Regulation of Mitogen-Activated Protein Kinases by Group I Metabotropic Glutamate Receptors

Thesis Submitted for The Degree of Doctor of Philosophy
February 2004

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Department of Cell Physiology and Pharmacology
University of Leicester
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ABSTRACT

The regulation of mitogen-activated protein kinase (MAPK) activities by the group I metabotropic glutamate (mGlu) receptors, mGlu1a and mGlu5a, has been studied in Chinese hamster ovary (CHO) cells, where receptor expression is under inducible control. Both mGlu receptors stimulated comparable, robust and transient increases in the activity of the extracellular signal-regulated kinase (ERK) subgroup. Further, the mGlu1a, but not the mGlu5a receptor was found to mediate an increase in the activity of c-Jun N-terminal kinase (JNK).

Examination of the signalling profile of mGlu1/mGlu5a receptor-mediated ERK activation revealed clear differences in the G-protein subpopulations involved, with only mGlu1a receptor-mediated ERK responses attenuated by pertussis toxin (PTx) pre-treatment. Both mGlu1a and mGlu5a receptor-mediated ERK activation occurred via mechanisms dependent on the non-receptor tyrosine kinase, Src, but independent of phosphoinositide 3-kinase activity, PKC and intracellular and/or extracellular Ca\(^{2+}\) concentration. Data also demonstrate a requirement for PDGF receptor tyrosine kinase activity in ERK activation by the mGlu1a, but not the mGlu5a receptor.

The mGlu1a receptor-mediated JNK response, unlike ERK activation by the same receptor, was insensitive to PTx pre-treatment and occurred via mechanisms independent of intracellular and/or extracellular Ca\(^{2+}\) concentration, Src kinase and was unaffected by PKC down-regulation. The delayed onset of JNK activation by the mGlu1a receptor was not found to be a result of earlier ERK activation.

Stimulation of mGlu1a/mGlu5a receptors, did not alter cellular proliferation, as measured by DNA synthesis, or have a marked effect on cytoskeletal organisation, as measured by immunocytochemistry, indicating that the activation of mitogenic signalling by these two mGlu receptors does not result in changes in growth in these cells. These studies highlight important differences in the activation and signalling pathways utilized to regulate MAP kinases.
ABSTRACTS


PAPERS


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ABBREVIATIONS

AC : Adenylyl Cyclase
AP-1 : Activating protein-1
ASK-1 : Apoptosis signal-regulated kinase-1
ATF2 : Activating transcription factor-2
BMK : Big MAPK (ERK 5)
CaM : Calmodulin
cAMP : 3', 5'-cyclic adenosine monophosphate
CaR : Calcium-sensing receptor
CHO : Chinese hamster ovary
CNS : Central nervous system
EGF : Epidermal growth factor
ERK : Extracellular signal-regulated kinase
FAK : Focal adhesion kinase
GEF : Guanine nucleotide exchange factor
GPCR : G protein-coupled receptor
GPT : Glutamic-pyruvic transaminase
Grb2 : Growth factor receptor binding protein 2
GRK : G protein-coupled receptor kinase
GST : Glutathione-S-transferase
HA : hemagglutinin
IP3 : Inositol 1,4,5-trisphosphate
IPTG : isopropyl-β-D-thiogalactoside
JIP1 : JNK-interacting protein 1
JNK : c-Jun N-terminal kinase
Ksr : Kinase-suppressor of ras
LTD : Long-term depression
LTP : Long-term potentiation
MAP2 : Microtubule-associated protein
MAPK : Mitogen-activated protein kinase
MEK : MAPK/ERK kinase
mGlu : Metabotropic glutamate
MKK : MAP kinase
MKKK : MAP kinase kinase
MLK : Mixed-lineage kinase
MP-1 : MEK partner-1
NMDA : N-methyl-D-aspartate
PAK : p21-activated kinase
PDBu : Phorbol-12,13-dibutyrate
PDGF : Platelet-derived growth factor
PI3-K : Phosphoinositide 3-kinase
PIP2 : Phosphatidylinositol 4,5-bisphosphate
PKC : Protein kinase C
PLA2 : Phospholipase A2
PLC : Phospholipase C
PLD : Phospholipase D
PTx : Pertussis toxin
PYK2 : Proline-rich tyrosine kinase 2
RGS : Regulator of G-protein signalling
RTK : Receptor tyrosine kinase
Shc : Src homology/collagen
Sos : Son-of-sevenless
TCA : Trichloroacetic acid
Chemically-mediated transmission in the central nervous system utilises a diverse array of neurotransmitters. These may exist in the form of simple amino acids such as glycine, neuropeptides such as somatostatin and substance P, or classical transmitters such as acetylcholine, noradrenaline and 5-hydroxytryptamine (5-HT). With regard to excitatory synapses, glutamate is recognised as the major excitatory neurotransmitter (Fonnum, 1984). This simple amino acid mediates its actions through a repertoire of pre- and post-synaptically located ionotropic and metabotropic glutamate receptors. These receptors contribute to the critical role that glutamatergic signalling plays in brain function via processes that include neuronal plasticity, neurodegeneration and pain transmission (Nakanishi, 1992; Conn & Pin, 1997). In addition, glutamate receptors have also been reported to be widely distributed outside the CNS in a variety of tissues, which allows glutamate, the classical neurotransmitter, to participate in signalling in non-excitable cells (Nedergaard et al. 2002).

Although it has been known since the 1950s that glutamate acts a neuroexcitant, it was not until the 1980s that glutamate-gated ion channels were identified (Collingridge & Lester, 1989). These ion channel receptors could be pharmacologically distinguished on the basis of agonist selectivity to structural analogues of L-glutamate, into NMDA (N-methyl-D-aspartate), AMPA (α-amino-5-methyl-4-isoxazole-propionic acid) and kainate subgroups (Nakanishi, 1992). AMPA and kainate receptors are often grouped together as non-NMDA receptors, and are typically permeable to K\(^+\) and Na\(^+\) and Ca\(^{2+}\). NMDA receptors are likewise permeable to K\(^+\), Na\(^+\) and Ca\(^{2+}\), but their unique activation not only involves glutamate binding, but also requires glycine as a co-agonist and membrane depolarisation to remove voltage-dependent block of the channel pore by magnesium ions (Johnson & Ascher, 1987).

For several years it was believed that glutamatergic signalling could be attributed solely to the action of these ionotropic receptors that responded to glutamate by eliciting a fast excitatory response, to facilitate rapid synaptic transmission. However, in the mid-1980s it was
discovered that glutamate could act at another new class of receptors coupled to GTP binding proteins (G-proteins). This discovery demonstrated that glutamate could also elicit slow synaptic responses through a novel family of proteins and highlighted the potential for modulation of the glutamatergic response and for activation of intracellular signalling cascades.

1.1 The metabotropic glutamate (mGlu) receptors
Sladeczek and colleagues (1985) were among the first to demonstrate that excitatory amino acids such as glutamate could directly stimulate inositol phosphate formation in striatal neurones. This study and subsequent observations by Sugiyama and colleagues (Sugiyama et al. 1987) identified that the characteristics of signalling to this second messenger cascade were consistent with that of a G-protein coupled receptor (GPCR). Subsequently, Masu et al. (1991) and Houamed and co-workers (1991) independently cloned the first novel metabotropic glutamate (mGlu) receptor, mGlul.

GPCRs mediate intracellular signalling by activating heterotrimeric G-proteins. These consist of three distinct subunits, α, β and γ, which in an inactive state are bound as a heterotrimer associated with the plasma membrane. Distinct genes encode approx. 20 α subunits, 5 β subunits and 13 γ subunits that are categorised, by reference to the α subunit, into four main classes of G protein, namely Gs, Gi0, Gq/11 and G12/13 (Simon, 1991; Hamm, 1998). The vast number of combinations of different α, β, γ isoforms in the heterotrimer allow potential for subunit composition to dictate the activation of specific pathways. Activation of G-proteins by GPCRs causes the exchange of bound GDP for GTP in the α subunit, which has intrinsic GTPase activity, and the dissociation of the α subunit and a βγ dimer. The dissociated α subunit and the βγ dimer can then independently activate or indeed inhibit target enzymes and channels. Regulation of GDP/GTP exchange, and hence the longevity of the response, is modulated by regulators of G-protein signalling (RGS) proteins that act as GTPase activating proteins (GAPs) for heterotrimeric G-proteins (De Vries et al. 2000; Ross & Wilkie, 2000). Hydrolysis of bound-GTP to GDP results in re-association of α and βγ subunits to form the heterotrimer. In addition to signalling through heterotrimeric G-proteins, the diversity of
signalling via GPCRs is increased by their ability to couple to downstream pathways by G-protein independent mechanisms through various other intracellular proteins (Heuss & Gerber, 2000; Brzostowski & Kimmel, 2001).

Typically G-protein coupled receptors (GPCRs) such as the adrenergic and muscarinic acetylcholine receptors possess a topology that exhibits a 7 transmembrane (7TM)-domain motif flanked by an extracellular N-terminus and an intracellular C-terminus (Ji et al. 1998). Structural homology allows grouping of these GPCRs into families: family 1 type GPCRs, include those of the rhodopsin-like and catecholamine receptors, whereas family 2 is composed of receptors activated by large peptides, such as the glucagon/VIP (vasoactive intestinal peptide) and calcitonin-type GPCRs (Bockaert & Pin, 1999; Pierce et al. 2002).

Analysis of the mGlula receptor amino acid sequence however, revealed no homology to other known G-protein coupled receptors except basic structural similarities. Although hydrophobicity profiles revealed that the receptor exhibited 7 TM spanning domains, it was a predicted polypeptide of 1199 amino acids and therefore much larger than other known GPCRs (Masu et al., 1991). The amino terminal sequence was also surprisingly large and composed of over 500 amino acids and in fact showed discernible sequence similarity to the AMPA/kainate family of ionotropic glutamate receptors. Since the cloning and characterisation of this receptor, a total of eight members of the mGlu receptor family have been identified to date. The observation that mGlu receptors were fundamentally different to other GPCRs, constituted their categorisation into a new third family of GPCRs that also encompasses the Ca²⁺-sensing receptor (CaR) and the GABA_B receptors. These similarly possess the distinct large extracellular domain, displayed by mGlu receptors, which is crucial for ligand binding and activation (Pin et al. 2003).

### 1.2 Classification of mGlu receptor subtypes

The eight cloned mGlu receptors (mGlu1-mGlu8) have been classified into three groups based on their sequence similarity, pharmacology and signal transduction mechanisms (Table 1.1). Members of the same group share approximately 70% sequence identity (Conn and
Pinn, 1997). Group I comprises the first mGlu receptor cloned by Masu and colleagues and Houamed et al. (1991) mGlu1a, and mGlu5, which are both coupled to phosphoinositide hydrolysis by phospholipase C activation and intracellular calcium signalling (Hermans & Challiss, 2001). Group II (mGlu2 & mGlu3) and Group III (mGlu4, mGlu6, mGlu7 and mGlu8) receptors couple negatively to adenylyl cyclase to inhibit cyclic AMP formation and also modulate a variety of ion channels (Ikeda et al. 1995).

<table>
<thead>
<tr>
<th>Group</th>
<th>Subtypes</th>
<th>Splice variants</th>
<th>Preferential signalling pathway</th>
<th>Selective agonists</th>
<th>Selective antagonists</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>mGlu1</td>
<td>a, b, c, d</td>
<td>Positively coupled to PLC to stimulate phosphoinositide turnover and calcium mobilisation</td>
<td>Quisqualate DHPG</td>
<td>CPCCOEt (1a) LY367385 (1a) MPEP (5a)</td>
</tr>
<tr>
<td></td>
<td>mGlu5</td>
<td>a, b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>mGlu2</td>
<td>-</td>
<td>Inhibit adenylyl cyclase activity</td>
<td>DCG-IV ACPD LGU</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mGlu3</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>mGlu4</td>
<td>a, b</td>
<td>Inhibit adenylyl cyclase activity</td>
<td>L-AP4 L-SOP LGU</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mGlu6</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>mGlu7</td>
<td>a, b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>mGlu8</td>
<td>a, b, c</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Table 1.1 Classification and pharmacology of the mGlu receptor family. Modified from Conn and Pin (1997) and Hermans and Challiss (2001). Abbreviations: ACPD, (2R,4R)-aminopyrrolidine-2,4-dicarboxylate; L-AP4, L-2-amino-4-phosphonobutyrate; CPCCOEt, DCG-IV, (2S,2′R,3′R)-2-(2′,3′-dicarboxycyclopropyl)glycine; DHPG, (S)-3,5-dihydroxyphenylglycine; EGLU, (2S)-α-ethylglutamic acid; MAP4, (S)-2-amino-2-methyl-4-phosphonobutanoate; MeSOP, (R,S)-α-methylserine-O-phosphate; MPEP, 2-methyl-6-(phenylethynyl)pyridine; LY341495, (2S)-2-amino-2-(1S,2S-2-carboxycyclopropan-1-yl)-3-(xanth-9-yl)propanoic acid; LY367385, (S)-(+-)α-amino-4-carboxy-2-methylbenzeneacetic acid; Quisqualate, L-quisqualic acid; L-SOP, L-serine-O-phosphate.
There are alternative splice variant forms of mGlu receptors mGlu1, mGlu5, mGlu4 (Iverson et al. 1994) and mGlu7 and 8 (Corti et al. 1998) and these essentially differ in the nature of the carboxy terminal tail. The group I mGlu receptors, mGlu1a and mGlu5a both possess a large carboxy terminus of over 350 residues that is absent from the other mGlu receptors, including the splice variants of mGlu1a, b, c and d. The mGlu5a receptor which, has the highest homology to the mGlu1a receptor with 60.1% sequence identity (Abe et al. 1992), also exists in a splice variant termed mGlu5b (Minakami et al. 1993) that differs in a 32 amino acid insertion in the cytoplasmic tail 49 residues after the 7th transmembrane segment (Joly et al. 1995). Although a specific role of the long carboxy terminus of mGlu1a and mGlu5 receptors has not been identified, a number of features have been attributed to these receptors owing to this structural feature (this is discussed further in Section 1.4.2).

1.3 The expression profile of mGlu receptors

The expression profiles of group I mGlu receptors, mGlu1a and mGlu5a were examined by Abe et al. (1992) by in situ-hybridisation. Although mRNA for both receptors was found to overlap in many brain regions, such as the dentate gyrus, striatum and CA2-CA4 regions of the hippocampus there were also distinct patterns of expression elsewhere. There were high levels of the mGlu1 receptor in the substantia nigra, superior colliculus and Purkinje cells of the cerebellum, whereas the mGlu5a receptor was highly expressed in cerebral cortex, nucleus accumbens, inferior colliculus and hippocampal pyramidal cells. Therefore, although similar in structural features and in their signal transduction, these receptors are specialised in their expression profiles in neuronal cells. Further studies have confirmed that mGlu1a and mGlu5a receptors are differentially distributed both at an anatomical level and with respect to their subcellular distributions (Hubert et al. 2001; Valenti et al. 2002), suggesting that they may fulfil different physiological functions.

At a synaptic level, group I mGlu receptors are generally localised at postsynaptic terminals away from active zones. This postsynaptic specialisation is regulated by the family of 'Homer' proteins, which contain PDZ (postsynaptic density (PSD)-95, Discs-Large, Zona occludens-1) interaction domains (see Section 1.4.2). In contrast, Group II and III mGlu
receptors are mostly presynaptically localised. Shigemoto et al. (1997) identified that group II mGlu receptors are found at peri-synaptic locations (either side of the synaptic cleft), whereas group III mGlu receptors are located in or near the active zone and function as autoreceptors to regulate neurotransmitter release (Cartmell & Schoepp, 2000). The mGlu6 receptor is exclusively expressed by ON bipolar cells in the retina (Masu et al. 1995).

It should be noted that metabotropic, and indeed ionotropic glutamate receptors, are also expressed outside the CNS in a variety of tissues. The role of these ‘peripheral’ glutamate receptors in non-CNS cells is likely to be important in the regulation of second messenger systems, rather than the modulation of rapid excitatory currents (Nedergaard et al. 2002).

Group I mGlu receptor expression has been reported in unmyelinated sensory afferent terminals (Bhave et al. 2001), where they have been implicated in the sensation of pain (see Section 1.6), in melanocytes (Frati et al. 2000), heart cells (Gill et al. 1999) and in the testis (Storto et al. 2001). The expression of mGlu5a receptors on nuclear membranes has also been reported, and shown to modulate oscillatory nuclear Ca^{2+} release (O’Malley et al. 2003).

1.4 Structural architecture of mGlu receptors

The cloned mGlu receptors possess large amino terminal domains that are 500-600 residues in length (Pin et al. 1999) and share marked resemblance to the large amino terminal domains of other major excitatory amino acid receptors (Nakanishi, 1992). As Masu and colleagues first identified (Masu et al. 1991), this region shares sequence similarity with the AMPA/kainate family of ionotropic glutamate receptors and not with other members of the G-protein coupled receptor family. Indeed it was believed that this region may have common evolutionary origin with AMPA/kainate receptors and therefore might be involved in glutamate binding. This is in contrast to the ligand binding domains of many GPCRs that are located in a pocket formed by the interaction of the 7TM domains (Savarese et al. 1992; Gether & Kobilka, 1998; Palczewski et al. 2000).

In 1993, O’Hara and colleagues reported evidence to show that the proposed ligand-binding region (LBR) of mGlu receptors was similar to an amino acid binding domain of bacterial
periplasmic binding proteins (PBPs). In this model the binding domain consists of two globular domains connected by three interdomain linkers (hinge region). Substrate binding occurs in a cleft between the two lobes and induces closure of the lobes trapping the ligand within a cavity. The proposal that glutamate may bind in a similar manner in mGlu receptors was subsequently confirmed by a detailed X-ray crystallographic study by Kunishima et al. (2000). Here, the ligand-binding N-terminal region of the mGlu1a receptor was crystallised and analysed in three different crystal structures: in a complex with glutamate itself and in two unliganded forms. They reported that glutamate did indeed bind in an interdomain crevice as found in ionotropic glutamate receptors and PBPs. This binding resulted in conformational changes in the ligand-binding domain that were transmitted to movements in interdomain and intersubunit interfaces thus resulting in receptor activation. Like PBPs, the LBR of the mGlu1a receptor oscillates between open and closed states even in the absence of ligand. The binding of glutamate within the cleft separating the lobes, stabilizes the closed state although a ligand-bound open state can also be observed (Jingami et al. 2003; Pin et al. 2003).

In addition to the LBR, the extracellular domain of mGlu receptors also exhibits a cysteine-rich region, a feature also displayed by the CaR, but not the GABAB receptor (Kaupmann et al. 1997). Nineteen cysteine residues are conserved in all members of the mGlu receptor family within the N-terminal domain and extracellular loops (Romano et al. 1996). These residues were originally proposed to be important in the formation of disulphide bonds leading to receptor dimerisation, but this is now known to be attributed to Cysteine 140 that contributes to an intermolecular disulphide-linked bridge (Ray et al. 2000).

Increasing evidence suggests that dimerised GPCRs, in the form of dimers, or indeed multimers, mediate intracellular signalling in contrast to the classical view of a single GPCR activated by binding of a single ligand (Angers et al. 2002; Pierce et al. 2002). The first evidence of mGlu receptor dimerisation came from a study by Romano and colleagues (1995), which demonstrated that the mGlu5a receptor formed a co-valently linked dimer by homodimerisation. Although, cysteine residues have been implied to be important in the dimerisation process, other regions of the mGlu receptor may also have a role in the
specificity of dimerisation as mGlu1a and mGlu1b splice variants, which differ in the length of the carboxy terminus, do not form heterodimers (Robbins et al. 1999). Further, heterodimerisation of mGlu1a and mGlu5a receptors does not appear to occur (Romano et al. 1995), and indeed heterodimerisation of other mGlu receptor subtypes has not been reported. However, the mGlu1a receptor has been shown to be capable of forming a heterodimer with the CaR (Gama et al. 2001) and the adenosine A1 receptor (Ciruela et al. 2001). As mGlu receptors appear to form constitutive dimers, the functional role of mGlu receptor dimerisation is still as yet unclear. In addition it is as yet unknown whether two ligands are required to activate the mGlu receptor dimer and indeed whether both subunits of the dimer are necessary for efficient G protein coupling. For GABA_B receptors, some of these questions have been addressed, as it is has been shown that G protein coupling requires molecular determinants from a receptor dimer (Galvez et al. 2001), and that heterodimerisation has been shown to be essential for signal transduction as well as for trafficking of functional receptors to the cell surface (Margeta-Mitrovic et al. 2000; Galvez et al. 2001).

1.4.1 mGlu receptors and G-protein coupling specificity

In prototypic GPCRs, the second, and in particular, third intracellular loops specify G protein coupling, with contributions from the carboxyl terminus (Savarese et al. 1992). In mGlu receptors the third intracellular loop is short and highly conserved and therefore unlikely to be involved in the G protein-coupling specificity of mGlu receptors to different G protein subpopulations. Pin et al. (1994) constructed chimeric receptors between the G_{i/o}-linked mGlu3 receptor and the PLC-linked mGlu1c receptor (although this receptor has a truncated C terminus, it still efficiently couples to PLC suggesting this region does not determine G-protein specificity), and showed that the C-terminal end of the second intracellular loop (i2) and a region downstream of the seventh transmembrane segment were important in the specificity of G-protein activation. Gomeza and colleagues (1996) further reported that chimeric receptors constructed between these two mGlu receptors did generate an mGlu3 receptor that coupled to PLC, but which had altered kinetics compared to those of the wild type mGlu1 receptor. In fact all intracellular domains were found in part to contribute to G
protein activation by mGlu receptors, thus facilitating the critical role of i2 in determining G protein coupling selectivity (Gomeza et al., 1996).

Figure 1.1 Schematic representation of mGlu receptor topology. All receptors of this family possess a large N-terminal domain containing the ligand-binding region (LBR) that is composed of two interconnected lobes, which close around the ligand in a manner analogous to a ‘Venus flytrap’ mechanism (inset). As with other GPCRs, mGlu receptors exhibit a topology of 7 transmembrane (TM) segments. The 7TM motif is labelled I-VII. Red circles represent a cysteine-rich region in the N-terminus. Unlike prototypic GPCRs, the domain important for G-protein coupling specificity is located in the second intracellular loop and is highlighted in green. See main text for further details. Modified from figure in Conn & Pin (1997).
1.4.2 Interaction of group I mGlu receptors with Homer proteins

A feature displayed by the closely related PLC-linked mGlu1a and mGlu5a receptors, is that they possess a long carboxyl-terminal intracellular tail. A feature that the three splice variants of mGlu1 and other members of the mGlu receptor family lack. Joly et al. (1995) noticed that in examining glutamate-stimulated chloride currents in Xenopus oocytes, the mGlu1a receptor generated a rapid and transient response, whereas the truncated isoforms mGlu1b and mGlu1c had slower oscillatory responses. Both mGlu5a and 5b receptor mediated transient responses that resembled those of the mGlu1a receptor. They also showed that in mGlu1a, 5a or 5b transfected LLC-PK1 cells, basal levels of phospholipase C (PLC) activity, as detected by phosphoinositide hydrolysis, were over two-fold higher than control cells. This basal or constitutive activity was not attributable to residual glutamate, and therefore suggested that these receptors are slightly active in the absence of agonist. Using a series of mutagenesis experiments, Mary and co-workers (1998) then reported that the absence of basal activity in the truncated isoforms of the mGlu1a receptor was indeed attributable to the lack of a long carboxyl-terminal domain.

A cluster of four basic residues (RRKK) 36 residues after the seventh transmembrane domain in short mGlu1 receptor variants was proposed to impair their PLC coupling efficacy and perhaps affect expression/trafficking of the receptor at the plasma membrane. The long carboxyl tail of mGlu1a was thought to mask this sequence element and prevent its inhibitory effects. Cell surface expression and targeting of the mGlu1a receptor to the plasma membrane compared to the shorter mGlu1b receptor was subsequently reported by Ciruela et al. (1999) to be regulated by the carboxyl tail. It was suggested that motifs within the different C-terminal tails of these splice variants could determine their differential delivery and anchoring in the plasma membrane. Further, the constitutive activity of mGlu1/5 receptors might be influences via C-terminal interactions with cytoskeletal elements. Indeed, this was confirmed and supported by the discovery of a novel family of ‘Homer’ proteins that selectively bind the carboxyl tail of mGlu1a and mGlu5 receptors (Brakeman et al. 1997; Ango et al. 2001).
Brakeman et al. (1997) first reported a novel gene termed Homer that encoded a 186 amino acid protein with a single PDZ-like domain, which specifically bound mGlu1a and mGlu5a receptors and was regulated by the level of synaptic activity. The expression of Homer was brain-specific and up-regulated in the hippocampus by seizure-induced neuronal activation. A family of Homer proteins has since been characterised (Xiao et al. 1998; Kato et al. 1998) that are distinct from other families of PDZ proteins. These proteins possess an Ena-VASP homology (EVH) domain that allows interaction with mGlu receptors. In contrast to the original Homer protein, Homer 1a, the variants 1b, 1c, 2 and 3 are constitutively expressed and have a C-terminal coil-coiled domain that allows dimerisation of the Homer protein. This structural feature allows these Homer proteins act as linkers to couple mGlu receptors to other intracellular proteins (Xiao et al. 1998; Fagni et al. 2002).

Tu and colleagues (1998) have provided evidence that Homer can physically tether mGlu receptors with inositol trisphosphate receptors (IP$_3$R). Using site-directed mutagenesis they identified a proline-rich PPXXFR motif, found in the C-terminal tail of group I mGlu receptors and in the IP$_3$R, as an important domain for this interaction. In contrast, Homer 1a antagonises this anchoring of mGlu receptors to IP$_3$ receptors and is thought to have a more prominent role in the regulation of intracellular mGlu receptor trafficking. Co-expression of Homer 1a with the mGlu1a receptor has also been shown to increase the cell surface expression of this receptor (Ciruela et al. 1999b) and all three Homer proteins have been shown to target the mGlu5a receptor to dendritic or axonal sites in a manner regulated by neuronal activity (Ango et al. 2000).

A later study by Tu and colleagues (Tu et al. 1999) used a yeast two-hybrid screen, to demonstrate that Homer proteins can also interact with the Shank family of PSD-95 proteins (Ehlers, 1999). Shank proteins are enriched at excitatory synapses and can co-localise with NMDA glutamate receptors (Naisbitt et al. 1999). The Shank-Homer protein interaction suggests that it would be possible to establish physical links between NMDA receptors and Homer-associated mGlu receptors allowing the formation of a microdomain of components necessary to transduce particular signalling cascades.
In addition to Homer proteins, group I mGlu receptors have also been shown to interact with a number of other intracellular proteins and cytoskeletal elements. The mGlu1a receptor has been shown to interact with tubulin via its C terminus, an interaction that can regulate cell surface expression of the receptor (Ciruela et al. 1999; 2001). Cupidin, an isoform of Homer, has been shown to interact with mGlu1a, actin and the Rho family protein Cdc42 thereby providing a molecular scaffold between this mGlu receptor and components of the actin cytoskeleton (Shiraishi et al. 1999). ‘Direct’ activation of small GTPases such as Rho by mGlu receptors has not been definitively reported, but associations such as those with Cupidin and the recently discovered Tamalin, allow speculation that such activation may occur. Tamalin is a PDZ-containing protein that binds group I mGlu receptors and cytohesins, which are GEFs for the ADP-ribosylation factor (ARF) family of small GTP-binding proteins (Kitano et al. 2002). The mammalian homologue of Drosophila seven-in-absentia (Siah-1A) has also been reported to interact with the C-terminal tail of mGlu1a and mGlu5a receptors. Siah-1A prevents calmodulin binding to mGlu1a/5a receptors, but the functional role of this interaction is as yet unknown (Ishikawa et al. 1999). Finally, calmodulin (CaM) has also been shown to interact with the C-terminus of mGlu1a/5 receptors in a Ca2+-dependent manner. For the mGlu5a receptor, this interaction prevents PKC-dependent phosphorylation (see below) of the receptor (Minakami et al. 1997).

1.5 Signalling through group I mGlu receptors

Group I mGlu receptors are known to preferentially couple to PLC activation through coupling to Gq/11 family G proteins. However, both receptors can additionally couple to other G protein classes to elicit several downstream signalling events. Although coupling of the mGlu5a receptor to this pathway is known to be relatively unaffected by PTx pre-treatment, linkage of the mGlu1a receptor to PLC has been shown to have a component of PTx sensitivity in some cell systems, suggesting that Gtp protein coupling may have a role in mGlu1a receptor-mediated phosphoinositide turnover. However, this is a complicated story with some reports of PTx attenuating mGlu1a receptor-phosphoinositide responses (Aramori & Nakinishi, 1992; Pickering et al. 1993; Thomsen et al. 1993), whereas others report an enhancement following PTx pre-treatment (Carruthers et al. 1997; Hermans et al. 2000).
The mGlu1a receptor, but not its shorter splice variants, has also been shown to couple to Gs family G proteins (Francesconi & Duvoisin, 1998) to mediate increases in cAMP accumulation via the activation of adenylyl cyclase in a number of cell lines, including CHO (Aramori & Nakanishi, 1992) and BHK (Hermans et al. 2000) cells. It is unclear however, if the mGlu1a receptor couples directly to Gs family G proteins to mediate this activation or whether it is a consequence of PLC activation. Although the mGlu5a receptor does not stimulate increases in cAMP in CHO cells, this receptor has been shown to mediate such responses in LLC-PK1 cells (Joly et al. 1995). Observations of increases in phospholipase A2 (PLA2) and phospholipase D (PLD) activities have also been reported in response to group I mGlu receptor stimulation (Nitsch et al. 1997; Servitja et al. 1999; Hermans & Challiss, 2001).

In addition to PLC/PKC activation influencing downstream signalling events, PKC also plays an important role in the feedback mechanism controlling mGlu1/5 receptor desensitisation (Alaluf et al. 1995; Gereau & Heinemann, 1998). Although other mechanisms of desensitisation exist (Dale et al. 2002), that mediated by PKC is believed to play a major role. PKC-mediated receptor phosphorylation selectively desensitises phosphoinositide hydrolysis; whereas mGlu receptor-mediated cAMP responses remain intact (Francesconi & Duvoisin, 2000). Desensitisation of mGlu receptors can also occur by phosphorylation via GPCR kinases (GRKs) (Dhami et al. 2002; Sorensen & Conn 2003), which enable interaction with β-arrestins and subsequent receptor internalisation (Ciruela & McIlhinney, 1997; Doherty et al. 1999).

PLC activation by group I mGlu receptors also results in the downstream increases in calcium mobilisation. The Ca²⁺ signatures generated following mGlu1a and mGlu5a receptor activation differ markedly. The mGlu1a receptor activates an agonist-induced single-peak of intracellular calcium release followed by a plateau phase, whereas the mGlu5a receptor elicits oscillatory patterns of calcium release (Kawabata et al. 1998; Hermans and Challiss 2001; Nash et al. 2001; 2002; Young et al. 2003). Kawabata and colleagues (1998) used the generation of several chimeras between these two receptors to show that this difference in calcium signalling was due to a critical amino acid residue (Thr⁸⁴⁰) in the C-terminus of
mGlu5a receptor which was different to that found at the same position in the mGlu1a receptor (Asp\textsuperscript{84}). The pattern of oscillatory calcium release was attributed to this difference in residues owing to the cyclical desensitisation and re-sensitisation of the receptor by PKC phosphorylation of this site.

These patterns of intracellular calcium signalling may have important consequences in subsequent signal transduction, but extracellular calcium [Ca\textsuperscript{2+}]\textsubscript{o} has also been shown to play an important role in mGlu receptor-mediated signalling. Manipulations in [Ca\textsuperscript{2+}]\textsubscript{o} have been shown to affect the efficacy of glutamate action (Kubowaka \textit{et al.} 1996) and indeed functional responses mediated by group I mGlu receptors, such as PLC activation (Kubo \textit{et al.} 1998; Saunders \textit{et al.} 1998). Whether extracellular Ca\textsuperscript{2+} itself directly stimulates mGlu receptors, in the absence of agonist, remains controversial. Such modulation has been reported by Kubo \textit{et al.} (1998) and more recently by Francescon & Duvoisin (2004). This latter study has reported that the activity of, transiently expressed, mGlu1a and the group II mGlu2 receptor are activated by extracellular Ca\textsuperscript{2+} in the absence of glutamate in HEK 293 cells.

In addition to phosphoinositide hydrolysis and calcium signalling, group I mGlu receptors can also regulate cell excitability by regulating specific ion channels. This regulation can occur directly via liberated G protein subunits or downstream of second messengers generated through coupling to intracellular signalling pathways. The inhibition of potassium channels, which are common targets of mGlu receptor activation, leads to increased cell excitability. For example the M-type potassium channel is inhibited by activation of the mGlu1a receptor, in a PTx-insensitive manner (Ikeda \textit{et al.} 1995). Both mGlu1a and mGlu5a receptors have also been shown to decrease currents through voltage-dependent calcium channels in a PTx-sensitive manner, in a number of cell systems (Swartz \textit{et al.} 1992; Rothe \textit{et al.} 1994; Choi & Lovinger, 1996). In HEK 293 cells, mGlu receptors modulate calcium conductances through a mechanism believed to involve G\betaγ subunits (McCool \textit{et al.} 1998). The group II mGlu2 receptor has been shown to activate a G-protein coupled inward rectifier potassium channel (GIRK) in \textit{Xenopus} oocytes, whereas mGlu1a and mGlu5a receptor activation inhibits the same channel (Sharon \textit{et al.} 1997). Regulation of ion channel activity by mGlu receptors has also been shown to occur via interaction with Homer proteins, as with modulation of N-type
calcium and M-type potassium channels by mGlu1a and mGlu5a receptors in cervical ganglion sympathetic neurons (Kammermeier et al. 2000).

Figure 1.2 Major signalling pathways activated by group I mGlu receptor activation. In addition to coupling to $G_{q11}$ G proteins to mediate PLC activation, mGlu1a and mGlu5 can couple to $G_{i/o}$ and $G_{s}$ proteins to activate multiple signalling cascades. These may be mediated by direct G protein association, or secondary to primary coupling to PLC activation. Block arrows represent direct associations whereas broken arrows represent multiple signalling steps or intermediary pathways not fully understood. See main text for details. Modified from figure in Hermans & Challiss (2001).
1.6 Roles of mGlu receptor signalling

As mentioned, the mGluRs show a heterogeneous expression in the central nervous system and also display a diverse range of functions and roles in physiological processes. The mGlu receptor subtype and the associated signal transduction pathways, therefore determine the nature and location of these functions. In the CNS, group I mGlu receptors play an important role in mediating events involved in synaptic plasticity including long-term potentiation (LTP) and long-term depression (LTD) (Lu et al. 1997; Bortolotto et al. 1999). In some brain regions, mGlu1a and mGlu5a receptors have been shown to differentially regulate such synaptic activity (Mannaioni et al. 2001; Poisik et al. 2003) and therefore there is the potential to target particular mGlu receptor subtypes to regulate specific CNS functions. In addition to the modulation of physiological functions, mGlu receptors have also been implicated in the pathology of the CNS. For instance, in excitotoxic events such as stroke and neurodegenerative disorders, agonists and antagonists of mGlu receptors have the potential to modulate the extent of excitotoxicity in cells and ultimately neuronal death (Nicoletti et al. 1995). The activation of mGlu receptors has also been demonstrated to regulate the morphology and development of neurones (Gomperts et al. 2000; Vanderklish & Edelman, 2002).

As mGlu receptors can be located pre- and post-synaptically, receptors from each group have the ability to function as autoreceptors to regulate transmitter release (Cartmell & Schoepp, 2000). However, there is also evidence to show that group I mGlu receptors are located peripherally, outside the CNS on unmyelinated sensory afferents in mice. This localisation is predominantly on nociceptive afferents and Bhave et al. (2001) have reported that these peripheral group I mGlu receptors can regulate nociceptive signalling. Consistent with this study, Karim and colleagues (2001) have shown that mGlu1a and mGlu5a receptors activate the extracellular signal-regulated kinase (ERK) family of mitogen-activated protein kinases (MAPKs) and increase nociceptive sensitivity in response to inflammation.

The activation and modulation of mitogen-activated protein kinases in response to mGlu receptor stimulation was first characterised by Fiore and co-workers (1993) in primary rat cortical cultures and demonstrated that these regulators of cell division and differentiation had a role in neuronal signalling in response to activation by glutamate.
1.7 The mitogen-activated protein kinases (MAPKs)

The mitogen-activated protein kinases are serine/threonine kinases that exhibit a high degree of evolutionary conservation and have many important roles in yeast, plants and mammalian cells. These include regulation of cell proliferation, differentiation, apoptosis and cell survival as well as processes such as mating and cell wall remodelling in yeast (Widmann et al. 1999).

The first MAPK was identified when the stimulation of various cell types with growth factors resulted in the phosphorylation of an unknown 42 KDa protein (Cooper et al. 1982). This protein was subsequently identified to be a serine/threonine kinase that phosphorylated microtubule-associated protein 2 (MAP2) in response to stimulation with insulin in adipocytes (Ray & Sturgill, 1987). When Boulton et al. (1990) subsequently cloned the cDNA encoding this MAPK, the term extracellular signal-regulated kinase (ERK) was adopted owing to the diverse nature of stimuli capable of its activation.

In mammals there are currently four distinct subfamilies of MAPKs recognised, which are grouped on the basis of the extracellular stimuli that activate them, sequence homology and activation by specific upstream kinases. These subfamilies are the extracellular signal-regulated kinases (ERKs 1-3) mentioned above, c-Jun NH$_2$ terminal kinases (JNKs 1-3) (also termed stress-activated protein kinases, SAPK1a, b, c), the p38 protein kinases (p38 $\alpha$, $\beta$, $\gamma$, $\delta$) and the big-mitogen activated protein kinase 1 (BMK1 or ERK 5). Additionally, ERK 7 (Abe et al. 1999), MOK kinases (Miyata et al. 1999) and a splice variant of ERK1, ERK1b (Yung et al. 2000) have also been identified.

Growth factors, hormones, cytokines, and neurotransmitters mediate the activation of ERK signalling, whereas JNK and p38 pathways are preferentially stimulated by cellular stress caused by UV irradiation, oxidative stress and heat or osmotic shock. Despite the diverse array of stimuli that activate these MAPKs, the actual mechanisms of activation are highly conserved (Widmann et al. 1999) by the existence of a sequential ‘three-kinase module’ of activation that is conserved in all eukaryotic cells (Figure 1.3A).
The assembly of this MAPK signalling pathway begins with the first kinase in the module, which is referred to as a MAPK kinase kinase (MKKK). In the ERK pathway, the prototypic MKKK in mammalian cells, is c-Raf (Kolch, 2000). The MKKK may itself be activated by an upstream kinase or be activated by the actions of the Ras/Rho family of small GTP binding proteins. Once, activated these serine/threonine kinases phosphorylate the second kinase in the module: the MAPK kinase (MKK). In the ERK cascade, the MKKs downstream of c-Raf are MEK 1 and 2, which proceed to phosphorylate and activate ERK 1/2.

The MKKs are dual specificity kinases that phosphorylate their target MAPK substrates at two critical threonine and tyrosine residues. This part of the pathway allows a degree of regulation, as only specific combinations of MKK and MAPK are possible (Widman et al. 1999; Garrington & Johnson, 1999). This specificity arises from the recognition of a tertiary structure surrounding the Thr-X-Tyr site of phosphorylation, in the activation loop of a specific MAPK. The activated MAPK can then be proline-directed to phosphorylate target proteins such as, other kinases, cytoplasmic substrates and transcription factors, at serine/threonine residues. The MKKs themselves are cytoplasmic proteins that are able to enter the nucleus, but a nuclear export sequence quickly removes them back to the cytoplasm.

To date, fourteen MKKK, seven MKK and twelve MAPKs have been identified. Although the MKKs are highly specific for the activation of particular MAPKs (Figure 1.3B), different MKKKs are able to activate many MKKs in the pathway. However, specificity is still achieved by association with scaffolding and anchoring proteins (Garrington & Johnson, 1999) that can co-ordinate the assembly of certain MKKK with specific downstream MKK/MAPK modules. The MKKKs themselves also exhibit regulatory motifs, including SH3 domains, phosphorylation sites and pleckstrin homology (PH) domains not found in MKK and MAPKs. These features allow the MAPK module to respond to a wide range of extracellular stimuli whilst maintaining a level of specificity in the signalling cascade.

It should be noted that the activation of MAPKs is a reversible process and therefore the actions of protein phosphatases also provide an important mechanism of control (Camps et al. 1999). As MAPKs require dual phosphorylation for activity, dephosphorylation at either
critical threonine or tyrosine residues by phosphatases is sufficient for inactivation (Keyse, 2000). Such regulation, determining the duration of MAPK activation is an important factor in governing downstream cellular effects. In cultured PC12 cells, for example, two different responses are observed on exposure to epidermal growth factor (EGF) or nerve growth factor (NGF). Whilst EGF stimulates cell proliferation, NGF causes cellular differentiation, and this differential outcome was shown to be accounted for by the ability of NGF, but not EGF, to cause sustained activation and subsequent nuclear translocation of MAPK (Marshall, 1995). As the translocation of MAPK to the nucleus is required for phosphorylation of transcription factors, which are major targets of all MAPK subfamilies, the duration of the MAPK response is important in determining their phosphorylation, as prolonged MAPK activation is generally associated with mediating these events. Further to the temporal activation of MAPK to identify specific signals and downstream effects, the spatial organisation of MAPK cascades are key to the efficiency of signalling by concentrating the components of the pathway (Pouyességur & Lenormand, 2003).

Such spatial organisation is mediated by specific scaffolding and anchoring proteins in each MAPK cascade. In the case of the ERK, MEK partner 1 (MP1) was identified using a yeast two-hybrid screen, as a scaffold protein that specifically binds MEK1 and ERK1 (Schaeffer et al. 1998). The kinase suppressor of Ras (KSR) is another scaffold for the ERK pathway that binds ERK and MEK. This scaffold binds the adapter protein 14-3-3 and dephosphorylation of KSR, in response to stimulation with growth factors; by a regulatory protein c-TAK1 allows liberation of KSR from 14-3-3 and translocation of KSR from the cytoplasm to the cell membrane where it brings ERK and MEK close to Raf (see review by Kolch, 2000). The 14-3-3 proteins are a family of ubiquitously expressed proteins that act as chaperones and scaffolds for a number of signalling proteins. 14-3-3 binds and enhances Raf signalling and binds to members of the MEKK family (Fanger et al. 1998; Hagemann & Blank, 2001). In addition to MEKK1 acting as an upstream kinase, this protein can also serve a scaffolding function for ERK and JNK cascades by binding MEK1 / ERK2 as well as JNK/ MKK4/7 (Karandikar & Cobb, 1999).
The JNK interacting proteins (JIPs) act as molecular scaffolds that organize JNK signal transduction cascades. JIP1-2 bind JNK, the JNK activator MKK and members of the mixed lineage kinases (MLKs) (Gallo & Johnson, 2002) but does not bind MEKK1, 3, 4 and MKK4. The structurally unrelated JIP3 also binds these kinases. Tethering of JNK signalling molecules to such scaffolds acts to facilitate and amplify the signal by concentrating key components in response to a specific stimulus (Davis, 2000). In addition to JIP, β-arrestin has also been shown to be an important scaffold in mediating the spatial organization of receptor activation of JNK3 (McDonald et al. 2000; Manning & Davis, 2003). Further to this, β-arrestin is also an important scaffold involved in activation and subcellular targeting of the ERK subgroup of MAPK, with particularly with reference to receptor internalisation.

Internalisation and subsequent redistribution of an activated receptor by endocytosis has been shown to be involved in mediating ERK activation. This may be β-arrestin-dependent and brings receptor and the three-kinase module in close proximity. Whilst some reports demonstrate receptor internalisation is necessary to co-localise receptors with components of the ERK cascade (Ignatova et al. 1999; Luttrell et al. 2001; Lavoie et al. 2002), others have shown that this is not a prerequisite for all receptor/ERK activation (Budd et al. 1999; Schramm & Limbird, 1999).

Upstream of the three-kinase module, numerous extracellular stimuli can preferentially activate MAPK signalling cascades by the stimulation of particular cell surface receptors. These include classical receptor tyrosine kinases (RTKs) and members of the GPCR family. Stimulation of RTKs, such as the platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptors, leads to the activation of their intrinsic tyrosine autophosphorylation mechanism and the recruitment of various adapter proteins. These proteins enable specific interactions between proteins through motifs such as Src homology-2 (SH2) and SH3 domains, and allow subsequent activation of the relevant downstream MAPK pathway (reviewed in Malarkey et al. 1995).
Figure 1.3 ‘Three-kinase module’ of MAPK activation and the three major MAPK kinase cascades. Left side panel A depicts the three-component hierarchy comprising of a MKKK, and an MKK upstream of the MAPK. Each is sequentially phosphorylated by the upstream kinase at specific residues. Right side panel B shows the three-kinase modules operating in the three main MAPK cascades: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK. See main text for details of MKKK and MKK specific to activation of these cascades.
1.8 GPCR activation of the ERK MAP kinases

The activation of the ERK cascade, by RTKs is well characterised and different RTKs commonly utilise the same repertoire of intracellular intermediates upstream of the ERK module (Figure 1.4A). Activation by the EGF receptor for example, leads to recruitment of the adapter protein Shc. Shc is subsequently tyrosine phosphorylated by the activated receptor, or by a non-receptor tyrosine kinase such as c-Src, and then itself recruits another adapter protein growth factor receptor binding protein 2 (Grb2), via its SH2 domain. Grb2 itself constitutively binds the guanine nucleotide exchange factor (GEF) Sos (Son-of-sevenless), which is thus also recruited and catalyses the exchange of GDP for GTP on the small GTP binding protein, Ras. GTP bound Ras is then able to co-localise and activate the first component of the ERK module: the MKKK Raf-1 (Raf-A and Raf-B isoforms also exist in mammals, but have a more limited expression pattern; Robinson & Cobb, 1997). Activated Raf-1 then phosphorylates MEK 1/2, the dual specificity MKK activators of ERK 1/2. Downstream targets of ERK activation include several microtubule-associated proteins, cytosolic phospholipase A$_2$, p90-S6 kinase and various transcription factors.

Many GPCRs have been shown to activate ERK, however, there is much heterogeneity in the mechanisms employed by GPCRs and further complexity arises from the number of different G-proteins through which GPCRs transmit their signals. The responses are often also cell type-specific and more complex than the well-characterised mechanisms of activation by RTKs. In addition, many of the pathways employed by GPCRs to activate ERK function in parallel with components of classical RTK cascades and involve a process that has been termed transactivation (see Section 1.8.1). Although mechanisms of ERK activation by GPCRs are diverse, a number of common themes can be discerned and one of the most important factors governing the intracellular signalling events is the class of G protein that the GPCR in question couples to.

When $G_{i/o}$ coupled GPCRs are involved, activation of ERK is primarily mediated through mechanisms involving released $\beta\gamma$ subunits. Crespo et al. (1994) demonstrated in COS-7 cells, that $\beta\gamma$ subunits could elicit both $G_i$-and $G_q$-mediated activation of ERK in a Ras-dependent manner. Released $\beta\gamma$ subunits are capable of activating ERK at a number of
different levels in the kinase module. This may be through the activation of the non-receptor tyrosine kinases, such as c-Src, as reported by Luttrell et al. (1996). In this study, a Src-like kinase was reported to be activated in response to lysophosphatidic acid (LPA) stimulation in COS-7 cells. LPA was shown to activate Src association with the adapter protein Shc, leading to PTx sensitive increases in Src tyrosine kinase activity. It was demonstrated that Gβγ subunits mediated the formation of these Src-Shc complexes, which subsequently lead to the activation of Ras via the recruitment of Grb2 and Sos to Shc.

In addition, routes of ERK activation mediated by βγ subunits have also been shown to involve phosphoinositide 3-kinases (PI3K) (Hawes et al. 1996). Gerhardt and colleagues (1999) demonstrated that in Chinese-hamster ovary cells, the human β3 adrenoceptor, which stimulated adenyl cyclase through coupling of Gs, could also activate ERK in a PTX-sensitive manner involving PI3K. In a study by Lopez-Illasaca and colleagues (1997) it was shown that the γ isoform of PI3K was specifically activated by βγ subunits and could activate ERK at the level of Src activation and Shc recruitment.

The involvement of Gβγ activation of ERK by GPCRs can also occur at the level of PLC activation, which is commonly envisaged to involve Gq-type G proteins. Liberated Gβγ subunits can activate PLCβ and lead to elevations in intracellular Ca^{2+}. These increases in Ca^{2+} can lead to activation of the calcium/calmodulin-dependent tyrosine kinase PYK2. PYK2 can then associate with Src kinases and therefore link Gi and Gq receptors to Grb2 and Sos pathways upstream of ERK activation (Dikic et al. 1996). This parallel nature of many of the pathways employed by GPCRs and RTKs to activate ERK demonstrate that there can be many points of convergence between these two pathways, especially involving non-receptor kinases, such as PYK2 and Src. GPCR signalling and cross-talk with RTK signalling to cause ERK activation are depicted in Figure 1.4B.

This convergence can occur downstream of receptor activation and G protein coupling to activate the same MAPK module. In a study by Blaukat et al. (2000), signalling pathways linking the bradykinin B2 receptor to ERK activation in HEK 293 cells were analysed. They showed that activation was not induced in a linear fashion involving either PTX-sensitive G
proteins or Gq, but found that co-operative actions of both Gq and Gi proteins were required for effective activation of ERK.

These pathways have all been demonstrated to involve receptor activation of ERK involving the small GTP binding protein, Ras. However, mechanisms of ERK activation also exist that are Ras-independent. For example, Gq-coupled GPCRs are able to activate the ERK MAPK, independently of Ras, by the direct activation of the ERK MKKK, Raf by PKC. Phorbol esters, which are potent activators of PKC, and over-expression of PLCβ have been shown to stimulate Raf activity (Marquardt et al. 1994). As non-receptor tyrosine kinases are not involved, pathways mediated in this manner are insensitive to protein tyrosine kinase (PTK) inhibitors. Hawes et al. (1997) demonstrated that Gq-mediated GPCR activation of ERK involved the αq subunit and was Ras-independent and unaffected by PTK inhibitors, but was inhibited by PKC down-regulation.

1.8.1 Transactivation of RTKs by GPCRs
Daub and colleagues (1996) were the first to use the term 'transactivation' in reference to the observation that a number of GPCR agonists could stimulate activation of the EGFR. This demonstrated a mechanism of ligand-independent activation of RTKs via cross-talk with GPCRs. Such a model suggested that RTKs could be recruited downstream of GPCRs to mediate coupling to MAPK pathways. Since this study a plethora of subsequent work by various groups has demonstrated that a number of GPCRs can transactivate RTKs as a means of transducing mitogenic signalling (Daub et al. 1997).

Proposed models of the mechanism of RTK transactivation by GPCRs (reviewed by Wetzker & Böhmer, 2003) include a sequence of membrane delimited signalling events or signalling mediated by non-receptor tyrosine kinases such as Src. The former involves a series of three steps that lead to transactivation involving membrane bound metalloproteinases. It was originally believed that transactivation occurred in a manner independent of an RTK ligand. However, Prenzel et al. (1999) demonstrated that GPCR stimulation of the EGFR involved release of a soluble ligand, heparin-binding EGF (HB-EGF):
Figure 1.4 Common intracellular signalling intermediates involved in RTK and GPCR activation of the ERK MAPK. Left side panel A depicts classical RTK components recruited upstream of the ERK module. Right side panel B shows common signalling upstream of ERK activation by $G_{i}$- or $G_{q}$-coupled GPCRs. The mechanism of activation by GPCRs often use kinases and scaffold proteins parallel those utilised by RTKs and there may be several points of convergence. GPCRs may also activate RTKs by means of transactivation and therefore activate components recruited downstream of the activated RTK.
HB-EGF is membrane-spanning protein which, is cleaved by membrane-bound metalloproteinases resulting in the release or ‘shedding’ of the HB-EGF ligand from the membrane allowing it to transactivate the RTK (Pierce et al. 2001). GPCR activation, cleavage of the latent HB-EGF ligand and the resultant activation of the EGFR are the essential events proposed to underly transactivation in this model. Although intracellular metalloproteinases are believed to mediate transactivation in this manner, the precise mechanism involved has not yet been elucidated. However, transactivation can also occur in a manner independent of metalloproteinase-mediated HB-EGF shedding by proteins such as Src, which have been shown to activate RTKs downstream of GPCR activation. Such mechanisms have been proposed for Src-mediated transactivation of the EGFR by the β-adrenoceptor (Kim et al. 2002) and transactivation of this RTK by the mGlu5a receptor (Peavy et al. 2001). Belcheva and co-workers (2001) have additionally demonstrated a role for calmodulin in the transactivation of the EGFR by the μ-opioid receptor.

In summary, the regulation of ERK activity is a complicated story and the nature and repertoire of signalling components involved is highly dependent on the class of receptor, whether an RTK or GPCR, activated by a particular ligand and the cell type, which is being investigated. In terms of GPCR activation of ERK, the class of G-protein, and indeed the G-protein subunits, involved is an important determinant of the subsequent intracellular signalling partners utilised in coupling to ERK as activation of G<sub>ı0</sub> or G<sub>q/11</sub> family proteins, as described above, can recruit the involvement of different kinases. The signal transduction mechanisms are also dependent upon the organisation and scaffolding of these pathways, the nature of which also depend on specific cell systems and the expression of the signalling components in different cell types. All these considerations are vital when elucidating and comparing mechanisms of ERK activation by specific receptors.

1.9 GPCR activation of the stress-activated MAP kinases
The c-Jun N-terminal kinase (JNK) MAP pathway is activated by cellular stresses such as ultraviolet radiation, heat and osmotic shock as well as by inflammatory cytokines. These different extracellular stimuli activate JNK through a number of cell surface receptors,
including GPCRs, TNF receptors and cytokine receptors. However, for GPCR activation of JNK much less is known about the events immediately downstream of receptor activation to couple to activation of the JNK module and there is much speculation about the pathways that may be involved.

When first identified in 1991 (Pulverer et al. 1991) it was shown that this kinase, like ERK, was activated by phosphorylation on threonine and tyrosine residues of the Thr-X-Tyr activation loop by specific MKKs (MKK4 and MKK7). However, JNK was able to phosphorylate the transcription factor c-Jun at its N-terminal domain rather than at C-terminal sites phosphorylated by ERK. To date three genes encoding JNK have been identified and these give rise to 10 different isoforms of this kinase. JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 displays a limited expression pattern and is mostly restricted to the brain (Gupta et al. 1996). The p38 kinase was first discovered in 1994 by Han and colleagues and it is now known that there are four primary isoforms of p38 MAP kinase, p38α, β, γ and δ. The p38 kinases are widely expressed in many tissues but only p38α and p38β isoforms are expressed in the brain (Harper & LoGrasso, 2001). Targets of p38 MAP kinase activation include many of the transcription factors also activated by JNK.

The JNKs were initially characterised by their ability to phosphorylate and activate the transcription factor c-Jun, a subunit of the transcription activator protein 1 (AP-1) (Pulverer et al. 1991), but other transcription factors, such as AFT-2, ELK1 and p53 are also activated downstream of JNK. In addition to transcription factors, JNK and p38 also have a number of cytosolic targets (Mielke & Herdegen, 2000). As the regulation of p38 activity is often similar to that of JNK, and indeed multiple stimuli that activate p38 also activate JNK, only upstream signalling to the former will be discussed here.

Several MKKKs, from a number of different groups, can activate the JNK module; these include the MEKK group (1-4), the mixed-lineage protein kinases (MLK1-3), apoptosis-inducing kinase (ASK1-2) and transforming growth factor β (TGFβ)-activated kinase 1 (TAK1) (Davis, 2000). In contrast to ERK activation, Raf does not play a direct role in JNK activation. The specificity with respect to which MKKK are activated in response to specific
physiological stimuli is unclear. Expression of wild-type MEKK1, also a weak activator of ERK, or overexpression of MLK3 (Teramoto *et al.* 1996) is sufficient for JNK activation; indeed much of the evidence for involvement of particular MKKK has involved overexpression of these proteins and subsequent analysis of the activation of downstream kinases MKK4 and MKK7. MEKK3 has been shown to be a specific activator of MKK7 and MKK6, involved in JNK and p38 pathways, respectively (Deacon & Blank, 1999).

MKK4 and MKK7 are dual specificity kinases that phosphorylate JNK on threonine and tyrosine residues. JNK can be activated by MKK4 and MKK7 in the nucleus or in the cytoplasm, as these MKKs are found in both locations (Tournier *et al.* 1999) unlike activators of ERK MEK1/2, which are cytoplasmic owing to their nuclear export sequence. MKK4, but not MKK7 can additionally activate p38, which is activated by upstream MKK3 and MKK6 (Enslen *et al.* 1998). Observations that MKK4 and MKK7 can be preferentially activated by cytokines or environmental stresses respectively imply that a degree of specificity for upstream MKKs to be activated in response to certain stimuli exists (Tournier *et al.* 1999). As previously discussed, scaffold molecules such as JIP, which binds JNK, MKK7 (but not MKK4) and MLKs, organise and facilitate the efficient activation of the JNK signal.

The phosphorylation of downstream substrates by JNK occurs at a Ser/Thr-X-Pro motif as with ERK, but an additional docking site is also required to properly direct JNK to the N-terminal phosphorylation motif of c-Jun. The docking and substrate specificities of JNK 1, and 2 have been shown to be different, such that c-Jun has been shown to be preferentially phosphorylated by JNK1 whereas ATF2 is preferentially phosphorylated by JNK2 (Kallunki *et al.* 1994). Such specificity may have implications for tissues where JNK isoforms are differentially expressed (Gupta *et al.* 1996).

Upstream of the three-kinase module, JNK can be activated by the small GTP-binding proteins of the Rho family, in particular, Rac and Cdc42 (Kyriakis & Avruch 1996; Teramoto *et al.* 1996; Coso *et al.* 1996). Little is known about how these GTPases are recruited to receptors or indeed how they regulate the activation of JNK cascades owing to the diverse nature of pathways upstream of this MAP kinase (Figure 1.5). The p21-activated kinase
(PAK) is thought to serve an intermediary role in coupling Rac/Cdc42 to JNK. Bagrodia and co-workers (1995) have shown that transfection of COS-1 cells with constitutively active PAK3 or Cdc42 increases JNK activity. Further to PAK being involved as a signalling intermediary between these GTP binding proteins, Teramoto et al. (1996) demonstrated that over-expression of MLK3 potently activated JNK via binding to Rac1 and Cdc42. A dominant-negative mutant of MLK3 abolished this activation, but MEKK-mediated JNK activity was not attenuated, suggesting that MLK3 was acting downstream of Rac/Cdc42.

Ras has also been shown to stimulate JNK activity via through the activation of Rac (Coso et al. 1995a, 1996). How Ras activates Rac is unclear, but it is thought that PI3 kinase may serve as an intermediate. PI3 kinase has been shown to mediate JNK activation by the m2 mACh receptor expressed in COS-7 cells (Lopez-Ilasaca et al. 1998) and by over-expression of the catalytic p110 subunit of the PI3 kinase in the same cell type (Klippel et al. 1996). It is thought that Ras, which binds to and activates the catalytic p110 subunit of PI3 kinase (Rodriguez-Viciona et al. 1994) leads to PI3 kinase activation resulting in the activation of Rac and JNK.

In terms of GPCRs, the Gq-coupled m1 mACh receptor has been shown to effectively activate JNK when expressed in NIH-3T3 cells (Coso et al. 1995b), and has also shown to activate this pathway in COS-7 cells in a Gβγ-dependent manner (Coso et al., 1996). This study demonstrated that over-expression of Gβγ subunits potently activated JNK, whereas expression of Gα subunits αs, αt2, αq or α13 did not mimic this effect. Other studies however, have found a role for Gα subunits in eliciting JNK activation. Thus, Prasad et al. (1995) have demonstrated that constitutively active forms of Gαt2 and Gαt3 elicit JNK activation, but not an ERK response NIH-3T3 cells. This was shown to be mediated by Ras, as expression of a competitive inhibitory form of this GTPase blocked Gαt2 and Gαt3-mediated JNK activation. A study by Nagao et al. (1999) has also shown that Gαt2 activates JNK in HEK 293 cells. Further, using a constitutively-active mutant of Gαt2 and dominant-negative RhoA, this activation was demonstrated to be mediated via Src kinase acting upstream of Rho activation. Rho involvement in JNK activation by Gt2 has also been demonstrated in NIH 3T3 cells by the m1 mACh receptor (Fromm et al. 1997).
The robust activation of JNK has also been demonstrated for the PLC-linked m3 mACh receptor expressed in CHO cells (Wylie et al. 1999; Burdon et al. 2002). This activation was shown to be calcium-sensitive and partially attenuated by PTx pre-treatment (Wylie et al. 1999). Calcium and PKC manipulations have been shown to influence JNK activation in a stimulatory (Ko et al. 1998) and inhibitory manner (Schwarzschild et al. 1999) in other systems. In Rat-1 cells lowering calcium and PKC inhibition has a stimulatory effect on JNK activation stimulated by endothelin (Cadwallader et al. 1997). Calcium sensitivity in the JNK pathway may involve Ras and Rho family GTPases as these are known to be calcium-sensitive. Further, the calcium-sensitive tyrosine kinase PYK2 has also been demonstrated to be a cell type-specific activator of JNK (Tokiwa et al. 1996). However, it is unclear how PKC modulates JNK activation.

Fig. 1.5 illustrates some of the major signalling intermediates of the JNK cascade linked to GPCRs coupled to a number of G protein classes and RTKs that have been discussed. In comparison to ERK activation, the signals transducing activation of JNK are far less well characterised and the complexity of signalling is increased by the diverse number of extracellular stimuli and MKKKs that are involved upstream of the JNK module. Mechanisms underlying JNK activation are therefore less universal and highly dependent on the cell type and stimulus.

1.10 mGlu receptor regulation of MAP kinase cascades

The regulation of MAPK signalling by growth factors is well documented and has been extensively examined in several cell systems. However, the regulation of these pathways in neuronal cells in response to neurotransmitters such as glutamate still remains less explored. Fiore and colleagues (1993) were the first to report metabotropic glutamate receptor-specific regulation of MAPK, specifically with respect to activation of ERK 1/2. This work was carried out in rat cortical cultures and since this study, several groups have identified mGlu receptor-mediated activation of the ERK cascade in various preparations and cell systems (Peavy & Conn, 1998; Ferraguti et al. 1999; Schinkmann et al. 2000; Peavy et al. 2001,
Although the intracellular signalling pathways coupling mGlu receptors to activation of ERK have been investigated in some of these studies, there is still much to elucidate in terms of the actual transduction mechanisms involved and the functional outcome of such activation. The components involved in mGlu receptor-ERK signalling are likely to involve many of the intermediates utilised in growth-factor and GPCR-mediated ERK activation, including kinases such as PKC, PI3-kinase, Src and PYK2, however the repertoire of signalling partners used will depend on upstream class of G-protein activated and the cell type.

The activation of the stress activated JNK and p38 MAP kinases has been far less investigated than activation of the ERK cascade. In fact, mGlu receptor activation of JNK has not been definitively reported in model systems or native preparations, but mGlu receptor-mediated p38 MAP kinase activation in the hippocampus has been described (Bolshakov et al. 2000). The activation of these two cascades by mGlu receptor stimulation can elicit different functional outputs to activation of the ERK cascade owing to their common association with cellular events, such as growth arrest and cell death, and therefore possible activation of JNK and p38 by mGlu receptors may have important implications in neuronal cells.

1.11 Summary
As discussed, the activation of group I metabotropic glutamate receptors leads to the activation of a number of intracellular signalling events that lead to a variety of cellular outputs. In addition to the preferential activation of phosphoinositide turnover by PLC-linked mGlu1a/mGlu5a receptors, the regulation of mitogenic signalling by these two mGlu receptors is documented in a number of models. However, as with many GPCRs, there still remains much to elucidate in the mechanisms that transduce signals from an activated receptor to the activation of MAPK cascades. Attempts to delineate such intracellular signalling mechanisms are further complicated by the diversity of pathways that are activated by mGlu receptors, which may influence activation of the MAPK module at various points downstream of mGlu receptor activation, but also owing to the cell type-specific nature of mGlu1a/mGlu5a receptor coupling to these pathways.
Figure 1.5 Activation of the JNK module by RTKs and GPCRs coupled to G\textsubscript{i}, G\textsubscript{q} and G\textsubscript{12/13} classes of G protein. Some of the common components involved in JNK activation by G\textsubscript{i}, G\textsubscript{q} and G\textsubscript{12/13}-coupled receptors are shown. Much of the signalling mechanisms upstream of the JNK module are far less well characterized than those for the ERK pathway, and broken arrows represent multiple steps not yet fully understood. Small GTPases of the Rho family including Rho, Rac and Cdc42 are generally regarded as common signalling molecules upstream of JNK activation. Spatial organisation and the efficiency of signal transduction of the JNK cascade is mediated by the JIP family of scaffolding proteins. See main text for details.
The use of cell models to investigate how mGlu receptors mediate coupling to intracellular cascades are not only useful in elucidating the signalling intermediates involved, but also aid in the understanding of how these GPCRs function in native cells.

1.12 Thesis Aims

The aims of this Thesis are to investigate the regulation of ERK, JNK and p38 MAP kinase activities in CHO cells mediated by the activation of the group I metabotropic glutamate receptors, mGlulα and mGlu5α. The magnitude and profile of these activations were determined and compared, and the intracellular signalling mechanisms underlying the transduction of these activations by both receptors were investigated using a pharmacological approach.
2.1 Materials

Cell culture medium, fetal calf serum, penicillin, streptomycin and geneticin sulphate (G-418) were obtained from Invitrogen (Paisley, UK). Glutamic-pyruvic transaminase (GPT), Triton-X-100, pertussis toxin (PTx), phorbol 12,13-dibutyrate (PDBu), sodium butyrate, dithiothreitol, pyruvate, isopropyl-β-D-thiogalactoside (IPTG), proline, leupeptin, aprotinin, phenylmethanesulphonyl fluoride (PMSF), dialyzed fetal calf serum, wortmannin, carbachol and protein G-Agarose were obtained from Sigma Chemical Co. (Poole, UK). Quisqualate, U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]-butadiene), PP1 (1-(1,1-dimethylethyl)-1-(4-methylphenyl)-1H-pyrazolo[3,4-d]-pyrimidin-4-amine), SP600125 (anthra[1,9-cd]pyrazol-6(2H)-one) and thapsigargin were from Tocris-Cookson (Bristol, UK). Protein A-Sepharose, myo-[3H]-inositol, [3H]-thymidine (TRK120), enhanced chemiluminescence (ECL) reagent and Hyperfilm was from Amersham Pharmacia (Little Chalfont, UK). [γ-32P]-ATP was from Perkin-Elmer Life Sciences (Boston, MA). Ro 31-8220 (3-[1-[3-(amidinothio)propyl]-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)-maleimide), Gö6976 (12H-indolo(2,3-a)pyrrolo(3,4-c)carbazole-12-propanenitrile,5,6,7,13-tetrahydro-13-methyl-5-oxo-[MESH]), AG 1296 (6,7-dimethoxy-3-phenylquinoxaline), AG 1478 (4-(3-chloroanilino)-6,7-dimethoxyquinazoline) and platelet-derived growth factor (PDGF) were from Calbiochem (Nottingham, UK). Glutathione-Sepharose was from Pharmacia LKB (Cambridge, UK). Nucleobond maxiprep kits were from Clontech/BD Biosciences (Oxford, UK). Nitrocellulose transfer membrane was from Schleicher & Schuell (London, UK). P81 cation-exchange paper was from Whatman (Maidstone, UK). GeneJuice was from Novagen/Merck Biosciences (Nottingham, UK).

For details of Plasmids and all antibodies used please refer to Section 2.3 and Table 2.1 respectively.
2.2 Cell culture

Chinese hamster ovary (CHO) cells, expressing either human mGlu1a or mGlu5a receptors under the control the LacSwitch II inducible expression system (Hermans et al. 1999), were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing GlutaMAX-1 (L-alanyl-L-glutamine) with sodium pyruvate and 4500 mg L\(^{-1}\) glucose, supplemented with 10% fetal calf serum, 50 units ml\(^{-1}\) penicillin, 50 µg ml\(^{-1}\) streptomycin, 2.5 µg ml\(^{-1}\) amphotericin B, 44 µg ml\(^{-1}\) proline, and 300 µg ml\(^{-1}\) G418 (passage medium) and grown in a humidified atmosphere of 95% air/5% CO\(_2\) at 37°C. For experiments, cells were washed using HEPES-buffed saline (154 mM NaCl, 10 mM HEPES, pH 7.4), then harvested using trypsin/EDTA and seeded on to 6 multiwell plates (9.6 cm\(^2\) well\(^{-1}\)) in medium composed as above, but devoid of G418 and containing 10% dialyzed fetal calf serum substituted for standard fetal calf serum (induction medium). To induce receptor expression, cells were treated with 100 µM IPTG (and 5 mM butyrate, in the case of CHO-lac-mGlu5a cells) 20 h prior to further experimentation unless otherwise indicated. To minimize effects of any endogenous glutamate release, for all experiments, cells were incubated in Krebs-Henseleit buffer (see Section 2.4) containing 3 units ml\(^{-1}\) of the glutamate-metabolizing enzyme, glutamic pyruvic transaminase (GPT), supplemented with the co-substrate pyruvate (5 mM) for 30 min prior to agonist additions.

CHO cells stably expressing m3 muscarinic acetylcholine receptors used to establish positive controls for ERK / JNK assays, immunoblot analyses and \[^{3}\text{H}\]thymidine incorporation were maintained in minimal essential medium alpha (MEM\(\alpha\)) supplemented with 10% new-born calf serum, 50 units ml\(^{-1}\) penicillin, 50 µg ml\(^{-1}\) streptomycin, 2.5 µg ml\(^{-1}\) amphotericin B.

2.3 Plasmids and cell transfections

HA-tagged ERK was a generous gift from Dr Jacques Pouyssegur (CNRS-UMR 6543, Nice, France) and provided in pCDNA-NEO (Brunet et al. 1999) and the construct for Go\(_4\), in the mammalian expression vector pCDNA3.1 was a kindly provided by Dr Gary Willars (Cell Physiology and Pharmacology, Leicester). Constructs were transformed into bacteria and purified using a maxiprep kit according to manufacturer’s instructions.
Sub-confluent CHO cells (30-60%) were grown on 6-multiwell plates and transiently transfected with GeneJuice according to manufacturer’s recommendations. For a single transfection (per well) 3 μl GeneJuice was combined with 1 μg of plasmid DNA in 100 μl serum-free medium and added to wells containing 2 ml passage medium. Cells were then incubated in a humidified atmosphere of 95% air/5% CO₂ at 37°C for 24 h (for co-transfections 6 μl of GeneJuice was combined with 1 μg of each of the two plasmid DNAs per well). After 24 h the medium was removed and the cell monolayers washed with 1 ml induction medium and then replaced with fresh induction medium before being induced with 100 μM IPTG (+ 5 mM butyrate for CHO-lac-mGlu5a cells) for a further 20 h before experimentation.

2.4 Measurement of [³H]-inositol phosphate accumulation

Cells grown to confluence on 24 multiwell plates were induced for receptor expression and labeled with 1 μCi ml⁻¹ [³H]-inositol for 24 h in culture medium. Thereafter, cells were washed three times with 0.5 ml of Krebs-Henseleit buffer (KHB: 10 mM HEPES, 118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 1.3 mM CaCl₂, 11 mM glucose, pH 7.4) and then incubated in 0.5 ml of this buffer (containing GPT/pyruvate). LiCl (10 mM final concentration) was added for 15 min prior to agonist addition. Experiments were performed at 37°C in a final volume of 0.5 ml per well and terminated by the addition of 0.5 ml ice-cold 1 M trichloroacetic acid (TCA). After extraction on ice, samples were collected from the well, mixed with 100 μl EDTA (10 mM, pH 7.0) and extracted with 1 ml of a 1:1 (v/v) mixture of tri-n-octylamine and 1,1,2-trichlorotrifluoroethane. A 400 μl sample of the upper aqueous phase was mixed with 100 μl NaHCO₃ (62.5 mM) and the [³H]-InsP fraction (containing inositol mono-, bis- and trisphosphates) was recovered by ion-exchange chromatography on Dowex AG1-X8 (formate form) columns as previously described (Challiss et al. 1993).

2.5 Treatment of cells and preparation of lysates

Before agonist stimulation, cells were washed, and incubated for 30 min at 37°C in freshly gassed (95% O₂/5% CO₂) KHB supplemented with GPT/pyruvate. CHO-lac-mGlu1a and
CHO-lac-mGlu5a cells were stimulated with the mGlu receptor agonist quisqualate; CHO-m3 cells were stimulated with carbachol. Additions were made directly to the KHB at concentrations and time-points indicated in the figure legends. Cell stimulations were terminated by placing plates on ice, rapid removal of the buffer/agonist solution and washing twice with 2 ml ice-cold phosphate-buffered saline (PBS, 140 mM NaCl, 2.68 mM KCl, 8.1 mM Na$_2$HPO$_4$, 1.47 mM KH$_2$PO$_4$, pH 7.4). Cells were subsequently solubilised in 300 µl per well lysis buffer (20 mM Tris-HCl, pH 7.4, 1% Triton-X100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM Na$_3$VO$_4$, 1 mM PMSF, 10 µg ml$^{-1}$ leupeptin, 10 µg ml$^{-1}$ aprotinin) and then centrifuged at 20,000 r.p.m. for 10 min at 4°C to remove insoluble material. Aliquots of the supernatant (100 µl) were removed and added to equivalent volumes of 2X Laemmli sample buffer (250 mM Tris-HCl, pH 6.8, 0.01% bromophenol blue, 2% SDS, 40% glycerol) containing 50 mM dithiothreitol for western blot analysis, or 200 µl aliquots were removed for use in protein kinase activity assays.

2.6 ERK activity assays
Following cell stimulation, lysates prepared as above were examined for ERK activity by the use of an in vitro immunocomplex kinase assay. ERK proteins were isolated from CHO cell lysates by immunoprecipitation with an anti-ERK rabbit polyclonal antibody or a HA-probe, for immunoprecipitation of HA-tagged ERK2 from transiently transfected CHO-lac cells, (see Table 2.1). Lysate (200 µl) was incubated at 4°C with a 1:100 dilution of anti-ERK antibody or a 1:20 dilution of anti-HA antibody (both 200 µg ml$^{-1}$) for 90 min. A 15% (w/v) slurry of protein A sepharose (70 µl) was added and incubated for a further 90 min at 4°C. The beads were then recovered by centrifugation (14,000 r.p.m for 1 min) and washed twice in 200 µl lysis buffer and twice in 200 µl kinase buffer (20 mM HEPES, pH 7.2, 20 mM β-glycerophosphate, 10 mM MgCl$_2$, 1 mM dithiothreitol, 50 µM Na$_3$VO$_4$). Kinase assays were then initiated by the addition of 40 µl of the kinase buffer containing 20 µM [$γ$-$^32P$]-ATP (2.5 µCi nmol$^{-1}$) and 200 µM of a synthetic peptide substrate corresponding to amino acids 662-681 of the epidermal growth factor receptor (RRELVEPLTPSGEAPNQALLR, Gardner et al. 1994). Reactions were performed at 30°C for 20 min and terminated by the addition of 10 µl of 25% (w/v) TCA, before centrifugation (14,000 r.p.m for 1 min) and spotting of 40 µl of the
kinase reaction mix on to Whatman P81 cation exchange filter paper. These papers were subsequently washed three times for 5 min each in 0.5% orthophosphoric acid and once for 3 min in acetone, left to dry and then added to 5 ml scintillation fluid. $[^{32}\text{P}]$-incorporation into the peptide was detected by liquid scintillation counting. Protein values (approx. 1 mg ml$^{-1}$) were obtained using the method of Bradford (1976) performed on cleared lysates, to enable the calculation of specific activities for all kinase assays as described in Section 2.13.

2.7 JNK activity assays
Following cell stimulation, lysates prepared as above were examined for JNK activity by the use of an in vitro immunocomplex kinase assay. JNK proteins were isolated from CHO cell lysates with an anti-JNK 1 rabbit polyclonal antibody (see Table 2.1). Lysate (200 μl) was incubated with 1:100 dilution of the anti-JNK 1 antibody (200 μg ml$^{-1}$) and 70 μl of a 15% (w/v) slurry of protein A-sepharose overnight at 4°C. The beads were recovered by centrifugation (14,000 r.p.m for 1 min) and washed twice in 200 μl lysis buffer and twice in 200 μl kinase buffer (20 mM HEPES, pH 7.2, 20 mM β-glycerophosphate, 10 mM MgCl$_2$, 1 mM dithiothreitol, 50 μM Na$_3$VO$_4$). Kinase assays were then initiated by the addition of 40 μl of the kinase buffer containing 20 μM $[^{32}\text{P}]$-ATP (2.5 μCi nmol$^{-1}$) and 5 μg of purified glutathione S-transferase (GST)-c-Jun as a substrate (for preparation see section 2.7.1). Reactions were performed at 30°C for 20 min and terminated by the addition of 40 μl Laemmli sample buffer and boiling at 100°C for 3 min. Phosphorylated proteins were resolved by 12% SDS-polyacrylamide gel electrophoresis, stained with Coomassie blue R250 and visualized by autoradiography. Radioactivity incorporated into c-Jun was quantified by liquid scintillation counting of the excised bands.

2.7.1 Preparation of glutathione S-transferase (GST)-c-Jun and ATF2
Dr. Roger Davis (University of Massachusetts, Worcester, USA) kindly provided constructs for the bacterial expression of c-Jun and ATF2 (for p38 MAP kinase assays, see below) as translational fusions with glutathione S-transferase (GST). The GST-fusion proteins were purified by affinity chromatography on glutathione-Sepharose. 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 albumin to construct a standard curve.

### 2.8 p38 MAPK activity assays

CHO cell lysates were prepared as for ERK and JNK assays were examined for p38 MAPK activity by the use of an *in vitro* immunocomplex kinase assay. p38 proteins were isolated from CHO cell lysates with an anti-p38 goat polyclonal antibody (see Table 2.1). Lysate (200 µl) was incubated with 1:20 dilution of the anti-p38 antibody (200 µg ml⁻¹) and 40µl of protein G-agarose overnight at 4°C. Recovered beads were then washed and assayed for p38 activity under identical conditions to those described for JNK, except that 5 µg of purified GST-ATF2 was used as a substrate. Reactions were performed at 30°C for 20 min and terminated by the addition of 40 µl Laemmli sample buffer and boiling. Samples were resolved by 12% SDS-polyacrylamide gel electrophoresis, stained with Coomassie blue R250 and visualized by autoradiography as for JNK assays. Radioactivity incorporated into ATF2 was quantified by liquid scintillation counting of the excised bands.

### 2.9 Src kinase activity assays

Lysates prepared as for ERK, JNK and p38 assays were examined for Src kinase activity by an *in-vitro* immunocomplex kinase assay as described by Cary *et al.* (2002). Src proteins were isolated from CHO cell lysates by immunoprecipitation with an anti-Src rabbit polyclonal antibody (see Table 2.1). Lysate (200 µl) was incubated at 4°C with 1:100 dilution of anti-Src antibody (200 µg ml⁻¹) for 90 min. A 15% (w/v) slurry of protein A sepharose (70 µl) was added and incubated for a further 90 min at 4°C. The beads were then recovered by centrifugation and washed twice in 200 µl lysis buffer and twice in 200 µl kinase buffer (20 mM HEPES, pH 7.2, 10 mM MnCl₂, 1 mM dithiothreitol, 50 µM Na₃VO₄). Kinase assays were then initiated by the addition of 30 µl of the kinase buffer containing 40 µM [γ⁻³²P]-ATP (5 µCi nmol⁻¹) and 7 µg of denatured (see Section 2.7.1) rabbit muscle enolase as a substrate (Feder & Bishop, 1990) for 10 min at 30°C. Reactions were terminated by the addition of 30 µl Laemmli sample buffer and boiling. Samples were resolved by resolved by 12% SDS-
polyacrylamide gel electrophoresis, stained with Coomassie blue R250 and visualized by autoradiography as for JNK and p38 assays.

2.9.1 Preparation of acid-denatured enolase
A 1 ml stock of rabbit muscle enolase (5.5 mg ml\(^{-1}\)) was dialyzed for 2 h at 4°C against 2 changes of distilled water (600 ml each). The dialyzed enolase (1.8 mg ml\(^{-1}\)) was divided into aliquots and stored at -20°C until required for Src kinase assays. Before kinase reactions were initiated, a 100 µl aliquot of enolase was denatured by the addition of an equal volume of 50 mM acetic acid for 5 min at 30°C and then neutralized using 1 M HEPES (pH 7.4). Denatured enolase (0.72 mg ml\(^{-1}\)) was then used in kinase reactions at 7 µg/sample.

2.10 Western blotting analysis
Lysates prepared with sample buffer were heated for 3 min at 100°C, centrifuged at 14000 r.p.m for 2 min, and 20 µl of each sample separated by 12% (unless otherwise indicated) SDS-polyacrylamide gel electrophoresis using the BioRad mini-gel system. Proteins were transferred to nitrocellulose membranes at 12 V for 30 min in transfer buffer (48 mM Tris, 39 mM glycine, 1.3 mM SDS, 20% methanol) using a semi-dry transfer apparatus. Membranes were blocked with 5% non-fat milk powder or bovine serum albumin (w/v) in TTBS (50 mM Tris, 150 mM NaCl, 0.1% Tween, pH 8.0) for 60 min at room temperature before incubation with primary antibody (see table 2.1 for all antibody dilutions and incubations times). Immunoblots were then washed using 3 x 5 min washes with TTBS followed by incubation for 60 min at room temperature with secondary anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated antibodies prepared at 1:1000 dilution in TTBS. Finally the membranes were washed with 5 x 5 min washes before developing using ECL and exposure to Hyperfilm.

2.10.1 Immunoprecipitation/western blot analysis (IP western)
For analysis of immunoprecipitated proteins (mGlu1a/mGlu5a receptors, the adapter protein Shc and focal adhesion kinase (FAK)) by western blot analysis: specific proteins were isolated from CHO cell lysates by immunoprecipitation with 1:100 dilution of anti-mGlu1a
receptor, anti-mGlu5a receptor, anti-Shc or anti-FAK rabbit polyclonal antibody (see Table 2.1) and 70 μl of a 15% (w/v) slurry of protein A-sepharose overnight at 4°C. The beads were recovered by centrifugation (14,000 r.p.m for 1 min) and washed three times in 200 μl lysis buffer. 50 μl Laemmli sample buffer was added to the immunoprecipitates and samples were heated for 3 min at 100°C, centrifuged at 14000 r.p.m for 2 min, and 20 μl of each sample separated by 12% (unless otherwise indicated) SDS-polyacrylamide gel electrophoresis and probed with appropriate primary and secondary antibodies as described in Section 2.10.

2.11 [3H]-thymidine incorporation

The method described by Levine et al. (1997) was followed, except for addition of the receptor induction step for CHO-lac-mGlu1/5 cells. Harvested cells were seeded into 24-well multidishes at a density of 50,000 cells per well and grown in a humidified atmosphere of 95% air/5% CO₂ at 37°C for 24 h. The cell monolayers were then washed with HEPES-buffered saline and replaced with medium containing or lacking 10% fetal calf serum (serum-free medium, SFM) as indicated in the figure legends. In the case of CHO-lac-mGlu1a and mGlu5a cells, receptor induction was also initiated at this stage by the addition of 100 μM IPTG well⁻¹ (+5 mM sodium butyrate for CHO-lac-mGlu5a cells). Cells were then incubated for a further 20 h before receptor stimulation. Subsequent agonist treatments, indicated in the results section, were carried out directly into the medium over a 24 h period. At 22 hrs after agonist addition, 2 μCi of [3H]-thymidine (25 μCi mmol⁻¹, TRK120; Amersham Pharmacia) was added per well during the final 2 h of agonist treatment. Termination of the assay proceeded by placing the plates on ice and washing wells three times with 2 ml of ice-cold SFM. Cells were then incubated for 1 h at 4°C with 5% ice-cold TCA. The TCA solution was then removed and wells were washed once with fresh TCA before incubation with 2 ml ice-cold ethanol containing 200 μM potassium acetate for 5 min. Cells were then incubated twice at 4°C for 15 min in a 2 ml 1:3 ether: ethanol mix. The mix was removed and cells were allowed to air dry before cellular precipitates were solubilized in 500 μl 0.5 M NaOH. Samples were then added to scintillation fluid and [3H]-thymidine incorporated into DNA measured by scintillation counting.
Table 2.1 Primary and secondary antibodies used for immunoprecipitations, western blot analysis and immunocytochemistry. Antibodies are given with clone numbers in brackets (), where applicable, and catalogue numbers in square brackets [ ]. Blocking agents and dilutions refer to antibodies diluted in non-fat milk powder or bovine serum albumin (BSA) (w/v) in TTBS. # Kind gift from Dr. A. Kazlauskas (Schepens Eye Research Institute, Harvard Medical School, Boston, USA).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
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<th>Dilution</th>
<th>Incubation time</th>
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<td>Anti-Rabbit (HRP) [A6154]</td>
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</table>

Antibodies for immunocytochemistry

Alexa Fluor 488 goat anti-mouse fluorophore conjugate [A-11001]
| Molecular Probes | Refer to Section 2.12 for details |

Alexa Fluor 488 goat anti-rabbit fluorophore conjugate [A-11034]
| Molecular Probes | Refer to Section 2.12 for details |

α tubulin [T5168] Mouse monoclonal
| Sigma | Refer to Section 2.12 for details |

F-Actin (Texas Red-X phalloidin) [T-7471]
| Molecular Probes | Refer to Section 2.12 for details |

Hoechst-33342 (nuclear stain) [H-3571]
| Molecular Probes | Refer to Section 2.12 for details |
2.12 Immunocytochemistry

Cells were seeded onto flame-sterilized 22 mm coverslips (thickness No.1) placed into 8-well plates and grown to 60% confluency before receptor induction (100 μM IPTG, +5 mM sodium butyrate for CHO-lac-mGlu5a cells, 20 h). Coverslips were then placed into a fresh 8-well plate and the cell monolayers washed twice with 1 ml phosphate-buffered saline (PBS) before fixation. For tubulin and mGlu receptor staining cells were fixed in 1 ml ice-cold methanol and then incubated at -20°C for 30 min. Cells were then washed three times in PBS before the addition of 500 μl well\(^{-1}\) of a 1% BSA/PBS (w/v) blocking solution for 30 min at room temperature. The blocking solution was then removed and primary antibodies added (anti-α-tubulin 1:1000, anti-mGlu1a 1:200 and anti-mGlu5a 1:200 in 1% BSA/PBS) for 1 h at room temperature. Antibodies were removed and cells washed three times in 1 ml PBS before incubation with secondary antibodies (goat anti-mouse Alexa Fluor 488 or goat anti-rabbit Alexa fluor 488 both at 1:1000 in 1% BSA/PBS). Antibodies were then removed and cells washed three times in 1 ml PBS before the addition of a nuclear stain, Hoechst-33342 (1:40,000 in PBS) for 2 min at room temperature. Cells were again washed three times with 1 ml PBS and then coverslips carefully removed and excess moisture removed before being inverted and mounted onto 20 μl of mounting medium (80% glycerol, 3% n-propyl gallate) placed onto microscope slides. The edges were then sealed using nail varnish and allowed to dry.

For actin staining, coverslips were washed twice in 1 ml PBS and then fixed using 1 ml formaldehyde (3.7% in PBS) for 20 min at room temperature. Cells were then washed three times in 1 ml PBS and then permeabilized using 1ml 0.1% Triton-X 100 in PBS for 2 min. Primary antibody (Texas Red-X phalloidin, 1:400 in PBS) was then added for 20 min at room temperature. Cells were then washed three times in PBS, stained for nuclei and mounted onto microscope slides as described above.

Cells were viewed on an inverted fluorescence microscope (TE3000; Nikon, Tokyo, Japan) and images were captured through a 100x objective (numerical aperture 1.4) by using an Orca ER charge-coupled device camera (Hamamatsu Photonics, Bridgewater, NJ). A krypton/argon laser was used for fluorescence excitation of Alexa flour (488 nm), Texas-Red
Phalloidin (568 nM) and Hoechst-33342 (364 nm). Images were merged using Openlab software (Improvision, Coventry, UK) and the Openlab TIFF images compiled in Photoshop (Adobe systems, Mountain view, CA) and Microsoft PowerPoint.

2.13 Data analysis
For ERK, JNK and p38 kinase assays, specific activity was calculated from conversion of radioactivity detected in cpm (counts per minute) to pmol/min/mg from protein values obtained from a Bradford assay of cleared lysates. Data were fitted as sigmoidal concentration curves with variable slope and statistical differences between data sets were determined by one-way analysis of variance (ANOVA) for multiple comparisons, followed by Bonferroni's multiple-range test at $P < 0.05$ using Prism III software (GraphPad, San Diego, CA).

Densitometry of immunoblots was carried out using a Bio-Rad densitometer and Microsoft Molecular Analyst software and quantitative analysis was performed using Kodak Digital Science software. Values are represented as fold-over basal.
CHAPTER 3  Characterization of the model cell system and activation of the ERK MAP kinase by mGlu1a and mGlu5a receptors

Introduction

The first metabotropic glutamate receptor, mGlu1a, cloned by Masu and colleagues and Houamed et al. in 1991, was initially identified by its ability to activate inositol phosphate metabolism in response to the application of glutamate, in a manner which Sugiyama et al. described as ‘fluctuating and having a delayed onset, suggesting the involvement of intracellular processes’ (Sugiyama et al. 1987). In 1992, Abe et al. reported the identification of another mGlu receptor, termed mGlu5a that displayed a high sequence homology to the mGlu1a receptor and similarly stimulated phosphoinositide hydrolysis. These two receptors and their splice variants constitute group I of the mGlu receptor family and are to date, the only mGlu receptors known to preferentially activate inositol phosphate metabolism (Hermans & Challiss, 2001).

In addition to PLC activation, these receptors also couple to the activation of mitogenic signalling. In recent years, there has been increasing interest in the way in which these receptors couple to these pathways and indeed how activation of these cascades might relate to the role of these receptors in neuronal signalling in the CNS. Since Fiore and co-workers first identified an mGlu receptor-mediated MAPK activation in primary rat cortical cultures (Fiore et al. 1993), several groups have reported activation of this cascade by mGlu receptors in native preparations and in cultured cells (Peavy & Conn, 1998; Ferraguti et al. 1999). However, few studies have investigated the activation of these pathways by both group I receptors in a common cell background and there remains many unanswered questions with respect to the intracellular signalling leading to the activation of these cascades.

The study of these receptors individually in native cells is challenging, not only due to their similar pharmacological profiles, but also owing to the distinct expression patterns of mGlu1a
and mGlu5a mRNA in specific neuronal cells (Abe et al. 1992). Although, there is overlap in some brain regions, the specialized expression of mGlu1a and mGlu5a receptors is likely to reflect the distinct functions these two subtypes may have in the CNS (Valenti et al., 2002). It follows that much research into the pharmacology and signal transduction mechanisms of these receptors has been carried out via transient or stable transfection of mGlu receptors into immortalised cell lines.

In this thesis, work has been carried out in stably transfected CHO cell lines, previously described by Hermans et al. (1998, 1999) in which the expression of human recombinant mGlu1a and mGlu5a receptors is under the control of an IPTG-inducible promoter. As expression of receptors can be regulated by the addition of the inducing agent, this system allows the minimization of problems related to the endogenous release of glutamate. As cultured cells can release significant amounts of glutamate (Desai et al. 1995; Nedergaard et al. 2002), this can result in desensitization and down-regulation events due to long-term exposure of the receptor to agonist when permanently expressed. Hermans et al. have shown (Hermans et al. 1999) that the pharmacology of receptors can be significantly influenced by receptor density, and therefore it is important that levels of receptor expression can be controlled and indeed matched when investigating receptor pharmacology and intracellular signalling. The use of sodium butyrate to further enhance receptor expression, induced by IPTG treatment, in CHO-lac-mGlu cells has also been described (Nash et al. 2001b). It is thought that sodium butyrate acts to enhance gene transcription by hyperacetylation of histone proteins via the inhibition of histone deacetylase (Kruh, 1982).

The aims of this Chapter were to examine receptor induction, the activation of the classical group I mGlu receptor inositol phosphate pathway in CHO-lac-mGlu1a and -mGlu5a cells, and to begin to investigate the coupling of these two receptors to the activation of the ERK subgroup of MAP kinases. The data presented show that activation of mGlu1a and mGlu5a receptors in these cells elicit different magnitudes of phosphoinositide hydrolysis, but that both receptors activate a robust and comparable increase in ERK activity both in terms of magnitude and profile. The effects of sodium butyrate treatment on both receptor induction and subsequent ERK activation are also discussed.
Results

3.1 Immunoblot analysis of receptor expression in the inducible CHO-lac model

Before the activation of any specific signalling cascades were investigated, the induction of receptor expression in CHO-lac-mGlu cells was examined. Treatment of CHO-lac-mGlu1a and -mGlu5a cells with IPTG resulted in a concentration and time-dependent increase in receptor expression as determined by immunoblot analysis with antisera specific to each receptor. Importantly, immunoblot analysis revealed no immunoreactivity detected in CHO-lac-mGlu5a lysates probed with anti-mGlu1a antisera (first lane of immunoblot Fig. 3.1A) and vice versa (first lane of immunoblot Fig. 3.1B).

Fig. 3.1A and B show that at a constant induction time of 20 h, increasing levels of receptor expression could be detected in response to increasing concentrations of IPTG, and that maximal receptor induction occurred at a concentration of 100 μM IPTG. As previously reported by Hermans et al. (1998, 1999), mGlu receptors expressed inducibly in CHO-lac cells migrate as two distinct bands of approximately 165 and 145 kDa for mGlu1a, and 148 and 130 kDa for mGlu5a. It is thought that the lower bands, which appear much earlier, represent an immature form of the receptor protein that has not yet undergone post-translational modification (such as glycosylation). It is worth noting that, although low percentage gels were routinely used to resolve receptor proteins for immunoblot analysis, the existence of the two separate receptor bands could not always be distinguished.

For CHO-lac-mGlu5a cells enhancement of receptor expression using sodium butyrate was required to increase receptor levels to that comparable with the mGlu1a receptor. Using a maximal concentration of IPTG, the induction of receptor expression in the absence and presence of 2, 5 and 10 mM sodium butyrate in CHO-lac-mGlu1a and -mGlu5a cells was also examined (Fig. 3.1C and D). Pre-treatment of cells with sodium butyrate, in the presence of IPTG, enhanced receptor expression in a concentration-dependent manner in both cell lines. Sodium butyrate pre-treatment alone (in the absence of IPTG) did not induce any receptor expression, as discerned by no immunoreactive bands, in CHO-lac-mGlu1a or CHO-lac-
Figure 3.1 Immunoblot analysis of the concentration-dependence of IPTG-induced receptor expression in CHO-lac-mGlu1a and CHO-lac-mGlu5a cells. Total cell extracts were prepared and resolved by 7% SDS-PAGE as described in Materials and Methods (Section 2.5 and 2.10), and receptor protein detected using receptor specific antisera (Table 2.1). A and B, increases in receptor expression during a 20h incubation period with increasing concentrations IPTG (0-100 μM) in CHO-lac-mGlu1a and -mGlu5a cells respectively. CHO-lac-mGlu5a cells were additionally treated with 5 mM sodium butyrate. * CHO-lac-mGlu5a lysates probed with anti-mGlu1a antisera and vice versa. C and D, increases in receptor expression in the absence and presence of sodium butyrate treatment in CHO-lac-mGlu1a and -mGlu5a cells respectively. Cells were induced for receptor expression with IPTG (100 μM, 20 h) in the presence and absence of increasing concentrations of sodium butyrate (0-10 mM). Representative immunoblots from 3 separate experiments are shown.
Figure 3.2 Immunoblot analysis of the time-course of IPTG-induced receptor expression in CHO-lac-mGlu1a and CHO-lac-mGlu5a cells. Total cell extracts were prepared and resolved by 7% SDS-PAGE as described in Materials and Methods (Section 2.5 and 2.10), and receptor protein detected using receptor specific antisera (Table 2.1). A and B, cells were treated with 100 μM IPTG for the indicated times in CHO-lac-mGlu1a and -mGlu5a cells, respectively. CHO-lac-mGlu5a cells were additionally treated with 5 mM sodium butyrate. Representative blots from 2-3 separate experiments are shown.
mGlu5a cells. The intermediate concentration of 5 mM sodium butyrate was chosen for all subsequent experiments using CHO-lac-mGlu5a expressing cells.

The length of time for which cells were exposed to IPTG to induce receptor expression was also investigated (Fig. 3.2). Cells treated with 100 μM IPTG for varying periods of incubation with IPTG revealed that maximal receptor expression could be detected 16-24 h following addition as described previously (Hermans et al., 1998, 1999). Therefore, an incubation period of 20 h was utilized for all future experiments. The induction of expression for 20 h using 100 μM IPTG (+ 5 mM sodium butyrate for mGlu5a expressing cells) revealed receptor levels of approx. 400 and 200 fmol mg protein$^{-1}$ for CHO-lac-mGlu1a and mGlu5a cells, respectively (Atkinson P.J., Nahorski S.R. & Challiss R.A.J, unpublished results).

3.2 Quisqualate-induced phosphoinositide hydrolysis in CHO-lac-mGlu1a and mGlu5a cells

As group I mGlu receptors are known to be primarily coupled to inositol phospholipid metabolism the activation of agonist-stimulated [3H]inositol phosphate ([3H]InsP) accumulations in CHO-lac-mGlu1a and mGlu5a expressing cells was examined. In the presence of LiCl, quisqualate stimulated time- and concentration-dependent increases in [3H]InsP accumulation in both cell lines (Fig. 3.3). The overall levels of [3H]InsP accumulation were consistently higher in CHO-lac-mGlu1a cells (8-10 fold over basal after 20 min incubation with 10 μM quisqualate), with responses being approximately twice those seen in mGlu5a receptor-expressing cells.

Analysis of concentration-effect curves for agonist-stimulated [3H]InsP accumulation gave EC$_{50}$ values of 0.7 μM (pEC$_{50}$ 6.2 ± 0.2) and 0.12 μM (pEC$_{50}$ 6.9 ± 0.3) for CHO-lac-mGlu1a and mGlu5a cells, respectively. This greater potency of quisqualate at the mGlu5a receptor compared with that of the mGlu1a receptor agrees with data reported by others (Pin et al. 1999). The coupling of both receptors to the activation of [3H]InsP accumulation was also assessed for sensitivity to pertussis toxin (PTx). Pre-incubation of either CHO-lac-mGlu1a or mGlu5a cells with PTx (100 ng ml$^{-1}$, 24 h) did not attenuate [3H]InsP accumulations in either
Figure 3.3 Time-course and concentration-dependencies of agonist-stimulated $[^{3}H]$-inositol phosphate responses in CHO-lac-mGlu1a and -mGlu5a cells. Before experimentation, $[^{3}H]$-inositol-labeled (24 h) cells were pre-treated with GPT/pyruvate, prior to LiCl (10 mM) addition as described in Materials and Methods (Section 2.4). A and B show agonist-stimulated $[^{3}H]$-InsP accumulations in response to stimulation with 30 nM quisqualate for the various times indicated. C and D show agonist-stimulated $[^{3}H]$-InsP accumulations in response to a 30 min stimulation with various concentrations of quisqualate. Data are shown as means ± SEM for 2-4 separate experiments performed in duplicate.
Figure 3.4 PTx sensitivity of agonist-stimulated [³H]-inositol phosphate responses in CHO-lac-mGlu1a and -mGlu5a cells. A and B show agonist-stimulated [³H]-InsP accumulations in CHO-lac-mGlu1a and -mGlu5a cells respectively. [³H]-inositol-labeled (24 h) cells were treated with PTx (100 ng ml⁻¹) or vehicle for 24 h. For experiments, cells were pre-treated with GPT/pyruvate prior to LiCl (10 mM) addition, as described in Materials and Methods (Section 2.5) and then stimulated with 30 µM quisqualate for the various times indicated. Data are shown as means ± SEM for 3 separate experiments performed in duplicate.
cell line (Fig. 3.4) suggesting that these receptors couple through non-PTx-sensitive (Gq/11) G proteins to activate PLC.

### 3.3 Activation of ERK MAP kinase by mGlu1a and mGlu5a receptors

The Group I mGlu receptors have been shown to activate ERK in native preparations, including cortical glia (Peavy & Conn 1998), primary astrocytes (Schinkmann et al. 2000) and in model cell systems (Ferraguti et al. 1999). Here, we have compared the activation of the ERK subfamily of MAP kinases by mGlu1a and mGlu5a receptors in CHO-lac cells. To our knowledge, no systematic study has directly compared mGlu1 versus mGlu5 receptor coupling to ERK in a common cell background in this manner.

The activation of ERK MAP kinase by receptor stimulation in CHO-lac-mGlu1a and -mGlu5a cells was assessed by both in vitro immunocomplex protein kinase assays by measuring [32P] incorporation into a synthetic EGF-receptor peptide substrate (Gardner et al. 1994), and by western blotting using a phospho-specific antibody capable of detecting the phosphorylated (active) form of the ERK protein (Table 2.1).

Fig. 3.5 shows time-courses of ERK activation in mGlu1a and mGlu5a receptor expressing cells in response to quisqualate stimulation. For both cell lines, maximal agonist-stimulated increases in ERK activity occurred at 5 min, with ERK activity declining towards basal levels at 30 min. The magnitude of the peak response in CHO-lac-mGlu1a cells was 4.9 ± 0.2-fold over basal, as measured by ERK activity assays (Fig. 3.5A, upper panel). Immunoblot analysis showed a similar profile and fold increase in levels of phosphorylated ERK (Fig. 3.5A, lower panel). This increase in ERK activity could not be attributed to any changes in ERK expression, as total ERK protein expression did not change over the time-course of the experiment. For CHO-lac-mGlu5a cells, the peak increase in ERK activity (4.8 ± 0.4-fold over basal) was similar to that seen in CHO-lac-mGlu1a cells (Fig. 3.5B, upper panel). This finding was mirrored by western blot analyses (Fig. 3.5B, lower panel). Analysis of the concentration-response curves of mGlu1a and mGlu5a receptor-stimulated ERK activation
Figure 3.5 Time-courses of agonist-stimulated ERK activation in CHO-lac-mGlu1a and -mGlu5a cells. A and B show time-courses of ERK activity in CHO-lac-mGlu1a and -mGlu5a cells respectively. Cells were stimulated with 10 μM quisqualate for the times indicated and then assayed for ERK activity (upper panels) by $[^{32}\text{P}]$ incorporation into the EGF-receptor peptide as described in Materials and Methods (section 2.4 and 2.6). Data are shown as means ± SEM for 5 separate experiments. The lower panels show representative ERK immunoblots for phospho-ERK and total ERK expression. Lysates were analyzed via 12% SDS-PAGE as described under Materials and Methods (Section 2.10) and probed with an antibody that recognizes the phosphorylated form of ERK1/2 (pERK), or with an anti-ERK antibody that allows visualization of total ERK protein (Anti-ERK, see Table 2.1). Representative blots from 4 separate experiments shown. NI; non-induced, C; basal activity, or +quisqualate for 2, 5, 10 or 30 min.
Figure 3.6 Concentration-dependencies of agonist-stimulated ERK activation in CHO-lac-mGlu1a and -mGlu5a cells. Cells were stimulated with various concentrations of quisqualate indicated for 5 min and then assayed for ERK activity by $[^{32}]$P incorporation into the EGF-receptor peptide as described in Materials and Methods (Section 2.4 and 2.6). A and B show the concentration-dependence of ERK activity in CHO-lac-mGlu1a and -mGlu5a cells, respectively. Data are shown as means ± SEM for 4 separate experiments performed in duplicate.
Figure 3.7 Time-course and concentration dependence of agonist-stimulated ERK activation in CHO-m3 cells. A cells were stimulated with 10 μM carbachol for the times indicated and then assayed for ERK activity by $[^{32}P]$ incorporation into the EGF-receptor peptide as described in Materials and Methods (Section 2.5 and 2.6). The lower panels show representative ERK immunoblots for phospho-ERK and total ERK expression. Lysates were analyzed via 12% SDS-PAGE as described under Materials and Methods (Section 2.10) and probed with an antibody that recognizes the phosphorylated form of ERK1/2 (pERK). B cells were stimulated with the various concentrations of carbachol indicated for 5min and assayed for ERK activity and immunoblot analysis as above. Data are shown as means ± SEM for 2-6 separate experiments. Representative blots from 2 separate experiments are shown. C; basal activity, or +carbachol for 2, 5, 10, 20 or 30 min.
(Fig. 3.6) revealed EC$_{50}$ values of 1.6 µM (pEC$_{50}$, 5.8 $\pm$ 0.1) and 0.9 µM (pEC$_{50}$ 6.0 $\pm$ 0.2) for CHO-lac-mGlu1a and -mGlu5a cells, respectively.

To compare the magnitude of the mGlu1a/mGlu5a receptor-mediated ERK response to that of another G$_{q/11}$ linked GPCR, ERK activation mediated by the m3 muscarinic acetylcholine receptor stably expressed in CHO cells was also examined. This receptor stimulated a robust, time- and concentration-dependent increase in ERK activation in response to stimulation with carbachol (Fig. 3.7). The profile of activation of m3 receptor-mediated ERK activation was similar to that of the mGlu1a and mGlu5a receptor response, in that maximal ERK activity occurred at 5 min, and declined towards basal levels over 30 min, consistent with previous reports of ERK activation by this receptor (Wylie et al. 1999; Burdon et al. 2002). However, the magnitude of the peak response was much greater than either mGlu receptor-mediated response (14.7 $\pm$ 2.7-fold over basal. EC$_{50}$ value of 1.7 µM, (pEC$_{50}$, 5.8 $\pm$ 0.5). This is likely to reflect the higher expression levels of this stably transfected receptor (2.2 $\pm$ 0.2 pmol mg$^{-1}$ protein, Wylie et al. 1999) compared to that of the inducibly expressed mGlu receptors.

3.4 Effects of sodium butyrate treatment on ERK activity and expression in CHO-lac-mGlu5a cells

When mGlu1a and mGlu5a receptor-mediated [H]InsP accumulation was assessed, the responses observed in CHO-lac-mGlu1a cells were almost twice that seen in CHO-lac-mGlu5a expressing cells. It was thought that this difference may be attributed to slight differences in receptor expression levels, but if so this was not reflected in the subsequent measurements of ERK activation by these two receptors as responses were similar in profile and in magnitude. This prompted investigation into whether the sodium butyrate treatment in CHO-lac-mGlu5a cells, used to boost receptor expression levels up towards those displayed by the mGlu1a receptor, may also enhance expression of other cellular proteins. In this instance, any effects on the expression of the ERK protein may help to explain the comparably robust magnitude of ERK activation in CHO-lac-mGlu5a compared to that of CHO-lac-mGlu1a cells despite the slightly lower level of receptor expression.
The effects of varying concentrations of sodium butyrate (added at the time of receptor induction) treatment on ERK activity and expression were therefore examined in CHO-lac-mGlu5a cells (Fig. 3.8). With increasing concentrations of sodium butyrate treatment (0-10 mM), increasing levels of ERK activation, as determined by immunoblot analysis, could be detected in CHO-lac-mGlu5a cells. Examination of the expression of the ERK protein using antisera that allows visualization of total ERK protein (anti-ERK) revealed that these increases were not attributable to increases in protein expression as a result of sodium butyrate treatment. In a study by Nash et al. (2001), the possible non-specific effects of sodium butyrate treatment, used to boost receptor expression in CHO-lac-mGlu1a cells, were assessed by examining possible effects on endogenous P2Y2 purinoreceptor mediated $[^{3}H]$InsP accumulation. Importantly, sodium butyrate was found not to potentiate PLC activation by this endogenously expressed receptor subtype.
Figure 3.8 Effects of sodium butyrate treatment on ERK activity and expression in CHO-lac-mGlu5a cells. Cells were induced with 100 μM IPTG (20 h) in the presence or absence of various concentrations of sodium butyrate (0-10 mM). Cells were then stimulated with 10μM quisqualate for the times indicated and then lysates were analysed via 12% SDS-PAGE as described under Materials and Methods (Section 2.10) and probed with an antibody that recognises the phosphorylated form of ERK1/2 (pERK), or with an anti-ERK antibody that allows visualization of total ERK protein (Anti-ERK, see Table 2.1). Representative immunoblots of phospho-ERK and total ERK expression from 2 separate experiments are shown. C; basal activity or +quisqualate for 2, 5, 10 or 30min.
Discussion

The present chapter has examined the induction of mGlu1a and mGlu5a receptors in CHO cells and explored the profile of coupling of these group I mGluRs to the activation of two important signalling cascades. Initially, phosphoinositide hydrolysis, known to be downstream of phospholipase C activation by these receptors, was assessed before the activation of the ERK MAP kinase was examined.

mGlu1a and mGlu5a receptor expression was IPTG-, time- and concentration-dependent and maximal receptor induction conditions were optimized prior to any investigations into signalling by these two receptors. The addition of sodium butyrate to CHO-lac-mGlu5a cells was additionally required to boost receptor expression levels comparable to those in CHO-lac-mGlu1a cells. The use of sodium butyrate to enhance gene expression has also been reported for dopamine D4 receptors expressed in CHO cells (Gazi et al. 1999) and for 5-HT1B/D receptors expressed in HEK cells (Lesage et al. 1998). As sodium butyrate is thought to act via increasing transgene expression (Kruh 1982) it is possible that this agent may have more general effects on enhancing gene transcription. As proceeding Chapters explore the activation of MAP kinase signalling in CHO-lac-mGlu1a and -mGlu5a cells it was important to ascertain if this treatment had any effects on the expression of proteins involved in these cascades. Specifically, the effects of sodium butyrate on the ERK protein in CHO-lac-mGlu5a cells were examined in this Chapter. Under increasing concentrations of sodium butyrate increases in agonist-stimulated ERK activity were detected, as would be expected from increased mGlu5a receptor expression. Importantly however, the treatment of cells with sodium butyrate was not found to influence the expression levels of the ERK protein itself.

As group I mGluRs are primarily coupled to phosphoinositide turnover, the activation of this pathway by both receptors in CHO-lac cells was then assessed. The mGlu receptor agonist quisqualate, stimulated time- and concentration-dependent increases in [3H]InsP accumulation in both cell lines, although responses were consistently higher in CHO-lac-mGlu1a cells. These responses were then examined for sensitivity to pertussis toxin (PTx) to examine the G protein subpopulations that may be involved in the activation of this pathway. Neither
mGlu1a nor mGlu5a receptor-stimulated \([^{3}H]\)InsP accumulations were found to be affected by PTx pre-treatment (100 ng ml\(^{-1}\), 24 h). This observation is indicative of these receptors coupling through PTx-insensitive \(G_{q/11}\) family G-proteins to mediate inositol phosphate metabolism in these cells. Previous studies have similarly reported that phosphoinositide responses mediated by agonist-stimulation of the mGlu5a receptor, and indeed splice variants of the mGlu1a receptor, are relatively unaffected by PTx pre-treatment (Abe et al. 1992, Pickering et al. 1993). The effects of PTx on mGlu1a receptor mediated responses are however more complicated and often cell type specific. The attenuation of mGlu1a receptor-mediated phosphoinositide hydrolysis has been reported in several cell types including transfected CHO cells (Aramori & Nakaniishi, 1992), BHK cells (Pickering et al. 1993; Thomsen et al. 1993) and in Xenopus oocytes (Kasahara & Sugiyama, 1994). This contrasts however, with other reports that have shown that coupling of the mGlu1a receptor to phosphoinositide hydrolysis, is actually enhanced by PTx pre-treatment in stably transfected BHK cells (Carruthers et al. 1997; Hermans et al. 2000) suggesting that this receptor couples to a subpopulation of PTx-sensitive G proteins that act to inhibit PLC activity. The mGlu1a receptor has additionally been shown to increase cAMP accumulation via the activation of adenyl cyclase in CHO (Aramori & Nakaniishi, 1992), BHK (Hermans et al. 2000) and HEK (Francesconi & Duvoisin, 1998) cells, although it is not certain whether the mGlu1a receptor couples directly to \(G_{s}\) family G proteins to mediate this activation, or whether it is an indirect consequence of PLC activation.

The G-protein coupling of these two mGlu receptors will continue to be the subject of some debate depending on the intracellular signalling being investigated and approach/cell model being used to investigate this coupling. The data presented here at least suggest that in the CHO-lac-mGlu model being used, there is not a PTx-sensitive component to the coupling of the mGlu1a or mGlu5a receptor to the activation of inositol phosphate metabolism. This G-protein coupling may influence the manner in which these two receptors couple to the activation of the ERK MAP kinase, which was also investigated in this Chapter.

The coupling of these two receptors to activation of the ERK MAP kinase has been investigated in model cell systems and in native preparations by several groups (Peavy &
Conn 1998; Ferraguti et al. 1999; Schinkmann et al. 2000), but the pathways linking the mGlu1/5 receptors to ERK activation have only been partially characterized. To our knowledge, no systematic study has directly compared mGlu1 versus mGlu5 receptor coupling to ERK, and the present CHO-lac cell system allows an investigation into these pathways and allows a direct comparison of the signalling from these two mGlu receptors in a common cell background.

The data provided in this chapter set the scene for a more in-depth investigation into the coupling of mGlu1a and mGlu5a receptors to the ERK cascade in the proceeding chapter. This will include the examination of the possible G-protein subpopulations that may be involved in mGlu1a/mGlu5a receptor mediated ERK activation and an assessment of the possible second messengers involved in the intracellular signalling from receptor to the ERK MAP kinase using pharmacological inhibitors of these pathways.
CHAPTER 4
CHAPTER 4 Dissecting the signalling pathways from mGlula and mGlu5a receptors to the activation of the ERK MAPK cascade

Introduction

The mGlula and mGlu5a receptors are closely related in structure, sharing approximately 60% homology (Abe et al. 1992) and are similar in their pharmacological profile (Pin et al. 1999; Herman & Challiss 2001) and in the signalling pathways that they regulate. There is however evidence that major differences between these GPCRs do exist. In particular, the Ca²⁺ signatures generated following mGlula and mGlu5a receptor activation differ markedly. The mGlula receptor activates an agonist-induced single-peak of intracellular calcium release followed by a plateau phase, whereas the mGlu5a receptor elicits oscillatory patterns of calcium release (Kawabata et al. 1998; Hermans & Challiss 2001; Nash et al. 2001, 2002; Young et al. 2003). This difference in calcium signalling has been shown to be attributed to a critical amino acid residue in the C-terminus of mGlu5a receptor which is different to that found at homologous position in the mGlula receptor (Kawabata et al. 1998). The pattern of oscillatory calcium release by the mGlu5a receptor arises from cyclical desensitisation and re-sensitisation of the receptor by PKC through phosphorylation/dephosphorylation at this site. This observation highlights the role of PKC, activated downstream of phosphoinositol hydrolysis by these two receptors, in the negative feedback control of mGlu receptor signalling by rapid desensitisation. In native cells these receptors also differ in that they are differentially distributed in the brain, both at an anatomical level and with respect to their subcellular distributions (Hubert et al. 2001; Valenti et al. 2002) suggesting they may fulfil different physiological functions.

In the nervous system, MAPK/ERK pathways have been implicated in many types of signalling, including cell survival and apoptosis (Bonni et al. 1999), as integrators of biochemical processes (Angenstein et al. 1998; Impey et al. 1999) and in specific pathways, such as those mediating inflammatory pain plasticity (Karim et al. 2001). There is also much
evidence supporting a role for MAPK/ERK signalling in the encoding of changes in synaptic efficacy that underlie the long-term changes in neuronal plasticity required for different types of memory (English & Sweatt, 1996; Bolshakov et al. 2000; Sweatt, 2001).

ERK activity is regulated by a diverse range of extracellular signals that include ligands acting at receptor-tyrosine kinases and G protein-coupled receptors (GPCRs). There is much heterogeneity in the mechanisms employed by different receptors to couple to activation of the MAPK/ERK module (Garrington & Johnson 1999; Pierce et al. 2001a; Sweatt, 2001). In the case of GPCR-regulated pathways, the signals from activated receptors may be transmitted through a number of different G protein subtypes, or indeed be mediated in a G protein-independent manner (Heuss et al. 1999), and often involve other non-receptor tyrosine kinases. Indeed, many of the pathways employed by GPCRs to couple to ERK utilize, and function in parallel with, components of classical receptor tyrosine kinase cascades, and/or may use cytoplasmic non-receptor tyrosine kinases in addition to G protein activation, to mediate ERK activation through a process that has been referred to as ‘transactivation’ (Daub et al. 1996). Transactivation of growth factor receptors may be mediated by membrane-bound metalloproteinases or by non-receptor tyrosine kinases, such as Src (Wetzker & Böhmer, 2003).

The aim of this Chapter was to examine and compare the signalling from the mGlula and mGlu5a receptors, to the activation of ERK MAP kinase. Various second messengers were investigated using pharmacological inhibitors to attempt to dissect the signalling mechanisms downstream of receptor activation to this pathway. Although it might be assumed that because mGlula and mGlu5a receptors are closely related, both structurally and with respect to the downstream signalling pathways they regulate, they are likely to utilize common pathways to link to ERK activation, however the data presented show that although some similarities exist in the profile of ERK activation by mGlula and mGlu5a, these two receptors utilize quite distinct mechanisms to couple to this cascade. Clear differences were found in the G-protein subpopulations involved and in the requirement for PDGF receptor tyrosine kinase activity to elicit ERK activation by the mGlula but not the mGlu5a receptor. This potential ‘transactivation’ mechanism of ERK activation by the mGlula receptor is also discussed.
Results

4.1 PTx sensitivity of mGlu1a and mGlu5a receptor-mediated ERK activation

For both cell-lines, maximal agonist-stimulated increases in ERK activity occurred at 5 min, with ERK activity declining towards basal levels at 30 min (Section 3.4). Therefore, in subsequent experiments these maximal and post-maximal time points of agonist-stimulated ERK activity were used to examine the effects of pharmacological inhibitors on mGlu receptor-mediated ERK activity.

Initial experiments (Fig. 3.3 and 3.4) demonstrated that quisqualate-stimulated [3H]-InsP accumulations were unaffected by pre-incubation of either CHO-lac-mGlu1a or -mGlu5a cells with PTx (100 ng ml\(^{-1}\), 24 h), suggesting that these receptors couple through non-PTx-sensitive (G\(_{q/11}\)) G proteins to activate PLC. In contrast to quisqualate-stimulated [3H]-InsP accumulation, striking differences in PTx sensitivity were observed with respect to mGlu receptor-mediated ERK activation in the two cell-lines (Fig. 4.1). Thus, whereas the mGlu1a-stimulated ERK response was almost completely abolished by PTx, (Fig 4.1A) that mediated by the mGlu5a receptor was totally unaffected by pre-treatment with the toxin (Fig. 4.1B). This suggests that mGlu1a and mGlu5a receptor coupling to ERK activation in CHO cells is dependent upon different G protein populations, with mGlu1 receptor-mediated responses requiring PTx-sensitive G\(_{i/o}\) proteins.

4.2 Calcium dependence of mGlu1a and mGlu5a receptor-mediated ERK activation

As both mGlu1a and mGlu5a are PLC-linked receptors, they elicit Ca\(^{2+}\) signalling downstream of phosphoinositide hydrolysis (Kawabata et al. 1998; Nash et al. 2002). The requirement of Ca\(^{2+}\) for the activation of the ERK cascade by the group I mGlu receptors in the CHO cell model was therefore investigated.

Extracellular and intracellular calcium concentrations were manipulated using EGTA and thapsigargin, respectively. Whereas EGTA removes the Ca\(^{2+}\) gradient across the plasma membrane by chelating extracellular Ca\(^{2+}\) preventing Ca\(^{2+}\) influx, thapsigargin, depletes
Figure 4.1 PTx sensitivity of agonist-stimulated ERK responses in CHO-lac-mGlula and mGlula5a cells. Cells were pre-treated with PTx (100 ng ml\(^{-1}\)) or vehicle for 24 h. For experiments, cells were pre-treated with GPT/pyruvate prior to stimulation with 10 \(\mu\)M quisqualate for either 5 min or 30 min. Cell lysates were assayed for ERK activity by \(^{32}\)P incorporation into the EGF-receptor peptide (upper panels) as described in Materials and Methods (Section 2.5 and 2.6). Data are shown as means ± SEM for 3 separate experiments. ***/?<0.001 (Bonferroni’s multiple comparison test). The lower panels show representative ERK immunoblots for phospho-ERK activity. Lysates were analyzed via 12% SDS-PAGE as described under Materials and Methods (Section 2.10) and probed with an antibody that recognizes the phosphorylated form of ERK1/2 (pERK, see Table 2.1). Representative immunoblots from 3 separate experiments are shown. C; basal activity, or +quisqualate for 5, or 30 min.
Figure 4.2 Ca\(^2+\) dependency of agonist-stimulated ERK activation in CHO-lac-mGlula and -mGlu5a cells. Experiments were performed in normal KHB (1.3 mM Ca\(^2+\)) or under conditions where extracellular Ca\(^2+\) (-[Ca\(^{2+}\)]\(_o\)), or both extracellular and intracellular Ca\(^2+\) (-[Ca\(^{2+}\)]\(_o\) +Thaps) were depleted prior to agonist challenge. Extracellular Ca\(^2+\) depletion was achieved by washing cells before the experiment in Ca\(^2+\)-free KHB supplemented with 100 µM EGTA. For intracellular Ca\(^2+\) depletion 2 µM thapsigargin was added under -[Ca\(^{2+}\)]\(_o\) conditions for 15 min prior to agonist addition. Cells were stimulated ±10 pM quisqualate for 5 min and then assayed for ERK activity by \(\text{[^{32}P]}\) incorporation into the EGF-receptor peptide (upper panels) as described in Materials and Methods (Section 2.5 and 2.6). Data are shown as means ± SEM for 4 separate experiments. The lower panels show representative ERK immunoblots for phospho-ERK activity. Lysates were analyzed via 12% SDS-PAGE as described under Materials and Methods (Section 2.10) and probed with an antibody that recognizes the phosphorylated form of ERK1/2 (pERK, see Table 2.1). Representative immunoblots from 2-4 separate experiments are shown. C; basal activity, or +quisqualate for 5, or 30 min.
intracellular Ca\(^{2+}\) stores by inhibition of the sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase. Reducing extracellular Ca\(^{2+}\) from 1.3 mM to approximately 100 nM, by incubation of cells in Ca\(^{2+}\)-free KHB containing EGTA, did not affect quisqualate-stimulated ERK activation in either CHO-lac-mGlu1a or -mGlu5a cells (Fig. 4.2). Similarly, addition of thapsigargin (2 μM; Wylie et al. 1999) to cells in Ca\(^{2+}\)-free KHB containing EGTA was also without effect on mGlu receptor-stimulated ERK activity (Fig. 4.2). These data clearly demonstrate that increases in [Ca\(^{2+}\)]\(_i\) are not required for ERK activation by either the mGlu1a or mGlu5a receptor in the CHO cell background.

4.3 Effects of protein kinase C inhibition on mGlu receptor-mediated ERK activity

In addition to activating IP\(_3/Ca\(^{2+}\)\) signalling, the group I mGlu receptors also activate PKCs through the generation of diacylglycerol (Newton, 1995; Toker, 1998)) and in addition, PKCs have been implicated in signalling upstream of the ERK cascade (Wylie et al. 1999). It follows that ERK activation by mGlu1a and mGlu5a receptors may occur through a PKC-dependent pathway. To examine this dependence two distinct approaches, using either PKC inhibitors or PKC down-regulation, were used to manipulate cellular PKC activities.

Prior to investigating the role of PKC in mGlu receptor-stimulated ERK activity, the effects of two PKC inhibitors on phorbol ester stimulated-ERK responses in CHO-lac-mGlu1a and mGlu5a cells were assessed. When added acutely, phorbol esters such as phorbol-12,13-dibutyrate (PDBu) are known to potently stimulate ERK activity through the activation of PKC. The effects of the bisindolylmaleimide PKC inhibitor Ro 31-8220 (Wilkinson et al. 1993) and the indolocarbazole PKC inhibitor Gö6976 (Martiny-Baron et al. 1993) on PDBu-stimulated ERK activation were examined (Fig. 4.3). Pre-incubation of cells with Ro 31-8220 (10 μM, 30 min) abolished the ERK response to the phorbol ester PDBu (1 μM, 5 min) in both cell-lines, whereas pre-incubation with Gö6976 (10 μM, 30 min) did not significantly attenuate PDBu stimulated ERK responses in either cell line. It is possible that this difference arises from differences in the specificity of these two inhibitors. Whereas, Ro 31-8220 is a broad-spectrum inhibitor of PKC isoforms (inhibits Ca\(^{2+}\)-dependent (conventional PKCs: α,βI, βII, γ) and Ca\(^{2+}\)-independent subtypes (novel PKCs: δ, ε, η, θ; atypical PKCs: ξ, ι, λ)),
Way et al. 2000), Gö6976 selectively inhibits Ca^{2+}-dependent subtypes of PKC (Martiny-Baron et al. 1993). It could be inferred that ERK activation by PDBu is being mediated by Ca^{2+}-independent PKC isoforms and therefore no inhibition of this response is observed when Gö6976 is used. Indeed it may be speculated that Ca^{2+}-independent PKC isoforms may be involved in mGlu receptor-mediated ERK activation owing to the calcium insensitivity of the ERK activation by both receptors. Therefore to investigate involvement of PKC in agonist-stimulated mGlu receptor activation only the Ro 31-8220 PKC inhibitor was used. The time-course of ERK activation by either mGlu receptor was not inhibited by Ro 31-8220 pre-addition (Fig. 4.4), suggesting that ERK activation proceeds via a PKC-independent pathway in both CHO-lac-mGlu1a and -mGlu5a cell-lines.

The involvement of PKC in mGlu receptor-mediated ERK activation was additionally investigated by using chronic treatment of cells with phorbol esters. Unlike, the PKC activator, diacylglycerol, phorbol esters are not readily metabolized and therefore prolonged treatment of cells with PDBu results in chronic activation of PKC and the subsequent down-regulation of the expression of conventional and novel PKC isozymes (Newton, 1995).

In contrast to data presented with inhibition of PKC using pharmacological inhibitors, down-regulation of PKC isoforms by chronic treatment of cells with PDBu (1 µM, 24 h) caused a near complete suppression of the peak ERK response stimulated by quisqualate in both cell-lines (Fig. 4.5A and B). To examine whether chronic PDBu treatment might suppress the ERK response through an indirect mechanism (e.g. by down-regulation of the mGlu receptors), agonist-stimulated [^{3}H]-InsP responses under conditions of chronic phorbol ester pre-treatment were also examined. Chronic PDBu pre-treatment did not reduce quisqualate-stimulated [^{3}H]-InsP accumulations in either CHO-lac-mGlu1a or -mGlu5a cells, but instead caused somewhat greater (by 22% and 76%, respectively) agonist-mediated responses (Fig. 4.5C and D). These increases in [^{3}H]-InsP accumulations in CHO-lac-mGlu1a and -mGlu5a cells might be explained by the removal of PKC-dependent receptor desensitization events.

The data presented highlight a discrepancy between the using PKC inhibitors and PKC down-regulation to investigate PKC involvement in mGlu receptor-mediated ERK activation. The
Figure 4.3 Effects of the PKC inhibitors Go6976 and Ro 31-8220 on phorbol ester-stimulated ERK activation in CHO-lac-mGlu1a and -mGlu5a cells. A and B show the effects of the PKC inhibitors on phorbol ester-stimulated ERK activity in the mGlu-expressing cell-lines. Following pre-incubation with GPT/pyruvate, cells were either treated with either Go6976 (10 μM), Ro 31-8220 (10 μM) or vehicle for 30 min prior to a 5 min PDBu stimulation. Lysates were assayed for ERK activity by [32P] incorporation into the EGF-receptor peptide as described in Materials and Methods (Section 2.5 and 2.6). Data are shown as means ± SEM for 2 separate experiments. ***p<0.001, (Bonferroni’s multiple comparison test).
Figure 4.4 Effects of the PKC inhibitor Ro 31-8220 on agonist-stimulated ERK activation in CHO-lac-mGlu1a and -mGlu5a cells. Following pre-incubation with GPT/pyruvate, cells were pre-treated ±Ro 31-8220 (10 µM), stimulated with 10 µM quisqualate for the times indicated, and assayed for ERK activity by [32P] incorporation into the EGF-receptor peptide assayed for ERK activity as described in Materials and Methods (Section 2.5 and 2.6). Data are shown as means ± SEM for 2-3 separate experiments performed in duplicate.
Figure 4.5 Effects chronic phorbol ester treatment on agonist-stimulated ERK activation and agonist-stimulated [3H]-inositol phosphate accumulation in CHO-lac-mGlula and -mGlu5a cells. A and B show the effect of longer-term pre-treatment with phorbol ester (1 μM PDBu for 24 h) on the subsequent ERK response to agonist challenge. For experiments, cells were pre-treated with GPT/pyruvate prior to stimulation with quisqualate (10 μM), added to vehicle or PDBu-pretreated CHO-lac-mGlula or -mGlu5a cells for 5 min. Cell lysates were assayed for ERK activity by [32P] incorporation into the EGF-receptor peptide (upper panels) as described in Materials and Methods (Section 2.5 and 2.6). Data are shown as means ± SEM for 3 separate experiments. ***p<0.001 (Bonferroni’s multiple comparison test). C and D show the effects of chronic phorbol ester treatment on agonist-stimulated [3H]-InsP accumulations in each cell-line. [3H]-inositol-labeled (24 h) cells were treated with PDBu (1 μM) or vehicle for 24 h. For experiments, cells were pre-treated with GPT/pyruvate prior to LiCl (10 mM) addition, as described in Materials and Methods (Section 2.4), and then stimulated with 10 μM quisqualate for 30min. Data are shown as means ± range for 2 separate experiments performed in duplicate.
present study does not allow us to identify the extent and/or effectiveness to which PKC isoforms are inhibited by these two methods and indeed whether chronic treatment with phorbol esters might down-regulate other components involved in the signalling mechanism between the mGlu receptors and the ERK cascade.

4.4 Investigating G protein βγ subunit involvement in mGlu receptor-mediated ERK activity

The observation that mGlulα receptor-mediated ERK activation required PTx-sensitive Gi/o proteins, suggested possible Gβγ subunit involvement. A common intermediate step in such pathways is involvement of phosphoinositide 3-kinase (PI3-kinase), that acts upstream of Ras (Crespo et al. 1994) with PI3-kinase-γ (Lopez-Ilasaca et al. 1997) and PI3-kinase-β (Yart et al. 2002) isoenzymes being modulated by Gβγ subunits. We investigated whether mGlulα or mGlu5a receptors couple to the ERK cascade via PI3-kinase by using the PI3-kinase inhibitor, wortmannin.

Cells were pre-treated with wortmannin (100 nM, 30 min) and stimulated with quisqualate prior to assay for ERK activity (Fig. 4.6). In addition, foetal calf serum (FCS, 10%) was used as a positive control. Pre-addition of wortmannin suppressed FCS-stimulated ERK activity by 50-70% in both cell-lines (Fig. 4.6A and B), but had no effect on ERK activation stimulated by agonist challenge at either the mGlulα or mGlu5a receptor. Thus, it is unlikely that mGlu receptors signal via PI3-kinase to mediate ERK activation in these cell-lines.

Further, to explore whether βγ subunits might be involved in ERK activation by mGlu receptor activation, independently of an intermediary step involving PI3 kinase, the sequestration of βγ subunits by over-expression of the βγ scavenger α-transducin (Gαt1) was also examined. The over-expression of Gαt1 in cells to sequester βγ subunits has been demonstrated by several groups in a number of cell types with varying receptor subtypes (Federman et al. 1992; Selbie et al. 1997; Olianas & Onali, 1999) and in this cell model a similar approach was utilized by co-transfection of Gαt1 with HA-tagged ERK2.
Figure 4.6 Effects of the PI3-kinase inhibitor wortmannin on agonist-stimulated ERK activation in CHO-lac-mGlu1a and -mGlu5a cells. A and B show the effects of wortmannin (100 nM) pre-treatment for 30 min on subsequent ERK activation by quisqualate (10 μM) addition for 0 (Ctrl), 5 or 30 min to the mGlu-expressing cell-lines. In addition, the effect of the PI3-kinase inhibitor on serum (FCS)-stimulated ERK activity was also assessed in each cell-line. Cell lysates were assayed for ERK activity by [³²P] incorporation into the EGF-receptor peptide (upper panels) as described in Materials and Methods (Section 2.5 and 2.6). Data are shown as means ± SEM for 3 separate experiments performed in duplicate. ***p<0.001 (Bonferroni’s multiple comparison test).
Figure 4.7 Agonist-stimulated HA-ERK2 activation and expression in CHO-lac-mGlu1a and -mGlu5a cells. Cells were transfected with mammalian expression vectors (1 µg) encoding HA-ERK2 and incubated for 44 h prior to experimentation as described under Materials and Methods (section 2.3). A and B, cells were pre-treated with GPT/pyruvate prior to stimulation with 10 µM quisqualate for either 5 min or 30 min. Cell lysates were assayed for HA-ERK activity by [³²P] incorporation into the EGF-receptor peptide (upper panels) as described in Materials and Methods (Section 2.5 and 2.6). Data are shown as means ± SEM for 5-7 separate experiments. C, D and E, immunoblot analysis of HA-ERK2 expression in transfected mGlu expressing cell lines. Lysates were analysed via 12% SDS-PAGE as described under Materials and Methods (Section 2.10) and probed with a specific anti-HA antibody to detect HA-ERK2, or with an anti-ERK antibody that allows visualization of total ERK protein (Anti-ERK, see Table 2.1). Representative blots from 2 separate experiments shown. NT; non-transfected, C; basal activity, or +quisqualate for 5, or 30 min, Butyrate; ± 5 mM sodium butyrate.
Figure 4.8 Effect of βγ subunit sequestration on HA-ERK2 activation and expression in CHO-lac-mGlu1a and -mGlu5a cells. Cells were co-transfected with mammalian expression vectors (1 μg) encoding HA-ERK2 and Goα11 or an empty vector (pCMV5) and incubated for 44 h prior to experimentation as described under Materials and Methods (Section 2.3). A and B, cells were pre-treated with GPT/pyruvate prior to stimulation with 10 μM quisqualate for either 5 min or 30 min. Cell lysates were assayed for HA-ERK activity by [32P] incorporation into the EGF-receptor peptide (upper panels) as described in Materials and Methods (Section 2.5 and 2.6). Data are shown as means ± SEM for 3 separate experiments. C and D, Immunoblot analysis of HA-ERK2 expression in transfected mGlu expressing cell lines. Lysates were analysed via 12% SDS-PAGE as described under Materials and Methods (Section 2.10) and probed with a specific anti-HA antibody to detect HA-ERK2, or with an anti-ERK antibody that allows visualization of total ERK protein (Anti-ERK, see Table 2.1). Representative blots from 2 separate experiments shown. NT; non-transfected, C; basal activity, or +quisqualate for 5 min.
Initially, expression and agonist-stimulated activation of transfected HA-ERK2 in CHO-lac-mGlu1a and −mGlu5a cells was assessed (Fig. 4.7A and B). Activation of HA-ERK2 by mGlu receptor stimulation revealed that the magnitude of peak responses were $2.4 \pm 0.1$ and $3.6 \pm 0.8$-fold over basal for CHO-lac-mGlu1a and −mGlu5a cells, respectively. The peak agonist-stimulated responses observed were thus lower than those for native ERK activation ($4.9 \pm 0.2$ and $4.8 \pm 0.4$-fold over basal for mGlu1a and mGlu5a receptors respectively, section 3.4) and were found to be consistently higher in mGlu5a expressing cells (by approx. 50%). To examine this discrepancy further, the expression of transfected HA-ERK2 in CHO-lac-mGlu1a and −mGlu5a cells was also examined (Fig. 4.7C and D). The expression of HA-ERK2 was found to be much greater in CHO-lac-mGlu5a than −mGlu1a cells. This increased expression in CHO-lac-mGlu5a cells was not found to be due to the presence of sodium butyrate, used to enhance receptor expression in these cells (Fig 4.7E).

Co-transfection of cells with HA-ERK2 and Goα41 resulted in an attenuation of the mGlu receptor-mediated HA-ERK2 response by approx. 46% and 67% in mGlu1a- and mGlu5a-expressing cells respectively (Fig. 4.8A and B), which would suggest βγ subunit involvement. However, immunoblot analysis of the expression profile of HA-ERK2 under these conditions revealed that co-transfection of Goα41 markedly reduced HA-ERK2 expression (Fig 4.8C and D) and therefore the attenuation of the mGlu-mediated HA-ERK2 response can be attributed to reduced expression by co-transfection with Goα41 rather than due to the removal of a key signalling component by scavenging of βγ subunits.

### 4.5 The involvement of Src tyrosine kinase in mGlu1a and mGlu5a receptor-mediated ERK activation

Considerable evidence has accumulated in recent years to implicate both receptor tyrosine kinases and non-receptor tyrosine kinases as common participants in GPCR-ERK signalling (Pierce et al. 2001a). Analysis of the effects of the general tyrosine kinase inhibitor genistein on agonist-stimulated mGlu ERK activity (Fig. 4.9) revealed that both mGlu1a and mGlu5a receptor-mediated ERK responses were attenuated by 53% and 50% respectively, suggesting
Figure 4.9 Effects of the tyrosine kinase inhibitor genistein on agonist-stimulated ERK activation in CHO-lac-mGlu1a and -mGlu5a cells. A and B Cells were pre-treated with genistein (100 μM) for 30 min prior to stimulation with quisqualate (10 μM) for 5 or 30 min prior to Immunoblot analysis. Lysates were analyzed via 12% SDS-PAGE as described under Materials and Methods (Section 2.10) and probed with an antibody that recognizes the phosphorylated form of ERK1/2 (pERK, see Table 2.1). Representative immunoblots from 3 separate experiments are shown. Lower panels show densitometric analysis of this data, as means ± SEM for C; basal activity, or +quisqualate for 5, or 30 min
Figure 4.10 Effects of the Src family kinase inhibitor PP1 on agonist-stimulated ERK responses in CHO-lac-mGlu1a and -mGlu5a cells. For A and B, cells were pre-incubated with GPT/pyruvate before treatment with the various concentrations of PP1 (0-10 μM) for 30 min prior to addition of quisqualate (10 μM) for 5 min. ERK activity was assessed by \(^{32}\)P incorporation into the EGF-receptor peptide (upper panels) and cell lysates were assayed for ERK activity by \(^{32}\)P incorporation into the EGF-receptor peptide (upper panels) as described in Materials and Methods (Section 2.5 and 2.6). Data are shown as means ± SEM for 4 separate experiments. The lower panels show representative ERK immunoblots for phospho-ERK activity. Lysates were analyzed via 12% SDS-PAGE as described under Materials and Methods (Section 2.10) and probed with an antibody that recognizes the phosphorylated form of ERK1/2 (pERK, see Table 2.1). Representative immunoblots of phospho-ERK from 2 separate experiments are shown. C; basal activity, or +quisqualate for 5 min in presence and absence of PP1 (0-10 μM).
Figure 4.11 Effects of Src tyrosine kinase inhibitor PP1 on agonist-stimulated $[^{3}H]$-inositol phosphate responses in CHO-lac-mGlu1a and -mGlu5a cells. Before experimentation, $[^{3}H]$-inositol-labeled (24 h) cells were pre-treated with GPT/pyruvate and PP1 (0.1 and 10 μM) for 30 min, prior to LiCl (10 mM) addition as described in Materials and Methods (Section 2.4). A and B show subsequent agonist-stimulated $[^{3}H]$-InsP accumulations in response to stimulation with 30 μM quisqualate for 30 min. Data are shown as means ± SEM for 2 separate experiments performed in duplicate.
Figure 4.12 Effects of the Src family kinase inhibitor PP1 on agonist-stimulated ERK responses in CHO-m3 cells. Cells were pre-incubated with the various concentrations of PP1 (0-10 μM) for 30 min prior to subsequent challenge with carbachol (10 μM). Cell lysates were assayed for ERK activity by [$^{32}$P] incorporation into the EGF-receptor peptide (upper panels) as described in Materials and Methods (Section 2.5 and 2.6). Data are shown as means ± SEM for 2 experiments performed in duplicate.
that intracellular signalling from these receptors to the ERK MAP kinase may utilize tyrosine kinases.

c-Src, a cellular homolog of the v-Src protein encoded by Rous sarcoma virus, is the prototypic member of a family of non-receptor tyrosine kinases and several studies have proposed a central role for Src in linking a variety of GPCRs, expressed in different cell backgrounds, to ERK activation (Luttrell et al. 1996; Della Rocca et al. 1999). To determine whether a Src family kinase may be involved in mGlu receptor-mediated ERK activation, the selective inhibitor, PP1 (Hanke et al. 1996) was used. CHO-lac-mGlu1a and -mGlu5a cells were pre-treated (30 min) with a range of PP1 concentrations before stimulation with quisqualate (10 μM). PP1 pre-treatment produced a marked inhibition of agonist-induced ERK activation in both cell lines, with IC50 values of 1.7 μM (pIC50, 5.8 ± 0.2) and 0.15 μM (pIC50, 6.8 ± 0.2) for mGlu1a and mGlu5a receptor-expressing cells, respectively (Fig. 4.10A and B). In contrast, PP1 had no effect on quisqualate-stimulated [3H]-InsP responses in either cell-line (Fig 4.11A and B). Furthermore, pre-treatment of CHO cells stably expressing the m3 muscarinic acetylcholine receptor with PP1 had no effect on the ERK response elicited by a subsequent challenge with carbachol (Fig. 4.12). These data strongly suggest that PP1 is having a specific effect on Src family kinases to inhibit the mGlu1/5-stimulated ERK response.

4.5.1 Exploring mGlu receptor activation of the Src tyrosine kinase using Src activity assays and immunoblot analysis

To further investigate the role of Src kinase in mGlu receptor/ERK coupling, Src kinase assays were used in an attempt to establish if an increase in Src activity could be detected in response to agonist challenge. Methods described by Feder and Bishop (1990) and Cary et al. (2002) were used to examine activity of Src kinase using phosphorylation of acid-denatured enolase (Cooper et al. 1984) as an exogenous substrate. Fig. 4.13A and B show Src tyrosine kinase activity in mGlu1a and -mGlu5a expressing cells, respectively (importantly, activity is only detected when acid-denatured enolase is used). Increases in Src tyrosine kinase activity could not be detected in response to stimulation of either mGlu receptor. However, further control experiments revealed that the detected phosphorylation of acid-denatured enolase
could not be increased with foetal calf serum, used as a positive, or indeed attenuated by pre-treatment with PP1 (Fig 4.13C and D). Over several experiments, no increases, or decreases were observed in the detected levels of activity and therefore these data suggest that the assay, under these conditions, was not capable of detecting changes in Src tyrosine kinase activity in these cells. As optimization of this method would be time-consuming, owing to the number of factors that may need to be addressed, a second strategy, using a phospho-specific Src antibody was utilised.

Src kinase is phosphorylated at a number of residues but not all of these phosphorylations coincide with an increase in kinase activity (Bjorge et al. 2000). It was therefore important to select an antibody that would detect phosphorylation that was indicative of increased activity. Therefore, a phospho-specific antibody (Table 2.1) that detects phosphorylation at a critical residue (Tyr 416) located in the activation loop of Src (Superti-Furga, 1995), which when phosphorylated is a positive regulator of Src activity, was used to examine mGlu receptor stimulation of Src kinase. Importantly, this antibody does not react with a second important phosphorylation site in the C-terminus of Src (Tyr 527) that allows physical interactions within the enzyme to suppress basal kinase activity (Bjorge et al. 2000).

Fig. 4.13E and F show a time-course of Src tyrosine kinase activity as measured by immunoblot analysis in CHO-lac-mGlu1a and -mGlu5a cells respectively. A small increase in Src phosphorylation, (approx. 2-3 fold-over basal by densitometric analysis, data not shown) apparent at 2 min after agonist-addition and that remained elevated, was detected in both cell lines, suggesting mGlu1a and mGlu5a receptors stimulate Src tyrosine kinase activity. However, this activation was not consistent across separate experiments and only two out of five experiments showed a detectable increase from basal.

Experiments examining the effects of PP1 on mGlu1a and mGlu5a receptor-stimulated ERK activity clearly suggested Src involvement in signalling from these two receptors, but the present data do not allow us to conclude categorically that these receptors increase the tyrosine kinase activity of this kinase.
### Figure 4.13 Analysis of agonist-stimulated Src tyrosine kinase activity in CHO-lac-mGlu1a and -mGlu5a cells.

A and B, cells were stimulated with 10 μM quisqualate for the times indicated and then assayed for Src activity using rabbit muscle enolase as a substrate as described in Materials and Methods (Section 2.9). Representative autoradiograms of enolase phosphorylation detected after exposure to autoradiography film (2-8 h) are shown for 2-4 separate experiments. C and D, as described for A and B, except cells were stimulated with serum (FCS)-stimulated or pre-incubated with PP1 (5 μM) for 30 min prior to stimulation with quisqualate (10 μM) for 5 min. E and F, lysates were analyzed via 12% SDS-PAGE as described under Materials and Methods (Section 2.10) and probed with an antibody that recognizes the phosphorylated form of Src (pSrc) or total Src protein (anti-Src, see Table 2.1). Representative immunoblots from 2 separate experiments are shown. NI; Non-Induced, C; basal activity, or +quisqualate for 2, 5, 10 or 30 min.

#### Table: Enolase phosphorylation

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4.6 Investigating the role of receptor tyrosine kinases in mGlu1a and mGlu5a receptor-mediated ERK activation

Recently, transactivation of the EGF receptor has been proposed as an important mechanism linking the mGlu5 receptor to ERK activation in astrocytes (Peavy et al. 2001). We therefore investigated whether tyrosine kinases might be employed in the mechanism of ERK activation by mGlu receptors.

It is understood that CHO cells lack endogenous expression of EGF receptors, but PDGF receptor expression has been reported (Duckworth & Cantley, 1997). Indeed, no stimulation of ERK activation by EGF (100 ng ml⁻¹) could be detected in either cell-line (Fig. 4.15A and B), whereas PDGF (20 ng ml⁻¹) elicited robust stimulations of ERK in CHO-lac-mGlu1a and -mGlu5a cells of 25 ± 8 and 20 ± 3 fold-over-basal, after PDGF addition, respectively (Fig. 4.14A and B). It is also known that this growth factor receptor is able to mediate GPCR transactivation (Herrlich et al. 1998). Therefore, we investigated whether the PDGF receptor tyrosine kinase inhibitor AG1296 (Kovalenko et al. 1994) affected mGlu1a and/or mGlu5a receptor-ERK signalling. Pre-incubation with AG1296 (10 µM, 20 min) differentially affected the ERK response stimulated by the two mGlu receptors (Fig. 4.14C and D). Thus, whilst the mGlu5a receptor-mediated ERK activation was unaffected by AG1296 pre-addition, the ERK response to mGlu1a receptor activation was significantly attenuated (by 49 ± 6 % at 5 min, Fig. 4.14C). We also investigated whether the EGF receptor kinase inhibitor AG1478 had any effect on mGlu1a and/or mGlu5a receptor-ERK signalling. In contrast to the marked inhibition of mGlu1a receptor-stimulated ERK responses by AG1296, AG1478 (10 µM) had no significant effect on either mGlu1a or mGlu5a receptor-stimulated ERK activity (Fig. 4.15C and D) suggesting that the observed effects of AG1296 occur through inhibition of PDGF receptor tyrosine kinase activity. Unexpectedly however, AG1478 did attenuate PDGF-stimulated responses in both CHO-lac-mGlu1a and -mGlu5a cells (by 45%, Fig 4.15A and 64%, Fig 4.15B, respectively).
Figure 4.14 Effects of the PDGF receptor tyrosine kinase inhibitor AG1296 on PDGF and agonist-stimulated ERK activation in CHO-lac-mGlu1a and -mGlu5a cells. A and B show the effects of AG1296 (10 μM) pre-treatment for 20 min on subsequent ERK activation by PDGF (20 ng ml⁻¹) for 5 min. Cell lysates were assayed for ERK activity by [³²P] incorporation into the EGF-receptor peptide as described in Materials and Methods (Section 2.5 and 2.6). Data are shown as means ± SEM for 2 separate experiments. ***p<0.001 (Bonferroni’s multiple comparison test). C and D, cells were pre-treated with AG1296 as above, stimulated with quisqualate (10 μM) for 5 or 30 min, and then assayed for ERK activity as described above. Data are shown as means ± SEM for 4 separate experiments performed in duplicate. *p<0.05 (Bonferroni’s multiple comparison test).
Figure 4.15 Effects of the EGF receptor tyrosine kinase inhibitor AG1478 on EGF, PDGF and agonist-stimulated ERK activation in CHO-lac-mGlu1a and -mGlu5a cells. A and B, Cells were pre-incubated with GPT/pyruvate before stimulation with either EGF (100 ng ml$^{-1}$) or PDGF (20 ng ml$^{-1}$) for 5 min in the presence and absence of pre-treatment with AG1478 (10 μM) for 20 min. Cell lysates were assayed for ERK activity by $[^{32}P]$ incorporation into the EGF-receptor peptide as described in Materials and Methods (Section 2.5 and 2.6). Data are shown as means ± SEM for 3 separate experiments. C and D show the effects of AG1478 (10 μM) pre-treatment for 20 min on subsequent ERK activation by quisqualate (10 μM) addition for 5 min in mGlu-expressing cell-lines. ERK activity was assessed as described above. Data are shown as means ± SEM for 2-6 separate experiments.
4.6.1 Exploring mGlu receptor transactivation of the PDGF receptor using co-immunoprecipitation

The observation that mGlu1a receptor-mediated ERK responses were attenuated by AG1296 prompted further investigation into possible PDGF receptor involvement. To examine if direct association of mGlu1a receptor with the PDGF receptor was occurring, as shown for the mGlu5a receptor and the EGF receptor in astrocytes (Peavy et al. 2001), a co-immunoprecipitation approach was used.

It was already observed that PDGF stimulated robust ERK activation in both cell lines but it was not known which PDGF receptor was involved. Immunoblot analysis revealed that CHO-lac-mGlu1a and -mGlu5a cells expressed both α and β forms of the PDGF receptor, (Fig. 4.16A and B), however subsequent experiments were only carried out with consideration to the PDGFRβ isoform (PDGF stimulation used above, stimulates both α and β forms of the PDGF receptor).

The mGlu1a and mGlu5a receptors were immunoprecipitated from cell lysates using specific antisera and the resulting immunoprecipitates separated by SDS-PAGE before western blot analysis with an anti-PDGFRβ antibody. The immunoblot analysis revealed that PDGFRβ co-immunoprecipitated with the mGlu1a, but not the mGlu5a receptor (Fig. 4.16C and D). No co-immunoprecipitation could be detected in control lanes where protein-A sepharose was added, but no mGlu receptor antisera (Fig. 4.16E and F), confirming that the protein-protein interaction is specific. Importantly, there was also no co-immunoprecipitation in non-induced lysates implying that the mGlu1a receptor must be present to detect the association with PDGFRβ (Fig. 4.16E and F). In addition to co-immunoprecipitation, the activity of the PDGFRβ in response to mGlu receptor stimulation was also assessed by examining autophosphorylation of PDGFRβ. However, increases in activity after agonist-challenge were inconsistent and difficult to detect owing to high levels of basal activity (data not shown).

The adapter protein Shc (Src homology/collagen) binds to phosphorylated receptor tyrosine kinases, such as the EGF/PDGF receptors and Src via its SH2 domain, and functions as an adapter protein by forming complexes via the SH2 domains of other proteins (Malarkey et al. 2001).
Figure 4.16 Co-immunoprecipitation of mGlu1a and mGlu5a receptors with the PDGF receptor in CHO-lac-mGlu1a and CHO-lac-mGlu5a cells. Cells were pre-incubated with GPT/pyruvate before stimulation with quisqualate (10 µM) for the various times indicated. mGlu receptor protein were then immunoprecipitated with receptor specific antisera (see Table 2.1) as described in Materials and Methods (Section 2.10.1). Immunoprecipitates were analyzed via 8% SDS-PAGE as described under Materials and Methods (Section 2.10) and probed with an antibody that recognizes the the PDGF receptor (see Table 2.1). A and B show PDGF receptor expression in CHO-lac-mGlu1a and-mGlu5a cells, respectively. C-F show mGlu receptor immunoprecipitates, treated as described above, probed with PDGF receptor β specific antiserum. Representative immunoblots from 2-3 separate experiments are shown. C; basal activity, or +quisqualate for 2-30 min, WCL; whole cell lysates/extracts, NI; Non-induced cell; P; protein A-sepharose beads only (no primary receptor antibody added).
1995; Cattaneo & Pelicci, 1998) to elicit downstream signalling. The Shc gene encodes three proteins of 46, 52, and 66 KDa. To examine whether Shc is tyrosine phosphorylated by mGlu receptor activation, a similar immunoprecipitation approach was used.

Shc was immunoprecipitated from CHO-lac-mGlu1a and -mGlu5a lysates using an antiserum that detects all three isoforms. Immunoprecipitates were then examined using immunoblot analysis using a phosphotyrosine antibody. Both mGlu1a and mGlu5a receptors stimulated an increase in the tyrosine phosphorylation of the 52 and 66 KDa proteins (Fig. 4.17A and B). Increases in tyrosine phosphorylation of the 46 KDa protein were not observed. These data strongly suggest that these two receptors increase the activity of this adapter protein and that this is likely to be upstream of ERK activation. It was not established if this activation was downstream of Src kinase (this could be identified by using PP1), or in the case of the mGlu1a receptor, whether this was a consequence of PDGF receptor activation.
Figure 4.17 Agonist-stimulated Shc tyrosine phosphorylation in CHO-lac-mGlu1a and CHO-lac-mGlu5a cells. A and B, cells were pre-incubated with GPT/pyruvate before stimulation with quisqualate (10 μM) for the various times indicated. Shc proteins were then immunoprecipitated with an anti-Shc antibody (see Table 2.1) as described in Materials and Methods (Section 2.10.1). Immunoprecipitates were analyzed via 12% SDS-PAGE as described under Materials and Methods (Section 2.10) and probed with an antibody that recognizes the phosphorylated tyrosine residues (anti-phosphotyrosine, see Table 2.1). Representative immunoblots of Shc phosphorylation from 3 separate experiments are shown. C; basal activity, or +quisqualate for 2-30 min, WCL; whole cell lysates/extracts.
Discussion

The present Chapter has examined the signalling profiles of mGlu1a and mGlu5a receptors to the activation of the ERK cascade, which was introduced in the preceding Chapter. The data presented some common signalling intermediates used by these two receptors to couple to ERK, but also some clear differences could be discerned. These differences might reflect differences in the G protein coupling specificities of mGlu1a and mGlu5a receptors and indeed the ability of these two receptors to couple to the signalling components available to them in native cells.

Initial experiments demonstrated that both mGlu1a and mGlu5a receptors activate ERK with similar time-courses and to comparable levels despite the somewhat higher expression of the mGlu1a relative to mGlu5a receptor in the CHO-lac cell-lines. However, experiments exploring the G protein sub-population(s) responsible for receptor-ERK coupling revealed a key difference between the group I mGlu receptors. Thus, while the agonist-stimulated ERK response in CHO-lac-mGlu1a cells was almost completely abolished by PTx pre-treatment, the ERK response of CHO-mGlu5a cells was unaffected by toxin treatment.

While these data confirm the PTx sensitivity of mGlu1a receptor-ERK signalling in this cell background (Ferraguti et al. 1999), they also demonstrate that mGlu1a and mGlu5a receptor coupling to ERK activation in CHO cells is dependent on different G protein populations. Whether mGlu1a receptor-ERK signalling is mediated exclusively by Gi/o proteins, or is dependent on Gli1/Gq11 cooperation, as has been shown for the B2 bradykinin receptor (Blaukat et al. 2000), has yet to be established. Previous studies have failed to detect mGlu1a-Gi/o coupling using [35S]-GTPyS and Ga-specific immunoprecipitation (Selkirk et al. 2001) in the CHO-lac cell line, although baby hamster kidney (BHK) cells stably expressing mGlu1a have provided evidence for both Gq11 and Gi/o activation (Hermans et al. 2000). A recent study by Conn and colleagues in astrocytes has shown that the mGlu5a receptor couples to ERK activation in astrocytes via a PTx-insensitive pathway, and this coupling is specifically dependent upon the activation of GqG by the receptor (Peavy et al. 2001). It is possible that the mGlu5a receptor may also mediate ERK activation by Gq in CHO cells, but
other PTx-insensitive mechanisms (e.g. via G_{12/13} proteins, or G protein-independent coupling) have not been explored.

The G_{i0} protein dependence of mGlu1a receptor-ERK coupling suggests possible Gβγ involvement. A common intermediate step in such pathways is PI3-kinase that acts upstream of Ras (Crespo et al. 1994) with PI3-kinase-γ (Lopez-Ilasaca et al. 1997) and PI3-kinase-β (Yart et al. 2002) isoenzymes being modulated by Gβγ subunits. However, the agonist-stimulated ERK response to either mGlu receptor was not affected by wortmannin suggesting that PI3-kinase is not involved in either mGlu1a or mGlu5a receptor signalling to ERK in this system.

The involvement of Gβγ subunits was further investigated using sequestration of βγ subunits by over-expression of the βγ ‘scavenger’ α-transducin (Gαt) with HA-tagged ERK2. However, transfected HA-ERK2 activity mediated by mGlu1a and mGlu5a receptor stimulation was approx. 51% lower in mGlu1a-, and 26% lower in mGlu5a receptor expressing cells, compared to the maximal stimulation of native ERK activation in these cell lines. This low level of fold-over basal activity made it difficult to utilize this approach in further investigations into intracellular signalling to ERK by these two receptors. Further, upon co-transfection with Gαt, this activity was significantly inhibited in both cell lines, suggesting Gβγ subunit involvement. However, any conclusions from this data cannot be made owing to the observation that co-transfection substantially reduced expression of HA-ERK2 in both cell lines. It is now common to use such co-transfection approaches, in particular with tagged proteins, to allow the exploration of signalling molecules that can be easily tracked and immunoprecipitated from cells. However, conclusions made from these approaches should perhaps be made cautiously and take into account any effects on expression levels made when co-transfecting two or more plasmids.

Recent studies have highlighted a role for Ca^{2+} as a positive modulator of group I mGlu receptor activation (Hermans et al., 2001) and for Ca^{2+}/calmodulin in the regulation of focal adhesion kinase activity and actin stress fibre formation as a consequence of mGlu1a receptor
activation in CHO cells (Shinohara et al. 2001). However, neither mGlu1a nor mGlu5a receptor linkage to ERK were dependent upon increases in intracellular Ca\(^{2+}\), as the removal of extracellular Ca\(^{2+}\) (in the presence or absence of an intracellular Ca\(^{2+}\) store) did not affect the ERK response to quisqualate. The dependency of receptor-ERK coupling on Ca\(^{2+}\) may vary with the cell background as both Ca\(^{2+}\)-dependent (Schinkmann et al. 2000) and Ca\(^{2+}\)-independent (Peavy et al. 2001) ERK activation in response to group I mGlu receptor stimulation has been reported. Previous data have shown that the G\(_q\)-coupled m3-mACh receptor also stimulates a robust activation of ERK via a Ca\(^{2+}\)-independent mechanism in the CHO cell background (Wylie et al. 1999).

Previous studies have shown that both G\(_{i/o}\)- and G\(_{q/11}\)-coupled GPCRs activate ERK via partially PKC-dependent pathways in CHO cells (Wylie et al. 1999). To investigate whether mGlu1a and/or mGlu5a receptor-ERK coupling is PKC-dependent two distinct approaches were used to manipulate cellular PKC activities. The broad-spectrum PKC inhibitor Ro 31-8820, at a concentration that blocks ERK activation by the phorbol ester PDBu, had no effect on receptor-mediated ERK activation in either CHO-lac-mGlu1a or -mGlu5a cells. In marked contrast, down-regulation of PKC by an overnight treatment with phorbol ester attenuated the receptor-stimulated ERK response in both cell-lines. It is possible that the pro-longed exposure of cell to phorbol esters, which firstly activate PKC prior to their down-regulation, may cause other non-specific effects. As quisqualate-stimulated \[^{3}\text{H}\]-InsP formation was actually increased in CHO-lac-mGlu1a and -mGlu5a cells following overnight treatment with PDBu, it is unlikely that any change in mGlu receptor expression accounts for the reduction in ERK activation under these conditions. The reason for the discrepancy between data obtained using acute PKC inhibition compared to a chronic pre-treatment to down-regulate (a subset of) PKC activities is presently unknown. Previous studies have been similarly inconclusive. Ferraguti et al. (1999) for example reported that some, but not all approaches pharmacologically to inhibit PKC attenuated mGlu1a receptor-mediated ERK activation in CHO cells. Similarly, ERK activation by group I mGlu receptors in astrocytes has been reported to be either PKC-dependent (Schinkmann et al. 2000) or -independent (Peavy & Conn 1998; Peavy et al. 2001; 2002). In contrast, PKC activation appears obligatory for
mGlu1 receptor-ERK linkage under pathophysiological conditions (Calabresi et al. 2001). Irrespective of whether mGlu1/5 receptor-ERK signalling proves ultimately to be PKC-dependent or -independent, the present data provide no evidence for distinct PKC sensitivities for mGlu1a versus mGlu5a receptor-ERK signalling.

Considerable evidence has accumulated in recent years to implicate both receptor tyrosine kinases and non-receptor tyrosine kinases as common participants in GPCR-ERK signalling (see Pierce et al. 2001a). Src is the prototypic member of a family of non-receptor tyrosine kinases that also includes Fyn, Yes, Fgr, Hck, Lyn, Lck, and Blk, which have been implicated as intermediaries in a variety of key cellular functions, including cell proliferation, adhesion and motility (Schwartzberg et al. 1998; Bjorge et al. 2000). Luttrell et al. (1996) showed that PTx-sensitive GPCR activation of ERK in COS-7 cells involved Src activation and the formation of complexes with the adaptor protein Shc. Subsequent studies by the same group proposed a central role for Src in linking a variety of GPCRs, expressed in different cell backgrounds, to ERK activation (Della Rocca et al. 1999). More recent studies have also highlighted the involvement of Src, and provided evidence for direct GPCR-Src interactions (Cao et al. 2000) reminiscent of the G protein-independent group I mGlu receptor-Src signalling interaction reported in CA3 pyramidal cells (Heuss et al. 1999).

In the present study we have shown a concentration-dependent inhibition of mGlu1a and mGlu5a receptor-stimulated ERK responses by the Src-selective inhibitor PP1 suggesting that both PTx-sensitive and -insensitive GPCR-ERK pathways are dependent on Src, or another PP1-sensitive Src-family kinase. The inhibitor was approx. 10 times more effective in the CHO-lac-mGlu5a cell line, while the specificity of the PP1 effect could be indicated by the complete lack of effect of this inhibitor on quisqualate-stimulated [³H]-InsP accumulation in either cell line. In addition, PP1 did not inhibit ERK activation by the Gq-coupled m3-mACh receptor in a CHO-m3 cell-line, which is consistent with data from others in the laboratory (Burdon D., personal communication). These findings are supported by studies reported by Peavy et al. (2001) that demonstrated a role for Src in mGlu5 receptor-stimulated ERK activation in astrocytes. In that study, Src was shown to be important in mediating ERK activation downstream of EGF receptor ‘transactivation’ (Daub et al. 1997; Pierce et al.
by the mGlu5 receptor. In addition, the Src-independence of m3-mACh receptor-ERK signalling has also been reported recently in SH-SY5Y cells, which endogenously express this muscarinic receptor subtype (Watcharasit et al. 2001).

Attempts to detect increases in tyrosine kinase activity mediated by mGlu receptor stimulation did not reveal significant increases in activity after challenge with quisqualate. It is feasible that the approaches used were not sensitive enough to consistently detect any changes in activity by mGlu receptor stimulation. However, it is also possible that actual increases in tyrosine kinase activity of Src are not required for its role in coupling these receptors to the ERK cascade. Src has an SH2 and SH3 domain (Bjorge et al. 2000), which allow it to serve as a scaffolding molecule. It has been suggested that the scaffolding and catalytic functions of Src may be considered separately, and indeed its role as a scaffold may be important in mediating signalling without the requirement for an increase in enzymatic activity (Schwartzberg et al. 1997), or vice versa (Cary et al. 2002). It is feasible that Src serves such a (scaffolding) function in mGlu receptor-mediated activation of ERK. Src has been shown to bind directly to GPCRs, including the β3-adrenoceptor (Cao et al. 2000), via proline-rich (PXXP) motifs in the third intracellular loop of the receptor, to couple the receptor to ERK activation. As both mGlu1a and mGlu5a receptors contain proline-rich sequences (including PXXP motifs) in their C-terminal tails it is possible that Src could bind directly to these regions and facilitate mGlu receptor/ERK coupling. This facilitation may be a scaffolding role, serving to recruits other key proteins, or one involving increased tyrosine kinase activity in response to agonist, resulting in phosphorylation and activation of subsequent components necessary for coupling to the ERK cascade. A recent study in striatal neurons has shown that the mGlu5a receptor itself can become tyrosine phosphorylated (Orlando et al. 2002) and suggests the potential for direct activation or recruitment of other proteins at this site to modulate downstream signalling.

In addition to Src requirement, mGlu1a receptor-mediated ERK activation also appeared to be dependent upon the PDGF receptor. Maudsley and co-workers (2000) have shown that the β2-adrenoceptor activates ERK via a multi-protein complex formed upon agonist addition and includes the receptor, the EGF receptor and Src kinase. The study by Peavy and colleagues
(2001) demonstrated that in astrocytes the mGlu5a receptor couples to ERK activation via a mechanism independent of PKC, Ca²⁺ and Gβγ subunits, similar to the data presented here. The activation was however, dependent upon mGlu5a receptor activation of Gαq leading to EGF receptor transactivation and the subsequent downstream activation of Src, resulting in ERK phosphorylation. We set out to determine whether such a mechanism of activation might account for mGlu receptor-mediated ERK activation in our CHO-cell system.

As CHO cells appear to lack endogenous EGF receptors, we addressed whether the PDGF receptor, known to be present in CHO and previously shown to be able to replace EGF receptor signalling in cells where the latter is not present (Herrlich et al. 1998), might be transactivated as a mechanism for mGlu receptor signalling to ERK. Similar to the situation with respect to PTx sensitivity a different effect of inhibiting PDGF receptor tyrosine kinase activity was observed in the two cell-lines. Thus, the PDGF receptor tyrosine kinase inhibitor AG1296 markedly attenuated mGlu1a, but not mGlu5a receptor-stimulated ERK activation. This suggested that the PDGF receptor may be important for mGlu1a receptor-stimulated ERK activation, but is not required by the pathway employed by the mGlu5a receptor.

The role of mGlu1a receptor-PDGF receptor coupling to ERK was further explored using a co-immunoprecipitation approach to examine physical interactions of these two receptors. In immunoprecipitates of the mGlu1a receptor from CHO-lac cells, co-immunoprecipitation of the PDGFβ receptor could be detected. Over a time-course of agonist challenge, no increase in this association could be discerned. No co-immunoprecipitation could be detected in non-induced cells indicating that co-immunoprecipitation of the PDGF receptor required the presence of the mGlu1a receptor, and this association was not dynamic as increased association was not detected after addition of agonist. Non-specific effects of co-immunoprecipitation were also unlikely due to the PDGF receptor not co-immunoprecipitating with the mGlu5a receptor. These data support the findings made using the tyrphostin AG1296, where sensitivity was observed with respect to mGlu1a receptor, but not mGlu5a receptor-mediated coupling to ERK. To confirm that the mGlu1a receptor was indeed being co-immunoprecipitated with PDGFβ receptor further investigation would
conversely involve examining PDGFβ receptor immunoprecipitates with immunoblot analysis for the mGlu1a receptor.

Collectively, observations of PDGF receptor and Src requirement strongly suggest a mechanism of transactivation in the coupling of the mGlu1a receptor to ERK activation. The insensitivity of mGlu5a receptor-mediated ERK responses to AG1296 and the lack of co-immunoprecipitation with the PDGF receptor clearly suggest that such a mechanism is not utilized in ERK coupling by this receptor. Further, the requirement of Src kinase for mGlu5a receptor ERK activation prompts speculation that Src recruitment by the mGlu5a receptor may occur via an entirely different mechanism to that utilized by the mGlu1a receptor, and may relate to the differences observed at the level of G protein-coupling for these two receptor subtypes.

Preliminary observations of increased Shc activity by both receptor subtypes suggest that this adapter protein may also be used in the coupling mechanism of both receptors upstream of ERK activation. Further investigations would however, need to establish if Shc activation is downstream of Src kinase, as Src is similarly activated by both mGlu receptors and is known to be capable of increasing Shc tyrosine phosphorylation.

In summary, we have investigated whether MAPK/ERK activation by mGlu1a and mGlu5a receptor subtypes involves a common signalling pathway(s), when these closely related family C GPCRs are expressed in the same cell background. The data presented show that while a number of pathway similarities can be identified (Src-family kinase-dependence, and Ca²⁺- and PI3-kinase-independence, increased Shc protein activity), clear differences can also be discerned (see Fig. 4.18). In particular, ERK activation by the mGlu1a, but not the mGlu5a, receptor requires a functioning G₁₀ protein population and is inhibited by a PDGF receptor tyrosine kinase inhibitor. The pharmacological approach used here does not allow us to distinguish whether signalling to ERK involves the activation of G₁₀ proteins by the mGlu1a receptor, or whether G₁₀ proteins play a ‘permissive’ role in mGlu1a receptor signalling. Similar uncertainties apply to the inhibitory effects seen using the Src and PDGF
receptor inhibitors, which not only prevent activation, but can also suppress basal kinase activity.

Over the past 10 years a plethora of data have been generated concerning GPCR signalling to MAPK/ERK pathways. One clear theme to emerge is that the precise pathway(s) employed by a GPCR can be highly dependent on cell background (Della Rocca et al. 1999; Pierce et al. 2001a). This may be especially the case for group I mGlu receptors as Homer scaffold proteins can influence the signalling properties of the mGlu1a and mGlu5a receptors (Hermans & Challiss, 2001). The dependence of mGlu receptor activation of ERK on receptor internalization is another consideration. Whilst some reports have shown that receptor internalization is necessary to co-localize the receptor with components of the ERK cascade (Ignatova et al. 1999; Luttrell et al. 2001; Lavoie et al. 2002), others have demonstrated that this is not a prerequisite for all receptor/ERK activation pathways (Budd et al. 1999). Attempts to investigate the requirement for receptor internalisation in mGlu receptor mediated ERK activation, using conditions of high osmolarity (data not shown), proved inconclusive and it therefore remains to be established whether internalization of mGlu1a and mGlu5a receptors is necessary for efficient coupling to the ERK cascade in CHO cells.

The most important aspect of the present data is not the components that constitute the mGlu1/5 receptor-ERK signalling pathway in CHO cells, but the fact that these group I mGlu receptors can recruit different subsets of downstream signalling components to execute this pathway. These data suggest that not only do mGlu1 and mGlu5 receptors exhibit different anatomical and cellular distributions within the CNS (Hubert et al. 2001; Valenti et al. 2002), but they also differ in their repertoires of downstream signalling partners.
Figure 4.18 Scheme showing possible linkage of mGlu1a and mGlu5a receptors to ERK activation. In CHO-lac cell lines, the heterologously expressed group I mGlu receptors differ in their PTx sensitivity and inhibition by AG1296, suggesting that mGlu1a but not mGlu5a receptors depend on Gq/0 protein activation and receptor tyrosine kinase (RTK) transactivation to cause ERK activation. In the CHO cell background the RK involved is the PDGF receptor. In addition, both mGlu1a and mGlu5a receptors stimulate ERK via Src-dependent, but PI3 kinase and Ca2+ independent pathways. The issue of PKC involvement remains to be established as both mGlu1a and mGlu5a receptor signalling to ERK is markedly attenuated by PKC down-regulation (by chronic phorbol ester treatment), but not by acute PKC inhibitor action.
CHAPTER 5 An investigation into the activation of c-Jun N-terminal kinase and p38 MAPKs by mGlu1a and mGlu5a receptors

Introduction

Whereas the ERK MAP kinases are commonly associated with cellular proliferation and differentiation, the other two major MAP kinase subgroups, the stress-activated protein kinases, or c-Jun N-terminal kinase (JNK) and p38 MAPKs are often associated with growth arrest and apoptosis (Mielke & Herdegen, 2000). In addition, they have been implicated in the regulation of cellular events such as embryonic morphogenesis, cell survival and differentiation (for review see Davies 2000; Lee et al. 2000). Yang and colleagues (Yang et al. 1997) have also highlighted the importance of JNK in excitotoxicity by examining stress-induced neuronal cell death by disruption of the JNK3 gene in mice.

JNK and p38 MAP kinases are typically activated in response to exposure to environmental stresses, such as ultraviolet radiation, osmotic stress, heat shock, and to inflammatory cytokines (for reviews see Minden & Karin, 1997; Widmann et al. 1999). The JNK subgrouping is encoded by three genes, which are alternatively spliced to produce at least ten JNK isoforms. JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 displays a limited expression pattern and is mostly restricted to the brain (Gupta et al. 1996). There are four primary isoforms of p38 MAP kinase, p38α, β, γ and δ. The p38 kinases are widely expressed in many tissues but only p38α, and p38β isoforms are expressed in the brain (Harper & LoGrasso, 2001).

The JNKs were initially characterised by their ability to phosphorylate and activate the transcription factor c-Jun, a subunit of the transcription activator protein 1 (AP-1) (Pulverer et al. 1991). The transcription factor ATF2 is also a target of both JNK (Gupta et al. 1995) and p38 MAP kinases (Raingeaurd et al. 1995). In addition to transcription factors, JNK and p38 can also phosphorylate a number of cytosolic protein targets (Mielke & Herdegen, 2000).
The activation of JNK MAP kinase pathways following addition of glutamate has been demonstrated in primary cultures of hippocampal neurones (Mukherjee et al. 1999), cortical neurones (Ko et al. 1998) and striatal neurones (Schwarzchild et al. 1997, 1999), and this activation has been attributed to the NMDA-receptor subgroup of ‘ionotropic’ glutamate receptors. The activation of p38 by glutamate application has also been reported in cerebellar granule cells and has similarly been attributed to the activation of NMDA-receptors (Kawasaki et al. 1997). However, to date, the activation of JNK by metabotropic glutamate receptors has not yet been documented. A study by Bolshakov and colleagues has suggested a role for p38 MAP kinases downstream of mGlur receptor-mediated induction of long-term depression (LTD) at CA3-CA1 synapses in the hippocampus (Bolshakov et al. 2000), but the specific subgroup of mGlur receptor involved was not determined.

Having ascertained that both the mGlur1a and mGlur5a receptor mediate a robust and comparable ERK activation in these cells, the aim of this Chapter was to investigate the coupling of the mGlur1a and mGlur5a receptors to the activation of JNK and p38 subgroups of MAP kinases. The data presented, reveal a robust activation of the JNK pathway by the mGlur1a receptor, but no significant activation of this cascade by the mGlur5a receptor. The signalling profile of the mGlur1a receptor to the JNK cascade was also explored. Initial observations also highlighted the potential for activation of the p38 MAP kinase by both mGlur receptors in these cells. The possible coupling mechanisms for mGlur receptor-mediated activation of ‘stress-activated’ MAP kinase signalling and the implications of this activation in native cells are also discussed.
Results

5.1 Agonist-stimulated JNK activation in CHO-lac-mGlu1a and -mGlu5a cells

JNK activation in CHO-lac cells was assessed by in vitro immunocomplex kinase assays using glutathione S-transferase (GST)-c-Jun as a substrate. Fig. 5.1 shows concentration-response curves for quisqualate-stimulated JNK activation in mGlu1a- and mGlu5a-expressing cells. For CHO-lac-mGlu1a cells, quisqualate stimulated a marked increase in JNK activation with an EC\textsubscript{50} value of 0.6 μM (pEC\textsubscript{50}, 6.2 ± 0.2). For CHO-lac-mGlu5a cells a small increase in JNK activity above basal was seen, but this failed to achieve statistical significance even at the highest concentration of quisqualate used (Fig. 5.1B). The response of CHO-lac-mGlu1a and -mGlu5a cells to anisomycin, a potent activator of JNK activity (Cano et al. 1994) was also assessed. Anisomycin stimulated a robust, comparable activation of JNK activity in both CHO-lac-mGlu1a (20.8 ± 3.5 fold-over-basal) and CHO-lac-mGlu5a (19.2 ± 2.4 fold-over-basal) cells (Fig 5.2).

The failure of the mGlu5a receptor to elicit an agonist-stimulated JNK response in CHO-lac cells was then investigated further. It is already known that the mGlu5a receptor undergoes cyclical desensitization/re-sensitization by phosphorylation of the receptor at a critical residue by PKC (Kawabata et al. 1998). As discussed later, a time-course of JNK activation in CHO-lac-mGlu1a expressing cells reveals that the profile of activation is much more delayed in onset and is more prolonged than the transient ERK activation observed by receptor stimulation in these cells. Therefore, a testable hypothesis was proposed: could the rapid cyclical desensitization of the mGlu5a receptor prevent this receptor from eliciting a prolonged activation, necessary for maximal JNK activation in these cells? To address this question, chronic phorbol ester treatment was used to down-regulate PKC isozymes in an attempt to limit desensitization of the receptor via PKC and therefore ascertain whether under these conditions, the mGlu5a receptor was now able to elicit a JNK response in these cells. Fig. 5.3 demonstrates that even under conditions of PKC down-regulation, the mGlu5a receptor is incapable of coupling to the activation of JNK in CHO cells. As no significant activation of this cascade by the mGlu5a receptor could be detected, only further investigations into the coupling of the mGlu1a receptor to JNK were carried out.
Figure 5.1 Concentration-dependencies of agonist-stimulated JNK activation in CHO-lac-mGlu1a and -mGlu5a cells. A and B show JNK activation in mGlu1a- and mGlu5a-expressing CHO-lac cell-lines, respectively. Cells were pre-incubated with GPT/pyruvate before stimulation with various concentrations of quisqualate indicated for 30 min and then assayed for JNK activity using GST-c-Jun as a substrate as described in Materials and Methods (Section 2.7). Representative autoradiograms of c-Jun phosphorylation detected after exposure to autoradiography film (24-48 h) are shown in lower panels and specific activity of JNK activity determined by quantification of this phosphorylation are shown in upper panels. Data are shown as means ± SEM for 2-4 separate experiments.
Figure 5.2 Anisomycin-stimulated JNK activation in CHO-lac-mGlul1a and -mGlul5a cells. A and B, cells were pre-incubated with GPT/pyruvate before stimulation with anisomycin for 30 min and then assayed for JNK activity using GST-c-Jun as a substrate as described in Materials and Methods (Section 2.7). Representative autoradiograms of c-Jun phosphorylation detected after exposure to autoradiography film (24-48 h) are shown in lower panels and specific activity of JNK activity determined by quantification of this phosphorylation are shown in upper panels. Data are shown as means ± SEM for 3 separate experiments.
**Figure 5.3** Effects chronic phorbol ester treatment on agonist-stimulated JNK activation in CHO-lac-mGlu5a cells. Cells were pre-treated with PDBu (1 μM for 24 h) prior to experimentation. Cells were pre-treated with GPT/pyruvate prior to stimulation with quisqualate (10 μM), added to vehicle or PDBu-pretreated CHO-lac-mGlu5a cells for 30 or 120 min. Cell lysates were assayed for JNK activity using GST-c-Jun as a substrate as described in *Materials and Methods* (Section 2.7). Representative autoradiograms of c-Jun phosphorylation detected after exposure to autoradiography film (24-48 h) are shown in lower panels and specific activity of JNK activity determined by quantification of this phosphorylation are shown in upper panels. Data are shown as means ± SEM for 2 separate experiments.
In CHO-lac-mGlu1a cells, analysis of the time-course of JNK activation by the mGlu1a receptor in response to quisqualate (10 μM) stimulation revealed that a maximal increase in JNK activity (4.2 ± 0.3 fold-over-basal) occurred at 30 min after agonist addition and declined towards basal at 4 h (Fig. 5.4A). No change in JNK1/2 expression (46 and 55 KDa, respectively) was observed over the time-course of the experiment (Fig. 5.4C), strongly suggesting that enzyme activation, rather than induction accounts for the observed agonist-stimulated changes in JNK activity.

The m3 muscarinic acetylcholine (mACH) receptor has also been shown to stimulate a robust increase in JNK activation in CHO cells (Wylie et al. 1999; Burdon et al. 2002). The magnitude of the mGlu1a receptor-stimulated JNK response was compared to that seen in CHO-m3 cells stimulated with carbachol. The m3 mACH receptor activated JNK with a similar time-course, with peak responses at 45 min following agonist addition, however, the magnitude of the peak response was greater (9.4 ± 1.0 fold-over-basal) than that seen in CHO-lac-mGlu1a cells (Fig. 5.5). Analysis of concentration-response curves for m3-receptor-mediated JNK activation revealed EC$_{50}$ values of 0.5μM (pEC$_{50}$, 6.3 ± 0.3).

5.2 PTx sensitivity of the mGlu1a receptor-mediated JNK response

In Chapter 4, analysis of the G protein populations involved in mGlu receptor coupling to ERK, using PTx pre-treatment revealed that the mGlu1a, but not the mGlu5a receptor-mediated response was significantly attenuated by this treatment and therefore indicated that $G_{i/o}$ family G proteins were important in the mechanism of activation of the ERK cascade by the mGlu1a receptor. Similarly, the mGlu1a receptor-mediated JNK response was examined for sensitivity to PTx. Pre-incubation of CHO-lac-mGlu1a cells with PTx (100 ng ml$^{-1}$, 24 h) revealed that increases in quisqualate-stimulated JNK activation were unaffected by this pre-treatment (Fig. 5.4B). Thus, in marked contrast to ERK activation by the same receptor, $G_{i/o}$ proteins are not involved in receptor coupling to the JNK cascade.
Figure 5.4 Time-course and PTx sensitivity of agonist-stimulated JNK activity in CHO-lac-mGlu1a cells. Cells were pre-incubated with GPT/pyruvate before stimulation with 10 μM quisqualate, in the absence (A) and presence (B) of PTx (100 ng ml⁻¹) or vehicle for 24 h, for the times indicated and then assayed for JNK activity using GST-c-Jun as a substrate as described in Materials and Methods (Section 2.7). Representative autoradiograms of c-Jun phosphorylation detected after exposure to autoradiography film (24-48 h) are shown in lower panels and specific activity of JNK activity determined by quantification of this phosphorylation are shown in upper panels. Data are shown as means ± SEM for 2-4 separate experiments performed in duplicate C shows a representative immunoblot of JNK expression. Lysates were analyzed via 12% SDS-PAGE as described under Materials and Methods (Section 2.10) and probed with an anti-JNK antibody (see Table 2.1). Representative immunoblot from 2 separate experiments is shown. C; control/basal cells, or +quisqualate for 10-240 min.
Figure 5.5 Concentration- and time-dependencies of agonist-stimulated JNK activation in CHO-m3 cells. A and B show a time-course and concentration-response curve of carbachol stimulated JNK activation in CHO-m3 cells, respectively. A, cells were stimulated with various concentrations of carbachol for 45 min and then assayed for JNK activity using GST-c-Jun as a substrate as described in the Materials and Methods (Section 2.7). Representative autoradiograms of c-Jun phosphorylation detected after exposure to autoradiography film (24-48 h) are shown in lower panels and specific activity of JNK activity determined by quantification of this phosphorylation are shown in upper panels. B, Cells were stimulated with 10 μM carbachol for the times indicated and assayed for JNK activity as described above. Data are shown as means ± SEM for 2-4 separate experiments.
5.3 Specificity of the relationship between the mGlu1a receptor and JNK activity

To demonstrate that the observed increases in c-Jun phosphorylation were JNK-mediated, the effects of the JNK inhibitor SP600125 (Bennett et al. 2001) on mGlu1a receptor-mediated JNK responses were assessed. Cells were pre-treated with various concentrations of SP600125 (Fig. 5.6A) prior to stimulation with quisqualate (10 µM). SP600125 completely inhibited agonist-stimulated JNK responses with an IC$_{50}$ of 1.86 µM (pIC$_{50}$, 5.7 ± 0.4). It should be noted that recent reports have questioned the specificity of SP600125 in inhibiting JNK, as it has also been shown to affect several other kinases with similar or greater potency (Bain et al. 2003). Although, the protein kinases reported to be inhibited by SP600125, in addition to JNK, are unlikely to be involved in mGlu receptor-mediated JNK responses, this study highlights the use of pharmacological inhibitors at concentrations at which they become non-selective to dissect signalling mechanisms. In this investigation, the compound was used to determine that the detected increases in activity were due to JNK and was not used to draw conclusions about any involvement of JNK in downstream signalling events.

To confirm the receptor-dependency of JNK activation, the effects of the competitive mGlu1 receptor-selective antagonist LY367385 (Schoepp et al. 1999) on agonist-stimulated JNK response in CHO-lac-mGlu1a cells were also examined (Fig. 5.6B). The mGlu1 receptor antagonist markedly attenuated quisqualate-stimulated JNK activity, but was without effect on JNK responses elicited by anisomycin. These data demonstrate that increases in c-Jun phosphorylation are wholly attributable to JNK activation and this increase in activity is conditional on mGlu1a receptor activation.

5.4 Investigating the Ca$^{2+}$-dependence and involvement of protein kinase C in mGlu1a receptor-mediated JNK activation

To further investigate the signalling mechanism of mGlu1a receptor coupling to JNK, the responses were then examined for sensitivity to calcium manipulations, and down-regulation of PKC, both of which have been shown to influence JNK activation in a stimulatory and inhibitory manner in other systems (Ko et al. 1998; Schwarzschild et al. 1999).
Figure 5.6 Effects of the JNK inhibitor SP600125 and the mGlul receptor-specific antagonist LY367385 on agonist-stimulated JNK activity in CHO-lac-mGlul1a cells. A, cells were pre-incubated with GPT/pyruvate before treatment with the various concentrations of SP600125 (0-100 pM) for 30 min prior to addition of quisqualate (10 μM) for 30 min. Cells were then assayed JNK activity using GST-c-Jun as a substrate as described in Materials and Methods (Section 2.7). Representative autoradiograms of c-Jun phosphorylation detected after exposure to autoradiography film (24-48h) are shown in lower panels and specific activity of JNK activity determined by quantification of this phosphorylation are shown in upper panels. B, cells were pre-incubated with GPT/pyruvate before treatment with LY367385 (100 μM) for 30 min prior to addition of various concentrations of quisqualate (0-10 μM) for 30 min. In addition, the effect of the mGlul1a receptor antagonist on anisomycin (50 ng ml⁻¹, 30min) - stimulated JNK activity was also assessed. Cells were then assayed for JNK activity as above. Data are shown as means ± SEM for 2-4 separate experiments.
Figure 5.7 Ca\(^{2+}\) dependency of agonist-stimulated JNK activation in CHO-lac-mGlu1a cells. Experiments were performed in normal KHB (1.3 mM Ca\(^{2+}\)), or under conditions where extracellular Ca\(^{2+}\) (-[Ca\(^{2+}\)]\(_o\)), or both extracellular and intracellular Ca\(^{2+}\) (-[Ca\(^{2+}\)]\(_o\) + Thaps) were depleted prior to agonist challenge (cells were additionally GPT/pyruvate treated as normal). Extracellular Ca\(^{2+}\) depletion was achieved by washing cells before the experiment in Ca\(^{2+}\)-free KHB supplemented with 100 \(\mu\)M EGTA. For intracellular Ca\(^{2+}\) depletion 2 \(\mu\)M thapsigargin was added under -[Ca\(^{2+}\)]\(_o\) conditions for 15 min prior to agonist addition. Cells stimulated with quisqualate (10 \(\mu\)M) for 30 or 120 min and then assayed for JNK activity using GST-c-Jun as a substrate as described in Materials and Methods (Section 2.7). Representative autoradiograms of c-Jun phosphorylation detected after exposure to autoradiography film (24-48 h) are shown in lower panels and specific activity of JNK activity determined by quantification of this phosphorylation are shown in upper panels. Data are shown as means ± SEM for 3 separate experiments.
Figure 5.8 Effects of PKC down-regulation by phorbol ester pretreatment on agonist-stimulated JNK activation in CHO-lac-mGlu1a cells. Cells were pre-treated with phorbol ester (1 μM PDBu for 24 h) prior to GPT/pyruvate treatment and subsequently stimulated with quisqualate (10 μM) for 0, 30 or 120 min and then assayed for JNK activity using GST-c-Jun as a substrate as described in Materials and Methods (Section 2.7). Representative autoradiograms of c-Jun phosphorylation detected after exposure to autoradiography film (24-48 h) are shown in lower panels and specific activity of JNK activity determined by quantification of this phosphorylation are shown in upper panels. Data are shown as means ± SEM for 3 separate experiments.
Reducing extracellular Ca\(^{2+}\) from 1.3 mM to approx. 100 nM, by incubation of CHO-lac-mGlula cells in Ca\(^{2+}\)-free KHB containing EGTA (100 μM), had no effect on quisqualate-stimulated JNK activation (Fig. 5.7). A requirement for intact intracellular Ca\(^{2+}\) stores was also excluded, as pre-addition of the sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase inhibitor thapsigargin (2 μM) to cells under Ca\(^{2+}\)-free conditions also had no effect on mGlula receptor-mediated JNK activation (Fig. 5.7). These data demonstrate that JNK activation by the mGlula receptor occurs via Ca\(^{2+}\)-independent mechanisms in CHO cells.

To investigate the possible role of PKC in mGlula receptor-mediated JNK activation a chronic down-regulation strategy was adopted. Pre-treating cells with PDBu (1 μM) for 24 h, to markedly reduce, both classical (PKCα) and novel (PKCδ, PKCε) isozyme expression (Hill et al. 2003), did not affect quisqualate-stimulated JNK responses in CHO-lac-mGlula cells (Fig. 5.8). This manipulation suggests that these PKC isozymes are not required for mGlula receptor-JNK signalling.

### 5.5 Investigating involvement of PI3 kinase and the non-receptor tyrosine kinase Src in mGlula1a receptor-mediated JNK activation

In addition we also investigated the possible role of PI3-kinase in JNK activation by the mGlula1a receptor using the PI3-kinase inhibitor wortmannin (Fig. 5.9). This kinase has been shown to activate JNK activity (Klippel et al. 1996), but agonist-stimulated JNK responses mediated by the mGlula1a receptor were not affected by wortmannin pre-treatment suggesting that PI3-kinase is not involved in signalling to JNK in this system.

As Src kinase was found to be important in receptor-mediated ERK activation in CHO-lac-mGlula1a and -mGlu5a cells, we used the same Src family kinase inhibitor PP1 to investigate whether this subset of non-receptor tyrosine kinases can be implicated in the mGlula1a receptor-JNK signaling pathway. PP1 (5 μM) at a concentration previously shown to attenuate markedly agonist-stimulated ERK responses did not affect quisqualate-stimulated JNK activation (Fig. 5.10). Therefore, a role for Src family kinases in this signaling pathway is unlikely. In addition, the PDGF receptor tyrosine kinase inhibitor, AG1296, which
Figure 5.9 Effects of the PI3-kinase inhibitor wortmannin on agonist-stimulated JNK responses in CHO-lac-mGlu1a cells. Cells were pre-incubated with GPT/pyruvate before treatment with wortmannin (100 nM) prior to addition of quisqualate for 0, 30 or 120 min and then assayed for JNK activity using GST-c-Jun as a substrate as described in Materials and Methods (Section 2.7). Representative autoradiograms of c-Jun phosphorylation detected after exposure to autoradiography film (24-48 h) are shown in lower panels and specific activity of JNK activity determined by quantification of this phosphorylation are shown in upper panels. Data are shown as means ± SEM for 2 separate experiments.
Figure 5.10 Effects of Src family kinase inhibitor PP1 on agonist-stimulated JNK responses in CHO-lac-mGlu1a cells. Cells were pre-incubated with GPT/pyruvate before treatment with PP1 (5 µM) for 30 min prior to addition of quisqualate (10 µM) for 0, 30 or 120 min and then assayed for JNK activity using GST-c-Jun as a substrate as described in Materials and Methods (Section 2.7). Representative autoradiograms of c-Jun phosphorylation detected after exposure to autoradiography film (24-48 h) are shown in lower panels and specific activity of JNK activity determined by quantification of this phosphorylation are shown in upper panels. Data are shown as means ± SEM for 2 separate experiments performed in duplicate.
**Figure 5.11** Effects of the PDGF receptor tyrosine kinase inhibitor AG1296 on agonist-stimulated JNK activation in CHO-lac-mGlu1a cells. Cells were pre-incubated with GPT/pyruvate before treatment with AG1296 (10 μM) for 20 min prior to addition of quisqualate (10 μM) for 0, 30 or 120 min and then assayed for JNK activity using GST-c-Jun as a substrate as described in *Materials and Methods* (Section 2.7). Representative autoradiograms of c-Jun phosphorylation detected after exposure to autoradiography film (24-48 h) are shown in lower panels and specific activity of JNK activity determined by quantification of this phosphorylation are shown in upper panels Data are shown as means ± SEM for 2 separate experiments.
markedly attenuated mGlu1a, but not mGlu5a receptor-mediated ERK activation was also used to examine whether this growth factor receptor might play a role in signaling to the activation of JNK. At a concentration of AG1296 that significantly attenuated both PDGF and quisqualate-stimulated ERK activities in CHO-lac-mGlu1a cells, there was no such inhibition on JNK responses elicited by the same receptor.

5.6 JNK activation by the mGlu1a receptor is not downstream of ERK activation
Finally, we investigated whether JNK activation by the mGlu1a receptor in these cells was a consequence of ERK activation. To determine whether ERK was involved, we used U0126, an inhibitor of MEK1/2, which are kinases upstream of ERK. U0126 (1 μM) did not attenuate agonist-stimulated increases in JNK activation (Fig. 5.12). The time-points chosen to investigate these effects enabled both JNK and ERK activities to be examined as the time-course of peak activation of these two kinases by the mGlu1a receptor differ. Therefore, to confirm that ERK activity was inhibited under these experimental conditions, cell lysates generated for examining JNK activity above were assessed for ERK activity using a phospho-ERK1/2-specific antibody. Immunoblot analysis revealed the increase in ERK activation (4.5 ± 0.9-fold over basal) 10 min after quisqualate addition was abolished by pre-treatment with U0126 (Fig 5.12B). These results demonstrate that the activation of JNK by the mGlu1a receptor is not simply a consequence of prior ERK activation by the same receptor. This is further supported by the previous data shown that demonstrate mGlu1a receptor-mediated JNK activation has no requirement for G_{i/o} family proteins, as determined by insensitivity to PTx, and Src kinase, both of which we have previously shown to be important for ERK activation by this receptor in these cells.

5.7 Investigating the activation of p38 MAP kinase by mGlu1a and mGlu5a receptors
The activation of the p38 MAP kinase by mGlu receptor activation was also explored. The activation of this kinase by quisqualate, and indeed anisomycin, a potent activator of p38 activity (Cano et al. 1994), in CHO-lac-mGlu1a and -mGlu5a cells proved to be variable due to inconsistencies in detecting any activation over basal levels. Out of five separate
Figure 5.12 Effects of the MEK inhibitor U0126 on agonist-stimulated JNK and ERK activation in CHO-lac-mGlu1a cells. A, cells were pre-incubated with GPT/pyruvate and then treated with U0126 (1 μM) for 30 min prior to agonist addition with 10 μM quisqualate for 0-60 min. In addition, the effect of the MEK inhibitor on anisomycin-stimulated JNK activity (50 ng ml⁻¹, 30 min) was also assessed. Cells were then assayed for JNK activity using GST-c-Jun as a substrate as described in Materials and Methods (Section 2.7). Representative autoradiograms of c-Jun phosphorylation detected after exposure to autoradiography film (24-48 h) are shown in lower panels and specific activity of JNK activity determined by quantification of this phosphorylation are shown in upper panels. Data are shown as means ± SEM for 3 separate experiments. B, whole cell lysates of the cells stimulated above were also analyzed for ERK activation in the absence and presence of U0126. Lysates were analyzed via 12% SDS-PAGE as described under Materials and Methods (Section 2.10) and probed with an antibody that recognizes the phosphorylated form of ERK1/2 (pERK, see Table 2.1). Representative immunoblots of ERK activation from 2 separate experiments are shown. C; control/basal cells, or +quisqualate for 10, 30 or 60 min.
experiments performed, only two showed a significant increase in levels of p38 activity in response to both mGlu receptor stimulation and to anisomycin.

From these two experiments, analysis of the time-course of p38 activation by the mGlu1a and mGlu5a receptor in response to quisqualate (10 μM) stimulation revealed that a maximal increase in p38 activity (9.7 ± 1.1 and 4.6 ± 0.9-fold-over-basal, respectively) occurred at 5 min after agonist addition and declined towards basal at 60 min (Fig. 5.13A and B). The levels of basal activity in CHO-lac-mGlu5a cells were approx. twice those detected in CHO-lac-mGlu1a cells. Anisomycin stimulated a robust, comparable activation of JNK activity in both CHO-lac-mGlu1a (18.2 ± 3.0 fold-over-basal) and CHO-lac-mGlu5a (17.7 ± 2.8 fold-over-basal) cells (Fig 5.13A and B, inset). The increases in p38 activity could not be attributed to any change in p38 expression, as immunoblot analysis demonstrated that total p38 protein expression did not change over the time-course of the experiment (Fig. 5.13C and D).

Others in the laboratory (Burdon D., unpublished results) have similarly found that both the m2 and the m3 mACh receptors mediate a carbachol-stimulated increase in p38 in CHO cells, which peaks at 5 min after agonist addition. Neither receptor however, appeared to be efficiently coupled to p38 activation and similar problems were encountered with consistency of data and measurement of activities over basal.
Figure 5.13 Time-course of agonist-stimulated p38 activity in CHO-lac-mGlu1a and -mGlu5a cells. Cells were pre-incubated with GPT/pyruvate before stimulation with 10 μM quisqualate for the times indicated and then assayed for p38 activity using GST-ATF2 as a substrate as described in Materials and Methods (Section 2.8). In addition, p38 activity stimulated by the addition of anisomycin (50 ng ml⁻¹, 30 min) was also assessed. Representative autoradiograms of ATF2 phosphorylation detected after exposure to autoradiography film (48 h) are shown in lower panels and specific activity of p38 activity determined by quantification of this phosphorylation are shown in upper panels. Data are shown as means ± SEM for 2 separate experiments. C shows a representative immunoblot of p38 expression in the mGlu expressing cell lines. Lysates were analyzed via 12% SDS-PAGE as described under Materials and Methods (Section 2.10) and probed with an anti-p38 antibody (see Table 2.1). Representative immunoblot from 2 separate experiments is shown. C; control/basal cells, or +quisqualate for 5-120 min, A; anisomycin.
Discussion

The activation of stress-activated MAP kinase pathways by application of glutamate is well documented (Ko et al. 1998; Schwarzchild et al. 1997, 1999; Mukherjee et al. 1999), and this activation has been attributed to the NMDA-receptor subgroup of ionotropic glutamate receptors. A recent study has also demonstrated divergent regulation of JNK and ERK signalling by ionotropic glutamate receptors via PI3-kinase and Src in neurones (Crossthwaite et al. 2004). However, the specific activation of mGlu receptor-mediated stress-activated MAP kinase activation has not yet been explored. There are reports in the literature of JNK activation in studies where mGlu receptor mediated ERK responses were primarily being investigated. For example, Schinkmann and co-workers (2000) reported a consistent, but modest increase in JNK activity in response to glutamate in primary astrocytes, but did not explore the subgroup of glutamate receptor, ionotropic or metabotropic, which might be involved. Mukherjee et al. (1999) have shown an element of cross-talk between mGlu receptors and the activation of JNK by NMDA receptors in hippocampal neurones. Activation of NMDA receptors by platelet-activating factor (PAF), a retrograde messenger of LTP that enhances glutamate release, was shown to mediate NMDA-receptor increases in JNK activities that were attenuated by activation of mGlu receptors. However, the subtype of mGlu receptor involved was not identified as the mixed mGlu group I and II antagonist MCPG (alpha-methyl-4-carboxyphenylglycine) was used.

In the present Chapter, the data presented demonstrate that in our CHO-lac cells, group I mGlu receptor stimulation can modulate JNK and p38 MAP kinase activities in response to agonist. Both, mGlu receptors (Nakanishi et al. 1998; Martin et al. 2000; Lanneau et al. 2002; Ziemsinska et al. 2003) and JNK (Manning & Davies 2003) have been implicated in neurodegenerative diseases and the finding that an mGlu receptor can activate pathways associated with cell death and apoptosis may have important implications for the role of these receptors in mediating these events in native cells. Further, we found that only the mGlu1a elicited a JNK response in these cells. This clear difference in signalling between these two receptors may also be important in native cells.
Levels of JNK activation displayed by the mGlu1a receptor were quite modest compared to another PLC-linked receptor, the m3 muscarinic acetylcholine receptor (mAChR). Like, group I mGlu receptors, the m1, m3 and m5 mAChRs are primarily coupled to phosphoinositide hydrolysis via PTx-insensitive G\textsubscript{q/11} family G-proteins, whereas the m2 and m4 mAChRs are coupled to the activation of potassium channels and the inhibition of adenylyl cyclase via PTx-sensitive G-proteins (Caulfield, 1993). When stably expressed in CHO cells, the m3 receptor elicits a robust agonist-stimulated JNK activation that has been reported to be approximately 10-20 fold over basal, whereas the m2 receptor fails to significantly stimulate JNK activity (Wylie \textit{et al} 1999; Burdon \textit{et al}. 2002). This difference in the ability of these two mAChRs to elicit a JNK response in response to agonist challenge is believed to be attributed to differences in G-protein coupling between these two receptors.

The m3 mAChR-mediated JNK activation, like the mGlu1a receptor-mediated JNK activation in CHO-lac cells, is delayed in onset with respect to ERK activation (peaks approx. 45min after agonist addition) and has a prolonged time-course, with levels of activity returning to basal after approx. 4 hours. However, unlike the mGlu1 receptor response, the activation was shown to be Ca\textsuperscript{2+} sensitive and partially attenuated by PTx pre-treatment (Wylie \textit{et al}. 1999). The variation in time-course between the transient ERK activation and sustained JNK activation in response to agonist may be important in the modulation of different intracellular signalling pathways, and in particular, this may be of significance in the central nervous system where differences in the temporal activation of mitogenic cascades by the same receptor may elicit either cell survival or cell death events. Conversely, this may allow different cells to respond to the same cellular stress in different ways (Harper & LoGrasso, 2001).

The possible intracellular coupling of the mGlu1a receptor to the activation of the JNK cascade was assessed using variety of pharmacological inhibitors. An initial assessment revealed a lack of involvement of G\textsubscript{i/o} family G-proteins in the mGlu1a receptor-mediated JNK activation in stark contrast to ERK activation by this receptor that was essentially abolished by PTx pre-treatment. The m3 mAChR-mediated ERK and JNK activities have both been shown to have major PTx-sensitive components in CHO-m3 cells (Wylie \textit{et al}.
1999), whereas like the mGlu mediated response in these cells, coupling to PLC is PTx-
insensitive.

These data imply that the mGlu1a receptor couples to distinct G protein populations to
activate phosphoinositide metabolism, ERK activation and to couple to JNK activation. It is
not known, if another subgroup of PTx-insensitive G proteins, such as the G_{12/13} subfamily,
mediates the actions of the mGlu1a receptor on JNK activation, or whether G protein-
independent mechanisms are involved (possible Rho GTPase family involvement, see later).
The G_{12} proteins have been shown to be upstream of JNK activation in HEK cells by the use
of a constitutively active mutant of G_{12} (Nagao et al. 1996), but it remains to be established
whether mGlu receptors can couple to this class of G proteins.

If the mGlu1a receptor were coupling through G_{q/11} G proteins to activate JNK, then it would
be feasible to suggest this coupling would activate PLC activity and the downstream
signalling events of this activation might influence subsequent JNK responses. The effects of
Ca^{2+} and PKC, both activated downstream of phosphoinositide hydrolysis by mGlu receptor
activation were therefore determined. Similar to ERK activation by the mGlu1a receptor,
manipulating intracellular/extracellular Ca^{2+} concentrations was without effect on receptor-
stimulated JNK responses. Further, down-regulation of PKC via chronic phorbol pre-
treatment did not affect JNK activation by the mGlu1a receptor. The literature surrounding
the dependence of JNK activation upon calcium and PKC is varied and appears to be highly
cell-type dependent. Whilst some reports reveal that calcium is stimulatory and necessary for
JNK activation (Zohn et al. 1995; Ko et al. 1998), others have shown that increasing calcium
concentrations have a negative effect on JNK responses. Indeed Schwarzschild et al. (1999)
reported that in cultured striatal neurones, increasing calcium has a stimulatory effect on ERK
activation, but an inhibitory effect on JNK activation elicited by the NMDA receptor.
Cadwallader and colleagues (1997) have also shown that lowering intracellular Ca^{2+} levels
actually accelerates the rate of onset of JNK activation and potentiates and prolongs the
maximal JNK response elicited by endothelin in Rat-1 cells. The same effects were observed
with inhibition of PKC. In contrast, Wylie et al. (1999) have shown that the m3 mACH
receptor-mediated JNK response in CHO cells is markedly attenuated by PKC down-

regulation by phorbol esters suggesting a stimulatory role for PKC in this pathway. In this study however, inhibition of increases in Ca\(^{2+}\) or PKC activity in mGlu1a receptor-expressing cells had neither an inhibitory or stimulatory effect on agonist-stimulated JNK activity, implying that these components are not involved in the signalling mechanism upstream of JNK activation by the mGlu1a receptor in these cells.

The mGlu1a receptor-mediated JNK response was then examined for sensitivity to wortmannin, a specific inhibitor of PI3 kinase. This kinase has been shown to mediate JNK activation by the m2 mACh receptor expressed in COS-7 cells (Lopez-Ilasaca et al. 1998) and by over-expression of the catalytic p110 subunit of the PI3 kinase, in the same cell type (Klippel et al. 1996). It is also thought that PI3 kinase may serve as an intermediate between Ras, which binds to and activates p110 (Rodriguez-Viciana et al. 1994), and the activation of Rac, a member of the Rho family of small GTPases that are activators of JNK signalling (see later). It is worth noting that following wortmannin pre-treatment, there was no observed increase in basal JNK activity, which has been reported previously (Kharbanda et al. 1995; Wylie et al. 1999).

In the previous Chapter, the tyrosine kinase Src was shown to have a role in both mGlu1a and mGlu5a receptor-mediated ERK activity by use of the specific inhibitor, PP1. Src kinase has also been implicated in the activation of JNK by several groups, in different cell types (Nagao et al. 1999; Yoshizumi et al. 2000; Krauss et al. 2003). This involvement may be downstream of receptor activation or as Yoshizumi and co-workers (2000) examined, receptor independent, as Src kinase was found to be involved in JNK activation mediated by reactive oxygen species in vascular smooth muscle cells, mouse embryo fibroblasts and in CHO cells. Recently, Miyamoto et al. (2003) have identified a novel guanine nucleotide exchange factor, that they termed FRG (FGD1-related Cdc42-GEF), for the small Cdc42 GTPase, that was directly phosphorylated and activated by Src kinase downstream of the endothelin-A receptor leading to JNK activation in HEK cells.

Using the Src inhibitor PP1 to elucidate a role for Src kinase in mGlu1a receptor-JNK activation, we found no attenuation of the maximal response using this inhibitor suggesting
that Src is not upstream of JNK activation by the mGlu1a receptor in these cells. Krauss and colleagues (2003) have shown that Src and EGF-receptor transactivation are both important in mediating ERK and JNK activation by the GnRH receptor in COS-7 cells. In this case, JNK activation required the EGF receptor, Go, Src, PI3 kinase, and was PKC-independent. ERK activation similarly involved the EGF receptor and Src, but required Ras activation with only minor roles for Gβγ subunits and β arrestin.

The PDGF receptor tyrosine kinase inhibitor, AG1296, attenuated mGlu1a receptor-mediated ERK activation, suggesting that transactivation of the PDGF receptor might be involved in the coupling mechanism to this pathway. We therefore investigated whether the JNK responses elicited by this receptor were similarly sensitive to this compound. JNK activation by this receptor was insensitive to this inhibition, suggesting that although ERK and JNK MAP kinase cascades often involve similar second messengers upstream of their activation, the pathways mediating ERK and JNK activation by the mGlu1a receptor in CHO cells are divergent.

To ascertain that these two pathways, activated by the mGlu1a receptor are divergent, and to confirm that the delayed onset of JNK activation by the mGlu1a receptor is not a result of earlier ERK activation, ERK activity was inhibited by using the MEK inhibitor U0126. There is evidence that cross-talk between ERK and JNK pathways can occur. Shen and colleagues (2003) have recently demonstrated a unique mechanism of negative cross-talk between JNK and ERK pathways in COS-7 cells. Sustained JNK activation in these cells was shown to uncouple ERK activation from upstream activation by MEK-1 through a mechanism involving active MLK3 and requiring Jun-mediated gene transcription. Pedram et al. (1998) have also shown ERK/JNK cross-talk where ERK activation by the VEGF receptor is a necessary prerequisite for subsequent JNK activation in bovine aortic endothelial cells. JNK activation was inhibited by the MEK1 inhibitor PD98059, and attenuated by transfection of cells with dominant-negative ERK2. Using U0126 pre-treatment, we found no attenuation of the mGlu1a receptor-mediated JNK activation under conditions where ERK activation by the same receptor was significantly inhibited. These data clearly demonstrate that JNK activation by the mGlu1a receptor in these cells is not simply downstream of ERK activation and
confirm that the signaling pathways to these two MAPKs by the mGlu1a receptor are divergent. Similarly, Schwarzschild et al. (1999) showed that NMDA receptor-mediated ERK and JNK activation in cultured striatal neurons had quite different ionic dependencies and sensitivities to Ca\textsuperscript{2+} suggesting that the mechanisms of modulation of these two pathways by the NMDA receptor are also quite distinct.

The present data, has not allowed us to establish the manner in which the mGlu1a receptor couples to the activation of the JNK cascade, although a number of common second messengers associated with signaling from GPCRs and tyrosine kinase receptors to the activation of MAP kinases in the activation of JNK, including Ca\textsuperscript{2+}, PKC, PI3-kinase and Src kinase have been eliminated (Fig. 5.14). However, there remains much to elucidate in the mechanism of this activation. The possible coupling mechanism between mGlu1a/JNK can only be speculated, but there are likely candidates that remain to be explored.

Firstly, the small GTP binding proteins of the Rho family, Rac and Cdc42 are known to be activators of the JNK pathway (Kyriakis & Avruch, 1996; Teramoto et al. 1996; Coso et al. 1996). Little is known about how these GTPases are recruited to receptors or indeed how they regulate the activation of JNK cascades owing to the diverse nature of pathways upstream of this MAP kinase. The p21-activated kinases (PAKs) are thought to serve an intermediary role in coupling Rac/Cdc42 to JNK. It is possible that members of this family may mediate the mGlu1a/JNK coupling but as yet there is no evidence that mGlu receptors are able to couple to the activation of these small GTPases in addition to heterotrimeric G proteins.

Scaffolding components have also been shown to be required for JNK MAP kinase activation. These not only arrange members of a cascade to allow specificity but also provide a potential mechanism of activation that may bypass common second messenger intermediates.

The JNK interacting proteins (JIPs) act as molecular scaffolds that organize JNK signal transduction cascades and act to increase the efficiency of signalling by concentrating the components of the pathway (Davis, 2000). β-arrestin has also been shown to be an important
Figure 5.14 Summary of mGlu receptor-mediated activation of JNK and p38 MAP kinase. In the CHO-lac cell-lines, the heterologously expressed group I mGlu receptors differ in their ability to activate JNK MAP kinase. The mGlu1a, but not the mGlu5a receptor activates JNK, although the exact mechanism upstream of coupling to the JNK cascade is presently unknown. The signaling profile however has been examined for Ca$^{2+}$ sensitivity and involvement of PKC, PI3 kinase, Src kinase and the PDGF receptor. None of these classical MAPK signaling intermediates were found to be involved in mGlu1a receptor-mediated activation of JNK. Preliminary data also showed that both mGlu receptors may be able to elicit p38 MAP kinase activation in these cells. The signaling profile to p38 MAP kinase was not investigated.
scaffold in mediating the spatial organization of receptor activation of ERK and more recently, JNK3 (McDonald et al. 2000; Manning & Davis, 2003). Unlike, JIPs the formation of the β-arrestin/JNK module is regulated by agonist stimulation of GPCRs. GRK dependent phosphorylation of an activated receptor promotes binding of β-arrestin along with components of the MAPK module, thereby facilitating receptor internalisation and co-localization to efficiently activate the MAPK involved.

It may be speculated that formation of such a complex, composed of a scaffolding protein such as JIP or β-arrestin, and the necessary components of a MAPK module might bind to an activated receptor, providing a G-protein independent mechanism of JNK activation. Although such an interaction poses a tempting proposal for a mechanism of JNK activation by the mGlu1a receptor, further detailed studies will need to establish if such direct recruitment to a receptor might occur and whether scaffolds such as JIP or β-arrestin act to spatially localize JNK components downstream of mGlu receptors.

Preliminary data also showed that both mGlu1a and mGlu5a receptors could couple to the activation of p38 MAP kinase. The time-course of this activation was similar to that of the ERK response mediated by these receptors, rather than the delayed onset of JNK activation displayed by the mGlu1a receptor. Bolshakov and colleagues (2000) have demonstrated that mGlu receptors mediate induction of LTD at CA3-CA1 synapses in the hippocampus, and that the mechanism involves activation of p38 MAP kinase. The exact subgroup of mGlu receptors involved however was not determined as the mixed group I/II antagonist MCPG was used. Rush et al. (2002) have similarly demonstrated that mGlu receptor-mediated LTD requires p38 activation in the rat dentate gyrus, but this form of LTD is dependent upon ‘previous’ mGlu receptor activation and subsequent activation of PKC. These reports clearly indicate an important role for p38 MAP kinase activation by mGlu receptors in native cells and the present data highlight the potential for group I mGlu receptors in mediating these events. Further investigations will be needed to confirm the activation of p38 by mGlu1a and mGlu5a receptors and indeed to confirm the relatively transient time-course of this activation. Further to this, there is the potential for cross-talk between the activation of p38 and ERK/JNK MAP kinases to allow cells to specifically elicit downstream signaling to targets that result in a specific cellular output. The study by Bolshakov and colleagues (2000) also
highlighted that whilst mGlu receptor-mediated induction of LTD was p38 dependent, the induction of LTP was mediated by ERK. These data demonstrate that two parallel MAP kinase pathways can contribute to opposing cellular effects. Pramanik et al. (2003) have recently demonstrated the potential for differential regulation of transcription factors by different p38 isoforms. Whereas p38β increased activity of the transcription activator protein 1 (AP-1), p38γ and p38δ isoforms had no or inhibitory effects. The effects of p38β were shown to be mediated by increased phosphorylation of c-Jun, whilst effects of p38γ/δ were mediated through regulation of c-Jun transcription. This study shows that different isoforms of this MAP kinase can transmit different signals to activate or inhibit transcription and ultimately the cellular output. Such differential regulation of isoforms by mGlu receptor activation is also a possibility that may lead to transmission of highly specific signals.

A common downstream target of ERK, JNK and p38 MAP kinases is the transcription factor Elk-1, which provides a point of convergence for these pathways (Zinck et al. 1995). Group I mGlu receptors have been shown to activate Elk-1 in striatal neurons (Choe & Wang, 2001) and although the activation of this transcriptional regulator was a consideration for investigation downstream of MAP kinase activation in our CHO-lac model. However, despite detecting high levels of Elk-1 in brain homogenate (used as a positive control) using a commercial antibody, only very low levels of this protein could be detected in CHO cells (data not shown).

Such transcriptional regulation by MAP kinase activation is however an important target downstream of these pathways, as a large number of the targets for ERK, JNK and p38 are indeed transcription factors. In the next Chapter, other functional cellular outputs, such as changes in cell proliferation and cytoskeletal changes in response to group I mGlu receptor stimulation will be examined and an attempt to ascertain what the downstream signalling of MAP kinase activation might be will be discussed.
CHAPTER 6 Investigating growth effects and cytoskeletal changes in CHO cells in response to mGlu receptor activation

Introduction

The activation of MAP kinase cascades are often associated with functional changes in cells including changes in cell morphology and cellular proliferation / anti-proliferation. The regulation of such events by MAPK regulation, allows a variety of extracellular stimuli to mediate long-lasting changes in cells, including protein synthesis and ultimately changes in cell growth. The regulation of cell growth by MAPK signalling occurs by the modulation of key cell-cycle proteins by these cascades, whereas effects on cellular morphology involve coupling of MAPKs to elements of the cytoskeleton. Indeed, the first MAPK (ERK) was identified by its ability to phosphorylate microtubule-associated protein 2 (MAP2), proteins that facilitate polymerisation of components of the cytoskeleton, in response to stimulation with insulin (Ray & Sturgill, 1987). It is now established that all three main subgroups of MAPKs, ERKs, JNKs and p38 MAP kinases are capable of eliciting changes in cell growth and mediation of cell survival and cell death events (Widmann et al. 1999). Coupling of MAPKs to the cytoskeleton also enables coupling to changes in cell structure and organisation without effects on actual cell growth.

Intracellular organisation, motility, and cell morphology is mediated by the cytoskeleton, which is composed of a network of microfilaments and microtubules (Janmey, 1998). Microfilaments are formed from filamentous (f) actin, whereas microtubules are composed of the protein tubulin. At a synaptic level, group I mGlu receptors are generally localized at postsynaptic terminals, where they can be found to interact, via their C-terminus to the scaffolding molecule, Homer. Other cytoskeletal proteins also found in the postsynaptic density include actin and tubulin. Indeed, both of these proteins have been shown to associate with group I mGlu receptors (see later) and are therefore potential targets for mGlu receptor signalling.
The aim of this Chapter was to explore the possible growth effects and changes in cellular morphology on CHO-lac cells by group I mGlu receptor activation and to examine if any such changes correlated with the observed MAPK activation described in Chapters 3-5. Changes in cellular proliferation were determined using $[^{3}\text{H}]$thymidine incorporation and cytoskeletal changes were examined by immunocytochemistry. The data presented shows that although, subtle changes in cell morphology were apparent by activation of both mGlu receptor subtypes, no significant increases or decreases in cellular proliferation were observed. An investigation into the cytoskeletal proteins, tubulin and actin using fluorescence imaging also revealed little effects of (transient) mGlu receptor activation, suggesting that signalling by mGlu1a/mGlu5a receptors does not lead to changes in cell growth in our CHO-lac cells, and it is therefore unlikely that altered cell growth is a cellular output of mGlu receptor-mediated mitogenic signalling in these cells.
6.1 Effects of group I mGlu receptor stimulation on DNA synthesis in CHO-lac cells

The activation of mitogenic signalling pathways is often correlated with growth effects such as increases or decreases in cellular proliferation. To determine whether mGlu receptor stimulation would result in such changes in our CHO-lac cells, changes in DNA synthesis using \[^{3}\text{H}]\text{thymidine incorporation were measured in response to agonist challenge. DNA synthesis was examined over a 24 h time-course of receptor activation in response to quisqualate in both cell lines. Activation of the mGlu1a receptor, in the absence or presence of serum, did not result in any significant increase or decrease in DNA synthesis (Fig. 6.1). Addition of foetal calf serum (FCS, 10%) used as a positive control, resulted in a modest increase in DNA synthesis of 2.4 ± 0.7-fold over basal and 1.9 ± 0.4-fold over basal in serum starved and serum containing CHO-lac-mGlu1a cells, respectively. Similarly, no significant increase/decrease in mGlu receptor stimulated DNA synthesis in CHO-lac-mGlu5a cells could be discerned (Fig. 6.2). FCS stimulated responses were slightly higher in CHO-lac-mGlu5a cells as compared to those in CHO-lac-mGlu1a cells, with an activation of 10.7 ± 1.7-fold over basal in serum replete conditions and 2.5 ± 0.9-fold over basal in the presence of serum. Further, basal levels of DNA synthesis were not attenuated by the addition of the mGlu1a receptor specific antagonist LY367385 in CHO-lac-mGlu1 cells, or with the mGlu5a receptor antagonist MPEP in CHO-lac-mGlu5a cells (data not shown).

In addition to the use of FCS, changes in DNA synthesis in response to agonist challenge were also examined in CHO-m3 and CHO-m2 cells as a positive control. These two receptors have previously been shown to exert opposing effects on growth in CHO cells (Burdon et al. 2002). In serum containing CHO-m3 cells, a marked decrease in DNA synthesis is observed in response to receptor activation by the addition of carbachol (Fig. 6.3A). In contrast, the m2 mACh receptor expressed in CHO cells stimulates a robust increase in DNA synthesis under conditions of serum starvation (Fig. 6.3B). Burdon et al. have demonstrated that significant increases in DNA synthesis (fold-over basal) are not apparent in ‘serum containing’ CHO-m2 cells whereas, a modest inhibition of DNA synthesis can still be discerned in ‘serum starved’ CHO-m3 cells.
**Figure 6.1** DNA synthesis in CHO-mGlul1a cells. Cells were induced (20h, 100 μM IPTG) in the presence (+ serum) or absence (- serum) serum prior to stimulation. Cells were stimulated, in the presence of GPT/pyruvate, with the indicated concentrations of quisqualate (μM) or serum (10% FCS) for 24 h, and incubated with 2 μCi of [³H]thymidine for the final 2 h. An equivalent volume of water was added to un-stimulated cells. DNA synthesis was measured using ether extraction as described in Materials and Methods (Section 2.11). Data are shown as means ± SEM for 4 separate experiments.
Figure 6.2 DNA synthesis in CHO-mGlu5a cells. Cells were induced (20h, 100 μM IPTG +5 mM butyrate) in the presence (+ serum) or absence (- serum) of serum prior to stimulation. Cells were stimulated, in the presence of GPT/pyruvate, with the indicated concentrations of quisqualate (μM) or serum (10% FCS) for 24 h, and incubated with 2 μCi of [3H]thymidine for the final 2 h. An equivalent volume of water was added to un-stimulated cells. DNA synthesis was measured using ether extraction as described in Materials and Methods (Section 2.11). Data are shown as means ± SEM for 4 separate experiments.
Figure 6.3 Concentration dependent effects of carbachol on DNA synthesis in CHO-m3 and -m2 cells. Cells were stimulated for 24 h with the indicated concentrations of carbachol and incubated with 2 μCi of [³H]thymidine for the final 2 h. Control values represent cells not stimulated with carbachol. DNA synthesis was measured using ether extraction as described in Materials and Methods (Section 2.11). A shows effects of carbachol treatment on a growing population of CHO-m3 cells (+ serum). Data are shown as means ± SEM for 4 separate experiments. B shows effects of carbachol treatment on serum starved CHO-m2 cells (- serum). Data are shown as means ± SEM for 2 separate experiments.
As changes in DNA synthesis, as determined by \[^{3}H\]thymidine incorporation, in response to mGlu receptor activation were not detected in either CHO-lac-mGlu1a or -mGlu5a cells it is unlikely that mGlu receptor activation of MAPK signalling in these cells results in changes in cellular proliferation. Therefore, other possible changes in cellular morphology in response to mGlu receptor activation were also examined.

6.2 Investigating the Effects of group I mGlu receptor stimulation on tubulin

It was noticed from observations of cultured CHO-lac-mGlu1a and -mGlu5a cells, that induction of mGlu receptor expression resulted in subtle changes in cellular morphology. In non-induced cells, CHO cells generally displayed a smooth rounded appearance, whereas upon receptor induction an elongation of cells to a more spindle-like morphology was observed. These effects seemed to become more prominent upon prolonged exposure to agonist. Previous reports have similarly demonstrated mGlu1a receptor-mediated changes in cell morphology (Kubo et al. 1998; Hermans et al. 1999; Huang & Hampson, 2000) and actual cell death (Dale et al. 2000), the data on \[^{3}H\]thymidine incorporation presented here however, do not show that there any actual changes in cell proliferation in response to mGlu receptor induction or agonist challenge in these cells. As rearrangements of the cytoskeleton may underlie the observed changes in cellular morphology, mentioned above, changes in the cytoskeletal proteins in response to mGlu receptor activation were examined.

The mGlu1a receptor has previously been shown to associate with tubulin via interactions through the C-terminus of this receptor by Ciruela and colleagues (1999c). Workers from the same group (Ciruela & McIlhinney, 2001) then went on to show that mGlu1a receptor activation induced depolimerization of tubulin and its translocation to the plasma membrane where it formed associations with the mGlu1a receptor in BHK-570 cells. The effects of receptor induction and receptor stimulation with agonist on tubulin were therefore assessed in CHO-lac-mGlu1a and -mGlu5a cells, using immunofluorescence.

Initially, immunocytochemical detection of mGlu receptor expression was assessed in CHO-lac-mGlu1a and -mGlu5a cells. In Chapter 3, mGlu receptor expression in response to
Figure 6.4 Detection of receptor expression in CHO-lac-mGlu1a and -mGlu5a cells using fluorescence imaging. A, B, C and D, E, F show receptor expression in CHO-lac-mGlu1a and -mGlu5a cells respectively. Cells were grown on cover slips and receptor expression induced using 100 μM IPTG (+ 5 mM butyrate for CHO-lac-mGlu5a cells) for 20 h. Cells were fixed using a methanol and washed prior to immunostaining for receptors using anti-mGlu1a or anti-mGlu5a receptor antisera (see Table 2.1) as described in Materials and Methods (Section 2.12). A secondary Alexa fluor 488 antibody was used to visualise receptor staining (coloured green) and Hoechst-33342 was used to stain cell nuclei (coloured red). Immunostaining was viewed using an inverted fluorescence microscope. Representative images of 3-6 images taken for each treatment over 2-3 separate experiments are shown. A and D; Non-specific staining (secondary antibody only), B and E; Non-induced cells, C and F; cells induced for receptor expression.
addition of IPTG was described using immunoblot analysis. Here, receptor expression was also examined using immunofluorescence (Fig. 6.4). No immunoreactivity was detected in the absence of IPTG (non-induced cells, Fig. 6.4B and E) or when the primary, mGlu receptor specific antibody was omitted (non-specific, Fig. 6.4A and D). Addition of the inducing agent, IPTG, resulted in a marked increase in immunofluorescence, indicating induction of receptor expression (induced cells, Fig. 6.4C and F). For both CHO-lac-mGlula and -mGlu5a cells (Fig. 6.4 C and D, respectively), immunofluorescence staining of receptors could be detected throughout the cytoplasm and intensive staining was observed close to the nucleus, as previously described by Hermans et al. (1999). It is thought that the latter represents newly synthesised receptors present after the induction period.

The effects of mGlu receptor stimulation with transient and pro-longed exposure to agonist on tubulin were then assessed. In both CHO-lac-mGlula (Fig. 6.5) and -mGlu5a cells (Fig. 6.6), similar effects were observed so these will be discussed collectively. In non-induced cells, tubulin staining displayed a dense microtubule network throughout the cell, with tubulin microtubules smooth in appearance (Fig 6.5/6.6B). Induction of receptor expression did not alter the appearance of tubulin microtubules, in either cell line (Fig. 6.5/6.6C). Cells were then treated with quisqualate. Agonist treatments of 10 and 30 min were used, in accordance with other studies also carried out into mGlu receptor-mediated changes in the cytoskeleton (Ciruela et al. 2001; Shinohara et al. 2001), and known time-courses of peak MAPK activation in these cells, described in Chapters 3-5. Additionally, the effect of prolonged (24 h) agonist exposure on tubulin in CHO-lac cells was also examined. Treatment of CHO-lac-mGlula1a and -mGlu5a cells for 10 and 30 min with quisqualate (Fig 6.5/6.6D and E, respectively) did not affect tubulin structure. Exposure of cells to agonist for 24 h however, produced a noticeable change in smooth tubulin microtubules, which were apparent in control cells, to a more granular appearance in chronically treated cells, in both cell lines (Fig. 6.5/6.6C and F). It is thought that these changes may possibly be indicative of tubulin depolimerization (personal communication R. Mistry, department of Biochemistry). The present investigation did not however explore if these changes in tubulin structure reflect cytotoxic effects of prolonged agonist exposure or mGlu receptor-mediated events.
Figure 6.5 Detection of tubulin staining in CHO-lac-mGlu1a cells using fluorescence imaging. Cells were grown on cover slips and receptor expression induced using 100 µM IPTG (20 h). Cells were treated with quisqualate (10 µM) for 10, 30 min or 24 h (in the presence of GPT/pyruvate) and then fixed using a methanol and washed prior to immunostaining for tubulin using a specific antiserum (see Table 2.1) as described in Materials and Methods (Section 2.12). A secondary Alexa fluor 488 antibody was used to visualise tubulin staining (coloured green) and Hoechst-33342 was used to stain cell nuclei (coloured blue). Immunostaining was viewed using an inverted fluorescence microscope. Representative images of 3-6 images taken for each treatment over 3 separate experiments are shown. A; non-specific staining (secondary antibody only), B; Non-induced cells, C; Induced cells, non-treated, D; Induced cells + quisqualate for 10 min, E; Induced cells + quisqualate for 30 min, F; Induced cells + quisqualate for 24 h.
Figure 6.6 Detection of tubulin staining in CHO-lac-mGlu5a cells using fluorescence imaging. Cells were grown on cover slips and receptor expression induced using 100 μM IPTG + 5 mM butyrate (20 h). Cells were treated with quisqualate (10 μM) for 10, 30 min or 24 h (in the presence of GPT/pyruvate) and then fixed using a methanol and washed prior to immunostaining for tubulin using a specific antiserum (see Table 2.1) as described in Materials and Methods (Section 2.12). A secondary Alexa fluor 488 antibody was used to visualise tubulin staining (coloured green) and Hoechst-33342 was used to stain cell nuclei (coloured blue). Immunostaining was viewed using an inverted fluorescence microscope. Representative images of 3-6 images taken for each treatment over 3 separate experiments are shown. A; non-specific staining (secondary antibody only), B; Non-induced cells, C; Induced cells, non-treated, D; Induced cells + quisqualate for 10 min, E; Induced cells + quisqualate for 30 min, F; Induced cells + quisqualate for 24 h.
6.3 Investigating the Effects of group I mGlu receptor stimulation on actin

A similar investigation into the effects of mGlulα/5a receptor stimulation on rearrangements in the actin cytoskeleton were then similarly assessed. The mGlulα receptor has not only been shown to interact with components of the actin cytoskeleton via Cupidin, an isoform of Homer, that provides a molecular scaffold between this mGlu receptor, actin and the Rho family protein cdc42 (Shiraishi et al. 1999), but Shinohara et al. (2001) have also demonstrated actin rearrangement by mGlulα receptor activation.

As with the tubulin data, results for CHO-lac-mGlulα and mGlul5a cells were similar and will therefore be discussed collectively. In non-induced CHO-lac-mGlulα and -mGlul5a cells, actin filaments appear as rod-like strands, which traverse across the cell (Fig. 6.7B and 6.8B, respectively). Different numbers of these strands can be observed between different cells. In induced cells (Fig. 6.7/6.8C) and cells treated acutely with agonist for 10 min (Fig. 6.7/6.8D) and 30 min (Fig. 6.7/6.8E), there is no apparent change in the morphology of actin filaments from control cells. However, in both CHO-lac-mGlulα and -mGlul5a cells treated with agonist for 24 h, the presence of these rod-like actin strands was almost completely abolished (Fig. 6.7/6.8E). In addition, filamentous protrusions from the cell membrane could be observed in many of the cells chronically treated with quisqualate. Again, it is unknown if these changes in cell morphology represent 'real' effects of mGlu receptor stimulation or due to cytotoxicity of prolonged agonist exposure.

6.4 Effects of group I mGlu receptor stimulation on focal adhesion kinase (FAK)

Focal adhesion kinase (FAK) is a 125kDa protein that is an important regulator of signal transduction mediated by integrins. Focal adhesions are points of adhesion to surfaces in cultured cells, and FAK is an intermediate in signalling from the extracellular matrix to the actin cytoskeleton at these focal adhesions, and therefore is able to regulate cellular events such as, cell migration, cell proliferation and cell survival (Girault et al. 1999; Parsons et al. 2000).
Figure 6.7 Detection of actin staining in CHO-lac-mGlu1a cells using fluorescence imaging. Cells were grown on cover slips and receptor expression induced using 100 μM IPTG (20 h). Cells were treated with quisqualate (10 μM) for 10, 30 min or 24 h (in the presence of GPT/pyruvate) and then fixed using formaldehyde and washed prior to immunostaining and visualisation of actin (coloured red) using a specific antiserum, Texas Red-X phalloidin (see Table 2.1) as described in Materials and Methods (Section 2.12). Hoechst-33342 stain was used to stain cell nuclei (coloured blue). Immunostaining was viewed using an inverted fluorescence microscope. Representative images of 3-6 images taken for each treatment over 3 separate experiments are shown. A; non-specific staining, B; Non-induced cells, C; Induced cells, non-treated, D; Induced cells + quisqualate for 10 min, E; Induced cells + quisqualate for 30 min, F; Induced cells + quisqualate for 24 h.
Figure 6.8 Detection of actin staining in CHO-lac-mGlu5a cells using fluorescence imaging. Cells were grown on cover slips and receptor expression induced using 100 μM IPTG + 5 mM butyrate (20 h). Cells were treated with quisqualate (10 μM) for 10, 30 min or 24 h (in the presence of GPT/pyruvate) and then fixed using formaldehyde and washed prior to immunostaining and visualisation of actin (coloured red) using a specific antiserum, Texas Red-X phalloidin (see Table 2.1) as described in Materials and Methods (Section 2.12). Hoechst-33342 stain was used to stain cell nuclei (coloured blue). Immunostaining was viewed using an inverted fluorescence microscope. Representative images of 3-6 images taken for each treatment over 3 separate experiments are shown. A; non-specific staining, B; Non-induced cells, C; Induced cells, non-treated, D; Induced cells + quisqualate for 10 min, E; Induced cells + quisqualate for 30 min, F; Induced cells + quisqualate for 24 h.
Regulation of FAK kinase activity by mGlu receptors has been demonstrated in model cells (Shinohara et al. 2001) and native preparations (Siciliano et al. 1996; Schinkmann et al. 2000). To further the investigation into mGlu1a/5a receptor activation on the cytoskeleton, and possible downstream effectors of mitogenic signalling by activation of these two receptors, the modulation of FAK activity by mGlu receptor activation in CHO-lac cells was explored using immunoblot analysis.

FAK was immunoprecipitated from CHO-lac-mGlu1a and -mGlu5a lysates using a FAK specific antiserum. Immunoprecipitates were then examined using immunoblot analysis using a phosphotyrosine antibody. In two out of five experiments, activation of mGlu1a and mGlu5a receptors stimulated a small increase in the tyrosine phosphorylation of FAK tyrosine kinase (Fig. 6.9A and B). However, this increase was small and inconsistent across separate experiments. In addition, a marked agonist-mediated increase in tyrosine phosphorylation of an unknown protein of approx. 50-60kDa in FAK immunoprecipitates was detected in CHO-lac-mGlu1a (Fig. 6.9C) but not CHO-lac-mGlu5a cells. This increase was observed in several experiments but the nature of this protein was not determined. Its is possible that the anti-FAK cross-reacts with another protein that is also immunoprecipitated and activated by mGlu1a receptor activation, or indeed that this protein is associated with FAK and is co-immunoprecipitated with it. Further investigations into activation of this unknown protein were not made.
Figure 6.9 Agonist-stimulated FAK tyrosine phosphorylation in CHO-lac-mGlu1a and mGlu5a cells. A and B, cells were pre-incubated with GPT/pyruvate before stimulation with quisqualate (10 μM) for the various times indicated. FAK was then immunoprecipitated with an anti-FAK antibody (see Table 2.1) as described in Materials and Methods (Section 2.10.1). Immunoprecipitates were analyzed via 8% SDS-PAGE as described under Materials and Methods (Section 2.10) and probed with an antibody that recognizes the phosphorylated tyrosine residues (anti-phosphotyrosine, see Table 2.1). C and D, as described for A and B, except tyrosine phosphorylation on an unknown protein immunoprecipitated with anti-FAK is shown. Representative immunoblots of FAK phosphorylation from 2 separate experiments are shown. C; basal activity, or +quisqualate for 2-30 min, WCL; whole cell lysates/extracts.
Discussion

The aim of this Chapter was to establish if group I mGlu receptor activation lead to growth and or cytoskeletal changes in CHO cells, in an attempt to correlate the robust activation of mitogenic signalling by mGlu1a/mGlu5a receptors to a physiological output in these cells. Initial investigations into the effects of mGlu1a/mGlu5a receptor activation on cell growth were determined by examining DNA synthesis via [³H]thymidine incorporation.

Stimulation of neither mGlu receptor resulted in any significant changes in [³H]thymidine incorporation indicative of no significant changes in cellular proliferation in CHO-lac cells. Although, mGlu receptor-mediated activation of MAPKs is well documented in cell models (Ferraguti et al. 1999) and native preparations (Peavy & Conn 1998; Peavy et al. 2001; 2002), actual cell growth effects of this activation are far less well documented. Schinkmann and colleagues (2000) have previously reported a small increase in DNA synthesis (1.7-1.9-fold) in response to mGlu receptor stimulation in astrocytes. This stimulation in DNA synthesis was shown to mediated by increases in ERK activation as the MKK inhibitor PD98059 inhibited the responses. Dale and co-workers (2000) have also demonstrated growth effects, mediated by the mGlu1a receptor, in HEK 293 cells. Here, expression of the mGlu1a receptor lead to agonist-independent cell death, an effect that was prevented by co-transfection with G protein-coupled receptor kinase 2 (GRK2). It was thought that GRK2-mediated desensitization of constitutive mGlu1a receptor activity resulting in protection against mGlu1a receptor-mediated cell death.

In contrast to the lack of growth effects in both serum starved and serum containing CHO-lac-mGlu1a/mGlu5a cells, the PLC-linked m3 mACh receptor elicits significant inhibition of growth responses in CHO cells (Burdon et al. 2002). However, this growth effect was not attributable to activation of ERK, JNK or p38 MAP kinases, all activated by this receptor when expressed in these cells, suggesting that although mitogenic signalling the observed growth responses are MAPK-independent. Gβγ subunits and PKC were however shown to be involved. Indeed it is known that PKC can also regulate changes in cell migration in CHO cells (Varker & Williams, 2002).
Having established that activation of neither group I mGlu receptor elicited growth responses in our CHO-lac cells, possible effects on cell morphology as a result of mGlu1a/mGlu5a receptor activation were explored by immunocytochemical analysis of the cytoskeletal elements, tubulin and actin.

It is already known that the extended C-terminal domain of group I mGlu receptors allows interaction with the Homer family of scaffolding proteins enabling interaction with other intracellular proteins (Tu et al. 1998, 1999). Ciruela and colleagues (1999) have also demonstrated that tubulin can associate with the mGlu1a receptor via its C terminus, an interaction that can regulate trafficking and cell surface expression of the receptor (Ciruela et al. 1999). Further to this study, quisqualate induced activation of the mGlu1a receptor in BHK-570 cells (Ciruela et al. 2001) resulted in tubulin depolimerization and its translocation to the plasma membrane. Immunocytochemical analysis showed that in addition to this disruption of the microtubule network, tubulin arrangement became punctate, and clustering of tubulin and the mGlu1a receptor could be discerned. Huang & Hampson (2000) have also demonstrated that mGlu1a receptor activation lead to the inhibition of microtubule-associated proteins (MAPs), which bind to and facilitate tubulin polymerization, leading to reduced microtubule stability. In data presented here, mGlu1a/mGlu5a receptor activation in CHO-lac cells only resulted in noticeable changes in the tubulin microtubule network after chronic (24 h) exposure to agonist, unlike the study by Ciruela et al. (2001) in BHK-570 cells, where changes in tubulin rearrangement were apparent after acute (20 min) agonist treatment. It may be that cytotoxic effects of chronic agonist exposure may account for the changes in tubulin in CHO-lac-mGlu1a/mGlu5a cells; this could be determined by further investigations in the presence of mGlu receptor specific antagonists to establish if the effects are indeed receptor-mediated. Additionally, differences in mGlu1a receptor-mediated effects on tubulin in this investigation and that by Ciruela et al. (2001) may reflect the different cell types used.

Similar effects to tubulin were observed for mGlu receptor activation on actin in CHO-lac-mGlu1a and -mGlu5a cells, with little effects of acute agonist treatment but a noticeable disruption of actin filaments after pro-longed exposure to quisqualate. Shinohara and co-workers (2001) have similarly investigated the effects of mGlu1a/mGlu5a receptor activation.
on actin in CHO cells. Here, acute treatment with glutamate (10 min) resulted in a marked change from a punctate distribution of actin to formation of actin filaments. This rearrangement was shown to be prevented by the calmodulin inhibitor, fluphenazine. Calmodulin/calcium signalling was demonstrated to mediate tyrosine phosphorylation of FAK leading to rearrangements in the actin cytoskeleton. Schinkmann et al. have also reported a time- and concentration-dependent increase in related adhesion focal adhesion kinase (RAFTK) phosphorylation in response to glutamate treatment in astrocytes. We similarly investigated FAK tyrosine phosphorylation in CHO-lac-mGlu1a/mGlu5a cells in response to agonist challenge but did not consistently find a significant increase in activity following mGlu receptor activation. It is therefore unlikely that activation of this kinase is downstream of mGlu receptor signalling in our CHO-lac cells.

The data presented in this chapter show that group I mGlu receptor activation shows little growth effects in the CHO-lac cell model. Although, immunocytochemical analysis did show subtle agonist-mediated changes in the arrangement of the cytoskeletal proteins, tubulin and actin, the extended time-course of these effects did not correlate with the activation of mitogenic signalling that has been investigated in this thesis. The main aims of investigating physiological changes such as cellular proliferation and changed cell morphology were to find downstream effects of MAPK activation by mGlu1a/mGlu5a receptors in CHO cells. However, as the observed effects do not coincide with peak ERK, JNK or p38 activation in these cells it can be concluded that mGlu1a/mGlu5a receptor-mediated MAPK activation has a divergent role to the classical regulation of cell division and growth, associated with activation of these pathways, in these cells. Further studies of group I mGlu receptor MAPK signalling in native preparations may provide more information about the physiological outputs of mGlu receptor coupling to these cascades.


CHAPTER 7 General Discussion and Critique

7.1 General summary of the regulation of MAPK activities by group I mGlu receptors in CHO cells

The main aim of this thesis was to carry out a systematic study comparing mGlu1 versus mGlu5 receptor coupling to ERK, JNK and p38 MAP kinase regulation in a common cell background. With respect to activation of the ERK MAPK, it might be assumed that because mGlu1a and mGlu5a receptors are closely related, both structurally and with respect to the downstream signalling pathways they regulate, they are likely to utilize common pathways to link to ERK activation, however the data presented show that although some similarities exist in the profile of ERK activation by mGlu1a and mGlu5a, these two receptors utilize quite distinct mechanisms to couple to this cascade. A clear difference was found in the G-protein subpopulations involved in coupling mGlu1a/mGlu5a receptors to ERK. The PTx sensitivity of the mGlu1a receptor-mediated response suggested G_{i0} coupling was involved. Whether mGlu1a receptor-ERK signalling is mediated exclusively by G_{i0} proteins or is dependent on G_{i0}/G_{q11} cooperation, as has been shown for the B2 bradykinin receptor (Blaukat et al. 2000), has yet to be established. Previous studies have failed to detect mGlu1a-G_{i0} coupling using [35S]-GTPyS and Ga-specific immunoprecipitation in CHO-lac cell membranes (Selkirk et al. 2001), although baby hamster kidney (BHK) cells stably expressing mGlu1a have provided evidence for both G_{q11} and G_{i0} activation (Hermans et al. 2000).

The PTx insensitivity of mGlu5a receptor-mediated ERK activation implies that PTx-insensitive G proteins from G_{q11}, or indeed G_{12/13} family proteins may be involved. As with the mGlu1a receptor, definitive immunoprecipitation studies of mGlu5a-G_{q11} and mGlu5a-G_{12/13} coupling have not been reported. Much of the data surrounding mGlu5a-G_{q11} coupling is assumed from the activation of the PLC pathway by this receptor. In this study, a role for G_{b\gamma} subunit involvement in mGlu1a/mGlu5a receptor-ERK coupling was also found to be unlikely owing to the lack of requirement for PI3-kinase, a common G_{b\gamma}-Ras-ERK intermediate (Crespo et al. 1994). Insensitivity of the mGlu1a/mGlu5a receptor-mediated
ERK response to the PI3-kinase inhibitor wortmannin suggests this kinase is not recruited by either mGlu receptor in signalling to ERK in CHO cells. It is possible that this may not be the case in native cells. PI3-kinase has been shown to be a key intermediate in coupling ionotropic glutamate receptors to ERK activation in neurons (Perkinton et al. 2002) and it is feasible that this kinase may also play role in mGlu receptor-ERK coupling in the CNS. The question of direct Gβγ subunit involvement could not be definitively answered using the approach of co-transfection of HA-tagged ERK and α-transducin (Gocti) due to effects on ERK expression following transfection with Gocti. In addition to G protein subunit involvement there also remains the possibility of G protein-independent mechanisms of ERK activation. Ascertaining the G protein coupling and/or G protein-independent mechanisms involved in linking these two mGlu receptors to ERK will aid our understanding of the signalling intermediates that are recruited to elicit activation of this pathway downstream of receptor activation. The problems with investigating such coupling in recombinant expression systems is that, although they provide important information on the repertoire of signalling partners that these receptors are able to utilize, these receptors may not functionally couple to the same G protein subpopulations and/or kinases in native cells.

The question of whether the mGlu1a receptor uses transactivation as a means of eliciting ERK activation is another important consideration. Importantly, data presented here suggest that not only does this receptor use intermediates such as Src kinase, but that another receptor is recruited to transduce the signal from the activated mGlu receptor to the ERK module. Support for this model comes from the proposed mechanisms of EGF receptor transactivation by the mGlu5a receptor in astrocytes reported by Peavy and colleagues (2001). As our CHO-lac cell model lacks EGF receptor expression, the mGlu5a receptor must be using a different pathway or signalling intermediate to elicit the robust ERK activation observed in these cells. It is evident from the data presented here that although the PDGFR seems to be a requirement for the mGlu1a receptor-mediated ERK response, this is not the case for the mGlu5a receptor, implying that differential signalling to this cascade can be adopted depending on the cell type. However, in this study, the most important aspect of the data presented is not the components that constitute the mGlu1/5 receptor-ERK signalling pathway in CHO cells, but the fact that these group I mGlu receptors can recruit different subsets of downstream signalling
components to execute this pathway. These data suggest that not only do mGlu1 and mGlu5 receptors exhibit different anatomical and cellular distributions within the CNS (Hubert et al. 2001; Valenti et al. 2002), but they also differ in their repertoires of downstream signalling partners.

Further evidence for the divergent nature of signalling partners for these two mGlu receptors came from the observed differential activation of the JNK pathway. The signalling from the mGlu1a receptor to JNK may be a much more complicated story than activation of the ERK cascade, as attempts to delineate this pathway provided little information about the key proteins that may be involved in eliciting this activation. At present very little information is available from other investigations into the activation of this cascade by mGlu receptors, and much further work will be needed to elucidate the transduction mechanism involved in mGlu receptor-JNK coupling. Again, a key aspect of this study is the observation that the mGlu1a, but not the mGlu5a receptor activates this pathway. Due to the heterogeneous expression of mGlu1a/mGlu5a receptors in the CNS, if such differential regulation of JNK also translates to native cells, then there is the potential for cell-type specific activation of this MAPK, which unlike ERK is associated with growth arrest and cell death (Mielke & Herdegen, 2000). Further to this, confirmation of the activation of p38 MAP kinase by mGlu1a/mGlu5a receptors also requires further study and investigation into characterizing the potential signalling mechanisms underlying this activation.

The findings of mGlu1a and mGlu5a receptor-mediated increases of ERK, JNK and p38 MAP kinase activities in CHO cells prompted investigations into the possible downstream effects of these activations. As MAP kinases are key regulators of cell division and differentiation, we examined growth effects of mGlu receptor stimulation in CHO cells. As no significant effects on growth in response to either mGlu1a/mGlu5a receptor stimulation could be discerned, the downstream effects of mGlu receptor-MAPK coupling can only be speculated. The first likely candidates are transcription factors, which are targets of ERK, JNK and p38 MAP kinase activation. The transcription factor Elk-1, which provides a point of convergence for these pathways (Zinck et al. 1995), was not a likely candidate as this protein could not be
detected in our CHO-lac cells. CREB, another transcription factor known to be a target of mGlu receptor activation in native cells (Choe & Wang, 2001) can be phosphorylated by mGlu1a and mGlu5a receptor activation but this occurs through an ERK-independent mechanism (Warwick H., unpublished results). Therefore, potential downstream targets of the robust mGlu receptor-mediated MAPK activation in these cells still remains to be determined.

7.2 Implications of mGlu receptor-MAPK signalling in native cells

Although, there is overlap in some brain regions, the specialized expression of mGlu1a and mGlu5a receptors is likely to reflect the distinct functions these two subtypes may have in the CNS (Valenti et al. 2002). The activation of MAPK signalling by these receptors may therefore have specific differential functions in different regions of the brain. The activation of mGlu receptors has been demonstrated to regulate the morphology and development of neurones and such effects may be the result of mGlu receptor-MAPK coupling (Gomperts et al. 2000; Vanderklish & Edelman, 2002). Perhaps one of the most important aspects of activating MAP kinase signalling in neuronal cells is the ability to regulate gene transcription, which ultimately provides the potential for involvement in processes involved in learning and memory (Sweatt, 2001). Indeed, components of the Ras/MAPK pathway are highly expressed in particular CNS regions implicated in the formation of memory, including the hippocampus, cerebellum and neo-cortex. The role of MAPKs in these phenomena may be to serve as molecular coincidence detectors that integrate a variety of extracellular signals to a common target, allowing an overall coordinated cellular output (Impey et al. 1999; Sweatt, 2001). As they have the ability to activate transcription factors, the changes in gene expression required for long-lasting forms of synaptic plasticity and memory formation can be mediated by activation of MAPK signalling cascades. MAP kinase activation has also recently been shown to play a role in regulating translation (Kelleher et al. 2004). Group I mGlu receptors have been shown to mediate events involved in synaptic plasticity, including long-term potentiation (LTP) and long-term depression (LTD) (Lu et al. 1997; Bolshakov et al. 2000; Fujii et al. 2003; 2004; Wu et al. 2004). Although, mGlu receptors have been shown to be involved in learning and memory formation (Salinska & Stafiej, 2003) the precise roles
of these receptors in the mechanisms underlying these processes are still unclear. In some brain regions, mGlu1a and mGlu5a receptors have been shown to differentially regulate synaptic activity (Mannaioni et al. 2001; Poisik et al. 2003). These studies demonstrate that in addition to the role of mGlu receptors in mediating events associated with changes in synaptic plasticity, the signal transduction pathways leading to the activation of these events can be differentially regulated by different mGlu receptor subtypes. Indeed, as with data presented here, differential coupling to MAP kinase activation by the mGlu1a and mGlu5a receptor demonstrate the potential for these two closely related mGlu receptor to elicit different physiological and indeed pathophysiological events in native cells in the CNS.

7.3 Critique

In this thesis, work has been carried out in stably transfected CHO cell lines, in which the expression of human recombinant mGlu1a and mGlu5a receptors is under the control of an IPTG-inducible promoter (Hermans et al. 1998; 1999). As expression of mGlu receptors can be regulated by the addition of IPTG, this system reduces the problems related to the endogenous release of glutamate, which can lead to mGlu receptor desensitization and down-regulation. Such effects have been observed in traditional stable expression systems (Desai et al. 1995; Nedergaard et al. 2002).

However, the main criticism concerning this work is the use of a model cell system in which mGlu receptors, found mostly expressed in neuronal and glial cells within the CNS, are expressed in a cell background quite different from that of native cells. CHO cells are very different in structural organisation to neurons and in particular, may lack expression of proteins, such as Homer, which play key roles in scaffolding and mediating signal transduction by group I mGlu receptors. This difference in cellular environment may influence the mechanism of, and preferential activation of, specific intracellular pathways by mGlu receptor activation. As the formation of signalling ‘microdomains’ is emerging as a key concept in mediating efficient signal transduction (Delmas et al. 2002; 2004), the organisation of cascades in particular cells, which might only express a certain repertoire of signalling molecules, is an important consideration.
Initial plans regarding exploration of the activation of mitogenic signalling by group I mGlu receptors in this thesis, prompted by the report by Dale et al. (2001), were to include an investigation into this signalling in native cells. However, time limitations did not allow a detailed examination into mGlu1a/mGlu5a receptor-mediated signalling to ERK, JNK and p38 MAP kinase beyond the CHO-lac cell model. The study of these receptors individually in native cells is however challenging, not only due to their similar pharmacological profiles, but also owing to the distinct expression patterns of mGlu1a and mGlu5a mRNA in specific neuronal cells (Abe et al. 1992). Investigations into group I mGlu receptors in native preparations could involve investigating mGlu5a receptor responses in astrocytes, which express a high level of the mGlu5a, but not the mGlu1a receptor (Schools & Kimelberg, 1999), whereas mGlu1a receptor-mediated MAPK activation would need to be examined in another cell type, such as cerebellar granule cells. These cells however, and many other neuronal preparations have a heterogeneous expression of both mGlu1a and mGlu5a receptors and may additionally express group II and III mGlu receptors. Further complications also arise from the expression of ionotropic glutamate receptor subtypes in these cells. Therefore, investigations into mGlu receptor signalling would likely require the use of various specific antagonists when examining signalling profiles of a particular mGlu receptor subtype.

It follows that much research into the pharmacology and signal transduction mechanisms of these receptors has been carried out via transient or stable transfection of mGlu receptors into immortalised cell lines, which allow comparisons in signalling to be made in a common background when looking at these two mGlu receptors. It is however, important to appreciate limitations when drawing physiological conclusions from data obtained in a model cell line. It is perhaps worth noting that data presented in this thesis, regarding the activation of the ERK cascade by the mGlu5a receptor, bear striking similarities in signalling characteristics to the work carried out by Peavy and colleagues (2001, 2002) into ERK activation by this receptor in astrocytes, and highlights similar findings in the intracellular signalling utilised by a particular receptor expressed in native cells and a model cell system. The advantages of a model cell system are that the signal transduction mechanisms of a particular receptor subtype can be investigated in detail. In addition, in the case of mGlu receptors, an inducible
expression system allows control of receptor expression and overcomes problems of endogenous glutamate affecting receptors permanently expressed in an immortalised cell line.

7.4 Future directions

Investigating the intracellular signalling involved in group I mGlu receptor coupling to MAPK activation in neuronal systems would be a long-term consideration, however the present model could be utilized as a starting point to address some questions that remain unanswered in the present study.

Perhaps one of the most important aspects for further investigation is examination of the striking differences in PTx sensitivity of the mGlu1a/mGlu5a receptor-mediated ERK response discussed in Chapter 4. As described previously, G_{q/11} coupling of mGlu1a/mGlu5a receptors is assumed from the activation of PLC. To explore whether this is indeed the case, and more importantly for this study whether the PTx-insensitive mGlu5a receptor-mediated ERK activation is G_{q/11}-dependent, might involve the approach of using G protein-specific interfering RNA (RNAi) (McManus & Sharp, 2002; Dykxhoorn et al. 2003), to dissect the G protein subpopulations involved. This rapidly developing field of technology is useful in silencing specific genes, and in this case could be used to target those responsible for expression of G_{q/11} G proteins, allowing dissection of their involvement in ERK activation downstream of mGlu receptor activation. Development of G_{q/11}-specific RNAi by others in the laboratory (Atkinson P., unpublished results) has produced positive results in knocking down G_{q/11} expression in CHO-lac and HEK 293 cells and future plans include extending these manipulations into neuronal cells. With respect to the robust mGlu receptor-mediated ERK response, this approach is currently being designed to be used in establishing whether knockdown of G_{q/11} affects the mGlu receptor-mediated ERK response. It is tempting to speculate that potential differential sensitivity of the mGlu1a and mGlu5a receptor-mediated ERK response to G_{q/11} knock-down might be an important step in understanding the G protein coupling dependency of these two receptors to this cascade. As the subtype of G protein is crucial in determining which signalling intermediates are subsequently recruited to receptors.
to mediate activation of MAPK pathways this approach will provide useful information in further delineation of mGlu-receptor ERK signalling.

The mGlu1a receptor-mediated JNK response also requires further investigation into the events upstream of the JNK module. As discussed in Chapter 5, a number of common second messengers including Ca$^{2+}$, PKC, PI3-kinase and Src kinase, have been eliminated in the signalling from the mGlu1a receptor to JNK in CHO cells. As GTP-binding proteins of the Rho family, Rac and Cdc42, are known to regulate JNK activity (Teramoto et al. 1996; Coso et al. 1996) analysis of their expression and possible activation in response to mGlu receptor activation in CHO cells, using activity assays would be an appropriate starting point. No current literature has explored whether these receptors are able to couple to the activation of these small GTPases in addition to heterotrimeric G proteins. In addition, the possibility of mGlu1a receptor-G$_{12/13}$ coupling to JNK is an important consideration owing to the lack of attenuation of the mGlu1a receptor/JNK response to manipulation of the G$_q$/PLC pathway. However, as yet coupling of these receptors to this G protein subfamily has similarly not been reported and an initial exploration of mGlu receptor-G$_{12/13}$ coupling using [$^{35}$S]-GTP$_\gamma$S immunoprecipitation proved inconclusive (Selkirk J., personal communication). Evidence of JNK activation by G$_{12/13}$ has been demonstrated (Nagao et al. 1996) and GPCR coupling to G$_{12/13}$ has been observed (Graler et al. 2003) making that mGlu receptor-G$_{12/13}$ involvement in JNK activation a possibility.

The idea of mGlu receptor transactivation of a growth factor receptor to elicit ERK activation is a concept that has already been described, with respect to the mGlu5a receptor, by Peavy and colleagues (2001) and has been also been investigated in this study. This aspect of the study requires much further work to establish whether the mGlu1a receptor does indeed use this mechanism to couple to the ERK module. Further exploration into whether mGlu1a receptor stimulation causes increased PDGFR tyrosine phosphorylation is a good starting point to examine such activation. Some initial experiments, of this nature were carried out in CHO-lac-mGlu1a cells, but these require further consideration and characterisation. In
particular, the use of Src kinase inhibition would help to establish whether such transactivation is downstream of Src kinase. Likewise, further analysis of increases in mGlu receptor-mediated Src kinase activity could be used to establish if Src kinase is involved downstream of the PDGFR by the use of the PDGFR kinase inhibitor AG1296. However, it is important to bear in mind that establishing transactivation as a mechanism for mGlu1a receptor activation of ERK is not a simple task. As demonstrated by a study by Rubio et al. (2003) and discussed by Downward (2003) and Shah & Catt (2004), the mechanisms underlying transactivation are complex and highly dependent upon the type of GPCR and RTK that are communicating, and the cell background. In addition to transactivation, the requirement for mGlu receptor internalisation in efficient MAPK coupling also requires further analysis. From immunocytochemistry in this study it is evident that a number of mGlu receptors are cytoplasmically located in addition to localisation at the plasma membrane. It is not yet established whether mGlu receptor internalization is necessary for coupling to ERK or indeed JNK activation.

An important consideration that has been mentioned previously is the involvement of scaffolding proteins that are key components in mGlu receptor signalling and in the organisation and transduction of MAPK cascades. The most important scaffolding component with regard to group I mGlu receptor signalling is the neuronal family of Homer proteins. Homer proteins act as linkers to couple mGlu receptors to other intracellular proteins (Xiao et al. 1998; Fagni et al. 2002) and Tu and colleagues (1998) have provided evidence that Homer can physically tether mGlu receptors with inositol trisphosphate receptors (IP3R). In addition, co-expression of Homer 1a with the mGlu1a receptor has also been shown to increase the cell surface expression of this receptor (Ciruela et al. 1999b). Owing to the structural differences in CHO cell and neuronal cell organisation, the effects of transfection of Homer isoforms into such a cell background would allow the question of how the presence of these scaffolding proteins impact on coupling mGlu receptors to ERK or indeed JNK activation. Current exploration of transient expression of Homer isoforms into cell lines and the effects on mGlu receptor IP3/Ca2+ signalling have been investigated by others in the laboratory and this work could be extended to include effects on mGlu receptor-MAPK regulation.
It is clear that although the present study has addressed a number of key issues regarding the intracellular signalling to MAP kinases from group I mGlu receptors, the picture emerging is complex, even when examining such signalling in a relatively simple cell background. It is important to understand the limitations of using cell lines, but to appreciate that the data and knowledge gained from such studies provide a platform from which to formulate and inform key questions when drawing physiological conclusions and examining MAPK signalling in neuronal cells.
REFERENCES


Harper S. J. & LoGrasso P. (2001). Signalling for survival and death in neurones, the role of


Hermans E. & Challiss R. A. J. (2001) Structural, signalling and regulatory properties of the
group I metabotropic glutamate receptors: prototypic family C G-protein-coupled

recombinant human mGlu1a receptors on the pharmacological properties of agonists and

Complex involvement of pertussis toxin-sensitive G proteins in the regulation of type 1α

1α metabotropic glutamate receptor expression level on phosphoinositide and Ca^{2+}

Ligand-independent activation of platelet-derived growth factor receptor is a necessary
USA* **95**, 8985-8990.


Houamed, K.M., Kuijper, J.L., Gilbert, T.L., Haldeman, B.A., O’Hara, P.J., Mulvihill, E.R.,

Huang X.-P. & Hampson D. R. (2000). Inhibition of microtubule formation by metabotropic


Schools G. P., & Kimelberg H. K. (1999). mGluR3 and mGluR5 are the predominant metabotropic glutamate receptor mRNAs expressed in hippocampal astrocytes acutely isolated from young rats. J. Neurosci. Res. 58, 533-543.


Siciliano J. C., Toutant M., Derkinderen P., Sasaki T. & Girault J. A. (1996). Differential regulation of proline-rich tyrosine kinase 2/cell adhesion kinase β (PYK2/CAKβ) and


Wu J., Rowan M. J. & Anwyl R. (2004). Synaptically stimulated induction of group I metabotropic glutamate receptor-dependent long-term depression and depotentiation is
inhibited by prior activation of metabotropic glutamate receptors and protein kinase C. *Neuroscience* **123**, 507-514.


