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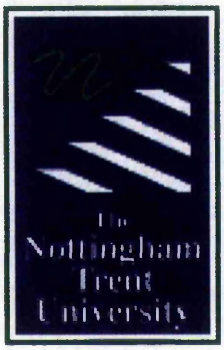

"Stimulation of the Mitogen-Activated Protein Kinase (MAPK) Pathway in DDT_1MF-2 Cells by Adenosine A_1 Receptors and Histamine H_1 Receptors "

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Thesis submitted in partial fulfilment for the degree of Doctor of Philosophy in Cell Signalling

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Declaration

I, the undersigned, declare that the work conducted as part of this thesis, and the thesis itself, was composed entirely by myself. No part of this thesis has been submitted as part of another degree. All sources are acknowledged as references.

Alexander John Robinson Date

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Finally I would like to thank God, without Whom nothing would be possible.

Abbreviations Used

ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ASK	Apoptosis signal-regulating kinase
ATF-2	Activating transcription factor-2
ATP	Adenosine triphosphate
A_1R	Adenosine A_1 receptor
BAD	Bcl-2-associated death protein
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CHO	Chinese hamster ovary
CNS	Central nervous system
CPA	N^6-cyclopentyladenosine
CREB	cAMP-response element-binding protein
DAG	Diacylglycerol
DMEM	Dulbeco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
FCS	Foetal calf serum
FGF	Fibroblast growth factor
GAP	GTPase-activating protein
GCK	Germinal centre kinase
GDP	Guanine diphosphate
GM-CSF	Granulocyte macrophage colony-stimulating factor
GPCR	Guanine nucleotide-binding regulatory protein-coupled receptor
GPS	G protein pathway suppressor
GRK	G protein-coupled receptor kinase
GTP	Guanine triphosphate
GTPase	Guanine triphosphatase
H_1R	Histamine H_1 receptor
H_2O_2	Hydrogen peroxide
HPK	Haematopoietic progenitor kinase
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor-1
IL-	Interleukin-
IP_3	Inositol 1,4,5-triphosphate
JNK	*c-jun* N-terminal kinase
JIP	JNK interacting protein
LPA	Lysophosphatidic acid
LPS	Lipopolysaccharide
MAP2	Microtubule-associated protein 2
MAPKAP	Mitogen-activated protein kinase activated-protein
MAPK	Mitogen-activated protein kinase

MAPKK/MKK	Mitogen-activated protein kinase kinase
MAPKKK/MTK	Mitogen-activated protein kinase kinase kinase
MEK	Extracellular signal-regulated kinase kinase
MKP	Mitogen-activated protein kinase phosphatases
MLK	Mixed-lineage kinage
MNK-1	MAPK interaction protein kinase-1
MSK	Mitogen- and stress-activated protein kinase
MTS	5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulphophenyl) tetrazolium
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor-κB
NGF	Nerve growth factor
NHE1	Na^+/H^+ exchanger 1
NMDA	*N*-methyl *D*-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
nNOS	Neuronal nitric oxide sythase
PAF	Platelet-activating factor
PAK	p21-activated kinase
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PIP_2	Phosphatidylinositol 4,5-biphosphate
PIP_3	Phosphotidylinositol-3,4,5-triphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PRAK	p38 regulated/activated kinase
pNa	*p*-Nitroalinine
PP-1	Protein phosphatase-1
PTX	Pertussis toxin
R-PIA	N^6-(R)-(phenylisopropyl)adenosine
RSK	p90kDa ribosomal S6 protein kinase
SAH	s-Adenosylhomocysteine
Sap1	SRF accessory protein 1
SAPK	Stress-activated protein kinase
SDS-PAGE	Sodium dodecylsulphate-polyacrylamide gel electrophoresis
SEK	SAPK/ERK kinase
SH2	Src-homology 2
SOS	Son of sevenless
SRF	Serum response factor
TAK	Transforming growth factor-beta-activated kinase
TM	Transmembrane-spanning domain
TNF	Tumour necrosis factor
TRAF	Tumour necrosis factor receptor-associated factor
UDP	Uridine diphosphate
UTP	Uridine triphosphate
UV	Ultra-violet
VEGF	Vascular endothelial growth factor

Abstract

The mitogen-activated protein kinases (MAPKs) are a group of serine/threonine protein kinases comprising of three main subfamilies. Extracellular signal-regulated kinases (ERKs 1/2, or p42/p44 MAPKs) are primarily associated with the regulation of cell proliferation and differentiation, whereas the *c-jun* N-terminal kinases (JNKs/SAPKs) and p38 MAPKs are involved in apoptosis, inflammation, and responses to environmental stress. Adenosine A_1 receptors (A_1Rs; $G_{i/o}$-coupled) have been implicated in cardiac and neuronal protection, and histamine H_1 receptors (H_1Rs; $G_{q/11}$-coupled) mediate various physiological effects, such as vascular smooth muscle contraction. In this study the coupling of these two G protein-coupled receptors (GPCRs) to the three main MAPK cascades, and the possible physiological consequences, were investigated in the smooth muscle cell line DDT_1MF-2.

Both A_1Rs and H_1Rs mediated ERK 1/2 activation in DDT_1MF-2 cells. ERK 1/2 stimulation by A_1Rs involved $G_{i/o}$ proteins, PI-3K, and MEK1, but appeared to be independent of tyrosine kinase activation. H_1R-mediated ERK 1/2 in DDT_1MF-2 cells involved PI-3K, tyrosine kinases, PKC, MEK1, and, unexpectedly, $G_{i/o}$ proteins. A_1Rs and H_1Rs also mediated p38 MAPK activation in DDT_1MF-2 cells. Similar to ERK 1/2 activation, p38 MAPK activation by both A_1Rs and H_1Rs involved $G_{i/o}$ proteins. However, neither the A_1R nor the H_1R activated the JNK/SAPK cascade in DDT_1MF-2 cells.

Both A_1R and H_1R stimulation had no significant effect on DDT_1MF-2 cell proliferation, and did not potentiate EGF-induced DDT_1MF-2 cell growth. A_1R stimulation also had no significant effect on FCS-mediated DDT_1MF-2 cell proliferation. A_1Rs and H_1Rs had no significant effect on both staurosporine-and hydrogen peroxide-induced cell death. Also, both receptors had no significant effect on staurosporine-induced caspase-3 activation. For comparison, EGF did significantly reduce staurosporine-induced caspase-3 activation.

In conclusion, this study has shown that A_1Rs and H_1Rs couple to the p42/p44 MAPK and p38 MAPK cascades in DDT_1MF-2 cells. Since neither receptor induced or potentiated DDT_1MF-2 cell proliferation, or inhibited caspase-3 activation, further experiments are required in order to establish the physiological roles of A_1Rs and H_1Rs in DDT_1MF-2 cells.

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Chapter 1

INTRODUCTION

1.0 – INTRODUCTION

Signal transduction across cell membranes is vital for regulation of intracellular activity (Sunhara *et al,* 1996). Changes in the extracellular environment, such as increases in hormone or neurotransmitter levels, provide chemical signals for cells to initiate an internal response (Linder & Gilman, 1992). This response is essential for the particular cell's function, and can be initiated by transmembrane-spanning structures such as ion channels, tyrosine kinase-linked receptors, or guanine nucleotide-binding regulatory protein- (G protein-) coupled receptors (GPCRs) (Graziano & Gilman, 1987).

1.1 - G Protein-Coupled Receptors (GPCRs)

GPCRs are a superfamily of proteins targeted via a wide range of stimuli, such as neurotransmitters, hormones, light, and odour (Ferguson, 2001). They represent the largest known receptor family, with over 1000 different subtypes found in vertebrates alone (Howard *et al,* 2001). Up to 5% of the genes comprising the human genome encode GPCRs, and 300 mammalian GPCR genes have been recognised (Bargmann, 1998). They are separated into three subfamilies, with Family A containing most of the known GPCRs that have been discovered so far (Marchese *et al,* 1999). However, GPCRs which bind ligands such as secretin, vasoactive intestinal peptide, and calcitonin, are grouped into Family B, and Family C comprises the taste receptors, $GABA_B$ receptors, and metabotropic glutamate receptors (Howard *et al*, 2001).

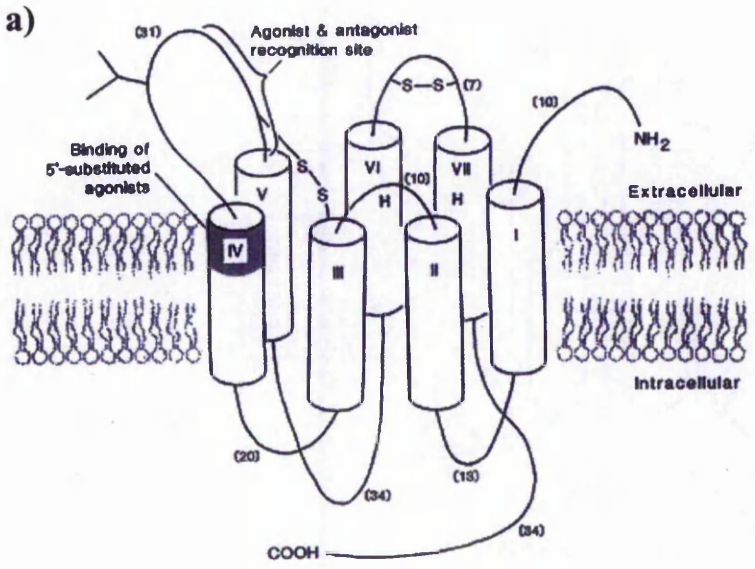
1.1.1 – GPCRs and Disease

GPCRs provide an excellent target for therapeutic intervention; around 50% of drugs available on the market target these receptors (Howard *et al*, 2001). Also, mutations within a GPCR can lead to a number of disease states. These include retinitis pigmentosa, which causes night blindness and eventually leads to complete blindness, and nephrogenic diabetes insipidus, which has been linked to mutations in the vasopressin V_2 receptor (Edwards *et al,* 2000). Therefore, further research into this superfamily of proteins will help identify further therapeutic strategies in the treatment of numerous medical conditions.

1.1.2 - Molecular Structure and Function of GPCRs

GPCRs are single polypeptide chains, between 400 and 700 amino acid residues long. There are seven membrane-spanning "segments", or α-helices, each between 20 and 25 residues long. There is a relatively short amine (N-) terminal in the extracellular fluid and a much longer carboxyl (C-) terminal in the cytosol. When inactive the G-protein is coupled via weak hydrogen bonds to the C-terminal and the loop of residues connecting the fifth and sixth transmembrane domains (TM5 and TM6) (Rang *et al*, 1995).

The G-protein itself consists of three subunits: the α-subunit, which has a molecular weight of between 39kDa and 52 kDa, the β-subunit (35-37 kDa), and the γ-subunit (7-10 kDa). Bound to the α-subunit is a guanosine diphosphate (GDP) molecule (Wingard *et al*, 1991). See Figure 1.1 for schematic diagrams of a GPCR.



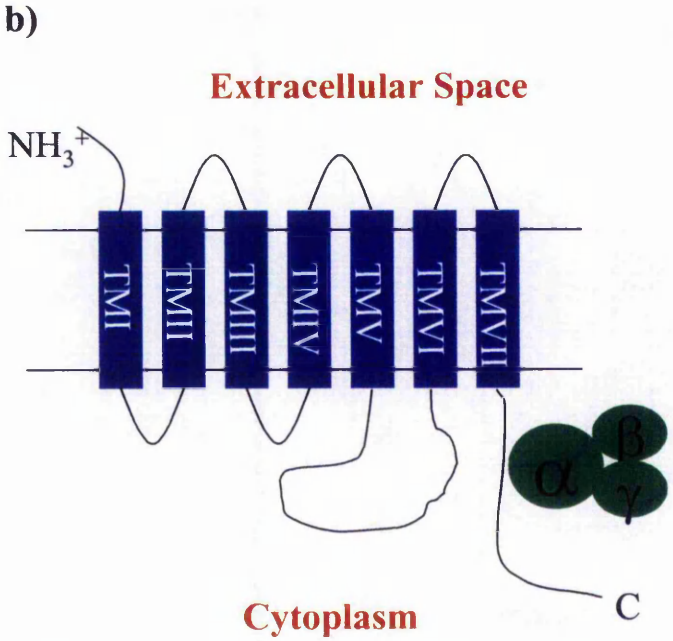


Figure 1.1 – Schematic diagrams of a G protein-coupled receptor. a) an adenosine A_1 receptor similar to how it would be actually be arranged in the cell membrane, the transmembrane α-helices (TMI-TMVII) being found in a circular pattern, the short amino (N-) terminal protruding into the extracellular space, and the long carboxy- (C-) terminal snaking into the cytosol. The G protein is not shown (Ralevic & Burnstock, 1998). b) a simplified version with the transmembrane a-helices "flattened out" over the entire cell membrane, and a representation of the G protein with its three subunits bound to the C-terminal. Note: the GDP molecule bound to the a-subunit of the G protein is not shown (Rang & Dale, 1995).

1.1.3 - Signal Transduction Mechanisms of GPCRs

A ligand binds to a GPCR by interacting with amino acids in TMIII, TMV, and TMVI (see Figure 1.2). This produces a conformational change of the receptor that causes a decrease in the GDP affinity of the α-subunit in the G protein. The GDP molecule breaks away, producing an increase in the α-subunit's affinity for guanosine triphosphate (GTP, Figure 1.2b). A molecule of GTP binds to the α-subunit, activating it and producing a conformational change that produces dissociation of the GTP-α-subunit complex from the βγ-subunits of the G protein. The GTP-α-complex then migrates along the plane of the intracellular membrane until it binds to its target e.g. enzymes such adenylyl cyclase, ion channels such as neuronal K^+ channels, or ion exchangers such as the H^+/Na^+ exchanger (Figure 1.2c). The species of targeted enzyme, and the effect produced (e.g. stimulation or inhibition) depends greatly on the molecular structure of the α-subunit, due to heterogeneity of the α-subunit (Neer, 1995, Eglen *et al*, 1994). Refer to section 1.1.4 for more detail. The βγ-subunits also have a number of protein targets which they can affect (see section 1.1.5)

Upon binding to the target enzyme a further conformational change causes the GTP-α-subunit complex to develop GTPase activity (Figure 1.2d). The GTP molecule bound to the α-subunit loses a phosphate group. This then breaks the bond between the target enzyme and the GDP-α-subunit complex, and another α-subunit conformational change takes place, increasing its affinity for the βγ-subunits. The G protein reforms, bound again to the GPCR and ready for restimulation (Wess, 1997, Simon *et al*, 1991).

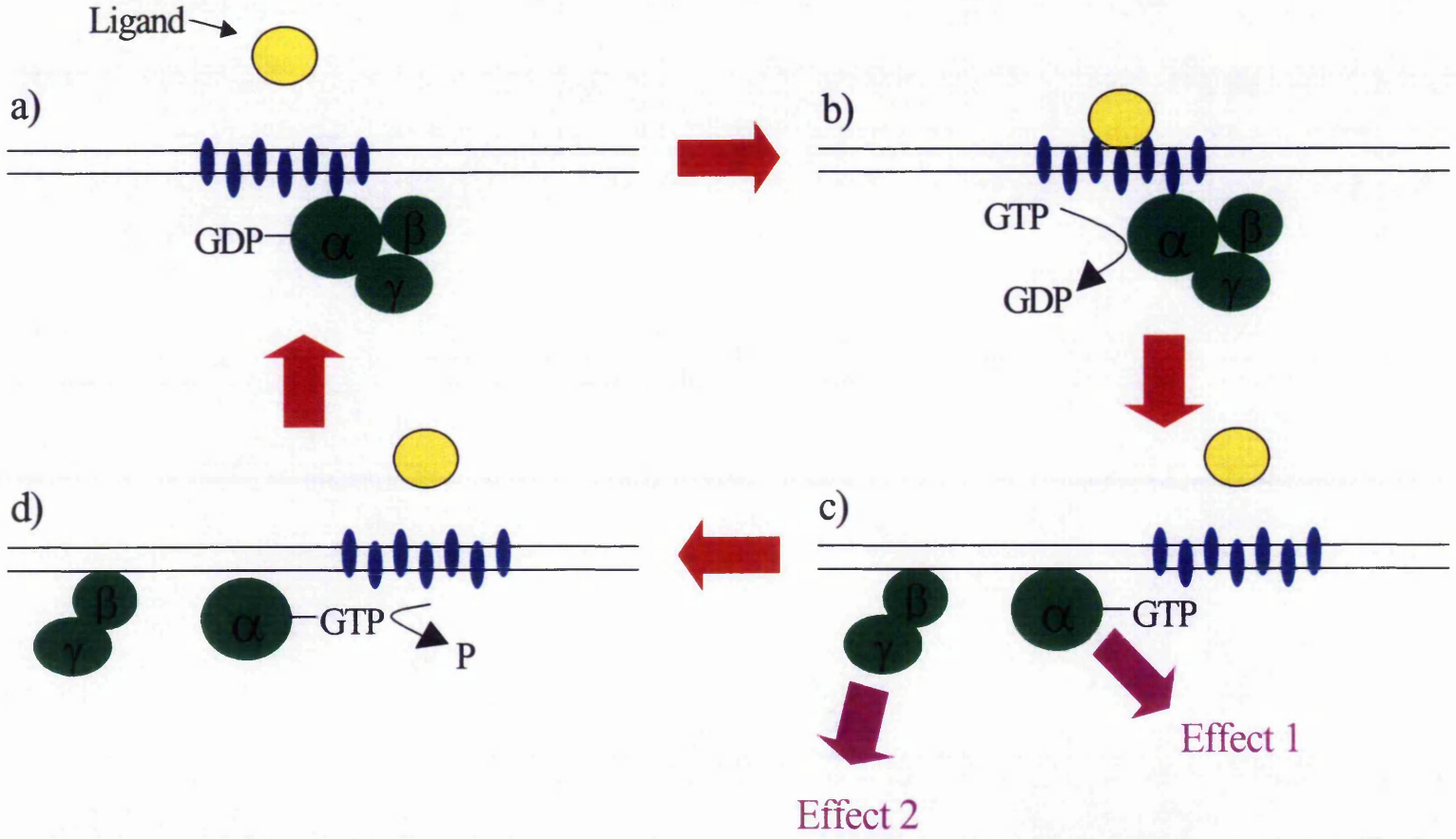


Figure 1.2 - The signal transduction cycle of a GPCR. The diagram shows the receptor in four stages: (a) its "resting" state, (b) ligand binding causing GDP-GTP exchange on the α-subunit, (c) dissociation of the GTP-α-subunit complex and βγ-subunits, allowing induction of their intracellular effects, and (d) GTPase activity of the target enzyme removing the phosphate group from the GTP-α-subunit complex, causing reassociation of the GDP-α-subunit and βγ-subunits and returning the receptor back to its resting state (Friessmuth et al, 1999).

1.1.4 – Regulation of GPCR Signal Transduction

A number of mechanisms exist to prevent excessive stimulation of GPCRs that may lead to cell damage or even death. The first line of defence is to reduce the GPCR agonist concentration in the extracellular space. Processes that achieve this role include uptake mechanisms that physically remove the agonist from the extra cellular space into either adjoining cells or those from which the agonist was originally secreted. The agonist is then stored in vesicles for re-release, or degraded for recycling (Neal, 1997). Another method is agonist degradation by specific enzymes found either in the extra cellular fluid or cell membrane (Bőhm *et al,* 1997). Mechanisms which inhibit agonist release also exist, including G protein βγ-subunits stimulating K^+ influx into cells, which inhibits Ca^{2+} influx and a reduction in agonist release (Clapham & Neer, 1997).

Other mechanisms involve changes to the GPCR itself. Phosphorylation of the receptor, usually at a cytosolic amino acid residue, causes uncoupling of the G protein from the receptor, severing the signal transduction pathway (Lefkowitz, 1998). Known as desensitisation this is a two-step process involving G protein-coupled receptor kinases (GRKs) and β-arrestins (Inglese *et al,* 1993). Agonist-bound GPCRs are phosphoylated by the GRK, which allows binding of β-arrestins to the receptor. This then prevents signalling via the G protein.

Also, activation of second messenger kinases such as protein kinase A (PKA) and protein kinase C (PKC) can phosphorylate the GPCR, usually on serine residues within the C-terminal or the loop between TMV and TMVI, causing a conformational change in the receptor and impaired signalling. These kinases, activated by G_s and G_q proteins respectively, can phosphorylate other GPCRs as well

as the GPCR that directly activated them. Hence this process is known as heterologous desensitisation (Sibley *et al,* 1987).

A further common pathway involves endocytosis of the GPCR. The agonist-receptor complex is desensitised by the GRK-β-arrestin two step process, as before, but is then internalised within vesicles (Koenig, 1997). If the vesicle is clathrin coated the receptor and ligand dissociate after internalisation, and the GPCR is relocated back on the cell membrane. If the vesicles are not clathrin coated the receptor is taken up into lysosomes and degraded. It should be noted, however, that GPCR endocytosis may also lead to activation of intracellular signalling pathways. (Lefkowitz, 1997).

1.1.5 - α-Subunits

The α-subunits of a GPCR show great molecular diversity. Over 20 different isoforms have currently been identified (Freissmuth *et al,* 1999), although this number is unlikely to increase since a new type of α-subunit has not been discovered for nearly ten years. Despite being so diverse, their similar biochemical and structural properties allow them to be split into four basic groups, described below in Table 1.1. The type of α-subunit that a GPCR is associated with determines the class of the whole G protein the GPCR is coupled to i.e α_s are found in G_s proteins, $\alpha_{i/o}$ in $G_{i/o}$ proteins, and so on.

Table 1.1 - The different α-subunit isoforms and their intracellular effects (Freissmuth *et al,* 1999, Simmonds, 1999).

α-Subunit Type	Further Isoforms	Action
α_s	-	Stimulates adenylyl cyclase production
$\alpha_{i/o/t}$	$\alpha_{i/o/z}$	(i) inhibition of adenylyl cyclase, (ii) inhibition of neuronal Ca^{2+} channels (iii) stimulation of neuronal K^+ channels
	$\alpha_{t/g}$	stimulation of retinal cGMP-phosphodiesterases
α_q	-	(i) activation of phospholipase Cβ (involved in Ca^{2+} mobilisation) (ii) activation of non-receptor tyrosine kinases of the BtK family
$\alpha_{12/13}$	-	(i) regulation of low-molecular-weight G proteins of the rho-family (which affect the cytoskeleton) (ii) regulation of the Na^+/H^+-exchanger

1.1.6- βγ-Subunits

β and γ subunits form the βγ-dimer which does not dissociate or undergo conformational change throughout the GPCR activation process outlined in section 1.1.3. There are multiple isoforms of each subunit, with 5 β-subunit and at least 12 γ-subunit isoforms known to exist (Clapham & Neer, 1997). Until recently only the α-subunit was thought to play a major role in signal transduction in GPCRs. The βγ-complex was merely thought of as an anchor to secure the G protein in place when the GPCR was in its resting state (Neer & Chapman, 1988). However, it has since

been shown that the βγ-dimer does have active and diverse roles in signal transduction too (see below), explaining the heterogeneity of each subunit.

1.1.7 – The Role of βγ-Subunits in Intracellular Signalling

The Na^+/K^+ exchange regulation by $G_{\alpha 12}$ (see Table 1.1) is due to the action of the βγ-complex, and there is evidence for βγ-complexes working in tandem with their corresponding α-subunits. Stimulation of α_2-adrenoceptors, due to being coupled to $G_{i/o}$ proteins, produces inhibition of cAMP production via the action of the α-subunit, but there is also a simultaneous increase in inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) production. βγ-subunits are thought to activate PLCβ which converts inositol-4,5-bisphosphate to IP_3 and DAG. There is also evidence to suggest the βγ-complex activates phospholipase A_2 in some cells, causing prostaglandin and thromboxane production (Milligan, 1993). Recently, it has been shown that the βγ-subunit can activate the mitogen-activated protein kinase (MAPK) pathway, which is involved in cell differentiation and proliferation (Koch *et al*, 1994, Hawes *et al*, 1995, Garnovskaya *et al,* 1996).

1.2 - Adenosine and Adenosine A_1 Receptors

Adenosine belongs to the family of molecules known as purines, a group which also includes ADP and ATP (Ralevic & Burnstock, 1998). Since the first studies by Drury and Szent--Gyögyi in 1929, adenosine has been identified as an extracellular signalling molecule that regulates a variety of physiological functions. Adenosine release from a number of different cell types leads to a diverse range of biological effects (see section 1.2.4).

1.2.1 - Adenosine Biosynthesis

Adenosine is biosynthesised via three processes when required. Firstly, from s-adenosylhomocysteine (SAH), by SAH hydrolase, secondly, from cytosolic AMP by cytosolic 5'-nucleotidase, and finally from AMP by ectosolic 5'- nucleotidase (Kroll *et al,* 1993). Conversely, it can be metabolised back to SAH, to ADP and ATP, or by various steps to uric acid (Miura & Tsuchida, 1999).

1.2.2 - Adenosine Receptors

Once released into the extracellular space adenosine can then interact with numerous cell surface receptors. Indications that adenosine had different properties to the other purines were first observed in 1934 when Gillespie demonstrated differences in potency and response, compared with ATP, on vascular tissue. Since then it has emerged that adenosine can activate at least four known receptor subtypes, collectively known as P1 receptors, all belonging to the GPCR superfamily (Ulah & Stiles, 1995). The other purines, primarily ATP, ADP, UTP, and UDP, activate a range of GPCRs and ion channel-linked receptors known as P2 receptors (Burnstock, 1978).

Adenosine receptors were initially divided into two subtypes, R_i and R_a, due to their ability to decrease and increase intracellular cAMP levels respectively (van Calker *et al,* 1979, Londos *et al,* 1980). However, this nomeclature then was changed to the A_1 and A_2 terms used today (van Calker *et al,* 1979). Evidence for further division of the A_2 group was presented by Daly *et al* (1983), who found high affinity A_2 receptors in the rat striatum but low affinity A_2 receptors elsewhere in the brain. Elfman *et al* (1984) then discovered high affinity A_2 receptors in neuroblastoma cells

but low affinity ones in glioma cells. These two distinct types of receptor were renamed A_{2A} and A_{2B} (Bruns *et al,* 1986). Finally, due to the advances in molecular cloning techniques, another adenosine receptor subtype was identified, the A_3 receptor (Zhou *et al,* 1992).

The four known subtypes of adenosine receptors are officially classified as A_1, A_{2A}, A_{2B}, and A_3 receptors (Fredholm *et al,* 1994, Fredholm *et al,* 1996, Fredholm *et al,* 1997). A_1 receptors are $G_{i/o}$ protein-linked, which mainly causes a decrease in intracellular cAMP levels when stimulated (Freissmuth *et al,* 1991, Munshi *et al,* 1991), but other intracellular effects also occur (see section 1.2.3). A_{2A} receptors are G_s-linked, producing an increase in intracelluler cAMP levels when activated (Ralevic & Burnstock, 1997). A_{2B} receptors are also G_s-linked, but have been shown to increase intracellular IP_3 and DAG levels as well, indicating coupling to G_q proteins (Feoktistov & Biaggoini, 1997). A_3 receptors couple to $G_{i/o}$ proteins, similar to A_1 receptors, but also couple to G_q proteins, like with the A_2 receptor (Palmer *et al,* 1995, Linden, 1995). Although classed in the same group of receptors there is relatively low sequence homology between each of the four subtypes, usually between 40-50% within species (Ralevic & Burnstock, 1997).

1.2.3 - Adenosine A_1 Receptors (A_1Rs) and Their Signal Transduction Mechanisms

Cloned human A_1Rs are 326 amino acid residues in length (Libert *et al,* 1992, Townsend-Nicholson & Shine, 1992), although this number of residues appears to be conserved throughout different species e.g. canines (Libert *et al,* 1989, Libert *et al,* 1991), rabbits (Bhattacharya *et al,* 1993) and bovines (Tucker *et al,* 1992). Further subdivision into A_{1a} and A_{1b} had been previously proposed due to high affinity agonist and antagonist binding in the brain, but low agonist and antagonist binding in

vas deferens and ileum tissue (Gustafsson *et al,* 1990). However, no cloned example of either the A_{1a} or A_{1b} receptor subtype has yet been found. The above may be due to high and low affinity states respectively of the same receptor (Ralevic & Burnstock, 1998).

As previously mentioned in section 1.2.2 A_1Rs are coupled to $G_{i/o}$ proteins, which classically inhibit adenylyl cyclase and reduce intracellular cAMP levels. This leads to the reduced activity of a number of cAMP-dependent enzymes, leading to the inhibition of various physiological processes, such as smooth muscle contraction, lypolysis, and neurotransmission (Olah & Stiles, 1995). However, A_1Rs also increase IP_3 and DAG production, and therefore Ca^{2+} mobilisation, by activating PLC. This action, similar to that exerted by G_q proteins, has been demonstrated in a number of cell lines, including DDT_1MF-2 cells (Weinberg *et al,* 1989, Dickenson & Hill, 1991, White *et al,* 1992, Peakman & Hill, 1995). Release of Ca^{2+} from the sarcoplasmic reticulum by IP_3 activates a variety of enzymes, such as PKC, phospholipase A_2 (leading to prostaglandin and thromboxane production), phospholipase D, and nitric oxide synthase (Berridge, 1993).

Other effects include activation of a variety of K^+ channels, including Ca^{2+}-dependent and ATP sensitive channels, increasing intracellular K^+ levels (Ralevic & Burnstock, 1998). Another main signal transduction pathway of A_1Rs is inhibition of Ca^{2+} influx via numerous types of Ca^{2+} channels (Fredholm, 1995). A_1Rs activate protein kinase A when stimulated, which phosphorlylates Ca^{2+} channels and reduces Ca^{2+} influx (Ralevic & Burnstock, 1998). There is also evidence for direct interaction of $G_{i/o}$ $\beta\gamma$ subunits with Ca^{2+} channels, which may also reduce Ca^{2+} influx via these channels (Mark *et al,* 2000). This has been shown to reduce neurotransmitter release in a variety of cell types, such as dorsal root ganglion neurones (Dolphon *et al,* 1986) and rat brain stem cells (Umemiya & Berger 1994).

Finally, a more recent discovery is the activation of the mitogen-activated protein kinase (MAPK) pathway by A_1Rs in Chinese hamster ovary (CHO) cells (Dickenson *et al,* 1998) and protein kinase B (PKB) activation in DDT_1MF-2 cells (Germack & Dickenson, 2000). These cascades are involved with cell differentiation and proliferation, apoptosis, inflammation, and responses to environmental stress (Sugden & Clerk, 1997, Ono & Han, 2000) and will be described in more detail later in this introduction i.e. see section 1.4.7 for a more detailed look at the role of PKB. For a summary of signal transduction pathways mediated by A_1Rs, see Figure 1.3.

1.2.4 - Distribution and Physiological Roles of Adenosine A_1 Receptors

A_1Rs are widely expressed in the CNS, in particular the hippocampus, thalamus, spinal cord, cerebellum, and cortex (Nyce, 1999). Their roles include inhibition of neurotransmission, induction of sleep, motor coordination and other side effects caused by ethanol ingestion, and inhibition of the pain response via the nociceptive pathway (Sebastião & Ribeiro, 2000).

A_1Rs are also found in the heart, and since the initial studies by Murry *et al* (1986) have been implicated in ischaemic preconditioning. Adenosine has been shown to be biosynthesised and released when the heart undergoes an ischaemic episode e.g. myocardial infarction. The time period between the onset of ischaemia and cardiac cell damage is increased in the presence of adenosine, and A_1Rs appear to play a major role in this process (Liu *et al*, 1991, Miura & Iimura, 1993). The activation of PKC by A_1Rs appears to be an important intracellular event (Miura & Tsuchida, 1999), as does activation of the MAPK cascade (Haq *et al*, 1998). Also, A_1Rs regulate cardiac function via their presence in the autonomic nervous system. Reduction of both heart rate and force of contraction mediated by A_1Rs help reduce

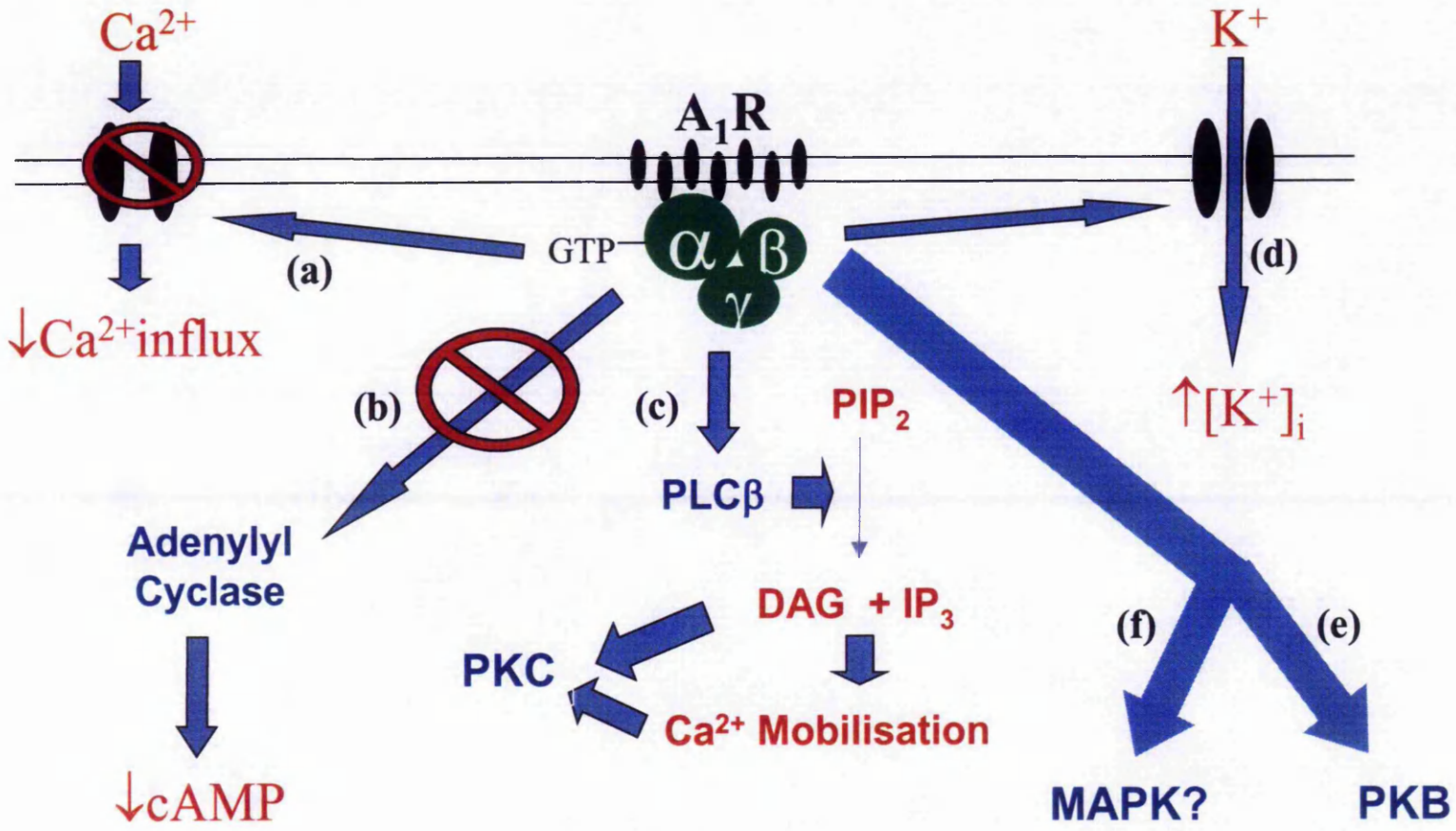


Figure 1.3 - The signal transduction pathways of the adenosine A_1 receptor, including (a) inhibition of Ca^{2+} influx via Ca^{2+} channels, (b) inhibition of adenylyl cyclase leading to reduced cAMP levels, (c) activation of PLCb causing Ca^{2+} mobilisation and PKC activation, (d) increased K^+ influx via different types of K^+ channel, (e) activation of PKB, and finally (f) possible activation of the MAPK cascade.

cardiac oxygen and energy requirements, prolonging onset of cardiac damage further (Nyce, 1999).

A_1Rs have a similar role in the brain, protecting it against dangerously low blood oxygen levels, which can lead to cerebral infarction and stroke (Ralevic & Burnstock, 1998). In conjunction with NMDA receptors, which are also widely distributed throughout the CNS, A_1Rs appear to have a neuroprotective role (Dawson & Dawson, 2000). NMDA receptors can indirectly activate the MAPK cascade in neurones, but via the production of nitric oxide, which activates the cascade directly. Stimulation of the NMDA receptor on the presynaptic cell produces an influx of Ca^{2+}, activating neuronal NOS (nNOS), and producing nitric oxide (NO). NO is released in to the synaptic cleft, and activates Ras in the post-synaptic neurone, leading to ERK phosphorylation (Gonzalez-Zulueta *et al*, 2000). NO-mediated Ras activation occurs via a redox sensitive mechanism, or through activation of a NO-dependent guanine nucleotide exchange factor. In both the heart and CNS adenosine is released shortly after cells undergo ischaemia. Adenosine activates post-synaptic A_1Rs, which activate Ra via $\beta\gamma$-subunts, augmenting NO-mediated Ras and ERK phosphorylation (Dawson & Dawson, 2000), and also activate pre-synaptic A_1Rs which reduces Ca^{2+} influx, leading to decreased neurotransmitter release, causing a reduction in neuronal activity and oxygen consumption.

Also found in the lungs, A_1Rs have been implicated in the development of asthma. Evidence shows that, when stimulated, they induce bronchoconstriction, particularly in asthmatics (Polosa, 2002). Studies have shown that both animals and patients suffering from asthma have both an increased amount of adenosine in their lungs and an elevated number of bronchial A_1Rs (Ali *et al,* 1994, El-Hashim *et al,* 1996, Pauwels & Joos, 1995). The use of methylxanthines such as theophylline, known

non-specific adenosine receptor antagonists, in the treatment of asthma is further evidence of this (Pauwels, 1989).

Other physiological roles for A_1Rs include regulation of various renal functions, including reducing blood flow to the kidneys, lowering glomerular filtration, and inhibiting renin secretion (Agmon *et al,* 1993, Barrett & Droppelman, 1993, Munger & Jackson, 1994). Also, A_1Rs appear to have a role in the human fertilisation process, as they have been found in the spermatozoa of various mammalian species (Allegrucci *et al,* 2000). Although unclear, it is thought A_1Rs may aid in sperm maturation and motilty.

1.3 – Histamine and Histamine H_1 Receptors

1.3.1 – Histamine

Histamine, or 2-(4-imidazolyl)-ethyl-amine, is a basic amine found in most tissues of the body (Rang et al, 1999). Formed from histidine by histidine decarboxylase, it is found in particularly high concentrations in the skin, lungs, gastrointestinal tract, mast cells and basophils. (Hill et al, 1997). It is metabolised by histaminase and/or imidazole N-methyltransferase (Rang et al, 1999). Histamine has a wide range of physiological functions, which have been investigated since the beginning of the last century (Dale & Laidlaw, 1910).

1.3.2 – Histamine Receptors

As mentioned the actions of histamine have been studied for nearly a century. Initially, its role in the allergic response was investigated, leading to the development

of so-called antihistamines, such as mepyramine, which blocked the action of histamine (Bovet, 1950). These compounds were found to inhibit bronchospasm and smooth muscle contraction, some "classical" actions of histamine, but had little effect on other histamine-mediated events (Loew, 1947). Both gastric secretion and the vasodilator response were not particularly affected after the addition of antihistamines (Ashford *et al,* 1949), leading to the idea that more than one type of histamine receptor existed (Folkow *et al*, 1948). The method developed by Schild (Arunlakshana & Schild, 1959) demonstrated a difference in the action of mepyramine on histamine-mediated increases in heart rate and guinea-pig ileum contraction (Arunlakshana & Schild, 1959, Trendelenburg, 1960). This lead to the receptors antagonised by the antihistamines being renamed histamine H_1 receptors (Ash & Schild, 1966) and further studies into the synthesis of compounds that antagonised the second type of histamine receptor.

This lead to the development of burimamide, discovered by Black *et al* (1972), the first recognised histamine H_2 receptor antagonist. It was found to reduce canine and human gastric acid secretion, and reduce feline blood pressure in response to histamine, which are now known to be classic actions of histamine H_2 receptor antagonism. Then, thanks to further development of new histamine H_2 receptor antagonists (see Cooper *et al,* 1990 for a detailed list), a further receptor was identified and classified, the histamine H_3 receptor (Arrang *et al,* 1983). It was found this receptor inhibited histamine synthesis and release in the CNS, indicating an autoinhibitory role for histamine (Arrang *et al,* 1987). Finally, molecular cloning techniques have indicated the presence of a fourth histamine receptor (Nakamura *et al,* 2000). Initially thought to be a histamine H_3 receptor subtype (West *et al,* 1990) this histamine H_4 receptor has been shown to be a distinct receptor in its own right. Although it's physiological role has yet to be determined (Oda *et al,* 2000. Nguyen *et*

al, 2001) it may have a role in the immune response as it is expressed in bone marrow (Liu *et al*, 2001).

Four histamine receptor subtypes have been identified to date. Histamine H_1, H_2, and H_3 receptors are G protein-linked, and evidence suggests the recently-cloned histamine H_4 receptor is G protein-coupled also (Hill *et al*, 1997, Oda *et al*, 2000). Histamine H_1 receptors are coupled to $G_{q/11}$ proteins, which activate PLC-β, triggering the production of IP_3 and DAG from plasma membrane phospholipid, and subsequent Ca^{2+} mobilisation and PKC activation (Hill, 1990, Leurs *et al*, 1995). H_2 receptors are G_s-linked, producing an increase in intracellular cAMP levels when activated (Johnson 1982, Hill *et al*, 1997). H_3 receptors appear to be $G_{i/o}$-linked, which decrease intracellular cAMP levels when stimulated (Lovenberg *et al*, 1999). H_4 receptors have only recently been identified and characterised (Oda *et al*, 2000. Nguyen *et al*, 2001) and as such which G protein they are linked to has yet to be determined, although evidence suggests they may be $G_{i/o}$-linked.

1.3.3 - Histamine H_1 Receptors (H_1Rs) and Their Signal Transduction Mechanisms

The human H_1R is 487 amino acids long with a molecular weight of 56 kDa, and displays many of the classic GPCR characteristics (Backer *et al*, 1993). It has seven transmemebrane-spanning domains, with an extracellular N-terminal and cytosolic C-terminal, and has N-terminal glycosylation sites (Hill *et al*, 1997). However, marked differences of the H_1R to other GPCRs include a larger third intracellular loop (which links TM5 and TM6) of 212 amino acids, and a very short C-terminal of 17 amino acids (Yamashita *et al*, 1991). Histamine binding to the H_1R is determined by two of the membrane-spanning domains, TM3 and TM5 (Timmerman, 1992). The aspartate residue 107 in TM3 (Ohta *et al*, 1994),

asparagine residue 207 (Leurs *et al,* 1994), and lysine residue 200 (Leurs *et al,* 1995) all appear to be essential for histamine binding. Subsequent to histamine binding to the H_1R, evidence suggests internalisation of the H_1R occurs (Hishinuma & Young, 1995).

As mentioned in section 1.3.2. H_1Rs are coupled to $G_{q/11}$ proteins. Activation of the receptor leads to activation of PLC-β by the α-subunit of the G protein, causing the conversion of phosphatidylinositol 4,5-biphosphate (PIP_2), a plasma memebrane phospholipid, into IP_3 and DAG. These compounds then cause release of Ca^{2+} from the sarcoplasmic reticulum, and activate PKC, respectively (Berridge, 1993). The subsequent increase in intracellular Ca^{2+} concentration activates a wide range of Ca^{2+}- and/or calmodulin-dependent enzymes, such as guanylyl cyclase and nitric oxide synthase (NOS) (Duncan *et al,* 1980, Schmidt *et al,* 1990, Leurs *at al,* 1991), and PLA_2 causing prostacyclin and thromboxane production (Resink *et al,* 1987). H_1Rs also potentiate adenylyl cyclase activation by other receptors, such as histamine H_2 and adenosine A_2 receptors, in a wide number of cells (Donaldson *et al,* 1989, Marley *et al,* 1991).

There is evidence for other enzymes that are activated by $G_{q/11}PCRs$. Protein kinase B (PKB), or Akt as it also known, has major roles in both protein synthesis and glucose metabolism (Coffer *et al,* 1998) and, as previously mentioned in section 1.2.3, has also been implicated in protecting cells from apoptosis (Downward *et al,* 1998, Mockridge *et al,* 2000). GPCRs coupled to $G_{q/11}$ proteins have been shown to activate PKB (Murga *et al,* 1998) and it is possible H_1Rs may do the same.

H_1Rs have been shown to activate two important transcription factors; NF-κB and NFAT (Boss *et al,* 1998, Bakker *et al,* 2001). Activation of NF-κB is an important step in inflammation, and forms part of the overall H_1R-mediated allergic response. NFAT is another important player in the immune response. After activation by rises

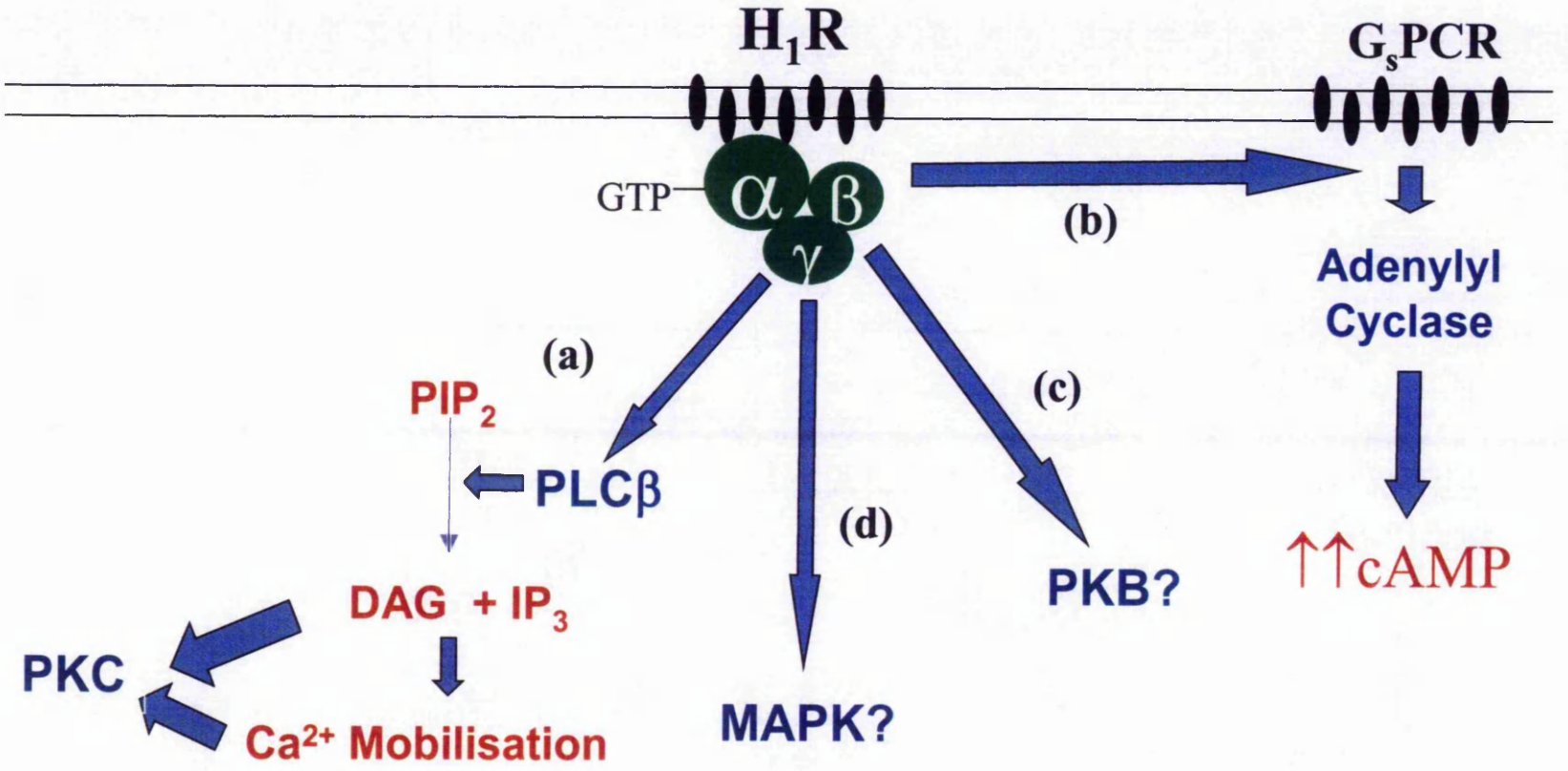


Figure 1.4 - The signal transduction pathways of the histamine H_1 receptor. (a) activation of PLCβ, causing Ca^{2+} mobilisation and PKC activation, (b) potentiation of adenylyl cyclase activation by G_sPCRs leading to further iincreased cAMP levels, (c) possible activation of PKB, and finally (d) possible activation of the MAPK cascade.

in intracellular Ca^{2+} levels it has a role in maturation of immature T lymphocytes (Aifantis *et al,* 2001).

$G_{q/11}$-coupled receptors have also been shown to activate the MAPK pathway (Sugden & Clerk, 1997, Xin *et al,* 1997) and this pathway may also be involved in the anti-apoptotic process, as mentioned before. Recent data indicates H_1Rs may activate the MAPK cascade in guinea-pig ileum smooth muscle, since MAPK inhibition reduced the H_1R-mediated contractile response (Koch *et al,* 2000). For a summary of H_1R-mediated signal transduction pathways, see Figure 1.4.

1.3.4 - Distribution and Physiological Roles of Histamine H_1 Receptors

H_1Rs are widely distributed throughout the mammalian body, being present in bronchial smooth muscle, the cardiovascular system, lymphocytes, endothelial cells, the adrenal medulla, genitourinary system, the gastrointestinal tract, and central nervous system (Hill *et al,* 1997). As previously mentioned histamine and the H_1R play a major role in the allergic response. The presence of H_1Rs on endothelial cells, particularly in post-capillary venuoles, is vital as their stimulation produces several key steps in the allergic response. These include vascular permeability due to contraction of the endothelial cells, allowing lymphocytes and other cells easy access to the location of the allergen (Majno *et al,* 1968, Killackey *et al,* 1986). Synthesis of prostacyclin and platelet-activating factor (PAF) by the H_1R cause increased vascular permeability as well as vasodilation which allows greater blood flow to the affected area (McIntyre *et al,* 1985, Resink *et al,* 1987). The release of Von Willebrand factor and nitric oxide is also mediated by H_1Rs (Toda, 1984, Hamilton & Sims, 1987, Van de Voorde & Leusen, 1993). These are required for platelet adhesion, to plug any tear in the blood vessel wall, and vasodilation respectively. Finally, H_1Rs have been located on T-lymphocytes, and their activation and

subsequent intracellular increase in Ca^{2+} is believed to have a role in chemokine release, an essential step in coordinating the immune response (Villemain, 1990, Kitamura *et al,* 1996).

H_1Rs are also found in high numbers in various types of smooth muscle, such as those found in the windpipe, blood vessels, and gastrointestinal tissue the ileum (Barger & Dale, 1910, Bülbring & Burnstock, 1960, Ash & Schild, 1966). Their role is smooth muscle contraction, either via modulation of action potentials which makes the muscle cell reach the "firing" potential easier (Bülbring & Burnstock, 1960, Bolton, 1979, Bolton *et al,* 1981), or by their ability to increase intracellular Ca^{2+} levels which increases activation of the enzymes required to initiate muscle cell contraction (Matsumoto *et al,* 1986, Kotlikoff *et al,* 1987).

H_1Rs are present in the heart, specifically in the atrial myocardium, and have been shown to decrease the rate of contraction (Guo, *et al,* 1984, Zavecz & Levi, 1978). The role of H_1Rs in the adrenal medulla is to stimulate hormone release, specifically adrenaline and noradrenaline (Emmelin & Muren, 1949, Noble *et al,* 1988). H_1R-mediated increases in intracellular Ca^{2+} cause release of the catecholamines from their intracellular vesicle stores, and also activate tyrosine hydroxylase that increases catecholamine biosynthesis (Bunn *et al,* 1995).

Finally, there is a wide distribution of H_1Rs in the brain, in particular the hippocampus, thalamus, nucleus accumbens, and posterior hypothalamus (Chang *et al,* 1979, Hill, 1990, Schwartz *et al,* 1991).

1.4 - The Mitogen-Activated Protein Kinase (MAPK) Pathway

The mitogen-activated protein kinases (MAPKs) are found, and highly conserved, in all eukaryotes (Guan, 1994). They are involved in a variety of intracellular

signalling processes that begin at the plasma membrane and cascade down to the nucleus (English *et al,* 1999). They were first investigated in the early 1980's after it was realised that several different types of growth factor caused the phosphorylation of a tyrosine residue located within a 42kDa kinase (Cooper *et al,* 1982, Cooper & Hunter, 1983). The isolated 42kDa kinase was subsequently found to phosphorylate the substrates microtubule-associated protein 2 (MAP2) and myelin (Ray & Sturgill, 1987, Hoshi *et al,* 1988, Price *et al,* 1989). Then, in the early 1990's, the first three isoforms of MAPK were cloned, specifically extracellular-regulated kinases (ERKs) 1, 2, and 3 (Boulton *et al,* 1990, Boulton *et al,* 1991). Further subgroups of MAPK were found, such as the p38 MAPKs (Han *et al,* 1994, Lee *et al,* 1994) and stress-activated protein kinases (SAPKs) (Kyriakis & Avruch, 1990, Hibi *et al,* 1993), as well as MAPK cascade activators other than growth factors, such as GPCRs (Sugden & Clerk, 1997).

1.4.1 - MAPK Structure

Although over a dozen different MAPK genes have been discovered to date (Hunter & Ploughman, 1997), and estimates of over 40 different MAPK isoforms existing being made (English *et al,* 1999), all MAPKs have a number of similar, distinct features. Structurally they are organised into two domains, similar to most protein kinases (Knighton *et al,* 1991). There is a smaller domain, which contains the N-terminal and is predominantly made up of β strands, and there is a larger C-terminal-containing domain, which mainly consists of α-helices (Zhang *et al,* 1994). The MAPK active site is located between these two domains, and at the very point where they meet a molecule of ATP binds. An activation loop, also known as a phosphorylation lip, branches out from the active site and contained within this loop

are the two residues required to be phosphorylated for MAPK activation (English *et al,* 1999). Both residues, tyrosine and threonine, are only separated by one amino acid, and the type of residue which resides between tyrosine and threonine determines which isoform of MAPK the enzyme is (see below).

1.4.2 - MAPK Isoforms

As previously mentioned 12 different isoforms of MAPK have been identified so far and these 12 types can be split into three distinct subgroups. The ERKs consist of three types of MAPK, ERKs 1 and 2 being the most widely studied of all. Also known as p42/p44 MAPKs, these enzymes have a glutamine residue between tyrosine and threonine on the activation lip, and are mainly involved in cell proliferation and differentiation (Kolch, 2000). A second group is the p38 MAPKs, of which there are four members, p38α, p38β, p38γ, and p38δ (Paul *et al*, 1997, Ono & Han, 2000). Each p38 MAPK isoform possesses a glycine residue separating tyrosine and threonine, and are activated in response to cellular stress e.g. UV light, osmotic stress (Malarkey *et al*, 1995). Other roles include cytokine biosynthesis, muscle differentiation, and B cell proliferation (Zhao *et al,* 1999, Craxton *et al,* 1998). The third group, SAPKs, contain three isoforms of molecular sizes 46 kDa and 55 kDa (Ip & Davis, 1998). Also known as *c-jun* N-terminal kinases (JNKs) their tyrosine and threonine residues are separated by proline, and are also activated by cellular stresses, similar to p38 MAPKs (Davis, 2000). JNK1 and JNK2 are widely expressed, whereas JNK3 are more localised to the heart, lungs and testis.

Other MAPK isoforms have been discovered, although the knowledge about them at present is very limited. ERK5, also known as BMK1, is believed to have a role in cell cycle control and transformation, hence its classification as an extracellular

signal-regulated protein kinase (Lee *et al,* 1995, Zhou *et al,* 1995). ERK5 has been shown to phosphorylate the transcriptional regulators MEF2C and Myc, and has a C-terminal extension that may act in an inhibitory manner (Kato *et al,* 1998, English *et al,* 1999b). ERK3 and ERK4 are highly expressed in the heart, but their physiological role has yet to be determined (Michel *et al,* 2001). ERK3, or p97 MAPK, is of particular interest as its phosphorylation lip has serine and glycine residues in place of the threonine and tyrosine residues that are dual phosphorylated in all the other MAPK isoforms discovered to date (Gonzalez *et al,* 1992, Zhu *et al,* 1994). The role of its C-terminal is also unknown. A thirteenth MAPK isoform, ERK7, has also been identified (Abe *et al,* 1999) and is also believed to have a role in cell proliferation, but it's exact function has yet to be identified.

1.4.3 - MAPK Cascade Activation

Although, as explained in the last section, there are a wide number of MAPK isoforms, the mechanism for their activation follows a relatively conserved route. The basic components of all MAPK cascades are three kinases, specifically MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and the MAPK isoform itself, which are sequentially activated by phosphorylation (Widmann *et al,* 1999). The initial, extracellular stimuli of the MAPK cascade can be very diverse (growth factors, GPCR ligands, UV light, osmotic stress, etc.), and then may go through a number of stages before the first part of the conserved pathway, MAPKKK, is activated by phosphorylation or via interaction with a member of the GTP-binding Ras or Rho family. MAPKKKs are also serine/threonine protein kinases, as are MAPKs, and when activated then phosphorylate, and thus activate, the next kinase in the cascade, MAPKK (Siouw *et al,* 1997). Once activated this kinase recognises and

then phosphorylates both the threonine and tyrosine residues on the phosphorylation lip of the MAPK isoform, activating it (Gartner *et al,* 1992). The activated MAPK isoform then proceeds to phosphorylate substrates on their serine and threonine residues. The identity of MAPK substrates varies considerably, ranging from, more commonly, transcription factors located within the nucleus, to cytosolic targets like other protein kinases, phospholipases, and proteins associated with the cytoskeleton. For a summary of the MAPK cascade, see Figure 1.5.

It should be noted, however, that although this pathway is conserved in terms of there always being the three protein kinases involved, the identity of each kinase can vary greatly, with numerous MAPKKK isoforms being able to activate the same MAPKK. In fact, currently there are more MAPKKK isoforms identified and characterised in mammalian cells than MAPK isoforms (Widmann *et al,* 1999). Also, regulation of the cascade can vary, depending on which MAPK is being activated (see individual MAPK isoform sections).

Although up to thirteen MAPK isoforms have been identified relatively little is known about the MAPK isoforms not classified within the three main groups i.e. ERKs 3, 4, 5, and 7. This study will therefore only concentrate on the three main subgroups of MAPK isoforms i.e. ERKs 1 and 2, p38 MAPKs, and SAPKs, and no further mention will be made about the other four MAPK isoforms.

1.4.4 - The Extracellular Signal-Regulated Protein Kinases (ERKs)

The most widely studied and clarified MAPK isoform cascade is the pathway that activates ERKs 1 and 2, or p42/p44 MAPKs. As previously mentioned these two isoforms are ubiquitous within all eukaryotic cells and are involved in cell proliferation and differentiation. As with all MAPK isoforms, the extracellular

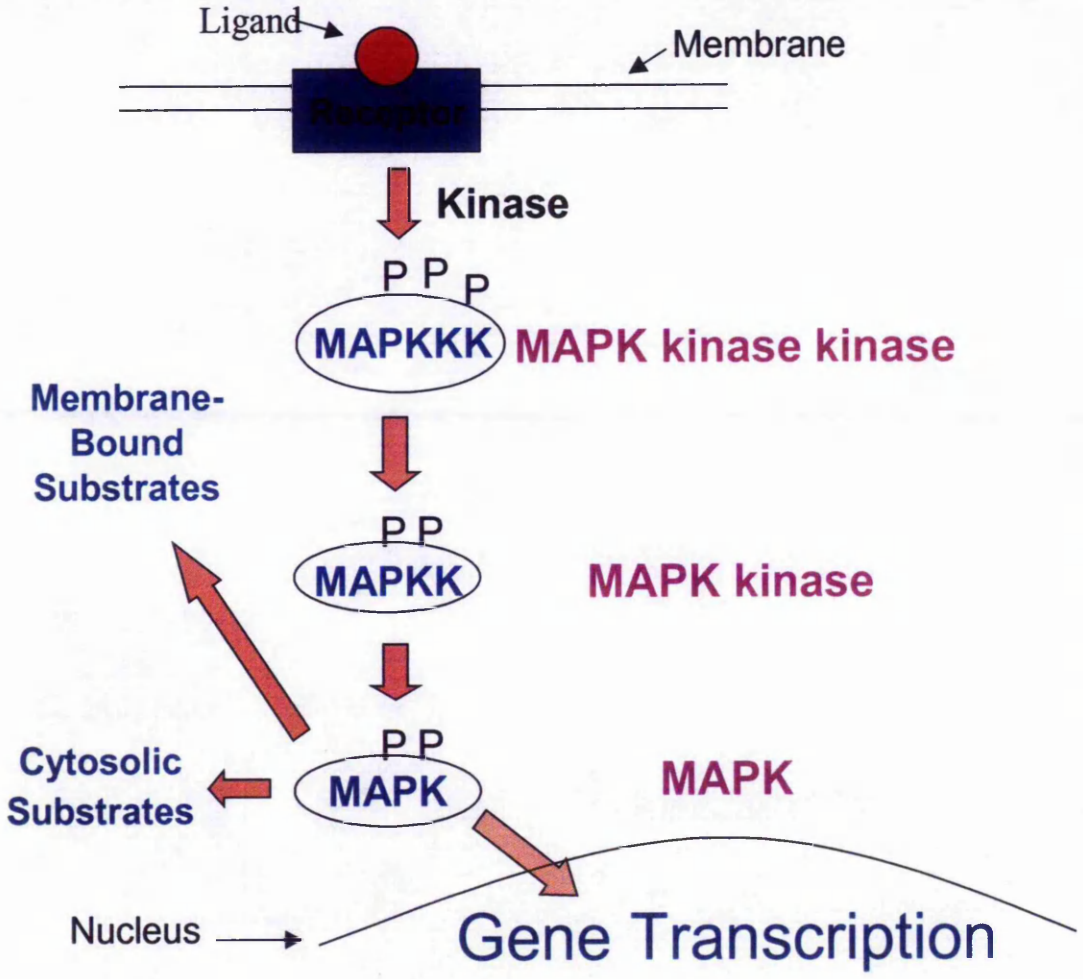


Figure 1.5 - The conserved three module MAPK pathway. Extracellular stimuli, via a number of steps, leads to phosphorylation of MAPK kinase kinase (MAPKKK). MAPKKK then causes the phosphorylation of MAPK kinase (MAPKK), which then dual-phosphorylates MAPK on its tyrosine and threonine residues found on the activation loop. MAPK goes on to cause gene transcription via phosphorylation of a number of transcription factors, and phosphrylates a number of cytosolic and membrane-bound substrates (Kolch, 2000).

stimuli which trigger the cascade can vary greatly. Classically, ERKs 1/2 are activated by growth factor receptor stimulation e.g. epidermal growth factor (EGF) and nerve growth factor (NGF) receptors (Cobb *et al*, 1994). However, more recent studies have shown GPCRs, T-cell antigens, and cytokines also activate this cascade (Guan, 1994).

1.4.4.1 - The ERK Cascade

Activation of the ERK cascade follows the conserved route described in section 1.4.3. Initialisation of the pathway, classically by growth factors e.g. EGF, leads to sequential tyrosine phosphorylation of the growth factor receptor, which is recognised by Src-homology 2 (SH2)-domain containing adaptor proteins, such as Shc and Grb2 (Chen *et al,* 1998). The Shc-Grb2 complex recruits SOS (son of sevenless), the guanine-nucleotide exchange factor, to the plasma membrane which catalyses GDP/GTP exchange on Ras, a small monomeric G-protein (Blenis, 1993). Ras becomes activated and then activates an isoform of Raf, a group of serine/threonine kinases, which are the MAPKKKs of this cascade. There are three known isoforms of Raf; Raf-1, A-Raf, and B-Raf (Kolch, 2000). It is believed Raf-1 is the most commonly involved isoform involved in the ERK cascade as it is fairly ubiquitous and has been implicated in tissue formation (Hagemann & Rapp, 1999). Raf-1 phosphorylates both MEK1 and MEK2 (the MAPK kinases of this pathway), a group of threonine/tyrosine kinases which possess the ability to dual-phosphorylate threonine and tyrosine residues, such as those found on the activation loops of MAPKs. MEKs 1 and 2 then phosphorylate ERKs 1 and 2, their only known target, which leads to cytoskeletal protein phosphorylation, serine/threonine kinase regulation (e.g. casein kinases and p90 ribosomal S6 kinase), activation of cell

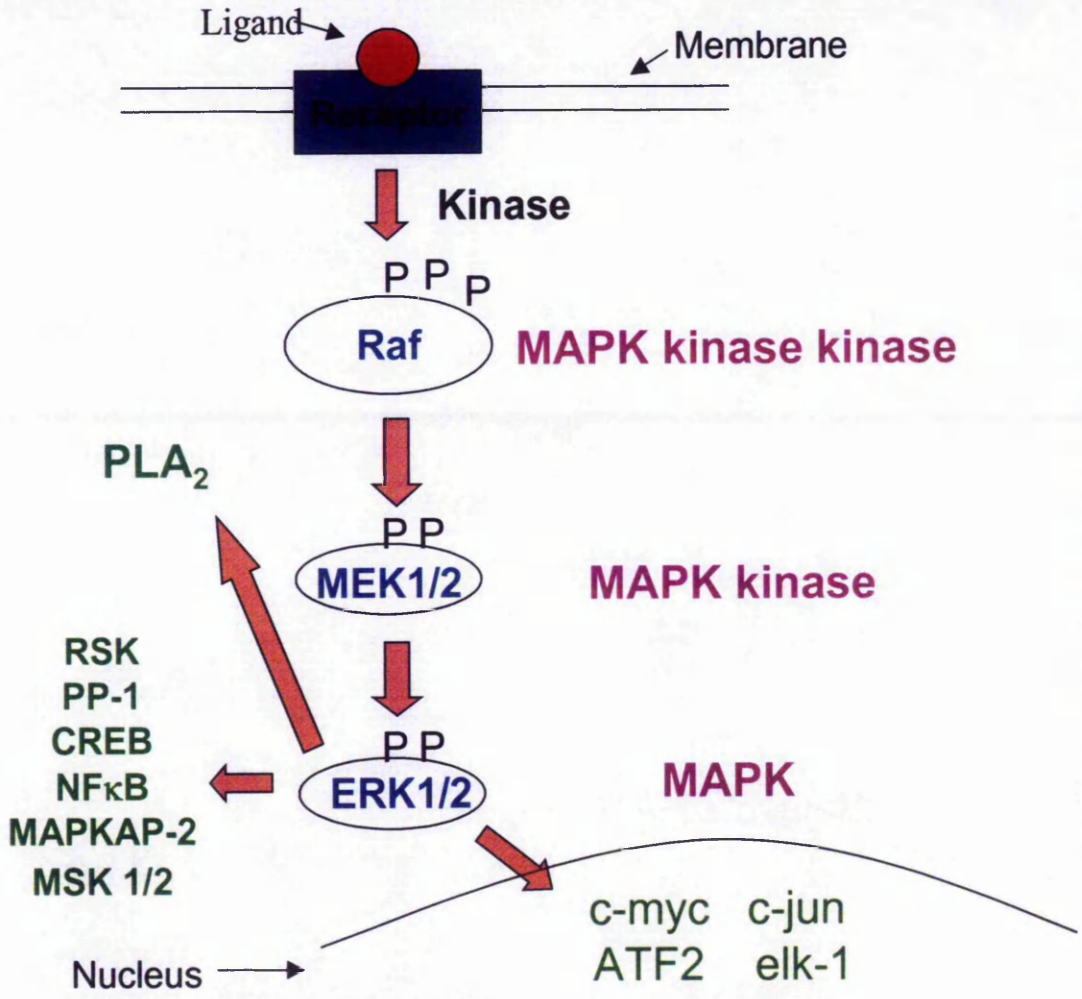


Figure 1.6 - The ERK, or p42/p44MAPK, pathway. Activation by growth factors, GPCRs, phorbol esters, cytokines, etc. leads to phosphorylation of a Raf isoform, usually Raf-1. Raf-1 then causes the phosphorylation of MEK 1/2, which then dual-phosphorylates ERKs 1/2 on its tyrosine and threonine residues found on the activation loop. ERKs 1/2 go on to cause gene transcription via phosphorylation of transcription factors c-myc, c-jun, ATF-2 and elk-1. It also phosphrylates cytosolic enzymes like CREB, RSK, PP-1, NFκB, MAPKAP-2 and MSKs 1/2, as well as membrane-bound PLA_2 which leads to increased prostaglandin and thromboxane production (Widmann, 1999).

signalling enzymes (e.g. PLA_2) and regulation of transcription factors e.g. c-myc, *c-jun*, ATF-2, and elk-1 (Blenis, 1993, Nemenoff *et al,* 1993, Karin, 1995). For a diagram showing the latter part of this pathway in comparison with the model described in section 1.4.3 and shown in Figure 1.5, see Figure 1.6.

1.4.4.2 - Downstream of the ERK cascade

ERKs 1 and 2 appear to be an important link between extracellular stimuli and a number of vital intracellular events. A number of substrates for the ERK isoforms exist, which can be split into three distinct groups (Davis, 1993).

1.4.4.2.1 - Protein Kinases Activated by ERK

The first group consists of protein kinases and includes the aforementioned p90 ribosomal S6 kinases, of which there are three mammalian isoforms; RSK1, RSK2, and RSK3 (Moller *et al,* 1994). Also known as $p90^{rsk}$ or MAPK-activated protein kinase-1 (MAPKAP-1), it has been implicated in a number of cellular processes (Frödin & Gammeltoft, 1999). $p90^{rsk}$ regulates gene expression by associating with and phosphorylating transcriptional regulators, such as *c-fos* (an immediate early gene), oestrogen receptors, NFκB and cAMP-response element-binding protein (CREB, a regulator of immediate early gene expression), and CREB-binding protein (Smith *et al,* 1999).

A second member of this group is MAPK-activated protein kinase-2 (MAPKAP-2). MAPKAP-2 has been shown to activate glycogen synthase, which goes on to phosphorylate the heat shock proteins hsp25 and hsp27 (Stokoe *et al,* 1992).

A third, more recently discovered members of this group are the mitogen- and stress-activated protein kinases 1 and 2 (MSK1 and 2). Predominantly located in the nucleus, these protein kinases also phosphorylates transcription factors, such as ATF-1, as well as also activating CREB (Deak *et al,* 1998).

1.4.4.2.2 - Protein Phosphatases Activated by ERK

The second group contains protein phosphatases, such as protein phosphatase-1 (PP-1). ERK has been shown to activate PP-1 via RSK, which phosphorylates PP-1 on its glycogen binding subunit. This then results in increased glycogen synthase phosphatase activity (Dent *et al*, 1990).

1.4.4.2.3 - Phopholipase A_2 (PLA_2) is Activated by ERK

Thirdly, as previously mentioned earlier in the introduction, ERK has the ability to activate PLA_2. It has been shown that PLA_2 is a substrate for ERK (Nemenoff *et al,* 1993, Lin *et al,* 1992) and can produce increased arachadonic acid release from cytosolically located PLA_2 enzymes. This, of course, leads to increased prostaglandin and thromboxane production.

1.4.4.2.4 - Nuclear Targets Activated by ERK

ERK also increases gene transcription via a number of nucleus-residing targets, such as those mentioned in section 1.4.4, i.e. *c-myc*, ATF-2, *elk-1,* and *c-jun*. Other transcription factor targets include *ets-1, sap1a* and *tal,* all of which are phosphorylated and activated by ERK (Michel *et al*, 2001). Conversely, however,

the transcription factor *myb* may be inhibited by the action of ERK (Widmann *et al*, 1999).

1.4.4.2.5 – The Role of the ERK Cascade in Cell Growth and Proliferation

Activation of the ERK cascade often leads to cell growth and proliferation. Various growth factors, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), which cause increases in both cell size and number, produce a corresponding stimulation of the ERK cascade (Widmann *et al,* 1999). Also, interfering with various components of the ERK pathway e.g. transfecting with dominant negative mutants, or antisense constructs for Raf-1 or ERK1, significantly reduces cell proliferation (Miltenberger *et al,* 1993, Pages *et al,* 1993, Seger *et al,* 1995). Conversely, constitiutively active MEK1 constantly activates ERK1, which leads to significantly increased cell proliferation (Pages *et al,* 1993).

1.4.4.2.5 – The Role of the ERK Cascade in Apoptosis

The ERK cascade also has an anti-apoptotic effect in a number of cell types. Constitutively active ERK1/2 in PC-12 cells inhibited apoptosis, and withdrawal of nerve growth factor (NGF) caused a reduction of ERK activation and resulted in cell death (Xia *et al,* 1995). Inhibition of ERK activation in L929 cells significantly reduces the anti-apoptotic effect after inducing apoptosis using TNF-α (Gardner & Johnson, 1996). This is due to ERK-mediated activation of RSK, which in turn, regulates transcription factors involved in mediating cell death e.g. *c-jun* (Frödin & Gammeltoft, 1999, Smith et al, 1999).

However, there is evidence for a pro-apoptotic role of ERK in some cells. Reduction of MEK1 activity inhibits crocidolite asbestos-induced and Fas-induced apoptosis (Goillot *et al,* 1997, Jimenez *et al,* 1997). This provides more evidence that the physiological actions of ERK are highly cell specific.

1.4.4.3 - Regulation of the ERK Cascade

Since activation of ERKs 1 and 2 is caused by dual phosphorylation, the main form of regulation is dephosphorylation by phosphatases. This is carried out by a group of phosphoprotein phosphatases known as MAPK phosphatases (MKPs), of which six isoforms have been discovered to date (English *et al,* 1999, Michel *et al,* 2001). MKP-1, also known as VH-1, specifically dephosphorylates ERKs 1 and 2 over the other MAPK isoforms. This appears to be a negative feedback mechanism since it has been shown that ERK phosphatases can be induced and/or activated by ERKs (Brondello *et al,* 1997, Camps *et al,* 1998).

Another regulation pathway is the effect of activated ERK on the Ras exchange factor, SOS. Dual-phosphorylated ERK disassembles the SOS/Grb-2/Shc complex formed upstream in the cascade by phosphorylating SOS, thus reducing further ERK activation (Kolch, 2000).

1.4.5 - The p38 MAPK Signal Transduction Pathway

First discovered when it was identified as an upstream kinase of MAPKAP-2 after cells were stimulated with interleukin-1 (IL-1) and LPS (Han *et al,* 1993, Han *et al,* 1994) this group of MAPKs are traditionally activated during cellular stress, such as

that caused by UV light, osmotic stress, and stimulation by pro-inflammatory cytokines (Brewster *et al,* 1993, Rouse *et al,* 1994). As previously mentioned there are currently four known isoforms of p38 MAPK, p38α, p38β, p38γ, and p38δ (Paul *et al,* 1997, Ono & Han, 2000). p38α and p38β are ubiquitous throughout all eukaryotes (Jiang *et al,* 1996), whereas p38γ (also known as ERK6 or SAPK3) and p38δ (also known as SAPK4) are distributed only in certain cell types (Ono & Han, 2000). p38γ MAPK is mainly found in skeletal tissue and is believed to be the p38 MAPK isoform responsible for skeletal muscle differentiation (Lechner *et al,* 1996, Li *et al,* 1996), while p38δ MAPK is predominantly distributed in the kidneys, lungs, testis, duodenum, and pancreas, and is developmentally regulated (Kumar *et al,* 1997, Hu *et al*, 1999).

1.4.5.1 - The p38 MAPK Cascade

Similarly to the ERK pathway, the p38 MAPK cascade can be activated by a wide variety of diverse stimuli. The most studied isoform of p38 MAPK is the p38α MAPK isoform, which has been shown to be activated by stimuli such as growth factors, like NGF (Morooka & Nishida, 1998), fibroblast growth factor (FGF, Xing *et al,* 1998), insulin-like growth factor-1 (IGF-1, Cheng & Feldman, 1998), vascular endothelial growth factor (VEGF, Rousseau & *et al,* 1997), platelet-derived growth factor (PDGF, Pyne & Pyne, 1997), granulocyte macrophage colony-stimulating factor (GM-CSF, Foltz *et al,* 1997), and interleukins 2, 3, and 7 (Nagata *et al,* 1997, Crawley *et al,* 1997). Other non-growth factor instigators include heat shock (Cuenda *et al,* 1997), cellular stretching (Kudoh *et al,* 1998), ischaemia and reperfusion (Bogoyevitch, *et al,* 1996), UV light, osmotic stress, and GPCRs (Ono &

Han, 2000). However, it should be noted that activation of the p38 MAPK cascade is not only dependent on the extracellular stimulus, but also the cell type on which the stimulus is having an effect. For example, in 3T3-L1 adipocytes insulin initiates the p38 MAPK cascade (Sweeney *et al,* 1999), but in chick forebrain neurone cells insulin reduces p38 MAPK activation (Heidenreich & Kummer, 1996).

As with nearly all the MAPK isoforms activation of p38 MAPKs follows the three module cascade described in section 1.4.3 and shown in Figure 1.5. However, identification of each member of the cascade is less defined compared to the ERK cascade. A diverse number of kinases have been isolated as potential MAPKKKs, which probably explains why the p38 MAPK cascade can be activated by such a wide range of extracellular stimuli (see above). One well-studied MAPKKK in the p38 MAPK cascade is MTK1. It has been shown to activate p38 MAPK (Takekowa *et al,* 1997, Cuenda & Darrow, 1998), but only mediates cellular stress signals e.g. UV light and osmotic stress, and not intracellular signals mediated by cytokines, such as TNF-α (Ogura & Kitamura, 1998). Other MAPKKKs shown to activate p38 MAPK include MLK2 and MLK3 (Hirai *et al,* 1997, Tibble *et al,* 1996), dlk (Fan *et al*, 1996, Hirai *et al,* 1997), ASK1 (Ichijo *et al*, 1997), and TAK1 (Moriguchi *et al,* 1996). All of these MAPKKKs have been shown to co-activate SAPKs and p38MAPKs when over-expressed, providing evidence as to why these two types of MAPK are often coactivation, although specific stimulation of one or the other MAPK has also been observed, again suggesting activation of either MAPK cascade is highly cell specific (Ogura & Kitamura, 1998).

The identity of MAPKKs that mediate dual-phosphorylation of p38 MAPKs is more defined. MKK3 and MKK6 both specifically activate p38 MAPKs, but whereas MKK6 can activate all four p38 MAPK isoforms, MKK3 preferentially activates only p38α, p38γ, and p38δ (Keesler *et al,* 1998). More non-specific activators of

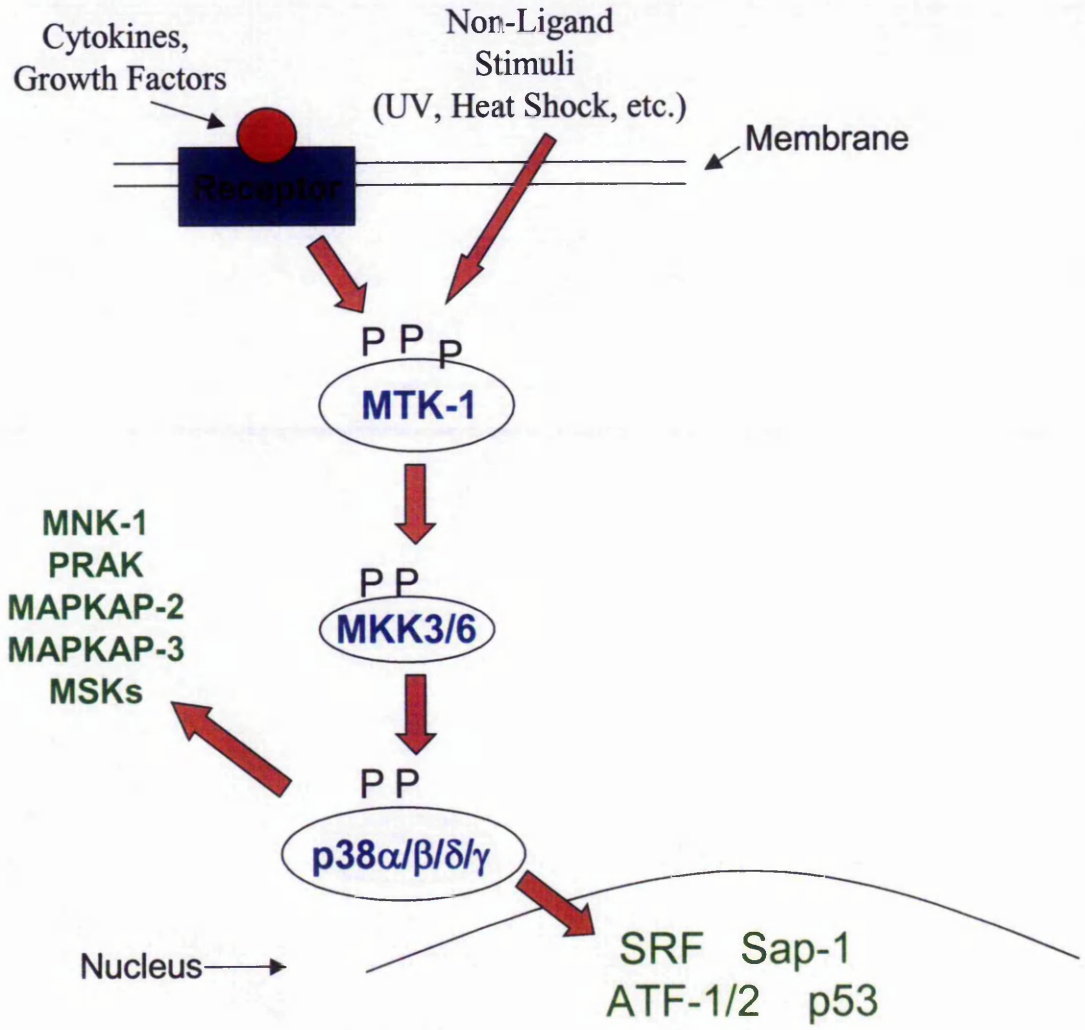


Figure 1.7 - The p38 MAPK pathway. Activation by growth factors, cytokines, GPCRs, and non-ligand stimuli (UV, heat shock, etc.) leads to phosphorylation of a MAPKKK e.g. MTK-1. MTK-1 then causes the phosphorylation of MAPKKs such as MKK3/6, which then dual-phosphorylates p38 MAPKs on their tyrosine and threonine residues found on their activation loops. p38 MAPKs go on to cause gene transcription via phosphorylation of transcription factors SRF, CHOP, ATF-1/2 and Sap-1. It also phosphorylates cytosolic enzymes like MAPKAP-2/3, MSKs, PRAK, and MNK-1 (Ono & Han, 2000).

p38 MAPKs include the SAPK dual-phosphorylators MKK4 and MKK7 (see section 1.4.6 for more detail). MKK4 has been shown to phosphorylate p38β and p38δ in one cell line, and MKK7 has been reported to phosphorylate p38δ also (Jiang *et al,* 1992, Hu *et al,* 1999). For a summary of the above, see Figure 1.7.

1.4.5.2 - Downstream of the p38 MAPK Cascade

Similar to the ERK cascade, the p38 MAPK cascade phosphorylates a wide variety of substrates (including some also activated by ERK), which include protein kinases and transcription factors (Ono & Han, 2000).

1.4.5.2.1 - Protein Kinases Activated by p38 MAPKs

As previously mentioned p38 MAPKs, specifically p38α, were first discovered after they were shown to phosphorylate MAPKAP-2, which is also activated by the ERK cascade (Han *et al,* 1993, Stokoe *et al,* 1992). A second, closely related protein kinase, MAPKAP-3, was then subsequently found to be a p38α substrate (McGlaughlin *et al,* 1996). These two protein kinases go on to phosphorylate various substrates, including the aforementioned hsp27 and CREB, and also lymphocyte-specific protein-1 (Huang *et al,* 1997), ATF-1 (Tan *et al,* 1996), SRF (Heidenreich *et al,* 1999), and tyrosine hydroxylase (Thomas *et al,* 1997).

Other downstream substrates shown to activate both p38α and p38β include MSKs, which are also activated by ERKs (Deak *et al*, 1998). MAPK interaction protein kinase-1 (MNK-1, Waskiewicz *et al,* 1997, Fukunaga & Hunter, 1997) is another substrate, which is involved in the initiation of translation. Finally there is p38

regulated/activated kinase (PRAK), which is preferentially activated by p38β and is capable of activating hsp27 (New *et al,* 1998).

1.4.5.2.2 - Nuclear Targets Activated by p38 MAPK

p38 MAPKs, specifically p38α, phosphorylate a number of transcription factors, including both ATF-1 and ATF-2, as opposed to the ERK cascade which only phosphorylates ATF-2 (Raingeraud *et al,* 1995, Abdel-Hafiz *et al*, 1992). Other transcription factors activated by p38α include SRF accessory protein 1 (Sap1), CHOP, and p53 (the latter two transcription factors are involved in regulating cell growth and differentiation). Also activated is NFAT, which, as previously mentioned, is an important transcription factor in the allergic response (Yang *et al,* 2002).

1.4.5.3 - Physiological Role of p38 MAPK Cascade Activation

As has already been mentioned, activation of the p38 MAPK cascade results in a number of biological actions. These actions can be split into five categories.

1.4.5.3.1 - p38 MAPK Cascade Activation and Apoptosis

One of the major areas of study currently is the part p38 MAPK activation is thought to play in apoptosis, which is also known as programmed cell death (Kinloch *et al,* 1999). Apoptosis is a highly conserved form of cell suicide involving a number of endogenous cellular enzymes, such as caspases and calpains (Fraser & Evan, 1996). As opposed to necrosis, which usually entails rapid cell lysis and the distribution of

the intracellular contents into the extracellular space, apoptosis involves the death of a cell while keeping the membrane integrity intact until very late in the denaturing process. It can be divided into five distinct steps.

The initial step after the apoptotic signal has been initiated is cellular membrane blebbing (Wylie *et al*, 1980). The second step involves chromatin that resides within the nucleus first condensing and then fragmenting. This is usually followed by DNA fragmentation, the most distinct and detectable step associated with apoptosis (Wyllie, 1980), although this does not always take place (Tomei *et al,* 1992). Finally, while the cytosolic organelles remain intact, fragments of the cell separate from the main structure to form apoptotic bodies, which are then engulfed by phagocytes.

p38 MAPK activation has been shown to mediate apoptosis initiated by a number of different stimuli, and in a variety of cell lines (Xia *et al,* 1995, Kummer *et al,* 1997, Ma *et al,* 1999). Also, inhibition of p38 MAPK activation, usually using the specific p38 MAPK inhibitor, SB 203580 (Cuenda *et al,* 1995), has been shown to have a protective effect on cells subjected to apoptotic stimuli (Schwenger *et al,* 1997, Ma *et al,* 1999). The precise role of p38 MAPK in apoptosis is still relatively unclear, but it is thought that p38 MAPKs are somehow involved with a group of cysteine proteases called caspases (Ono & Han, 2000). This very specific group of enzymes have numerous roles in the apoptotic process, including breaking down cytoskeletal proteins and inhibiting cellular survival processes (Cohen 1997, Thornberry & Lazebnik, 1998). How p38 MAPK stimulation causes caspase activation has yet to be clarified.

1.4.5.3.2 - Cell Specific Actions of p38 MAPK Activation

Although p38 MAPKs predominantly appear to mediate apoptosis in most cells, there is evidence to suggest they may have an opposite role in some cell types. Although it has been shown that p38 MAPK is involved in cardiomyocyte apoptosis (Ma *et al,* 1999) another study has demonstrated a role for p38 MAPK in cardiomyocyte hypertrophy (Wang *et al,* 1998). These opposing effects appear to be due to the different isoforms of p38 MAPK present. In cardiomyocytes, p38α appears to be responsible for mediating apoptosis, whereas p38β appears to cause cell hypertrophy. Further studies into how each isoform is activated and why could explain differences in responses to p38 MAPK activation in other cell lines, and may provide valuable therapeutic information into finding new treatments for cardiac patients.

1.4.5.3.3 - p38 MAPK Cascade Activation and Cell Differentiation

In some cell lines p38 MAPKs may, like the ERKs, play a part in cell differentiation. 3T3-L1 cells differentiating into adipocytes, and the differentiation of PC12 cells into neurones both appear to involve activation of either or both p38α and p38β MAPKs (Morooka & Nishida, 1998, Engelman *et al*, 1998). It is thought transcription factors found downstream of the p38 MAPK cascade, such as CREB, may be responsible for this action (Ono & Han, 2000).

1.4.5.4 - Regulation of the p38 MAPK Cascade

To date, only a group of dual phosphatases have been identified as p38 MAPK cascade regulators. MKP-1, which also regulates the ERK pathway, has the ability to dephosphorylate isoforms p38α and p38β (Sun *et al,* 1993). Two other members of this group have also been shown to specifically inhibit both these p38 MAPK isoforms, namely MKP-4 and MKP-5 (Muda *et al,* 1996, Camps *et al,* 1998). All three phosphatases are inducible, and, as yet, no regulatory mechanism has been identified for the other two known p38 MAPK isoforms.

1.4.6 - The JNK/SAPK Protein Kinase Pathway

The first member of this final MAPK subgroup was first identified as a "p54 microtubule-associated protein kinase" that was activated by cyclohexamide (Kyriakis & Avruch, 1990). It was found to bind to the N-terminal activation domain of the transcription factor *c-jun* (Adler *et al,* 1992, Hibi *et al,* 1993), and dual phosphorylate it at residues Ser^{63} and Ser^{73} (Pulverer *et al,* 1991). The kinase was thus named *c-jun* N-terminal kinase (JNK, Davis, 2000). Further studies showed that this kinase could be activated by treatment of cells with cytokines, or with a number of environmental stress stumuli, such as osmotic and redox stress, and UV radiation, similar to p38 MAPKs. JNKs were then also named as stress-activated protein kinases (SAPKs, Ip & Davis, 1998).

To date, three distinct types of JNKs/SAPKs have been identified. JNK1 and JNK2 are ubiquitous in eukaryotes, whereas JNK3 is only expressed in the brain, heart, and testis (Ip & Davis, 1998). These three JNKs/SAPKs can be further divided into four JNK1 isoforms, four JNK2 isoforms, and two JNK3 isoforms (Gupta *et al,* 1996).

Each of the three types of JNK/SAPK is expressed as both 46kDa and 54kDa kinases, due to differential processes at the genetic level, but the physiological implications of this are still unclear (Davis, 2000).

1.4.6.1 - The JNK/SAPK Cascade

The JNK/SAPK cascade is activated by very similar stimuli to the p38 MAPK cascade, such as the aforementioned osmotic stress, UV radiation, and inflammatory cytokines such as TNF-α and IL-1. This leads to activation of a number of MAPKKKs, some of which are mentioned in section 1.4.5.1 since they also activate the p38 MAPK pathway. How the extracellular stimuli leads to MAPKKK activation is down to a number of proteins, and is somewhat dependent on the nature of the stimulus. The Rho family of GTPases have been shown to induce the JNK/SAPK cascade via MAPKKK activation for a number of different extracellular stimuli (Fanger *et al*, 2000). Rho activation is, in turn, mediated by tyrosine kinases, similar to those involved in the ERK cascade (Schlessinger, 2000). Other mediators of the JNK/SAPK cascade are the TRAF adaptor proteins (Lui *et al*, 1996), which are mainly responsible for cytokine-induced MAPKKK activation e.g. IL-1 activates TRAF6 which then binds to a MAPKKK (Lomaga *et al*, 1999).

As well as the MAPKKs mentioned in section 1.4.5.1 a number of other proteins act as MAPKKs in the JNK/SAPK cascade. These include MEKK1 and Tpl-1/2 (Fanger *et al*, 1997), MEKK2 (Blank *et al*, 1996, Deacon & Blank, 1997)), MEKK3 (Ellinger-Ziegelbauer *et al*, 1997, Gerwins *et al*, 1997), and MEKK5 (Wang *et al*, 1996). These proteins act as MAPKKKs in the three module cascade described in 1.4.3. However, other mediators of the JNK/SAPK cascade include the Ste20 homologues, such as p21-activated kinase (PAK), germinal centre kinase (GCK),

and hematopoietic progenitor kinase (HPK), which activate JNK/SAPK via a different and, as yet, unknown mechanism (Fanger *et al,* 1997).

Two MAPKKs are involved in dual phosphorylating JNK/SAPKs, namely MKK4 (also known as SAPK/ERK kinase 1, or SEK1) and MKK7 (Sanchez *et al,* 1994, Tournier *et al,* 1997). To date, three isoforms of MKK4 have been identified and are mainly activated by environmental stress (Davis, 2000). Six MKK7 isoforms have so far been discovered, and are usually activated by cytokines (Tournier *et al,* 1999). For a summary of the above, see Figure 1.8.

1.4.6.2 - Downstream of the JNK/SAPK Cascade

Unlike both the ERK and p38 MAPK cascades, targets of activated JNK/SAPK isoforms appear mainly to be transcription factors within the cell nucleus, with no cytosolic targets currently having been discovered. JNK/SAPKs do have similar targets to both ERK (Elk-1) and p38 MAPK (Elk-1 and ATF-2), but also have specific targets exclusive to this pathway.

1.4.6.2.1 - JNK/SAPK Activation of Transcription Factors

The JNK/SAPK cascade has been shown to increase the transcriptional activity of a particular group of transcription factors, namely the Ets domain proteins. This group includes the aforementioned Elk-1 (Whitmarsh *et al,* 1998), and also Sap-1 (Strahl *et al,* 1996, Janknecht *et al,* 1997). Finally, it has been demonstrated that the JNK/SAPK pathway activates p53 (Milne *et al,* 1995, Hu *et al,* 1997), Dpc4 (Afti *et al,* 1996), and nuclear factor of T cells-4, NFAT4 (Chow *et al*, 1997).

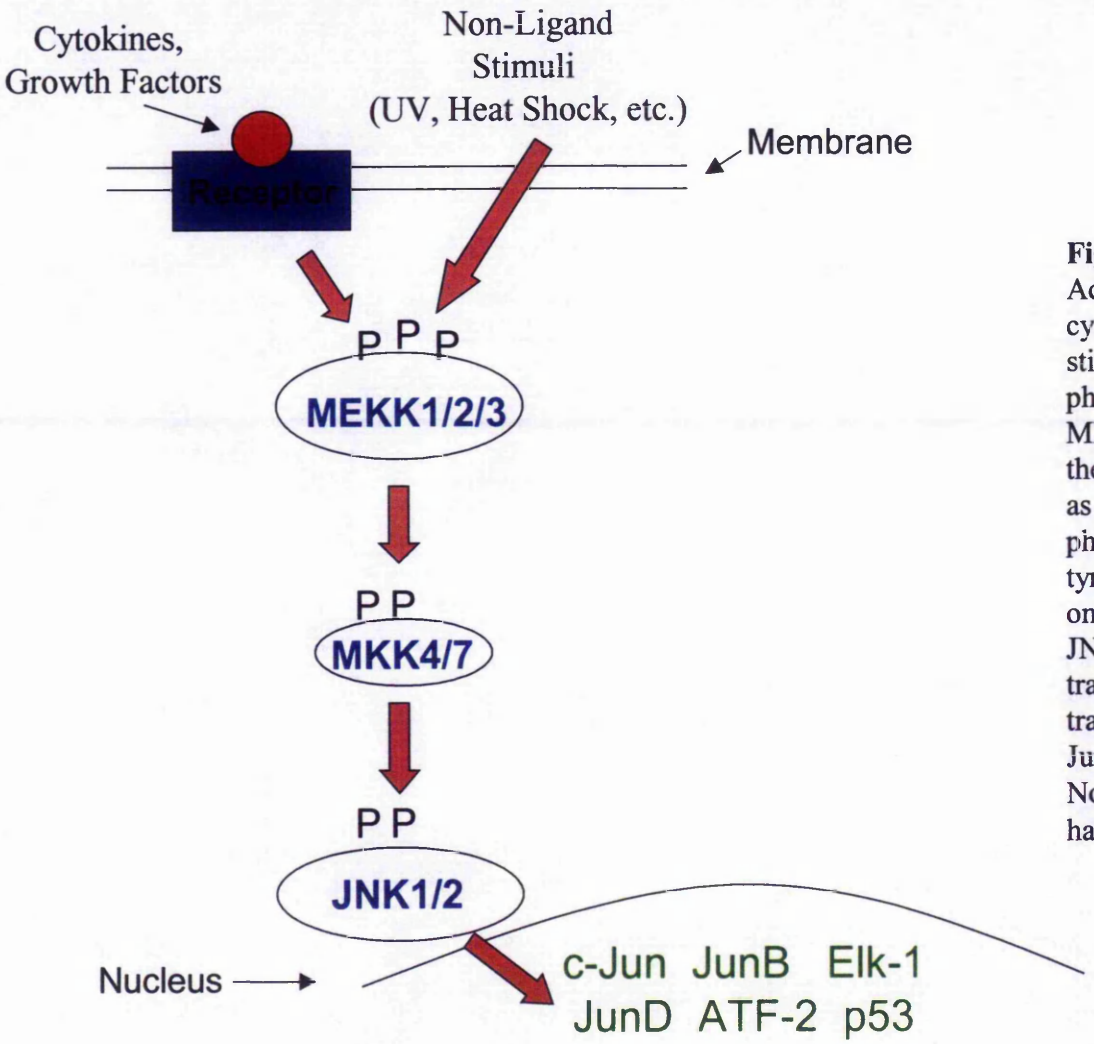


Figure 1.8 - The JNK/SAPK pathway. Activation by growth factors, cytokines, GPCRs, and non-ligand stimuli (UV, heat shock, etc.) leads to phosphorylation of a MAPKKK e.g. MEKK1/2/3. MEKK1/2/3 then causes the phosphorylation of MAPKKs such as MKK4/7, which then dual-phosphorylate JNK/SAPKs on their tyrosine and threonine residues found on their activation loops. JNKs/SAPKs go on to cause gene transcription via phosphorylation of transcription factors c-Jun, JunB, JunD, Elk-1, ATF-2, p53, and Sap-1. No cytosolic targets for JNKs/SAPKs have yet been identified (Davis, 2000).

1.4.6.3 - Physiological Implications of JNK/SAPK Cascade Activation

Activation of the JNK/SAPK pathway has a number of physiological effects. Three of the main processes that JNKs/SAPKs are currently known to be involved in are described below.

1.4.6.3.1 - The JNK/SAPK Cascade and Apoptosis

The JNK/SAPK pathway was initially implicated in mediating apoptosis by Xia *et al* (1995), when they discovered the cascade had a role in neuronal cell death. Following exposure to a pro-apoptotic signal the JNK/SAPK cascade is activated, although whether this happens at an early or late stage is still unclear (Xia *et al,* 1995, Virdee *et al,* 1997). Activation of JNK-/SAPK-mediated AP-1 transcription factors has also been shown to occur during programmed cell death (Herdegen *et al,* 1997), but the mechanism via which this leads to apoptosis is still unclear. However, this pro-apoptotic pathway appears to be recessive when co-activated with anti-apoptotic mechanisms, such as activation of the ERK cascade or PKB stimulation (Xia *et al,* 1995, Datta *et al,* 1997). It should be noted though that the above applies to apoptosis mediated by stress stimuli, such as UV radition or osmotic stress. Cell surface receptor-mediated apoptosis e.g. Fas or TNF-α, is dependent on caspases, not the JNK/SAPK cascade. However, JNKs/SAPKs do have a role in this mechanism, as they mediate caspase-9 activation by the mitochondria (Tournier *et al,* 2000).

Conversely, JNK/SAPK activation has also been shown to have an anti-apoptotic effect. One mechanism is JNK-/SAPK-mediated p53 phosphorylation (Fuchs *et al,*

1998a). p53 arrests the cell cycle and allows various repair processes to take place e.g. DNA reconstruction (Potapova *et al,* 1997).

1.4.6.4 - Regulation of the JNK/SAPK Cascade

Similar to both ERK and p38 MAPK, JNK/SAPKs are regulated by the action of the MAPK phosphatases, MKPs. MKP-1, which dephosphorylates ERK and p38 MAPK isoforms, can inactivate JNK/SAPKs also (Franklin & Craft, 1997). The JNKs/SAPKs have also been shown to induce MKP-1 in some cell lines, forming a negative feedback loop (Bokemeyer *et al,* 1996, Widmann *et al*, 1999). Other MKPs that regulate the JNK/SAPK cascade include M3/6, also known as hVH-5 (Muda *et al,* 1996). However, it is not known if M3/6 is a specific regulator of the JNK/SAPK cascade, or if it is involved in other pathways (Widmann *et al*, 1999).

A second mechanism of regulation also exists. JNK interacting proteins (JIPs), found in the cytosol, decrease transcription factor activation mediated by the JNK/SAPk pathway (Dickens *et al,* 1997). This group of proteins contains three isoforms, JIP-1, JIP-2, and JIP-3 (Davis, 2000).

Finally, a third mechanism has been suggested. G protein pathway suppressor-2 (GPS2) has been shown to suppress JNK/SAPK activation mediated by TNF-α and Tax (Jun *et al,* 1997).

1.4.7 - Phosphoinositol 3-Kinase (PI-3K) and Protein Kinase B (PKB)

PI-3Ks are a group of enzymes that generate inositol phospholipids e.g. phosphotidylinositol-3,4,5-triphosphate (PIP_3), that are involved in cell growth regulation (Vanhaesebroeck & Alessi, 2000). Multiple isoforms of PI-3K exist,

which can be split into three distinct classes; class I PI-3Ks, class II PI-3Ks, and class III PI-3Ks (Vanhaesebroeck *et al,* 1997, Fruman *et al,* 1998). Class I PI-3Ks are heterodimers consisiting of a 110kDa catalytic subunit (p110) and a regulatory/adapter subunit (Wyman & Pirola, 1998). This class can be further subdivided into 2 groups; class I_A PI-3Ks are linked to tyrosine kinases, while class I_B PI-3Ks are linked to GPCRs (Vanhaesebroeck & Alessi, 2000).

Class I PI-3K, but not classes II or III, has been shown to activate protein kinase B (PKB, or Akt), a 57kDa serine/threonine kinase located in the cytosol (Coffer *et al,* 1998). This activation can be direct, or indirect via PDK1 (Vanhaesebroeck & Alessi, 2000). PKB has a number of intracellular targets, found in both the cytosol and the nucleus, but three of these are of particular interest with respect to MAPK cascades.

One target is Raf, which is a MAPKKK in the ERK cascade. PKB has been shown to interact with and inhibit Raf, via phosphorylation on residue Ser^{259}, and thus reduce ERK phosphorylation (Zimmermann & Moeling, 1999). However, PI-3K inhibitors e.g. wortmannin and LY 294002, have no effect on or inhibit Raf activation in some cell lines, suggesting PKB may activate Raf in certain conditions, leading to increased ERK phosphorylation (Duckworth & Cantley, 1997, Wennstrom & Downward, 1999).

The second target of interest is caspase-9, a protease vital in the initiation of apoptosis (Wolf & Green, 1999). One of the main pathways to programmed cell death, caspase-9 is activated by mitochondrial cytochrome c following an apoptotic stimulus (Thorberry & Lazebnik, 1998). Caspase-9 then goes on to cleave a domain off caspase-3, activating it and instigating the characteristic biological processes of apoptosis e.g. membrane blebbing, chromatin condensation, etc. (Villa *et al,* 1997). PKB has been identified as a possible regulator of caspase-9 activity via

phosphorylation of the caspase, thus inhibiting the action of cytochrome c (Cardone *et al,* 1998).

Finally, PKB can phosphorylate the pro-apoptotic BCL-2 family member, BAD (del Peso *et al,* 1997, Datta *et al,* 1997). BAD has been shown to heterodimerise with other Bcl-2 family members i.e. Bcl-2 and Bcl-X_L, promoting their survival and therefore inducing apoptosis (Downward, 1998). Phosphorylation of BAD at residue Ser^{136} by PKB creates a binding site on BAD for 14-3-3, a family of highly expressed adaptor proteins (Coffer *et al,* 1998). When bound to 14-3-3, BAD cannot heterodimerise with Bcl-2 and Bcl-X_L, leading to inhibition of survival of these Bcl-2 family members, and thus protection of the cell from denaturing via apoptosis (Zha *et al,* 1996).

1.5 - G Protein-Coupled Receptors and the MAPK Cascade

A few years after the ERK cascade had been identified (Ray & Sturgill, 1987) a number of studies demonstrated activation of the ERK cascade by GPCRs (L'Allemain *et al,* 1991, Albas *et al,* 1993, Howe & Marshall, 1993). Since then it has been found that the p38 MAPK and JNK/SAPK cascades can also be activated by GPCRs (van Biesen *et al,* 1996), and that the mechanism of activation for all three MAPK pathways varies depending on the type of G protein the receptor is coupled to and the type of cell the GPCR is expressed on (English *et al,* 1999).

1.5.1 - $G_{i/o}$ Protein-Coupled Receptor Activation of the MAPK Cascades

$G_{i/o}$PCRs, such as the lysophosphatidic acid (LPA) and muscarinic m2 acetylcholine receptors, were the first group of GPCRs to be identified as MAPK cascade

activators (L'Allemain *et al,* 1991, Albas *et al,* 1993, Howe & Marshall, 1993). How $G_{i/o}$PCRs activate each MAPK pathway is described below, and is summarised in Figure 1.9.

1.5.1.1 - $G_{i/o}$ Protein-Coupled Receptor Activation of the ERK Cascade

$G_{i/o}$PCR activation of the ERK cascade is mediated via their βγ subunits (van Biesen *et al,* 1995). Activation of the $G_{i/o}$PCR by ligand binding leads to dissociation of the α- and βγ-subunits, and the βγ-subunits then proceed to mediate the tyrosine phosphorylation of Shc via a member of the Src-family of tyrosine kinases. The Src-family member then recruits either a receptor tyrosine kinase e.g. an EGF receptor, or a non-receptor tyrosine kinase e.g. Pyk2, to produce Shc phosphorylation (Dikic *et al,* 1996, Daub *et al,* 1996). Activation of ERK then follows a similar pathway to that activated by growth factors i.e. Shc phosphorylation leads to the formation of the Shc-Grb2-Sos complex on the plasma membrane, which causes Ras to lose its GDP molecule and gain a GTP molecule (English *et al,* 1999). Ras-GTP phosphorylates, and thus activates, an isoform of Raf (usually Raf-1), which then proceeds to activate MEK1/2, which dual phosphorylates ERK1/2 (Sugden & Clerk, 1997).

How the βγ-subunits of the $G_{i/o}$PCR mediates Src activation is still unclear. However, it is thought that an isoform of phosphoinositol 3-kinase (PI-3K), specifically PI-3Kγ, is recruited by βγ-subunits to the plasma membrane, and causes production of phosphotidylinositol-3,4,5-triphosphate, PIP_3 (Lopez-Ilasaca *et al,* 1997). PIP_3 can then activate Src via the SH2 domain contained within the tyrosine kinase (Vanhaesebroeck & Alessi, 2000). The PI-3K inhibitors, wortmannin and LY294002, inhibited $G_{i/o}$PCR-mediated ERK activation in a number of studies,

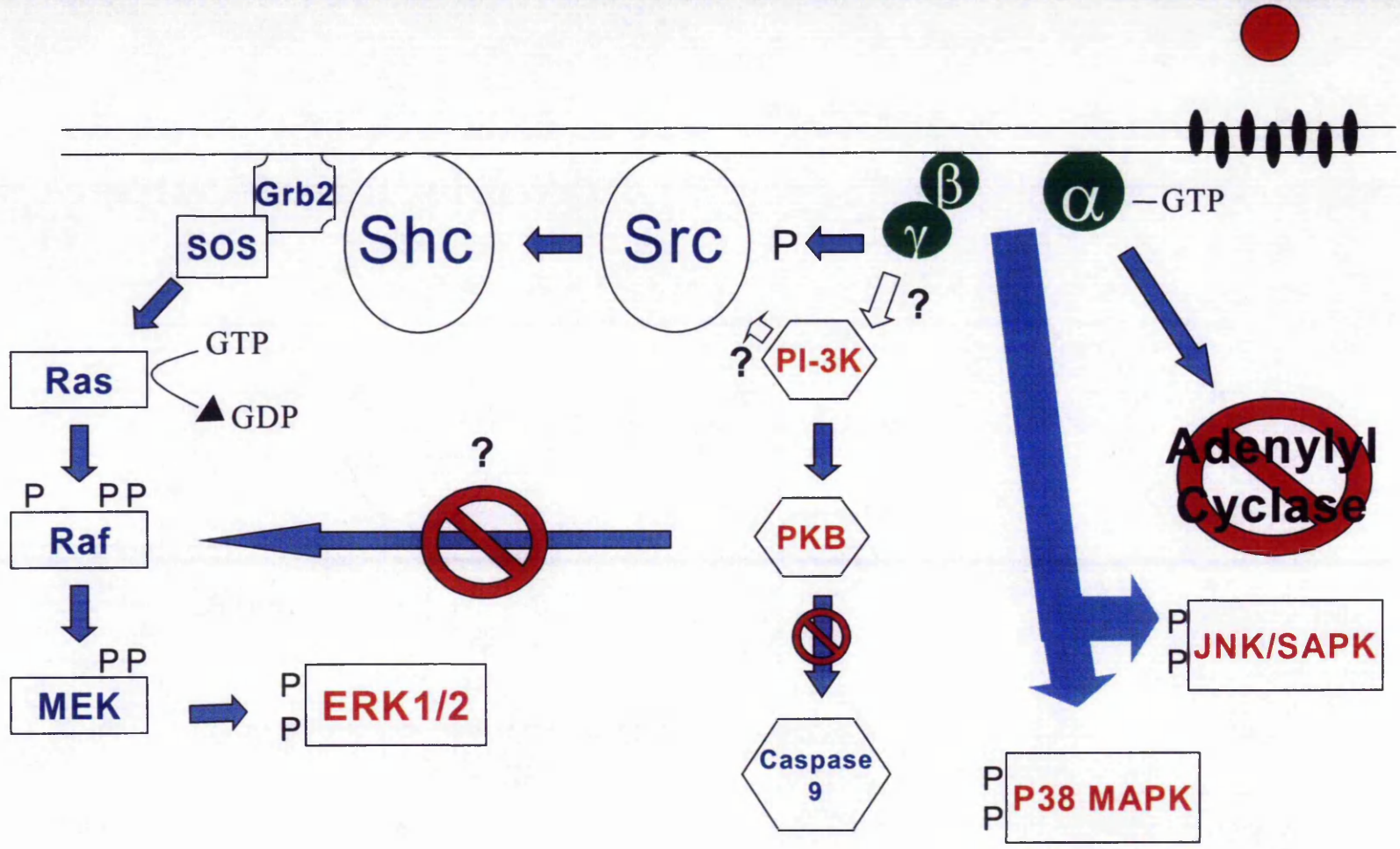


Figure 1.9 – Activation of MAPK cascades by Gi/o protein-coupled receptors. Activation of ERK involves βγ-subunits, Src family protein kinases, formation of the Shc-Grb2-SOS complex, guanine nucleotide exchange on Ras, followed by Raf activation, MEK activation, and ERK dual phosphorylation. p38 MAPK may also involve gb-subunits, and MKK3/6 probably dual phosphorylates the MAPK. JNK/SAPK activation invloves possibly both α- and βγ-subunits, and are dual phosphorylated by MKK4/7 (Widmann, 1999, English *et al,* 1999).

supporting this theory (Touhara *et al,* 1995, Knall *et al,* 1996, Hawes *et al,* 1996). One study, though, has suggested another pathway involving PI-3K, giving evidence for $G_{i/o}$PCR-mediated ERK activation mediated by PI-3Kζ activation of PKC, which is independent of Ras (Takeda *et al,* 1999).

However, there have been recent studies to propose a tyrosine kinase/Ras-independent pathway may exist for $G_{i/o}$PCR-mediated ERK activation. The GTP-binding protein, Rap1, can inhibit Ras-dependent ERK activation by $G_{i/o}$PCRs (Zwartkruis & Bos, 1999). A study by Mochizuki *et al* (1999) has suggested $G_{i/o}$ proteins recruit a Rap1 GTPase-activating protein (GAP), rap1GAPII, to the plasma membrane, causing a decrease of GTP-bound Rap1 levels. This may imply that some $G_{i/o}$PCRs activate ERK by inactivating Rap1 and not via Ras activation.

1.5.1.2 - $G_{i/o}$ Protein-Coupled Receptor Activation of the p38 MAPK Cascade

$G_{i/o}$PCR-mediated activation of the p38 MAPK pathway is less understood. $G_{i/o}$PCRs have been shown to activate the p38 MAPK cascade (Yamauchi *et al,* 1997, Clerk *et al,* 1998, Pellieux *et al,* 2000), but the exact intracellular signalling mechanisms are unclear. Initial data from the study by Yamauchi *et al* (1997) gives evidence for the involvement of $\beta\gamma$-subunits, but not α-subunits, similar to $G_{i/o}$PCR-mediated activation of the ERK cascade. Which MAPKKKs and MAPKKs are involved in $G_{i/o}$PCR-mediated activation of the p38 MAPK cascade is currently unclear, as is which specific p38 MAPK isoforms are activated.

1.5.1.3 - $G_{i/o}$ Protein-Coupled Receptor Activation of the JNK/SAPK Cascade

$G_{i/o}$PCR-mediated activation of the JNK/SAPK cascade has been demonstrated by a variety of GPCRs in a number of cell lines (Coso *et al,* 1995, Mitchell *et al,* 1995, Coso *et al,* 1996). It has been reported that, similar to the ERK pathway, βγ-subunits are involved in activating the cascade, and the small G proteins Ras and Rac, as well as PI-3Kγ, are also involved (Coso *et al,* 1996, Lopex-Ilasaca *et al,* 1998). Activation of JNK/SAPK by this route appears to be mediated by MKK4 (Yamauchi *et al*, 1999). Another possible pathway for $G_{i/o}$-βγ-subunit activation of JNK/SAPK involves a non-Src protein tyrosine kinase, the small GTPases Rho and Cdc42, and MKK7 (Yamauchi *et al,* 2000).

A role for $G_{i/o}$ proteins has been proposed also. Evidence suggests $G_{i/o}$-α-subunit activation of Src, leading to Rho and Cdc42 phosphorylation, and finally JNK/SAPK activation by a currently unidentified MAPKK (Yamauchi *et al,* 2000). Evidence has shown this pathway can be activated simultaneously with the $G_{i/o}$-βγ-subunit-mediated cascade.

1.5.2 - $G_{q/11}$ Protein-Coupled Receptor Activation of the MAPK Cascades

$G_{q/11}$PCRs, e.g., α_{1B}-adrenoceptors and bradykinin B_2 receptors, were the next group of GPCRs identified as stimulators of the MAPK cascade (Bogoyevitch *et al,* 1996a, Clerk *et al*, 1996). The pathways for each MAPK subfamily are described below, and summarised in Figure 1.10.

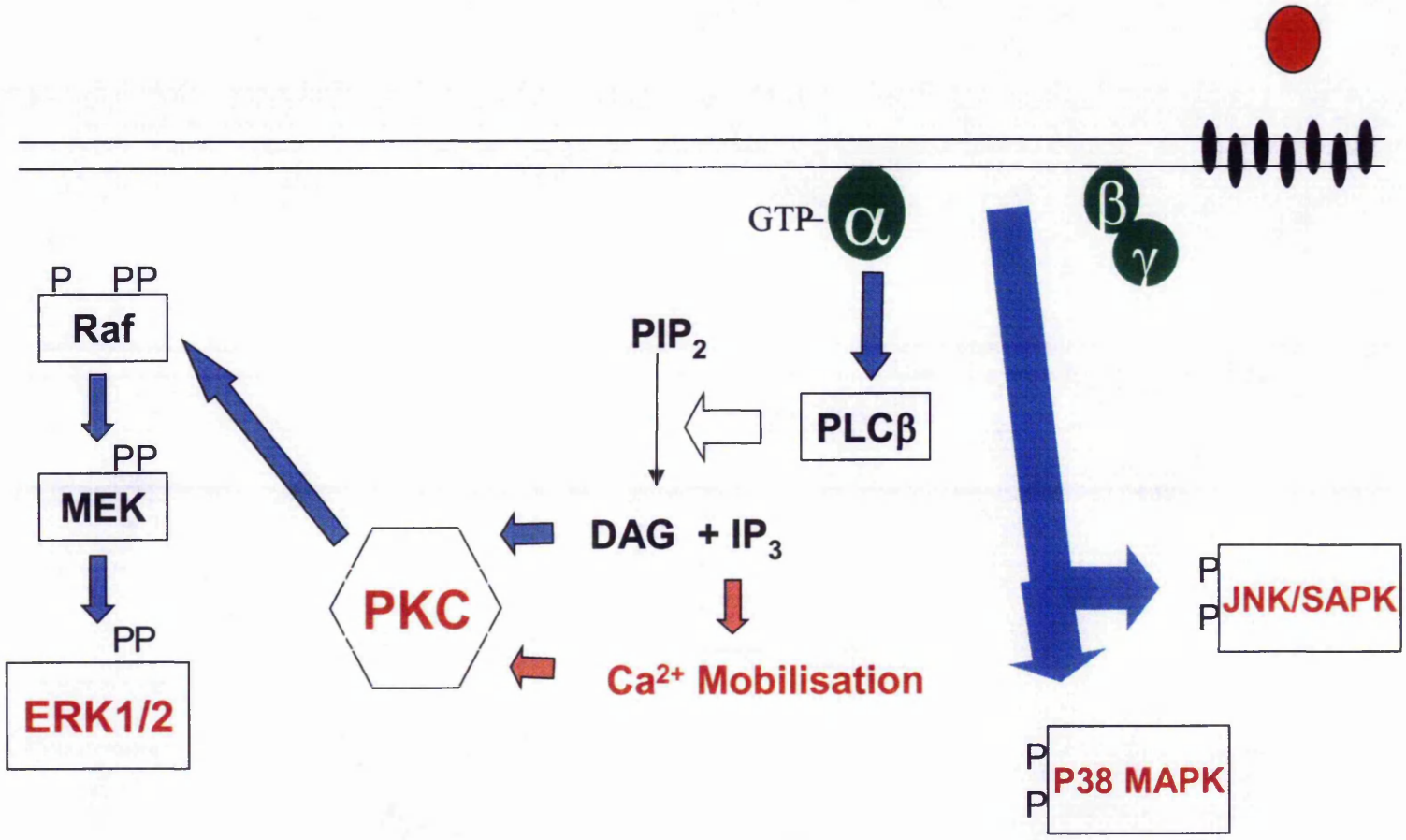


Figure 1.10 – Activation of MAPK cascades by $G_{q/11}$ protein-coupled receptors. Activation of ERK involves α- subunits, activation of PLCβ, leading to IP_3 and DAG formation, Ca^{2+} mobilisation and PKC activation, followed by Raf activation, MEK activation, and ERK dual phosphorylation. p38 MAPK may also involve α-subunits, and MKK3/6 probably dual phosphorylates the MAPK. JNK/SAPK activation involves α-subunits, as well as Ca^{2+} mobilisation and PKC activation, and are dual phosphorylated by MKK4/7 (Yamauchi *et al,* 1997, English *et al,* 1999).

1.5.2.1 - $G_{q/11}$ Protein-Coupled Receptor Activation of the ERK Cascade

$G_{q/11}$PCR-mediated activation of the ERK pathway usually involves PKC. It starts via direct activation of PLCβ by $G_{q/11}$-α-subunits, causing production of IP_3 and DAG. DAG and the resultant increase in intracellular Ca^{2+} levels activate conventional isoforms of PKC, which are able to phosphorylate Raf-1 (and possibly A-Raf) and thus activate ERKs (Bogoyevitch *et al,* 1995, English *et al*, 2000).

There is evidence for other pathways that cause ERK activation however. In one cell line $G_{q/11}$PCRs can activate Pyk2, a Src family tyrosine kinase, and may follow a similar pathway to $G_{i/o}$PCR-mediated ERK activation (Della Rocca *et al,* 1999a). Endothelin-1, a $G_{q/11}$PCR agonist, stimulates the EGF receptor, leading to ERK activation via transactivation, which is also similar to one route to $G_{i/o}$PCR-mediated ERK activation (Daub *et al*, 1996)

1.5.2.2 - $G_{q/11}$ Protein-Coupled Receptor Activation of the p38 MAPK Cascade

$G_{q/11}$PCRs can also activate the p38 MAPK cascade, such as the α_{1B}-adrenoceptors on cardiac myocytes (Clerk *et al*, 1998, Sugden & Clerk, 1998). The cascade appears to be activated by $G_{q/11}$-α-subunits and does not involve $G_{q/11}$-βγ-subunits (Yamauchi *et al,* 1997, Michel *et al*, 2001), but relatively little else is known about the identity or order of this cascade's constituents. However, since α_{1B}-adrenoceptors can activate both the p38 MAPK and JNK/SAPK cascades (see below), this suggests that MKK4 and/or MKK7 are involved, since these MKKs can activate both MAPK subfamilies.

1.5.2.3 - $G_{q/11}$ Protein-Coupled Receptor Activation of the JNK/SAPK Cascade

The JNK/SAPK cascade can be activated by a number of $G_{q/11}$PCRs, such as the prostaglandin $F_{2\alpha}$ receptor and the α_{1B}-adrenoceptor (Adams *et al,* 1998, Zhong & Minneman, 1999). Although still somewhat unclear, some members of the $G_{q/11}$PCR-mediated JNK/SAPK cascade appear to have been identified. Evidence suggests activation is via $G_{q/11}$-α-subunits (Yamauchi *et al,* 2001) and studies have implied that both PKC and increased intracellular Ca^{2+} are also needed for JNK/SAPK activation by $G_{q/11}$PCRs (Kudoh *et al,* 1997, Pellieux *et al,* 2000). As mentioned above, the fact that both p38 MAPKs and JNKs/SAPKs can be activated by the same $G_{q/11}$PCR suggests MKK4 and/or MKK7 may be responsible for JNK/SAPK phosphorylation.

1.5.3 - G_s Protein-Coupled Receptor Activation of the MAPK Cascades

G_sPCR-mediated activation of the MAPK cascades is less well defined than $G_{i/o}$PCR- or $G_{q/11}$PCR-mediated activation, but evidence shows that it may be somewhat distinctive compared to the latter two mechanisms (see below).

1.5.3.1 - G_s Protein-Coupled Receptor Activation of the ERK Cascade

Unlike $G_{i/o}$PCRs or $G_{q/11}$PCRs, the influence of G_sPCRs on the ERK cascade may be stimulatory or inhibitory (English *et al,* 1999). The stimulatory pathway involves activation of protein kinase A (PKA) a cAMP-dependent protein kinase, which then activates Rap-1 (Erhardt *et al,* 1995). Although Rap-1 inhibits activation of the ubiquitously distributed Raf-1 isoform, it does promote activation of another Raf

isoform, B-Raf, whose distribution is restricted to the brain and neuroendocrine glands (Vossler *et al,* 1997). Activation of ERKs then occurs via the usual three module system.

The first inhibitory mechanism of ERK activation by G_sPCRs is via the increase in PKA activity, leading to inhibition of Raf-1 phosphorylation, mentioned above. PKA phosphorylates Raf-1 at residues Ser^{43} and Ser^{621}, reducing but not completely eliminating binding to Ras (Kikuchi & Williams, 1996). A reduction in ERK activation thus ensues.

A second proposed inhibitory pathway is PKA-independent. Activation of Rap-1 still occurs, but via a cAMP-dependent Rap-1 guanine nucleotide exchange factor, Epac, activated by the increase in cAMP levels due to G_s-α-subunits (de Rooij *et al,* 1998, Kawasaki *et al,* 1998).

1.5.3.2 - G_s Protein-Coupled Receptor Activation of the p38 MAPK Cascade

Activation of the p38 MAPK cascade by G_sPCRs has been shown, specifically by the β_1-adrenoceptor (Yamauchi *et al,* 1997, Sugden & Clerk, 1998). Very little else is known about this pathway, but overexpression of G_s-α-subunits in HEK293 cells did not activate p38 MAPKs, suggesting G_sPCR-mediated p38 MAPK activation involves G_s-βγ-subunits (Yamauchi *et al,* 1997). However, it should be noted that the β_1-adrenoceptor did not activate p38 MAPKs in transfected PC12 cells (Williams *et al,* 1998).

1.5.3.3 - G_s Protein-Coupled Receptor Activation of the JNK/SAPK Cascade

Activation of the JNK/SAPK cascade by G_sPCRs has also been reported, again by the β_1-adrenoceptor (Yamauchi *et al,* 1997, Sugden & Clerk, 1998). Knowledge of this pathway is also very poorly defined but, similar to G_sPCR-mediated p38 MAPK activation, G_s-$\beta\gamma$-subunits may be involved (Yamauchi *et al,* 1997).

1.5.4 - $G_{12/13}$ Protein-Coupled Receptor Activation of the MAPK Cascades

Activation of any of the three main MAPK cascades by $G_{12/13}$PCRs is the least defined of the four GPCR subtypes. $G_{12/13}$PCRs stimulate the amiloride-sensitive Na^+/H^+ exchanger 1 (NHE1), which is PKC-dependent (Dhanasekaran *et al,* 1994, Voyno-Yasenetskaya *et al,* 1994). Therefore, any MAPK activation due to $G_{12/13}$PCRs may be indirect. However, overexpression of $G_{12/13}$-α-subunits has been shown to activate the ERK cascade (Faure *et al,* 1994). Currently, there is no evidence for $G_{12/13}$PCR-mediated activation of either the p38 MAPK or JNK/SAPK cascades.

1.6 - DDT_1MF-2 Cells

DDT_1MF-2 cells are derived from a steroid-induced leimyosarcoma tumour of Syrian hamster vas deferens smooth muscle (Norris *et al,* 1974), and are a well-classified and studied cell line. This cell line was used due to the high levels of both adenosine A_1 and histamine H_1 receptors found on the cell surface (Gerwins *et al,* 1990, Ramkumar *et al,* 1990, Dickenson & Hill, 1994).

A_1Rs were first characterised on the DDT_1MF-2 cell surface by Ramkumar *et al* (1990), using a number of A_1R-selective radioligands. This study also found the molecular weight of the receptor to be approximately 38 kDa, and that isopretenerol-induced cAMP production was inhibited upon A_1R stimulation by selective agonists. Port *et al* (1992) also reported A_1R agonist-mediated inhibition of cAMP levels in DDT_1MF-2 cells, and this reduction in cAMP levels was abolished in the presence of the selective A_1R antagonist, DPCPX (Shryock *et al,* 1993). This indicated coupling of A_1Rs to $G_{i/o}$ proteins in this cell line. Further studies revealed A_1R-mediated increases in intracellular Ca^{2+}, and coupling of A_1Rs to PLCβ, in DDT_1MF-2 cells (Dickenson & Hill, 1993a, Dickenson & Hill, 1993b). Desensitisation of A_1Rs in DDT_1MF-2 cells due to overexposure to agonists involves phosphorylation of the receptor, probably via GRKs, and results in clustering of A_1Rs on the cell surface (Ciruela *et al,* 1997, Nie *et al,* 1997). Chronic overexposure to the A_1R-selective agonist, N^6-(R)-(phenylisopropyl)adenosine (R-PIA) causes desensitisation, clustering, and internalisation of A_1Rs in DDT_1MF-2 cells, and this process is enhanced upon pre-treatment with adenosine deaminase (Saura *et al,* 1998). Finally, expression of A_1Rs on DDT_1MF-2 cell membranes is increased when they undergo oxidative stress, and this process is mediated via NFκB activation (Nie *et al,* 1998).

Evidence for the existence of H_1Rs in DDT_1MF-2 cells was initially presented by Mitsuhashi & Payan (1988) when a mepyramine binding site was detected, using a radiolabelled form of the specific H_1R antagonist i.e. [H^3] mepyramine (Hill *et al,* 1977). The binding protein was purified and found to have a molecular weight of 38-40kDa (Mitsuhashi & Payan, 1989). Further studies showed that inositol phospholipid hydrolysis and increases in intracellular Ca^{2+} levels are mediated by H_1R stimulation, and antagonised by mepyramine, in DDT_1MF-2 cells (Dickenson & Hill, 1991,

Dickenson & Hill, 1992, White *et al,* 1993). This suggests that the functional H_1Rs found in DDT_1MF-2 cells are typical of the "classical" H_1Rs found in guinea-pig tissue i.e. coupled to G_q proteins (Hill *et al,* 1990). Another classical H_1R-mediated action, activation of PLCβ, was also found to occur in DDT_1MF-2 cells and is regulated by increases in intracellular cAMP levels (Dickenson & Hill, 1993b).

It has also been shown that there is intracellular cross-talk between A_1Rs and H_1Rs in DDT_1MF-2 cells (Dickenson & Hill, 1993b). As previously mentioned, both receptors are coupled to PLCβ, and co-stimulation of both receptors in DDT_1MF-2 cells produced a synergistic increase in intracellular Ca^{2+} levels.

Although there is evidence for stimulation of the MAPK pathway by A_1Rs and H_1Rs in other cell lines (Dickenson *et al,* 1998, Haq *et al,* 1998, Koch *et al,* 2000), a link between endogenous $G_{i/o}$- and G_q-coupled receptors and the MAPK cascade in DDT_1MF-2 had yet to be established. These two receptors were investigated not only due to their physiological significance, but because they show the "classical" responses of their GPCR subtype i.e. adenosine A_1 receptors reduce intracellular cAMP levels typically for a $G_{i/o}$PCR (Gerwins *et al,* 1990, Ramkumar *et al,* 1991), and histamine H_1 receptors stimulates inositol phospholipid hydrolysis and Ca^{2+} mobilisation as is expected of a $G_{q/11}$PCR (Dickenson & Hill 1991, Dickenson & Hill 1992, White *et al,* 1993).

1.7 - Aims

The two main aims of this study were:

1) To determine whether the three main MAPK cascades are activated by adenosine A_1-receptors and histamine H_1 receptors in DDT_1MF-2 cells.
2) To determine the physiological consequences of adenosine A_1 and histamine H_1 receptor-induced MAPK activation. For example, are adenosine A_1 and histamine H_1 receptors linked to cell proliferation and/or modulation of cell survival.

Chapter 2

MATERIALS AND METHODS

2.0 - MATERIALS AND METHODS

2.1 - Cell Culture

Hamster vas deferens smooth muscle cells (DDT_1MF-2 cells) were utilised for this study due to having an abundance of A_1Rs and histamine H_1-receptors in their cell surface (Dickenson & Hill, 1993c, Dickenson & Hill, 1994b), and were obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). The cells were cultured in $75cm^2$ flasks in Dulbeco's modified Eagles Medium (DMEM) supplemented with 2mM glutamine, 10% (v/v) foetal calf serum, and 5 Units/ml penicillin and 5μg/ml streptomycin. Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere until confluency and were subcultured (1:10 split ratio) using trypsin (0.05% w/v)/EDTA (0.02% w/v). Cells for the determination of MAPK activity were grown in 6 well plate cluster dishes (Iwaki). Cells for the determination of cell growth were grown in 96 well plate cluster dishes (Iwaki). Cells for the determination of caspase-3 activity were grown in $25cm^3$ flasks (Iwaki).

2.2 - Production of Cell Lysates for MAPK/PKB Analysis

MAPK or PKB activity was measured using a modified version of a previously used protocol (Dickenson *et al*, 1998). DDT_1MF2 cells were grown in 6 well plates and, when the cells were 80-90% confluent, were placed in DMEM containing 0.1% bovine serum albumin for 16 hours. Serum starved cells were incubated for 30 min at 37°C in 500μl $well^{-1}$ Hanks/HEPES buffer, pH 7.4, and where appropriate, the relevant concentration of pharmacological inhibitor. Agonists were subsequently

added in 500µl of medium/well and were incubated at 37°C for 5 mins unless otherwise stated. Stimulations were terminated by aspiration of the medium and then addition of 300µl lysis buffer [1% (v/v) IGEPAL CA-630, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS, 1mM benzamidine, 5µg/ml leupeptin, 5µl/ml aprotinin, 0.1mM PMSF, 100µM sodium vanadate, 1mM sodium fluoride] before centrifugation of the cell lysate to remove insoluble proteins. One of the cell lysis buffer constituents, IGEPAL CA-630 or (octylphenoxy)polyethoxyethanol, is a detergent and aids cell membrane lysis. Cell lysates were mixed 1:1 with Laemmli sample buffer (9.5ml contained 3.55ml deionised water, 1.25ml 0.5 M Tris-HCl, pH 6.8, 2.5ml glycerol, 2.0ml 10% (w/v) SDS, and 0.2ml bromophenol blue) in Eppendorf microcentrifuge tubes and incubated at 95°C for 5 mins, before being stored at -20°C until required.

2.3 - Western Blot Analysis of MAPK/PKB activation

Protein samples (20µg), determined using the method of Lowry (1951, see section 2.5), were separated by SDS-PAGE (10% acrylamide gel) using the BioRad Mini-Protean II system. The BioRad apparatus was constructed as per the instructions, and 10ml of the 10% acrylamide gel was then made up (4ml distilled water, 3.3 ml Acrylamide mix (Sigma), 2.5 ml 1.5M Tris buffer (pH 8.8), 0.1 ml 10% SDS aqueous solution, 0.004 ml TEMED (Sigma), and 0.1 ml 10% ammonium persulphate (APS) solution (aqueous,freshly made). The APS was added last to start the polymerisation process, and the unpolermerised gel was pipetted into the BioRad apparatus, leaving a 2-3 cm gap from the top of the plates for the stacking gel (see later). The 2-3 cm gap was filled with distilled water, to level off the gel, and left to polymerise for 20-30 mins. The distilled water was then poured off, and the constiuents for 2 ml stacking

gel were then mixed (1.4 ml distilled water, 0.33 ml acrylamide mix, 0.25 ml 1 M Tris buffer (pH 6.8), 0.02 ml 10% SDS solution, 0.002 ml TEMED, and finally 0.02 ml APS solution). The unpolymerised stacking gel was pipetted on top of the running gel, and a while plastic comb (supplied with apparatus) placed into the gel. The stacking gel was allowed to polymerise for a further 20-30 mins, before the comb was removed and the gel washed with distilled water to remove any unpolymerised residue. The whole apparatus was then placed into the buffer chamber, and filled with electrode buffer (a 10 times concentrate, 1L contained 30.3g Tris, 144.0g glycine and 10.0g SDS, was kept at 4°C and diluted with distilled water on the day). 0.02 ml of each sample were loaded into each "well", the electrodes were connected, and the power switched on (200V for 50 mins). Separated proteins were then transferred to a nitrocellulose membrane using a BioRad Trans-Blot system (100V for 60 mins in 25mM Tris, 192mM glycine and 20% methanol). The membrane was then stained with Ponceau S (Sigma), and a 1.5cm strip of the membrane containing the required protein band was washed with phosphate buffered saline (PBS, 10 times concentrate contained 136.98 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4.12H_20$, and 1.5 mM KH_2PO_4. Diluted with distilled water when required). The membrane was blocked with 5% (w/v) skimmed milk powder in PBS, and probed with primary mouse phospho-specific anti-p42/p44 MAPK antibody (Thr^{202}/Tyr^{204}), rabbit phospho-specific anti-p38 MAPK (Thr^{180}/Tyr^{182}) (both Sigma, 1:1000 dilution in 5% (w/v) skimmed milk powder in PBS), rabbit phospho-specific anti-JNK/SAPK (Thr^{183}/Tyr^{185}, Promega, also 1:1000 dilution), or rabbit phospho-specific anti-PKB (Ser^{473}, New England Biolabs, 1:500) for 16 hours at 4°C. The primary antibody was removed, and blots washed using 0.5% Tween 20 in PBS (protocol: washed twice immediately, then three times, each period lasting five mins). Then, the blots were

incubated with secondary goat anti-mouse IgG (for mouse-derived primary antibodies) or goat anti-rabbit IgG (for rabbit derived primary antibodies). Both secondary antibodies were coupled to horseradish peroxidase and were incubated for two hours at room temperature (Dako, 1:1000 dilution in 5% skimmed milk powder dissolved in PBS/0.5% Tween 20). The blots were washed as before in PBS/0.5% Tween 20, and were developed using the Enhanced Chemiluminescence (ECL) detection system (Amersham).

2.4 – Determination of Protein Content

Using a modified version of the Lowry protein determination assay (Lowry *et al*, 1951) the protein content of each sample was determined. First, a BSA calibration curve was prepared, as in table 2.1.

Table 2.1 – Components required for BSA calibration curve preparation

µg Protein Tube^{-1} (mg ml^{-1})	µl 1mg ml^{-1} BSA	µl Deionised Water
0 (0)	-	500
10 (0.05)	25	475
20 (0.10)	50	450
30 (0.15)	75	425
40 (0.25)	100	400
50 (0.25)	125	375
100 (0.50)	250	250

200µl of each standard were dispensed into eppendorfs, in duplicate. 2µl of each sample from the caspase-3 experiments were then added to 198µl deionised water. 250µl 2% potassium tartrate and 250µl 1% $CuSO_4.5H_2O$ were added to 50ml 0.1M

NaOH containing 0.2% SDS and 2% Na_2CO_3. 1ml of this solution was added to each standard BSA solution and sample tube. Each standard and sample tube were vortex mixed, and left for 10 mins. At room temperature Folin and Ciocalteu's phenol reagent was diluted 1:1 with deionised water, and 100µl of this were added to each BSA standard and sample tube. The tubes were left for one hour at room temperature, before 200µl of each tube in triplicate were dispensed into a well in a 96 well plate. Both BSA standards and samples were read at 750nm using a spectrophotometer (see earlier), and a protein calibration curve constructed with absorbance units on the y-axis and µg protein $tube^{-1}$ on the x-axis. This curve was used to determine the protein content in each sample.

2.5 - Cell Growth Assay

DDT_1MF-2 cell growth was measured using a modified version of a previously used protocol (Law *et al,* 1997). In a typical assay 10000 cells were seeded into each well of the 96 well plate and cultured in serum-free DMEM for 24 hours, to arrest growth and keep cells in the quiescent G_0 phase of the cell cycle. The media was then removed and replaced with 100µl DMEM containing 2mM glutamine, 5 Units/ml penicillin, 5µg/ml streptomycin, and pre-incubation compounds (e.g. MAPK inhibitors) where necessary, and incubated at 37°C for 30 mins. Varying concentrations of agonist (e.g. CPA, histamine), dissolved in 100µl DMEM supplemented with 2mM glutamine, 5 Units/ml penicillin, and 5µg/ml streptomycin, were added to the cells after the 30 mins pre-incubation period, and incubated for 72 hours at 37°C (200µl per well).

2.5.1 - Cell Growth Analysis

Initially, two colourimetric assays were utilised for the determination of cell growth - the acid phosphatase (AP) assay and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The AP assay involves conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol, which absorbs light maximally at 405nm (Yang *et al,* 1996). The MTT assay depends upon the intracellular-reduction of soluble MTT by mitochondrial dehydrogenase, to a formazan product, which absorbs light at 570nm (Petty *et al,* 1995). It was observed that the MTT assay was more accurate in determining cell growth than the AP assay, despite evidence which suggested the AP assay demonstrates higher sensitivity and reproducibility in some cell lines (Yang *et al,* 1996). Therefore, only the MTT assay was subsequently used.

2.5.2 - MTT Assay

After the incubation period with growth factors (e.g. FCS, EGF) or inducers of cell death (e.g. staurosporine, H_2O_2) 20µl of 5mg/ml MTT dissolved in PBS were added to each well. The cells were incubated for one hour at 37°C, before the medium was removed and the cells were washed with 50µl PBS. 200µl of dimethyl sulphoxide (DMSO) were added to each well and the plate was agitated for a few minutes. Colour development was assayed using a TECAN SPECTRA FLUOR spectrophotometer set at an absorbance of 570nm, and XFLUOR4 software.

2.6 - Caspase-3 Activity Assay

Caspase-3 activity was determined based on the protocol supplied with the CaspACE™ colourimetric assay system (Promega) and the method given by (Blöm *et al,* 1999). DDT_1MF-2 cells were grown in $25cm^3$ flasks in serum-containing DMEM supplemented with 2mM glutamine, 10% (v/v) foetal calf serum, and 5 Units/ml penicillin and 5μg/ml streptomycin. Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere until 80-90% confluent, similar to the method described in section 2.2. Once confluent the media was removed and replaced with serum-free DMEM containing 0.1% bovine serum albumin only. The cells were incubated for 16 hours in a 5% CO_2 atmosphere at 37°C.

After this time the media was replaced again, with DMEM containing 2mM glutamine, 5 Units/ml penicillin and 5μg/ml streptomycin, and the compounds to be assayed, for 30 mins. Cell death was induced by staurosporine (1μM) for 4 hours, or H_2O_2 (100μM) for up to 8 hours.

2.6.1 - Sample Preparation

The cells were then harvested using trypsin (0.05% w/v)/EDTA (0.02% w/v) and spun in a centrifuge at 1800 rpm for 10 mins at 4°C. The supernatant was removed, the cells washed once with ice-cold PBS, and then 50μl of cell lysis buffer (10mM HEPES, 40mM β-glycerophosphate, 50mM NaCl, 2mM $MgCl_2$, and 5mM EGTA) was added. Lysing of the cells was accomplished by freezing them at minus 70°C for 20 mins, thawing them, and then putting them on ice for 15 mins. The cycle was then

repeated. Each sample was then spun at 19 000 rpm at 4°C for 20 mins and the supernatant removed and stored at minus 70°C overnight.

In each well of a 96 well plate 32µl of caspase assay buffer (supplied with CaspACE™ assay system), 2µl DMSO, and 10µl 100mM DTT dissolved in water were added. 5µl of sample and 49µl of deionised water (54µl deionised water only in the blank) was then placed in each well, before starting the assay with addition of 2µl 10mM Ac-DEVD-pNA (dissolved in DMSO), the caspase-3 substrate. Each sample was assayed in triplicate, and incubated for 4 hours at 37°C, sealing the well plate with Parafilm. The amount of pNA product present, which was proportional to the activity of caspase-3 in each sample, was recorded using a TECAN SPECTRA FLUOR spectrophotometer set at an absorbance of 405nm, and XFLUOR4 software.

2.6.2 – Calculation of Caspase-3 Activity

Caspase-3 activity was then calculated, which also required the construction of a pNa standard curve, and determination of protein content in each sample, using a modified version of the Lowry method (Lowry *et al*, 1951, see section 2.4).

2.6.2.1 – pNa Calibration Curve

A 100mM pNa standard solution was supplied with the CaspACE™ kit. 10µl of this solution were mixed with 90µl DMSO, to produce a 10mM stock solution on the day the assay was to be carried out. Using the same technique, further serial dilutions of 1mM, 100µM, and 10µM pNa were made up. 100µl volume of a range of pNa

concentrations i.e. 0, 1, 2, 5, 10, 50, and 100 pmol/µl were prepared in a 96 well plate, in duplicate, as follows:

Table 2.2 - Components required for pNa calibration curve in 96 well plates

pNa Stock Solution			DMSO	Deionised Water	pNa Standard	
10µM	100µM	1mM			µM	pmol μl^{-1}
-	-	-	20µl	80µl	0	0
10µl	-	-	10µl	80µl	1	1
20µl	-	-	0	80µl	2	2
-	5µl	-	15µl	80µl	5	5
-	10µl	-	10µl	80µl	10	10
-	-	5µl	15µl	80µl	50	50
-	-	10µl	10µl	80µl	100	100

The absorbance of each sample was read using the aforementioned spectrophotometer (see section 2.6) set at 405nm. A calibration curve was constructed, with absorbance units on the y-axis and pNa concentration, in pmol μl^{-1}, on the x-axis. The slope was then calculated using the Microsoft Excel package.

2.6.2.2 – Calculation of Specific Caspase-3 Activity

First, the relative absorbance (ΔA) of the caspase-3 samples was calculated by subtracting the blank absorbance value at 405nm from the sample values. Then, the activity of caspase-3 present in each sample was calculated as below, where X = pmol pNa liberated $hour^{-1}$.

$$X = \frac{\Delta A - (\text{y intercept of pNa standard curve})}{\text{Incubation time in hrs}} \times \frac{100\mu l \text{ (sample volume)}}{\text{slope of pNa standard curve}}$$

Finally the specific activity of caspase-3 in each sample (SA) was calculated using the equation below, which incorporates the protein content of the sample calculated in section 2.4.

$$SA = \frac{\text{pmol pNa liberated hour}^{-1}}{\mu\text{g protein}} = \frac{X}{\mu\text{g protein}}$$

Specific activity of caspase-3 is expressed as pmol pNa liberated $hour^{-1}$ $(\mu g\ protein)^{-1}$.

2.7 - Data Analysis

MAPK and PKB activity was quantified by scanning the developed films into a computer and running Quantiscan™. The developed film was placed on a white lit background and positioned under a camera, which was connected to a computer. Using the Grab-IT program (Synoptics Ltd.) the blots were scanned, using the camera's focus and aperture adjustments to sharpen the image and reduce background optical density respectively. The scanned blots were then quantified by using Quantiscan™. "Lanes" were superimposed over each band on the blot, ensuring each individual band was completely contained within each lane. The band within the lane was then quantified, using the "Quantify Lane" command found in the Quantiscan™ package. The net area of the band was recorded, which was equivalent to the gross band area minus background optical intensity. If necessary, the net area of the control value band (labelled 0 or "Con" in the results sections") was subtracted from the other sample bands' net areas, and percentage values were then calculated. Agonist EC_{50} values and antagonist IC_{50} values were obtained using the Prism 3.02 computer package. Statistical significance was determined using Student's unpaired *t* test and ANOVA (analysis of variance) test, and P values below 0.05 were considered

significant. The data presented in the results section are given as mean ± S.E.M, with the character *n* referring to the number of separate experiments completed.

The antagonist dissociation constant (K_D) was calculated using a modified version of the method given by Lazerano & Roberts (1987). Dose-response curves to an agonist were constructed, and the various values obtained from these graphs were used in the equation below.

$$\frac{C}{EC_{50}} = \frac{IC_{50}}{K_D + 1}$$

where:

C = concentration of agonist which gave a response greater then 50% of the maximum
IC_{50} = concentration of antagonist required to reduce the response due to C by 50%
EC_{50} = Concentration of agonist which gave a response equivalent to 50% of the maximum without the antagonist present
K_D = Antagonist dissociation constant

The equation above was then rearranged and the apparent K_D value was then calculated.

2.8 - Materials

Adenosine, Mouse phospho-specific p42/p44 MAPK (Thr^{202}/Tyr^{204}) and p38 MAPK (Thr^{180}/Tyr^{182}) antibodies, aprotinin, bovine serum albumin (BSA), DPCPX (1,3-dipropylcyclo-pentlyxanthine), Dulbeco's modified Eagles Medium (DMEM), EDTA (ethylenediaminetetraacetic acid), foetal calf serum (FCS), histamine, IGEPAL CA-630 (octylphenoxy)polyethoxyethanol), insulin, leupeptin, mepyramine, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), N^6-cyclopentyladenosine (CPA), penicillin/streptomycin, staurosporine, and trypsin, were purchased from

Sigma Chemical Co. (Poole, Dorset, UK). Rabbit phospho-specific JNK/SAPK antibodies were purchased from Promega (Southampton, UK). Pertussis toxin (PTX) was obtained from Porton Products Limited. AG 1478 (4-(3-chloroanilo)-6,7-dimethyloxyquinalozine), BAPTA/AM ([1,2-bis(*o*-amino-5-bromophenoxy)ethane-*N,N,N', N'*-tetraacetic acid tetra(acetyl-methyl)ester]), cytochalasin D, daidzein, epidermal growth factor (EGF), genistein, GF 109203X (2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide), LY 294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), PD 98059 (2'-amino-3'methoxyflavone), PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine), Ro-31-8220 (3-{1-[3-(2-isothioureido) propyl]indol-3-yl}-4-(1-methylindol-3-yl)-3-pyr-rolin-2,5-dione), SB 203580 (4-(4-fluoro-phenyl)-2-(4--methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole), tyrphostin A47 (α-cyano-(3,4-dihydroxy)thiocynnamide), and wortmannin were purchased from Calbiochem (Nottingham, UK). Anti-rabbit phospho-specific PKB (Ser^{473}) antibodies were purchased from New England Biolabs (Beverley, MA, U.S.A.). Tiotidine and thioperamide were obtained from Tocris (Semat Technical (U.K.) Ltd.). Other chemicals used were all of analytical grade.

Chapter 3

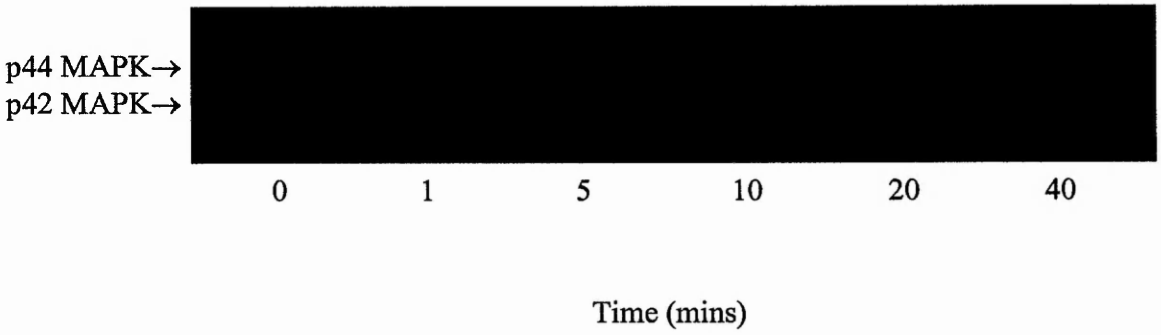
ACTIVATION OF p42/p44 MITOGEN-ACTIVATED PROTEIN KINASES BY ADENOSINE A_1 RECEPTOR STIMULATION

3.0 – ACTIVATION OF p42/p44 MITOGEN-ACTIVATED PROTEIN KINASES BY ADENOSINE A1 RECEPTOR STIMULATION

3.1 – p42/p44 MAPK Phosphorylation by Adenosine A_1 Receptors

The effect of adenosine A_1 receptors (A_1Rs) on the p42/p44 mitogen-activated protein kinase (p42/p44 MAPK) pathway in DDT_1MF-2 cells was investigated using the selective A_1R agonist, N^6-cyclopentyladenosine (CPA), and the endogenous agonist, adenosine. Stimulation of DDT_1MF-2 cells with maximally-effective concentrations of CPA (1μM) and adenosine (100μM) over a period of 40 mins both produced a maximal activated p42/p44 MAPK response at 5 mins, before decreasing back to basal levels (see Figures 3.1 and 3.2). With both agonists there was dominant phosphorylation of p42 MAPK compared to p44 MAPK (Figures 3.1a and 3.2a). Activation of p42/p44 MAPK by CPA and adenosine was also concentration dependent, producing $p[EC_{50}]$ values of 8.98 ± 0.24 (n=6) and 7.18 ± 0.36 (n=4) respectively (see Figures 3.3 and 3.4). The selective adenosine A_1 receptor antagonist, DPCPX (1,3-dipropylcyclopentylxanthine), antagonised CPA-mediated increases in p42/p44 MAPK phosphorylation, with an apparent K_D value of 0.9 ± 0.2 nM (n=5, see Figure 3.5).

(a)



(b)

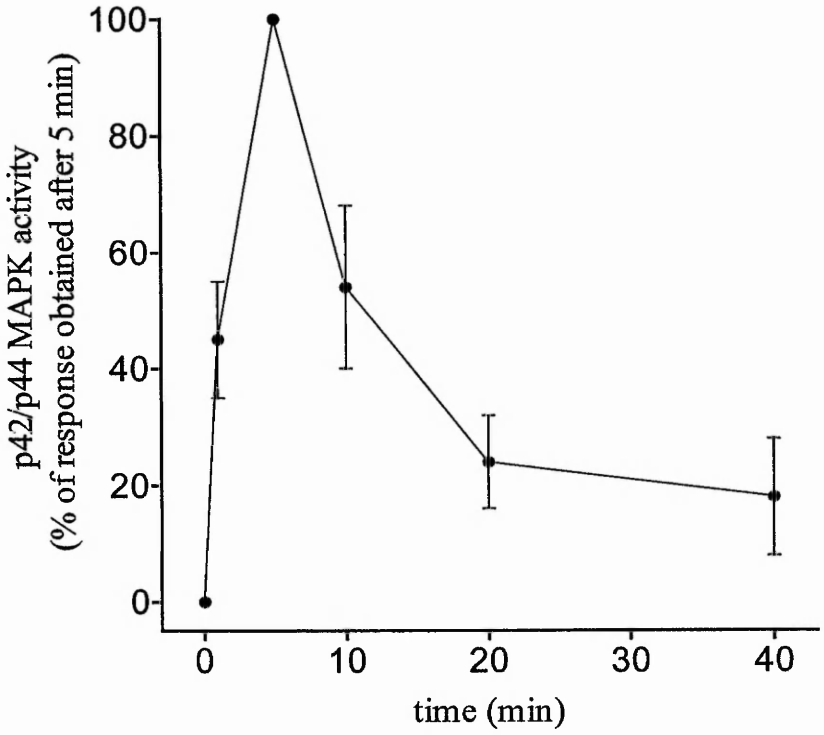
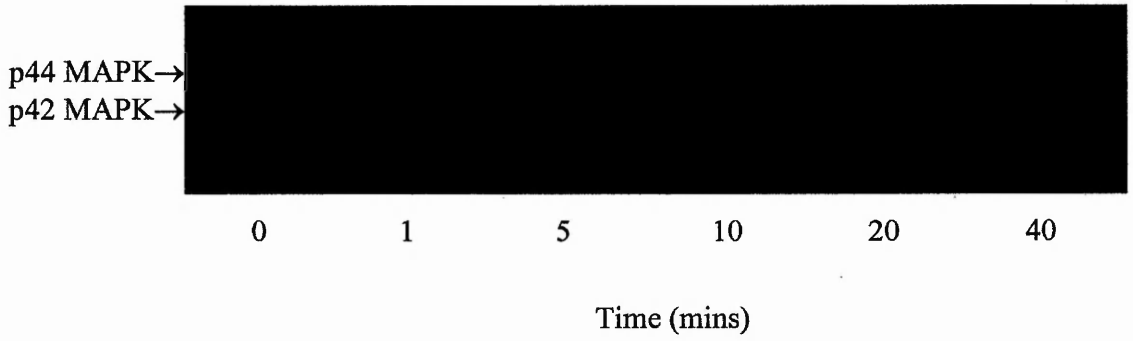


Figure 3.1 – CPA-mediated adenosine A_1 receptor stimulation of the p42/p44 MAPK pathway in DDT_1MF-2 cells - time course. (a) Representative Western blot and (b) Time course plot (n=6) of 1μM CPA-mediated stimulation of p42/p44 MAPK in DDT_1MF-2 cells. 20μg of cell lysate per time point was analysed for p42/p44 MAPK phosphorylation by Western blotting using a phospho-specific p42/p44 MAPK antibody. Cells were stimulated for up to forty minutes.

(a)



(b)

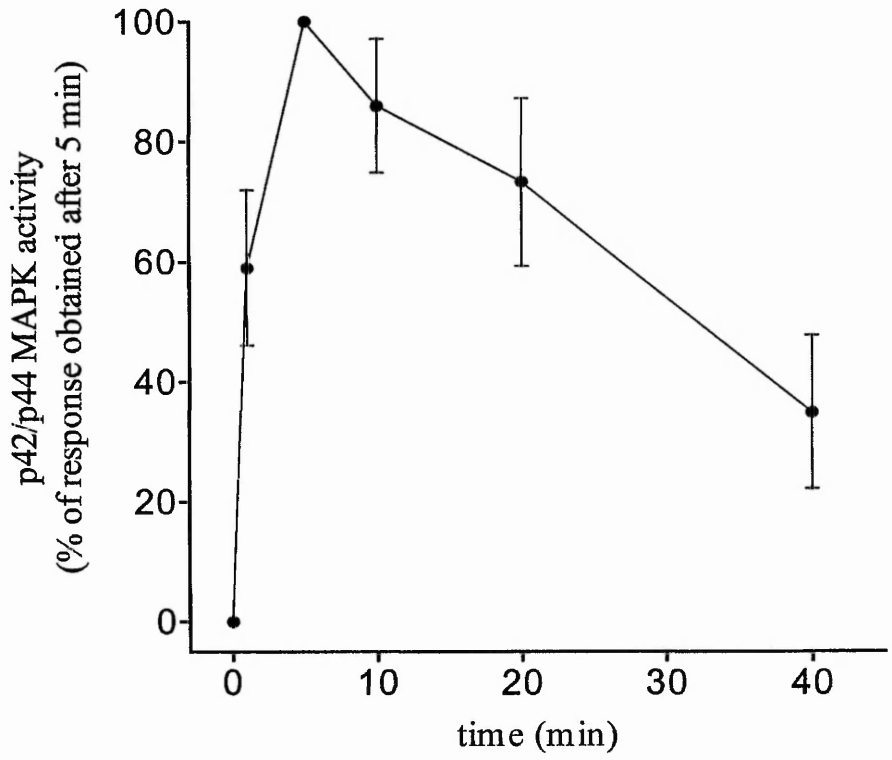
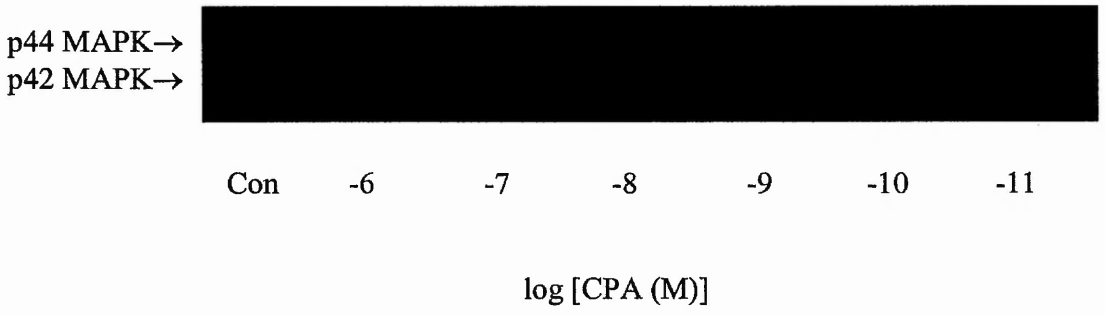


Figure 3.2 – Adenosine-mediated adenosine A_1 receptor stimulation of the p42/p44 MAPK pathway in DDT_1MF-2 cells - time course. (a) Representative Western blot and (b) Time course plot (n=4) of 100μM adenosine-mediated stimulation of p42/p44 MAPK in DDT_1MF-2 cells. 20μg of cell lysate per time point was analysed for p42/p44 MAPK phosphorylation by Western blotting using a phospho-specific p42/p44 MAPK antibody. Cells were stimulated for up to forty minutes. Values represent mean ± S.E.M. of four independent experiments.

(a)



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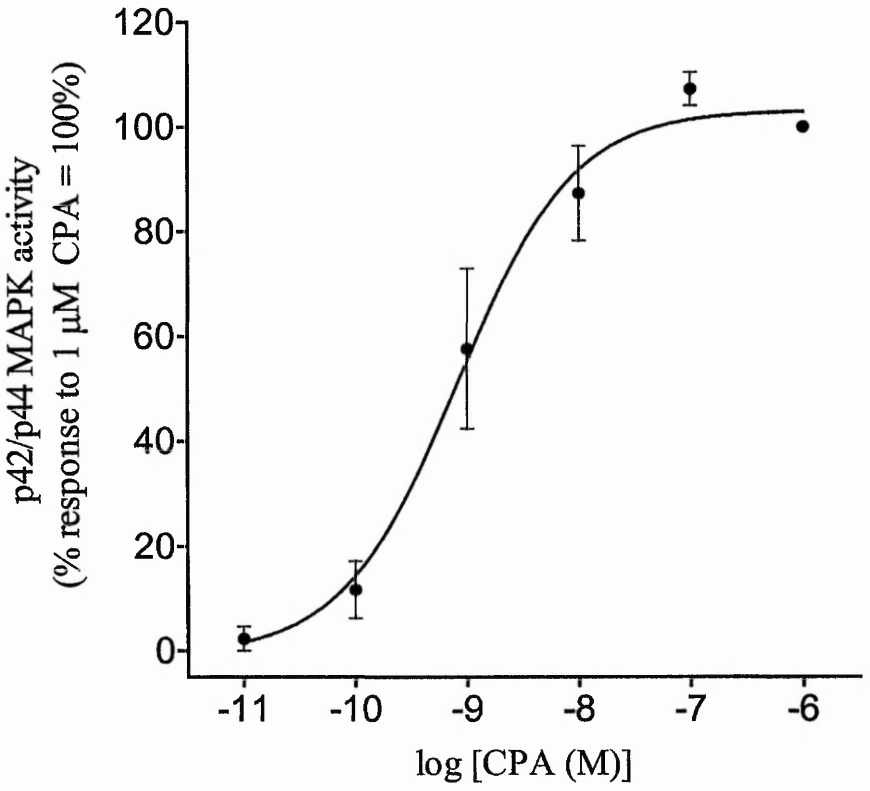


Figure 3.3 – CPA-mediated adenosine A_1 receptor stimulation of the p42/p44 MAPK pathway in DDT_1MF-2 cells - concentration response. (a) Representative Western blot and (b) concentration-response curve of CPA-mediated stimulation of p42/p44 MAPK in DDT_1MF-2 cells. 20μg of cell lysate per concentration of CPA was analysed for p42/p44 MAPK phosphorylation by Western blotting using a phospho-specific p42/p44 MAPK antibody. Values represent mean ± S.E.M. of six independent experiments. Con = control sample (0% response).

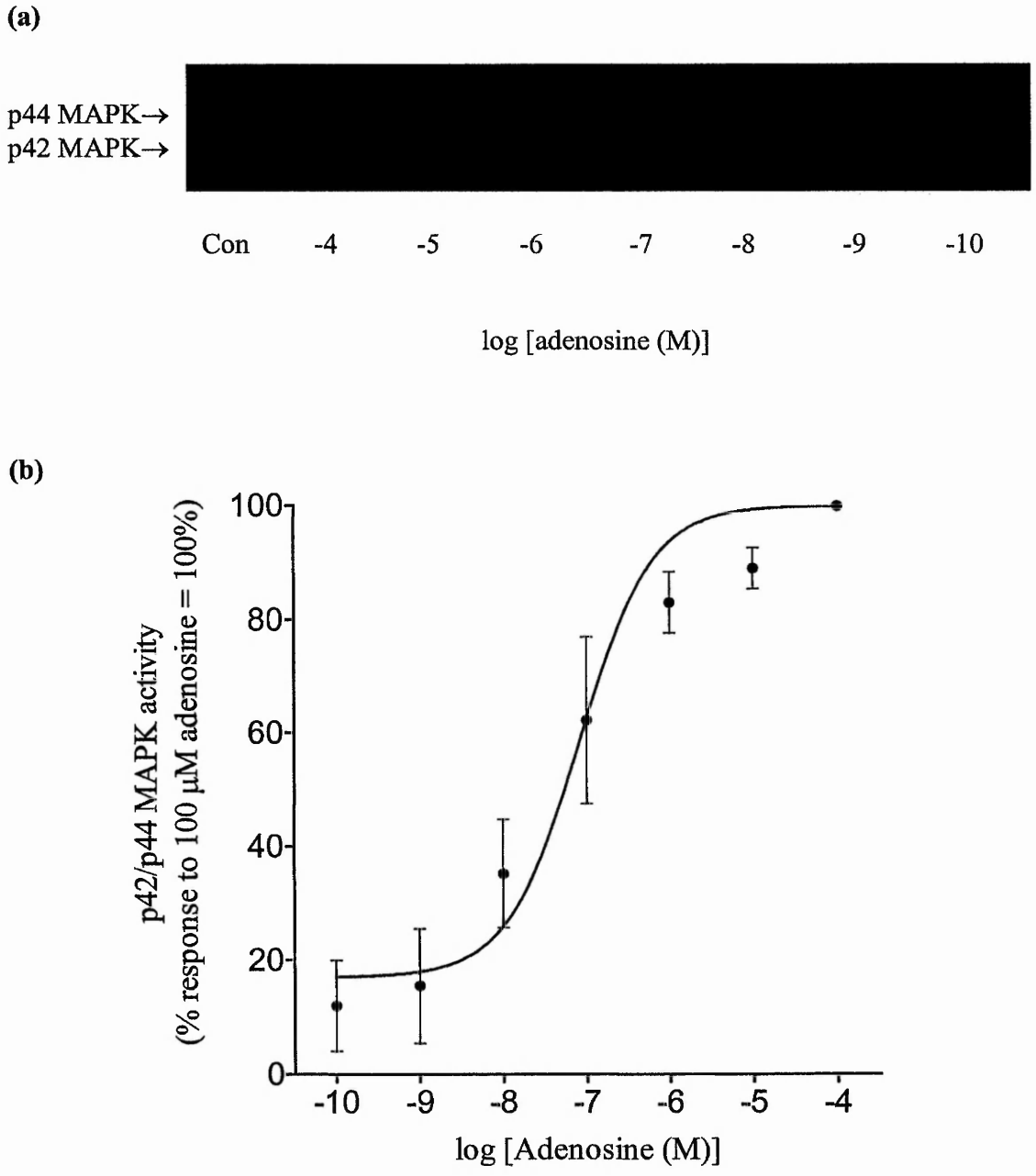


Figure 3.4 – Adenosine-mediated adenosine A_1 receptor stimulation of the p42/p44 MAPK pathway in DDT_1MF-2 cells - concentration response. (a) Representative Western blot and (b) concentration-response curve of adenosine-mediated stimulation of p42/p44 MAPK in DDT_1MF-2 cells. 20μg of cell lysate per concentration of CPA was analysed for p42/p44 MAPK phosphorylation by Western blotting using a phospho-specific p42/p44 MAPK antibody. Values represent mean ± S.E.M. of four independent experiments. Con = control sample (0% response).

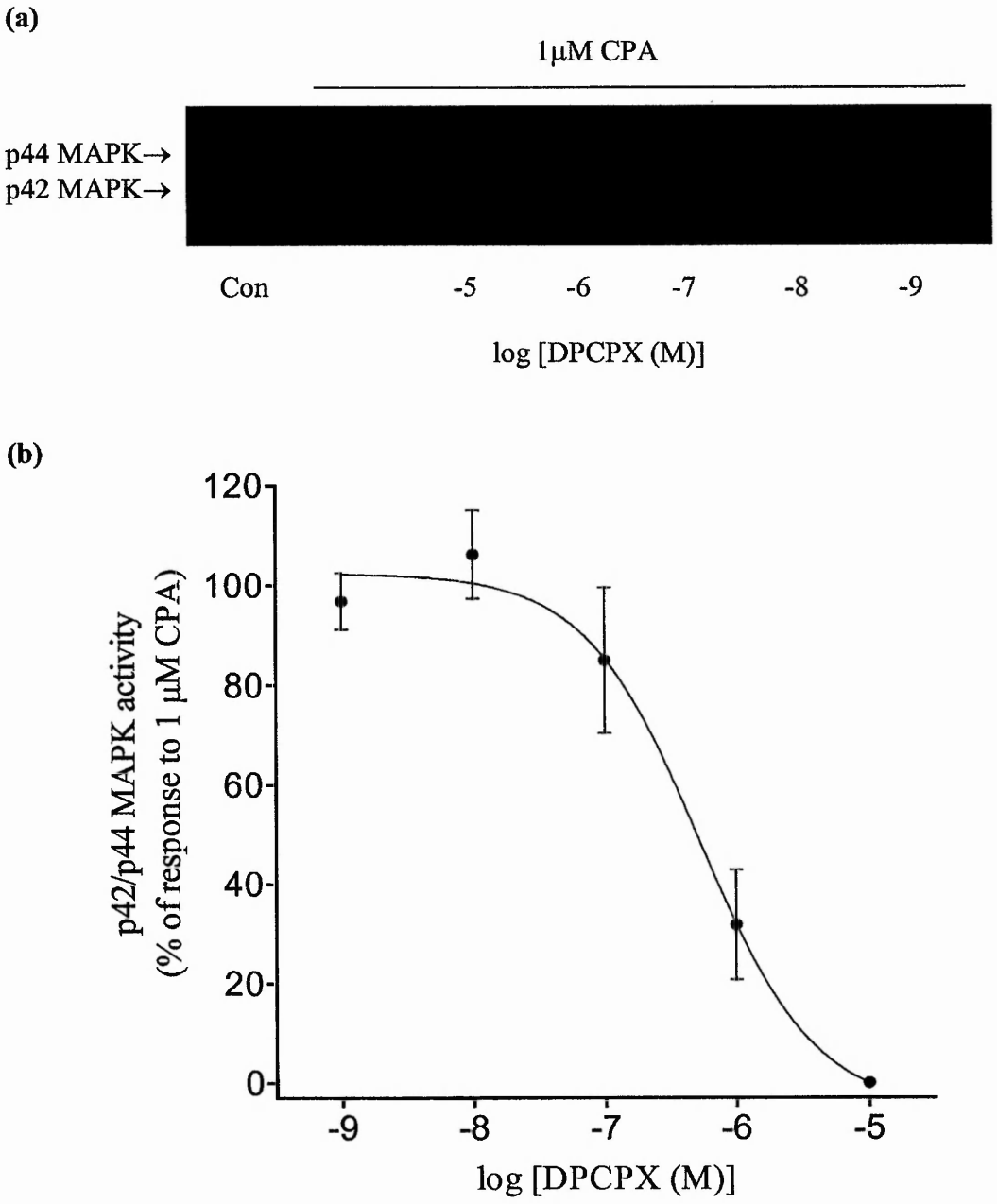


Figure 3.5 – Effect of the specific A_1R antagonist, DPCPX, on adenosine A_1 receptor stimulation of the p42/p44 MAPK pathway in DDT_1MF-2 cells. (a) Representative Western blot and (b) concentration-response curve (n=5) of antagonism by DPCPX of CPA-mediated stimulation of p42/p44 MAPK in DDT_1MF-2 cells. Cells were pre-incubated for 30 mins with the indicated concentrations of DPCPX, before being exposed to 1μM CPA for a further 5 mins. 20μg of cell lysate per concentration of CPA was analysed for p42/p44 MAPK phosphorylation by Western blotting using a phospho-specific p42/p44 MAPK antibody. Apparent K_D value = 0.9 ± 0.2 (n=5). Values represent mean ± S.E.M. of five independent experiments. Con = control sample (0% response).

3.2 - The Effect of PTX and PD 98059 on A_1R-Mediated p42/p44 MAPK Activation

As has already been stated, A_1Rs couple to the $G_{i/o}$ family of G proteins, which are PTX sensitive (Ralevic & Burnstock, 1998). Therefore, pre-incubation with PTX should reduce both CPA- and adenosine-mediated phosphorylation of p42/p44 MAPK in DDT_1MF-2 cells. Pretreatment with PTX (100 ng ml^{-1}, 16 hours) completely inhibited p42/p44 MAPK phosphorylation by both CPA (n=4) and adenosine (n=3) in this cell line (see Figures 3.6a and 3.6b).

MEK1 (MAPK kinase kinase) is responsible for the dual phosphorylation, and activation, of p42/p44 MAPK (Widmann *et al,* 1999). DDT_1MF-2 cells were preincubated with the specific MEK1 inhibitor, PD 98059 (50 μM, 30 mins, Dudley *et al,* 1995) before exposing cells to CPA (1μM) and insulin (100nM) for five mins. Insulin binds to the insulin growth factor receptor and has been shown to phosphorylate p42/p44 MAPK via MEK1 (Foncea *et al*, 1997, Lavendero *et al,* 1998), and so provides a positive result with which to compare. PD 98059 significantly reduced CPA-mediated p42/p44 MAPK phosphorylation (79% ± 11% inhibition, n=4, $p<0.05$) and, as expected, insulin-mediated p42/p44 MAPK phosphorylation. The reduction in phosphorylation for both agonists is of a similar magnitude, as shown in Figure 3.6c.

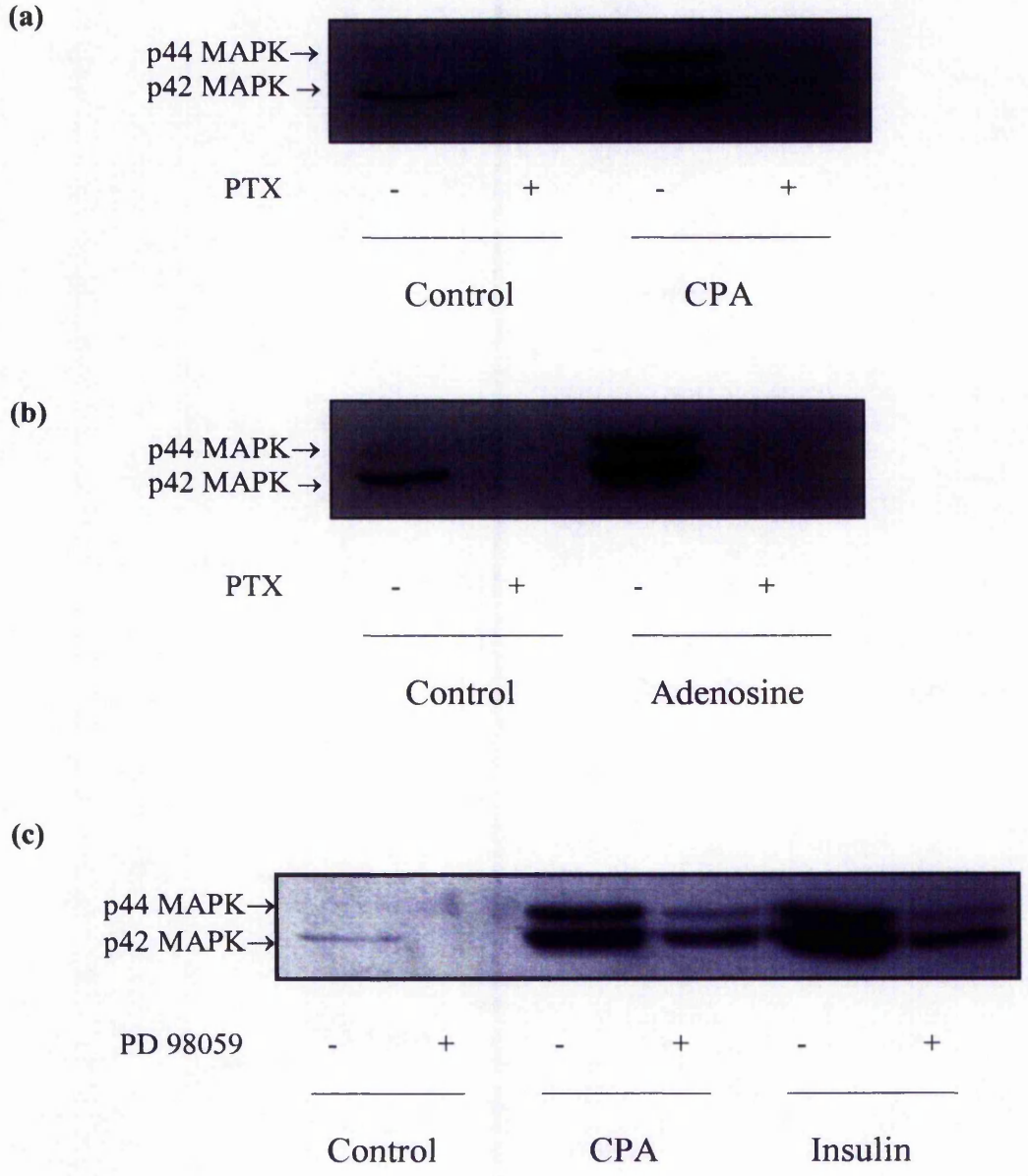
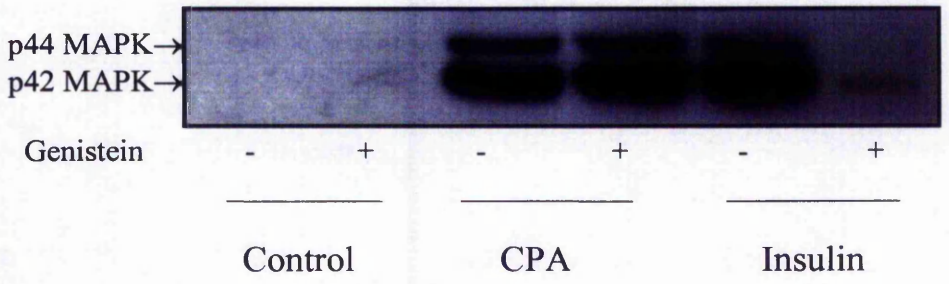


Figure 3.6 – Effect of pertussis toxin (PTX) and the specific MEK1 inhibitor, PD 98059, on adenosine A_1 receptor stimulation of the p42/p44 MAPK pathway in DDT_1MF-2 cells. Representative Western blots showing the effect of pre-treatment of 100ng ml^{-1} PTX for 16 hours on (a) 1μM CPA-mediated (n=4) and (b) 100μM adenosine-mediated p42/p44 MAPK phosphorylation in DDT_1MF-2 cells (n=3). (c) The effect of 50μM PD98059, pre-incubated for 30 mins, on 1μM CPA- and 100nM insulin-mediated p42/p44 MAPK phosphorylation (n=4). CPA, adenosine, and insulin were all incubated for 5 mins after the pre-treatment protocols. 20μg of cell lysate per agonist was analysed for p42/p44 MAPK phosphorylation by Western blotting using a phospho-specific p42/p44 MAPK antibody.

3.3 - The Involvement of Tyrosine Kinases in A_1R-Mediated p42/p44 MAPK Activation

Previous studies have shown that tyrosine kinases (both receptor and non-receptor) are involved in activation of p42/p44 MAPK by $G_{i/o}$PCRs (Lopez-Ilasaca, 1998). Previous studies have shown that genistein, a non-specific tyrosine kinase inhibitor, significantly reduced human A_1R-induced p42/p44 MAPK phosphorylation in transfected CHO cells (Dickenson *et al*, 1998). The involvement of tyrosine kinases in A_1R-induced phosphorylation of p42/p44 MAPK in DDT_1MF-2 cells was investigated using genistein, tyrphostin A47 (both broad-range, non-specific tyrosine kinase inhibitors), and PP2 (specifically inhibits Src and not the other members of the Src family of tyrosine kinase family) (Hanke *et al*, 1996). Daidzein, the inactive analogue of genistein, was also used as a control. As shown in Figures 3.7 and 3.8 pretreatment with genistein (100 μM, 30 mins), daidzein (100 μM, 30 mins), tyrphostin A47 (100 μM, 30 mins), and PP2 (10 μM, 30 mins) had no significant effect on CPA-mediated p42/p44 MAPK phosphorylation. In contrast, p42/p44 MAPK phosphorylation induced by insulin was significantly reduced by genistein and tyrphostin A47. The data obtained from these experiments are summarised in Table 3.1, and provides evidence that A_1R stimulation of the p42/p44 MAPK cascade in DDT_1MF-2 cells does not require tyrosine kinase activation.

(a)



(b)

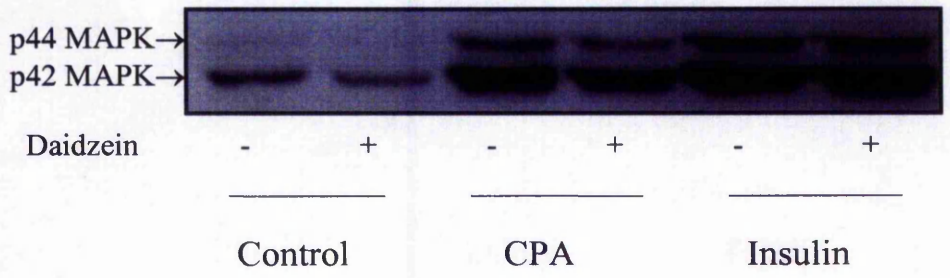


Figure 3.7 – Effect of the tyrosine kinase inhibitor, genistein, and its inactive analogue, daidzein, on adenosine A_1 receptor stimulation of the p42/p44 MAPK pathway in DDT_1MF-2 cells. Representative Western blots showing the effect of 30 mins pre-treatment with (a) 100μM genistein and (b) 100μM daidzein, on 1μM CPA and 100nM insulin-mediated p42/p44 MAPK phosphorylation in DDT_1MF-2 cells. Cells were incubated for 5 mins after the pre-treatment protocols. 20μg of cell lysate per agonist was analysed for p42/p44 MAPK phosphorylation by Western blotting using a phospho-specific p42/p44 MAPK antibody. Results were obtained for at least three independent experiments (see Table 3.1 for a summary of the data).

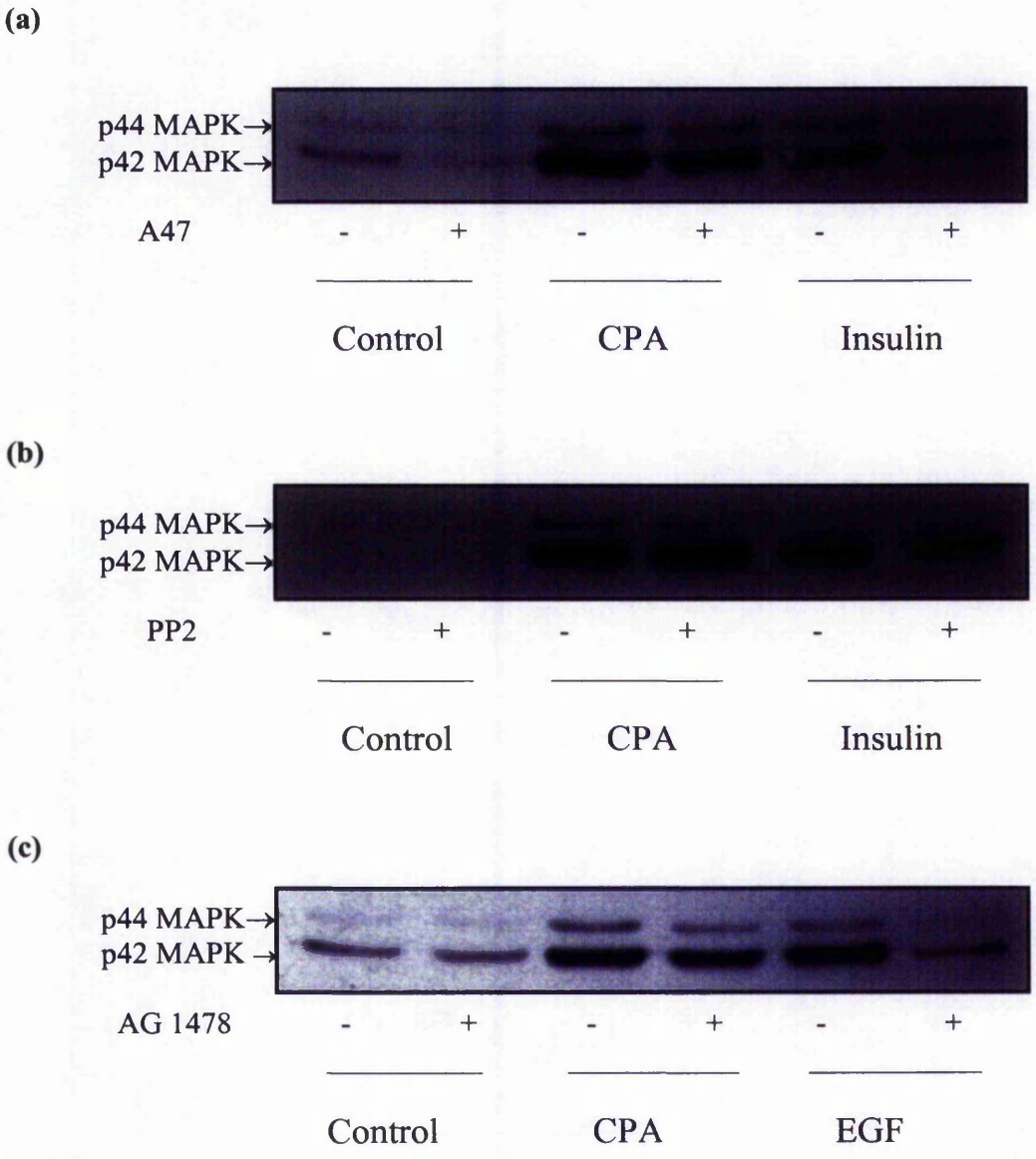


Figure 3.8 – Effect of the tyrosine kinase inhibitors tyrphostin A47 and PP2, and the EGF receptor tyrosine kinase inhibitor, AG 1478, on adenosine A_1 receptor stimulation of the p42/p44 MAPK pathway in DDT_1MF-2 cells. Representative Western blots showing the effect of 30 mins pre-treatment with (a) 100μM tyrphostin A47, a non-specific tyrosine kinase inhibitor, and (b) 10μM PP2, a Src family tyrosine kinase inhibitor, on 1μM CPA and 100nM insulin-mediated p42/p44 MAPK phosphorylation in DDT_1MF-2 cells. (c) The effect of 30 mins pre-treatment with 1μM AG 1478, on 1μM CPA- and 10nM EGF-mediated p42/p44 MAPK phosphorylation in DDT_1MF-2 cells. CPA, EGF, and insulin were all incubated for 5 mins after the pre-treatment protocols. 20μg of cell lysate per agonist was analysed for p42/p44 MAPK phosphorylation by Western blotting using a phospho-specific p42/p44 MAPK antibody. Results were obtained for four independent experiments (see Table 3.1 for a summary of the tyrphostin A47 and PP2 data).

Table 3.1 – The effect of various tyrosine kinase inhibitors on CPA- and insulin-mediated p42/p44 MAPK phosphorylation. Serum-starved DDT_1MF-2 cells were pre-incubated for 30 mins with genistein (100 μM), daidzein (100 μM), tyrphostin A47 (100 μM), and PP2 (10 μM), before stimulating with CPA (1μM) or insulin (100nM). Data is expressed as a percentage of the response obtained with 1μM CPA (100%) and 100 nM insulin (100%) in control cells (independent controls were used for each inhibitor). Control cells were pre-incubated with 0.1% dimethylsuphoxide (DMSO) as a vehicle for 30 mins. Values represent mean ± S.E.M. of *n* experiments.

* Significantly ($p<0.05$, student's *t*-test) different compared to the control response.

Treatment	CPA (% of control)	*n*	Insulin (% of control)	*n*
Genistein (100μM)	94 ± 12	4	4 ± 2*	3
Daidzein (100μM)	98 ± 7	3	95 ± 9	3
Tyrphostin A47 (100μM)	103 ± 8	4	8 ± 6*	4
PP2 (10μM)	95 ± 10	4	86 ± 9	4

GPCRs have been shown to phosphorylate p42/p44 MAPK via a process known as transactivation (Daub *et al*, 1996, Zwick *et al,* 1999). In some studies GPCR ligand binding e.g. lysophosphatidic acid (LPA) or angiotensin II, leads to epidermal growth factor (EGF) receptor tyrosine kinase activation and subsequent p42/p44 MAPK activation, without EGF binding to the receptor (Daub *et al,* 1997, Li *et al*, 1998). In this study it was investigated whether transactivation occurred in DDT_1MF-2 cells by using the specific EGF tyrosine kinase inhibitor, AG 1478 (Levitzki & Gazit, 1995).

Cells were pre-incubated with AG 1478 (1μM, 30 mins) and then exposed to CPA (1μM) or EGF (10nM) for 5 mins. No significant reduction in CPA-mediated p42/p44 MAPK phosphorylation was observed but, as expected, a marked inhibition was seen with EGF (10% ± 8% compared to control response, n=4, $p<0.05$, see Figure 3.8c). This would suggest that transactivation of the EGF receptor by A_1Rs is not involved in the activation of the p42/p44 MAPK cascade by A_1Rs.

3.4 – Involvement of Protein Kinase C and Ca^{2+} in p42/p44 MAPK Phosphorylation by Adenosine A_1 Receptors

Previous work has shown A_1R-mediated activation of phospholipase C (PLC) in DDT_1MF-2 cells is PTX sensitive (White *et al,* 1992). As explained in the introduction section, this enzyme is responsible for the production of IP_3 and DAG, which release Ca^{2+} from intracellular stores and activates PKC respectively (Berridge, 1993). Previous studies have shown that A_1Rs stimulate intracellular Ca^{2+} release and activate PKC in DDT_1MF-2 cells (Dickenson & Hill, 1993b, Gerwins & Friedholm, 1995). Therefore, the role of Ca^{2+} and PKC in the regulation of p42/p44 MAPK phosphorylation by the A_1R was investigated.

Ca^{2+} influx during these experiments was reduced by using Ca^{2+}-free Hanks/HEPES buffer containing 0.1 mM EGTA, instead of the Ca^{2+}-containing Hanks/HEPES buffer. However, removal of extracellular Ca^{2+} had no significant effect on CPA-mediated p42/p44 MAPK phosphorylation (98% ± 9% of control response, $P > 0.05$, n=4). To monitor the role of Ca^{2+} released from intracellular stores in A_1R-mediated phosphorylation of p42/p44 MAPK, the Ca^{2+} chelator BAPTA/AM (50μM, 30 mins) was preincubated with the DDT_1MF-2, again in the absence of extracellular Ca^{2+}.

Loading cells with BAPTA in the absence of extracellular Ca^{2+} had no significant effect on p42/p44 MAPK phosphorylation induced by CPA (94% ± 12% of control response, $P > 0.05$, n=4). These data would indicate that increases in intracellular Ca^{2+} (either via Ca^{2+} influx or Ca^{2+} release) are not required for A_1R-mediated p42/p44 MAPK phosphorylation in DDT_1MF-2 cells.

The role of PKC in p42/p44 MAPK phosphorylation by A_1Rs was investigated by utilising a number of selective PKC inhibitors. Preincubation with either 10μM Ro 31-8220 (Davis *et al*, 1989), 1μM GF 109203X (Matiny-Brown *et al*, 1993), or 10μM chelerythrine (Herbert *et al,* 1990) for 30 mins all had no significant effect on CPA-mediated stimulation of p42/p44 MAPK. See Table 3.2 for summarised data, and Figure 3.9a for representation Western blots. In comparison, all three PKC inhibitors significantly reduced p42/p44 MAPK activation induced by the phorbol ester phorbol 12-myristate 13-acetate (PMA, 1μM) (Table 3.2 and Figure 3.9b). Finally, PKC levels were reduced by downregulation with 1μM PMA for 36 hours (Mene *et al,* 1993). PKC-downregulation had no significant effect on CPA-mediated stimulation of p42/p44 MAPK, while PMA-mediated p42/p44 MAPK activation was significantly reduced (Table 3.2 and Figure 3.9c). These data indicate that PKC does not play a role in A_1R-mediated p42/p44 MAPK phosphorylation in DDT_1MF-2 cells.

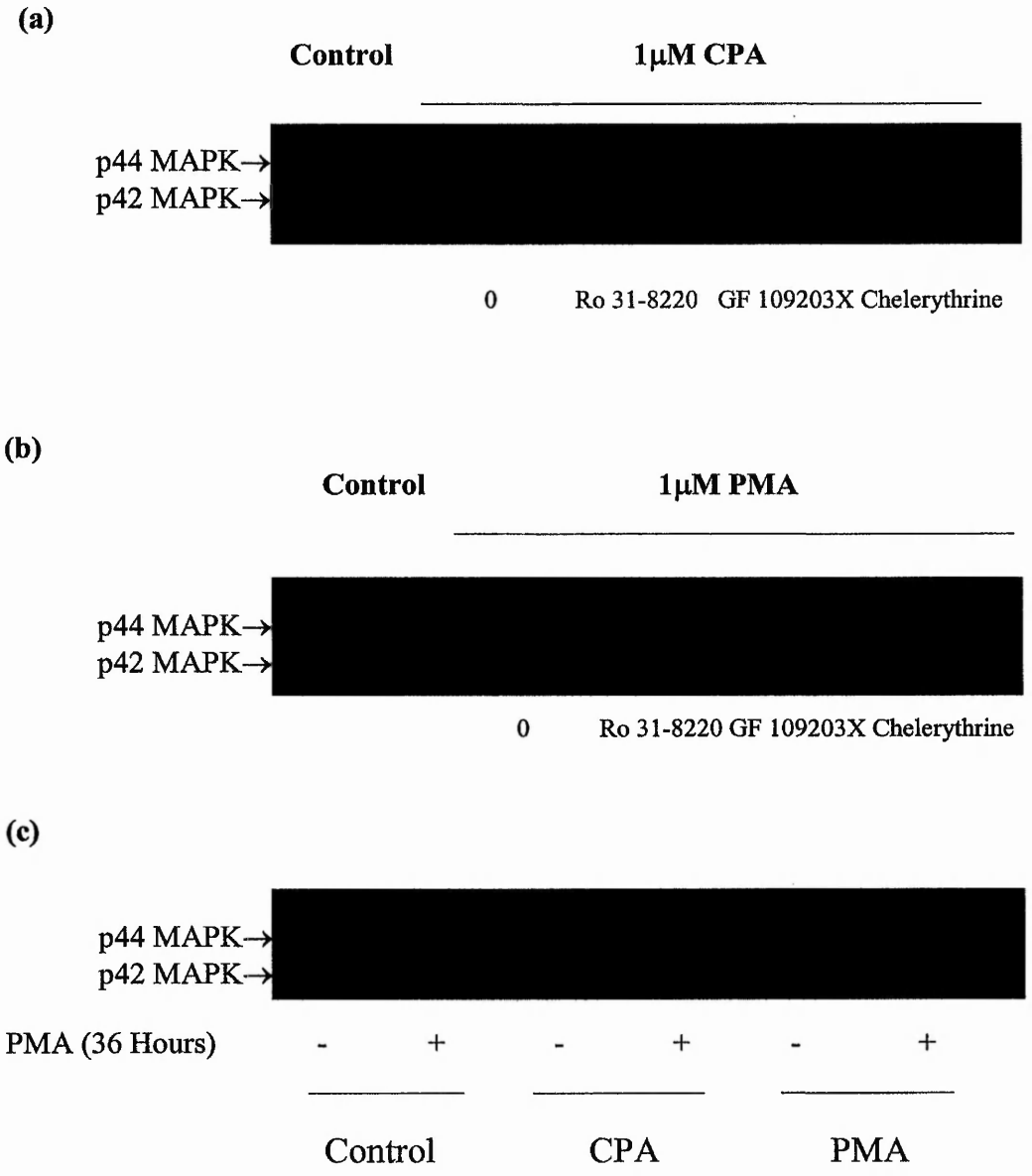


Figure 3.9 – Effect of the protein kinase C inhibitors Ro 31-8220, GF 109203X and chelerythrine, and protein kinase C downregulation, on adenosine A_1 receptor stimulation of the p42/p44 MAPK pathway in DDT_1MF-2 cells. Representative Western blots showing the effect of 30 mins pre-treatment with the PKC inhibitors 10μM Ro 31-8220, 1μM GF 109203X, and 1μM chelerythrine on (a) 1μM CPA-mediated and (b) 1μM PMA-mediated p42/p44 MAPK phosphorylation in DDT_1MF-2 cells. (c) The effect of 36 hours pre-treatment with 1μM PMA (PKC downregulation) on 1μM CPA- and 1 μM PMA-mediated p42/p44 MAPK phosphorylation in DDT_1MF-2 cells. CPA and PMA were all incubated for 5 mins after the pre-treatment protocols. 20μg of cell lysate per agonist was analysed for p42/p44 MAPK phosphorylation by Western blotting using a phospho-specific p42/p44 MAPK antibody. Results were obtained for at least four independent experiments (see Table 3.2 for a summary of the data).

Table 3.2 – The effect of various specific PKC inhibitors and PKC downregulation on CPA- and PMA-mediated p42/p44 MAPK phosphorylation. Serum-starved DDT_1MF-2 cells were pre-incubated for 30 mins with Ro 31-8220 (10 μM), GF 109203X (1 μM), and cheleryhrine (10 μM). Preincubation with PMA (1μM, 36 hours) resulted in PKC downregulation. Cells were then stimulated with CPA (1 μM) or PMA (1 μM) for 5 mins. Data is expressed as a percentage of the response obtained with 1 μM CPA (100%) and 1 μM PMA (100%) in control cells (independent controls were used for each inhibitor and PKC downregulation). Control cells were pre-incubated with 0.1% dimethylsuphoxide (DMSO) as a vehicle for 30 mins. Values represent mean ± S.E.M. of *n* experiments.

* Significantly ($p<0.05$, student's *t*-test) different compared to the control response.

Treatment	CPA (% of control)	*n*	PMA (% of control)	*n*
Ro 31-8220 (10μM)	99 ± 8	8	30 ± 5*	5
GF 109203X (1μM)	107 ± 8	6	25 ± 9*	4
Chelerythrine (10μM)	102 ± 10	7	19 ± 6*	4
Downregulation using PMA	90 ± 4	5	20 ± 6*	4

3.5 – Involvement of phosphatidylinositol 3-kinase (PI-3K) in p42/p44 MAPK Phosphorylation by Adenosine A_1 Receptors

PI-3K appears to have a prominent role in $G_{i/o}$PCR-mediated activation of the p42/p44 MAPK cascade (van Biesen *et al*, 1996, Michel *et al*, 2001), and it has been shown to be involved in p42/p44 MAPK activation by the human A_1R in transfected CHO cells (Dickenson *et al*, 1998). The role of PI-3K in A_1R-mediated

phosphorylation of p42/p44 MAPK in DDT_1MF-2 cells was explored using the PI-3K inhibitors, wortmannin (Yano *et al,* 1993) and LY 294002 (Vlahos *et al,* 1994). Preincubation of cells for 30 mins with wortmannin and LY 294002 reduced 1μM CPA-induced p42/p44 MAPK phosphorylation in a dose-responsive manner. At maximal CPA concentration, 1 μM, both compounds produced significant inhibition. Wortmannin (100nM) reduced the response by 57% ± 8% (n=5, $p<0.05$), while LY 294002 (100μM) reduced the response by 55% ± 11% (n=5, $p<0.05$). Representative Western blots and dose-response inhibition curves are shown in Figures 3.10 (wortmannin) and 3.11 (LY 294002). These data clearly indicate that a PI-3K-depedent pathway is important in the phosphorylation of p42/p44 MAPK by A_1Rs in DDT_1MF-2 cells.

3.6 – p38 MAPK and JNK/SAPK Phosphorylation by Adenosine A_1 Receptors

In previous studies it has been demonstrated that $G_{i/o}$PCRs can activate both the p38 MAPK (Yamauchi *et al,* 1997) and the JNK/SAPK cascades (Coso *et al,* 1995, Yamauchi *et al,* 2000.) Experiments determined whether p38 MAPK and JNK/SAPK could be activated in DDT_1MF-2 cells by using the osmotic stress inducer, sorbitol (0.5 M). Sorbitol induced time-dependent increases in phosphorylated p38 MAPK, and both 46 and 54 kDa isoforms of JNK/SAPK, although with different time course profiles (see Figure 3.12). Similar to p42/p44 MAPK, stimulation of DDT_1MF-2 cells by 1μM CPA induced time-dependent increases in p38 MAPK phosphorylation with peak activation occurring at five mins before returning to basal levels (see Figure 3.13). Phosphorylation of p38 MAPK by CPA was concentration-dependent, producing a $p[EC_{50}]$ value of 8.1 ± 0.1 (n=4, see Figure 3.14). CPA did not, however,

appear to stimulate increases in JNK/SAPK phosphorylation above basal levels in DDT_1MF-2 cells. Basal time-course profiles were recorded up to a period of 40 mins. CPA-mediated increases in p38 MAPK phosphorylation were inhibited in a dose-responsive manner by the specific A_1R antagonist, DPCPX, with an apparent K_D value of 1.2 ± 0.1 nM (n=5, see Figure 3.15). Finally, CPA-mediated increases in p38 MAPK phosphorylation were significantly inhibited by the specific p38 MAPK inhibitor, SB 203580 (20μM, 91% ±.7% inhibition, n=4, $p<0.05$; see Figure 3.16; Cuenda *et al,* 1995) and by pre-treatment for 16 hours with 100ng ml^{-1} PTX (95% ±.7% inhibition, n=4, $p<0.05$; see Figure 3.16).

3.7 - Summary

In conclusion, we have shown that stimulation of the endogenous adenosine A_1 receptor in DDT_1MF-2 cells induces p42/p44 MAPK phosphorylation via MEK1, PI-3K, and G_i/G_o proteins. Endogenous adenosine A_1 receptors in DDT_1MF-2 cells also induced phosphorylation of p38 MAPKs in a PTX-sensitive manner, indicating the receptor is coupled to $G_{i/o}$ proteins in this cell line. Adenosine A_1 receptor stimulation did not phosphorylate the 46kDa or 54kDa isoforms of JNK/SAPK in DDT_1MF-2 cells.

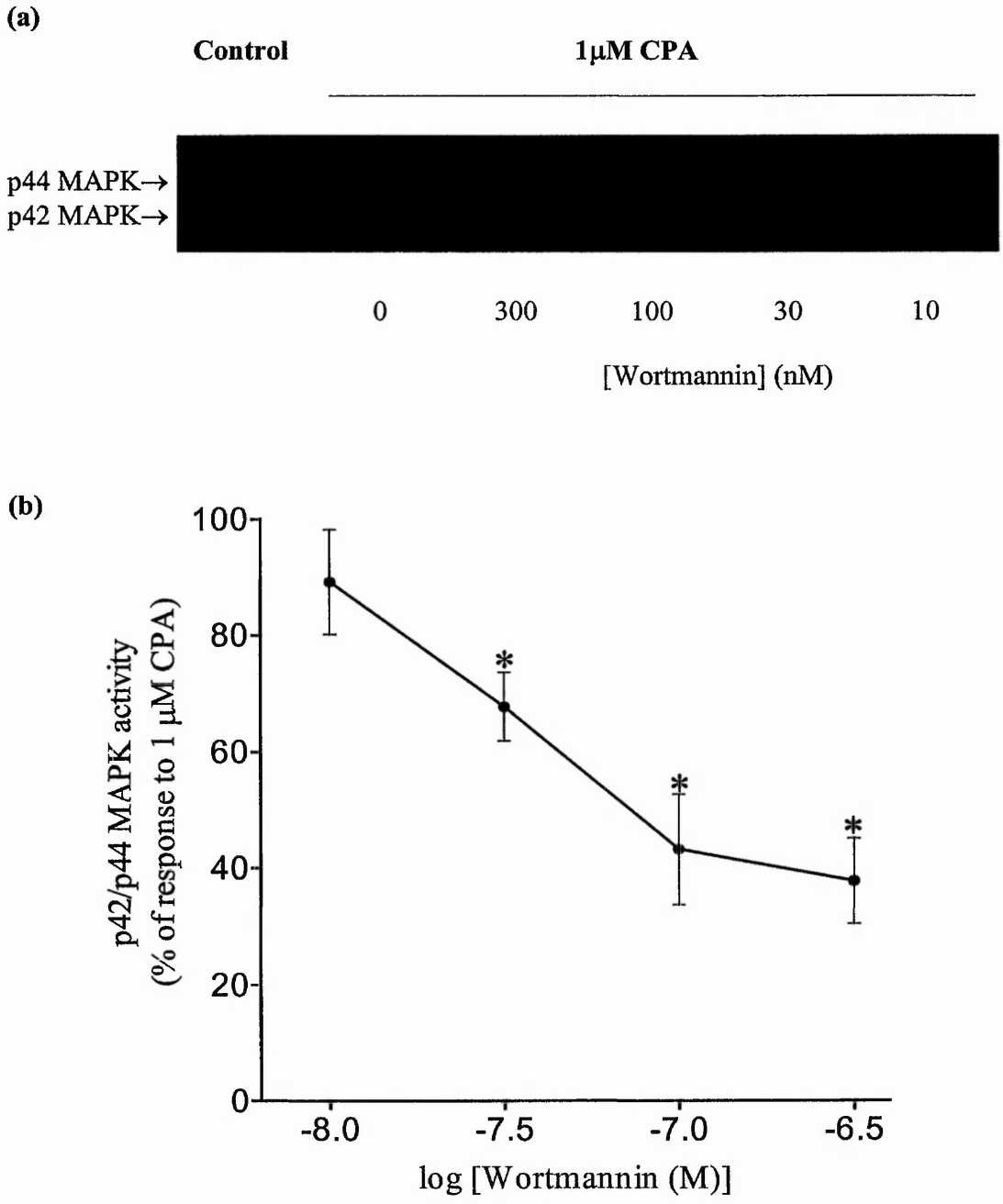


Figure 3.10 – Effect of the specific phosphoinositol 3-kinase inhibitor, wortmannin, on adenosine A_1 receptor stimulation of the p42/p44 MAPK pathway in DDT_1MF-2 cells. (a) Representative Western blot and (b) dose-inhibition curve (n=5) for wortmannin on p42/p44 MAPK phosphorylation mediated by 1μM CPA in DDT_1MF-2 cells. 20μg of cell lysate per concentration was analysed for p42/p44 MAPK phosphorylation by Western blotting using a phospho-specific p42/p44 MAPK antibody. * denotes significant differences ($p<0.05$, ANOVA test) for the specific inhibitor concentration compared to control (1μM CPA only).

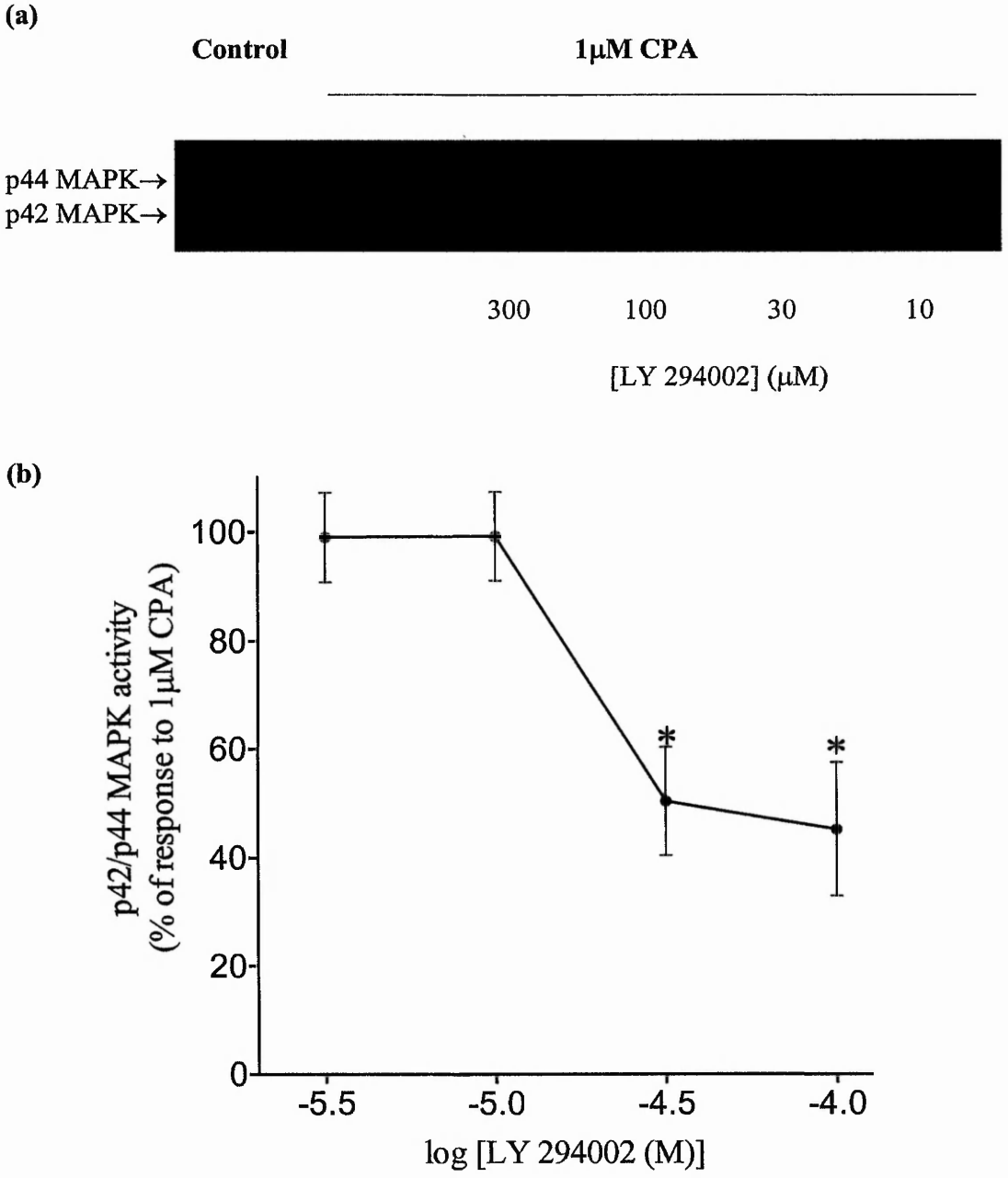


Figure 3.11 – Effect of the specific phosphoinositol 3-kinase inhibitor, LY 294002, on adenosine A_1 receptor stimulation of the p42/p44 MAPK pathway in DDT_1MF-2 cells. (a) Representative Western blot and (b) dose-inhibition curve (n=5) for LY 294002 on p42/p44 MAPK phosphorylation mediated by 1μM CPA in DDT_1MF-2 cells. 20μg of cell lysate per concentration was analysed for p42/p44 MAPK phosphorylation by Western blotting using a phospho-specific p42/p44 MAPK antibody. * denotes significant differences ($p<0.05$, ANOVA test) for the specific concentration compared to control (1μM CPA only).

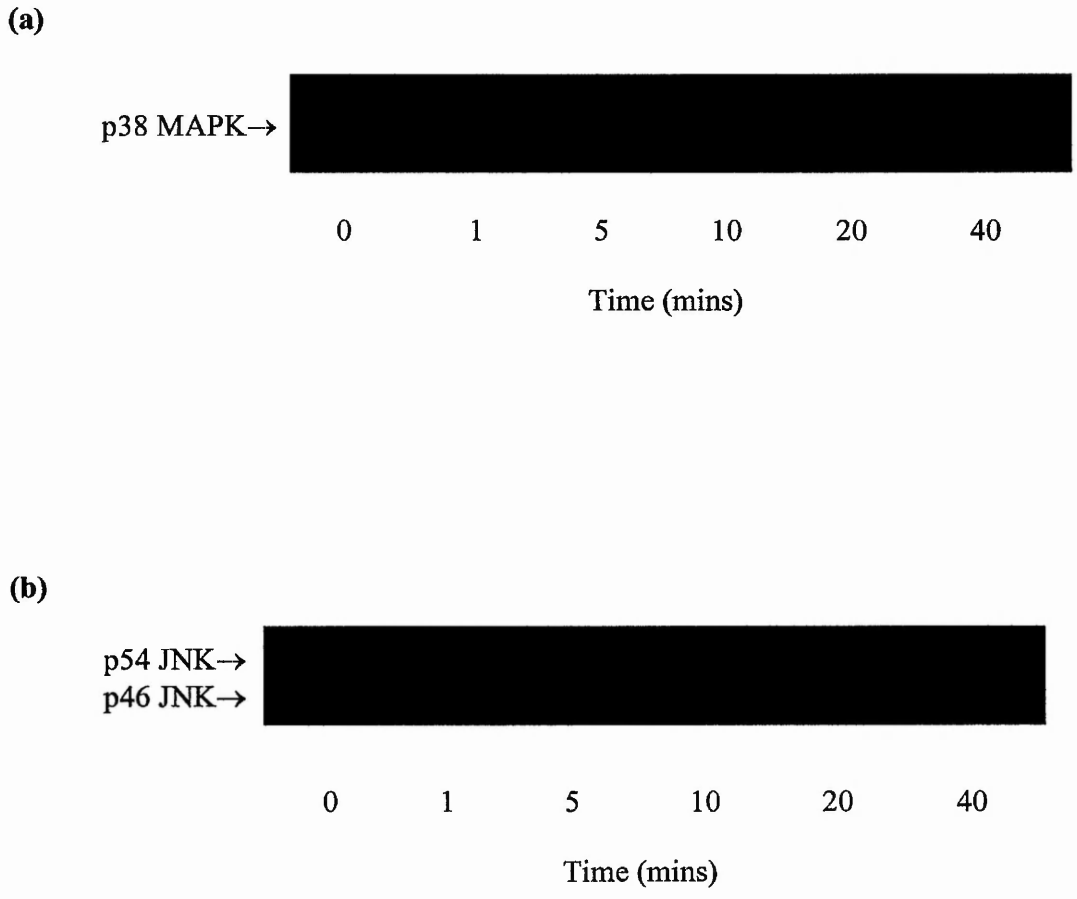


Figure 3.12 – Sorbitol-induced activation of the p38 MAPK and p46/p54 JNK/SAPK pathways in DDT_1MF-2 cells - time course. Representative Western blots showing the effect over a forty minute period of 0.5M sorbitol on (a) p38 MAPK phosphorylation and (b) p46/p54 JNK phosphorylation in DDT_1MF-2 cells. 20μg of cell lysate per time point was analysed for p38 MAPK and p46/p54 JNK phosphorylation by Western blotting using a phospho-specific p38 MAPK and p46/p54 JNK antibody respectively. Similar results were obtained for two further independent experiments.

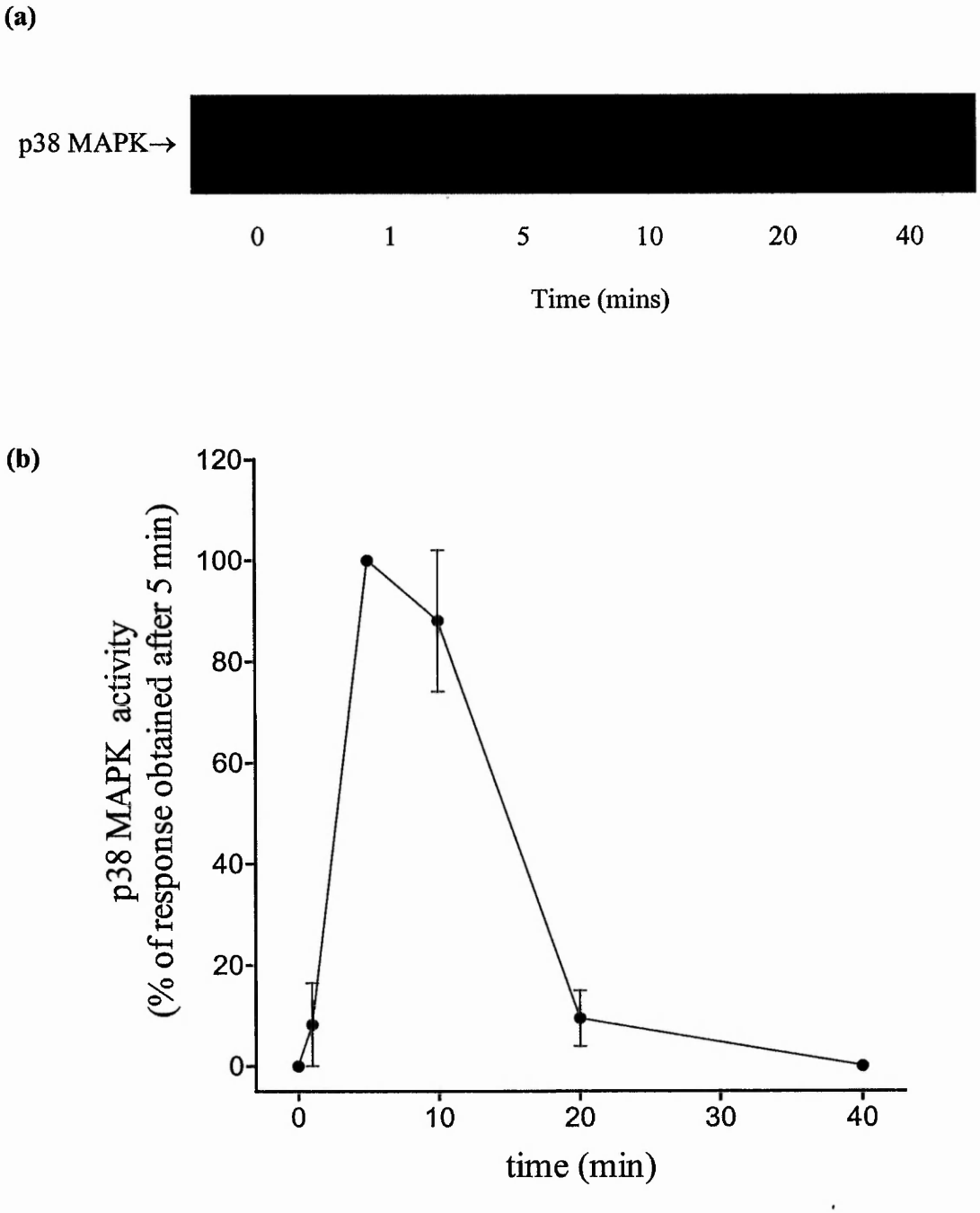
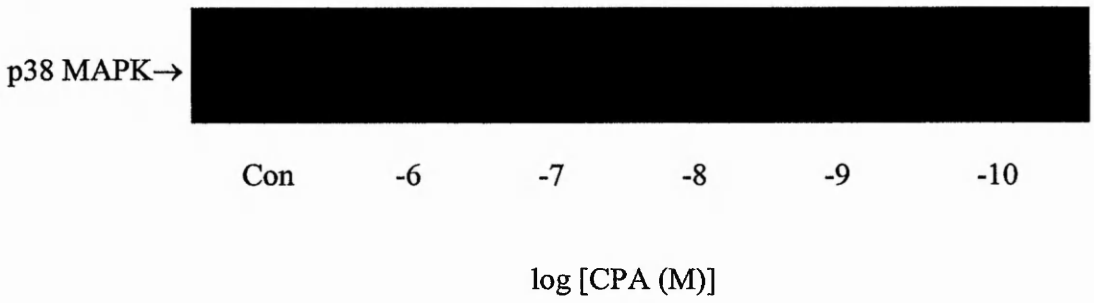


Figure 3.13 – Adenosine A_1 receptor stimulation of the p38 MAPK pathway in DDT_1MF-2 cells - time course. (a) Representative Western blot and (b) Time course plot (n=4) of 1μM CPA-mediated stimulation of p38 MAPK in DDT_1MF-2 cells. 20μg of cell lysate per time point was analysed for p38 MAPK phosphorylation by Western blotting using a phospho-specific p38 MAPK antibody. Cells were stimulated for up to forty minutes.

(a)



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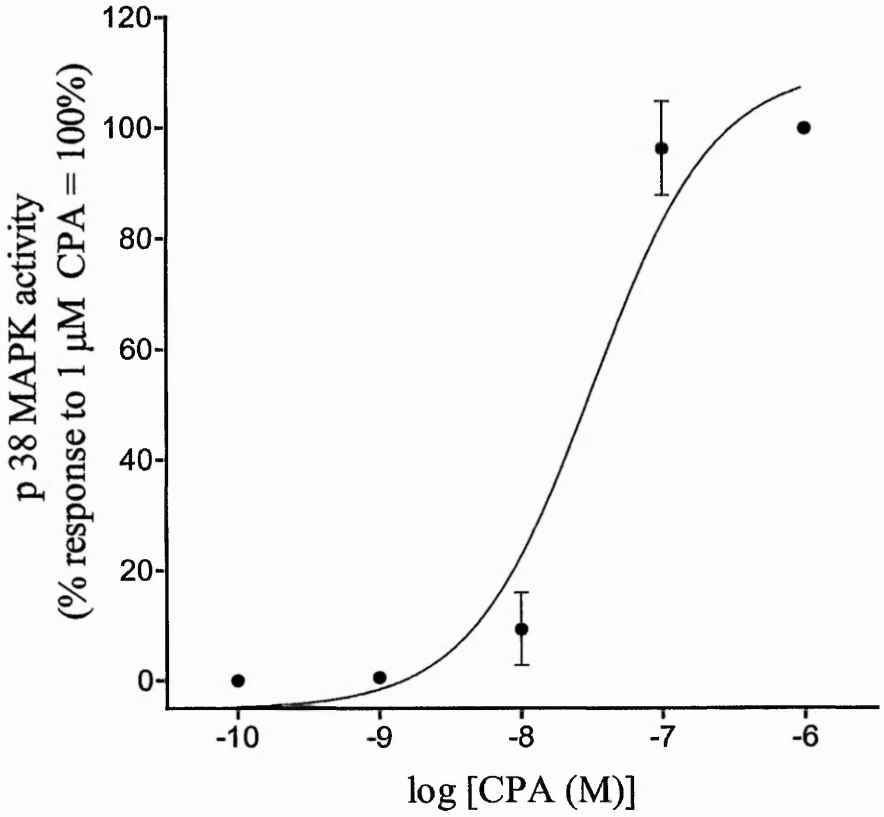
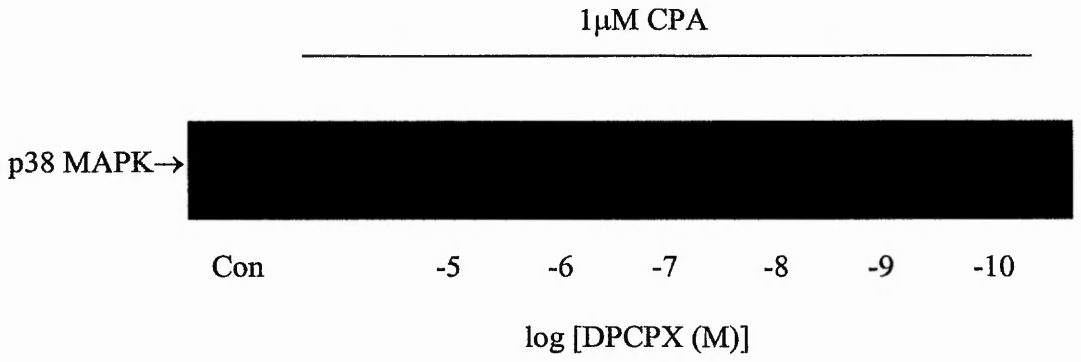


Figure 3.14 – CPA-mediated adenosine A_1 receptor stimulation of the p38 MAPK pathway in DDT_1MF-2 cells - concentration response. (a) Representative Western blot and (b) concentration-response curve of CPA-mediated stimulation of p38 MAPK in DDT_1MF-2 cells. 20μg of cell lysate per concentration of CPA was analysed for p38 MAPK phosphorylation by Western blotting using a phospho-specific p38 MAPK antibody. p[EC_{50}] value = 8.1 ± 0.3 (n=4). Values represent mean ± S.E.M. of four independent experiments. Con = control sample (0% response).

(a)



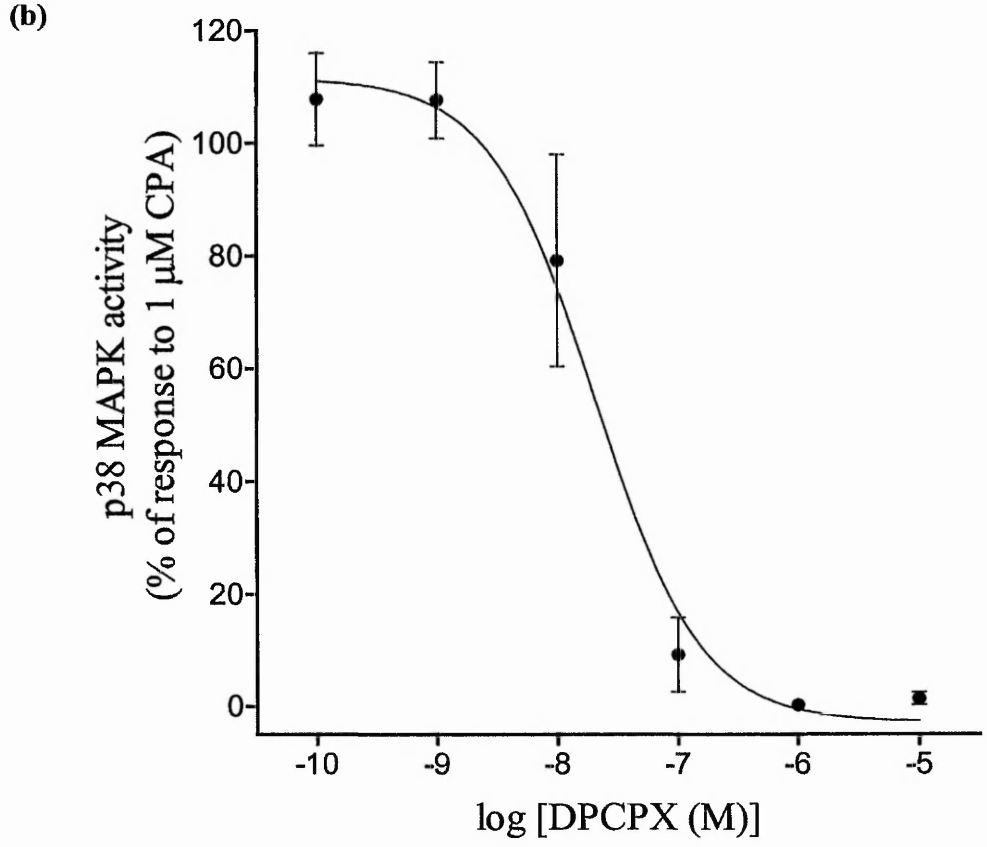


Figure 3.15 – Effect of the specific A_1R antagonist, DPCPX, on CPA-mediated adenosine A_1 receptor stimulation of the p38 MAPK pathway in DDT_1MF-2 cells. (a) Representative Western blot and (b) concentration-response curve (n=5) of antagonism by DPCPX of CPA-mediated stimulation of p38 MAPK in DDT_1MF-2 cells. Cells were pre-incubated for 30 mins with the indicated concentrations of DPCPX, before being exposed to 1μM CPA for a further 5 mins. 20μg of cell lysate per concentration of CPA was analysed for p38 MAPK phosphorylation by Western blotting using a phospho-specific p38 MAPK antibody. Apparent K_D value = 1.2 ± 0.1 (n=5). Values represent mean ± S.E.M. of five independent experiments. Con = control sample (0% response).

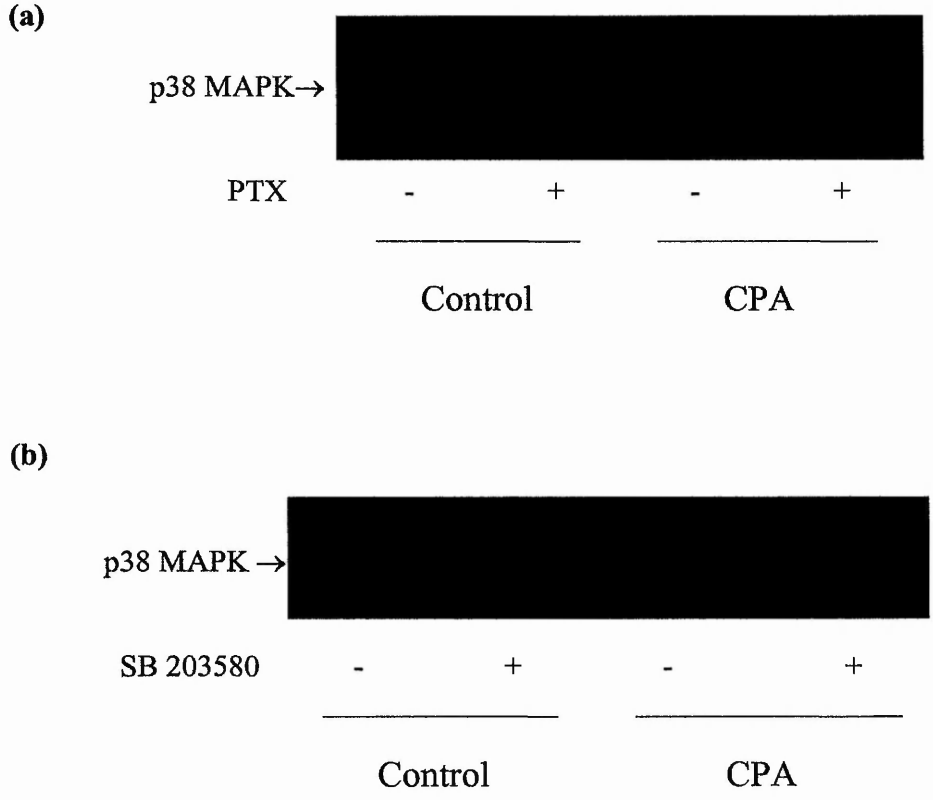


Figure 3.16 – Effect of pertussis toxin (PTX) and the specific p38 MAPK inhibitor, SB 203580, on adenosine A_1 receptor stimulation of the p38 MAPK pathway in DDT_1MF-2 cells. Representative Western blots showing the effect of pre-treatment with (a) 100ng ml^{-1} PTX for 16 hours and (b) 20μM SB 203580 (30 min) on 1μM CPA-mediated p38 MAPK phosphorylation in DDT_1MF-2 cells. Cells were exposed to CPA for 5 mins after the pre-treatment protocols. 20μg of cell lysate per time point was analysed for p38 MAPK phosphorylation by Western blotting using a phospho-specific p38 MAPK antibody. PTX and SB 203580 inhibited the CPA-mediated response by 95% ±.7% (n=4, $p<0.05$) and 91% ± 7% (n=5, $p<0.05$) respectively. Con = control sample (0% response).

Chapter 4

ACTIVATION OF p42/p44 MITOGEN-ACTIVATED PROTEIN KINASES BY THE HISTAMINE H_1 RECEPTOR

4.0 – ACTIVATION OF p42/p44 MITOGEN-ACTIVATED PROTEIN KINASES BY THE HISTAMINE H_1 RECEPTOR

4.1 – p42/p44 MAPK Phosphorylation by Histamine H_1 Receptors

Histamine H_1 receptors (H_1Rs) are a "classical" type of G_qPCR, and a recent study presented evidence for H_1R coupling to the MAPK cascade in smooth muscle cells (Koch *et al,* 2000). Therefore, the effect of histamine H_1 receptors (H_1Rs) on the p42/p44 mitogen-activated protein kinase (p42/p44 MAPK) pathway in DDT_1MF-2 cells was investigated using the endogenous agonist, histamine. Stimulation of DDT_1MF-2 cells with maximally-effective concentrations of histamine (100μM) over a period of 40 min produced a maximal activated p42/p44 MAPK response at 5 min, before decreasing back to basal levels (see Figure 4.1). Similar to CPA and adenosine (see section 3.1) there was dominant phosphorylation of p42 MAPK by histamine, compared to p44 MAPK (Figure 3.1a). Activation of p42/p44 MAPK by histamine was also concentration dependent, with a $p[EC_{50}]$ value of 6.1 ± 0.3 (n=4) (see Figure 3.2). The selective histamine H_1 receptor antagonist, mepyramine, antagonised histamine-mediated increases in p42/p44 MAPK phosphorylation, yielding an apparent K_D value of 3.1 ± 0.8 nM (n=3, see Figure 4.3). Pretreatment with the selective histamine H_2 and histamine H_3 receptor antagonists, tiotidine (1 μM) and thioperamide (1 μM) respectively, did not significantly reduce histamine-induced p42/p44 MAPK phosphorylation (see Figure 4.4). Only one concentration of each agonist was used, since, at 1μM and for 30 mins pre-incubation, both tiotidine and thioperamide have been shown to be potent H_2 and H_3 receptor antagonists respectively (Cooper *et al,* 1990, Hill, 1990). Also, since there is currently no

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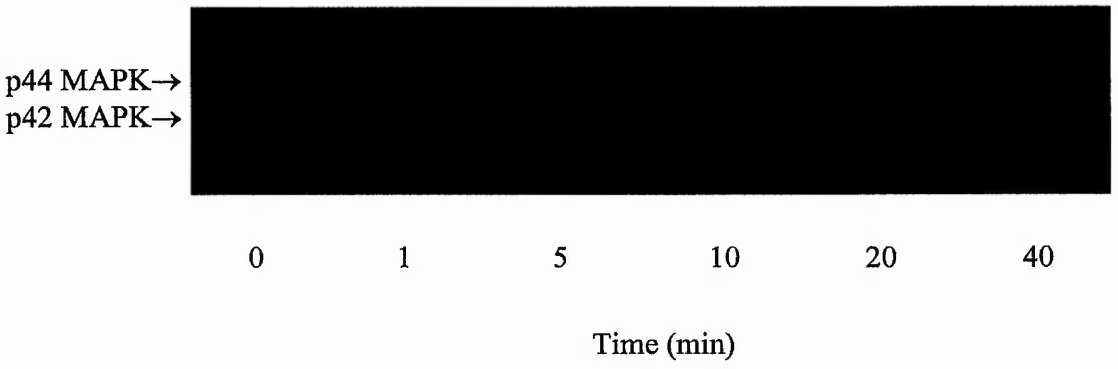

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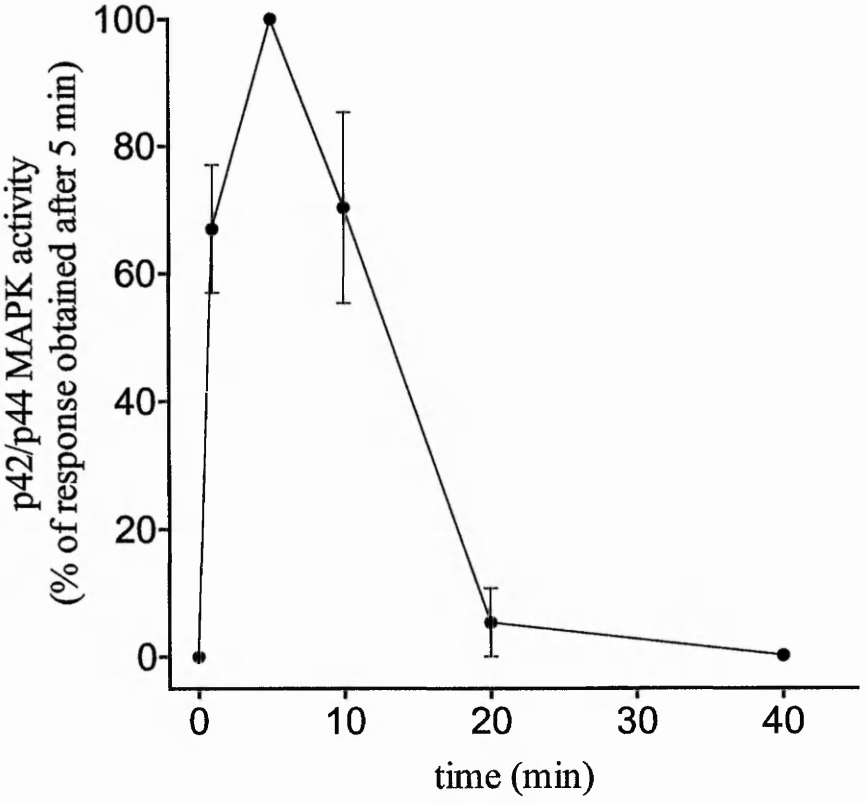
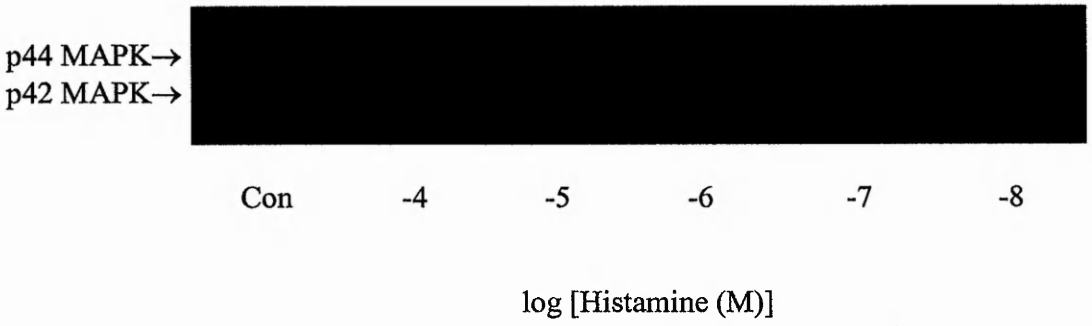


Figure 4.1 – Histamine-mediated stimulation of the p42/p44 MAPK pathway in DDT$_1$MF-2 cells - time course. (a) Representative Western blot and (b) Time course plot (n=4) of 100μM histamine-mediated stimulation of p42/p44 MAPK in DDT$_1$MF-2 cells. 20μg of cell lysate per time point was analysed for p42/p44 MAPK phosphorylation by Western blotting using a phospho-specific p42/p44 MAPK antibody. Cells were stimulated for up to forty minutes. Control values are given as 0.

(a)



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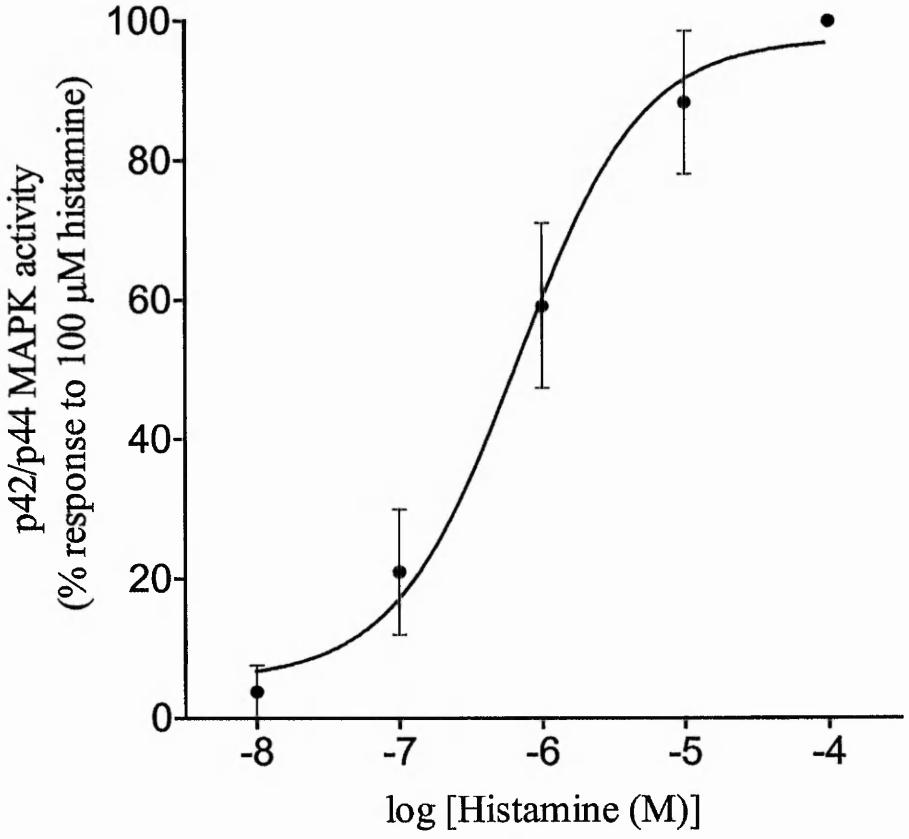


Figure 4.2 – Histamine-mediated stimulation of the p42/p44 MAPK pathway in DDT$_1$MF-2 cells - concentration response. (a) Representative Western blot and (b) concentration-response curve of histamine-mediated stimulation of p42/p44 MAPK in DDT$_1$MF-2 cells. 20µg of cell lysate per concentration of histamine was analysed for p42/p44 MAPK phosphorylation by Western blotting using a phospho-specific p42/p44 MAPK antibody. Values represent mean ± S.E.M. of six independent experiments. Con = control sample (0% response).

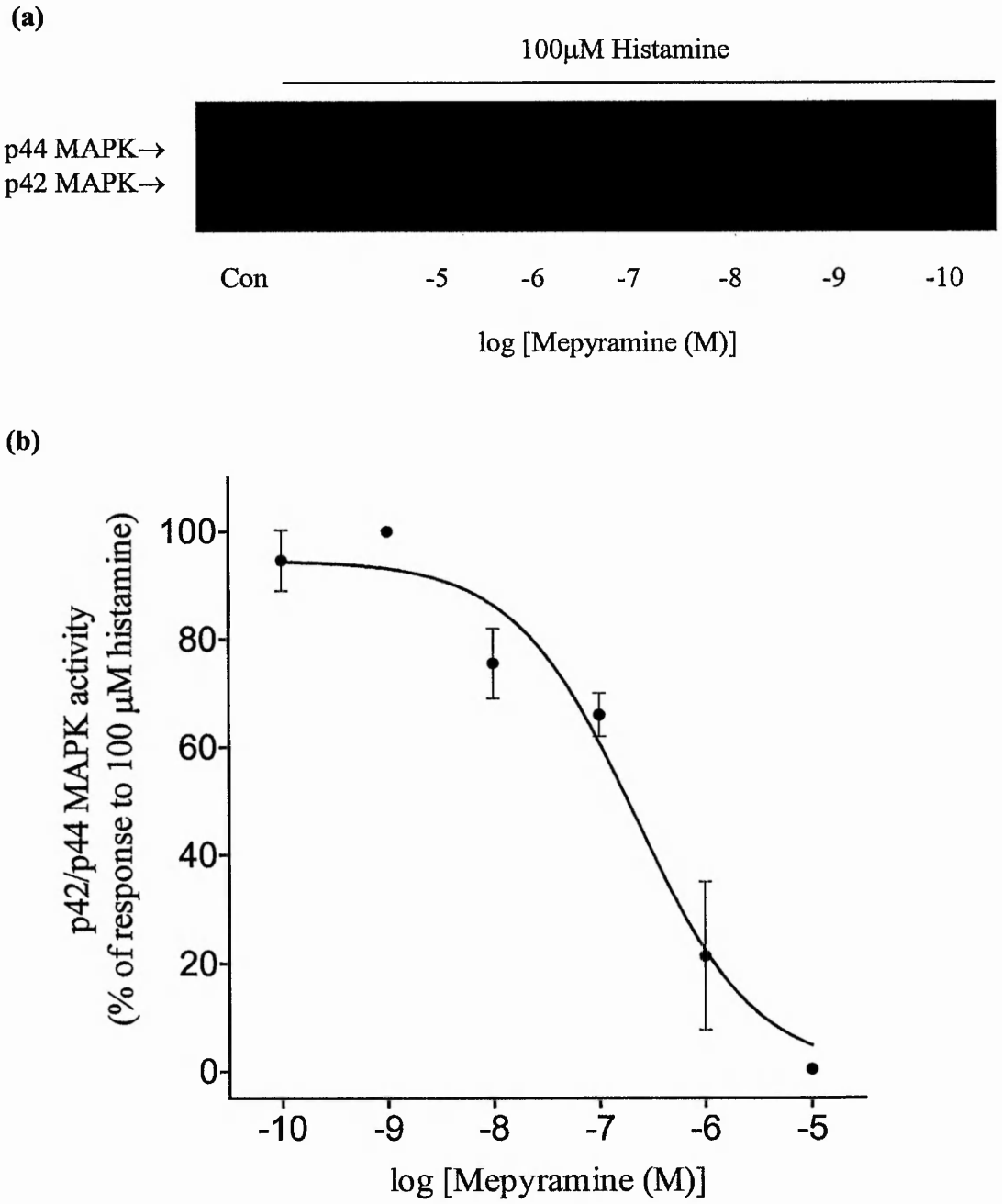


Figure 4.3 – Effect of the specific H_1R antagonist, mepyramine, on histamine H_1 receptor stimulation of the p42/p44 MAPK pathway in DDT_1MF-2 cells. (a) Representative Western blot and (b) concentration-response curve (n=3) of antagonism by mepyramine of histamine-mediated stimulation of p42/p44 MAPK in DDT_1MF-2 cells. Cells were pre-incubated for 30 min with the indicated concentrations of mepyramine, before being exposed to 100μM histamine for a further 5 min. 20μg of cell lysate per concentration of histamine was analysed for p42/p44 MAPK phosphorylation by Western blotting using a phospho-specific p42/p44 MAPK antibody. Apparent K_D value = 3.1 ± 0.8 nM (n=3). Values represent mean ± S.E.M. of three independent experiments. Con = control sample (0% response).

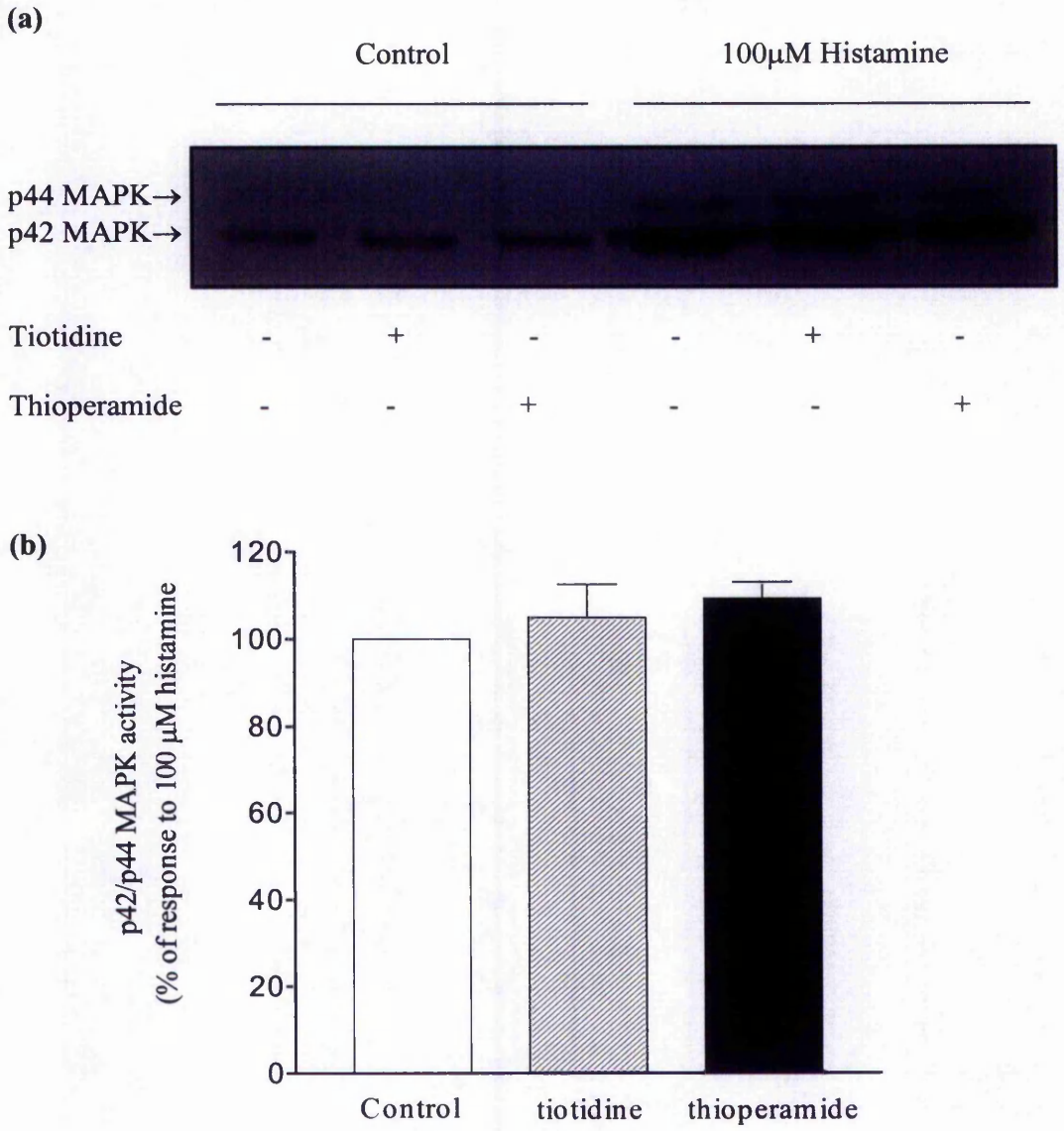


Figure 4.4 – Effect of the specific histamine receptor antagonists tiotidine (H_2) and thioperamide (H_3) on histamine-mediated stimulation of the p42/p44 MAPK pathway in DDT_1MF-2 cells. (a) Representative Western blot and (b) bar graph (n=3) of antagonism by tiotidine (1 μM) and thioperamide (1 μM) of histamine-mediated stimulation of p42/p44 MAPK in DDT_1MF-2 cells. Cells were pre-incubated for 30 min with the indicated histamine antagonist, before being exposed to 100μM histamine for a further 5 min. 20μg of cell lysate per sample was analysed for p42/p44 MAPK phosphorylation by Western blotting using a phospho-specific p42/p44 MAPK antibody. Values represent mean ± S.E.M. of three independent experiments.

evidence for H_2 or H_3 receptors on DDT_1MF-2 cells, one known highly inhibitory concentration of each antagonist was used to preserve resources. These data indicate that increases in p42/p44 MAPK phosphorylation following stimulation of DDT_1MF-2 cells with histamine are mediated via H_1Rs.

4.2 - The Effect of PTX and PD 98059 on H_1R-Mediated p42/p44 MAPK Activation

As has already been stated, H_1Rs couple to the G_q family of G proteins, which are PTX-insensitive (Hill *et al*, 1997). Previous studies have shown that histamine H_1 receptor activation in DDT_1MF-2 cells stimulates PTX-insensitive increases in inositol phosphate accumulation and calcium mobilization (Dickenson & Hill, 1991, Dickenson & Hill, 1992, White *et al,* 1993). These observations reflect the proposed coupling of histamine H_1 receptors to phospholipase C activation via G_q-proteins (Hill *et al.*, 1997). However, pre-treatment with PTX (100 ng ml^{-1} for 16 h) significantly reduced histamine-induced p42/p44 MAPK activation (57 ± 9% inhibition; n=7; $p<0.05$; Figure 4.5a) suggesting the involvement of $G_{i/o}$ proteins. In addition PTX pre-treatment inhibited basal p42/p44 MAPK phosphorylation (60 ± 6% inhibition; n=7). MEK1 dual phosphorylates, and activates, p42/p44 MAPK (Widmann *et al,* 1999). DDT_1MF-2 cells were preincubated with the specific MEK1 inhibitor, PD 98059 (50 μM, 30 min, Dudley *et al,* 1995) before exposing cells to histamine (100μM) for five min. PD 98059 significantly reduced histamine-mediated p42/p44 MAPK phosphorylation (89% ± 5% inhibition, n=3, $p<0.05$, Figure 4.5b).

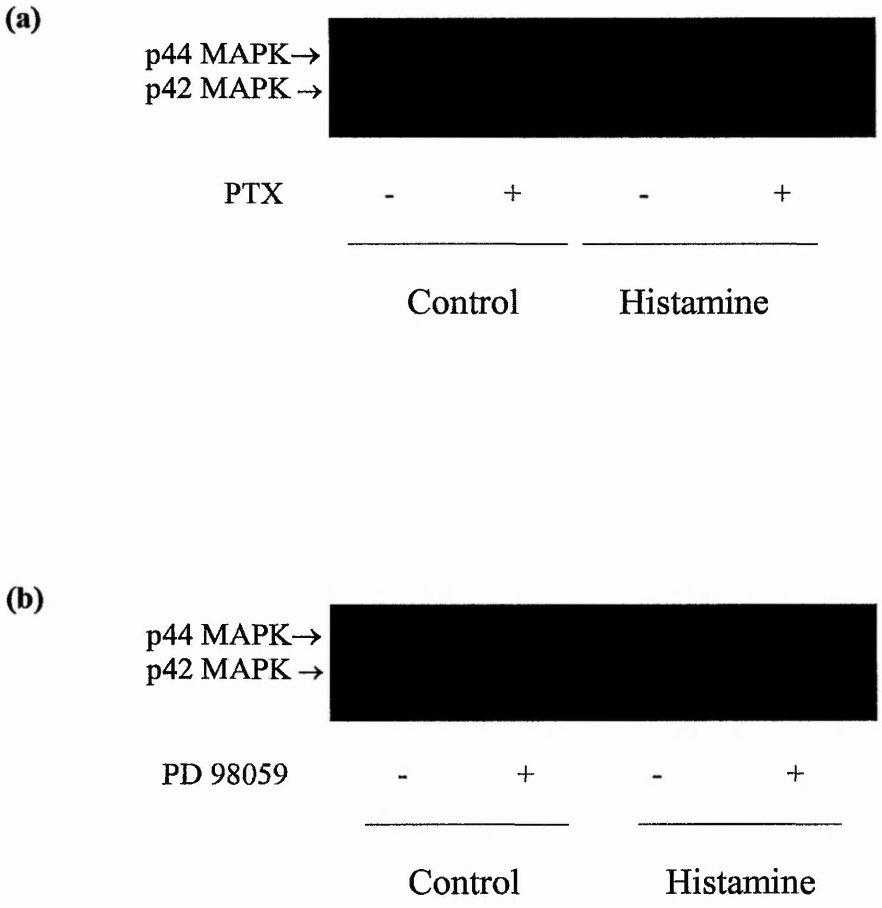


Figure 4.5 – Effect of pertussis toxin (PTX) and the specific MEK1 inhibitor, PD 98059, on histamine H_1 receptor stimulation of the p42/p44 MAPK pathway in DDT_1MF-2 cells. Representative Western blots showing (a) the effect of pre-treatment of 100ng ml^{-1} PTX for 16 hours (n=7), and (b) the effect of pre-treatment of 50μM PD98059 for 30 min, (n=3), on 100 μM histamine-mediated p42/p44 MAPK phosphorylation in DDT_1MF-2 cells. Histamine was incubated for 5 min after the pre-treatment protocols. 20μg of cell lysate per agonist was analysed for p42/p44 MAPK phosphorylation by Western blotting using a phospho-specific p42/p44 MAPK antibody.

4.3 - The Involvement of Tyrosine Kinases and "Transactivation" in H_1R-Mediated p42/p44 MAPK Activation

Tyrosine kinases (both receptor and non-receptor) have been shown to be involved in activation of p42/p44 MAPK by both G_qPCRs and $G_{i/o}$PCRs (Lopez-Ilasaca, 1998). The involvement of tyrosine kinases in H_1R-induced phosphorylation of p42/p44 MAPK in DDT_1MF-2 cells was investigated using genistein, tyrphostin A47, and PP2 (PP2 specifically inhibits the Src family of tyrosine kinases, Hanke *et al*, 1996). Daidzein, the inactive analogue of genistein, was again used as the control. As shown in Figure 4.6a pretreatment with genistein (100 μM, 30 min) significantly inhibited (87% ± 8%, n=6, $p<0.05$) histamine-mediated p42/p44 MAPK phosphorylation, but daidzein had no significant effect (95% ± 8% of control response, n=4, $p>0.05$). Pretreatment with tyrphostin A47 (100 μM, 30 min) significantly reduced p42/p44 MAPK phosphorylation (95% ± 9% inhibition, n=6, $p<0.05$, see figure 4.6b), but pre-treatment with the Src-family tyrosine kinase inhibitor, PP2 (10 μM, 30 min), did not (95% ± 8% of control response, n=4, $p>0.05$, see Figure 4.6b). Previous studies have shown that this concentration of PP2 for the specified pre-incubation period is sufficient to inhibit Src signalling (Dckenson *et al,* 1998). The data obtained from these experiments provides evidence that H_1R stimulation of the p42/p44 MAPK cascade in DDT_1MF-2 cells requires activation of tyrosine kinases, but not activation of the Src family of tyrosine kinases.

As previously explained recent studies have demonstrated that certain GPCRs activate the p42/p44 MAPK pathway via transactivation (ligand-independent) of the epidermal growth factor receptor tyrosine kinase (Zwick *et al.*, 1999). Pre-incubation of cells with AG1478 (1 μM; 30 min), an inhibitor specific for the epidermal growth

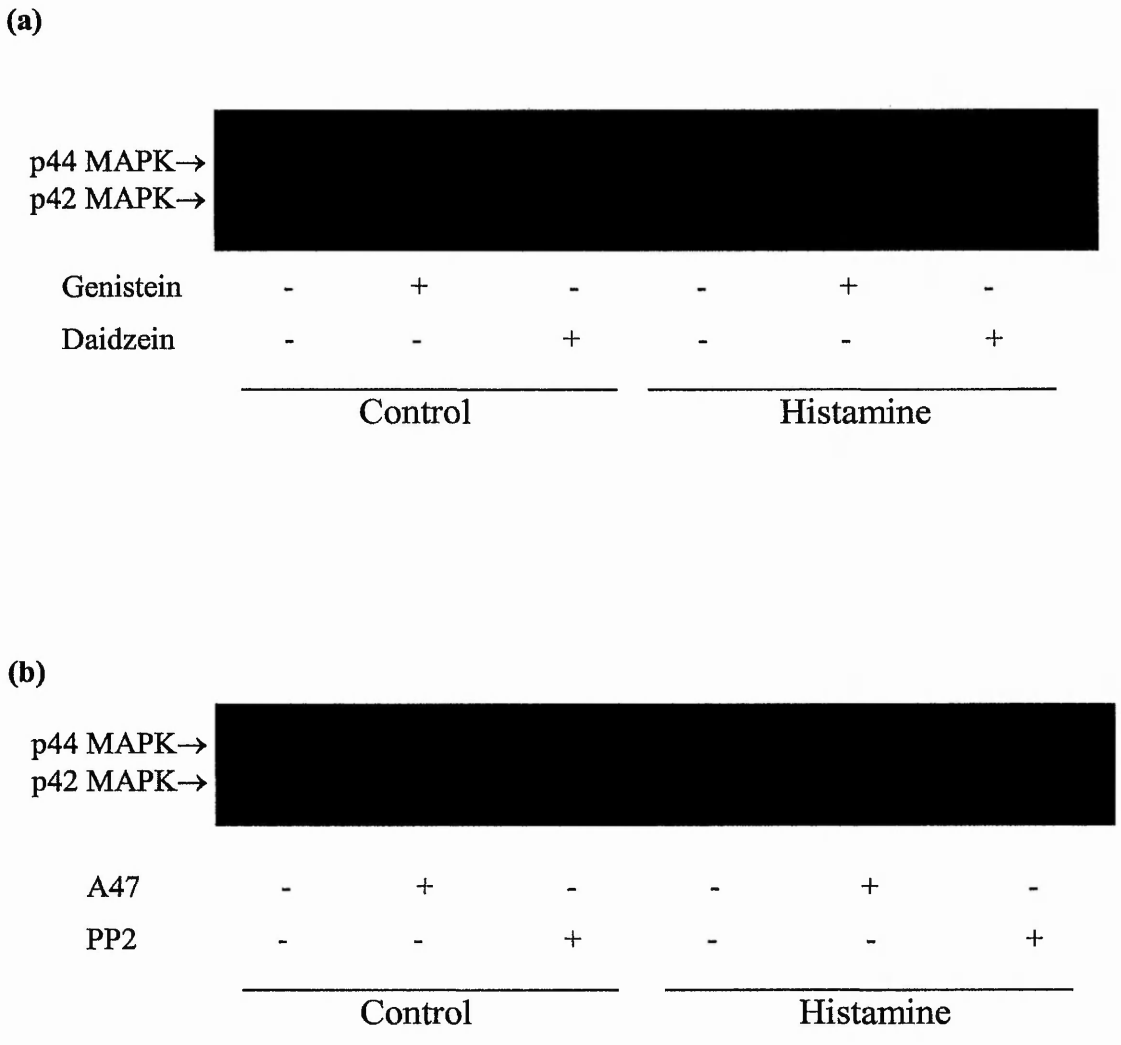


Figure 4.6 – Effect of the tyrosine kinase inhibitors, genistein and tyrphostin A47, and PP2, on histamine H_1 receptor stimulation of the p42/p44 MAPK pathway in DDT_1MF-2 cells. Representative Western blots showing the effect of 30 min pre-treatment with (a) 100μM genistein (n=6) and 100μM daidzein (n=4), and (b) 100μM tyrphostin A47 (n=6) and 10μM PP2 (n=4), on 100μM histamine-mediated p42/p44 MAPK phosphorylation in DDT_1MF-2 cells. Cells were incubated for 5 min after the pre-treatment protocols. 20μg of cell lysate per agonist was analysed for p42/p44 MAPK phosphorylation by Western blotting using a phospho-specific p42/p44 MAPK antibody.

factor receptor tyrosine kinase (Levitzki & Gazit, 1995) had no significant effect on histamine (100 μM)-mediated p42/p44 MAPK activation. In contrast, epidermal growth factor (10 nM)-induced p42/p44 MAPK activation was completely inhibited (10 ± 8% of control response; n=4; Figure 4.7) by AG 1478 (1 μM). These data suggest that epidermal growth factor receptor transactivation is not involved in histamine H_1 receptor-mediated p42/p44 MAPK activation in DDT_1MF-2 cells.

4.4 - Role of Ca^{2+} and PKC in Histamine H_1 Receptor-Mediated p42/p44 MAPK activation.

PLC activation generates IP_3 and DAG, which mobilise intracellular Ca^{2+} and activate certain PKC isoforms, respectively (Berridge, 1993). Histamine H_1 receptor activation in DDT_1MF-2 cells stimulates intracellular Ca^{2+} release and Ca^{2+} influx (Dickenson & Hill, 1991, Dickenson & Hill, 1992). As before, the role Ca^{2+} influx was explored by measuring p42/p44 MAPK responses in the absence of extracellular Ca^{2+} (using nominally Ca^{2+}-free Hanks/HEPES buffer containing 0.1 mM EGTA). As shown in Figure 4.8a, removal of extracellular Ca^{2+} had no significant effect on histamine (100 μM; 81 ± 9% of control response; *n*=8; p>0.05) induced p42/p44 MAPK activation. The potential role of Ca^{2+} derived from intracellular stores was investigated using the Ca^{2+} chelator BAPTA (cells pre-incubated for 30 min with 50 μM BAPTA/AM) in the absence of extracellular Ca^{2+}. Loading cells with BAPTA in the absence of extracellular Ca^{2+} did not inhibit histamine-induced p42/p44 MAPK activation (90 ± 12% of control; *n*=4,see Figure 4.8b). These observations demonstrate that p42/p44 MAPK activation by histamine H_1 receptors is

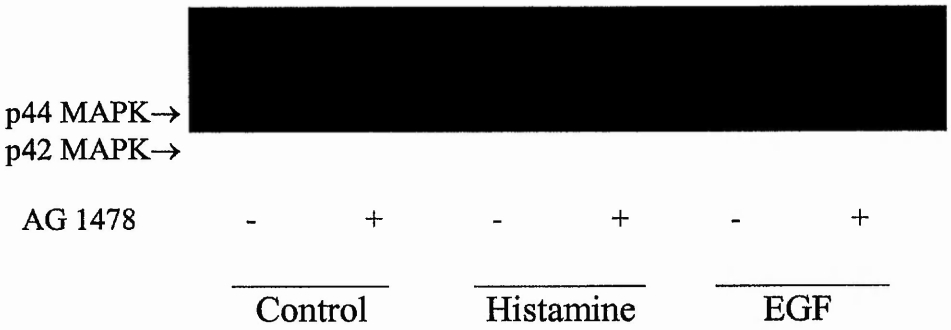


Figure 4.7 – Effect of the EGF receptor tyrosine kinase inhibitor, AG 1478, on histamine H_1 receptor stimulation of the p42/p44 MAPK pathway in DDT_1MF-2 cells. Representative Western blots showing the effect of 30 min pre-treatment with 1μM AG 1478, on 100μM histamine-and 10nM EGF-mediated p42/p44 MAPK phosphorylation in DDT_1MF-2 cells. Histamine and EGF were incubated for 5 min after the pre-treatment protocols. 20μg of cell lysate per agonist was analysed for p42/p44 MAPK phosphorylation by Western blotting using a phospho-specific p42/p44 MAPK antibody. Similar results were obtained in three other independent experiments.

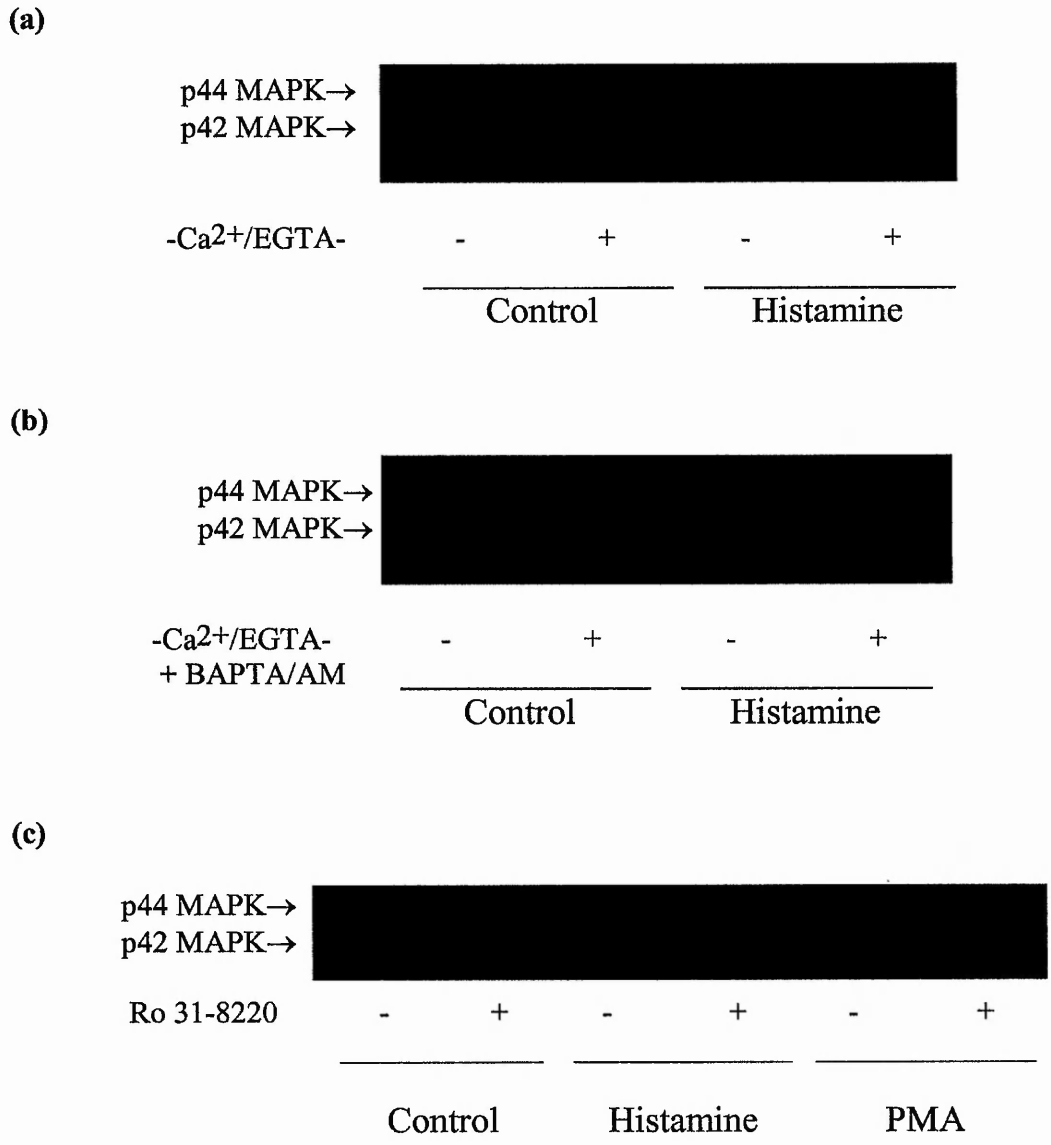


Figure 4.8 – Effect of Ca^{2+} influx restriction, intracellular Ca^{2+} release depravation, and protein kinase C inhibition on histamine H_1 receptor stimulation of the p42/p44 MAPK pathway in DDT$_1$MF-2 cells. Representative Western blots showing the effect of (a) reduction of Ca^{2+} influx, (b) reduction of intracellular Ca^{2+} levels by pretreating cells for 30 min with the Ca^{2+} chelator, BAPTA/AM (50 μM), and (c) PKC inhibition, via 30 min pre-treatment with the PKC inhibitor, Ro 31-8220 (10 μM). Histamine (100 μM) and PMA (100 nM) were incubated for 5 min after the pre-treatment protocols. 20μg of cell lysate per agonist was analysed for p42/p44 MAPK phosphorylation by Western blotting using a phospho-specific p42/p44 MAPK antibody. Results were obtained for at least four independent experiments.

independent of Ca^{2+} elevation in DDT_1MF-2 cells.

The role of PKC in the regulation of p42/p44 MAPK by the histamine H_1 receptor was explored using the PKC inhibitor Ro 31-8220 (Davis *et al.*, 1989). The PKC activator, phorbol 12-myristate 13-acetate (PMA, 100 nM) induced p42/p44 MAPK activation in DDT_1MF-2 cells and as expected Ro 31-8220 (10 μM) inhibited this response (85 ± 5% inhibition; n=5, $p<0.05$). Responses to histamine (100 μM) were partially sensitive to PKC inhibition (41 ± 7% inhibition; n=6, $p<0.05$) indicating the possible involvement of a PKC-dependent pathway (Figure 4.8c).

4.5 - Role of Focal Adhesion Kinases in Histamine H_1 Receptor-Mediated p42/p44 MAPK Activation.

Recent studies have shown that depolymerisation of the actin cytoskeleton using cytochalasin D blocks GPCR-mediated p42/p44 MAPK activation suggesting the involvement of focal adhesion based signalling (Luttrell *et al.*, 1997; Della Rocca *et al.*, 1999; Luttrell *et al.*, 1999). However, as shown in Figure 4.9, pre-treatment of DDT_1MF-2 cells with cytochalasin D (1 μM for 1 h) had no significant effect on histamine (100 μM; 95 ± 7% of control response; n=4) induced p42/p44 MAPK activation.

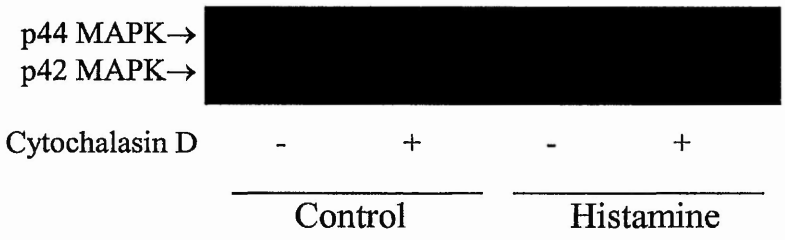


Figure 4.9 – Effect of the focal adhesion kinase inhibitor, cytochalasin D, on histamine H_1 receptor stimulation of the p42/p44 MAPK pathway in DDT_1MF-2 cells. Representative Western blot showing the effect of 1 hour pre-treatment with 1μM cytochalasin D, on 100μM histamine-mediated p42/p44 MAPK phosphorylation in DDT_1MF-2 cells. Histamine was incubated for 5 min after the pre-treatment protocol. 20μg of cell lysate per agonist was analysed for p42/p44 MAPK phosphorylation by Western blotting using a phospho-specific p42/p44 MAPK antibody. Similar results were obtained in three other independent experiments.

4.6 – Involvement of Phosphatidylinositol 3-Kinase (PI-3K) in p42/p44 MAPK Phosphorylation by Histamine H1 Receptors

Phosphatidylinositol 3-kinase (PI-3K) has been implicated in G_i-PCR-induced regulation of p42/p44 MAPK (Sudgen & Clerk, 1997; van Biesen *et al.*, 1996). In this study the role of PI-3K in the regulation of p42/p44 MAPK by the histamine H_1 receptor in DDT_1MF-2 cells was examined. As shown in Figures 4.10 and 4.11, responses to histamine (100 μM) were inhibited following pre-treatment (30 min) with wortmannin ($p[IC_{50}] = 7.6 \pm 0.06$; n=4) and LY 294002 ($p[IC_{50}] = 4.8 \pm 0.1$; n=4) respectively. In these experiments, wortmannin (30 nM) and LY 294002 (30 μM) inhibited histamine (100 μM) p42/p44 MAPK responses by 64 ± 10% (n=4) and 85 ± 8% (n=4) respectively. These observations clearly demonstrate that a PI-3K-dependent pathway is involved in histamine H_1 receptor-mediated p42/p44 MAPK activation in DDT_1MF-2 cells.

4.7 - Effect of Histamine H_1 Receptor Activation on JNK/SAPKs and p38 MAPKs.

As previously explained, the p38 MAPK and JNK/SAPK signalling pathways are activated by stimuli such as UV irradiation, osmotic stress and inflammatory cytokines (Paul *et al.*, 1997). Having previously established that osmotic stress activates p38 MAPK and JNK in DDT_1MF-2 cells, using sorbitol (0.5 M, see section 3.6 and Figure 3.12), it was determined whether histamine H_1 receptor activation in this cell line could activate the p38 MAPK and/or the JNK/SAPK cascade.

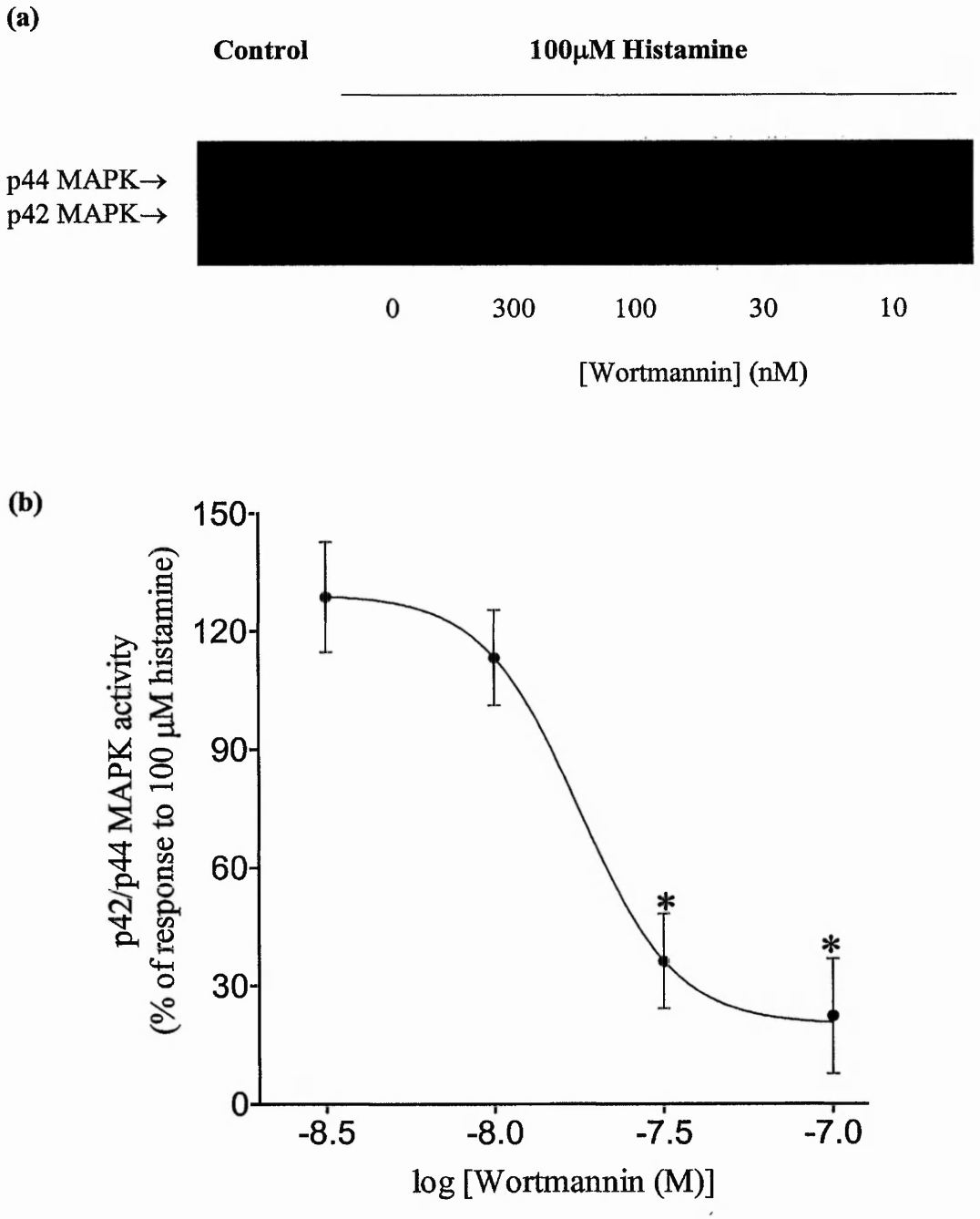


Figure 4.10 – Effect of the specific phosphoinositol 3-kinase inhibitor, wortmannin, on histamine H_1 receptor stimulation of the p42/p44 MAPK pathway in DDT_1MF-2 cells. (a) Representative Western blot and (b) dose-inhibition curve (n=4) for wortmannin on p42/p44 MAPK phosphorylation mediated by 100μM histamine in DDT_1MF-2 cells. 20μg of cell lysate per concentration was analysed for p42/p44 MAPK phosphorylation by Western blotting using a phospho-specific p42/p44 MAPK antibody. * denotes significant differences ($p<0.05$, ANOVA test) for the specific inhibitor concentration compared to control (100μM histamine only).

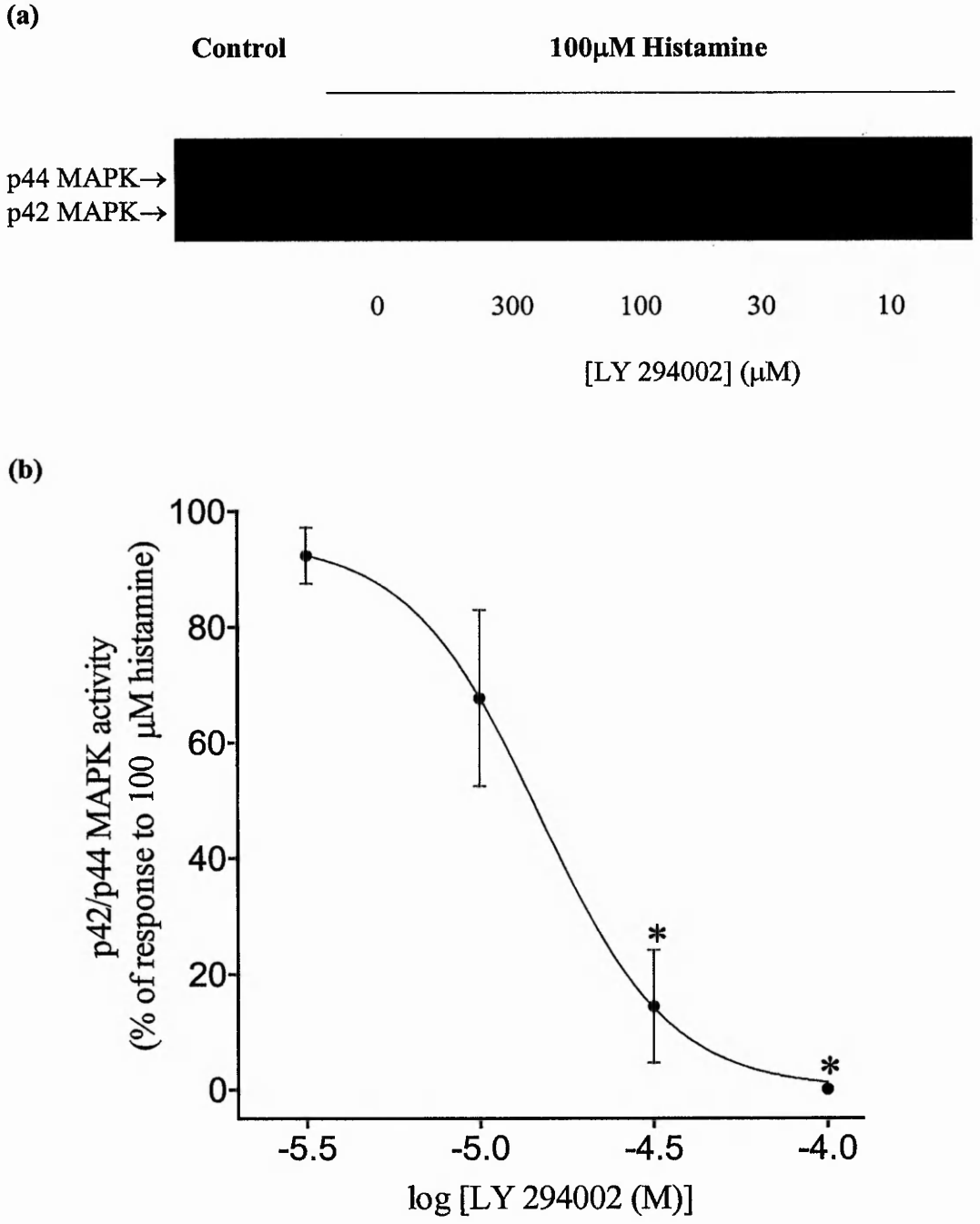


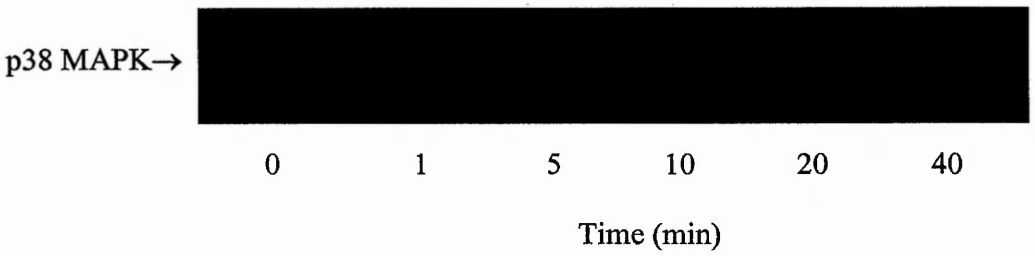
Figure 4.11 – Effect of the specific phosphoinositol 3-kinase inhibitor, LY 294002, on histamine H_1 receptor stimulation of the p42/p44 MAPK pathway in DDT_1MF-2 cells. (a) Representative Western blot and (b) dose-inhibition curve (n=4) for LY 294002 on p42/p44 MAPK phosphorylation mediated by 100μM histamine in DDT_1MF-2 cells. 20μg of cell lysate per concentration was analysed for p42/p44 MAPK phosphorylation by Western blotting using a phospho-specific p42/p44 MAPK antibody. * denotes significant differences ($p<0.05$, ANOVA test) for the specific concentration compared to control (100 μM histamine only).

Stimulation of DDT_1MF-2 cells with histamine (100 μM) produced time-dependent (peak activation occurring at 5 min; Figure 4.12), and concentration-dependent ($p[EC_{50}]$ = 5.8 ± 0.4; *n*=5; Figure 4.13) increases in p38 MAPK activation. In contrast, histamine did not stimulate JNK/SAPK phosphorylation in DDT_1MF-2 cells (data not shown). The histamine H_1 receptor antagonist mepyramine (1 μM) blocked histamine (100 μM)-induced p38 MAPK activation (95 ± 9% inhibition; n=4; Figure 4.14a), whereas tiotidine (1 μM) and thioperamide (1 μM), histamine H_2 and H_3 receptor antagonists respectively, had no effect. These data indicate that histamine stimulated increases in p38 MAPK activation are mediated through the histamine H_1 receptor in DDT_1MF-2 cells. The p38 MAPK inhibitor SB 203580 (20 μM) blocked 100 μM histamine-induced p38 MAPK activation (95 ± 7% inhibition; n=4; Figure 4.14b). Finally, pre-treatment with PTX (100 ng ml^{-1}) significantly reduced histamine-induced p38 MAPK activation (74 ± 11% inhibition; n=6; $P<0.05$; Figure 4.14c).

4.7 - Summary

In conclusion, we have shown that stimulation of the endogenous histamine H_1 receptor in DDT_1MF-2 cells induces p42/p44 MAPK phosphorylation via MEK1, PI-3K, and PKC. Interestingly endogenous histamine H_1 receptors activates p42/p44 MAPK in a PTX-sensitive manner, indicating the receptor is coupled to G_i/G_o proteins. Endogenous histamine H_1 receptors in DDT_1MF-2 cells also phosphorylated p38 MAPK in a PTX-sensitive manner, giving further evidence that the receptor is coupled to $G_{i/o}$ proteins in this cell line.

(a)



(b)

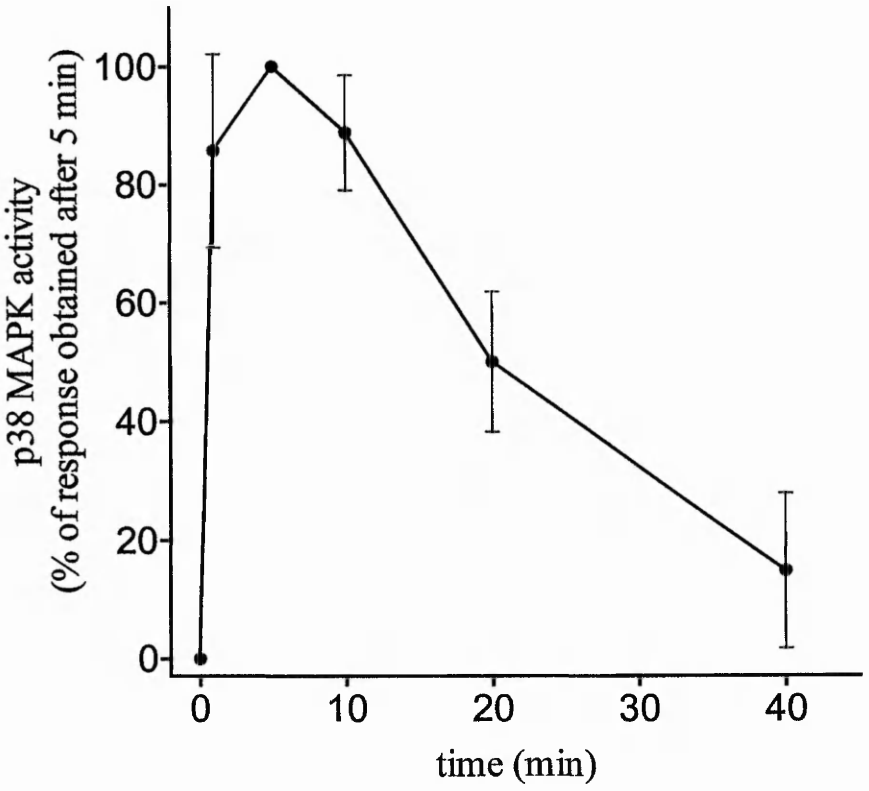


Figure 4.12 – Histamine H_1 receptor stimulation of the p38 MAPK pathway in DDT_1MF-2 cells - time course. (a) Representative Western blot and (b) Time course plot (n=5) of 100μM histamine-mediated stimulation of p38 MAPK in DDT_1MF-2 cells. 20μg of cell lysate per time point was analysed for p38 MAPK phosphorylation by Western blotting using a phospho-specific p38 MAPK antibody. Cells were stimulated for up to forty minutes.

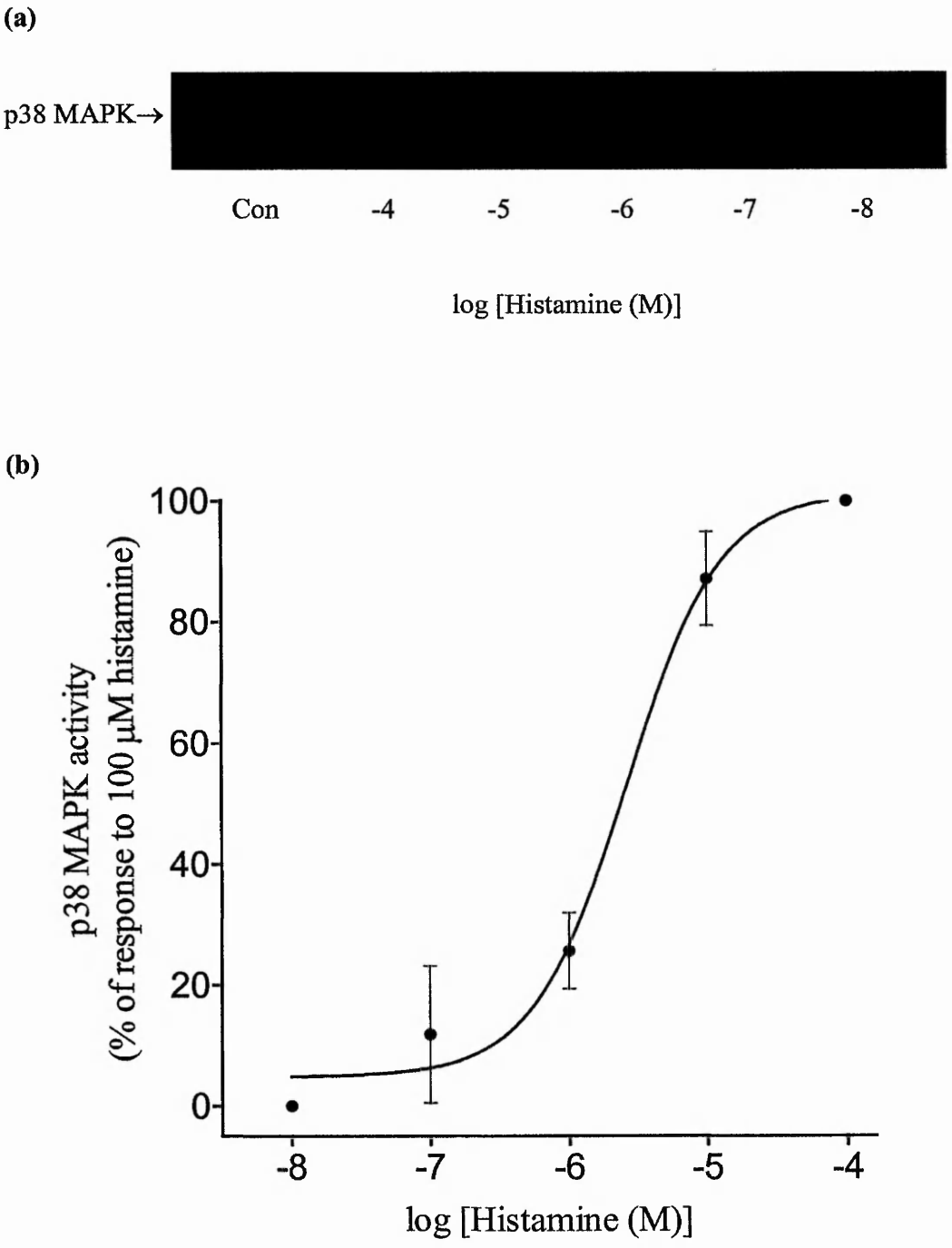


Figure 4.13 – Histamine H_1 receptor stimulation of the p38 MAPK pathway in DDT_1MF-2 cells - concentration response. (a) Representative Western blot and (b) concentration-response curve of histamine-mediated stimulation of p38 MAPK in DDT_1MF-2 cells. 20µg of cell lysate per concentration of histamine was analysed for p38 MAPK phosphorylation by Western blotting using a phospho-specific p38 MAPK antibody. $p[EC_{50}]$ value = 5.8 ± 0.4 (n=5). Values represent mean ± S.E.M. of five independent experiments. Con = control sample (0% response).

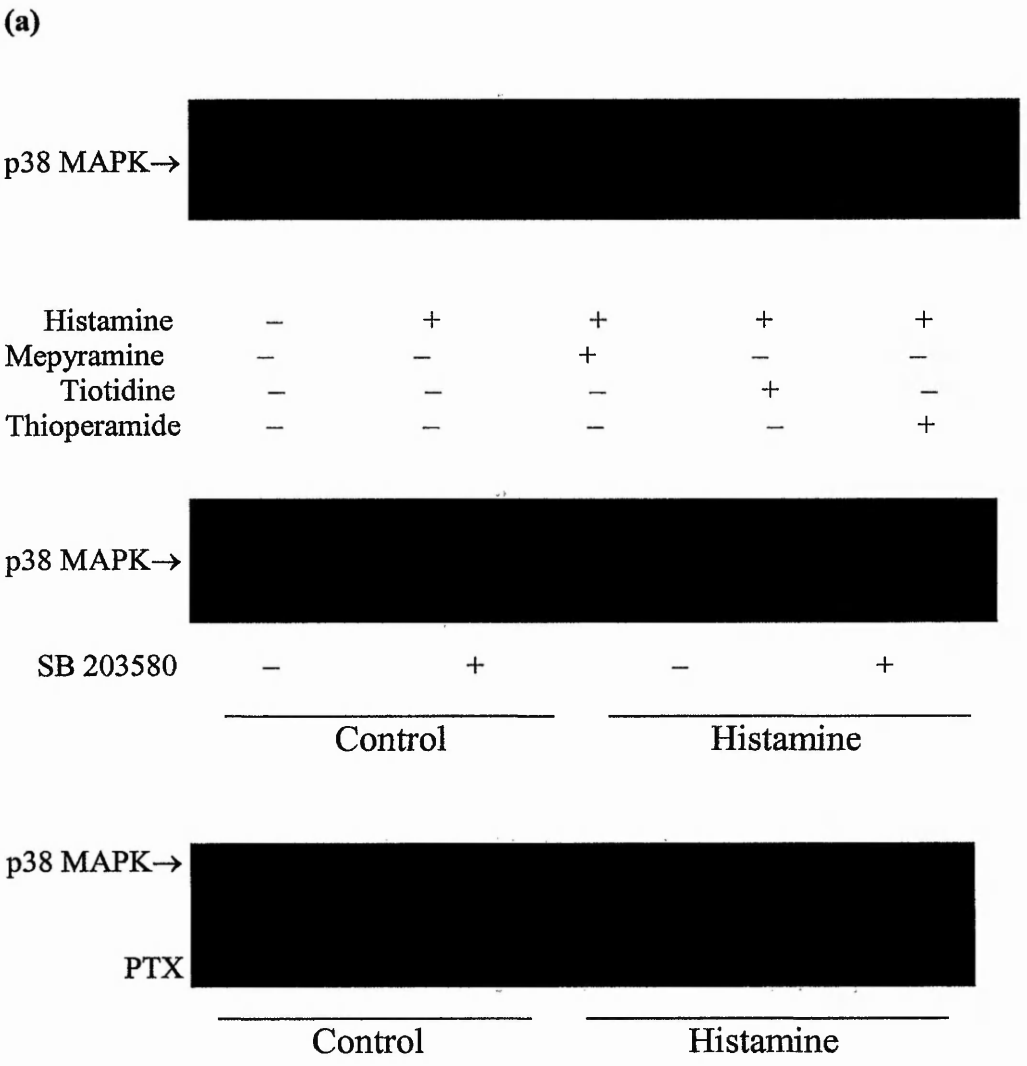


Figure 4.14 – Effect of specific histamine receptor antagonists, the specific p38 MAPK inhibitor SB 203580, and pertussis toxin (PTX) on histamine-mediated p38 MAPK activation. Representative Western blots showing the effect of pre-treatment with (a) the specific histamine receptor antagonists mepyramine (H_1, 1 μM, 30 min), tiotidine (H_2, 1 μM, 30 min), and thioperamide (H_3, 1 μM, 30 min), (b) 20μM SB 203580 (30 min), and (c) 100ng ml^{-1} PTX for 16 hours, on histamine mediated p38 MAPK phosphorylation. Cells were exposed to histamine (100 μM) for 5 min after the pre-treatment protocols. 20μg of cell lysate per time point was analysed for p38 MAPK phosphorylation by Western blotting using a phospho-specific p38 MAPK antibody. Similar results were obtained in at least four independent experiments. Con = control sample (0% response).

Histamine H_1 receptor stimulation did not phosphorylate the 46kDa or 54kDa isoforms of JNK/SAPK in DDT_1MF-2 cells.

Chapter 5

EFFECT OF ADENOSINE A_1 AND HISTAMINE H_1 RECEPTORS ON DDT_1MF-2 CELL PROLIFERATION AND CELL VIABILITY

5.0 - Effect of Adenosine A_1 and Histamine H_1 Receptors on DDT1MF-2 Cell Proliferation and Cell Viability

The physiological implications of A_1R- and H_1R-mediated activation of the p42/p44 MAPK and p38 MAPK cascade were analysed, specifically their effect on DDT_1MF-2 cell proliferation and cell viability.

5.1 – Effect of Adenosine A_1 and Histamine H_1 Receptor Stimulation on DDT_1MF-2 Cell Proliferation

The MTT protocol described in section 2.6 was used to determine whether A_1Rs and H_1Rs induce DDT_1MF-2 cell proliferation. Previous studies have shown that $G_{i/o}$PCRs, including A_1Rs, and G_qPCRs induce cell proliferation in other cell lines via activation of the p42/p44 MAPK cascade (Lelièvre *et al*, 1998a, Lelièvre *et al*, 1998b, Williams *et al*, 1998). DDT_1MF-2 cells were incubated in DMEM (serum-free) for 72 hours with CPA, histamine, and foetal calf serum (FCS), which acted as a positive control. FCS produced a concentration-dependent increase in cell proliferation, inducing a four-fold increase in cell number at the highest concentration used (Figure 5.1). However, neither CPA (Figure 5.2a, n=5), or histamine (Figure 5.2b, n=5), caused a significant increase in DDT_1MF-2 cell proliferation ($p>0.05$).

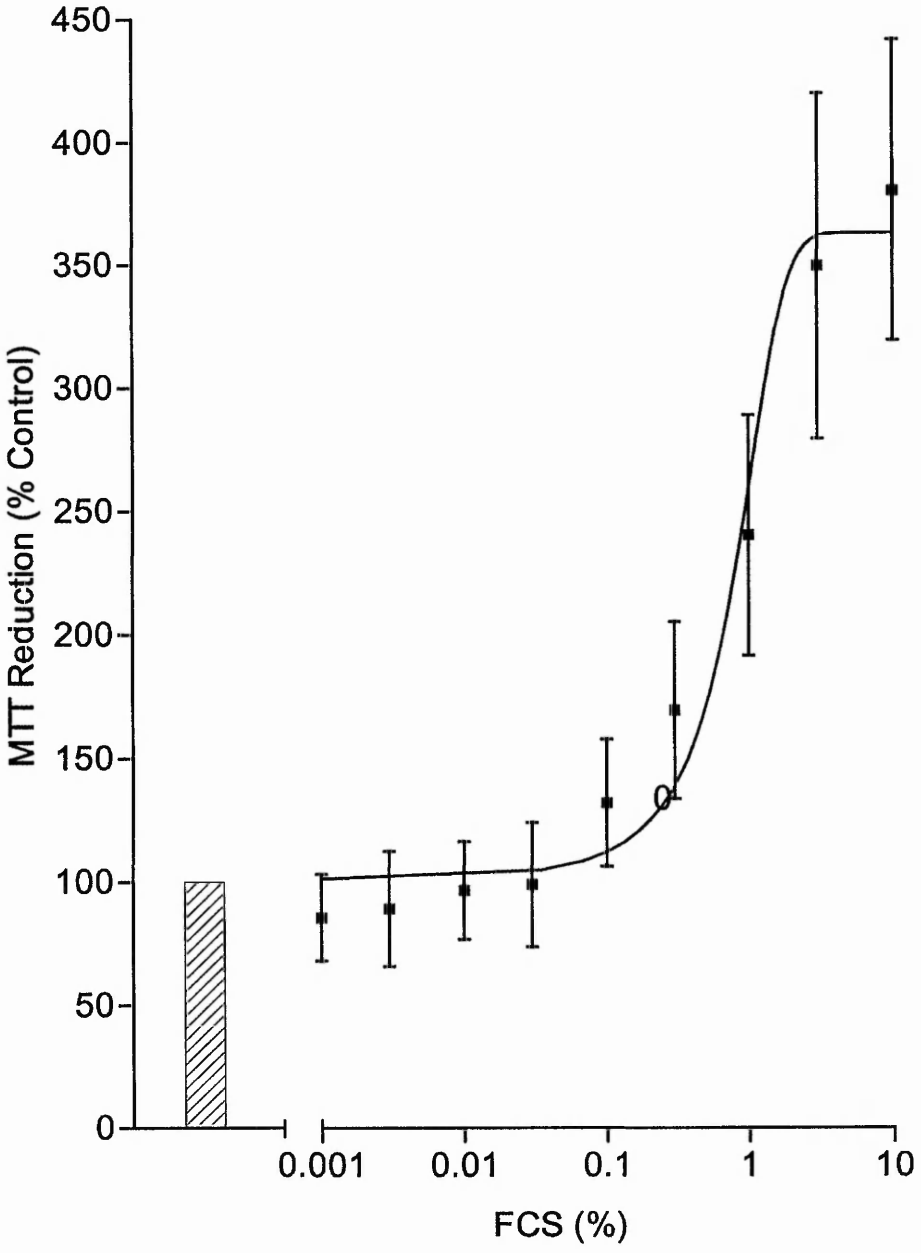


Figure 5.1 - Foetal calf serum-mediated stimulation of cell proliferation. Concentration-response curve for FCS in DDT_1MF-2 cells treated with vehicle (control=100%) or indicated concentrations of FCS for 72 hours. Cells were incubated with 5mg ml^{-1} MTT in PBS for one hour after incubation period, lysed with DMSO, and optical density recorded using a spectrophotometer. The graph represents the combined results of six independent experiments, each measured in triplicate.

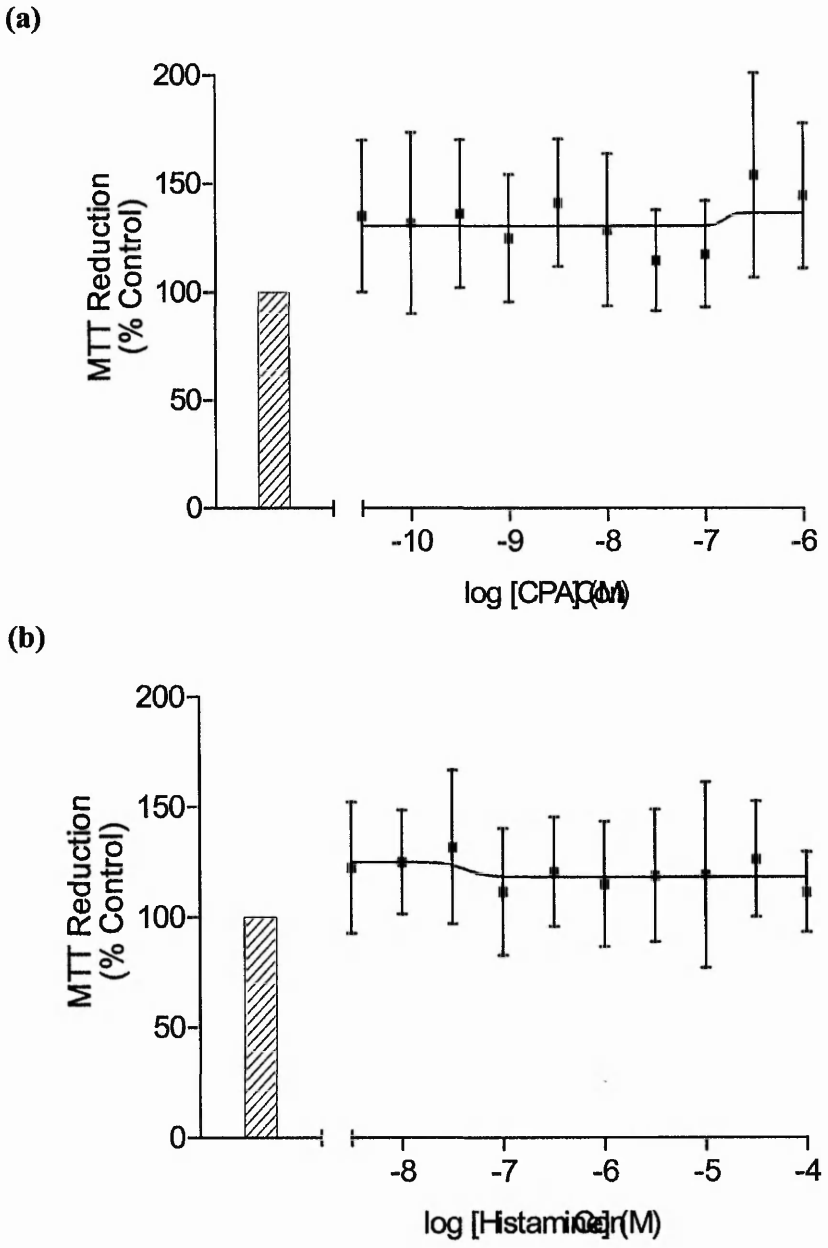


Figure 5.2 – Effect of adenosine A_1 and histamine H_1 receptor stimulation of DDT_1MF-2 cell proliferation. Concentration-response curves for (a) CPA and (b) histamine in DDT_1MF-2 cells treated with vehicle (control=100%) or indicated concentrations of CPA or histamine for 72 hours. Cells were incubated with 5mg ml^{-1} MTT in PBS for one hour after incubation period, lysed with DMSO, and optical density recorded using a spectrophotometer. Both graphs represent the combined results of five independent experiments, each measured in triplicate.

5.1.1 – Effect of Adenosine A1 and Histamine H1 Receptors on EGF- and FCS-Mediated DDT1MF-2 Cell Proliferation

Law *et al* (1997) observed $G_{i/o}$–coupled δ-opioid receptors potentiated FCS-mediated cell proliferation in transfected CHO cells. DDT_1MF-2 cells were pretreated with for 30 min. with 1 μM CPA before incubation with increasing concentrations of FCS in DMEM for 72 hours. CPA failed to induce a significant increase in FCS-mediated DDT_1MF-2 cell proliferation (Figure 5.3, n=3, $p>0.05$).

Some GPCRs require the co-activation of a growth factor receptor to induce cell proliferation, such as the $G_{q/11}$–coupled thromboxane A_2 receptor which required platelet-derived growth factor (PDGF) receptor co-stimulation, and the $G_{i/o}$–coupled endothelin-1 requiring co-stimulation of EGF receptors (Grosser *et al,* 1997, Buist *et al,* 1998). DDT_1MF-2 cells were pre-incubated for 30 min. with 1 μM CPA and 100 μM histamine before incubation with increasing concentrations of EGF in DMEM for 72 hours. Neither CPA (Figure 5.4a, n=3), or histamine (Figure 5.4a, n=3), produced a significant increase in DDT_1MF-2 cell proliferation in the presence of varying EGF concentrations ($p>0.05$).

5.1.2 - The Effect of PD 98059 and SB 203580 on DDT_1MF-2 Cell Viability

Both the p42/p44 MAPK and p38 MAPK cascades have a role in cell regulation (Widmann *et al,* 1999, Ono & Han, 2000). The ERK cascade has a proliferative effect in some cells (Miltenberger *et al,* 1993, Seger *et al,* 1995), and the p38 MAPK cascade mediates apoptosis (Xia *et al,* 1995, Ma *et al,* 1999). It is possible that these cascades are contradicting each other when activated by either A_1Rs or H_1Rs in

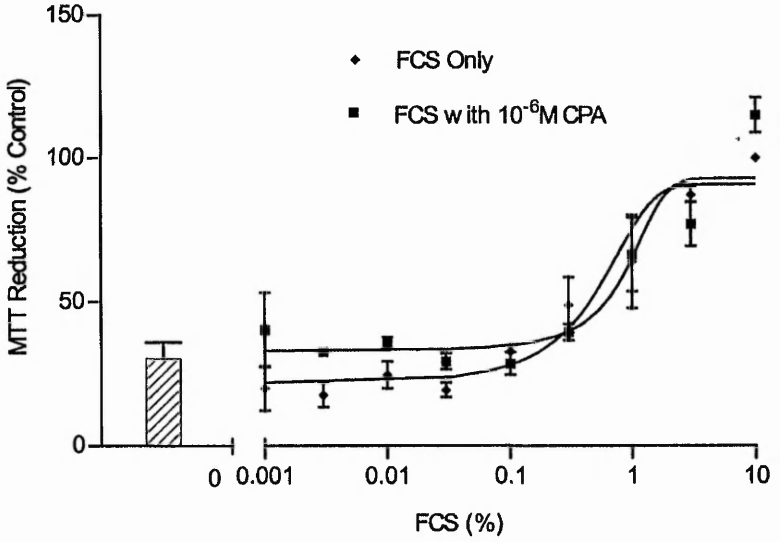
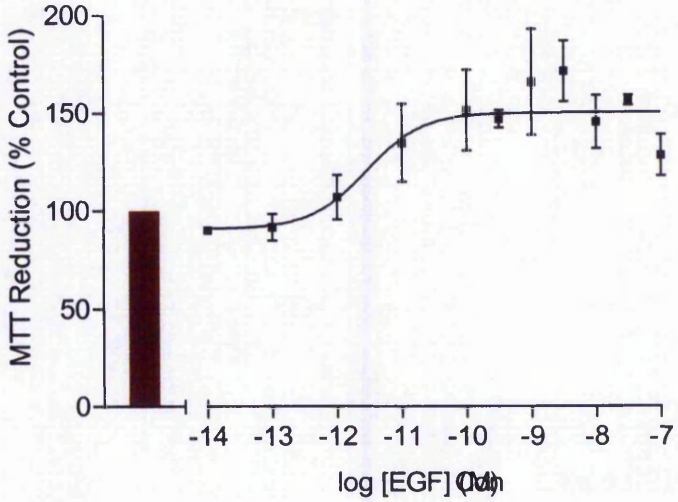


Figure 5.3 – Effect of adenosine A_1 receptor stimulation on foetal calf serum-mediated DDT_1MF-2 cell proliferation. FCS-induced proliferation in the absence and presence of CPA (1 μM, 30 min preincubation), in DDT_1MF-2 cells treated with vehicle or indicated concentrations of FCS for 72 hours. Cells were incubated with 5mg ml^{-1} MTT in PBS for one hour after incubation period, lysed with DMSO, and optical density recorded using a spectrophotometer. The graph represents the combined results of three independent experiments, each measured in triplicate.

(a)



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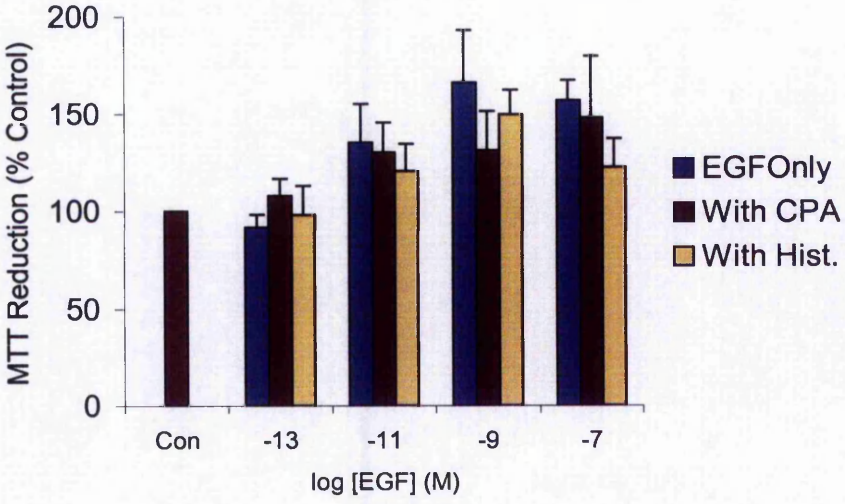


Figure 5.4 – Effect of adenosine A_1 and histamine H_1 receptor stimulation on epidermal growth factor-mediated cell proliferation in DDT_1MF-2 cells. Concentration-response curve for (a) EGF alone, and (b) EGF in the presence of 1μM CPA and 100 μM histamine (30 min preincubation). DDT_1MF-2 cells were treated with vehicle (control=100%) or indicated concentrations of EGF for 72 hours. Cells were incubated with 5mg ml^{-1} MTT in PBS for one hour after incubation period, lysed with DMSO, and optical density recorded using a spectrophotometer. Both graphs represent the combined results of three independent experiments.

DDT_1MF-2 cells. Therefore, the role of each MAPK cascade was investigated, using the MEK inhibitor, PD 98059 and p38 MAPK inhibitor, SB230580, to block the other cascade. DDT_1MF-2 cells were pretreated with the specific MEK1 inhibitor, PD 98059 (50 μM, 30 min., Dudley *et al,* 1995) and the specific p38 MAPK inhibitor, SB 203580 (20μM, 30 min., Cuenda *et al,* 1995). The cells were then exposed to CPA (1 μM), histamine (100 μM), and EGF (10nM) for 72 hours. PD 98059 had no significant effect on cell proliferation with all three agonists, compared to control (*n*=3, p.0.05), but SB 203580 did significantly reduce DDT_1MF-2 cell levels when pre-incubated with all three agonists and in the control (*n*=3, p.0.05). See figure 5.5.

5.2 – Effect of Adenosine A_1 and Histamine H_1 Receptors on Cell Viability

Studies have shown that activation of the p42/p44 MAPK cascade can inhibit cell death (Xia *et al,* 1995, Gardner & Johnson, 1996). Conversely, activation of the p38 MAPK pathway usually induces cell death, predominantly via apoptosis (Xia *et al,* 1995, Kummer *et al,* 1997, Ma *et al,* 1999). The effect of A_1R- and H_1R-mediated p42/p44 MAPK and p38 MAPK activation on cell viability was investigated.

5.2.1 – Effect of Adenosine A1 and Histamine H1 Receptor Stimulation on Chemically-Induced Cell Death

In this section the effect of A_1R- and H_1R- mediated p42/p44 MAPK and p38 MAPK activation on chemically-induced cell death was determined. Previous studies have shown that staurosporine and H_2O_2 can induce cell death (Meyer *et al,*

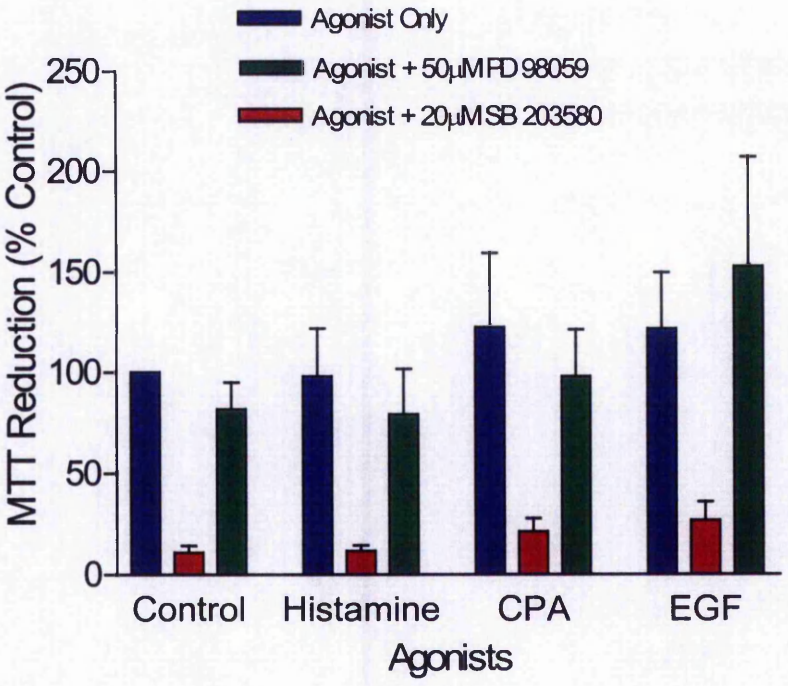


Figure 5.5 - Effect of the MAPK inhibitors, PD 98059 and SB 203580, on DDT$_1$MF-2 cell proliferation in the presence and absence of CPA, histamine, and EGF. DDT$_1$MF-2 cells were pretreated for 30 min. with the MEK inhibitor, PD 98059 (50 μM), and p38 MAPK inhibitor, SB 203580 (20 μM), before incubating the cells for 72 hours with CPA (1 μM), histamine (100 μM), and EGF (10nM). Cells were incubated with 5mg ml^{-1} MTT in PBS for one hour after incubation period, lysed with DMSO, and optical density recorded using a spectrophotometer. The graph represents the combined results of three independent experiments.

1989, Burdon, 1996). Initially, DDT_1MF-2 cells were incubated with staurosporine (0.1 nM – 1000 nM) and H_2O_2 (100 μM), over a period of 32 hours. A significant reduction in cell number was observed with 100 nM and 1000 nM staurosporine, and 100 μM H_2O_2 (Figure 5.6).

DDT_1MF-2 cells were then pretreated for 30 min. with CPA (1 μM), adenosine (100 μM), histamine (100 μM), and EGF (10 nM), before incubation in the absence and presence of staurosporine (0.1 μM, 16-20 hours) or H_2O_2 (100 μM, 1-6 hours). CPA, adenosine, histamine, and EGF, had no significant effect on either staurosporine-mediated or H_2O_2-mediated cell death (Figures 5.7 and 5.8).

The MTT assay was used to record DDT_1MF-2 cell viability. However, this method is not able to distinguish between the type of cell death induced i.e. necrosis or apoptosis. Further experiments were performed to assess whether DDT_1MF-2 cell death was mediated by apoptosis, and the effect of A_1R and H_1R stimulation on this mechanism.

5.2.2 – Effect of Adenosine A_1 and Histamine H_1 Receptor Stimulation on Caspase-3 Activation in DDT_1MF-2 Cells.

Caspase-3 is one of the main mediators of apoptosis (Cohen, 1997). The effect of A_1R- and H_1R- mediated p42/p44 MAPK and p38 MAPK activation on staurosporine-induced caspase-3 activation was determined. Staurosporine is a potent but non-specific tyrosine kinase inhibitor that initiates apoptosis (Meyer *et al,* 1989, Rüegg & Burgess, 1989). Initially, staurosporine was incubated with DDT_1MF-2 cells for increasing time periods to determine the maximal level of caspase-3 activation. Maximal caspase-3 activation occurred at 4 hours (see Figure

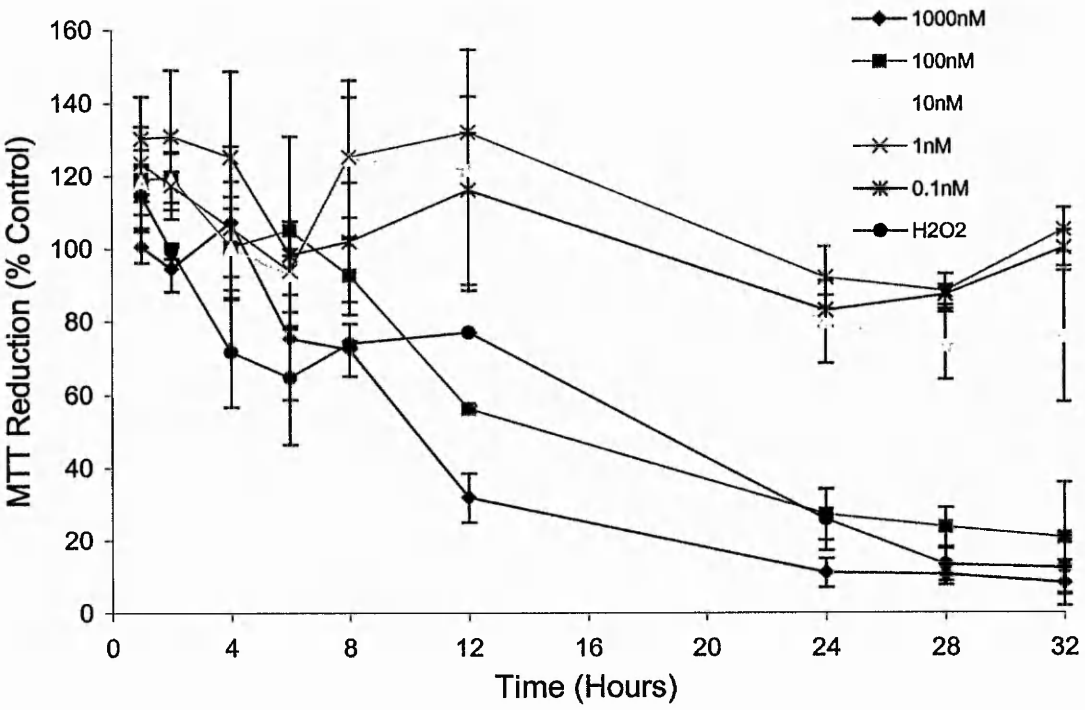


Figure 5.6 – Effect of varying concentrations of staurosporine and hydrogen peroxide on DDT$_1$MF-2 cell viability – time course. DDT$_1$MF-2 cells were incubated with varying concentration of staurosporine and H_2O_2 (100 μM) for up to 32 hours. Cells were incubated with 5mg ml^{-1} MTT in PBS for one hour after incubation period, lysed with DMSO, and optical density recorded using a spectrophotometer. The graph represents the combined results of five independent experiments, each measured in triplicate.

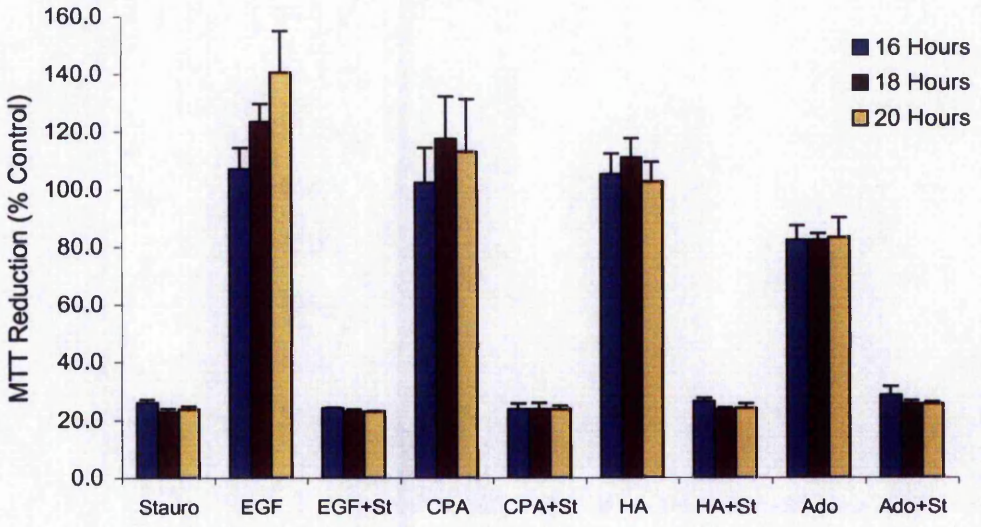


Figure 5.7 – Effect of adenosine A_1, histamine H_1, and epidermal growth factor receptor stimulation on staurosporine-induced DDT$_1$MF-2 cell death. Cells were pretreated (30 min) with CPA (1 μM), histamine (100 μM), and EGF (10 nM) prior to exposure with staurosporine (1 μM) for 16, 18, and 20 hours. Cells were incubated with 5mg ml^{-1} MTT in PBS for one hour after incubation period, lysed with DMSO, and optical density recorded using a spectrophotometer. The graph represents the combined results of three independent experiments, each measured in triplicate.

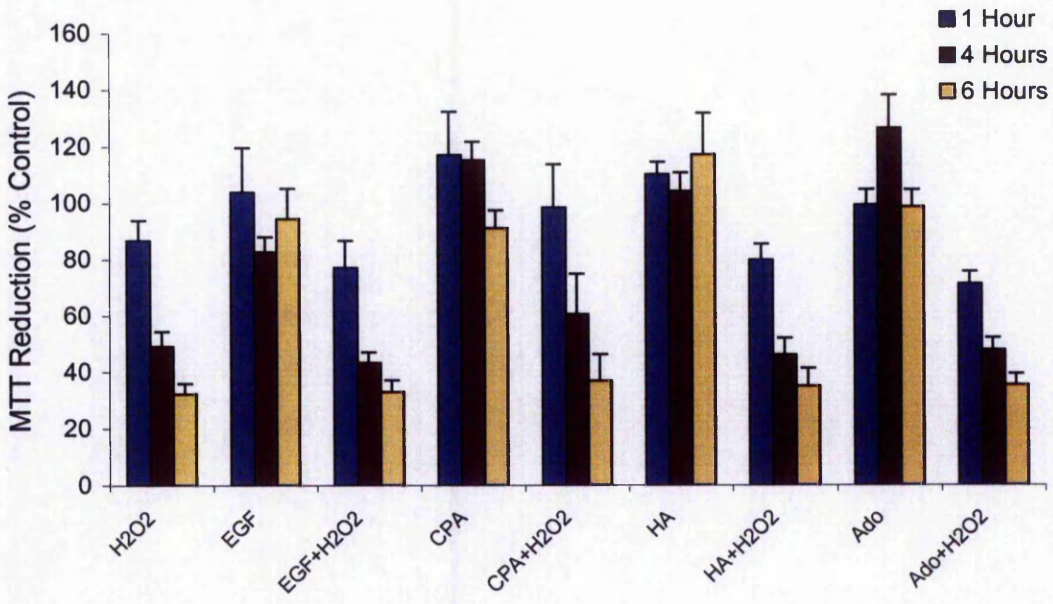


Figure 5.8 – Effect of adenosine A_1, histamine H_1, and epidermal growth factor receptors on hydrogen peroxide- (H_2O_2-) induced DDT$_1$MF-2 cell death. Pretreatment with CPA (1 μM), histamine (100 μM), and EGF (10 nM) was followed by treatment with H_2O_2 (100 μM) for 1, 4, and 6 hours. Cells were incubated with 5mg ml^{-1} MTT in PBS for one hour after incubation period, lysed with DMSO, and optical density recorded using a spectrophotometer. The graph represents the combined results of five independent experiments, each measured in triplicate.

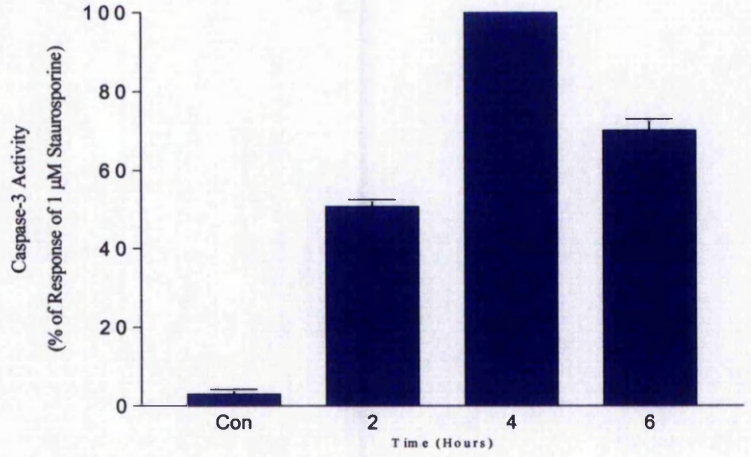
5.9a). Pretreatment for 30 min. of DDT_1MF-2 cells with CPA (1 μM), adenosine (100 μM), histamine (100 μM), and EGF (10 nM), a known inhibitor of cell death (Hackel *et al,* 1999), was succeeded by staurosporine (1 μM, 4 hours). Caspase-3 levels were recorded using the protocol outlined in chapter 2. Histamine had no effect on staurosporine-mediated caspase-3 activation, CPA and adenosine did appear to inhibit caspase-3 activation, but not significantly (n=4, $p>0.05$), whereas EGF significantly reduced levels of activated caspase-3 (44.5 ± 15.3 % inhibition, n=4, $p<0.05$). See Figure 5.9b for graph.

A similar study using hydrogen peroxide (H_2O_2), a known inducer of apoptosis in some cell lines (Burdon, 1996), was attempted. However, H_2O_2 did not mediate activation of caspase-3 in DDT_1MF-2 cells.

5.2.2 – Activation of Protein Kinase B by Adenosine A_1 and Histamine H_1 Receptors

Protein kinase B (PKB) regulates apoptosis by inhibiting caspase-9, an important enzyme in the process of programmed cell death. PKB is activated by GPCRs via Class I_B PI-3Ks (Vanhaessebroeck & Alessi, 2000). Previous work has already demonstrated activation of PKB by A_1Rs in DDT_1MF-2 cells (Germack & Dickenson, 2000). The ability of H_1Rs to activate PKB in this cell line was investigated. PKB was activated by histamine (100 μM) in DDT_1MF-2 cells, with maximal activation occurring at five min. (Figure 5.10, n=3). Histamine-mediated PKB activation was also concentration-dependent, yielding a $p[EC_{50}]$ value of 5.8 ± 0.6 (Figure 5.11, n=3), a value, as expected, very similar to concentration-dependent histamine-mediated p42/p44 MAPK and p38 MAPK activation (see chapter 4).

(a)



(b)

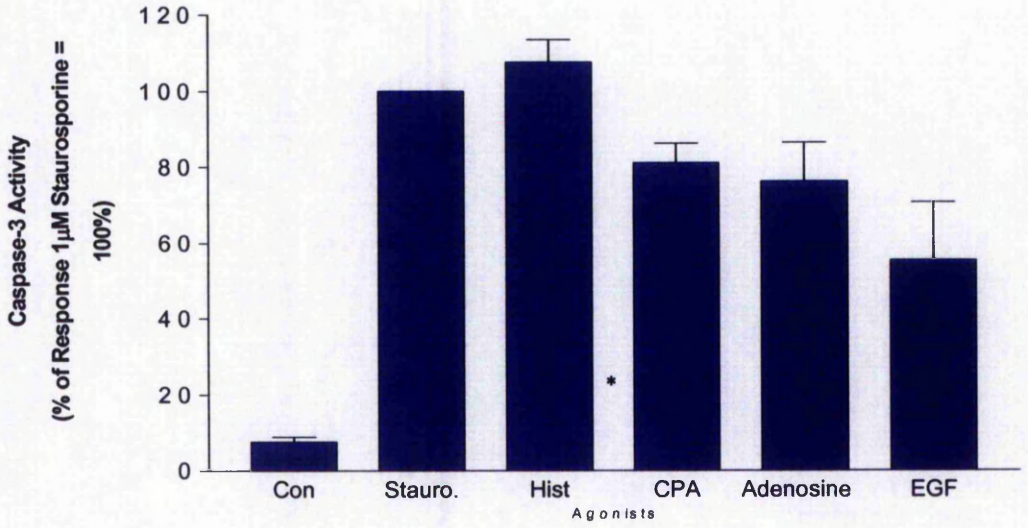
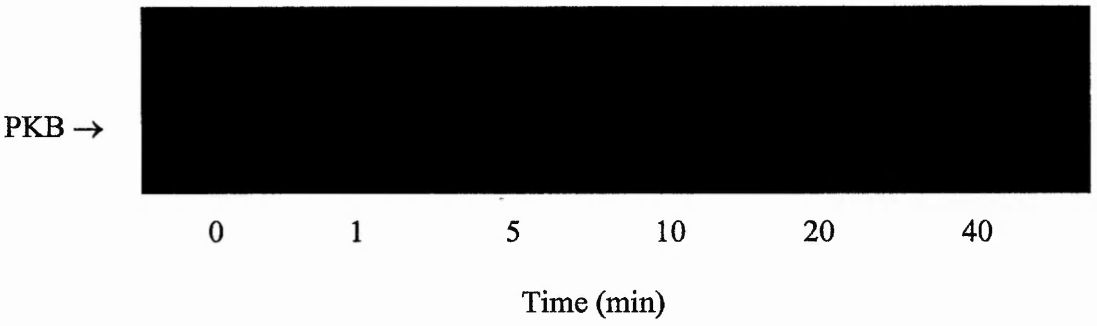


Figure 5.9 – Effect of adenosine A_1, histamine H_1, and EGF receptor stimulation on staurosporine-mediated caspase-3 activation in DDT_1MF-2 cells. (a) Time course for staurosporine-(1 µM)-induced caspase-3 activation, and (b) pretreatment of DDT_1MF-2 cells for 30 min. with histamine (100 µM), CPA (1 µM), adenosine (100 µM), and EGF (10 nM) before activation of caspase-3 using staurosporine (1 µM, 4 hours). Cells underwent the caspase-3 protocol described in chapter 2. Results are expressed as % of caspase-3 activity in cells treated with staurosporine alone. Each graph represents the combined results of four independent experiments, measured in duplicate.

(a)



(b)

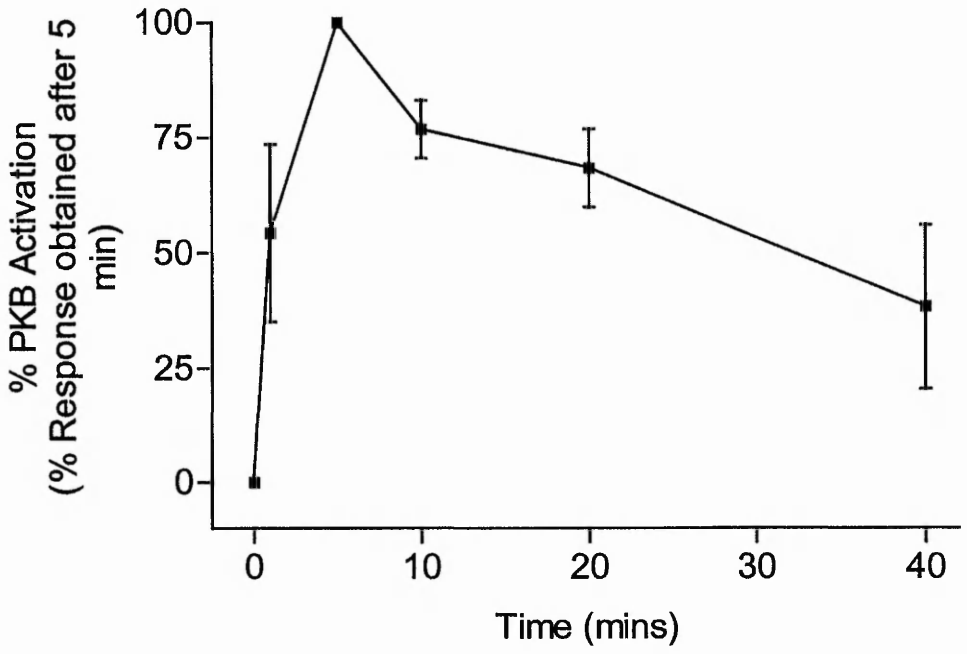
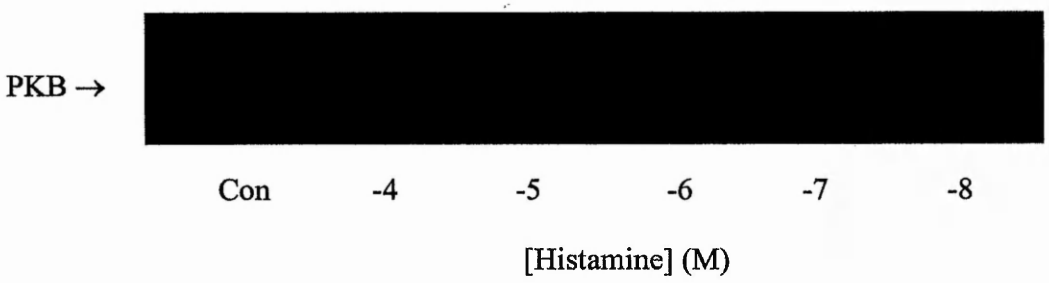


Figure 5.10 – Histamine H_1 receptor-mediated stimulation of protein kinase B in DDT_1MF-2 cells - time course. (a) Representative Western blot and (b) Time course plot (n=3) of 100 μM histamine-mediated stimulation of PKB in DDT_1MF-2 cells. 20μg of cell lysate per time point was analysed for PKB phosphorylation by Western blotting using a phospho-specific PKB (Ser^{473}) antibody. Cells were stimulated for up to forty minutes.

(a)



(b)

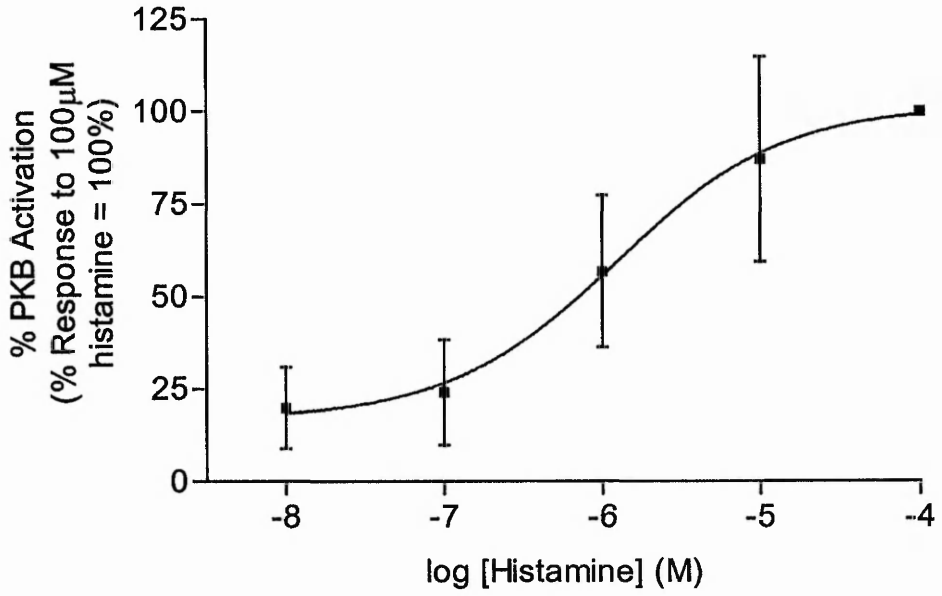


Figure 5.11 – Histamine H_1 receptor-mediated stimulation of protein kinase B in DDT_1MF-2 cells – concentration response. (a) Representative Western blot and (b) concentration response curve (n=3) of histamine-mediated stimulation of PKB in DDT_1MF-2 cells. 20μg of cell lysate per time point was analysed for PKB phosphorylation by Western blotting using a phospho-specific PKB (Ser^{473}) antibody. Values represent mean ± S.E.M. of three independent experiments. Con = control sample (0% response).

5.6 – Summary

A_1R and H_1R activation did not stimulate DDT_1MF-2 cell growth, either in the absence or presence of EGF. Both receptors mediate PKB activation in a time and dose responsive manner. However, neither A_1Rs or H_1Rs inhibit staurosporine-induced caspase-3 activation, or inhibit both staurosporine- and H_2O_2-mediated cell death. In conclusion, the data presented indicate that neither the A_1R or H_1R is involved in DDT_1MF2 cell viability.

Chapter 6

DISCUSSION

6.0 -DISCUSSION

It has been shown in this study that, for the first time, endogenous adenosine A_1 receptors expressed in DDT_1MF-2 cells can activate both ERK 1/2 and p38 MAPK. A_1R-mediated ERK 1/2 activation involved PTX-sensitive $G_{i/o}$ proteins, PI-3K, and MEK. Similarly, A_1R-mediated p38 MAPK activation also involved $G_{i/o}$ proteins. However, A_1Rs did not activate the JNK/SAPK cascade in DDT_1MF-2 cells. Stimulation of the ERK and p38 MAPK cascades by A_1Rs did not cause DDT_1MF-2 cell proliferation, or potentiate proliferation mediated by either FCS or EGF. A_1R-mediated MAPK cascade stimulation does not appear to have any effect on DDT_1MF-2 cell viability.

This study has also shown that, for the first time, endogenous histamine H_1 receptors can activate both ERK1/2 and p38 MAPK in any cell line. ERK 1/2 activation by these receptors in DDT_1MF-2 cells involved non-receptor tyrosine kinases, PI-3K, PKC, MEK, and most surprisingly $G_{i/o}$ proteins. H_1R-mediated p38 MAPK activation in this cell line also involves $G_{i/o}$ proteins. H_1Rs did not activate the JNK/SAPK cascade in DDT_1MF-2 cells. Similar to A_1Rs, stimulation of the ERK and p38 MAPK cascades by H_1Rs did not cause DDT_1MF-2 cell proliferation, or potentiate EGF-mediated cell proliferation. Finally, H_1R-mediated MAPK cascade stimulation does not appear to have any effect on DDT_1MF-2 cell viability.

6.1 - Adenosine A_1 Receptor Activation of MAPK Cascades in DDT1MF-2 Cells

It has previously been reported that transfected human A_1Rs in CHO cells activate the ERK cascade (Dickenson *et al,* 1998). Other studies have demonstrated $G_{i/o}$PCR-

mediated activation of the ERK cascade is via the βγ-subunits of the G protein, one or more genistein-sensitive *Src*-related protein tyrosine kinases, Shc phosphorylation, and PI-3K-dependent Ras activation (Koch *et al,* 1994, Hawes *et al,* 1995, Hawes *et al,* 1996, Garnovskaya *et al,* 1996, Igishi & Gutkind, 1998). Indeed, A_1R-induced ERK 1/2 activation is mediated by $G_{i/o}$-βγ-subunits in COS-7 cells (Faure *et al,* 1994). The involvement of Shc phosphorylation in A_1R-mediated ERK 1/2 activation in DDT_1MF-2 was investigated. However, the data obtained was inconclusive.

Dickenson *et al* (1998) also showed A_1R-mediated ERK 1/2 activation in transfected CHO cells involved genistein-sensitive protein tyrosine kinases and activation of PI-3K, demonstrated using the PI-3K inhibitors, wortmannin and LY 294002. Wortmannin and LY 294002 also reduced ERK 1/2 activation in this study, indicating a role for PI-3K activation in A_1R-mediated stimulation of the ERK cascade in DDT_1MF-2 cells too. One major difference in this study compared to that carried out in CHO cells, however, was the inability of the broad-range tyrosine kinase inhibitors, genistein and tyrphostin A47, to significantly inhibit A_1R-mediated ERK 1/2 activation. Also, neither the selective Src tyrosine kinase inhibitor, PP2, or the EGF receptor tyrosine kinase inhibitor, AG1478, significantly reduced A_1R-mediated ERK 1/2 activation in DDT_1MF-2 cells. Data would suggest that A_1R-mediated ERK 1/2 activation in this cell line involves either genistein- and tyrphostin A47-insensitive protein tyrosine kinases, or is independent of receptor/non-receptor tyrosine kinases altogether.

Genistein-insensitive ERK 1/2 activation has also been reported for other $G_{i/o}$PCRs, such as the dopamine D_3 receptor (Cussac *et al,* 1999) and the 5-HT_{1A} receptor (Cowen *et al,* 1996). It has also been shown in other GPCR subtypes, such as the G_s-

coupled adenosine A_{2B} receptor, and the $G_{q/11}$-coupled $P2Y_2$ receptor (Gao *et al,* 1999).

Recent studies have shown inhibition of ERK cascade activation by the GTP-binding protein, Rap1 (Zwartkruis & Ros, 1999). It has been proposed that $G_{i/o}$-α-subunits recruit the Rap1 GTPase activating protein, rap1GAPII, from the cytosol to the membrane (Mochizuki *et al,* 1999). Studies with the $G_{i/o}$-coupled muscarinic M_2 receptor showed rap1GAPII translocation to the cell membrane, causing a reduction in GTP-bound Rap1 levels (Mochizuki *et al,* 1999). A_1R-mediated ERK 1/2 activation in DDT_1MF-2 cells may, therefore, involve Rap1 inhibition, and therefore explain genistein- and tyrphostin A47-insensitive A_1R-mediated ERK 1/2 activation in DDT_1MF-2 cells.

As mentioned above, studies have demonstrated a role for PKC in ERK 1/2 activation mediated by $G_{i/o}$PCRs. Currently, there are two known pathways resulting in PKC-dependent ERK 1/2 activation by $G_{i/o}$PCRs. The first involves $G_{i/o}$-βγ-subunit-mediated Ca^{2+}-dependent PLCβ activation, causing IP_3 and DAG formation, Ca^{2+} mobilsation, resulting in PKC activation (Sugden & Clerk, 1997). The second pathway incorporates $G_{i/o}$-βγ-subunit activation of PI-3Kγ, which activates an atypical PKC isoform, PKCζ (Lopez-Ilasaca *et al,* 1997). $G_{i/o}$-coupled receptors, such as the LPA and dopamine D_3 receptors, mediate ERK 1/2 activation via βγ-subunits and PKCζ (Cussac *et al,* 1999, Takeda *et al,* 1999). In both mechanisms, PKC then mediates activation of Raf, resulting in ERK 1/2 phosphorylation. Both processes appear to be independent of Ras (English *et al,* 1999, Takeda *et al,* 1999). A_1Rs can activate phospholipase C (PLC) in DDT_1MF-2 cells (Dickenson & Hill, 1993b). Therefore the involvement of both PKC and Ca^{2+} in A_1R-mediated ERK 1/2 activation was investigated. However, the inability of a range of PKC inhibitors, to

inhibit ERK 1/2 activation implied A_1R-mediated ERK 1/2 activation in DDT_1MF-2 cells is PKC independent. Reduction of extracellular Ca^{2+}, and intracellular Ca^{2+} chelation, did not inhibit A_1R-mediated activation in these cells either, suggesting Ca^{2+} influx is not involved either.

A_1Rs in DDT_1MF-2 cells were also shown to activate the p38 MAPK cascade. SB 203580, the p38 MAPK inhibitor used in this study, acts by binding to the inactive form of p38 MAPK, preventing phosphorlyation of p38 MAPK and thus inhibiting activation (Cuenda *et al,* 1995). Upon binding, SB 203580 also reduces the catalytic activity of p38 MAPK, thus reducing activation of the p38 MAPK substrate, MAPKAP2 (Kumar *et al,* 1999). Although typically activated by stimuli such as inflammatory cytokines, osmotic stress, and UV radiation, an increasing number of GPCRs have demonstrated coupling to this cascade (Paul *et al,* Ono & Han, 2000). PTX sensitivity to A_1R-mediated activation of p38 MAPK in this cell line indicates a role for $G_{i/o}$ proteins in this process.

As stated in the introduction, the signalling pathways involved in A_1R-mediated activation of p38 MAPK by GPCRs, is relatively unclear. Yamauchi *et al* (1997) demonstrated that the $G_{i/o}$-coupled muscarinic M_2 receptor mediated p38 MAPK activation via its $\beta\gamma$-subunits, and it is possible a similar mechanism is involved in A_1R stimulation of p38 MAPK activation in DDT_1MF-2 cells.

The EC_{50} for CPA-mediated p38 MAPK phosphorylation, approximately 20nM, is similar to values from previous studies showing A_1R-stimulated inositol phosphate accumulation (i.e. 26 nM, White *et al,* 1992), and Ca^{2+} mobilisation (i.e. 19 nM, Dickenson & Hill, 1993a) in DDT_1MF-2 cells. However, this study and previous work (Dickenson & Hill, 1993a) showed lower EC_{50} values for A_1R-mediated ERK 1/2 activation, and inhibition of adenylyl cyclase, in DDT_1MF-2 cells (i.e. 1.3 nM and

2.8 nM respectively). Previous data suggests that $G_{i/o}$PCRs, like the A_1R, inhibit adenylyl cyclase via their $G_{i/o}$ protein α-subunits, while PLC activation involves $G_{i/o}$ βγ-subunits (Dickenson *et al,* 1998). The lower concentrations of CPA required to activate ERK 1/2 and inhibit adenylyl cyclase, compared to stimulation of inositol phosphate accumulation, Ca^{2+} mobilisation and p38 MAPK, may suggest involvement of $G_{i/o}$-α-subunits and $G_{i/o}$-βγ-subunits respectively. Therefore, the EC_{50} values for CPA-mediated ERK 1/2 and p38 MAPK activation implies signalling pathways involving $G_{i/o}$-α-subunits and $G_{i/o}$-βγ-subunits, respectively. This could be investigated by using the chimeric molecule, CD8-βARK, which is generated by fusing the transmembrane domain of CD8 to the carboxy-terminal domain of βARK. This chimera binds to and sequesters free βγ-subunits, inhibiting βγ-mediated pathways (Koch *et al,* 1994) i.e. Faure *et al* (1994) used this technique to identify $G_{i/o}$-βγ-subunit-mediated ERK 1/2 activation by A_1Rs in COS-7 cells.

Activation of the p38 MAPK cascade, but not the JNK/SAPK cascade, by A_1Rs in DDT_1MF-2 cells may be due to A_1Rs activating MKK3 and/or MKK6. Both of these cascades are activated by similar extracellular stimuli (UV radiation, inflammatory cytokines, osmotic stress, etc.), but significant differences are seen at the MAPKK level (English *et al,* 1999). MKKs 4 and 7 have both been shown to activate isoforms of JNK/SAPK and p38 MAPK (Jiang *et al,* 1992, Hu *et al,* 1999, Wingard *et al,* 1999). However, previous studies have demonstrated that MKKs 3 and 6 exclusively activate p38 MAPK isoforms, and not JNK/SAPK isoforms (Keesler *et al,* 1998, Ono & Han, 2000). Whether A_1R-mediated p38 MAPK activation is mediated by MKK3 and/or MKK6 in DDT_1MF-2 cells could be investigated using commercially available phospho-specific MKK3 and MKK6 antibodies.

Receptor internalisation, involving endocytosis of the receptor and β-arrestins, is required for various GPCR-mediated signal transduction pathways in a number of GPCR subtypes. β-Arrestins uncouple the receptor from the associated G protein and target the receptor for internalisation in clathrin-coated pits (Lefkowitz, 1998). Recent studies have observed that MAPK activation mediated by GPCRs involves receptor endocytosis and β-arrestins, which including the G_s-coupled β_2-adrenoceptor and $G_{i/o}$-coupled muscarinic M_2 receptor (Daaka *et al,* 1998, Vogler *et al,* 1999). However, MAPK activation mediated by the α_2-adrenoceptor does not require receptor internalisation (Schramm & Linbird, 1999). It is not known whether internalistion is required for A_1R-induced MAPK activation in DDT_1MF-2 cells. This can be investigated by using K^+ free media, which blocks the receptor internalisation process, or using negative mutants of β-arrestin or dynamin, which inhibit GPCR endocytosis (Schramm & Linbird, 1999).

Finally, the reduction in both ERK and p38 MAPK activation mediated by A_1Rs over time (figures 3.1, 3.2, and 3.13) may have been due to reduced MAPK protein levels and not reduced MAPK phosphorlyation. However, studies using non-activated MAPK antibodies showed no reduction in MAPK protein levels over the agonist time period used in this study (data not shown).

This study has shown endogenous A_1R-mediated activation of the ERK and p38 MAPK cascades in DDT_1MF-2 cells. Whether these results are similar in other cells that express endogenous A_1Rs e.g. human mesangial cells and neuronal cells (Libert *et al,* 1992, MacGlaughlin *et al,* 1997) needs further investigation.

6.2 - Histamine H_1 Receptor Activation of MAPK Cascades in DDT_1MF-2 Cells

This study showed, for the first time, the histamine H_1 receptor activates the ERK cascade. As previously explained in the introduction, $G_{q/11}$PCRs activate PKC via Ca^{2+}-dependent PLCβ activation. PKC then activates Raf, resulting in ERK 1/2 phosphorylation in a Ras-independent manner (Sugden & Clerk, 1997). Therefore, the role of Ca^{2+} and PKC in H_1R-mediated ERK 1/2 activation in DDT_1MF-2 cells was investigated. The results presented in chapter 4 suggest that ERK 1/2 activation via H_1Rs is independent of Ca^{2+} released from intracellular stores, such as the endoplasmic reticulum. However, a small, albeit non-significant decrease (approximately 20%) in H_1R-mediated ERK 1/2 activation was observed following the removal of extracellular Ca^{2+}, suggesting a possible role for Ca^{2+} influx in this process. ERK 1/2 activation mediated by the H_1R was partially reduced (approximately 40% inhibition) by the selective PKC inhibitor, Ro 31-8220, indicating the involvement of a PKC-dependent pathway to ERK 1/2 activation by H_1Rs. Previous studies have shown that removal of extracellular Ca^{2+} reduced inositol phosphate accumulation mediated by the H_1R in this cell line (White *et al,* 1993). Therefore, the small but non-significant reduction in H_1R-mediated ERK 1/2 activation observed in the absence of extracellular Ca^{2+} could be due to a decrease in inositol phospholipid metabolism, causing a reduction in DAG-dependent PKC activation. Given that PKC does seem to be involved in ERK 1/2 activation by H_1Rs, this would seem logical.

Unexpectedly, this study found that H_1R-mediated activation of the ERK cascade, was sensitive to PTX (approximately 60 % inhibition). This suggests that H_1Rs are also coupled to $G_{i/o}$ proteins in DDT_1MF-2 cells. Previous studies have demonstrated

that H_1R-induced increases in inositol phosphate accumulation and Ca^{2+} mobilsation in this cell line are PTX-insensitive, indicating coupling of H_1Rs to $G_{q/11}$ proteins (Dickenson & Hill, 1993, White *et al,* 1993). Other studies have also shown coupling of H_1Rs to $G_{i/o}$ proteins in other cell lines. Leurs *et al* (1994) showed arachadonic acid (AA) release by the transfected guinea-pig H_1R in CHO cells is partially PTX sensitive. Likewise, AA release mediated by H_1Rs was also found to be PTX-sensitive in rabbit platelets, although in this study release of AA was completely blocked (Murayama *et al,* 1990). This would indicate that different intracellular signalling pathways stimulated by H_1Rs are activated by different subtypes of G protein i.e. $G_{q/11}$-mediated PLCβ activation and $G_{i/o}$-mediated PLA_2 and MAPK activation.

Given the evidence for PI-3K involvement in βγ-subunit-dependent $G_{i/o}$PCR-mediated ERK 1/2 activation (Hawes *et al,* 1996, Lopez-Ilasaca *et al,* 1997), and having shown in this study $G_{i/o}$PCR-coupled A_1R-mediated activation of ERK 1/2 in a PI-3K-dependent manner in DDT_1MF-2 cells, the involvement of PI-3K in H_1R-mediated ERK 1/2 activation was investigated. Both wortmannin and LY 294002 inhibited ERK 1/2 phosphorylation induced by histamine, indicating H_1Rs activate the ERK cascade via PI-3K in this cell line. PKC-dependent H_1R-mediated ERK 1/2 activation may involve activation of the PI-3Kγ isoform, leading to phosphorylation of the atypical PKCζ isoform, causing subsequent Raf, MEK, and ERK 1/2 activation

Non-receptor tyrosine kinases (e.g. the Src family of protein kinases and the calcium-regulated focal adhesion kinase, Pyk2) are known to play a role in GPCR-mediated ERK cascade activation (Sugden & Clerk, 1997). The involvement of non-receptor tyrosine kinases in H_1R-mediated ERK 1/2 activation in DDT_1MF-2 was investigated. Genistein and tyrphostin A47, two broad range tyrosine kinase inhibitors, inhibited

ERK 1/2 phosphorylation induced by histamine. However, the specific Src tyrosine kinase inhibitor, PP2, had no significant effect. This implies that tyrosine kinases other than Src mediate H_1R activation of the ERK cascade in DDT_1MF-2 cells. Also, since it was shown that H_1R-mediated ERK 1/2 activation did not involve intracellular Ca^{2+} or Ca^{2+} influx, this would suggest the Ca^{2+}-dependent tyrosine kinase Pyk2 is not involved either. A non-Ca^{2+}-dependent focal adhesion kinase, p125FAK, has been implicated in GPCR-mediated ERK 1/2 activation (Lutrell *et al,* 1997, Della Rocca *et al,* 1999a). However, depolymerisation of the actin cytoskeleton by cytochalasin D had no significant effect on histamine-mediated ERK 1/2 activation, indicating p125FAK is not involved in H_1R-coupling to ERK 1/2.

As previously described, receptor tyrosine kinase transactivation (e.g. the EGF receptor) has been implicated in ERK cascade stimulation by GPCRs (Daub *et al,* 1996, Zwick *et al,* 1999). In this study AG1478, the specific EGF receptor tyrosine kinase inhibitor, was used to try and inhibit histamine-mediated ERK 1/2 activation in DDT_1MF-2 cells. No significant reduction in ERK 1/2 phosphorylation was observed, suggesting transactivation of EGF receptors by H_1Rs does not mediate stimulation of the ERK cascade in these cells.

Activation of the p38 MAPK and JNK/SAPK cascades by H_1R in DDT_1MF-2 cells was also investigated, and produced similar data to that obtained from the A_1R. Histamine induced p38 MAPK phosphorylation in these cells, but had no significant effect on activated JNK/SAPK levels. H_1R-mediated p38 MAPK activation was also PTX-sensitive, indicating activation of this pathway is mediated through $G_{i/o}$ proteins. Recent studies have shown activation of both the ERK 1/2 and p38 MAPK cascades via $G_{q/11}PCRs$ (Bogoyevitch *et al,* 1995, Clerk *et al,* 1998, Sugden & Clerk, 1998), although $G_{q/11}PCR$-mediated p38 MAPK activation is relatively undefined, compared

to $G_{q/11}$PCR-mediated ERK 1/2 activation. Recent data implicates $G_{q/11}$-coupled receptors may activate p38 MAPK via both $G_{q/11}$-α-subunits and $G_{q/11}$-βγ-subunits (Yamauchi *et al,* 1997). Furthermore, it has also been shown that Src family kinases and PKC are involved in $G_{q/11}$-α-subunit mediation of p38 MAPK activation (Yamauchi *et al,* 1997, Nagao *et al,* 1998).

Activation of the p38 MAPK cascade, but not the JNK/SAPK casacde, by H_1Rs in DDT_1MF-2 cells suggests the activation of p38 MAPKs via this receptor is mediated by MKK3 and/or MKK6, similar to A_1Rs in this cell line. As previously explained these two MAPKK isoforms only phosphorylate p38 MAPKs, as opposed to MKK4 and MKK7 which can activate both p38 MAPKs and JNKs/SAPKs (Jiang *et al,* 1992, Keesler *et al,* 1998, Hu *et al,* 1999, Wingard *et al,* 1999). Repetition of the SDS-PAGE/Western Blot experiments, probing instead with phospho-specific MKK3 and MKK6 antibodies, would determine whether H_1R-mediated p38 MAPK in DDT_1MF-2 cells is MKK3- and/or MKK6-dependent.

Similar to the A_1R data, the reduction in both ERK and p38 MAPK activation mediated by H_1Rs over time (figures 4.1 and 4.12) may have been due to reduced MAPK protein levels and not reduced MAPK phosphorlyation. However, studies using non-activated MAPK antibodies showed no reduction in MAPK protein levels over the agonist time period used in this study (data also not shown).

As previously discussed, GPCR internalisation is required for MAPK cascade activation by some GPCR subtypes (Lefkowitz, 1998). Previous studies have shown that the $G_{q/11}$-coupled muscarinic M_1 and M_3 receptors require internalisation to activate the ERK cascade (Vogler *et al,* 1999). Whether H_1R-dependent ERK 1/2 and p38 MAPK activation in DDT_1MF-2 cells involves H_1R internalisation could be investigated using K^+ free media, or negative mutants of β-arrestins or dynamin.

6.3 – Critical Analysis of A_1R- and H_1R-mediated MAPK Activation Studies

In some cases only one concentration of compound was used to demonstrate whether certain enzymes or processes were involved in A_1R- or H_1R-mediated MAPK activation e.g. 50μM PD 98059, 20μM SB 203580. The reasons for this were time efficiency, reduction of resources used, and that these concentrations of compound had all been previously shown to be effective at the stated concentration, either in DDT_1MF-2 cells themselves or other cell lines. However, it could be argued that where no inhibition was detected, it may be due to use of the compond at too low a concentration, or when inhibition was detected, due to a high concentration of compound producing a stearic effect instead of specificity. Therefore, a range of concentrations of the compound would have been preferable. However, as explained, resources were limited, and given that there was plenty of evidence in the literature for the compounds being effective at the concentrations used, this was not thought to be a problem.

Cytochalasin D was used in this study to determine the role of focal adhesion-based signalling, as it has been shown to inhibit actin, thus depolymerising the actin cytoskeleton and inhibiting GPCR-mediated ERK activation (Lutrell *et al,* 1999). No significant inhibition of A_1R- or H_1R-mediated ERK activation by cytochalasin D was detected, but there is no corroborating evidence to prove that this compound has the abilty to depolymerise the actin cytoskeleton in DDT_1MF-2 cells. Despite evidence in other cell lines that cytochalasin D causes microfillament disassembly (Lutrell *et al,* 1997, Della Rocca *et al,* 1999) it cannot be stated that this compound has the same effect in DDT_1MF-2 cells. Further experiments, such as rhodamine phalloidin

staining, would demonstrate whether cytochalasin D causes depolymerisation of the actin cytoskeleton, and then it could be stated whether the results given here demonstrate no involvement for focal adhesion kinases in A_1R- and H_1R-mediated ERK activation.

Finally, although the method of developing, scanning, and quantifying the blots is a very time efficient, relatively easy to complete, and, above all, a well-established method in recording MAPK activation, it does have its limitations. The main disadvantage is preparing the blots for quantitfication. Setting up the camera so that the bands are focussed and there is minmal background is prone to human error, since it relies on the judgment of the user. Therefore, the size of the bands can vary greatly, and the background optical density fluctuates too. This leads to a relatively large error margin, hence the data given in chapters 3 and 4 is only given to two significant figures. Although the methods used are quick, easy, and fairly accurate, other assays such as immunoflourescence, could be used to give more accurate and more sensitive data. However, this sort of procedure is more time consuming, particularly with multiple samples, and more difficult to accomplish.

6.4 - Physiological Implications of Adenosine A_1 Receptor Activation of MAPK Cascades in DDT_1MF-2 Cells

In this study it was investigated whether A_1Rs affect DDT_1MF-2 cell proliferation. $G_{i/o}$-coupled receptors, in general, have been shown to play a role in cell growth regulation (Williams *et al,* 1998, Keffel *et al,* 2000). However, studies have shown that A_1Rs may stimulate or inhibit cell growth, depending on the cell line they are expressed in (Dubey *et al,* 1996, MacLaughlin *et al,* 1997, Lelièvre *et al,* 1998a, Lelièvre *et al,* 1998b). A_1Rs mediated cell proliferation in messangial and human

colonic carcinoma cells, but inhibited vascular smooth muscle cell growth. GPCR transactivation of growth factor receptors is another GPCR-mediated pathway leading to ERK 1/2 activation and cell proliferation (Daub *et al,* 1997). GPCRs are able to activate growth receptor tyrosine kinases, producing ERK cascade stimulation. The $G_{i/o}$-coupled endothelin-1 and angiotensin-II receptors both transactivated the EGF receptor in Rat-1 fibroblasts and epithelial cells respectively (Daub *et al,* 1996, Li *et al*, 1998). However, stimulation of A_1Rs in DDT_1MF-2 cells had no significant effect on cell growth. Previous data has shown GPCRs potentiate growth factor-mediated cell proliferation, but fail to induce cell proliferation in the absence of growth factors e.g. thromboxane A_2 receptors potentiate PDGF-mediated bovine coronary artery smooth muscle cells, but fail to induce cell growth in the absence of PDGF (Grosser *et al,* 1997). In this study, A_1Rs and EGF receptors were co-stimulated on DDT_1MF-2 cells, but CPA did not potentiate EGF-mediated cell proliferation. As mentioned above, A_1Rs inhibited cell proliferation in vascular smooth muscle cells (Dubey *et al*, 1996). However, A_1R stimulation during both EGF- and FCS-mediated cell growth had no significant effect on DDT_1MF-2 cell proliferation.

Stimulation of the p38 MAPK cascade often results in cell death, usually via apoptosis (Ono & Han, 2000). Activation of this pathway by A_1Rs in DDT_1MF-2 cells, in the presence of PD98059 to block the ERK cascade, does not significantly reduce cell numbers. p38 MAPK activation in DDT_1MF-2 cells may contradict a proliferative signal from ERK cascade stimulation. However, A_1R-mediated ERK cascade activation, in the presence of the p38 MAPK inhibitor, SB 203580, did not increase DDT_1MF-2 cell number, but significantly reduced it. This would indicate that p38 MAPK inhibition in DDT_1MF-2 cells causes cell death. Therefore, the p38 MAPK cascade may have a protective role in this cell line, as demonstrated in other

some other cell types e.g. 3T3-L1 and PC12 cells (Engelman *et al*, 1998, Morooka & Nishida, 1998). This evidence is supported by the cell viability experiments, as SB 203580 significantly reduced DDT_1MF-2 cell number, even in the absence of any GPCR stimuli. Previous studies have suggested the balance of activated MAPK isoforms may decide the fate of a cell (Xia *et al,* 1995). Inhibition of the seemingly protective p38 MAPK pathway in DDT_1MF-2 cells may shift the balance towards a pro-apoptotic cascade, inducing programmed cell death. Although the ERK cascade has been shown to induce cell death in some studies (Goillot *et al,* 1997, Jimenez *et al,* 1997), it is more likely that the JNK/SAPK cascade is producing this pro-apoptotic effect. Having been shown to be a mainly apoptosis-inducing pathway (Ip & Davis, 1998, Davis, 2000), the balance shift towards JNK/SAPK activation following p38 MAPK inhibition, even when at basal levels, may cause DDT_1MF-2 cell death via apoptosis. This could be investigated by using the specific JNK inhibitor, SP600125 (Bennett *et al,* 2001), which would reverse the shift from the proposed pro-apoptotic JNK/SAPK cascade.

There was no evidence for A_1R-mediated DDT_1MF-2 cell proliferation, or potentiation of FCS- and EGF-mediated cell growth. Co-stimulation of the ERK and p38 MAPK cascades by A_1Rs in DDT_1MF-2 cells may have induced opposing effects i.e. ERK-mediated proliferation and p38 MAPK-mediated cell death, which resulted in no significant change in DDT_1MF-2 cell numbers. However, inhibition of the p38 MAPK cascade during A_1R stimulation in DDT_1MF-2 cells did not induce cell proliferation but, as previous mentioned, significantly reduced cell numbers.

Although there is no evidence for A_1R-induced cell proliferation, activation of the ERK cascade by the A_1R may protect against cell death. ERK 1/2 activates RSK, which phosphorylates CREB and BAD (Frödin & Gammeltoft, 1999). BAD

phosphorylation causes it to bind to 14-3-3, preventing BAD-mediated inhibition of the Bcl-2 family of proteins (Zha *et al,* 1996). CREB is an important regulator of early gene transcription, and its activation has been shown to induce both cell proliferation and inhibit apoptosis (Barton *et al,* 1996, Walton & Dragunow, 2000). However, A_1R stimulation had no significant effect on staurosporine- or H_2O_2-mediated DDT_1MF-2 cell death. As previously mentioned, the MTT assay is unable to determine the mechanism of cell death i.e necrosis or apoptosis. Therefore, the effect of A_1R stimulation on caspase-3 activation was investigated. CPA had no significant effect on staurosporine-induced caspase-3 activation in DDT_1MF-2 cells. This evidence would suggest that, if A_1Rs have a protective role in DDT_1MF-2 cells, it is independent of caspase-3 inhibition.

Activation of PKB is a well-studied anti-apoptotic pathway, due to its ability to inhibit caspase-9 (Vanhaesebroeck & Alessi, 2000). Previous data had shown that A_1Rs mediated PKB activation in DDT_1MF-2 cells (Germack & Dickenson, 2000). The effect of A_1R-mediated PKB activation in these cells on one of the main pro-apoptotic caspases, caspase-3, was investigated. CPA-induced A_1R activation had no significant effect on staurosporine-induced caspase-3 activation in DDT_1MF-2 cells. PKB is known to be involved in some of the actions of insulin, such as stimulation of glycolysis and glycogen synthase activity (Downward, 1998). PKB also has a role in cell cycle regulation (Vanhaesebroeck & Alessi, 2000). A_1R-mediated PKB activation in DDT_1MF-2 cells may have a role in some of these essential processes. Further investigation into the physiological implications of A_1R-induced MAPK activation in DDT_1MF-2 cells would clarify the molecular mechanisms involved in the role of A_1Rs in both cardiac and neuronal protection.

6.5 - Physiological Implications of Histamine H_1 Receptor Activation of MAPK Cascades in DDT_1MF-2 Cells

The effect of H_1Rs on DDT_1MF-2 cell growth and proliferation was also investigated. Previous studies have shown that members of the $G_{q/11}PCRs$ induce cell proliferation in a number of cell lines (Williams *et al,* 1998, Keffel *et al,* 2000). H_1Rs mediate cell growth in human astrocytoma U373 MG cells and intestinal mucosal lymphocytes (Roberts *et al,* 1994, Hernandez-Angeles *et al,* 2001). In addition, studies have shown inhibition of cell proliferation by $G_{q/11}PCRs$ i.e. muscarinic M_1 and M_3 receptors (Yamauchi *et al,* 2001), and H_1Rs (Valencia *et al,* 2001). Since this study presents data showing H_1R-mediated activation of potential cell proliferative pathways in DDT_1MF-2 cells, i.e. stimulation of the ERK and p38 MAPK cascades, the effect of H_1Rs on DDT_1MF-2 cell proliferation was investigated. H_1Rs had no significant effect on DDT_1MF-2 cell growth, both with and without co-stimulation of EGF receptors. H_1R-mediated activation of both ERK 1/2 and p38 MAPK must therefore have an alternative role in these cells. Activation of the ERK cascade has been implicated in smooth muscle contraction, a well-known H_1R-mediated response (Watts, 1996). It has also been shown that H_1R-mediated bovine trachea smooth muscle contraction is inhibited by the specific MEK-1 inhibitor, PD 98059 (Koch *et al,* 2000). One substrate of ERKs 1 and 2 is the actin-binding protein, caldesmon (Gerthoffer *et al,* 1997). This protein, which is involved in the contractile response in a range of smooth muscles cells (Gerthoffer & Paul, 1994, Gerthoffer *et al,* 1996, Dessy *et al,* 1998), is reversibly phosphorylated at proline-directed serine residues, characteristic of the consensus sequence for ERK 1/2 phosphorylation (Adam & Hathaway, 1993). A second actin-binding protein, calponin, has also been suggested as being regulated by the ERK cascade during smooth muscle contraction (Menice *et*

al, 1997). It is therefore possible that H_1R-mediated activation of the ERK cascade in DDT_1MF-2 cells, possibly via its $G_{i/o}$-$\beta\gamma$-subunits, may regulate the contractile response mediated by the $G_{q/11}$-α-subunits of the H_1R (Hill *et al,* 1997, Koch *et al,* 2000).

It was shown in this study, for the first time, that the p38 MAPK cascade is activated by H_1Rs. Similar to the A_1R study, inhibition of p38 MAPK with SB 203580 during H_1R stimulation of DDT_1MF-2 cells, did not increase cell numbers, indicating H_1R-mediated p38 MAPK activation was not antagonising a H_1R-induced ERK 1/2-mediated cell proliferation signalling pathway. The physiological implications of H_1R-mediated p38 MAPK activation are relatively unclear but, like the A_1R, it may have an anti-apoptotic role in DDT_1MF-2 cells. Inhibition of the p38 MAPK pathway by SB 203580 in the presence of histamine produced a dramatic decrease in cell number, indicating that, similar to the A_1R studies, p38 MAPK inhibition in this cell line causes cell death. Since the JNK/SAPK cascade was not activated by H_1Rs in this cell line, p38 MAPK phosphorylation may provide a protective mechanism for the smooth muscles cells during contraction e.g. preventing apoptosis when the smooth muscle cells are overly exposed to an excess of histamine, leading to over-stimulation of H_1Rs and a potentially dangerous contractile response.

Similar to the A_1R data, there was no evidence for H_1R-mediated DDT_1MF-2 cell proliferation, or potentiation of EGF-mediated cell growth. Co-stimulation of the ERK and p38 MAPK cascades by H_1Rs in DDT_1MF-2 cells may have induced opposing effects i.e. ERK-mediated proliferation and p38 MAPK-mediated cell death, which resulted in no significant change in DDT_1MF-2 cell numbers. However, inhibition of the p38 MAPK cascade during A_1R stimulation in DDT_1MF-2 cells did

not induce cell proliferation but, as previous mentioned, significantly reduced cell numbers.

Again, an additional role for H_1R-induced ERK 1/2 activation may be to protect against cell death. Similar to that described for A_1Rs, H_1Rs may play a role in cell protection via ERK 1/2-mediated activation of RSK, leading to BAD and CREB phosphorylation. However, H_1R stimulation had no significant effect on staurosporine- or H_2O_2- mediated DDT_1MF-2 cell death. Due to the MTT assay being unable to distinguish which type of cell death is being executed i.e. necrosis or apoptosis, the effect of H_1R stimulation on caspase-3 activation was investigated. However, H_1R stimulation did not significantly inhibit staurosporine-induced caspase-3 activation. If H_1Rs do play a role in DDT_1MF-2 cell survival, it does not involve inhibition of caspase-3. Further investigation into these molecular mechanisms would clarify any part H_1Rs play in cell survival

This study also showed coupling of H_1Rs in DDT_1MF-2 cells to PKB activation. As previously explained, this is one anti-apoptotic mechanism in cells, via activated PKB-mediated inhibition of caspase-9, which in turn would normally activate the main apoptosis caspase isoform, caspase-3 (Cohen, 1997, Vanhaesebroeck & Alessi, 2000). The ability of histamine to prevent staurosporine-induced caspase-3 activation was investigated. Caspase-3 activation induced by staurosporine was not significantly inhibited by histamine, suggesting H_1R-mediated PKB activation does not have an anti-apoptotic effect. Therefore, H_1R stimulation in DDT_1MF-2 cells may result in other physiological events mediated by PKB, such as glycolysis stimulation, increased glycogen synthase activation, and regulation of the cell cycle (Downward, 1998). Further investigation into these ideas would give a more-defined idea as to the role of PKB activation by H_1Rs in this cell line.

6.6 – Critical Analysis of MTT Assay

Although the MTT assay is a widely recognised, relatively quick, easy, and safe method of recording cell viability and proliferation, it does have a number of restrictions. The first is that the assay only measures total cell population, and is not able to detect any cells that are in the process of division (Petty *et al*, 1995). Therefore, if cells are undergoing division, but have not yet formed separate cells, the MTT assay would not detect this. A more accurate way of detecting both cells undergoing division and pre-divided cells is the tritiated thymodine ([^{3}H] thymodine) assay. Cells require thymidine for DNA synthesis in preparation for mitosis (Stanners & Till, 1960). The [^{3}H] thymidine assay involves incubation of a [^{3}H] thymodine solution with the cells being assayed, and then detecting the level of reactivity present in the cells, which is assumed to be proportional to the number of viable cells. There are two major disadvantages to this assay however, which is why it was not chosen in this study. The first is safety, due to using radiolabelled material, and would therefore require a number of precautionary measures before attempting the experiments themselves. The second is time efficiency. Due to the nature of the assay, both preparing the samples to be detected and recording them is much more time consuming than the MTT assay, particularly when it comes to recording multiple samples, as was completed in this study (Yang *et al,* 1996). Therefore, [^{3}H] thymidine assays could be completed in conjunction with the MTT experiments to confirm whether cell division is taking place. However, [^{3}H] thymidine incorporation should only be attempted initially on the highest agonist concentrations used (e.g. 10^{-6}M CPA, 10^{-4}M histamine, 10^{-8} EGF) to restrict exposure to radiation and improve

time efficiency. If no [^{3}H] thymidine incorporation is detected at these concentrations, there would be no need to complete assays with lower agonist concentrations.

Also, in comparison to other colourimetric assays, such as the aforementioned acid phosphatase assay (Martin & Clynes), as well as the neutral red and sulforhodamine assays (Fiennes *et al,* 1987, Cook & Mitchell, 1989), the MTT assay has been shown to be both less accurate and less sensitive (Petty *et al,* 1995). However, the MTT assay does have a greater linear range than the other assays mentioned earlier, and this was important in measuring the wide range of concentrations used in this study. Furthermore, recent improvements to the MTT assay include using the substrate analogue 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulphophenyl) tetrazolium (MTS), which is converted to the required formazan product easier than MTT (Barltrop & Owen, 1991). Repeating the experiments using MTS instead of MTT may reveal any significant increase in cell numbers not detected by MTT reduction.

6.7 – Summary

In summary, this study has shown activation of both the extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (p38 MAPK) cascades by the $G_{i/o}$ protein-coupled adenosine A_1 receptor and $G_{q/11}$ protein-coupled histamine H_1 receptor in the smooth muscle cell line, DDT_1MF-2. However, neither receptor activated the c-Jun N-terminal protein kinase (JNK) cascade. Activation of the two MAPK pathways by histamine H_1 receptors involved coupling to $G_{i/o}$ proteins. Histamine H_1 receptors also induced protein kinase B activation in DDT_1MF-2 cells, similar to data obtained for the A_1R in a previous study (Germack & Dickenson, 2000). Both adenosine A_1 and histamine H_1 receptors did not mediate DDT_1MF-2

cell proliferation, or inhibit staurosporine/H_2O_2-induced cell death. Summaries of the intracellular signalling pathways activated by adenosine A_1 and histamine H_1 receptors in DDT_1MF-2 cells are given in Figures 6.1 and 6.2 respectively. The physiological consequences of adenosine A_1 and histamine H_1 receptor-induced ERK 1/2 and p38 MAPK activation remain to be established since neither receptor induced DDT_1MF-2 cell proliferation, or prevented staurosporine/H_2O_2-mediated cell death. Clearly further experiments are required in order to establish the physiological role for A_1Rs and H_1Rs in DDT_1MF-2 cells.

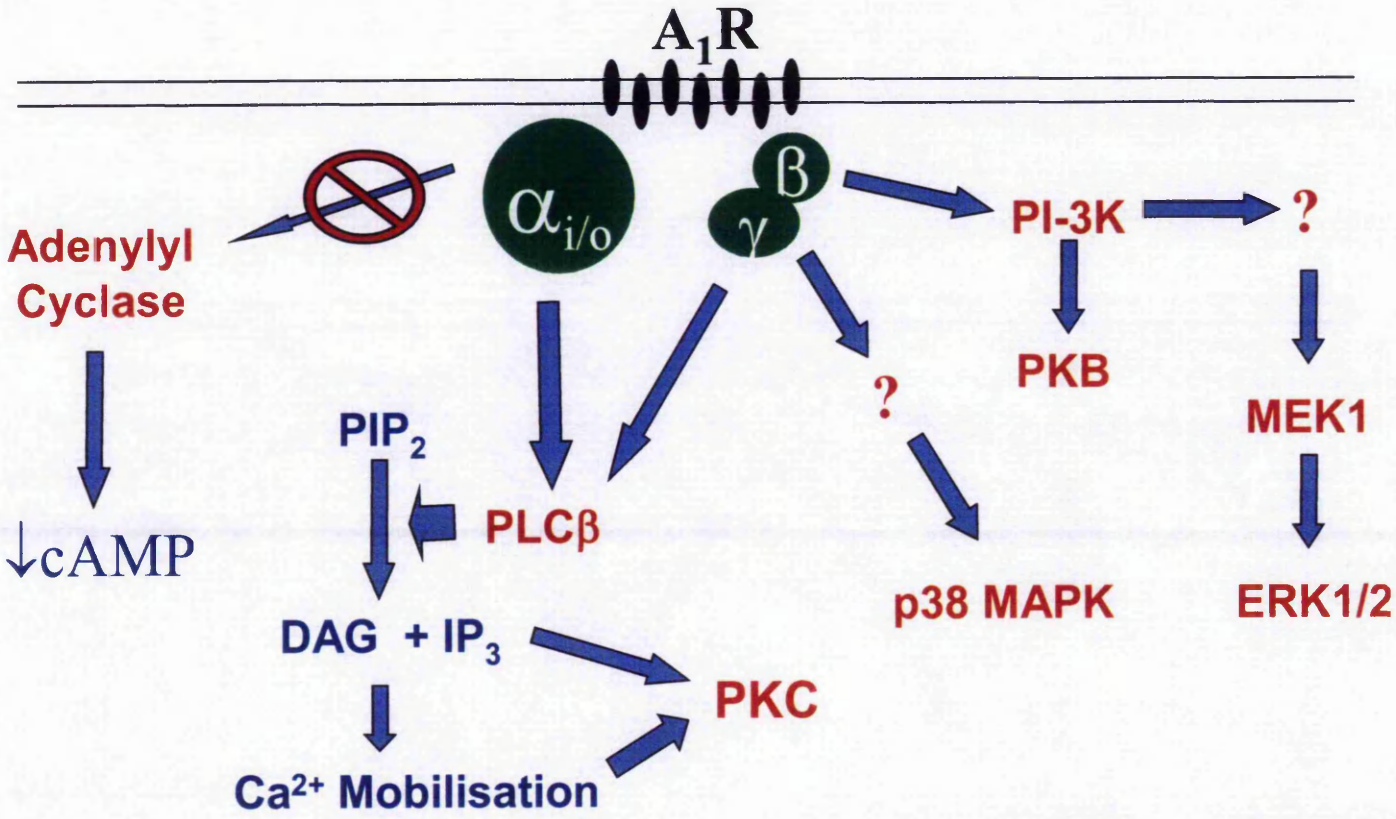


Figure 6.1 - Signal transduction pathways of the adenosine A_1 receptor in DDT_1MF-2 cells. Inhibition of adenylyl cyclase and activation of PLCβ, causing Ca^{2+} mobilisation and PKC activation, are mediated by $G_{i/o}$-α-subunits and $G_{i/o}$-βγ-subunits, respectively (Dickenson & Hill, 1991, 1992). Recent studies have also shown that A_1Rs also couple to PKB (Germack & Dickenson, 2000), ERK 1/2 via PI-3K and MEK1 activation (Robinson & Dickenson, 2000a), and p38 MAPK (Robinson & Dickenson, 2000b). Activation of both MAPK cascades probably involves $G_{i/o}$-βγ-subunits.

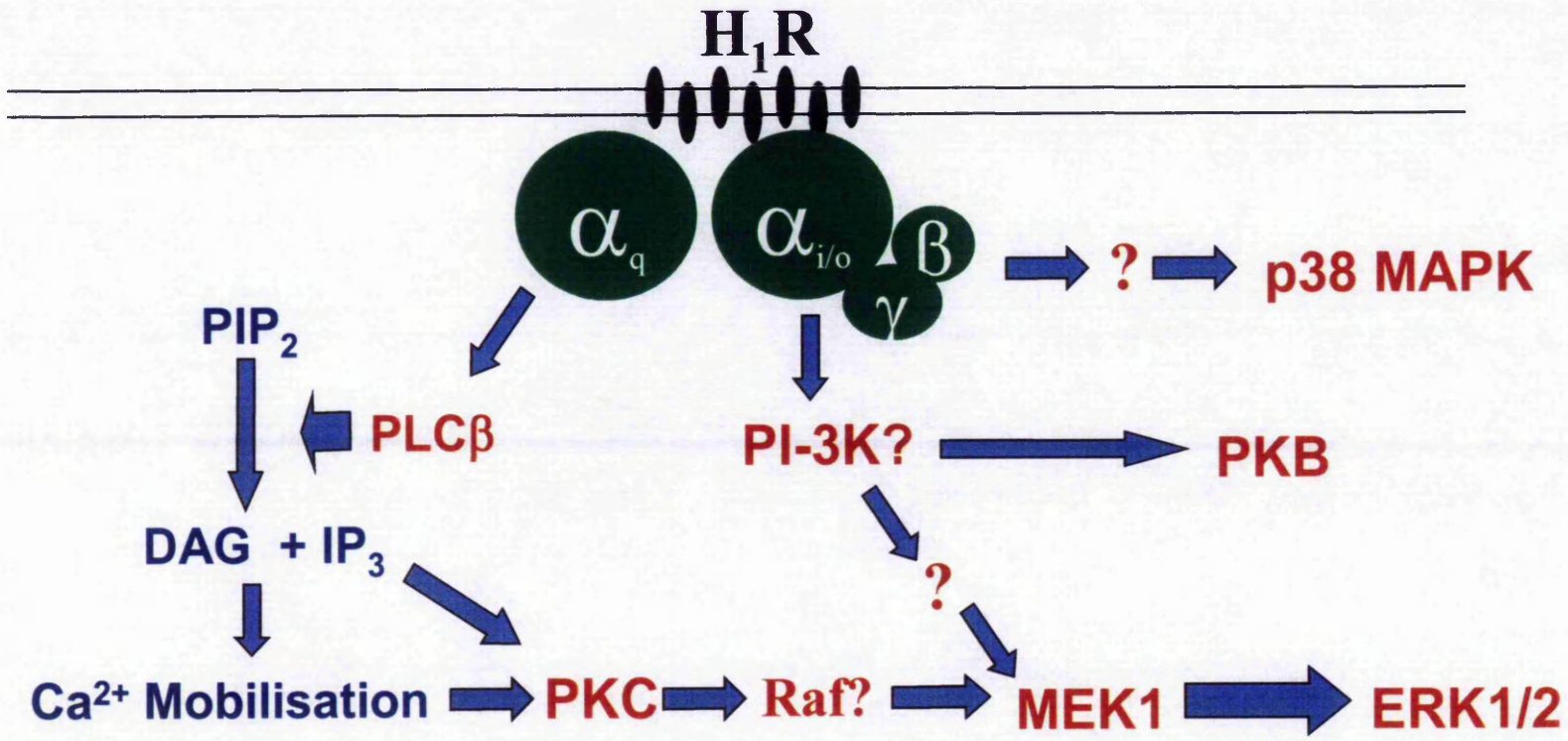


Figure 6.2 - Signal transduction pathways of the histamine H_1 receptor in DDT$_1$MF-2 cells. Activation of PLCβ, causing Ca^{2+} mobilisation and PKC activation, are mediated by $G_{q/11}$-α-subunits (White *et al,* 1993). Results from this study are included, showing activation of PKB by H_1Rs, probably via PI-3K. Activation of the ERK cascade by H_1Rs involves PI-3K, PKC, and MEK1 activation, and may involve both $G_{q/11}$ and $G_{i/o}$ proteins. Also shown is H_1R-mediated p38 MAPK activation, which involves PTX-sensitive $G_{i/o}$ proteins.

Chapter 7

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7.0 - REFERENCES

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8.0– APPENDIX

8.1 – Publications (Papers)

ROBINSON, A.J., & DICKENSON, J.M. (2001). Regulation of p42/p44 MAPK and p38 MAPK by the adenosine A_1 receptor in DDT_1MF-2 cells. *Eur. J. Pharmacol.*, **413**, 151-161.

ROBINSON, A.J., & DICKENSON, J.M. (2001) Activation of the p38 and p42/p44 mitogen-activated protein kinase families by the histamine H_1 receptor in DDT_1MF-2 cells. *Br. J. Pharmacol.*, **133,** 1378-1386.

8.2 – Publications (Abstracts)

ROBINSON, A.J., & DICKENSON, J.M. (1999). Adenosine A_1 receptor-mediated activation of the MAP kinase signalling pathway in DDT_1MF-2 cells. *Br. J. Pharmacol,* **128**, 151P.

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ROBINSON, A.J., & DICKENSON, J.M. (2000). A_1 Adenosine receptor activation of p38 MAP kinase in DDT_1MF-2 cells. *Drug Devel. Res.*, **50,** 127.