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The Regulation Of The Human A_1 Adenosine Receptor And The Sphingosine 1-Phosphate Receptor, EDG1

Kenneth Robert Watterson BSc.

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Institute of Biomedical and Life Sciences
University of Glasgow

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Abbreviations

AC	Adenylate cyclase
ADA	adenosine deaminase
AP	Adaptor protein
Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
AMP	adenosine 5' monophosphate
cAMP	cyclic adenosine 5' monophosphate
ASMC	aortic smooth muscle cell
ATP	adenosine 5' triphosphate
ADP	adenosine 5' diphosphate
AR	adenosine receptor
A_1AR	A_1 adenosine receptor
$A_{2A}AR$	A_{2A} adenosine receptor
$A_{2B}AR$	A_{2B} adenosine receptor
β_2AR	β_2 adrenergic receptor
βARK	β-adrenergic receptor kinase
BCA	Bicinchonic acid
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
E-Cadherin	epithelial cadherin
P-Cadherin	placental cadherin
VE-Cadherin	vascular endothelial cadherin
CCK	Cholecystokinin
CFTR	Cystic fibrosis transmembrane regulator
CHAPS	3-[(3-Cholamidopropyl)-dimethylammino]-1-propane sulfonate
CHO	Chinese hamster ovary
CNS	Central nervous system
CO_2	carbon dioxide
CPA	N^6-cyclopentyladenosine
CTx	Cholera toxin
Da	Dalton
DAG	Diacylglycerol

DHS	DL-*threo*-dihydrosphingosine
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPCPX	1,3-dipropyl-8-cyclopentylxanthine
DTT	dithiothreitol
EC_{50}	Concentration of the drug required to induce a half-maximal response
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
ECV	Endosomal carrier vesicles
EDG	Endothelial differentiation gene
EDTA	Diaminoethanetetra-acetic acid disodium salt
ER	Endoplasmic reticulum
ERK	extracellular-regulated protein kinase
ET	Endothelin
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
FRAP	Fluorescence recovery after photobleaching
FRET	Fluorescence resonance energy transfer
GAP	GTPase-activating protein
GDP	Guanosine 5' diphosphate
GDI	GDP dissociation inhibitor
GEP	Guanine nucleotide exchange protein
GFP	Green fluorescent protein
GnRHR	Gonadotrophin releasing hormone receptor
GPCR	G-protein-coupled receptor
GRK	G-protein-coupled receptor kinase
GTP	Guanosine 5' triphosphate
HA	Haemagglutinin
HBSS	HEPES buffered saline solution

HCl	Hydrochloric acid
HEK	human embryonic kidney
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanosulfonic acid
HIF-1	hypoxia inducible factor-1
HMC	Human mastocytoma
hmw	High molecular weight
HRP	horseradish peroxidase
HSP	heat shock protein
HUVEC	human umbilical vein endothelial cell
IB-MECA	N^6-(3-iodobenzyl)-5'-(N-methylcarbomoyl)adenosine
IC_{50}	Concentration of the competitor required to inhibit half the specific binding
IgG	Immunoglobulin G
IL-2	Interleukin-1
IL-8	Interleukin-2
IP_3	inositol-1,4,5-trisphosphate
JNK	c-Jun N-terminal kinase
K_d	concentration of ligand that will bind to half the receptors at equilibrium
K_i	Affinity of the receptor for the competing drug
Kan	Kanomycin
K^+_{ATP}	ATP-sensitive K^+ channel
KRHB	Krebs-Ringer-HEPES-BSA
LB	*Luria-Bertani* medium
lmw	Low molecular weight
LPA	Lysophosphatidic acid
mAchR	muscarinic acetylcholine receptor
MAP Kinase	Mitogen-activated protein kinase
MBP	Myelin basic protein
MEF	Mouse embryonic fibroblast
$MgCl_2$	Magnesium chloride
fMIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MnSOD	Mitochondrial superoxide dismutase

MVB	multivesicular body
NBCS	New born calf serum
NECA	5'-N-ethylcarboxamidoadenosine
NO	Nitric oxide
NOS	Nitric oxide synthase
eNOS	Endothelial nitric oxide synthase
iNOS	Inducible nitric oxide synthase
dNTP	deoxynucleoside triphosphate
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinase
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
PKB	Protein kinase B
PKG	Protein kinase G
PBS	Phosphate-buffered saline
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulphonylfluoride
RBL	Rat basophilic leukemia
RGS	Regulator of G-protein signalling
RIPA	Radioimmunoprecipitation buffer
R-PIA	R-N^6-(phenylisopropyl)adenosine
PTx	Pertussis toxin
RNA	Ribonucleic acid
mRNA	Message ribonucleic acid
SAH	S-adenosylhomocysteine
SAM	S-adenosyl methionine
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SE	Standard error
SH	Src homology
Shc	Src homology 2 domain containing protein
Sos	Son of sevenless
SSP/S1P	Sphingosine-1-phosphate
$t_{½}$	time required to see 50% effect
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF	Transforming growth factor
TM	Transmembrane domain
TRH	Thyrotropin-releasing hormone
TRITC	Tetramethylrhodamine isothiocyanate
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VSMC	Vascular smoooth muscle cell
WT	Wild type

Standard one and three letter amino acids codes have been used throughout

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Abstract

The adenosine A_1 receptor (A_1AR) and the sphingosine-1-phosphate (SSP/S1P) receptor, endothelial differentiation gene 1 (EDG1) are members of the large superfamily of cell surface G-protein-coupled receptors (GPCRs). A_1AR activation by the purine, adenosine results in a number of cardio- and neuroprotective effects and has been implicated in the process of ischaemic preconditioning. S1P-mediated activation of the EDG1 receptor also elicits a range of biological effects and has recently been shown to be heavily involved in the process of new blood vessel formation called angiogenesis. Following a sustained agonist exposure, many GPCRs are desensitised such that their responses plateau and then diminish. The relative ability or inability of a GPCR to undergo agonist-mediated receptor phosphorylation and subsequent internalisation away from the cell surface is an important measure of a GPCR's ability to become desensitised. This study has characterised the phosphorylation and internalisation of the human A_1AR and the human EDG1 receptor.

Whole cell receptor phosphorylation assays demonstrated that A_1ARs stably expressed in CHO cells were not phosphorylated in response to the agonist, R-PIA. In contrast, the A_3AR, which is similar in terms of structure, G-protein coupling specificity and biological effects was rapidly phosphorylated following R-PIA exposure. Additionally, cell surface biotinylation assays showed that, whereas the A_3AR was internalised rapidly following R-PIA exposure ($t_{1/2}$= 10 min), the agonist-dependent loss of A_1AR from the cell surface was much slower ($t_{1/2}$= 90 min) and less complete. Using confocal analysis, it was shown that the mutation of Cys309, a site of palmitoylation within the C-terminal, had no visible effect on the cell surface expression of A_1ARs tagged with green fluorescent protein (GFP) following a 1 hour agonist exposure. In contrast, parallel studies within the lab demonstrated that mutation of Cys302 and Cys305 within the C-terminal of the A_3AR resulted in a marked increase in basal receptor phosphorylation and an increased rate of internalisation.

Whole cell phosphorylation studies on hamster lung CCL-39 fibroblasts stably expressing human EDG1 receptors showed that EDG1 is phosphorylated in response to agonist (S1P) and also PMA, a phorbol ester that activates PKC subtypes. However, lysophosphatidic acid (LPA), a bioactive lipid similar in structure and biological effects to EDG1, had no effect on EDG1 phosphorylation. Phosphoamino acid analysis showed that S1P and PMA stimulate the accumulation of phosphoserine and phosphothreonine but not

phosphotyrosine. Preincubation with the PKC inhibitor, GF109203X abolished EDG1 phosphorylation in the presence of PMA but failed to block S1P-mediated EDG1 phosphorylation, suggesting that these processes are mechanistically distinct. Additionally, the removal of 12 amino acids from the C-terminal tail of EDG1 significantly reduced S1P- but not PMA-stimulated phosphorylation, providing further evidence of two distinct mechanisms of receptor phosphorylation. However, incubation of S1P and PMA together did not result in a significant increase in EDG1 phosphorylation when compared to that achieved by S1P treatment alone. Therefore, although PMA- and S1P-mediated EDG1 phosphorylation were distinct, each pathway utilised overlapping phosphorylation sites within EDG1. *In vitro* assays implicated a role for GRK2 in S1P-induced EDG1 phosphorylation observed in intact cells. Also, the region between the last 12 and the last 32 amino acids of the EDG1 C-terminal was shown to be responsible for the *in vitro* S1P-dependent phosphorylation of EDG1 in the presence of GRK2.

Phosphorylation of EDG1 by S1P and PMA were related to the loss of cell surface EDG1. Two separate mechanisms of EDG1 internalisation were observed: an S1P-dependent loss of cell surface EDG1 receptor and a less complete yet still significant agonist-independent, PMA-mediated loss of cell surface EDG1 which is abolished in the presence of GF109203X. Removal of the last 12 amino acids completely abolished S1P-mediated EDG1 internalisation. Interestingly, this region is not required for the *in vitro* phosphorylation of EDG1 by GRK2 in the presence of S1P, suggesting that another kinase may be involved in agonist-dependent EDG1 internalisation. Internalisation of EDG1 due to a 30 min S1P exposure was also shown to be irreversible following a 2 hour agonist removal. In contrast, whole cell receptor phosphorylation studies demonstrated that EDG1 phosphorylation is reversible. Immunoblot analysis of total EDG1 receptor expression following a 24 hour agonist treatment also demonstrated that EDG1 is not significantly downregulated. Therefore, EDG1 is phosphorylated following agonist exposure. The receptor is then internalised where it is dephosphorylated but is neither returned to the cell surface nor degraded. Alternatively, EDG1 receptor recycling may be slow and hence requires a period of agonist removal longer than 2 hours.

Chapter 1
Introduction

Adenosine was first shown to act as an extracellular signalling molecule by Drury and Szent-Gyorgyi in 1929. This study demonstrated that extracellular application of the purines, adenosine and adenosine 5'-monophosphate (AMP), extracted from heart muscle, resulted in various biological effects such as heart block, arterial dilatation, lowering of blood pressure and inhibition of intestinal contraction. Adenosine is now fully established as a ubiquitous physiological regulator and neuromodulator that exerts its actions in a number of tissues, such as the brain, heart, kidney, vasculature and adipocytes *via* a specific sub-family of G-protein-coupled receptors (GPCRs) expressed on the plasma membrane (Olah and Stiles, 1995; Linden, 2001; Ralevic and Burnstock, 2000).

In contrast to adenosine, the concept of the bioactive phospholipid, sphingosine-1-phosphate (SSP/S1P), as both an extracellular signalling molecule as well as an intracellular second messenger has only been recognised within the last decade (Cuvellier *et al.*, 1996; Lee *et al.*, 1998b; Spiegel and Milstein, 2000a). Zhang et al in 1991 were one of the first groups to demonstrate the importance of S1P in cell growth regulation. A number of studies have since shown that S1P is a potent mitogen in a number of diverse cell types and elicits various biological effects such as the mobilisation of intracellular calcium, regulation of cytoskeletal organisation and cell growth, differentiation, survival and motility (Im *et al.*, 1997; An *et al.*, 1999; Hong *et al.*, 1999; Pyne and Pyne, 2000a, 2000b). As with adenosine signalling, S1P acts as an extracellular mediator by binding to a distinct sub-family of plasma membrane GPCRs.

1.1 Adenosine Structure, Synthesis And Metabolism

Adenosine is a ribonucleoside consisting of a D-ribose sugar and the nitrogenous base, adenine (Figure 1.1) and is a constitutive metabolite of all cells, involved in key pathways such as purinergic nucleic acid base synthesis, amino acid metabolism and the modulation of cellular metabolic status (Stone, 1985). The most common stimulus for adenosine formation is ischaemia, whereby inadequate blood flow results in tissue hypoxia (Berne, 1963; Belardinelli and Shyrock, 1992; Schrader, 1990). This has been demonstrated in cardiac myocytes, which rely almost entirely upon aerobic metabolism to generate the adenosine 5' triphosphate (ATP) required for contractile activity (Mullane and Bullough, 1995) (Figure 1.2). Although changes in ATP should be the initial sensor of metabolic imbalance, ATP production is so tightly coupled to oxidative phosphorylation that only pronounced metabolic changes result in changes in intracellular ATP concentration. However, the intracellular ATP concentration is in the millimolar range whereas the intracellular concentration of AMP is in the low nanomolar range. Therefore,

AMP acts as a control signal to adapt primary metabolism to metabolic imbalance (Cunha, 2001; Fell and Sauro, 1985). Because AMP cannot cross the plasma membrane, it is not suited to rapidly signalling stressful situations, such as ischaemia, to neighbouring cells (Cunha, 2001). Hence, a substrate cycle between AMP and adenosine through the opposing effects of 5'nucleotidase and adenosine kinase regulates the formation of intracellular adenosine upon changes in intracellular AMP concentration (Cunha, 2001; Dunwiddie and Masino, 2001). Intracellular adenosine may also be formed by the action of S-adenosylhomocysteine (SAH) hydroxylase (Dunwiddie and Massino, 2001). Additionally, substantial amounts of adenosine may be formed from the breakdown of adenine nucleotides that are present in the granules of autonomic nerves, platelets and mast cells (Linden, 2001). Adenine nucleotides are rapidly converted to adenosine by a family of ecto-ATP/ADPases including CD39 (NTPDase 1) and ecto-5' nucleotidases including CD73 (Cunha, 2001; Dunwiddie and Massino, 2001; Linden, 2001; Mullane and Bullough, 1995). Extracellular adenosine then acts upon G-protein-coupled cell surface adenosine receptors (ARs) to produce specific biological effects. Excess adenosine can be degraded to inosine and finally uric acid *via* adenosine deaminase (Cunha, 2001; Mullane and Bullough, 1995). Non-concentrative bi-directional adenosine (or nucleoside) transporters equilibrate changes in the intracellular and extracellular adenosine concentrations (Cunha, 2001; Mullane and Bullough, 1995).

1.2 Sphingosine-1-Phosphate Synthesis, Structure And Metabolism

The structure of S1P consists of one long hydrocarbon chain on a three carbon backbone containing a phosphate group and is a phosphorylated derivative of sphingosine, an integral sphingolipid component present in the membranes of most mammalian cells (Spiegel, 2000b) (Figure 1.3). S1P may be biosynthesised by cells either *de novo* through pathways of intermediate lipid metabolism or *via* stimulus-coupled liberation of the respective precursor glycerophospholipids and sphingolipids and subsequent enzymatic conversions (Goetzl and An, 1998, Pyne and Pyne, 2000a; Pyne and Pyne, 2000b; Vesper, 1999. *De novo* synthesis of S1P begins with the condensation of a fatty acid-CoA and serine to form 3-ketosphinganine, which is reduced and converted to a dihydroceramide in the endoplasmic reticulum (ER) (Goetzl and An, 1998). This is then sequentially converted to ceramide, sphingosine and finally S1P (Goetzl and An, 1998; Vesper, 1999). However, the contribution from stored sphingomyelin turnover constitutes the main source of free and secreted S1P (Goetzl and An, 1998).

Sphingolipid metabolism is a dynamic process resulting in the formation of a number of bioactive metabolites including ceramide, sphingosine and S1P (Pyne and Pyne, 2000a; Pyne and Pyne, 2000b) (Figure 1.4). Sphingomyelin degradation occurs in the membranes of lysosomes and endosomes and in the plasma membrane in response to growth factors, pro-inflammatory cytokines and arachidonic acid and also following cellular stress (Pyne and Pyne, 2000a; Pyne and Pyne, 2000b; Spiegel, 1999; Spiegel and Milstien, 2000b). Following sphingomyelinase activation, sphingomyelin is hydrolysed to ceramide, thought to be involved in cell growth arrest, differentiation and apoptosis (Hannun, 1996; Kolesnick *et al.*, 1998). Ceramide is then converted to sphingosine in the presence of ceramidase. Sphingosine has been shown to inhibit protein kinase C (PKC) and induce apoptosis (Spiegel and Milstien, 2000a; Spiegel and Milstien, 2000b). Finally, sphingosine can be phosphorylated by sphingosine kinase to produce S1P, implicated in cell growth and the inhibition of ceramide-mediated apoptosis (Spiegel, 1999; Spiegel and Milstien, 2000a; Spiegel and Milstien, 2000b). Metabolism of S1P is catalysed by both a pyridoxal phosphorylation-dependent lyase located in the ER, which degrades S1P to phosphoethanolamine and palmitaldehyde, and by a phosphatase which converts S1P back to sphingosine (Goetzl and An, 1998). Therefore, the dynamic balance between the concentration of all the bioactive sphingolipid metabolites helps determine cell fate. This is commonly known as the "sphingolipid rheostat" model (Spiegel, 1999; Pyne and Pyne, 2000b) (Figure 1.4).

Following the intracellular production of S1P, S1P can be released into the extracellular space where it is present in an albumin-bound form (Hla *et al.*, 2001; Igarishi and Yatomi, 1998,). The main extracellular source of S1P within the blood is derived from platelets activated either *via* stress stimuli, phorbol esters or thrombin (Igarishi and Yatomi, 1998). Extracellular S1P can also be derived from other cell types such as mast cells and monocytes (Spiegel and Merrill, 1996; Hannun *et al.*, 2001). The accumulation of generated S1P within platelets can be attributed to the unique lack of the major S1P hydrolysing enzyme, S1P lyase in platelets (Pyne and Pyne, 2000a). However, the precise mechanisms regarding S1P release remain poorly understood. A recent study has also suggested that as well as newly released S1P, the extracellular S1P content could also be derived from by the extracellular metabolism of sphingomyelin since the biosynthetic enzymes, namely sphingomyelinase, ceramidase and sphingosine kinase have been shown to be secreted by cells (Hla *et al.*, 2001; Romiti *et al.*, 2000; Tabas, 1999). A recent study also demonstrated that the cystic fibrosis transmembrane regulator (CFTR), a member of

the ATP binding cassette family of proteins, is involved in the uptake of extracellular S1P and other related phosphorylated lipids (Boujaoude *et al.*, 2001). This uptake would therefore influence the balance between extracellular and intracellular S1P concentrations and hence, affect the ability of S1P to modulate biological activity via its interactions with cell surface GPCRs.

1.3 G-Protein-Coupled Receptors

The GPCR superfamily includes more than 1000 genes encoding receptors (Marinissen and Gutkind, 2001). This represents the largest family of transmembrane receptors responsible for the transduction of a diverse array of extracellular signals, including light, Ca^{2+}, odorants, amino acids, nucleotides, peptides, fatty acid derivatives and various polypeptide ligands (Howard *et al.*, 2001; Ulrik, 2000).

GPCRs generally exist in equilibrium between an inactive (R) and active (R^*) conformation (Milligan and Bond, 1997; Strange, 2000). Preferential agonist ligand binding to the R^* state promotes an isomerisation step that stabilises the receptor in a relaxed state and shifts the equilibrium toward the active R^* conformation, leading to the activation of intracellular heterotrimeric G proteins. The receptors can then mediate a variety of intracellular responses to regulate cellular function. There is a degree of variability amongst GPCRs concerning the equilibrium between the R and R* conformations. In the basal state of most GPCRs, the majority of receptors are in the inactive (R) state (Milligan and Bond, 1997). Therefore, true antagonist ligands with no preference for the R or R* receptor conformation are difficult to differentiate from inverse agonists, where only a small proportion of R* GPCRs can be preferentially converted to an R state by an inverse agonist. On the other hand, a substantial fraction of R* state receptors are found with other GPCRs, such as the histamine H_2 receptor, and therefore display higher levels of basal effector activity (Milligan and Bond, 1997; Smit *et al.*, 1996). Hence, it is easier to separate full inverse agonists of the H_2 receptor (cimetidine, ranitidine) and antagonists (burinamide) (Smit *et al.*, 1996). A select group of GPCRs, including the Kaposi's sarcoma-associated herpesvirus, exhibit full constitutive activity and may not require or possess an endogenous ligand (Rosenkilde *et al.*, 2001; Arvanitakis *et al.*, 1997).

GPCRs can be classified into three major subfamilies, based on their relationship to rhodopsin (class I), the calcitonin receptor (class II) and metabotropic receptors (class III) (Ulloa-Aguirre *et al.*, 1999; Ulrik, 2000). The subfamily of rhodopsin-like receptors (class I) is by far the largest and the most studied. The structure of class I GPCRs is defined by

seven transmembrane (TM) helices linked by three extracellular and three intracellular loops (Howard *et al.*, 2001; Ulloa-Aguirre *et al.*, 1999; Ulrik, 2000) (Figure 1.5). The extracellular N-terminus sequence contains N-linked glycosylation sites, thought to be involved in the trafficking of many GPCRs to the cell surface and implicated in the stabilisation of protein conformation, protection of proteins from proteases and modulation of protein function (Davidson *et al.*, 1996; Davidson *et al.*, 1995; Davis *et al.*, 1995; George *et al.*, 1986; Ulloa-Aguirre *et al.* 1999). The intracellular C-terminus usually contains phosphorylation and palmitoylation sites that are intimately involved in regulating receptor trafficking and signal transduction (Ferguson, 2001; Ulloa-Aguirre *et al.*, 1999). TMs I, IV and VII contain only one hydrophilic residue and are therefore more hydrophobic than TMs II, III, V, and VI, which contain several ionic and/or neutral residues (Ulloa-Aguirre, 1999). The overall homology among all the class I GPCRs is low and restricted to a number of highly conserved key residues. These include a single conserved cysteine residue in the first two extracellular loops, which is responsible for protein stability, and a GPCR signature triplet sequence (typically DRY) found downstream from TM3, which is involved in G-protein interaction (Howard *et al.*, 2001; Ulloa-Aguirre, 1999). From this point on, the term GPCR will refer to class I GPCRs.

1.4 G-Proteins

G-proteins are comprised of three subunits, termed α, β, and γ (Downes and Gautam, 1999; Willard and Crouch, 2000). α subunits contain two domains; a domain involved in binding and hydrolysing guanosine 5' triphosphate (GTP) that is structurally identical to the large superfamily of GTPases, and a unique helical domain that buries the GTP in the core of the protein (Hamm, 1998). The β subunit consists of a seven-membered β-propeller structure based on its seven WD-40 repeats. The γ subunit interacts with β through an N-terminal coiled coil and then all along the base of β, forming a functional unit under physiological conditions that is not dissociable except upon denaturation (Hamm, 1998).

The binding of agonist to a GPCR changes the conformation of the receptor which promotes the exchange of guanosine 5' diphosphate (GDP) for GTP on the G protein α-subunit. This allows the dissociation of α from βγ subunits (Downes and Gautam, 1999, Ham, 1998). The free α and βγ subunits are then able to interact with effector molecules to evoke cellular responses. The intrinsic GTPase activity of the α subunit hydrolyses GTP to GDP, allowing reassociation of the α and βγ subunits. The inactive G protein is

subsequently reformed and signalling is terminated (Downes and Gautam, 1999, Ham, 1998) (Figure 1.6). The family of G proteins have been subdivided into four categories based upon the α-subunit composition: $G\alpha_i$, which was originally shown to result in adenylate cyclase inhibition; $G\alpha_s$, which stimulates adenylate cyclase; $G\alpha_q$, which activates phospholipase C; and $G\alpha_{12/13}$, implicated in the activation of small G-proteins, such as Rac and Rho (Downes and Gautam, 1999; Offermans, 2001; Radhika and Dhanasekaran, 2001; Ulloa-Aguirre *et al.*, 1999). A comprehensive list of the Gα family is shown in Table 1. The βγ subunits also play a significant role in signal transduction by regulating the activity of several effectors such as the adenylyl cyclase types I, II, and IV, isoforms 1-3 of phospholipase Cβ (PLCβ) and phospholipase A_2 (PLA_2), as well as the activity of the muscarinic-gated K^+ channels (Hamm, 1998; Radhika and Dhanasekaran, 2001; Ulloa-Aguirre *et al.*, 1999)

G-proteins are also sensitive to a number of covalent modifications. Most Gα subunits undergo N-myristoylation and/or palmitoylation (Chen and Manning, 2001; Willard and Crouch, 2000). In addition, Gγ subunits are subject to prenylation (Chen and Manning, 2001). Each of these lipid modifications has been implicated in membrane targeting and to the interactions of these subunits with each other and other proteins (Chen and Manning, 2001; Hamm, 1998). Some Gα subunits and a Gγ subunit undergo phosphorylation, important in signal amplitude and duration (Chen and Manning, 2001). Also, a valuable experimental tool is the selective susceptibility to endotoxins of certain Gα subunit members. For example, $G\alpha_s$ subunits are adenosine 5' diphosphate (ADP)-ribosylated in the presence of cholera toxin, resulting in protein activation due to inhibition of its GTPase activity (Chen and Manning, 2001; Hamm, 1998; Willard and Crouch, 2000). Similarly, $G\alpha_i$ proteins undergo ADP-ribosylation in the presence of pertussis toxin (PTx) (Chen and Manning, 2001; Hamm, 1998; Willard and Crouch, 2000). This occurs at a cysteine residue close to the C-terminus and is thought to uncouple the G-protein from its receptor (Willard and Crouch, 2000).

1.5 Small G-Proteins

As mentioned above, activation of $G_{12/13}$–coupled receptors can result in the activation of small G-proteins. Small GTP-binding proteins are monomeric G-proteins with a molecular weight of 20-40kDa (Takai *et al.*, 2001). The superfamily of small G-proteins consists of more than 100 members found in eukaryote systems ranging from yeast to human and is made of at least 5 families: - Ras, Rab, Sar1/Arf and Ran (Bourne *et*

al., 1990; Hall, 1990; Takai *et al.*, 2001). The Ras family consists of 6 members and regulates gene expression (Takai *et al.*, 2001). Rho/Rac/Cdc42 regulate cytoskeletal reorganisation and gene expression (Evers *et al.*, 2000; Fukata *et al.*, 2001; Sah *et al.*, 2000; Takai *et al.*, 2001). Rab and Sar1/Arf regulate intracellular vesical trafficking (Ferguson, 2001; Takai *et al.*, 2001) and Ran regulates nucleocytoplasmic transport during G_1, S and G_2 phase (Takei *et al.*, 2001).

All small G-proteins have consensus amino acid sequences responsible for specific interaction with the GDP and GTP, for GTPase activity for the hydrolysis of bound GTP to GDP and P_i and a region for interacting with downstream effectors (Bourne *et al.*, 1991; Takai *et al.*, 1992, Takai *et al.*, 2001). Ras, Rho/Rac/Cdc42 and Rab have sequences at their COOH terminus that undergo posttranslational modifications with lipid. These include farnesyl, geranylgeranyl, palmitoyl and methyl moieties and proteolysis (Casey and Seabra, 1996; Glomset and Farnsworth, 1994; Takai *et al.*, 1992; Takai *et al.*, 2001). The small G-proteins exist in two interconvertible forms: - GDP-bound inactive and GTP-bound active (Benard *et al.*, 1999; Takai *et al.*, 2001). Following stimulation from an upstream signal, GDP dissociates from the GDP-bound form followed by the binding of GTP. A conformational change of the downstream effector-binding region leads to interaction with downstream effectors, thereby altering their function. The GTP-bound form is then converted back to the inactive GDP-bound form *via* the intrinsic GTPase activity of the small G-protein, resulting in the release of the bound downstream effectors (Benard *et al.*, 1999; Takai *et al.*, 2001).

The rate-limiting step of GDP/GTP exchange is the dissociation of GDP from the GDP-bound form (Benard *et al.*, 1999; Takai *et al.*, 2001). Regulators called guanine nucleotide exchange proteins (GEPs), which are, in turn, regulated by an upstream signal, can increase the dissociation rate. GEPs interact with the GDP-bound form and release bound GDP to form a binary complex of small G-protein and GEP. GTP then replaces GEP, resulting in the formation of the active GTP-bound form (Benard *et al.*, 1999; Takai *et al.*, 2001). Most GEPs, such as son of sevenless (Sos), a Ras GEP and Rab3GEP are specific for each member or subfamily of small G-proteins (Boguski and McCormick, 1993; Buday and Downward, 1993; Wada *et al.*, 1997). However, some GEPs, such as Dbl, a GEP active on Rho/Rac/Cdc42 proteins, exhibit wider substrate specificity (Hart *et al.*, 1991; Yaku *et al.*, 1994). GDP/GTP exchange reactions of Rho/Rac/Cdc42 and Rab are also regulated by the GDP dissociation inhibitors (GDIs), Rho GDI and Rab GDI respectively (Araki *et al.*, 1990; Fukui *et al.*, 1997; Ueda *et al.*, 1990). These molecules

inhibit the basal and GEP-stimulated dissociation of GDP from the GDP-bound form and keep the small G-protein in the inactive GDP-bound form. Rho GDI and RabGDI exhibit a wider substrate specificity than GEPs and GTPase-activating proteins (GAPs) and are active on all Rho/Rac/Cdc42 and Rabs respectively (Takai *et al.*, 2001). Hence, Rho/Rac/Cdc42 and Rab activation are susceptible to positive and negative regulators. The GTPase activity of each small G-protein is variable but relatively slow and is stimulated by GTPase-activating proteins (GAPs). Most GAPs, such as Ras GAP and Rab3 GAP, are highly specific for each member or subfamily of small G-protein (Boguski and McCormick, 1993; Fukui *et al.*, 1997). However, some GAPs, such as p190, a GAP active on Rho/Rac/Cdc42 proteins have wider substrate specificity (Settleman *et al.*, 1992) (Figure 1.7).

1.6 Adenosine Receptors

Many of the extracellular effects of adenosine are mediated *via* a GPCR subfamily of cell surface adenosine receptors. At present, four subtypes have been recognised; the A_1AR, A_{2A}AR, A_{2B}AR and A_3AR, with each subtype isolated from a number of species (Palmer and Stiles, 1995; Ralevic and Burnstock, 1998; Tucker and Linden; 1993) (Table 2). An overall amino acid identity of 87% exists between the various A_1ARs. A similar high degree of homology is observed between the different A_{2A}AR subtypes (92% between the human and dog receptors). The identity between the human and rat A_{2B}AR is around 86% whereas the A_3AR exhibits a relatively low level of identity (72% between each species), reflected in the distinct pharmacological characteristics of each type of A_3AR. There is also a distinct lack of amino acid sequence homology between the different AR subtypes. For example, the homology between the rat A_1 and rat A_3 receptors is only 45% whereas the human A_3 receptor only shows approximately 50%, 43% and 40% homology with the human A_1, A_{2A} and A_{2B} receptors, respectively. This is indicative of the fact that each subtype represents a distinct receptor (Palmer and Stiles, 1995; Ralevick and Burnstock, 1998).

The protein structure of each adenosine receptors is typical of most GPCRs. Each member consists of seven α-helical transmembrane domains of hydrophobic amino acids composed of 20-27 amino acids. The amino-terminus of the receptor is positioned toward the extracellular space whereas the C-terminus is orientated intracellularly. The transmembrane domains are connected by three extracellular and three intracellular loops of unequal size and the subsequent orientation of the transmembrane domains forms a

pocket for ligand binding. Consensus sites for N-linked glycosylation exist upon the extracellular regions of ARs although the precise location of the appropriate sites varies amongst AR subtypes. The "DRY" sequence present in the second intracellular loop of almost all GPCRs is present in all the AR subtypes and is implicated in the mediation of G-protein activation (Olah and Stiles, 2000; Ralevick and Burnstock, 1998).

A conserved cysteine residue representing a potential site of palmitoylation exists in the carboxyl-terminal tail of all AR subtypes, with the exception of the A_{2A}AR. The A_2 and A_3 receptors also have an abundance of serine and threonine residues distal to this site of palmitoylation. In contrast, the A_1AR has only one such site although other sites are located in the third intracellular loop. Phosphorylation of this region has been shown to be crucial in the regulation of the processes of desensitisation and internalisation of many GPCRs (Ferguson, 2001). The role of the C-terminal tail in GPCR regulation will be discussed extensively elsewhere.

1.7 Adenosine Receptor Subtypes

1.7.1. A_1 Receptors

A_1ARs are ubiquitously expressed in most species, and are highly expressed in the central nervous system (CNS), with the greatest expression in brain observed in cortex, cerebellum, hippocampus and thalamus (Dixon *et al.*, 1996; Ralevick and Burnstock, 1998; Reppert *et al.*, 1991; Tucker and Linden, 1993). A_1 receptor mRNA is also widely distributed in peripheral tissues, having been localised in vas deferens, testis, white adipose tissue, stomach, spleen, pituitary, adrenal, heart, aorta, liver, eye and bladder (Dixon *et al.*, 1996; Ralevick and Burnstock, 1998; Reppert *et al.*, 1991; Tucker and Linden, 1993). Low levels of expression are also found in lung, kidney and small intestine (Dixon *et al.*, 1996; Ralevick and Burnstock, 1998; Reppert *et al.*, 1991). A_1ARs mediate a variety of signalling responses primarily through its coupling to different G proteins within the $G_{i/o}$ family (Bevan *et al.*, 1991; Linden, 2001; Olah and Stiles; 1995; Ralevick and Burnstock, 1998; Waldhoer *et al.*, 1999) (Table 3). Traditionally, the A_1AR has been associated with the inhibition of adenylate cyclase, resulting in a decrease in the second messenger cAMP. This then modulates the activity of cAMP-dependent protein kinase (PKA) (Londos *et al.*, 1980; Van Calker *et al.*, 1978; Ralevic and Burnstock, 1998; Linden, 2001). In addition, the inhibition of adenylate cyclase *via* A_1ARs can attenuate the effect of other receptors, such as the β_2 adrenergic receptor (β_2AR), that result in an increase in adenylate cyclase activity (McIntyre Jr. *et al.*, 1994; Perlini *et al.*, 1998; Snyder *et al.*, 1998; Song and

Belardinelli, 1996). A_1AR activation has also been shown, in different cell types, to increase, decrease or have no effect on inositol phosphate accumulation, a measure of PLC activity (Dickenson and Hill, 1998; Linden, 2001; Selbie and Hill, 1998; Tomura *et al.*, 1997). This is because G_i/G_0 derived $\beta\gamma$ subunits are involved in the A_1AR-mediated potentiation of G_q-coupled receptor stimulated PLC responses elicited, for example, by bradykinin, ATP and also *via* activation of $A_{2B}ARs$ (Dickenson and Hill, 1998; Linden, 2001; Selbie and Hill, 1998; Tomura *et al.*, 1997). PLC activation increases inositol phospholipid hydrolysis, leading to the formation of inositol-1,4,5- trisphosphate (IP_3) and diacylglycerol (DAG), and calcium mobilisation via interactions with specific receptors located on the endoplasmic reticulum. Elevation of cytosolic Ca^{2+} by IP_3 stimulates a variety of signalling pathways, including PKC, phospholipase A_2 (PLA_2), Ca^{2+}-dependent K^+ channels and nitric oxide synthase (NOS) (Linden, 2001; Ralevic and Burnstock, 1998).

A_1AR stimulation can also activate ATP-sensitive K^+ channels (K_{ATP} channels) that are regulated by metabolic demand and become closed when intracellular ATP levels are high (Baxter and Yellon, 1999; Bryon and Marshall, 1999; Heurtaux *et al.*, 1995). K_{ATP} channels mediate a reduction in action potential duration, vasodilatation and an increase in blood flow (Baxter and Yellon, 1999; Bryon and Marshall, 1999; Heurtaux *et al.*, 1995). Subsequently, activation of these receptors has been shown to be involved in protective mechanisms against ischaemia, specifically ischaemic preconditioning. This is defined as a protective, acute adaptation to brief periods of ischaemia (Carr *et al.*, 1997; Ferninandy *et al.*, 1998; Heurteaux, 1995; Rubino and Yellon, 2000; Sumeray and Yellon, 1997). The coupling of A_1ARs with K_{ATP} channels occurs through the G-protein in a membrane-delimited manner, although coupling via cytosolic factors is possible since A_1ARs, K_{ATP} channels and PKC are intrinsically linked with ischaemic preconditioning (Carr *et al.*, 1997; Ferninandy *et al.*, 1998; Heurteaux, 1995; Rubino and Yellon, 2000; Sumeray and Yellon, 1997).

It has been discovered that activation of a protein tyrosine kinase by ligands of GPCRs might be mediated by $\beta\gamma$ subunits of G-proteins. This represents a novel system whereby signal transduction via GPCRs can be linked to MAP kinase activation. Mitogen-activated protein (MAP) kinase activation requires the interaction of SH2-containing proteins with phospho-tyrosine residues present in receptors with tyrosine kinase activity or in receptors associated with src-related tyrosine kinases. Recently, it has been

discovered that A_1ARs may undergo tyrosine phosphorylation and that the physiological role of this may be related to signalling via SH2-containing proteins.

1.7.2. A_{2A} Receptors

A_{2A}ARs are highly expressed in intermediate spiney neurons of the striatum (Linden, 2001; Ongini and Fredholm, 1996). Expression of A_{2A}ARs is also found in neutrophils, monocytes, macrophages, mast cells, platelets and vascular smooth muscle and endothelium (Linden, 2001; Ralevic and Burnstock, 1998). A_{2A}AR activation commonly results in adenylate cyclase activation via coupling with the G_s G protein (Linden, 2001; Palmer and Stiles, 1995; Ralevic and Burnstock, 1998) (Table 3). However, receptors in the striatum may interact predominantly with G_{olf} as G_{olf} is more abundant in the striatum than G_s (Herve *et al.*, 1993). In striatal nerve terminals, A_{2A}ARs mediate dual signalling via P- and N-type Ca^{2+} channels linked to G_s/ adenylate cyclase/PKA and cholera toxin-insensitive G protein/PKC respectively (Gubitz *et al.*, 1996) (Table 3). Functionally, the activation of A_{2A}ARs opposes the action of D2 dopamine receptors in spiney neurons and antagonists of A_{2A}ARs are being investigated for possible use in Parkinson's disease (Aoyama *et al.*, 2000; Dunwiddie and Masino, 2001; Linden, 2001). In addition, A_{2A}AR activation of produces profound anti-inflammatory responses (Sullivan and Linden, 1998) and also results in vasodilatation of coronary arteries and other blood vessels (Akatsuka *et al.*, 1994).

1.7.3. A_{2B} Receptors

Functional studies have found A_{2B}ARs in airway smooth muscle, fibroblasts, glial cells, the gastrointestinal tract and the vasculature (Ralevic and Burnstock, 1998). In addition, A_{2B}ARs have been shown to activate human mast cells, implying a possible role in allergic and inflammatory disorders, such as asthma (Meade *et al.*, 2001). The A_{2B}AR receptor signals through Gs to activate adenylate cyclase (Linden, 2001; Ralevic and Burnstock, 1998). Recent studies have also indicated that A_{2B}ARs can couple to G_q/G_{11} to produce a PLC and IP_3-dependent increase in $[Ca^{2+}]_i$ and MAPK activation (Feoktistov and Biaggioni, 1995, Meade *et al.*, 2001). Ca^{2+} mobilisation is not limited to cells that overexpress A_{2B} receptors because the endogenous A_{2B} receptors of human embryonic kidney-293 (HEK-293) cells produce a robust A_{2B}-mediated Ca^{2+} mobilisation (Gao *et al.*, 1999). The PKA inhibitor, H89, blocks forskolin but not NECA-stimulated MAPK activation in HEK cells, suggesting that the Gq pathway contributes to MAPK activation through a pathway including MEK and Ras (Gao *et al.*, 1999). A_{2B} receptors on vascular

endothelial cells may contribute to a nitric oxide (NO)-dependent component of vasodilation mediated by Ca^{2+}-dependent NOS activation (Linden, 2001) (Table 3).

1.7.4. A_3 Receptors

The A_3AR is widely distributed, with the highest levels of human A_3AR mRNA found in lung and liver and lower levels in aorta and brain (Olah and Stiles, 1995; Ralevik and Burnstock, 1998). The A_3AR is also found on mast cells where it facilitates the release of allergic mediators including histamine, suggesting a role in inflammation (Meade *et al.*, 2001; Ralevik and Burnstock, 1998). Additionally, A_3AR activation has been shown to be involved in ischaemic preconditioning (Carr *et al.*, 1997; Liang and Jacobson, 1998; Thourani *et al.*, 1999). As with the A_1AR, the A_3AR signals primarily through a G_i G protein to inhibit adenylate cyclase activity (Zhou *et al.*, 1992). In rat basophilic leukemia cells (RBL-2H3, a cultured mast cell line) and in rat brain, the A_3AR stimulates PLC and elevates IP_3 levels and intracellular Ca^{2+} (Ralevic and Burnstock, 1998). A_3AR activation can also result in protein kinase B (PKB) phosphorylation, mediating the protection of RBL-2H3 mast cells from apoptosis. (Table 3).

1.8 Physiological Effects Of A_1AR

As described previously, the A_1AR is distributed in a wide variety of mammalian tissues. Therefore, A_1AR activation mediates a diverse set of biological effects. However, the main roles of the A_1AR are as a cardioprotective and neuroprotective agent, with evidence also implicating a role in asthma.

1.8.1. Cardioprotective Effects

Several studies have demonstrated that, under conditions of ischaemia, adenosine is released to act as a cardioprotective agent (Auchampach and Boli, 1999; Fraser *et al.*, 1999; Lasley and Smart, 2001; Marwick, 1997, McIntyre *et al.*, 1994). Many of the adenosine-induced cardioprotective effects have been attributed to activation of the A_1AR. Decreased heart rate was attributed to the activation of A_1ARs located on the sinoatrial and atrioventricular nodes, resulting in bradycardia and heart block respectively (Olsson and Pearson, 1990). This aspect of A_1AR signalling is used clinically to terminate supraventricular tachycardias.

Adenosine has been shown to be an important mediator of the endogenous defence against ischaemia-induced injury in the heart. A well-established myocardial action of adenosine is the reduction of harmful metabolic and contractile responses induced by β-adrenergic stimulation of cAMP signalling during periods of ischaemia (Auchampach and

Bolli, 1999; McIntyre Jr. *et al.*, 1994; Perlini *et al.*, 1998; Song and Belardinelli, 1996). In recent years, A_1AR, as well as A_3AR, stimulation has been shown to mediate ischaemic preconditioning, defined as a protective, acute adaptation to brief periods of ischaemia (Carr *et al.*, 1997; Ferninandy *et al.*, 1998; Heurteaux, 1995; Liang and Jacobson, 1998; Rubino and Yellon, 2000; Sumeray and Yellon, 1997; Thourani *et al.*, 1999) (Figure 8). This is where a brief period of sublethal ischaemia, consisting of either a single five minute period or a cycle of two or more 5 minute periods, is then followed by reflow, rendering the heart resistant to infarction from a subsequent, more sustained period of ischaemia (Rubino and Yellon, 2000; Sumeray and Yellon, 1997). As a result, there is a reduction in arrhythmias and cardiac cell death from the prolonged, potentially injurious period of ischaemia. The phenomenon is biphasic. The first phase is termed classical preconditioning and occurs within a few minutes of the initial preconditioning ischaemia. If the period of time between preconditioning ischaemia is extended beyond 120 minutes, no protection is observed. Delayed preconditioning, also referred to as the second window of protection, is where the preconditioning effect is observed 24 hours after the initial period of ischaemia and is thought to be associated with the induction of cytoprotective proteins, such as heat shock protein and endogenous anti-oxidants (Rubino and Yellon, 2000; Sumeray and Yellon, 1997).

A_1AR activation has a significant role in both forms of ischaemic preconditioning, alongside other mediators such as NO, free radicals and bradykinin. As outlined previously, A_1AR-linked effector mechanisms include the stimulation of potassium conductance, inhibition of calcium conductance, activation of PKC, activation of MAP kinase and the activation of ATP-dependent potassium channels. All of these processes have been strongly implicated in ischaemic preconditioning (Carr *et al.*, 1997; Ferninandy *et al.*, 1998; Heurteaux, 1995; Liang and Jacobson, 1998; Rubino and Yellon, 2000; Sumeray and Yellon, 1997; Thourani *et al.*, 1999). For example, there is evidence that the binding of agonist to A_1ARs leads to translocation of PKC subtypes to the nucleus and that this results in alterations in gene regulatory processes. This results in the altered expression of cytoprotective proteins important in mediating delayed preconditioning. Studies with isolated human atrial trabeculae have provided evidence that K^+_{ATP} channels are involved in mediating human ischaemic preconditioning *via* activation of PKC. Recent research has also shown that ischaemia activates p38 MAP kinase but not the extracellular-regulated protein kinase (ERK) cascade. p38 was also shown to be activated after exposure to adenosine. In addition, the late phase of preconditioning in response to A_1AR

receptor activation in the rabbit heart appears to be mediated in part by the induction of manganese superoxide dismutase (Figure 1.8).

1.8.2. Neuroprotective Effects

A_1ARs play an important role in signalling within the CNS. For example, A_1AR activation results in sleep induction, antinociception and the mediation of ethanol-induced motor incoordination (Dunwiddie and Masino, 2001; Nikodijevic *et al.*, 1991; Porkka-Heiskanen *et al.*, 1999; Ralevic and Burnstock, 1998). It is also well established that endogenous adenosine released by hypoxia, ischaemia, electrical activity or hypoglycaemia acts as a neuroprotective agent *via* stimulation of A_1ARs located pre and postsynaptically on cell bodies and on axons (Dunwiddie and Masino, 2001; Mitchell *et al.*, 1995; Ralevic and Burnstock, 1998). In contrast, exposure to A_1AR antagonists under these conditions exacerbates neuronal damage (Arvin *et al.*, 1989; Mitchell *et al.*, 1995). A_1AR-mediated neuroprotection occurs via a number of mechanisms, including the inhibition of neurotransmission by decreasing the release of excitatory transmitters such as glutamate, hyperpolarizing neuronal membranes, reducing excitability and firing rate, directly inhibiting Ca^{2+} channels and altering axonal transmission (Dunwiddie and Masino, 2001; Ralevic and Burnstock, 1998; Santos *et al.*, 2000. These actions reduce excitotoxicity by limiting Ca^{2+} entry and by reducing metabolic demand, which would preserve ATP stores that are essential for pumping Ca^{2+} out of the cell. The tissue expression of A_1ARs may also be a limiting factor for acute protection. For example, an allosteric enhancer of A_1AR binding offered neuroprotection in neonates (Halles *et al.*, 1997). Similarly, A_1AR receptor expression in monocytes and macrophages in the brain was shown to be reduced in patients with multiple sclerosis (Johnston *et al.*, 2001)

A_1AR activation also mediates ischaemic preconditioning in neuronal tissue, as well as cardiac tissue. In the brain, adenosine release, A_1 receptor activation and the opening of K^+_{ATP} channels play a central role in preconditioning (Heurteaux *et al.*, 1995). It has recently been demonstrated that cross-tolerance exists between potentially damaging stimuli and many of these interactions involve adenosine receptors. For example, a sublethal kainate seizure protects against a subsequent ischaemic episode and vice versa (Plamondon *et al.*, 1999). This suggests that similar molecular mechanisms may be utilised in both responses.

1.8.3. Role Of A_1AR Activation In Asthma

Aeorosolized adenosine can cause mast-cell-dependent bronchoconstriction in asthmatic subjects but bronchodilatation in nonasthmatics (Cushley *et al.*, 1983; Meade *et*

al., 2001; Nyce, 1999). In addition, the nonselective adenosine receptor antagonist, theophylline, is widely used as an antiasthmatic drug, although its precise mechanism remains unknown (Linden, 2001). The A_1AR represents a desirable therapeutic target for the treatment of asthma for several reasons. Firstly, evidence suggests that asthma may result in a disease-associated upregulation of A_1ARs, with A_1ARs found overexpressed in allergic rabbits and rats (Ali *et al.*, 1994; El-Hashim *et al.*, 1996). Secondly, bronchial smooth muscle of human asthmatics was also shown to contract in an A_1-dependent manner (Nyce, 1999). Thirdly, the A_1AR is rapidly upregulated in bronchial smooth muscle tissue exposed to human asthmatic serum (Nyce, 1999). Finally, selective inhibition of the synthesis of A_1AR with antisense oligonucleotides demonstrated a marked reduction in the number of A_1ARs in the lung and attenuation of airway constriction to adenosine, histamine and dustmite allergen (Nyce, 1999). Recent evidence, however, shows that degranulation of rat RBL 2H3 mast-like cells and perivascular mast cells of the hamster cheek pouch is mediated by activation of the A_3AR (Ramkumer *et al.*, 1993). In contrast, the A_{2B}AR has been implicated as the receptor subtype that facilitates the release of allergic mediators from canine BR and human HMC-1 mastocytoma cells (Auchampach *et al.*, 1997; Feoktistov and Biaggioni, 1995). Occupation of the A_{2A}AR generally resulted in either no bronchospasm or relaxation (Linden, 2001; Meade *et al.*, 2001). Therefore, a precise role for the A_1AR in the treatment of asthma remains to be determined. Recent evidence suggests that at least two different mechanisms of bronchospasm exist. One, involving the A_1AR, functions in mast cell depleted animals and involves a direct interaction with nerve or muscle. The second mechanism involves mast cells stimulated via activation of A_{2B}ARs or A_3ARs (Meade *et al.*, 2001).

1.9 S1P Receptors

Over the past decade, a degree of confusion has arisen over the intracellular and extracellular actions of S1P. While the intracellular targets of S1P have yet to be elucidated, intracellular S1P has been implicated in the mobilisation of intracellular calcium independently of IP_3, activation of ERK, inhibition of stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) and suppression of apoptosis (Spiegel and Milstien, 2000). However, the discovery in 1998 that S1P acts on cell surface G-protein-coupled receptors showed that extracellular S1P mediates a number of biological effects, including some effects previously attributed to intracellular S1P (Lee *et al.*, 1998).

The extracellular effects of S1P are due to its binding to specific members of the Endothelial Differentiation Gene (EDG) family of GPCRs. The EDG receptors are a sub-

family of GPCRs consisting of 8 members, EDG1-8. However, only EDG1, 3,5,6 and 8 are selective S1P receptors (Hla *et al.*, 2001; Pyne and Pyne, 2000a, Pyne and Pyne, 2000b). EDG2, 4 and 7 are selective for lysophosphatidic acid (LPA), a bioactive phospholipid with similar biological effects and structure to S1P (Contos *et al.*, 2000; Fukushima *et al.*, 2001). The EDG family of proteins can be subdivided, according to amino acid sequence similarity, into three groups: (1) EDG1, 3, 5 and 8 (around 50% identical); (2) EDG2, 4, and 7 (around 55% identical); and (3) EDG6 which is about 35-42% identical to the other EDG proteins (Lynch and Im, 1999). The EDG2, 4, 7 group is about 35% identical to the EDG1, 3, 5, 8 group. In addition, the EDG2, 4, 7 group each contain an intron in the region of the gene encoding the 6^{th} transmembrane domain (TM6) which is not present in the EDG1, 3, 5, 8 group (Contos and Chun, 1998). The EDG proteins also share partial homology with the cannabinoid receptor subfamily (<30%), indicative of the EDG proteins being lipid-selective receptors and also suggestive of a possible common ancestral gene (Lynch and Im, 1999).

As described for the adenosine receptors, the EDG proteins are integral membrane proteins that are glycosylated and are predicted to have seven transmembrane-spanning domains. However, each EDG receptor possesses distinguishing structural elements that have yet to be fully related to any aspects of ligand binding or signalling. For example, the substitution of alanine for proline in the usual seventh transmembrane NPXXY sequence of EDG4, which is conserved in the other EDG proteins and most GPCRs (Goetzl and An, 1998). In addition, a recent study has shown that the basic amino acids within EDG1, Arg^{120} and Arg^{292}, ion pairs with the phosphate of S1P (Parrill *et al.*, 2000). Also, the S1P receptors, EDG1, 3, 5, 6, and 8 all share an anionic residue corresponding to the Glu^{121} residue defined in EDG1 to interact with the ammonium moiety of S1P (Parrill *et al.*, 2000). In contrast, the LPA-specific receptors, EDG2, 4 and 7 have a neutral glutamine residue at this position which could interact with the neutral hydroxyl group in LPA (Parrill *et al.*, 2000). The C-terminal of EDG3 is unique amongst the S1P-specific group of EDG receptors in that it contains a putative class I SH3 interaction motif (RASPIQP), important in tyrosine kinase signalling. Also, the last three amino acids of the EDG5 C-terminal (TVV) represent a consensus PDZ domain interaction motif.

1.9.1. The EDG1 Receptor

EDG1 was the first S1P receptor to be cloned and was originally identified as an early immediate gene product induced in phorbol ester-differentiated human umbilical vein endothelial cells (HUVECs) (Hla and Maciag, 1990). It is expressed in most mammalian

tissues with the highest expression found in skeletal structures undergoing ossification, in endothelial cells and in the Purkinje cell layer of the cerebellum (Fukushima *et al.*, 2001; Spiegel and Milstien, 2000). The EDG1 receptor was also the first EDG receptor shown to be specific for S1P, therefore providing the impetus for the recent surge of interest in S1P as an extracellular mediator (Lee *et al.*, 1998; Okamoto *et al.*, 1998; Zondag *et al.*, 1998). EDG1 was also reported to act as a low-affinity receptor for LPA to induce EDG1 phosphorylation (Lee *et al.*, 1998). However, a separate study using membranes of Sf9 cells co-expressing EDG1 and G_{i2} failed to elicit any biological effects (Windh *et al.*, 1999). Other studies have not observed competition of [^{32}P]S1P binding by LPA (Lee *et al.*, 1998; Van Brocklyn *et al.*, 1999). Additionally, LPA did not function as an agonist for the murine analog of EDG1, lp_{B1}, when transfected into RH7777 cells (Zhang *et al.*, 1999).

EDG1 signalling is involved in cell migration, the formation of new blood vessels and vascular maturation (Hla *et al.*, 2001, Pyne and Pyne, 2000a, Pyne and Pyne, 2000b, Spiegel and Milstien, 2000) (Table 4). A recent study using EDG1-expressing Sf9 cells demonstrated that EDG1 activation by S1P results in the activation of a variety of G-protein family members, including G_{i1}, G_{i2}, G_{i3}, G_o and G_z but not G_s, G_q, G_{12} or G_{13} (Windh *et al.*, 1999). EDG1 signalling *via* a $G_{i/0}$-coupled mechanism has been demonstrated in a number of cell types, such as transfected Chineses hamster ovary (CHO), HEL, Cos-7 and Sf9 cells, and often results in extracellular signal-regulated kinase (ERK) activation and the inhibition of adenylyl cyclase activity (Okamoto *et al.*, 1998; Pyne and Pyne, 2000b; Zondag *et al.*, 1998). EDG1 activation by S1P activates phosphoinositide 3-kinase (PI3K) via the heterotrimeric G_i protein, leading to the activation of the serine/threonine kinase Akt and phosphorylation of the Akt substrate, endothelial nitric oxide synthase (eNOS), shown to be involved in endothelial cell chemotaxis (Lee *et al.*, 2001; Morales-Ruiz *et al.*, 2001; Igaraishi and Michel, 2000; Igaraishi and Michel, 2001).

EDG1 activation also regulates the activation state of small GTPases of the Rho family, namely Rac and Rho, which are downstream of the heterotrimeric G-proteins and are involved in the regulation of cytoskeletal rearrangements (Lee *et al.*, 2001; Paik *et al.*, 2001; Hobson *et al.*, 2001). It has recently been shown that the EDG1-induced G_i – and PI3K- dependent activation of Akt leads to the phosphorylation of EDG1 at Thr^{236} located within the third intracellular loop (Lee *et al.*, 2001). This activates Rac *via* an unknown mechanism and the subsequent signalling pathways required for cortical actin assembly, lamellopodia formation and chemotaxis (Lee *et al.*, 2001). In addition, HEK293 cells

transfected with EDG1 have also been shown to stimulate PTx-insensitive, $G_{12/13}$-mediated Rho pathways that regulate morphogenesis, such as adherens junction assembly and induction of placental (P)- and epithelial (E)- cadherin expression (Lee *et al.*, 1998, Lee *et al.*, 1999; Liu *et al.*, 2000). In contrast, it has recently been observed that EDG3 and EDG5, but not EDG1, mediate an increase in the amount of GTP-bound Rho in CHO cells (Takuwa *et al.*, 2001). Therefore, it is possible that, since EDG1 receptor cannot couple to $G_{12/13}$, the EDG1 receptor-dependent activation of Rho must be through a different mechanism which has yet to be defined and which may also be dependent upon cell type. A recent study showed that EDG1, along with EDG3, regulates signalling pathways required for HUVEC morphogenesis into capillary-like networks (Lee *et al.*, 1999). Therefore, one possible mechanism of EDG1 activation of Rho could therefore be through a cross-talk mechanism with EDG3. Interestingly, EDG1 has recently been shown to be involved in a cross-talk mechanism with the platelet-derived growth factor (PDGF) receptor (Hobson *et al.*, 2001; Rosenfeldt *et al.*, 2001). The concept of EDG1 cross-talk will be discussed elsewhere in the physiological context of angiogenesis.

1.9.2 The EDG3 and EDG5 Receptors

Both the EDG3 and the EDG5 receptors are widely expressed, with the EDG3 receptor being primarily expressed in the heart, lung, kidney and brain whereas the EDG5 receptor is abundant in the heart and lung but less so in the brain of the adult rat and mouse (Fukushima *et al.*, 2001; Pyne and Pyne, 2000a; Pyne and Pyne, 2000b; Spiegel and Milstien, 2000; Takuwa *et al.*, 2001). However, the EDG5 receptor is more prominent in the brain during embryonic development, suggesting a role for EDG5-mediated signalling in neuronal development (Fukushima *et al.*, 2001; MacLennan *et al.*, 1994). The EDG3 and EDG5 receptors couple to the G_i, G_q, G_{12} and G_{13} heterotrimeric G-proteins (An *et al*, 1998; An *et al*, 1999, Ancellin and Hla, 1999). Consequently, it has been demonstrated in CHO cells, HEL cells, Jurkat T cells and HTC4 hepatoma cells that EDG3 and EDG5 are coupled to the stimulation of phospholipase C and Ca^{2+} mobilisation *via* both PTx-sensitive and PTx-insensitive G proteins, most likely G_i and $G_{q/11}$ respectively (An *et al.*, 1999; Gonda *et al*, 1999; Kon *et al*, 1999; Okamoto *et al.*, 1999). EDG3 and EDG5 also mediate MAPK activation almost exclusively via G_i in CHO cells (Takuwa *et al*, 2001). EDG5 was observed to activate JNK and p38MAPK in a PTx-insensitive manner (Gonda *et al.*, 1999). A recent study demonstrated that EDG5 activation resulted in an increase in adenylate cyclase activity in CHO cells (Kon *et al.*, 1999). However, direct coupling of

EDG5 to Gs was not observed in membranes of Sf9 cells (Windh *et al.*, 1999). It remains to be determined whether or not EDG5 is directly coupled *via* Gs to adenylate cyclase.

EDG3 and EDG5 also regulate the activity of small GTPases. Both EDG3 and EDG5 have been shown to activate Rho through a $G_{12/13}$-dependent mechanism, resulting in stress fibre formation, cell rounding, neurite retraction and serum response element-driven transcriptional activation (Buhl *et al.*, 1995; Kozasa *et al.*, 1998; Pyne and Pyne, 2000a; Pyne and Pyne, 2000b; Takuwa *et al.*, 2001). Interestingly, a recent study using transfected CHO cells has shown that, whereas EDG1 and EDG3 result in a PI3K-dependent activation of Rac, EDG5 inhibited Rac activation and subsequently membrane ruffling and cell migration (Okamoto *et al.*, 2000). The physiological significance of this observation is illustrated by the fact that EDG5 is expressed in cells in which S1P is an inhibitor of cell migration, such as melanoma cells and vascular smooth muscle cells (Okamoto *et al.*, 2000) (Table 4).

1.9.3. The EDG6 And EDG8 Receptors

The EDG6 and EDG8 receptors represent the most recently identified and therefore the most poorly characterised S1P receptors. The EDG6 receptor exhibits the most restricted expression pattern of all the S1P receptors, being expressed primarily in lymphoid and haematopoeitic tissues as well as the lung (Fukushima *et al.*, 2001; Takuwa *et al.*, 2001). The EDG8 receptor is expressed in a variety of tissue types but is highly expressed in the brain, specifically in white matter, and in the spleen (Fukushima *et al.*, 2001; Im *et al.*, 2000; Im *et al.* 2001; Takuwa *et al.*, 2001). The EDG6 receptor has been shown to mediate S1P-induced PLC activation, intracellular Ca^{2+} mobilisation and MAPK activation, all of which are blocked by PTx treatment (Fukushima *et al.*, 2001). EDG8 has been shown to couple to $G_{i/0}$ and G_{12} but not G_s or $G_{q/11}$ (Im *et al.*, 2000; Malek *et al.*, 2001). In a recent study using CHO cells transfected with EDG8, S1P treatment resulted in a PTx-sensitive inhibition of forskolin-induced cAMP accumulation and a PTx-insensitive activation of JNK and inhibition of serum-induced activation of ERK1/2 (Malek *et al.*, 2001). The inhibitory effect of S1P on ERK1/2 activity was abolished by treatment with orthovanadate, suggesting the involvement of a tyrosine phosphatase (Malek *et al.*, 2001) (Table 4).

1.10. The LPA Receptors

The remaining members of the EDG family of GPCRs, namely EDG2, 4, and 7, are activated by LPA. EDG2 is expressed widespread outside the nervous system and is expressed prominently in testis and intestine (Contos *et al.*, 2000; Fukushima *et al.*, 2001;

Pyne and Pyne, 2000b; Takuwa *et al.*, 2001). EDG2 is also prevalent in the myelinating cells of the adult nervous system where LPA promotes the G_i-mediated PI3K/Akt-dependent survival of myelinated Schwann cells from the peripheral nervous system (Weiner and Chun, 1999). In addition, EDG2 is also expressed in several cancers, suggesting a pathological role for receptor-mediated LPA signalling (Furui *et al.*, 1999). EDG2 couples to $G_{i/o}$, which leads to cell proliferation (Fukushima *et al.*, 2001, Hla *et al.*, 2001). In addition, EDG2 is also coupled to the Rho pathway, leading to actin rearrangement and cell proliferation (Fukushima *et al.*, 2001, Hla *et al.*, 2001; Weiner and Chun, 1999). EDG4 is a high-affinity LPA receptor that activates the G_q pathway (Contos *et al.*, 2000; Fukushima *et al.*, 2001). It is constitutively expressed in $CD4^+$ T cells and inhibits the secretion of interleukin-2 (IL-2) (Hla *et al.*, 2001). EDG4 couples to G_i and G_q, which mediates LPA-induced PLC activation and leads to intracellular Ca^{2+} increases and inositol phosphate production (Contos *et al.*, 2000; Fukushima *et al.*, 2001; Hla *et al.*, 2001). EDG4 receptor expression is strongly induced in ovarian cancer cell lines where it regulates the transcription of immediate-early genes and cellular proliferation (Goetzl *et al.*, 1999). EDG7 is abundantly expressed in testis, heart and frontal regions of the cerebral cortex (Contos *et al.*, 2000; Fukushima *et al.*, 2001). Studies of EDG7 function within mammalian and insect cell lines have demonstrated an LPA-dependent, PTx-insensitive increase in PLC activity and intracellular Ca^{2+} concentration, suggesting that EDG7 is coupled primarily to G_q (Fukushima *et al.*, 2001) (Table 4).

1.11 The Role Of S1P-Dependent Activation Of EDG1 In Angiogenesis

One of the most important biological roles of EDG1 is in the process of angiogenesis, defined as the formation of new blood vessels from pre-existing ones (Figure 1.9). This process constitutes an integral component of many physiological events, such as embryonic development, wound healing and the menstrual cycle, each of which are defined by a requirement for new vessel formation to simultaneously supply oxygen and nutrients and remove waste products (Cross and Claesson-Welsh, 2001; Griffioen and Molema, 2000; Richard *et al.*, 2001). Angiogenesis is also critically important in a number of pathological conditions associated with blood vessel formation. For example, excessive angiogenesis has been linked with solid and haematologic tumour progression, chronic inflammation present in rheumatoid arthritis and Crohn's disease, endometriosis, and diabetic retinopathy (Carmeliet and Jain, 2000; Cross and Claesson-Welsh, 2001; Griffioen and Molema, 2000; Richard *et al.*, 2001). The process of angiogenesis involves a number of steps; 1) initiation; 2) endothelial cell migration and proliferation 3) differentiation and

4) maturation of the neovasculature (Cross and Claesson-Welsh, 2001; Griffioen and Molema, 2000) (Figure 1.9). These steps are regulated by a number a factors, with the control of each factor representing a clinically important challenge (Carmeliet and Jain, 2000; Fan *et al.*, 1995; Griffioen and Molema, 2000). Recent studies have suggested that the regulation of S1P-dependent activation of EDG1 represents such a challenge (Hobson *et al.*, 2001; Kimura *et al.*, 2000; Lee *et al.*, 1999; Lee *et al.*, 2001; Liu *et al.*, 2000; Rosenfeldt *et al.*, 2001).

1.11.1. Initiation of Angiogenesis

Conditions of hypoxia or ischaemia provide the most common initiation stimulus for angiogenesis to occur (Carmeliet and Jain, 2000; Griffioen and Molema, 2000). This usually results in a NO-mediated relaxation of the vascular muscle and, in turn, causes morphological changes of the endothelial cells that facilitate the binding of mitogens (Griffioen and Molema, 2000). Vascular endothelial growth factor (VEGF), an important mediator of angiogenic initiation, is known to act on VEGF receptors (VEGFRs) to induce vasodilatation via NO production and increased endothelial cell permeability, allowing plasma proteins to enter the tissue and form a fibrin-rich provisional network (Cross and Claesson-Welsh, 2001; Dvorak, 1986; Griffioen and Molema, 2000; Ziche *et al.*, 1997). In hypoxic tissues, the hypoxia inducible factor-1 (HIF-1) has a central role in inducing the transcription of genes that are involved in glycolysis and angiogenesis, especially VEGF (Cross and Claesson-Welsh, 2001; Griffioen and Molema, 2000; Ryan *et al.*, 2000; Saaristo *et al.*, 2000). To date, there are three known VEGF tyrosine kinase receptors; VEGFR-1 (Flt-1), VEGFR-2 (KDR or Flk-1) and VEGFR-3 (Flt-4) (Cross and Claesson-Welsh, 2001). VEGFR-1 and VEGFR-2 are expressed mainly in the vascular endothelium whereas VEGFR-3 is mostly restricted to the lymphatic endothelium (Cross and Claesson-Welsh, 2001). Endothelial cell penetration into new areas of the body is then achieved by degradation of the basal membrane by matrix metalloproteinases (MMPs) (Cross and Claesson-Welsh, 2001; Griffioen and Molema, 2000). Recent studies have also demonstrated that S1P activation of EDG1 results in an Akt-dependent phosphorylation of eNOS (Igaraishi and Michel, 2001; Kwon *et al.*, 2001; Lee *et al.*, 2001; Morales-Ruiz *et al.*, 2001). This suggests that EDG1 activation may affect vasodilatation in conjunction with VEGF and would therefore implicate EDG1 activation in the initiation of angiogenesis.

1.11.2. Endothelial cell migration and proliferation

Directional endothelial cell motility is driven by a number of chemoattractants that bind GPCRs (interleukin-8 and fMIP) or growth factors, such as VEGF and fibroblast growth factor (FGF) (Cross and Claesson-Welsh, 2001; Griffioen and Molema, 2000; Richard *et al*, 2001). EDG1 has been shown in a number of studies to regulate cell migration (Hobson *et al.*, 2001; Kimura *et al.*, 2000; Lee *et al.*, 2000; Lee *et al.*, 2001; Morales-Ruiz *et al.*, 2001; Rosenfeldt *et al.*, 2001). For example, low concentrations of S1P increased migration of endothelial cells and EDG1-expressing HEK293 cells whereas high S1P concentrations inhibited cell migration (Wang *et al.*, 1999). A study using CHO cells expressing either EDG1, EDG3 or EDG5 showed that CHO/EDG5 cells did not migrate whereas CHO/EDG1 and CHO/EDG3 cells migrated in a PTx-sensitive manner following S1P exposure (Kon *et al.*, 1999). A recent study showed that EDG1-induced endothelial cell chemotaxis was mediated via a Gi/PI3K/Akt-dependent activation of Rac (Lee *et al.*, 2001). However, the inhibition of NO production had no effect on S1P-induced endothelial cell chemotaxis whereas VEGF-dependent chemotaxis was blocked (Morales-Ruiz *et al.*, 2001).

The plasmin-mediated mobilisation of FGF from the endothelial cell matrix represents an important step in endothelial cell migration and proliferation (Cross and Claesson-Welsh, 2001; Griffioen and Molema, 2000; Richard *et al*, 2001). The classical FGFs, FGF-1 and FGF-2, mediate their biological effects via 4 tyrosine kinase receptors, namely FGFR-1, 2, 3, and 4 (Cross and Claesson-Welsh, 2001). FGF-2 consists of a high molecular weight (hmw) and a low molecular weight (lmw) form of FGF (Griffioen and Molema, 2000). lmw FGF binding to the endothelium during angiogenesis causes FGFR down-regulation, increased motility, proliferation and proteinase activity and also modulates levels of integrins (Griffioen and Molema, 2000). Integrins are transmembrane proteins which bind to extracellular matrix (ECM) proteins or cell surface ligands where they influence the activity of the basal cell cycle machinery consisting of cyclin-dependent kinase complexes and can mediate anchorage-dependent cell death (Cross and Claesson-Welsh, 2001; Griffioen and Molema, 2000). hmw FGF may act upon endothelial cell proliferation after nuclear translocation in the endothelial cells (Cross and Claesson-Welsh, 2001; Griffioen and Molema, 2000). In addition, VEGF has been shown to also affect endothelial cell proliferation by activating MAPK via a NO/cGMP dependent process (Griffioen and Molema, 2000). Interestingly, S1P has been shown to act synergistically with FGF and VEGF in promoting angiogenesis (Lee *et al.*, 1999). Also, recent evidence

suggests that S1P-dependent activation of EDG1, along with EDG3, results in the Rho-dependent activation of integrin $\alpha_v\beta_3$ and β_1-containing integrins, leading to the formation of initial focal contacts required for cell spreading and migration (Paik *et al.*, 2001). $\alpha_v\beta_3$ has also been shown to function in a synergistic manner with VEGF in the processes of cell migration and proliferation (Soldi *et al.*, 1999).

1.11.3. Differentiation Of The Neovasculature

Following endothelial cell proliferation, the newly formed neovasculature is remodelled into capillary-like networks by a process of morphogenesis (Cross and Claesson-Welsh, 2001; Griffioen and Molema, 2000) (Figure 1.10). Morphogenesis involves the folding of epithelial cell sheets into tubes and other related structures and is typically regulated *via* connection sites for actin filaments known as adherens junctions (Alberts *et al.*, 1994). Cell-matrix adherens junctions allow cells to grip the extracellular matrix. Cell-to-cell adherens junctions occur in various forms. In non-epithelial tissues, these cell-to-cell adherens junctions form small punctate/streak-like attachments that connect actin filaments in the cortical cytoplasm of adjacent cells (Alberts *et al.*, 1994; Evers *et al.*, 2000; Griffioen and Molema, 2000). In epithelial sheets, cell-to-cell adherens junctions make up a continuous adhesion belt (zonula adherens) around each of the interacting cells in the sheet, located near the apex of each cell (Evers *et al.*, 2000; Alberts *et al.*, 1994). Adhesion belts in adjacent epithelial cells are directly opposed and the interacting plasma membranes are held together by transmembrane linker proteins that are members of the family of Ca^{2+}-dependent cell-cell adhesion molecules called cadherins (Evers *et al.* 2000; Alberts *et al.*, 1994). The family of cadherins consists of around 12 members, including vascular endothelial (VE)-cadherin, found in the vascular endotheliam; E-cadherin, found in epithelial cells; N-cadherin, found in nerve, muscle and lens cells and P-cadherin, found in placenta and epidermal cells (Alberts *et al.*, 1994). Each cell contains a contractile bundle of actin filaments adjacent to the adhesion belt and running parallel to the plasma membrane that are attached via a set of intracellular attachment proteins; α-, β-, and γ-catenin, vinculin, α-actinin and plakaglobin (Alberts *et al.*, 1994; Griffioen and Molema, 2000; Jones *et al.*, 2001). The actin bundles in adjacent cells are linked via cadherins and attachment proteins, resulting in an extensive transcellular network. It is the contraction of this network that mediates morphogenesis.

EDG1 activation has been shown to regulate many of the above components that are involved in morphogenesis and this typically involves the activation of the small

GTPases, Rac and Rho (Hla *et al.*, 2001; Pyne and Pyne, 2000a, Pyne and Pyne, 2000b, Lee *et al.*, 1999). Rac and Rho are known to be involved in the S1P-stimulated translocation of VE-cadherin and β-catenin to cell-cell junctions (Lee *et al.*, 1999). Also, S1P treatment in HUVECs results in the activation of EDG1 and EDG3 receptors which, in turn, activate Rac and Rho dependent adherens junction assembly and cytoskeletal rearrangement which ultimately results in the morphogenesis of HUVECs into capillary-like networks (Lee *et al.*, 1999). Interestingly, the action of S1P is in contrast to the action of VEGF, which is known to disrupt adherens junctions (Hla *et al.*, 2001; Lee *et al.*, 1999). As described previously, S1P stimulation of EDG1 and EDG3 receptors expressed in HUVECs results in the activation of $\alpha_v\beta_3$- and β_1-containing integrins (Paik *et al.*, 2001). In addition to regulating cell spreading and migration, antagonists of $\alpha_v\beta_3$ and β_1-containing integrins inhibited S1P-induced endothelial cell morphogenesis in a three-dimensional fibrin matrix (Paik *et al.*, 2001).

1.11.4. Maturation of Neovasculature

Once the neovasculature has been formed, endothelial cells must deposit a new basement membrane and recruit surrounding vessel layers composed of mural cells, such as pericytes in small vessels and smooth muscle cells in large vessels (Griffioen and Molema, 2000; Richard *et al.*, 2001; Saaristo *et al.*, 2000). The recruitment of mural cells is largely dependent upon the synthesis and secretion of PDGF within endothelial cells (Cross and Claesson-Welsh, 2001; Griffioen and Molema, 2000). On endothelial cell-mural cell contact, a latent form of transforming growth factor-β (TGF-β), produced by both endothelium and mural cells, is activated in a plasmin-mediated process (Griffioen and Molema, 2000). Activated TGF-β induces changes in myofibroblasts and pericytes, leading to the formation of a quiescent vessel, ECM production and maintenance of growth control (Griffioen and Molema, 2000). Angiopoietins and receptor tyrosine kinases Tie1 and Tie2 are critical for the communication of endothelial cells with the surrounding mesenchyme (Griffioen and Molema, 2000; Jones *et al.*, 2001; Lin *et al.* 1997). Tie1 function is related to endothelial cell differentiation and establishing blood vessel integrity whereas Tie2 is important for vascular network formation and is only expressed on endothelial cells (Jones *et al.*, 2001). Angiopioetin-1 (Ang-1) and Angiopioetin-2 (Ang-2) are Tie2-specific ligands that activate or antagonize Tie2 signalling respectively (Davis *et al.*, 1996; Jones *et al.*, 2001; Maisonpierre *et al.*, 1997). Vessels in embryos lacking Tie2 or Ang1 demonstrated that endothelial cells failed to associate properly with underlying

support cells, indicating that Tie2 signalling may facilitate recruitment of, and tight association with, adjacent periendothelial cells (Patan, 1998; Suri *et al.*, 1996). The persistent expression and phosphorylation of Tie2 in quiescent adult endothelium also suggests a role for Tie2 in transducing a sustained survival signal (Jones *et al.*, 2001; Wong *et al.*, 1997). In human glioblastomas, a cell-specific up-regulation of Tie2, Ang-1 and Ang-2 during tumour progression was detected in a pattern compatible with a role in tumour-induced angiogenesis (Griffioen and Molema, 2000; Stratmann *et al.*, 1998).

Studies on EDG1 knockout mice showed that EDG1 is essential for vascular maturation as EDG1 gene disruption resulted in impaired vascular maturation due to the failure of mural cells to migrate to arteries and capillaries to reinforce them (Liu *et al.*, 2000). In fact, although EDG1 null embryos died *in utero* due to massive hemorrage, they exhibited normal vasculogenesis and a substantially normal blood vessel network, yet were severely impaired in the recruitment of smooth muscle cells and pericytes to the vessel walls and this was attributed to their defective migration (Liu *et al.*, 2000). Extracellular S1P can directly stimulate EDG1 on VSMCs, facilitating their migration to vessel walls or, alternatively, can stimulate EDG1 expressed in endothelial cells that in turn may recruit vascular smooth muscle cells (VSMCs) (Liu *et al.*, 2000) (Figure 1.11). Recent studies have demonstrated that the effect of EDG1 on vascular maturation can be largely attributed to the cross-talk between EDG1 and PDGF receptor signalling mentioned previously (Hobson *et al.*, 2001; Rosenfeldt *et al.*, 2001). Cell migration toward PDGF, which stimulates sphingosine kinase and increases intracellular S1P, was dependent upon EDG1 expression in a number of cell types, including HEK293 cells, human aortic smooth muscle cells (ASMCs) and mouse embryonic fibroblasts (MEFs) (Hobson *et al.*, 2001). It was therefore suggested that spatially and temporally localised generation of S1P by activation of sphingosine kinase in response to PDGF results in restricted activation of EDG that in turn activates Rac, resulting in an increase in cell motility (Hobson *et al.*, 2001). A recent study has subsequently demonstrated that the PDGF-induced cytoskeletal rearrangements, lamelipodia extensions and cell motility are abrogated in EDG1 null fibroblasts (Rosenfeldt *et al.*, 2001). Also, PDGF-induced focal adhesion formation and activation of FAK, Src and SAPK 2 were disregulated in the absence of EDG1 (Rosenfeldt *et al.*, 2001). However, EDG1 was not involved in mitogenicity and survival effects induced by S1P or PDGF (Rosenfeldt *et al.*, 2001). Hence, it was suggested that EDG1 acted as an integrator linking the PDGFR to lamellipodia extension and cell migration. Cells then migrate towards PDGF, enhancing PDGF receptor signalling and therefore

acting as a positive feedback mechanism (Hobson *et al.*, 2001; Rosenfeldt *et al.*, 2001) (Figure 1.12).

1.12 GPCR Regulation

GPCR signal transduction must be properly regulated in order to prevent overstimulation, achieve signal termination and render the receptor responsive to subsequent stimuli. Desensitisation of GPCRs is defined as the process whereby receptor signalling responses plateau and then diminish despite the continuous presence of agonist (Ferguson, 2001). The mechanism of GPCR desensitisation involves a number of different processes. Rapid, homologous desensitisation is thought to involve receptor uncoupling from its associated G-protein(s) within a few minutes of agonist exposure and seems to involve receptor phosphorylation (Appleyard *et al.*, 1999; Bouvier *et al.*, 1998; Jockers *et al.*, 1996; Palmer *et al.*, 1995; Small *et al.*, 2001; Xiao *et al.*, 1999). Receptor internalisation, or sequestration, away from the cell surface that not only reduces the number of cell surface receptors available but may also facilitate receptor dephosphorylation and subsequent resensitisation upon agonist removal (Cavalli *et al.*, 2001; Ferguson, 2001; Koenig and Edwardson, 1997; Mukherjee *et al.*, 1997). After several hours of agonist exposure, receptor down-regulation may occur where there is a decrease in the total number of receptors expressed (Bouvier *et al.*, 1989; Clark, 1986; Ferguson, 2001; Law *et al.*, 1982; Tsao *et al.*, 2001). This requires an increase in gene expression to compensate for the loss of expressed receptor protein (Collins *et al.*, 1989; Tsao *et al.*, 2001).

1.12.1 GPCR Desensitisation

GPCR desensitisation can vary from complete inhibition (visual/olfactory systems) to attenuation (β_2AR) (Ferguson, 2001; Sakmar, 1998; Zhang *et al.*, 1997). In the case of many GPCRs, the ability or inability to undergo agonist-induced receptor phosphorylation is integral to the subsequent rate and extent of receptor desensitisation (Clark *et al.*, 1999; Ferguson, 2001; Palmer *et al.*, 1996). GPCR desensitisation has been shown in a number of cases to be regulated by phosphorylation by G-protein receptor kinases, arrestins and second messenger-dependent kinases (Appleyard *et al.*, 1999; Ferguson, 2001; Jockers *et al.*, 1996; Oakley *et al.*, 1999; Tang *et al.*, 1998; Yuan *et al.* 1994). Desensitisation can also occur at the G-protein level. For example, RGS proteins (regulators of G protein signalling) can increase the rate of GTP hydrolysis bound to G_i and G_q α-subunits and therefore attenuates signalling *via* G_i-and G_q- regulated signalling pathways (Dohlman and

Thorner, 1997; Ferguson, 2001; Hepler, 1999). The most well defined example of rapid GPCR desensitisation is that of the β_2-adrenergic receptor (Jockers *et al.*, 1996; Luttrell *et al.*, 1999; McLean *et al.*, 1999; Menard *et al.*, 1997; Moffett *et al.*, 1993). Some GPCRs, although not all, seem to share similar regulatory mechanisms, including the thrombin, m_2-muscarinic, and rhodopsin receptors (Ferguson, 2001). In this case, the predominant form of agonist-induced β_2AR desensitisation is caused by a conformational change of the agonist-occupied receptor that facilitates receptor phosphorylation by G-protein receptor kinases (GRKs) (Ferguson *et al.*, 2001; Jockers *et al.*, 1996; Menard *et al.*, 1997; Pierce and Lefkowitz, 2001). Following β_2AR phosphorylation, a scaffold protein, β-arrestin, binds to the phosphorylated receptor and uncouples the receptor from heterotrimeric G-proteins (Giadarov *et al.*, 1999; Luttrell *et al.*, 1999; Menard *et al.*, 1997, Miller and Lefkowitz, 2001). β-arrestin not only desensitises the receptor but also functions as a clathrin adaptor, mediating receptor sequestration *via* clathrin-coated vesicles (Giadarov *et al.*, 1999; Luttrell *et al.*, 1999; Menard *et al.*, 1997, Miller and Lefkowitz, 2001; Takei and Haucke; 2001) (Figure 1.13).

1.12.2. Second Messenger-Dependent Kinases

The activation of second messenger-dependent kinases results in receptor phosphorylation independently of agonist occupation. This subsequently causes so-called "heterologous" receptor desensitisation as agonist occupancy is not required (Ferguson, 2001). In the case of the β_2AR, phosphorylation by PKA of a consensus site within its third intracellular loop causes partial uncoupling of the receptor from G_s (about 40-60%) (Yuan *et al.*, 1994). This mechanism is usually triggered by very low occupancy of receptor (2-5nM epinephrine) as it requires only small increases in cAMP to fully activate PKA, and is rapid, with a $t_{1/2}$ of 1-2 min (January *et al.*, 1997). This is in contrast to GRK-mediated phosphorylation, where the EC_{50} for GRK-mediated phosphorylation approaches the K_d for agonist binding (50-200 nM epinephrine) (Hausdorff *et al.*, 1997; January *et al.*, 1997; Clark *et al.*, 1988). Therefore, as the concentration of agonist increases, β_2AR desensitisation moves from being almost exclusively PKA-mediated towards a progressively larger GRK-mediated mechanism. Additionally, the time course of GRK-mediated phosphorylation usually occurs within seconds (Clark *et al.*, 1999; Ferguson *et al.*, 2001). The relatively slower time-course of second messenger-mediated receptor phosphorylation can be attributed to the time required to activate the second messenger kinase whereas GRK-mediated phosphorylation only requires the appropriate

conformational change of the receptor. Interestingly, phosphorylation of β_2ARs by PKA has also been shown to switch the receptor from G_s to G_i coupling (Daaka *et al.*, 1997). In addition to PKA, a number of other second messenger-dependent kinases are involved in GPCR desensitisation. For example, PKC has been shown to phosphorylate and desensitise a number of G_i- and G_q-coupled GPCRs, including α_{1B}-adrenoceptor and the type 1A angiotensin II receptor (Diviani *et al.*, 1997; Liang *et al.*, 1998; Tang *et al.*, 1998).

1.12.3. G-Protein Receptor Kinases

GRKs constitute a family of receptor kinases consisting of seven members, namely GRK1-7 (Ferguson, 2001; Pierce and Lefkowitz, 2001). Each member contains a central common catalytic domain, an N-terminal domain that controls substrate recognition and contains a conserved RGS domain, and a C-terminal domain responsible for targeting GRKs to the plasma membrane. The GRK family can be subdivided into three groups: 1) GRK1 (rhodopsin kinase) and GRK7 (cone opsin kinase); 2) GRK2 (β-adrenergic kinase 1, βARK1) and GRK3 (β-adrenergic kinase 2, βARK2) and 3) GRK4, GRK5, and GRK6. GRK1 and GRK7 are farnesylated at CAAX motifs in their carboxyl termini, with GRK1 also shown to be regulated by the Ca^{2+} sensor protein, recoverin (Iacovelli *et al.*, 1999; Inglese *et al.*, 1992). The translocation of GRK2 and GRK3 to the plasma membrane are each regulated by phosphatidylinositol 4,5-bisphosphate binding to a region that exhibits sequence homology to a pleckstrin homology (PH) domain contained within the carboxyl-terminal domains of GRK2 and GRK3 (Pitcher *et al.*, 1992; Touhara *et al.*, 1994). The GRK5 carboxyl-terminal domain contains a stretch of 46 basic amino acids that mediates interactions with plasma membrane-phospholipid . The activity of GRK5 decreases in the presence of PKC whereas GRK2 activity is increased (Chaung *et al.*, 1995; Chuang *et al.*, 1996; Winstel *et al.*, 1996). Calmodulin associates with the N-terminal of GRK5 to decrease the ability of the kinase to bind the receptor and phospholipids and decreases GRK5 activity by promoting the autophosphorylation of serine and threonine residues distinct from those involved in kinase activation (Pronin and Benovic, 1997; Pronin *et al.*, 1997; Iacovelli *et al.*, 1999). The plasma membrane localisation of GRK4 and GRK6 is regulated by the palmitoylation of cysteine residues within their C-termini (Stoffel *et al.*, 1994; Stoffel *et al.*, 1998) (Figure 1.14).

The GRKs can selectively phosphorylate serine and threonine residues within the 3rd intracellular loop (m2 mAchR and α_{2A}AR) or the C-terminal tail (rhodopsin and β_2AR) of agonist-occupied GPCRs (Ferguson *et al.*, 2001). In addition, GPCRs can isomerise to

an activated conformation in the absence of agonist and therefore GRKs may also contribute to basal GPCR phosphorylation (Pie *et al.*, 1994; Rim and Oprian, 1995). An agonist-induced conformational change in GPCRs exposes two physically and functionally distinct domains. One contains the sequence that is phosphorylated by GRK and the second acts as an activator of these kinases (Chen *et al.*, 1993; Iacovelli *et al.*, 1999). For example, the sites within the m_2-muscarinic receptor phosphorylated by GRK2 and the domains able to activate this kinase were found to be located in different intracellular regions of the receptor (Iacovelli *et al.*, 1999; Nakata *et al.*, 1994).

1.12.4. Arrestins

The GRK-mediated phosphorylation of clusters of serine and threonine residues in C-tails may regulate stability of receptor/arrestin complexes (Miller and Lefkowitz, 2001; Oakley *et al.*, 1999). The arrestins are adaptor proteins that preferentially bind agonist-activated and GRK-phosphorylated GPCRs, with which where they form a complex that results in receptor uncoupling from G-proteins, can target the receptor for internalisation *via* clathrin-coated vesicles and can mediate the activation of alternative signalling pathways (Ferguson, 2001; Gaidarov *et al.*, 1999; Luttrell *et al.*, 1999; Miller and Lefkowitz, 2001; Pierce and Lefkowitz, 2001). The arrestin family is subdivided into 2 groups: a) visual arrestin (S antigen) and cone arrestin (X-arrestin/C-arrestin) and b) β-arrestins (β-arrestin1 and β-arrestin2) (Ferguson, 2001; Miller and Lefkowitz, 2001; Pierce and Lefkowitz, 2001). Visual arrestin is found within rod outer segments and is localised primarily to the retina (Smith *et al.*, 1994). C-arrestin is highly enriched in retina and pineal gland but is localised primarily within cone photoreceptors in the retina (Craft *et al.*, 1994). β-arrestins are ubiquitously expressed, with the highest expression found within neuronal tissues and the spleen where they regulate signalling of many different GPCRs (Attramadal *et al.*, 1992; Pierce and Lefkowitz, 2001). Studies on crystal structure, as well as mutagenesis studies, of visual arrestin identified 3 functional domains (a secondary receptor-binding domain, a receptor activation domain and a phosphate sensor domain) and two regulatory domains, located at the amino terminal and carboxyl-terminal (Ferguson, 2001; Gurevich *et al.*, 1995; Granzin *et al.*, 1998). Within the N-terminal domain of β-arrestin1 and β-arrestin2, but not the visual arrestins, there is a proline-rich region (Luttrell *et al.*, 1999). The C-terminal region contains clathrin- and β-adaptin-binding domains that are conserved among nonvisual arrestins (Krupnicke *et al.*, 1997; Laporte *et al.*, 2000) (Figure 1.15).

1.12.5. The Role Of Arrestins In Clathrin-Mediated GPCR Internalisation

Many receptors undergo agonist-induced endocytosis and recycling back to the plasma membrane (Ferguson, 2001; Pierce and Lefkowitz, 2001; Takei and Haucke, 2001). For the β_2AR and many other GPCRS, such as the angiotensin $AT_{1A}R$, the endothelin ET_A receptor and the D2 dopamine receptor, endocytosis involves GRK- and arrestin-dependent recruitment of GPCRs to plasma-membrane clathrin-coated pits and then their invagination and pinching off to form intracellular clathrin-coated vesicles (Brodin *et al.*, 2000; Ferguson, 2001; Pierce and Lefkowitz, 2001; Takei and Haucke, 2001) (Figure 1.13). Clathrin is composed of three light and three heavy chains that form a three-legged structure called a triskelion (Brodin *et al.*, 2000; Takei and Haucke, 2001) (Figure 1.16). The triskelions then assemble into a basket-like convex framework of hexagons and pentagons that form the coated pits on the cytoplasmic surface of the plasma membranes (Brodin *et al.*, 2000; Schmid, 1997; Takei and Haucke, 2001). One of the main components of the coats formed during membrane endocytosis is an adaptor protein (AP) called AP2. The AP2 complex consists of four subunits: two large 100kDa subunits (α-adaptin, which binds to clathrin, dynamin and Eps15 and β2-adaptin, essential for clathrin coat formation), one medium size 50kDa subunit (μ2, which recognises tyrosine-based internalisation signals) and a small 17kDa subunit (σ2) (Brodin *et al.*, 2000; Ferguson, 2001; Takei and Haucke, 2001) (Figure 1.16). In clathrin, the β-arrestin binding domain is localised to residues 89-100 of the amino-terminal globular region in the terminal domain of the clathrin heavy chain at the distal end of each clathrin triskelion (Goodman *et al.*, 1997). The β-arrestin domain involved in binding to the β2-adaptin subunit of the AP2 complex is also localised to the carboxyl terminus (Ferguson, 2001; Brodin *et al.*, 2001). In particular, two arginine residues (R394and R396) in β-arrestin2 mediate binding to β2-adaptin *in vitro* (Laporte *et al.*, 2000). The binding of β-arrestins to β2-adaptin is independent of clathrin binding (Laporte *et al.*, 2000). In addition, whereas β_2AR/β-arrestin complexes lacking the β-arrestin clathrin binding motif redistributed to coated pits, receptor/β-arrestin complexes lacking the β2-adaptin binding site did not (Laporte *et al.*, 2000). Therefore, β-arrestin interactions with the AP-2 complex, rather than with clathrin, are necessary for the initial targeting of receptors to coated pits (Laporte *et al.*, 2000). β-arrestins bind to both the clathrin heavy chain and the β2-adaptin subunit of AP2 (Ferguson, 2001; Brodin *et al.*, 2000). The coat also contains a monomeric adaptor protein, AP180 (Figure 1.16), which interacts with AP2 and may regulate vesicle size

(Brodin *et al.*, 2000). The formation of clathrin-coated pits is assisted by synaptotagmin (Figure 1.16), an AP-2 binding protein that facilitates vesicle recycling by promoting coated pit nucleation (Brodin *et al.*, 2000; Ferguson, 2001; Takei and Haucke, 2001).

The pinching off of clathrin-coated vesicles is largely dependent upon the action of a large GTPase called dynamin (Figure 1.16) and is ATP-dependent (Brodin *et al.*, 2000; Takei and Haucke, 2001). Overexpression of Dynamin K44A, a dominant negative form of dynamin lacking GTPase activity, blocked both β_2AR and $AT_{1A}R$ internalisation (Brodin *et al.*, 2000; Ferguson, 2001; Gagnon *et al.*, 1998; Tsao *et al.*, 2001; Zhang *et al.*, 1996). Dynamin self-assembles into a helical structure that wraps around the necks of forming vesicles and facilitates their pinching off from the membrane (Brodin *et al.*, 2000; Takei and Haucke, 2001). Dynamin can interact with amphiphysin and syndapins, which link endocytosis with the actin cytoskeleton (Brodin *et al.*, 2001; Takei and Haucke, 2001). The accessory protein amphiphysin (Figure 1.16) acts as a binding partner for clathrin, AP-2 and dynamin and has been shown to recruit dynamin to clathrin-coated pits (Brodin *et al.*, 2001; Takei and Haucke, 2001). A number of other accessory proteins are involved in clathrin-mediated endocytosis. These include: synaptojanin, an inositol phosphatase that regulates PIP_2 metabolism and the stability of clathrin-AP2 coats; endophilin, a lysophosphatidic acid acyl transferase involved in pit maturation and vesicle fission, and epsin and Eps15, both of which are interacting partners for the α-adaptin subunit of AP2 (Brodin *et al.*, 2001; Cavalli *et al.*, 2001; Ferguson, 2001; Mukherjee *et al.*, 1997; Simpson *et al.*, 1999; Takei and Haucke, 2001) (Figure 1.16).

Components of the endocytic machinery described above, particularly dynamin and clathrin, have been shown to be regulated as a result of the β-arrestin-mediated activation of ERK (Ahn *et al.*, 1999; Ferguson, 2001; Miller and Lefkowitz, 2001; Miller *et al.*, 2000; Pierce and Lefkowitz, 2001). Recent evidence has shown that several components of the ERK pathway form complexes with β-arrestins and are then recruited to GPCRs in an agonist-dependent manner (DeFea *et al.*, 2000; Luttrell *et al.*, 1999; Miller and Lefkowitz, 2001). The role of β-arrestins as molecular adapter proteins was extended to components of the ERK pathway with the discovery that β-arrestin could recruit the non-receptor tyrosine kinase Src to activated β_2ARs (Luttrell *et al.*, 1999). Src molecules associated with β-arrestin and activated β_2ARs were found to be dephosphorylated on Tyr530 and thus catalytically active (Luttrell *et al.*, 1999; Miller and Lefkowitz, 2001). Recruitment of active Src to agonist-occupied receptor leads to phosphorylation of the adaptor protein Shc,

formation of Shc-Grb2 complexes and mediates, as well as ERK activation, the phosphorylation of dynamin and clathrin (Luttrell *et al.*, 1999; Miller and Lefkowitz, 2001).

1.12.6. Trafficking Through Endocytic Organelles

Following internalisation, receptors are delivered to peripheral early endosomes (Cavalli *et al.*, 2001; Ferguson, 2001; Mukherjee *et al.*, 1997). The small GTPase Rab5 is one of the key regulators of this process and cycles between GTP- and GDP-bound form, and GTP hydrolysis depends on a specific GEP, Rabex-5 (Cavalli, *et al.*, 2001; Ferguson, 2001; Takai *et al.*, 2001). Rab5 often contributes to the formation of endocytic vesicles, the trafficking of vesicles to early endosomes and the fusion of endocytic vesicles with early endosomes (Cavalli *et al.*, 2001; Ferguson, 2001). Once delivered to early endosomes, recycling receptors such as the β_2AR and the transferrin receptors are returned to the cell suface, at least in part *via* recycling endosomes (Ferguson, 2001; Pierce and Lefkowitz, 2001). The small GTPase, Rab4 is also involved in the recycling pathway where it regulates the budding and/or recycling of receptor-bearing recycling vesicles (Cavalli *et al.*, 2001; Ferguson, 2001; Seachrist *et al.*, 2000). In contrast to recycling receptors, some endocytosed receptors targeted for downregulation are transported from early to late endosomes where they are then targeted to lysosomes for degradation (Gruenberg and Maxfield, 1995). Transport from early to late endosomes is mediated through intermediates exhibiting a characteristic multivesicular appearance called multivesicular bodies (MVBs) or endosomal carrier vesicles (ECVs). In mammalian cells, ECVs/MVBs, once formed on early endosomes, move towards late endosomes on microtubules and then dock onto and fuse with late endosomes (Cavalli *et al.*, 2001; Gruenberg and Maxfield, 1995) (Figure 1.17). Little is known about the possible cross-talk between late endosomes/lysosomes and signalling pathways. However, recent studies identified a novel 14 kDa protein that interacts with the MAPK scaffold protein MP1 on late endosomes/lysosomes but its function remains unclear (Cavalli *et al.*, 2001). In addition, protein ubiquitination has been implicated at multiple steps of the endocytic pathway from the internalisation reaction to the maturation of endosomes and lysosomal delivery (Cavalli *et al*, 2001; Ferguson, 2001; Shenoy *et al.*, 2001). For example, a β_2AR mutant lacking lysine residues, which was not ubiquitinated, was internalised normally but was degraded ineffectively (Shenoy *et al.*, 2001).

1.12.7. Alternative Pathways Of Internalisation

Although clathrin-mediated internalisation represents the most common mechanism for GPCR internalisation, receptors can also internalise *via* pathways that are independent of both clathrin and β-arrestin (Anderson, 1998; Cavalli, 2001; Ferguson, 2001; Mukherjee *et al.*, 1997). One possible route of entry involves cell surface microdomains containing cholesterol and glycosphingolipids (rafts), which are believed to play an important role in the internalisation of the IL-2 receptor (Anderson, 1998; Cavalli, 2001; Ferguson, 2001; Mukherjee *et al.*, 1997). Rafts can form flask-shaped invaginations smaller than clathrin-coated pits termed caveolae, when associated with caveolin (Anderson, 1998). These structures have been reported in a variety of cell types, including smooth muscle cells, fibroblasts, adipocytes, endothelial cells and many epithelial cells (Anderson, 1998; Kogo and Fujimoto, 2000; Mineo *et al.*, 1996; Oh *et al.*, 1998; Parton, 1996). Although much less is known about the molecular mechanism involved, both the agonist-occupied β_2AR and the bradykinin B2 receptors were shown to be localised in caveolae as determined by electron microscopy studies (de Weerd *et al.*, 1997; Ferguson, 2001; Haasemann *et al.*, 1998; Okamoto *et al.*, 2000). Other GPCRs, such as the angiotensin type 1 and m2 muscarinic acetylcholine receptors also undergo agonist-dependent sequestration in this microdomain, as demonstrated by the recovery of receptor proteins in caveolin-rich fractions (Feron *et al.*, 1997; Ishizaka *et al.*, 1998). In many cases, cell-type is crucial in determining the favoured pathway of receptor internalisation. For example, the β_2AR internalises in some cell types *via* clathrin-coated pits but internalises *via* caveolae in other cell types, such as A431 cells (Ferguson, 2001; Kallal and Benovic, 2000; Raposo *et al.*, 1989). The endothelin ET_A receptor has also been shown to internalise *via* both pathways in a cell-type dependent manner (Okamoto *et al.*, 2000). The form of internalisation pathway can also determine the intracellular trafficking of the receptor (Okamoto *et al.*, 2000).

1.13 The Study Of GPCR Cell Surface Distribution And Agonist-Induced Internalisation Using Green Fluorescent Protein (GFP)

Classical approaches to studying GPCR internalisation include radioligand binding, physical fractionation techniques and immunofluorescence (Kallal and Benovic, 2000). However, the recent application of green fluorescent protein (GFP) as a tool in the study of agonist-induced GPCR internalisation has proved invaluable (Drmota *et al.*, 1998; Kallal and Benovic, 2000; McLean *et al.*, 1999; Milligan, 1999). The gene encoding GFP was

originally isolated from the jellyfish *Aequoria victoria* in 1992 (Prasher *et al.*, 1992). GFP is an autofluorescent protein of 238 amino acids that emits green light with an emission maximum of 509nm upon fluorescent excitation at 488nm derived from either standard fluorescence microscope light sources or fluorescein isothiocyanate (FITC) excitation and emission filters (Tsien, 1998). The use of GFP in the study of receptor internalisation has been used for a variety of GPCRs, including the cholecystokinin CCK_1 receptor, β_2AR, thyrotropin-releasing hormone TRH_1 receptor and the vasopressin V_2 receptor (Drmota *et al.*, 1998; McLean *et al.*, 1999; Schulein *et al.*, 1998; Tarasova *et al.*, 1997).

There are a number of advantages gained from using GFP (Kallal and Benovic, 2000; Milligan, 1999). Since there are no cofactors or substrates required for fluorescence, the time and expense of using primary and secondary antibodies on fixed cells can be avoided. GFP-expressing cells can also be studied on living cells in real time so that the dynamics of protein trafficking can be observed. Cells expressing GFP can also be fixed, as GFP is relatively chemically resistant. Also, since GFP is covalently attached to the protein of interest, nonspecific fluorescence is avoided. One of the major disadvantages of using GFP is that the expression of GPCR-GFP chimeras in cells results in the labelling of protein biosynthetic compartments, such as the endoplasmic reticulum and Golgi, which can influence the interpretation of results (Kallal and Benovic, 2000). Many examples of GFP-tagging of GPCRs, such as the β_2AR and TRH_1 receptor, have shown that the addition of GFP has no effect on the receptor's ability to bind ligand and that the ability to generate second messengers and to desensitise was unaffected (Drmota *et al.*, 1998; McLean *et al.*, 1999; Schulein *et al.*, 1998; Tarasova *et al.*, 1997). However, GFP-tagging may result in altered properties of the receptor. Hence, careful experimental comparisons to untagged proteins should be made prior to visualisation studies using confocal microscopy.

A number of studies have used GPCR-GFP chimeras in conjunction with red fluorescent markers that label specific cellular organelles or membranes. Also, antibodies or proteins conjugated to rhodamine, Texas Red or other red fluorescent compounds that localise to specific organelles or membranes can be used in combination with the GPCR-GFP chimeras (Kam *et al.*, 2001; Kallal *et al.*, 1998; Kallal and Benovic, 2000; McConalogue *et al.*, 1999; Tarasova *et al.*, 1998). By observation of the GPCR-GFP and the red fluorescent component in the same cell, any potential co-localisation can be readily ascertained. Compounds that are commonly used with GPCR-GFP chimeras include

rhodamine-dextran and LysoTracker Red, which label late endosomes and lysosomes, respectively and rhodamine-transferrin, which labels early endosomes and endocytic recycling compartments. Most GPCR-GFP chimeras, including the β_2AR and the TRH_1 receptor, were shown to enter transferrin-containing endosomes rapidly after exposure to an agonist and co-localise with tetramethylrhodamine isothiocyanate (TRITC)-transferrin or Texas-Red transferrin (Kallal and Benovic, 2000). Also, studies using fluorescent dextran- or Lysotracker red-tagged lysosomes demonstrated that receptors, such as the CXCR-4-GFP receptor, accumulate in lysosomes whereas other receptors, such as the CCK_1-GFP receptor, did not show any lysosomal co-localisation (Tarasova *et al.*, 1997; Tarasova *et al.*., 1998).

In addition to co-localisation studies, GPCR-GFP chimeras can be used for FRAP (fluorescence recovery after photobleaching) in order to examine receptor mobility in the membrane. This is where a small area of the cell membrane is bleached with a high intensity laser and recovery of fluorescence into the bleached area is used to measure protein diffusion rate within membranes (Barak and Benovic, 2000; Milligan, 1999). The FRAP technique has demonstrated that β_2AR-GFP has a rapid diffusion rate, similar to other plasma membrane proteins (Barak *et al.*, 1997). In contrast, the gonadotrophin releasing hormone receptor tagged with GFP (GnRHR-GFP) membrane diffusion rate was reduced in the presence of agonists and antagonists (Nelson *et al.*, 1999). Because some receptors have been shown to oligomerize upon activation (Herbert and Bouvier, 1998), changes in membrane mobility might reflect the formation of receptor oligomers. Another application of GFP relies on the use of newer GFPs, such as the cyan and yellow GFPs, in the technique of FRET (fluorescence resonance energy transfer) (Barak and Benovic, 2000; Milligan, 1999; Pollok and Heim, 1999). This is where the excitation of one fluorophore results in an emission that excites a second fluorophore. Such emission is a measure of the interaction between two proteins as energy transfer is inversely proportional to distance and is detectable only if fluorophores are within 7 nm of each other (Barak and Benovic, 2000). The FRET technique could potentially be an important measure of transient agonist-mediated GPCR-protein interactions.

1.14 Aim

The aim of this project is to examine the phosphorylation and internalisation of the adenosine A_1 receptor and the sphingosine-1-phosphate receptor, EDG1. Distinct differences exist amongst GPCRs in relation to the processes of receptor phosphorylation and internalisation. However, as described previously, one of the most well characterised examples of GPCR phosphorylation and internalisation is the β_2AR. Therefore, this will provide a basis for studying the regulation of EDG1 and A_1 receptors. Studies using the A_1AR will also be compared with concurrent studies using the A_3AR, an adenosine receptor with a similar structure and exhibiting similar biological effects.

Figure 1.1: The Structure Of The Ribonucleoside, Adenosine

Adenosine is a ribonucleoside consisting of a D-ribose sugar and the nitrogenous base, adenine. The sugar and the base are attached by an N-glycoside bond between the C-1' of the sugar and N^9 of adenine. The 5' position of the ribose group and the N^6 position of the adenine ring, important in ligand binding, are illustrated.

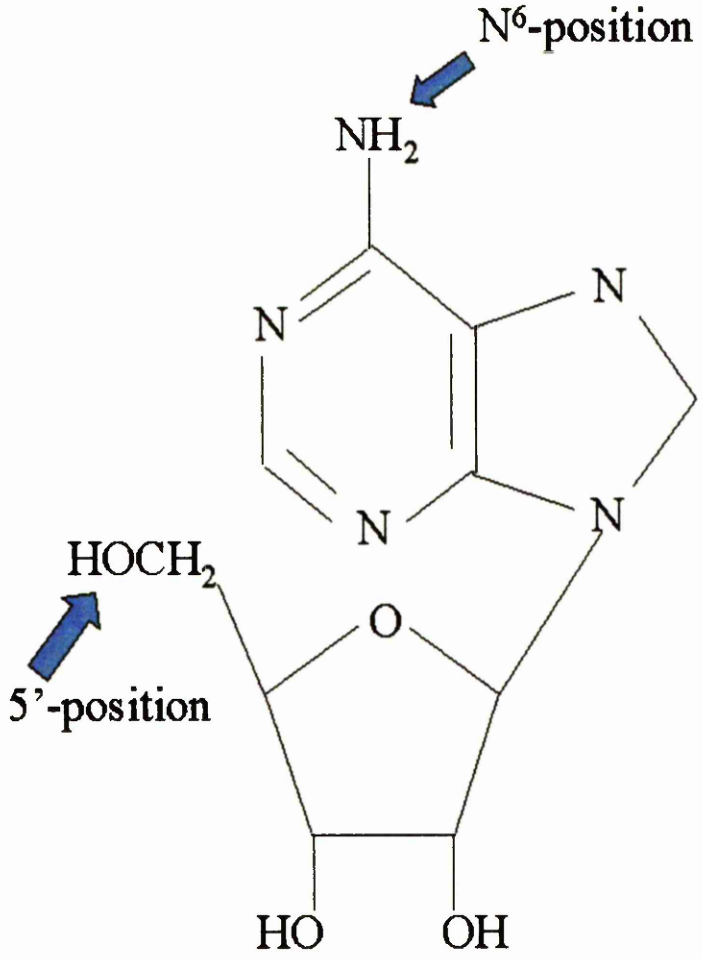

N6-position
NH2
N
N
N
N
HOCH2
O
5'-position
HO
OH

Figure 1.2: Synthesis And Metabolism Of Adenosine

A substrate cycle exists between AMP and adenosine through the opposing effects of 5'nucleotidase and adenosine kinase, thereby regulating intracellular adenosine formation upon changes in intracellular AMP concentration. Additionally, substantial amounts of adenosine may be formed from the breakdown of adenine nucleotides that are present in the granules of autonomic nerves, platelets and mast cells. Adenine nucleotides are rapidly converted to adenosine by a family of ecto-ATP/ADPases including CD39 (NTPDase 1) and ecto-5' nucleotidases including CD73. Extracellular adenosine then acts upon G-protein-coupled cell surface adenosine receptors to produce specific biological effects. Excess adenosine can be degraded to inosine and finally uric acid *via* adenosine deaminase. Non-concentrative bi-directional adenosine (or nucleoside) transporters equilibrate changes in intracellular and extracellular adenosine concentrations. Figure adapted from Mullane and Bullough, 1995

Key: 1) 5'nucleotidase; 2) ecto-5' nucleotidase; 3) adenosine deaminase 4) AMP deaminase; 5) adenosine kinase; 6) purine nucleoside phosphorylase; 7) hypoxanthine phosphoribose transferase; 8) ecto-ATP/ADPases including CD39 (NTPDase 1) and ecto-5' nucleotidases including CD73.

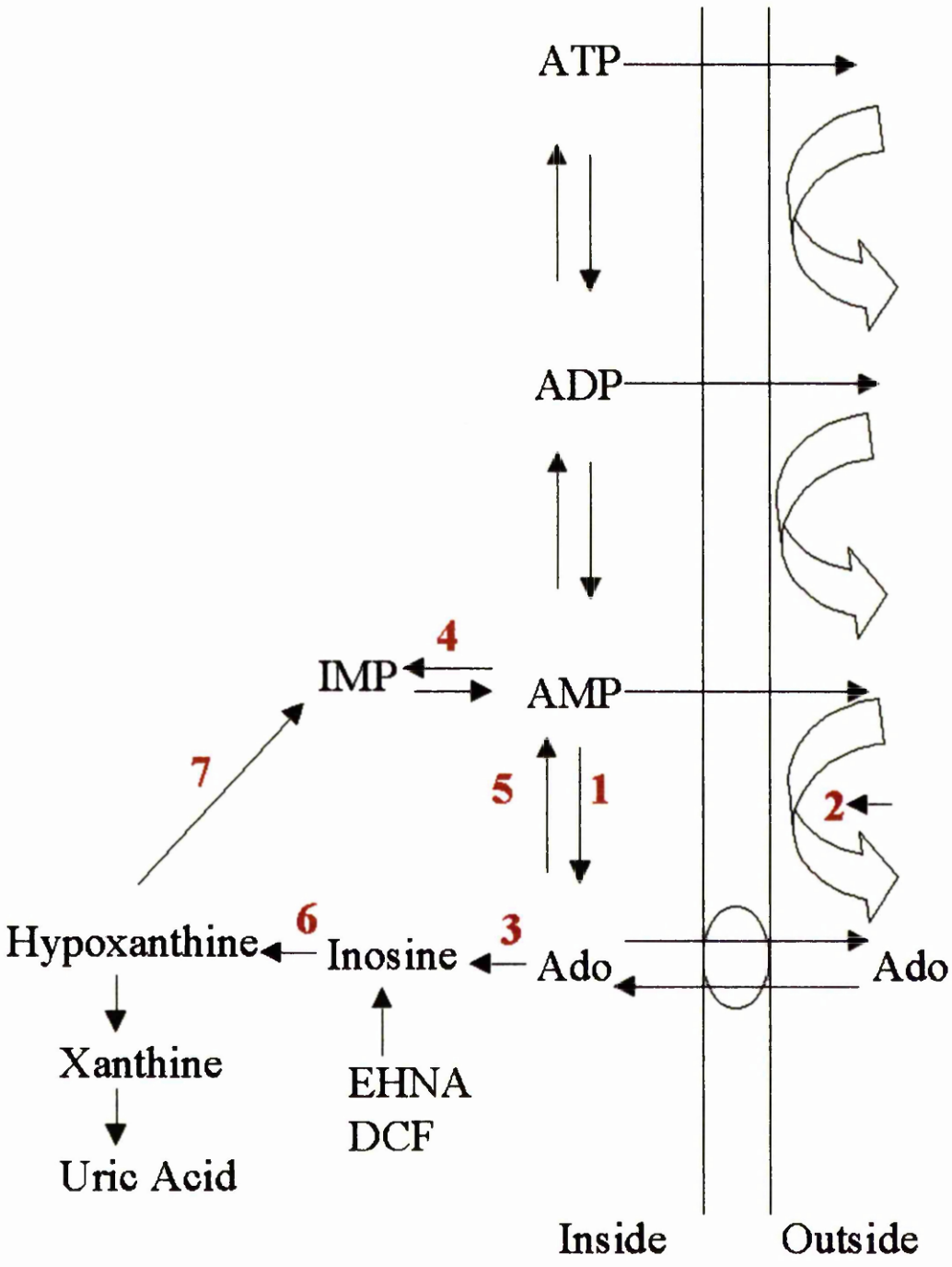

ATP
ADP
AMP
IMP
4
7
5
1
2
Hypoxanthine
6
Inosine
3
Ado
Ado
Xanthine
Uric Acid
EHNA
DCF
Inside
Outside

Figure 1.3: Structure Of The Bioactive Phospholipid, Sphingosine-1-Phosphate

Sphingosine-1-phosphate (S1P) is a naturally occuring bioactive lysophospholipid. Its structure consists of one long hydrocarbon chain on a three-carbon backbone containing a phosphate group.

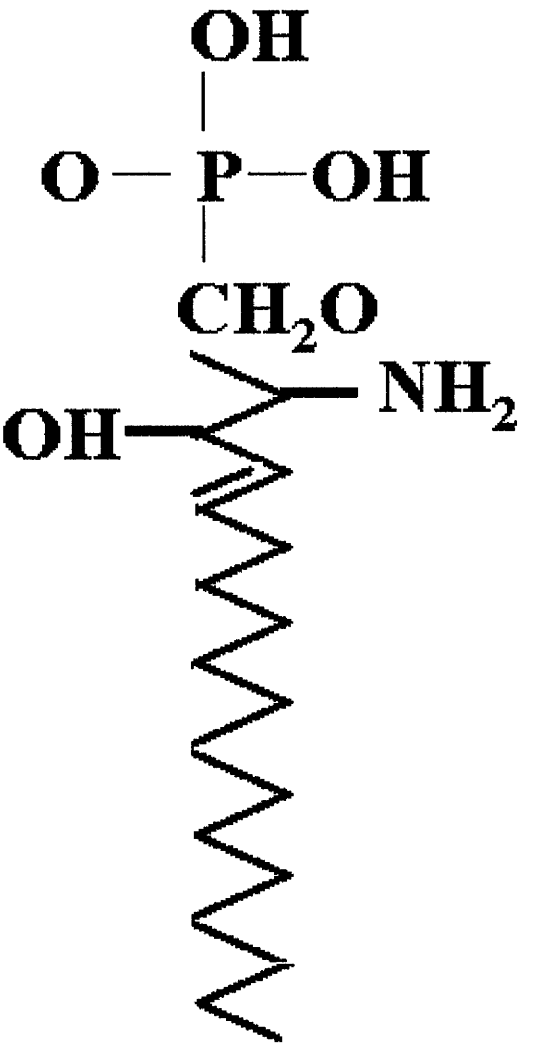
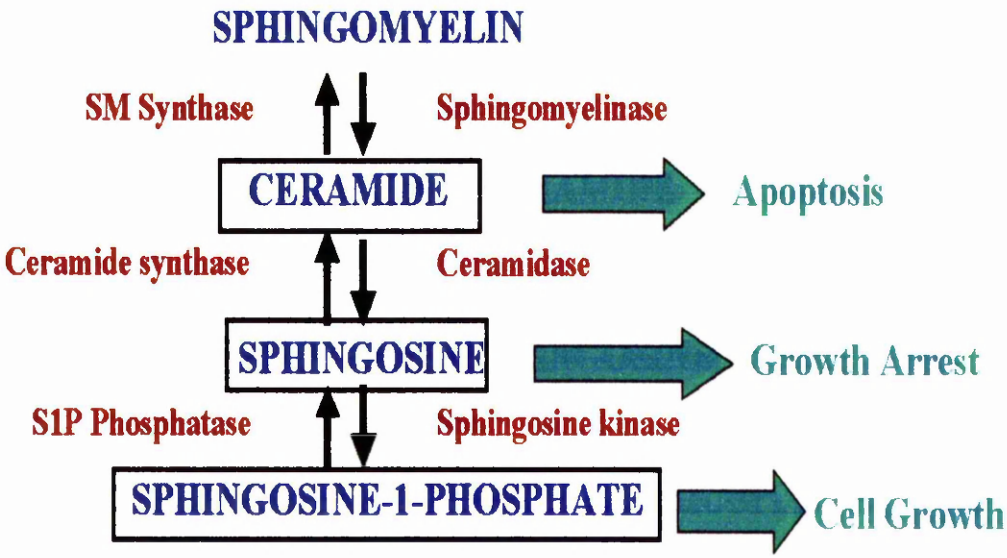

OH
O
P
OH
CH2O
NH2
OH

Figure 1.4: S1P Production And The Sphingolipid Rheostat Model

Sphingolipid metabolism is a dynamic process resulting in the formation of a number of bioactive metabolites including ceramide, sphingosine and S1P. Following sphingomyelinase activation, sphingomyelin is hydrolysed to ceramide, thought to be involved in cell growth arrest, differentiation and apoptosis. Ceramide is then converted to sphingosine by ceramidase. Sphingosine has been shown to inhibit PKC and induce apoptosis. Finally, sphingosine can be phosphorylated by sphingosine kinase to produce S1P, implicated in cell growth and inhibition of ceramide-mediated apoptosis. Metabolism of S1P is catalysed by both a pyridoxal phosphorylation-dependent lyase located in the ER, which degrades S1P to phosphoethanolamine and palmitaldehyde, and by a phosphatase which converts S1P back to sphingosine. Therefore, the dynamic balance between the concentration of all the bioactive sphingolipid metabolites helps determine cell fate. This is commonly known as the "sphingolipid rheostat".

Sphingolipid Production And Cell Fate



Balance Of Each Lipid Determines Cell Fate

Figure 1.5: The Prototypical Structure Of A G-Protein-Coupled Receptor

A prototypical GPCR consists of seven transmembrane-spanning α-helical domains, with an N-terminal extracellular domain and a C-terminal intracellular domain. The diagram illustrates the representative structure of a GPCR for small ligands such as biogenic amines and nucleosides. The central core, essential for ligand binding, is comprised mainly of domains II, III, V, and VI. Domains I and IV are peripherally sequestered. The sites of glycosylation, thought to be involved in receptor trafficking to the membrane, are also shown. Figure adapted from Ulloa-Aguirre *et al*, 1999.

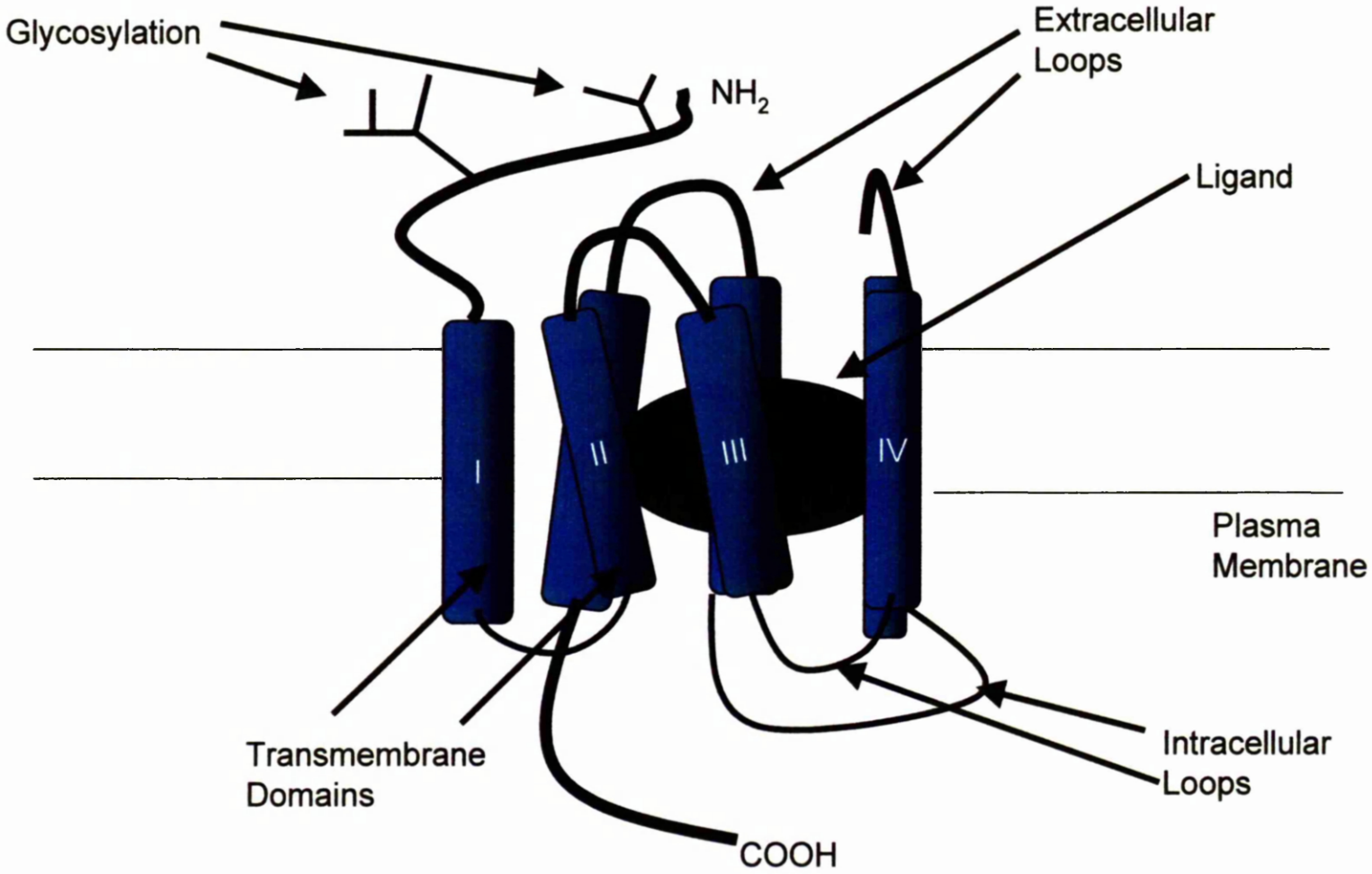

Glycosylation
NH2
Extracellular
Loops
Ligand
I
II
III
IV
Plasma
Membrane
Transmembrane
Domains
Intracellular
Loops
COOH

Figure 1.6: The Function Of The G-Protein

The binding of agonist to a GPCR changes the conformation of the receptor which promotes the exchange of GDP for GTP on the G protein α-subunit to allow the dissociation of the α and βγ subunits. The free α and βγ subunits are then able to interact with effector molecules to evoke cellular responses. The intrinsic GTPase activity of the α subunit hydrolyses GTP to GDP, allowing reassociation of the α and βγ subunits. The inactive G protein is subsequently reformed and signalling is terminated.

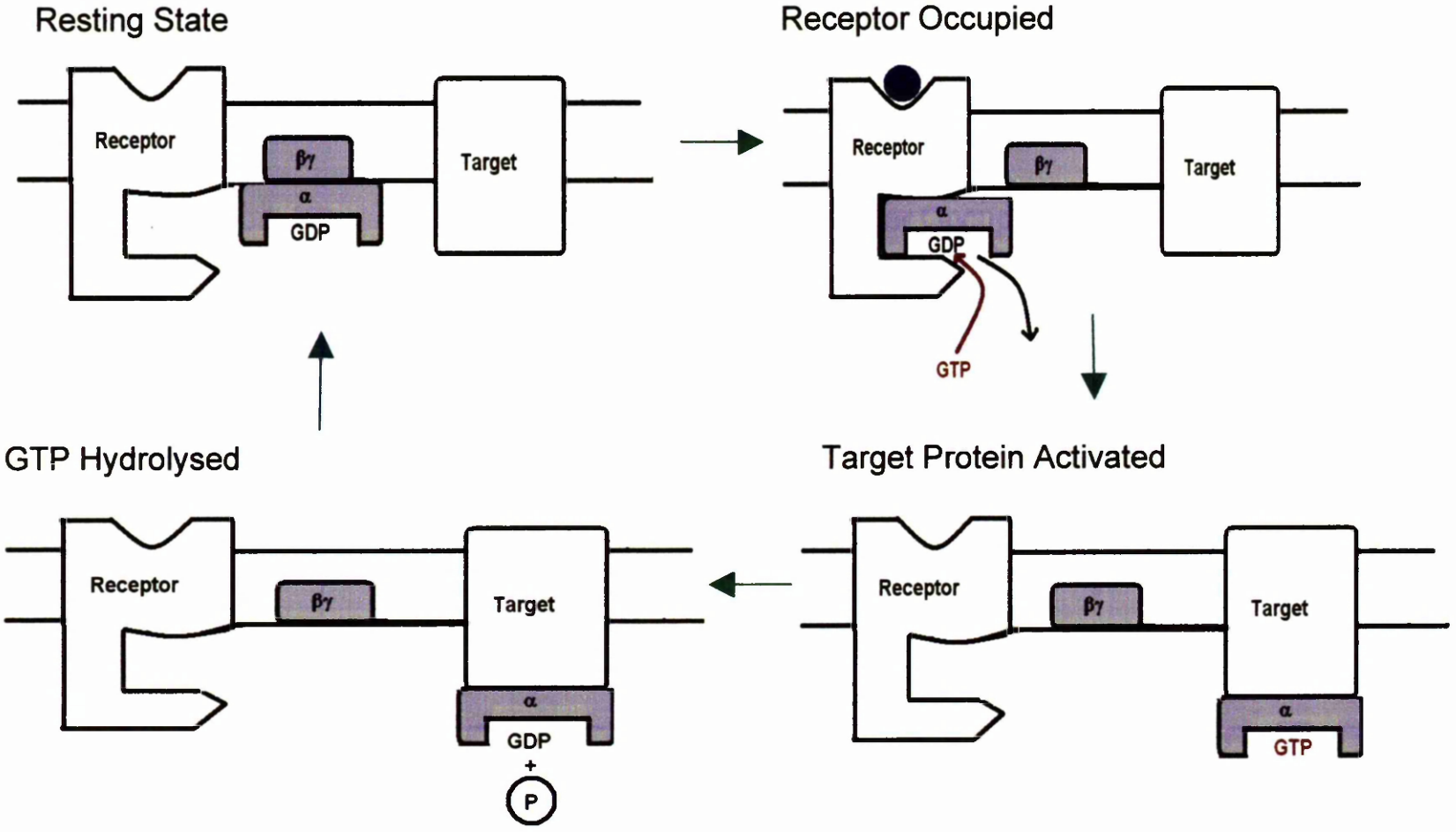

Resting State
Receptor
βγ
α
GDP
Target
Receptor Occupied
Receptor
βγ
Target
α
GDP
GTP
Target Protein Activated
Receptor
βγ
Target
α
GTP
GTP Hydrolysed
Receptor
βγ
Target
α
GDP
+
P

Table1: The Subfamily Of Gα-Subunits

The table shows the main members of the Gα subfamily.

Table adapted from Ulloa-Aguirre *et al.*, 1999

Gα-subunit	Size (kDa)	Effect(s)	Tissue Distribution	Sensitivity To Toxins
G_s class				
$G\alpha_s$ 1-4	45-52	↑ AC, Regulate Ca^{2+} Channels	Ubiquitous	CTx
$G\alpha_{olf}$			Olfactory Neurons	
G_i Class				
$G\alpha_i$ 1-3	40-41	↓ AC	Ubiquitous	PTx
$G\alpha_0$ 1 and 2	39	Ca^{2+} channels	Neural, endocrine	PTx
$G\alpha_t$ 1 and 2	39-40	↑ cGMP-PDE	Retina	PTx/CTx
$G\alpha_{gust}$	41	↑ cGMP-PDE	Taste Buds	PTx
$G\alpha_z$	41	↓ AC ↓ K^+ Channel	Neural, Platelets	—
G_q Class				
$G\alpha_{q/11}$	41-43	↑ PLCβ	Ubiquitous	—
$G\alpha_{14}$			Liver, lung, kidney	—
$G\alpha_{15/16}$			Blood cells	—
G_{12} Class				
$G\alpha_{12/13}$	44	Na^+, H^+ antiporter?, cell growth	Ubiquitous	—

Figure 1.7: The Regulation Of Small G-Protein Activity

Small G-proteins exist in two interconvertible forms: - GDP-bound inactive and GTP-bound active. Following stimulation from an upstream signal, GDP dissociates from the GDP-bound form followed by the binding of GTP, resulting in the activation of downstream effectors. The GTP-bound form is then converted back to the inactive GDP-bound form *via* the intrinsic GTPase activity of the small G-protein, resulting in the release of the bound downstream effectors. The rate-limiting step of GDP/GTP exchange is the dissociation of GDP from the GDP-bound form. A regulator, termed a GEP, which is, in turn, regulated by an upstream signal, can increase the rate of this dissociation. The GEP interacts with the GDP-bound small G-protein to release bound GDP to form a binary complex of small G-protein and GEP. GEP is then replaced by GTP, resulting in the formation of the active GTP-bound form. GDP/GTP exchange reactions of Rho/Rac/Cdc42 and Rab are also regulated by Rho GDI and Rab GDI respectively, which inhibit both the basal and the GEP-stimulated dissociation of GDP from the GDP-bound form and keeps the small G-protein in the inactive GDP-bound form. Figure adapted from Takai *et al.*, 2001.

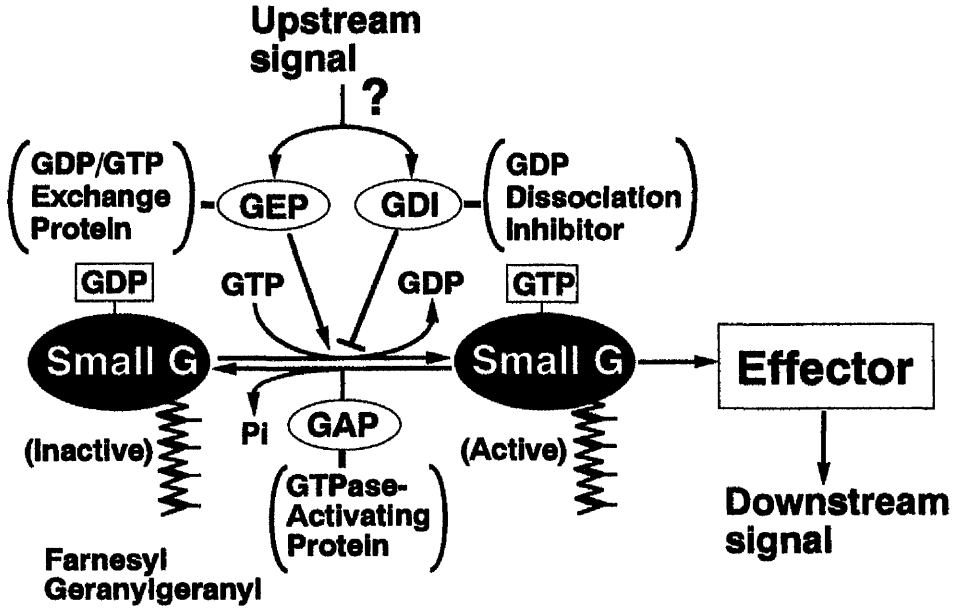

Upstream signal
?
GDP/GTP Exchange Protein
GEP
GDI
GDP Dissociation Inhibitor
GDP
GTP
GDP
GTP
Small G
Small G
Effector
Pi
GAP
(Inactive)
(Active)
GTPase-Activating Protein
Farnesyl
Geranylgeranyl
Downstream signal

Table 2: The Adenosine-Specific Family Of GPCRs

The table demonstrates the main family members of the adenosine-specific family of GPCRs, namely the A_1AR, $A_{2A}AR$, $A_{2B}AR$ and A_3AR. The G-protein coupling, effects and main agonists and antagonists are shown for each receptor subtype.

	A_1	A_{2A}	A_{2B}	A_3
cDNA Library source	Human, canine, bovine, rabbit, mouse, Guinea-pig, rat	Human, canine, rat, guinea-pig, mouse	Human, rat, mouse	Human, sheep, rabbit, rat
G-protein-coupling	$G_{i/o}$	G_s	G_s G_q	G_i G_q
Effects	↓cAMP, ↑IP_3, ↑K^+, ↓Ca^{2+}	↑cAMP	↑cAMP ↑IP_3	↓cAMP ↑IP_3
Selective agonists	CPA, CCPA CHA, R-PIA	CGS21680, HE-NECA, APEC, CV1808, WRC-0470	-	IB-MECA, 2Cl-IB-MECA
Selective anatagonists	DPCPX, XAC, KW-3902, ENX, KFM 19, N 0861, FK 453, WRC 0571	KF17837, ZM241385, CSC, SCH 58261	-	I-ABOPX, L-268605, L-249313, MRS 1067, MRS 1097

Figure 1.8: The Role Of A_1ARs In The Process Of Early And Delayed Ischaemic Preconditioning

A short ischaemic insult results in the release of adenosine. Subsequent A_1AR activation then results in the stimulation of PKC, tyrosine kinase and MAPK pathways which, in turn, open K_{ATP} channels in the sarcolemma, mitochondria, or both, or in translocation to the nucleus of NF-κB and the subsequent synthesis of protective proteins such as heat shock proteins (HSPs) and the mitochondrial form of the antioxidant superoxide dismutase (MnSOD). Synthesis and release of nitric oxide (NO) from both endothelial (eNOS) and inducible (iNOS) forms of nitric oxide synthase from both myocardial and non-myocardial sources can also result in early and delayed protection respectively. Figure adapted from Rubino and Yellon, 2000.

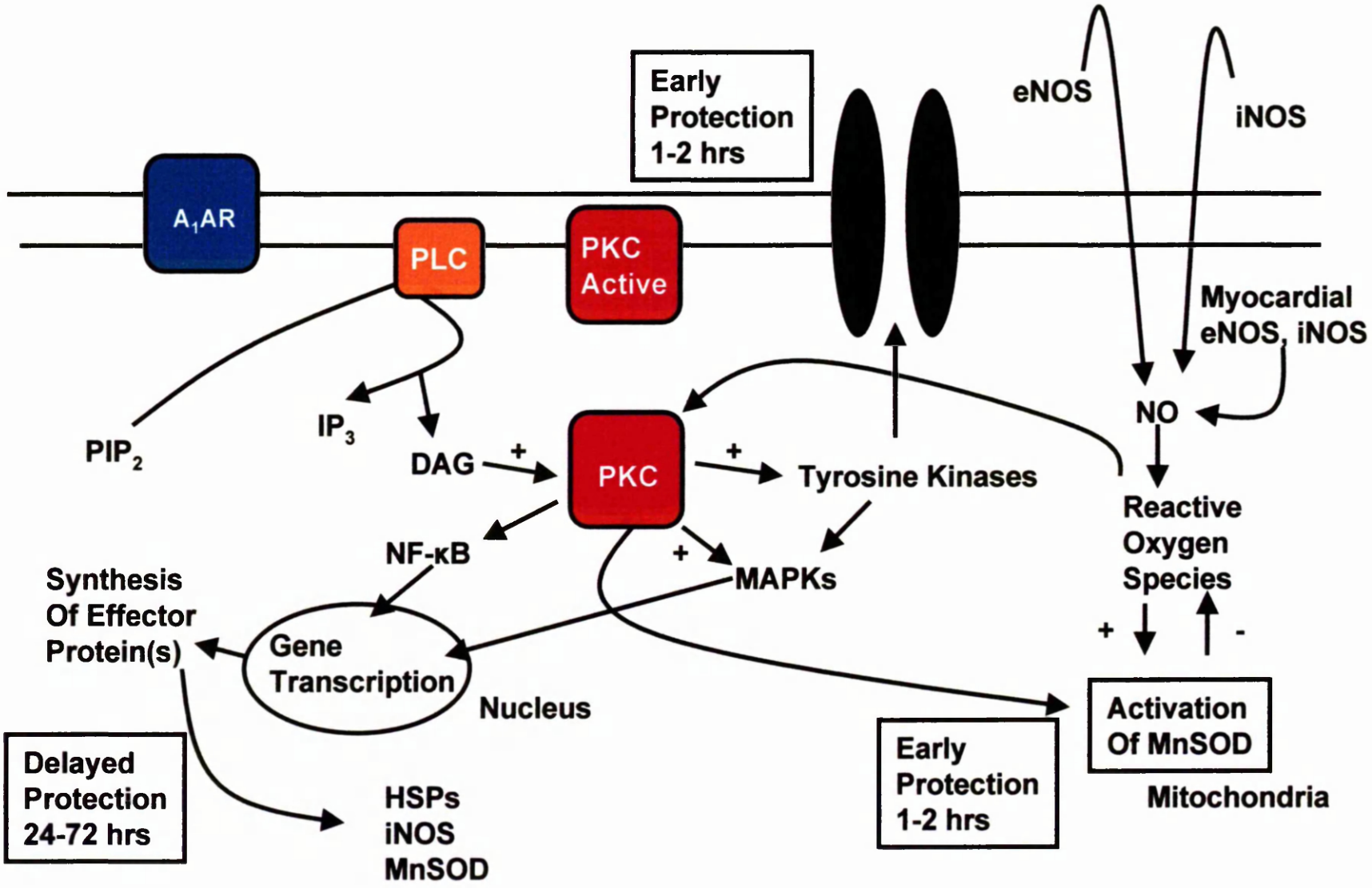

Early
Protection
1-2 hrs
eNOS
iNOS
A1AR
PLC
PKC
Active
Myocardial
eNOS, iNOS
NO
PIP2
IP3
DAG
+
PKC
+
Tyrosine Kinases
Reactive
Oxygen
Species
NF-κB
+
MAPKs
Synthesis
Of Effector
Protein(s)
Gene
Transcription
Nucleus
+
-
Activation
Of MnSOD
Mitochondria
Delayed
Protection
24-72 hrs
HSPs
iNOS
MnSOD
Early
Protection
1-2 hrs

Table 3: The EDG Family Of GPCRs

The table demonstrates the main family members of the S1P- and LPA-specific family of GPCRs, namely EDG1-7. For each receptor subtype, the main agonist, G-protein coupling, effects and tissue distribution are shown.

Receptor	Agonist	Tissue Distribution	Coupled G-proteins	Cellular Function
EDG1	S1P	Widely distributed	$G_{i/o}$ G_z	↓ AC, ↑ ERK, ↑ PLC, ↑ Rac, Rho
EDG2	LPA	Widely distributed	$G_{i/o}$	↓ AC, ↑ PLC, ↑ Rho,
EDG3	S1P	Widely distributed	$G_{i/o}$ $G_{q/12/13}$	↓ AC, ↑ PLC, ↑ ERK, ↑ Rac, Rho
EDG4	LPA	Kidney, testis, prostate	$G_{i/o}$ $G_{q/12/13}$	↓ IL-2 secretion, ↑ PLC, ↓ AC,
EDG5	S1P	Widely distributed	$G_{i/o}$ $G_{q/12/13}$	↑ ↓ AC, ↑ PLC, ↑ JNK, ↑ Rho, ↓ Rac
EDG6	S1P	Lymphoid tissues	$G_{i/o}$	↑ ERK, ↑ PLC,
EDG7	LPA	Heart, pancreas, prostate, testis, lung	$G_{i/o}$ $G_{q/12/13}$	↑ PLC, ↑ ERK,
EDG8	S1P	Brain, spleen	$G_{i/o}$ G_{12}	↓ AC, ↑ JNK, ↑ ERK

Figure 1.9: Schematic Of The Process Of Angiogenesis

Angiogenesis, the formation of new blood vessel from pre-existing ones, involves a number of steps; 1) initiation; 2) endothelial cell migration and proliferation 3) differentiation and 4) maturation of the neovasculature. These steps are regulated by a number of factors, such as PDGF, VEGF, and angiopoietin. Recently, S1P-dependent activation of EDG1 has been shown to be heavily involved in specific parts of this process. Figure adapted from Griffioen and Molema, 2000.

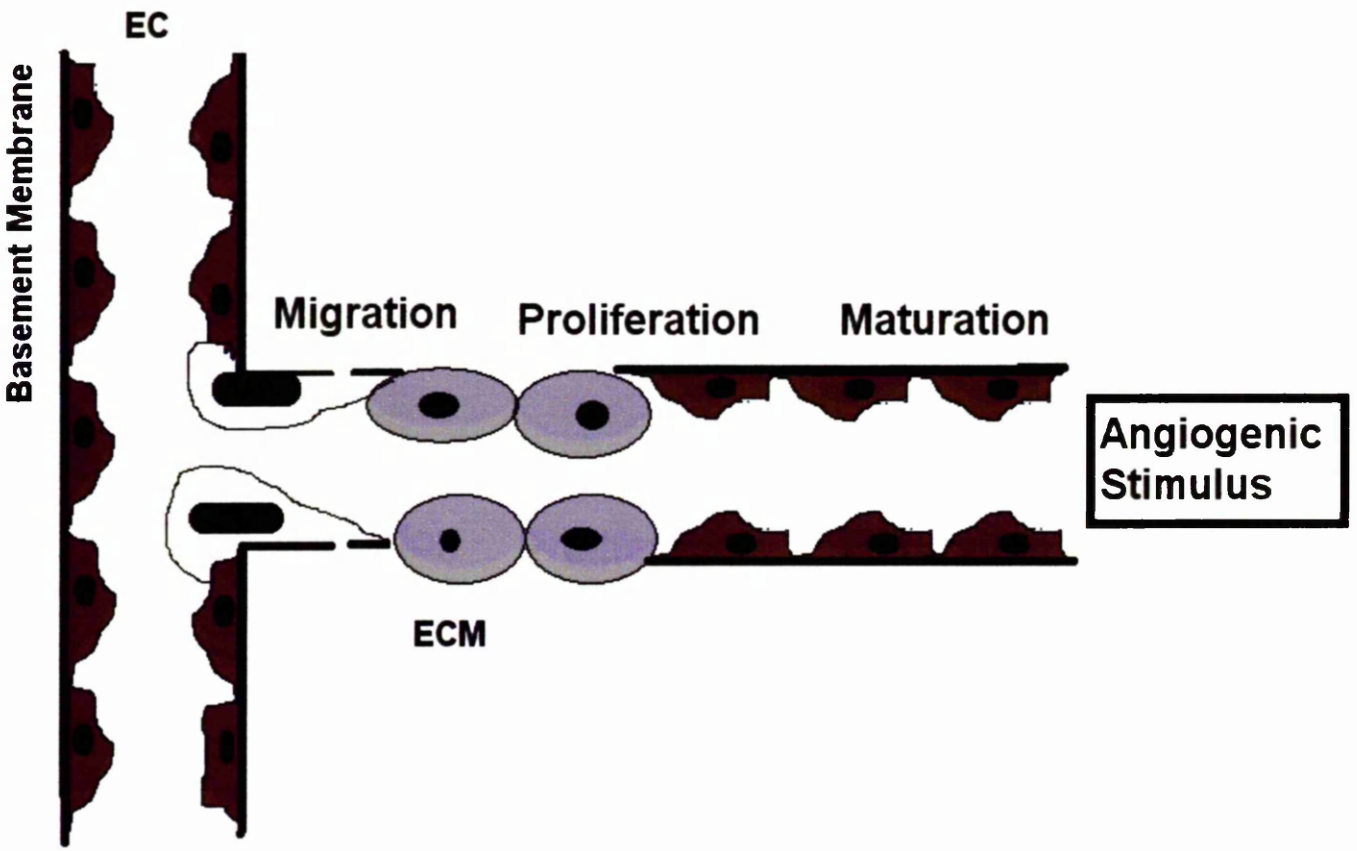

EC
Basement Membrane
Migration
Proliferation
Maturation
Angiogenic Stimulus
ECM

Figure 1.10: The Role Of EDG1 In The Process Of Endothelial Cell Morphogenesis

In epithelial sheets, cell-to-cell adherin junctions make up a continuous adhesion belt (zonula adherens) around each of the interacting cells in the sheet, located near the apex of each cell (Panel A). Adhesion belts in adjacent epithelial cells are directly opposed and the interacting plasma membranes are held together by linker proteins called cadherins. Each cell contains a contractile bundle of actin filaments, adjacent to the adhesion belt parallel to the plasma membrane, that are attached *via* a set of intracellular attachment proteins; α-, β-, and γ-catenin, vinculin, α-actinin and plakaglobin (Panel B). The actin bundles in adjacent cells are linked *via* cadherins and attachment proteins, resulting in an extensive transcellular network. It is the contraction of this network that mediates morphogenesis (Panel A).

EDG1 activation regulates many of the components involved in morphogenesis *via* the activation of Rac and Rho. Rac and Rho are known to be involved in the S1P-stimulated translocation of VE-cadherin and β-catenin to cell-cell junctions. Also, S1P treatment in HUVECs results in the activation of EDG1 and EDG3 receptors which, in turn, activate Rac and Rho dependent adherens junction assembly and cytoskeletal rearrangement which ultimately results in the morphogenesis of HUVECs into capillary-like networks. Panel A adapted from Alberts *et al.*, 1994. Panel B adapted from Jones *et al.*, 2001.

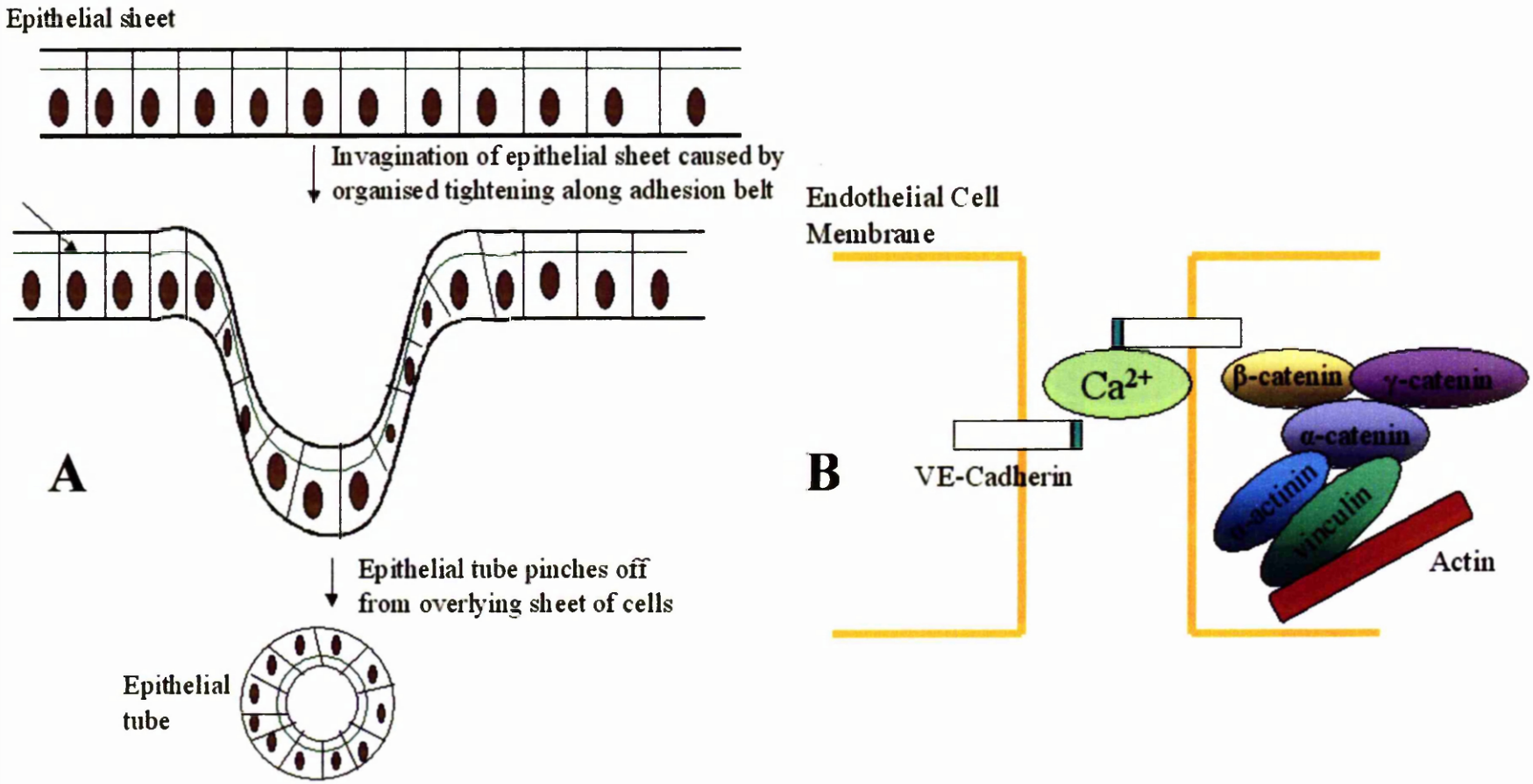

Epithelial sheet
Invagination of epithelial sheet caused by organised tightening along adhesion belt
A
Epithelial tube pinches off from overlying sheet of cells
Epithelial tube
Endothelial Cell Membrane
B
VE-Cadherin
Ca2+
β-catenin
γ-catenin
α-catenin
α-actinin
vinculin
Actin

Figure 1.11: The Role Of EDG1 In The Process Of Endothelial Cell Migration

Extracellular S1P can directly stimulate EDG1 on VSMCs, facilitating their migration to vessel walls or, alternatively, can stimulate EDG1 expressed in endothelial cells that in turn may recruit VSMCs. This results in the recruitment of smooth muscle cells and pericytes to the vessel walls and contributes to endothelial cell maturation. Figure adapted from Liu *et al.*, 2000.

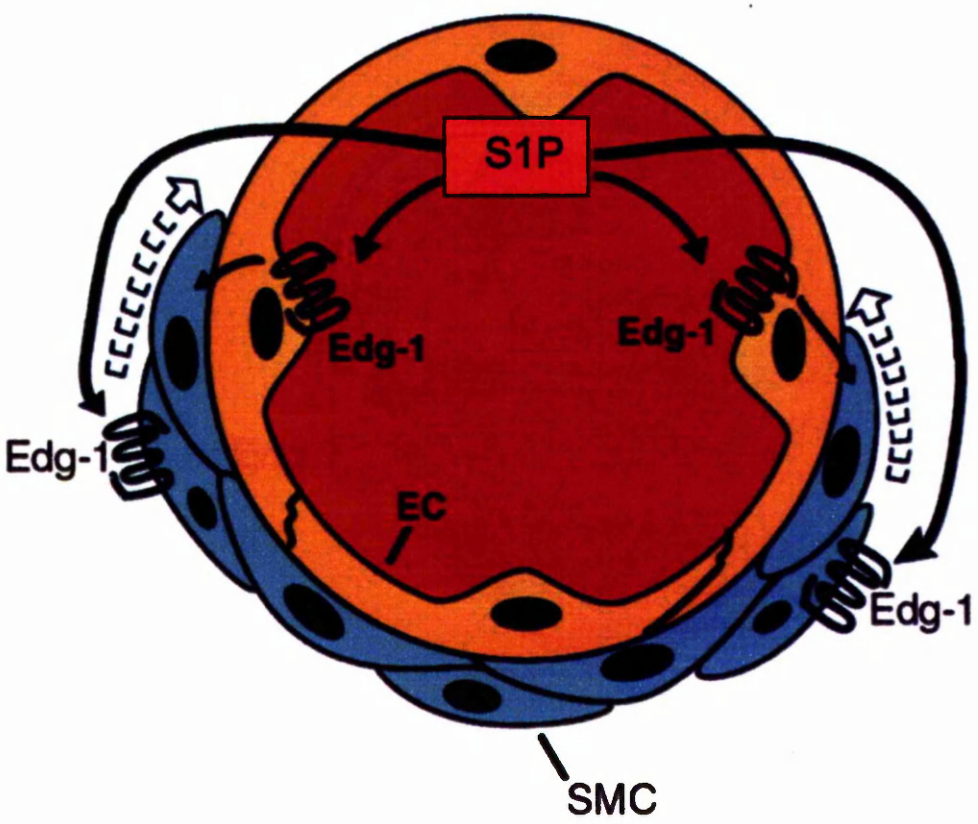

S1P
Edg-1
Edg-1
Edg-1
Edg-1
EC
SMC

Figure 1.12: Cross-Talk Between The PDGF Receptor And The EDG1 Receptor And Its Role In Cell Migration

Cell migration toward PDGF, which stimulates sphingosine kinase and increases intracellular S1P, has been shown to be dependent upon EDG1 expression. PDGF-dependent-generation of S1P *via* activation of sphingosine kinase results in the EDG1-dependent activation of Rac, leading to cell migration towards PDGF. Figure adapted from Hobson *et al.*, 2001.

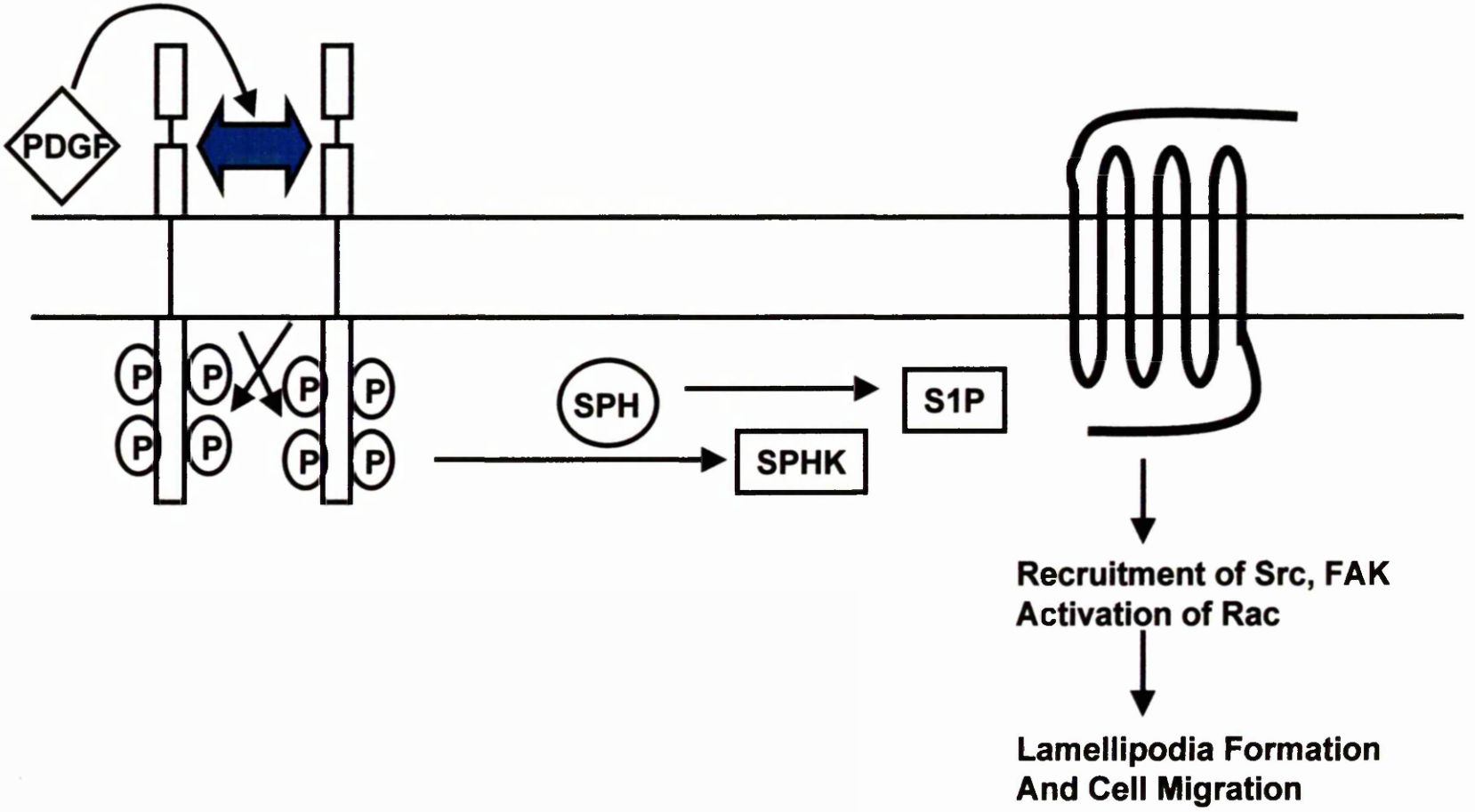

PDGF
P
P
P
P
P
P
P
P
SPH
SPHK
S1P
Recruitment of Src, FAK
Activation of Rac
Lamellipodia Formation
And Cell Migration

Figure 1.13: Internalisation Of The β_2AR Receptor

Agonist-induced β_2AR desensitisation is caused by a conformational change of the agonist-occupied receptor that facillitates receptor phosphorylation by G-protein receptor kinases (GRKs). Following β_2AR phosphorylation, the scaffold protein β-arrestin binds to the phosphorylated receptor and uncouples the receptor from heterotrimeric G-proteins. β-arrestin not only desensitises the receptor but also functions as a clathrin adaptor, mediating receptor sequestration *via* clathrin-coated vesicles. Figure adapted from Pierce *et al.*, 2001.

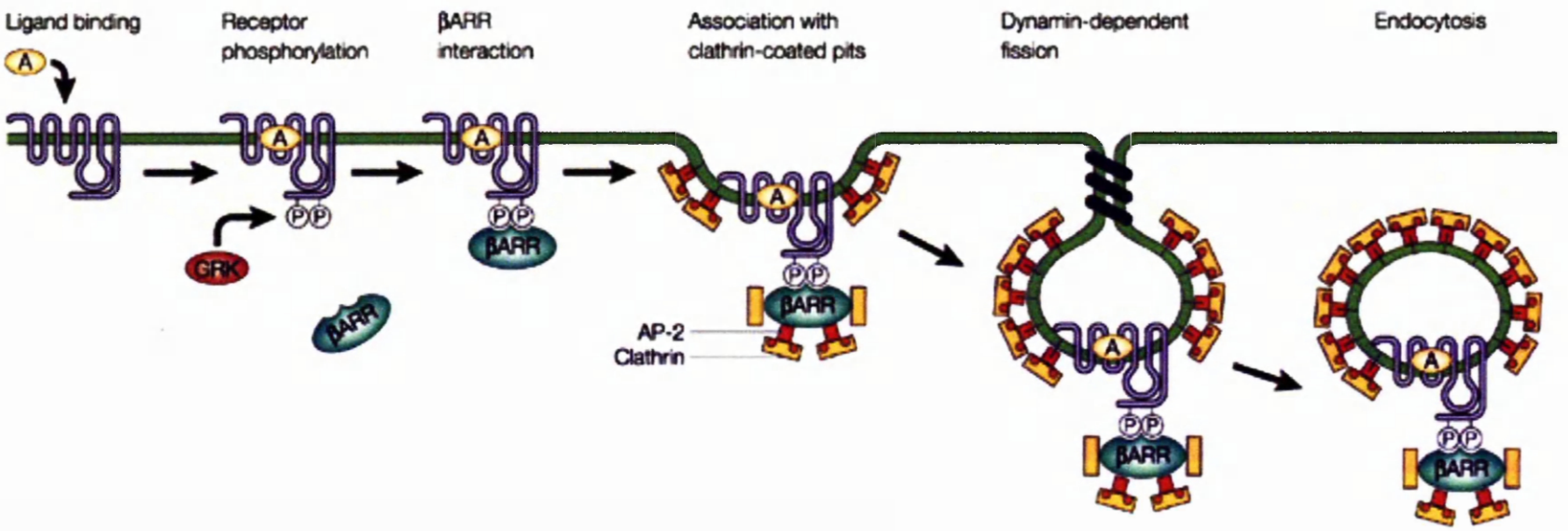

Ligand binding
Receptor phosphorylation
βARR interaction
Association with clathrin-coated pits
Dynamin-dependent fission
Endocytosis
A
GRK
βARR
AP-2
Clathrin
P

Figure 1.14: The Family Of G-Protein Receptor Kinases (GRKs)

GRKs consist of 7 members, GRK1-7. Each member contains a central catalytic domain, an N-terminal domain for substrate recognition and containing a conserved RGS domain and a C-terminal domain for targeting GRKs to the plasma membrane. The GRK family can be subdivided into 3 groups: 1) GRK1 (rhodopsin kinase) and GRK7 (cone opsin kinase); 2) GRK2 (β-adrenergic kinase 1, βARK1) and GRK3 (β-adrenergic kinase 2, βARK2) and 3) GRK4, GRK5 and GRK6. Figure adapted from Ferguson, 2001.

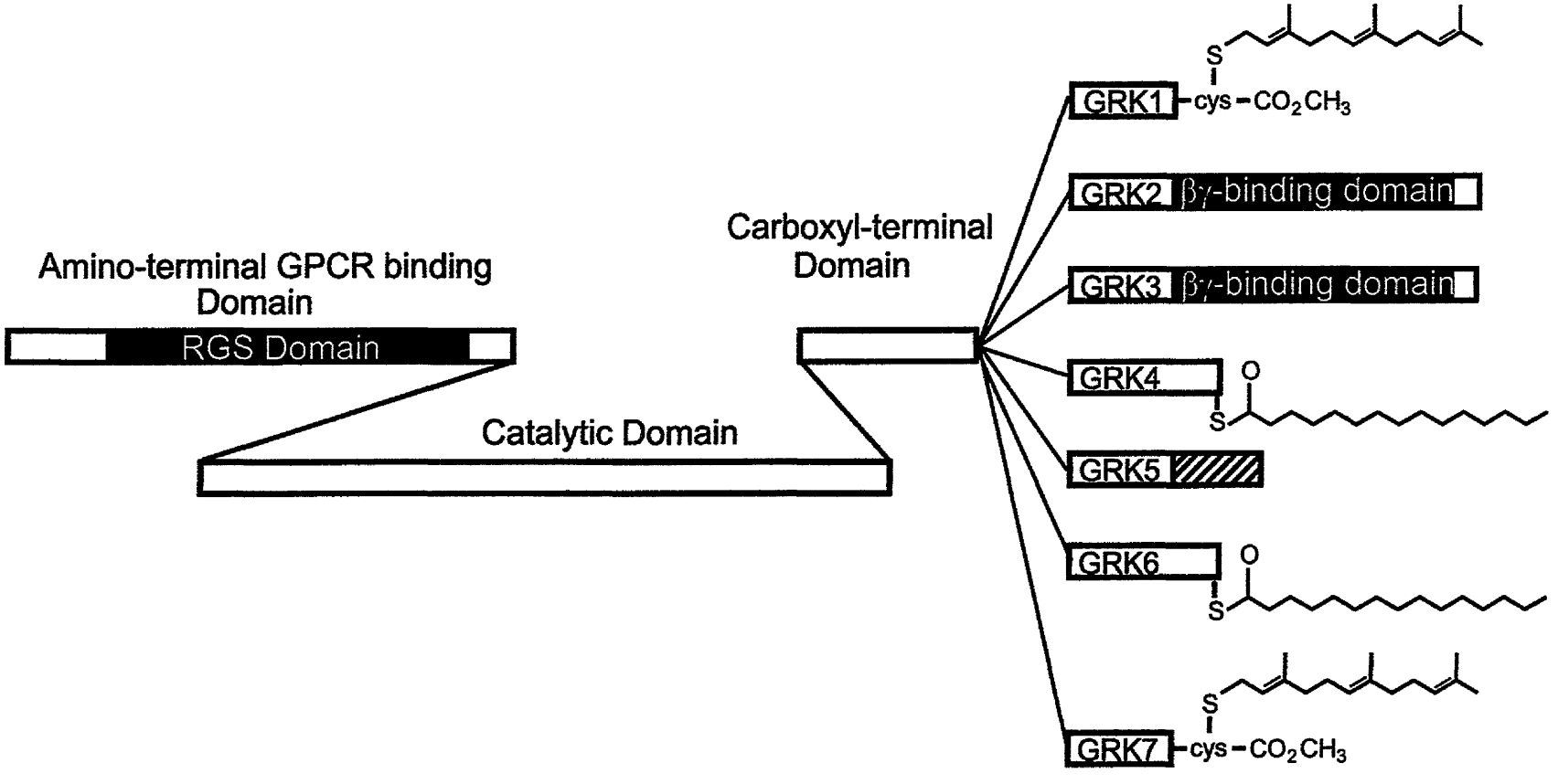

Amino-terminal GPCR binding Domain
RGS Domain
Catalytic Domain
Carboxyl-terminal Domain
GRK1
cys – CO_2CH_3
GRK2 βγ-binding domain
GRK3 βγ-binding domain
GRK4
GRK5
GRK6
GRK7
cys – CO_2CH_3

Figure 1.15: The Family Of Arrestins

The arrestins are adaptor proteins that preferentially bind agonist-activated and GRK-phosphorylated GPCRs where they form a scaffold complex that results in receptor uncoupling from G-proteins, often targets the receptor for internalisation *via* clathrin-coated vesicles and can mediate the activation of alternative signalling pathways. The arrestin family is subdivided into 2 groups: a) visual arrestin (S antigen) and cone arrestin (X-arrestin/C-arrestin) and b) β-arrestins (β-arrestin1 and β-arrestin2). The structure of arrestins constitute 3 functional domains (a secondary receptor-binding domain, a receptor activation domain and a phosphate sensor domain) and two regulatory domains, located at the amino terminal and carboxyl-terminal. Within the N-terminal domain of β-arrestin1 and β-arrestin2, but not the visual arrestins, there is a proline-rich region (underlined in panel A). The black box in panel A illustrates the clathrin- and β-adaptin-binding domains within the C-terminus of nonvisual arrestins. Panal B demonstrates the functional differences between visual and nonvisual arrestins that arise as the consequence of the clathrin (bold) and β2-adaptin (bold and asterisked) binding domains amongst nonvisual arrestins. Figure adapted from Ferguson, 2001.

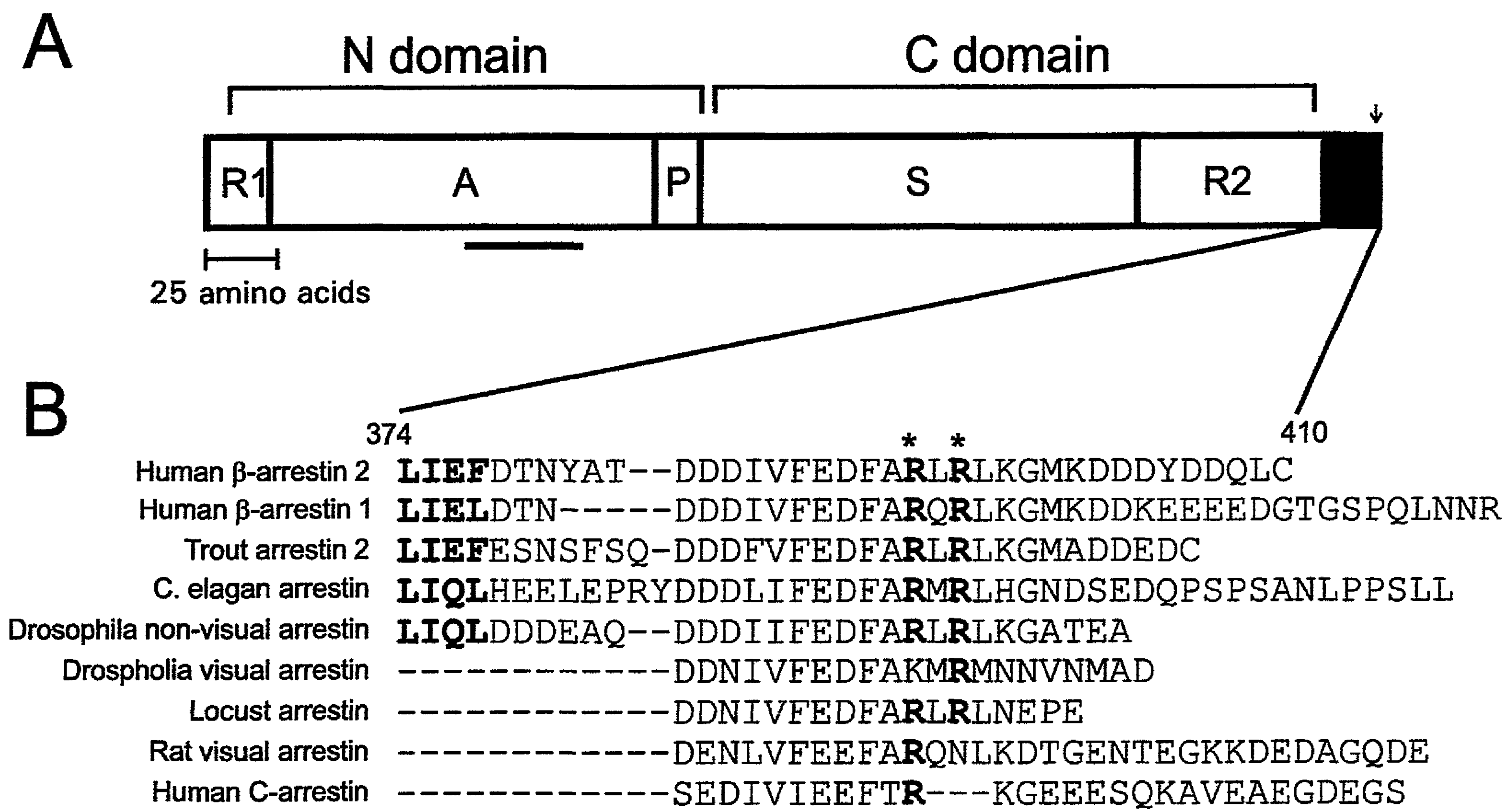

A
N domain
C domain
R1
A
P
S
R2
25 amino acids
B
374
410
* *
Human β-arrestin 2 LIEFDTNYAT--DDDIVFEDFARLRLKGMKDDDYDDQLC
Human β-arrestin 1 LIELDTN-----DDDIVFEDFARQRLKGMKDDKEEEEDGTGSPQLNNR
Trout arrestin 2 LIEFESNSFSQ-DDDFVFEDFARLRLKGMADDEDC
C. elagan arrestin LIQLHEELEPRYDDDLIFEDFARMRLHGNDSEDQPSPSANLPPSLL
Drosophila non-visual arrestin LIQLDDDEAQ--DDDIIFEDFARLRLKGATEA
Drospholia visual arrestin ------------DDNIVFEDFAKMRMNNVNMAD
Locust arrestin ------------DDNIVFEDFARLRLNEPE
Rat visual arrestin ------------DENLVFEEFARQNLKDTGENTEGKKDEDAGQDE
Human C-arrestin ------------SEDIVIEEFTR---KGEEESQKAVEAEGDEGS

Figure 1.16: Components Of The Endocytic Machinery

A. Clathrin: three-legged triskelion, with each leg containing a heavy and a light chain. The N-terminal globular region of the heavy chain interacts with β-arrestin.

B. AP-2: tetrameric adapter protein that links the clathrin shell to the membrane via interactions between its μ2 and σ2 subunits with membrane proteins. β-arrestin interacts with the β2 subunit.

C. AP180: Involved in regulating the size of the clathrin-coated pit. Contains PIP_2-binding epsin amino-terminal homology (ENTH) domain and clathrin assembly domains.

D. Synaptotagmin: binds AP-2 within synaptic vescles and allows vesicle recycling via coated pit nucleation. Contains protein kinase C homology 2 (C2A, C2B) domains.

E. Dynamin: multi-domain protein with GTPase domain, a phospholipid-binding pleckstrin homology (PH) domain, GTPase effector domain (GED) and a proline-rich domain within its C-terminal (PRD) which interacts with SH3 domains of other accessory proteins such as amphiphysin. Main role of dynamin is in the budding of the pits from the membrane.

F. Endophilin: Converts lysophosphatidic acid within membrane to phosphatidic acid via LPA-ATas domain and facilitates invagination of the coated pit.

G. Amphiphysin: Interacts with dynamin to facilitate fission. Contains clathrin/AP-2 binding and SH3 domain.

H. Epsin: bind to clathrin, AP-2 and PIP_2. Contains epsin amino-terminal homology (ENTH), Asp-Pro-Trp (DPW) and Asp-Pro-Phe (NPF) domains.

I. Synaptojanin: inositol phosphatase that regulates PIP_2 metabolism and the stability of clathrin-AP-2 coats. Contains suppressor of actin 1 (Sac1), 5'phosphatase, PRD and NPF domains.

J. Auxilin: J-domain protein that assists the ATPase heat shock cognate protein 70kDa (hsp70) in the uncoating and disassembly of clathrin coated vesicles.

Figure adapted from Brodin *et al.*, 2000; Takei and Haucke, 2001

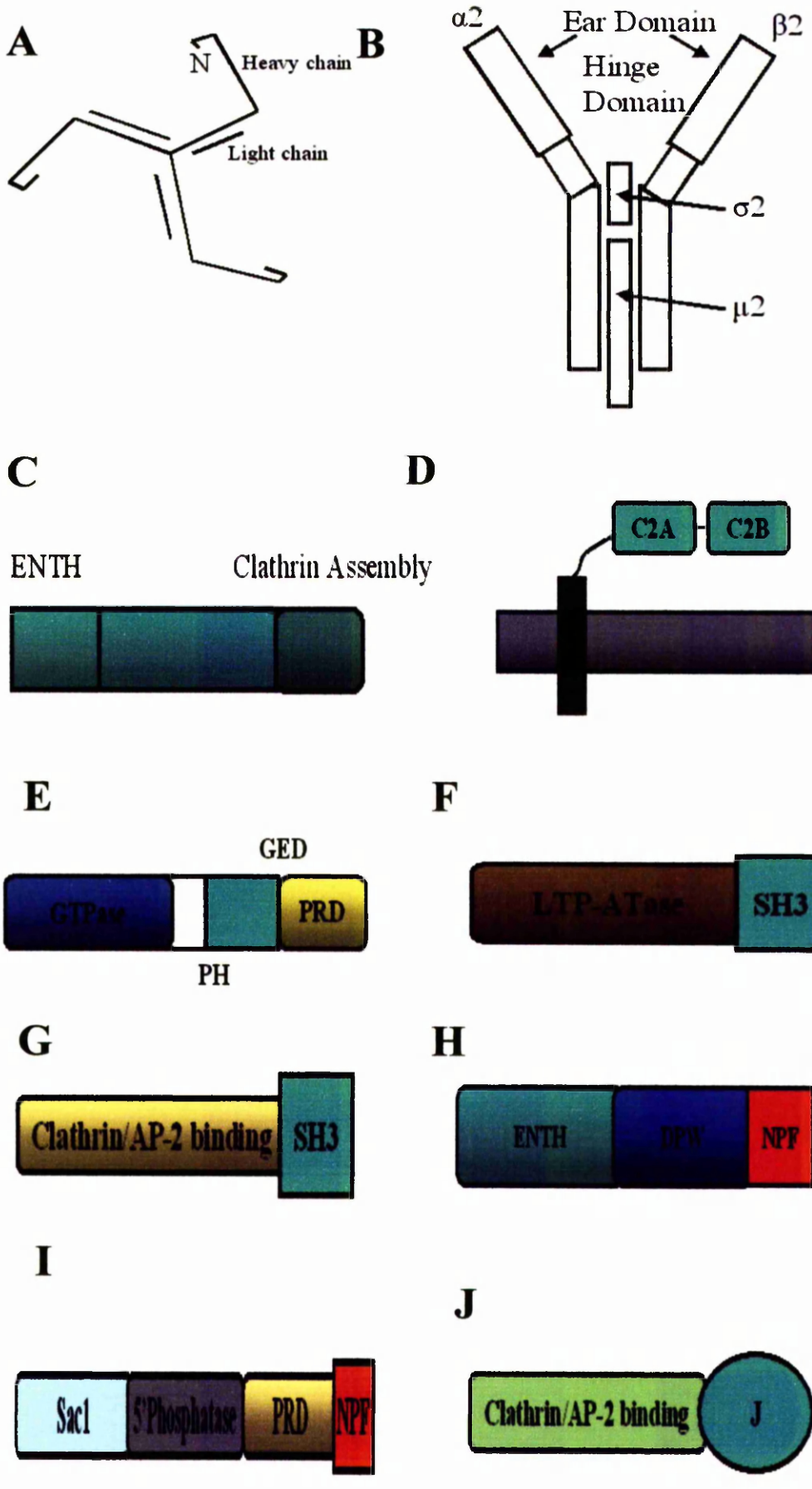
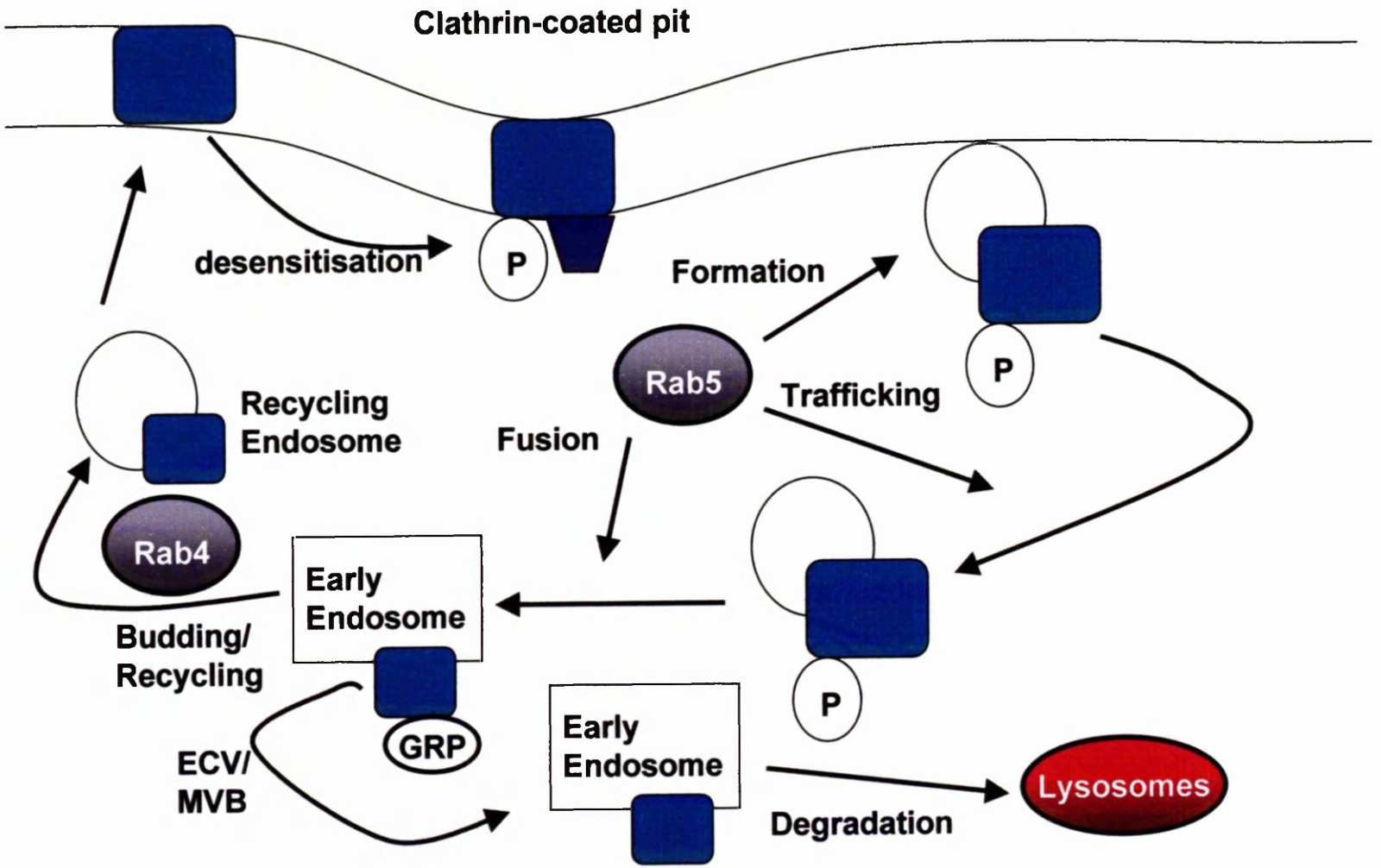

A
N
Heavy chain
Light chain
B
α2
Ear Domain
β2
Hinge
Domain
σ2
μ2
C
ENTH
Clathrin Assembly
D
C2A
C2B
E
GED
GTPase
PRD
PH
F
LTP-ATase
SH3
G
Clathrin/AP-2 binding
SH3
H
ENTH
DPW
NPF
I
Sac1
5'Phosphatase
PRD
NPF
J
Clathrin/AP-2 binding
J

Figure 1.17: Trafficking Of Internalised GPCRs

Agonist-activated receptors (green) are phosphorylated by GRK (P), facilitating their interaction arrestin molecules (blue). Once internalised, receptors are delivered to peripheral early endosomes where they are dephosphorylated by a GPCR-specific phosphatase (GRP). The small GTPase Rab5 often contributes to the formation of endocytic vesicles, the trafficking of vesicles to early endosomes and the fusion of endocytic vesicles with early endosomes. Once delivered to early endosomes, recycling receptors such as the β_2AR and the transferrin receptors are returned to the cell surface via recycling endosomes. The small GTPase, Rab4 regulates the budding and/or recycling of receptor-bearing recycling vesicles. In contrast to recycling receptors, some endocytosed receptors targeted for downregulation are transported from early to late endosomes where they are then targeted to lysosomes for degradation through the action of intermediates with a characteristic multivesicular appearance called multivesicular bodies (MVBs) or endosomal carrier vesicles (ECVs). In mammalian cells, ECVs/MVBs, once formed on early endosomes, move towards late endosomes on microtubules and then dock onto and fuse with late endosomes.

Figure adapted from Cavalli *et al.*, 2001; Ferguson, 2001


Clathrin-coated pit
desensitisation
P
Formation
Rab5
Trafficking
P
Recycling
Endosome
Fusion
Rab4
Early
Endosome
Budding/
Recycling
P
GRP
Early
Endosome
ECV/
MVB
Degradation
Lysosomes

Chapter 2
Materials and Methods

2.1 Materials

All reagents used were of the highest grade commercially available and obtained from the following suppliers:

Alexis Corporation, San Diego, CA, USA

Dithiothreitol

BDH Chemicals Ltd., Poole, UK

Acrylamide, coverslips

Calbiochem-Novabiochem (UK) Ltd., Nottingham, UK

Forskolin, PMA, S1P

Costar, Cambridge, MA, USA

75cm^2 tissue culture flasks, 60mm and 100mm tissue culture dishes, 6-, 12- and 24- well tissue culture plates, cryovials

Cruachem, Glasgow, UK

Oligonucleotides

Fisher Scientific, Loughborough, Leicestershire, UK

HEPES, sodium dodecyl sulphate, EDTA, DMSO, ethidium bromide solution, glacial acetic acid, methanol, ethanol, concentrated HCl, sodium fluoride, sodium phosphate

GIBCO BRL Life Technologies, Paisley, UK

Phenol:chloroform:isoamyl alcohol, LipofectAMINE, newborn calf serum, OptiMEM, phosphate-free Dulbecco's Modified Eagle's Medium (PF-DMEM)

Melford, Chelsworth, Ipswich, Suffolk, UK

Kanamycin

Merck, Darmstadt, Germany

Bactotryptone, agar

Molecular Probes

Alexa^{TM}594-conjugated goat-anti-mouse IgG

New England Biolabs Inc., Beverley

Protein molecular weight marker, restriction enzymes

NEN Life Science Products Inc., Boston

ECL reagents, ^{32}P-orthophosphate, X-ray film

Pierce, Rockford, IL 61105, USA

EZ-Link^{TM} Biotin-LC-Hydrazide, HRP-streptavidin

Promega, Southampton, UK

T4 DNA ligase, SV mini-prep kit, G-418 sulphate, restriction enzymes

Qiagen, Crawley, West Sussex

Gel purification kit, plasmid maxi kit

Research Biochemicals International, Natick, MA, USA

(R)-PIA, NECA

Roche Molecular Biochemicals/Boehringer-Mannheim, Mannheim, Germany

Tris, DNA molecular weight marker, restriction enzymes, anti-HA mouse monoclonal IgG (clone 12CA5), adenosine deaminase

Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA

GFP rabbit polyclonal IgG

Sigma-Aldrich Company Ltd., Poole, Dorset, UK

Triton X-100, soybean, benzamidine, pepstatin A, bovine serum albumin, fatty acid free bovine serum albumin, protein A-Sepharose, sodium periodate, bisacrylamide, HRP-conjugated goat anti-rabbit IgG, HRP-conjugated goat anti-mouse IgG, HRP-streptavidin, thimerosal, bromophenol blue, bichinchonic acid, sodium azide, 3-[(3-Cholamidopropyl)-dimethylammino]-1-propane sulfonate (CHAPS), agarose, deoxycholic acid, myelin basic

protein (MBP), polyethylenimine, ninhydrin, ammonium hydroxide, isobutyric acid, ampicillin, adenosine deaminase, paraformaldehyde, N,N,N',N'-tetramethylethylenediamine (TEMED), Phenylmethylsulphonylfluoride (PMSF), chlorpromazine, 8-bromo-cGMP, Ham's F-12, Dulbecco's Modified Eagle's Medium (DMEM), Phosphate-buffered saline (PBS) (sterile), foetal bovine serum (FBS), trypsin, penicillin/streptomycin, L-glutamine

Stratagene

P*fu* Turbo DNA polymerase

Tocris/Semat Technical (UK) Ltd., St. Albans, Herts., UK

A23187

Whatman International Ltd., Maidstone, UK

GF/C glass fibre filters

A mutant human A_1AR cDNA in which the Cys 309 was changed to Ala was a gift from Dr Mark Olah, University of Cincinnati College of Medicine, Cincinnati, OH

9E10 monoclonal antibody specific to myc-epitope, was prepared in-house at Duke University, Durham, NC by Dr Tim Palmer

Purified GRK2 and GRK5 were a gift from Jeffrey L. Benovic, Kimmel Cancer Centre, Department of Microbiology and Immunology, Thomas Jefferson University, Philadelphia PA19107

2.2 Cell Culture And Transfections

2.2.1 Cell Maintenance

CHO cells were maintained in Ham's F-12 medium and HEK293 cells were maintained in DMEM, supplemented with 10%(v/v) FBS, penicillin (100units/ml), streptomycin (100μg/ml) and 1% L-glutamine in a 37°C humidified atmosphere containing 5% CO_2. Cells stably expressing adenosine or EDG1 receptors were maintained in the appropriate medium supplemented with G-418 in order to optimise receptor expression by maintaining selection pressure. Cells were routinely passaged 1:8. Confluent T-75 flasks were washed with PBS without $CaCl_2$ and $MgCl_2$. Cells were detached by the addition of 2ml of trypsin, followed by incubation at 37°C. 6mls of medium were then added to the flasks and the cells pipetted gently to allow resuspension. Cells were either passaged into flasks to maintain the cell line or seeded into dishes for experimental analysis.

2.2.2 Transient Expression Of cDNA Expression Constructs

cDNA expression constructs were transiently transfected into either HEK293 or CHO cells using a Lipofectamine-mediated transient transfection protocol. Cells were plated into 6-well dishes at the appropriate density such that they would be 70-80% confluent the next day. Transfection mixes of 0.24ml OptiMEM, 2μg plasmid DNA and 4μl lipofectamine were prepared in sterile microfuge tubes for each well. The tubes were then incubated at room temperature for 15-45 minutes. During this incubation, each cell monolayer was washed once with 2 ml/well OptiMEM and then given 0.75ml/well of OptiMEM. Following the 15-45 minute incubation, the Lipofectamine-DNA-OptiMEM mixes were added to each well and incubated for 3 hours at 37°C. The mixture from each well was then removed and replaced with 3ml/well of growth medium. Cells were analysed 48-72 hours post-transfection.

2.2.3 Stable Expression of cDNA Expression Constructs

CHO or hamster lung CCL-39 fibroblast stable cell lines were generated by cotransfecting cells with the appropriate cDNAs subcloned into pcDNA3.1 and pSVNeo in a 20:1 ratio using a modified calcium phosphate precipitation/glycerol shock procedure. A confluent T-75 of plain CCL-39 fibroblasts, routinely grown in Dulbecco's modified Eagle's medium, or CHOs, maintained in Ham's F-12 medium, were split 1:5, adding $1/5^{th}$ of cells

to a 100mm dish. The next morning, after reaching 50-70% confluency, the cells were given fresh media. In the afternoon, the following transfection mix was added to a sterile Falcon tube: 30μg pcDNA3.1 and 1.5 μg of the appropriate cDNA containing pSVNeo to confer G418 resistance. This was made up to a final volume of 375 μl with sterile water. 125 μl 1M $CaCl_2$ solution was then added. Finally, using a sterile 1ml pipette, 0.5ml 2X Hepes buffered saline solution (HBSS) (280mM NaCl, 50mM HEPES, 1.5mM Na_2HPO_4, pH 7.13) was added slowly to the tube, and mixed by gently bubbling air into the mix in the tube for approximately 30 seconds until the solution becomes slightly milky due to the formation of a fine calcium phosphate precipitate. The mix was left at room temperature for 40 minutes and then added dropwise to the cells in the 100mm dish. Following a 4-5 hour incubation at 37°C, the media was aspirated and the cells rinsed with 5ml PBS. The cells then underwent glycerol shock by incubating with 2ml of a 15% (v/v) glycerol solution (1.5 ml sterile glycerol, 3.5ml sterile water, 5ml 2XHBSS) for exactly 2 minutes at 37°C. The glycerol solution was then aspirated off and the cells washed three times with PBS and incubated with 10ml regular medium overnight. On day 3, the medium was changed on the cells. On day 4, the cells were split for selection with 0.4mg/ml G418 into 5 dishes representing a 2:5, 1:5, 1:10, 1:20 and a 1:40 cell suspension. After selection in G418, resistant colonies were isolated, expanded and screened for receptor expression by western blotting and, in the case of the GFP constructs, fluorescence following excitation at 488 nm using an argon/krypton laser.

2.3 Molecular Biology

2.3.1 Preparation Of Antibiotic Agar Plates

LB agar (1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, with 1.5% (w/v) agar) was prepared, autoclaved and allowed to cool before the addition of the appropriate antibiotic (either ampicillin at 50μg/ml or kanamycin at 30μg/ml). The liquid LB was poured into 90mm diameter Petri dishes and allowed to solidify and sweat overnight at room temperature. Plates were then stored at 4°C until required.

2.3.2 Preparation of competent XL1 Blue E.coli

An overnight culture of XL1 Blue E.coli was grown in 3ml of LB broth containing 50μg/ml tetracycline. The next day, 250ml of LB broth was inoculated with the culture and grown with aeration until the cells reached log phase. The cells were then transferred

into two 250ml centrifuge tubes on ice and left for 1 hour. Log phase was defined when the optical density (OD_{600}) reached approximately 0.35-0.375. Cells were spun at 3468g at 4°C for 20 minutes, the supernatant decanted and the cells resuspended and washed in $^1/_4$ starting volume of ice-cold 0.1M $MgCl_2$. Following a second 20 min centrifugation, the cells were resuspended in $^1/_4$ starting volume of ice-cold 15% (v/v) glycerol with 0.1M $CaCl_2$. 250µl of resuspended cells were each aliquoted into sterile microfuge tubes on dry ice/methanol, to induce rapid freezing, and stored at –80°C until required.

2.3.3 Transformation Of Competent *E.Coli*

Approximately 30-50 ng of DNA was added to a plastic Falcon tube on ice. 50µl of thawed competent *E.coli* were added and the DNA and *E.coli* mix incubated on ice for 10 min. The tubes were then incubated for 5 minutes in a 37°C water bath. 0.5ml LB/tube was then added and the tubes were then incubated for 45 min at 37°C. 200µl from each transformation was then plated out onto an LB agar plate supplemented with the appropriate selection antibiotic and incubated overnight at 37°C.

2.3.4 Preparation Of Plasmid DNA

Transformed colonies were picked from agar plates and grown overnight in 5-10 mls of LB broth containing the appropriate antibiotic. Plasmid DNA was prepared using the Promega™ Wizard Plus SV miniprep purification system as per the manufacturer's instructions. Larger quantities of DNA were obtained by transferring an initial overnight culture into 500ml of LB broth containing the appropriate antibiotic and grown overnight. DNA purification was achieved obtained using the Qiagen plasmid maxi kit system. The concentration of DNA obtained was determined by measuring the absorbance at 260nm (A_{260}) of a 1:50 dilution of each preparation in sterile H_20, assuming that 1 absorbance unit was equivalent to 50µg/ml of double stranded DNA.

2.3.5 Digestion Of Plasmid DNA

1-2 µg of plasmid DNA was digested in a volume of 15µl using the buffer conditions recommended by the manufacturer with 2-4 units of the appropriate restriction enzyme. Digested DNA was analysed by agarose gel electrophoresis in which samples were prepared by the addition of a 1:3 dilution of loading buffer. Electrophoresis took place on a 1% (w/v) gel containing 2.5mg/ml ethidium bromide at 75 V for 20-30 minutes

in a TAE buffer (40mM Tris-acetate, 1mM EDTA, glacial acetic acid). DNA purification from excised agarose gel chips was achieved using Qiagen QIAquick gel purification kit, as per manufacturer's instructions.

2.3.6 Ligation of DNA fragments

Ligation of vector DNA was carried out overnight at 4°C in a reaction volume of 10µl containing 30mM Tris-HCl, pH 7.8, 10mM $MgCl_2$, 10mM DDT, 1mM ATP, T4 DNA ligase vector and insert DNA at a ratio of 1:4. Ligated DNA was then transformed into competent *E.coli* as described in section 2.3.3.

2.3.7 Construction Of HA-A_1AR-GFP And HA-A_1(Cys309-Ala)AR-GFP Receptor Constructs

The HA-A_1AR-GFP and HA-A_1(Cys309-Ala)AR-GFP constructs were generated by PCR using the pCMV5/HA epitope-tagged WT (Ren and Stiles, 1994) and Cys309-Ala human A_1AR cDNAs as templates. Standard PCR reactions contained, in a volume of 100µl, 100ng template DNA, 100µM dNTP's, 50pmol sense/antisense primers, 0.002 units P*fu* turbo, 10µl 10-fold concentrated amplification buffer and 5% (v/v) DMSO. The reaction was initiated by a denaturation cycle of 95°C for 5 min followed by 30-40 cycles of a 95°C (1 min) denaturing step, 55°C (1 min) annealing step and 72°C (1.5 min) extension step. A final cycle of 95°C (1 min), 55°C (1 min) and 72°C (10 min) was added before reactions were placed at 4°C until required. For each receptor, the primers used were: -

5'-ATTTG**GAATTC**CCACC*ATG*CCGCCCTCCATCTCAGC-3' (sense) and

5'-ATTTG**GGTACC**GCAGCGTAGTCTGGGAC-3' (antisense)

The sense primer was designed to remove the N-terminal HA epitope tag sequence and add an *Eco*RI site (bold) upstream of a consensus Kozak sequence (underlined) and the A_1AR initiating Met (italics). The antisense primer was designed to remove the A_1AR stop codon and add an *Xba*I site (bold). This allowed in-frame fusion of the C-terminally HA epitope-tagged A_1AR open reading frames with GFP following subcloning of the *Eco*RI/*Xba*I-digested PCR products with the similarly digested modified pEGFP-N1 cDNA in which the initiating Met of the GFP open reading frame was mutated to Ala.

2.3.8 Construction Of EDG1 Receptor Constructs

(i) Generation Of Human MycEDG1 cDNA Expression Construct: - This was generated by PCR using a pcDNA/EDG1 template. The following primers were used: -

dCATTG**AAGCTT**CCACC*ATG*GGGCCCACCAGCGT (sense)
dCATTG**TCTAGA**GGAAGAAGAGTTGA (antisense)

The sense primer incorporated a *Hind*III site (bold) upstream of a consensus Kozak sequence (underlined) and the EDG1 initiating methionine (italics). The antisense primer was designed to remove the EDG1 stop codon and add an XbaI site. This allowed in-frame ligation of the EDG1 coding region with that of the myc-His epitope tag following ligation of the *Hind*III/*Xba*I-digested PCR product with a similarly digested pcDNA3.1/ myc-HisA vector (Invitrogen).

(ii) Generation Of MycEDG Δ51, Δ32 and Δ12 cDNA Expression Constructs: - These were generated by PCR using the pcDNA3.1/myc-His-EDG1 receptor as a template. The primers are shown below: -

dTCTGGCTAACTAGAGAACC(sense)
dATTTGCTCTAGAGCACTTGCAGCAGGACATGAT (antisense (Δ51))
dATTTGCTCTAGAGAATTCCCATGCCGGCGATGAT (antisense (Δ32))
dATTTGCTCTAGACTCTGGGTTGTCCCCTTCGTCTTTCTG (antisense (Δ12))

The same sense primer was used to for all three truncation mutants and was designed to anneal upstream of the *Hind*III site of the EDG1 receptor. The antisense primers for each of the truncation mutant were designed to anneal either 12-, 32-, or 51 amino acids upstream of the EDG1 C-terminal tail for the appropriate truncation mutant and to add an *Xba*I site (bold). This allowed in-frame ligation of the *Hind*III/*Xba*I-digested PCR products with a similarly digested pcDNA3.1/myc-HisA vector.

(iii) Generation Of MycEDG1-GFP And MycEDG1Δ51-GFP cDNA Expression constructs:- These were generated by PCR using the pcDNA3.1/myc-His-EDG1 receptor

and the pcDNA3.1/myc-His-EDG1Δ51 receptor as templates for the pcDNA3.1/myc-His-EDG1-GFP and pcDNA3.1/myc-His-EDG1Δ51-GFP constructs respectively. The primers used are shown below: -

dTCTGGCTAACTAGAGAACC	(sense)
dCATTG**GGATCC**CGATGGTGATGGTGATGATG	(antisense)

The sense primer was as described for the truncation mutants above. The antisense primer was designed to remove the stop codon in the myc-His tag of the EDG1 and EDG1Δ51 receptors with that of GFP following ligation of *Hind*III/*Bam*HI-digested PCR product with a similarly digested pEGFPAla1 vector.

2.3.9 Confirmation Of cDNA Receptor Subcloning

All the above reactions sites were confirmed by overnight double digestion of the constructs with the appropriate restriction enzymes. The completed digests were ran out on 1% agarose gels at 75 V in TAE buffer. Correct subcloning of the receptors was confirmed by the use of dideoxynucleotide sequencing.

2.4 Experimental Techniques

2.4.1 Preparation Of S1P

1mg of commercially supplied S1P (Calbiochem) was resuspended in 0.66ml of methanol to give a final S1P concentration of 4mM. The tube was capped tightly and transferred to an 80°C oven. The S1P was then vortexed every 5 min until it had gone visibly into solution. The S1P solution was then pipetted into 25µl aliquots in brown glass vials. The methanol solvent was then evaporated off using a nitrogen gas stream. The tubes were then capped and stored at –80°C. Reconstitution of S1P was achieved by adding 0.25 ml serum-free medium supplemented with 0.5mg/ml fatty acid-free BSA to give a working S1P stock concentration of 400µM. Following addition of "medium + BSA", the vial was vortexed repeatedly to resuspend the S1P.

2.4.2 Preparation Of Cell Extracts For Immunoblotting

Confluent monolayers in 6-well dishes were kept on ice and washed three times with PBS. Cells were solubilized by scraping into 250µl of immunoprecipitation buffer (50mM

Hepes, pH 7.5, 5mM EDTA, 10mM sodium fluoride, 10mM sodium phosphate, 0.1mM phenylmethylsulfonyl fluoride, and 10μg/ml each of soybean trypsin inhibitor, leupeptin, and pepstatin A). The lysate was then transferred into ice-cold microfuge tubes. The cells were solubilized by a 1 hour incubation on a rotating wheel at 4°C. Lysates were clarified by centrifugation (14000g for 15 minutes, 4°C) and supernatants assayed for protein content. BSA standards ranging from 0-2mg/ml were used to obtain a best-fit straight line of a plot of A_{492} versus protein concentration in a bicinchonic acid (BCA) based protein assay using the graph package "Prism v20." The protein concentrations of 10μl samples of each unknown extract were calculated from the equation of the best-fit straight line obtained from the BSA standards.

2.4.3 SDS-PAGE And Immunoblotting

Samples solubilised in immunoprecipitation buffer were equilibrated for protein concentration. The appropriate quantity of Laemmli sample buffer (50nM Tris (pH 6.8), 10% (v/v) glycerol, 12% (w/v) SDS, few grains of bromophenol blue, 1.6mg/ml dithiothreitol) was then added to each to sample to give a total volume of 30μl per sample. Samples were separated by SDS-PAGE using a 10% acrylamide resolving gel (10% (w/v) acrylamide, 0.3% (w/v) bisacrylamide, 0.4M Tris (pH 8.8), 0.1% (w/v) SDS, 3% (v/v) glycerol, 0.01% (w/v) ammonium persulphate and 0.001% (v/v) TEMED) and 3% acrylamide stacking gel (3% (v/v) acrylamide, 0.1% (v/v) bisacrylamide, 0.1M Tris (pH 6.8), 0.1% (w/v) SDS, 0.01% (w/v) ammonium persulphate and 0.001% (v/v) TEMED). Electrophoresis of the samples was carried out at 150 V in conjunction with prestained protein markers (6.5-175 kDa) in a running buffer containing 27.4mM Tris, 0.19M glycine and 0.1% (w/v) SDS until the dye front reached the end of the gel. The resolved proteins were then transferred to nitrocellulose at 400mA for 45 min in transfer buffer containing 24.7mM Tris, 0.19M glycine and 20% (v/v) methanol. Following transfer, the nitrocellulose was washed briefly in PBS and incubated for 1 hour in Blotto (5% (w/v) skimmed milk in PBS supplemented with 0.2% (v/v) Triton X-100) to block non-specific protein binding sites. Membranes were then incubated for 1 hour with the appropriate dilution of primary antibody in fresh Blotto. The membranes were then washed three times for 10min with Blotto followed by two brief washes in PBS. Membranes were then incubated for 1 hour with HRP-conjugated secondary antibody made in high-detergent blotto (10% (v/v) Blotto in PBS supplemented with 1.02% (v/v) Triton X-100 and 0.1%

(w/v) SDS). The membranes were then washed three times for 10 min in Blotto, followed by three washes with PBS for 10 min. Membranes were then incubated for 1 min at room temperature with ECL reagents (1ml solution A, 1ml solution B). Immunostained protein was then visualised by autoradiography.

2.4.4 Immune Complex Kinase Assay Of ERK Activation

Quiescent CCL39 and CCL39/mycEDG1 cells in 100mm dishes were stimulated with increasing concentrations of S1P. Reactions were quenched by placing the cells on ice and washing rapidly with ice-cold PBS then quenched reactions. Cells were then solubilised in 0.7ml detergent lysis buffer and clarified extracts equalised for protein content prior to immunoprecipitation for 2 hours at 4°C with 50µl of a 50% suspension of protein A-Sepharose beads and 5µl ERK1-specific antiserum (Cook et al., 1993). Immune complexes were isolated by centrifugation and washed three times (1ml/wash) with lysis buffer (10mM Hepes, pH 7.5, 2mM EDTA, 0.25 M sodium chloride supplemented with 0.1 mM PMSF, 10µg/ml soybean trypsin inhibitor and 10µg/ml benzamidine). All remaining buffer was removed from immune complexes following the last rinse using a fine-gauge Hamilton syringe. Immune-complex kinase assays were then performed by adding 30µl kinase buffer containing 10 µM ATP, 100 µCi/ml [γ-^{32}P]ATP, and 100µg/ml myelin basic protein (MBP). Kinase reactions took place at 30°C for 30 min and were stopped by adding 10µl of 4xSDS-PAGE sample buffer and boiling for 10 min. MBP samples were fractionated by SDS-PAGE using 14% (w/v) polyacrylamide resolving gels and stained with Coomassie Brilliant Blue. Gels were then dried and the amount of ^{32}P incorporated into MBP determined by phosphorimaging.

2.4.5 Phosphoamino Acid Analysis

Following SDS-PAGE, resolved proteins were transferred to a polyvinylidene difluoride membrane. After overnight autoradiography, the region of the membrane corresponding to phosphorylated EDG1 was excised, hydrated and, following purging with nitrogen, hydrolysed at 110°C in 0.2ml 5.7 M hydrochloric acid for 90 min. The hydrolysate was then lyophilised and resuspended in chromatography buffer supplemented with phosphoamino acid standards. After spotting onto cellulose-coated plastic-backed plates, samples were subjected to ascending chromatography in an isobutyric acid, 0.5M ammonium hydroxide (5:3, v:v) buffer system (Duclos et al., 1991). Standards were

visualised by ninhydrin staining and ^{32}P-labelled amino acids identified by autoradiography. Phosphorylated amino acids were identified by comparison with ninhydrin-stained standards.

2.4.6 Whole Cell Receptor Phosphorylation

Cells were plated into 6-well dishes at a density of $1x10^6$ cells/well and cultured overnight. When using cell lines expressing one of the EDG1 constructs, the cells were serum-starved for 16-20 hours in serum-free Dulbecco's modified Eagle's medium. The next day, the cells were washed twice with 3ml phosphate-free Dulbecco's modified Eagle's medium and incubated for 90 minutes at 37°C with 0.75ml of the same medium supplemented with 50μCi/well [^{32}P] orthophosphate. After stimulation with the indicated agonists, added as a 2X concentrated dose in 0.75ml, reactions were terminated by placing the cells on ice and washing the monolayers twice with 3ml of ice-cold phosphate-buffered saline. All subsequent procedures were carried out at 4°C unless indicated otherwise. Cells were washed three times with PBS and solubilized by scraping into an initial 250μl of immunoprecipitation buffer. The lysate was then transferred into ice-cold microfuge tubes. The wells were then washed in another 250μl of immunoprecipitation buffer which was subsequently transferred to the appropriate microfuge tube. The cells were then solubilized and analysed for protein content as described in 2.4.2. Equivalent amounts of soluble protein from each sample were then made up to 400μl with immunoprecipitation buffer and added to microfuge tubes containing 100μl 0.2% (w/v) IgG-free bovine serum albumin and, in the case of myc-tagged constructs, 50μl of a 50% (v/v) suspension of 9E10-conjugated protein G-Sepharose beads for 1 hour. HA-tagged constructs were incubated with 20μl 50% suspension of protein-A Sepharose beads, 100μl 0.2% (w/v) IgG-free BSA in the presence of 1μg 12CA5. Following overnight incubation on a rotating wheel, immune complexes were isolated by brief centrifugation, washed three times with 1ml immunoprecipitation buffer and eluted from the beads by the addition of 30μl Laemmli sample buffer and incubation at 37°C for 1 hour, vortexing every 15 minutes. In the case of lysates containing one of the adenosine receptors, the immune complexes were washed twice with immunoprecipitation buffer supplemented with 0.2M ammonium sulphate and once with imunoprecipitation buffer alone. Analysis was by SDS-PAGE using 10% (w/v) polyacrylamide resolving gels as described in section 3.3 and

autoradiography for between 16 and 40 hours at –80°C. Quantitation was by either densitometric scanning of autoradiographs or phosphorimaging.

2.4.7 In Vitro Receptor Phosphorylation Assays With Purified GRKs

Confluent monolayers of transfected cells in 100 mm dishes were washed with ice-cold PBS and scraped into 5ml/dish of lysis buffer (10mM HEPES, pH 7.5, 2mM EDTA, 0.25M sodium chloride supplemented with 0.1mM PMSF, 10μg/ml soybean trypsin inhibitor and 10μg/ml benzamidine). The cells were then transferred to a tight-fitting glass-on-glass Dounce homogeniser on ice and homogenised by 20 up-and-down strokes. Following a 15 min incubation on ice, the membranes were pelleted by centrifugation at 14,000g for 15 min. The supernatant was then discarded and the pellet resuspended in 5ml lysis buffer as before. The membranes were then re-homogenised, left on ice and centrifuged as described previously. The pellet was then resuspended in 5 ml GRK assay buffer (25mM Hepes, pH 7.5, 2.5mM EDTA and 7.5mM $MgCl_2$ supplemented with protease inhibitors) by homogenisation and re-centrifuged. The pellet was then resuspended in 220μl GRK assay buffer. Assays consisted of 40μl membrane suspension, 40μl kinase mix (GRK assay buffer supplemented with 0.25mM ATP, 0.88mM dithiothreitol, 0.15 μM okadeic acid and 10 μCi [γ-^{32}P]ATP), 10μl vehicle or 50nM purified GRK, and 10μl of vehicle or S1P. After incubation at 30°C for 5 min, reactions were terminated by placing the tubes on ice and adding 0.5 ml/tube stop solution (0.1M sodium phosphate, pH 7.5, 10mM EDTA). Membranes were pelleted by microcentrifugation (14,000g, 10 min) and the resulting pellets solubilised in 0.3ml immunoprecipitation buffer by rotation for 60 min at 4°C. After the removal of insoluble material by centrifugation, detergent extracts were equalised by protein assay prior to receptor immunoprecipitation with 9E10 and analysis by SDS-PAGE and autoradiography as described in Section 2.5.5. For rhodopsin phosphorylation experiments, urea-treated bovine rod outer segments (ROS) were employed. In this case, each assay consisted of GRK assay buffer containing 0.5μl ROS and 2μCi [γ-^{32}P] ATP. In these experiments, reactions were terminated by the addition of 15μl Laemmli sample buffer prior to analysis of rhodopsin phosphorylation by SDS-PAGE and autoradiography.

2.4.8 Biotin labelling-immunoprecipitation Assay Of Cell Surface Receptor Expression

Cells were plated into 6-well dishes at a density of $1x10^6$ cells/well and cultured overnight. Cell lines expressing EDG1 constructs were then serum-starved for 16-20 hours in serum-free Dulbecco's modified Eagle's medium. The next day, the cells were washed in the appropriate medium followed by the addition of 0.75 ml/well of the same medium. Incubations were initiated by the addition of 0.75ml medium supplemented with either vehicle or 2X concentrated dilution of drug as indicated in the Figure Legends. Reactions were terminated by placing the cells on ice and washing the monolayers three times with 3ml ice-cold PBS. All subsequent procedures were performed at 4°C unless stated otherwise. The alcohol groups on the cell surface glycoproteins were oxidised to aldehydes by a 30 minute incubation with 0.75ml/well 10mM sodium periodate in PBS. Following the removal of periodate and washing with PBS, the monolayers were washed twice with 3ml 0.1M sodium acetate, pH 5.5, and incubated for 30 minutes in 0.75ml/well of the same buffer supplemented with 1mM biotin-LC-hydrazide. This reacts with the newly formed alcohol groups thereby labelling all cell-surface glycoproteins with biotin. Labelling was terminated by removal of the biotin-LC-hydrazide solution and washing the monolayers three times with 3ml PBS. Cells were then solubilised for receptor immunoprecipitation as described for the whole cell phosphorylation assay (Section 2.4.6). Following fractionation of immunoprecipitated receptors by SDS-PAGE, proteins were transferred to a nitrocellulose membrane. Non-specific protein binding sites were blocked by incubation in Blotto and cell-surface biotin-labelled receptors were then identified by incubation of the membrane with 1μg/ml HRP–conjugated streptavidin for 60 minutes at room temperature. Following three 10min washes with Blotto and two washes with PBS, reactive proteins were visualised by enhanced chemiluminescence. Agonist-induced loss of cell-surface receptor was quantitated by densitometric scanning of non-saturating blots

2.4.9 Saturation radioligand binding assays with ^{3}H-DPCPX in isolated membranes

Confluent 75cm^2 flasks of COS cells transfected with either HA-A_1AR or HA-A_1(C309-Ala)AR receptor constructs were placed on ice and washed three times with 10ml ice-cold PBS. The cells were scraped from the bottom of the flask into the PBS and transferred to a pre-chilled 13ml non-sterile centrifuge tube on ice. Cells were pelleted by a 17640g for 10 minute spin at 4°C. The supernatant was then removed and the pellet resuspended in 1ml

of lysis buffer (10mM Tris, 5mM EDTA, pH 7.5 at 4°C). Cells were disrupted by 20 up-and-down strokes in a tight-fitting glass-on-glass Dounce homogeniser. The homogenate was removed to a microfuge tube and membranes pelleted by centrifigutation at 14 000g for 15 minutes.

On removal of the supernatant, the pellet was resuspended in 4mls of radioligand binding buffer (50mM Tris, 10mM $MgCl_2$, 1mM EDTA, pH 8.26 at 4°C) and transferred to the homogeniser. 1µl of stock ADA, added to give a final concentration of 0.47units/ml, was used to degrade endogenous adenosine prior to resuspension of the membrane pellet by Dounce homogenisation (20 up-and-down strokes). 150µl of the membrane suspension was added immediately to duplicate assay tubes containing tritiated radioligand, ranging from 0.25-8.0nM, and incubated at 37°C for 45-60 minutes in a shaking water bath at which point equilibrium was reached. Non-specific binding was defined in parallel by the inclusion of (R)-PIA to a final concentration of 10µM. Reactions were terminated by filtration using a Brandel cell harvester and rapid washing with three washes with wash buffer (binding buffer supplemented with 0.01% (w/v) CHAPS) over glass fiber filters pretreated with 0.3% (v/v) polyethyleneimine. Filter discs for each sample were then incubated in 5ml/disc of scintililant overnight at 4°C in scintillation vials and counted on a scintillation counter (Beckmann Instrumnents Inc., Fullerton, CA).

Non-specific counts were subtracted from the total counts and the resulting values plotted against [^{3}H-DPCPX] nM. To determine the total number of receptors expressed (B_{max}) and the equilibrium dissociation constant (K_d), the data was fitted to a nonlinear regression equation using the graph package "Prism". A bichinchonic acid (BCA) protein assay, as described in section 3.2, was used to determine the µg of protein added per tube. Combining the calculated B_{max}, final assay volume in litres and µg of protein added per tube, the receptor level was expressed in pmol/mg. An example of this calculation is shown in Appendix 1.

2.4.10 Confocal laser microscopy

Live cell analysis of HA-A_1AR and HA-A_1(Cys309-Ala)AR cell surface expression was carried out using CHO cells grown on glass coverslips. The coverslips were mounted on the imaging chamber and maintained at 37°C in Krebs-Ringer-HEPES-BSA (KREB) buffer (120mM NaCl, 5mM KCl, 1.2mM $MgSO_4$, 1.2mM $CaCl_2$, 20mM HEPES, 1.2mM Na_2HPO_4, 10mM glucose, 0.1% (w/v) BSA). (R)-PIA was applied in KRHB buffer. For

fixed cell analysis of GFP-tagged receptors, cells were grown on coverslips and treated with vehicle or agonist. The cells on the coverslips were then washed with PBS and fixed for 20 min at room temperature using 4% (w/v) paraformaldehyde in 5% (w/v) sucrose/PBS (pH 7.2).

Cell surface expression of EDG1 truncation mutants was visualised by immunofluorescence. Following two washes with 3ml/coverslip of PBS, the cells were fixed in 1.5ml/coverslip of 4% (w/v) paraformaldehyde as described above. Cells were then washed again with PBS and permeabilised for 3 min with 0.4% (v/v) Triton X-100 in PBS. Antibody dilutions were prepared in 0.1% (v/v) new born calf serum (NBCS)/0.2% (w/v) gelatin/PBS. The primary anti-myc 9E10 antibody was used at a 1:200 dilution and Alexa 594-conjugated anti-mouse IgG was used at 1:400 dilution. 100µl of antibody dilution for each coverslip was placed onto Nescofilm, before the coverslips were placed onto the antibody solution with the cells facing downwards and incubated at room temperature for 1 hour. Cells were then washed twice with PBS/NBCS/gelatin and placed onto nescofilm with secondary antibody for a further 1 hour. Coverslips were then washed twice with 3ml/coverslip of PBS/NBCS/gelatin and once with 3ml/coverslip of PBS prior to mounting on microscope slides with 7µl/coverslip of 40% (v/v) glycerol in PBS.

Cells were visualised using a Zeiss Axiovert 100 laser scanning confocal microscope (Zeiss, Oberkochen, Germany) using a Zeiss Plan-Apo 63 x 1.4 NA oil immersion objective, pinhole of 20 and electronic zoom between 1 and 4. GFP was excited using a 488 nm argon / krypton laser and detected with 515-540 nm band pass filter. The Alexa 594-conjugated anti-mouse antibody was excited at 543 nm and detected with a long pass band filter 590 nm. The images were manipulated with Zeiss LSM or MetaMorph software (Universal Imaging Corporation, West Chester, PA).

2.4.11 Statistical Analysis

All statistical analysis, unless stated otherwise was carried out using the Student t-test was as described in the Graphpad software, "Prism 3.0". Elsewhere, statistical analysis using ANOVA was as carried out using the "Graphpad" software as indicated in the figure legends.

Chapter 3

Functional Analysis Of The Regulation Of The Human A_1AR Receptor

Introduction

In cardiac myocytes, adenosine exerts protective effects by binding to both the A_1 and the A_3 AR subtypes (Ralevic *et al.*, 1998; Lozza *et al.*, 1997, Carr *et al.*, 1997; Mullane *et al.*, 1995). Both of these receptors are coupled to G_i-proteins to elicit similar cardioprotective effects (Nyce *et al.*, 1999; Ralevic *et al.*, 1998). However, the A_1AR and A_3AR are controlled by distinct regulatory mechanisms designed to dampen, or desensitise, receptor signalling (Palmer *et al.*, 1996). In this Chapter, the regulation of A_1AR function and the basis for this differential pattern in desensitisation between the A_1AR and A_3AR will be examined.

Desensitisation is defined as the process whereby GPCR signalling responses plateau and then diminish despite the continuous presence of agonist (Palmer and Stiles, 1997). This can involve a number of several temporally and mechanistically distinct processes:- 1) receptor uncoupling from its associated G-protein. This occurs within a few minutes and is thought to involve receptor phosphorylation (Palmer and Stiles, 1997; Gao *et al.*, 1999; Nie *et al.*, 1997; Menard *et al*, 1997); 2) receptor internalisation away from the cell surface. This reduces the number of cell surface receptors available for agonist binding and also facilitates receptor dephosphorylation and resensitisation following agonist removal (Lefkowitz, R.J. *et al.* 1998; von Zastrow, M. and Kobilka, B.K., 1992; Pitcher, J.A. *et al.*, 1995); and 3) receptor down-regulation which occurs after several hours of agonist exposure and describes a decrease in receptor expression (Palmer and Stiles, 1997; Gao *et al.*, 1999; Jockers *et al.*, 1999; Pak *et al.*, 1999).

A_3AR desensitisation occurs rapidly within minutes of agonist exposure and seems to involve pathways of phosphorylation and internalisation similar to those described for the β_2AR (Palmer and Stiles, 1997; Palmer *et al.*, 1996; Palmer *et al.*, 1995). In the β_2AR model, agonist exposure leads to a conformational change in the receptor, facilitating receptor phosphorylation by a GRK (Ciruela *et al.*, 1997; Ferguson, 2001). It has recently been proposed that GRK2, or a kinase of similar substrate specificity, phosphorylates the agonist-occupied A_3AR (Palmer and Stiles, 1997; Palmer *et al*, 1996). The binding of GRK to the receptor leads to the recruitment and binding of the scaffolding protein, "β-arrestin" to the phosphorylated receptor, resulting in receptor uncoupling (Benovic *et al.*, 1987; Menard *et al* 1997). β-arrestin not only desensitises the receptor but also directs receptor trafficking by functioning as an adaptor protein and targeting the β_2AR to clathrin-coated vesicles where the receptor is sequestered into the cell (Menard *et al*,

1997). The receptor is then either degraded or recycled back to the cell surface (Menard *et al*, 1997; Ferguson, 2001; Tsao *et al*, 2001).

In contrast to the A_3AR, desensitisation of the A_1AR requires prolonged agonist exposure, ranging from 15 minutes to several hours or even days (Ralevic *et al.*, 1998; Gao *et al.*, 1999; Palmer and Stiles, 1997; Ciruela *et al.*, 1997; Saura *et al.*, 1998). Differences in the amino acid sequences between the C-terminal tails of the A_1AR and A_3AR appear to be crucial to the differences in receptor regulation. Whereas the C-terminal region of the A_3AR is rich in serine and threonine residues, the A_1AR has only one such phosphorylation site (Figure 3.1). The importance of the C-terminal region in determining subtype-specific desensitisation was demonstrated in a study using stably expressing wild type (WT) and chimeric A_1 and A_3ARs in CHO cells (Palmer *et al*, 1996). While human A_1ARs were neither phosphorylated or desensitised following 10 minutes of R-PIA treatment, a chimeric A_1-A_3 adenosine receptor in which the C-terminus domain of the A_1AR distal to the predicted site of palmitoylation was replaced by the corresponding region of the A_3AR was able to undergo rapid functional desensitisation and agonist-stimulated phosphorylation (Palmer *et al.*, 1996). This suggested that differences in the amino acid sequences between the A_1AR and the A_3AR C-terminal domains are crucial to the differences in agonist-dependent phosphorylation. More specifically, the inability of the A_1AR to undergo rapid agonist-dependent phosphorylation in CHO cells was attributed to a lack of potential GRK phosphorylation sites in its cytosolic domains (Palmer *et al.*, 1996; Palmer and Stiles, 1997). Therefore, the lack of potential phosphorylation sites within the C-terminus of the A_1AR may result in differences in receptor desensitisation and internalisation.

In this chapter, the A_1AR has been characterized in terms of its structure and function in relation to the phenomenon of receptor desensitisation. This has been achieved using CHO cell lines stably expressing epitope-tagged human A_1ARs (Figure 3.2) and human A_1ARs tagged with GFP (Figures 3.8, 3.9). This facilitated the examination of the sub-cellular localisation of any internalised A_1AR following sustained agonist exposure. Concurrent studies using epitope- and GFP- tagged rat A_3ARs (Figures 3.8, 3.11) also allowed comparisons to be made between the A_1AR and the A_3AR. Finally, the role of palmitoylation in A_1AR regulation was addressed by using an A_1(Cys309-Ala)AR-GFP construct (Figures 3.15, 3.16).

Results

To facilitate identification and isolation of recombinant receptors for these studies of the A_1AR, the human A_1AR and rat A_3AR open reading frames were tagged with a six-amino acid haemagglutinin epitope (DVPDYA) and then stably expressed in CHO cells as described in the Methods section. This epitope is recognized by the monoclonal antibody, 12CA5, enabling immunoprecipitation of the epitope-tagged receptors in studies of phosphorylation and cell surface expression to be carried out. Expression of the HA-A_1AR and HA-A_3AR was confirmed by cell-surface biotinylation (Figure 3.2). Receptor expression levels were determined using cell surface biotinylation (Figure 3.2), immunostaining (Figure 3.3) and saturation binding studies (Figures 3.10, 3.12). Immunostaining gave a measure of total receptor expression whereas cell surface biotinylation measured glycosylated receptors expressed on the cell surface. Saturation binding studies using the A_3-selective agonist radioligand, ^{125}I-AB-MECA selectively bound the population of A_3ARs in the active (R*) conformation. By comparison, the A_1AR-selective antagonist radioligand [^{3}H]DPCPX binds irrespective of the G-protein-coupling status of the receptor and will therefore give a more representative reflection of the entire A_1AR population. Previous assays of adenylate cyclase inhibition have also shown that the WT HA-A_1AR and WT HA-A_3AR cell lines represent functional, viable cell lines (Palmer *et al.*, 1995; Palmer *et al.*, 1996).

Whole cell phosphorylation studies were performed to demonstrate the distinct effects of agonist stimulation on the phosphorylation of the HA-A_1AR and the HA-A_3AR. A 10 min treatment of 5μM R-PIA resulted in a strong phosphorylation of the HA-A_3AR. However, in the same experiment, 12CA5 failed to immunoprecipitate any phosphorylated HA-A_1AR following treatment with R-PIA for 10mins at 37^{0}C, suggesting that the HA-A_1AR is not phosphorylated in the presence of agonist (Figure 3.3, n=3). This is consistent with previously published data showing that, whereas the HA-A_3AR is phosphorylated rapidly following agonist stimulation, the HA-A_1AR is resistant to phosphorylation (Palmer *et al.*, 1996). The same study suggests that these differences may be due to a lack of serine and threonine phosphorylation sites within the C-terminal domain of the A_1AR distal to its site of palmitate attachment at Cys 309 (Palmer *et al.*, 1996).

Cell-surface receptor biotinylation assays were carried out to determine what effects the differential patterns of agonist-dependent phosphorylation between the HA-A_1AR and the HA-A_3AR had on receptor internalisation. After 7 hours of sustained 5μM

R-PIA treatment, a loss of 55±4% of biotinylated cell surface HA-A_1ARs was observed as compared with vehicle-treated controls ($p<0.05$, $n=3$) with a $t_{1/2}$ of 90 mins (Figure 3.4). In contrast, a 1 hour treatment of 1μM R-PIA resulted in a loss of 78±6% of HA-A_3ARs as compared with vehicle-treated controls ($p<0.05$, $n=3$) with a $t_{1/2}$ of 10 mins (Figure 3.5). This shows that, in contrast to the HA-A_3AR, loss of HA-A_1AR from the cell surface following sustained agonist exposure is a slower, less extensive process.

Changes in HA-A_1AR cell surface expression were then characterized using biotinylation studies in the presence of R-PIA and the selective A_1AR antagonist, DPCPX. Given that HA-A_1AR loss from the cell surface was maximal after a 2 hour agonist exposure, this time point was therefore chosen for all subsequent studies of HA-A_1AR loss from the cell surface. Figure 3.6 shows a concentration-response of cell surface loss using the A_1AR agonist, R-PIA. Significant cell surface loss required a dose of around 1μM (IC_{50}=0.71μM) with a maximal dose of 10μM R-PIA resulting in a loss of 44±5 % cell surface HA-A_1AR (*versus* vehicle treated controls (set at 100%), $p<0.05$, $n=3$, Figure 3.6). The effect of 5μM R-PIA was completely antagonised by a 30 minute pretreatment with 10nM DPCPX, a selective A_1AR antagonist (100±19% A_1AR cell surface expression as compared with vehicle treated controls (set at 100%), $p>0.05$, N/S, $n=3$, Figure 3.7), suggesting that the loss of cell surface receptors induced by R-PIA is agonist-specific. Interestingly, pretreatment with 1μM DPCPX resulted in a significant increase in the level of cell surface receptors (122±17% A_1AR cell surface expression *versus* vehicle-treated controls (set at 100%), $p<0.05$, $n=3$, Figure 3.7).

Fluorescent tagging of GPCRs with GFP has aided the characterisation of many examples of GPCR regulation, most notably the β_2-adrenergic receptor (Kallal *et al,* 1998; Barak *et al.*, 1997; Drmota *et al.*, 1998; Lee *et al.*, 1998). Whereas biochemical assays such as biotinylation of cell surface receptors provide a good quantitative measure of agonist-dependent changes in receptor cell surface expression, GFP tagging of the receptor provides a means of visualising agonist-dependent receptor trafficking. Therefore, the addition of a GFP tag onto the C-terminal allowed visualisation of the movement of the HA-A_1AR and the HA-A_3AR in response to agonist exposure using laser-scanning confocal microscopy (Figure 3.8). In particular, GFP tagging could provide further support for the distinct differences in agonist-dependent loss of cell surface receptor observed between the HA-A_1AR and the HA-A_3AR subtypes using cell surface biotinylation.

Expression of the HA-A_1AR-GFP protein was confirmed by immunoblotting using an anti-GFP antibody against the GFP tag (Figure 3.9). Expression of the HA-A_3AR-GFP construct was illustrated using a cell surface biotinylation assay (Figure 3.11). Saturation binding studies on COS-P cells transiently transfected with either HA-A_1AR or HA-A_1AR-GFP using the A_1AR-selective radioligand [^{3}H]DPCPX showed that fusion of GFP on to the C-terminus of the WT HA-A_1AR had no significant effect on either the B_{max} or K_d value (Figure 3.10, Table 4). In addition, radioligand binding studies on COS-P cells transiently expressing either A_3AR or A_3AR-GFP using the A_3-selective agonist radioligand ^{125}I-AB-MECA showed no significant differences between the K_d or B_{max} values of each receptor (Figure 3.12, Table 4). Therefore, as with the HA-A_1AR, the addition of a GFP tag onto the C-terminal tail of the HA-A_3AR had no significant effect on receptor binding. As mentioned previously, the detection of the A_3AR population using ^{125}I-AB-MECA is selective to G-protein coupled receptors and is therefore influenced by the proportion of A_3ARs in the active state under basal conditions.

The HA-A_1AR-GFP construct was transiently transfected into CHO cells and then exposed to a 1 hour treatment of 5μM R-PIA and examined in live cells in real-time by laser-scanning confocal microscopy. Figure 3.13 shows that, prior to agonist treatment, the HA-A_1AR-GFP is expressed exclusively on the cell surface. Following a 1 hour agonist treatment, the HA-A_1AR-GFP remained expressed on the cell surface and no pools of internalised receptor were detectable (Figure 3.13). This is in contrast to the HA-A_3AR-GFP receptor where a 30 minute exposure to agonist resulted in the almost complete re-distribution of the cell surface receptors from the plasma membrane into punctate intracellular vesicles (Figure 3.14).

A crucial link in the process of A_1AR short-term desensitisation and internalisation could be between palmitoylation of the receptor and the structure of the cytosolic tail. Like most GPCRs, such as rhodopsin, β_2-adrenergic and vasopressin V_{1a} receptors (Hawtin *et al.*, 2001, Konig *et al.*, 1989; Moffet *et al.*, 2001) the human A_1AR has a cysteine residue representing a potential palmitoylation site in its C-terminal domain (Tucker *et al.*, 1993). In addition, a recent study has shown that the A_1AR is palmitoylated following agonist stimulation and that this palmitoylation is abolished following the mutation of Cys309 to Ala, which cannot be palmitoylated (Gao *et al.*, 1999). However, the effect of A_1AR palmitoylation in terms of receptor internalisation has yet to be determined.

In order to test whether the integrity of Cys309 had any effect on A_1AR cell surface expression, the residue was mutated to a non-palmitoylatable Ala residue. A Cys309-Ala mutated HA-A_1AR-GFP construct was then produced to allow visualisation of any changes in cell surface expression in the presence of agonist (Figure 3.15). Analysis by immunoblotting using the monoclonal anti-GFP antibody showed that the HA-A_1AR-GFP and the HA-A_1(Cys309-Ala)AR-GFP receptors are expressed to the same levels and migrate at the same relative molecular mass (Figure 3.16). The HA-A_1(Cys309-Ala)AR-GFP construct was transiently expressed in CHO cells and exposed to a 1 hour treatment of 5μM R-PIA and examined in live cells in real-time by laser-scanning confocal microscopy. Figure 3.17 shows that there is no visible internalisation of the mutated receptor following 60 minutes of 5μM R-PIA exposure. The fact that mutation of Cys309 to Ala had no visible effect on the internalisation of the receptor suggests that palmitoylation of Cys309 in the C-terminus of the A_1AR has no bearing on the short-term rate of A_1AR internalisation.

Both cell surface biotinylation and confocal microscopy confirm that, in CHO cells, the R-PIA-dependent loss of HA-A_1AR from the cell surface is markedly slower and less complete than the HA-A_3AR. It was therefore important to determine whether the loss of cell surface receptors observed in the biotinylation assays represented a change in total receptor expression as opposed to receptor internalisation away from the cell surface. Radioligand binding studies using the A_1AR-selective radioligand [^{3}H]DPCPX were carried out on total cell membranes prepared from CHO/HA-A_1ARs following a 20 hour treatment with 5μM R-PIA. 20 hours of agonist exposure resulted in a significant HA-A_1AR down-regulation, with a 44.7±12.4% decrease in B_{max} in comparison to untreated controls observed following 7 hours of 5μM R-PIA exposure ($p<0.05$, $n=3$, Figure 3.18). Interestingly, the time course of this decrease in B_{max} is similar to the time course of the loss of cell surface receptor, with a $t_{1/2}$ of approximately 60 mins (Figure 3.18). This suggests that the loss of HA-A_1AR from the cell surface may be due to down-regulation of the receptor.

Discussion

As stated in the introduction, activation of the adenosine A_1AR is important in the biological defence against ischaemic damage. Therefore, A_1AR regulation may provide a potentially useful therapeutic target for drugs aimed at combating cardiac disease.

However, very little is known about the mechanisms regulating A_1AR signalling. To address this, the current study has used point-mutated and GFP-fused chimeric human A_1ARs in order to characterize the processes regulating the sub-cellular distribution of the A_1AR in response to sustained agonist exposure. The regulation of the human A_1AR was also compared with the regulation of the rat A_3AR using epitope- and GFP- tagged rat A_3ARs.

The rat and human forms of the A_3AR exhibit a relatively low level of identity (72% between each species) and this is reflected in distinct pharmacological characteristics of each type of A_3AR (Olah and Stiles, 2000; Palmer and Stiles, 1995). For example, the agonists R-PIA and NECA are equipotent at the rat A_3AR (Olah and Stiles, 2000). Importantly, however, studies examining stable cell lines of either the rat or the human forms of A_3AR transfected into CHOs demonstrated that both receptors have a similar desensitisation profile (Palmer *et al.*, 1997). Additionally, the adenosine human A_1AR and rat A_3AR subtypes are both Gi-coupled, are similar in structure and elicit similar biological responses upon exposure to adenosine. Therefore, the rat A_3AR provided a good model with which to compare the regulation of human A_1AR.

Despite the similarities exhibited between the human A_1AR and the rat A_3AR, each subtype exhibits very distinct regulatory patterns of phosphorylation and internalisation. For instance, in CHO cells, the HA-A_3AR is strongly phosphorylated when exposed to agonist, whereas the HA-A_1AR appears to be phosphorylation-resistant (Figure 3.3). A possible explanation for the lack of phosphorylation observed with the A_1AR is that the receptor may be partially glycosylated resulting in a differential processing of the receptor compared to the A_3AR. However, consensus sites for N-linked glycosylation (N-X-S/T where "X" may be an amino acid except P) exist on the second extracellular loops of all the ARs, with the A_3AR possessing two additional sites within the N-terminal domain. Studies have shown that the A_1AR is a single subunit glycoprotein with a molecular mass of 34-41 kDa which varies among the tissues or species (Gonzalezcalero *et al.*, 1992; Nakata, 1992; Schwabe *et al.*, 1993). For example, the molecular mass of purified A_1AR from human brain membranes shown to be 35kDa (Nakata, 1992). Additionally, endoglycosidase F treatment on purified A_1AR reduces the molecular mass to around 30kDa (Nakata, 1992). Figures 3.2 and 3.3 demonstrate that the HA-tagged human A_1AR has a molecular mass of around 40kDa. Additionally, cell surface A_1ARs can be easily detected using cell surface biotinylation of glycosylated receptors (Figure 3.2). This would suggest that the lack of phosphorylation of A_1ARs observed following agonist exposure is

due to the lack of phosphorylation sites within the C-terminal tail, as opposed to partial processing of the receptor.

A previous study has shown that a chimeric A_1-A_3AR form of the A_1AR in which the last 14 amino acids of the A_3AR have been fused distal to the predicted palmitoylation site within the A_1AR behaves pharmacologically like the A_1AR but is rapidly phosphorylated and undergoes internalisation in a similar way to the A_3AR (Palmer *et al.*, 1996). Since GPCR phosphorylation is often a prerequisite for internalisation, this difference implies that differences in the regulatory processes of phosphorylation and internalisation are due to the lack of serine and threonine phosphorylation sites within the C-terminal of the A_1AR. It appears, however, that phosphorylation and subsequent desensitisation for the A_1AR is tissue-specific. For example, an agonist-stimulated increase in phosphorylation of the A_1AR has been described in DDT_1MF-2 cells in association with receptor uncoupling from G-proteins and desensitisation (Ciruela *et al.*, 1997; Saura *et al.*, 1998; Nie *et al.*, 1997). Interestingly, the lack of serine and threonine residues in the carboxyl tail did not prevent rapid, ligand-induced desensitisation in DDT_1MF-2 cells. However, the short term desensitisation and phosphorylation of the A_1AR in DDT_1MF-2 cells demonstrates that the appropriate cellular environment is important in conferring specific mechanisms of receptor regulation. There is also evidence that receptor phosphorylation may not always be required for GPCR internalisation. A study of a mutant α_{1B}-adrenergic receptor with a 147 amino acid truncation of the C-terminal tail showed no decrease in agonist-induced inositol-1,4,5,-trisphosphate accumulation, compared to the wild-type receptor (Garrard *et al.*, 1998). Interestingly, while the truncated receptor was resistant to desensitisation and was not phosphorylated in response to epinephrine, it was able to undergo agonist-dependent internalisation, albeit more slowly than the wild-type receptor.

Previous studies have demonstrated that A_1AR internalisation is a slow, incomplete process requiring hours of agonist exposure. For example, despite the fact that short term phosphorylation and desensitisation of the A_1AR was found using DDT_1MF-2 cells, maximal internalisation was still only seen after several hours (Ciruela *et al.*, 1997; Saura *et al.*, 1998; Nie *et al.*, 1997). In CHO cells, there is a 55±4% loss of cell surface A_1ARs after 7 hours of R-PIA exposure (Figure 3.4). This is also a slow process ($t_{1/2}$=90 hours, Figure 4) as compared with the A_3AR ($t_{1/2}$= 10 mins, Figure 3.5) and is in contrast to most of the other GPCRs studied to date where maximal internalisation is typically achieved by

around 30 minutes (Ciruela *et al.*, 1997; Ralevic *et al.*, 1998). In addition, the loss of cell surface A_1ARs expressed in CHOs is an agonist-dependent process, where an R-PIA-dependent decrease in biotin-labelled cell surface receptors was shown to be inhibited in the presence of the A_1AR antagonists, DPCPX (Figure 3.7).

Visualisation of AR-GFP chimeras using laser-scanning confocal microscopy demonstrated that a sustained exposure to 5μM R-PIA does not result in any detectable movement of HA-A_1AR-GFP from the cell surface (Figure 3.13). In contrast, there is a marked translocation of HA-A_3AR-GFP from the plasma membrane into distinct punctate intracellular vesicles following a 30 minute exposure to 5μM R-PIA (Figure 3.14). This correlates well with the biotinylation studies of cell surface receptor expression and provides further evidence that, whereas there is rapid, significant loss of A_3AR from the cell surface, agonist exposure results in a far slower, less complete loss of cell surface A_1AR. In addition, mutation of the site of A_1AR palmitoylation had no effect on the translocation of the A_1AR from the cell surface following a 1 hour exposure of 5μM R-PIA (Figure 3.17). This result is consistent with a previous study showing that point mutation of Cys309 had no effect on receptor-G-protein coupling, effector activation or down-regulation in response to sustained agonist exposure of stably transfected HEK293 cells (Gao *et al.*, 1999).

The effect of palmitoylayion on receptor signalling and regulation varies amongst GPCRs. For example, it was shown in rhodopsin that a synthetic polypeptide from the fourth intracellular loop created by palmitoylation of Cys residues in the membrane-proximal portion of the C-terminus is capable of interacting with Gt (Konig *et al.*, 1989). Additionally, the β_2AR has been shown to be palmitoylated at a cysteine residue at position 341 in its C-terminus (Moffet *et al.*, 1993; Mouillac *et al.*, 1992; Horstmeyer *et al.*, 1996) and it was proposed that β_2AR palmitoylation permits the anchoring of the C-terminal tail of the receptor to the membrane to form a fourth intracellular loop (Moffet *et al.*, 1993; Mouillac *et al.*, 1992). Hence, the absence of a palmitate anchor may lead to a structural organisation of the carboxylic tail, exposing otherwise unavailable phosphorylation sites for kinases such as PKA and βARK (Moffet *et al.*, 1993). The observation that agonist treatment did not significantly increase the high basal level of receptor phosphorylation of a β_2(Cys341-Ala)AR mutant is consistent with a role for palmitoylation in regulating agonist-promoted phosphorylation (Moffet *et al.*, 1993). Consistent with this hypothesis, it has been shown that agonist exposure enhances the

incorporation of palmitate into human β_2ARs overexpressed in Sf9 cells (Mouillac *et al.*, 1992). This suggests that receptor activation increases the turnover rate of receptor-linked palmitate. Hence, a rapid acylation-deacylation cycle may provide a mechanism for the regulation of protein function and that dynamic regulation of receptor palmitoylation may serve as a mechanism for revealing phosphorylation sites.

However, Cys palmitoylation of other GPCRs has been shown to have no effect on G-protein coupling. For example, disruption of α_{2A}-adrenergic receptor palmitoylation was shown to have no effect on receptor phosphorylation but instead abolished receptor down-regulation (Eason *et al.*, 1994). Additionally, the 5-$HT_{4(a)}$ receptor is know to be palmitoylated (Ponimaskin *et al.*, 2001; Ponimaskin *et al*, 2002). However, non-palmitoylated 5-HT_{4A} receptors were indistinguishable from the wild type in their ability to interact with Gs, to stimulate adenyly cyclase activity and to activate cyclic nucleotide-sensitive cation channels following agonist stimulation (Ponimaskin *et al*, 2002). Interestingly, a palmitoylation-deficient mutant of the vasopressin V_{1a} receptor exhibited decreased phosphorylation under both basal and agonist-stimulated conditions when compared to the wild type receptor yet the mutant receptor has an increased rate of sequestration (Hawtin *et al.*, 2001). Hence, the apparent inability of the Cys309 mutation to alter A_1AR signalling is in contrast to the β_2-adrenergic receptor, where mutation of Cys341 within the β_2AR C-terminal tail increases the accessibility of a PKA phosphorylation site upon agonist stimulation and therefore increases receptor phosphorylation (Moffet *et al.*, 1993; Mouillac *et al.*, 1992). However, other examples such as the 5-HT_{4A}, α_{2A}-adrenergic, and vasopressin V_{1a} receptors illustrate the varied role of palmitoylation in GPCR signalling and regulation. In essence, the effect of palmitoylation on GPCR signalling is dependent upon the residues surrounding the site of palmitoylation and is therefore receptor-specific. Indeed, studies within our lab have shown that mutation of Cys302 and Cys305 within the C-terminal domain of the A_3AR resulted in a marked increase in receptor phosphorylation and rate of internalisation. This difference presumably reflects the ability of the WTA_3AR to be phosphorylated by GRKs in comparison to the phosphorylation-resistant A_1AR.

The fact that a slow agonist-dependent loss of cell surface A_1AR expression is observed using either CHO or DDT1-MF2 cells would suggest that any A_1AR internalisation either utilises a unique molecular mechanism involving the clathrin/dynamin endocytic machinery or is through a clathrin-independent mechanism. In

LLC-PK$_1$ cells, it has been suggested that A_1AR internalisation occurs following their translocation to rafts enriched in caveolin and that the putative caveolin binding motif within the C-terminal of the A_1AR (**YAFRIHKF**) is involved (Gines *et al.*, 2001). In contrast, a separate study showed that 67±5% of adenosine A_1 receptors were isolated with caveolae from unstimulated rat cardiac ventricular myocytes and, following incubation with the A_1AR agonist, CCPA, there was rapid translocation of the A_1 receptors from caveolae into non-caveolae plasma membrane (Lasley *et al.*, 2000). Alternatively, the loss of cell surface expression may be due to down-regulation. In CHO cells, a radioligand binding assay to measure receptor down-regulation demonstrated that the loss of cell surface expression and the loss of total receptor expression were temporally similar ($t_{1/2}$=60 mins, Figure 3.18). Therefore, the concomitant decrease in total receptor expression level would suggest that, in CHO cells, receptor down-regulation is the major determinant of A_1AR cell surface expression. This down-regulation could be the result of modulation of receptor gene transcription, RNA stability or receptor proteolysis.

In conclusion, the A_1AR and the A_3AR undergo distinct regulatory pathways of phosphorylation and internalisation. The loss of cell surface A_1AR due to agonist exposure is a slower, less complete process than the A_3AR and this can be attributed to its inability to undergo receptor phosphoryation in response to agonist. It also been shown that mutation of Cys309, a site of palmitoylation within the A_1AR C-terminal tail, does not visibly increase the agonist-dependent effect on the translocation of the A_1AR from the cell surface. Future work should be aimed at examining the precise mechanisms involved in the slow loss of A_1AR from the cell surface observed in CHO cells, focusing primarily upon mechanisms of receptor down-regulation. This may also help to explain the reported variability in A_1AR regulation found within different cell types. Future studies should examine the potential role of caveolin both in terms of A_1AR cell surface expression and also in terms of A_1AR signalling. The C-terminal tail of the A_1AR contains a RxxPxxP class I Src Homology 3 (SH3) binding motif (**R^{308}CQPAPP**) proximal to the putative caveolin binding site. Therefore, one potential area of interest involves examining the role of caveolin in regulating the attachment of the A_1AR with proteins containing SH3 domains. In addition, it remains unclear why it is physiologically beneficial to have the A_1AR subtype comparatively resistant to regulatory mechanisms of phosphorylation and internalisation in comparison to the A_3AR. Therefore, future work should also involve

cardiac myocytes infected with an adenoviral construct of the A_1AR in order to study the effect of sustained agonist treatment of the A_1AR under conditions of ischaemia.

Figure 3.1: Alignment Of A_1AR And A_3AR C-Terminal Domains

The primary sequences of the C-terminal domains of the human A_1AR and the rat A_3AR are shown. Phosphorylation sites at Thr 307, 318 and 319 of the A_3AR are shown in blue.

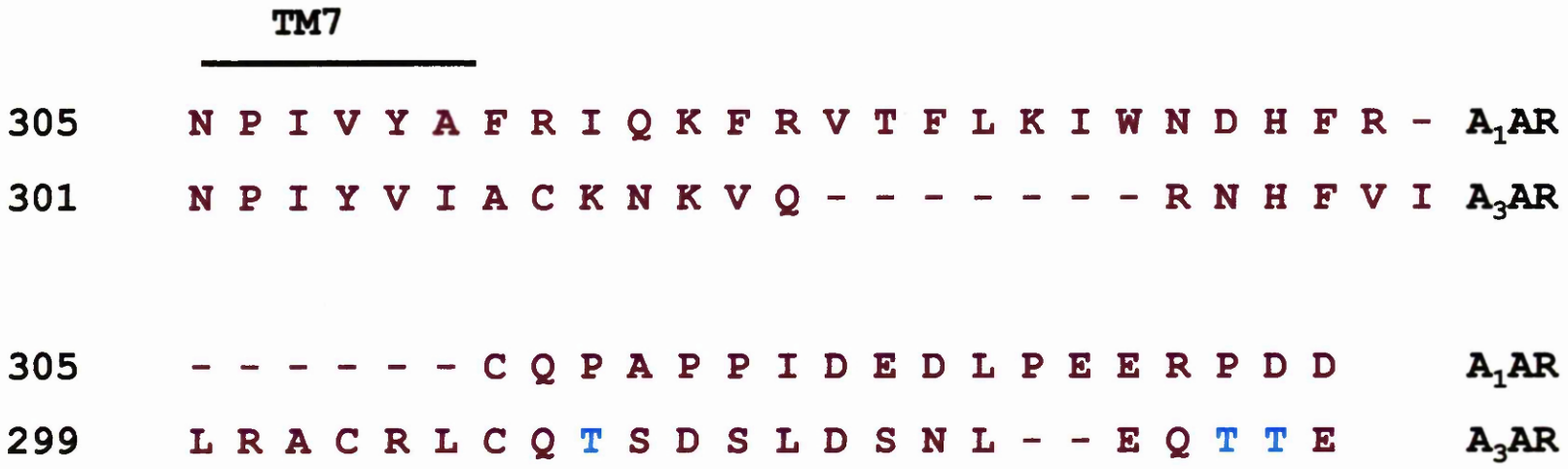

TM7
305 N P I V Y A F R I Q K F R V T F L K I W N D H F R - A_1AR
301 N P I Y V I A C K N K V Q - - - - - - - - R N H F V I A_3AR
305 - - - - - - C Q P A P P I D E D L P E E R P D D A_1AR
299 L R A C R L C Q T S D S L D S N L - - E Q T T E A_3AR

Figure 3.2: Cell-Surface Biotinylation Of HA-A_1AR And HA-A_3AR

CHO cells stably expressing either the HA-A_1AR or the HA-A_3AR were subjected to cell surface biotinylation and solubilised, followed by receptor immunoprecipitation with 12CA5. This represents one of multiple experiments.

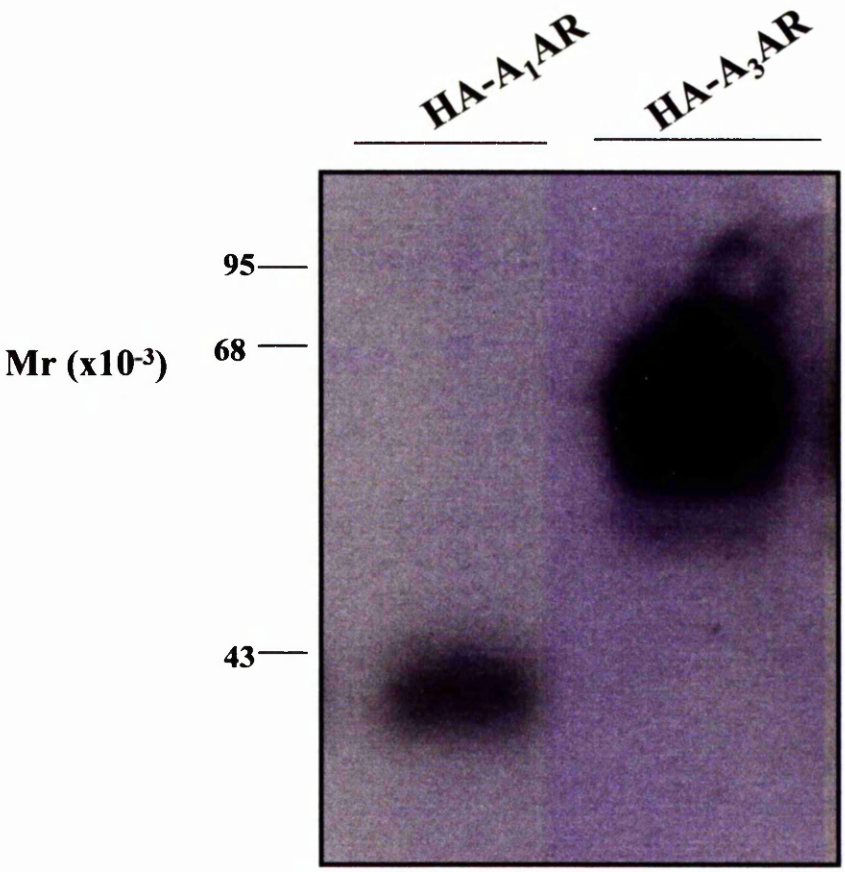
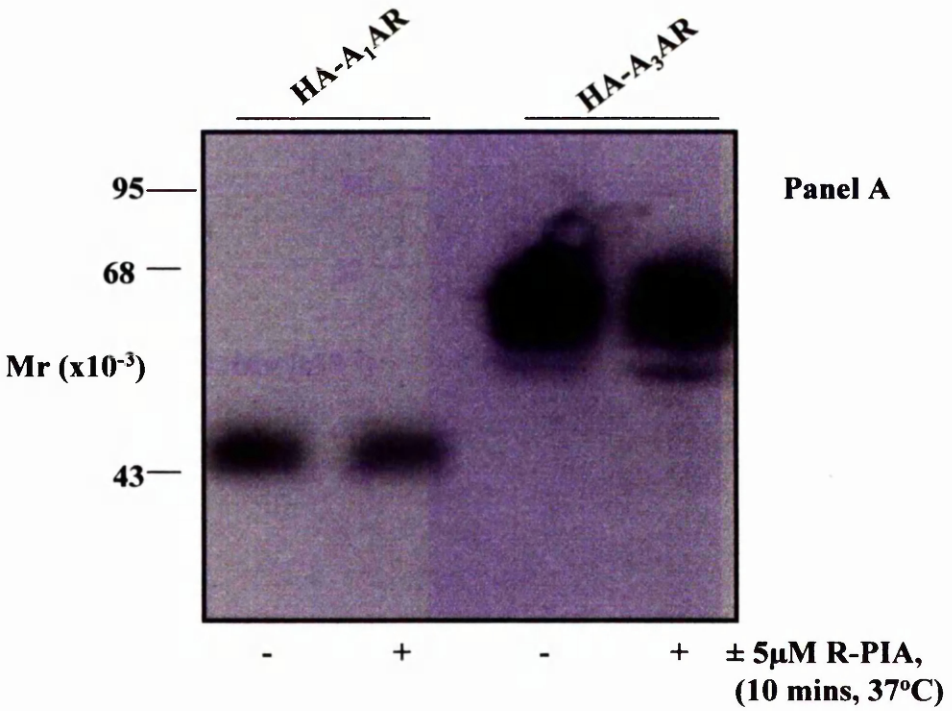

HA-A1AR
HA-A3AR
Mr (x10-3)
95
68
43

Figure 3.3: Comparison Of Agonist-Mediated Phosphorylation Of WT A_1 And A_3ARs

^{32}P-labelled stably transfected CHO/HA-A_1AR and CHO/HA-A_3AR cells were treated for 10 mins with 5μM R-PIA at 37^{0}C in the presence of 0.5units/ml of adenosine deaminase. The cells were then solubilised for analysis of A_1AR and A_3AR phosphorylation by immunoprecipitation followed by SDS-PAGE and phosphorimaging. Panel (A) shows an immunoblot of the immunoprecipitated samples, demonstrating equal loading of each receptor construct. The A_1AR is not phosphorylated in the presence of agonist whereas the A_3AR is strongly phosphorylated, as shown in the autoradiograph in panel (B). Typical data is shown from one of three experiments.


HA-A1AR
HA-A3AR
95
68
Mr (x10-3)
43
Panel A
- + - +
± 5μM R-PIA,
(10 mins, 37°C)

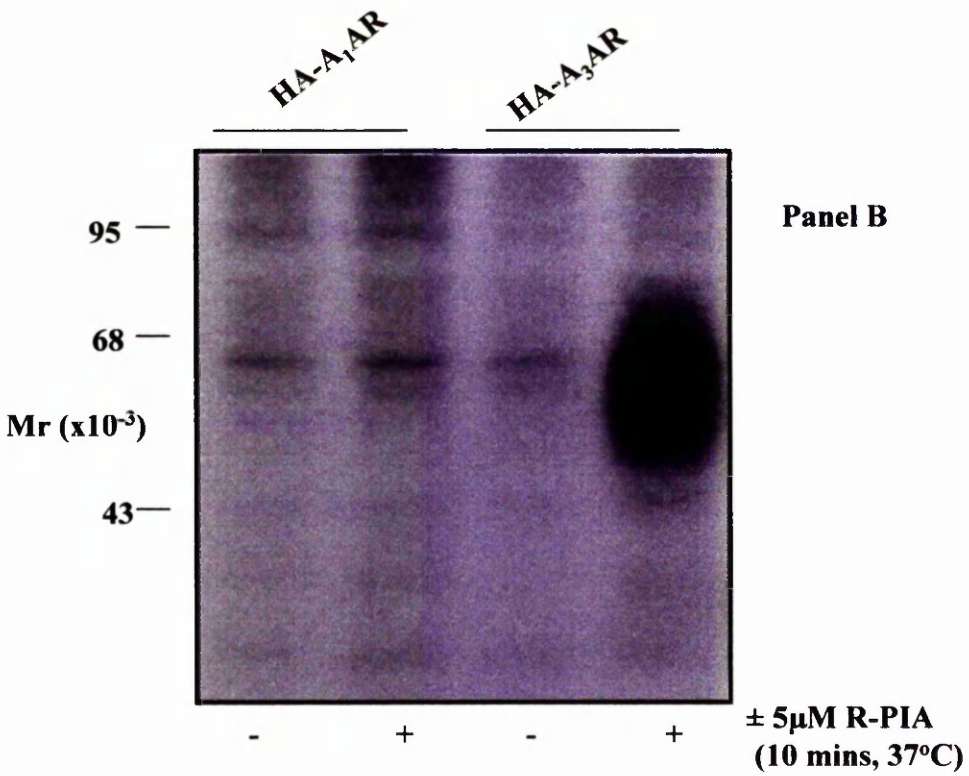
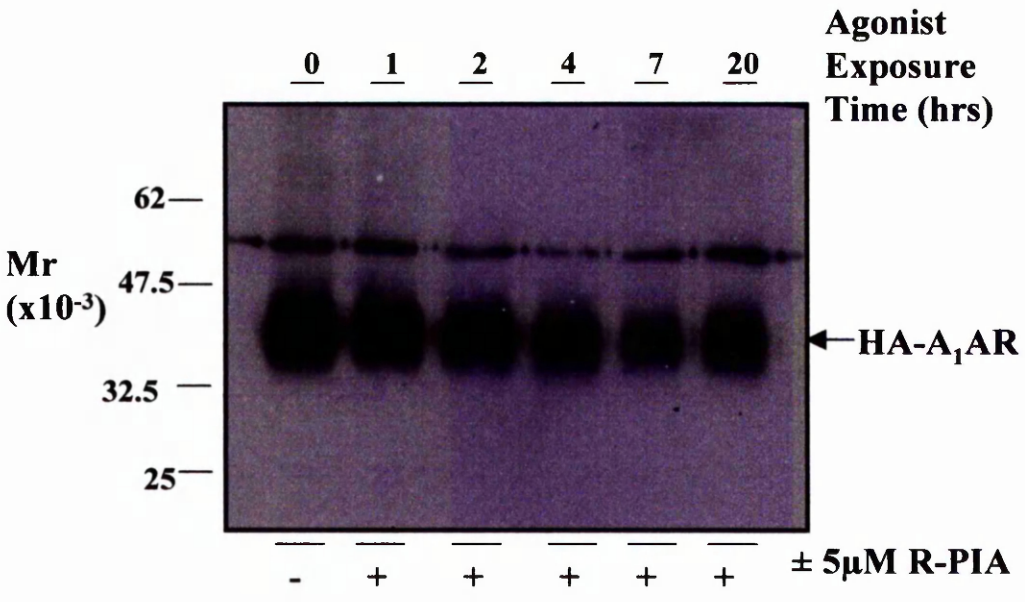

HA-A1AR
HA-A3AR
95
68
Mr (x10-3)
43
Panel B
- + - +
± 5μM R-PIA
(10 mins, 37°C)

Figure 3.4: Time Course Of Agonist-Mediated Loss Of Cell Surface HA-A_1AR

CHO cells stably transfected with the HA-A_1AR expression construct were treated with vehicle or 5μM R-PIA for 1,2,4,7,or 20 hours at 37^0C in the presence of 0.5units/ml adenosine deaminase as indicated in the graph. The cells were examined for internalisation by cell surface labelling with biotin-LC-hydrazide, followed by immunoprecipitation with the 12CA5 antibody. Biotin labelling of untreated A_1ARs was set at 100% and the results following agonist treatment expressed as a ratio of the control. The data from three experiments is shown in the graph and expressed as mean±SEM. The graph shows that agonist-dependent cell surface loss of A_1AR is a slow process ($t_{1/2}$=90 mins) with a loss of 60% of cell surface A_1AR after 7 hours.


Agonist
Exposure
Time (hrs)
0
1
2
4
7
20
62
47.5
32.5
25
Mr
(x10-3)
HA-A1AR
-
+
+
+
+
+
± 5μM R-PIA

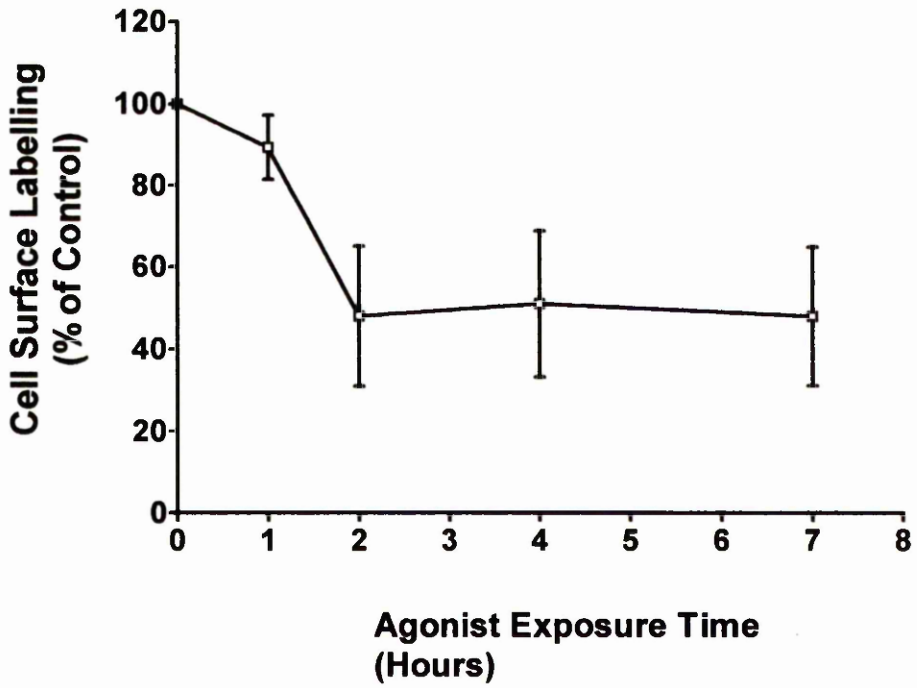
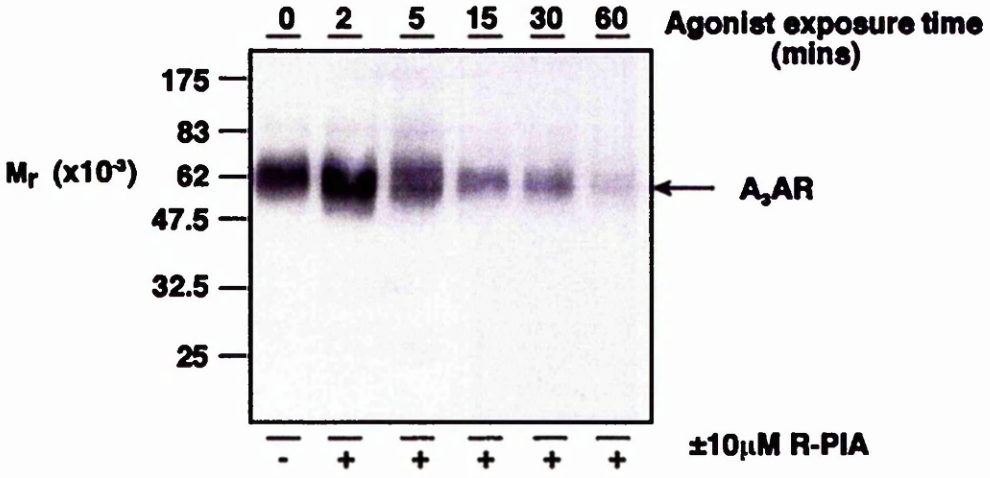

Cell Surface Labelling
(% of Control)
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
Agonist Exposure Time
(Hours)

Figure 3.5: Time Course Of Agonist-Mediated Loss of Cell Surface HA-A_3AR

CHO/HA-A_3AR cells were treated with vehicle or 5μM R-PIA for 2,5,15,30 or 60 minutes at 37^{0}C in the presence of 0.5units/ml adenosine deaminase as indicated in the graph. The cells were examined for internalisation by cell surface labelling with biotin-LC-hydrazide, followed by immunoprecipitation with the 12CA5 antibody. Biotin labelling of untreated A_3AR was set at 100% and the results following agonist treatment expressed as a ratio of the control. The data from three experiments is shown in the graph and expressed as mean ± SEM. The graph shows that, in contrast to the A_1AR, agonist-dependent cell surface loss of A_3AR is a relatively rapid process ($t_{1/2}$=10mins) with a loss of around 75% of cell surface A_3AR after 1 hour.


0
2
5
15
30
60
Agonist exposure time (mins)
175
83
62
47.5
32.5
25
M_r (x10^{-3})
A_3AR
-
+
+
+
+
+
±10μM R-PIA

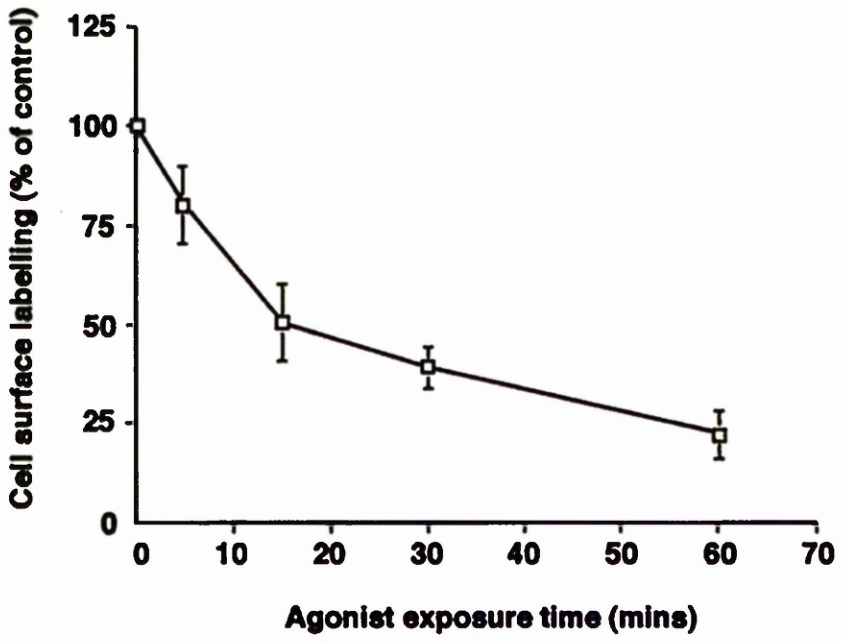
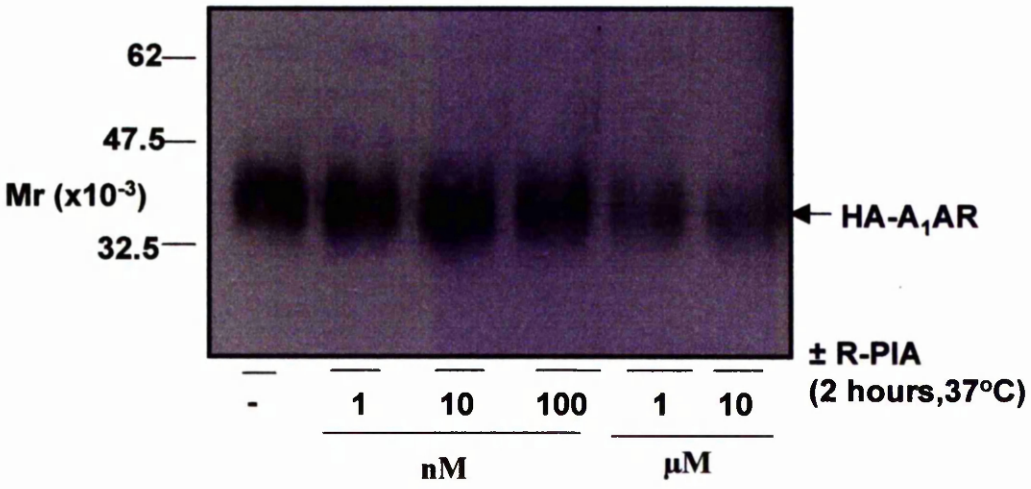

Cell surface labelling (% of control)
125
100
75
50
25
0
0
10
20
30
40
50
60
70
Agonist exposure time (mins)

Figure 3.6: Effect Of Increasing Agonist Concentration On Loss Of Cell Surface A_1AR

Stably transfected CHO/HA-A_1AR cells were treated for 2 hours with either vehicle or 1nM, 10nM, 100nM, 1μM or 10μM R-PIA at 37^0C, supplemented with 0.5units/ml adenosine deaminase. Cell surface expression was then examined by cell surface labelling with biotin-LC-hydrazide, followed by immunoprecipitation with the 12CA5 antibody. Biotin labelling of untreated A_1ARs was set at 100% and the results following agonist treatment expressed as a ratio of the control. The data from three experiments is shown in the graph and expressed as mean±SEM. The figure shows that a significant loss of cell surface A_1AR was found following treatment with doses greater than or equal to 1μM R-PIA (EC_{50}=0.71μM).


62
47.5
Mr (x10-3)
32.5
HA-A1AR
± R-PIA
(2 hours,37°C)
-
1
10
100
1
10
nM
µM

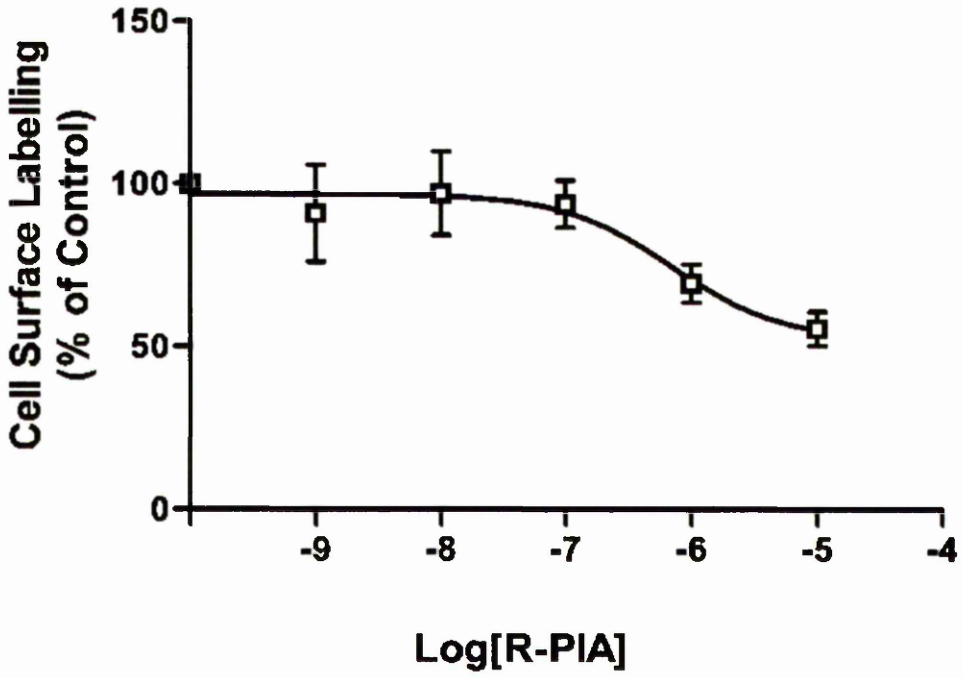
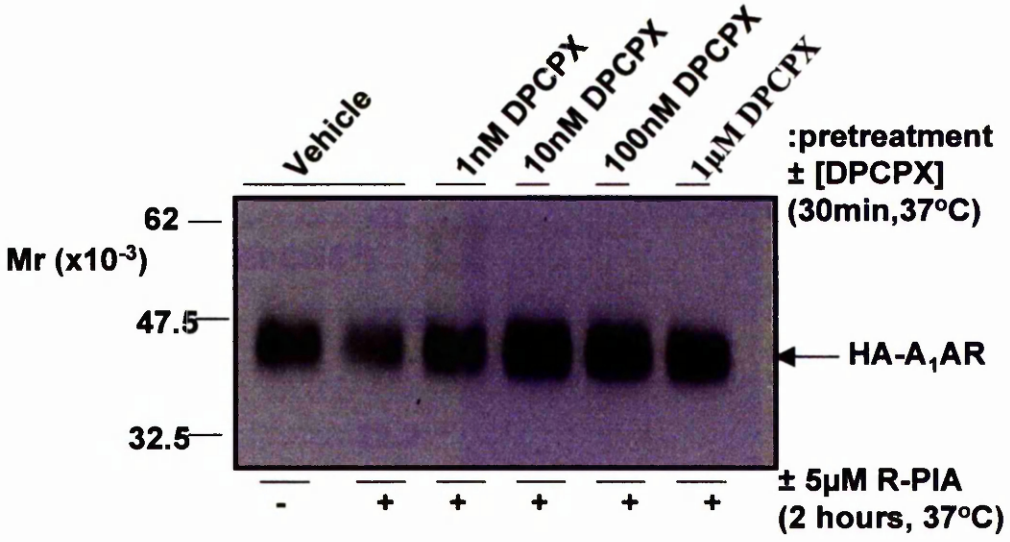

Cell Surface Labelling
(% of Control)
150
100
50
0
-9
-8
-7
-6
-5
-4
Log[R-PIA]

Figure 3.7: Effect Of The A_1AR-Selective Antagonist DPCPX On Agonist-Mediated Loss Of Cell Surface A_1AR

CHO/HA-A_1AR cells were pretreated for 30mins with vehicle, 1nM, 10nm, 100nM, or 1μM DPCPX. The cells were then given a 2 hour treatment with either vehicle or 5μM R-PIA. All additions were at 37^0C and were supplemented with 0.5units/ml of adenosine deaminase. Cell surface expression was then examined by cell surface labelling using biotin-LC-hydrazide. Biotin labelling of untreated A_1ARs was set at 100% and the results following agonist and antagonist treatment were expressed as a ratio of the control. The data from three experiments is shown in the graph and expressed as mean±SEM. * denotes statistically significant changes in cell surface expression versus untreated controls ($p<0.05$). R-PIA-dependent loss of cell surface A_1AR was completely antagonised following pretreatment with 10nM DPCPX. 1μM DPCPX pretreatment resulted in a significant increase in cell surface receptor levels.


Vehicle
1nM DPCPX
10nM DPCPX
100nM DPCPX
1μM DPCPX
:pretreatment
± [DPCPX]
(30min,37°C)
62
Mr (x10-3)
47.5
32.5
HA-A1AR
- + + + + +
± 5μM R-PIA
(2 hours, 37°C)

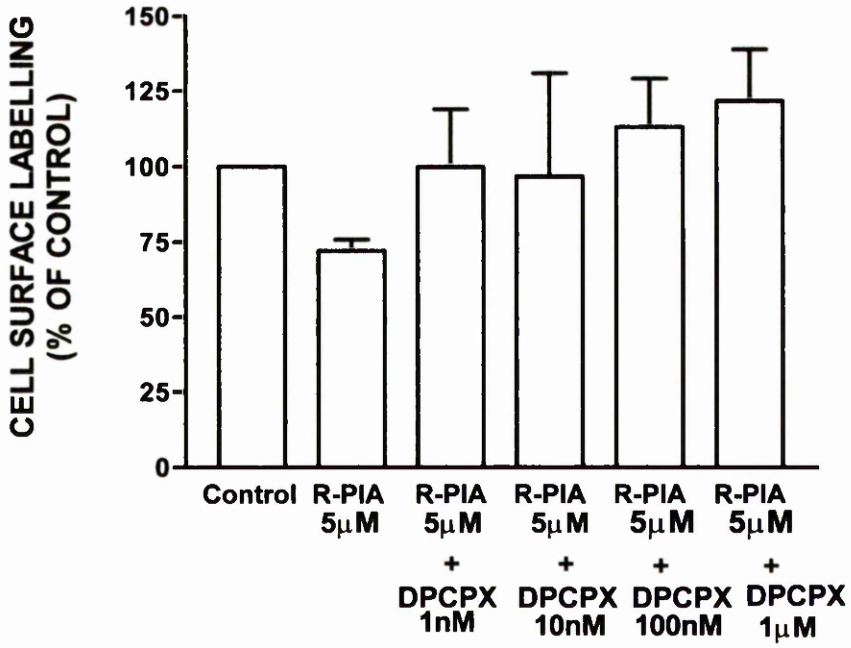
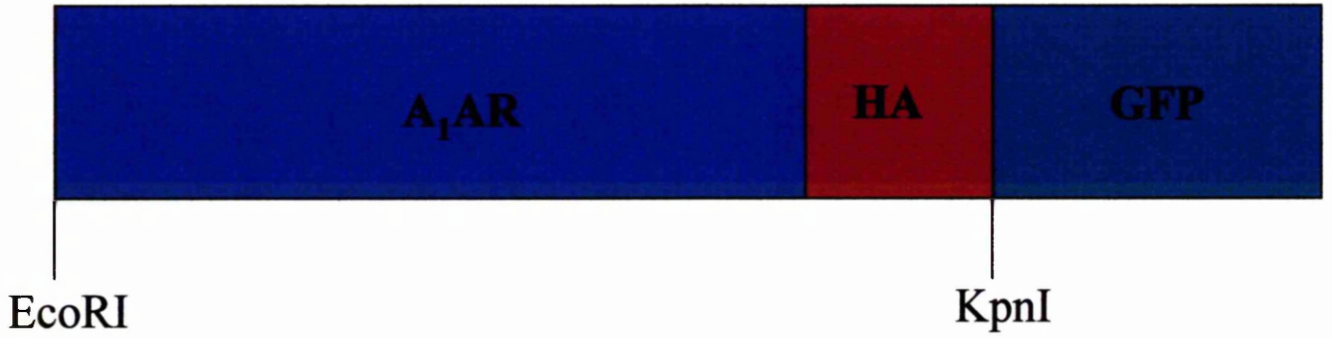

CELL SURFACE LABELLING
(% OF CONTROL)
150
125
100
75
50
25
0
Control
R-PIA 5μM
R-PIA 5μM + DPCPX 1nM
R-PIA 5μM + DPCPX 10nM
R-PIA 5μM + DPCPX 100nM
R-PIA 5μM + DPCPX 1μM

Figure 3.8: Schematic Diagram Of HA-A_1AR-GFP And HA-A_3AR-GFP Construct

Epitope-tagged forms of the human A_1AR and rat A_3AR were tagged with green fluorescent protein using either a pCMV5/human HA-A_1AR or pCMV5/rat A_3AR cDNA respectively as a template. The C-terminal HA-tag present on the both the A_1AR and the A_3AR were removed by PCR and ligated into the multiple cloning site of pEGFP-N1 at *Hind*III/*Kpn*I for the A_1AR and at *Hind*III / *Sma*I for the A_3AR. The addition of the GFP tag allowed visualisation of any movement of the cell surface A_1AR and A_3AR following sustained agonist exposure.


A1AR
HA
GFP
EcoRI
KpnI


A3AR
HA
GFP
HindIII
SmaI

Figure 3.9: Immunoblotting Analysis Of HA-A_1AR-GFP

CHO cells transiently expressing either the HA-A_1AR-GFP or WT-GFP construct were solubilised in electrophoresis sample buffer and analysed by SDS-PAGE and immunoblotting with the anti-GFP antibody against the GFP tag. This represents one of multiple experiments.

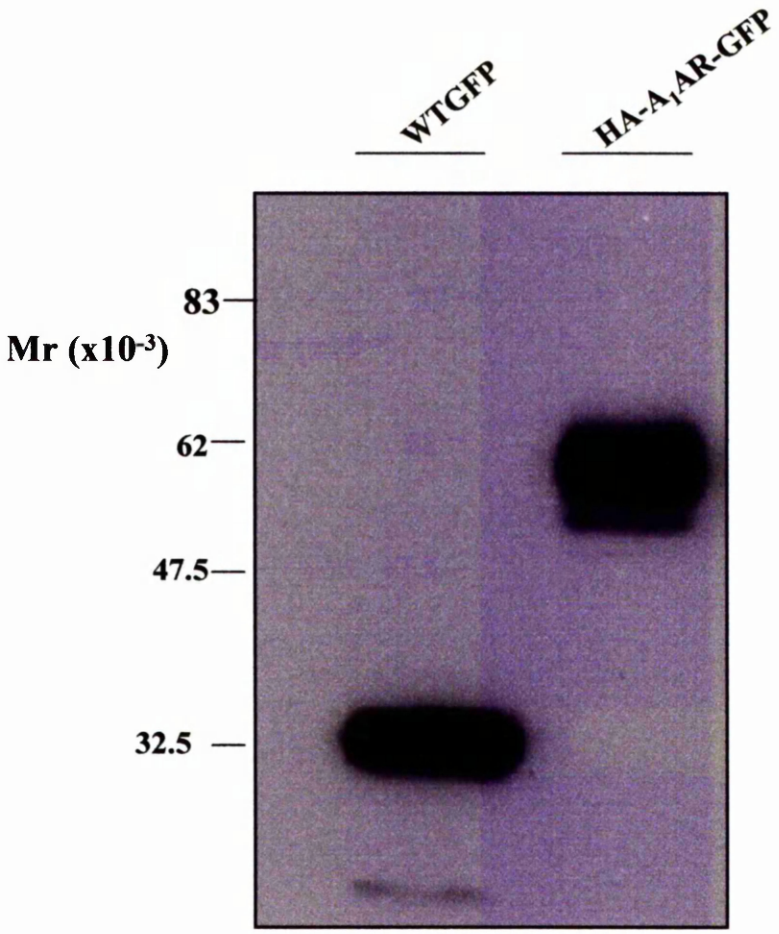

WTGFP
HA-A1AR-GFP
Mr (x10-3)
83
62
47.5
32.5

Figure 3.10: Saturation Analysis Of [^{3}H] DPCPX Binding Of HA-A_1AR and HA-A_1AR-GFP

Membranes prepared from COS-P cells transiently transfected with either A_1AR or A_1AR-GFP expression constructs were used for saturation radioligand binding assays with increasing concentrations of the A_1AR antagonist radioligand [^{3}H]DPCPX as described in the material and methods. This is one of three experiments, composite data from which are presented in table 1.

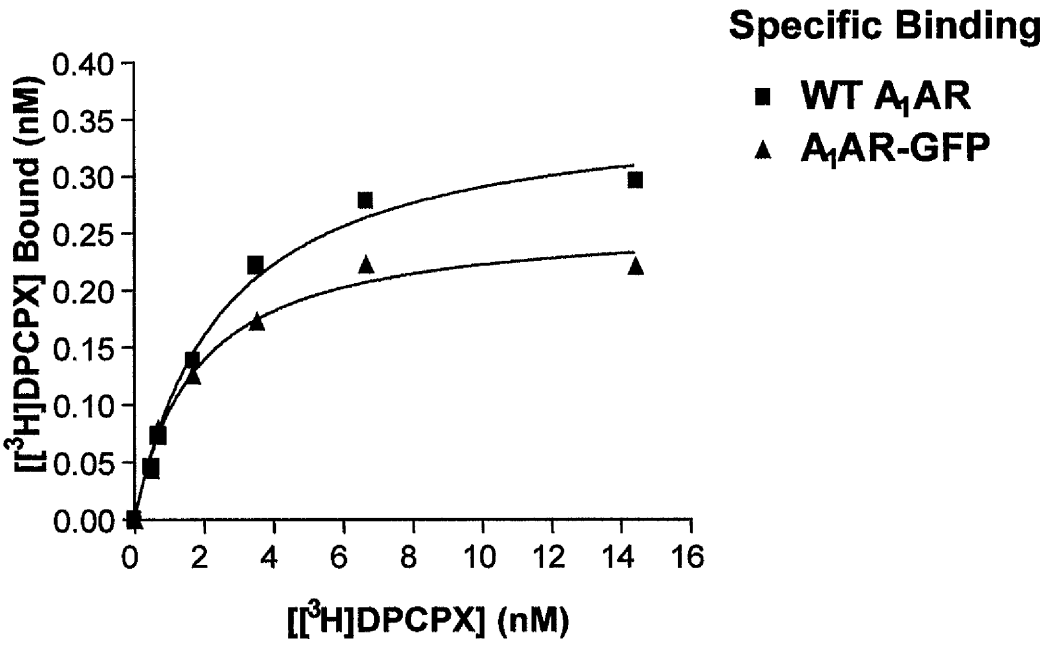

Specific Binding
WT A1AR
A1AR-GFP
[[3H]DPCPX] Bound (nM)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
2
4
6
8
10
12
14
16
[[3H]DPCPX] (nM)

Figure 3.11: Cell-Surface Labelling Analysis Of HA-A_3AR-GFP

Non-transfected CHO cells or CHO cells transiently expressing either the HA-A_3AR or the HA-A_3AR-GFP construct were subjected to cell surface biotinylation and receptor immunoprecipitation with 12CA5 as outlined in the methods section. The samples were solubilised in electrophoresis sample buffer and analysed by SDS-PAGE and probing with HRP-streptavidin. Each figure represents one of multiple experiments.

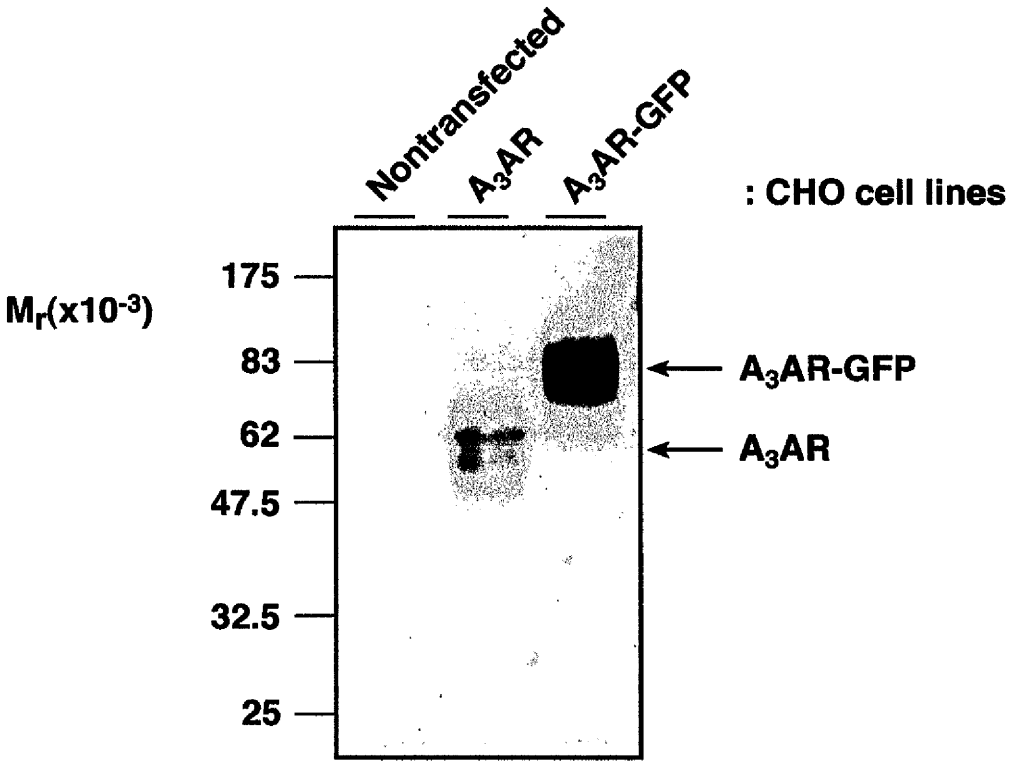

Nontransfected
A3AR
A3AR-GFP
: CHO cell lines
Mr(x10-3)
175
83
62
47.5
32.5
25
A3AR-GFP
A3AR

Figure 3.12: Saturation Analysis Of ^{125}I-ABMECA Binding Of HA-A_3AR and HA-A_3AR-GFP

Membranes prepared from COS-P cells transiently transfected with either A_3AR or A_3AR-GFP expression constructs were used for saturation radioligand binding assays with increasing concentrations of the A_3AR agonist radioligand ^{125}I-ABMECA as described in the methods section. This is one of three experiments, composite data from which are presented in table 1.

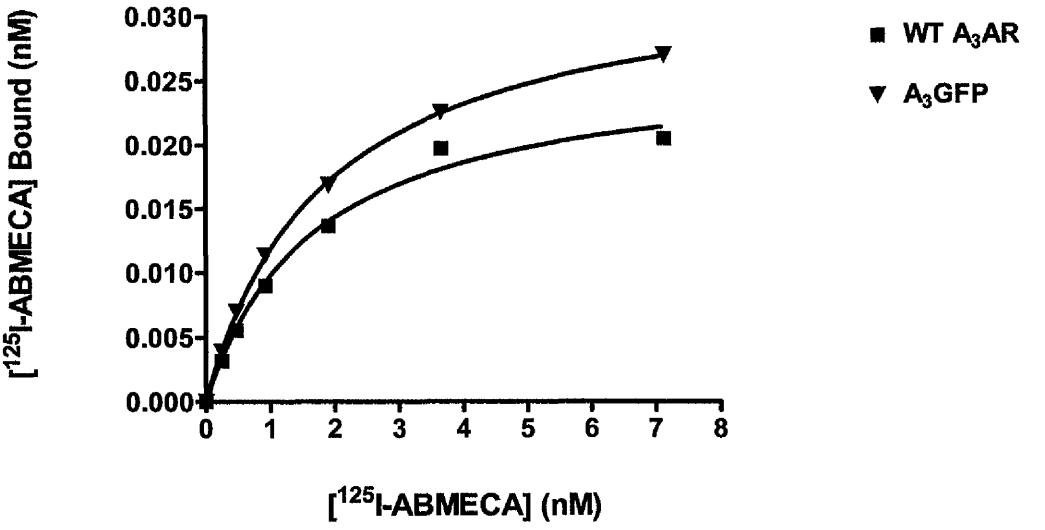

[125I-ABMECA] Bound (nM)
0.030
0.025
0.020
0.015
0.010
0.005
0.000
0
1
2
3
4
5
6
7
8
[125I-ABMECA] (nM)
WT A3AR
A3GFP

Table 4: Pharmacological Characterisation of HA-A_1AR, HA-A_1AR-GFP, HA-A_3AR And HA-A_3AR-GFP Receptors

Membranes prepared from COS-P cells transiently expressing the indicated HA-A_1ARs were used for saturation radioligand binding assays employing increasing concentrations of the A_1AR-selective antagonist ligand [^{3}H]DPCPX as described in section. COS-P membranes expressing the indicated HA-A_3ARs were used for saturation radioligand binding assays employing increasing concentrations of the A_3AR agonist radioligand ^{125}I-ABMECA. Results are presented as means ± standard error from three experiments. * indicates no significant differences between the wild type and GFP tagged receptors (p>0.05, n=3)

Receptor	K_d (nM)	B_{max} (pmol/mg protein)
A_1AR	2.75±0.11	5.32±1.09
A_1AR-GFP*	2.29±0.39	3.98 ±0.52
A_3AR	2.18±0.54	1.20 ±0.30
A_3AR-GFP *	2.03±0.31	1.43 ±0.20

Figure 3.13: Real-Time Visualisation Of HA-A_1AR-GFP Cell Surface Expression Following Sustained Agonist Treatment

CHO cells transiently transfected with the HA-A_1AR-GFP construct were exposed to a sustained treatment of 5μM R-PIA and examined under live cell conditions by confocal microscopy. Under conditions of no agonist treatment, the HA-A_1AR-GFP construct was expressed on the cell surface. Even after 1 hour of agonist exposure, the HA-A_1AR-GFP remained on the cell surface with no internal pools of internalised receptor found within the cytoplasm. This represents a typical example of three similar experiments.

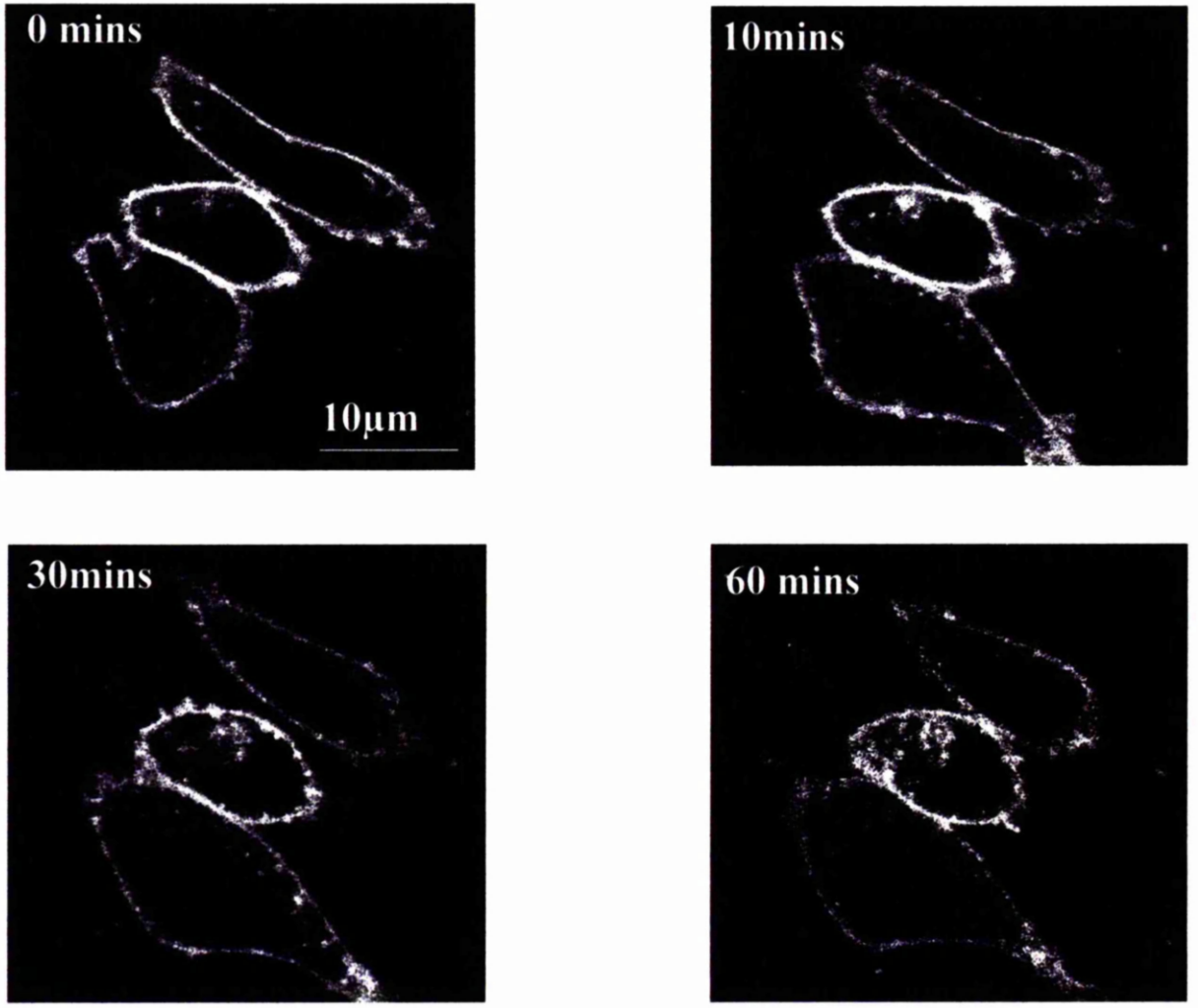

0 mins
10μm
10mins
30mins
60 mins

Figure 3.14: Real-Time Visualisation Of HA-A_3AR-GFP Cell Surface Expression Following Sustained Agonist Treatment

CHO cells transiently transfected with the HA-A_3AR-GFP expression construct were exposed to a sustained treatment of 5μM R-PIA and examined under live cell conditions by confocal microscopy. When exposed to vehicle alone, the HA-A_3AR-GFP construct was expressed on the cell surface. In contrast to the HA-A_1AR-GFP construct, a 30 minute exposure to agonist resulted in a dramatic re-distribution of the HA-A_3AR-GFP receptor away from the cell surface and into intracellular pools.

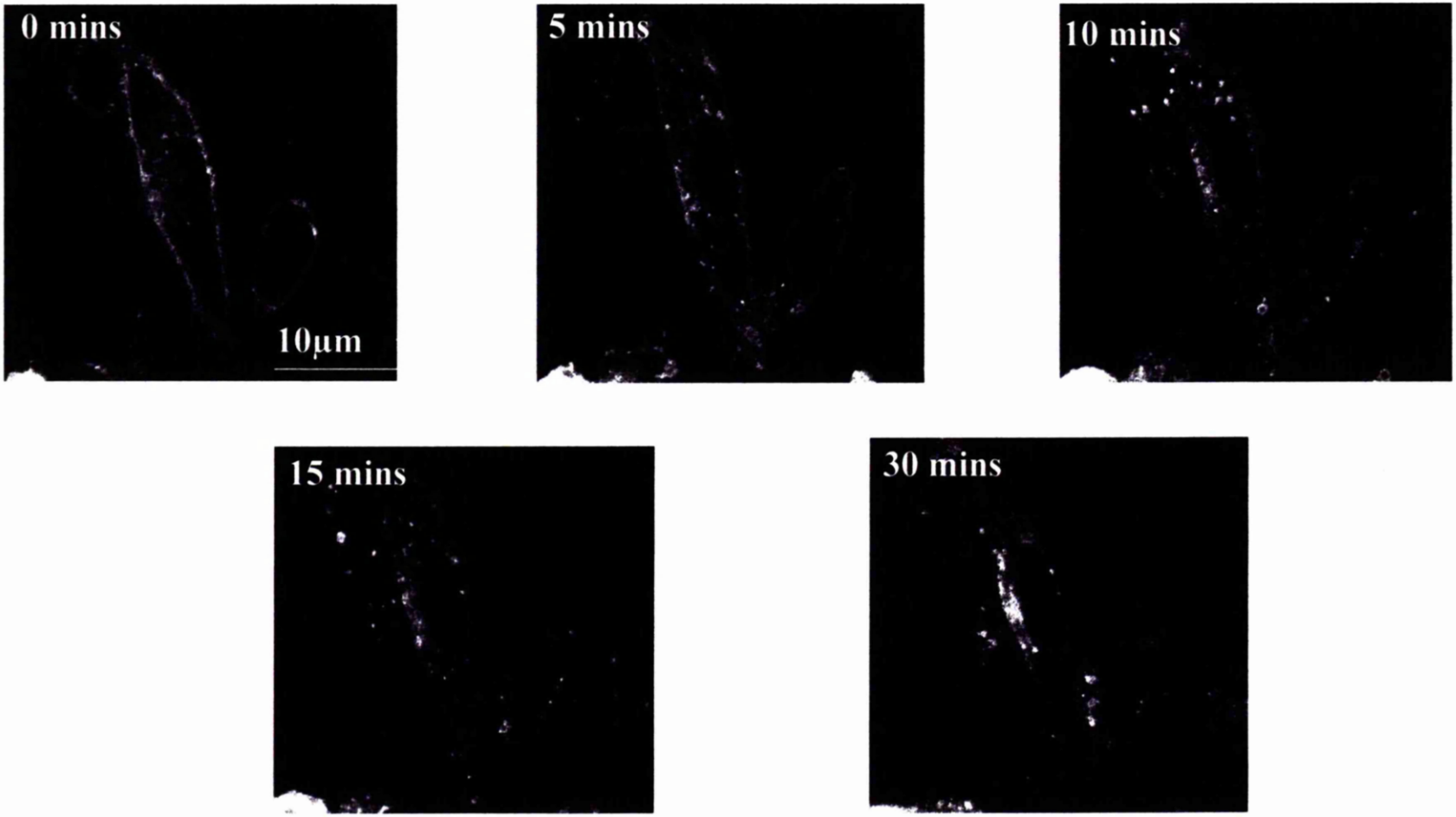

0 mins
10µm
5 mins
10 mins
15 mins
30 mins

Figure 3.15: Schematic Diagram Of The HA-A_1(Cys309-Ala)AR-GFP Expression Construct

An epitope-tagged form of the human A_1AR containing a mutation at the cysteine residue in the C-terminal domain, thought to be a possible site of palmitoylation, to alanine was tagged with GFP using a pCMV5/HA-A_1(Cys309-Ala)AR template. The C-terminal HA-tag present on the A_1(Cys309-Ala)AR was removed by PCR and ligated into the multiple cloning site of pEGFP-N1 at *Hind*III/*Kpn*I. The addition of the GFP tag allowed visualisation studies to be carried out to determine the potential effect of palmitoylation on the internalisation of the cell surface A_1AR following sustained agonist exposure.

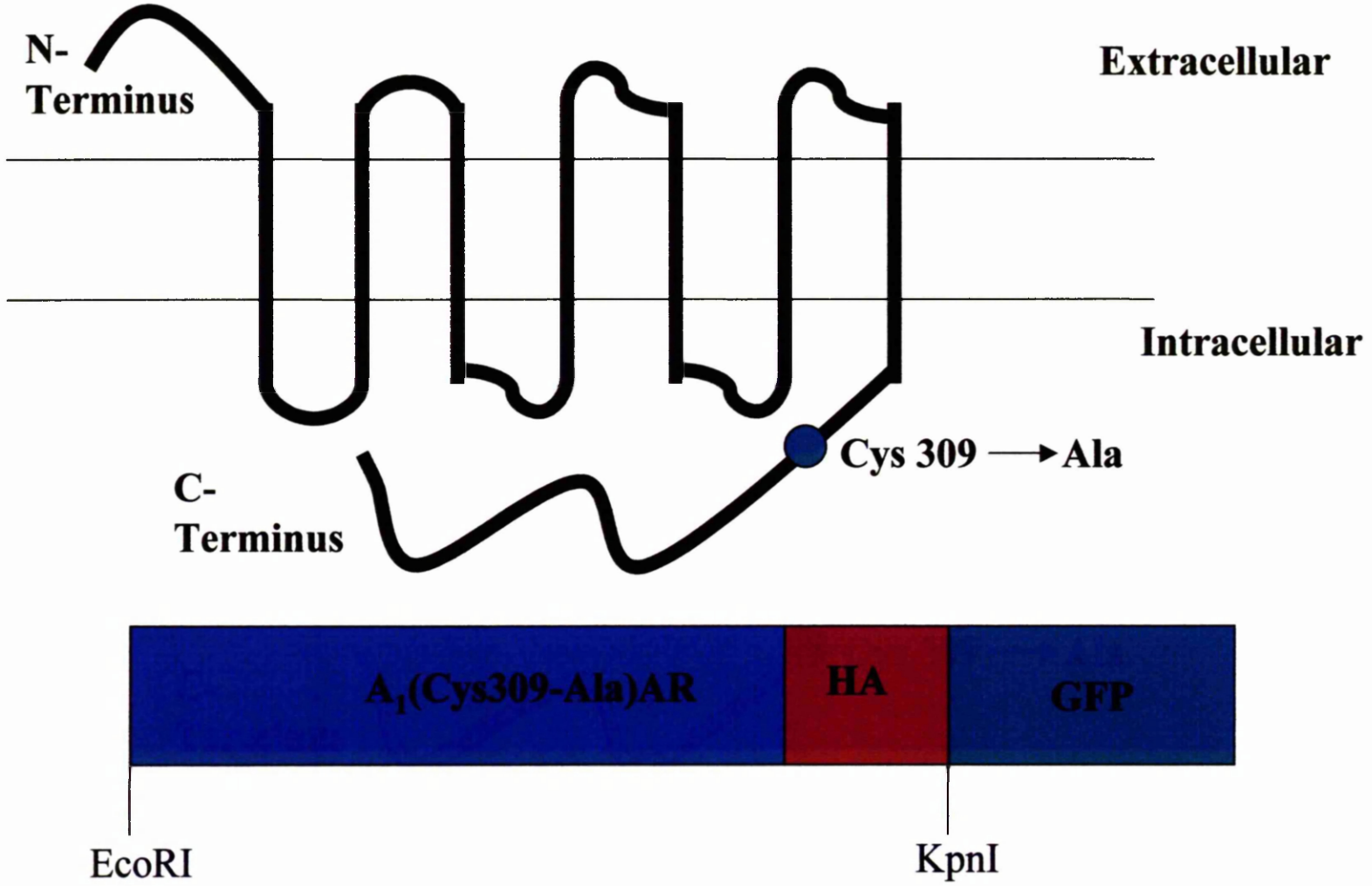

N-
Terminus
Extracellular
Intracellular
Cys 309 → Ala
C-
Terminus
A1(Cys309-Ala)AR
HA
GFP
EcoRI
KpnI

Figure 3.16: Immunoblot Analysis Of HA-A_1AR-GFP And HA-A_1(Cys309-Ala)AR-GFP

Samples of CHO cells transiently expressing either the HA-A_1AR-GFP or the HA-A_1(Cys-309-Ala)AR-GFP construct were solubilised in electrophoresis sample buffer and analysed by SDS-PAGE and immunoblotting with the anti-GFP antibody against the GFP tag. This represents one of multiple experiments.

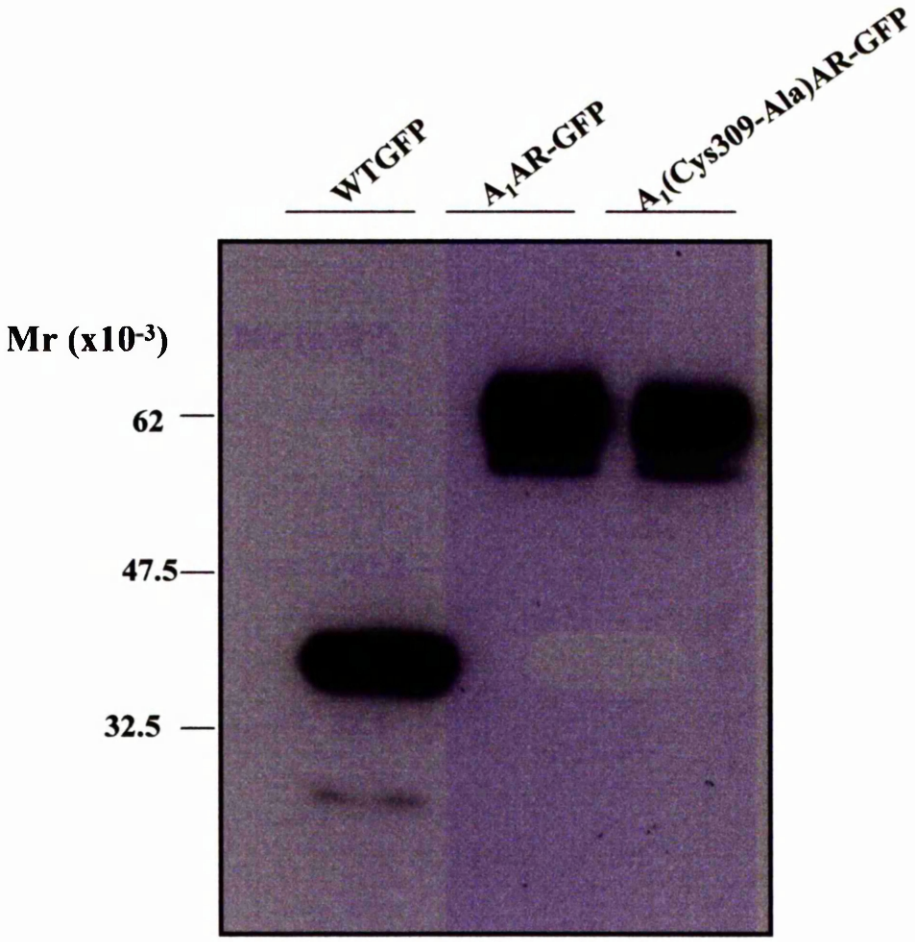
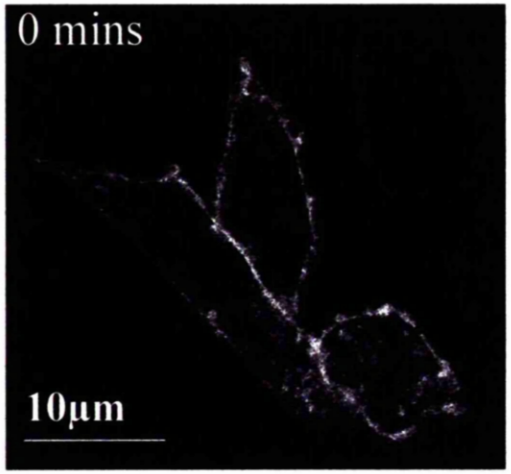
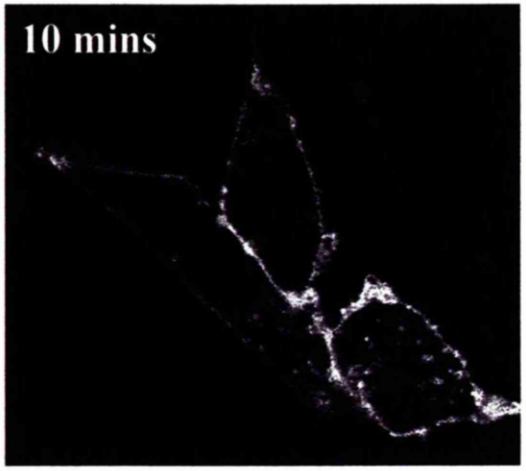
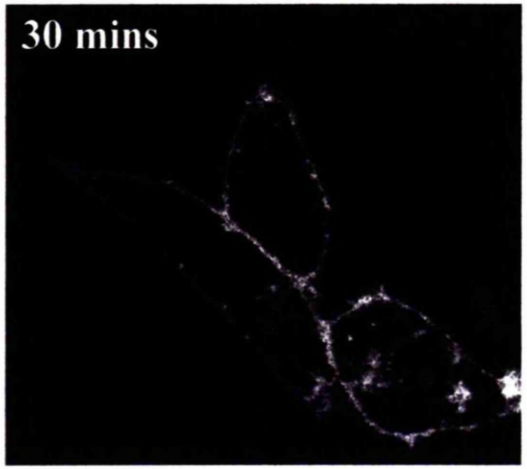

WTGFP
A1AR-GFP
A1(Cys309-Ala)AR-GFP
Mr (x10-3)
62
47.5
32.5

Figure 3.17: Real-Time Visualisation Of The Effect Of Palmitoylation Of Cys309 On A_1AR-GFP Cell Surface Expression Following Sustained Agonist Treatment

The Cys309Ala-mutated A_1AR-GFP construct was transiently transfected into CHO cells and observed under live cell conditions by confocal microscopy for any agonist-dependent changes in cell surface distribution. With no agonist treatment, the receptor is expressed solely on the cell surface. As with the wild type A_1AR-GFP construct, a 1 hour treatment with 5μM R-PIA did not result in any visible changes in cellular distribution. This suggests that palmitoylation of Cys309 within the C-terminus of the A_1AR does not influence internalisation of the receptor in response to sustained agonist exposure. This represents a typical example of three similar experiments.


0 mins
10µm


10 mins


30 mins

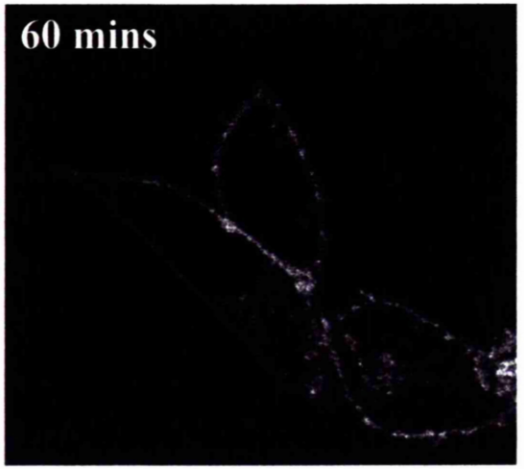
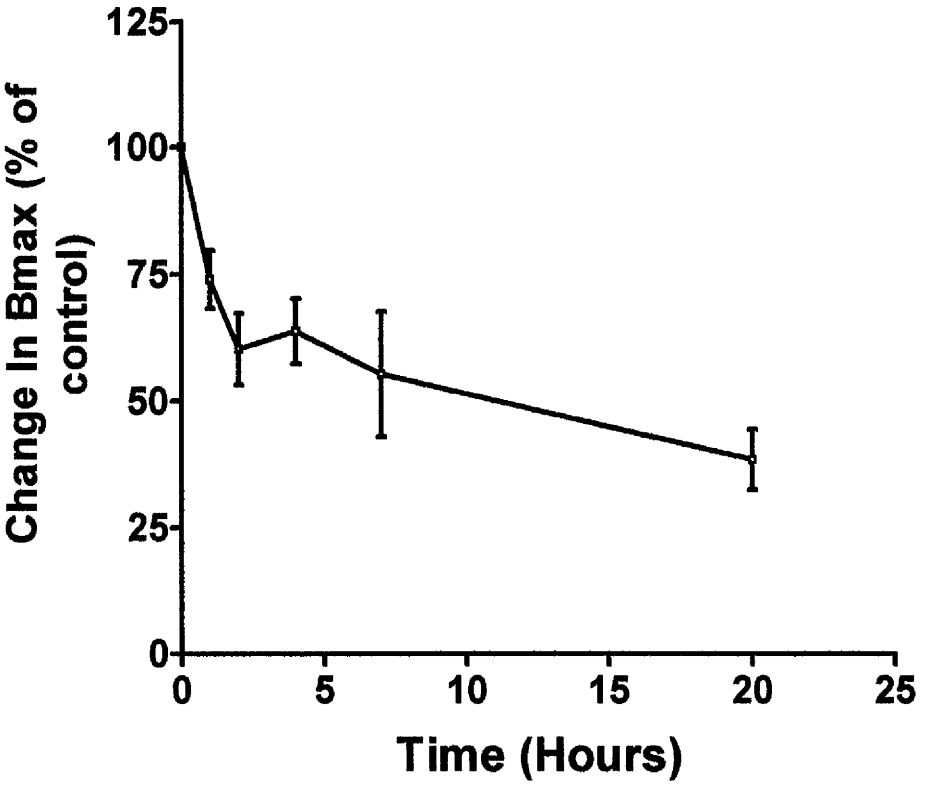

60 mins

Figure 3.18: Saturation Analysis of [^{3}H] DPCPX Binding To HA-A_1ARs Following A 20 Hour Agonist Time Course

Membranes prepared from CHO cells stably transfected with the HA-A_1AR expression construct were treated with vehicle or R-PIA for 1,2,4,7 or 20 hours in the presence of 0.5 units/ml adenosine deaminase as indicated in the graph. The membranes were then used for saturation binding assays as described in the materials and methods using 8nM [^{3}H] DPCPX. This represented a maximal dose of the A_1AR antagonist. Data is presented as mean±SEM. The graph of three similar experiments shows that there is significant down-regulation over 20 hours, with a 44.7 ± 12.4% decrease in B_{max} observed after 7 hours in comparison to untreated controls. The time course of this decrease in B_{max} ($t_{1/2}$=60 mins) is similar to the time course of the loss of cell surface A_1AR, suggesting that the loss of A_1AR from the cell surface may be due to down-regulation of the receptor.


Change In Bmax (% of control)
125
100
75
50
25
0
0
5
10
15
20
25
Time (Hours)

Chapter 4

Analysis Of The Phosphorylation Of The Human EDG1 Receptor

Introduction

S1P is a bioactive lipid released from a number of cells, such as platelets and fibroblasts (Goetzl *et al.*, 1998; Van Brocklyn *et al.*, 1998; Liu *et al.*, 1999; Olivera *et al.*, 1999). S1P initiates a number of cellular effects such as mobilisation of intracellular calcium, regulation of cytoskeletal organisation and cell growth, differentiation, survival and motility (Goetzl *et al.*, 1998; Im *et al.*, 1997; Zondag *et al.* 1998; Van Brocklyn *et al.*, 1998; Okamoto *et al.*, 1998; An *et al.*, 1999; Kon *et al.*, 1999; Liu *et al.*, Olivera *et al.*, 1999). These effects have been attributed to both the intracellular and extracellular actions of S1P (Van Brocklyn *et al.*, 1998; An *et al.*, 1999; Kon *et al.*, 1999; Sato *et al.*, 1999; Olivera *et al.*, 1999).

The extracellular effects of S1P are due to its binding to specific members of the EDG family of GPCRs (Olivera *et al.*, 1999; Sato *et al.*, 1999; Kon *et al.*, 1999). While this family consists of eight members, termed EDG1-8, only EDG1, 3, 5, 6 and 8 are high affinity S1P receptors (Lynch *et al.*, 1999; Hla *et al.*, 2001, Malek *et al.*, 2001, Im *et al.*, 2001). The binding of S1P to the EDG1 receptor results in pertussis toxin (PTx)-sensitive, Gi-dependent activation of ERK2 and the inhibition of adenylyl cyclase (Sato *et al.*, 1999; Lee *et al.*, 1998; Lynch *et al.*, 1999; Ancillin *et al.*, 1999). In addition, EDG1 also stimulates the PTx-insensitive, $G_{12/13}$-mediated activation of Rho-coupled pathways that regulate morphogenesis, such as adherens junction assembly and translocation of P- and E-cadherin (Kon *et al.*, 1999; Liu *et al.*, 1999; Lee *et al.*, 1999; Ancellin *et al.*, 1999). EDG1, along with EDG3, also regulates signalling pathways required for human umbilical vein endothelial cell morphogenesis into capillary-like networks (Lee *et al.*, 1998). The same study also suggested that antagonists of EDG1 and EDG3 could attenuate the enhanced angiogenesis process associated with solid tumour growth, rheumatoid arthritis and diabetic retinopathy (Lee *et al.*, 1998).

Given the importance of EDG1 signalling in processes such as angiogenesis, the molecular mechanisms regulating EDG1 signalling therefore have tremendous therapeutic potential. As described previously, sustained agonist occupation of many GPCRs can result in the desensitisation of receptor function; internalisation of the receptor away from the cell surface and coupling of GPCRs to alternative signalling pathways (Ferguson, 2001; Cao *et al.*, 1998; Zhang *et al.*, 1999). In many cases, each of these processes are dependent upon GPCR phosphorylation on serine and threonine residues within either the third intracellular loop and/or C-terminal tail by both second messenger-dependent protein kinases, such as PKA and PKC, and GRKs (Ferguson, 2001: von Zastrow *et al.*, 1994;

Goodman *et al.*, 1996). In the case of GRK-mediated phosphorylation, the agonist-dependent conformational change in the receptor promotes the selective binding of arrestins to agonist-activated receptors (Jie Zhang *et al.*, 1999; Barak *et al.*, 1997; Miller and Lefkowitz, 2001; Pierce and Lefkowitz, 2001). The binding of arrestin sterically uncouples the receptor from heterotrimeric G-proteins, targets many GPCRs for internalisation in clathrin coated vesicles and, in some cases, can initiate alternate signalling pathways such as the arrestin-mediated increase in ERK and JNK signalling found with the β_2AR (Jie Zhang *et al.*, 1999; Barak *et al.*, 1997; Miller and Lefkowitz, 2001; Pierce and Lefkowitz, 2001, Ferguson, 2001).

GPCR phosphorylation may therefore represent a crucial step in both the rapid desensitisation of GPCR function and in the subsequent processes of internalisation. This was highlighted in the previous Chapter for the A_1 and A_3 adenosine receptors where the phosphorylation-deficient A_1AR internalised much slower compared with the rapidly phosphorylated A_3AR. Hence, in order to begin characterising the regulation of EDG1 signalling, it is important to first fully characterise EDG1 receptor phosphorylation. In this Chapter, the molecular mechanisms regulating EDG1 phosphorylation in stably transfected hamster lung CCL-39 fibroblasts have been characterised in detail. In addition, truncation mutants of the EDG1 C-terminus were used to define specific regions of EDG1 phosphorylation

Results

Due to the lack of a selective commercially available antibody against endogenous EDG1 receptors, a myc epitope tag was incorporated into a human EDG1 cDNA expression construct (Figure 4.1). This facilitated the identification and isolation of recombinant receptors using the monoclonal 9E10 antibody which recognises the myc tag. The construct was stably expressed in CCL-39 hamster lung fibroblasts using a modified calcium phosphate precipitation/glycerol shock transfection procedure and stable expression of the mycEDG1 receptor in CCL-39 cells was then confirmed by immunoblotting using the 9E10 (Figure 4.2). Analysis of ERK activation showed that mycEDG1 activated ERK in the presence of S1P when stably expressed in CCL-39 cells. Concentration-response curve analysis of ERK activation at 10 min demonstrated that S1P produced a 6-10 fold activation of ERK (EC_{50}=0.4μM) in mycEDG1-expressing, but not parental, CCL39 cells (Figure 4.3, n=3). This demonstrated that the recombinant human

EDG1 receptor remained functional despite the addition of the myc epitope to its C-terminus.

To begin the characterisation of EDG1 phosphorylation, a whole cell phosphorylation study using serum-starved CCL-39/mycEDG1 cells was carried out in the presence of the agonist, S1P and a number of activators of second messenger-regulated kinases (Figure 4.4, n=3). These included 1μM PMA, a phorbol ester that activates conventional and novel PKC subtypes; 10μM A23187, a calcium ionophore; 10μM forskolin, an activator of adenylyl cyclase and 100μM 8-Br-cGMP, non-hydrolysable analogue of cGMP that activates protein kinase G (PKG). A 12-25-fold increase in EDG1 phosphorylation was observed following exposure to 10μM S1P exposure (range from 20 experiments) whereas a weaker but still significant increase in EDG1 phosphorylation in the region 4-8 fold over basal levels was observed under the same conditions following 1μM PMA treatment (range from 16 experiments) (Figure 4.4). The other second messenger activators failed to induce EDG1 phosphorylation (*versus* a vehicle-treated control where S1P-induced EDG1 phosphorylation set at 100%, p>0.05, n=3, Figure 4.4).

Previous studies have produced conflicting results with respect to the role of LPA in EDG1 signalling, with one group suggesting that LPA is a partial agonist for EDG1 (Lee *et al.*, 1998) whereas other groups have found LPA to have no effect on EDG1 activation (Windh *et al.* 1998; Zondag *et al.*, 1998). To resolve this issue, a whole cell phosphorylation study of serum-starved CCL-39/mycEDG1 cells exposed to S1P, PMA or LPA demonstrated that, under conditions where both S1P and PMA resulted in a strong phosphorylation of EDG1, no significant increase in phosphorylation was observed over basal levels following 100μM LPA exposure (4.4 ± 2.4 % *versus* vehicle-treated controls where S1P-induced EDG1 phosphorylation set at 100%, p>0.05, N/S, Figure 4.5a). The inability of LPA to induce significant EDG1 phosphorylation was not caused by any discrepancies in the integrity of the LPA because LPA was shown to strongly phosphorylate EDG2 in related studies within the lab (Figure 4.5b). Hence, EDG1 selectively undergoes phosphorylation in the presence of S1P and also following activation of PKC. In addition, a phosphoamino acid analysis demonstrated that both S1P and PKC result in the phosphorylation of serine and threonine residues within the EDG1 receptor (Figure 4.6).

To further characterise S1P-induced EDG1 phosphorylation, a whole cell phosphorylation study on serum-starved CCL-39/mycEDG1 cells was carried out in the

presence of a range of S1P concentrations. S1P produced a concentration-dependent increase in EDG1 phosphorylation (EC_{50}= 1.9±0.37μM, *n*=3, Figure 4.7). A concentration of 10μM S1P was subsequently chosen for the remaining experiments as this was shown to induce maximal EDG1 phosphorylation but remained physiologically relevant. A time course of EDG1 phosphorylation in the presence of 10μM S1P showed that phosphorylation is a rapid process, with significant EDG1 phosphorylation observed after the earliest time point examined (15 seconds) and near maximal EDG1 phosphorylation achieved after 60 seconds (*n*=3, Figure 4.8). Maximal EDG1 phosphorylation was then maintained for a further 20 min in the continuous presence of 10μM S1P (*n*=3, Figure 4.8). 10μM S1P-induced EDG1 phosphorylation was also shown to be a reversible process, with a significant decrease in EDG1 phosphorylation observed after only 30mins of agonist removal (44.3±15.6% reduction in S1P-induced EDG1 phosphorylation *versus* maximal S1P-induced EDG1 phosphorylation (set at 100%), $p<0.05$, *n*=3, Figure 4.9). After 2 hours of agonist removal, phosphorylation of EDG1 remained significantly greater than basal EDG1 phosphorylation but was dramatically reduced from maximal S1P-induced EDG1 phosphorylation. (86.1±2.5% reduction in S1P-induced EDG1 phosphorylation *versus* maximal S1P-induced EDG1 phosphorylation, $p<0.05$, *n*=3, Figure 4.9).

Using other examples of agonist-dependent GPCR phosphorylation, a potential candidate for the kinase involved in S1P-induced EDG1 phosphorylation was a member of the GRK family. Purified forms of GRK2, representing the GRK2 subfamily of GRKs, and GRK5, representing the GRK5 subfamily were chosen to examine the role of GRKs in S1P-induced EDG1 phosphorylation (Figure 4.10). An *in vitro* study using membranes from serum-starved CCL-39/mycEDG1 cells incubated in the presence or absence of GRK2 or GRK5 showed that a strong S1P-induced EDG1 phosphorylation was observed following incubation with GRK2 (*n*=3, Figure 4.11). However, no EDG1 phosphorylation was observed in the presence of purified GRK5 (*n*=3, Figure 4.11). The purified forms of GRK2 and GRK5 were shown to be functional in a similar *in vitro* study using the light receptor, rhodopsin. A 15 min exposure of light induced receptor phosphorylation in the presence of both GRK2 and GRK5, suggesting that both forms were functional (Figure 4.12, *n*=3). Therefore, S1P-induced EDG1 phosphorylation is specific for GRK2 *in vitro*.

PMA-induced EDG1 phosphorylation was then characterised and compared with S1P-induced receptor phosphorylation. A concentration-response of PMA-induced EDG1 phosphorylation showed that this was concentration-dependent (EC_{50} =0.1μM, *n*=3, Figure

4.13). Interestingly, a time course of PMA-induced phosphorylation showed that, in contrast to S1P-induced phosphorylation, there is a delayed onset of EDG1 phosphorylation. Whereas S1P-induced EDG1 phosphorylation is near maximal after 1 min, there appears to be a bi-phasic effect following 1μM PMA exposure where receptor phosphorylation peaks at around 50% of maximal phosphorylation after 5 min, followed by a second phase towards maximal phosphorylation after 10mins (n=3, Figure 4.14).

The question remained whether or not S1P-induced EDG1 phosphorylation was PKC dependent. In other words, could S1P activate PLC that could, in turn, increase intracellular calcium and activate DAG. This would result in PKC activation, causing EDG1 phosphorylation (Figure 4.15). In order to address this point, whole cell phosphorylation assays were carried out where serum-starved CCl-39/myc-EDG1 cells were treated with vehicle, 10μM S1P or 1μM PMA in the presence or absence of 5μM GF109203X, an inhibitor of conventional and novel PKC subtypes (Martiny-Baron *et al.*, 1993; Way *et al.*, 2000). Figure 4.16 shows that, in the absence of GF109203X, there is a strong S1P-induced phosphorylation and a weaker, but still significant, PMA-induced EDG1 phosphorylation, as described before. However, in the presence of GF109203X, the effect of PMA is virtually abolished (phosphorylation reduced by 78±8% *versus* vehicle-preincubated PMA-treated cells (set at 100%), p<0.05, n=3) whereas S1P-induced receptor phosphorylation is barely affected (phosphorylation reduced by 18±10%, p<0.05, n=3) (Figure 4.16). This suggests that two phosphorylation pathways exist: an S1P, agonist-dependent pathway and an agonist-independent phosphorylation pathway, mediated by PKC.

PMA-induced EDG1 phosphorylation was then examined in the presence or absence of Go6976, an inhibitor of conventional PKC subtypes and rottlerin, an inhibitor of novel PKC subtypes, primarily PKCδ, (Martiny-Baron *et al.*, 1993; Gschwendt *et al.*, 1994) in order to elucidate the subtypes of PKC involved in EDG1 phosphorylation . Figure 4.17 shows a preincubation with 5μM GFX inhibits PMA-induced EDG1 phosphorylation by 90.3±2.3% (*versus* maximal PMA-induced receptor phosphorylation (set at 100%), p<0.05, n=3). However, preincubation with Go6976 resulted in a reduction in PMA-induced phosphorylation of 47.6±1.5% (*versus* maximal PMA-induced receptor phosphorylation (set at 100%), p<0.05, n=3) and preincubation with rottlerin reduced PMA-induced EDG1 phosphorylation by 58.6±8.1% (*versus* maximal PMA-induced receptor phosphorylation (set at 100%), p<0.05, n=3), suggesting the involvement of

typical and atypical subtypes of PKC (Figure 4.17). In addition, the effects of Go6976 and rottlerin were shown to be additive. Preincubation of Go6976 and rottlerin together reduced PMA-induced receptor phosphorylation by 85.1±8.6% (*versus* maximal PMA-induced receptor phosphorylation, $p<0.05$, $n=3$), reinforcing the suggestion that both groups of PKC subtypes are involved in PMA-induced EDG1 phosphorylation (Figure 4.18). However, despite an additive effect being observed following incubation with Go6976 and rottlerin together, this was not significantly different to either Go6976 or rottlerin alone ($p>0.05$, N/S, $n=3$, Figure 4.18)

Since there are two distinct pathways of phosphorylation, it was possible that the effect of PMA and S1P on EDG1 phosphorylation may involve different phosphorylation sites and could therefore be additive. To address this possibility, whole cell phosphorylation assays were carried out on CCL-39/mycEDG1 cells either treated for 20 mins with vehicle; pretreated for 10 minutes with 1μM PMA, followed by a 10 minute treatment with 10μM S1P; pretreated with 10μM S1P followed by a 10 min treatment of 1μM PMA; or treated for 10 min with 10μM S1P and 1μM PMA together. Figure 4.19 shows that a 10 min pretreatment with 1μM PMA, followed by a 10 minute treatment with 10μM S1P resulted in 89.8±8.2% receptor phosphorylation (*versus* maximal S1P-induced receptor phosphorylation (set at 100%), $p>0.05$, N/S, $n=3$). A 10 min treatment of 10μM S1P and 1μM PMA together induced 122±34% receptor phosphorylation (*versus* maximal S1P-induced receptor phosphorylation (set at 100%), $p>0.05$, N/S, $n=3$). A 10 minute pretreatment with 10μM S1P followed by a 10 min treatment of 1μM PMA resulted in 127.4±17.5% receptor phosphorylation (*versus* maximal S1P-induced receptor phosphorylation (set at 100%), $p>0.05$, N/S, $n=3$). Therefore, all the treatments involving the addition of both S1P and PMA together resulted in a phosphorylation similar to EDG1 phosphorylation in the presence of S1P alone. Hence, although S1P and PKC induce EDG1 phosphorylation by distinct mechanisms, each pathway may involve the phosphorylation of overlapping sites within the EDG1 receptor.

Previous studies have demonstrated that sphingosine kinase, the enzyme responsible for the conversion of sphingosine to sphingosine-1-phosphate within cells, can be stimulated by phorbol esters such as PMA (Cuvillier *et al.*, 1996, Pyne and Pyne, 2000a; Pyne and Pyne, 2000b). This could potentially increase the concentration of intracellular sphingosine-1-phosphate, which in turn would be released into the extracellular space, and lead to an agonist-mediated phosphorylation of EDG1 (Figure

4.20). One of the most commonly used inhibitors of sphingosine kinase is D,L-*threo*-dihydrosphingosine (DHS). However, at high doses, DHS inhibits PKC subtypes in addition to sphingosine kinase. A previous study has shown that 1μM DHS can inhibit sphingosine kinase activity (Kohama *et al.*, 1998). Importantly, this concentration of DHS had no effect on PKC activation (Tolan *et al.*, 1999). Figure 4.21 shows that, in a whole cell phosphorylation study, PMA treatment induced 93.5±12.5% EDG1 phosphorylation in the presence of 1μM DHS (*versus* maximal PMA-induced EDG1 phosphorylation (set at 100%), $p>0.05$, N/S, $n=3$). Immunoblotting of PMA-dependent ERK activation was carried out in parallel to each whole cell phosphorylation study. These acted as a measure of PMA-stimulated PKC activity in the presence of DHS. Figure 4.22 demonstrates that 1μM DHS had no significant effect on PKC activity (103.5±12.2% *versus* PMA-treated EDG1 cells (set at 100%), $p>0.05$, N/S, $n=3$). This is consistent with previous studies (Tolan *et al.*, 1999) and suggests that, at a concentration of 1μM, DHS selectively inhibited sphingosine kinase. However, because of the non-selective nature of DHS, this should be confirmed in future studies using other approaches, such as oligodeoxynucleotide anti-sense against sphingosine kinase.

Other examples of GPCR phosphorylation, such as the β_2AR, have shown that phosphorylation of the C-terminal tail is an important precursor for mechanisms of receptor desensitisation and internalisation (Ferguson, 2001: von Zastrow *et al.*, 1994; Goodman *et al.*, 1996). As shown in the previous Chapter, the lack of potential phosphorylation sites within the C-terminal tail of the A_1AR was linked with receptor's slow rate of internalisation. In contrast, the A_3AR, which is G_i-coupled like the A_1AR and exhibits similar biological effects, is rapidly internalised following phosphorylation of its C-terminal domain. Within the C-terminal tail of the EDG1 receptor, there are a number of serine and threonine residues distal to the predicted sites for palmitate attachment (Figure 4.23). Therefore, a truncation mutant removing the last 51 amino acids of the C-terminal tail of the mycEDG1 receptor was designed to remove all the potential phosphorylation sites within the EDG1 C-terminal (Figure 4.24).

The mycEDG1Δ51 receptor was generated as described in the Materials And Methods Chapter and then stably expressed in CCL-39 hamster lung fibroblasts. Stable expression of the mycEDG1Δ51 receptor was confirmed by immunoblotting using 9E10 (Figure 25). Whole cell phosphorylation studies were then carried out using WT CCL-39/mycEDG1 and CCL-39/mycEDG1Δ51 cells exposed to a 10 min treatment of vehicle, 10μM S1P or

1μM PMA. Figure 4.26 shows that whereas the WT EDG1 receptor is phosphorylated in the presence of S1P and PMA, neither S1P or PMA were able to stimulate phosphorylation of the truncated receptor (Figure 4.26, n=3). Hence, agonist-dependent and agonist-independent phosphorylation require the integrity of the last 51 amino acids.

As shown in Figure 4.11, EDG1 is preferentially phosphorylated *in vitro* by GRK2 rather than GRK5. Within the last 51 amino acids of the C-terminal of EDG1, there are two distinct clusters of potential serine/threonine phosphorylation sites, which can be removed by the truncation of the last 12 and the last 32 amino acids of the C-terminal of the mycEDG1 receptor (Figure 4.23). Importantly, distinct clusters of these potential phosphorylation sites are located proximal to acidic amino acids, representing potential GRK2 phosphorylation sites (Ferguson, 2001). Therefore, the truncation mutants, mycEDG1Δ32 and mycEDG1Δ12 were generated to remove these potential GRK2 phosphorylation sites (Figure 4.24). Expression of the mycEDG1Δ12 and mycEDG1Δ32 receptors was confirmed by immunoblotting using the anti-myc antibody, 9E10 (Figure 4.27). Wild type mycEDG1, mycEDG1Δ12 and mycEDG1Δ32 receptor cDNA constructs were then transfected into HEK 293 cells and the cells given a 10 minute exposure of either vehicle, 10μM S1P or 1μM PMA in a whole cell phosphorylation study. Receptor phosphorylation quantitated using autoradiography was then normalised using a parallel blot of receptor expression. Truncation of the last 32 amino acids completely abolished both S1P- and PMA- induced phosphorylation. There was no significant difference between WTEDG1 and EDG1Δ12 PMA-induced phosphorylation (28.9±5.4% *versus* PMA-induced WT mycEDG1 phosphorylation (S1P-induced WT EDG1 phosphorylation set at 100%), p<0.05, n=3,) (Figure 4.28). However, although S1P-induced phosphorylation is maintained following the truncation of the last 12 amino acids, maximal phosphorylation was significantly less when compared to WT EDG1 S1P-induced EDG1 phosphorylation (47.9±8.7% *versus* S1P-induced WT mycEDG1 phosphorylation (set at 100%), p<0.05, n=3,) (Figure 4.28).

As described above, S1P induces EDG1 phosphorylation *in vitro* in the presence of purified GRK2. An *in vitro* study was therefore carried out on membranes of HEK293 cells transfected with WTEDG1, EDG1Δ32 or EDG1Δ12 receptor cDNA constructs in order to examine the effect of removing the potential GRK2 phosphorylation sites more fully. In the presence of purified GRK2, no significant difference was observed between S1P-induced EDG1 phosphorylation in membranes containing either WTEDG1 or

EDGΔ12 (Figure 4.29). In contrast, no significant S1P-induced phosphorylation was observed using EDG1Δ32-expressing membranes (Figure 4.29). Hence, truncation of the last 32 amino acids abolishes S1P-induced phosphorylation in the presence of purified GRK2 although phosphorylation is maintained following the truncation of the last 12 amino acids within the EDG1 C-terminal.

Discussion

An important regulatory process of GPCR signalling is receptor phosphorylation. In many cases, such as the β_2AR and the A_3AR, phosphorylation is regarded as the critical step necessary to observe both receptor desensitisation and internalisation (Ferguson, 2001; (Ferguson, 2001: von Zastrow *et al.*, 1994; Goodman *et al.*, 1996). For example, the previous chapter has demonstrated that the A_1AR's inability to undergo receptor phosphorylation is associated with a relatively slow, incomplete loss of cell surface receptors following sustained agonist exposure. This chapter has characterised the phosphorylation of the S1P receptor, EDG1 using a myc-tagged human EDG1 receptor and a series of C-terminal truncation mutants.

In CCL-39 hamster lung fibroblasts, there is a strong, reversible, agonist-dependent phosphorylation of EDG1 (Figures 4.7, 4.8 and 4.9). The EC_{50} value (1.9±0.4μM, Figure 4.7) for S1P-induced EDG1 phosphorylation related to the physiological range of S1P concentration in the blood which can reach μM concentrations upon platelet activation (Pyne and Pyne, 2000). However, previous studies have shown that the K_D of S1P for the EDG1 receptor is between 8.1nM (Lee *et al.*, 1998) and 13.2 nM (Kon *et al.*, 1999). One reason for the differences between the observed EC_{50} value and the reported K_D values may be due to differences in the preparation of S1P between research groups. It should also be stressed that many of the biological responses of S1P, such as the mobilisation of Ca^{2+} in HEK293 cells transfected with EDG1 (Van Brocklyn *et al.*, 1998) and in the rat mast cell line RBL-2H3, require μM concentrations of S1P. Due to the unavailability of a reliable, radiolabelled form of S1P, it was not possible to carry out binding studies to determine the K_D of S1P for EDG1 in relation to this set of experiments. Future measurement of the K_D for S1P would allow for a more accurate reflection of the calculated EC_{50} value in relation to the comparatively low published K_D value. A measured K_D value may also explain why S1P-induced phosphorylation does not mirror the agonist-occupation curve related to the published K_D.

S1P also induced EDG1 phosphorylation *in vitro* using CCL-39/mycEDG1 membranes in the presence of purified GRK2 (Figure 4.11). In contrast, no EDG1 phosphorylation was observed in the presence of GRK5 (Figure 4.11). This suggests that S1P-induced EDG1 phosphorylation may be mediated by GRK2. In addition to an agonist-mediated phosphorylation of EDG1, a weaker, but still significant, agonist-independent phosphorylation of EDG1 that was mediated by PKC (Figure 4.16). Whereas agonist-dependent EDG1 phosphorylation is a rapid process, with a significant phosphoryation observed after only 15 seconds (Figure 4.8), PKC-mediated EDG1 phosphorylation is a more delayed process, with half-maximal phosphorylation only observed after 5 min (Figure 4.14). A role for PKC in GPCR phosphorylation and desensitisation has been demonstrated in other studies for a number of G_i-and G_q- linked GPCRs, including the α_{1B}-adrenoceptor and the type 1A angiotensin II receptor (Diviani *et al.*, 1997; Liang *et al.*, 1998; Tang *et al.*, 1998).

Interestingly, LPA exposure failed to induce EDG1 phosphorylation (Figure 4.5a). This is in contrast to a previous study that showed that EDG1 is a low-affinity receptor for LPA and that LPA increased EDG1 phosphorylation (Lee *et al.*, 1998). However, it has also been shown that LPA failed to elicit any biological effects in membranes of Sf9 cells co-expressing EDG1 and G_{12}, whereas S1P was effective (Windh *et al.*, 1999, Zondag *et al.*, 1998). Other studies have not observed competition of [^{32}P]S1P binding by LPA (Lee *et al.*, 1996; Van Brocklyn *et al.*, 1999). Additionally, LPA did not function as an agonist for the murine analog of EDG1, lp_{B1}, when transfected into RH7777 cells (Zhang *et al.*, 1999). One possible explanation for the LPA-induced phosphorylation observed by Lee *et al.* may be that LPA stimulated endogenous LPA receptors, such as EDG2. This could have resulted in an indirect phosphorylation of EDG1, possibly mediated by EDG2 activation of PKC. Alternatively, the activation of endogenous LPA receptors by LPA may have induced the release of S1P which subsequently activated EDG1.

A series of PKC inhibitors were used in order to investigate the PKC subtypes involved in EDG1 phosphorylation. GF109203X, an inhibitor of conventional and novel PKC subtypes abolished PMA-induced EDG1 phosphorylation (Figure 4.16). Both Go6976, an inhibitor of conventional subtypes, and rottlerin, an inhibitor of the novel PKC subtype, PKCδ inhibited PMA-induced EDG1 phosphorylation by around 50% (Figure 4.17). This suggested that both conventional and novel subtypes of PKC were involved. However, significant inhibition of PMA-induced EDG1 phosphorylation was only

observed at doses of 100μM. At this dose, it is known that, in addition to inhibiting PKCδ, rottlerin also inhibits conventional subtypes as well as other protein kinases such as casein kinase II (Way *et al.*, 2000). Hence, the inhibition observed with rottlerin may be, at least in part, due to a kinase other than PKC. Future work should therefore be aimed at the possible role of other kinases in PMA-induced EDG1 phosphorylation such as casein kinase II. Interestingly, a potential site of casein kinase II phosphorylation was identified within the area of the EDG1 C-terminus implicated in the process of receptor internalisation (Liu *et al.*, 1999). However, casein kinase II is constitutively active and would therefore be unlikely to be involved in agonist-dependent processes of phosphorylation and desensitisation.

This Chapter has demonstrated that PMA-induced phosphorylation was not the result of an increase in intracellular S1P production (Figure 4.21). In addition, S1P- and PMA- mediated EDG1 phosphorylation are not additive (Figure 4.19). This suggests that, although S1P and PMA regulate EDG1 phosphorylation by distinct mechanisms, they may ultimately be competing for overlapping phosphorylation sites. Phosphoamino acid analysis of EDG1 demonstrated that S1P and PMA incerased incorporation of phosphate into serine and threonine residues (Figure 4.6). In order to narrow down the region of phosphorylation within EDG1, a series of EDG1 truncation mutants removing clusters of potential Ser/Thr phosphorylation sites were analysed. Importantly, both these sites are situated proximal to acidic residues, making them potential targets for aciditrophic kinases such as GRK2 (Ferguson, 2001) (Figure 4.23). PMA-induced EDG1 phosphorylation was abolished by the truncation of the last 32 amino acids but was unaffected by the truncation of the last 12 amino acids (Figure 4.28). S1P-mediated EDG1 phosphorylation was also abolished by the truncation of the last 32 amino acids and was significantly attenuated following the truncation of 12 amino acids (Figure 4.28). This suggests that residues within the last 12 amino acids, as well as residues between the last 32 and the last 12 amino acids, are involved in S1P-mediated EDG1 phosphorylation. In contrast, PMA-induced EDG1 phosphorylation did not involve the last 12 amino acids of the C-terminal. This provides further evidence that S1P- and PMA- induced EDG1 phosphorylation occurs via distinct mechanisms. In addition, GRK2-dependent S1P-mediated phosphorylation in the membranes HEK293 cells transfected with mycEDG1 is abolished by the truncation of the last 32 amino acids but is maintained following the truncation of the last 12 amino

acids, suggesting that GRK2-mediated EDG1 phosphorylation is located between the last 12 and the last 32 amino acids of the C-terminal domain of EDG1 (Figure 4.29).

Interestingly, truncation of the last 12 amino acids had no significant effect on PMA-induced EDG1 phosphorylation. Figure 4.23 illustrates that the only threonine present within the C-terminus is removed following truncation of the last 12 amino acids. However, the phosphoamino acid analysis shows that PMA phosphorylates both serine and threonine residues. One other possible target of phosphorylation, therefore, may be threonine 236 within the third intracellular loop of EDG1, which also conforms to a consensus PKC phosphorylation site. A recent study has implicated this residue in the Akt-mediated phosphorylation of EDG1 and have shown a role for this phosphorylation in S1P-induced Rac activation, chemotaxis and angiogenesis (Lee *et al.*, 2001). However, truncation of the last 32 amino acids abolished both agonist-dependent and agonist-independent EDG1 phosphorylation. It should be recognised that one possibility that may explain the differences in EDG1 phosphorylation between the wild type and the truncated forms of the receptor could be the loss or disruption of relevant potential kinase docking domains for kinases such as GRK2. This also raises the question of whether truncation of the C-terminal should affect phosphorylation of residues within the third loop, perhaps through changes within the structure of EDG1. Therefore, future studies should be aimed at more restricted site-directed mutagenesis studies, particularly within the last 32 amino acids.

The existence of a mechanism of EDG1 phosphorylation which is independent of agonist suggests that EDG1 phosphorylation can potentially be regulated by signalling *via* other receptors through the activation of PKC. Since EDG1 plays an important role in angiogenesis, many of the receptors integral to the control of angiogenesis could potentially regulate EDG1 phosphorylation. EDG1 signalling has been shown to regulate and be regulated by a number of such receptors. For example, cell migration toward PDGF, which stimulates sphingosine kinase and increases intracellular S1P production, was dependent upon expression of EDG1 (Hobson *et al.*, 2001). Therefore, future work should be aimed at examining the role of cross-talk mechanisms between EDG1 and other receptors, such as other EDG members and receptor tyrosine kinases for PDGF, VEGF and FGF2. Another possible candidate could be the angiopoietin receptors, Tie1 and Tie2. As described previously, Tie1 and Tie2 are receptor tyrosine kinases found solely within the epithelium that are critical for the communication of endothelial cells with the surrounding mesenchyme and are particularly important in blood vessel maturation (Jones *et al.*, 2001).

Since the specific involvement of EDG1 in blood vessel maturation has recently been established, cross-talk between EDG1 and Tie1 and Tie2 signalling represents a potentially important area of future research.

In conclusion, EDG1 phosphorylation is regulated by two independent mechanisms: 1) an agonist-dependent mechanism acting *via* GRK2 *in vitro* and 2) an agonist-independent mechanism regulated by the activation of PKC. The sites of phosphorylation for both regulatory mechanisms of EDG1 phosphorylation are located within the last 32 amino acids of the C-terminal tail of EDG1. Future work should be able to elucidate the sites of phosphorylation more fully using site-directed mutagenesis. The discovery of two regulatory pathways of EDG1 phosphorylation implies that EDG1 may be regulated through signalling *via* other receptors through the activation of PKC. This has potential significance in terms of EDG1 regulation in the context of receptor internalisation and also in physiological processes such as angiogenesis.

Figure 4.1: Schematic Diagram Of The Myc-tagged Human EDG1 Receptor

A myc epitope (orange) containing 6 histidine residues (blue) was added to the C-terminus of the human EDG1 receptor using pcDNA/EDG1 as a template. The sense primer incorporated a HindIII site upstream of a consensus Kozak sequence and the EDG1 initiating methionine, as indicated. The diagram also shows that the antisense primer was designed to remove the EDG1 stop codon and add an XbaI site. This allowed in-frame ligation of the EDG1 coding region with that of the myc-His epitope tag following ligation of the HindIII/XbaI-digested PCR product with a similarly digested pcDNA3.1/ myc-HisA vector.

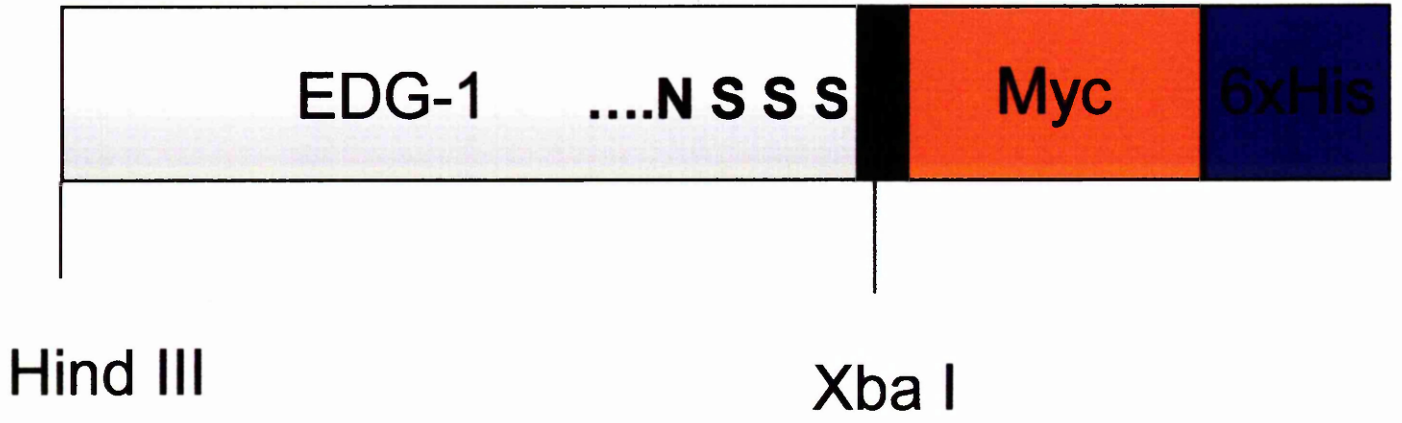

EDG-1
....N S S S
Myc
6xHis
Hind III
Xba I

Figure 4.2: Immunoblot Analysis Of The Myc-Tagged Human EDG1 Receptor

CCL-39 fibroblasts stably expressing the mycEDG1 receptor were solubilised in electrophoresis sample buffer and analysed by SDS-PAGE and immunoblotting using the anti-myc 9E10 monoclonal antibody.

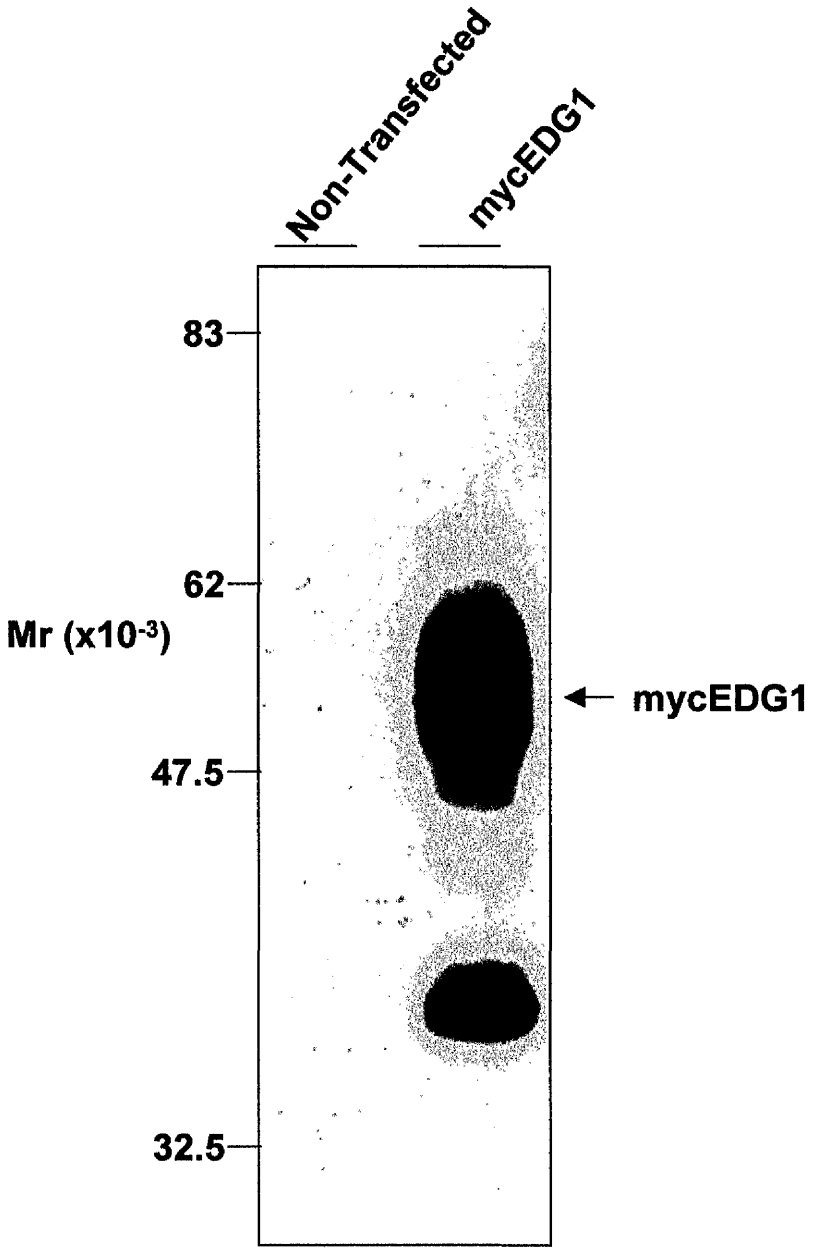

Non-Transfected
mycEDG1
83
62
Mr (x10-3)
47.5
32.5
mycEDG1

Figure 4.3: Effect Of Increasing SSP Concentrations On ERK Activation In CCL-39 and CCL-39/MycEDG1 Cells

Serum-starved parental and mycEDG1 expressing CCL-39 cells were stimulated for 10 min with increasing concentrations of SSP. Endogenous ERK1 was then immunoprecipitated from soluble cell extracts for immune-complex kinase assay of ERK1 activity using MBP as the substrate as described in the Methods section. Quantification of ^{32}P incorporation into MBP was determined by phosphorimaging. Data represents the mean ± SEM of three similar experiments. SSP produced a 6-10 fold activation of ERK (EC_{50}=0.4μM) in mycEDG1-expressing, but not parental, CCL-39 cells.

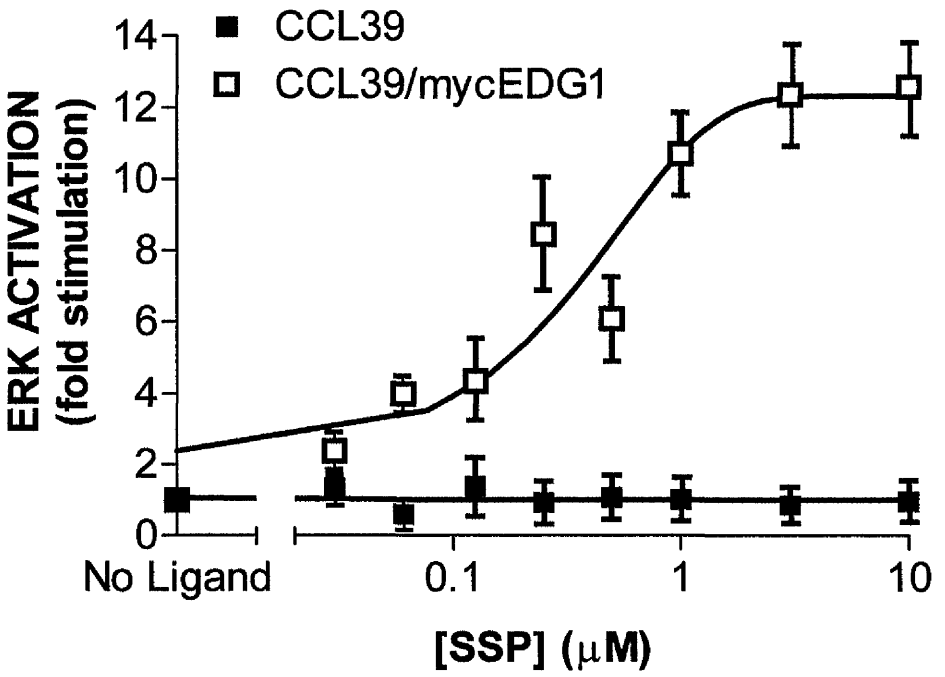

CCL39
CCL39/mycEDG1
ERK ACTIVATION
(fold stimulation)
14
12
10
8
6
4
2
0
No Ligand
0.1
1
10
[SSP] (μM)

Figure 4.4: Effect Of SSP And A Range Of Activators Of Second Messenger Kinases On The Phosphorylation Of The Human Myc-EDG1 Receptor

Serum-starved CCL-39/mycEDG1 cells were treated for 10 min at 37°C with 10μM of the agonist, SSP and a range of second messenger activators; 1μM phorbol 12-myristate 13-acetate (PMA), an activator of PKC; 10μM A23187, a calcium ionophore; 10μM Forskolin, an activator of adenylyl cyclase and 100μM 8-bromo-cGMP, an activator of cGMP. The cells were then solubilised for analysis of EDG1 phosphorylation by immunoprecipitation followed by SDS-PAGE and phosphorimaging. Typical data is shown from one of three experiments.

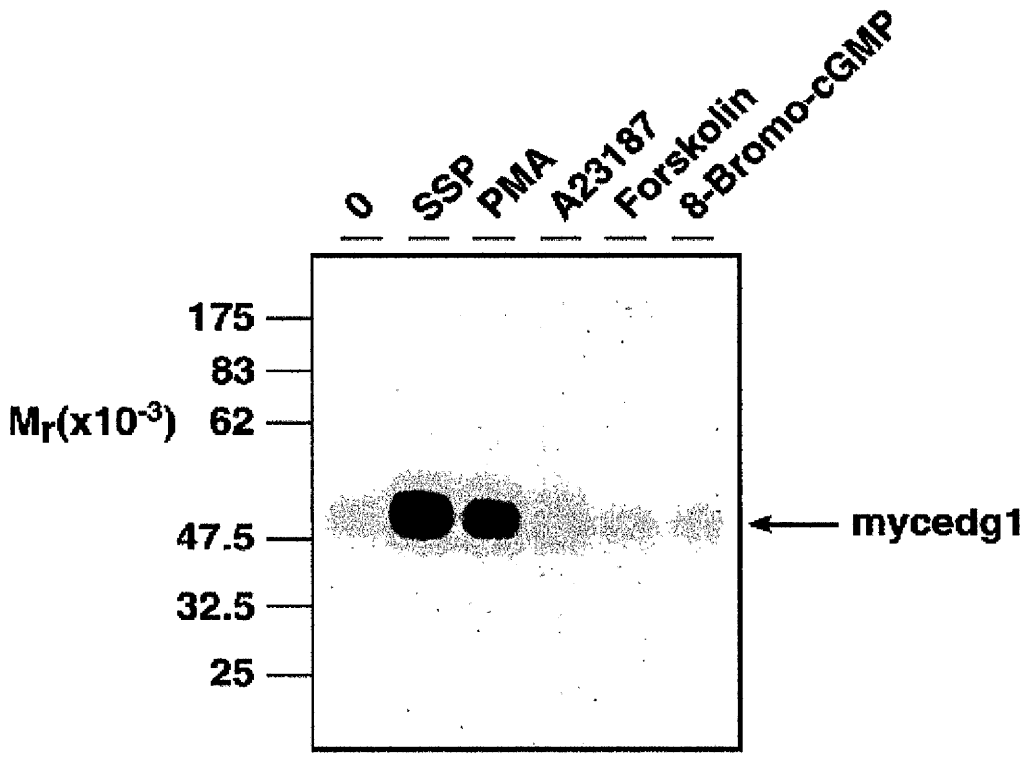
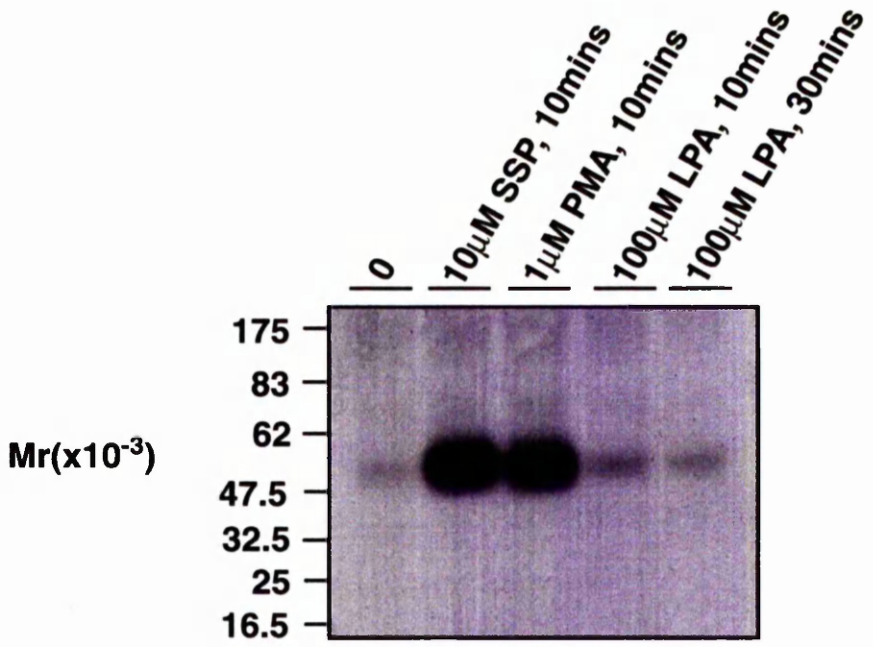

0
SSP
PMA
A23187
Forskolin
8-Bromo-cGMP
175
83
62
47.5
32.5
25
$M_r(x10^{-3})$
mycedg1

Figure 4.5a: Comparison Of The Effects Of SSP And LPA On EDG1 Phosphorylation

^{32}P-labelled serum starved stably transfected CCL-39/mycEDG1 cells were treated with either vehicle, 10μM SSP, 1μM PMA or 100μM LPA for 10 minutes or with 100μM LPA for 30minutes at 37°C. The cells were then solubilised for analysis of EDG1 phosphorylation by immunoprecipitation followed by SDS-PAGE and phosphorimaging. Phosphorylation of CCL-39/mycEDG1 cells treated with S1P was set at 100% and results from other treatments were expressed as a ratio of the control. The data represents the mean ± SEM of three similar experiments. * Denotes a significant decrease ($p<0.05$) *versus* the level of phosphorylation observed for SSP-induced WT EDG1 phosphorylation. SSP and PMA both result in phosphorylation of the receptor, with SSP resulting in the strongest stimulation. LPA, however, failed to induce phosphorylation, even after a 100μM, 30 minute exposure.


0
10μM SSP, 10mins
1μM PMA, 10mins
100μM LPA, 10mins
100μM LPA, 30mins
Mr(x10-3)
175
83
62
47.5
32.5
25
16.5

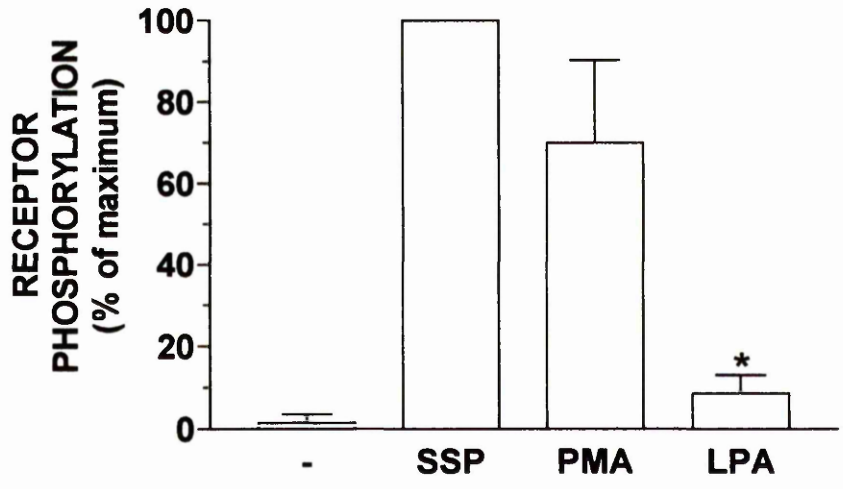

RECEPTOR PHOSPHORYLATION (% of maximum)
100
80
60
40
20
0
*
-
SSP
PMA
LPA

Figure 4.5b: Comparison Of The Effects Of SSP And LPA On EDG2 Phosphorylation

The mycEDG2 receptor construct was transiently transfected into HEK293 cells. The cells were subsequently serum-starved, labelled with ^{32}P-orthophosphate and then treated with either vehicle, 10μM SSP, 1μM PMA, 100μM LPA or the Ca^{2+} ionophore, A23187 for 15 minutes at 37°C. The cells were then solubilised for analysis of EDG2 phosphorylation by immunoprecipitation followed by SDS-PAGE and phosphorimaging. Parallel immunoblot analysis of each sample was carried out for each phosphorylation study using the 9E10 antibody in order to normalise receptor phosphorylation with receptor expression. A strong, LPA-dependent EDG2 phosphorylation was observed, demonstrating that were no discrepancies in the integrity of the stock LPA used in Figure 4.5a. Typical data is shown from one of three experiments.

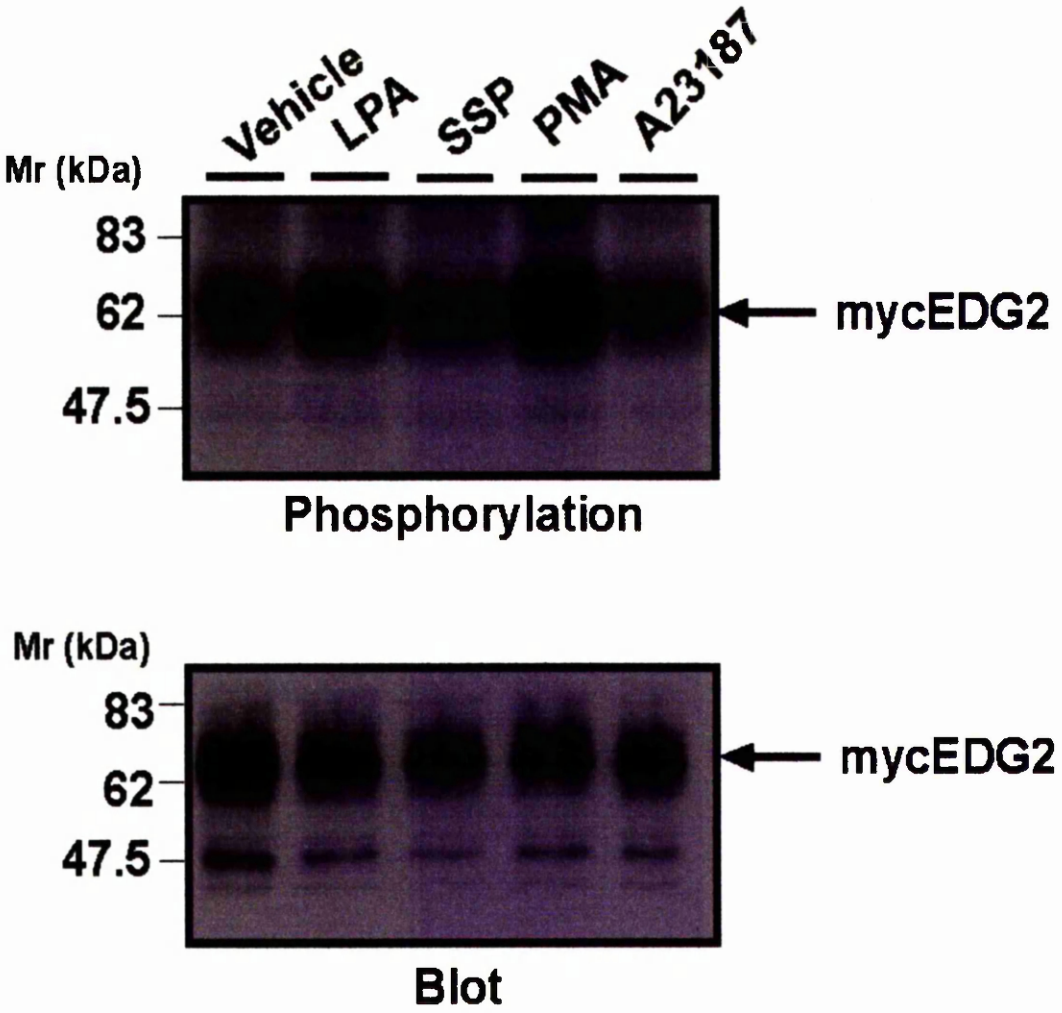

Vehicle
LPA
SSP
PMA
A23187
Mr (kDa)
83
62
47.5
mycEDG2
Phosphorylation
Mr (kDa)
83
62
47.5
mycEDG2
Blot

Figure 4.6: Phosphoamino Acid Analysis Of The Human Myc-EDG1 Receptor

Serum-starved CCL-39/myc-EDG1 cells were treated with either 20μM S1P or 1μM PMA for 10mins at 37°C. The samples were then immunoprecipitated, ran on SDS-PAGE and transferred to a PVDF membrane. The region of membrane containing the EDG1 receptor was then excised, hydrated and hydrolyzed with acid. Phosphorylated amino acids were visualised using chromatography by ninhydrin staining and ^{32}P-labelled amino acids visualised by autoradiography. The autoradiograph shows that both S1P and PMA treatment result in the phosphorylation of serine and threonine residues.

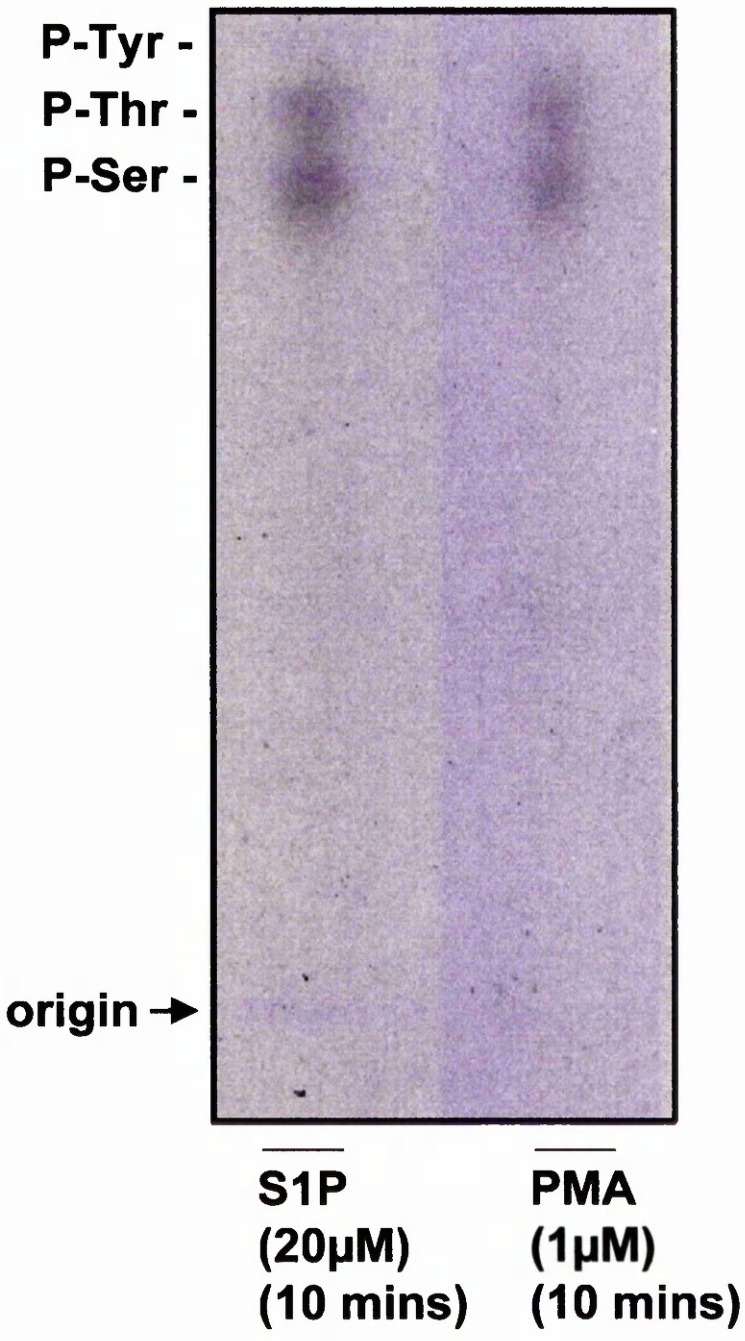
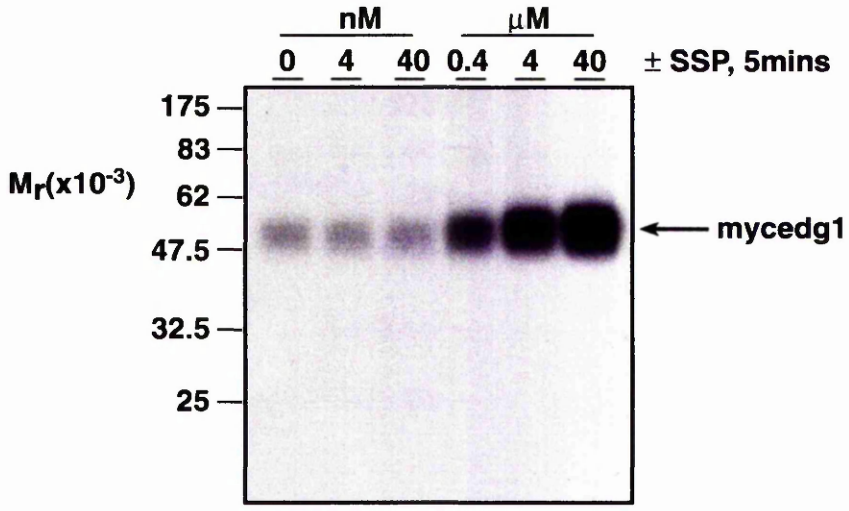

P-Tyr -
P-Thr -
P-Ser -
origin
S1P
(20μM)
(10 mins)
PMA
(1μM)
(10 mins)

Figure 4.7: Effect Of Increasing SSP Concentrations On EDG1 Phosphorylation

^{32}P-labelled serum-starved stably transfected CCL-39/mycEDG1 cells were treated with vehicle or increasing concentrations of SSP for 5 minutes at 37°C. The cells were then solubilised for analysis of EDG1 phosphorylation by immunoprecipitation followed by SDS-PAGE and phosphorimaging. Maximal SSP-induced EDG1 phosphorylation was set at 100% and the results from other treatments expressed accordingly. These data represent the mean ± SEM of three similar experiments. The results show that SSP-induced EDG1 phosphorylation is concentration-dependent (EC_{50} =1.9±0.37μM).


nM
μM
0
4
40
0.4
4
40
± SSP, 5mins
175
83
62
47.5
32.5
25
$M_r(x10^{-3})$
mycedg1

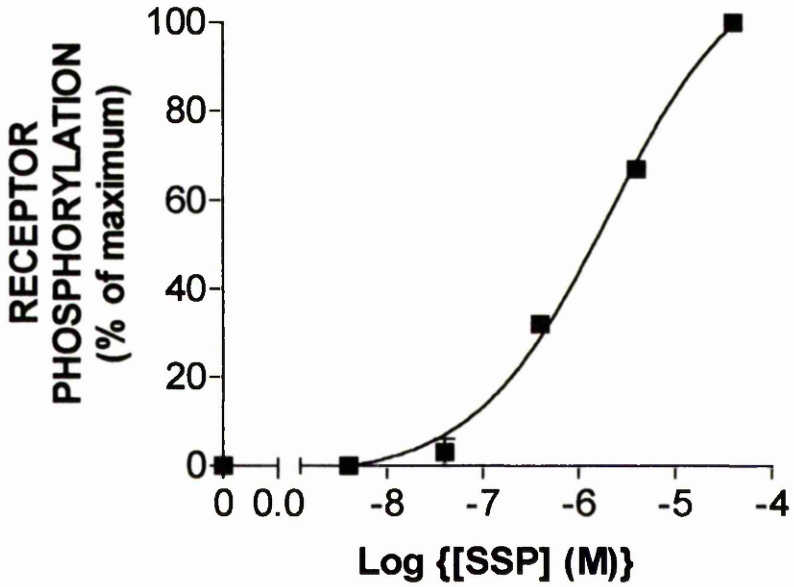
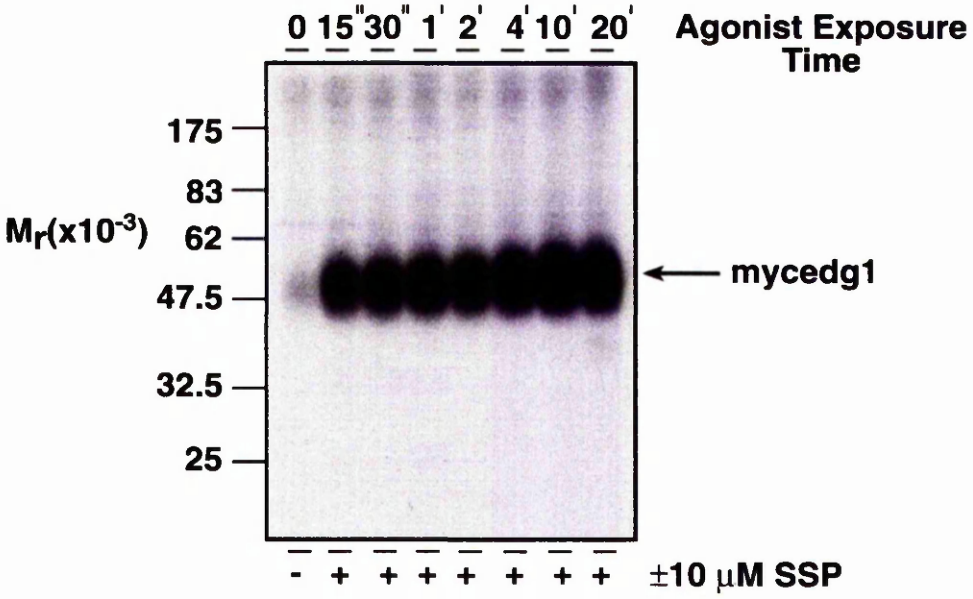

RECEPTOR PHOSPHORYLATION (% of maximum)
100
80
60
40
20
0
0 0.0
-8
-7
-6
-5
-4
Log {[SSP] (M)}

Figure 4.8: Time-Course Of SSP-Induced EDG1 Phosphorylation

^{32}P-labelled serum starved stably transfected CCL-39/mycEDG1 cells were treated with either vehicle or 10μM SSP at 37°C for the times indicated in the graph. The cells were then solubilised for analysis of EDG1 phosphorylation by immunoprecipitation followed by SDS-PAGE and phosphorimaging. Maximal SSP-induced EDG1 phosphorylation was set at 100% and the results from other treatments were expressed as a ratio of the control. These data represent the mean ± SEM of three similar experiments. SSP-induced EDG1 phosphorylation is a rapid process, with a significant phosphorylation observed after 15 seconds and is maximal after 10 minutes.


0 15" 30" 1' 2' 4' 10' 20'
Agonist Exposure Time
175
83
62
47.5
32.5
25
Mr(x10-3)
mycedg1
- + + + + + + +
±10 μM SSP

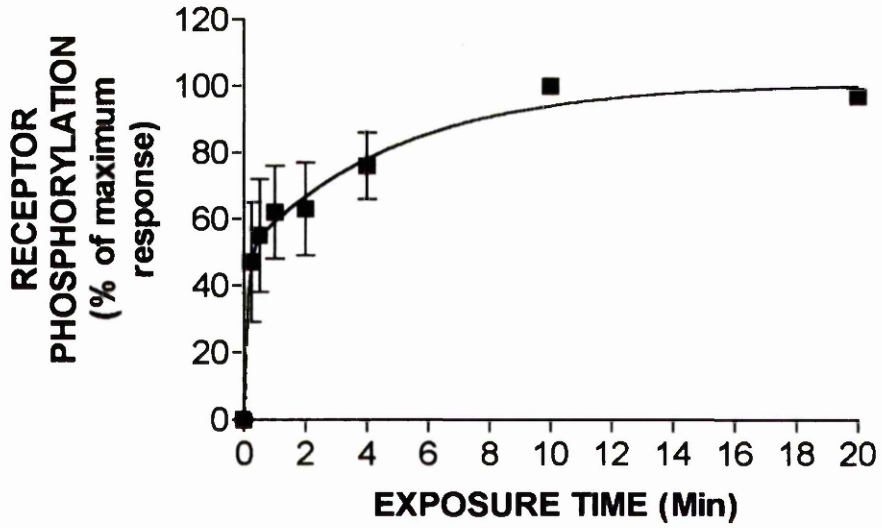
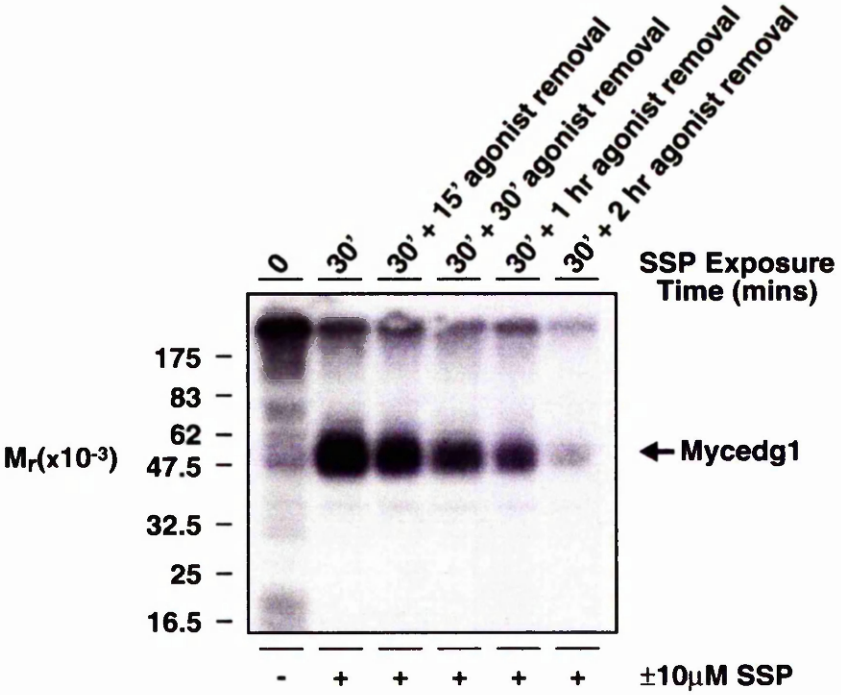

RECEPTOR PHOSPHORYLATION (% of maximum response)
120
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16 18 20
EXPOSURE TIME (Min)

Figure 4.9: Effect Of SSP Removal On SSP-Induced EDG1 Phosphorylation

^{32}P-labelled serum starved stably transfected CCL-39/mycEDG1 cells were treated with either vehicle or 10μM SSP at 37°C for 30 minutes. Agonist was subsequently removed for 15, 30, 60 or 120 minutes. The cells were then solubilised for analysis of EDG1 phosphorylation by immunoprecipitation followed by SDS-PAGE and phosphorimaging. Maximal SSP-induced EDG1 phosphorylation was set at 100% and the results from other treatments were expressed as a ratio of the control. These data represent the mean ± SEM of three similar experiments. * Denotes a significant decrease ($p<0.05$) *versus* the level of phosphorylation observed for SSP-induced WT EDG1 phosphorylation. The graph shows that SSP-induced EDG1 phosphorylation is a reversible process with a significant reduction in EDG1 receptor phosphorylation observed after 30 minutes of agonist removal. After 2 hours of agonist removal, EDG1 phosphorylation is close to basal levels.


0
30'
30' + 15' agonist removal
30' + 30' agonist removal
30' + 1 hr agonist removal
30' + 2 hr agonist removal
SSP Exposure Time (mins)
175
83
62
47.5
32.5
25
16.5
Mr(x10-3)
Mycedg1
- + + + + +
±10μM SSP

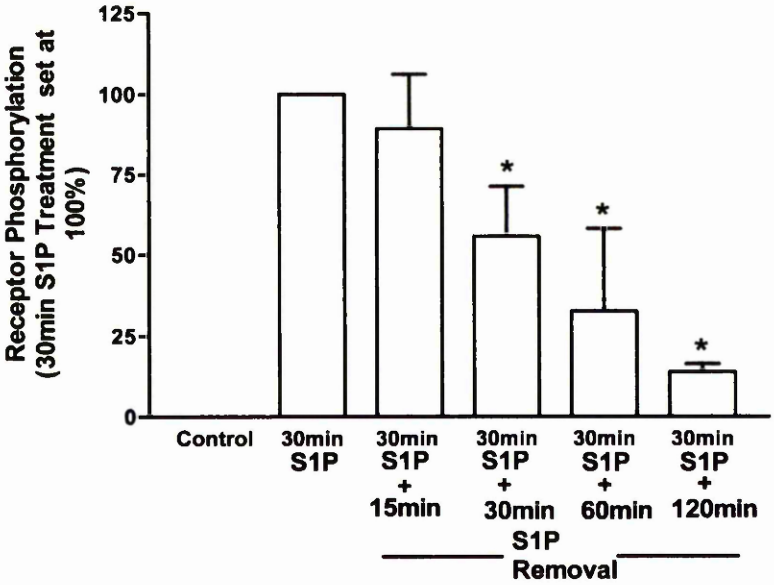

Receptor Phosphorylation (30min S1P Treatment set at 100%)
125
100
75
50
25
0
*
*
*
Control
30min S1P
30min S1P + 15min
30min S1P + 30min
30min S1P + 60min
30min S1P + 120min
S1P Removal

Figure 4.10: Schematic Of The G-Protein Receptor Kinase (GRK) Family

The GRK family of kinases is comprised of 7 members. Each kinase selectively phosphorylates agonist-activated receptors, facilitating the binding of cytosolic cofactor proteins called arrestins, which not only uncouple receptors from heterotrimeric G-proteins but also targets many GPCRs for internalisation *via* clathrin-coated pits. The family is subdivided into 3 catagories based on sequence and functional homology: - 1) GRK1 (rhodopsin kinase) and GRK7 (a new candidate cone opsin kinase); 2) GRK2 (β-adrenergic receptor kinase 1, βARK1) and GRK3 (β-adrenergic receptor kinase 2, βARK2); and 3) GRK4, GRK5 and GRK6. Since both GRK1 and GRK7 are highly specific, they were unlikely to have a role in agonist-dependent phosphorylation of EDG1. Therefore, to examine the role of GRKs in S1P-induced EDG1 phosphorylation, GRK2 and GRK5 were chosen to represent ubiquitously expressed members of the 2^{nd} and 3^{rd} subfamilies.

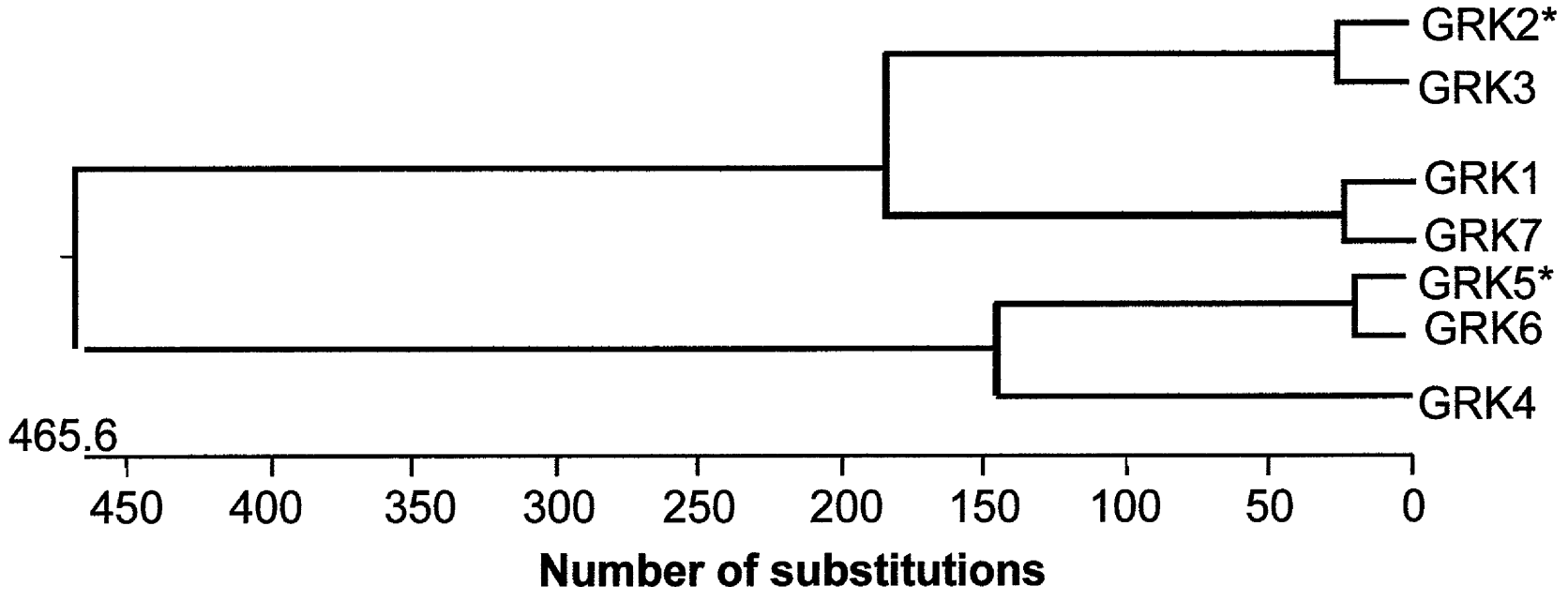
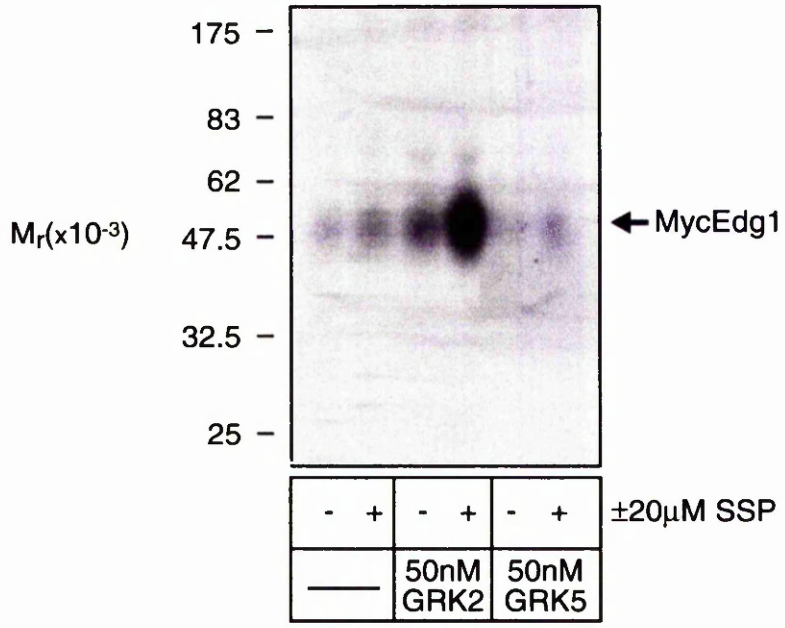

GRK2*
GRK3
GRK1
GRK7
GRK5*
GRK6
GRK4
465.6
450
400
350
300
250
200
150
100
50
0
Number of substitutions

Figure 4.11: Effects OF GRK2 And GRK5 On S1P-Induced EDG1 Phosphorylation *In Vitro*

Membranes from serum-starved CCL-39/mycEDG1 cells were incubated with [γ-^{32}P] ATP in the presence or absence of 20μM SSP alone or in the presence of either 50nM purified GRK2 or GRK5 at 37°C. These data represent the mean ± SEM of three similar experiments. * Indicates a significant increase ($p<0.05$) *versus* the level of phosphorylation observed in the absence of agonist. SSP-induced phosphorylation of EDG1 occurs in the presence of GRK2 but there is no phosphorylation in the absence of GRK or in the presence of GRK5, indicating a specific role for GRK2 in SSP-induced EDG1 phosphorylation *in vitro*.


175
83
62
47.5
32.5
25
$M_r(x10^{-3})$
MycEdg1
- + - + - +
±20μM SSP
50nM GRK2
50nM GRK5

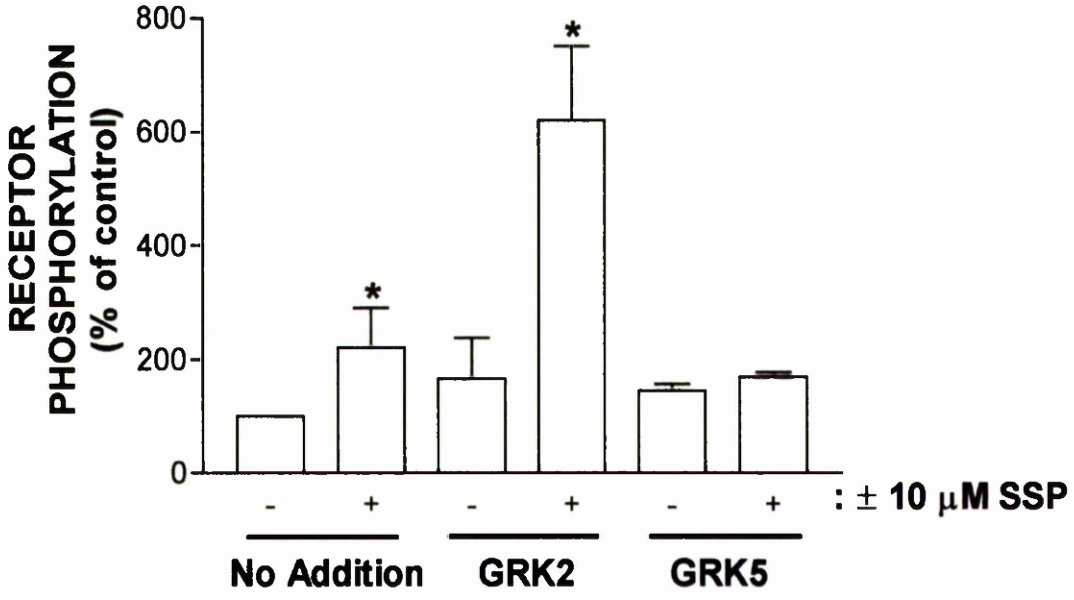

RECEPTOR PHOSPHORYLATION (% of control)
800
600
400
200
0
*
*
- + - + - +
: ± 10 μM SSP
No Addition
GRK2
GRK5

Figure 4.12: Effects Of GRK2 And GRK5 On Light-Stimulated Rhodopsin Phosphorylation *In Vitro*

Urea-treated bovine rod outer segments were incubated either in the dark or illuminated in the absence or presence of GRK2 or GRK5. Reactions were stopped by the addition of SDS-PAGE sample buffer prior to analysis of light-stimulated rhodopsin phosphorylation by SDS-PAGE and autoradiography. A strong light-dependent phosphorylation was detectable in the presence of both GRK2 and GRK5, showing that the purified forms of GRK2 and GRK5 were both active under the *in vitro* phosphorylation assay conditions. Typical data is shown from one of three experiments.

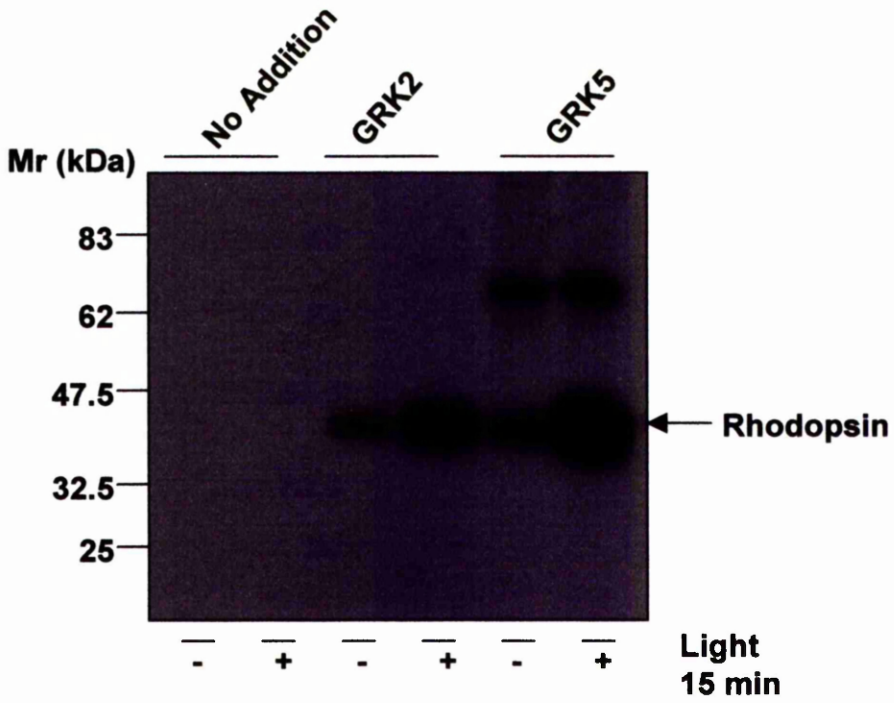
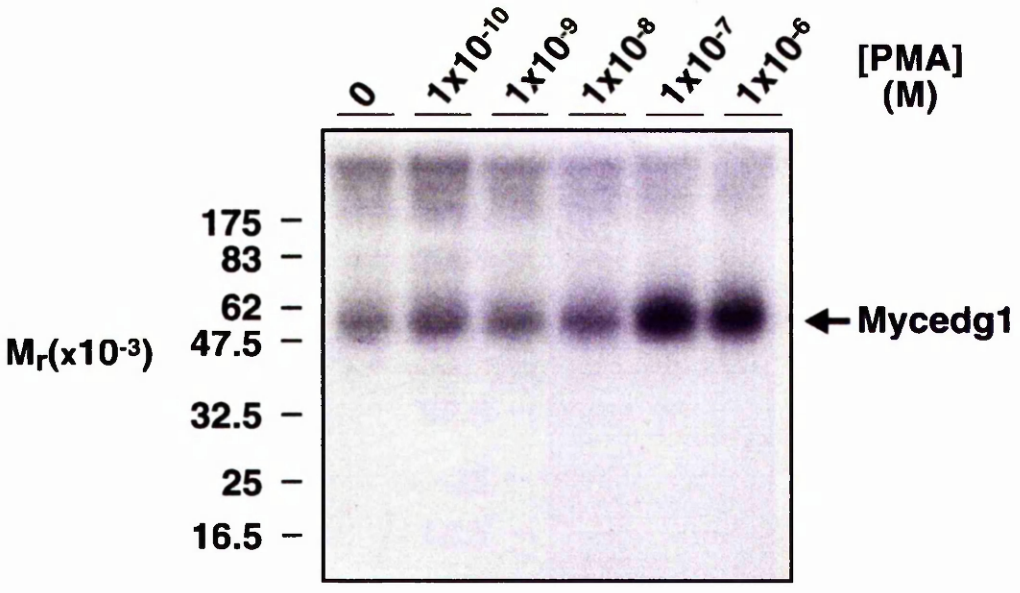

No Addition
GRK2
GRK5
Mr (kDa)
83
62
47.5
32.5
25
Rhodopsin
- + - + - +
Light
15 min

Figure 4.13: Effect Of Increasing Concentrations Of PMA On EDG1 Phosphorylation

^{32}P-labelled serum-starved stably transfected CCL-39/mycEDG1 cells were treated with either vehicle or increasing concentrations of PMA for 10 minutes at 37°C. The cells were then solubilised for analysis of EDG1 phosphorylation by immunoprecipitation followed by SDS-PAGE and phosphorimaging. Maximal PMA-induced EDG1 phosphorylation was set at 100% and the results from other treatments were expressed as a ratio of the control. These data represent the mean ± SEM of three similar experiments. There is a concentration dependent PMA-induced EDG1 phosphorylation (EC_{50}= 0.1μM)


0
1x10-10
1x10-9
1x10-8
1x10-7
1x10-6
[PMA]
(M)
175
83
62
47.5
32.5
25
16.5
Mr(x10-3)
Mycedg1

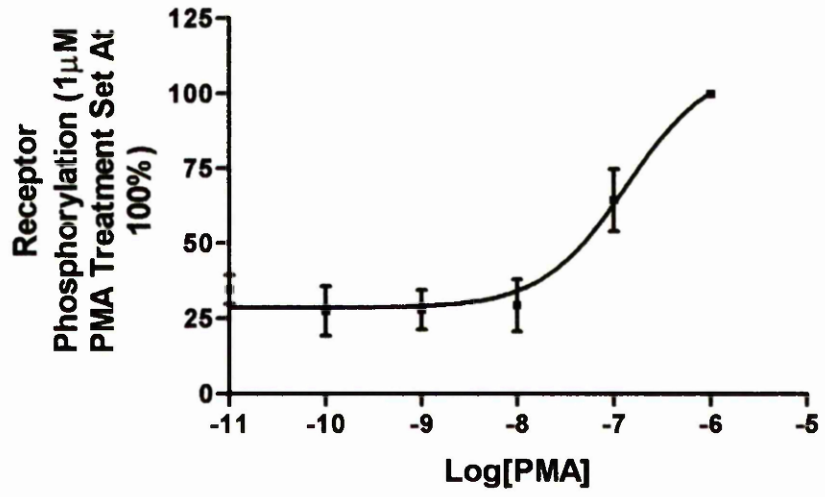
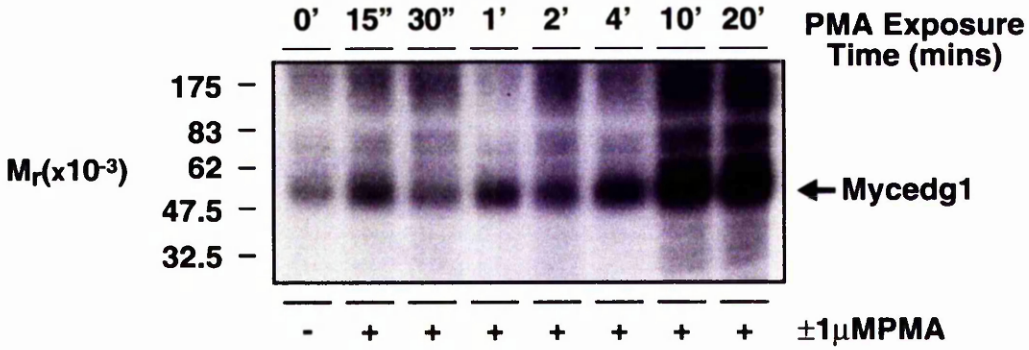

Receptor
Phosphorylation (1μM
PMA Treatment Set At
100%)
125
100
75
50
25
0
-11
-10
-9
-8
-7
-6
-5
Log[PMA]

Figure 4.14: Time-Course Of PMA-Induced EDG1 Phosphorylation

^{32}P-labelled serum starved stably transfected CCL-39/mycEDG1 cells were treated with either vehicle or 1μM PMA at 37°C for the times indicated in the graph. The cells were then solubilised for analysis of EDG1 phosphorylation by immunoprecipitation followed by SDS-PAGE and phosphorimaging. Maximal PMA-induced EDG1 phosphorylation was set at 100% and the results from other treatments expressed accordingly. The data represents the mean ± SEM of three similar experiments.


0'
15"
30"
1'
2'
4'
10'
20'
PMA Exposure Time (mins)
175
83
62
47.5
32.5
Mr(x10-3)
Mycedg1
-
+
+
+
+
+
+
+
±1μMPMA

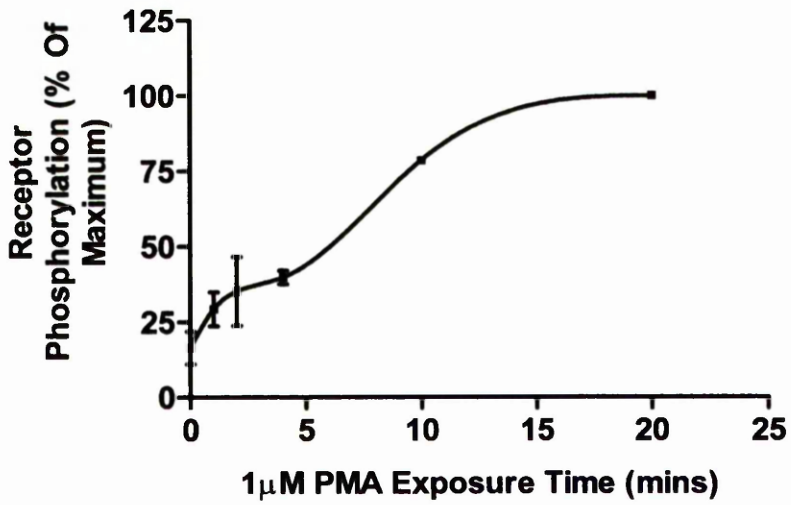

Receptor Phosphorylation (% Of Maximum)
125
100
75
50
25
0
0
5
10
15
20
25
1μM PMA Exposure Time (mins)

Figure 4.15: Schematic Of The Potential Role Of PKC In S1P-Induced EDG1 Phosphorylation

The question remained whether or not S1P-induced EDG1 phosphorylation was PKC dependent. In other words, could S1P activate PLC which could, in turn, increase both intracellular calcium and DAG levels. This would result in PKC activation, causing EDG1 phosphorylation. To investigate this, S1P- and PMA- induced EDG1 phosphorylation was measured in the presence of the PKC inhibitor, GF109203X.

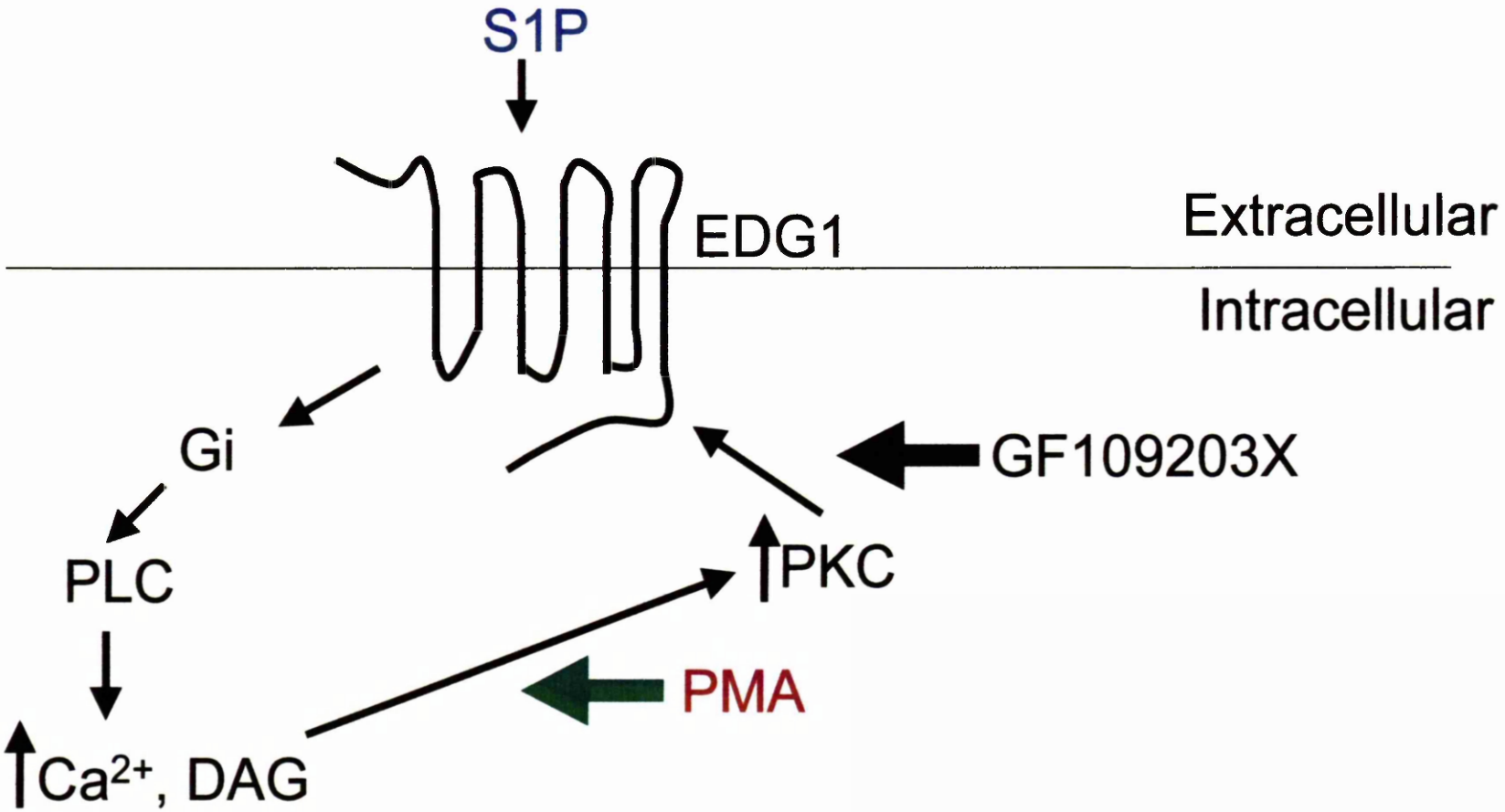
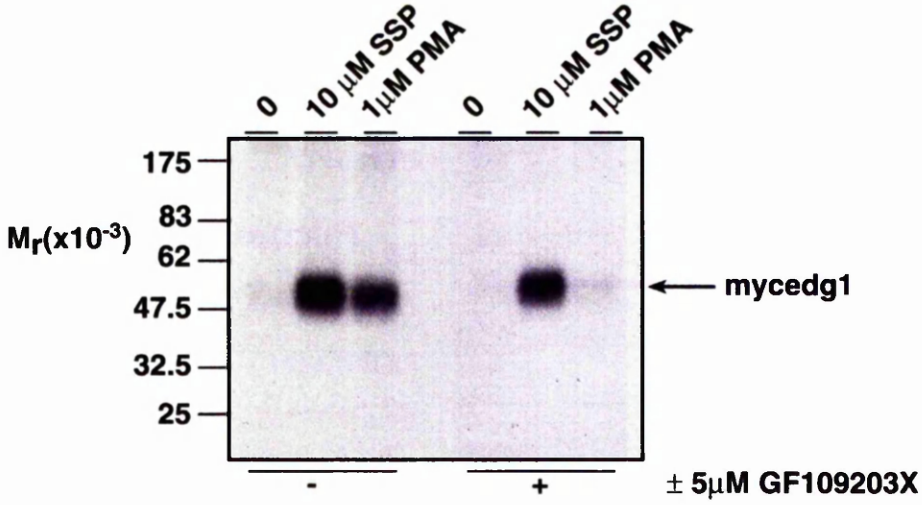

S1P
EDG1
Extracellular
Intracellular
Gi
PLC
Ca2+, DAG
PKC
GF109203X
PMA

Figure 4.16: Effect Of An Inhibitor Of Conventional And Novel PKC Subtypes On S1P- And Protein Kinase C-Induced EDG1 Phosphorylation

^{32}P-labelled serum-starved stably transfected CCL-39/mycEDG1 cells were preincubated with vehicle or 5μM of the PKC inhibitor, GF109203X (GFX) for 30 min. The cells were then incubated for 10 min in the absence of ligand or in the presence of 10μM SSP or 1μM PMA. All drug treatments were carried out at 37°C. The cells were analysed by immunoprecipitation followed by SDS-PAGE and phosphorimaging. Maximal SSP-induced EDG1 phosphorylation was set at 100% and the results from other treatments were expressed as a ratio of the control. These data represent the mean ± SEM of three similar experiments. * Denotes a significant inhibition ($p<0.05$) between GFX-preincubated PMA-treated cells and vehicle-preincubated PMA-treated cells. ψ Indicates a significant inhibition ($p<0.05$) between GFX-preincubated SSP treated cells and vehicle-preincubated SSP-treated cells.


0
10 μM SSP
1μM PMA
0
10 μM SSP
1μM PMA
175
83
62
47.5
32.5
25
Mr(x10-3)
mycedg1
-
+
± 5μM GF109203X

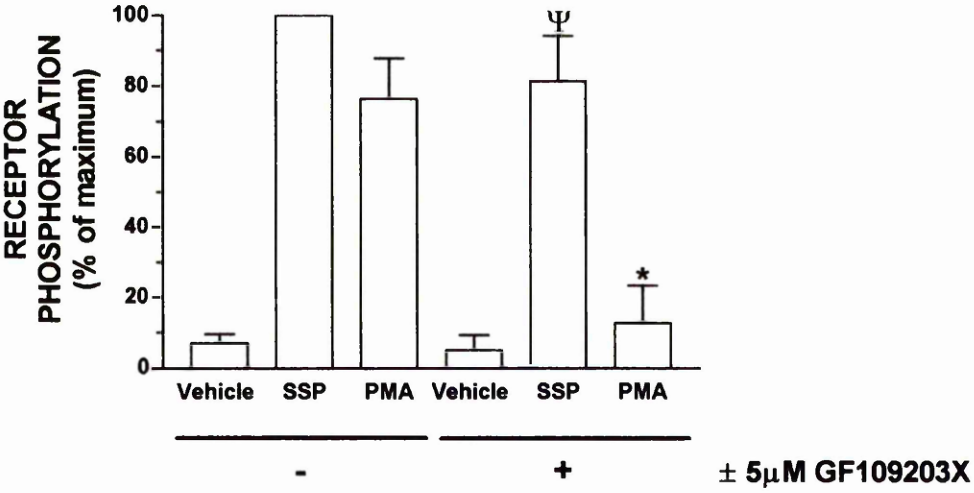
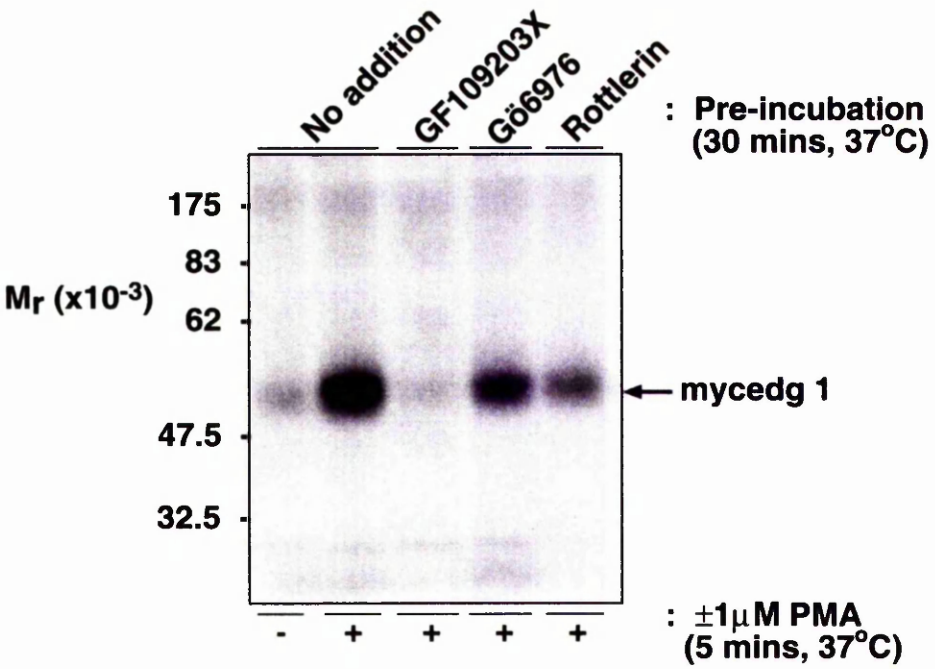

RECEPTOR PHOSPHORYLATION (% of maximum)
100
80
60
40
20
0
Ψ
*
Vehicle
SSP
PMA
Vehicle
SSP
PMA
-
+
± 5μM GF109203X

Figure 4.17: Effect Of Inhibitors Of Conventional And Non-Conventional PKC Subtypes On PMA-Induced EDG1 Phosphorylation

^{32}P-labelled serum-starved stably transfected CCL-39/mycEDG1 cells were preincubated for 30 min at 37°C with vehicle or 5μM GF109203X (GFX), an inhibitor of conventional and novel PKC isoform; 10μM Go6976, an inhibitor of conventional PKC isoforms or 100μM rottlerin, an inhibitor of novel PKC isoforms, particularly PKCδ. The cells were then incubated at 37°C for 10 min with vehicle or in the presence of 1μM PMA. The cells were analysed by immunoprecipitation followed by SDS-PAGE and phosphorimaging. Maximal PMA-induced EDG1 phosphorylation was set at 100% and the results from other treatments were expressed as a ratio of the control. The data represents the mean ± SEM of three similar experiments. * Indicates $p<0.05$ *versus* phosphorylation observed in PMA-treated cells. ψ Indicates $p<0.05$ *versus* inhibition produced by GF109203X. 5μM GF109203X pretreatment abolishes PMA-induced EDG1 phosphorylation, whereas an approximately half-maximal reduction in PMA-induced phosphorylation was observed following a preincubation with either Go6976 or rottlerin. This suggests that conventional and novel subtypes of PKC are involved in the PMA-induced phosphorylation of EDG1.


No addition
GF109203X
Gö6976
Rottlerin
: Pre-incubation
(30 mins, 37°C)
175
83
62
47.5
32.5
Mr (x10-3)
mycedg 1
- + + + +
: ±1μM PMA
(5 mins, 37°C)

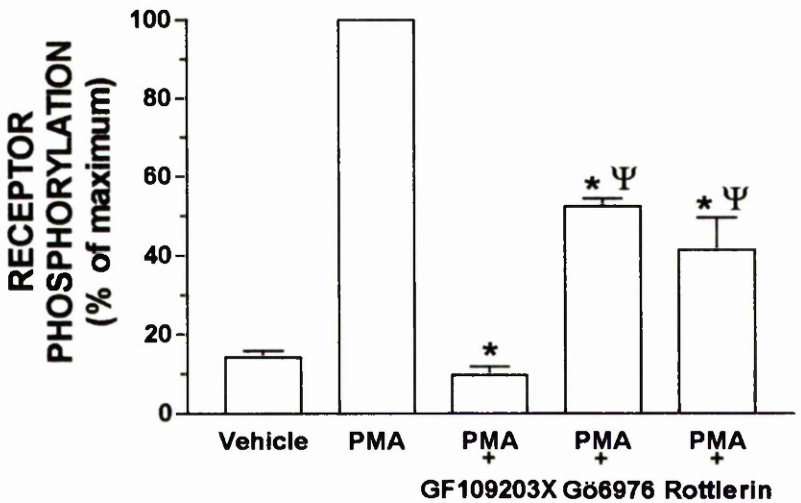
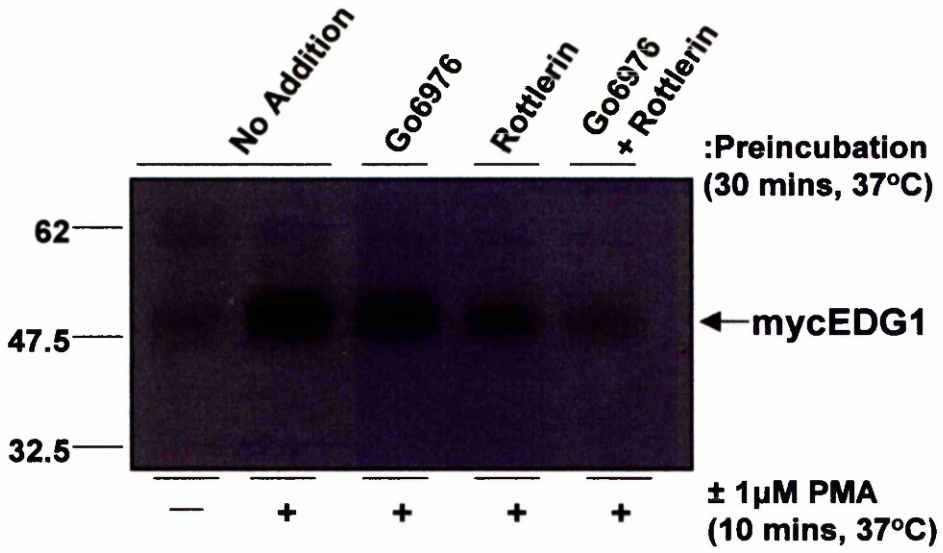

RECEPTOR
PHOSPHORYLATION
(% of maximum)
100
80
60
40
20
0
*
*Ψ
*Ψ
Vehicle
PMA
PMA +
GF109203X
PMA +
Gö6976
PMA +
Rottlerin

Figure 4.18: Effect Of Inhibitors Of Conventional And Novel PKC subtypes On PMA-Induced EDG1 Phosphorylation

^{32}P-labelled serum-starved stably transfected CCL-39/mycEDG1 cells were preincubated at 37°C for 30 min with vehicle or 10μM Go6976, an inhibitor of conventional PKC isoforms or 100μM rottlerin, an inhibitor of novel PKC isoforms, particularly PKCδ either alone or together. The cells were then incubated for 10 min in the absence of ligand or in the presence of 1μM PMA. The cells were analysed by immunoprecipitation followed by SDS-PAGE and phosphorimaging. Maximal PMA-induced EDG1 phosphorylation was set at 100% and the results from other treatments were expressed as a ratio of the control. The data represents the mean ± SEM of three similar experiments. * denotes a significant difference versus PMA-treated cells. ~ indicates a siginificant difference versis Go6976-pretreated cells. # indicates a significant difference between rottlerin-pretreated cells.


No Addition
Go6976
Rottlerin
Go6976 + Rottlerin
:Preincubation (30 mins, 37°C)
62
47.5
32.5
mycEDG1
— + + + +
± 1μM PMA (10 mins, 37°C)

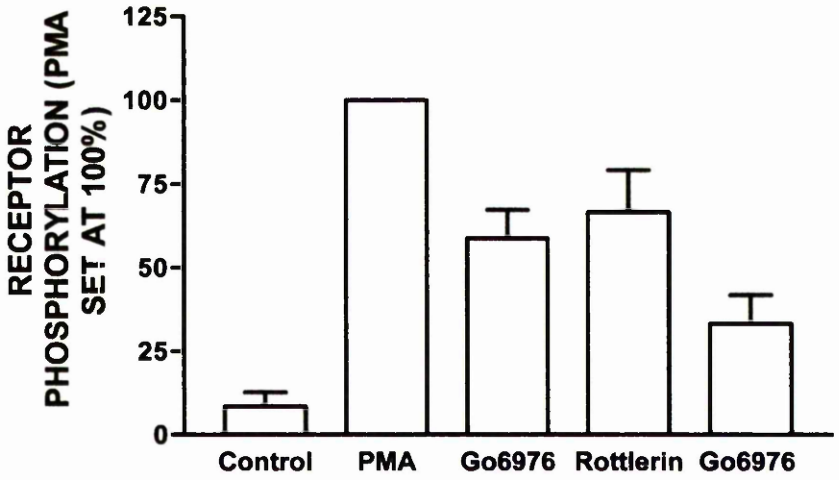
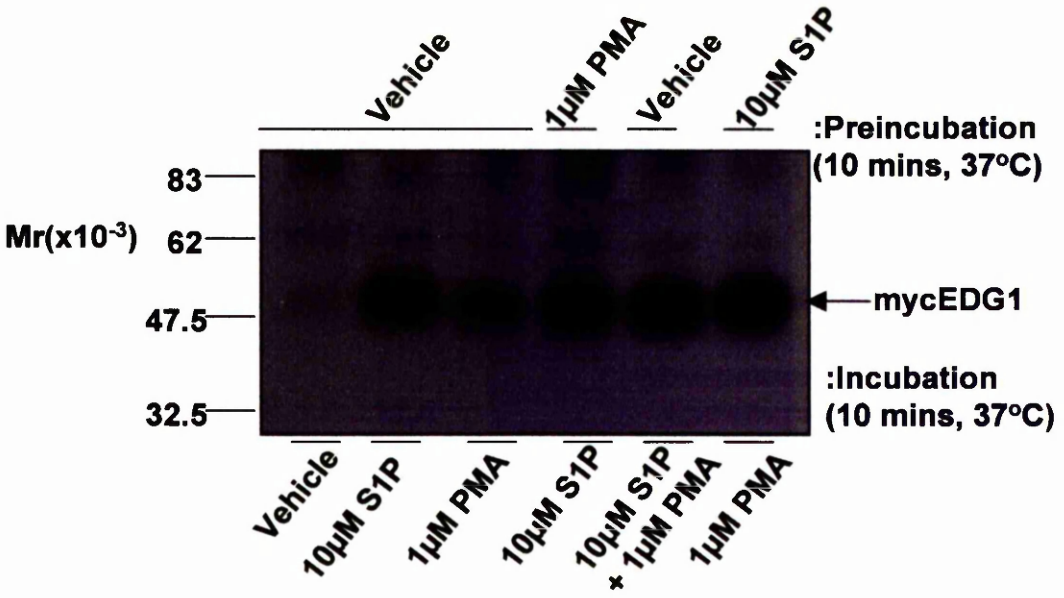

RECEPTOR PHOSPHORYLATION (PMA SET AT 100%)
125
100
75
50
25
0
Control
PMA
Go6976
Rottlerin
Go6976

Figure 4.19: S1P- And PMA-Induced EDG1 Phosphorylation Are Not Additive

^{32}P-labelled serum-starved stably transfected CCL-39/mycEDG1 cells were exposed to either a 10 min treatment of vehicle, 10μM S1P, 1μM PMA, or 10μM S1P and 1μM PMA together. In addition, cells were also either pretreated for 10 minutes with vehicle, 1μM PMA, or 10μM S1P together as indicated. All drug treatments were at 37°C. The cells were analysed by immunoprecipitation followed by SDS-PAGE and phosphorimaging. Maximal S1P-induced EDG1 phosphorylation was set at 100% and the results from other treatments were expressed as a ratio of the control. The data represents the mean ± SEM of three similar experiments. * Denotes a significant difference *versus* S1P-treated cells. No significant differences were observed between EDG1 phosphorylation following treatments with both S1P and PMA together compared with EDG1 phosphorylation in the presence of S1P alone.


Vehicle
1μM PMA
Vehicle
10μM S1P
:Preincubation
(10 mins, 37°C)
Mr(x10-3)
83
62
47.5
32.5
mycEDG1
:Incubation
(10 mins, 37°C)
Vehicle
10μM S1P
1μM PMA
10μM S1P
10μM S1P
+ 1μM PMA
1μM PMA

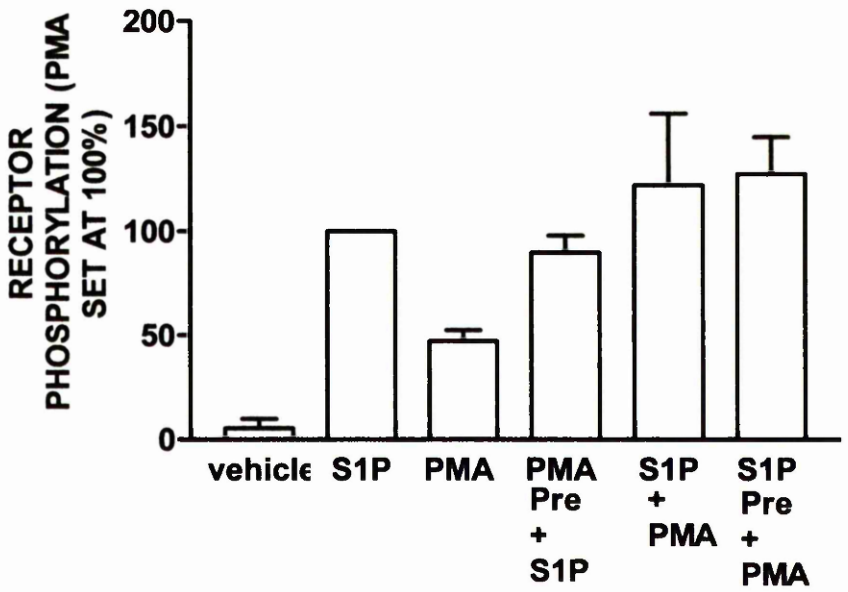

RECEPTOR PHOSPHORYLATION (PMA SET AT 100%)
200
150
100
50
0
vehicle
S1P
PMA
PMA Pre + S1P
S1P + PMA
S1P Pre + PMA

Figure 4.20: Schematic Of The Potential Role Of PMA On The Release Of Intracellular SSP *Via* Activation Of Sphingosine Kinase

It is known that, as well as stimulating PKC subtypes, PMA can also activate sphingosine kinase, the enzyme responsible for the conversion of sphingosine to sphingosine-1-phosphate within cells. It is therefore possible that PMA-induced EDG1 phosphorylation may be the result of an increase in intracellular SSP. The resultant S1P is then released, activating the extracellular EDG1 receptor. To examine this, PMA-induced EDG1 phosphorylation was observed following a preincubation with an inhibitor of sphingosine kinase, L-*threo*-dihydrosphingosine (DHS).

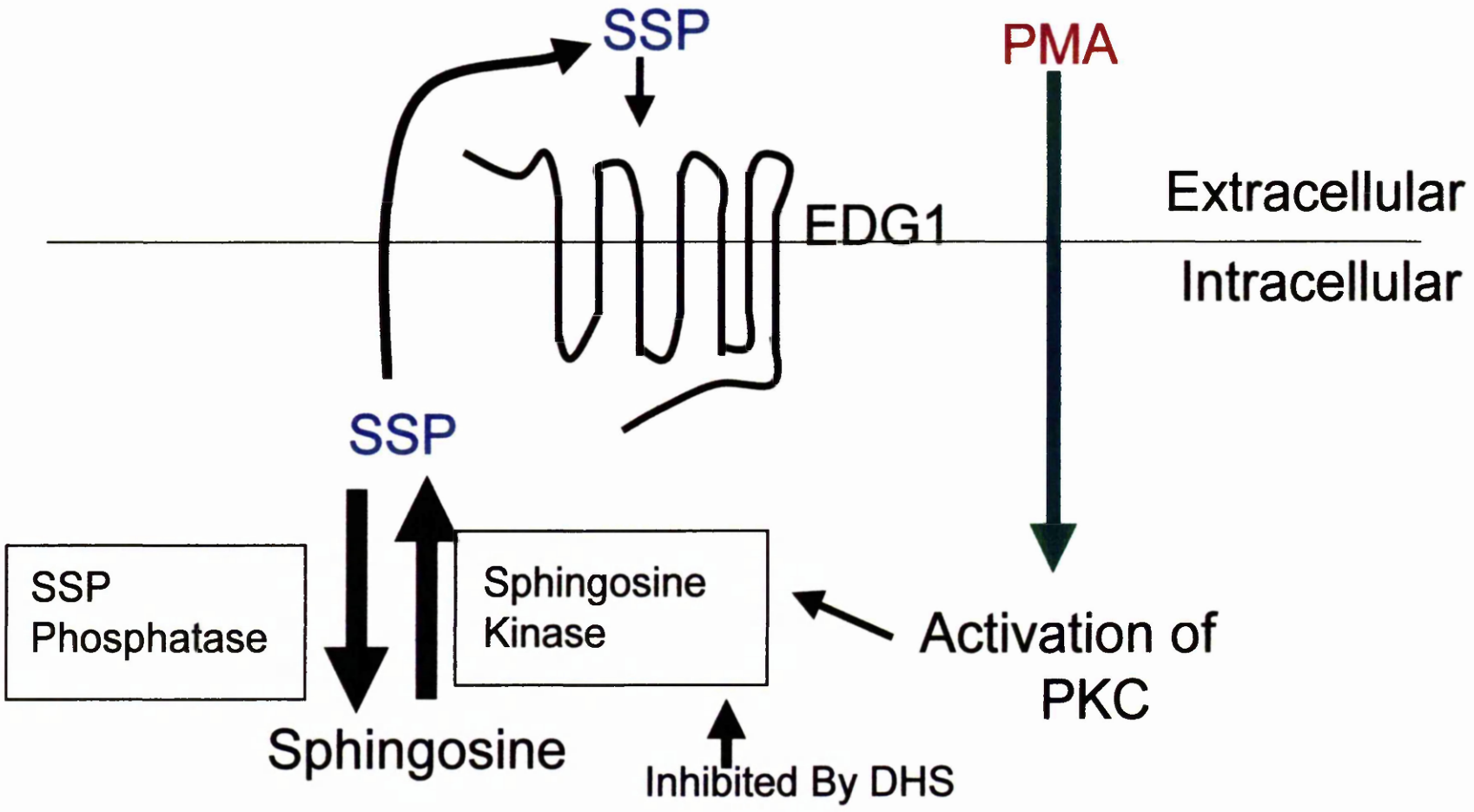
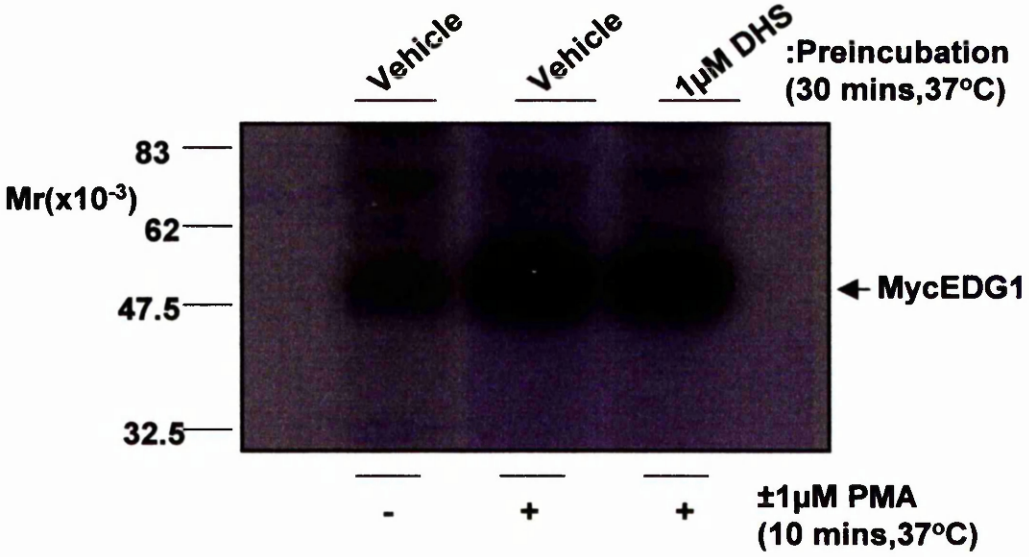

SSP
PMA
EDG1
Extracellular
Intracellular
SSP
SSP
Phosphatase
Sphingosine
Kinase
Activation of
PKC
Sphingosine
Inhibited By DHS

Figure 4.21: Effect Of L-*Threo*-Dihydrosphingosine, An Inhibitor Of Sphingosine Kinase, On PMA-Induced EDG1 Phosphorylation

^{32}P-labelled serum-starved stably transfected CCL-39/mycEDG1 cells were preincubated with either vehicle or 1μM L-*threo*-dihydrosphingosine (DHS) for 30 min. The cells were then exposed to either a 10 min treatment of vehicle or 1μM PMA. All treatments were carried out at 37°C. The cells were analysed by immunoprecipitation followed by SDS-PAGE and phosphorimaging. The data is presented as mean ± SEM of three similar experiments. * Denotes a significant difference ($p<0.05$) versus vehicle-preincubated PMA-treated cells. The presence of DHS had no significant effect on PMA-induced EDG1 phosphorylation.


Vehicle
Vehicle
1μM DHS
:Preincubation
(30 mins,37°C)
83
Mr(x10-3)
62
47.5
32.5
MycEDG1
-
+
+
±1μM PMA
(10 mins,37°C)

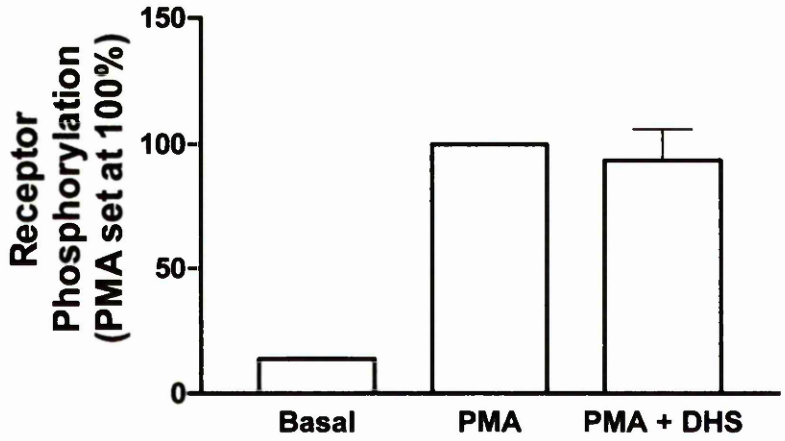
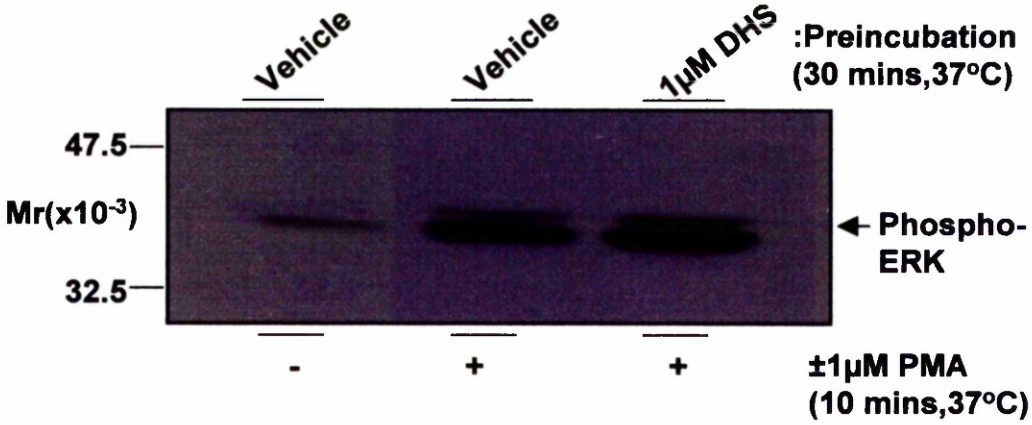

Receptor
Phosphorylation
(PMA set at 100%)
150
100
50
0
Basal
PMA
PMA + DHS

Figure 4.22: Effect Of L-*Threo*-Dihydrosphingosine On PMA-Induced Stimulation Of ERK

Samples of ^{32}P-labelled serum-starved stably transfected CCL-39/mycEDG1 cells from the experiments described in Figure 20 were solubilised in electrophoresis sample buffer and analysed by SDS-PAGE and immunoblotting with an anti-phospho-ERK monoclonal antibody. The data is presented as mean ± SEM of three similar experiments. * Denotes a significant difference *versus* vehicle-preincubated PMA-treated cells. DHS had no significant effect on PMA-induced ERK activation, suggesting that, at doses of 1μM, dihydrosphingosine did not inhibit PKC activation by PMA.


Vehicle
Vehicle
1μM DHS
:Preincubation
(30 mins,37°C)
47.5
Mr(x10-3)
32.5
Phospho-
ERK
-
+
+
±1μM PMA
(10 mins,37°C)

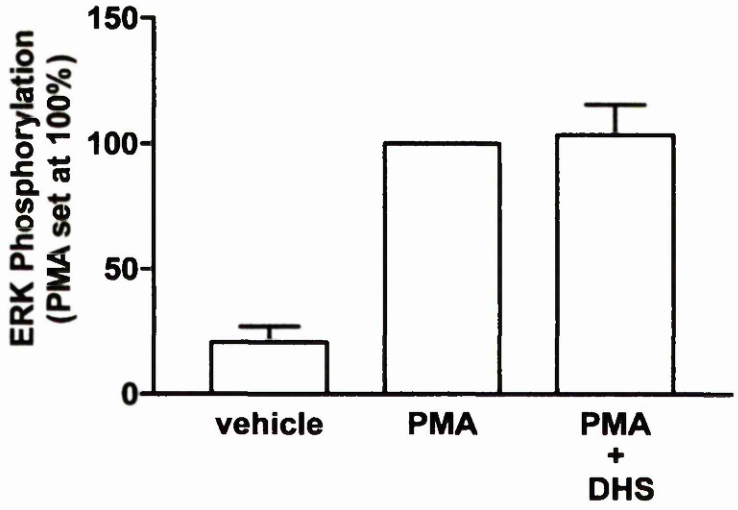

ERK Phosphorylation
(PMA set at 100%)
150
100
50
0
vehicle
PMA
PMA
+
DHS

Figure 4.23: Schematic Diagram Of The Regulatory C-Terminal Domain Of The Human EDG1 Receptor

The C-terminal region of many GPCRs, such as the β_2-adrenergic receptor, is critical to the processes of receptor phosphorylation and internalisation. In the case of the human EDG1, the C-terminal tail contains thirteen potential serine/threonine phosphorylation sites distal of the three potential sites of palmitoylation. Truncation of the last 51 amino acids of the C-terminal domain (mycEDG1Δ51) removed all thirteen potential serine/threonine phosphorylation sites.

Additionally, there are two distinct clusters of potential serine/threonine phosphorylation sites which can be removed by the truncation of the last 12 (EDG1Δ12) and the last 32 (EDG1Δ32) amino acids of the C-terminal. Each cluster is preceded by acidic amino acid residues, making both clusters potential sites of phosphorylation by the aciditrophic kinase GRK2 which was shown in Figure 11 to selectively phosphorylate EDG1 *in vitro*.

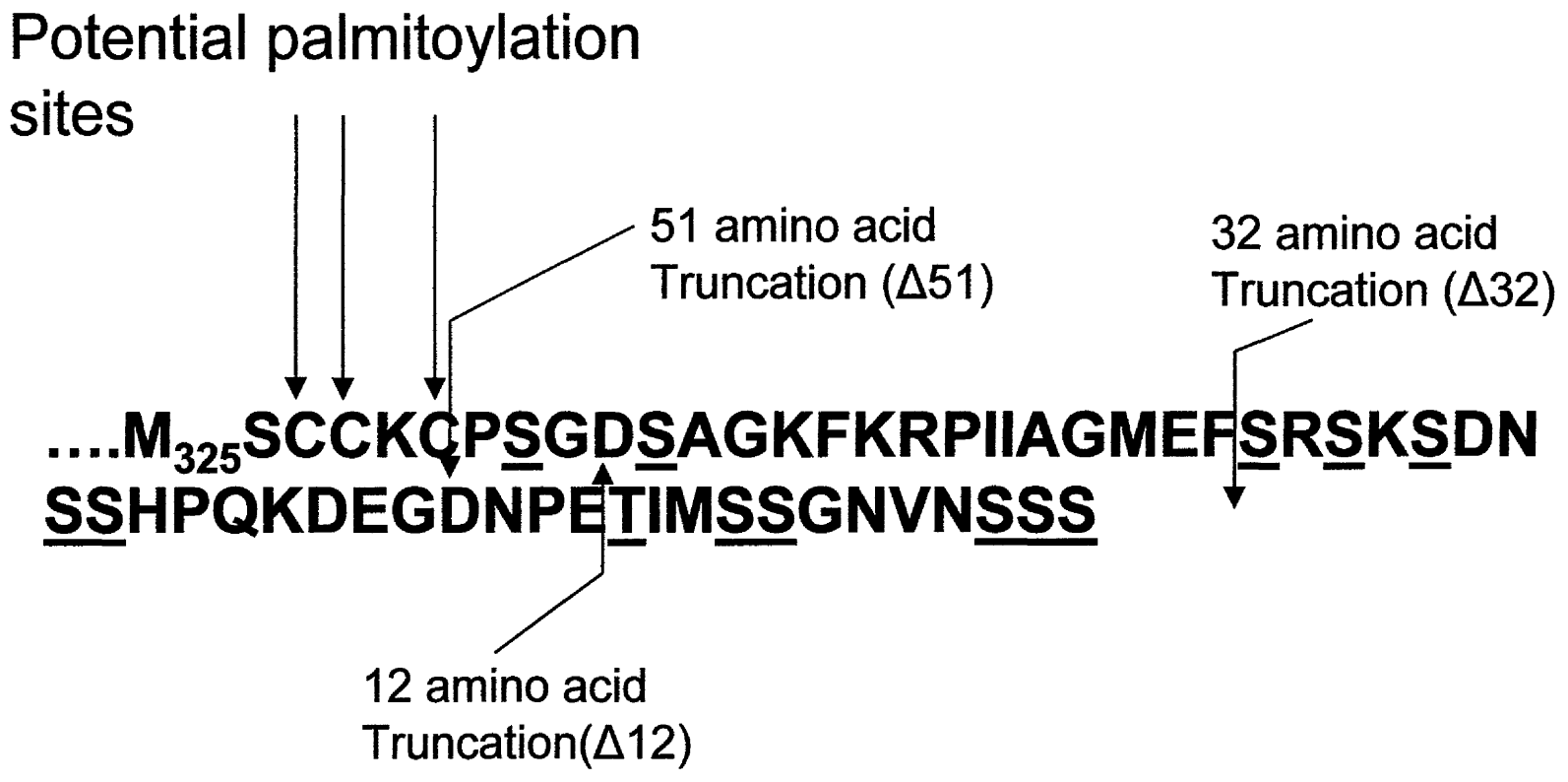

Potential palmitoylation sites
51 amino acid Truncation (Δ51)
32 amino acid Truncation (Δ32)
….M325SCCKCPSGDSAGKFKRPIIAGMEFSRSKSDN
SSHPQKDEGDNPETIMSSGNVNSSS
12 amino acid Truncation(Δ12)

Figure 4.24: Schematic Of The MycEDG1Δ51 , MyDG1Δ32 And MycEDG1Δ12 Receptors

The human mycEDG1 receptor was truncated using a human pcDNA3.1/human mycEDG1 template. The sense primer was designed to anneal upstream of the HindIII site (indicated) of the EDG1 receptor. The same sense primer was used to for all three truncation mutants and was designed to anneal upstream of the HindIII site of the EDG1 receptor, as indicated in the diagram. The antisense primers for each of the truncation mutant were designed to anneal either 12-, 32-, or 51 amino acids upstream of the EDG1 C-terminal tail for the appropriate truncation mutant and to add an XbaI site, as shown. Each receptor was then ligated into a pcDNA3.1/mycHisA vector at *Hind*III/*Xba*I to incorporate a myc tag (orange) with 6 histadine residues (blue) at the C-terminus.

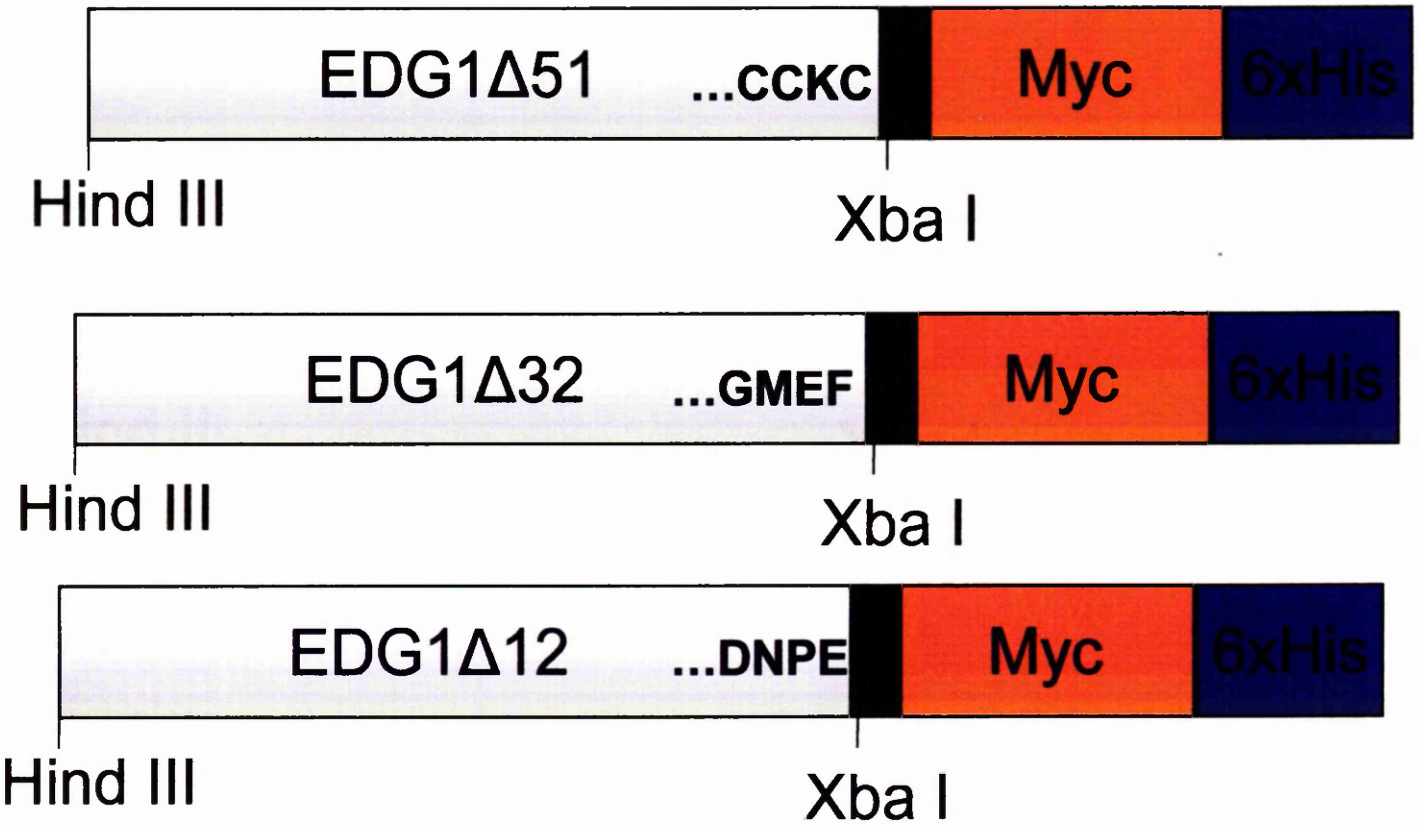

EDG1Δ51
...CCKC
Myc
6xHis
Hind III
Xba I
EDG1Δ32
...GMEF
Myc
6xHis
Hind III
Xba I
EDG1Δ12
...DNPE
Myc
6xHis
Hind III
Xba I

Figure 4.25: Cell Surface Expression Of MycEDG1 and MycEDG1Δ51 Receptors

CCL-39 cells stably expressing either the mycEDG1 or mycEDG1Δ51 receptor were subjected to cell surface biotinylation and receptor immunoprecipitation using the anti-myc 9E10 antibody as demonstrated in the Materials and Methods. The samples were then solubilised in electrophoresis sample buffer, transferred to nitrocellulose following SDS-PAGE and visualised by probing with HRP-streptavidin. This represents one of multiple experiments.

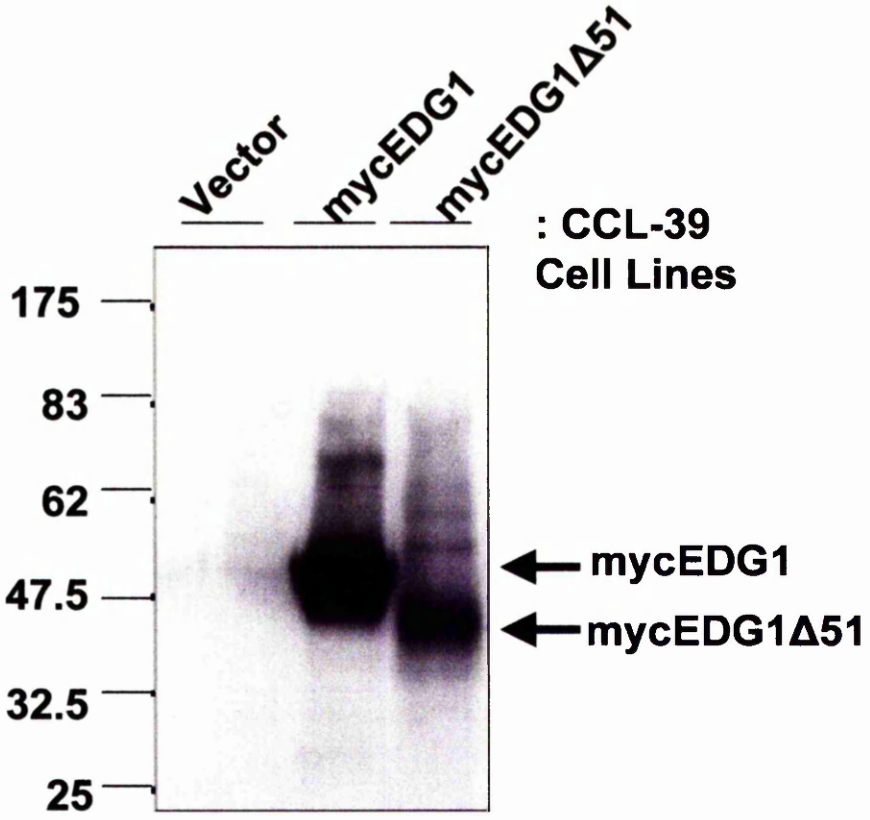
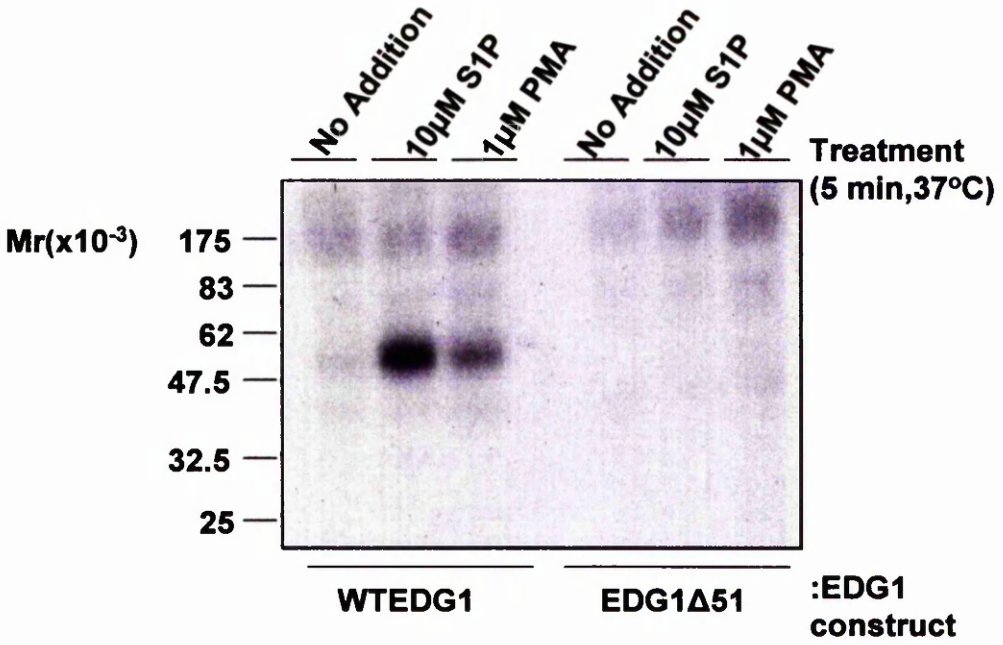

Vector
mycEDG1
mycEDG1Δ51
: CCL-39
Cell Lines
175
83
62
47.5
32.5
25
mycEDG1
mycEDG1Δ51

Figure 4.26: Comparison Of The Effects S1P And PMA Exposure On The Phosphorylation Of The MycEDG1 And MycEDG1Δ51

^{32}P-labelled serum-starved stably transfected CCL-39/MycEDG1 and CCL39/mycEDG1 truncated cells were exposed to either a 10 min treatment of vehicle, 10μM S1P or 1μM PMA at 37°C. The cells were then analysed by immunoprecipitation followed by SDS-PAGE and phosphorimaging. These data represent the mean ± SEM of three similar experiments. * Denotes a significant decrease ($p<0.05$) *versus* S1P-induced WT EDG1 phosphorylation. ~ Denotes a significant decrease ($p<0.05$) *versus* PMA-induced WT EDG1 phosphorylation. Statistical significance was determined using the one-way Analysis of Variance (ANOVA) Dunnett multiple comparisons test. The mycEDG1 receptor is phosphorylated by S1P and PMA as described previously. However, truncation of the last 51 amino acids of the C-terminal of EDG1 abolished both S1P- and PMA-mediated EDG1 phosphorylation.


No Addition
10μM S1P
1μM PMA
No Addition
10μM S1P
1μM PMA
Treatment
(5 min,37°C)
Mr(x10-3)
175
83
62
47.5
32.5
25
WTEDG1
EDG1Δ51
:EDG1
construct

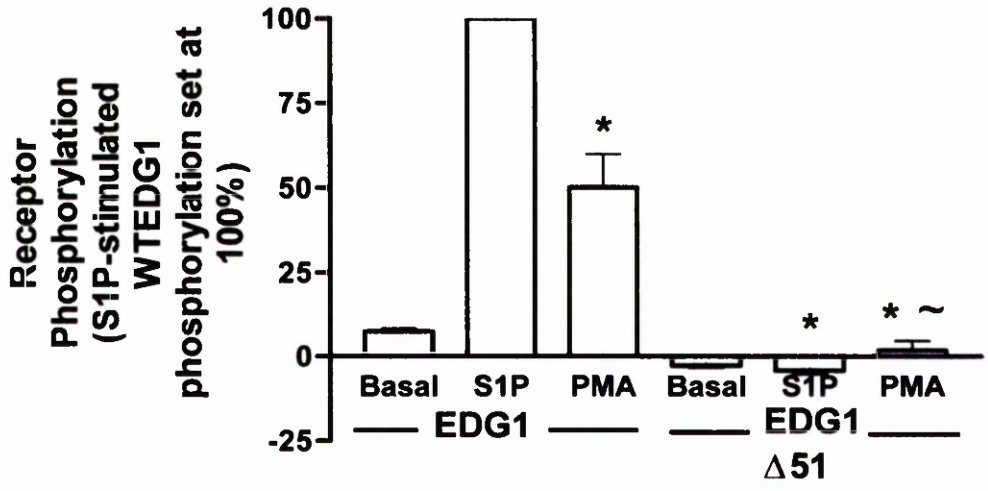

Receptor
Phosphorylation
(S1P-stimulated
WTEDG1
phosphorylation set at
100%)
100
75
50
25
0
-25
*
*
* ~
Basal
S1P
PMA
Basal
S1P
PMA
EDG1
EDG1
Δ51

Figure 4.27: Immunoblot Analysis Of The MycEDG1, MycEDG1Δ12 And MycEDG1Δ32 Receptors

HEK293 cells transiently expressing either the mycEDG1, mycEDG1Δ12 or mycEDG1Δ32 receptor were solubilised and then analysed by SDS-PAGE and immunoblotting with the anti-myc 9E10 monoclonal antibody. Truncation of the C-terminal results in the EDG1 receptor migrating faster down the SDS-PAGE gel, with the truncation of the last 32 amino acids resulting in the furthest migration. This reflects the decrease in molecular weight caused by truncation of the C-terminal tail.

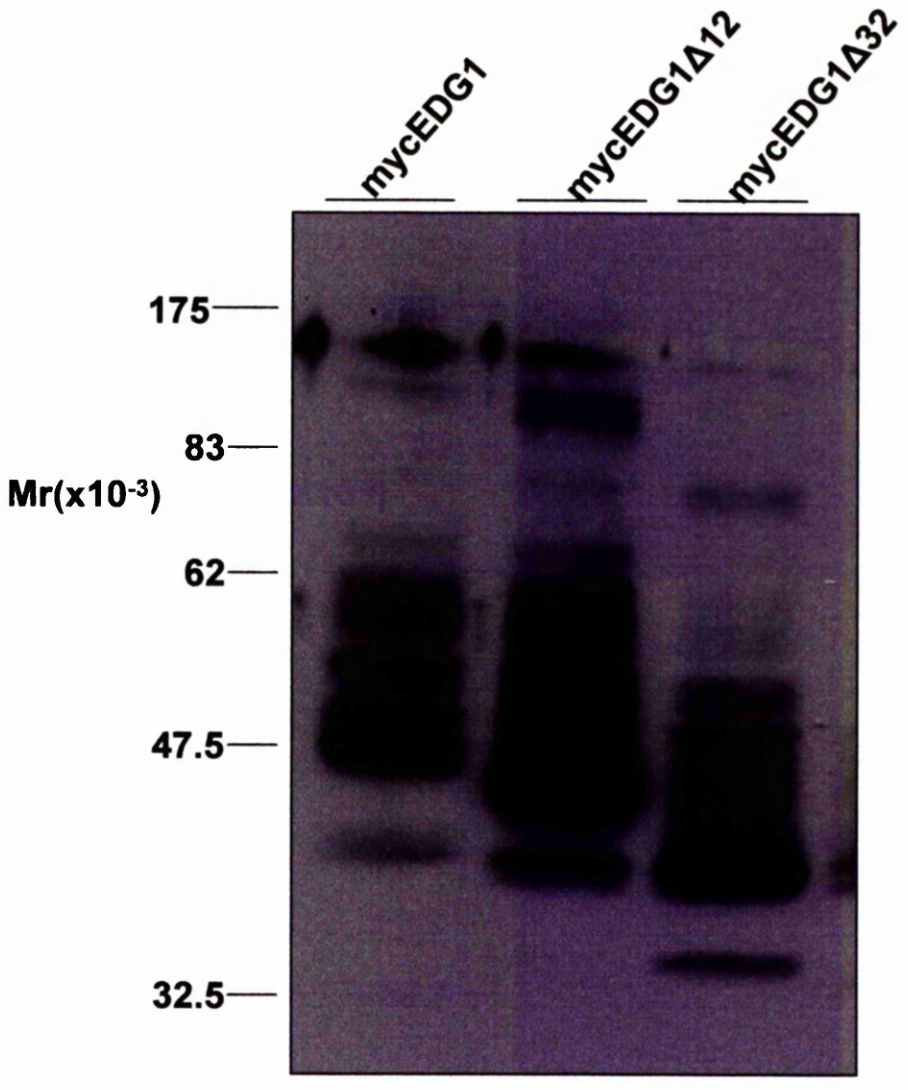
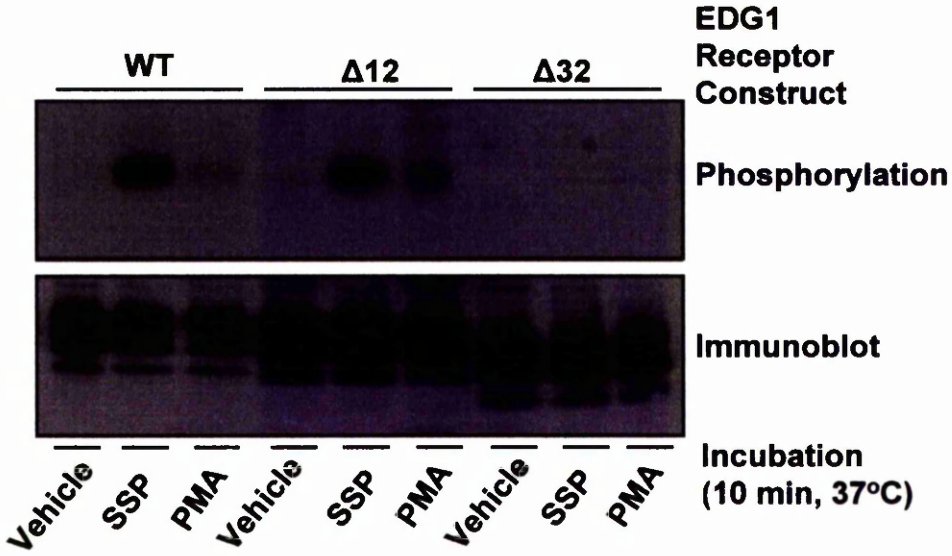

mycEDG1
mycEDG1Δ12
mycEDG1Δ32
175
83
Mr(x10-3)
62
47.5
32.5

Figure 4.28: Comparison Of The Effects Of SSP And PMA Exposure On The Phosphorylation Of The MycEDG1, MycEDG1Δ32 And MycEDG1Δ12 Receptors

Wild type EDG1, EDG1Δ32 and the EDG1Δ12 receptor constructs transiently transfected into HEK293 cells. The cells were then serum-starved, incubated with ^{32}P-orthophosphate and then given a 10 min treatment with vehicle, 10μM SSP or 1μM PMA at 37°C. The cells were then analysed by immunoprecipitation followed by SDS-PAGE and phosphorimaging. Parallel immunoblot analysis of each sample was carried out for each phosphorylation study using the 9E10 antibody in order to normalise receptor phosphorylation with receptor expression (Figure 28a, panel B). Data is presented as the mean ± SEM of three similar experiments. * Denotes a significant decrease ($p<0.05$) *versus* SSP-induced WT EDG1 phosphorylation. ~ Indicates a significant decrease ($p<0.05$) *versus* PMA-induced WT EDG1 phosphorylation Both SSP- and PMA- induced phosphorylation were abolished with the 32 amino acid truncated mutant (Figure 29a, panel A and Figure 28b). Statistical significance was determined using the one-way Analysis of Variance (ANOVA) Dunnett multiple comparisons test


WT
Δ12
Δ32
EDG1 Receptor Construct
Phosphorylation
Immunoblot
Vehicle
SSP
PMA
Vehicle
SSP
PMA
Vehicle
SSP
PMA
Incubation (10 min, 37°C)

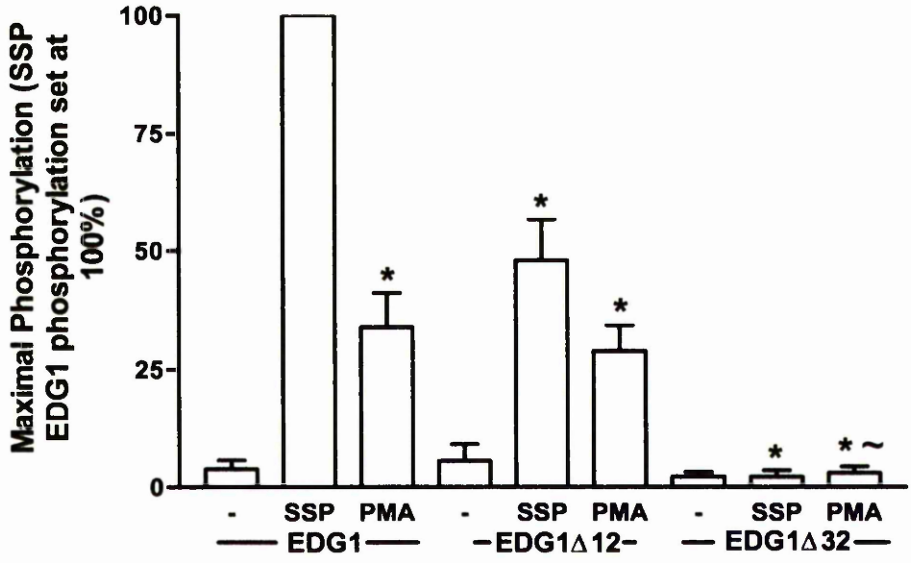
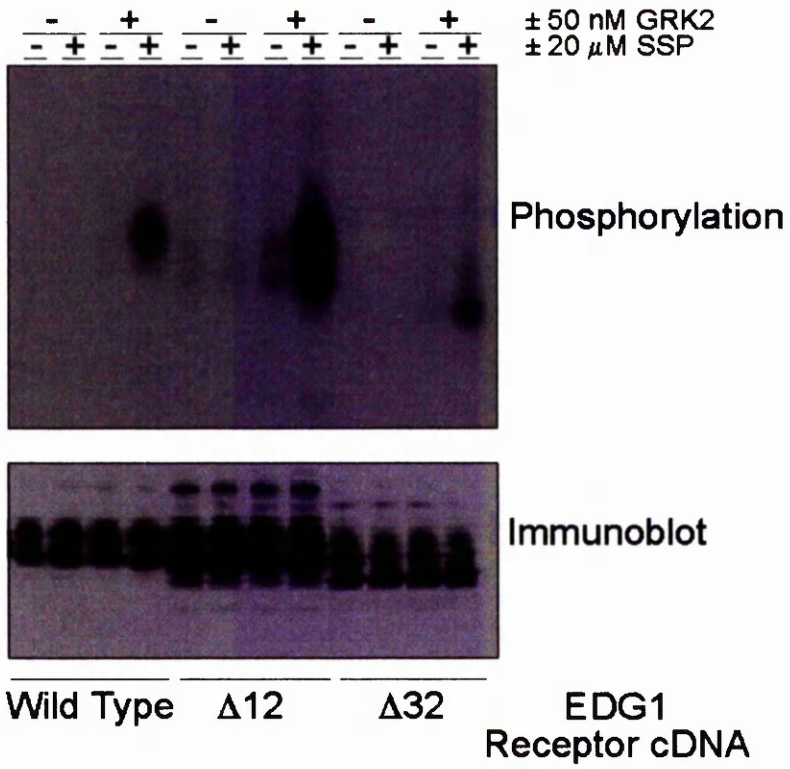
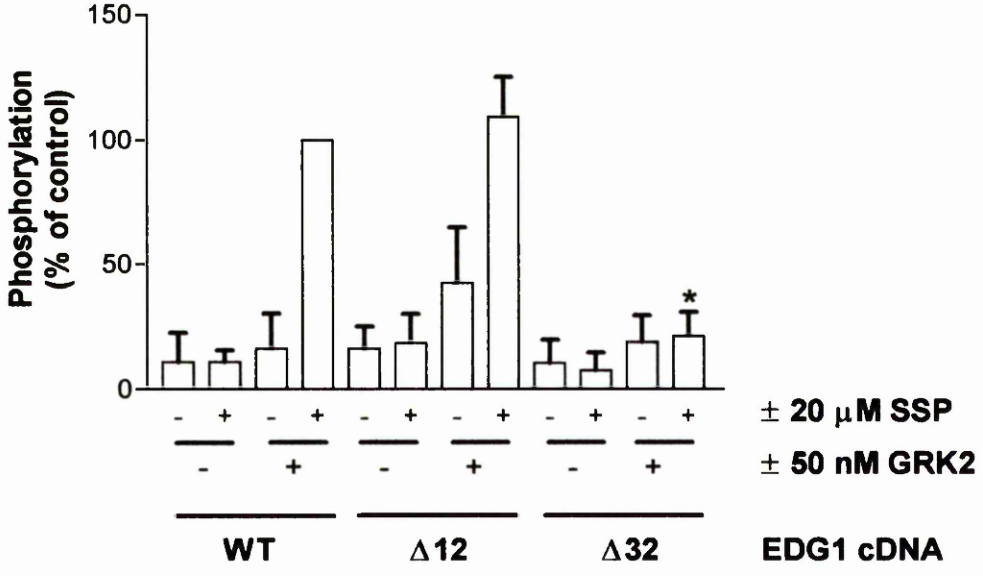

Maximal Phosphorylation (SSP EDG1 phosphorylation set at 100%)
100
75
50
25
0
*
*
*
*
*
*~
-
SSP
PMA
-
SSP
PMA
-
SSP
PMA
EDG1
EDG1Δ12
EDG1Δ32

Figure 4.29: Comparison Of The Effects Of GRK2 And GRK5 On SSP-Induced Phosphorylation Of The MycEDG1, MycEDG1Δ32 And MycEDG1Δ12 Receptors *In Vitro*

Membranes prepared from serum-starved HEK293 cells transiently expressing either the WT, Δ12 or Δ32 mutated mycEDG1 receptors were incubated with [γ-^{32}P] ATP in the absence or presence of 10μM SSP for 10 min and in the absence or presence of 50nM purified recombinant GRK2 or GRK5 at 37°C as indicated. Solubilised extracts were then prepared for receptor immunoprecipitation with 9E10 and analysis of receptor phosphorylation. Aliquots from each extract (4μg/lane) were also fractionated by SDS-PAGE for immunoblotting with 9E10 to normalise to receptor expression in each experimental condition. The data represents the mean ± SEM of 3 similar experiments. * Denotes a significant decrease ($p<0.05$) *versus* the level of phosphorylation observed for WT EDG1 under the same conditions. Statistical significance was determined using the one-way Analysis of Variance (ANOVA) Dunnett multiple comparisons test.


± 50 nM GRK2
± 20 μM SSP
Phosphorylation
Immunoblot
Wild Type
Δ12
Δ32
EDG1
Receptor cDNA


Phosphorylation
(% of control)
150
100
50
0
*
± 20 μM SSP
± 50 nM GRK2
WT
Δ12
Δ32
EDG1 cDNA

Chapter 5

Characterisation Of The Internalisation Of The Human EDG1 Receptor

Introduction

The process of GPCR internalisation represents an important aspect of receptor activity and regulation. For many GPCRs, receptor phosphorylation is a critical precursor to receptor internalisation. This has been described for many GPCRs, including the β_2AR, the A_3AR and the thrombin receptor, to name but a few (Ferguson, 2001; Palmer and Stiles, 1997; Xiao *et al.*, 1999). In most cases, receptor phosphorylation increases the receptor's affinity for arrestin molecules, which uncouple the receptors from their associated G-proteins and also direct receptor trafficking by functioning as an adapter protein and targeting the receptors to clathrin-coated vesicles from where the receptor is sequestered into the cell (Giadarov *et al.*, 1999; Miller and Lefkowitz, 2001; Pierce and Lefkowitz; 2001). This process of GPCR internalisation is thought to promote dephosphorylation of receptors by bringing them near to an endosome-associated phosphatase (Di Fiore and De Camilli, 2001; Ferguson, 2001). Dephosphorylation and subsequent recycling of receptors back to the plasma membrane contributes to a reversal of the desensitised state (resensitisation), which is required for full recovery of cellular signalling potential following agonist withdrawal (Di Fiore and De Camilli, 2001; Krueger *et al.*, 1997; Cao *et al*; 1998). Alternatively, the internalised receptor can be targeted to lysosomes where the receptor is degraded (Di Fiore and De Camilli, 2001; Ferguson, 2001).

The importance of receptor phosphorylation on receptor internalisation has been outlined in Chapter 3 where the loss of cell surface adenosine A_1 receptor following agonist exposure was slow ($t_{1/2}$=90 min) compared to the A_3 receptor ($t_{1/2}$=19 min). This difference was attributed to the lack of phosphorylation observed with the A_1 receptor in comparison to the rapidly phosphorylated A_3 receptor. As described in the previous Chapter, EDG1 is phosphorylated in the presence of agonist and also in an agonist-independent manner, through the activation of PKC. Both regulatory mechanisms of EDG1 phosphorylation involve phosphorylation of serine and threonine residues within the last 32 amino acids of the C-terminal tail. A previous study has also shown that the EDG1 receptor tagged with GFP is internalised following a 2 hour exposure of S1P (Lee *et al.*, 1998).

In this Chapter, the internalisation of the myc-tagged human EDG1 receptor stably expressed in CCL-39 hamster lung fibroblasts was characterised using cell surface biotinylation and confocal microscopic analysis. The relationship between EDG1 receptor

phosphorylation and receptor internalisation was also investigated using the previously demonstrated EDG1 C-terminal truncation mutants.

Results

CCL-39 cells stably expressing the human mycEDG1 receptor, as described in the previous Chapter, represents a good model in which to characterise EDG1 internalisation. Cell surface receptor biotinylation was carried out on CCL-39/mycEDG1 cells exposed to a range of S1P concentrations. Figure 5.1 shows that there is a concentration-dependent loss of mycEDG1 receptor from the cell surface (IC_{50}=0.24μM, n=3), with a maximal loss of 75 ± 16% following a 2 hour 40μM S1P exposure (versus vehicle-treated CCL-39/mycEDG1 cells (set at 100%), $p<0.05$, n=3). Cell surface biotinylation assays of CCL-39/mycEDG1 over a 2 hour time-course of 10μM S1P treatment demonstrated that loss of cell surface mycEDG1 is a fairly rapid process ($t_{1/2}$=15mins, n=3), with a total loss of 70 ± 3% cell surface EDG1 receptors observed after 2 hours (versus vehicle-treated CCL-39/mycEDG1 cells (set at 100%), $p<0.05$, n=3) (Figure 5.2). Interestingly, 10μM S1P-induced EDG1 internalisation was also shown to be an irreversible process. A 30 minute exposure of 10μM S1P resulted in a loss of 51± 8% of cell surface mycEDG1 as compared with vehicle-treated EDG1 cells (set at 100%, $p<0.05$, n=3) (Figure 5.3). However, no significant difference in mycEDG1 cell surface expression was observed following agonist removal for 2 hours (68±10% reduction in total cell surface mycEDG1 expression (set at 100%) versus agonist-treated CCL-39/mycEDG1 cells, $p>0.05$, N/S, n=3) (Figure 5.3). Interestingly, an immunoblot analysis of total mycEDG1 receptor expression on CCL-39/mycEDG1 cells treated with vehicle or 10μM S1P for 24 hours showed that there is no significant decrease in total mycEDG1 receptor expression following a chronic S1P exposure (92±5% versus vehicle-treated EDG1 cells (set at 100%), $p>0.05$, N/S, n=3) (Figure 5.4).

As described in the previous Chapter, EDG1 is phosphorylated in the presence of S1P and PMA via distinct mechanisms. It was therefore important to examine whether PMA-induced EDG1 phosphorylation resulted in EDG1 internalisation and if so whether this was also via a mechanism distinct from S1P-induced internalisation. Thus, a cell surface biotinylation assay was carried out on CCL-39/mycEDG1 cells exposed for 2 hours with either vehicle, 10μM S1P or 1μM PMA in the presence or absence of a 30 min pretreatment with 5μM GF109203X, an inhibitor of conventional and novel PKC subtypes

(Way et al., 2000; Martiny-Baron et al., 1993). Figure 5.5 shows that, in the absence of GF109203X, S1P induced a 46% loss of cell surface EDG1 receptors (versus vehicle-treated CCL-39/MycEDG1 cells (set at 100%), $p<0.05$, n=3) whereas PMA exposure resulted in a significantly smaller reduction in mycEDG1 cell surface expression (25 ± 5%, significantly less ($p<0.05$) than S1P-mediated effect, n=3). Preincubation with GF109203X abolished the PMA-induced effect (100±14% mycEDG1 cell surface expression versus vehicle-treated mycEDG1 cell surface expression (set at 100%), $p<0.05$, n=3), whereas S1P-induced internalisation was unaffected (45±14% loss of mycEDG1 cell surface expression versus vehicle-treated CCL-39/mycEDG1 cells, $p<0.05$, n=3), suggesting the existence of two distinct pathways of receptor internalisation (Figure 5.5).

In order to visualise S1P- or PMA- induced changes in EDG1 receptor trafficking, a mycEDG1-GFP construct was generated and stably expressed in CCL-39 hamster lung fibroblasts as described in the Methods section (Figure 5.6). Stable expression of the mycEDG1-GFP receptor was confirmed by immunoblotting using the anti-myc monoclonal antibody 9E10 and a monoclonal anti-GFP antibody (Figure 5.7). Both receptors were shown to be expressed at similar levels as visualised by the 9E10 antibody. Incubation with the anti-GFP antibody selectively identified the EDG1-GFP protein. The observed difference in molecular mass between the wild type EDG1 receptor and the EDG1-GFP receptor (around 28kDa) was due to the GFP tag. Having confirmed that the mycEDG1 receptor was GFP-tagged, it was important to determine whether the addition of the GFP tag influenced mycEDG1 receptor function. Thus, a whole cell phosphorylation study was carried out on CCL-39/mycEDG1 and CCL-39/mycEDG1-GFP cells in the presence of a 10 min treatment of vehicle, 10μM S1P or 1μM PMA. A strong, S1P-induced phosphorylation and a weaker, but still significant, PMA-induced phosphorylation was observed with both the wild type EDG1 and EDG1-GFP receptors (Figure 5.8). This indicated that GFP tagging of the EDG1 receptor had no discernable effect on its ability to be phosphorylated by S1P or PMA. The CCL-39/mycEDG1GFP cell line therefore represented a relevant model in which to study changes in the trafficking of the EDG1 receptor following exposure to agonist and PMA.

In order to visualise the changes in agonist-dependent changes in Edg1 receptor trafficking demonstrated with the cell surface labelling assays, CCL-39/mycEDG1GFP cells were then exposed to 10μM S1P over a 2 hour time course. The cells were then fixed, mounted on coverslips and examined by confocal microscopy. A 2 hour treatment

of 10μM S1P results in a significant translocation of mycEDG1-GFP from the cell surface into distinct punctate intracellular vesicles within the cytoplasm. The formation of intracellular pools of mycEDG1GFP was observed after only 15 min. After 30 min, there was a significantly larger movement of mycEDG1GFP from the cell surface into the cytoplasm (Figure 5.9). These data correlate with the cell surface biotinylation experiments, with cell surface loss observed after 15 mins and the greatest loss of cell surface EDG1 observed following 30 minutes of 10μM S1P treatment. Following 2 hours of agonist exposure, a large quantity of receptor protein has translocated from the cell surface into the cytoplasm.

As shown in Figure 5.5, there is a small, but still significant, loss of mycEDG1 receptor from the cell surface following a 2 hour treatment of 1μM PMA and that this process is distinct from S1P-induced EDG1 internalisation. CCL-39/mycEDG1GFP cells were therefore given a 2 hour treatment with vehicle, 10μM S1P or 1μM PMA to visualise any differences in EDG1 receptor trafficking between S1P- and PMA- induced EDG1 internalisation. The cells were then fixed, mounted on coverslips and examined by confocal microscopy. A 2 hour exposure to 10μM S1P resulted in a significant redistribution of EDG1 from the cell surface into punctate vesicles within the cytoplasm. In contrast, no significant translocation of EDG1 was observed following a 2 hour treatment with 1μM PMA. One possibility is that the loss of cell surface EDG1 receptor observed with the biochemical cell surface assays may be the result of EDG1 moving into distinct plasma membrane compartments, in which the receptor becomes inaccessible to biotin hydrazide used for labelling (Figure 5.10). Therefore, while S1P and PMA exposure both result in the loss of EDG1 from the cell surface, the extent and nature of each type of EDG1 re-distribution are distinct.

As described in the previous chapter, truncation of the last 51 amino acids within the C-terminal tail of EDG1 abolishes both S1P- and PMA- induced receptor phosphorylation (Figure 4.26). Since phosphorylation is often a crucial determinant in GPCR internalisation (Ferguson, 2001; Palmer and Stiles, 1997; Xiao et al., 1999), it was therefore important to investigate the effect of how this truncation might affect EDG1 internalisation. The mycEDG1Δ51 receptor was GFP-tagged and stably expressed in CCL-39 cells to visualise the effect of a non-phosphorylated EDG1 receptor on receptor trafficking (Figure 5.11). Figure 5.12 shows an immunoblot analysis of the mycEDG1-GFP, mycEDG1Δ51, and mycEDG1Δ51-GFP receptors each stably expressed in CCL-39

hamster lung fibroblasts. Truncation of the last 51 amino acids of the C-tail resulted in a faster migration of the protein on SDS-PAGE, reflecting the approximately 8kDa reduction in molecular mass of the mutated receptor.

CCL-39/mycEDG1cells and CCL-39/mycEDG1Δ51-GFP cells were then exposed to either vehicle or 10μM S1P to visualise any differences in S1P-induced EDG1 receptor trafficking. The cells were then fixed, mounted on coverslips and examined by confocal microscopy. As demonstrated previously in Figure 5.9 and 5.10, a 2 hour treatment with 10μM S1P resulted in a significant redistribution of WT mycEDG1-GFP from the cell surface into punctate intracellular vesicles within the cytoplasm. In contrast, no changes in the subcellular distribution of the mycEDG1Δ51-GFP receptor were observed throughout a 2 hour time-course (Figure 5.13). This suggests that the integrity of the last 51 amino acids are critical to observe S1P stimulation of EDG1 internalisation.

As shown in the last Chapter, truncation of the last 12 and the last 32 amino acids from the C-terminal region of EDG1 was shown to have distinct effects upon receptor phosphorylation. While the EDG1Δ32 receptor was not phosphorylated in the presence of either S1P or PMA, truncation of the last 12 amino acids had no effect on PMA-induced EDG1 phosphorylation but significantly impaired S1P-induced EDG1 phosphorylation (Figure 4.28). A study was therefore carried out to examine the effects of each of these truncations on EDG1 receptor internalisation. Before studying the effect of C-terminal EDG1 truncations on EDG1 receptor internalisation, it was important to observe whether truncation of the C-terminal had any effect on the cell surface expression of EDG1. HEK293 cells transiently expressing the EDG1, EDG1Δ12 and EDG1Δ32 cDNA receptors were grown on coverslips, washed and then fixed using paraformaldehyde. After permeabilisation of the cells, the receptors were immunostained using the 9E10 anti-myc antibody followed by incubation with an Alexa 594-conjugated anti-mouse IgG. No significant differences in the cell surface expression between each receptor were observed (Figure 5.14). Therefore, truncation of the C-terminus had no visible effect on the cell surface expression of EDG1 under non-stimulated conditions.

In order to quantitate the effect of the removal of 32 amino acids from the EDG1 C-terminal domain on receptor internalisation, a cell surface biotinylation was carried out on HEK293 cells transiently expressing either the mycEDG1 or the mycEDG1Δ32 receptor exposed to either vehicle or 10μM S1P over a 2 hour time course. Truncation of 32 amino acids from the C-terminal abolished mycEDG1 receptor internalisation (111.7±23.3% total

mycEDG1 cell surface expression versus vehicle-treated HEK293 cells expressing the mycEDG1Δ32 receptor (set at 100%), $p>0.05$, N/S, n=3) whereas a 2 hour exposure to 10μM S1P resulted in a 46.61 ± 3.6% loss of the wild type EDG1 receptor (versus vehicle-treated HEK293 cells expressing the mycEDG1 receptor, $p<0.05$, n=3) (Figure 5.15)

Finally, the effect of the removal of the last 12 amino acids from the EDG1 C-terminal domain on receptor internalisation was carried out on HEK293 cells transiently expressing either the mycEDG1 or the mycEDG1Δ12 receptor exposed to either vehicle or 10μM S1P over a 2 hour time course. As with the mycEDG1Δ32 receptor, truncation of the last 12 amino acids from the C-terminal abolished mycEDG1 receptor internalisation (102±16.5% total mycEDG1 cell surface expression versus vehicle-treated HEK293 cells expressing the mycEDG1Δ12 receptor (set at 100%), $p>0.05$, N/S, n=3) whereas a 2 hour exposure to 10μM S1P resulted in a 56.1±11.7% loss of the wild type EDG1 receptor (versus vehicle-treated HEK293 cells expressing the mycEDG1 receptor, $p<0.05$, n=3) (Figure 5.16)

Discussion

As shown in the previous Chapter, the myc-tagged human EDG1 receptor is not only phosphorylated in the presence of S1P but also via an agonist-independent mechanism mediated by PKC activation. In many cases, GPCR phosphorylation is a prerequisite for receptor internalisation away from the cell surface (Ferguson, 2001; Palmer and Stiles, 1997; Xiao et al., 1999). Receptor internalisation represents an important mechanism for regulating the signalling of many GPCRs following a sustained agonist exposure. For example, GPCR internalisation is involved in the resensitisation of GPCRs. Internalisation is also often a prerequisite for receptor degradation. Studies primarily involving the β_2AR have also shown that internalisation can mediate alternative signalling pathways. Since EDG1 receptor activation is important in the process of angiogenesis, the regulation of EDG1 signalling therefore represents a potentially important target for generating therapeutics to combat diseases such as cancer and ischaemic heart disease where the control of angiogenesis is critical. In this Chapter, the cell surface distribution of the myc-tagged human EDG1 receptor following either S1P or PMA treatment was characterised and compared with the patterns of EDG1 phosphorylation established in the previous chapter.

Cell surface biotinylation assays demonstrated that there is a concentration-dependent loss of cell surface EDG1 receptor (IC_{50}=0.24μM) after a 2 hour agonist exposure (Figure 5.1). In comparison, the EC_{50} of S1P-induced EDG1 phosphorylation was 1.9±0.37μM (Figure 4.7). This suggests that only relatively low levels of EDG1 phosphorylation are required for EDG1 internalisation. Time-course experiments demonstrated that the loss of EDG1 receptor from the cell surface was a relatively rapid process ($t_{1/2}$=15 min), with a maximal loss of 70 ± 3% cell surface EDG1 receptors observed after 2 hours of 10μM S1P exposure (Figure 5.2). As shown in the previous Chapter, S1P-induced EDG1 phosphorylation is a reversible process (Figure 4.9). However, EDG1 internalisation was shown to be irreversible over a 2 hour time course (Figure 5.3). In addition, total EDG1 expression was shown to be unchanged following a 24 hour agonist exposure (Figure 5.4). Taken together, this would suggest that dephosphorylation of EDG1 occurs within the cell and that the EDG1 receptor remains within the cytoplasm following agonist removal, possibly within selected intracellular compartments, but that the receptor is not degraded.

Since PMA has been shown to phosphorylate EDG1, there may also be a PKC-mediated internalisation of EDG1 that was independent of agonist. Figure 5.5 shows that whereas a 2 hour exposure to 10μM S1P decreased cell surface EDG1 expression by 45±8%, a 2 hour exposure to 1μM PMA resulted in a smaller, yet still significant, loss of cell surface EDG1 receptor (25±5%). The PMA-induced effect was completely abolished by preincubation with the PKC inhibitor GF109203X, whereas the same preincubation had no effect on S1P-induced EDG1 internalisation. This suggests that, as with EDG1 phosphorylation, there is an agonist-dependent pathway of EDG1 internalisation and an agonist-independent mechanism mediated through the activation of PKC. As mentioned in the previous Chapter, other studies have shown that PKC can phosphorylate and subsequently desensitise a number of Gi- and G_q- linked GPCRs, including the α_{1B}-adrenoceptor and the type 1A angiotensin II receptor (Diviani et al., 1997; Liang et al., 1998; Tang et al., 1998). Other studies have also shown a role for PKC in receptor internalisation. For example, there is evidence of a PLC-β- and PKC- dependent mechanism of FcγRI internalisation (Norman and Allan, 2000). As discussed for EDG1 phosphorylation, the existence of an agonist-independent mechanism of EDG1 internalisation mediated through activation of PKC implies a role for other GPCRs in the regulation of EDG1 internalisation through a PKC-dependent cross-talk mechanism. For

example, receptors that play an integral part in the regulation of angiogenesis, such as the PDGF receptor, represent potential candidates and future work should be designed to examine this further. Future work should also aim to examine the physiological significance of the PMA-induced internalisation since the observed effect is relatively small.

Confocal analysis of a CCL-39/mycEDG1-GFP stable cell line demonstrated that there is a dramatic re-distribution of mycEDG1-GFP from the cell surface to distinct intracellular vesicles following a 2 hour exposure of 10μM S1P. A significant translocation of receptor into the cytoplasm could be observed as early as a 15 min treatment with 10μM S1P (Figure 5.9). This is in agreement with the cell surface biotinylation studies ($t_{1/2}$=15 min) (Figure 5.2) and also with a previous study where HEK293 cells expressing an EDG1GFP construct demonstrated significant receptor internalisation from the cell surface following a 2 hour exposure of 100nM S1P (Lee et al, 1998). In contrast, a 2 hour treatment with 1μM PMA did not result in a significant accumulation of intracellular mycEDG1-GFP (Figure 5.10). Confocal analysis only permits the observation of dramatic changes in receptor trafficking whereas cell surface labelling will reflect changes in the accessibility of alcohol groups belonging to cell surface receptors. Therefore, one explanation for this may be that PMA treatment results in the translocation of mycEDG1 into selective compartments within the plasma membrane where the receptor is inaccessible to the cell surface biotin. Hence, it is possible that the differences in cell surface EDG1 expression observed following PMA exposure are due to the increased sensitivity of cell surface biotinylation over immunocytochemistry. Previous studies have shown that the cholecystokinin (CCK) receptor can undergo an agonist-dependent translocation to a basolateral plamalemmal compartment similar to caveolin in CHO and pancreatic acinar cells where it does not translocate deeper into the cell (Roettger et al., 1995; Rosenzweig et al., 1983). Therefore, it is possible that the EDG1 receptor may translocate into a similar pathway following PMA exposure. Interestingly, the CCK receptor exhibits a clathrin-dependent mechanism involved in the lysosomal degradation and resensitisation of the receptor and a clathrin-independent mechanism via a vesicular compartment in the plasmalemma involved in a more more rapid resensitisation of the CCK receptor (Roettger et al., 1995). Therefore, a similar physiological role may exist for the PMA-dependent internalisation observed for EDG1.

Like a number of GPCRs, such as the A_3 adenosine receptor and the β_2-adrenergic receptor, the region of the EDG1 C-terminal domain distal to the three predicted sites of palmitoylation is enriched in potential phosphorylation sites. An EDG1 mutant (mycEDG1Δ51-GFP), in which all these potential phosphorylation sites have been removed by the truncation of the last 51 amino acids, was GFP-tagged and any agonist-induced changes in receptor trafficking observed by confocal microscopy. Whereas the wild-type mycEDG1-GFP receptor undergoes significant internalisation following a 2 hour exposure of 10μM S1P, there is no significant translocation of the mycEDG1Δ51-GFP receptor from the plasma membrane is observed (Figure 5.13). This is in agreement with a similar study where a GFP-tagged EDG1 receptor in which all the potential phosphorylation sites were removed was not internalised following agonist exposure (Liu et al., 1999). This provides strong evidence for a link between EDG1 receptor phosphorylation within the C-terminal tail and subsequent EDG1 receptor internalisation.

As discussed in the previous Chapter, the deletion of the last 12 and the last 32 amino acids removed two clusters of serine/threonine residues, each of which constituted a potential GRK2 phosphorylation site. Cell surface biotinylation studies demonstrated that the removal of both the last 12 and the last 32 amino acids from the C-terminal abolished EDG1 receptor internalisation (Figure 5.15, 5.16). This would suggest that the last 12 amino acids of the C-terminal tail are crucial for S1P-mediated internalisation to be observed. However, the previous Chapter demonstrated that GRK2-mediated EDG1 phosphorylation was maintained following the truncation of the last 12 amino acids. One possibility is that the truncation of the C-terminal hinders the binding of arrestin molecules that are essential for receptor internalisation. It should also be noted that the stoichiometry of GRK phosphorylation differs, depending upon the GPCR studied such that the primary sites of GRK phosphorylation observed in vitro are not necessarily the sites of phosphorylation in vivo (Siebold et al., 1998). For example, the mutation of the primary GRK phosphorylated residues identified in vitro on the β_2AR did not prevent the GRK-mediated β_2AR desensitisation in cells (Siebold et al., 1998). Alternatively, the phosphorylation of secondary GRK phosphorylation sites may compensate for the loss of the primary site of GRK-mediated phosphorylation. Additionally, the muscarinic m_2 receptor is phosphorylated by GRK2 and β-arrestins subsequently desensitise the receptor. However, the m_2 receptor internalises primarily through β-arrestin-independent mechanisms (Pais-Rylaarsdam et al., 1997; Pierce and Lefkowitz, 2001). Another

possibility is that EDG1 internalisation may be dependent upon receptor phosphorylation in vivo by an acidotrophic kinase distinct from GRK2, as has been shown for the casein kinase-1α-phosphorylated m_3 muscarinic acetylcholine receptor (Budd et al., 2000). As mentioned in the previous Chapter, there is also a potential site of casein kinase II phosphorylation present within the region of the EDG1 C-terminus implicated in the process of receptor internalisation (Liu et al., 1999).

This Chapter has established that there is an agonist-mediated internalisation of EDG1. In addition, there is also a smaller, but still significant agonist-independent EDG1 receptor internalisation mediated by PKC. Confocal microscopic analysis of a mutated EDG1-GFP chimera in which all the potential phosphorylation sites within the area of the EDG1 C-terminal domain distal to the three predicted sites of palmitoylation were removed completely inhibited agonist-mediated receptor trafficking towards the cytoplasm. Further sequential EDG1 truncation studies demonstrated that removal of both the last 12 and the last 32 amino acids completely inhibited EDG1 internalisation.

To establish the amino acids responsible for EDG1 internalisation, future work should be aimed at site-directed mutagenensis, specifically within the last 12 amino acids. Identification of the kinase(s) responsible for EDG1 internalisation should also be examined. The role of GRK2 should be fully examined, in addition to other kinases such as casein kinase-1α and casein kinase II. The role of the scaffolding proteins, arrestins in EDG1 internalisation should also be studied.

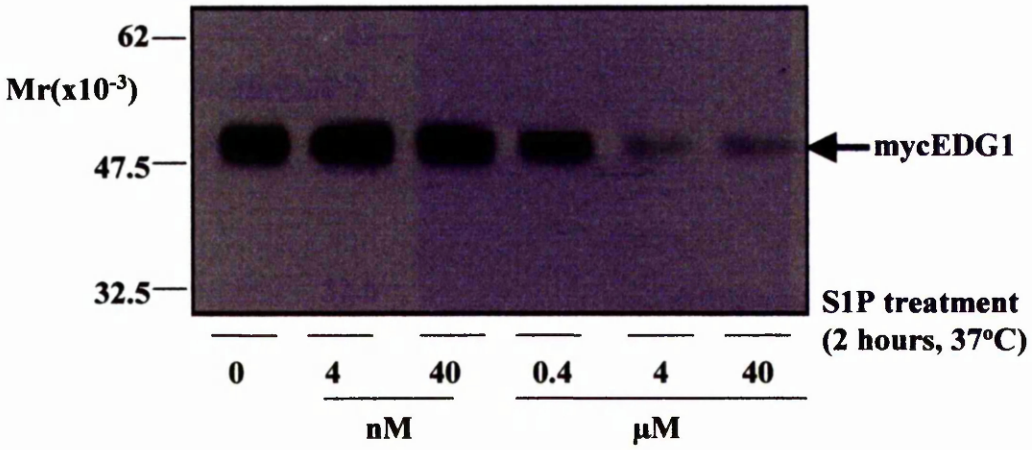
A previous study has shown that S1P results in the translocation of β-arrestin2 towards activated EDG1 in HEK293 cells (Hobson et al., 2001). However, the role of arrestins in EDG1 internalisation has yet to be fully characterized. Additionally, little is known about the possible effect of arrestins on EDG1 signal transduction. Interestingly, a recent study has suggested a role for β-arrestin in a cross-talk mechanism between EDG1 and PDGF (Hobson et al., 2001). Recent evidence has strongly implicated the PDGF-dependent activation of EDG1 via the intracellular production of S1P. This activation of EDG1 results in the recruitment of β-arrestin2 and the activation of Rac-dependent processes of cell migration. This results in the migration of cells towards PDGF, causing a potentiation of PDGF receptor signalling (Hobson, et al., 2001; Rosenfeldt et al., 2001). However, it is not known how the internalisation of EDG1 affects PDGF signalling. As described previously, the recruitment of arrestin molecules towards activated β_2AR can result in the activation of alternative signalling pathways, such as the activation of JNK

and ERK. Therefore, the effect of the recruitment of arrestin by EDG1 on the signalling of EDG1 and PDGF should be examined.

Additionally, very little is known about the intracellular trafficking of the EDG1 receptor. As shown previously, the EDG1 receptor is dephosphorylated upon agonist removal but does not return to the cell surface. Also, the receptor is not down regulated following a chronic S1P exposure. Therefore, immunostaining experiments should be carried out to determine the fate of the EDG1 receptor following internalisation. As mentioned earlier, the discovery of an agonist-independent mechanism of receptor internalisation via PKC suggests the involvement of other Ca^{2+}-mobilising GPCRs in the regulation of the EDG1 receptor. Therefore, future work should also be aimed at examining the potential role of other GPCRs in regulating EDG1 signalling, such as the thrombin and PDGF receptors, as well as Tie2, Flt and Flk, all of which are heavily involved in the angiogenic process.

Figure 5.1: Effect Of Increasing Concentrations Of S1P On Cell Surface Expression Of EDG1

Serum-starved stably transfected CCL-39/mycEDG1 cells were treated with vehicle or increasing concentrations of S1P for 2 hours at 37°C. The cells were examined for internalisation by cell surface labelling with biotin-LC-hydrazide and solubilised followed by immunoprecipitation with the anti-myc 9E10 antibody. Biotin labelling of vehicle-treated CCL-39/mycEDG1 cells was set at 100% and the results following agonist treatment were expressed as a ratio of the control. The data represents the mean ± SEM of three similar experiments. The results show a concentration-dependent S1P-induced loss of cell surface EDG1 receptor (EC_{50}= 0.24μM).


62
Mr(x10-3)
47.5
32.5
mycEDG1
S1P treatment
(2 hours, 37°C)
0
4
40
0.4
4
40
nM
µM

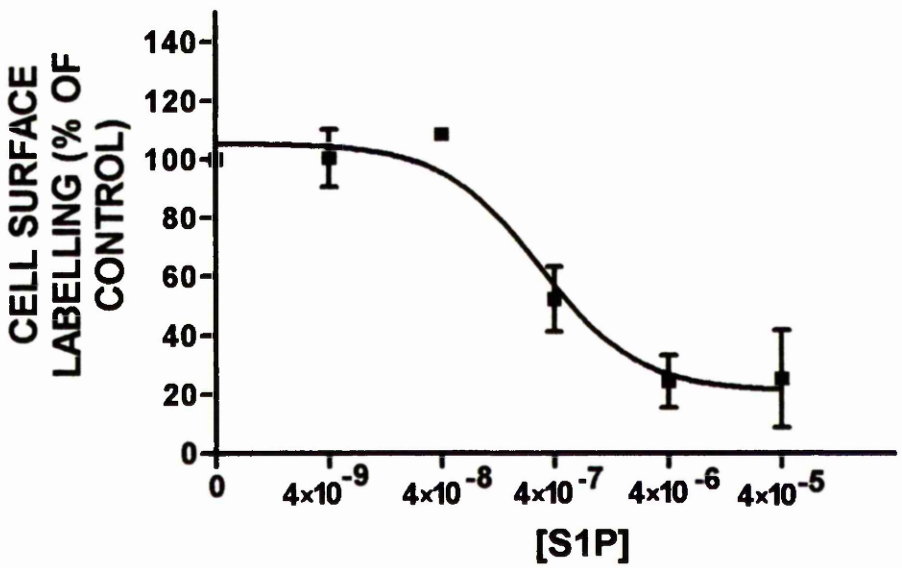
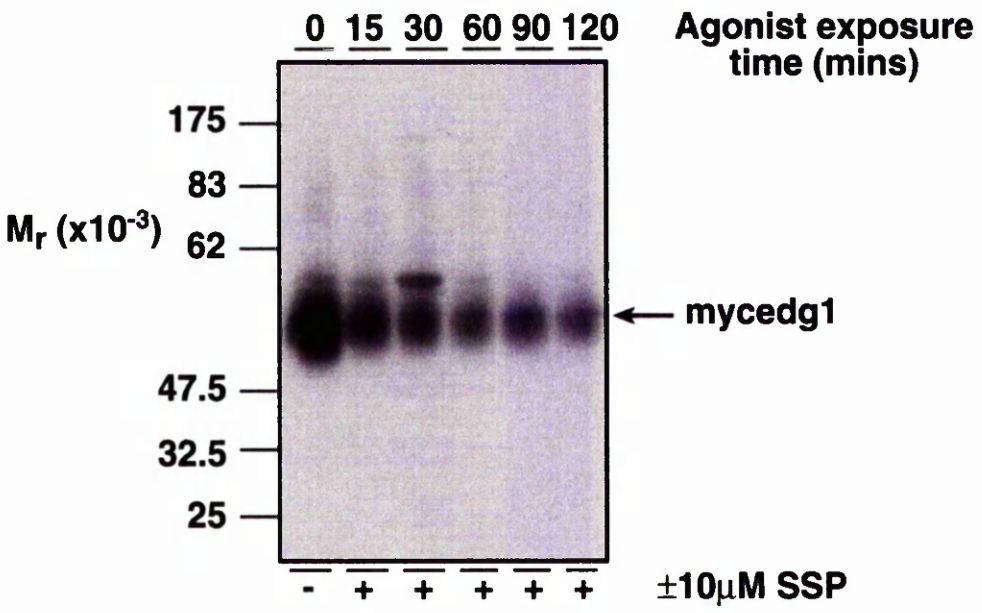

CELL SURFACE LABELLING (% OF CONTROL)
140
120
100
80
60
40
20
0
0
4x10-9
4x10-8
4x10-7
4x10-6
4x10-5
[S1P]

Figure 5.2: Time-Course Of SSP-Mediated Loss Of Cell Surface EDG1 Receptor

Serum-starved stably transfected CCL-39/mycEDG1 cells were treated at 37°C with vehicle or 10μM SSP for the times indicated. The cells were examined for internalisation by cell surface labelling with biotin-LC-hydrazide and solubilised, followed by immunoprecipitation with the anti-myc 9E10 antibody. Biotin labelling of vehicle-treated CCL-39/mycEDG1 cells was set at 100% and the results following agonist treatment expressed as a ratio of the control. The data represents the mean ± SEM of three similar experiments. The results show a time-dependent SSP-induced EDG1 internalisation ($t_{1/2}$= 15 min).


0 15 30 60 90 120
Agonist exposure time (mins)
175
83
62
47.5
32.5
25
M_r (x10^{-3})
mycedg1
- + + + + +
±10μM SSP

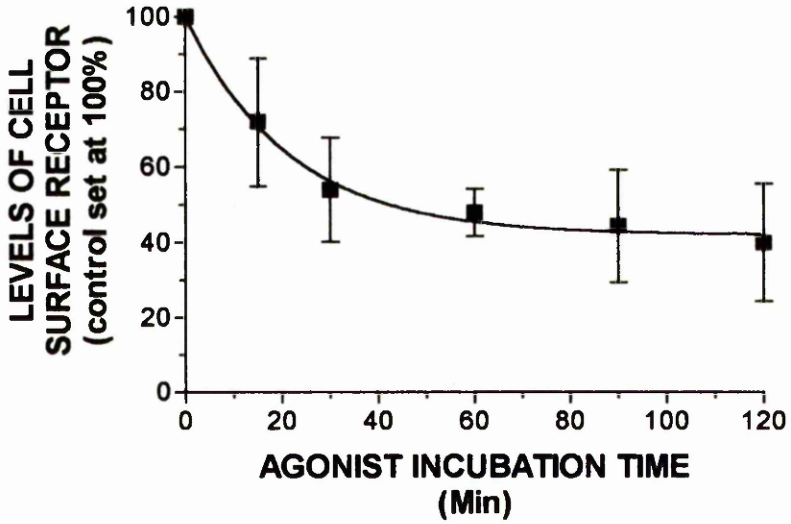
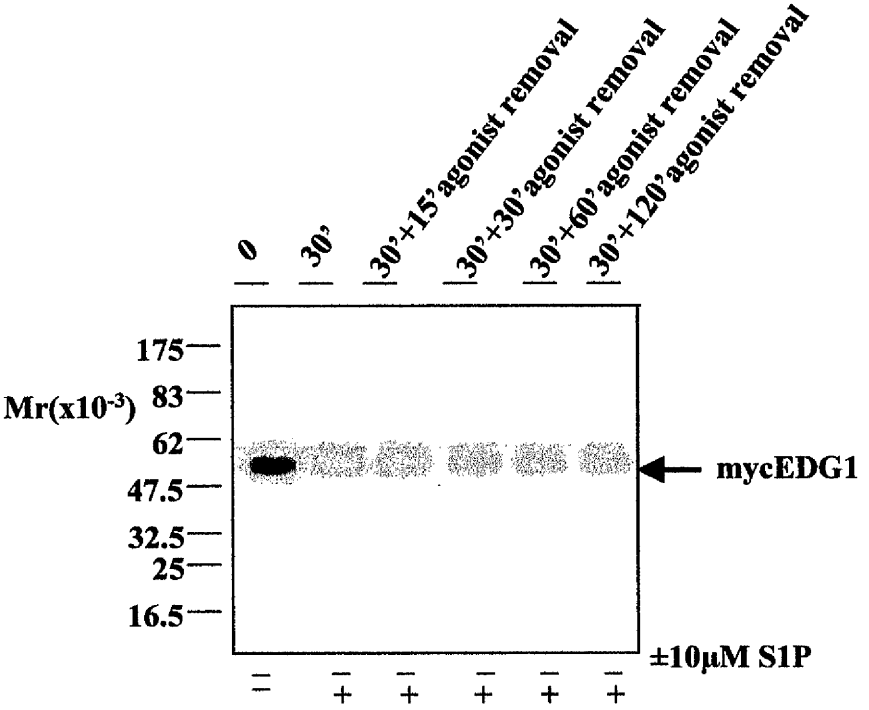

LEVELS OF CELL SURFACE RECEPTOR (control set at 100%)
100
80
60
40
20
0
0 20 40 60 80 100 120
AGONIST INCUBATION TIME (Min)

Figure 5.3: Effect Of S1P Removal On S1P-Mediated Loss Of EDG1 Receptor From The Cell Surface

Serum starved stably transfected CCL-39/mycEDG1 cells were treated with either vehicle or 10μM S1P for 30 minutes at 37°C. Agonist was then removed after 15, 30, 60 or 120 minutes. The cells were examined for internalisation by cell surface labelling with biotin-LC-hydrazide and solubilised followed by immunoprecipitation with the anti-myc 9E10 antibody. Biotin labelling of vehicle-treated CCL-39/mycEDG1 cells was set at 100% and the results following agonist treatment expressed as a ratio of the control. The data represents the mean ± SEM of three similar experiments. * Denotes a significant difference ($p<0.05$) *versus* vehicle-treated EDG1 cells. # Indicates a significant difference ($p<0.05$) *versus* S1P-treated EDG1 cells.


0
30'
30'+15' agonist removal
30'+30' agonist removal
30'+60' agonist removal
30'+120' agonist removal
175
83
62
47.5
32.5
25
16.5
Mr(x10-3)
mycEDG1
±10μM S1P
-
-
+
+
+
+
+

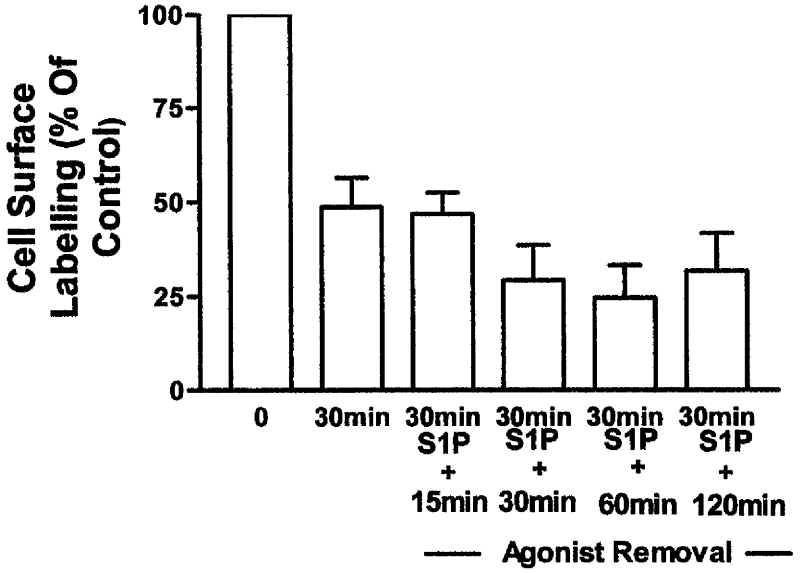
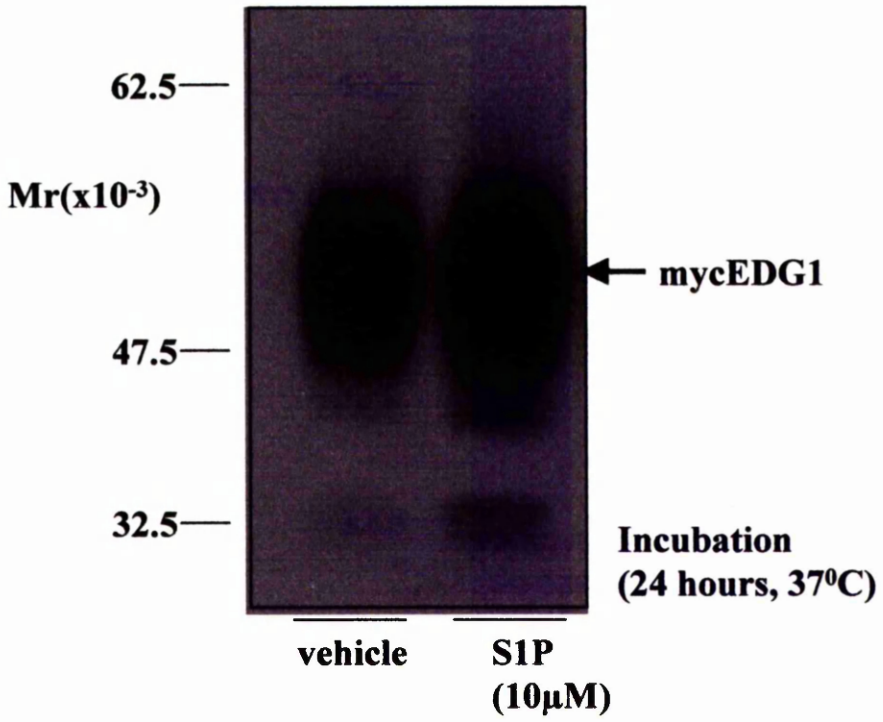

Cell Surface Labelling (% Of Control)
100
75
50
25
0
0
30min
30min S1P + 15min
30min S1P + 30min
30min S1P + 60min
30min S1P + 120min
Agonist Removal

Figure 5.4: Effect Of Sustained S1P Treatment On Total EDG1 Receptor Expression

Serum starved stably transfected CCL-39/mycEDG1 cells were treated with either vehicle or 10μM S1P for 24 hours at 37°C. The cells were then solubilised and analysed by SDS-PAGE and immunoblotting with anti-myc monoclonal antibody 9E10. The data represents the mean ± SEM of three similar experiments. * denotes a significant difference ($p<0.05$) between intreated EDG1-expressing cells. No significant differences in total EDG1 expression were observed following a chronic S1P exposure.


62.5
Mr(x10-3)
47.5
32.5
mycEDG1
Incubation
(24 hours, 37°C)
vehicle
S1P
(10μM)

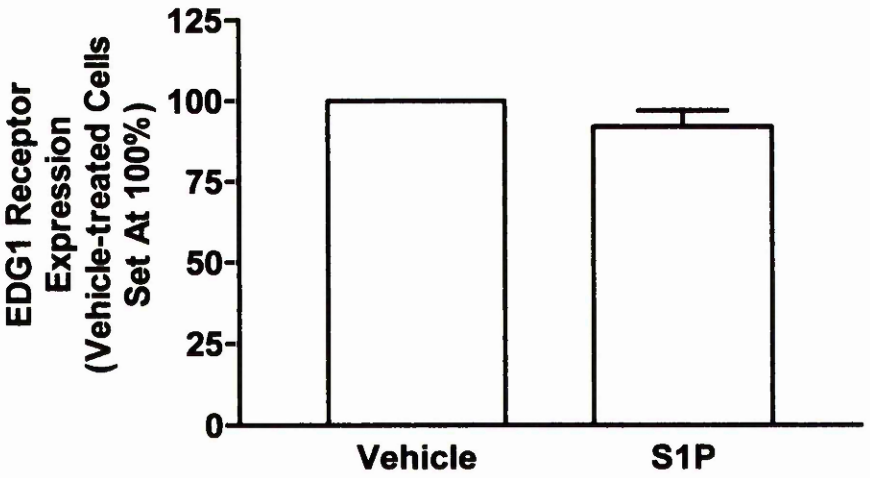
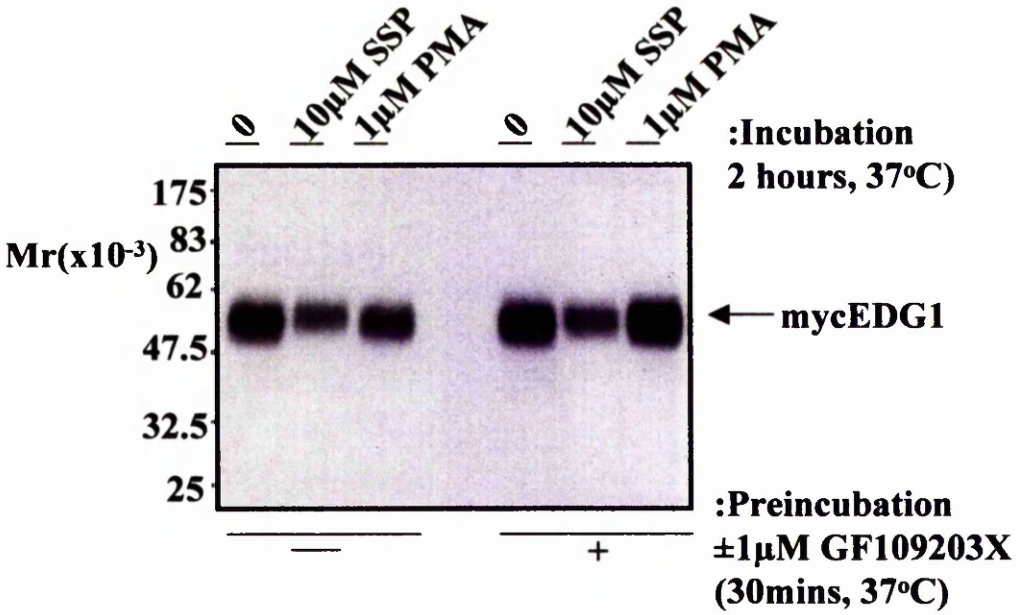

EDG1 Receptor
Expression
(Vehicle-treated Cells
Set At 100%)
125
100
75
50
25
0
Vehicle
S1P

Figure 5.5: Comparison Of The Effects Of SSP And PMA Treatment On Cell Surface EDG1 Receptor Expression

Serum-starved CCL-39/mycEDG1 cells were preincubated with vehicle or 1μM of the PKC inhibitor, GF109203X (GFX) for 30 min. The cells were then incubated for 2 hours with vehicle or in the presence of 10μM SSP or 1μM PMA. All drug treatments were carried out at 37°C. The cells were analysed for receptor internalisation by cell surface labelling with biotin-LC-hydrazide, followed by immunoprecipitation with the anti-myc 9E10 antibody. Biotin labelling of vehicle-treated CCL-39/MycEDG1 cells was set at 100% and the results following agonist treatment normalised accordingly. The data represents the mean ± SEM of three similar experiments. * Denotes a significant difference ($p<0.05$) *versus* vehicle-treated EDG1 cells. Whereas a 2 hour 10μM SSP incubation decreased cell surface EDG1 levels by 45±8%, a 1μM PMA exposure produced a significantly smaller reduction of 25±5% in cell surface expression. Preincubation with GF109203X abolished PMA-induced EDG1 cell surface loss but SSP-induced EDG1 cell surface loss was completely preserved.


0
10μM SSP
1μM PMA
0
10μM SSP
1μM PMA
:Incubation
2 hours, 37°C)
Mr(x10-3)
175
83
62
47.5
32.5
25
mycEDG1
:Preincubation
±1μM GF109203X
(30mins, 37°C)
—
+

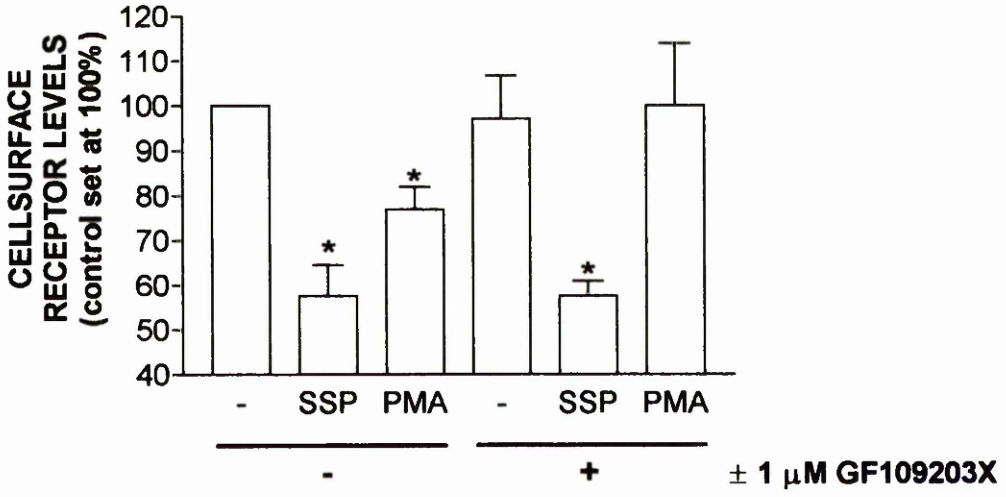
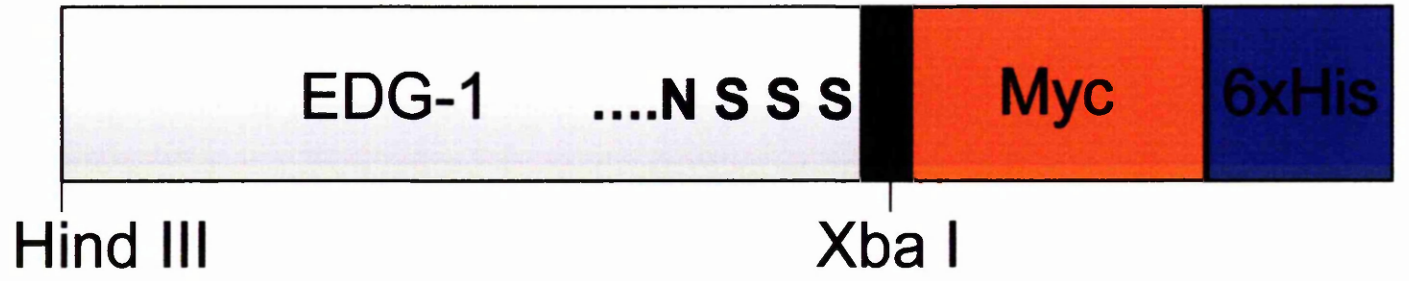

CELLSURFACE
RECEPTOR LEVELS
(control set at 100%)
120
110
100
90
80
70
60
50
40
-
SSP
PMA
-
SSP
PMA
-
+
± 1 μM GF109203X

Figure 5.6: Schematic Of The MycEDG1-GFP Receptors

The human mycEDG1 receptor was tagged with green fluorescent protein using a pcDNA/human mycEDG1 template. The mycEDG1 receptor was ligated into the multiple cloning site of pEGFP-N1 at *Hind*III/*Bam*HI. The addition of the GFP tag allowed visualisation of any movement of the cell surface EDG1 receptor following sustained agonist exposure.


EDG-1
....N S S S
Myc
6xHis
Hind III
Xba I

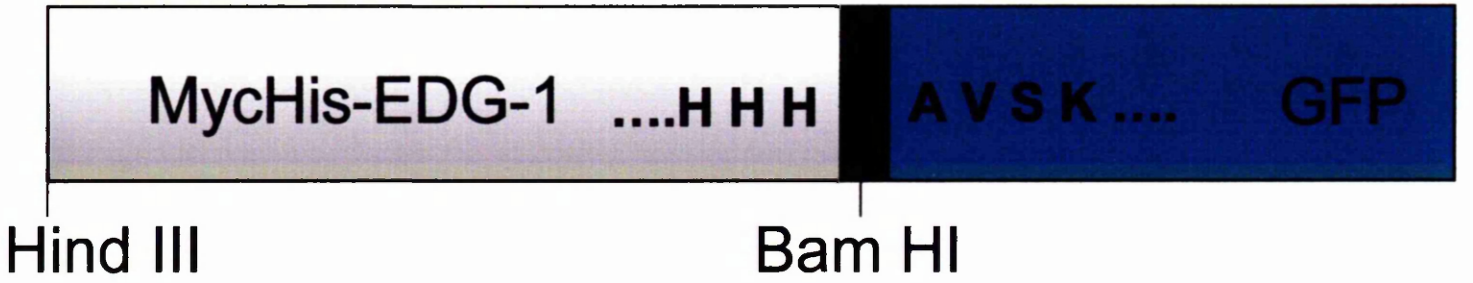

MycHis-EDG-1
....H H H
A V S K ....
GFP
Hind III
Bam HI

Figure 5.7: Immunoblot Analysis Of MycEDG1 And MycEDG1-GFP Receptor Expression

Cell extracts prepared from nontransfected CCL-39 fibroblasts or CCL-39 cells stably expressing either mycEDG1 or mycEDG1-GFP were solubilised, normalised for protein content and analysed by SDS-PAGE and immunoblotting with either an anti-myc monoclonal 9E10 antibody or a monoclonal anti-GFP antibody as indicated. mycEDG1 and mycEDG1-GFP bands are indicated.

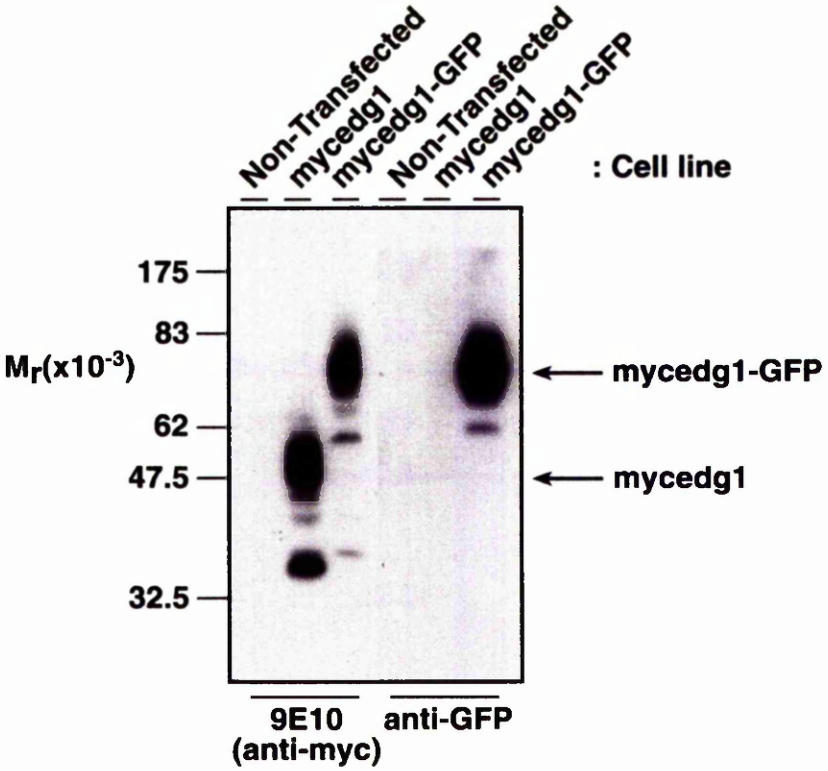

Non-Transfected
mycedg1
mycedg1-GFP
Non-Transfected
mycedg1
mycedg1-GFP
: Cell line
175
83
$M_r(x10^{-3})$
62
47.5
32.5
mycedg1-GFP
mycedg1
9E10
(anti-myc)
anti-GFP

Figure 5.8: Comparison Of The Effects Of SSP And PMA Exposure On MycEDG1 And MycEDG1-GFP Phosphorylation

^{32}P-labelled serum-starved stably transfected CCL-39/mycEDG1 and CCL-39/mycEDG1-GFP cells were incubated at 37°C for 10 min with either vehicle, 10μM SSP, or 1μM PMA. Receptor phosphorylation was analysed by immunoprecipitation followed by SDS-PAGE and phosphorimaging. The figure shows that both receptors are phosphorylated in the presence of either SSP or PMA, with SSP resulting in the strongest phosphorylation. Typical data is shown from one of three experiments.

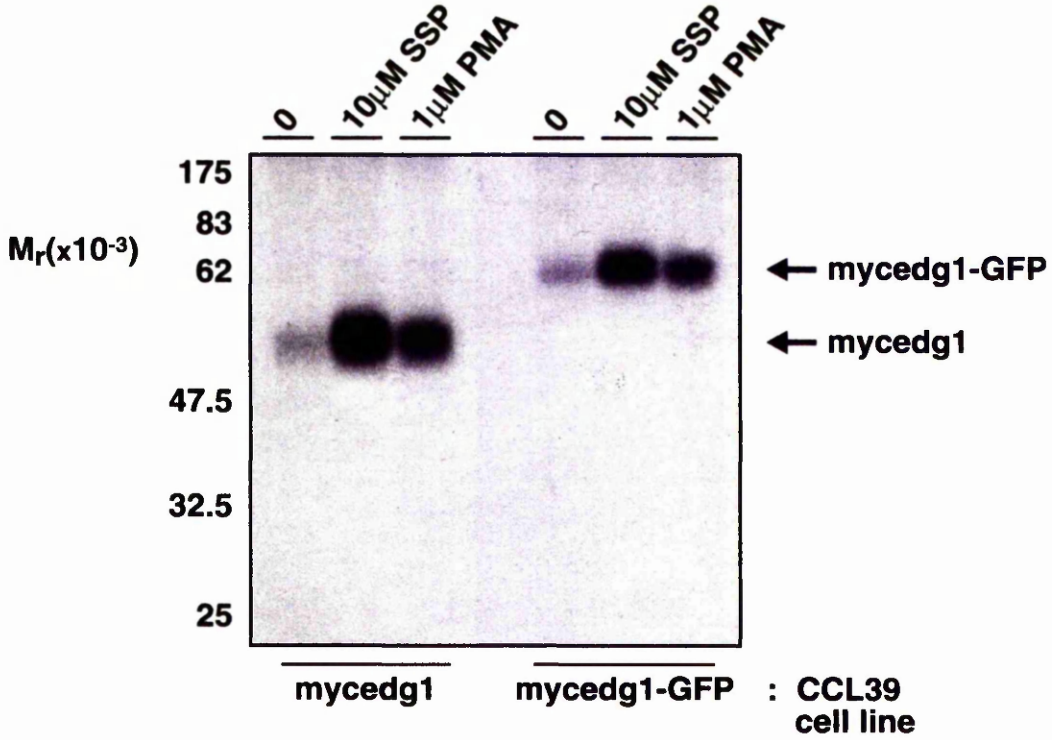

0
10μM SSP
1μM PMA
0
10μM SSP
1μM PMA
175
83
62
47.5
32.5
25
Mr(x10-3)
mycedg1-GFP
mycedg1
mycedg1
mycedg1-GFP
: CCL39 cell line

Figure 5.9: Fixed-Cell Analysis By Confocal Microscopy Of MycEDG1-GFP Cell Surface Expression Following Sustained S1P Treatment

Serum-starved CCL-39/mycEDG1-GFP cells were plated onto coverslips and exposed to either vehicle or 10μM R-PIA for the times indicated. The cells were then washed and fixed in paraformaldehyde prior to visualisation of receptor distribution by confocal microscopy. Under conditions of no agonist treatment, the mycEDG1-GFP construct was expressed on the cell surface. After only 15 min of S1P exposure, small, punctate spots of internalised receptor were detected within the cytoplasm. Following two hours of agonist exposure, a significant redistribution of cell surface mycEDG1-GFP receptor from the cell surface into internalised perinuclear pools within the cytoplasm was observed. This is a representative example of three experiments.

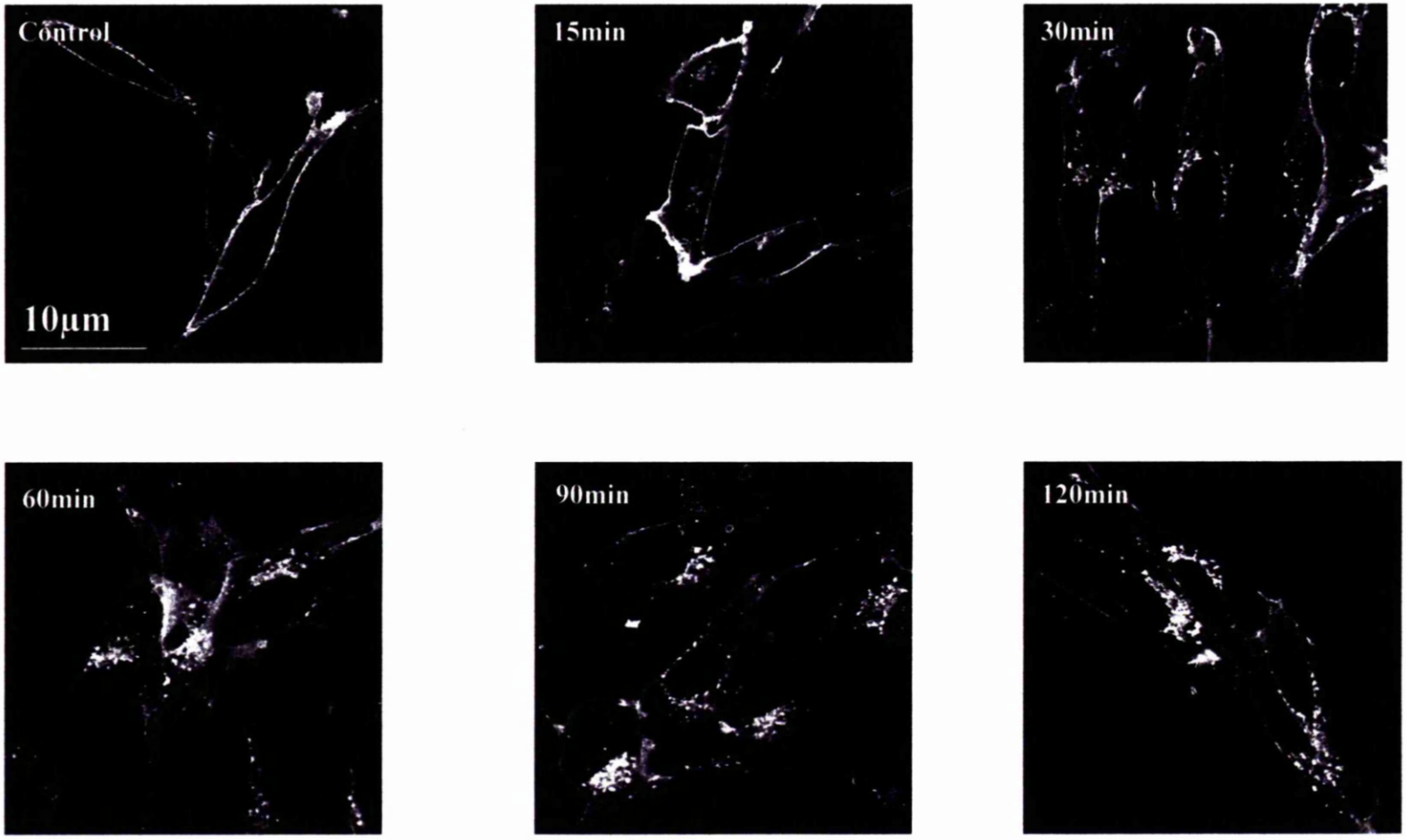

Control
10μm
15min
30min
60min
90min
120min

Figure 5.10: Fixed-Cell Analysis By Confocal Microscopy Of MycEDG1-GFP Cell Surface Expression Following S1P And PMA Exposure

Serum-starved CCL-39/mycEDG1-GFP cells were plated onto coverslips and exposed to vehicle, 10μM S1P or 1μM PMA for 2 hours at 37°C. The cells were then washed and fixed in paraformaldehyde prior to visualisation of receptor distribution by confocal microscopy. When treated with vehicle alone, the mycEDG1-GFP receptor was expressed primarily on the cell surface. A 2 hour exposure to 10μM S1P resulted in a significant redistribution of mycEDG1-GFP from the cell surface into punctate intracellular vesicles. In contrast, a 2 hour treatment with 1μM PMA produced only a small increase in the number of detectable intracellular receptors.

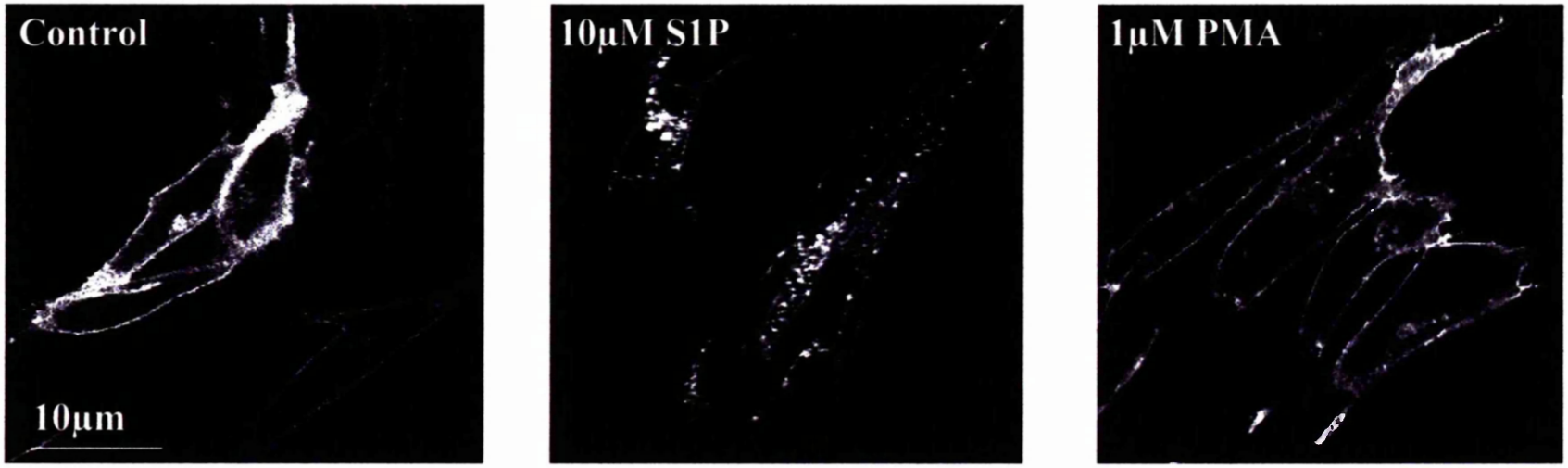

Control
10μm
10μM S1P
1μM PMA

Figure 5.11: Schematic Of The MycEDG1Δ51-GFP Receptor

The human mycEDG1Δ51 receptor was tagged with green fluorescent protein using a pcDNA/human mycEDG1Δ51 template. The mycEDG1Δ51 receptor was ligated into the multiple cloning site of pEGFP-N1 at *Hind*III/*Bam*HI. The addition of the GFP tag allowed visualisation of any movement of the cell surface mycEDG1Δ51-GFP receptor following sustained agonist exposure.

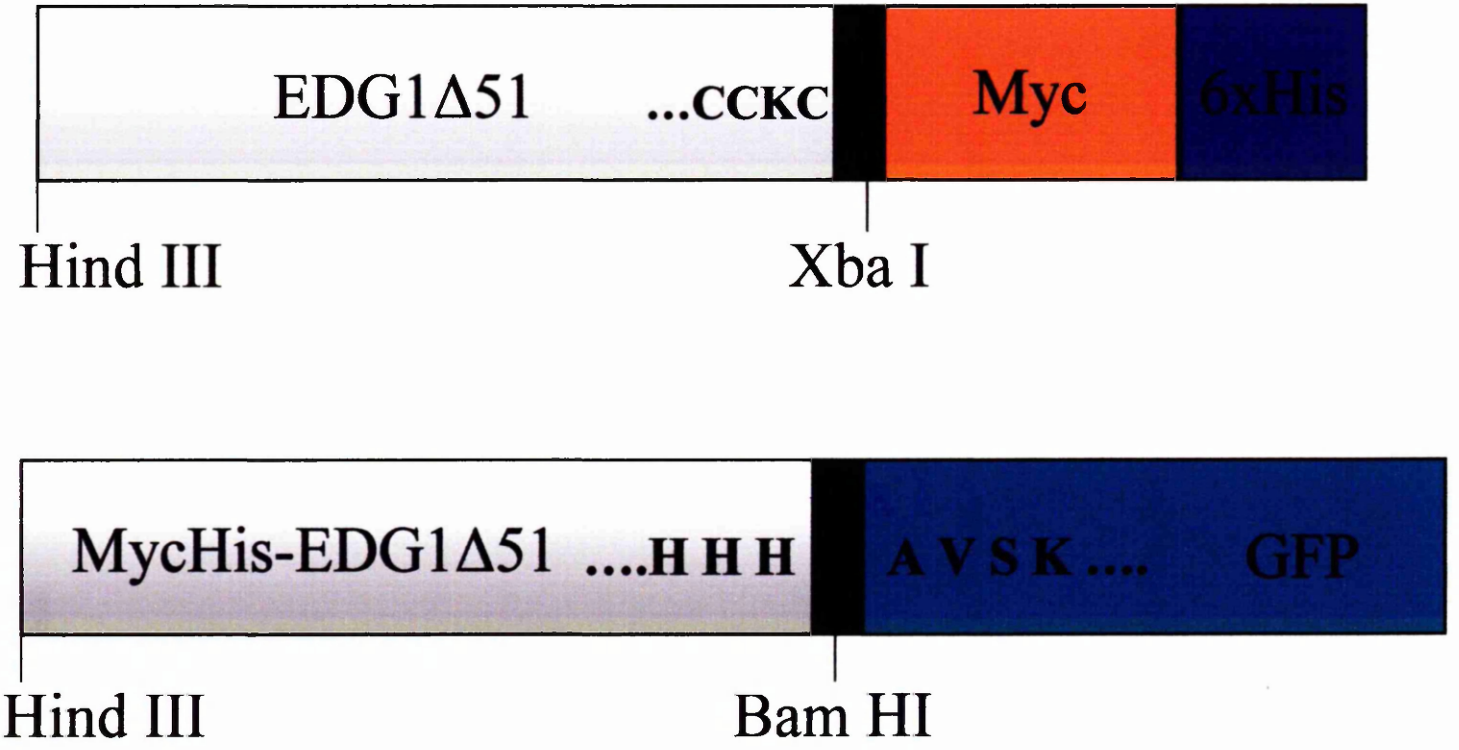

EDG1Δ51
...CCKC
Myc
6xHis
Hind III
Xba I
MycHis-EDG1Δ51
....H H H
A V S K ....
GFP
Hind III
Bam HI

Figure 5.12: Immunoblot Analysis Of The MycEDG1, MycEDG1Δ51 And MycEDG1Δ51-GFP Receptor

CCL-39 cells stably expressing either the mycEDG1-GFP, the mycEDG1Δ51 or the mycEDG1Δ51GFP receptor were solubilised, normalised for protein content and analysed by SDS-PAGE and immunoblotting with the anti-myc monoclonal 9E10 antibody.

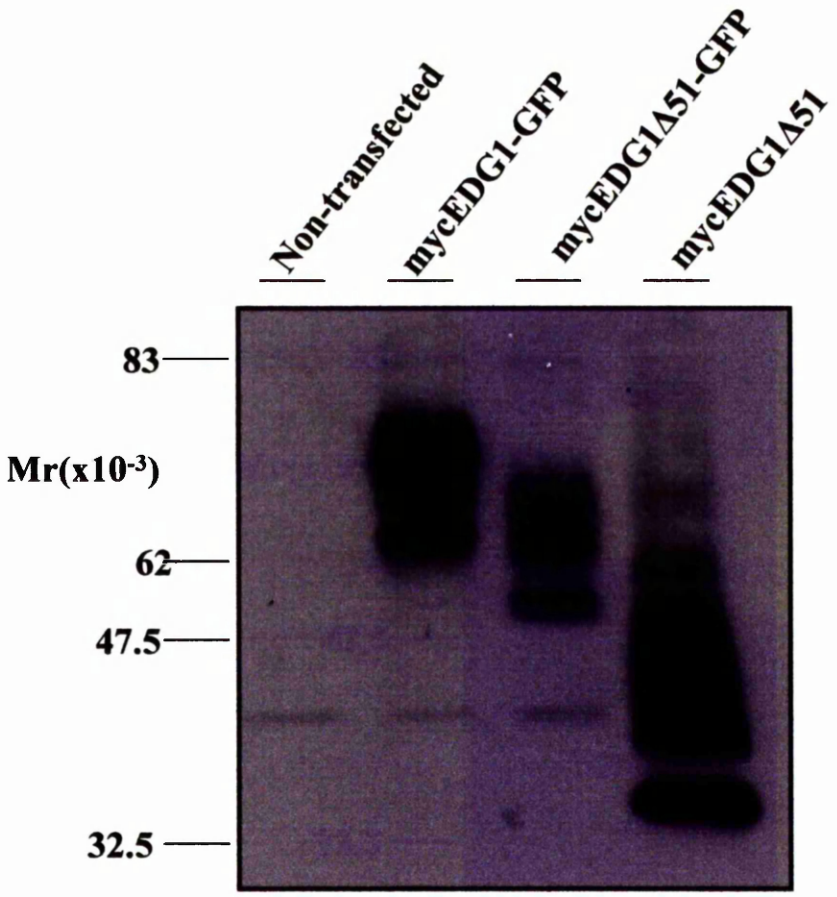

Non-transfected
mycEDG1-GFP
mycEDG1Δ51-GFP
mycEDG1Δ51
83
Mr(x10-3)
62
47.5
32.5

Figure 5.13: Fixed Cell Analysis By Confocal Microscopy Of MycEDG1Δ51-GFP Cell Surface Expression Following S1P Exposure

Serum-starved CCL-39/mycEDG1-GFP or CCL-39/mycEDG1Δ51-GFP cells were plated onto coverslips and exposed to either vehicle or 10μM S1P at 37°C for the times indicated. The cells were then washed and fixed in paraformaldehyde prior to visualisation of receptor distribution by confocal microscopy. A 2 hour exposure to 10μM S1P resulted in a redistribution of mycEDG1-GFP from the cell surface into punctate intracellular vesicles. In contrast, no significant trafficking of the mycEDG1Δ51-GFP receptor was observed throughout a 2 hour time-course in the presence of 10μM S1P.

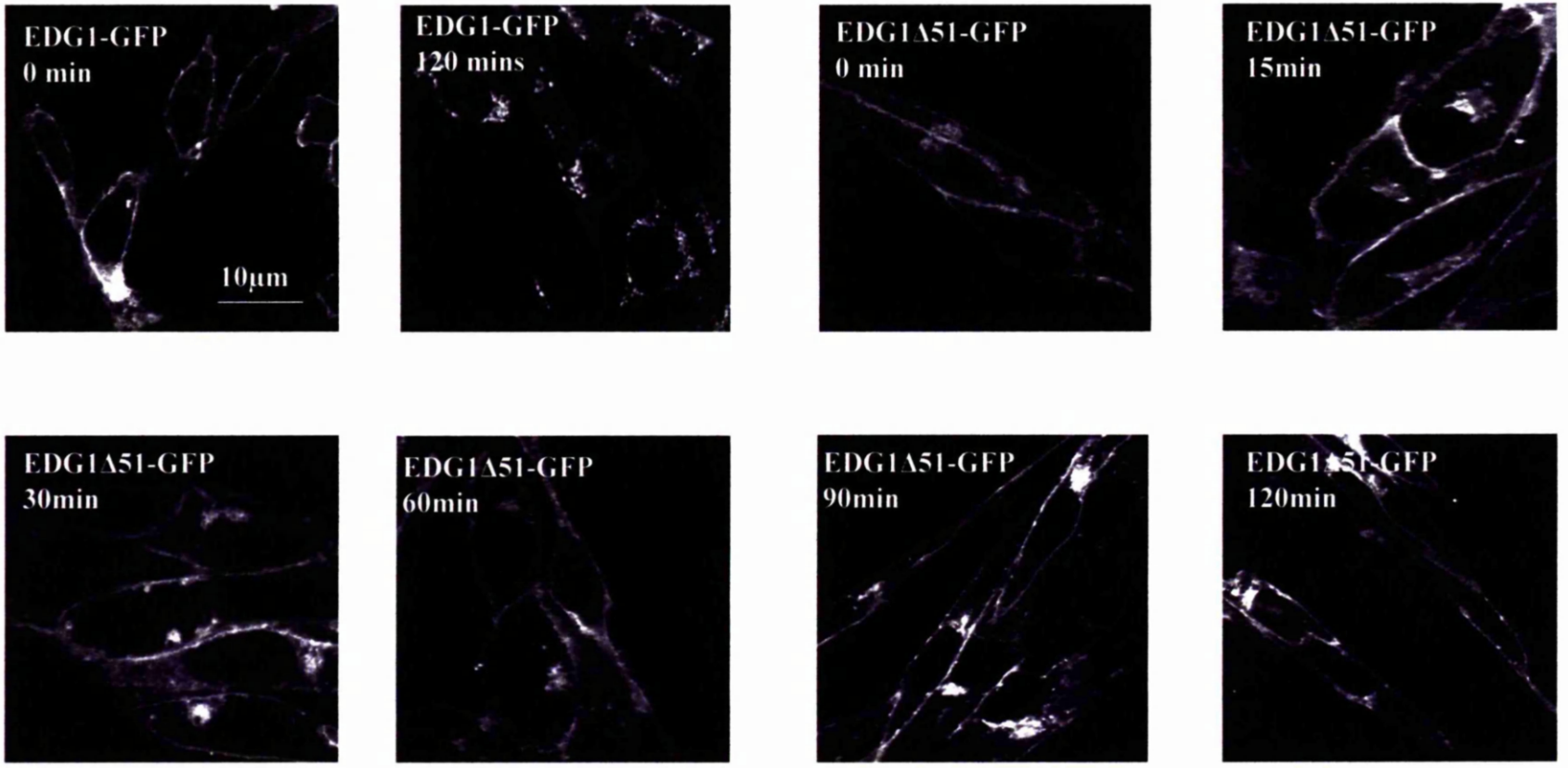

EDG1-GFP
0 min
10μm
EDG1-GFP
120 mins
EDG1Δ51-GFP
0 min
EDG1Δ51-GFP
15min
EDG1Δ51-GFP
30min
EDG1Δ51-GFP
60min
EDG1Δ51-GFP
90min
EDG1Δ51-GFP
120min

Figure 5.14: Identification Of The MycEDG1, MycEDG1Δ32 And MycEDG1Δ12 Receptors By Immunofluorescence

HEK293 cells transiently expressing either the mycEDG1, mycEDG1Δ32 or the mycEDG1Δ12 receptor were grown on coverslips, washed and then fixed using paraformaldehyde. After permeabilisation, the cells were incubated with the 9E10 anti-myc antibody followed by an anti-mouse Alexa-red 594 conjugated IgG. Receptor expression was then visualised by confocal microscopy. The figure shows that truncation of the last 12 and the last 32 amino acids had no effect on EDG1 cell surface expression.

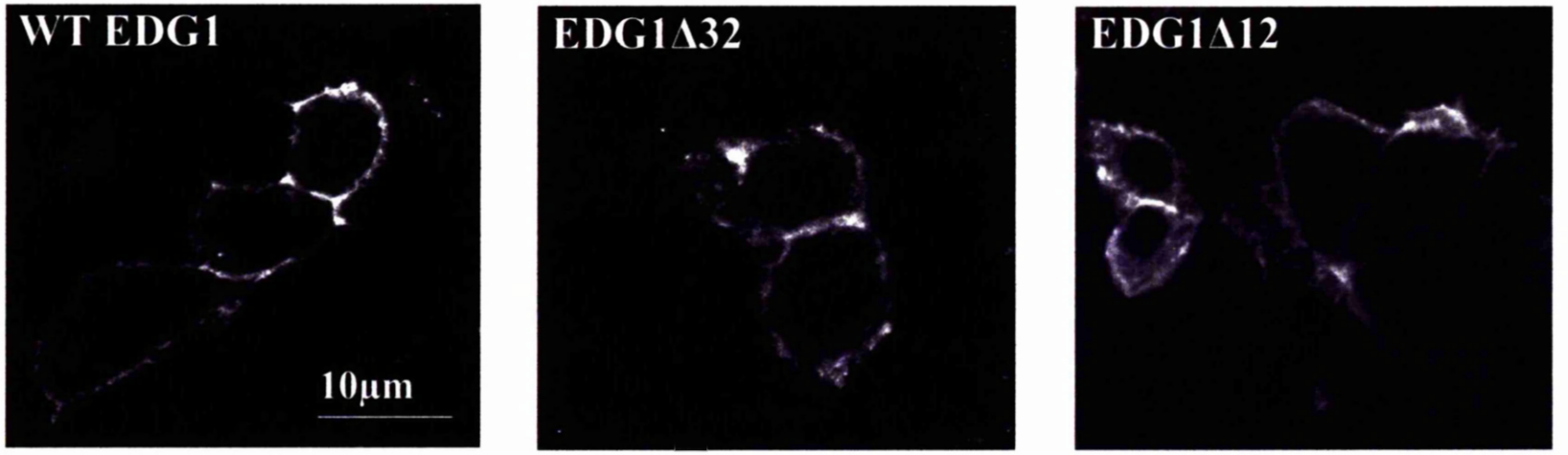
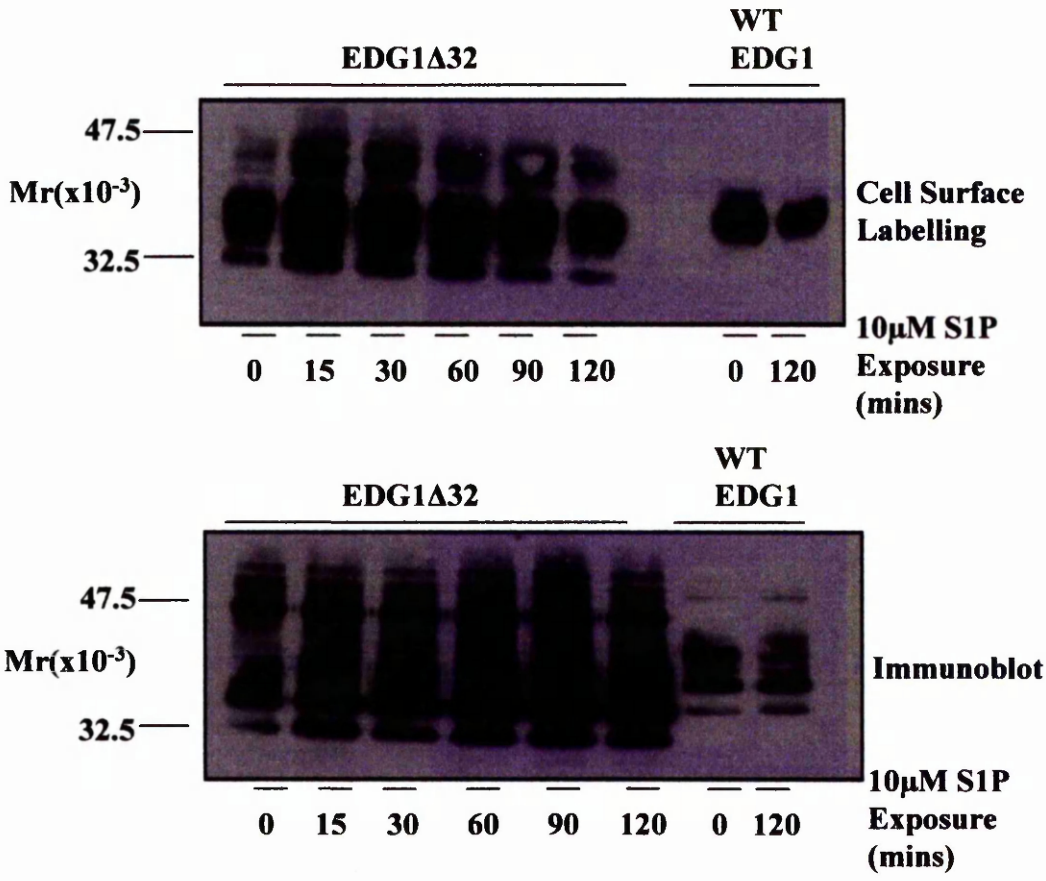

WT EDG1
10μm
EDG1Δ32
EDG1Δ12

Figure 5.15: Effect Of S1P Exposure On The Cell Surface Expression Of The MycEDG1Δ32 Receptor

Serum-starved HEK293 cells transiently expressing either mycEDG1 or mycEDG1Δ32 receptor were treated with vehicle or 10μM S1P at 37°C for the times indicated. The cells were examined for internalisation by cell surface labelling with biotin-LC-hydrazide, solubilised and then immunoprecipitated with the anti-myc 9E10 antibody. Biotin labelling of vehicle-treated controls was set at 100% and the results for each receptor following agonist treatment expressed as a ratio of the control. The data represents the mean ± SEM of three similar experiments. Cell surface biotinylation of mycEDG1 and mycEDG1Δ32, as shown in panel (B), were normalised for total receptor expression using an immunoblot analysis of the immunoprecipitated samples, as shown in Panel (A). * Indicates a significant decrease ($p<0.05$) *versus* level of internalisation as compared with vehicle-treated controls.


EDG1Δ32
WT
EDG1
47.5
Mr(x10-3)
32.5
Cell Surface
Labelling
10μM S1P
Exposure
(mins)
0 15 30 60 90 120 0 120
EDG1Δ32
WT
EDG1
47.5
Mr(x10-3)
32.5
Immunoblot
10μM S1P
Exposure
(mins)
0 15 30 60 90 120 0 120

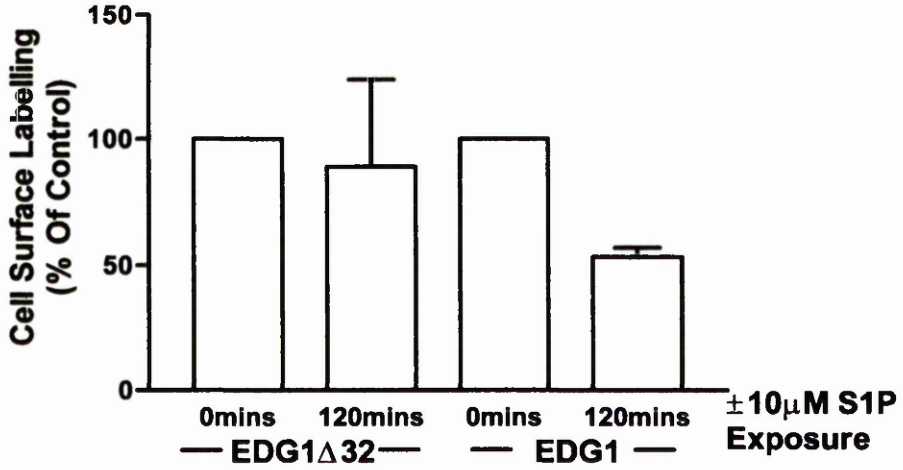
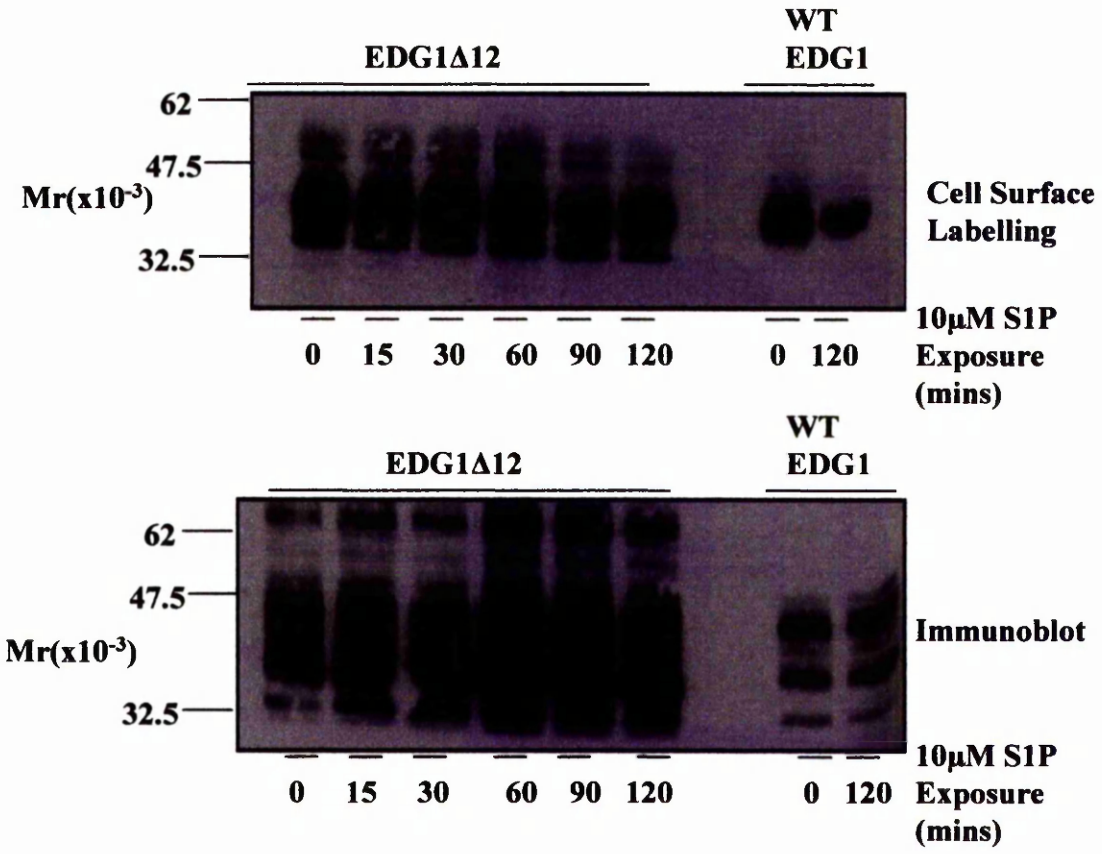
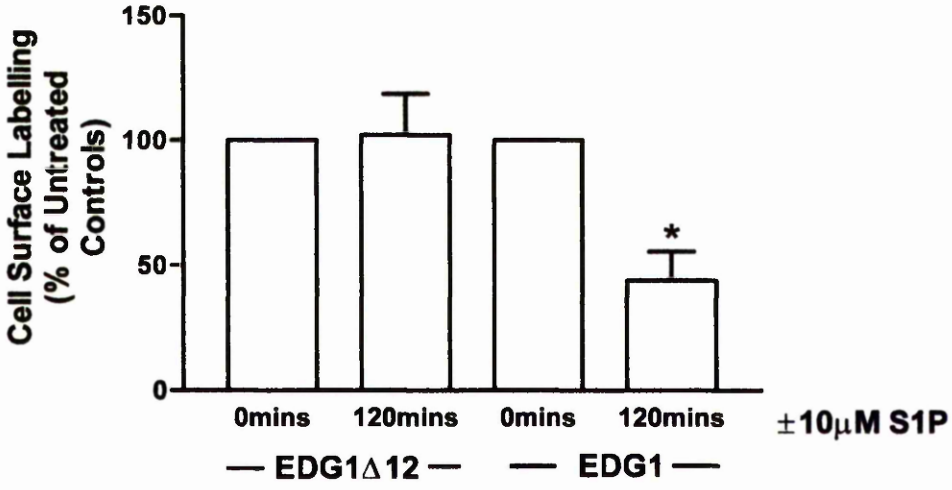

Cell Surface Labelling
(% Of Control)
150
100
50
0
0mins
120mins
0mins
120mins
±10μM S1P
Exposure
EDG1Δ32
EDG1

Figure 5.16: Effect Of S1P Exposure On The Cell Surface Expression Of The MycEDG1Δ12 Receptor

Serum-starved HEK293 cells transiently expressing either the mycEDG1 or the mycEDG1Δ12 receptor were treated with vehicle or 10μM S1P at 37°C for the times indicated. The cells were solubilised and examined for internalisation by cell surface labelling with biotin-LC-hydrazide, followed by immunoprecipitation with the anti-myc 9E10 antibody. Biotin labelling of vehicle-treated controls was set at 100% and the results for each receptor following agonist treatment normalised accordingly. The data represents the mean ± SEM of three similar experiments. Cell surface biotinylation of mycEDG1 and mycEDG1Δ12, as shown in panel (B), were normalised for total receptor expression using an immunoblot analysis of the immunoprecipitated samples, as shown in Panel (A). * Indicates a significant decrease ($p<0.05$) *versus* level of internalisation as compared with vehicle-treated controls.


WT
EDG1
EDG1Δ12
62
47.5
Mr(x10-3)
32.5
Cell Surface
Labelling
10μM S1P
0 15 30 60 90 120 0 120
Exposure
(mins)
WT
EDG1
EDG1Δ12
62
47.5
Mr(x10-3)
32.5
Immunoblot
10μM S1P
0 15 30 60 90 120 0 120
Exposure
(mins)


Cell Surface Labelling
(% of Untreated
Controls)
150
100
50
0
*
0mins
120mins
0mins
120mins
±10μM S1P
— EDG1Δ12 —
— EDG1 —

Chapter 6

Final Discussion

Receptor phosphorylation and receptor internalisation represent two important regulatory processes in the signalling of many GPCRs. As described previously, one of the most well-characterised models of GPCR phosphorylation and internalisation is the β_2AR (Jockers *et al.*, 1996; Luttrell *et al.*, 1999; McLean *et al.*, 1999; Menard *et al.*, 1997; Moffett *et al.*, 1993). Using this model as a starting point, this project has characterised the regulation of two GPCRs: the human A_1 adenosine receptor and the human S1P receptor EDG1. Additionally, the A_1AR has been compared with the rat A_3AR, an AR subtype similar in structure and biological effects containing a number of potential serine and threonine phosphorylation sites not present in the A_1AR. The A_1AR, A_3AR and EDG1 receptor each display distinct differences in terms of their rate and their extent of phosphorylation and internalisation. In fact, these receptors highlight the diverse pathways of phosphorylation and internalisation that exist between members of the GPCR family and demonstrate that all GPCRs are regulated uniquely in a manner that is not identical to the β_2AR. This study therefore emphasises the need to characterise the regulation of each GPCR individually. However, each example also illustrates the importance of receptor phosphorylation in the regulation of receptor internalisation

The link between receptor phosphorylation and internalisation has been established for a number of receptors and is generally attributed to a synergistic relationship between GRK-mediated receptor phosphorylation and the binding of endocytic adaptor proteins such as β-arrestin (Ferguson, 2001). For example, GRK2-mediated phosphorylation is known to facilitate the internalisation of the m2 muscarinic receptor (Moro *et al.*, 1993), angiotensin AT_{1A}R (Smith *et al.*, 1998), endothelin A receptor (Bremmes *et al*, 2000), D2 dopamine receptor (Itokawa *et al.*, 1996), and the follitropin receptor (Lazari *et al.*, 1999). Studies using a β_2AR-Y326A mutant, which was internalisation-defective and did not serve as a substrate for GRK-mediated phosphorylation, also demonstrated that overexpression of GRK2 not only promoted internalisation but also re-established GRK-mediated phosphorylation of the mutant receptor (Tsuga *et al.*, 1994). However, this model does not apply to all GPCRs. Early studies using β_2AR mutants lacking sites for both second-messenger-dependent protein kinase- and GRK-mediated phosphorylation showed no significant differences in the internalisation of the wild-type and mutant β_2ARs (Bouvier *et al.*, 1988; Hausdorff *et al.*, 1989). Also, m2 muscarinic receptor internalisation is GRK-dependent yet appears to be β-arrestin-independent in some cell types (Tsuga *et al.*, 1994; Schlador and Nathanson, 1997; Vogler *et al.*, 1999; Werbonat *et al.*, 2000). Additionally, in COS7 cells, which express relatively little GRK and β-arrestin protein, the

maximal extent of $AT_{1A}R$ internalisation is virtually indistinguishable from that observed in HEK293 cells whereas the maximal extent of β_2AR internalisation is markedly lower in COS7 cells (Zhang *et al.*, 1996; Menard *et al.*, 1997).

However, the role of phosphorylation in GPCR internalisation is reinforced by the differential rates of internalisation of the phosphorylation-deficient A_1AR and the rapidly-phosphorylated A_3AR. In Chapter 3, it was shown that A_1ARs expressed in CHOs were not phosphorylated in response to the agonist, R-PIA. In contrast, the A_3AR, which is similar in terms of structure, G-protein coupling specificity and biological effects, was rapidly phosphorylated following R-PIA exposure. This agreed with a previous study which demonstrated that a chimeric A_1-A_3 adenosine receptor in which the C-terminal domain of the A_1AR distal to the predicted site of palmitoylation was replaced by the corresponding region of the A_3AR was able to undergo rapid functional desensitisation and agonist-stimulated phosphorylation (Palmer *et al.*, 1996). The differential patterns of A_1AR and A_3AR phosphorylation were reflected in the internalisation rates of both receptors. Whereas the A_3AR is internalised rapidly following R-PIA exposure ($t_{1/2}$=10 min), the agonist-dependent loss of A_1AR from the cell surface was much slower ($t_{1/2}$=90min) and was also smaller in magnitude. Interestingly, the type I GnRH-receptors (GnRH-Rs) lack C-terminal tails and do not undergo agonist-induced phosphorylation and are resistant to receptor desensitisation and internalise slowly (McArdle *et al.*, 2002a; McArdle *et al.*, 2002b). In contrast, the type II GnRH-Rs posses C-terminal tails and show rapid desensitisation and internalisation with comcomitant receptor phosphorylation (McArdle *et al.*, 2002a; McArdle *et al.*, 2002b).

Palmitoylation of GPCRs has diverse effects on GPCR regulation and signalling. For example, as discussed in Chapter 3, mutation of Cys341 within the β_2AR C-terminal tail increases the accessibility of a PKA phosphorylation site upon agonist stimulation and therefore increases receptor phosphorylation (Moffet *et al.*, 1993; Mouillac *et al.*, 1992). In contrast, the disruption of α_{2A}-adrenergic receptor palmitoylation was shown to have no effect on receptor phosphorylation but instead abolished receptor down-regulation (Eason *et al.*, 1994). Additionally, non-palmitoylated $5\text{-}HT_{4(a)}$ receptors were indistinguishable from the wild type in their ability to interact with Gs, to stimulate adenyly cyclase activity and to activate cyclic nucleotide-sensitive cation channels following agonist stimulation (Ponimaskin *et al.*, 2002). The mutation of Cys309, a site of palmitoylation within the A_1AR C-terminal tail, had no visible agonist-dependent effect on the rate of translocation

of the A_1AR from the cell surface during a 1 hour treatment with 10μM R-PIA. In contrast, parallel studies within the lab have demonstrated that mutation of Cys302 and Cys305 within the C-terminal of the A_3AR resulted in a marked increase in basal receptor phosphorylation and an increased rate of internalisation. This difference presumably reflects the ability of the WTA_3AR to be phosphorylated by GRKs in comparison to the phosphorylation-resistant A_1AR. However, longer time-course experiments should be carried out using the Cys309 mutant of the A_1AR to examine the potential effect in relation to the $t_{1/2}$ of wild type receptor internalisation ($t_{1/2}$= 90 mins).

As discussed in Chapter 3, previous studies have suggested that the A_1AR could internalise *via* a unique molecular mechanism involving the clathrin/dynamin endocytic machinery or through a clathrin-independent mechanism (Ciruela *et al.*, 1997; Gines *et al.*, 2001; Saura *et al.*, 1998; Nie *et al.*, 1997). In LLC-PK_1 cells, it was suggested that A_1AR internalisation occurs following their translocation to rafts enriched in caveolin and that the putative caveolin binding motif within the C-terminal of the A_1AR (**YAFRIHKF**) is involved (Gines *et al.*, 2001). In contrast, a separate study showed that 67±5% of adenosine A_1 receptors were isolated with caveolae from unstimulated rat cardiac ventricular myocytes and, following incubation with the A_1AR agonist, CCPA, there was rapid translocation of the A_1 receptors from caveolae into non-caveolar plasma membrane compartments (Lasley *et al.*, 2000). Alternatively, the A_1AR may move into distinct plasma membrane compartments similar to those described for the CCK receptor. The CCK receptor has been shown to translocate to a basolateral plasmalemmal compartment similar to caveolin in CHO and pancreatic acinar cells yet does not translocate deeper into the cell (Roettger *et al.*, 1995; Rosenzweig *et al.*, 1983). As mentioned previously, the translocation of the CCK receptor to these membranous compartments is thought to result in a rapid desensitisation of the receptor (Roettger *et al.*, 1995). Therefore, the translocation of A_1ARs to compartments within the membrane may result in a similar physiological effect. Interestingly, a radioligand binding assay to measure receptor down-regulation demonstrated that time-courses of the loss of cell surface expression and the loss of total receptor expression were similar ($t_{1/2}$=60 mins). Therefore, the loss of cell surface A_1AR may be attributable to a down-regulation of the receptor. One important further line of investigation would be to determine the exact trafficking characteristics of the A_1AR using a CHO cell line stably expressing the A_1AR-GFP construct. This would allow fixed cell confocal analysis to be used to determine the precise movements of the A_1AR

following a long-term agonist treatment and permit visualisation of the changes in A_1AR expression determined here biochemically by biotin-labelling and radioligand binding assays.

It remains unclear why it is physiologically beneficial to have two subtypes of receptor that are so similar in terms of structure, ligand and biological effects but which exhibit markedly different regulatory mechanisms of phosphorylation and internalisation. As described in the Introduction, both the A_1AR and the A_3AR are involved in the process of ischaemic preconditioning (Carr *et al.*, 1997; Ferninandy *et al.*, 1998; Heurteaux, 1995; Liang and Jacobson, 1998; Rubino and Yellon, 2000; Sumeray and Yellon, 1997; Thourani *et al.*, 1999). Ischaemic preconditioning is defined as a cardio- and neuroprotective, acute adaptation to brief periods of ischaemia. This is where a brief period of sublethal ischaemia, consisting of either a single five minute period or a cycle of two or more 5 minute periods, is then followed by reflow, rendering the heart resistant to infarction from a subsequent, more sustained period of ischaemia (Rubino and Yellon, 2000; Sumeray and Yellon, 1997). As a result, there is a reduction in arrythmias and cardiac cell death from the prolonged, potentially injurious period of ischaemia. The process is bi-phasic. The first phase is termed classical preconditioning and occurs within a few minutes of the initial preconditioning ischaemia. If the period of time between preconditioning ischaemia is extended beyond 120 minutes, no protection is observed. Delayed preconditioning, also referred to as the second window of protection, is where the preconditioning effect is observed 24 hours after the initial period of ischaemia and is thought to be associated with the induction of cytoprotective proteins, such as heat shock protein and endogenous anti-oxidants (Rubino and Yellon, 2000; Sumeray and Yellon, 1997). However, it is not known how the regulation of the A_1AR and the A_3AR is reflected in their mediation of ischaemic preconditioning. For example, how does the slow, incomplete loss of A_1AR from the cell surface influence the sustained protection observed with ischaemic preconditioning? Therefore, future research could involve cardiac myocytes infected with an adenoviral construct of either the A_1AR, A_3AR or the chimeric A_1/A_3AR described above in order to ensure efficient receptor overexpression since primary cells are difficult to transfect. This could then be used to study how the different regulatory patterns observed between the A_1AR and the A_3AR influence the process of ischaemic preconditioning. For example, how does the regulation of each receptor affect the activation of K^+_{ATP} channels? Additionally, previous studies have demonstrated that a significant proportion of the cardioprotective effect elicited by the A_1AR is due to the inhibition of the β_2AR-dependent

increase in cAMP activity (Auchampach and Bolli, 1999; McIntyre Jr. *et al.*, 1994; Perlini *et al.*, 1998; Song and Belardinelli, 1996). Hence, it would be beneficial to examine potential cross-talk mechanisms between the A_1AR and the β_2AR and also to determine whether the regulation of each receptor has any bearing on the signalling and the regulation of the other.

EDG1 is activated by the bioactive phospholipid S1P and is heavily involved in the process of angiogenesis (Hla *et al.*, 2001, Pyne and Pyne, 2000a, Pyne and Pyne, 2000b, Spiegel and Milstien, 2000). Chapter 4 demonstrated that EDG1 is phosphorylated in response to agonist (S1P) and also in response to PMA, a phorbol ester that activates PKC subtypes and that these processes are mechanistically distinct. Additionally, incubation of PMA and S1P together did not result in a significant increase in EDG1 phosphorylation when compared to that achieved by S1P treatment alone. This suggested that although PMA- and S1P- mediated EDG1 phosphorylation were distinct, each pathway utilised overlapping phosphorylation sites within EDG1. Interestingly, the removal of 12 amino acids from the C-terminal tail of EDG1 significantly reduced S1P- but not PMA-stimulated phosphorylation. Hence, S1P treatment induces the phosphorylation of sites within the last 12 amino acids of the EDG1 C-terminal tail that are not phosphorylated following PMA exposure. This implies that each mechanism of phosphorylation is distinct. Future work might employ the use of 2D gels to examine the patterns of EDG1 phosphorylation following S1P and PMA exposure. If the patterns observed with each activator are different, then this further suggests that each mechanism of EDG1 phosphorylation is distinct. Site-directed mutagenesis studies within the last 32 amino acids of the C-terminal tail should also determine the exact the sites of EDG1 phosphorylation following S1P and PMA treatment. In addition, the removal of 12 amino acids from C-terminal tail of EDG1 significantly reduced S1P- but not PMA-stimulated phosphorylation. *In vitro* assays implicated a role for GRK2 in S1P-induced EDG1 phosphorylation observed in intact cells. Also, the region between the last 12 and the last 32 amino acids was shown to be responsible for the *in vitro* S1P-dependent phosphorylation of EDG1 in the presence of GRK2. Again, the precise sites of GRK2 phosphorylation *in vitro* could be examined using site-directed mutagenesis of the EDG1 C-terminus.

The patterns of EDG1 phosphorylation established in Chapter 4 were related to loss of EDG1 cell surface receptor studies presented in Chapter 5. Two separate mechanisms of EDG1 internalisation were observed: an S1P-dependent loss of cell surface EDG1 receptor and a less complete but still significant agonist –independent loss of cell

surface EDG1 mediated by PMA. Confocal analysis demonstrated that, whereas S1P induces the trafficking of EDG1 from the cell surface into perinuclear pits, PMA exposure did not visibly affect receptor trafficking. As discussed in Chapter 5, PMA-induced EDG1 internalisation may involve the trafficking of EDG1 into membranous compartments, similar to those described for the CCK receptor (Roettger *et al.*, 1995; Rosenzweig *et al.*, 1983). Whether the PMA-induced internalisation of EDG1 is physiologically significant, given the relatively small effect observed in Chapter 5, should be addressed in future studies.

The removal of the 12 residues from the carboxyl terminal of EDG1 completely inhibited S1P-induced EDG1 internalisation, implicating this domain in the control of EDG1 internalisation while retaining sensitivity to S1P phosphorylation. Interestingly, this region is not required for the *in vitro* phosphorylation of EDG1 by GRK2 in the presence of S1P. Hence, this suggests that another kinase may be responsible for EDG1 internalisation. Interestingly, the muscarinic m2 receptor is phosphorylated by GRK2 and β-arrestins subsequently desensitise the receptor. However, the m_2 receptor internalises primarily through β-arrestin-independent mechanisms (Pais-Rylaarsdam *et al.*, 1997; Pierce and Lefkowitz, (2001). Future work should therefore be aimed at examining the role of other aciditrophic kinases, such as casein kinase-1α. Alternatively, the sites of GRK2-dependent phosphorylation of EDG1 *in vitro* may not be the sites phosphorylated *in vivo*. For example, the mutation of the primary GRK phosphorylated residues on the β_2AR did not prevent the GRK-mediated β_2AR desensitisation in cells (Siebold *et al.*, 1998). Therefore, a role for GRK2 in the internalisation of EDG1 *in vivo* cannot be completely ruled out. However, the possibility that EDG1 may internalise *via* a mechanism that is independent of GRK phosphorylation again highlights the need to examine the regulation of a given GPCR independently. Additionally, if GRK2 is found to phosphorylate EDG1 *in vivo* then the physiological role of GRK2-dependent EDG1 phosphorylation must be addressed. Elucidation of the exact sites of EDG1 phosphorylation stimulated by S1P and PMA through site-directed mutations and phosphopeptide mapping of EDG1 should address these issues.

Chapter 5 also demonstrated that a 30 min agonist-dependent internalisation of the EDG1 receptor was irreversible following 2 hours of agonist removal. Additionally, no significant decrease in total EDG1 receptor expression was observed following a 24 hour agonist exposure, suggesting that the receptor is not down-regulated. Chapter 4 also

demonstrated that EDG1 phosphorylation was reversible following a 2 hour agonist removal. Together, this would suggest that the EDG1 receptor is phosphorylated following agonist exposure. The receptor is then internalised where it is then dephosphorylated but not degraded. Alternatively, EDG1 may be slowly recycled back to the cell surface, requiring a period of agonist removal longer than 2 hours. Although GPCRs are generally rapidly recycled back to the cell surface or targeted for degradation following internalisation, some GPCRs are retained within the cell. For example, both the angiotensin AT_{1A} and vasopressin V2 receptor are retained within endocytic vesicles, resulting in a prolonged state of functional desensitisation (Zhang *et al.*, 1999; Anborough *et al.*, 2000; Innamorati *et al.*, 1998; Oakley *et al.*, 1999). Future studies using confocal microscopy should therefore determine the exact trafficking pathway of the internalised EDG1 receptor. Future work should also investigate why the EDG1 receptor is dephosphorylated and then remains within the cell. As mentioned previously, S1P has been shown to be an intracellular messenger yet the precise targets for EDG1 within the cell remain unknown (Spiegel and Milstien, 2000). Therefore, does the EDG1 receptor elicit S1P-dependent biological responses within the cell? The elicitation of signalling responses by internalised receptors has been shown previously. For example, it has been proposed that internalised β_2ARs activate MAP kinase signalling via a Src-associated endocytic signalling complex containing the activated receptor (Luttrell *et al.*, 1999). Additionally, the protease-activated receptor, PAR2 and the neurokinin NK_1 receptor have also been shown to activate MAPK following their translocation into endocytic vesicles (DeFea *et al.*, 2000a; DeFea *et al.*, 2000b; Luttrell *et al.*, 2001).

The demonstration of agonist-independent processes of phosphorylation and internalisation, mediated by PKC activation, suggest the existence of potentially important physiological cross-talk mechanisms between the EDG1 receptor and other receptors. For example, recent studies have revealed an important role for EDG1 in a cross-talk mechanism with the PDGF receptor (Hobson *et al.*, 2001; Rosenfeldt *et al.*, 2001). It has been shown that activation of the PDGF receptor can, in turn, increase S1P production through the activation of sphingosine kinase. This results in the activation of EDG1 and the subsequent activation of Rac-dependent cell migration pathways. Consequently, cells migrate towards PDGF, thus enhancing PDGF receptor signalling in cell maturation (Hobson *et al.*, 2001; Rosenfeldt *et al.*, 2001). Therefore, the discovery of agonist-independent pathways of EDG1 phosphorylation and internalisation has important implications in the role of the EDG1 receptor in cross-talk mechanisms with other

receptors, such as the PDGF receptor. For example, can PDGF receptor activation directly phosphorylate EDG1 by the activation of PKC to enhance EDG1 signalling as well as indirectly *via* an increase in S1P production? Also, the effect on PDGF receptor signalling of a phosphorylation and/or internalisation-deficient mutant of EDG1 should be examined. Additionally, future research should be aimed at examining the potential effects of EDG1 signalling in terms of other angiogenic receptors such as the angiopoietin receptors, Tie1 and Tie2, and other receptor tyrosine kinase receptors, such as the VEGF receptors, Flt and Flk. Future experiments should also relate the phosphorylation and internalisation patterns of EDG1 established in Chapters 4 and 5 to a more physiologically relevant environment. This could be achieved by generation of adenoviral constructs of the WT EDG1, EDG1Δ12 and EDG1Δ32 receptors. These could be used to infect human umbilical vein endothelial cells as a means of overexpressing these receptors in endothelial cells. A viral construct of the EDG1Δ32 receptor represents both a phosphorylation- and an internalisation-deficient mutant of the EDG1 receptor whereas an EDG1Δ12 viral construct would represent an internalisation-deficient mutant that can still be significantly phosphorylated by agonist. The effect of overexpressing these receptors on angiogenesis could then be examined using a matrigel system. Additionally, the possible generation through site-directed mutagenesis of an EDG1 mutant that differentiates between PMA, but not S1P induced EDG1 phosphorylation could be used to elucidate any potential physiological consequences of each phosphorylation pathway.

The regulation of small G-proteins, such as Rac and Rho, represents an integral part of EDG1 signalling (Lee *et al.*, 2001; Paik *et al.*, 2001; Hobson *et al.*, 2001). For instance, the activation of Rac has been shown to mediate EDG1-dependent cell migration pathways (Lee *et al.*, 2001; Hobson *et al.*, 2001). In addition, EDG1, along with EDG3, has been shown to stimulate Rac- and Rho-coupled pathways that regulate morphogenesis, such as adherens junction assembly and translocation of P- and E-cadherin (Lee *et al.*, 1998, Lee *et al.*, 1999; Liu *et al.*, 2000). The activation of Rac by EDG1 has also recently been shown to be dependent upon EDG1 phosphorylation by Akt (Lee *et al.*, 2001). Future studies should examine the consequences of EDG1 phosphorylation and internalisation for small G-protein activation. Conversely, the effect of small G-proteins on the regulation of GPCRs should be examined. A number of reports have indicated that Rho family GTPases are involved in regulating endocytic traffic. For example, the microinjection of constitutively activated forms of RhoA and Rac1 have been shown to block clathrin-mediated endocytosis of the transferrin receptor in fibroblasts (Lamaze *et al.*, 1996).

Additionally, a constitutively active form of Rac1 has been shown to induce the formation of large intracellular vesicles around which Rac1 and E-cadherin tightly colocalise (Nakagawa *et al.*, 2001).

The physiological significance of EDG1 regulation can be related primarily to the process of angiogenesis (Hla *et al.*, 2001, Pyne and Pyne, 2000a, Pyne and Pyne, 2000b, Spiegel and Milstien, 2000). The regulation of angiogenesis represents an important process in a number of disease states where blood vessel formation is critical such as ischaemic heart disease, solid tumour growth and diabetic retinopathy (Carmeliet and Jain, 2000; Fan *et al.*, 1995; Griffioen and Molema, 2000; Isner, 2002; Yancopoulos *et al.*, 2000). This is reflected in the current interest in designing clinical treatments aimed at controlling angiogenesis (Carmeliet and Jain, 2000). For example, gene transfer of plasmid DNA encoding VEGF in patients with peripheral artery disease resulted in a variety of clinical benefits such as the abolition of rest pain, limb salvage and the healing of ischaemic ulcers (Isner, 2002). Recently, phase I clinical trials have began using similar gene transfer strategies in the treatment of myocardial ischaemia (Carmeliet and Jain, 2000; Isner, 2002; Yancopoulos *et al.*, 2000). Interestingly, out of 30 individuals receiving plasmid DNA encoding VEGF, 29 experienced reduced angina (Isner, 2002). Similar trials are underway using gene transfer strategies with plasmid DNA encoding FGF and a constitutively active form of HIF-1α (Carmeliet and Jain, 2000; Isner, 2002; Yancopoulos *et al.*, 2000).

Alternatively, the inhibition of angiogenesis represents a potential target against solid tumour growth and diabetic retinopathy (Carmeliet and Jain, 2000; Fan *et al.*, 1995). Tumours cannot grow beyond a critical size or metastasize to another organ without blood vessels (Cameliet and Jain, 2000). In 1971, Folkman proposed that tumour growth and metastasis are angiogenesis-dependent and hence, blocking angiogenesis could be a strategy to arrest tumour growth (Cameliet and Jain, 2000). It is widely accepted that a change in the balance of anti- and pro-angiogenic factors in favour of the latter through, for example, metabolic stress or genetic mutations, regulates tumour formation (Bouck *et al.*, 1996; Carmeliet and Jain, 2000; Fan *et al.*, 1995; Hanahan and Weinberg; 2000). A number of antiangiogenic agents are now being investigated clinically. These include treatments aimed at specific angiogenic ligands, their receptors and downstream signalling and also at the upregulation or deliverance of endogenous inhibitors (Carmeliet and Jain, 2000; Fan *et al.*, 1995). However, a major side-effect of antiangiogenic treatments may be the inhibition of bone fracture healing (Hausman *et al.*, 2001). This is particularly

important in the treatment of skeletal tumours, where bone fractures are a frequent complication (Hausman *et al.*, 2001).

As discussed in the Introduction, regulation of blood vessel formation can be attributed to the action of a number of growth factors being carefully orchestrated in terms of space, time and dose. Hence, future treatments controlling angiogenesis will most likely involve some form of combination therapy involving more than one growth factor. This is highlighted by studies showing that vessels formed in ischaemic tissue following the delivery of VEGF are leaky and tortuous (Carmeliet and Jain, 2000). The orchestration of growth factors is also demonstrated by the cross-talk mechanism discussed elsewhere between the PDGF receptor and EDG1 which mediates cell maturation (Hobson *et al.*, 2001; Rosenfeldt *et al.*, 2001). At present, no selective EDG1 agonists or antagonists are available. However, studies using antisense EDG1 and knockout mice have demonstrated that EDG1 plays a crucial role in angiogenesis, particularly at the stage of cell maturation (Lee *et al.*, 1999; Liu *et al.*, 2000). Hence, the regulation of EDG1-dependent angiogenic changes in blood vessel formation represents a potentially important clinical target. Future treatments involving EDG1 control of angiogenesis will most likely be used in combination with treatments affecting other growth factors, such as VEGF and PDGF.

Understanding how both the adenosine A_1AR and the S1P receptor EDG1 are regulated represents an area of research with potentially important clinical implications. In terms of A_1AR signalling, understanding how the receptor is regulated will help to fully establish the role of the A_1AR in physiologically important processes such as ischaemic preconditioning, an important cardio- and neuroprotective process. The EDG1 receptor plays an important role in the process of angiogenesis. Enhanced angiogenesis has been implicated in solid tumour growth, diabetic retinopathy and endometriosis (Carmeliet and Jain, 2000; Fan *et al.*, 1995; Griffioen and Molema, 2000; Isner, 2002; Yancopoulos *et al.*, 2000). Hence, future antagonists of EDG1 could prove useful in the treatment of these disease states. On the other hand, EDG1 agonists could be clinically useful in the treatment of diseases where the promotion of angiogenesis would be beneficial, such as ischaemic heart disease. A more complete understanding about the regulation of both A_1AR and EDG1 signalling will aid the future production of more selective and more effective treatments involving these receptors. For example, the identification of novel drug targets based on an understanding of how the A_1AR and EDG1 respond to agonist treatment in terms of receptor desensitisation, changes in total receptor expression and receptor resensitisation.

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