Muscarinic Acetylcholine Receptor Regulation of ERK and JNK in CHO Cells.

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

by

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I would like to dedicate this Thesis to my family and friends, without whose support I would not have succeeded.

ABSTRACT

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Paul Wylie

Extracellular signal-regulated kinases (ERKs) and the c-Jun N-terminal kinases/stress-activated protein kinases (JNKs/SAPKs) are activated by an array of extracellular signals to mediate a variety of cellular responses e.g. mitogenesis, differentiation, hypertrophy and apoptosis. The study investigated the regulation of ERK and JNK by agonist-mediated stimulation of the human m2-AChR or m3-AChR stably expressed in CHO cells. Stimulation of both receptors dramatically activated ERK, although stimulation by the m3-AChR was more sustained. The m3-AChR is efficiently coupled to JNK activation, whereas the m2-AChR is not. Activation of JNK in CHO-m3 cells was delayed and more sustained relative to that of ERK in either CHO-m2 or CHO-m3 cells. The dose-dependence for methacholine (MCh)-stimulated JNK activation by m3-AChR and ERK activation by both receptor subtypes were similar. Although pertussis toxin (PTX) had no effect on $Ins(1,4,5)P_3$ accumulation in CHO-m3 cells, there was significant inhibition of agonist-induced ERK and JNK activation in CHO-m3 cells, suggesting that the m3-AChR was able to couple to $G_{i/o}$ in addition to G_q. ERK activation was entirely PTX-sensitive in CHO-m2 cells. ERK activation in both cell types was shown to be independent of Ca^{2+}. However, JNK activation by m3 receptors was shown to have both a Ca^{2+}-dependent and a Ca^{2+}-independent component. PKC inhibition studies demonstrated a novel PKC- and an atypical PKC-component, but not a classical PKC-component in ERK activation in CHO-m2 cells, whereas, there appears to be a cPKC and an aPKC-component in CHO-m3 cells. In contrast to this, PKC appears to have an inhibitory role in m3-AChR-mediated JNK activation. Inhibiting PI3K resulted in a moderate inhibition of agonist-induced ERK activation in CHO-m2 cells only. However, examining the role of PI3K on JNK activation was inconclusive due to receptor-independent effects of wortmannin. The results presented demonstrate that the m2-AChR and m3-AChR activate ERK and JNK via divergent mechanisms.

ABBREVIATIONS

AC	Adenylyl Cyclase
ACh	Acetylcholine
AChR	Acetylcholine Receptor
ANF	Arterial Natriuretic Factor
AngII	Angiotensin II
AP-1	Activating Protein-1
AR	Adrenergic Receptor
ATF	Activating Transcription Factor
ATP	Adenosine Triphosphate
BAPTA	1,2-Bis (2-aminophenoxy) ethane-*N,N,N',N'* -tetraacetic acid
Ca^{2+}	Calcium
cAMP	cyclic adenosine monophosphate
CCh	Carbachol
CDP	Cytidine diphosphate
CHO	Chinese Hamster Ovary
CRE	cAMP-Response Element
CSBP	CSAID-binding protein
CTP	Cytosine triphosphate
DAG	Diacylglycerol
DLK	Dual Leucine zipper Kinase
DNA	Deoxyribonucleic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGTA	Ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetate
ERK	Extracellular signal-Regulated protein Kinase
FAK	Focal Adhesion Kinase
FCS	Foetal Calf Serum
GAP	GTPase Activating Protein
GDP	Guanosine diphosphate
GEF	Guanine-nucleotide Exchange Factor
GFP	Green Fluorsecent Protein
GPCR	G-protein coupled receptor
G-protein	Guanine-nucleotide binding protein
GRB2	Growth-factor-Receptor Binding protein 2
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
HEK	Human Embryonic Kidney
HRE	Hormone Response Element
IL-1	Interleukin-1
IP	Inositol Phosphate
IP_3	Inositol 1,4,5-trisphosphate
IP_3R	Inositol 1,4,5-trisphosphate receptor
IPTG	Isopropylthiogalactosidase
JIP-1	JNK-Interacting binding Protein-1
JNK	c-Jun NH2-terminal kinase
JNKK	JNK Kinase

KHB	Krebs-Henseleit buffer
LPA	Lysophosphatidic acid
mAChR	Muscarinic Acetylcholine receptor
m2-AChR	m2-acetylcholine receptor
m3-AChR	m3-acetylcholine receptor
MAPK	Mitogen-Activated Protein Kinase
MAPKK	Mitogen-Activated Protein Kinase Kinase
MAPKKK	Mitogen-Activated Protein Kinase Kinase Kinase
MCh	Methacholine
MEF	Myocyte Enhancer Family
MEK	MAPK/ERK kinase
MEKK	MAPK/ERK kinase kinase
MEM-α	Minimal Essential Medium α
MKK	MAPK Kinase
MLK	Mixed-lineage kinase
NMS	N-methyl scopolamine
PA	Phosphatidic acid
PAK	p21 activated protein kinase
PBS	Phosphate Buffered Saline
PC-12	Phaeochromocytoma cells
PDBu	Phorbol 12,13-dibutyrate
PDGF	Platlet Derived Growth Factor
PI	Phosphatidylinositol
PI3K	Phosphoinositide 3-Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PtdIns(3,4,5)P_3	Phosphatidyl inositol 3,4,5-trisphosphate
PtdIns(4,5)P_2 / PIP2	Phosphatidyl inositol 4,5-bisphosphate
PTX	Pertussis Toxin
RK	Reactivating Kinase
RNA	Ribonucleic acid
RTK	Receptor Tyrosine Kinase
SAPK	Stress-activated protein kinase
SEK1	MKK4
SH2	Src-homology-2
SHC	SH2-domain-containing α2-collagen-related
SOS	Son-of-Sevenless
SRE	Serum Response Element
TAB	(TGF-β-activated kinase)-binding protein
TAK	TGF-β-activated kinase
TCA	Tri-carboxcyclic caid
TCF	Ternary Complex Factor
TGF	Transforming growth factor
TK	Tyrosine Kinase
TNF	Tumour Necrosis Factor
TPA	4β-12-*O*-tetradecanoylphorbol-13-acetate
TRE	TPA-responsive element

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Chapter 1

Introduction

1.1 Receptors

Receptors are utilised by cells as a means of eliciting an internal response to an external stimulus, e.g. a hormone via an effector system (Berridge, 1993b). Receptors are proteins that have a binding site with a high affinity for a specific signalling substance. They can couple to a variety of effectors to enable a response to cross the plasma membrane. Certain effector systems allow the transduction of the signal from the plasma membrane to the nucleus. There are a large range of effector systems that act in a variety of ways, for example in terms of speed, synaptic transmission is in the μs range, whilst some hormone responses take many hours for the effect to be elicited. Receptors can been classified into four superfamilies, the ligand gated ion channels, steroid receptors, tyrosine kinase receptors and G-protein coupled receptors (Rang, Dale and Ritter, 1999).

Direct ligand-gated ion channels are receptors that are involved in allowing selective ion movements across the plasma membrane and are often hetero-oligomers made up of subunits which are themselves integral membrane proteins. They elicit the fastest response of all the receptor superfamilies and are found in processes involved in synaptic transmission that induce an increase in ion permeability in cells such as nerve and muscle cells. The most well-defined receptor in this family is the nicotinic acetylcholine

receptor (Changeux *et al.*, 1987).

Steroid receptors are ligand-activated proteins that regulate gene transcription, e.g. the oestrogen receptor and thyroid hormone receptors (Moore 1989). These are intracellular receptors that are present in the cytosol and/or nucleus. They bind to their respective ligands and then exhibit a conformational change (transformation) to the active form. The activated receptor can then bind to specific DNA sequences, termed hormone-response elements (HREs), to allow either activation or repression of transcriptional initiation by RNA polymerase II (for general review see Parker, 1993).

Tyrosine kinase (TK) receptors are involved with a range of growth factors, that stimulate mitogenesis (Rosen 1987). They generally have a large intracellular catalytic domain and an extracellular-ligand binding domain (Fantl *et al.*, 1993). An example of a tyrosine kinase receptor is the insulin receptor. In this example, the majority of the extracellular domain is a separate polypeptide chain that is bound to the transmembrane region by a series of disulphide bonds. In contrast, other types of growth factor receptor are composed of a single polypeptide chain. Upon stimulation by a ligand, there is a conformational change that allows two individual chains to dimerise and become activated. Once activated, specific tyrosine residues in the intracellular region of the receptor become autophosphorylated; this then allows for the formation of a high affinity binding site. Some groups of intracellular proteins contain a region called the SH2 (Src-homology-2) domain which enables them to bind specifically to certain phosphotyrosine receptors. Cytokine receptors possess a similar structure to the tyrosine kinase receptors, except that they do not have the intracellular kinase domain. However, upon activation, they dimerise as described above for the receptor tyrosine kinases.

1.2 G-protein-coupled receptors (GPCRs)

There have now been nearly 2000 guanine-nucleotide binding protein (G-proteins) coupled receptors identified since bovine opsin (a light-sensitive GPCR) was first cloned (Nathans and Hogness, 1983). These highly conserved proteins are composed of a single polypeptide chain that spans the plasma membrane seven times utilizing seven α-helix structures (Dohlman *et al.*, 1987), with an extracellular NH_2-terminal domain, an intracellular COOH terminal tail and often a large third intracellular loop which are all highly variable, giving both ligand and effector specificity (for review see Selbie and Hill, 1998). Upon activation by an external stimulus (e.g. hormone, neurotransmitter or drug), the receptor becomes activated and the respective G-protein couples the receptor to the relevant effector molecule (e.g. adenylyl cyclase (AC), or phospholipase C (PLC)), possibly via a conformational change in the receptor which enables the variable third intracellular loop and carboxy terminal tail to interact with the G-protein (Lambright *et al.*, 1996; Lefkowitz *et al.*, 1993).

1.3 Guanine nucleotide-binding proteins

As previously mentioned, G-proteins mediate the coupling of a stimulated GPCR to an effector, thus allowing an external stimulus to elicit an intracellular response. G-proteins are heterotrimeric membrane-associated proteins composed of three subunits: α- (45 kDa), β- (35 kDa) and γ- (7 kDa), (Gilman, 1987). G-proteins exist in 2 states: an inactive form and an active form. In the absence of hormone or agonist, the G-protein is bound to guanosine diphosphate (GDP). Once the receptor-hormone complex has been formed at the plasma membrane, there is a conformational change in the G-protein and

GDP is replaced with intracellular free guanosine triphosphate (GTP), which binds non-covalently to the G-protein α-subunit. This conformational change causes the dissociation of the α-subunit on the G-protein from the βγ-subunit (Caulfield and Birdsall, 1998). These subunits are then able to regulate effector proteins, for example, activation of G_s G-proteins stimulate adenylyl cyclase activity, resulting in an increased production of cAMP. cAMP can then act on cAMP-dependent-protein kinases, e.g. PKA (Neer 1995; Simonds, 1999) or ion channels (for review see Yamada *et al.,* 1998).

There are four main classes of G-proteins identified by their α-subunits (Dhanasekaran *et al.*, 1995). These have been termed: G_s, which stimulates adenylyl cyclase activity, $G_{i/o}$ which induces adenylyl cyclase inhibition, $G_{q/11}$ which stimulates certain isoforms of PLC, and the $G_{12/13}$ class whose function has yet to be fully elucidated, but has been demonstrated to be involved in Na^+/H^+ exchange (Sternweis, 1996). The β and γ subunits remain associated together, but this complex also has the ability of regulating effectors (Birnbaumer, 1992), such as PLCβ (Blank *et al.,* 1992). It has also been demonstrated that both the α-subunit and the βγ-subunit may activate the MAP kinase pathway via Ras (Crespo *et al.,* 1994; Faure *et al.,* 1994; Coso *et al.,* 1996).

1.4 Muscarinic Acetylcholine Receptors

Muscarinic receptors are members of the GPCR family. In smooth muscle, the regulation of muscle contraction is evoked by the release of the neurotransmitter acetylcholine (ACh) from the parasympathetic nerves acting on the muscarinic acetylcholine receptors. The m3-AChR has been shown to be involved in the contraction of smooth muscle (Roux *et al.,* 1998) via PLC (see Fig 1.1). However, the m2-AChR

inhibits the relaxation of smooth muscle via inhibition of adenylyl cyclase and therefore reducing levels of cAMP (Eglen *et al.*, 1994).

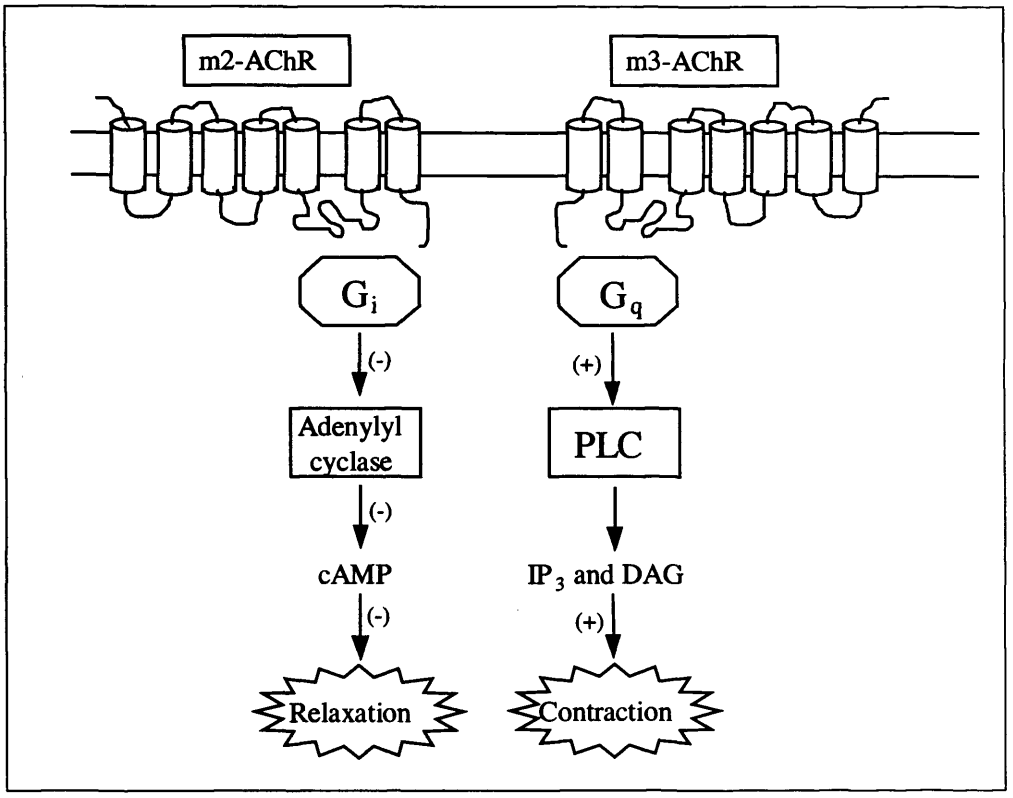

Figure 1.1 A diagram demonstrating the route, and role of m2- and m3-AChR stimulation in smooth muscle.

Stimulation of the m2-AChR results in activating G_i G-proteins which inhibits the increased levels of adenylyl cyclase activity, that occurs via activation of the G_s-coupled β-adrenergic receptor. Stimulation of the β-adrenergic receptor increases the cellular level of cAMP thereby activating cAMP-mediated protein kinase. This increases the phosphorylation of myosin LC (light chain) kinase, which decreases the binding affinity of myosin LC kinase for the Ca^{2+}-calmodulin complex. This prevents the myosin regulatory pair to be phosphorylated and the muscle stays in a relaxed state. Therefore reduced levels of adenylyl cyclase results in relaxation being inhibited, thereby contributing to muscle contraction. Stimulation of the m3-AChR results in the activation of G_q G-proteins which activate PLC to yield IP_3 and diacylglycerol (DAG), inducing muscle contraction via releasing the binding of caldesmon from actin, and thereby allowing actin to bind myosin.

By using binding studies, muscarinic cholinergic receptors were initially classified into two subtypes by their distribution, the neural m1 and the cardiac, m2 (Hammer, 1980), then the m3 was identified in glandular smooth muscle (Birdsall and Hulme, 1987). Five distinct mACh receptor subtypes have since been cloned, termed, m1, m2, m3, m4 and m5 (Kubo *et al.*, 1986a; Kubo *et al.*, 1986b; Bonner *et al.*, 1987; Bonner *et al.*, 1988; Peralta *et al.*, 1987a; Peralta, *et al.*, 1987b).

Functionally, however, they can be divided into two main groups. The ml-, m3- and m5-AChR are positively coupled to PLC-β, via G_q, members of a pertussis toxin-insensitive class of G-proteins (Blank *et al.*, 1992; Van Giersbergen and Leppik., 1995). This hydrolyses phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2 or PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 is water soluble and thus diffuses into the cytosol, mobilizing calcium from the intracellular stores by binding to intracellular IP_3 receptors (IP_3Rs). DAG can activate PKC (in conjunction with Ca^{2+} in the case of classical PKC isoforms; see section 1.5.3), resulting in a variety of cellular responses (see Fig. 1.2).

In contrast, the m2 and m4 receptors are negatively coupled to adenylyl cyclase (Hulme *et al.*, 1990) via G_i, a pertussis toxin-sensitive G-protein (Van Giersbergen and Leppik., 1995). This is generally achieved by direct interaction of the adenylyl cyclase with the free α-subunit (Fig. 1.3), although a mechanism involving the βγ subunit binding to the free α_s-subunit, preventing it from activating the adenylyl cyclase cannot be discounted (Gilman., 1994).

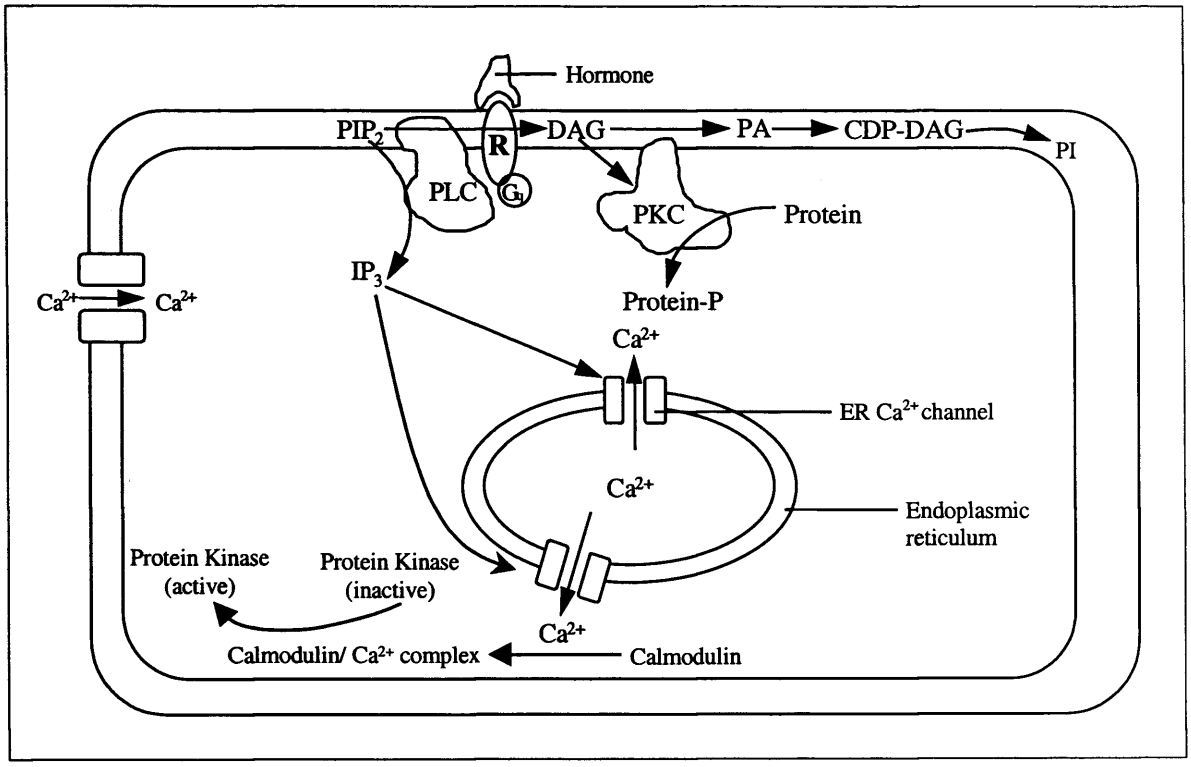

Figure 1.2 A diagram showing the activation of the IP_3 pathway and the mechanism for the release of Ca^{2+} by $G_{q/11}$- coupled GPCRs.

Upon stimulation of the receptor by agonist, PLC is activated and hydrolyses PIP_2 to IP_3 and DAG. DAG activates PKC in association with Ca^{2+} and phosphatidylserine for some isoforms. DAG is then phosphorylated to phosphatidic acid (PA)which reacts with CTP, forming CDP-DAG and eventually phosphatidylinositol (PI). However, IP_3 stimulates IP_3Rs on the endoplasmic reticulum and causes the release of Ca^{2+} from calcium stores enabling Ca^{2+} to act as an activator of Ca^{2+} dependent proteins, e.g. calmodulin.

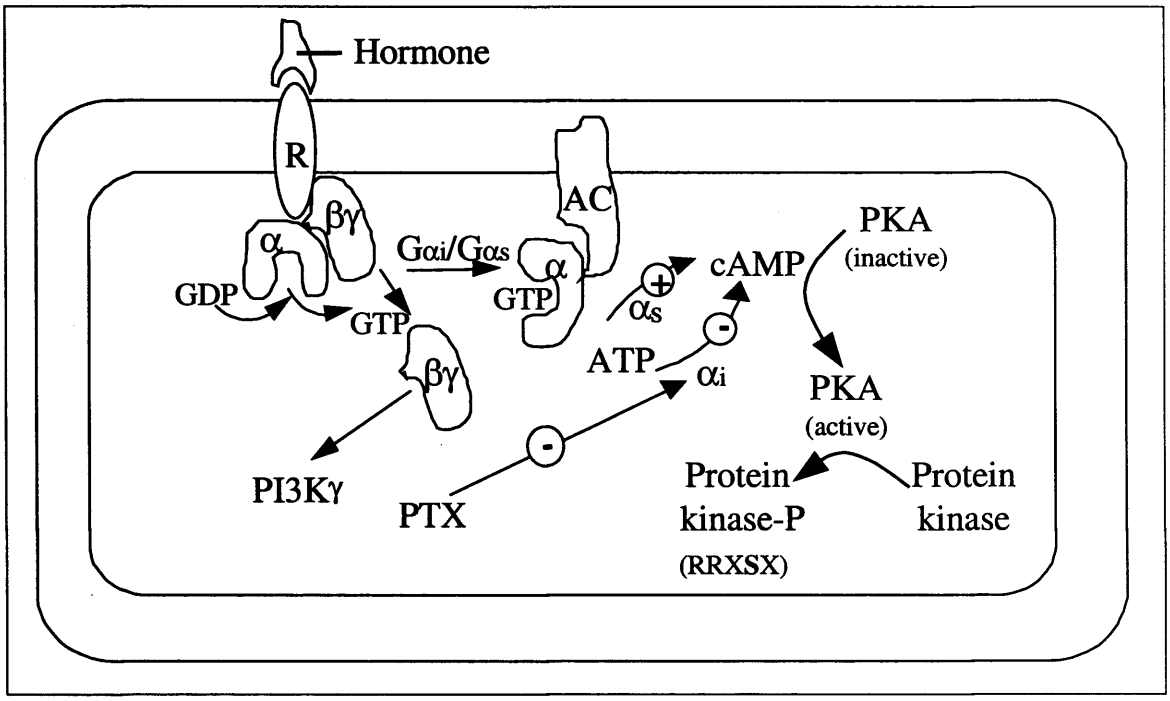

Figure 1.3. A diagram showing the regulation of adenylyl cyclase and PI3K by G_i-coupled receptors.

Stimulation of the $G_{i/s}$ linked receptors leads to a GDP/GTP exchange on the α-subunit of the G-protein. The α-subunit acts to either inhibit (α_i) or stimulate (α_s) adenylyl cyclase (AC) to regulate levels of cAMP, which then activates protein kinase A (PKA) which is then able to phosphorylate protein kinases on a Ser residue in the motif, RRXSX. The free βγ subunit has also been shown to have a role in signalling, an example of which is demonstrated by the direct activation of PI3Kγ (and also PI3Kβ), which can then elicit a number of responses, including activating the MAP kinase pathways.

1.5 Mitogen-activated protein kinases (MAP Kinases)

Stimulation of tyrosine kinase receptors were shown to lead to the activation of a family of serine-threonine kinases, termed the MAP kinases (mitogen-activated protein kinases), to enable an extracellular signal to control gene expression and lead to the control of cell differentiation and proliferation (Cano and Mahadevan 1995; Marshall, 1995). Initial studies in the yeast, *Saccaromyces cerevisiae,* demonstrated a pathway that is involved in pheromone-response termed the FUS3/KSS1 pathway (Cairns *et al.*, 1992; Gartner *et al.*, 1993). This was the first study to demonstrate the fundamental significance of a MAP kinase pathway. The MAP kinases are a highly conserved group of proteins that form part of a protein cascade of sequential phosphorylations that regulate mitogenesis so that from an extracellular signal, e.g. a growth factor or hormone binding to a membrane bound receptor, the transcription of individual genes can be either up regulated or down regulated to enhance cell differentiation or induce cell proliferation (Blenis 1993). In mammalian cells three main MAP kinase pathways, have been characterised. The ERK (extracellular signal-regulated protein kinase) pathway, the SAPK/JNK (stress-activated protein kinase/c-Jun NH_2 terminal kinase) pathway and the p38 pathway (Robinson and Cobb 1997). There has also been an ERK6 (p38-like) pathway (Lechner *et al.*, 1996), an ERK5 (English *et al.*, 1999; Marinissen *et al.*, 1999) and ERK3 pathway identified (Cheng *et al.*, 1996). The three main pathways are composed of a cascade, characterised by three kinases; a MAPK which is activated by a dual phosphorylation on threonine (T) and tyrosine (Y) in a T-X-Y motif by the upstream kinase, MAPK kinase (MAPKK). MAPKK is itself activated by a dual phosphorylation by a serine/threonine-specific MAPKK kinase (MAPKKK) (see Fig. 1.4.).

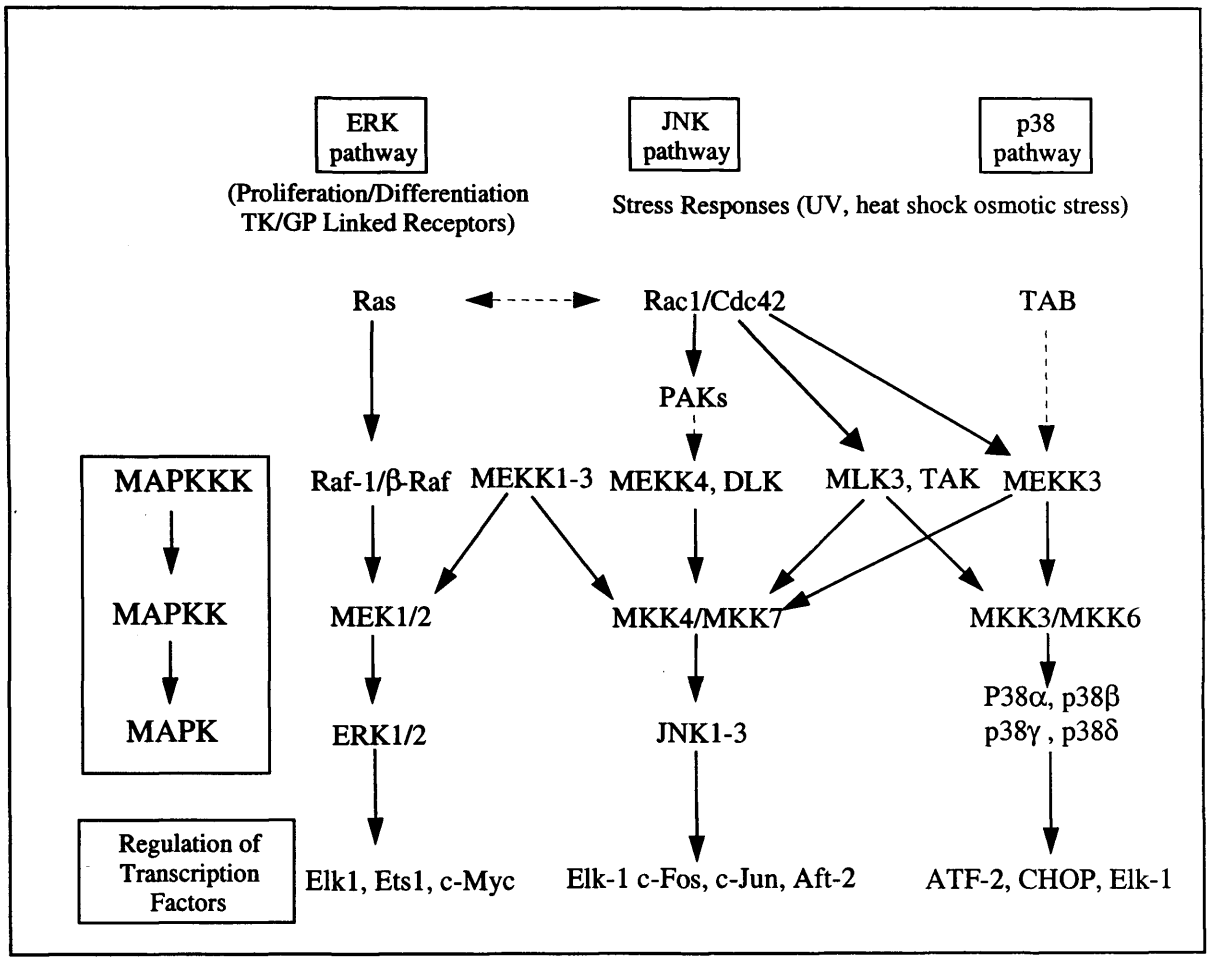

Figure 1.4. Diagram showing the MAP kinase cascade of the ERK, JNK and p38 pathways.

The ERK, JNK and p38 pathways are all composed of a three kinase cascade to enable an extracellular signal to elicit regulation of transcription factors. The ERK pathway is activated by growth factors and agonists for GPCRs. The JNK and p38 pathways are activated by GPCR stimulation and stress responses. The pathways have the ability to cross-talk within each other as shown in the diagram. Key: PAK; p21 activated protein kinase, TAB; TAK1 (TGF-β-activated kinase)-binding protein, MLK3; Mixed-Lineage Kinase-3; DLK:, Dual Leucine zipper Kinase.

It is thought that the duration of MAP kinase activity is a major factor in the decision of the cell to either proliferate or differentiate (Cano and Mahadevan, 1995; Marshall, 1995; Lavoie, *et al.*, 1996; Cohen, 1997), via the tyrosine kinase signalling pathway (for further discussion see section 1.7.2.). Upon sustained activation of the MAP kinase it translocates to the nucleus and acts on the regulation of transcription factors (e.g. Elk-1) (Chen *et al.*, 1992).

1.5.1 MAP kinase terminology

To date, there are contrasting views on the terminology for the MAP kinase family. The two main classifications have been suggested primarily by R. Davis, and P. Cohen, and in the absence of a consensus within the scientific community, the different terms used are summarised below in Table 1.1.

R. Davis	P. Cohen
ERK1	MAP kinase 1
ERK2	MAP kinase 2
JNK2	SAPK1a
JNK3	SAPK1b
JNK1	SAPK1c
p38α	SAPK2a
p38β	SAPK2b
ERK5	SAPK5

Table 1.1 A summary of the MAP kinase family terminology.

1.5.2 Extracellular-signal regulated protein kinase 1/2 (ERK1/2) Pathway

The MAP kinase pathway, is a cascade composed of a number of levels, in which there is the potential for amplification of the signal. As discussed, a MAPKKK activates a MAPKK, which then in turn activates a MAPK (for reviews see Cobb and Goldsmith, 1995; Cohen, 1997; Robinson and Cobb, 1997; Lopez-Llasaca, 1998). The MAP kinase, ERK has two distinct isoforms: ERK1 is a 44kDa protein also termed $p44^{MAPK}$ and ERK2 is a 42kDa protein, termed $p42^{MAPK}$ which are phosphorylated and therefore activated by the MAPKKs, MEK1 and MEK2 (Dhansekaran and Reddy, 1998). The dual phosphorylation by MEK1 and MEK2 on ERK1/2 is on a TEY motif 7 residues upstream from the APE sequence situated in kinase subdomain VIII (Blenis, 1993). The MAPKKs are activated by the MAPKKKs Raf-1 (Kyriakis *et al.,* 1992, 1993) and B-Raf, with an equal affinity, by dual phosphorylating the serine/threonine kinases. In contrast, A-Raf is selective for MEK1 over MEK2 (Wu *et al.*, 1996). One of the first comparative studies between the activation of the ERK and JNK pathways showed that Raf-1 activated the ERK pathway and MEKK activated the JNK pathway via two distinct Ras-dependent pathways (Minden *et al.,* 1994). The upstream activator of the MAPKKKs (in the ERK pathway) has been shown to be Ras and indeed the first MAPK pathway to be discovered involved Ras activation via tyrosine kinase receptors (Avruch *et al.*, 1994; Lange-Carter and Johnson, 1994), although, more recently, Ras has also been implicated in the activation of the ERK pathway via G-protein linked receptors (Coso *et al.*, 1995a).

The cDNAs for MEK1 and MEK2 were cloned in 1993 by Zheng and Guan; they demonstrated that there was 80% homology between the two proteins. MEKK1,

MEKK2 and more preferentially, MEKK3 have been shown to activate the ERK pathway when transfected into HEK293 cells (Lange-Carter and Johnson, 1994; Blank *et al.,* 1996). MEKK1, when transiently overexpressed, has also been shown to constitutively activate both the ERK and JNK pathway, but not the p38 pathway (Lin *et al.,* 1995; Blank *et al.,* 1996), the first demonstration that one protein can activate more than one MAP kinase pathway. There have also been reports of other MAPKKKs activating the ERK pathway e.g. Mos (Lange-Carter *et al.,* 1993; Posado *et al.,* 1993). Interestingly, it has been shown that the relative amounts of MEK and ERK are similar, with the MEK proteins, sometimes being in excess (Ferrell, 1996). Therefore, this implies that the pathway may not have solely an amplification role, but the complexity of the pathway exists for specific regulation.

In the last few years, there have been reports that describe evidence of the importance of scaffold proteins in the MAP kinase pathways (Choi *et al.,* 1994, Whiteway *et al.,* 1995, Schaeffer *et al.,* 1998). Cells have a multitude of intracellular signalling pathways that have the ability to interact with one another, leading to a potential difficulty in obtaining pathway activation specificity. These scaffold proteins (or anchor proteins) may target proteins to the correct pathway, allowing for the ability to specifically target interactions. To date, most of the literature pertains to scaffold proteins that have been isolated in yeast cells. For example, Choi *et al.,* (1994) and Whiteway *et al.,* (1995) isolated a scaffold protein called Ste5p. When the GPCR was stimulated by a pheromone, Ste5p bound the three members of the ERK pathway FUS, STE7 and STE11 causing the initiation of mating. Later, Schaeffer *et al.,* (1998) identified a mammalian scaffold protein specific for MEK1 and ERK1 called MP1 (MEK Partner 1), which upon

binding, increased the activation of these enzymes. Therefore, these studies indicate that there is an important regulatory component of the MAP kinase pathways via these newly identified scaffold proteins (for review see Garrington and Johnson, 1999). Fukuda, *et al.,* (1997) determined a direct association between ERK2 and MEK1 by a identifying a binding site for ERK2 in the N-terminus on MEK1, that aided association. They hypothesised that binding of MEK1 to ERK2 retained ERK2 in the cytoplasm, and therefore inhibited its ability to enter the nucleus and have an effect on transcription factors. Later experiments, following on from the realisation that MEK1 contains two phosphorylation sites for ERK2 (Xu, *et al,.* 1999), it was demonstrated that deletion of the ERK2 binding site in MEK1, prevented ERK activation (*in vivo*) and also ERK2 phosphorylating MEK1 on Thr^{292} and Thr^{386} (*in vitro*). In later experiments, Rubinfield *et al.,* (1999) created a GFP-ERK2 fusion protein in CHO cells, which, when expressed in isolation, was found to be localised in the nucleus. However, where the fusion protein was co-expressed with MEK1, this caused cytoplasmic retention of the fusion protein due to the association of ERK2 with MEK1, which was reversible upon stimulation. From their results obtained using amino acid substitutions, they conclude that in the ERK2 protein, residues 312-320 were important for MEK1 binding and thus the retention of ERK2 in the cytoplasm, whilst residues 321-327 are important in causing ERK2 to translocate to the nucleus upon stimulation. Therefore, these observations suggest that formation of enzyme complexes are important in the activation of the pathways, thus reducing the possibility of the signal being greatly amplified.

Recently, there has been increasing evidence for the need for ERK to homodimerise. When the crystal structure of ERK2 was first revealed, it was shown that there was a

dimer interface structure (Canagarajah *et al.*, 1997). Following on from this work, it was then shown that when ERK2 was microinjected into quiescent rat embryo fibroblast cells there was a requirement for ERK2 to be phosphorylated for it to enter the nucleus. However, preventing ERK2 to homodimerise (by using mutagenesis) resulted in a reduction of ERK2 accumulation in the nucleus (Khokhlatchev *et al.*, 1998). These results demonstrate the potential for dimerisation of ERK2 in determining the localisation of the protein.

1.5.2.1 Receptor Tyrosine Kinase (RTK) Activation of ERK1/2

Initial studies investigated the role of ERK activation in growth factor receptor mediated responses of mammalian cells (for review see Malarkey *et al.*, 1995). A well-studied tyrosine kinase receptor (RTK) that activates the ERK pathway is the platelet derived growth factor (PDGF) receptor. Upon activation of the receptor, there is a phosphorylation of serine residues in the intracellular domain, which enables two PDGF receptors to dimerise. An important feature of RTK signalling is the involvement of adapter proteins that contain an SH2-binding domain. These are structures that allow signalling molecules, such as PI3K, PLCγ and adapter proteins to bind to phosphorylated tyrosine residues determined by a 3-6 amino acid sequence on the C-terminal side of the phosphotyrosine on the receptor. Two examples of such adapter proteins are the SH2-domain-containing α2-collagen-related (SHC), a non-enzymatic adapter protein and growth-factor-receptor binding protein 2 (GRB2).

In the case of the activated PDGF receptor, GRB2 binds, by virtue of its SH2-domain, directly to the receptor which then increases the affinity for GRB2 for the guanine-

nucleotide exchange factor (GEF), son of sevenless (SOS), via the SH3-domain that it contains (Huang and Erikson, 1996). This complex then catalyses the exchange of GDP for GTP on Ras (Johnson and Vaillancourt, 1994; Levers, 1996) by SOS. Once Ras has GTP bound to it, Raf translocates to the membrane, leading to activation of Raf-1 and B-Raf and subsequently the ERK pathway (Marais *et al.*, 1997). The activation of the ERK pathway by TrkA is similar, however GRB2 is unable to directly bind to the TrkA receptor, and the presence of SHC is required for the activation of GRB2 (Zhou *et al.*, 1995, de Vries-Smits *et al.*, 1995) and therefore the formation of the GRB2-SOS complex which is required for the GDP/GTP exchange on Ras (see Fig. 1.5).

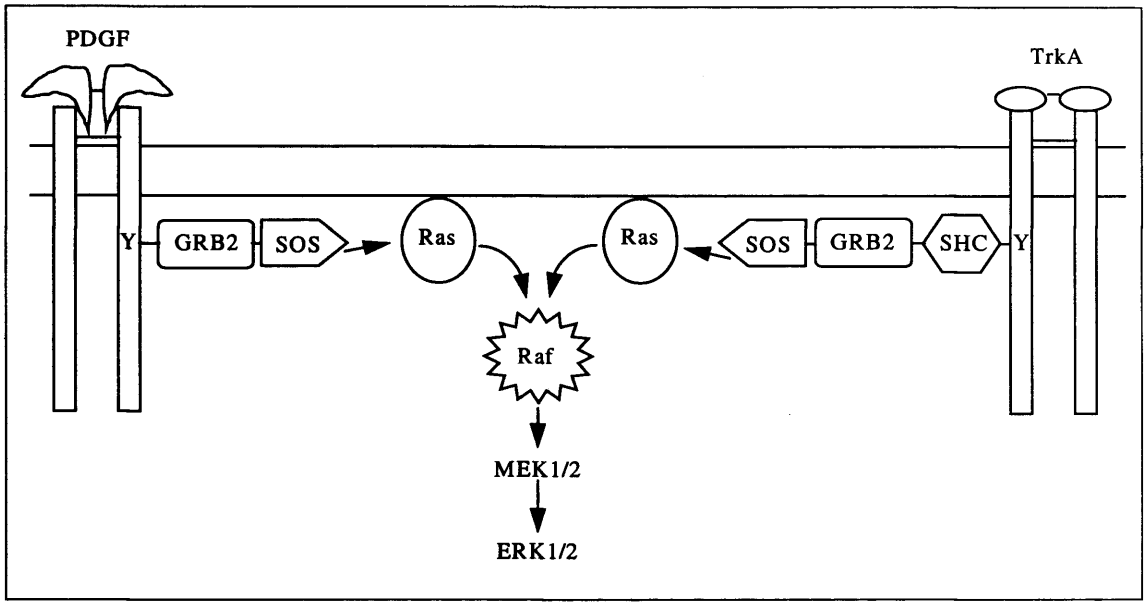

Figure 1.5. A diagram of the signaling of the PDGF receptor and the TrkA receptor to the activation of ERK.

Upon stimulation of the receptors, autophosphorylation occurs, followed by dimerisation of the receptors. Then adapter proteins with the relevant SH2-domains bind to phosphotyrosines, with PDGF receptor, GRB2 binds directly to Y^{716}, however, in the case of TrkA, SHC is required to bind GRB2 to the receptor. Then, the ERK pathway is activated via a SOS-Ras interaction.

1.5.2.2 G-protein Coupled Receptor (GPCR)-regulation of ERK1/2

Following on from research on the ERK pathway via activation of RTKs, there is now much research on GPCR-mediated activation of the ERK pathway. Studies have described a mechanism that is similar to that of RTKs, whereby SHC is tyrosine phosphorylated via $G_{\beta\gamma}$ subunit-dependent mechanism upon stimulation of the α2-C-10 adrenergic receptor (Touhara *et al.,* 1995). This tyrosine phosphorylation results in the formation of a SHC-GRB2-SOS complex, which as described in the previous section can then catalyse the exchange of GDP to GTP on Ras (van Biesen *et al.,* 1995). They also reported that disruption of the SHC-GRB2-SOS complex, blocked ERK activation via $G_{\beta\gamma}$ subunits, suggesting that the GPCR- and RTK-mediated activation of the ERK pathway, maybe via a common route. Luttrell *et al.,* (1996) described a role for the non-receptor tyrosine kinase c-Src in the activation of ERK via LPA receptors in COS-7 cells. Further to this work, they then showed that $G_{\beta\gamma}$-subunits from G_i-coupled G-proteins (via LPA receptors) increased phosphorylation of EGFR in a Src-dependent manner (Luttrell *et al.,* 1997). More recently, Della Rocca *et al.,* (1999a) demonstrated a role for focal adhesions (by the use of cytochalasin D, a disrupter of the assembly of focal adhesions) in PC12 cells and a RTK (by the use of AG1478, a EGFR-specific tyrphostin) in HEK-293 cells but not PC12 cells in the activation of ERK1/2. However, the Src kinase inhibitor 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo-D-3,4-pyrimidine (PP1), inhibited GPCR-mediated ERK activation in both cell lines, indicating a possible point of signal convergence.

Additional evidence of a mechanism whereby GPCRs can regulate TKs (for review see Luttrell *et al.,* 1999), and therefore elicit an effect on the ERK pathway is

demonstrated by Wan *et al,* (1996). In the study, they showed that ERK activation via stimulation of muscarinic receptors in avian B cells (DT40), in which there is a deficiency in Lyn (a Src-related tyrosine kinase) activation of the ERK pathway by the G_q-coupled m1-AChR, was attenuated whereby, in contrast, activation of the ERK pathway by G_i-coupled m2-AChR was unaffected. However in experiments repeated in DT40 cells that were deficient in Syk, the ERK pathway was unable to be activated by either of the muscarinic receptor subtypes Wan *et al.,* (1996).

Lev *et al.,* (1995) demonstrated that the Ca^{2+}-dependent TK, Pyk2 activated ERK1/2 via G_q-coupled receptors (Della Rocca *et al.,* 1997). In 1996, the focal adhesion kinases (FAK) were identified as having a role in interleukin (IL) signalling to the MAP kinase pathway via recruitment of GRB2 and SHC (Schlaepfer and Hunter 1997). The role of FAK on the MAP kinases was further investigated and found that activation of FAK which then phosphorylates SHC is sufficient to activate the ERK pathway in a PI3K-independent manner (Igishi *et al.,* 1999).

It has been reported that for some G-protein linked receptor agonists, the involvement of PKC (Raf is a substrate for PKC) is important in the activation of MAP kinase (Bogoyevitch *et al.,* 1994), which was demonstrated by the down-regulation of PKC by prolonged TPA pre-treatment. There have also however, been examples of PKC independent activation of the MAP kinase pathways, via a pertussis toxin sensitive activation of nucleotide exchange on p21ras.

Some groups have demonstrated that expression of p110α PI3K activates ERK (Hu *et al.,* 1995b) whereas other groups have shown no role for PI3K in ERK activation (Klippel *et al.,* 1996; Frevert, and Kahn 1997) including CHO cells (Welsh *et al.,*

1994). However, to emphasise the discrepancies of research in this field, Lopez-Ilasaca *et al.*, (1997) demonstrated a role for p110γ PI3K, but not p110α PI3K in ERK activation via the m2-AChR. Other groups have shown that incubating cells with the PI3K inhibitor, wortmannin does inhibit ERK activation in some cell lines (Hu *et al.*, 1995a; von Willebrand *et al.*, 1996). More recently, it has been shown that PDGF receptor-mediated PI3K activation is efficient at stimulating the ERK pathway, but it appears dependent upon the levels of receptor expression (Duckworth and Cantley, 1997). In Swiss 3T3 cells where the PDGF receptor was expressed at high levels, wortmannin had little effect on ERK activity, but in CHO cells where few PDGF receptors are expressed, wortmannin blocked PDGF-dependent ERK activation. Therefore they postulate that when high numbers of receptor are expressed MAP kinase can be activated via redundant pathways.

In COS-7 cells, PI3K has also been shown to have a role, upstream of Ras, in LPA mediated ERK activation via G_i (Hawes *et al.*, 1996). PI3Kγ has also been shown to have a role in $G_{\beta\gamma}$-mediated phosphorylation of SHC and the subsequent activation of the ERK pathway (Lopez-Ilasaca *et al.*, 1997). In contrast, there is evidence to suggest that the dissociated βγ subunits from $G_{q/11}$ G-proteins bind to the SH2 domain of p85; the regulatory subunit of PI3K (Stephens *et al.*, 1993; Hawes *et al.*, 1996). In addition to this, there has recently been evidence of receptors coupled to $G_{q/11}$, activating the ERK pathway via activation of PI3Kβ and PKCε (Graneß *et al.*, 1998). They demonstrate that activation of ERK by stimulation of the bradykinin receptor in SW-480 cells is dependent on PKCε in agreement with studies that $G_{q/11}$-linked G-proteins can activate PI3Kβ via stimulation by βγ subunits (Leopoldt *et al.*, 1998).

1.5.2.3 Muscarinic Acetylcholine receptor-regulation of ERK1/2

Stimulation of muscarinic acetylcholine receptors have been demonstrated to activate the ERK pathway (Winitz *et al.*, 1993; Lopez-Ilasaca *et al.*, 1997; Gutkind *et al.*, 1997). Winitz *et al*, (1993) demonstrated the ability of the G_i-coupled m2-AChR to activate the ERK pathway via Ras and Raf with agonist challenge. This activation was attenuated in cells pre-treated with pertussis toxin. In COS-7 cells, Gutkind *et al*, (1997), also demonstrated that incubating cells with pertussis toxin attenuated ERK activation via the m2-AChR, but not via m1-AChRs. Some groups have reported that in COS-7 cells, overexpression of $\beta_1\gamma_2$ subunits was able to activate ERK1 and ERK2 downstream of the m2-AChR (Hawes *et al.*, 1995; Faure *et al.*, 1994; Crespo *et al.*, 1994a; Koch, *et al.*, 1994). It was found that overexpression of α_{i2} had no effect on ERK1 activation (Hawes *et al.*, 1995; Faure *et al.*, 1994; Gupta *et al.*, 1992) whereas constitutively activated α_{i2} caused ERK2 activation in COS-7 cells (Faure *et al.*, 1994).

Stimulation of the m3-mAChR has also been previously demonstrated to activate the ERK pathway (Offermans *et al.*, 1993; Larocca, *et al.*, 1997), although the mechanism of m3-mediated ERK activation is poorly understood at this time. Kim *et al.*, (1999), demonstrated a role for PKCε, upstream of Ras in m3-AChR-mediated ERK activation in the human neuroblastoma cell line, SK-N-MC. Most of the muscarinic receptor work in this field has focused on the m1-AChR and thus, less is known about m3-AChR-mediated ERK activation. Both the m1-AChR and m3-AChR couple to $G_{q/11}$ G-proteins, so therefore the m1-AChR is likely to be a good model to use as a comparison to what may occur downstream of m3-AChR stimulation. The literature is currently conflicting

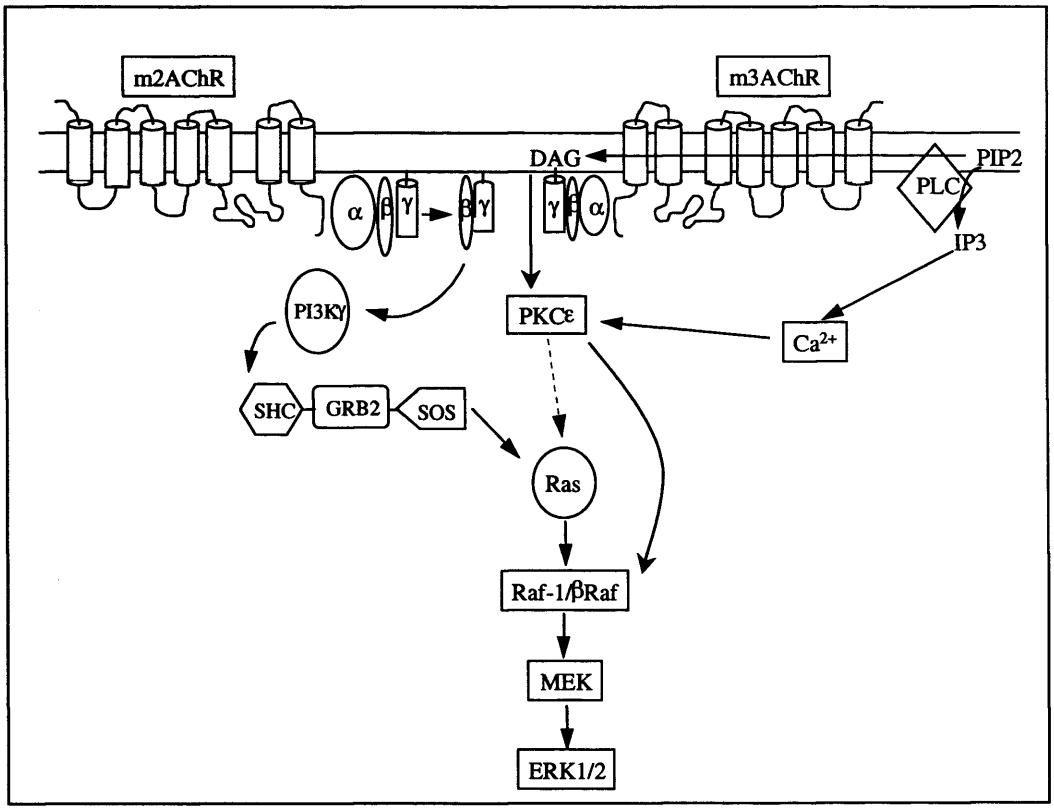

Figure 1.6. Signalling to the ERK Pathway via the m2-AChR and m3-AChR.

Upon stimulation of the m2-AChR, the βγ-subunit of the G_i G-protein activates PI3Kγ, to aid the formation of the SHC-GRB2-SOS complex, which is then able to activate Ras and the ERK pathway. When the m3-AChR is activated, PLC hydrolyses PIP2 to yield IP_3 and DAG. DAG activate PKCε, which has been shown to activate the ERK pathway in a Ras-dependent manner (Kim *et al.*, 1999). IP_3 liberates Ca^{2+} from intracellular stores and this can also activate PKCε.

about the mechanism of m1-mediated ERK activation, which is probably a result of cell specific pathway regulation. It has been shown that there is a PKC-dependent Raf activation that is Ras-independent (Hawes *et al.,* 1995; van Biesen *et al.,* 1996), whilst other researchers have described a Raf-dependent, Ras-independent mechanism that is only partly PKC dependent (Crespo *et al.,* 1994b). However, Gutkind *et al,* (1997) postulated a PKC-independent and PKC-dependent mechanism, whereby $G_{\beta\gamma}$ subunits were responsible for the PKC-independent pathway, that converges at the level of Ras. Mattingly and Macara (1996) showed that Ras GRF/CDC25^{Mm} exchange factor was phosphorylated when m1-AChR and m2-AChRs were stimulated with carbachol, potentially demonstrating a mechanism by which m1-AChR and m2-AChRs could activate the ERK pathway via Ras (see Fig 1.6).

1.5.3 Protein Kinase C

Protein Kinase C (PKC) was first identified as a histone protein kinase in rat brain, that was activated by limited proteolysis (Inoue *et al.,* 1977), Ca^{2+} and phospholipids (Takai *et al.,* 1979) or phospholipids and phorbol esters (Castagna *et al.,* 1982). PKC is composed of a single polypeptide chain that has a regulatory domain in the NH_2-terminal and a catalytic domain at the COOH-terminal. There are three classes of PKC isoform (for review see Newton 1995a): cPKCs (conventional PKCs: PKCα, PKCβI, PKCβII and PKCγ), nPKCs (novel PKCs: PKCδ, PKCε, PKCη (L), and PKCθ), and aPKCs (atypical PKCs: PKCζ, PKCι and PKCλ). All PKC isoforms are activated by phosphatidylserine (an acidic phospholipid), however, the conventional and novel PKC isoforms require DAG, whilst cPKCs also require Ca^{2+} to bind to the C2 domain which contains a Ca^{2+}

binding motif for optimal activity (Ryves *et al.,* 1991; Newton, 1995b). DAG and phorbol esters recruit cPKCs to the plasma membrane by causing a dramatic increase in the affinity of the enzyme for the plasma membrane (Mosior and Newton 1996). As phorbol esters act like DAG, but are not metabolised they have been used extensively as constitutive activators of cPKCs and nPKCs (Gschewendt *et al.,* 1991).

PKC has been demonstrated to play an important role in ERK activation. Total inhibition of ERK activation (via the m1-AChR) has been shown in response to either prolonged PKC activation by phorbol ester (which down-regulates PKC), or direct inhibition of PKC (Gutkind 1998; Crespo *et al.,* 1994b; Hawes *et al.,* 1995; Rossomando *et al.,* 1989). When cPKCs or nPKCs are activated by phorbol ester treatment, or overexpression of a PKC isoform, ERK activation was induced (Cobb and Goldsmith 1995; Schönwasser *et al.,* 1998; Marais, *et al.,* 1998). Different PKC isoforms have been demonstrated to modulate ERK activity via growth factors (Cai *et al.,* 1997), phorbol esters (Mackenzie *et al.,* 1997) and hormones (Schönwasser *et al.,* 1998). There has also been evidence that the activation of ERK can occur concurrently in both a PKC-dependent and PKC-independent manner in response to agonist stimulation in macrophages (Qui and Leslie 1994).

As previously described, receptors that couple to G_q G-proteins, stimulate PI hydrolysis by PLC to yield IP_3 and DAG, which can lead to PKC activation (Nishizuka, 1982, Nishizuka 1984). DAG activates cPKC and nPKC isoforms, and PKCα has been shown to activate Raf-1 *in vitro* (Kolch *et al.,* 1993) and therefore activate the ERK pathway independently of Ras. CHO cells expressing the G_q-coupled m1-AChR were found to activate the ERK pathway, upon receptor stimulation in a PKC-dependent

manner, whereas in contrast, receptor stimulation of G_i-coupled α2A adrenergic receptor caused ERK activation in a PKC-independent manner (Hawes *et al.,* 1995). The same group later showed that m1-AChRs in CHO cells were coupled to G_o G-proteins in addition to G_q and activated Ras in a PKC-dependent manner (van Biesen *et al.,* 1996). Agonist-induced ERK activation was found to be inhibited upon PKC downregulation in cells expressing the m1-AChR but PKC downregulation had no effect on agonist-induced ERK activation in cells expressing the m2-AChR (Gutkind *et al.,* 1997). However, more recently, Wang *et al,* (1999) demonstrated the ability of m2-AChRs to couple to nonselective cation channels via stimulation of an atypical PKC (PKCζ). Collectively, these results demonstrate the importance of PKC in MAP kinase signalling, but that ERK activation in some cell lines is not entirely dependent on PKC.

1.5.4 c-Jun NH_2-terminal protein kinase (JNK)/Stress-activated protein kinase (SAPK) pathway

Initial studies identified the requirement for the transcription factor c-Jun to be dual phosphorylated on two serine residues in the N-terminal region of the protein for it to be activated. However, it was also noted that ERK1/2 was a poor activator of this transcription factor (Davis 1994). Activated JNK was found to bind to the delta domain of c-Jun (Dai *et al.,* 1995). Proteins that activated c-Jun were termed JNK and were initially identified as a response to stress factors, e.g.: exposure to U.V. light (Dérijard, *et al.,* 1994; Sluss *et al.,* 1994), osmotic shock (Galcheva-Gargova *et al.,* 1994), protein synthesis inhibitors (Kyriakis *et al.,* 1994), ceramide (Westwick *et al.,* 1995), and γ-radiation (Dérijard *et al.,* 1994, Kharbanda *et al.,* 1995). The activation of JNK and p38

by c-Abl and the subsequent inhibition in proliferation, in response to ara-C, a chemical that damages DNA has also been previously demonstrated (Kharbanda *et al.,* 1995). JNK has also been demonstrated to be activated by the inflammatory cytokines (Lee, *et al.,* 1994): interleukin-1β (Waskiewicz and Cooper, 1995) and tumour necrosis factor-α (Huang *et al.,* 1999), growth factors such as epidermal growth factor (Moriguchi *et al.,* 1995), in addition to stimulation of GPCRs by agonist (Coso, *et al.,* 1995a; Mitchell, *et al.,* 1995; Coso, *et al.,* 1996; Wylie *et al.,* 1999). JNK has been reported to phosphorylate transcription factors, other than, c-Jun such as ATF2 (Gupta *et al.,* 1996) and Elk-1 (Whitmarsh *et al.,* 1995), which then result in the regulation of gene transcription.

There are ten JNK isoforms which are originated from three homologous genes; *jnk1, jnk2 and jnk3.* The transcripts of these genes are alternatively spliced to form four JNK1 isoforms, four JNK2 isoforms and two JNK3 isoforms (Gupta *et al.,* 1996; Kyriakis, *et al.,* 1995). JNK was shown to be activated by a dual phosphorylation on both a threonine and tyrosine residue in the sequence Thr-Pro-Tyr in kinase domain VIII (Dérijard *et al.,* 1994). This is achieved by upstream kinases, JNKK1 (JNK kinase)/MKK4 (MAPK kinase 4) (Sanchez *et al.,* 1994; Lin *et al.,* 1995) and JNKK2/MKK7 (MAPK Kinase 7) (Lu *et al.,* 1997; Tournier *et al.,* 1997; Yao *et al.,* 1997; Foltz, *et al.,* 1998; Tournier *et al.,* 1999;). JNKK2 when fused to JNK1 has also been recently demonstrated to act as a constitutively active c-Jun kinase (Zheng *et al.,* 1999). This is itself activated by further upstream kinases as shown in Fig. 1.4. The first study identified MEKK1 as activating SEK1 (MKK4) and subsequently JNK (Yan *et al.,* 1994). Subsequently, other MAPKKK proteins have been identified; MEKK1 (Xu, *et al.,* 1997), MEKK2, MEKK3

(Blank *et al.*, 1996), MEKK4 (Gerwins, *et al.*, 1997), TAK1 (Yamauchi *et al.*, 1995) and ASK1 (Ichijo *et al.*, 1997) all activate MKK4 and/or MKK7. MEKK1 is a protein of 195kDa, that when overexpressed has been shown to induce apoptosis (Johnson *et al.*, 1996). However, more recently, transfected MEKK1 has been shown to be required for selective JNK activation and seems to be important in protecting a cell from apoptosis (Yujiri *et al.*, 1998). For example, MEKK1 is not involved in JNK activation by anisomycin, but is required for hyperosmolarity. MEKK1 has also been shown to bind directly to JNK *in vitro* (Xu and Cobb, 1997). MEKK3 is an important protein that was isolated and found to directly induce both the ERK and JNK pathways, but not the p38 pathway (Ellinger-Ziegelbauer *et al.*, 1997), which compares to results demonstrated using MEKK2 and MEKK3 expressed in HEK293 cells (Blank *et al.*, 1996).

The mixed-lineage kinases (MLK), MLK3 (Rana *et al.*, 1996; Teramoto, *et al.*, 1996;) and MLK2 (Hirai, *et al.*, 1997) have also been demonstrated to activate the MAPKK level. MLK2 and MLK3 are themselves Ser/Thr protein kinases, that are structurally similar to tyrosine kinases, in that they contain a SH3 domain and have been demonstrated to interact with activated Cdc42 and Rac (Nagata, *et al.*, 1998).

Once activated, the JNK protein kinase phosphorylates two serine residues, Ser^{63} and Ser^{73}, in the NH_2 terminus of c-Jun (Pulverer *et al.*, 1991, Smeal *et al.*, 1991), causing an increase in c-Jun activity. Activating protein-1 (AP-1) is composed of dimeric complexes formed by components of the Jun and Fos families. The AP-1 complex is responsible for mediating the expression of the immediate-early genes in response to external stimulation of the cell and has been shown to be activated by phosphorylation of Jun and Fos (Hunter and Karin 1992) and is essential for proliferation and differentiation.

Therefore, activation of JNK has a regulatory role in the level of immediate-early gene expression in the cell.

As previously discussed, there is emerging evidence for the importance of scaffold protein in the MAP kinase pathways (Garrington and Johnson, 1999). JIP-1 (JNK interacting protein-1) was isolated by yeast two hybrid analysis (Dickens *et al.,* 1997), and it was shown that when overexpressed, there was an inhibition of JNK-mediated gene expression due to the retention of JNK in the cytoplasm. JIP-1 was then demonstrated to be a mammalian scaffold protein for the JNK pathway (Whitmarsh *et al.,* 1998) that selectively bound JNK (MAPK), MKK7 (MAPKK) (but not MKK4) and the mixed-lineage kinases, MLK3 and DLK (MAPKKK). Therefore there is increasing evidence for a group of proteins that can mediate the association of pathway components to increase specificity. This potential for specificity is discussed as a potential reason for selective stress-dependent JNK isoform activation. Butterfield *et al,* (1999), observed that different JNK isoforms have specific activations, dependent on which cell line they are in. For example, they report that UV activated JNK1α and JNK2β in small-cell lung cancer cells, but not in PC12 cells.

1.5.4.1 G-protein Coupled Receptor-Regulation of JNK

Coso *et al,* (1995a) showed that the muscarinic agonist, carbachol, was able to induce mRNA expression of the nuclear proto-oncogenes, *jun* and *fos* in NIH 3T3 cells that contained the m1-AChR, independently of PKC or ERK activation. They showed that the response was due to JNK activation, which was independent of TK activation, providing the first evidence for GPCR-mediated JNK activation. Since then JNK

activation has been demonstrated in response to activation of a wide variety of GPCRs, examples of which are thrombin receptors (Shapiro *et al.,* 1996) and α_1 adrenergic receptors (Ramirez *et al.,* 1997). Although, initial hypotheses seemed to link Ras to JNK activation, this was difficult to resolve, as when receptors were stimulated that were known to activate Ras, there was little resulting JNK activation, for example, it has now been shown that activation of PDGF receptors (a known Ras activator) does not activate JNK (Coso *et al.,* 1995a). In a separate study, Coso *et al.,* (1996) demonstrated that overexpression of $\beta_1\gamma_2$ subunits was able to activate the JNK pathway in addition to $\beta\gamma$-sequestering agents inhibiting JNK activation by both m1- and m2-AChR stimulation. However, overexpression of the α_{12} subunit had no effect on agonist-mediated JNK activation by either of the two muscarinic receptors. Therefore, the data suggest that this α-subunit has no role in JNK activation, however, there have been other groups whose studies suggest that $G_{\alpha 12}$ is involved in muscarinic receptor-mediated JNK activation in COS-1 cells (Prasad *et al.,* 1995) and $G_{\alpha q}$ is involved in muscarinic receptor-mediated JNK activation in PC12 cells (Heasley *et al.,* 1996). Further evidence for the role of G_α-subunits in JNK activation is the observation that overexpression of $G_{\alpha q}$ subunits activates apoptosis, which has been shown to be caused by JNK stimulation (Voyno-Yasenetskaya *et al.,* 1996). In addition to this work, studies in which constitutively active α_{12} and α_{13} subunits have been examined, it was found that they were able to activate JNK via a Ras (Prasad *et al.,* 1995; Collins *et al.,* 1996), Rac (Mitsui, *et al.,* 1997; Collins *et al.,* 1996) or a Cdc42 (Voyno-Yasenetskaya *et al.,* 1996) pathway (Coso *et al.,* 1995b). However, interestingly, ERK was not activated even though JNK activation was via a Ras-dependent mechanism. Further work demonstrating the role of

Rac1 and Cdc42 in the activation of JNK by IL-1β (Minden *et al.*, 1994b), showed a distinct pathway when compared with GPCR-mediated ERK activation. These GTP-binding proteins are related to Ras, and were initially identified as proteins that control the actin cytoskeleton structure (Ridley *et al.*, 1992; Kozma *et al.*, 1995; Nobes and Hall, 1995) [for a summary see Fig. 1.7].

The first evidence of a G_i-coupled receptor activating JNK was provided by another muscarinic receptor, the m2-AChR, where it was shown that pre-treatment of Rat1a cells expressing the m2-AChR with pertussis toxin, completely inhibited agonist-induced JNK activation (Mitchell *et al.*, 1995). They also reported that chelation of Ca^{2+} with BAPTA (an intracellular calcium chelator) completely inhibited both m1-AChR and m2-AChR-mediated JNK activation, however, there was no effect on m2-AChR-mediated ERK activation. Thus, in Rat1a fibroblasts, it appears that muscarinic receptor activation of JNK is Ca^{2+}-dependent, whereas ERK activation is not.

JNK1 activation in endothelian-stimulated Rat-1 fibroblasts is inhibited by PKC (Cadwaller *et al.*, 1997), although this contrasts with other reports that activation of PKC stimulates JNK activity (Zohn *et al.*, 1995). However, they report that JNK activation was not affected by TPA-induced down-regulation of PKC, suggesting the involvement of atypical PKC isoforms (which are unaffected by TPA treatment). Also in NIH3T3 cells, activation of the JNK pathway by agonist-stimulation of the m1-AChR, was found to be independent of PKC (Coso *et al.*, 1995a).

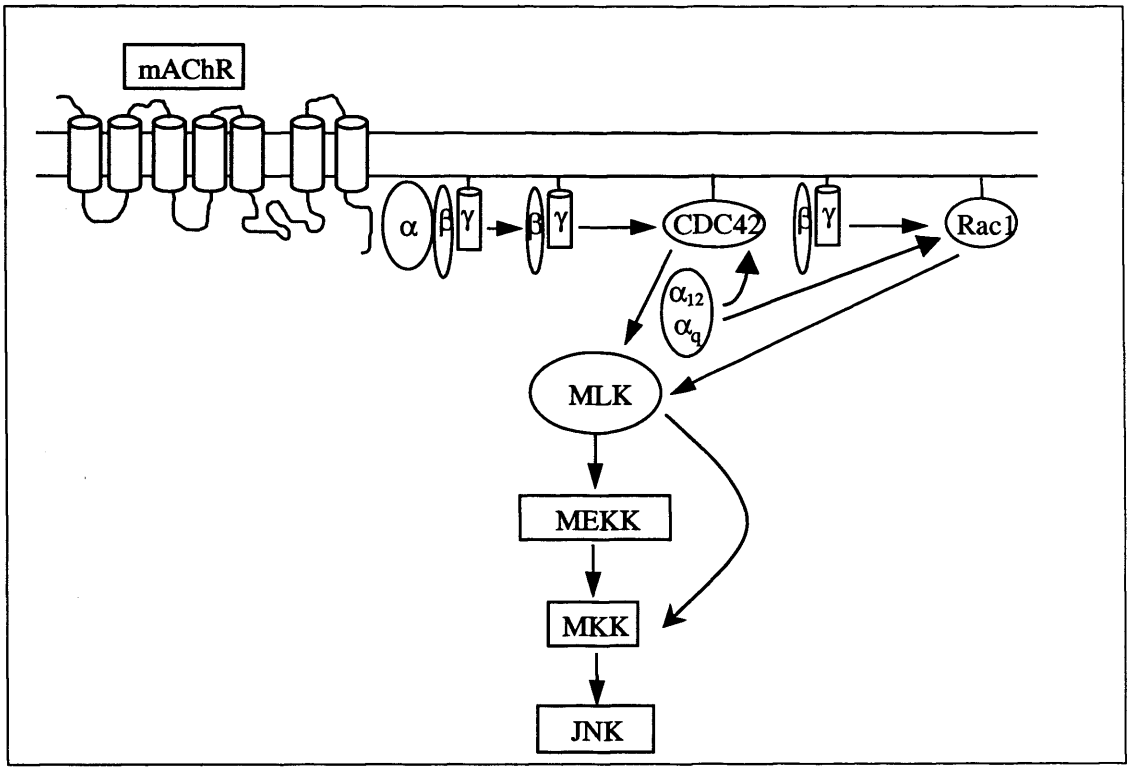

Figure 1.7 Possible Mechanism for Muscarinic Receptor Activation of the JNK Pathway.

Stimulation of the muscarinic receptor leads to the activation of CDC42 and also Rac, however, there is emerging evidence for the role of α_{12} subunits in the activation of CDC42 and Rac. These proteins then activate the mixed-lineage kinase MLK and the subsequent JNK pathway. α_q subunits have also been implicated to have a role in the activation of the JNK pathway, but by, as yet, an unknown mechanism, but presumably involving Ca^{2+} and/or PKC.

1.5.5 p38 MAPK pathway

The most recent member of the MAP kinase family to have been identified is the p38 MAP kinase (Han *et al.,* 1994). It is a mammalian homologue of the high osmolarity glycerol response (HOG-1) kinase (Davis 1994) identified in *S. cerevisiae* (Han *et al.,* 1994, Raingeaud, *et al.,* 1995) and it is also known as CSBP (CSAID-binding Protein) and RK (Reactivating Kinase). The stress-activated protein kinase, p38 was initially identified by it being activated by lipopolysaccharides, which cause cellular stress by releasing TNF-α and IL-1β (Minden and Karin, 1997, Ellinger-Ziegelbauer *et al.,* 1997). There are to date, four isoforms of p38 MAP kinase: p38α (Lee *et al.,* 1994, Raingeaud *et al.,* 1995), p38β (Jiang *et al.,* 1996), p38γ (Lechner *et al.,* 1996, Cuenda *et al.,* 1997a) and p38δ (Wang *et al.,* 1997, Goedert *et al.,* 1997). These are activated by a range of physical and chemical cellular stresses, including, UV-radiation (Raingeaud *et al.,* 1995), heat stress (Raingeaud *et al.,* 1995), osmotic stress (Shapiro and Dinerallo, 1995) and by the pro-inflammatory cytokine, interleukin-1β (Kyriakis and Avruch 1996). p38 MAP kinase is activated by a dual phosphorylation on a TGY motif in subdomain VIII (Raingeaud *et al.,* 1995; Doza *et al.,* 1995) of the catalytic domain by the upstream kinases MKK3, (Dérijard *et al.,* 1995), [it was later determined that MKK3 activates only p38α and p38γ (Enslen *et al.,* 1998)] and MKK6 which activates p38α, p38β2 and p38γ (Raingeaud *et al.,* 1996; Enslen *et al.,* 1998). However, MKK4 does not appear to activate any of the p38 isoforms in vivo, but it can activate p38 *in vitro* (Lin *et al.,* 1995; Dérijard *et al.,* 1995). Little is known about the upstream kinase of MKK3 and MKK6, however, TAK-1 was demonstrated to be an upstream activator of MKK3 and MKK6 (Moriguchi *et al.,* 1996). MEKK3 was also demonstrated to activate MKK3 *in vivo*

(Deacon and Blank, 1997) and MKK6 *in vitro* (Deacon and Blank 1999). Dominant negative studies have shown that activation of Rac1 (Coso *et al.,* 1996; Minden *et al.,* 1995) and Cdc42 (Li and Smithgall, 1998) activate p38 MAP kinase activity, as well as the mixed lineage kinases (Hirai *et al.,* 1996, Fan *et al.,* 1996). p38 MAP kinase has been demonstrated to phosphorylate and consequently activate transcription factors, including CHOP (GADD153) (Freshney *et al.,* 1994, Wang and Ron, 1996), ELK-1 (Cavigelli *et al.,* 1995), ATF-2 (Gupta *et al.,* 1996). There is also evidence that stress responses and GPCRs are not exclusively responsible for p38 activation. Pandey *et al.,* (1999), recently discovered that the tyrosine kinase, PYK2 was important in p38 activation. Therefore, p38 MAP kinase, being a stress related protein kinase, is a distinct MAP kinase enzyme to JNK, but there is the potential for certain components of the pathways to overlap at the level of MAPKKK, but not MAPKK. p38 MAP kinase and JNK have been implicated in programmed cell death, or apoptosis, as demonstrated in the absence of NGF in differentiated PC12 cells (see section 1.7.3) (Ruckenstein *et al.,* 1991; Xia *et al.,* 1995).

Studies of the role of p38 MAP kinase have been aided by the discovery that pyridinyl imidazole compounds act as specific inhibitors of the p38 MAP kinase pathway. SB-203580 is one of these compounds, however it has been previously reported to inhibit only p38 MAPKα and p38 MAPKβ but not p38 MAPKγ or p38 MAPKδ (Lee *et al.,* 1994; Cuenda *et al.,* 1995; Goedert *et al.,* 1997; Kumar *et al.,* 1997). However, Clerk and Sugden (1998) reported that SB-203580 inhibited JNK1 in adult perfused hearts and neonatal ventricular myocytes, so the specificity of the compound for p38 MAP kinase is not absolute.

1.5.5.1 G-protein-Coupled Receptor regulation of p38

In comparison with the other members of the MAP kinase family discussed here, the current understanding of p38 activation is not as well understood. The first demonstration of GPCR-mediated p38 activation was in HEK293 cells expressing muscarinic receptors and β-adrenergic receptors (coupled to G_s G-proteins) in a study by Yamanuchi *et al.*, (1997). They showed that stimulation of the m1- and m2-AChRs (and also β-adrenergic receptors) led to p38 activation. In addition to this, they also demonstrated that m2-AChR-mediated activation was completely inhibited when $G_{\alpha o}$ was co-expressed, but m1-AChR-mediated activation was only partially attenuated. They also investigated receptor-independent activation of p38 and found overexpression of $G_{\beta\gamma}$ or a constitutively activated $G_{\alpha 11}$ was also able to activate p38. However, overexpression of $G_{\alpha s}$ or $G_{\alpha i}$ was not able to activate p38, indicating that p38 activation via the m2-AChR was mediated by $G_{\beta\gamma}$ subunits, in contrast to the activation of p38 by m1-AChR stimulation, where the data suggest that both the $G_{\beta\gamma}$ and $G_{\alpha q/11}$ subunits are important.

1.6 Roles of MAPK pathway

There are many substrates for the MAP kinases as may be expected based on their ability to regulate a number of cellular responses including hypertrophy, differentiation and mitogenesis. This is achieved by ERK phosphorylating either intracellular enzymes (e.g. p90 ribosomal protein S6 kinase) or transcription factors (e.g. Elk-1). The stress-activated protein kinases have been demonstrated to have a role in apoptosis, immune responses and oncogenic transformation. MAPKs generally have also been shown to

phosphorylate transcription factors, such as: ERK1/2; Elk-1 c-fos, egr-1, junB and c-myc (Cavigelli *et al.*, 1995; Hodge *et al.*, 1998; Gupta and Davis, 1994), JNK; c-Jun, Elk-1 and ATF-2 (Derijard *et al.*, 1994; Whitmarsh *et al.*, 1995; Gupta *et al.*, 1996), and p38; ATF-2, Elk-1, SAP1a (Gupta *et al.*, 1995; Freshney *et al.*, 1994).

1.7 Transcriptional regulation by the MAP kinases

The ERKs are proteins that regulate the induction of specific genes (Cavigelli *et al.*, 1995) by phosphorylating transcription factors e.g. Elk-1. Elk-1 is a member of the Ternary Complex Factor (TCF) family of transcription factors (Triesman 1994) and has been shown to be activated by growth hormone (Liao, J. *et al.*, 1997). In unstimulated cells, ERKs are mainly located in the cytoplasm, however, upon activation, they translocate to the nucleus (Chen *et al.*, 1992; Chen *et al.*, 1996; Fukuda *et al.*, 1997). ERK2 is retained in the cytoplasm to a greater extent that ERK1, where is it associated with the cytoskeleton (Reska *et al.*, 1995), although a more recent study has demonstrated that phosphorylated ERK2 forms homodimers and this assists in ERK being translocated into the nucleus in REF52 cells (Khokhlatchev *et al.*, 1998).

As discussed, JNK binds c-Jun and this interaction is believed to regulate c-Jun phosphorylation (Hibi *et al.*, 1993; May *et al.*, 1998a). This interaction occurs via multiple binding sites *in vivo*, and c-Jun can then phosphorylate its substrates without the requirement for dissociation from the JNK-c-Jun complex (May *et al.*, 1998b). It is an important pathway in the activation of proteins that form the AP-1 complex that is a transcription factor and can therefore regulate gene activation. AP-1 is a family of transcription factors that can be composed of either homodimers or heterodimers of

members of the Jun (e.g. c-jun, junB and junD), or Fos (c-fos, fosB, fra-1) family or members of the family of activating transcription factor (ATF2, ATF3) ((Vogt and Bos 1990; Angel and Karin, 1991; for review see Karin *et al.,* 1997 and Karin 1995)). Jun proteins are able to heterodimerise with Fos, ATF and other Jun proteins. ATF proteins are also capable of heterodimerisation, but Fos proteins are unable to form heterodimers with ATF proteins (Ziff, 1990). There are two distinct response elements (which are both composed of a palindromic sequence) that will bind to the gene depending on which AP-1 dimer is formed. For example, the TRE (phorbol 12-O-tetradecanoate 13-acetate (TPA)-responsive element) is composed of the sequence TGACTCA and is bound by Jun-Jun and Jun-Fos dimers, in contrast, Jun-ATF and ATF-ATF dimers, bind to the CRE (c-AMP-responsive element) which is composed of the sequence TGACGTCA (Hai and Curran, 1991). In addition, however, the JNK and p38 pathways can also activate c-fos transcription by activating the serum response element (SRE) via activation of the ternary complex factor (Dérijard *et al.,* 1994, Whitmarsh *et al.,* 1997). Indeed in NIH 3T3 cells expressing the m1-AChR it has been shown that the α–subunit of $G\alpha_{12}$ strongly induces the SRE, potentially via JNK activation (Fromm *et al.,* 1997). There is evidence that the length of JNK activation is important in determining if AP-1 is stimulated. In studies carried out with tumour-necrosis-factor-α, prolonged JNK activation was required for stimulation of AP-1 activity (Westwick *et al.,* 1994). To demonstrate that JNK is not the sole activator of the c-Jun promoter, it has been demonstrated that stimulation of the m1-AChR in NIH 3T3 cells causes c-jun promoter expression independently of MEKK-JNK activation (Coso *et al.,* 1997). They suggested that MEF2 (myocyte enhancer family) was able to mediate the c-jun promoter,

independently of JNK via stimulation of GPCRs.

The role of p38 in the regulation of gene expression is less well understood than for the other stress-activated protein kinase, however known substrates for p38 phosphorylation are ATF-2 (Dérijard *et al.,* 1995), Max (Zervos *et al.,* 1995) CHOP (Freshney *et al.,* 1994; Rouse *et al.,* 1994) and Elk-1 (Cavigelli *et al.,* 1995; Whitmarsh *et al.,* 1995; Gille *et al.,* 1995, Price *et al.,* 1996.).

1.7.1 Proliferation

It has been known for some time that mitogenic signals can be transduced from cell surface receptors of TKs (Pelach and Sanghera, 1993; Schlessenger 1993). It was first demonstrated that ERK1 and ERK2 could cause cell proliferation in Rat-1 fibroblasts in response to the growth factor EGF and LPA (Cook *et al.,* 1993). Although there is less known about the role of GPCR-mediated mitogenic signalling, the first evidence for muscarinic-mediated signalling was by Gutkind *et al.,* (1991) whereby, m1-, m3- and m5-AChRs transformed NIH3T3 cells when the receptors were continuously activated. However, it has also been shown that in NIH3T3 cells, the IP_3-linked receptor-subtypes, m1-, m3- and m5-AChRs can transform cells, whereas, the m2- and m4-AChRs (which inhibit adenylyl cyclase, and therefore reduces cAMP production) are not able to transform NIH3T3 cells (Gutkind *et al.,* 1991; Burstein *et al.,* 1997). This mitogenic response appears to be cell specific, as in 1321N1 astrocytoma cells, activation of the m3-AChR caused PLC stimulation, but was unable to induce a mitogenic response (Heller Brown *et al.,* 1997). They do concede, however, that the numbers of muscarinic receptors present was low (<50 fmol/mg protein) and suggest that the receptors are

required to be present at a much higher density; Gutkind *et al.,* (1991) had a receptor density of ~400 fmol/mg protein in the NIH3T3 cells. Inhibition of the ERK pathway has been shown to inhibit cell proliferation in response to growth factors (Pages *et al.,* 1993), and more recently, constitutive activation of the ERK pathway has been shown to induce tumorogenesis (Mansour *et al.,* 1994; Schlessenger, 1993). There is an apparent conflict in that both G_i and G_q-coupled (m1-, m2- and m3-AChR subtypes) have been shown to activate the ERK pathway (Gardner *et al.,* 1994; Coso *et al.,* 1995a; Wylie *et al.,* 1999) and ERK can induce cell proliferation, but G_i-coupled muscarinic receptors do not seem to induce mitogenesis. A possible reason to account for this difference is whether there is co-activation of the JNK and p38 pathways together with ERK. Although some groups have demonstrated JNK activation by stimulation of m2-AChRs (Mitchell *et al.,* 1995), other groups have reported a lack of JNK activation, including work presented in this Thesis (Coso *et al.,* 1995; Wylie *et al.,* 1999).

In rat ventricular cardiomyocytes, the m2-AChR is the most abundant muscarinic subtype and inhibits adenylyl cyclase (Caulfield, 1993). Pre-treatment with PTX attenuated carbachol-induced ERK activation, consistent with G_i-mediated ERK activation via $\beta\gamma$ subunits. Ventricular myocytes are non dividing cells (i.e. they are post-mitotic), and it has been demonstrated that activation of ERK by different receptors can result in different effects upon gene transcription in these terminally differentiated cells. ERK1 and ERK2 were activated by both the m2-AChR and the α1-adrenergic receptor (α1-AR) by stimulation with carbachol (CCh) or phenylephrine (PE) respectively. However, activation of ERK via the α1-AR was able to induce ANF (atrial natriuretic factor), which is characteristic of the molecular and phenotypic changes

associated with cardiac hypertrophy. In contrast to that, stimulation of the m2-AChR with CCh had no effect on hypertrophy. These results demonstrate that activation of the MAP kinase pathway alone is insufficient for hypertrophy (Post *et al.*, 1996). In contrast to this, Shapiro *et al.*, 1996 demonstrated that stimulation of endothelian and thrombin receptors caused proliferation of airways smooth muscle, and also activation of JNK, in a Ras-independent manner, suggesting a potential for the relationship of JNK and ERK pathway activation in cell proliferation. This demonstrates the specificity of proliferative responses that are dependent on the relevant agonist and tissue.

1.7.2 Differentiation

In embryogenesis, certain precursor cell types will proliferate until they stop dividing and undergo differentiation. Such cells can be found in the development of the pituitary gland (for review see Dasen and Rosenfeld, 1999), formation of the liver (Darlington 1999), fat cells (Wu *et al.*, 1999) and muscle development (Naya and Olson, 1999). There have been some recent studies that have demonstrated the role of the MAP kinases in muscle cell differentiation (for review see Naya and Olson, 1999). In C2C12 myoblasts, the MAP kinase phosphatase MKB-1, inactivates ERK2, by reducing cyclin D1 expression and causing exit from the cell cycle (Bennet and Tonks, 1997). However at later stages of differentiation, MKB-1 is down-regulated and ERK activation appears to maintain the myogenesis. In contrast however, the p38 MAP kinase has been shown to have a stimulatory role in L8 myoblast differentiation, as the pyrimidazole SB-203580, an inhibitor of p38α and p38β, inhibits myogenesis (Zetser *et al.*, 1999). However, as in the ERK regulation, constitutive activation of p38 or MKK6 maintains the myogenic response. These data suggest that the timing of the activation of the MAP kinase

pathway is important. In the early stages of differentiation (in muscle cells), ERK inhibition induces cells to differentiate, whereas activation of p38 MAP kinase stimulates differentiation. But later in differentiation, ERK activation or continued p38 activation maintains myogenesis.

There have however been studies to suggest that the duration of the ERK response alone is sufficient for the cell to differentiate or proliferate (for review see Marshall, 1995). In PC12 cells, stimulation of the cells with NGF caused a sustained level of activated Ras, but stimulation with EGF caused a transient activation of Ras (Muroya *et al.,* 1992). Further studies demonstrated that stimulation of PC12 cells with NGF resulted in a sustained ERK activation, whereas, EGF, resulted in a transient activation of ERK (Healey and Johnson, 1992; Traverse *et al.,* 1992; Nguyen *et al.,* 1993). Therefore the conclusion from these results may be that sustained ERK activation results in a cell undergoing differentiation. Further evidence to support this conclusion comes from data also in PC12 cells, using mutants of the MEK1 phosphorylation sites. It was found that mutating these sites caused a reduction in ERK activation and also blocked differentiation (Cowley *et al.,* 1994). As previously discussed, upon activation of ERK, ERK translocates to the nucleus (Chen *et al.,* 1992). However, there is further evidence to suggest that only with sustained ERK activation, does it translocate to the nucleus (Traverse *et al.,* 1992, Nguyen *et al.,* 1993; Dikic *et al.,* 1994). It therefore follows that with sustained ERK activation, the ERK translocates to the nucleus and can induce gene transcription, whereas, with transient ERK activation, there is no ERK translocation and therefore there is no effect on gene transcription. However, this model appears to be a cell specific phenomenon, the reverse has also been seen in other cell types. In fibroblasts, sustained ERK activation has been shown to cause cell proliferation and not

differentiation (Meloche *et al.*, 1992; Mansour *et al.*, 1994; Cowley *et al.*, 1994).

1.7.3 Apoptosis

Apoptosis was first described in 1970, when it was distinguished from necrosis (a condition where the cell is depleted of energy and protein synthesis is terminated) by Kerr *et al.*, (1972). Apoptosis is programmed cell death or cell suicide, which is important in a number of pathological disease states such as the formation of tumours, immune responses and neurodegeneration (for review see Kinloch *et al.*, 1999). Common features of apoptosis in cells are cell shrinkage, nuclear condensation and DNA fragmentation (Kerr *et al.*, 1972; Wyllie, 1980). It is thought that a cell is always in a state ready for apoptosis, but extracellular signals binding to receptors e.g. integrins, growth factors, cytokines and agonists for GPCRs maintain the survival of the cell and prevent it from entering apoptosis (Wyllie, 1980).

The ERK, JNK and p38 MAP kinase pathways, have all been implicated in apoptosis (Xia *et al.*, 1995). ERK and JNK activation has been demonstrated to have an important role in the induction of apoptosis in sympathetic neurons (Ham *et al.*, 1995) and T lymphocytes (Chen *et al.*, 1996). In differentiated PC12 cells, the removal of NGF increases JNK and p38 activity, prior to the induction of apoptosis, leading to the suggestion that JNK and p38 activation are required for apoptosis (Xia *et al.*, 1995). However, JNK and p38 have been shown to have opposing roles in apoptosis where IGF-1 (Insulin-like Growth Factor-1) prevents apoptosis by activating p38, whilst inhibiting JNK activation in the neuronal cell line SH-SY5Y (Cheng and Feldman, 1998). A further example of apoptosis via a p38 pathway is the recent report that overexpression of

constitutively active MKK6 inhibits anisomycin-induced apoptosis in cardiac myocytes in a p38-dependent manner (Zechner *et al.*, 1998). Supporting evidence for the differences in the role of the MAP kinases is the study that demonstrate in Jurkat cells, the pyridinyl imidazole SB202190 (an inhibitor of p38α (Lee *et al.*, 1994) and p38 β (Jiang *et al.*, 1996)) induces apoptosis by inhibiting p38β (Nemoto *et al.*, 1998). However, they also showed that expression of p38α induced cell death, suggesting a complicated and cell specific role for the MAP kinases in apoptosis.

1.8 Aims

The aim of the work was to investigate receptor-mediated ERK and JNK signalling in CHO cells that had been stably transfected to express the m2-AChR or the m3-AChR. The initial aims were to investigate the time-dependence of ERK and JNK activation to MCh and the concentration-response to MCh of ERK and JNK activation. The m2-AChR and m3-AChR were chosen to be investigated because they are coupled to different G-proteins are therefore different effector systems. As described previously, the m2-AChR is coupled to G_i G-proteins which act to inhibit adenylyl cyclase and reduce the production of cAMP, while the m3-AChR is coupled to PLC activation, Ca^{2+} mobilisation and PKC activation. Due to this difference in effector coupling, the roles of Ca^{2+}, PKC and PI3K in receptor-mediated ERK and JNK activation were investigated.

Chapter 2

Methods

2.1 Tissue Preparation

2.1.1 Cell Culture

Chinese Hamster Ovary cells (CHO-K1) stably expressing, the cDNA of the m3-AChR (CHO-m3) or the cDNA encoding human m2-AChR receptor (CHO-slm2) were obtained from Dr. N. Buckley and Dr. S. Larenzo, respectively (National Institute for Medical Research, Mill Hill, London, U.K.). CHO cells were cultured in Minimal Essential Medium α (MEMα) supplemented by 10% (v/v) newborn calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2.5μg/ml amphoteracin B. The CHO cells were routinely passaged using trypsin-EDTA.

Initial experiments determined the effects of incubating cells with methacholine in the presence of freshly gassed Krebs-Henseleit Buffer (KHB) compared to cell culture media so that future experiments could be performed under conditions in which the buffer constituents could be easily modified. Experiments were limited to measurement of JNK activity in CHOm3-AChR cells. The results demonstrated that the basal level of c-Jun phosphorylation was constant under both conditions, whereas the level of maximal activation increased from 4 fold over basal in the presence of media compared to 11 fold over basal in the presence of that KHB respectively (Figure 2.1).

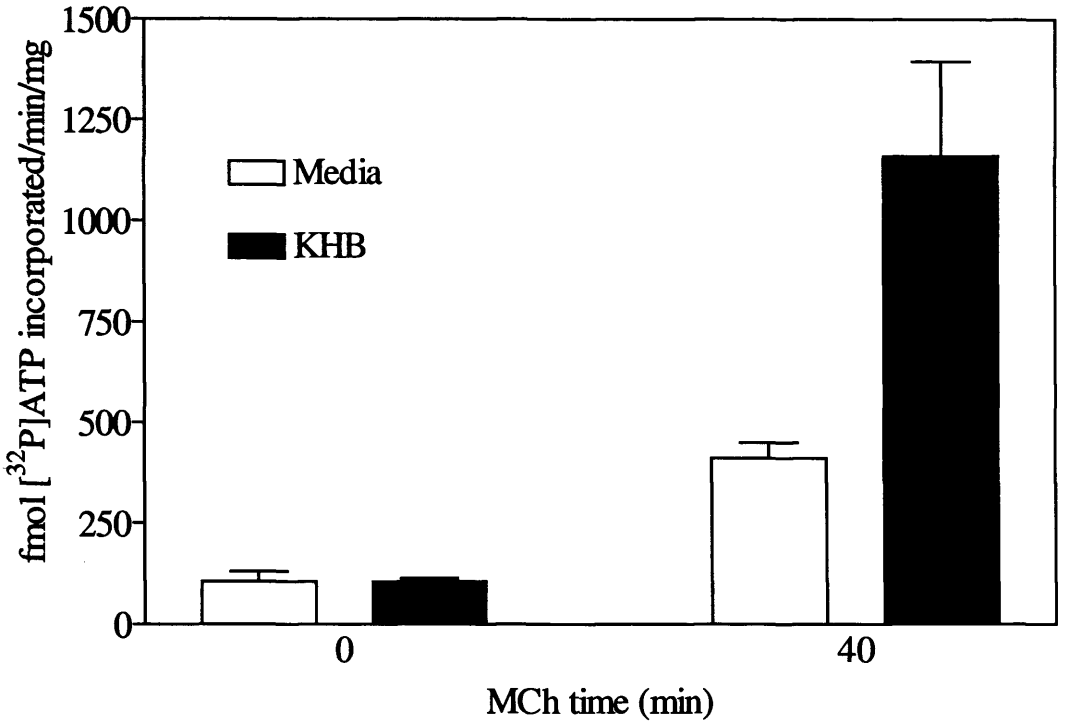

Figure 2.1 Effect of a 30 min pre-incubation with freshly gassed KHB on JNK activation in CHO-m3 cells.

CHO-m3 cells were incubated for 30 min in the presence of freshly gassed KHB or fresh MEMα media followed by a 30 min stimulation with 100 μM MCh. Lysates containing 200 μg of protein were assayed for JNK activity by GST-Jun phosphorylation (see section 2.2.3.2). Data represent means ± S.E.M. of three separate experiments.

2.2 *in vitro* Kinase Assays

2.2.1 Preparation of Cells for Stimulation

CHO cells were grown to confluency in a 175 cm^2 flask, washed once in 10 ml sterile HBS and then gently lifted from the flask by a 5 min treatment with 5 ml per flask trypsin/EDTA. The cells were the spun at 1500 r.p.m. for 3 min and the pellet resuspended in 25 ml MEMα supplemented as described above. A 6 well plate had 2 ml of media added to each well, then 1 ml of resuspended cells, was added to each well. Cells were then grown to confluency in 6 well plates, (surface area, 9.6 cm^2/well). Prior

to stimulation, the medium was removed and the cells washed once in 3 ml freshly gassed (95% CO_2, 5% O_2) Krebs-Henseleit Buffer (KHB, 0.9% NaCl/10 mM Hepes, pH 7.45, 1.15% KCl, 3.82% $MgSO_4$, 2.11 % KH_2PO_4, 0.11 mM $CaCl_2$, 11 mM glucose) and replaced with 3ml of freshly gassed KHB. Following incubation for 30 min at 37°C, the cells were stimulated with 100 μM methacholine for the indicated times at 37°C. Variations to this procedure are given in the text.

2.2.2 ERK1/2

Following agonist stimulation, cells were washed once in ice cold PBS (140 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4) and scraped into 500 μl of ice cold lysis buffer (20 mM Tris-HCl, pH 8, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM phenylmethylsulphonylfluoride, 2 mM sodium orthovanadate, 20 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM dithiothreitol). The lysates were cleared by centrifugation at 20,000 r.p.m. for 10 min at 4°C and split into two 200 μl aliquots for assays of ERK1 and ERK2. Rabbit anti-ERK1 and anti-ERK2 polyclonal antibodies (ERK1 (C-16) sc-93, ERK2 (C-14) sc-94, Santa Cruz) were added to lysates at a dilution of 1:100 and incubated on ice for 90 min. 70 μl of a 15% slurry of protein A sepharose (Pharmacia-LKB) was then added and the samples were rolled at 4°C for 90 min. The beads were retained by centrifugation and washed twice in 200 μl of lysis buffer and twice in 200μl of kinase buffer (20 mM Hepes, pH 7.2, 20 mM β-glycerophosphate, pH 7.2, 10 mM $MgCl_2$). To initiate the reaction, beads were resuspended in 40 μl of kinase buffer containing 1 μCi [γ-^{32}P]ATP, 20 μM unlabelled ATP and 200 μM of a synthetic peptide substrate corresponding to amino acids of 662-681 of the epidermal growth factor receptor (Sequence; RRELVEPLTPSGEAPNQALL. (PNACL) Gardner *et al.*,

(1994)) as the substrate and incubated for 20 min at 30°C. The reaction was terminated by the addition of 10 µl 25% TCA and the samples were spun for 2 min at 14,000 r.p.m. after which 40 µl of sample was blotted onto p81 Whatman chromatography paper. The blots were then washed four times in 400 ml of 0.5% orthophosphoric acid (five min per wash), followed by a final wash for 2 min in 400 ml of acetone. The papers were then allowed to dry at room temperature and placed in scintillation vials with 5 ml Scintillation Plus fluid and counted on a Packard Liquid Scintillation Analyser for 3 min.

2.2.3 c-Jun Kinase

2.2.3.1 Production of GST-c-Jun Fusion Protein

The activity of c-Jun kinase was assayed from cell lysates by affinity purification using a GST-c-Jun$_{(1-79)}$ fusion protein coupled to glutathione-Sepharose (Gupta et al., 1996), prepared by the following protocol. The GST-c-Jun fusion protein (a kind gift from Dr. Roger J. Davis; Gupta *et al.,* 1996) was produced using the GST (Glutathione S-transferase) Gene Fusion System (Pharmacia Biotech), which allows, in *E. coli,* expression and purification of fusion proteins. The system uses the pGEX plasmid vector in which contains the lac I_q gene (to induce expression), a Amp^r gene (for selection) and the GST gene upstream of the multiple cloning site (MCS) into which the c-Jun encoding amino acids 1-79 gene has been inserted. Glycerol stocks of *E. coli* (BLC21(DE3)LysS) transformed with the pGEX-c-Jun plasmid were stored at -80°C. To 10 ml of sterile Luzia Broth (LB), (10 g/1 tryptone, 5 g/1 yeast extract, 170 mM NaCl), containing 50 µg/ml ampicillin, a scrape of the GST-c-Jun bacterial stock was added and incubated overnight on a shaker at 37°C. The following day, the culture was added to 500 ml of LB containing 50 µg/ml ampicillin and left to grow at 37°C for approximately

3 hours until the OD, measured at 600 nm, reached 0.5 units (on a UV-1201 spectrophotometer (Shimadzu). The expression of GST-c-Jun was induced by incubation with 1 mM IPTG (isopropyl P-D-thiogalactopyranoside) for 3 hours at 30°C shaking. The culture was then centrifuged for 10 min at 8,000 r.p.m. and the bacterial pellet resuspended in 5 ml PBS, containing 1% Triton X100, 20 μg/ml aprotinin, 50 μg/ml leupeptin. The resuspended pellets were then stored at -20°C overnight.

The resuspended culture was defrosted and sonicated for 30 seconds on ice. The sample was then spun for 15 min at 10,000 r.p.m. and the supernatant retained and added to 2ml of packed glutathione Sepharose 4B beads, (Pharmacia Biotech) that had been prewashed twice in 30 ml of ice cold PBS containing 1% Triton X-100. The samples were then allowed to incubate with the beads by rolling for 30 min at room temperature. The beads were then washed twice in 50 ml of PBS/Triton, and separated into aliquots in PBS containing 1% Triton. Proteins were analysed by boiling 5 μl of beads in 2 × Laemmli sample buffer (see Appendix 2 for composition) and subjecting them to 12% SDS PAGE using a BioRad minigel apparatus.

2.2.3.2 c-Jun Kinase Activity Assay

The cells from a 6 well plate were washed in ice cold PBS then scraped into 500 μl lysis buffer as described for the ERK assay as above. The lysates were centrifuged at 20,000 r.p.m. for 10 min at 4°C and 400 μl aliquots of the supernatant removed for assay. 20 μl of a 1:3 slurry of GST-c-Jun beads were added to the samples, and rolled at 4°C for 60 min. The beads were washed twice in 200 μl lysis buffer and twice in 200 μl kinase buffer (as described previously). Reactions were initiated by the addition of 40 μl of kinase buffer containing 1 μCi [γ-^{32}P]ATP and 20 μM unlabelled ATP and incubated for

20 min at 30°C. Reactions were terminated by the addition of 40 µl of 2 × Laemmli sample buffer. The samples were heated at 100°C for 5 min and centrifuged for 5 min at 14,000 r.p.m. after which 70 µl of sample was loaded on a 12% PAGE gel, together with 20 µl of low molecular weight marker (Sigma; see Table 2.1). Electrophoresis was performed at 18 V/gel through the stacking gel and 24 V/gel through the resolving gel, or at 4 V per gel overnight. The gels were stained in a solution of 0.25% (w/v) Coomassie blue containing 50% methanol and 10% acetic acid to detect the GST-c-Jun, destained (40% methanol, 10% acetic acid, 1% glycerol) and dried for 1 hour at 80°C under a dry vacuum, prior to autoradiography on Hyperfilm MP, (Amersham) using an intensifying screen. The film was developed using a Hyperprocessor (Amersham). After the autoradiography, the individual bands were excised from the gel and placed in scintillation vials with 5 ml scintillation plus and counted for 3 min (Packard Liquid Scintillation Analyser).

MWt (kDa)	**Marker**
66	Albumin, Bovine
45	Albumin, Egg
36	glyceraldehyde-3-P-dehydrogenase
29	carbonic anhydrase, bovine
24	trypsinogen. bovine pancreas
20	trypsin inhibitor, soybean
14.2	α-lactalbumin, bovine milk

Table 2.1. Summary of Low Molecular weight markers (Sigma).

2.3 Western Blot Analysis

The lysates were prepared as described above, normalised for protein and run on a 12% SDS-PAGE minigel, with one lane containing 5 µl of prestain broad range markers (Biorad). The protein was transferred to nitrocellulose paper in transfer buffer (40 mM Tris, 39 mM glycine, 1.3 mM SDS, 20% methanol) using a Transblot semi-dry transfer cell system (Biorad) run at 12V for 30 min. Once the transfer was complete, the paper was stained with Ponceau Red stain (0.2% Ponceau S in 3% TCA and 3% sulfosalicyclic acid), to confirm equal protein loading, then destained using distilled water. The blots were blocked for one hour in 25 ml of blocking buffer 5% Marvel in TTBS (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1 % Tween 20) and then washed once for 5 min in TTBS. Membranes were incubated overnight with mixing at 4°C in primary antibody at the indicated dilutions in blocking buffer. The blots were then washed three times for 5 min in TTBS to remove the primary antibody. The secondary antibody (α-rabbit for polyclonal primary antibodies, or α-mouse for monoclonal antibodies) was diluted 1:1000 in TTBS and incubated with the membranes with shaking for 1 hour at room temperature. The antibody was then removed and the blot was washed five times for 5 min with TTBS. Blots were then incubated in ECL Reagent (Amersham) for 80 seconds, drained and wrapped in clingfilm. Blots were then exposed to Hyperfilm MP (Amersham) for times varying from 5 seconds to 15 min, or as otherwise indicated and the film developed using a Hyperprocesser (Amersham).

2.4 Measurement of Intracellular Calcium

2.4.1 Cell Preparation

CHO cells were grown to confluency in a 175cm^2 flask, washed in 10 ml of HBS and then harvested in 5 ml of harvest buffer (10 mM Hepes, pH 7.4, 154 mM NaCl, 0.54 mM EDTA). The cells were gently spun (1500 r.p.m. for 3 min) and then the pellet was washed in 10 ml KHB, and then resuspended in 3.5 ml/flask of KHB.

The level of intracellular calcium was measured by using a previously described protocol (Tobin *et al.*, 1995), with some modifications. A 500 µl aliquot was removed and added to 1.5 ml of KHB, to measure cellular autofluorescence. To the remaining cells, 2 µM fura-2-AM was added and the cells left at room temperature with rolling for 45 min. For each experiment, 500 µl of these cells was spun at 4,000 r.p.m. for 1 min, to discard the fura-2-AM that had not been taken up by the cells. The cells were then resuspended in 1.5 ml of KHB (+/-Ca^{2+} as indicated), at room temperature. The 340/380 nm ratio was recorded every second and methacholine concentrations were added as indicated in the presence and absence of 2 mM $[Ca^{2+}]_e$. At the end of each trace, in the presence of 2 mM $[Ca^{2+}]_e$ 0. 1% Triton X-100 was added to determine the R_{max} followed by 80 mM EGTA added to determine R_{min}. The level of $[Ca^{2+}]_i$ was determined by converting the 340/380 ratio using the Grynkiewicz equation (Grynkiewicz *et al.*, 1985).

$$[Ca^{2+}]_i = Kd \times \{(R-R_{min})/R_{max}-R)\} \times \{F_{min}(380\ nm)/F_{max}(380\ nm)\}$$

where, Kd is the dissociation constant of Ca^{2+} for FURA-2 (224 nM at 37°C). F_{min}(380 nm) and F_{max}(380 nm) are the fluorescent intensities after excitation at 380 nm, in the absence and presence of Ca^{2+} respectively.

2.5 [^{3}H]Thymidine Incorporation

2.5.1 Cell preparation

Cells were plated out at a density of 50,000 cells/well into 1 ml of media in a 24 well plate and left to adhere overnight. The following day, the media was removed and the cells washed in 1 ml HBS (pH 7.4) prior to addition of 1ml of serum free MEMα supplemented by, 0.01 units/ml penicillin, 0.01 μg/ml streptomycin, and 2.5 μg/ml amphoteracin. The cells were then incubated overnight (for 20 hours) at 37°C.

2.5.2 [^{3}H]Thymidine Incorporation Assay

The following day after the cells had been serum starved, the cells were treated with agonist for 20 hours or as described in the results section. In experiments where inhibitors were used, the inhibitor was added to the cells at the concentration described for 30 min prior to agonist addition. For the final 2 hours, 2 μCi of [^{3}H]thymidine was added to each well and the cells incubated at 37°C. The cells were the placed on ice and the media was removed, followed by 3 washes in 1 ml of serum free media. After the final wash, 2 ml of ice cold 5% TCA was added and the cells were for 1 hr at 4°C. The TCA was then removed, and the cells washed in fresh 2 ml of TCA. This was then followed by a 5 min incubation in 2 ml of ice cold ethanol, supplemented with 200 μM potassium acetate. The cells were the incubated twice for 15 min with a 3:1 mix of ethanol:ether. The cells were then allowed to air dry for approximately 30 min, and then 1 ml of 0.1 M NaOH was added to each well. The cells were scraped off the plate and placed into a scintillation vial with 9 ml of Goldscint. The samples were then counted on a liquid scintillation counter.

2.6 Western Blotting for PKC Isoforms

CHO cells were split into 6 well plate plates and allowed to adhere overnight, the cells were then serum starved for 20 hours. Prior to agonist stimulation of the cells, they were washed in 1ml of KHB and pre-incubated for 5 min at 37°C. After agonist stimulation, the cells were lysed in 400µl of ice-cold lysis buffer (20 mM Tris/HCl, 5 mM EGTA, 2 mM EDTA, 1 mM DDT, 0.5 mM PMSF, 10 µM benzamidine hydrochloride, 5 µM iodoacetamide, pH 7.4), extracted on ice for 30 min and scraped. Samples were mixed in an equal volume of 2 × Lamelli buffer and the samples were run down an 8% SDS PAGE gel with high molecular weight markers (Sigma) (Table 2.2). Immunoblotting was as described in the section 'Western Blot Analysis'. Antibodies to PKC isoforms were used at the following dilutions in TTBS/1% dry milk: PKCα (1:5000), PKCβ (1:2500), PKCγ (1:250), PKCε (1:500), PKCδ (1:1000), PKCθ (1:250), PKCι (1:500) λ PKCµ (1:1000), PKCζ (1:500).

MWt (kDa)	Marker
205	myosin, Rabbit Muscle
116	β-galactosidase, E. coli
97.4	phosphorylase b, Rabbit Muscle
66	Albumin, Bovine
45	Albumin, Egg
20	Carbonic Anyhydrase, Bovine Erthrocytes

Table 2.2. High Molecular Weight Markers (Sigma).

2.7 N-methyl-[^{3}H]-scopolamine (NMS) Binding

2.7.1 Membrane Preparation

The required number of 175 cm^2 flasks were grown to confluency and the media aspirated off before the cells were washed with HBS. The cells were then lifted with 10 ml EDTA (10 mM HEPES, 0.9% NaCl, 0.2% EDTA. pH 7.4) per flask. The cells were centrifuged at 200 g for 5 min at 4°C, and the supernatant discarded. The cells were then homogenised in 2 ml per flask with wash buffer A (10 mM HEPES, 10 mM EDTA, pH 7.4) with a Polytron homogeniser, (speed 5) for 4 x 5 sec bursts separated by 30 sec on ice, in Sorvall centrifuge tubes. The homogenates were then centrifuged at 40,000 g in a Sorvall RC-5 centrifuge (rotor SS-34) for 15 min at 4°C. The supernatant was then discarded and the pellet was re-homogenised, as above in buffer B (10 mM HEPES, 0.1 mM EDTA, pH 7.4). The tubes were again centrifuged at 40,000g in Sorvall RC-5 centrifuge for 15 min at 4°C, the supernatant discarded and the pellet was dispersed in buffer B, (1ml per confluent flask of cells), using the Polytron homogeniser. The protein concentration of the membranes was determined by a Lowry assay, the membrane samples were either aliquoted or diluted with wash buffer B to the desired concentration and then snap frozen in liquid nitrogen.

2.7.2 [^{3}H]-NMS saturation binding

[^{3}H]-NMS binding was performed on membrane samples prepared as described above. Two total binding and one non-specific binding, samples were prepared for each concentration of [^{3}H]-NMS. Samples were prepared in test tubes with a final assay volume of 200 µl, total binding samples were 140 µl wash/assay buffer (10 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl), 40 µl of [^{3}H]-NMS at the stated concentration and 20 µl

of the membranes (added last). Non-specific binding samples were 100 µl wash/assay buffer, 40 µl of 1 µM atropine, 40 µl of [^{3}H]-NMS at the stated concentration and 20 µl of the membrane (added last). [^{3}H]-NMS concentrations were prepared from 0.05 nM - 3 nM, in wash/assay buffer, two 40 µl samples of the standards were taken of each concentration in order to calculate [^{3}H]-NMS added to each sample. The samples were incubated in a shaking water bath after membrane addition for 60 min at 37°C. The samples were then rapidly filtered onto Whatman GF/B filter paper with 3 × 3 ml washes with ice cold wash/assay buffer. The filter discs were then placed into picovials, 4ml of Emulsifier Safe scintillant was added and they were left overnight before being counted.

2.8 Inositol 1,4,5-trisphosphate (IP_3) Mass Accumulation

2.8.1 Cell Preparation

Following the removal of the medium, the intact cells, confluent in 24 well plates (surface area 1.9 cm^2/well) were washed twice in 1 ml freshly gassed (95% CO_2, 5% O_2) Krebs-Henseleit Buffer (KHB) (composition as previously described) and this was replaced with 450 µl KHB and allowed to stabilize for 15 min at 37°C.

2.8.2 Time Course Experiments

Cells were stimulated by the addition of 100 µM methacholine (MCh) at 37°C. The reaction was terminated by aspiration of the KHB/drug solution and the addition of 300 µl 0.5 M TCA to each well. The plate was then left on ice for 30 min after which all 300 µl in each well was transferred to Eppendorf tubes. Blank buffer for the mass assay was prepared using 10 Eppendorfs of 300 µl 0.5 M TCA, 75 µl 10 mM EDTA (pH 7.0) was

then added to each tube, the samples were vortexed and 600 µl of a 1:1 solution of tri-n-octylamine and trichlorofluoroethane added and vortexed again. These samples were then left for 15 min at room temperature, vortexed again, centrifuged for 2 min in a bench centrifuge at full speed. Then, 200 µl of supernatant was transferred to a fresh Eppendorf and 50 µl $NaHCO_3$ was added and the samples re-vortexed and stored at 4°C.

2.8.3 Ins(1,4,5)P_3 Mass Assay

IP_3 binding protein was prepared from fresh bovine adrenal glands, obtained from a local abattoir. 30 µl Tris (100 mM)/EDTA (4 mM) pH 8.0 was added to 30 µl of sample/standard on ice. 30 µl [^{3}H]-IP_3 (at a concentration where 30 µl gives approximately 8000 d.p.m. (approximately 10µl [^{3}H]-IP_3 : 1ml H_2O) was then added before 30µl binding protein. Standards were prepared in the range 1.2 nM - 1.2 µM IP_3, using 40 µM IP_3 standard, diluted with blank buffer. The samples were the vortexed and left on ice for 40 min before rapid filtration on manifolds using 3 × 3 ml washes with ice-cold wash buffer (25 mM Tris, 1 mM EDTA, 5 mM $NaHCO_3$, pH 8.0) onto GF/B Whatman filter discs which were then placed in picovials with 4.2 ml Scintillant Plus and left overnight before counting.

2.9 Protein Assays

2.9.1 Bradford Protein Assay

All protein assays were carried out in duplicate with standard curve range of 1-0.025 mg/ml. The samples, blank and standard curve had 10 µl d.H_2O and 10 µl (final volume) of lysis buffer added, as the lysis buffer had a significant effect of the absorbance of the samples. To the final samples was added 0.71 M NaOH and 1 ml of dye reagent (8.5%

phosphoric acid, 4.75% ethanol, 0.01% Serva blue G (Serva,Westbury,NY) filtered with a buchner funnel and Whatman No 1 filter paper) and the samples were incubated for 5 min. The absorbance was measured at 595 nm and 465 nm on a UV-1201 spectrophotometer (Shimadzu) and the difference between these was calculated and plotted against protein concentration.

2.9.2 Lowry Protein Assay

Protein assays were carried out in quadruplicate on 500 µl samples by the Lowry method (Lowry *et al.,* 1951). A standard curve was set up in duplicate and unknown protein samples were diluted by a factor of 1:2 in 0.1 M NaOH to obtain a 500 µl volume. To each tube, 1 ml of assay solution (see below) was then added (made in a ratio of; 100:1:1, 2% Na_2CO_3, 0.4% NaOH, 2% K^+/Na^+ tartrate:1% $CuSO_4$) and the tubes vortexed and left for 10 min. To each tube was then added, 100 µl of a 3-fold diluted Folin and Ciocalteus Phenol reagent and the tubes were re-vortexed and left for 15 min. 1 ml of d.H_2O was the added to each tube, the samples vortexed and the absorbance was measured at 750 nm on a UV-1201 spectrophotometer (Shimadzu).

Composition of 2 × Lamelli Buffer:

Solution A: 30% glycerol (in 90 ml final volume)

Solution B. 10g/l Tris Base
3.33g/l SDS
48g/l Glycine

Solution C. 0.75g/50ml Tris-HCl
3.6g/50ml Imidazole
0.1g/50ml Bromophenol Blue

20g/50ml Sucrose

5g/50ml SDS

Add 48 ml of Solution B to all of Solution A and mix.

Add 12 ml of Solution C to mixture of A and B and mix well.

Divide into 1ml aliquots and store at -20°C. Add 50 µl of 1M DTT prior to use.

2.10 Concentration- and inhibition-response curves.

The data were plotted on Graphpad Prism® (GraphPad Software Inc.) using the sigmoidal dose-response equation with no pre-set parameters. For each individual experiment, a line was plotted and the EC_{50}/IC_{50} value obtained and recorded. The mean EC_{50}/IC_{50} values and the associated errors that are quoted, were obtained by using these individual values and calculating the mean ± S.E.M from those data. The figures presented in this Thesis, show the combination of the individual data sets to produce a representative graphical plot, but not to generate the EC_{50}/IC_{50} values and associated means.

2.11 Statistics

The raw data in cpm obtained from experiments were converted into enzyme activities (see next section). The control unstimulated cells were meaned and this mean enzyme activity value was used to calculate fold activity for all the results obtained on a particular day, and subsequently plotted on GraphPad Prism® (GraphPad Software Inc.). The statistical differences between data sets was assessed by one-way analysis of variance for multiple comparisons, followed by Duncan's multiple-range test at $P < 0.05$ using SPSS version 6.1 software (Chicago, Il. U.S.A).

2.12 Calculation of kinase activity.

A spreadsheet was set up to calculate the actual kinase activity of the enzymes. During the kinase assay, a 4 µl aliquot of [^{32}P]ATP was taken and counted at the same time as the samples from the assay (this equates to 1/10th of the volume used in the assay which is a safer level of radioactivity to use). This therefore means that the concentration of the cold ATP stock is known, and the number of counts of radiolabelled ATP from that concentration can be calculated (the increase in ATP concentration from the addition of the radiolabelled ATP is so small that it is ignored). An example calculation is shown below:

Cold ATP stock is 2 mM, which is diluted 1/100 fold in the final kinase buffer stock.

Therefore the actual concentration of ATP = 2 mM/100
= 0.02 mM ≡ 20 µM

The volume of ATP/assay is 40 µl.
Therefore amount of ATP/assay = 20 µM × 40 µl
= 800 pmoles
If the average count from the 4 µl aliquots was 455705 cpm, therefore in the 40 µl reaction there would be 4557050 cpm.

From this and the number of counts obtained from 4 µl aliquot, the specific activity can be calculated as follows:

Specific Activity = 4557050 ÷ 800 pmoles
=5696.31 cpm/pmol

From this, the following example calculation is shown to determine the fmol/min/mg of ATP incorporated using the following variables.

cpm = 100 cpm
specific activity = 5696.31 cpm/pmol
time of reaction = 20 min

protein = 1mg/ml in 200 µl
= 1mg/ml × 0.2 ml
= 0.2 mg

cpm	pmols ATP/20min	pmols ATP/min	pmol ATP/min/mg	fmol/min/mg
	=cpm/specific activity	=pmolATP/20min/20	pmol ATP/min/0.2mg	pmol ATP/min/mg × 1000
1000	0.176	0.009	0.05	50

Table 2.3 Example calculation from cpm to incorporation of ATP (fmol/min/mg).

2.13 Materials

anti- PKC antibodies	Affinity Laboratories
ECL reagent	Amersham
Glutathione sepharose™ 4B	Amersham
Hyperfilm MP	Amersham
[^{3}H]-IP_3	Amersham
[^{3}H]-thymidine	Amersham
anti JNK-1 antibody	Autogenbioclear
anti p38 antibody	Autogenbioclear
anti-ERK 2 antibody	Autogenbioclear
anti-ERK1 antibody	Autogenbioclear
$MgSO_4$	BDH
Prestain Broad range markers	Biorad
Bisindolylmaleimide	Calbiochem
Gö-6976	Calbiochem
LY-294002	Calbiochem
PD-98059	Calbiochem
Ro-31-8220	Calbiochem
acetic acid	Fisher
acetone	Fisher
Ammonium persulphate	Fisher
Coomassie Blue	Fisher
$CuSO_4$	Fisher
Glycerol	Fisher
Hepes	Fisher
KCl	Fisher
KH_2PO_4	Fisher
Methanol	Fisher
Na_2HPO_4	Fisher
NaCl	Fisher
$NaHCO_3$	Fisher
SDS	Fisher
sulfosalicyclic acid	Fisher
Tri-n-octylamine	Fisher
Tris Buffer	Fisher
$CaCl_2$	Fisons
D-glucose	Fisons
EDTA	Fisons
EGTA	Fisons

$MgSO_4$	Fisons
NaCl	Fisons
$NaCO_3$	Fisons
Na_2HCO_3	Fisons
NaOH	Fisons
TCA	Fisons
	Fisons
Amphoteracin B	Gibco BRL
MEMα	Gibco BRL
Newborn Calf Serum	Gibco BRL
penicillin	Gibco BRL
streptomycin	Gibco BRL
Trypsin-EDTA	Gibco BRL
Folin and Ciocalteus phenol	ICN
fura 2-AM	Molecular Probes (Leiden, NL)
Ultrapure Protogel	National Diagnostics
$[\gamma^{32}P]ATP$	NEN
Scintillation Plus	Packard
GST	Pharmacia
Protein A sepharose	Pharmacia
EGFR peptide	PNACL (MRC Toxicology Unit, University of Leicester)
Serva blue G	Serva, Westbury, NY.
α-mouse secondary antibody	Sigma
α-rabbit secondary antibody	Sigma
Anisomycin	Sigma
Aprotinin	Sigma
ATP	Sigma
β-glycerophosphate	Sigma
benzamidine hydrochloride	Sigma
$CaCl_2$	Sigma
deoxycholate	Sigma
DTT	Sigma
ethanol	Sigma
Ether	Sigma
High Molecular weight markers	Sigma
Imidazole	Sigma
iodoacetamide	Sigma

IPTG	Sigma
leupeptin	Sigma
Low Molecular weight markers	Sigma
methacholine	Sigma
Na_2VO_4	Sigma
NP-40	Sigma
orthophosphoric acid	Sigma
PDBu	Sigma
PMSF	Sigma
Ponceau Red	Sigma
potassium acetate	Sigma
TEMED	Sigma
trichlorofluoroethane	Sigma
tryptone	Sigma
Tween 20	Sigma
Wortmannin	Sigma
Yeast extract	Sigma
SB-203580	SmithKline-Beecham
p81 paper	Whatman

Chapter 3

General characterisation of the ERK and JNK pathways in CHO-m2 and CHO-m3 cells

3.1 Introduction.

It has previously been demonstrated that the ERK and JNK pathways can be activated by GPCRs. With respect to mACh receptors, Crespo and colleagues (1994a) demonstrated that m1- and m2- acetylcholine receptors coupling to G_q and G_i proteins respectively in COS7 cells, are able to activate ERK. It has also been previously demonstrated that GPCR-mediated JNK activation can occur via the m1- and m2-AChRs (Coso *et al.*, 1995a). In previous studies it had been reported that carbachol-induced JNK activity appeared to occur more slowly with respect to ERK activation for the m1 receptor expressed in NIH3T3 cells (Coso *et al.*, 1995a). Also, Mitchell and co-workers demonstrated that the m1-AChR activated JNK, whereas the m2-AChR poorly activated JNK when stably expressed in Rat-1a fibroblasts (Mitchell *et al.*, 1995). In this Chapter, the activation of the ERK and JNK pathways by receptor stimulation with agonist in the CHO-m2 and CHO-m3 cell lines is described.

The initial studies, which are described in this Chapter, set out to characterise the time- and concentration- dependence of agonist-induced ERK and JNK activation in the CHO-m2 and CHO-m3 cell lines, in addition to other experiments designed to characterise related responses, in both cell lines.

3.2 [^{3}H]-NMS saturation binding in CHO-m2 and CHO-m3 membranes.

The model cell line used for the studies were CHO-K1 cells that have been transfected stably to express the human m2-AChR (from Dr. S. Larenzo, National Research Institute, Mill Hill, London) or the human m3-AChR (from Dr. N. Buckley, National Research Institute, Mill Hill, London). In order to draw comparisons of agonist-induced ERK and JNK activation between the m2-AChR and m3-AChR subtypes, it was important initially to establish the relative levels of receptor expression in the CHO-m2 and CHO-m3 cell lines.

3.2.1 Introduction to saturation binding.

Saturation binding experiments involve adding a known range of radioligand concentrations to a known level of membrane protein preparation and this analysis gives the values B_{max} and K_d. B_{max} is the maximal binding capacity and can be defined as the total receptor density in the membrane protein preparation. K_d is the equilibrium dissociation constant, and it is defined as the concentration of free radioligand required to occupy 50% of the receptor sites.

The K_d and B_{max} values can be determined graphically in two ways. Previously, due to the absence of reliable graphical computer packages, the most common way of analysis was by a linear plot derived from the Michaelis-Menten equation termed the Scatchard plot. The Scatchard plot is obtained by plotting bound radioligand/free radioligand (on the y-axis) against bound radioligand (on the x-axis). In this plot, the gradient of the line is $-1/K_d$ and B_{max} is defined where the line intersects the x-axis. The

alternative method of analysis, which is often more accurate with reliable graphical packages, is by the use of a binding isotherm plot, obtained by plotting specific binding of radioligand against the concentration of free radioligand, the data are then fitted to a rectangular hyperbola.

3.2.2 N-methyl-[^{3}H]-scopolamine (NMS) saturation binding in CHO-m2 and CHO-m3 membranes.

Ligand binding studies were carried out and the results obtained demonstrated that the m2-AChR and the m3-AChR were expressed at comparable levels. By analysing the data by means of a binding isotherm plot (Figure 3.1), the [^{3}H]-NMS binding studies demonstrated that the m2-AChR was expressed at 1.38 ± 0.2 pmol/mg protein, and the m3-AChR was expressed at 1.53 ± 0.2 pmol/mg protein (mean ± S.E.M., n=8). The dissociation constant values (K_d) for the m2-AChR and the m3-AChR were 0.56 ± 0.2 nM and 0.39 ± 0.1 nM respectively. The equivalent Scatchard plots are shown in Figure 3.2. therefore, as the expression levels of the m2- and m3- AChRs were similar, the potential for differences in ERK and JNK activation between the cell lines being due to variations in receptor number was unlikely.

3.3 Time- and agonist concentration- dependence of ERK and JNK activation in CHO-m2 and CHO-m3 cells.

The time-course and concentration-dependence of agonist-induced ERK and JNK activation was investigated in both CHO-m2 and CHO-m3 cell lines. Throughout this Chapter, the enzyme activity values for ERK and JNK are expressed as fmol of [γ^{32}P]-ATP incorporated per min per mg protein (fmol/min/mg), calculated as described in the Methods section.

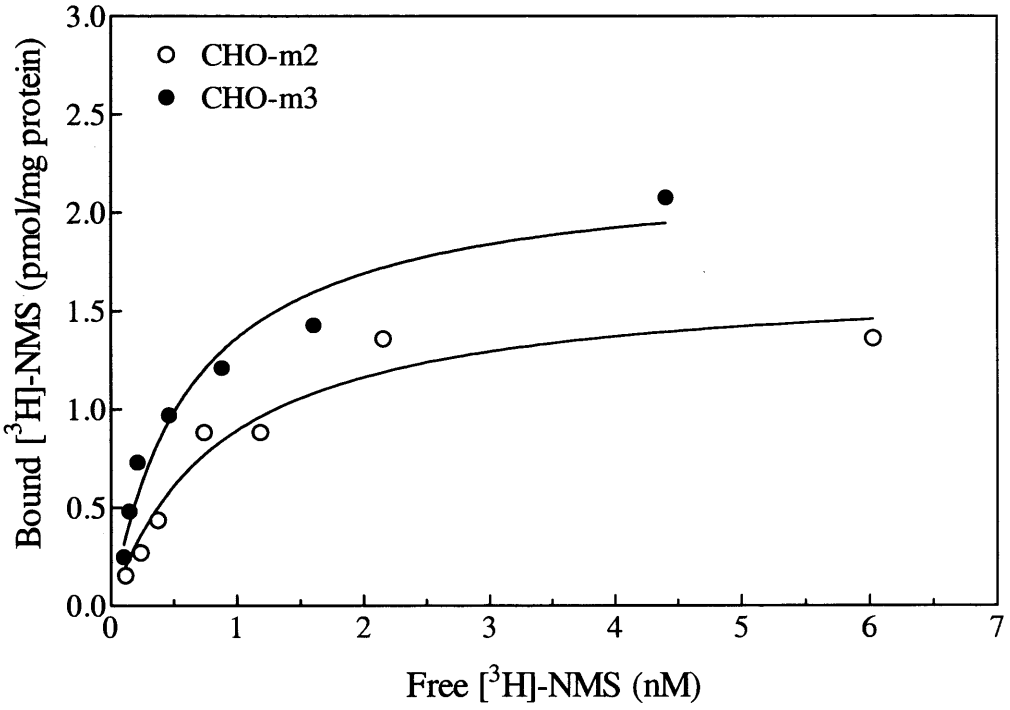

Figure 3.1. Saturation curve of [^{3}H]-NMS binding in CHO-m2 and CHO-m3 cells.

Cell membranes were prepared as described in the Methods section. Membranes were incubated (approximately 25 μg) in assay buffer (see Methods), with the indicated concentrations of N-methyl-[^{3}H]-scopolamine (Free [^{3}H]-NMS), in the presence and absence of 1 μM atropine (to define non-specific binding) at 37°C for 60 min. Bound [^{3}H]-NMS was calculated by subtracting non-specific binding from the total radioligand bound. Each curve was fitted by using a rectangular hyperbola in Graphpad Prism. Data represent mean of duplicate results from a single experiment which is representative of 4 separate experiments.

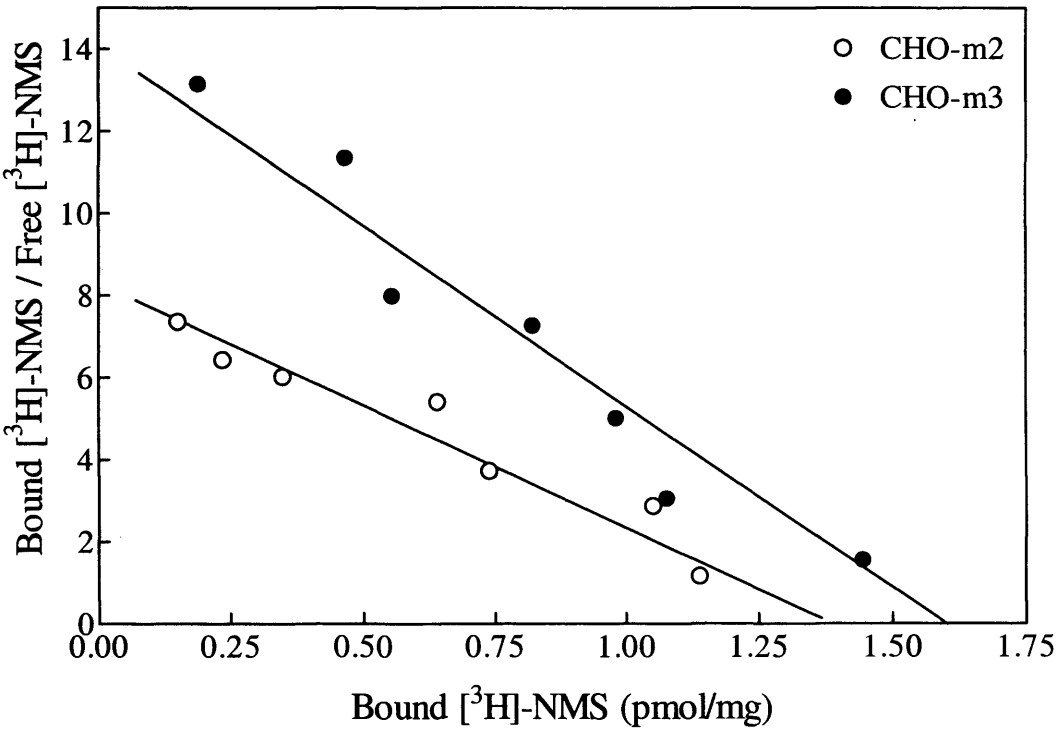

Figure 3.2 Scatchard analysis of [^{3}H]-NMS binding in CHO-m2 and CHO-m3 cells.

The data represented here are from the same data presented in Figure 3.1. However a linear plot is produced when bound radioligand divided by free radioligand is plotted against bound radioligand. Each plot was fitted by using a linear regression in Graphpad Prism. Data represent mean of duplicate results from a single experiment which is representative of 4 separate experiments.

3.3.1 Time-dependence of agonist-induced ERK1 and ERK2 activation in CHO-m2 and CHO-m3 cells.

The time- dependence of p44-ERK1 and p42-ERK2 activation by receptor stimulation with maximal agonist concentration (100 μM MCh) was investigated in both cell lines. Stimulation of the m2- and m3- ACh receptors by 100 μM methacholine (MCh), produced a large and rapid stimulation of ERK1 and ERK2 (Figure 3.3 and Figure 3.4). In both CHO-m2 and CHO-m3 cells, a large initial activation of ERK1 and ERK2 was

observed at 2 min after agonist stimulation which continued to rise to give a maximal activation at 5 min after agonist addition. In m2-AChR expressing cells (Figure 3.3), in the experiments shown, the peak ERK1 and ERK2 activities were 4715 ± 369 fmol/min/mg (32 fold over basal) and 2058 ± 204 fmol/min/mg (16 fold over basal) respectively. This compared with values obtained, shown in Figure 3.4 of 7050 ± 373 fmol/min/mg (38 fold over basal) and 4528 ± 272 fmol/min/mg (16 fold over basal) for ERK1 and ERK2 activities respectively in the m3-AChR expressing cells. In the CHO-m2 cells, both the ERK1 and ERK2 responses had returned to basal values by 20 min after MCh stimulation, whereas, in CHO-m3 cells, the ERK1 and ERK2 activation was more sustained, whereby at 40 min after agonist addition in the experiments shown, the ERK1 activity was 3145 ± 198 fmol/min/mg (17 fold over basal).

3.3.2 Time-dependence of agonist-induced JNK activation in CHO-m2 and CHO-m3 cells.

The time course of agonist-induced JNK activation was also examined in both cell lines. The results demonstrated that stimulation of the m2-AChR in CHO cells with 100 μM MCh caused only a small JNK activation. By 5 min after MCh addition, a small activation of JNK was observed, and at 15 min, the optimum activity of two fold over basal had been achieved, with a peak activity in the experiment shown of 341 ± 43 fmol/min/mg (Figure 3.5). This response decreased over the subsequent 30 min after which the response returned to a constant level just above basal values. In contrast to the m2-AChR, JNK activation by the m3-AChR was greater and much more sustained than that observed in CHO-m2 cells (Figure 3.5). By 10 min after agonist addition, an increase of 4 fold over basal was observed and as shown in Figure 3.5, the maximal

response of 1672 ± 143 fmol/min/mg (10 fold over basal) was reached at 30 min following agonist stimulation of the receptor. This was sustained for approximately 60 min after agonist addition, before the response started to slowly decline.

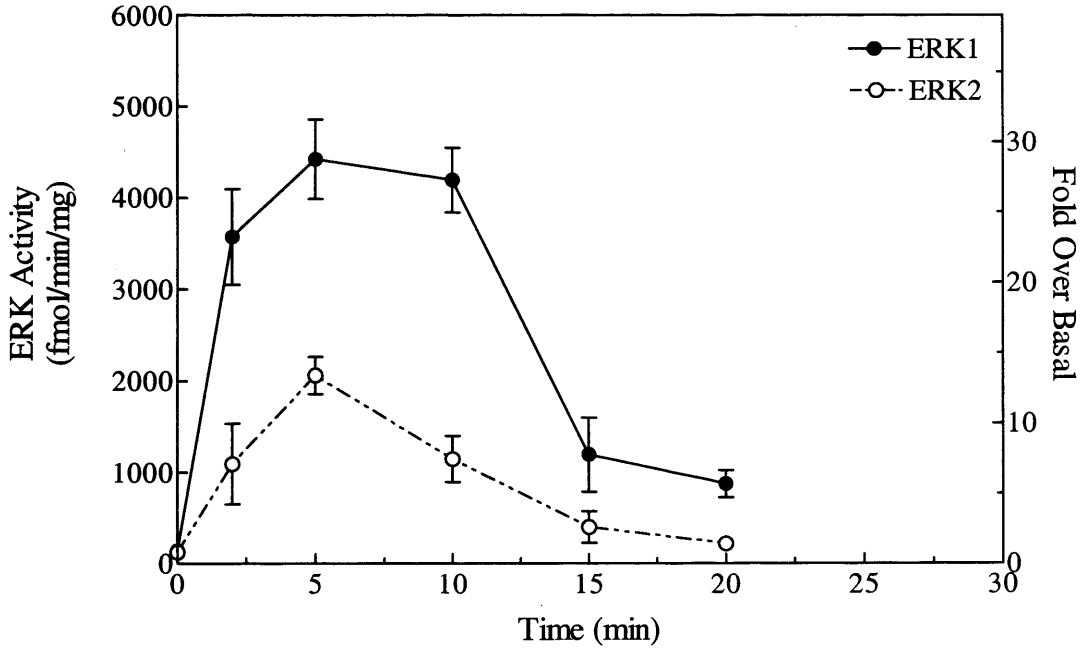

Figure 3.3 Time- course of ERK1 and ERK2 activation in CHO-m2 cells.

CHO cells expressing the m2-AChR were stimulated with 100 μM MCh for the times indicated. ERK1 and ERK2 activity was isolated (approx. 200 μg protein) using anti-ERK1 (p44) or anti-ERK2 (p42) antisera and assayed following immunoprecipitation as described in the Methods section. Basal ERK1 and ERK2 activities were 149 ± 13 fmol/min/mg and 195 ± 17 fmol/min/mg, respectively. Data represent mean ± S.E.M. of 5-9 separate experiments.

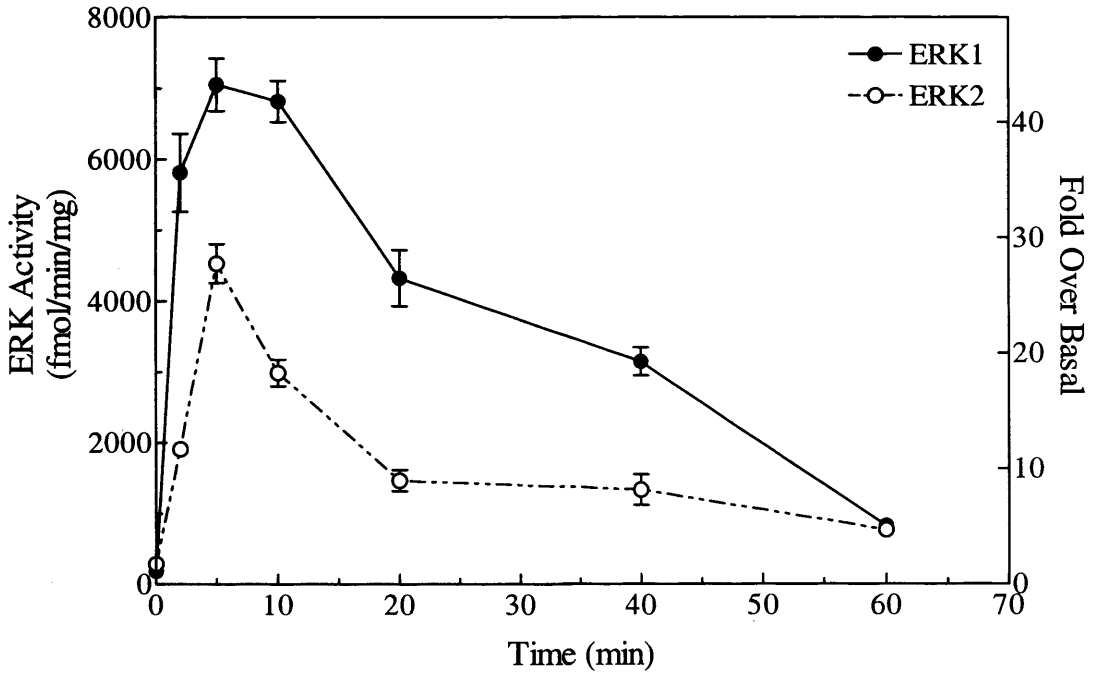

Figure 3.4. Time- course of ERK1 and ERK2 activation in CHO-m3 cells.

CHO cells expressing the m3 AChR were stimulated with 100 μM MCh for the times indicated. ERK1 and ERK2 activity was isolated (approx. 200 μg protein) using anti-ERK1 (p44) or anti-ERK2 (p42) antisera and assayed following immunoprecipitation as described in the Methods section. Basal ERK1 and ERK2 activities were 187 ± 15 fmol/min/mg and 288 ± 51 fmol/min/mg, respectively. Data represent mean ± S.E.M. of 4-9 separate experiments.

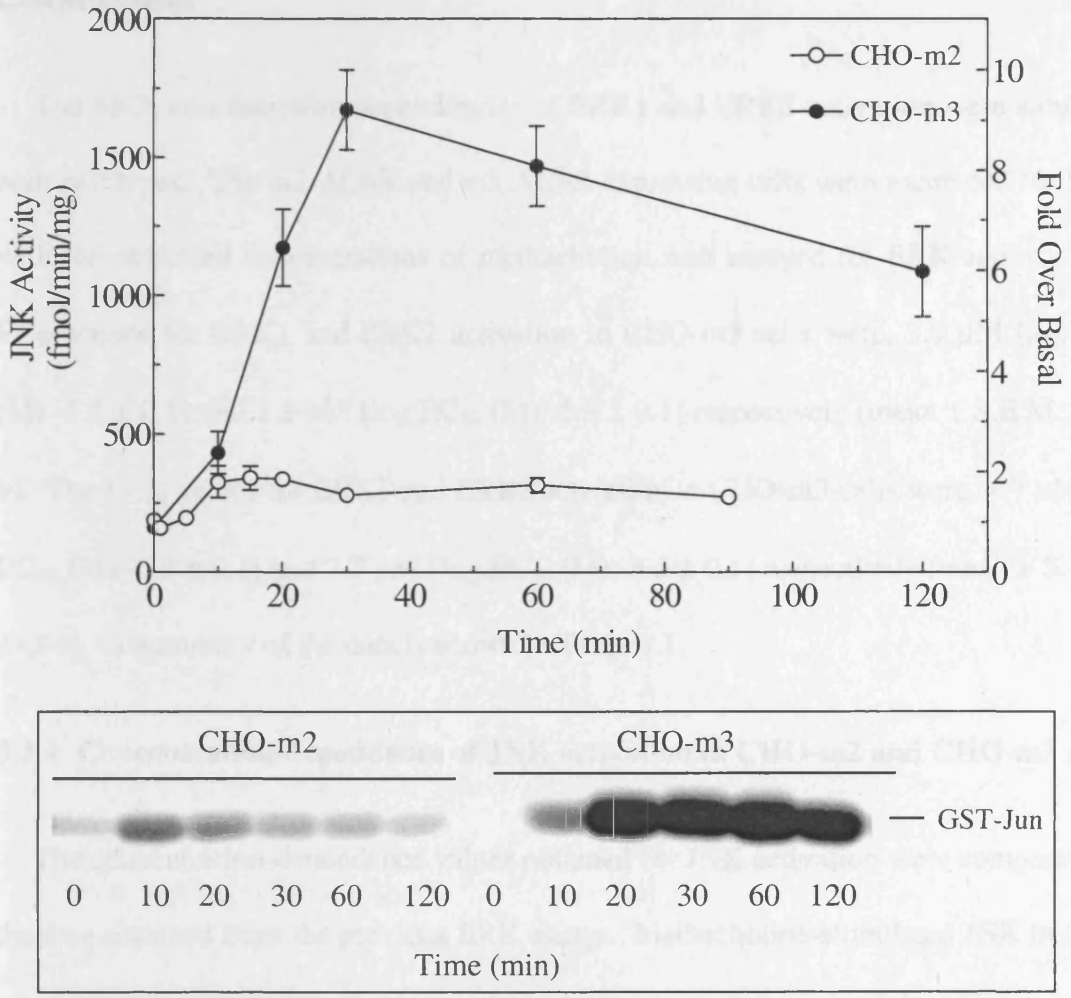

Figure 3.5 Time- course of JNK activation in CHO-m2 and CHO-m3 cells.

CHO cells expressing either the m2- or m3-AChR were stimulated with 100 μM MCh for the times indicated. CHO cell lysates (approx. 400 μg protein) were assessed for JNK activity by GST-Jun phosphorylation as described in the Methods section. Basal JNK activities in CHO-m2 and CHO-m3 cells were 187 ± 15 and 161 ± 17 fmol/min/mg, respectively. The lower panel shows a representative autoradiogram of GST-Jun phosphorylation. Data represent mean ± S.E.M. 3-7 separate experiments.

3.3.3 Concentration-dependence of ERK1 and ERK2 activation in CHO-m2 and CHO-m3 cells.

The MCh concentration-dependencies of ERK1 and ERK2 activation were similar in both cell types. The m2-AChR and m3-AChR expressing cells were incubated for 5 min with the indicated concentrations of methacholine, and assayed for ERK activity. The EC_{50} values for ERK1 and ERK2 activation in CHO-m2 cells were, 3.9 μM [log EC_{50} (M) -5.4 ± 0.1] and 1.8 μM [log EC_{50} (M) -5.8 ± 0.1] respectively (mean ± S.E.M., n=3-9). The EC_{50} values for ERK1 and ERK2 activation in CHO-m3-cells were, 4.7 μM [log EC_{50} (M) -5.3 ± 0.1] and 2.7 μM [log EC_{50} (M) -5.6 ± 0.1] respectively (mean ± S.E.M., n=3-9). A summary of the data is shown in Table 3.1.

3.3.4 Concentration-dependence of JNK activation in CHO-m2 and CHO-m3 cells.

The concentration-dependence values obtained for JNK activation were comparable to the data obtained from the previous ERK assays. Methacholine-stimulated JNK in CHO-m3 cells with an EC_{50} of 1.8 μM [log EC_{50} (M) -5.8 ± 0.2], and in CHO-m2 cells with an EC_{50} of 0.2 μM [log EC_{50} (M) -6.71 ± 0.4] (Figure 3.8.). However, because the maximal level of c-Jun kinase activation by m2-AChR was only two-fold above basal it was difficult to determine accurately the EC_{50} for JNK activation. A summary of the data is shown in Table 3.1.

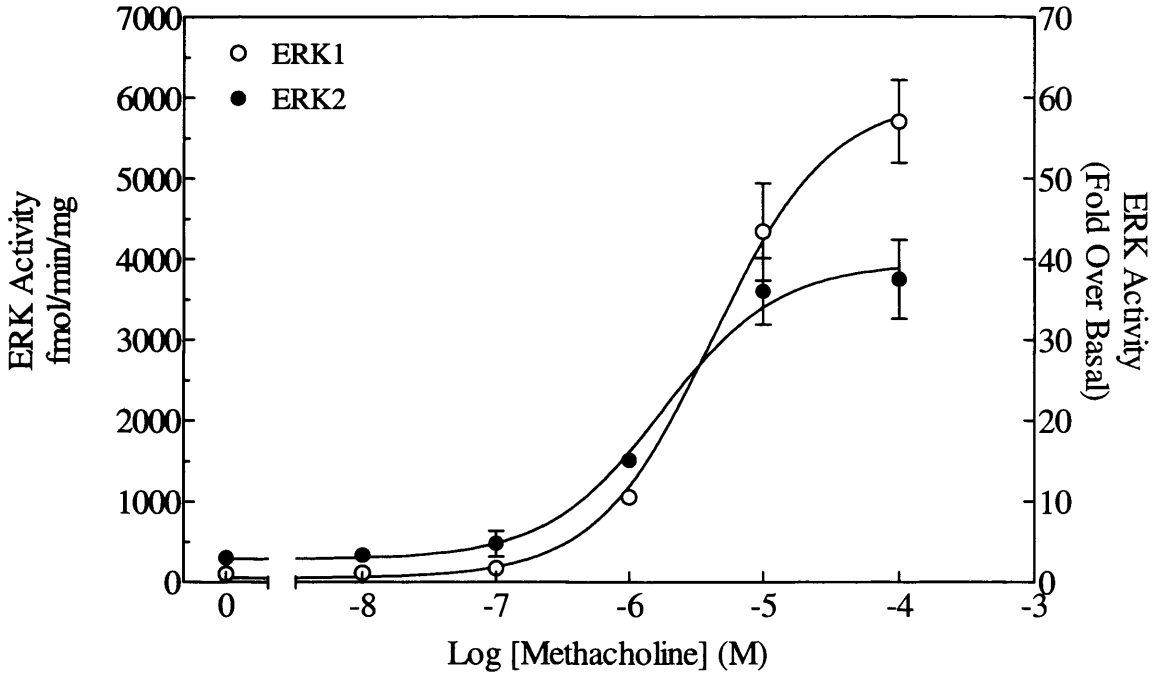

Figure 3.6. Concentration-dependence of ERK1 and ERK2 activation in CHO-m2 cells.

CHO cells were incubated with various concentrations of MCh for 5 min. ERK1 and ERK2 activity was isolated from lysate (approx. 200 μg protein) using anti-ERK1 (p42) or anti-ERK2 (p44) antisera and assayed following immunoprecipitation as described in the Methods section. Basal ERK1 activity was 102 ± 2 fmol/min/mg and basal ERK2 activity was 142 ± 36 fmol/min/mg. Data represent mean ± S.E.M. of three separate experiments.

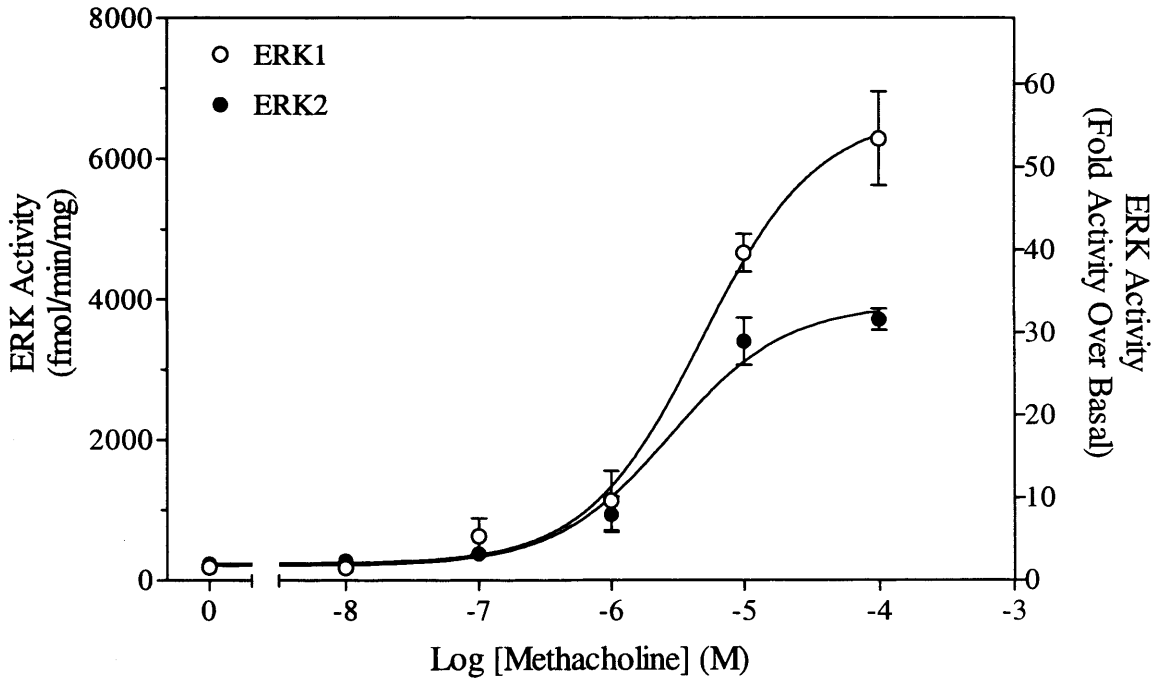

Figure 3.7. Concentration-dependence of ERK1/2 Activation in CHO-m3 cells.

CHO cells were incubated with various concentrations of MCh for 5 min and lysed. ERK1 and ERK2 activity was isolated from lysate (approx. 200 μg protein) using anti-ERK1 (p42) or anti-ERK2 (p44) antisera and assayed following immunoprecipitation as described in the Methods section. Basal ERK1 activity was 102 ± 2 fmol/min/mg and basal ERK2 activity was 142 ± 36 fmol/min/mg. Data represent mean ± S.E.M. of three separate experiments.

	Time of agonist exposure for peak activation			log EC_{50} (EC_{50} μM)		
AChR	**ERK1**	**ERK2**	**JNK**	**ERK1**	**ERK2**	**JNK**
m2	5 min	5 min	10 min	-5.5 ± 0.2 (3.1×10^{-6}M)	-5.8 ± 0.1 (1.8×10^{-6}M)	-6.7 ± 0.4 (2.0×10^{-7}M)
m3	5 min	5 min	30 min	-5.3 ± 0.1 (4.7×10^{-6}M)	-5.6 ± 0.1 (2.7×10^{-6}M)	-5.8 ± 0.2 (1.5×10^{-6}M)

Table 3.1. Table summarising the time required for agonist exposure to obtain maximal ERK1, ERK2 and JNK activation, and EC_{50} values for MCh in the activation of ERK1, ERK2 and JNK in CHO-m2 and CHO-m3 cells.

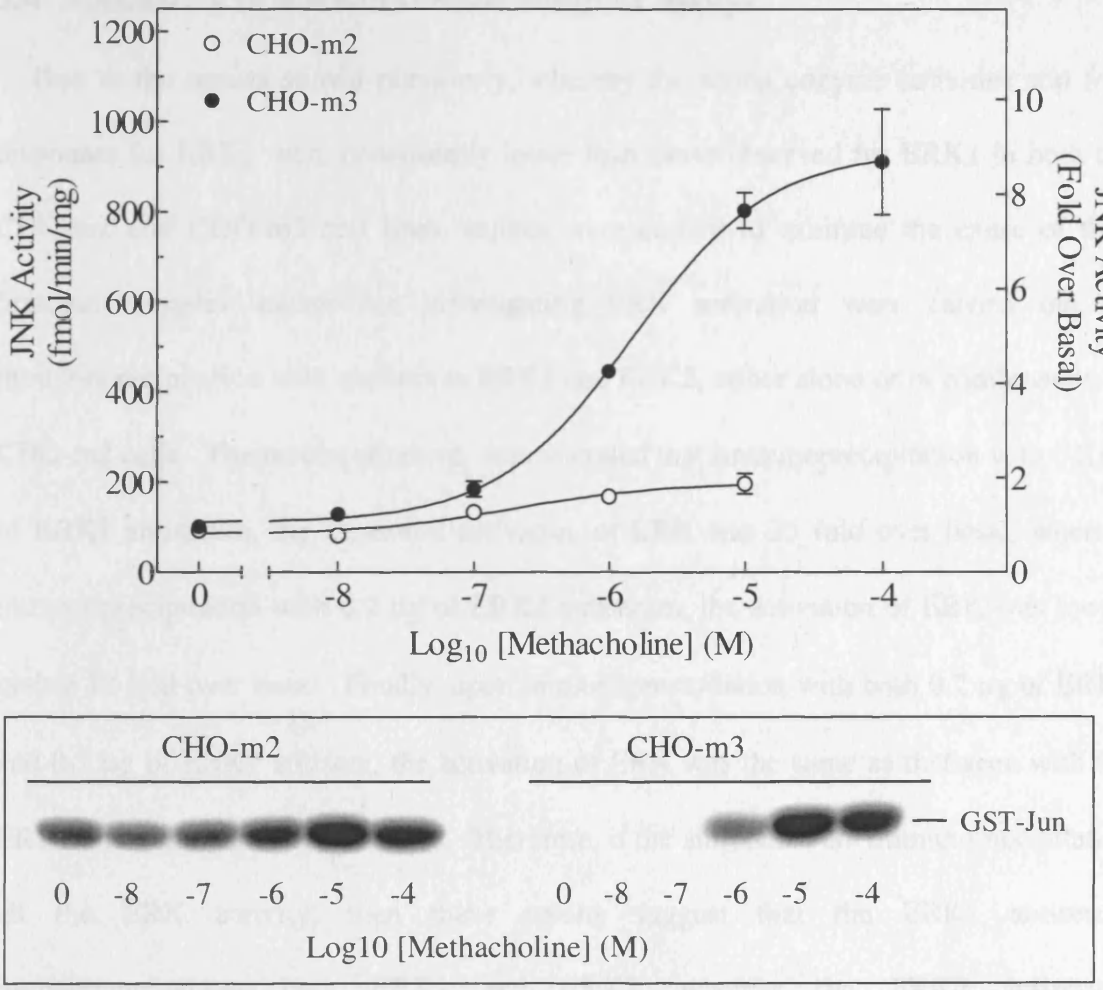

Figure 3.8 Concentration-dependence of JNK activation in CHO-m2 and CHO-m3 cells.

CHO-m2 cells and CHO-m3 cells were stimulated for either 15 min or 30 min respectively with various concentrations of MCh, and lysates containing 400 μg protein were assayed for JNK activity by GST-Jun phosphorylation (see Methods). Basal JNK activity was 224 ± 10 fmol/min/mg and was 101 ± 15 fmol/min/mg for CHO-m2 and CHO-m3 respectively. The lower panel shows a representative autoradiogram of GST-Jun phosphorylation (CHO-m2 exposed for 12 hr, CHO-m3 exposed for 2.5 hr). Data represent mean ± S.E.M. of three separate experiments.

3.4 Specificity of ERK- immune complex assays.

Due to the results shown previously, whereby the actual enzyme activities and fold responses for ERK2 were consistently lower than those observed for ERK1 in both the CHO-m2 and CHO-m3 cell lines, studies were carried to examine the cause of this. Immune complex assays for investigating ERK activation were carried out by immunoprecipitation with antisera to ERK1 and ERK2, either alone or in combination in CHO-m2 cells. The results obtained, demonstrated that immunoprecipitation with 0.2 μg of ERK1 antiserum, the measured activation of ERK was 25 fold over basal, whereas immunoprecipitation with 0.2 μg of ERK2 antiserum, the activation of ERK was lower, giving 12 fold over basal. Finally, upon immunoprecipitation with both 0.2 μg of ERK1 and 0.2 μg of ERK2 antisera, the activation of ERK was the same as that seen with the ERK1 antiserum alone (Figure 3.9). Therefore, if the antibodies are immunoprecipitating all the ERK activity, then these results suggest that the ERK1 antiserum immunoprecipitates both ERK1 and ERK2, whereas the ERK2 antiserum, immunoprecipitates ERK2 only in the m2-AChR expressing cells. However, these experiments do not show clearly the efficiencies of the antibodies to immunoprecipitate the different ERK isoforms. As a result, it is not possible to make a conclusion regarding which ERK isoforms are being immunoprecipitated (and thus the proportions of ERK activities from each antibody) from this data (for further discussion see pg 96).

By immunoblot analysis, it was observed that the levels of ERK proteins in the two cell lines were similar and also, as predicted, there was no effect on protein expression upon receptor stimulation. However, it was also found that the ERK1 antiserum cross-reacted with both p44 ERK1 and p42 ERK2, whereas, in contrast, the ERK2 antiserum

directed to the C-terminal sequence of ERK2 was specific for this isoenzyme (Figure 3.10). Thus, the immunoblot data in conjunction with the immunoprecipitation data suggest that results obtained using the ERK1 antiserum are likely to represent the combined activity of both ERK1 and ERK2. Therefore the ERK1 antiserum could be used to assay the combined activity of both ERK1 and ERK2 to give a total ERK activity.

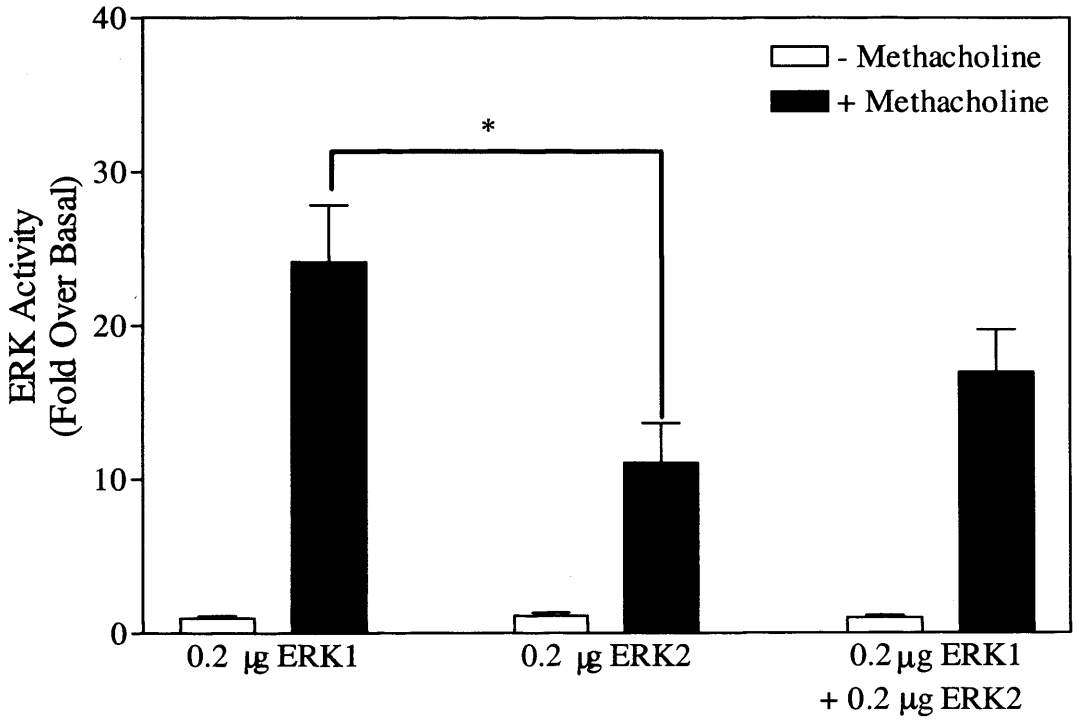

Figure 3.9 Determination of the specificity of ERK1 and ERK2 antisera.

CHO-m2 cells were stimulated for 5 min with 100 µM MCh, lysed and approximately 200 µg of protein in cleared lysates were assayed following immunoprecipitation for ERK activity using ERK1 and ERK2 antisera as shown (as described in the Methods). Data represent means ± S.E.M. of three separate experiments. * indicates inhibition of ERK activation at $P < 0.05$, by Duncan's multiple-range test, that is statistically significantly different from the activation in the control, agonist-stimulated cells. In all cases, the MCh stimulation of ERK was statistically significantly different from the corresponding basal ERK activation at $P < 0.05$, by Duncan's multiple-range test.

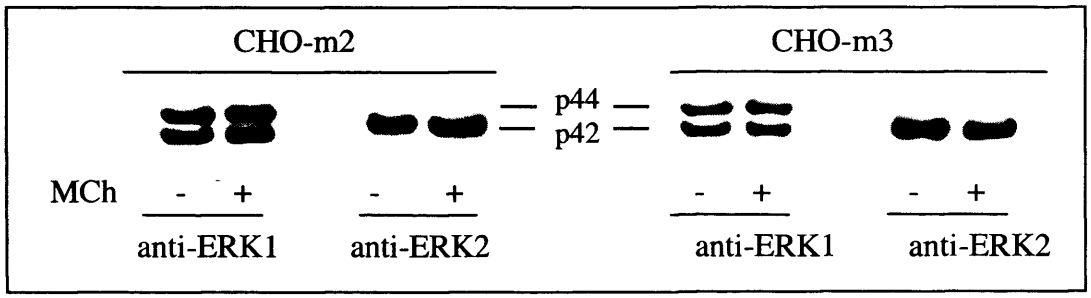

Figure 3.10 Western blot of p44-ERK1 and p42-ERK2 in CHO -m2 and CHO-m3 cells.

CHO-m2 and CHO-m3 cells were stimulated with 100 μM MCh for 5 min and cleared lysates were run on a SDS-PAGE gel (10 μg protein). p44-ERK1 and p42-ERK2 were detected using antisera for ERK1 and ERK2 as shown. Blots shown are representative of at least three separate experiments.

3.5 Expression of JNK-1 and p38 in CHO-m2 and CHO-m3 cells by western blot analysis.

In order to determine the expression of the other MAP kinase proteins, JNK and p38 in the two CHO cell lines used, an immunoblot experiment was carried out on CHO cell lysates. By western blot analysis, it was shown that JNK-1 (p46) and p38 were also present in both the CHO-m2 and CHO-m3 cells (Figure 3.11). As an equivalent amount of protein was loaded in all the lanes, it can be concluded that there was no apparent difference in the relative expression of either p38 or JNK in both of the cell lines. In the experiment, the cells were either stimulated or unstimulated with 100 μM MCh, for 5 min in the immunoblot for p38, or 15 min for the immunoblot for JNK, in CHO-m2 and 30 min for CHO-m3 cells. In all cases, there was no effect on the expression of the proteins upon activation of the receptor, which due to the time required for *de novo* protein synthesis, may be predicted.

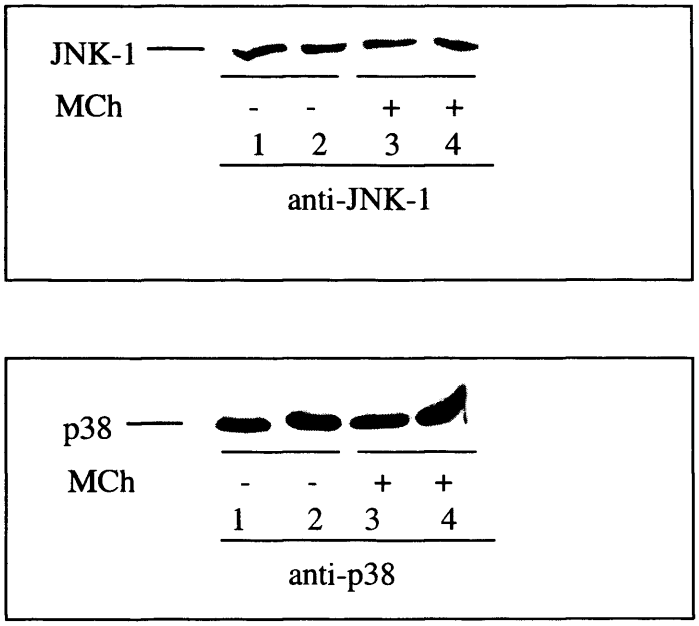

Figure 3.11. Western blot of JNK-1 and p38 in CHO-m2 and CHO-m3 cells.

The upper panel show the results for JNK-1 and the lower panel shows the results for p38. CHO-m2 and CHO-m3 cells were stimulated with 100 μM MCh for 5 min (for p38), 15 min (CHO-m2, JNK-1) and 30 min (CHO-m3, JNK-1) and cleared lysates were run on a SDS-PAGE gel (10 μg protein). JNK-1 and p38- were detected using selective antisera for JNK-1 and p38 as shown. Lane 1, CHO-m2 -MCh, lane 2, CHO-m3 -MCh, lane 3 CHO-m2 +MCh, lane 4 CHO-m3 +MCh. Blots shown are representative of at least three separate experiments.

3.6 Effect of atropine on agonist-stimulated ERK and JNK activation in CHO-m2 and CHO-m3 cells.

The muscarinic antagonist, atropine, was used to determine whether the agonist-induced response were via the muscarinic receptors in CHO-m2 and CHO-m3 cells. Atropine has K_i values of -8.9 and -9.5 for m2-AChRs and m3-AChRs respectively (Eglen *et al.,* 1996). In both the CHO-m2 and CHO-m3 cells there was no significant effect ($P < 0.05$ by Duncan's multiple-range test) on the basal activation of ERK by atropine in the absence of MCh (Figure 3.12). In CHO-m2 cells , upon a 5 min agonist stimulation in the absence of atropine, ERK was activated to 13 fold over basal, in contrast to the activation being completely inhibited in the presence of 5 μM atropine (Figure 3.12). In CHO-m3 cells, similar effects of atropine were also observed. Upon a 5 min agonist stimulation in the absence of atropine, ERK was activated to 13 fold over basal, whereas, the response was completely inhibited in the presence of 5 μM atropine (Figure 3.13).

In CHO-m3 cells upon a 30 min agonist stimulation in the absence of atropine, an 8 fold over basal JNK activation was observed, whereas, the response was almost completely inhibited in the presence of 5 μM atropine (Figure 3.14). As previously described in the results observed in both cell types for ERK activation, there was no significant effect of atropine on the basal JNK response ($P < 0.05$ by Duncan's multiple-range test).

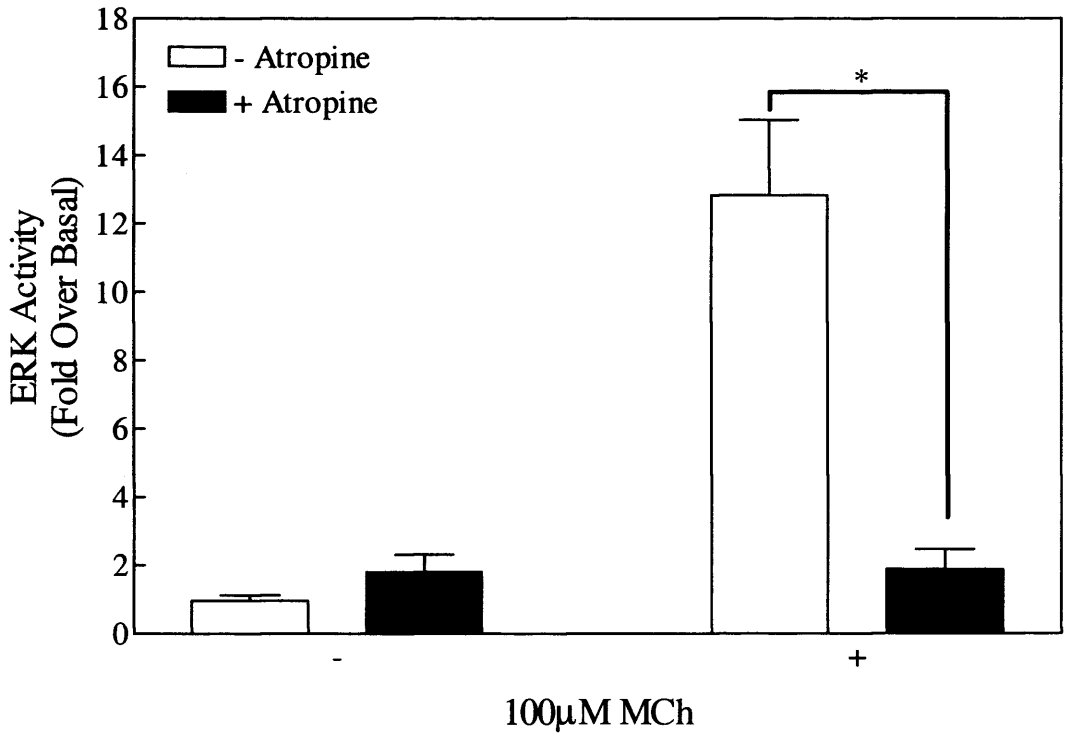

Figure 3.12. Effect of a 30 min pre-incubation with 5 μM atropine on agonist-stimulated ERK activation in CHO-m2 cells.

CHO-m2 cells were incubated with 5 μM atropine or vehicle for 30 min at 37°C prior to stimulation with 100 μM methacholine (MCh) for 5 min. Lysates containing 200 μg protein were assayed for ERK activity following immunoprecipitation (see Methods). Data represent means ± S.E.M. of three separate experiments. * indicates inhibition of ERK activation at $P < 0.05$, by Duncan's multiple-range test, that is statistically significantly different from the activation in the control, agonist-stimulated cells.

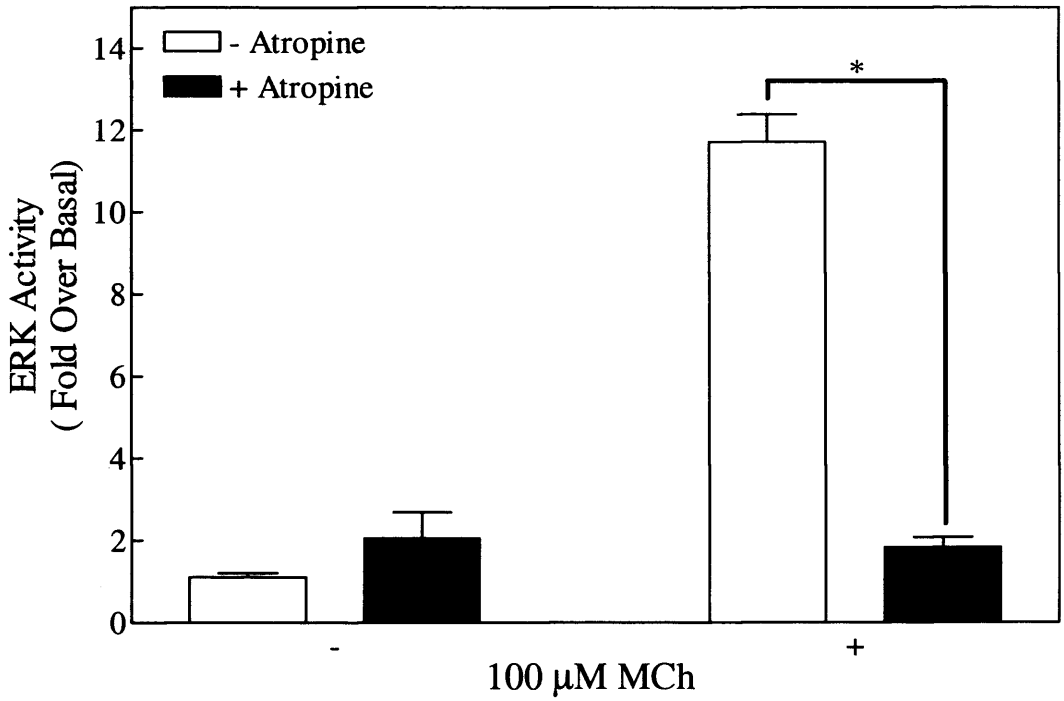

Figure 3.13 Effect of a 30 min pre-incubation with 5 μM atropine on agonist-stimulated ERK activation in CHO-m3 cells.

CHO-m3 cells were incubated with 5 μM atropine or vehicle for 30 min at 37°C prior to stimulation with 100 μM methacholine (MCh) for 5 min. Lysates containing 200 μg protein were assayed for ERK activity following immunoprecipitation (see Methods). Data represent means ± S.E.M. of three separate experiments. * indicates inhibition of ERK activation at $P < 0.05$, by Duncan's multiple-range test, that is statistically significantly different from the activation in the control, agonist-stimulated cells.

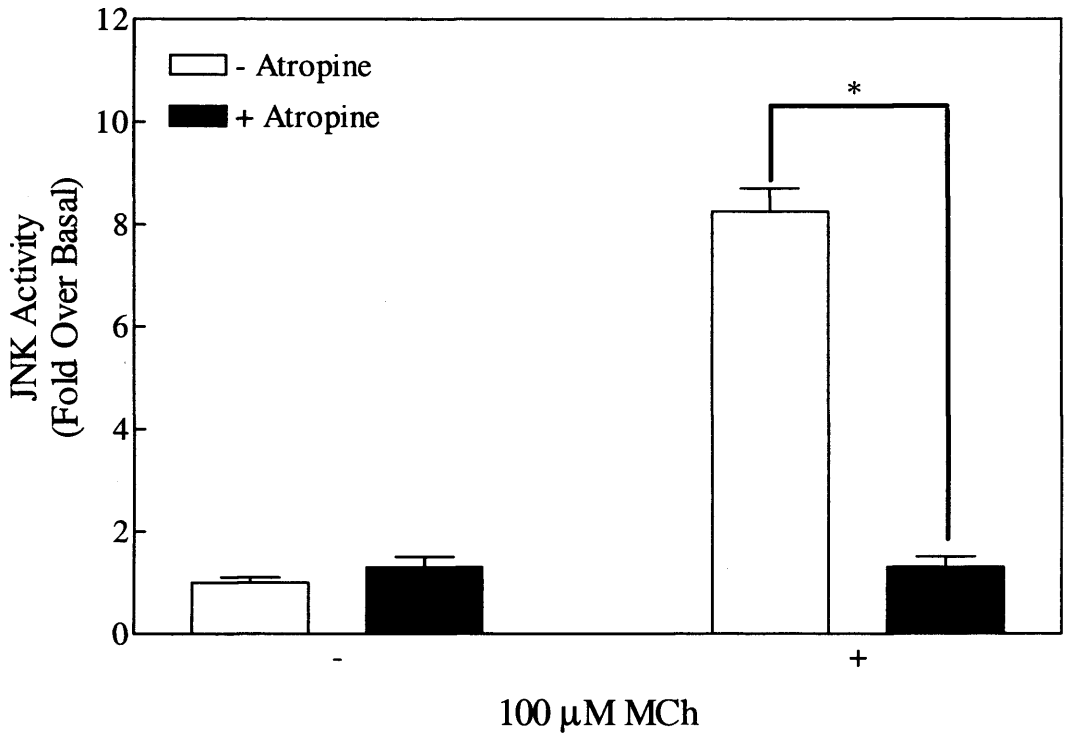

Figure 3.14. Effect of a 30 min pre-incubation with 5 μM atropine on agonist-stimulated JNK activation in CHO-m3 cells.

CHO-m3 cells were incubated with 5 μM atropine or vehicle for 30 min at 37°C prior to stimulation with 100 μM methacholine (MCh) for 30 min. Lysates containing 400 μg protein were assayed for JNK activity by GST-Jun phosphorylation (see Methods). Data represent means ± S.E.M. of three separate experiments. * indicates inhibition of JNK activation at $P < 0.05$, by Duncan's multiple-range test, that is statistically significantly different from the activation in the control, agonist-stimulated cells.

3.7 Time-dependence of ERK at EC_{50} value MCh concentrations.

In order to assess whether the time of peak ERK and JNK activation was similar at all agonist concentrations, the time-dependence of ERK and JNK activities were investigated using an EC_{50} concentration of MCh. As an approximate EC_{50} value, 5 μM MCh was used in all experiments. It was important to establish the time-course for ERK and JNK activation at a sub-maximal agonist concentration to ensure the validity of concentration-response curves. Furthermore, the use of a sub-maximal agonist concentration may allow detection of changes in agonist potency (rather than necessarily differences in maximal responses) in inhibitor studies. The results show that in CHO-m2 cells, the peak ERK activity following challenge with 5μM MCh was observed at 5 min after agonist addition. This is then followed by a rapid drop in activity, so that by 15 min after agonist addition, the response had decreased to basal values and remained at a constant level at 60 min after stimulation. (Figure 3.15). In CHO-m3 cells a rapid rise was also observed giving a peak activity at 5 min after agonist addition. There was then a sustained phase, whereby at 60 min after agonist addition, the ERK activity was 14 fold over basal (Figure 3.15). Therefore, the time to peak in both cell lines was similar at maximal and sub-maximal agonist concentrations, which therefore justifies the results from the concentration-response curves.

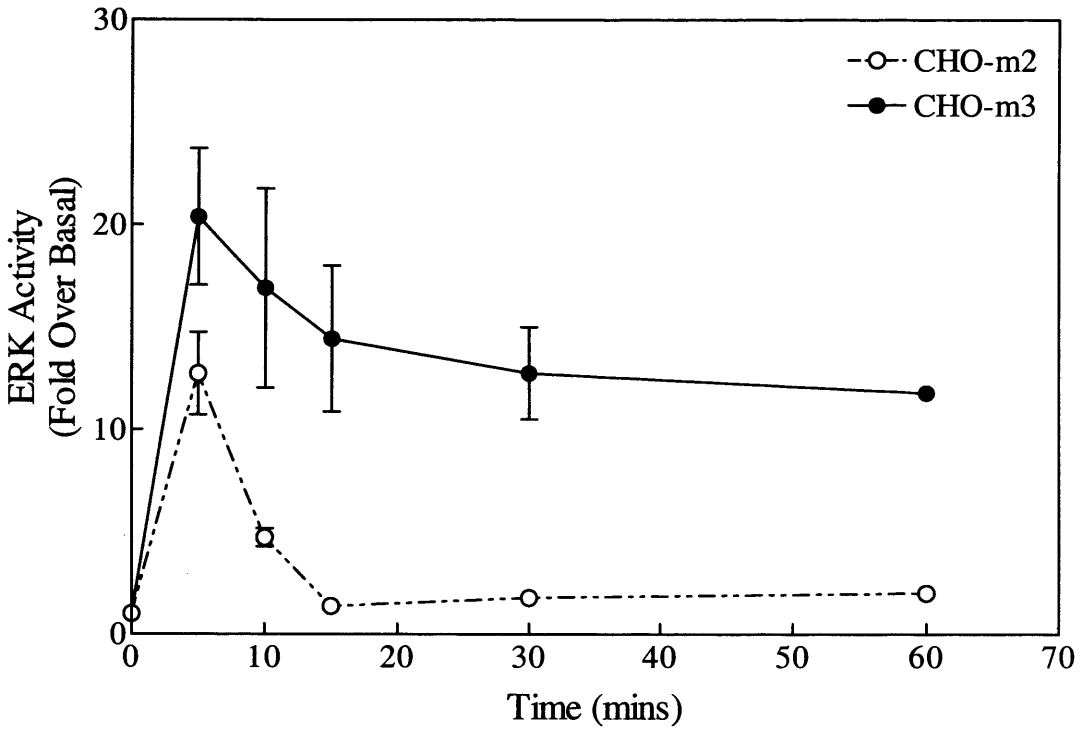

Figure 3.15 Time -dependence of ERK activation in CHO-m2 and CHO-m3 cells with 5 μM MCh.

CHO cells expressing either the m2-AChR or m3-AChR were stimulated with 5 μM MCh for the times indicated. ERK activity was immunoprecipitated from lysate containing approx. 200 μg protein using anti-ERK1 and assayed as described in the Methods section. Data represent mean ± S.E.M. of 3-4 separate experiments.

3.8 Importance of receptor occupancy by agonist in the stimulation of JNK in CHO-m3 cells.

Initial characterisation studies showed that JNK activation was considerably delayed relative to that of ERK in CHO-m3 cells. It was therefore of interest to determine whether JNK activation at later times (i.e. 30 min) required the continuous presence of agonist. In order to examine this, the muscarinic antagonist atropine was added to CHO-m3 cells at different times following addition of MCh and lysates were prepared 30 min after MCh addition. As shown in Figure 3.16, the time-course seems to be very similar

to previous time-courses with MCh alone (Figure 3.5). Assuming that there is no decay in JNK activity after the addition of atropine, then prolonged binding of agonist to the m3-AChR may be required for maximal JNK activation. Therefore, it is potentially unlikely that there is a slow activation of JNK in response to brief receptor stimulation.

In addition to this experiment, a second experiment was carried out to further investigate the role of receptor occupancy in agonist-induced JNK activation, whereby the decrease in JNK activity following removal of agonist stimulation was examined. In this experiment, CHO-m3 cells were incubated with MCh for 30 min, then atropine was added for a range of times that are indicated, prior to cell lysate being prepared in the continued presence of MCh. In the absence of atropine, by 60 min following MCh addition, the fold agonist-induced JNK activation was 7 fold over basal (Figure 3.17). In the presence of atropine, after a 30 min stimulation with MCh, followed by a 30 min incubation with atropine in the presence of agonist, the fold agonist-induced JNK activation was 5 fold over basal. These results, as shown in Figure 3.17, demonstrate that there appears to be no discernible effect on the decrease in JNK activity in the presence of atropine, compared with the decrease in JNK activity observed in the absence of atropine. These results indicate that the decrease in JNK activity is unlikely to be dependent on receptor occupation. Therefore the results from these experiments demonstrate that JNK is maximally activated at 30 min and following this, receptor occupation is not involved, perhaps due to the receptor or pathway becoming desensitised.

3.9 [^{3}H]-thymidine incorporation.

The ability of the two receptor subtypes to induce cell division when stimulated were investigated. This was carried out as by using the incorporation of [^{3}H]-thymidine as a measure of DNA synthesis. The results are perhaps somewhat surprising in that they show that stimulation of the m3-AChR with MCh has no proliferative effect whereas, stimulation of the m2-AChR with MCh does increase [^{3}H]-thymidine incorporation to up to 2 fold over basal levels in a concentration-dependent manner. However, 10% FCS strongly induces [^{3}H]-thymidine incorporation in the CHO-m3 cells, giving a fold increase of 6-7 fold over basal, in contrast the CHO-m2 cells do induce [^{3}H]-thymidine incorporation, but much more weakly (approximately 3 fold over basal). Also, the effect of LPA was investigated and found to be able to induce [^{3}H]-thymidine incorporation equally well in both the CHO-m2 and -m3 receptors, with a fold over basal value of approximately 2 fold (Figure 3.18).

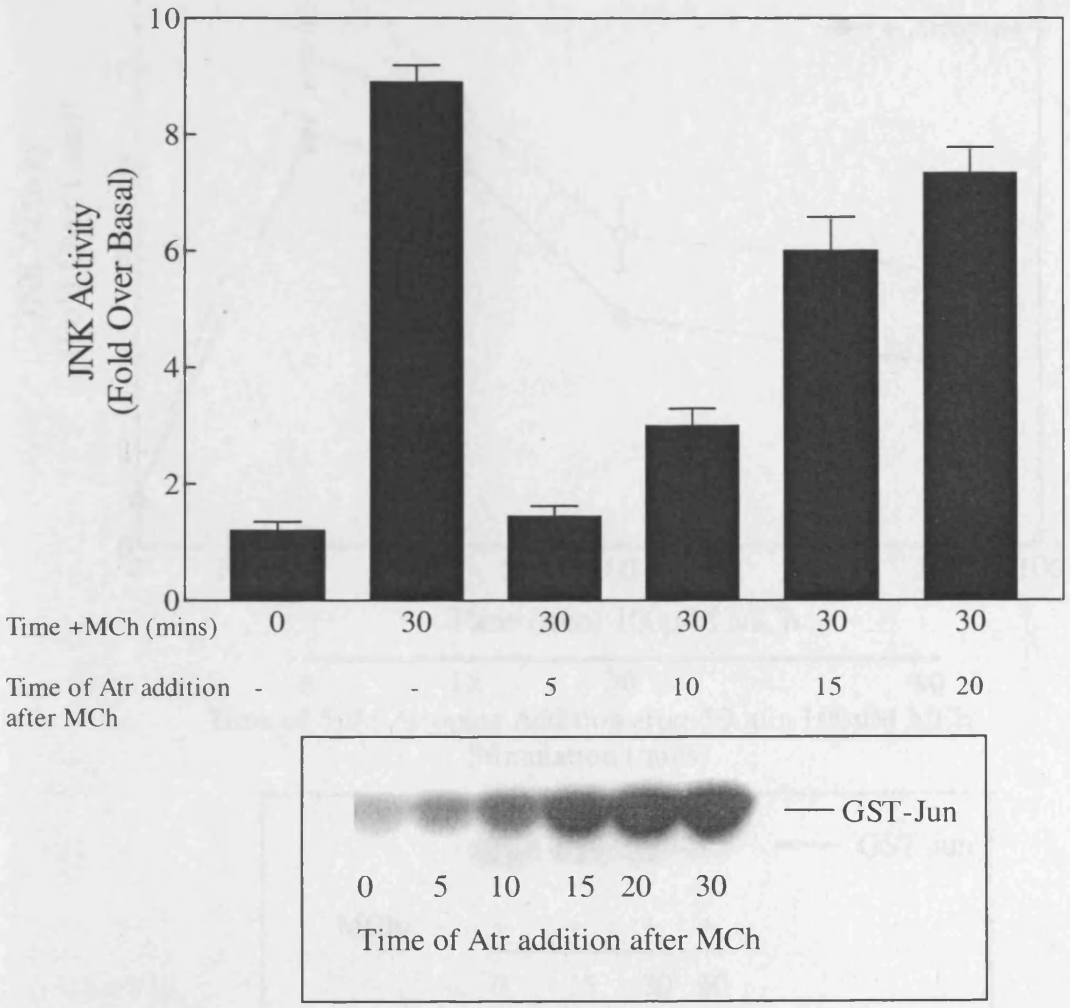

Figure 3.16 Effect of atropine on the time-dependence of JNK activation in CHO-m3 cells.

CHO cells expressing the m3 receptor were stimulated with 100 μM MCh, and then incubated with 5 μM atropine (Atr) for the times indicated prior to lysis. 400 μg of protein was assayed for JNK activity by GST-Jun phosphorylation (see Methods). The lower panel shows a representative autoradiogram of GST-Jun phosphorylation. Data represent mean ± S.E.M of three separate experiments.

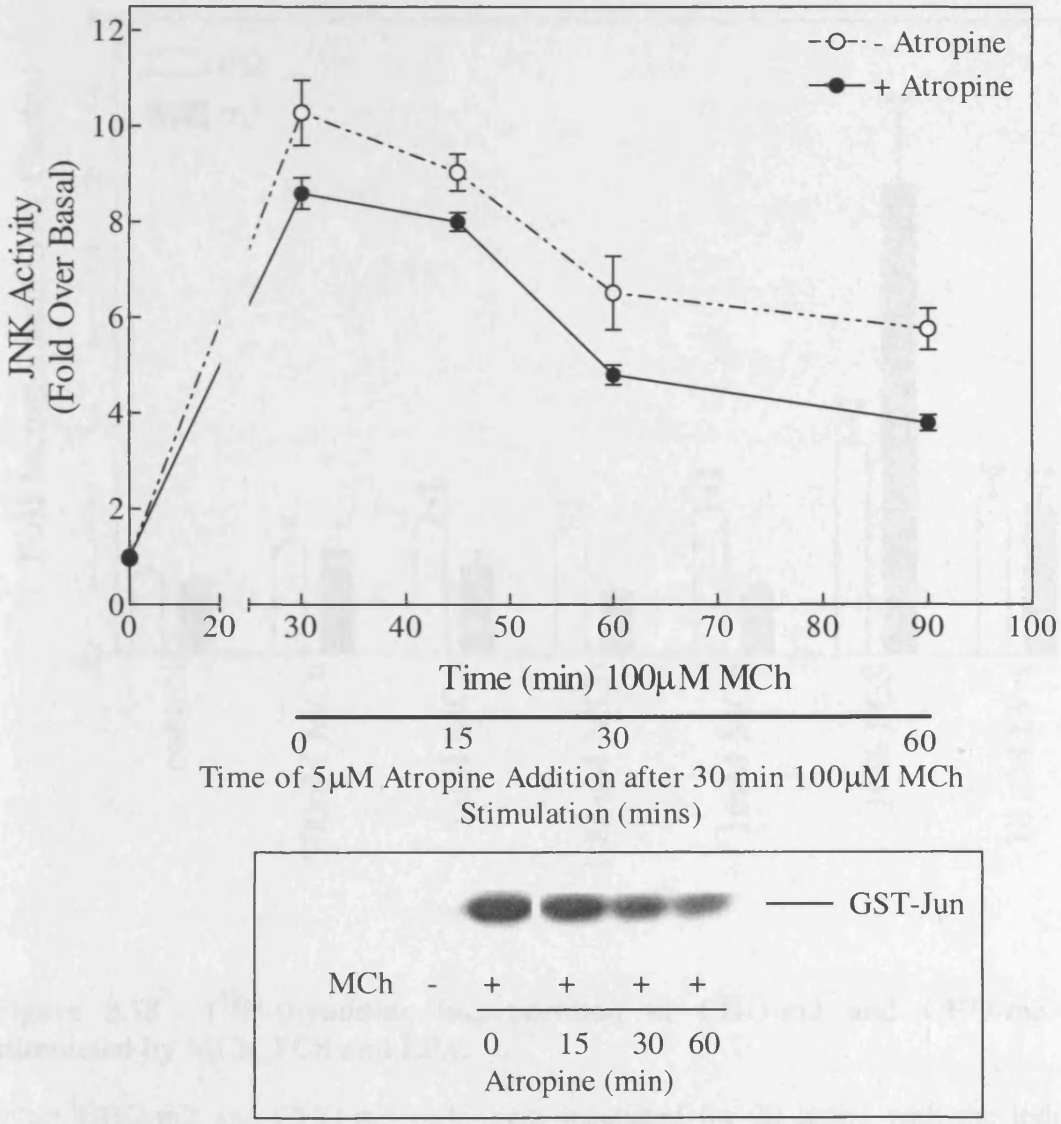

Figure 3.17 Effect of atropine on the time- dependence of JNK activation in CHO-m3 cells following agonist stimulation.

CHO cells expressing the m3 receptor were stimulated with 100 μM MCh and incubated at 37°C for 30 min. The cells were then incubated with 5 μM atropine for the times indicated prior to lysis. 400 μg of protein was assayed for JNK activity by GST-Jun phosphorylation (see Methods). The lower panel shows a representative autoradiogram of GST-Jun phosphorylation. Data represent means ± S.E.M. of three separate experiments.

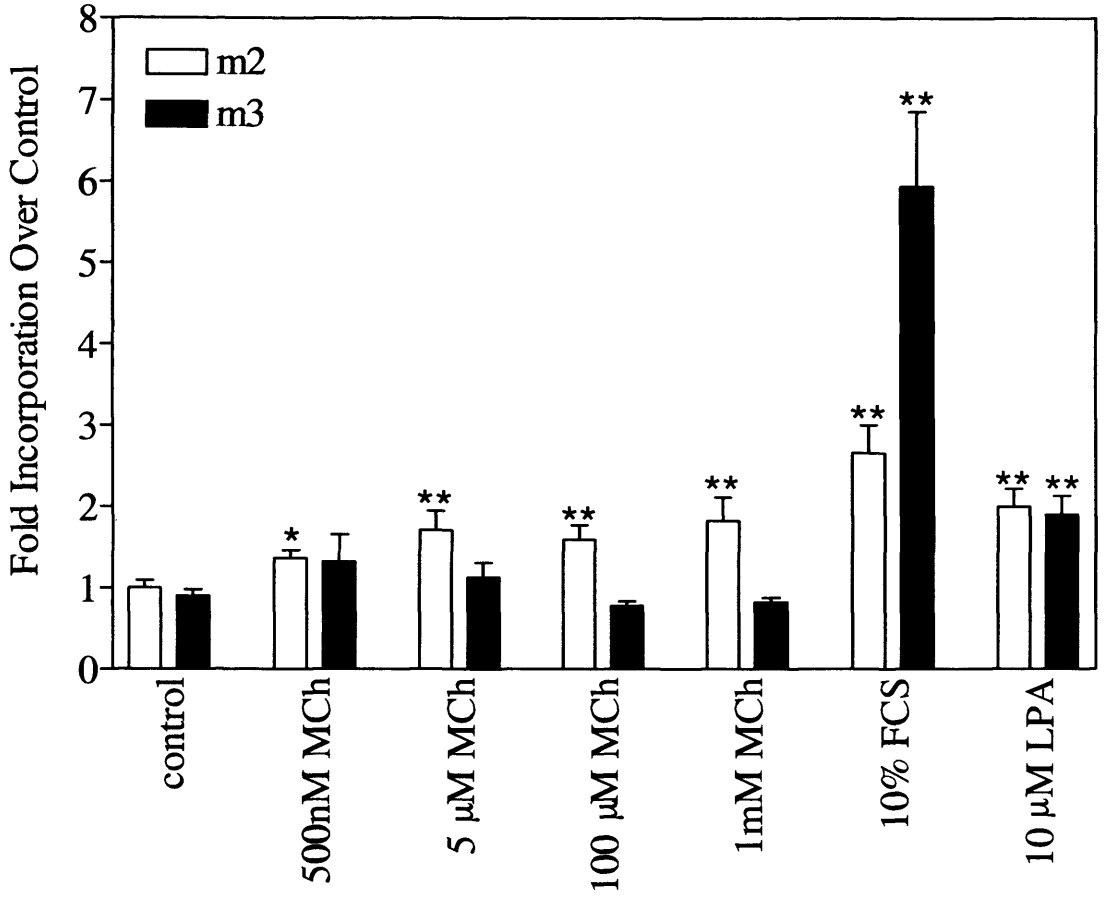

Figure 3.18 [^{3}H]-thymidine incorporation in CHO-m2 and CHO-m3 cells stimulated by MCh, FCS and LPA.

Intact CHO-m2 and CHO-m3 cells were incubated for 20 hours with the indicated concentrations of MCh, 10% FCS (Foetal Calf Serum) or 10 μM LPA. For the final 2 hours, cells were incubated with 2 μCi of [^{3}H]-thymidine and treated as described in the materials and methods section. Data represent the means ± S.E.M of 3-4 separate determinates in triplicate or quadruplicate. * indicates an increase at $P < 0.05$ by Duncan's multiple-range test, of [^{3}H]-thymidine incorporation that is statistically significantly different from control cells. ** indicates an increase at $P < 0.01$ by Duncan's multiple-range test, of [^{3}H]-thymidine incorporation that is statistically significantly different from control cells.

3.10 Discussion

In this Chapter, the relative levels of receptor expression in both the CHO-m2 and CHO-m3 cell lines has been examined. The time and agonist concentration-dependencies of ERK1, ERK2 and JNK activation have been determined, including looking at the effect on the time- dependence using a sub-maximal agonist concentration. In addition, the relative levels of protein expression between the CHO-m2 and CHO-m3 cell lines have been investigated. It has also been shown that the responses obtained are due to activation of the muscarinic receptors since inhibition of the responses was observed in the presence of the muscarinic antagonist atropine. Finally the role of receptor occupation by agonist via the m3-AChR on JNK activation in CHO-m3 cells and the possibility that they play a role in DNA synthesis was investigated.

The saturation binding experiments were analysed in two ways, the Scatchard plot and the binding isotherm plot. It has been suggested that Scatchard plots should only be used as a way of visualising saturation analysis data, as it is very easy to obtain a plot that mis-represent the data (Klotz, 1982). Klotz criticises some elements of the Scatchard plot; the plot does not use an independent variable caused by having to transform all the data to provide a linear variable. This is solved by using the free radioligand concentration as the independent variable, and the dependent variable is the bound radioligand, therefore the variable radioligand is subject to uncertainty. A second reason is that there can be significant errors where the line intercepts the X-axis (this can be accentuated when the line is extrapolated). Finally, the distribution of data points can be scattered widely making an accurate attempt at drawing the line of best fit very difficult and inaccurate.

The results from the saturation binding experiments obtained, demonstrated that the receptor expression levels were essentially the same in both of the cell lines. Binding studies were carried out periodically throughout the project and the results presented are a mean of these results demonstrating that the receptor expression levels remained constant for the duration of the project. Also as the receptor expression levels are similar, this allows for more valid comparisons to be drawn between the different receptor subtypes with respect to ERK and JNK activation. For example, the differences in ERK and JNK activation are due to the nature of the receptors and their distinct coupling to intracellular pathways rather than purely due to differences in receptor number.

In order to confirm that the activation of ERK and JNK by agonist was acting via the m2- and m3-ACh receptors, studies were carried out using the muscarinic antagonist atropine. The results demonstrated that the agonist-induced ERK activation and JNK activation observed were completely inhibited in the presence of 5 μM atropine, therefore confirming that the ERK and JNK activation observed was due to stimulation of the m2-AChR and m3-AChRs.

In this Chapter, a base characterisation of CHO cells stably expressing the m2-AChR and m3-AChR with respect to ERK1, ERK2 and JNK activity has been provided, upon stimulation with the muscarinic agonist, methacholine. The results demonstrate that both the recombinant m3- and m2-AChRs are able to activate the JNK and ERK1/2 pathways when stably expressed in CHO cells. Distinct time course characteristics between the ERK1/2 and JNK pathways in both CHO-m2 and CHO-m3 cells were observed.

In both the ERK1 and the ERK2 pathways, the maximal time for stimulation with methacholine was 5 min, with the specific kinase activity of ERK1 and ERK2 being comparable in both cell lines. By 20 min after stimulation, the response had returned to near basal values in the m2 expressing cells and 50% of peak activity in the CHO-m3 cells. A noticeable difference in ERK1 activation between the two cell lines is that the ERK1 response in the CHO-m2 cells lines, ERK activation had returned to basal values by 20 min after agonist stimulation. In contrast to this, the m3-AChR mediated response was much more sustained, whereby at 20 min after agonist stimulation, the ERK response was still 26 fold over basal and at 60 min, the response was still observed to be 5 fold over basal levels. This difference in time profile allows for the possibility of different cellular responses to be elicited upon activation of ERK when stimulated by receptors coupled to different classes of G-protein. A chronic ERK activation has been demonstrated to decrease cdk2 activity and DNA synthesis in hepatocytes, in addition to an acute ERK activation promoting G1 progression to the S phase of the cell cycle, therefore causing increased DNA synthesis (Tombes *et al.,* 1998).

As discussed in the Introduction section of this Thesis, there is evidence suggesting that the time of ERK activation is important in determining a cellular response. Sustained ERK activation has been shown to be important (in PC12 cells) for ERK translocation to the nucleus, and therefore affecting gene transcription (Traverse *et al.,* 1992; Nguyen *et al.,* 1993). However, sustained ERK activation has, conversely in fibroblasts, been shown to be responsible for cell proliferation, and not differentiation (Meloche *et al.,* 1992, Mansour *et al.,* 1994; Cowley *et al.,* 1994). Therefore, the differences in ERK activation observed in this Thesis may indicate a point of divergence

for the muscarinic receptors and their mode of action on determining whether the cell undergoes proliferation.

There was also a significant contrast between the CHO-m2 and CHO-m3 cell lines with respect to JNK activation by the muscarinic agonist methacholine. The G_i-coupled m2-AChR did activate JNK, but not very efficiently. The results showed that a maximal JNK activation of two fold over basal was achieved at 10 min following agonist stimulation. This activation however, was sustained for at least 90 min after agonist stimulation. In the CHO-m3 cells, an initial JNK activation was recorded at 10 min after agonist stimulation of the receptor. This activation peaked at 30 min giving a maximal activation of approximately 10 fold over basal. This response then begun to decline, though slowly, so that 2 hr after agonist stimulation, there was a 6 fold over basal JNK activity recorded.

These data compare to similar data reported for the m1-mACh receptor expressed in NIH3T3 cells (Coso *et al.,* 1995a) where the carbachol induced JNK activity appeared delayed with respect to ERK activity. An important observation in the results presented in this Thesis was the relative fold activation of JNK by agonist in CHO-m2 and CHO-m3 cells. As previously described data presented here show that in cells expressing the m2-AChR, maximal activation of JNK was 2 fold over basal, however, in cells expressing the m3-AChR, a maximal activation of 10 fold over basal was observed, occurring 30 min after stimulation. These data compare to a study in which the m1-AChR and m2AChR had been stably expressed in Rat la fibroblasts (Mitchell *et al.,* 1995) where they reported that m2-AChR mediated activation of JNK was poor compared with m1-AChR-mediated JNK activation. Mitchell also demonstrated that

pre-treatment of the cells with pertussis toxin with m2R completely inhibited JNK activation, providing the first evidence that a G_i coupled receptor can activate JNK, a response that has been observed and presented here in the CHO cell lines. A noticeable difference in the data here and those of Mitchell, is the absolute JNK activation times, whereby Mitchell *et al.*, report that maximal levels of JNK activation were reached by 15 min and returned to near basal levels by 30 min. Similar results were reported for JNK activation by sorbitol in ventricular myocytes cultured from neonatal rat hearts. A peak activation of 10-20 fold was observed after 30 min stimulation, this was sustained for up to 4 hours after sorbitol stimulation (Bogoyevitch *et al.*, 1995). It should be noted though, that the difference in the data presented here, and those reported by Mitchell *et al.*, and Bogoyevitch *et al.*, demonstrates the high degree of specificity of the activation of these enzymes with respect to both cell line and receptor subtypes.

There is currently no data in the literature regarding the EC_{50} values for JNK or ERK activation by muscarinic agonists in any cell type, though it has been previously reported that carbachol stimulates JNK activity in a concentration dependent manner in Rat 1a cells expressing the m1-AChR (Mitchell *et al.*, 1995). The data presented here shows that JNK and ERK activity stimulated by methacholine in CHO-m2 and CHO-m3 cells is concentration dependent, the EC_{50} values presented are in the low micromolar range, between 1-5μM. In agonist displacement studies with carbachol, the K_d values are 1 μM and 40 μM for CHO-m2 and CHO-m3 cells respectively (Burford *et al.*, 1995), and are therefore in a similar range to the EC_{50} value for ERK and JNK activation by agonist. This is interesting to note, as it is commonly thought that these pathways are towards the end of an amplification cascade, therefore, it would be predicted that the EC_{50} values for

the activation of these proteins would be much lower than the EC_{50} values of the agonist binding to the receptor. Therefore, these observations suggest that the potential for amplification of ERK or JNK mediated by second-messengers may be limited by additional factors, for example the relative levels of intracellular second messengers compared with the overexpression of the muscarinic receptors.

Recently, reports have emerged providing evidence of the importance of scaffold proteins in the MAP kinase pathways. In cells, there are a multitude of intracellular signalling pathways that interact with each other, providing a potential problem in obtaining specificity of pathway activation. However, scaffold proteins allow for the potential for proteins in these complex pathways to be accurately targeted to the correct pathways. Generally, scaffold proteins (or anchor proteins) have been demonstrated to be involved in the regulation of signal transduction pathways e.g., in yeast cells, Choi *et al.,* (1994) and Whiteway *et al.,* (1995) describe a scaffold protein called Ste5p that binds the three members of the MAP kinase pathway FUS, STE7 and STE11 in order to activate the pathway and initiate mating upon a stimulus via GPCRs to pheromones. In 1997, Dickens *et al.,* isolated a protein by yeast two hybrid analysis called JIP-1 (JNK interacting protein-1) that when overexpressed inhibited JNK mediated gene expression by causing JNK to be retained in the cytoplasm. This work was followed up by Whitmarsh *et al.,* in 1998 (and subsequently Yasuda *et al.,* 1999)where they identified JIP-1 as a mammalian scaffold protein for the JNK pathway that selectively binds JNK (MAPK), MKK7 (MAPKK) (but not MKK4) and the mixed-lineage kinases, MLK3 and DLK (MAPKKK). The work also suggest that there is an association with HPK1, which is has been suggested to be an upstream activator of the mixed-lineage kinases. In

addition to this work, Schaeffer *et al.*, (1998) identified a protein called MP1 (MEK Partner 1) again by a yeast two hybrid system, which when bound to MEK1 and ERK1, increased the activation of these enzymes. Assuming that these proteins are present and required for the activation of the MAP kinase pathways in the cells used in this study, and if there is a requirement for the presence of a scaffold protein, to which the kinases in the pathways can be bound, then the potential for an amplification cascade would be reduced.

The data obtained from immunoblot analysis showed that the ERK1 antibody was cross reactive with both ERK1 and ERK2, whereas, the ERK2 antibody was highly specific for the ERK2 protein. These results suggested that in the ERK assays, what in fact was assayed as ERK1 was a combination of ERK1 and ERK2. This was investigated in the ERK assays by immunoprecipitation using ERK1, ERK2 and a ERK1/ERK2 combination. The results showed that immunoprecipitating with ERK1 alone gave similar results as immunoprecipitating with ERK1 and ERK2. This may indicate that the ERK1 antiserum is immunoprecipitating both ERK1 and ERK2. As was shown in the time-dependence experiments, immunoprecipitation with ERK2 alone gave 50% of the ERK1 values. As previously discussed, the data presented does not confirm that the ERK1 antibody is immunoprecipitating both ERK1 and ERK2, while the ERK2 antibody is immunoprecipitating ERK2 only. This could be further investigated by immunoprecipitating with each ERK antibody, and then western blotting the remaining lysate in addition to the immunoprecipitate with both antibodies. For example, if a lysate was immunoprecipitated with ERK1 and the subsequent western blot was shown to have both ERK1 and ERK2 this would demonstrate that ERK1 did indeed immunoprecipitate

both ERK1 and ERK2, and would give an indication of the relative efficiencies for both isoforms. Therefore using the data presented in this Thesis, although the immunoprecipitation efficiency of the ERK1 antibody is unknown, by western blot the ERK1 antibody has been shown to recognise both ERK1 and ERK2, and therefore the ERK1 antibody was subsequently used to assay for total ERK activity.

Detection of ERK1, ERK2, JNK and p38 by western blot analysis demonstrated that the proteins are all expressed in CHO cells. As lysates of CHO-m2 and CHO-m3 were loaded on the gel at similar protein concentrations, it was also shown that ERK1, ERK2, JNK and p38 were expressed at similar levels when comparing expression of a protein between the two cell lines. However, unsurprisingly, the western blot analysis did show that the levels of ERK1, ERK2, JNK and p38 did not increase from unstimulated compared with stimulated samples, therefore demonstrating that the increase of activity is due to the protein becoming activated, not an increase in the amount of protein.

As the JNK response was delayed with respect to ERK activity and peaked at 30 min after agonist addition, it seemed that there may have been a possibility of a requirement for the agonist to occupy the receptor for the duration of the JNK response. This was investigated by two different experiments and the data together suggest that the m3-AChR requires constant receptor occupation by agonist. By 30 min after agonist addition, it would be expected that a large proportion of the m3-AChR would be desensitised, therefore this data suggests that JNK may be partly dependent on receptor internalisation. The internalisation of GPCRs have been previously demonstrated to have a role in the activation of MAP kinases, in HEK293 cells expressing the β_2-adrenergic receptor, by using inhibitors of receptor internalisation (e.g. Concanavalin A), activation

of ERK1/2 via Raf was inhibited (Daaka *et al.*, 1998)

The initial experiment to investigate the role of receptor occupancy was carried out by stimulating the receptor with agonist, then adding atropine for a range of times, and subsequently assaying for JNK activation 30 min after initial agonist addition. The results from this experiment suggest that occupancy of the receptor is required for agonist-induced JNK activation. In the absence of any atropine, a time-course of agonist stimulated JNK activation showed that 30 min of agonist stimulated JNK maximally. However, if the receptor was stimulated with agonist for less than 30 min but the JNK activation assayed at 30 min after MCh addition, this gave the same results to those obtained in the absence of atropine. Therefore, these results suggest that sustained receptor occupation is required for maximal JNK activation and the delayed JNK activation is not due to a delay via intracellular mechanisms.

The second experiment to investigate receptor occupancy examined the decrease in JNK activity in the absence of MCh. In this experiment, the CHO-m3 cells were incubated with 100 μM MCh for 30 min, then atropine was added at a range of times in the continued presence of agonist and the lysates assayed. The results demonstrated that in the absence of agonist, JNK activity decreased at approximately the same rate as that seen in the continuous presence of agonist. When the decrease in JNK activity is expressed as a percentage of the maximal, then this analysis gives a decrease of JNK activity at 60 min following the peak activation of 30 % in the absence of atropine, compared with a decrease of 40 % in the presence of atropine, which with the data presented was not statistically significant (by a Duncan's multiple range test).

These results together may lead to the suggestion that agonist occupation is at least partially required for JNK activation as any addition of atropine (i.e. if the agonist was not present for the entire 30 min) lead to a decreased activation of JNK with time, that was similar to when just agonist was present as described earlier. If receptor occupancy by agonist is required for JNK activation, it may be expected that addition of atropine after a pre-stimulation with MCh should completely inhibit JNK activation or at least increase the rate of decay. The data presented demonstrate that the decrease in JNK activation is unchanged when compared with the absence of atropine. A possible reason for this may be due to receptor internalisation, so that the receptors that are internalised in the cell are inaccessible to the atropine, and therefore are still able to activate JNK, and the decrease in activity is not able to be reduced with atropine. In conclusion the results do not provide a conclusive answer to the role of receptor occupation on JNK activation. There may be some involvement, but with the results presented, it is probable that the decrease in JNK activation is independent of the receptor and is likely to be derived from some other mechanism such as phosphatase activity.

Finally, the ability of the m2-AChR and m3-AChR in inducing DNA synthesis was investigated. The results showed that the m2-AChR was able to induce [^{3}H]-thymidine incorporation, whereas the m3-AChR was unable to do this. This was perhaps a surprising result, however, there may be a number a possible explanations for this. The first is to look at the differences between the activation of the ERK and JNK pathways by the two receptors. As previously discussed, the length of time that ERK is activated may be responsible for determining whether the cell undergoes proliferation or differentiation. However, apart from the differences in the decrease of ERK activation after

approximately 20 min, both of the receptors activate ERK in a very similar manner, but the activation of the JNK pathway in both cell lines is very different. Therefore, it may be reasonable to suggest that the JNK pathway is the major component involved. It would be of interest to investigate the role of JNK in [^{3}H]-thymidine incorporation, to determine if JNK activation inhibits DNA synthesis. However, recent experiments carried out by Dr. R. Patel (Dept of Biochemistry, University of Leicester) using bromodeoxyuridine as a visual measure of DNA synthesis, suggested that although the cells were serum starved for a total of 48 hours, approximately 50 % of the cells were still undergoing cell division. This inability to quesce by serum starvation the cells may also be a contributing factor to the results obtained and presented in this Thesis. It is reasonable to assume that the cell division mechanism is working at an almost maximal rate and further activation of the m2- or m3-AChR is unable to further induce [^{3}H]-thymidine incorporation. Further experiments will have to be performed to investigate this in more detail.

Chapter 4

Modulation of ERK and JNK Activities in CHO-m2 and CHO-m3 cells by Intracellular and Extracellular Ca^{2+}

4.1 Introduction.

Many extracellular signals mediate their effects within the cell by inducing an increase in intracellular Ca^{2+} levels by either allowing the entry of Ca^{2+} via plasma membrane Ca^{2+} channels and/or Ca^{2+} mobilisation from intracellular stores (Berridge, 1993b). In this Chapter, the effects of Ca^{2+} on the ERK and JNK responses in the two cell lines were investigated, where the m3-AChR is classically coupled to PLC and the mobilisation of intracellular Ca^{2+}, compared with the m2-AChR where the receptor is coupled to G_i G-proteins and does not have a Ca^{2+} mobilising role.

GPCRs linked to the phosphoinositide pathway (via G_q-proteins) and adenylyl cyclase inhibition (via G_i-proteins) have been shown to be involved in the activation of the MAP kinases (Koch *et al.*, 1994; Crespo *et al.*, 1994). The m3-mACh receptor has been shown to couple to the PTX-insensitive G_q G-protein, which is coupled to PLC activation to elicit hydrolysis of PIP_2 to IP_3 (and DAG), thereby evoking Ca^{2+} release. Conversely, m2-AChRs couple to PTX-sensitive G_i G-proteins, that act to inhibit adenylyl cyclase and decrease cAMP levels. Receptors that couple to Ca^{2+} mobilising pathways have

been shown to regulate ERK via Ras-dependent and Ras-independent pathways (Gutkind, 1998). Recent studies have identified roles for Ca^{2+} in the regulation of JNK (Mitchell *et al.*, 1995; Zohn *et al.*, 1995). Therefore, the aim of the experiments reported in this Chapter was to examine the roles of Ca^{2+} in the agonist-induced activation of ERK and JNK in CHO cells stably expressing the m2- or m3-AChRs.

4.2 Intracellular Ca^{2+} release in CHO-m2 and CHO-m3 cells.

Before an assessment could be made of the potential roles of Ca^{2+}_e and/or Ca^{2+}_i in ERK and JNK activation, it was important to determine the effect of MCh on the release of Ca^{2+} in the two CHO cell lines. This was investigated by the use of the Ca^{2+} sensitive dye fura-2. In addition, later experiments were carried out to investigate the role of Ca^{2+} on ERK and JNK activation. This was achieved by decreasing the levels of Ca^{2+}_e alone, (thereby preventing Ca^{2+} influx across the plasma membrane), and by decreasing the levels of Ca^{2+}_e and Ca^{2+}_i, (which prevented the influx of Ca^{2+} across the plasma membrane and the release of Ca^{2+} from intracellular stores). In order to obtain this condition, thapsigargin was added, to the buffer, which acts to inhibit the Ca^{2+} ATPase (which pumps Ca^{2+} back into the stores) on the membrane of the Ca^{2+} stores. This prevents the Ca^{2+} stores from being refilled and therefore they become empty as the Ca^{2+} leaks out down the concentration gradient (or via release by activation of IP_3 receptors). Therefore, it was important to investigate the effect of thapsigargin on agonist-mediated Ca^{2+} release directly in the two cell lines.

4.2.1 Agonist-induced intracellular Ca^{2+} (Ca^{2+}_i) release in CHO-m3 cells.

Intracellular Ca^{2+} release was investigated in whole cells upon stimulation by 100 μM MCh in KHB +Ca^{2+}, nominally Ca^{2+}- free KHB and KHB - Ca^{2+} + 100 μM EGTA. Upon receptor stimulation, in CHO-m3 cells in KHB +Ca^{2+}, a maximum cytosolic Ca^{2+} concentration of approximately 600 nM was achieved within 10 sec of agonist addition (Figure 4.1) which was then followed by a sustained plateau phase between 200-300 nM. This sustained plateau was due to the influx of extracellular Ca^{2+} from the KHB into the

cell. Agonist stimulation in nominally Ca^{2+}- free KHB (KHB with no $CaCl_2$ added) caused a cytosolic Ca^{2+} peak of approximately 300 nM, however there was no sustained plateau phase due to the absence of Ca^{2+}_e in the KHB, and the level of Ca^{2+}_i returned to basal levels within 90 sec after agonist addition (Figure 4.1). The result demonstrating the reduction in the peak Ca^{2+} release upon agonist addition indicates that the Ca^{2+} influx across the plasma membrane is also an important component of the agonist-induced peak in Ca^{2+}_i. In the absence of Ca^{2+}_e by adding nominally Ca^{2+}- free KHB and further Ca^{2+} chelation by EGTA, the cytosolic Ca^{2+} release gave a peak Ca^{2+}_i level of only 100-150 nM, which also did not have a plateau phase and returned to basal values within 60 sec after agonist addition (Figure 4.1).

4.2.2 Agonist-induced Ca^{2+}_i release in CHO-m2 cells.

The intracellular Ca^{2+} levels upon stimulation by 100 μM MCh were investigated in CHO-m2 cells using fura-2-AM and using cells incubated in KHB +Ca^{2+}, nominally Ca^{2+}- free KHB, and KHB -Ca^{2+} + 100 μM EGTA. Upon receptor stimulation in the presence of Ca^{2+}_e, a maximum 2 fold increase in the cytosolic [Ca^{2+}] value to approximately 100 nM was obtained (Figure 4.2) which was then followed by a plateau phase probably due to the presence of Ca^{2+}_e influx from the buffer into the cells. Surprisingly, agonist stimulation in nominally Ca^{2+}- free KHB (KHB with no $CaCl_2$ added) gave a greater increase in cytosolic Ca^{2+} to approximately 160 nM, but with no sustained plateau phase; whilst in the complete absence of Ca^{2+}_e by chelation with EGTA, the peak cytosolic Ca^{2+} level in response to MCh was greater still (~195 nM). As the m2-AChR is coupled to G_i and inhibition of adenylyl cyclase, which has no effect on Ca^{2+} mobilisation, this result is somewhat surprising. It is even more surprising that the

Ca^{2+} elevation induced by MCh was reproducibly enhanced when Ca^{2+}_e was chelated. Therefore in these cells, the m2-AChR, is at least to a small extent, coupled to a Ca^{2+} mobilising pathway.

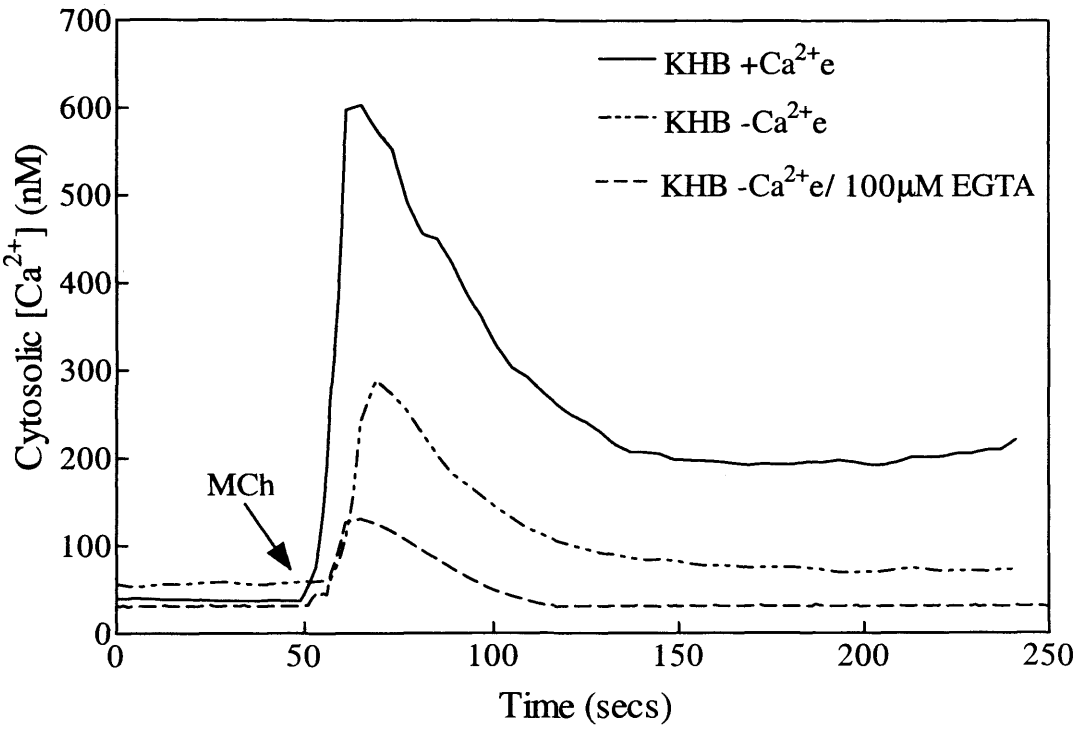

Figure 4.1 Intracellular Ca^{2+} release in CHO-m3 cells.

Cytosolic Ca^{2+} concentration was measured in fura-2/AM-loaded intact CHO-m3 cells stimulated with 100 μM MCh as indicated, in the presence of 1.3 mM $CaCl_2$, or under calcium-depleted conditions; i.e. nominally-free calcium, or under removal of total extracellular Ca^{2+} (- Ca^{2+} /+EGTA (100 μM)). Data are representative of results obtained from four separate experiments.

4.3 Effect of thapsigargin on Ca^{2+}_i release in CHO-m2 and CHO-m3 cells.

In experiments to investigate the role of Ca^{2+} in ERK and JNK activation it was necessary to completely prevent Ca^{2+}_i elevation and therefore cells needed to be incubated with thapsigargin. It was therefore important to determine the effect of thapsigargin *per se* on Ca^{2+} release in the two cell lines.

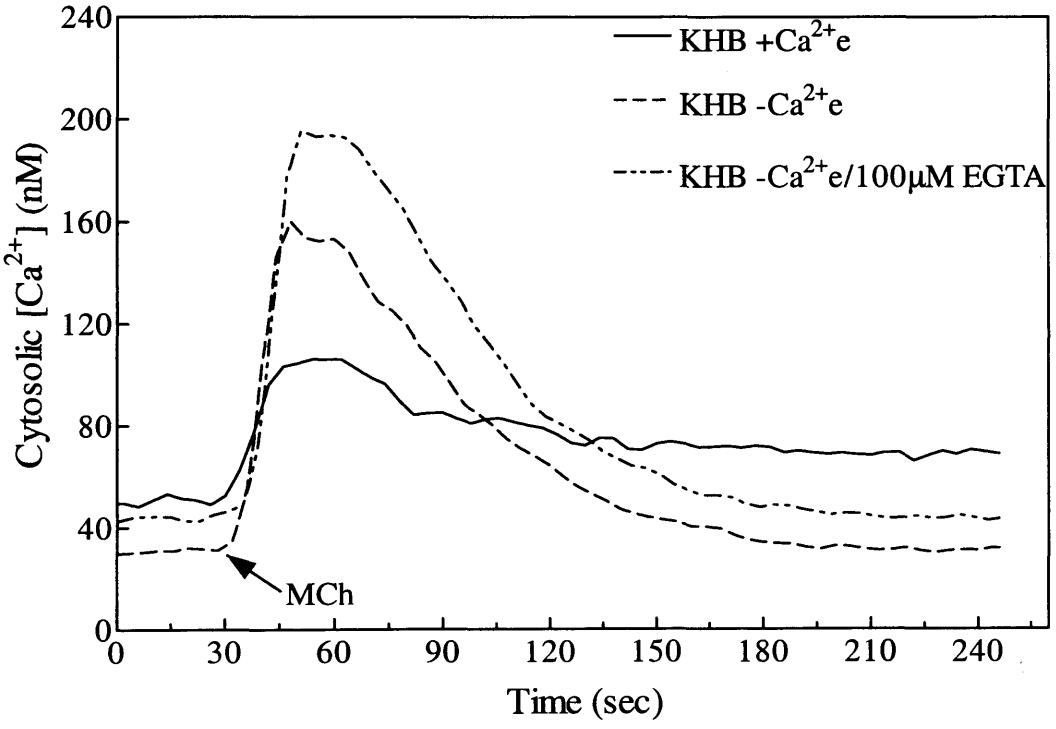

Figure 4.2. Intracellular Ca^{2+} release in CHO-m2 cells.

Cytosolic Ca^{2+} concentration was measured in fura-2/AM-loaded intact CHO-m2 cells stimulated with 100 μM MCh as indicated, in the presence of 1.3 mM $CaCl_2$, or under calcium-depleted conditions; i.e. nominally Ca^{2+}- free, or under removal of total extracellular Ca^{2+} (-Ca^{2+} /+ EGTA (100 μM). Data are representative of results obtained from three separate experiments.

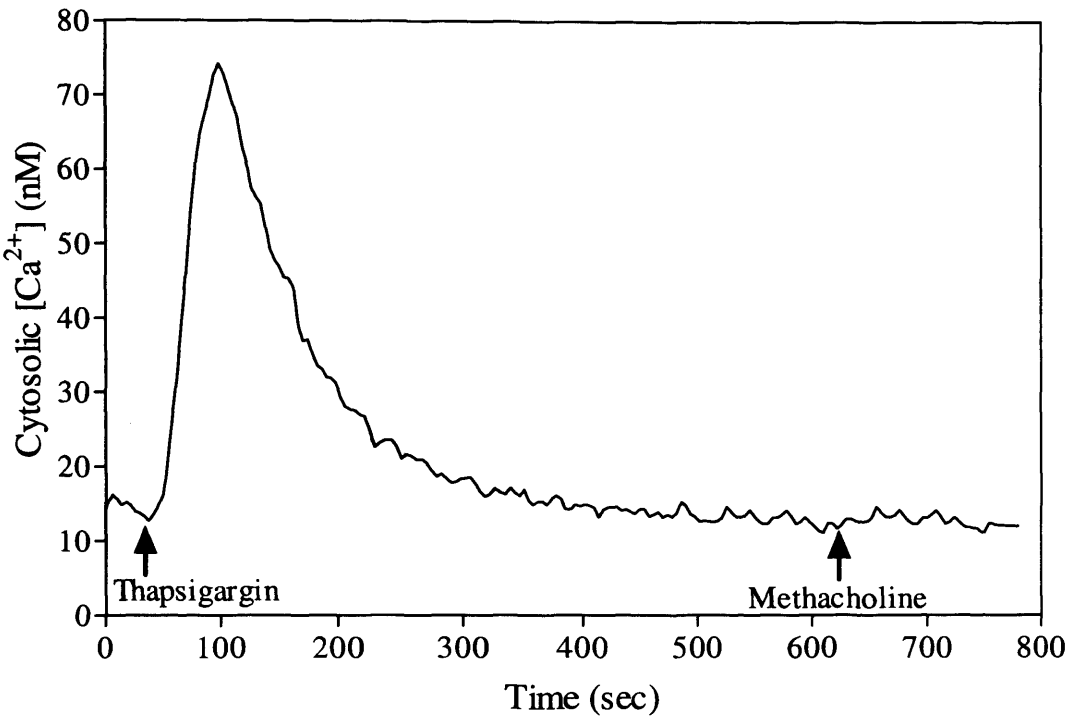

Figure 4.3. Effect of thapsigargin on $[Ca^{2+}]_i$ release in CHO-m3 cells in the absence of Ca^{2+}.

Cytosolic Ca^{2+} concentration was measured in fura-2/AM-loaded intact CHO-m3 cells in the presence of 2 μM thapsigargin, under calcium-depleted conditions followed by 100 μM MCh. Data are representative of results obtained from at least three separate experiments.

4.3.1 Effect of thapsigargin on $Ca^{2+}{}_i$ release in CHO-m3 cells

When cells were incubated in buffer in the absence of $Ca^{2+}{}_e$ (i.e. no $CaCl_2$) and the addition of 100 μM EGTA, stimulation of the cells with 2 μM thapsigargin gave a rapid rise in intracellular Ca^{2+} to approximately 100 nM by 50 sec which then slowly returned to basal levels. 10 min after the cells were treated with thapsigargin, they were stimulated by the addition of 100 μM methacholine. The results show that this gave no further increase in cytosolic Ca^{2+} (Figure 4.3). When cells were incubated in KHB in the

presence of Ca^{2+} upon addition of 2 μM thapsigargin, a peak Ca^{2+} release of approximately 700 nM was reached by 40 sec. If MCh (100 μM) was added at this point, no further increase in Ca^{2+}_i was observed (Figure 4.4). These data demonstrate that in the absence of Ca^{2+}_e a 10 min stimulation with thapsigargin completely depletes the Ca^{2+} stores, as there is no further Ca^{2+} release upon agonist stimulation. In the presence of Ca^{2+}_e, there is a sustained increase in cytosolic Ca^{2+} concentration due to intracellular store depletion, inducing an increased Ca^{2+}-influx probably via a capacitative Ca^{2+}-entry pathway (Van Breeman 1989; Putney, 1990; Putney and Bird 1993). These results are unsurprising, as the m3-AChR is known to couple to PLC-β, which has been demonstrated to hydrolyse phosphatidylinositol 4,5-bisphosphate (PIP_2) to IP_3, which then acts via IP_3 receptors to mobilise Ca^{2+} (Caulfield *et al.*, 1993).

4.3.2 Effect of thapsigargin on Ca^{2+}_i release in CHO-m2 cells.

The results of these experiments were essentially similar to those described for the CHO-m3 cells, as the experiments are involving manipulations that are independent of the receptor. When CHO-m2 cells were incubated in buffer in the absence of Ca^{2+}_e (i.e. no $CaCl_2$) and the addition of 100 μM EGTA, stimulation of the cells with 2 μM thapsigargin gave a rapid rise in intracellular Ca^{2+} to approximately 100 nM by 50 sec which then slowly returned to basal levels by 400 sec (Figure 4.5). 10 min after stimulation with thapsigargin, addition of 100 μM methacholine gave no further cytosolic Ca^{2+} release, indicating that the Ca^{2+} stores had been emptied. When cells were incubated in KHB in the presence of Ca^{2+}, upon addition of 2 μM thapsigargin, a peak Ca^{2+} release of 700 nM was reached by 40 sec, which remained constant following the addition of 100 μM MCh, added 2 min later (Figure 4.6).

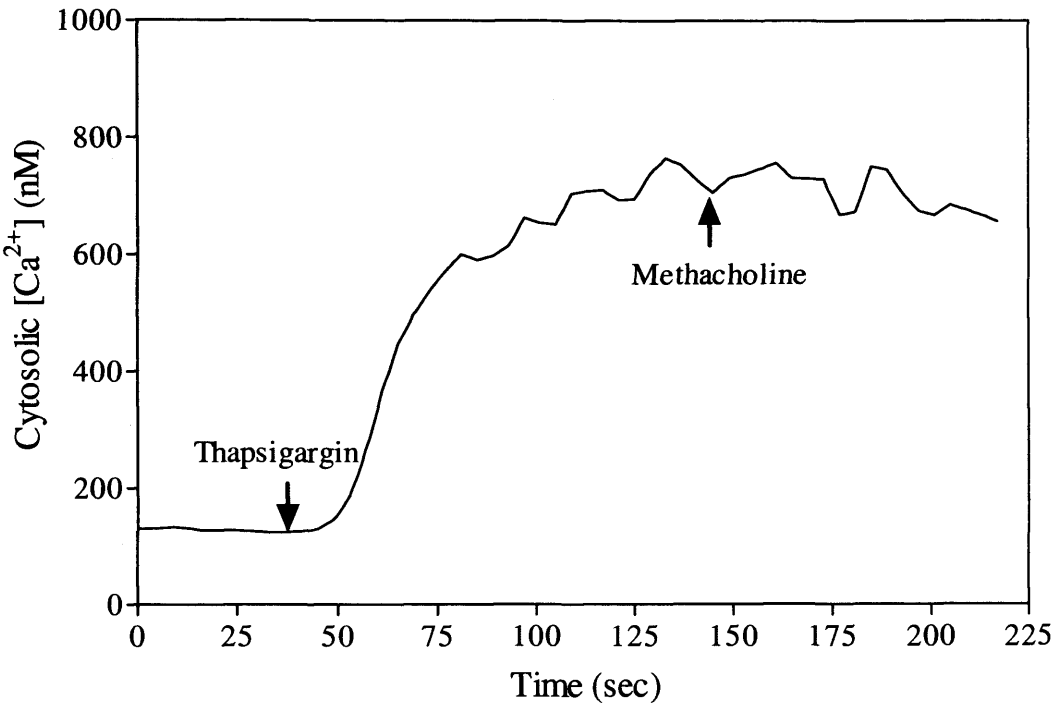

Figure 4.4 Effect of thapsigargin on $[Ca^{2+}]_i$ release in CHO-m3 cells in the presence of Ca^{2+}.

Cytosolic Ca^{2+} concentration was measured in fura-2/AM-loaded intact CHO-m3 cells in the presence of 2 μM thapsigargin, in the presence of 1.3 mM Ca^{2+} followed by 100 μM MCh. Data are representative of results obtained from at least three separate experiments.

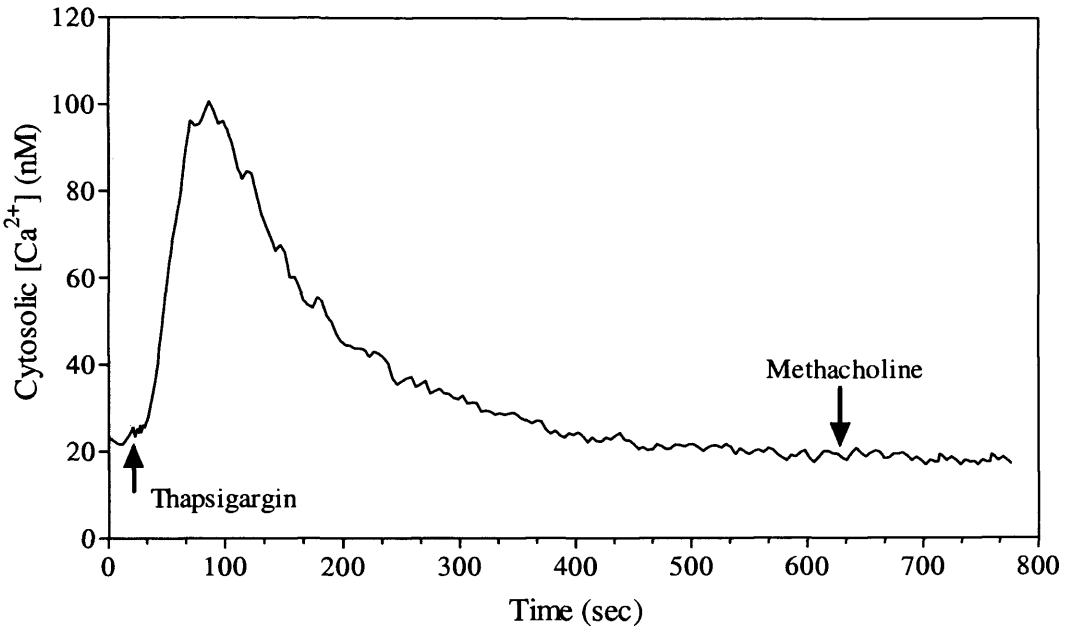

Figure 4.5. Effect of thapsigargin on $[Ca^{2+}]_i$ release in CHO-m2 cells in the absence of Ca^{2+}.

Cytosolic Ca^{2+} concentration was measured in fura-2/AM-loaded intact CHO-m2 cells in the presence of 2 μM thapsigargin, under calcium-depleted conditions (KHB-Ca^{2+} + 100 μM EGTA) followed by 100 μM MCh. Data are representative of results obtained from three separate experiments.

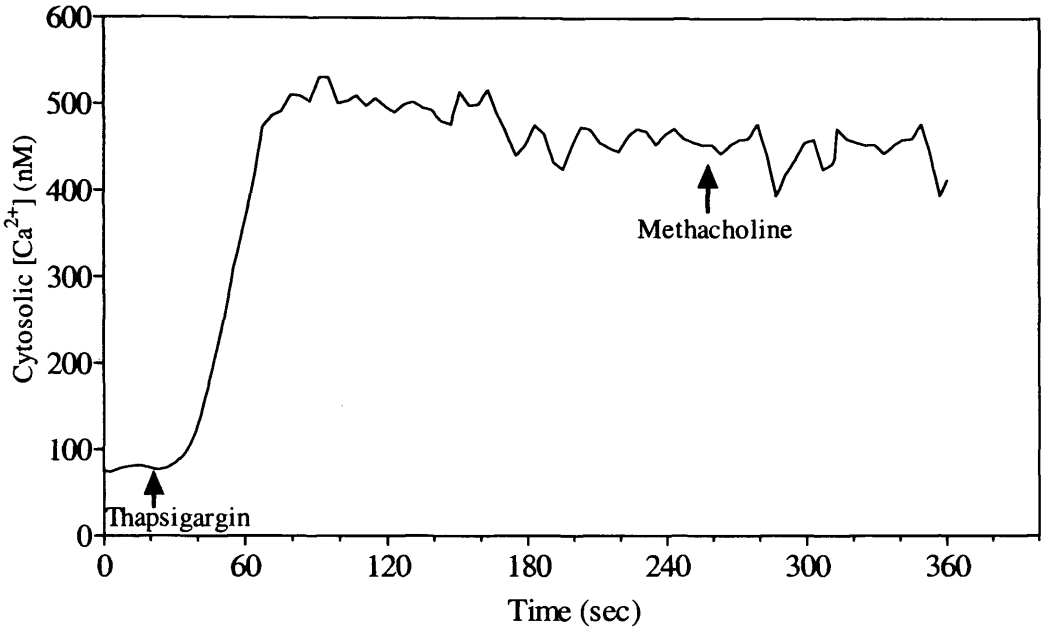

Figure 4.6. Effect of thapsigargin on $[Ca^{2+}]_i$ release in CHO-m2 cells in the presence of Ca^{2+}.

Cytosolic Ca^{2+} concentration was measured in fura-2/AM-loaded intact CHO-m2 cells in the presence of 2 μM thapsigargin, in the presence of 1.3 mM Ca^{2+} followed by 100 μM MCh. Data are representative of results obtained from at least three separate experiments.

4.4 Ca^{2+}- dependence of agonist-stimulated ERK and JNK activities in CHO-m2 and CHO-m3 cells.

Since the m3-AChR is a Ca^{2+}-mobilising receptor coupled to PLC, the contribution of Ca^{2+} to the activation of ERK and JNK was investigated in CHO-m3 cells, but as the m2-AChR also mobilised Ca^{2+}, but to a smaller degree, it was of interest also to examine the role of Ca^{2+} in agonist-stimulated ERK (and JNK) activation in CHO-m2 cells.

4.4.1 Ca^{2+}- dependence of agonist-stimulated ERK activity in CHO-m2 and CHO-m3 cells.

CHO cells were pre-incubated for 20 min in 'normal' KHB (i.e. KHB containing 1.3 mM $CaCl_2$), then 10 min prior to agonist stimulation, the cells were incubated under one of the following conditions; (i) KHB $+Ca^{2+}$, (ii) KHB $-Ca^{2+}$ (i.e. KHB $-CaCl_2$) + 100 μM EGTA, or (iii) KHB $-Ca^{2+}$ +100 μM EGTA + 2 μM thapsigargin. The cells were then either treated with 100 μM methacholine or vehicle. The results demonstrated that in CHO m2 cells there was no effect upon ERK activation of removing either Ca^{2+}_e (i.e. Ca^{2+} from the buffer alone) or removing both Ca^{2+}_e (from the buffer) and Ca^{2+}_i from within the cell (by depletion of Ca^{2+} stores) on the basal or the agonist-induced ERK activities (Figure 4.7). A similar result was also observed in the CHO-m3 cells, whereby, the removal of Ca^{2+}_e or the depletion of Ca^{2+}_i using the Ca^{2+}- ATPase inhibitor thapsigargin, had no effect on basal or agonist-induced ERK activities (Figure 4.8).

4.4.2 Ca^{2+}-dependence of agonist-stimulated JNK activity in CHO-m2 and CHO-m3 cells.

CHO cells were pre-incubated for 20 min in 'normal' KHB (i.e. KHB containing 1.3mM $CaCl_2$), then 10 min prior to agonist stimulation, the cells were incubated under one of the following conditions; KHB $+Ca^{2+}$, KHB $-Ca^{2+}$ (i.e. KHB $-CaCl_2$) + 100 μM EGTA, or KHB $-Ca^{2+}$ + 100 μM EGTA + 2 μM thapsigargin. The cells were then treated either with 100 μM methacholine or vehicle. The results demonstrated that in CHO-m3 cells, the basal level of JNK activation was unaffected by any of the three treatments described. Upon agonist stimulation under control conditions, a 9 fold-over-basal JNK activity was

observed, when the Ca^{2+}_e was removed by the addition of 100 μM EGTA, the fold JNK activity was reduced to 5 fold-over-basal, which was comparable to the effect demonstrated when both Ca^{2+}_e + Ca^{2+}_i were removed (Figure 4.9). These results indicate that there are both Ca^{2+}-dependent and Ca^{2+}- independent components to m3-AChR-mediated JNK activation.

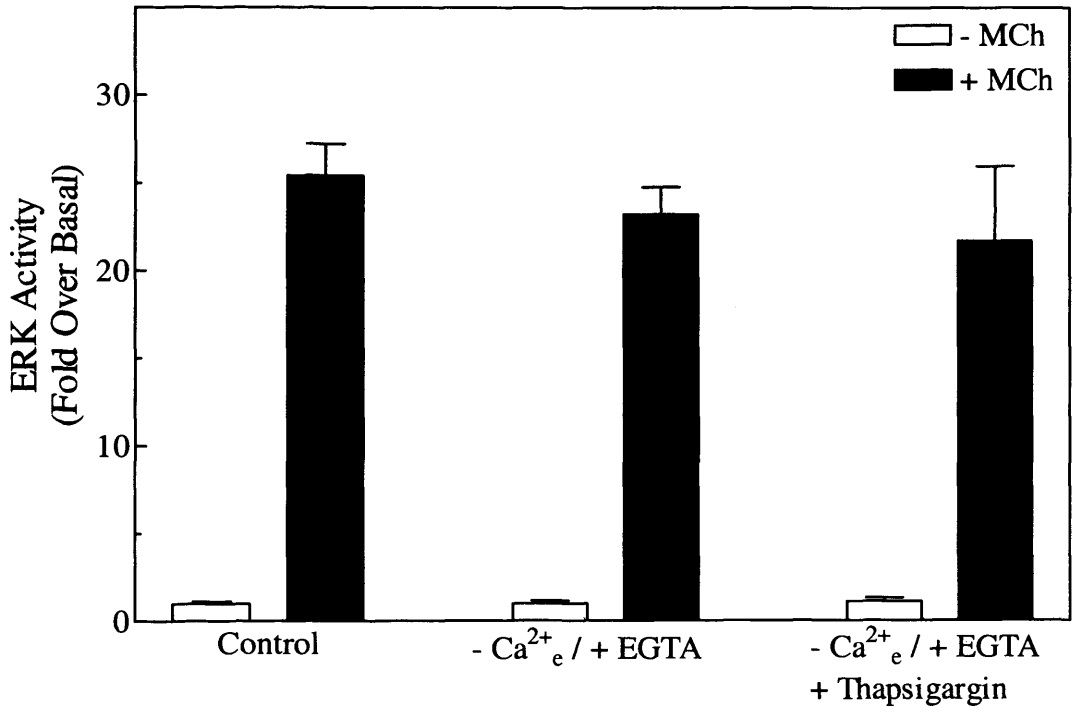

Figure 4.7. Role of Ca^{2+} in agonist-stimulated ERK activation in CHO-m2 cells.

CHO-m2 cells were incubated for 30 min in KHB, control (containing; 1.3 mM $CaCl_2$), under removal of total extracellular Ca^{2+} (KHB -Ca^{2+} / + EGTA (100 μM) or in the absence of Ca^{2+}_e and Ca^{2+}_i (KHB - Ca^{2+} / + EGTA (100 μM) / + 2 μM thapsigargin) and stimulated for 5 min with 100 μM MCh. Lysates containing 200 μg protein were assayed for ERK activity following immunoprecipitation (see Methods). Data represent means ± S.E.M. of three separate experiments.

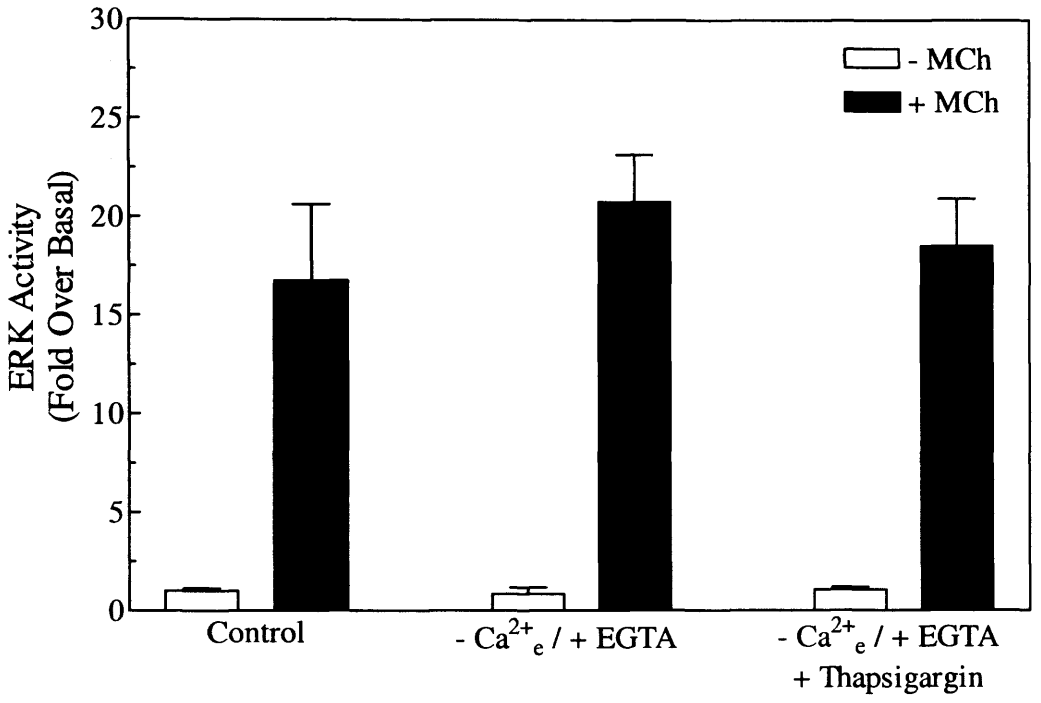

Figure 4.8. Role of Ca^{2+} in agonist-stimulated ERK activation in CHO-m3 cells.

CHO-m3 cells were incubated for 30 min in KHB, control (containing; 1.3 mM $CaCl_2$), under removal of total extracellular Ca^{2+} (KHB -Ca^{2+} / + EGTA (100 μM) or in the absence of Ca^{2+}_e and Ca^{2+}_i (KHB -Ca^{2+} / +EGTA (100 μM) / + 2 μM thapsigargin) and stimulated for 5 min with 100 μM MCh. Lysates containing 200 μg protein were assayed for ERK activity following immunoprecipitation (see Methods). Data represent means ± S.E.M. of 3-6 separate experiments.

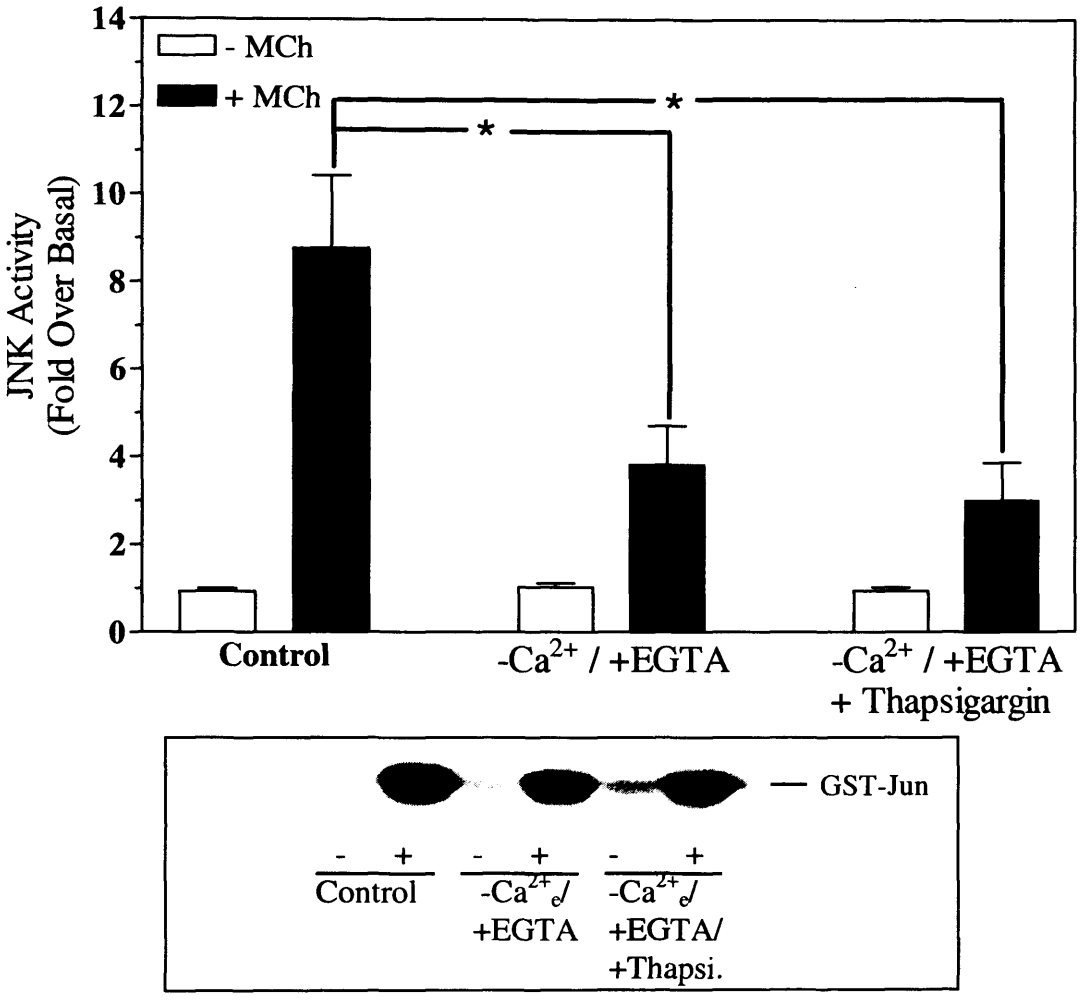

Figure 4.9. Role of Ca^{2+} in agonist-stimulated JNK activation in CHO-m3 cells.

CHO-m3 cells were incubated for 30 min in KHB, control (containing; 1.3 mM $CaCl_2$), under removal of total extracellular Ca^{2+} (KHB -Ca^{2+} / + EGTA (100 μM) or in the absence of Ca^{2+}_e and Ca^{2+}_i (KHB -Ca^{2+} / + EGTA (100 μM) / + 2 μM thapsigargin) and stimulated for 30 min with 100 μM MCh. Lysates containing 400 μg protein were assayed for JNK activity by GST-Jun phosphorylation (see Methods). The lower panel shows a representative autoradiogram of GST-Jun phosphorylation. Data represent means ± S.E.M. of 3-6 separate experiments. * indicates inhibition of JNK activation was statistically significantly different from the response in the agonist-stimulated control cells by Duncan's multiple-range test ($P< 0.05$). In all cases, the MCh stimulation of JNK was statistically significantly different from the corresponding basal ERK activation at $P < 0.05$, by Duncan's multiple-range test.

Experiments to determine the role of Ca^{2+} on JNK activation in CHO-m2 cells, demonstrated that there was a statistically significant agonist-induced JNK activation compared with control conditions when cells were stimulated with maximal MCh concentrations in the absence of Ca^{2+}_e (Figure 4.10). However, when the same cells were stimulated with MCh in the absence of both Ca^{2+}_e and Ca^{2+}_i, agonist-induced JNK activation was completely attenuated (Figure 4.10). However, due to the very low levels of agonist-induced JNK activation (1.8 fold over basal JNK activation in control conditions) the formulation of meaningful conclusions from these results is difficult.

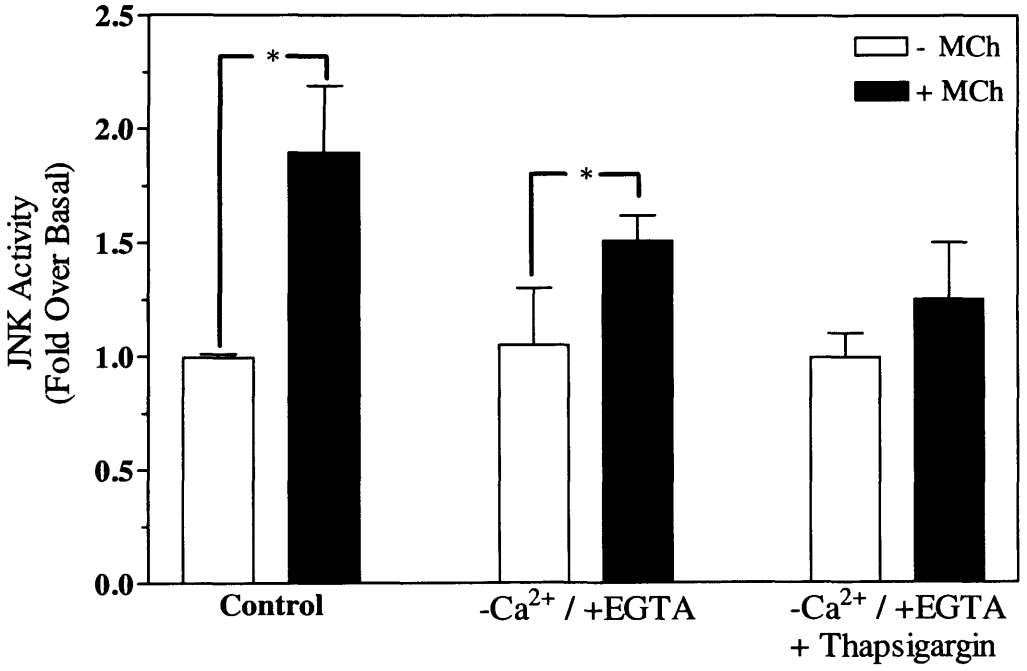

Figure 4.10 Role of Ca^{2+} in agonist-stimulated JNK activation in CHO-m2 cells.

CHO-m2 cells were incubated for 30 min in KHB, control (containing; 1.3 mM $CaCl_2$), under removal of total extracellular Ca^{2+} (KHB -Ca^{2+} / + EGTA (100 μM) or in the absence of Ca^{2+}_e and Ca^{2+}_i (KHB -Ca^{2+} / +EGTA (100 μM) / + 2μM thapsigargin) and stimulated for 10 min with 100 μM MCh. Lysates containing 400 μg protein were assayed for JNK activity by GST-Jun phosphorylation (see Methods). Data represent mean ± S.E.M. of 4-8 separate experiments. * indicates JNK activation was statistically significant in response to agonist-stimulation by Duncan's multiple-range test ($P< 0.05$).

4.4.3 Effect of agonist-independent Ca^{2+} elevation on ERK and JNK activities in CHO-m2 and CHO-m3 cells.

In the presence of Ca^{2+}_e, thapsigargin evokes a release of Ca^{2+}-stores which essentially maximally activates capacitative Ca^{2+}-entry. This produces a large and sustained increase in $[Ca^{2+}]_i$ – see Figs. 4.4 and 4.6. Therefore, the effect of adding thapsigargin to the cells and thus stimulating a sustained elevation of Ca^{2+}_i to potentially stimulate ERK and JNK activation was investigated.

CHO-m2 and CHO-m3 cells were incubated with 2 μM thapsigargin for a time period of 0-70 min, and cell lysates assayed for either ERK or JNK activity. ERK assays showed that at 5 min following stimulation, there was a maximal activity of approximately 200 fmol/min/mg in both m2-AChR and m3-AChR expressing cells (basal values were approximately 25-30 fmol/min/mg in both m2-AChR and m3-AChR expressing cells). This represented a fold activation of 7-8 fold over basal, compared with which, agonist-stimulation gave an activation of approximately 30 fold in both cell lines (Figure 4.11 and Figure 4.12). The thapsigargin effect was observed in the presence of extracellular calcium, but no effect was seen in the absence of extracellular calcium where the increase in Ca^{2+} is transient (see Figs. 4.3 and 4.5). Thus, elevation of $[Ca^{2+}]$ *per se* can activate ERK but to a much smaller that that achieved by receptor activation.

JNK activity assays demonstrated that in the absence of extracellular Ca^{2+}, addition of thapsigargin up to 70 min had no effect on the basal activation of JNK in both the m2-AChR (Figure 4.13) and m3-AChR-expressing cells (Figure 4.14). However, in the presence of extracellular Ca^{2+}, there was a gradual increase in the rate of activation that was the same in both the CHO-m2 and CHO-m3 cells, with 70 min thapsigargin incubation giving a 2-4 fold increase over basal in both cells.

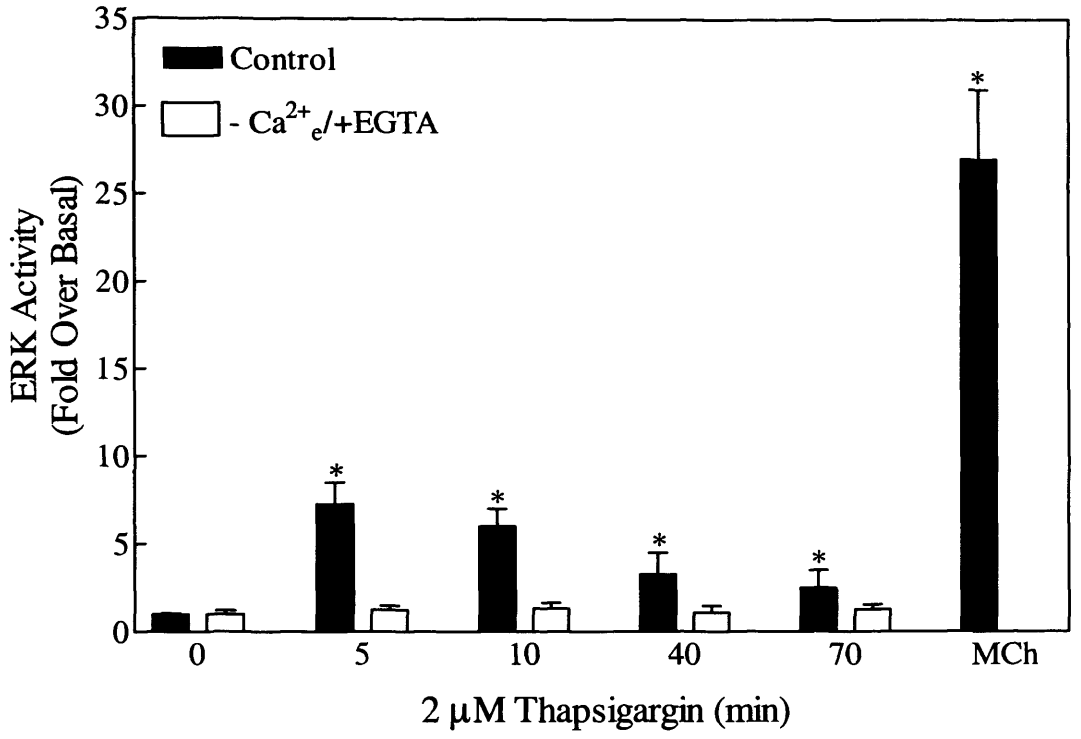

Figure 4.11 Effect of thapsigargin-mediated intracellular Ca^{2+} elevation on ERK activity in CHO-m2 cells.

CHO-m2 cells were pre-incubated in KHB + 1.3 mM Ca^{2+} or KHB -Ca^{2+} / + 100 μM EGTA prior to stimulation. Cells were treated for the indicated times with 2 μM thapsigargin under both conditions or with 100 μM MCh for 5 min in the presence of KHB + 1.3 mM Ca^{2+} (solid bar). Lysates were assayed for ERK activity as described in the Methods section. Data represent means ± S.E.M. of four separate experiments. * indicates ERK activation that was statistically significantly different from the response in the unstimulated control (KHB + 1.3 mM Ca^{2+}) cells by Duncan's multiple-range test ($P< 0.05$).

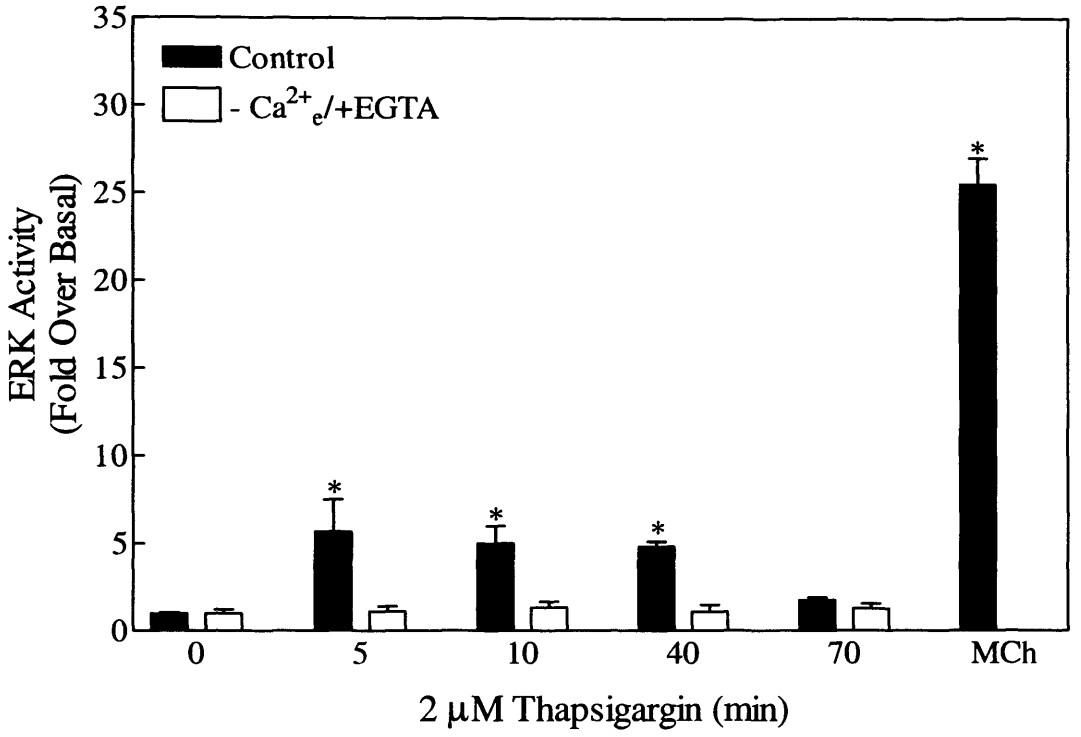

Figure 4.12. Effect of thapsigargin-mediated intracellular Ca^{2+} elevation on ERK activity in CHO-m3 cells.

CHO-m3 cells were pre-incubated in KHB + 1.3 mM Ca^{2+} or KHB -Ca^{2+} / + 100 μM EGTA prior to stimulation. Cells were treated for the indicated times with 2 μM thapsigargin under both conditions or with 100 μM MCh for 5 min in the presence of KHB + 1.3 mM Ca^{2+} (solid bar). Lysates were assayed for ERK activity as described in the Methods section. Data represent means ± S.E.M. of four separate experiments. * indicates ERK activation that was statistically significantly different from the response in the unstimulated control (KHB + 1.3 mM Ca^{2+}) cells, by Duncan's multiple-range test ($P< 0.05$).

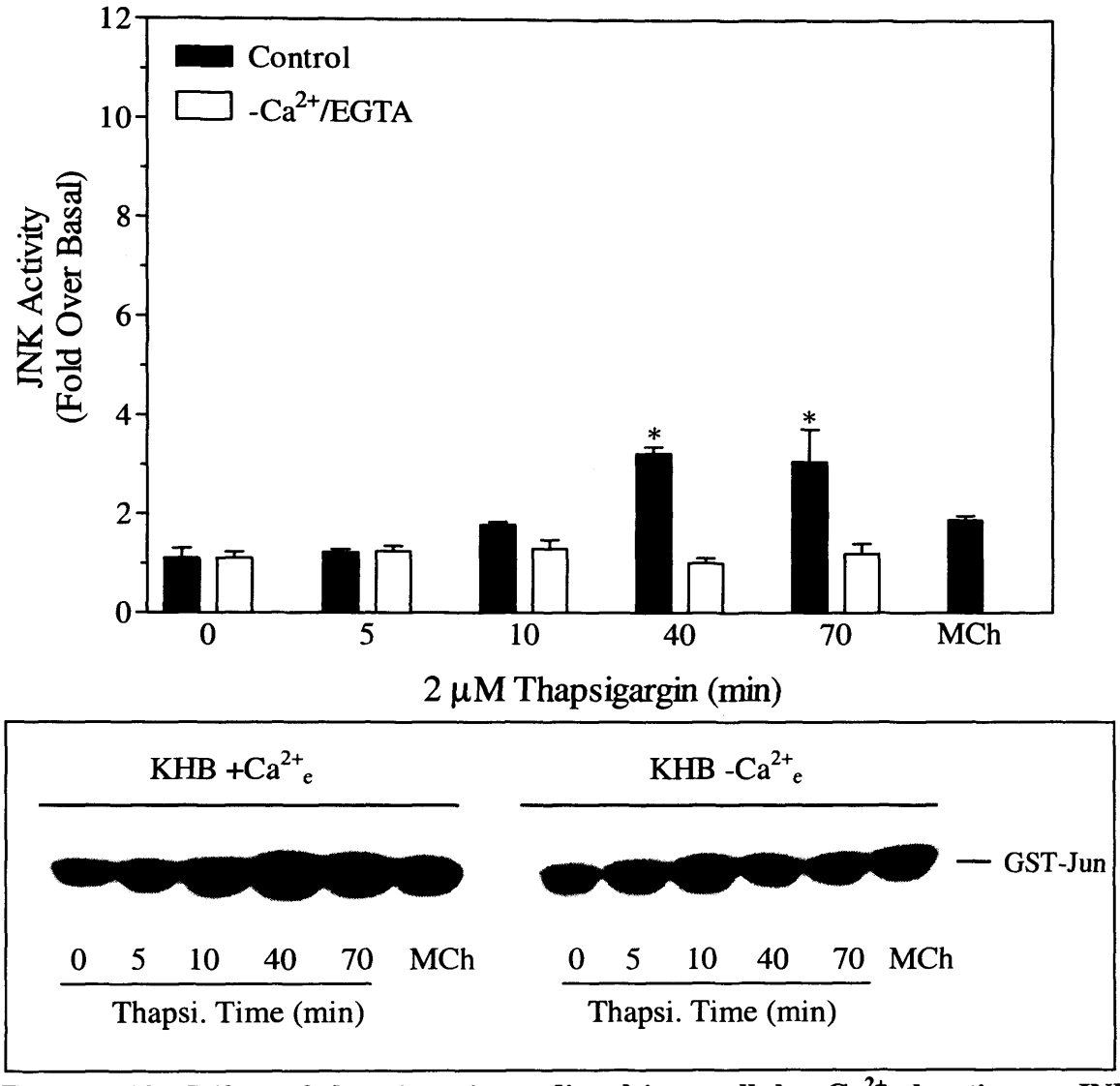

Figure 4.13. Effect of thapsigargin-mediated intracellular Ca^{2+} elevation on JNK activity in CHO-m2 cells.

CHO-m2 cells were pre-incubated in KHB + 1.3 mM Ca^{2+} or KHB -Ca^{2+} / + 100 μM EGTA prior to stimulation. Cells were treated for the indicated times with 2 μM thapsigargin under both conditions or with 100 μM MCh for 10 min in the presence of KHB + 1.3 mM Ca^{2+} (solid bar). Lysates containing 400 μg protein were assayed for JNK activity by GST-Jun phosphorylation (see Methods). The lower panel shows a representative autoradiogram of GST-Jun phosphorylation. Data represent means ± S.E.M. of four separate experiments. * indicates JNK activation that was statistically significantly different from the response in the unstimulated control (KHB + 1.3 mM Ca^{2+}) cells, by Duncan's multiple-range test ($P< 0.05$).

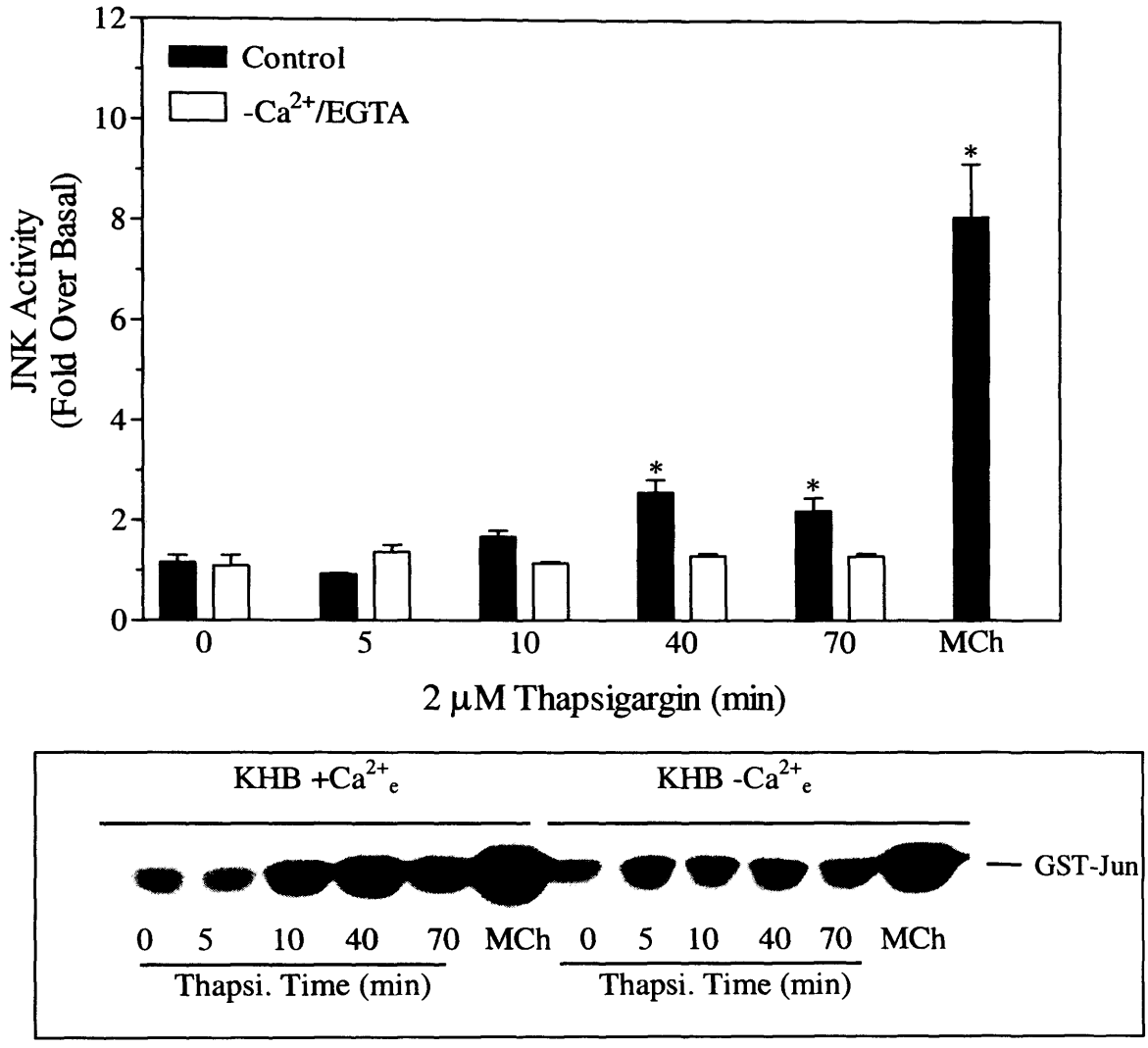

Figure 4.14. Effect of thapsigargin-mediated intracellular Ca^{2+} elevation on JNK activity in CHO-m3 cells.

CHO-m3 cells were pre-incubated in KHB + 1.3 mM Ca^{2+} or KHB -Ca^{2+} / + 100 μM EGTA prior to stimulation. Cells were treated for the indicated times with 2 μM thapsigargin under both conditions or with 100 μM MCh for 30 min in the presence of KHB + 1.3 mM Ca^{2+} (solid bar). Lysates containing 400 μg protein were assayed for JNK activity by GST-Jun phosphorylation (see Methods). The lower panel shows a representative autoradiogram of GST-Jun phosphorylation. Data represent means ± S.E.M. of four separate experiments. * indicates JNK activation that was statistically significantly different from the response in the unstimulated control (KHB + 1.3 mM Ca^{2+}) cells by Duncan's multiple-range test ($P< 0.05$).

4.5 PTX sensitivity of mAChR-mediated responses.

Pertussis toxin (PTX) catalyses the ADP-ribosylation of the α- subunits of G_i and G_o G-proteins. This uncouples them from the activated receptor, and therefore prevents $G_{i/o}$-mediated effector activation. As previously discussed, the m3 receptor is coupled to phosphoinositide-specific phospholipase C (PLC) activation via PTX-insensitive G_q G-proteins, whereas, the m2 receptor is coupled to PTX-sensitive G_i-proteins. Therefore, the effect of PTX pre-treatment was investigated to determine the specificity of the ERK and JNK responses to the coupling of receptor subtypes to specific G-proteins.

4.5.1 Effect of PTX on the agonist-induced activation of the IP_3 pathway in CHO-m3 cells.

Agonist-stimulated Ins(1,4,5)P_3 (IP_3) accumulation mediated by the m3 receptor, was rapid and maximal at 15 sec. This was shown to be unaffected by a 75 ng/ml PTX 20 hour pre-treatment (Figure 4.15). In addition, the EC_{50} of agonist-stimulated IP_3 accumulation was unaffected by PTX pre-treatment, log EC_{50} (M) -5.6 ± 0.2 and -6.0 ± 0.1 in the absence and presence of PTX, respectively (Figure 4.16). This demonstrated that the m3-AChR couples to a PTX-insensitive G-protein (G_q) to activate the IP_3 pathway via PLC.

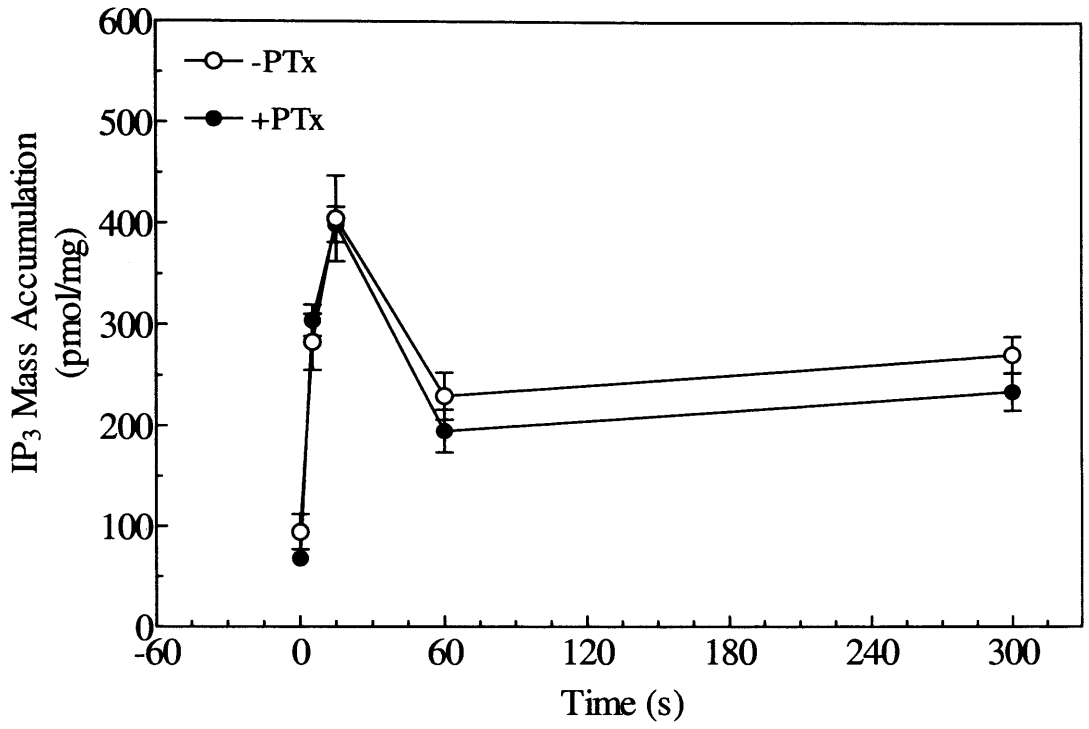

Figure 4.15. Effect of pertussis toxin pre-treatment on the agonist-induced time-course of IP_3 accumulation in CHO-m3 cells.

CHO-m3 were pre-treated with 75 ng/ml pertussis toxin (PTx) or carrier for 20 h prior to cells being washed and incubated for 10 min in KHB. Cells were then stimulated with 100 μM MCh for the indicated times. The reaction was terminated by aspiration of the KHB/drug solution and the addition of 300 μM 0.5 M TCA. IP_3 mass levels were the analysed as described in Methods. Data represent means ± S.E.M. of three separate experiments performed in duplicate.

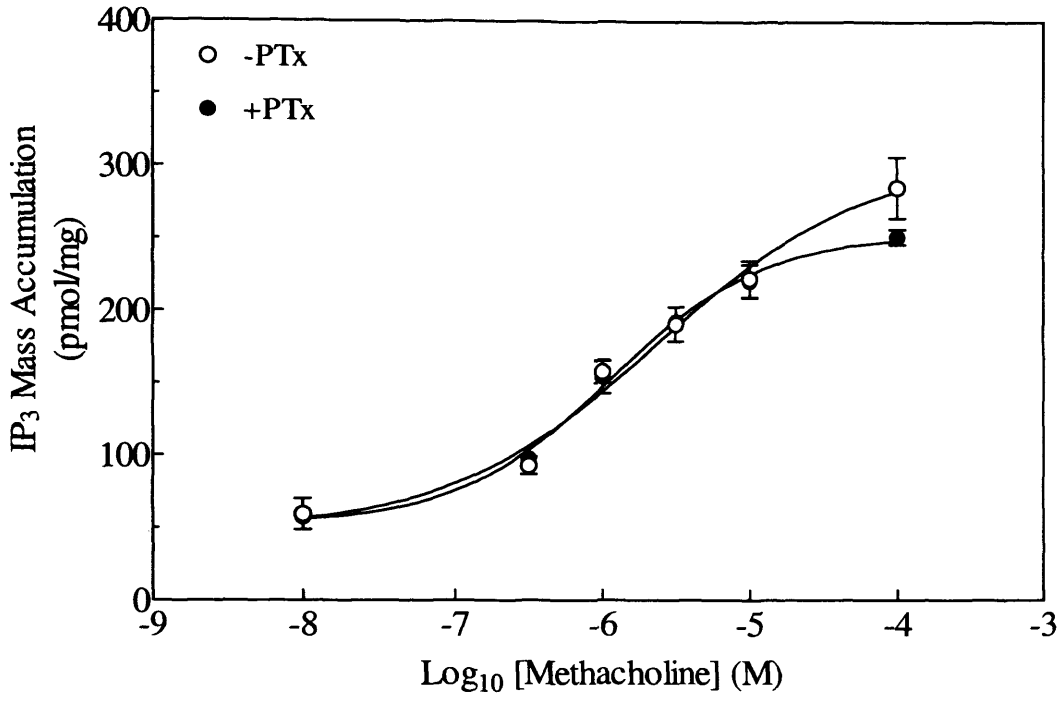

Figure 4.16 Effect of pertussis toxin pre-treatment on the agonist concentration-dependency of IP_3 accumulation in CHO-m3 cells.

CHO-m3 cells were pre-treated with 75 ng/ml pertussis toxin (PTX) or carrier for 20 h and stimulated with the indicated concentrations of MCh for 10 sec. The reaction was terminated by aspiration of the KHB/drug solution and the addition of 300 µM 0.5 M TCA. IP_3 mass levels were the analysed as described in Methods. Data represent means ± S.E.M. of three separate experiments performed in duplicate.

4.5.2 PTX-sensitivity of agonist-stimulated ERK activity in CHO-m2 and CHO-m3 cells.

In contrast to the results reported above for the activation of the IP_3 pathway, pre-treatment of CHO-m3 cells for 20 hr with 75 ng/ml PTX significantly inhibited agonist-stimulated ERK activation by the m3-AChR, (with a reduction from 16 fold over basal in untreated cells to 5 fold over basal in treated cells; Figure 4.17). It was also demonstrated that JNK activation by the m3 receptor was also partially sensitive to PTX pre-treatment, reducing the agonist-stimulated activity from 6 fold over basal to 4

fold over basal (Figure 4.19). As expected, PTX completely abolished the agonist-induced ERK and JNK activation in CHO-m2 cells (Figure 4.18 and Figure 4.20 respectively).

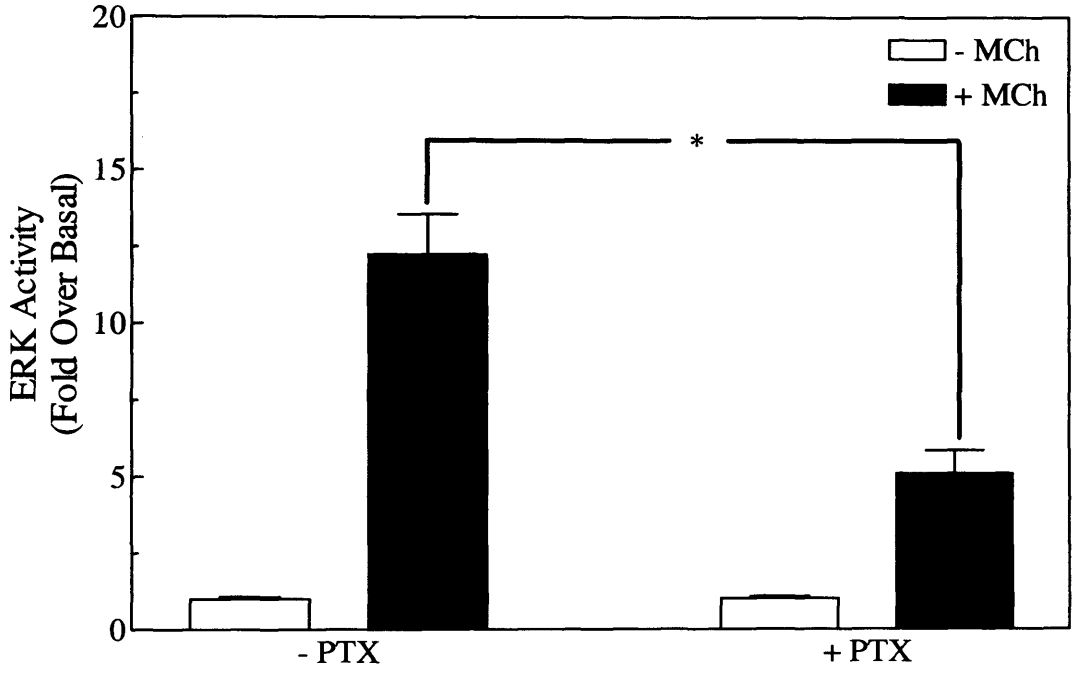

Figure 4.17 The effect of PTX on agonist-induced ERK activity in CHO-m3 cells.

CHO-m3 cells were pre-treated with 75 ng/ml pertussis toxin (PTX) or carrier for 20 h prior to stimulation with 100 μM MCh for 5 min. ERK activity was isolated (approx. 200 μg protein) using anti-ERK1 and assayed as described in the Methods section). Data represent means ± S.E.M. of 9 separate experiments. * indicates statistically significant inhibition of ERK activation by PTX compared with the agonist-stimulated control cells, by Duncan's multiple-range test ($P < 0.05$). Under both conditions, the MCh stimulation of ERK was statistically significantly different from the corresponding basal ERK activation at $P < 0.05$, by Duncan's multiple-range test.

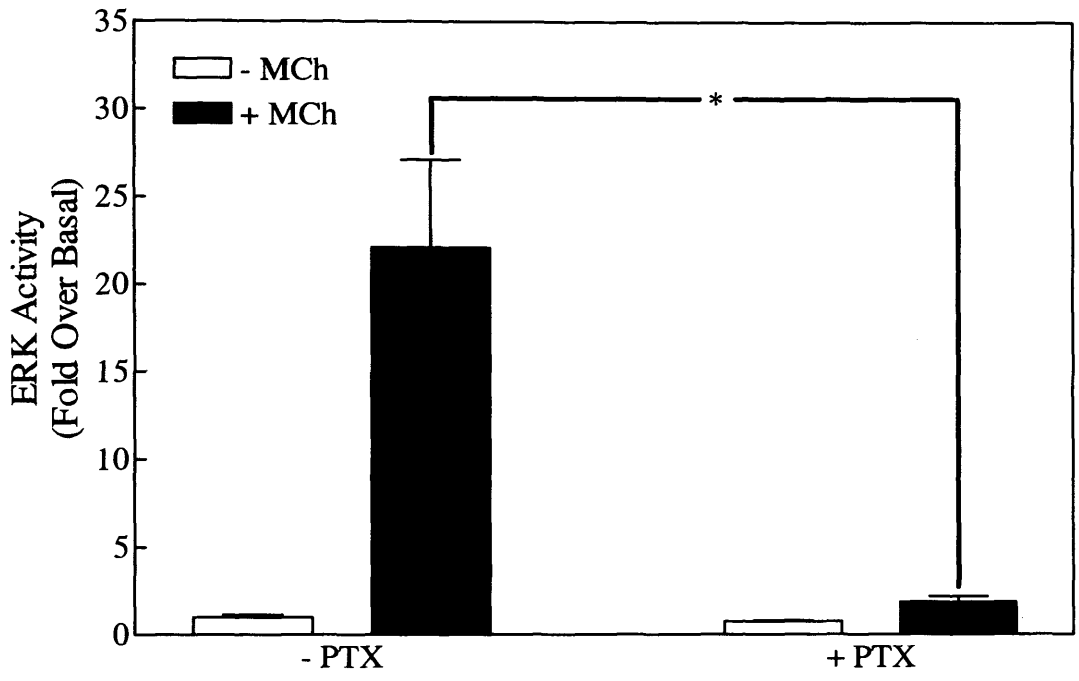

Figure 4.18 The effect of PTX on agonist-induced ERK activity in CHO-m2 cells.

CHO-m2 cells were pre-treated with 75 ng/ml pertussis toxin (PTX) or carrier for 20 h prior to stimulation with 100 μM MCh for 5 min. ERK activity was isolated (approx. 200 μg protein) using anti-ERK1 and assayed as described in the Methods section). Data represent means ± S.E.M. of four separate experiments. * indicates statistically significant inhibition of ERK activation by PTX compared with the agonist-stimulated control cells, by Duncan's multiple-range test ($P < 0.05$). In the absence of PTX only, the MCh stimulation of ERK was statistically significantly different from the corresponding basal ERK activation at $P < 0.05$, by Duncan's multiple-range test.

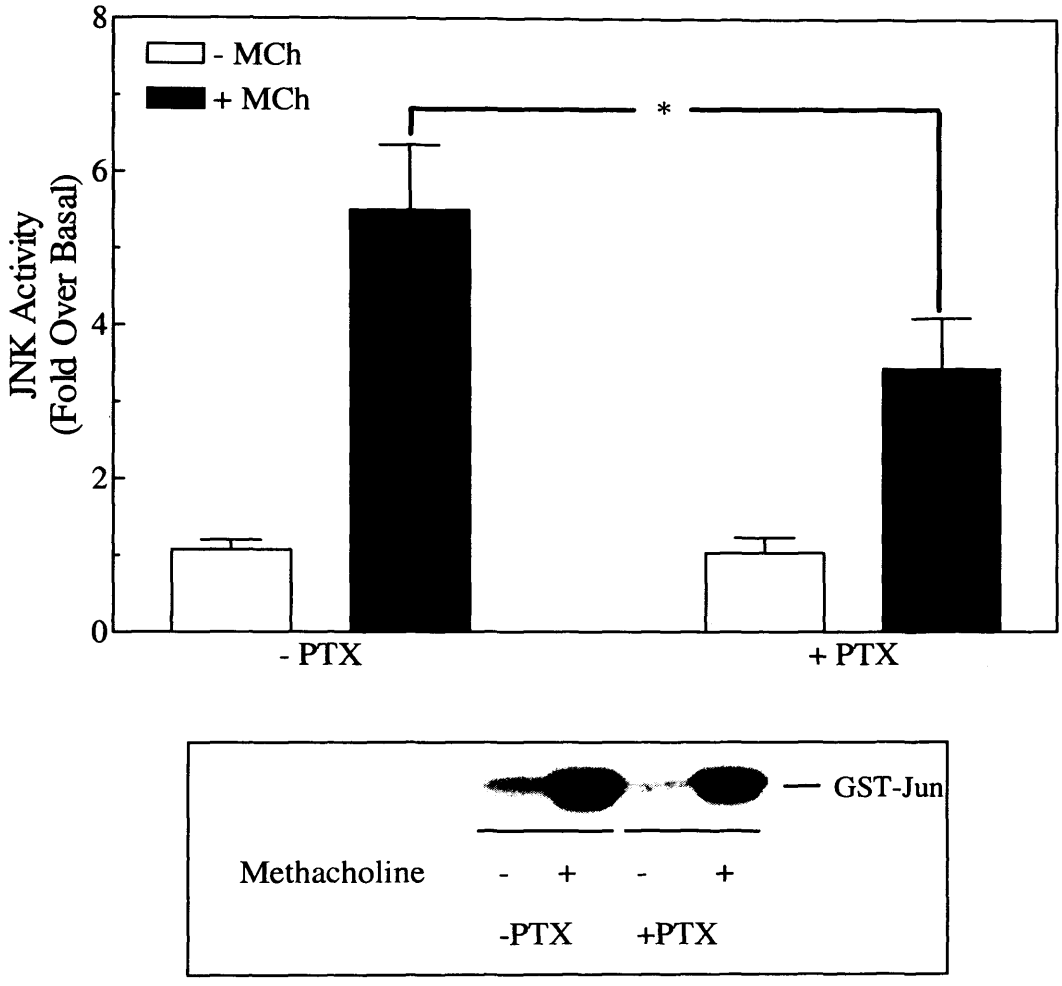

Figure 4.19 The effect of PTX on agonist-induced JNK activity in CHO-m3 cells.

CHO-m3 cells were pre-treated with 75 ng/ml pertussis toxin (PTX) or carrier for 20 h prior to stimulation with 100 μM MCh for 30 min. JNK activity was assayed (approx. 400 μg protein) by GST-Jun phosphorylation as described in the Methods section. The lower panel shows a representative autoradiogram of GST-Jun phosphorylation. Data represent means ± S.E.M. of 5-6 separate experiments. * indicates statistically significant inhibition of JNK activation by PTX compared with the agonist-stimulated control cells, by Duncan's multiple-range test ($P< 0.05$). Under both conditions, the MCh stimulation of ERK was statistically significantly different from the corresponding basal ERK activation at $P < 0.05$, by Duncan's multiple-range test.

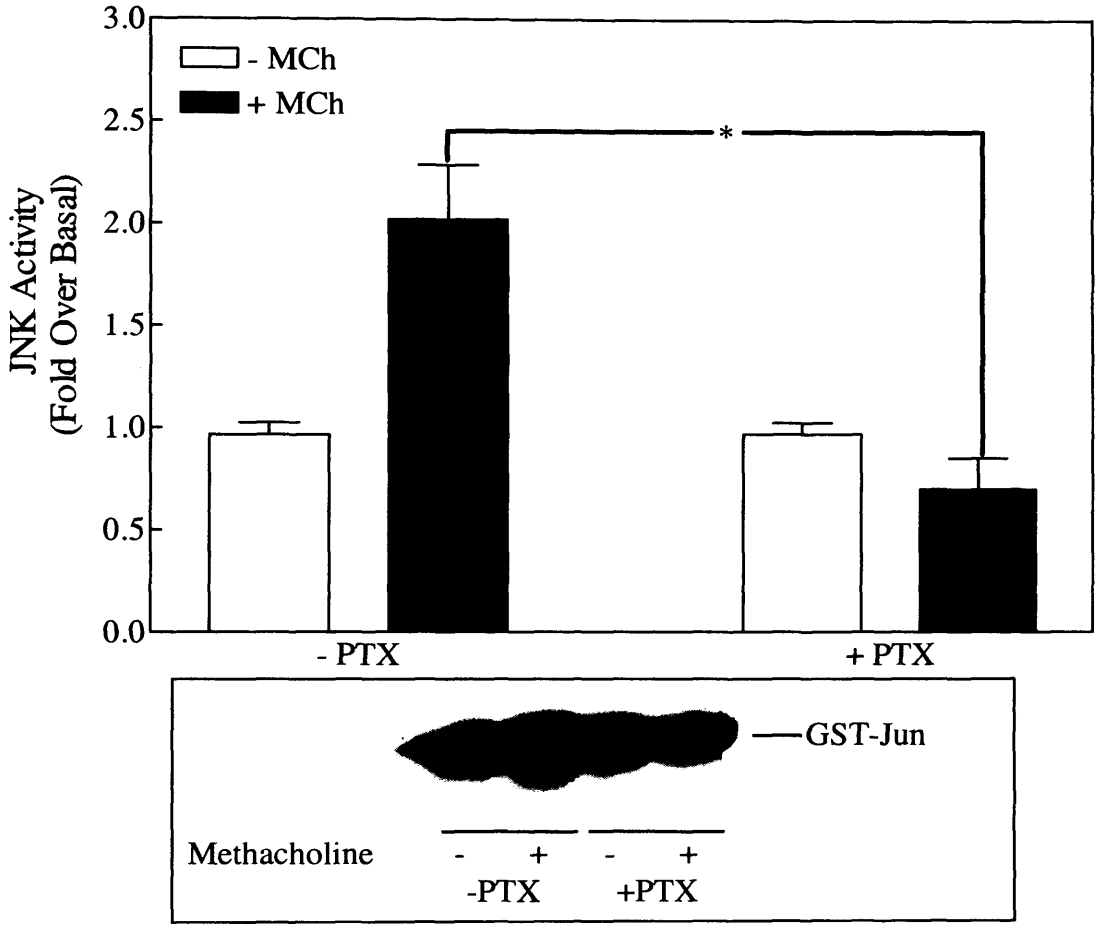

Figure 4.20 The effect of PTX on agonist-induced JNK activity in CHO-m2 cells.

CHO-m2 cells were pre-treated with 75 ng/ml pertussis toxin (PTX) or carrier for 20 h prior to stimulation with 100 μM MCh for 15 min. JNK activity was assayed (approx. 400 μg protein) by GST-Jun phosphorylation as described in the Methods section. The lower panel shows a representative autoradiogram of GST-Jun phosphorylation. Data represent means ± S.E.M. of 3-5 separate experiments. * indicates statistically significant inhibition of JNK activation by PTX compared with the agonist-stimulated control cells, by Duncan's multiple-range test ($P< 0.05$). In the absence of PTX only, the MCh stimulation of ERK was statistically significantly different from the corresponding basal ERK activation at $P < 0.05$, by Duncan's multiple-range test.

4.6 Discussion

In this Chapter, the role of Ca^{2+} in the agonist-mediated activation of ERK and JNK in CHO cells expressing the m2-AChR or the m3-AChR was examined. The effect of intracellular Ca^{2+} release by MCh stimulation of the receptors was investigated in addition to the effect by thapsigargin. The role of intracellular Ca^{2+} release was examined in the presence of thapsigargin, in order to determine the time-course required to fully deplete the intracellular Ca^{2+} stores. The receptor-independent effect of thapsigargin-mediated elevation of intracellular Ca^{2+} on ERK and JNK activation was also determined in both the cell lines. Having established the profile of Ca^{2+} release, the role of intracellular Ca^{2+} and extracellular Ca^{2+} on ERK and JNK activation in both of the cell lines was examined. By utilising three different conditions, in the presence of 1.3 mM Ca^{2} nominally free Ca^{2+}, and the absence of intracellular and extracellular Ca^{2+}, the role of Ca^{2+} on agonist-induced ERK and JNK activation could be investigated. It has also been shown that the responses in the CHO-m2 cells are entirely PTX-sensitive, however, surprisingly, in the CHO-m3 cells there is also a PTX-sensitive component for ERK and JNK activation.

Prior to investigating the role of Ca^{2+} on ERK and JNK responses, it was important to carry out initial studies that set out to examine agonist-mediated Ca^{2+} release in both the cell lines. It was demonstrated that in the G_q-coupled, PLC-activating CHO-m3 cells, agonist stimulation in the presence of extracellular Ca^{2+} resulted in a typical peak-plateau phase due to release of intracellular Ca^{2+} followed by an influx of extracellular Ca^{2+} as previously reported (Tobin *et al.*, 1995). Previously, they provided evidence suggesting a pre-stimulation of the m3-AChR with agonist caused receptor

desensitisation which was found partially to inhibit Ca^{2+} mobilisation (Tobin *et al.*, 1992). Interestingly, it was found that there was also some intracellular Ca^{2+} release via stimulation of the G_i-coupled m2-AChR. The α-subunit of a G_i-protein, acts to inhibit the activity of adenylyl cyclase and therefore reduce cAMP levels, it does not have a Ca^{2+} mobilising role like G_q coupled G-proteins. However, in the results presented, the stimulation of the m2-AChR mobilised Ca^{2+}, to a much lesser extent when compared with the m3-AChR. A potential reason for this ability to mobilise Ca^{2+} may be due to a high level of receptor expression causing promiscuous coupling of the receptor to other classes of G-protein other than the G_i class. This could be easily investigated, by comparing the effect observed here against CHO cells that contain the m2-AChR expressed at lower levels.

However, there is evidence in the literature that supports the data here that the m2-ACh receptor may mediate Ca^{2+}-mobilisation. In HEK-293 cells, stimulation of the m2-AChR resulted in a similar $Ca^{2+}{}_i$ peak to that observed with m3-AChR stimulation (Schmidt *et al.*, 1995). However, the Ca^{2+} release was unaffected by EGTA, and therefore they conclude that the release is entirely from intracellular stores, in contrast to the m3-AChR-mediated response. Subsequent to this study, Meyer zu Heringdorf *et al.*, (1998) demonstrated that sphingosine kinase was stimulated by both m2- and m3-AChRs, in HEK-293 cells. They also showed that inhibitors of sphingosine kinase, DL-*threo*-dihydrosphingosine and *N,N*-dimethylsphingosine inhibited m2- and m3-AChR-mediated $[Ca^{2+}]_i$.

An interesting result in the m2-AChR-mobilisation of Ca^{2+} is the feature that Ca^{2+} mobilisation is increased (although to a small degree) as $Ca^{2+}{}_e$ is removed. This may

occur due to a number of possible distinct or combined reasons. A potential mechanism is via βγ-mediated activation of PLC-beta isoenzymes in a G_i-dependent manner (Blank *et al.,* 1993). As previously discussed, it may be that the mobilisation of Ca^{2+} is entirely derived from the intracellular stores and under the conditions used, the Ca^{2+} store is more accessible. It is also possible that the sphingosine-1-phosphate pathway used to mobilise Ca^{2+} by stimulation of the m2-AChR (Meyer zu Heringdorf *et al.,* 1998) is more efficient under the conditions where there is a depletion of Ca^{2+}_e. A final possibility is that the mechanisms in the cell to remove Ca^{2+} (e.g. plasmalemmal Ca^{2+}-ATPase) become less effective under Ca^{2+} free conditions.

In experiments to investigate the role of Ca^{2+} in ERK and JNK activation, $[Ca^{2+}]_i$ was removed (the intracellular Ca^{2+} stores can be emptied by the addition of 2 μM thapsigargin) in addition to Ca^{2+}_e by chelation with EGTA. However, prior to the ERK and JNK assays being carried out, it was important to examine the time required for thapsigargin to empty the stores. In both CHO-m2 and CHO-m3 cells, addition of thapsigargin under conditions where Ca^{2+}_e had been removed and chelated with EGTA, a peak Ca^{2+}_i of approximately 100 nM was obtained which slowly fell to basal levels. Therefore, it was concluded that a 10 min pre-incubation with thapsigargin completely emptied the Ca^{2+} stores and under these conditions agonist did not effect a further Ca^{2+}_i rise.

It was interesting to note that in the presence of Ca^{2+}_e upon addition of 2 μM thapsigargin (in the absence of agonist), a large rise in intracellular Ca^{2+} was recorded, which peaked between 500- 700 nM and remained at a constant level in both the CHO-m2 and CHO-m3 cells. These results demonstrate that there may be a mechanism

whereby upon release of intracellular Ca^{2+}, there is a refilling of intracellular Ca^{2+} stores that originates from the extracellular buffer, this mechanism has been previously identified as capacitative Ca^{2+} entry (Berridge *et al.,* 1993; Berridge *et al.,* 1995; Putney 1990). Upon the depletion of Ca^{2+} from the intracellular stores by activation of the IP_3 pathway with agonist, Ca^{2+} influx across the plasma membrane is increased by voltage-independent mechanisms (Van Breeman, 1989; Putney and Bird, 1993). Ca^{2+} release-activated Ca^{2+} channels (CRAC) have been identified (Putney, 1986) in some cell lines and are thought to be activated by the depletion of Ca^{2+} stores. To date, the mechanism of Ca^{2+} entry stimulated by Ca^{2+} elevation from intracellular stores is unclear. There have been reports demonstrating that Ca^{2+} release is agonist-concentration dependent (Willars and Nahorski, 1995a/b) in a population of cells. However, there have also been reports that the release of Ca^{2+} is an all or nothing effect (Shao and McCarthy, 1993), at the single cell level.

As thapsigargin is acting via a receptor-independent mechanism, the observation that the results are similar in both cell types would be expected. The data presented showing that addition of thapsigargin in the presence of 1.3mM Ca^{2+} in the extracellular buffer gives a large sustained Ca^{2+}_i suggests that capacitative Ca^{2+} entry occurs to similar extents in both CHO-m2 and CHO-m3 cells.

The role of Ca^{2+} in agonist-induced ERK and JNK responses were then examined in both CHO-m2 and CHO-m3 cells. Cells were pre-incubated as described in the results section and the data showed that there was no significant effect on agonist-induced ERK activation in either m2-AChR, or m3-AChR expressing cells under conditions where extracellular calcium chelation and/or intracellular Ca^{2+} had been depleted. Therefore

these results, suggest that in agonist-induced activation of ERK in both of the two cell lines, neither intracellular Ca^{2+} nor extracellular Ca^{2+}, play a role. This has also been demonstrated for the m2-AChR by Mitchell and colleagues (1995), whereby Ca^{2+} chelation had no effect on ERK1/2 activation. However other groups have demonstrated a role of Ca^{2+} in ERK activation via Ras in neuronal cell lines (Finkbeiner and Greenberg, 1996) and via receptor tyrosine kinase activation (Ebinu *et al.,* 1998). A postulated mechanism for this has been suggested to involve the calcium-regulated guanine-nucleotide releasing factor Ras-GRF although this is neuronal (Farnsworth *et al.,* 1995). Alternative suggested mechanisms described are via activation of the tyrosine kinases Src (Rusanescu *et al.,* 1995), Pyk2 (Lev *et al.,* 1995) and also via inhibition of Ras-GTPase by CaM kinase II (Chen *et al.,* 1998).

The role of Ca^{2+} in JNK activation was then investigated. In CHO-m3 cells, the results demonstrate that removal of extracellular Ca^{2+} reduced JNK activation by up to 50%, and subsequent depletion of intracellular Ca^{2+} had no further effect. Therefore the data suggest that Ca^{2+} derived from the extracellular buffer is important for part of the agonist-induced JNK activation by the m3-AChR. This depletion of intracellular Ca^{2+} and/or extracellular Ca^{2+} demonstrated that JNK activation via stimulation of the m3-AChR was regulated by both a Ca^{2+}-dependent and Ca^{2+}-independent component (in contrast to ERK activation) in agreement with previous studies in GN4 rat liver epithelial cells by angiotensin II (Zohn *et al.,* 1995). In COS-7 cells, stimulation of the m1-AChR and m2-AChR have been shown to activate JNK via $\beta\gamma$ subunits acting on Ras and Rac1 (Coso *et al.,* 1996). Therefore the results observed in this study for JNK activation suggests two distinct regulatory pathways; the Ca^{2+}-dependent component being derived

from G_q activation of PLC and subsequent Ca^{2+} mobilisation, whereas the Ca^{2+}-independent component being possibly derived from βγ subunits acting via Ras and/or Rac1. In the CHO-m2 cells, the results are difficult to interpret due to the small JNK activation. However, the results seem to suggest that the absence of extracellular Ca^{2+} and intracellular Ca^{2+} is required to inhibit JNK activation. Stimulation of the m2-AChR has already been shown to mobilise Ca^{2+} to a moderate level previously in this Chapter. Therefore the results suggest that m2-AChR-mediated JNK activation is via the ability of the receptor to cause a small Ca^{2+} mobilisation.

To ascertain if thapsigargin directly activated ERK or JNK, the time-dependence of thapsigargin-activation of ERK and JNK was investigated. The results demonstrated peak ERK activation was achieved 5 min following addition of thapsigargin in both cell types which subsequently dropped towards basal values. However, in the absence of extracellular Ca^{2+} there was no activation of ERK following thapsigargin addition. Thapsigargin-induced activation of JNK in both CHO-m2 and CHO-m3 cells showed a peak activation by 40 min in the presence of extracellular Ca^{2+}. However, as in the results presented for the activation of ERK, under conditions where extracellular Ca^{2+} had been removed by chelation with EGTA, there was no thapsigargin-induced JNK activation. Therefore, as the activation of ERK and JNK in both the CHO-m2 and CHO-m3 cells are the same, these results demonstrate that these responses to thapsigargin are mediated via a receptor-independent mechanism, and are representative of a CHO cell response. However, the activation of both the ERK and JNK by thapsigargin only occurs when the cells are in the presence of 1.3mM Ca^{2+}. Therefore, the data seem to suggest that Ca^{2+} alone is sufficient to cause a small activation of ERK and JNK provided that

Ca^{2+}_i levels rise greater than approximately 100 nM (from Fig 4.3 thapsigargin caused approximately a 75 nM increase in Ca^{2+}_i which caused no ERK or JNK activation) and/or the Ca^{2+}_i is sustained. Capacitative Ca^{2+} entry has already been briefly discussed in this Chapter, but the results lead to the suggestion that depletion of the intracellular Ca^{2+} stores by thapsigargin leads to the influx of Ca^{2+} from the extracellular buffer to refill the intracellular stores, which then has a stimulatory effect on ERK and JNK.

Data presented previously in this Chapter has shown that there is no role for Ca^{2+} in the activation of ERK in either the CHO-m2 or CHO-m3 cells upon stimulation of the receptor with agonist. In both of the experiments shown, a positive control of 100 μM MCh was used, causing a much greater ERK activation than that by thapsigargin alone. Therefore this conflicting data seems to suggest that there may be a small Ca^{2+}-dependent component in ERK activation, but this only occurs in conditions where there are high and sustained levels of intracellular Ca^{2+}. A potential mechanism for this may involve the proposed calcium-regulated ERK activators discussed previously e.g. the guanine-releasing factor Ras-GRF (Farnsworth *et al.*, 1995). In the activation of JNK in both cell lines, there was also a Ca^{2+}-dependent component that upon capacitative Ca^{2+} entry, gave a significant increase in JNK activity, that was greater than by receptor stimulation in the poorly coupled CHO-m2 cells. In the CHO-m3 cells which have already been shown to activate the JNK pathway via a partially Ca^{2+}-dependent mechanism there is also a significant increase in JNK activity, though as for the ERK data, it is small when compared with the activation seen by receptor stimulation, again suggesting that a high sustained level of Ca^{2+}_i is sufficient to cause some JNK activation.

The first evidence demonstrating calcium regulation for JNK arose from studies in

Jurkat cells (a human T-cell line), whereby calcium ionophore strongly activated JNK (Su *et al.*, 1994). Later, Mitchell *et al,* (1995) demonstrated that pre-treatment of Rat 1a cells with 20 μM BAPTA (an intracellular calcium chelator) resulted in m1-AChR- and m2-AChR- mediated JNK activation being completely inhibited, but there was no effect on ERK1/2 activation via the m2-AChR. This complete inhibition of JNK activity was not observed in work for this Thesis, however, there was an inhibition of approximately 40% of JNK activity in m3-AChR expressing cells upon calcium chelation.

The results presented here also compare to those obtained by Zohn *et al* (1995), in which GN4 rat liver epithelial cells were pre-treated with thapsigargin and EGTA 30 min prior to angiotensin II (AngII) stimulation. Angiotensin II stimulates proliferation through G-protein-dependent activation of PKC and via angiotensin II-dependent stimulation of tyrosine kinase activity. Depletion of intracellular calcium resulted in a 50% inhibition of AngII-stimulated JNK activation, whereas the additional chelation of extracellular calcium inhibited the JNK activation by 90%. The failure to completely block JNK activation by removal of intracellular calcium or extracellular calcium, suggests that the m3-AChR also acts via both calcium-dependent and calcium-independent mechanisms in these cells. Therefore, Zohn *et al.*, (1995), provided evidence for a role for Ca^{2+} in JNK activation by G-protein coupled receptor agonists, supporting data presented in this Chapter. In later reports, Li *et al* (1997) using the same GN4 cell system, reported that thapsigargin strongly stimulated JNK activity after 10 min, giving a 28 fold activation which subsequently peaked at 20 min at 46 fold over basal. In the results presented here, in the CHO-m3 cells, as previously discussed, it was observed that thapsigargin stimulated JNK activity in the presence of extracellular

calcium, but to a modest extent compared with the observations in the GN4 cells. At 40 min thapsigargin stimulation, a 3-4 fold activity over basal was recorded in both CHO cell lines. However, in absence of extracellular calcium, there was no effect on the addition of thapsigargin in either cell line, suggesting that the JNK response was dependent on sustained extracellular calcium entry.

In 1996, Yu *et al*, identified a soluble protein in GN4 rat liver epithelial cells called calcium-dependent tyrosine kinase (CADTK) which when sequenced was found to be a rat homologue of PYK2. They found that LPA and thapsigargin activated CADTK, however, CADTK was found not be involved in the activation of the ERK pathway by angiotensin II in the same cells. Therefore, this may provide evidence for the role of Ca^{2+} in the activation of JNK, but not ERK upon stimulation of GPCRs by agonists.

Pertussis toxin (PTX) uncouples G_i and G_o G-proteins from their receptor by catalysing the ADP-ribosylation of the α-subunit, 'locking' the α-subunit in the GDP form. This inhibits the ability of the receptor to couple to an effector. Initial experiments investigated the effect of a 20 h PTX pre-treatment on the agonist-induced time-course of IP_3 mass accumulation and the agonist-concentration-dependency of IP_3 accumulation in CHO-m3 cells. The results showed that peak IP_3 levels were achieved by 15 s following agonist addition, followed by a sustained plateau phase in both the presence and absence of PTX. Experiments which examined the effects of PTX on the agonist-induced concentration-dependency of IP_3 accumulation, demonstrated that there was no effect on the concentration-dependency in the presence of PTX. As the m3-AChR couples to G_q G-proteins, these results were expected, as the PTX only affects G_i and G_o G-proteins. Therefore, these data demonstrate that the m3-AChR is coupled to G_q G-proteins that

activate the IP_3 pathway, via PLC activation. The role of $G_{i/o}$ proteins on agonist-induced ERK activation in CHO-m2 and CHO-m3 cells was then examined. Pre-treatment with PTX caused a complete inhibition of ERK activation in CHO-m2 cells, demonstrating that the m2-AChR is coupled to the ERK pathway exclusively through a PTX sensitive G-protein. This result was expected, due to, as previously discussed, the m2-AChR (and m4-AChR) having been demonstrated to couple to PTX-sensitive G_i G-proteins (Caulfield *et al.,* 1993; Dell'Acqua *et al.,* 1993). Surprisingly however, the results presented with CHO-m3 cells showed that there was also an inhibition of approximately 60% of agonist-induced ERK activation following PTX pre-treatment, which suggests that ERK is activated in these cells by PTX-insensitive G-protein (G_q), but also, unexpectedly by a PTX-sensitive G-protein (G_i/G_o). In CHO-m2 cells, pre-treatment with PTX completely attenuated the activation of JNK by agonist stimulation, however, in the CHO-m3 cells, the activation of JNK was inhibited by approximately 20% in the presence of PTX. These results, as with the ERK data, suggest that in the CHO-m3 cells, the m3-AChR is coupled to both G_q and also a G_i/G_o G-protein. Although these results were unexpected, they are not unprecedented. Studies have demonstrated the potential for muscarinic receptors that have been expressed at high levels to couple divergently to multiple G-protein-species (Askenazi *et al.,* 1987; Peralta *et al.,* 1988; Richards *et al.,* 1991). In addition to these studies, previous investigations into this phenomenon have demonstrated that the m2-AChR stably expressed in CHO cells couple to PTX-sensitive G-proteins, however, the m1-AChR and m3-AChR can couple to both PTX-sensitive G-proteins and PTX-insensitive G-proteins (Burford *et al.,* 1995; van Biersen *et al.,* 1996). Therefore, these data, in conjunction with the data presented in this Thesis support the potential for divergent coupling of receptor to G-protein in systems where the receptor

has been expressed at high levels. However, it may be possible that the m3-AChR is also coupled to G_i proteins under physiological conditions.

In order to determine this, the experiments described above could be performed in the CHO-m3 cells used in this study, in addition to a CHO cell line in which the m3-AChR is expressed at lower levels to compare the coupling of the m3-AChR to G_i and G_q pathways. Alternatively, studies could be carried out in tissue that have the endogenous m2- and m3-ACh-receptors, for example bovine tracheal smooth muscle, which would require the use of an m2-AChR muscarinic antagonist that is selective over the m3-AChR such as methoctramine. It would also be of interest to determine if there was a role for the m3-AChR in cAMP down-regulation (via G_i), this can be achieved by treating cells with forskolin (which acts directly on adenylyl cyclase) to increase cAMP levels. This would potentially demonstrate if the m3-AChR was coupled to G_i G-proteins.

Chapter 5

Modulation of ERK and JNK Activities in CHO-m2 and CHO-m3 cells by Protein Kinase C (PKC) and Phosphoinositide 3-Kinase (PI3K).

5.1 Introduction.

The protein kinase C (PKC) family are proteins that have a regulatory region in the N-terminus and a catalytic region at the C-terminus. PKC has been demonstrated to play a role in ERK activation (Gutkind 1998) in studies whereby PKC down-regulation by prolonged phorbol ester pre-treatment or by the inhibition of PKC by inhibitors either completely inhibited or partially inhibited ERK activation by phospholipase C (PLC)-coupled receptors (Gutkind 1998, Crespo *et al.*, 1994, Hawes *et al.*, 1995). In addition to this work, overexpression of PKC isoforms, caused ERK activation (Schönwasser *et al.*, 1998; Marais *et al.*, 1998) and JNK activation (Jun *et al.*, 1999). However, phorbol ester treatment has been shown to have no effect on JNK activation (Kyriakis et al., 1994), which implies that PKC activation alone is not able to activate JNK. However, Zohn *et al.*, (1995) demonstrated an attenuation of JNK activation when PKC was down-regulated by prolonged TPA treatment in GN4 cells. Further demonstrating the receptor- and cell-specific nature of these responses, Coso *et al.*, (1995) showed that in NIH3T3 cells, inhibition of PKC had no effect on the ability of the m1-AChR to activate JNK.

Phosphoinositide 3-kinase (PI3K) phosphorylates inositol phospholipids to yield phosphatidylinositol 3-phosphate (PtdIns3P), phosphatidylinositol (3,4)-bisphosphate PtdIns(3,4)P_2 and phosphatidylinositol (3,4,5)-trisphosphate (PtdIns (3,4,5)P_3) (Duronio *et al.*, 1998). The role of PI3K in the activation of ERK seems to be cell type- and ligand- specific. Duckworth and Cantley (1997), demonstrated that the PI3K inhibitor wortmannin inhibited platelet-derived growth factor (PDGF)-dependant activation of ERK in CHO cells, but had no effect on ERK activation via PDGF receptors in Swiss 3T3 cells. In 1996, Hawes *et al.*, demonstrated a role of PI3K in MAPK activation via GPCRs, this observation was followed by further evidence for PI3Kγ linking G_i- coupled receptors and $G_{\beta\gamma}$ to the MAPK pathway (Lopez-Ilasaca *et al.*, 1997).

Therefore, from this work, the aims of this Chapter are to investigate the role of PKC and PI3K in the agonist-induced activation of ERK and JNK in CHO cells expressing the m2- and m3- AChRs.

5.2 PKC Isozyme expression in CHO-m2 and CHO-m3 cells.

In order to determine which PKC isoforms were present in both the CHO-m2 and CHO-m3 cell lines, a western blot was carried out. The results demonstrate that the same isoforms are present in both cell lines. However, the results demonstrate that whilst PKCα, γ, δ, ε, ζ, λ, ι and μ could be shown, PKCβ and PKCθ were not detected. However, in the data presented, a positive control such as rat brain extract was not run, therefore the experiment provides no evidence that the antibodies used were viable. Therefore, it is possible that the reason the PKC isoforms could not be detected may be due to the low specificity of the antibodies, rather than the absence of the isoenzymes.

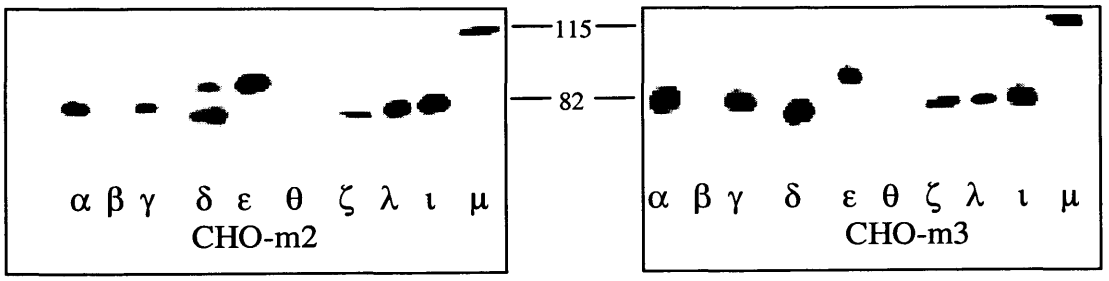

Figure 5.1 Immunoblot of PKC isoforms in CHO-m2 and CHO-m3 cells
CHO-m2 and CHO-m3 cells were lysed and 150 μg of protein run down a 10% SDS-PAGE gel. Using a multi-lane blotter, PKC primary antibodies were incubated for 2 h at the dilutions indicated in the Methods section (see section 2.6).

PKC Isoform	Apparent Molecular weight (kDa)	Literature Value (kDa) (from SDS/PAGE)
α (alpha)	82	82 (a)
β (beta)	-	80 (b)
γ (gamma)	82	79-84 (c)
δ (delta)	78	74-79 (d)
ε (epsilon)	89	90 (e)
θ (theta)	-	79 (f)
ζ (zeta)	76	76-80 (g)
λ (lambda)	78	74 (h)
ι (iota)	78	74 (i)
μ (mu)	114	115 (j)

Table 5.1 Apparent molecular weights of PKC isoforms detected in CHO cells compared with literature values. (Refs: (**a**); Parker *et al., (1986).*, Marias and Parker (1989), Burns *et al.*, (1990), (**b**); Marias and Parker (1989), Ono *et al.*, (1987), *(c);* Coussens *et al.*, (1986) Patel and Stabel (1989), Marais and Parker (1989), Burns *et al.*, (1990). (**d**); Ono *et al., (1988),* (**e**); Wang *et al.*, 1993, Mischak, *et al.*, (1993). (**f**); Osada *et al.*, (1992), Chang *et al.*, (1993). (**g**); Ono *et al., (1989)* Nakanashi and Exton *(1992),* McGlynn *et al., (1992*), Liyange *et al., (1992).* (**h**); Akimoto *et al.*, (1994), (**i**); Selbie *et al.*, 1993, (**j**); Johannes *et al.*, 1994.

5.2.1 Phorbol ester treatment induces ERK activation in CHO-m2 and CHO-m3 cells.

As has been previously reported by Cobb and Goldsmith, (1995), PKC stimulation by treatment of the cells with phorbol ester (1 μM phorbol dibutyrate (PDBu)) activated ERK. This was demonstrated for both the CHO-m2 and CHO-m3 cells lines, (Figure 5.2 and Figure 5.3 respectively). However, incubation of CHO-m3 cells with PDBu did not stimulate JNK activation (Figure 5.4) in agreement with previous studies (Kyriakis *et al.*, 1994).

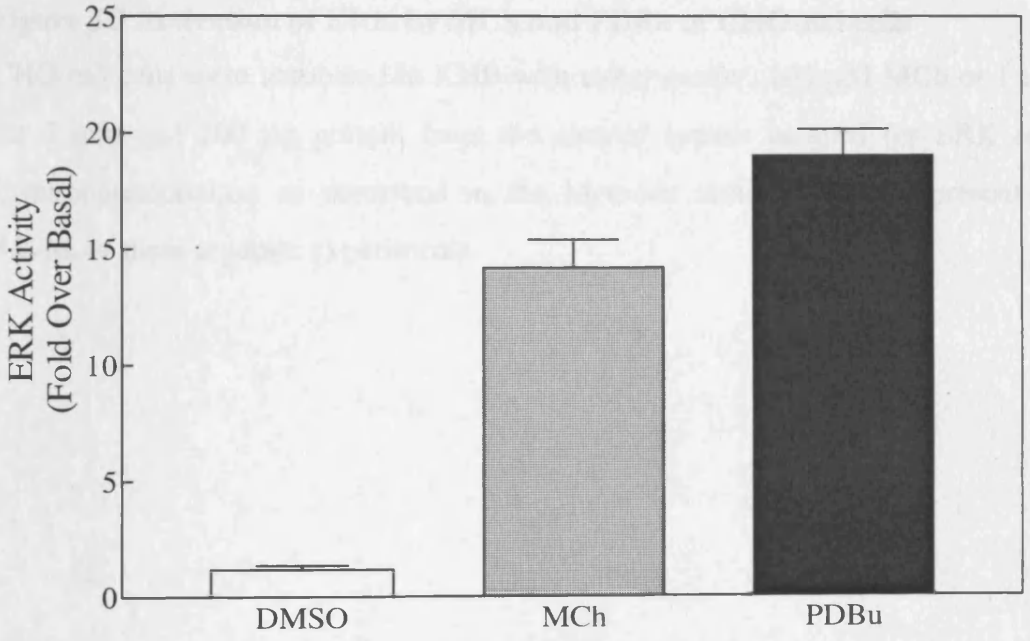

Figure 5.2 Activation of ERK by MCh and PDBu in CHO-m2 cells

CHO-m2 cells were incubated in KHB with either carrier, 100 μM MCh or 1 μM PDBu for 5 min, and 200 μg protein from the cleared lysates assayed for ERK activity by immunoprecipitation as described in the Methods section. Data represent means ± S.E.M. of three separate experiments.

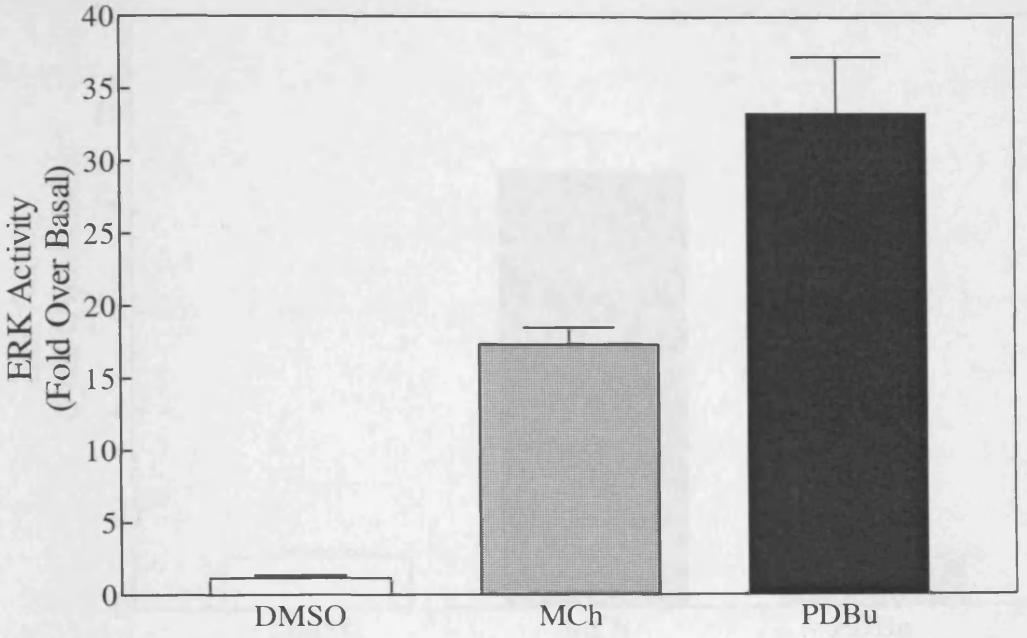

Figure 5.3 Activation of ERK by MCh and PDBu in CHO-m3 cells

CHO-m3 cells were incubated in KHB with either carrier, 100 μM MCh or 1 μM PDBu for 5 min and 200 μg protein from the cleared lysates assayed for ERK activity by immunoprecipitation as described in the Methods section. Data represent means ± S.E.M. of three separate experiments.

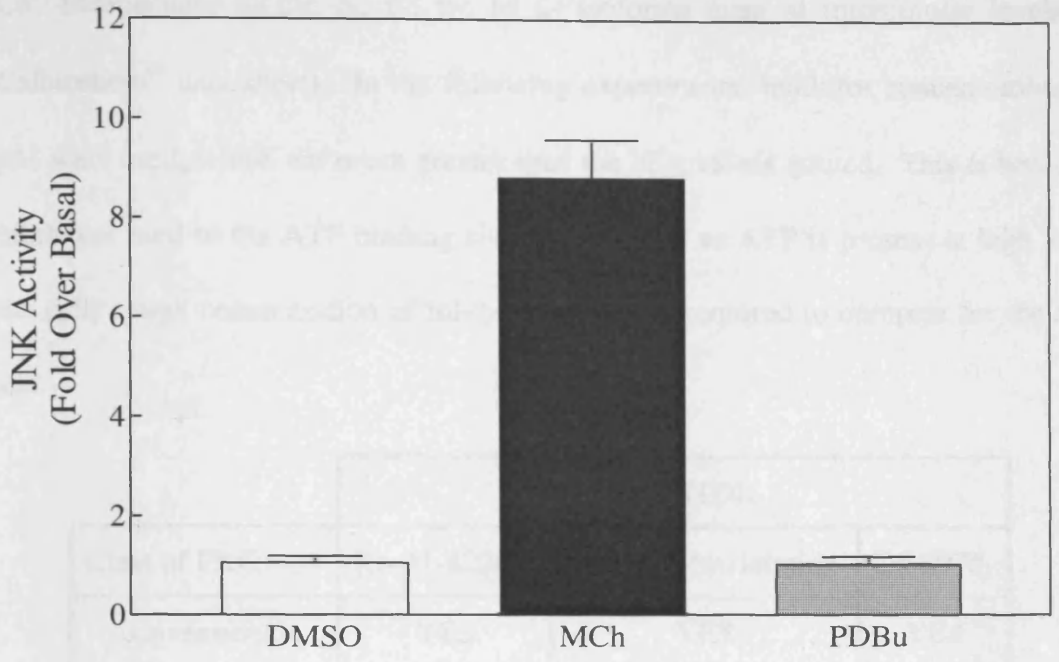

Figure 5.4 Activation of JNK by MCh and PDBu in CHO-m3 cells.
CHO-m3 cells were incubated in KHB with either vehicle, 100 μM MCh or 1 μM PDBu for 30 min, and cleared lysates containing 400 μg protein assayed for JNK activity by GST-Jun phosphorylation as described in the Methods section. Data represent means ± S.E.M. of 5-7 separate experiments.

5.2.2 The role of PKC in ERK activation in CHO-m2 and CHO-m3 cells.

The role of PKC in the agonist-induced activation of ERK and JNK in the CHO cells expressing the m2-AChR or m3-AChR was then investigated by using inhibitors and prolonged pre-treatment with phorbol ester. The compound Ro-31-8220 is a general PKC inhibitor with an IC_{50} value of 10 nM, and bisindolylmaleimide (also known as GF 109203X or Gö 6850) is a inhibitor of conventional and novel PKCs, which acts by binding competitively at the ATP-binding site with IC_{50} values of 8.4 nM, 18 nM, 210 nM, 132 nM for PKC α, β-, δ-, ε-, respectively (from Calbiochem® data sheet). The compound, Gö 6976, is a specific inhibitor for PKCα and PKCβI, with IC_{50} values (with respect to ATP) of 2.3 nM and 6.2 nM, respectively, in contrast to having no effect on the

Ca^{2+}-independent PKCs; δ-, ε-, η-, or ζ- isoforms even at micromolar levels (from Calbiochem® data sheet). In the following experiments, inhibitor concentrations of 10 μM were used, which are much greater than the IC_{50} values quoted. This is because, the inhibitors bind to the ATP binding site of PKC, and as ATP is present at high levels in the cell, a high concentration of inhibitor would be required to compete for the binding site.

	INHIBITOR		
Class of PKC	Ro-31-8220	Bisindolylmaleimide	Gö 6976
conventional (α, β, γ)	YES	YES	YES
novel (δ, ε, θ)	YES	YES	NO
atypical (ζ, ι, λ, μ)	YES	NO	NO

Table 5.2 Summary of PKC isoforms and the action of the inhibitors, Ro-31-8220, bisindolylmaleimide and Gö 6976.

CHO-m2 cells were pre-incubated with KHB as described in the Methods section for 30 min, and for the final 10 min the cells were pre-treated with 10 μM Ro-31-8220, 10 μM bisindolylmaleimide or 10 μM Gö 6976 prior to stimulation with 100 μM MCh. In CHO-m2 cells, the general PKC inhibitors Ro-31-8220 and bisindolylmaleimide inhibited agonist-stimulated ERK activation by 75% and 50% respectively. In addition, ERK inhibition by bisindolylmaleimide when compared with that by Ro-31-8220 was also significantly different ($P < 0.05$ by Duncan's multiple-range test), suggesting that there is a role of aPKCs in the activation of ERK and also a role for nPKCs and cPKCs.

However, the conventional PKC inhibitor, Gö 6976 had no inhibitory effect on ERK activity (Figure 5.5). Therefore indicating that there is a PKC component in m2-AChR-mediated ERK activity which is independent of the conventional PKC isoforms.

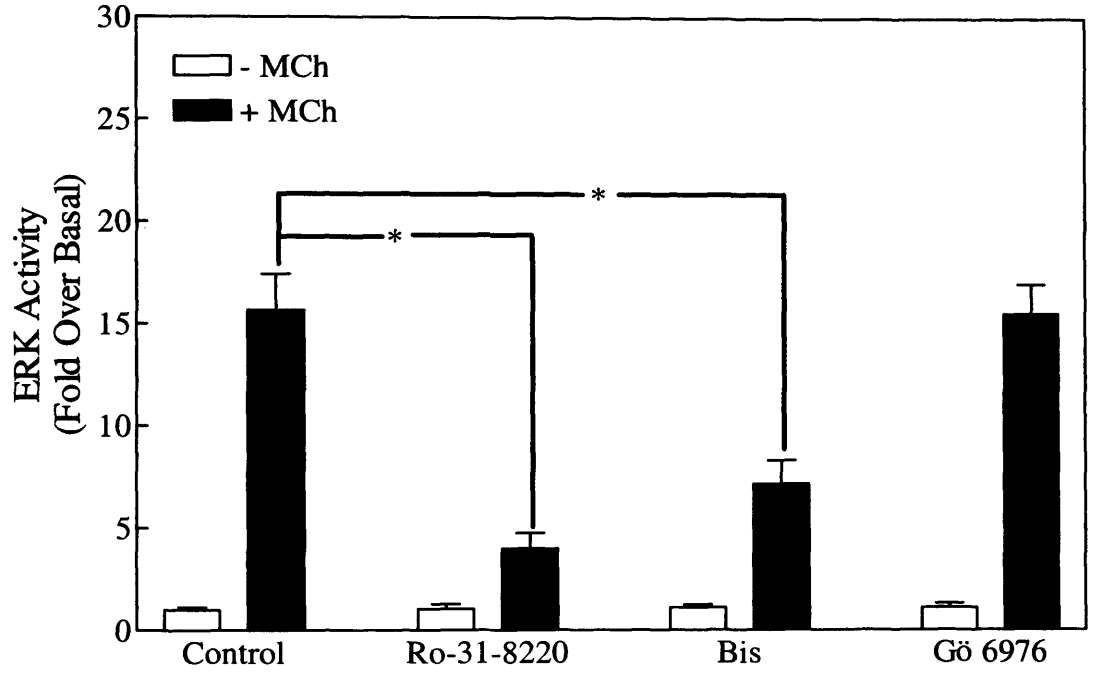

Figure 5.5 Role of PKC in ERK activation in CHO-m2 cells.

CHO-m2 cells were incubated in freshly gassed KHB with carrier (DMSO), 10 μM Ro-31-8220, 10 μM bisindolylmaleimide (Bis) or 10 μM Gö 6976 for 10 min prior to stimulation with 100 μM MCh for 5 min. Cleared lysates containing 200 μg protein were assayed for ERK activity following immunoprecipitation as described in the Methods section. Data represent means ± S.E.M. of 5-7 separate experiments. * indicates inhibition of agonist-induced ERK activation at $P < 0.05$, by Duncan's multiple-range test that is statistically significantly different from the activation by agonist in control cells. Under all conditions, the MCh stimulation of ERK was statistically significantly different from the corresponding basal ERK activation at $P < 0.05$, by Duncan's multiple-range test.

In contrast, pre-incubation of CHO-m3 cells with Ro-31-8220 essentially abolished the ERK activation induced by MCh (Figure 5.6). However, bisindolylmaleimide only

inhibited the ERK activity by 50%, similar to the effect seen in the CHO-m2 cells. Gö 6976 also inhibited the ERK activation by agonist by 50%. These results indicate that m3-AChR-mediated ERK activation is PKC-dependent, and in contrast to the m2-AChR-mediated response has a conventional PKC isoform component.

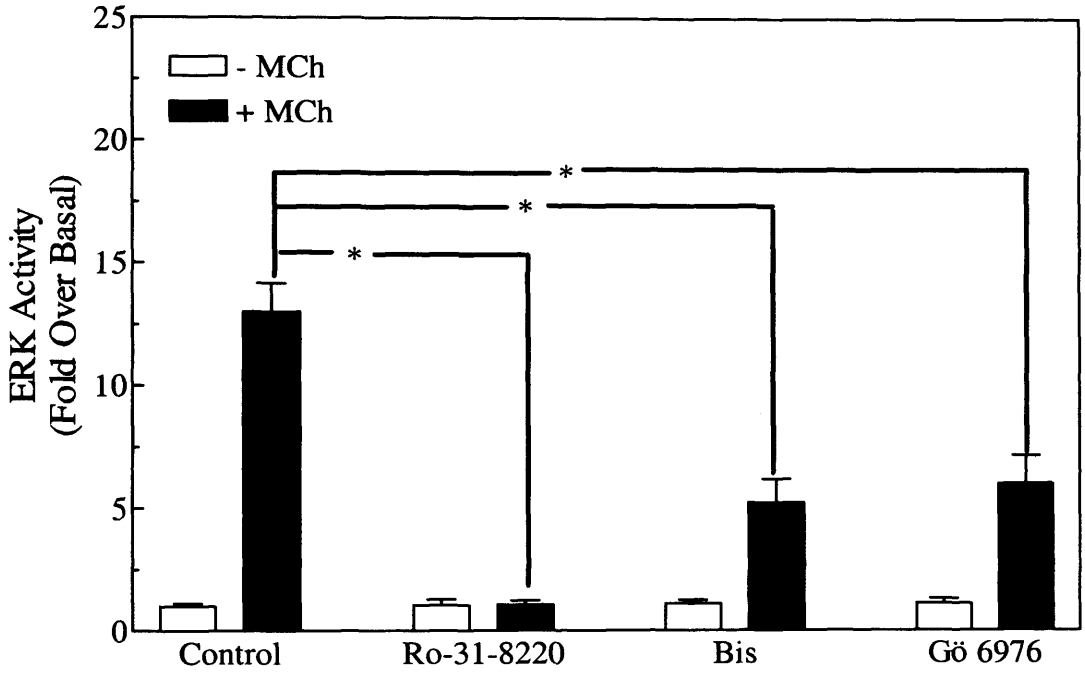

Figure 5.6 Role of PKC in ERK activation in CHO-m3 cells.

CHO-m3 cells were incubated in freshly gassed KHB with carrier (DMSO), 10 μM Ro-31-8220, 10 μM bisindolylmaleimide (Bis) or 10 μM Gö 6976 for 10 min prior to stimulation with 100 μM MCh or carrier (DMSO at a final concentration of 0.1%) for 5 min. Cleared lysates containing 200 μg protein were assayed for ERK activity following immunoprecipitation as described in the Methods section. Data represent means ± S.E.M. of 4-7 separate experiments. * indicates inhibition of agonist-induced ERK activation at $P < 0.05$, by Duncan's multiple-range test that is statistically significantly different from the activation by agonist in control cells. Under all conditions, except with Ro-31-8220, the MCh stimulation of ERK was statistically significantly different from the corresponding basal ERK activation at $P < 0.05$, by Duncan's multiple-range test.

In order to determine the concentration-dependence of PKC inhibitor effects on agonist-induced ERK activation, an inhibition curve experiment was carried out, and this determined that 1 μM Ro-31-8220 was maximal for inhibiting agonist-induced ERK activation in both CHO-m2 and CHO-m3 cells (Figure 5.7 and Figure 5.8), with log EC_{50} values of -6.72 ± 0.21 (EC_{50} (M) 1.88×10^{-7}) and -6.82 ± 0.24 (EC_{50} (M) 1.52×10^{-7}).

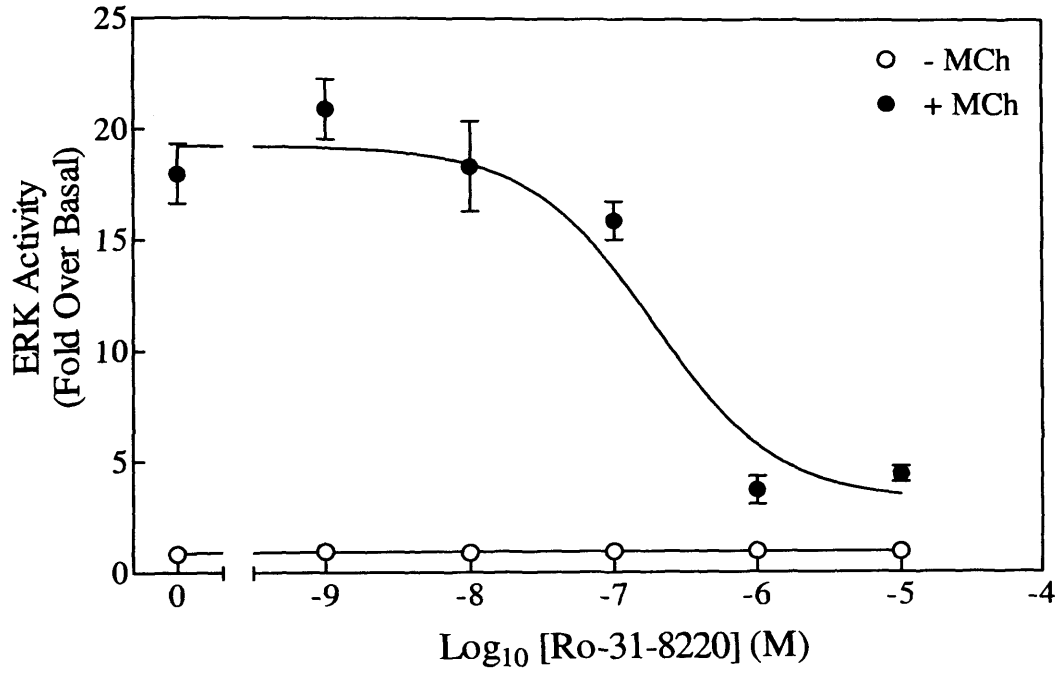

Figure 5.7 Concentration-inhibition curve for the effects of Ro-31-8220 on ERK activation in CHO-m2 cells.

CHO-m2 cells were incubated in freshly gassed KHB with the indicated concentrations of Ro-31-8220 for 10 min prior to stimulation with 100 μM MCh or vehicle for 5 min. Cleared lysates containing 200 μg protein were assayed for ERK activity following immunoprecipitation as described in the Methods section. Data represent mean ± S.E.M. of 4-9 separate experiments. The IC_{50} value for Ro-31-8220 mediated inhibition was 0.2 μM (log IC_{50} -6.7 ± 0.2).

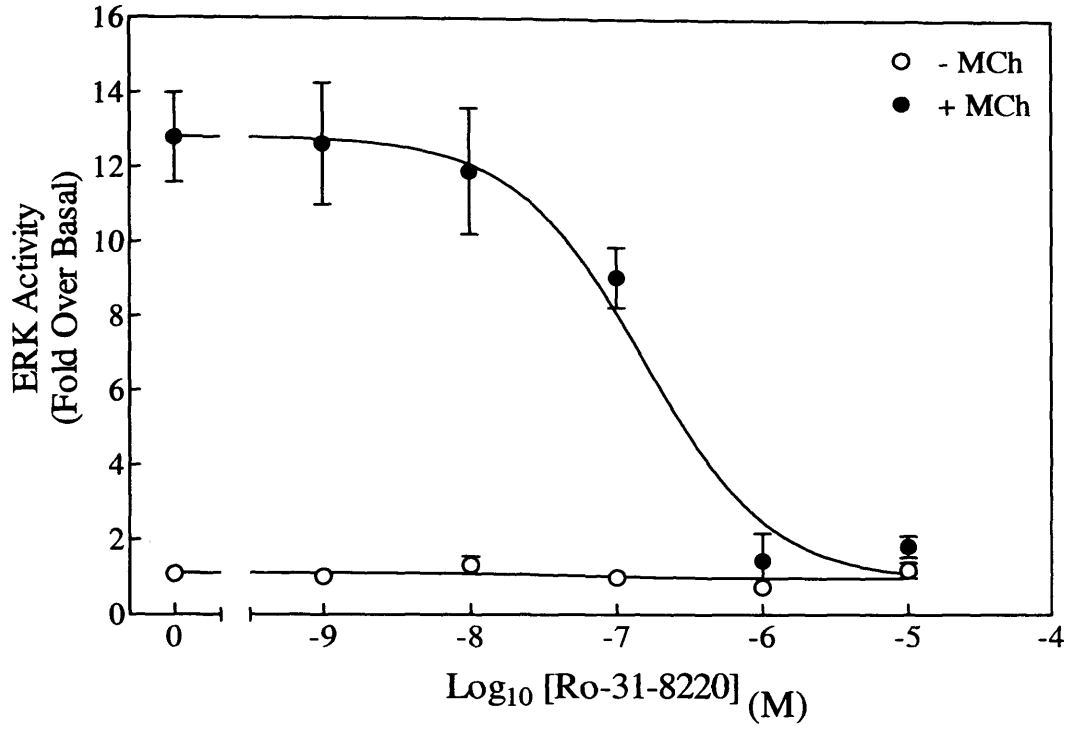

Figure 5.8 Concentration-inhibition curve for the effects of Ro-31-8220 on ERK activation in CHO-m3 cells.

CHO-m3 cells were incubated in freshly gassed KHB with the indicated concentrations of Ro-31-8220 for 10 min prior to stimulation with 100 μM MCh or vehicle for 30 min. Cleared lysates containing 200 μg protein were assayed for ERK activity following immunoprecipitation as described in the Methods section. Data represent means ± S.E.M. of 4-9 separate experiments. The IC_{50} value for Ro-31-8220 mediated inhibition was 0.2 μM (log IC_{50} -6.8 ± 0.2).

As high concentrations of Ro-31-8220 have been reported to cause muscarinic receptor antagonism, we thought it prudent to confirm that 1 μM Ro-31-8220 had the same effect as 10 μM Ro-31-8220 as observed in the concentration-inhibition data. In this experiment, cells were incubated in the presence of 1 μM or 10 μM Ro-31-8220 for 10 min prior to agonist stimulation. The results from both the CHO-m2 and CHO-m3 cells lines show that 1 μM Ro-31-8220 has the same inhibitory effect on agonist-induced ERK activation as 10 μM Ro-31-8220 (Figure 5.9 and Figure 5.10). Data presented in

the following section (5.2.3) also provide evidence that Ro-31-8220 does not act as a muscarinic receptor antagonist in these cells. This might be predicted as MCh was used at a much greater concentration than the Kd value for the receptor (Burford *et al.*, 1995), and so Ro-31-228 was used at too low a concentration to compete with MCh for the receptor.

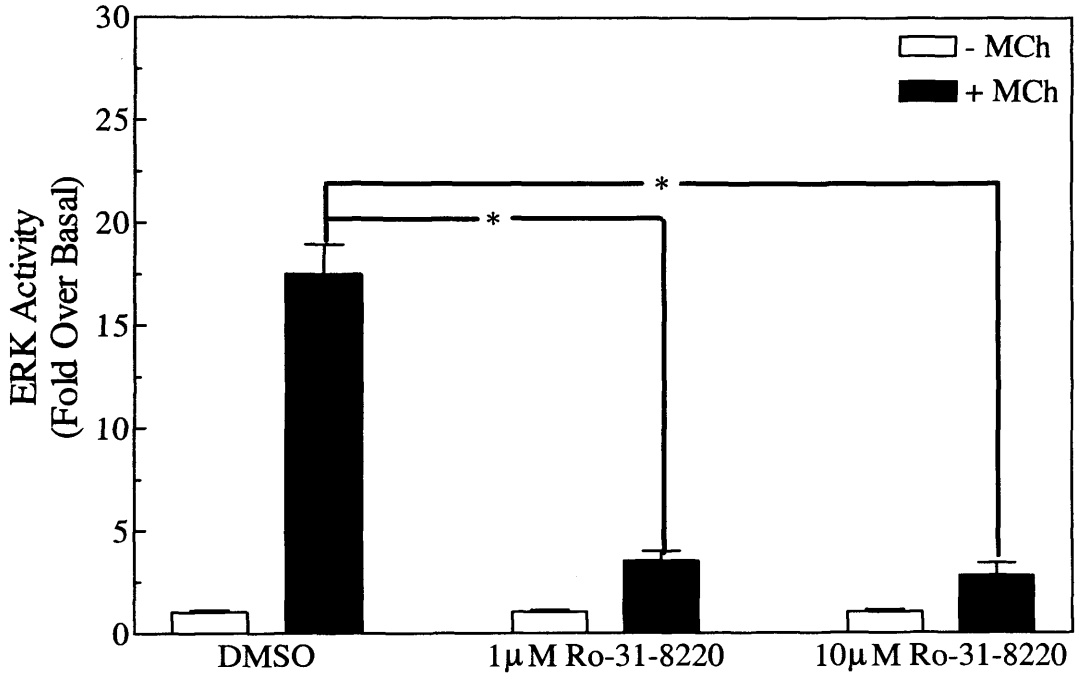

Figure 5.9 Effect of 1 μM versus 10 μM Ro-31-8220 to inhibit agonist-induced ERK activation in CHO-m2 cells.

CHO-m2 cells were incubated in freshly gassed KHB with vehicle (DMSO), 1 μM Ro-31-8220 or 10 μM Ro-31-8220 for 10 min prior to stimulation with 100 μM MCh for 5 min. Cleared lysates containing 200 μg protein were assayed for ERK activity following immunoprecipitation as described in the Methods section. Data represent means ± S.E.M. of 7-11 separate experiments. * indicates inhibition of agonist-induced ERK activation at $P < 0.05$, by Duncan's multiple-range test that is statistically significantly different from the activation by agonist in control cells. Under all conditions, the MCh stimulation of ERK was statistically significantly different from the corresponding basal ERK activation at $P < 0.05$, by Duncan's multiple-range test.

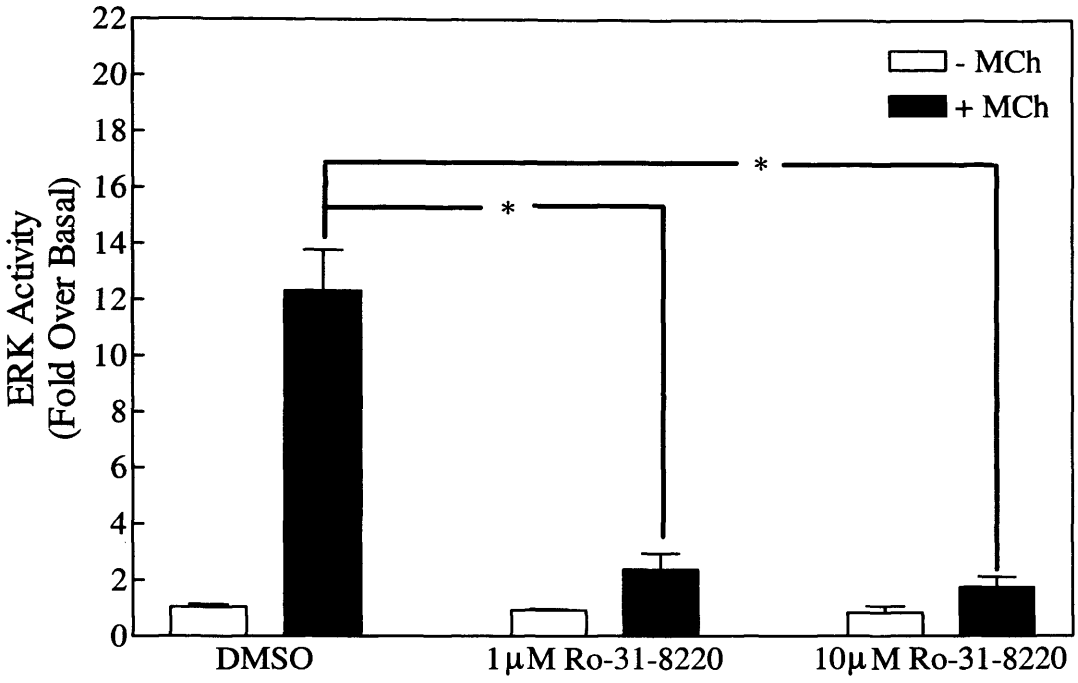

Figure 5.10 Effect of 1 μM Ro-31-8220 versus 10 μM Ro-31-8220 to inhibit agonist-induced ERK activation in CHO-m3 cells.

CHO-m3 cells were incubated in freshly gassed KHB with vehicle (DMSO), 1 μM Ro-31-8220 or 10 μM Ro-31-8220 for 10 min prior to stimulation with 100 μM MCh for 5 min. Cleared lysates containing 200 μg protein were assayed for ERK activity following immunoprecipitation as described in the Methods section. Data represent means ± S.E.M. of 7-11 separate experiments. * indicates inhibition of agonist-induced ERK activation at $P < 0.05$, by Duncan's multiple-range test that is statistically significantly different from the activation by agonist in control cells. Under all conditions, except with 10 μM Ro-31-8220, the MCh stimulation of ERK was statistically significantly different from the corresponding basal ERK activation at $P < 0.05$, by Duncan's multiple-range test.

To further confirm the role of PKC in the ERK pathway, CHO-m3 cells were pre-treated with 1 μM PDBu for 18 h prior to MCh stimulation. Prolonged activation of PKC down-regulates many of the PKC isoforms (Kennedy *et al.*, 1997) and is a common method used to inhibit PKC activity. The results demonstrate that ERK activation by

MCh was significantly reduced by 50% following PKC-down-regulation, confirming a stimulatory role for PKC in the ERK pathway (Figure 5.11 and Figure 5.12 respectively).

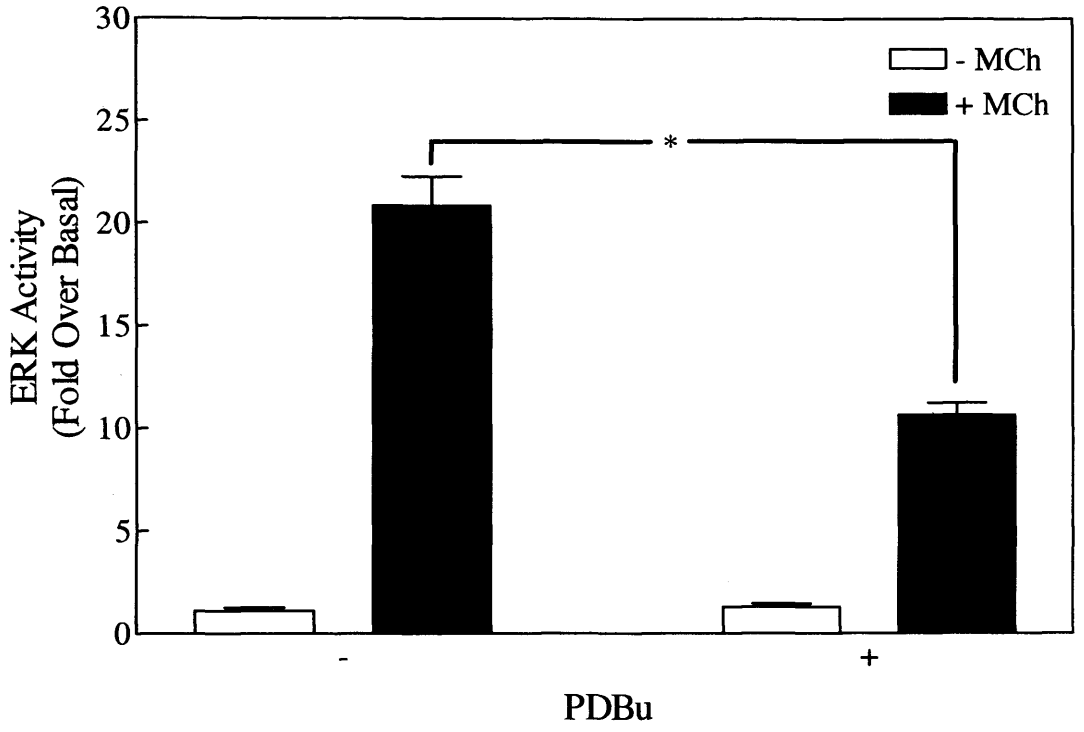

Figure 5.11 Effect of PKC down-regulation on agonist-induced ERK activation in CHO-m2 cells.

CHO-m2 cells were incubated in medium with 1 μM PDBu for 18 h prior to stimulation with 100 μM MCh for 5 min. Cleared lysates containing 200 μg protein were assayed for ERK activity following immunoprecipitation as described in the Methods section. Data represent means ± S.E.M. of 3 separate experiments. * indicates inhibition of agonist-induced ERK activation at $P < 0.05$, by Duncan's multiple-range test that is statistically significantly different from the activation by agonist in control cells. Under both conditions, the MCh stimulation of ERK was statistically significantly different from the corresponding basal ERK activation at $P < 0.05$, by Duncan's multiple-range test.

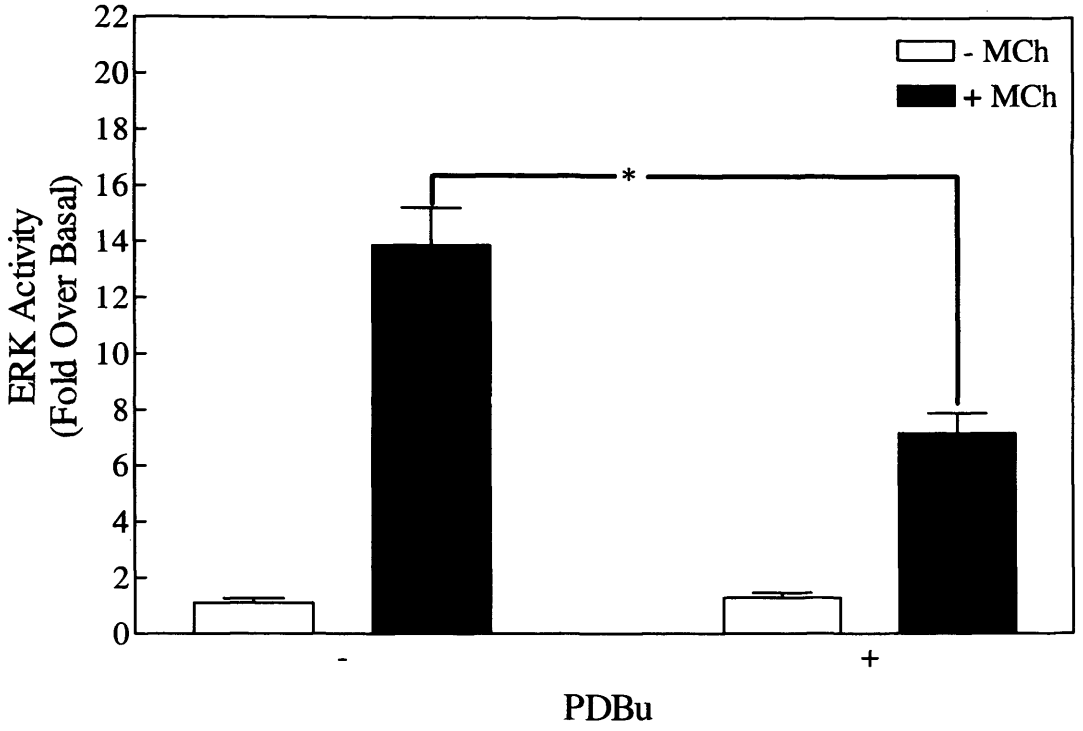

Figure 5.12 Effect of PKC down-regulation on agonist-induced ERK activation in CHO-m3 cells.

CHO-m3 cells were incubated in medium with 1 μM PDBu for 18 h prior to stimulation with 100 μM MCh for 5 min. Cleared lysates containing 200 μg protein were assayed for ERK activity following immunoprecipitation as described in the Methods section. Data represent means ± S.E.M. of 4 separate experiments. * indicates inhibition of agonist-induced ERK activation at $P < 0.05$, by Duncan's multiple-range test that is statistically significantly different from the activation by agonist in control cells. Under both conditions, the MCh stimulation of ERK was statistically significantly different from the corresponding basal ERK activation at $P < 0.05$, by Duncan's multiple-range test.

5.2.3 Effect of PKC inhibition on JNK activation in CHO-m2 and CHO-m3 cells.

As has been previously discussed, JNK can be activated by a number of cellular stresses including the inhibitor of protein synthesis, anisomycin (Kyriakis *et al.,* 1994). However, treatment of cells with the PKC activator PDBu has no effect on JNK activity (Kyriakis *et al.,* 1994). Figure 5.14 and Figure 5.15 demonstrate that these results are also observed in the CHO-m3 cells used in this study.

Interpretation of experiments to determine the involvement of PKC in the activation of JNK by AChRs was complicated by the marked stimulatory effect Ro-31-8220 had on basal JNK activity in both CHO-m2 and CHO-m3 cells. (Figure 5.13 and Figure 5.14). In CHO-m3 cells, there was also a marked enhancement of JNK activity by 10 μM Ro-31-8220 when the cells were stimulated with 100 μM MCh (Figure 5.14).

To investigate whether this activation was due to the inhibition of PKC, CHO-m3 cells were chronically treated with 1 μM PDBu for 18 h to down-regulate PKC. Under these conditions, MCh caused a larger JNK activation (Figure 5.15) than that observed in control cells. Indeed the response following PKC-downregulation was similar to that seen under conditions of Ro-31-8220 inhibition of PKC. As a final experiment to confirm PKC inhibition of the JNK pathway, CHO-m3 cells were pre-incubated for 5 min with 1 μM PDBu to activate PKC. This resulted in a 50 % inhibition on agonist-induced JNK activity (Figure 5.16), further suggesting that PKC is a negative regulator of the JNK pathway.

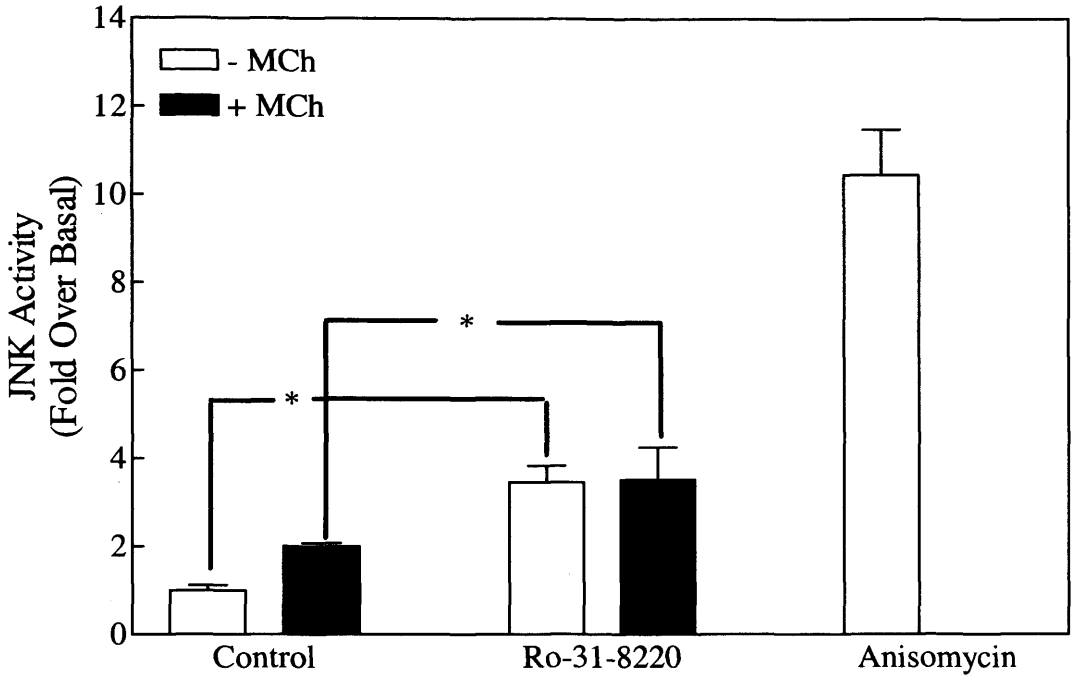

Figure 5.13 Effect of PKC inhibition on JNK activation in CHO-m2 cells.

CHO-m2 cells were incubated in freshly gassed KHB with carrier (DMSO at a final concentration of 0.1 %) or 10 μM Ro-31-8220 for 10 min prior to stimulation with 100 μM MCh for 15 min. As a positive control, cells were incubated with 100 ng/ml anisomycin for 30 min. Cleared lysates containing 400 μg protein were assayed for JNK activity by GST-Jun phosphorylation as described in the Methods section. Data represent means ± S.E.M. of 4-9 separate experiments. * indicates an increase in JNK activity at $P < 0.05$ by Duncan's multiple-range test that is statistically significantly different from the activity in the corresponding control cells, as indicated.

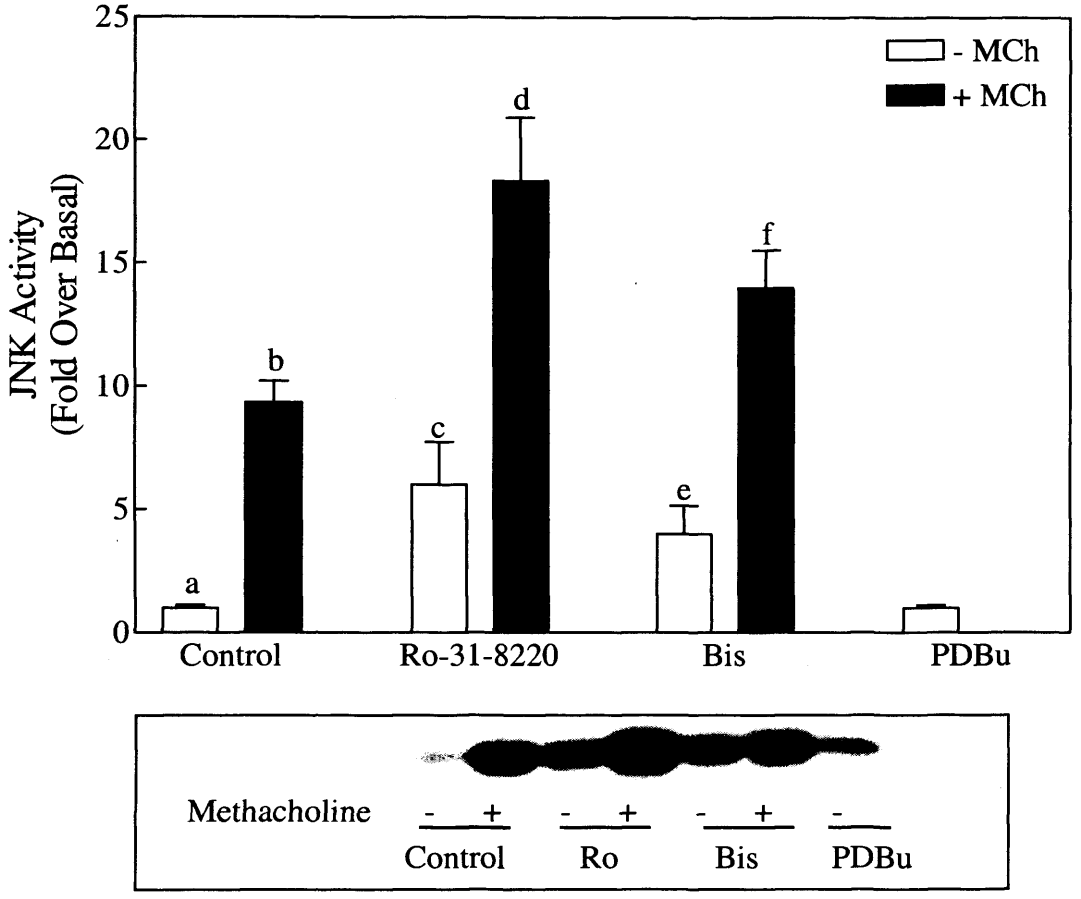

Figure 5.14 Effects of PKC inhibition or activation on JNK activation in CHO-m3 cells.

CHO-m3 cells were incubated in freshly gassed KHB with carrier (DMSO), 10 μM Ro-31-8220 or 10 μM bisindolylmaleimide (Bis) for 10 min prior to stimulation with 100 μM MCh for 30 min. Cleared lysates containing 400 μg protein were assayed for JNK activity by GST-Jun phosphorylation as described in the Methods section. The lower panel shows a representative autoradiogram of GST-Jun phosphorylation. Data represent means ± S.E.M. of 3-8 separate experiments. a vs c and e are significantly different at $P < 0.05$; b vs d and f are significantly different at $P < 0.05$ by Duncan's multiple-range test.

Finally, to confirm that Ro-31-8220 was not having a non-specific effect on the IP_3 pathway, IP_3 mass assays were carried out in the CHO-m3 cells in the presence and absence of Ro-31-8220 at 10 μM. The results obtained demonstrate (Figure 5.17) that with a 100 μM MCh challenge, there was no effect on the IP_3 accumulation, therefore

demonstrating that the results observed in the CHO-m3 cells by Ro-31-8220 were likely to be caused by inhibition of PKC rather than due to a non-specific inhibition of the m3-AChR-mediated signalling pathway such as might be predicted by muscarinic receptor antagonism.

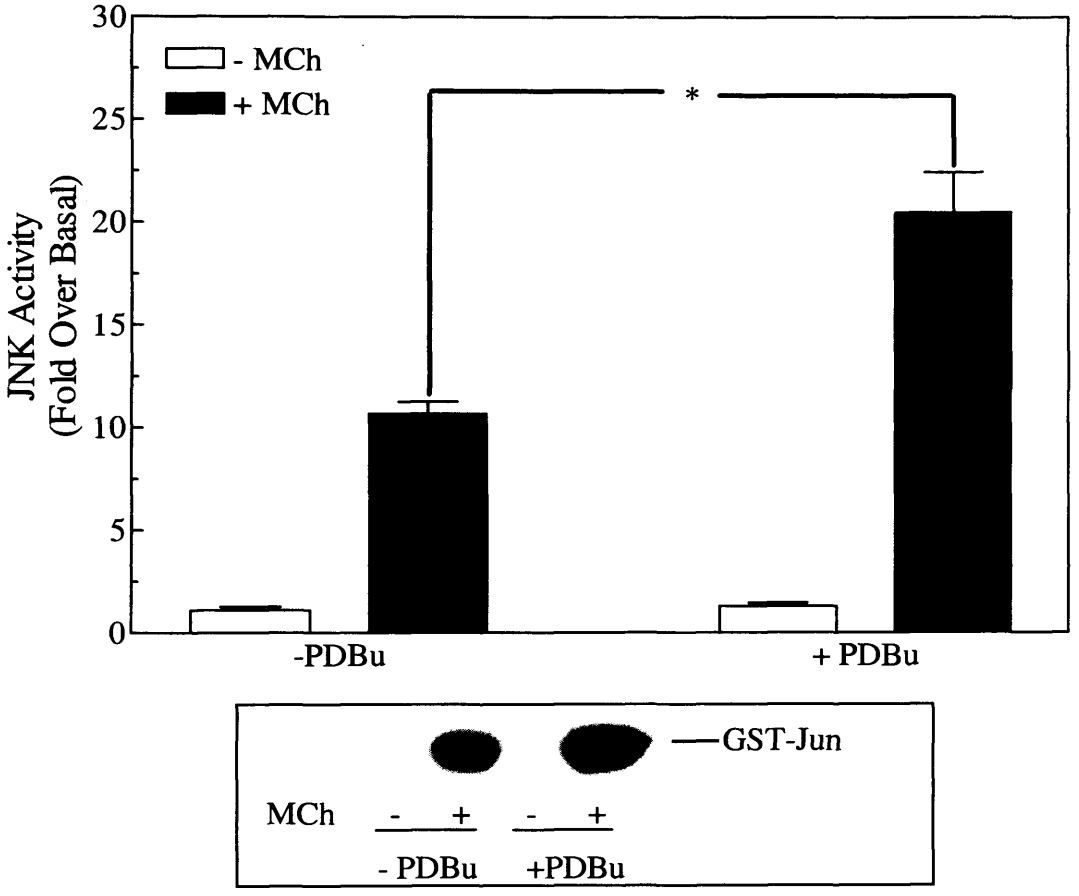

Figure 5.15 Effect of PKC down-regulation on JNK activation in CHO-m3 cells. CHO-m3 cells were incubated in medium with 1 μM PDBu for 18 hr prior to stimulation with 100 μM MCh for 30 min. Cleared lysates containing 400 μg protein were assayed for JNK activity by GST-Jun phosphorylation as described in the Methods section. The lower panel shows a representative autoradiogram of GST-Jun phosphorylation. Data represent means ± S.E.M. of four separate experiments. * indicates an increase in JNK activation at $P < 0.05$, by Duncan's multiple-range test that is statistically significantly different from the activation by agonist in control cells. Under both conditions, the MCh stimulation of JNK was statistically significantly different from the corresponding basal JNK activation at $P < 0.05$, by Duncan's multiple-range test.

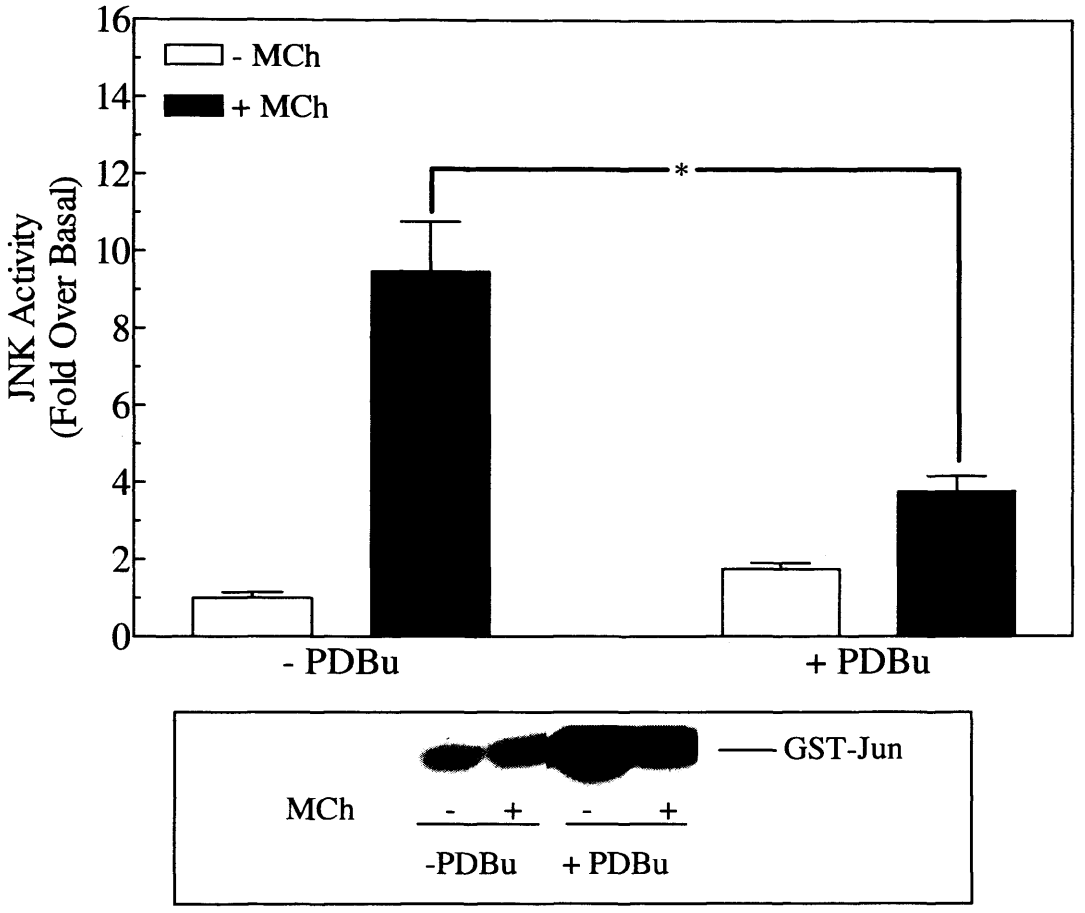

Figure 5.16. Effect of brief PDBu pre-treatment on JNK activity in CHO-m3 cells. CHO-m3 cells were incubated in KHB with 1 μM PDBu or carrier for 5 min prior to stimulation with 100 μM MCh for 30 min. Cleared lysates containing 400 μg protein were assayed for JNK activity by GST-Jun phosphorylation as described in the Methods section. The lower panel shows a representative autoradiogram of GST-Jun phosphorylation. Data represent means ± S.E.M. of 3 separate experiments. * indicates inhibition of agonist-induced JNK activation at $P < 0.05$, by Duncan's multiple-range test that is statistically significantly different from the activation by agonist in control cells. Under both conditions, the MCh stimulation of JNK was statistically significantly different from the corresponding basal JNK activation at $P < 0.05$, by Duncan's multiple-range test.

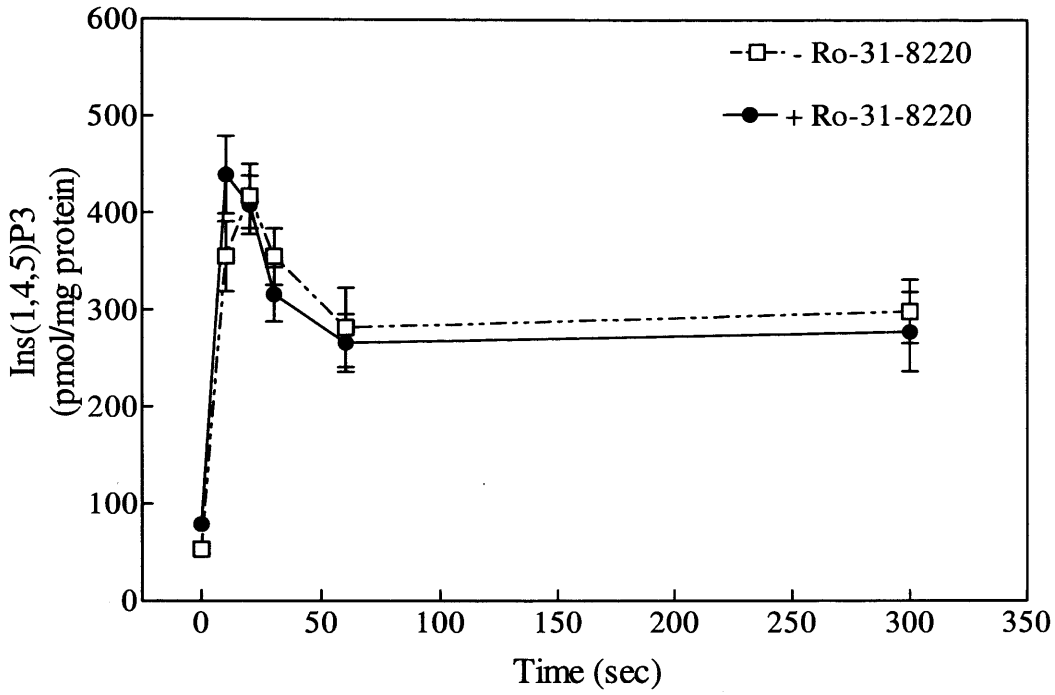

Figure 5.17 Effect of Ro-31-8220 on MCh-stimulated IP_3 accumulation in CHO-m3 cells.

CHO-m3 were pre-treated with 10 μM Ro-31-8220 or carrier for 20 min. Cells were then stimulated with 100 μM MCh for the indicated times. The reaction was terminated by aspiration of the KHB/drug solution and the addition of 300 μM 0.5 M TCA. IP_3 mass levels were the analysed as described in Methods. Data represent means ± S.E.M. of three separate experiments performed in duplicate.

5.3 Role of PI3K in agonist-induced ERK and JNK activation in CHO-m2 and CHO-m3 cells.

5.3.1 Role of PI3K in agonist-induced ERK activation.

To investigate the role of PI3K in the agonist-induced activation of the ERK and JNK pathways, cells were pre-incubated with either 10 μM wortmannin or 5 μM LY-294002 for 10 min prior to agonist stimulation. The results in the CHO-m2 cells demonstrated an inhibition of 60% in agonist stimulated ERK activation following treatment with 10 μM wortmannin, and a 50% inhibition with 5 μM LY-294002 (Figure 5.18), suggesting a role of PI3K in the activation of ERK via the m2 receptor. In contrast, however, pre-treatment of CHO-m3 cells with 10 μM wortmannin inhibited agonist-induced ERK activation completely, compared with a 50% inhibition observed with LY-294002 (Figure 5.19).

The concentration of wortmannin used in this experiment may have effects beyond PI3K inhibition. A concentration of 100 nM is probably sufficient to inhibit PI3K completely, whereas a concentration of 10 μM will not only completely inhibit PI3K, but may also reduce levels of PI4K (Carpenter and Cantley, 1996, Nakagawa *et al.*, 1996). To determine the effect of the concentration of the inhibitors on agonist-induced ERK activation, an inhibition curve for LY-294002 and wortmannin was performed. The results of these experiments gave an IC_{50} values for wortmannin of 2.8 μM [log IC_{50} (M) -5.55 ± 0.3] and 1.15 μM [log IC_{50} (M) -5.94 ± 0.3] in CHO-m2 and CHO-m3 cells respectively (Figure 5.20, Figure 5.21). This compared with IC_{50} values with LY-294002 26 μM [log IC_{50} (M) -4.58 ± 0.2] and 30 μM [log IC_{50} (M) -4.53 ± 0.3] for CHO-m2 and CHO-m3 cells respectively (Figure 5.22 and Figure 5.23).

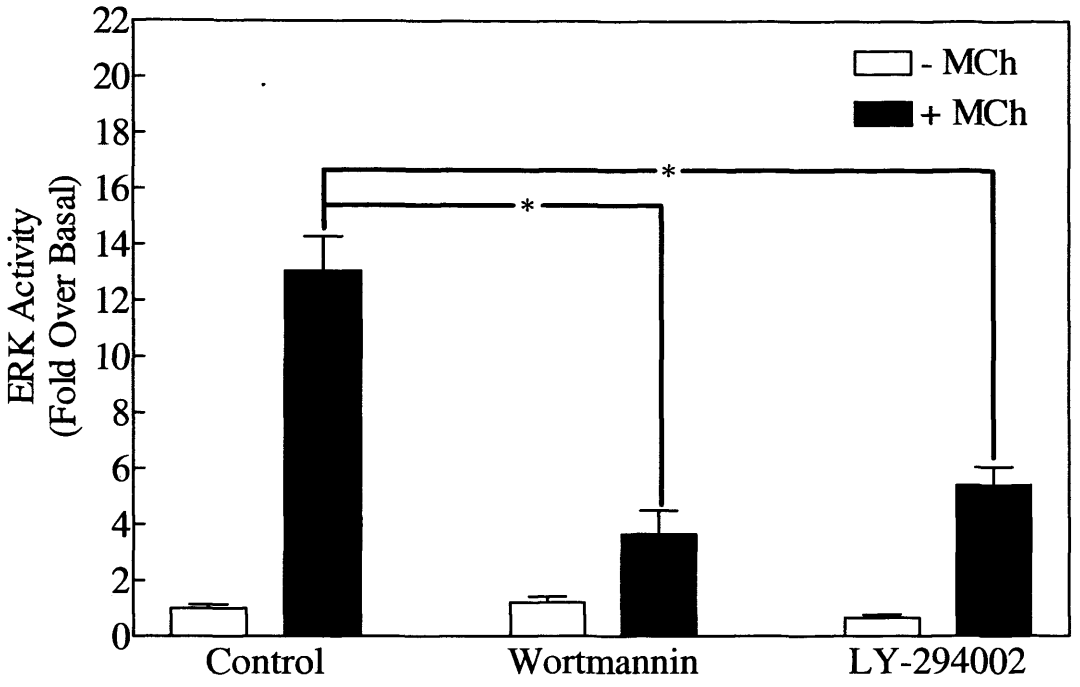

Figure 5.18 Effect of PI3K inhibitors on ERK activation in CHO-m2 cells.

CHO-m2 cells were incubated in freshly gassed KHB with carrier (DMSO), 10 μM wortmannin or 5 μM LY-294002 for 10 min prior to stimulation with 100 μM MCh (solid bars) or carrier (white bars) for 5 min. Cleared lysates containing 200 μg protein were assayed for ERK activity by immunoprecipitation as described in the Methods section. Data represent means ± S.E.M. of 4-6 separate experiments. * indicates inhibition of agonist-induced ERK activation at $P< 0.05$, by Duncan's multiple-range test that is statistically significantly different from the activation by agonist in control cells. Under all conditions, the MCh stimulation of ERK was statistically significantly different from the corresponding basal ERK activation at $P < 0.05$, by Duncan's multiple-range test.

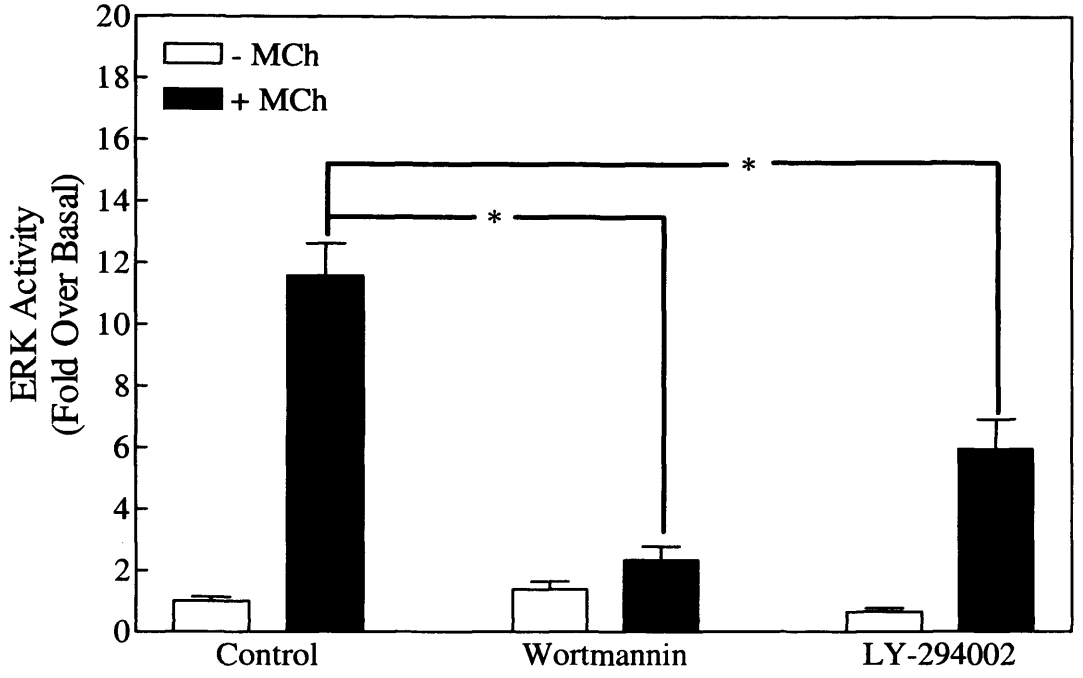

Figure 5.19 Effect of PI3K inhibitors on ERK activation in CHO-m3 cells.

CHO-m3 cells were incubated in freshly gassed KHB with carrier (DMSO), 10 μM wortmannin or 5 μM LY-294002 for 10 min prior to stimulation with 100 μM MCh (solid bars) or carrier (clear bars) for 5 min. Cleared lysates containing 200 μg protein were assayed for ERK activity by immunoprecipitation as described in the Methods section. Data represent means ± S.E.M. of 3-7 separate experiments. * indicates inhibition of agonist-induced ERK activation at $P < 0.05$, by Duncan's multiple-range test that is statistically significantly different from the activation by agonist in control cells. In control cells and those treated with 5 μM LY-294002 (but not 10 μM wortmannin), the MCh stimulation of ERK was statistically significantly different from the corresponding basal ERK activation at $P < 0.05$, by Duncan's multiple-range test.

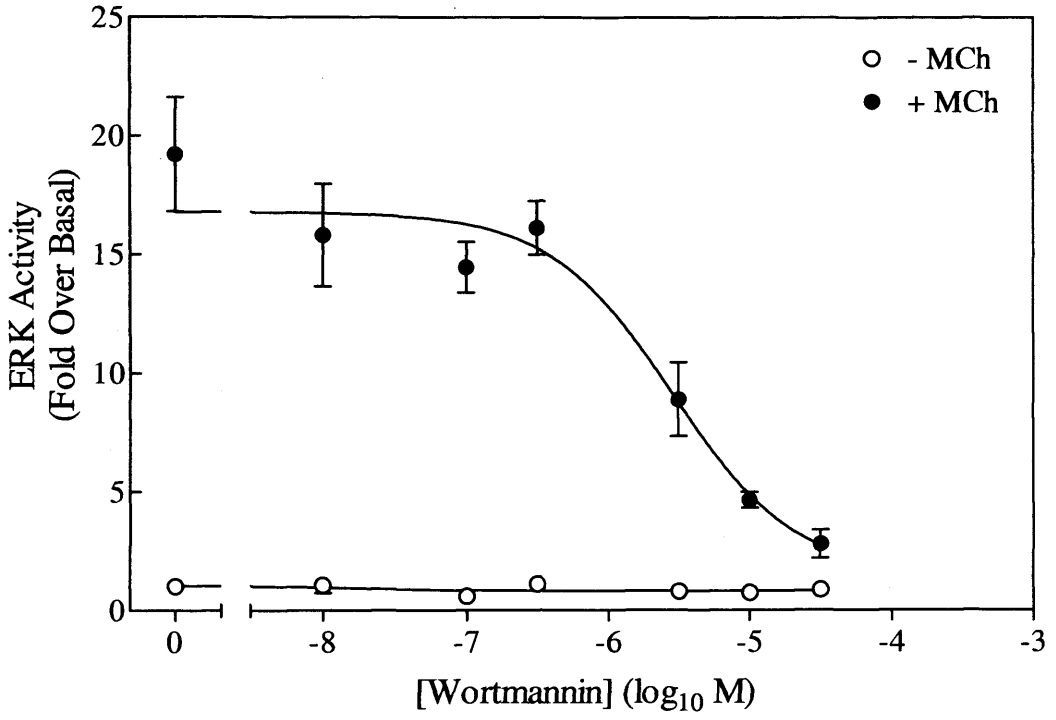

Figure 5.20 Concentration-dependent inhibition of agonist-stimulated ERK activation by wortmannin in CHO-m2 cells.

CHO-m2 cells were pre-incubated for 10 min with various concentrations of wortmannin or carrier (DMSO) prior to stimulation with 100 μM MCh for 5 min. 200 μg of protein was assayed for ERK activity by immunoprecipitation (see Methods). Data represent means ± S.E.M. of three separate experiments.

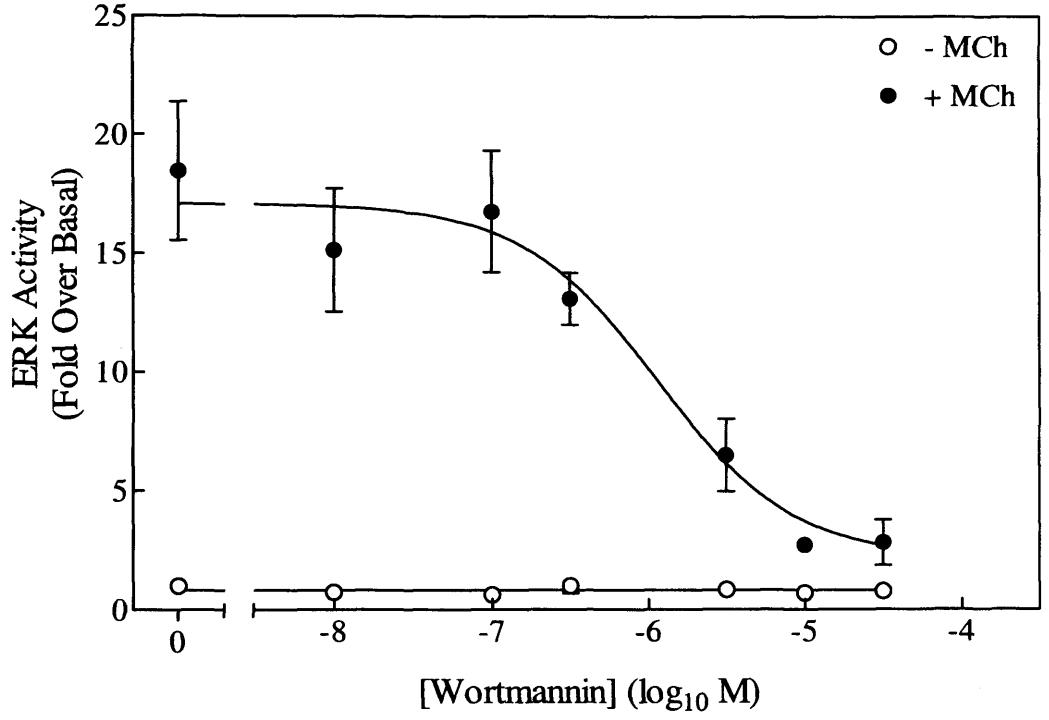

Figure 5.21 Concentration-dependent inhibition of agonist-stimulated ERK activation by wortmannin in CHO-m3 cells.

CHO-m3 cells were pre-incubated for 10 min with various concentrations of wortmannin or carrier (DMSO) prior to stimulation with 100 μM MCh for 5 min. 200 μg of protein was assayed for ERK activity by immunoprecipitation (see Methods). Data represent means ± S.E.M. of three separate experiments.

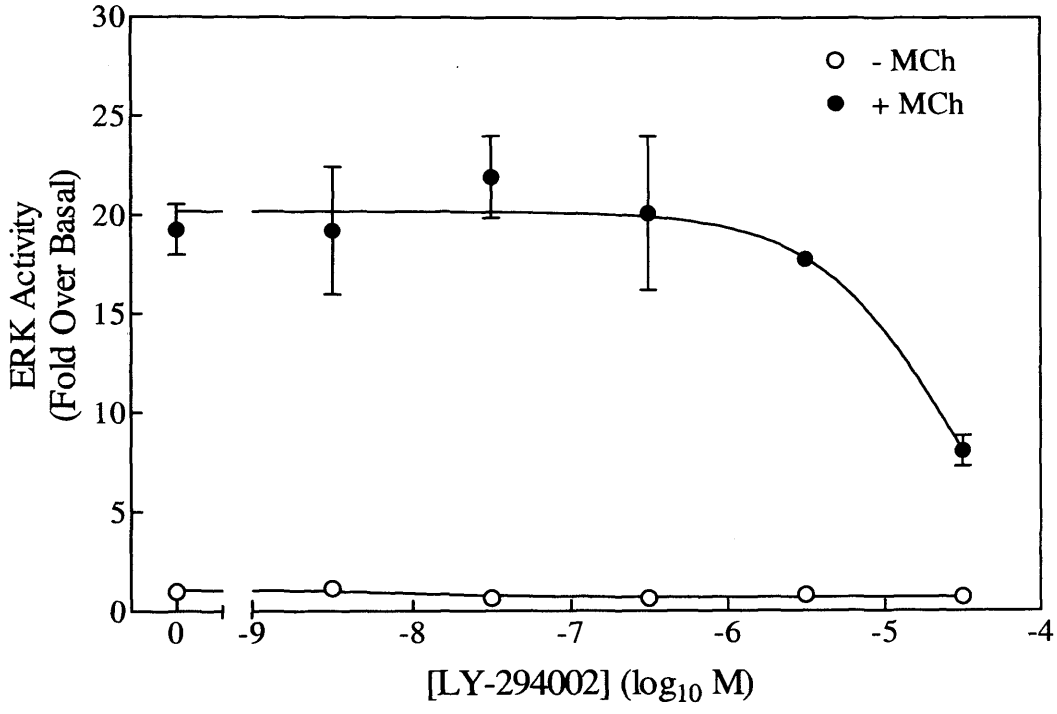

Figure 5.22 Concentration-dependent inhibition of agonist-stimulated ERK activation by LY-294002 in CHO-m2 cells.

CHO-m2 cells were pre-incubated for 10 min with various concentrations of LY-294002 or carrier (DMSO) prior to stimulation with 100 μM MCh for 5 min. 200 μg of protein was assayed for ERK activity by immunoprecipitation (see Methods). Data represent means ± S.E.M. of three separate experiments.

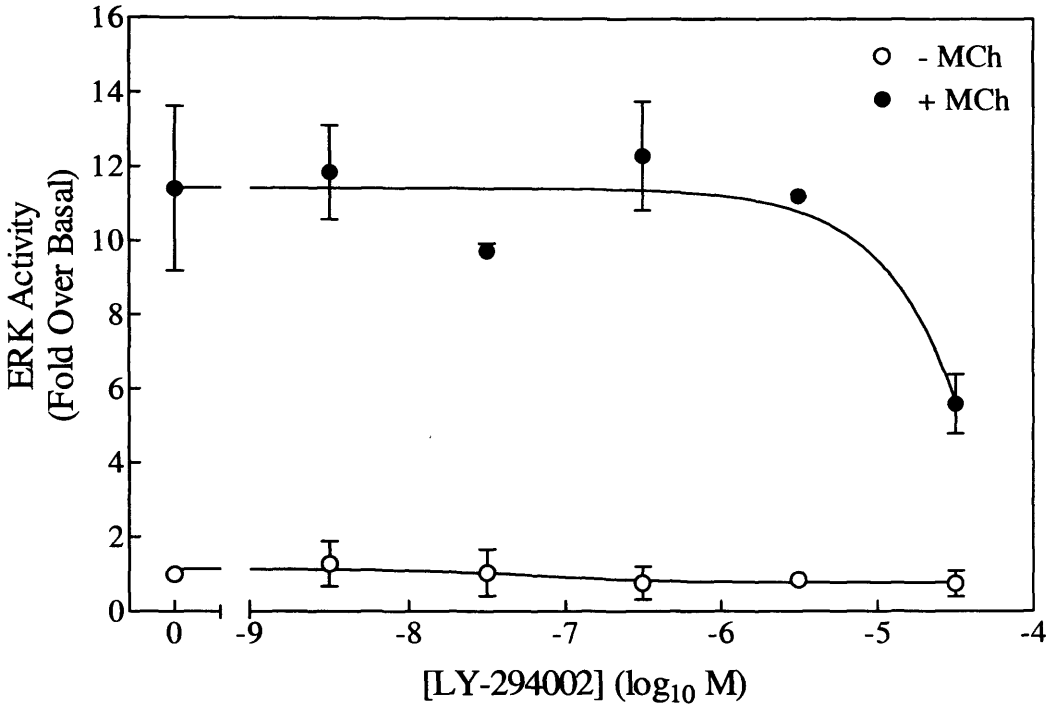

Figure 5.23 Concentration-dependent inhibition of agonist-stimulated ERK activation by LY-294002 in CHO-m3 cells.

CHO-m3 cells were pre-incubated for 10 min with various concentrations of LY-294002 or carrier (DMSO) prior to stimulation with 100 μM MCh for 5 min. 200 μg of protein was assayed for ERK activity by immunoprecipitation (see Methods). Data represent means ± S.E.M. of three separate experiments.

The results of this experiment demonstrate that at the concentrations used in the study, wortmannin does inhibit ERK activation. At the higher wortmannin concentration (10 μM), the inhibition of agonist-induced ERK activation was approximately 80 % in both CHO-m2 and CHO-m3 cell lines. However, pre-incubation with 100 nM wortmannin prior to agonist addition resulted in a significant inhibition of 30% on agonist-induced ERK activation in CHO-m2 cells, but there was no significant inhibition observed in the CHO-m3 cells. Therefore, as 100 nM wortmannin is known to be sufficient to inhibit PI3K, the results obtained from the results using this concentration can be used as a measure to determine the role of PI3K in ERK activation. Together, these data suggest

that there is a PI3K-dependent and PI3K-independent-component in the m2-AChR-mediated activation of ERK, whereas there is not a PI3K dependent role in the m3-AChR-mediated activation of ERK.

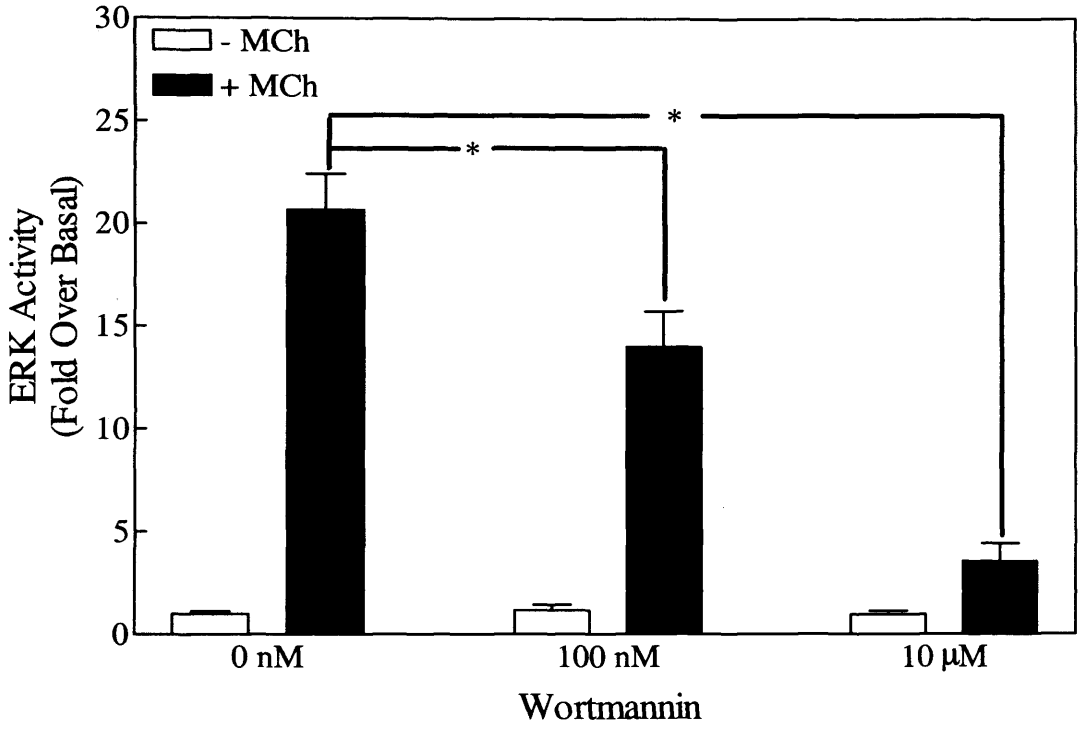

Figure 5.24 Effect of wortmannin on agonist-induced ERK activation in CHO-m2 cells.

CHO-m2 cells were incubated in freshly gassed KHB with vehicle (0.1 % DMSO), and either 100 nM or 10 μM wortmannin for 10 min prior to stimulation with 100 μM MCh or carrier for 5 min. Cleared lysates containing 200 μg protein were assayed for ERK activity by immunoprecipitation as described in the Methods section. Data represent means ± S.E.M. of 4-6 separate experiments. * indicates inhibition of agonist-induced ERK activation at $P < 0.05$ by Duncan's multiple-range test that is statistically significantly different from the activation by agonist in control cells. Under all conditions, the MCh stimulation of ERK was statistically significantly different from the corresponding basal ERK activation at $P < 0.05$, by Duncan's multiple-range test.

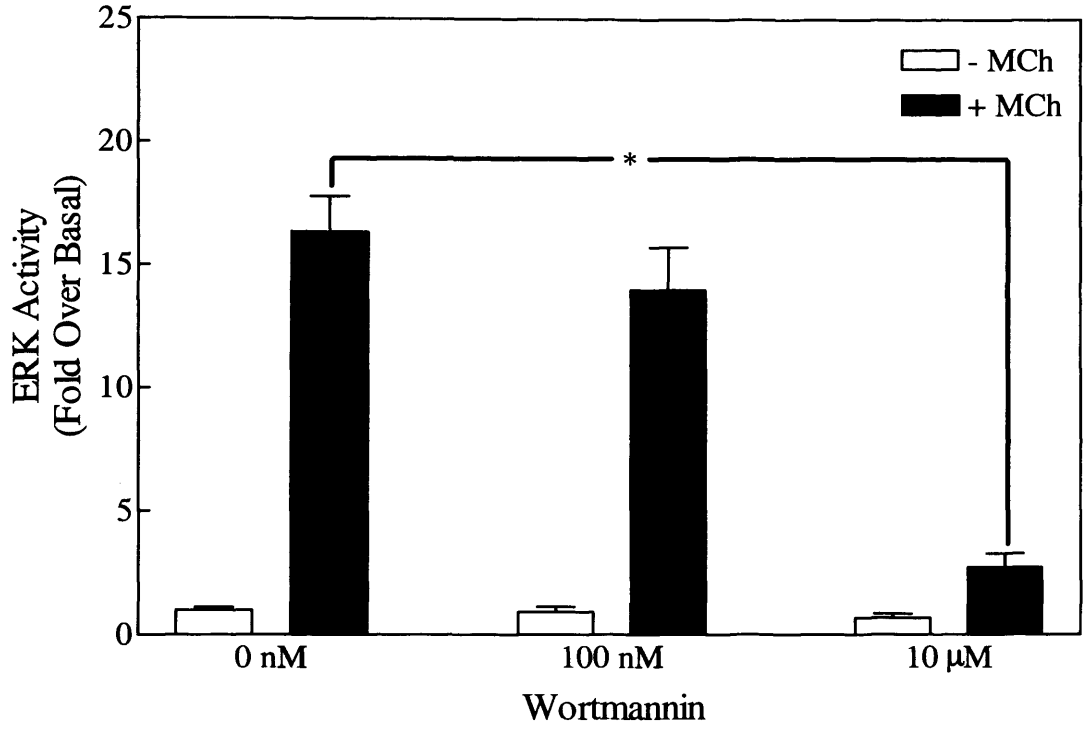

Figure 5.25 Effect of wortmannin on agonist-induced ERK activation in CHO-m3 cells.

CHO-m3 cells were incubated in freshly gassed KHB with vehicle (0.1 % DMSO), and either 100 nM or 10 μM wortmannin for 10 min prior to stimulation with 100 μM MCh for 5 min. Cleared lysates containing 200 μg protein were assayed for ERK activity by immunoprecipitation as described in the Methods section. Data represent means ± S.E.M. of 5-6 separate experiments. * indicates inhibition of agonist-induced ERK activation at $P < 0.05$ by Duncan's multiple-range test that is statistically significantly different from the activation by agonist in control cells. Under all conditions, the MCh stimulation of ERK was statistically significantly different from the corresponding basal ERK activation at $P < 0.05$, by Duncan's multiple-range test.

5.3.2 Role of PI3K in agonist-induced JNK activation

As was observed in the experiments investigating the effects of PKC inhibition described previously in this Chapter, the determination of the effects of PI3K on JNK activation was complicated by the fact that the inhibitors had a marked stimulatory effect *per se* on JNK activation in both CHO-m2 and CHO-m3 cells. In the CHO-m2 cells (Figure 5.26) the basal JNK activity rose to 1.7 fold-over-basal when cells were pre-incubated with 10 μM wortmannin, which was a significant increase over control levels. However, in the presence of MCh, there was no significant attenuation of agonist-induced JNK activation, possibly due to the effect of wortmannin on basal JNK activity. However, as previously noted due to the m2-AChR poorly coupling to the JNK pathway, the degree of activation is very small, and this makes analysis of the data very difficult. In CHO-m3 cells however, pre-incubation with 10 μM wortmannin in the absence of MCh, caused an activation of JNK of 3-4 fold over control cells, representing a significant increase, however, in the presence of MCh, there was no significant inhibition or stimulation of agonist-induced JNK activation (Figure 5.27). Pre-treatment of CHO-m3 cells with 5 μM LY-294002 did not increase basal JNK values in the absence of agonist, however, in the presence of agonist, the JNK activation was attenuated by 55 % when compared with control cells. Therefore, providing evidence of a PI3K component in the activation of JNK by receptor stimulation in CHO-m3 cells.

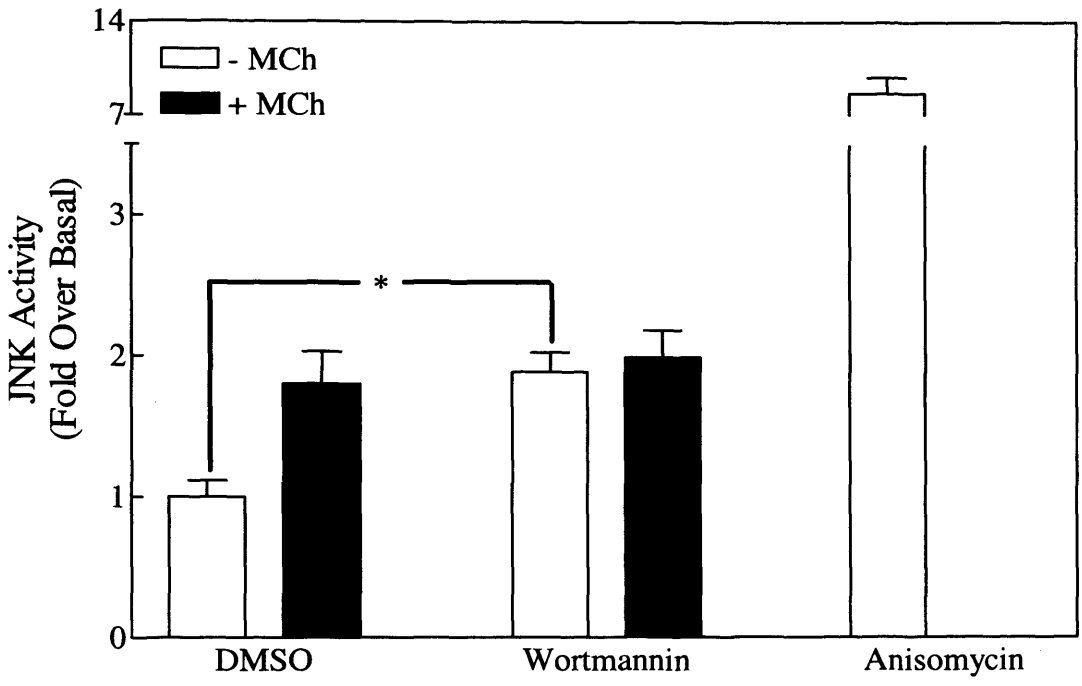

Figure 5.26 Effect of PI3K inhibition on JNK activation in CHO-m2 cells.

CHO-m2 cells were incubated in freshly gassed KHB with vehicle (0.1 % DMSO), 10 μM wortmannin for 10 min or 50 ng/ml Anisomycin for 10 min prior to stimulation with 100 μM MCh or carrier. Cleared lysates containing 400 μg protein were assayed for JNK activity by GST-Jun phosphorylation as described in the Methods section. Data represent means ± S.E.M. of 3 separate experiments. * indicates an increase in JNK activation at $P< 0.05$, by Duncan's multiple-range test that is statistically significantly different from the activation in unstimulated control cells.

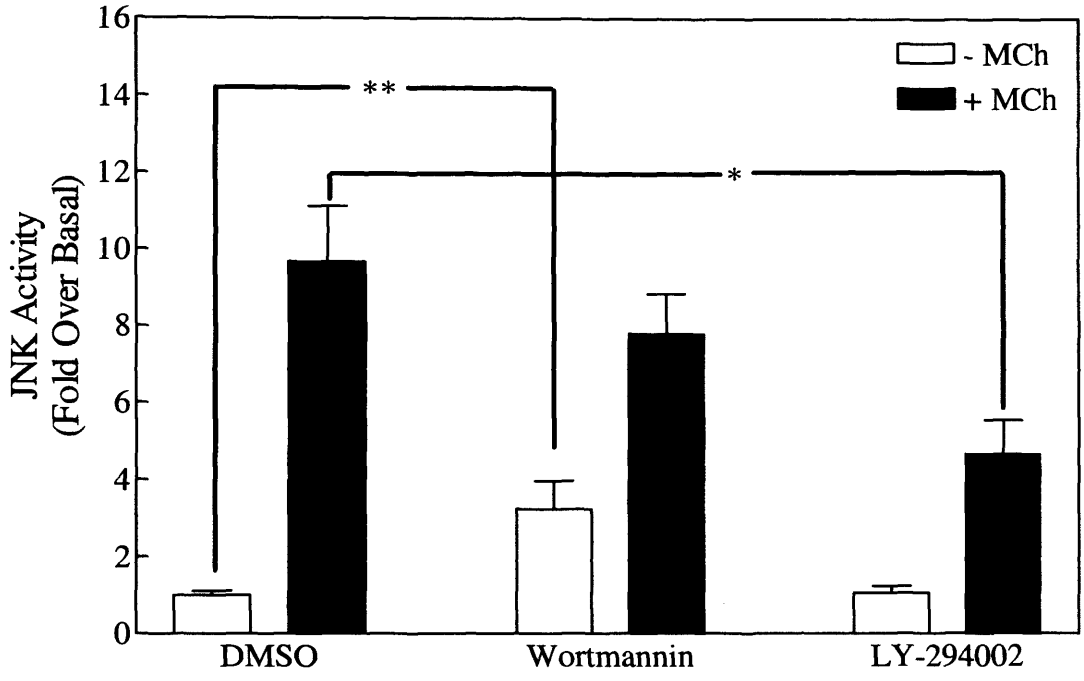

Figure 5.27 Effect of PI3K inhibitors on JNK activation in CHO-m3 cells.

CHO-m3 cells were incubated in freshly gassed KHB with vehicle (0.1 % DMSO), 10 μM wortmannin or 5 μM LY-294002 for 10 min prior to stimulation with 100 μM MCh (solid bars) or carrier (white bars) for 30 min. Cleared lysates containing 400 μg protein were assayed for JNK activity by GST-Jun phosphorylation as described in the Methods section. Data represent means ± S.E.M. of 3-7 separate experiments. * indicates inhibition of agonist-induced JNK activation at $P < 0.05$ by Duncan's multiple-range test that is statistically significantly different from the activation by agonist in control cells. ** indicates an increase in basal JNK activation by 10 μM wortmannin at $P < 0.05$ by Duncan's multiple-range test that is statistically significantly different from the level of activity in control cells in the absence of MCh. Under all conditions, the MCh stimulation of JNK was statistically significantly different from the corresponding basal JNK activation at $P < 0.05$, by Duncan's multiple-range test.

5.4 Role of PI3K in [^{3}H]-thymidine incorporation in CHO-m2 cells.

The role of PI3K was investigated in DNA synthesis by using [^{3}H]-thymidine incorporation as a measure of DNA synthesis in CHO-m2 cells (CHO-m3 cells were not investigated due to the lack of receptor-mediated [^{3}H]-thymidine incorporation; see Chapter 3). The results demonstrate that in control cells, there is an approximate 2 fold stimulation over basal levels, and 10 % FCS gave a robust 3-4 fold activation. The MEK1 inhibitor PD-98059 reduced [^{3}H]-thymidine incorporation in both stimulated and unstimulated cells, which suggests that the ERK pathway has a stimulatory role in DNA synthesis under both basal and MCh-stimulated conditions. The reason that the unstimulated cells show an inhibition relative to the control cells is a probably a reflection that as it is not possible to completely quesce the cells by serum-starvation, the ERK pathway has a constitutive activation that is large enough to induce a high level of DNA synthesis (Figure 5.28). Interestingly however, pre-incubation of cells with the PI3K inhibitors wortmannin or LY-294002 gave similar results to that seen with PD-98059. These results suggest that PI3K has an important role in ERK-mediated DNA synthesis in CHO cells under normal culture conditions or when DNA synthesis is stimulated by m2-mAChR activation.

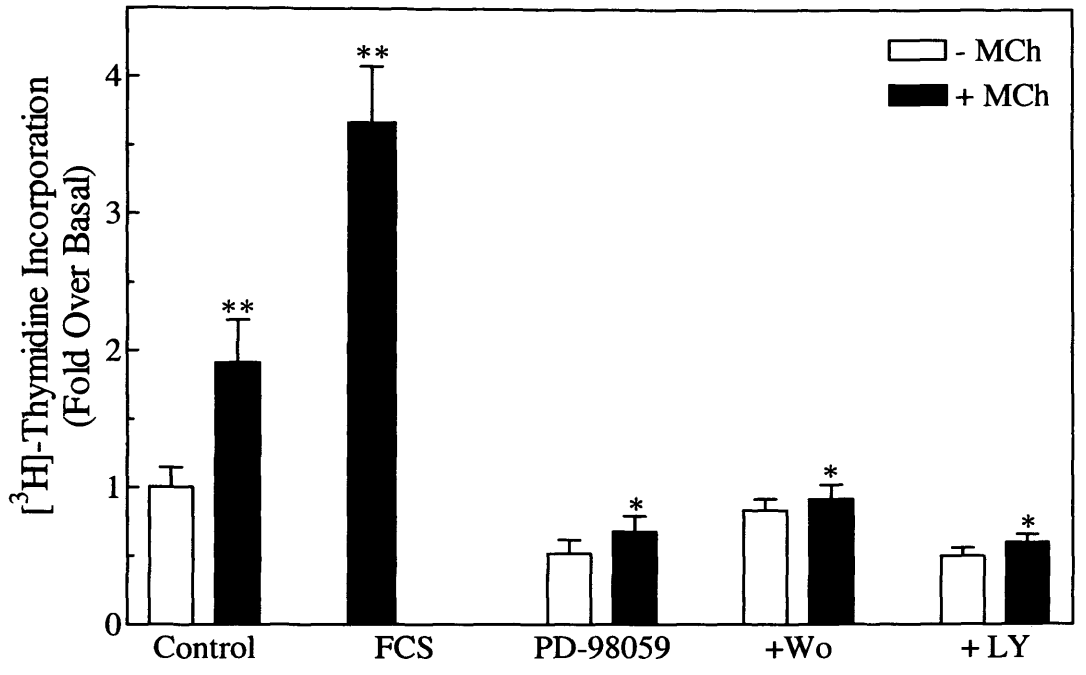

Figure 5.28. Role of PI3K on [^{3}H]-thymidine incorporation in CHO-m2

Intact CHO-m2 cells were incubated for 30 min with 1μM PD-98059, 100nM wortmannin (Wo) or 5 μM LY-294002 (LY) prior to incubating the cells for 20 hours with 100 μM MCh or 10 % FCS only. For the final 2 hours, cells were incubated with 2 μCi of [^{3}H]-thymidine and treated as described in the materials and methods section. Data represent the means ± S.E.M of 3-6 separate determinates in triplicate. * indicates inhibition of agonist-induced [^{3}H]-thymidine incorporation at $P< 0.05$ by Duncan's multiple-range test that is statistically significantly different from the activation by agonist in control cells. ** indicates stimulation of [^{3}H]-thymidine incorporation by MCh or FCS at $P< 0.05$ by Duncan's multiple-range test that is statistically significantly different from the [^{3}H]-thymidine incorporation in the absence of treatment in control cells.

5.5 Discussion

In this Chapter, the role of protein kinase C (PKC) and phosphoinositide 3-kinase (PI3K) in the agonist-induced activation of ERK and JNK in CHO-m2 and CHO-m3 cells has been examined. Initial experiments determined which PKC isoforms were present in both of the CHO cell lines. This was followed by confirming that phorbol ester treatment of the CHO cells stimulated ERK activation, but not JNK activation. The role of PKC in agonist-induced ERK activation was then investigated by the use of inhibitors in both CHO-m2 and CHO-m3 cells lines. These results demonstrated that in CHO-m2 cells there is a PKC-dependent component of the ERK activation response, but it was not via a Ca^{2+}- sensitive PKC (i.e. a cPKC), however, in the CHO-m3 cells, 50% of the agonist-induced ERK activation was attenuated by inhibition of Ca^{2+}- sensitive PKCs, and by inhibiting total PKC activity, the ERK response by receptor stimulation was totally attenuated. The role of PKC in agonist-induced JNK activation was also investigated by the use of inhibitors in both CHO-m2 and CHO-m3 cell lines. These results demonstrated that in CHO-m2 cells there is a potential PKC-dependent component, but due to the small agonist-evoked JNK activation, clear results were not established. In CHO-m3 cells, inhibition of PKC caused an induction of JNK activation, independently of receptor stimulation. However, in the presence of agonist, JNK activation was stimulated to a greater extent with PKC inhibition, compared with control cells. An alternative method to determine the role on PKC in the activation of ERK and JNK by inhibiting PKC by down-regulation with prolonged PKC activation by phorbol ester was utilised. The results from these experiment confirmed the data obtained previously with the PKC inhibitors.

Following on from the studies investigating the role of PKC, the role of PI3K on agonist-induced ERK and JNK activation was examined, by the use of PI3K inhibitors. These data showed that there appeared to be a PI3K-dependent component in the activation of ERK by stimulation of the m2-AChR, but there was no PI3K component in ERK activation via the m3-AChR. The role of PI3K in agonist-induced JNK activation was difficult to establish as the inhibitors used caused marked JNK activation independent of receptor stimulation. Due to these non-specific effects of the inhibitors on JNK activation in CHO-m3 cells, a definitive answer to the role of PI3K in JNK is difficult to establish with the data presented. Therefore, this requires further investigation such as determining the effect of lower concentrations of the inhibitors.

As previously described in both section 5.2.2 and also in the Introductory Chapter of this Thesis, there are a number of PKC isoforms that are differentially inhibited by a variety of commercially available inhibitors. Initial immunoblots showed that out of the ten PKC isoforms blotted for, only PKCβ and PKCθ were not detected. PKCβ is a classical PKC (cPKC) (i.e. is a Ca^{2+}-sensitive PKC) which is expressed in high levels in the brain and haematopoietic cells, and therefore may have been predicted to not have been present in CHO cells. PKCθ is a novel PKC (nPKC) that would have been predicted to have been present as it is a widely expressed PKC isoform. However, as a positive control lane (e.g. rat brain lysate) was not run there is no evidence that either the antibody used was viable or that the western blot analysis was not sensitive enough to detect those isoforms. Therefore, the results presented demonstrate that the two isoforms could not be detected, but it does not confirm that the isoforms are not present in CHO cells.

In agreement with previous studies in Swiss 3T3 cells (Cobb and Goldsmith 1995; Schönwasser, *et al.,* 1998; Marais *et al.,* 1998), it was demonstrated that treatment of cells with phorbol ester (PDBu) activates ERK. Phorbol esters, such as PDBu, activate PKC, by binding to the DAG binding site of cPKCs and nPKC (for review see Newton, 1995). However, unlike DAG, phorbol esters are not metabolised and therefore prolonged treatments constitutively activate PKC. The results presented have demonstrated that in CHO-m2 and CHO-m3 cells, PDBu activates ERK either equally or better than activation by stimulation of muscarinic receptors. It was also established that a 30 min pre-incubation with 1 μM PDBu does not activate JNK, which was an expected result, as JNK is activated by cellular stress, and has not been reported to be activated by phorbol ester treatment (Kyriakis *et al.,* 1995).

In order to establish which classes of PKC isoforms were involved in agonist-mediated ERK activation, the effect of three PKC inhibitors were investigated in CHO-m2 and CHO-m3 cells. Ro-31-8220 is a general PKC inhibitor that will inhibit all PKC isoforms. Bisindolylmaleimide is a PKC inhibitor that inhibits cPKCs and nPKCs, but has no effect on aPKCs (i.e. PKCζ, PKCι, PKCλ and PKCμ). Finally, Gö 6976 is a PKC inhibitor that will inhibit only the Ca^{2+}-sensitive cPKCs; PKCα and PKCβ_I.

Ro-31-8220 has been previously reported to be an antagonist at the muscarinic receptor (Willars *et al.*, 1996) although this appeared to be at low agonist concentrations. In the data discussed later in this Chapter, Ro-31-8220 was used at 10 μM, which is a high concentration, and if there was an antagonist effect, this may occur at these concentrations. Therefore experiments were carried out to determine if lower concentrations of Ro-31-8220 had similar effects on ERK activation. Inhibition curves

of Ro-31-8220 on ERK activation in both CHO-m2 and CHO-m3 showed that the IC_{50} was 0.2 μM in both cell lines. However, the data showed that a concentration of 1 μM Ro-31-8220 had the same effects as 10 μM Ro-31-8220 in both cell lines. These experiments have only investigated the effect on ERK activation, and it would be of interest to determine the IC_{50} value of Ro-31-8220 on JNK activation in CHO-m3 cells.

As an alternative method of investigating if the concentrations of Ro-31-8220 used had an antagonist effect on the muscarinic receptors, an IP_3 mass assay was carried out in CHO-m3 cells. The results demonstrated that in the presence of 10 μM Ro-31-8220, there was no effect on IP_3 accumulation. In addition to this evidence, it has already been shown that a pre-treatment of CHO-m3 cells with Ro-31-8220 results in an increase in JNK activity both in the absence and presence of agonist. Therefore these data suggest that under the conditions used, it would seem unlikely that Ro-31-8220 is acting as an antagonist at the muscarinic receptor in this system.

Results in CHO-m2, demonstrated that Gö 6976 had no effect on either the agonist-unstimulated ERK activity or the agonist-stimulated ERK activity, therefore, this suggests that the PKCα isoform is not involved in the ERK activation. As PKCα is Ca^{2+} sensitive, these results are consistent with data presented in Chapter 3 where it was shown that Ca^{2+} had no effect on agonist-induced ERK activation. Bisindolylmaleimide inhibition showed that there is a significant inhibition of agonist-induced ERK activation compared with control values. Therefore, these data suggests that there is a nPKC component in the activation of ERK, but not a cPKC component.

Pre-incubation with Ro-31-8220 prior to agonist addition, gave no effect on unstimulated ERK activation, there was however, a significant inhibition of 75% on

agonist-stimulated ERK activation. This result was unexpected, as the m2-AChR is coupled to G_i G-proteins, but is not generally coupled to PLC and therefore DAG generation to give PKC activation. However, in the Chapter 3 it was demonstrated that stimulation of the m2-AChR was able to mobilise some Ca^{2+} which might be consistent with PLC activation via βγ subunits, therefore providing a potential route for PKC activation. An interesting point to note is that there was significantly more attenuation of ERK by Ro-31-8220 than bisindolylmaleimide. Therefore, this suggests that there is also an aPKC component involved in ERK activation via stimulation of the m2-AChR. Therefore in summary, in the activation of ERK via the m2-AChR, cPKCs are probably not involved, whereas nPKCs and aPKCs are involved.

CHO-m3 cells pre-incubated with PKC inhibitors prior to receptor stimulation demonstrated that there was no effect on the unstimulated ERK activity with Gö 6976 but there was a significant attenuation of 50% with agonist challenge. As Gö 6976 inhibits PKCα and PKCβ, but as previously discussed, the PKCβ isoform was not detected in these CHO cells, this implies that the PKCα isoform may be the major component in ERK activation. These data are somewhat surprising as it contradicts data presented in Chapter 4 where it was shown that Ca^{2+} had no effect on agonist-induced ERK activation in CHO-m3 cells (Figure 4.7). This is surprising, as the PKCα isoform is a Ca^{2+}-sensitive isoform, and therefore if Ca^{2+} was not a requirement for ERK activation, a Ca^{2+}-sensitive PKC isoform would not predicted to be required for ERK activation. A possible explanation for this, is that in the experiments presented in Chapter 4 (i.e. Figure 4.8) there were three separate Ca^{2+}-containing buffers in which the assays were carried out. Under conditions where $Ca^{2+}{}_i$ and $Ca^{2+}{}_e$ had been depleted, there was still enough Ca^{2+} present to activate Ca^{2+}-binding PKC isoforms. Data that demonstrates this can be

seen in Figure 4.3. The level of cytosolic Ca^{2+} can be seen to be constantly at approximately 15 nM once the intracellular Ca^{2+} stores had been completely depleted. This low concentration of Ca^{2+} may be adequate for the activation of Ca^{2+}-sensitive PKC isoforms (Sheu *et al.*, 1989), and this provides the most likely reason for the apparent contradiction in the data.

Pre-incubation with bisindolylmaleimide in CHO-m3 cells showed that there was a significant inhibition of agonist-induced ERK activation compared with control values. Therefore these data suggest in conjunction with the Gö 6976 data that there is a cPKC component in the agonist-induced activation of ERK. However, there was no statistical difference between the attenuation of ERK activation by Gö 6976 and bisindolylmaleimide, leaving the issue of nPKC open to question. Pre-incubation with Ro-31-8220 completely inhibited agonist-induced ERK activation in CHO-m3 cells. As bisindolylmaleimide did not completely inhibit the agonist-induced ERK activation and has no effect on aPKC isoforms, the data suggest that there may also may be an aPKC component involved.

These data all require the assumption that the PKC inhibitors are maximally effective at inhibiting PKC at the concentrations used, although this was not tested, it would be useful in future experiments to determine the IC_{50} values for the inhibitors used to the PKC isoforms in these cells.

An alternative method of investigating the role of PKC other than by the use of inhibitors is by a prolonged pre-treatment with phorbol ester as previously described. In CHO-m2 cells, down-regulation of PKC with 1 μM PDBu had no effect on ERK activation in the absence of agonist, however, in the presence of agonist, the ERK

activation was decreased by 50 % (from approximately 20 fold to 10 fold over basal). Therefore these data further suggest a role for PKC in the agonist-induced activation of ERK via the m2-AChR. Also, the degree of inhibition observed in control cells was similar to that observed in the inhibitor studies, whereby cells were treated with bisindolylmaleimide, which inhibits the same classes of PKC as prolonged phorbol ester treatment. Therefore both sets of data imply that nPKC and aPKC isoforms are involved in agonist-mediated ERK activation. In CHO-m3 cells, there was a 50% attenuation of the agonist-induced ERK activation, with a PDBu pre-incubation which also corresponded with the bisindolylmaleimide data. Therefore these data together strongly suggest that cPKCs and nPKCs are important in ERK activation via the m3-AChR.

Schönwasser *et al.,* (1998) demonstrated that in Cos-7 cells, all three groups of the PKC family were involved in ERK activation by constructing constitutively activated PKC mutants. In further experiments, they also showed that these mutants activated the upstream ERK activator, MEK1. However, although these experiments demonstrated that PKCs can activate ERK they were conducted in the absence of receptor stimulation. However, van Biersen *et al.,* (1996) demonstrated in CHO cells expressing the m1-AChR (that are generally thought to couple exclusively to G_q), that they are also able to couple to G_o to activate ERK via a PKC-mechanism. In these experiments PKC was down-regulated by prolonged phorbol ester treatment. In addition to this, the $G_{i/o}$-coupled human dopamine D3 receptor has been shown to couple to ERK activity via an aPKC isoform (Cussac *et al.,* 1999) which compares to the $G_{i/o}$ m2-AChR data presented here. They also demonstrated a PI3K component via stimulation of the D3-receptor (for further discussion see later).

In 1999, Wang *et al.*, demonstrated that the m2-AChR can activate PI3Kγ via $G_{\beta\gamma}$ subunits (see later discussion for PI3K), PIP_3 and activation of an atypical PKC isoform. They investigated the coupling of the m2-AChR to membrane ion channels, and found that PKCζ was involved. They concluded that stimulation of PKCζ was required and also sufficient to open chloride channels following m2-AChR activation. Although this is not evidence for activation of the ERK pathway via an m2-AChR-PKC-dependent pathway, it does provide evidence that the m2-AChR can activate aPKCs, and thus supports data presented in this Chapter. Further evidence for the role of PKC via G_i-coupled receptors is the demonstration that PKCζ can be activated by PI3Kγ via activation of MEK and ERK in a Ras-independent manner in CHO cells (Takeda *et al.*, 1999). Therefore, these demonstrate a role for PKC in ERK activation via stimulation of receptors that are coupled to $G_{i/o}$.

Studies into the signalling of bradykinin in SW-480 cells (a human colon carcinoma cell line) showed that PKCε was implicated in the activation of ERK via PI3Kβ (Graneß, *et al.*, 1998). In addition to this, ERK activation via the m1-AChR has been previously shown to act via a PKC-dependent pathway via the α–subunit activating PKC to activate Raf (Kolch *et al.*, 1993; Troppmair *et al.*, 1994). Here we have shown that the m3-AChR can couple to cPKC isoforms, and there is data in the literature demonstrating that the m3-AChR can couple to cPKCs (Strassheim *et al*, 1999). They demonstrated that stimulation of the m3-AChR induced the phosphorylation of myosin light chains in CHO cells via cPKC isoforms. However, they also showed that all three PKC isoform families were activated with receptor stimulation by using PKC translocation studies, in contrast to the data presented in this Thesis. It would be of interest to repeat those PKC translocation experiments here to try and characterise further, the role of the individual

PKC isoforms in ERK activation. Therefore, these data demonstrate a role of PKC in $G_{q/11}$-stimulated activation of ERK.

The investigation into the role of PKC in the agonist-induced activation of JNK is impaired by agonist-independent effect of the inhibitors on JNK activation. As the JNK activation by receptor stimulation of the m2-AChR is poor (approx. 2 fold over basal), only the effect of Ro-31-8220 was investigated. Ro-31-8220 significantly stimulated JNK by up to 3 fold in both the absence and presence of agonist, suggesting that this increase is independent of receptor stimulation.

In the absence of inhibitors in CHO-m3 cells, stimulation with a maximal MCh concentration gave a robust JNK activation. CHO-m3 cells pre-incubated with Ro-31-8220 in the absence of MCh, gave a large JNK activation. However, in the presence of agonist, the level of JNK activation was significantly greater than in the presence of agonist alone. These data suggest that PKC inhibits activation of JNK in CHO-m3 cells. Further experiments to dissect the role of the three PKC classes were then undertaken in CHO-m3 cells. Bisindolylmaleimide resulted in receptor-independent JNK activation, (5 fold activation over basal), however it also resulted in a greater and significant increase in agonist-induced JNK activation. As the JNK activation in the presence of agonist with Ro-31-8220 or bisindolylmaleimide were not significantly different, these data suggest that aPKCs are not involved in agonist-induced JNK activation. The latter contrasts to the ERK data, where aPKCs have been implicated to be involved in agonist-induced ERK activation. However, it could be argued that the marked increase in JNK activation shown here is due to the combined effects of receptor-independent effects of the inhibitors and the receptor-dependent effects of the agonist.

To investigate this further, experiments to down-regulate PKC by prolonged treatment with phorbol ester were carried out. The data presented show that in the absence of PDBu pre-treatment, MCh stimulates a 10 fold over basal JNK activation, and in the presence of prolonged PDBu incubation, there is no effect on JNK activation in the absence of agonist. However, with PKC down-regulation in the presence of agonist, there is a significant (2 fold) increase over JNK activation with agonist alone. Therefore, these data support the inhibitor data, and demonstrate that the increase in JNK activation with the inhibitors is not an additive phenomenon. As a result, these data further suggest that PKC has an inhibitory role in agonist-induced JNK activation, which contrasts to the ERK data, whereby PKC has a stimulatory effect on ERK activation. As a final experiment to determine the inhibitory role of PKC on JNK activation, PKC was activated by briefly stimulating the cells with PDBu prior to MCh stimulation. The results show that in the presence of PDBu the agonist-induced JNK activation decreased significantly compared with control values. Therefore, these combined data suggest that activation of PKC tends to oppose agonist-induced JNK activation, and therefore upon inhibition of PKC, JNK activation is increased above control levels.

There have been few studies investigating the role of PKC in JNK activation, however, Beltman *et al.* (1996) used Ro-31-8220 to investigate the role of PKC in JNK-1 activation by growth factors. They reported that Ro-31-8220 could activate JNK1 via a PKC-independent mechanism. Rat-1 fibroblasts were treated for 48 hours with PMA and cells subsequently incubated with 5 μM Ro-31-8220, down-regulation of PKC had no effect on Ro-31-8220 activation of JNK1, although they admit that Ro-31-8220 could act via an aPKC mechanism, as down-regulation experiments do not affect aPKC isoforms. However, they conclude that this is unlikely, as PKC down regulation and

bisindolylmaleimide had similar effects. These data agree with the data presented in this Chapter, that experiments with bisindolylmaleimide, Ro-31-8220 and PKC-down-regulation give similar levels of JNK activation. Therefore, Beltman *et al.* conclude that Ro-31-8220 may have effects other than those on PKC. However, the conclusions presented in this Thesis are not dependent entirely on experiments using Ro-31-8220, and so therefore it is unlikely that the Ro-31-8220 results are due to some other effects in this system.

There are a number of reports to show that PKC is important in JNK activation. We have shown that PKC appears to have a inhibitory effect in JNK activation. These data are in agreement with a study in Rat-1 fibroblasts, where inhibition of PKC was shown to potentiate JNK activation by endothelian-1, (Cadwaller *et al.,* 1996). In addition to this Zohn *et al,* (1995) have shown that PKC inhibits JNK activation by angiotensin II in GN4 epithelial cells. However, there is also evidence in NIH3T3 cells, that PKC does not have a role in JNK activation by stimulation of the m1-AChR (Coso *et al.,* 1995) in contrast to data in this Thesis.

The role of PI3K in phosphorylating inositol phospholipids has been well documented (for review see Duronio *et al.,* 1998). The role of PI3K in ERK activation has been controversial, with some groups reporting that when p110α PI3K is activated this stimulates the ERK pathway (Hu *et al.,* 1995) while others report there is no role (Klippel *et al.,* 1996; Kauffmann-Zeh *et al.,* 1997). However, these discrepancies are probably due to cell specific regulation, as more recently PI3K has been shown to be involved in ERK activation via G_i-coupled G-proteins (Hawes *et al.,* 1996; Cussac et al., 1999). Also, Lopez-Ilasca *et al.,* (1997) demonstrated the role of the $G_{\beta\gamma}$ subunit

activating ERK in COS-7 cells via PI3Kγ. Recently in CHO-ΔSOS cells, PI3Kγ was shown to activate ERK in a Ras-independent manner via LPA (Takeda et al., 1999). Also, PI3K has been suggested to mediate JNK activation via stimulation of the m2-AChR in Cos-7 cells (Lopez-Ilasaca *et al.,* 1998). More recently, the G_i-coupled D_3 receptor expressed in CHO cells has been shown to have a PI3K component in ERK activation (Cussac *et al.,* 1999). Pre-incubation with 1 μM wortmannin for 30 min prior to agonist stimulation significantly reduced ERK activation, and 10 μM wortmannin did not completely inhibit ERK activation. These results show a role of PI3K for G_i-coupled receptors, however, the effect of PI3K inhibition via stimulation of the m2-AChR was not as marked. Therefore as a role for PI3K in the activation of ERK and JNK has been previously demonstrated, it was of interest to examine whether there was a role via activation of the m2-AChR or m3-AChR.

Two PI3K inhibitors were used to investigate the role of PI3K in the ERK and JNK pathways. Firstly, wortmannin, a highly specific PI3K inhibitor (Nakanishi *et al.,* 1995;), and secondly, LY-294002, which is also highly selective inhibitor of PI3K (Vlahos *et al.,* 1994) of which the mode of inhibition for each agent is to block the ATP-binding site of PI3K. In initial experiments to determine the effect of wortmannin on agonist-induced ERK activation, 10 μM wortmannin was used (which is in excess of that required to inhibit PI3K). In the initial studies, 10 μM wortmannin and 5 μM LY-294002 partially inhibited agonist-induced ERK in CHO-m2 cells. In CHO-m3 cells, 10 μM wortmannin completely inhibited agonist-induced ERK activation. However, LY-294002 inhibited ERK activation by 50%, similar to that seen in the CHO-m2 cells. With these results, it was concluded that in CHO-m3 cells, inhibition of PKC completely inhibited ERK

activation, but also inhibition of PI3K, also completely attenuated the ERK activation. Clearly ERK activation could not be entirely dependent on PKC and PI3K, so the conclusion was that at the concentrations used, wortmannin was having a non-specific inhibitory effect. Indeed, high concentrations of wortmannin have been previously demonstrated to have many non-specific effects, e.g. inhibiting PI4K (Nakanishi *et al.*, 1995). From the literature, 100 nM wortmannin has been shown to inhibit PI3K (Arcaro and Wymann, 1993) and from this, the experiments were repeated using this concentration.

Results show that in CHO-m2 cells, pre-incubation with wortmannin moderately inhibits agonist-induced ERK activation, suggesting that there is a minor PI3K component in m2-AChR-mediated ERK activation. Previous studies have also demonstrated the role of PI3K in m2-mediated ERK activation, (Lopez-Ilasaca, 1997) and also the ability of m2-AChRs to couple to PI3K, but not the $G_{i/o}$-linked m4-AChRs (Wang *et al.*, 1999). A point of interest is that Ro-31-8220 inhibited ERK activation by 75 %, and it would be of interest to inhibit PKC and PI3K at the same time, to determine if this completely inhibits total ERK activation. In addition to these results, Graneß *et al.*, (1998) demonstrated a role for PI3Kβ activation in bradykinin stimulation of the ERK pathway in the SW-480 cell line via a pertussis toxin insensitive G-protein of the $G_{q/11}$ family (and subsequent activation of PKCε downstream from PI3Kβ). In CHO-m3 cells, 100 nM wortmannin has no effect on agonist ERK activation, which as Ro-31-8220 completely inhibited ERK activation agrees with the hypothesis that the m3-AChR is entirely dependent on PKC activation only. In agreement with these results, the m1-AChR has been demonstrated to act via PKC, independently of Ras and PI3K (Hawes *et al.*, 1995). Therefore, the data presented suggest that there may be a PI3K component to

m2-AChR-mediated ERK activation, but there is no PI3K component in m3-AChR-mediated ERK activation.

The role of PI3K on JNK activation was then investigated in CHO-m2 and CHO-m3 cells. The interpretation of the data in both cell lines was made difficult due to the fact that 10 μM wortmannin has a significant stimulatory effect on JNK activation independently of the receptor. However, as previously discussed, 10 μM wortmannin is too high a concentration to inhibit PI3K alone, and therefore an experiment of interest is to repeat these studies in the presence of 100 nM wortmannin and also generate an inhibition curve for JNK activation similar to those previously described for ERK activation. However, 5 μM LY-294002 significantly inhibited agonist-induced JNK activation in CHO-m3 cells by approximately 50 %, but importantly had no effect on basal JNK activation in the absence of agonist. Therefore, these data suggest that there may be a PI3K component in the agonist-mediated activation of JNK in CHO-m3 cells. PI3K has been previously shown to have a role in JNK activation. A potential PI3K isoform for this is PI3Kγ, which has been demonstrated to mediate $G_{\beta\gamma}$-dependent activation of JNK in Cos7 cells (Lopez-Ilasaca *et al.,* 1998) via activation of the m2-AChR.

Finally, the role of PKC and PI3K in DNA synthesis was investigated by using the inhibitors already described. However, the inhibitors in the protocol used were present for 20 hours and this caused cytotoxicity problems. Ro-31-8220, Gö 6976 and bisindolylmaleimide all caused the cells to die and therefore there were no meaningful results obtained for PKC involvement in [^{3}H]-thymidine incorporation. However, the

PI3K inhibitors did not seem to exhibit the same degree of cytotoxicity and the results suggest that PI3K may have a stimulatory role in DNA synthesis.

The interesting result is that the unstimulated cells in the presence of PD-98059 or the PI3K inhibitors showed a [^{3}H]-thymidine incorporation inhibition relative to the control cells. This fits in with results discussed in Chapter 3 from results by Dr. R. Patel who found that the serum starvation of the cells did not growth arrest the cells, and a large degree of DNA synthesis was occurring. Therefore this allows for the possibility of the ERK pathway being constitutively activated to some degree, which is enough to induce a high level of DNA synthesis. Therefore, inhibition of the ERK pathway by either PD-98059 or the PI3K inhibitors could be predicted to attenuate unstimulated receptor-mediated DNA synthesis.

As it can be seen from the text, the evidence currently available in the literature to dissect the role of PKC and PI3K in the regulation of ERK and JNK is very contradictory. It appears that the specificity of the activation of the pathways are entirely dependent on the receptor subtypes being investigated, in addition to which cell lines the receptors are expressed.

Chapter 6

SUMMARY

In this Thesis, CHO cells stably expressing the m2- or m3- muscarinic acetylcholine receptors have been used as a model system to examine the regulation of ERK and JNK activities by G-protein coupled receptors. The study demonstrates that stimulation of both the recombinant m3-AChRs and m2-AChRs are able to activate ERK1/2 pathways, however, only stimulation of the m3-AChR activated JNK. It has been demonstrated that there are distinct time course characteristics between the ERK1/2 and JNK pathways in both m2-AChR and m3-AChR cells. The activation of the ERK pathway was rapid and robust in both cell lines, whereas only the m3-AChR caused significant activation of the JNK pathway which was more sustained than that observed for ERK activation. Although to date there has not been a study to investigate the methacholine dose-dependence for activation of ERK and JNK, the study shows that the EC_{50} values for both activities are similar and in the low micromolar range in both cell types. Further characterisation has demonstrated that only stimulation of the m2-AChR appears to induce DNA synthesis, although there is the possibility that due to the inability of the CHO cells to fully serum starve, DNA synthesis is only modestly stimulated in CHO-m2 cells and activation by the m3-AChR may be obscured by constitutive action of the cell cycle. In addition to this, PTX pre-treatment prior to stimulation by agonist has shown to totally inhibit m2-AChR-mediated ERK activation consistent with its known coupling to G_i proteins (Caulfield *et al.*, 1993; Dell'Acqua *et al.*, 1993). However, PTX-pre-

treatment of CHO-m3 cells also partially attenuated agonist-induced ERK and JNK activation, possibly due to high receptor expression allowing for the receptor to promiscuously couple to G_i/G_o proteins (Askenazi *et al.,* 1987; Peralta *et al.,* 1988; Richards *et al.,* 1991; Burford *et al.,* 1995).

In experiments to determine the ability of the stimulated receptors to mobilise Ca^{2+}, it has been demonstrated that in agreement with other studies (Tobin *et al.,* 1995) stimulation of the m3-AChR mobilises Ca^{2+} but stimulation of the m2-AChR also mobilised Ca^{2+}, albeit to a much lesser extent. It has been shown that there appears to be no role for Ca^{2+} in the activation of the ERK pathway by either receptor, in agreement with some studies (Mitchell *et al.,* 1995) and in disagreement with others (Finkbeiner and Greenberg 1996). However, there does appear to be a role for Ca^{2+} in receptor-mediated JNK activation via the m3-AChR receptor as has been reported previously by others (Mitchell *et al.,* 1995; Zohn *et al.,* 1995).

Following on from this work, it has been demonstrated using inhibitor studies that ERK activation by the m3-AChR, contains a PKC component, probably via cPKC (potentially PKCα) and aPKC isoforms. However, ERK activation by m2-AChR is probably via nPKC and aPKC isoforms in agreement with other studies (Ueda *et al.,* 1996; Clerk *et al.,* 1994; Corbit *et al.,* 1999; Graneß *et al.,* 1998) though further work is required to confirm these observations. It would also be of interest to investigate the role of PKCζ in ERK activation, as it has been previously demonstrated to have effects in other systems (Liao *et al.,* 1997; Schönwasser *et al.,* 1998, Wang *et al.,* 1999). Investigation into the effect of PKC on JNK activation in the CHO-m3 cells was found to be difficult, due to the effect of the inhibitors on JNK activation. However, the JNK data

presented suggest that cPKCs and nPKCs have an inhibitory role in agonist-induced JNK activation in agreement with Beltman *et al.*, 1996, who demonstrated Ro-31-8220 activated JNK1 via a PKC-dependent mechanism. Therefore different PKC family members may provide divergent routes for the m3-AChR to activate the ERK and JNK pathways.

Investigations into the role of PI3K demonstrated that PI3K has a permissive role in ERK activation via of the m2-AChR, but not via the m3-AChR. These data suggest that βγ subunits derived from G_i proteins that couple to the m2-AChR are capable of activating PI3K (Hawes *et al.*, 1996; Lopez-Ilasca *et al.*, 1997). This mechanism may also account for the involvement of PKC in this pathway, as it is known that the PIP3-dependent kinase, PDK1 is involved in activating PKC enzymes (Parker *et al.*, 1999). However, experiments to investigate the role of PI3K in m3-AChR agonist-mediated JNK activation were impaired due to marked effects of the inhibitors on basal JNK activity. Therefore, it will be of interest to further investigate the role of PI3K in JNK-activation by using different concentrations of the inhibitors, different PI3K inhibitors or by using dominant-negative interfering mutants of the PI3K enzymes. Similar studies could also be designed to identify the different PKC isoforms involved in ERK and JNK regulation by m2- and/or m3-acetylcholine receptors.

References

Akimoto, K., Mizuno, K., Osada, S., Hirai, S., Tanuma, S., Suzuki, K., Ohno, S. (1994). A new member of the 3rd class in the Protein Kinase C family, PKC-lambda, expressed dominantly in an undifferentiated mouse embryonal carcinoma cell-line and also in many tissues and cells. *J. Biol. Chem.* **269:** 12677-12683.

Angel, P., Karin, M. (1991). The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim. Biophys. Acta.* **1072:** 129-157.

Arcaro A and Wymann., M.P. (1993). Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. *Biochem. J.* **296:** 297-301.

Askenazi, A., Winslow, J.W., Peralta, E.G., Peterson, G.L., Schimerlik, M.I., Capon, D.J., Ramachandran, J. (1987) An M2 muscarininc receptor subtype coupled to both adenylyl cyclase and phosphoinositide turnover. *Science.* **238:** 672-675.

Avruch, J., Zhang, X-F., Kyriakis, J.M. (1994). Raf meets Ras: Completing the framework of a signal transduction pathway. *Trends. Biochem. Sci.* **19:** 279-283.

Beltman, J., McCormick, F., Cook, S.J (1996). The selective Protein Kinase C inhibitor, Ro-31-8220, inhibits Mitogen-activated Protein Kinase Phosphatase-1 (MKP-1) expression, induces c-Jun expression, and activates Jun N-terminal Kinase. *J. Biol. Chem.* 271: 27018-27024.

Bennet, A.M., Tonks, N.K. (1997). Regulation of distinct stages of skeletal muscle differentation by mitogen-activated protein kinses. *Science.* **278:** 1288-1291.

Berridge, M.J. (1993a). The molecular basis of communication within the cell. *Sci. Am.* **253:** 142-150.

Berridge, M.J. (1993b). Inositol trisphosphate and calcium signalling. *Nature.* **361:** 315-325.

Berridge, M.J (1995). Capacitative calcium entry. *Biochem. J.* **312:** 1-11.

Binetruy, B., Smeal, T., Karin, M. (1991). Ha-Ras augments c-Jun activity and stimulates phosphorylation of its activation domain. *Nature.* **351:** 122-127.

Birdsall, N.J.M., Hulme, E.C. (1987). Characterization of muscarinic acetylcholine receptors and their subtypes. *Isi Atlas Sci: Pharmacol.* **1(2):** 98-100.

Birnbaumer, L. (1992). Receptor-to-effector signaling through G proteins: Roles for betagamma dimers as well as alpha subunits. *Cell* **71:** 1069-1072.

Blank, J.L., Gershwin, P., Elliot, E.M., Sather, S., Johnson, G.L (1996). Molecular cloning of mitogen-activated protein/ERK kinase kinases (MEKK) 2 and 3. *J. Biol. Chem* **271:** 5361-5368.

Blank, J.L., Shaw, K., Ross, A.H., Exton, J.H. (1993). Purification of a 110-kDa phosphoinositide phospholipase C that is activated by G-protein βγ-subunits. *J. Biol. Chem.* **268:** 25184-25191.

Blank, J.L., Brattain, K.A., Exton, J.H. (1992). Activation of cytosolic phosphoinositide phospholipase C by G-protein βγ-subunits. *J. Biol. Chem.* **267:** 23069-23075.

Blenis, J. (1993). Signal transduction via map kinases:-Proceed at your own RSK. *Proc. Natl. Acad. Sci.* **90:** 5889-5892.

Bogoyevitch, M.A., Glennon, P.E., Andersson, M.B., Clerk, A., Lazou, A., Marshall, C.J., Parker, P., Sugden, P.H. (1994). Endothelian-1 and fibroblast growth factors stimulate the Mitogen-activated Protein Kinase signalling cascade in cardiac myocytes. *J. Biol. Chem.* **269:** 1100-1119.

Bogoyevitch, M.A., Marshall, C.J., Sugden, P.H. (1995). Cellular stresses differentially activate c-Jun N-terminal protein kinases and Extracellular signal-Regulated Protein Kinases in cultured ventricular myocytes. *J. Biol. Chem.* **270:**29710-29717.

Bonner, T.I., Buckley, N.J., Young A.C., Brann, M.R. (1987). Identification of a family of muscarinic acetylcholine receptor genes. *Science.* **237:** 527-534.

Bonner, T.I., Young A.C., Brann, M.R., Buckley, N.J. (1988). Cloning and expression of the human and rat m5 muscarinic receptor gene. *Neuron.* **1:** 403-410.

Burford, N.T., Tobin, A.T., Nahorski, S.R. (1995). Coupling of muscarinic m1, m2, and m3 acetylcholine receptors, expressed in Chinese Hamster Ovary cells, to pertussis toxin-sensitive/insensitive guanine nucleotide-binding proteins. *Eur. J. Pharmacol.* **289:** 343-351.

Burns, D.J., Bloomenthal, J., Lee, M.-H., Bell, R.M. (1990). Expression of the alpha, beta II, and gamma Protein Kinase C isozymes in the baculovirus-insect cell expression system. Purification and characterization of the individual isoforms *J. Biol. Chem.* **265:** 12044-12051.

Burstein, E.S., Bräuner-Osbourne, H., Spalding, Conklin, B.R., Brann, M.R. (1997). Interactions of muscarinic receptors with the heterotrimeric G proteins G_q and G12: Transduction of proliferative signals. *J. Neurochem.* **68:** 525-533.

Butterfield, L., Zentrich, E., Beekman, A., Heasley, L.E. (1999). Stress- and cell type-dependent regulation of transfected c-Jun N-terminal kinase and mitogen-activated protein kinase. *Biochem. J.* **338:** 681-686.

Cadwaller, K., Beltman, J., McCormick, F., Cook, S. (1997). Differential regulation of extracellular signal-regulated protein kinase 1 and Jun N-terminal kinase 1 by Ca^{2+} and Protein Kinase C in endothelin-stimulated Rat-1 cells. *Biochem. J.* **321:** 795-814.

Cai, H., Smola, U., Wixler, V., Eisenmann-Tappe, I., Diaz-Meco, M.T., Moscat, J., Rapp, U, Cooper, G.M. (1997). Role of diaceylglycerol-regulated Protein Kinase C isotypes in growth factor activation of Raf-1 protein kinase. *Mol. Cell. Biol.* **17:** 732-741.

Cairns BR. Ramer SW. Kornberg RD. (1992). Order of action of components in the yeast pheromone response pathway revealed with a dominant allele of the STE11 kinase and the multiple phosphorylation of the STE7 kinase. *Genes &Development.* **6(7)**: 1305-1318.

Canagarajah, B., Khokhlatchev, A., Cobb, M., Goldsmith, E.J. (1997). Activation mechanism of the MAP kinase ERK2 by dual phosphorylation. *Cell.* **90:** 859-869.

Cano, E., Mahadevan, L.C., (1995). Parallel Signal processing among mammalian MAPKs. *Trends. Biochem. Sci.* **7:** 353-361.

Carpenter, C.L., Cantley, L.C. (1996). Phosphoinositide kinases. *Curr. Opin. Cell. Biol.* **8:** 153-158.

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., Nishizuka, Y. (1982). Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.* **257:** 7847-77851.

Caulfield, M.P. (1993). Muscarinic Receptors-characterisation, coupling and function. *Pharmacol. Ther.* **58:** 319-379.

Caulfield, M.P., Birdsall, N.J.M. (1998). International Union of Pharmacology. XVII. Classification of Muscarinic Acetylcholine Receptors. *Pharmacol. Rev.* **50:** 279-290.

Cavigelli, M., Dolfi, F., Claret, F.X., Karin, M. (1995). Induction of c-fos expression through JNK-mediated TCF/Elk-1 phosphorylation. *EMBO. J.* **14:** 5957-5964.

Chang, J.D., Xu, Y.P., Raychowdhury, M.K., Ware, J.A. (1993). Molecular-cloning and expression of a cDNA-encoding a novel isoenzyme of Protein Kinase C (nPKC) - a new member of the nPKC family expressed in skeletal-muscle, megakaryoblastic cells, and platelets. *J. Biol. Chem.* **268:** 14208-14214.

Changeux, J-P, Giraudat, J., Dennis, M. (1987). The nicotinic acetylcholine receptor: molecular architecture of a ligand-regulated ion channel. *Trends. Pharmacol. Sci.* **8:** 459-465.

Chen, H.J., Rojas-Soto, M., Oguni, A., Kennedy, M.B. (1998). A synaptic Ras-GTPase activating protein (p135 synGAP) inhibited by CaM kinase II. *Neuron.* **20:** 895-904.

Chen, R.H., Sarnecki, C., Blenis, J. (1992). Nuclear translocation and regulation of ERK and RSK encoded protein kinases. *Mol. Cell. Biol.* **12:** 915-927.

Chen, Y.R., Wang, X., Templeton, D., Davis, R.J., Tan, T-H. (1996). The role of c-Jun N-terminal Kinase (JNK) in apoptosis induced by ultraviolet C and -radiation. Duration of JNK activation may determine cell death and proliferation. *J. Biol. Chem.* **271:** 31929-31936.

Cheng, H-L., Feldman, E.L. (1998). Bidirectional regulation of p38 kinase and c-Jun N-terminal protein kinase by insulin-like growth factor-I. *J. Biol. Chem.* **273:** 14560-14565.

Cheng, M., Boulton, T.G., Cobb, M.H. (1996). ERK3 is a constitutively nuclear protein kinase. *J. Biol. Chem.* **271:** 8951-8958.

Choi, K-Y., Satterberg, B., Lyons, D.M., Elion, E.A. (1994). Ste5 tethers multiple protein kinases in the MAP Kinase cascade required for mating in *S. cerevisiae. Cell.* **78:** 499-512.

Clerk, A., Bogoyevitch, M.A., Andersson, M.B., Sugden, P.H. (1994). Differential activation of Protein Kinase C isoforms by endothelin-1 and phenylephrine and subsequent stimulation of p42 and p44 Mitogen-Activated Protein Kinases in ventricular myocytes cultured from neonatal rat hearts. *J. Biol. Chem.* **269:** 32848-32857.

Clerk, A., Sugden, P.H. (1998). The p38-MAPK inhibitor, SB203580, inhibits cardiac stress-activated protein kinases/c-Jun N-terminal kinases (SAPKs/JNKs). *FEBS Lett.* **426:** 93-96.

Cobb, M.H., Goldsmith, E.J. (1995). How MAP kinases are regulated. *J. Biol. Chem.* **270:** 14843-14846.

Cohen, P. (1997). The search for physiological; substrates or MAP and SAP kinases in mammalian cells. *Trends in Cell Biol.* **7:** 353-361.

Collins, L.R., Minden, A., Karin, M., Brown, J.H. (1996). $G_{\alpha 12}$ stimulates c-Jun NH_2-terminal kinase through the small G proteins Ras and Rac. *J. Biol. Chem.* **271:** 17349-17353.

Cook, S.J., Rubinfeld, B., Albert, I., McCormick, F. (1993). Rap V12 antagonizes Ras-dependent activation of ERK1 and ERK2 by LPA and EGF in Rat-1 fibroblasts. *EMBO J.* **12:** 3475–3485.

Corbit, K.C., Foster, D.A., Rosner, M.R. (1999). Protein Kinase C δ mediates neurogenic but not mitogenic activation of mitogen-activated protein kinase in neuronal cells. *Mol. Cell. Biol.* **19:** 4209-4218.

Coso, O.A., Chiariello, M., Kalinec, G., Kyriakis, J.M., Woodget, J., Gutkind, J.S. (1995a). Transforming G-protein-coupled receptors potently activate JNK (SAPK). *J. Biol. Chem.* **270:** 5620-5624.

Coso, O.A., Chiariello, M., Yu, J.C., Teramoto, H., Crespo, P., Xu, N., Miki, T., Gutkind, J.S. (1995b). The small GTP-binding proteins Rac1 and Ccd42 regulate the activity of the JNK/SAPK signalling pathway. *Cell.* **81:** 1137-1146.

Coso, O.A., Teramoto, H., Simonds, W.F., Gutkind, J.S. (1996). Signaling from G protein-coupled receptors to c-Jun kinase heterotrimeric G proteins acting on a Ras and Rac1-dependent pathway. *J. Biol. Chem.* **271:** 3963-3966.

Cowley, S., Paterson, H., Kemp, P., Marshall, C.J. (1994). Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH-3T3 cells. *Cell.* **77:** 841-852.

Crespo, P., Xu, N., Simonds, W.F., Gutkind, J.S. (1994a). Ras dependent activation of MAP kinase pathway mediated by G-protein βγ subunits. *Nature.* **369:** 418-420.

Crespo, P., Daniotti, J.L., Troppmair, J., Rapp, U.R., Gutkind, J.S. (1994b). Signaling through transforming G protein-coupled receptors in NIH 3T3 cells involves c-Raf activation. Evidence for a Protein Kinase C- independent pathway. *J. Biol. Chem.* **269:** 21103-21109.

Cuenda, A., Rouse, J., Doza, Y.N., Meier, R., Cohen, P., Gallagher, T.F., Young, P.R., Lee, J.C. (1995). SE 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.* **364:** 229-233.

Cuenda, A., Cohen, P., Buee-Scherrer, V., Goedert, M. (1997). Activation of stress-activated protein kinase-3 (SAPK3) by cytokines and cellular stresses is mediated via SAPKK3 (MKK6); Comparison of the specificities of SAPK3 and SAPK2 (RK/p38). *EMBO. J.* **16:** 295-305.

Cussac, D., Newman-Tancredi, A., Pasteau, V., Millan, M.J. (1999). Human dopamine D3 receptors mediate mitogen-activated protein kinase activation via a phosphatidyl 3-kinase and an atypical Protein Kinase C-dependent mechanism. *Mol. Pharmacol.* **56:** 1025-1030.

Daaka, Y., Luttrell, L.M., Ahn, S., Della Rocca, G.J., Ferguson, S.G., Caron, M.G., Lefkowitz, R.J. (1998). Essential role for G Protein-coupled receptor endocytosis in the activation of Mitogen-activated Protein Kinase. *J. Biol. Chem.* **273:** 685-688.

Dai, T., Rubie, E., Franklin, C.C., Kraft, A., Gillespie, D.A.F., Avruch, J., Kyriakis, J.M., Woodgett, J.R. (1995). Stress-activated protein kinases bind directly to the delta domain of c-Jun in resting cells: Implications for repression of c-Jun function. *Oncogene.* **10:** 849-855.

Darlington, G.J. (1999). Molecular mechanisms of liver development and differentiation. *Curr. Op. Cell. Biol.* **11** :678-682.

Dasen, J.S., Rosenfeld, M.G. (1999). Signaling mechanisms in pituitary morphogenesis and cell fate determination. *Curr. Op. Cell. Biol.* **11:** 669-677.

Davis, R.J., (1994). MAPKs: new JNK expands the group. *Trends. Biochem. Sci.* **19:** 470-473.

Deacon, K and Blank, J. L. (1997). Characterization of the mitogen-activated protein kinase kinase 4 (MKKK4)/c-Jun NH2-terminal kinase 1 and MKK3/p38 pathways regulated by MEK kinases 2 and 3: MEK kinase 3 activates MKK3 but does not cause activation of p38 kinase In vivo. *J. Biol. Chem.*. **272:** 14489-14496.

Deacon, K and Blank, J.L. (1999). MEK kinase 3 directly activates MKK6 and MKK7, specific activators of the p38 and c-Jun NH2-terminal kinases. *J. Biol. Chem.* **274:** 16604-16610.

Dell'Acqua, M.L., Carroll, R.C., Peralta, E.G. (1993). Transfected m2 muscarinic acetylcholine receptors couple to Gαi2 and Gαi3 in Chinese Hamster Ovary cells. *J. Biol. Chem.* **268:** 5676-5685.

Della Rocca, G.J., Maudsley, S., Daaka, Y., Lefkowitz, R.J., Luttrell, L.M. (1999). Pleitropic coupling of G protein-coupled receptors to the mitogen-activated Protein Kinase Cascade. *J. Biol. Chem.* **274:** 13978-13984.

Della Rocca, G.J., van Biesen, T., Daaka, Y., Luttrell, D.K., Luttrell, L.M., Lefkowitz, R.J. (1997). Ras-dependent Mitogen-activated Protein Kinase activation by G Protein-coupled receptors. *J. Biol. Chem.* **272:** 19125-19132.

Dérijard, B., Hibi, M., Wu, I-H., Barrett, T., Su, B., Deng, T., Karin, M, Davis, R.J. (1994). JNK1: A protein kinase stimulated by U.V. light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell.* **76:** 1025-1037.

Dérijard, B., Raingeaud, J., Barrett, T., Wu, I-H., Han, J., Ulevitch, R.J., Davis, R.J. (1995). Independent human MAP Kinase signal transduction pathways defined by MEK and MKK isoforms. *Science.* **267:** 682-685.

Dhanasekaran, N., Heasley, L.E., Johnson, G.L. (1995). G Protein-Coupled Receptor systems involved in cell growth and oncogenesis. *Endocr. Rev.* **16:** 259-270.

Dhanasekaran, N., Reddy, E.P. (1998). Signaling by dual specificity kinases *Oncogene.* **17:** 1447-1455.

Dickens, M., Rogers, J.S., Cavanagh, J., Raitano, A., Xia, Z., Halpern, J.R., Greenberg, M.E., Sawyers, C.L., Davis, R.J. (1997). A cytoplasmic Inhibitor of the JNK signal transduction pathway. *Science.* **277:** 693-696.

Dikic, I., Schlessinger, J., Laz, I. (1994). PC12 cells overexpression the insulin receptor undergo insulin-dependent neuronal differentiation. *Curr. Biol.* **4:** 702-708.

Dohlman, H.G., Caron, M.G., Lefkowitz, R.B. (1987). A family of receptors coupled to guanine nucleotide regulatory proteins. *Biochemistry.* **26:** 2657-2664.

Dohlman HG, Bouvier M, Benovic JL, Caron, M.G., Lefkowitz., R.J. (1987). The multiple membrane spanning topography of the beta 2-adrenergic receptor. Localization of the sites of binding, glycosylation, and regulatory phosphorylation by limited proteolysis. *J. Biol. Chem.* **262:** 14282-14288.

Doza, Y.N., Cuenda, A., Thomas, G.M., Cohen, P., Nebreda, A.R. (1995). Activation of the MAP kinase homologue RK requires the phosphorylation of Thr-180 and Tyr-182 and both residues are phosphorylated in chemically stressed KB cells. *FEBS Lett.* **364:** 223-228.

Duckworth, B.C., Cantley, L.C. (1997). Conditional inhibition of the Mitogen-activated Protein Kinase Cascade by wortmannin. *J. Biol. Chem.* **272:** 27665-27670.

Durino, V., Scheid, M.P., Ettinger, S. (1998). Downstream signalling events regulated by phosphatidylinositol 3-kinase activity. *Cell. Signal.* **10:** 233-239.

Ebinu, J.O., Bottorf, D.A., Chan, E.Y.W., Stang, S.L., Dunn, R.J., Stone, J.C. (1998). RasGRP, a Ras guanyl nucleotide- releasing protein with calcium- and diacylglycerol-binding motifs. *Science.* **280:** 1082-1086.

Eglen, R.M., Reddy, H., Watson, N., Challiss, R.A.J. (1994). Muscarinic acetylcholine receptor subtypes in smooth muscle. *Trends. Pharmacol. Sci.* **15:** 114-119.

Ellinger-Ziegelbauer, H., Brown, K., Kelley, K., Siebenlist, U. (1997). Direct activation of the stress-activated protein kinase (SAPK) and extracellular signal-regulated protein kinase (ERK) pathways in an inducible mitogen-activated protein kinase/ERK kinase kinase 3 (MEKK3) derivative. *J. Biol. Chem.* **272:** 2668-2674.

English, J.M., Pearson, G., Hockenberry, T., Shivakumar, L., White, M.A., Cobb, M.H. (1999). Contribution of the ERK5/MEK5 pathway to Ras/Raf signaling and growth control. *J. Biol. Chem.* **274:** 31588-31592.

Enslen, H., Raingeaud, J., Davis, R.J. (1998). Selective activation of p38 mitogen-activated protein (MAP) kinase isoforms by the MAP kinase kinases MKK3 and MKK6. *J. Biol. Chem.* **273:** 1741-1748.

Fan, G., Merritt, S.E., Kortenjann, M., Shwa, P.E., Holzman, L.B. (1996). Dual Leucine Zipper-bearing Kinase (DLK) activates $p46^{SAPK}$ and $p38^{mapk}$ but not ERK2. *J. Biol. Chem.* **271:** 24788-24793.

Fantl, W.J., Johnson, D.E., Williams, L.T. (1993). Signalling by receptor tyrosine kinases. *Ann. Rev. Biochem.* **62:** 453-481.

Farsworth, C.L., Freshney, N.W., Rosen, L.B., Ghosh, A., Greenberg, M.E., Feig, L.A. (1995). Calcium activation of Ras mediated by neuronal exchange factor Ras-GRF. *Nature.* **376:** 524-527.

Faure, M., Voyno-Yasenetskaya, T.A., Bourne, H.R. (1994). cAMP and βγ subunits of heterotrimeric G-proteins stimulate the mitogenic activated protein kinase pathway in COS7 cells. *J. Biol. Chem.* **269:** 7851-7854.

Ferrell, J.E. (1996). MAP kinases in mitogenesis and development. *Curr. Top. Dev. Biol.* **33:** 1-60.

Finkbeiner, S., Greenberg, M.E. (1996). Ca^{2+}-dependent routes to Ras: Mechanisms for neuronal survival, differentiation, and plasticity? *Neuron.* **16:** 233-236.

Foltz, I.N., Gerl, R.E., Wieler, J.S., Luckach, M., Salmon, R.A., Schrader, J.W. (1998). Human Mitogen-activated Protein Kinase Kinase 7 (MKK7) is a highly conserved c-Jun N-terminal Kinase/Stress-activated Protein Kinase (JNK/SAPK) activated by environmental stresses and physiological stimuli. *J. Biol. Chem.* **273:** 9344-9351.

Freshney, N.W., Rawlinson, L., Guesdon, F., Jones, E., Cowley, S., Hsuan, J., Saklatvala, J. (1994). Interleukin-1 activates a novel Protein Kinase Cascade that results in the phosphorylation of Hsp27. *Cell.* **78:** 1039-1049.

Frevert, E.U., Kahn, B.B. (1997) Differential effects of constitutively active phosphatidylinositol 3- kinase on glucose transport, glycogen synthase activity, and DNA synthesis in 3T3-L1 adipocytes. *Mol. Cell. Biol.* **17:** 190-198.

Fromm, C., Coso, O.A., Montaner, S., Xu, N., Gutkind, J.S. (1997). The small GTP-binding protein Rho links G protein-coupled receptors and $G\alpha_{12}$ to the serum response element and to cellular transformation. *Proc. Natl. Acad. Sci.* **94:** 10098-10103.

Fukuda, M., Gotoh, Y., Nishida, E. (1997). Interaction of MAP kinase with MAP kinase kinase: its possible role in the control of nucleocytoplasmic transport of MAP kinase. *EMBO. J.* **16:** 1901-1908.

Galcheva-Gargova, Z., Dérijard, B., Wu, I-H., Davis, R.J. (1994). An osmosensing signal transduction pathway in mammalian cells. *Nature.* **265:** 806-808.

Gardner, A.M., Vaillancourt, R.R., Lange-Carter, C.A., Johnson, G.L. (1994). MEK-1 phosphorylation by MEK kinase, Raf, and mitogen-activated protein kinase: analysis of phosphopeptides and regulation of activity. *Mol. Biol. Cell.* **5:** 193-201.

Garrington, T.P., Johnson, G.L. (1999). Organization and regulation of mitogen-activated protein kinase signaling pathways. *Curr. Op. Cell Biol.* **11**: 211-218.

Gartner, A., Zhou, Z., Ammerer, G., Errede, B. (1993). Reconstitution of a pheromone induced kinase cascade in saccharomyces-cerevisiae - activation of the fus3 kinase by the Ste7 kinase in vitro. *J. Cellular Biochem.* **S17A:** 267.

Gerwins, P., Blank, J.L., Johnson, G.L. (1997). Cloning of a novel Mitogen-activated Protein Kinase Kinase Kinase, MEKK4, that selectively regulates the c-Jun amino terminal Kinase Pathway. *J. Biol. Chem.* **272:** 8288-8295.

Gille, J., Kortenjann, M., Thomas, O., Moomaw, C., Slaughter, C., Cobb, M.H., Shaw, P.E. (1995). ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation. *EMBO. J.* **14:** 951-962.

Gilman, A.G. (1987). G-proteins:transducers of receptor generated signals. *Ann. Rev. Biochem.* **56:** 615-649.

Glassberg, M.K., Ergul, A., Wanner, A., Puett, D. (1992). Endothelian binding and stimulation in ovine airway smooth muscle cells. *Am. Rev. Respir. Dis.* **145:** A124(Abstract).

Glassberg, M.K., Eegul, A., Wanner, A., Puett, D. (1994). Endothelin-1 promotes mitogenesis in airway smooth-muscle cells. *Am. J. Resp. Cell and Mol. Biol.* **10:** 316-321.

Goedert, M., Cuenda, A., Craxton, M., Jakes, R., Cohen, P. (1997). Activation of the novel stress-activated protein kinase SAPK4 by cytokines and cellular stresses is mediated by SKK3 (MKK6); Comparison of its substrate specificity with that of other SAP kinases. *EMBO J.* **16:** 3563-3571.

Graneß, A., Adomeit, A., Heinze, R., Wetzker, R., Liebmann, C. (1998). A novel mitogenic signalling pathway of bradykinin in the human colon carcinoma cell line SW-480 involves sequential activation of a G_q/11 Protein, phosphatidylinositol 3-kinase, and Protein Kinase Cε. *J. Biol. Chem.* **273:** 32016-32022.

Grynkiewicz, G., Poenis, M., Tsien, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260:** 3440-3450.

Gschewendt, M., Kittstein, W., Marks, F. (1991). Protein Kinase C activation by phorbol esters: Do cysteine-rich regions and pseudosubstrate motifs play a role? *Trends Biochem. Sci.* **16:** 167-169.

Gupta, S., Gallego, C., Johnson, G.L., Heasley, L.E. (1992). MAP kinase is constitutively activated in gip2 and src transformed rat 1a fibroblasts. *J. Biol. Chem.* **267:** 7987-7990.

Gupta, S., Davis, R.J. (1994). MAP kinase binds to the NH_2-terminal activation domain of c-Myc. *FEBS Lett.* **353:** 281-285.

Gupta, S., Barrett, T., Whitmarsh, A.J., Cavanagh, J., Sluss, H.K., Dérijard, B., Davis, R.J. (1996). Selective interaction of JNK protein kinase isoforms with transcription factors. *EMBO. J.* **15:** 2760-2770.

Gutkind, J.S., Novotny, E.A., Brann, M.R., Robbins, K.C. (1991). Muscarinic acetylcholine receptor subtypes as agonist-dependent oncogenes. *Proc. Natl. Acad. Sci.* **88:** 4703-7407.

Gutkind, J.S., Crespo, P., Xu, N., Teramoto, H., Coso, O.A. (1997). The pathway connecting m2 receptors to the nucleus involves small GTP-binding proteins acting on divergent MAP kinase cascades. *Life Sci.* **60:** 999-1006.

Gutkind, J.S. (1998). Pathways connecting G protein-coupled receptors to the nucleus through divergent mitogen-activated Protein Kinase Cascades. *J. Biol. Chem.* **273:** 1839-1842.

Hai, T., Curran, T. (1991). Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc. Natl. Acad. Sci.* **88:** 3720-3724.

Ham, J., Babij, C., Whitfield, J., Pfarr, C.M., Lallemand, D., Yaniv, M., Rubin, L.L. (1995). A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death. *Neuron.* **14:** 927-939.

Hammer, R., Berrie, C.P., Birdsall, N.J.M., Burgen, A.S.V., Hulme, E.C. (1980). Pirenzepine distinguishes between different subclasses of muscarinic receptors. *Nature.* **283:** 90-92.

Han, J., Lee, J-D., Bibbs, L., Ulevitch, R.J. (1994). A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Nature.* **265:** 808-811.

Hawes, B.E., van Biesen, T., Koch, W.J., Luttrell, L.M., Lefkowitz, R.J. (1995). Distinct pathways of G_i- and G_q- mediated Mitogen-activated Protein Kinase activation. *J. Biol. Chem.* **270:** 17148-17153.

Hawes, B.E., Luttrell, L.M., van Biesen, T., Lefkowitz, R.J. (1996). Phosphatidylinositol 3-Kinase is an Early Intermediate in the $G_{\beta\gamma}$- mediated Mitogen-activated Protein Kinase Signalling Pathway. *J. Biol. Chem.* **271:** 12133-12136.

Heasley, L.E, Johnson, G.L. (1992). The β-PDGF receptor induces neuronal differentiation of PC12 cells. *Mol. Cell. Biol.* **3:** 545-553.

Heasley, L.E., Storey, B., Fanger, G.R., Butterfield, L., Zamarripa, J., Blumberg, D., Maue, R.A. (1996). GTPase-deficient $G_{\alpha 16}$ and $G_{\alpha q}$ induce PC12 cell differentiation and persistent activation of cJun NH2-terminal kinases. *Mol. Cell. Biol.* **16:** 648-656.

Heidenreich, K.A., and Kummer, J.L. (1996). Inhibition of p38 Mitogen-activated Protein Kinase by insulin in cultured fetal neurons. *J. Biol. Chem.* **271:** 9891-9894.

Hella Brown, J., Sah, V., Moskowitz, S., Ramirez, T., Collins, L., Post, G., Goldstein, D. (1997). Pathways and roadblocks in muscarinic receptor-mediated growth regulation. *Life Sci.* **60:** 1077-1084.

Hibi, M., Lin, A., Smeal, T., Minden, A., Karin, M. (1993). Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes. Dev.* **7:** 2135-2148.

Hirai, S., Izawa, M., Osada, S., Spyrou, G., Ohno, S. (1996). Activation of the JNK pathway by distantly related protein kinases, MEKK and MUK. *Oncogene.* **12:** 641-650.

Hirai, S., Noda, K., Moriguchi, T., Nishida, E., Yamashita, A., Deyama, T., Fukuyama, K., Ohno, S. (1998). Differential activation of two JNK activators, MKK7 and SEK1, by MKN28-derived nonreceptor serine/threonine kinase/Mixed Lineage Kinase 2. *J. Biol. Chem.* **273:** 7406-7412.

Hirai, S-I., Katoh, M., Terada, M., Kyriakis, J.M., Zon, L.I., Rana, A., Avruch, J., Ohno, S. (1997). MST/MLK2, a member of the mixed lineage kinase family, directly phosphorylates and activates SEK1, an activator of c-Jun N-terminal kinase/stress-activated protein kinase. *J. Biol. Chem.* **272:** 15167-15173.

Hodge, C., Liao, J., Stofega, M., Guan, K., Carter-Su, C., Schwartz, J. (1998). Growth hormone stimulates phosphorylation and activation of c-fos, egr-1 and junB through activation of Extracellular Signal-regulated Kinases 1 and 2. *J. Biol. Chem.* **273:** 31327-31336.

Hu, Q., Milfay, D., Williams, L.T (1995a). Binding of NCK to SOS and activation of Ras-dependent gene expression. *Mol. Cell. Biol.* **15:** 1169-1174.

Hu, Q., Klippel, A., Muslin, A.J., Fantl, W.J., Williams, L.T (1995b). Ras-dependent induction of cellular responses by constitutively active phosphatidylinositol-3 kinase. *Science.* **268:** 100-102.

Huang, C., Li, J., Ma, W-Y., Dong, Z. (1999). JNK activation is required for JB6 cell transformation induced by tumor necrosis factor-alpha but not by 12-O-tetradecanoylphorbol-13-acetate. *J. Biol. Chem.* **274:** 29672-29676.

Huang, W., Erikson, R.L. (1996). MAP Kinases in multiple signalling pathways. pg 159-172. Signal Transduction. Editors. Heldin and Purton, Nelson Thornes, UK.

Hulme, E.C., Birsdall, N.J., Buckley, N.J. (1990). Muscarinic receptor subtypes. *Ann. Rev. Pharm. Toxicol.* **30:** 633-673.

Hunter, T., Karin, M. (1992). The regulation of transcription by phosphorylation. *Cell.* **70:** 375-387.

Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K., Gotoh, Y. (1997). Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 Signalling Pathways *Science.* **275:** 90-94.

Igishi, T., Fukuhara, S., Patel, V., Katz, B-Z., Yamada, K.M., Gutkind, J.S. (1999). Divergent signalling pathways link Focal Adhesion Kinase to Mitogen-activated Protein Kinase Cascades. Evidence for a role of paxillin in c-Jun NH_2-terminal kinase activation. *J. Biol. Chem.* **274:** 30738-30746.

Inoue, M., Kishimoto, A., Takai, Y., Nishizuka, Y. (1977). Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. II. Proenzyme and its activation by calcium-dependent protease from rat brain. *J. Biol. Chem.* **252:** 7610-7616.

Jiang, Y., Chan, C., Li, X., Guo, W., Gegner, J.A., Lin, S., Han, J. (1996). Characterization of the structure and function of a new Mitogen-activated Protein Kinase (p38). *J. Biol. Chem.* **271:** 17920-17926.

Johannes, F.J., Prestle, J., Eis, S., Oberhagemann, P., Pfizenmaier, K. (1994). PKCμ is a novel, atypical member of the Protein Kinase C family. *J. Biol. Chem.* **269:** 6140-6148.

Johannes, F.J., Prestle, J., Dieterich, S., Oberhagemann, P., Link, G., Pfizenmaier, K. (1995). Characterization of activators and inhibitors of Protein Kinase Cμ. *Eur. J. Biochem.* **227:** 303-307.

Johnson, G.L., Vaillancourt, R.R. (1994). Sequential protein kinase reactions controlling cell growth and differentiation. *Curr. Op. Cell. Biol.* **6:** 230-238.

Johnson N.L., Gardiner, A.M., Diener, K.M., Lange-Carter, C.A., Gleavy, J., Jarpe, M.B., Minden, A., Karin, M., Zon, L.I., Johnson, G.L. (1996). Signal transduction pathways regulated by mitogen activated/extracellular response kinase kinase kinase induce cell death. *J. Biol. Chem.* **271:** 3229-3237.

Jun, C.D., Oh, C.D., Kwak, H.J. Pae, H.O., Yoo, J.C., Choi, B.M., Chun, J.S., Park, R.K., Chung, H.T. (1999). Overexpression of Protein Kinase C isoforms protects RAW 264.7 macrophages from nitric oxide-induced apoptosis: Involvement of c-Jun N-terminal kinase/stress-activated protein kinase, p38 kinase, and CPP-32 protease pathways. *J. Immun.* **162:** 3395-3401.

Karin. M., (1995). The regulation of AP-1 activity by Mitogen-Activated Protein Kinases *J. Biol. Chem.* **270:** 16483-16486.

Karin, M., Liu, Z-G., Zandi, E. (1997). AP-1 function and regulation. *Curr. Op. Cell. Biol.* **9:** 240-246.

Kauffmann-Zeh, A., Rodriguez,-Viciana, P., Ulrich, E., Gilbert, C., Coffer, P., Downward, J., Evan, G. (1997). Suppression of c-Myc-induced apoptosis by Ras signalling through PI3K and PKB. *Nature.* **385:** 544-548.

Kennedy, C.R.J., Hebert, R.L., Do, M.T., Proulx, P.R. (1997). Bradykinin-stimulated arachidonic acid release from MDCK cells is not Protein Kinase C dependent. *Am. J. Physiol.* **42:** C1605-C1612

Kerr, J.F.R., Wyllie, A.H., Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer.* **26:** 239-257.

Kharbanda, S., Pandey, P., Ren, R., Mayer, B., Zon, L., Kufe, D. (1995). c-Abl activation regulates induction of the SEK1/stress-activated protein kinase pathway in the cellular response to 1-β-D-Arabinofuranosylcytosine. *J. Biol. Chem.* **270:** 30278-30281.

Kharbanda, S., Saleem, A., Shafman, T., Emoto, Y., Taneja, N., Ruben, E., Weichselbaum, R., Woodgett, J., Avruch, J., Kyriakis, J., Kufe, D. (1995). Ionizing radiation stimulates a Grb2-mediated association of the Stress-activated Protein Kinase with phosphatidylinositol 3-kinase. *J. Biol. Chem.* **270:** 18871-18874.

Khokhlatchev, A.V., Canagarajah, B., Wilsbacher, J., Robinson, M., Atkinson, M., Goldsmith, E., Cobb, M.H. (1998). Phosphorylation of the MAP Kinase ERK2 promotes its homerdimerization and nuclear translocation. *Cell.* **93:** 605-615.

Kim, J-Y., Yang, M-S., Oh, C-D., Kim, K-T., Ha, M.J., Kang, S-S., Chun, J-S. (1999). Signalling pathway leading to an activation of mitogen-activated protein kinase by stimulating M3 muscarinic receptor. *Biochem. J.* **337:** 275-280.

Kinloch, R.A., Treherne, J.M., Furness, L.M., Hajimohamadreza, I. (1999). The pharmacology of Apoptosis. *Trends. Pharmacol. Sci.* **20:** 35-42.

Klippel, A., Reinhard, C., Kavanaugh, W.M., Apell, G., Escobedo, M.A., Williams, L.T. (1996). Membrane localization of phosphatidylinositol 3-kinase is sufficient to activate multiple signal-transducing kinase pathways. *Mol. Cell. Biol.* **16:** 4117-4127.

Klotz, I.M. (1982). Numbers of receptor-sites from Scatchard graphs:-Facts and fantasies. *Science.* **217:** 1247-1249.

Koch, W.J. Hawes, B.E., Allen, L.F., Lefkowitz, R. J. (1994). Direct evidence that G_i-coupled receptor stimulation of mitogen-activated protein kinase is mediated by $G_{\beta\gamma}$ activation of p21ras. *Proc. Natl. Acad. Sci.* **91:** 12706-12710.

Kolch., W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marmé, D., Rapp, U.R. (1993). Protein Kinase C α activates Raf-1 by direct phosphorylation. *Nature.* **364**: 249-252.

Kozma, R., Ahmed, S., Best, A., Lim, L., (1995). The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. *Mol. Cell. Biol.* **15:** 1942-1952.

Kubo, T., Fukuda, K., Mikami, A., Maeda, A., Takahashi, H., Mishina, M., Haga, T., Haga, K., Ichiyama, A., Kangawa, K., Kajima, M., Matsuo, H., Hirose, T., Numa, S. (1986a). Cloning, sequencing and expression of complimentary DNA encoding the muscarinic acetylcholine receptor. *Nature.* **323:** 411-416.

Kubo, T., Maeda, A., Sugimoto, K., Akiba, I., Mikami, A., Takahashi, H., Haga, T., Haga, K., Ichiyama, A., Kangawa, K., Matsuo, H., Hirose, T., Numa, S. (1986b). Primary structure of porcine cardiac muscarinic acetylcholine receptor deduced from the cDNA sequence. *FEBS Lett.* **209:** 367-372.

Kumar, S., McDonnell, P.C., Gum, R.J., Hand, A.T., Lee, A.C., Young, P.R. (1997). Novel homologues of CSBP/p38 MAP kinase: Activation, substrate specificity and sensitivity to inhibition by pyridinyl imidazoles. *Biochem. Biophys. Res. Commum.* **235:** 533-538.

Kyriakis, J.M., App, H., Zhang, X., Banerjee, P., Brautigan, D.L., Rapp, U.R., Avruch, J. (1992). Raf-1 activates MAP kinase-kinase. *Nature.* **358:** 417-421.

Kyriakis, J.M., Force, T.L., Rapp ,U.R., Bonventre, J.V., Avruch, J. (1993). Mitogen regulation of c-Raf-1 protein kinase activity toward mitogen- activated protein kinase-kinase. *J. Biol. Chem.* **268:** 16009-16019.

Kyriakis, J.M., Bannerjay, P., Nikolakaki, E., Dai, T.A., Rubie, E.A., Ahmad, M.F., Avruch, J., Woodget, J.R. (1994). The stress-activated Protein Kinase Subfamily of c-Jun Kinases. *Nature.* **369:** 156-160.

Kyriakis, J.M., and Avruch, J. (1996). Sounding the alarm: Protein Kinase Cascades activated by stress and inflammation. *J. Biol. Chem.* **271:** 24313-24316.

Kyriakis, J.M., Woodgett, J.R., Avruch, J. (1995). The stress-activated protein kinases. A novel ERK subfamily responsive to cellular stress and inflammatory cytokines. *Ann. NY. Acad. Sci.* **766:** 303-319.

Lambright, D.G, Sondek, J., Bohm, A., Skiba, N.P., Hamm, H.E., Sigler, P.B. (1996). The 2.0 angstrom crystal structure of a heterotrimeric G protein. *Nature.* **379:** 311-319.

Lange-Carter, CA., Pleiman, C.M., Gardner, A.M., Blumer, K.J., Johnson, G.L. (1993). A divergence in the MAP Kinase regulatory network defined by MEK Kinase and Raf. *Science*, **260**: 315–319.

Lange-Carter, C.A., Johnson, G.L. (1994). Ras-dependent growth factor regulation of MEK kinase in PC12 cells. *Science.* **265:** 1458-1461.

Larocca, J.N., Almazan, G. (1997). Acetylcholine agonists stimulate mitogen-activated protein kinase in oligodendrocyte progenitors by muscarinic receptors. *J. Neurosci. Res.* **50:** 743-754.

Lavoie, J.N., L'Allemain, G., Brunet, A., Müller, F., Pouysségur, J. (1996). Cyclin D1 expression is regulated positively by the p42/p44^{MAPK} and negatively by the p38/HOG^{MAPK} pathway. *J. Biol. Chem.* **271:** 20608-20616.

Lechner, C., Zahalker, M.A., Giot, J-F., Moller, N.P., Ullrich, A. (1996). ERK6, a mitogen-activated protein kinase involved in C2C12 myoblast differentiation. *Proc. Natl. Acad. Sci.* **93:** 4355-4359.

Lee, J.C., Laydon, J.T., McDonnell, P.C., Gallagher, T.F., Kumar, S., Green, D., McNulty, D., Blumenthal, M.J., Heys, R.J., Landvatter, S.W., Strickler, J.E., McLaughlin, M.M., Siemens, I.R., Fisher, S.M., Livi, G.P., White, J.R., Adams, J.L., Young, P.R. (1994). A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature.* **372:** 739-746.

Leeu, T., Fourestlieuvin, A., Wu, C., Chenevert J., Clark, K., Whiteway, M., Thomas, D.Y., Leberer, E. (1995). Pheromone response in yeast - association of Bem1p with proteins of the map kinase cascade and actin. *Science.* **270:** 1210-1213.

Leevers, S.J. (1996). Ras Signalling. pg. 143-158. Signal Transduction. Editors. Heldin and Purton, Nelson Thornes, UK.

Lefkowitz, R.J., Cotecchia, S., Samama, P., Costa, T. (1993). Constitutive activation of receptors coupled to guanine-nucleotide regulatory proteins. *Trends. Pharmacol. Sci.* **14:** 303-307.

Leopoldt, D., Hanck, T., Exner, T., Maier, U., Wetzker, R., Nürnberg, B. (1998). $G_{\beta\gamma}$ stimulates phosphoinositide 3-kinase-γ by direct interaction with two domains of the catalytic p110 subunit. *J. Biol. Chem.* **273:** 7024-7029.

Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J.M., Plowman, G.D., Rudy, B., Schlessinger, J. (1995). Protein tyrosine kinase PYK2 involved in Ca^{2+}-induced regulation of ion channel and MAP Kinase functions. *Nature.* **376:** 737-745.

Li, J., Smithgall, T.E. (1998). Fibroblast transformation by Fps/Fes tyrosine kinases requires Ras, Rac, and Cdc42 and induces extracellular signal-regulated and c-Jun N-terminal kinase activation. *J. Biol. Chem.* **273:** 13828-13834.

Li, X.O, Yu, H., Graves, L.M., Earp, H.S. (1997). Protein Kinase C and protein kinase A inhibit calcium-dependent but not stress-dependent c-Jun N-terminal kinase activation in rat liver epithelial cells. *J. Biol. Chem*, **272:** 14996-15002.

Liao, D-F., Monia, B., Dean, N., Berk, B.C. (1997). Protein Kinase C-δ mediates angiotensin II activation of ERK1/2 in vascular smooth muscle cells. *J. Biol. Chem.* **272:** 6146-6150.

Liao, J., Hodge, C., Meyer, D., Ho, P.S., Rosenspire, K., Schwartz, J. (1997). Growth hormone regulates ternary complex factors and serum response factor associated with the c-*fos* serum response element *J. Biol. Chem.* **272:** 25951-25958.

Lin, A., Minden, A., Martinetto, H., Claret, F.X., Lange-Carter, C., Mercurio, F., Johnson, G.L., Karin, M. (1995). Identification of a dual specificity kinase that activates the Jun kinases and p38-Mpk2. *Science.* **268:** 286-290.

Liyanage, M., Frith, D., Livnen, E., Stabel, S. (1992). Protein Kinase C group-B members PKC-Delta, PKC-Epsilon, PKC -Zeta And PKC –L (Eta)-Comparison of properties of recombinant proteins *in vitro* and *in vivo. Biochem. J.* **283:** 781-787.

Lopez, Ilasaca, M., Gutkind, J.S., Wetzker, R. (1998). Phosphoinositide 3-Kinase γ is a mediator of $G_{\beta\gamma}$-dependent Jun Kinase activation. *J. Biol. Chem.* **273:** 2505-2508.

Lopez-Ilasaca, M. (1998). Signalling from G-Protein-coupled Receptors to Mitogen-activated Protein (MAP)-Kinase cascades. *Biochem. Pharmacol.* **56:** 269-277.

Lopez-Ilasaca, M. Crespo, P., Pellici, P.G., Gutkind, J.S., Wetzker, R. (1997). Linkage of G-Protein Coupled Receptors to the MAPK signalling pathway through PI 3-Kinase γ. *Science.* **275:** 394-397.

Lowry, O.H., Rosebrough, N. J., Farr, A. L., Randall, R.J. (1951). Protein measurement with the Folin-Phenol reagents. *J. Biol. Chem.* **193:** 265-275.

Lu, X., Nemoto, S., Lin, A. (1997). Identification of c-Jun NH2-terminal Protein Kinase (JNK)-activating Kinase 2 as an Activator of JNK but not p38. *J. Biol. Chem.* **272:** 24751-24754.

Luttrell, L.M., Hawes, B.E., van Biesen, T., Luttrell, D.K., Lansing, T.J., Lefkowitz, R.J. (1996). Role of c-Src tyrosine kinase in G protein-coupled receptor-and $G_{\beta\gamma}$ subunit-mediated activation of Mitogen-Activated Protein Kinases. *Nature.* **271:** 19443-19450.

Luttrell, L.M., Della Rocca, G.J., van Biesen, T., Luttrell, D.K., Lefkowitz, R.J. (1997). $G_{\beta\gamma}$ subunits mediate Src-dependent phosphorylation of the epidermal growth factor receptor. *Nature.* **272:** 4637-4644.

Luttrell, L.M., Daaka, Y., Lefkowitz, R.J. (1999). Regulation of tyrosine kinase cascades by G-protein-coupled receptors. *Curr. Op. Cell. Biol.* **11:** 177-183.

Mackenzi, S., Fleming, I., Houslay, M.D., Anderson, N.G., Kilgour, E. (1997). Growth hormone and phorbol esters require specific Protein Kinase C isoforms to activate Mitogen-Activated Protein Kinases in 3T3-F442A cells. *Biochem. J.* **324:** 159-165.

Majerus, P.W. (1992). Inositol phosphate biochemistry. *Ann. Rev. Biochem.* **61:** 225-250.

Majerus, P.W., Kisseleva, M.V., Anderson Norris, F. (1999). The role of phosphatases in inositol signalling reactions. *J. Biol. Chem.* **274:** 10669-10672.

Malarkey, K., Belham, C.M., Paul, A., Graham, A., McLees, A., Scott, P.H., Plevin, R. (1995). The regulation of tyrosine kinase signalling pathways by growth factor and G-protein-coupled receptors. *Biochem. J.* **309:** 361-375.

Mansour, S.J., Matten, W.T., Hermann, A.S., Candia, J.M., Rong, S., Fukasawa, K., Vande Woude, G.F., Ahn, N.G. (1994). Transformation of mammalian cells by constitutively active MAP kinase kinase. *Science.* **265:** 966-970.

Marais, R., Light, Y., Paterson, H.F., Mason, C.S., Marshall, C.J. (1997). Differential regulation of Raf-1, A-Raf and B-Raf by oncogenic Ras and tyrosine kinases. *J. Biol. Chem.* **272:** 4378-4383.

Marais, R., Light, Y., Mason, C., Paterson, H., Olson, M.F., Marshall, C.J. (1998). Requirement of Ras-GTP-Raf complexes for activation of Raf-1 by Protein Kinase C. *Science.* **280:** 109-111.

Marais, R., Parker, P.J. (1989). Purification and characterization of bovine brain Protein Kinase-C Isotype-Alpha, Isotope-Beta and Isotope-Gamma. *Eur. J. Biochem.* **182:** 129-137.

Marinissen, M.J., Chiariello, M., Pallante, M., Gutkind, J.S. (1999). A network of Mitogen-Activated Protein Kinases links G protein-coupled receptors to the c-jun promoter: A role for c-Jun NH2-terminal kinase, p38s, and Extracellular signal-Regulated Kinase 5. *Mol. Cell. Biol.* **19:** 4289-4301.

Marshall, C.J. (1995). Specificity of receptor tyrosine kinase signaling: Transient versus sustained Extracellular signal-Regulated Kinase activation. *Cell.* **80**: 179–185.

Mattingly, R.R., Macara, I.G. (1996). Phosphorylation-dependent activation of the Ras-GRF/CDC25^{Mm} exchange factor by muscarinic receptors and G-protein βγ subunits. *Nature.* **382:** 268-272.

May, G.H., Funk, M., Black, E.J., Clark, W., Hussain, S., Woodgett, J.R., Gillespie, D.A. (1998a). An oncogenic mutation uncouples the v-Jun oncoprotein from positive regulation by the SAPK/JNK pathway in vivo. *Curr. Biol.* **8:** 117-120.

May, G.H., Allen, K.A., Clark, W., Funk, M., Woodgett, J.R., Gillespie, D.A. (1998b). Analysis of the interaction between c-Jun and c-Jun N-terminal kinase *in vivo*. *J. Biol. Chem.* **273:** 33429-33435.

McGlynn, E., Liebetanz, J., Reutner, S., Wood, J., Lydon, N.B., Hofstetter, H., Vanek, M., Meyer, T., Fabbro, D. (1992). Expression and partial characterization of rat Protein Kinase C-delta and Protein Kinase C-zeta in insect cells using recombinant baculovirus. *J. Cell. Biochem.* **49:** 239-250.

Meloche, S., Seuwen, K., Pagès, G., Pouysségur, J. (1992). Biphasic and synergistic activation of p44 (MAPK) (ERK1) by growth factors: correlation between late activation and mitogenicity. *Mol. Endocrinol.* **658:** 845-854.

Meyer zu Heringdorf, D., Lass, H., Alemany, R., Laser, K.T., Neumann, E., Zhang, C., Schmidt, M., Rauen, U., Jakobs, K.H., van Koppen, C.J. (1998). Sphingosine kinase-mediated Ca^{2+} signalling by G-protein-coupled receptors. *EMBO J.* **17:** 2830-2837.

Minden, A., Lin, A., McMahon, M., Lange-Carter, C.A., Déijard, B., Davis, R.J., Johnson, G.L., Karin, M. (1994a). Differential activation of ERK and JNK Mitogen-Activated Protein Kinases by Raf-1 and MEKK. *Science.* **266:** 1719-1723.

Minden, A., Lin, A., Smeal, T., Derijard, B., Cobb, M.H., Davis, R.J., Karin, M. (1994b). c-Jun N-terminal phosphorylation correlates with activation of the JNK subgroup but not the ERK subgroup of Mitogen-Activated Protein Kinases. *Mol. Cell. Biol.* **14:** 6683-6688.

Minden, A., Lin, A., Claret, F-X., Abo, A., Karin, M. (1995). Selective Activation of the JNK Signalling Cascade and c-Jun Transcriptional Activity by the small GTPases Rac and Cdc42Hs. *Cell.* **81:** 1147-1157.

Minden, A., Karin, M. (1997). Regulation and function of the JNK subgroup of MAP Kinases. *B.B.A.* **1333:** F85-F104.

Mischak, H., Goodnight, J., Kolch, W., Martinybaron, G., Schaechtle, C., Kazanietz, M.G., Blumberg, P.M., Pierce, J.H., Mushinski, J.F. (1993). Overexpression of Protein Kinase C-delta and -epsilon in NIH 3T3 cells induces opposite effects on growth, morphology, anchorage dependence, and tumorigenicity. *J. Biol. Chem.* **268:** 6090-6096.

Mitchell, F.M., Russell, M., Johnson, G.L. (1995). Differential calcium dependence in the activation of c-Jun Kinase and Mitogen-Activated Protein Kinase by muscarinic acetylcholine receptors in Rat 1a cells. *Biochem. J.* **309:** 381-384.

Mitsui, H., Takuwa, N., Kurokawa, K., Exton, J.H., Takuwa, Y. (1997). Dependence of activated $G\alpha_{12}$-induced G_1 to S Phase cell cycle progression on both Ras/Mitogen-activated Protein Kinase and Ras/Rac1/Jun N-terminal Kinase cascades in NIH3T3 fibroblasts *J. Biol. Chem.* **272:** 4904-4910.

Moore, D.D. (1989). Promiscuous behaviour in the steroid hormone receptor superfamily. *Trends. Neurosci.* **12:** 165-168.

Moriguchi, T., Gotoh, Y., Nishida E. (1995). Activation of two isoforms of Mitogen-activated Protein Kinase Kinase in response to epidermal growth factor and nerve growth factor. *Eur. J. Biochem.* **234:** 32-38.

Moriguchi, T., Kuroyanagi, N, Yamaguchi, K., Gotoh, Y., Irie, K., Kano, T., Shirakabe, K., Muro, Y., Shibuya, H., Matsumoto, K., Nishida, E., Hagiwara, M. (1996). A Novel kinase cascade mediated by Mitogen-activated Protein Kinase Kinase 6 and MKK3. *J. Biol. Chem.* **271:** 13675-13679.

Mosior, M., Newton, A.C. (1996). Calcium-independent binding to interfacial phorbol esters causes Protein Kinase C to associate with membranes in the absence of acidic lipids. *Biochemistry.* **35:** 1612-1623.

Muroya, K., Hattori, S., Nakamura, S. (1992). Nerve growth factor induces rapid accumulation of the GTP bound form of p21(ras) in rat pheochromocytoma PC12 cells. *Oncogene.* **7:** 277-281.

Nagata, K-I., Puls, A., Futter, C., Aspenstrom, P., Schaefer, E., Nakata, T., Hirokawa, N., Hall, A. (1998). The MAP Kinase Kinase Kinase MLK2 co-localises with the activated JNK along microtubules and associates with kinesin superfamily motor KIF3. *EMBO. J.* **17:** 149-158.

Nakagawa, T., Goto, K., Kondo, H. (1996). Cloning, expression, and localization of 230-kDa Phosphatidylinositol 4-Kinase *J. Biol. Chem.* **271:** 12088-12094.

Nakanishi, H,. Exton, J.H. (1992). Purification and characterization of the zeta isoform of Protein Kinase C from bovine kidney. *J. Biol. Chem.* **267:** 16347-16354.

Nakanishi, S., Catt, K.J., Balla, T. (1995). A wortmannin-sensitive phosphatidylinositol 4-kinase that regulates hormone-sensitive pools of inositolphospholipids. *Proc. Natl. Acad. Sci.* **92:** 5317-5321.

Nathans, J., Hogness, D.S. (1983). Isolation, sequence analysis, and intron-exon arrangement of the gene encoding bovine rhodopsin. *Cell.* **34:** 807-814.

Naya, F.J., Olson, E. (1999). MEF2: a transcriptional target for signaling pathways controlling skeletal muscle growth and differentiation. *Curr. Op. Cell Biol.* **11:** 683-688.

Neer, E.J. (1995). Heterotrimeric G-proteins - organizers of transmembrane signals. *Cell.* **80:** 249-257.

Nemoto, S., Xiang, J., Huang, S., Lin, A. (1998). Induction of apoptosis by SB202190 through inhibition of p38β Mitogen-activated Protein Kinase. *J. Biol. Chem.* **273:** 16415-16420.

Newton, A.C. (1995a). Protein Kinase C: Structure, function, and regulation. *J. Biol. Chem.* **270:** 28495-28498.

Newton, A.C. (1995b). Protein Kinase C - Seeing 2 domains. *Curr.Op. Cell. Biol.* **5:** 973-976.

Newton, A.C. (1997). Regulation of Protein Kinase C. *Curr.Op. Cell. Biol.* **9:** 161-167.

Nguyen, T.T., Scimeca, J.C., Filloux, C., Peraldi, P., Carpentier, J.L., van Obberghen, E. (1993). Co-regulation of the Mitogen-activated Protein Kinase, Extracellular signal-Regulated Kinase 1 and the 90 kDa ribosomal S6 kinase in PC12 cells. *J. Biol. Chem.* **268:** 9803-9810.

Nishizuka, Y. (1982). Intracellular signalling by hydrolysis of phospholipids and activation of Protein Kinase C. *Nature.* **258:** 607-614.

Nishizuka, Y. (1984). The role of Protein Kinase C in cell surface signal transduction and tumour promotion. *Nature.* **308:** 693-698.

Nobes, C.D., Hall, A. (1995). Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipedia, and filopodia. *Cell.* **81:** 53-62.

Offermanns S, Bombien E, Schultz G (1993). Stimulation of tyrosine phosphorylation and Mitogen-Activated-Protein (MAP) kinase activity in human SH-SY5Y neuroblastoma cells by carbachol. *Biochem. J.* **294:** 545-550.

Ono, Y., Kikkawa, U., Ogita, K., Fujii, T., Kurokawa, T., Asaoka, Y., Sekiguchi, K., Ase, K., Igarashi, K., Nishizuka, Y. (1987). Expression and properties of 2 types of Protein Kinase C-alternative splicing from a single gene. *Science.* **236:** 1116-1120.

Ono, Y., Fujii, T., Ogita, K., Kikkawa, Igarashi, K., Nishizuka, Y. (1988). The structure, expression, and properties of additional members of the Protein Kinase C family. *J. Biol. Chem.* **263:** 6927-6932.

Ono, Y., Fujii, T., Ogita, K., Kikkawa, Igarashi, K., Nishizuka, Y. (1989). Phorbol ester binding to Protein Kinase-C requires a cysteine-rich zinc-finger-like sequence *Proc. Natl. Acad. Sci.* **86:** 3099-3103.

Osada, S-I., Mizuno, K., Saido, T.C., Suziki, K., Kuroki, T., Ohno, S. (1992). A new member of the Protein Kinase C family, nPKC-θ, Predominantly expressed in skeletal-muscle. *Mol. Cell. Biol.* **12:** 3930-3983.

Pages, G., Lenormand, P., L'Allemain, G., Chambard, J.C., Meloche, S., Pouyssegur, J. (1993). Mitogen-Activated Protein Kinases p42(mapk) and p44(mapk) are required for fibroblast proliferation. *Proc. Natl. Acad, Sci.* **90:** 8319-8323.

Pandey, P., Avraham, S., Kumar, S., Nakazawa, A., Place, A., Ghanem, L., Rana, A., Kumar, V.,. Majumder, P.K., Avraham, H., Davis, R.J., Kharbanda, S. (1999). Activation of p38 Mitogen-Activated Protein Kinase by PYK2/related adhesion focal tyrosine kinase-dependent mechanism. *J. Biol. Chem.* **274:** 10140-10144.

Parker. M. (1993). Steroid and related receptors. *Curr. Op. Cell. Biol.* **5:** 499-504.

Parker, P.J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M.D., Ullrich, A. (1986). The complete primary structure of Protein Kinase C - the major phorbol ester receptor. *Science.* **233:** 853-859.

Patel, G., Stabel, S. (1989). Expression of a functional Protein Kinase C-gamma using a baculovirus vector: purification and characterisation of a single Protein Kinase C iso-enzyme. *Cell. Signalling.* **1:** 227-240.

Pelach, S.L., Sanghera, J.S. (1993). MAP kinases: charting the regulatory pathways. *Science.* **257:** 1355-1356.

Peralta, E.G., Ashkenazi, A., Winslow, J.W., Smith, D., Ramachandran, J., Capon, D.J. (1987a). Distinct primary structures, ligand-binding properties and tissue-specific expression of four human muscarinic acetylcholine receptors. *EMBO. J.* **6:** 3923-3929.

Peralta, E.G., Winslow, J.W., Peterson, G., Smith, D., Ashkenazi, A., Ramachandran, J., Schimerlik, M., Capon, D.J. (1987b). Primary structure and biochemical properties of an M2 muscarinic receptor. *Science* **236:** 600-605.

Posado, J., Yew, N., Ahn, N.G., Van de Woude, G.F., Cooper, J.A. (1993). Mos stimulates MAP Kinase in *Xenopus* Oocytes and activates a MAP Kinase Kinase *in vitro*. *Mol. Cell. Biol.* **13:** 2546–2553.

Post, G.R., Goldstein, D., Theurauf, D., Glembotski, C.C. Brown, J.H. (1996). Dissociation of p44 and p42 Mitogen-activated Protein Kinase activation from receptor-induced hypertrophy in neonatal rat ventricular myocytes *J. Biol. Chem.* **271:** 8452-8457.

Prasad, M.V.V.S.V., Dermott, J.M., Heasley, L.E., Johnson, G.L., Dhanasekaran, N. (1995). Activation of Jun Kinase/Stress-activated Protein Kinase by GTPase-deficient mutants of G_{12} and$G\alpha_{13}$. *J. Biol. Chem.* **270:** 18655-18659.

Price, M.A., Cruzalegui, F. H., Treisman, R. (1996). The p38 and ERK MAP kinase pathways cooperate to activate ternary complex factors and c-fos transcription in response to U.V. light. *EMBO. J.* **15:** 6552-6563.

Pulverer BJ. Kyriakis JM. Avruch J. Nikolakaki E. Woodgett JR. (1991). Phosphorylation of c-Jun mediated by MAP kinases. *Nature.* **353:** 670-674.

Putney, J.W. (1986). A model for receptor-regulated calcium entry. *Cell Calcium.* 7: 1-12.

Putney, J.W. (1990). Capacitative calcium entry revisited. *Cell Calcium.* **11:** 611-624.

Putney, J.W. and Bird (1993). The inositol phosphate-calcium signaling system in nonexcitable cells. *Endocrine reviews.* **14:** 610-631.

Qui, Z.H., Leslie, C.C (1994). Protein kinase-C dependent and -independent pathways of Mitogen-Activated Protein Kinase activation in macrophages by stimuli that activate phospholipase-A2. *J. Biol. Chem.* **269:** 19480-19487.

Rana, A., Gallo, K., Godowski, P., Hirai, S-I., Ohno, S., Zon, L., Kyriakis, J.M., Avruch, J., (1996). The mixed lineage kinase SPRK phosphorylates and activates the stress-activated protein kinase activator, SEK-1. *J. Biol. Chem.* **271:** 19025-19028.

Raingeaud, J., Gupta, S., Rogers, J.S., Dickens, M., Han, J., Ulevitch, R.J. (1995). Pro-inflammatory cytokines and environmental stress cause p38 Mitogen-activated Protein Kinase activation by dual phosphorylation on tyrosine and threonine. *J. Biol. Chem.* **270:** 7420-7426.

Raingeaud, J., Whitmarsh, A., Barrett, T, Dérijard, B., Davis, R.J. (1996). MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen- activated protein kinase signal transduction pathway. *Mol. Cell. Biol.* **16:** 1247-1255.

Ramirez, M.T., Sah, V.P., Zhao, X-L., Hunter, J.J., Chien, K.R., Brown, J.H. (1997). The MEKK-JNK pathway is stimulated by $_1$ adrenergic receptor and Ras activation and is associated with *in vitro* and *in vivo* cardiac hypertrophy. *J. Biol. Chem.* **272:** 14057-14061.

Rang, H.P., Dale, M.M., Ritter, J.M. (1999). *Pharmacology* (4th Ed.) p31. Churchill Livingstone. Edinburgh, London, Melbourne, New York, Tokyo and Madrid.

Reska, A.A., Seger, R., Diltz, C.D., Krebs, E.G., Fischer, E.H. (1995). Association of the Mitogen-activated Protein Kinase with the microtubule cytoskeleton. *Proc. Natl. Acad. Sci.* **92:** 8881-8885.

Richards, M.H., (1991). Pharmacology and 2nd messenger interactions of cloned muscarinic receptors. *Biochem. Pharmacol.* **42:** 1645-1653.

Ridley, A.J., Paterson, H.F., Johnston, C.L., Diekman, D., Hall, A. (1992). The small GTP-binding protein Rac regulates growth factor-induced membrane ruffling. *Cell.* **70:** 401-410.

Robinson, M.J., Cobb, M. H. (1997). Mitogen-activated Protein Kinase Pathways. *Curr. Op. Cell. Biol.* **9:** 108-186.

Rosen, O.M. (1987). After insulin binds. *Science.* **237:** 1452-1458.

Rossomando, A.J., Payne, D.M., Weber, M.J., Sturgill. T.W. (1989). Evidence that pp42, a major tyrosine kinase target protein, is a itogen-activated serine/threonine protein kinase. *Proc. Natl. Acad. Sci.* **86:** 6940-6943.

Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T., Nebreda, A. (1994). A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell.* **78:** 1027-1037.

Roux, E., Molimard, M., Savineau, J.P., Marthan, R., (1998). Muscarinic stimulation of airway smooth muscle cells. *General Pharmacology.* **31:** 349-356).

Rubinfield, H., Hanoch, T., Seger, R. (1999). Identification of a cytoplasmic retention sequence in ERK2. *J. Biol. Chem.* **274:** 30349-30352.

Rukenstein, A., Rydel, R.E., Greene, L.A., (1991). Multiple agents rescue PC12 cells from serum-free cell death by translation- and transcription-independent mechanisms. *J. Neurosci.* **11:** 2552-2563.

Rusanescu, G., Qi, H., Thomas, S.M., Brugge, J.S., Halegoua, S. (1995). Calcium influx induces neurite growth through a Src-Ras signalling cassette. *Neuron.* **15:** 1415-1425.

Ryves, W.J., Evans, E.T., Oliver, A.R., Parker, P.J., Evans, F.J. (1991). Activation of the PKC-isotypes alpha, beta1, gamma, delta and epsilon by phorbol esters of different biological activities. *FEBS Lett.* **288:** 5-9.

Sanchez, I., Hughes, R.T., Mayer, B.J., Yee, K., Woodgett, J.R., Avruch, J., Kyriakis, J.M., Zon, L.I. (1994). Role of SAPK/ERK Kinase-1 in the Stress-activated Pathway regulating the transcription factor c-Jun. *Nature.* **372:** 794-798.

Schaeffer, H.J., Catling, A.D., Eblen, S.T., Collier, L.S., Krauss, A., Weber, M.J. (1998). MP1: A MEK binding partner that enhances enzymatic activation of the MAP Kinase cascade. *Nature.* **281:** 1668-1671.

Schlaepfer, D.D., Hunter, T. (1997). Focal Adhesion Kinase overexpression enhances Ras-dependent integrin signaling to ERK2/Mitogen-activated Protein Kinase through interactions with and activation of c-Src. *J. Biol. Chem.* **272:** 13189-13195.

Schlessenger, J. (1993). How receptor tyrosine kinases activate Ras. *Trends. Biochem. Sci.* **18:** 273-275.

Schönwasser , D.C., Marais, R.M., Marshall, C.J., Parker, P.J. (1998). Activation of the Mitogen-activated Protein Kinase/Extracellular signal- Regulated Kinase pathway by conventional, novel and atypical Protein Kinase C isotypes. *Mol. Cell. Biol.* **18:** 790-798.

Selbie, L.A., Schmitzpeiffer, C., Sheng, Y.H., Biden, T.J., (1993). Molecular cloning and characterization of PKC iota, an atypical isoform of Protein Kinase C derived from insulin-secreting cells. *J. Biol. Chem.* **268:** 24296-24302.

Selbie , L.A., Hill, S.J. (1998). G Protein-coupled-receptor cross-talk:The fine-tuning of multiple receptor-signalling pathways. *Trends. Pharmacol. Sci.* **19:** 87-93.

Shapiro, L., Dinerallo, C.A. (1995). Osmotic regulation of cytokine synthesis in vitro. *Proc. Natl. Acad. Sci.* **92:** 12230-12234.

Shapiro, P.S., Evans, J.N., Davis, R.J., Posada, J.A. (1996). The seven-transmembrane-spanning receptors for endothelian and thrombin cause proliferation of airway smooth muscle cells and activation of the Extracellular Regulated Kinase and c-Jun NH2-terminal Kinase Groups of Mitogen-Activated Protein Kinases. *J. Biol. Chem.* **271:** 5750-5754.

Shao, Y.P, McCarthy, K.D. (1993). Quantitative relationship between alpha (1)-adrenergic receptor density and the receptor-mediated calcium response in individual astroglial cells. *Mol. Pharmacol.* **44:** 247-254.

Sheu, H-M., Kitajima, Y., Yaoita, H. (1989). Involvement of Protein Kinase C in translocation of desmoplakins from cytosol to plasma membrane during desmosome formation in human squamous cell carcinoma cells grown in low to normal calcium concentration. *Exp. Cell Res.* **185:** 176-190.

Simonds, W.F. (1999). G protein regulation of adenylate cyclase. *Trends. Pharmacol. Sci.* **20:** 66-73.

Sluss, H.K., Barrett, T., Derijard, B., Davis, R.J. (1994). Signal transduction by tumor necrosis factor mediated by JNK protein kinases. *Mol. Cell. Biol.* **14:** 8376-8384.

Smeal T. Binetruy B. Mercola D.A. Birrer M. Karin M. (1991). Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serines 63 and 73. *Nature.* **354:** 494-496.

Stephens, L., Eguinoa, A., Corey, S., Jackson, T., Hawkins, P.T. (1993). Receptor stimulated accumulation of phosphatidylinositol (3,4,5)-trisphosphate by G-protein mediated pathways in human myeloid derived cells. *EMBO. J.* **12:** 2265-2273.

Sternweiss, P.C. (1996). G-proteins in signal transduction. pg. 287-301. Signal Transduction. Editors. Heldin and Purton, Nelson Thornes, UK.

Strassheim, D., May, L.G., Varker, K.A., Puhl, H.L., Phelps, S.H., Porter, R.A., Aronstam, R.S., Noti, J.D., Williams, C.L. (1999). M_3 muscarinic acetylcholine receptors regulate cytoplasmic myosin by a process involving RhoA and requiring convential Protein Kinase C isoforms. *J. Biol. Chem.* **274:** 18675-18685.

Su, B., Jacinto, E., Hibi, M., Kallunki, T., Karin, M., Ben-Neriah, Y. (1994). JNK is involved in signal integration during costimulation of T-lymphocytes. *Cell.* **77:** 727-736.

Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T., Nishizuka, Y. (1979). Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids. *J. Biol. Chem.* **254:** 3692-3695.

Takeda, H., Matozaki, T., Takade, T., Noguchi, T., Yamao, T., Tsuda, M., Ochi, F., Fukunaga, K., Inagaki, K., Kasuga, M. (1999). PI 3kinase γ and Protein Kinase C-ζ mediate Ras-independent activation of MAP kinase by a G_i protein-coupled receptor. *EMBO. J.* **18:** 386-395.

Teramoto, H., Coso, O.A., Miyata, H., Igishi, T., Miki, T., Gutkind, J.S (1996). Signaling from the small GTP-binding proteins Rac1 and Cdc42 to the c-Jun N-terminal Kinase/Stress-activated Protein Kinase pathway. A role for mixed lineage kinase 3/protein-tyrosine kinase 1,a novel member of the mixed lineage kinase family. *J. Biol. Chem.* **271**: 27225-27228.

Tobin, A.B., Lambert, D.G., Hahorski, S.R. (1992). Rapid desensitation of muscarinic m3 receptor-stimulated polyphosphoinositide responses. *Mol. Pharmacol.* **42:** 1042-1048.

Tobin, A.B., Willars, G.B., Burford, N.T., Nahorski, S.R. (1995). Relationship between agonist binding, phosphorylation and immunoprecipitation of the m3-muscarinic receptor, and 2nd messenger responses. *Br. J. Pharmacol.* **116:** 1723-1728.

Tombes, R.M., Auer, K.L., Mikkelsen, R., Valerie, K., Wymanns, M.P., Marshall, C.J., McMahon, M., Dent, P. (1998). The Mitogen-activated Protein (MAP) kinase cascade can either stimulate or inhibit DNA synthesis in primary cultures of rat hepatocytes depending upon whether its activation is acute/phasic or chronic. *Biochem. J.* **330:** 1451-1460.

Touhara, K., Hawes, B.E., van Biesen, T., Lefkowitz, R.J. (1995). G protein βγ subunits stimulate phosphorylation of Shc adapter protein. *Proc. Natl. Acad. Sci.* **92:** 9284-9287.

Tournier, C., Whitmarsh, A.J., Cavanagh, J., Barrett. T., Davis, R.J. (1999). The MKK7 gene encodes a group of c-Jun NH_2-terminal kinase kinases. *Mol. Cell. Biol.* **19:** 1569-1581.

Tournier, C., Whitmarsh, A.J., Cavanagh, J., Barrett, T., Davis, R.J. (1997). Mitogen-activated Protein Kinase Kinase 7 is an activator of the c-Jun NH_2-terminal kinase. *Proc. Natl. Acad. Sci.* **94:** 7337-7342.

Traverse, S., Gomez, N., Paterson, H., Marshall, C., Cohen, P. (1992). Sustained activation of the Mitogen-activated Protein (MAP) Kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor. *Biochem. J.* **288:** 351-355.

Triesman, R. (1994). Ternary complex factors: Growth factor regulated transcriptional activators. *Curr. Op. Genet. Dev.* **4:** 96-101.

Troppmair, J., Bruder, J.T., Munoz, H., Lloyd, P.A., Kyriakis, J., Bannerjee, P., Arvruch, J., Rapp, U.R. (1994). Mitogen-activated Protein Kinase/Extracellular signal-Regulated protein Kinase activation by oncogenes, serum, and 12-O-tetradecanoylphorbol-13-acetate requires Raf and is necessary for transformation. *J. Biol. Chem.* **269:** 7030-7035.

Ueda, Y., Hirai, S-I., Osada, S-I., Suzuki, A., Mizuno, K., Ohno, S. (1996). Protein Kinase C δ activates the MEK-ERK pathway in a manner independent of Ras and dependent on Raf. *J. Biol. Chem.* **271:** 23512-23519.

van Biesen, T., Hawes, B.E., Luttrell, D.K., Krueger, K.M., Touhara, K., Porfiri, E., Sakaue, M., Luttrell, L.M., Lefkowitz, R.J. (1995). Receptor-tyrosine-kinase- and $G_{\beta\gamma}$-mediated MAP kinase activation by a common signalling pathway. *Nature.* **376:** 781-784.

van Biesen, T., Hawes, B.E., Raymond, J.R., Luttrell, L.M., Lefkowitz, R.J. (1996). G_o-protein α-subunits activate Mitogen-activated Protein Kinase via a novel Protein Kinase C-dependent mechanism. *J. Biol. Chem.* **271:** 1266-1269.

van Breemen, C. (1989). Cellular mechanisms regulating $[Ca^{2+}]_i$ smooth muscle. *Annual Review of Physiology.* **51:** 315-329

Van Giersbergen, P.L.M and Leppik, J. (1995). Modulation of agonist binding by guanine nucleotides in CHO cells expressing muscarinic m1-m5 receptors. *Naun. Schm. Arch. Pharm.* **352:** 166-172.

Vlahos, C.J., Matter, W.F., Hui, K.Y., Brown, F.H. (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.* **269:** 5241-5248.

Vogt, P.K., Bos, T.J. (1990). jun: Oncogene and transcription factor. *Adv. Cancer. Res.* **55:** 1-35.

Von Willebrand, M., Jascur, T., Bonnefroy-Bernard, N., Yano, H., Altman, A., Matsuda, Y., Mustelin, T. (1996). Inhibition of phosphatidylinositol 3-kinase blocks T cell antigen receptor/CD3-induced activation of the mitogen-activated kinase ERK2. *Eur. J. Biochem.* **235:** 828-835.

Voyno-Yasenetskaya, T.A., Faure, M.P., Ahn, N.G., Bourne, H.R. (1996). $G_{\alpha 12}$ and $G_{\alpha 13}$ regulate Extracellular signal-Regulated Kinase and c-Jun kinase pathways by different mechanisms in COS-7 Cells. *J. Biol. Chem.* **271:** 21081-21087.

de Vries-Smits, A.M.M., Pronk, G.J., Medema, J.P., Burgering, B.M.T., Bos, J.L. (1995). Shc associates with an unphosphorylated form of the p21ras guanine nucleotide exchange factor mSOS. *Oncogene.* **10:** 919-925.

Wan, Y., Kurosaki, T, Huang, X-Y. (1996). Tyrosine kinases in activation of the MAP kinase cascade by G-protein-coupled receptors. *Nature.* **380:** 541-544.

Wang, C., Constantinescu, S.N., Macewan, D.J., Strulovici, B., Dekker, L.V., Parker, P.J, Pfeffer, L.M. (1993). Interferon-alpha induces Protein Kinase C-epsilon (PKC-epsilon) gene-expression and a 4.7-kb pkc-epsilon-related transcript. *Proc. Natl. Acad. Sci.* **90:** 6944-6948.

Wang, X and Ron, D. (1996). Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP Kinase. *Science.* **272:** 1347-1349.

Wang, X.S., Diener, K., Manthey, C.L., Wang, S., Rosenzweig, B., Bray, J., Delaney, J., Cole, C.N., Chan-Hui, P-Y., Mantlo, N., Lichenstein, H.S., Zukowski, M., Yao, Z. (1997). Molecular cloning and characterization of a novel p38 Mitogen-activated Protein Kinase. *J. Biol. Chem.* **272:** 23668-23674.

Wang, Y-X., Dhulipa P.D.K., Li, L., Benvovic, J.L., Kotlikoff, M.I. (1999). Coupling of M2 muscarinic receptors to membrane ion channels via Phosphoinositide 3-Kinase γ and atypical Protein Kinase C. *J. Biol. Chem.* **274:** 13859-13864.

Waskiewicz, A.J., Cooper, J.A. (1995). Mitogen and stress-response pathways: MAP Kinase cascades and phosphatase regulation in mammals and yeast. *Curr. Op. Cell. Biol.* **7:** 798-805.

Welsh, G.I., Foulstone, E.J., Young, S.W., Tavaré, J.M., Proud, C.G. (1994). Wortmannin inhibits the effects of insulin and serum on the activities of glycogen synthase kinase-3 and Mitogen-activated Protein Kinase. *Biochem. J.* **303:** 15-20.

Westwick, J.K., Weitzel, C., Minden, A., Karin, M., Brenner, D.A. (1994). Tumour necrosis factor α stimulates AP-1 activity through prolonged activation of c-Jun kinase. *J. Biol. Chem.* **269:** 26396-26401.

Westwick, J.K., Bielawska, Dbaibo, G., Hannun, Y.A., Brenner, D.A. (1995). Ceramide activates the Stress-activated Protein Kinases *J. Biol. Chem.* **270:** 22689-22692.

Whiteway, M.S., Wu, C., Leeuw, T., Clark, K., Fourest-Lieuvin, A., Thomas, D.Y., Leberer, E. (1995). Association of the yeast pheromone response G protein βγ subunits with the MAP kinase scaffold Ste. *Science.* **269:** 1572-1575.

Whitmarsh, A.J., Shore, P., Sharrocks, A.D., Davis, R.J. (1995). Integration of MAP kinase signal transduction pathways at the serum response element. *Science.* **269:** 403-406.

Whitmarsh, A.J., Yang, S.H., Su, M.S.S., Sharrocks, A.D., Davis, R.J. (1997). Role of p38 and JNK Mitogen-Activated Protein Kinases in the activation of ternary complex factors. *Mol. Cell. Biol.* **17:** 2360-2371.

Whitmarsh, A.J., Cavanagh, J., Tournier, C., Yasuda, J., Davis, R.J. (1998). A mammalian scaffold complex that selectively mediates MAP Kinase activation. *Science.* **281:** 1671-1674.

Willars, G.B., Nahorski, S.R. (1995a). Quantitative comparisons of muscarinic and bradykinin receptor-mediated Ins (1,4,5)P3 accumulation and Ca^{2+} signalling in human neuroblastoma cells. *Br. J. Pharmacol.* **114:** 1133-1142.

Willars, G.B., Nahorski, S.R. (1995b). Heterologous desensitization of both phosphoinositide and Ca^{2+} signaling in SH-SY5Y neuroblastoma cells: A role for intracellular Ca^{2+} store depletion? *Mol. Pharmacol.* **47:** 509-516.

Willars, G.B., Nahorski, S.R., Challiss, R.A.J. (1998). Differential regulation of muscarinic acetylcholine receptor-sensitive polyphosphoinositide pools and consequences for signalling in human neuroblastoma cells. *J. Biol. Chem.* **273:** 5037-5046.

Willars, G.B., Challiss, R.A.J., Stuart, J.A., Nahorski, S.R. (1996). Contrasting effects of phorbol ester and agonist-mediated activation of Protein Kinase C on phosphoinositide and Ca^{2+} signalling in a human neuroblastoma. *Biochem. J.* **316:** 905-913

Winitz, S., Russell, M., Qian, N.X., Gardner, A., Dwyer, L., Johnson, G.L. (1993). Involvement of Ras and Raf in the G_i-coupled acetylcholine muscarinic receptor activation of Mitogen-activated Protein (MAP) kinase kinase and MAP kinase. *J. Biol. Chem.* **268:** 19196-19199.

Wu, X., Noh, S.J., Zhou, G., Dixon, J.E., Guan, K.L. (1996). Selective activation of MEK1 but Not MEK2 by A-Raf from epidermal growth factor-stimulated HeLa cells. *J. Biol. Chem.* **271:** 3265-3271.

Wu, Z., Puigserver, P., Spiegelman B.M. (1999). Transcriptional activation of adipogenesis. *Curr. Op. Cell Biol.* **11**: 689-694.

Wylie, P.G., Challiss, R.A.J., Blank, J.L. (1999). Regulation of Extracellular-signal Regulated Protein Kinase and c-Jun N-terminal Kinase by G-protein-linked muscarinic acetylcholine receptors. *Biochem. J.* **338:** 619-628.

Wyllie, A.H. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature.* **284:** 555-556.

Xia, Z., Dickens, M., Raingeaud, J., Davis, R.J., Greenberg, M.E. (1995). Opposing effects of ERK and JNK p38 MAP Kinases on apoptosis. *Science.* **270:** 1326-1331.

Xu, B., Wilsbacher, J.L., Collisson, T., Cobb, M.H. (1999). The N-terminal ERK-binding site of MEK1 is required for efficient feedback phosphorylation by ERK2 *in vitro* and ERK activation *in vivo*. *J. Biol. Chem.* **274:** 34029-34035.

Xu, S., Cobb, M.H. (1997). MEKK1 binds directly to the c-Jun N-terminal Kinases/Stress-activated Protein Kinases. *J. Biol. Chem.* **272:** 32056-32060.

Yamada, M, Inanobe, A, Kurachi, Y. (1998). G Protein Regulation of potassium ion channels. *Pharmacol. Rev.* **50:** 723-757.

Yamaguchi, K., Shirakabe, K., Shibuya, H., Irie, K., Oishi, I., Ueno, N., Taniguchi. T., Nishida, E., Matsumoto, (1995). Identification of a member of the MAPKKK family as a potential mediator of TGF- signal transduction *Science.* **270:** 2008-2011.

Yamauchi, J., Nagoa, M., Kaziro, Y., Itoh, H. (1997). Activation of p38 Mitogen-activated Protein Kinase by Signalling through G-protein-coupled receptors. *J. Biol. Chem.* **272:** 27771-27777.

Yan, M., Dai, T., Deak, J.C., Kyriakis, J.M., Zon, L.I., Woodgett, J.R., Templeton, D.J. (1994). Activation of stress activated protein kinase by MEKK1 phosphorylation of its activator SEK1. *Nature.* **372:** 798-800.

Yao, Z., Diener, K., Wang, X.S., Zukowski, M., Matsumoto, G., Zhou, G., Mo, R., Sasaki, T., Nishina, H., Hui, C., Tan, T-H., Woodgett, J.P., Penninger, J.M. (1997). Activation of Stress-activated Protein Kinases/c-Jun N-terminal Protein Kinases (SAPKs/JNKs) by a novel Mitogen-activated Protein Kinase Kinase (MKK7). *J. Biol. Chem.* **272:** 32378-32383.

Yasuda, J., Whitmarsh, A.J., Cavanagh, J. Sharma, M., Davis, R.J. (1999). The JIP group of Mitogen-activated Protein kinase scaffold proteins. *Mol. Cell. Biol.* **19:** 7245-7254.

Yu, H., Li, X., Marchetto, G.S., Dy, R., Hunter, D., Calvo, B., Dawson, T.L., Wilm, M., Anderegg, R.J., Graves, L.M., Earp, H.S. (1996). Activation of a novel calcium-dependent protein-tyrosine kinase. correlation with c-Jun N-terminal kinase but not mitogen-activated protein kinase activation. *J. Biol. Chem.* **271:** 29993-29998.

Yujiri, T., Sather, S., Fanger, G.R., Johnson, G.L. (1998). Role of MEKK1 in cell survival and activation of JNK and ERK pathways defined by targeted gene disruption. *Science.* **282:** 1911-1914.

Zechner, D., Craig, R., Hanford, D.S., McDonough, P.M., Sabbadini, R.A., Glembotski, C.C. (1998). MKK6 activates myocardial cell NF-κB and inhibits apoptosis in a p38 Mitogen-activated Protein Kinase-dependent manner. *J. Biol. Chem.* **273:** 8232-8239.

Zervos, A.S., Faccio, L., Kyriakis, J.M., Brent, R. (1995). Mxi2, a Mitogen-activated Protein kinase that recognizes and phosphorylates Max protein. *Proc. Natl. Acad. Sci.* **92:** 10531-10534.

Zetser, A., Gredinger, E., Bengal, E. (1999). p38 mitogen-activated protein kinase pathway promotes skeletal muscle differentiation. *J. Biol. Chem.* **274:** 5193-5200.

Zheng, C-F., Guan, K-L. (1993). Cloning and characterisation of two distinct human Extracellular signal-Regulated Kinase activator kinases, MEK1 and MEK2. *J. Biol. Chem.* **268:** 11435-11439.

Zheng, C., Xiang, J., Hunter, T., Lin. A. (1999). The JNKK2-JNK1 fusion protein acts as a constitutively active c-Jun kinase that stimulates c-Jun Transcription activity. *J. Biol. Chem.* **274:** 28966-28971.

Zhou, J., Valletta, J.S., Grimes, M.L., Mobley, W.C. (1995). Multiple levels for regulation of TrkA in PC12 cells by nerve growth-factor. *J. Neurochem.* **65:** 1146-1156.

Ziff, E.B. (1990). Transcription factors: a new family gathers at the cAMP response site. *Trends. Genet.* **6:** 69-72.

Zohn, I.E., Yu, H., Li, X., Cox, A.D., Earp, H.S. (1995). Angiotensin II stimulates calcium-dependent activation of c-Jun N-Terminal Kinase. *Mol. Cell. Biol.* **15:** 6160-6168.

Regulation of extracellular-signal regulated kinase and c-Jun N-terminal kinase by G-protein-linked muscarinic acetylcholine receptors

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Extracellular signal-regulated kinases (ERKs) and c-Jun N-terminal kinases (JNKs, or stress-activated protein kinases) are activated by diverse extracellular signals and mediate a variety of cellular responses, including mitogenesis, differentiation, hypertrophy, inflammatory reactions and apoptosis. We have examined the involvement of Ca^{2+} and protein kinase C (PKC) in ERK and JNK activation by the human G-protein-coupled m2 and m3 muscarinic acetylcholine receptors (mAChR) expressed in Chinese hamster ovary (CHO) cells. We show that the Ca^{2+}-mobilizing m3 AChR is efficiently coupled to JNK and ERK activation, whereas the m2 AChR activates ERK but not JNK. Activation of JNK in CHO-m3 cells by the agonist methacholine (MCh) was delayed in onset and more sustained relative to that of ERK in either CHO-m2 or CHO-m3 cells. The EC_{50} values for MCh-induced ERK activation in both cell types were essentially identical and similar to that for JNK activation in CHO-m3 cells, suggesting little amplification of the response. Agonist-stimulated Ins(1,4,5)P_3 accumulation in CHO-m3 cells was insensitive to pertussis toxin (PTX), consistent with a G_q/phosphoinositide-specific phospholipase C-β mediated pathway, whereas a significant component of ERK and JNK activation in CHO-m3 cells was PTX-sensitive, indicating $G_{i/o}$ involvement. Using manipulations that prevent receptor-mediated extracellular Ca^{2+} influx and intracellular Ca^{2+}-store release, we also show that ERK activation by m2 and m3 receptors is Ca^{2+}-independent. In contrast, a significant component (> 50%) of JNK activation mediated by the m3 AChR was dependent on Ca^{2+}, mainly derived from extracellular influx. PKC inhibition and down-regulation studies suggested that JNK activation was negatively regulated by PKC. Conversely, ERK activation by both m2 and m3 AChRs required PKC, suggesting a novel mechanism for PKC activation by PTX-sensitive m2 AChRs. In summary, mAChRs activate JNK and ERK via divergent mechanisms involving either Ca^{2+} or PKC respectively.

Key words: calcium, G-protein-coupled receptor, mitogen-activated protein kinase, protein kinase C, stress-activated protein kinase.

INTRODUCTION

Mitogen-activated protein kinases (MAPKs) are activated by a diverse array of extracellular stimuli and regulate a variety of cellular responses (for reviews see [1–4]). MAPK family members include the extracellular signal-regulated kinases (ERKs) [1,2], the c-Jun N-terminal kinases (JNKs) and p38 kinases [3–6]. MAPKs are activated by parallel sequential protein kinase cascades, comprising the MAPK (e.g. ERK1), a MAPK kinase (e.g. MEK1), and a MAPK kinase kinase (e.g. Raf-1) [2–4]. The ERK pathway is activated by receptor tyrosine kinases and G-protein-coupled receptors (GPCRs) that promote mitogenesis, differentiation or hypertrophy. ERKs mediate these effects by phosphorylating various substrates, including cytoplasmic enzymes (e.g. phospholipase A_2, p90 ribosomal protein S6 kinase) and nuclear transcription factors (e.g. ELK-1) [1,2]. JNKs and p38 kinases are activated by cellular stress (e.g. UV- and γ-radiation, osmotic and heat shock, protein synthesis inhibitors) and inflammatory cytokines (e.g. tumour necrosis factor-α, interleukin-1), but also weakly by growth factors (e.g. epidermal growth factor) [3–6]. JNK activation has been implicated in the immune response, oncogenic transformation and apoptosis. Current evidence suggests that JNKs mediate these effects by increasing gene expression; transcription factors activated by JNK include c-Jun, activating transcription factor 2 and ELK-1 [3–6].

MAPK regulation by GPCRs appears to be a widespread phenomenon and is likely to mediate the proliferative and hypertrophic responses of cells to various hormones, neurotransmitters and local mediators that act at this class of receptors [7]. Receptors involved include those linked to phosphoinositide-specific phospholipase C (PLC) activation via pertussis toxin (PTX)-insensitive G_q-proteins and those preferentially coupled to inhibition of adenylyl cyclase via PTX-sensitive G_i-proteins. In most cases, ERK activation by PTX-sensitive G-proteins is independent of protein kinase C (PKC), which is consistent with a lack of involvement of receptor-mediated phosphoinositide hydrolysis [7–9]. Transient expression studies of α_2 adrenergic and m2 muscarinic acetylcholine receptors (mAChRs) in COS-7 cells suggest that $\beta\gamma$ subunits liberated from PTX-sensitive G-proteins couple these receptors to ERK activation via a pathway requiring the monomeric G-protein Ras [7–12]. Indeed, ex-

Abbreviations used: $[Ca^{2+}]_i$, intracellular calcium ion concentration; CHO, Chinese hamster ovary; ERK, extracellular signal-regulated protein kinase; fura-2/AM, fura-2 acetoxymethyl ester; GPCR, G-protein-coupled receptor; GST, glutathione S-transferase; JNK, c-Jun N-terminal kinase; KH buffer, Krebs–Henseleit buffer; mAChR, muscarinic acetylcholine receptor; MCh, methacholine; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MAPKKK, MAPK kinase kinase; MEKK, MAPK/ERK kinase kinase; [^{3}H]NMS, *N*-[^{3}H]methylscopolamine; PDBu, phorbol dibutyrate; PI-3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLC, phosphoinositide-specific phospholipase C; PTX, pertussis toxin.

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pression of free $\beta_1\gamma_2$ subunits in COS-7 cells, but not mutationally activated α_{i2}, is sufficient to activate ERK1 [9,11] and ERK2 [8] in a Ras-dependent manner [8,9]. In contrast, activated α_{12}can transform Rat-1 fibroblasts [12] and activate ERK2 [11], and may contribute to Ras-dependent ERK activation by the m2 AChR in these cells [13].

Heterogeneity also exists in the mechanisms by which PTX-resistant Ca^{2+}-mobilizing receptors activate ERK, and both Ras-dependent and -independent pathways have been implicated [7]. PKC appears to be important, as its down-regulation or inhibition can partly attenuate or fully inhibit ERK activation by receptors coupled to PLC [7–9]. Consistent with this, acute treatment of cells with tumour-promoting phorbol esters, or overexpression of conventional or novel PKC isoenzymes, causes ERK activation [2,14,15]. Persistent activation of m1, m3 or m5 receptors [7], or expression of mutationally activated α_q [16,17], can induce proliferation and neoplastic transformation of NIH3T3 cells, although expression of activated α_q alone causes modest [11] or no [8] activation of ERK in COS-7 cells. However, manipulations to sequester $\beta\gamma$ subunits have provided evidence for both α_q and $\beta\gamma$ subunit involvement in ERK activation mediated by the G_q-coupled m1 AChR [8], α_{1B}-adrenoceptor [9,18] and bombesin receptor [11] in COS-7 cells. The mechanisms by which $\beta\gamma$ subunits activate the ERK pathway may include Ras guanine nucleotide exchange factors [19] and cytoplasmic tyrosine kinases [18,20–22].

Recently, JNK activation has also been demonstrated for several GPCRs, including m1 and m2 ACh [23–25], angiotensin II [26], α_1 adrenergic [27], thrombin [28] and endothelin-1 [28,29] receptors. For example, the m1 AChR has been shown to activate both JNK and ERK pathways in NIH3T3 cells and cause expression of immediate early genes involved in cell growth (*c-Jun, Jun D, c-Fos, Fos B*) [23]. In COS-7 cells, the m1 and m2 AChRs stimulate JNK by a mechanism involving $\beta\gamma$ subunits acting via Ras and Rac1 [25]. Consistent with this, overexpression of $\beta_1\gamma_2$ is sufficient to activate JNK in COS-7 cells, whereas constitutively active α_{i2} is without effect [25]. α_q has also been reported to activate JNK in COS-1 [30] and PC-12 [31] cells, but does not appear to do so in COS-7 cells [26]. Phorbol esters have little effect on JNK activity [5], indicating that PKC stimulation alone is insufficient to activate JNK. Some studies support a role for Ca^{2+} in receptor-mediated JNK activation [24,26], and the Ca^{2+}-dependent tyrosine kinase Pyk2 has been implicated in JNK activation by cellular stress in PC12 cells but not in COS or HEK293 cells [32]. Finally, mutationally active α_{12} and α_{13} are also able to activate JNK, but not ERK, via Ras [30] and Rac1 [33] or Cdc42 [34]. Although the effectors for α_{12} and α_{13} have not been defined, this pathway may account for thrombin-induced AP1-dependent gene expression and/or mitogenesis [35,36].

Five distinct mAChRs show preferential coupling to at least two classes of G-protein [37]. Thus, the m1, m3 and m5 AChRs are primarily linked to PLC activation via PTX-resistant G_q-proteins. Subsequent hydrolysis of PtdIns(4,5)P_2 provides the second messengers Ins(1,4,5)P_3 and 1,2-diacylglycerol, which mobilize intracellular calcium and activate certain PKC isozymes respectively [38,39]. Conversely, m2 and m4 AChRs are coupled to the activation of potassium channels and to the inhibition of adenylyl cyclase via PTX-sensitive G_i-proteins, thereby lowering cAMP levels [37]. Clearly, MAPK regulation by GPCRs may be downstream or independent of second messenger generation mediated by individual G-protein subunits. We have examined the role of Ca^{2+} and PKC in the regulation of JNK and ERK by human m2 and m3 AChRs expressed in Chinese hamster ovary (CHO) cells. We find that the m3 AChR is coupled to JNK and ERK activation in these cells, whereas the m2 receptor only activates ERK. We demonstrate that Ca^{2+}, mainly derived from extracellular influx, contributes to m3-mediated JNK activation, whereas ERK activation by both receptors is Ca^{2+}-independent. We also demonstrate that PKC is necessary for ERK activation by both m2 and m3 receptors, suggesting a novel mechanism for PKC activation by PTX-sensitive m2 AChRs.

MATERIALS AND METHODS

Cell culture

Chinese hamster ovary (CHO-K1) cells stably expressing either the recombinant human m2 or m3 AChR [40] were maintained in minimal essential medium-α (Gibco-BRL) at 37 °C in a 5% CO_2/95% air mixture. The medium was supplemented with 10% (v/v) newborn calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin and 2.5 μg/ml amphotericin B (Gibco-BRL). Before agonist addition, cells were incubated in a Krebs-Henseleit (KH) buffer (pre-equilibrated with 5% CO_2/95% O_2 and containing 5 mM Hepes, pH 7.4/10 mM glucose/25 mM $NaHCO_3$/1.2 mM K_2HPO_4/118 mM NaCl/4.7 mM KCl/1.2 mM $MgSO_4$/1.3 mM $CaCl_2$) for 30 min at 37 °C under 5% CO_2/95% air.

ERK immunoprecipitation and assay

ERK proteins were isolated by immunoprecipitation from CHO cell lysates and assayed for activity according to the following procedure. Cells were solubilized with lysis buffer containing 20 mM Tris/HCl (pH 8.0)/0.5% Nonidet P40/250 mM NaCl/3 mM EDTA/3 mM EGTA/2 mM Na_3VO_4/1 mM dithiothreitol/1 mM PMSF/20 μg/ml aprotinin/5 μg/ml leupeptin. Insoluble material was removed by centrifugation at 14000 *g* for 10 min at 4 °C. Lysates were incubated for 90 min at 4 °C with a 1:100 dilution of a rabbit polyclonal antiserum (200 μg/ml) directed against ERK1 or ERK2 [C16: sc-93 or C14: sc-94 respectively (Santa Cruz)]. Immune complexes were incubated for a further 90 min at 4 °C with 70 μl of a 15% (v/v) slurry of Protein A–Sepharose (Pharmacia-LKB) and collected by centrifugation. Immunoprecipitates were washed twice in 200 μl of lysis buffer and twice in 200 μl of kinase buffer containing 20 mM Hepes, pH 7.2/20 mM β-glycerophosphate/10 mM $MgCl_2$/1 mM dithiothreitol/50 μM Na_3VO_4. Immune complex ERK assays were initiated by addition of 40 μl of kinase buffer containing 20 μM [γ-^{32}P]ATP (2.5 μCi/nmol) and 200 μM of a synthetic peptide substrate corresponding to amino acids 662–681 of the epidermal growth factor receptor [41]. Reactions were incubated for 20 min at 30 °C and terminated by addition of 10 μl of 25% (w/v) trichloroacetic acid. Mixtures were centrifuged at 14000 *g* for 2 min and spotted onto P81 cation-exchange paper (Whatman). Papers were washed as described [41] and counted by liquid-scintillation counting.

JNK assay

JNK activity was assessed using a recombinant protein fragment of c-Jun as an affinity ligand and substrate {glutathione S-transferase (GST)–c-Jun [1–79]} [6]. Cleared cell lysates were prepared exactly as described above and incubated for 60 min at 4 °C with 20 μl of a 25% (v/v) slurry of glutathione–Sepharose (Pharmacia-LKB), pre-coupled to GST–c-Jun (5 μg of protein). Beads were collected by centrifugation and washed twice in 200 μl of lysis buffer and twice in 200 μl of kinase buffer. Reactions were initiated by addition of 40 μl of kinase buffer containing 20 μM [γ-^{32}P]ATP (2.5 μCi/nmol). After incubation for 20 min at 30 °C, reactions were terminated by addition of

40 μl of Laemmli sample buffer and boiling. Phosphorylated proteins were resolved by PAGE through 12% acrylamide in the presence of 0.1% SDS, stained with Coomassie Blue R250 and visualized by autoradiography. Radioactivity incorporated into GST–c-Jun was quantified by liquid-scintillation counting of the excised bands.

Measurement of intracellular calcium

The concentration of intracellular calcium, $[Ca^{2+}]_i$, was estimated as described previously [42], with some modifications. Confluent cells from a 175 cm^2 flask were collected in 5 ml of buffer containing 10 mM Hepes, pH 7.4, 154 mM NaCl and 0.54 mM EDTA, and then washed and resuspended in 3.5 ml of KH buffer. A 0.5 ml aliquot of this suspension was diluted to 2 ml in KH buffer to measure cellular autofluorescence. To the remainder, fura-2 acetoxymethyl ester (fura-2/AM) was added to a final concentration of 2 μM. After incubation for 45 min at room temperature, 0.5 ml aliquots of the cell suspension were centrifuged at 4000 *g* for 1 min and the cell pellet was resuspended for analysis in 1.5 ml of KH buffer at room temperature. To make the intracellular and extracellular Ca^{2+} concentrations approximately equivalent, some cells were resuspended in KH buffer which lacked added $CaCl_2$ but contained 100 μM EGTA. Routinely, 100 μM methacholine (MCh) was added and the 340/380 nm excitation ratio was recorded at 509 nm every second using a luminescence spectrometer (Perkin-Elmer). After data collection, maximal and minimal 340/380 nm ratios were recorded in the presence 2 mM $CaCl_2$/0.1% Triton X-100 and 80 mM EGTA respectively. $[Ca^{2+}]_i$ was determined using the equation described by Grynkiewicz et al. [43].

Ins(1,4,5)P_3 mass assay

Ins(1,4,5)P_3 mass was determined using a radioligand-receptor competition assay [44], with slight modifications. Briefly, incubations of cells were terminated in 300 μl of 0.5 M trichloroacetic acid and subsequently processed by addition of 75 μl of 10 mM EDTA and extraction with 600 μl of tri-n-octylamine/1,1,2-trichlorotrifluoroethane (1:1, v/v). To 200 μl of the upper aqueous phase was added 50 μl of 10 mM $NaHCO_3$ and the mixture was stored overnight at 4 °C for subsequent mass measurement of Ins(1,4,5)P_3 using 30 μl of this mixture as described [44].

Measurement of *N*-[^{3}H]methylscopolamine ([^{3}H]NMS) binding

Total mAChR number was determined by [^{3}H]NMS saturation-binding to cell membrane preparations [45]. Confluent cells from a 175 cm^2 flask were collected in buffer containing 10 mM Hepes, pH 7.4, 154 mM NaCl and 0.54 mM EDTA, resuspended in buffer A (10 mM Hepes, pH 7.4/10 mM EDTA) at 4 °C and homogenized using a Polytron tissue disrupter. The particulate fraction was obtained by centrifugation at 30000 *g* for 15 min at 4 °C and resuspended in buffer B (10 mM Hepes, pH 7.4/0.1 mM EDTA) at 4 °C using the Polytron homogenizer. The cell membrane preparation was resuspended in buffer B following centrifugation to a final concentration of 1 mg of protein/ml and stored at −80 °C. Radioligand binding was performed for 1 h at 37 °C in a final volume of 200 μl of buffer C (10 mM Hepes, pH 7.4/100 mM NaCl/10 mM $MgCl_2$) containing 20 μg of membrane protein and various concentrations of [^{3}H]NMS (0.03–3 nM). For each concentration, non-specific binding was determined in the presence of 1 μM atropine and was less than 10% of total [^{3}H]NMS binding. Bound and free ligand were separated by rapid filtration through GF/B paper (Whatman). Filters were washed in buffer C and then dried and counted by liquid-scintillation counting. Maximum binding capacity (B_{max}) and equilibrium dissociation constant (K_d) values were determined by saturation analysis using the Prism program (GraphPad Software, San Diego, CA, U.S.A.).

Western blot analysis

Analysis was performed either on cleared cell lysates or on proteins isolated from CHO-m3 cells that specifically bound to GST–c-Jun using the incubation and washing procedure described in the JNK assay section. Proteins were separated by SDS/12%-PAGE and transferred onto a nitrocellulose membrane for 30 min at 12 V in transfer buffer (48 mM Tris/HCl/39 mM glycine/1.3 mM SDS/20% methanol) using a semi-dry transfer apparatus (Bio-Rad). Membranes were blocked for 1 h at room temperature with 5% non-fat milk powder in TTBS [50 mM Tris/HCl (pH 8.0)/0.1% Tween-20/150mM NaCl] and incubated overnight at 4 °C with primary antibody [anti-ERK1 (C16: sc-93), 1:1000; anti-ERK2 (C14: sc-94), 1:1000; anti-JNK1 (C17: sc-474), 1:500; (Santa Cruz)] in TTBS. After washing in TTBS, blots were incubated for 1 h at room temperature with a 1:1000 dilution in TTBS of anti-rabbit IgG coupled to peroxidase (Sigma). Immunoblots were developed by enhanced chemiluminescence (Amersham International).

Fusion protein expression

The construct for bacterial expression of c-Jun (amino acids 1–79) fused to GST was a gift from Dr. Roger J. Davis [6]. The GST–c-Jun protein was isolated on a glutathione–Sepharose affinity matrix (Pharmacia-LKB). Purified protein was resolved by SDS/PAGE through 12% polyacrylamide and quantified by comparative Coomassie Blue R250 staining using BSA as a standard. In all other cases, protein concentrations were determined by a modified Bradford procedure [46].

Statistical analysis

The statistical differences between data sets was assessed by one-way analysis of variance for multiple comparisons, followed by Duncan's multiple-range test at $P < 0.05$ using SPSS version 6.1 software (Chicago, IL, U.S.A.).

RESULTS

Time- and concentration-dependence of agonist-stimulated ERK and JNK activities in CHO-m2 and CHO-m3 cells

CHO cells separately expressing recombinant human m2 and m3 AChRs at comparable levels of 1.5±0.1 pmol/mg and 2.2±0.2 pmol/mg (mean±S.E.M., $n = 6$) were stimulated with the muscarinic agonist MCh. ERK was isolated using an antiserum to ERK1 and assayed using a synthetic peptide substrate [41]. Stimulation of either m2 or m3 AChRs with 100 μM MCh caused dramatic ERK activation (Figure 1A). ERK activation by both m2 and m3 AChRs was observed 2 min after agonist addition and was maximal by 5 min. In CHO-m2 cells, ERK activity returned to basal by 20 min of MCh stimulation, whereas ERK activation in CHO-m3 cells was more sustained. The MCh concentration dependence for ERK activation was similar in both cell types (Figure 1B). Mean EC_{50} values for the m2- and m3-mediated responses were 4 μM MCh [log EC_{50} (M) −5.4±0.1] and 5 μM MCh [log EC_{50} (M) −5.3±0.1] respectively (mean±S.E.M., $n = 3$–9), where the M in parentheses refers to molarity.

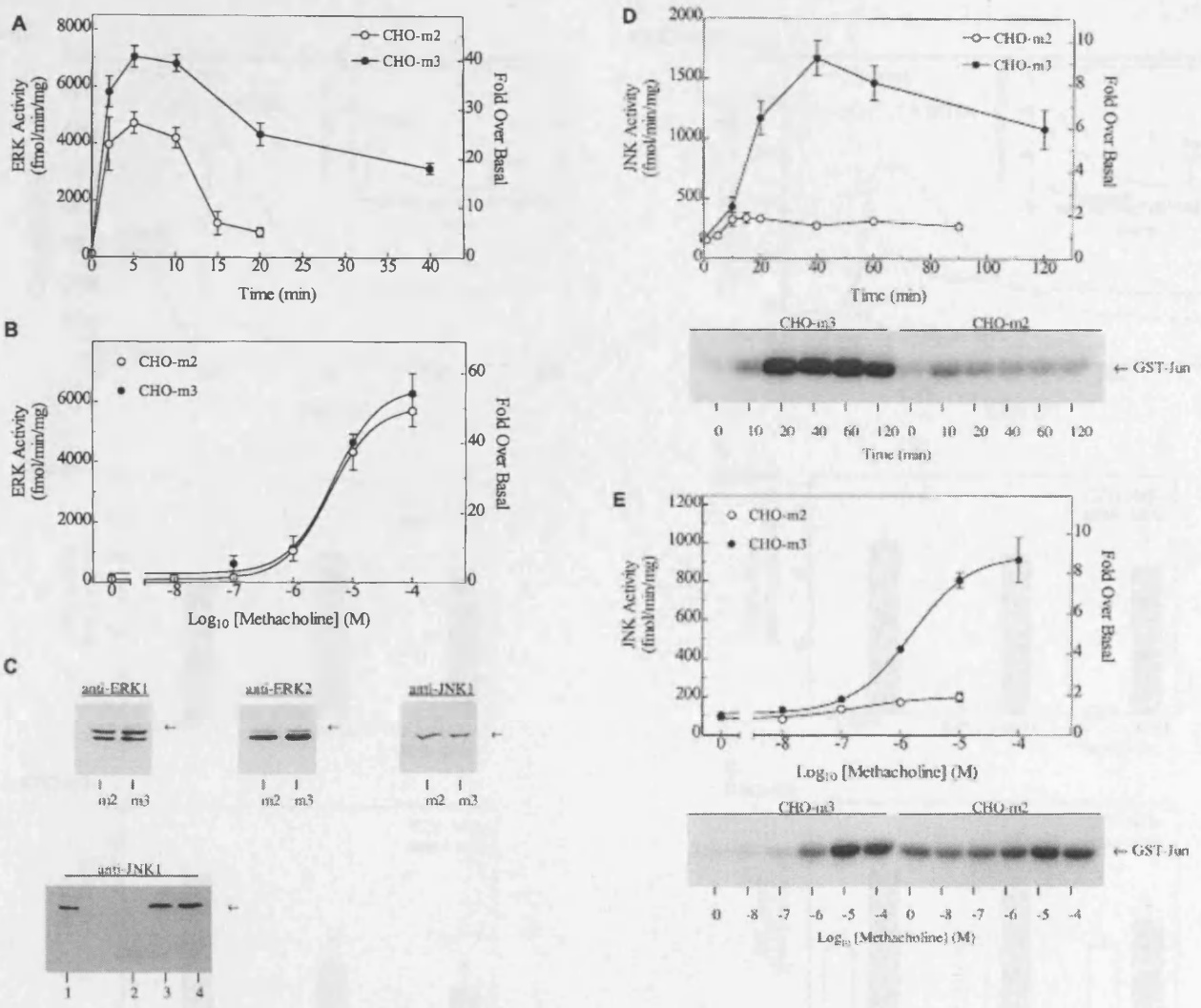

Figure 1 Time- and concentration-dependence of agonist-stimulated ERK and JNK activities in cells expressing human m2 and m3 AChRs

(**A**) CHO cells expressing either the m2 or m3 AChR were stimulated with 100 μM MCh for the times indicated. ERK activity was isolated using anti-ERK1 and assayed as described in the Materials and methods section. Basal ERK activity in CHO-m2 and CHO-m3 cells was 149 ± 38 and 186 ± 30 fmol $\cdot$ min^{-1} $\cdot$ mg^{-1} respectively. (**B**) Cells were incubated with various concentrations of MCh for 5 min and assayed for ERK activity. Basal ERK activity in CHO-m2 and CHO-m3 cells was 102 ± 35 and 187 ± 88 fmol $\cdot$ min^{-1} $\cdot$ mg^{-1}re spectively. (**C**) p44-ERK1, p42-ERK2 and JNK1 were detected in CHO-m2 (m2) and CHO-m3 (m3) lysates (10 μg of protein) by Western blot analysis using selective antisera. The lower portion of panel (**C**) shows immunoblot analysis using anti-JNK1 antiserum of GST–c-Jun affinity captured proteins isolated from lysis buffer as a control (lane 2), from unstimulated CHO-m3 cells (50 μg of protein, lane 3) and from CHO-m3 cells stimulated for 30 min with MCh (50 μg of protein, lane 4.). Immunoblot analysis was also performed on CHO-m3 cell lysate (10 μg of protein) using the same antiserum (lane 1). The relative position of the 45 kDa ovalbumin marker is indicated by an arrow. (**D**) Cells were stimulated with 100 μM MCh for the times indicated and JNK activity was assessed by GST–Jun phosphorylation. Basal JNK activity in CHO-m2 and CHO-m3 cells was 187 ± 36 and 161 ± 43 fmol $\cdot$ min^{-1} $\cdot$ mg^{-1}re spectively. (**E**) CHO-m2 and CHO-m3 cells were stimulated for either 10 min or 30 min respectively with the indicated concentrations of MCh and assayed for JNK activity. Basal JNK activity in CHO-m3 cells was 101 ± 10 fmol $\cdot$ min^{-1} $\cdot$ mg^{-1}. The lower portions of panels (**D**) and (**E**) show representative autoradiograms of GST–Jun phosphorylation. Activities represent the means $\pm$ S.E.M. for 3–7 separate determinations.

Immunoblot analysis indicated that the level of ERK proteins in the two cell types was similar (Figure 1C). The ERK1 antiserum recognized both p44 ERK1 and p42 ERK2, whereas an antiserum directed to the C-terminal sequence of ERK2 was specific for this isoenzyme (Figure 1C). Immune complex assays of kinases isolated by each antibody, either alone or in combination, suggested that the ERK1 antiserum measured both ERK1 and ERK2 activities, whereas the ERK2 antiserum was more selective (results not shown). Thus, results obtained using the ERK1 antiserum are likely to represent the combined activity of both ERK1 and ERK2.

JNK1, JNK2 and JNK3 are indistinguishable based on their ability to bind and phosphorylate the N-terminal domain of c-Jun [6]. Therefore, JNK activity was measured using a recombinant fragment of c-Jun (amino acids 1–79) fused to GST as an affinity ligand and substrate. Immunoblot analysis using an antiserum to JNK1 showed the presence of similar amounts of the 46 kDa enzyme in CHO-m2 and CHO-m3 cells (Figure 1C). This analysis also showed that the affinity ligand, GST–c-Jun, bound equal quantities of the 46 kDa JNK1 protein from unstimulated and MCh-stimulated CHO-m3 cells (Figure 1C). As shown in Figure 1(D), MCh induced a marked and sustained JNK activation in CHO-m3 cells that was delayed in onset relative to ERK activation (Figure 1A). In contrast, the m2 receptor failed to activate JNK greatly, giving a maximal stimulation of 2-fold. JNK was activated in CHO-m3 cells with

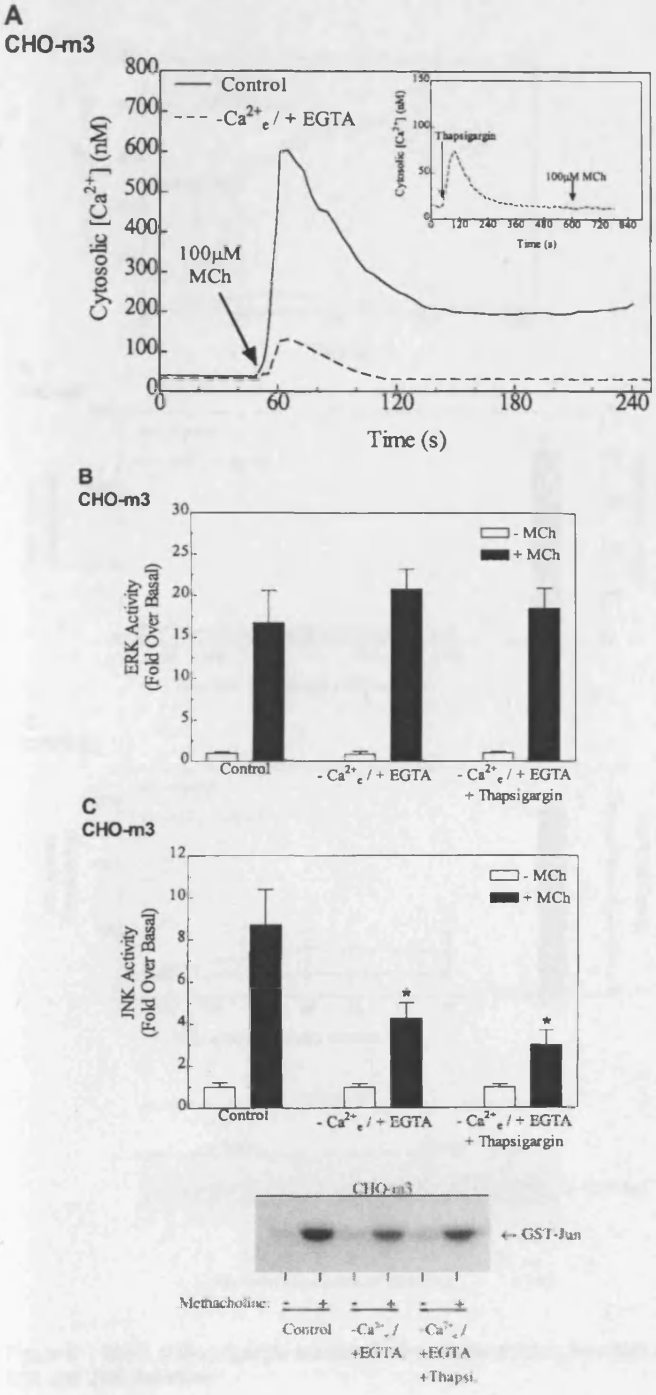

Figure 2 Calcium-dependence of agonist-stimulated ERK and JNK activities in CHO-m3 cells

(**A**) Cytosolic Ca^{2+} concentration was measured in fura-2/AM-loaded CHO-m3 cells stimulated with 100 μM MCh in the presence of 1.3 mM $CaCl_2$ (control, solid line) or under calcium-depleted conditions [i.e. $-Ca^{2+}_e$/ + EGTA (100 μM) broken lines]. The inset shows the effect of 2 μM thapsigargin under calcium-depleted conditions. (**B**) and (**C**) CHO-m3 cells were prepared in the presence of 1.3 mM $CaCl_2$(control) or under conditions to prevent Ca^{2+} entry [$-Ca^{2+}_e$/ + EGTA (100 μM)] and intracellular Ca^{2+} release [i.e. $-Ca^{2+}_e$/ + EGTA (100 μM) + thapsigargin (2 μM)]. ERK and JNK activities were assayed at 5 min and 30 min respectively after addition of 100 μM MCh (solid bars) or carrier (open bars). Non-stimulated activities for each condition were assigned a fold activity of 1. A representative autoradiogram of JNK activity is shown below (**C**). Results are means ± S.E.M. of 3–6 separate determinations. $^*P < 0.05$ by Duncan's multiple-range test.

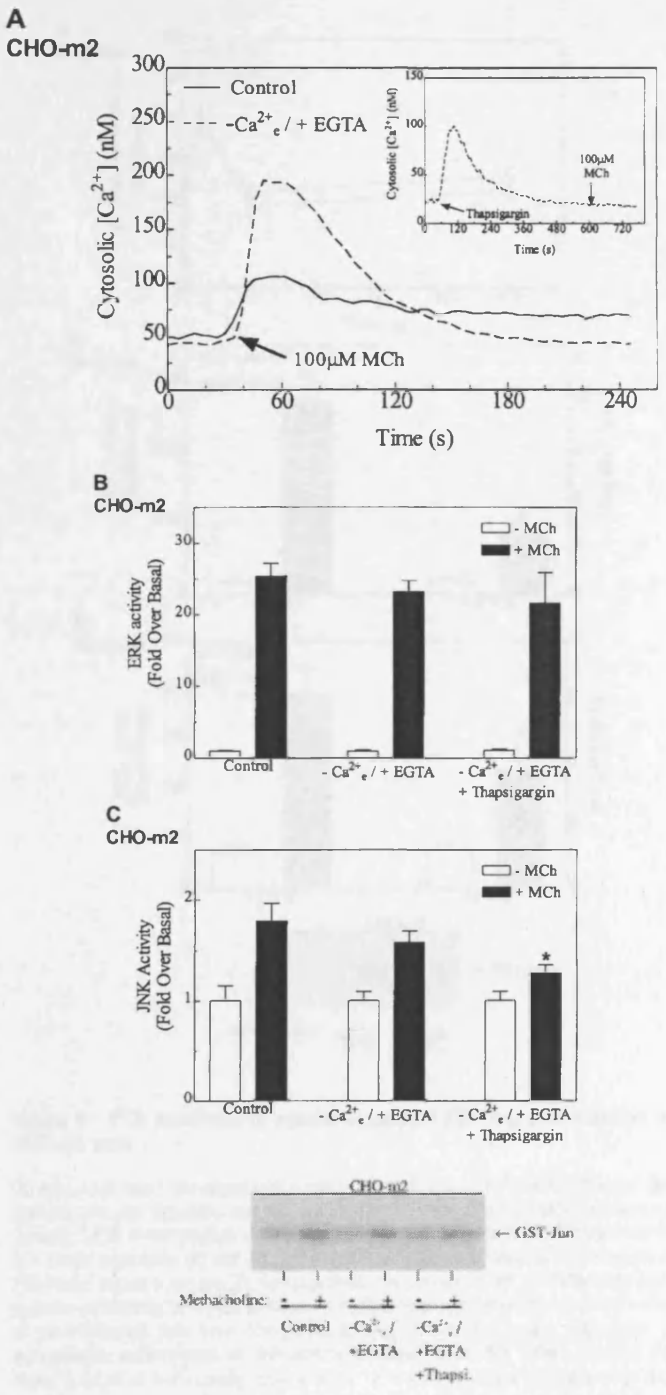

Figure 3 Calcium-dependence of agonist-stimulated ERK and JNK activities in CHO-m2 cells

(**A**) Fura-2/AM-loaded CHO-m2 cells were analysed for agonist-induced intracellular Ca^{2+} elevation as described in the legend to Figure 2. (**B**, **C**) MCh-induced ERK and JNK activities in CHO-m2 cells were measured under the conditions described in the legend to Figure 2, except that JNK activity was measured at 10 min after addition of 100 μM MCh (solid bars) or carrier (open bars). A representative autoradiogram of JNK activity is shown below (**C**). Results are means ± S.E.M. of 4–8 separate determinations. $^*P < 0.05$ by Duncan's multiple-range test.

an EC_{50} value of 2 μM MCh [log EC_{50} (M) -5.8 ± 0.2; mean ± S.E.M., $n = 3$–9] (Figure 1E). An equivalent EC_{50} value could not be determined in CHO-m2 cells. MCh-stimulated ERK and JNK activities in both cell types were abolished by the antagonist

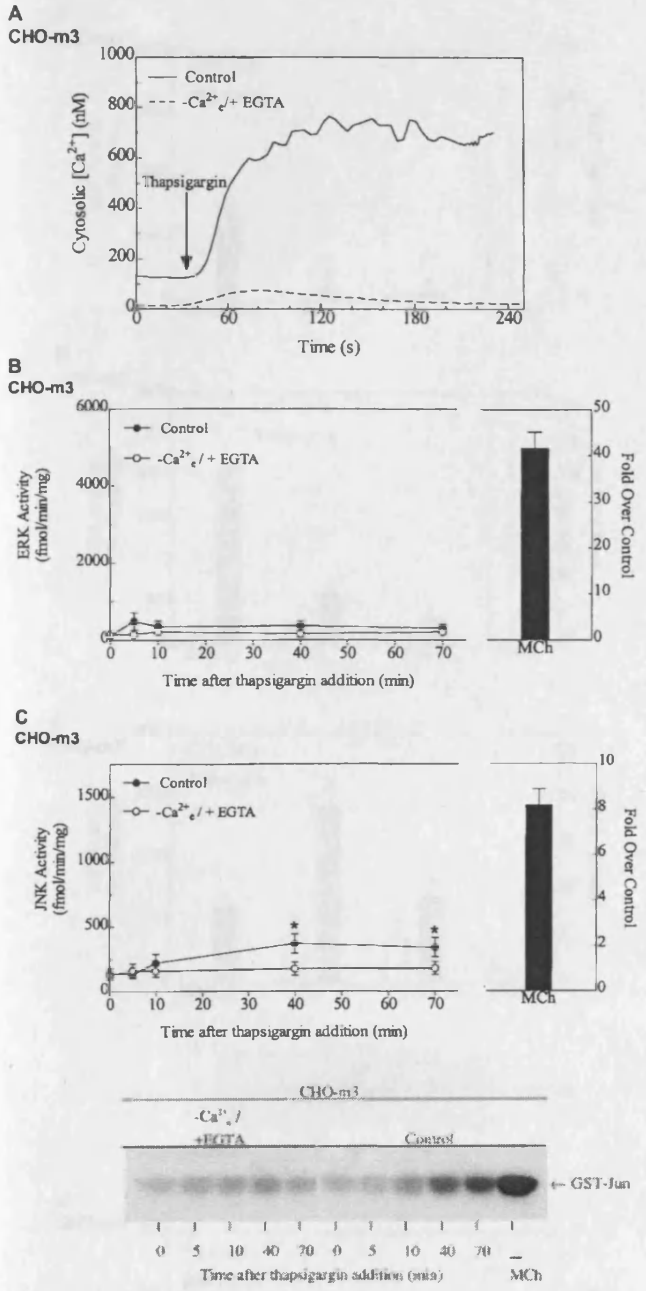

Figure 4 Effect of thapsigargin-mediated intracellular calcium elevation on ERK and JNK activities

(**A**) The effect of 2 μM thapsigargin on $[Ca^{2+}]_i$ was measured in fura-2/AM-loaded CHO-m3 cells in the presence of 1.3 mM $CaCl_2$ (control, solid line) or under Ca^{2+}-depleted conditions [i.e. $-Ca^{2+}_e/+$EGTA (100 μM), broken line]. (**B**) and (**C**) ERK and JNK activities were measured in the absence (○) and presence (●) of 1.3 mM $CaCl_2$, after addition of 2 μM thapsigargin. ERK and JNK activities were also determined after 5 and 30 min of 100 μM MCh stimulation in the presence of 1.3 mM $CaCl_2$(sol id bars). An autoradiogram of JNK activity is shown below (**C**). Results are means of four separate determinations. *Indicates JNK activation (●) at $P < 0.05$ by Duncan's multiple-range test.

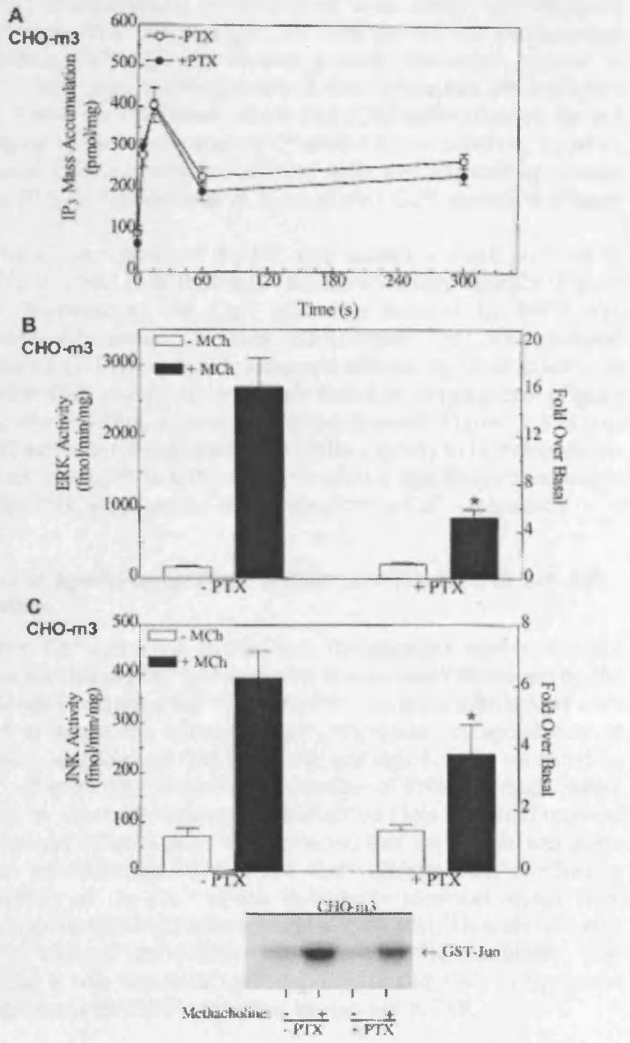

Figure 5 PTX sensitivity of agonist-stimulated ERK and JNK activities in CHO-m3 cells

(**A**) Ins(1,4,5)P_3mas s was determined in either untreated (○) or PTX-treated (75 ng/ml, ●) CHO-m3 cells after stimulation with 100 μM MCh for the times indicated. Values represent the means ± S.E.M. of four separate assay points and were essentially identical with those obtained in a similar experiment. (**B**) and (**C**) ERK and JNK activities were measured in untreated and PTX-treated cells at 5 min and 30 min respectively after addition of 100 μM MCh (white bars) or carrier (solid bars). *$P < 0.05$ by Duncan's multiple-range test. Basal ERK and JNK activities in non-PTX-treated cells were 159 ± 34 and 81 ± 39 fmol·min^{-1}·mg^{-1} respectively. A representative autoradiogram of JNK activity is shown below (**C**). Values represent the means ± S.E.M. of 4–9 separate determinations. *$P < 0.05$ by Duncan's multiple-range test.

atropine (results not shown), confirming that the responses were muscarinic receptor-mediated. Immunoblot analysis also showed that the amounts of ERK and JNK were unchanged by MCh (results not shown).

Calcium dependence of agonist-stimulated ERK and JNK activities in CHO-m3 and CHO-m2 cells

Since m1, m3 and m5 AChRs are Ca^{2+}-mobilizing receptors coupled to PLC [37], we determined the contribution of Ca^{2+} to ERK and JNK activation in CHO-m3 cells. $[Ca^{2+}]_i$ was measured using the Ca^{2+}-sensitive dye fura-2 and, for comparative purposes, CHO-m2 cells were analysed similarly. MCh (100 μM) induced a rapid elevation of $[Ca^{2+}]_i$ in CHO-m3 cells that was maximal within 10 s of agonist addition (Figure 2A). Removal of the Ca^{2+} gradient across the plasma membrane using 100 μM EGTA in the extracellular buffer indicated that Ca^{2+} influx contributed the major component to the agonist-induced rise in

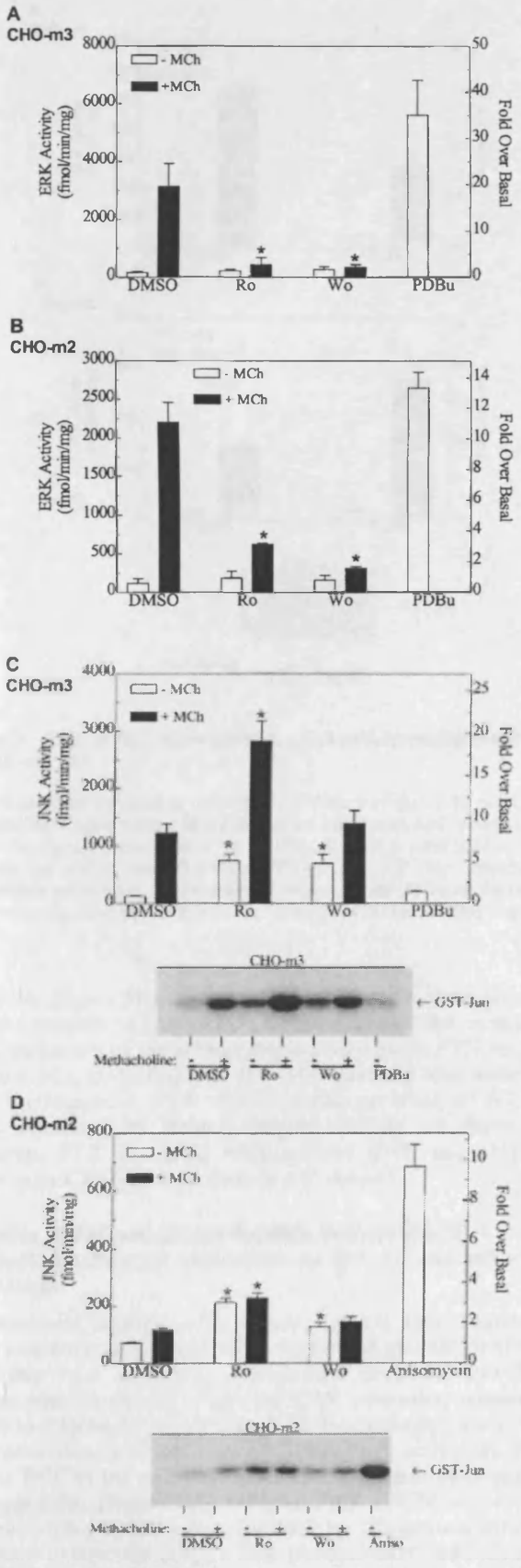

Figure 6 Role of PKC in agonist-stimulated ERK and JNK activities in CHO-m2 and CHO-m3 cells

Cells were pre-treated for 10 min with 10 μM Ro-31-8220 (Ro), 10 μM wortmannin (Wo) or the carrier DMSO before agonist addition. (**A**) and (**B**) ERK activity was measured in CHO-m3 and CHO-m2 at 5 min after addition of 100 μM MCh (white bars) or carrier (solid bars). Cells were separately treated for 10 min with 1 μM PDBu. Basal ERK activities in CHO-m3 and CHO-m2 cells were 157 ± 80 and 118 ± 106 fmol·min^{-1}·mg^{-1} respectively. (**C**) and (**D**) JNK activity was measured in CHO-m3 and CHO-m2 at 30 min and 10 min respectively after addition of 100 μM MCh (white bars) or carrier (solid bars). Cells were separately treated for 10 min with 1 μM PDBu (**C**) or for 15 min with 50 ng/ml anisomycin (**D**). Basal JNK activities in CHO-m3 and CHO-m2 cells were 148 ± 30 and 71 ± 6 fmol·min^{-1}·mg^{-1}. Representative autoradiograms of JNK activity are shown (**C** and **D**). Values represent the means $\pm$ S.E.M. of 3–9 separate experiments. *Indicates significant effects of Ro-31-8220 or wortmannin on basal and agonist-stimulated activities at $P < 0.05$ by Duncan's multiple-range test.

$[Ca^{2+}]_i$. Furthermore, treatment of cells under Ca^{2+}-depleted conditions with thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, caused a small transient increase in $[Ca^{2+}]_i$ but, importantly, abolished that induced by MCh (Figure 2A). These manipulations show that ERK activation by the m3 receptor is Ca^{2+}-independent (Figure 2B). In contrast, agonist-induced JNK activity in CHO-m3 cells was reduced by greater than 50% in the absence of intracellular Ca^{2+} elevation (Figure 2C).

The G_i -coupled m2 AChR also caused a small increase in $[Ca^{2+}]_i$ in CHO cells that was abolished by thapsigargin (Figure 3A). Surprisingly, the Ca^{2+} elevation induced by MCh was reproducibly enhanced when extracellular Ca^{2+} was chelated (Figure 3A). ERK activation was not affected by these conditions or when Ca^{2+} mobilization was abolished by thapsigargin (Figure 3B), whereas JNK activation was attenuated (Figure 3C). Thus, ERK activation by m2 and m3 AChRs appears to be independent of Ca^{2+} elevation in CHO cells, whereas a significant component of the JNK response to these receptors is Ca^{2+}-dependent.

Effect of agonist-independent calcium elevation on ERK and JNK activities

Under Ca^{2+}-depleted conditions, thapsigargin causes a small transient rise in $[Ca^{2+}]_i$ that can be dramatically enhanced by the presence of external Ca^{2+} (Figure 4A). These manipulations were used to assess the effect of $[Ca^{2+}]_i$ elevation, independently of agonist, and showed that ERK was not significantly activated by Ca^{2+} (Figure 4B). However, activation of JNK by thapsigargin could be observed, although the effect was less than that induced by agonist (Figure 4C). Furthermore, this activation was abolished by depletion of external Ca^{2+} (Figure 4C), confirming specificity of the Ca^{2+} effect. Essentially identical results were obtained in CHO-m2 cells (results not shown). Thus elevation of $[Ca^{2+}]_i$ alone is not sufficient to activate JNK maximally, supporting a role for both Ca^{2+}-dependent and Ca^{2+}-independent components in JNK activation by the m3 AChR.

PTX sensitivity of mAChR-mediated responses

PTX catalyses the ADP-ribosylation of α subunits of G_i and G_o, uncoupling them from activated receptors [47]. Although the m1 AChR can be considered a prototypic GPCR linked to PTX-insensitive G_q-proteins, ERK activation by this receptor may be mediated by G_o in CHO cells [48]. In CHO-m3 cells, MCh stimulated a rapid Ins(1,4,5)P_3 accumulation which was maximal at 15 s and which was unaffected by PTX (Figure 5A). Similarly, the EC_{50} for MCh-induced Ins(1,4,5)P_3 accumulation was unaffected by PTX [log EC_{50} (M) -5.6 ± 0.2 and -6.0 ± 0.1 in the absence and presence of PTX; results not shown]. Thus coupling of the m3-AChR to PLC probably involves G_q. In contrast, a large component of ERK activation by the m3 AChR was PTX-

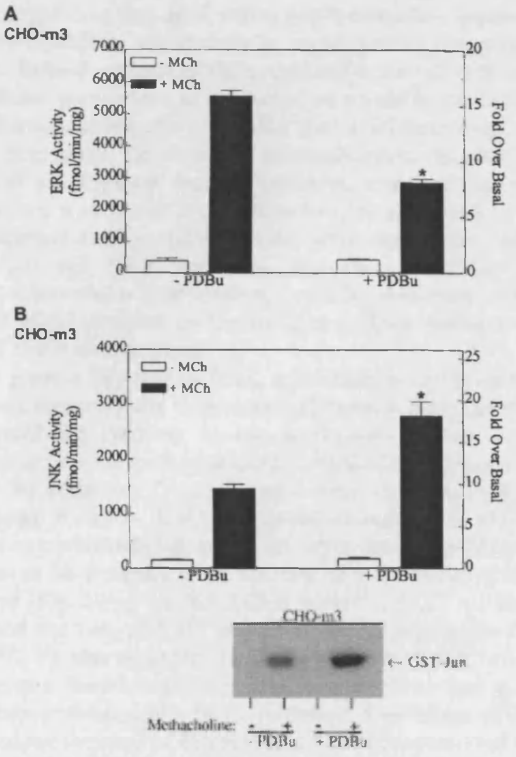

Figure 7 Effect of PKC down-regulation on m3 receptor-mediated activation of ERK and JNK

CHO-m3 cells were pre-treated for 18 h with 1 μM PDBu (+PDBu) or the carrier DMSO (−PDBu) before agonist addition. (**A**) and (**B**) ERK and JNK activities were measured at 5 min and 30 min respectively after addition of 100 μM MCh (white bars) or carrier (solid bars). Basal ERK and JNK activities were 385 ± 108 and 171 ± 55 fmol·min^{-1}·mg^{-1} respectively. A representative autoradiogram of JNK activity is shown in below (**B**). Values represent the means ± S.E.M. of 3–5 separate experiments. *$P < 0.05$ by Duncan's multiple-range test.

sensitive (Figure 5B), indicating that distinct G-proteins couple the m3 receptor to Ins(1,4,5)P_3 formation and ERK activation. JNK activation by the m3 receptor was also partly PTX-sensitive (Figure 5C), indicating that the JNK pathway also involves a G_i/G_o component. PTX treatment had no effect on ERK or JNK expression by Western blotting (results not shown). As expected, PTX abolished MCh-induced ERK and JNK activation in CHO-m2 cells (results not shown).

Inhibition of PKC and phosphoinositide 3-kinase (PI-3K) demonstrates divergent mechanisms for ERK and JNK activation by mAChRs

In agreement with previous reports [2,14,15], PKC stimulation with phorbol ester activates ERK (Figure 6A and 6B). Inhibition of either PKC by 10 μM Ro-31-8220 or PI-3K by 10 μM wortmannin essentially abolished ERK activation induced by MCh in CHO-m3 cells (Figure 6A). Interestingly, Ro-31-8220 also dramatically reduced m2-mediated ERK activation, implicating PKC in the pathway by which PTX-sensitive G-proteins activate ERK (Figure 6B). Whereas JNK can be activated by cellular stress, such as that induced by the protein synthesis inhibitor anisomycin (Figure 6D), phorbol ester had little effect on JNK (Figure 6C), which is consistent with previous work [5]. Furthermore, elevation of $[Ca^{2+}]_i$ by thapsigargin concurrent with activation of PKC by phorbol dibutyrate (PDBu) had less than an additive effect on JNK activity (results not shown). Experiments to determine the involvement of PKC and PI-3K in activation of JNK by mAChRs were confused by the marked stimulatory effects Ro-31-8220 and wortmannin had on basal JNK activity (Figures 6C and 6D). However, PI-3K inhibition had no effect on the level of JNK activity in the presence of MCh in either CHO-m3 (Figure 6C) or CHO-m2 (Figure 6D) cells, whereas a marked enhancement of JNK activity by Ro-31-8220 was apparent in CHO-m3 cells stimulated with MCh (Figure 6C). As the stimulatory effect on JNK of Ro-31-8220 may not be mediated by PKC inhibition, CHO-m3 cells were alternatively treated for 18 h with 1 μM PDBu to down-regulate PKC. Under these conditions, ERK activation induced by MCh was significantly attenuated, confirming a stimulatory role for PKC in this pathway (Figure 7A). In contrast, JNK activation was enhanced by MCh after chronic PDBu treatment (Figure 7B), further suggesting that PKC is a negative regulator of the JNK pathway.

DISCUSSION

Evidence to date points to considerable heterogeneity in the mechanisms by which MAPK signalling pathways are regulated by GPCRs [7]. This heterogeneity can be attributed in part to the nature of the heterotrimeric G-proteins to which individual receptors couple, and also to cell-type-specific differences in the complement of intracellular molecules that participate in the signalling cascades. The current study has used CHO cells as a model to examine the regulation of two distinct subfamilies of MAPK by recombinant human m2 and m3 AChRs that preferentially couple to inhibition of adenylyl cyclase and activation of PLC respectively [37]. We found that the Ca^{2+}-mobilizing m3 AChR is efficiently coupled to both ERK and JNK activation in CHO cells, whereas the m2 AChR activates ERK to a comparable level yet fails to activate JNK significantly. The EC_{50} values for MCh-induced ERK activation in CHO-m2 and CHO-m3 cells were essentially identical (4 μM and 5 μM respectively), and similar to that for JNK activation in CHO-m3 cells (2 μM). Agonist displacement studies of NMS binding in membranes derived from CHO-m2 and CHO-m3 cells have provided agonist occupation curves with K_D values of 1 μM and 40 μM carbachol respectively [40]. In addition, the EC_{50} values for carbachol-stimulated early- and late-phase Ins(1,4,5)P_3 production in intact CHO-m3 cells are approx. 5 μM carbachol [42], whereas this agonist is more potent at elevating intracellular Ca^{2+} by at least one order of magnitude [42]. These observations suggest that the potential for second-messenger-mediated amplification of ERK or JNK activities may be limited by additional factors, such as the stoichiometry of intracellular proteins that are required for activation of these pathways.

We also find that JNK activation by the m3 AChR is delayed in onset and more sustained relative to ERK activation induced by either the m3 or m2 AChR subtype. In Rat1 cells, the m1 AChR activates JNK with a similar time-course as described here, although in these cells ERK is not activated [13,24]. However, in NIH3T3 cells where m1 AChR activates both JNK and ERK, a similar temporal pattern of rapid ERK activation with delayed JNK activation has been reported [23]. Although the G_i-coupled m2 AChR can cause a modest (3-fold) activation of JNK in Rat1 [24] and COS-7 [25] cells, receptors that cause significant JNK activation are primarily coupled to PtdIns(4,5)P_2 hydrolysis, including angiotensin II [26], α_1 adrenergic [27], thrombin [28] and endothelin-1 [28,29] receptors, and induce JNK activity with a similar time-course to that observed here [23–27]. Interestingly, maximal activation of JNK mediated by the m3 receptor requires constant receptor occupation by agonist (P. G. Wylie, R. A. J. Challiss and J. L. Blank, unpublished

work), suggesting that JNK activation is not solely dependent on an early signalling event such as rapid second messenger generation. Indeed maximal JNK activation occurred at times when a significant proportion of m3 receptors would be predicted to be desensitized, raising the possibility that JNK activation may be partly dependent on receptor internalization. In this regard, Daaka et al. [49] have recently provided evidence that receptor endocytosis is required for ERK activation mediated by the β_2-adrenoceptor and lysophosphatidic acid receptor in HEK 293 cells. Although ERK and JNK activation pathways involve distinct intracellular components, it will be of interest to examine the role of m3 receptor internalization in JNK activation in the light of these observations.

Ca^{2+} plays a key role in ERK activation in neuronal cells via pathways requiring the monomeric G-protein Ras, and mechanisms involving tyrosine kinase activation and activation of guanine nucleotide exchange factors for Ras have been proposed [50,51]. In addition, T-lymphocytes have an apparently unique mechanism to allow Ca^{2+}-dependent activation of JNK when PKC is co-activated [52], and JNK activation in B-lymphocytes appears to be dependent on the size and duration of the Ca^{2+} transient [53]. Since the m3 AChR activates PLC, we have also examined the role of Ca^{2+} and PKC in the regulation or ERK and JNK by this receptor. In CHO-m3 cells, MCh can release Ca^{2+} from a thapsigargin-sensitive internal store and activate a large extracellular Ca^{2+} entry pathway. Depletion of internal Ca^{2+} and/or removal of extracellular Ca^{2+} demonstrated that m3 AChR-mediated JNK activation involved both a Ca^{2+}-dependent and a Ca^{2+}-independent component, whereas muscarinic receptor activation of ERK did not require Ca^{2+}. Similar manipulations that should abolish Ca^{2+} mobilization by angiotensin II in GN4 rat liver epithelial cells have also provided evidence for a Ca^{2+}-dependent and a Ca^{2+}-independent mechanism of JNK activation [26], whereas JNK activation by the m1 and m2 AChR has been reported to be entirely Ca^{2+}-dependent in Rat 1 cells [24]. Our observation that increasing $[Ca^{2+}]_i$ independently of agonist can cause significant JNK activation, yet negligible ERK activation, relative to that induced by agonist, supports a differential role for Ca^{2+} in activation of these two MAPK pathways by the m3 AChR. In COS-7 cells, the m1 and m2 AChRs stimulate JNK through G-protein $\beta\gamma$ subunits acting via Ras and Rac1 [25], and such a mechanism may account for the Ca^{2+}-independent component of the m2- and m3-mediated signal in CHO cells. Activating mutants of α_q have also been reported to activate JNK in COS-1 [30] and PC-12 [31] cells, presumably by stimulating β isoenzymes of PLC with consequent elevation of $[Ca^{2+}]_i$. The Ca^{2+}-activated protein tyrosine kinase Pyk2 can be activated by GPCRs for bradykinin, lysophosphatidic acid and carbachol in PC12 cells [22], and by angiotensin II in GN4 cells [54], and can mediate ERK [22] and JNK [32] activation. We have yet to establish whether activation of JNK in CHO-m3 cells requires a Ca^{2+}-dependent tyrosine kinase or whether a Ca^{2+}/calmodulin-dependent protein kinase may be involved [55].

As demonstrated here, agonist-stimulated Ins(1,4,5)P_3 formation in CHO-m3 cells is resistant to PTX, consistent with G_q/PLC-β involvement, whereas a significant component of ERK and JNK response was toxin sensitive. Measurements of agonist-stimulated guanosine 5′-[γ-thio]triphosphate (GTP[S]) binding in CHO-m1 and CHO-m3 cells membranes following PTX treatment has indicated that these G_q-coupled receptors are also linked to G_i and/or G_o[4 0]. Interestingly, $G_o$is not widely expressed outside the nervous system [56] but is present in CHO cells where it has been implicated in coupling the m1 AChR to ERK activation [48]. These observations indicate that both the m1 and m3 receptor can couple to at least two distinct classes of G-protein to mediate activation of separate but overlapping signalling pathways.

As evidence exists for the involvement of PI-3K in ERK activation by GPCRs [57], we examined the effect of the PI-3K inhibitor wortmannin on muscarinic regulation of ERK and JNK. Our results suggest that ERK activation by m2 and m3 AChRs is dependent on the activity of PI-3K, which is consistent with the notion that specific isoforms of PI-3K link G-protein $\beta\gamma$ subunits to activation of the ERK pathway [57]. Very recently, PI-3K has also been proposed to mediate JNK activation by the m2 AChR in COS-7 cells [58] and can apparently form a complex with JNK in γ-irradiated U937 myeloid leukaemia cells [59]. Our studies examining PI-3K involvement in JNK activation by muscarinic receptors were inconclusive due to the marked stimulatory effects of wortmannin on JNK as reported previously [59].

Finally, we have also examined the involvement of PKC in muscarinic activation of the ERK and JNK pathways by using the PKC inhibitor Ro-31-8220 or by prolonged phorbol ester treatment to deplete the protein. These experiments indicate that ERK activation by the m3 AChR is mainly mediated via a PKC-dependent pathway and are consistent with work demonstrating that PKC activation by phorbol ester or overexpression of the novel or conventional isoforms of PKC is sufficient alone to activate ERK [2,14,15]. Surprisingly, we also found that ERK activation by the m2 AChR required PKC, despite operating exclusively through a PTX-sensitive pathway. Our observation that MCh could increase intracellular Ca^{2+} in CHO-m2 cells is consistent with PLC activation being mediated by $\beta\gamma$ subunits derived from PTX-sensitive G-proteins, thereby providing a mechanism to account for this PKC-dependence. In support of our observations, the PTX-sensitive G_o protein has been shown to couple the m1 AChR and platelet-activating factor receptor to ERK activation via PKC in CHO cells [48].

In NIH3T3 cells, JNK activation by the m1 AChR does not require PKC [23]. However, we find that PKC inhibition by Ro-31-8220 or by chronic phorbol ester treatment results in enhanced JNK activity in CHO-m3 cells stimulated with MCh, suggesting that PKC contributes an inhibitory component to this pathway. In CHO-m3 cells that were pretreated with PDBu for 5 min to activate PKC, stimulation of JNK by MCh was attenuated by approx. 50% (P. G. Wylie, R. A. J. Challiss and J. L. Blank, unpublished work), whick is consistent with an inhibitory effect of PKC on JNK. In accord with our observations, other studies have shown that PKC inhibition can potentiate JNK activation by endothelin-1 in Rat 1 fibroblasts [60] and angiotensin II in GN4 epithelial cells [26]. Although Ro-31-8220 may also have an effect on JNK activity that is independent of PKC inhibition [61], these results suggest that PKC plays opposing roles in the regulation of JNK and ERK by m3 AChRs in CHO cells.

Biochemical and molecular biological studies have identified several intracellular protein kinase cascades that provide the potential for parallel regulation of the ERK, JNK and p38 subgroups of MAPKs in response to extracellular stimuli and various forms of cellular stress [1–4,7]. Whereas the role of PKC and Ca^{2+} in ERK regulation via pathways involving Ras and the Raf family of MAPKKKs have been examined extensively, the upstream elements involved in JNK and p38 regulation by GPCRs remain to be determined. Recent evidence has established that Rac1 and Cdc42, two members of the Rho family of monomeric G-proteins, are key intracellular regulators of JNK and p38 [3,4,7]. In addition, a family of MAPK/ERK-activating kinase kinases (MEKKs) have been identified as MAPKKKs that preferentially regulate JNK and p38 [2–4,7]. This distinguishes MEKKs from Raf, which activates ERK but not JNK

or p38 [4]. Importantly, Rho family G-proteins have also been implicated in muscarinic receptor signalling [7,25,62] and in the regulation of MEKKs [63,64] and functionally related MAPKKKs [3,4]. Future studies will examine the involvement of MEKKs and of Rho family G-proteins in JNK regulation by muscarinic receptors in CHO cells and address the mechanisms by which Ca^{2+} and PKC may influence this pathway.

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REFERENCES

1 Davis, R. J. (1993) J. Biol. Chem. **268**, 14553–14556
2 Cobb, M. H. and Goldsmith, E. J. (1995) J. Biol. Chem. **270**, 14843–14846
3 Kyriakis, J. M. and Avruch, J. (1996) J. Biol. Chem. **271**, 24313–24316
4 Minden, A. and Karin, M. (1997) Biochim. Biophys. Acta **1333**, F85–F104
5 Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J. and Woodgett, J. R. (1994) Nature (London) **369**, 156–160
6 Gupta, S., Barrett, T., Whitmarsh, A. J., Cavanagh, J., Sluss, H. K., Dérijard, B. and Davis, R. J. (1996) EMBO J. **15**, 2760–2770
7 Gutkind, J. S. (1998) J. Biol. Chem. **273**, 1839–1842
8 Crespo, P., Xu, N., Simonds, W. F. and Gutkind, J. S. (1994) Nature (London) **369**, 418–420
9 Hawes, B. E., van Biesen, T., Koch, W. J., Luttrell, L. M. and Lefkowitz, R. J. (1995) J. Biol. Chem. **270**, 17148–17153
10 Koch, W. J., Hawes, B. E., Allen, L. F. and Lefkowitz, R. J. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 12706–12710
11 Faure, M., Voyno-Yasenetskaya, T. A. and Bourne, H. R. (1994) J. Biol. Chem. **269**, 7851–7854
12 Gupta, S. K., Gallego, C., Johnson, G. L. and Heasley, L. E. (1992) J. Biol. Chem. **267**, 7987–7990
13 Winitz, S., Russell, M., Qian, N.-X., Gardner, A., Dwyer, L. and Johnson, G. L. (1993) J. Biol. Chem. **268**, 19196–19199
14 Schonwasser, D. C., Marais, R. M., Marshall, C. J. and Parker, P. J. (1998) Mol. Cell. Biol. **18**, 790–798
15 Marais, R., Light, Y., Mason, C., Paterson, H., Olson, M. F. and Marshall, C. J. (1998) Science **280**, 109–112
16 De Vivo, M., Chen, J., Codina, J. and Iyengar, R. (1992) J. Biol. Chem. **267**, 18263–18266
17 Kalinec, G., Nazarali, A. J., Hermouet, S., Xu, N. and Gutkind, J. S. (1992) Mol. Cell. Biol. **12**, 4687–4693
18 Della Rocca, G. J., van Biesen, T., Daaka, Y., Luttrell, D. K., Luttrell, L. M. and Lefkowitz, R. J. (1997) J. Biol. Chem. **272**, 19125–19132
19 Mattingly, R. R. and Macara, I. G. (1996) Nature (London) **382**, 268–272
20 van Biesen, T., Hawes, B. E., Luttrell, D. K., Krueger, K. M., Touhara, K., Porfiri, E., Sakaue, M., Luttrell, L. M. and Lefkowitz, R. J. (1995) Nature (London) **376**, 781–784
21 Luttrell, L. M., Della Rocca, G. J., van Biesen, T., Luttrell, D. K. and Lefkowitz, R. J. (1997) J. Biol. Chem. **272**, 4637–4644
22 Dikic, I., Tokiwa, G., Lev, Courtneidge, S. A. and Schlessinger, J. (1996) Nature (London) **383**, 547–550
23 Coso, O. A., Chiariello, M., Kalinec, G., Kyriakis, J. M., Woodgett, J. and Gutkind, J. S. (1995) J. Biol. Chem. **270**, 5620–5624
24 Mitchell, F. M., Russell, M. and Johnson, G. L. (1995) Biochem. J. **309**, 381–384
25 Coso, O. A., Teramoto, H., Simonds, W. F. and Gutkind, J. S. (1996) J. Biol. Chem. **271**, 3963–3966
26 Zohn, I. E., Yu, H., Li, X., Cox, A. D. and Earp, H. S. (1995) Mol. Cell. Biol. **15**, 6160–6168
27 Ramirez, M. T., Sah, V. P., Zhao, X.-L., Hunter, J. J., Chien, K. R. and Brown, J. H. (1997) J. Biol. Chem. **272**, 14057–14061
28 Shapiro, P. S., Evans, J. N., Davis, R. J. and Posada, J. A. (1996) J. Biol. Chem. **271**, 5750–5754
29 Bogoyevitch, M. A., Ketterman, A. and Sugden, P. H. (1995) J. Biol. Chem. **270**, 29710–29717
30 Prasad, M. V. V. S. V., Dermott, J. M., Heasley, L. E., Johnson, G. L. and Dhanasekaran, N. (1995) J. Biol. Chem. **270**, 18655–18659
31 Heasley, L. E., Storey, B., Fanger, G. R., Butterfield, L., Zamarripa, J., Blumberg, D. and Maue, R. A. (1996) Mol. Cell. Biol. **16**, 648–656
32 Tokiwa, G., Dikic, I., Lev, S. and Schlessinger, J. (1996) Science **273**, 792–794
33 Mitsui, H., Takuwa, N., Kurokawa, K., Exton, J. H. and Takuwa, Y. (1997) J. Biol. Chem. **272**, 4904–4910
34 Voyno-Yasenetskaya, T. A., Faure, M. P., Ahn, N. G. and Bourne, H. R. (1996) J. Biol. Chem. **271**, 21081–21087
35 Collins, L. R., Minden, A., Karin, M. and Brown, J. H. (1996) J. Biol. Chem. **271**, 17349–17353
36 Aragay, A. M., Collins, L. R., Post, G. R., Watson, A. J., Feramisco, J. R., Brown, J. H. and Simon, M. I. (1995) J. Biol. Chem. **270**, 20073–20077
37 Caulfield, M. P. (1993) Pharmacol. Ther. **58**, 319–379
38 Berridge, M. J. (1993) Nature (London) **361**, 315–325
39 Newton, A. C. (1995) J. Biol. Chem. **270**, 28495–28498
40 Burford, N. T., Tobin, A. B. and Nahorski, S. R. (1995) Eur. J. Pharmacol. **289**, 343–351
41 Gardner, A. M., Lange-Carter, C. A., Vaillancourt, R. R. and Johnson, G. L. (1994) Methods Enzymol. **238**, 258–270
42 Tobin, A. B., Willars, G. B., Burford, N. T. and Nahorski, S. R. (1995) Br. J. Pharmacol. **116**, 1723–1728
43 Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985) J. Biol. Chem. **260**, 3440–3450
44 Challiss, R. A. J., Batty, I. H. and Nahorski, S. R. (1988) Biochem. Biophys. Res. Commun. **157**, 684–691
45 Lambert, D. G., Ghataorre, A.S and Nahorski, S. R. (1989) Eur. J. Pharmacol. **165**, 71–77
46 Stoscheck, C. M. (1990) Methods Enzymol. **182**, 62–63
47 Simon, M. I., Strathmann, M. P. and Gautam, N. (1991) Science **252**, 802–808
48 van Biesen, T., Hawes, B. E., Raymond, J. R., Luttrell, L. M., Koch, W. J. and Lefkowitz, R. J. (1996) J. Biol. Chem. **271**, 1266–1269
49 Daaka, Y., Luttrell, L. M., Ahn, S., Della Rocca, G. J., Ferguson, S. G., Caron, M. G. and Lefkowitz, R. J. (1998) J. Biol. Chem. **273**, 685–688
50 Finkbeiner, S. and Greenberg, M. E. (1996) Neuron **16**, 233–236
51 Ebinu, J. O., Bottorff, D. A., Chan, E. Y. W., Stang, S. L., Dunn, R. J. and Stone, J. C. (1998) Science **280**, 1082–1086
52 Su, B., Jacinto, E., Hibi, M., Kallunki, T., Karin, M. and Ben-Neriah, Y. (1994) Cell **77**, 727–736
53 Dolmetsch, R. E., Lewis, R. S., Goodnow, C. C. and Healy, J. I. (1997) Nature (London) **386**, 855–858
54 Yu, H., Li, X., Marchetto, G. S., Dy, R., Hunter, D., Calvo, B., Dawson, T. L., Wilm, M., Anderegg, R. J., Graves, L. M. and Earp, H. S. (1996) J. Bol. Chem. **271**, 29993–29998
55 Enslen, H., Tokumitsu, H., Stork, P. J. S., Davis, R. J. and Soderling, T. R. (1996) Proc. Natl. Acad. Sci. U.S.A. **93**, 10803–10808
56 Strathmann, M., Wilkie, T. M. and Simon, M. I. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 6477–6481
57 Lopez-liasaca, M., Crespo, P., Pellici, P. G., Gutkind, J. S. and Wetzker, R. (1997) Science **275**, 394–397
58 Lopez-liasaca, M., Gutkind, J. S. and Wetzker, R. (1998) J. Biol. Chem. **273**, 2505–2508
59 Kharbanda, S., Saleem, A., Shafman, T., Emoto, Y., Taneja, N., Rubin, E., Weichselbaum, R., Woodgett, J., Avruch, J., Kyriakis, J. and Kufe, D. (1995) J. Biol. Chem. **270**, 18871–18874
60 Cadwallader, K., Beltman, J., McCormick, F. and Cook, S. (1997) Biochem. J. **321**, 795–804
61 Beltman, J., McCormick, F. and Cook, S. J. (1996) J. Biol. Chem. **271**, 27018–27024
62 Mitchell, R., McCulloch, D., Lutz, E., Johnson, M., MacKenzie, C., Fennell, M., Fink, G., Zhou, W. and Sealfon, S. C. (1998) Nature (London) **392**, 411–414
63 Gerwins, P., Blank, J. L. and Johnson, G. L. (1997) J. Biol. Chem. **272**, 8288–8295
64 Fanger, G. R., Lassignal, N. and Johnson, G. L. (1997) EMBO J. **16**, 4961–4972

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