Cell Growth Regulation by Muscarinic Acetylcholine Receptors

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ABSTRACT

The growth response of Chinese hamster ovary (CHO) cells to activation of recombinantly expressed G protein-coupled muscarinic M₂ or M₃ acetylcholine (ACh) receptors has been assessed. Activation of these receptors leads to divergent growth responses: M₂ ACh receptor activation causes an increase in DNA synthesis, whereas M₃ ACh receptor activation causes a dramatic inhibition of DNA synthesis.

The M₃ ACh receptor-mediated growth inhibition has been characterised, and shown to comprise a G₁-phase cell cycle arrest, involving an increase in p21Cip1/Waf1 protein expression. Further, a receptor-mediated increase in p21Cip1/Waf1 association with cyclin-dependent kinase 2 (CDK2) leads to a decrease in CDK2 activity and an accumulation of hypophosphorylated retinoblastoma protein (pRb). The increase in p21Cip1/Waf1 expression is due at least in part to an increase in p21Cip1/Waf1 mRNA although no receptor-mediated change in candidate transcription factor activities could be detected.

Extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) activation profiles suggested two alternative hypotheses to account for the receptor-mediated growth arrest. However, pharmacological and biochemical data demonstrate that ERK, JNK and p38 MAPK are not involved in the growth regulation, whilst inhibition of PKC partially ablates the growth arrest. Data demonstrate that both M₃ ACh receptor-mediated ERK and JNK activities may be dependent on liberated G-protein βγ subunits, whilst the growth arrest is not perturbed by βγ subunit sequestration. Data presented reveal a MAPK-independent mechanism of growth regulation that may involve coupling of the M₃ ACh receptor to heterotrimeric G-protein families other than Gq/11.

Work presented in this thesis appears in the following publication:

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<td>ACh</td>
<td>Acetylcholine</td>
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<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PIP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>RGS</td>
<td>Regulator of G-protein signalling</td>
</tr>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Inositol 1,4,5-trisphosphate</td>
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<td>DAG</td>
<td>sn-1,2-diacylglycerol</td>
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<td>PKC</td>
<td>Protein kinase C</td>
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<td>Protein kinase A</td>
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<td>PI</td>
<td>Phosphatidyl inositol</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>ATF-2</td>
<td>Activating transcription factor-2</td>
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<td>SRE</td>
<td>Serum-response element</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
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<td>JNK</td>
<td>c-jun N-terminal kinase</td>
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<tr>
<td>BMK</td>
<td>Big MAPK (ERK5)</td>
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<td>MKK</td>
<td>MAPK kinase</td>
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<td>M KK</td>
<td>MAPK kinase kinase</td>
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<td>MEK</td>
<td>MAPK/ERK kinase</td>
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<td>MAPK/ERK kinase kinase</td>
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<td>MP-1</td>
<td>MEK partner-1</td>
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<td>Ksr</td>
<td>Kinase-suppressor of ras</td>
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<td>JIP&lt;sub&gt;1&lt;/sub&gt;</td>
<td>JNK-interacting protein 1</td>
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<td>MLK</td>
<td>Mixed-lineage kinase</td>
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<td>Grb2</td>
<td>Growth-factor receptor binding protein 2</td>
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<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<td>Sos1</td>
<td>Son-of-sevenless</td>
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<td>LPA</td>
<td>Lysophosphatidic acid</td>
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<td>FAK</td>
<td>Focal adhesion kinase</td>
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<td>CHO</td>
<td>Chinese hamster ovary</td>
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<td>Retinoblastoma protein</td>
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<td>Cyclin-dependent kinase inhibitor</td>
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<td>NLS</td>
<td>Nuclear localisation sequence</td>
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<td>PCNA</td>
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<td>PKB</td>
<td>Protein kinase B</td>
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<td>PTX</td>
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<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
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<td>GST</td>
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<td>TCA</td>
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<td>Fluorescence-activated cell sorting</td>
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<td>Polymerase chain reaction</td>
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<td>Carboxymido-triazole</td>
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<tr>
<td>PDBu</td>
<td>Phorbol-12,13-dibutyrate</td>
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<td>MKP</td>
<td>MAPK phosphatase</td>
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Chapter 1

Introduction
1.1. Muscarinic ACh Receptors

The muscarinic acetylcholine (ACh) receptors are members of the family of G protein-coupled receptors (GPCRs) that are defined by their heptahelical structure and membrane topology (Fig. 1.1). The receptors possess an extracellular amino-terminal region, which for some GPCRs can be the site of agonist/hormone binding, and an intracellular carboxy-terminal region. The highest degree of sequence homology exists within the seven-transmembrane domains of the GPCR family (Wess, 1998; Bockaert and Pinn, 1999). GPCRs elicit their cellular effects primarily by coupling through heterotrimeric G-proteins (Simon et al., 1991).

![Diagram of M2 and M3 ACh receptors and their coupling to G-proteins and second messenger pathways]
Heterotrimeric G-proteins consist of three distinct subunits, namely α, β, and γ. There are many distinct genes encoding each subunit, resulting in approximately 20 known α subunits, 5 β subunits and 13 γ subunits (Simon et al., 1991; Hamm, 1998). These have been subdivided into four main classes of G-protein, namely Gαs, Gαo, Gq/11 and G12/13, all of which elicit distinct effects on second messenger pathways. G-proteins transduce signals received by the cell surface receptor to intracellular effectors, usually enzymes or ion channels. Functionally, heterotrimeric G-proteins act as two subunits, the α subunit, and the βγ subunit. The α subunit contains the guanine nucleotide-binding domain and intrinsic GTPase activity. In the inactive state, the α and βγ subunits associate with each other and the plasma membrane (through post-translational lipid modifications to α and γ subunits).

Agonist binding to the GPCR catalyses GTP for GDP exchange on the α subunit, which results in its dissociation from the βγ subunits enabling α-GTP and/or βγ subunits to interact with effectors located within the cell (Clapham and Neer, 1997). Temporally, the signal persists until GTP is hydrolysed to produce Ga-GDP, which rapidly re-sequesters a free βγ subunit. The intrinsic GTPase activity of the α subunit, effector GAP activity and/or interactions with regulators of G-protein signalling (RGS) proteins determine the rate of this hydrolysis (Ross and Wilkie, 2000).

It has recently been appreciated that GPCRs can activate signalling pathways independently of heterotrimeric G-proteins (Hall et al., 1999). For example, a number of GPCRs including the M3 ACh receptor have been shown to activate phospholipase D (PLD) independently of heterotrimeric G-protein signalling (Mitchell et al., 1998). PLD activation appears to occur by direct receptor activation of the small GTPases ARF and RhoA. This phenomenon increases the number of potential effector pathways activated by GPCRs.

The mammalian muscarinic ACh receptor family is encoded by 5 genes (Bonner et al., 1987; Caulfield and Birdsall, 1998). The five members are divided into two sub-families based on their effector coupling. The M1, M3, and M5 ACh receptors preferentially couple to pertussus toxin-insensitive G-proteins of the Gq/11 family (Caulfield and Birdsall, 1998). Activation of this family of
G-proteins leads to activation of PLC, resulting in the increased generation of inositol 1,4,5-trisphosphate (IP$_3$) and sn-1,2-diacylglycerol (DAG) (Rhee, 2001). Increases of IP$_3$ and DAG cause a rise in intracellular Ca$^{2+}$ and activation of certain ('classical' and 'novel') isoforms of protein kinase C (PKC) respectively (Fig. 1.1). The liberated $G_\text{q}$ $\beta$\gamma subunits can also act on effectors and can activate the $\beta$\gamma-sensitive forms of PLC as well as other effector proteins such as isoforms of phosphatidylinositol 3-kinase (PI3-K) (Stephens et al., 1997; Krugmann et al., 2002).

The $M_2$ and $M_4$ ACh receptors couple via the pertussis toxin-sensitive $G_i$ family of G-proteins to modulate a number of cellular activities including the inhibition of adenylyl cyclase (AC) activity, resulting in the lowering of cyclic AMP levels. This reduction of cAMP may impede protein kinase A (PKA) function (Fig. 1.1). Like other G-protein families, liberated $\beta$\gamma subunits from $G_i$ proteins can act on effectors. For example $G_i$-liberated $\beta$\gamma subunits can activate the $\beta$\gamma subunit-sensitive forms of PLC (Rhee et al., 2001).

Muscarinic ACh receptors are expressed in a wide variety of mammalian cells and mediate effects in the parasympathetic nervous system as well as in non-neuronal tissues (Wessler et al., 1998). The parasympathetic system is primarily concerned with 'functions of energy conservation in periods of minimal activity', and many of the physiological effects mediated by muscarinic ACh receptors reflect this function. Muscarinic effects range from mediating contraction of the iris and ciliary muscles of the eye to lowering heart rate and contractility of the atria. Dilation of arterioles and lowering of blood pressure as well as the contraction of airways smooth muscle are effects mediated by muscarinic receptor stimulation. Muscarinic activation also increases motility and tone of the stomach and intestine as well as having effects on the gallbladder, male sexual organs, exocrine glands, the bladder and the central nervous system (Berstein and Haga, 1990; Eglen et al., 1996).

The $M_2$ and $M_3$ ACh receptors are differentially expressed throughout the mammalian system. The $M_2$ ACh receptor is expressed in smooth muscle, and in the medulla oblongata and pons regions of the
The \( M_2 \) ACh receptor is the only muscarinic subtype that has been identified in the heart, being expressed in the atrium. Like the \( M_2 \) ACh receptor, the \( M_3 \) ACh receptor is expressed in smooth muscle. The \( M_3 \) ACh receptor is also expressed in exocrine glands such as the pancreas and salivary glands, as well as the cerebral cortex of the brain.

It is clear that activation of the \( M_2 \) and \( M_3 \) ACh receptors leads to regulation of distinct signalling pathways in distinct glands and organs and thus, although they are members of the same family of GPCRs, their activation leads to distinct physiological effects (Eglen et al., 1996; Caulfield and Birdsall, 1998).

1.2. Mitogen-Activated Protein Kinases (MAPKs)

The Mitogen-activated protein kinases (MAPKs) are a family of ubiquitously expressed proline-directed serine/threonine kinases that are involved in the transduction of intracellular signalling pathways. They are activated by a number of external stimuli, as diverse as growth factors, hormones, and cellular stresses, and stimulate diverse cellular effects such as differentiation, proliferation and apoptosis by acting upon numerous transcription factors (e.g. ATF-2, c-jun, ELK-1, SRF and p53), as well as other kinases (e.g. MAPKAP-K2, S6 kinase), enzymes (e.g. phospholipase A\(_2\)) and upstream regulators (e.g. EGF receptor) (Garrington and Johnson, 1999).

The MAPKs can be grouped into subfamilies on the basis of sequence similarity, their activation by cellular stimuli and their sensitivity to activation by upstream dual specificity kinases. There are twelve members of the MAPK family to date, which can be grouped into five main families. Of these, three families constitute the main areas of research interest in the field of MAPK signal transduction to date. These are extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38 MAPK. Five isoforms of ERK have been identified, although a splice variant of ERK1 has recently been discovered (ERK1b) (Yung et al., 2000). Three isoforms of JNK have been identified, and four p38 isoforms have been reported (Garrington and Johnson, 1999). ERK5 (Big MAPK (BMK1))
represents another family of MAPKs that has shown to be regulated differentially than ERK and JNK sub-families (Fukuhara et al., 2000).

The cascade of events leading to MAPK activation is characterised by a three-protein kinase cascade often referred to as the MAPK module (Fig. 1.2). The module consists of the MAPK, a MAPK kinase (MKK) and a MAPK kinase kinase (MKKK), and is found in all eukaryotic cells. The MAPKs must be phosphorylated on two residues, to become fully activated. This is achieved by dual specificity kinases (MKKs), which are capable of phosphorylating both tyrosine and threonine residues on the MAPK proteins. The most defined MAPK module is the \(\text{c-raf-1} / \text{MEK1/2} / \text{ERK1/2}\) pathway. In this system \(\text{c-raf-1}\) is a serine/threonine protein kinase, functioning as the MKKK that specifically phosphorylates the MKKs, MEK (MAPK/ERK kinase) 1/2. MEK1/2 can then phosphorylate and activate ERK1/2 on, for example tyrosine 185 and threonine 183 of ERK2, enabling the MAPK to phosphorylate its substrates.

There are fourteen MKKK proteins of which \(\text{raf}\) was the first to be identified in mammalian cells. The MKK\(\text{s}\) contain proline-rich domains for SH2 domain binding, GTPase binding sites, leucine zipper domains, and phosphorylation sites for tyrosine and serine/threonine kinases. There are therefore potentially a number of ways of activating the MKKKs. In terms of substrate binding, the MKKKs show little specificity for the MKKs that they activate. For example MAPK/ERK kinase kinase 1 (MEKK1) was initially discovered as an activator of the ERK pathway, but has more recently been shown to strongly activate JNK. MEKK2/3 will activate ERK, JNK, p38 and ERK5 although MEKK2 may be selective for ERK and MEKK3 is selective for JNK. The exception to this rule is \(\text{raf}\), which through phosphorylation of MEK1/2 only leads to ERK activation (reviewed in Hagemann and Blank, 2001).

The MKKs are cytoplasmic proteins of which seven have been reported. They can enter the nucleus but contain a nuclear export sequence, which quickly removes them back into the cytoplasm.
Figure 1.2. The MAPK ‘Module’. Schematic representation of the MAPK cascades showing the general MAPK module (left hand panel), and some key proteins that are involved in the ERK, JNK, and p38 MAPK cascades. Stimuli are shown for the three classes of MAPK as well as some biological responses.

Specificity of the MAPK module is mainly determined at this level. The MKKs show great specificity for MAPK molecules. MEK1/2 will only activate ERK1/2. MEK1/2 contain proline rich inserts in their carboxy-terminal domains, which are not found in any other MKKs. Deletion of this region has been shown to prevent c-raf-1 binding, and therefore elicits a certain level of specificity. MKK3/6 specifically activate p38 isoforms, and MKK4/7 are specific to JNK activation. Less is known about MKK5, but it is involved in ERK5 activation (reviewed in Robinson and Cobb, 1997), and may be directly regulated by MEKK3 (Chao et al., 1999).
Another level of specificity in MAPK signalling is conferred by protein scaffolds capable of interacting with multiple members of a signalling cascade (Burack and Shaw, 2000). MP-1 (MEK-partner-1) performs this scaffold function in the ERK pathway by interacting directly with MEK1 and ERK1. A second ERK scaffold, kinase-suppressor of ras (Ksr) has also been described. Direct interactions of Ksr with c-raf-1, MEK1/2 and ERK2, as well as the adapter protein 14-3-3, allows organised progression of activation in this ERK signalling cascade. Scaffolding proteins also regulate the JNK pathway. JNK-interacting protein 1 (JIP1) specifically interacts with mixed lineage kinase 3 (MLK3), M KK7 and JNK encompassing the entire JNK activation module. JIP1 does not interact with MEKK1, 3 or 4 or M KK4 demonstrating that JIP1 can confer specificity to a single MAPK module. MEKK1 can perform a scaffolding function for either the ERK or JNK signalling cascades. Interactions of MEKK1 with JNK and M KK4/7 as well as with MEK1 and ERK2 demonstrate that even a member of the core MAPK module can regulate the cascade in a way other than simple phosphorylation of its substrate (reviewed in Karandikar and Cobb, 1999).

1.3. GPCR Activation of ERK

Activation of the ERK pathway is well established down-stream of growth factor/tyrosine kinase receptors. Receptor activation leads to dimerisation and autophosphorylation of an intrinsic tyrosine kinase domain, which then becomes a docking region for a variety of target proteins containing SH2 domains. One such protein is the growth factor receptor binding protein 2 (Grb2), which acts as an adapter protein, recruiting the guanine nucleotide exchange factor (GEF), son-of-sevenless (Sos1). The small GTPase ras binds to Sos1 resulting in ras activation through GDP-GTP exchange. Interaction of activated ras with c-raf-1 leads to raf activation and progression of the MAPK phosphorylation cascade.

Activation of ERK via GPCRs is a more complicated story. Over the last ten years or so, many groups have addressed the question of GPCR-mediated ERK activation. Experiments have been performed in many different cell lines, and many GPCRs have been investigated. The general impression is that
ERK activation is dependent on both receptor and cell type, although some common themes can be found.

Having described the differences in effector coupling between $G_i$ and $G_q$ proteins it is not surprising that the potential pathways leading to ERK activation from these $G$-proteins are not the same. Over-expression of constitutively active mutants of $G\alpha_2$ or $G\alpha_q$ in COS-7 cells lead to inhibition of adenylyl cyclase and increased PI turnover respectively, but did not induce ERK activity (Crespo et al., 1994). G-protein $\beta\gamma$ subunit-sequestration using $G\alpha_i$ (a-transducin) blocked the M$_2$ and M$_3$ ACh receptor-mediated ERK activation, demonstrating that both $G_i$- and $G_q$-mediated ERK activation involved $\beta\gamma$ subunits. Over-expression of a dominant-negative ras construct (N17-ras) showed that the ERK activation was dependent on ras function (Crespo et al., 1994). The activation of ERK by $G_i$- and $G_q$-protein generated $\beta\gamma$ subunits is a theme that is reported throughout the literature. The involvement of G-protein $\alpha$ subunits appears to be more variable. Experiments using transiently transfected COS-7 cells, and Rat-1 fibroblasts expressing endogenous $G_i$-coupled receptors, demonstrated that $G_i$-mediated ERK and ras activation was blocked by expression of a $\beta\gamma$ subunit antagonist (carboxyl terminal region of $\beta$ARK) (Koch et al., 1994). $\beta\gamma$ subunit antagonism had no effect on either $G_q$-mediated or receptor tyrosine kinase-mediated ERK activation, indicating that the $G_q$-mediated ERK activation occurred through $G\alpha_q$ and not $\beta\gamma$ subunit liberation. This report also showed that $G_i$-mediated PI turnover and inhibition of adenylyl cyclase activity was not affected by $\beta\gamma$ sequestration providing evidence for two independent $G_i$ pathways being activated.

These reports generate two important questions. How do $\beta\gamma$ subunits cause ERK activation, and how does $G_q$ cause ERK activation through both $\alpha$ and $\beta\gamma$ subunits?

In apparent contradiction with the work of Crespo et al. (1994), constitutively active $G\alpha_q$, over-expression of PLC$\beta$, and phorbol esters (PKC activators), all caused ERK activation in COS-7 cells (Faure et al., 1994), suggesting that $G_q$ activates MAPK via $G\alpha$ subunits, an increase in PI turnover,
and PKC activation. One possibility is that PLC activation and a subsequent increase in DAG, results in PKC activation. PKC can directly activate c-raf-1 (Kolch et al., 1993), thus creating a potential entry point from GPCR stimulation to ERK activation. This is an example of a pathway that could mediate ras-independent activation of ERK. Data also demonstrated that expression of constitutively active Go4 did not result in increased ERK activity, although expression of β and γ isoforms did elicit a MAPK response, again revealing that Gj-mediated ERK activation occurred through βγ subunits. One possibility is that liberated βγ subunits could activate βγ-sensitive PLCβ isoforms, which would then stimulate the DAG/PKC/c-raf-1 pathway.

Other examples of ras-independent ERK activation have been reported. Takahashi et al. (1997) used vascular smooth muscle cells (VSMCs) to show that angiotensin 2 (ANG 2)-mediated ras activation occurred through a pertussis toxin-sensitive pathway, that could be blocked by protein tyrosine-kinase inhibitors (genistein, or herbimycin A) or a PI3-K inhibitor (wortmannin). Neither of these inhibitors had an effect on ANG 2-mediated ERK activity, which was pertussis toxin-insensitive, and ras-independent. As both raf and MEK are activated in VSCM by ANG-2 it is likely that the Gq-mediated ras-independent ERK activity was occurring through a similar PKC-dependent pathway as described previously (Faure et al., 1994).

These data, and other reports provide a possible method of βγ-mediated ras activation. The βγ subunit could interact, directly or indirectly with a non-receptor tyrosine kinase, potentially of the c-src family, that would allow entry into the receptor tyrosine-kinase pathway of ERK activation.

Lysophosphatidic acid (LPA) induced MAPK activity was Gi-mediated and sensitive to dominant negative forms of ras, raf, and mSos (Luttrell et al., 1996). ERK activation was mediated by βγ subunits and LPA stimulation lead to 'transient recruitment of tyrosine kinase activity into Shc complexes'. Immunoblotting of Shc immunoprecipitates revealed an LPA-mediated increase in Shc-c-src association, but not of other c-src kinase family members, Lyn or Yes. The c-src inhibitor, Csk (c-
src kinase), which inactivates c-src by phosphorylating the regulatory C-terminal tyrosine residue, inhibited LPA induction of Shc activity. These data lead to a potential pathway of βγ-mediated ERK activation. Liberated βγ subunits activate and cause autophosphorylation of c-src, which proceeds to phosphorylate Shc. This phosphorylation localises Shc to the membrane, and creates a docking site for Grb2. The Grb2-Sos complex can then recruit and activate ras, which in turn will activate c-raf-1 and allow progression of the MAPK module.

A Gαq-mediated ERK pathway has been reported in HEK293 cells that was dependent on ras, and possibly involved PKC (Della Rocca et al., 1997). A βγ subunit-mediated Gαi pathway was also described that was dependent on ras, but biochemically separable from its PI and AC effector activities. Data showed that both βγ and Gαq induced MAPK responses were sensitive to PLC inhibition, down regulation of ras, raf, and Sos, dominant negative c-src, calcium chelation, inhibition of protein tyrosine-kinases, chemical inhibition of calmodulin, and dominant-negative Pyk2. These data give rise to a more complete ERK activation pathway, and suggest that Gαq and βγ subunits converge at the level of PLC. Pyk2 is a member of the focal adhesion kinase (FAK) protein tyrosine kinase family, and interacts directly with c-src. After LPA or bradykinin stimulation of PC12 cells, tyrosine phosphorylation of Pyk2 at tyrosine 402 allowed docking of c-src via its SH2 domain (Dikic et al., 1996). Pyk2 activation provides a possible regulatory point, upstream of c-src. Once PLC is activated either by Gαq, or by Gγ-liberated βγ subunits, the increase in intracellular calcium would cause calmodulin binding and Pyk2 activation, leading to association with c-src and progression through the Shc/Grb2-Sos/ras/raf/MEK/ERK pathway.

Blaukat et al. (1999) developed this idea further and showed that PLC-generated calcium increases (via calmodulin) or PKC activation caused autophosphorylation of Pyk2 on tyrosine 402. Activated c-src, which could then phosphorylate Shc leading to pathway progression. Activated c-src can also phosphorylate a second tyrosine residue (881) on Pyk2, which further promoted Pyk2 activity. This site could also act as a docking region for Grb2, again resulting in pathway progression.
It is clear that GPCR-mediated activation of c-src can result in ERK activation. Reports have indicated that receptor tyrosine-kinases can also contribute to GPCR-mediated ERK activity. Activation of the $G_{i}$-coupled $\beta_{2}$-adrenoceptor in COS-7 cells caused EGF receptor dimerisation, tyrosine phosphorylation, and formation of a multi-receptor complex containing the $\beta_{2}$-adrenoceptor and the EGF receptor (Maudsley et al., 2000). Selective inhibition of EGF receptor and c-src function perturbed the GPCR-mediated ERK activation, demonstrating their importance in the ERK activation cascade downstream of this GPCR. $\alpha_{2a}$-adrenoceptor stimulation caused ERK activation in COS-7 cells by two distinct pathways (Pierce et al., 2001). One pathway was dependent on EGF receptor transactivation, whereas the second pathway was independent of EGF receptor function.

Interpreting all of these data presents a complex signal transduction story (Fig. 1.3). It appears to be dependent both on cell type and receptor class. In some systems ras appears to be involved, and in some systems it does not. PKC is important in feeding into the ERK pathway in selected cell systems, and the involvement of receptor and non-receptor tyrosine-kinases in ERK activation also appears significant downstream of some GPCRs. It seems certain that elucidation of other regulatory mechanisms such as scaffolding, compartmentalisation, as well as other kinases and phosphatases, will create a clearer picture of this scheme of signal transduction. Indeed, there are numerous other proteins that have been implicated in GPCR-mediated ERK activation. Experiments in COS-7 cells demonstrated the importance of an isoform of PI3-K (PI3-Kγ) in ERK activation (Lopez-Illasaca et al., 1997). This isoform, like some other PI3-K isoforms, can be activated by G-protein $\beta_{Y}$ subunits and therefore has various potential feed-in routes to ERK activation. PI3-Kγ was proposed to cause c-src activation, and progression through the standard pathway. PI3-Kγ has been shown to mediate ras-independent ERK activation downstream of a $G_{i}$-coupled receptor in Chinese hamster ovary (CHO) cells that was also dependent on the atypical PKC isoform, PKC$\zeta$ (Takeda et al., 1999). Activation of a $G_{q11}$ receptor in a human colon carcinoma cell line (SW-480) resulted in ERK activation that was dependent on PI3-K$\beta$ activating PKCe, implicating another PI3-K isoform in ERK activation (Graneß et al., 1998).
Figure 1.3. Experimentally determined methods of ERK activation by G_q and G_j G-proteins. The figure emphasises both Ca^{2+}-dependent and Ca^{2+}-independent mechanisms for ERK activation, and also highlights the involvement of PKC and PI3-K.
The JNK pathway was originally characterised as a stress-activated pathway. Protein synthesis inhibitors, heat shock, and changes in osmolarity, as well as cytokines such as TNFα and IL-1 all lead to activation of the JNK pathway (reviewed in Kyriakis et al., 1996; Ichijo, 1999). The actual mechanisms underlying JNK activation have proven difficult to characterise, and even now there is speculation about the pathways. Experiments in a number of cell lines demonstrated the involvement of two small GTPases, rac1 and cdc42, both members of the Rho family of small GTPases, in JNK activation mediated by growth-factor receptors or v-src (Minden et al., 1995). Experiments also demonstrated the involvement of ras in JNK activation downstream of growth-factor stimulation although cytokine-induced JNK activity was independent of ras function. Expression of a dominant-negative rac1 construct attenuated the JNK response by growth factors, v-src, and ras. Co-expression of ras and rac1 led to a greater JNK activation compared to that obtained from expression of either rac1 or ras individually, suggesting that both of these monomeric G-proteins could be involved in the growth factor-induced activation of JNK. Similar findings were reported in COS-7 cells (Coso et al., 1995a). Constitutively activated ras led to a poor JNK response, but a strong ERK response, whereas constitutively activated rac1 or cdc42 produced a robust JNK response, with no significant ERK activation. These experiments demonstrated that ras alone would elicit only a small JNK response, but the rho family of monomeric G-proteins coupled effectively to the JNK pathway. Rho family inhibitor proteins were used to block the JNK response demonstrating the selectivity of the rho family GTPases for JNK activation (Coso et al., 1995a).

It has been reported that the serine/threonine protein kinase p21-activated kinase (PAK) can lead to JNK activation. Over-expression of PAK-3 or cdc42 caused an increase in JNK activity in COS-1 cells (Bagrodia et al., 1995). Data also showed that over-expression of activated cdc42 caused an increase in recombinant PAK-3 activity suggesting that PAK-3 was a downstream target of cdc42. Another report showed that rac1 and cdc42 can specifically bind to and activate PAK demonstrating a direct functional interaction between these proteins (Manser et al., 1994). These data suggest a pathway...
linking rac1 and cdc42 to JNK through activation of an intermediate protein kinase, PAK. However co-expression of PAK with rac/cdc42 in COS-7 cells diminished rather than enhanced JNK activity (Teramoto et al., 1996). Data implicated an alternative protein, mixed-lineage kinase 3 (MLK3) that when over-expressed caused an increase in JNK activity without affecting ERK or p38 activation. MLK3 was shown to bind to rac1 and cdc42 in vivo, an association that enhanced MLK3's ability to phosphorylate MBP. A kinase-deficient form of MLK3 inhibited the active rac1- and cdc42-mediated JNK activity, but had no effect on MEKK-mediated JNK activation, suggesting that MLK3 is acting downstream of rac1 and cdc42, and independently of MEKK. A kinase-deficient from of MKK4, the upstream kinase of JNK inhibited both MLK3- and MEKK-mediated JNK activation supporting the role of MLK3 acting upstream of the classical JNK signalling cascade (Teramoto et al., 1996).

More recently it has been shown that GPCRs can cause JNK activation independently of the ERK pathway. Direct G-protein activation using aluminium fluoride increased JNK activity independently of Raf-1 activation (Shapiro et al., 1996). Stimulation of the M1 ACh receptor stably expressed in NIH 3T3 cells led to an increase in c-jun phosphorylation and activating protein-1 (AP-1) transcriptional activity that was not detected after PDGF receptor-stimulation of the cells (Coso et al., 1995b). These effects were shown to be a consequence of M1 ACh receptor-mediated JNK activation, which was temporally distinct from both carbachol (CCh)- and PDGF-stimulated ERK activities. Activation of transiently transfected M1 or M2 ACh receptors induced JNK activity in COS-7 cells that was dependent on G protein-liberated βγ subunits (Coso et al., 1996). Over-expression of activated forms of αo, α2, αq, or αt3 did not mimic the receptor-mediated JNK activation, whereas JNK was activated by over-expression of βγ subunits. Co-transfection of dominant-negative ras or rac1 inhibited M1 and M2 receptor-mediated, as well as βγ subunit-mediated JNK activation, defining a role for these monomeric G-proteins in the βγ subunit-mediated activation of JNK. Dominant-negative cdc42 inhibited the M1 ACh receptor-mediated JNK activation, but had no effect on M2 ACh receptor or βγ subunit-mediated JNK activation. PI3-Kγ has been linked with G-protein βγ subunit-mediated JNK activation adding to the plethora of proteins involved in GPCR-JNK signalling. Pharmacological
inhibition of PI3-K in COS-7 cells perturbed M2 ACh receptor and βγ subunit-induced JNK activation (Lopez-Ilasaca et al., 1998). Transient transfection of dominant-negative forms of ras, rac, and PAK interfered with the PI3-K to JNK pathway determining a detailed signalling cascade linking GPCR activation to JNK.

In disagreement with the findings of Coso et al. (1996), Go subunits have also been shown to cause JNK activation. Constitutively active forms of Go12 and Go13 caused JNK activation in NIH 3T3 and COS-1 cells without inducing ERK activation (Prasad et al., 1995). Expression of a competitive, inhibitory form of ras blocked JNK activation mediated by activated Go12 and Go13, defining a role for ras in this GPCR-mediated signalling cascade. Over-expression of activated Goq, but not Goq or Goq also caused an increase in JNK activation. Transient expression of activated Go12 resulted in increased ras and JNK activation as well as increased c-jun transcriptional activity (Collins et al., 1996). A dominant-negative ras construct blocked this activation in NIH 3T3 cells, but not in HEK293 cells, whereas dominant-negative rac1 blocked JNK activity in all cell lines that were investigated (NIH 3T3, HEK293, HeLa, and COS-1). These data demonstrate a level of cell-specificity in the activation of JNK by Go12. This report also showed that AP-1 activity induced either by thrombin or activated Go12 in 1321N1 astrocytoma cells could be perturbed by dominant-negative rac1 or MEKK1, demonstrating a link between the G-protein and an upstream activating kinase of JNK.

Multiple signalling pathways linking Gt to JNK have been reported in HEK293 cells (Yamauchi et al., 2000). A βγ subunit-mediated pathway was shown to cause JNK activation and involved rho, rac1 and cdc42 as well as MKK4 and MKK7, the MKK proteins classically involved in JNK activation. Conversely, the Goq-mediated component of the pathway involved rho and cdc42 but not rac. Dominant-negative co-transfection experiments also revealed that the αt-mediated signalling cascade did not involve MKK4 or MKK7, but did involve at least one member of the c-src family of non-receptor tyrosine kinases. The report postulated that JNK was being activated in the αt-mediated pathway by an as yet unidentified MKK.
It is clear that the regulation of JNK both by GPCRs and by stress, cytokines and growth factors, is a research area requiring continued investigation. Several generalisations however can be made (see Fig. 1.4).

Figure 1.4. G_{i3}- and G_{i4}-coupled receptor activation of JNK. Solid lines indicate direct associations and dashed lines indicate multiple interactions or steps that are not yet fully understood. Both ras and rho-family GTPases are sensitive to Ca^{2+}, determining how PLC activation could mediate JNK activity. PKC may have a role in JNK activation although possible targets of PKC are yet to be identified.
JNK activation ubiquitously involves members of the rho family of monomeric G-proteins. Ras is involved in the signalling cascade in some cellular systems although like ERK activation its function is not always required. c-src family members and PI3-K appear to have roles upstream of the monomeric G-proteins in some cell systems. Both G-protein α and βγ subunits can mediate JNK activation, and there is growing evidence that kinases, for example PAK and MLK3 are involved in coupling the rho family G-proteins to the classical JNK activation cascade.

1.5. The Eukaryotic Cell Cycle

The eukaryotic cell cycle is a spatially and temporally regulated process that allows cells to synthesise DNA and divide. Appropriate regulation of this cycle is critical for normal cell growth, and mutations in many cell cycle proteins are associated with cancers (Catzavelos et al., 1997; Schutte et al., 1997) and other diseases (Howie et al., 1985).

Temporally, the cell cycle is organised into four discrete phases that allow appropriate regulation of the cycle. The phases are called G1, S, where DNA is synthesised, G2, and M, where mitosis occurs. Progression through these stages is regulated by checkpoints. Two checkpoints exist, the G1/S checkpoint after which cells are committed to replicate DNA, and the G2/M checkpoint, progression through which defines the start of mitosis. During G1, the accumulation of proteins required for the G1/S transition occurs, and as long as specific growth factors and essential amino acids are present, progression from G1 to S phase will take place. Once the G1/S checkpoint is passed, cells progress through S phase independently of external stimuli. During G2, newly replicated DNA is checked for successful synthesis, before progression through the G2/M checkpoint and commitment to mitosis resulting in the production of two daughter cells, which can then progress through their own cell cycles. Cells can also exist in a state of quiescence (G0) after serum withdrawal, and can re-enter G1 after a mitogenic stimulus.
Progression through the cell cycle is mediated by cyclin-dependent kinases (CDKs) which drive ordered transition through the different cell cycle phases. CDKs have various substrates depending on the stage of the cell cycle. For example at the G1/S phase checkpoint, the tumour suppressor retinoblastoma protein (pRb), and the pRb-related pocket proteins, p107 and p130 are phosphorylated by CDKs, whereas at the G2/M checkpoint, structural proteins involved in the execution of mitotic events are targets of CDK activity. CDKs are activated by their association with cyclins. Specific cyclins are expressed at specific times throughout the cell cycle and it is the differential expression of these proteins that regulate CDK activity and allow an ordered cell cycle progression. Cyclin D and cyclin E are involved in G1/S transition, cyclin A is expressed in late G1 and early S phase, whereas cyclin B is involved in progression through the G2/M checkpoint. CDK regulation is also achieved by phosphorylation/dephosphorylation events. After cyclin-CDK association, the CDK is phosphorylated on an activating phosphorylation site (Thr 160 for CDK2) by CDK-activating kinase (CAK) itself made up of a cyclin/CDK complex (cyclin H/CDK7). CDKs also contain conserved inactivating phosphorylation sites (Tyr 15 and Thr 14 for CDK2) that must be dephosphorylated for complete kinase activity. This is accomplished by protein phosphatases of the cdc25 family. CDK activity can be negatively regulated by cyclin-dependent kinase inhibitors (CDKIs) that interfere with the CDK complex (reviewed in Morgan, 1995; Obaya and Sedivy, 2002). There are two families of CDKI, the Cip/Kip family, which inhibit all cyclin/CDK complexes and the Ink4 family that specifically inhibit cyclin D-associated kinases (Sherr and Roberts, 1995)(Fig. 1.5)

Figure 1.5. Factors that contribute to the regulation of CDKs. Increases in cyclin expression, cdc25 activity, and CAK activity, all result in an increase in CDK activity. Increases in CDKI expression have an inhibitory effect on CDK activation.
Cell cycle progression through the $G_{i}/S$ checkpoint precedes DNA synthesis and requires hyperphosphorylation of $pRb$ (Harbour and Douglas, 2000) in order to pass the checkpoint otherwise known as the restriction point (R) (Fig. 1.6).

Figure 1.6. Schematic representation of the cell cycle showing the key proteins involved in the $G_{i}/S$-phase checkpoint. R represents the restriction point, which must be passed in order for S-phase progression to occur.
Phosphorylation of pRb at the G1/S checkpoint is primarily mediated by CDK4/6 and CDK2, which are activated by their interactions with cyclin D1 and cyclin E, respectively (Sherr, 1996). Dual phosphorylation of pRb is required for a successful G1/S transition and this is achieved by sequential phosphorylation by CDK4/6 followed by CDK2 (Zarkowska and Mittnacht, 1997). This phosphorylation allows the dissociation of transcription factors such as E2F from discrete binding pockets within the tumour suppressor (Dyson, 1998), which mediate the onset of DNA synthesis. Three members of the CDKIs are important at the G1/S checkpoint, p21\textsuperscript{Cip1/Waf1}, p27\textsuperscript{Kip1}, and p16\textsuperscript{Ink4a}.

1.6. The Structure and Function of the CDKIs

The cyclin-dependent kinase inhibitors (CDKIs) are a family of proteins that are involved in cell cycle regulation. CDKIs inhibit CDK activity by binding either to the CDK protein or to the cyclin-CDK complex, thus preventing CDK activation and cell cycle progression. As regulators of the cell cycle, they have been implicated in many pathophysiological conditions such as cancers (Catzavelos \textit{et al.}, 1997; Loda \textit{et al.}, 1997; Schutte \textit{et al.}, 1997), angiogenesis (Zhang \textit{et al.}, 2000), and age-related conditions such as Alzheimer's disease (Howie \textit{et al.}, 1985) and atherosclerosis (Nachtigal \textit{et al.}, 1998).

There are two distinct families of CDKI that are distinguishable both structurally and functionally. The Ink4 family, which comprises p16\textsuperscript{Ink4a}, p15\textsuperscript{Ink4b}, p18\textsuperscript{Ink4c}, and p19\textsuperscript{Ink4d} specifically interact with cyclin D associated kinases, CDK4 and CDK6. The Cip/Kip family, which includes p21\textsuperscript{Cip1/Waf1}, p27\textsuperscript{Kip1}, and p57\textsuperscript{Kip2}, interact with all reported cyclin-CDK complexes (Sherr \textit{et al.}, 1995).

The Cip/Kip family of CDKI, often referred to as the universal CDKI, interact with many cyclin-CDK complexes as well as individual cyclins and CDKs. Many groups have concerned themselves with identifying binding domains and structural motifs within these proteins that enable their interaction with cyclins and CDKs, as well as other downstream targets of the Cip/Kip proteins.
Fig. 1.7 is a schematic representation of the binding domains of p21\textsuperscript{Cip1/Waf1}, the best-studied Cip/Kip family member. The amino terminal region is highly conserved within this family of CDKI and contains an interaction domain for cyclin/CDK. Within this amino-terminal region, p27\textsuperscript{Kip1} has a 42 % amino acid sequence homology with p21\textsuperscript{Cip1/Waf1}, and a 47 % homology with p57\textsuperscript{Kip2}. Chen et al. (1995) expressed the amino- and carboxy-terminal domains of p21\textsuperscript{Cip1/Waf1} as GST-fusion proteins and evaluated their binding to CDK2. CDK2 bound as well to the GST-p21\textsuperscript{Cip1/Waf1} amino-terminal domain, containing amino acids 1-90, as it did to the full-length p21\textsuperscript{Cip1/Waf1} protein. CDK2 did not bind to the carboxy-terminal fragment (amino acids 87-164), indicating the requirement of the p21\textsuperscript{Cip1/Waf1} amino-terminal domain for CDK interaction.

Figure 1.7. Schematic representation of the p21\textsuperscript{Cip1/Waf1} protein showing various binding domains (adapted from Rousseau et al., 1999). The blue outline represents the amino terminal segment of the protein that interacts with cyclins and cyclin-dependent kinases. The light blue shaded region represents the area where cyclin binding occurs and the grey shaded area is important in CDK binding. The darker strip within this region has been shown to be critical for cyclin binding. The red outline represents the carboxy-terminus segment of the protein that interacts with PCNA. The red strip represents a putative bipartite nuclear localisation signal (NLS) that is common to all Cip/Kip family members. The amino acids that represent the boundaries of each domain are numbered.
The Cip/Kip family have also been shown to interact directly with cyclins (Chen et al., 1996). Experiments demonstrated that p21\textsuperscript{Cip1/Waf1} could interact with cyclins independently of CDK2 through a cyclin binding motif (Cyl) in the amino terminal region of p21\textsuperscript{Cip1/Waf1}. Manipulation of the Cyl site in p21\textsuperscript{Cip1/Waf1} using either a monoclonal antibody specific to the Cyl site, or deletion mutants of p21\textsuperscript{Cip1/Waf1} lacking this site, showed that this cyclin binding motif was required for optimum inhibition of CDK \textit{in vitro} and for growth suppression \textit{in vivo} (Chen et al., 1996).

The mechanism of Cip/Kip-mediated CDK inhibition has been reported from X-ray crystallography studies (Russo et al., 1996). Structural resolution of the N-terminal 69 amino acids of p27\textsuperscript{Kip1} bound to cyclinA-CDK2, showed that p27\textsuperscript{Kip1} interaction caused a large conformational change in and around the catalytic cleft of CDK2, resulting in a destabilising effect on ATP binding. p27\textsuperscript{Kip1} also bound to a peptide groove in cyclin A which prevented CDK2 binding. As the N-terminal domain is highly conserved in the Cip/Kip family, it is likely that this mechanism of CDK inhibition is also utilised by p21\textsuperscript{Cip1/Waf1} and p57\textsuperscript{Kip2}. A second conserved region of the Cip/Kip proteins is the nuclear localisation signal (NLS) found within their carboxy-terminal domains (Fig. 1.7). This sequence is thought to be important in the nuclear localisation of the Cip/Kip proteins, possibly allowing cyclin/CDK into the nucleus as part of a complex where it can be activated by CAK.

p21\textsuperscript{Cip1/Waf1} contains an additional domain that distinguishes it from p27\textsuperscript{Kip1} and p57\textsuperscript{Kip2}. This domain in p21\textsuperscript{Cip1/Waf1} allows binding of a DNA polymerase δ replication factor, termed proliferating cellular nuclear antigen (PCNA). Using \textsuperscript{3}H-PCNA, the PCNA binding domain has been mapped to the carboxy-terminus of p21\textsuperscript{Cip1/Waf1} (Luo et al., 1995). Furthermore, a peptide spanning amino acids 139-164 of p21\textsuperscript{Cip1/Waf1} has been shown to bind to PCNA and inhibit p21\textsuperscript{Cip1/Waf1} binding (Chen et al., 1995). Evidence is also available suggesting that the PCNA-binding domain of p21\textsuperscript{Cip1/Waf1} is important for DNA replication (Luo et al., 1995).
Unlike the Cip/Kip family of CDKI, Ink4 family members only interact with cyclin D-associated kinases, and are not thought to bind directly to cyclins. The Ink4 family members contain significant amino acid sequence homology and share similar tertiary structures. Luh et al. (1997) defined the structure of p19\textsuperscript{ink4a} using NMR spectroscopy. The group reported that the protein consisted of five ankyrin repeats that each shared 47\% sequence homology. Ankyrin repeats are motifs involved in protein-protein interactions and are likely to confer CDK binding. Ankyrin repeats are found in all Ink4 proteins and represent the regions of highest sequence homology between the family members. Further information was obtained from the X-ray crystallographic structure of p19\textsuperscript{ink4a} (Bauringer et al., 1998). Resolution of the structure revealed that each ankyrin repeat consisted of 32 amino acid residues and showed that a specific region of these ankyrin repeats, the helical stacks, contained the highest degree of structural homology between the family members. These structural similarities suggest that Ink4 family members share overlapping regulatory properties.

Although the Ink4 proteins only directly interact with CDK4/6, they can influence other cell cycle proteins indirectly. A series of elegant experiments showed how induction of p16\textsuperscript{ink4a} caused a decrease in CDK2 activity (McConnell et al., 1999). This inhibition was an indirect effect of p16\textsuperscript{ink4a} expression resulting from a reassortment of cyclins and CDKIs bound to the cellular CDK molecules. Thus, although Ink4 family members can only bind to CDK4/6, they can have a greater influence on cell cycle regulation than this interaction alone suggests.

The CDKIs were initially identified as inhibitors of CDK proteins and therefore negative regulators of the cell cycle. Over-expression of members of either CDKI family leads to cell cycle arrest. p21\textsuperscript{Cip1/Waf1} inhibits CDK2 activity by directly binding to the kinase, preventing phosphorylation by CAK on threonine 160 (Hitomi et al., 1998). Using purified full length CDKI proteins of known concentration, and cyclin A-CDK2 of known specific activity, a molar ratio of 1:1 was sufficient to inhibit kinase activity in vitro, suggesting that CDKIs have a single functional role in cell cycle regulation (Adams et al., 2000). However a more subtle role for the CDKI has been suggested from numerous independent studies. Although it is widely accepted that over-expression of the CDKIs will
cause a cell cycle arrest, several groups have described a second role for CDKIs as positive regulators of the cell cycle. At high concentrations p21\textsuperscript{Cip1/Waf1} has been shown to inhibit cell cycle progression, whereas low concentrations of p21\textsuperscript{Cip1/Waf1} promotes the assembly of cyclin-CDK complexes and cell cycle progression (LaBaer et al., 1997). Many reports have described how CDKI proteins can be found in active cyclin-CDK complexes (Zhang et al., 1994). If the relationship of CDKIs with CDKs were simply an inhibitory one, this finding would be difficult to explain. One possibility is that in order to inhibit the CDK activity, a number of CDKI molecules must associate with the complex. In other words, there would be a stoichiometric increase of CDKI in the complex, which would lead to CDK inhibition, whereas a single bound CDKI would not inhibit CDK activity. This would suggest that the CDKI in the active complex may have a function other than its inhibitory role. Transfection of several cell lines with antisense oligodeoxynucleotides for p21\textsuperscript{Cip1/Waf1} has been shown to cause a decreased association of the cyclin D1-CDK4 but not the cyclin E-CDK2 complex (Weiss et al., 2000). Antisense nucleotides to p21\textsuperscript{Cip1/Waf1} also led to a dose-dependent inhibition of vascular smooth muscle cell proliferation and DNA synthesis. These observations suggest that p21\textsuperscript{Cip1/Waf1} acts as a scaffold protein to facilitate assembly of cyclin D1-CDK4 complexes and is therefore critical for cell cycle progression.

The suggestion that CDKIs may have positive roles in cell cycle progression is supported by other work suggesting that p21\textsuperscript{Cip1/Waf1} and p27\textsuperscript{Kip1} are 'essential activators' of cyclin D-dependent kinases (Cheng et al., 1999). In primary embryonic fibroblasts that do not express p21\textsuperscript{Cip1/Waf1} and/or p27\textsuperscript{Kip1}, numerous defects were identified: cyclin D-CDK4/6 assembly was disrupted, cyclin D1 expression was reduced, and cells were unable to direct cyclin D1 to the nucleus. All of these defects were reversed by restoring p21\textsuperscript{Cip1/Waf1}/p27\textsuperscript{Kip1} expression. These data suggests that in addition to having the function of scaffold proteins or assembly factors, p21\textsuperscript{Cip1/Waf1} and p27\textsuperscript{Kip1} may also serve as nuclear transport proteins. The Cip/Kip family of CDKI contain a NLS that is absent from cyclins and CDKs. It may be that the p21\textsuperscript{Cip1/Waf1} or p27\textsuperscript{Kip1} are required to ensure that the cyclin-CDK complex can enter the nucleus where it can be phosphorylated and activated by CAK.
These observations suggest that CDKIs may have multiple roles in regulating the cell cycle, the subtlety and diversity of which has yet to be fully identified.

1.7. Regulation of CDKI Protein Expression

Regulation of cellular protein can occur at any stage from the initial stimulus for transcription, to the degradation of the protein. This is exemplified by CDKI expression where complex processes control the level of the protein, thereby regulating cell cycle progression. In this respect, protein regulation is critical in determining cell fate.

The first point at which protein expression can be regulated is at the level of the promotor region. For example the p21\textsuperscript{Cip1/Waf1} promotor contains numerous transcription factor-binding sites (Gartel and Tyner, 1999) and the transcription of p21\textsuperscript{Cip1/Waf1} can be influenced both positively and negatively by interaction with these factors (Fig. 1.8).

![Figure 1.8](image.png)

**Figure 1.8.** Schematic representation of the p21\textsuperscript{Cip1/Waf1} promotor showing selected transcription factor binding regions (adapted from Gartel and Tyner (1999)). Binding regions for STAT (light blue), p53 (black), AP-1 (green), and SP-1/3 (red) are shown as well as a binding region for SP-1/3 and SMAD (red/dark blue). The numbers indicate the number of base pairs away from the START site where transcription is initiated.

In theory, any pathway or response that can activate, inactivate, stabilise, or destabilise a factor capable of interacting with the p21\textsuperscript{Cip1/Waf1} promotor can influence transcription of the gene. The
tumour suppressor protein p53, is the classical upstream regulator of p21Cip1/Waf1, and can bind to a region within the p21Cip1/Waf1 promoter. p53 is normally activated in response to DNA damage, although other signals such as ras and CBP/p300 can also lead to p53-dependent activation of p21Cip1/Waf1 (Gartel and Tyner, 1999). Phosphorylation of p53 activates the tumour suppressor increasing its ability to bind to the p21Cip1/Waf1 promoter resulting in increased transcriptional activity. Activation of kinases capable of phosphorylating specific sites within the p53 protein, for example ERK and JNK have been shown to phosphorylate and activate p53 leading to p21Cip1/Waf1 induction (Milne et al., 1994, 1995; Buschmann et al., 2001). p53 phosphorylation can also be influenced by phosphatase activity. Inhibition of the serine/threonine phosphatase 5 (PP5) has been shown to have an anti-proliferative effect in A549 human lung carcinoma cells (Zuo et al., 1998, 1999). This effect was mediated by an increase in p53 phosphorylation without affecting protein levels.

Transcriptional regulation of p21Cip1/Waf1 can also occur independently of p53 (Park et al., 2001), and numerous other transcription factors can influence promoter activity. SP-1 and SP-3 are two related transcription factors capable of binding to distinct regions within the p21Cip1/Waf1 promoter region and inducing its transcription. There are 6 regions within the p21Cip1/Waf1 promoter where SP-1 and SP-3 can interact, named SP-1-1, to SP-1-6 (Gartel and Tyner, 1999). SP-1 can be superactivated by a second transcription factor, c-jun, forming a complex which will bind to and activate the p21Cip1/Waf1 promoter (Kardassis et al., 1999). Other reports have shown the importance of SP-3 in mediating p21Cip1/Waf1 gene transcription. In the Caco-2 colon adenocarcinoma cell line, SP-1 and SP-3 transactivated the p21Cip1/Waf1 promoter via independent SP-1 binding sites, although SP-3 acted as a more efficient transactivator (Gartel et al., 2000).

Other transcription factors can also influence p21Cip1/Waf1 gene expression. AP-1 has a discrete binding site within the p21Cip1/Waf1 promoter region (Crowe et al., 2000). Expression of dominant negative c-jun, a component of AP-1, caused G1-arrest, which was proposed to be acting via the CDKI promoter (Hennigan et al., 2000). These reports both demonstrated a role for AP-1 binding in the suppression of p21Cip1/Waf1 transcriptional activity. A second transcription factor that represses p21Cip1/Waf1 protein
expression is c-Myc. Treatment of HaCaT cells with TGFβ caused a decrease in c-Myc protein with a reciprocal increase in p21Cip1/Waf1 protein levels (Claassen et al., 2000). c-Myc was shown to regulate p21Cip1/Waf1 levels at a specific region within the promoter region of the CDKI, corresponding to a region from −62 to +16 base pairs of the start site of transcription, although no direct association was described. Experiments utilising BALB/c mouse fibroblast cells demonstrated a mechanism whereby c-Myc influenced p21Cip1/Waf1 gene transcription by sequestering SP-1/SP-3, rather than directly interacting with the p21Cip1/Waf1 promoter (Gartel et al., 2001). A region within the promoter that did not contain a c-Myc binding pocket, but containing SP-1 binding sites was shown to be critical to the c-Myc repression of gene expression. Another report described the importance of direct protein-protein binding between c-Myc and p21Cip1/Waf1 rather than transcriptional interactions (Kitaura et al., 2000). Both proteins were able to regulate the activities of the other in a reciprocal manner. Therefore, c-Myc provides an example of a protein that influences the expression of p21Cip1/Waf1 without directly binding to the p21Cip1/Waf1 promoter.

Although it is clear that the interaction of transcription factors with promoter sequences is very important in regulating protein expression, there are several other mechanisms that can affect transcription rates. One of these mechanisms, known as gene silencing, involves the methylation of discrete ‘CpG’ islands within the promoter region. This methylation suppresses gene expression and is a general method of controlling mRNA levels without requiring transcription factors. Inhibition of DNA methyltransferase (MeTase), the enzyme responsible for the transfer of methyl groups to specific bases within the promoter, induced p21Cip1/Waf1 expression without affecting any other known tumour suppressors (Milutinovic et al., 2000). Although the p21Cip1/Waf1 promoter contains ‘CpG’ islands they were not methylated in this report suggesting that the methylation was occurring on an upstream transactivating protein of p21Cip1/Waf1. Acetylation of nucleosomal histones allows unfolding of associated DNA, access to transcription factors and changes in gene expression. Experiments have shown that inhibition of histone deacetylase, an enzyme responsible for the removal of acetyl groups from histone molecules, causes an increase in gene selective acetylation of the p21Cip1/Waf1 promoter,
leading to an increase in mRNA and protein expression (Richon et al., 2000). This is a second example of how transcription can be altered without the direct involvement of transcription factors.

An alternative method of regulating protein levels is to either stabilise or destabilise mRNA, thus influencing the initiation of translation. After a 70% partial hepatectomy, p21Cip1/Waf1 mRNA was up-regulated (Albrecht et al., 1997). [\(\alpha\)-\(\text{\textsuperscript{32}P}\)]-UTP run-off experiments indicated that this induction was not due to increases in transcription rates, but experiments did show a significant increase in p21Cip1/Waf1 mRNA stability as measured by transcript half-lives before and after partial hepatectomy.

A second report analysed the ability of ERK to mediate growth responses in primary hepatocytes (Park et al., 2000). Experiments emphasised the importance of prolonged ERK activation to stabilise both p21Cip1/Waf1 mRNA and protein levels, resulting in increased CDKI expression. These data emphasised the importance of multiple regulatory mechanisms in influencing the expression of a protein. The combination of transcriptional and post-transcriptional regulation is apparent in this example.

It is possible to regulate the levels of cellular protein even after the functional protein has been produced. Protein kinase B (PKB) has been shown to directly phosphorylate p21Cip1/Waf1 on two residues, one of which that significantly increases p21Cip1/Waf1 protein stability (Li et al., 2002). The increase in protein stability would result in a general increase in cellular p21Cip1/Waf1.

Once a protein is no longer required its expression must be reduced by degradation. The classical non-lysosomal degradative pathway is the ubiquitin-proteasome pathway. Experiments in ML-1 cells underlined the importance of this pathway in p21Cip1/Waf1 regulation. Ubiquitinated p21Cip1/Waf1 is quickly degraded by the 26S proteosome resulting in lowered p21Cip1/Waf1 protein levels (Fukuchi et al., 1999). Pharmacological inhibition of the proteosome increased the half-life of p21Cip1/Waf1 more than 4-fold, emphasising the importance of this regulation in protein stability (Sheaft et al., 2000). The closest known *Xenopus laevis* homologue to mammalian p21Cip1/Waf1 and p27Kip1, Xic1, requires either nuclear accumulation or phosphorylation to be ubiquitinated and subsequently degraded (Swanson et
The phosphorylation step, which can overcome the need for nuclear accumulation, was performed by CDK2/cyclin E. Mammalian p27<sup>Kip1</sup> is also regulated in this manner by phosphorylation by CDK2/cyclin E (Sheaff <i>et al.</i>, 1997). The idea that phosphorylation is required to initiate degradation opens up many possibilities concerning upstream regulation of this pathway. Experiments designed to determine the importance of the N- and C-terminal regions of p21<sup>Cip1/Waf1</sup> in growth inhibition showed that the level of inhibition was dependent on the stability of the peptide, which was dependent on its degradation by the ubiquitin-proteasome pathway (Rousseau <i>et al.</i>, 1999). This report emphasised the importance of this degradative regulation showing that it can influence the overall cellular response. p21<sup>Cip1/Waf1</sup> can also be degraded by the proteosome without prior ubiquitination. Mutant versions of p21<sup>Cip1/Waf1</sup> that could not be ubiquitinated were produced and experiments showed that they could still be degraded by the proteosome (Sheaff <i>et al.</i>, 2000). It may be that other cellular signals can also contribute to proteasomal activation and ubiquitin-independent degradation of p21<sup>Cip1/Waf1</sup>.

In conclusion, it is clear that the regulation of p21<sup>Cip1/Waf1</sup> expression is a complex process that can be undertaken at a number of levels. It is likely therefore that the cellular level of p21<sup>Cip1/Waf1</sup> is under the influence of many regulatory proteins, pathways and mechanisms that could result in a change in p21<sup>Cip1/Waf1</sup> expression.

1.8 GPCR Involvement in Cell Growth, Differentiation, and Tumorigenesis

Many different types of GPCR have been identified by traditional pharmacological and biochemical approaches, and by molecular cloning studies. These receptors are expressed in diverse cellular settings, couple through distinct G-proteins, and elicit cellular responses by coupling through an intricate network of effector and second messenger systems.

The finding that a quiescent population of fibroblasts could be driven back into mitogenesis by α-thrombin and 5-HT, as well as the classical growth factors, provided strong evidence that GPCRs have
a role in cellular proliferation (Pouyssegur et al., 1988). An elegant series of experiments indicated the involvement of a pertussis toxin (PTX)-sensitive G-protein in cellular proliferation in response to LPA receptor activation (van Corven et al., 1989), an effect attributed to a reduction in cAMP mediated by the G-protein.

The discovery that activating mutations in certain G-protein α subunits can transform cells to a malignant phenotype has also revealed the potential role of GPCRs in cellular growth. A constitutively active form of Goα, known as the gsp oncogene, was discovered in human pituitary tumours (Landis et al., 1989). Further work described a similar mutation in the Goα2 subunit, known as the gip2 oncogene (Lyons et al., 1990). This oncogene was discovered in tumours in the adrenal cortex and in ovarian granulosa cells. Thus, activation of Gs and Gi can lead to cellular transformation even though they elicit opposing effects on AC. This suggests that G-protein regulation of mitogenesis is not predictable simply on the basis of G-protein-effector coupling. A hyperactive form of Goα12 was cloned based on its potent oncogenic effects when expressed in NIH 3T3 cells (Chan et al., 1993). The transforming ability of this mutant was demonstrated by its ability to cause cells to form foci when grown in semi-solid medium and by its tumorigenic capability in nude mice (Xu et al., 1993).

Microinjection studies have also provided evidence of G-protein involvement in proliferation. Injection of an antibody specific to the Goα2 subunit inhibited serum-induced cell proliferation (LaMorte et al., 1992). Treatment of the BALB/c 3T3 cells with PTX inhibited DNA synthesis by 55% suggesting that serum-stimulated DNA synthesis was due in part to the PTX-sensitive G12 protein, with the PTX-insensitive component, likely to represent PDGF. The mitogenic stimulus appears to be dependent on multiple pathways rather than just one. This is an example of how G-proteins and growth factors can interact to mediate cellular growth. Further microinjection studies were used to assess the involvement of Goαq in agonist-induced DNA synthesis (LaMorte et al., 1993). In the BALB/c 3T3 cell system both bradykinin and thrombin caused increases in intracellular Ca2+ and DNA synthesis. Microinjection of the Goαq antibody blocked both bradykinin- and thrombin-induced
Ca\(^{2+}\) elevation and DNA synthesis, indicating a role for this G-protein in growth stimulation by these agonists. Microinjection of the Go\(\alpha_2\) antibody had no effect on agonist-mediated Ca\(^{2+}\) increases, or on the bradykinin-mediated increase in DNA synthesis. However the thrombin-induced DNA synthesis was abolished by Go\(\alpha_2\) antibody injection. These data suggest that although Go\(\alpha_q\) is sufficient to mediate bradykinin-induced DNA synthesis, thrombin requires the activity of both Go\(\alpha_q\) and Go\(\alpha_2\), highlighting the diversity of growth regulation by GPCRs.

Studies have also shown that G-proteins can influence cell differentiation (Wang et al., 1992). Dexamethasone-induced differentiation of 3T3-L1 fibroblasts can be blocked by addition of cholera toxin, known to activate G\(\alpha_s\), whereas antisense oligodeoxynucleotides to G\(\alpha_q\) can increase the rate of differentiation. These observations suggest that activation of G\(s\) can prevent cells from undergoing differentiation, probably by increasing proliferation.

It is clear that G-proteins that couple to a variety of different second messenger pathways have a role in cellular proliferation. It is also apparent that \(\alpha\) subunits derived from each of the four main classes of G-protein have a potential role in cell proliferation. This suggests that more than one G-protein-mediated pathway can lead to proliferation and eventual transformation. This is particularly significant since a vast number of receptors have been described that can activate them.

One such class of receptor is the muscarinic ACh receptor. Experiments using transiently transfected muscarinic ACh receptors expressed in NIH 3T3 cells demonstrated an agonist-dependent, subtype-specific transformation of the cells (Gutkind et al., 1991). Only activation of the G\(q\)-coupled M\(_1\), M\(_3\), and M\(_5\) ACh receptors caused foci formation in soft agar whereas activation of the G\(t\)-coupled M\(_2\) and M\(_4\) ACh receptors had no transforming effect. Further work confirmed these findings and also demonstrated that although carbachol potently stimulated DNA synthesis through muscarinic receptors linked to PIP\(_2\) hydrolysis, additional growth factors were required for a sustained increase in DNA synthesis (Stephens et al., 1993).
While these studies link muscarinic receptors to proliferation, activation of muscarinic ACh receptors has also been shown to inhibit DNA synthesis and even serve a potential tumour suppressor function. Activation of the M₁ and M₃ ACh receptors stably expressed in A9 L cells can inhibit mitogenesis in this cell system (Conklin et al., 1988). This was proposed to be due to an increase in agonist-induced arachidonic acid accumulation. Muscarinic ACh receptors coupled to PIP₂ hydrolysis are also capable of inhibiting density-independent growth and suppressing tumours in nude mice (Felder et al., 1993). Activation of the wild-type receptor and a mutated receptor resistant to down-regulation, lead to a decrease in DNA synthesis in CHO cells (Detjen et al., 1995). The inhibition by the mutated receptor was sustained over a two-week period, but the inhibition mediated by the wild-type receptor was transient. In other words, desensitisation of the receptor led to restoration of cell cycle progression. The authors proposed that the variability of the time-course and extent of desensitisation of receptors, and of other proteins including effectors within different cellular systems, could account for the variability of G-protein-mediated growth responses.

The finding that a receptor coupled to a single G-protein type can either stimulate DNA synthesis (LaMorte et al., 1993) or inhibit DNA synthesis (Felder et al., 1993) prompts some important questions. In particular, what are the factors that contribute to the growth response mediated by a GPCR and what determines whether a specific G-protein will contribute to a positive or negative growth response? There are a number of possibilities, some of which are outlined in Table 1.1.

Another possibility was described in experiments using brain-derived glial cells. Activation of specific muscarinic ACh receptors was found to induce DNA synthesis in perinatal rat brain depending on the age of development (Ashkenazi et al., 1989). This would suggest that receptors and other proteins could be specifically expressed during development or periods of differentiation when the tissue requires them. It may also be possible that receptors could be modified at specific times, or that other proteins could be temporally expressed to impair or enhance the receptor/G-protein function, depending on when the particular response is required.
CONTRIBUTING FACTORS THAT MAY DETERMINE THE GROWTH OUTCOME

| Type and subtype of receptor expressed (e.g. mAChR, 5-HT-R, LPA-R etc.) |
| Promiscuous coupling between receptor and G-proteins |
| Specific G-protein expression (e.g. G₆, G₁₀, G₉ etc.) |
| Liberation of α and βγ subunits and their relative importance as intracellular messengers |
| Expression of specific isoforms of α and βγ subunits |
| Expression of specific isoforms of effectors (e.g. PLC/AC/PI3-K) |
| Specific second messenger pathways that are activated |
| Transactivation of growth factor pathways |
| Desensitisation/down-regulation of receptors and effectors |
| Developmental or cell cycle stage-dependent expression of proteins |

Table 1.1. Factors that could determine the role of a G-protein in cellular growth regulation. Some ideas presented are described in the text.

G-proteins appear to be an intracellular tool used to mediate cellular growth responses. Whether a specific G-protein causes proliferation, differentiation, tumorigenesis or acts as a tumour suppressor appears to depend on cellular context and is likely to be modulated by many other factors (Table 1.1).

It is likely that the G-proteins transduce signals that modulate the effects of other factors responsible for the regulatory “decision making”. Whether or not a single “rule” will be defined to allow predictions about specific G-protein involvement in a particular growth response is open to debate. At the moment the most likely explanation is that a large number of determinants will contribute to G-protein-mediated growth responses.

1.9. GPCR-Mediated Regulation of Cell Growth: The Involvement of Signalling Cascades and Their Influence on the Cell Cycle

The involvement of GPCRs in cell growth is now well established. The discovery that all known subclasses of G-protein are activated by many classes of GPCR can cause cellular growth effects has led to a considerable interest in the field. However, although there are many examples of receptors mediating growth effects, mechanistic data connecting receptor activation to specific intracellular signalling pathways and to changes in expression or activity of cell cycle proteins are limited. GPCRs
have been shown to be critical both in physiological and pathophysiological growth. Perturbation of the cholecystokinin B (CCK-B) receptor by gene targeting caused atrophy in the gastric muscle cells of receptor-deficient mice (Nagata et al., 1996). The mice had only 70% of normal protein levels. Inactivation of the 5-HT\textsubscript{2B} receptor by gene targeting caused embryonic and neonatal death in mutant mice resulting from heart defects (Nebigil et al., 2000b). The mutant embryos lacked specific expression of the tyrosine kinase ErbB-2, suggesting that cardiac differentiation was mediated by 5-HT\textsubscript{2B} GPCRs acting on ErbB-2 receptors. In this study, surviving mice suffered severe hypoplasia due to a reduction in proliferation.

Many intracellular signalling cascades are triggered by GPCR activation, resulting in a plethora of cellular events. The challenge lies in determining which of these pathways are specifically involved in growth regulation and how they control the cell cycle machinery.

The G\textsubscript{q}-coupled CCK-B receptor has opposing effects on cell growth depending on the cellular setting (Detjen et al., 1997). Activation of the receptor in growing CHO cells caused a reduction in DNA synthesis, whereas stimulation of the same receptor in serum-starved Swiss 3T3 cells caused an increase in proliferation. These effects could not be explained by differential coupling of the receptor to second messenger pathways, or differences in receptor number, suggesting that cell-specific expression of proteins may be responsible for the differences in cellular growth. Receptor activation of selective second messenger pathways has been shown to determine growth responses. Pituitary adenylate cyclase-activating peptide-1 (PAC\textsubscript{1}) receptor activation caused increases in cAMP production and activation of PI signalling in peripheral sympathetic neuroblasts that led to an increase in proliferation (Lu et al., 1998). Neuropeptide activation of the PAC\textsubscript{1}-receptor endogenously expressed in cerebral cortical precursor cells caused only an increase in cAMP production, and resulted in a decrease in proliferation. This study demonstrates the importance of activation of second messenger pathways in determining a GPCR-mediated growth response.
Several studies have attempted to determine the involvement and importance of signalling cascades in growth regulation. Activation of the G\textsubscript{1}-coupled somatostatin 4 (sst\textsubscript{4}) receptor caused an increase in cellular proliferation that was dependent on the level of ERK activation (Sellers 1999a). A src- and ras-dependent acute phase of ERK activation could be abolished without affecting the proliferative response. The proliferation was caused by PTX-sensitive, prolonged PKC-dependent ERK activation. This study emphasises the importance, not only of the kinases involved, but also of the pathways leading to their activation and the duration of their activation. Further work on this receptor-mediated growth response (Sellers et al., 1999b) showed that the sustained ERK activation led to a PTX-sensitive serine-phosphorylation of signal transducer and activator of transcription 3 (STAT3) that could not be seen after the transient ERK activation. This activating phosphorylation demonstrates a link between GPCR, ERK and transcriptional regulation and demonstrates how a single receptor can couple to multiple G-proteins to mediate distinct effects. Over-expression of PKD and subsequent activation of the G\textsubscript{q}-coupled bombesin GPCR caused an increase in PKD phosphorylation and activation, and a subsequent increase in DNA synthesis compared with control cells (Zhukova et al., 2001). This PKD involvement was downstream of PKC, although downstream targets of PKD were not identified.

The involvement of ERK and of other MAPK classes, specifically JNK and p38, in GPCR-mediated growth effects is a common theme. This is a predictable phenomenon, as MAPKs are well known for their involvement in positive and negative growth regulation by other receptor classes and by cellular stress. ERK activation downstream of the neurokinin-1 (NK\textsubscript{1}) receptor was shown to mediate increases in DNA synthesis by G\textsubscript{1}-coupling in U-373 astroglia cells (Castagliulo et al., 2000). Receptor activation caused tyrosine phosphorylation of the EGF receptor, and induced formation of an active EGF receptor complex containing the adapter proteins Shc and Grb2. Activation of either the \( \alpha_{1B} \) or the \( \alpha_{1D} \)-adrenoceptors caused a decrease in DNA synthesis in Rat 1 fibroblasts (Waldrop et al., 2001). The \( \alpha_{1B} \)-adrenoceptor-mediated growth effects were shown to be dependent on its ability to
activate p38, whereas growth inhibition by the α1D-adrenoceptor subtype was independent of p38 activity.

p38 has also been shown to be important in growth responses mediated by somatostatin receptors. Activation of the G\textsubscript{j0}-coupled sst\textsubscript{2a} and sst\textsubscript{2b} receptors led to a decrease and increase in DNA synthesis respectively (Sellers \textit{et al.}, 2000). The sst\textsubscript{2a} receptor-mediated growth arrest was due to the receptor activating p38 over a sustained time period. The sst\textsubscript{2b} receptor preferentially coupled to PI3-K activation and subsequent PKB and p70\textsuperscript{S6K} phosphorylation. Although both receptors activated the ERK pathway, the decrease in DNA synthesis in response to sst\textsubscript{2a} activation was due to increased expression of the CDK1 p2\textsuperscript{Cip1/Waf1}, which was dependent on both ERK and p38 activation. The authors suggested that subtle differences in the carboxyl tails of the receptors might allow coupling to different isoforms of G-protein βγ subunits, which could explain the differential pathway activation and growth effects. LPA and sphingosine-1-phosphate (S1P) caused increases in DNA synthesis through G\textsubscript{i}-coupled endothelial differentiation gene-encoded (edg) receptor in human breast cancer cells (Goetzl \textit{et al.}, 1999). This effect was caused both by direct agonist-dependent increases in serum response element (SRE) transcriptional activity, and by indirectly increasing SRE activity by inducing insulin-like growth factor 2 (IGF2) release from the cells. Both of these effects were ERK and Rho-dependent suggesting the involvement of multiple signalling pathways in the response.

The last two studies have described mechanisms linking GPCRs to components that can influence the cell cycle or transcriptional regulation thus providing a more direct link between the receptor and growth regulation. Work progressing from that of Goetzl \textit{et al.} (1999) demonstrated that the edg\textsubscript{3} and 5 receptor activation and binding of SRE to a putative site within the c-fos promotor, resulting in its activation and the formation of an active AP-1 complex (An \textit{et al.}, 2000). AP-1 is known to have specific binding regions in cell cycle proteins such as cyclin D1 (Albanese \textit{et al.}, 1995) resulting in increased expression and a positive influence on the cell cycle.
Studies have shown a direct relationship between receptor activation and changes in cell cycle protein expression. Activation of the G<sub>i</sub>-coupled receptor Smoothened, caused an increase in expression of cyclins D1, D2 and E, resulting in sustained cell cycle progression (Kenney et al., 2000). Activation of the G<sub>q</sub>-coupled 5-HT<sub>2B</sub> receptor caused an induction of cyclin D1 and cyclin E expression, resulting in increases in CDK2 and CDK4 activity and hyperphosphorylation of pRb (Nebigil et al., 2000a). The increase in cyclin D1 was dependent on src transactivating the PDGF receptor and activating ERK, whereas the cyclin E induction was independent of PDGF and ERK, but still required src activity. This is an important phenomenon that describes divergent signalling pathways coupling the same GPCR to different cyclin proteins resulting in cellular proliferation. β-migrating very low-density lipoprotein (βVLDL) has been shown to be involved in mediating cell proliferation via a PTX-sensitive GPCR transactivating the EGF receptor resulting in ERK activation (Zhao et al., 2001). The ERK activation resulted in increased cyclin D1 expression and decreased expression of the CDKI p27<sup>Kip1</sup>. Both EGF and βVLDL addition to the smooth muscle cells caused an increase in ERK activity, but only concomitant addition resulted in the changes in cell cycle proteins and cell proliferation. This suggests that a larger more sustained ERK activation produced by addition of both agonists was required to drive cells to proliferate. Studies of the G<sub>q</sub>/11-coupled angiotensin 2-type 1A (AT<sub>1A</sub>) receptor demonstrated the importance of increases in cyclin D1 promotor activity in cell cycle progression in CHO cells (Guillemot et al., 2000). This increase in activity was dependent on the ras, c-raf-1, ERK pathway as well as PI3-K and the protein tyrosine phosphatase SHP-2. These experiments provide strong mechanistic data as well as demonstrating how a GPCR can directly regulate the cyclin D1 promotor.

The involvement of tyrosine phosphatases has also been described in GPCR-mediated cell growth arrest. Experiments investigating the effects of sst<sub>2</sub> receptor activation on insulin-induced proliferation of CHO cells showed that GPCR activation caused a G<sub>1</sub>-phase cell cycle arrest through a transient induction of p27<sup>Kip1</sup> and a resulting decrease in CDK2 activity (Pagès et al., 1999). Over-expression of
an inactive form of the protein tyrosine phosphatase SHP-1 reversed the G1-phase arrest demonstrating its importance in the pathway linking the receptor to the cell cycle.

GPCR-mediated induction of CDKIs appears to be involved in growth arrest in response to activation of a number of receptors. Inhibition of bFGF-induced proliferation by sst2 and sst4 receptors was a result of an increase in p21Cip/Waf1 expression (Alderton et al., 2001). This p21Cip/Waf1 induction was dependent on a sustained ERK and p38 activation. Inhibition of either MAPK ablated the increased p21Cip/Waf1 expression demonstrating the requirement of both kinases in this growth response. The p21Cip/Waf1 expression was only induced in the presence of bFGF suggesting that the GPCR-mediated p38 activity alone was not pronounced enough to mediate the growth effects. A G2-phase cell cycle arrest resulting from an induction of p21Cip/Waf1 protein levels downstream of the calcitonin receptor was shown to be dependent on transcriptional regulation of the p21 Cip/Waf1 promotor (Evdokiou et al., 2000). A calcitonin receptor-mediated increase in SP-1 transcription factor activity was shown to be responsible for the increase in CDKI protein levels. Activation of the M3 ACh receptor caused opposing effects on cell growth depending on the growth condition of the cell (Nicke et al., 1999). Under serum-containing conditions, cell growth was arrested as a result of increases in p21Cip/Waf1 protein levels and a decrease in cyclin D1 expression. The decrease in cyclin D1 expression was dependent on proteasomal degradation, and the induction of p21Cip/Waf1 had an upstream PKC-dependent component. The authors dismissed the involvement of p21Cip/Waf1 in the growth arrest stating that the growth inhibition occurred before the induction of p21Cip/Waf1 protein. This would reveal the importance of the cyclin D1 degradative pathway in this growth response. The importance of proteasomal degradation in mediating growth arrest has also been shown after β2-adrenoceptor activation in human airways smooth muscle cells (Stewart et al., 1999). Activation of a Gq-coupled receptor, or direct cell permeant cAMP analogue addition led to a growth arrest of thrombin-induced proliferation. This arrest was caused by a decrease in ERK activity and cyclin D1 expression, which was accounted for by proteasomal degradation of the cyclin.
Fig. 1.9 summarises the main pathways that link GPCRs to the cell cycle. Clearly MAPKs play a key role in this regulation, although examples of MAPK-independent pathways are being discovered (Burdon et al., unpublished data). The majority of reports to date have shown receptor-mediated changes in cyclin or CDKI expression through transcriptional regulation and/or by regulation of protein degradation, although other methods of cell cycle regulation have been described.

Figure 1.9. GPCR regulation of growth signalling pathways by cell cycle protein regulation. Dashed lines represent pathways involving multiple steps or factors (for example, transcription factors, degradative pathways). MAPK represents ERK, JNK, and p38.
1.10. Summary

It is clear that GPCR regulation of cell growth is an extensive field of research in which more mechanistic data are being compiled that links GPCRs to effects on cell cycle proteins. Elucidation of the pathways from a GPCR through its signalling components all the way through transcriptional regulation and control of the cell cycle is a large task. As more components of intracellular signalling pathways are identified that link GPCRs to the cell cycle machinery in various cell models and systems, our understanding of one of the most significant of cellular responses to G protein-coupled receptor activation, that of growth regulation, will be enhanced.

1.11. Thesis Aims

The aims of this thesis were to investigate the growth regulation mediated by the M₂ and M₃ ACh receptors stably expressed in CHO cells. The growth responses to activation of these receptors were determined, and pharmacological, biochemical, and molecular biological approaches were used to investigate the mechanisms underlying the growth effects, both at the level of the cell cycle and at the level of intracellular protein kinase pathways that transduce the signal.
Chapter 2

Materials and Methods
MATERIALS AND METHODS

2.1. Cell culture

Chinese hamster ovary (CHO-K1) cells stably expressing either the recombinant human M2 or M3 ACh receptor (1.5 ± 0.1 and 2.2 ± 0.2 pmol/mg of total cellular protein respectively (n=3 and errors are S.E.M.), personal communication from R.A.J.C.) were maintained in minimal essential medium-α (Gibco-BRL) at 37 °C in a 5 % CO₂ / 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). Where indicated, cells were made quiescent by serum starvation for 24 h prior to agonist addition.

2.2. Plasmids

Constructs for bacterial expression of c-jun and ATF-2 as translational fusions with glutathione S-transferase (GST) were generously provided by Dr. Roger Davis. The GST-fusion proteins were purified by affinity chromatography on glutathione-Sepharose (Pharmacia LKB). Purified fusion proteins were resolved by SDS-polyacrylamide gel electrophoresis using 12 % polyacrylamide in the presence of 0.1 % SDS and quantified by comparative Coomassie Blue staining using bovine serum albumen to construct a standard curve. Hamster CDK2 3* Myc-tagged (N-terminal fusion) in pEFTSIGN (ires-GFP) was a kind gift from Dr. Clare Hall. HA-tagged JNK1 in the mammalian expression vector pSRα was kindly provided by Dr. Gary Johnson. HA-tagged ERK2 was a generous gift from Dr. Jacques Pouyssegur and provided in pCDNA-NEO (Brunet et al., 1999). The kinase-inactive mutant of MEK1 in pCMV5 was provided by Dr. Gary Johnson. The kinase-inactive mutant of Flag-MKK4 in pCMV5 was prepared by substituting the sites of activating phosphorylation (Ser²²¹ and Thr²⁰⁲) with alanine (Deacon and Blank, 1997). The corresponding double point mutant of Flag-MKK7 (Ser¹⁹⁸ and Thr²⁰² replaced with alanine) was provided by Dr. Davis in pCDNA3 (Tournier et
Flag-JIP1 and Flag-JNK-binding domain of JIP1 (JBD) were kindly provided by Dr. Martin Dickens. Flag-MKK3 mutants containing double point substitutions of Ser\(^{189}\) and Thr\(^{193}\) with alanine were expressed from pCMV5 (Deacon and Blank, 1997). A signalling-inactive mutant of Flag-MKK6 was created by substitution of Ser-207 and Thr-211 with alanine and provided in pCDNA3 by Dr. Roger Davis (Raingeaud et al., 1996). The construct for G\(\alpha_4\) in the mammalian expression vector pCDNA3.1 was a generous gift from Dr. Gary Willars. All constructs were transformed into bacteria and purified using a Maxiprep (Nucleobond) according to the manufacturers instructions.

2.3. ERK immunoprecipitation and assay

ERK proteins were isolated by immunoprecipitation from CHO cell lysates and assayed for enzyme activity. Cells were solubilized with lysis buffer containing 20 mM Tris-HCl, pH 7.4, 1 % Triton X-100, 10 % glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM \(\beta\)-glycerophosphate, 1 mM Na\(_3\)VO\(_4\), 1 mM phenylmethylsulfonyl fluoride, and 10 \(\mu\)g/ml leupeptin. Insoluble material was removed by centrifugation at 14000 \(g\) for 10 min at 4 °C. Lysates were incubated for 60 min at 4 °C with a 1:100 dilution of a rabbit polyclonal antiserum (200 \(\mu\)g/ml) directed against ERK1 [C16: sc-93 (Santa Cruz)]. Immune complexes were incubated for a further 60 min at 4 °C with 70 \(\mu\)l of a 15 % (v/v) slurry of Protein A-Sepharose (Pharmacia-LKB) and collected by centrifugation. Immunoprecipitates were washed twice in 200 \(\mu\)l of lysis buffer and twice in 200 \(\mu\)l of kinase buffer containing 20 mM Hepes, pH 7.2, 20 mM \(\beta\)-glycerophosphate, 10 mM MgCl\(_2\), 1 mM dithiothreitol, and 50 \(\mu\)M Na\(_3\)VO\(_4\). Immune complex ERK assays were initiated by addition of 40 \(\mu\)l of kinase buffer containing 20 \(\mu\)M \([\gamma\text{-}^{32}\text{P}]\)ATP (2.5 \(\mu\)Ci / nmol; New England Biolabs) and 200 \(\mu\)M of a synthetic peptide substrate corresponding to amino acids 662–681 of the epidermal growth factor receptor. Reactions were incubated for 20 min at 30 °C and terminated by addition of 10 \(\mu\)l of 25 % (w/v) trichloroacetic acid (TCA). Mixtures were centrifuged at 14000 \(g\) for 2 min and the resulting supernatant was spotted onto P81 cation-exchange paper (Whatman). Papers were washed three times in 0.5 % orthophosphoric
acid, once in acetone, and allowed to dry before being counted in a Tri-Carb liquid scintillation counter.

2.4. JNK immunoprecipitation and assay

CHO cell lysates, prepared exactly as for ERK immunoprecipitation, were incubated for 60 min at 4 °C with a 1:100 dilution of a rabbit polyclonal antiserum (200 μg/ml) directed against JNK1 [C17: sc-474 (Santa Cruz)]. Immune complexes were incubated overnight at 4 °C with 70 μl of a 15 % (v/v) slurry of Protein A–Sepharose and collected by centrifugation. JNK was assayed under identical conditions to those described for ERK except that 5 μg of purified GST-c-jun was used as a substrate. Reactions were terminated by addition of 40 μl of Laemmli sample buffer and boiling. Phosphorylated proteins were resolved by polyacrylamide gel electrophoresis (PAGE) through 12 % acrylamide in the presence of 0.1 % SDS, stained with Coomassie Blue R250 and visualized by autoradiography. Radioactivity incorporated into c-jun was quantified by liquid scintillation counting of the excised bands.

2.5. p38 immunoprecipitation and assay

CHO cell lysates, prepared exactly as for JNK immunoprecipitation, were precleared with 20 μl of Protein G-agarose (Santa Cruz) before an overnight incubation at 4 °C with a 1:20 dilution of a goat polyclonal antiserum (200 μg/ml) directed against p38 [C-20g: sc-535g (Santa Cruz)]. Immune complexes were incubated for 2 hours at 4 °C with 20 μl of Protein G-agarose and collected by centrifugation. p38 was assayed under identical conditions to those described for JNK except that 5 μg of purified GST-ATF2 was used as a substrate. Reactions were terminated by addition of 40 μl of Laemmli sample buffer and boiling. Phosphorylated proteins were resolved by polyacrylamide gel electrophoresis (PAGE) through 15 % acrylamide in the presence of 0.1 % SDS, stained with
Coomassie Blue R250 and visualized by autoradiography. Radioactivity incorporated into ATF-2 was quantified by liquid scintillation counting of the excised bands.

2.6. CDK and cyclin immunoprecipitation and assay

Rabbit polyclonal antibodies (all 200 µg/ml) to CDK2 (M2: sc-163), CDK4 [C22: sc-260], cyclin E (M20: sc-481) (Santa Cruz), and mouse monoclonal antibodies (all 200 µg/ml) to cyclin D1 (R124: sc-6281) (Santa Cruz) and cyclin A (05-155) (Upstate Biotechnologies) were used for immunoprecipitation from CHO cell lysates prepared as described for ERK immunoprecipitation except that lysates were precleared with 70 µl of a 15 % (v/v) slurry of Protein A–Sepharose prior to antibody addition. Immunoprecipitates were washed twice in 200 µl of a wash buffer containing 25 mM Hepes pH 7.4, 150 mM NaCl, and 0.1 % (v/v) Triton-X-100, and twice in 200 µl of an assay buffer containing 50 mM Hepes pH 7.4, 1 mM DTT, 15 mM EGTA, and 20 mM MgCl₂. Immune complex CDK2 reactions were performed at 30 °C for 20 min in this assay buffer containing 20 pM [γ-³²P]ATP (2.5 µCi/nmol) and 25 µg of histone H1 as a substrate. Reactions were terminated by addition of 40 µl of Laemmli sample buffer and boiling. Phosphorylated proteins were resolved by PAGE through 15 % acrylamide in the presence of 0.1 % SDS, stained with Coomassie Blue R250 and visualized by autoradiography. Phosphorylated histone H1 was quantified by liquid-scintillation counting of the excised bands.

2.7. Western blot analysis

Proteins were separated by SDS/PAGE and transferred onto a nitrocellulose membrane for 60 min at 60 mA 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, H 8.0, 0.1 % Tween-20, 150 mM NaCl] and incubated overnight at 4 °C with primary antibody: [anti-ERK1 (C16: sc-93), 1:500; anti-JNK1 (C17:
G\alpha t (K20: sc-389), 1:500, all purchased from Santa Cruz; anti-cyclin A (05-155), 1:1000, (Upstate Biotechnologies); anti-pRb (M032700), 1:500, (Pharmingen); and anti-Myc-tag (2272), 1:1000, (Cell Signalling Technology)) all in TTBS. After washing 3 times in TTBS, blots were incubated for 1 h at room temperature with a 1:1000 dilution in TTBS of anti-rabbit or anti-mouse IgG coupled to peroxidase (Sigma). Immunoblots were developed by enhanced chemiluminescence (Amersham International). To detect p21\textsuperscript{Cip1/Waf1} associated with CDK2, immunoprecipitates of CDK2 were prepared (as described under \textit{CDK and cyclin immunoprecipitation and assay}) and resuspended in 100 \mu l of Laemmli sample buffer.

2.8. Thymidine incorporation

CHO cells were plated at 50,000 cells/well and incubated overnight under normal culture conditions. Cells were then incubated for a further 24 hours in medium either containing or lacking 10 % newborn calf serum as indicated in the Figure legends. Carbachol incubations were for 24 hours unless otherwise stated. 2 hours prior to lysis, 2 \mu Ci of \textsuperscript{3}H\textsuperscript{thymidine (25 Ci/mmol: Amersham Pharmacia Biotech; TRK120) was added to each well. Cells were washed three times with minimal essential medium-\alpha, then incubated with 2 ml of 5 % (w/v) TCA for 1 hour at 4 \degree C. TCA solution was removed and cells were washed once in fresh TCA solution. 2 ml of ice-cold ethanol containing 200 \mu M potassium acetate was placed in each well for 5 min, following which the cells were incubated twice for 15 min in a 2 ml mixture of ethanol:ether. Plates were allowed to air dry before the cellular precipitates were solubilized with 0.5 ml of 0.5 M NaOH. \textsuperscript{3}H\textsuperscript{thymidine incorporated into DNA was measured by adding samples to scintillation fluid and counting on a Tri-Carb liquid scintillation counter.
2.9. Fluorescence-activated cell sorting (FACS) analysis

Exponentially growing CHO cells were treated with 10 μM CCh for either 24 hours, 48 hours, or left untreated. Cells were washed twice in PBS, lifted using Cell Dissociation Agent™ (Sigma), and fixed in 70 % ethanol at -20 °C for 15 min. Cells were collected using a bench top centrifuge, allowed to dry and then stained overnight with propidium iodide (200 μg/ml), before being analysed using a Becton Dickenson FACScan.

2.10. RNA extraction and Reverse Transcription

Exponentially growing CHO-m3 cells were stimulated with 10 μM CCh for the times indicated or left untreated as controls before total RNA was extracted using RNAzol™ B according to the manufacturers instructions (Biogenesis Ltd). After photometric quantification 200 ng of RNA was used for a 20 μl reverse transcriptase reaction in buffer containing 200 U of Superscript™ II RNase H+ reverse transcriptase (Invitrogen), random primers, and dNTPs as recommended by the manufacturer (Invitrogen).

2.11. Polymerase chain reaction (PCR)

PCRs were performed using 2 μl of the transcribed RNA as a template. For the p21\textsuperscript{Cip1/Waf1} reaction, 200 nM each of the sense primer, 5'-GTG GCC TTG TCG CTG TCT TGC AC-3' and antisense primer, 5'-TTT CTC TTG CAG AAG ACC AAT C-3' were used in the presence of Taq DNA polymerase, dNTPs and MgCl₂ (Invitrogen). After a 2 min denaturing period at 94 °C, PCRs were performed over 40 cycles (94 °C for 1 min, 45 °C for 1 min, and 72 °C for 2 min), with a final extension step at 72 °C for 7 min. For the β-actin reaction, 100nM of the sense primer, 5'-TTC AAC TTC ATC ATG AAG ACC AAT C-3' and antisense primer 5'-CTA AGT CAT AGT CAT AGT CCG CCT AGA AGC ATT-3' were used. After a 3 min denaturing period at 95 °C, reactions were performed
over 25 cycles (95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min), with a final extension step at 72 °C for 5 min. 4 μl of the product was added to 20 μl of 6 times loading buffer and run at 110 mA on a 1 % agarose gel containing propidium iodide. Bands were visualised using UV light.

2.12. Caspase-3 Assay

Exponentially growing CHO-m3 cells were treated with 10 μM CCh for 24 hours or with 25 μM etoposide overnight. Cells were harvested in PBS/0.5 mM EDTA and collected in a bench-top centrifuge. Pellets were resuspended in lysis buffer (50 mM HEPES, pH 7.4, 0.1 % CHAPS, 1 mM DDT, 0.1 mM EDTA) and left on ice for 10 mins, before being cleared by centrifugation at 10000 g for 1 min. A Bradford protein assay was performed on the lysates and 200-400 μg of cell lysate was diluted 1:2 in reaction buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1 % CHAPS, 10 mM DTT, 1 mM EDTA, 10 % glycerol) in plastic 96-well plates. A 1:10 v:v of 4 mM DEVD-pNA was added to each well of the 96-well plate and the plate was placed at 37 °C for 2-4 hours. Cleavage of the DEVD-pNA substrate was monitored colorimetrically at 405 nm in a microplate reader.

2.13. Transient transfection of CHO cells

Sub-confluent (20-50 %) CHO cells were grown on 10 cm² plates and transiently transfected using FuGeneVI (Roche Molecular Biochemicals) according to the manufacturers recommendations. For a single transfection, 12 μl of FuGeneVI was combined with 4 μg of plasmid DNA and incubated in 4 ml of complete medium at 37 °C in a 5 % CO₂/ 95 % air mixture for 24 hours, at which time a further 6 ml of complete medium was added. After a further 24-hour incubation period, cells were treated with 10 μM CCh for the appropriate time. For co-transfections 24 μl of FuGeneVI was combined with 4 μg of each of the 2 plasmid DNAs, per 10 cm² plate. Where serum-starvation was required it was performed after the 48-hour transfection period, before the appropriate CCh stimulation time.

Transiently transfected CHO-m3 cells were stimulated with CCh and lysates prepared as for ERK (for HA-tagged proteins) or CDK2 (for Myc-tagged proteins) immunoprecipitation. Cleared lysates were precleared with 70 μl of a 15 % (v/v) slurry of Protein A–Sepharose, before overnight incubation at 4 °C with a 1:40 dilution of a rabbit antiserum (200 μg/ml) directed against HA-probe [Y11: sc-805 (Santa Cruz)], or a 1:50 dilution of a rabbit antiserum directed against Myc-tag [2272 (Cell Signalling Technology)]. Immune complexes were incubated for 2 hours at 4 °C with 70 μl of a 15 % (v/v) slurry of Protein A–Sepharose and collected by centrifugation. Washes and kinase assays were performed as previously described for ERK, JNK or CDK2 immunoprecipitates depending on the over-expressed protein.

2.15. Reporter gene assays

CHO-m3 cells were transiently transfected with pAP-1-luciferase, p53-luciferase (Stratagene) or luciferase construct pGL2-143/+8p21-luciferase with the human p21<sub>Cip1/Waf1</sub> promotor (kind gift from Dr. Aristidis Moustakes, Ludwig Institute for Cancer Research, Uppsala, Sweden). After a 48-hour incubation period cells were serum-starved for 24 hours prior to stimulation with 10 μM CCh for the appropriate times as indicated in the Figure legends. Cells were washed 3 times in PBS and lifted in PBS containing 4 mM CaCl₂ and 4 mM MgCl₂ before being lysed and assayed in black 96-well plates using the LucLite™ reporter gene system according to the manufacturers instructions (Packard Bioscience). Lysates were dark-adapted for 10 min before being monitored for luciferase activity in a microplate reader.
2.16. Adenoviral infection of CHO cells

Replicant-deficient adenoviral-JBD and adenoviral-eGFP were kind gifts from Dr. J. Uney (University of Bristol). Both DNAs were inserted into a transfer vector (pXCXCMV), and were supplied at titres of $8 \times 10^{10}$ (JBD) and $9 \times 10^{9}$ (eGFP) plaque forming units (PFU). CHO-m3 cells were plated at 50,000 cells/well (24-well plate for DNA synthesis) or 100,000 cells/well (6-well plate for JNK immunoprecipitation and assay) and incubated overnight under normal culture conditions. Cells were then infected at a multiplicity of infection (MOI) of 400, and incubated in complete medium at 37 °C in a 5% CO$_2$ / 95% air mixture for 48 hours prior to treatment. Infected cells were washed 3-4 times in PBS and stimulated with 10 μM CCh for the appropriate time, before being assayed appropriately.
Chapter 3

Cell Growth Regulation by $M_2$ and $M_3$ ACh Receptors
Every eukaryotic cell has a finite life span. The ability to control this life span by regulating the rate at which a cell proliferates and eventually dies is the basis of the ability of an organism to develop and survive. Uncontrolled regulation of cells results in a host of growth disorders disrupting the ability of an organism to function. Growth disorders such as cancers (Catzevelos et al., 1997; Loda et al., 1997; Schutte et al., 1997), angiogenesis-related illness (Zhang et al., 2000), Alzheimer's disease (Howie et al., 1985) and atherosclerosis (Nachtigal et al., 1998), are all at least in part due to dysregulation of the cell cycle. Indeed any disease condition that involves hyper- or hypoplastic growth can be attributed to an unsuccessful cell division cycle. Chromosomal disorders such as Down Syndrome are caused by errors within the cell cycle, and the thickening of the airways smooth muscle, a factor contributing to asthma, is a result of ineffective cell cycling. Understanding the mechanisms that regulate the cell cycle is therefore very important, and the ability to manipulate the cell cycle, or cell cycle components is essential if we are to target therapies against many of these disease conditions.

Muscarinic ACh receptors have been implicated in growth control in a number of cellular settings. The receptors have been shown to transform cells (Gutkind et al., 1991; Stephens et al., 1993) as well as acting as tumour suppressors (Felder et al., 1993; Detjen et al., 1995), and thus have a wide-ranging impact on growth regulation. Activation of the M₃ ACh receptor has been shown to have divergent effects on cell growth depending on the growth condition under which cells are cultured (Nicke et al., 1999), emphasising the complexity of the involvement of these receptors in cellular growth control. Muscarinic ACh receptor sub-types are differentially expressed and so differences in growth response to receptor activation are likely to be dependent on both the sub-type of receptor and its tissue-specific expression.

The aim of this Chapter was to determine the growth effects mediated by M₂ and M₃ ACh receptors expressed in CHO cells and to investigate the cell cycle regulatory events involved in the growth responses. Identification of cell cycle proteins involved in the receptor-mediated growth effects would
allow a focused investigation into the pathways influencing the cell cycle, including specific protein kinases and transcription factors. The data presented demonstrate that activation of the M₂ and M₃ ACh receptors lead to divergent growth effects that are dependent on the cell growth conditions. The receptor-mediated effects involve changes in cell-cycle protein expression and activities, and the possible regulation of these cell cycle effects are discussed with particular emphasis placed on the M₃ ACh receptor subtype.
RESULTS

3.1. The Effect of CCh on DNA Synthesis in CHO-m2 and CHO-m3 cells

Activation of muscarinic $M_3$ ACh receptors has been shown to exert opposing effects on DNA synthesis in NIH3T3 fibroblasts, depending on the growth condition of the cell (Nicke et al., 1999). Fig. 3.1 shows the effect of the ACh receptor agonist carbachol (CCh) on DNA synthesis, as measured by $[^{3}H]$thymidine incorporation, in CHO cells expressing either the muscarinic $M_2$ or $M_3$ ACh receptor. Activation of the $M_2$ ACh receptor under serum-starved conditions (Fig. 3.1A) resulted in a 2.9 fold increase in DNA synthesis over the basal level at a maximally effective concentration of 10 $\mu$M CCh. By contrast, $M_3$ ACh receptor activation in growing CHO-m3 cells (Fig. 3.1B) caused a decrease in DNA synthesis. At 10 $\mu$M CCh, DNA synthesis was maximally inhibited by 77% compared to control levels. DNA synthesis was examined over a 24 h time-course of receptor activation in the two cell lines in the presence of 10 $\mu$M CCh (Fig. 3.2.) In serum-starved CHO-m2 cells, an increase in DNA synthesis could be detected after 12 h of CCh addition, and was maximal by 24 h of agonist treatment (Fig 3.2A). The inhibitory effect of CCh on DNA synthesis in growing CHO-m3 cells (Fig. 3.2B) occurred more rapidly than the stimulation observed in the CHO-m2 cells. For example, 6 h after CCh addition, DNA synthesis was inhibited by 48% in CHO-m3 cells, whereas no effect was seen after 6 h of agonist stimulation of CHO-m2 cells. By 12 h after agonist addition to CHO-m3 cells, inhibition was almost maximal (72%) and by 18 h the DNA synthesis was inhibited by 82% compared to control levels (Fig. 3.2B). In growing CHO-m2 cells (Fig. 3.3A), 10 $\mu$M CCh did not cause any change in DNA synthesis compared to control levels demonstrating that CCh is only mitogenic under serum-starved conditions. Under serum-starved conditions, a modest inhibition of DNA synthesis by CCh could still be observed in CHO-m3 cells (38% inhibition compared to 76% inhibition in the presence of serum at 24 h (Fig. 3.3B)), suggesting that serum starvation is insufficient to drive CHO-m3 cells fully into quiescence, at least within the time-frame of the experiments reported here. Indeed, cyclin D1 protein expression could still be detected by Western blotting of
Figure 3.1. Concentration-dependent effects of CCh on DNA synthesis in CHO-m2 and CHO-m3 cells. Cells were stimulated for 24 h with the indicated concentrations of CCh and incubated with 2 μCi of [3H]thymidine for the final 2 h. DNA synthesis was measured using the ether extraction method as outlined under Materials and Methods. Control values represent cells that were not treated with CCh. A, CHO-m2 cells were serum-starved for 24 h prior to CCh addition. B, a growing population of CHO-m3 cells was treated with CCh. All values represent the means of 4 independent determinations, and errors shown are standard errors of the mean. Data shown are representative of 3-10 separate experiments.
Figure 3.2. 24 h time-courses showing the effect of 10 μM CCh on DNA synthesis in CHO-m2 and CHO-m3 cells. Cells were stimulated with 10 μM CCh, or an equivalent addition of water (Control) for the times indicated and incubated with 2 μCi of [3H]thymidine for the final 2 h. DNA synthesis was measured using the ether extraction method as outlined under Materials and Methods. A, CHO-m2 cells were serum-starved 24 h before CCh addition. B, Growing CHO-m3 cells were treated with CCh. All values represent the means of 4 independent determinations, and errors shown are standard errors of the mean. Data shown are representative of 3-10 separate experiments.
Figure 3.3. Effect of CCh on DNA synthesis in CHO-m2 and CHO-m3 cells. Cells were stimulated for 24 h with 10 μM CCh and incubated with 2 μCi of [3H]thymidine for the final 2 h. DNA synthesis was measured using the ether extraction method as outlined under Materials and Methods. Control values represent cells that were not treated with CCh. A, a growing population of CHO-m2 cells was treated with CCh. B, CHO-m3 cells were serum-starved for 24 h prior to CCh addition. All values represent the means of 4 independent determinations, and errors shown are standard errors of the mean. Data shown are representative of 3-10 separate experiments.
serum-starved CHO-m3 cells (data not shown). Pre-incubation with atropine ablated the M3 (Fig 3.4A) and M2 (Fig. 3.4B) ACh receptor-mediated growth responses demonstrating that the changes in DNA synthesis are a direct consequence of receptor activation. In these experiments and all subsequent experiments, CCh has been used as the ACh receptor agonist. Preliminary experiments utilized an alternative muscarinic agonist, methacholine (MCh), but discrepancies were discovered between MCh and CCh on the growth effects in CHO cells. Fig. 3.5 shows concentration-dependent effects of CCh and MCh on DNA synthesis in CHO-m2 and CHO-m3 cells. Under serum-starved conditions there was no significant difference between the two agonists in promoting cell proliferation in CHO-m2 cells (Fig. 3.5A). However, the M3-mediated inhibition of DNA synthesis in growing cells appears to be dependent on the agonist used (Fig. 3.5B). At 10 μM CCh, DNA synthesis was inhibited by 77% compared to control levels, whereas only a 10% decrease in DNA synthesis was seen after stimulation with 10 μM MCh. It is likely that the discrepancy in agonist efficacies and potencies arises from the growth conditions of the cells as differences are only seen in growing cells. It is possible that the newborn calf serum contains cholinesterase activity capable of enzymically degrading ACh and MCh, but not CCh (whose structure makes it resistant to degradation). Rapid degradation of MCh would effectively reduce the concentration of the agonist present to stimulate the receptor until high concentrations were added, as seen in Fig. 3.5B.

3.2. The Nature of the Receptor-Mediated Inhibition of DNA Synthesis Observed in CHO-m3 Cells

Having ascertained that activation of the M3 ACh receptor causes a dramatic decrease in DNA synthesis, it was important to determine the nature of this inhibition. It could result from the induction of a pro-apoptotic response, a general decrease in protein synthesis, or a specific block of the cell cycle. Fig. 3.6 shows the results of FACS analysis performed on exponentially growing CHO-m3 cells stimulated with CCh for 48 h. The profiles indicated an increase in the proportion of cells in G1 with a resulting decrease of cells in S phase and in G2/M. The Table summarises data from a number of independent experiments in which CHO-m3 and CHO-m2 cells were stimulated with CCh for 24 or 48
Figure 3.4. Effect of atropine on CCh-mediated DNA synthesis in CHO-m2 and CHO-m3 cells. Cells were treated with 1 or 10 μM atropine for 30 min prior to stimulation with 10 μM CCh (+) for 24 h. Cells not stimulated with CCh (-) were given an equivalent atropine incubation. Cells were incubated with 2 μCi of [3H]thymidine for the final 2 h. DNA synthesis was measured using the ether extraction method as outlined under Materials and Methods. Control values represent cells that were not treated with atropine. A, a growing population of CHO-m3 cells was treated with CCh. B, CHO-m2 cells were serum-starved for 24 h prior to CCh addition. All values represent the means of 4 independent determinations, and errors shown are standard errors of the mean. Data shown are representative of 3-10 separate experiments.
Figure 3.5. Concentration-dependent effects of CCh and MCh on DNA synthesis in CHO-m2 and CHO-m3 cells. Cells were stimulated for 24 h with the indicated concentrations of agonist and incubated with 2 μCi of [³H]thymidine for the final 2 h. DNA synthesis was measured using the ether extraction method as outlined under Materials and Methods. Control values represent cells that were not treated with agonist. A, CHO-m2 cells were serum-starved for 24 h prior to CCh addition. B, a growing population of CHO-m3 cells was treated with CCh. All values represent the means of 4 independent determinations, and are represented as the percentage of control values, where the control is 100 %. Standard errors of the mean have been calculated and are represented within the symbols. Data shown are representative of 3-10 separate experiments.
Figure 3.6. **Fluorescence-Activated Cell Sorting (FACS) Analysis** of growing CHO-m2 and CHO-m3 cells. Growing CHO-m2 and CHO-m3 cells were treated with 10 μM CCh for 24 and 48 h. Control cells were not treated with CCh. Cells were lifted, fixed in methanol at -20 °C, and then stained overnight with propidium iodide for FACS analysis. 2n represents the normal diploid number and 4n the replicated DNA. A, representative profiles of control and 48 h CCh-treated CHO-m3 cells respectively. B, tabulated data showing the percentage of CHO-m3 and CHO-m2 cells in G₁, S and G₂/M phases of the cell cycle. Errors shown represent standard errors of the mean.
h in the presence of serum (Fig. 3.6B). In the control CHO-m3 cells, around 56 % of cells are in G\textsubscript{1} with 25 % in S phase and 18 % in G\textsubscript{2}/M. After 48 h of CCh stimulation 80 % of cells were in G\textsubscript{1}, a proportional increase of 24 % over the control cell population. Only 10.6 % and 9.2 % of cells were in S phase and G\textsubscript{2}/M respectively after incubation in the presence of CCh for 48 h. This profile is indicative of a population of cells that have been growth arrested at the G\textsubscript{1}-phase of the cell cycle. The Table also includes data for the CHO-m2 cells which show no corresponding changes in cell cycle phase in the presence of CCh and which serve as a control. To confirm these findings, it was important to exclude the possibility of a pro-apoptotic response contributing to the inhibition of growth. Fig. 3.7 shows the results of a caspase-3 assay performed on an asynchronous population of CHO-m3 cells. The Figure demonstrates that incubation with CCh for 24 h did not increase caspase-3 activity, and by inference, apoptotic activity, compared to a positive control (etoposide). This was confirmed by demonstrating that the inhibition of DNA synthesis is a reversible phenomenon, as shown by the re-establishment of DNA synthesis upon atropine addition (Fig. 3.8). The induction of an apoptotic pathway by CCh would result in an irreversible effect that is clearly not seen.

### 3.3. Muscarinic ACh Receptor Activation Inhibits CDK2 and Cyclin-Associated Kinase Activity in CHO-m3 Cells

Because growth inhibition of CHO-m3 cells by CCh is due to a G\textsubscript{1}-phase block of the cell cycle, proteins known to be involved in the regulation of G\textsubscript{1}-phase progression were investigated. We therefore assessed the activation of CDKs following CCh stimulation of CHO-m3 cells, because they exert their regulatory effects on the cell cycle by direct phosphorylation of retinoblastoma protein (pRb). Fig. 3.9A shows a time-course of CDK2 activity following immunoprecipitation from CHO-m3 cells stimulated with CCh. CCh inhibited CDK2 with a time-course similar to that observed for DNA synthesis (Fig 3.2B). By 6 h of CCh incubation the CDK2 activity was inhibited by 38 % compared to control, and at 24 h the activity was inhibited by 68 %. This observation suggests that activation of the M\textsubscript{3} ACh receptor inhibits CDK2 activity, leading to an inhibition of DNA synthesis, and the accumulation of cells in G\textsubscript{1}. Because other CDKs are known to be involved in this transitional point,
Figure 3.7. **Effect of CCh on the induction of apoptosis in growing CHO-m3 cells.** Cells were treated for 24 h with 10 μM CCh or left untreated as control cells. An overnight addition of 250 μM etoposide was made as a positive control. Cells were lifted, and assayed for caspase activity as described in *Materials and Methods.* Data represent n=3 and errors shown are standard errors of the mean. Data presented represent the fold-over-basal activity with respect to control cells.
Figure 3.8. The CHO-m3-mediated inhibition of DNA Synthesis is a reversible phenomenon. Growing CHO-m3 cells were stimulated with 10 μM CCh or left untreated as control cells. 24 h after CCh addition one population of cells were treated with 10 μM atropine. 48 h after CCh addition a second population of cells were treated with 10 μM atropine. A third population of cells were left without atropine addition. Cells were incubated with 2 μCi of [3H]thymidine for the final 2 h. DNA synthesis was measured using the ether extraction method as outlined under Materials and Methods. A, cells stimulated with CCh or left unstimulated (control) were challenged with atropine for 24 or 48 h before DNA synthesis was measured. Data points represent n=4 and errors shown are standard errors of the mean. B, schematic figure describing the methodology of the experiment. The 3 populations of cells (A, B and C) have all been treated with CCh (0 h) before atropine addition at different time points. This protocol was also performed on cells not treated with CCh (control).
Figure 3.9. Effect of CCh on cyclin-dependent kinase activities in growing CHO-m3 cells. CHO-m3 cells were left untreated (0 time point) or stimulated with 10 µM CCh for the indicated times. Cyclins and CDKs were immunoprecipitated from lysed cells using appropriate antisera and kinase activities were measured using histone H1 as a substrate. Specific activity was calculated after determination of the protein content of cleared cell lysates using a Bradford protein assay. All values represent n=4 and errors are standard errors of the mean. A, CDK2 was assayed from CHO-m3 cell lysates after addition of CCh at the time-points shown. B, CDK2, CDK4, cyclin A, cyclin D1 and cyclin E were immunoprecipitated and associated kinase activity was measured. Control (-) and 24 h CCh (+) treated samples were assayed. Data shown represent percentage kinase activity of each protein with respect to its individual control. A one-tailed, paired Student’s t-test was used as a measure of significance. Significant difference (p<0.05) is indicated (*).
the effects of CCh on CDK2 and CDK4 activity, and on cyclin D1-, cyclin E- and cyclin A-associated kinase activities were also examined in parallel (Fig. 3.9B). CCh had a significant inhibitory effect ($p < 0.05$; assessed by Student's $t$-test) on CDK2 activity as well as on both cyclin E- and cyclin A-associated kinase activities. CDK2 activity was inhibited by 66 %, whereas cyclin A- and cyclin E-associated kinase activities were inhibited by 42 % and 40 %, respectively. Cyclin E and cyclin A are known to associate with, and activate, CDK2 during G1/S-phase and therefore the CDK activity associated with these cyclins is likely to reflect CDK2 activity. However, it is also likely that the inhibition of cyclin A- and cyclin E-associated kinase activities by CCh is not as great as that observed in CDK2 immunoprecipitates because additional CDK proteins may associate with these cyclins. CCh did not have a significant effect ($p > 0.05$) on CDK4 activity or on the kinase activity associated with cyclin D1, which specifically activates CDK4 and CDK6 at the G1/S checkpoint. Thus, CDK2 activity is inhibited by M3 ACh receptor activation as assessed by direct kinase assays either of immunoprecipitated CDK2 or of CDK2 associated with immunoprecipitated cyclins A and E. CCh had no significant effect on CDK4 activity in CHO-m3 cells, and M3 ACh receptor activation is also unlikely to inhibit CDK6 based on the lack of inhibition of cyclin D1-associated kinase activity.

It is possible that changes in CDK activity may also contribute to the CHO-m2-mediated increase in DNA synthesis. Fig. 3.10 shows CDK2 and CDK4 activity after immunoprecipitation from serum-starved CHO-m2 cells following incubation with CCh or FCS. There is no detectable change in CDK activity after receptor stimulation compared with the FCS positive control. The observation that basal CDK2 activity is much greater in these cells than basal CDK4 activity agrees with findings in the CHO-m3 cell line. An obvious explanation for this is that the CDK4 antibody is not as efficient as the CDK2 antibody at immunoprecipitation. However the finding that any immunoprecipitation performed either directly or indirectly (cyclin D1-associated kinase) to measure CDK4 activity results in lower activities than any CDK2 or CDK2-associated immunoprecipitation suggests that CDK2 activity is greater than CDK4 activity in these cells.
Figure 3.10.  Effect of CCh on cyclin-dependent kinase activities in serum-starved CHO-m2 cells. CHO-m2 cells were serum-starved for 24 h before stimulation with 10 μM CCh or 10 % FCS for 24 h. Control cells were left untreated after serum starvation. CDK2 (dark bars) and CDK4 (light bars) were immunoprecipitated from lysed cells using appropriate antisera and kinase activities were measured using histone H1 as a substrate. Specific activity was calculated after determination of the protein content of cleared cell lysates using a Bradford protein assay. CDK2 activity is represented on the left hand axis and CDK4 activity on the right hand axis. All values represent n=4 and errors are standard errors of the mean.

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3.4. Muscarinic Receptor Activation Selectively Induces p21^{Cip1/Waf1} Expression in CHO-m3 and CHO-m2 Cells

Changes in cyclin expression can regulate CDK activity. Therefore Western blot analysis was performed to detect changes in expression of cyclin D1, cyclin E and cyclin A following agonist stimulation of CHO-m3 cells. No changes in protein levels of these cyclins were detected after 24 h of CCh treatment (Fig. 3.11, first three panels) or over a 24 h time-course (data not shown). Fig. 3.11 also shows that the levels of CDK2 or CDK4 protein did not change following agonist addition. Thus, M₃ ACh receptor-mediated CDK2 inhibition does not appear to involve expression changes of CDK2 or the cyclins with which it associates. CDKIs are another class of cell cycle protein capable of regulating CDKs. Fig. 3.11 (middle three panels) shows three CDKI proteins that were investigated by Western blot analysis. Although no changes in expression of p27^{Kip1} or p16^{Ink4a} were observed following CCh treatment, the agonist clearly increased the expression of p21^{Cip1/Waf1}. Stimulation of CHO-m3 cells with CCh for 24 h led to a 4.5-fold increase in expression of p21^{Cip1/Waf1}, as measured by densitometry. Investigations of cell cycle protein expression changes were also performed in lysates prepared from serum-starved CHO-m2 cells (Fig 3.11). As with the CHO-m3 cells no detectable changes in cyclins or CDKs were observed. No changes in p27^{Kip1} were found however a modest increase in p21^{Cip1/Waf1} was detected. p16^{Ink4a} expression could not be detected due a lack of antibody availability at the time the experiments were performed.

The final panel shown in Fig 3.11 shows changes in electrophoretic mobility of pRb in CHO-m2 and CHO-m3 cells. pRb is a tumour suppressor protein that must be phosphorylated for G₁/S progression to occur. When cells are cycling, pRb exists exclusively in its hyperphosphorylated form (ppRb), releasing transcription factors that promote growth. pRb was hyperphosphorylated in the control growing CHO-m3 cells where it displayed a retarded electrophoretic mobility (0 time-point). After 24 h incubation in the presence of CCh, pRb became hypophosphorylated as shown by the appearance of the lower molecular weight form, indicative of a population of cells becoming growth arrested in G₁. Although the Western blot shown for the CHO-m2 cells is less clear, it appears to correlate with the
Figure 3.11. Effect of CCh on cell cycle protein expression in CHO-m2 and CHO-m3 cells. Growing CHO-m3 cells and serum-starved CHO-m2 cells were stimulated for 24 h with 10 µM CCh or left untreated as controls (0 time-point). Three independent cell lysates were generated per condition as shown. Cell lysates were prepared and Western Blotting analysis was performed. Proteins were detected using the appropriate antibodies as described in Materials and Methods. Data shown are representative of 3-10 Western Blots.
increase in DNA synthesis seen in this cell line. After the 24 h incubation in the presence of CCh an increase in expression of the hyperphosphorylated form of pRb was detectable.

p21^{Cipl/Waf1} can inhibit both CDK2 and CDK4/6 by direct association and this may account for the inhibition of DNA synthesis described in growing CHO-m3 cells. Western blot analysis of p21^{Cipl/Waf1} was performed on CDK2 immunoprecipitates to test whether CCh stimulates p21^{Cipl/Waf1} association with CDK2 in CHO-m3 cells. Agonist treatment increased the association of p21^{Cipl/Waf1} with CDK2 without affecting the amount of CDK2 immunoprecipitated (Fig. 3.12), confirming this as the likely mechanism for CDK2 inhibition.

3.5. Investigating the Mechanisms Underlying p21^{Cipl/Waf1} Protein Induction

As the G1-arrest mediated by the M3 ACh receptor involved an increase in expression of p21^{Cipl/Waf1} protein, it was important to investigate how this was occurring. Increases in p21^{Cipl/Waf1} protein levels can be mediated by an increase in protein stability (Park et al., 2000). To investigate this as a possible mechanism, protein synthesis in control and CCh-treated CHO-m3 cells was disrupted by cycloheximide and the rate of p21^{Cipl/Waf1} protein degradation measured in both cell populations by Western blot analysis of p21^{Cipl/Waf1} (Fig. 3.13). These data provided no indication of a difference in the rate of degradation between the control and CCh-treated cells, demonstrating that protein stability is not likely to be affected by M3 ACh receptor activation.

A second mechanism that could account for the receptor-mediated increase in p21^{Cipl/Waf1} protein expression is transcriptional regulation of p21^{Cipl/Waf1}. To address this possibility, RT-PCR was performed to measure receptor-mediated changes in p21^{Cipl/Waf1} mRNA. Preliminary experiments were designed to optimise the technique, the results of which are shown in Fig. 3.14. In order to detect receptor-mediated changes in mRNA it was important that the products were not saturated prior to receptor activation. For this reason 40 cycles were run for subsequent p21^{Cipl/Waf1} reactions and 25 cycles were completed for β-actin reactions. 0.2 µg of RNA was used for both sets of reactions.
Figure 3.12. Effect of CCh on \( p21^{Cp1/Waf1} \) association with CDK2 in CHO-m3 cells. Growing CHO-m3 cells were stimulated for 24 h with 10 μM CCh or left untreated as controls (0 time-point). Three independent cell lysates were generated per condition as shown. CDK2 was immunoprecipitated from control and 24 h CCh-stimulated cell lysates. \( p21^{Cp1/Waf1} \) and CDK2 were detected in these immunoprecipitates by Western blotting with appropriate antisera. Total cell lysate from a growing population of CHO-m3 cells stimulated with CCh were also blotted for \( p21^{Cp1/Waf1} \) and CDK2.
Figure 3.13. The involvement of protein stability in \( p21^{Cip1/Waf1} \) protein expression. Growing CHO-m3 cells were stimulated with 10 \( \mu M \) CCh for 24 h or left untreated as control cells. After 24 h cells were incubated further with 0.25 mg/ml cycloheximide and lysed at the times indicated. The 0 time-point represents cells with no cycloheximide addition. A, Western blots of \( p21^{Cip1/Waf1} \) were analysed densitometrically for the times indicated after cycloheximide addition. The data represent \( n=3-4 \) and errors shown are standard errors of the mean. B, representative Western blots of \( p21^{Cip1/Waf1} \) in the control and CCh treated cell populations following cycloheximide treatment.
Characterisation of RT-PCR performed on p21<sup>Cip1/Waf1</sup> and β-actin RNA templates. Reverse transcription was performed on the total RNA extracted from an asynchronous population of CHO-m3 cells. p21<sup>Cip1/Waf1</sup> mRNA and β-actin mRNA was detected by PCR using the transcribed RNA as a template. PCR was performed on 0.2 and 1 μg RNA at increasing PCR cycles as shown. Also shown is the pUC19 DNA/Msp1 marker (MBI Fermentas) that contains 13 DNA fragments ranging from 501 to 26 base pairs.
Following 2 h CCh stimulation of CHO-m3 cells, p21^{Cip1/Waf1} mRNA was increased by 2.6-fold relative to the control (Fig. 3.15) as measured by densitometry, and returned to basal levels by 24 hours (data not shown). No changes in β-actin mRNA could be seen after CCh incubation demonstrating that the increase in p21^{Cip1/Waf1} mRNA was specific.
Figure 3.15. Effect of CCh on p21\textsuperscript{Cip1/Waf1} mRNA levels in growing CHO-m3 cells. Growing CHO-m3 cells were stimulated with 10 μM CCh for 1 or 2 h or left untreated as controls. Reverse transcription was performed on the total RNA extracted from the cells, and p21\textsuperscript{Cip1/Waf1} mRNA was detected by PCR using the transcribed RNA as a template. Control reactions were also performed using primers specific for β-actin. Each individual lane for duplicate conditions was generated from a separate RNA extraction of two independent wells of cells. Also shown is the pUC19 DNA/MspI marker (MBI Fermentas) that contains 13 DNA fragments ranging from 501 to 26 base pairs.
**DISCUSSION**

The present Chapter has presented data that examine the growth regulatory effects of the M₂ and M₃ ACh receptors stably expressed in CHO cells. The data presented indicate that activation of the M₃ ACh receptor leads to growth arrest. This arrest occurs during the G₁-phase of the cell cycle and appears to be a consequence of increased p21Cip1/Waf1 mRNA and protein levels, decreased CDK2 activity and hypophosphorylation of pRb (see Figure 3A). These effects are consistent with the reported role of the M₃ ACh receptor as a tumour suppressor (Detjen *et al.*, 1995). The finding that activation of the M₂ ACh receptor, which preferentially couples to G₁/o rather than G₉, evokes a proliferative response may have important physiological implications and shows the diverse cellular functions mediated by a single sub-family of GPCR.

![Diagram](image)

**Figure 3A.** Summary of the experimentally determined findings described in Chapter 3. pRb-Phos represents the hyperphosphorylated form of pRb. See text for details.
Of the cell cycle proteins examined only the expression of p21\textsuperscript{Cip1/Waf1} was found to change in response to M\textsubscript{3} ACh receptor activation. M\textsubscript{3} ACh receptor stimulation also increased p21\textsuperscript{Cip1/Waf1} association with CDK2, suggesting this as the mechanism for CDK2 inhibition and G\textsubscript{1} arrest. Although the agonist-induced increase in total p21\textsuperscript{Cip1/Waf1} protein expression was relatively modest, the activity of CDKs is dependent on small stoichiometric changes within the CDKI/CDK complexes, rather than large changes in total cellular protein expression (LaBaer \textit{et al.}, 1997). For example, a 1.8-fold increase in p21\textsuperscript{Cip1/Waf1} protein expression was sufficient to arrest IEC-6 cells in G\textsubscript{1} after polyamine depletion (Ray \textit{et al.}, 1999). Similarly a 2.5-fold increase in p21\textsuperscript{Cip1/Waf1} association with CDK2 caused G\textsubscript{1} arrest of bladder carcinoma cells (Schnier \textit{et al.}, 1996). p21\textsuperscript{Cip1/Waf1} has been shown to have a role in G\textsubscript{1} arrest in many cell systems and it can bind to and inhibit the kinase activity of cyclin D1/CDK4 and cyclin E/CDK2 complexes (Hennigan and Stambrook, 2001). Experiments using p21\textsuperscript{Cip1/Waf1} antisense RNA to block a TGF-\textbeta-mediated G\textsubscript{1} arrest indicated that expression of p21\textsuperscript{Cip1/Waf1} was sufficient to cause G\textsubscript{1} arrest of gastric-carcinoma cells (Yoo \textit{et al.}, 1999). Although the induction of p21\textsuperscript{Cip1/Waf1} by M\textsubscript{3} ACh receptor activation caused a marked inhibition of CDK2 activity, it appeared to have little effect on CDK4 activity. In an asynchronous population of growing CHO-m3 cells, the activity of CDK2 was high compared to that of CDK4. One possibility is that p21\textsuperscript{Cip1/Waf1} will bind and inhibit both CDK complexes equally well, but that the low basal CDK4 activity is insufficient to detect any significant effects mediated by p21\textsuperscript{Cip1/Waf1}. However, our current studies suggest that CDK2 activity is more important than CDK4 in regulating G\textsubscript{1} progression and growth arrest in this cell line.

Although we believe the data presented provide strong evidence for the involvement of p21\textsuperscript{Cip1/Waf1} in the growth inhibition, other mechanisms of CDK2 regulation could be contributing to the growth inhibition. An increase in p21\textsuperscript{Cip1/Waf1} expression was difficult to detect by Western blot analysis after a 6 h CCh stimulation in CHO-m3 cells (data not shown). This would suggest either that any increase in p21\textsuperscript{Cip1/Waf1} expression was too small to detect at this time or that there is no increase in p21\textsuperscript{Cip1/Waf1} protein levels at a 6 h time-point. After a 6 h CCh incubation period, CDK2 activity was decreased by 38 % compared with control cells (Fig. 3.9A). If the level of p21\textsuperscript{Cip1/Waf1} is not increased by this time-point another mechanism is likely to be causing the initial decrease in CDK2 activity, with p21\textsuperscript{Cip1/Waf1}
contributing to the reduction in activity at later time-points. Experiments have determined that CCh does not affect the expression of cdc25A, the protein phosphatase responsible for CDK dephosphorylation at the G1/S checkpoint (data not shown), although experiments have not determined whether the CDK2 phosphorylation state changes after M3 ACh receptor stimulation. A receptor-mediated change in CDK2 phosphorylation could implicate other proteins for example cyclin-activating kinase (CAK) in the growth inhibition.

The increased p21\textsuperscript{Cipl/Waf1} protein levels induced by the M\textsubscript{3} ACh receptor appear to be mediated by an increase in p21\textsuperscript{Cipl/Waf1} mRNA rather than by increasing protein stability because the rate of p21\textsuperscript{Cipl/Waf1} degradation in the presence of the protein synthesis blocker cycloheximide was unaffected by agonist addition. However, other regulatory mechanisms such as protein translation may also contribute to the increased p21\textsuperscript{Cipl/Waf1} expression. There are many potential sites of transcriptional regulation at the p21\textsuperscript{Cipl/Waf1} promoter, and factors such as p53, AP-1 and SP-1 can influence p21\textsuperscript{Cipl/Waf1} transcription (Gartel and Tyner, 1999). The tumour suppressor p53 is induced by agents that cause growth arrest such as DNA damage, and is an upstream transcriptional activator of p21\textsuperscript{Cipl/Waf1} (Agarwal et al., 1998). In either stimulated or unstimulated CHO-m3 cells it has been difficult to detect p53 using commercially available antisera (data not shown), suggesting that p53 expression may be very low in these cells. Incubation with the DNA damaging agent cis-platinum did not cause an increase in p53 expression in the CHO-m3 cells suggesting that the p53 pathway is not functional in these cells (data not shown). It is possible that the M\textsubscript{3} ACh receptor may induce changes in p53 phosphorylation, although further work will be required to examine this possibility. The transcription factor SP-1 can be activated by increased association with c-jun as well as JunB, JunD and ATF-2, leading to transactivation of the p21\textsuperscript{Cipl/Waf1} promoter (Kardassis et al., 1999). Western blot analysis has shown that SP-1 expression in CHO-m3 cells is not changed after agonist addition (data not shown). However, preliminary experiments suggest that CCh enhances the association of SP-1 and c-jun in CHO-m3 cells (not shown) and this may contribute to activation of the p21\textsuperscript{Cipl/Waf1} promoter.
It is possible that M₃ ACh receptor activation could induce Gᵢ arrest by disrupting a growth factor signalling pathway that acts to suppress p21^Cip1/Waf1 promoter activity in the presence of serum factors. The ability of GPCRs to influence growth factor receptor signalling has been well documented (Leserer et al., 2000). For example, MAPK activation by G_q and G_i-coupled receptors can be mediated through agonist-independent transactivation of the epidermal growth factor (EGF) receptor (Daub et al., 1997). Similarly, the G_q-coupled 5-HT₂B receptor has been shown to cause cell proliferation through activation of the platelet-derived growth factor (PDGF) receptor via a mechanism involving c-src (Nebigil et al., 2000). The AP-1 complex has been implicated in cell proliferation by interaction with the cyclin D1 promoter, and expression of c-jun or another component of AP-1, c-fos, has been shown to induce cyclin D1 mRNA expression (Albanese et al., 1991; Miao and Curren, 1994). However, recent reports suggest that AP-1 can also stimulate growth by repressing p21^Cip1/Waf1 expression. A conditionally active component of AP-1, c-fos, was shown to down-regulate p21^Cip1/Waf1 promoter activity leading to cell cycle progression (Crowe et al., 2000). Furthermore, dominant-negative c-jun has been proposed to cause a Gᵢ arrest by affecting AP-1 interaction with the promoter of the p21^Cip1/Waf1 gene rather than by altering the expression of cyclin or CDK proteins (Hennigan and Stambrook, 2001). Therefore, activation of the M₃ ACh receptor could be alleviating an AP-1-mediated suppression of the p21^Cip1/Waf1 promoter under the tonic control of a growth factor signalling pathway. However, the simplest hypothesis would be that p21^Cip1/Waf1 regulation is due to the activation of a transcription factor enhancing p21^Cip1/Waf1 promotional activity.

The M₂ ACh receptor-mediated increase in proliferation occurred over a longer time-course than the M₃ ACh receptor-mediated inhibition of DNA synthesis. No significant increase in DNA synthesis occurred until 18 h of CCh incubation. It may be that M₂ ACh receptor activation stimulates the production of a mitogen (e.g. growth factor) essential to CHO cell growth that was removed by serum withdrawal. Stimulation of the M₃ ACh receptor caused an 80 % decrease in DNA synthesis, whereas the M₂ ACh receptor-mediated increase in proliferation was more modest at 2.9 fold-over basal (Fig. 3.1). This meant that any change in cell cycle protein expression or activity was likely to be more
modest and therefore more difficult to detect in the CHO-m2 cells. The only effect mediated on the cell cycle proteins investigated in response to $M_2$ ACh stimulation was an increase in $p21^{CIP/WAF1}$. This was unexpected as increased CDKI expression is normally associated with growth inhibition rather than increased proliferation. However there are examples of CDKIs being involved in proliferation and their involvement as scaffolds for the assembly of cyclin/CDK complexes as well as reports demonstrating their occurrence in active CDK complexes suggest that they may have more diverse functions than simple inhibitors of CDKs (Zhang et al., 1994; LaBaer et al., 1997; Weiss et al., 2000). If this were the case in the CHO-m2 cells, a more modest increase of $p21^{CIP/WAF1}$ would be expected compared to the CHO-m3 cells where $p21^{CIP/WAF1}$ inhibits the CDK activity. This is a very difficult question to address as it relies on densitometric analysis that is not particularly quantitative. A second way to investigate this idea would be to perform immunoprecipitation/Western blot analysis to measure the association of $p21^{CIP/WAF1}$ with CDK complexes in the CHO-m2 and CHO-m3 cells. This is a very difficult technique to perform and again relies on densitometry for quantitative analysis. The increase in $p21^{CIP/WAF1}$ may be involved in the increase in proliferation after $M_2$ ACh receptor activation, however no increase in CDK2 or CDK4 activity was detected, contradicting the role of $p21^{CIP/WAF1}$ in CDK complex assembly. As with the CHO-m3 cells the level of basal CDK4 activity was much lower than basal CDK2 activity. This may be a genuine effect in CHO cells, however if the immunoprecipitation was less efficient for CDK4 than for CDK2 any receptor-mediated change in CDK4 activity would be more difficult to detect. This leaves the possibility that the modest increase in $p21^{CIP/WAF1}$ could be aiding the assembly of CDK4 complexes resulting in increased CDK activity and increased proliferation. Transfection of antisense $p21^{CIP/WAF1}$ into several cell lines caused a decrease in association of cyclin D1/CDK4 complexes, but not cyclin E/CDK2 complexes (Wiess et al., 2000), which could account for the findings presented in this Chapter.

In summary this chapter has shown that activation of the $M_3$ ACh receptor causes a rapid inhibition of DNA synthesis in growing CHO-m3 cells. An induction of $p21^{CIP/WAF1}$ protein expression in response to $M_3$ ACh receptor activation involves an increase in $p21^{CIP/WAF1}$ mRNA and results in an increased association of the CDKI with CDK2. Enhanced formation of CDK2/$p21^{CIP/WAF1}$ complexes in response
to M₃ ACh receptor activation correlates with a decrease in CDK2 activity and is likely to contribute to the accumulation of hypophosphorylated pRb and the G₁-phase arrest. Our data have also shown that M₂ ACh receptor activation causes an increase in cellular proliferation that correlates with a modest increase in p2¹Cipl/Wraf protein expression that may be involved in the receptor-mediated growth effects.

In Chapter 4, M₂ and M₃ ACh receptor-mediated activation of MAPK cascades will be investigated and pharmacological inhibitors of these pathways used to assess their involvement in the receptor-mediated growth effects.
Chapter 4

A Pharmacological Investigation into the Involvement of MAPK Signalling Cascades in the M₃ ACh Receptor-Mediated Inhibition of Growth
INTRODUCTION

MAPKs have been widely implicated in cellular growth control, and can mediate processes as diverse as proliferation, differentiation and apoptosis. MAPKs can influence growth, and in particular the cell cycle, at a number of levels and as the research area progresses, more possible MAPK-mediated mechanisms for growth regulation arise.

The first evidence for MAPK involvement in growth regulation came from experiments demonstrating that transient expression of either the complete ERK1/2 antisense RNA or a kinase-deficient form of ERK1/2 was sufficient to abrogate growth factor-induced fibroblast proliferation (Pages et al., 1993). MAPKs have been implicated in CDK regulation by a number of mechanisms. Cyclin D1 protein expression can be regulated both positively and negatively by ERK and p38 respectively, at the level of the cyclin D1 promoter (Lavoie et al., 1996). In addition, JNK can directly phosphorylate and activate c-jun, a subunit of the transcription factor activator protein 1 (AP-1). c-jun appears to have a critical role in G1 progression through direct transcriptional control of cyclin D1 via an AP-1 site in the promoter region of the gene (Wisdom et al., 1999). CDK regulation by MAPKs can also occur independently of cyclin expression. For example, inhibition of ERK activity has been shown to correlate with CDK2 inhibition in NIH 3T3 cells via a mechanism involving CDK-activating kinase (CAK), the enzyme responsible for CDK phosphorylation at its activating phosphorylation site, threonine-160 (Chiariello et al., 2000). MAPKs may also influence the cell cycle via CDKIs. For example, the direct association between p21\textsuperscript{Cip1/Waf1} and JNK may contribute to cell cycle regulation by sequestering p21\textsuperscript{Cip1/Waf1} from CDKs (Patel et al., 1998). Alternatively ERK activation can increase the population of cells in S phase by decreasing p27\textsuperscript{Kip1} expression (Greulich et al., 1998). ERK appears to affect p27\textsuperscript{Kip1} expression by altering its rate of degradation, possibly via a proteasomal pathway, rather than by affecting its synthesis (Delmas et al., 2001). Concomitant ERK and p38 activity are required to growth arrest cells through the induction of p21\textsuperscript{Cip1/Waf1} protein levels (Alderton et al., 2001).
The aim of this Chapter is to assess the involvement of MAPK family members in the $M_3$ ACh receptor-mediated inhibition of DNA synthesis. Pharmacological inhibitors have been used to address this question including a recently developed JNK inhibitor that may be useful in assessing JNK function in CHO and other cell lines. The data presented demonstrate that the $M_2$ ACh receptor-mediated induction of proliferation is dependent of MAPK function whereas the $M_3$ ACh receptor-mediated growth arrest is mediated by an alternative mechanism. Data suggest that PKC may have a role in the growth inhibition, and the benefits and limitations of pharmacological inhibitors as investigative tools are discussed.
RESULTS

4.1. Muscarinic Receptor Stimulation Activates ERK in CHO-m2 and CHO-m3 cells

ERK has been widely implicated in growth control responses to extracellular stimuli in many different cellular systems. Stimulation of the M2 and the M3 ACh receptors in CHO cells has been shown to activate ERK (Wylie et al., 1999). However, it was important in the context of this study to re-examine the extent of ERK activation by these muscarinic receptors under the growth conditions where perturbations of DNA synthesis have been shown. Fig. 4.1 shows the ERK activation profiles in CHO-m2 and CHO-m3 cells over a 24 h period of CCh stimulation under serum-starved conditions. Peak ERK activation was observed after 5 min of agonist stimulation in both cell lines (specific ERK activities of 7.62 and 7.23 pmol ATP/min/mg in CHO-m2 and CHO-m3 cells, respectively), although a lower basal ERK activity in the CHO-m2 cells resulted in a higher fold-over basal value (20.9 compared with 12.1 for the CHO-m3 cells). However, ERK activation was more sustained in CHO-m3 cells. After 60 min of CCh treatment, ERK activity had returned towards basal levels in CHO-m2 cells whereas it remained elevated in the CHO-m3 cells (4.5 fold over basal). This may represent a model for transient versus sustained ERK activation that could account for the differential effects of CCh on the growth responses of CHO-m2 and CHO-m3 cells under serum-starved conditions.

In the presence of serum, basal ERK activity was high, resulting in small fold-over-basal increases in activity at 5 min in both cell lines in response to CCh (2.3-fold and 1.7-fold in CHO-m2 and CHO-m3 cells) (Fig. 4.2). The peak specific activities in both cell lines in the presence of serum (7.80 and 5.35 pmol ATP/min/mg in CHO-m2 and CHO-m3 cells, respectively) were similar to those obtained under serum-starved conditions, suggesting that the main difference with respect to ERK activation between the growth conditions is the dramatically increased basal activity seen in growing cells. However, a key difference between the two cell lines was apparent over the longer ERK time-course in the presence of serum. Whereas ERK activity returned to basal values by 2 h of CCh stimulation of CHO-m2 cells, ERK activity dropped significantly below basal levels in CHO-m3 cells at this time-point.
Figure 4.1. Time-course of ERK activation in serum-starved CHO-m2 and CHO-m3 cells. CHO-m2 (▲) and CHO-m3 (●) cells were serum-starved for 24 h prior to incubation with 10 μM CCh for the appropriate times as shown. Cells were lysed and ERK1 was immunoprecipitated using a selective antibody as described under Materials and Methods. Specific activity has been calculated for each time-point using a protein value obtained from a Bradford protein assay of the cleared cell lysates. Each data point represents n=4-8 and standard errors of the mean are shown. Western blot analysis of total ERK protein was also performed on lysates prepared for ERK assay and representative blots are shown.
Figure 4.2. Time-course of ERK activation in growing CHO-m2 and CHO-m3 cells. Asynchronous populations of CHO-m2 (△) and CHO-m3 (●) cells were incubated with 10 μM CCh for the appropriate times as shown. Cells were lysed and ERK1 was immunoprecipitated using a selective antibody as described under Materials and Methods. Specific activity has been calculated for each time-point using a protein value obtained from a Bradford protein assay of the cleared cell lysates. Each data point represents n=4-8 and standard errors of the mean are shown. Western blot analysis of total ERK protein was also performed on lysates prepared for ERK assay and representative blots are shown.
(0.38-fold compared to basal). At 6 h of agonist incubation, ERK activity in the growing CHO-m3 cells was 0.85 pmol ATP/min/mg, representing 0.27-fold of the basal value, and approaching the basal activity obtained under serum-starved conditions in this cell line (0.60 pmol ATP/min/mg). Thus, the high ERK activity remains elevated in growing CHO-m2 cells following CCh stimulation, whereas ERK activity in growing CHO-m3 cells is reduced by CCh to levels approaching those observed under serum-starved conditions over the long term. Western blot analysis of ERK1 and ERK2 proteins indicated that the total amount of ERK protein was not affected by CCh stimulation of CHO-m2 or CHO-m3 cells under either growth condition (Fig. 4.1 and 4.2, lower panels).

4.2. The Involvement of ERK in the M<sub>3</sub>-Mediated Decrease in DNA Synthesis

These time-course data suggest a testable working hypothesis: In growing cells, the M<sub>3</sub> receptor-mediated inhibition of basal ERK activity is causing the inhibition of growth. To test this hypothesis the MEK1 inhibitors U0126 and PD98059 were used to inhibit ERK activity in growing CHO-m3 cells. Fig. 4.3 shows the concentration-dependent effects of U0126 on DNA synthesis measured at 24 h, and ERK activation measured at 5 min of agonist stimulation. At 1 μM U0126 both basal and CCh-stimulated ERK activities were significantly inhibited (90 % and 85 % respectively compared to control cells) (Fig. 4.3B). At this concentration U0126 had no significant effect on the inhibition of DNA synthesis (Fig. 4.3A) suggesting that ERK is not involved in the growth inhibition. It was surprising to find that basal DNA synthesis was maintained under conditions where ERK was inhibited. However it may be that other pathways contribute to the maintenance of growth rates and can maintain basal rates of proliferation when ERK is inhibited. These findings were confirmed using PD98059 (Fig. 4.4). Although PD98059 was less effective at inhibiting ERK, the data support the idea that ERK is not involved in the growth inhibition.

These experiments demonstrate that the MEK1 inhibitors block the 5 min peak of ERK activation, but they do not determine how effective the inhibitors are over the 24 h time period assessed in the DNA synthesis experiments. Fig 4.5 shows a 24 h time-course of ERK activation in CHO-m3 cells.
Figure 4.3. **Concentration-dependent effects of U0126 on DNA synthesis and ERK activity in growing CHO-m3 cells.** Cells were treated with the indicated concentrations of U0126 for 30 min prior to CCh stimulation (▲). Basal cells (●) were treated with U0126 but not stimulated with CCh. Control values represent cells that were not treated with U0126. A, cells were stimulated for 24 h with 10 μM CCh and incubated with 2 μCi of [3H]thymidine for the final 2 h. DNA synthesis was measured using the ether extraction method as outlined under Materials and Methods. B, cells were stimulated for 5 min with 10 μM CCh, lysed and ERK1 was immunoprecipitated using a selective antibody as described under Materials and Methods. Specific activity has been calculated for each time-point using a protein value obtained from a Bradford protein assay of the cleared cell lysates. Each data point represents n=4-8 and standard errors of the mean are shown.
Figure 4.4. Concentration-dependent effects of PD98059 on DNA synthesis and ERK activity in growing CHO-m3 cells. Cells were treated with the indicated concentrations of PD98059 for 30 min prior to CCh stimulation (▲). Basal cells (●) were treated with PD98059 but not stimulated with CCh. Control values represent cells that were not treated with PD98059. A, cells were stimulated for 24 h with 10 μM CCh and incubated with 2 μCi of [3H]thymidine for the final 2 h. DNA synthesis was measured using the ether extraction method as outlined under Materials and Methods. B, cells were stimulated for 5 min with 10 μM CCh, lysed and ERK1 was immunoprecipitated using a selective antibody as described under Materials and Methods. Specific activity has been calculated for each time-point using a protein value obtained from a Bradford protein assay of the cleared cell lysates. Each data point represents n=4-8 and standard errors of the mean are shown.
Figure 4.5. **Effect of U0126 on long-term ERK activation in growing CHO-m3 cells.** Cells were treated with 1 μM U0126 for 30 min prior to stimulation with 10 μM CCh (○) at the time-points as shown. A second population of cells were treated with U0126 but not stimulated with CCh (▲), and a third population were only stimulated with CCh (●). Cells were lysed and ERK1 was immunoprecipitated using a selective antibody as described under *Materials and Methods*. Specific activity has been calculated for each time-point using a protein value obtained from a Bradford protein assay of the cleared cell lysates. Each data point represents n=4 and standard errors of the mean are shown.
incubated with CCh, U0126, or CCh with a 30 min pre-treatment with U0126. These data demonstrate that even at the later time-points, U0126 inhibits both basal and receptor-mediated ERK activation. If the receptor-mediated reduction in basal ERK activity were responsible for the reduction in DNA synthesis, then basal proliferation in the absence of agonist would be inhibited by addition of 1 µM U0126 over a 24 h time period. This is clearly not the case (Fig. 4.3A) and these observations refute the hypothesis that changes in ERK activity are causing the M₃-mediated inhibition of DNA synthesis.

The MEK1 inhibitors were also used to assess the contribution of ERK activation to the increase in proliferation seen in CHO-m2 cells. U0126 blocks the increase in proliferation in a concentration-dependent manner (Fig. 4.6A). U0126 (at 100 nM) reduced both basal and receptor-mediated proliferation. It is likely that the reduction of basal DNA synthesis is due to the incomplete serum-starvation of these cells observed throughout this project. Higher concentrations of U0126 reduced the receptor-stimulated DNA synthesis up to a maximally effective concentration of 3 µM U0126. These data were supported using an alternative MEK inhibitor with complete inhibition of DNA synthesis occurring at 10 µM PD98059 (Fig. 4.6B). Thus, ERK activation appears to be responsible for agonist-induced proliferation in CHO-m2 cells.

4.3. The Involvement of Other Protein Kinases and Second Messenger Pathways in the M₃-Mediated Inhibition of DNA Synthesis

Stimulation of the M₃ ACh receptor results in the activation of specific second messenger pathways that can influence the activity of many intracellular proteins such as PKC, PI3-K, and receptor and non-receptor tyrosine kinases. Any of these changes could be contributing to the growth inhibition seen in CHO-m3 cells. Activation of the Gₛ-coupled M₃ ACh receptor causes an increase in IP₃ and a subsequent rise in intracellular Ca²⁺ in CHO cells (Tobin et al., 1995; Wylie et al., 1999). Many proteins and transcription factors require Ca²⁺ to function and therefore, elucidation of the involvement of intracellular Ca²⁺ in the growth inhibition would provide further focus the research. EGTA,
Figure 4.6. Concentration-dependent effects of U0126 and PD98059 on DNA synthesis in serum-starved CHO-m2 cells. CHO-m2 cells were serum-starved for 24 h prior to inhibitor additions. Cells were treated with the indicated concentrations of A, U0126 or B, PD98059 for 30 min prior to CCh stimulation (+). Basal cells (-) were treated with U0126 (A) or PD98059 (B) but not stimulated with CCh. Control values represent cells that were not treated with U0126 or PD98059. Cells were stimulated for 24 h with 10 μM CCh and incubated with 2 μCi of [3H]thymidine for the final 2 h. DNA synthesis was measured using the ether extraction method as outlined under Materials and Methods. Each data bar represents n=4 and standard errors of the mean are shown.
thapsigargin, and BAPTA (Fig. 4.7A, B, and C) prevent M₃ ACh receptor-mediated increases in intracellular Ca²⁺ by different mechanisms. EGTA removes the Ca²⁺ gradient across the plasma membrane by chelating extracellular Ca²⁺, thus preventing Ca²⁺ influx. 2 mM EGTA caused complete inhibition of both CCh-stimulated and basal cell growth, and no concentration used effected the receptor-mediated inhibition of growth (Fig. 4.7A). Treatment of cells with an inhibitor of the endoplasmic reticulum Ca²⁺-ATPase, thapsigargin had a similar effect on the cells (Fig. 4.7B). 100 nM thapsigargin abolished both receptor-stimulated and basal cell growth. 30 μM and higher concentrations of BAPTA, a compound that chelates intracellular Ca²⁺, were cytotoxic to growing CHO-m3 cells without reversing the M₃ ACh receptor-mediated inhibition of growth (Fig. 4.7C). These data show that inhibiting increases in intracellular Ca²⁺ can have profound effects on basal growth, but does not prevent the receptor-mediated inhibition of DNA synthesis. Ionomycin (Fig. 4.7D) allows essentially unregulated Ca²⁺ entry into the cells and so maintains a high intracellular Ca²⁺ concentration. Ionomycin addition did not affect basal or CCh-inhibited growth suggesting along with the other data in Fig. 4.7, that Ca²⁺ is not involved in the growth inhibition. An alternative way to inhibit increases in intracellular Ca²⁺ is to use carboxamido-triazole (CAI). CAI has been shown to inhibit muscarinic receptor-mediated Ca²⁺ influx in CHO cells (Felder et al., 1991; Rodland et al., 1997) and is therefore a useful tool in manipulating intracellular Ca²⁺ in this cell system. Fig. 4.8 reports data that agree with the reported anti-proliferative function of CAI, but does not show any reversal of the receptor-mediated growth inhibition, providing further evidence that Ca²⁺ is not important in the inhibition of DNA synthesis by the M₃ ACh receptor.

c-Src is a non-receptor tyrosine kinase that is involved in GPCR-mediated ERK activation via transactivation of growth factor receptors (Maudsley et al., 2000; Pierce et al., 2001), as well as being involved in other pathways and processes. Data presented demonstrate that c-src is not involved in the inhibition of DNA synthesis (Fig. 4.9). At concentrations of PP1 that are completely effective in inhibiting src kinase activity, no changes in basal or CCh-inhibited DNA synthesis were seen. Use of PP1 also indicates that c-src is not involved in M₃-mediated activation of ERK (Fig. 4.9B) although c-src has been shown to be involved in ERK activation by other GPCRs studied in the laboratory.
Figure 4.7. Concentration-dependent effects of [Ca^{2+}]-manipulating drugs on DNA synthesis in growing CHO-m3 cells. Cells were treated with the indicated concentrations of A, EGTA, B, thapsigargin, C, BAPTA-AM or D, ionomycin for 30 min prior to CCh stimulation (▲). Basal cells (●) were treated with the appropriate drug, but not stimulated with CCh. Control values represent cells that were not treated with drugs. Cells were stimulated for 24 h with 10 μM CCh and incubated with 2 μCi of [³H]thymidine for the final 2 h. DNA synthesis was measured using the ether extraction method as outlined under Materials and Methods. Each data point represents n=4 and standard errors of the mean are shown.
Figure 4.8. Concentration-dependent effects of CAI on DNA synthesis in growing CHO-m3 cells. Cells were treated with the indicated concentrations of CAI for 30 min prior to CCh stimulation (▲). Basal cells (●) were treated with CAI, but not stimulated with CCh. Control values represent cells that were not treated with CAI. Cells were stimulated for 24 h with 10 μM CCh and incubated with 2 μCi of [³H]thymidine for the final 2 h. DNA synthesis was measured using the ether extraction method as outlined under Materials and Methods. Each data point represents n=4 and standard errors of the mean are shown.
Figure 4.9. Concentration-dependent effects of PP1 on DNA synthesis in growing CHO-m3 cells. Cells were treated with the indicated concentrations of PP1 for 30 min prior to CCh stimulation (▲). Basal cells (●) were treated with PP1 but not stimulated with CCh. Control values represent cells that were not treated with PP1. A, cells were stimulated for 24 h with 10 μM CCh and incubated with 2 μCi of [3H]thymidine for the final 2 h. DNA synthesis was measured using the ether extraction method as outlined under Materials and Methods. B, cells were either not stimulated (-) or stimulated for 5 min with 10 μM CCh (+), lysed and ERK1 was immunoprecipitated using a selective antibody as described under Materials and Methods. Where indicated cells were pre-treated for 30 min with 10 μM PP1 prior to agonist addition. Specific activity has been calculated for each time-point using a protein value obtained from a Bradford protein assay of the cleared cell lysates. Each data point represents n=4-8 and standard errors of the mean are shown.
The PI3-K inhibitors LY294002 and wortmannin were used to assess the involvement of PI3-K and its downstream target PKB on the inhibition of DNA synthesis (Fig. 4.10). At concentrations that are specific for PI3-K inhibition (1 μM LY294002 and 0.1 μM wortmannin), no significant effects were seen on basal or receptor-inhibited DNA synthesis. Western blot analysis of phosphorylated PKB in control experiments indicated that both compounds were inhibiting PKB phosphorylation, suggesting that PI3-K activity was impaired (data not shown).

PKCs are activated by increased cellular DAG levels (e.g. following PIP2 hydrolysis by PLC's), and PKC isoenzymes have been implicated in growth control (Zhou et al., 1993). Chronic treatment of cells with phorbol esters, for example phorbol-12,13-dibutyrate (PDBu), down-regulates expression of a number of conventional and novel PKC isoenzymes. Fig. 4.11A shows the effect of this down-regulation on the inhibition of DNA synthesis seen in CHO-m3 cells. PDBu pre-treatment affords partial protection against the receptor-mediated decrease in DNA synthesis, although PDBu treatment alone lowers the basal DNA synthesis by 18% relative to basal control values. CCh causes a 43% decrease in DNA synthesis in PDBu treated cells compared to a 78% decrease in control cells. However, pre-treatment of cells with the general PKC inhibitor Ro-31-8220, did not have any effect on M3 ACh receptor-mediated inhibition of DNA synthesis (Fig. 4.11B). These contradictory results can be explained by considering the extent of PKC inhibition by these two methods. Fig. 4.12 demonstrates the ability of 1 μM Ro-31-8220 or chronic PDBu pre-treatment to inhibit M3 receptor-mediated and direct PKC-stimulated ERK activity. Previous studies (Wylie et al., 1999) demonstrated that ERK activation in CHO-m3 cells is entirely dependent on PKC and so ERK activity can be used as a positive control for PKC function. These data show that both CCh and acute PDBu treatment result in robust ERK activation (15.9 and 25.4 fold-over-basal, respectively), which is entirely blocked with chronic PDBu treatment, but only partially blocked with Ro-31-8220 treatment (Fig. 4.12). Increasing concentrations of Ro-31-8220 would produce a more complete inhibition, although high concentrations of Ro-31-8220 became cytotoxic to the CHO cells over the long incubation time.
Figure 4.10. Concentration-dependent effects of LY294002 and Wortmannin on DNA synthesis in growing CHO-m3 cells. Cells were treated with the indicated concentrations of A, LY294002 or B, wortmannin for 30 min prior to CCh stimulation (▲). Basal cells (●) were treated with LY294002 (A) or wortmannin (B) but not stimulated with CCh. Control values represent cells that were not treated with LY294002 or wortmannin. Cells were stimulated for 24 h with 10 μM CCh and incubated with 2 μCi of [3H]thymidine for the final 2 h. DNA synthesis was measured using the ether extraction method as outlined under Materials and Methods. Each data point represents n=4 and standard errors of the mean are shown.
Figure 4.11. Effects of PKC inhibitors on DNA synthesis in growing CHO-m3 cells. A, cells were treated with 1 μM PDBu for 24 h prior to CCh stimulation (+). Basal cells (-) were treated with PDBu but not stimulated with CCh. Control values represent cells that were not treated with PDBu. Also shown is a population of cells only treated with PDBu (24 h) B, cells were treated with the indicated concentrations of Ro-31-8220 for 30 min prior to CCh stimulation (▲). Basal cells (●) were treated with Ro-31-8220 but not stimulated with CCh. Control values represent cells that were not treated with Ro-31-8220. All cells (A and B) were stimulated for 24 h with 10 μM CCh and incubated with 2 μCi of [³H]thymidine for the final 2 h. DNA synthesis was measured using the ether extraction method as outlined under Materials and Methods. Each data point represents n=4 and standard errors of the mean are shown.
Figure 4.12. Effects of PKC inhibitors on CCh- and PDBu-mediated ERK activity in serum-starved CHO-m3 cells. Cells were serum-starved for 24 h prior to inhibitor additions. Cells were treated for 24 h with 1 μM PDBu, for 30 min with 1 μM Ro-31-8220, or left untreated as control cells prior to stimulation for 5 min with 10 μM CCh, 1μM PDBu or left unstimulated (-). Cells were lysed and ERK1 was immunoprecipitated using a selective antibody as described under Materials and Methods. Specific activity has been calculated for each time-point using a protein value obtained from a Bradford protein assay of the cleared cell lysates. Each data point represents n=4-8 and standard errors of the mean are shown.
required for DNA synthesis experiments (Fig. 4.11B). These data suggest that there may be a PKC component to the $M_3$ ACh receptor-mediated decrease in DNA synthesis.

4.4. The Involvement of p38 in the $M_3$-Mediated Inhibition of DNA Synthesis

p38 MAPK activation has been implicated in growth regulation, particularly in growth arrest and apoptosis (Xia et al., 1995). Fig. 4.13A shows a short-term time-course of p38 activity in growing CHO-m2 and CHO-m3 cells. Both the $M_2$ and $M_3$ ACh receptors caused an increase in p38 activity after 5 min of CCh incubation. The $M_3$ ACh receptor caused a more sustained p38 activation, however neither receptor appears to couple efficiently to p38 in the CHO cell background. The representative autoradiograms shown are from a one-week exposure, and the radioactivity incorporated into the substrate was too low to quantify suggesting very low basal and receptor-stimulated levels of activity. This suggests that p38 is unlikely to be involved in the growth arrest seen in CHO-m3 cells. Western blot analysis of p38 protein indicated that the total amount of p38 protein was not affected by CCh stimulation of CHO-m2 or CHO-m3 cells (Fig. 4.13A, lower panel). To confirm that p38 was not involved in the inhibition of DNA synthesis in CHO-m3 cells a specific p38 inhibitor, SB203580 was used and DNA synthesis was measured (Fig. 4.13B). SB203850 did not reverse the inhibition of DNA synthesis caused by CCh stimulation confirming that p38 is not a component of the growth regulation pathway. An alternative p38 inhibitor, SB202190 was also used to inhibit p38 activity at the level of DNA synthesis (data not shown). The results obtained were similar to those obtained using SB203850 as shown.

4.5. Muscarinic Receptor Stimulation Differentially Activates JNK in CHO-m2 and CHO-m3 Cells

JNK, like ERK has been implicated in growth control responses to extracellular stimuli in a multitude of different cellular systems. Stimulation of the $M_2$ and the $M_3$ ACh receptors has been shown to differentially activate JNK in CHO cells (Wylie et al., 1999). As with ERK, it was important to perform longer-term time-courses of activation under the conditions where the growth inhibition was
Figure 4.13. The involvement of p38 in DNA synthesis in growing CHO-m3 cells. A, CHO-m2 and CHO-m3 cells were incubated with 10 μM CCh for the appropriate times as shown. Cells were lysed and p38 was immunoprecipitated using a selective antibody as described under Materials and Methods. Kinase activity was measured using GST-ATF2 as a substrate and representative autoradiograms of ATF2 phosphorylation are shown. Representative Western blots of total p38 protein in cleared cell lysates prepared for p38 assay are also shown. B, cells were treated with the indicated concentrations of SB203580 for 30 min prior to CCh stimulation (▲). Basal cells (○) were treated with SB203580 but not stimulated with CCh. Control values represent cells that were not treated with SB203580. Cells were stimulated for 24 h with 10 μM CCh and incubated with 2 μCi of [3H]thymidine for the final 2 h DNA synthesis was measured using the ether extraction method as outlined under Materials and Methods. Each data point represents n=4 and standard errors of the mean are shown.
occurring. Under serum-starved conditions, CHO-m3 cells displayed a robust JNK response to CCh, peaking at 45 min and returning close to basal levels by 4 h after agonist addition (Fig. 4.14). The peak activity of 7.49 pmol ATP/min/mg, represented a 23.9-fold JNK activation over the basal level. CCh did not elicit a large JNK response in CHO-m2 cells, giving a peak value at 15 min of 0.99 pmol ATP/min/mg. The lower part of Fig. 4.14 shows representative autoradiograms of GST-c-jun phosphorylation by JNK immunoprecipitates. They also show Western blots using an antibody to JNK1 indicating that the total JNK protein does not change over the time-course of the experiments. In the presence of serum, CCh produced a large JNK activation in CHO-m3 cells, compared to CHO-m2 cells (Fig. 4.15), confirming that the M2 ACh receptor couples relatively poorly to JNK activation (Wylie et al., 1999). The main difference between the growth conditions was observed with respect to basal JNK activities which were higher in growing cells, giving lower fold responses to CCh in the presence of serum (Fig. 4.15) compared with its absence (Fig 4.14). Thus, the M3 ACh receptor is more effectively coupled to JNK activation than the M2 ACh receptor regardless of the growth conditions.

4.6. The Involvement of JNK in the M3-Mediated Inhibition of DNA Synthesis

These JNK time-course data suggest a testable working hypothesis: The ability of the M3 ACh receptor to couple to JNK causes the CHO-m3 cell growth arrest, whereas in the absence of significant JNK activation the CHO-m2 cells continue to proliferate. Unlike for ERK and p38 there are no well-established commercially available inhibitors of JNK. Recently, preliminary reports of pharmacological JNK inhibitors have been described (Maroney et al., 1999; Bonny et al., 2001). Fig. 4.16 shows the characterisation of two JNK inhibitors produced by Alexis ® Biochemicals. Both L- and D- (a more stable analogue) JNK inhibitor peptides had small inhibitory effects on JNK activation in CHO-m3 cells (Fig. 4.16A). The L-JNK inhibitor reduced the peak of JNK activation in control cells from 12.3 to 10.0 pmol ATP/min/mg, and the D-JNK inhibitor reduced the peak activation to 8.5 pmol ATP/min/mg. The lower part of Fig. 4.16A shows representative autoradiograms of GST-c-jun phosphorylation by JNK1 immunoprecipitates. As peptide inhibitors can disassociate from proteins
Figure 4.14. Time-course of JNK activation in serum-starved CHO-m2 and CHO-m3 cells. CHO-m2 (▲) and CHO-m3 (●) cells were serum-starved for 24 h prior to incubation with 10 μM CCh for the appropriate times as shown. Cells were lysed and JNK1 was immunoprecipitated with a specific antibody as described under Materials and Methods. Kinase activity was measured using GST-c-jun as a substrate and representative autoradiograms of c-jun phosphorylation are shown. Specific activity has been calculated for each time-point using a protein value obtained from a Bradford protein assay of the cleared cell lysates. Each data point represents n=4-8 and standard errors of the mean are shown. Western blot analysis of total JNK protein was also performed on lysates prepared for JNK assay and representative blots are shown.
Figure 4.15. Time-course of JNK activation in growing CHO-m2 and CHO-m3 cells. CHO-m2 (▲) and CHO-m3 (●) cells were incubated with 10 µM CCh for the appropriate times as shown. Cells were lysed and JNK1 was immunoprecipitated with a specific antibody as described under Materials and Methods. Kinase activity was measured using GST-c-jun as a substrate and representative autoradiograms of c-jun phosphorylation are shown. Specific activity has been calculated for each time-point using a protein value obtained from a Bradford protein assay of the cleared cell lysates. Each data point represents n=4-8 and standard errors of the mean are shown. Western blot analysis of total JNK protein was also performed on lysates prepared for JNK assay and representative blots are shown.
Figure 4.16. Effects of L- and D-JNK inhibitors on JNK activation and c-jun phosphorylation in growing CHO-m3 cells. Cells were treated with the indicated concentrations of inhibitor for 30 min prior to CCh stimulation (+). Basal cells (-) were treated with inhibitor but not stimulated with CCh. Control values represent cells that were not treated with inhibitors. Cells were incubated with 10 μM CCh for 45 min. A, cells were lysed and JNK1 was immunoprecipitated with a specific antibody as described under Materials and Methods. Kinase activity was measured using GST-c-jun as a substrate and a representative autoradiogram of c-jun phosphorylation is shown. Specific activity has been calculated for each time-point using a protein value obtained from a Bradford protein assay of the cleared cell lysates. Each data point represents n=4-8 and standard errors of the mean are shown. B, Western blot analysis of c-jun protein was also performed on lysates prepared for JNK assay and representative blots are shown.
during the process of immunoprecipitation and kinase assay, it was important to test whether the inhibitors could interfere with activation of a downstream target of JNK. Western blot analysis of cleared lysates from the experiment in Fig. 4.16A was performed to detect expression of the JNK substrate, c-jun (Fig. 4.16 B). The Western blots show a CCh-stimulated shift in c-jun (caused by phosphorylation) represented by the upper bands in the figure. Both L- and D-JNK inhibitors reduced the level of CCh-stimulated c-jun phosphorylation without completely blocking it. Fig. 4.17 shows the effects of these JNK inhibitors on the inhibition of DNA synthesis in CHO-m3 cells. Neither inhibitor affected the growth inhibition, which either suggests that JNK is not a component of the growth regulation, or that the incomplete inhibition of JNK activity by the inhibitors allows sufficient JNK activity to cause the observed growth arrest.

Another inhibitor thought to block JNK activity is the non-commercially available Cephalon Corporation inhibitor CEP-11004-2 (Maroney et al., 1999). CEP-11004-2 inhibits MLK, an upstream activator of JNK and so JNK immunoprecipitation and assay is sufficient to test the effectiveness of the inhibitor. Fig. 4.18 shows that even at low concentrations, CEP-11004-2 inhibited JNK activation in growing CHO-m3 cells. 100 nM CEP-11004-2 inhibited CCh-stimulated JNK activation by 89 % compared to CCh-stimulated control cells. At this concentration, JNK activation was reduced from a 10.8 fold-over-basal increase in control cells to a 3.4 fold-over basal increase. At 1 μM CEP-11004-2 this fold-over-basal value was further reduced to 1.6, confirming CEP-11004-2 as an effective inhibitor of JNK activated by M3 ACh receptor in CHO cells. CEP-11004-2 was also used in DNA synthesis experiments, the results of which are shown in Fig. 4.19. Over the long incubation period required, CEP-11004-2 showed cytotoxic effects on basal cellular growth rates (Fig. 4.19). However the receptor-mediated decrease in DNA synthesis was still apparent, suggesting that JNK is not involved in the growth regulation.
Figure 4.17. Effects of L- and D-JNK inhibitors on DNA synthesis in growing CHO-m3 cells. Cells were treated with the indicated concentrations of A, D-JNKI or B, L-JNKI inhibitors for 30 min prior to CCh stimulation (+). Basal cells (-) were treated with D-JNKI (A) or L-JNKI (B) but not stimulated with CCh. Control values represent cells that were not treated with inhibitors. Cells were stimulated for 24 h with 10 µM CCh and incubated with 2 µCi of [3H]thymidine for the final 2 h. DNA synthesis was measured using the ether extraction method as outlined under Materials and Methods. Each data point represents n=4 and standard errors of the mean are shown.
Figure 4.18. Effect of CEP-11004-2 on JNK activation in growing CHO-m3 cells. Cells were treated with the indicated concentrations of CEP-11004-2 for 24 h prior to CCh stimulation (+). Basal cells (-) were treated with inhibitor but not stimulated with CCh. Control values represent cells that were not treated with the inhibitor. Cells were incubated with 10 μM CCh for 45 min. Cells were lysed and JNK1 was immunoprecipitated with a specific antibody as described under Materials and Methods. Kinase activity was measured using GST-c-jun as a substrate and a representative autoradiogram of c-jun phosphorylation is shown. Specific activity has been calculated for each time-point using a protein value obtained from a Bradford protein assay of the cleared cell lysates. Each data point represents n=4-8 and standard errors of the mean are shown.
Figure 4.19. Effect of CEP-11004-2 on DNA synthesis in growing CHO-m3 cells. Cells were treated with the indicated concentrations of CEP-11004-2 for 24 h prior to CCh stimulation (+). Basal cells (-) were treated with CEP-11004-2 but not stimulated with CCh. Control values represent cells that were not treated with the inhibitor. Cells were stimulated for 24 h with 10 μM CCh and incubated with 2 μCi of [3H]thymidine for the final 2 h. DNA synthesis was measured using the ether extraction method as outlined under Materials and Methods. Each data point represents n=4 and standard errors of the mean are shown.
DISCUSSION

The present Chapter has examined the involvement of protein kinases, in particular MAPKs, and second messenger pathways in the M₃ ACh receptor-mediated inhibition of DNA synthesis using pharmacological inhibitors. The data presented demonstrate that ERK is not involved in the inhibition of growth by M₃ ACh receptors, although inhibition of ERK activity does ablate the M₂ ACh receptor-mediated increase in proliferation. Results demonstrate that PI3-K, intracellular Ca²⁺, src-family tyrosine kinases, and p38 MAPK do not contribute to the growth inhibition by M₃ ACh receptors, but phorbol ester induced down-regulation of PKC affords partial protection against the M₃ ACh receptor-mediated inhibition of growth. Recently developed JNK inhibitors have also been assessed and the results obtained suggest that JNK activity does not contribute to the growth control by M₃ ACh receptor activation.

The finding that inhibition of ERK, JNK or p38 had no effect on the M₃ ACh receptor-mediated inhibition of DNA synthesis was unexpected. All three MAPK sub-families are involved in a variety of growth regulatory pathways and were all candidates to mediate the G₁-phase growth arrest. ERK activation is classically involved in the induction of cellular proliferation (Pages et al., 1993; Lavoie et al., 1996), although reports have demonstrated that ERK activation can also lead to growth arrest (Pumiglia and Decker, 1997; Tang et al., 2002). The M₃ ACh receptor-stimulated ERK activity could have been contributing to the growth inhibition by two distinct mechanisms. Firstly, the ERK time-course in serum-starved CHO-m3 cells was more sustained compared to the transient ERK response in CHO-m2 cells and was thereby growth arresting the CHO-m3 cells. There are many reports demonstrating that the duration of ERK activity determines its effect on cellular growth (see Marshall, 1995). Experiments in primary cultures of rat hepatocytes demonstrated a general model where acute ERK activity caused an increase in DNA synthesis via increased CDK2 activity (Tombes et al., 1998). In contrast, chronic ERK activation reduced DNA synthesis by increased expression of p21Cip/Waf1 and p16Ink4a, emphasising the importance of the temporal pattern of ERK activation (Tombes et al., 1998). The second hypothesis was that the M₃ ACh receptor-mediated decrease in basal ERK activity in
growing CHO cells was suppressing proliferation. This was the more likely possibility as it was based on an ERK time-course obtained under the conditions where the largest inhibition of DNA synthesis was detected (i.e. in growing cells). The finding that both receptor-stimulated and basal ERK activities were inhibited by U0126 over a 24 h time-course without affecting DNA synthesis refutes these hypotheses, and provides strong evidence that ERK is not involved in the receptor-mediated growth inhibition. In contrast, the M_2 ACh receptor-mediated increase in proliferation does appear to involve ERK activation, as increasing concentrations of the MEK1 inhibitors perturbed the growth response.

JNK and p38 MAPK are generally considered as negative regulators of growth, mediating responses such as growth arrest, differentiation, and apoptosis, although JNK also has been implicated in cellular proliferation (Potapova et al., 2000). Sustained p38 activation has been shown to cause cell cycle arrest by inducing p21Cip1/Waf1 expression downstream of GPCR activation (Alderton et al., 2001), as well as causing a reduction in cyclin D1 expression in airways smooth muscle cells (Page et al., 2001). Neither the M_2 nor the M_3 ACh receptor coupled efficiently to p38 in CHO cells, and inhibitor studies confirmed that p38 was not contributing to the growth arrest in CHO-m3 cells. Although JNK is classically linked with apoptosis (Xia et al., 1995) it is also reported to mediate growth arrest (Ray et al., 1999). The differential effects of M_2 and M_3 ACh receptors on JNK activation provided a potential mechanism for the inhibition of DNA synthesis in CHO-m3 cells. The lack of a well-characterised, commercially available JNK inhibitor hindered this investigation. However, although the Alexis ® Biochemical’s inhibitors were only partially effective in the CHO cells, the MLK inhibitor CEP-11004-2 inhibited JNK activity in CHO-m3 cells but had no effect on the receptor-mediated decrease in DNA synthesis. These studies suggest that JNK is not involved in the growth inhibition. Further, the discovery that CEP-11004-2 inhibits JNK activity in CHO-m3 cells provides insight into the pathway linking the M_3 ACh receptor to JNK, by suggesting the involvement of an MLK family member as an upstream JNK kinase kinase.

These MAPK inhibitor studies suggest an uncommon mechanism of growth regulation in CHO-m3 cells, which is independent of MAPK activation. MAPK activation by a wide variety of structurally...
distinct receptors is a common phenomenon and the MAPK is often assumed to mediate the growth effects of the receptor stimulus. These studies demonstrate that although the M₃ ACh receptor couples to ERK and JNK activation, neither pathway is involved in the growth modulation. TGF-β₁ is mitogenic in Swiss 3T3 cells and Balb 3T3 cells in a MAPK-independent manner (Chatani et al., 1995). Data showed that TGF-β₁ did not stimulate ERK in either cell line, suggesting that other factors or pathways were modulating these growth effects. Data presented in this Chapter support the idea that pathways other than MAPK cascades can mediate growth responses and determine cell fate.

Cross-talk between MAPK family members is important in mediating cellular responses. Co-operation between ERK and JNK pathways is critical in modulating proliferation in vascular endothelial cells (Pedram et al., 1998), whereas ERK/p38 co-operation is involved in growth arresting CHO cells (Sellers et al., 2000). The idea that one MAPK pathway can influence another could explain the ERK time-course data described in this Chapter. It is possible that the M₃ ACh receptor-mediated decrease in ERK activity seen at later time-points in growing cells, is caused by the receptor-mediated activation of JNK. Thus, it has been shown that JNK activation causes an increase in the gene expression of MAPK phosphatase 1 (MKP-1), the dual specificity phosphatase responsible for dephosphorylating and inactivating ERK (Bokemeyer et al., 1996). A second MKP, MKP-3 has also been shown to inactivate ERK and provides a second candidate that could perform this MAPK-linking function (Muda et al., 1996). The peak JNK activation occurs prior to the reduction in ERK activity seen between 60-120 min after agonist challenge, which would support this model of interaction. Although ERK and JNK do not appear to be involved in the inhibition of DNA synthesis, understanding the interactions between the MAPK families could give a greater understanding of the intricacies underlying their role in cellular physiology.

As MAPKs appeared not to be involved in the inhibition of DNA synthesis, other possibilities were investigated. Of the proteins examined only inhibition of PKC afforded any protection against the receptor-mediated decrease in DNA synthesis. Down-regulation of PKC by chronic phorbol ester
treatment partially reduced the M₃ ACh receptor-mediated growth inhibition. PKC has been implicated in both positive and negative growth control, responses that are mediated by complex regulation of CDK signalling pathways (Zhou et al., 1993). PKC can phosphorylate a broad range of substrates and could link the M₃ ACh receptor to changes in the cell cycle machinery. For example, an M₃ ACh receptor-mediated increase in p21^{Cip1/Waf1} expression in NIH 3T3 cells was blocked by PKC inhibition (Nicke et al., 1999).

The data presented support the role for PKC(s) in growth control in the CHO cell model, however over the long time-period of phorbol ester incubation, other non-specific effects could be occurring that could perturb the growth inhibition. The PKC investigation highlights the practical limitations of pharmacological inhibitors as tools for pathway investigations. The general PKC inhibitor Ro-31-8220 had no effect on the inhibition of DNA synthesis, but perhaps could not be used at a sufficiently high concentration due to cytotoxic effects to the CHO cells. The highest concentration of inhibitor that could be used during the long incubation period required for the DNA synthesis experiment did not completely inhibit PKC activity. It is likely that higher concentrations of Ro-31-8220 would block PKC activation but these concentrations might also affect other non-specific pathways in the DNA synthesis experiments that would invalidate any data obtained. This is an experimental limitation apparent when working with any pharmacological agent over longer time-periods. All pharmacological agents are likely to be cytotoxic or insufficiently selective at higher concentrations, which limits their practical use. The conclusion made concerning the lack of involvement of JNK in the growth inhibition must be made cautiously. Basal growth was affected even at low CEP-11004-2 concentrations suggesting a level of cytotoxicity or non-specificity. The relative novelty of this inhibitor must also be taken into consideration when making conclusive statements. Until a full characterisation of the compound in CHO cells is performed data must be treated as preliminary findings to be confirmed using alternative approaches.

As a consequence of the DNA synthesis investigation certain aspects of the M₃ ACh receptor-mediated ERK activation have been elucidated. The receptor-mediated ERK activation is entirely
dependent on PKC, confirming previous studies from the laboratory (Wylie et al., 1999). Data revealed that src-family kinases are not involved in the activation of ERK, whilst PI3-K may have a role in basal but not receptor-mediated ERK activation (data not shown).

In summary the data presented in this Chapter have demonstrated that the M₃ ACh receptor-mediated inhibition of DNA synthesis is likely to be independent of ERK, JNK or p38 MAPK family member activities. These data were obtained using pharmacological inhibitors and alternative approaches are required to confirm the findings. Data showed that the M₂ ACh receptor-mediated increase in DNA synthesis was dependent on ERK activation and that PKC appears to be partly involved in the inhibition of DNA synthesis, in CHO-m3 cells although other unidentified factors must also contribute to the growth inhibition. The data also demonstrated that the MLK inhibitor CEP-11004-2 is effective at inhibiting JNK activity in CHO-m3 cells and could be a useful tool for examining the physiological consequences of JNK activation.

In Chapter 5, molecular biological approaches will be taken to further examine and confirm the involvement of MAPK cascades in the M₃ ACh receptor-mediated inhibition of DNA synthesis.
Chapter 5

Molecular Biological Approaches to Assessing the
Biochemical Basis of the M₃ ACh Receptor-Mediated Growth
Inhibition
INTRODUCTION

There are many ways to investigate the involvement of an individual protein or biochemical pathway in a cellular response. One well-established technique is to block a specific pathway by introducing a protein into the cell that will interfere with the downstream signalling cascade. Such a protein, known as a dominant-negative protein, can be used to interfere at any level of a signalling pathway. For example, kinase-inactive mutants of an MEKK or MKK would be dominant-negative and selectively block activation of downstream MAPKs. This technique produces reliable data as over-expression of a protein that disrupts a specific pathway can potentially eliminate non-specific effects seen with more traditional approaches using pharmacological inhibitors. There are a number of ways of introducing recombinant protein into a cell of which transient transfection and viral infection are two commonly used examples.

The transient transfection of cells represents a powerful technique in pathway investigation, and many studies to investigate intracellular signalling pathways have relied on this approach. The limitation of this technique is that a relatively small percentage of cells will be transiently transfected and will express the desired protein. This means that whole cell population assays are of limited value, as any effect mediated by the dominant-negative protein will be masked by low transfection efficiency. There are experimental designs to over-come this problem. For example, co-transfection of a dominant negative construct with an epitope-tagged version of the downstream protein of interest would allow immunoprecipitation and assay to be performed on transfected cells only, allowing any effects of the inhibitor protein to be detected.

Alternatively, the problem of poor transfection efficiency can be overcome by using viral infection protocols (e.g. using genetically modified adenovirus constructs). Infection of cells can result in as much as 100 % efficiency of protein expression and so whole cell population assays can be performed. The limitation of this technique is that preparation of (adeno)viruses expressing specific DNA
constructs is a time consuming and expensive process, and great care must be taken in their use to prevent inadvertent contamination.

The aim of this Chapter was to assess the involvement of MAPKs in the M₃ ACh receptor-mediated growth inhibition by interfering with individual MAPK signalling cascades using dominant-negative constructs. Immunoprecipitation and assay of over-expressed Myc-CDK2 as an index of growth inhibition provided a technique to address this question, and conclusions concerning MAPK involvement as well as the involvement of G-protein subunits in the growth inhibition are made. The potential involvement of p53, AP-1, and SP-1 transcription factors in the induction of p21<sup>Cip1/Waf1</sup> mRNA are also assessed and other transcription factors and signalling cascades that may be important in the growth inhibition are discussed.
RESULTS

5.1. The Effect of CCh on Transcription Factor Activation in CHO-m3 Cells

Data from Chapter 3 demonstrated that p21\(^{Cip1/Waf1}\) expression in CHO-m3 cells was regulated, at least in part, at the level of transcription. The p21\(^{Cip1/Waf1}\) promotor contains many transcription factor sites that could cause an induction of promotor activity and account for the increase in mRNA and protein detected in growing CHO-m3 cells in response to receptor activation. If any transcription factors were activated by the M\(_3\) ACh receptor, dominant-negative co-transfection approaches would allow a more direct biochemical investigation of the pathways involved in the growth inhibition. Studies have focused on transcription factors that can be activated by ERK or JNK and that have putative binding sites within the p21\(^{Cip1/Waf1}\) promotor region. Fig. 5.1A shows a 24 h time-course of c-jun expression in growing CHO-m3 cells. These data demonstrate an increase in c-jun expression and phosphorylation that correlate closely with the JNK activation time-courses shown in Chapter 4, supporting the role of c-jun as a down-stream target of JNK. c-Jun is a constituent of the AP-1 family of transcription factors that have binding regions within the p21\(^{Cip1/Waf1}\) promotor (Crowe et al., 2000). Fig. 5.1B shows a reporter gene assay measuring AP-1 activity after CCh stimulation in CHO-m3 cells. No receptor-mediated induction of AP-1 activity was seen compared with a positive control co-transfection (pFC-MEKK). Both ERK and JNK can also activate p53 (Milne et al., 1994, 1995), the classical transcriptional activator of p21\(^{Cip1/Waf1}\) that is sensitive to DNA damage (Gartel and Tyner 1999). As with AP-1, no increase in p53 activity was detected in response to CCh stimulation compared with a positive control (pFC-p53) (Fig. 5.2A). c-Jun has also been shown to transactivate the p21\(^{Cip1/Waf1}\) promotor by acting as an activator of the transcription factor SP-1 (Kardassis et al., 1999). Data showed that CCh incubation did not increase SP-1 activity in CHO-m3 cells (Fig. 5.2B). Constitutively active MEKK did not increase SP-1 transcriptional activity suggesting that SP-1 was not activated by JNK, and therefore c-jun in this cell line.
Figure 5.1. Activation of AP-1 in CHO-m3 cells. A, growing CHO-m3 cells were incubated with 10 μM CCh for the time points as shown. Western blot analysis of c-jun protein was performed on cleared lysates and representative blots are shown. B, growing CHO-m3 cells were transiently transfected with a mammalian expression vector (4 μg) encoding pAP-1-luciferase using Fugene VI as described in Materials and Methods. Cells were incubated for 48 h before serum-starvation for 24 h prior to stimulation with 10 μM CCh at the time points shown. Control values represent cells that were not stimulated with CCh. As a positive control, cells were co-transfected with a mammalian expression vector (4 μg) encoding pFC-MEKK (as supplied by the manufacturer). Luciferase activity was detected using a luminometer. Each value represents n=3-4 and errors shown are standard errors of the mean.
Figure 5.2. **Activation of p53 and SP-1 in CHO-m3 cells.** Growing CHO-m3 cells were transiently transfected with mammalian expression vectors (4 μg) encoding A, p53-luciferase or B, SP-1-luciferase using Fugene VI as described in Materials and Methods. Cells were incubated for 48 h before serum-starvation for 24 h prior to stimulation with 10 μM CCh at the time points shown. Control values represent cells that were not stimulated with CCh. As a positive control, cells were co-transfected with mammalian expression vectors (4 μg) encoding A, pFC-p53 or B, pFC-MEKK (as supplied by the manufacturer). Luciferase activity was detected using a luminometer. Each value represents n=3-4 and errors shown are standard errors of the mean.
5.2. Inhibition of JNK Activity by Adenoviral Infection of CHO Cells with JBD

Infection of cells with adenoviral-JBD (JNK binding-domain of JNK-interacting protein (JIP)) has been reported to inhibit JNK activity (Xia et al., 2001). Initial experiments determined the most efficient multiplicity of infection (MOI) for CHO-m3 cells using a recombinant adenovirus expressing eGFP. At an MOI of 400, 92% of cells were infected as assessed by fluorescence microscopy (data not shown). At an MOI of 200, 51% of cells were infected, and at an MOI of 100, 11% of cells expressed eGFP (data not shown). Therefore, all subsequent experiments used an MOI of 400 to infect cells. Fig. 5.3 shows the effects of adenoviral-JBD on native JNK activation in growing CHO-m3 cells. Receptor-stimulation caused an 8.2 fold-over-basal activation in control cells that was reduced to a 5.5 fold-over-basal increase following adenoviral-JBD infection. The lower part of Fig. 5.3 shows a representative autoradiogram of GST-c-jun phosphorylation by JNK immunoprecipitates using an anti-JNK1 antibody. Adenoviral-JBD infection of CHO-m3 cells inhibited JNK activation by 44% compared with non-infected cells. The effect of adenoviral-JBD infection on receptor-mediated DNA synthesis was also assessed (Fig. 5.4). Adenoviral-mediated expression of JBD had no effect on the M₃ ACh receptor-mediated inhibition of DNA synthesis, although as JBD expression only inhibited JNK activation by 44% (Fig. 5.3), interpretation of these data is difficult.

5.3. Manipulation of the M₃-Mediated Inhibition of DNA Synthesis Using Co-Transfection Approaches

Data from Chapter 3 demonstrated that M₃ ACh receptor activation caused a reduction in native CDK2 activity with a time-course similar to that of the DNA synthesis inhibition. This inhibitory effect on endogenous CDK2 can be reproduced by assaying over-expressed Myc-CDK2 in CHO-m3 cells (Fig. 5.5). CCh stimulation reduced Myc-CDK2 activity by 57%, a value comparable to the inhibition of endogenous CDK2 reported in Fig. 3.9A (68%). A representative autoradiogram of histone H1 phosphorylation by Myc-CDK2 immunoprecipitates is shown. Western blot analysis of total Myc-CDK2 indicated that the amount of protein was not affected by CCh stimulation (Fig. 5.5, lower
Figure 5.3. Effect of adenoviral-JBD infection on JNK activation in growing CHO-m3 cells. Cells were infected with adenoviral-JBD at an MOI of 400 and incubated for 48 h prior to stimulation with 10 \( \mu \text{M CCh} \) (+) for 45 min. Basal cells (-) were not stimulated with CCh. Control values represent cells that were not infected with adenoviral-JBD. Cells were lysed and JNK1 was immunoprecipitated with a specific antibody as described under Materials and Methods. Kinase activity was measured using GST-c-jun as a substrate and a representative autoradiogram of c-jun phosphorylation is shown. Specific activity has been calculated for each condition using a protein value obtained from a Bradford protein assay of the cleared cell lysates. Each data point represents \( n=3 \) and standard errors of the mean are shown.
Figure 5.4. Effect of adenoviral-JBD infection on DNA synthesis in growing CHO-m3 cells. Cells were infected with adenoviral-JBD at an MOI of 400 and incubated for 48 h prior to stimulation with CCh (+). Basal cells (-) were not stimulated with CCh. Control values represent cells that were not infected with adenoviral-JBD. Cells were stimulated for 24 h with 10 μM CCh and incubated with 2 μCi of \(^{3}H\)thymidine for the final 2 h. DNA synthesis was measured using the ether extraction method as outlined under Materials and Methods. Each data point represents n=4 and standard errors of the mean are shown.
Figure 5.5. Effect of CCh on Myc-CDK2 activity in growing CHO-m3 cells. Cells were transiently transfected with a mammalian expression vector (4 µg) encoding Myc-CDK2 using Fugene VI as described in Materials and Methods. Cells were incubated for 48 h before stimulation with 10 µM CCh for 24 h (+). Basal cells were not stimulated with CCh (-). Cells were lysed and Myc-CDK2 was immunoprecipitated with a specific anti-Myc antibody as described under Materials and Methods. Kinase activity was measured using Histone H1 as a substrate and a representative autoradiogram of Histone H1 phosphorylation is shown. Specific activity has been calculated for each condition using a protein value obtained from a Bradford protein assay of the cleared cell lysates. Each data point represents n=3-6 and standard errors of the mean are shown. Western blot analysis of total Myc-tagged protein was also performed on transfected cell lysates prepared for Myc-CDK2 assay and a representative blot is shown.
panel). This technique can therefore be used in co-transfection experiments to assess the involvement of specific MAPK components in the growth inhibitory response to M₃ ACh receptor activation.

Co-transfection of HA-JNK1 and constructs capable of interfering with the JNK pathway are shown in Fig. 5.6. Full-length JIP and the JNK-binding domain (JBD) of JIP blocked HA-JNK1 activation, most effectively, inhibiting the receptor-mediated activation by 87 % and 83 % respectively compared with an empty vector (pCMV5) co-transfection. Dominant-negative (DN) MKK7 was also effective, inhibiting HA-JNK activation by 76 % compared with an empty vector co-transfection, whereas dominant-negative MKK4 was less effective at inhibiting the activation of HA-JNK1. A representative autoradiogram of GST-c-jun phosphorylation by HA-JNK1 immunoprecipitates is shown. Western blot analysis of total HA-JNK1 protein indicated that the amount of protein was not affected by CCh stimulation. The expression of dominant-negative Flag-tagged constructs was also not affected by CCh stimulation as shown (Fig 5.6, lower panels).

The effect of a kinase-inactive form of MEK1 on HA-ERK2 activation in CHO-m3 cells was also assessed (Fig. 5.7). The MEK1 construct caused a full block of receptor-mediated HA-ERK2 activation. Both basal and stimulated HA-ERK2 activity was inhibited by the dominant-negative construct (54 % and 84 % respectively compared with an empty vector (pCMV5) co-transfection), indicating the effectiveness of the construct. CCh did not affect the expression of total HA-ERK2 protein or MEK1 over-expression as shown (Fig 5.7, lower panels).

These dominant-negative constructs can be used to evaluate directly the involvement of ERK and JNK in the growth inhibition of CHO-m3 cells using Myc-CDK2 activity as a marker for the inhibition of DNA synthesis. Dominant-negative ERK, JNK, and p38 constructs were co-transfected with Myc-CDK2 and the effects on the inhibition of DNA synthesis were determined. Fig. 5.8 illustrates data that demonstrate that dominant-negative MKK7, MKK6, or kinase-dead MEK1 had no significant effect on the inhibition of DNA synthesis confirming that ERK, JNK and p38, respectively, are not involved in the growth inhibition. A representative autoradiogram of histone H1 phosphorylation by
Figure 5.6. Effects of dominant-negative (DN) constructs on HA-JNK1 activation in growing CHO-m3 cells. Cells were co-transfected with mammalian expression vectors (4 μg) encoding HA-JNK and MKK4(DN), MKK7(DN), JIP, or JBD or an empty vector (pCMV5) and incubated for 48 h prior to stimulation with 10 μM CCh for 45 min (+). Basal cells were not stimulated with CCh (−). Cells were lysed and HA-JNK1 was immunoprecipitated with a specific anti-HA antibody as described under Materials and Methods. Kinase activity was measured using GST-c-jun as a substrate and a representative autoradiogram of c-jun phosphorylation is shown. Specific activity has been calculated for each condition using a protein value obtained from a Bradford protein assay of the cleared cell lysates. Each data point represents n=3-4 and standard errors of the mean are shown. Western blot analysis of total HA-JNK1 and Flag-tagged proteins was also performed on transfected cell lysates prepared for HA-JNK1 assay and representative blots are shown.
Figure 5.7. **Effects of kinase-dead (KD) MEK1 on HA-ERK2 activation in growing CHO-m3 cells.** Cells were co-transfected with mammalian expression vectors (4 µg) encoding HA-ERK and MEK1(KD) or an empty vector (pCMV5) and incubated for 48 h prior to stimulation with 10 µM CCh for 5 min (+). Basal cells were not stimulated with CCh (-). Cells were lysed and HA-ERK2 was immunoprecipitated with a specific anti-HA antibody as described under Materials and Methods. Specific activity has been calculated for each condition using a protein value obtained from a Bradford protein assay of the cleared cell lysates. Each data point represents n=3 and standard errors of the mean are shown. Western blot analysis of total HA-ERK2 and MEK1 protein was also performed on transfected cell lysates prepared for HA-ERK2 assay and representative blots are shown.
Figure 5.8. Effects of dominant-negative constructs on Myc-CDK2 activity in growing CHO-m3 cells. Cells were co-transfected with mammalian expression vectors (4 μg) encoding Myc-CDK2 and MKK7(DN), MKK6(DN) or MEK1(KD) or an empty vector (pCMV5) and incubated for 48 h prior to stimulation with 10 μM CCh for 24 h (+). Basal cells were not stimulated with CCh (-). Cells were lysed and Myc-CDK2 was immunoprecipitated using a specific anti-Myc antibody as described under Materials and Methods. Kinase activity was measured using Histone H1 as a substrate and a representative autoradiogram of Histone H1 phosphorylation is shown. Specific activity has been calculated for each condition using a protein value obtained from a Bradford protein assay of the cleared cell lysates. Each data point represents n=4 and standard errors of the mean are shown. Western blot analysis of total Myc-CDK2 and Flag-MKK6 protein was also performed on transfected cell lysates prepared for Myc-CDK2 assay and representative blots are shown.
Myc-CDK2 immunoprecipitates is shown. CCh did not affect the expression of total Myc-CDK2 (Fig. 5.8, lower panel) and also shown is the expression of Flag-tagged MKK6, unaffected by CCh-stimulation. To confirm these findings, other constructs were investigated as shown in Fig. 5.9. Two alternative dominant-negative JNK constructs, signalling-inactive MKK4, and JIP, as well as signalling-inactive MKK3, a dominant-inhibitor of p38 activation, had no effect on the inhibition of Myc-CDK2 activity. JIP- and MKK3- transfected cells resulted in a greater Myc-CDK2 activity compared with an empty vector co-transfection, both in stimulated and unstimulated cells (as shown in the representative autoradiogram), although this is likely to be an artifact of the experimental design, rather than a genuine cellular effect. Western blot analysis of total Myc-CDK2 indicated that the amount of protein was not affected by CCh stimulation (Fig 5.9, lower panel). Flag-tagged MKK3 expression could not be detected by Western blot analysis.

Reports have demonstrated the ability of MAPK family members to act concomitantly in mediating growth responses (Pedram et al., 1998; Sellers et al., 2000; Zentrich et al., 2002). As ERK and JNK activities are most strongly activated by the M3 ACh receptor, co-inhibition of both proteins could potentially ablate the inhibition of DNA synthesis. Blocking JNK activation by JIP expression, and ERK activation using U0126 pre-incubation afforded slight protection against the inhibition of Myc-CDK2 activity (38 % inhibition compared to 25 % inhibition in empty vector (pCMV5) co-transfected cells) (Fig. 5.10), however this protection is likely not to be significant. A representative autoradiogram of histone H1 phosphorylation by Myc-CDK2 immunoprecipitates is shown. Western blot analysis of total Myc-CDK2 indicated that the amount of protein was not affected by CCh stimulation (Fig. 5.10, lower panel).

5.4. The Effect of β Subunit Sequestration on the Inhibition of Myc-CDK2 Activity

As inhibition of individual, or a combination of MAPKs did not perturb the inhibition of Myc-CDK2 activity it was important to assess alternative mechanisms that could potentially contribute to the
Figure 5.9. Effects of other dominant-negative constructs on Myc-CDK2 activity in growing CHO-m3 cells. Cells were co-transfected with mammalian expression vectors (4 µg) encoding Myc-CDK2 and MKK4(DN), JIP or MKK3(DN) or an empty vector (pCMV5) and incubated for 48 h prior to stimulation with 10 µM CCh for 24 h (+). Basal cells were not stimulated with CCh (-). Cells were lysed and Myc-CDK2 was immunoprecipitated with a specific anti-Myc antibody as described under Materials and Methods. Kinase activity was measured using Histone H1 as a substrate and a representative autoradiogram of Histone H1 phosphorylation is shown. Specific activity has been calculated for each condition using a protein value obtained from a Bradford protein assay of the cleared cell lysates. Each data point represents n=4 and standard errors of the mean are shown. Western blot analysis of total Myc-CDK2 protein was also performed on transfected cell lysates prepared for Myc-CDK2 assay and a representative blot is shown.
Figure 5.10. Effect of inhibiting ERK and JNK on Myc-CDK2 activity in growing CHO-m3 cells. Cells were co-transfected with mammalian expression vectors (4 µg) encoding Myc-CDK2 and JIP or an empty vector (pCMV5), and incubated for 48 h prior to stimulation with 10 µM CCh for 24 h (+). Basal cells were not stimulated with CCh (-). One population of JIP transfectants was treated with 1 µM U0126 (U0) for 30 min prior to CCh stimulation. Cells were lysed and Myc-CDK2 was immunoprecipitated with a specific anti-Myc antibody as described under Materials and Methods. Kinase activity was measured using Histone H1 as a substrate and a representative autoradiogram of Histone H1 phosphorylation is shown. Specific activity has been calculated for each condition using a protein value obtained from a Bradford protein assay of the cleared cell lysates. Each data point represents n=3 and standard errors of the mean are shown. Western blot analysis of total Myc-CDK2 protein was also performed on transfected cell lysates prepared for Myc-CDK2 assay and a representative blot is shown.
inhibitory effects. Fig. 5.11 demonstrates that G protein βγ subunit sequestration by overexpression of α-transducin (Gαi1) had no effect on the receptor-mediated inhibition of Myc-CDK2 activity, suggesting that the M3 ACh receptor-mediated growth effects involve G-protein α subunits. A representative autoradiogram of histone H1 phosphorylation by Myc-CDK2 immunoprecipitates is shown. Western blot analysis of total Myc-CDK2 indicated that the amount of CDK2 expressed was not affected by CCh stimulation, although in this experiment Gαi1 did affect Myc-CDK2 expression. CCh did not affect the expression of Gαi1 as shown (Fig. 5.11, lower panels). To ensure that βγ subunit sequestration was occurring, the effects of Gαi1 on HA-ERK2 and HA-JNK1 activation were investigated as shown in Fig. 5.12. Gαi1 had profound inhibitory effects on both HA-ERK2 (Fig. 5.12A) and HA-JNK1 (Fig. 5.12B) activation in growing CHO-m3 cells. βγ subunit sequestration inhibited HA-ERK2 activation from a 5.6 fold-over-basal activation in control cells to a 1.5 fold-over-basal activation, and inhibited HA-JNK1 activation from a 4.7 fold-over-basal activation in control cells to a 1.6 fold-over-basal activation. These data suggest that both ERK and JNK activation downstream of the M3 ACh receptor is mediated by G protein βγ subunits. Western blot analysis of total HA-ERK2 and HA-JNK1 indicated that the amount of protein was not affected by CCh stimulation, although as in the previous experiment Gαi1 did affect HA-ERK2 and HA-JNK1 expression. CCh did not affect the expression of Gαi1 as shown.
Figure 5.11. Effect of $\beta$ subunit sequestration on Myc-CDK2 activity in growing CHO-m3 cells. Cells were co-transfected with mammalian expression vectors (4 $\mu$g) encoding Myc-CDK2 and $G_{o_1}$ or an empty vector (pCMV5) and incubated for 48 h prior to stimulation with 10 $\mu$M CCh for 24 h (+). Basal cells were not stimulated with CCh (-). Cells were lysed and Myc-CDK2 was immunoprecipitated with a specific antibody as described under Materials and Methods. Kinase activity was measured using Histone H1 as a substrate and a representative autoradiogram of Histone H1 phosphorylation is shown. Specific activity has been calculated for each condition using a protein value obtained from a Bradford protein assay of the cleared cell lysates. Each data point represents $n=3$ and standard errors of the mean are shown. Western blot analysis of total Myc-CDK2 protein and $G_{o_1}$ was also performed on transfected cell lysates prepared for Myc-CDK2 assay and representative blots are shown.
Figure 5.12. Effect of Gγ subunit sequestration on HA-ERK2 and HA-JNK1 activation in growing CHO-m3 cells. Cells were co-transfected with mammalian expression vectors (4 µg) encoding A, HA-ERK2 and B, HA-JNK1 and Gαt1 or an empty vector (pCMV5) and incubated for 48 h prior to stimulation with 10 µM CCh (+). Basal cells were not stimulated with CCh (-). Specific activity has been calculated for each condition using a protein value obtained from a Bradford protein assay of the cleared cell lysates. Each data point represents n=3 and standard errors of the mean are shown. A, cells were stimulated with CCh for 5 min, lysed and HA-ERK1 was immunoprecipitated using a selective anti-HA antibody as described under Materials and Methods. B, cells were stimulated for 45 min with CCh, lysed and HA-JNK1 was immunoprecipitated with a specific anti-HA antibody as described under Materials and Methods. Kinase activity was measured using GST-c-jun as a substrate and a representative autoradiogram of c-jun phosphorylation is shown. Western blot analysis of total HA-ERK2 and HA-JNK2 protein and Gαt1 was also performed on transfected cell lysates prepared for HA-ERK2 and HA-JNK1 assays and representative blots are shown.
DISCUSSION

In the current Chapter direct molecular biochemical approaches have been utilised to confirm observations concerning the involvement of MAPKs in the M₃ ACh receptor-mediated decrease in DNA synthesis. Experiments involving dominant-negative protein expression, demonstrated that ERK, JNK, and p38 activities are not required for the receptor-mediated inhibition of growth. The growth inhibition was also unaffected by G-protein βγ subunit sequestration, although the M₃ ACh receptor-mediated ERK and JNK activities may be dependent on liberated βγ subunits. This result supports previous reports (Crespo et al., 1994; Coso et al., 1996), although the dramatic effect of Gα₁ plasmid expression on the expression of co-transfected plasmids makes conclusions difficult to draw. Reporter gene assays demonstrated that AP-1, p53, or SP-1 transcription factors are not activated by the M₃ ACh receptor, demonstrating that in CHO cells AP-1 is not a target of M₃ ACh receptor-mediated JNK activation.

Although data presented in Chapter 4 suggested that ERK, JNK and p38 made no contribution to the M₃ ACh receptor-mediated growth inhibition, it relied on pharmacological inhibitors that are often cytotoxic to cells over long incubation periods and may elicit non-specific effects. It was important, particularly for the relatively uncharacterised JNK inhibitors, to use more direct approaches to confirm these inhibitor data. The discovery that the M₃ ACh receptor inhibited over-expressed Myc-tagged CDK2 to a similar level as that of endogenous CDK2 provided a technique to address this pharmacological problem. Co-expression of dominant-negative components of the ERK, JNK or p38 pathways did not abrogate the receptor-mediated inhibition of Myc-tagged CDK2 activity suggesting that the inhibitor data were accurate. This technique relied on the assumption that CDK2 activity could be used as a marker for DNA synthesis. We believe that as CDK2 activity is the only CDK affected by the M₃ ACh receptor at the G₁/S phase transition in CHO cells, and that basal CDK4 activity is low in this cell line, it is likely that CDK2 is critical in the CHO cell growth inhibition, and can be used as a marker for DNA synthesis. These data have also provided further insight into the M₃ ACh receptor to
JNK signalling cascade in CHO cells. JIP, and dominant-negative MKK7 most efficiently inhibited JNK activity compared to a much less effective dominant-negative MKK4 construct. This suggests that in CHO cells JIP may perform a scaffold function for a JNK cascade involving MKK7 (Karandikar and Cobb, 1999). Data in Chapter 4 showed that the MLK inhibitor CEP 11004-2 effectively inhibited JNK activity after M₃ ACh receptor stimulation. MLK3 is another protein capable of binding JIP (Karandikar and Cobb, 1999), thus completing the full JNK ‘module’ of signalling in CHO cells, i.e. JIP provides a scaffold function for MLK3 and MKK7 to facilitate JNK activation by the M₃ ACh receptor. MKK4 cannot bind to JIP, which would explain why it appears to be less significant in the JNK signalling pathway downstream of this receptor (Karandikar and Cobb, 1999).

An attempt to confirm that JNK was not involved in the growth inhibition at the level of DNA synthesis was made using adenoviral-JBD. Although the virus efficiently infected the CHO cells, JNK activity was not completely inhibited, limiting the value of the technique in this study.

As MAPKs were not involved in the growth inhibition, an attempt to elucidate alternative mechanisms that could be involved was made. Experiments showed that G-protein βγ subunit sequestration had no effect on the Myc-tagged CDK2 activity suggesting that the inhibition of growth is likely to be mediated by Gα subunits. This was an interesting finding as Gq, the G-protein that is preferentially activated by the M₃ ACh receptor activates second messenger pathways that could not account for the growth inhibition. Data in Chapter 4 showed that Ca²⁺ was not involved in the growth inhibition and that PKC activity could only partially account for the reduction in DNA synthesis. These data together imply that either Gα₁₂ is activating alternative second messenger pathways that are important in the growth regulation or that the M₃ ACh receptor is coupling to other G-proteins. It is unlikely that the growth effects are due to the receptor coupling promiscuously to G, as the M₂ ACh receptor does not mediate a growth inhibition. Reports have demonstrated that another phosphoinositide-hydrolysing muscarinic receptor, the M₁ ACh receptor, elicits cellular effects through Gα₁₂ signalling to small G-proteins of the Rho family of GTPases (Fromm et al., 1997). If this were the case for the M₃ ACh...
receptor in CHO cells, alternative pathways, for example, Rho family-mediated pathways could be involved in the growth inhibition. This promiscuity would explain why the mechanism of growth regulation has been difficult to clarify in the current study.

The discovery that both ERK and JNK activation in CHO-m3 cells is mediated by βγ subunits adds to the evidence supporting the MAPK-independent growth inhibition as G-protein βγ subunits do not appear to be involved in the growth regulation. These data also provide further mechanistic information into the ERK and JNK signalling cascades in CHO-m3 cells.

The M₃ ACh receptor caused an increase in phosphorylation and expression of c-jun that correlated well with the time-course of JNK activation. This prompted an investigation into specific transcription factors that could be regulating the p21Cip1/Waf1 promotor. AP-1 was not activated by the M₃ ACh receptor. This was unexpected as AP-1 is the classical down-stream target of JNK, and c-jun is part of the heterodimer that constitutes the functional transcription factor (Leppa and Bohmann, 1999). It may be that other components of the AP-1 complex that are required for functional activation, for example fos-family or ATF-2-family transcription factors (Angel and Karin, 1991) are not activated by the M₃ ACh receptor, or expressed in CHO cells. c-Jun is just one component of AP-1, which can consist of numerous homo- or heterodimer combinations. It is likely that multiple signalling cascades must be stimulated to activate the constituent transcription factors to form a functional AP-1 complex. The fact that M₃ ACh receptor-mediated JNK activity is increasing c-jun phosphorylation suggests that this pathway has a functional role in CHO cells, but data suggest that this role does not involve AP-1.

The finding that SP-1 is not activated by the M₃ ACh receptor, or by constitutively active MEKK refutes the hypothesis that c-jun could superactivate SP-1 resulting in increased p21Cip1/Waf1 mRNA and protein expression as reported (Kardassis et al., 1999). As direct activation of JNK by MEKK does not increase SP-1 activity, it is unlikely that this transactivation pathway occurs in CHO cells. p53 was not activated by M₃ ACh receptor suggesting that it is not involved in the induction of p21Cip1/Waf1 mRNA.
Data also demonstrated that the DNA damaging agent cis-platinum did not cause an increase in p53 expression in CHO-m3 cells (not shown) supporting previous reports suggesting that p53 is not functional in these cells (Lee et al., 1997).

Two possibilities remain that would explain the increase in p21<sup>Cipl/Waf1</sup> mRNA. Either a different transcription factor is activating the p21<sup>Cipl/Waf1</sup> promotor, or the increase in mRNA is due to stabilisation of the mRNA, a possibility that has been previously reported (Park et al., 2000). Other transcription factors, for example signal transducers and activator of transcription (STAT) and Smad have binding regions within the p21<sup>Cipl/Waf1</sup> promotor (Gartel and Tyner, 1999), and are both candidates to mediate the increase in p21<sup>Cipl/Waf1</sup> mRNA. For example, STAT3 has been shown to functionally interact with p21<sup>Cipl/Waf1</sup> (Coqueret and Gascan, 2000) and has also been shown to mediate antiproliferative growth effects through activation of p21<sup>Cipl/Waf1</sup> (Su et al. 1997; Sahni et al., 1999). STAT3 can be activated after GPCR activation (Sellers et al., 1999b) and is therefore a candidate for the growth effects seen after M<sub>3</sub> ACh receptor activation in this study.

In summary the data reported in this Chapter have confirmed that the M<sub>3</sub> ACh receptor-mediated inhibition of DNA synthesis is independent of ERK, JNK or p38 activities. These data were generated using direct biochemical approaches, and the techniques used provide examples of how the function of a protein can be investigated at a biochemical level. Data imply that the growth inhibition is mediated by G-protein α subunits, and also show that ERK and JNK are activated by liberated βγ subunits in CHO-m3 cells. The M<sub>3</sub> ACh receptor did not activate any transcription factor that was investigated leaving the issue of p21<sup>Cipl/Waf1</sup> mRNA regulation unanswered.
Chapter 6

General Discussion and Critique
6.1. The M₃ ACh Receptor-Mediated Inhibition of Growth

The discovery that stimulation of the M₃ ACh receptor caused a population of CHO cells to become growth-arrested prompted investigations into the cell cycle mechanisms mediating this effect, and upstream signalling cascades involved in the growth regulation. Data obtained present an apparently paradoxical problem. The inhibition of growth is not mediated by G-protein βγ subunits, and yet second messenger pathways initiated by Gα₉, the classical M₃ ACh receptor-activated G-protein cannot fully account for the growth effects. As mentioned in Chapter 5, other possibilities exist that can explain this inconsistency, the most likely of which involves receptor-G-protein promiscuity.

Although the M₃ ACh receptor primarily couples through G₉, it can also activate other classes of G-protein. For example pharmacological data demonstrated that Gα₉ was preferentially activated by the M₃ ACh receptor as compared with the M₁ ACh receptor, depending on the agonist used (Akam et al., 2001). The G₁₂/₁₃ class of G-protein can also be activated by M₃ ACh receptor stimulation (Brown et al., 1997; Rumenapp et al., 2001). This class of G-protein primarily activates members of the Rho family of small GTPases, including Rho, Rac, and Cdc42, which have all been implicated in Gₛ/S phase cell cycle progression (Olsen et al., 1995, 1998). It is possible that these monomeric G-proteins could be involved in the receptor-mediated growth inhibition described in this Thesis. It is likely that Rac and Cdc42 are expressed in CHO cells, as they are the classical activators of the JNK cascade, a pathway that is strongly induced in CHO-m3 cells. In addition, experiments in CHO and HEK cells showed that M₃ ACh receptor-mediated pathways involved Rho, suggesting that activation of this receptor functionally activates the monomeric G-protein in CHO and other cell lines (Slack, 1998; Strassheim et al., 1999).

Activation of Rho family GTPases also causes effects on the cytoskeleton, including the formation of actin stress fibres and focal adhesion complexes, formation of lamellipodia and filopodia and membrane ruffling (Hall, 1998; Mackay and Hall, 1998), resulting in changes of cell morphology and...
motility. Rho family-mediated changes in cytoskeletal organisation have been implicated in cellular growth arrest. Experiments showed that cell attachment to the extracellular matrix caused a decrease in p21\textsuperscript{Cip1/Waf1} expression in a number of cell lines (Bao \textit{et al.}, 2002). Over-expression of dominant-negative Rac1 and Cdc42 constructs interfered with the anchorage-dependent increase in degradation of p21\textsuperscript{Cip1/Waf1}, implying that these monomeric G-proteins are involved in the signalling pathway regulating the expression of this CDKI. Disruption of cell adhesion in BALB/c 3T3 cells caused a G\textsubscript{i}/S phase arrest mediated by an increase in p21\textsuperscript{Cip1/Waf1} expression and a resultant reduction in CDK2 activity (Kuzumaki and Ishikawa, 1997). These reports provide a link between Rho family GTPases, cytoskeletal re-arrangement, p21\textsuperscript{Cip1/Waf1} expression and growth regulation that can be incorporated into a hypothesis pertaining to this Thesis. The hypothesis would state that: Activation of the M\textsubscript{3} ACh receptor, coupling through Go\textsubscript{12/13}, activates Rho family GTPases that cause re-arrangement of the cytoskeleton. This re-organisation causes an increase in p21\textsuperscript{Cip1/Waf1} expression and results in a G\textsubscript{i}-phase growth arrest.

One further piece of evidence obtained during the course of this project would support this hypothesis. Stimulation of a growing population of CHO-m3 cells with CCh for 24 h caused a distinct change in cell morphology compared with unstimulated cells (data not shown). Receptor activation caused a reduction in cell number, and an elongation of these enlarged cells. This piece of evidence would suggest that receptor activation is causing some changes in cytoskeletal arrangement or composition. Such a phenomenon has been described before. Thus, M\textsubscript{3} ACh receptor activation has been shown to inhibit CHO cell migration (Varker and Williams, 2002). These authors showed that receptor stimulation caused the CHO cells to become elongated, and suggested that this morphology change was 'reminiscent of migrating cells that cannot detach their trailing edges from the substratum.' This report also described the involvement of novel PKC isoforms in the inhibition of cell migration, a finding that correlates with the partial protection of growth inhibition by PKC described in this Thesis. The involvement of RhoA and PKC in cytoskeletal organisation downstream of the M\textsubscript{3} ACh receptor in CHO cells has also been described (Strassheim \textit{et al.}, 1999). Receptor activation caused
phosphorylation of the myosin light chain, a process involved in stress fibre formation and muscle contraction.

Figure 6.1 shows a schematic representation of the hypothesis encompassing the involvement of $G_{13}$, Rho family GTPases and cytoskeletal re-arrangement in the M$_3$ ACh receptor-mediated growth arrest.

Figure 6.1. Schematic representation of the hypothesis generated from the data presented in this thesis. The hypothesis states that activation of the M$_3$ ACh receptor activates both $G_{q/11}$ and $G_{12/13}$ G-protein families. Both ERK and JNK are activated by liberated $\beta\gamma$ subunits whereas the growth inhibition results from activation of $G_{q12/13}$ that causes changes in cytoskeletal organisation and consequent growth arrest. Experiments determined that PKC is involved in the growth inhibition, although the point of involvement has not been established.
Although it is impossible to say, without further experimental investigation, what effect receptor activation is having on cytoskeletal organisation, it is clear that the M₃ ACh receptor has multiple roles in the regulation and organisation of the cytoskeleton. Whatever effects the M₃ ACh receptor activation is having on the CHO cell cytoskeleton in this study could be causing the growth-arrest. Cell attachment is required for anchorage-dependent growth in cell lines that cannot grow in suspension (Le Gall et al., 1998; Bao et al., 2002), so it may be that the receptor-mediated change in CHO cell morphology is disrupting this basic requirement for cell proliferation. The cytoskeletal re-arrangement could be generating a signal for the cell that proliferation must be stopped, and the cell is simply using its cell cycle machinery to elicit that response. It was assumed that the growth arrest was the primary physiological effect of M₃ ACh receptor activation in this study. It may be that the inhibition of growth is a ‘secondary’ effect of receptor activation that is primarily concerned with cytoskeletal organisation and cell motility/morphology.

The involvement of Rho family GTPases, specific Gα subunits, and PKC in the growth inhibition could be investigated using the Myc-CDK2 approach. Co-expression of dominant-interfering mutants of these proteins would determine if this hypothesis is valid, and provide further information about the proteins involved in the growth/cytoskeletal regulation.

6.2. M₃ ACh Receptor-Mediated ERK and JNK Signalling Cascades

Whilst the main thrust of experimentation presented within this Thesis has been concerned with receptor-mediated growth responses, information about activation of the ERK and JNK pathways by the M₃ ACh receptor has also been obtained. Figure 6.2 shows experimentally determined schemes of ERK and JNK activation by the M₃ ACh receptor established in this Thesis. Both MAPKs are activated by liberated G-protein βγ subunits. ERK activation is entirely dependent on PKC as determined by pharmacological data presented in Chapter 4. Although the Ca²⁺-dependence of ERK activation was not investigated, previous work in the laboratory showed that the M₃ ACh receptor-
mediated ERK activation was independent of intracellular Ca$^{2+}$ concentration (Wylie et al., 1999). As ERK activation is dependent on G-protein $\beta\gamma$ subunits and PKC it is probable that the $\beta\gamma$ subunit-sensitive forms of PLC, PLC$\beta_{1,4}$ may be involved in the pathway. ERK activation is mediated by the M KK, MEK1 as over-expression of kinase-inactive MEK1 blocked HA-ERK2 activation as described in Chapter 5. Although c-raf-1 function was not investigated in this study it is likely to be involved in the ERK pathway. Both c-raf-1 and B-raf are expressed in CHO-m3 cells (data not shown), and c-raf-1 classically links PKC to MEK1 and ERK function. Neither PI3-K nor c-src family members are
involved in the ERK activation, as determined pharmacologically, so a relatively simple receptor-ERK signalling cascade can be predicted (Fig. 6.2).

M₃ ACh receptor-mediated JNK activation may involve JIP scaffolding for a JNK signalling module involving MLK (as the MLK inhibitor CEP-11004-2 blocked JNK activation, as described in Chapter 4), and M KK7 (established in Chapter 5). The discovery that signalling-inactive MKK4 partially inhibits HA-JNK1 activation suggests that other mechanisms may link the M₃ ACh receptor to JNK, as MKK4 cannot bind to JIP (Karandikar and Cobb, 1999). Other upstream kinases, for example PAK, may have a role in this signalling cascade. It is likely that Rho family GTPases link the G-protein βγ subunits to the JNK signalling module. Although these proteins were not investigated in this study they are universally involved in JNK activation, are expressed in CHO cells (Strassheim et al., 1999), and can activate MLK (Burbelo et al., 1995; Teramoto et al., 1996; Bock et al., 2000).

6.3. The Significance of Differential Growth Effects Mediated by Two Muscarinic Receptor Sub-types

Although one must be careful when drawing conclusions about the physiological significance of receptors from data obtained in model cells (see: 6.4. Critique) it is fair to discuss some possibilities. The fact that two receptor sub-types elicit opposing growth responses is likely to reflect where they are expressed and the second messenger pathways that they activate. Other than in a variety of smooth muscle types, the M₂ and M₃ ACh receptors are differentially expressed. It may be that organisms have evolved so that the sub-types of receptor expressed in a specific tissue contribute to the growth regulation of cells in that tissue. For example, the M₂ ACh receptor stimulates cell growth under serum-starved conditions so may be involved in cell survival and continuous proliferation during times of cellular stress or growth factor deprivation. The M₃ ACh receptor most efficiently inhibits DNA synthesis under normal growth conditions and so may regulate the extent of growth in tissue with a high proliferative capacity. It is difficult to understand the importance of the opposing growth effects in smooth muscle where both sub-types are co-expressed. It may be that the two receptor sub-types provide a balanced control over the regulation of growth in smooth muscle cells, or that one receptor
dominates the growth regulatory response, and which does so varies with the (patho)physiological circumstance. It is more likely however that the receptors act independently of each other eliciting their effects only under the conditions where their respective growth outcomes are required.

6.4. Critique

The main criticisms concerning this Thesis surround presumptions made during the early stages of the study. It was immediately presumed that effects mediated through the M₃ ACh receptor occurred via G₉/₁₁ coupling. If the question regarding G-protein subunit involvement had been addressed earlier in the study, more time would have been available to explore other possibilities; for example, the involvement of the Rho family of GTPases in the growth arrest. The change in CHO cell morphology after CCh stimulation was a very early observation, but it was not until the end of the study that it was considered important in terms of the growth inhibition. It was initially considered as an effect of the receptor-mediated growth arrest when in fact it is more likely to be causing the growth arrest. Investigating the change in cell morphology as a cause rather than as a consequence of the growth inhibition would have directed the research towards proteins involved in cytoskeletal organisation, which may have provided information on the regulation of growth from a different perspective.

The main limitation of this study was the use of a model cell system throughout the investigation. Although the CHO cell is a good model to investigate muscarinic receptor function (as it has no endogenous muscarinic receptors), it is an immortalized cell line and so physiologically relevant conclusions are difficult to draw from the data obtained. The advantage of this cell system is that it allows a detailed study of the effects of the activation of a single receptor sub-class. If more physiologically relevant cells, for example airways smooth muscle cells, were used, conclusions surrounding the effects of individual muscarinic receptor sub-types would be difficult to make as the cells express a heterogeneous population of muscarinic receptor sub-types that are not easy to individually stimulate through the use of available selective agonists/antagonists. In the model CHO cell system, physiological outcomes, as well as the activities of individual proteins and signalling
pathways can be attributed to activation of single muscarinic receptor sub-types. The use of the model
cell system is therefore an invaluable tool from which valuable information can be generated as long
as the limitations of the system are appreciated.
References


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Koch, W.J., Hawes, B.E., Allen, L.F., and Lefkowitz, R.J. (1994) Direct evidence that G\textsubscript{i}-coupled receptor stimulation of mitogen-activated protein kinase is mediated by G\textsubscript{b}G\textsubscript{y} activation of p21\textsuperscript{ras}. *Proceedings of the National Academy of Sciences of the USA*, **91**, 12706-12710


Kuzumaki, T., and Ishikawa, K. (1997) Loss of cell adhesion to substratum up-regulates p21\textsuperscript{Cip1/WAF1} expression in BALB/c 3T3 fibroblasts. *Biochemical and Biophysical Research Communications*, **238**, 169-172


Rhee, S.G., (2001) Regulation of phosphoinositide-specific phospholipase C. Annual Reviews of Biochemistry, 70, 281-312


Rumenapp, U., Asmus, M., Schablowksi, H., Woznicki, M., Han, L., Jakobs, K.H., Fahimi-Vahid, M.,
expressed in HEK293 cells signals to phospholipase D via Gi12 but not Gq-type G proteins: regulators
of G proteins as tools to dissect pertussis toxin-resistant G proteins in receptor-effector coupling. The
Journal of Biological Chemistry, 276, 2474-2479


signalling inhibits chondrocyte proliferation and regulates bone development through the STAT-1
pathway. Genes and Development, 13, 1361-1366

cyclin E/cyclin-dependent kinase 2 by the protein kinase inhibitor staurosporine are dependent on the
retinoblastoma protein in the bladder carcinoma cell line 5637. Proceedings of the National Academy
of Sciences of the USA, 93, 5941-5946

Schutte, M., Hruban, R.H., Geradts, J., Maynard, R., Hilgers, W., Rabindran, S.K., Moskaluk, C.A.,
Abrogation of the Rb/p16 tumour suppressive pathway in virtually all pancreatic cancers. Cancer
Research, 57, 3126-3130

C-dependent and N17-ras-insensitive mechanism mediates the proliferative response of G12-coupled
somatostatin sst4 receptors. The Journal of Biological Chemistry, 274, 24280-24288

isoforms mediate opposing proliferative effects through Gβγ-activated p38 or Akt pathways.
Molecular and Cellular Biology, 20, 5974-5985

receptor induces phosphorylation of STAT3 and agonist-selective serine phosphorylation via sustained
stimulation of mitogen-activated protein kinase. The Journal of Biological Chemistry, 274, 16423-
16430
receptors for endothelin 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


Proteasomal turnover of p21Cip1 does not require p21Cip1 ubiquitination. *Molecular Cell*, 2, 401-410


and Development*, 9, 1149-1163

*Science*, 252, 802-808

Slack, B.E. (1998) Tyrosine phosphorylation of paxillin and focal adhesion kinase by activation of
muscarinic m3 receptors is dependent on integrin engagement by the extracellular matrix. *Proceedings
of the National Academy of Sciences of the USA*, 95, 7281-7286

receptors transduce potent mitogenic signals in NIH 3T3 cells independent of cAMP inhibition or
conventional protein kinase C. *Oncogene*, 8, 19-26

Stephens, J.R., Eguinoa, A., Erdjument-Bromage, H., Lui, M., Cooke, F., Coadwell, J., Smrcka, A.S.,
dependent upon a tightly associated adapter p101. *Cell*, 89, 105-114

Stewart, A.G., Harris, T., Fernandes, D.J., Schachte, L.C., Koutsoubos, V., Guida, E., Ravenhall, C.E.,
smooth muscle cells in the G1 phase of the cell cycle: Role of proteosome degradation of cyclin D1.
*Molecular Pharmacology*, 56, 1079-1086


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