 ↑	~2.7 ↓
post cell desensitisation with 3.16 units/ml Thrombin					
wt-$hPAR_2$:	Trypsin	4.00 nM **± 1.53**	55.95 % **± 2.513**		
	SLIGKV	32.69 µM **± 1.34**	58.70 % **± 3.519**		
$hPAR_2$C361A:	Trypsin	22.76 nM **± 1.39**	27.01 % **± 1.781**	~5.7 ↑	~2.1 ↓
	SLIGKV	109.88 µM **± 1.77**	31.66 % **± 5.332**	~3.4 ↑	~1.9 ↓

Table 4.3.6(ii) - $hPAR_2$ agonist stimulated Ca^{2+} mobilisation Curve Values
Table giving EC_{50} values for wt-$hPAR_2$ and $hPAR_2$ C361A agonist concentration effect curves shown in **Fig 4.3.6[A]/[B]**. ↑ indicates a fold increase over wt-$hPAR_2$, and ↓ indicates a fold decrease over wt-$hPAR_2$. EC_{50} values given to 2 decimal places with SEM displayed in **bold**. Slope maximum response values expressed as percentage of A23187 response.

4.3.7 Calcium Signalling: Activation kinetics with trypsin

In an effort to characterise the effect on the C361A mutation on $hPAR_2$ signalling kinetics Ca^{2+} signalling data was recorded following addition of 3.16 nM trypsin to wt-PAR_2 and 316 nM trypsin to $hPAR_2$C361A cell lines to match maximum signal (**fig 4.3.7 (i)**).

The graphs shown are plotted with the time in secs on the *x* axis and signal, expressed as a percentage of the A23187 response, on the *y* axis. **Fig 4.3.7(i)** shows the data recorded for the resulting calcium signalling. The signal curve shown here for wt-$hPAR_2$ reaches a signal maximum of ~37% within 10 secs plateauing for only a few seconds before beginning to return to baseline. In contrast the signal curve for $hPAR_2$C361A reaches a signal maximum of ~36% in 23 secs and plateaus for a comparably longer before beginning to return to baseline (see **fig 4.3.7(i)**). In an effort to compare the rate activation, the pre-plateau data was plotted separately (see **fig 4.3.7(ii)**) and a linear regression fitted the signal plateau in wt-$hPAR_2$ is reached in less than ½ of the time of $hPAR_2$C361A (9 sec compared to 23 sec). The data from 3 repeats was plotted for the initial rate of activation for wt-$hPAR_2$, shown on **Fig 4.3.7(ii)**, produces a linear regression fit (shown in blue) with a steep slope reaching a maximum of ~38% in 7 secs. The linear regression fitted to $hPAR_2$C361A initial rate of activation data (shown in red) results in a shallow slope running to a maximum of ~30% in 18 secs.

The slope of the line fit ($\delta y/\delta x$) gives the initial rate of activation/deactivation for the receptors in *percent* sec^{-1}. These data show that the activation rate for wt-$hPAR_2$ calcium signalling is approximately 4 fold faster than for $hPAR_2$C361A (6.303 *percent* sec^{-1} compared to 1.602 *percent* sec^{-1} a significant difference ($P<0.0001$).

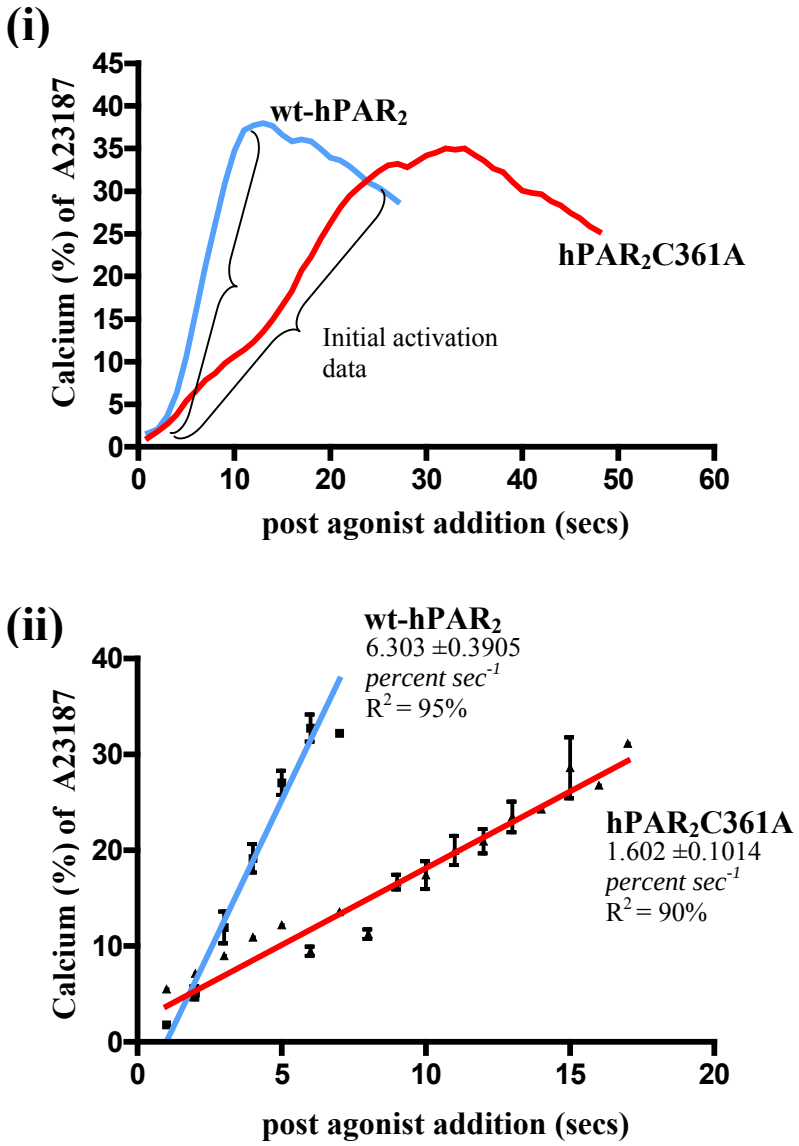


Figure 4.3.7 - Activation Kinetics with Trypsin
(i) Graph showing the Ca^{2+} signalling produced on addition of trypsin to wt-$hPAR_2$ and $hPAR_2$C361A. The graph is representative of 3 repeats **(ii)** Graph showing a linear regression fit against the data produced for initial activation, the slope of which gives the initial activation rate in *percent* sec^{-1}, results plotted as mean +/- SEM for 3 repeats.
wt-$hPAR_2$ $hPAR_2$C361A
Results are expressed as percentage of A23187.

4.3.8 MAPK Signalling: Time Course

In an effort to characterise the effect on the C361A mutation on $hPAR_2$ ERK1/2 (p44/42) MAPK signalling, post agonist activation curves for wt-PAR_2 and $hPAR_2$C361A cell lines with PAR_2 agonists (trypsin and SLIGKV) were produced. Cell protein preparations were analysed on immunoblot for phosphorylated p44/42 (**fig 4.3.8a (i)**) and then reprobed for total p44/42 (**fig 4.3.8a (ii)**). Phosphorylated p44/42 bands densities are expressed as a percentage of the total p44/42 and plotted versus minutes post agonist activation (**fig 4.3.8b (i)/(ii)**).

The phosphorylated p44/42 blot produced for wt-$hPAR_2$ (shown in **fig 4.3.8a(i)**), shows an increase in band intensity, over "no treatment", 5 min after agonist addition for both SLIGKV-NH_2 and trypsin. The band intensity then rapidly returns to being equivalent to "no treatment" (at ~ 10-20 min) for SLIGKV-NH_2 treated cells, and more slowly (~30 min) for trypsin treated cells. The phosphorylated p44/42 blot produced for $hPAR_2$C361A (shown in **fig 4.3.8a(i)**), shows an increase in band intensity, over "no treatment", peaking at 10 min after agonist addition for both SLIGKV-NH_2 and trypsin. The band intensity then slowly reduces but remains increased compared to that of "no treatment" at 60 mins post treatment. The band intensities for trypsin at all time points post-agonist addition with both wt-$hPAR_2$ and $hPAR_2$C361A are greater than that of SLIGKV-NH_2. **Fig 4.3.8a(ii)** shows total ERK for each sample which is approximately uniform for all treatments. **Fig 4.3.8b(i)/(ii)** show the densitometry values taken from the western blot and plotted versus time post-agonist addition, examples of bands produced at each time point are displayed above their respective graph point. The MAP Kinase activation curve post-SLIGKV-NH_2 addition for both wt-$hPAR_2$ and $hPAR_2$C361A, shown in **fig 4.3.8b(i)**, show greater band densities for $hPAR_2$C361A

samples at all time points, and are significantly different when analysed by two-way ANOVA table (P=0.0010). The MAP Kinase activation curve post-trypsin addition for both wt-hPAR$_2$ and hPAR$_2$C361A addition, shown in **fig 4.3.8b(ii)**, show greater band densities for hPAR$_2$C361A samples at all time points and are significantly different when analysed by two-way ANOVA table (P=0.0024).

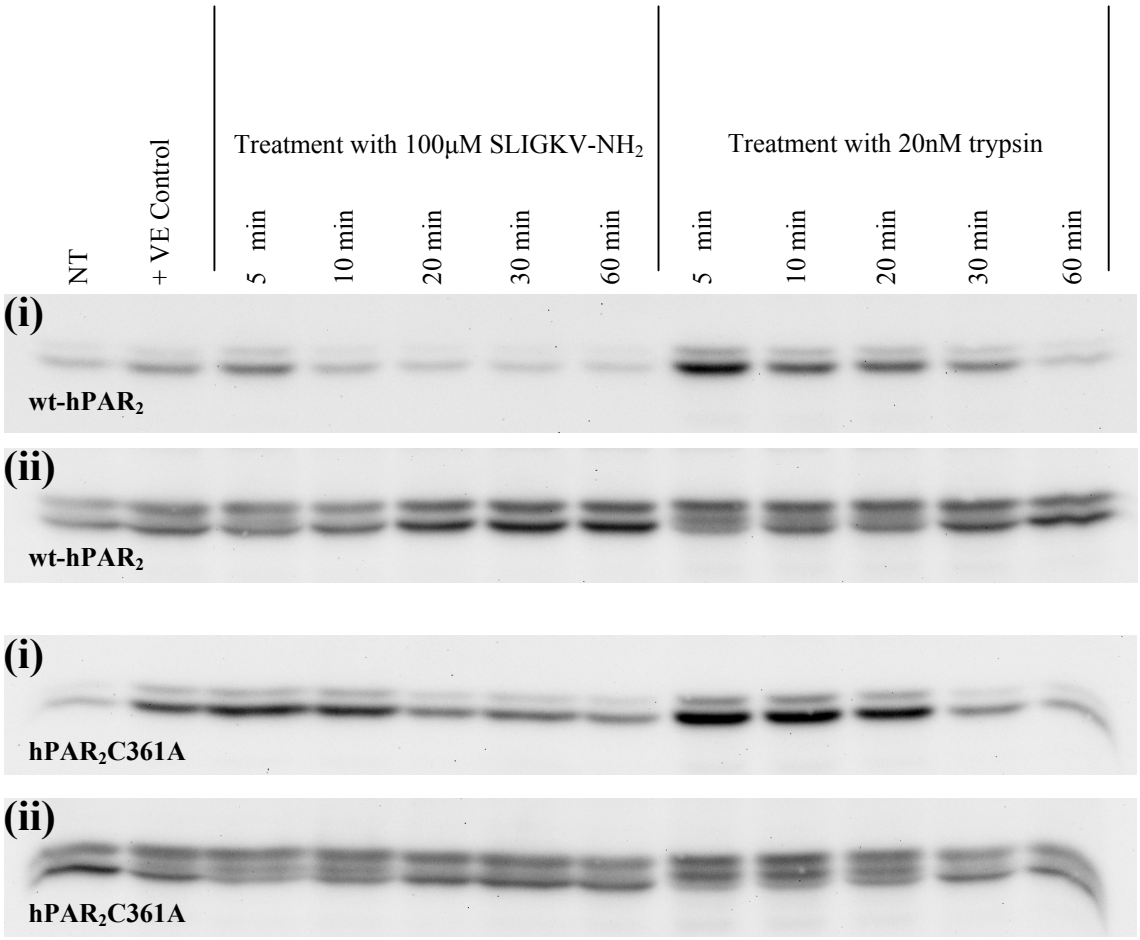


Figure 4.3.8a – Agonist Stimulated MAPK Phosphorylation Blots
Western blots produced for wt-hPAR$_2$ and hPAR$_2$C361A cells treated with agonist and protein harvested at set time points.
NT- no treatment, +VE control – 3.16 units/ml thrombin for 10 min.
(i) – Have been probed with anti-phosphorylated p44/42 antibody.
(ii) – Have been re-probed with anti-total p44/42 antibody.
A 10 min exposure time was used for all blots.
The blots shown are a representation of 3 repeats.

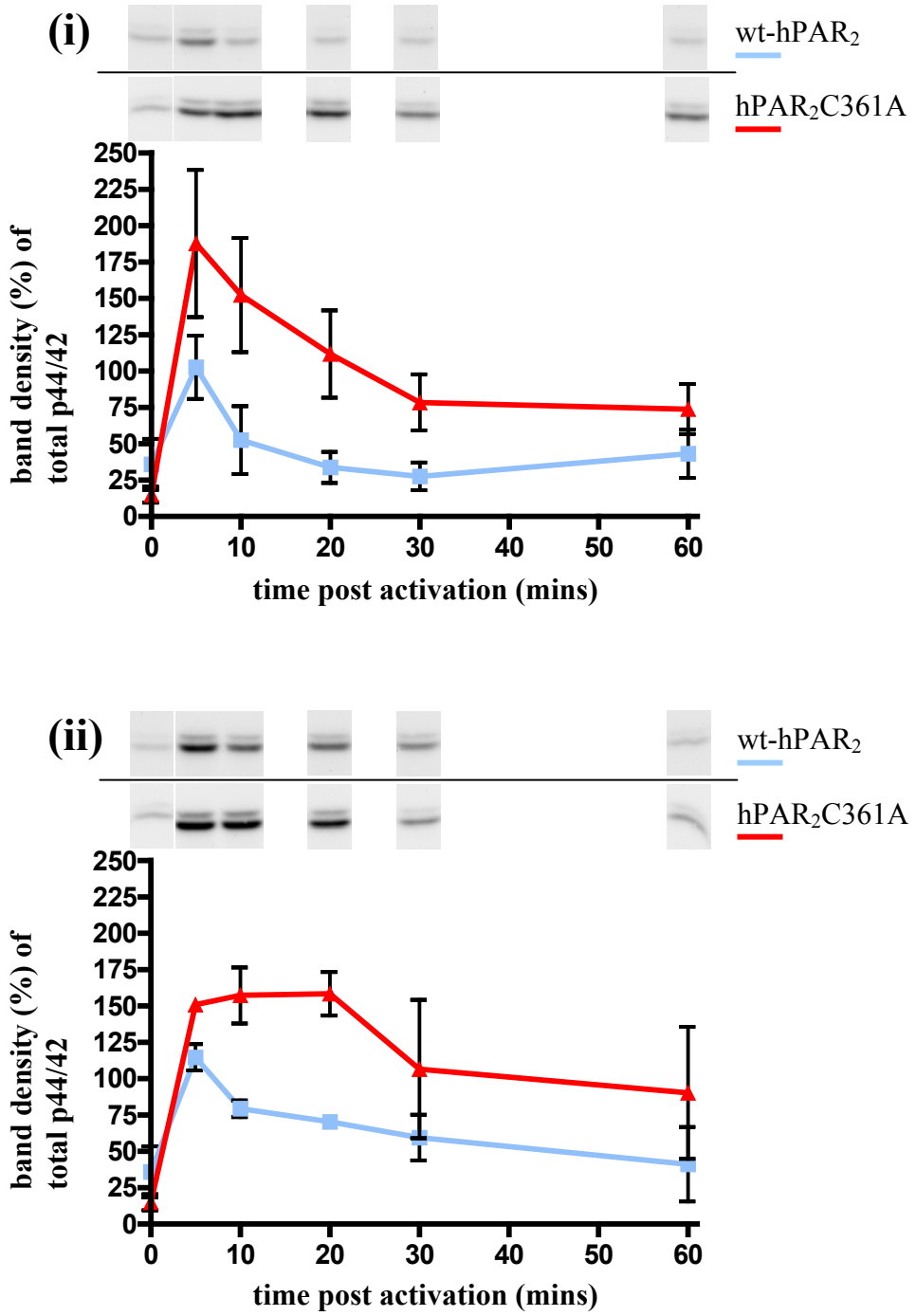


Figure 4.3.8b – Agonist Stimulated MAPK Phosphorylation Curves
Graphs showing densitometric values for bands produced by western blot (see **fig 4.3.8a**) for phosphorylated p44/42 post-SLIGKV-NH_2 treatment **(i)** and post trypsin treatment **(ii)**. Values expressed as a percentage of total p44/42 measured on reprobed blot (see **fig 4.3.8a**).

Results are expressed as the mean +/- SEM of 3 separate experiments, while the bands above are representative of the blots.

4.3.9 Inhibition of Trypsin Mediated Calcium Signal by Pertussis Toxin

To ascertain what proportion of the total IP_3 induced Ca^{2+} flux is due to signalling through G_i, wt-$hPAR_2$ and $hPAR_2$C361A cells were preincubated with pertussis toxin (PTX) before assessing calcium signalling in response to trypsin. PTX dose dependently, inhibited wt-$hPAR_2$ and $hPAR_2$C361A mediated calcium signalling. wt-$hPAR_2$ generates maximum calcium signal ~70% of A23187 decreasing to ~50% of A23187 at the maximum PTX concentration of 316 ng/ml (**fig 4.3.9[A]**). Where as, $hPAR_2$C361A produces a maximum calcium signal of ~30% of A23187 reducing down to <10% of A23187 at the maximum PTX concentration of 316 ng/ml. As such both curves reduce by ~20% of their A23187 signal (**fig 4.3.9[A]).** In **fig 4.3.9[B]** at the maximum PTX concentration wt-$hPAR_2$ generated Ca^{2+} mobilisation is reduced to ~75% of its untreated signal, where as Ca^{2+} mobilisation for $hPAR_2$C361A is reduced to ~35% of it's untreated signal. Curve fit values for both figures are included in **table 4.3.9** showing the IC_{50} values for PTX with wt-$hPAR_2$ to be higher than that for $hPAR_2$C361A (13.17 ±2.50 ng/ml compared to 2.313 ±3.78 ng/ml). There is a significant difference between the concentration effect curves (P=0.0002) when analysed by teo-way ANOVA table.

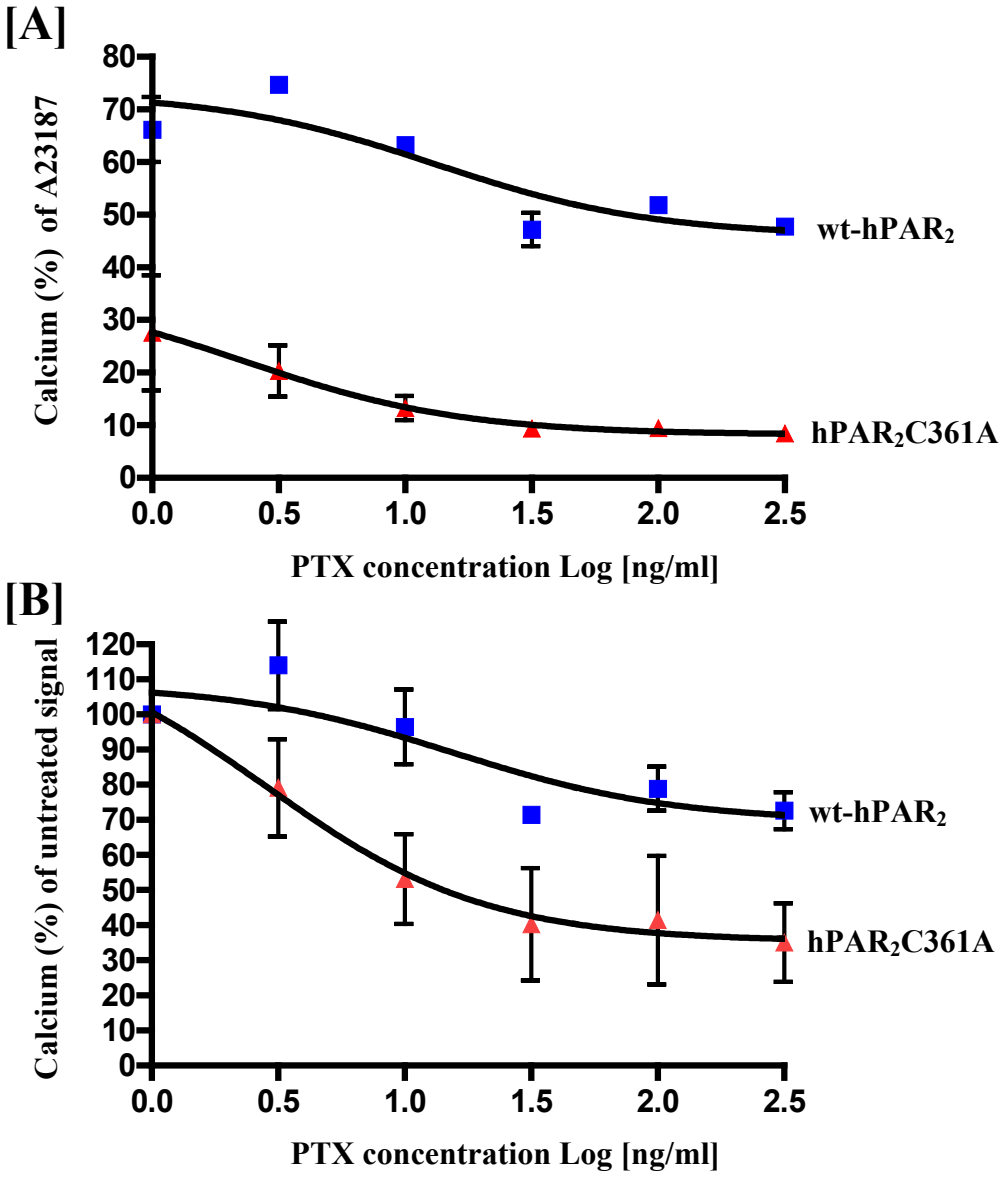


Figure 4.3.9 - Graph showing inhibition of trypsin mediated Ca^{2+} release in wt- $hPAR_2$ and $hPAR_2C361A$
wt- $hPAR_2$ and $hPAR_2C361A$ cells incubated with different concentrations of PTX prior to addition of 100 nM trypsin. Values shown as a percentage of A23187 [A] and as a percentage of untreated signal [B].
■ wt-$hPAR_2$ ▲ $hPAR_2C361A$
Results expressed as the mean +/- SEM of 3 separate experiments (2 only for $hPAR_2C361A$). Each preformed in duplicate.

A	**wt-hPAR$_2$**	**hPAR$_2$C361A**
Bottom	**45.94%**	**8.182%**
Top	**73.26%**	**36.11%**
IC$_{50}$	**13.17 ng/ml** ± 2.50	**2.313 ng/ml** ± 3.78

B	**wt-hPAR$_2$**	**hPAR$_2$C361A**
Bottom	**69.52%**	**35.32%**
Top	**108.6%**	**123.7%**
IC$_{50}$	**15.67 ng/ml** ± 3.09	**2.838 ng/ml** ± 2.77

Table 4.3.9 - Table showing curve fit values for PTX inhibition of trypsin induced calcium signalling graphs.
A – Shows curve fit for values for **fig 4.3.9** [**A**]. *y* axis percentage of A23187.
B – Shows curve fit for values for **fig 4.3.9** [**B**]. *y* axis percentage of PTX untreated signal.

4.3.10 Inhibition of MAP Kinase Signalling by Pertussis Toxin

To ascertain what proportion of the total MAP kinase signalling is due to signalling through G_i, wt-hPAR$_2$, (**[B]**) hPAR$_2$C361A (**[C]**) and empty vector control (**[A]**)cells were preincubated with pertussis toxin (PTX) before treating PAR$_2$ agonists (trypsin and SLIGKV: **4.2.2.9**). Cell protein preparations were analysed on immunoblot for phosphorylated p44/42 and then reprobed for total p44/42. Phosphorylated p44/42 bands densities are expressed as a percentage of the total p44/42 and plotted versus agonist (**fig 4.3.10**). Blots were produced within the Biomedical Research Lab by Dr L.R. Sadofsky of the Department of Cardiovascular-Respiratory Medicine from previously produced protein preparations.

ERK1/2 response for empty vector control cell (**fig 4.3.10 [A]**) for untreated and SLIGKV-NH$_2$ remains less than 10% of the total ERK1/2, rising to ~20% of total ERK1/2 following trypsin treatment. When pre-treated with PTX ERK signals remain the same with no significant difference between datasets (P = 0.8751, 0.9616, 0.9584 for untreated, SLIGKV-NH$_2$ treated and trypsin treated, respectively) , untreated and SLIGKV-NH$_2$ remain below 10% of total ERK1/2 and trypsin treatment results in an increase to ~20% of total ERK1/2. For wt-hPAR$_2$ (**fig 4.3.10 [B]**) untreated ERK1/2 signalling has a mean response of 100% of total ERK1/2 signal increasing to a mean value of ~200% of total ERK1/2 signal when treated with SLIGKV-NH$_2$, and ~130% of total ERK1/2 signal when treated with trypsin. Following pre-treatment with PTX there appears to be a slight decrease in ERK1/2 signalling with all treatments, though no significant difference is noted (P= 0.7638, 0.6611, 0.9911, for untreated, SLIGKV-NH$_2$ treated and trypsin treated respectively) . Signalling through ERK1/2 for hPAR$_2$C361A (**fig 4.3.10 [C]**) increases from ~50% of total ERK1/2 signal when untreated, to a mean of ~100% of total ERK1/2 signal on treatment with SLIGKV-NH$_2$, and to a mean of

~200% of total ERK1/2 signal on treatment with trypsin. When pre-treated with PTX ERK1/2 signalling in $hPAR_2C361A$ deceases a small amount following no treatment (~50% → ~25%) and trypsin (~200% → ~150%) in comparison to samples not pre-treated with PTX. However, a large apparent drop in ERK1/2 signalling may be seen in the PTX pre-incubated SLIGKV-NH_2 treated samples (~100% → ~35%) compared those not pre-incubated with PTX, though the changes are not statistically significant (P = 0.2952, 0.2415, 0.6757 for untreated, SLIGKV-NH_2 treated and trypsin treated respectively) .

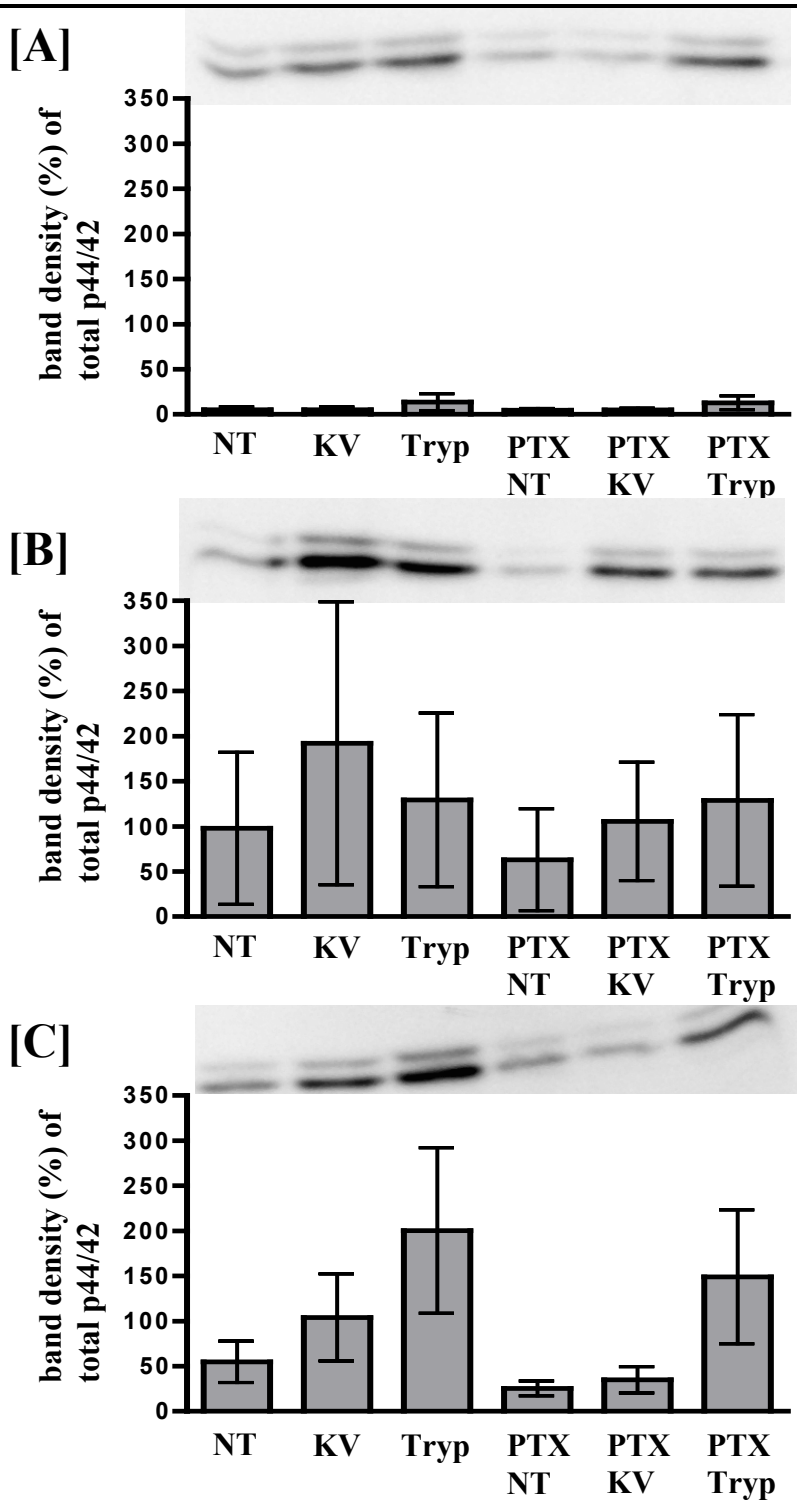


Figure 4.3.10 – Inhibition of MAPK with PTX
wt- $hPAR_2$ **[B]**, $hPAR_2C361A$ **[C]**, and empty vector **[A]** control cells incubated with and without 100 ng/ml PTX prior to addition of 20 nM trypsin (**Tryp**) or 100 μM SLIGKV-NH_2 (**KV**) or no treatment (**NT**) for 10 mins. Values expressed as a percentage of total p44/42 measured on reprobed blot. A representative anti-phospho p44/42 blot is shown above the respective histogram. Blots were given a 20 min exposure time.
Results expressed as the mean +/- SEM of 3 separate experiments..
for $hPAR_2C361A$ and 2 separate experiments for wt-$hPAR_2$ and empty

4.3.11 FACS Analysis: Receptor Internalisation

FACS analysis was performed on wt-hPAR$_2$ and hPAR$_2$C361A cell lines to determine the post-agonist activation receptor internalisation rate, data was analysed for significance using a two-way ANOVA for comparison between datasets(cell lines or treatments), and a one-way ANOVA table for between time points. A P value of <0.05 is considered significant. The data generated from this experiment showed that a significant difference exists between internalisation post activation with SLIGKV-NH$_2$ (**Fig 4.3.11 (i) [A] and [B]**) and post-activation with trypsin (**Fig 4.3.11 (i) [C] and [D]**) for both wt-hPAR$_2$ ($P<0.0001$) and hPAR$_2$C361A ($P<0.0001$). No significant receptor internalisation (reduction in cell surface expression) was observed up to 60 mins post-activation with 100 μM SLIGKV-NH$_2$ for either wt-hPAR$_2$ ($P=0.1166$) or hPAR$_2$C361A ($P=0.3903$). However, both cell-lines displayed a rapid receptor internalisation post-activation with 100 nM trypsin (**Fig 4.3.11 (ii)**) though internalisation of hPAR$_2$C361A post trypsin treated is significantly different to that of wt-hPAR$_2$ ($P=0.0155$). Within 10 mins of activation with trypsin wt-hPAR$_2$ receptor cell surface expression dropped to 60% of initial expression and reaching a plateau at 47.83% of initial expression at $t=\infty$. For hPAR$_2$C361A, receptor cell surface expression dropped to 20% of initial levels within 10 mins of trypsin activation, reaching a plateau at 12.51% of initial expression at $t=\infty$. As well as internalising to a greater extent than wt-PAR$_2$, hPAR$_2$C361A has a larger exponential decay rate constant than wt-hPAR$_2$ (0.21 compared to 0.13).

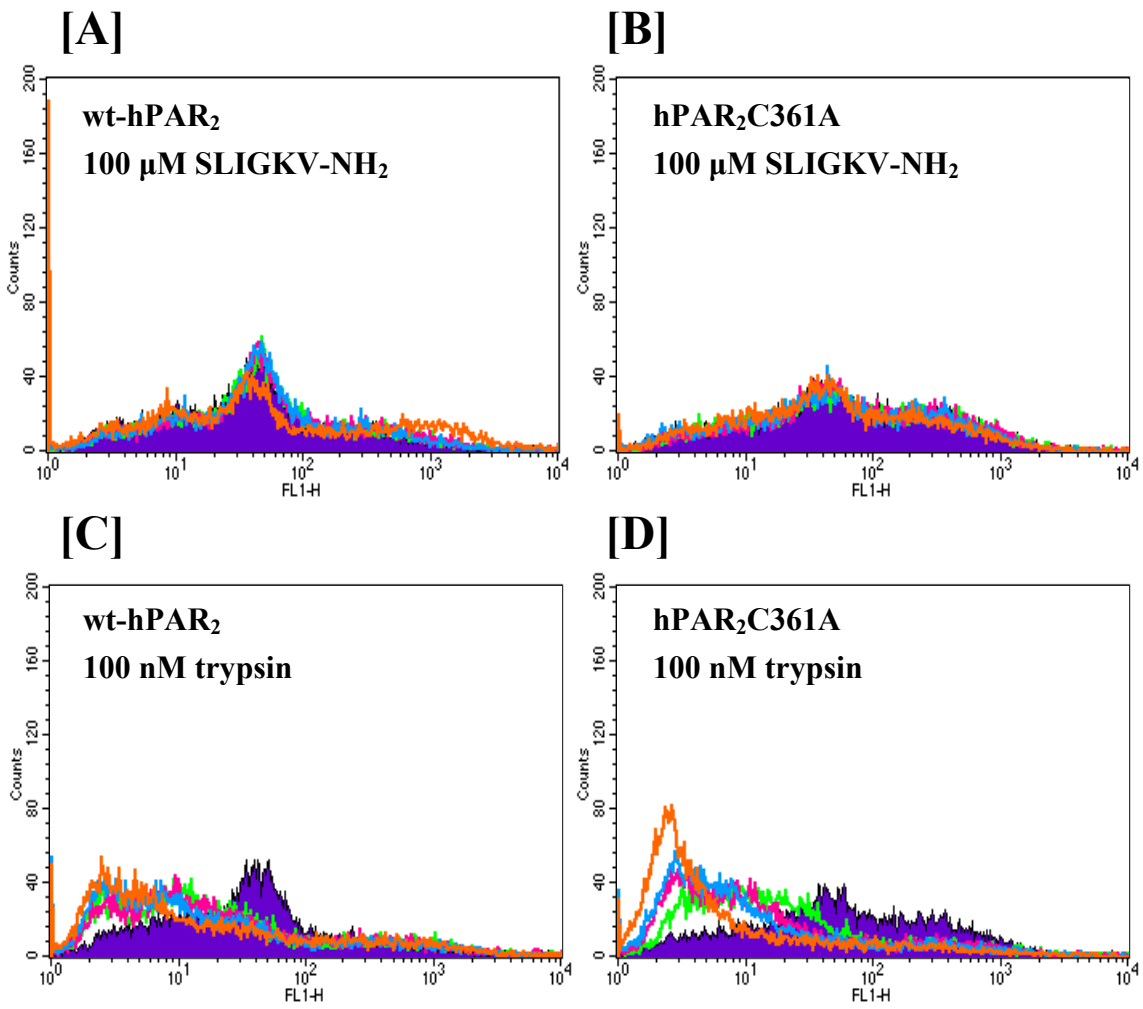
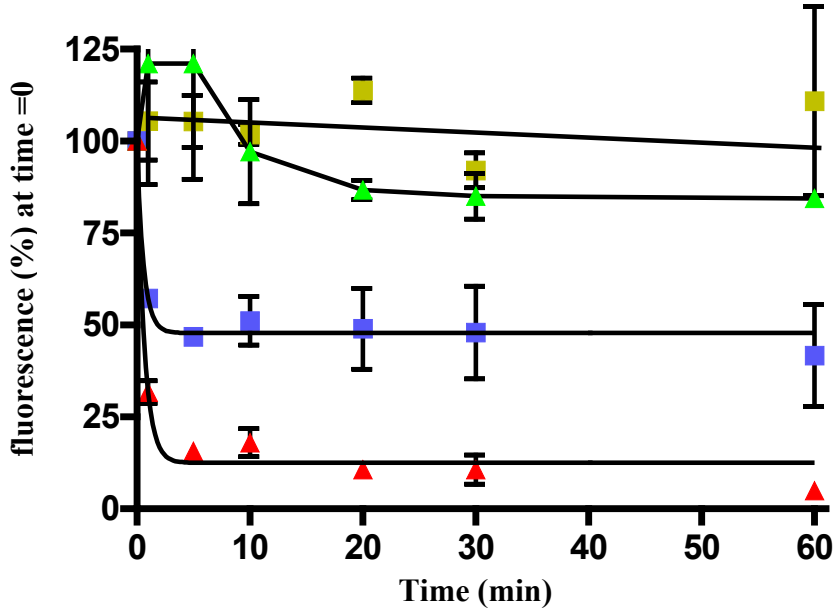


Figure 4.3.11(i) - Receptor Internalisation: FACS Traces
FACS trace showing fluorescence post addition of 100 µM SLIGKV-NH_2 to wt-hPAR_2 **[A]** and hPAR_2C361A **[B]** cells, and post addition of 100 nM trypsin to wt-hPAR_2 **[C]** and hPAR_2C361A **[D]** cells.

- 0 min post addition of agonist
- 10 min post addition of agonist
- 20 min post addition of agonist
- 30 min post addition of agonist
- 60 min post addition of agonist

FACS traces shown are representative of three experiments.



	hPAR$_2$ wild-type	hPAR$_2$C361A
Plateau at time = ∞ ±SEM	47.83 % **±3.402**	12.51 % **±1.517**

Figure 4.3.11(ii) - Receptor Internalisation: Curve Fit
Graphs showing the hPAR$_2$ wild-type and hPAR$_2$ C361A receptor cell surface expression post treatment with 100 nM Trypsin, or 100 µM SLIGKV-NH$_2$, expressed as a percentage of expression prior to treatment, versus time.

wt-hPAR$_2$: **with trypsin** **with SLIGKV** **hPAR$_2$C361A :** **with trypsin** **with SLIGKV**

Results are expressed as the mean +/- SEM of 3 separate experiments.

4.3.12 Internalisation: Internal staining of receptor

Since the loss of staining for $hPAR_2$ at the cell surface (seen in **4.3.11**) can occur for a number of reasons total cellular expression of receptor was assessed alongside cell surface receptor expression. Cells were treated with either 20 nM trypsin, 100 μM SLIGKV-NH_2 or no treatment for 10 mins before staining with Sam-11 anti-$hPAR_2$ antibody for un-permeabilised cells or anti-HA11 for permeabilised cells. Datasets were comared using a two-tailed t-test as a measure of significant change, where $P<0.05$ is significant. In **figure 4.3.12** it can be seen that treatment with trypsin resulted in a significant reduction in Sam-11 anti-$hPAR_2$ antibody staining in non-permeabilsed cells for both wt-$hPAR_2$ (shown in blue, P=0.0005) and $hPAR_2$C361A (show in red, P=0.0020) to ~60% and ~50% respectively of that of untreated cells. Whilst no significant change was noted in Sam-11 staining of non-permeabilsed cells (compared to untreated) for both wt-$hPAR_2$ (yellow bar, P=0.1042) and $hPAR_2$C361A (green bar, P=0.6696) following SLIGKV-NH_2 treatment. Additionally, staining with anti-HA11 antibody following permeabilisation shows no reduction in whole cell staining of receptor for both wt-$hPAR_2$ and $hPAR_2$C361A with either trypsin or SLIGKV-NH_2 compared to untreated control.

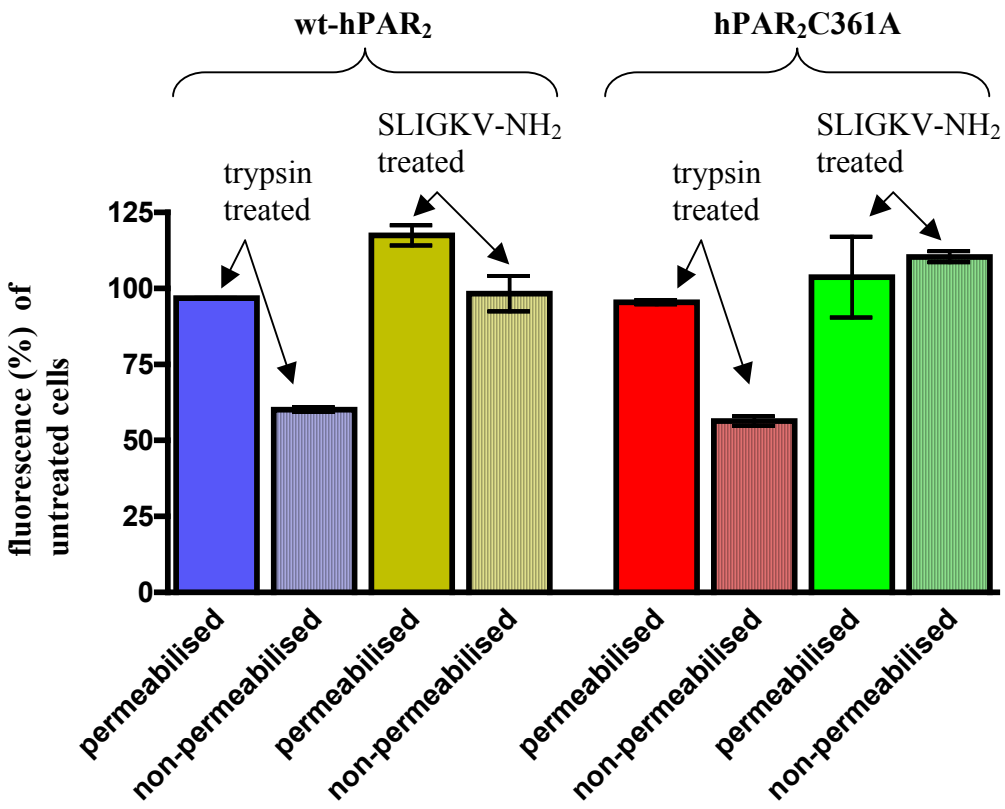
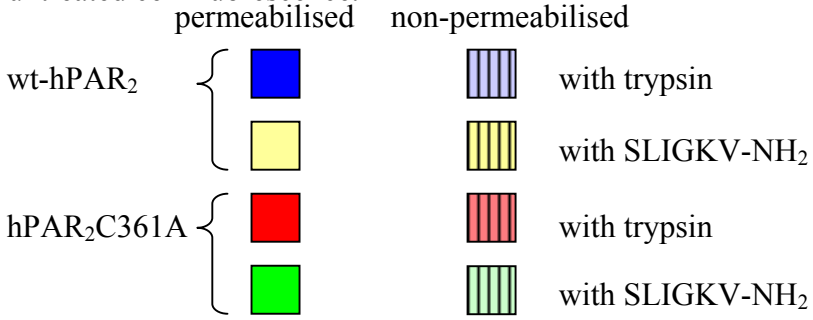


Figure 4.3.12 - Flow cytometry of permeabilised and non-permeabilised cells to show total compared to cell surface expression of receptor post-agonist addition. wt-hPAR$_2$ and hPAR$_2$ C361A cells treated with either 20 nM trypsin or 100 μM SLIGKV-NH$_2$ for 10 mins before staining with Sam-11 anti-PAR$_2$ antibody and anti-mouse FITC conjugate. The experiment was then repeated permeabilising the cells using 4% formaldehyde prior to labelling with anti-HA11 antibody and anti-mouse FITC conjugate. Results are expressed as a percentage of untreated cell fluorescence.



Results are expressed as the mean +/- SEM of 3 separate experiments

4.3.13 Assessment of hPAR$_2$-eYFP Agonist Induced Internalisation by Confocal Microscopy

Confocal imaging of KNRK cells expressing wt-hPAR$_2$ eYFP following treatment with trypsin and SLIGKV-NH$_2$ was used to compare post-activation internalisation between agonists (**figure 4.3.13**). Cells were treated with either 100 nM trypsin, 100 μM SLIGKV-NH$_2$, or no treatment for 30 min. Both agonist treated (**[B]** and **[C]**) and untreated (**[A]**) cells show clear eYFP signal ringing the cell membrane as well as some eYFP signal internally. Where as there appears to be no difference in eYFP signal pattern between SLIGKV-NH$_2$ treated (**[B]**) and untreated (**[A]**) cells. The trypsin treated (**[C]**) cells appear to have a greater punctate eYFP signal presence just within the cell membrane.

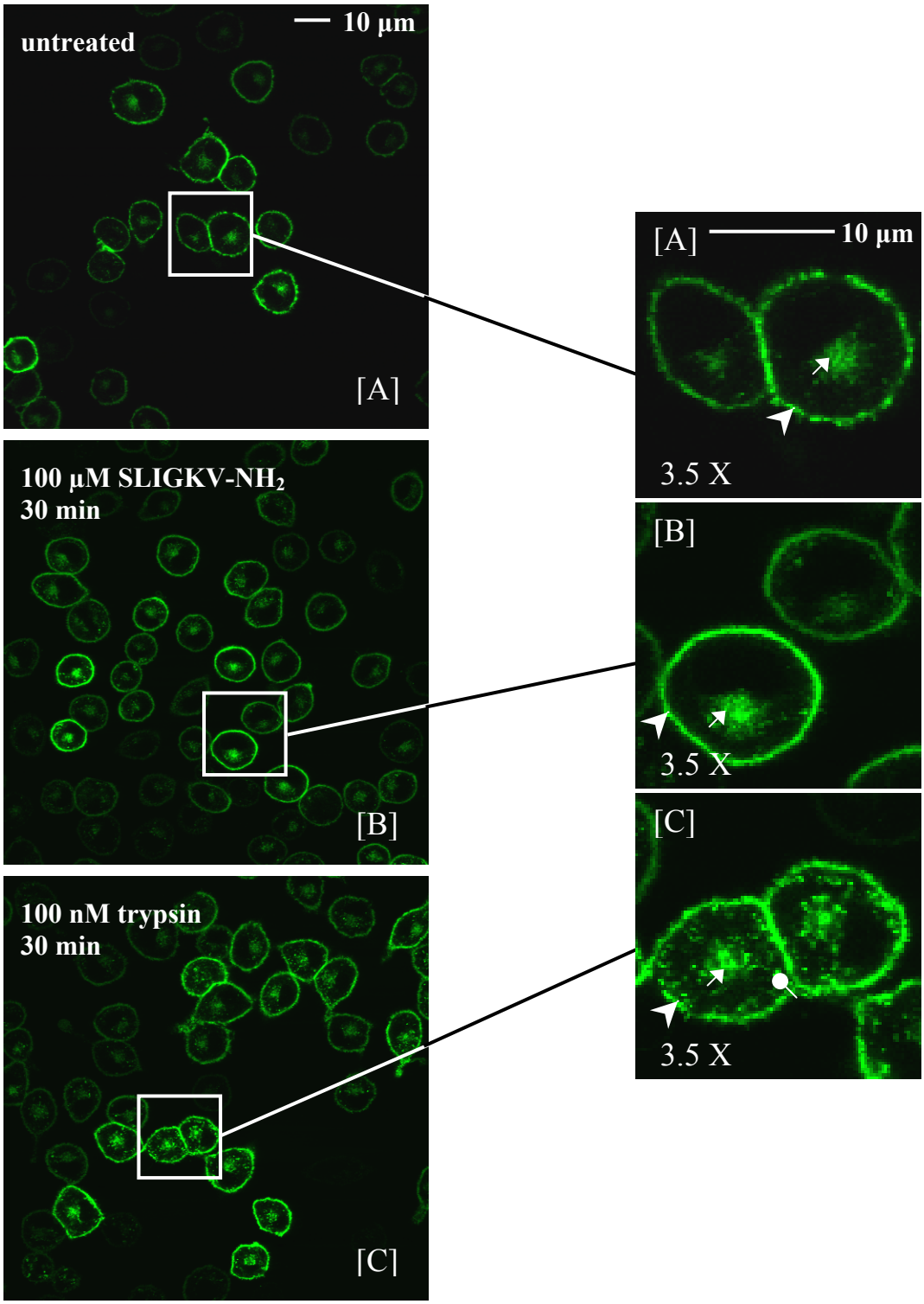


Figure 4.3.13 - Confocal imaging of KNRK hPAR$_2$ eYFP cells Images of cells following treatment with 100 nM trypsin [C], 100 µM SLIGKV-NH$_2$ [B], or no treatment [A] for 30 mins. Zoomed regions are indicated by a white box. Presence of PAR$_2$ at the plasma membrane is indicated with arrowheads, PAR$_2$ contained internally is indicated with arrows, punctate PAR$_2$ below the plasma membrane is indicated with a circle ended marker. Scale bar shown in white on image [A] indicates 10 µm. Images shown are representative of 5 experiments repeated in triplicate.

4.3.14 Assessment of HA11 Tagged hPAR$_2$ Agonist Induced Internalisation by Confocal Microscopy

As a means of visualising the putative post-agonist internalisation of both wt-hPAR$_2$ and hPAR$_2$C361A, cells were grown on coverslips and treated with agonist prior to fixing and permeabilisation. Cells were then stained with anti-HA11 antibody and analysed using confocal microscopy. **Figure 4.3.14** shows six representative confocal images for wt-hPAR$_2$ and hPAR$_2$C361A cell lines following treatment with 100 μM SLIGKV-NH$_2$, 100 nM trypsin or no treatment for 10 min. Confocal images of untreated cells for both wt-hPAR$_2$ (**A1**) and hPAR$_2$C361A (**B1**) show predominantly ring staining around the cell membrane with some intracellular staining. Treatment of wt-hPAR$_2$ cells with SLIGKV-NH$_2$ (**A2**) results similar cellular staining to that of untreated cells (**A1**). In the confocal images produced for hPAR$_2$C361A with SLIGKV-NH$_2$ an apparent small decrease in ring staining and an equivalent increase in intracellular staining can be perceived (**B2**). However, this small change may be due cell-cell variation. Following treatment with trypsin both wt-hPAR$_2$ (**A3**) and hPAR$_2$C361A (**B3**) cell lines show a clear decrease in ring staining and an increase in intracellular staining, primarily located close to the plasma membrane, compared to that on the untreated cells (**A1** and **B1** respectively).

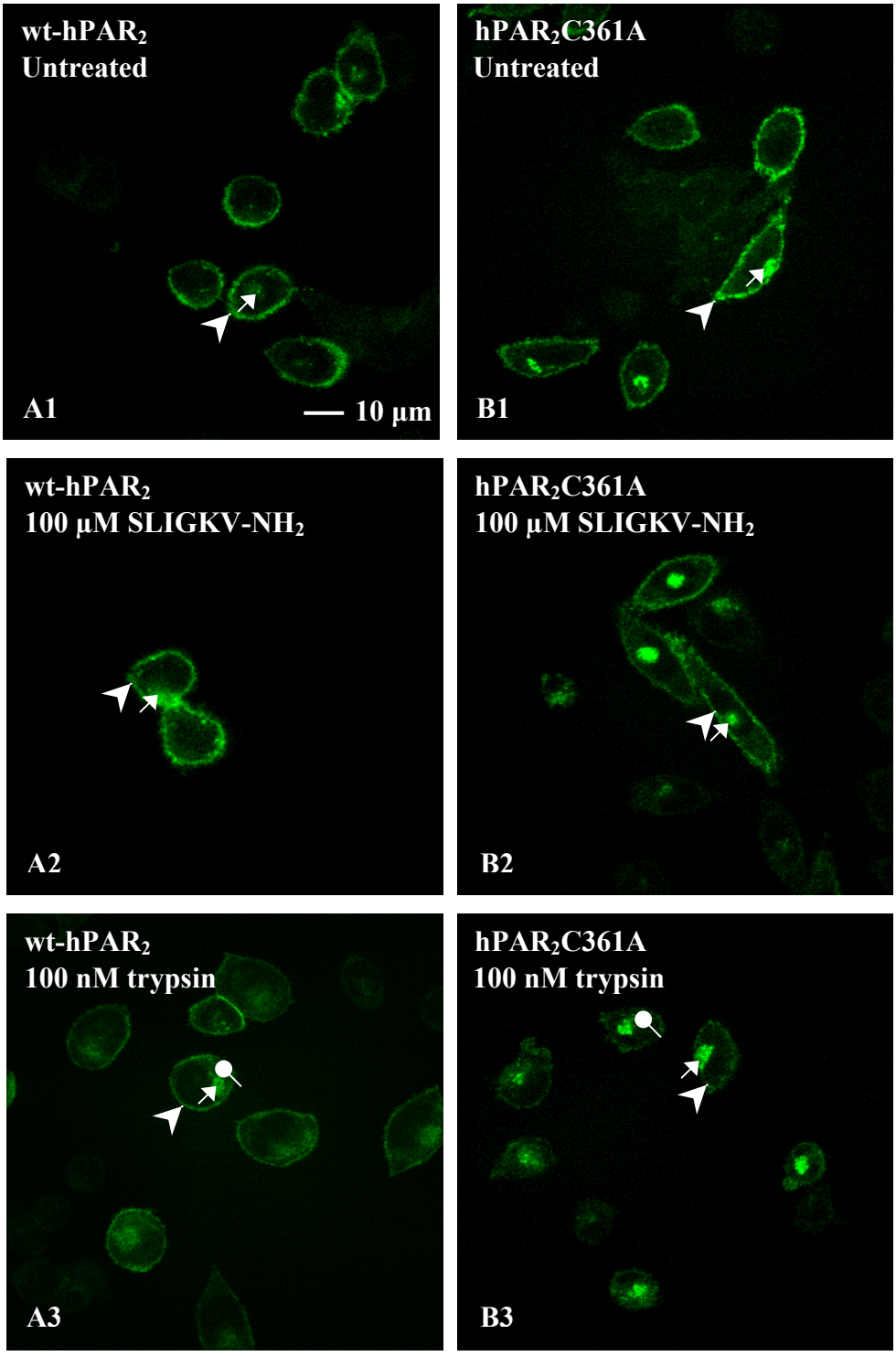


Figure 4.3.14 - Confocal Imaging Receptor Internalisation
Confocal Images of wt-hPAR$_2$ and hPAR$_2$C361A treated with 100µM SLIGKV-NH$_2$ (**A2** and **B2** respectively), 100nM trypsin (**A3** and **B3** respectively), or no treatment (**A1** and **B1** respectively) for 10 min prior to fixing, permeabilisation and staining with anti-HA11 antibody. Presence of PAR$_2$ at the plasma membrane is indicated with arrowheads, PAR$_2$ contained internally is indicated with arrows, punctate PAR$_2$ below the plasma membrane is indicated with a circle ended marker. Scale bar shown in white on image A1 indicates 10 µm.
Pictures shown are representative of each treatment taken from two pictures on triplicate slides from 3 separate experiments

4.3.15Inhibition of PAR_2 Internalisation

Known inhibitors of GPCR internalisation were used to treat to cells in an effort to abate the loss of cell surface $hPAR_2$ staining observed upon treatment with trypsin, and so demonstrate loss of signal was due to receptor internalisation. wt-$hPAR_2$ and $hPAR_2$C361A cells were treated with 20 nM trypsin for 10 min prior to staining with Sam-11 anti-$hPAR_2$ mAb and analysed by flow-cytometry. Dataset were compared using two-tailed t-test as a measure of significant change where $P<0.05$ is considered significant. The combination of GF109203X and trypsin repeatedly resulted in excessive cell death leaving insufficient cells for acquisition (data not shown), as such its effect of Sam-11 cell surface immunoreactivity could not be assessed. Acquisition of data for GF109203X and SLIGKV-NH_2 was possible and showed no change in cell surface staining compared to cells not treated with GF109203X (data not shown). Treatment with PAO consistently failed to inhibit receptor internalisation following trypsin treatment (data not shown).

Figure 4.3.15 shows the effect of five treatments on anti-$hPAR_2$ Sam-11 mAb staining of trypsin treated cells. Both wt-$hPAR_2$ (shown in blue) and $hPAR_2$C361A (shown in red) cells displayed a significant reduction in cell surface Sam-11 staining upon treatment with trypsin (**A**) compared to the untreated control (55.93 ±2.99% P=0.0205, and 49.06 ±13.78% P=0.0001 respectively: **fig 4.3.15 A**). However, if cells were fixed with 4% paraformaldehyde prior to treatment with trypsin, no significant loss of cell surface Sam-11 staining occurred with both wt-$hPAR_2$ and $hPAR_2$C361A cell lines (96.84 ±4.07% P=0.4407 and 99.75 ±0.93% P=0.5580 respectively: **fig 4.3.15 B**). When cells are fixed after treatment with trypsin (**C**) the loss of cell surface Sam11 staining is equivalent to that of **A** (P=0.0657 and P=0.8946 for wt-$hPAR_2$ and $hPAR_2$C361A respectively). Treatment with hypertonic sucrose (**D**) inhibited trypsin induced loss of

cell surface Sam-11 staining, retaining 78.59 ±10.73% of untreated signal for wt-hPAR$_2$ (though there is no significant change from **A** P=0.1118, also no significant difference from untreated P=0.1844) and 91.08 ±1.49% for hPAR$_2$C361A (a significant inhibition over **A** P=0.0387, and no significant difference from untreated P=0.4523). Finally, treatment with concanavalin A prior to addition of trypsin (**E**) abolished the ability of trypsin induced loss of cell surface Sam-11 staining for both wt-hPAR$_2$ and hPAR$_2$C361A (99.97 ±2.97% P=0.9091 and 104.94 ±5.33% P= 0.4375 respectively: **fig 4.3.15 E**).

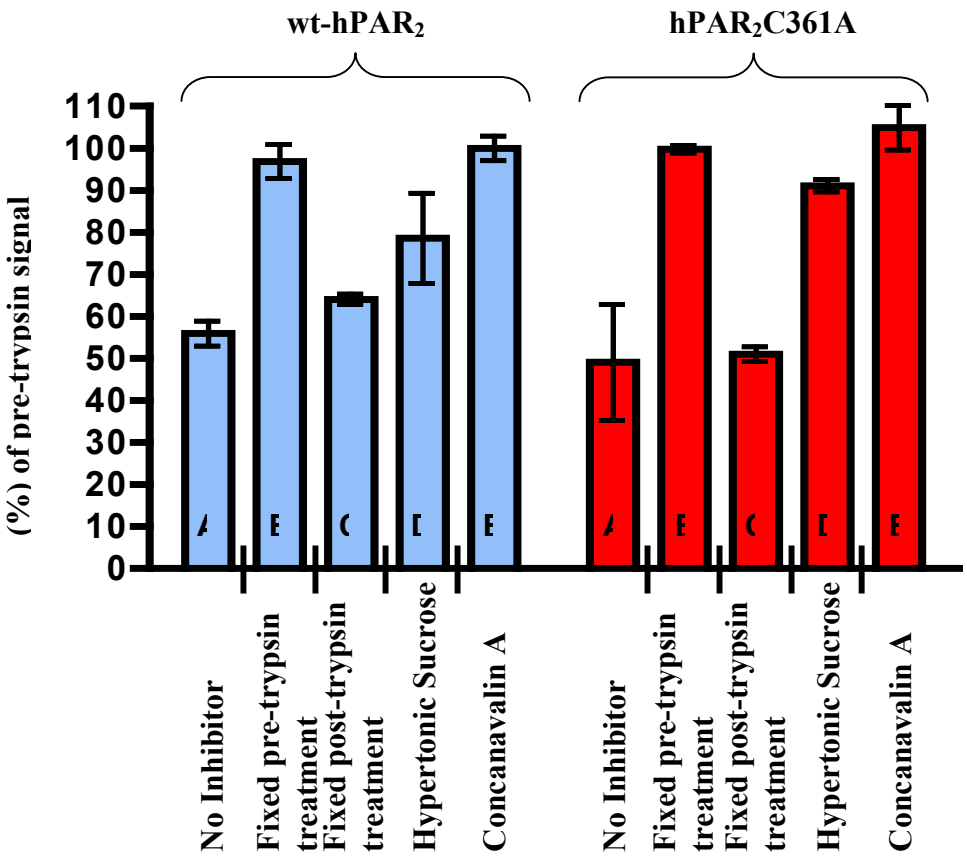


Figure 4.3.15 - Inhibition of Internalisation
Bar chart showing the $hPAR_2$ cell surface staining, as assessed by FACS, of wt-$hPAR_2$ and $hPAR_2$C361A, following treatment with 20 nM trypsin for 10 min, pre and post treatment with known inhibitors of receptor internalisation. **A**– No inhibitor, **B**– Fixed with 4% paraformaldehyde pre-trypsin treatment, **C**– Fixed with 4% paraformaldehyde post-trypsin treatment, **D**– incubated and treated in 0.5 sucrose (hypertonic solution), **E**– incubated in 250 µg/ml concanavalin A.
Results are shown as a percentage of untreated control. Results are expressed as the mean +/- SEM of 3 separate experiments

4.4 DISCUSSION

The data presented in this chapter provides the first direct biochemical and molecular evidence that $hPAR_2$ is both palmitoylated and that cysteine 361 is likely to be the primary site of palmitoylation. In addition evidence is presented here which demonstrates that palmitoylation of $hPAR_2$ is a dynamic process that is influenced by agonist activation. Further, palmitoylation of $hPAR_2$ was shown to regulate constitutive receptor expression, agonist triggered internalisation and more importantly inversely regulate receptor signalling to two major signalling pathways, namely calcium and MAPK. Thus, palmitoylation of PAR_2 may be an attractive target for modifying $hPAR_2$ function in a number of cardiovascular diseases where this receptor has been implicated.

The $hPAR_2$C361A cell line consistently displayed 2 fold greater receptor expression than that of wt-$hPAR_2$, at all levels of confluence. The increased receptor expression observed for $hPAR_2$ on removal of palmitoylation is in contrast to that seen with many GPCR's such as the V_2 vasopressin receptor (Sadeghi et al, 1997). In that study, saturation binding assays showed a decrease in receptor cell surface expression in the non-palmitoylated receptor compared to wild-type. Similarly, the CCR5 receptor displays a profound decrease in cell surface expression in non-palmitoylated mutants compared to wild-type receptor (Blanpain et al, 2001; Percherancier et al, 2001). However, this is the first study to find an increase in receptor expression resulting from the mutagenic removal of a palmitoylated cysteine. Possible reasons for increased receptor expression are covered following discussion of agonist mediated Ca^{2+} mobilisation.

Confocal microscopy was next employed to compare the cellular distribution of wt-$hPAR_2$ and $hPAR_2$C361A. Initially this was carried out using Sam-11 staining, these

images confirmed cell surface expression receptor in both wt-hPAR$_2$ and hPAR$_2$C361A. Although it appears that hPAR$_2$C361A produces a greater fluorescence signal in the images shown, the low level of fluorescence seen with this staining method required a high level of photomultiplier gain so it was concluded that this antibody was not a reliable indication of expression level. To give a better confocal signal and localise receptor expression, cells were permeabilised and stained using anti-HA11 antibody. Sam-11 labelling was only of use in the staining of non-permeabilised cells, in permeabilised cells Sam-11 associated non-specifically. Confocal imaging shows that PAR$_2$ is located at the plasma membrane along with some internal localisation (which is greater in hPAR$_2$C361A), this is presumably, at least in part, PAR$_2$ contained in golgi stores, which is confirmatory of previous confocal imaging of PAR$_2$ (Bohm et al, 1996a; Dery et al, 1999; DeFea et al, 2000b).

Immunoprecipitation of wt-hPAR$_2$ and hPAR$_2$C361A was carried out using the HA11 epitope tag, and western blotting carried out using anti-HA11 antibody. The banding pattern seen is in keeping with the previous immunoblotting of hPAR$_2$ (Compton et al, 2002) and the C361A appears to have no perceptible effect on the receptor molecular weight compared to wild-type. Interesting considerably more PAR$_2$ protein was isolated from the wt-hPAR$_2$ cells than those of the hPAR$_2$C361A. Indeed, three times more cells were required to achieve matching quantities of receptor. This is contrary to what might be expected based on the FACs data showing hPAR$_2$C361A to have a higher level of receptor expression. However, if hPAR$_2$ is palmitoylated at C361, the C361A mutation will result in a reduced C-terminal association with the cell membrane and potentially leave the C-terminus available to be more easily cleaved by endogenous proteases so removing the HA11 tag. Alternatively, the epitope maybe obscured by the C-terminus

becoming associated with different receptor domains, or sterically by an accessory protein binding to a nearby region on the C-terminus (Bockaert et al, 2004a; Bockaert et al, 2004b).

Using direct biochemical labelling we provide compelling evidence that $hPAR_2$ is palmitoylated. There is evidence that GPCRs can be palmitoylated in regions other than the C-terminal tail (Hawtin et al, 2001), seen by the agonist mediated palmitate turnover in V_{1A} receptor where C-terminal palmitoylation sites have been mutagenically substituted. Additionally, when all cysteines of the rat μ-opioid receptor were mutated there was no effect on palmitate incorporation (Chen et al, 1998). However, since no [^{3}H]-palmitate signal is evident in $hPAR_2$C361A it seems likely this is sole palmitoylation site for $hPAR_2$.

In some GPCRs found to be palmitoylated, it has been noted that agonist stimulation of the receptor results in an change in receptor turnover of palmitate (**table 1.5.6.1a**) as demonstrated by an increase or decrease increase in [^{3}H]-palmitate incorporation when cells are treated with agonist during labelling (**table 1.5.6.1a**. Treatment with both agonists tested of wt-$hPAR_2$ in the last 10 min of radio-labelling resulted in a decrease in [^{3}H]-palmitate incorporation compared to wild-type which is contrary to what is reported for α2-adrenergic (Kennedy & Limbird, 1993), β_2-adrenergic (Mouillac et al, 1992), M_2-muscarinic (Hayashi & Haga, 1997), and 5-HT_{4a} (Ponimaskin et al, 2002) receptors. A decrease in [^{3}H]-palmitate incorporation on agonist stimulation was seen with the vasopressin V_2 receptor (Sadeghi et al, 1997; Charest & Bouvier, 2003). Other GPCR show no alteration in palmitoylation state upon agonist binding such as the A_1 adenosine (Gao et al, 1999) and 5-hydroxytrytamine (1A) (Papoucheva et al, 2004) receptors An observed increase/decrease in the rate of palmitate turnover at the receptor

can be an indication of a change in the palmitoylation/depalmitoylation cycle occurring from agonist stimulation. The ability of agonist to effect palmitate turnover suggests a regulatory influence on $hPAR_2$ function consistent with dynamic palmitoylation. Dynamic palmitoylation occurs sometime post-protein synthesis (Escriba et al, 2007), where constitutive palmitoylation occurs during or very shortly after protein-synthesis and is primarily concerned with membrane targeting (Escriba et al, 2007). Interestingly additional bands at approximately 90 and 50 kDa appear in the untreated wt-$hPAR_2$ lane compared to SLIGKV-NH_2 and trypsin treated lanes. This could be as a result of reduced signal intensity for the agonist treated samples, or as a result of changes due of agonist activation. However, on a longer exposure these bands still failed to develop in the agonist activated lanes. It is therefore possible that these bands are as a result of hetero or homo dimerisation of PAR_2 that is disrupted following activation, resulting in a co-immunoprecipitation of a second palmitoylated receptor. Alternatively it maybe the co-immunoprecipitation of the G protein α subunit, which are known to be palmitoylated in order to allow membrane association(Linder & Deschenes, 2007). Upon activation the receptor becomes dissociated from the Gα subunit and therefore it wouldn't appear in the agonist treated lanes. Nevertheless we have shown by direct biochemical evidence that $hPAR_2$ is predominantly, and most likely to be solely palmitoylated on C361 and that it is regulated by agonist activation.

The agonist concentration effect curves for $hPAR_2$C361A and wt-$hPAR_2$ show the removal of palmitoylation to have significant effect on receptor mediated calcium signalling. The decreased signal and agonist sensitivity observed for the $hPAR_2$C361A mutant maybe due to the revealing of phosphorylation sites within the C-terminus, which may normally be obscured by association with the membrane when

palmitoylation is present. This is similar to the β-adrenergic receptor where substitution of the palmitoylated cysteine appears to allow agonist-independent phosphorylation of receptor PKA and GRK sites which would normally only phosphorylate upon activation (Moffett et al, 2001). There is also a similarity in what is seen in other GPCRs where desensitisation/internalisation is driven by the phosphorylation-dependant interaction of the receptor with β-arrestin (Moffett et al, 1996; Moffett et al, 2001; Munshi et al, 2001; Ferguson et al, 2002). Mutagenic replacement of the $hPAR_2$ palmitoylation site may alternatively have resulted in a conformational change in the receptor leaving the TL and AP incapable of correctly binding to the ligand binding site. Although this has not previously been seen in the mutagenic replacement of palmitoylation, removal and mutagenesis of areas of the C-terminal tail in some GPCR have been shown to affect receptor folding (Piersen et al, 1994; Klinger et al, 2002). The reduction in receptor mediated calcium signalling may be as a result of altered G-protein coupling. The C-terminal tail of many GPCRs are associated with G-protein coupling as such removal of the palmitoylation site affects G-protein coupling in many GPCRs (Mouillac et al, 1992; Moffett et al, 1996; Hayashi & Haga, 1997; Okamoto et al, 1997; Hukovic et al, 1998; Doi et al, 1999; Fukushima et al, 2001; Moffett et al, 2001). In some this is mostly likely because of with increased phosphorylation (Moffett et al, 1996; Palmer & Stiles, 2000; Moffett et al, 2001; Munshi et al, 2001) but some mutagenically depalmitoylated GPCRs have decreased G-protein coupling without displaying increased phosphorylation (Okamoto et al, 1997; Doi et al, 1999) suggesting another mechanism.

Experiments assessing MAPK activation revealed that palmitoylation of PAR_2 inversely regulates MAPK activation. This increased ERK1/2 signalling seen with the non-palmitoylated mutant may be due to increased β-arrestin association. It is well known

that phosphorylation of the C-terminus of PAR_2 results in an increased affinity for β-arrestin binding which in turn uncouples the receptor from it's G-protein by a steric mechanism (Bohm et al, 1996a; Dery et al, 1999; DeFea et al, 2000b). Additionally, PAR_2 expressed alongside a β-arrestin mutant incapable of receptor binding shows diminished ability to activate ERK1/2 (DeFea et al, 2000b). Studies on a number of GPCRs have now demonstrated that β-arrestins can mediate a number of signalling pathways independent of G-proteins (Wei et al, 2003; Hunton et al, 2005; Shenoy et al, 2006). Recently PAR_2 activation has also been shown to promote β-arrestin actions-independent of G-proteins by direct inhibition of $G\alpha_{q/11}$, as well as receptor coupling to $G\alpha_{q/11}$ (Zoudilova et al, 2007). There is additional evidence to suggest that residues in the C-terminus of PAR_2 define specificity of β-arrestin binding and the duration of ERK1/2 association with β-arrestin (DeFea et al, 2000b; Seatter et al, 2004; Stalheim et al, 2005). This combined with β-arrestin 1 and 2 association with ERK1/2 activation in the early and intermediate phases (Kumar et al, 2007) supports the hypothesis that ERK1/2 signalling in $hPAR_2C361A$ maybe enhanced due an increase in β-arrestin association. This increased β-arrestin association may also go to explain the increased receptor expression seen in $hPAR_2C361A$ compared to wt-$hPAR_2$. MEF from β-arrestin knockout mice displayed lower levels of PAR_2 expression than normal (Zoudilova et al, 2007) and over-expression of β-arrestin 1 alongside wt-$hPAR_2$ enhances restoration of PAR_2 post-activation (Kumar et al, 2007) suggesting a role for β-arrestin 1 in trafficking of PAR_2 from golgi stores. Alternatively, palmitoylation is linked with the targeting of receptors and signalling molecules alike to specific membrane microdomains such as lipid rafts (Okamoto et al, 1998; Melkonian et al, 1999). It has been shown that $G\alpha_q$ interacts with caveolin, where as $G\alpha_i$ does not suggesting they may therefore be targeted to different lipid regions (Oh & Schnitzer, 2001). The oxytocin receptor (OTR) is

known to couple to both G_q and G_i but differentially activates one or the other depending on it's membrane localisation (Rimoldi et al, 2003). When located within lipid rafts OTR activation results in cell growth through a G_q mediated pathway, when outside lipids rafts it results in G_i mediated inhibition of cell growth. This effect on cell growth is shown to be as a result of a different temporal pattern of EGFR and ERK1/2 phosphorylation, which is shown to be more persistent when receptors are located outside of lipid raft microdomains (Rimoldi et al, 2003). As such palmitoylation in $hPAR_2$ may target the receptor to specific lipid raft microdomains, removal of which results in movement outside of these lipid rafts resulting in greater G_i association and prolonged ERK1/2 signalling.

Palmitoylation has been shown to affect a GPCRs ability to couple to its respective G-protein α subunit. For some GPCRs palmitoylation has been shown to selectively effect coupling to specific Gα subunits (Okamoto et al, 1997; Doi et al, 1999), removing coupling to one or more subunits whilst maintaining coupling with another. In order to further understand the effect of palmitoylation on $hPAR_2$ signalling due to G-protein coupling Ca^{2+} signalling was carried out on wt-$hPAR_2$ and $hPAR_2$C361A cells pre-treated with pertussis toxin (PTX), an inhibitor of signalling through G_i. PTX has a larger inhibitory effect on the Ca^{2+} signalling for $hPAR_2$C361A than for wt-$hPAR_2$. Conventionally, G_i has not been known to result in calcium signalling, however these results clearly show that inhibition of G_i results in inhibition of calcium signalling. As such the mechanism of signalling through calcium maybe related to interaction between $hPAR_2$C361A and other receptors (as discussed in **3.4**) bringing their respective G-protiens into close association. Alternatively some as yet unknown mechanism maybe in place, further work using inhibitors of specific points in the calcium signalling

cascade is required to elucidate the exact mechanism of action. Interestingly the signalling reduction in wt-hPAR$_2$ as a result of PTX treatment is approximately the same as the total signalling seen for hPAR$_2$C361A. Since the remaining signalling seen in hPAR$_2$C361A at the top PTX dose is equivalent to what can be attributed to trypsin activation of PAR$_1$ it seems likely that the signalling seen in hPAR$_2$C361A is solely due to coupling with G_i and that coupling to G_q has been entirely removed. Although previous GPCRs have shown selective uncoupling of Gα subunits (Okamoto et al, 1997; Doi et al, 1999) these have preferentially lost G_i and maintained G_q coupling which was suggested by Okomoto et al as being due to G_i requiring a higher level of palmitoylation. However, if palmitoylation of hPAR$_2$ targets the receptor to G_q containing lipid rafts, as with the OTR (Rimoldi et al, 2003), and removal of palmitoylation results in the receptor locating outside of the raft then association with G_q would be disrupted. Pre-treatment of wt-hPAR$_2$ with PTX prior to evaluation of ERK signalling indicates that PAR$_2$ activates ERK1/2 through a PTX-insensitive pathway, so having little or no involvement by G_i. This is concurrent with previous investigation of ERK1/2 activation in PAR$_2$ (DeFea et al, 2000b). However, the reduced ERK phosphorylation post-SLIGKV-NH$_2$ activation seen in hPAR$_2$C361A pre-treated with PTX suggests that ERK activation is G_i-mediated in the non-palmitoylated mutant. Numerous studies have shown ERK activation to be independent of G-protein signalling (see introduction) and in PAR$_2$ ERK signalling has been shown to be mediated by β-arrestin association (Kumar et al, 2007; Zoudilova et al, 2007). It maybe the increased association with G_i results in shift in the preferred pathway for ERK1/2 activation. The signalling pathway responsible for activation of ERK in PAR$_2$ has previously been shown to "switch" in the case of PAR$_2$δST363/366A, a phosphorylation deficient mutant, which activated ERK1/2 via a proline-rich tyrsoine kinase-2 (PYK2) mediated

pathway (DeFea et al, 2000b). It was hypothesized that the interaction with the PYK2 pathway was a result of the prolonged Ca^{2+}-mobilisation observed in this mutant. As stated earlier Ca^{2+}-mobilisation following $hPAR_2C361A$ activation takes longer to reach a signalling plateau so it maybe that the G_i-dependant activation of ERK1/2 activation observed is PYK2-mediated.

The rate and extent of loss of cell surface staining with Sam-11 anti-$hPAR_2$ mAb in response to trypsin is increased in the palmitoylated mutant compared to wt-$hPAR_2$. This loss of immunoreactivity to Sam-11 suggests receptor post-activation internalisation. However, neither mutant nor wild-type receptor showed a loss of cell surface staining with Sam-11 following treatment with SLIGKV-NH_2. This is contrary to what has previously been reported for PAR_2 in studies showing SLIGKV-NH_2 treatment to stimulate PAR_2 endocytosis (Bohm et al, 1996a; Dery et al, 1999; DeFea et al, 2000b). In order to ascertain whether this loss of immunoreactivity is as a result of internalisation or degradation of receptor, total cellular receptor expression of permeabilised cells was compared with cell surface expression, post-agonist addition. The fact that total cellular receptor levels remains at 100% that of untreated cells shows no reduction in total cellular receptor compliment and indicates only a loss of cell surface expression concurrent with internalisation of receptor post-activation. Since these data are contrary to published data we used confocal imaging of KNRK $hPAR_2$ eYFP cells post agonist treatment to further investigate the differential agonist effect. The data produced shows a translocation of cell surface receptor into internal compartments, concurrent with receptor internalisation on post-activation with trypsin, but not with SLIGKV-NH_2 so supporting our previous findings. Similar results were found using the same Pro5 cell lines permeabilised and stained for HA11 post-agonist

treatment. These images also show a greater translocation of $hPAR_2C361A$ receptor into the cytosol compared to wt-$hPAR_2$ which further supports the previous FACs data. To further illustrate that loss of cell surface immunoreactivity to Sam-11 was as a result of receptor internalisation by endocytosis, a number of known endocytotic inhibitors were investigated along with an inhibitor of PKC. The reduction or abolition of the loss of Sam-11 cell surface immunoreactivity following treatment with concanavalin A and hyperosmolar glucose concentrations, as well as fixing the membrane, further supports the previous evidence that this loss is as a result of receptor endocytosis. Although this study is the first to report a differential effect of receptor cell surface expression and post-agonist endocytosis with trypsin and SLIGKV-NH_2, the internalisation seen post-SLIGKV-NH_2 treatment in previous publications maybe be due to over-expression of β-arrestin in the cell lines used (Plevin, 2006; Personal Communication to Dr S. Compton; DeFea, 2007; Personal Communication to Dr S. Compton). A recent study has shown over-expression of β-arrestin to result in its constitutive association with cellular components (Zoudilova et al, 2007). Hypertonic sucrose is known to prevent the recruitment of clathrin and interferes with normal coated-pit formation and endocytosis (Heuser & Anderson, 1989; Hansen et al, 1993), and concanavalin A is known to prevent the formation of coated pits (Jiang et al, 2001; Lafleur et al, 2006). As such we have also demonstrated that $hPAR_2$ internalises by clathrin-coated pits, and $hPAR_2C361A$ internalises by the same mechanism albeit more rapidly.

The enhanced post-activation endocytosis seen with $hPAR_2C361A$ may be for a number of different reasons. If as previously discussed $hPAR_2C361A$ is constitutively phosphorylation then β-arrestin may associate with the receptor C-terminus. For the conformational change to occur in β-arrestin that allows high-affinity binding of

accessory protein-2 (AP-2) and clathrin, resulting in endocytosis, it must be bound to a receptor that is both phosphorylated and activated (Moore et al, 2007). Since β-arrestin is already associated with the receptor upon activation, endocytosis can occur with fewer binding steps. If $hPAR_2$ functions similarly to the OTR, then following activation the receptor depalmitoylates and needs to translocate from the caveolae domain before being endocytosed (Rimoldi et al, 2003). The removal of $hPAR_2$ palmitoylation would mean the receptor was already located outside of the caveolae domain allowing clathrin binding and endocytosis without the need for receptor translocation. The increase is the amount of receptor endocytosed post-activation may occur as a result of uncoupling from G_q, phospholipase C (PLC) activity which has previously been shown to inhibit endocytosis via clathrin-coated pits (Carvou et al, 2007). Since $hPAR_2C361A$ has reduced/abolished coupling to G_q, PLC activity would be significantly reduced/abolished so allowing increased endocytosis via clathrin-coated pits in the absence of inhibition from PLC.

Upon activation the receptor must become depalmitoylated and the phosphorylated before β-arrestin binding. In order to mediate receptor endocytosis β-arrestin must undergo a conformational change which increases binding affinity of clathrin and AP-2 (Moore et al, 2007). For this conformational change to occur the receptor must be both in an activated confirmation and phosphorylated (Moore et al, 2007). Additionally, receptor phosphorylation has previously been shown to be the rate limiting step for β-arrestin association (Krasel et al, 2004). As stated earlier it may be that the removal of palmitoylation results in the receptor being phosphorylated due the increased availability of the C-terminal phosphorylation sites, thus allowing β-arrestin binding but endocytosis does not occur until the receptor is activated. Since β-arrestin is already

associated with the receptor, endocytosis can occur immediately without further β-arrestin association.

This is the first study investigating the palmitoylation status of $hPAR_2$ and its effect on receptor function. We have demonstrated that $hPAR_2$ is palmitoylated pre-dominantly, if not entirely on C361. We have further demonstrated the multifaceted importance of palmitoylation on receptor expression, agonist sensitivity, desensitisation and internalisation.

5 GENERAL DISCUSSION

5.0 GENERAL DISCUSSION

5.1 Introduction

The major hypothesis of this thesis was that *"Extracellular and intracellular cysteines of $hPAR_2$ undergo modifications that regulate receptor function"*. The major findings in this research support this hypothesis, namely: **1)** C361 is the major and most probably only site of palmitoylation in $hPAR_2$ and removal of palmitoylation dramatically effects $hPAR_2$ expression, agonist sensitivity, agonist mediated Ca^{2+}-mobilisation, ERK1/2 signalling, and agonist-stimulated internalisation, **2)** The palmitoylation of C361 serves as a differential regulator of G-protein association. This is demonstrated by the shift of association from $G\alpha_q$ with wt-$hPAR_2$ to $G\alpha_i$ with $hPAR_2$C361A, and the $G\alpha_i$-mediated ERK1/2 signalling seen with $hPAR_2$C361A and not with wt-$hPAR_2$, **3)** Mutagenesis of conserved cysteine residues which are putative sites of disulphide bridging (C148 and C226) result in either an inability to successfully express the receptor (for $hPAR_2$C226A and $hPAR_2$C148A/C226A) or a receptor incapable agonist-mediated Ca^{2+}-mobilisation. Thus showing this modification to have a significant role in receptor expression and function and finally, **4)** Mutagenic removal of C22 results in a receptor with altered agonist sensitivity and a novel trypsin sensitising response to thrombin. Although this study failed to elucidate the modification occurring at this site, given the nature of the reactive sulfhydryl group attached to cysteine residues it is probable that some covalent modification is present.

Taken together the findings within this Thesis clearly show an importance of post-translational modifications in the function of $hPAR_2$.

5.2 Caveolae and PAR_2 signalling

The findings in this Thesis combined with that already known regarding the signalling of PAR_2 leads us to the following hypothesis: Prior to activation $hPAR_2$ is primarily located within the caveolae domain coupling to $G\alpha_q$. Upon activation G_q mediated Ca^{2+} mobilisation results, and $hPAR_2$ becomes depalmitoylated and rapidly phosphorylated. In addition $hPAR_2$ signals partially through $G\alpha_i$, this could occur either through a small amount of $G\alpha_i$ being present with caveolae, a small percentage of $hPAR_2$ being located outside of the caveolae leaving it able to associate with $G\alpha_i$, or $hPAR_2$ becoming depalmitoylated upon activation translocates to outside of caveolae and once there becomes associated with $G\alpha_i$ as with OTR (Rimoldi et al, 2003). β-arrestin binds to the activated, phosphorylated receptor sterically inhibiting further G-protein association and resulting in signalling through ERK. The resulting change in β-arrestin conformation increases clathrin and AP-2 binding affinity causing receptor internalisation. This process is schematically represented in **fig 5a**. Since $hPAR_2C361A$ is not palmitoylated it is located outside of the caveolae and is therefore able to readily associate with $G\alpha_i$ (equivalent to step ❹ seen in **fig 5a**). If $hPAR_2C361A$ is intrinsically phosphorylated, as discussed earlier, β-arrestin may be partially associated with the receptor prior to activation by agonist, so reducing G-protein association or the percentage of receptor associated with the G-protein. Upon activation $hPAR_2C361A$ stimulates Ca^{2+} mobilisation through G_i. Since no translocation out of caveolae or initial β-arrestin binding is required, β-arrestin need only fully bind to the now activated as well as phosphorylated receptor to achieve the conformational change required for clathrin association and internalisation. Since G-protein coupling is not required for ERK activation the full receptor population stimulate ERK signalling and not just the

proportion associated with G_i. Internalisation for both receptors appears to be independent of Ca^{2+}-mobilisation (so probably G-protein coupling) as seen by the rapid internalisation kinetics of $hPAR_2C361A$ versus the reduced Ca^{2+} mobilisation kinetics. The G-protein independence of receptor internalisation is further supported by the post-trypsin endocytosis of $hPAR_2C148A$ which is incapable of agonist-mediated Ca^{2+} mobilisation. The fact that $hPAR_2C148A$ still signals through ERK1/2 further demonstrates the independence of ERK1/2 signalling from G_q.

5.3 Palmitoylation and the PAR family

Previous studies exploring the role of palmitoylation have suggested that data from one receptor cannot always be translated to another, even closely related receptor. However, this may not be the case for the PARs. The absence of a putative palmitoylation site in PAR_4 acts as an interesting comparison to PAR_2. PAR_4 when transfected into cells does not signal well through intercellular Ca^{2+}-mobilisation but is capable of robust signalling through ERK1/2 (Hollenberg and Ramachandran; Xiao and Compton; unpublished observations)(Ritchie et al, 2007). Thus, PAR_4 appears to act similarly to the $hPAR_2C361A$ mutant, and a lack of this palmitoylation site maybe responsible for such differences in PAR_4 signalling. Inserting the palmitoylation site back into PAR_4 would make an interesting study. PAR_1 possesses three putative palmitoylation sites within its C-terminal tail. Removal of two of these putative palmitoylation sites in PAR_1 leads to a complete ablation of all agonist-stimulated Ca^{2+}-mobilisation (Guo and Compton; unpublished observations). Thus it appears that palmitoylation may play an important role in the signalling properties of the PAR family and not just specifically to PAR_2.

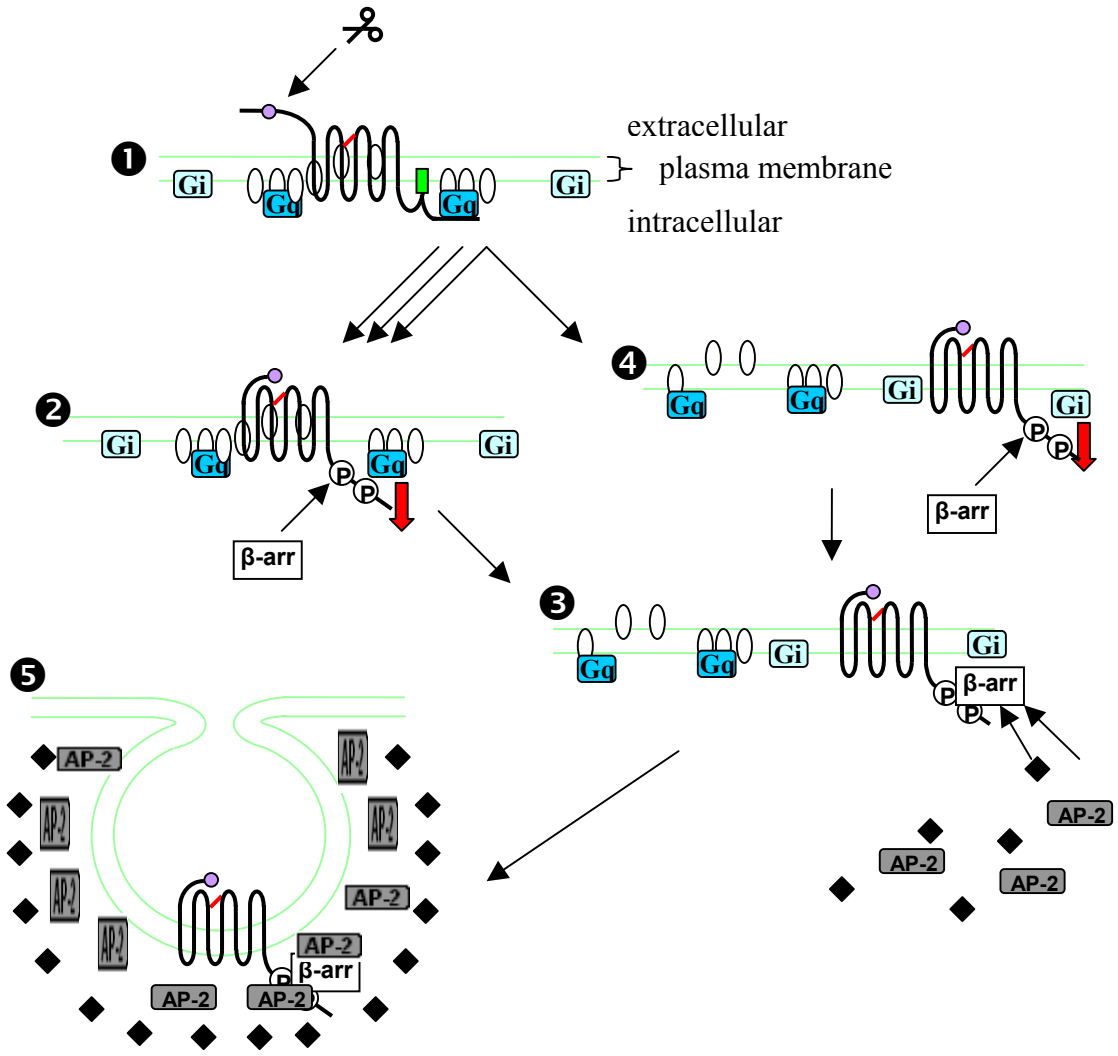


Figure 5a - Schematic of hPAR$_2$ internalisation
Schematic showing the possible route of endocytosis of hPAR$_2$ following activation with trypsin. Molecular ratios are representative only. ❶- Cleavage of N-terminus resulting receptor activation. The receptor then either ❷- binding of TL to binding domain, receptor is depalmitoylated, activates Gq and is phosphorylated increasing β-arrestin binding affinity, ❸- the receptor translocates out of caveolae and β-arrestin binding disrupts G-protein coupling. Conformational changes in β-arrestin result in AP-2 and clathrin binding with greater affinity, or to a smaller extent ❹- binding of TL to binding domain, receptor is depalmitoylated and translocates out of caveolae before activating Gi and becoming phosphorylated increasing β-arrestin binding affinity then proceeding to ❸ having already translocated. ❺- the receptor is endocytosed into early endosomes remaining associated with β-arrestin.

✂-trypsin, ○ -tethered ligand, ▮ -palmitoylation, ⬯ -caveolae Ⓟ-phosphorylation, Gq -$G\alpha_q$, Gi -$G\alpha_i$, ⬇ -G-protein mediated signalling, β-arr -β-arrestin, AP-2 - Accessory Protein-2, ◆- Clathrin.

5.4 Therapeutic Significance

Though intracellular, the possibility of therapeutically targeting palmitoylation is supported by the development of novel cell-penetrating peptides shown capable of intracellularly interacting with ICL3 and C-terminal regions of both PAR_1 and PAR_4 (Kuliopulos & Covic, 2003; Wielders et al, 2007). The fact that palmitoylation appears to be able to selectively control the signalling pathway through which signalling will occur (in the case of PAR_1) and as we show here with PAR_2, shift its predisposition or bias between pathways, makes it an interesting therapeutic target capable of dissecting out particular functional consequences instead of broadly antagonising a receptor. Traditional views in GPCRs function are that ligand binding stimulated or inhibited all receptor functions to an equal extent (Benovic et al, 1988). In this model using an agonist of low potency results in a reduction in all downstream signalling effects compared an agonist of higher potency, so allowing an assay of any receptor function to be able to fully characterise any ligand as agonist, antagonist, partial agonist, or inverse agonist. However, more recently the concept of "biased agonism" also known as "functional selectivity" or "collateral efficacy"has come to light (Kenakin, 2007; Violin & Lefkowitz, 2007). This concept is based on ligand binding affecting the affinity of a receptor for particular conformations, which further confer the receptors ability to traffic information selectively to some, but not all, cellular signalling pathways. This was first suggested by the observation that although acetylcholine activates both G_q-mediated PLC and G_s-mediated cAMP, several ligands of the muscarinic acetylcholine receptor were capable of preferentially activating PLC whilst inhibiting cAMP (Fisher et al, 1993; Gurwitz et al, 1994). This concept was further expanded to include β-arrestin function through the discovery of non-desensitising agonists capable of triggering G-protein signalling without β-arrestin associated desensitisation and internalisation

(Lewis et al, 1998; Whistler & von Zastrow, 1998; Kohout et al, 2004). Since then the inverse has also been shown with other ligands capable of stimulating β-arrestin recruitment and/or internalisation occurring completely independently of G-protein signalling (Blanpain et al, 2002; Wei et al, 2003; Gesty-Palmer et al, 2006). This is indeed similar to what we have shown in this study with $hPAR_2C148A$ where this mutation resulted in a receptor incapable of G-protein mediated Ca^{2+}-mobilisation but still capable of β-arrestin mediated ERK1/2 activation showing also that modifications within the receptor affect ligand bias, possible by changing the receptor conformation stabilised by the ligand (Kenakin, 2007). This paradigm may also go some way to explaining the observation within this study that $hPAR_2$ activation by TL results in G-protein and ERK1/2 signalling as well as receptor internalisation, where as activation by SLIGKV-NH_2 results is comparable signalling but is deficient in stimulating receptor internalisation. The nature of TL activation may allow the stabilisation of a pronounced, or least distinct, conformation in PAR_2 that is not possible using SLIGKV-NH_2. In this study we therefore demonstrated a difference in ligand bias between the TL and SLIGKV-NH_2 but have also demonstrated two cases of altering ligand bias, with $hPAR_2C148A$ and $hPAR_2C361A$, by targeting specific sites of post-translational modification. This is particularly significant given the nature of activation of PARs, where although different proteinases may cleave the receptor the same TL results. Also, although antagonists have been successfully synthesized for PAR_1 and PAR_4 (see introduction), no inhibitors or modulators of PAR_2 signalling are available. Selective targeting of therapeutic agents to these regions designed to disrupt such modifications may therefore open a new field of PAR_2 directed drug discovery. In addition to this, our findings showing agonist-activation to result in receptor depalmitoylation provides an additional therapeutic possibility based on an endogenous agonist driven effect. It may

be possible to screen current agonist peptide libraries or design new APs in order to identify an AP that is capable of depalmitoylating PAR_2 to a greater or lesser extent. Ultimately, this would provide a bank of agonists capable of preferentially driving either ERK1/2 or Ca^{2+}-signalling within PAR_2, thus selectively activating or inhibiting their specific downstream effects. This may provide an agonist that would stabilise a non-palmitoylated conformation of PAR_2 without triggering signalling and thus providing a "molecular switch" extracellularly targeting an intracellular post-translational modification, resulting in a receptor with pharmacology similar to $hPAR_2C361A$.

An example would be the ability to selectively inhibit PAR_2-mediated activation of ERK1/2, a pathway strongly associated with inflammation, whilst leaving its ability to cause vascular relaxation through a Ca^{2+}-mobilisation induced NO dependant mechanism intact. PAR_2 is however linked to a number of other intracellular signalling pathways which may show changes in agonist bias following these, or indeed other, modifications. Thus further characterisation is required in order to uncover all the cellular signalling changes and which disease states would benefit from invention in PAR_2 modifications.

5.5 Significance in Disease

It is our contention in this study that palmitoylation of $hPAR_2$ targets the receptor to specific cholesterol-rich membrane domains called caveolae, and thus determining G-protein selectivity by the proximity of particular G_α-subunits. The altered receptor pharmacology seen in $hPAR_2C361A$ may therefore be significant in diseases that require the therapeutic usage of cholesterol depleting drugs. A group of drugs called filipins (Bornig & Geyer, 1974) which are potent antifungal drugs, are capable of

binding cholesterol and disrupting caveolae domains. Though most are too toxic for therapeutic use a few are used clinically: amphotericin B (administered intravenously for systemic fungal infections)(Almirante & Rodriguez, 2007) and nystatin A1 (used clinically for gastrointestinal fungal infections)(Wong et al, 2007) could potentially alter PAR_2 signalling bias by depleting available caveolae domains switching G_α-subunit predisposition. Both of these drugs certainly have side-effects that could in part be credited to actions through PAR_2 (Le et al, 1996; Bishara et al, 2001), which have been attributed partially to histamine liberation and increased prostaglandin synthesis.

It is now widely accepted that high serum cholesterol is a negative risk factor for a variety of CVDs (Grundy et al, 2004; Reid et al, 2007). Plasma lipoproteins have been shown capable of altering endothelial cell function *in vivo* (Gotto, 2001), with elevated low-density lipoprotein (LDL) and triglyceride-rich lipoproteins being associated with abnormal vasodilatory function and development of atherosclerosis (Gotto, 2001). Conversely elevated high-density lipoproteins (HDL) are antiatherogenic and appear to modulate endothelial function beneficially (Gotto, 2001). The mechanism of action of lipoprotein interactions are however unclear, though they appear to be readily reversible. Indeed, statins are used clinically to lower serum cholesterol levels in patients with/or at risk of cardiovascular diseases (Shepherd et al, 2003). They inhibit cholesterol production and stimulate LDL receptors in the liver resulting in increased clearance of circulating LDL (Shepherd et al, 2003). Additionally dietary sources of certain fatty acids have been shown to lower LDL and have further cardioprotective effects (Ascherio, 2002; Sacks & Katan, 2002). Thus, cholersterol lowering drugs may alter cellular function by reducing the abundance of cholesterol rich caveolae. It follows that the presence of high cholesterol and altered fatty acid composition may result in

significantly altered PAR_2 signalling, by virtue of their ability to influence receptor function and cell surface location. Furthermore, altered PAR_2 expression and function in some cardiovascular diseases where cholesterol is reportedly high may therefore be a result of altered receptor palmitoylation and/or location within cholesterol rich caveolae. Further studies are required to explore these interesting ideas.

5.6 Future Work

To further investigate disulphide bridging in $hPAR_2$, treatment with DTT would pharmacologically remove the disulphide bridge (as well as any other extracellular covalent modifications) to enable the analysis of $hPAR_2$ deficient in disulphide bridging but already expressed at the plasma membrane.

In order to elucidate the cause of thrombin associated trypsin sensitisation of $hPAR_2$C22A, this receptor should be expressed in a cell lines not containing endogenously expressed PAR_1. This would enable the investigation of whether or not the endogenously expressed PAR_1 in Pro5 cells is a factor in this phenomenon. This is cell line has already been produced within our laboratory and work is ongoing.

Considering the interesting results seen following the mutation of C22 further investigation in the possible dimerisation of $hPAR_2$ should be carried out using BRET and co-expression of $hPAR_2$C22A with wt-$hPAR_2$ and $hPAR_1$. This is in order to show whether or not $hPAR_2$ is closely associated enough with either itself or $hPAR_1$ to allow for dimerisation. Co-immunoprecipitation studies (using cells co-expressing receptors with different C-terminal epitope) could also be used to demonstrate an association between receptors and investigate whether it is still intact in mutagenically altered receptors such as $hPAR_2$C22A.

Mutation of C-terminal phosphorylation sites in wt-hPAR$_2$ and hPAR$_2$C361A (namely S363 and T366) would enable us to see the function or C-terminal phosphorylation in the observed differences in receptor function and internalisation. It would also enable us to see if our hypothesis regarding the basal phosphorylation of hPAR$_2$C361A is true. These constructs and cell lines have already been produced within our laboratory and work in ongoing. However, since hPAR$_2$δST363/366A displayed a "switch" in pathways through which its ERK1/2 signalling is controlled this data should be used in combination with pharmacological means. Use of inhibitors of PKC-mediated phosphorylation, such as GF109203X should be further attempted. Other inhibitors of components of PAR$_2$ downstream signalling should also be used to investigate their role in Ca^{2+}-mobilisation, ERK1/2 signalling, and receptor internalisation in the depalmitoylated mutant compared to wild-type e.g. BAPTA-AM as a blocker of Ca^{2+}-mobilisation, U73122 to block PLC activity, GP2A an antagonist of $G\alpha_q$ activity, additionally gene silencing of different components with siRNA may be possible such as those used for β-arrestin by (Kumar et al, 2007). The effect of PTX on wt-hPAR$_2$ and hPAR$_2$C361A post-agonist internalisation may also be interesting to see if the increased G_i association has an effect, in the same way it does in ERK1/2 signalling. The co-expression of fluorescently labelled hPAR$_2$C361A with β-arrestin labelled with a difference fluorescent tag would allow the visualisation of any β-arrestin-receptor association at any and all points pre- and post-activation of receptor.

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6.0 REFERENCES

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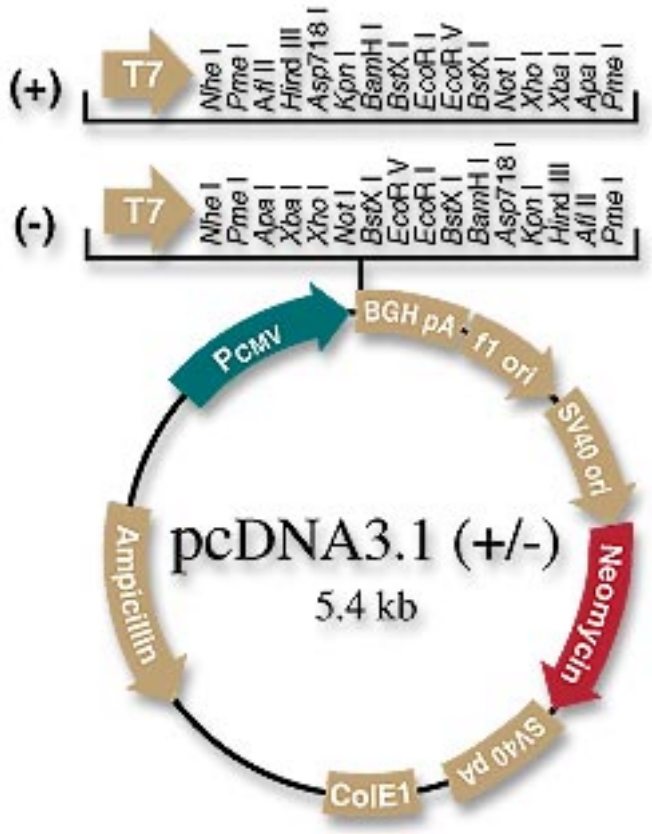
7 APPENDIX

APPENDIX: 1 pcDNA3.1(+/-) Plasmid Map

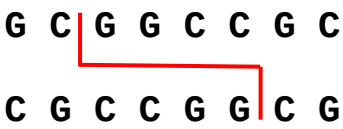
pcDNA3.1(+/-) Plasmid Map



APPENDIX: 2 Restriction Enzyme Recognition Sites

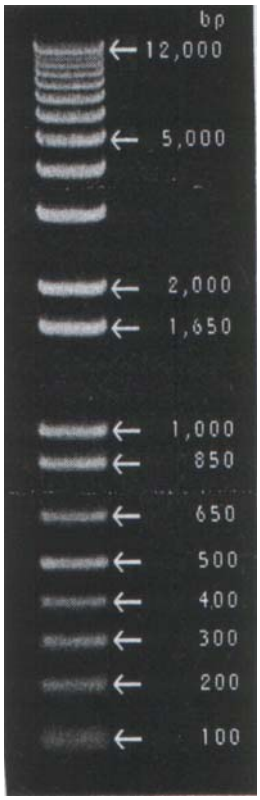
*Hind*III

*Not*I

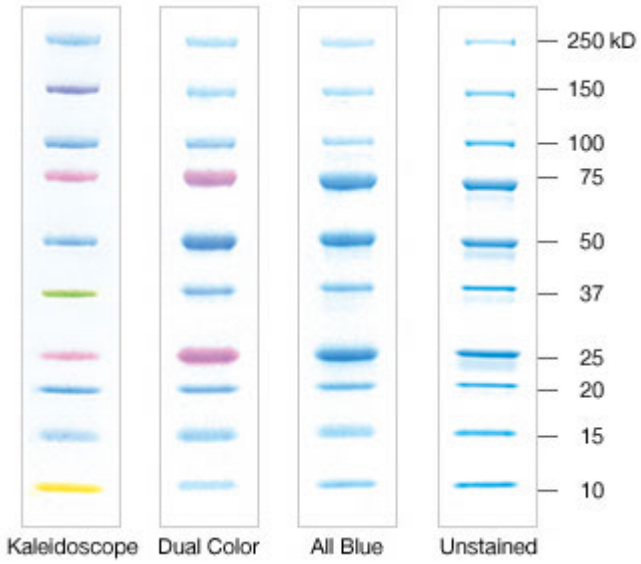


APPENDIX: 3 DNA and protein standards

1kb Plus DNA ladder



Precision Plus Protein Standard



APPENDIX: 4 Human Proteinase Activated Receptor 2

mRNA complete coding sequence

Taken from NCBI Accession : U34038

Size : 1193 bp

ATGcggagccccagcgcggcgtggctgctgggggccgccatcctgctagcagcctctctcctcTGCagtg
gcaccatccaaggaaccaatagatcctctaaaggaagaagccttattggtaaggttgatggcacatccca
cgtcactggaaaaggagttacagttgaaacagtcttttctgtggatgagttttctgcatctgtcctcact
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caacaactggatttatggggaagctcttTGTaatgtgcttattggctttttctatggcaacatgtactgt
tccattctcttcatgacctgcctcagtgtgcagaggtattgggtcatcgtgaaccccatggggcactcca
ggaagaaggcaaacattgccattggcatctccctggcaatatggctgctgattctgctggtcaccatccc
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gagcagctcttggtgggagacatgttcaattacttcctctctctggccattggggtcttt
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ctgcttcactcctagtaaccttctgcttgtggtgcattattttctgattaagagccagggccagagccat
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gcagatgcaagtatccctcacctcaaagaaacactccaggaaatccagctcttactcttcaagttcaacc
actgttaagacctcctatTGA

TGC	-	Cysteine 22 codon	TGT	-	Cysteine 148 codon
TGT	-	Cysteine 226 codon	TGC	-	Cysteine 361 codon
ATG	-	**Start Codon**	**TGA**	-	**Stop Codon**

Human Proteinase Activated Receptor 2 Amino-acid sequence

Taken from Swiss Prot Accession : P55085

Size : 397 aa

MRSPSAAWLL GAAILLAASL SCSGTIQGTN RSSKGRSLIG KVDGTSHVTG KGVTVETVFS
VDEFSASVLT GKLTTVFLPI VYTIVFVVGL PSNGMALWVF LFRTKKKHPA VIYMANLALA
DLLSVIWFPL KIAYHIHGNN WIYGEALCNV LIGFFYGNMY CSILFMTCLS VQRYWVIVNP
MGHSRKKANI AIGISLAIWL LILLVTIPLY VVKQTIFIPA LNITTCHDVL PEQLLVGDMF
NYFLSLAIGV FLFPAFLTAS AYVLMIRMLR SSAMDENSEK KRKRAIKLIV TVLAMYLICF
TPSNLLLVVH YFLIKSQGQS HVYALYIVAL CLSTLNSCID PFVYYFVSHD FRDHAKNALL
CRSVRTVKQM QVSLTSKKHS RKSSSYSSSS TTVKTSY

C	-	**Cysteine 22**	C	-	**Cysteine 148**
C	-	**Cysteine 226**	C	-	**Cysteine 361**
M	-	**Start**	Y	-	**Stop**

APPENDIX: 5 Sequencing Results for Selected Clones

$hPAR_2C22A$ Selected Clone Sequencing Results using T7 Primer

48..768 of trace file

Cysteine TGC -> Alanine GCC

GGTGGAATTCGATTCCAGGAGGATGCGGAGCCCCAGCGCGGCGTGGCTGCTGGGGGCCGC

CATCCTGCTAGCAGCCTCTCTCTCCGCCAGTGGCACCATCCAAGGAACCAATAGATCCTC

TAAAGGAAGAAGCCTTATTGGTAAGGTTGATGGCACATCCCACGTCACTGGAAAAGGAGT

TACAGTTGAAACAGTCTTTTCTGTGGATGAGTTTTCTGCATCTGTCCTCACTGGAAAACT

GACCACTGTCTTCCTTCCAATTGTCTACACAATTGTGTTTGTGGTGGGTTTGCCAAGTAA

CGGCATGGCCCTGTGGGTCTTTCTTTTCCGAACTAAGAAGAAGCACCCTGCTGTGATTTA

CATGGCCAATCTGGCCTTGGCTGACCTCCTCTCTGTCATCTGGTTCCCCTTGAAGATTGC

CTATCACATACATGGCAACAACTGGATTTATGGGGAAGCTCTTTGTAATGTGCTTATTGG

CTTTTTCTATGGCAACATGTACTGTTCCATTCTCTTCATGACCTGCCTCAGTGTGCAGAG

GTATTGGGTCATCGTGAACCCCATGGGGCACTCCAGGAAGAAGCAAACATTGCCATTGGC

ATCTCCCTGGCAATATGGCTGCTGATTCTGCTGGTCACCATCCCTTTGTATGTCGTGAAG

CAGACATCTTCATTCCTGCCCTGAACATCACGACCTGTCATGATGTTTTGCCTGAGCAGC

$hPAR_2C148A$ Selected Clone Sequencing Results using T7 Primer

39..661 of trace file

Cysteine TGT -> Alanine GCT

GGATCCACTAGTCCAGTGTGGTGGAATTCGATTCCAGGAGGATGCGGAGCCCCAGCGCGG

CGTGGCTGCTGGGGGCCGCCATCCTGCTAGCAGCCTCTCTCTCCTGCAGTGGCACCATCC

AAGGAACCAATAGATCCTCTAAAGGAAGAAGCCTTATTGGTAAGGTTGATGGCACATCCC

ACGTCACTGGAAAAGGAGTTACAGTTGAAACAGTCTTTTCTGTGGATGAGTTTTCTGCAT

CTGTCCTCACTGGAAAACTGACCACTGTCTTCCTTCCAATTGTCTACACAATTGTGTTTG

TGGTGGGTTTGCCAAGTAACGGCATGGCCCTGTGGGTCTTTCTTTTCCGAACTAAGAAGA

AGCACCCTGCTGTGATTTACATGGCCAATCTGGCCTTGGCTGACCTCCTCTCTGTCATCT

GGTTCCCCTTGAAGATTGCCTATCACATACATGGCAACAACTGGATTTATGGGGAAGCTC

TTGCTAATGTGCTTATTGGCTTTTTCTATGGCAACATGTACTGTTCCATTCTCTTCATGA

CCTGCCTCAGTGTGCAGAGGTATTGGGTCATCGTGAACCCCATGGGGCACTCCAGGAAGA

AGGCAAACATTGCCATTGGCAT

hPAR$_2$C226A Selected Clone Sequencing Results using C148 mutagenic primer

69..630 of trace file

Cysteine TGT -> Alanine GCT

ACCCCATGGGGCACTCCAGGAAGAAGGCAAACATTGCCATTGGCATCTCCCTGGCAATATGGCTGCTGAT
TCTGCTGGTCACCATCCCTTTGTATGTCGTGAAGCAAACCATCTTCATTCCTGCCCTGAACATCACGACC
GCTCATGATGTTTTGCCTGAGCAGCTCTTGGTGGGAGACATGTTCAATTACTTCCTCTCTCTGGCCATTG
GGGTCTTTCTGTTCCCAGCCTTCCTCACAGCCTCTGCCTATGTGCTGATGATCAGAATGCTGCGATCTTC
TGCCATGGATGAAAACTCAGAGAAGAAAAGGAAGAGGGCCATCAAACTCATTGTCACTGTCCTGGCCATG
TACCTGATCTGCTTCACTCCTAGTAACCTTCTGCTTGTGGTGCATTATTTTCTGATTAAGAGCCAGGGCC
AGAGCCATGTCTATGCCCTGTACATTGTAGCCCTCTGCCTCTCTACCCTTAACAGCTGCATCGACCCCTT
TGTCTATTACTTTGTTTCACATGATTTCAGGGATCATGCAAAGAACGCTCTCCTTTGCCGAAGTGTCCGC
A

hPAR$_2$C148A/C226A Selected Clone Sequencing Results using T7 primer

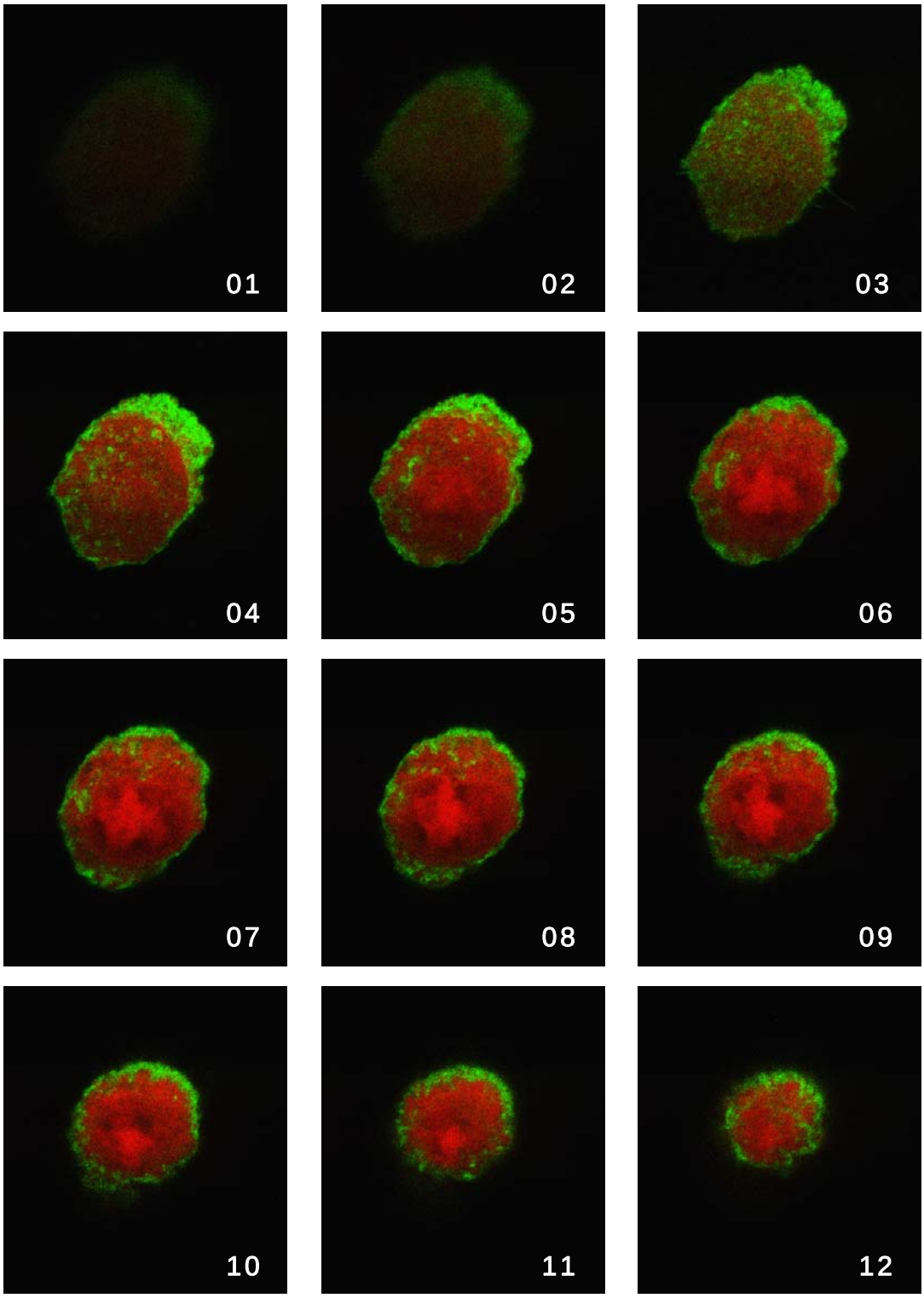
67..830 of trace file

Cysteine TGT -> Alanine GCT

CAGGAGGATGCGGAGCCCCAGCGCGGCGTGGCTGCTGGGGGCCGCCATCCTGCTAGCAGCCTCTCTCTCC
TGCAGTGGCACCATCCAAGGAACCAATAGATCCTCTAAAGGAAGAAGCCTTATTGGTAAGGTTGATGGCA
CATCCCACGTCACTGGAAAAGGAGTTACAGTTGAAACAGTCTTTTCTGTGGATGAGTTTTCTGCATCTGT
CCTCACTGGAAAACTGACCACTGTCTTCCTTCCAATTGTCTACACAATTGTGTTTGTGGTGGGTTTGCCA
AGTAACGGCATGGCCCTGTGGGTCTTTCTTTTCCGAACTAAGAAGAAGCACCCTGCTGTGATTTACATGG
CCAATCTGGCCTTGGCTGACCTCCTCTCTGTCATCTGGTTCCCCTTGAAGATTGCCTATCACATACATGG
CAACAACTGGATTTATGGGGAAGCTCTTGCTAATGTGCTTATTGGCTTTTTCTATGGCAACATGTACTGT
TCCATTCTCTTCATGACCTGCCTCAGTGTGCAGAGGTATTGGGTCATCGTGAACCCCATGGGGCACTCCA
GGAAGAAGGCAAACATTGCCATTGGCATCTCCCTGGCAATATGGCTGCTGATTCTGCTGGTCACCATCCC
TTTGTATGTCGTGAAGCAGACCATCTTCATTCCTGCCCTGAACATCACGACCGCTCATGATGTTTTGCCT
GAGCAGCTCTTGGTGGGAGACATGTTCAATTACTTCCTCTCTCTGGCCATTGGGGTCTTTCTG

APPENDIX: 6 Z Axis Confocal Imaging of wt-hPAR$_2$

Confocal Imaging Z axis scan at 100 nm intervals through Pro5 wt-hPAR$_2$ demonstrating cell surface expression.



APPENDIX: 7 Z Axis Confocal Imaging of hPAR$_2$C148A

Confocal Imaging Z axis scan at 100 nm intervals through Pro5 hPAR$_2$C148A demonstrating cell surface expression.