Characterisation of Proteinase-activated receptor-4 (PAR-4) Signalling in NCTC-2544 cells stably expressing PAR-4 (Clone 10H9) and PAR-4 expressing cells, EAhy-926

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Mary Francis Nilsson

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University of Strathclyde, Glasgow, UK

Copyright Statement

Abstract

Proteinase-activated receptor-4, (PAR-4), is the most recently identified member of a family of G-protein coupled receptors, which have a specific mechanism of receptor activation. This receptor undergoes proteolytic cleavage of the amino-terminus, thereby giving rise to a tethered ligand, which binds to the receptor, resulting in intramolecular activation. PAR-4 is endogenously activated by serine proteases such as thrombin, trypsin and cathepsin G and is also suitably activated by synthetic peptides which are derived from the tethered ligand sequence, which can activate the receptor without proteolytic cleavage. This study sought to determine the signalling characteristics of PAR-4 following its activation in NCTC-2544 cells, stably expressing PAR-4 (clone 10H9) and EAhy-926 cells. In particular, activation of NFκB and the MAP kinase pathways were assessed. Initial findings have demonstrated that PAR-4 is capable of stimulating the NFκB pathway in an IKK-independent manner, at the level of both p65 NFκB phosphorylation and NFκB-DNA binding in clone 10H9 cells, but not in EAhy-926 cells. This may suggest further investigation is required into the signalling characteristics of PAR-4 to determine if it can activate NFκB, or if this is possibly an artefactual consequence of overexpression of PAR-4 in a heterologous expression system. It has also been demonstrated that PAR-4 is capable of mediating phosphorylation of both ERK and p38 MAP kinase in both clone 10H9 and EAhy-926 cells. In addition to this, the study has included work to determine the effects of the the $G\alpha_{q/11}$ inhibitor, YM-254890 as well as EGFR tyrosine kinase inhibitor, AG-1478 to determine the mechanisms whereby PAR-4 couples to the NFκB and MAP kinase pathways. These inhibition studies demonstrated that PAR-4 stimulates the MAPK pathway, in part by coupling with $G\alpha_{q/11}$, while mediating transactivation of the EGFR, suggesting that in the case of the MAPK pathway, PAR-4 can mediate activation via both G protein-dependent and –independent mechanisms. Activation of NFκB has shown that whilst it utilises both $G\alpha_{q/11}$ and the EGFR, it is more likely to have effect in a redundant fashion, whereby loss of one mechanism is compensated by the other.

PUBLICATIONS

Journal Publications

Goon Goh, F., Sloss, C.M., Cunningham, M.R., Nilsson, M., Cadalbert, L. & Plevin, R. (2008). G-protein-dependent and -independent pathways regulate proteinase-activated receptor-2 mediated p65 NFkappaB serine 536 phosphorylation in human keratinocytes. *Cell Signal*. **20**:1267-174.

Goon Goh, F., Pei Yuen, Ng., Nilsson, M., Kanke, T., & Plevin, R. (2009). Dual effect upon Proteinase-Activated Receptor-2 (PAR-2)-Mediated Signalling by the novel peptide antagonist K-14585. *Br J Pharmacol*. **158**:1695-1704.

Sekiguchi, F., Nilsson, M., Cadalbert, L. & Plevin, R. (2010). $G\alpha_{q/11}$ -dependent and independent proteinase-activated receptor 4 stimulation of mitogen-activated protein kinase signalling pathways. **Article in preparation**.

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For Hugh Francis Gallagher
and
Douglas Reilly

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March 2010

Once upon a time, a God asked a great king, “What is the most amazing thing in the world”? The king replied, “That everyday, a million souls pass into the abode of death and yet none believes that they shall follow…”

(Crispian Mills & Alonza Bevan, 2007)

ABBREVIATIONS

Adv	adenovirus
ANOVA	analysis of variance
AP	activating peptide
APS	ammonium persulphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine 3', 5'-monophosphate
cDNA	complimentary DNA
DAG	diacylglycerol
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescence
ECL-2	extracellular loop-2
EGFR	epidermal growth factor receptor
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol-bis (β-aminoethyl ether) tetraacetic acid
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
GAP	GTPase-activating protein
GDP	Guanosine diphosphate
GM-CSF	granulocyte macrophage-colony stimulating factor
GPCR	G protein coupled receptor
G-protein	GTP-binding protein
GRK	G protein-coupled receptor kinase
GTP	Guanosine triphosphate
HEK	human embryonic kidney cell
HEPES	N-[2-hydroxymethyl] piperazine-N'-[2-ethaesulphonic Acid]

HRP	horseradish peroxidase
HLH	helix-loop-helix
HSF	heat shock factor
HSP	heat shock protein
HPAEC	human pulmonary artery endothelial cells
ICAM-1	intracellular adhesion molecule-1
IKAP	IKK complex-associated protein
IκB	inhibitory kappa-B
IKK	inhibitory kappa-B kinase
IL-8	interleukin-8
IP	inositol phosphate
IP_3	inositol 1,4,5-triphosphate
JNK	c-jun amino-terminal kinase
kDa	kilo-Dalton
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MEK	MAP kinase kinase
MEKK	MAP kinase kinase kinase
MMP	matrix metalloproteinase
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
MSK	mitogen and stress kinase
MyD88	Myeloid differentiation primary reponse protein-88
NEMO	NFκB essential modulator
NFκB	nuclear factor kappa-B
NIK	nuclear factor kappa-B inducing kinase
NLS	nuclear localistion sequence
NO	nitric oxide
NOS	nitric oxide synthase
PAF	platelet activating factor

PAGE	polyacrylamide gel electrophoresis
PAR	Proteinase-activated receptor
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PFU	plaque forming unit
PGE_2	prostaglandin E-2
PI3K	phosphatidyl inositol-3 kinase
PIP_3	phosphatidyl inositol 3,4,5-triphosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLA_2	phospholipase A-2
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonylfluoride
PTX	pertussis toxin
RGS	regulator of G protein signalling
RHD	rel homology domain
RIP	receptor-interacting protein
RNA	ribonucleic acid
ROI	reactive oxygen intermediate
ROS	reactive oxygen species
RSK	ribosomal S6 kinase
RTK	receptor tyrosine kinase
RT-PCR	reverse transcriptase-polymerase chain reaction
SAPK	stress activated protein kinase
SDS	sodium dodecyl sulphate
siRNA	small interfering RNA
STAT	signal transducer and activator of transcription
TEMED	N,N,N',N'-tetramethylenediamine

TNFα	Tumour necrosis factor α
TXA_2	thromboxane A-2
VCAM-1	vascular cell adhesion molecule-1
VEGF	vscular endothelial growth factor

AMINO ACID ABBREVIATIONS

Symbol	3-letter code	Full name
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Asparic
E	Glu	Glutamic
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
X	Xxx	Unknown/Any
Y	Tyr	Tyrosine

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CHAPTER 1
INTRODUCTION

1.1 INTRODUCTION

In order to govern cell activities and co-ordinate cellular functions, eukaryotic cells have evolved certain systems to help maintain the overall homeostasis of the particular organism. For this purpose, cells have evolved to receive extracellular information via specialised membrane receptors. Each receptor has its own classification, including ionotropic receptors, G protein coupled recepotors (GPCR), kinase-linked receptors and nuclear receptors, all of which have their own purpose in the generalised function of the cell. As such, via a process of ligand-receptor interaction, receptors serve as a communicatory mechanism, which is necessary for any changes associated with the intracellular environment.

1.1.1 The G protein coupled receptor superfamily

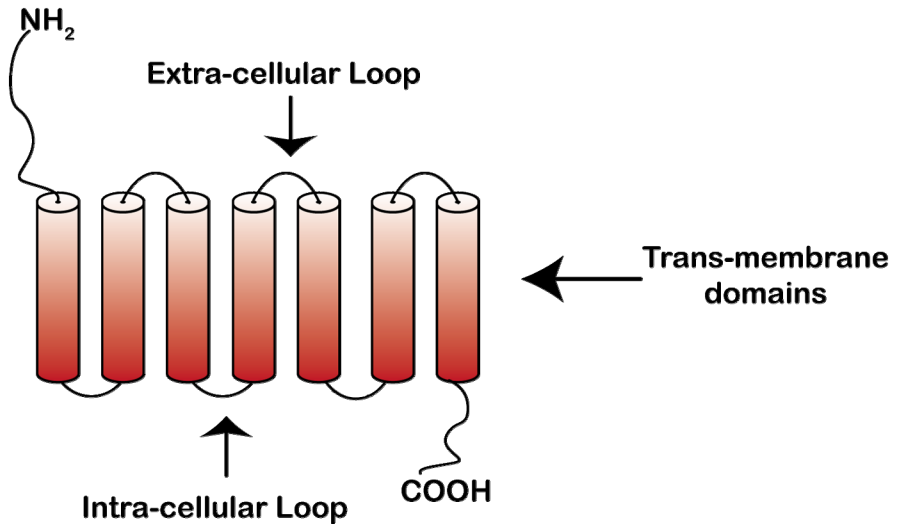
There have been several different families of membrane-bound receptors identified to date. These include kinase-linked receptors (Johnson & Ingram, 2005), cytokine receptors (Grotzinger, 2002) and ligand-gated ion channels (Colquhoun, 2006). The largest of all the receptor superfamilies is known as the family of Guanosine-nucleotide binding protein (G-protein) coupled receptors or GPCRs (reviewed by Davies *et al,* 2007).

The class of receptors which are coupled to G proteins are so far found to be in excess of 1000 proteins. One of the main conserved features that are common to all GPCRs includes a set of seven hydrophobic transmembrane spanning domains, which are found to be interconnected by three intracellular and three extracellular loops (Baldwin, 1993) (see figure 1.1). Providing an interesting focal point for study, GPCRs are found to be associated with the regulation of a wide range of physiological processes, mainly due to the variation of ligands that are available to bind to and activate these receptors. Some of these include biological molecules such as hormones, neuropeptides and proteases (Xing *et al*, 2009, Bounoutas *et al*, 2006, Sonmez *et al*, 2009, Yu *et al*, 2009). However, as these receptors are overwhelming in their structure and diversity, it has become necessary to devise a classification system which will allow for the GPCRs to be organised according to sequence homology, receptor function and ligand binding, (Foord *et al,* 2005).

1.1.2 Sub-classes of GPCR

The largest of these families, is known as class 1 or family A receptors, and is found to comprise the rhodopsin-like receptors. This particular family, due to the nature of its size, can be readily broken down into a further 6 subgroups. The first of these sub-groups include biogenic amine receptors, such as the adrenergic receptors, dopamine receptors and 5-HT receptors. (Reviewed by Gether, 2000). The conserved features of class 1 are found to be rather restricted. The most common conserved areas of these receptors include a small disulphide bridge which aids the connection between extra-cellular loops 2 and 3. The second most prominent feature is a palmitoylated cysteine residue, which is found in the carboxyl tail. This site is able to aid the formation of a putative fourth intracellular loop. Such specific areas of conservation suggest that these features are necessary for the structural and functional abilities of the receptor.

Class 2 receptors, otherwise known as family B, comprises of the secretin-like receptors, which includes glucagon and vaso-active intestinal peptide (VIP), receptors. Like class 1, class 2 receptors also have a conserved area of a disulphide bridge between extra-cellular loops 2 and 3. One of the more notable features of class 2 GPCRs is the characteristic amino terminus, which is found to contain several cysteine residues. It is thought that the purpose of these residues is to form disulphide bridges at intermediate points throughout the amino terminus (Ulrich *et al*, 1998). Class 3 receptors or family C, are another GPCR family which are found to be of pharmacological interest. One of the most distinctive features of this family of GPCR is the presence of an extremely long amino terminus, which is approximately 600 amino acids in length. Notably, the ligand binding site is located in the amino terminus, and as with class 1 and 2, class 3 receptors also have the conserved cysteine residues which give rise to the formation of the disulphide bridge, which links extra cellular loops 2 and 3. In addition, family C receptors are also found to have a unique feature, which is the presence of a highly conserved, but extremely short 3rd intracellular loop (Conn & Pinn, 1997)



(Adapted from Gether, 2000)

Figure 1.1 Diagrammatical representation of the features of a G protein coupled receptor (GPCR). The above diagram shows the features common to members of the GPCR super family of proteins. Each GPCR includes an extracellular amino (NH_2) terminus, seven membrane spanning domains, which are linked by the presence of extracellular and intracellular loops, as well as the presence of an intracellular carboxyl (COOH) terminus.

This receptor family is found to comprise of receptors such as the metabotropic glutamate receptors, taste receptors, γ-amino butyric acid ($GABA_B$) receptors and calcium-sensing receptors. Also included in the super-family of GPCRs are groups of less pharmacological significance, and as such, are not as well documented. These latter families, known as class D and E are found to consist of receptors relating to the fungal pheromone receptors and cAMP receptors. Finally, class F consists of non-mammalian receptors and bacteriorhodopsin receptors respectively (Wess *et al*, 1998, Horn *et al*, 1998).

The above classification system is not the only system available to researchers. A second model exists which serves to place GPCRs into 4 groups, categorised on the basis of the binding mode of the endogenous ligand of the receptor (reviewed by Ji *et al*, 1998). When using this classification system, the first group is found to consist of rhodopsin and biogenic-amine receptors, as these receptors require their ligand to bind to the trans-membrane core in order to become activated. However, other receptors, which require short-peptide ligands to bind at both the trans-membrane region and extra-cellular loops for their activation, such as the angiotensin receptors and formyl receptors form the second group within this particular classification system. Receptors such as VIP receptors or calcitonin receptors, while requiring ligand binding at the trans-membrane and the extra-cellular loops for their activation, fall into the third group of this classification system. This is because, unlike the receptors of the second group, which are activated by short-peptide ligands, the third group receptors are activated by long-peptide ligands (Ji *et al*, 1998).

Finally, the fourth group within this classification system consists of receptors which are found to be activated by initial binding of the ligand at the amino-terminal domain of the receptor. Following this, the amino terminus makes contact with the ligand binding domain of the extracellular loops, therefore activating the receptor. These include receptors such as glycoprotein-hormone receptors, neurotransmitter receptors and proteinase-activated receptors, which are the main focal point of this study.

1.1.3 G-proteins

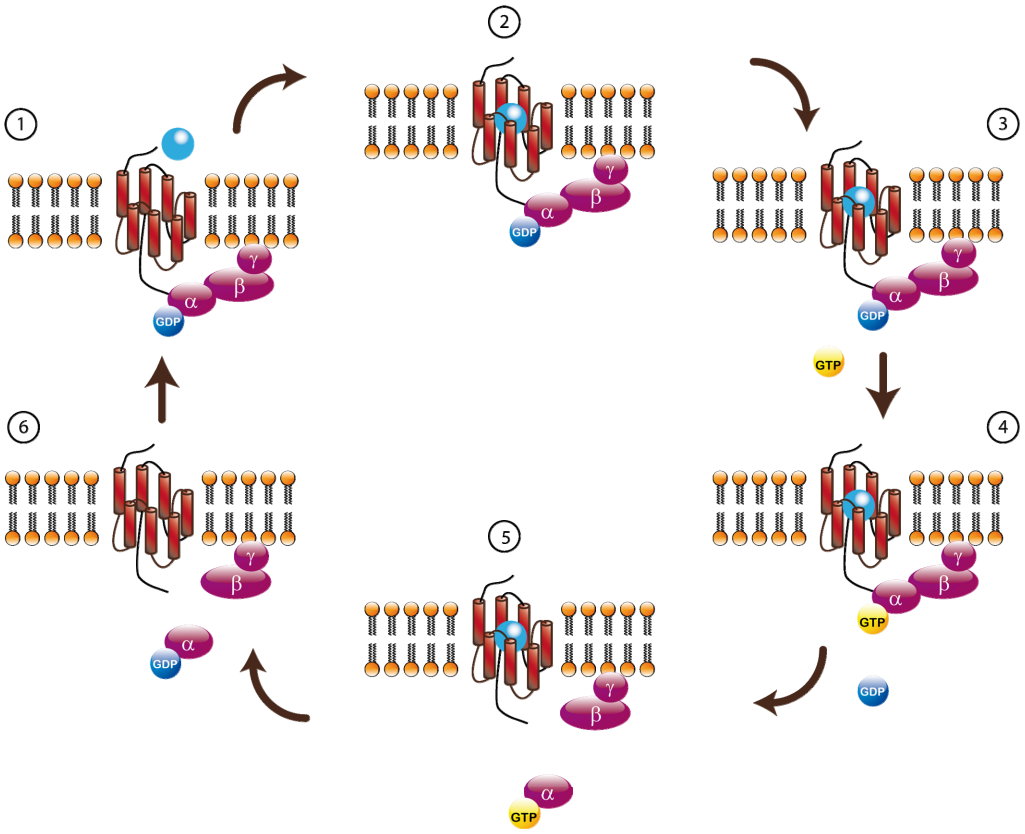
The pathways that are regulated by GPCRs are varied and abundant, and are responsible not only for transmitting signals from the external environment of the organism (eg. hormone secretion, olfactory sensors and light transmission) (reviewed in Hermanns, 2003), but they are also a means of relaying receptor-mediated signals originating from the membrane in order to stimulate intracellular signalling pathways. To carry out their specific functions, all GPCRs are found to be associated with a G-protein, with which its interaction is usually direct. G-proteins are extrinsic membrane proteins that are involved in second-messenger cascades. They are named as such as they act as a molecular switch mechanism, whereby they alternate between an inactive guanosine-diphosphate (GDP) bound state to an active guanosine-triphosphate (GTP) bound state. The term G-protein can be applied to two distinct families of protein, namely the heterotrimeric G-proteins, also referred to as "large G-proteins", or the monomeric G-proteins (small G-proteins), which are also referred to as GTPases (Bar-Sagi & Hall, 2000).

Heterotrimeric G-proteins consist of 3 sub-units, the first of which is known as G alpha (Gα). At approximately 39-46 kDa, this is the sub-unit which contains the binding site for the guanine nucleotides (reviewed by Hermanns 2003). To date, the α subunit has had at least 23 subtypes identified, which have been classified into 4 groups which include, $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{q12/13}$. The other units are known as G beta (Gβ), which is around 36 kDa, and G gamma (Gγ), which is the smallest of the sub-units at only 8 kDa. Together, beta and gamma form the βγ complex, which is found to associate tightly with the membrane. There have also been 6 Gβ and 12 Gγ subunits identified to date, all of which are associated in a selective manner with GPCR signalling. The function of heterotrimeric G-proteins can be demonstrated by the involvement of 2 co-dependent cycles which involve the exchange of guanine nucleotides and dissociation of the G-protein subunits. In an inactive state, the Gα subunit is found to be associated with the Gβγ subunit. At this point, the complex is normally found to be within close proximity of the GPCR. Upon activation of the receptor, a conformational change occurs, which allows the GPCR to become associated with its selective G protein. GDP, which is bound to Gα, becomes dissociated from the subunit and the GDP is exchanged for GTP.

The Gα-GTP subunit then dissociates from the Gβγ complex. Gα and Gβγ can then interact specifically with a vast array of effector molecules. Termination of the signal finally occurs when the Gα subunit, which has an intrinsic GTPase, catalyses the hydrolysis of GTP, to yield GDP, which results in the re-association of Gα with Gβγ, (Bourne *et al*, 1991, Hamm *et al*, 1998) (see figure 1.2).

This particular process is regulated by specialised proteins known as regulators of G-protein signalling (RGS) proteins. Initial characterisation of RGS proteins indicated that they had the ability to inhibit the signal transduction which is initiated by GPCRs. This is because they have demonstrated an ability to increase the intrinsic GTPase activity of heterotrimeric G proteins (Bansal *et al*, 2007). GTPase accelerating (GAP) activity mediates a more rapid deactivation of the G protein, therefore promoting desensitisation of the receptor (Blumer, 2004, He *et al*, 1998).

Early RGS studies determined the function of these proteins by assessing the mating response of the yeast known as *Saccharomyces cerevisiae* (Dohlman *et al*, 1998). This study which identified the RGS protein, SSt2p, demonstrated inhibition of pheromone-induced mating of the yeast, by binding directly to the Gα protein, subsequently increasing the GTPase activity of the subunit. This action resulted in the deactivation of the G protein (Dohlman *et al*, 1998). To date, more than 30 RGS proteins have been identified in mammalian cell types, with specificity for different Gα subtypes. Identification of RGS/Gα subunit selectivity has been shown by means which include RGS over expression studies. For example, over expression of RGS4 has successfully shown a decrease in receptor coupling with $G\alpha_{q/11}$ with various GPCRs, which include muscarinic receptors (Rumenapp *et al,* 2001), cholecytokinin receptors (Tovey & Willars, 2004) and bombesin receptors (Xu *et al*, 1999).



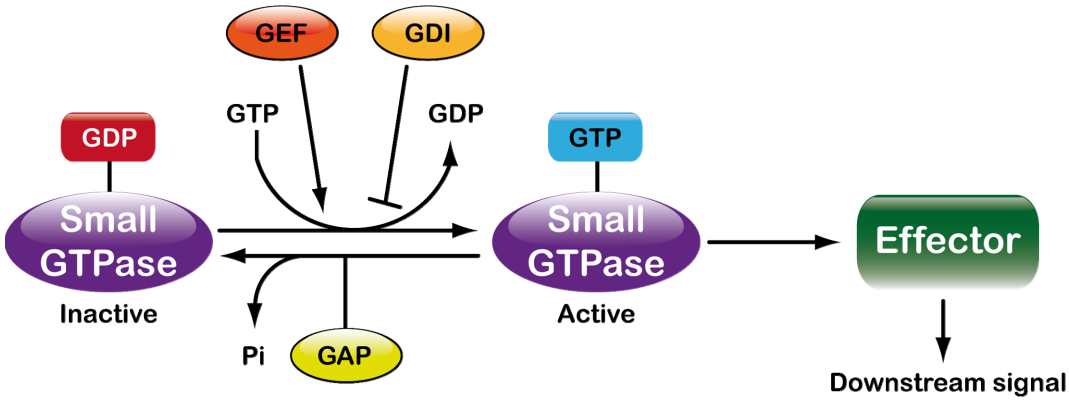
(Adapted from Rang, Dale, Ritter & Moore, 5th Edition, 2003)

Figure 1.2 Representation of interaction of a GPCR with a hetero-trimeric G protein. The above diagram represents the different stages whereby a GPCR interacts with a selective G protein in order to signal intra-cellularly. **1**. Prior to agonist binding, the hetero-trimeric G protein is in a GDP bound resting conformation. **2**. An agonist becomes bound to the GPCR mediating its activation, resulting in a conformational change. This allows for the receptor to act as a Guanine nucleotide exchange factor (GEF). **3**. The GEF then allows for the exchange of GDP for GTP on the Gα subunit. **4**. GTP now replaces GDP on the Gα subunit. **5**. Binding of GTP mediates the separation of the Gα subunit from the Gβγ complex. **6**. The Gα subunit mediates hydrolysis of the GTP, aided by a GTPase- accelerating protein (GAP), which is exchanged for GDP. This results in the rejoining of the Gα subunit with the Gβγ complex, which results in the heterotrimeric G protein returning to its resting conformation.

As previously mentioned, other than heterotrimeric G proteins, monomeric G proteins (small G proteins) have also been well documented (reviewed by Lundquist *et al* 2006, Wennerberg *et al*, 2005). The small G proteins are GTPases which are homologous to the alpha subunit of the heterotrimeric G proteins (see figure 1.3). The most abundant of these proteins are the Ras superfamily of GTPases (Wennerberg *et al*, 2005). This protein superfamily can be further classified into sub-families which include, Ras, Rho, Rab, Ran and Rheb (Munemitso *et al*, 1990). The Ras superfamily are the key regulators of a diverse range of cellular events, which include cell division, differentiation, vesicle transport and nuclear assembly (Lundquist *et al*, 2006). The following describes each of the subfamilies of small G proteins and their functions:

i. Ras – is a small GTPase, which is related in structure to the G_{α} subunit of heterotrimeric G proteins. As previously mentioned, when in the "off" state, it is bound to the nucleotide guanosine diphosphate (GDP). While in the "on" state, Ras is bound to GTP, which has an extra phosphate group compared to that of GDP. It is this extra phosphate which holds the two switch regions in a configuration similar to that of a loaded spring. When released, the switch regions mediate a conformational change into the activated state. Hence, activation and deactivation of Ras can be controlled by cycling between the active GTP-bound and inactive GDP-bound forms.

The process of exchanging the bound nucleotide is facilitated by guanine nucleotide exchange factors (GEFs) and GAP proteins. Ras has an intrinsic GTPase activity, which means that the protein on its own will hydrolyze the bound GTP into GDP. However this process is not fast enough to function in an efficient manner. Therefore, the GAP selective to Ras, known as RasGAP is able to bind to and stabilize Ras, thus supplying additional catalytic residues. A water molecule is then efficiently positioned for nucleophilic attack on the gamma-phosphate of GTP. An inorganic phosphate is released and the Ras molecule becomes bound to GDP. Therefore, GAP proteins are capable of mediating the regulation of Ras inactivation (Takai *et al*, 2001).



(Adapted from Takai *et al,* 2001)

Figure 1.3 Diagrammatical representation of the functions of monomeric G proteins (Small GTPase). Small G proteins are capable of independent function. As shown above, the protein when in the inactive conformation is GDP bound. GTP hydrolysis is helped by GTPase accelerating proteins (GAP), whereas GDP exchange is mediated by Guanine nucleotide exchange factors (GEF). Guanine nucleotide dissociation inhibitors (GDI) maintain small GTPases in the inactive state.

The function of Ras-regulated signal pathways are to control various cellular functions, including actin cytoskeletal integrity, cellular proliferation and differentiation, as well as cell adhesion and apoptosis. Unfortunately, in pathological conditions such as cancer. Ras and ras-related proteins are often over expressed, which in turn can lead to abberant cell division and cellular hyperplasia in the tissue involved (Wennerberg *et al*, 2005).

ii. Rho – a subfamily of the Ras superfamily, are small G proteins which are approximately 21kDa in size. The members of the Rho GTPase family have been shown to regulate many aspects of intracellular actin dynamics, and are found in all eukaryotic organisms including yeasts and plants. Rho proteins have been found to contribute to cellular proliferation, apoptosis and gene expression (Bouroux *et al*, 2007, Bustelo *et al*, 2007). The three general classes of regulating proteins which are associated with Rho signalling include GEFs, GAPs and another type of protein known as guanine nucleotide dissociation inhibitors (GDIs). As previously described, GEFs control the release of GDP from the protein, in this case, Rho, thus promoting the replacement with GTP. Like Ras, the GAPs control the ability of the GTPase to hydrolyze GTP to GDP, thus rate controlling the movement from active state to the inactive state. However, the final group of proteins, known as GDI proteins, are found to form a large complex with Rho, thereby preventing its diffusion from the membrane into the cytosol. Such proteins are therefore suggested to act as an anchor which allow for spatial control of Rho activation (Ellenbroek & Collard, 2007).

iii. Rab – Again, the Rab family of proteins is also a member of the Ras superfamily of monomeric G proteins. To date, there have been around 70 types of Rab proteins identified in humans. The Rab GTPases regulate various steps of membrane traffic, which have been found to include vesicle formation, vesicle movement along actin and tubulin networks, as well as membrane fusion. These processes make up the route through which cell surface proteins are trafficked from the Golgi to the plasma membrane and are recycled.

Rab proteins are characterised as peripheral membrane proteins, ie, they are anchored to a membrane via a lipid group which is covalently linked to an amino acid. In fact, Rabs are specifically anchored via prenyl groups on two cysteines in the C-terminus. Specialised proteins, known as Rab escort proteins (REPs) deliver newly-synthesized and prenylated Rab to its destination membrane by binding the hydrophobic, insoluble prenyl groups and carrying Rab through the cytoplasm. The lipid prenyl groups can then insert into the membrane, anchoring Rab at the cytoplasmic face of a vesicle or the plasma membrane (Stenmark & Olkkonen, 2001).

In the case of Rab, hydrolysis of GTP to GDP is also catalyzed by GAP proteins. REPs carry only the GDP-bound form of Rab. Rab effector proteins only bind the GTP-bound form of Rab. Rab effectors are very heterogeneous, and as such, each Rab isoform has many effectors through which it carries out multiple functions. Finally, a GDI protein binds the prenyl groups of the inactive GDP-bound form of Rab, and inhibits the exchange of GDP for GTP, thus preventing the reactivation of Rab thus delivering it to its original membrane location (Stenmark, 2009).

iv. Ran – is an abbreviation, which represents RAS-related Nuclear protein. Ran are small proteins, approximately 25kDa in size, and are found to be involved in transport into and out of the cell nucleus during interphase and also involved in mitosis. It is also a member of the Ras superfamily (Moore & Blobel, 1994, Avis & Clarke, 1996).

Ran is another type of GTP binding protein that is a necessary cellular component for the translocation of RNA and proteins through the nuclear pore. The Ran protein is involved in control of DNA synthesis and cell cycle progression. Nuclear localization of Ran requires the presence of regulator of chromosome condensation 1 (RCC1). Mutations in Ran have been found to disrupt DNA synthesis (Sazer & Dasso, 2000). In activation of this protien, RanGDP is converted into RanGTP through the action of RCC1, also known as RanGEF. Ran's intrinsic GTPase-activity is activated via the interaction with RanGAP, facilitated by complex formation with Ran-binding protein (RanBP). GTPase-activation leads to the conversion of RanGTP to RanGDP, hence completing the Ran cycle (Reviewed by Rensen *et al*, 2008).

As such, the Ras superfamily of GTPases demonstrate an extremely important role in the regulation of cellular functions, by promoting the regulation of molecular signalling, and controlling functions which are important to the overall homeostasis of the organism.

1.1.4 Role of G proteins in signalling pathways

To date, it has been demonstrated that Gα subtypes show considerable diversity in their ability to activate signalling in a variety of pathways by means of activating specific target enzymes, which in turn activate cellular mechanisms via their effectors (Milligan & Kostenis, 2006). A well characterized example of G protein interaction with a second messenger system is that of $G\alpha_{q/11}$. It is possible to understand the mechanism of operation of $G\alpha_{q/11}$ by assessment of the inositol-1-4-5, trisphosphate (IP_3) pathway (Ghosh *et al*, 2004). The exchange of GTP for a molecule of bound GDP on the Gα subunit results in its activation. These events mediate the concurrent activation of phospholipase-Cβ (PLCβ). PLCβ then mediates an increase of GTPase activity of its G protein activator, which in turn switches off the Gα-GTP signal. These events result in the cleavage and subsequent catalysis of phosphatidylinositol-4,5-bisphosphate (PIP_2), resulting in the generation of IP_3 and diacylglycerol (DAG) (Hubbard & Hepler, 2006).

Recruitment of the $G\alpha_s$ protein results in the activation of adenylyl cyclase (AC) (Ulens *et al*, 2004). Adenylyl cyclase is a membrane bound enzyme that is responsible for the conversion of ATP to 3', 5'-cyclic adenosine monophosphate (cAMP). An increase in the levels of cAMP leads to the activation of protein kinase A (PKA), a cAMP-dependent kinase (D'Angelo *et al*, 1997, Ulens *et al,* 2001). This in turn results in the regulation of lipid metabolism as well as regulation of glycogen. Over activation of $G\alpha_s$ proteins in particular have been associated with pathological conditions such as pituitary tumours (Radhika & Dhanasekaran, 2001). In such cases, activating mutations result in a consistent production of cAMP, thus resulting in abnormal cellular proliferation patterns. However when $G\alpha_{i/o}$ is stimulated, it results in the inhibition of AC, meaning that the overall production of cAMP will also be inhibited (Hurley, 1999).

While Gα activation is well recognised, its functions cannot always be assessed in isolation. It has been demonstrated that with the heterotrimeric group of G proteins, the physiological role of the Gβγ subunits also need to be taken into consideration. Originally, it was thought that the βγ subunits functioned to terminate Gα activation; but it has now been shown that this is not necessarily the case. Recent findings have shown that Gβγ can bind to effector molecules and result in their activation. These include several effector molecules normally under control of a variety of Gα subtypes, including adenylyl cyclase and PLCβ (isoforms 1-4 activation depends on the Gα family member) and (Hur & Kim, 2002, Hubbard & Hepler, 2006). This therefore suggests that G protein subunits result in a dual method of intracellular signalling due to the nature of the heterotrimeric group of G proteins.

1.1.5 Diversity of signalling via GPCR/G protein coupling

Due to the size and diverse nature of the GPCR family, it is hardly surprising that the cellular signalling is also complex. Through a variety of biochemical studies, it has been shown that specificities of various Gβγ subunits are able to interact in a specific fashion with specific Gα subunits, leading to signal transduction pathways of a very selective nature (Cabrera-Vera *et al*, 2003). It has also been found that a single GPCR is able to couple at a functional level with more than one GPCR (Kostenis *et al*, 2005). An example of this looks at the α_2-adrenergic receptor, which has been reported to couple with both $G\alpha_s$ and $G\alpha_{i/o}$ (Eason *et al*, 1992). Furthermore, there is emerging evidence that certain GPCRs are able to form homo- and/or heterodimers and it is not unusual to find them as part of a complex oligomeric structure (Terrillon & Bouvier, 2004). This type of receptor behaviour is regarded as a means of cross-talk and is of importance in many aspects of cellular regulation, including surface expression of the receptor and signalling specificities.

An example which helps to demonstrate GPCR oligomerisation is exemplified in the study of metabotropic $GABA_B$ receptors. Formed by heterodimerisation of $GABA_BR1$ and $GABA_BR2$ subunits, it is found that expression of $GABA_BR1$ without $GABA_BR2$ results in retention of the subunit in the endoplasmic reticulum (ER) (Margeta *et al,* 2000). However, whilst $GABA_BR2$ alone can show surface expression, it is not functionally active. Therefore, it would seem that expression of both $GABA_BR1$ and $GABA_BR2$ are inter-dependent and both are required to generate the expression of a functional receptor, as the binding of $GABA_BR2$ to $GABA_BR1$ (Kaupmann, 1997), results in masking of the ER retention motif, allowing it to travel to the cell surface (White *et al*, 1998).

1.1.5.1 GPCR mediated activation of the MAP kinase pathway

The diversity of GPCR agonist directed signalling can be attributed to the understanding of a variety of intracellular signalling pathways. For example, GPCRs have been attributed to the stimulation of certain pathways such as the mitogen-activated protein kinase (MAPK) pathway via G protein-dependent pathways, some of which include adrenergic receptors, proteinase activated receptors and bradykinin receptors. To date, several mechanisms which show GPCR mediated regulation of the MAP kinase pathway have been demonstrated. The MAP kinase pathway is associated with several intracellular regulatory mechanisms including growth and differentiation, inflammation and apoptosis. Currently, the MAP kinase pathway is found to consist of 5 subfamilies within mammalian cells. These subgroups consist of the extracellular signal-regulated kinase (ERK)-1 and 2, c-Jun amino terminal kinases (JNKs), p38 MAP kinase, ERK 3/4 and ERK-5 also known as big MAP kinase-1 (BMK-1) (Pearson *et al,* 2001).

Collectively, JNK, p38 MAP kinase and BMK-1 are known as the stress-activated protein (SAP) kinases. Mechanisms whereby GPCRs mediate stimulation of the MAP kinase pathway include G protein-independent mechanisms, including transactivation of the epidermal growth factor receptor (EGFR) as well as G protein-dependent mechanisms, such as second messengers (reviewed by Luttrell, 2008).

Generally, GPCR mediated activation of the MAP kinase pathway is dependent on the selectivity of the GPCR/G protein coupling and involves downstream effectors such as protein kinase C (PKC), cAMP or phospholipase C (PLC), or changes in intracellular calcium (Luttrell, 2008). It can also be suggested that these signalling components are found to encompass Ras or Raf within their pathway. Many studies that assess MAP kinase relate to receptor coupling with Gα subunits (Anger *et al*, 2007, Vazquez-Prado *et al*, 2003). The gonadotropin-releasing hormone receptor (GnRH), for example has been demonstrated to mediate the activation of the MAP kinase pathway via coupling with $G\alpha_{q/11}$(Caunt *et al*, 2006). Studies carried out in HeLa cells which had been transfected with xenopus GnRH receptors (XGnRHR) delinneated a pathway which resulted in production of PLC and an increase of intracellular Ca^{2+}. These events in turn stimulated the activation of a PKC-dependent pathway, which resulted in the activation of ERK via Raf and MEK. Interestigly, in comparison to type 1 human GnRH receptors, which lack the C terminus associated with other mammalian GPCRs, the XGnRHR underwent β-arrestin assisted internalisation – a protein which can also act as a scaffolding protein to facilitate ERK activation (Caunt et al, 2006).

Receptor cross-talk has also been attributed to GPCR dependent activation of the MAP kinase pathway. An example of this is the transactivation of the tyrosine kinase receptor known as the EGFR (reviewed by Luttrell, 2008), which has been shown to mediate activation of the MAP kinase pathway by activation of selected GPCRs including PAR-1 and PAR-2 (Darmoul *et al*, 2004a, Darmoul *et al*, 2004b). Such instances of an increase in EGFR and ERK activity can result in the over proliferation of particular cells, including breast cancer cells (Arora *et al,* 2008). Also in non-small cell lung carcinomas, an increase in EGFR and ERK activity has been associated with proliferation of the tumour (Lupi *et al*, 2007). In cases such as these the GPCR involved, induces the acivity of matrix metalloproteinases (MMPs), which in turn cleaves the heparin-binding EGF-like growth factor (HB-EGF) precursor. Following this, the HB-EGF binds to the extracellular domain of the EGFR, which activates the receptor, which in turn stimulates activation of ERK (Yan *et al,* 2002, Chansel *et al,* 2006).

Ligand -independent mechanisms work in a different fashion, whereby the involvement of intracellular components are required. Downstream effectors of GPCRs such as protein kinase C (PKC) and $pp60^{src}$ have been suggested to cause direct phosphorylation of the EGFR, therefore resulting in activation of the MAP kinase pathway (Slomiany & Slomiany, 2005), (see section 1.1.5.2).

As previously mentioned, another means of GPCR regulation of ERK is mediated via a specialised molecule known as β-arrestin (Luttrell *et al*, 2008). Initially characterised as a part of a conserved two-step mechanism for regulating the activity of GPCRs, β-arrestins are a small family of proteins which are important for the regulation of signal transduction (Moore *et al,* 2007, Lefkowitz & Shenoy, 2005). Arrestins can be found in an inactive conformation, where the molecules are found to be localized to the cytoplasm. Initiated by a response to an external stimulus, GPCRs interact with selective G proteins (see section 1.1.3). In order to regulate this response, or adapt to a persistent stimulus, activated receptors require an efficient mechanism with which to mediate desensitisaton. Initially in the desensitisation process, a class of serine/threonine kinases called G protein coupled receptor kinases (GRKs) mediate phosphorylation of the receptor. Phosphorylation of the receptor via GRK not only prepares the activated receptor for the binding of β-arrestin molecules, but also prompts a change in the conformational state of β-arrestin into an active state. As such, the arrestin molecules are recruited from the cytoplasm to the plasma membrane. Conformational change of β-arrestin mediates the release of its C-terminal, which contains both clathrin and adaptor-protein 2 (AP2) binding sites.

The binding of the arrestin molecule to the receptor prevents further G protein-mediated signalling, thus resulting in the targeting of the receptor for internalisation. This occurs by the receptor-arrestin complex having increased accessibility to clathrin-coated vesicles, which are membrane structures involved in receptor-mediated endocytosis. Once internalised, the clathrin-coated vesicle becomes uncoated and individual vesicles fuse to form the early endosome (Howe, 2005). Alternatively it can redirect signalling to alternative G protein-independent pathways (Gurevich & Gurvich, 2004).

Whilst β-arrestin has been successfully shown to mediate the desensitisation of GPCRs, it has also been shown to act as a scaffolding protein, which mediates the signalling pathway of molecules such as ERK (DeFea *et al*, 2000 a and b). As a scaffolding protein, β-arrestin ensures that the selective signalling components of the pathway being regulated are maintained in the proper location.

Further to this, β-arrestin ensures that the components of the signalling pathway are within the proper proximity of each other, thus ensuring adequate downstream activation of the pathway at hand. Finally, scaffold proteins ensure that there is specificity within signalling pathways, bringing into close proximity the specific kinases which will lead to the appropriate downstream signals. This co-localization may also enhance mutual interactions between the kinases and thus enhance signaling efficiency (DeFea, 2008, Pierce *et al*, 2001). Taking the angiotensin II receptor as an example (AT1aR), a study by Luttrell *et al* demonstrated that activated ERK can become co-localised with β-arrestin following receptor activation by angiotensin (Luttrell *et al,* 2001). This particular study initially demonstrated that the $AT_{1A}R$/β-arrestin complex was successfully redistributed to the endosomal compartments following stimulation with angiotensin. However, following these findings, the group determined by use of ERK tagged with red fluorescent protein (RFP) that ERK2 was also found to form part of the complex with the receptor and β-arrestin. Prior to stimulation by angiotenisn, anti-phospho ERK anti-bodies demonstrated that there was very little RFP-ERK2 available in the cell. However, following stimulation, whilst some RFP-ERK2 was found to have translocated to the nucleus, a high quantity was found to be localized within the endosomal vesicles, therefore suggesting that the angiotensin II mediated interaction of the AT_1AR/β-arrestin complex results in the production of activated ERK (Luttrell *et al*, 2001).

1.1.5.2 GPCR mediated activation of the epidermal-growth factor receptor (EGFR)

Whilst it is common for G protein coupled receptors to mediate their effects via coupling with selected G proteins and activation of second messenger systems, it is now well characterised that GPCRs can also stimulate the activation of cell signalling pathways via G protein-independent mechanisms. As previously mentioned, one such mechanism is by the GPCR mediated transactivation of the EGFR. GPCRs have been found capable of utilizing receptor-tyrosine kinases (RTKs) such as the EGFR to mediate important cellular processes, which include proliferation, differentiation and cell survival mechanisms (Piiper & Zeuzem, 2004). Interactions have been demonstrated by various receptors which include chemokine receptors (Kodali *et al*, 2006), P2Y1 receptor (Buvinic *et al,* 2007) and the angiotensin II receptor (Yahata *et al,* 2006). Assessment of the angiotensin II receptor found that it was a process selective to the Ang II type 1 receptor (AT1R) which was associated with the transactivation of the EGFR in a G protein-independent fashion (Yahata *et al*, 2006).

In a study using an *in vivo* $^{-/-}$AT1R murine keratinocyte model as well as primary human keratinocyte cultures (NHEK), this particular study by Yahata *et al*, 2006 demonstrated that angiotensin II (Ang II) can promote wound healing via transactivation of the EGFR. Initial result demonstrated a decrease of Ang II mediated wound healing in the $^{-/-}$AT1R model as opposed to the wild type murine model, which displayed a significantly higher level of wound healing when stimulated with angiotensin. The Ang II mediated wound healing was later demonstrated to be associated with cell migration which had been induced by HB-EGF-mediated transactivation of the EGFR. This is a process found to involve a mechanism, whereby activation of the AT1R results in the recruitment of a specialized group of MMPs known as ADAMs (A Desintegrin and Metalloproteinase), which have the ability to shed extracellular portions of transmembrane proteins (Wolfsberg *et al*, 1995).

Results obtained using a phospho EGFR antibody for Western blotting, demonstrated that addition of Ang II to cultured NHEKs resulted in phosphorylation of the EGFR – a process which was successfully inhibited by pre-treatment with the Ang II inhibitor valsartan. Later studies demonstrated the involvement of HB-EGF as the membrane bound ligand which mediated activation of the EGFR. Cell migration assay demonstrated that NHEK and skin fibroblasts were capable of migration when stimulated with Ang II. This particular cell migration was successfully inhibited by the introduction of an anti-HB-EGF antibody, therefore suggesting that the stimulation of the AT1R by Ang II mediated transactivation of the EGFR via the membrane shedding of HB-EGF.

This was further confirmed by the use of an HB-EGF specific inhibitor, CRM197, which also successfully resulted in the inhibition of cell migration. Final experiments performed in this particular study demonstrated that the MMP inhibitor GM6001 and EGFR-tyrosine kinase inhibitor, AG1478 both completely inhibited NHEK migration, therefore suggesting a specific transactivation pathway. This pathway begins by activation of the AT1R. This mediates activation of ADAMs, which results in the shedding of the membrane bound fraction of the HB-EGF ligand, which upon release is capable of mediating phosphorylation and activation of the EGFR (Yahata *et al*, 2006).

As previously mentioned, other GPCRs which have been demonstrated as capable of mediating transactivation of the EGFR include the $P2Y_1R$, which has been shown to mediate mitogenesis in epithelial cells via the EGFR (Buvinic *et al*, 2007). P2Y receptors are a family of purinergic receptors, G protein-coupled receptors stimulated by nucleotides such as ATP, ADP, UTP, UDP and UDP-glucose (Abbrachio *et al,* 2006). The $P2Y_1R$ has been demonstrated to have a high affinity for the nucleotide ADP (Chhatriwala *et al*, 2004, Takasaki *et al*, 2001). In this particular study, which has utlised both tumoral HeLa cells and FRT epithelial cells, it is demonstrated that $P2Y_1R$ is capable of mediating mitogenesis via transactivation of the EGFR (Buvinic *et al,* 2007). Immunoprecipitation studies demonstrated that activation of the $P2Y_1R$ by the synthetic ADP agonist, 2-MeSADP in both cell types resulted in the phosphorylation of the EGFR in a concentration-dependent manner.

Activation of ERK1/2 was also observed, therefore suggesting the importance of the EGFR as an upstream activator of the MAP kinase pathway. The study then utilised a variety of inhibitors, which included MRS2179 ($P2Y_1R$ inhibitor), AG1478 (EGFR tyrosine kinase inhibitor), PP2 (Src inhibitor) and Ilomostat (MMP inhibitor) and Ro318220 (PKC inhibitor) all of which demonstrated a concentration-dependent inhibition of $P2Y_1R$ mediated activation of the EGFR, as well as inhibition of ERK1/2. As a whole, these particular results suggest that $P2Y_1R$ mediated transactivation of the EGFR occurs via a specific pathway, whereby Src, PKC and MMPs will mediate the shedding of EGFR ligands from the cell membrane (Buvinic *et al,* 2007).

One other set of receptors which have been characterised as being capable of mediating transactivation of the EGFR include the chemokine receptors (Kodali *et al*, 2006). Chemokines are a type of GPCR which have been characterised as cytokine receptors and they are normally found on the surface of leukocytes (Murphy *et al*, 2000), however, the chemokine receptors CCR3 and CXCR4 are both found to be present in smooth muscle cells (SMC), and become upregulated and functional upon vascular injury (Li *et al*, 1999). In the case of vascular pathology, it has been demonstrated that MMP-2 is associated with the *in vivo* migration of SMC, which have been found to be a contributory factor in conditions including arterial remodeling and the formation of atherosclerotic plaques (Galis *et al*, 2002, Li *et al*, 1996). However, whilst many other studies concentrate on finding an MMP-dependent mechanism to determine a GPCR mediated pathway for the activation of the EGFR, this particular study has been conducted to determine if the EGFR is responsible for the production of MMP-2 (Kodali *et al,* 2006). In this particular case, a G protein-dependent mechanism was also demonstrated.

Using the chemokine receptor agonists, eotaxin and SDF-1, it was demonstrated by means of Western blotting, that both agonists are capable of inducing production of proMMP2 in both human and murine SMC. This proMMP2 production was found to be dependent on receptor coupling with $G\alpha_i$, as pre-incubation with PTx caused part inhibition of the proMMP-2 production, therefore suggesting the possibility of a second mechanism.

Therefore, immunoprecipitation studies demonstrated that both chemokine agonists were capable of mediating transactivation of the EGFR. Activation of the EGFR was successfully inhibited by means of pre-incubation with AG1478 and another EGFR inhibitor, PD153035. However, determination of the EGFR ligand responsible for the activation of the receptor demonstrated a difference in results. Whilst HB-EFG inhibitor CRM197 demonstrated inhibition of SDF-1 mediated production of proMMP2, there was no inhibition when stimulated by eotaxin. These results suggest that whilst SDF-1 can mediate transactivation of the EGFR via an extracellular ligand-dependent mechanism, eotaxin follows a ligand-independent route (Kodali *et al,* 2006). Taken together, these particular studies demonstrate that the EGFR can be successfully transactivated by the actions of a variety of GPCRs by mechanisms that are both G protein-dependent and – independent.

1.2 Nuclear Factor kappa B (NFκB)

Initially identified in the laboratory of David Baltimore, the transcription factor, Nuclear Factor kappa B (NFκB) is a transcription factor important for the transcription of immunoglobulin kappa light chain in B lymphocytes (Sen & Baltimore, 1986). Since this initial identification, it has become apparent that NFκB is also a ubiquitously expressed cytoplasmic signalling molecule, which upon activation, is capable of nuclear translocation and subsequent transcription of selective target genes.

1.2.1 General structure of NFκB

NFκB belongs to a group of proteins collectively known as Rel. To date, five members of this group have been identified in eukaryotic cells, known as p50 (NFκB-1), p52 (NFκB-2), p65 (Rel A), Rel B and c-Rel (reviewed in Ghosh *et al,* 2008), which can be selectively sub-divided into two distinct groups. The first of these groups includes the proteins which are synthesised as fully functional proteins, which are p65 (Rel A), Rel B and c-Rel. The second group employs the other proteins, namely p50 and p52, which are produced *in vivo* as their precursor proteins, p105 and p100, and require proteolytic cleavage in order to produce the active molecules (reviewed by Perkins & Gilmore, 2006).

NFκB is found to exist in many cell types, and is normally found as homo- or hetero-dimers, which share a highly conserved 300 amino acid long amino terminus. This is known as the Rel-Homology Domain (RHD). Within the RHD not only are the DNA-binding and dimerization domains to be found, but also the Nuclear Localisation Sequence (NLS), which becomes apparent upon the proteosomal degradation of IκBα (reviewed by Ghosh *et al,* 2008). The first heterodimer to be identified was p65/p50. This is the most abundant form of NFκB dimer and as such is classically referred to as NFκB, although further forms of dimer have been identified (Ghosh & Baltimore, 1990).

Specific DNA-binding sites for NFκB are found to have a decameric sequence, identified as 5'-GGGRNNYYCC-3', referred to as κB sites (Kunsch *et al*, 1992, Parry & Mackman, 1994). This particular sequence has been identified in the promoter region of various inducible genes, which can give rise to a variety of pathological conditions which NFκB has been found to be associated with. However, each individual NFκB dimer is found to have distinct DNA-binding specificity and transactivation potential, (Ghosh *et al*, 2008, Perkins & Gilmore, 2006). For example, p65 NFκB, otherwise known as Rel A, contains two transactivation domains (TADs). TAD1 found within the terminal 30 amino acids (521-551), whilst TAD2 is found to be located within the adjacent 90 amino acid residues (430-520) (Schmitz *et al*, 1995). Published evidence has suggested that phosphorylation of p65 NFκB in the TAD regions serves as a method of post-translational modification in order to regulate transcriptional activation (Viatour *et al,* 2005). So far, several inducible phosphorylation sites have been identified in p65 NFκB, of which the most commonly studied is serine 536 (Viatour *et al*, 2005, Oh *et al*, 2007).

1.2.2 Inhibitory Kappa B (IκB)

Under normal physiological conditions, the inactive form of NFκB is found to be located within the cytoplasm, where it is physically sequestered by an inhibitory protein known as Inhibitory Kappa B (IκB). It is the presence of the IκB proteins that results in the masking of the NLS, rendering NFκB incapable of translocating to the nucleus (Beg *et al*, 1992).

Like NFκB, IκB also comprises a conserved family of structurally related proteins, which to date is found to include IκBα, IκBβ, IκBε, IκBγ and Bcl-3 (Ghosh *et al*, 2008). All of the IκB subtypes include a domain which encode multiple copies of ankyrin motifs, which are known by the term ankyrin repeats. These are responsible for the protein-protein interaction whereby IκB can bind to the RHD of NFκB (Ghosh *et al*, 2008). It has been found that different IκB subtypes bind in a selective manner to the varied NFκB heterodimers, for example, IκBα and IκBβ are found to predominantly interact with the classical p50/p65 dimer as well as the p50/c-Rel dimer, whilst other subtypes such as IκBε is found to interact with dimers containing p52 (Whiteside *et al*, 1997, Thompson *et al*, 1995).

The intial IκB protein to be identified was IκBα (Ishikawa *et al,* 1995). Of the IκB subtypes that have been identified, IκBα is probably the most well characterised of this family. Activation of IκBα involves a series of phosphorylation events, followed by ubiquitination and proteosomal degradation (Traenckner *et al*, 1995). The normal series of events which have been indentified include the specific phosphorylation of serine residues 32 and 36 by the upstream kinase inhibitory kappa B kinase β (IKKβ) (Traenckner *et al*, 1995). Following phosphorylation, the ubiquitination of the two neighbouring amino-terminal lysine residues, lysine 21 and 22, occurs (Scherer *et al*, 1995). These events mediate rapid proteosomal degradation of IκBα, which unmasks the NLS of NFκB. In the absence of IκBα, NFκB is no longer sequestered in the cytoplasm and is free to translocate into the nucleus where it regulates the transcription of its selected target genes (Yaron *et al,* 1997, Mercurio *et al*, 1997).

1.2.3 Inhibitory kappa B kinase (IKK) and IKK kinase

As mentioned in section 1.2.2, phosphorylation of IκBα occurs at selective serine residues, and occurs by means of an upstream kinase known as IKKβ. This particular kinase belongs to a signalosome complex which is known as the IKK complex, which comprises of three subunits, namely; IKKα (IKK1), IKKβ (IKK2) and IKKγ (Ghosh *et al*, 2008, Perkins & Gilmore, 2006, Mercurio *et al*, 1999).

IKKα (85kDa) and IKKβ (87kDa) are the subunits of the complex which are catalytically active and are found to share approximately 52% sequence homology (Mercurio *et al,* 1997, Woronicz *et al*, 1997). IKKα and IKKβ are ubiquitously expressed in most eukaryotic cells, and both share a common feature whereby each of the proteins have an amino-terminal kinase which are flanked by a leucine zipper (LZ) region, which allows for IKKα and IKKβ to form either homo- or heterodimers (Mercurio *et al* 1997, Zandi *et al,* 1997). Also present at the C-terminus is helix-loop-helix (HLH) domain, (Ghosh *et al*, 1998), which has proven to be important for the activation of the IKK complex (Zandi *et al*, 1997).

With the alternative names of NFκB essential modulator (NEMO) or IKK-associated protein 1 (IKKAP1), IKKγ is the regulatory component of the IKK complex (Rothwarf *et al*, 1998, Mercurio *et al*, 1999, Mercurio & Manning, 1999). Activation of the IKK complex can be mediated by other molecules, including PKC (Sanz *et al*, 1999, Lallena *et al*, 1999, Tojima *et al*, 2000), as well as NFκB-inducing kinase (NIK), which is a member of the MEKK family (Malinin *et al,* 1997). Other molecules responsible for the activation of the IKK complex include MEKK-1-3 (Mercurio *et al*, 1997, Zhao & Lee, 1997, Hirano *et al,* 1996), as well as Akt (Ozes *et al*, 1999) (see section 1.2.4). As previously mentioned, the activation of NFκB occurs by proteolytic cleavage of IκBα. Upstream of IκBα, functional IKKα or IKKβ mediate the phosphorylation of IκBα, which is thought to be ubiquitinated by an E3 ubiquitin ligase, which is part of a cascade of ubiquitin-conjugating enzymes (Hershko *et al,* 1992). Therefore, by IKK mediated phosphorylation of the IκBα subunit, it is possible for IκBα to become degraded by the 26S proteosome (Alkalay *et al,* 1995), where unmasking of the NLS allows for the nuclear translocation of NFκB.

1.2.4 The three distinctive pathway of NFκB activation

It has taken many years to elucidate that there are at least three intracellular mechanisms which can mediate NFκB activation. These pathways are known collectively as the classical, alternative and atypical pathways (Viatour *et al*, 2005).

i. The Classical Pathway

The classical pathway, otherwise known as the canonical pathway, was the initial pathway to be characterised. Activation of the classical pathway can be achieved by means of inflammatory mediators, which include LPS, IL-1 and TNFα, (Zhang *et al,* 2000, Endo *et al,* 2009, Soloff *et al*, 2006). TNFα for example, results in activation of NFκB by the molecule of TNFα binding a trimerized TNF protein to one of two cell-surface receptors, known as p55 (TNFR-1, TNFRSF1A) or p75 (TNFR-2, TNFRSF1B) (Gilberts *et al*, 2004, Ding & Yin, 2004). Activation of the TNF receptor then results in the recruitment of the TNF receptor associated death domain (TRADD). TRADD in turn recruits TRAF-2 along with a serine/threonine kinase known as the ring-finger interacting protein (RIP). It is thought that TRAF-2 is the protein responsible for binding to and recruiting IKKβ thus mediating its activation. This mediates IKKβ–dependent phosphorylation of IκBα at serine 32 and 36 (Hacker & Karin, 2006, Karin & Ben-Neriah, 2000). Subsequently, upon phosphorylation, IκBα becomes ubiquitinated and undergoes proteosomal degradation. As NFκB is no longer sequestered by IκBα, it is able to translocate to the nucleus where it initiates the transcription of its target genes (Mercurio & Manning, 1999) (see figure 1.4).

ii. The Alternative Pathway

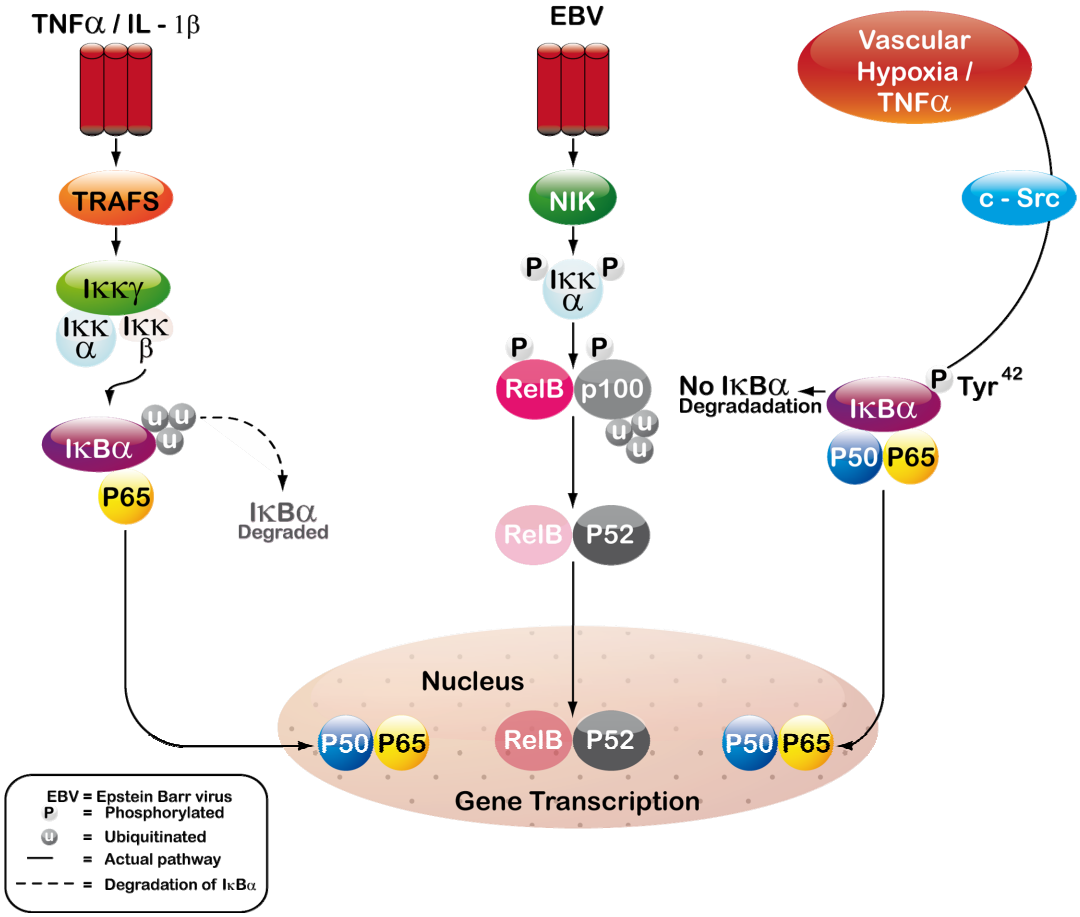
The alternative pathway, or non-canonical pathway, is found to be activated by various stimuli. Some of these include viral activators, such as the human T-cell leukaemia virus or Epstein - Barr virus (EBV) (reviewed by Chuang *et al*, 2007) (see figure 1.4). Other means of activation include cytokines such as B-cell activating factor (BAFF) and CD40 (Brink, 2006, Dejardin, 2006). These cytokine activators mediate the activation of TRAF proteins, subsequently resulting in the activation of NIK. It is the activation of NIK which mediates the activation of the IKKα subunit of the IKK signalosome. Activation of IKKα mediates a site-specific phosphorylation of C-terminus of the pre-cursor protein, p100. Found within the C-terminus of p100 are the ankyrin repeats normally found within the IκB proteins. As such, phosphorylation of p100 by IKKα results in the processing of p100 into the resultant product of p52.

Forming a heterodimers with Rel-B, the p52/Rel-B heterodimer translocates to the nucleus in order to regulate gene transcription (Claudio *et al*, 2002, Coope *et al*, 2002, Eliopoulos *et al*, 2003, Perkins & Gilmore, 2006).

iii. The Atypical Pathway

When referring to the atypical pathway, it is notable that there is more than one method of activation. However, the best characterised method of atypical activation suggests an IKK-independent method, which results in tyrosine phosphorylation of IκBα on tyrosine 42 as opposed to the classical residues, serine 32 and 36 (see figure 1.4). Stimuli other than cytokines and viral components are required for the activation of this pathway.

These include hypoxia and reperfusion injury, as well as induction by hydrogen peroxide or tyrosine phosphatase inhibitor pervanadate (Schoonbroodt *et al,* 2000, Imbert *et al*, 1996, Mukhopadhyay *et al*, 2000). Interestingly, phosphorylation of IκBα at Tyr^{42} results in translocation of NFκB without any degradation of the IκBα protein (Bui *et al,* 2001, Yakovlev *et al*, 2007, Sethi *et al,* 2007).



(Adapted from cellsignal.com)

Figure 1.4 Representation of the canonical, non-canonical and atypical activation of the NFκB pathway. The above diagram represents the pathways involved in varied types of NFκB activation. It demonstrates the differences in the chosen pathway, and translocated dimers depending on the type of stimulus.

1.2.5 PI3K/Akt regulation of NFκB

It has been demonstrated that Akt/PKB (protein kinase B), is a serine/threonine kinase family, which have been found to be associated with various cellular functions, including cellular proliferation as well as cell survival (Gang-Song *et al,* 2007, Kane *et al*, 1999). Comprising of three known family members, Akt/PKB consists of Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ. Of these three family members, Akt1/PKBα has found to be involved in the regulation of cell survival pathways whereby it works to inhibit the process of apoptosis, as well as mediate protein synthesis (reviewed by Kim & Chung, 2002). Akt1 has been found to contribute not only to tissue hypertrophy, but due to its anti-apoptotic properties and ability to mediate cell proliferation, Akt1 has also been implicated as a contributing factor of various cancerous growths (Staal *et al,* 1977). Akt has been shown to be successfully activated by means of PI3K, which itself is a well characterised upstream regulator of cell survival as well as cellular trafficking and cell transformation (Varticovski *et al*, 1989). The most abundant form of PI3K exists as a heterodimer of a catalytic subunit and a regulatory subunit, and can be activated by a variety of membrane bound receptors (reviewed by Fruman *et al*, 1998).

These receptors, including growth factor receptors, are normally activated by means of tyrosine autophosphorylation, following which they recruit PI3K, which becomes localised to the plasma membrane (Mandelker et al, 2009, Procko & McCall, 2005). Upon reaching the plasma membrane, PI3K phosphorylates membrane bound phosphoinositides, thus generating production of PIP_2 and PIP_3. Generation of PIP_2 and PIP_3 results in the PI3K-dependent activation of Akt (Franke *et al*, 1997, Stokoe *et al*, 1997). This activation of Akt is thought to occur not only by recruiting Akt to the plasma membrane, but also by recruiting a protein known as 3-phosphoinositide dependent protein-kinase-1 (PDK1), a known activator of Akt, to the membrane. This recuitment of both Akt and PDK1 is thought to occur as they have a specialised region known as the Pleckstrin Homology (PH) domain, a region which has a particularly high affinity for both PIP_2 and PIP_3 (Alessi *et al,* 1997).

Once PDK1 and Akt are localised at the membrane, the binding of Akt with PIP_2 and PIP_3 results in a conformational change in Akt, which allows it to become phosphorylated by PDK1 and a second protein, PDK2, thus resulting in the activation of Akt (Alessi *et al*, 1997, Stokoe *et al,* 1997).

As mentioned, Akt is activated by PI3K activity, and becomes a regulator of cellular growth and apoptosis. It achieves this by being able to phosphorylate oncogenic targets, whereby promoting cell growth or cell death. However, it can be inhibited by a tumour supressor known as PTEN (Hay, 2005, Dan *et al*, 2007), inhibition of which results in the activation of a molecule known as mTOR (mammalian target of rapamycin) (Dreyer *et al*, 2009, Jiang *et al*, 2009). mTOR itself controls cell growth by means of phosphorylating the mRNA regulators S6K and 4EBP1 (Dan *et al,* 2007). mTOR control is regulated by Akt-mediated phosphorylation of TSC-2 (tuberous sclerosis complex-2) (Roux *et al*, 2004). TSC1 and 2 together result in the formation of a Rheb GTPase inhibitor, thus preventing the inhibition of mTOR (Hodges *et al,* 2001, Aicher *et al*, 2001). Interaction with another regulatory protein known as Raptor results in the formation of another complex known as rapamycin-sensitive complex-1 (TORC1), helping to control cellular functions downstream of mTOR.

However, a second mTOR complex, containing a protein known as Rictor (TORC2), has been shown to possibly provide PDK2 activity, which is required for the phosphorylation of Akt at serine 473 (Hresko *et al*, 2003, Chan & Tsichilis, 2001). Therefore, taking these molecular interactions into consideration, it has been well characterised that Akt is shown to be highly expressed in many cancers, and as it has been shown to mediate both pro- and anti-apoptotic properties, it can be suggested that Akt is capable of control of the transcription factor, NFκB (Hay, 2005). It has, in fact, been demonstrated that Akt is capable of mediating the phosphorylation of p65 NFκB, and its subsequent nuclear translocation, via a pathway that involves IKKα, and mTOR (Dan *et al*, 2007, Dan *et al*, 2008, Sizemore *et al*, 2002, Gustin *et al,* 2004).

The study by Dan *et al*, 2007 demonstrated this particular pathway in prostate cancer cells that were nulled for PTEN. This particular study also investigated the ability of Akt to activate mTOR in an IKKα-dependent fashion. In order to demonstrate that Akt required IKKα for the activation of mTOR, this particular group employed a $^{-/-}$IKKα mouse embroyonic fibroblast (MEF) cell line, which were transfected with constitutively active Akt. For this purpose, the phosphorylation of S6K was investigated by means of Western blotting. These experiments determined that in $^{-/-}$IKKα cells, Akt resulted in poor phosphorylation of S6K. However, upon re-introduction of active IKKα into these particular cells, successful Akt mediated phosphorylation of S6K was demonstrated. As S6K is under the control of mTOR, which in turn is activated by Akt, these results may suggest the relationship between Akt and mTOR occurs by an IKKα–dependent mechanism (Dan *et al*, 2007).

This relationship was later found to extend beyond the regulation of IKKα, and it was demonstrated by the same group in a follow-on study, that the Akt/mTOR pathway was also capable of mediating the activation of p65 NFκB(Dan *et al,* 2008). Using PTEN inactive prostate cancer cells, Akt is capable of mediating the activation of NFκB via stimulation of IKKα. One way in which this particular group investigated the relationship between the Akt pathway and the NFκB pathway was to determine whether or not NFκB-dependent genes were controlled by the mTOR/Raptor complex in the prostate cancer cells. In order to achieve this, selective siRNA for either mTOR or Raptor were employed, and NFκB dependent genes Bcl-2, cIAP1, IκBα and cyclin D1 were measured by PCR.

Results indicated that the mTOR siRNA demonstrated a loss of NFκB-dependent gene expression of approximately 30-40%, whilst loss of Raptor resulted in a loss of around 50% of these genes. Taken together, these results display that mTOR and Raptor do mediate NFκB-dependent gene expression – a pathway suggesting the involvement of Akt (Dan *et al*, 2008). Further to these finding, this particular group also ascertained that mTOR and Raptor are also capable of control of both NFκB DNA-binding and phosphorylation of p65-NFκB in prostate cancer cells.

Again, siRNA for mTOR and Raptor were used to determine these perameters. Analysis of nuclear extracts by means of electrophoretic mobility shift assay (EMSA), demonstrated that nuclear binding of p65 NFκB was reduced by both Raptor and mTOR siRNA. Later EMSA studies, whereby mTOR and Raptor were re-transfected into the prostate cancer cells demonstrated an increase in nuclear binding, which was successfully inhibited by Rapamycin. Again, taken together, these results suggest that mTOR and Raptor are capable of mediating control of NFκB at various levels within these particular cells. Examination of whole cell extracts, which were examined by Western blotting, demonstrated that phosphorylation of p65 NFκB at Ser^{536}, along with correlating phosphorylation of IκBα was also apparent. Knockdown of several key components of the Akt pathway, which included knockdown of Akt and IKKα, along with the PI3K inhibitor LY294002 and mTOR inhibitor Rapamycin all demonstrated a reduction in both the phosphorylation of p65 NFκB and IκBα. Taken together, these particular studies have demonstrated a pathway mediated by Akt in regulation of the NFκB pathway.

1.2.6 Cellular importance of NFκB

NFκB has been reported to transcribe a wide variety of genes, which include many cytokines such as IL-1, IL-6, IL-8, TNFα, as well as adhesion molecules such as ICAM-1, VCAM-1 and E-selectin (Nam *et al*, 2009, Song *et al*, 2009, Chiu *et al*, 2007, Reed *et al*, 2005). From these examples, it is suggested that NFκB has a regulatory role in the transcription of inflammatory mediators, therefore, the role of NFκB can be exemplefied by assessement of inflammatory disorders associated with the body (Hayden & Ghosh, 2004). However, NFκB has also been associated with mediating processes such as cell growth and apoptosis, as well as responding to viral infiltration (Gato *et al*, 2008, Naijar *et al*, 2005, reviewed by Sen, 2006).

In studies carried out on p50 and c-Rel knockout mice, it was determined that certain members of the NFκB family are necessary for inflammatory or immune responses. The results indicated that mice null for these NFκB subtypes were found to develop abnormal B-cells and T-cells. The immune system of these animals failed to defend the host organism against bacterial infections (Ghosh *et al,* 1998). NFκB has also been found to be associated with inflammatory diseases such as Crohn's disease (Gelbmann *et al*, 2003), asthma (Hewitt *et al*, 2008) and psoriasis (McKenzie & Sabin, 2003). Studies carried out in p65 NFκB knockout mice demonstrated hepatic difficulties, whereby the murine hepatocytes underwent apoptosis. These findings suggested that while NFκB has been linked to inflammatory responses, it is also necessary for cell survival (Beg *et al*, 1995). However, it has been shown to be apoptotic in a variety of cells including endothelial cells, B cells and T cells (Shishodia & Aggarwal, 2002).

It has also been demonstrated that several members of the NFκB family are oncogenic (Gilmore *et al*, 2004). One study in particular has shown that c-Rel results in cell transformation while still in culture. Activation of c-Rel occurs via a retroviral promoter insertion found in avian B-cell lymphoma, therefore, it is not uncommon for large quantities of c-Rel to be found in conditions such as Hodgkin's lymphoma and diffuse large B-cell lymphomas (Gilmore *et al*, 2004). As such it can be suggested that the diversity of NFκB is wide and varied and is found to be an important regulator of both physiological and pathological responses, and such regulation may be due to the ability of NFκB to mediate these events with such specificty due to the properties of the varied NFκB dimers.

1.2.7 GPCR mediated regulation of NFκB

Regulation of NFκB by GPCRs is an area that has not been well documented or fully defined. However, certain studies have emerged which demonstrate the ability of certain GPCRs as being capable of regulating this transcription factor (see figure 1.5).

Evidence to support GPCR mediated activation of NFκB include Kaposi's sarcoma herpes virus (KSHV)-GPCR (Dadke *et al,* 2003). In this particular study, it was found that transformation of the KSHV-GPCR required the activation of Rac-1, which is a small G protein, along with the effector, p21-activated kinase (Pak-1). Furthermore, it was shown that KSHV-GPCR induced transformation of the cell was dependent on NFκB-induced transcriptional activity. Dominant negative forms of the Pak-1 effector demonstrated inhibition of transcriptional activity. However, wild type Pak-1 resulted in the phosphorylation of IKKβ, showing that in this case, the KSHV-GPCR mediated NFκB activation occurs via an IKK-dependent mechanism (Dadke *et al*, 2003).

Other G protein coupled receptors that are found to be involved in the regulation of NFκB activity include the bradykinin receptor (Pan *et al,* 1996 – see chapter 5) and proteinase-activated receptors, including PAR-1 (Rahman *et al*, 2002), PAR-2 (Kanke *et al*, 2001) and PAR-4 (Suo *et al*, 2003). The study by Dadke *et al*, 2003 which examined the way in which the KSHV-GPCR administered transcriptional control of NFκB, suggests that activation of this transcription factor by agents other than cytokines may be achieved in an alternative fashion. As such, GPCRs utilise pathways attributed to the initiation of G proteins, which can converge with upstream mediators of the NFκB pathway, including the IKK signalosome, thus intiating activation of NFκB. Therefore, while the KSHV-GPCR has been shown to activate NFκB via an IKK-dependent mechanism, other receptors, such as the bradykinin receptor have been shown to mediate its activation by induction of IκB degradation as well as expressing an increased production of IL-8, which is a product of NFκB activation (Zhu *et al,* 2003).

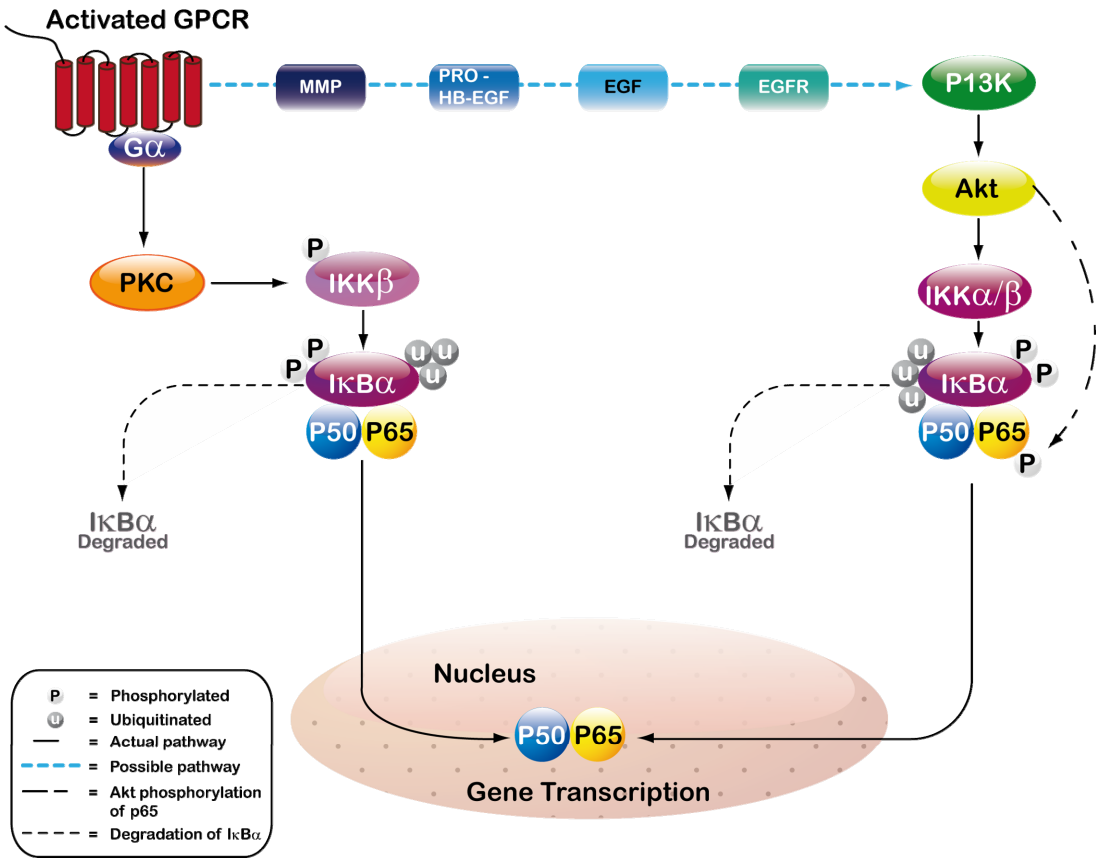
NFκB has also been reported to be regulated by a variety of GPCR/G protein coupling selectivities. In the case of this particular transcription factor, reports have shown that activation can occur via $G\alpha_{i/o}$ mediated mechanisms, $G\alpha_s$ and $G\alpha_{q/11}$ subtypes. Assessment of $G\alpha_{i/o}$ regulation of NFκB employed traditional methods, whereby identification of the subunit occurred by means of PTx sensitive mechanisms. One study assessed the role of $G\alpha_{i/o}$ mediated regulation of the NFκB pathway when stimulated by the lysophosphatidic acid receptor (Shahrestanifar *et al*, 1999). This study demonstrated that in Swiss 3T3 cells, stimulation with LPA resulted in nuclear binding of NFκB, along with a corresponding cellular degradation of IκBα. Nuclear binding and IκBα loss were found to be inhibited when cells were pre-treated with PTx, therefore demonstrating a $G\alpha_{i/o}$ -dependent mechanism to stimulate activation of the NFκB pathway (Shahrestanifar *et al*, 1999).

$G\alpha_s$ has been reported to regulate NFκB in a negative manner via the β-adrenergic receptor (Farmer & Pugin, 2000). In a study by Farmer & Pugin, it was demonstrated that in cells stimulated with LPS, there was a marked increase in cytokine production including IL-8 and TNFα. These results coincided with a time dependent cellular degradation of IκBα along with NFκB nuclear binding. However, addition of β-adrenergic agonists showed inhibition of NFκB nuclear binding, along with a reduction in the degradation of IκBα. Employment of cAMP elevating agents showed comparable results, suggesting that the β-adrenergic receptor inhibits activation of NFκB via a $G\alpha_s$ -dependent mechanism (Farmer & Pugin, 2000).

Increased cAMP has also been reported to inhibit NFκB in both monocytes and endothelial cells (Parry & Mackman, 1997). However, this has been suggested to be without inhibiting translocation of NFκB to the nucleus. In this study, it was demonstrated that a PKA-dependent pathway, in particular via the cAMP response element binding protein (CREB) is responsible for this inhibition. It was suggested that PKA induces phosphorylation of CREB, therefore resulting in recruitment of the CREB binding protein (CBP).

It was found that CBP was able to interact with the carboxyl terminus of p65 NFκB in both *in vivo* and *in vitro* conditions. This interaction renders NFκB inactive, therefore showing a means of $G\alpha_s$/PKA-dependent inhibition of this transcription factor (Parry & Mackman, 1997).

Recent examples of GPCR mediated activation of NFκB can be shown with a family of GPCR known as proteinase-activated receptors (PARs) (section 1.3), which are the focus of this current study. Most recently, it has been demonstrated that PAR-2 has been found to regulate the activation of NFκB via both G protein-dependent and -independent mechanisms (Goh *et al*, 2008) (see section 1.3.4.4). Another member of this family, PAR-1 has also been demonstrated to activate NFκB. However, the entire mechanism has not yet been fully elucidated. One study by Tantivejkul and colleagues in 2005 demonstrated that PAR-1 is able to mediate activation of NFκB in prostate cancer cells (Tantivejkul et *al*, 2005). Previous findings have shown that PAR-1 is increased in metastatic cells (Salah *et al*, 2007). In androgen-independent DU145 and PC-3 cells, it was found that activation of PAR-1 by either thrombin or activating peptide showed an increase of p65 NFκB nuclear binding, as well as an associated increase in cytokine production, including IL-6 and IL-8. Such results suggest that PAR-1 may contribute to the progressive metastasis of malignant prostate cancer cells via the activation of NFκB (Tantivejkul *et al*, 2005).



(Adapted from cellsignal.com)

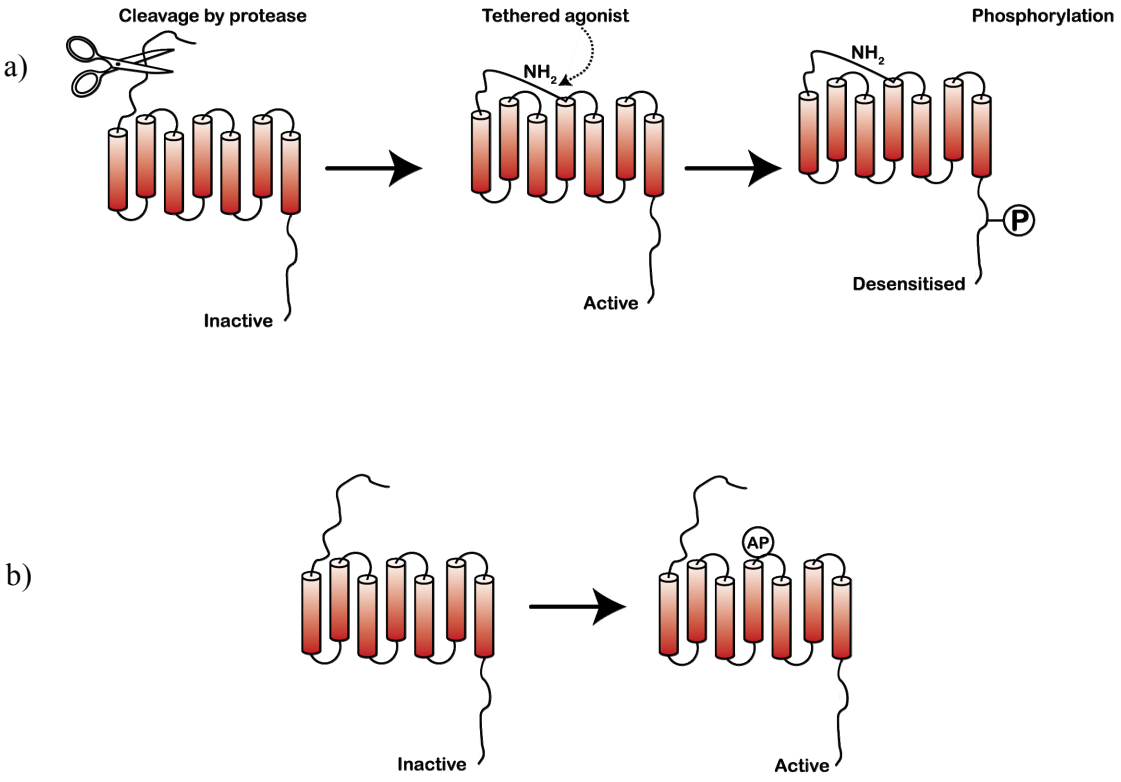
Figure 1.5 GPCR mediated activation of the NFκB pathway. The above diagram represents the different pathways whereby GPCRs can mediate activation of the NFκB pathway. The G protein-dependent pathway demonstrates downstream activation of PKC, which directly activates IKKβ to phosphorylate IκBα, thus ultimately activate NFκB. However, the G protein-independent pathway displays the ability for the GPCR to mediate activation of PI3K, which may occur via activation of MMP, with possible cleavage of membrane bound proHB-EGF. Cleavage of HB-EGF results in the release of EGF with subsequent activation of the EGFR. EGFR activation results in the activation of PI3K, which in turn activates Akt. Akt is not only found to directly phosphorylate p65 NFκB, but also mediates activation of the IKK complex in order to activate IκBα, resulting in subsequent nuclear translocation of NFκB.

1.3 PROTEINASE-ACTIVATED RECEPTORS

Proteinase activated receptors (PARs) are a novel family of G protein coupled receptors, which are activated by proteolytic cleavage of the extracellular amino terminus. This results in the unmasking of a newly generated amino terminus, which acts as a tethered ligand. This tethered ligand then interacts with a conserved region located on extracellular loop 2 (ECL-2) of the transmembrane regions, resulting in activation of the receptor (see figure 1.6) (reviewed by Hollenberg *et al*, 2008, Kawabata *et al*, 2008). Initially characterised in 1991, proteinase activated receptor-1 (PAR-1) was found to be cleaved by the serine protease thrombin, and was therefore known as the thrombin receptor (Vu *et al*, 1991). Three more receptor subtypes have since been identified, including PAR-2 (Nystedt *et al*, 1994), PAR-3 (Ishihara *et al*, 1997) and PAR-4 (Xu *et al*, 1998). Of these four PARs identified, PARs 1, 3 and 4 are preferentially found to be cleaved by thrombin, whereas PAR-2 required the serine protease trypsin for cleavage and subsequent receptor activation.

1.3.1 Identification of proteinase activated receptor-1 (PAR-1)

PAR-1 was successfully identified by an expression cloning method, which involved the isolation of cDNA which was found to encode the human thrombin receptor (Vu *et al*, 1991a). This particular study employed a technique, whereby messenger RNA (mRNA) from cell types (HEL and Dami), which were highly responsive to thrombin, was injected into *Xenopus oocytes*, which had been labelled with $^{45}Ca^{2+}$. From this assay, this particular group were able to measure the response as a thrombin-dependent release of $^{45}Ca^{2+}$. Following this, a cDNA library using the fractionated mRNA from the Dami cells was constructed, from which 50 pools of around 20,000 expression clones were generated. cRNA from these pools was injected into the oocytes, and the thrombin response assessed, from which, finally, a cDNA which encoded the thrombin receptor was identified.

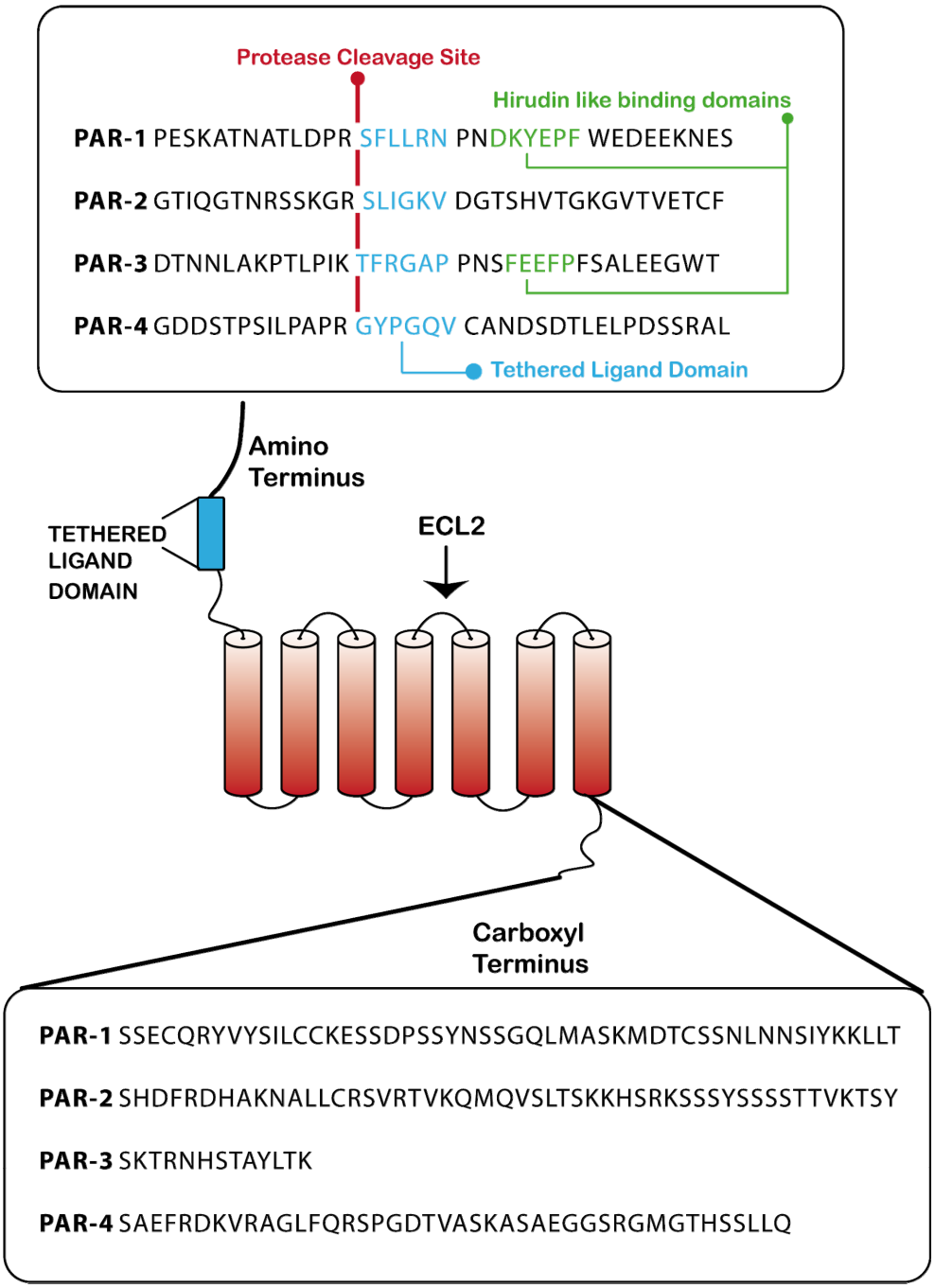


(Adapted from Range, Dale, Ritter & Moore, 5th Edition, 2003)

Figure 1.6 Activation Methods of Proteinase Activated Receptors (PARs). Panel A illustrates proteolytic cleavage of the receptor, while panel B illustrates activation of the receptor by synthetic activating peptide.

Further studies performed by reverse-transcription polymerase chain reaction (RT-PCR), ascertained that mRNA of the thrombin receptor was present in platelets and vascular endothelial cells. A 3.5kb insert was identified when nucleotide sequencing was performed on the receptor, with an open reading frame which encoded a 425 amino acid protein, as well as the presence of seven transmembrane spanning domains - a cardinal feature of a GPCR. Further study of the sequence located other GPCR features; including an extracellular amino-terminus, found to be 75 amino acids long, which contained a specialised anion-binding site, which came to be known as the hirudin-like binding site.

Site directed mutagenesis uncovered a thrombin cleavage point on the extracellular amino terminus located at $LDPR^{41}$ $\downarrow S^{42}FLL$ (see figure 1.7). This was found to be necessary for receptor activation (Vu *et al*, 1991b). Upon cleavage by thrombin at Arg^{41} of the PAR-1 amino terminus, a newly exposed amino terminus acting as a tethered ligand was able to interact with the second extracellular loop of the receptor transmembrane domains, resulting in activation of the receptor. Also in 1991, Rasmussen *et al,* reported similar findings in a study conducted on the hamster thrombin receptor, which too, had been expressed in Xenopus oocytes. It was found that around 79% sequence homology was shared between the hamster thrombin receptor and the human thrombin receptor. Also found within the hamster thrombin receptor was the presence of a thrombin consensus cleavage site, as well as an anionic cluster found within the amino terminus (Rasmussen *et al*, 1991).



(Adapted from Ossovskaya & Bunnett, 2004)

Figure 1.7 General features of a proteinase activated receptor. Note the tethered ligand domain featured in blue and hirudin-like binding site of PAR-1 & PAR-3 represented in green. The cleavage site is located above in red.

1.3.1.1 Identification of PAR-1 activating peptides (AP)

The mechanism of activation of PAR-1 was confirmed by using a synthetic peptide which effectively mimicked the new amino terminal tethered ligand. This knowledge has allowed a series of structure activity relationships (SAR) to be developed. Development of such relationships is crucial for finding potent and selective agonists and antagonists.

There are prominent advantages to using the peptide model of activation, mainly being the fact that activation with a selective peptide not only allows activation without proteolytic cleavage, but also means that for experimental design,that it is the selected PAR being activated, and does not involve the action of the protease enzyme on another type of receptor. Through experimental design of the tethered ligand sequence of PAR-1, SFLLRN, a series of agonist peptides of varied affinity were developed, which aided in the understanding of the structure-activity relationship for the receptor (Scarborough *et al*, 1992, Nose *et al,* 1993).

It was from these studies it was found, that like naturally cleaved PAR-1, the selective binding site for the PAR-1 activating peptide was also ECL-2 (Lerner *et al*, 1996). However, it was reported that activating peptides had a far lower potency when activating the receptor, as opposed to thrombin (Lasne *et al*, 1995). This particular study demonstrated a difference in kinetics between use of activating peptide, SFLLRN and thrombin in the rise of platelet calcium. Initially, it was found that SFLLRN and thrombin displayed a similar calcium release response. However, upon removal of the extra-cellular calcium, thrombin mediated a far higher calcium response to that of SFLLRN. It was also demonstrated that the SFLLRN induced calcium release reached basal levels much faster than that associated with the thrombin mediated calcium response. This resulted in thrombin mediating a more robust activation of platelets as to that mediated by SFLLRN. Therefore, not only did this particular study demonstrate the importance of extracellular calcium in the activation of platelets, but it also demonstrated that in the absence of extracellular calcium, there are considerable differences between the potency of thrombin and synthetic activating peptides.

The study suggests that differences in experimental environments may in fact contribute to a poorer performance of the activating peptide as opposed to that of thrombin (Lasne *et al*, 1995).

A further explanation for this phenomenon may be that while in nature, the serine protease may be able to cause the activation of more than one receptor at a time, whereas this would not be the same for activating peptides. It has also been suggested that there are conformational differences between the peptide and natural tethered ligand which may account for the lack of potency. Interestingly, a study by Blackheart *et al* 2000, illustrated a distinction in receptor activation by either SFLLRN or thrombin. This was demonstrated by deletion of amino acid residues 68-93 of the PAR-1 receptor, which are located in the amino terminus, past the thrombin cleavage site (position 41/42). This particular deletion resulted in complete inhibition of peptide mediated activation of the receptor. However, as the thrombin cleavage site had not been affected, thrombin was still capable of mediating activation of the receptor (Blackhart *et al,* 2000). These findings suggest that activating peptides, other than binding to the activation site on ECL-2, may interact with an additional site on the amino terminus in order to activate the receptor. However, to date, very little evidence has been published which may substantiate these claims.

Bonafide antagonists have also been developed for PAR-1. Examples of these include RWJ-56110 (Andrade-Gordon *et al*, 1999), RWJ-58259 (Zhang *et al*, 2001), which were both based on the tethered ligand sequence of PAR-1. Following this, there were also SCH-79797 (Ahn *et al*, 2000) and SCH-203099 (Soslau *et al*, 2001), representative of a class of non-peptide PAR-1 antagonists. Studies carried out by using these selective antagonists were performed by both *in vivo* and *in vitro* approaches, whereby it has been found that they are selective for PAR-1 and not any other member of the PAR family (Andrade-Gordon *et al*, 1999, Maryannoff *et al*, 2003). Such drugs were found to be useful in studying a variety of cardiovascular disorders, for example, angiogenesis (Di Serio *et al*, 2008), thrombosis (Wu & Teng, 2006) and associated stenosis (Andrade-Gordon *et al*, 1999).

1.3.1.2 PAR-1 mediated signalling

From its identification in 1991, various roles for PAR-1 have been elucidated which are notably dependent on the nature of the G protein coupling associated with the receptor (reviewed by Ossovskaya & Bunnett, 2004, Macfarlane *et al*, 2001). PAR-1 has been found to interact with several Gα subunits, including $G\alpha_{q/11}$, $G\alpha_{12/13}$ and $G\alpha_{i/o}$. Various studies have assessed the coupling of PAR-1 with $G\alpha_{q/11}$ (Hung *et al,* 1992, Baffy *et al*, 1994). It was found that PAR-1 signalling was impaired in both fibroblasts and platelets when pre-incubated with antibodies to $G\alpha_{q/11}$. Furthermore, studies have demonstrated that mice deficient in $G\alpha_{q/11}$ have decreased platelet aggregation accompanied with prolonged periods of bleeding which is thought to be due to impairment of the thrombin response associated with PAR-1 (Offermans *et al*, 1997). It was also found that PAR-1 coupling with $G\alpha_{q/11}$ mediated the hydrolysis of phosphoinositides resulting in the generation of IP_3 and DAG (Brass *et al*, 1991, Hung *et al*, 1992). However, PAR-1 does not only regulate phosphoinositide hydrolysis via Gα subunits, but can also perform this via Gβγ subunits, whereby PAR-1 was found to have a role in the procedure regulating cell motility and survival, as well as being linked with changes in cyto-skeletal structure and mitogenesis (Wang *et al,* 2002).

As mentioned previously, PAR-1 has been found to couple with more than one type of Gα protein and as such, characterisation of PAR-1 coupling with $G\alpha_{i/o}$ has also been assessed. Functional coupling of PAR-1 to $G\alpha_{i/o}$ was found to result in inhibition of adenylyl cyclase (AC), therefore inhibition of cAMP formation. However in studies using fibroblasts which had been pre-incubated with PTx, activation of PAR-1 did not affect cAMP formation. Taken together, these results suggest that PAR-1 is capable of coupling with $G\alpha_{i/o}$ (Hung *et al,* 1992). Further evidence of PAR-1 coupling with $G\alpha_{i/o}$ was achieved by means of expressing a mutant Gi2 in Chinese hamster ovary (CHO) cells. From this mutation, release of thrombin-stimulated arachidonic acid was inhibited, thus suggesting that PAR-1 stimulation was coupled to phospholipase A_2 via Gi2 (Winitz *et al*, 1994, Rickart & McHowat, 2002).

Characterisation of PAR-1 coupling with $G\alpha_{12/13}$ has also been assessed. Platelet studies demonstrated that $G\alpha_{12/13}$ contributes to platelet shape change from discoid to spheroid (Offermanns *et al*, 1994). This shape change occurs under normal physiological conditions. Following thrombin stimulation, platelets undergo a cytoskeletal shape change, which is necessary for their full activation. $^{-/-}G\alpha_{12/13}$ mice displayed impairment in thrombin-mediated platelet aggregation (Offermanns *et al*, 1997). This was because the platelets were unable to undergo the necessary morphological changes in order to mediate aggregation. $G\alpha_{12/13}$ has also been found to be involved in PAR-1 mediated regulation of the endothelial barrier via the downstream effectors p115RhoGEF and Rho-associated coiled-coil forming kinase (ROCK) (Klages *et al*, 1999, Majumdar *et al,* 1999, Nguyen *et al*, 2002).

Studies have also revealed that stimulation of PAR-1 can result in activation of the MAP kinase pathway. The association of PAR-1 mediated activation of this pathway goes back prior to the identification of the PAR-1 receptor, via studies that investigated thrombin as a mitogenic factor (Seuwen *et al,* 1990). It was demonstrated that initiation of DNA synthesis by thrombin was dependent on activation of ERK (Pages *et al,* 1993). In addition to this, it was also found that stimulation with thrombin results in stimulation of the upstream regulator of ERK known as $p21^{ras}$. This occurred by the exchange of GDP for GTP on p21. This process was found to be PTx-sensitive, therefore suggesting that it is a process not only regulated by PAR-1 coupling to $G\alpha_{i/o}$, but also that PAR-1 is involved in tyrosine kinase-mediated mitogenesis (Vancorven *et al,* 1993).

1.3.1.3 Inactivation/Desensitisation of PAR-1

To prevent unwanted cellular effects by PAR-1, it is necessary to have an efficient termination system. For PARs, more so than other types of GPCR, this is particularly important, as once cleaved, the tethered ligand is able to have continuous interaction with the receptor (Bohm *et al,* 1996). Desensitization by phosphorylation occurs by means of GRKs (see section 1.1.3).

Such enzymes are of particular importance, as once they have bound to their specific site, the GRKs become bound by β-arrestin which then prevents any further interaction between the G-protein and in this case, PAR-1. This action inhibits any further activation of the receptor. GRKs have also shown a means of targeting PAR-1 for internalisation (Ferguson *et al*, 1998). The binding consensus for specific GRK phosphorylation is normally found either within the 3rd extracellular loop or the C-terminus of the receptor. The involvement of GRK was suggested via studies using rat fibroblasts, which had been transfected with a PAR-1 expressing plasmid. Rapid PKC-independent phosphorylation of the receptor occurred in response to thrombin. This along with the identification of a GRK binding site on serine residues 391 and 395, suggested the involvement of GRK in this particular desensitisation process (Ishii *et al,* 1994). This study further demonstrated that an increase of GRK-3 was capable of abrogating thrombin-stimulated calcium signalling in these particular cells, (Ishii *et al,* 1994). More recently, it has been demonstrated that in endothelial cells, GRK-5 mediates desensitisation of PAR-1 (Tiruppathi *et al*, 2000).

Other studies have determined that the C-terminus is of importance for the process of GRK phosphorylation. A study by Vouret-Craviari *et al* demonstrated GRK-mediated phosphorylation of the PAR-1 C-terminus by utilising chimeric receptors. The chimeric receptors that were generated were 5-hydroxytryptamine receptors (5-HT_2), containing the C-terminus replacement of a PAR-1 receptor C-terminus. 5-HT_2 receptors like PAR-1 are also GPCRs that are found within human platelets and blood vessels, and have been shown to mediate the response to agonists such as serotonin and ketanserin. 5-HT receptors are also found to couple with PLC, a mediator of IP_3 and DAG production. Normally, 5-HT_2 receptors are desensitized slowly by the action of serotonin. However, in the chimeric receptor, rapid desensitisation was found to occur, which is a characteristic more readily associated with PAR-1. These chimeric studies therefore suggested that GRK-mediated phosphorylation of PAR-1 may be attributed to GRK binding at the C-terminus (Vouret-Craviari *et al,* 1995).

Trejo & Coughlin also devised experiments, whereby a chimeric neurokinin receptor with a PAR-1 C-terminus and a PAR-1 receptor with a neurokinin C-terminus were generated. From these studies, the PAR-1 C-terminus was found to be responsible for the internalisation of the receptor to clathrin coated pits, after which it becomes engaged in the process of lysosomal sorting. However, a normal neurokinin receptor C-terminus is found to induce recycling of the receptor to the cell membrane rather than initiate receptor degradation. From these studies, it was ascertained that the chimeric PAR-1 receptor with the neurokinin C-terminus resulted in the receptor undergoing membrane recycling, whereas the chimeric neurokinin receptor with the PAR-1 C-terminus became rapidly inactivated, and transported for lysosomal degradation These results therefore suggest that PAR-1 inactivation can be mediated via the C-terminus (Trejo & Coughlin, 1999).

It is not unusual for certain cell types to have an intracellular pool of PAR-1 along with a cell surface population of PAR-1. Inactive receptors find themselves constantly cycling back and forth between the cell membrane and the intracellular pool of PARs. Such receptors are not triggered into activation by ligands and neither are they subject to C-terminus phosphorylation procedures (Shapiro *et al*, 1996). However, location between lysine^{397} and tyrosine^{807} on the C-terminus has been shown to be significant in a process known as 'tonic' cycling of the receptor (Shapiro *et al*, 1996, Shapiro & Coughlin, 1998). Tonic receptor cycling of PARs is a fast and efficient means of replacing receptors without having to undergo receptor re-synthesis. It is uncertain if this procedure is associated with all cell types. It is, however, found to be a characteristic of endothelial cells, whereby pools of PAR-1 are found to be located within the Golgi apparatus (Storck *et al*, 1997). The presence of this PAR-1 receptor pool is thought to account for the partial recovery of PAR-1 within 90 minutes of the initial internalization (Ellis *et al*, 1999). Certain other cell types however, have been shown to have a slow recovery of PAR-1, including megakaryoblastic cells, as they do not have a receptor pool, and therefore, rely entirely on re-synthesis (Hoxie *et al*, 1993, Brass *et al*, 1994).

1.3.1.4 Physiological Role of PAR-1

Coagulation is highly conserved cellular process whereby bloodclots are formed. Part of the process known as haemostasis helps to prevent blood loss from a damaged vessel (Furie & Furie, 2005). It results in the damaged endothelial wall of blood vessels becoming covered by a specialised platelet and fibrin-containing clot, helping to impede bleeding, thus allow for the repair of the damaged vessel to begin (Furie & Furie, 2005). Coagulation begins instantly following injury within the endothelium. Almost immediately, platelets form a plug at the site of injury; a process which is known as primary haemostasis (reviewed by Lippi *et al*, 2009).

Further to this, another part of the coagulation cascade, known as secondary haemostasis occurs. This has two pathways, known as the contact activation pathway (or intrinsic pathway), as well as the tissue factor pathway (or extrinsic pathway), ultimately leading to the formation of fibrin. Fibrin (also known as Factor Ia), is a fibrous protein derived from the pre-cursor known as Fibrinogen (Muszbeck *et al*, 2008). Formation of fibrin is of upmost importance within the process of secondary haemostasis. The process of fibrin formation occurs initially by the activation of the zymogen known as prothrombin – which is the precursor to the serine protease thrombin (Karimi *et al*, 2009). Fibrin is then cross linked by fibrin stabilising factor (factor XIII) in order to form a clot. Once activated by thrombin, factor XIIIa further stabilises the fibrin by incorporation of the fibrinolysis inhibitors alpha-2-antiplasmin and thrombin activatable fibrinolysis inhibitor, procarboxypeptidase B (Muszbeck *et al,* 2008). Both the activation of Factor XIII by thrombin and plasminogen activator (t-PA) are catalyzed by fibrin.

Fibrin specifically binds the activated coagulation factors thrombokinase (also known as factor Xa), and thrombin, encompassing them within the network of fibers. This functions as a temporary inhibitor of these enzymes which stay active and can be released during fibrinolysis (Muszbeck *et al*, 2008, Kaiser 2003). However, since the identification of PAR-1 in 1991, thrombin has been found to contribute to a more diverse role within mammalian homeostasis (Vu *et al*, 1991). By means of studies using both thrombin and activating peptides, it has become possible to identify the functional abilities and expression characteristics of this receptor.

With the identification of PAR-4 in 1998, it was found that the role of PAR-1 in the facilitation of platelet aggregation was not exclusive, but was facilitated by PAR-4 (Kahn *et al*, 1999); therefore, a level of caution is required in attributing all thrombin mediated effects to PAR-1.

Clearly as a thrombin-sensitive receptor, it is not surprising that PAR-1 is highly expressed in the vascular endothelium (see table 1.1), where it has been found to stimulate nitric oxide mediated vaso-relaxation. This particular role is associated with human pulmonary artery endothelial cells (HPAEC) (Hamilton *et al*, 2001) and isolated rat aorta (Magazine *et al*, 1996). PAR-1 within the endothelium, however, does tend to play a rather versatile role. It has been found that over and above its ability to stimulate NO release, it has also been found to be associated with the up-regulation of vascular adhesion molecules such as ICAM-1 (Chan & Sukhatme, 2008) and has also been found to play a role in the protection of the endothelial barrier during sepsis (Kaneider *et al*, 2007). However, even when the endothelium has been damaged, PAR-1 is still able to stimulate an effect.

PAR 1	TISSUE	CELL TYPE	AGONIST	EFFECT	REFERENCE
	Airway	Epithelium, trachea, fibroblasts, SM	Thrombin, TRAP-9, TRAP-14, SFLLRN-NH_2, AparafluroFRChaCit-y-NH_2, TFLLR-NH_2	↑ PDGF release, relaxation/contraction, ↑ prostanoid release, ↑ procollagen production, cellular proliferation	Cicala *et al,* 1999; Cocks *et al*., 1999, Hauck *et al,* 1999, Lan *et al*, 2000, Saifeddine *et al*, 2001, Shapiro *et al*, 1996; Shimizu *et al.,* 2000
	Blood	Platelets	Thrombin, SFLLRN, TRAP (activating peptide), SFLLRN	Activation; degranulation; ↑ aggregation: ↑ $[Ca^{2+}]_{(i)}$; ↑ TXA_2	Andersen *et al*., 1999; Henriksen *et al*., 1997; Kinlough-Rathbone *et al.,* 1993; Vu *et al*., 1991
	Bone	Osteoblasts	Thrombin, SFFLRNPSENTFELVPL	↑ $[Ca^{2+}]_{(i)}$; ↑ proliferation	Abraham & Mackie, 1999; Jenkins *et al*., 1993
	Cardiovascular	Vascular SM; endothelium	Thrombin, TRAPs 5-14, SFLLRN, FLLRN, SFLLRNPNDKYEPF, TRP-14	Relaxation/contraction; mitogenesis; ↑ $[Ca^{2+}]_{(i)}$; ↑ von Willebrand factor release; ↑ NO release; ↑ P-selectin and ICAM expression	Emilsson *et al*., 1997; Garcia *et al.,* 1993; Godin *et al*., 1995; Hwa *et al*., 1996; Jiang *et al.,* 1998; Storck & Zimmermann, 1996; Sugama *et al*., 1992
	Epidermal	Keratinocytes	Thrombin, TRAP42-55, TRAP42-46, SFLLRN	↑ $[Ca^{2+}]_{(i)}$; ↑ proliferation; inhibits differentiation	Algermissen *et al.,* 2000; Derian *et al.,* 1997
	Immune System	Monocytes; mast cells; T cells	SFLLRNP, thrombin, TRAP-6	↑ $[Ca^{2+}]_{(i)}$; ↑ IL-6; ↑ IL-1; ↑ TNF-α; degranulation; activation	Mari *et al*., 1994; Naldini *et al*., 1998; Umarova *et al*., 2000
	Intestinal	Smooth Muscle	TFLLR-NH_2, thrombin	Relaxation/contraction	Cocks *et al*., 1999b; Kawabata *et al.,* 2000b
	Nervous System	Glia; astrocytes; neurones	Thrombin, TRAP-14, SFLLRN	↑ $[Ca^{2+}]_{(i)}$; ↑ proliferation; stellation reversal; neuronal apoptosis	Corvera *et al*., 1999; Debeir *et al.,* 1997; Pindon *et al*., 1998; Turgeon *et al*., 1998
	Skeletal Muscle	Myocytes	Thrombin	↑$[Ca^{2+}]_{(i)}$	Mbebi *et al.,* 2001
	Stomach	Smooth Muscle	Thrombin, SFLLRN, TRP42-46, TRP42-55	Relaxation/contraction	Cocks *et al*., 1999b; Hollenberg *et al*., 1992; Yang *et al*., 1992

Table 1.1 Expression/Distribution of PAR-1 (Adapted from MacFarlane *et al*, 2001).

Recent studies have shown that even in the absence of the endothelial layer, selective PAR-1 agonists were able to induce vasoconstriction, with no attenuation of platelet activation (Gudmundsdottir *et al*, 2008). Therefore, vascular PAR-1 is not only found to be expressed in the endothelial tissue, but also found within the smooth muscle layers, where it has been associated with contraction (Gudmunsdottir *et al*, 2008, Cleator & Vaughn, 2008, Laniyonu & Hollenberg, 1995). Primarily, the focus of PAR-1 has been associated with the vasculature and platelet studies. However, expression of PAR-1 is varied, and is expressed in other areas of physiological importance, including the gastro-intestinal tract (GIT). PAR-1 expression in the GIT as been found to be associated with modulation of GIT smooth muscle motility, suppression of gastric acid production, ion transport and electrolyte secretion (reviewed by Kawabata *et al,* 2007).

As previously mentioned, identification of PAR-1 has also exemplified the role of thrombin as a mitogenic factor (Zania *et al*, 2008). For example, it has been shown that in HUVECs, both thrombin and the activating peptide SFLLRN are capable of increasing endothelial cell DNA synthesis as well as mediating an increase in the phosphorylation of ERK. Use of SFLLRN determined that this particular process was selective for PAR-1, which was further established by the use of PAR-1 antagonist SCH79797. Pre-incubation with this antagonist resulted in the inhibition of ERK phosphorylation. Further to this, studies demonstrated that hirudin, a potent inhibitor of thrombin also resulted in the loss of ERK phosphorylation, thus suggesting a role for PAR-1 in endothelial mitogenesis (Zania *et al*, 2008). Other studies by Marrinessen have demonstrated that in certain cancers, thrombin mediated activation of PAR-1 can mediate cell transformation by activation of c Jun (Marrinissen *et al*, 2003).

Assessment of NIH 3T3 cells demonstrated that thrombin mediated activation of PAR-1 resulted in the rapid accumulation of c-jun mRNA, which is thought to be associated with the production of malignant cell types (Marrinessen *et al*, 2003, Shaulian & Karin, 2002). Taken together, it is thought that thrombin, via the actions of PAR-1 is thought to contribute cellular proliferation, therefore suggesting that PAR-1 has a role within a variety of activities within the cellular environment.

1.3.2 Proteinase activated receptor-2 (PAR-2)

The second member of the PAR family to be identified was PAR-2 (Nystedt *et al*, 1994). PAR-2 was initially found by Nystedt *et al* in 1994 primarily in the search for novel neurokinin receptors. PAR-2, unlike PAR-1 was not cleaved by thrombin, but instead underwent proteolytic cleavage by the serine protease trypsin. PAR-2 was identified by means of assessing the DNA sequence of a GPCR which was found to have structural similarities to that of the thrombin receptor. Again, the use of Xenopus oocytes was employed for expression of the receptor. While insensitive to concentrations of thrombin up to 100nM, PAR-2 was found to be cleaved by low concentrations of trypsin, normally found to be within the nanomolar range. An open reading frame encoding for a protein of 395 amino acids was identified, which included the 7-TM domains associated with GPCRs. PAR-2 was found to share around 30% sequence homology with PAR-1 (Nystedt *et al,* 1994).

Further study also indicated that the amino terminus of PAR-2 was found to be composed of 46 amino acids and was 29 amino acids shorter than that of PAR-1. It was also found that PAR-2 was not equipped with an anion-binding sequence required to facilitate thrombin binding associated with PAR-1 (Nystedt *et al*, 1994). Alignment of PAR-1 and PAR-2 demonstrated that ECL-2 was identified as a highly conserved site, therefore suggesting the importance of this particular feature in the activation of this receptor family. Murine PAR-2 was found to be activated by a similar mechanism to that of PAR-1, which involved proteolytic cleavage of the extracellular terminus. An activating peptide based on the murine PAR-2 tethered ligand activating sequence, SLIGRL was used (Nystedt *et al*, 1995).

Eventual studies utilising human PAR-2, found that there was an 83% sequence similarity between the murine and human receptors (Nystedt *et al,* 1995, Bohm *et al,* 1996). Studies of human PAR-2 determined that the cleavage site of PAR-2 was located at $SKGR^{36}\downarrow S^{37}LIGKV$ (see figure 1.7), (Nystedt *et al*, 1995). From this, an activating peptide based in the human sequence was developed, known as SLIGKV. Northern blot analysis determined that there is expression of PAR-2 in physiological locations which have a high level of vasculature. This included the pancreas, gastro-intestinal tract, hepatobiliary system and secretory organs such as the kidneys (Nystedt *et al,* 1995, Bohm *et al* 1996) (see table 1.2).

1.3.2.1 PAR-2 selective activating peptides (APs)

The advent of PAR-2 selective activating peptides was able to contribute to the overall understanding of the pharmacological mechanisms of the receptor. They also helped in the development of PAR-2 antagonists. Initial studies assessed the human hexapeptide sequence, SLIGKV and murine hexapeptide, SLIGRL. From these studies, a relative order of potency was established, where it was found that SLIGRL was more potent at activating PAR-2 than the human SLIGKV variant (Nystedt *et al,* 1994, Nystedt *et al*, 1995). However, as it was found with PAR-1 activating peptides, PAR-2 activating peptides were also found to have considerably less potency than trypsin. Trypsin has an EC_{50} of 1nM and can activate PAR-2 at far lower concentrations than SLIGKV or SLIGRL, (EC_{50}s of 12μM and 6μM respectively) (Nystedt *et al,* 1994, Nystedt *et al,* 1995, Maryanoff *et al*, 2001).

A series of SAR studies were carried out to assess the importance of each amino acid residue within the peptide sequences (Blackhart *et al*, 1996, Hollenberg *et al,* 1997). These particular studies revealed that amidation of the carboxyl terminal of the peptides resulted in an increase in the potency of the peptide as opposed to those that were not. Therefore, it was found that SLIGKV-NH_2 had a greater potency than SLIGKV-OH. In follow up to these original agonist studies, several modifications have resulted in a variety of peptides being available for activation of PAR-2.

PAR-2	TISSUE	CELL TYPE	AGONIST	EFFECT	REFERENCE
	Airway	Bronchial SM; endothelial; lung fibroblast	Tryptase; MT-SP-1; HAT	Bronchorelaxation/bronchoconstriction; ↑ PGE_2 release; proliferation	Akers *et al.,* 2000; Cicala *et al*., 1999; Cocks *et al*., 1999; Ricciardolo *et al.,* 2000; Takeuchi *et al*., 2000; Yamaoka *et al*., 1998
	Cardiovascular	Vascular SM; endothelium; cardiac myocytes	Tryptase	Relaxation/contraction; mitogenesis; ↑ $[Ca^{2+}]_{(i)}$; ↑ von Willebrand factor release; ↑ NO release	Al-Ani *et al.,* 1995; Bono *et al.,* 1997; Mirza *et al*., 1996; Roy *et al*., 1998; Sabri *et al*., 2000; Storck et al., 1996
	Epidermal	Keratinocytes	Tryptase; SCTE, Trypsin, 2f-LIGKV	↑ $[Ca^{2+}]_{(i)}$; inhibits proliferation and differentiation; ↑ IL-6 and GM-CSF expression; ↑ pigmentation; ↑ melanin ingestion; ↑ phagocytosis	Derian *et al*., 1997; Ekholm *et al*., 2000; Santulli *et al*., 1995; Seiberg *et al*., 2000, Sharlow *et al*., 2000; Wakita *et al*., 1997, Goon-Goh *et al,* 2008
	Immune System	Leukocytes; mast cells	Tryptase	↑$[Ca^{2+}]_{(i)}$; ↑ adhesion; ↑ rolling; ↑ migration	Howells *et al*., 1997; Vergnolle, 1999; Vergnolle et al., 1999
	Intestine	Epithelium; SM	Tryptase; trypsin; MT-SP-1	PGE_2 release, ↑$[Ca^{2+}]_{(i)}$; contraction; ↓ motility	Bohm *et al.,* 1996; Corvera *et al*., 1997; Kong *et al*., 1997; Takeuchi *et al*., 2000
	Nervous System	Glia; astrocytes; neurones	Tryptase; P22; SFRB60; neurosin	↑$[Ca^{2+}]_{(i)}$	Corvera *et al.,* 1999; Hollenberg *et al.,* 2000; Kaufmann *et al*., 1999; Sawada *et al*., 2000; Ubl *et al.,* 1998; Yamashiro *et al.,* 1997
	Pancreas	Duct epithelial cells; MIAPaCa-2 tumor cells	Trypsin; tryptase; TMPRSS3	↑ Cl^- and K^+ conductance; ↑ IP turnover and ↑ $[Ca^{2+}]_{(i)}$; ↑ proliferation; ↑ pancreatic juice secretion	Kaufmann *et al*., 1998; Kawabata *et al.,* 2000; Nguyen *et al.,* 1999; Wallrapp *et al*., 2000

Table 1.2 Expression/Distribution of PAR-2, (Adapted from Macfarlane *et al,* 2001)

One of these PAR-2 selective compounds known as *trans*-cinnamoyl-LIGRL-Orn-NH_2 was developed by Vergnolle *et al,* in 1998. Interestingly, it has also been shown that substitution of the fifth Arginine of SLIGRL for hydrophobic residues such as Phe, p-ClPhe or 1-Nal, have shown an increased ability to activate the receptors. These findings suggested the presence of a specialised binding pocket for amino acids of a positively charged or hydrophobic nature, which results in increased agonist selectivity (Maryanoff *et al,* 2001).

Later studies have further investigated the binding of PAR-2 agonists, and have resulted in modifications providing improvements for peptide induced receptor activation. One such study has shown that substitution of the serine residue located at the amino terminus of the peptide with a furoyl group resulted in the formation of a highly selective PAR-2 activating peptide, 2-furoyl-LIGKV-NH_2 (2f-LIGKV-NH_2) (Ferrell *et al*, 2003). The increase in potency was found to be partly due to a decrease in degradation of the peptide by means of aminopeptidases (Kawabata *et al*, 2004). This led to the development of one of the most potent PAR-2 activating peptides to date. In 2004, a murine variant of this peptide, known as 2-furoyl-LIGRL-NH_2 (2-f-LIGRL-NH_2), was developed (Kawabata *et al,* 2004).

1.3.2.2 PAR-2 selective antagonists

While selective PAR-1 antagonists have been developed, limited studies have been apparent in the quest to develop a selective antagonist relative to PAR-2. Early work showed that scrambled peptides such as LLRY-NH_2 and LSIGRL-NH_2 were effective as antagonists in the action of trypsin when activating PAR-2. However these have not proved successful in antagonising the action of activating peptides (Al-Ani *et al*, 2002). This study has therefore revealed the possibility of an unidentified mechanism whereby there is an interaction between the tethered ligand and the receptor. This information would most certainly suggest that there is a variation in the binding sites of the putative tethered ligand as opposed to that of the activating peptide, further suggesting that there is a degree of difference in receptor activation, with the possibility of signalling variations (Al-Ani *et al,* 2002a, Al-Ani *et al,* 2002b).

More recently, a compound known as N'-3 methylbutyryl-N^4-6-aminohexanoyl-piperazine (ENMD-1068) has been utilised. This compound has the characteristics of a selective PAR-2 antagonist (Kelso *et al,* 2006). ENMD-1068 has been found to cause an inhibition of calcium signalling induced by PAR-2 agonists *in vitro*, without any effect on PAR-1 induced activity. This particular study demonstrated a significant inhibition of trypsin induced swelling in an *in vivo* arthritis model by ENMD-1068. However, this study failed to provide any direct evidence relating to ENMD-1068 induced antagonism of swelling induced by PAR-2 activating peptide in the same model.

Therefore, this study seemed to be left open ended as to the role of this particular compound as a PAR-2 specific antagonist. However, a following study from this group again looked at the specificity of this particular novel antagonist within an *in vivo* model of rheumatoid arthritis. In this study, PAR-2 was found to be substantially up-regulated in the rheumatoid arthritis model as opposed to that of normal tissue. In this case, ENMD-1068 resulted in a dose-dependent inhibition (0.02-8mM), of PAR-2 mediated release of TNFα and IL-1β (Kelso *et al*, 2007). Taken together, these findings suggest the novel compound ENMD-1068 not only to be a novel antagonist of PAR-2, but also show the possibility of being a potential therapeutic compound in the treatment of arthritis.

1.3.2.3 PAR-2 mediated signalling

When compared with PAR-1, it is notable that the signal transduction mechanisms regulated by PAR-2 are not as well characterised. So far, PAR-2 has been demonstrated to couple with various signalling molecules which include selective G proteins, the MAP kinase pathway (Chokki *et al*, 2005, Kawao *et al*, 2005), as well as mediating activation of the NFκB pathway (Goh *et al*, 2008, Larsen *et al*, 2008, Kanke *et al,* 2001). One of the initial signalling characteristics of PAR-2 to be assessed was the production of IP_3 and subsequent mobilisation of calcium. This was demonstrated in a variety of cell types, including Xenopus oocytes (Nystedt *et al*, 1994), keratinocytes (Santulli *et al*, 1995) and epithelial cells (Bohm *et al*, 1996).

These characteristics suggested that the possibility of PAR-2 to couple with $G\alpha_{q/11}$ or $G\alpha_{i/o}$ was likely. To date, in regard of PAR-2, this is a signalling pathway which few studies have directly addressed. Study of this pathway has occurred by employment of $G\alpha_{q/11}$ inhibitor, YM-254890 (Taniguchi *et al*, 2003, Takasaki *et al*, 2004).

It has been shown that pre-incubation of NCTC-2544 cells, which stably expressed a PAR-2 construct (Clone G), with YM-254890, demonstrated a concentration-dependent inhibition of [^{3}H] IP accumulation. These results suggest that PAR-2 has the potential to couple with $G\alpha_{q/11}$ (Goh *et al,* 2008). Coupling with $G\alpha_{q/11}$ is not the only PAR-2 G protein coupling that has been assessed. Studies to determine the ability of PAR-2 to couple with $G\alpha_{i/o}$ were performed using PTx. PTx has been found to inhibit receptor-$G\alpha_{i/o}$ coupling by ADP-ribosylation of the $G\alpha$ subunit of G_i (Wang *et al,* 2007). One study demonstrated PAR-2 induced calcium signalling was inhibited by a PTx, therefore suggesting PAR-2 coupling with $G\alpha_{i/o}$ (Schultheiss *et al*, 1997). Further studies have shown that in PAR-2 transfected HEK-293 cells, stimulation of PAR-2 by activating peptide SLIGKV increases the activity of the c-fos promoter. However, the c-fos promoter activity did not show an increase in activity in cells that had been pre-incubated with PTx. These results provide further evidence to suggest that PAR-2 can successfully couple with $G\alpha_{i/o}$ (Yu *et al*, 1997). Unfortunately, at present, there is little published data which confirms PAR-2 coupling with $G\alpha_{i/o}$.

Many studies have associated PAR-2 activation with activation of ERK. One study has demonstrated that in coronary artery smooth muscle cells (CASMC) the activity of factor Xa is mediated by the PAR-2 activation of ERK (Koo *et al*, 2002). One of the mechanisms whereby PAR-2 couples with ERK is a process mediated by the scaffolding molecule, β-arrestin (see section 1.1.5.1). In a study by De Fea *et al,* 2000, it was proposed that agonists of PAR-2 mediate the formation of a multi-protein signalling complex, which is found to contain the internalised PAR-2 receptor, β-arrestin, raf-1 and activated ERK. Use of $^{-/-}$β-arrestin in assessing PAR-2 mediated ERK activation demonstrated a significant decrease in phosphorylation of ERK.

This suggests a requirement for PAR-2 to undergo endocytosis by β-arrestin as being necessary for the normal activation of ERK (DeFea *et al*, 2000). Further study by Stalheim *et al,* 2005 again demonstrated the involvement of β-arrestin in PAR-2 mediated activation of ERK. This particular study assessed the regulation of PAR-2 signalling by examination of the involvement of the C-terminus. Findings demonstrated that PAR-2 mutants that had been truncated at the C-terminus displayed a difference in ERK1/2 signalling as opposed to wild type PAR-2. These differences in ERK signalling occurred due to the fact that the truncated PAR-2 C terminus had lost the ability to stably associate with β-arrestin, which has been demonstrated previously by DeFea *et al,* to be a pre-requisite in the PAR-2 mediated activation of ERK (Stalheim *et al*, 2005). Therefore, these studies suggest that the specific interaction of β-arrestin with the PAR-2 C terminus, are important in the regulation of PAR-2 mediated activation of ERK.

Further to its ability to regulate the MAP kinase pathway, PAR-2 has also been found to be associated with the activation of the transcription factor NFκB. It is now known that NFκB, which has been previously characterised by means of cytokine activation, can in undergo a level of regulation by certain GPCRs, including PAR-2. As such, it has been shown that both specific PAR-2 activating peptides and trypsin are capable of inducing significant nuclear translocation of NFκB in various cellular models, including human keratinocytes (Kanke *et al*, 2001) and vascular smooth muscle cells (Bretschneider *et al*, 1999). Taken together, the studies above show that PAR-2 has the ability to signal to an array of effector molecules and as such, gives this particular receptor the ability to elicit a variety of cellular effects through regulation of a variety of downstream signalling pathways.

1.3.2.4 Inactivation/Desensitisation of PAR-2

Like PAR-1, the activation response for PAR-2 is rapidly terminated. It has also been found that with repeated exposure, it can become desensitised. However, at present, the full mechanisms as to the control of desensitisation have not been fully elucidated. One mechanism which has been found to be important in the regulation of PAR-2 is the interaction of PKCβ (Bohm *et al*, 1996, DeFea *et al*, 2000).

This has been demonstrated by observing the activation of PKBβ by phorbol esters. This process has been found to result in the attenuation of PAR-2 induced Ca^{2+} signalling in both cells that have been transfected, or naturally express the receptor. Further study has confirmed that inhibition of PKC will result in a normal PAR-2 mediated response. DeFea *et al*, have established that mutation of the PKC binding site located in the C-terminus of PAR-2, resulted in the receptor being unable to be inhibited by phorbol esters. This particular mutation also resulted in the inability of the receptor to undergo desensitisation or internalisation. Therefore, in response to this mutation, trypsin and the Ca^{2+} signalling associated with PAR-2, showed prolonged and exaggerated responses to agonist stimulation (DeFea *et al*, 2000).

Although the current role of GRKs are not fully understood in PAR-2 desensitisation, it has been found that PAR-2 becomes internalised into clathrin coated pits and transported to cellular lysosomes for degradation (Bohm *et al*, 1996, Dery *et al,* 1999). However, it has been shown that PAR-2 requires dynamin for complete endocytosis. This was clarified by means of a GTPase-defective dynamin, K44E, which was found to inhibit PAR-2 endocytosis. (Roosterman *et al*, 2003). It was thought that during the process of endocytosis, PAR-2 utilises β-arrestin. Upon activation of PAR-2, β-arrestin-1 was found to translocate from the cytoplasm to the plasma membrane. This was found to result in the co-localisation of PAR-2 and β-arrestin-1 in the early endosomes (DeaFea *et al*, 2000, Dery *et al,* 1996).

Following this co-localisation, PAR-2 and β-arrestin-1 become compartmentalised, whereby, PAR-2 becomes targeted for lysosomal degradation. At the same time, β-arrestin-1 returns unaltered to the cytoplasm. Therefore, by the interaction of these two membrane molecules, it can be suggested that β-arrestin-1 is important in regulating endocytosis of PAR-2. These observations were presented in a study by DeFea *et al* 2000, which utilised a dominant negative β-arrestin $^{319-419}$. This particular dominant negative molecule prevented the process of endocytosis, suggesting β-arrestin is a feature of the PAR-2 desensitisation process.

This was further clarified by use of a specificPAR-2 mutant (δST363/6→A), in which the amino acids serine and threonine at positions 363 and 366 respectively, were substituted with alanine. This mutation demonstrated the inability of PAR-2 to become internalised, as it no longer had the ability to interact with β-arrestin (DeFea *et al*, 2000).

However, other mechanisms have also been found necessary for the receptor trafficking of PAR-2. These include the family of Rab GTPases. As previously mentioned, these particular molecules are responsible for the transport of various proteins from the plasma membrane to the organelles. The specific Rab GTPase associated with PAR-2 and found to be located in the early endosomes is Rab5a. This molecule is found to co-localise with PAR-2 rapidly following receptor activation (Roosterman *et al*, 2003). By the utilisation of a $^{-/-}$Rab GTPase, rab5S34N, a molecule which does not show any GTPase activity, it was found that the early endosomes were disrupted, and endocytosis of PAR-2 was inhibited. These results suggested that PAR-2 is dependent on Rab5a for transportation from the clathrin pits to the endosomes (Marchese & Benovic, 2001). However, while Rab GTPases are found to be associated with the endocytosis of PAR-2, they are also utilised in the re-sensitization process. It has been suggested that Rab11a is responsible for the transport of PAR-2 from the Golgi apparatus to the cell surface.

In the Golgi, it has been reported that RabIIa co-localises with PAR-2. It has been suggested that stimulation of PAR-2 may mediate the re-distribution of Rab11a into PAR-2 containing vesicles, therefore resulting in aiding PAR-2 transportation back to the cell surface (Roosterman *et al,* 2003). Mutation studies determined that by using GTPase defective $^{-/-}$rab11aS25N, resulted in PAR-2 being retained in the Golgi apparatus, unable to return to the cell surface. Further to this, it has been demonstrated that overexpression of RabIIa results in an express recovery of PAR-2. This allows for re-sensitization of the receptor and re-instatement of the receptor signal. Taken together, these studies suggest that Rab GTPases are an important factor in maintenance of PAR-2 signalling.

1.3.2.5 Physiological Actions of PAR-2

PAR-2 is found to be expressed in a variety of tissues (see table 1.2). Within these tissues, it contributes to both physiological and pathological conditions, as well as having both pro- and anti-inflammatory properties (reviewed by Macfarlane *et al,* 2001). For example, the expression of PAR-2 within the pulmonary system has been found within various cell types, which include the pulmonary epithelium (Lin *et al*, 2008), bronchial fibroblasts (Ramachandran *et al*, 2007) and pulmonary vascular smooth muscle cells (Trian *et al,* 2006). However, there has been controversy in published literature as to whether or not the expression of PAR-2 in pulmonary tissue mediates a protective or pathological role. Certain studies have ascertained that PAR-2 is protective in its role within bronchial tissue, whereby activation of PAR-2 caused an epithelium and prostanoid-dependent relaxation in the isolated bronchial tissue of a variety of animal models (Cocks *et al*, 1999).

However, when similar experiments were carried out on isolated human bronchial rings, it was found that the activation of PAR-2 resulted in contraction (Chambers *et al,* 2001, Schmidlin *et al,* 2001), therefore suggesting that the effect of activated PAR-2 within the bronchial environment may be a species-dependent phenomenon. Immunolocalisation studies found PAR-2 to be expressed in several layers of skin tissue, including basal membrane, spinous layer, epidermal granular layers (D'Andrea *et al*, 1998) as well as hair follicles and myoepithelial cells of sweat glands (Steinhoff *et al*, 1999). However, the role of PAR-2 as an inflammatory mediator in skin cells was not apparent until it was found to be highly expressed in inflammatory dermatological conditions, including psoriasis (Steinhoff *et al*, 1999). Further studies performed in PAR-2 knockout mice showed a decreased inflammatory response, whereby there was a reduction in oedema and a decrease in inflammatory infiltrate (Kawagoe *et al,* 2001). In a variety of studies, it has been notable that there is an increase in the level of tryptase-containing mast cells within the dermal layers, which in turn leads to a powerful activation of PAR-2 in skin and subsequent infiltration of inflammatory mediators including IL-6 and IL-8 (Steinhoff *et al,* 1999).

PAR-2 is also found to be highly expressed in the gastro-intestinal (GI) tract. Areas of expression within the GI tract include small intestine, large intestine as well as the pancreas and hepatobiliary organs such as the liver (Nystedt *et al*, 1994, Nystedt *et al,* 1995). However, at a cellular level, expression of PAR-2 was found to be mostly abundant in enterocytes, the myenteric and submucosal neurones and vascular smooth muscle cells (D'Andrea *et al*, 1998, Kawabata *et al*, 2006, reviewed by Kawabata *et al,* 2007). Taken as a whole, PAR-2 has been found to be a diverse receptor associated with an abundance of physiological and pathological processes. Also, due to the fact that PAR-2 has been found to play a pivotal role within the inflammatory process, including the secretion of a variety of cytokines, it can be suggested that PAR-2 is an ideal candidate to be assessed for therapeutic targeting.

1.3.3 Identification/Pharmacological Significance of PAR-3

Despite the identification of PAR-2 in 1994, it was likely that further members of the PAR family were still to be identified. With the generation of a $^{-/-}$PAR-1 mouse, it was discovered that there was still a thrombin mediated response, suggesting the presence of other thrombin-sensitive receptors (Connolly *et al,* 1996). By utilising murine platelets, PAR-3 was identified in 1997 (Ishihara *et al*, 1997). Comparative analysis found that PAR-3 shared 28% sequence homology its other family members. PAR-3 was also found to have a thrombin cleavage site within its extra-cellular amino terminus, at the location LPIK^{38}↓T^{39}FRGAP (see figure 1.7). Cleavage at this site resulted in the formation of a newly exposed amino terminus with the tethered ligand sequence, TFRGA (Ishihara *et al*, 1997). It was found that substitution of Thr^{39} with Pro, led to thrombin insensitivity, which confirmed the cleavage site of PAR-3 was indeed found at residues Lys-^{38}/Thr^{39} within the exodomain.

Further studies have confirmed the presence of a hirudin-like binding domain, a feature that PAR-3 shares in common with the original thrombin receptor, PAR-1. This was found to be located at Phe^{40} (Ishihara *et al*, 1997). However, while it has the presence of the hirudin-like binding domain for thrombin, PAR-3 is found to be lacking an essential proline residue sequence known as P1-P4. While the other thrombin receptors, namely PAR-1 and PAR-4 contain P2 prolines, which help optimise their structural specificity for thrombin, PAR-3 contains a P3 proline at position 34 of its amino acid sequence (L^{34}TIK), resulting in steric hindrance in its cleavage and subsequent activation by thrombin (Steinberg, 2004).

PAR-3 was closely tested for activation with varied protease enzymes, but the only protease capable of causing cleavage of the receptor was thrombin, which with an EC_{50} of 0.2nM, seemed to have an extremely high affinity for this receptor. So far, it has proven difficult for researchers to generate an activating peptide for this receptor, as it does not share the characteristics of receptor activation apparent with PAR-1 and PAR-2, where it has been possible to generate a peptide able to mimic the activation which normally occurs with the tethered ligand. Studies carried out by Hollenberg *et al*, determined that human (TFRGAP-NH_2) and murine (SFNGGP-NH_2) derived PAR-3 activating peptides have been found to activate both PAR-1 and PAR-2 in Jurkat T-leukaemia cells (Hansen *et al*, 2004), highlighting the difficulty in finding a suitable activating peptide which can be utilised in PAR-3 studies.

PAR-3 has been found to be expressed in various models of lymphatic vessels, including human bone marrow (Ishihara *et al,* 1997) and murine splenic cells (Schmidt *et al*, 1998) (see table 1.3). However the only physiological function that has been determined for PAR-3 to date is the possibility that it may act as a co-factor in the activation of PAR-4 via a thrombin response in murine platelets (Nakinishi-Matsui *et al*, 2000). This particular study demonstrated that in the platelets of $^{-/-}$PAR-3 mice resulted in a decrease in platelet aggregation when stimulated by lower concentrations of thrombin, although it was not completely inhibited.

Therefore, while PAR-3 itself has not been demonstrated to signal intracellularly, the notable decrease in the platelet response suggests that it facilitates the response mediated by PAR-4, which is the other thrombin receptor expressed in murine platelets (Nakanishi-Matsui *et al,* 2000).

1.3.4 Identification of proteinase-activated receptor-4 (PAR-4)

As outlined previously, it has been noted that PAR-1 is not involved in mediating murine platelet aggregation, but was possibly mediated in part by PAR-3 (Connolly *et al,* 1996). However, murine platelets that were deficient in PAR-3 still displayed a response to thrombin, albeit a delayed aggregation response (Nakanishi-Matsui *et al*, 2000, Kahn *et al*, 1998). These observations suggested the presence of another thrombin receptor, which was identified as PAR-4 (Kahn *et al*, 1998, Xu *et al*, 1998). With a 33% sequence homology to the other members of the group, PAR-4 was identified by means of assessing PAR-like sequences via libraries of expressed sequence tags. From this, PAR-4 was found to be a protein of 385 amino acids, with a cleavage site which had the potential to be cleaved by both either thrombin or trypsin (Xu *et al,* 1998).

However, while PAR-4 shared homology with other family members, there were a variety of differences found in certain areas of the amino terminus in the respect that it had very little amino acid similarities to its other family members. The presence of an anion binding site was not present on the amino terminus of PAR-4, therefore suggesting a lower potency of thrombin binding (Xu *et al,* 1998). Differences in amino acid sequence were also notable within the carboxyl terminus, as the PAR-4 carboxyl terminus shared very little amino acid sequence similarity with that of either PAR-1 or PAR-2. Finally, within the ECL-2 of the transmembrane regions, it was found that in the conserved area of this loop, there were only 3 amino acids that shared any similarity within the conserved region (CHD) with PAR-1, PAR-2 and PAR-3 (Xu *et al,* 1998).

PAR 3	Tissue	Cell Type	Agonist	Effect	Reference
	Airway	Smooth Muscle cells	Thrombin	Possible Bronchial Contraction with PAR-1	Hauck *et al*, 1999
	Blood	Platelets, murine platelets	Thrombin	Platelet activation, co-factor for Activation of PAR-4	Kahn *et al,* 1998, Nakanishi-Matsui *et al,* 2000
	Bone	Bone marrow	Thrombin	$\uparrow IP_3$ production	Ishihara *et al*, 1997
	Cardio-vascular Tissue	Vascular endothelium	Thrombin	Regulation of PAR-1 by receptor Dimerization, identification of PAR-3 in human endothelial cells	McLaughlin *et al,* 2007, Schmidt *et al*, 1998
	Immune Tissue	Murine megakaryocytes, Dendritic cells	Thrombin	$\uparrow IP_3$ production, possible regulation of chemotaxis & induction of chemo-kines with PAR-1, maturation of megakaryocytes, activation of PKC	Ishihara *et al*, 1997, Xuehau *et al*, 2008, Cupit *et a*l, 2004
	Nervous Tissue	Astrocytes, microglia	Thrombin, plasmin, Endothlin	Possible $\uparrow Ca^{2+}$ signalling with PAR-1, post-ischaemic inflammation	Bartha *et al*, 2000, Henrich-Noack *et al*, 2006

Table 1.3 Expression/Distribution of PAR-3,(style adapted from Macfarlane *et al,* 2001)

Using site directed mutagenesis; the cleavage site for PAR-4 was identified and found to lie at $PAPR^{47}\downarrow G^{48}YPGQV$ (see figure 1.7). Studies performed in COS cells showed that activation of PAR-4, like PAR-1 and PAR-2 was able to be achieved by means of an activating peptide based on the putative tethered ligand sequence of the murine model, known as GYPGQV. However, in the case of PAR-4, it was found that while the peptide was capable of activating the receptor at a functional level, it was with a very low potency (EC_{50} = 100μM) (Xu *et al,* 1998). Further functional studies have also determined that PAR-4, unlike the other members of the PAR family, is a receptor that displays poor desensitisation, although as yet, few studies have concentrated on investigating this mechanism. As previously stated, PAR-4 has large differences in amino acid sequence of its C-terminus compared to that of PAR-1 or PAR-2 (Xu *et al*, 1998). One particular study assessed the differences in activation between PAR-1 and PAR-4 in fibroblasts and human platelets (Shapiro *et al*, 2000).

Initial findings within this particular study suggested that PAR-4 mediated a higher level of total inositol phosphate accumulation than that accumulated by PAR-1. In this case, the authors have suggested that this may be due to PAR-4 being uncoupled from the G protein in a slower manner than PAR-1. These findings suggest a possible reason why the rate of PAR-4 internalisation is much slower than that of its other family members (Shapiro *et al*, 2000). This study also assessed the calcium signalling associated with PAR-4. Results displayed in human platelets determined that there was a difference in the kinetics of both receptors. The rapid calcium response induced by PAR-1 demonstrated the transient signalling characteristics associated with the receptor, whereas with PAR-4 a more gradual response was observed (Shapiro *et al,* 2000). These results were parallel with findings from another study which assessed the persistent signalling associated with PAR-4 (Suo *et al*, 2003). This study assessed the signalling of PAR-1 and PAR-4 in N9 murine microglial cells, which again demonstrate differences in the kinetics of PAR-1 and PAR-4. Again, assessing the calcium kinetics of both these receptors, PAR-1 was found to induce a transient response which rapidly returned to basal, whereas PAR-4 produced a calcium signal which was both gradual and prolonged.

This particular study also suggested that while the C-terminus of PAR-1 is able to interact with GRK-5 and GRK-2, which results in rapid desensitisation of the receptor, the prolonged desensitisation of PAR-4 suggests poor interaction of the receptor C-terminus with GRK-5 or GRK-2 (Suo *et al*, 2003). To date, there is a relative lack in studies which have focused on desensitisation of PAR-4. As such, it can be suggested that much more study is required in this area in order to ascertain the functional abilities of this receptor.

1.3.4.1 PAR-4 Selective Activating Peptides

As previously mentioned in section 1.3.4, the initial activating peptide that was designed for activation of PAR-4 was a hexapeptide based on the murine tethered ligand sequence, GYPGQV (Xu *et al*, 1998). However, it was found that early PAR-4 selective activating peptides did not prove to be particularly potent; therefore, modifications have been applied to provide a pharmacological tool for activating the receptor at a more acceptable concentration. It was also notable that GYPGQV was a hexapeptide based on the murine receptor sequence and not human. Therefore, generation of an amidated synthetic peptide based on the human tethered ligand sequence was designed as GYPGKF-NH_2 (Hollenberg *et al*, 1999). However the EC_{50} of this particular peptide was reported to be within the region of 300 to 400μM, therefore large concentrations were required for activation of the receptor, making selectivity a problem.

Therefore, it was necessary to design a peptide that could initiate receptor activation more effectively. To this end, the synthetic peptide AYPGKF-NH_2 was designed. It was found that by substituting the glycine residue in position 1 for that of an alanine residue resulted in the generation of a peptide which was at least 10 fold more potent than that of its predecessor (Faruqi *et al*, 2000). While many studies still utilise the parent peptide (Wu *et al*, 2005, Lee & Huang, 2008), it has been notable that many PAR researchers prefer the more potent AYPGKF-NH_2. This may not necessarily be due to the order of peptide potency though.

Certain studies have reported the lack of platelet activation by GYPGKF in certain animal models, including guinea pig platelets, suggesting that either PAR-4 is not expressed in this particular model, or that the peptide is not potent enough to achieve activation in these models (Nishikawa *et al*, 2000).

More recently, the development of some rather low potency antagonists have been reported. These antagonists include low potency peptide mimetics such as trans-cinnamoyl-YPGKF-NH_2, which with its modified sequence, prevents the receptor from becoming activated (Hollenberg & Saifeddine, 2001). However, it has been suggested in a study by Hollenberg that antagonists derived from PAR-4 peptides may unfortunately, work on other receptors that are not PAR-4. Therefore, the true nature of these antagonists has not yet been fully elucidated (Hollenberg *et al*, 2004). Other novel cell penetrating molecules have been developed, known as pepducins, which are designed to interrupt the association of PAR-4 with its G protein, thus inhibiting any downstream effects that may be a consequence of PAR-4 activation (Covic *et al*, 2002). A study by Covic demonstrated the use of PAR-4 selective pepducins in murine platelets, which is a model firmly associated with the expression of PAR-4. Addition of the pepducin resulted in extended bleeding times, which is a condition reported in PAR-4 deficient mice (Covic *et al*, 2002).

1.3.4.2 Physiological roles of PAR-4

Initial analysis of PAR-4 expression was performed upon its identification. By means of Northern blot analysis it was found that PAR-4 was expressed in a variety of tissues, including the lungs, pancreas, thyroid gland, testicular tissue and the small intestine (Xu *et al*, 1998) (see table 1.4). Further analysis has found it to be associated with the vascular endothelium, where it has been shown to be expressed in a variety of tissue, particularly aortic endothelium and pulmonary artery endothelial tissue (Fujiwara *et al*, 2004), as well as showing expression within neuronal tissue (Suo *et al*, 2003).

PAR 4	Tissue	Cell Type	Agonist	Effect	Reference
	Airway	Pulmonary endothelia Cells, fibroblasts, Pulmonary smooth Muscle cells, pulmonary epithelial cells	Thrombin, AYPGKF, GYPGKF, Cathepsin G, AYPGQV	↑pulmonary fibrosis, ↑EGFR phosphorylation, ↑inflammatory cytokines ↑epithelial relaxation, ↑inflammatory infiltrate, regulation of ERK signalling	Ando *et al*, 2007, Tran & Stewart, 2003, Chow *et al,* 2000, Ramachandran *et al*, 2007, Kataoka *et al*, 2003.
	Blood	Platelets	Thrombin, AYPGKF, GYPGKF, Plasmin	↑Platelet adhesion, ↑ platelet spreading, ↑ Ca^{2+} mobilisation, ADP secretion, p38 activation, platelet accumulation, blood clot elasticity, ↑PLC signalling, ↑ phosphorylation of Akt	Mazharian *et al*, 2007, Vandendries *et al*, 2007, Vretenbrant *et al,* 2007 Hall *et al*, 2007, Resendiz *et al*, 2007, Mao *et al*, 2009.
	Cardio-vascular Tissue	Endothelial cells, Cardiomyocytes	Cathepsin G, AYPGKF, thrombin	↑MAP kinase activation, ↑ PLC activation, ↑IP_3 accumulation, cardiomyocyte hypertrophy, transactivates EGFR, ↑Ca^{2+} mobilisation, ↑NO production	Sabri *et al*, 2003a, Sabri *et a*l 2003b, Hirano *et al*, 2007, Momota *et al,* 2006
	GIT	Colon longitudinal muscle, submucosa, oesophagus	AYPGKF, GYPGKF, thrombin	↑contraction/relaxation of smooth muscle	Mule *et al,* 2004, Kawabata,, 2003
	Nervous System	Microglial cells, Cerebral endothelial cells	Thrombin, GYPGKF	↑TNFα, ↑ERK phosphorylation, ↑NFκB activation, neural degeneration	Suo *et al,* 2003, Henrich-Noack *et al,* 2006

Table 1.4 Expression/Distribution of PAR-4, (style adapted from Macfarlane *et al,* 2001)

PAR-4 was also found to be expressed in platelets, where it functions to facilitate platelet aggregation along with PAR-1 (Kahn *et al*, 1999). In human platelets, PAR-4 was found to have a far lower affinity for thrombin as opposed to that of PAR-1 and requires a higher concentration of thrombin for activation. Due to its sustainable response, PAR-4 is found to contribute to the late phase of platelet aggregation, compensating for the transient nature of PAR-1 (Covic *et al*, 2000). Surprisingly, it is via the expression of PAR-4 on murine platelets that provides PAR-3 with a physiological role, whereby it is found to contribute as a co-factor to PAR-4 in order to potentiate its activation (Nakanishi-Matsui *et al*, 2000).

However, it has been found that PAR-4 plays a more definitive role within endothelial cells. Studies by Fujiwara determined that each one of the PAR family is found to have preferential expression within selected endothelial cells, suggesting a distinct physiological or pathological role for each PAR. Examinations of various endothelial cells were investigated for PAR expression. Incorporation of Bromodeoxyuridine (BrdU) acted as an indicator for DNA replication of the various members of the PAR family. From these results, while it was shown that PAR-1 was found to be expressed in human aortic endothelial cells (HAEC) and high expression of PAR-2 was found to be associated with HUVECs. However, stimulation by PAR-4 AP, GYPGKF displayed the highest level of BrdU incorporation in human pulmonary artery endothelial cells (HPAECs), confirming the presence of PAR-4 within the pulmonary endothelium (Fujiwara *et al,* 2004).

Early studies that were attributed to the functional activity of PAR-4 in lung cells assessed the role of PAR-4 in the release of the inflammatory mediators IL-8 and IL-6 along with prostaglandin E2 (PGE2) in respiratory epithelial cells (Asokananthan *et al*, 2002). In the case of human bronchial epithelial cells, as well as A549 cells, it was found that PAR-4 worked in conjunction with PAR-2 when activated with their respective peptides, to generate the secretion of IL-6 and IL-8, suggesting a possible pro-inflammatory role for PAR-4 within the pulmonary environment (Asokananthan *et al*, 2002).

Various studies over the past few years, have suggested a possible role for PAR-4 as an inflammatory mediator within the respiratory system. A study by Ramachandran *et al*, suggested a role for PAR-4, which by activation with either thrombin or cathepsin G, results in an inflammatory response in human bronchial fibroblasts (Ramachandran *et al,* 2006). Under normal circumstances, PAR-1, PAR-2 and PAR-3 are found within these fibroblasts, but PCR reported no expression of PAR-4. However, it was the intention of this study to determine if it was possible to up-regulate a receptor not normally expressed, by means of pharmacological manipulation. It was hypothesised that due to the nature of PAR-2 within pulmonary fibroblasts, that the inflammatory mediators may actually cause a change in receptor expression and result in an inflammatory mediated expression of PAR-4.

Stimulation of pulmonary fibroblasts by TNFα demonstrated by PCR that both PAR-2 and PAR-4 mRNA were being expressed. These results suggested that PAR-4 may have an association with the inflammatory response in pulmonary tissue (Ramachandran *et al*, 2006). Other studies have demonstrated the expression of PAR-4 in the pulmonary environment. For example, by PCR, expression of PAR-4 mRNA has been found to be expressed on murine primary lung alveolar cells (Ando *et al,* 2007). In this particular study, PAR-4 was shown to induce an increase in intracellular calcium, which is possibly consistent with the desensitisation of PAR-4, as it took a considerable length of time to return to baseline.

While this particular study has not attributed any inflammatory role to PAR-4 within the alveolar environment, it does show a trend which suggests that PAR-4 is likely to be of significance within the respiratory system (Ando *et al*, 2007). However, a degree of caution needs to be applied to the suggestion that PAR-4 is a pro-inflammatory mediator, as no firm evidence has been presented as yet to confirm this role. The findings are, however, consistent with studies conducted in other species, which have demonstrated PAR-4 expression in pulmonary tissue including rabbit (Jesmin *et al*, 2004) and rat (Jesmin *et al,* 2007).

PAR-4 has also been found to be associated with contraction of the small intestine, a procedure which is necessary for the movement of the bolus through the GI tract (Kawabata, 2003, reviewed by Vergnolle, 2008). Thrombin is found to mediate the stimulatory role of PAR-4. Thrombin has been shown to produce opposing actions via PAR-1 and PAR-4 in order to retain a level of homeostasis (Kawabata *et al,* 2000). This action of PAR- 4 was assessed in a study by Kawabata, whereby rat oesophageal tissue was found to be contracted by thrombin via actions of PAR-1. This was verified by use of the PAR-1 selective peptides TFLLR-NH_2 and SFLLR-NH_2, which were found to mediate oesophageal contraction, whereas the PAR-4 activating peptide did not. However, in sections of muscle that had been pre-contracted by carbachol, it was found that while PAR-1 selective peptides were not able to cause any relaxation the PAR-4 activating peptide, GYPGKF-NH_2 was found to mediate an effective relaxation, therefore suggesting a dual role for thrombin within the modulation of oesophageal muscle (Kawabata *et al*, 2000).

It has also been demonstrated that PAR-4 has a functional role within the rat colon. Mule *et al*, provided evidence of a PAR-4 induced contractile response within the rat colon (Mule *et al*, 2004). Following PCR to determine the location of PAR-4 within the colon, it was found that it was highly expressed within the submucosa and surface epithelial tissue. Further to these findings, it was demonstrated that both PAR-4 activating peptides evoked a concentration-dependent contraction of the colonic muscle, which was later found to be inhibited by the addition of a tetrodotoxin (TTX), a potent Na^{2+} channel blocker, therefore suggesting that this contractile response was partly mediated by a neurological mechanism (Mule *et al*, 2004). Taken together, these studies suggest a varied role for PAR-4 within the gastrointestinal tract. However, as one of the initial roles attributed to PAR-4 was identified in platelets, along with the fact it is a thrombin receptor, it can be suggested that PAR-4 does play a role within the cardiovascular system.

To date, the functional role of PAR-4 within the vasculature has not been fully elucidated. However, it has been suggested that thrombin elicits its inflammatory response via proteinase activated receptors (Vergnolle *et al*, 2002). Some studies in particular focussed on the differences in calcium requirement between PAR-1 and PAR-4 in the production of NO in endothelial cells. It has been reported previously that PAR-1 requires a calcium-dependent mechanism to produce NO (Mizuno *et al*, 2000), whereas, the mechanism underlying PAR-4 has been suggested to be via a non-calcium-dependent mechanism which involves the coupling of PAR-4 with $G\alpha_{i/o}$ (Hirano *et al*, 2007). This study was a follow up to a previous study which assessed the role of PAR-4 in endothelial cells (Momoto *et al,* 2006). By means of fluorescence studies using a detector of NO, known as DAR-4M, PAR-4 activating peptide, AYPGKF-NH_2 demonstrated a concentration dependent increase of NO release, comparable to that produced by thrombin. This NO production was attenuated when cells were treated with PTx which demonstrated the ability of PAR-4 to couple with $G\alpha_{i/o}$.

However, in both studies, the use of AYPGKF-NH_2, as opposed to PAR-1 activating peptides, have demonstrated that PAR-4 does not result in any increase of intracellular calcium (Momota *et al,* 2006, Hirano *et al*, 2007). These results therefore suggest that PAR-4 is responsible for a calcium-independent rise in NO production. Taken together, these studies have demonstrated that this mechanism was attributed to both bovine and porcine aortic endothelial cells, suggesting a cross-species role for PAR-4 in the regulation of vascular tone via NO mediated vasorelaxation (Hirano *et al,* 2007).

Other studies relating to the role of PAR-4 within the vasculature include an assessment of a possible pro-inflammatory role for PAR-4 in thrombin induced leukocyte rolling (Vergnolle *et al,* 2002). This role for PAR-4 was determined via pharmacological inhibition of PAR-1 in isolated rat mesenteric arteries. Through initial topical application of thrombin, leukocyte rolling and adherence to vascular endothelial cells was observed, which is a characteristic of vascular inflammation. However, this leukocyte rolling was not observed when repeated with PAR-1 activating peptide.

Further to these findings, tissue pre-treated with the PAR-1 selective antagonist RWJ-56110 did not show any inhibition of leukocyte rolling when thrombin was again topically applied to the tissue. However, upon application of PAR-4 activating peptide, GYPGQV-NH_2, leukocyte rolling and endothelial adhesion comparable to that induced by thrombin, was observed within the rat mesenteric artery. Although these findings have not been applied to further animal models, it can be suggested that PAR-4 is the receptor whereby thrombin mediates certain characteristics of the inflammatory response within endothelial cells (Vergnolle *et al*, 2002).

1.3.4.3 PAR-4 mediated signalling

Unlike PAR-3, PAR-4 has been found to have a more definitive role within the PAR family. Currently, there is less known about the signalling characteristics of PAR-4 as opposed to that of PAR-1 and PAR-2. Numerous studies have concentrated on the functional activation of this receptor, but only a few have looked at the G protein -dependent and -independent coupling events that couple PAR-4 to its downstream effectors, including the MAP kinase pathway (Sabri *et al*, 2003a, Sabri *et al*, 2003b) and NFκB pathway (Suo *et al*, 2003). However, when PAR-4 was initially identified, it was found in transfected COS cells to mediate an increase of IP_3. Such results suggest the ability to couple with $G\alpha_{q/11}$ or $G\alpha_{i/o}$ (Xu *et al,* 1998).

More recently, studies which have assessed PAR-4 mediated signalling have concentrated on cell types including cardiac myocytes (Sabri *et al,* 2003a, Sabri *et al*, 2003b). Steinberg and colleagues provided evidence to suggest that PAR-4 is expressed at a functional level within cardiac myocytes, in which it was found to result in a strong and sustained phosphorylation of p38 MAP kinase, as well as activation of ERK, albeit the ERK response was much more transient. This particular study was carried out in $^{-/-}$ PAR-1 cardiac myocytes, thus demonstrating a role for thrombin mediated PAR-4 activation which was independent of PAR-1 (Sabri *et al*, 2003a).

Further experiments within this study demonstrated via use of Src and EGFR tyrosine kinase inhibitors, that both thrombin and AYPGKF were able to mediate activation of p38 MAP kinase via Src-dependent transactivation of the EFGR. These results therefore suggested that PAR-4 can mediate activation of MAP kinase occurs in a G protein - independent fashion (Sabri *et al*, 2003a). Taken as a whole, these particular studies have demonstrated that the PAR-4 mediated activation of the MAP kinase pathway is partly mediated via transactivation of the EGFR, although the downstream effectors of PAR-4 in MAP kinase activation are not yet fully known. As such, in order to define the full mechanism which underlies PAR-4 coupling to the MAP kinase pathway, more investigation is required.

Other studies that have assessed the functional signalling mechanisms of PAR-4 have been carried out in a murine microglial cell line known as N9 microglial cells (Suo *et al*, 2003). This particular group had previously reported thrombin induced inflammation within these cells, and although it appeared to be a PAR sensitive-mechanism, it was not attributed to PAR-1 (Suo *et al,* 2002). The prior study demonstrated that PAR-1 was in fact, associated with the proliferation of microglial cells, as opposed to inflammation. Therefore, this group were able to determine that the inflammatory response within microglial cells was attributed to another thrombin sensitive receptor, which was found to be PAR-4 (Suo *et al,* 2003). In addition, this group also demonstrated the calcium signalling characteristics mediated by PAR-4. Looking at this phenomenon, it was found that PAR-1 activating peptide showed a sharp transient calcium spike, which rapidly returned to basal level. However, PAR-4 activating peptide, GYPGKF-NH_2 displayed a smaller spike, which although reached a peak in a time-dependent manner was found to have a significantly slower decline – a feature that is suggested to be a characteristic of PAR-4 desensitisation. This study not only demonstrated variation of thrombin receptors within a selected cell type, but also displayed a difference in certain aspects of PAR-4 signalling in comparison to that of PAR-1 (Suo *et al*, 2003).

More recently, it has been demonstrated that PAR-4 co-operates with PAR-1 via coupling with $G\alpha_{i/o}$ in hepatocellular carcinoma cells (Kauffman *et al*, 2007). This particular study demonstrated PTx mediated inhibition of both PAR-1 and PAR-4-dependent cell migration, suggesting that PAR-1 and PAR-4 may be part of a thrombin induced signalling network, necessary for the migration of tumour cells (Kauffman *et al,* 2007). These studies suggest a possible role for PAR-4 in respect of coupling with $G\alpha_{q/11}$ and $G\alpha_{i/o}$, as well as being associated with varied intracellular signalling as a result of transactivation of the EGFR. However, to date, no studies have looked at signalling possibilities relating to other G protein coupling, including $G\alpha_s$ or $G\alpha_{12/13}$. Other signalling possibilities that have also been overlooked to date include $G\beta\gamma$ subunits.

1.3.4.4 PAR-4 mediated regulation of Nuclear Factor kappa B (NFκB)

As previously mentioned, nuclear factor kappa B (NFκB) is a ubiquitously expressed transcription factor which can be found in the majority of eukaryotic cells (reviewed by Perkins & Gilmore, 2006, Ghosh *et al*, 2008). So far, it has been shown that certain GPCRs, including PAR-2 are capable of mediating activation of this pathway (Goh *et al*, 2008, Macfarlane *et al*, 2005, Kanke *et al*, 2001), however, it is unknown if PAR-4 is also capable of this regulating this pathway. This report has suggested that while much is known about the activation of PAR-4, very few studies have concentrated on the intracellular signalling pathways that are regulated via this receptor. While some studies have ascertained that there may be a possibility of PAR-4 regulation of the MAP kinase pathway (Sabri *et al*, 2003 a, Sabri *et al,* 2003b) and others have demonstrated the ability of PAR-4 to mediate release of endothelial NO via a PTx-dependent mechanism (Momota *et al*, 2006), literature to support PAR-4 mediated activation of NFκB is very sparce.

Only one published study has demonstrated the ability of PAR-4 to mediate activation of NFκB (Suo *et al*, 2003), however with no following studies to support these findings, the exact mechanisms whereby PAR-4 mediates activation of this transcription factor are yet to be elucidated. The study by Suo *et al*, demonstrated that PAR-4 is able to mediate a prolonged activation of NFκB in N9 murine microglial cells (Suo *et al*, 2003).

It also demonstrated that both thrombin and the PAR-4 activating peptide GYPGKF-NH_2, stimulated an increased and prolonged nuclear binding of NFκB along with a corresponding increase of TNFα release. However, this study did not provide any evidence to support an intermediate signalling pathway to determine the manner in which PAR-4 causes this activation. Interestlingly, while the possiblity of a pathway induced by PAR-4 in order to mediate activation of NFκB has not yet been elucidated, other GPCRs, including PAR-2 have demonstrated the ability to mediate activation of NFκB via both G protein dependent and independent mechanisms (Goh *et al,* 2008). The study by Goh *et al*, 2008 has demonstrated the abilty of PAR-2 to mediate activation of NFκB at the level of NFκB-DNA binding as well as the phosphorylation of p65 NFκB. Current literature suggests that phosphorylation of p65 at Ser^{536} is an important form of post-translational modification in order to regulate transcriptional activity (Viatour *et al,* 2005). The ability of PAR-2 to phosphorylate p65 NFκB at this critical serine residue therefore suggests that post-transcriptional control of NFκB can be controlled by GPCRs and in this particular case, it can be controlled by PAR-2.

This particular study also assessed the functional coupling of PAR-2 to the NFκB pathway, whereby it displayed a $G\alpha_{q/11}$ -dependent mechanism, which suggested that this pathway would encompass a variety of downstream effectors such as intracellular Ca^{2+}, PLC and PKC. Involvement of $G\alpha_{q/11}$ was confirmed by the use of siRNA and $G\alpha_{q/11}$ inibitor, YM-254890 (Taniguchi *et al*, 2003), which not only resulted in a concentration-dependent inhibition of $[^3H]$-IP_3 accumulation, but also demonstrated a decrease in the nuclear binding of NFκB in primary human keratinocytes (Goh *et al*, 2008).

Prior to this, it has been demonstrated that in the case of PAR-2, activation of NFκB is via an IKK-dependent mechanism. Pre-treatment of the cell line NCTC-2544, which contains a PAR-2 expressing plasmid (clone G) (Kanke *et al,* 2001), with a calcium chelator known as BAPTA-AM, demonstrated an inhibition of IKKα and IKKβ when stimulated with trypsin, the natural agonist of PAR-2.

These results suggest activation of an IKK-dependent nature that is mediated by an increase in intracellular calcium (Macfarlane *et al*, 2005, Kanke *et al,* 2001). However, while such pathways have been elucidated for PAR-2, no such literature exists to support the NFκB-dependent pathway that has been utilised by PAR-4. As such, it can therefore be suggested that as the signalling pathway that is associated with the PAR-4 mediated activation has not yet been elucidated, further study is required in this area in order to identify the mechanisms whereby PAR-4 mediates activation of NFκB.

1.4 AIMS

Proteinase-activated receptor 4 (PAR-4) is the most recently identified member of a unique group of G protein coupled receptors that are activated by means of proteolytic cleavage (Xu *et al,* 1998). To date, there is less known about the signalling characteristics of PAR-4 as opposed to that of other members of the PAR family. While numerous studies have concentrated on functional activation of this receptor, few have looked at the G protein-dependent and -independent mechanisms which couple PAR-4 to the MAP kinase pathway (Sabri *et al*, 2003a, Sabri *et al*, 2003b) and the NFκB pathway (Suo *et al*, 2003).

Therefore, the aims of this study were to characterise PAR-4 mediated MAP kinase and NFκB activation. For this purpose, an expression clone was generated from a human keratinocyte parental cell, NCTC-2544, which stably expressed PAR-4 (Clone 10H9). Clone 10H9 was used in comparison with a cell type which naturally expresses PAR-4 – EAhy-926. This study has employed the use of a selective $G\alpha_{q/11}$ inhibitor YM-254890 (Taniguchi *et al*, 2003), and tyrosine kinase inhibitor AG-1478, to determine if the mechanism whereby PAR-4 couples with these two pathways is dependent on $G\alpha_{q/11}$ and/or transactivation of the epidermal growth factor receptor (EGFR). Further to this, PKC inhibitor GF109203X was used to determine the interaction of PAR-4 with PKC in activation of these pathways. Use of dominant negative adenovirus incorporating an isoform of IKKβ has also been employed to determine the role of the IKK signalosome within this pathway.

Through these particular mechanisms, this study has sought to delineate the pathway and mechanisms whereby PAR-4 may mediate the activation of both the MAP kinase and NFκB pathways, which as previously mentioned still remain largely undefined.

Chapter 2
Materials and Methods

2.1 MATERIALS

2.1.1 General Reagents

All materials and reagents used are of the highest commercial purity available, and were supplied by the list of companies that follows:

Amaxa, Colongne, Germany

Nucleofection Optimisation Transfection kit

Amersham International Plc, Aylsbury, Buckinghamshire, UK.

ECL detection reagents

BDH Laboratory Supplies, Poole, UK

Formic Acid

Bio-Rad Laboratories, Hertfordshire, UK.

Bio-Rad AG ^{TM} Protein Assay kit, pre-stained SDS-PAGE molecular weight markers, AG^{R} 1-X8 (Dowex) Resin, Bradford Protein Dye

Boehringer Mannheim, East Sussex, UK.

Bovine Serum Albumin (BSA, Fraction V), Dithiothreitol (DTT).

Calbiochem, UK

AG1478 (EGFR tyrosine kinase inhibitor)

SB203528 (p38 MAP kinase inhibitor)

U0126 (ERK inhibitor)

SC2154 (IKK2 inhibitor)

BAY11-7082 (IKK2 inhibitor)

BAY11-7085 (IKK2 inhibitor)

GF 109203X (PKC inhibitor)

Corning Costar, Buckinghamshire, UK

All tissue culture plastics, including graduated pipettes, T25, T75 and T150 tissue culture flasks, 6 well tissue culture plates, 12 well tissue culture plates, 24 well tissue culture plates and 96 well ELISA plates.

Gibco Life Technologies Ltd, Renfrewshire, UK.

Tissue culture growth media, DulBeccos Modified Eagle MEM (DMEM), antibiotics (Penicillin/Streptomycin), Geneticin (G418), Fetal Calf Serum (FCS), L-Glutamate, Inerleukin-8 standard.

Perkin Elmer Life Sciences, UK

EasyTides [γ^{32}P]-Adenosine 5'-tri-phosphate

[^{3}H]-myo-D-inositol

Optiphase Hi-safe ^{TM} scintillant

Promega Corporation, Madison, WI, USA

NFκB consensus oligonucleotide

T4 Polynucleotide Kinase Buffer

T4 Polynucleotide Kinase

5X Gel Shift Binding Buffer

Sigma Aldrich, Chemical Company Ltd, Dorset, UK.

Tissue culture growth media M199 (with Earl's Salts), Sodium Dodecyl Sulphate (SDS), Tween-20, Sodium Chloride, Glycine, Lithium Chloride, Acrylamide, bis-Acrylamide, Trizma Base, Sigmacote, Tetramethylenediamine, (TEMED), Sodium Tetraborate, Pepstatin, Aprotinin, Ethylene diamine tetraacetic acid, (EDTA), Ethylene glycol tetraacetic acid, (EGTA), Glycine, Kodak X-Omat Film, Hydrochloric acid, Tetramethylbenzidine (TMB).

Rectapur ™

Chloroform

Whatmann, Kent, UK.

3MM blotting paper, Nitrocellulose Membranes.

2.1.2 Antibodies

Amersham International Plc, Alyesbury, Buckinghamshire, UK.

Horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG.

Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG

Horseradish peroxidase (HRP)-conjugated donkey anti-goat IgG

Biosource EU, Belgium, Europe

Rabbit polyclonal anti-phospho p38

Mouse monoclonal Anti-human Interleukin-8 Capture Antibody

Human Interleukin-8 Biotin Conjugate Detection Antibody

ELISA grade Streptavidin-Horseradish Peroxidase (HRP) Conjugate

Cell Signalling Technology Inc, New England Biolabs, Hertfordshire, England, UK.

Rabbit polyclonal anti-phospho p65 (Ser^{536})

Santa Cruz Biotechnology Inc, CA, USA.

Mouse monoclonal anti-phospho ERK

Rabbit polyclonal anti-IκBα.

Rabbit polyclonal anti-IKKα/β

Rabbit polyclonal anti-p65

Rabbit polyclonal anti-p38

Rabbit polyclonal anti-PY20

Rabbit polyclonal anti-ERK1

Jackson Immunoresearch Laboratories Inc, PA, USA

HRP-conjugated goat anti-rabbit IgG

2.1.3 PAR-4 Activating Peptide

University of Calgary Peptide Service, Calgary, Canada

AYPGKF-NH_2

2.1.4 Protease Enzymes

Sigma Aldrich, Chemical Company Ltd, Dorset, UK

α-Thrombin (Human Plasma).

Trypsin (Human pancreatic)

2.1.5 $G\alpha_{q/11}$ inhibitor

Astellas Pharmaceuticals, Japan

YM-254890

2.2 CELL CULTURE METHODS

All cell culture work was carried out in a class II cell culture hood, following strict aseptic conditions. Unless otherwise stated, all cells were grown in 75cm^2 tissue culture flasks.

2.2.1 Generation of a PAR-4 stably expressing cell line, using NCTC-2544 cells

Human skin epithelial cells, NCTC-2544 cells were transfected with a wild type PAR-4 plasmid (see figure 2.1) by means of electroporation (Amaxa Nucleofection system). Coagulation factor II (thrombin) receptor-like 3 (F2RL3/PAR-4) wild type was cloned into pcDNA3.1 at restriction site EcoRI(5') and Xhol (3'). The open reading frame was amplified by PCR from human lung cDNA, and the insert of 1.2 Kb was sequenced.

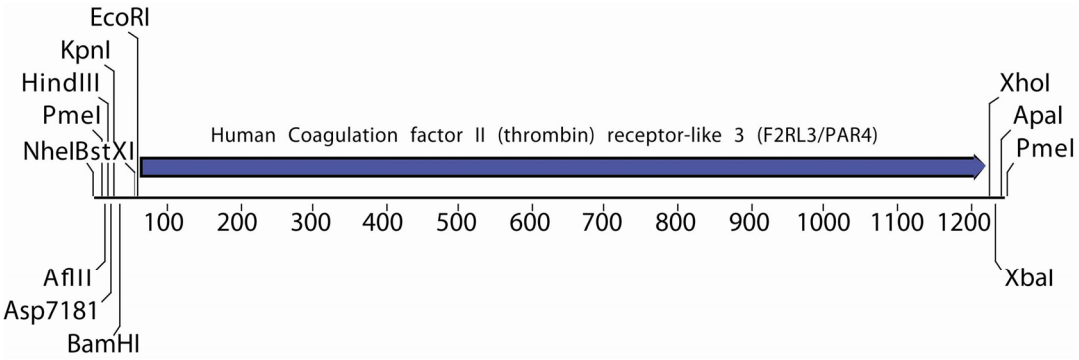


Figure 2.1 Coagulation factor II (thrombin) receptor-like 3 (PAR-4)

2.2.1.1 Electroporation of NCTC-2544 cells by use of Nucleofection

NCTC 2544 cells were grown to 80% confluency on a 12 well plate. Cells were supplemented with 1ml of M199 media (heated to 37^{o}C). Upon reaching confluency, 1 well was used for cell count procedure using haemocytometer, to determine the required cell density (1x10^{6}/100µl sample). Cells were collectively placed in a fresh 15 ml tissue culture tube and subjected to centrifugation at 90 x g for 10 minutes. Cells were re-suspended in 1ml of Nucleofection^{TM} solution. 100 µl of this solution was then removed to be kept for control purposes. 800µl was taken and 16µg of PAR-4/pcDNA3.1 was added. Sample was then aliquoted into 8 separate samples for transfection purposes.

Samples of 100µl were aliquoted into specialised Amaxa certified cuvettes. Nucleofection^{TM} programme was selected as per manufacturer's instructions. Cuvette was inserted into cuvette holder and electroporation procedure was initiated. Once display read 'OK', samples were removed from carousel and 500µl of warmed M199 media (37^{o}C) was added to the cuvette. Samples were then transferred back into a fresh 12 well plate. For this purpose, it was necessary to use the plastic pipettes supplied with Nucleofection^{TM} kit to ensure minimal loss or damage to cells. Cells were then subjected to incubation in a humidified incubator (37^{o}C, 5% CO_2). Cells were then subjected to clone selection pressure using Geneticin (G418), to ensure that cells expressing the PAR-4 plasmid survived. Cells were left in optimised incubation conditions until reaching 90% confluency, from which they could be sub-cultured.

2.2.2 Sub-culturing by use of Accutase

At around 90% confluency, cells were sub-cultured by enzymatic dissociation using a collagenase enzyme substitute for trypsin known as Accutase. The medium was removed and cells washed once with 2ml of sterile phosphate buffered saline (PBS), (150nM NaCl, 5.4 mM KCl, 10mM Na_2PO_4, 1.5mM KH_2PO_4, pH 7.4). This solution was then aspirated and replaced with 2 ml of fresh, sterile 1X Accutase. Cells were then placed in an incubator at 37^{o}C humidified at 5% CO_2 for approximately 2-5 minutes.

Gentle tapping of the flask resulted in complete detachment of the cells, after which cells were washed with M199 medium, supplemented with 250units/ml penicillin, 100 μg/ml streptomycin, 27mg/ml glutamine and 10% fetal calf serum. To continue cell selection, Geneticin was added every second sub-culture. The mixture of cells and M199 was then diluted 1:3 with additional M199 and placed in a fresh flask. Cells were maintained in an incubator at 37^{o}C, 5% CO_2, and media replaced every second day.

2.2.3 EAhy 926 cells

Low passage EAhy 926 cells were maintained in Dulbecco's Modified Eagle's Medium (D-MEM), supplemented with 250units/ml penicillin, 100μg/ml streptomycin, 27mg/ml glutamine, 10% fetal calf serum and 0.5% HAT supplement. Cells were grown to approximately 80% confluency and passaged using Versene, (0.2% EDTA in sterile PBS). Cells were passaged at a ratio of 1:5 and placed in a fresh tissue culture flask and supplemented with fresh DMEM. Cells were maintained in an incubator at 37^{o}C, 5% CO_2, and media replaced every second day.

2.2.4 Human Embryonic Kidney (HEK) 293 cells

Low passage HEK 293 cells were maintained in modified eagle's medium (MEM), supplemented with 250unit/ml penicillin, 100μg/ml streptomycin, 27mg/ml glutamine and 10% fetal calf serum. Cells were grown to approximately 80% confluency and passaged using trypsin-EDTA solution (0.25% trypsin, 1mM EDTA). Cells were passaged at a ratio of 1:5 and placed in a fresh tissue culture flask, supplemented with fresh MEM. Cells were maintained in an incubator at 37^{o}C, 5% CO_2, and media replaced every second day.

2.3 PREPARATION OF RECOMBINANT ADENOVIRUS

Replication deficient adenoviral vectors encoding dominant negative inhibitory kappa B kinase β (Adv IKKβ $^{+/-}$) were utilised in this project. HEK 293 cells were used for the propagation of this adenovirus. The purification of this virus was achieved by means of Clontech Adeno X Viral Purification Kit. Following purification, a method known as the end point dilution method was employed in order to determine the titration of viral stock, (Nicklin & Baker, 1999).

2.3.1 Generation of Crude Adenoviral Stock

Crude $^{+/-}$IKKβ Adenoviral stock was generated in HEK 293 cells, by infection of a 75 cm^2 flask which had been grown to confluency. 0.5µl of purified viral stock was used to infect the cells were infect in a class II adenoviral tissue culture hood. Cells were then incubated at 37^oC, 5% CO_2 for a period of 5 days, by which time cellular plaques were visible. Cells were removed from the flask and centrifuged at 1500 x g for 5 min. The viral pellet was washed twice with PBS, and again centrifuged at 1500 x g for 5 min. Pellet was re-suspended in HE buffer (10mM HEPES, pH 7.5, 1mM EDTA), followed by placement in liquid nitrogen and thawed at 37^OC. The freeze-thaw cycle was repeated for a total of 3 times. Cells were centrifuged for a final time at 1500 x g for 5 min, and the supernatant produced, containing the crude adenoviral stock was transferred into a sterile tube and stored at -80^oC until required.

2.3.2 Generation of Purified High-Titre Stocks of $^{+/-}$IKKβ Adenovirus

Large scale generation of $^{+/-}$IKKβ adenovirus was achieved by amplification of the crude adenoviral lysate as described in section 2.3.1. Twenty-five 150 cm^2 flasks of HEK 293 cells were grown to 80 % confluency, each of which was infected with 100µl of crude adenoviral stock. Flasks were incubated at 37^oC, 5% CO_2 for 5-7 days, again until cellular plaques were apparent. Cells were removed from the flasks and centrifuged at 1500 x g for 5 min. 100ml of the viral supernatant (supernatant A) was kept for purification purposes. Cells were pooled into a single large pellet and washed twice with PBS.

As with the preparation of crude adenoviral stock, the large pellet was also underwent a total of 3 freeze thaw cycles. Following this, the supernatant, (Supernatant B), was collected in a fresh tissue culture tube, ready to undergo purification (see section 2.3.3).

2.3.3 Purification of Adenovirus by Adeno X Viral Purification Method

Purification of the adenovirus was performed by the use of Takara Adeno X Purification Kit, closely following manufacturer's instructions. Following the 3 x freeze thaw cycles (section 2.3.2), supernatant A was pooled with supernatant B, and by means of filtration, the pooled supernatant was transferred into a sterile container as per manufacturer's instructions. Once filtered, 40µl of benzonase was added, and incubated at 37^{o}C for exactly 30 min. During incubation, 100 ml of 1 X dilution buffer and 60ml of 1 X Wash buffer were prepared from 5 X stock buffers, as per manufacturer's instructions. Following 30 min incubation, the 100 ml 1X dilution buffer was added to the filtered supernatant. The diluted supernatant was then subjected to filtration by employment of the Takara mega filtration syringe system. Following filtration of the supernatant, the 60 ml of 1 X wash buffer was then filtered through the syringe system. Finally, the virus was eluted from the mega filter by addition of 3ml of manufacturer's 1 X elution buffer, which was collected in a fresh tissue culture tube. The virus was then aliquoted into working amounts, and kept at -80^{o}C prior to titration.

2.3.4 Titration of Adenovirus by End-Point Dilution

The end point dilution method was used to determine the titration value of the generated adenovirus (Nicklin & Baker, 1999). HEK 293 cells were grown to approximately 80% confluency in a 96 well tissue culture plate in MEM contaning 10% fetal calf serum. A serial dilution of the virus was used when infecting cells (see Figure 2.2). During infection, the media containing 10% fetal calf serum was replaced with MEM containing only 2% fetal calf serum. The plate was then incubated at 37^{o}C, 5% CO_2 for 10 days, until the viral cytopathic effect had been optimised. Wells containing the plaques were then counted on a scale known as plaque forming units (pfu), and calculated as shown in the example of figure 2.3.

2.3.5 Infection of Cells with Adenovirus

Cells were grown to confluency on a 6 well tissue culture plate, and the number of cells present was determined by means of a haemocytometer. Following the count, cells were incubated with the appropriate plaque forming units of the necessary adenoviral construct. Cells were incubated for 24 hours at 37^{o}C, 5% CO_2 in normal growth medium, after which cells were quiesced overnight before stimulation.

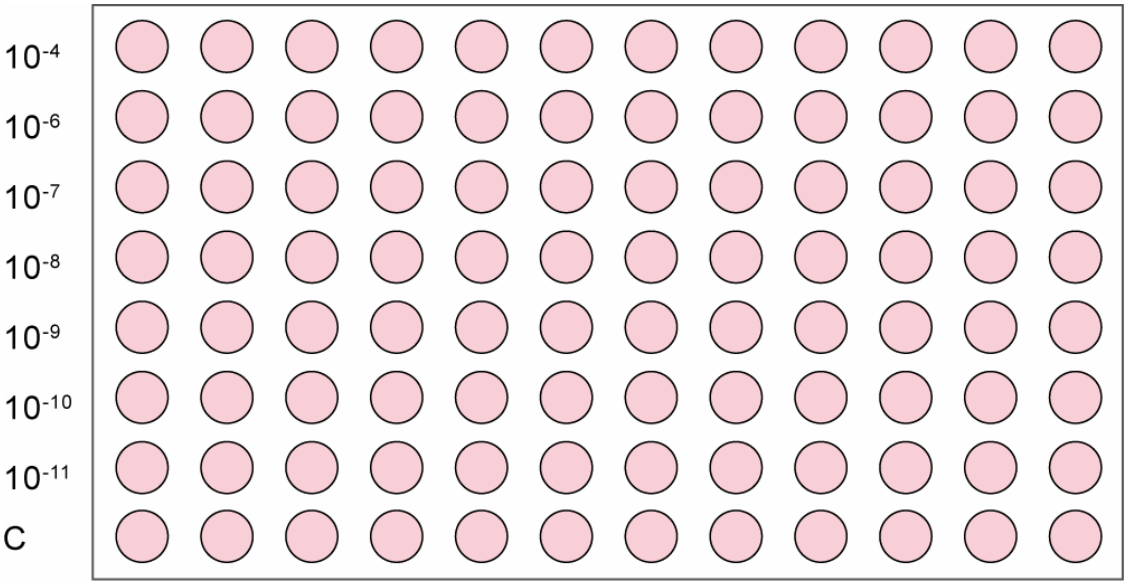


Figure 2.2 Layout of 96 well plate for plaque purification by end-point dilution titration method. $^{+/-}$ IKKβ adenovirus was added to the middle 10 wells of each row in a serial dilution fashion. Only the bottom row of wells was left blank for control purposes.

Number of Wells in 96 well plate Containing plaques:

10^{-4} = 10/10 = 100%
10^{-6} = 10/10 = 100%
10^{-7} = 10/10 = 100%
10^{-8} = 10/10 = 100%
10^{-9} = 10/10 = 100%
10^{-10} = 5/10 = 50%
10^{-11} = 2/10 = 20%

Proportionate distance = $\frac{\text{(\% wells positive above 50\%) – 50\%}}{\text{(\% wells positive above 50\%)-(\%wells positive below 50\%)}}$

$= \frac{100-50}{100-50} = 1$

Log ID_{50} = log dilution factor at % wells positive above 50% + (proportionate distance x -1)

= -9+(-1) = -10

ID_{50} = 10^{-10}

TCID (tissue culture infectivity dose)$_{50}$/100µl = $\frac{1}{10^{-10}}$ = 10^{10}/100µl

$TCID_{50}$/ml = 10^{10} x dilution factor

= 10^{10} x 10

= 1x10^{11}/ml

1 TCID50 ≈ 0.7 plaque forming units (pfu). Referring to this figure, final titre for virus = 1 x 1011 x 0.7 = 7 x 10^{10} pfu/ml

= $\frac{7 \times 10^{7}}{}$ pfu/µl

Figure 2.3 Example of the End Point Titre method to establish adenovirus titre

2.4 MEASUREMENT OF [^{3}H]-INOSITOL PHOSPHATE ACCUMULATION

NCTC-2544 cells stably expressing PAR-4 were individually screened for the accumulation of total inositol phosphate. Cells of each expression clone were grown to confluence on a 24 well plate and quiesced overnight in the presence of ^{3}H-2-Myo-inositol (0.5μCi/well). Lithium Chloride (0.5%, 0.5M), was added to the media 15 minutes prior to addition of agonists, in order prevent inositol-1-phosphatase activity (Oldham, 1990).

Following stimulation with agonists, cells were washed once with ice cold PBS to stop the reaction. Cells were then scraped twice in the presence of 1 ml of methanol, after which they were removed to a fresh scintillation vial. Chloroform (0.5ml) was then added to give a 2:1 methanol:chloroform ratio. Vials were then incubated on ice for 90 min. Following ice bath incubation, 0.8ml of distilled water and a further 0.5ml of chloroform were added, and the vials were vortexed and then allowed to settle for a further 60 min at 4^{o}C. Once the organic separation was complete, the upper aqueous phase (1ml) was carefully removed and added to vials which contained 1ml of ion exchange resin (AG 1-X8 Resin). Vials were gently vortexed, after which the resin was allowed to settle. The liquid layer was then aspirated leaving only the sedimented resin at the bottom. Resin was washed with 2.5ml of distilled water, allowing resin to settle, and the water was then aspirated. The resin was then washed in 2.5ml of sodium tetraborate solution (5mM $Na_2B_4O_7.10H_2O$, 60mM ammonium formate).

Once aspirated, the resin was once again washed in 2.5 ml of distilled water, after which 1ml of ammonium formate solution, (1M ammonium formate, 0.427% formic acid, in distilled water), was added in order to elute the total phosphoinositides ([^{3}H]-IP_{1-4}) from the DOWEX resin, (Berridge *et al*, 1982). Vials were vortexed and resin allowed to settle. Aqueous phase was gently removed and added to a fresh scintillation vial. Further to this, 4ml of Optiphase Hi-safe^{TM} scintillation fluid was added. Finally, vials were vortexed and read on a Packard 1500 Tri-carb liquid scintillation analyser for 2 min per sample.

2.5 WESTERN BLOT ANALYSIS

2.5.1 Preparation of whole cell extracts

Cells were grown to confluency in 6 or 12 well tissue culture plates, and quiesced overnight in serum-free media. Following this, cells were exposed to appropriate concentrations of selective agonists or inhibitors for relevant time periods, after which the cells were placed on ice. Cells were then washed twice with 1ml of ice cold PBS after which 150µl of SDS-PAGE sample buffer (63mM Tris-HCL, pH 6.8, 2mM $Na_4P_2O_7$, 5mM EDTA, 10% (v/v) glycerol, 2% (w/v) SDS, 50 mM DTT, 0.007% (w/v) bromophenol blue) was added. Cells were then scraped and passed through a 21 gauge needle repeatedly in order to shear the chromosomal DNA. Samples were then transferred into pierced Eppendorf tubes, after which they were boiled for 5 minutes, to achieve protein denaturation. Samples were then stored at -20^{o}C until required.

2.5.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Resolving gels containing appropriate amounts (7.5% (w/v), 9% (w/v), 10% (w/v), 11% (w/v) of acrylamide: N'-methylenebis-acrylamide (30:0.8), 0.375M Tris pH 8.8, 0.1% (w/v) SDS and 0.05% (w/v) ammonium persulfate (APS) were prepared. Polymerisation was initiated by the addition of 0.05% (v/v) N,N,N,N', N'-tetramethylethylenediamine (TEMED). This solution was then poured between 2 glass plates which had previously been assembled on the appropriate vertical slab configuration according to manufacturer's instructions, after which they were overlaid with 200 µl of 0.1% (w/v) SDS. Once polymerised, a stacking gel containing 10% (v/v) acrylamide. N,N'-methylenebisacrylamide (30:0.8), in 125 mM Tris, pH 6.8, 0.1% (w/v), SDS, 0.05% APS and 0.05% (v/v) TEMED was poured directly on top of the resolving gel, and a comb inserted. Following polymerisation, the comb was removed and the polyacrylamide gels were then assembled in a Bio-Rad Mini-PROTEAN II ^{TM} gel running kit. Aliquots of the prepared samples were then loaded into the wells using a Hamilton micro-syringe. Pre-stained SDS-PAGE molecular weight markers of known molecular weights were run concurrently with the samples in order to identify the polypeptide of interest. Samples were electrophoresed at a constant voltage of 130 V until the bromophenol blue sample dye had reached the bottom of the gel (approximately 90 min).

2.5.3 Electrophoretic Transfer of Proteins to Nitrocellulose Membrane

The proteins that had been separated by SDS-PAGE were then transferred to a nitrocellulose membrane by means of a standard electrophoretic transfer blotting method (Towbin *et al*, 1979). The gel was firmly pressed against a nitrocellulose sheet and assembled in a transfer cassette, where it was sandwiched between 3MM paper and 2 sponge pads. The cassette was then immersed in blotting buffer, (25mM Tris, 19mM Glycine, 20% (v/v) Methanol) in a Bio-Rad Mini Trans-Blot ^{TM} tank, where a constant current of 350mA was applied for 2 hours. The tank was cooled during this period by inclusion of an ice reservoir.

ANTIBODY	COMPANY	CATALOGUE	DILUTION	SECONDARY
Total ERK	Santa Cruz	sc-153	1:5000	HRP α-Rabbit
Total p38	Santa Cruz	sc-7149	1:15000	HRP α-Rabbit
Total NFκB	Santa Cruz	sc-372	1:7500	HRP α-Rabbit
Total IκBα	Santa Cruz	sc-371	1:7500	HRP α-Rabbit
PY20	Santa Cruz	sc-508	1:5000	HRP α-Mouse
Phospho ERK	Santa Cruz	sc-81492	1:5000	HRP α-Mouse
Phospho p38	Biosource	368500	1:15000	HRP α-Rabbit
Phospho p65-NFκB (ser^{536})	Cell Signalling Technologies	3033L	1:5000	HRP α-Rabbit
Total IKKα/β	Santa Cruz	sc-101707	1:15000	HRP α-Rabbit

Table 2.1 Antibodies Used in Study

2.5.4 Immunological Detection of Proteins

Following the protein transfer to nitrocellulose membrane, the membrane was removed after which it was blocked by incubating the membrane in a solution of 2% (w/v) BSA in Sodium Chloride-Trizma Base-Tween (NaTT) buffer (150mM NaCl, 20mM Tris, 0.2% (v/v) Tween 20, pH 7.4), for 2 hours. The 2% BSA blocking buffer was then removed and replaced with 0.2% (w/v) BSA in NaTT buffer, and incubated overnight at room temperature on a platform shaker, with an appropriate amount of primary antibody added, which was specific to the protein of interest.

The following morning, the incubated blots were washed for 90 minutes, at intervals of 15 minutes with NaTT buffer on a platform shaker, after which a secondary horseradish peroxidase (HRP) conjugated IgG antibody was added in NaTT buffer containing 0.2% BSA. The nitrocellulose membrane was then incubated with the secondary antibody at room temperature on the platform shaker for a period of 90-120 min. The membranes were once again washed at room temperature for 90 minutes, at intervals of 15 min with NaTT buffer. Following this, the membranes were exposed to enhanced chemiluminescence (ECL) reagent for 1-2 min and blotted onto paper towel to remove any excess liquid. The blots were then mounted onto an exposure cassette and covered with cling film. Blots were exposed to Kodak X-OMAT LS film for 1-5 min under darkroom conditions and developed using a Kodak M35-M X-OMAT processor.

2.6 SCANNING DENSITOMETRY

Results obtained via Western blotting were scanned at 150 DPI on a Lexmark P4350 scanner utilising Lexmark Studio Imaging Software for calibration purposes. Using Scion Image Software (Scion Corp, Maryland, USA), an identical sized area was chosen from each protein band (10mm x 7mm), and density measurement taken for quantification. Identical amounts of background area were also measured in order to subtract background from total protein. Background subtraction was performed by transferring measurements into Microsoft Excel, where measured values were normalised against control values.

2.7 ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

2.7.1 Preparation of Nuclear Extracts

Cells were grown to 90% confluency on 6 well plates, and quiesced overnight with serum free media. While cells were exposed to appropriate agonists, buffering solutions for preparation of nuclear extracts were made. 10 ml of Buffer 1 (10mM HEPES (pH 7.9), 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM 1,4 Dithiothreitol (DTT), 0.5mM PMSF, Aprotinin 10μg/ml, Leupeptin 10μg/ml, Pepstatin 10μg/ml), was prepared as a low salt solution in order to swell the cells. 10 ml of Buffer 2 (20mM HEPES (pH7.9), 25% (v/v) glycerol, 0.4M NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT, 0.5mM PFSF, Aprotinin 10μg/ml, Leupeptin 10μg/ml, Pepstatin 10μg/ml), was prepared as a high salt solution to cause cell lysis. Once stimulated, cells were washed twice in ice cold PBS. Cells were then scraped into fresh Eppendorf tubes with 500 μl of ice cold PBS. Centrifugation at 13000 rpm for 2 min allowed for recovery of cells. Supernatant was aspirated and cells re-suspended in 400μl of Buffer 1. Samples were then incubated on ice for 15 min. 25 μl of 10% (v/v) NP-40 was added to each sample and vortexed at full speed for 10 seconds. Samples were centifuged at 13000 rpm for 2 min and supernatant aspirated. Following this, 20 μl of buffer 2 was added, and samples were then vortexed briefly to loosen cell pellet from the wall of Eppendorf tube. Samples were then placed on a shaker at 4^{o}C for 15 min. Following this, samples were placed in a cooled sonicator and subject to sonication 2 x 30 seconds. Finally, samples were centrifuged at 13000 rpm for 15 min at 4^{o}C, and supernatants transferred into sterile Eppendorf tube, and stored at -20^{o}C until required.

2.7.2 Determination of protein concentrations by Bradford Reaction

The protein content of the nuclear extracts was quantified using BioRAD^{TM} protein assay kit, using the Bradford method. Duplicate aliquots of samples were made up to 800μl with water, following which 200μl of concentrated protein dye reagent was added. Samples were then vortexed and allowed to stand for 15 min before reading the sample absorbance at 595nm. Protein concentrations of the samples were quantified using a standard curve of Bovine Serum Albumin (BSA) at 0-20 μg/ml^{-1} prepared in nuclear extract buffer 2.

2.7.3 Labelling of Oligonucleotides with ^{32}P-γ-ATP

An oligonucleotide containing the consensus binding sequence for the transcription factor Nuclear Factor kappa B (NFκB) (5'-AGTTGAGGGGACTTTCCCAGGC-3'), was purchased from Promega. Oligonucleotide was labelled at the 5'-end via incubation with T4 polynucleotide kinase (Promega), nuclease free water and ^{32}P-γ-ATP (1μCi/μl) for 30 min at 37^{o}C. Termination of the reaction occurred by addition of 0.5M EDTA. Following this, the labelled oligonucleotide was diluted 1:10 with TE buffer (10mM Tris base, pH 8.0, 1mM EDTA). Efficiency of ^{32}P-γATP incorporation into the oligonucleotide was determined by aliquoting spots of the labelled primer onto DE81 filters. Two of the filters were subject to scintillation counting to determine the total amount of label in each aliquot. The other two filters were subjected to washing with 0.5M Na_2HPO_4 (pH6.8), resulting in the removal of any unincorporated label. Following this, all 4 filter papers were subjected to scintillation counting, from which the percentage of label incorporation could be determined. Determination of the label incorporation was achieved by using the following calculation:

$$\text{Percentage incorporation} = \frac{\text{cpm incorporated}}{\text{cpm total}} \times 100$$

Removal of unincorporated oligonucleotide was achieved by running probe through G25 spin columns at 11000 x g for 3 min. Although this step is optional, it is recommended as quality of shift assay is improved. Once prepared, the oligonucleotide was stored at 4^{o}C until required.

2.7.4 NFκB Transcription Factor Binding Assay

Non-denaturing EMSA gels, using thick spacers and combs (1.5mm) were cast (10X TBE buffer, 2% (w/v) bis-acrylamide, 25% (w/v) acrylamide, 50% (v/v) glycerol, distilled H_2O, 0.05% TEMED, 10% (w/v) APS) and run for 30 minutes at 100 volts in 0.5% TBE buffer, (10 X TBE buffer: 45mM Tris, 1mM EDTA, 45mM boric acid, pH 8.3).

While empty gel was running, 5μg of nuclear extract samples were placed into fresh Eppendorf tubes and made up to a final volume of 7 μl using dH_2O. 2μl of DNA binding buffer (20% glycerol, 5mM EDTA, 2.5mM DTT, 250mM Tris-HCL (pH 7.5), 0.25mg/ml poly(dI-dC), was added to samples, and left to incubate at room temperature for 20 minutes.

Samples were transferred to radiation bench following incubation, where 1μl (15,000 cps) [γ-^{32}P]-labelled consensus oligonucleotide was added and samples were left to incubate for a further 20 minutes at room temperature. Following this, incubation of the samples was terminated by the addition of 1μl of non-denaturing loading dye buffer (250mM Tris-HCL (pH 7.5), 0.2% (w/v) bromophenol blue, 40% (v/v) glycerol). Working at a radiation-bench, samples were loaded onto the non-denaturing gels. The gels were then run in a BioRad electrophoresis tank at 100 Volts for 55 minutes only, ensuring that radioactive samples did not migrate from the bottom of the gel into surrounding buffer. Gels were then sandwiched between 2 cellophane sheets and dried for 2 hours in gel dryer. To determine the level of binding of the transcription factor, dried gels were subjected to overnight autoradiography, and placed in -80^{o}C. Film was then developed using Kodak X-Omat film developer. Scanning densitometry was performed as described in section 2.6.

2.8 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

2.8.1 Preparation of Samples to assess IL-8 production

Cells were grown to confluency in 6 or 12 well tissue culture plates, and quiesced overnight in serum-free media. Following this, cells were exposed to appropriate concentrations of selective agonists or inhibitors for relevant time periods, after which the cells were placed on ice. Supernatants were collected in clean Eppendorf tubes, and centrifuged at 13000 rpm for 5 min to pellet any cellular debris that may have been present. Again, the supernatant was transferred into fresh Eppendorf tubes, and samples stored at -20^{o}C until required.

2.8.2 Preparation of ELISA plate

48 hours prior to assay, preparation of the ELISA plate was started by coating 96 well plate with 100µl per well Interleukin-8 Capture Antibody (1:2000), diluted in manufacturer's coating buffer. This plate was then wrapped in tin foil, and incubated for a minimum of 18 hours at 4^{o}C. Antibody was then aspirated, and replaced with manufacturer's blocking buffer (200µl/well). Again, plate was covered with tin foil, and incubated at 4^{o}C overnight.

2.8.3 ELISA Assay

On day of assay, a range of IL-8 standards were made, ranging from 0-1000pg, as per manufacturer's instructions. Supernatant samples were then diluted 1:10 with manufacturer's assay diluent buffer. Blocking buffer was aspirated, and both standards and samples of unknown IL-8 content were loaded onto the prepared 96 well plate, and incubated at 37^{o}C, 5% CO_2 for 1 hour. Following incubation, samples were aspirated and washed 3 times with manufacturer's wash buffer, and wells tapped dry on paper towel. Biotinylated Interleukin-8 Detection antibody (1:2500), was applied to both standard and unknown wells, and incubated on a platform shaker for 90 min at room temperature. Following 90 min incubation, antibody was aspirated and wells were again washed 3 times with manufacturer's wash buffer, and tapped dry on paper towel. The final antibody, Streptavidin HRP conjugate (1:2000), was applied to all wells and incubated on a platform shaker for 90 min.

Following incubation, antibody was aspirated and wells washed 3 times with manufacturer's wash buffer. Substrate composed of 6mg/ml TMB (dissolved in DMSO) was applied to all wells, and incubated on a platform shaker for 30 min at room temperature. Due to light sensitive nature of substrate, plate was covered prior to placement on rotator. When cover was lifted, IL-8 production was apparent by a colour change from clear to light blue (see figure 2.4). In order to terminate reaction, 100µl stop solution (10% sulphuric acid in dH_20) was applied, showing further colour change from blue to yellow (see figure 2.4). Plate was then read at 450nm to determine concentrations of IL-8 present.

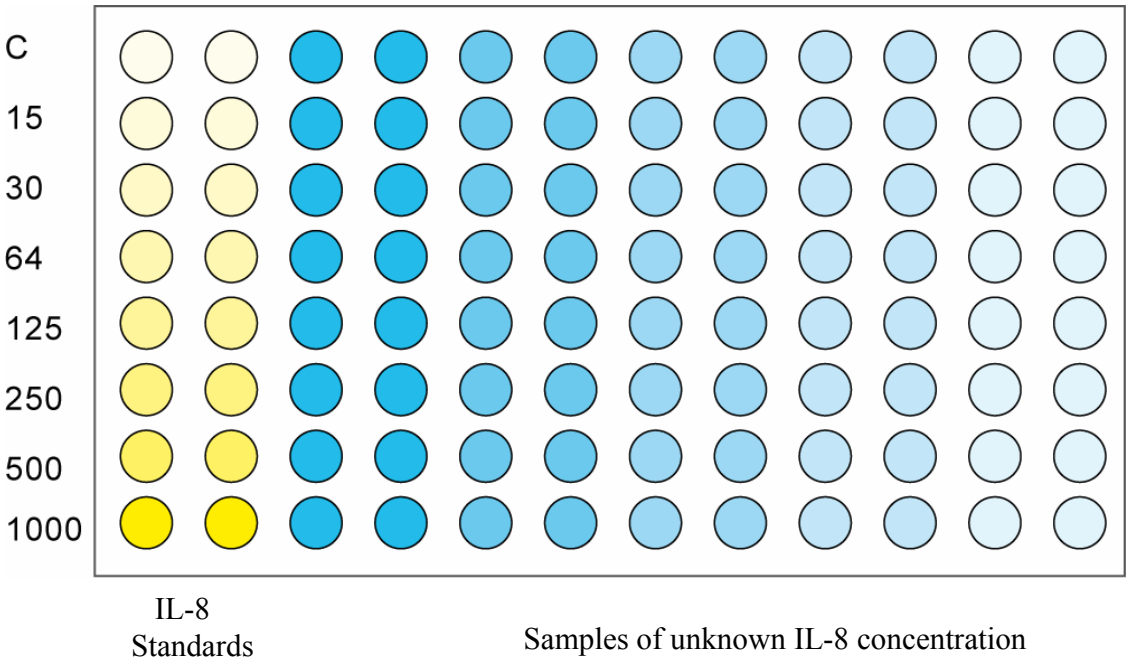


Figure 2.4 Layout of 96 well plate for Enzyme linked immunosorbent assay (ELISA). IL-8 standards are present as demonstrated by graduated yellow samples (indicating that stop solution had been added), whereas, graduated blue samples show varied concentrations of IL-8 in assayed samples (blue colour indicating stop solution had not yet been added).

2.9 IMMUNOPRECIPITATION

To determine if PAR-4 stimulates phosphorylation of IκBα at Tyrosine 42, an immunoprecipitation assay was performed, which employed the phospho-tyrosine antibody, PY20 in order to assess tyrosine phosphorylation of IκBα. Clone 10H9 cells were grown to confluency in 6 wells and rendered quiescent overnight in serum-free media. Cells were then stimulated with AYPGKF-NH_2 (100μM) for 30 min. During stimulation, fresh eppendorf tubes were filled with 20μl of glutathione-sepharose beads, resuspended in 20% EtOH. Tubes were centrifuged at 13,000 rpm for 1 min to pellet beads after which EtOH was aspirated and 10μl of IκBα antibody added to each tube. These tubes were then placed on cooled shaker for 30 min. While the tubes were shaking, stimulated cells were placed onto ice and washed twice with ice cold PBS.

Cells were then scraped in solubilisation buffer (25mM HEPES-NaOH, 5mM β-glycerophosphate, 0.1mM Na_3VO_4, 2mM DTT, pH 7.6, 0.5mg/ml leupeptin, 0.5mg/ml aprotinin, 0.2mM PMSF) into fresh eppendorf tubes and incubated for 30 min on ice. Following 30 min incubation, beads were again centrifuged at 13,000 rpm and samples transferred onto the beads. Samples were then placed onto a cooled shaker for 60 min after which they were centrifuged for 1 min at 13,000 rpm. Samples were washed twice in solubilisation buffer and then vortexed prior to placing samples onto rotating wheel for 1 hour at 4^{o}C. Following this, samples were centrifuged at 13,000 rpm for 2 min and samples washed twice in ice cold solubilisation buffer. Finally, following final centrifugation at 13,000 rpm, 100μl of the supernatant was mixed 1:1 with Western blot sample buffer. The pellets were also mixed with Western blot sample buffer 1:4 and all samples were boiled for 5 min and stored at -20^{o}C until resolved by Western blotting.

2.10 STATISTICAL ANALYSIS

The data presented were expressed as mean $\pm$ s.e.m. For applicable cases, statistical analysis was performed using GraphPad Prism 4, by means of one-way ANOVA with Dunnet's post-test or paired student's t-test. In the case of the data presented, differences were considered to be significant at $p<0.05$.

Chapter 3

Characterisation of PAR-4 mediated activation of the MAP kinase pathway in NCTC-2544 cells stably expressing PAR-4, and in naturally expressing cells, EAhy-926

3.1 INTRODUCTION

Identified in 1998, PAR-4 is the most recently identified member of the family of proteinase-activated receptors (Xu *et al*, 1998). PAR-4 has been identifed in a variety of tissues within the body, including pulmonary (Ando *et al*, 2007), gastro-intestinal (Mule *et al*, 2004) and endothelial tissues (Fujiwara *et al*, 2004). To date, the intracellular signalling mechanisms of PAR-4 have not been fully characterised and as such, there are fewer relating to this receptor as that of its other family members. Few studies have concentrated on the downstream signalling events mediated by PAR-4, although it has been shown in a model of cardiomyocytes to activate the MAP kinase pathway, in particular p38 MAP kinase (Sabri *et al,* 2003a, Sabri *et al*, 2003b), as well as having a potential role in the regulation of the NFκB pathway (Suo *et al*, 2003). This study therefore sought to examine the signalling characteristics of PAR-4 by utilising an NCTC-2544 skin cell line which expresses PAR-4 in a stable manner (Clone 10H9) and EAhy-926 cells, which naturally express PAR-4.

Previously in this laboratory, a cell line which expressed PAR-2 in a stable manner (clone G) was generated through use of NCTC-2544 cells (Kanke *et al*, 2001). Use of clone G cells demonstrated that while these cells show selective expression for PAR-2, NCTC-2544 cells express negligible amounts of endogenous PAR-1, PAR-2, PAR-3 or PAR-4 mRNA (Kawabata *et al*, 2004). As such, the use of NCTC-2544 cells for the study of PAR-4 may be suggested as a useful cell type for initial characterisation of the receptor. This is because the responses gained from the use of selective PAR-4 agonists can be attributed to PAR-4 activation with little interference from other members of the PAR family. Further to these initial characterisation studies, a cell type found to express PAR-4 endogenously was used, EAhy-926 cells. Edgell *et al,* (1983) established EAhy-926 cells by the fusing of human umbilical vein endothelial cells (HUVEC's) with an epithelial derived cell type, A549, in order to establish the function of factor VIII-related antigen within the vascular endothelium, which in these cells, is easily immunofluoresced (Edgell *et al*, 1983).

No published studies have selected this cell type to assess characterisation of PAR-4; however, studies within this laboratory have shown successful PAR-2 expression (Ritchie, PhD thesis, 2005). Initial data has shown that PAR-4 is also expressed naturally in this cell type, and can be successfully associated with the activation of ERK and p38 MAP kinase.

The signalling pathways which have been the topic of interest for this project included the mitogen-activated protein kinase (MAPK) pathway, in particular ERK and p38 MAP kinase. The second pathway of interest that has been investigated in this study is the NFκB pathway. Documented evidence has established NFκB as a key transcription factor in the transcription of inflammatory genes (Reviewed by Mercurio & Manning, 1999, Ghosh *et al*, 1998) and has previously been shown to be regulated by PAR-2, another member of the PAR receptor family (Goh *et al,* 2008, Macfarlane *et al,* 2005, Kanke *et al*, 2001). This study has examined PAR-4 activation by means of its natural protease activator, thrombin (Xu *et al*, 1998) and has also utilised PAR-4 selective activating peptide AYPGKF-NH_2 (Faruqi *et al,* 2000). Also used within this study was YM-254890 (see table 3.1). This is a selective inhibitor of $G\alpha_{q/11}$ coupling, which was used to determine that PAR-4 mediated [^{3}H]-inositol phosphate ([^{3}H]-IP) accumulation as well as phosphorylation of ERK and p38 MAP kinase occur via PAR-4 coupling with $G\alpha_{q/11}$.

AGONIST	EC_{50}	INHIBITOR	IC_{50}
Thrombin (non-selective PAR-4 agonist)	3.2nM (platelets) (Nylander & Mattsson, 2003)	YM254890 (selective inhibitor of $G\alpha_{q/11}$)	<0.6μM (Taniguchi *et al*, 2003)
AYPGKF-NH_2 (selective peptide based PAR-4 agonist)	14μM (Hollenberg & Saifeddine, 2001)	AG1478 (selective EGFR tyrosine kinase inhibitor)	3nM (Cole, 1999)
PMA (direct activator of PKC)	1nM (McFerran *et al*, 1995)	GF109203X (selective inhibitor of classic PKC isoforms)	2μM (Toullec *et al*, 1991)

Table 3.1 Agonists and Inhibitors used for PAR-4 mediated activation of the MAP kinase pathway.

3.2 CHARACTERISATION OF PAR-4 MEDIATED SIGNALLING IN NCTC-2544 CELLS, STABLY EXPRESSING PAR-4 (CLONE 10H9)

3.2.1 Generation of PAR-4 stable expression cell line in NCTC-2544 cells

In order to investigate the role of PAR-4 mediated activation of the MAP kinase pathway, it was necessary to generate a cell line which was able to express PAR-4 in a stable manner. This was achieved by utilising a human keratinocyte cell line, NCTC-2544 which was used as a parental cell (see section 3.1). PAR-4 pcDNA was transfected into NCTC-2544 cells (see section 2.2.1.1) thus resulting in the generation of PAR-4 expressing cells, which were assessed for incorporation of the plasmid by assessing accumulation of total [^{3}H]-IP.

3.2.2 Screening of PAR-4 expression cell types by assessment of total [^{3}H]-inositol phosphate accumulation

The transfection of PAR-4 pcDNA into parental NCTC-2544 cells resulted in the generation of 100 clones. A number of these cell types were assessed for total [^{3}H]-IP by activation with thrombin. Only 3 of these cell types namely 7H6, 10B9 and 10H9 respectively, demonstrated an increase of total [^{3}H]-IP (See figure 3.1). Clone 10H9 demonstrated a stimulation of 17.7$\pm$5.4 (**p<0.01, n=4), fold above basal at 60 min when stimulated with thrombin (5units/ml), which was significantly higher than any other expression clone generated. Clone 10H9 was therefore sub-cultured and used for the remainder of the project.

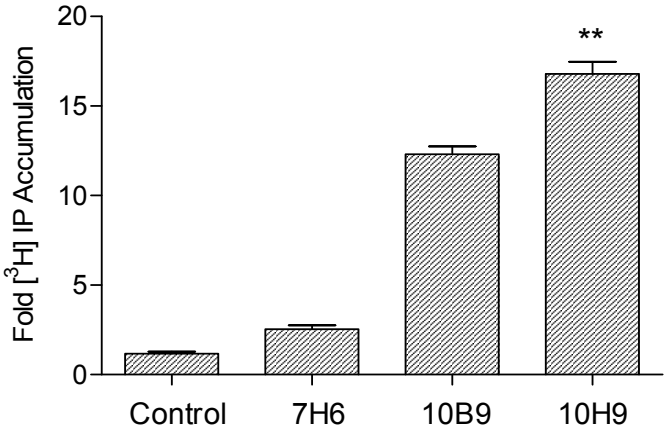


Figure 3.1 Screening of PAR-4/NCTC expression clones by inositol phosphate accumulation assay. Expression clones 7H6, 10B9 and 10H9 were grown to confluency on a 12 well plate, and quiesced overnight in the presence of [^{3}H]-myo-D-inositol (0.5μCi/ml). Following pre-incubation with LiCl (10mM), cells were stimulated with thrombin (5units/ml) for 60 min. Cells were then assessed for accumulation of inositol phosphate as outlined in section 2.4. Each value represents the mean $\pm$ s.e.m (fold accumulation, **p<0.01, different from unstimulated control) of 4 individual experiments performed in triplicate.

3.2.2.1 Effect of AYPGKF-NH_2 and Thrombin on PAR-4 mediated total IP accumulation in 10H9 cells

Following identification of clone 10H9 it was necessary to determine that activating peptide AYPGKF-NH_2 was capable of producing an accumulation of total [^{3}H]-IP in a manner comparable to that produced by thrombin, the endogenous agonist for PAR-4. Therefore, clone 10H9 cells were quiesced overnight in the presence of [^{3}H]-myo-inositol, after which cells were stimulated with AYPGKF-NH_2 (200μM) or thrombin (5units/ml). Results demonstrated that both agonists were capable of generating a comparable accumulation of total [^{3}H]-IP (14$\pm$0.75, 12.5$\pm$1.2 fold increase, n=3, t=60min, for thrombin and AYPGKF-NH_2 respectively) (see figure 3.2). These results obtained by assessing total inositol phosphate accumulation, suggest that PAR-4 is linked to this second messenger system.

3.2.3 Phosphorylation of ERK stimulated by thrombin or AYPGKF-NH_2 in clone 10H9 and NCTC-2544 cells

Western blot analysis was carried out to determine the expression of PAR-4 mediated activation of the MAP kinase pathway in clone 10H9 cells, in comparison to parental NCTC-2544 cells. For this purpose both cell types were stimulated with thrombin, (3units/ml) or AYPGKF-NH_2, (100μM), for a time period of 0-120 minutes. Using total ERK as a means of determining equal protein loading, relative ERK phosphorylation was assessed over the time course. Results in NCTC-2544 cells suggested a low endogenous background of PAR-4, as phosphorylation of ERK which was stimulated by thrombin or peptide was negligible. However, results in clone 10H9 cells showed a transient increase in ERK activity (6.4$\pm$1.16, 4.7$\pm$1.23 fold, n=3 in response to thrombin and AYPGKF-NH_2 respectively at t=5 min), quickly returning to basal levels within 30 min.

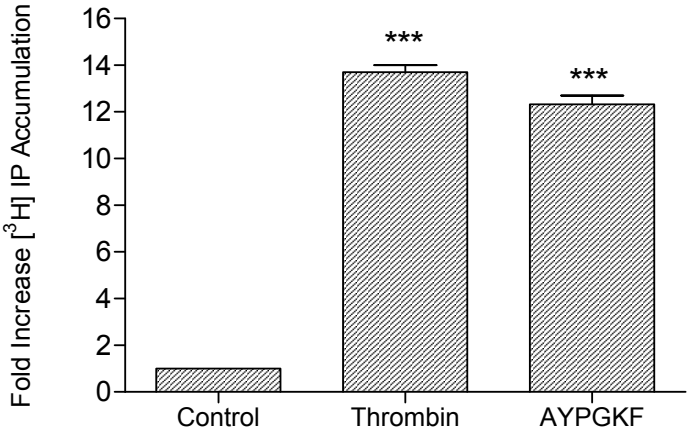
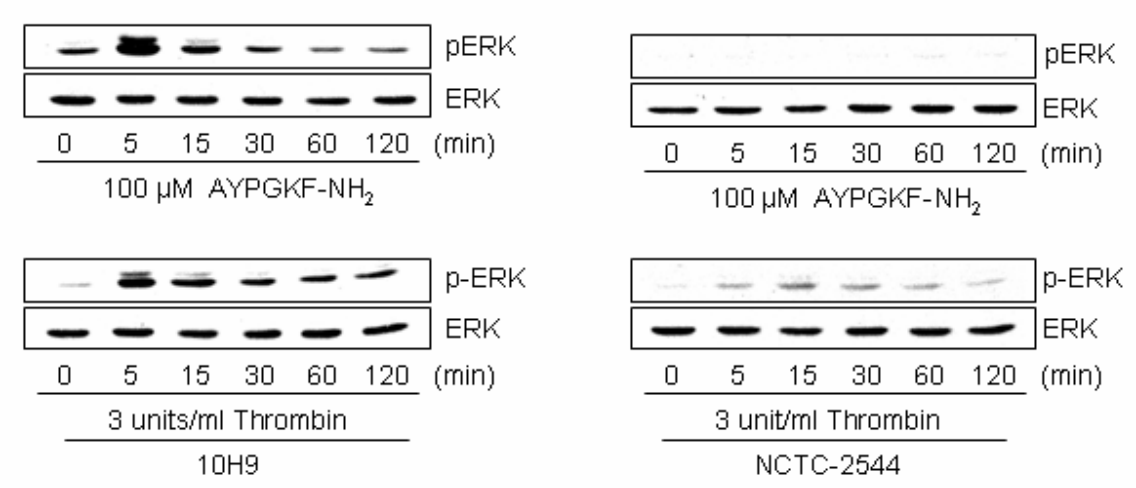


Figure 3.2 Effect of Thrombin and AYPGKF-NH_2 on PAR-4 mediated increase of inositol phosphate in clone 10H9 cells. Expression clone 10H9 was grown to confluency on a 12 well plate, and quiesced overnight in the presence of [^{3}H]-myo-D-inositol (0.5μCi/ml). Following pre-incubation with LiCl (10mM), cells were stimulated with thrombin (5units/ml) or AYPGKF-NH_2 (200μM) for 60 min. Cells were then assessed for accumulation of inositol phosphate as outlined in section 2.4. Each value is representative of mean $\pm$ s.e.m (fold increase total IP, ***$p<0.001$, different from unstimulated control) of 3 individual experiments performed in triplicate.

3.2.3.1 Phosphorylation of p38 MAP kinase stimulated by thrombin or AYPGKF-NH_2 in clone 10H9 and NCTC-2544 cells

Western blot analysis was carried out to further investigate the role of PAR-4 in the activation of the MAP kinase pathway in clone 10H9 cells, in comparison to parental NCTC-2544. Both cell types were stimulated with thrombin, (3units/ml) or AYPGKF-NH_2, (100μM), for a time period of 0-120 minutes. Using total p38 MAP kinase as a means of determining equal protein loading, relative p38 MAP kinase phosphorylation was assessed over the time course. Again, results in NCTC-2544 cells suggested a low endogenous background of PAR-4, as phosphorylation of p38 MAP kinase was negligible. However, whilst, like ERK, p38 MAP kinase displayed a maximum response at 5 minutes (7.2$\pm$1.12, 5.2$\pm$1.23 fold, n=3 for thrombin and AYPGKF-NH_2 respectively at t=5 min), it also resulted in a more sustained PAR-4 mediated activation, which was still apparent up to and including 120 min, suggesting a strong coupling of PAR-4 in association with activation of p38 MAP kinase (see figure 3.4). Taken together these results suggest that not only does PAR-4 mediate activation of ERK and p38 MAP kinase, but due to the low background levels of endogenous PAR-4 in NCTC-2544 cells, results may suggest that NCTC-2544 cells are a plausible parental cell with which to determine the signalling characteristics of PAR-4.

a)



b)

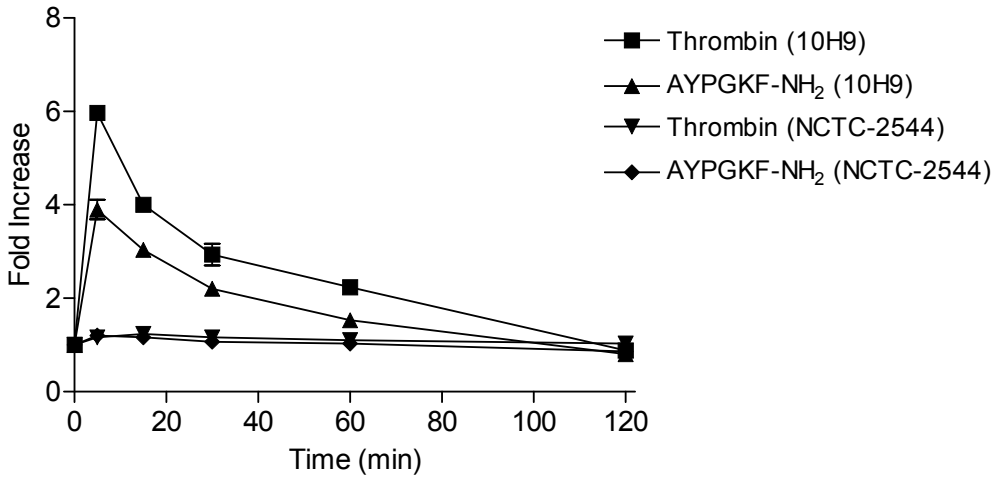
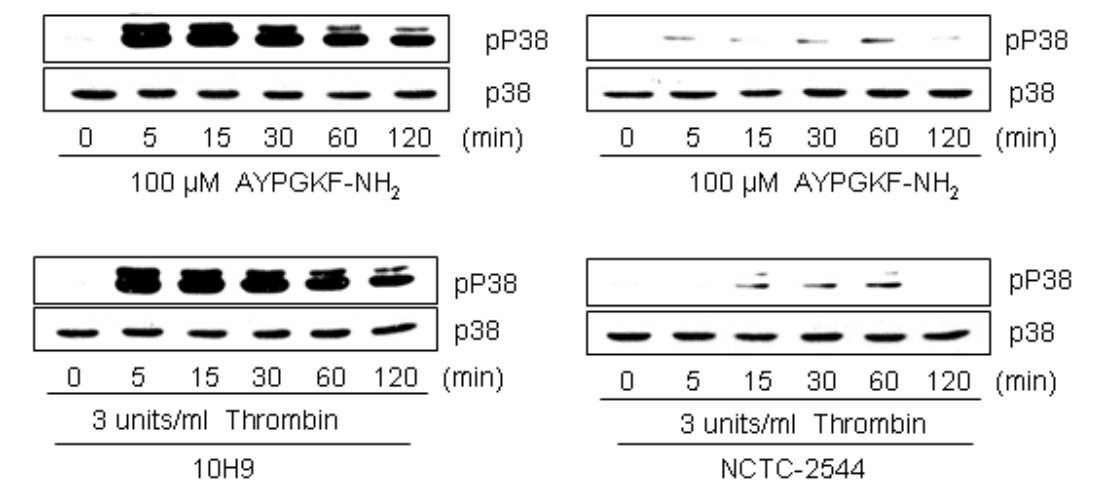


Figure 3.3 Phosphorylation of ERK stimulated by thrombin or AYPGKF-NH_2 in clone 10H9 and NCTC-2544 cells. Both cell types were grown to confluency on 12 well plates and quiesced overnight in serum free media. Cells were then stimulated with either thrombin (3units/ml) or AYPGKF-NH_2 (100µM) over a time period ranging from 0-120min. Whole cell extracts were resolved by SDS-PAGE and examined by Western blotting as outlined in chapter 2. Panel A demonstrates relative phosphoryation of ERK in both cell types, while B indicates the quantification by densitometry expressed as mean $\pm$ s.e.m (fold increase). Each blot is representative of 3 individual experiments.

a)



b)

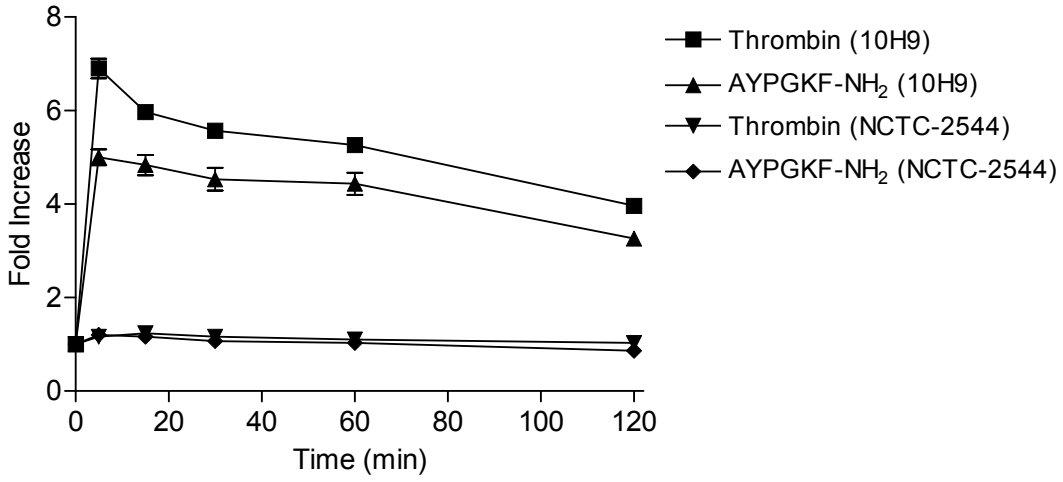


Figure 3.4 Phosphorylation of p38 MAP kinase stimulated by thrombin or AYPGKF-NH_2 in clone 10H9 and NCTC-2544 cells. Both cell types were grown to confluency on 12 well plates and quiesced overnight in serum free media. Cells were then stimulated with either thrombin (3units/ml) or AYPGKF-NH_2 (100µM) over a time period ranging from 0-120min. Whole cell extracts were resolved by SDS-PAGE and examined by Western blotting as outlined in chapter 2. Panel A demonstrates relative phosphoryation of p38 MAPK in both cell types. Panel B illustrates quantification by densitometry expressed as mean $\pm$ s.e.m (fold stimulation). Each blot is representative of 3 separate experiments.

3.3 G-PROTEIN COUPLING ASSOCIATED WITH PAR-4-MEDIATED ACTIVATION OF MAP KINASE IN CLONE 10H9 CELLS

To date, few studies have concentrated on the G protein coupling that is attributed to the intracellular signalling mediated by PAR-4. Certain studies have attributed PAR-4 mediated production of endothelial nitric oxide to be via a pertussis (PTx)-dependent mechanism, suggesting the ability of PAR-4 to couple with $G\alpha_{i/o}$ (Momota *et al,* 2006). Studies that have demonstrated PAR-4 mediated activation of the MAP kinase pathway have shown that PAR-4 is capable of causing accumulation of total [^{3}H]-IP (Sabri *et al*, 2003a). The results by Sabri *et al,* 2003a coincide with findings that have been demonstrated within this study (see section 3.2.2). Therefore, this would suggest that PAR-4 is likely to cause activation of intracellular pathways either by coupling with $G\alpha_{q/11}$ or $G\alpha_{i/o}$. This study has employed PTx to determine the ability of PAR-4 to couple with $G\alpha_{i/o}$, but has also utilised a pharmacological inhibitor, YM-254890, which is capable of inhibiting $G\alpha_{q/11}$ (Kawasaki *et al*, 2003, Takasaki *et al,* 2004).

3.3.1 Effect of PTx on PAR-4 mediated total [^{3}H]-inositol phosphate accumulation in clone 10H9 cells

As previous results have shown the ability of PAR-4 activating peptide to mediate an increase in total inositol phosphate, this assay was applied to determine if PAR-4 mediated this response via coupling with $G\alpha_{i/o}$. Therefore, clone 10H9 cells, which had been grown to confluency, were quiesced overnight in the presence of PTx (100ng/ml)(Hirano *et al,* 2007) and [^{3}H]-myo-D-inositol (0.5 μCi/ml). Results obtained from assessing accumulation of total inositol phosphate demonstrated a fold increase of total inositol phosphate mediated by AYPGKF-NH_2, (fold increase 15.2$\pm$1.5 above basal for AYPGKF-NH_2 (100μM), n=3, t=60min). However, PTx caused a small, but insignificant loss (fold increase 14.3$\pm$1.2 above basal, for AYPGKF-NH_2 (100μM) in the presence of PTx (100ng/ml), n=3, t=60 min) (see figure 3.5). These initial results suggest that PAR-4 mediated increase of total [^{3}H]-inositol phosphate is not mediated by PAR-4 coupling with $G\alpha_{i/o}$.

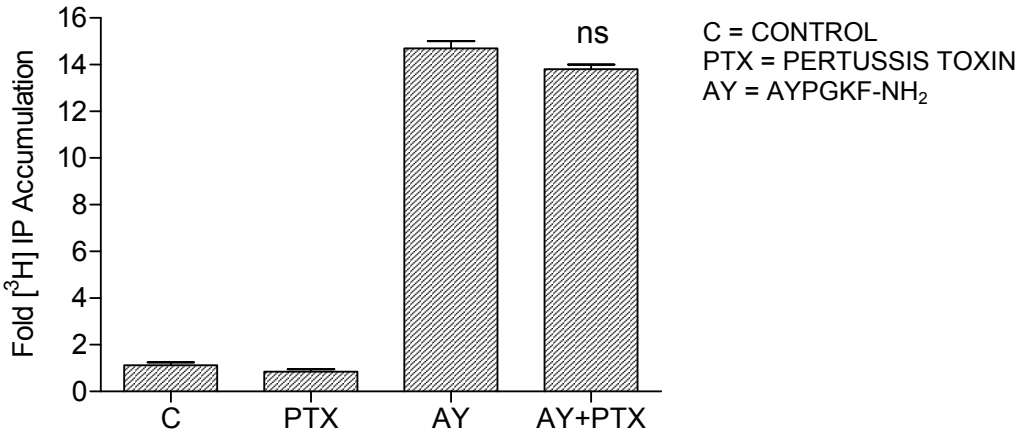


Figure 3.5 Effect of PTx on PAR-4 mediated accumulation of [^{3}H] total inositol phosphate in clone 10H9 cells. Clone 10H9 cells were grown to confluency and quiesced overnight in the presence of 0.5μCi of [^{3}H]-myo-D-inositol and PTx (100ng/ml). Subsequently, cells were stimulated with AYPGKF-NH_2 (100μM) for 60min. Accumulation of total [^{3}H]-IP was measured as outlined in section 2.4. Each value represents mean $\pm$ s.e.m of 3 separate experiments which were performed in triplicate.

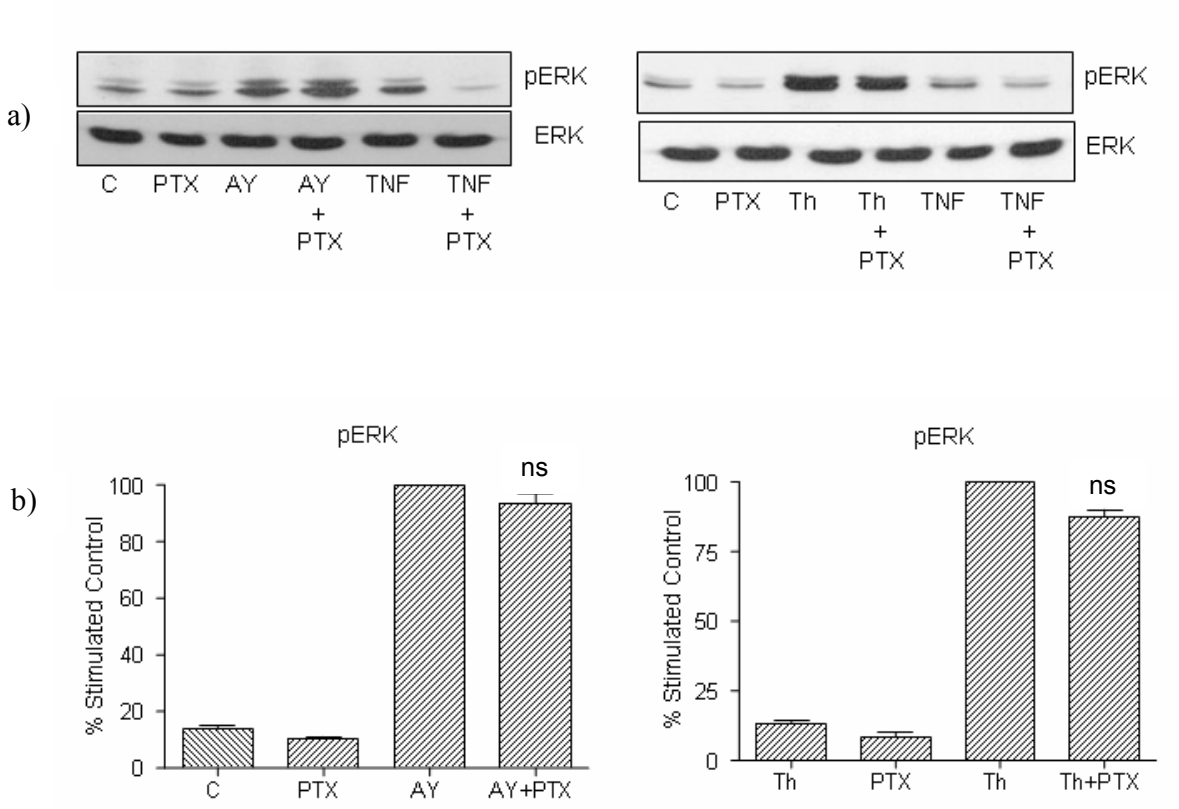
3.3.2 Effect of PTx on PAR-4 mediated phosphorylation of ERK in clone 10H9 cells

To properly determine the role of $G\alpha_{i/o}$ in PAR-4 stimulated ERK activation in clone 10H9 cells, the cells were grown to confluency and quiesced in the presence of 100ng/ml PTx for 15 hours prior to stimulation with either thrombin (3units/ml) or AYPGKF-NH_2 (100μM) for 5 min. Following preparation of samples, relative phosphorylation of ERK was assessed by Western blotting, with total ERK being used to determine equal protein loading. Stimulation by thrombin and AYPGKF-NH_2 demonstrated a transient phosphorylation of ERK (6.1$\pm$1.2 and 3.8$\pm$0.5 for thrombin and AYPGKF-NH_2 respectively). Cells which underwent pre-incubation with PTx demonstrated a small and insignificant inhibition of ERK phosphorylation (% inhibition: 6.3$\pm$1.5%, 8$\pm$2.4%, (*$p>0.05$) n=3), for thrombin and AYPGKF-NH_2 respectively, t=5 min), therefore suggesting PAR-4 may not couple with $G\alpha_{i/o}$, and it is not by this mechanism that it mediates phosphorylation of ERK (see figure 3.6).

3.3.3 Effect of PTx on PAR-4 mediated phosphorylation of p38 MAP kinase in clone 10H9 cells

In a similar fashion to ERK, the investigation of $G\alpha_{i/o}$ PAR-4 mediated p38 MAP kinase activation in clone10H9 cells by a PTx sensitive mechanism was carried out in clone 10H9 cells. The cells were grown to confluency and quiesced in the presence of 100ng/ml pertussis toxin for 15 hours prior to stimulation with either thrombin (3units/ml) or AYPGKF-NH_2 (100μM) for 5 min. Following preparation of samples, relative phosphorylation of p38 MAP kinase was assessed by Western blotting, with total p38 being used to determine equal protein loading. Control results demonstrated phosphorylation of p38 MAP kinase (3.5$\pm$0.7 and 6.5$\pm$1.5 fold increase for AYPGKF-NH_2 and thrombin respectively).

Cells which underwent pre-incubation with PTx displayed a small but insignificant inhibition of p38 MAP kinase phosphorylation (% inhibition: 7$\pm$2.3%, 10$\pm$3%, ($p>0.05$) n=3), for thrombin and AYPGKF-NH_2 respectively, t=5 min), again suggesting that PAR-4 coupling with $G\alpha_{i/o}$ is not the mechanism whereby PAR-4 mediates regulation of the MAP kinase pathway in clone 10H9 cells (see figure 3.7).



C = CONTROL
PTX = PERTUSSIS TOXIN
AY = AYPGKF-NH_2
Th = THROMBIN

Figure 3.6 Effect of PTx on PAR-4 mediated phosphorylation of ERK in clone 10H9 cells. Clone 10H9 cells were grown to confluency on 12 well plates and quiesced overnight in serum free media containing PTx (100ng/ml). Cells were then stimulated with AYPGKF-NH_2 (100μM) for 5 min or TNFα (10ng/ml) for 15 min. Whole cell extracts were resolved by SDS-PAGE and examined by Western blotting as outlined in chapter 2. Relative phosphorylation of ERK is outlined in panel A, while panel B illustrates quantification by densitometry expressed as mean $\pm$ s.e.m (% AYPGKF-NH_2 or thrombin stimulated control). Each blot is representative of 3 separate experiments.

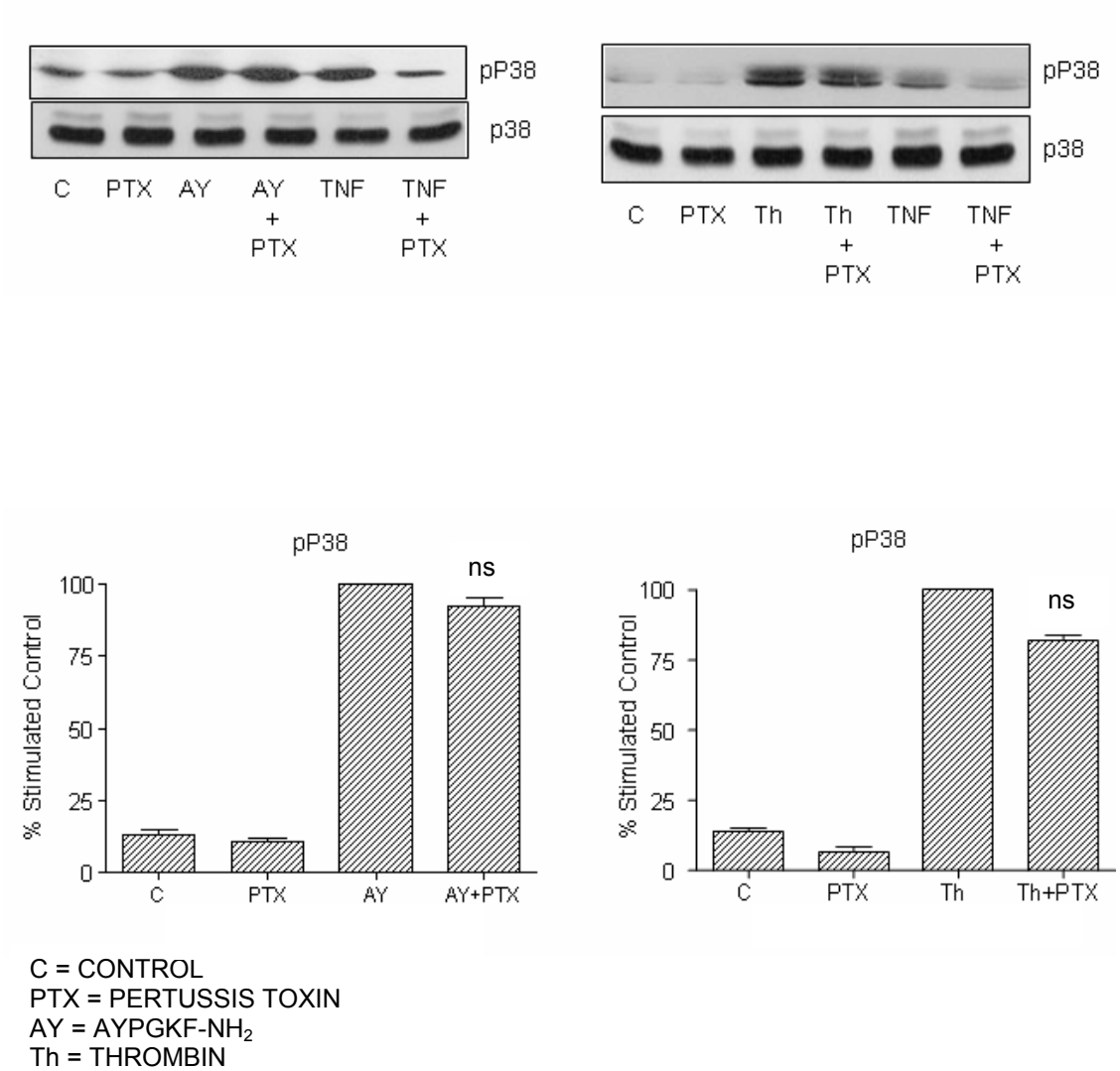


Figure 3.7 Effect of PTx on PAR-4 mediated phosphorylation of p38 in clone 10H9 cells. Clone 10H9 cells were grown to confluency on 12 well plates and quiesced overnight in serum free media containing PTx (100ng/ml). Cells were then stimulated with AYPGKF-NH_2 (100μM) for 5 min and TNFα (10ng/ml) for 15 min. Whole cell extracts were resolved by SDS-PAGE and examined by Western blotting as outlined in chapter 2. Relative phosphorylation of p38 MAP kinase is outlined in panel A, while panel B illustrates the quantification by densitometry, expressed as mean $\pm$ s.e.m (% AYPGKF-NH_2 or thrombin stimulated control). Each blot is representative of 3 separate experiments.

3.3.4 PAR-4 mediates intracellular signalling via a $G\alpha_{q/11}$-dependent mechanism in clone 10H9 cells

The initial means of characterising the effect of compound YM-254890 in clone 10H9 cells, employed inhibition studies which assessed PAR-4 mediated [^{3}H]-inositol phosphate accumulation. Results displayed an increase in total inositol phosphate, stimulated by both thrombin and AYPGKF-NH_2 (fold increase, 16.5±2.2 and 14.8±1.5 for thrombin and AYPGKF-NH_2 respectively, n=4, t=60 min). Further to this, results showed that YM-254890 (0-100nM) was capable of inducing a concentration-dependent inhibition of [^{3}H]-IP accumulation. These results were applicable to stimulation by either thrombin (3units/ml) or AYPGKF-NH_2 (100μM). Maximum effect takes place within the mid nanomolar range (IC_{50}: thrombin = 1.35±0.12nM, AYPGKF-NH_2 = 1.40±0.47nM, n=4). Therefore, it may be suggested that PAR-4 induced inositol phosphate accumulation may be mediated by coupling with $G\alpha_{q/11}$.

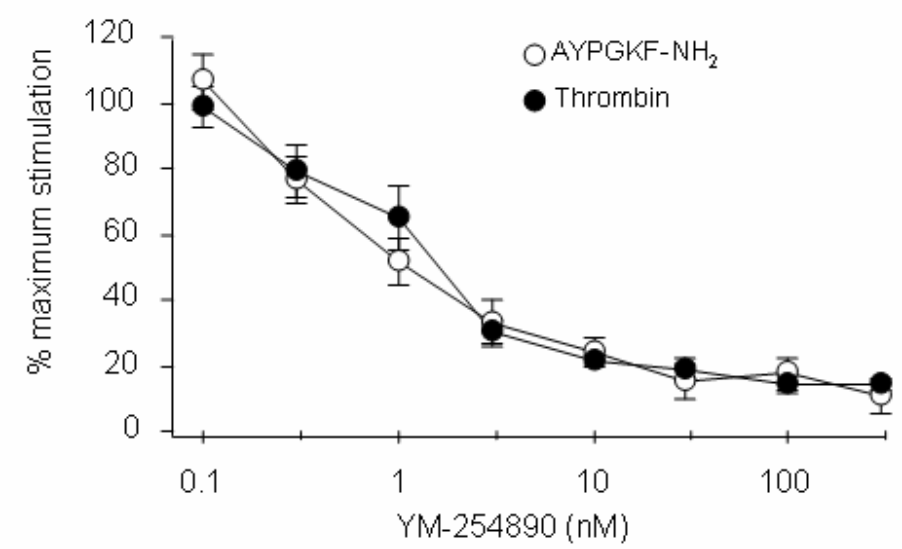


Figure 3.8 Effect of YM-254890 on accumulation of [^{3}H]-inositol phosphate in clone 10H9 cells. Expression clone10H9 was grown to confluency on a 12 well plate, and quiesced overnight in the presence of [^{3}H]-myo-D-inositol (0.5μCi/ml). Following pre-incubation with LiCl (10mM), cells were again pre-incubated with increasing concentrations of YM-254890 (0.1-100nM) for 30 min and then stimulated with thrombin (3units/ml) or AYPGKF-NH_2 (100μM) for 60 min. Cells were then assessed for accumulation of inositol phosphate as outlined in section 2.4. Each value represents the mean ± s.e.m (% maximum AYPGKF-NH_2 or thrombin stimulated control) and representatve of 3 individual experiments.

3.3.4.1 PAR-4 mediates activation of ERK via a $G\alpha_{q/11}$-dependent mechanism in clone 10H9 cells

To determine the role of $G\alpha_{q/11}$ in PAR-4 mediated activation of ERK in clone10H9 cells, relative phosphorylation of ERK was assessed by Western blotting. Cells were pre-11 incubated with YM-254890 (100nM) for 30 min prior to stimulation with thrombin (3units/ml) or AYPGKF-NH_2 (100μM) for 5 min. Thrombin and AYPGKF-NH_2 stimulated an increase of ERK phosphorylation 6.2±1.5 and 5.8±1.7 fold (for thrombin and AYPGKF-NH_2 respectively, t=5min) above basal. The cells which underwent pre-incubation with YM-254890 showed partial inhibition of the ERK signal at the level of ERK phosphorylation (% inhibition: 34±4.5%, 23±5.2%, (*$p<0.05$) n=3, for thrombin and AYPGKF-NH_2 respectively, t=5 min), therefore suggesting PAR-4 mediates activation of ERK, in part, by coupling with $G\alpha_{q/11}$ (see figure 3.9) .

3.3.4.2 PAR-4 mediates activation of p38 MAP kinase via a $G\alpha_{q/11}$-dependent mechanism in clone 10H9 cells

Phosphorylation of p38 MAP kinase was investigated in the same fashion as ERK. Again, clone 10H9 cells were pre-incubated with YM-254890 (100nM) for 30 min prior to stimulation with either thrombin (3units/ml) or AYPGKF-NH_2 (100μM) for 5 min. Stimulation with thrombin or AYPGKF-NH_2 mediated phosphorylation of p38 MAP 4.8±0.7 and 4.2±1 fold (for thrombin and AYPGKF-NH_2 respectively, t=5min) above basal. However, pre-incubation of cells with YM-254890 demonstrated inhibition of phosphorylation of p38 MAP kinase (% inhibition: 31±5.7%, 44±5.6%, (*$p<0.05$) n=3, for thrombin and AYPGKF-NH_2 respectively, t=5 min) (see figure 3.10). Taken together, these results suggest that PAR-4 mediates activation of the MAP kinase pathway in part via $G\alpha_{q/11}$- dependent mechanism but may employ other mechanisms in coupling to the MAP kinase pathway.

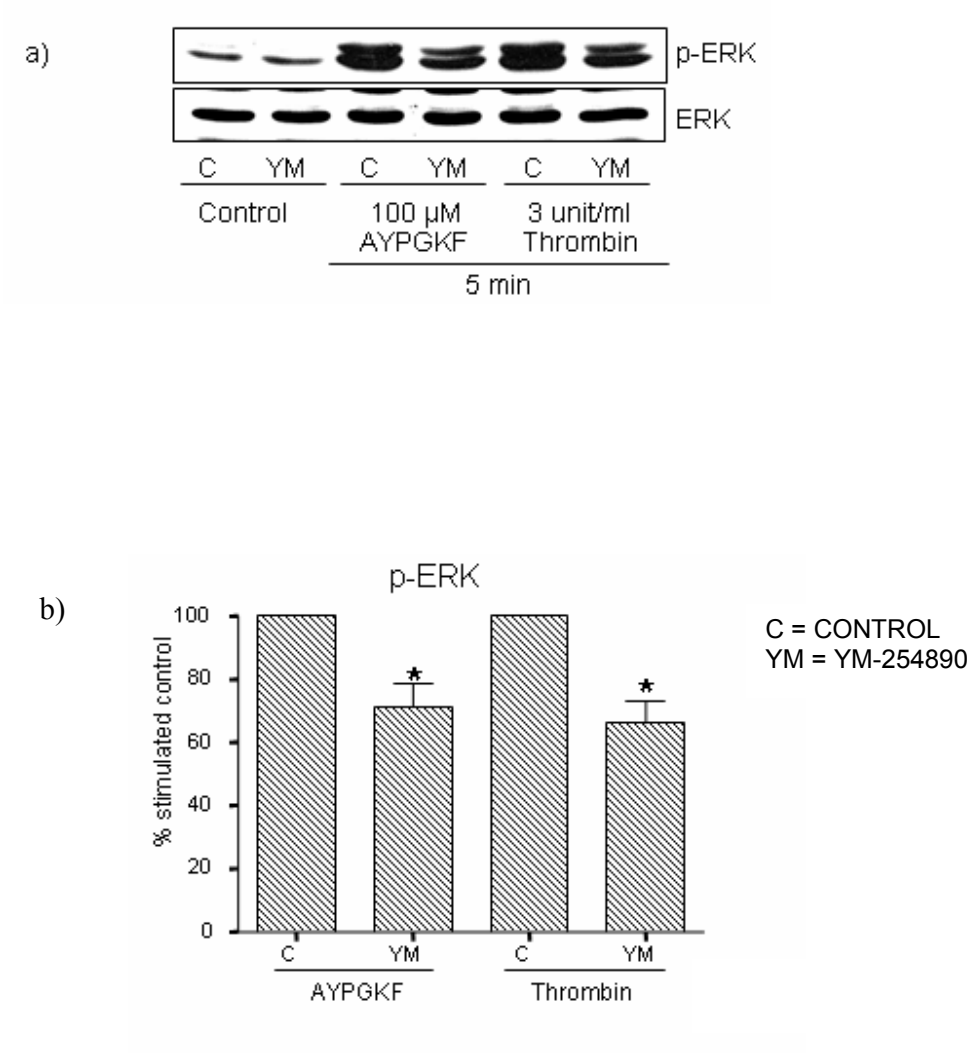


Figure 3.9 Effect of YM-254890 on PAR-4 mediated phosphorylation of ERK in clone 10H9 cells. Confluent clone 10H9 cells, which were rendered quiescent overnight in serum free media, and pre-incubated with YM-254890 (100nM) for 30 min. Cells were then stimulated for 5 min with AYPGKF-NH_2 (100µM) or thrombin (3units/ml). Whole cell extracts were resolved by SDS-PAGE and examined by Western blotting for phosphorylation of ERK Panel A shows relative phosphorylation of ERK. Panel B illustrates quantification by densitometry expressed as mean $\pm$ s.e.m (% AYPGKF-NH_2 or thrombin stimulated control, *$p<0.05$). Each blot is representative of 3 separate experiments.

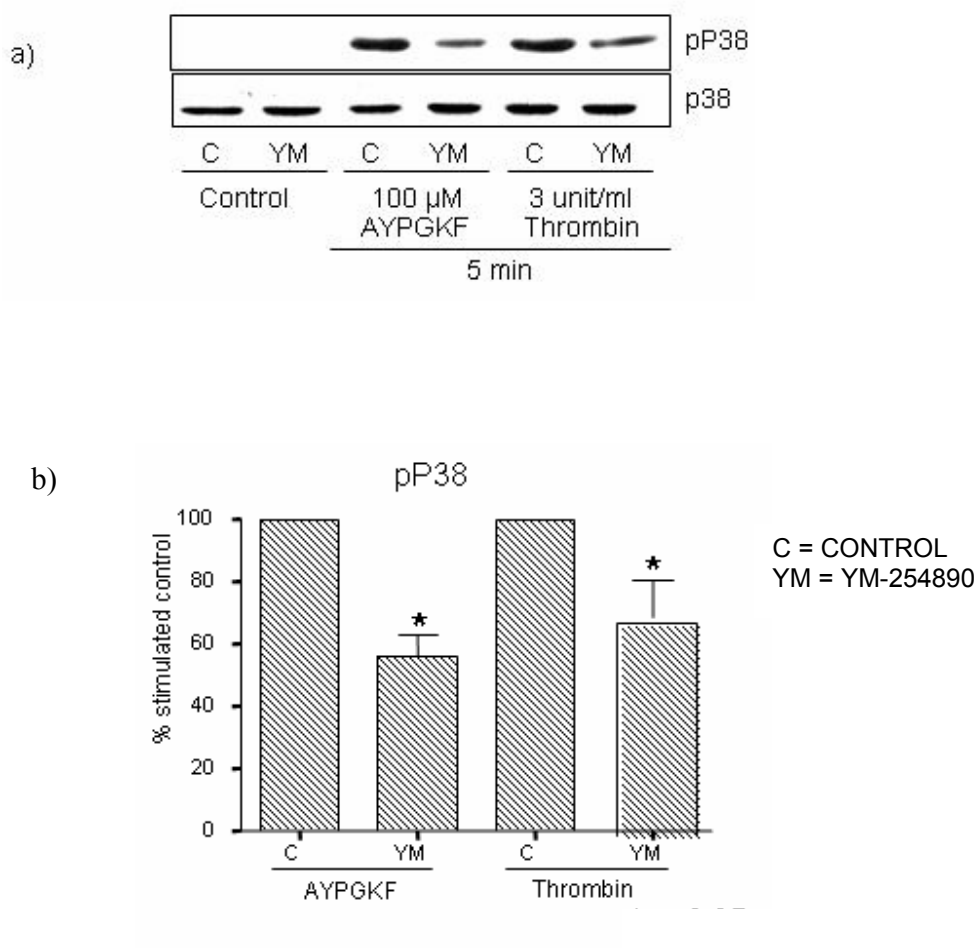


Figure 3.10 Effect of YM-254890 PAR-4 mediated phosphorylation of p38 MAP kinase in clone 10H9 cells. Panel A shows confluent clone 10H9 cells, which were rendered quiescent overnight in serum free media, and pre-incubated with YM-254890 (100nM) for 30 min. Cells were then stimulated for 5 min with AYPGKF-NH_2 (100µM) or thrombin (3units/ml). Whole cell extracts were then resolved by SDS-PAGE and examined by Western blotting for phosphorylation of p38 MAP kinase. Panel B illustrates quantification by densitometry expressed as mean±s.e.m (% AYPGKF-NH_2 or thrombin stimulated control, *$p<0.05$). Each blot is representative of at least three individual experiments.

3.4 PAR-4 MEDIATES ACTIVATION OF THE MAP KINASE PATHWAY VIA TRANSACTIVATION OF THE EGFR IN CLONE 10H9 CELLS

Previous studies have shown the ability of PAR-1 and PAR-2 to mediate activation of downstream pathways via transactivation of the EGFR (Darmoul *et al*, 2004a, Darmoul *et al*, 2004b, Bergmann *et al*, 2006, Moriyuki *et al*, 2008). Sabri *et al*, (2003) have also shown that PAR-4 is also capable of MAP kinase activation, in particular p38 MAP kinase, via transactivation of the EGFR in cardiac myocytes. Therefore, to determine if PAR-4 causes activation of the MAP kinase pathway in clone 10H9 cells by transactivation of the EGFR, a tyrosine kinase inhibitor, AG-1478 was utilised (see table 3.1).This inhibitor has been previously shown to be a selective inhibitor of the EGFR (Hodges *et al,* 2007, Kim & Akaike, 2007). Results obtained by Western blotting were used to assess the relative phosphorylation of ERK and p38 MAP kinase in clone 10H9 cells which had been pre-incubated with AG-1478.

3.4.1 PAR-4 mediated activation of ERK via transactivation of the EGFR in clone 10H9 cells.

To determine if PAR-4 mediates activation of ERK via transactivation of the EGFR, 10H9 cells were pre-incubated with AG-1478 (1μM) for 30 min, prior to stimulation of cells with either thrombin (3units/ml) or AYPGKF-NH_2 (100μM), for 5 min. Thrombin and AYPGKF-NH_2 stimulated a phosphorylation of ERK 4.5$\pm$0.75 and 4$\pm$1.2 fold (for thrombin and AYPGKF-NH_2 respectively, t=5min), above basal. Unlike inhibition with YM-254890, AG-1478 demonstrated a much more significant level of inhibition of ERK phosphorylation (% inhibition 50$\pm$3.7%, 70$\pm$4.6%, (**$p<0.01$) n=3, for thrombin and AYPGKF-NH_2 respectively), was observed in the presence of AG-1478, suggesting that PAR-4 mediated activation of ERK did not only occur by a G protein-dependent mechanism, but also by transactivation of the EGFR (see figure 3.11). Epidermal-growth factor (EGF) (50ng/ml) was utilised as a control, which mediated a stimulation of 5.3$\pm$0.65 fold above basal. Stimulation was inhibited in the presence of AG-1478 (% inhibition 93$\pm$3.2, ***$p<0.001$).

3.4.2 PAR-4 mediated activation of p38 MAP kinase via transactivation of the EGFR in clone 10H9 cells.

Again, in the same fashion as ERK, inhibition of p38 MAP kinase phosphorylation was investigated. Cells were pre-incubated with AG-1478 (1μM) for 30 min, prior to stimulation of cells with either thrombin (3units/ml) or AYPGKF-NH_2 (100μM), for 5 min. Stimulation by thrombin or AYPGKF-NH_2 mediated phosphorylation of p38 MAP kinase 5.2±1.3 and 5.5±0.8 fold (for thrombin and AYPGKF-NH_2 respectively, t=5min) above basal. Inhibition of p38 MAP kinase phosphorylation in the presence of AG-1478 demonstrated a significant level of inhibition (% inhibition 40±2.3%, 50±5.6%, (**$p<0.01$) n=3, for thrombin and AYPGKF-NH_2 respectively) (see figure 3.12). EGF (50ng/ml) which was used as a control, mediated phosphorylation of p38 MAP kinase 5.6±1.2 fold above basal, which was successfully inhibited by AG-1478 (% inhibition 90±4.8 *** $p<0.001$).

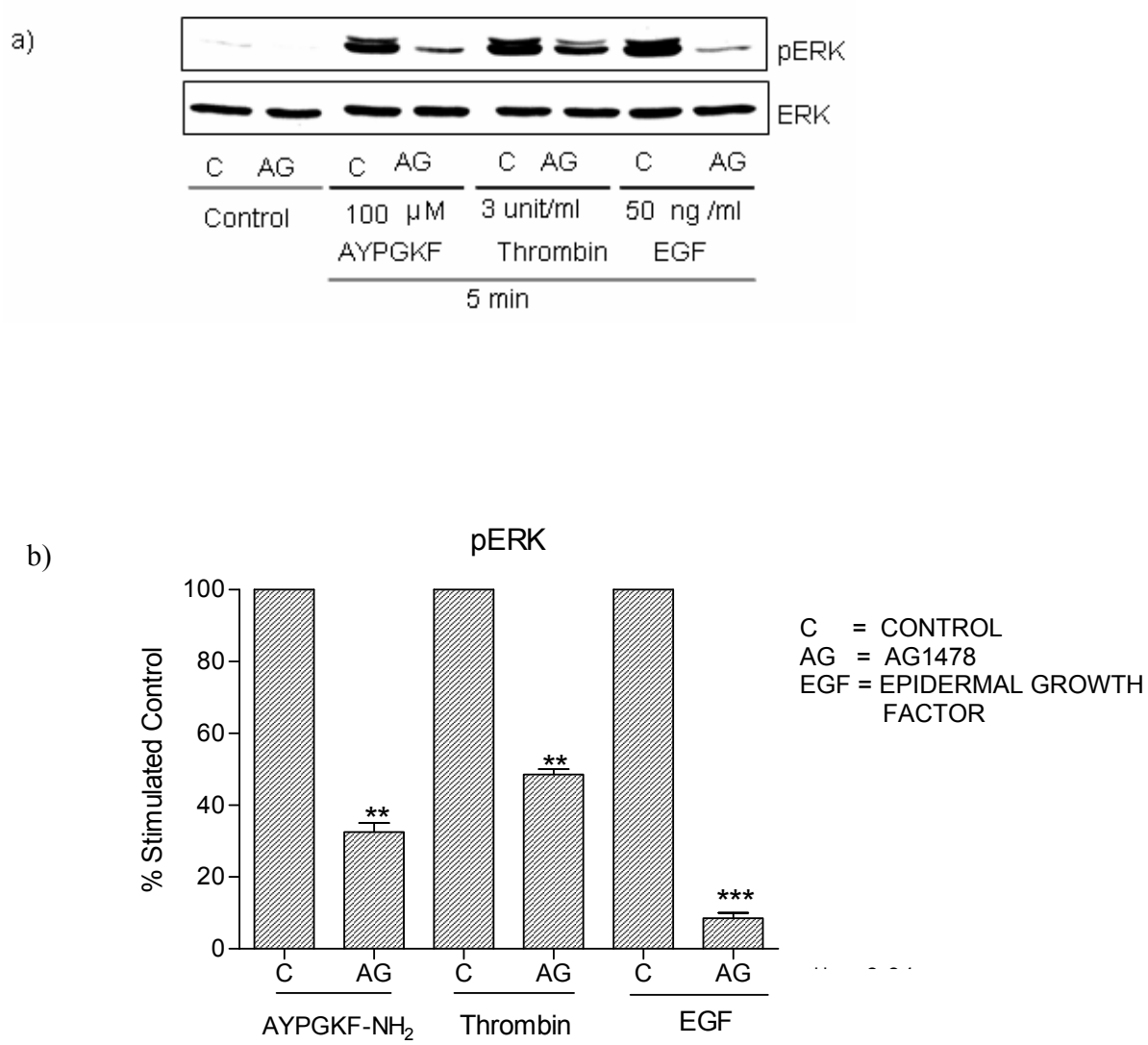


Figure 3.11 Effect of AG-1478 PAR-4 mediated phosphorylation of ERK in clone 10H9 cells. Confluent clone 10H9 cells were rendered quiescent overnight in serum free media, and pre-incubated with AG1478 (1µM) for 30 min. Cells were stimulated for 5 min with AYPGKF-NH_2 (100µM), thrombin (3units/ml) or EGF (50ng/ml). Whole cell extracts were then resolved by SDS-PAGE and exmined by Western blotting phosphorylation of ERK as observed in panel A. Panel B illustrates quantification by densitometry expressed as mean+s.e.m (% AYPGKF-NH_2, thrombin or EGF stimulated control, **$p<0.01$, ***$p<0.001$). Each blot is representative of 3 individual experiments.

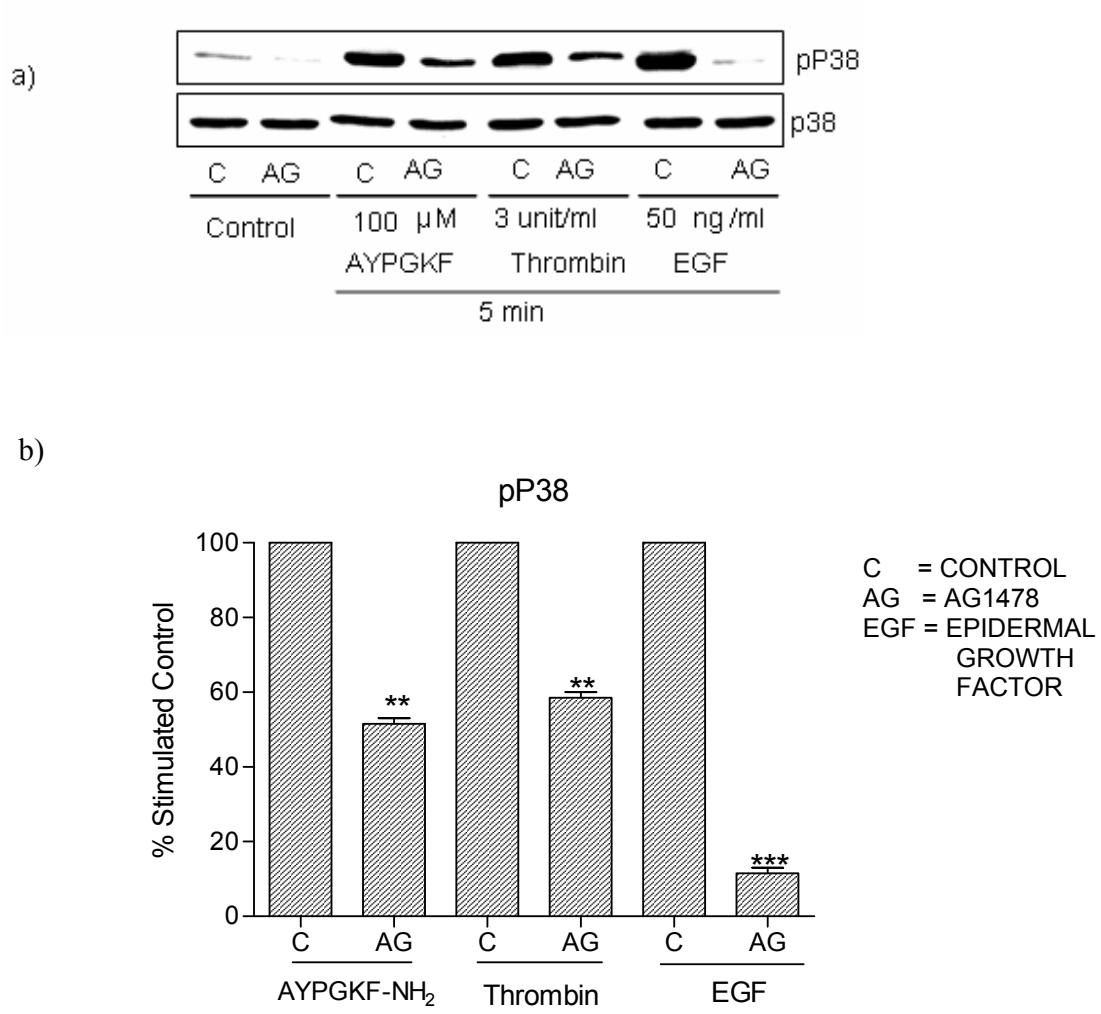


Figure 3.12 Effect of AG1478 on PAR-4 mediated phosphorylation of p38 MAP kinase in clone 10H9 cells. Clone 10H9 cells were rendered quiescent overnight in serum free media, and pre-incubated with AG1478 (1µM) for 30 min. Cells were then stimulated for 5 min with AYPGKF-NH_2 (100µM), thrombin (3units/ml) or EGF (50ng/ml). Whole cell extracts were resolved by SDS-PAGE and examined by Western blotting for phosphorylation of p38 MAP kinase as observed in panel A. Panel B illustrates quantification by densitometry expressed as mean±s.e.m (% AYPGKF-NH_2, thrombin or EGF stimulated control, **$p<0.01$, ***$p<0.001$). Each blot is representative of three individual experiments.

3.4.3 Additive inhibitory effect of YM-254890 and AG-1478 on phosphorylation of ERK and p38 MAP kinase in clone 10H9 cells

So far, results have demonstrated the ability of PAR-4 to mediate activation of ERK and p38 MAP kinase via two distinct mechanisms. It was therefore necessary to determine if these responses showed an additive effect, which would suggest PAR-4 is capable of signalling simultaneously via these two separate mechanisms. Therefore, clone 10H9 cells were pre-incubated with both YM-254890 (100nM) and AG-1478 (1μM) either separately or together for 30 min, prior to stimulation with either thrombin (3units/ml) or AYPGKF-NH_2 (100μM) for 5 min.

As with previous results, both thrombin and AYPGKF-NH_2 stimulated the phosphorylation of both ERK (5.3$\pm$0.5 and 4.2$\pm$0.75 fold above basal for thrombin and AYPGKF-NH_2 respectively) and p38 MAP kinase (5.2$\pm$1.1 and 5$\pm$0.4 fold above basal for thrombin and AYPGKF-NH_2 respectively). Cells which had been pre-incubated with YM-254890 or AG-1478 displayed the same inhibition as previously. However, the cells which had been incubated with both compounds demonstrated a more significant level of inhibition of both ERK and p38 MAP kinase when compared to stimulated controls. Inhibition of ERK was found to be 60$\pm$5.7%, 80$\pm$4.6%, (***$p<0.001$) n=3, for thrombin and AYPGKF-NH_2 respectively and inhibition of p38 MAP kinase was 75$\pm$4.2%, 86$\pm$5.6%, (***$p<0.001$) n=3, for thrombin and AYPGKF-NH_2 respectively (see figure 3.13). This additive inhibitory response demonstrates that PAR-4 is capable of mediating activation of the MAP kinase pathway via both G protein-dependent and -independent pathways.

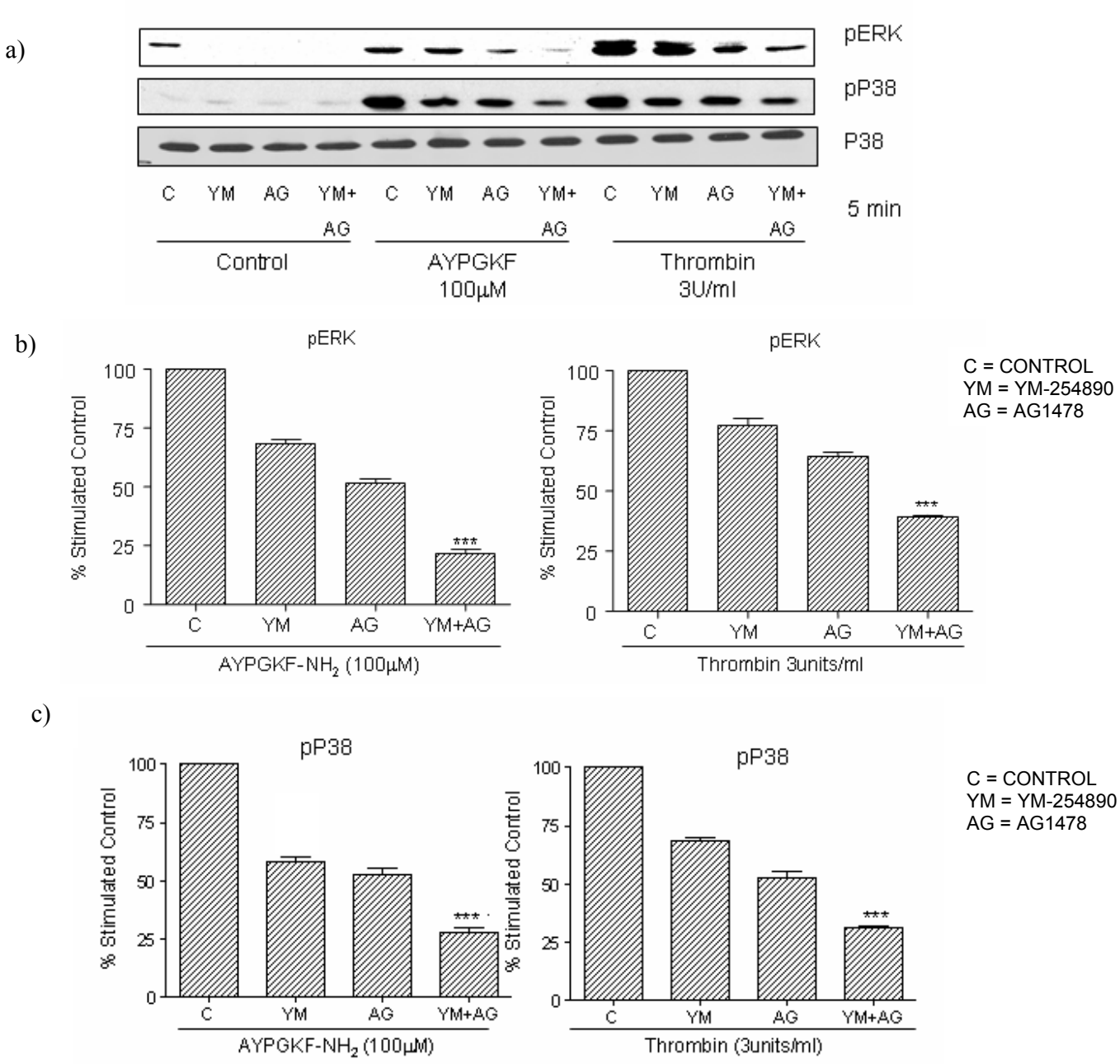


Figure 3.13 Additive inhibitory effect of YM-254890 and AG1478 on phosphorylation of ERK and p38 MAP kinase in clone 10H9 cells. Confluent clone 10H9 cells were rendered quiescent overnight in serum free media, and pre-incubated with YM-254890 (100nM) or AG1478 (1μM) or a combination of both for 30 min prior to stimulation over a period of 5 min with AYPGKF-NH_2 (100μM) or thrombin (3units/ml). Results were examined by Western blotting for phosphorylation of ERK and p38 MAP kinase. Panel A shows relative phosphorylation of ERK and p38 MAP kinase and panels B and C show quantification of blots by densitometry, expressed as mean±s.e.m (% AYPGKF-NH_2 or thrombin stimulated control, ***$p<0.001$). Each blot is representative of 3 separate experiments

3.5 PAR-4 MEDIATED ACTIVATION OF THE MAP KINASE PATHWAY IS NOT MEDIATED VIA PKC IN CLONE 10H9 CELLS

Protein kinase C (PKC) is a kinase family which consists of a various isoforms. Conventional isoforms become activated via Ca^{2+}- and diacylglycerol (DAG)-dependent mechanisms and include PKCα, β_I, β_{II} and γ. The other non-conventional isoforms include PKCδ, η, θ and ε and are found to become activated by Ca^{2+}-independent mechanisms (Nishizuka, 1995). As PAR-4 is capable of coupling with $G\alpha_{q/11}$, it could be hypothesized that with the generation of IP_3, and subsequent increase of intracellular Ca^{2+} and DAG, that PAR-4 may mediate activation of ERK and p38 MAP kinase via a conventional PKC-dependent mechanism. Therefore, a selective inhibitor of conventional PKC isoforms, GF-109203X, was utilised to determine the role of PKC in PAR-4 mediated activation of the MAP kinase pathway. For this purpose clone 10H9 cells were pre-incubated alone with either GF-109203X (10μM) or AG-1478 (1μM) or in combination for 30 min, prior to incubation with either thrombin (3units/ml) or AYPGKF-NH_2 (100μM) for 5 min.

Stimulation by thrombin and AYPGKF-NH_2 mediated phosphorylation of both ERK (4.1$\pm$0.5 and 4.3$\pm$0.6 fold above basal for thrombin and AYPGKF-NH_2 respectively) and p38 MAP kinase (4.4$\pm$0.45 and 4.2$\pm$1 fold above basal for thrombin and AYPGKF-NH_2 respectively). The results also demonstrated that, as expected, inhibition was apparent in the samples pre-incubated with AG-1478. However, samples pre-incubated with GF-109203X did not demonstrate any inhibition or contribute to any further inhibition in samples which were also pre-incubated with AG-1478. These results suggest that while PAR-4 seems to couple with $G\alpha_{q/11}$ as well as transactivate the EGFR to activate the MAP kinase pathway, it would appear that this pathway does not activate ERK and p38 MAP kinase via a conventional PKC isoform (see figure 3.14).

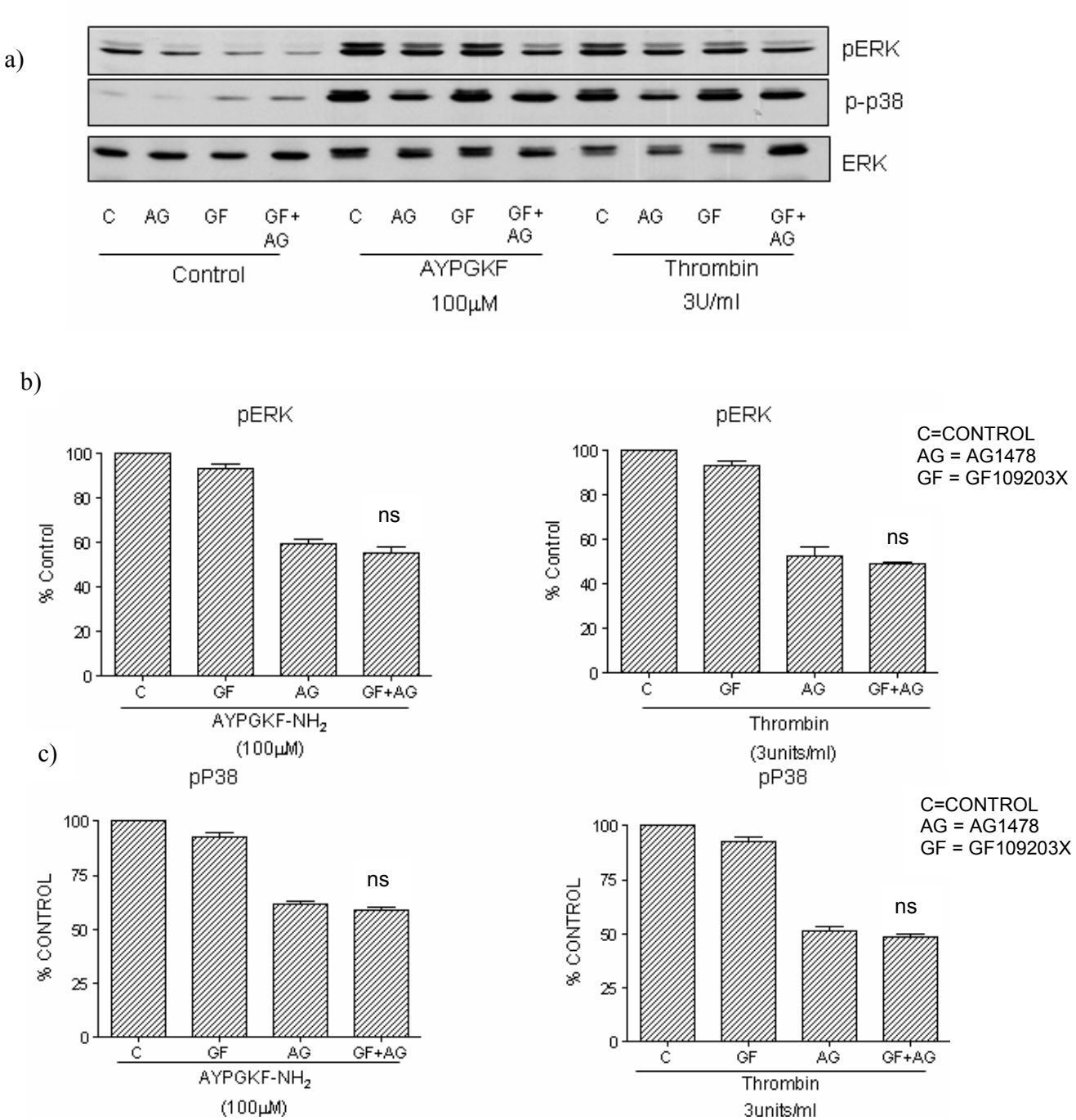


Figure 3.14 Additive inhibitory effect of GF-109203X and AG1478 on phosphorylation of ERK and p38 in clone 10H9 cells. Confluent clone 10H9 cells were rendered quiescent overnight in serum free media, and pre-incubated with GF-109203X (10μM) or AG1478 (1μM) or a combination of both for 30 min prior to stimulation over a period of 5 min with AYPGKF-NH_2 (100μM) or thrombin (3units/ml). Whole cell extracts were examined by Western blotting and assessed for phosphorylation of ERK and p38 MAP kinase. Panel A shows relative phosphorylation of ERK and p38 MAP kinase, while panels B and C show quantification by densitometry, expressed as mean+ s.e.m (% AYPGKF-NH_2 or thrombin stimulated control). Each blot is representative of at least 3 individual experiments.

3.6 PAR-4 MEDIATES PRODUCTION OF IL-8 IN CLONE 10H9 CELLS

Previous studies have shown that proteinase-activated receptors are capable of mediating an increase in the production of interleukins, particularly IL-6 and IL-8 (Chiu *et al,* 2008, Tanaka *et al*, 2004). More recently, it has been demonstrated that PAR-2 is capable of increasing production of IL-8 in pulmonary fibroblasts via a MAP kinase-dependent mechanism by activation of JNK (Ostrowska & Reiser, 2008). However, previous findings in this study have shown that PAR-4 does not stimulate activation of JNK (colleague's observation, results not shown), therefore, it is unlikely that any production of IL-8 would be via a JNK- dependent mechanism. Therefore, in order to determine if PAR-4 was capable of mediating the production of IL-8 in clone 10H9 cells, the cells were stimulated with AYPGKF-NH_2 (100μM) over a period of 0-6 hours. Cell supernatants were collected in order to determine the production of IL-8. Results demonstrated that PAR-4 mediates production of IL-8 in a time-dependent manner, with maximum production of IL-8 being shown at 6 hours (750±75.3pg/ml n=3), which was found to be comparable with the TNFα control (990±25pg/ml n=3, t=60 min) (see figure 3.15). Subsequent experiments used a 6 hour time point.

3.6.1 PAR-4 mediates IL-8 production via a $G\alpha_{q/11}$-dependent mechanism in clone 10H9 cells

In order to determine the intracellular mechanisms whereby PAR-4 mediates production of IL-8, clone 10H9 cells were pre-incubated with YM-254890 (100nM) for 30 min prior to stimulation with AYPGKF-NH_2 (100μM) for 6 hours. Supernatants were collected and assessed by IL-8 specific ELISA as per manufacturer's instuctions. Results indicate that PAR-4 activating peptide stimulated the production of IL-8 (780±70pg/ml, n=3, t=6hr). However, it was also demonstrated that PAR-4 mediates IL-8 production in part, via a $G\alpha_{q/11}$-dependent mechanism. YM-254890 caused inhibition of IL-8 (% inhibition 28±3.5, n=3, t=6hrs, *$p<0.05$). This again suggests that more than one mechanism is required for PAR-4 to mediate production of IL-8 (see figure 3.16).

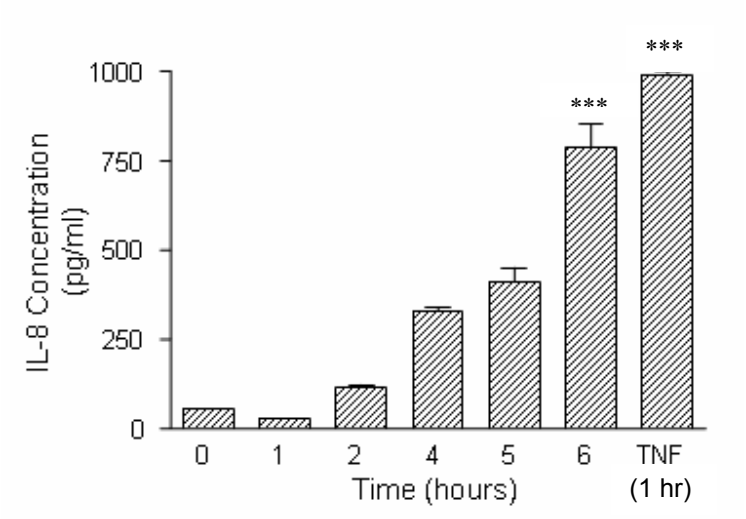


Figure 3.15 PAR-4 mediates production of IL-8 in clone 10H9 cells. Clone 10H9 cells were grown to confluency on a 12 well plate and quiesced overnight in serum free media. Cells were stimulated with AYPGKF-NH_2 (100μM) and TNFα (10ng/ml) over a time period of 0-6 hours. Following stimulation, supernatant was collected in fresh eppendorf tubes. ELISA specific for IL-8 was performed as described in section 2.8. Quantification was expressed as mean + s.e.m. (pg/ml, ***$p<0.001$ as compared to unstimulated control). Each column is representive of 3 individual experiments.

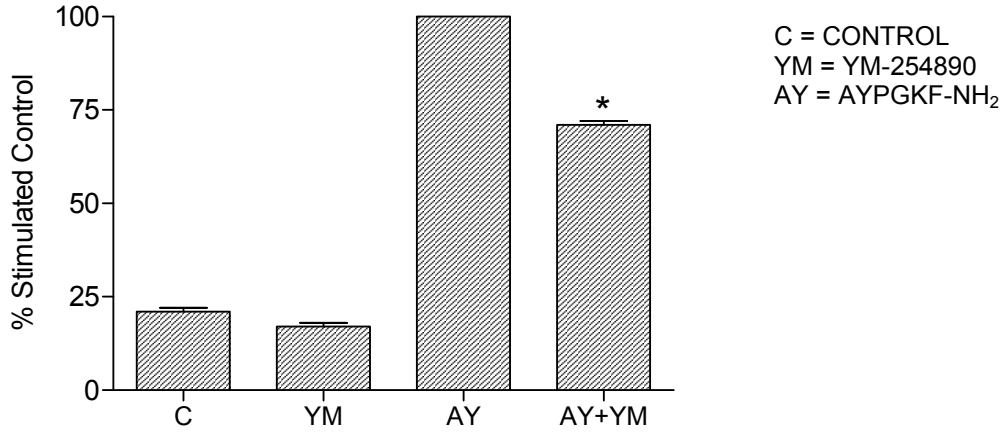


Figure 3.16 Effect of YM-254890 on PAR-4 mediated IL-8 production in clone 10H9 cells. Clone 10H9 cells were grown to confluency on a 12 well plate and quiesced overnight in serum free media. Cells were pre-incubated with YM-254890 (100nM) for 30 min prior to being stimulated with AYPGKF-NH_2 (100μM) for 6 hrs. Following stimulation, supernatant was collected in fresh eppendorf tubes. ELISA specific for IL-8 was performed as outlined in section 2.8. Quantification was expressed as mean $\pm$s.e.m (% AYPGKF-NH_2 stimulated control, *$p<0.05$). Each column is representitive of 3 individual experiments.

3.6.2 PAR-4 mediates IL-8 production via transactivation of the EGFR

As previous findings in this study have shown that PAR-4 is capable of mediating activation of MAP kinase via an EGFR-dependent mechanism, it was hypothesised that this may also be involved in the production of IL-8. Therefore, clone 10H9 cells were pre-incubated with AG-1478 (1μM) for 30 min prior to stimulation of cells with AYPGKF-NH_2 (100μM) for 6 hours. PAR-4 activating peptide, AYPGKF-NH_2, stimulated production of IL-8 (755±30.75pg/ml, t=6hr, n=3). However, results also indicated that PAR-4 mediates IL-8 production in part by transactivation of the EGFR. Samples pre-incubated with AG-1478 demonstrated inhibition of IL-8 (% inhibition 40±7.8 n=3, t=6hrs, **p<0.01). Taken together with the previous result, these data suggest that PAR-4 may mediate IL-8 production via 2 distinct mechanisms (see figure 3.17).

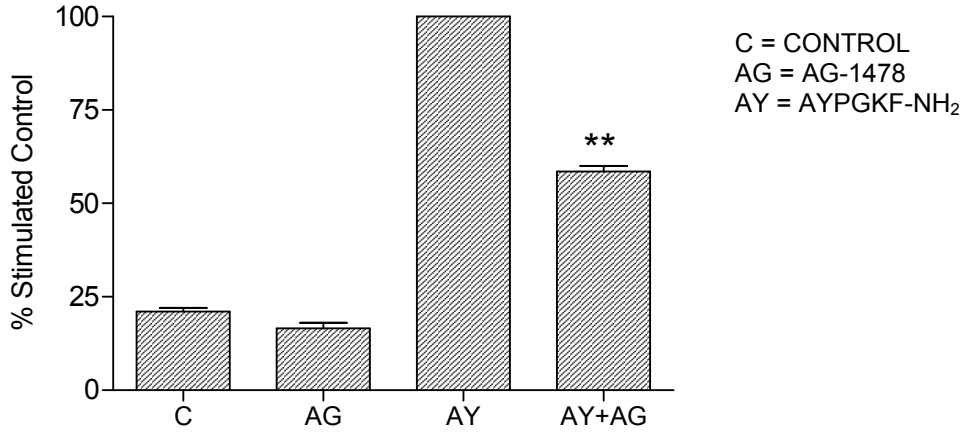


Figure 3.17 Effect of AG-1478 on PAR-4 mediated IL-8 production in clone 10H9 cells. Clone 10H9 cells were grown to confluency on a 12 well plate and quiesced overnight in serum free media. Cells were pre-incubated with AG-1478 (1μM) for 30 min prior to being stimulated with AYPGKF-NH_2 (100μM) for 60 min. Following stimulation, supernatant was collected in fresh eppendorf tubes. ELISA specific for IL-8 was performed as outlined in section 2.8. Quantification was expressed as mean±s.e.m (% AYPGKF-NH_2 stimulated control, **p<0.01). Each column is representative of 3 individual experiments.

3.6.3 Additive effect of YM-254890 and AG-1478 on PAR-4 mediated production of IL-8

Assessment of PAR-4 mediated production of IL-8 has so far suggested the utilisation of two distinct mechanisms, which are PAR-4 coupling with $G\alpha_{q/11}$ and by transactivation of the EGFR. Therefore, to determine if an additive response was functionally relevent for PAR-4 mediated production of IL-8, clone 10H9 cells were pre-incubated with YM-254890 (100nM) and AG-1478 (1μM), either separately or in combination 30 minutes prior to stimulation of cells with AYPGKF-NH_2 (100μM) for 6 hours. Results indicated that AYPGKF-NH_2 stimulated production of IL-8 (735.5±25pg/ml). Cells pre-incubated with YM-254890 or AG-1478 demonstrated the same level of inhibition as had been previously shown (see figures 3.16 and 3.17). In combination, these inhibitors showed an additive inhibitory effect (% inhibition 45±6.3, n=3, t=6hrs, **$p<0.01$). However, while an inhibitory effect was shown, it did not reflect the additive response which had been previously shown when assessing the activation of ERK and p38 MAP kinase. Therefore, while this data demonstrates an additive effect, it would appear PAR-4 mediates IL-8 production via another mechanism (figure 3.18).

3.6.4 PAR-4 mediates IL-8 production via a p38 MAP kinase-dependent mechanism

In response to previous studies, it was necessary to determine if PAR-4 mediated production of IL-8 via a MAP kinase-dependent mechanism. Selective MAP kinase inhibitors were employed for this assay and included p38 MAP kinase inhibitor SB203528 (10μM), JNK inhibitor SP600125, (10μM) and ERK inhibitor U0126 (10μM). As with previous experiments, clone 10H9 cells were pre-incubated with each of these inhibitors 30 min prior to stimulation with AYPGKF-NH_2 (100μM) for 6 hours. AYPGKF-NH_2 stimulated IL-8 production (770±35pg/ml). Cells treated with either the selective JNK or ERK inhibitors did not demonstrate a significant level of inhibition (% inhibition 8±2.5 and 4±2.3 for SP600125 and U0126 respectively).

p38 MAP kinase inhibitor SB203528, demonstrated a significant level of inhibition (% inhibition 53$\pm$5.5, n=3, t=6hrs, **$p<0.01$), which suggests that PAR-4 not only mediates production of IL-8 via $G\alpha_{q/11}$ and transactivation of the EGFR, but also demonstrates its ability to mediate IL-8 production via a p38 MAP kinase-dependent mechanism (see figure 3.19).

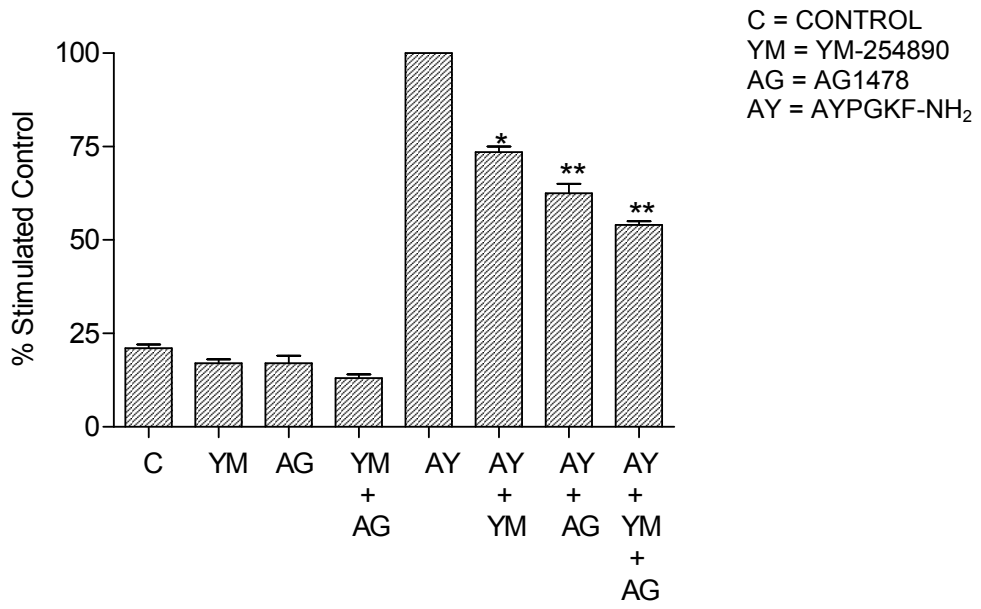


Figure 3.18 Additive effect of YM-254890 and AG-1478 on PAR-4 mediated IL-8 production in clone 10H9 cells. Clone 10H9 cells were grown to confluency on a 12 well plate and quiesced overnight in serum free media. Cells were pre-incubated with YM-254890 (100nM) or AG-1478 (1μM) either alone or in combination for 30 min prior to being stimulated with AYPGKF-NH_2 (100μM) for 6 hours. Following stimulation, supernatant was collected in fresh eppendorf tubes. ELISA specific for IL-8 was performed as mentioned in section 2.8. Quantification was expressed as mean$\pm$s.e.m (% AYPGKF-NH_2 stimulated control, *$p<0.05$, **$p<0.01$). Each column is representative of 3 individual experiments.

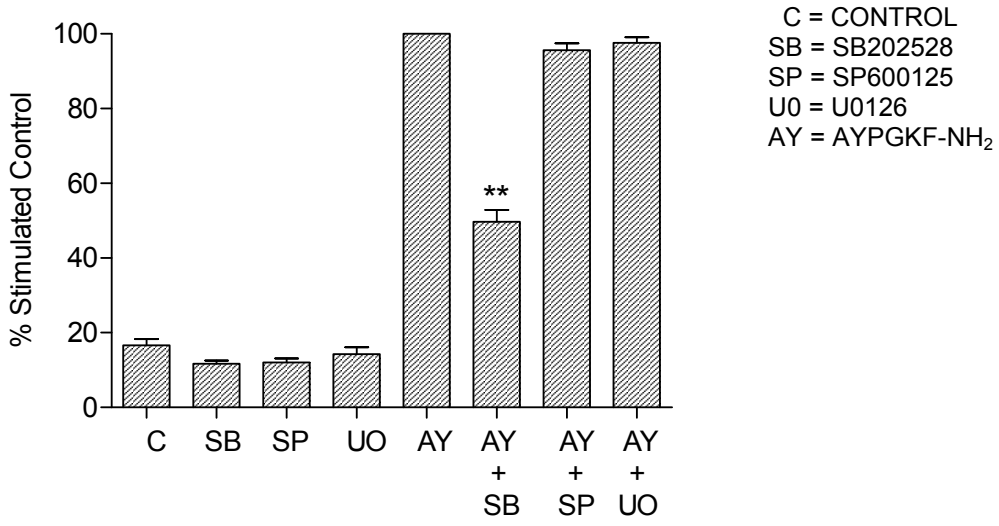


Figure 3.19 Effect of SB203528, SP600125 and U0126 on PAR-4 mediated IL-8 production in clone 10H9 cells. Clone 10H9 cells were grown to confluency on a 12 well plate and quiesced overnight in serum free media. Cells were pre-incubated with SB203528 (10μM), SP600125 (10μM) or U0126 (10μM) for 30 min prior to being stimulated with AYPGKF-NH_2 (100μM) for 6 hours. Following stimulation, supernatant was collected in fresh eppendorf tubes. ELISA specific for IL-8 was performed as outlined in section 2.8. Quantification was expressed as mean$\pm$s.e.m (% AYPGKF-NH_2 stimulated control, **$p<0.01$). Each column is representative of 3 individual experiments.

3.7 PAR-4 MEDIATES PHOSPHORYLATION OF ERK in EAhy-926 CELLS

The results obtained so far in this study have been generated in a cell system created to over express PAR-4. However, it was necessary to show that the results obtained in clone 10H9 cells were reproducible in a cell type which naturally express PAR-4. For this purpose, EAhy-926 cells (see section 3.1) were stimulated with either thrombin (3units/ml) or AYPGKF-NH_2 (100μM) for a time course ranging from 0-90 min. While thrombin mediated a transient phosphorylation of ERK, with a maximum response at 5 minutes (5.1±2.8 fold increase, n=3, t=5min), the response to AYPGKF-NH_2 was delayed (3.9±1.2 fold increase, n=3, t=15min), showing a variation in the kinetics of PAR-4 mediated response in the over expression cell type, as opposed to those cells which endogenously express the receptor (see figure 3.20).

3.7.1 PAR-4 mediates phosphorylation of p38 MAP kinase in EAhy-926 cells

For this purpose, EAhy-926 cells were stimulated with thrombin (3units/ml) or AYPGKF-NH_2 (100μM) for a time course ranging from 0-90 min. As with ERK, thrombin again mediated a transient phosphorylation of p38 MAP kinase (2.5±1.6 fold increase, n=3), with maximum phosphorylation at 5 min, whereas AYPGKF-NH_2 again did not mediate a response until 15 min (3.3±0.9 fold increase, n=3) (see figure 3.21). These particular results which display a difference in activation times may be due to the expression of another thrombin receptor within EAhy-926 cells. As previously mentioned, EAhy-926 is a hybrid cell type which incorporates the fusion of both HUVECs and A549 cells (Edgell *et al,* 1986). Previous studies have shown that PAR-1 is expressed in HUVECs and is involved in COX-2 activation (Bruekmann *et al*, 2005), as well as resulting in the upregulation of chemokine CXC3 (Popvic *et al*, 2008). It is therefore possible that whilst the selective PAR-4 activating peptide does demonstrate the ability of PAR-4 to mediate activation of the MAP kinase pathway in this cell type, the early onset of the thrombin response may in fact be due to the co-expression of PAR-1 in these particular cells.

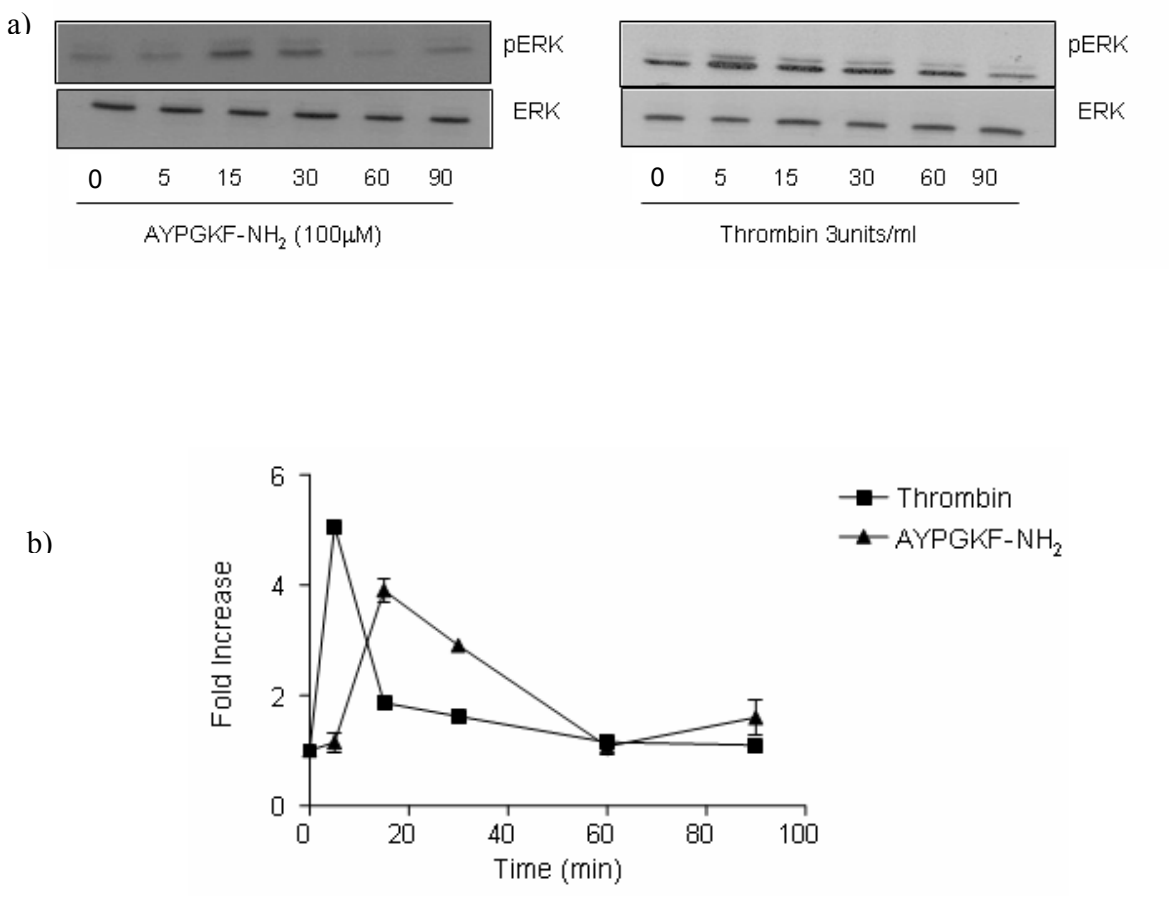
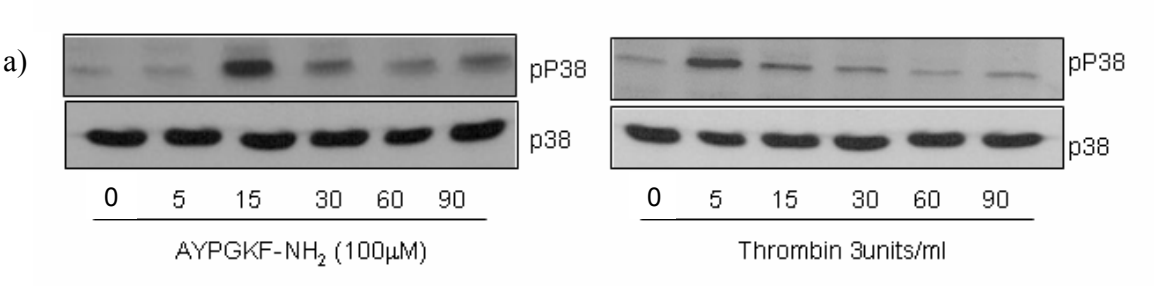


Figure 3.20 Phosphorylation of ERK stimulated by thrombin or AYPGKF-NH_2 in EAhy-926 cells. EAhy-926 cells were grown to confluency on 12 well plates and quiesced overnight in serum free media. Cells were then stimulated with either thrombin (3units/ml) or AYPGKF-NH_2 (100μM) over a time period ranging from 0-90min. Whole cell extracts were resolved by SDS-PAGE and examined by Western blotting as outlined in section 2.5. Panel A demonstrated relative phosphorylation of ERK while panel B illustrates quantification, which was performed by densitometry and expressed as mean±s.e.m (fold increase). Each blot is representative of 3 individual experiments.



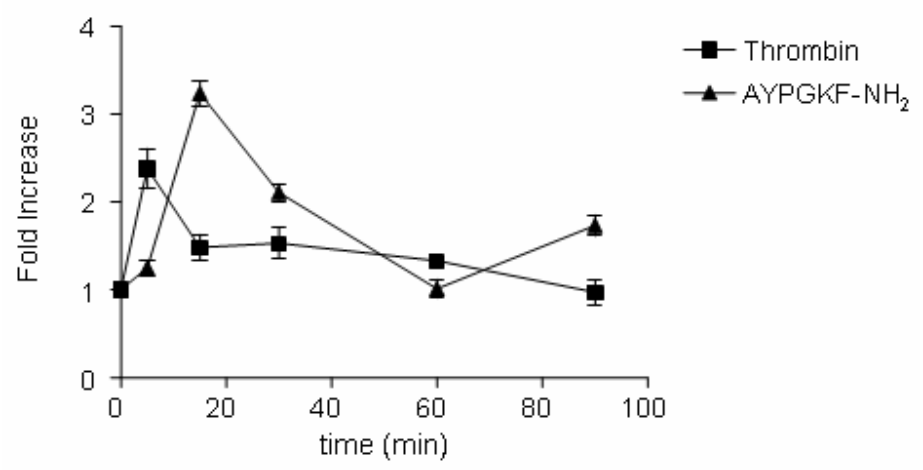


Figure 3.21 Phosphorylation of p38 MAP kinase stimulated by thrombin or AYPGKF-NH_2 in EAhy-926 cells. EAhy-926 cells were grown to confluency on 12 well plates and quiesced overnight in serum free media. Cells were then stimulated with either thrombin (3units/ml) or AYPGKF-NH_2 (100μM) over a time period ranging from 0-90min. Whole cell extracts were resolved by SDS-PAGE and examined by Western blotting as outlined in section 2.5. Panel A demonstrates the relative phosphorylation of p38, while panel B illustrates quantification which was performed by densitometry and expressed as mean±s.e.m (fold increase). Each blot represents 3 individual experiments.

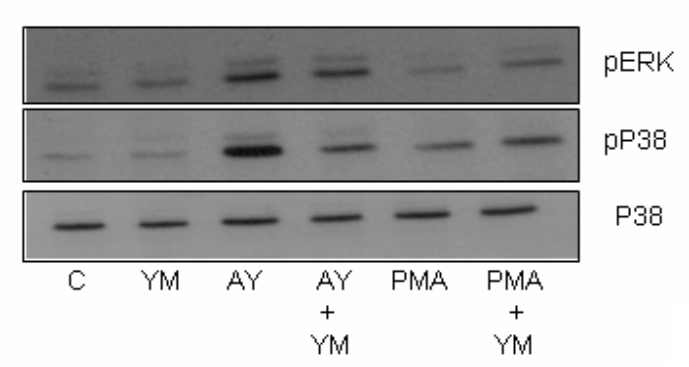
3.8 PAR-4 MEDIATES PHOSPHORYLATION OF ERK AND p38 MAPK VIA $G\alpha_{q/11}$-DEPENDENT MECHANISM IN EAhy-926 CELLS

Previous results in clone 10H9 cells have demonstrated that PAR-4 is capable of stimulating activation of ERK and p38 MAP kinase, in part, by coupling with $G\alpha_{q/11}$. These results complimented the results shown in cardiac myocytes, which demonstrated the ability of the PAR-4 activating peptide GYPGKF-NH_2, to an increase in total inositol phosphate, a mechanism associated with receptor coupling with $G\alpha_{q/11}$ (Sabri *et al*, 2003a). Therefore experiments were carried out to determine if PAR-4 is capable of stimulating phosphorylation of the MAP kinase pathway in EAhy-926 cells via the same mechanism.

3.8.1 Effect of YM-254890 on PAR-4 mediated phosphorylation of ERK and p38 MAP kinase in EAhy-926 cells

For this purpose, EAhy-926 cells were pre-incubated with YM-254890 (100nM) for 30 min prior to stimulation with AYPGKF-NH_2 (100μM) for 15 min. In order to ensure that the results obtained from the remaining experiments were selective for PAR-4, only the PAR-4 activating peptide AYPGKF-NH_2 was used. Results obtained by Western blotting assessed the relative phosphorylation of both ERK and p38 MAP kinase, with normalization of protein loading determined by total p38. AYPGKF-NH_2 stimulated phosphorylation of both ERK and p38 MAP kinase (4.2$\pm$1 and 4.8$\pm$1.4 fold above basal for ERK and p38 MAP kinase respectively, n=3) Results demonstrated that PAR-4 mediates the phosphorylation of ERK and p38 MAP kinase, again in part, by means of PAR-4 coupling with $G\alpha_{q/11,}$ (% inhibition, 30$\pm$3.5, 29$\pm$2.9, n=3, t=15min, *$p<0.05$ for ERK and p38 MAP kinase respectively) (see figure 3.22). Therefore, it can again be inferred that a second mechanism is involved in the signalling mechanism coupling PAR-4 with the MAP kinase pathway.

a)



C = CONTROL
AY = AYPGKF-NH_2
YM = YM-254890
PMA = PHORBOL 12-MYRISTATE 13-ACETATE

b)

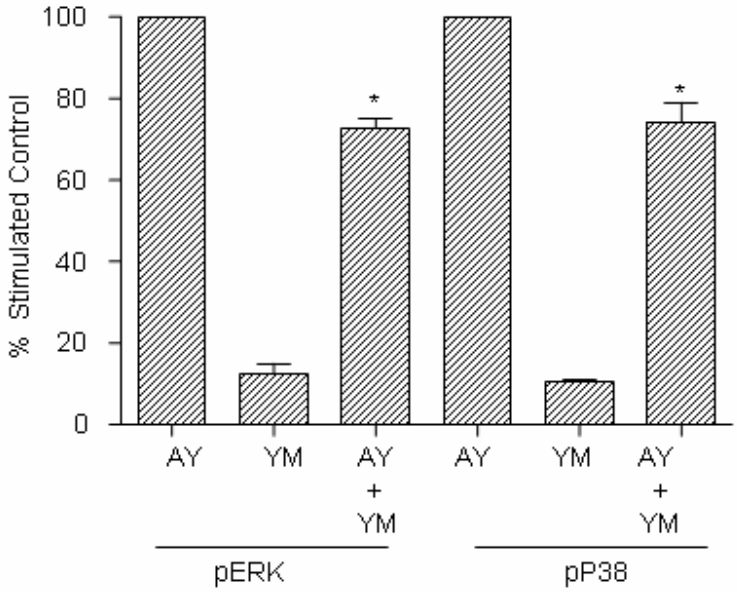


Figure 3.22 Effect of YM-254890 on PAR-4 mediated phosphorylation of ERK p38 MAP kinase in EAhy-926 cells. EAhy-926 cells were grown to confluency on 12 well plates and quiesced overnight in serum free media. Cells were pre-incubated with YM-254890 (100nM) for 30 min prior to stimulation with AYPGKF-NH_2 (100μM) or PMA (100nM) for 15 min. Whole cell extracts were resolved by SDS-PAGE and examined by Western blotting as outlined in section 2.5. Panel A demonstrates inhibition of ERK and p38 MAP kinase, while panel B illustrates quantification by densitometry, expressed as mean±s.e.m (% AYPGKF-NH_2 stimulated control, *$p<0.05$). Each blot is representative of 3 separate experiments.

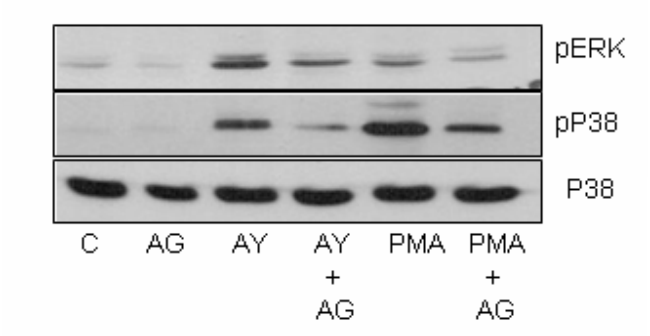
3.9 PAR-4 MEDIATES PHOSPHORYLATION OF ERK AND p38 MAPK BY TRANSACTIVATION OF THE EGFR IN EAhy-926 CELLS

Previous results in this study have demonstrated that the additive mechanism whereby PAR-4 mediates activation of the MAP kinase pathway is by coupling with $G\alpha_{q/11}$ while simultaneously initiating transactivation of the EGFR. Therefore, experiments were carried out to determine if PAR-4 also mediates activation of the MAP kinase pathway by means of transactivation of the EGFR in EAhy-926 cells.

3.9.1 Effect of AG-1478 on PAR-4 mediated phosphorylation of ERK and p38 MAP kinase in EAhy-926 cells

For the purpose of these experiments, EAhy-926 cells were pre-incubated with AG-1478 (1μM) for 30 min prior to stimulation with AYPGKF-NH_2 (100μM) for 15 min. Results shown in figure 3.23 were obtained by Western blotting and determined the relative phosphorylation of ERK and p38 MAP kinase, with equal protein loading being determined by total p38. AYPGKF-NH_2 stimulated phosphorylation of ERK and p38 MAP kinase (3.5$\pm$0.3 and 4$\pm$0.45 fold increase above basal for ERK and p38 MAP kinase respectively). Results demonstrated that AG-1478 caused inhibition of PAR-4 mediated phosphorylation of ERK and p38 MAP kinase (% inhibition, 45$\pm$0.9, 55$\pm$2.2, n=3, t=15min for ERK and p38 MAP kinase respectively), therefore suggesting that PAR-4 also mediates activation of the MAP kinase pathway via transactivation of the EGFR in cells which naturally express PAR-4 (see figure 3.23).

a)



C = CONTROL
AY = AYPGKF-NH_2
AG = AG1478
PMA = PHORBOL 12-MYRISTATE 13-ACETATE

b)

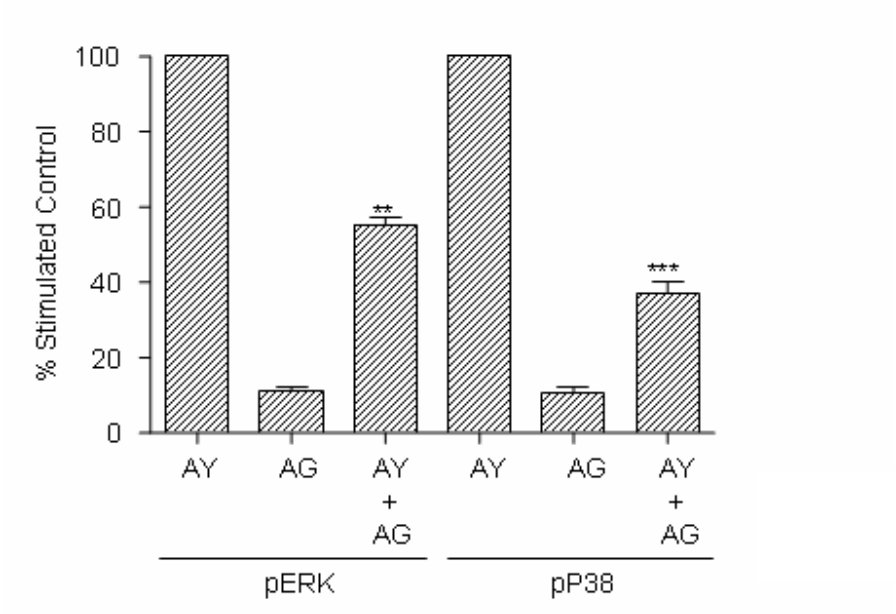


Figure 3.23 Effect of AG-1478 on PAR-4 mediated phosphorylation of ERK and p38 MAP kinase in EAhy-926 cells. EAhy-926 cells were grown to confluency on 12 well plates and quiesced overnight in serum free media. Cells were pre-incubated with AG-1478 (1μM) for 30 min prior to stimulation with AYPGKF-NH_2 (100μM) or PMA (100nM) for 15 min. Whole cell extracts were resolved by SDS-PAGE and examined by Western blotting as outlined in section 2.5. Panel A demonstrates AG-1478 mediated inhibition of ERK and p38 MAP kinase. Panel B illustrates the quantification performed by densitometry, expressed as mean±s.e.m (%AYPGKF-NH_2 stimulated control, **p<0.01, ***p<0.001). Each blot represents 3 individual experiments.

3.10 ADDITIVE INHIBITORY EFFECT OF YM-254890 AND AG1478 ON PAR-4 MEDIATED ACTIVATION OF ERK AND p38 MAPK IN EAhy-926 CELLS

Results from sections 3.8 and 3.9 have demonstrated that PAR-4 is capable of mediating phosphorylation of ERK and p38 MAP kinase in EAhy-926 cells in a similar fashion to clone 10H9 cells, whereby PAR-4 coupling with $G\alpha_{q/11}$ and transactivation of the EGFR are involved. Therefore, to determine if these mechanisms work in an additive fashion in EAhy-926, confluent cells were pre-incubated with YM-254890 or AG-1478 either alone or both inhibitors together. Cells were then stimulated with AYPGKF-NH_2 (100μM) for 15 min. Figure 3.24 demonstrates phosphorylation of ERK by AYPGKF-NH_2 (3.5$\pm$0.5 fold above basal, n=3, t=15min). Phosphorylation of p38 MAP kinase was also apparent, (4.2$\pm$0.4 fold above basal, n=3, t=15min). The results further demonstrate that both YM-254890 and AG-1478 together caused an additive inhibitory effect on the phosphorylation of ERK and p38 MAP kinase in EAhy-926 cells. This occurred in a manner similar to that found in clone 10H9 cells. As AYPGKF-NH_2 stimulated cells which had been pre-incubated with both inhibitors displayed a significant inhibition of both ERK and p38 MAP kinase (% inhibition 92$\pm$2.2, 95$\pm$1.5, n=3, t=15min ***$p<0.001$, for ERK and p38 MAP kinase respectively) (see figure 3.24), it may be suggested that PAR-4 coupling to Gαq/11 and transactivation of the EGFR both result in the activation of ERK and p38 MAP kinase.

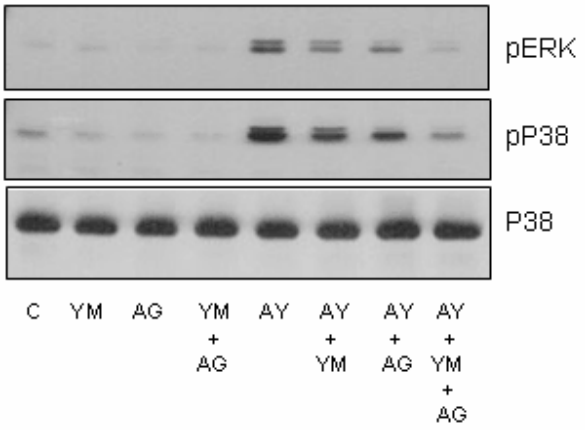
3.11 PAR-4 mediates activation of the MAP kinase pathway via PKC in EAhy-926 cells

As previously mentioned, receptor coupling with $G\alpha_{q/11}$ normally mediates the production of IP_3 with a subsequent increase in intracellular Ca^{2+} and a DAG-dependent increase in PKC activity as a mediator of MAP kinase activation. However, this intermediate action was not demonstrated in the PAR-4 expression clone 10H9. Therefore, it was necessary to determine if the PAR-4 mediated activation of the MAP kinase pathway involves the interaction of PKC in cells which naturally express PAR-4. For this purpose, the PKC inhibitor GF-109203X was employed to investigate the interaction of PKC in EAhy-926 cells.

3.11.1 Additive inhibitory effect of GF-109203X and AG-1478 on PAR-4 mediated phosphorylation of ERK and p38 MAP kinase in EAhy-926 cells

To demonstrate that PKC mediates activation of the MAP kinase pathway in a manner that is downstream of $G\alpha_{q/11}$, the PKC inhibitor, GF-109203X along with AG-1478 were used. Therefore EAhy-926 cells were pre-incubated with GF-109203X (10μM) or AG-1478 (1μM) either alone or together prior to stimulation with AYPGKF-NH_2 (100μM) or PMA (100nM) for 15 min. Results obtained by Western blotting demonstrated stimulation of both ERK and p38 MAP kinase by both AYPGKF-NH_2 and PMA (stimulation of ERK, 4.8$\pm$0.75 and 5$\pm$1.2 for AYPGKF-NH_2 and PMA respectively, n=3, t-15 min, stimulation of p38, 5.1$\pm$0.5 and 4.7$\pm$0.3 for AYPGKF-NH_2 and PMA respectively, n=3, t=15 min) (see figure 3.25). GF-109203X and AG-1478 both displayed inhibition individually, however no additive inhibitory effect with both GF-109203X and AG-1478 was apparent with phosphorylation of ERK. Interestingly, an additive inhibitory effect was apparent when assessing phosphorylation of p38 MAP kinase when stimulated with either AYPGKF-NH_2 or PMA (% inhibition, 75$\pm$1.5 and 73$\pm$2.4 for AYPGKF-NH_2 and PMA respectively, n=3), suggesting that classical PKC isoforms may be involved PAR-4 mediated phosphorylation of p38-MAP kinase in EAhy-926 cells.

a)



C = CONTROL
YM = YM-254890
AG = AG1478
AY = AYPGKF-NH_2

b)

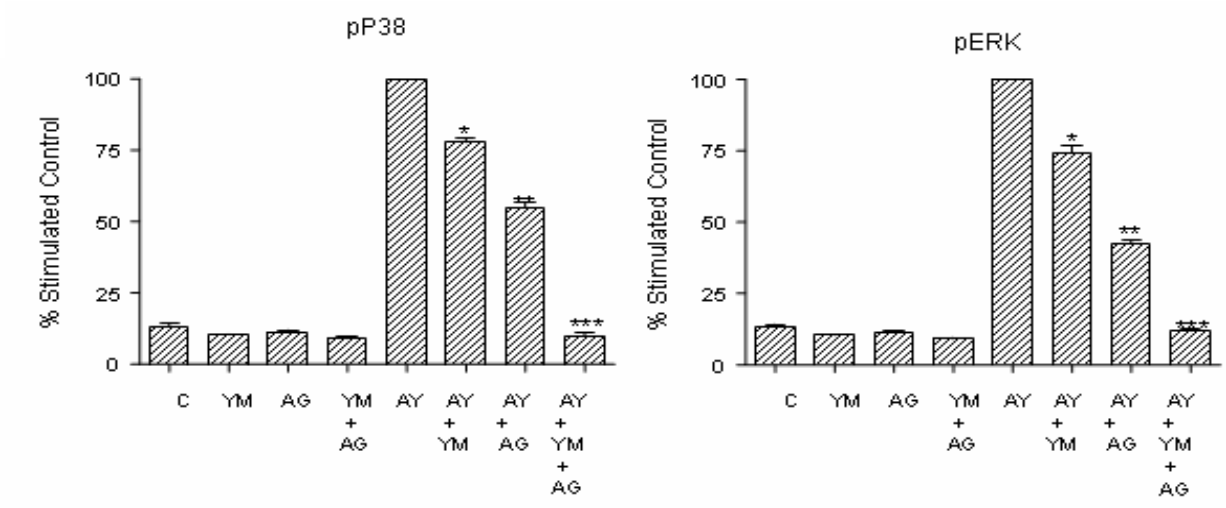


Figure 3.24 Additive inhibitory effect of YM-254890 and AG-1478 on PAR-4 mediated phosphorylation of ERK and p38 MAP kinase in EAhy-926 cells. EAhy-926 cells were grown to confluency on 12 well plates and quiesced overnight in serum free media. Cells were pre-incubated with YM-254890 (100nM) or AG-1478 (1μM) either alone or in combination for 30 min prior to stimulation with AYPGKF-NH_2 (100μM)for 15 min. Whole cell extracts were examined by Western blotting as outlined in section 2.5. Panel A demonstrates relative phosphorylation of ERK and p38 MAP kinase, while panel B illustrates the quantification by densitometry, expressed as mean±s.e.m (% stimulated control, *p<0.05, **p<0.01, ***p<0.001).

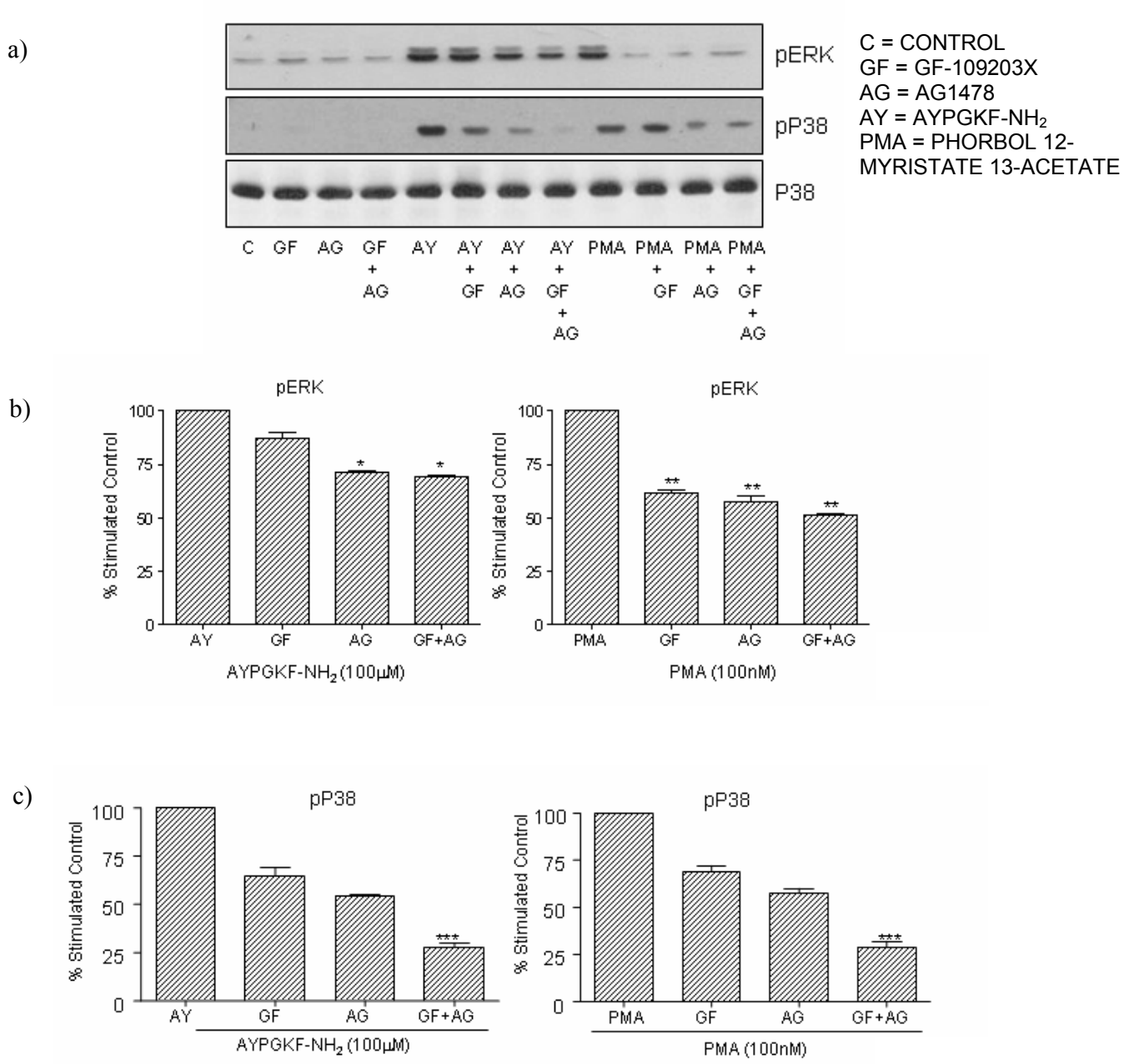


Figure 3.25 Additive inhibitory effect of GF-109203X and AG-1478 on PAR-4 mediated phosphorylation of ERK and p38 MAP kinase in EAhy-926 cells. EAhy-926 cells were grown to confluency on 12 well plates and quiesced overnight in serum free media. Cells were pre-incubated with GF-109203X (10μM) or AG-1478 (1μM) either alone or in combination for 30 min prior to stimulation with AYPGKF-NH_2 (100μM) or PMA (100nM) for 15 min. Whole cell extracts were examined by Western blotting as outlined in section 2.5. Panel A demonstrates inhibition of ERK and p38 MAP kinase, while panels B & C illustrate quantification by densitometry, expressed as mean±s.e.m (% stimulated control, *$p<0.05$, **$p<0.01$, ***$p<0.001$). Each blot is representative of 3 individual experiments.

3.12 DISCUSSION

So far, this project has studied the coupling of PAR-4 to ERK and p38 MAP kinase in NCTC-2544 cells stably expressing PAR-4 (clone 10H9) and EAhy-926 cells which express PAR-4 naturally. The ability of PAR-4 to mediate production of IL-8 was also assessed. The effects of several inhibitors, including $G\alpha_{q/11}$ inhibitor YM-254890, EGFR tyrosine kinase inhibitor AG-1478 and PKC inhibitor GF-109203X were employed to determine the intracellular mechanisms whereby PAR-4 is capable of mediating activation of the MAP kinase pathway.

There are few studies which have investigated the signalling characteristics of PAR-4. Therefore, it was necessary to understand the signalling characteristics of the other members of the PAR family. For example, PAR-1 has been shown to couple with $G\alpha_{q/11}$, a mechanism which has been demonstrated by means of selective antibody studies (Hung *et al,* 1992a, Baffy *et al*, 1994). These particular studies illustrated that PAR-1 signalling was impaired in both fibroblasts and platelets when pre-incubated with antibodies to $G\alpha_{q/11}$. Furthermore, other studies have shown that mice deficient in $G\alpha_{q/11}$ display decreased platelet aggregation accompanied with prolonged periods of bleeding due to impairment of the response associated with PAR-1 (Offermanns *et al*, 1997). PAR-1 coupling with $G\alpha_{q/11}$ has also been shown to stimulate an increase of total inositol phosphate (Brass *et al,* 1991, Hung *et al*, 1992a). It has also been shown that PAR-1 is capable of mediating activation of the MAP kinase pathway via coupling with $G\alpha_{q/11}$. This was demonstrated in a study by Marrinissen *et al*, 2003, who illustrated that thrombin mediated activation of the receptor results in PAR-1 coupling with $G\alpha_{q/11}$, which in turn resulted in MAP kinase phosphorylation, in particular p38 MAP kinase and JNK (Marrinissen *et al,* 2003).

PAR-1 has also been found to couple with other G proteins, including $G\alpha_{i/o}$. Assessment of coupling with $G\alpha_{i/o}$ was found to result in an increase in the production of total inositol phosphate production. Inhibition studies were carried out in CCL-39 fibroblasts, whereby pre-incubation of cells with PTx resulted in a decrease in total inositol phosphate stimulated by PAR-1 agonists, therefore suggesting the ability of PAR-1 to couple with $G\alpha_{i/o}$ (Hung *et al,* 1992b). However, association of such signalling characteristics have been poorly defined for PAR-4. In this current study, initial assessment of PAR-4 activation in clone 10H9 cells demonstrated the ability to mediate an increase in total inositol phosphate accumulation. These results suggest the ability of PAR-4 to couple with either $G\alpha_{q/11}$ or $G\alpha_{i/o}$. Whilst the ability to mediate an increase in total inositol phosphate had previously been demonstrated upon initial identification of PAR-4 (Xu *et al,* 1998) few studies have illustrated the subsequent G protein coupling utilized by PAR-4 following its activation. Therefore, it was shown in this project that PAR-4 was able to stimulate a concentration-dependent increase in the accumulation of total [^{3}H]-IP, in clone 10H9 cells, suggesting that the ability of the receptor to couple with $G\alpha_{q/11}$ or $G\alpha_{i/o}$ (see figure 3.2). This data is consistent with previous studies which have shown that PAR-4 agonists such as thrombin or activating peptide, have the ability to mediate an increase in total [^{3}H]-IP (Sabri *et al*, 2003a, Shapiro *et al*, 2000).

To determine if PAR-4 mediated accumulation of total inositol phosphate was dependent on coupling with either $G\alpha_{q/11}$ or $G\alpha_{i/o}$, inhibition studies employed the use of PTx to assess coupling to $G\alpha_{i/o}$, while use of YM-254890 was employed to determine possible receptor coupling with $G\alpha_{q/11}$. YM-254890 is a recently introduced pharmacological inhibitor, cultured from the broth of *Chromobacterium* sp. QS3666, (Taniguchi *et al*, 2003). With the ability to prevent receptor coupling to $G\alpha_{q/11}$, YM-254890 works in a selective manner, whereby it prevents the exchange of GDP for GTP, while at the same time, does not interfere with the calcium mobilization which is stimulated by $G\alpha_{i/o}$ (Takasaki *et al*, 2004).

This particular inhibitor has already been successfully employed within this laboratory to demonstrate that PAR-2 is capable of activating the MAP kinase pathway via coupling with $G\alpha_{q/11}$ (Goh *et al*, 2008). Results from this current study suggested that at the level of total inositol phosphate accumulation, PAR-4 did not appear to couple with $G\alpha_{i/o}$, but did successfully demonstrate the ability to couple with $G\alpha_{q/11}$. YM-254890 caused a concentration-dependent inhibition of PAR-4 mediated inositol phosphate accumulation in clone 10H9 cells (see figure 3.8), further demonstrating that PAR-4 is capable of mediating an increase of total inositol phosphate accumulation via coupling with $G\alpha_{q/11}$. This data also suggests that YM-254890 provides the potential of serving as a useful pharmacological inhibitor of PAR-4 associated $G\alpha_{q/11}$ activity.

Further data within this study has also demonstrated that PAR-4 is capable of mediating the phosphorylation and activation of ERK and p38 MAP kinase in clone 10H9 and EAhy-926 cells (see figures 3.3, 3.4, 3.20 & 3.21). While few studies have looked at PAR-4 mediated activation of this pathway, other GPCRs including PAR-1 and PAR-2 have been documented as being able to stimulate MAP kinase activation. For example, PAR-2, has been shown to activate ERK in several cell types, including rat aortic smooth muscles (RASMCs) (Belham *et al*, 1996), human umbilical vein endothelial cells (HUVECS), (Woolkalis *et al*, 1996), astrocytes (Ge *et al*, 2003) and human colon cells (Nishibori *et al,* 2005), while PAR-1 has been shown to mediate prolonged phosphorylation of ERK within the epithelial cells of the rat mucosa (Sekiguchi *et al,* 2007).

Many other GPCRs have been associated with activation of the MAP kinase pathway, which include the β-adrenergic receptor, which results in the activation of ERK by mediating the transactivation of the EGFR in COS-7 cells (Maudsley *et al*, 2000). Also the muscarinc (M2) receptor has been found to activate ERK via the Ras-ERK pathway, by initially coupling with $G\alpha_{i/o,}$(Mochizuki *et al*, 1999) and the δ-opioid receptor, which like the β-adrenergic receptor, has also been documented to mediate activation of ERK by means of initiating transactivation of the EGFR. Some studies have assessed PAR-4 mediated activation of both ERK and p38 MAP kinase.

One particular study has determined that in N9 rat microglial cells, PAR-4 was capable of a sustained activation of ERK, which displays activation characteristics which are completely varied from that of either 10H9 or EAhy-926 cells. Initially in this study it was demonstrated by PCR that PAR-4 mRNA was expressed in N9 microglial cells (Suo *et al*, 2003). To follow these findings, this group demonstrated that PAR-4 peptide mediated phosphorylation of ERK was apparent up to and including 6 hours in these particular cells. However, in this current study, it was demonstrated that clone 10H9 and EAhy-926 cells displayed a difference in the signalling mediated by the selective PAR-4 activating peptide, AYPGKF-NH_2, whereby clone 10H9 cells displayed phosphorylation of ERK at 5 minutes (see figure 3.3), whilst in EAhy-926 cells demonstrated optimum phosphorylation at 15 minute (see figure 3.20). Taken together, these current data, along with the sustained phosphorylation demonstrated in microglial cells, it may be suggested that the mechanism whereby PAR-4 mediates activation of ERK may be a cell specific mechanism rather than a receptor specific feature.

Another cell type in which PAR-4 has been found to regulate ERK and p38 MAP kinase are cardiomyocytes. Sabri *et al*, 2003a, demonstrated that in murine cardiomyocytes, PAR-4 mediated a transient phosphorylation of ERK and sustained phosphorylation of p38 MAP kinase. These particular results correlate with the data demonstrated by both clone 10H9 and EAhy-926 cells. The study by Sabri *et al*, 2003a, also determined that PAR-4 mediated activation of the MAP kinase pathway was also in part, mediated by PAR-4 transactivation of the EGFR. This study, which utilised EGFR inhibitor, AG1478 illustrated that in cardiomyocytes which were pre-incubated with AG1478 displayed an inhibition of the phosphorylation of both ERK and p38 MAP kinase. These results suggest that PAR-4 mediates activation of the MAP kinase via activation of the EGFR in this cell type. Again, this coincides with the results demonstrated in clone 10H9 and EAhy-926 cells, which suggests that EGFR transactivation may be a possible signalling characteristic whereby PAR-4 mediates activation of the MAP kinase pathway (see figures 3.12 & 3.23).

As such, these results suggest that PAR-4 has the ability to activate the MAP kinase pathway by 2 distinct mechanisms, namely by coupling with $G\alpha_{q/11}$ and also by transactivation of the EGF receptor. Further experiments assessed the combined response of both inhibitors. Both clone 10H9 and EAhy-926 cells which had been pre-incubated with both YM-254890 and AG1478 displayed an additive inhibitory effect, which almost completely inhibited the PAR-4 mediated activation of both ERK and p38 MAP kinase (see figures 3.13 & 3.24). Several previous studies have demonstrated that activation of the MAP kinase pathway via transactivation of the EGFR may actually be a common feature shared by the PAR family. To date, such evidence has been shown for PAR-1 (Darmoul *et al*, 2004a, Kalmes *et al*, 2000 and Bergmann *et al*, 2006), PAR-2 (Darmoul *et al*, 2004b) and PAR-4 (Sabri *et al*, 2003a, Ando *et al,* 2008).

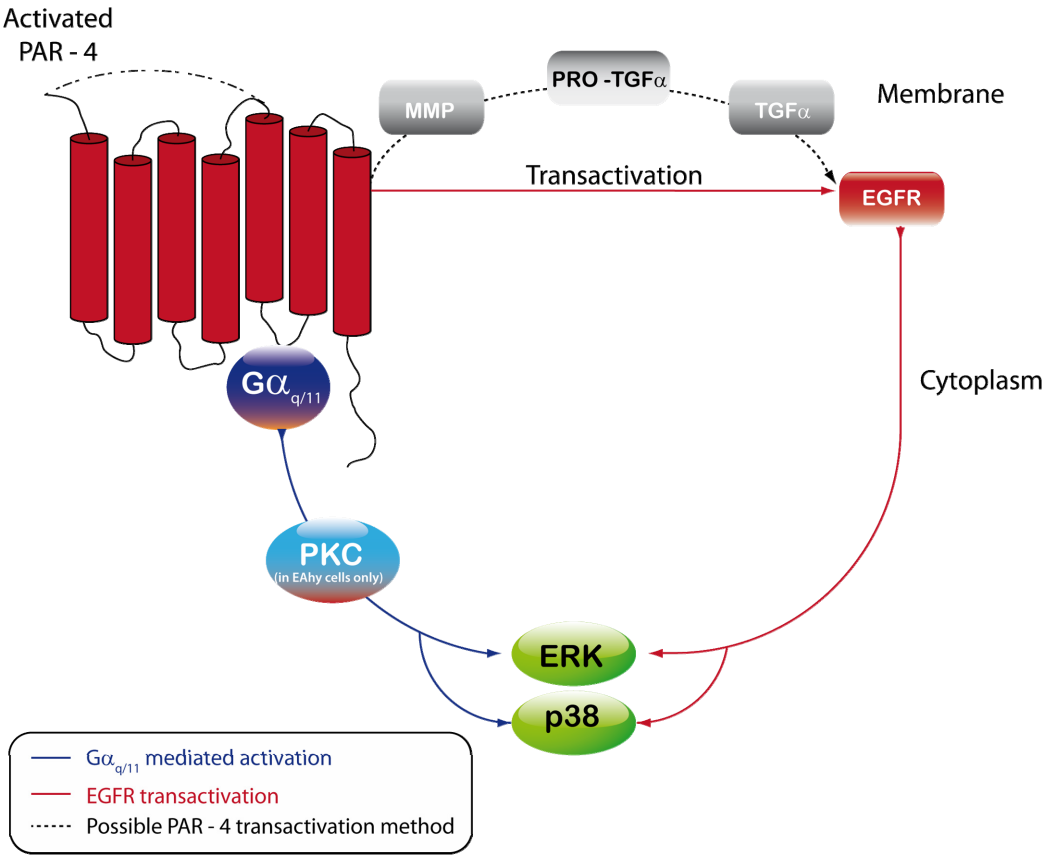


Figure 3.26 Diagrammatical representation of the possible PAR-4 mediated activation of ERK & p38 MAP kinase in clone 10H9 and EAhy-926 cells. The above diagram represents the possible pathway characterising the PAR-4 mediated activation of the MAP kinase pathway. Activation of PAR-4 has so far been found to activate this pathway in part by coupling with $G\alpha_{q/11}$ and by transactivation of the EGFR. The above diagram also contains the possible pathway involved in the transactivation of the EGFR, whereby possible PAR-4 mediated activation of matrix-metalloproteinases (MMP) may result in the cleavage of the membrane bound pre-cursor of TGFα. Shedding of this pre-cursor will result in the release of TGFα, which may be the ligand responsible for the activation of the EGFR. Previous studies have suggested that PAR mediated activation of MMP; with subsequent release of EGFR ligands is a common method of transactivation of the EGFR (Darmoul *et al*, 2004a, Darmoul *et al,* 2004b, Bergmann *et al*, 2006). Downstream of $G\alpha_{q/11}$ in EAhy-296 cells also appeared to involve a PKC-dependent mechanism in activation of MAP kinase. This was not demonstrated clone 10H9 cells, possibly due to the artefactual limitations of using a heterologous over expression system.

Recent evidence from a study by Ando *et al*, 2008, has shown that PAR-4 is capable of mediating transactivation of the EGFR in the human alveolar-epithelial cell line known as A549 cells, which are one of the cell types incorporated in EAhy-926 cells. In this particular study, stimulation of PAR-4 with either thrombin or activating peptide resulted in morphological changes of these epithelial cells. Through inhibition studies, this group demonstrated that this was via the involvement of the EGFR. Using cellular markers α-SMA and E-cadherin, which are associated with epithelial mesenchymal transition (EMT), both thrombin and AYPGKF-NH_2 demonstrated that activation of PAR-4 is capable of mediating the elongation of the epithelial cells from their normal cobblestone-like appearance. Both markers of EMT were significantly inhibited when cells were pre-incubated with EGFR tyrosine kinase inhibitor AG1478, suggesting that PAR-4 mediated EMT in these epithelial cells was likely to occur via transactivation of the EGFR (Ando *et al,* 2008). Further to demonstrating that the EGFR was involved with this EMT process, transactivation of the EGFR was significantly inhibited by addition of the Src inhibitor AG1879 (PP-2), suggesting that Src may also be involved in the activation of the EGFR (Ando *et al*, 2008). These results did not coincide with the data demonstrated by clone 10H9 cells, which when pre-incubated with PP-2, did not display any level of inhibition when stimulated with AYPGKF-NH_2 (observation by colleague, data not shown). These observed results could account for the limitations of such experiments being carried out in a heterologous expression system such as clone 10H9. In such a cell type, activation of PAR-4 is not under the level of cellular control that would normally be associated with its naturally expressing environment.

As previously mentioned, current literature has suggested that transactivation of the EGFR may be a general characteristic of PAR signalling. PAR-1 has also been shown to mediate transactivation of the EGFR via the interaction of endopeptidase known as matrix-metalloproteinase (MMP) (Darmoul *et al*, 2004, Bergmann *et al*, 2006). The study by Darmoul *et al*, 2004a, demonstrated that PAR-1 is highly expressed in the human colon cancer cell line, HT-29 cells. In this particular study, it was suggested that activation of PAR-1 results in the involvement of MMPs, which in turn result in the cleavage of the membrane bound form of transforming growth factor-a (TGFα).

TGFα is a member of the EGF family of cytokines that are synthesised as transmembrane pre-cursors. The soluble form of TGFα is released from its membrane bound protein by means of proteolytic cleavage (Luetteke & Lee, 1991). The study by Darmoul *et al*, 2004a, demonstrated that TGFα was responsible for PAR-1 mediated transactivation of the EGFR in colon cancer cells. This was demonstrated by use of TGFα neutralising antibodies. Studies whereby PAR-1 activating peptide mediated cellular proliferation, demonstrated a decrease in proliferation when pre-incubated with TGFα antibody. Use of selective MMP inhibitor batimistat also resulted in a decrease in the phosphorylation of ERK, which suggests that the membrane bound cleavage of the TGFα pro-form is likely to be dependent on the involvement of MMPs (Darmoul *et al*, 2004a). However, the role of MMP involvement in PAR-1 mediated transactivation of the EGFR was further established in a study by Bergmann *et al*, 2006.

In the study by Bergmann *et al*, it was shown that PAR-1 mediates the transactivation of the EGFR in A948 renal-carcinoma cells with involvement of matrix-metalloproteinase. Initial results showed that through use of the EGFR inhibitor AG1478, and selective PAR-1 antagonist, SCH 79797, that thrombin or PAR-1 selective activating peptide mediated phosphorylation of the EGFR was significantly inhibited. Further to this, the Bergmann study utilised selective MMP inhibitor GM-6001. These experiments demonstrated that GM-6001 resulted in partial inhibition of EGFR phosphorylation, suggesting that MMPs may play a role in the PAR-1 mediated transactivation of the EGFR (Bergmann *et al*, 2006). However, this particular study did not concentrate on the specific ligand which was released via MMP interaction. One further study by Kalmes *et al*, 2000), has suggested that MMPs may be involved in the PAR-1 mediated transactivation of the EGFR in rat smooth muscle cells. In the study by Kalmes *et al*, 2000, it was demonstrated that thrombin receptors, such as PAR-1 can be responsible for the transactivation of the EGFR by heparin-binding epidermal growth factor (HB-EGF). HB-EGF is a member of the epidermal-growth factor family, and like TGFα, is synthesised as a transmembrane pre-cursor.

HB-EGF becomes an active soluble growth factor by means of proteolytic cleavage by endopeptidase enzymes, including MMPs (reviewed by Raab & Klagsburn, 1997). As the previous studies mentioned, involvement of the EGFR was shown by utilising the EGFR tyrosine-kinase inhibitor, AG1478, which showed an inhibition of both the thrombin and PAR-1 activating peptide mediated phosphorylation of ERK. Further to this, it was established that MMPs may be the type of endopeptidase responsible for the membrane-bound shedding of HB-EGF, as the thrombin mediated phosphorylation of ERK was markedly reduced in the presence of MMP inhibitor batimastat (Kalmes *et al,* 2000). However, whilst it has been suggested that the PAR mediated transactivation of the EGFR is possibly dependent on the MMP mediated membrane shedding of pro-forms of EGFR ligands, as yet, the relationship between the proteinase-activated receptor and the matrix-metalloproteinase remains elusive.

Taken together, these particular studies appear to show a trend whereby PARs are capable of mediating the transactivation of the EGFR. This would suggest that the results generated in both clone 10H9 cells and EAhy-926 cells have shown that it is likely that a general signalling characteristic of PAR-4 may be to mediate the activation of the MAP kinase pathway, in part by transactivation of the EGFR. Further to the data generated within this current study, it would be advantageous to assess the possibility of the involvement of MMPs in this pathway by selective inhibition studies. Should this be the case, it would be interesting to determine which membrane-bound EGF ligand is generated upon cleavage by MMPs by means of antibody studies in order to provide a more detailed description of PAR-4 mediated transactivation of the EGFR in both these cell types.

It has been documented that the activation of conventional PKC is a process dependent on diacylglycerol (DAG), (Naor, 2008, Das Evcimen & King, 2007, Godoy & Cukierman, 1994). The production of DAG normally occurs as a concurrent product of phosphatidylinositol hydrolysis, which in turn can occur via receptor coupling with $G\alpha_{q/11}$. In this current study, it has been demonstrated that the PAR-4 mediated activation of the MAP kinase pathway is partly dependent on $G\alpha_{q/11}$. Therefore, it was hypothesised that PKC may act as a mediator in the pathway stimulated by PAR-4 activation.

For this purpose, clone 10H9 and EAhy-926 cells were stimulated with activating peptide after being pre-incubated with a broad spectrum PKC inhibitor, GF-109203X. It has been previously documented that this inhibitor has been proven to be a potent inhibitor of Ca^{2+}-dependent and -independent PKC isoforms (Macfarlane *et al*, 2005, Wang *et al,* 2008, Ihara *et al*, 2007). However, GF-109203X did not demonstrate inhibition of PAR-4 mediated ERK or p38 MAP kinase signalling either individually, or as part of an additive effect with AG-1478 in clone 10H9 cells. These results suggest that in these particular cells, conventional PKC is not involved in the PAR-4 mediated activation of the MAP kinase pathway. However, in PAR-4 naturally expressing cells, EAhy-926, GF-109203X did cause inhibition of both ERK and p38 MAP kinase (% inhibition 70 ± 1.5, 72 ± 3.5 for ERK and p38 MAP kinase respectively) (see fig 3.25). This would suggest that PAR-4 may mediate activation of the MAP kinase pathway via a PKC-dependent mechanism.

The fact that AG1478 caused an inhibition of p38 MAP kinase when stimulated by PMA suggests that PKC may be involved in cross-talk with the EGFR in this particular cell type. Whilst few studies have concentrated on this area of research, it has been shown that the cell migration of human keratinocytes, which is dependent on tissue kallikrein (TK) may utilise a similar mechanism (Gao *et al*, 2009). PAR-1 mediated activation of TK has been shown to mediate an increase in the phosphorylation of the EGFR in keratinocytes, thus promoting their migration.

However, this phosphorylation of the EGFR was attenuated upon PKC inhibition, which in turn appeared to be instrumental in the activation of Src. Therefore, activation of PAR-1 in this instance has suggested the possibility of a pathway whereby it causes phosphorylation of the EGFR via activation of PKC and Src (Gao *et al*, 2009).

Further evidence has demonstrated that GPCR mediated activation of PKC can mediate activation of matrix-metalloproteinases, which as previously discussed, have the ability to result in the activation of the EGFR. One study by Sinclair *et al*, 2002 demonstrated that in gastric cells, the peptic enzyme gastrin is capable of regulating activation of the EGFR via a PKC-dependent pathway. In this pathway, GPCR activation of PKC results in the activation of MMP-3, which in turn will mediate cleavage of the membrane bound form of HB-EGF. Membrane shedding of EGF finally results in the activation of the EGFR (Sinclair *et al,* 2002). Whilst PMA by-passes any activation of the GPCR and concurrent action of G proteins to directly activate PKC, it would appear that PKC may still be of importance in this pathway. No evidence has as yet been established as to which method PAR-4 utilises to mediate activation of the EGFR, but it would be of future relevance to investigate this possibility in order to help characterise this receptor.

As previously mentioned, there was a difference in the utilisation of PKC between the two cell types. Again, this may represent a difference in the expression systems used. Clone 10H9 cells are a heterologous expression system, and as such, study of PAR-4 in these cells is not under the normal control of a natural expression system. It may therefore be suggested that clone 10H9 may not be the cell of choice when assessing the involvement of PKC in the PAR-4 mediated activation of the MAP kinase pathway. The ability of PAR-4 to couple with the MAP kinase pathway suggests that PAR-4 may be implicated in mediating varied cellular responses, although these results reflect a difference in the sensitivity of PAR-4's ability to couple with both ERK and p38 MAP kinase.

It would appear that while thrombin is able to elicit a prolonged ERK response via activation of PAR-1 in certain cells, including astrocytes and cardiomyocytes respectively (Wang *et al*, 2002, Suo *et al,* 2003), PAR-4 mainly seems to mediate a transient ERK response, and in this current study, activation of PAR-4 demonstrated a prolonged p38 MAP kinase response.

This would suggest that PAR-4 may be capable of mediating cellular stress responses and possibly stimulate an inflammatory response. These are responses which are typically associated with PAR-2. For example, PAR-2 mediates inflammatory responses in cell types including synovial mast cells (Palmer *et al,* 2007), pulmonary fibroblasts (Ramachandran *et al,* 2007) and human primary keratinocytes (Goh *et al,* 2008). Therefore, to demonstrate the ability of the receptor to mediate a possible inflammatory response, PAR-4 mediated production of an inflammatory cytokine was investigated. IL-8 is part of the chemokine family which is normally produced in macrophages and epithelial cells (Baggiolini *et al*, 1992). The current data from this project correlates with the ability of other GPCRs that have been associated with the production of IL-8, including the Kaposi's sarcoma-associated herpes virus G protein coupled receptor (KSHV GPCR), (Schwarz & Murphy, 2001), while in prostate cancer cells, thrombin-mediated activation of PAR-1 has been found to be associated with the production of IL-8 (Tantivejkul, *et al,* 2005). PAR-2 has also been shown to mediate the production of IL-8 in human bronchial epithelial cells (Pages *et al*, 2004); although more recently, activation of PAR-2 has demonstrated an increase of IL-8 production via a JNK-dependent mechanism in human bronchial fibroblasts (Ostrowska & Reiser 2008).

Taken together, these results have already demonstrated a role for PAR-2 in the pathogenesis of airway disorders. To date, few studies have identified PAR-4 as a mediator of inflammatory responses. Interestingly, while IL-8 production may be suggested as an end product of PAR activation, not all GPCRs are capable of mediating the production of IL-8, and instead regulate IL-8 activity in a negative manner.

For example, β_2-adrenergic agonists when used in synergy with corticosteroids have been found, in an additive manner, to inhibit the production of certain inflammatory mediators, including IL-8 and TNFα in human airway smooth muscle cells (Nie *et al*, 2005).

The results generated in this study demonstrated that in clone 10H9 cells, activation of PAR-4 mediated a substantial increase in the production of IL-8 (750$\pm$75.3pg/ml). Furthermore, PAR-4 was found to stimulate this response via G protein-dependent and -independent mechanisms. Employment of selective MAP kinase inhibitors demonstrated that PAR-4 mediated production of IL-8 appeared to be achieved via a p38 MAP kinase-dependent mechanism (see figure 3.19). Inhibitors YM-254890 and AG-1478 displayed a small additive response whereby partial inhibition of IL-8 production was also demonstrated (see figure 3.18). Involvement of the EGFR is not uncommon, as this particular receptor has been implicated in such responses, particularly in pathological conditions such as prostate cancer (Araki *et al*, 2007). The study by Araki *et al*, (2007), demonstrated a correlation between increase in EGFR phosphorylation and increased production of IL-8, which was successfully inhibited in the presence Src inhibitors. However, previous to the findings of this study, p38 MAP kinase has already been shown to be a regulator of the production of IL-8.

A study by Cheng *et al*, 2008, determined by use of a dominant-negative isoform adenovirus of p38 MAP kinase (Adv $^{+/-}$p38), resulted in the attenuation of IL-8 production. This particular study also determined the involvement of ERK and JNK in mediating IL-8 production in HUVECs (Cheng *et al*, 2008). However, this current study has not found any involvement from other MAP kinase subunits. It has been further demonstrated that IL-8 production can be under the regulatory control of a p38 MAP kinase dependent mechanism in human proximal tubule kidney cells without any involvement of ERK or JNK (Li & Nord, 2008). Further study has also established in human lung epithelial cells that introducing fluoride can result in the increased production of IL-8 (Refsnes *et al*, 2007).

Interestingly, this study in particular shows a response similar to that of the results found within this current project, where it has suggested that EGFR-dependent activation of the MAP kinase pathway is necessary for p38 MAP kinase mediated production of IL-8. Therefore, the signalling characteristics with which PAR-4 mediates the production of IL-8 seem to correlate with studies which assess the involvement of similar mechanisms which implement the production of IL-8.

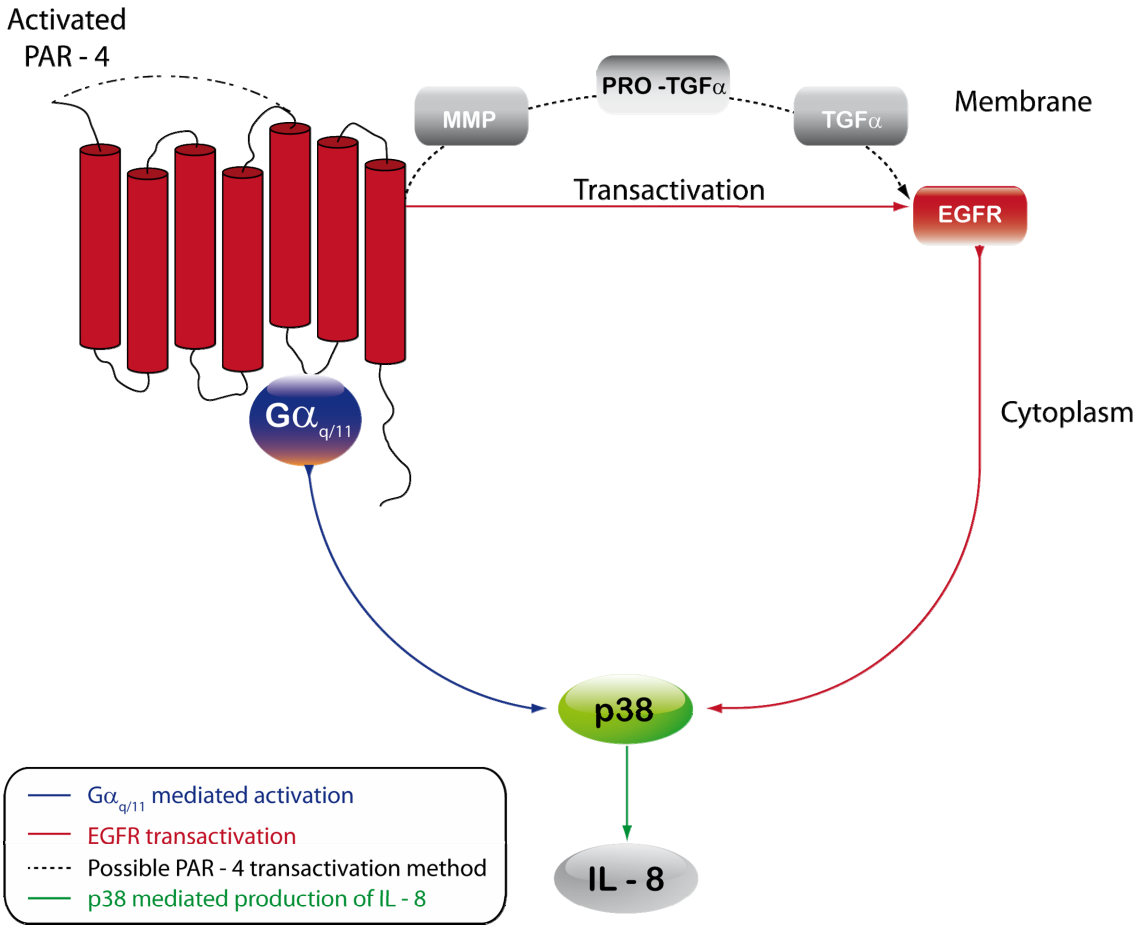


Figure 3.27 Diagrammatical representation of the possible pathway whereby PAR-4 mediates IL-8 production in clone 10H9 cells. The above diagram illustrates the possible pathway mediated by PAR-4 in order to generate production of IL-8. As with the general activation of the MAP kinase pathway, inhibition studies again suggested the involvement of both coupling with $G\alpha_{q/11}$ and transactivation of the EGFR. However, use of selective p38 MAP kinase inhibitor, SB203528 caused significant inhibition of IL-8 in clone 10H9 cells.

So far, it has been demonstrated that PAR-4 is capable of coupling to $G\alpha_{q/11}$, ERK and p38 MAP kinase and has also shown the possibility of mediating transactivation of the EGFR. It has also been found that PAR-4 is capable of stimulating the production of IL-8 in clone 10H9 cells. It may be that PAR-4 is able to utilise these signalling components to administer cellular effects including cellular proliferation and/or inflammation when expressed endogenously in endothelial or epithelial cell types. Unfortunately, current literature has not yet fully defined the signalling pathways that are activated by PAR-4; therefore its complete biological roles remain largely undefined. From these current results, it can be suggested that further investigation is required to elucidate the signalling characteristics which are components of PAR-4 mediated signalling.

Chapter 4

PAR-4 mediated activation of Nuclear Factor kappaB (NFκB) in NCTC-2544 cells stably expressing PAR-4 and in naturally expressing EAhy-926 cells

4.1 INTRODUCTION

Nuclear factor kappa B (NFκB) is ubiquitously expressed transcription factor that is present as homo- or heterodimers in the overall majority of eukaryotic cells. Originally identified in the laborartory of David Baltimore, NFκB was identified as a nuclear factor that was responsible for immunoglobulin kappa light chain transcrption in B cells (Sen & Baltimore, 1986). NFκB has since been found to be responsible for the transcription of a variety of genes, particularly those found to be associated with inflammation and cell survial (reviewed by Perkins & Gilmore, 2006). Under resting conditions, NFκB is localised to the cytoplasm, where it is held in a complex with its inhibitory protein, IκBα. Through a series of phosphorylation events, IκBα becomes degraded, which unmasks the NFκB nuclear localisation sequence, allowing it to translocate to the nucleus, where it modulates transcription of its target genes (Mercurio & Manning, 1999).

Classical characterisation of the NFκB pathway has been predominantly associated with cytokine activation, including molecules such as TNFα or Il-1β. However, the signalling events associated with GPCR mediated activation of NFκB have not been fully elucidated. To date, there have been some studies which have assessed the GPCR mediated activation of NFκB, including the bradykinin receptor and PAF receptor (Pan *et al,* 1996) (further dicussion in chapter 5). Studies within this laboratory have also partly elucidated the mechanisms whereby PAR-2 is able to mediate activation of NFκB via both G protein-dependent and -independent mechanisms (Goh *et al,* 2008).

One study has suggested the possible involvement of PAR-4 in the activation of NFκB (Suo *et al,* 2003), and as such, the mechanisms surrounding the PAR-4 mediated activation of NFκB remain poorly defined. A better understanding of GPCR mediated activation of NFκB may produce possibilities for the identification of novel therapeutics. Therefore, this study sought to understand the mechanisms whereby PAR-4 results in the nuclear localisation of NFκB.

For this purpose, the stable cell line expressing PAR-4, 10H9, was utilised to help elucidate these mechanisms. Pharmacological tools, such as dominant negative IKKβ adenovirus were utilised to determine if PAR-4 mediated activation of NFκB was via an IKK-dependent mechanism. The possible roles of PAR-4 coupling to $G\alpha_{q/11}$ and the transactivation of the EGFR were also assessed by use of YM-254890 and AG1478. The possibility of a PKC-dependent mechanism was assessed by utilsing selective PKC inhibitor GF-109203X (see table 4.1). The process of immunoprecipitation was also employed to determine the possiblity of IκBα phosphorylation at Tyr[42].

AGONIST	EC_{50}	INHIBITOR	IC_{50}
Thrombin (non-selective PAR-4 agonist)	3.2nM (platelets) (Nylander & Mattsson, 2003)	YM254890 (selective inhibitor of $G\alpha_{q/11}$)	<0.6μM (Taniguchi *et al*, 2003)
AYPGKF-NH_2 (selective peptide based PAR-4 agonist)	14μM (Hollenberg & Saifeddine, 2001)	AG1478 (selective EGFR tyrosine kinase inhibitor)	3nM (Cole, 1999)
PMA (direct activator of PKC)	1nM (McFerran *et al*, 1995)	GF109203X (selective inhibitor of classic PKC isoforms)	2μM (Toullec *et al*, 1991)
		SC514 (selective inhibitor of IKKβ)	12μM (Baxter A, 2004)
		BAY11-7082 (selective inhibitor of IKKβ)	5-10μM (Pierce *et al*, 1997)
		BAY11-7085(selective inhibitor of IKKβ)	5-10μM (Pierce *et al,* 1997)

Table 4.1 Agonists and Inhibitors used to determine PAR-4 mediated activation of the NFκB pathway.

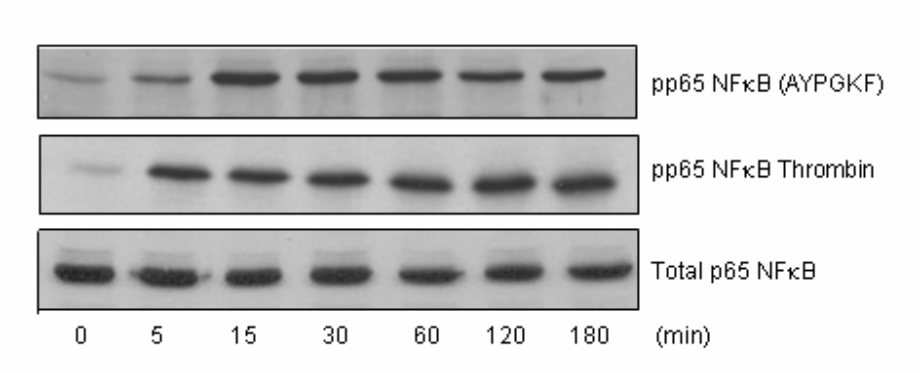
4.2 PAR-4 MEDIATED ACTIVATION OF NUCLEAR FACTOR kappa B (NFκB)

This area of study determined PAR-4 mediated activation of the NFκB pathway by assessment of phosphorylation of p65 at Ser^{536}. Observation of the cellular degradation of IκBα and the level of NFκB-DNA binding activity found within cellular nuclear extracts were also examined.

4.2.1 PAR-4 mediated phosphorylation of p65 NFκB

In order to investigate phosphorylation of p65 NFκB by PAR-4 clone 10H9 cells were stimulated with thrombin (3units/ml) or AYPGKF-NH_2 (100μM) for a time period which ranged from 0-180 min. By means of Western blotting with an antibody selective for phosphorylation of Ser^{536} of p65 NFκB, a time dependent phosphorylation of p65 NFκB was apparent from 15 min up to and including 180 min, with a maximum response at 15-30 min (5$\pm$1.2 fold and 6.5$\pm$0.59 fold for AYPGKF-NH_2 and thrombin respectively, n=3, t=30 min), (see figure 4.1). These results suggest that PAR-4 is capable of mediating regulation of the NFκB response at the level of p65 NFκB phosphorylation. From this data, it can also be suggested that PAR-4 mediates a prolonged phosphorylation of p65 NFκB, suggesting a longer desensitisation process as a characteristic of this receptor.

a)



b)

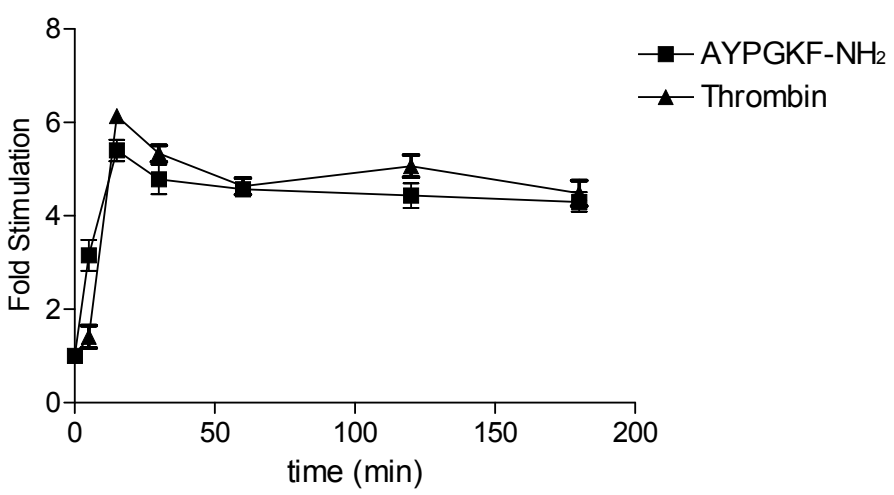


Figure 4.1 PAR-4 mediates phosphorylation of p65 NFκB in clone 10H9 cells. Clone 10H9 cells were grown to confluency on a 12 well plate and quiesced overnight. Following this, cells were stimulated over a time course ranging from 0-180min with either thrombin (3units/ml) or AYPGKF-NH_2 (100μM). Whole cell extracts were resolved by SDS-PAGE and assessed by Western blotting for phosphorylation of p65. Panel A demonstrates the relative phosphorylation of p65 NFκB, while panel B illustrates the quantification by densitometry, expressed as mean $\pm$ s.e.m (fold stimulation). Each blot is representative of three separate experiments.

4.2.1.2 Effect of PAR-4 on cellular degradation of IκBα in clone 10H9 cells

Degradation of IκBα is normally found with the corresponding phosphorylation of p65 NFκB. Therefore, it would be expected that as PAR-4 was able to mediate phosphorylation of p65 NFκB, it would also mediate a cellular degradation of IκBα. Clone 10H9 cells were stimulated with thrombin (3units/ml) or AYPGKF-NH_2 (100μM) over a time period of 0-180 min and control samples were stimulated with TNFα (10ng/ml) for 30 min. Samples were assessed by Western blotting and cellular degradation of IκBα investigated (See figure 4.2). However, in this case, PAR-4 did not appear to mediate any loss of IκBα, suggesting the possibility of a non-canonical means of PAR-4 mediated activation of NFκB. TNFα stimulation caused the expected loss of IκBα in clone 10H9. This loss was almost 90±2.3% inhibition at 30 min stimulation.

4.2.1.3 Effect of PAR-4 activation on phosphorylation of IκBα at Tyr^{42}

When NFκB is classically activated via the canonical IKK-dependent pathway it normally encompasses the cellular degradation of IκBα. However, activation can also be achieved by an IKK-independent mechanism, whereby IκBα becomes phosphorylated at Tyr^{42} as opposed to the normal amino acid residues, Ser^{32} and Ser^{36} (Bui *et al,* 2001). Interestingly, this results in the phosphorylation of p65 and subsequent translocation of NFκB to the nucleus. Therefore, assessment of PAR-4 mediated phosphorylation of IκBα in clone 10H9 cells at Tyr^{42} was performed by means of immunoprecipitation of total cellular IκBα (see figure 4.3). Using a tyrosine selective antibody, Western blot analysis displayed negligible IκBα loss and poor tyrosine phosphorylation of the protein. These results suggest that PAR-4 does not mediate activation of NFκB by this axis.

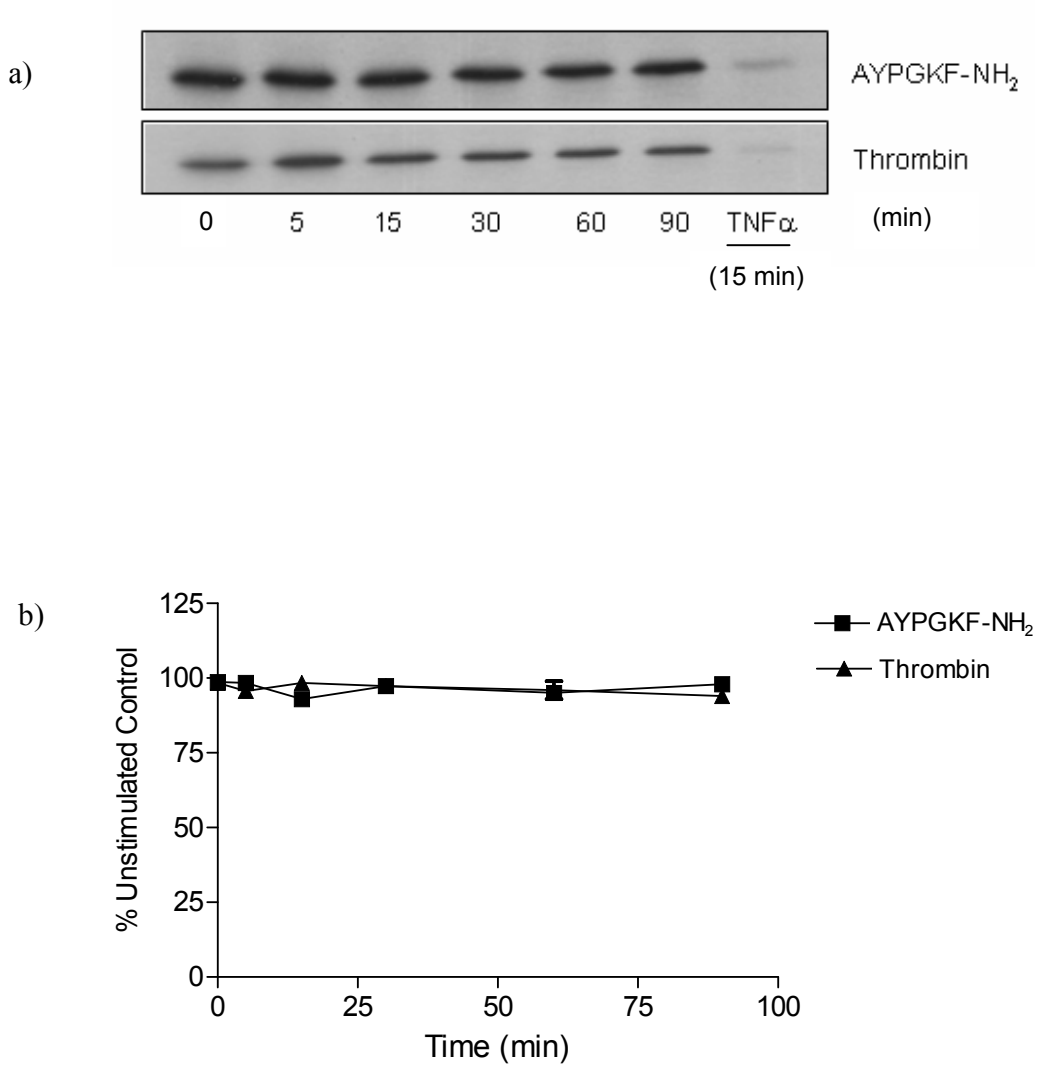


Figure 4.2 Effect of PAR-4 activation on cellular degradation of IκBα in clone 10H9 cells. Clone 10H9 cells were grown to confluency on a 12 well plate and quiesced overnight. Following this, cells were stimulated over a time course ranging from 0-90min and then stimulated with either thrombin (3units/ml) or AYPGKF-NH_2 (100μM), with control samples stimulated with TNFα (10ng/ml) for 15 min. Whole cell extracts were resolved by SDS-PAGE and assessed by Western blotting for cellular degradation of IκBα. Panel A demonstrates relative IκBα loss, while panel B illustrates quantification by densitometry, expressed as mean ± s.e.m (% unstimulated control). Each blot is representative of 3 individual experiments.

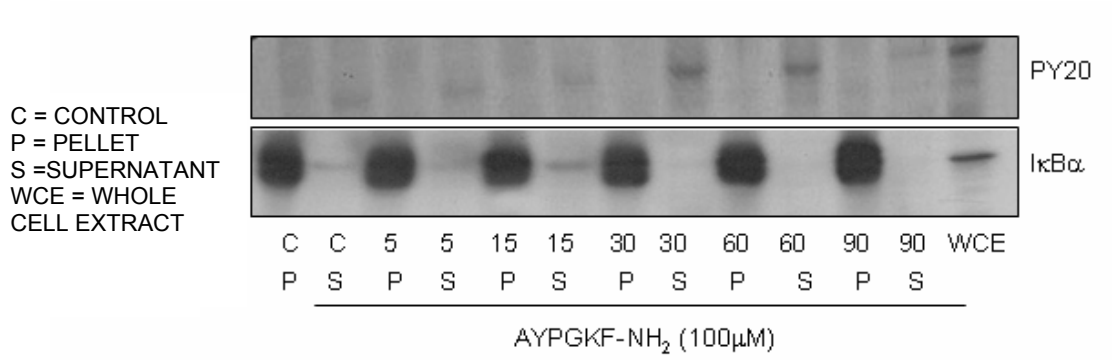


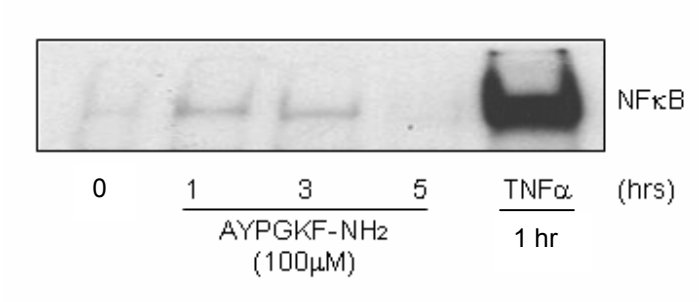
Figure 4.3 Effect of PAR-4 activation on phosphorylation of IκBα at Tyr[42]. Clone 10H9 cells were grown to confluency on a 12 well plate and rendered quiescent overnight. Following this, cells were stimulated with AYPGKF-NH_2 (100μM) over a time course ranging from 0-90 min, following which samples were solubilised and incubated with IκBα antibody and prepared for immunoprecipitation. Samples were resolved by SDS-PAGE and assessed by Western blotting for tyrosine phosphorylation with PY20 antibody. Cellular degradation of IκBα was assessed by IκBα specific antibody. Above blot is representative of 3 individual experiments, as outlined in section 2.9.

4.2.1.4 PAR-4 mediated NFκB-DNA binding in clone 10H9 and NCTC-2544 cells

To further investigate the possibility that PAR-4 may mediate NFκB activation, it was necessary to assess nuclear binding of the transcription factor when stimulated by AYPGKF-NH_2 (100μM) or thrombin (3units/ml) compared with controls stimulated with TNFα (10ng/ml). Nuclear extracts were prepared from both 10H9 cells and NCTC-2544 cells over a 0-7 hour period and bound with a selective NFκB oligonucleotide and run on a non-denaturing gel to determine the level of nuclear binding associated with PAR-4 mediated activation of NFκB. As expected, NCTC-2544 cells stimulated with AYPGKF-NH_2 (100μM), displayed no visible nuclear binding, suggesting that PAR-4 is not naturally present in these parental cells (see figure 4.4). Positive control samples stimulated by TNFα (10ng/ml) mediated a significant increase in nuclear binding 5.8$\pm$0.75 (n=3, t= 60min) above basal.

Initial results in NCTC-2544 cells expressing PAR-4 (clone 10H9) cells indicate that there is a time-dependent effect of PAR-4 mediated NFκB DNA binding when activated by either thrombin or activating peptide (see figure 4.5). Apparent binding reaches a maximum response at 60 min for AYPGKF-NH_2 (4.2$\pm$1.4 fold stimulation, n=4, t=1hr), while thrombin displays a more sustained response, peaking from 1-3 hours (3.9$\pm$0.75 fold stimulation, n=4, t=1hr), before declining towards basal levels. These results suggest that PAR-4 is capable of stimulating activation of NFκB at the level of DNA binding.

a)



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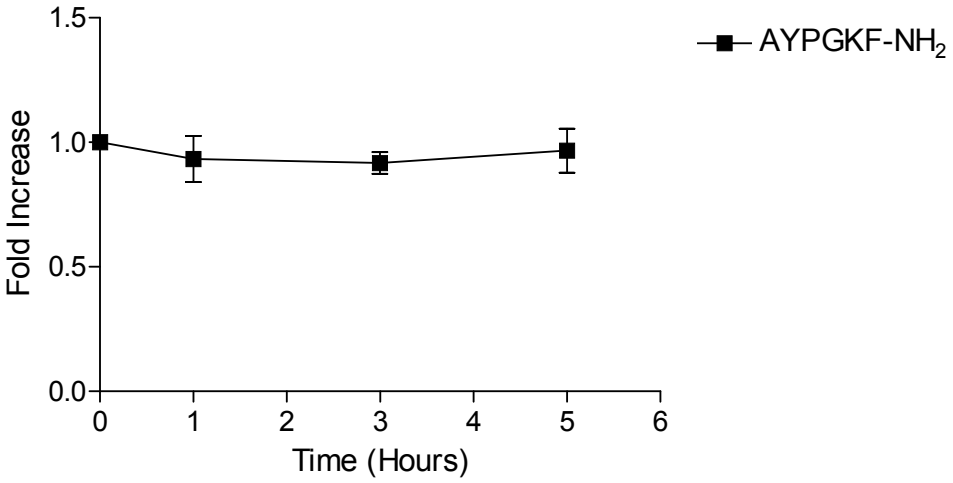
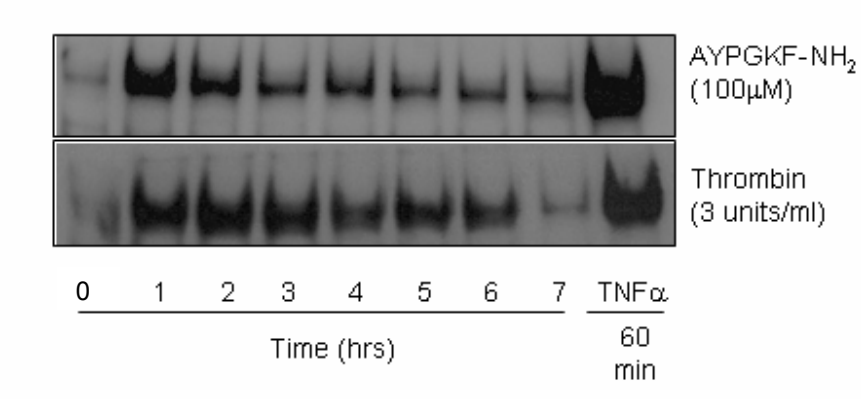


Figure 4.4 PAR-4 mediated NFκB-DNA binding in NCTC-2544 cells. NCTC-2544 cells were grown to confluency and quiesced overnight. Cells were then stimulated with AYPGKF-NH_2 (100μM) for 0-5 hours, with control sample stimulated by TNFα (10ng/ml) for 60 min. Samples were prepared as nuclear extracts and bound with selective NFκB oligonucleotide and then resolved by EMSA. Autoradiograph on panel A is representative of 3 individual experiments, while panel B shows quantification by densitometry, expressed as s.e.m $\pm$ (fold increase).

a)



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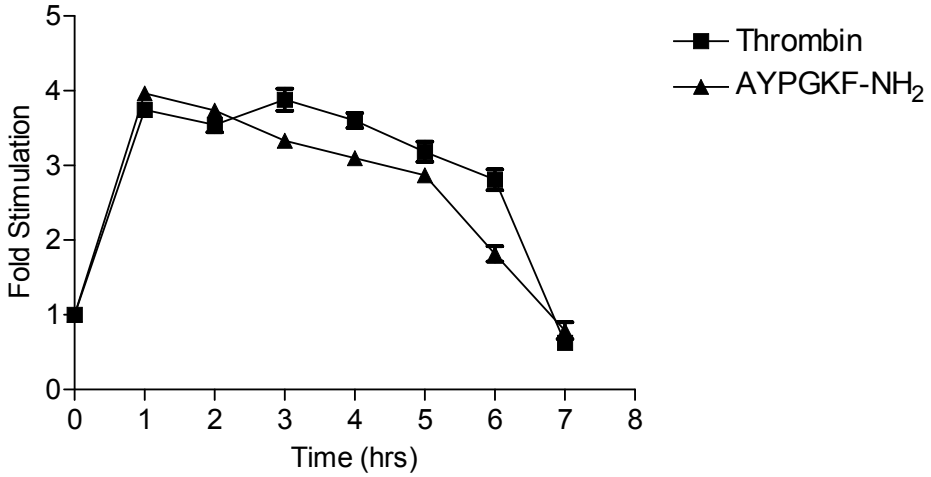


Figure 4.5 PAR-4 mediated NFκB-DNA binding in clone 10H9 cells. Clone 10H9 cells were grown to confluency on 6 well plates and rendered quiescent overnight. Following this, cells were stimulated with thrombin (3units/ml) or AYPGKF-NH_2 (100μM) over a time course of 0-7 hours, with TNFα (10ng/ml) 60 min used for control. Nuclear extracts were prepared and binding activity measured by EMSA (see section 2.7). Auto-radiograms shown are representative of 3 more experiments. Quantification was performed by densitometry and expressed as mean ± s.e.m of 4 separate experiments.

4.3 POSSIBLE ROLE OF AN IKKβ-DEPENDENT MECHANISM IN PAR-4 MEDIATED ACTIVATION OF NFκB

Since the identification of NFκB (Sen & Baltimore, 1986), it has been demonstrated that activation of this transcription factor is regulated by a series of upstream phosphorylation events, ultimately leading to the freeing of NFκB from IκBα in the cytoplasm (Mercurio & Manning, 1999). So far, this study has concentrated on the downstream events, including the phosphorylation of p65 NFκB as well as NFκB-DNA binding. However, cellular degradation of IκBα is known to be mediated by phosphorylation of critical serine residues by upstream kinases known as inhibitory kappa B kinases (IKK).

The IKK signalosome consists of 3 identified members, namely IKKα, IKKβ and IKKγ. Classical activation of NFκB usually comprises of IKKβ mediated phosphorylation of the IκB protein prior to translocation of NFκB (Mercurio & Manning, 1999). Therefore, as PAR-4 has demonstrated a relative lack of IκBα cellular degradation or even phosphorylation of IκBα at Tyr^{42}, it was necessary to investigate the possibility of PAR-4 being able to stimulate activation of NFκB via an IKK-dependent axis. For this purpose, the use of adenovirus which incorporated a dominant negative isoform of IKKβ ($Adv^{+/-}$ IKKβ) was used to determine the role of this upstream mediator in the PAR-4 stimulated pathway. Pharmacological inhibitors BAY11-7082, BAY11-7082 and SC-514 were also utilised as a means of secondary validation.

4.3.1 Expression of Adv $^{+/-}$IKKβ in clone 10H9 cells

Before examination of the phosphorylation of p65, NFκB-DNA binding or PAR-4 mediated production of IL-8 could be investigated with $^{+/-}$IKKβ Adv, studies validating the virus were initiated by checking the level of expression in clone 10H9 cells. For this purpose, cells were infected with increasing concentrations of the virus ranging from 100-400 pfu/cell 48 hours prior to stimulation with AYPGKF-NH_2 (100μM) for 15-30 min. Control samples were stimulated with TNFα (10ng/ml) for 30 min.

By means of Western blotting and use of an IKKα/β specific antibody, these results demonstrated that the $^{+/-}$IKKβ Adv was capable of being expressed in clone 10H9 cells at a level comparable to the TNFα mediated control samples (see figure 4.6).

4.3.2 Effect of Adv $^{+/-}$IKKβ on PAR-4 mediated phosphorylation of p65 NFκB in clone 10H9 cells

In order to determine if PAR-4 mediated phosphorylation of p65 NFκB was via an IKK-dependent mechanism, clone 10H9 cells were infected with Adv $^{+/-}$IKKβ (200-400pfu/cell), 48 hours prior to stimulation with thrombin (3units/ml) or AYPGKF-NH_2 (100μM) for 15-30 min. Results obtained by Western blotting, demonstrated that even increasing concentrations of virus did not present any visual inhibition of PAR-4 mediated phosphorylation of p65 NFκB. Therefore, early indications suggest that PAR-4 mediated activation of NFκB may not be via an IKKβ-dependent mechanism (see figure 4.7).

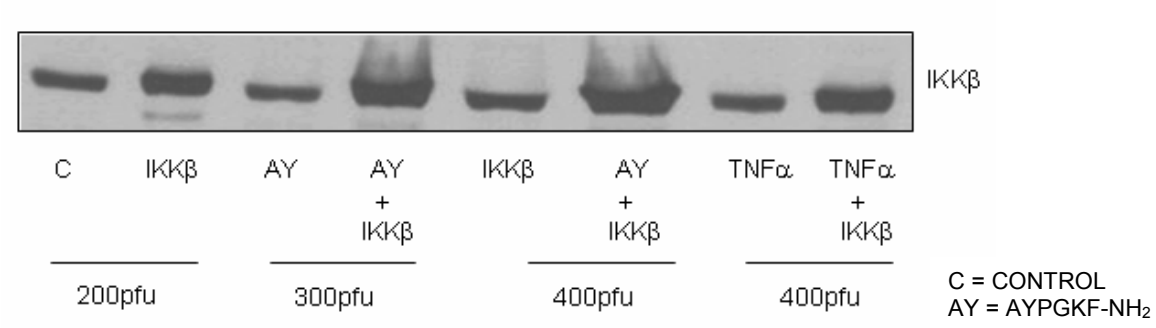
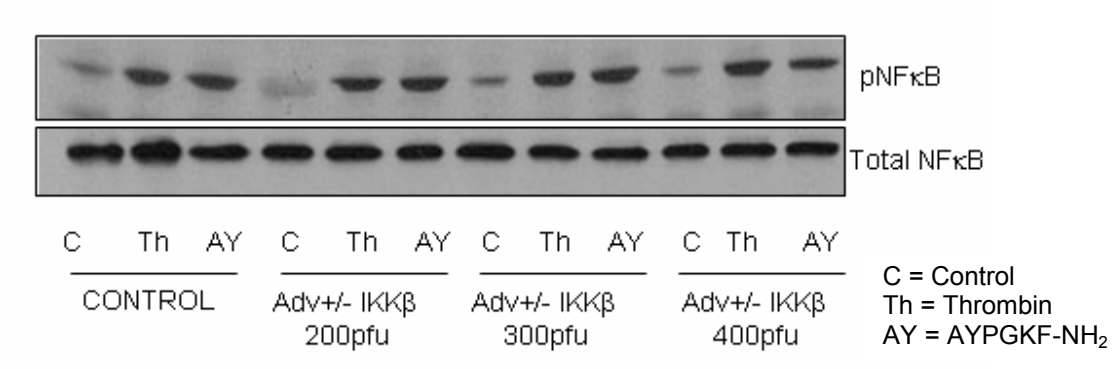


Figure 4.6 Expression of $^{+/-}$ IKKβ adenovirus in clone 10H9 cells. Clone 10H9 cells were grown overnight on a 12 well plate and infected with increasing amounts of $^{+/-}$IKKβ Adv. Cells were rendered quiescent 24 hours post infection. Cells were stimulated the following morning with AYPGKF-NH_2 (100μM) and TNFα (10ng/ml) for 30 min. Samples were resolved by SDS-PAGE and examined by Western blotting. Employment of a selective IKKβ antibody was used to determine viral expression.

a)



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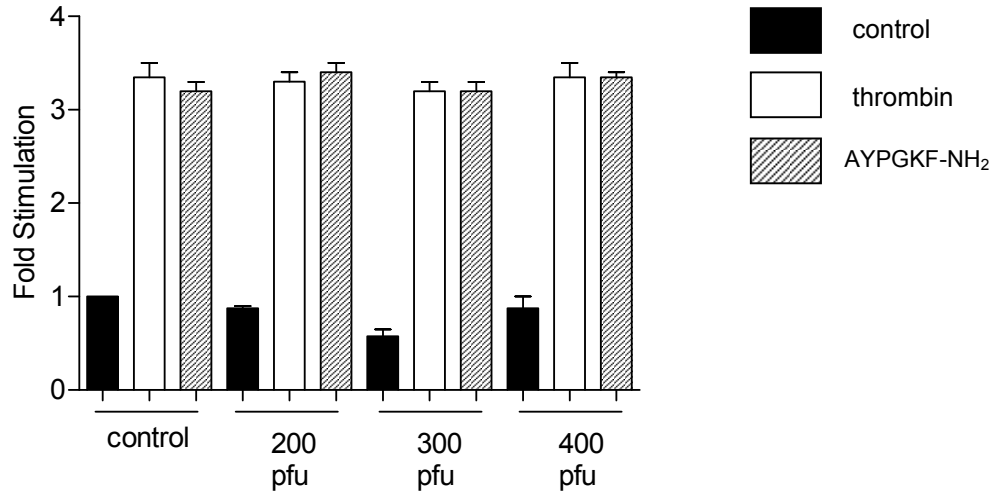

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Figure 4.7 Effect of $^{+/-}$ IKKβ adenovirus on PAR-4 mediated phosphorylation of p65 NFκB in clone 10H9 cells. Clone 10H9 cells were grown overnight on a 12 well plate and infected with increasing amounts of $^{+/-}$IKKβ Adv. Cells were rendered quiescent 24 hours post infection. Cells were stimulated the following morning with thrombin (3units/ml) or AYPGKF-NH_2 (100μM) for 30 min. Samples were resolved by SDS-PAGE and examined by Western blotting for relative phosphorylation of p65. Panel A displays p65 NFκB phosphorylation, while panel B illustrates quantification by densitometry, expressed as mean ± s.e.m (fold stimulation). Each blot is representative of 3 experiments.

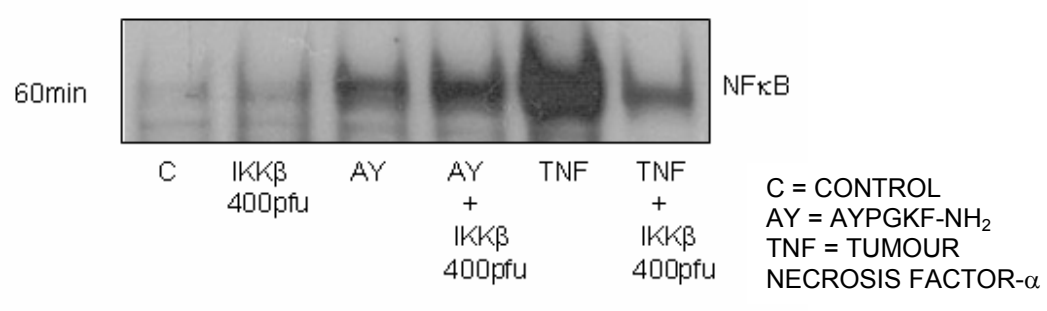
4.3.3 Effect of Adv $^{+/-}$ IKKβ on PAR-4 mediated NFκB-DNA binding in clone 10H9 cells.

To determine if PAR-4 mediated NFκB-DNA binding was dependent on the IKKβ axis, clone 10H9 cells were again infected with Adv $^{+/-}$IKKβ (400pfu/cell), 48 hours prior to stimulation with AYPGKF-NH_2 (100μM) for 60 min and TNFα (10ng/ml) for 60 min. Nuclear extract samples were incubated with NFκB oligonucleotide and resolved by EMSA. Results obtained from overnight autoradiography displayed NFκB-DNA binding mediated by both AYPGKF-NH_2 and TNFα (3.4±0.4 and 5.7±1 for AYPGKF-NH_2 and TNFα respectively, n=3, t=60 min). No loss of NFκB-DNA binding was observed by AYPGKF-NH_2, suggesting that at the level of DNA binding, PAR-4 does not mediate activation of NFκB via an IKKβ-dependent mechanism (see figure 4.8). Adv $^{+/-}$IKKβ reduced the positive control samples stimulated with TNFα by 55%.

4.3.4 Effect of Adv $^{+/-}$ IKKβ on PAR-4 mediated production of IL-8 production

Results presented in chapter 3 demonstrated that PAR-4 is capable of mediating IL-8 production via activation of p38 MAP kinase. It has been documented that IL-8 is an NFκB-dependent inflammatory mediator (Karimi *et al*, 2006). Cytokine production would suggest that the classical NFκB pathway had been employed for the production and subsequent secretion of IL-8. Therefore, to determine if PAR-4 mediated IL-8 production was via an IKKβ –dependent mechanism, 10H9 cells were infected with $^{+/-}$ IKKβ Adv (400pfu/cell), 48 hours prior to stimulation with AYPGKF-NH_2 (100μM) or TNFα (10ng/ml) for 6 hrs. Supernatants were collected and subjected to IL-8 specific ELISA. Results displayed a rise in the production of IL-8 by AYPGKF-NH_2 (760±10pg/ml, n=3) comparable with that of TNFα (890±28pg/ml, n=3). However a relative lack of IL-8 inhibition was displayed by IKKβ samples stimulated with AYPGKF-NH_2, therefore suggesting that PAR-4 mediated IL-8 production is not achieved via an IKK-dependent mechanism. However, IKKβ samples stimulated with TNFα showed a loss in IL-8 production (% inhibition 35±4.8, n=3, *$p<0.05$), which confirms TNFα mediates activation of the NFκB pathway via the classical IKK-dependent pathway (see figure 4.9).

a)



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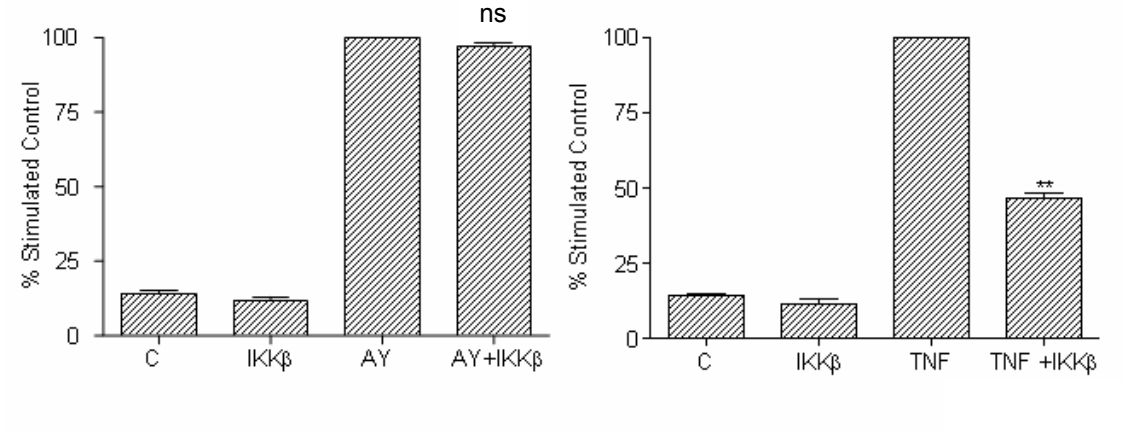


Figure 4.8 Effect of Adv $^{+/-}$IKKβ on PAR-4 mediated NFκB-DNA binding in clone 10H9 cells. Cells were grown in 6 well plates and infected 24 hours after plating. Cells were rendered quiescent overnight, 24 hours post-infection. Cells were then stimulated with AYPGKF-NH_2 (100μM) for 60 min; with control samples stimulated with TNFα (10ng/ml) also for 60 min. Samples were prepared as nuclear extracts and resolved by EMSA (see section 2.7). Panel A illustrates nuclear binding by autoradiography, while panel B illustrates the quantification by densitometry, expressed as mean ± s.e.m (% AYPGKF-NH_2 or TNFα stimulated control, **$p<0.01$). Each autoradiograph represents 3 separate experiments.

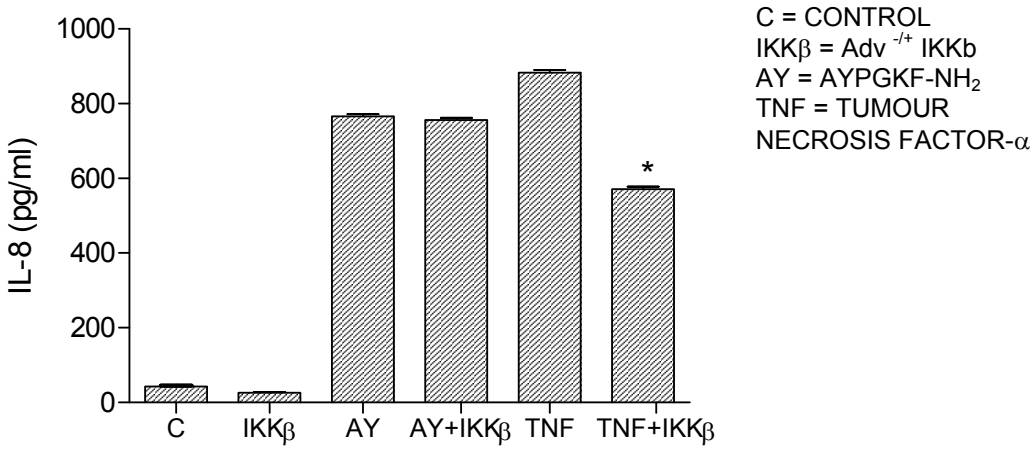


Figure 4.9 Effect of Adv $^{+/-}$ IKKβ on PAR-4 mediated production of IL-8 in clone 10H9 cells. Clone 10H9 cells were grown overnight in a 12 well plate and infected with increasing amounts of $^{+/-}$IKKβ Adv. 24 hours post infection, cells were rendered quiescent overnight and stimulated with AYPGKF-NH_2 (100μM) for 6 hours. Supernatants were collected and subject to IL-8 specific ELISA (as outlined in section 2.8). Figure 4.15 represents quantification of IL-8 production, expressed as mean $\pm$ s.e.m (IL-8 pg/ml, *$p<0.05$ as compared with stimulated control). Each of the above columns is representative of 3 separate experiments.

4.3.5 Effect of BAY11-7082 and BAY11-7085 on PAR-4 mediated activation of NFκB

As employment of Adv $^{+/-}$IKKβ did not demonstrate an IKK-dependent mechanism to represent PAR-4 mediated activation of NFκB, it was necessary to determine the possibility of this pathway via pharmacological means. Therefore, for this purpose two selective IKKβ inhibitors were utilised. Both BAY11-7082 and BAY11-7085 inhibit translocation of NFκB specifically by inhibiting the IKKβ-dependent phosphorylation of IκBα, (Kamthong & Wu, 2001). These compounds were examined by means of EMSA and Western blotting.

4.3.5.1 Effect of BAY11-7082 and BAY11-7085 on PAR-4 mediated NFκB DNA binding

For this purpose, clone 10H9 cells were pre-incubated with BAY11-7082 (50μM) and BAY11-7085 (50μM) for 30 min prior to stimulation with AYPGKF-NH_2 (100μM) for 60 min, with control samples being stimulated with TNFα (10ng/ml) also for 60 min. Samples which had been prepared as nuclear extracts were incubated with NFκB consensus oligonucleotide and resolved by EMSA. Overnight radiography indicated that both AYPGKF-NH_2 and TNFα stimulated NFκB-DNA binding in clone 10H9 cells (6.3$\pm$1.2 and 5.8$\pm$0.75 fold stimulation for AYPGKF-NH_2 and TNFα respectively, n=3, t=60, for panel A and 6.1$\pm$0.45 and 5.6$\pm$0.32 fold stimulation for AYPGKF-NH_2 and TNFα respectively, n=3, t=60, for panel B) (see figure 4.10). Pre-treatment with BAY11-7082 did not inhibit NFκB-DNA binding stimulated by either AYPGKF-NH_2 or TNFα. However, original characterisation of these compounds demonstrated a significant *in vivo* anti-inflammatory effect by inhibition of TNFα mediated IκBα phosphorylation (Pierce *et al*, 1997). In the study by Pierce *et al,* a significant inhibition of the phosphorylation of IκBα was apparent at a concentration of 20μM for both compounds. These results suggest that these compounds are not only selective to inhibit TNFα mediated IκBα phosphorylation, but have been able to be readily transported across the cell membrane in order to carry out their selective task.

In this current study, even at a concentration of 50μM, no notable inhibition is visible, even for the samples stimulated with TNFα. Unfortunately, higher concentrations did not prove to be possible for investigation purposes, as concentrations above 50μM proved to be cytotoxic in clone 10H9 cells. Therefore, these results may suggest that there is poor cell permeability of these compounds in NCTC-2544 cells, and as such, it can be suggested that these compounds are not suitable to investigate the possibility of PAR-4 mediated NFκB activation in clone 10H9 cells.

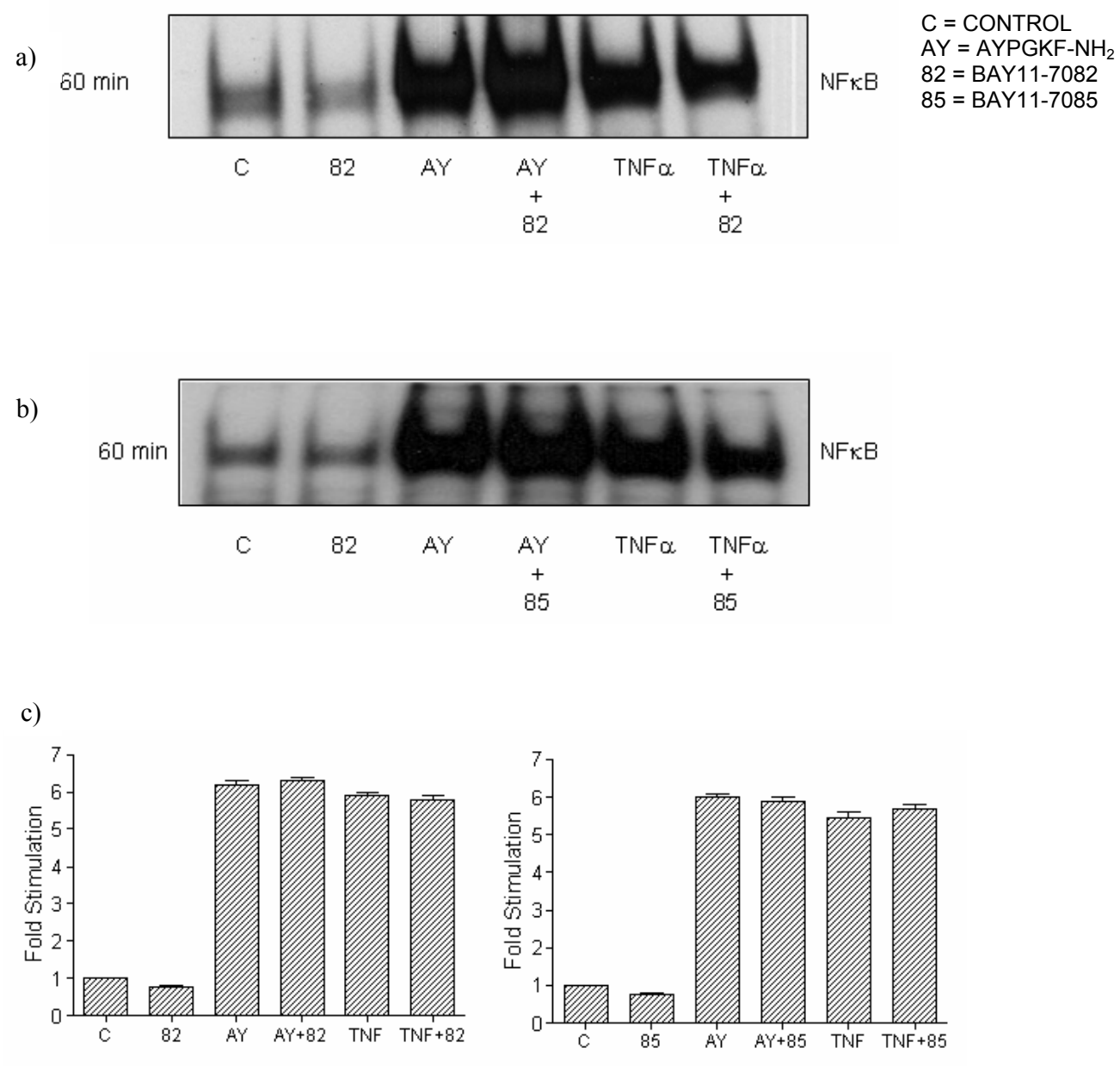


Figure 4.10 Effect of BAY11-7082 and BAY11-7085 on PAR-4 mediated NFκB-DNA binding in clone 10H9 cells. Clone 10H9 cells were grown to confluency on 6 well plates and rendered quiescent overnight. Cells were then pre-incubated with either BAY11-7082 or BAY11-7085 for 30 min prior to stimulation with AYPGKF-NH_2 (100μM) for 60 min and prepared as nuclear extracts and were resolved by EMSA (see section 2.7). Panels A & B demonstrate NFκB- DNA binding while panel C illustrates the quantification by densitometry, expressed as mean $\pm$ s.e.m (fold stimulation) Each autoradiograph is indicative of 3 individual experiments.

4.3.5.2 Effect of BAY11-7082 and BAY11-7085 on PAR-4 mediated phosphorylation of p65 NFκB

In order to determine the viability of this compound, assays were repeated using clone 10H9 cells, by means of Western blotting. Cells were again pre-incubated with BAY11-7082 (50μM) and BAY11-7085 (50μM) for 30 min prior to stimulation with AYPGKF-NH_2 (100μM) for 30 min and TNFα (10ng/ml) for 30 min. Results obtained by Western blotting showed phosphorylation of p65 NFκB by AYPGKF-NH_2 and TNFα (1.3±0.2 and 1.4±0.3 fold above basal for AYPGKF-NH_2 and TNFα respectively, n=3, t=30min for panel A and 1.5±0.5 and 1.6±0.2 for AYPGKF-NH_2 and TNFα respectively, n=3, t=30min for panel B) no loss of p65 phosphorylation (see figure 4.11). Again, these results suggest that in NCTC-2544 cells, BAY11-7082 and BAY11-7085 are not the choice compounds to investigate either IKKβ-dependent phosphorylation of IκBα or p65 NFκB.

4.3.6 Effect of SC-514 on PAR-4 mediated activation of NFκB in clone 10H9 cells

Further studies were performed using another IKKβ selective inhibitor, SC-514, which acts by inhibiting the ability of IKKβ to phosphorylate IκBα within cells. Again, this inhibitory compound was examined by means of NFκB-DNA binding and Western blotting.

4.3.6.1 Effect of SC-514 on PAR-4 mediated NFκB-DNA binding in clone 10H9 cells

In order to further investigate the possibility of PAR-4 mediating NFκB activation via an IKKβ–dependent mechanism, clone 10H9 cells were pre-incubated with SC-514 (100μM) for 30 min prior to stimulation with AYPGKF-NH_2 (100μM) for 60 min, with control samples being stimulated with TNFα (10ng/ml) also for 60 min. Samples were prepared as nuclear extracts, which were incubated with NFκB specific oligonucleotide. Results obtained by EMSA displayed NFκB-DNA binding stimulated by both AYPGKF-NH_2 and TNFα (5.7±1.1 and 5±0.75 for AYPGKF-NH_2 and TNFα respectively, n=3, t=60min) (see figure 4.12).

Samples stimulated with AYPGKF-NH_2 showed no loss of DNA binding when cells were pre-incubated with SC-514, which suggests that PAR-4 may activate NFκB through an IKK-independent manner. A small loss of NFκB-DNA binding was apparent in TNFα stimulated samples, (% inhibition 15$\pm$3.5, n=3, *$p<0.05$). As the inhibitory compound used in this case was originally characterised by showing inhibition of cytokine mediated activation of NFκB, this current loss was poor in comparison. In a study by Kishore *et al*, 2003, SC-514 was shown to mediate a highly significant inhibition of IL-1β induced NFκB activation in synovial fibroblasts. In the study by Kishore *et al*, 2003 SC-514 almost completely abolished the RNA expression of both IL-8 and cycloxygenase-2 (COX-2), which are genes readily under the control of activated NFκB. This inhibition occurred at the same 100μM concentration used within this current study. Therefore, it is possible that in the same fashion as the BAY11-7082 and BAY11-7085, the possibility lies that this inhibitor is also unable to properly penetrate the cellular membrane, leaving it unable to carry out its selective inhibition in clone 10H9 cells.

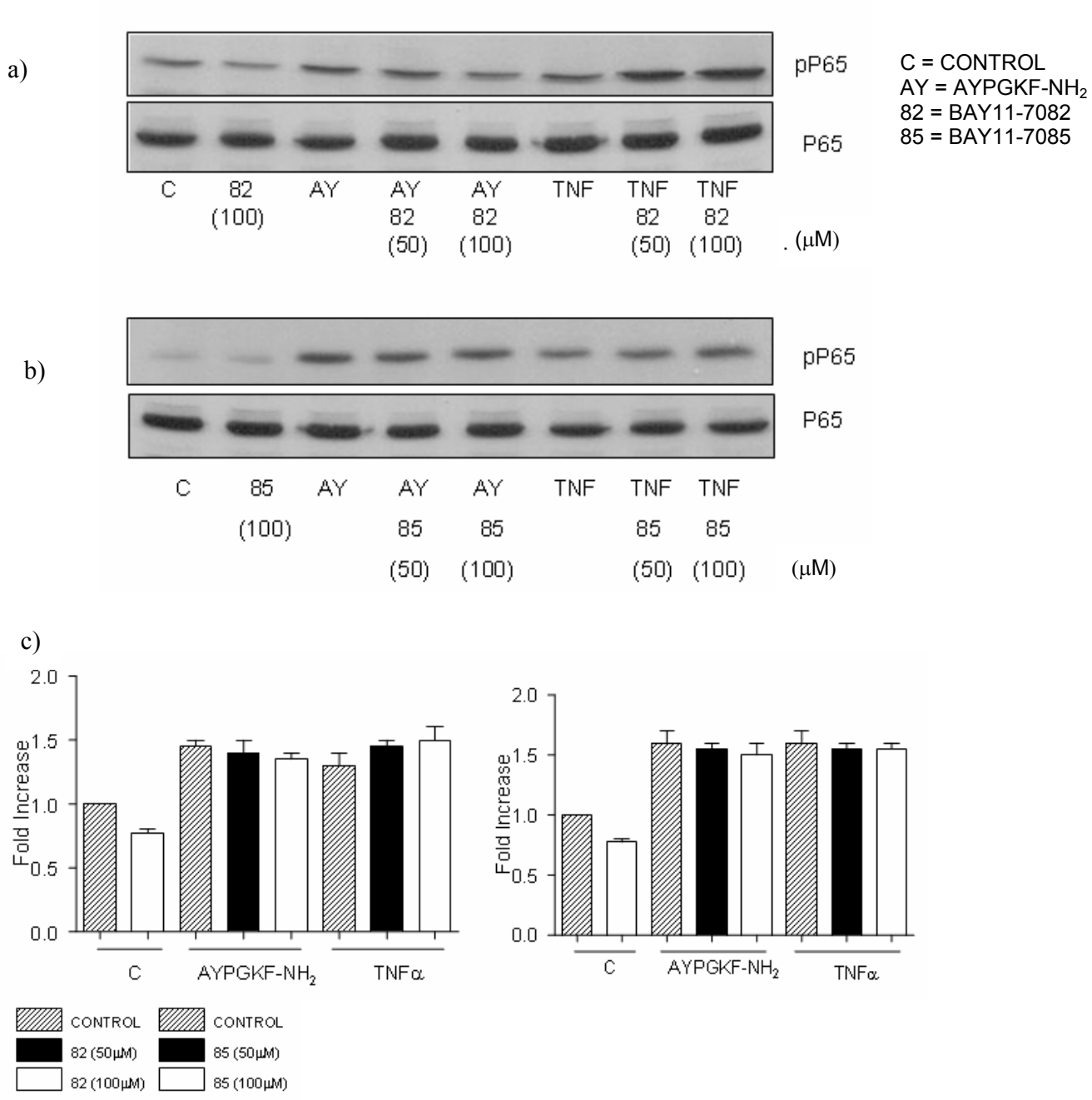


Figure 4.11 Effect of BAY11-7082 and BAY11-7085 on PAR-4 mediated phosphorylation of p65 NFκB in clone 10H9 cells. Clone 10H9 cells were grown to confluency on 12 well plates and rendered quiescent overnight. Cells were then pre-incubated with BAY11-7082 (50-100μM) or BAY11-7085 (50-100μM) for 30 min prior to stimulation with AYPGKF-NH_2 (100μM) for 30 min. Samples were examined by Western blotting for phosphorylation of p65 as shown in section 2.5. Panel A and B demonstrate phosphorylation of p65 while panel C illustrates quantification by densitometry, expressed as mean ± s.e.m (fold increase). Each blot represents 3 separate experiments.

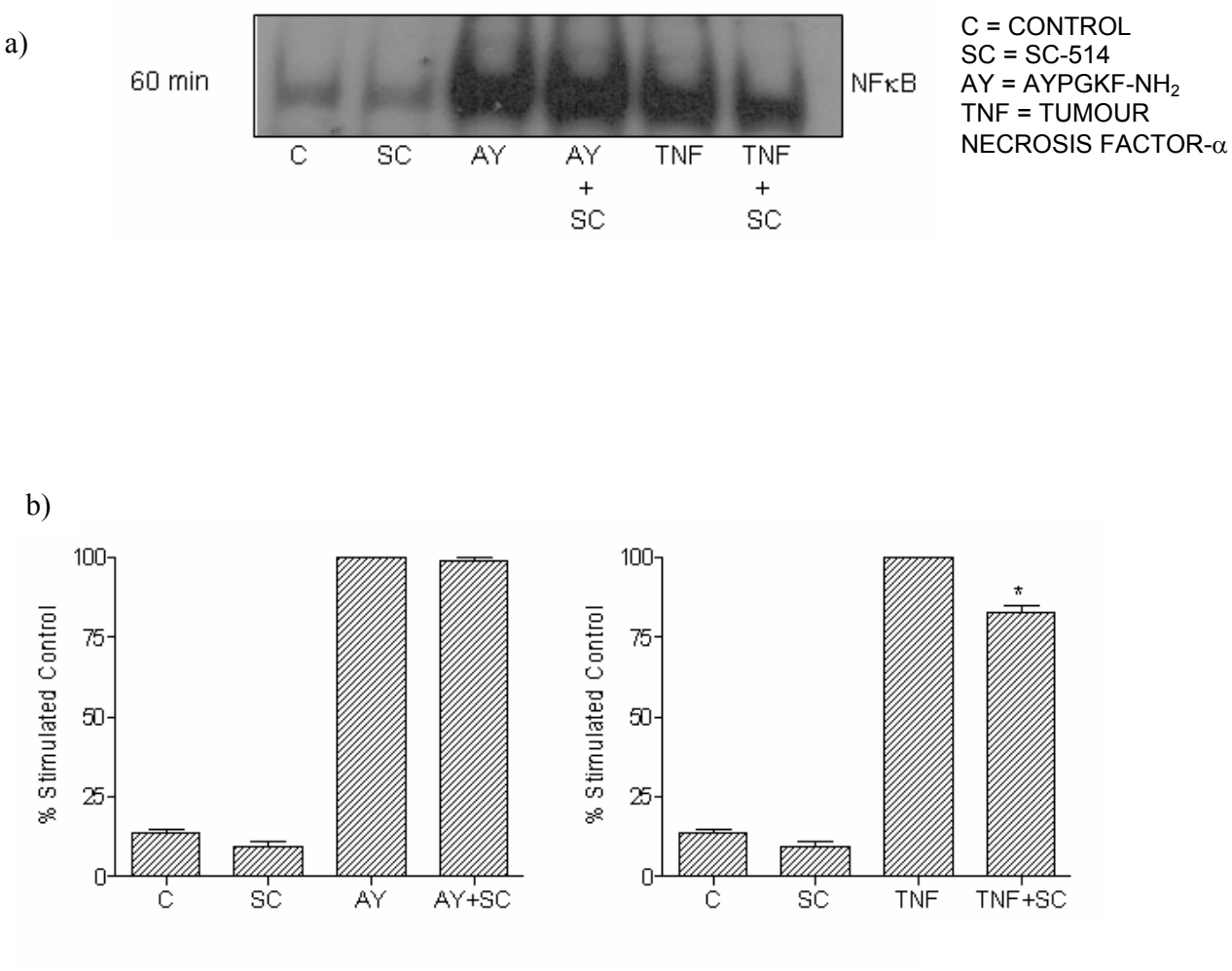
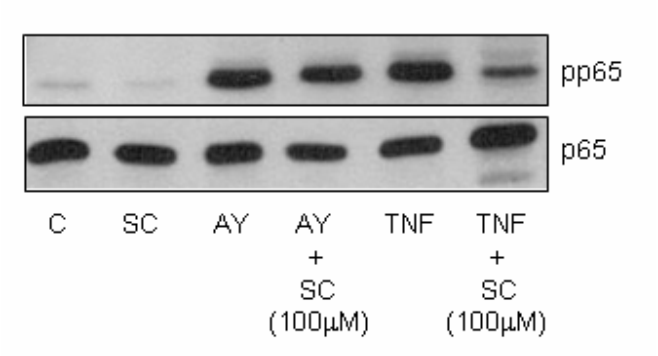


Figure 4.12 Effect of SC-514 on PAR-4 mediated NFκB-DNA binding in clone 10H9 cells. Cells were grown to confluency in 6 well plates and rendered quiescent overnight. Cells were then pre-treated with SC-514 (100μM) for 30 min prior to stimulation with AYPGKF-NH_2 (100μM). Samples were prepared as nuclear extracts and resolved by EMSA (see section 2.7). Panel A shows nuclear binding via autoradiography and is representative of 3 individual experiments, while panel B shows quantification by densitometry, expressed as mean $\pm$ s.e.m (% AYPGKF-NH_2 or TNFα stimulated control, *$p<0.05$).

a)



C = CONTROL
SC = SC-514
AY = AYPGKF-NH_2
TNF = TUMOUR NECROSIS FACTOR-α

b)

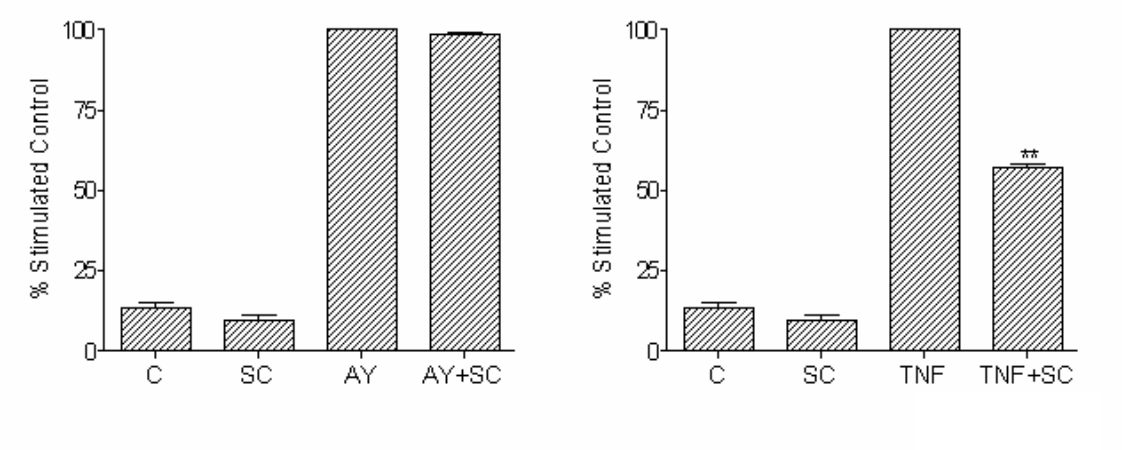


Figure 4.13 Effect of SC-514 on PAR-4 mediated phosphorylation of p65 NFκB in clone 10H9 cells. Cells were grown to confluency on 12 well plates and rendered quiescent overnight. Cells were then pre-incubated with SC-514 (100μM) for 30 min prior to stimulation with AYPGKF-NH_2 (100μM) for 30 min with control samples prepared with TNFα (10ng/ml). Samples were then resolved by SDS-PAGE and examined by Western blotting for phosphorylation of p65 NFκB, as outlined in section 2.5. Panel A demonstrates the phosphorylation of p65 NFκB and panel B illustrates quantification by densitometry, expressed as mean $\pm$ s.e.m (% stimulated control, **$p<0.01$). Above blot is representative of 3 individual experiments.

4.3.6.2 Effect of SC-514 on PAR-4 mediated phosphorylation of p65 NFκB in clone 10H9 cells

To clarify the possibility of a role for IKKβ in PAR-4 mediated NFκB activation, clone 10H9 cells were pre-incubated with SC-514 (100μM) for 30 min prior to stimulation with AYPGKF-NH_2 (100μM) for 30 min with control samples being stimulated with TNFα (10ng/ml) for 30 min. Results demonstrated phosphorylation of p65 NFκB by both AYPGKF-NH_2 and TNFα (4.8±1.2 and 5.1±0.6 for AYPGKF-NH_2 and TNFα respectively, n=3, t=30). Pre-treatment of cells with SC-514 caused no significant inhibition of p65 NFκB phosphorylation, TNFα demonstrated a significant level of inhibition (% inhibition, 42±0.3, n=3, **$p<0.01$) (see figure 4.13), which may suggest that PAR-4 does not mediate activation of NFκB via an IKKβ-dependent mechanism. However, as this compound did not show the same level of inhibition of the TNFα stimulated samples in the previous figure (figure 4.12) further validation may be required in order to ascertain this suggestion.

4.4 PAR-4 MEDIATED ACTIVATION OF NFκB BY G PROTEIN - DEPENDENT AND –INDEPENDENT MECHANISMS

NFκB is one of the major transcription factors which is able to contribute to the cellular inflammatory response. The results in this chapter have so far demonstrated that there is poor cellular degradation of IκBα, which is a process normally associated with canonical NFκB activation. The results also suggest that the PAR-4 mediated activation of NFκB may also occur via an IKK-independent mechanism. However, NFκB still displays an increase in activation mediated by PAR-4, whereby activation has been shown at the level of phosphorylation of p65 NFκB, as well as an increase of NFκB-DNA binding. However, as yet, it has not been ascertained as to whether or not this PAR-4 mediated NFκB activation is caused by the coupling of the receptor to a particular G protein, or if it is due to a G protein-independent mechanism. The following section will concentrate on the mechanisms utilised by PAR-4 in order to generate NFκB activity.

For this purpose, YM-254890 was used to determine the role of $G\alpha_{q/11}$, while EGFR tyrosine kinase inhibitor, AG-1478 was used to determine if PAR-4 mediated transactivation of the EGFR was involved in the regulation of this pathway.

4.4.1 Effect of YM-254890 on PAR-4 mediated phosphorylation of p65 NFκB

Previously, it was demonstrated that PAR-4 resulted in activation of ERK and p38 MAP kinase by coupling to $G\alpha_{q/11}$ as well as by transactivation of the EGF receptor. It was therefore investigated whether or not $G\alpha_{q/11}$ played a possible role in coupling PAR-4 to the NFκB cascade by means of pharmacological inhibition of this G-protein. This was achieved by utilising the compound, YM-254890. Initial experiments to determine the role of $G\alpha_{q/11}$ in PAR-4 mediated activation of NFκB, assessed inhibition at the level of phosphorylation of p65 NFκB. As such, clone 10H9 cells were pre-incubated with YM-254890 (100nM) for 30 min prior to stimulation with thrombin (3units/ml) or AYPGKF-NH_2 (100μM) for time points including 15 and 30 min. Control samples were stimulated with TNFα (10ng/ml) for 30 min. Figure 4.14 illustrates that PAR-4 mediates phosphorylation of p65 NFκB (2.6$\pm$0.3 and 2.1$\pm$0.15 fold above basal for thrombin and AYPGKF-NH_2 respectively, n=3, t=15-30 min) (see figure 4.14). TNFα mediated phosphorylation of p65 NFκB 4.3$\pm$0.5 fold above basal (n=3, t=30). While YM-254890 previously demonstrated a partial inhibition of PAR-4 stimulated activation of the MAP kinase pathway, in the case of PAR-4 mediated activation of NFκB, early results indicate that activation of NFκB may not occur via a $G\alpha_{q/11}$-dependent mechanism in clone 10H9 cells as YM-254890 did not demonstrate inhibition of p65 NFκB phosphorylation. As expected, the YM-254890 pre-incubated samples stimulated with TNFα did not display any loss of phosphorylation of p65 NFκB.

4.4.2 Effect of AG-1478 on PAR-4 mediated phosphorylation of p65 NFκB

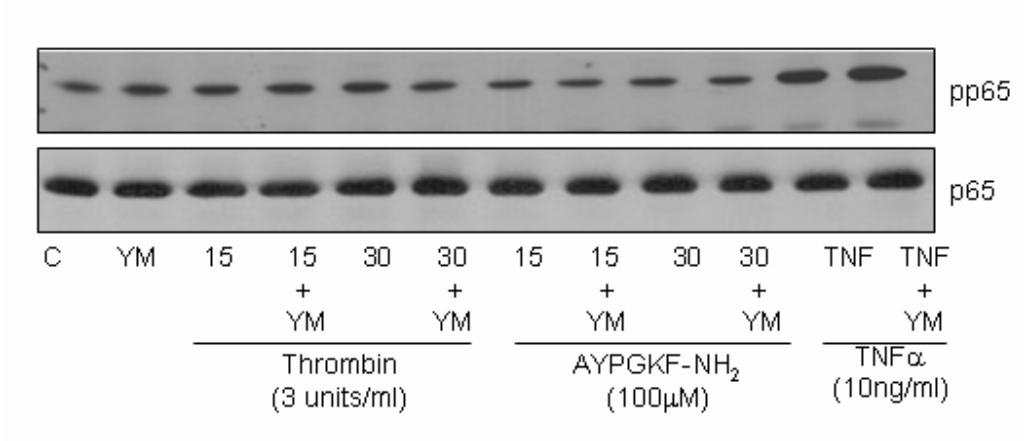
Again, initial experiments to determine the role of EGFR in PAR-4 mediated activation of NFκB, assessed inhibition at the level of p65 phosphorylation. For this purpose, 10H9 cells were pre-incubated with AG-1478 (1μM) for 30 min prior to stimulation with thrombin (3units/ml) or AYPGKF-NH_2 (100μM) for time points including 15 and 30 min. Control samples were again prepared with TNFα (10ng/ml). Figure 4.15 illustrates thrombin and AYPGKF-NH_2 mediated phosphorylation of p65 NFκB (3.5$\pm$0.4 and 3.2 $\pm$0.5 fold increase above basal for thrombin and AYPGKF-NH_2 respectively, n=3, t=30 min). Previously, it had been demonstrated that PAR-4 was capable of activating the MAP kinase pathway, in part, via transactivation of the EGFR. However, in the assessment of the ability of PAR-4 to mediate phosphorylation of p65 via transactivation of the EGFR, AG-1478 did not demonstrate any level of inhibition of p65 NFκB phosphorylation. These results suggest that PAR-4 may not mediate activation of NFκB via an EGFR-dependent mechanism. TNFα control samples stimulated an increase in the phosphorylation of p65 NFκB 5.3$\pm$2.2 above basal and were unaltered by AG-1478.

4.4.3 Combined inhibitory effect of YM-254890 and AG-1478 on PAR-4 mediated phosphorylation of p65 NFκB

Previous findings have shown that PAR-4 may cause activation of the MAP kinase pathway via the additive response of coupling with $G\alpha_{q/11}$ along with transactivation of the EGFR. However, while it was shown that this shared an additive effect, PAR-4 was able to successfully activate ERK and p38 MAP kinase, at least in part, via each of these pathways individually. It therefore remained unknown whether PAR-4 mediated activation of NFκB depended on activation of both of these mechanisms to achieve successful phosphorylation of p65 NFκB. Therefore, clone 10H9 cells were pre-incubated with YM-254890 (100nM) and AG-1478 (1μM) either alone or together for 30 min prior to stimulation with AYPGKF-NH_2 (100μM) for a time period of 30 min.

Results shown in figure 4.16 demonstrated phosphorylation of p65 NFκB by AYPGKF-NH_2 and TNFα (3.3±0.4 and 5.8±0.8 fold above basal for AYPGKF-NH_2 and TNFα respectively, n=3, t=30). No loss was observed in samples which were pre-incubated with only YM-254890 or AG-1478 alone, but surprisingly, both compounds together caused an inhibition of PAR-4 mediated p65 NFκB phosphorylation (% inhibition 55±3.5 n=3, t=30 min, **$p<0.01$). These results suggest that PAR-4 may couple with $G\alpha_{q/11}$ and mediate transactivation of the EGFR in a redundant fashion in order to stimulate phosphorylation of p65 NFκB. This would suggest that whilst activation of both mechanisms will result in the activation of NFκB, inhibition of one or the other will not result in its inhibition. Therefore, in order to cause inhibition of PAR-4 mediated NFκB activation, it may be necessary to block both the coupling of PAR-4 with $G\alpha_{q/11}$ and any involvement of the EGFR at the same time.

a)



b)

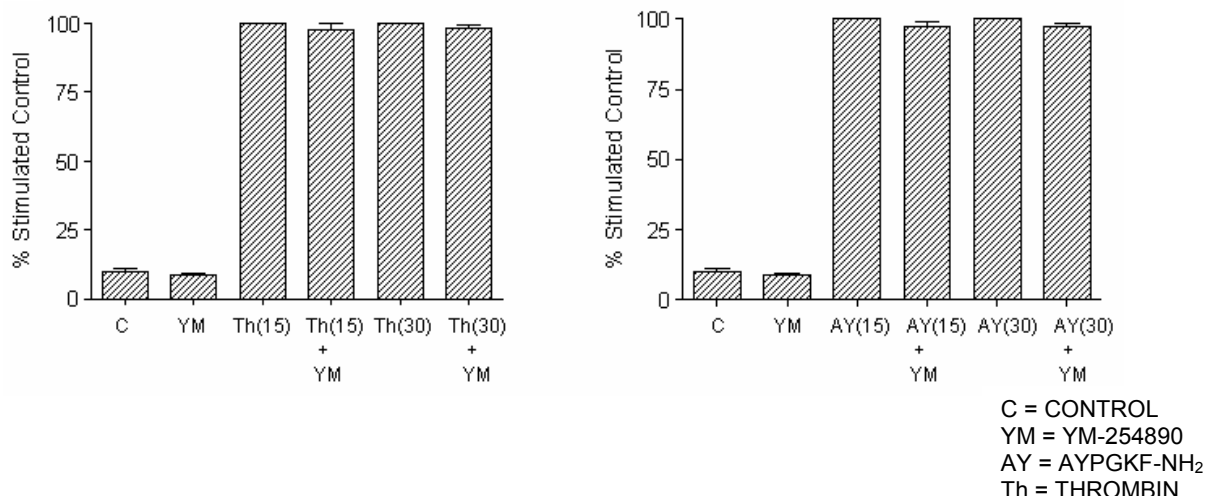


Figure 4.14 Effect of YM-254890 on PAR-4 mediated phosphorylation of p65 NFκB in clone 10H9 cells. Clone 10H9 cells were grown to confluency on 12 well plates and rendered quiescent overnight. Following this, cells were pre-incubated with YM-254890 (100nM) for 30 min prior to each time point. Cells were then stimulated with AYPGKF-NH_2 (100μM) for 15-30 min with TNFα (10ng/ml) employed for control samples. Panel A shows relative phosphorylation of p65, while panel B illustrates quantification by densitometry, expressed as mean $\pm$ s.em (% stimulated control). Each blot is representative of 3 separate experiments.

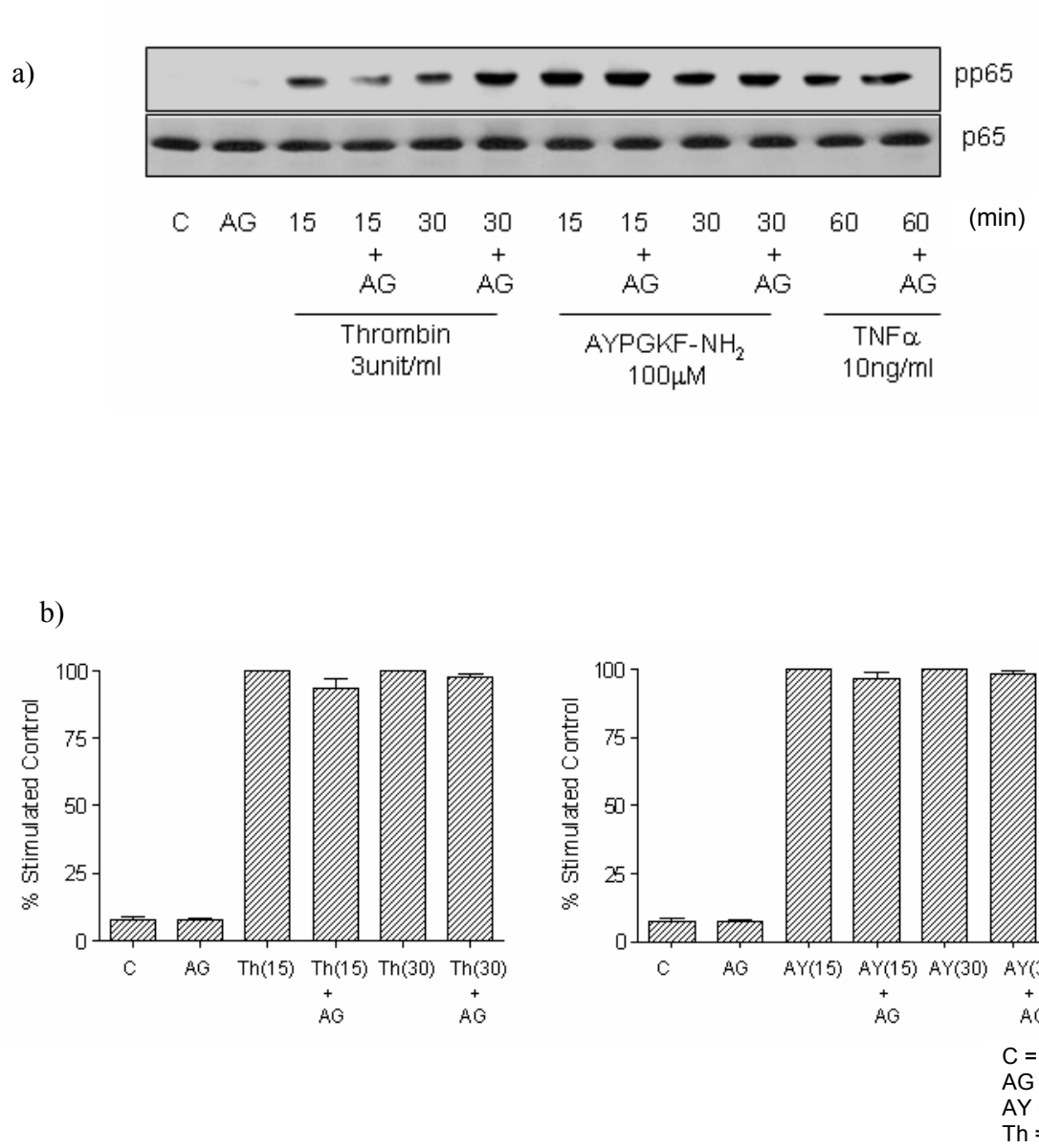
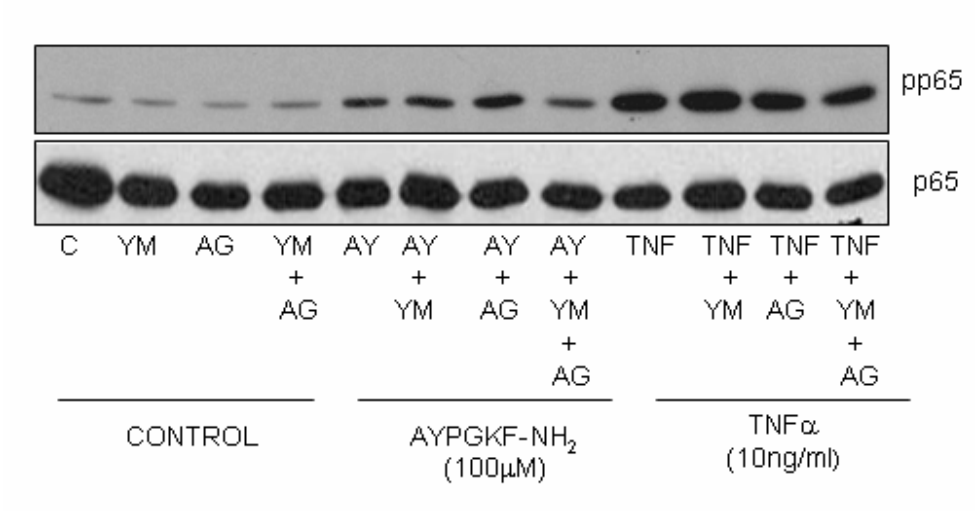
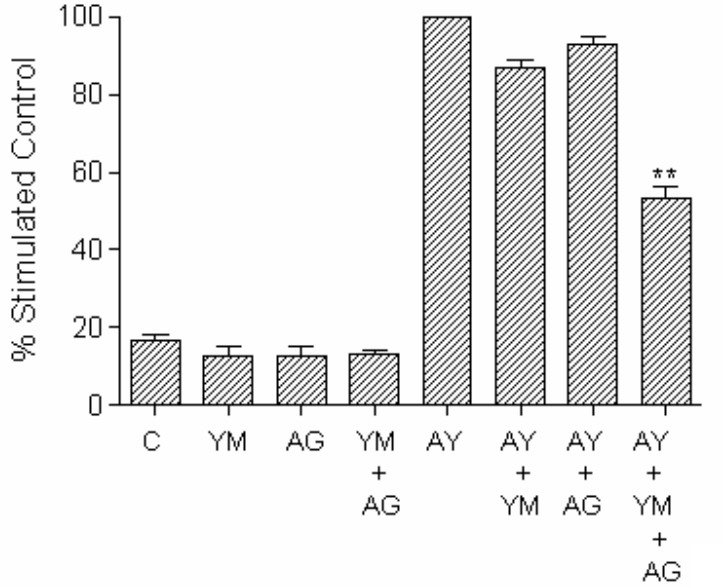


Figure 4.15 Effect of AG-1478 on PAR-4 mediated phosphorylation of p65 NFκB in clone 10H9 cells. Clone 10H9 cells were grown to confluency on 12 well plates and rendered quiescent overnight. Following this, cells were pre-incubated with AG-1478 (1μM) for 30 min prior to each time point. Cells were then stimulated with AYPGKF-NH_2 (100μM) for 15-30 min with TNFα (10ng/ml) employed for control samples. Panel A demonstrates relative phosphorylation of p65 NFκB, while panel B illustrates quantification by densitometry, expressed as mean ± s.e.m (% stimulated control). Each blot is representative of 3 individual experiments.

a)



b)



C = CONTROL
YM = YM-254890
AG = AG1478
AY = AYPGKF-NH_2
Th = THROMBIN

Figure 4.16 Combined inhibitory effect of YM-254890 & AG-1478 on PAR-4 mediated phosphorylation of p65 NFκB in clone 10H9 cells. Clone 10H9 cells were grown to confluency on 12 well plates and rendered quiescent overnight. Following this, cells were pre-incubated with YM-254890 (100nM) or AG-1478 (1μM) alone or in combination for 30 min prior to each time point. Cells were then stimulated with AYPGKF-NH_2 (100μM) for 30min with TNFα (10ng/ml) employed for control samples. Panel A shows relative phosphorylation of p65, while, panel B illustrates the quantification by densitometry, expressed as mean ± s.e.m (% AYPGKF-NH_2 stimulated control, **$p<0.01$). Above blots are representative of 3 individual experiments.

4.4.4 Effect of YM-254890 on PAR-4 mediated NFκB-DNA binding in clone 10H9 cells

So far, it has been shown that PAR-4 requires coupling with $G\alpha_{q/11}$ and transactivation of the EGFR in order to stimulate a phosphorylation of p65 NFκB. However, this has not yet been shown at the level of NFκB-DNA binding in clone 10H9 cells. Therefore, 10H9 cells were pre-incubated with YM-254890 (100nM) for 30 min prior to stimulation with thrombin (3units/ml) or AYPGKF-NH_2 (100μM) for 60 min. Control samples were prepared with TNFα (10ng/ml) for 60 min. Nuclear extracts were prepared and labelled with specific NFκB oligonucleotide and run on a non-denaturing gel to assess the level of NFκB- DNA binding. Results show that both AYPGKF-NH_2 and TNFα stimulated NFκB DNA binding (5±0.75 and 6.5±1.2 fold above basal for AYPGKF-NH_2 and TNFα respectively, n=3). Again, in much the same way as with p65 NFκB phosphorylation, no inhibition of PAR-4 mediated NFκB-DNA binding is visible, suggesting either no involvement of $G\alpha_{q/11}$ (see figure 4.17), or again, the possibility of a redundancy mechanism. As expected, no inhibition of NFκB-DNA binding was observed in YM-254890 samples which were stimulated with TNFα.

4.4.5 Effect of AG-1478 on PAR-4 mediated NFκB-DNA binding in clone 10H9 cells

In order to investigate involvement of EGFR transactivation in PAR-4 mediated NFκB-DNA binding, clone 10H9 cells were pre-incubated with AG-1478 (1μM) for 30 min prior to stimulation with thrombin (3units/ml) or AYPGKF-NH_2 (100μM) for 60 min. Control samples were prepared with TNFα (10ng/ml) for 60 min. Samples were prepared as nuclear extracts and NFκB-DNA binding assessed by EMSA. Results displayed an increase in NFκB-DNA binding mediated by AYPGKF-NH_2 and TNFα (5.8±0.9 and 5.2±0.52 fold above basal for AYPGKF-NH_2 and TNFα respectively, n=3, t=60min).

No inhibition of NFκB-DNA binding was observed in samples pre-treated with AG-1478 which had been stimulated by AYPGKF-NH_2, suggesting no involvement of EGFR transactivation in PAR-4 mediated NFκB-DNA binding (see figure 4.18). Again, the possibility of the same redundancy mechanism previously observed with p65 NFκB phosphorylation may also be a possible effect. To investigate this possibility, it was therefore necessary to use both inhibitors together.

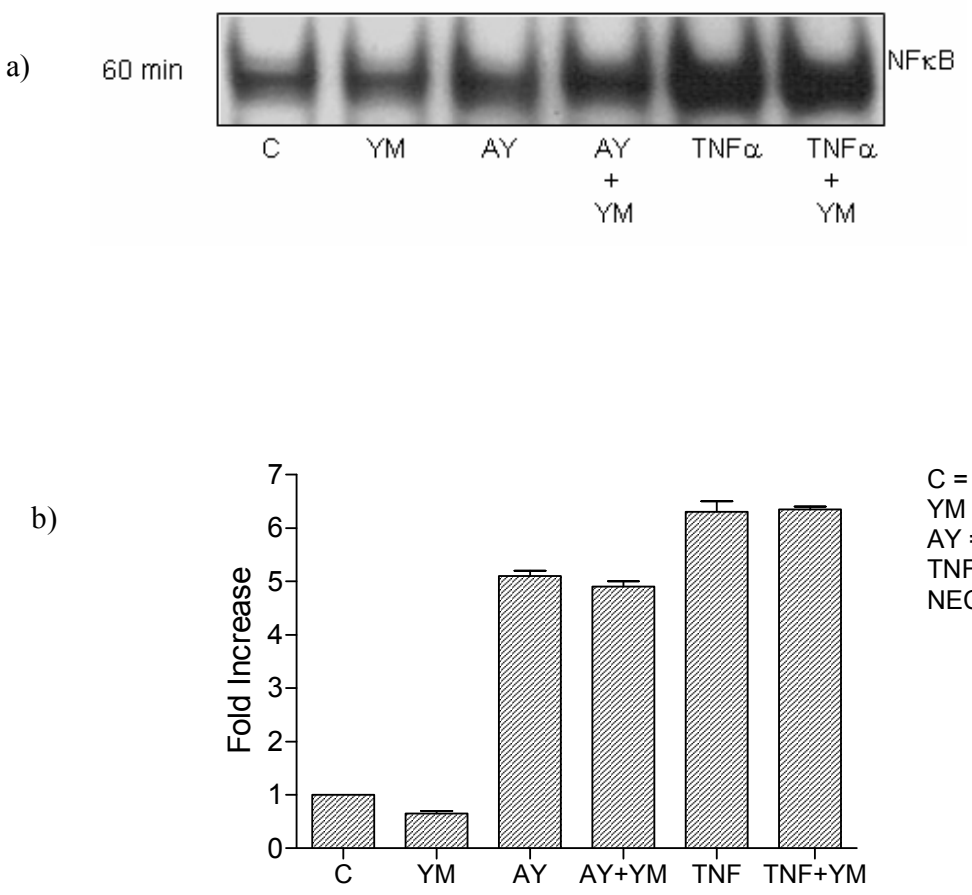
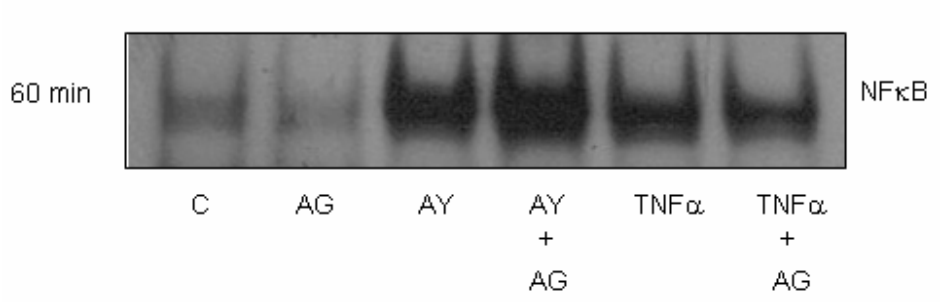
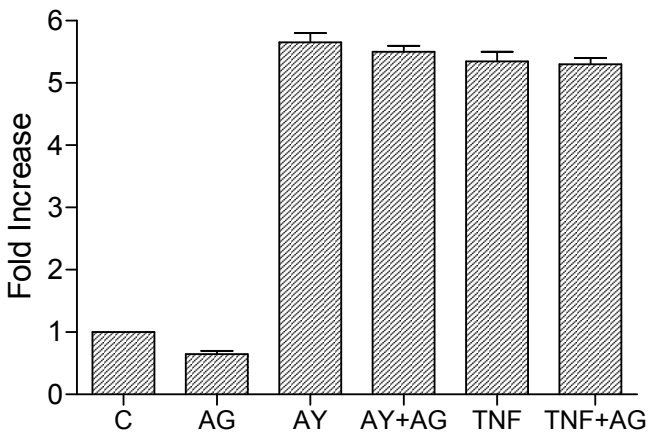


Figure 4.17 Effect of YM-254890 on PAR-4 mediated NFκB-DNA binding in clone 10H9 cells. Clone 10H9 cells were grown to confluency on 6 well plates and rendered quiescent overnight. Cells were then pre-incubated with YM-254890 (100nM) for 30 min prior to stimulation with AYPGKF-NH_2 (100μM) for 60 min. Prepared nuclear extracts were incubated NFκB specific oligonucleotide and resolved by EMSA (see section 2.7) subject to overnight autoradiography. Panel A demonstrates NFκB-DNA binding while panel B illustrates quantification by densitometry, expressed as mean $\pm$ s.e.m (fold increase). Each autoradiograph is indicative of 3 separate experiments.

a)



b)



C = CONTROL
AG = AG1478
AY = AYPGKF-NH_2
TNF = TUMOUR NECROSIS FATOR-α

Figure 4.18 Effect of AG-1478 on PAR-4 mediated NFκB-DNA binding in clone 10H9 cells. Clone 10H9 cells were grown to confluency on 6 well plates and rendered quiescent overnight. Cells were then pre-incubated with AG-1478 (1μM) for 30 min prior to stimulation with AYPGKF-NH_2 (100μM) for 60 min and. Prepared nuclear extracts were incubated with NFκB specific oligonucleotide and resolved by EMSA then subject to overnight autoradiography. Panel A demonstrates NFκB- DNA binding while panel B shows quantification as determined by densitometry and expressed as mean ± s.e.m (fold increase).

4.4.6 Combined inhibitory effect of YM-254890 and AG-1478 on PAR-4 mediated NFκB-DNA binding in clone 10H9 cells

In the same manner in which the phosphorylation of p65 NFκB was investigated, the combined effect of these two inhibitory compounds was investigated to determine if PAR-4 mediated NFκB-DNA binding in same fashion in which it demonstrated phosphorylation of p65 NFκB. Therefore, clone 10H9 cells were pre-incubated with YM-254890 or AG-1478 either alone or together, for 30 min prior to stimulation with AYPGKF-NH_2 (100μM) for 60 min. Nuclear extracts were incubated with NFκB oligonucleotide and run on a non-denaturing gel. Results obtained by EMSA and overnight auto-radiography demonstrated, that PAR-4 mediates NFκB-DNA binding by coupling with $G\alpha_{q/11}$, as well as mediating transactivation of the EGFR (see figure 4.19). Stimulation by AYPGKF-NH_2 resulted in NFκB-DNA binding 5.3$\pm$0.45 above basal (n=3, t=60min). While samples incubated with only one of the inhibitors displayed no inhibition, samples treated with both inhibitors demonstrated a significant level of inhibition (% inhibition 80$\pm$4.7, n=3, t=60min ***$p<0.001$) from the peptide stimulated control. Again, these results suggest a redundancy mechanism, whereby PAR-4 is equally capable of mediating activation of NFκB either by coupling with $G\alpha_{q/11}$ or by transactivation of the EGFR.

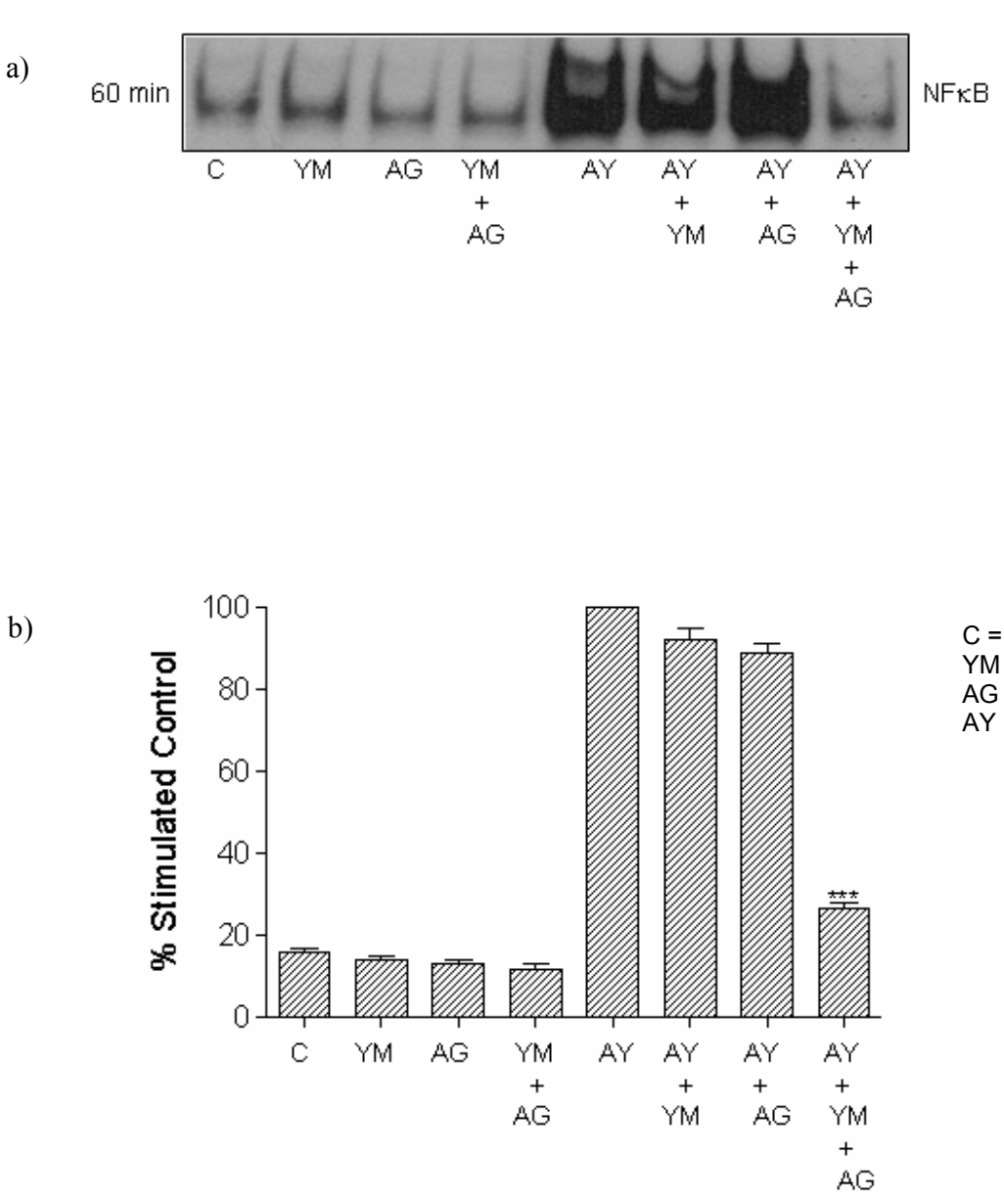


Figure 4.19 Combined inhibitory effect of YM-254890 & AG-1478 on PAR-4 mediated NFκB-DNA binding in clone 10H9 cells. Clone 10H9 cells were grown to confluency on 6 well plates and rendered quiescent overnight. Cells were then pre-incubated with YM-254890 (100nM) or AG-1478 (1μM) alone or in combination for 30 min prior to stimulation with AYPGKF-NH_2 (100μM) for 60 min and prepared as nuclear extracts. Samples were incubated with NFκB specific oligonucleotide and resolved by EMSA (see section 2.7) and subject to overnight autoradiography. Panel A demonstrates NFκB DNA binding while panel B illustrates quantification by densitometry, expressed as mean $\pm$ s.e.m (% AYPGKF-NH_2 stimulated control, ***$p<0.001$). Each autoradiograph represents 3 other experiments.

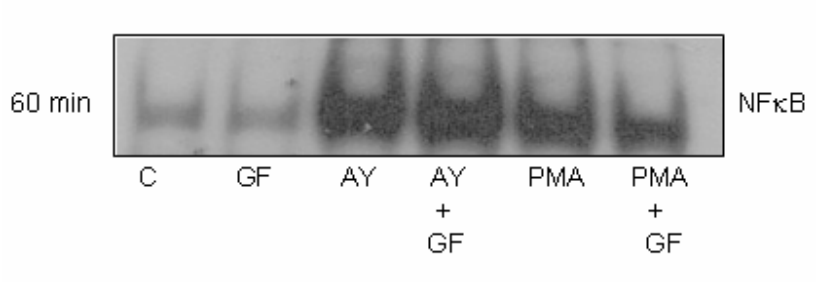
4.5 PKC DOES NOT MEDIATE PAR-4 REGULATED ACTIVATION OF NFκB IN CLONE 10H9 CELLS

In previous studies, it has been demonstrated that PAR-2 is able to mediate NFκB-DNA binding via a PKC-dependent mechanism (Macfarlane *et al*, 2005, Goh *et al*, 2008). These findings have been supported by evidence that PAR-2 is capable of mediating phosphorylation of p65 NFκB at Ser^{536} in human primary keratinocytes. The study by Goh *et al*, 2008 has demonstrated successful inhibition of p65 NFκB phosphorylation in samples which had been pre-incubated with broad spectrum PKC inhibitor GF-109203X. However, any studies relating to PAR-4 mediated activation of PKC as means of activating NFκB have not been published. This area of study aimed to demonstrate that PAR-4 does not mediate phosphorylation of p65 NFκB or NFκB DNA binding via a PKC-dependent mechanism in clone 10H9 cells. For this purpose, the PKC inhibitor GF-109203X was employed to assess the involvement of PKC.

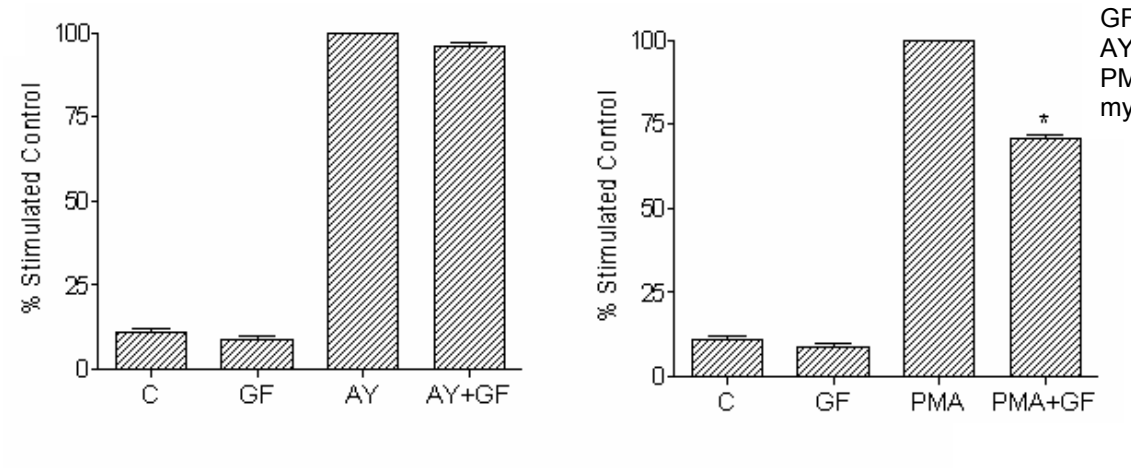
4.5.1 Effect of GF-109203X on PAR-4 mediated NFκB-DNA binding in clone 10H9 cells

To assess PAR-4 mediated NFκB-DNA binding, clone 10H9 cells were pre-incubated with GF-109203X (10μM) for 30 min prior to stimulation with AYPGKF-NH_2 (100μM) for 60 min. Control samples were prepared using PMA (100nM). Samples were prepared as nuclear extracts and incubated with NFκB specific oligonucleotide and resolved by EMSA. Results displayed NFκB-DNA binding mediated by both AYPGKF-NH_2 and PMA (5.6$\pm$1.25 and 4.9$\pm$0.75 fold stimulation for AYPGKF-NH_2 and PMA respectively, n=3, t=60). However, it was apparent that unlike PMA stimulated samples, which exhibited a loss of NFκB-DNA binding when incubated with GF-109203X (% inhibition 30$\pm$2.5, n=3, *$p<0.05$) (see figure 4.20), no loss of binding was exhibited by AYPGKF-NH_2 samples which had been pre-incubated with GF-109203X, therefore suggesting that PKC is not involved in PAR-4 mediated nuclear binding of NFκB. Taken together, these results suggest that unlike PAR-2, PAR-4 does not mediate activation of NFκB via a PKC-dependent pathway.

a)



b)



C = Control
GF = GF-109203X
AY = AYPGKF-NH_2
PMA = Phorbol 12-myristate 13-acetate

Figure 4.20 Effect of GF-109203X on PAR-4 mediated NFκB-DNA binding in 10H9 cells. Clone 10H9 cells were grown to confluency on 6 well plates and rendered quiescent overnight. Cells were then pre-incubated with GF-109203X (10μM) for 30 min prior to stimulation with AYPGKF-NH_2 (100μM) or PMA (100nM) for 60 min. Samples were prepared as nuclear extracts which were resolved by EMSA (see section 2.7). Panel A demonstrates NFκB-DNA binding, while panel B illustrates quantification by densitometry, expressed as mean±s.e.m (% AYPGKF-NH_2 or PMA stimulated control, *$p<0.05$). Above autoradiography is indicative of 3 separate experiments.

4.5.2 Effect of GF-109203X on PAR-4 mediated phosphorylation of p65 NFκB in clone 10H9 cells.

For this purpose, clone 10H9 cells were grown to confluency and following quiescence, were pre-incubated with GF-109203X (10μM) for 30 min. Following this, cells were either stimulated with AYPGKF-NH_2 (100μM) or PMA (100nM) for 15-30 min. Results obtained by Western blotting demonstrated phosphorylation of p65 NFκB by both AYPGKF-NH_2 (4.8±1 and 5.1±0.55 fold above basal, for 15min and 30 min respectively, n=3) and PMA (4.5±0.3 fold above basal, n=3, t=30min). However, these results also showed that PAR-4 mediated phosphorylation of p65 NFκB was not inhibited by GF-109203X in a manner similar to that of PMA, which was significantly reduced by the compound (% inhibition, 62±3.5) (see figure 4.21). These data have support the suggestion that PAR-4 mediated activation of NFκB is not achieved by a PKC-dependent mechanism.

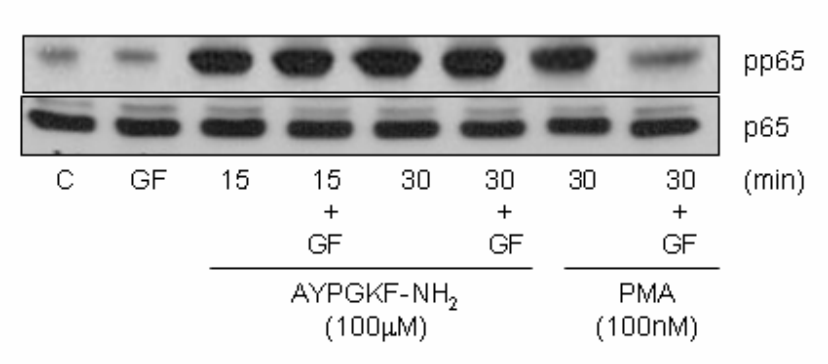
4.6 PAR-4 MEDIATED ACTIVATION OF NFκB IN EAhy-926 CELLS

As it has been demonstrated in this study that PAR-4 is capable of mediating NFκB activation in clone 10H9 cells, it was necessary to determine if PAR-4 is also capable of producing comparable responses in cells found to naturally express the receptor.

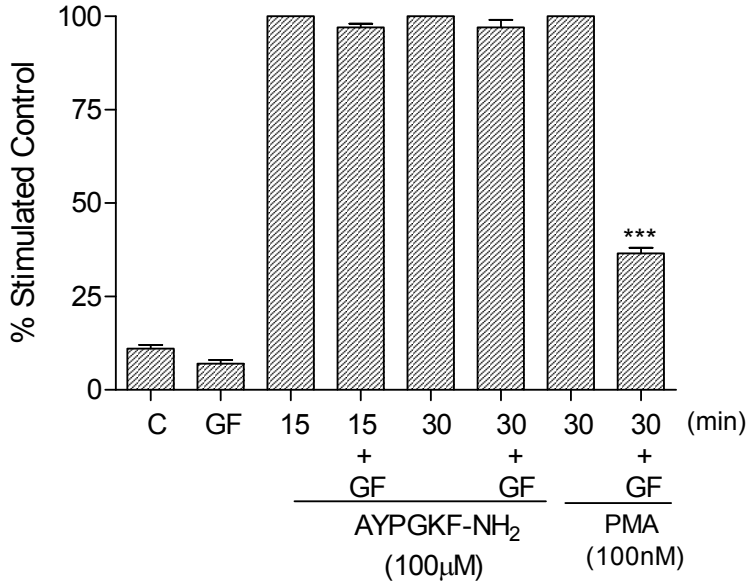
4.6.1 PAR-4 mediated NFκB-DNA binding in EAhy-926 cells

To assess PAR-4 mediated activation of NFκB, EAhy-926 cells were stimulated with AYPGKF-NH_2 (100μM) for a time course ranging from 0-6 hrs with control samples stimulated with TNFα (10ng/ml) for 60 min. Samples were then prepared as nuclear extracts which were incubated with NFκB specific oligonucleotide. Results obtained by EMSA demonstrated a relative lack of DNA binding by PAR-4 activating peptide, whereas, TNFα demonstrated a significant level of DNA binding (6.5±2.7 fold above basal). These results suggest that PAR-4 may not mediate activation of NFκB in EAhy-926 cells (see figure 4.22).

a)



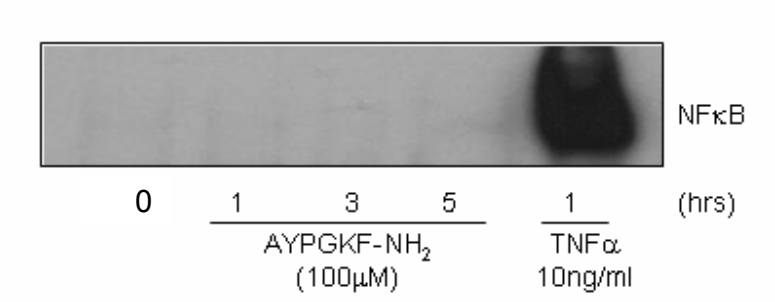
b)



C = Control
GF = GF-109203X
AY = AYPGKF-NH_2
PMA = Phorbol 12-myristate 13-acetate

Figure 4.21 Effect of GF-109203X on PAR-4 mediated phosphorylation of p65 NFκB in clone 10H9 cells. Clone 10H9 cells were grown to confluency on 12 well plates and rendered quiescent overnight. Following this, cells were pre-incubated with GF-109203X (10μM) for 30 min prior to stimulation with AYPGKF-NH_2 (100μM) or PMA (100nM). Samples were resolved by SDS-PAGE and examined by Western blotting for phosphorylation of p65 NFκB. Panel A demonstrates the phosphorylation of p65, while panel B illustrates quantification by densitometry, expressed as mean±s.e.m (% inhibition). Each blot is representative of 3 separate experiments.

a)



b)

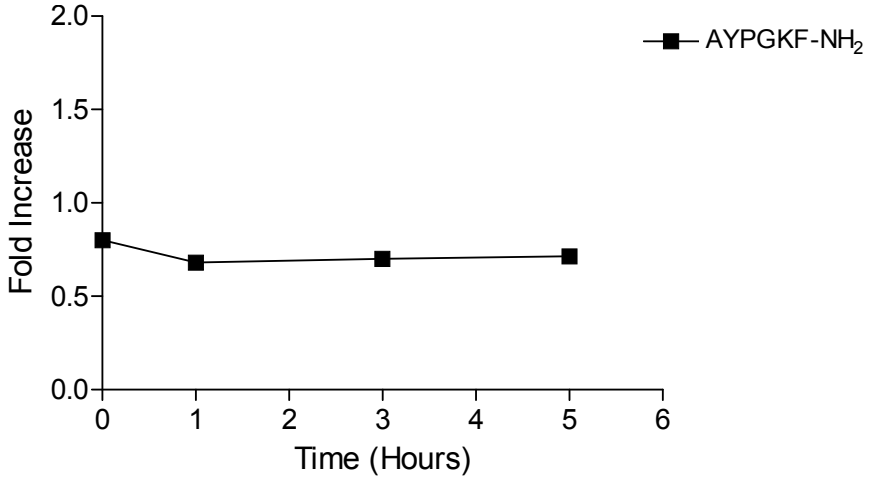
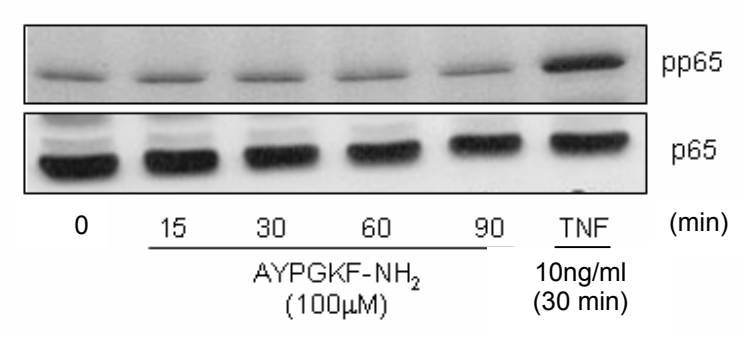


Figure 4.22 Lack of PAR-4 mediated NFκB-DNA binding in EAhy-926 cells. EAhy-926 cells were grown to confluency in 6 well plates and rendered quiescent overnight. Cells were then stimulated with AYPGKF-NH_2 (100μM) from 0-5 hours, with TNFα (10ng/ml) stimulation for 1hr. Cells were prepared as nuclear extracts and incubated NFκB specific oligonucleotide and resolved by EMSA (see section 2.7). Panel A shows results of overnight autoradiography, while panel B illustrates quantification by densitometry expressed as mean±s.e.m (fold increase). The above is representative of 3 individual experiments.

4.6.2 PAR-4 mediated phosphorylation of p65 NFκB in EAhy-926 cells

Further investigation was required to determine if PAR-4 mediates phosphorylation of p65 NFκB in EAhy-926 cells. Therefore, EAhy-926 cells were stimulated with AYPGKF-NH_2 (100μM) for 15-30 min with control samples being stimulated with TNFα (10ng/ml) for 30 min. Results obtained by Western blotting (see figure 4.23) demonstrated a lack of phosphorylation of p65 NFκB in samples stimulated with PAR-4 activating peptide. These results were not comparable with TNFα stimulated samples, which generated phosphorylation of p65 NFκB 5.5±2.5 fold above basal (n=3, t=30min). Taken together, these results suggest that PAR-4 may not mediate activation of NFκB in cells which endogenously express the receptor as opposed to cells generated to over express PAR-4. However, lack of PAR-4 mediated activation of NFκB in EAhy-926 cells may not necessarily suggest that the same is so of other cells in which PAR-4 is naturally expressed. To date, endothelial cells including human pulmonary artery endothelial cells and human aortic endothelial cells have been reported to express PAR-4 endogenously (Fujiwara *et al*, 2004), although as yet, no published studies have properly assessed the functional coupling of PAR-4 with the NFκB pathway.

a)



b)

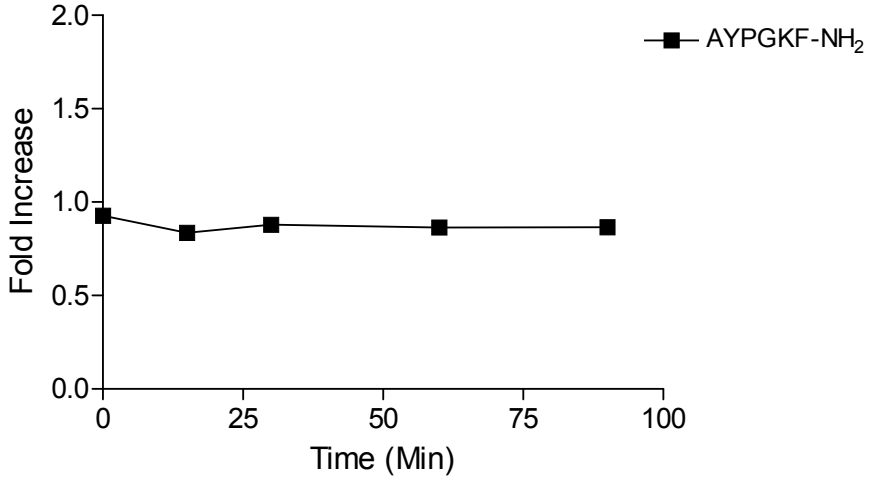


Figure 4.23 Lack of PAR-4 mediated phosphorylation of p65 NFκB in EAhy-926 cells. EAhy-926 cells were grown to confluency and rendered quiescent overnight. Following this, cells were stimulated over a time course of 0-90 min with AYPGKF-NH_2 (100μM), with control samples being stimulated with TNFα (10ng/ml) for 30 min. Samples were resolved by SDS-PAGE and examined by Western blotting for relative phosphorylation of p65 NFκB. The above blot demonstrates the phosphorylation of p65 NFκB by AYPGKF-NH_2 and TNFα. Panel A demonstrates relative phosphorylation of p65 NFκB, while panel B illustrates quantification by densitometry expressed as mean $\pm$ s.e.m (fold increase) and is representative of 3 individual experiments.

4.7 DISCUSSION

This particular area of study has employed varied approaches in order to investigate the involvement of IKKβ, $G\alpha_{q/11}$, EGFR transactivation and PKC in coupling PAR-4 to the activation of the NFκB pathway in NCTC-2544 cells which stably express PAR-4 (clone 10H9) and EAhy-926 cells, which express PAR-4 at an endogenous level. For this purpose, pharmacological inhibitors such as YM-254890, AG-1478 and GF-109203X have been employed, along with $^{+/-}$IKKβ adenoviral constructs and selective pharmacological IKKβ inhibitory compounds including SC-514, BAY11-7082 and BAY11-7085.

The study itself has focussed on the regulation of NFκB activity at different levels, including phosphorylation of p65 NFκB at Ser^{536}, phosphorylation and degradation of IκBα and NFκB-DNA binding, as well as determining whether PAR-4 mediated production of IL-8 is achieved via an IKK-dependent mechanism. Current literature supports the identification of 5 members of the NFκB family which are found in most eukaryotic cell types. These include Rel-A (p65), Rel-B and c-Rel, all of which are produced as transcriptionally active proteins, as well as NFκB-1 (p50) and NFκB-2 (p52). Of these 5 members, the most commonly studied, and best characterised is p65 NFκB, which has been one of the focal points of this study.

To date, at least two members of the PAR family, PAR-1 and PAR-2 have been linked to regulation of the NFκB pathway. PAR-2 for example, has been shown to mediate regulation of NFκB via both G protein-dependent and -independent mechanisms in primary human keratinocytes (Goh *et al,* 2008). Other studies conducted in PAR-2 transfected NCTC-2544 cells (Clone G) have elicited an NFκB response which occurs via an IKK-dependent mechanism, which is caused by the direct phosphorylation of IKKβ by PKC (Macfarlane *et al,* 2005). Although the GPCR mediated stimulation of the NFκB pathway is not as well characterised as that of classical mediators, it has been documented that activation of the NFκB pathway is a characteristic of certain GPCRs.

For example, a study by Leon-Ponte *et al,* 2007, demonstrated that in T cells, 5-HT specific agonists mediated rapid phosphorylation and subsequent degradation of IκBα, which suggests activation of NFκB via an IKK-dependent pathway. In the same study, pre-incubation of T cells with selective 5-HT antagonists prior to stimulation by serotonin, displayed inhibition of NFκB (Leon-Ponte *et al*, 2007).

Earlier studies have also shown that the β_2-adrenoceptor is also capable of mediating activation of NFκB activation (Storm & Khawaja, 1999). The study by Storm & Khawaja demonstrated that in rat glioma cells, activation of the β_2-adrenoceptor was capable of mediating phosphorylation of IκBα as well as mediating nuclear translocation of NFκB. Furthermore, the Somatostatin Type 2 receptor (SSTR2) has been found to mediate activation of NFκB in both HEK-293 cells and pancreatic acinar cells, via coupling with $G\alpha_{14}$ (Lui & Wong, 2005). While the trend of these studies has demonstrated GPCR mediated activation of NFκB via the classical IKK-dependent pathway, the mechanism whereby PAR-4 mediates activation of NFκB has yet to be elucidated.

Thus far, PAR-4 has displayed signalling characteristics, which have shown the ability to mediate sustained activation of NFκB, particularly at the level of NFκB-DNA binding, which may suggest a prolonged activation of the receptor. This is most likely due to the poor desensitisation process that is thought to be associated with PAR-4. This may be due to the characteristics of the receptor, whereby the possibility lies that it may not uncouple from its selected G protein as quickly as other GPCRs, hence resulting in a slower receptor internalisation (Shapiro *et al*, 2000). However, this prolonged activation may occur due to the fact that clone 10H9 cells are a heterologous cellular expression system. Whilst heterologous expression systems allow for the study of selected proteins, forcible expression of a protein in a cell type which does not normally express it, may result in improper regulation of the cellular signalling mechanisms induced by the protein.

Therefore, while clone 10H9 has shown to be a partly suitable model for the characterisation of the PAR-4 mediated activation of the MAP kinase pathway (see chapter 3), the limitations of the cell system may contribute to inconsistencies in signalling when studying PAR-4 mediated activation of NFκB. In the study by Suo *et al,* 2003, it was demonstrated that PAR-4 is capable of mediating NFκB-DNA binding at 7 hours in N9 microglial cells (Suo *et al,* 2003). Suo *et al,* 2003 determined by means of PCR that both PAR-1 and PAR-4 mRNA were present in this particular cell type.

Whilst prior studies by this group had determined that thrombin mediated activation of PAR-1 mediated proliferation of microglial cells, it did not mediate the thrombin stimulated inflammatory response which was also apparent. As such, this particular group provided the follow on study to determine if PAR-4 was therefore responsible for microglial inflammation. Results displayed by both thrombin and activating peptide, GYPGKF-NH_2 in the Suo study suggested that PAR-4 does have a long activation period, not only by mediating prolonged NFκB activation, but it was also shown that the calcium response associated with PAR-4 were much slower than that displayed by PAR-1 (Suo *et al*, 2003).

In this current study, while PAR-4 demonstrated the ability to regulate NFκB at the level of both p65 NFκB phosphorylation and NFκB-DNA binding (see fig 4.1 & 4.5), it did not prove to be via the cellular degradation of IκBα, a process that is normally associated with the nuclear translocation of NFκB (Traenckner *et al,* 1995). This data also suggests that due to lack of IκBα cellular degradation, the mechanism leading to the activation of NFκB may not be mediated through the classical IKK-dependent pathway. Under normal circumstances, IκBα is degraded, which enables NFκB to translocate to the nucleus and is therefore thought to be used as an indicator of NFκB activation. However, this would appear to be a phenomenon more closely associated with the classical pathway of NFκB activation (reviewed by Perkins & Gilmore, 2006).

It is however, possible to mediate activation of NFκB via an atypical pathway. Classical activation of NFκB requires IκBα to become phosphorylated at serine residues 32 and 36 in order to initiate ubiquitination and degradation. However, the method of atyptical activation includes an IKK-independent mode of regulation, which results in tyrosine phosphorylation of IκBα on tyrosine 42 as opposed to the regular serine residues. Stimuli other than cytokines or viral components are required for the activation of this mechanism. These include hypoxia and reperfusion injury, as well as induction by hydrogen peroxide or tyrosine phosphatase inhibitor pervanadate (Schoonbroodt *et al,* 2000, Imbert *et al*, 1996, Mukhopadhyay *et al*, 2000). Interestingly, phosphorylation of IκBα at Tyr^{42} results in translocation of NFκB without any degradation of the IκBα protein (Bui *et al,* 2001, Imbert et al, 1996, Abu-Amer, 1998). Therefore, in this project, as the results demonstrated no IκBα loss, investigation of IκBα phosphorylation at Tyr^{42} was assessed via immunoprecipitation studies. Again, this proved an unsuccessful means of elucidating the mechanism whereby PAR-4 mediates the activation of NFκB. Very poor phosphorylation of Tyr^{42} was visible, therefore, the immunoprecipitation assay illustrates that this is not how PAR-4 regulates the NFκB pathway (see fig 4.3). These results therefore suggest that phosphorylation of p65 NFκB alone may be sufficient to modulate nuclear translocation of NFκB.

Irrespective of the lack of direct IκBα regulation, the possible role of IKK was assessed. As such, an $^{+/-}$IKKβ adenovirus in clone 10H9 cells was utilised. General characterisation of the NFκB pathway has demonstrated that IKKβ is one of the main upstream mediators of the classical pathway, (Li *et al,* 2001, reviewed by Perkins & Gilmore, 2006) (see section 1.2.4). It would therefore be expected, due to the nature of IKKβ, that use of a dominant negative form of this molecule would inhibit the activation of NFκB by inhibiting not only the IKKβ dependent phosphorylation of IκBα, but also the IKKβ dependent phosphorylation of p65 NFκB. Previously, in HeLA cells, it has been shown that IKKβ is not only responsible for the phosphorylation of IκBα, but also p65 NFκB at Ser^{536} (Sakurai *et al*, 1999), suggesting a dual role for the IKKβ subunit in mediating activation and translocation of p65 NFκB.

However, in the case of PAR-4 mediated p65 NFκB phosphorylation in clone 10H9 cells (see fig 4.7), the virus did not demonstrate any level of inhibition of phosphorylation, suggesting that PAR-4 does not require the involvement of IKKβ in this particular pathway. Observations which were similar in finding to that of p65 NFκB phosphorylation in control samples were displayed. $^{+/-}$ IKKβ Adv also did not display any inhibitory effect on PAR-4 mediated NFκB-DNA binding. Similar findings were observed when pharmacological inhibitors of IKKβ were employed. All three inhibitors, SC-514 (fig 4.12 & 4.13), BAY11-7085 and BAY11-7082 (fig 4.10 &4.11), failed to display any significant loss of either p65 NFκB phosphorylation or NFκB-DNA binding. Taken as a whole, these findings have suggested that in the case of PAR-4 mediated regulation of NFκB, IKK-dependent mechanisms may not be involved. However, it cannot be ignored that whilst the findings provided by the use of adenovirus are valid, the pharmacological inhibitors used within this project may have had poor membrane permeability. This would suggest further research may be required within this area to positively determine that PAR-4 does mediate activation of the NFκB pathway via an IKK-independent manner.

Whilst there was no apparent role for IKK in this NFκB regulation, nevertheless, it was important to demonstrate that PAR-4 mediated activation of NFκB was not regulated by the upstream mediator IKKβ. Therefore, it was necessary to determine if PAR-4 resulted in regulation of NFκB by G protein-dependent or -independent mechanisms. Previous studies from this laboratory have successfully demonstrated that PAR-2 is capable of mediating activation of the NFκB pathway through various mechanisms (Goh FG, PhD thesis, 2006). This study demonstrated via specific siRNA studies and use of the pharmacological inhibitor YM-254890, that PAR-2 stimulates activation via a $G\alpha_{q/11}$ -dependent mechanism. Furthermore it was also shown that PAR-2 is able to mediate NFκB by means of an IKKβ-dependent mechanism (Goh *et al*, 2008, Kanke *et al*, 2001).

From the findings relating to PAR-2, it was therefore suggested that PAR-4, as it has previously demonstrated coupling with $G\alpha_{q/11}$, may regulate NFκB in a manner similar to that of its other family member. As shown previously in chapter 3, YM-254890, a selective pharmacological inhibitor of $G\alpha_{q/11}$, was utilised to help characterise PAR-4 mediated activation of the MAP kinase pathway. However, in the assessment of the phosphorylation of p65 NFκB, YM-254890 alone did not mediate an inhibitory effect (fig 4.14). Therefore, initial results suggested that PAR-4 may not couple to the NFκB pathway in the same manner as that of the MAP kinase pathway. NFκB-DNA binding further assessed the actions of YM-254890 on PAR-4 mediated activation of NFκB. Again, no inhibitory effect was observed (fig 4.17). As shown by these results, it was initially thought that PAR-4 coupling with $G\alpha_{q/11}$ may not be the mechanism whereby PAR-4 mediates NFκB-DNA binding in clone 10H9 cells. Thus far, this chapter has established that PAR-4 mediates both phosphorylation of p65 NFκB at Ser^{536}, as well as mediating nuclear binding of NFκB-DNA. However, no particular mechanism has been elucidated to determine these actions of the receptor. No IKKβ-dependent mechanism has been demonstrated and no observaton of either classical or atypical activation has been shown via IκBα phosphorylation or cellular degradation.

Also, no G protein-dependent mechanism has been demonsrated as yet in this case. It was previously demonstrated in chaper 3 that PAR-4 can mediate activation of the MAP kinase pathway via transactivation of the EGFR in both clone 10H9 cells as well as EAhy-926 cells. These findings correlate with previous studies which demonstrated the ability of PAR-4 to mediate transactivation of the EGFR and activate the MAP kinase pathway in cardiomyocytes (Sabri *et al*, 2003a). However, no studies have been published which investigate this ability in the PAR-4 stimulated activation of NFκB. Therefore this study has assessed the abilty of PAR-4 to cause activation of NFκB via transactivation of the EGFR.

In clone 10H9 cells, EGFR tyrosine kinase inhibitor AG-1478 was utilised to investigate this hypothesis at the level of phosphorylation of p65 NFκB at Ser^{536} and at the level of PAR-4 mediated NFκB-DNA binding (see figs 4.15 & 4.18). Initial findings showed that AG-1478 alone did not cause inhibition of p65 NFκB phosphorylation, indicating that transactivation of the EGFR may not be the mechanism of PAR-4 mediated activation of NFκB. Assessment of PAR-4 mediated NFκB-DNA binding was also performed. Again, AG-1478 did not mediate any inhibition. Taken together, these results suggest that PAR-4 mediated NFκB-DNA binding may not be governed by PAR-4 stimulated transactivation of the EGFR.

Findings presented in chapter 3 also demonstrated that PAR-4 mediated activation of ERK and p38 MAP kinase was achieved via a combined-mechanism, whereby the receptor coupled with $G\alpha_{q/11}$ and also mediated the transactivation of the EGFR. Therefore, the final assessment of these two mechanisms investigated the combined effect of YM-254890 and AG-1478 on PAR-4 mediated activation of NFκB. From these experiments, it was shown that together, YM-254890 and AG-1478 caused a significant level of inhibition at the level of both NFκB-DNA binding and phosphorylation of p65 NFκB (see figs 4.16 & 4.19).

These observations demonstrated that PAR-4 may mediate activation of NFκB via both $G\alpha_{q/11}$ and the EGFR in a redundant fashion. This would suggest that inhibition of one or the other mechanisms is not enough to inhibit PAR-4 mediated activation of NFκB. As such, inhibition of NFκB would require for both mechanisms to be inhibited together. As PAR-4 has shown the possibility to mediate activation of NFκB via the EGFR, it can be compared with previous studies where the EGFR has been utilised in the activation of NFκB. Such studies have mainly concentrated on the activation of the inflammatory mediator COX-2, which is under NFκB control. One study assessed thrombin mediated activation of COX-2 via inhibition studies. It was found from this study that thrombin stimulates the increased production of NFκB and subsequent increase of COX-2 via a Src/EGFR-dependent pathway.

Selective Src inhibitor PP-1 and EGFR inhibitor AG-1478 caused a time-dependent decrease in both NFκB and COX-2 production in vascular smooth muscle cells (Hsieh *et al*, 2008). Further studies conducted on EGFR mediated activation of NFκB have suggested that in human bronchial primary epithelial cells, lipo-phosphatidic acid (LPA) mediates an EGFR-dependent increase in COX-2 which was successfully attenuated by AG-1478 (He *et al*, 2008), thus suggesting the possibility that the activation of NFκB may involve activation of the EGFR in this particular cell type. Generally, PAR-4 has been found to be highly expressed in pulmonary tissues (Fujiwara *et al*, 2004, Ramachandran *et al*, 2006). This may suggest that investigation of PAR-4 mediated activation of NFκB via transactivation of the EGFR in varied pulmonary cell types may be a useful investigation.

Thus far, the assessment within this study has looked at PAR-4 mediated activation of NFκB in clone 10H9 cells, which are NCTC-2544 cells overexpressing PAR-4. However, results in chapter 3 also incorporated the use of a hybrid cell type, EAhy-926, which naturally express PAR-4 . Therefore, to determine if PAR-4 can mediate the activation of NFκB in a natural expression environment, PAR-4 mediated phosphorylation of p65 NFκB and NFκB-DNA binding were assessed in EAhy-926 cells. Unlike the ability of PAR-4 to mediate activation of the MAP kinase pathway in this cell type, activation of NFκB in EAhy-926 cells by thrombin or AYPGKF-NH_2 was not successful (see figs 4.22 & 4.23). These data would therefore suggest that it may be possible that the PAR-4 mediated activation of NFκB in this current study may be attributed to the heterologous expression system utilised by clone 10H9. As such, activation of NFκB may not actually be a regular signalling characteristic of this receptor at an endogenous level. As only one previous study has assessed the ability of PAR-4 to successfully mediate NFκB activation in murine microglial cells, which express both PAR-1 and PAR-4 (Suo *et al*, 2003), it is therefore perhaps a cell specific expression characteristic as opposed to being a receptor selective function.

Further to this investigation, it was necessary to determine the effect of PKC in this particular pathway. As previously mentioned other studies have demonstrated that PAR-2 can mediate the activation of NFκB via PKC-dependent phosphorylation of IKKβ (Macfarlane *et al,* 2005, Kanke *et al*, 2001, Goh *et al*, 2008). Other studies have also reported that PKC is capable of NFκB activation by directly phosphorylating IKKα or IKKβ catalytic subunits (Lallena *et al,* 1999). However as this study has suggested that PAR-4 may mediate activation of NFκB via an IKKβ-independent mechanism, PKC mediated phosphorylation of IKKβ was not thought to be likely. Involvement of PKC in the PAR-4 mediated activation of NFκB was assessed by broad spectrum PKC inhibitor GF-109203X in clone 10H9 cells. Results obtained by PAR-4 mediated NFκB-DNA binding and phosphorylation of p65 NFκB, demonstrated no level of inhibition (see figs 4.20 & 4.21), therefore suggesting that PKC was not an intermediate molecule in the pathway whereby PAR-4 mediates activation of NFκB.

In conclusion, this study has shown that PAR-4, within a heterologous cell system, demonstrates the ability to mediate activation of the transcription factor NFκB. This has been initially demonstrated to be via a possible IKK-independent pathway. However, further investigation has shown that PAR-4 can mediate the activation of NFκB via coupling with $G\alpha_{q/11}$ or by transactivation of the EGFR (see figure 4.24). This has been shown to occur via a redundancy mechanism, whereby inhibition of either pathway individually does not result in the inhibition of NFκB. Inhibition can only be shown successfully when both pathways have been inhibited. However, as the intracellular signalling characteristics of PAR-4 have not been fully elucidated, it can therefore be determined that this study has only started to determine the effects displayed by this receptor. As such, it can be suggested that more investigation is required to fully determine the functional abilities of this receptor.

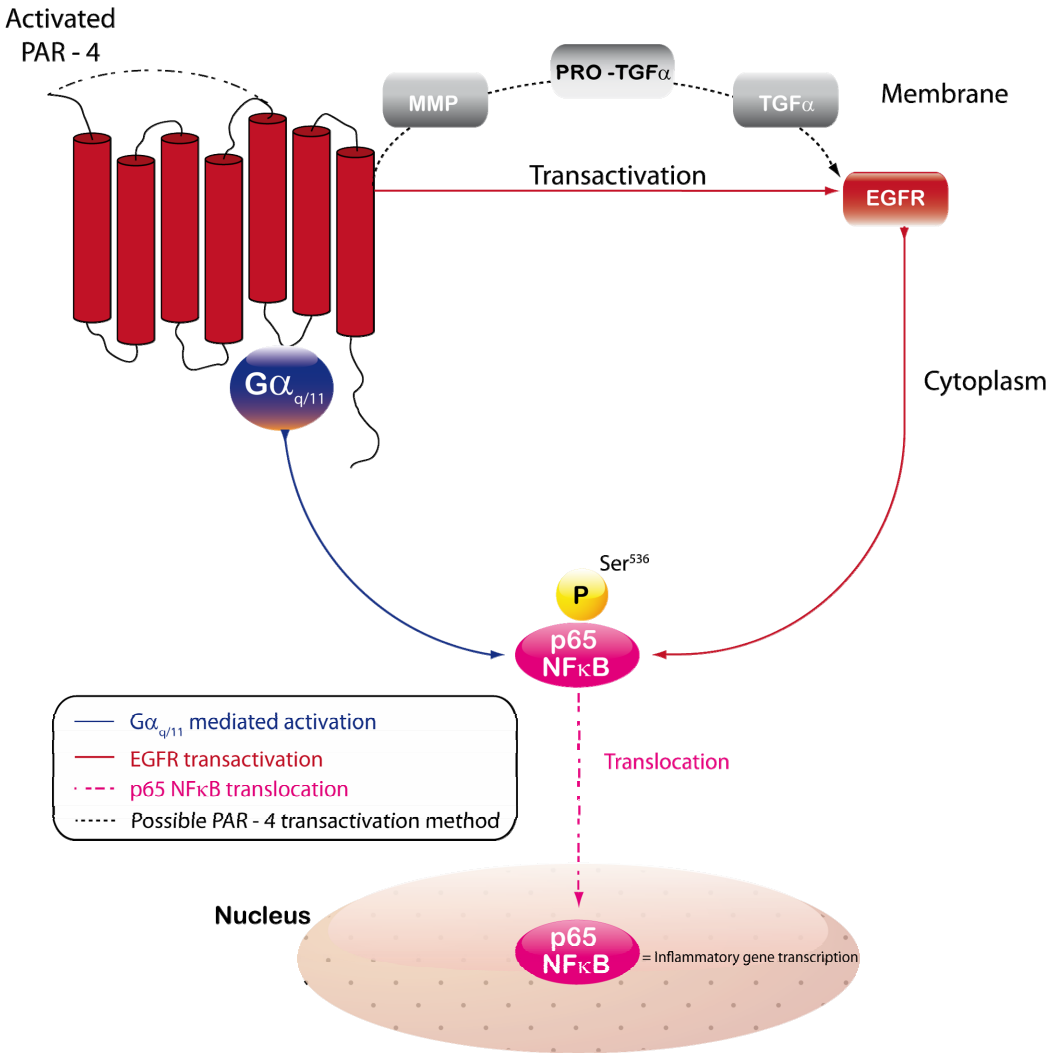


Figure 4.24 Diagrammatical representation of possible PAR-4 mediated activation of NFκB in clone 10H9 cells. The above diagram illustrates the mechanisms whereby PAR-4 may mediate the phosphorylation and subsequent translocation of p65 NFκB in the heterologous expression system, clone 10H9. Both $G\alpha_{q/11}$ and the EGFR were found to be utilised in a redundant fashion. This suggests that in the absence of one mechanism, activation of NFκB may still occur via the un-inhibited mechanism. Due to transactivation of EGFR, possible pathway, including MMPs, membrane bound TGFα and released TGFα, which may act as an activator of the EGFR, have been included.

Chapter 5
General Discussion

PAR-4, which is the most recently identified member of the PAR family (Xu *et al*, 1998), has been found to be located within a variety of tissues of the body, including pulmonary (Ando *et al*, 2007, Jesmin *et al*, 2007, Fujiwara *et al*, 2004, Momota *et al*, 2006), gastric tissue (Mule *et al,* 2004) and the vascular endothelium (Hirano *et al*, 2007). To date, there are few published studies which have properly assessed the functional role of PAR-4, and as such, they have not been fully defined. Therefore, this project has sought to investigate PAR-4 and help identify the mechanisms whereby it regulates activation of certain pathways, including the MAP kinase pathway and NFκB pathway. The results presented herein have helped to demonstrate the ability of PAR-4 to mediate an increase in total inositol phosphate, as well as mediate activation of ERK and p38 MAP kinase in NCTC-2544 cells, which stably express PAR-4, (clone 10H9) as well using EAhy-926 cells, which naturally express PAR-4.

Results have shown that PAR-4 is capable of mediating the phosphorylation of both ERK and p38 MAP kinase in both cell types, suggesting a role for PAR-4 in activation of the MAP kinase pathway, although there were minor differences between the cell types. The phosphorylation of ERK in clone 10H9 was shown to be transient, lasting little more than 5 minutes, as opposed to that of p38 MAP kinase, which demonstrated a more sustained response. The transient ERK response in this cell type shows similarities to the reduced PAR-2 mediated ERK activation which was apparent in $^{-/-}$β-arrestin MEFs (Stalheim *et al*, 2005). The involvement of β-arrestin has been shown to be associated with PAR-2 mediated activation of ERK (DeFea *et al*, 2000, Stalheim, *et al*, 2005). PAR-2 stably interacts with β-arrestin, whereby the receptor/arrestin complex becomes endocytosed. Use of PAR-2 mutants with C terminal truncations demonstrated that the PAR-2 C terminus was of importance in mediating an interaction with β-arrestin. Previously demonstrated by Oakley *et al*, 2001, PAR-2 has been shown to contain three distinct clusters of serine and threonine residues, which are of importance in mediating the stable binding of β-arrestin.

Loss of these clusters resulted in the loss of co-localisation of the PAR-2/β-arrestin complex within the endocytic vesicles. These results demonstrate the importance of these specific sites within the PAR-2 C terminus in mediating the binding of β-arrestin (Stalheim *et al,* 2005). As such, PAR-2 receptors that cannot successfully form a stable complex with β-arrestin do not mediate overall activation of ERK, as there is a loss of the scaffolding complex necessary for this process. Assessment of PAR-4 has been shown to have very few similarities in comparison to PAR-2. Investigation of the amino acid sequence of the PAR-4 C terminus has demonstrated that there are no suitable serine or threonine clusters that could facilitate an interaction with β-arrestin (reviewed by Ossovskaya & Bunnett, 2004, Xu *et al*, 1998). Whole receptor amino acid analysis has also illustrated a lack of suitable binding sites for human, rat or mouse C termini (Hoogerwerf *et al*, 2002). It may, therefore, be possible that as PAR-4 lacks the suitability to interact with such molecules, it cannot undergo β-arrestin facilitated endocytosis. As such, utilisation of the β-arrestin-containing scaffolding complex is not available for PAR-4 for mediating the activation of ERK.

In comparison to clone 10H9 cells, results obtained by using EAhy-926 cells demonstrated that whilst thrombin mediated a response similar to that displayed in clone 10H9 cells, AYPGKF-NH_2 resulted in a delay in the phosphorylation of ERK, which was not apparent until 15 min. These results suggest that the over expression of PAR-4 in a heterologous cell system may lead to limitations on the level of receptor control. It may therefore be of benefit for future research on this receptor, to find a heterologous expression system more capable of mediating responses which are more comparable with a naturally expressing cell type.

As mentioned previously, over and above the transient phosphorylation of ERK, a sustained phosphorylation of p38 MAP kinase was shown in both cell types. Also known as a member of the stress activated protein kinase (SAPK) family, p38 MAP kinase can be associated with both physiological and pathological conditions. Under pathological conditions, it can become activated by inflammatory stimuli such as cytokines, ultraviolet light and reactive oxygen species (Lui *et al*, 2008, Lewis & Spandau, 2008, Jameel *et al*,

2008). However, a degree of caution should be applied to the suggestion that all cellular processes mediated by p38 MAP kinase are of an inflammatory nature. It has also been found to be involved in haemostatic mechanisms, whereby it has been shown to be responsible for mediating the regulation of platelet-activating factor in neutrophils (Baker *et al,* 2002), and has also been shown to increase endostatin-mediated regulation of the retinal barrier function by upregulation of the tight junction protein, occludin (Campbell *et al,* 2006).

Further evidence which supports a physiological role for p38 MAP kinase is illustrated in normal murine mammary glands. In this environment, p38 MAP kinase is suggested to be one of the pathways which mediates TGFβ induction of connexin 43. Responsible for the regulation of the epithelial cell cycle, connexin 43 was shown to inhibit cell cyle progression in a manner which prevents malignant transformation, therefore protecting the organism from the progression of epithelial cancers (Tacheau *et al*, 2008). Loss of function of several signalling molecules including PI3K/Akt and p38 MAP kinase resulted in a decrease of connexin 43, thus suggesting that p38 MAPK may be partly responsible for governing the signalling pathways mediating cellular homeostasis in mammary epithelial cells (Tacheau *et al*, 2008).

The results displayed in this thesis have suggested that PAR-4 may mediate p38 MAP kinase in a more robust manner to that of ERK. It also became apparent in this project that PAR-4 was capable of stimulating production of IL-8 via a pathway which involved p38 MAP kinase. IL-8 is a member of the cytokine family and is a well established inflammatory mediator, which is found to be in particular abundance in conditions such as asthma or other chronic pulmonary conditions (Ramachandran *et al*, 2007, Ostrowska & Reiser, 2007). Under normal physiological conditions, the alveoli of the lungs allow the respiratory system to mediate the process of gaseous exchange (reviewed by Scarpelli, 2003). However conditions such as asthma have been shown to involve excessive inflammation within the airways, therefore resulting in the impairment of such processes (reviewed by Hida, 1999).

Part of the inflammatory infiltate that has been found to be associated with conditions including asthma has been found to include increased levels of IL-8, which as a chemokine, is capable of chemically attracting neutrophils. Neutrophils then undergo degranulation, which releases primary, secondary or tertiary protein granules, the latter which mediates the release of cathepsin, which has been characterised as an activator of PAR-4 (Sabri *et al*, 2003b, Ramachnadran *et al*, 2007). It has now been demonstrated that whilst PAR-4 is not normally expressed in pulmonary fibroblasts in a constitutive manner, expression of the receptor is upregulated in response to the inflammatory infiltrate associated with pulmonary disease (Ramachandran *et al*, 2007).

Other studies have shown that both IL-8 and p38 MAP kinase are also been associated with chronic airway inflammation. For example, Nath *et al*, 2006 have shown that p38 MAP kinase plays a role in airway remodelling, hyperplasia of goblet cells and bronchial hyper-reactivity. Further studies have illustrated that p38 MAP kinase has been shown to mediate an increase in VEGF, which is associated with airway remodelling of a number of pulmonary disorders including asthma, pulmonary hypertension, lung cancer and pulmonary fibrosis (Raidl, *et al,* 2007). Therefore, as PAR-4 is constitutively expressed in human pulmonary artery endothelial cells (HPAECs), (Fujiwara *et al,* 2004), as well as being upregulated in response to pulmonary inflammation (Ramachandran *et al*, 2007) it could be a possibility that PAR-4 may be involved in the inflammatory response associated with chronic airway diseases. As such, the study of PAR-4 in models of airway disease may indicate an area of novel anti-inflammatory strategies which could be valuable in future pharmacological research.

Further to establishing the ability of PAR-4 to stimulate the MAP kinase pathway, this particular study has also concentrated on characterising the various signalling mechanisms which result in PAR-4 mediated phosphorylation of this pathway. As PAR-4 displayed the ability to mediate an increase in $[^3H]$-IP_3 the possibility that the receptor coupled with either $G\alpha_{q/11}$ or $G\alpha_{i/o}$ was likely. Previous studies have shown that PAR-4 was capable of mediating nitric oxide production by coupling with $G\alpha_{i/o}$ in bovine aortic endothelial cells as NO release was inhibited by pre-incubation of the cells with PTx

(Momota *et al*, 2006). In this current study, assessment of clone 10H9 cells which had been pre-incubated with PTx and [^{3}H]-inositol phosphate did not show any inhibition of total [^{3}H]-IP_3, suggesting that in this particular cell type, PAR-4 did not mediate an increase in IP_3 via coupling with $G\alpha_{i/o}$. However, this may be a cell specific feature, and does not rule out the possiblity that in eondothelial cells as opposed to the cell types used within this study, PAR-4 may couple with $G\alpha_{i/o}$. The fact that in certain endothelial cells, including bovine and porcine cell types, shows the ability of PAR-4 to mediate NO production, suggests another possible physiological role for PAR-4. NO is an important messenger molecule involved in both physiological and pathological processes within the mammalian body (Hou *et al*, 1999). Vascular endothelium utilises NO in order to mediate relaxation of the vascular smooth muscle, thus regulate vascular tone (reviewed by Dessy & Ferron, 2004). It is a highly reactive molecule which has been shown to contribute to vessel regulation by inhibiting smooth muscle contraction, reducing platelet aggregation and regulating the ashesion of leukocytes to the walls of blood vessels, all of which can be disrupted during pathological conditions such as hypertension or atherosclerosis (Dessy & Ferron, 2004).

Previous studies have determined that PAR-1 and PAR-2 have been shown to modulate endothelial-dependent vasodilation, possibly via production of NO in human blood vessels (Ballerio *et al*, 2007). One paricular study utlised the PAR-1 and PAR-2 activating peptides, TFLLR-NH_2 and SLIGKV-NH_2, to assess the changes in vascular tone of patients undergoing coronary artery bypass grafting. Pre-contracted arteries showed relaxation mediated by these activating peptides were shown to be both endothelial and NO dependent. Mechanical removal of the endothelium not only demonstrated loss of vascular relaxation, but vessels with an intact endothelium which were pre-treated with the NO inhibitor N^G-nitro-L arginine methyl ester (L-NAME), also demonstrated loss of vascular relaxation upon addition of the peptides. Other studies have demonstrated that both PAR-2 and PAR-4 are also capable of mediating NO dependent relaxation in response to inflammatory stimuli in the human coronary artery (Hamilton *et al*, 2001).

Exposure of isolated human coronary artery to IL-1 resulted in endothelial dependent relaxation when stimulated by the PAR-4 activating peptide GYPGQV. This suggests that PAR-4 may be capable of mediating endothelial dependent relaxation via the production of NO. To date no further published studies have pursued this particular subject area. Therefore, it may be useful for future characterisation studies of PAR-4 to further investigate the role of PAR-4 in the regulation of vascular tone by production of NO.

Further to investigation of coupling with $G\alpha_{i/o}$, the coupling of PAR-4 with $G\alpha_{q/11}$ was also assessed. Previous studies have established that other members of the PAR family, including PAR-1 and PAR-2 are capable of mediating activation of MAP kinase pathway by coupling with $G\alpha_{q/11}$ (Coughlin *et al*, 2001, Deng *et al*, 2008), as well as mediating transactivation of the EGFR (Arora *et al*, 2008, Darmoul *et al*, 2004, Wiiger *et al*, 2004). Previous studies within this laboratory have also demonstrated via use of $G\alpha_{q/11}$ inhibitor YM-254890 (Taniguchi *et al*, 2003), that PAR-2 was capable of mediating an increase in IP_3 via coupling with $G\alpha_{q/11}$ (Goh *et al*, 2008). Therefore, in clone 10H9 cells YM-254890 mediated a concentration dependent inhibition of $[^3H]$-IP_3, as well causing partial inhibition of PAR-4 mediated phosphorylation of both ERK and p38 MAP kinase. YM-254890 mediated inhibition of both ERK and p38 MAP kinase was also apparent in EAhy-926 cells. As YM-254890 did not completely inhibit the phosphorylation of the MAP kinase pathway, it was thought a second mechanism was involved. Inhibition studies which utilised the tyrosine kinase inhibitor AG1478, demonstrated that PAR-4 also mediated activation of the MAP kinase pathway via transactivation of the EGFR. Moreover, pre-incbation with both YM-254890 and AG-1478 was found to result in an additive inhibitory response in both clone 10H9 and EAhy-926 cells.

Further to this current study, a new direction to verify that PAR-4 is capable of coupling with $G\alpha_{q/11}$ may include the use of $G\alpha_{q/11}$ specific siRNA. RNA interference has been implemented as a post-translational tool which targets the mRNA of interest, thus resulting in its degradation. This occurs by inhibiting expression of the protein of interest (reviewed by Stanislawska & Olszewski, 2005). Previous studies have shown that transient transfection of specific siRNA has successfully inhibited expression of $G\alpha_{q/11}$ in signal transduction induced by muscarinic receptors (Atkinson *et al*, 2006). Investigation of metabotropic glutamate (mGlu) receptors determined that reduced expression of $G\alpha_{q/11}$ resulted in significant reduction in the level of IP_3 and Ca^{2+} signalling generated by both mGlu1 and mGlu5 in CHO cells (Atkinson *et al*, 2006).

Another study by Barnes *et al*, 2005 demonstrated the effect of $G\alpha_{q/11}$ siRNA in angiontensin receptor ($AT_{1A}R$) signalling (Barnes *et al,* 2005). Run down of the expression of $G\alpha_{q/11}$ in the CHO cells again resulted in the impairment of IP_3 production, but interestingly, was also found to work with β-arrestin-1 to mediate the activation of RhoA, as well as stimulate the formation of stress fibres (Barnes *et al*, 2005). CHO cells were stimulated with angiotensin following preparation with $G\alpha_{q/11}$ siRNA. Western blotting demonstrated a partial loss of RhoA signalling, which was later found to be inhibited in an additive fashion when β arrestin-1 siRNA was also used. Use of both $G\alpha_{q/11}$ and β arrestin-1 siRNA also resulted in the inhibition of stress fibre formation in the CHO cells (Barnes *et al*, 2005). It would therefore be useful to utilise $G\alpha_{q/11}$ siRNA in cell types which express PAR-4. From such studies, it may be possible to determine if such pharmacolgical intervention will inhibit the PAR-4 mediated increase in $[^3H]$-IP_3, as well as interfere with both activation of the MAP kinase pathway and NFκB pathway.

Further to the use of specific siRNA, other investigative tools have been introduced with can also investigate the selective coupling between GPCRs and G proteins. Recent advances have led to the introduction of real time investigative tools such as Forster (or Fluorescence) Resonance Energy Transfer (FRET) or Bioluminescent Resonance Energy Transfer (BRET). Such tools can allow for the investigation of protein to protein relationships, and as such can investigate areas including the coupling of a GPCR to a selctive G protein.

One particular study investigated the coupling of PAR-1 with $G\alpha_{i1}$ (Ayoub *et al*, 2007). This study utilised a PAR-1 receptor which was successfully tagged at the C terminus with yellow fluorescent protein (YFP). Corresponding to this, the G protein, $G\alpha_{i1}$ was tagged with another fluorescent protein known as Rluc. Using BRET analysis, this group found that a significant level of energy transfer was apparent between the YFP tagged PAR-1 and Rluc tagged $G\alpha_{i1}$. Pre-incubation of cells with PTx resulted in the inhibition of the BRET signal, validating the previous results that had shown that PAR-1 couples successfully with $G\alpha_{i1}$ (Ayoub *et al*, 2007). Such investigative techniques may provide a means of validation to confirm the suggestion that PAR-4 couples with $G\alpha_{q/11}$ in both clone 10H9 cells and EAhy-926.

As previously mentioned, PAR-4 can mediate the activation of both the MAP kinase pathway and the NFκB pathway by coupling with $G\alpha_{q/11}$ and by mediating transactivation of the EGFR. Such results suggest that it is possible that the receptor may be capable of mediating these pathways by both G protein-dependent and –independent mechanisms. Whilst few studies have investigated PAR-4 mediated transactivation of the EGFR, the discussion in section 1.1.5.2 has illustrated that GPCR mediated transactivation of the EGFR in a well characterised method of intracellular signalling associated with certain pathways including the MAP kinase pathway and Akt/PI3K pathway. Transactivation of the EGFR has been reported within a variety of GPCRs, which include the angiotensin II receptor (Yahata *et al*, 2006), P2Y1 receptor (Buvinic *et al*, 2007), chemokine receptors (Kodali *et al,* 2006), as well as other members of the PAR family, including PAR-1 and PAR-2 (Darmoul *et al*, 2002, Darmoul *et al*, 2003).

Such evidence along with the results presented within this study may therefore support the notion that transactivation of the EGFR may be a signalling characteristic associated with PAR-4.

The second part of this study investigated PAR-4 mediated regulation of the NFκB pathway. It has been well established in literature that NFκB is a transcription factor found within many eukaryotic cell types, which is responsible for the transcription of inflammatory mediators including cytokines, chemokines and cell adhesion molecules (reviewed by Perkins & Gilmore, 2006, Brasier, 2006). As previously discussed in chapter 4, canonical activation of this pathway has been characterised mainly through cytokine interactions. It has been poorly represented by studies investigating its regulation by GPCRs. Certain aspects of GPCR regulated NFκB have been established, including receptors such as the bradykinin receptor (Pan *et al*, 1996). This particular study demonstrated the pro-inflammatory nature of bradykinin in normal human keratinocytes (NHEK). The study illustrated that the bradykinin-2 (BK2) receptor mediates the production of IL-1β via activation of the NFκB pathway. This was found to be achieved via a G protein-dependent mechanism. Pre-incubation of NHEK cells with PTx resulted in the inhibition of NFκB and the corresponding production of IL-1β which was demonstrated by both ELISA and Western blotting. Such results suggest that the BK2 receptor mediates activation of the NFκB pathway and the subsequent production of IL-1β via coupling with $G\alpha_i$ (Pan *et al,* 1996).

Other studies have demonstrated that GPCRs such as muscarinic receptors are also capable of mediating activation of the NFκB pathway (Guizzetti *et al*, 2003, Todisco *et al,* 1999). The study by Guizzetti *et al*, 2003 demonstrated that carbachol, a cholinergic agonist, is able to mediate activation of NFκB in astrocytoma cells. Results provided evidence that the M3 muscarinic receptor was capable of mediating phosphorylation of p65 NFκB and NFκB-DNA binding. Inhibition of NFκB was demonstrated by use of BAY11-7082, suggesting that this particular GPCR mediates the activation of NFκB via an IKK-dependent pathway (Guizzetti *et al*, 2003).

Prior to this study, Todisco *et al*, 1999 demonstrated by use of luciferase assay in gastric parietal cells, that carbachol mediated an increase in NFκB activity. Along with mediating a time-dependent activation of the IKK signalosome, PKC inhibitor GF109203X inhibited the activaton of NFκB, suggesting PKC mediated activation of the IKK isoforms (Todisco *et al*, 1999). These results therefore represent a possbile pathway of GPCR mediated activation of NFκB, which are similar to that utilised by PAR-2 (see section 1.3.4.4) (Macfarlane *et al*, 2005).

As it is possible for GPCRs, including members of the PAR family to regulate activation of NFκB, this project also sought to investigate the possibility of PAR-4 mediated NFκB activation. This included investigation at the level of p65 NFκB phosphorylation and NFκB-DNA binding and the mechanisms whereby it couples with this particular transcription factor. Upon finding that PAR-4 could mediate activation of NFκB at both the level of NFκB-DNA binding and phosphorylation of p65 NFκB in clone 10H9 cells, it may be possible that PAR-4 is involved in the regulation of this cellular response. One previous study has implicated the involvement of prolonged PAR-4 stimulated activation of NFκB in N9 microglial cells, which have been shown to express PAR-4 naturally. Unfortunately, the mechanism whereby PAR-4 activates the NFκB pathway was not elucidated (Suo *et al*, 2003). However, results from this current study demonstrated that unlike PAR-2, which mediates NFκB activation via a PKC and IKKβ-dependent pathway, PAR-4 mediated activation of NFκB did not occur via the canonical IKK-dependent mechanism. Furthermore, phosphorylation or degradation of IκBα were not apparent either.

This study has so far demonstrated that in clone 10H9 cells, PAR-4 mediated activation of the NFκB pathway may occur by PAR-4 coupling with $G\alpha_{q/11}$ and by transactivation of the EGFR. However, in the activation of NFκB, a redundancy mechanism was apparent, suggesting that inhibition of one or the other mechanisms was not enough to inhibit the overall activation of NFκB. Whilst PAR-4 mediated activation of NFκB was apparent in the heterologous 10H9 cells, no activation was demonstrated in the naturally

expressing EAhy-926 cells. Such results may suggest that the activation of NFκB in clone 10H9 cells may in fact be an artifactual activation based on the overexpression of PAR-4 in a foreign environment. Therefore, such results would suggest that futher study in other cell types thought to naturally express PAR-4 is required to determine whether or not activation of NFκB is a genuine characteristic of PAR-4 signalling.

Interestingly, certain studies have associated the EGFR with NFκB activation. Such findings have been illustrated in a model of human breast cancer cells (Singh *et al*, 2007, Biswas *et al,* 2000). Over expression of the EGFR is found to be common in many cancers, including breast cancer. It is particularly over expressed in the oestrogen negative (ER-) cell type (Biswas *et al*, 2006). It was apparent in some studies that NFκB also appeared to be active within this cell type, contributing to the possible role of irregular cell cycle activity. Results obtained by EMSA demonstrated NFκB-DNA binding was apparent upon addition of EGF, which was later inhibited by pre-incubation of cells with anti-EGF antibody. These results suggest possible EGF mediated activation of NFκB. Involvement of PI3K was found downstream of the EGFR, as NFκB-DNA binding was also successfully inhibited by PI3K inhibitor, LY294002. Furthermore, it was shown that PI3K resulted in the activaton of PKC, and PKC mediated phosphorylation of IKK in order to complete the EGFR stimulated pathway ending in NFκB activation. These results have helped to define a possible connection between the activation of the EGFR and eventual activation of NFκB (Biswas *et al*, 2000). Later studies by this group utilised a $^{-/-}$IKKβ mouse model, where it was found that interruption of the pathway at the level of IKKβ resulted in a decrease in the level of NFκB activity (Singh *et al*, 2007).

In this current study, PAR-4 did not demonstrate the ability to mediate activation of the NFκB pathway via an IKK-dependent pathway. However, as it has been shown within the model of breast cancer that a pathway between the EGFR and the activation of NFκB has been defined (Singh *et al*, 2007), it would be prudent to investigate the possibility of the involvement of PI3K/Akt in order to help delinneate the pathway whereby PAR-4 mediates activation of NFκB.

Certain studies have suggested the possibility of p38 MAP kinase stimulating activation of the NFκB pathway - which may be a possible suggestion as to why PAR-4 mediates a robust phosphorylation of p38 MAP kinase. One study has demonstrated that p38 MAP kinase is capable of mediating nuclear translocation of NFκB in THP-1 monocytes (O'Sullivan *et al*, 2008). This study in particular demonstrated that the nuclear translocation of NFκB was attenuated by selective p38 MAP kinase inhibitor SB202190 in these cells. These results suggest that p38 MAP kinase may have an important role mediating nuclear translocation of NFκB (O'Sullivan *et al*, 2008). Another study by Sinke *et al*, 2008, demonstrated in astrocytes by means of NFκB-DNA binding, that iNOS mediated translocation of NFκB was successfully attenuated by another selective inhibitor for p38 MAP kinase, SB239063 (Sinke *et al*, 2008). SB239063 resulted in a significant decrease in NFκB signalling as demonstrated by Western blotting. NFκB mediated induction of iNOS ultimately results in the production of NO, which in astrocytes is thought to mediate astrocyte swelling. A significant reduction of swelling was apparent in cells pre-treated with the p38 MAP kinase inhibitor, which were comparable with the reduction of swelling demonstrated by IKK inhibitors BAY11-7082 and NFκB inhibitor SN-50. Such findings are suggestive of the ability of p38 MAP kinase to be involved in the regulation of NFκB activation (Sinke *et al*, 2008).

In other studies Sen *et al*, 2008, V79 lung fibroblasts demonstrated that over a period of four weeks an increase in NFκB translocation was apparent. This was mediated by IKK-dependent phosphorylation of IκBα. Increased phosphorylation of p65 NFκB was also apparent. Use of specific p38 MAP kinase inhibitor SB203580 resulted in the inhibition of p65 NFκB phosphorylation as well as inhibiting upstream IKK activity. The importance of p38 MAP kinase in the activation of NFκB was confirmed by using selective p38 MAP kinase siRNA, which resulted in the complete inhibition of NFκB activity. These results suggest that p38 MAP kinase is of significant importance to the NFκB response in V79 lung fibroblasts (Sen *et al*, 2008).

As the results in this thesis have demonstrated, PAR-4 mediates a strong activation of p38 MAP kinase, which was further demonstrated to lend itself to the PAR-4 mediated production of IL-8. Taken together with these aforementioned studies, which show the importance of p38 MAP kinase in the activation of NFκB, it may be useful to investigate the possibility that PAR-4 may mediate activation of NFκB via a p38 MAP kinase-dependent mechanism. Inhibition studies which assess the involvement of p38 MAP kinase in PAR-4 mediated phosphorylation of p65 NFκB, NFκB-DNA binding and NFκB transcriptional activity would merit potential investigation.

In conclusion, this study has shown that PAR-4 is able to mediate intracellular signalling via distinct mechanisms in both NCTC-2544 cells which stably express PAR-4 (clone 10H9) and EAhy-926 cells which naturally express the receptor. It has been demonstrated that while PAR-4 is capable of mediating activation of the MAP kinase pathway in both cell types, there are distinct differences between thrombin and activating peptide in the naturally expressing cells (see section 3.7). Such results may simply be due to the presence of another member of the PAR family in the EAhy-926 cells, which is also being cleaved by thrombin to mediate activation of ERK and p38 MAP kinase. However, it may be due to the limitations of using a heterologous expression system such as 10H9 to investigate the signalling characteristics.

Future studies may benefit by investigative techniques such as PCR, which can determine the expression of other PAR mRNA in EAhy-926 cells. Such steps could at least determine the differences between the thrombin mediated response and activating peptide response, therefore allowing for a better measure of accuracy by overseeing the selectivity of PAR-4 agonists. Further to activation of the MAP kinase pathway, this study has suggested that PAR-4 is also capable of mediating the activation of NFκB.

Activation of both pathways have demonstrated the involvement of both $G\alpha_{q/11}$ and transactivation of the EGFR. Nevertheless, identification of intermediate molecules between PAR-4 and its downstream effectors remains undefined. Involvement of both $G\alpha_{q/11}$ and the EGFR suggests the possibility of PAR-4 to mediate activation of these pathways via both G protein-dependent and –independent mechanisms, prompting an investigation more complex than intially anticipated. It can therefore be suggested that in regard of PAR-4 much more investigative research is required to delineate the signalling pathways associated with this receptor.

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