Modulation of type 1α metabotropic glutamate receptor signalling by extracellular Ca$^{2+}$

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This Thesis describes experiments in which the modulation of type 1α metabotropic glutamate receptor (mGluR1α) signalling by \([\text{Ca}^{2+}]_e\) has been studied. These experiments set out to investigate the \([\text{Ca}^{2+}]_e\)-sensitivity of mGluR1α expressed in a baby hamster kidney cell line.

Increasing \([\text{Ca}^{2+}]_e\) was demonstrated to enhance agonist-stimulated phosphoinositide signalling via mGluR1α, but not the M3-muscarinic receptor, expressed in baby hamster kidney (BHK) cells, suggesting that the modulatory effect of Ca\(^{2+}\) is selective to signalling via mGluR1α. The limitation of this modulatory effect of Ca\(^{2+}\) in BHK-\(S^166A\)-mGluR1α lends support for the involvement of this region in the Ca\(^{2+}\) sensitivity of mGluRs, as demonstrated by the serine 166 residue of mGluR1α being essential for Ca\(^{2+}\) to behave as an agonist of mGluR1α (Kubo et al., 1998). However, no conclusive evidence for the site of modulation by Ca\(^{2+}\) was obtained.

The ability of Ca\(^{2+}\) to modulate signalling via mGluR1α inducibly expressed in a Chinese hamster ovary (CHO) cell line, was then investigated. As in BHK cells increasing \([\text{Ca}^{2+}]_e\) resulted in a graded increase in agonist-stimulated \([^3\text{H}]\)-InsP\(_1\) accumulation via mGluR1α, not observed in CHO cells expressing the M3-muscarinic receptor, suggesting that the modulatory effect of \([\text{Ca}^{2+}]_e\) is selective to signalling via mGluR1α. Studies then investigated the site at which Ca\(^{2+}\) had its modulatory effect. Varying \([\text{Ca}^{2+}]_e\) had no effect on membrane phospholipid levels. Measurement of \([\text{Ca}^{2+}]_e\) elevation in different \([\text{Ca}^{2+}]_e\) was not conclusive in determining the site of action of Ca\(^{2+}\). Manipulations of the levels of PKC and CaM in the cells suggest these proteins are not involved in the modulation of phosphoinositide signalling via mGluR1α by \([\text{Ca}^{2+}]_e\).

To investigate the importance of the serine 166 residue for the modulatory effect of Ca\(^{2+}\), the cDNAs for mGluR1α and \(S^{166D}\)-mGluR1α were inserted into a suitable plasmid; pcDNA 3, and transiently transfected into CHO cells using FuGene-6. A graded decrease in the EC\(_{50}\) for quisqualate-stimulated \([^3\text{H}]\)-InsP\(_1\) accumulation as \([\text{Ca}^{2+}]_e\) was increased was observed in cells transfected with wild type mGluR1α, but not \(S^{166D}\)-mGluR1α. Thus, these initial data provide evidence that the modulatory effect of Ca\(^{2+}\) on agonist-stimulated signalling via mGluR1α is, like the agonist activity of Ca\(^{2+}\) on mGluR1α expressed in Xenopus oocytes, dependent on the presence of the serine 166 residue. Further studies are required to confirm and extend these data.
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CONTENTS

CHAPTER 1 - INTRODUCTION

1.1 Cloning of mGluRs 2
1.2 Classification of mGluRs 4
  1.2.1 Sequence homology 5
  1.2.2 Signal transduction mechanisms upon expression in heterologous systems 5
  1.2.3 Pharmacological profiles 9
1.3 G-protein coupling of mGluRs 13
1.4 The G-protein cycle and phosphoinositide hydrolysis 16
1.5 Structural features and functional domains of mGluRs 17
  1.5.1 The amino-terminal domain 18
  1.5.2 Intramolecular transduction 22
  1.5.3 Regions involved in G-protein coupling 23
  1.5.4 The carboxy-terminal domain 25
1.6 Localisation of mGluRs in the mammalian central nervous system 28
1.7 Transduction mechanisms in native preparations 29
1.8 Physiological roles of mGluRs 32
  1.8.1 Neuronal excitability 32
  1.8.2 Presynaptic effects 34
  1.8.3 Modulation of other neurotransmitters 35
  1.8.4 Synaptic plasticity 37
1.9 Pathophysiological roles of mGluRs 42
  1.9.1 Roles of mGluRs in neurotoxicity 42
  1.9.2 Nociception 46
1.10 Aims 48

CHAPTER 2 - METHODS

2.1 Materials 49
2.2 Cell culture techniques 50
  2.2.1 BHK cell culture 50
  2.2.2 CHO cell culture 50
2.3 Determination of \([3\text{H}]-\text{inositol monophosphate accumulation}\) 51
  2.3.1 Poly-L-lysine coating of plasticware 54
2.4 Inositol 1,4,5-trisphosphate mass determination 55
  2.4.1 Generation of samples for Ins(1,4,5)P\(_3\) mass determination 55
  2.4.2 Ins(1,4,5)P\(_3\) mass assay 55
2.5 Preparation of cell membranes 56
2.6 Membrane phospholipid determination 57
2.7 Determination of protein concentration 58
2.8 Calcium phosphate transfections 58
2.9 \([^3\text{H}]-\text{NMS (1-[N-methyl-}^{3}\text{H] scopolamine methyl chloride) binding}\) 61
  2.9.1 On intact cells 61
  2.9.2 Using cell membranes 61
2.10 \([\text{Ca}^{2+}]\), measurements 63
2.10.1 Cell preparation
2.10.2 Fura-2 as an indicator of changes in $[Ca^{2+}]_{i}$
2.10.3 Loading cells with fura-2 AM
2.10.4 Measurement of the fluorescence changes
2.11 SDS polyacrylamide gel electrophoresis, Western blotting and immunodetection of proteins
2.11.1 Preparation of cell lysates
2.11.2 SDS-PAGE
2.11.3 Western blotting
2.11.4 Immunodetection of protein bands
2.12 Cloning and maintenance of plasmid DNA
2.12.1 DNA quantification
2.12.2 Large scale plasmid purification (for subcloning only)
2.12.3 Large scale plasmid purification (for mammalian cell transfection only)
2.12.4 Small scale plasmid purification
2.12.5 Glycerol stock maintenance
2.12.6 Restriction endonuclease digestion of DNA
2.12.7 Isolation and gel purification of DNA
2.12.8 Ligation of DNA
2.12.9 Transformation of E. Coli
2.12.10 DNA sequencing
2.12.11 DNA sequence analysis
2.12.12 COS7 transient transfection with DEAE Dextran / Chloroquine
2.12.13 Transient transfection with Fugene-6
2.13 Data Analysis

CHAPTER 3 - MODULATION OF RAT TYPE 1α METABOTROPIC GLUTAMATE RECEPTOR SIGNALLING IN BABY HAMSTER KIDNEY CELLS
3.1 Investigations into the effect of varying $[Ca^{2+}]_{e}$ on phosphoinositide signalling in BHK cells stably expressing mGluR1α
3.1.1 Concentration-dependence of agonist-stimulated $[^3H]$,InsP₁ accumulation in BHK-mGluR1α cells in the presence of different $[Ca^{2+}]_{e}$
3.2 Investigations into the effect of varying $[Ca^{2+}]_{e}$ on phosphoinositide signalling in BHK cells stably expressing the muscarinic-M₃ receptor
3.2.1 Screening of BHK-570 cells transfected with the M₃-muscarinic receptor using $[^3H]$,NMS binding
3.2.2 Methacholine-stimulated $[^3H]$,InsP₁ accumulation in BHK-m3 cells
3.2.3 $[^3H]$,NMS saturation binding using BHK-m3 cell membranes
3.2.4 Concentration-dependence of methacholine-stimulated $[^3H]$,InsP₁ responses in BHK-m3 cells in the presence of different $[Ca^{2+}]_{e}$
3.3 Investigations into the site of action of $[Ca^{2+}]_{e}$
3.3.1 Effects of polyvalent cations on $[^3H]$,InsP₁ accumulation in BHK-mGluR1α cells
3.3.2 Time-dependence of agonist-stimulated Ins(1,4,5)P₃ mass responses in BHK-mGluR1α cells in the presence of different $[Ca^{2+}]_{e}$
3.3.3 Intracellular Ca²⁺ measurements in BHK-mGluR1α cells following agonist stimulation of mGluR1α expressed in BHK cells
3.3.4 Concentration-dependence of agonist-stimulated $[^3H]$,InsP₁ accumulation in BHK-$^{S165A}$-mGluR1α cells in the presence of different
3.4 Investigations into the pertussis toxin sensitivity of BHK-m3 cells compared to BHK-mGluR1α cells

3.4.1 Effects of pertussis toxin pre-treatment on [3H]-InsP₁ accumulation in BHK-mGluR1α cells in response to stimulation with quisqualate, AlF₄⁻ or ionomycin

3.4.2 Effects of pertussis toxin pre-treatment on [3H]-InsP₁ accumulation in BHK-m3 cells in response to stimulation with methacholine

3.5 Discussion

CHAPTER 4 - MODULATION OF HUMAN TYPE 1α METABOTROPIC GLUTAMATE RECEPTOR SIGNALLING IN CHINESE HAMSTER OVARY CELLS

4.1 Investigations into the effect of varying [Ca²⁺]ₑ on phosphoinositide signalling in CHO cells inducibly expressing mGluR1α

4.1.1 [Ca²⁺]ₑ-dependence of basal and agonist-stimulated [³H]-InsP₁ accumulation in CHO-lac-hmGluR1α cells

4.2 Investigations into the effect of varying [Ca²⁺]ₑ on phosphoinositide signalling in CHO cells stably expressing the M₃-muscarinic receptor

4.2.1 [Ca²⁺]ₑ-dependence of basal and agonist-stimulated [³H]-InsP₁ accumulation in CHO-m3 cells

4.3 Membrane phospholipid measurements in CHO-lac-hmGluR1α cells following agonist stimulation of mGluR1α inducibly expressed in CHO cells

4.4 Intracellular Ca²⁺ measurements in CHO-lac-hmGluR1α cells following agonist-stimulation of mGluR1α

4.4.1 Reproducibility of [Ca²⁺]ₑ elevation following repeated stimulation of mGluR1α with quisqualate

4.4.2 Changes in peak [Ca²⁺]ₑ elevation following stimulation of mGluR1α with quisqualate in the presence of different [Ca²⁺]ₑ

4.4.3 Changes in the plateau phase of [Ca²⁺]ₑ elevation following stimulation of mGluR1α with quisqualate in the presence of different [Ca²⁺]ₑ

4.5 Intracellular Ca²⁺ measurements in CHO-m3 cells following agonist stimulation of the M₃-muscarinic receptor

4.5.1 Reproducibility of [Ca²⁺]ₑ elevation following repeated stimulation of the M₃-muscarinic receptor with methacholine

4.5.2 Changes in peak [Ca²⁺]ₑ elevation following stimulation of the M₃-muscarinic receptor with methacholine in the presence of different [Ca²⁺]ₑ

4.5.3 Changes in the plateau phase of [Ca²⁺]ₑ elevation following stimulation of the M₃-muscarinic receptor with methacholine in the presence of different [Ca²⁺]ₑ

4.6 Investigations to determine the importance of calmodulin (CaM) and protein kinase C (PKC) in the modulation of signalling via mGluR1α by [Ca²⁺]ₑ

4.6.1 Comparison of the effects of a PKC activator (PdBu) and a PKC inhibitor (Ro-318220) on the concentration-dependence of agonist-stimulated [³H]-InsP₁ accumulation in CHO-lac-hmGluR1α and CHO-m3 cells

4.6.2 Effect of a PKC activator (PdBu) and PKC inhibitors on [³H]-InsP₁ accumulation in the presence of different [Ca²⁺]ₑ in CHO-lac-hmGluR1α cells
4.6.3 Effect of CaM antagonists on [H]-InsP₁ accumulation in the presence of different \([\text{Ca}^{2+}]_e\) in CHO-lac-hmGluR1α cells

4.7 Discussion

CHAPTER 5 - MANIPULATION OF WILD-TYPE RAT mGluR1α AND S₁₆₆₆-mGluR1α MUTANT cDNA FOR INVESTIGATION INTO THE INVOLVEMENT OF THE SERINE 166 RESIDUE IN THE CA²⁺-SENSITIVITY OF mGluR1α.

5.1 The pmGR1 plasmid

5.2 Isolation of the cDNA for mGluR1α and S₁₆₆₆-mGluR1α from pmGR1 and pmGR1-S₁₆₆₆

5.3 Engineering of DNA with a Bam HI restriction endonuclease site followed by the sequence for the amino-terminal region of mGluR1α and S₁₆₆₆-mGluR1α

5.4 Isolation of the 3560 bp region of both mGluR1α and S₁₆₆₆-mGluR1α from pmGR1 and pmGR1-S₁₆₆₆ between the Eco RI and Not I restriction endonuclease sites

5.5 Ligation of the amino-terminal fragment of mGluR1α into pcDNA 3 containing the 3560 bp fragments of mGluR1α or S₁₆₆₆-mGluR1α

5.6 Transient transfection of the pcDNA 3 mGluR1α and pcDNA 3 S₁₆₆₆-mGluR1α into COS 7 cells

5.7 Introduction of the S₁₆₆₆ mutation into pcDNA 3 containing the cDNA for mGluR1α

5.8 Transient transfection of pcDNA 3 mGluR1α and pcDNA 3 S₁₆₆₆-mGluR1α into HEK cells

5.9 Stable transfection of WT mGluR1α and S₁₆₆₆-mGluR1α cDNA into CHO cells

5.10 Transient transfection of WT mGluR1α and S₁₆₆₆-mGluR1α cDNA into CHO cells

5.11 Effect of varying \([\text{Ca}^{2+}]_e\) on quisqualate-stimulated [H]-InsP₁ accumulation in CHO cells transiently transfected with mGluR1α or S₁₆₆₆-mGluR1α

5.12 Discussion

CHAPTER 6 - CONCLUDING DISCUSSION

BIBLIOGRAPHY
Glutamate is used as a neurotransmitter by most of the excitatory synapses in the central nervous system. Glutamate was initially thought to act solely on ionotropic receptors (iGluRs): N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) and kainate (KA) receptors to mediate fast excitatory transmission (Monaghan et al, 1989; Hollmann and Heinemann, 1994). However, since the cloning of the first metabotropic glutamate receptor (mGluR) in 1991 (Houamed et al, 1991; Masu et al, 1991) the existence of a new family of G-protein coupled glutamate receptors has become clear. These metabotropic glutamate receptors (mGluRs) have since become the focus of intensive research.

mGluRs are located at postsynaptic sites, and as autoreceptors at presynaptic sites, where they act to modulate the fast excitatory neurotransmission mediated by the ionotropic glutamate receptors in the central nervous system. The diversity of transduction mechanisms among mGluRs enables them to enhance or inhibit glutamate-mediated neurotransmission. As a result of this modulation the mGluRs have been implicated to have roles in many physiological (e.g. synaptic plasticity) and pathophysiological (e.g. brain ischaemia, epilepsy) processes.

The development of sub-type selective agonists and antagonists is aiding the characterisation of the subtypes of mGluRs involved in these processes, and the mechanisms by which they act. It is becoming clear that the development of therapeutic agents which target specific mGluRs may be important in the prevention of glutamate-induced neuronal death, and the treatment of illnesses such as; epilepsy, Alzheimer's disease and Parkinson's disease.
1.1 Cloning of mGluRs

The ability of glutamate, but not the ionotropic glutamate receptor agonists NMDA and kainate, to activate phospholipase C (PLC) in a number of cell preparations, e.g. cultured mouse striatal neurones, (Sladeczek et al, 1985), rat hippocampal slices (Nicoletti et al, 1986a; 1986b) and rat cerebellar granule cells (Nicoletti et al, 1986c) suggested that glutamate was activating a novel class of G-protein coupled glutamate receptor (GPCR). Direct evidence for this hypothesis was provided following injection of rat brain mRNA into Xenopus oocytes. Quisqualate and glutamate evoked a fluctuating response of delayed onset which was sensitive to pertussis toxin (PTx) and could be mimicked by inositol trisphosphate (Ins(1,4,5)P3) injection, suggesting the involvement of intracellular metabolic processes, Gproteins and the phosphoinositide cascade, respectively (Sugiyama et al, 1987).

By screening for functional expression in Xenopus oocytes, the cDNA of the first mGluR, named mGluR1α, was cloned simultaneously by 2 groups from rat cerebellum cDNA libraries (Houamed et al, 1991; Masu et al, 1991). mGluR1α sequence analysis indicated shared topology, but little homology, with other members of the G protein-coupled receptor family (Houamed et al, 1991; Masu et al, 1991). Thus, it seemed likely that mGluR1α belonged to a new sub-family of GPCRs. mGluR1α cDNA was used as a probe for cross-hybridisation screening of a rat brain cDNA library to aid the identification of genes for additional subtypes of mGluR. This led to the identification of 3 novel mGluRs; mGluR2, 3 and 4, and a smaller splice variant of mGluR1, mGluR1β (Tanabe et al, 1992), confirming the existence of a new sub-family of GPCRs. Further cloning studies led to the
identification of mGluR5 (Abe et al, 1992), mGluR6 (Nakajima et al, 1993) and mGluR7 (Okamoto et al, 1994; Saugstad et al, 1994). An additional subtype, mGluR8 was originally cloned from mouse brain (Duvoisin et al, 1995), and subsequently from rat brain (Saugstad et al, 1997). Homologues to mGluRs 1-8 have been cloned from human brain, the genes for these human mGluRs (hmGluRs) encode amino acid sequences highly homologous (93 - 96 %) to their rat counterparts (Daggett et al, 1995; Desai et al, 1995; Flor et al, 1995a; Flor et al, 1995b; Emile et al, 1996; Makoff et al, 1996; Flor et al, 1997; Laurie et al, 1997; Scherer et al, 1997). An invertebrate homologue of mGluRs from Drosophila melanogaster has also been isolated; this mGluR has 43 % and 45 % homology with mammalian mGluRs 2 and 3, respectively (Parmentier et al, 1996).

Cloning studies have also led to the identification of a number of mGluR splice variants, namely mGluR1 β, c, d, mGluR4 a and b, mGluR5 a and b, mGluR7 a and b and mGluR8 a and b (Pin et al, 1992; Tanabe et al, 1992; Minakami et al, 1993; Iversen et al, 1994; Joly et al, 1995; Mary et al, 1997; Thomsen et al, 1997; Corti et al, 1998). Human homologues to all except mGluR1c have been identified (Minakami et al, 1994; Desai et al, 1995; Laurie et al, 1996; Stephan et al, 1996; Flor et al, 1997; Malherbe et al, 1999). The insertion of an 85 base pair (bp) sequence into the coding region for the carboxy-terminal domain of the original mGluR1 (now mGluR1α) sequence results in the generation of mGluR1β with the 20 amino acids unique to mGluR1β replacing the carboxy-terminal 312 amino acids of mGluR1α (Tanabe et al, 1992). mGluR1c is generated via the replacement of the carboxy-terminal 312 amino acids of mGluR1α, with 10 amino acids unique to mGluR1c (Pin et al, 1992). mGluR1d is generated following the deletion of 35 bp, and the
replacement of the 313 carboxyl-terminal residues of mGluR1α with 26 residues unique to mGluR1d (Mary et al., 1997). Thus, the alternative splicing of mGluR1 results in splice variants lacking the long carboxy-terminal domain of mGluR1α (see figure 1.1). As the distribution of the splice variants in rat brain slices only differs subtly, such splicing may have important consequences for the function and targeting of the receptor within the same cell, allowing different patterns of \([\text{Ca}^{2+}]_e\) elevation within the same cell (Pin et al., 1992).

The alternative acceptor splice site of mGluR5 is found in an intron conserved in a similar position with that of mGluR1α. However in contrast to mGluR1α, alternative splicing of mGluR5 results in the generation of longer carboxy-terminal domains, the insertion of 96 bp into the coding sequence for mGluR5a resulting in the generation of a longer splice variant; mGluR5b, with an additional 32 amino acids within its carboxy-terminal domain (Minakami et al., 1993; Joly et al., 1995).

1.2 Classification of mGluRs

The mGluR sub-family of GPCRs have been sub-divided further into 3 groups based on their amino acid sequence homology (see figure 1.2). This classification is also supported by their signal transduction mechanisms and their pharmacological profiles.
Fig. 1.1 Schematic representation of the different lengths of the C-terminal domains of the mGluR1 splice variants. The C-terminal coding region is represented by white boxes. The 7th transmembrane domain is represented by the black boxes. The alternative acceptor splice sites for these variants are found in an intron conserved in position among the different variants. This intron is represented by $V$. Adapted from Mary et al, (1997).
Fig. 1.2 Schematic representation of the sequence homology (expressed as a percentage) of the mGluR subtypes with each other and the CaSR. Adapted from Conn and Pin (1997) and Duvoisin et al., (1995).
1.2.1 Sequence homology

The 60% sequence identity between mGluR subtypes 1 and 5 placed them in a distinct group, the group I mGluRs (Abe et al, 1992). Although mGluRs 1 - 4 share sequence homology in the extracellular amino-terminal and membrane spanning domains (43 - 47 %), less homology is seen in the carboxy-terminal region of these receptors (Tanabe et al, 1992). Thus, mGluRs 2 and 3 were assigned to a separate group, the group II mGluRs, due to their high overall sequence homology of 67 %, and mGluR4 was assigned to a separate sub-family, the group III mGluRs. The ~ 70 % sequence similarity between mGluR4 and both mGluR6 and mGluR7 (Nakajima et al, 1993; Saugstad et al, 1994) placed these receptors into group III, with the later addition of mGluR8 which shares ~ 75 % sequence identity with mGluRs 4 and 7 and 70 % identity with mGluR6 (Duvoisin et al, 1995; Saugstad et al, 1997). Sequence alignments confirmed this sub-division, demonstrating a much lower level of homology between, compared to within, groups; 43% between group I and groups II and III, and 46 % between group II and III (Duvoisin et al, 1995).

1.2.2 Signal transduction mechanisms upon expression in heterologous systems

mGluRs 1 and 5, isolated from both rat (Houamed et al, 1991; Masu et al, 1991; Abe et al, 1992; Aramori and Nakanishi, 1992; Pin et al, 1992; Thomsen et al, 1993; Joly et al, 1995) and human (Minakami et al, 1994; Daggett et al, 1995; Desai et al, 1995; Laurie et al, 1996; Stephan et al, 1996) brain couple to phosphoinositide hydrolysis upon expression in heterologous systems. Both rat mGluR1β and mGluR1c
generate a slower longer lasting intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{\text{i}}\)]) elevation following phosphoinositide hydrolysis compared to mGluR1\(\alpha\) (Pin et al, 1992; Pickering et al, 1993) and rat mGluR1d is unable to evoke [Ca\(^{2+}\)] elevation (Mary et al, 1997). In addition mGluRs 1\(\beta\), c and d are not constitutively active in contrast to mGluR1\(\alpha\) (Prezeau et al, 1996; Mary et al, 1997). Although no differences were seen in the [Ca\(^{2+}\)] elevation characteristics of hmGluR1\(\alpha\) activation compared to hmGluR1\(\beta\) activation (Stephan et al, 1996), in common with their rat homologues, hmGluR1\(\alpha\), but not hmGluR1\(\beta\) and hmGluR1d, exhibited constitutive activity (Hiltscher et al, 1998). Thus, the longer carboxy-terminal domain of mGluR1\(\alpha\) has been implicated in increasing the coupling efficiency of mGluR1 (Pin et al, 1992; Pickering et al, 1993). Such rapid transient [Ca\(^{2+}\)], elevation and constitutive activity are also characteristics of mGluR5 a and b activation (Joly et al, 1995). As both mGluR5 splice variants have a long carboxy-terminal domain, this adds further support to the proposed role of the longer carboxy-terminal domain of mGluR1\(\alpha\) in enhancing the coupling efficiency of the receptor. Perhaps surprisingly, deletion of the final 19 residues from mGluR1c results in a receptor with the properties of mGluR1\(\alpha\). This deletion includes a cluster of 4 basic residues which are found in all mGluR1 splice variants. In an attempt to rationalise this finding an interaction of the long carboxy-terminal domain of mGluR1\(\alpha\) with this region has been proposed to counteract an inhibitory action of this region resulting in the increased coupling efficiency of mGluR1\(\alpha\) compared to mGluR1c (Mary et al, 1998).

Different patterns of [Ca\(^{2+}\)], elevation have been observed upon expression of the different group I subtypes in human embryonic kidney (HEK) cells. mGluR1\(\alpha\) stimulation resulted in a peak of [Ca\(^{2+}\)], elevation from intracellular Ca\(^{2+}\) stores,
followed by a plateau or oscillatory phase, due to Ca\(^{2+}\) influx into the cell. In contrast, mGluR5a stimulation resulted in continuous Ca\(^{2+}\) oscillations via intracellular Ca\(^{2+}\) store mobilisation (Kawabata et al, 1998). This may have implications for a divergence in signal transduction following activation of mGluR1 and mGluR5, both of which are PLC-coupled mGluRs.

The group I mGluRs couple to a number of additional signal transduction pathways. mGluR1\(\alpha\) evokes cyclic AMP (cAMP) accumulation upon expression in Chinese hamster ovary (CHO) or baby hamster kidney (BHK) cells (Aramori and Nakanishi, 1992, Pickering et al, 1993; Thomsen et al, 1996). cAMP accumulation is also stimulated in porcine kidney epithelial (LLC-PK1) cells expressing mGluR1\(\alpha\) and both mGluR5a and b (Joly et al, 1995), although not upon expression of mGluR5 in CHO cells (Abe et al, 1992). mGluR1\(\alpha\) inhibits M-type K\(^+\) channels following injection into superior cervical ganglion neurones (Ikeda et al, 1995). In addition mGluR1\(\alpha\) activation results in the inhibition of a G-protein-coupled inwardly rectifying K\(^+\) channel (GIRK) in Xenopus oocytes (Sharon et al, 1997). In both cases it is not clear whether channel inhibition occurs secondary to phosphoinositide hydrolysis as a result of protein kinase C (PKC) activation. The group I mGluRs also inhibit both N-type and P/Q type Ca\(^{2+}\) channels in a voltage-dependent manner (McCool et al, 1998). mGluR1\(\alpha\) also mediates the phosphorylation and activation of extracellular signal-regulated protein kinases (ERKs) upon expression in CHO cells (Ferraguti et al, 1999). The ability of mGluR1\(\alpha\) and mGluR5 to couple to additional signal transduction pathways provides further evidence for an increase in coupling efficiency conferred upon these receptors by their long carboxy-terminal tails.
The group II mGluRs, and their invertebrate homologue (DmGluRA) inhibit cAMP accumulation upon expression in heterologous systems (Tanabe et al, 1992; Tanabe et al, 1993; Flor et al, 1995a; Emile et al, 1996; Parmentier et al, 1996). mGluR2 also inhibits N-type Ca\(^{2+}\) channels following injection into superior cervical ganglion neurones (Ikeda et al, 1995). Both mGluR2 and mGluR3 inhibit this channel upon transient transfection into HEK cells stably expressing the channel (McCool et al, 1996) as well as activating GIRK upon expression in Xenopus oocytes (Sharon et al, 1997). mGluR2 also mediates the phosphorylation and activation of ERK upon expression in CHO cells (Ferraguti et al, 1999).

The group III subtypes; 4, 6, 7 and 8, are also coupled to the inhibition cAMP accumulation upon expression in heterologous systems (Nakajima et al, 1993; Tanabe et al, 1993; Okamoto et al, 1994; Saugstad et al, 1994; Duvoisin et al, 1995; Flor et al, 1995b; Flor et al, 1997; Laurie et al, 1997; Malherbe et al, 1999). mGluRs 4, 6, 7 and 8 have also been reported to activate a G-protein coupled inwardly rectifying K\(^+\) channel upon expression in Xenopus oocytes (Saugstad et al, 1997; Sharon et al, 1997). In addition mGluR4 mediates the activation of ERK (Ferraguti et al, 1999). No difference was observed in the coupling characteristics of rat mGluR4a and b, expressed in Sf-9 cells (Thomsen et al, 1997), hmGluR7a compared to hmGluR7b expressed in CHO cells (Flor et al, 1997) or hmGluR8a compared to hmGluR8b expressed in CHO cells (Malherbe et al, 1999).

Thus, studies in heterologous expression systems have been useful in characterising the signal transduction pathways activated by the different mGluR
subtypes. The modulation of various effectors following mGluR activation suggested that they may have widespread importance in the central nervous system.

1.2.3 Pharmacological profiles

The pharmacological profiles of the cloned mGluR subtypes also supported their classification into different groups. Upon expression of mGluR1α in heterologous systems the rank order of agonist potency for phosphoinositide hydrolysis is: quisqualate > L-glutamate ≥ ibotenate > L-homocysteine sulphinate > trans-(±)-1-amino-1,3-cyclopentanedicarboxylic acid (trans-ACPD) (Masu et al, 1991; Aramori and Nakanishi, 1992; Thomsen et al, 1993). This pharmacological profile is similar for the mGluR1β, mGluR1c and mGluR1d splice variants (Pin et al, 1992; Pickering et al, 1993; Mary et al, 1997), however these agonists are less potent at the shorter splice variants of mGluR1, again giving credence to a role for the longer carboxy-terminal tail of mGluR1α in increasing coupling efficiency to G-proteins. The same agonist rank order of potency is seen at the other group I mGluR, mGluR5 upon expression in CHO cells (Abe et al, 1992), however upon expression of mGluR1α, mGluR5 a and b in LLC-PK1 cells, ibotenate is seen to be more potent than glutamate at mGluR5, with no difference observed between the pharmacological profiles of the mGluR5 splice variants (Joly et al, 1995). Such differences could be due to different receptor expression levels or the different cell types used. Quisqualate is also the most potent agonist at hmGluR1α, consistent with its rat counterpart, followed in potency by 3,5-dihydroxyphenylglycine (3,5-DHPG) > (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine (L-CCG-I) ≥ 1-aminocyclopentane-1S,3R-dicarboxylate (1S,3R-ACPD) (Desai et al, 1995). A study of [Ca²⁺], elevation
as a measure of phosphoinositide hydrolysis has revealed no difference in the rank order of agonist potencies between the hmGluR1α and hmGluR1β splice variants; quisqualate > glutamate > ACPD (Stephan et al., 1996) in contrast to the rat mGluR1α and 1β splice variants. That of hmGluR1d is also typical of group I mGluRs upon expression in CHO cells (Hiltscher et al., 1998). The pharmacological profile of hmGluR5 is similar to that of its rat counterpart; quisqualate > L-glutamate = ibotenate > 1S,3R-ACPD, with no difference seen between the a and b splice variants (Minakami et al., 1994; Daggett et al., 1995).

Both mGluR1α-mediated stimulation of cAMP accumulation and inhibition of the M-type K⁺ channel display a pharmacological profile comparable to that seen for phosphoinositide hydrolysis (Aramori and Nakanishi, 1992; Ikeda et al., 1995; Thomsen et al., 1996).

The inhibition of cAMP accumulation by mGluR2 and 3 shares the same rank order of agonist potency; L-glutamate > t-ACPD > ibotenate > quisqualate (Tanabe et al., 1992; Tanabe et al., 1993). Upon expression in CHO cells the pharmacological profile of hmGluR2 was found to be directly comparable to rat mGluR2; L-CCG-I > L-glutamate ≥ 1S,3R-ACPD (Flor et al., 1995a). That of hmGluR3 also compares to its rat counterpart (Emile et al., 1996). A similar pharmacological profile is observed in mGluR2 mediated inhibition of the N-type Ca²⁺ channel; 2-(2,3-dicarboxycyclopropyl)glycine (DCG-IV) > L-glutamate > 1S,3R-ACPD > quisqualate (Ikeda et al., 1995).
The inhibition of cAMP accumulation by the group III mGluRs, mGluR4 a and b, 6, 7 share the following rank order of agonist potency: L-2-amino-4-phosphonobutyrate (L-AP4) > L-serine-O-phosphate (L-SOP) > L-glutamate >> quisqualate (Nakajima et al, 1993; Tanabe et al, 1993; Okamoto et al, 1994). This pharmacological profile is the same for mGluRs 7 a and b and 8 a and b when transiently co-expressed in HEK cells with a PLC activating chimeric Gαqι9 G-protein in which the nine carboxy-terminal residues of Gαq are replaced with those of Gαi2 (Corti et al, 1998). That of mGluR8 isolated from mouse brain differs in that L-glutamate is more potent than L-AP4 (Duvoisin et al, 1995). However, although mGluR8 isolated from rat brain shares the highest sequence homology with the other group III mGluRs, L-CCG-I was found to be as potent as L-AP4 at the receptor followed by glutamate then ACPD (Saugstad et al, 1997). The pharmacological profiles of the rat group III mGluRs is closely mimicked by hmGluR4, 6, 7 a and b (Flor et al, 1995b; Flor et al, 1997; Laurie et al, 1997). For both hmGluR8 a and b L-AP4 was the most potent agonist with L-CCG-I being equipotent to L-SOP, followed by glutamate (Malherbe et al, 1999).

Thus, it can be seen that within groups the pharmacological profiles of the mGluR subtypes are very similar. However, as some agonists are active at more than one subtype or even group of mGluRs, the development of selective agonists at different mGluR subtypes has been an area of intensive research, to aid the characterisation of the roles of mGluRs in physiological and pathophysiological processes.

To date there are still few selective agonists for the group I mGluRs, the most selective being 3,5-DHPG, although this still has some activity at NMDA receptors at
high concentrations (Schoepp et al, 1994; Brabet et al, 1995). (RS)-2-chloro-5-hydroxyphenylglycine (CHPG), is a putative selective agonist for mGluR5, but has a very low potency (Doherty et al, 1997). A number of selective agonists for the group II mGluRs exist; the highly potent (1S,2S,5R,6S)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylate (LY354740) and 1R,4R,5S,6R-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate (LY379268) (Schoepp et al, 1997; Monn et al, 1998), 2R,4R-4-aminopyrrolidine-2,4-dicarboxylate (2R,4R-APDC) (Schoepp et al, 1995) and DCG-IV (Brabet et al, 1998), although DCG-IV also acts as an antagonist at the group III receptors and iGluRs (Brabet et al, 1998). N-acetyl-aspartyl-glutamate (NAAG) shows some degree of discrimination between the subtypes of the group II mGluRs, being more potent at mGluR3 than mGluR2 (Wroblewska et al, 1997; Schaffhauser et al, 1998). Several selective agonists for the group III mGluRs exist; (1S,3R,4R)-1-aminocyclopentane-1,3,4-tricarboxylate (ACPT-I), (3S,4S)-1-aminocyclopentane-1,3,4-tricarboxylate ((+)-ACPT-III) (Acher et al, 1997) and 4-phosphonophenylglycine (4-PPG) (Gasparini et al, 1999a).

The identification of mGluR subtype-selective antagonists is also important in understanding the roles of mGluRs. α-thioxanthylmethyl-3-carboxycyclobutylglycine (LY393675) is a group I-selective competitive antagonist (Baker et al, 1998), with 4-carboxy-3-hydroxyphenylglycine (4C3HPG), 2-(3′-carboxybicyclo[1.1.1]pentyl)glycine (CBPG) (Pellicciari et al, 1996) and (S)-2-methyl-4-carboxyphenylglycine (LY367385) (Clark et al, 1997) being the most potent mGluR1-selective competitive antagonists. NPS 2390 has been identified as a group I mGluR non-competitive antagonist (Van Wagenen et al, 1998) and selective non-competitive antagonists for both mGluR1 and mGluR5 exist, 7-(hydroxy-imino)cyclopropan[b]chromen-1-a-
carboxylic acid ethylester (CPCCOEt) (Hermans et al, 1998a; Litschig et al, 1999) and 2-methyl-6-(phenylethynyl)pyridine (MPEP) (Gasparini et al, 1999b), respectively. No antagonists selective for either mGluR2 or mGluR3 have been identified, although a number of antagonists selective for the group II mGluRs exist; α-methyl-L-CCG-I (MCCG-I) (Jane et al, 1994), (2S,1'S,2'S,3'R)-2-(2'-carboxy-3'-phenylcyclopropyl)glycine (PCCG-IV) (Thomsen et al, 1996), 2S-2-amino-2-(1S,2S-2-carboxycycloprop-1-yl)-3-(xanth-9-yl)propanoate (LY341495) (Kingston et al, 1998) and (2S,4S)-2-amino-4-(4,4-diphenylbut-1-yl)-pentane-1,5-dioate (LY307452) (Wermuth et al, 1996). A number of antagonists exist for the group III mGluRs, α-methyl-AP4 (MAP4) (Jane et al, 1994) being the first to be described, however at high concentrations these antagonists have activity at other mGluR subtypes, e.g. α-methyl-4-phosphonophenylglycine (MPPG) (Jane et al, 1995) and DCG-IV (Brabet et al, 1998).

1.3 G-protein coupling of mGluRs

Studies of the G proteins involved in the coupling of mGluR1α to PLC give conflicting results. Stimulation of PLC following mGluR1α activation, upon expression in Xenopus oocytes, CHO and BHK cells, has been reported to be partially (40-80%) inhibited by PTx treatment (Houamed et al, 1991; Masu et al, 1991; Aramori and Nakanishi 1992; Pickering et al, 1993) indicating some degree of coupling of mGluR1α to members of the PTx-sensitive Gι0 family of G-proteins. The other component of the response is likely mediated by members of the PTx-insensitive PLC coupled Gq11 family of G-proteins. However, PTx treatment has also been shown to enhance the coupling of mGluR1α to PLC, in BHK cells, under
conditions in which glutamate produced endogenously by the cells is removed from the buffer (Carruthers et al, 1997). The earlier conflicting results may be explained by PTx treatment sensitising the cells to μM concentrations of glutamate released into the buffer by the cells prior to agonist stimulation, resulting in an apparent increase in basal values. Subsequent expression of responses as fold over basal therefore suggest a decrease in the extent of the response in the presence of PTx compared to control conditions, thus masking the effect reported by Carruthers et al (1997). The enhancement of mGluR1α coupling to PLC following PTx treatment reported by Carruthers et al (1997) suggests there may be a dual regulation of PLC activity by both Gv0 and Gq11 G-proteins following mGluR1α activation. It is proposed that Gv0 proteins have a negative modulatory influence on the activation of PLC by Gq11. The PLC isoform activated most readily by the α subunit of Gq11 proteins is PLCβ1, although PLCβ2 and PLCβ3 can also be activated by Gq011 (Berstein et al, 1992; Jhon et al, 1993).

The coupling of mGluR1β to PLC has been demonstrated to be insensitive to PTx treatment in mammalian cells (Pickering et al, 1993), suggesting specific coupling via Gq proteins, that of mGluR1c does however show some degree of PTx-sensitivity upon expression in Xenopus oocytes (Pin et al, 1992) suggesting some coupling via Gv0 proteins. The coupling of mGluR5 to PLC is slightly PTx-sensitive, thus a small component of the mGluR5 response may also be coupled via Gv0 proteins (Abe et al, 1992). In common with mGluR1α, both hmGluR5 a and b isoforms appear to undergo dual regulation upon co-expression with bovine homologues of Gα11, and Gα14. Thus, phosphoinositide hydrolysis is stimulated via the Gα11 homologue and inhibited via the Gα14 homologue (Minakami et al, 1994).
Although direct involvement of $G_s$ proteins in the activation of adenylyl cyclase (AC) by mGluR1α, 5 a and b has not been demonstrated, several lines of evidence support this theory. PLC activation by mGluR1α is sensitive to PTx and phorbol ester treatment whereas cAMP production is unaffected by phorbol esters and enhanced by PTx, suggesting AC is activated via a signal transduction pathway distinct from that involved in PLC activation (Aramori and Nakanishi, 1992). mGluR5 stimulates PLC, but not AC activation, in CHO cells suggesting that AC activation does not occur secondary to PLC activation (Abe et al, 1992). The inhibition of N-type and P/Q-type calcium channels occurs almost exclusively via the $G_{iv}$ family of G-proteins, however the lack of total abolishment of the inhibition of N-type $Ca^{2+}$ channel activity following PTx treatment suggests the involvement of another subtype(s) of G-protein(s) (McCool et al, 1998). mGluR1α-mediated activation of ERK is completely blocked following PTx treatment further suggesting the involvement of $G_{iv}$ proteins (Ferraguti et al, 1999).

The inhibition of AC by group II and III mGluRs and *Drosophila* metabotropic glutamate receptor A (DmGluRA) is totally inhibited following PTx treatment, suggesting coupling via $G_i$ proteins (Tanabe et al, 1992; Nakajima et al, 1993; Tanabe et al, 1993; Okamoto et al, 1994; Saugstad et al, 1994; Duvoisin et al, 1995). In addition, the inhibition of N-type $Ca^{2+}$ channels by mGluRs 2 and 3, the activation of the G-protein activated $K^+$ channel GIRK by mGluR2 and the activation of ERK by mGluR2 are all sensitive to PTx suggesting coupling via $G_{iv}$ proteins (Ikeda et al, 1995; McCool et al, 1996; Sharon et al, 1997; Ferraguti et al, 1999).
Thus, the group II and III mGluRs appear to couple exclusively via $G_{i\alpha}$ proteins, whereas the group I mGluRs, in particular the longer mGluR1$\alpha$, 5 a and b isoforms couple via multiple $G\alpha$ subtypes, providing further justification for a role of the long C-terminal domain in increasing the efficiency of G-protein coupling of these receptors.

1.4 The G-protein cycle and phosphoinositide hydrolysis

GPCRs share a common method of information transfer across the plasma membrane. Following agonist binding a conformational change in the receptor allows interaction with a G protein. This results in the exchange of GDP for GTP at the $\alpha$ subunit of the heterotrimeric G protein ($\alpha\beta\gamma$) and a decrease in the affinity of the receptor for the G protein and the agonist. Subsequently the $\beta\gamma\alpha$-GTP form of the G protein dissociates into $G\alpha$-GTP and $G\beta\gamma$ which are free to activate different effectors. The hydrolysis of $G\alpha$GTP to $G\alpha$GDP returns the G protein to its heterotrimeric resting state. This hydrolysis is catalysed by the inherent GTPase activity of the $G\alpha$ subunit (see Taylor 1990; Lefkowitz et al, 1993; Lambright et al, 1996) and can be facilitated by regulators of G protein signalling (RGS) proteins (Dohlman and Thorner, 1997; Berman and Gilman, 1998).

G-protein mediated activation of PLC results in the hydrolysis of the membrane located phosphatidylinositol 4,5-bisphosphate (PIP$_2$) into Ins(1,4,5)P$_3$ and diacylglycerol. The water soluble Ins(1,4,5)P$_3$ diffuses into the cytosol, mobilising $Ca^{2+}$ from intracellular stores via binding to intracellular inositol trisphosphate receptors (IP$_3$Rs). The lipophilic diacylglycerol remains in association with the
membrane to activate PKC, which phosphorylates many cellular proteins (see Berridge and Irvine, 1984, Berridge, 1993).

1.5 Structural features and functional domains of mGluRs

The primary structure of the first cloned mGluR, mGluR1α revealed that the receptor had no sequence similarity to other known GPCRs. Analysis of the 1199 amino acid sequence of mGluR1α revealed several structural characteristics (see figure 1.3), leading to the proposal of the following topology; the presence of a putative signal peptide at the amino terminus followed by a large hydrophilic domain, indicates that mGluR1α has a large extracellular amino-terminal domain of ~ 570 amino acids, the following cluster of seven hydrophobic segments indicates the presence of seven transmembrane domains (characteristic of all GPCRs), thus the carboxy-terminal domain of ~ 360 residues is located intracellularly (Masu et al, 1991). Sequence analysis has revealed that other members of the mGluR family share the large amino-terminal domain of mGluR1α and lack of sequence similarity with other GPCRs. In addition, other members of this distinct GPCR family (family 3) have been identified; the Ca$^{2+}$-sensing receptor (CaSR) (Brown et al, 1993), the salmon bifunctional receptor (sBimR) (Kubokawa et al, 1996), the γ-aminobutyric acid type B receptor (GABA_bR) (Kaupman et al, 1997) and some pheromone receptors (Bargmann, 1997). In common with the mGluRs these receptors are larger than, and do not share sequence homology with, other GPCRs, although they do share significant sequence homology within the mGluR gene family; the CaSR shares ~ 30 % sequence similarity with the mGluRs (Brown et al, 1993), the sBimR shares 69 % homology with mGluR1α and 24 % homology with the CaSR (Kubokawa et al, 1996).
Glutamate

LIVBP* like domain
(ligand binding
and Ca\(^{2+}\) sensing)

Cysteine rich
domain
(dimerisation)

7-TMD
g-protein
coupling

Interaction with intracellular proteins
(receptor targeting
trafficking and regulation)

* LIVBP = Leucine, isoleucine and valine binding protein.

Fig 1.3 Schematic representation of mGluR1\(\alpha\). The different functional domains and associated roles are indicated by [ ]. Adapted from Conn and Pin, (1997).
the GABA_βR shares > 40 % sequence similarity with the mGluRs and the CaSR (Kaupmann et al, 1997).

1.5.1 The amino-terminal domain

Conserved cysteine residues

21 cysteine residues are conserved between all mGluRs, 19 of which are found in the extracellular amino-terminal domain and extracellular loops. These extracellular cysteine residues may be involved in the formation of mGluR dimers at the plasma membrane (Romano et al, 1996). mGluRs 1_α, 2, 3, 4 and 5 can form covalently linked dimers in a subtype-specific manner. In the case of mGluR5 this dimerisation occurs via the formation of disulphide bonds between conserved cysteine residue(s) located in the amino-terminal 17 kDa of the receptor. mGluR1_α also dimerises via disulphide bond formation within the amino-terminal domain (Robbins et al, 1999). However, the inability of the 1_α and β splice variants of mGluR1, which differ only in their C termini, to heterodimerise suggests the involvement of other domains or cellular proteins, such as Homer, in regulating/preventing dimerisation (Robbins et al, 1999). The CaSR can also dimerise (Bai et al, 1998). 20 of the 21 cysteine residues conserved in all mGluRs have been shown to be conserved in the CaSRs (Brown et al, 1993; O’Hara et al, 1993; Ruat et al, 1995) and 5 of these have been shown to be essential for dimerisation of the CaSR (Pace et al, 1999; Ray et al, 1999). Most of the conserved cysteine residues are not present in GABA_βR1 (Kaupmann et al, 1997), indeed, an interaction between GABA_βR1 and GABA_βR2
subtypes has been shown to occur via their carboxy-terminal domain and to be essential for formation of a functional GABA<sub>B</sub> receptor (White et al, 1998).

**N-linked glycosylation sites**

The mGluRs and other members of the family 3 GPCRs also have a number of N-linked glycosylation consensus sites within the extracellular domain (Masu et al, 1991; Brown et al, 1993; White et al, 1998). The extensive N-linked glycosylation of the human Ca<sup>2+</sup>-sensing receptor is important for receptor cell-surface expression (Ray et al, 1998). In contrast, the cell surface expression of hmGluR1<sub>α</sub> which has 4 putative N-linked glycosylation sites (Desai et al, 1995), does not appear to be dependent on N-linked glycosylation (Mody et al, 1999). Thus, despite being closely related members of a distinct GPCR sub-family, the trafficking of mGluR1<sub>α</sub> and the CaSR is differentially affected by receptor glycosylation.

**The ligand binding domain**

The amino-terminal domain of mGluRs contains a highly conserved region of hydrophobicity proposed to form the ligand binding domain (Duvoisin et al, 1995). Following the exchange of the extracellular domains of mGluR1 and mGluR2, there is a resultant exchange of the pharmacological profiles of these receptors. Thus, this region was proposed to encompass the ligand binding domain of the receptor (Takahashi et al, 1993), the homology observed in this region indicating a common mechanism for ligand binding within the mGluR family. This extracellular domain shares some sequence homology with bacterial periplasmic binding proteins,
especially when the region comparable to the binding pocket of the leucine, isoleucine and valine binding protein (LIVBP) is considered (81%). Thus, in contrast to many other GPCRs, especially those for small hydrophilic ligands, in which a ligand binding pocket is formed by the seven transmembrane domain segments (Savarese and Fraser, 1992), an alternative model for the ligand binding domain of mGluRs has been constructed based on the structures of several bacterial periplasmic binding proteins (O'Hara et al, 1993). The ligand binding domain of mGluRs is thus proposed to consist of two globular domains linked by a hinge region, ligand binding occurring in the cleft between these two globular domains. Following binding of the ligand to one domain, the hinged region folds trapping it in the pocket. This model is supported by the introduction of two mutations into the putative ligand binding site (at serine 165 and threonine 188), both of which alter the functional affinities of mGluR1α for glutamate and quisqualate (O'Hara et al, 1993). In addition, the mutation of serine 159 and threonine 182 of mGluR4, which correspond to serine 165 and threonine 188 of mGluR1α, and in addition arginine 78, all result in a decrease in ligand binding to mGluR4. The conservation of these residues between all members of the mGluR family suggests that they are fundamental in ligand binding to mGluRs (Hampson et al, 1999). The ligand binding characteristics of the amino terminal domain of mGluR1α are equivalent to those of the full length receptor suggesting that this region is sufficient to determine the affinity and selectivity of ligand binding (Okamoto et al, 1998). However, a truncated form of mGluR4 exhibits higher affinities for agonists and lower affinities for antagonists, suggesting that amino acids downstream of the amino terminal domain may also have some influence on the affinity of ligand binding to mGluRs (Han and Hampson, 1999). In the highly conserved amino-terminal domains of mGluR1 and
mGluR5, one non-conserved residue, corresponding to proline 369 of mGluR1 and glutamine 356 of mGluR5 is proposed to affect the steric environment of the ligand binding site and may contribute to the ligand subtype specificity of some agonists and antagonists (Costantino et al, 1999).

The large extracellular domain of the CaSR is also involved in ligand binding, and it is suggested that this occurs between two hinged lobes as for mGluRs (Goldsmith et al, 1999; Hammerland et al, 1999). In the CaSR, serine 165 of mGluR1α is conserved as serine 147 and serine 170 is substituted in place of threonine 188 of mGluR1α, both residues are involved in ligand binding to the CaSR (Bräuner-Osborne et al, 1999). In addition, a mutation of arginine 186 of the human PCaR1 to glutamic acid has been identified in patients suffering from familial hypocalciuric hypercalcaemia and neonatal severe hyperparathyroidism (Pollak et al, 1993).

Studies of the GABA\textsubscript{b} receptor amino-terminal domain have revealed sequence similarity with the periplasmic binding protein like domain of mGluRs. Modelling of this region suggests that it also consists of two hinged lobes. Mutagenesis has revealed that serine 246 and serine 269, which correspond to serine 165 and threonine 188 of mGluR1α, are important for ligand binding (Galvez et al, 1999). Thus, similarities exist between the ligand binding domains of mGluR1α, the CaSR and the GABA\textsubscript{b}R.
Ca\textsuperscript{2+}-sensing by mGluRs

The amino-terminal domain is also implicated in Ca\textsuperscript{2+}-sensing by mGluRs. mGluRs 1\textalpha, 3 and 5 have been reported to be activated by Ca\textsuperscript{2+} as well as glutamate (Kubokawa et al., 1996; Kubo et al., 1998). Furthermore, the presence of a serine residue at the site corresponding to serine 166 of mGluR1\textalpha, within the ligand binding domain referred to above, is essential in conferring Ca\textsuperscript{2+} sensitivity upon mGluR1\textalpha and 3 expressed in Xenopus oocytes (Kubo et al., 1998). In addition, the GABA\textsubscript{B} receptor has been shown to have Ca\textsuperscript{2+}-sensing properties, with Ca\textsuperscript{2+} allosterically enhancing GABA responses at the receptor, however mutation of the conserved serine residue, corresponding to serine 166 of mGluR1\textalpha has no effect on this Ca\textsuperscript{2+}-sensitivity (Wise et al., 1999).

1.5.2 Intramolecular transduction

Based on intramolecular transduction in bacterial periplasmic binding proteins, Conn and Pin (1997) proposed a model by which glutamate binding to mGluRs evokes the conformational change required in the receptor in order to activate G-proteins. Following binding to the ligand binding domain as previously described glutamate could be delivered to an extracellular pocket formed by the seven transmembrane domain segments as for most GPCRs. Alternately the presence of cysteine residues in the amino-terminal domain, which may influence the tertiary structure of the receptor, enables the conformational change in the ligand binding domain to be transduced to the membrane spanning regions (Conn and Pin, 1997). The resultant conformational change in the membrane spanning regions allows information
transfer across the membrane by enabling the intracellular domains to interact with and activate G-proteins.

1.5.3 Regions involved in G-protein coupling

The mGluRs also differ in the regions involved in G-protein activation compared to other GPCRs. A tripeptide (DRY or ERW) sequence at the amino-terminal end of the second intracellular loop is important in G-protein coupling in most GPCRs, but is not found in any intracellular loops in mGluRs (Conn and Pin, 1997). The third intracellular loop contains proposed α helices at its amino- and carboxy-terminal ends which are proposed to be important in the specificity of G protein coupling for most GPCRs (Blümml et al, 1994), however the sequence of the third intracellular loop in the mGluR family is highly conserved, thus it is unlikely to be involved in the selectivity of G-protein coupling (Pin et al, 1994). A pseudo-fourth intracellular loop formed by palmitoylation of cysteine residues in the carboxy-terminal domain and subsequent anchorage to the membrane has also been implicated as being important for G-protein coupling in some GPCRs (Moench et al, 1994a; 1994b; Bouvier et al, 1995). Although mGluR4 has been shown to be palmitoylated upon expression in BHK cells, mGluR1α is not (Alaluf et al, 1995a). Interestingly mGluR4, like other receptors shown to be palmitoylated, is coupled to AC, whereas mGluR1α is primarily coupled to the phosphoinositide cascade (Alaluf et al, 1995a). Thus, it is possible that palmitoylation modulates G-protein interactions of AC-coupled receptors.
Construction of chimeric receptors between PLC-coupled mGluR1c and AC-coupled mGluR3 has revealed that the second intracellular loop and the region of the carboxy-terminal tail proximal to the membrane are involved in the specificity of coupling of mGluR1c to PLC, and thus G-protein interactions. These regions are rich in basic amino acid residues and could therefore form amphipathic α helices in common with the regions involved in G-protein interactions in most other GPCRs (Pin et al, 1994). Within the second intracellular loop of mGluR1α cysteine 694 and threonine 695 which are conserved in the group I, but not group II and III, mGluRs, are required for coupling to Gs, but not Gq (Francesconi and Duvoisin, 1998). In addition, lysine 690 is important in determining the efficiency of coupling to different G proteins; mutation of this residue resulting in decreased ability to activate Gq, increased ability to activate Gs and the additional ability to activate Gi (Francesconi and Duvoisin, 1998). However, most intracellular domains of mGluR3 have to be replaced with those of mGluR1c in order to gain optimal G-protein coupling, indicating co-operation between the different intracellular domains in efficient G-protein coupling (Gomez et al, 1996). The conservation of the short first and third intracellular loop sequences among mGluRs suggests an important role in G-protein activation (Pin and Duvoisin, 1995). Indeed, within the third intracellular loop arginine 775 and phenylalanine 781, conserved between all mGluRs, are required for coupling of the receptor to either the PLC or AC pathway. More specifically proline 778 is required for coupling to Gq, and asparagine 782 is required for coupling to Gs (Francesconi and Duvoisin, 1998).

The second intracellular loop has also been implicated in G-protein coupling in both the GABA receptors and α₂-adrenoceptors. Although the second intracellular loop of the
GABA<sub>B</sub>R1a has no significant sequence similarity to the second intracellular loop in mGluRs, it is rich in basic residues, and is likely to form an amphipathic α helix which is predicted to be involved in G protein interactions (Kaupman et al, 1997). Investigation into the mechanism behind the ability of α2-adrenoceptors to couple both negatively and positively to adenylyl cyclase has shown that the second intracellular loop is important for the specificity of G-protein coupling in these receptors (Nasman et al, 1997).

1.5.4 The carboxy-terminal domain

The carboxy terminus of mGluRs has been implicated in receptor targeting. The last 86 residues of the carboxy terminus of mGluR1<sub>α</sub> are proposed to be involved in its plasma membrane targeting, as co-expression of a peptide composed of these residues with mGluR1<sub>α</sub> results in a reduced amount of mGluR1<sub>α</sub> at the membrane surface (Ciruela et al, 1999a). The interaction of tubulin with these residues suggests its involvement in stabilising mGluR1<sub>α</sub> at specific cellular locations (Ciruela et al, 1999b). In addition, the differential targeting of mGluR2 to dendrites and mGluR7 to axons is dependent on motifs within the last ~ 60 amino acid carboxy-terminal tails (Stowell and Craig, 1999).

The carboxy-terminals of the group 1 mGluRs are also the target for the Homer protein. Both mGluR1<sub>α</sub> and mGluR5, but not mGluR2 or mGluR4, bind Homer. This binding involves the carboxy-terminal 10 amino acids of mGluR5 (Brakeman et al, 1997). Homer belongs to a family of proteins which share a putative enabled/VASP homology 1 (EVH1) domain involved in binding the C-terminus of the group 1
mGluRs (Xiao et al, 1998). This includes Homer 1a, 1b and 1c. Homer 1b, but not Homer 1a, regulates the trafficking of and inhibits cell-surface expression of mGluR5 resulting in its accumulation in the endoplasmic reticulum. Point mutations within the Homer 1b binding site of mGluR5 enabled cell-surface expression of mGluR5 suggesting a direct interaction between the two proteins (Roche et al, 1999). Co-expression of mGluR1α with Homer 1a results in an increase in the cell-surface expression of the receptor compared to mGluR1α expressed alone (Ciruela et al, 1999c). Homer 1a also blocks the association of mGluRs with other members of the Homer family (Xiao et al, 1998). Co-expression of mGluR1α or mGluR5 with Homer 1c results in the clustering of these mGluRs at the cell surface, this is dependent on a carboxy-terminal leucine-zipper motif (a heptad leucine repeat). Interestingly, Homer 1c and mGluR1α are co-localised to synapses in the cerebellum (Tadokoro et al, 1999). Homer proteins are also implicated in forming complexes between the group I mGluRs and the IP₃R, via proline rich domains in both receptors (Tu et al, 1998). Thus, the Homer family of proteins appear to be important in regulating the trafficking and targeting of the group I mGluRs and the interaction of mGluRs with intracellular proteins. This could have implications for the formation of multimeric signalling complexes (Ciruela et al, 1999c) and spatial aspects of cellular signalling.

Calmodulin (CaM) has also been shown to interact with the carboxy terminus of mGluR1α, mGluR5 and mGluR7 and may thus regulate their properties. CaM binds to mGluR5 at the amino-terminal end of the carboxy-terminal domain adjacent to the 7th trans-membrane domain. As this region is involved in G-protein interactions, the binding of CaM has implications for G-protein coupling (Minakami et al, 1997; Nakajima et al, 1999).
A role for the carboxy terminus of the mGlul receptors in receptor internalisation, following desensitisation of the receptor, has also been proposed. Following agonist challenge the shorter splice variant mGluR1β, but not the longer mGluR1α, expressed in BHK cells, rapidly disappears from the cell surface (Ciruela and McIlhinney, 1997). In contrast mGluR1α has been reported to undergo rapid internalisation following agonist exposure in HEK cells (Doherty et al, 1999). As the internalisation of GPCRs coupled to PLC has been shown to be dependent on cell type this discrepancy could be due to the different cell types used (Doherty et al, 1999). Obviously further studies are required to clarify the role of the carboxy-terminal tail of mGluR1 in receptor internalisation.

The presence of serine and threonine residues and putative protein kinase phosphorylation sites also suggests that the large carboxy-terminal domains of mGluR1α, 5 a and b are targets for regulation by protein kinases. Indeed, a number of studies suggest agonist-mediated desensitisation of group I mGluRs by PKC activation (Alaluf et al, 1995b; Gereau and Heinemann, 1998). Both PKC and CaM have been shown to bind to the same sites in the C-terminus of mGluR5 and mGluR7, the binding of CaM and PKC phosphorylation being mutually antagonistic. This may have implications for cellular responses (Minakami et al 1997, Nakajima et al, 1999).
1.6 Localisation of mGluRs in the mammalian central nervous system

The identification of the expression patterns of the different subtypes of mGluRs provides clues as to the physiological processes in which the receptors may be involved. RNA blot hybridisation and in situ hybridisation techniques have been used extensively to determine the expression patterns of the mGluRs. Rat mGluR1 mRNA is most highly localised in the cerebellum; with prominent expression in Purkinje cells, and the olfactory bulb and thalamic nuclei. Expression in the hippocampus is low, although higher mGluR1 expression can be detected in distinct regions, i.e. granule cells and CA4 cells of the dentate gyrus, CA2-3 pyramidal cells (Masu et al, 1991). In humans mGluR1 is also most highly localised in the cerebellum (Stephan et al, 1996). Rat and human mGluR5 expression is highest in the hippocampus, cerebral cortex, striatum, olfactory bulb and thalamus (Abe et al, 1992; Daggett et al, 1995).

Rat mGluR2 mRNA is most highly localised in the olfactory bulb and the cerebral cortex, with some localisation in the cerebellum and the dentate gyrus (Tanabe et al, 1992). Human mGluR2 has a different expression pattern, being most prominently expressed in the cerebellum, thalamus and hypothalamus (Flor et al, 1995a), possibly indicating different roles for mGluR2 interspecies homologues. mGluR3 mRNA is most prominently found in the cerebral cortex, thalamus, dentate gyrus and glial cells throughout the CNS, with some expression in the cerebellum (Tanabe et al, 1993, Emile et al, 1996).
Both rat and human mGluR4 mRNA are most highly localised in the cerebellum, with rat mGluR4 expression additionally detected in the thalamus and olfactory bulb (Tanabe et al., 1993; Flor et al., 1995b). mGluR6 mRNA is found only in the retina (Nakajima et al., 1993; Laurie et al., 1997). Rat mGluR7 mRNA has a widespread distribution; it is most highly localised in the cerebral cortex, olfactory bulb, hippocampus, striatum, thalamus, hypothalamus, brainstem, midbrain and dorsal root ganglion cells (Okamoto et al., 1994; Saugstad et al., 1994). hmGluR7 is most highly localised in the hippocampus, cerebral cortex and cerebellum and is also expressed at low levels in many regions (Makoff et al., 1996; Flor et al., 1997). mGluR8 mRNA is most highly localised in the olfactory bulb, olfactory tubercle, mammillary body and thalamus (Duvoisin et al., 1995; Saugstad et al., 1997).

As the extensive and differential expression of mGluRs in the mammalian CNS has become apparent it has become increasingly clear that mGluRs are likely to play important roles within the CNS. It is therefore important to consider the mechanisms by which mGluRs couple in native systems and relate this to their roles in physiological and pathophysiological processes within the CNS.

1.7 Transduction mechanisms in native preparations

The development of selective agonists and antagonists using heterologous mGluR expression systems has aided the characterisation of the transduction mechanisms of mGluR subtypes in native preparations, however the interpretation of data from native systems still remains complicated due to possible interactions between
different subtypes, and possible differences in the specificity of drugs between cloned and native systems.

Phosphoinositide hydrolysis in brain slices and cultured cells is mediated primarily by the group I mGluRs, the agonist and antagonist pharmacological profiles of the response agreeing with that of cloned group I mGluRs (Eaton et al, 1993a; Schoepp and Conn, 1993; Schoepp et al, 1994; Gereau and Conn 1995; Miller et al, 1995; Toms et al, 1995; Mistry and Challiss, 1996). However, the antagonism of PLC-linked mGluRs by L-AP3, L-AP4 and L-SOP in brain slices (Schoepp et al, 1990), and the dual inhibition of (at < 300 μM) and stimulation of phosphoinositide hydrolysis by L-AP3 in cerebral cortex slices (Mistry et al, 1996) could indicate the presence of additional subtypes of mGluRs linked to phosphoinositide hydrolysis.

mGluR-mediated IP₃ production has been reported to be both insensitive (Sladeczek et al, 1985) and sensitive to PTx in cerebellar granule cells and striatal neurones (Nicoletti et al, 1988; Ambrosini and Meldolesi, 1989). Thus it appears that, as in heterologous expression systems, PLC-coupled mGluRs can couple to both G₉ and Gᵥ₀ proteins in native systems.

mGluR agonists can stimulate cAMP accumulation in brain slices (Casabona et al, 1992; Winder and Conn 1992; 1993), the potent stimulation by quisqualate implicating the involvement of the group I mGluRs (Schoepp and Johnson, 1993). However, direct activation of Gₛ by mGluRs in native systems has not been demonstrated (Pin and Duvoisin, 1995).
In hippocampal preparations from rats group I mGluRs have been shown to act in synergy with group II mGluRs to increase cAMP accumulation (Schoepp et al, 1996a). Such synergy is also seen for phosphoinositide hydrolysis in hippocampal and cerebral cortical slices (Schoepp et al, 1996b, Challiss et al, 1998).

Group I mGluRs are implicated in the increase in phospholipase A₂ (PLA₂) activity and arachidonic acid (AA) release seen in cortical neuron-glia cultures and in cortical astrocytes (Stella et al, 1994; Kim et al, 1995) and PLA₂-dependent AA release following co-activation of AMPA receptors and mGluRs in striatal neurones (Dumuis et al, 1993). The activation of group I mGluRs also results in both the inhibition and potentiation of L-type Ca²⁺ channels in hippocampal and neocortical neurones and cerebellar granule cells (Lester and Jahr, 1990; Sayer et al, 1992; Chavis et al, 1996). In addition, the group I mGluRs are implicated in the inhibition of N-type Ca²⁺-currents in cortical neurones (Choi and Lovinger, 1996) as well as the enhancement of N-type Ca²⁺-currents in retinal ganglion neurones (Rothe et al, 1994).

Group I mGluR activation is implicated in the inhibition of both the voltage-dependent K⁺ current, IKMK, and the Ca²⁺-activated K⁺-current, IKAHP, in hippocampal neurones (Charpak et al, 1990; Gerber et al, 1992) and neurones of the nucleus of the tractus solitarius (Glaum and Miller, 1992) and the activation of the Ca²⁺-activated K⁺-channel BKₓ in cerebellar granule cells (Fagni et al, 1991). Selective agonists of the group I or group II mGluRs, respectively; 3,5-DHPG and DCG-IV, suppress a slowly inactivating potassium current IKD in cultured hippocampal pyramidal neurones (Wu et al, 1999). The group I and II mGluR agonists quisqualate
and L-CCG-I have also been shown to activate p42 MAP kinase in primary cortical cultures (Fiore et al, 1993). In rat cortical glia preparations p44/p42 MAP kinases (ERK1/2) are activated by the group I mGluR agonists 3,5-DHPG and quisqualate, the exclusive expression of mGluR5 strongly suggests mediation by this group I mGluR (Peavy and Conn, 1998).

The use of selective agonists and antagonists has, therefore, provided evidence that, as in heterologous expression systems, the group I mGluRs can couple to multiple transduction mechanisms in native preparations. Thus, the coupling of the group I mGluRs to multiple G-proteins in heterologous expression systems seems unlikely to be due to spurious coupling as a result of receptor over-expression. The widespread distribution of group I mGluRs in the central nervous system, and their ability to couple to multiple signal transduction pathways suggests they have important and varied roles in physiological and pathophysiological processes.

1.8 Physiological roles of mGluRs

1.8.1 Neuronal excitability

mGluRs are involved in both potentiating and repressing neuronal excitability. The inhibition of various K⁺ channels, (e.g. IK_leak, IK_M and IK_AHP) by mGluRs results in an increase in cell excitability (Stratton et al, 1989; 1990; Charpak et al, 1990; Hu and Storm, 1991; McCormick and Krosigk, 1992; Guerineau et al, 1994). Studies in a number of brain regions suggest the involvement of group I mGluRs. In ventro-basal thalamic neurones, selective antagonists of the group I mGluRs, 4C3HPG and (S)-4-
carboxyphenylglycine (4CPG), are able to block 1S,3R-ACPD-induced excitation (Eaton et al, 1993b). In the CA1 area of the hippocampus the effects of ACPD can be reproduced with the group I-selective agonist 3,5-DHPG and blocked by the selective antagonist 4CPG (Davies et al, 1995; Gereau and Conn, 1995), which also inhibits agonist-induced excitation in the cerebellum (Lingenhöhl et al, 1993). More specifically in CA1 neurones of mGluR1-deficient mice, ACPD could still evoke excitatory effects (Aiba et al, 1994a; Conquet et al, 1994) compared to mGluR5-deficient mice where it could not (Lu et al, 1997), thus suggesting a role for mGluR5 in neuronal excitability in CA1 neurones.

mGluRs also induce depolarisation via inward currents mediated by a Na⁺/Ca²⁺ exchanger, a Ca²⁺-activated non-specific cation (CAN) current and Ca²⁺-independent non-specific cation channel (Glaum et al, 1992; Staub et al, 1992; Crepel et al, 1994; McBain et al, 1994; Guerineau et al, 1995; Congar et al, 1997; Keele et al, 1997). Mediation by group I mGluRs is suggested by the activation of these currents by quisqualate and the selective group I mGluR agonist 3,5-DHPG (Guerineau et al, 1995; Congar et al, 1997; Keele et al, 1997). The group I mGluRs are also implicated in decreasing neuronal excitability. Hyperpolarisation is observed subsequent to both ACPD- and quisqualate-induced depolarisation in cerebellar Purkinje cells (Staub et al, 1992; Vranesic et al, 1993).

Thus, evidence suggests that the group I mGluRs may have important roles in regulating neuronal excitability. By mediating an increase in excitability they could have roles in the development of epileptic seizures and neurodegenerative
disorders. In contrast their ability to decrease neuronal excitability could be important for neuroprotection under excitotoxic conditions.

1.8.2 Presynaptic effects

One of the principal roles of mGluRs is that of autoreception. A number of different mGluR subtypes have been implicated in the reduction of glutamate release and thus transmission at glutamatergic synapses, via a presynaptic location (Conn and Pin, 1997). Pharmacological and immunochemical evidence implicates that mGluR5 behaves as an autoreceptor in the CA1 area of the hippocampus (Gereau and Conn, 1995; Manzoni and Bockaert, 1995; Romano et al, 1995). In addition, the group I mGluRs have been reported to potentiate glutamate release in the cerebral cortex and hippocampus (Herrero et al, 1992; Rodriguez-Moreno et al, 1998), as this is unaffected in mGluR1-deficient mice, mediation by mGluR5 is suggested (Sistiaga et al, 1998). This potentiation is inhibited by high levels of glutamate suggesting that the facilitation of glutamate release by group I mGluR activation is controlled to prevent glutamate reaching neurotoxic levels at the synapse (Herrero et al, 1998; Rodriguez-Moreno et al, 1998; Sistiaga et al, 1998). Such a mechanism may also account for limited observations of presynaptic mGluRs potentiating presynaptic glutamate release (Herrero et al, 1998).

However, the majority of studies implicate the group II and group III mGluRs in mediating autoreception. The group II mGluRs are believed to serve as autoreceptors at a number of synapses including those in the dentate gyrus, corticostriatal synapses and mossy fiber-CA3 synapses (Hayashi et al, 1993; Jane et
1994; Lovinger et al, 1994a; Lovinger and McCool, 1995; Manzoni et al, 1995; Ugolini and Bordi, 1995; Macek et al, 1996; Kilbride et al, 1998). The group III mGluRs are also implicated as autoreceptors at the above synapses and, in addition, at synapses in the CA1 region of the hippocampus (Koerner and Cotman, 1981; Gereau and Conn, 1995; Johansen et al, 1995; Manzoni et al, 1995; Bradley et al, 1996; Macek et al, 1996; Pisani et al, 1997b). Thus, evidence suggests that autoreception is mediated primarily by group II and III mGluRs. The limited role of the group I mGluRs in autoreception, as identified to date, suggest their primary roles are mediated via a postsynaptic location. This is supported by immunohistochemical studies in which group II and III mGluRs are predominantly located presynaptically and group I mGluRs are located postsynaptically (Baude et al, 1993; Shigemoto et al, 1997).

1.8.3 Modulation of other neurotransmitters

The group I mGluRs are implicated in the regulation of the ionotropic receptor activity. The antagonism of 1S,3R-ACPD mediated inhibition of GABA\textsubscript{A} receptor-mediated currents in neurones of the tractus solitarius nucleus by phenylglycine derivatives suggests the involvement of group I mGluRs (Glaum and Miller, 1993; Glaum et al, 1993). Group I mGluRs are also implicated in the reduction of GABA release and thus inhibitory synaptic transmission via both presynaptic and postsynaptic locations in the hippocampus (Gereau and Conn, 1995; Doherty and Dingledine, 1998; Morishita and Alger, 1999). ACPD has also been shown to activate GABAergic interneurons in the hippocampus (McBain et al, 1994;
Jouvenceau et al., 1995), this can be mimicked by quisqualate suggesting the involvement of group I mGluRs (Poncer et al., 1995).

NMDA and AMPA-mediated currents are also potentiated by mGluR agonists in a number of brain regions (Aniksztejn et al., 1992; Bleakman et al., 1992; Glaum and Miller, 1993; Harvey and Collingridge, 1993; Kinney and Slater, 1993; Mannaioni et al., 1996; Rahmann and Neumann, 1996; Wang and Daw, 1996; Pisani et al., 1997a; Ugolini et al., 1997). Group I mGluR involvement is suggested in the potentiation of NMDA responses in the hippocampus and striatum and both NMDA and AMPA responses in the spinal cord, as the effects of ACPD are mimicked by 3,5-DHPG and are PKC-dependent in both the striatum and spinal cord (Fitzjohn et al., 1996; Pisani et al., 1997a; Ugolini et al., 1997). The potentiation of NMDA responses in the hippocampus, as well as NMDA and AMPA responses in the spinal cord by CHPG indicates the involvement of mGluR5 (Doherty et al., 1997; Ugolini et al., 1999). However, the antagonism of ACPD, but not CHPG, -induced facilitation of NMDA and AMPA responses in the spinal cord by group I-selective antagonists or PKC blockers suggests the involvement of both group I mGluR subtypes (Ugolini et al., 1999). In contrast, group I mGluR agonists have also been shown to inhibit NMDA responses in some studies (Colwell and Levine, 1994; Yu et al., 1997). Interestingly, NMDA receptor activation has been demonstrated to potentiate mGluR5 responses via dephosphorylation of PKC phosphorylation sites on mGluR5 (Alagarsamy et al., 1999).

mGluRs are also implicated in the regulation of a number of other neurotransmitters. Group I mGluR activation by 3,5-DHPG increases levels of NPY mRNA in rat dentate
gyrus neurones (Schwarzer and Sperk, 1998), attenuates the inhibitory effects of
adenosine A1 receptor activation in the hippocampus (Budd and Nicholls, 1998) and
enhances cAMP accumulation induced by $\beta$-adrenoceptor stimulation in cultured
astrocytes (Balazs et al, 1998). Group I mGluR activation is implicated in either
increasing or decreasing the excitability of dopaminergic neurones (Mercuri et al,
1993; Meltzer et al, 1997; Wigmore and Lacey, 1998), the potentiation of cAMP
formation following activation of a D1-like dopamine receptor in striatal neuronal
cultures (Paolillo et al, 1998) and increasing dopamine release in the rat striatum
(Bruton et al, 1999).

The above observations provide evidence for the regulation of multiple
neurotransmitters following group I mGluR activation and for complex interactions
between mGluRs and ionotropic receptors. Thus mGluRs are implicated in having
diverse roles in the regulation of both inhibitory and excitatory neurotransmission in
the central nervous system.

1.8.4 Synaptic plasticity

Synaptic plasticity involves either long-term potentiation (LTP) or long-term
depression (LTD) of glutamatergic synapses, and is proposed to be involved in
learning and memory processes. Most research on LTP has been carried out on the
Schaffer collateral-CA1 pyramidal cell synapses of the hippocampus, these findings
have been brought together in a recent review resulting in the simplified model
presented below (Malenka and Nicoll, 1999). The induction of LTP requires the
concomitant release of glutamate from presynaptic terminals and postsynaptic
depolarisation. This allows Ca\(^{2+}\) influx via the NMDA receptor, with the resultant postsynaptic [Ca\(^{2+}\)]\(_i\) elevation. The binding of Ca\(^{2+}\) to CaM results in the activation of CaMKII which allows LTP expression via the phosphorylation of AMPA receptors increasing the conductance of ions through these receptors, and an increase in the number of AMPA receptors delivered to the cell membrane. The long-term maintenance of LTP is proposed to involve gene transcription and new protein synthesis.

ACPD can induce or potentiate LTP in the hippocampus (McGuiness et al., 1991; Otani and Ben-Ari, 1991; Aniksztein et al., 1992; Behnisch and Reymann, 1993). The inhibition of induction by NMDA receptor antagonists, PKC inhibitors and thapsigargin suggests mediation via a PKC/NMDA receptor interaction and the involvement of [Ca\(^{2+}\)]\(_i\) elevation (Aniksztein et al., 1992; Bortolotto and Collingridge, 1993).

In addition, MCPG inhibits the induction of LTP in the hippocampus in some cases (Bashir et al., 1993; Breakwell et al., 1998; Fitzjohn et al., 1998; Grover, 1998; Wilsch et al., 1998) but not others (Chinesta et al., 1993; Manzoni et al., 1994). In explanation, MCPG has been demonstrated to block the induction of LTP in naive hippocampal slices, but not in slices in which LTP has been previously induced (Bortolotto et al., 1994). Thus it is proposed that the mGluRs activate a molecular switch turned on during the first instance of LTP, resulting in a persistant change, dependent on the activity of PKC and CaMKII, such that mGluR activation is not required for subsequent LTP induction (Bortolotto et al., 1994; Bortolotto and Collingridge, 1998).
Studies to elucidate the subtypes of mGluR involved in the induction of LTP have given conflicting results. CHPG pre-treatment results in enhanced LTP in the dentate gyrus suggesting a role for mGluR5 (O'Leary and O'Connor, 1998). In mGluR1 knockout mice LTP in the CA1, and dentate gyrus areas has been shown to be unaffected whereas LTP in the CA3 area was reduced (Conquet et al, 1994). However, LTP was shown to be reduced in the CA1 region in another study using a different set of mice (Aiba et al, 1994a). In mGluR5 knockout mice LTP is reduced in the CA1 and dentate gyrus, but not the CA3 region, suggesting a role for mGluR5 in some regions (Lu et al, 1997). Behavioural studies support a role for group I mGluRs in learning and memory formation; spatial learning tasks known to depend on an intact hippocampus being impaired in mGluR1 and 5 knockout mice (Aiba et al, 1994a; Conquet et al, 1994; Lu et al, 1997). In addition the group I mGluR antagonist AIDA affects working memory in rats (Ohno and Watanabe, 1998). The effects of the mGluR antagonists; MCPG and AIDA, were shown to be accentuated by NMDA receptor antagonists and lessened by NMDA receptor agonists, suggesting an interactive involvement of these receptors in memory processes (Ohno and Watanabe, 1996, 1998). Presynaptic group I mGluRs have also been implicated in the expression of LTP in the CA1 region via enhanced presynaptic glutamate release (Manahan-Vaughan et al, 1999). The proposed involvement of group I mGluRs in LTP is illustrated in figure 1.4.

In contrast to the group I mGluRs, the group II and III mGluRs appear to have a negative modulatory influence on the induction of LTP. In the dentate gyrus DCG-IV and PCCG-IV inhibit LTP induction (Ishida et al, 1993; Huang et al, 1997) as do
Fig 1.4 Schematic representation of the involvement of the group I mGluRs in the induction of LTP. The Mg\(^{2+}\) block of the NMDAR seen under resting membrane potential is relieved during depolarisation allowing activation of both AMPA and NMDA receptors by glutamate released from the presynaptic cell. The resultant rise in \([Ca^{2+}]_j\) triggers the induction of LTP. This results in the binding of Ca\(^{2+}\) to CaM and the activation of CaMKII. CaMKII then (i) autophosphorylates, maintaining its activity after Ca\(^{2+}\) levels return to basal, (ii) phosphorylates AMPARs increasing their single-channel conductance and, (iii) increases the number of AMPARs present at the cell surface. The group I mGluRs are proposed to mediate the potentiation of such induction in the hippocampus via a PKC/NMDAR interaction and an increase in \([Ca^{2+}]_j\). + denotes a positive modulatory effect. Adapted from Malenka and Nicoll (1999).
1S,3S-ACPD, L-CCG-I and DCG-IV in the CA1 region (Holscher et al, 1997; Behnisch et al, 1998). The group III mGluRs have also been implicated in the reduction of CA1 and dentate gyrus long-term potentiation, L-AP4 reducing the amplitude of LTP in these brain regions (Manahan-Vaughan and Reymann, 1995).

LTD has been most extensively studied in parallel fibre-Purkinje cell synapses of the cerebellum and involves an attenuation of this synaptic connection upon dual activation of parallel and climbing fibres (Ito, 1989). LTD is induced as a result of the postsynaptic depolarisation and activation of voltage-sensitive Ca$^{2+}$ channels resulting in Ca$^{2+}$ influx into the postsynaptic neuron following climbing fibre activation, in combination with the activation of mGluRs and Na$^{+}$ influx via the AMPA receptor or voltage-gated Na$^{+}$ channels resulting from parallel fibre stimulation. Evidence suggests that these events then lead to the expression of LTD via a PKC-dependent mechanism, resulting in an alteration in the number or sensitivity of AMPA receptors (Linden, 1994).

The co-activation of VSCCs or AMPA receptors and ACPD-sensitive mGluRs results in the induction of cerebellar LTD (Ito and Karachot, 1990a; 1990b; Linden et al, 1991; Daniel et al, 1992). The localisation of mGluR1 in Purkinje cells (Masu et al, 1991), the dependence of induction on PKC activation (Crepel and Krupa, 1988, Linden and Connor, 1991), the inhibition of LTD induction by mGluR1 antibodies in cultured Purkinje neurones (Shigemoto et al, 1994) and the impairment of LTD in mGluR1, but not mGluR5, knockout mice (Aiba et al, 1994b; Conquet et al, 1994; Lu et al, 1997) all suggest the involvement of this mGluR subtype. Indeed, the induction of LTD in mGluR1 knockout mice can be recovered by the photolytic
release of caged-InsP$_3$ in conjunction with a pairing protocol, confirming the involvement of mGluR1 gene inactivation, and not developmental abnormalities, in blocking LTD induction in mGluR1 knockout mice (Daniel et al., 1999).

In addition to a role in cerebellar LTD, the involvement of mGluRs has been suggested in hippocampal LTD. Both NMDA receptor -dependent and -independent forms of LTD in hippocampal Schaffer collateral-CA1 synapses can be induced by the group I mGluR agonist 3,5-DHPG (Palmer et al., 1997; Fitzjohn et al., 1999). This can be mimicked by the mGluR5-selective agonist CHPG, and is unaffected by the selective mGluR1 antagonist LY367385, providing evidence for the involvement of mGluR5 (Fitzjohn et al., 1999). mGluR5 is also implicated in the induction of LTD in the dentate gyrus, as 3,5-DHPG, and the mGluR5-selective agonists CHPG and (S)-(-)-2-(3'-carboxy-bicyclo[1.1.1]pentyl)-glycine (CBPG) can induce LTP in this brain region (Camodeca et al., 1999). In another study group I and II mGluR antagonists inhibit LTD induction in the CA1 region of the hippocampus (Manahan-Vaughan, 1997). The group II mGluRs have also been implicated in the induction of LTD in the dentate gyrus and the CA3 region of the hippocampus (Yokoi et al., 1996; Huang et al., 1997; Huang et al., 1999a; 1999b). The ability of the group II mGluR antagonists to modulate LTD when applied after high-frequency tetanus, implies a role for group II mGluRs in the maintenance of LTD as well (Manahan-Vaughan, 1997). Thus, different mGluRs may have different roles at different stages of LTD.

The use of mGluR subtype-selective agonists and antagonists has enabled the characterisation of the roles of different mGluR subtypes in synaptic plasticity. Due to the importance of the group I mGluRs in the induction of LTP and LTD it has been
proposed that agonists of these receptors may be of therapeutic benefit to patients with learning and memory impairments (Conn and Pin, 1997).

1.9 Pathophysiological roles of mGluRs

1.9.1 Roles of mGluRs in neurotoxicity

In addition to being the major excitatory neurotransmitter glutamate is also a neurotoxic agent. Over-stimulation of glutamate receptors can lead to neuronal cell death, via excessive Ca\(^{2+}\) influx into cells via NMDA receptors and delayed toxicity resulting from glutamate-induced glutamate release (Monyer et al, 1992; Pin and Duvoisin, 1995). Such neuronal death has been implicated in a number of pathological conditions.

The group I mGluRs are implicated in enhancing glutamate-mediated neurodegeneration; the potentiation of NMDA-induced neurodegeneration by group I agonists in murine cortical cells (Bruno et al, 1995a; Buisson and Choi, 1995; Strasser et al, 1998) being blocked by group I mGluRs antagonists (Orlando et al, 1995; Strasser et al, 1998). The mGluR1-selective antagonists (+)-2-methyl-4-carboxyphenylglycine (LY367385), AIDA and CBPG are neuroprotective against NMDA-mediated neurodegeneration, oxygen-glucose deprivation and following transient global ischaemia (Bruno et al, 1999; Pellegrini-Giampietro et al, 1999). mGluR1 is also implicated in post-traumatic neuronal death. The group I mGluR agonist DHPG increasing, and the group I mGluR antagonist 4CPG and an mGluR1 antisense oligodeoxynucleotide reducing, such neuronal death (Mukhin et al, 1996).
However group I mGluR agonists have also been shown to be neuroprotective when present prior to hypoxic/hypoglycaemic insult of hippocampal slices, in a PKC-dependent manner (Schroder et al., 1999), and against excitotoxic damage in cerebellar granule cells (Pizzi et al., 1996). In primary cultures of cerebellar neurones CHPG protects against glutamate- and NMDA-mediated neurodegeneration suggesting the involvement of mGluR5 (Montoliu et al., 1997). In addition, the inactivation of mGluR1 was shown not to protect against, and mGluR1 knockout mice displayed no difference in the degree of, neurodegeneration following ischaemia and excitotoxic brain injuries, providing evidence against a neurotoxic role of mGluR1 (Ferraguti et al., 1997).

Several hypotheses have been proposed to explain the contradictory evidence for the role of group I mGluRs in excitotoxic neuronal death. The heteromeric composition of NMDA receptors may influence whether group I mGluR activation is neuroprotective or neurotoxic, the activity of NMDA receptors containing the NR2C subunit being reduced by group I mGluR activation, resulting in neuroprotection (Pizzi et al., 1996, Nicoletti et al., 1999). Another theory is that a functional switch, such as that described in Section 1.8.2 (Herrero et al., 1998; Rodriguez-Moreno et al., 1998; Sistiaga et al., 1998) may also occur postsynaptically. Indeed, an initial application of DHPG to hippocampal neuronal cultures was shown to potentiate NMDA-mediated toxicity, whereas application of DHPG 5 min after a brief pre-exposure protected against NMDA-mediated neuronal death, in a PKC-dependent manner (Nicoletti et al., 1999). An alternative explanation is that glial mGluR5 activation results in the secretion of a neurotoxic factor, as DHPG has a
neuroprotective effect on cultured granule cells alone, but a neurotoxic effect on
cells cultured with a monolayer of astrocytes (Nicoletti et al, 1999)

In addition, group II and III mGluR activation has been reported to be
neuroprotective against NMDA- and kainate-mediated neurodegeneration,
hypoxic/ischaemic injury and global cerebral ischaemia (Bruno et al, 1994; 1995b;
Buisson et al, 1996; Miyamoto et al, 1997; Battaglia et al, 1998; Bond et al, 1998;
Bruno et al, 1998; Gasparini et al, 1999a; Kingston et al, 1999; Lafon-Cazal et al,
1999a). Like the group I mGluRs, group III mGluR activation has also been
implicated in the mediation neurodegeneration (Staton et al, 1998; Lafon-Cazal,
1999b).

At present there is conflicting evidence suggesting roles for different mGluR
subtypes in both preventing and accentuating excitotoxic cell death. However,
further studies to elucidate and explain the roles of specific mGluRs in excitotoxicity
should be useful in developing therapeutic agents to prevent or lessen neuronal
death under excitotoxic conditions.

The group I mGluRs are also implicated in a number of neurodegenerative
disorders. Quinolinic acid treatment results in striatal lesions resembling those seen
in Huntington's disease (Beal et al, 1986). 4C3HPG protects against such lesions
(Orlando et al, 1995), however, as this compound is also an agonist of group II
mGluRs, this could suggest a toxic role of group I mGluRs or a protective role of
group II mGluRs in this process.
Overactivity of the subthalamic nucleus, which influences locomotion and voluntary movement control via excitation of the basal ganglia (Albin et al, 1989) is a major property of Parkinson’s disease. This nucleus can be activated by ACPD and 3,5-DHPG indicating a role for mGluRs, possibly group I mGluRs in this aspect of the disease (Abbott et al, 1997).

The β amyloid protein which accumulates in the brain of individuals with Alzheimer’s disease potentiates glutamate induced neurodegeneration (Choi, 1992; Mattson et al, 1992). β amyloid formation is reduced following mGluR1α activation in HEK cells, and following stimulation of cultured hippocampal neurones with glutamate, quisqualate or t-ACPD, via the breakdown of the amyloid precursor protein (Lee et al, 1995; 1996).

Further characterisation of the subtypes of mGluRs involved in these neurodegenerative processes could aid the development of therapeutic agents to treat these disorders.

Epileptogenesis has also been reported to involve group I mGluRs. 1S,3R-ACPD-induced seizures have been reported to involve Ca²⁺ store mobilisation and the group I-selective agonist 3,5-DHPG induces limbic seizures followed by neurodegeneration in mice or rats and increases epileptogenic activity in the CA3 region of guinea pig hippocampal slices and basolateral amygdala neurones (Sacaan and Schoepp, 1992; McDonald et al, 1993; Tizzano et al, 1993; 1995; Camon et al, 1998; Keele et al, 1999; Merlin, 1999). In addition, the group I mGluR antagonist 4C3HPG and the mGluR1-selective antagonists LY367385 and AIDA
have anticonvulsant activity in mice and rats (Thomsen et al., 1994; Dalby and Thomsen, 1996; Tang et al., 1997; Chapman et al., 1999). Thus, the development of therapeutic agents which antagonise the group I mGluRs could be useful in preventing epileptic seizures.

1.9.2 Nociception

ACPD has been shown to facilitate nociceptive transmission in the dorsal horn neurones of the spinal cord (Bleakmann et al., 1992). The attenuation of the ACPD effect by the group I-selective antagonist 4C3HPG (Budai and Larson, 1998), the greater excitation of dorsal horn neurones by the group I-selective agonist 3,5-DHPG compared to group II- and III-selective agonists, and the dependence of the excitation on PKC and CaMKII activation (Young et al., 1997) strongly suggest the involvement of group I mGluRs. Responses to noxious thermal somatosensory stimuli, and 1S,3R-ACPD and DHPG application, in the thalamus are also decreased upon application of group I mGluR antagonists, and the mGluR1-selective antagonist LY367385 (Eaton et al., 1993 a; b; Salt and Turner, 1998). Further evidence for the involvement of mGluR1 is provided by the incomplete antagonism of the response to ACPD by the mGluR5-selective antagonist MPEP (Salt et al., 1999). Behavioural studies in mice and rats, also indicate a role for mGluRs in nociception. The group I-selective agonist 3,5-DHPG is shown to increase, and the group I-selective antagonists 4CPG, 4C3HPG and the mGluR1-selective antagonist AIDA, to decrease nociceptive responses (Fisher andCoderre, 1996; Corsi et al., 1997; Moroni et al., 1997). In addition, intrathecal administration of antibodies to both mGluR1 and mGluR5 reduced 3,5-DHPG-induced spontaneous nociceptive
behaviour in rats, indicating the involvement of both group I mGluRs in nociceptive processing in persistent pain (Fundytus et al., 1998). mGluR1 activation is also implicated in both brief and prolonged nociceptive processing in primate spinothalamic tract (STT) cells. The group I agonist, 3,5-DHPG, but not the mGluR5 agonist, CHPG, was demonstrated to enhance the response to brief noxious stimuli. This 3,5-DHPG-induced response and also prolonged capsaicin-induced central sensitisation were inhibited by the mGluR1-selective antagonist AIDA (Neugebauer et al., 1999). Thus, the development of therapeutic agents which block the activity of the group I mGluRs may be important in the development of analgesics for the treatment of chronic and acute pain.
The research presented in this study will focus on investigating the Ca\(^{2+}\) sensitivity of phosphoinositide signalling via mGluR1\(\alpha\). This was prompted by the observation that mGluR1\(\alpha\) could be activated by both Ca\(^{2+}\) and glutamate upon expression in *Xenopus* oocytes (Kubokawa *et al.*, 1996). This study extends these observations to address the 'Ca\(^{2+}\)-sensing' ability of mGluR1\(\alpha\) expressed in mammalian model cell systems. Following the observation that Ca\(^{2+}\) modulates agonist-stimulated phosphoinositide signalling via mGluR1\(\alpha\) expressed in BHK cells, a BHK cell line expressing another phosphoinositide-coupled GPCR, the M\(_3\)-muscarinic receptor, was generated in order to investigate the receptor selectivity of this Ca\(^{2+}\) modulation. The selectivity of Ca\(^{2+}\) modulation to phosphoinositide signalling via mGluR1\(\alpha\) and not the M\(_3\)-muscarinic receptor led to subsequent studies to establish the site at which such Ca\(^{2+}\) modulation occurs.

The generation of the M\(_3\)-muscarinic receptor-expressing BHK cell line also allowed investigation of the receptor specificity of the enhancement of agonist-stimulated phosphoinositide signalling via mGluR1\(\alpha\) observed following PTx pre-treatment (Carruthers *et al.*, 1997).
CHAPTER 2 - METHODS

2.1 Materials

All chemicals were from Sigma Chemical Co. Ltd. (Poole, U.K.) or Fisher Scientific Ltd (Loughborough, U.K.) except: GPT (glutamic-pyruvic transaminase) from Boehringer Mannheim (Mannheim, Germany), 1S,3R-ACPD and CPCCOEt from Tocris - Cookson (Bristol, U.K.), quisqualate from Sigma/Tocris-Cookson, hygromycin B, fura-2 AM, bisindolylmaleimide and Gö 6976 from CalBiochem (Nottingham, U.K.), Ro-31-8220 from Roche Bioscience (Palo Alto, California), myo-[3H]-inositol, D-myo-[3H]-inositol 1,4,5-trisphosphate and l-[N-methyl-3H]-scopolamine methyl chloride from Amersham (Little Chalford, U.K.), Dowex anion exchange resin AG1-X8 (200-400 mesh, formate form) and Dowex 50 H\textsuperscript{+} form resin from BioRad (Watford, U.K.), LiCl from BDH (Poole, U.K.), Protogel from Flowgen (Kent, U.K.), nitrocellulose membranes from Schleicher and Schuell, carboxy-terminal mGluR1\textalpha{} primary antibody from Chemicon International (Harrow, U.K.) and DNA restriction and modifying enzymes from GIBCO-BRL (Paisley, U.K.). The pCEP4 vector containing cDNA for the M\textsubscript{3}-muscarinic receptor was provided by Dr. A. B. Tobin (University of Leicester, U.K.). The pmGR1-mGluR1\textalpha{} and pmGR1-\textsuperscript{S166D}-mGluR1\textalpha{} plasmids were provided by Prof. Y. Kubo (Tokyo Metropolitan Institute for Neuroscience, Japan). BHK-570 and BHK-mGluR1\textalpha{} cells were provided by Dr. C. Thomsen (Novo Nordisk, Denmark). CHO-lac-hmGluR1\textalpha{} cells were provided by Dr. E. Hermans (University of Leicester, U.K.). All cell culture media and reagents were from GIBCO-BRL unless otherwise indicated.
2.2 Cell culture techniques

2.2.1 Baby hamster kidney (BHK) cell culture

BHK cells were cultured in Dulbecco's modified Eagle's medium containing Glutamax-1, sodium pyruvate and 4500 mg L\(^{-1}\) glucose supplemented with 5% dialysed foetal calf serum. The medium was additionally supplemented with; 0.6 mg ml\(^{-1}\) G418, and 1 μM methotrexate (for BHK cells stably expressing the rat type 1α mGluR (Pickering et al, 1993; Thomsen et al, 1993)), or 50 μg ml\(^{-1}\) gentamicin (for BHK-570 vector control cells (Pickering et al, 1993)), or 50 μg ml\(^{-1}\) gentamicin and 300 μg ml\(^{-1}\) hygromycin B (for BHK-570 cells stably transfected with the muscarinic m\(_3\) receptor and the vector control cells for this transfection) or 50 μg ml\(^{-1}\) gentamicin and 1 μM methotrexate (for BHK-\(^{S165A}\)-mGluR1α cells).

2.2.2 Chinese hamster ovary (CHO) cell culture

CHO-lac-hmGluR1α cells developed by Dr. E. Hermans (Hermans et al, 1998b) were cultured in modified Eagle’s medium alpha containing Glutamax-1, supplemented with 10% foetal calf serum (FCS), 0.3 mg ml\(^{-1}\) G418, 100 μg ml\(^{-1}\) streptomycin and 10\(^5\) U L\(^{-1}\) penicillin. The development of CHO-lac-hmGluR1α cells enabled hmGluR1α to be studied in a better model system. The induction of hmGluR1α expression when required means hmGluR1α is exposed to endogenously produced glutamate for as little time as possible. This induction occurs following the application of isopropyl-β-D-thiogalactoside (IPTG) which relieves the lac repression of hmGluR1α expression. CHO-m3 cells were cultured in modified Eagle’s medium
alpha supplemented with 10% new born calf serum (NBCS) and 100 μg ml\(^{-1}\) streptomycin and 10\(^5\) U L\(^{-1}\) penicillin. Wild-type CHO (CHO-WT) cells were adapted to grow in a glutamate-free medium consisting of minimum essential medium containing Earle’s salts and Glutamax-1 supplemented with 10% FCS, 100 μg ml\(^{-1}\) streptomycin and 10\(^5\) U L\(^{-1}\) penicillin. CHO-mGluR1\(\alpha\)-WT and CHO\(^{S166D}\)-mGluR1\(\alpha\) cells were grown as above with the addition of 0.6 mg ml\(^{-1}\) G418.

All cells were grown at 37°C in a humidified atmosphere (95% air, 5% CO\(_2\)) and were passaged every 4-6 days in a 1 in 4 to 1 in 20 ratio and were fed every 2-4 days. Cells were harvested in 2 ml trypsin-EDTA solution (0.5g trypsin, 0.2 g EDTA per litre of modified Puck’s saline A) following 2 x 1.5 ml washes with sterile HBS (10 mM HEPES, 0.9% NaCl, pH 7.4).

2.3 Determination of \(^{[3]H}\)-inositol monophosphate (\(^{[3]H}\)-InsP\(_1\)) accumulation

BHK cells were seeded into 24 well multidishes (Nunc), 1ml medium per well, in the presence of 1 μCi ml\(^{-1}\) myo\(^{[3]H}\)-inositol, and were incubated for 48 h before experimentation. Pertussis toxin pre-treatment of cells occurred 22-24 h before experimentation by addition of pertussis toxin to a final concentration of 100 ng ml\(^{-1}\) to the cell culture medium.

CHO cells were seeded into 24 well multidishes, 0.5 ml medium per well, in the presence of 2.5 μCi ml\(^{-1}\) myo\(^{[3]H}\)-inositol, and were incubated for 48 h before experimentation. The expression of mGluR1\(\alpha\) in CHO-lac-hmGluR1\(\alpha\) cells was induced by treatment with 100 μM IPTG for ~20 h prior to experimentation (Hermans
et al, 1998b). Pre-treatment with GPT (glutamic - pyruvic transaminase; 3 U ml⁻¹)/pyruvate (5 mM) to remove glutamate present in medium, or additions of 1 μM PdBu or 30 μM CPCCOEt were performed overnight by addition of these reagents to the growth medium. Growth medium was removed from confluent cells by aspiration and the cells were washed with 3 x 1 ml Krebs-Henseleit buffer (KHB; in mM: NaCl, 118; KCl, 4.7; NaHCO₃, 25; KH₂PO₄, 1.2; MgSO₄, 1.2; HEPES, 5 and D-glucose, 10, pH 7.4 following equilibration with 95% O₂, 5% CO₂ at 37°C. N.B. - CaCl₂, 1.3; unless otherwise stated). Where [Ca²⁺]ᵢ was manipulated the cells were then washed with KHB containing the appropriate [Ca²⁺]ᵢ. 10 mM LiCl (in KHB of appropriate [Ca²⁺]), was added to the cells 15 - 30 min before agonist application, this inhibits the activity of inositol monophosphatase to allow measurement of the accumulation of inositol monophosphate as an index of PLC activation (Nahorski et al, 1991). Cells were challenged with agonists for 30 min (15 min; CHO cells) in a total volume of 300 μl. Experiments were carried out at 37°C. Figure 2.1 illustrates the phosphoinositide signal transduction cascade and the influence of [³H]-inositol labelling and LiCl treatment.

Where cells were treated with GPT (3 U ml⁻¹) and pyruvate (5 mM) this occurred 10 - 20 min before addition of LiCl (see figure legends). GdCl₃, MnCl₂, CaCl₂, MgCl₂ additions occurred 15 min after LiCl and 5 min before agonist/vehicle (see Section 3.3.1). AlCl₃ was added 10 min prior to NaF, the addition of which occurred 40 min before termination of the reaction with trichloroacetic acid (TCA), (see Section 3.4.1). PKC inhibitors and W-5, W-7 and W-13 were added 35 min prior to agonist challenge. PdBu was added 5 min prior to agonist challenge (see Section 4.3.4).
Fig. 2.1 Schematic representation of the phosphoinositide signal transduction cascade (black arrows) with reference to [3H]-inositol labelling and Li+ blockade (grey arrows). Labelling with [3H]-inositol for 48 h results in its incorporation into all inositol-containing elements of the phosphoinositide cycle. Subsequent Li+ blockade of the cycle during experimentation prevents the conversion of inositol phosphate to inositol and thus results in the accumulation of [3H]-inositol phosphate for later separation by affinity chromatography and measurement by scintillation counting.
For ether extraction of TCA, reactions were then stopped by rapid aspiration of the reaction mixture followed by addition of 300 μl of 0.5 M ice cold TCA. Plates were left on ice for approximately 30 min before transfer of the samples to polypropylene tubes. TCA was extracted by thoroughly mixing the sample with 2 ml water-saturated diethylether. The sample was allowed to settle out before the upper phase was removed by aspiration. This was repeated 3 times and any ether remaining was allowed to evaporate. The samples were neutralised by addition of 100/50 μl 60 mM NaHCO₃ and 50/25 μl 30 mM EDTA, pH 7.0, to 540/260 μl of sample after transfer to microfuge tubes before storage at 0 - 4°C until further analysis. The amounts vary depending on whether samples from 2 wells were pooled or not.

For freon mix extraction of TCA reactions were stopped by rapid aspiration of the reaction mixture followed by addition of 250 or 500 μl of 0.5 M ice cold TCA depending on whether the 2 samples were to be pooled or not. Plates were left on ice for approximately 30 min before transfer of the samples to microfuge tubes. 100 μl of 10 mM EDTA, pH 7.0 was added to each sample, followed by 600 μl of a 1:1 mixture of tri-n-octylamine and 1,1,2-trichlorotrifluoroethane. The samples were vortexed and left at room temperature for 15-30 min. The samples were then vortexed again before being spun in a bench top centrifuge for 2.5 min. 450 μl of the upper aqueous phase of each sample was then transferred to an Eppendorf tube and 50 μl of 60 mM NaHCO₃ was then added before vortexing and storage at 0-4°C until further analysis.

The [³H]-InsP₃ fraction was resolved by ion-exchange chromatography on Dowex AG1-X8 formate form columns; 200-400 mesh, 1.0 ml bed volume (as described by
Challiss et al., 1993). Columns were filled with water which was allowed to wash through. The samples were then added to the columns and the microfuge tubes washed out with water which was also added to the columns and allowed to wash through. 10 ml 60 mM ammonium formate / 5 mM Na$_2$B$_4$O$_7$ was then added to each column. Once this had washed through 12 ml of 0.2 M ammonium formate was added to the columns and the eluate collected in scintillation vials placed beneath the columns. 5 ml of the eluate was retained and 15 ml Flo-Scint IV scintillant and 1 ml 1 M HCl added before vortexing and storage in the dark overnight before counting. Columns were regenerated with 5 ml 2 M ammonium formate / 0.1 M formic acid, washed through with water and stored at 0-4°C until further use. Cell protein was quantified using the Lowry method (Lowry et al., 1951) to allow data to be expressed as disintegrations per minute (d.p.m.) mg$^{-1}$ protein.

2.3.1 Poly-L-lysine coating of plasticware

Where CHO-m3 cells were used it was necessary to coat 24 well multidishes with poly-L-lysine prior to addition of cells to minimise cell loss during experimentation. Under sterile conditions poly-L-lysine was diluted to 5 μM with filter-sterilised dH$_2$O. 0.5 ml was added to each well and incubated at room temperature for 15-30 min before removal by aspiration. The wells were then washed twice with 0.5 ml sterile HBS, which was removed by aspiration. The multidishes were then ready to use.
2.4 Ins(1,4,5)P₃ mass determination

2.4.1 Generation of samples for Ins(1,4,5)P₃ mass determination

Cells were seeded into 24 well multidishes, 1 ml medium per well. Following an incubation period of 24 h samples for measurement of Ins(1,4,5)P₃ mass were generated as in Section 2.3, except for the exclusion of 10 mM LiCl. Ether extraction of TCA from samples was carried out as described in Section 2.3. In addition extraction was carried out on 10 ml 0.5 M TCA for preparation of a 'buffer blank' to be used in the Ins(1,4,5)P₃ mass assay. 200 µl of sample/8 ml of buffer blank were then neutralised with 50 µl/2 ml 60 mM NaHCO₃ followed by 50 µl/2 ml 30 mM EDTA, pH 7.0 before vortexing and storage at 0-4°C until the Ins(1,4,5)P₃ mass assay was carried out to determine the concentration of Ins(1,4,5)P₃ in the samples.

2.4.2 Ins(1,4,5)P₃ mass assay

Ins(1,4,5)P₃ mass was assayed as described by Challiss et al (1988) using a radioligand binding assay. The following reagents, in order of addition, were added to 5 ml tubes on ice: 60 µl sample, 30 µl [³H]-Ins(1,4,5)P₃ (prepared in Ins(1,4,5)P₃ buffer: 100 mM Tris/HCl, 4 mM EDTA, pH 8.0), 30 µl Ins(1,4,5)P₃ binding protein (∼15 mg ml⁻¹ bovine adrenal cortex crude 'P₂' membrane preparation, prepared in the laboratory (see D.K. Boxall, 1998)). In addition to assaying the samples a number of other tubes were prepared. To calculate the Ins(1,4,5)P₃ mass present in the samples a standard curve was produced with: 36, 12, 3.6, 1.2, 0.36, 0.12 and 0.036
pmol Ins(1,4,5)P₃ standard. The non-specific binding (NSB) was determined using 60 μl of the 20 μM Ins(1,4,5)P₃ standard. Total binding was determined using 60 μl of buffer blank.

To ensure the correct amount of [³H]-Ins(1,4,5)P₃ was to be used in the assay 30 μl of the [³H]-Ins(1,4,5)P₃ / buffer mix (~8 μl ml⁻¹) was added to 4.2 ml of emulsifier safe scintillant and counted to ensure the counts obtained were between 6000 and 8000 d.p.m.

Tubes were then vortexed gently and incubated on ice for 30-45 min. Samples were then rapidly vacuum filtered using Millipore manifolds onto GF/B Whatman filter discs. Each tube was washed 3 times with 3 ml ice-cold Ins(1,4,5)P₃ wash buffer (25 mM Tris, 1 mM EDTA and 5 mM NaHCO₃, pH 8.0) and the filtrate collected on the filters. The filters were then placed in scintillation vials, 4.2 ml of emulsifier safe scintillant was then added, the vials were then capped and left overnight before vortexing at least 30 min before counting.

2.5 Preparation of cell membranes

Cells were grown to confluence in 175 cm³ flasks and subsequently washed with 10 ml HBS. The HBS was removed and the cells harvested by addition of 5 ml HBS EDTA (10 mM HEPES, 0.9% NaCl, 0.2% EDTA, pH 7.4) for approximately 15 min. The contents of 3-4 flasks of 1 cell type were then pooled and centrifuged at 210 X g for 4 min such that a loose pellet of cells formed. The supernatant was then discarded and 10 ml wash buffer A (10 mM HEPES, 10 mM EDTA, pH 7.4) was
added to the pellet, which was then tritutated. For membrane preparations in which protease inhibitors were used the cells were incubated in wash buffer A, containing protease inhibitors, for 15 min. The cells were then homogenised on ice using a polytron homogeniser (speed 5, 4 X 5 s bursts at 30 s intervals). The homogenate was centrifuged at 40,000 X g for 15 min at 4°C. The supernatant was discarded and the pellet re-tritutated and homogenised in 10 ml wash buffer B (10 mM HEPES, 0.1 mM EDTA, pH 7.4, containing protease inhibitors where appropriate). The homogenate was re-centrifuged as above. The pellet was resuspended in wash buffer B to a concentration of 1 mg ml⁻¹ and subsequently snap frozen in liquid nitrogen and stored at -80°C until required. The protease inhibitors were present at the following concentrations where used; PMSF (phenylmethylsulfonylfluoride): 1 mM, leupeptin: 1 μg ml⁻¹, aprotinin: 20 μg ml⁻¹ and pepstatin A: 1 μg ml⁻¹.

2.6 Membrane phospholipid determination

After recovery of samples for determining [³H]-InsP₃ accumulation and aspiration of any fluid remaining in the wells of 24 well plates of CHO-lacmGluR1α and CHO-m3 cells, membrane phospholipids were extracted as follows (see Batty et al, 1997): 1 ml (0.5 ml if 2 samples to be pooled) acidified methanol/chloroform (methanol:chloroform:HCl; 80:40:1) was added to each well for 45 s. Following transferral to polycarbonate tubes, 300 μl chloroform, then 500 μl 0.1 M HCl was added to each sample. The samples were then vortexed and left at room temperature for 15-30 min. The samples were then centrifuged at 3500 X g for 5 min at 4°C, and the aqueous upper phase removed by aspiration.
For total membrane phospholipid determination 450 μl of the lower phase was transferred to a scintillation vial and evaporated to dryness overnight in a fume hood. 4 ml Safe-Fluor scintillant was then added to each of the vials, which were then vortexed thoroughly before being counted.

2.7 Determination of protein concentration (Lowry et al, 1951)

Cell protein from 24 well multidishes were incubated for 2 h with and membrane preparations added to 0.5 ml 0.1 M NaOH. 1 ml Lowry C reagent (100 parts 2% Na₂CO₃, 0.4% NaOH, plus 1 part 1% CuSO₄ and 1 part 2% sodium potassium tartrate) was then added before vortexing and incubation at room temperature for 10 min. 100 μl of a 1:3 dilution of Folin/Ciocalteau reagent was then added to the samples which were vortexed and incubated at room temperature for a further 15-20 mins. 1 ml of dH₂O was then added to the samples which were then vortexed. The samples become different shades of blue depending on the concentration of protein present (the darker the blue, the more protein). Spectrophotometric measurement of the absorbance of the samples at 750 nm was then used to calculate protein concentration from a bovine serum albumin standard calibration curve constructed and measured as above.

2.8 Calcium phosphate transfections

BHK-mGluR1α cells and BHK-570 cells were transfected with the pCEP4 vector and pCEP4 vector containing cDNA for the human muscarinic m₃ receptor using calcium phosphate precipitation (described by Sambrook et al, 1989). The pCEP4 vector
confers hygromycin B resistance to the cells, allowing selection of transfected clones with hygromycin B. CHO-WT cells were transfected with the pcDNA 3 vector containing cDNA for the rat type 1α metabotropic glutamate receptor and the cDNA for the same receptor with serine 166 mutated to an aspartate residue. The pcDNA 3 vector confers G418 resistance to the cells, allowing selection of transfected clones with G418.

Death curves were constructed to determine the concentration of selection agents required to kill all untransfected cells. The cells were seeded to 50% confluence into 10 cm petri dishes containing 10 ml medium plus a range of hygromycin B/G418 concentrations from 200 μg ml⁻¹ to 700 μg ml⁻¹ in 100 μg ml⁻¹ increments, the cells were then maintained at 37°C in a humidified atmosphere (95% air, 5% CO₂) and fed every 2-3 days until the lowest concentration of hygromycin B/G418 required to kill all untransfected cells was established.

Cells were then seeded into 10 cm petri dishes in 10 ml medium, in various ratios (1:5, 1:10, 1:20) to obtain a dish with cells present at 50% confluence, after overnight incubation at 37°C in a humidified atmosphere (95% air, 5% CO₂).

10 μg of DNA was taken to a final volume of 440 μl in 0.1 x TE (in mM TRIS, 1; EDTA, 0.1; pH 8, sterile filtered). 500 μl 2 x HBS (in mM NaCl, 280; KCl, 10; Na₂HPO₄, 1.5; glucose, 11; HEPES, 50; pH 7.05, sterile filtered) was then added but not mixed. 67 μl 2 M CaCl₂ (sterile filtered) was then added slowly as air was bubbled through the solution. The mixture was incubated at room temperature for 30 min before being added to the cells which were incubated for 24 h at 37°C in a
humidified atmosphere (95% air, 5% CO₂). The medium was then changed to regular medium and the cells incubated as above for a further 24 h. The cells were then washed twice with 4 ml HBS, harvested in 2 ml trypsin-EDTA solution then seeded in various ratios (1:5, 1:6, 1:10, 1:20) into 10 cm petri dishes containing 10 ml selection medium (regular medium supplemented with 300 μg ml⁻¹ hygromycin B or 600 μg ml⁻¹ G418).

The cells were then maintained at 37°C in a humidified atmosphere (95% air, 5% CO₂), and fed every 2-3 days until clones resistant to hygromycin/G418 could be observed, i.e. 1-2 weeks. 24 clones from each cell type were then transferred to 24 well multidishes using sterile toothpicks, and were maintained as above until the wells became confluent. The cells from each well were harvested using trypsin-EDTA solution and then transferred to 80 cm² tissue culture flasks in 12 ml medium and maintained as above until reaching confluence. Once confluent the cells were screened for M₃-muscarinic receptors using [³H]-NMS binding and mGluR1αS₁₈₆D- mGluR1α using Western blotting. Transfected clones were also screened for their ability to increase InsP₁ accumulation in response to agonist application by the method described previously. Three BHK-570 clones expressing the muscarinic m₃ receptor coupled to InsP₁ accumulation (BHK-m3 cells) were then characterised further by constructing MCh concentration effect curves.
2.9 [³H]-NMS (1-[N-methyl-³H] scopolamine methyl chloride) binding

2.9.1 On intact cells

Cells were seeded into 24 well multidishes, in 1 ml medium to give a confluence of 40-70% after overnight incubation at 37°C in a humidified atmosphere (95% air, 5% CO₂).

The cells were then washed with 2 x 1 ml KHB at 37°C. 2 total binding and 1 NSB (non-specific binding) values were obtained for each clone. To obtain total binding values the cells were incubated with ~ 1 nM [³H]-NMS, in KHB to a final volume of 500 µl. For the NSB the cells were incubated with 1 nM [³H]-NMS and 1 µM atropine in KHB to a final volume of 500 µl. After 1 h the incubation mixture was aspirated off and the cells were washed with 2 x 1 ml ice cold KHB. 250 µl 0.1 M NaOH was then added to the wells which were then left on ice for 1 h. The contents of the wells were then pipetted to scintillation vials and the wells washed out with 250 µl 0.1 M HCl, which was also added to the vials. 2 x 100 µl of the [³H]-NMS used was also added to scintillation vials as standards. 4 ml emulsifier safe scintillant was then added to each vial before counting. For each clone the protein concentration was determined using the Lowry method to enable the binding to be expressed as pmol mg protein⁻¹.

2.9.2 Using cell membranes

The expression levels of the muscarinic-m3 receptor in the BHK cells transfected with the cDNA for the muscarinic-m3 receptor were determined using [³H]-NMS
saturation binding to determine the $B_{\text{max}}$ and $K_D$, which can be used to estimate receptor number.

Membranes of BHK-m3 cells were prepared as described previously. The concentrations of $[^3\text{H}]$-NMS used were: 3, 1, 0.57, 0.38, 0.19, 0.13 and 0.07 nM. For each concentration of $[^3\text{H}]$-NMS total and non-specific binding were determined using the following reaction mixtures, in order of addition;

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Total (µl)</th>
<th>NSB (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAB*</td>
<td>140</td>
<td>100</td>
</tr>
<tr>
<td>Atropine (1µM)</td>
<td>-</td>
<td>40</td>
</tr>
<tr>
<td>$[^3\text{H}]$-NMS</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Membranes (1 mg ml$^{-1}$)</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

*(MAB = Membrane assay buffer; 10 mM HEPES, 10 mM MgCl$_2$, 100 mM NaCl, pH 7.4)*

The samples were vortexed and incubated at 37°C for 1 h in a shaking water bath. Using a Brandel cell harvester the filtrate was then collected on wet GF/B Whatman filter paper (moistened with MAB) and the tubes washed with 3 X 3ml ice-cold MAB. The filters were then transferred to scintillation vials and 4ml Emulsifier Safe scintillant added. The samples were then left overnight and vortexed at least 30 min before counting.
2.10 [Ca\textsuperscript{2+}]\textsubscript{j} measurements

2.10.1 Cell preparation

Cells were prepared for experimental use the day before experiments were performed. Cells were harvested as described in Section 2.2 and subsequently reseeded in 2 ml medium on sterile type 0 22 mm diameter coverslips in 35 mm petri dishes, at a density of approximately 1:30, to allow the presence of single cells on the coverslips. CHO-lac-hmGluR1\textsubscript{a} cells were induced as described in Section 2.3. Cells were incubated at 37\textdegree C in 5% CO\textsubscript{2}/ humidified air to allow attachment of cells to the coverslips overnight. Cells were treated with GPT/pyruvate or CCPCOEt as described in Section 2.3.

2.10.2 Fura-2 as an indicator of changes in [Ca\textsuperscript{2+}],

Fura-2 is a highly fluorescent Ca\textsuperscript{2+} chelator, used to detect changes in intracellular Ca\textsuperscript{2+} concentration. Free fura emits maximally upon excitation at 380nm. Upon binding Ca\textsuperscript{2+} its excitation spectrum shifts to the left, so that it emits maximally, when excited, at 340nm (Gryniewicz et al, 1985). Emission is measured at 509nm. By using the ratio of emission when the cells are excited at 340nm and 380nm, changes in intracellular Ca\textsuperscript{2+} concentration can be measured independent of the concentration of fura-2 within the cell, and the sensitivity of the instrument used to measure the emission. This ratiometric technique also allows accurate calibration to be performed, in which loss of fura-2 from the cell, or a change in the sensitivity of the instrument used to measure the emission have no effect. Free fura-2 (the
lipophobic pentacarboxylic form) is however membrane impermeable. Loading of cells therefore takes place using fura-2 AM (the membrane permeable acetoxy-methyl form of the compound) allowing the fluorescent dye to enter the cells. The dye is prevented from leaking out of the cell by non-specific intracellular esterases which cleave off the acetoxy-methyl groups. Fura-2 can also be photobleached, therefore loading and loaded cells are kept in the dark.

2.10.3 Loading cells with fura-2 AM

The prepared coverslips were washed twice in 2 ml of KHB to remove medium and then placed in 2 ml of KHB. The cells were then incubated with 2 μM fura-2 AM for 1-2 h room temperature in the dark. Unloaded fura-2 AM was removed by washing the coverslip twice with 2 ml KHB, the coverslip was then incubated at room temperature for a further 15 min to enable intracellular esterases to remove the acetoxy-methyl groups from the fura, allowing retention of the fura inside the cell. The coverslip was then kept at room temperature in the dark for use within 1 h. Loading and loaded CHO-lac-hmGluR1α cells were incubated in the presence of 3 U ml⁻¹ GPTand 5 mM pyruvate to remove glutamate present in medium.

2.10.4 Measurement of the fluorescence changes

*Photon Technology Deltascan International system:*

The PTI (Photon Technology Deltascan International) system consists of a fluorescence microscope and a photomultiplier connected to FeliX Software loaded
on a computer. Fluorescence readings were obtained by exciting the cells at 340 nm and 380 nm at a frequency of 1 Hz and then measuring the emission at 509 nm. The FeliX Software recorded the fluorescence at 340 nm and 380 nm and calculated the ratio, which was displayed on the screen with respect to time as the experiment is carried out, and saved for subsequent analysis. Background fluorescence occurs due to the cells, the coverslip they are attached to, and the coverslip in the Peltier chamber. At the beginning of each days experiments the background fluorescence at 340 nm and 380 nm was measured for 3 unloaded cells. The mean value for each wavelength was manually entered into the FeliX Software to be subtracted automatically by the software from all subsequent fluorescence readings. For each experiment carried out a fragment of coverslip with loaded cells attached was placed in the Peltier chamber of the fluorescence microscope and was continually perfused with KHB (22°C) at a flow rate of 1 ml min⁻¹. Single cells were isolated for fluorescence measurements using shutters in the photomultiplier system. Agonists were applied via the perfusion lines.

*Applied Imaging QuantiCell-700 Calcium Imager*

Loaded coverslips were mounted in a metal casing to form a chamber in which the cells could be continually perfused with KHB (22°C) at a flow rate of 5 ml min⁻¹. This was inserted onto the stage of the Nikon Diaphot inverted epifluorescence microscope. Agonists were again applied via the perfusion lines. Background fluorescence was automatically subtracted from the fluorescence measurements which were obtained as described above. These measurements were collected with an intensified charge-coupled device camera and displayed and analysed using the
Applied Imaging QuantiCell-700 Calcium Imager software, ratiometric values being converted to approximate $[\text{Ca}^{2+}]$, using the Grynkiewicz equation (Grynkiewicz et al., 1985).

2.11 SDS polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and immunodetection of proteins

2.11.1 Preparation of cell lysates

Adherent mammalian cells were cultured on 6 well multidishes (Nunc) see Section 2.1. Cells were washed with 3 ml PBS (Phosphate buffered saline: 136 mM NaCl, 3.5 mM Na$_2$HPO$_4$, 2.6 mM KCl and 1.76 mM KH$_2$PO$_4$) at 4°C. Cells were then lysed in 300 µl of R.D.L. buffer (20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM PMSF, 10 µg ml$^{-1}$ leupeptin) and transferred to a 1.5 ml microfuge tube, 2 µl of 10% SDS was then added before vortexing for 2 X 10 s. Lysates were cleared by centrifugation at 20,000 X g for 2 min at 4°C.
2.11.2 SDS-PAGE

SDS-PAGE (Laemmli, 1970; Sambrook et al, 1989) was performed using a Biorad MINI-PROTEAN II apparatus. 7% polyacrylamide gels were used:

<table>
<thead>
<tr>
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<th>Stacking phase</th>
<th>Resolving phase</th>
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<tr>
<td>% Acrylamide</td>
<td>3%</td>
<td>7%</td>
</tr>
<tr>
<td>dH₂O</td>
<td>4.6 ml</td>
<td>9.9 ml</td>
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<tr>
<td>30% Protogel</td>
<td>800 µl</td>
<td>4.7 ml</td>
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<tr>
<td>Tris: 1 M, pH 6.8</td>
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<tr>
<td>1.5 M, pH 8.8</td>
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<td>5 ml</td>
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<tr>
<td>10% SDS</td>
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<tr>
<td>10% AMPS</td>
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<td>200 µl</td>
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<tr>
<td>TEMED</td>
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Location of protein bands was estimated using low and high molecular weight markers: low; α lactalbumin (14.2 kDa), trypsin inhibitor (20 kDa), trypsinogen (24 kDa), carboxic anhydrase (29 kDa), glyceraldehyde-3-phosphate-dehydrogenase (36 kDa), albumin (45 kDa) and bovine albumin (66 kDa), high; carboxic anhydrase (29 kDa), albumin (45 kDa), bovine albumin (66 kDa), phosphorylase B (97.4 kDa), β-galactosidase (116 kDa) and myosin (205 kDa).

Samples and markers were heated to 70°C for 3 min, in an equal volume of gel-loading buffer (100 mM Tris HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue and 200 mM DTT (added just before use)) i.e. 15 µl sample + 15 µl gel-loading buffer, prior to loading onto the gel, mounted in the electrophoresis tank containing
running buffer (25 mM Tris base, 250 mM glycine and 0.1% SDS). Gels were run at 150 V for ~ 1 h.

### 2.11.3 Western blotting (Harlow and Lane, 1985)

Following SDS-PAGE, proteins were transferred, in transfer buffer (150 mM glycine, 20 mM Tris base, 0.037% SDS), by electrophoresis to nitrocellulose membranes, at a maximum of 5.5 mA/cm² for 15 min using a BioRad Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell. Transferred proteins were visualised with Ponceau S solution (0.5% Ponceau S, 1% acetic acid) and following removal of excess stain with deionised water, marker positions were recorded with ink pen. The remaining Ponceau S stain was removed by washing the nitrocellulose membranes with TBS-Tween (4 mM Tris base, 100 mM NaCl, pH 7.5 and 0.1% Tween 20). Nitrocellulose membranes were then transferred to blocking buffer (TBS-Tween plus 5% Marvel) overnight at 4°C or at room temperature for 30 min.

### 2.11.4 Immunodetection of protein bands

Following Western blotting the nitrocellulose membranes were incubated in blocking solution containing 1% Marvel, containing appropriate amounts of primary antibody (mGluR1α 1; 1:5000) overnight at 4°C, on a bottle roller in 50 ml centrifuge tubes. The nitrocellulose membranes were then washed 3 X 250 ml in TBS-Tween, prior to incubation with a HRP (horse radish peroxidase) linked goat anti-rabbit secondary antibody (1:1000) for 1 h at room temperature and further 3 X 250 ml washing with TBS-Tween. The membranes were then incubated with ECL (enhanced...
chemiluminescence) Western blotting detection reagents (Amersham) and chemiluminescence detected on hyperfilm MP (Amersham X-ray film) following various exposure times.

2.12 Cloning and maintenance of plasmid DNA

2.12.1 DNA quantification

DNA concentrations were quantified by spectrophotometric measurement of the absorbance of DNA solutions at 260 nm, one absorbance unit being equivalent to 50 \( \mu g \) ml\(^{-1} \); double stranded DNA and 20 \( \mu g \) ml\(^{-1} \); single stranded DNA.

DNA concentrations were also quantified using ethidium bromide agarose gel electrophoresis (Sambrook et al, 1989). The intensity of ethidium bromide/UV-fluorescence of the DNA solution of unknown concentration was compared with that of \( \lambda \) phage, Hind III digested DNA standards. 10 \( \mu g \) of the standard digest was loaded per lane giving concentrations for comparison of 4.76 \( \mu g \) at 23.1 Kb, 1.94 \( \mu g \) at 9.41 Kb, 1.35 \( \mu g \) at 6.55 Kb, 0.89 \( \mu g \) at 4.36 Kb, 0.47 \( \mu g \) at 2 Kb, 0.11 \( \mu g \) at 0.56 Kb and 0.025 \( \mu g \) at 0.125 Kb.

2.12.2 Large scale plasmid purification (for subcloning only)

Single colonies of \( E. \ Coli \) (DH5\( \alpha \)) containing the relevant plasmids were inoculated to 10 ml cultures of 2 X YT bacterial culture medium (10 g NaCl, 10 g yeast extract and 16 g bacto-trypotone in 1 L de-ionised water, pH 7.5 plus 100 \( \mu g \) ml\(^{-1} \) ampicillin),
and incubated overnight at 37°C with constant shaking at 200 r.p.m. 1 ml of this
overnight culture was then inoculated to 200 ml of 2 X YT and incubated for 18 h as
above. This culture was then centrifuged at 10000 X g for 5 min at 25 °C, the culture
medium was removed by aspiration and the pellet resuspended in 10 ml of
solution 1(50 mM glucose, 25 mM Tris-HCl, pH 7.5 and 10 mM EDTA). The cultures
were then lysed by addition of 20 ml of ice-cold solution 2 (0.2 M NaOH and 1%
SDS) followed by gentle inversion. The culture lysate was then cleared by addition
of 15 ml of solution 3 (0.8 M potassium acetate, 2 M acetic acid) followed by
centrifugation at 10000 X g for 10 min at 4°C and filtering of the supernatant (cleared
lysate) through polyallomer wool into a 30 ml centrifuge tube. The total nucleic acid
present in the lysate was then precipitated with an equal volume of propan-2-ol and
pelleted by centrifugation at 10000 X g for 10 min at 4°C and the supernatant
discarded prior to resuspension of the pellet in 3 ml de-ionised water. An equal
volume of 8 M LiCl was then added to precipitate any RNA present prior to its
removal by centrifugation in a 30 ml centrifuge tube at 9000 X g for 10 min at 4°C.
The supernatant was then transferred to another 30 ml centrifuge tube and mixed
with an equal volume of propan-2-ol and the plasmid DNA precipitate collected by
centrifugation at 9000 X g for 10 min at 4°C. The pellet was then resuspended in
400 μl Q water (Millipore) and any remaining low Mw RNA removed by addition of
20 μl of RNase A solution (10 mg ml⁻¹ in 100 mM Tris-HCl, pH 7.5) and incubation at
37°C for 30 min, followed by removal of RNase by the addition of 400 μl of
phenol:chloroform (phenol:chloroform:isoamylalcohol; 25:24:1) prior to vortexing and
centrifugation at 12000 X g for 5 min. The upper aqueous phase was re-extracted
with an equal volume of phenol:chloroform, and again with an equal volume of water-
saturated chloroform. Plasmid DNA was then precipitated with 1 M ammonium
acetate and 2.5 volumes of ethanol at -80°C for 10 min, prior to centrifugation at 12000 X g for 10 min. The supernatant was then discarded and plasmid DNA washed with 1 ml 70 % ethanol prior to air drying. This purified plasmid DNA was then resuspended in 100 μl of Q water and quantified spectrophotometrically. The quality of plasmid DNA was assessed by restriction endonuclease digestion followed by agarose gel electrophoresis. Plasmid DNA was then suspended at 1 μg ml⁻¹ in sterile double distilled water and stored at -20°C.

2.12.3 Large scale plasmid purification (for mammalian cell transfection only)

Large scale plasmid purification for mammalian cell transfection was performed using a Qiagen Plasmid Maxi Kit in accordance with the manufacturer's guidelines. DNA was quantified spectrophotometrically.

2.12.4 Small scale plasmid purification

Small scale plasmid purification was performed for sub-cloning steps only using the WIZARD preparation from Promega in accordance with the manufacturer's guidelines.

2.12.5 Glycerol stock maintenance

Glycerol stocks of transformed bacterial cultures were produced by the addition of 0.5 ml glycerol stock solution (60% Nutrient broth, 40% glycerol) to 0.5 ml of the
appropriate culture grown overnight. The stocks were then snap frozen in liquid nitrogen and stored at -80°C until further use.

2.12.6 Restriction endonuclease digestion of DNA

DNA of ≤ 20 µg was digested in a final volume of 20 µl with 10 units of enzyme for 1 h. DNA of > 20 µg was digested in a volume of 100 µl with 50 units of enzyme overnight. In both cases the buffer and temperature conditions used were those recommended by the manufacturer (GIBCO-BRL).

2.12.7 Isolation and gel purification of DNA

Isolation and gel purification of DNA was carried out using the QIAEX II system (Qiagen) in accordance with the manufacturer’s guidelines.

2.12.8 Ligation of DNA

Plasmid and insert DNA was ligated with 1 unit T4 DNA ligase in a volume no greater than 10 µl (DNA, T4 DNA ligase, T4 DNA ligase buffer, Q water). Where compatible “sticky end” ligations were performed plasmid and insert DNA were present in an equimolar ratio. Ligations were performed at room temperature for 24 h prior to transformation into E. Coli.
2.12.8 Transformation of E. Coli

Transformation of *E. Coli* was carried out using Library Efficiency DH5α Competent Cells supplied by GIBCO-BRL.

Competent cells were defrosted on ice, gently mixed and 100 µl aliquots placed in the required number of transformation tubes on ice. 1-10 µl of ligation mix of plasmid and insert DNA were gently added to the aliquoted competent cells, tapped to mix, and incubated on ice for 30 min. Transformation mixes were then heat-shocked at 42°C for 90 s prior to incubation on ice for 2 min. 2 X YT medium was then added to a final volume of 1 ml prior to incubation at 37°C with shaking at 200 r.p.m. for 1 h. Transformation mixes were then centrifuged at 700 X g for 30 s and 800 µl of supernatant removed. The remaining 200 µl of transformation mix was spread on solid 2 X YT medium (2 X YT with 15% bacterial agarose) with 100 µg ml⁻¹ ampicillin. Plated transformed DH5α *E. Coli* were then incubated at 37°C overnight.

2.12.9 Selection of recombinants

PCR (polymerase chain reaction) was used to screen colonies potentially containing recombinant DNA. Potential recombinant colonies from transformation reactions were picked from the solid medium plates and incubated overnight at 37°C in 5 ml of 2 X YT containing 100 µg ml⁻¹ ampicillin, with continuous shaking at 200 r.p.m. 5 µl of each culture was then added to 50 µl PCR reaction mix (1.0 unit Taq polymerase, 1 mM each dNTP, 50 mM Tris-HCl, pH 8.8, 12 mM NH₄SO₄, 5 mM MgCl₂, 1 mM 2-
mercaptoethanol, 5 μM EDTA, 0.1 mg ml⁻¹ BSA and 160 ng of primer (18 mer)). The primers complemented sequences internal to the cloned insert DNA or the external sequence of the plasmid in use. PCR reactions were carried out using a 2400 model Perkin Elmer Cetus thermocycler. The following step cycles were performed 25-30 times: denaturing; 94°C, 30 s, annealing; 55°C, 60 s, extension; 74°C, 90 s. PCR products were then characterised using gel electrophoretic analysis to determine which of the overnight 5 ml cultures were positive recombinants containing the appropriate plasmid DNA to be used in further work.

2.12.10 DNA sequencing

All DNA sequencing was performed by P.N.A.C.L. (Protein and Nucleic Acid Chemistry Laboratory of the University of Leicester) using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit. Sequences were analysed on a PE Biosystems 377 sequencer.

2.12.11 DNA sequence analysis

Sequences were viewed using the ABI Autoassembler, on the P.N.A.C.L. fileserver site (University of Leicester).

2.12.12 COS7 transient transfection with DEAE Dextran/Chloroquine

COS7 cells were seeded into 10 cm petri dishes 18 h prior to transfection with 10 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FCS, 100 μg
streptomycin, $10^5 \text{ U L}^{-1}$ penicillin, 2.5 $\mu\text{g ml}^{-1}$ amphotericin B, 2mM L-glutamine.

Prior to transfection the medium was removed, the cells washed once with PBS and the medium replaced with 4 ml DMEM supplemented as above, except Nu-Serum is used in place of FCS. 6 $\mu\text{g}$ of the DNA for transfection was added to 120 $\mu\text{l}$ of solution C, which comprises 10% solution A and 1% solution B in Milli Q water (solution A: 8.0 % NaCl, 0.38 % KCl, 0.2 % Na$_2$HPO$_4$, 3.0 % TRIS-base, pH 7.4, solution B: 1.5 % CaCl$_2$ and 1.0 % MgCl$_2$). 120 $\mu\text{l}$ chloroquine solution (10 mM chloroquine in solution C) was mixed with 480 $\mu\text{l}$ DEAE solution (10 mg ml$^{-1}$ DEAE dextran in solution C). This was then mixed with the 120 $\mu\text{l}$ of solution C and DNA. 240 $\mu\text{l}$ of this mixture was then added drop-wise to each 10 cm petri dish of COS7 cells. The cells are then incubated at 37 °C in a humidified atmosphere (95 % air, 5 % CO$_2$) for 4 h. The media and DNA are then removed by aspiration and the cells washed with 4 ml PBS. A 10 % solution of DMSO in PBS was then added to the cells for 2 min at room temperature. This solution was then rapidly removed by aspiration and the cells washed with 4 ml of standard COS7 media (with FCS). The cells are then incubated with 10 ml COS7 media for 72 h prior to cell lysis.

### 2.12.13 Transient transfection with FuGene-6

Wild-type CHO cells were seeded into 12 well multidishes to a confluence of 30 to 40 % prior to transfection. The appropriate amount of FuGene-6* was added to 1200 $\mu\text{l}$ minimal essential medium (containing Earle's salts and Glutamax-1). *For each transfection, reagent sufficient for addition of 50 $\mu\text{l}$ to each of 24 wells was mixed, thus for 300 ng DNA well$^{-1}$, 10.8 $\mu\text{l}$ FuGene-6 was added to 1200 $\mu\text{l}$ minimal essential medium, for 600 ng DNA well$^{-1}$, 21.6 $\mu\text{l}$ FuGene-6 was used and for 900 ng
DNA well$^{-1}$, 32.4 μl FuGene-6 was used. This mixture was then incubated at room temperature for 5 min. The FuGene-6 mixture was then added to the appropriate amount of DNA in 20 μl minimal essential medium, i.e. for 300 ng DNA well$^{-1}$, 7.2 μg, for 600 ng DNA well$^{-1}$, 14.4 μg, for 900 ng DNA well$^{-1}$, 21.6 μg. This mixture was incubated at room temperature for 15 min. 50 μl of this mixture was then added to each well. The cells were then incubated at 37 °C in a humidified atmosphere (95 % air, 5 % CO$_2$) for 24 - 72 h prior to experimentation.

2.13 Data Analysis

Data were analysed using GraphPad Prism 2.1 for Microsoft Windows for Workgroups Version 3.11 and SPSS for Windows. Statistical tests used are stated in the text. For parametric data where single comparisons were made t-tests were used, where two independent variables e.g. quisqualate concentration and [Ca$^{2+}$]$_{o}$ were measured two-way ANOVA was used, where multiple comparisons were made one-way ANOVA followed by a Duncans multiple range test was used. For non-parametric data where single comparisons were made a Mann-Whitney test was used, where multiple comparisons were made a Kruskal-Wallis test followed by a Dunn's post test was used.
CHAPTER 3 - MODULATION OF RAT TYPE 1α METABOTROPIC GLUTAMATE RECEPTOR SIGNALLING IN BABY HAMSTER KIDNEY CELLS

3.1 Investigations into the effect of varying \([\text{Ca}^{2+}]_e\) on phosphoinositide signalling in BHK cells stably expressing mGluR1α.

The identification of \(\sim 30\%\) sequence homology between CaSRs and mGluRs raised questions as to the ability of mGluRs to respond to changes in \([\text{Ca}^{2+}]_e\) (Brown et al., 1993). The identification of sBimR, which is activated by both glutamate and \(\text{Ca}^{2+}\) when expressed in Xenopus oocytes and shares sequence homology with both the CaSR (24%) and mGluR1α (69%), was therefore very interesting (Kubokawa et al., 1996). Due to the high sequence homology of sBimR with mGluR1α, mGluR1α was expressed in Xenopus oocytes and was also shown to be activated by both glutamate and \(\text{Ca}^{2+}\). My investigations have extended these observations to encompass the ability of \(\text{Ca}^{2+}\) to modulate signalling via rat mGluR1α stably expressed in a mammalian baby hamster kidney cell-line.

3.1.1 Concentration-dependence of agonist-stimulated \([^3\text{H}]-\text{InsP}_1\) accumulation in BHK-mGluR1α cells in the presence of different \([\text{Ca}^{2+}]_e\).

The influence of \([\text{Ca}^{2+}]_e\) on the coupling of mGluR1α to PLC in BHK cells was studied by measuring \([^3\text{H}]-\text{InsP}_1\) accumulation in the presence of 10 mM LiCl in cells which had been incubated with 1 μCi ml⁻¹ \([^3\text{H}]-\text{inositol}\) for 48 h.
Fig. 3.1. shows basal $[^3\text{H}]-\text{InsP}_1$ accumulation and concentration-dependent increases in $[^3\text{H}]-\text{InsP}_1$ accumulation in response to stimulation of mGluR1$\alpha$ with quisqualate, a full agonist of mGluR1$\alpha$, for 30 min, in the presence of nominal, 0.5, 1.3, 2.5 and 4 mM Ca$^{2+}_e$ (n = 3). Previous studies in this laboratory have shown that omission of CaCl$_2$ in KHB plus addition of 100 $\mu$M EGTA reduces $[^3\text{H}]-\text{InsP}_1$ accumulation in response to 300 $\mu$M glutamate by $>$80% (A.M. Carruthers, University of Leicester, PhD thesis). In support of these data omission of CaCl$_2$ (without addition of EGTA), reduced the $[^3\text{H}]-\text{InsP}_1$ accumulation in response to 30 $\mu$M quisqualate to 47.0 ± 6.6% of that in the presence of 1.3 mM Ca$^{2+}_e$. In addition, increasing [Ca$^{2+}_e$] over the range stated, above nominally Ca$^{2+}_e$-free conditions, results in a graded increase in the $[^3\text{H}]-\text{InsP}_1$ accumulation in response to 30 $\mu$M quisqualate, from 30153 ± 6662 (nominally Ca$^{2+}_e$-free) through 51173 ± 10708 (0.5 mM Ca$^{2+}_e$), 62575 ± 6915 (1.3 mM Ca$^{2+}_e$), 70272 ± 6052 (2.5 mM Ca$^{2+}_e$) to 76886 ± 1140 (4 mM Ca$^{2+}_e$) d.p.m. mg$^{-1}$ protein. Increasing [Ca$^{2+}_e$] also results in a graded decrease in the EC$_{50}$ values for quisqualate-stimulated $[^3\text{H}]-\text{InsP}_1$ accumulation, from -5.2 ± 0.4 (6.9 $\mu$M, nominally Ca$^{2+}_e$-free) through -5.6 ± 0.4 (2.7 $\mu$M, 0.5 mM Ca$^{2+}_e$), -5.8 ± 0.3 (1.5 $\mu$M, 1.3 mM Ca$^{2+}_e$), -6.0 ± 0.3 (1.1 $\mu$M, 2.5 mM Ca$^{2+}_e$) to -6.1 ± 0.3 (0.8 $\mu$M, 4 mM Ca$^{2+}_e$). Increasing [Ca$^{2+}_e$] per se did not however result in a significant increase in basal $[^3\text{H}]-\text{InsP}_1$ accumulation; 4839 ± 1208 (nominally Ca$^{2+}_e$-free) through 5166 ± 737 (0.5 mM Ca$^{2+}_e$), 5156 ± 886 (1.3 mM Ca$^{2+}_e$), 7088 ± 1945 (2.5 mM Ca$^{2+}_e$) to 9516 ± 2873 (4 mM Ca$^{2+}_e$) d.p.m. mg$^{-1}$ protein. As these initial experiments, with limited concentration-effect curves, proved interesting, full $[^3\text{H}]-\text{InsP}_1$ accumulation concentration-effect curves to quisqualate were constructed, in the presence of nominally Ca$^{2+}_e$-free buffer, 1.3 mM Ca$^{2+}_e$ and 4 mM Ca$^{2+}_e$. 

78
Fig. 3.1 Concentration-dependency of quisqualate-stimulated $[^3\text{H}]$-InsP$_1$ accumulations in the presence of different $[\text{Ca}^{2+}]_o$ (nominally $\text{Ca}^{2+}$-free; ■, 0.5 mM; O, 1.3 mM; ◆, 2.5 mM; ▼, 4 mM; ●) in BHK-mGluR1α cells. BHK cells stably expressing mGluR1α were incubated with 1 μCi ml$^{-1}$ $[^3\text{H}]$-inositol for 48 h, washed with KHB and incubated with KHB + 10 mM LiCl for 15 min before challenge with the indicated concentrations of quisqualate for 30 min. $[^3\text{H}]$-InsP$_1$ data are expressed as d.p.m. mg$^{-1}$ protein. Data are shown as mean ± standard error for 3 separate experiments performed in duplicate.
Fig. 3.2 shows basal $[^3]$H-InsP$_1$ accumulation and concentration-dependent increases in $[^3]$H-InsP$_1$ accumulation in response to stimulation of mGluR1$\alpha$ with quisqualate for 30 min, in the presence of nominal, 1.3 and 4 mM Ca$^{2+}$, (n = 5). The results confirm those obtained from construction of the limited concentration-effect curves shown in Fig. 3.1. $[^3]$H-InsP$_1$ accumulation in response to a maximal concentration of quisqualate (30 μM) increased as [Ca$^{2+}$]$_e$ was increased; 28.2 ± 3.3 (nominal Ca$^{2+}$), 89.9 ± 4.6 (1.3 mM Ca$^{2+}$), and 101.4 ± 3.1% (4 mM Ca$^{2+}$) of the $[^3]$H-InsP$_1$ accumulation in response to 30 μM quisqualate in the presence of 4 mM Ca$^{2+}$, corresponding to 15855 ± 5526 (nominal Ca$^{2+}$), 53073 ± 19660 (1.3 mM Ca$^{2+}$) and 59755 ± 24532 (4 mM Ca$^{2+}$) d.p.m. mg$^{-1}$ protein. The EC$_{50}$ values for quisqualate-stimulated $[^3]$H-InsP$_1$ accumulation decreased as [Ca$^{2+}$]$_e$ was increased; log EC$_{50}$ (M): -5.2 ± 0.6 (5.7 μM), -5.7 ± 0.4 (1.95 μM), -5.9 ± 0.5 (1.22 μM), in the presence of nominally Ca$^{2+}$ free buffer, 1.3 mM Ca$^{2+}$, and 4 mM Ca$^{2+}$, respectively. Increasing [Ca$^{2+}$]$_e$ per se had no significant effect on basal $[^3]$H-InsP$_1$ accumulation; 5972 ± 1320 (nominal Ca$^{2+}$), 4977 ± 704 (1.3 mM Ca$^{2+}$) and 6408 ± 1432 (4 mM Ca$^{2+}$) d.p.m. mg$^{-1}$ protein.

Fig. 3.3 shows basal $[^3]$H-InsP$_1$ accumulation and concentration-dependent increases in $[^3]$H-InsP$_1$ accumulation in response to stimulation of mGluR1$\alpha$ with 1S,3R-ACPD for 30 min, in the presence of nominal, 1.3 mM and 4 mM Ca$^{2+}$, (n = 4). In contrast to stimulation of mGluR1$\alpha$ with quisqualate, no $[^3]$H-InsP$_1$ accumulation was seen in response to 1S,3R-ACPD in the presence of nominally Ca$^{2+}$ free buffer; 10670 ± 3221 (basal) compared to 11540 ± 1855 (3 mM 1S,3R-ACPD) d.p.m. mg$^{-1}$ protein. However $[^3]$H-InsP$_1$ accumulation in response to stimulation of mGluR1$\alpha$ with a maximal concentration of 1S,3R-ACPD (3 mM) increased as [Ca$^{2+}$]$_e$ was
increased; 45.5 ± 6.2 (1.3 mM Ca²⁺ₜ) to 65.6 ± 4.2% (4 mM Ca²⁺ₜ) of the [³H]-lnsP₁ accumulation in response to stimulation of mGluR₁α with 30 μM quisqualate in the presence of 4 mM Ca²⁺ₜ, corresponding to 41775 ± 2657 (1.3 mM Ca²⁺ₜ) and 66418 ± 3896 (4 mM Ca²⁺ₜ) d.p.m. mg⁻¹ protein (p<0.01, unpaired t-test). The EC₅₀ values for 1S,3R-ACPD-stimulated [³H]-InsP₁ accumulation decreased as [Ca²⁺ₜ] was increased; -3.6 ± 0.2 (180 μM) and -4.2 ± 0.2 (63 μM) in the presence of 1.3 mM and 4 mM Ca²⁺ₜ respectively (p<0.05, unpaired t-test). Basal [³H]-InsP₁ accumulation did not increase significantly as Ca²⁺ₜ was increased over the range nominally Ca²⁺-free to 4 mM; 10670 ± 3221 (nominally Ca²⁺-free), 7270 ± 615 (1.3 mM Ca²⁺ₜ) and 18890 ± 5737 (4 mM Ca²⁺ₜ) d.p.m. mg⁻¹ protein. Analysis of the concentration-effect curves by two-way ANOVA (with or without subtraction of respective basal values) reveals statistically significant effects (p<0.01) of increasing [Ca²⁺ₜ] from 1.3 to 4 mM. In the presence of equivalent [Ca²⁺ₜ]; 3 mM 1S,3R-ACPD generated 53% of the response to 30 μM quisqualate in 1.3 mM Ca²⁺ₜ, and 69% of the response to 30 μM quisqualate in the presence of 4 mM Ca²⁺ₜ. These data confirm that 1S,3R-ACPD behaves as a partial agonist in the presence of both 1.3 and 4 mM Ca²⁺ₜ.

These data suggest that rather than having a stimulatory effect per se, Ca²⁺ₜ has a modulatory effect on agonist-stimulated [³H]-InsP₁ accumulation in BHK-mGluR₁α cells.
**Fig. 3.2** Concentration-dependency of quisqualate-stimulated \[^{3}\text{H}]\text{InsP}_1\) accumulations in the presence of different \([\text{Ca}^{2+}]_e\) (nominally \(\text{Ca}^{2+}\)-free; ■, 1.3 mM; △, 4 mM; ●) in BHK-mGluR1α cells. BHK cells stably expressing mGluR1α were incubated with 1 μCi ml\(^{-1}\) \[^{3}\text{H}]\text{-inositol for 48 h, washed with KHB and incubated with KHB + 10 mM LiCl for 15 min before challenge with the indicated concentrations of quisqualate for 30 min. \[^{3}\text{H}]\text{-InsP}_1\) data are expressed as a percentage of the response of cells to 30 μM quisqualate in the presence of 4 mM \(\text{Ca}^{2+}_e\). Data are shown as mean ± standard error for 5 separate experiments.
Fig. 3.3 Concentration-dependency of 1S,3R-ACPD-stimulated [\(^3\)H]-InsP\(_1\) accumulations in the presence of different [Ca\(^{2+}\)]\(_e\) (nominally Ca\(^{2+}\)-free; ■, 1.3 mM; △, 4 mM; ○) in BHK-mGluR1α cells. BHK cells stably expressing mGluR1α were incubated with 1 μCi ml\(^{-1}\) [\(^3\)H]-inositol for 48 h, washed with KHB and incubated with KHB + 10 mM LiCl for 15 min before challenge with the indicated concentrations of 1S,3R-ACPD for 30 min. [\(^3\)H]-InsP\(_1\) data are expressed as a percentage of the response of cells to 30 μM quisqualate in the presence of 4 mM Ca\(^{2+}\). Data are shown as mean ± standard error for 4 separate experiments.
3.2 Investigations into the effect of varying \([\text{Ca}^{2+}]_e\) on phosphoinositide signalling in BHK cells stably expressing the muscarinic-M₃ receptor.

In order to determine whether the modulatory effect of \([\text{Ca}^{2+}]_e\) is receptor- (mGluR1α) or cell-type (BHK) selective, the PLC-coupled M₃-muscarinic (acetylcholine) receptor was stably transfected into a BHK cell background.

3.2.1 Screening of BHK-570 cells transfected with the M₃-muscarinic receptor using \(^3\text{H}\)-NMS binding.

A number of hygromycin-resistant clones positive for M₃-muscarinic receptor expression (BHK-m3 cells) were identified using \(^3\text{H}\)-NMS binding. The specific \(^3\text{H}\)-NMS binding, used as an indication of the level of expression of the M₃-muscarinic receptor, ranged from 0.22 pmol mg protein\(^{-1}\) in clone *2 to 1.96 pmol mg protein\(^{-1}\) in clone *21, as shown in Table 3.1.

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<tr>
<th>Clone</th>
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<td>21</td>
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Table 3.1: \(^3\text{H}\)-NMS binding in BHK-570 cells transfected with the M₃-muscarinic receptor. Data are expressed as pmol \(^3\text{H}\)-NMS bound (mg protein\(^{-1}\)), for a single experiment.
3.2.2 Methacholine-stimulated $[^{3}H]$-InsP$_1$ accumulation in BHK-m3 cells.

BHK-m3 clones identified as being positive for M$_3$-muscarinic receptor expression were challenged with methacholine for 30 min in the presence of 10 mM LiCl, following incubation with 1 $\mu$Ci ml$^{-1}$ $[^{3}H]$-inositol for 48 h. $[^{3}H]$-InsP$_1$ accumulation was measured to give an indication of the efficiency of coupling in these clones. Fig. 3.4 shows concentration-dependent increases in $[^{3}H]$-InsP$_1$ accumulation of three BHK-m3 clones, shown to couple to PLC, and a vector control clone in response to challenge with methacholine. No increase in $[^{3}H]$-InsP$_1$ accumulation above basal was seen in vector control cells (Clone * V3); 150 d.p.m. well$^{-1}$ (basal) and 126 d.p.m. well$^{-1}$ (+10 $\mu$M methacholine). Fold increases in $[^{3}H]$-InsP$_1$ accumulation over basal, following stimulation with a maximal concentration of methacholine (100 $\mu$M) for each BHK-m3 clone were as follows; clone * 16; 8.9, clone * 20; 7.9, clone * 5; 11.4. The EC$_{50}$ ($\mu$M) values for methacholine-stimulated $[^{3}H]$-InsP$_1$ accumulation in each clone were; clone * 16; 8.1, clone * 20; 3.0, clone * 5; 2.5. Clones * 5 and * 20 had EC$_{50}$ values consistent with those previously reported for methacholine stimulation of the M$_3$-muscarinic receptor (D.K. Boxall, 1998) and appeared to show good coupling efficiency. These clones were chosen for further experimentation.

3.2.3 $[^{3}H]$-NMS saturation binding using BHK-m3 cell membranes.

$[^{3}H]$-NMS saturation binding using BHK cell membranes expressing the M$_3$-muscarinic receptor (clone * 5) was carried out to obtain $B_{\text{max}}$ and $K_D$ values allowing further characterisation of this clone. The $[^{3}H]$-NMS saturation binding data obtained were analysed using both the curve-fitting GraphPad prism software and the method
Fig. 3.4 Concentration-dependency of methacholine-stimulated [³H]-InsP₃ responses in isolated clones of BHK cells stably transfected with the M₃-muscarinic receptor (clone # 5; ■, clone # 20; ▽, clone # 16; ●) compared to a vector control clone (clone # V3; ○). Cells were cultured in the presence of 1 μCi ml⁻¹ [³H]-inositol for 48 h, washed with KHB and incubated with KHB + 10 mM LiCl for 15 min before challenge with the indicated concentrations of methacholine for 30 min. [³H]-InsP₃ data are expressed as fold increase over basal. Data are shown as means for a single experiment performed in duplicate.
of Scatchard, (1949). Fig. 3.5 shows an example of a [³H]-NMS saturation binding isotherm and a Scatchard plot. The B\textsubscript{max} obtained from the binding isotherm was 0.96 ± 0.08 pmol mg\textsuperscript{-1} protein and the K\textsubscript{D} was 0.45 ± 0.08 nM, the B\textsubscript{max} from Scatchard analysis was 0.99 ± 0.07 pmol mg\textsuperscript{-1} protein and the K\textsubscript{D} 0.49 ± 0.10 nM (n=3). Both the B\textsubscript{max} and K\textsubscript{D} values obtained from the 2 different methods of analysis are in close agreement with each other. The B\textsubscript{max} is an estimate of the total number of [³H]-NMS binding sites per mg protein for the membranes, and is taken to represent the density of M\textsubscript{3} -muscarinic receptor expression per mg protein. The K\textsubscript{D} value represents the concentration of [³H]-NMS required to occupy 50% of the total number of [³H]-NMS binding sites, and is in close agreement with previous values obtained for [³H]-NMS binding to M\textsubscript{3} -muscarinic receptors (D.K. Boxall, 1998).

3.2.4 Concentration-dependence of methacholine-stimulated [³H]-InsP\textsubscript{1} responses in BHK-m3 cells in the presence of different [Ca\textsuperscript{2+}]\textsubscript{e}.

The influence of [Ca\textsuperscript{2+}]\textsubscript{e} on the coupling of the M\textsubscript{3} -muscarinic receptor to PLC in BHK cells was studied by measuring [³H]-InsP\textsubscript{1} accumulation in the presence of 10 mM LiCl in cells which had been incubated with 1 μCi ml\textsuperscript{-1} [³H]-inositol for 48 h.

Fig. 3.6 shows concentration-dependent increases in [³H]-InsP\textsubscript{1} accumulation in response to stimulation of the M\textsubscript{3} -muscarinic receptor (BHK-m3, clone * 5) with methacholine for 30 min, in the presence of nominal, 1.3 mM and 4 mM Ca\textsuperscript{2+} (n = 4). The log EC\textsubscript{50} (M) value for methacholine-stimulated [³H]-InsP\textsubscript{1} accumulation in the presence of 1.3 mM Ca\textsuperscript{2+} was -5.9 ± 0.2 (1.26 μM) which is appropriate for methacholine stimulation of the M\textsubscript{3} -muscarinic receptor and in close agreement with
Fig. 3.5 [3H]-N-methylscopolamine saturation binding in BHK-m3 (clone # 5) cell membrane preparations. Cell membranes were prepared as described in Methods. Bound and free ligand were separated using vacuum filtration. Data are from a typical experiment performed in duplicate, A; Scatchard analysis, B; Hyperbolic curve-fitting.
Fig. 3.6 Concentration-dependency of methacholine-stimulated $[^3]$H-InsP$_1$ accumulations in the presence of different [Ca$^{2+}$]$_e$ (nominally Ca$^{2+}$-free; ■, 1.3 mM; △, 4 mM; ●) in BHK-m3 cells. BHK cells stably expressing M$_3$-muscarinic receptor were incubated with 1 μCi ml$^{-1}$ $[^3]$H-inositol for 48 h, washed with KHB and incubated with KHB + 10 mM LiCl for 15 min before challenge with the indicated concentrations of methacholine for 30 min. $[^3]$H-InsP$_1$ data are expressed as a percentage of the response of cells to 300 μM methacholine in the presence of 4 mM Ca$^{2+}$$_e$. Data are shown as mean ± standard error for 4 separate experiments.
the value obtained with a limited concentration-effect curve during screening. [$^3$H]-InsP$_1$ accumulation in response to a maximal concentration of methacholine (300 μM) was decreased in nominally Ca$^{2+}$ free buffer compared to in the presence of 1.3 and 4 mM Ca$^{2+}$; 37.5 ± 4.7% (nominal Ca$^{2+}$), compared to 99.6 ± 6.9% (1.3 mM) and 100% (4 mM), of the response to 300 μM methacholine in the presence of 4 mM Ca$^{2+}$. Thus, in common with BHK-mGluR1α cells, [$^3$H]-InsP$_1$ accumulation was decreased under nominally Ca$^{2+}$-free conditions, however increasing [Ca$^{2+}$]$_e$ from 1.3 mM to 4 mM has no effect (p>0.05, unpaired t-test) on the [$^3$H]-InsP$_1$ accumulation in response to a maximal concentration of methacholine. The log EC$_{50}$ (M) value for methacholine-stimulated [$^3$H]-InsP$_1$ accumulation was higher in nominally Ca$^{2+}$ free buffer; -5.5 ± 0.2 (3.3 μM), but was unaffected (p>0.05, unpaired t-test) by increasing the [Ca$^{2+}$]$_e$ from 1.3 to 4 mM Ca$^{2+}$; -5.9 ± 0.2 (1.26 μM) and -5.7 ± 0.2 (1.85 μM), respectively. Analysis of the concentration-effect curves by two-way ANOVA revealed no significant effect of increasing [Ca$^{2+}$]$_e$ from 1.3 to 4 mM.

To ensure that these data were not peculiar to clone 5 another M$_3$-muscarinic receptor expressing clone was studied. Fig. 3.7 shows concentration-dependent increases in [$^3$H]-InsP$_1$ accumulation in response to stimulation of the M$_3$-muscarinic receptor (BHK-m3 clone 20) with methacholine for 30 min, in the presence of nominal, 1.3 mM and 4 mM Ca$^{2+}$ (n = 1). [$^3$H]-InsP$_1$ accumulation in response to a maximal concentration of methacholine (300 μM) was decreased in nominally Ca$^{2+}$-free buffer; 41.7%, compared to 102.5% (1.3 mM) and 100% (4 mM), of the response to 300 μM methacholine in the presence of 4 mM Ca$^{2+}$. The log EC$_{50}$ value (M) for methacholine-stimulated [$^3$H]-InsP$_1$ accumulation, in the presence of 1.3 mM Ca$^{2+}$, was -5.8 (1.6 μM) which is appropriate for methacholine stimulation of the M$_3$-
Fig. 3.7 Concentration-dependency of methacholine-stimulated $[^3\text{H}]$-InsP$_1$ accumulation in BHK-m3 (clone # 20) cells, in the presence of different [Ca$^{2+}$]$_o$ (nominally Ca$^{2+}$-free; ■, 1.3 mM; △, 4 mM; ○). BHK cells stably expressing the M$_3$-muscarinic receptor were incubated in the presence of 1 μCi ml$^{-1}$ $[^3\text{H}]$-inositol for 48 h, washed with KHB and incubated with KHB + 10 mM LiCl for 15 min before challenge with the indicated concentrations of methacholine for 30 min. $[^3\text{H}]$-InsP$_1$ data are expressed as a percentage of the response of cells to 300 μM methacholine in the presence of 4 mM Ca$^{2+}$, for a single experiment.
muscarinic receptor. As for clone # 5 this value was increased in nominally Ca\(^{2+}\) free buffer; -5.1 (7.3 \(\mu\)M) compared to -5.8 (1.6 \(\mu\)M) and -5.7 (2.2 \(\mu\)M), in the presence of 1.3 and 4 mM Ca\(^{2+}\), respectively.

Fig. 3.8 shows concentration-dependent increases in \([^{3}\text{H}]\)-InsP\(_1\) accumulation in response to stimulation of the M\(_3\)-muscarinic receptor (BHK-m3, clone # 5) with arecoline, a partial agonist of the receptor, for 30 min, in the presence of nominal, 1.3 mM and 4 mM Ca\(^{2+}\) (n = 4). \([^{3}\text{H}]\)-InsP\(_1\) accumulation in response to a maximal concentration of arecoline (100 \(\mu\)M) was decreased in nominally Ca\(^{2+}\) free buffer compared to in the presence of 1.3 and 4 mM Ca\(^{2+}\); 24.9 \(\pm\) 4.1\% (nominal Ca\(^{2+}\)), compared to 57.8 \(\pm\) 6.2\% (1.3 mM) and 52.8 \(\pm\) 6.1\%, (4 mM), of the response to 300 \(\mu\)M methacholine in the presence of 4 mM Ca\(^{2+}\). Thus, in common with the response to methacholine \([^{3}\text{H}]\)-InsP\(_1\) accumulation is decreased under nominally Ca\(^{2+}\)-free conditions, and increasing [Ca\(^{2+}\)]\(_e\) from 1.3 mM to 4 mM has no effect (p>0.05, unpaired t-test) on the \([^{3}\text{H}]\)-InsP\(_1\) accumulation in response to a maximal concentration of arecoline. The log EC\(_{50}\) (M) value for arecoline-stimulated \([^{3}\text{H}]\)-InsP\(_1\) accumulation was unaffected by changes in [Ca\(^{2+}\)]\(_e\); -4.9 \(\pm\) 0.6 (11.6 \(\mu\)M, nominal Ca\(^{2+}\)), -5.0 \(\pm\) 0.4 (9.8 \(\mu\)M, 1.3 mM) and -5.1 \(\pm\) 0.4 (8.3 \(\mu\)M, 4 mM), thus, in common with the log EC\(_{50}\) (M) values for methacholine-stimulated \([^{3}\text{H}]\)-InsP\(_1\) accumulation, increasing [Ca\(^{2+}\)]\(_e\) in the millimolar range has no effect on the log EC\(_{50}\) (M) value for arecoline-stimulated \([^{3}\text{H}]\)-InsP\(_1\) accumulation (p>0.05). Furthermore, analysis of the concentration-effect curves by two way ANOVA revealed no significant effect of increasing [Ca\(^{2+}\)]\(_e\) from 1.3 to 4 mM.
**Fig. 3.8** Concentration-dependency of arecoline-stimulated \[^{3}\text{H}]-\text{InsP}_1\) accumulations in the presence of different \([Ca^{2+}]_e\) (nominally \(Ca^{2+}\)-free; ■, 1.3 mM; △, 4 mM; ●) in BHK-m3 cells. BHK cells stably expressing \(M_3\)-muscarinic receptor were incubated with 1 μCi ml\(^{-1}\) \[^{3}\text{H}\]-inositol for 48 h, washed with KHB and incubated with KHB + 10 mM LiCl for 15 min before challenge with the indicated concentrations of arecoline for 30 min. \[^{3}\text{H}]-\text{InsP}_1\) data are expressed as a percentage of the response of cells to 300 μM methacholine in the presence of 4 mM \(Ca^{2+}\). Data are shown as mean ± standard error for 4 separate experiments.
Thus, varying Ca\(^{2+}\) in the millimolar range has no effect on phosphoinositide signalling via the M\(_3\)-muscarinic receptor. These data suggest that the modulatory effect of Ca\(^{2+}\) in the millimolar range is selective to signalling via mGluR1\(\alpha\) rather than the BHK cell-type.

3.3 Investigations into the site of action of [Ca\(^{2+}\)]\(_e\).

The modulatory effects of Ca\(^{2+}\) on signalling via mGluR1\(\alpha\) could be due to Ca\(^{2+}\) interacting directly with the receptor or at an intracellular site in the signal transduction pathway downstream of the receptor, via entry into the cell, e.g. via the activation of a distinct sub-type of PLC with different Ca\(^{2+}\) requirements to that activated by the M\(_3\)-muscarinic receptor. This Section includes a number of experiments carried out in an attempt to distinguish between these two possibilities.

3.3.1 Effects of polyvalent cations on \([^3\text{H}]\)-InsP\(_1\) accumulation in BHK-mGluR1\(\alpha\) cells.

Several groups have reported activation of CaSRs by Gd\(^{3+}\) and Mg\(^{2+}\) as well as Ca\(^{2+}\) (Brown et al, 1993; Riccardi et al, 1995; Bai et al, 1996). sBimR and mGluR1\(\alpha\) have been shown to be activated by Mn\(^{2+}\) as well (Kubokawa et al, 1996).

The ability of Gd\(^{3+}\), Mg\(^{2+}\) and Mn\(^{2+}\) to behave as agonists of mGluR1\(\alpha\) was studied by measuring \([^3\text{H}]\)-InsP\(_1\) accumulation in the presence of 10 mM LiCl in BHK-mGluR1\(\alpha\) cells which had been incubated with 1 \(\mu\)Ci ml\(^{-1}\) myo-[^3H]-inositol for 48 h. The cells were treated with Gd\(^{3+}\), Mg\(^{2+}\) or Mn\(^{2+}\), 5 min prior to addition of vehicle / quisqualate.
(30 μM) for a further 30 min (n ≥ 4). Figs. 3.9 - 3.11 show the effect of treatment with Gd³⁺, Mg²⁺ or Mn²⁺ on [³H]-InsP₁ accumulation under basal conditions and in response to 30 μM quisqualate in the presence of nominally Ca²⁺-free media. Gd³⁺ and Mg²⁺ had no effect per se on [³H]-InsP₁ accumulation in BHK-mGluR1α cells (Gd³⁺: control; 645 ± 77, 500 μM Gd³⁺; 575 ± 73, Mg²⁺: control; 562 ± 118, 12 mM Mg²⁺; 943 ± 155 d.p.m. well⁻¹) in addition, when these ions are present instead of Ca²⁺ the ability of mGluR1α to respond to 30 μM quisqualate is virtually abolished (1.3 mM Ca²⁺; 4834 ± 433, 500 μM Gd³⁺; 977 ± 126, 12 mM Mg²⁺; 1141 ± 154 d.p.m. well⁻¹). In the presence of 5 mM Mn²⁺ the basal [³H]-InsP₁ accumulation increases 4.5 fold compared to basal [³H]-InsP₁ accumulation in the absence of Mn²⁺ (2821 ± 378 compared to 623 ± 86 dpm well⁻¹, respectively). When Mn²⁺ is used in place of Ca²⁺ mGluR1α is still able to respond to quisqualate, (2739 ± 192, 2920 ± 507 and 3711 ± 364 dpm well⁻¹, in the presence of 0.5, 2 and 5mM Mn²⁺ respectively, compared to 1244 ± 148 dpm well⁻¹ in the absence of Mn²⁺). These results indicate that Mn²⁺ (0.5 or 2 mM) can substitute for Ca²⁺ in enabling mGluR1α to bring about a response to quisqualate, while at higher [Mn²⁺] (5 mM) the results are more difficult to interpret due to the basal effect.

3.3.2 Time-dependence of agonist-stimulated Ins(1,4,5)P₃ mass responses in BHK-mGluR1α cells in the presence of different [Ca²⁺]ₑ.

Fig. 3.12 shows the time-course of Ins(1,4,5)P₃ mass accumulation following addition of 100 μM quisqualate in the presence of nominally Ca²⁺-free media, 1.3 mM and 4 mM Ca²⁺. Ins(1,4,5)P₃ mass accumulation followed the peak and plateau pattern as previously reported in these cells (Carruthers et al, 1997) peaking at 20 s in
nominally Ca\(^{2+}\)-free buffer and 40 s in the presence of 1.3 and 4 mM Ca\(^{2+}\), reaching a lower elevated plateau by 5 min at all [Ca\(^{2+}\)]. As expected both the peak and plateau phases of Ins(1,4,5)P\(_3\) mass accumulation were reduced in nominally Ca\(^{2+}\)-free buffer compared to that seen in 1.3 mM Ca\(^{2+}\) (peak; 79 ± 9, plateau, 5 min; 37 ± 4 pmol mg\(^{-1}\) protein). Increasing [Ca\(^{2+}\)] from 1.3 to 4 mM had no significant effect on the peak of Ins(1,4,5)P\(_3\) mass accumulation (1.3 mM Ca\(^{2+}\); 134 ± 27, 4 mM Ca\(^{2+}\); 139 ± 50 pmol mg\(^{-1}\) protein), however there was a slight but not significant increase in the plateau level of accumulation at 5 and 15 min (5 min: 1.3 mM Ca\(^{2+}\); 66 ± 10, 4 mM Ca\(^{2+}\); 80 ± 16, 15 min: 1.3 mM Ca\(^{2+}\); 52 ± 5, 4 mM Ca\(^{2+}\); 70 ± 8 pmol mg\(^{-1}\) protein).

3.3.3 Intracellular Ca\(^{2+}\) measurements in BHK-mGluR1\(\alpha\) cells following agonist stimulation of mGluR1\(\alpha\) expressed in BHK cells.

Intracellular Ca\(^{2+}\) measurements in response to stimulation with 30 \(\mu\)M quisqualate were measured with the aim of investigating the influence of [Ca\(^{2+}\)] on the response seen. It was not possible to obtain reproducible elevation of [Ca\(^{2+}\)] in response to stimulation with 30 \(\mu\)M quisqualate as measured using the Photon Technology Deltascan International system (data not shown) and the Applied Imaging Quanticell-700 Calcium Imager (data not shown). This could be due to heterogeneous expression of mGluR1\(\alpha\) in the BHK cell culture or down-regulation of elements of the phosphoinositide signalling cascade downstream of the phosphoinositide cycle due to persistent activation of mGluR1\(\alpha\) by the low levels of glutamate released by the cells into the culture medium. Thus, [Ca\(^{2+}\)] elevation characteristics could not be used to elucidate the site at which Ca\(^{2+}\) modulates signalling via mGluR1\(\alpha\).
Fig. 3.9 Effects of different concentrations of Gd$^{3+}$ on basal $[^3$H]-InsP$_1$ accumulation (unshaded bars) and $[^3$H]-InsP$_1$ accumulation in response to challenge with 30 µM quisqualate (shaded bars) in BHK-mGluR1α cells. Cells were cultured in the presence of 1 µCi ml$^{-1}$ $[^3$H]-inositol for 48 h, washed with KHB (-Ca$^{2+}$) and incubated with KHB (-Ca$^{2+}$) + 10 mM LiCl for 15 min before addition of Gd$^{3+}$. Vehicle / quisqualate was added after a further 5 min and cells incubated at 37°C for a further 30 min. Experiments were carried out in the presence of nominal Ca$^{2+}$ (free [Ca$^{2+}$] < 5 µM). $[^3$H]-InsP$_1$ data are expressed as d.p.m. well$^{-1}$ and are shown as mean ± standard error for 4 separate experiments.
Fig. 3.10 Effects of different concentrations of Mn$^{2+}$ on basal [$^3$H]-InsP$_1$ accumulation (unshaded bars) and [$^3$H]-InsP$_1$ accumulation in response to challenge with 30 μM quisqualate (shaded bars) in BHK-mGluR1α cells. Cells were cultured in the presence of 1 μCi ml$^{-1}$ [$^3$H]-inositol for 48 h, washed with KHB (-Ca$^{2+}$) and incubated with KHB (-Ca$^{2+}$) + 10 mM LiCl for 15 min before addition of Mn$^{2+}$. Vehicle / quisqualate was added after a further 5 min and cells incubated at 37°C for a further 30 min. Experiments were carried out in the presence of nominal Ca$^{2+}$ (free [Ca$^{2+}$] < 5 μM). [$^3$H]-InsP$_1$ data are expressed as d.p.m. well$^{-1}$ and are shown as mean ± standard error for 4 separate experiments.
Fig. 3.11 Effects of different concentrations of Mg$^{2+}$ on basal [$^3$H]-InsP$_1$ accumulation (unshaded bars) and [$^3$H]-InsP$_1$ accumulation in response to challenge with 30 µM quisqualate (shaded bars) in BHK-mGluR1α cells. Cells were cultured in the presence of 1 µCi ml$^{-1}$ [$^3$H]-inositol for 48 h, washed with KHB (-Ca$^{2+}$) and incubated with KHB (-Ca$^{2+}$) + 10 mM LiCl for 15 min before addition of Mg$^{2+}$. Vehicle / quisqualate was added after a further 5 min and cells incubated at 37°C for a further 30 min. Experiments were carried out in the presence of nominal Ca$^{2+}$ (free [Ca$^{2+}$] < 5 µM). [$^3$H]-InsP$_1$ data are expressed as d.p.m. well$^{-1}$ and are shown as mean ± standard error for 4 separate experiments.
Fig. 3.12  Time-course of Ins(1,4,5)P₃ production in response to challenge with 100 μM quisqualate in the presence of different [Ca²⁺]₀ (nominally Ca²⁺-free; ■, 1.3 mM; ▼, 4 mM; •) for the times indicated. Ins(1,4,5)P₃ data are expressed as a percentage of the Ins(1,4,5)P₃ production in the presence of 1.3 mM Ca²⁺₀ at 20 s. Data are shown as mean ± standard error for 6 separate experiments. Basal values were 24 ± 4; 38 ± 6 and 38 ± 3 pmol mg⁻¹ protein in nominally Ca²⁺-free conditions, 1.3 mM and 4 mM Ca²⁺₀, respectively.
3.3.4 Concentration-dependence of agonist-stimulated \([^{3}\text{H}]\)-InsP\(_1\) accumulation in BHK-S165A-mGluR1\(\alpha\) cells in the presence of different \([\text{Ca}^{2+}]_e\).

The importance of the S165 residue in the Ca\(^{2+}\) sensitivity of mGluR1\(\alpha\) was studied following the determination of S166 as an essential residue for the Ca\(^{2+}\) sensitivity of rat mGluR1\(\alpha\) (Kubo et al., 1998) due to the close proximity of S165 to this residue. The influence of \([\text{Ca}^{2+}]_e\) on receptor-coupling to PLC in BHK-mGluR1\(\alpha\) and BHK-S165A-mGluR1\(\alpha\) cells was studied by measuring \([^{3}\text{H}]\)-InsP\(_1\) accumulation in the presence of 10 mM LiCl in cells which had been incubated with 1 \(\mu\)Ci ml\(^{-1}\) \([^{3}\text{H}]\)-inositol for 48 h. Figs. 3.13 A and B show concentration-dependent increases in \([^{3}\text{H}]\)-InsP\(_1\) accumulation in response to stimulation with glutamate for 30 min in the presence of 0.5, 1.3, 2.5 and 4 mM \([\text{Ca}^{2+}]_e\) in BHK-mGluR1\(\alpha\) and BHK-S165A-mGluR1\(\alpha\) cells respectively. \([^{3}\text{H}]\)-InsP\(_1\) accumulation in response to a maximal concentration of glutamate (300 \(\mu\)M) in BHK-mGluR1\(\alpha\) cells increased in a graded manner as \([\text{Ca}^{2+}]_e\) was increased; 80 \(\pm\) 6 (0.5 mM Ca\(^{2+}\)\(e\)), 87 \(\pm\) 5 (1.3 mM Ca\(^{2+}\)\(e\)), 93 \(\pm\) 4 (2.5 mM Ca\(^{2+}\)\(e\)) and 100 \(\pm\) 0 (4 mM Ca\(^{2+}\)\(e\)) \% of the \([^{3}\text{H}]\)-InsP\(_1\) accumulation in response to 300 \(\mu\)M glutamate in the presence of 4 mM Ca\(^{2+}\)\(e\) (corresponding to 15734 \(\pm\) 4546 d.p.m. mg\(^{-1}\) protein). The EC\(_{50}\) values for glutamate-stimulated \([^{3}\text{H}]\)-InsP\(_1\) accumulation decreased in a graded manner as \([\text{Ca}^{2+}]_e\) increased; log EC\(_{50}\) (M): \(-4.5 \pm 0.4\) (35.6 \(\mu\)M), \(-4.6 \pm 0.4\) (26.8 \(\mu\)M), \(-4.7 \pm 0.4\) (19.8 \(\mu\)M), \(-4.8 \pm 0.4\) (16.8 \(\mu\)M) in the presence of 0.5 mM, 1.3 mM, 2.5 mM and 4 mM Ca\(^{2+}\)\(e\), respectively.
Fig. 3.13 Concentration-dependency of glutamate-stimulated $[^3]H$-InsP$_1$ accumulations in the presence of different [Ca$^{2+}$]$_e$ (0.5 mM; $\bigcirc$, 1.3 mM; ♦, 2.5 mM; ▼, 4 mM; ●) in BHK-mGluR1α cells (A) and BHK$^{S165A}$ cells (B). BHK cells stably expressing mGluR1α or $^{S165A}$-mGluR1α were incubated with 1 μCi ml$^{-1}$ $[^3]H$-inositol for 48 h, washed with KHB and incubated with KHB + 10 mM LiCl for 15 min before challenge with the indicated concentrations of glutamate for 30 min. $[^3]H$-InsP$_1$ data are expressed as a percentage of the response of cells to 300 μM (A) and 30 mM (B) glutamate in the presence of 4 mM Ca$^{2+}$. Data are shown as mean ± standard error for 4 (A) and 5 (B) separate experiments.
[3H]-InsP₁ accumulation in response to a maximal concentration of glutamate (30 mM) in BHK-S¹⁶⁵A-mGluR₁α cells was unaffected as [Ca²⁺]ₑ increased; 105 ± 5 (0.5 mM Ca²⁺ₑ), 97 ± 5 (1.3 mM Ca²⁺ₑ), 106 ± 6 (2.5 mM Ca²⁺ₑ) and 100 (4 mM Ca²⁺ₑ) % of the [3H]-InsP₁ accumulation in response to 30 mM glutamate in the presence of 4 mM Ca²⁺ₑ (corresponding to 13886 ± 1682 d.p.m. mg⁻¹ protein).

Thus, Ca²⁺ has a modulatory effect on the and [3H]-InsP₁ accumulation in response to a maximal concentration of glutamate in BHK-mGluR₁α cells in comparison to BHK-S¹⁶⁵A-mGluR₁α cells in which only the EC₅₀ value of glutamate-stimulated [3H]-InsP₁ accumulation is affected.

3.4 Investigations into the pertussis toxin sensitivity of BHK-m3 cells compared to BHK-mGluR₁α cells.

Previous studies in the laboratory have investigated the G-protein(s) involved in the coupling of mGluR₁α to PLC. In contrast to earlier studies in CHO cells, BHK cells and Xenopus oocytes (Aramori and Nakanishi; 1992, Pickering et al, 1993; Thomsen et al, 1993) in which agonist stimulation of mGluR₁α was reported to be inhibited following PTx pre-treatment, PTx pre-treatment was shown to enhance agonist-stimulated [3H]-InsP₁ accumulation in the presence of GPT/pyruvate (to remove endogenously produced glutamate from the medium) causing an increase in [3H]-InsP₁ accumulation in response to a maximal concentration of agonist and a decrease in the EC₅₀ for agonist-stimulated [3H]-InsP₁ accumulation (Carruthers et al, 1997). This suggested that mGluR₁α couples to PLC via both PTx-insensitive G₉₁₁
proteins and PTx-sensitive $G_{i/o}$ proteins. The present studies have confirmed these earlier observations and have investigated the effect of PTx pre-treatment on receptor-independent mechanisms by which PLC activity can be stimulated. Stable transfection of the M3-muscarinic receptor into BHK cells has also enabled comparison of the pertussis toxin sensitivity of the coupling of mGluR1$\alpha$ to PLC with that of the M3-muscarinic receptor expressed in the same cell background.

3.4.1 Effects of pertussis toxin pre-treatment on $[^3H]£€InsP_1$ accumulation in BHK-mGluR1$\alpha$ cells in response to stimulation with quisqualate, AlF$_4^-$ or ionomycin.

The influence of pertussis toxin pre-treatment (100 ng ml$^{-1},$ for 24 h) on the coupling of mGluR1$\alpha$ to PLC in BHK cells was studied by measuring $[^3H]£€InsP_1$ accumulation in the presence of 10 mM LiCl in cells which had been incubated with 1 $\mu$Ci ml$^{-1}$ myo-$[^3H]£€inositol$ for 48 h. Fig. 3.14 demonstrates concentration-dependent increases in $[^3H]£€InsP_1$ accumulation in control and PTx pre-treated BHK-mGluR1$\alpha$ cells in response to stimulation with quisqualate for 30 min. In agreement with previous studies (Carruthers et al, 1997) PTx treatment caused a significant increase in the response to a maximal concentration of quisqualate (30 $\mu$M); control cells; 10719 ± 3631, PTx-treated cells; 13221 ± 2993 d.p.m. well$^{-1},$ 132 ± 14 % of control, p< 0.05 (Mann-Whitney test) and a decrease in the EC$_{50}$ value for quisqualate-stimulated $[^3H]£€InsP_1$ accumulation (log EC$_{50}$ (M): control cells; -5.9 ± 0.5 (1.4$\mu$M), PTx-treated cells; -6.7 ± 0.4 (0.2 $\mu$M), p< 0.01, unpaired t-test).
Fig. 3.14 Concentration-dependence of quisqualate-stimulated \[^3\text{H}\]-InsP\(_1\) accumulations in control (\(\nabla\)) and PTx pre-treated (\(\blacksquare\)) BHK-mGluR1\(\alpha\) cells, in the presence of GPT (3 U ml\(^{-1}\)) and pyruvate (5 mM). BHK cells stably expressing mGluR1\(\alpha\) were incubated with 1 \(\mu\text{Ci ml}^{-1}\) \[^3\text{H}\]-inositol for 48 h, pre-treated with 100 ng ml\(^{-1}\) PTx for 24 h, washed with KHB and incubated with KHB + 10 mM LiCl for 15 min before challenge with the indicated concentrations of quisqualate for 30 min. \[^3\text{H}\]-InsP\(_1\) data are expressed as a percentage of the response to 30 \(\mu\text{M}\) quisqualate in control cells. Data are shown as mean ± standard error for 3-7 separate experiments.
In an attempt to examine the effects of PTx pre-treatment on receptor-independent mechanisms of PLC activation, control and PTx-treated BHK-mGluR1α cells were challenged with AlF₄⁻, (a non-selective G-protein activator) and ionomycin (a Ca²⁺ ionophore). AlF₄⁻ stimulated a 2-3 fold increase in [³H]-InsP₁ accumulation compared to basal [³H]-InsP₁ accumulation, this was significantly enhanced following PTx pre-treatment of the cells (see Table 3.2). Ionomycin also stimulated a modest increase in [³H]-InsP₁ accumulation compared to basal [³H]-InsP₁ accumulation, however this was not significantly enhanced following PTx pre-treatment (see Table 3.2).

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Control cells (dpm well⁻¹)</th>
<th>PTx-treated cells (dpm well⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>821 ± 142</td>
<td>942 ± 78</td>
</tr>
<tr>
<td>NaF/AICl₃</td>
<td>1932 ± 71</td>
<td>2495 ± 174*</td>
</tr>
<tr>
<td>No addition</td>
<td>612 ± 34</td>
<td>800 ± 151</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>1846 ± 110</td>
<td>2706 ± 436</td>
</tr>
</tbody>
</table>

Table 3.2: [³H]-InsP₁ accumulations in control or pertussis toxin-treated BHK-mGluR1α cells in response to stimulation with NaF (50 mM) and AICl₃ (10 μM) or ionomycin (5 μM), or vehicle, in the presence of GPT (3 U ml⁻¹) and pyruvate (5 mM). Cells were cultured in the presence of myo-[³H]-inositol (1 μCi ml⁻¹) for 48 h and were either exposed to PTx (100 ng ml⁻¹) or vehicle for the last 24 h of the labelling period. [³H]-InsP₁ data are expressed as dpm well⁻¹. Data are shown as means ± standard error for 3 separate experiments performed in duplicate. Statistically significant differences between +/- PTx conditions are indicated as *p<0.05.
3.4.2 Effects of pertussis toxin pre-treatment on $[^3]$H-InsP$_1$ accumulation in BHK-m3 cells in response to stimulation with methacholine.

The influence of pertussis toxin pre-treatment (100 ng ml$^{-1}$, for 24 h) on the coupling of the M$_3$-muscarinic receptor to PLC in BHK-m3 cells was studied by measuring $[^3]$H-InsP$_1$ accumulation in the presence of 10 mM LiCl in cells which had been incubated with 1 µCi ml$^{-1}$ myo-$[^3]$H]-inositol for 48 h. Fig. 3.15 shows concentration-dependent increases in $[^3]$H-InsP$_1$ accumulation in control and PTx pre-treated BHK-m3 cells in response to stimulation with methacholine for 5 and 30 min. $[^3]$H-InsP$_1$ accumulation in response to a maximal concentration of methacholine (300 µM) was unaffected by PTx pre-treatment at 5 min (control; 2939 ± 384, PTx-treated; 2652 ± 359 d.p.m. well$^{-1}$), and was increased by PTx pre-treatment at 30 min (control; 13093 ± 1167, PTx-treated; 16865 ± 1111 d.p.m. well$^{-1}$, p<0.05, paired t-test). The EC$_{50}$ values for methacholine-stimulated $[^3]$H-InsP$_1$ accumulation after 5 or 30 min, were unaffected by PTx pre-treatment (log EC$_{50}$ (M)): 5 min; control: -5.6 ± 0.2 (2.4 µM), PTx-treated: -6.0 ± 0.3 (1.1 µM), 30 min; control: -5.4 ± 0.1 (4.2 µM), PTx-treated: -5.5 ± 0.1 (3.0 µM)).
Fig. 3.15  Concentration-dependence of methacholine-stimulated $[^3$H]-InsP$_1$ accumulations in BHK-3 cells after challenge with methacholine for 5 min (control; ▼, PTx pre-treated; ■) and 30 min (control; O, PTx pre-treated; ♦). BHK cells stably expressing the M$_3$-muscarinic receptor were incubated with 1 μCi ml$^{-1}$ $[^3$H]-inositol for 48 h, pre-treated with 100 ng ml$^{-1}$ PTx for 24 h, washed with KHB and incubated with KHB + 10 mM LiCl for 15 min before challenge with the indicated concentrations of methacholine for 30 min. $[^3$H]-InsP$_1$ data are expressed as d.p.m. well$^{-1}$. Data are shown as mean ± standard error for 5 (5 min) and 3 (30 min) separate experiments.
3.5 Discussion.

As stated in the Introduction, mGluRs are larger than, and do not share sequence homology with, other GPCRs, and are therefore believed to belong to a new sub-family of GPCRs (family 3) which also includes the CaSRs, the GABA$_	ext{B}$ receptor and some pheromone receptors. Evidence is emerging to suggest that this sub-family of GPCRs shares another characteristic distinct from other GPCRs, that of Ca$^{2+}$ sensitivity. In addition, previous studies provide evidence for another interesting form of modulation of phosphoinositide signalling in BHK-mGluR1$\alpha$ cells, i.e. the dual regulation of PLC by $G_{q11}$ and $G_{o}$ proteins following mGluR1$\alpha$ activation (Carruthers et al, 1997).

These present studies have investigated the Ca$^{2+}$ sensitivity of mGluR1$\alpha$ expressed in BHK cells and have demonstrated a modulatory effect of Ca$^{2+}$ on phosphoinositide signalling via mGluR1$\alpha$. Transfection of the phospholipase C-coupled M$_{3}$-muscarinic receptor into BHK cells has enabled investigations to determine whether the modulatory effect of Ca$^{2+}$ and the dual regulation of PLC are selective to signalling via mGluR1$\alpha$ or general phenomena of signalling via GPCRs in the BHK cell type.

Following 24h pre-treatment of BHK-mGluR1$\alpha$ cells with PTx (100 ng ml$^{-1}$) a reduction in the EC$_{50}$ value for agonist-stimulated [$^{3}$H]-InsP$_{1}$ accumulation and an increase in [$^{3}$H]-InsP$_{1}$ accumulation elicited by a maximal concentration of agonist was observed, in the presence of GPT/pyruvate, in PTx-treated compared to control cells (Carruthers et al, 1997). These data prompted Carruthers and colleagues
(1997) to propose that mGluR1α associates with both stimulatory, PTx-insensitive
Gq/11 and inhibitory, PTx-sensitive Gi/o proteins, resulting in the dual regulation of
PLC activity. The stimulatory effect of Gq/11 activation on PLC activity is thus limited
by the inhibitory effect of Gi/o activation, removal of this negative influence by PTx
pre-treatment therefore results in the observed potency shift and increase in maximal
response of mGluR1α to agonist-stimulation. This model is supported by studies in
which glutamate-stimulated [35S]-GTPγS binding in BHK-mGluR1α cell membranes
has been shown to involve both PTx-sensitive and -insensitive G-proteins (Akam et
al, 1997), these observations have been extended using [35S]-GTPγS binding with
immunoprecipitation techniques to demonstrate agonist-stimulated PTx-sensitive
[35S]-GTPγS binding to Gi1/2α and G13αα proteins and agonist-stimulated PTx-
insensitive [35S]-GTPγS binding to Gq/11α proteins (Hermans et al, submitted). A
number of other studies also suggest that mGluR1α can associate with members of
several G protein families (Aramori and Nakanishi, 1992; Thomsen et al, 1996;
Kammermeier and Ikeda, 1999). This study has confirmed the effects of PTx pre­
treatment on [3H]-InsP1 accumulation in BHK-mGluR1α cells. Experiments were then
carried out in which mGluR1α activation was bypassed to investigate the effects of
PTx on mGluR1α-independent activation of PLC. Measurement of [3H]-InsP1
accumulation revealed a small, but significant, enhancement of PLC activity by AlF4−
in PTx-treated compared to control cells. Although the lack of specificity of G-protein
activation by AlF4− means that clear interpretation of these data is difficult, this
observation is consistent with the above model proposed from previous studies.

Further studies (Hermans et al, submitted) have examined the time-dependency of
the effect of PTx treatment on agonist-stimulated [3H]-InsP1 accumulation. The EC50
shift observed following agonist-stimulation of mGluR1α for 30 min is also observed after stimulation for 5 min, however there is no increase in the response to a maximal concentration of agonist at this earlier time-point compared to that seen at the 30 min time-point. This suggests that PTx pre-treatment has two temporally distinct effects on phosphoinositide signalling via mGluR1α; an effect on the potency of agonist activation of mGluR1α which occurs immediately, and an effect on the response of mGluR1α to a maximal concentration of agonist which only becomes evident at longer time-points.

The present study has also investigated the influence of PTx pre-treatment on phosphoinositide signalling via the M3-muscarinic receptor expressed in the BHK cell type. PTx pre-treatment has been demonstrated to have no effect on the EC50 of the [3H]-InsP1 accumulation in response to methacholine-stimulation at either the 5 min or 30 min time-point. However, in common with the effect of PTx pre-treatment on phosphoinositide signalling via mGluR1α, a small increase in the [3H]-InsP1 accumulation is observed following stimulation of the M3-muscarinic receptor with a maximal concentration of methacholine for 30 min, but not at the 5 min time-point. Thus it is proposed (Hermans et al, submitted) that PTx pre-treatment has two temporally distinct effects on phosphoinositide signalling via mGluR1α; an immediate effect on the potency of agonist-activation of mGluR1α (specific to mGluR1α), and an effect on the response of mGluR1α to a maximal concentration of agonist (a phenomenon of the BHK cell type), evident at longer time points. This may be a general feature of phosphoinositide-coupled GPCRs expressed in a BHK cell line (Hermans et al, submitted). PTx-treatment has been shown to dramatically reduce desensitisation of the agonist-stimulated [3H]-InsP1 response, and the idea that PTx

96
pre-treatment may affect the time-course of receptor desensitisation/sequestration is currently under investigation (Hermans et al, submitted).

These studies suggest that the coupling of mGluR1α to both Gq/11 and Gi0 proteins may be important in determining the net PLC activity, however the mechanism by which such dual regulation of PLC occurs remains elusive. It could occur via direct inhibition of PLC activity by Gi0 proteins; such inhibition has been observed in a number of studies (Bizzarri et al, 1990; Litosch et al, 1993; Nakamura et al, 1994; Watkins et al, 1994). It is possible that the inhibitory effect of Gi0 coupling on PLC activity is mediated by βγ subunits of these proteins; again there is some evidence to suggest that βγ subunits derived from Gi0 proteins can have inhibitory effects on PLC activity (Litosch, 1996).

The possibility that mGluRs may be able to sense [Ca2+]i was raised following the identification of 30% sequence homology between mGluRs and CaSRs. This seemed increasingly likely following the identification of sBimR which was activated by both Ca2+ and glutamate, when expressed in Xenopus oocytes, and shares 69% sequence homology with mGluR1α. mGluR1α was then shown to be activated by Ca2+ when expressed in Xenopus oocytes (Kubokawa et al, 1996). In situ hybridisation analysis revealed similar brain distributions of both receptors, thus sBimR is believed to be the salmon equivalent of mGluR1α. Further studies then revealed the importance of a single amino acid residue, S166, in the Ca2+ sensitivity of mGluR1α, expressed in Xenopus oocytes (Kubo et al, 1998). This study has investigated the Ca2+ sensitivity of mGluR1α expressed in a mammalian, baby hamster kidney, cell type. Indeed, in support of the observations of Kubokawa et al
(1996), we have demonstrated sensitivity of mGluR1α to Ca^{2+} in a mammalian cell system. However, Ca^{2+} has not been shown to act as an agonist of mGluR1α, rather it acts to modulate agonist-stimulated signalling via mGluR1α.

Initial experiments investigating the effect of varying [Ca^{2+}]_e on receptor coupling to PLC in BHK-mGluR1α cells, by assessing [^{3}H]-InsP₁ accumulation in the presence of 10 mM LiCl, demonstrated that as [Ca^{2+}]_e was increased in the mM range a graded increase in the response to a maximal concentration of quisqualate (30 μM) was observed, as well as a graded decrease in the EC_{50} value for quisqualate-stimulated [^{3}H]-InsP₁ accumulation. However, in contrast to the observations of Kubokawa et al (1996), Ca^{2+} did not act as an agonist of mGluR1α; basal [^{3}H]-InsP₁ accumulation did not increase in a graded manner as [Ca^{2+}]_e was increased. As these initial observations proved interesting, more extensive concentration-effect curves were constructed in the presence of nominally Ca^{2+}-free buffer, 1.3 mM Ca^{2+}_e and 4 mM Ca^{2+}_e. The results confirmed the modulatory effects of varying [Ca^{2+}]_e in the mM range on [^{3}H]-InsP₁ accumulation in response to quisqualate-stimulation of mGluR1α. The effects of varying [Ca^{2+}]_e on the response of mGluR1α to stimulation with 1S,3R-ACPD, a partial agonist of the receptor were then investigated. Use of the partial agonist 1S,3R-ACPD may afford a broader window for the effects of varying [Ca^{2+}]_e to be seen (i.e. changes in the amplitude of the response indicate a change in the coupling efficiency of mGluR1α and a change in the EC_{50} indicates a change in the sensitivity of the receptor to the agonist). The results are therefore more conclusive in defining how [Ca^{2+}]_e is influencing phosphoinositide signalling via mGluR1α in these cells. Both the increase in [^{3}H]-InsP₁ accumulation in response to a maximal concentration of 1S,3R-ACPD and the decrease in the EC_{50} value for
1S,3R-ACPD stimulated $[^3H]\text{InsP}_1$ accumulation as $[\text{Ca}^{2+}]_o$ increases are more pronounced in response to stimulation with 1S,3R-ACPD. Thus, it appears that increasing $[\text{Ca}^{2+}]_o$ increases both the efficiency of coupling of the receptor and the sensitivity of the receptor to agonists. There is a slight increase in basal $[^3H]\text{InsP}_1$ accumulation in the presence of 4 mM Ca$^{2+}$ in these experiments, however increasing $[\text{Ca}^{2+}]_o$ over the range from nominally Ca$^{2+}$-free ($\leq$ μM) to 4 mM does not result in a graded increase in basal $[^3H]\text{InsP}_1$ accumulation, indeed increasing $[\text{Ca}^{2+}]_o$ from nominally Ca$^{2+}$-free to 1.3 mM causes no change in basal $[^3H]\text{InsP}_1$ accumulation. In addition, analysis of the concentration-effect curves by two-way ANOVA revealed statistically significant effects of increasing $[\text{Ca}^{2+}]_o$ from 1.3 mM to 4 mM with or without subtraction of the respective basal values. Thus, these experiments provide support for the observations in Figs. 3.1 and 3.2 that Ca$^{2+}$, in the physiological range acts as a modulator of $[^3H]\text{InsP}_1$ accumulation in response to agonist-stimulation of mGluR1α rather than as an agonist of mGluR1α in its own right.

In order to determine whether the modulatory effect of $[\text{Ca}^{2+}]_o$ was selective to signalling via mGluR1α, or a general phenomenon of the BHK cell-type, it was necessary to transfect a different PLC coupled GPCR into a BHK cell line. Thus, the M₃-muscarinic acetylcholine receptor was transfected into BHK-570 cells, using the pCEP4 vector containing cDNA for the human M₃-muscarinic receptor. BHK-570 cells were also transfected with the pCEP4 vector without the cDNA for the M₃-muscarinic receptor to generate a vector control cell line. The pCEP4 vector confers hygromycin B resistance to transfected cells. A number of hygromycin B resistant clones were tested for M₃-muscarinic receptor expression using $[^3H]\text{NMS}$ binding.
Expression levels ranged from 0.22 to 1.96 pmol mg protein\(^{-1}\). \[^{3}\text{H}\]-InsP\(_1\) accumulation in response to agonist-stimulation was then measured in these clones, to give an indication of the efficiency of coupling of the M\(_3\)-muscarinic receptor to PLC. \[^{3}\text{H}\]-InsP\(_1\) accumulation in response to methacholine-stimulation was also measured in a vector control clone, as expected no increase in \[^{3}\text{H}\]-InsP\(_1\) accumulation above basal was seen in vector control cells. Of the clones tested 2 M\(_3\)-muscarinic receptor expressing clones coupled efficiently with the expected EC\(_{50}\) value for methacholine-stimulated \[^{3}\text{H}\]-InsP\(_1\) accumulation and were thus used for further study. \[^{3}\text{H}\]-NMS saturation binding was then carried out on BHK-m3 (clone \# 5) cell membranes to obtain more accurate estimates of the B\(_{\text{max}}\) and K\(_D\) values for this clone. The modulatory effect of \([\text{Ca}^{2+}]_o\) on the M\(_3\)-muscarinic receptor was then investigated. As in BHK-mGluR1\(\alpha\) cells \[^{3}\text{H}\]-InsP\(_1\) accumulation was reduced greatly in nominally Ca\(^{2+}\)-free buffer compared to in the presence of 1.3 and 4 mM Ca\(^{2+}\). However, in contrast to BHK-mGluR1\(\alpha\) cells increasing the Ca\(^{2+}\) from 1.3 to 4 mM had no effect on the \[^{3}\text{H}\]-InsP\(_1\) accumulation following stimulation of the M\(_3\)-muscarinic receptor with a maximal concentration of methacholine (300 \(\mu\)M). The EC\(_{50}\) value for methacholine-stimulated \[^{3}\text{H}\]-InsP\(_1\) accumulation was also unaffected by increasing \([\text{Ca}^{2+}]_o\) from 1.3 to 4 mM. This was demonstrated for two separate clones, so the possibility that the effects are unique to one clone is unlikely. As the use of partial agonists provides a broader window for the effects of varying \([\text{Ca}^{2+}]_o\) to be seen, the effect of varying \([\text{Ca}^{2+}]_o\) on \[^{3}\text{H}\]-InsP\(_1\) accumulation following stimulation of the M\(_3\)-muscarinic receptor with arecoline, a partial agonist of the receptor, was investigated. As for methacholine-stimulated \[^{3}\text{H}\]-InsP\(_1\) accumulation increasing \([\text{Ca}^{2+}]_o\) from 1.3 to 4 mM had no effect. Thus, using both a full and partial agonist of the M\(_3\)-muscarinic receptor, no effect on agonist-stimulated \[^{3}\text{H}\]-InsP\(_1\) accumulation
is seen as \([\text{Ca}^{2+}]_e\) was increased from 1.3 mM to 4 mM. These data suggest that the modulatory effect of \(\text{Ca}^{2+}\) observed in BHK-mGluR1\(\alpha\) cells is likely be selective to mGluR1\(\alpha\) rather than the BHK cell-type.

It was therefore necessary to address the question of whether \(\text{Ca}^{2+}\) was interacting directly with mGluR1\(\alpha\) or another element of the signal transduction pathway downstream of the receptor, via entry into the cell. The results of Kubokawa et al (1996), in which \(\text{Ca}^{2+}\) was shown to activate mGluR1\(\alpha\) per se, suggest that the sBim and mGlu1\(\alpha\) receptors may contain \(\text{Ca}^{2+}\) binding sites in the extracellular domain of the receptors enabling \(\text{Ca}^{2+}\) to behave as an agonist of these receptors. A number of polyvalent cations including \(\text{Gd}^{3+}\), \(\text{Mn}^{2+}\) and \(\text{Mg}^{2+}\), which can substitute for \(\text{Ca}^{2+}\) to activate the CaSR (Brown et al, 1993; Riccardi et al, 1995) were also shown to activate the sBim and mGlu1\(\alpha\) receptors expressed in Xenopus oocytes (Kubokawa et al, 1996), providing further evidence for the existence of \(\text{Ca}^{2+}\) /polyvalent cation binding sites in the extracellular region of the receptors.

The ability of these polyvalent cations to activate mGluR1\(\alpha\) (expressed in a mammalian, BHK, cell line) per se and to modulate the quisqualate-stimulated response has been investigated. In experiments in which \([\text{Mn}^{2+}]_e\) was increased in place of \(\text{Ca}^{2+}\), this divalent cation, unlike \(\text{Gd}^{3+}\) and \(\text{Mg}^{2+}\), could substitute for \(\text{Ca}^{2+}\) in allowing mGluR1\(\alpha\) to respond to quisqualate, and also caused an increase in basal \([^3\text{H}]\)-InsP\(_1\) accumulation as \([\text{Mn}^{2+}]_e\) was increased, albeit at high concentrations (> 2mM). \(\text{Mn}^{2+}\) like \(\text{Ca}^{2+}\) can enter the cell, so the possibility that \(\text{Ca}^{2+}\) and \(\text{Mn}^{2+}\) are entering the cell to bring about their effects cannot be excluded, therefore these data
do not provide conclusive evidence that mGluR1α contains a binding site for Ca\(^{2+}\) and some other polyvalent cations.

The effect of varying \([\text{Ca}^{2+}]_e\) on another index of mGluR1α activation, \(\text{Ins}(1,4,5)P_3\) mass accumulation, was then measured to attempt to determine the site of action of Ca\(^{2+}\). In accordance with previous work in the laboratory (Carruthers et al, 1997) \(\text{Ins}(1,4,5)P_3\) mass accumulation was biphasic with an initial peak phase followed by a sustained plateau phase, this biphasic response was reduced in the presence of nominally Ca\(^{2+}\)-free buffer, however no difference in the peak of \(\text{Ins}(1,4,5)P_3\) mass accumulation was seen as \([\text{Ca}^{2+}]_e\) was increased from 1.3 mM to 4 mM, although there was a slight increase in the plateau phase.

Intracellular Ca\(^{2+}\) measurements in response to stimulation with 30 μM quisqualate were also made with the aim of investigating the influence of \([\text{Ca}^{2+}]_i\) on the response seen. It was not possible to obtain reproducible \([\text{Ca}^{2+}]_i\) elevations in response to stimulation of BHK-mGluR1α cells with 30 μM quisqualate, as measured using the Photon Technology Deltascan International system and the Applied Imaging Quanticell-700 Calcium Imager.

If the peaks of \(\text{Ins}(1,4,5)P_3\) mass accumulation or \([\text{Ca}^{2+}]_i\) elevation had varied in the presence of different \([\text{Ca}^{2+}]_e\), Ca\(^{2+}\) channel blockers were to be used to investigate the dependency of variation in peak height on the entry of Ca\(^{2+}\) into the cell and thus elucidate whether Ca\(^{2+}\) acted extracellularly or intracellularly. In addition, the EC\(_{50}\) of agonist-stimulated peak \(\text{Ins}(1,4,5)P_3\) mass accumulation in the presence of different \([\text{Ca}^{2+}]_e\) could have been examined. An effect of \([\text{Ca}^{2+}]_e\) on the potency of such a
response, following agonist-stimulation for seconds (rather than minutes in the measurement of \[^{3}\text{H}]\text{-InsP}_1\) accumulation) would suggest that the modulatory effect of varying \([\text{Ca}^{2+}]_e\) is not mediated via an effect on the activity of \(\text{Ca}^{2+}\)-dependent PLCs via entry into the cell. However, \(\text{Ins}(1,4,5)P_3\) mass accumulation measurements were variable from day-to-day and not considered suitable for a detailed quantitative study. Thus, measurement of \(\text{Ins}(1,4,5)P_3\) mass accumulation and \([\text{Ca}^{2+}]_i\) elevation was unsuccessful in elucidating whether \(\text{Ca}^{2+}\) modulates signalling via mGluR1\(\alpha\) at an intracellular or an extracellular site.

Following the demonstration of the importance of the serine 166 residue in the \(\text{Ca}^{2+}\) sensitivity of mGluR1\(\alpha\) expressed in \textit{Xenopus} oocytes (Kubo \textit{et al}, 1998), and the involvement of serine 165 and threonine 188 residues in glutamate binding in mGluR1\(\alpha\) (O'Hara \textit{et al}, 1993), the \(\text{Ca}^{2+}\) sensing ability of mGluR1\(\alpha\) in which serine 165 is mutated to an alanine residue was investigated. The modulatory effect of \(\text{Ca}^{2+}\) on glutamate-stimulated \[^{3}\text{H}]\text{-InsP}_1\) accumulation in BHK-mGluR1\(\alpha\) and BHK-S\(165^A\)-mGluR1\(\alpha\) cells was compared. As for quisqualate-stimulated \[^{3}\text{H}]\text{-InsP}_1\) accumulation in BHK-mGluR1\(\alpha\) cells, varying \([\text{Ca}^{2+}]_e\) over the mM range resulted in a decrease in the \(\text{EC}_{50}\) of glutamate-stimulated \[^{3}\text{H}]\text{-InsP}_1\) accumulation and an increase in \[^{3}\text{H}]\text{-InsP}_1\) accumulation in response to stimulation with a maximal concentration of glutamate (300 \(\mu\text{M}\)). However, varying \([\text{Ca}^{2+}]_e\) over the mM range had no effect on the response of the mutant S\(165^A\)-mGluR1\(\alpha\) to challenge with a maximal concentration of glutamate (30 mM), although a slight decrease in the \(\text{EC}_{50}\) of glutamate-stimulated \[^{3}\text{H}]\text{-InsP}_1\) accumulation was still observed. Thus, the serine 165 residue, in addition to being important for high affinity agonist binding also appears to be important for the modulatory effect of \(\text{Ca}^{2+}\) on the coupling efficiency
of, but not the agonist potency at, mGluR1α. This observation lends support for the involvement of this region in binding Ca\(^{2+}\), in agreement with the observations of Kubo et al. (1998), thus studies of the importance of the serine 166 mutation in the Ca\(^{2+}\) sensitivity of mGluR1α expressed in mammalian cell system were examined and will be presented in a later Chapter.

In summary, these studies have demonstrated that increasing [Ca\(^{2+}\)]\(_o\) in the mM range has differential effects on agonist-stimulated \[^{3}H\]-InsP\(_1\) accumulation in BHK-mGluR1α compared to BHK-m3 cells. Increasing [Ca\(^{2+}\)]\(_o\) from 1.3 to 4 mM has a positive modulatory effect on \[^{3}H\]-InsP\(_1\) accumulation following agonist-stimulation of mGluR1α, the effects being more pronounced following stimulation with a partial as compared to a full agonist of the receptor. In contrast, increasing [Ca\(^{2+}\)]\(_o\) over the same range has no effect on \[^{3}H\]-InsP\(_1\) accumulation following stimulation of the M3-muscarinic receptor with either a full or partial agonist. Thus, it appears that the modulatory effect of Ca\(^{2+}\) is selective to signalling via mGluR1α. Studies to determine whether Ca\(^{2+}\) is acting extracellularly or intracellularly have unfortunately been inconclusive so far. However, the modulatory effect of Ca\(^{2+}\) is limited in BHK-S165A-mGluR1α compared to BHK-mGluR1α cells, lending support for the importance of this region in the Ca\(^{2+}\) sensitivity of mGluRs.

Thus, there is a growing body of evidence to suggest that the CaSR is not the only member of the family 3 GPCRs that is able to "sense" [Ca\(^{2+}\)]\(_o\). Studies in Xenopus oocytes have shown that both sBimR and mGluR1α can be activated by Ca\(^{2+}\) as well as glutamate (Kubokawa et al., 1996), the results in this Chapter also provide evidence for a modulatory effect of Ca\(^{2+}\) on mGluR1α expressed in a mammalian
cell system, however in this study it has proved difficult to elucidate the site at which 
Ca$^{2+}$ has its modulatory effect on agonist-stimulated phosphoinositide signalling via mGluR1α. The studies of Kubokawa et al (1996) in which Ca$^{2+}$ (as well as other polyvalent cations) behaves as an agonist of mGluR1α suggest that mGluR1α may contain Ca$^{2+}$ binding sites within its extracellular domain. Indeed, studies leading on from the observations of Kubokawa et al (1996) have been successful in elucidating the site which confers Ca$^{2+}$ sensitivity upon mGluR1α. Mutation of a single amino acid residue, serine 166, was shown to be sufficient to reduce dramatically the sensitivity of mGluR1α to Ca$^{2+}$ (Kubo et al, 1998). In addition, this Ca$^{2+}$ sensitivity was shown not to be unique to mGluR1α, both mGluR3 and 5 were also shown to share this Ca$^{2+}$ sensitivity. An homologous serine residue, serine 152, is shown to be essential for the Ca$^{2+}$ sensitivity of mGluR3 (i.e. this residue corresponds to serine 166 of mGluR1α). Thus, in a non-mammalian expression system mGluR1α has been shown to contain a site within its extracellular domain which is responsible for the Ca$^{2+}$ sensitivity of the receptor, allowing Ca$^{2+}$ to behave as an agonist of mGluR1α (Kubo et al, 1998).

Interestingly the serine 166 residue, essential for the Ca$^{2+}$ sensitivity of mGluR1α, as well as serine 165 and threonine 188, involved in glutamate binding, (O'Hara et al, 1993), all fall within the large amino-terminal domain of the receptor, a structural feature which is shared by other members of the family 3 GPCRs (Bräuner-Osborne et al, 1999). Studies to investigate the importance of this region in Ca$^{2+}$ binding to the CaSR have shown that serine 165 of mGluR1α is homologous to serine 147 in the CaSR and that serine 170, is substituted in place of threonine 188. Due to the homology of these amino acids with those of mGluR1α which have been shown to be
important in ligand binding the importance of these residues in the binding of \( \text{Ca}^{2+} \) to
the \text{CaSR} were tested by Brauner-Osborne and colleagues (1999). Both residues
were shown to be important in the activation of the receptor by \( \text{Ca}^{2+} \). Thus, similarieties exist between the ligand binding domains of mGluR1\( \alpha \) and the \text{CaSR}
(Brauner-Osborne \textit{et al}, 1999). Studies of the \text{GABA}\(_{\beta} \) receptor amino-terminal
domain have also revealed the importance of serine residues in this region for ligand
binding, these residues include serine 246 and serine 269 which correspond to
serine 165 and threonine 188 of mGluR1\( \alpha \) (Galvez \textit{et al}, 1999). More recent studies
have also demonstrated the ability of \( \text{Ca}^{2+} \) to modulate the response of the \text{GABA}\(_{\beta} \) receptor to agonist-stimulation (Wise \textit{et al}, 1999). In light of the similarity of residues
and the positioning of these residues involved in ligand binding in different members
of the family 3 GPCRs, and the shared \( \text{Ca}^{2+} \) sensitivity of the \text{CaSR} and some
mGluRs, the sequences of the GABA\(_{\beta} \) receptors were aligned with those of mGluRs
1,2,3 and 5 (Wise \textit{et al}, 1999). Interestingly, a serine is conserved in both GABA\(_{\beta} \)
receptor sub-types (serine 248 in GABA\(_{\beta}\)-R1\( \alpha \) and serine 137 in GABA\(_{\beta}\)-R2) at the
site corresponding to serine 166 in mGluR1\( \alpha \). However, when these residues were
mutated to aspartate residues the ability of \( \text{Ca}^{2+} \) to modulate the GABA response
was unaffected (Wise \textit{et al}, 1999). Thus, residues corresponding to serine 166 in
mGluR1\( \alpha \) appear not to be important in the \( \text{Ca}^{2+} \) sensitivity of the GABA\(_{\beta} \) receptor
(Wise \textit{et al}, 1999). The affinity of GABA for the GABA\(_{\beta} \) receptor and the potency of
GABA in stimulating GTP\(_{\gamma} \text{S} \) binding in rat brain membranes is also decreased in the
absence of \( \text{Ca}^{2+} \). This effect of \( \text{Ca}^{2+} \) on GABA\(_{\beta} \) receptor function, which enables a
better interaction of GABA with the GABA\(_{\beta} \) receptor, is dependent on the presence of
a serine residue at position 269, which corresponds to threonine 188 of mGluR1\( \alpha \)
(Galvez \textit{et al}, 2000).
4.1 Investigations into the effect of varying $[\text{Ca}^{2+}]_o$ on phosphoinositide signalling in CHO cells inducibly expressing mGluR1α.

The observation that, when expressed in *Xenopus* oocytes, mGluR1α could be activated by both glutamate and $\text{Ca}^{2+}_o$ (Kubokawa et al, 1996) prompted investigations to extend the above observations by encompassing the ability of $\text{Ca}^{2+}_o$ to modulate signalling via rat mGluR1α stably expressed in a mammalian, baby hamster kidney, cell-line (see Chapter 3). $[\text{Ca}^{2+}]_o$ was shown to have a modulatory effect on agonist-stimulated phosphoinositide signalling via mGluR1α expressed in BHK cells (Saunders et al, 1998). Thus, $\text{Ca}^{2+}_o$ can act as an agonist of rat mGluR1α (Kubokawa et al, 1996) or as a modulator of agonist-stimulated signalling via mGluR1α (Saunders et al, 1998), suggesting that mGluR1α may contain a $\text{Ca}^{2+}$ binding site within its extracellular domain. Unfortunately studies described in Chapter 3 were inconclusive in elucidating the site of action of $\text{Ca}^{2+}$. The development and characterisation of a CHO cell line in which mGluR1α expression is inducible, and $[\text{Ca}^{2+}]_i$ measurements are possible (Hermans et al, 1998b) has enabled the investigation of the ability of $\text{Ca}^{2+}_o$ to modulate signalling via mGluR1α in an additional mammalian cell line: CHO-lac-hmGluR1α cells.
4.1.1 $[\text{Ca}^{2+}]_e$-dependence of basal and agonist-stimulated $[^3\text{H}]-\text{InsP}_1$ accumulation in CHO-lac-hmGluR1$\alpha$ cells.

The influence of $[\text{Ca}^{2+}]_e$ on the coupling of mGluR1$\alpha$ to PLC in an inducible CHO cell system was studied by measuring $[^3\text{H}]-\text{InsP}_1$ accumulation in the presence of 10 mM LiCl in cells which had been incubated with 2.5 $\mu$Ci ml$^{-1}$ $[^3\text{H}]-\text{inositol}$ for 48 h, the expression of mGluR1$\alpha$ occurring in the last 20 h following treatment with 100 $\mu$M IPTG. Data were analysed using one-way ANOVA followed by a Duncan's multiple range test.

Fig. 4.1. shows basal and quisqualate-stimulated $[^3\text{H}]-\text{InsP}_1$ accumulation (1 $\mu$M and 30 $\mu$M) in the presence of nominal, 0.5, 0.9, 1.3, 2.5 and 4 mM $\text{Ca}^{2+}_e$ ($n \geq 4$). Increasing $[\text{Ca}^{2+}]_e$ over the range stated had little effect on basal $[^3\text{H}]-\text{InsP}_1$ accumulation (nominally $\text{Ca}^{2+}$-free; 433 ± 116, 4 mM $\text{Ca}^{2+}_e$; 666 ± 92 d.p.m. well$^{-1}$, p>0.05). $[^3\text{H}]-\text{InsP}_1$ accumulation in response to stimulation with a maximal concentration of quisqualate (30 $\mu$M) increased as $[\text{Ca}^{2+}]_e$ was increased across the nominally $\text{Ca}^{2+}$-free to 0.9 mM range (nominally $\text{Ca}^{2+}$-free; 936 ± 316, to 0.5 mM; 5984 ± 580, (p<0.05) to 0.9 mM; 8499 ± 804 d.p.m. well$^{-1}$ (p<0.05)). Increasing $[\text{Ca}^{2+}]_e$ above 0.9 mM caused no further significant increase in $[^3\text{H}]-\text{InsP}_1$ accumulation in response to stimulation with 30 $\mu$M quisqualate. A similar trend was seen upon stimulation with a sub-maximal concentration of quisqualate (1 $\mu$M); significant increases (p<0.05) in $[^3\text{H}]-\text{InsP}_1$ accumulation were seen as $[\text{Ca}^{2+}]_e$ was increased from nominally-$\text{Ca}^{2+}$ free to 0.5 mM and then from 0.5 to 2.5 mM.
Fig. 4.1 $[Ca^{2+}]_o$-dependency of basal (■) and quisqualate-stimulated (1 μM; ▼, 30 μM; ♦) $[^3H]$-InsP$_1$ accumulations in CHO-lac-hmGluR1α cells. CHO cells inducibly expressing mGluR1α were incubated with 2.5 μCi ml$^{-1}$ $[^3H]$-inositol for 48 h, mGluR1α expression was induced with 100 μM IPTG for the last 20 h. Cells were then washed with KHB and incubated with KHB + 3 U ml$^{-1}$ GPT and 5 mM pyruvate 15 min prior to addition of LiCl to a final concentration of 10 mM for a further 15 min prior to challenge with the indicated concentrations of quisqualate for 15 min. $[^3H]$-InsP$_1$ data are expressed as d.p.m. well$^{-1}$. Data are shown as means ± standard error for at least 4 separate experiments.
Fig. 4.2 shows basal and 1S,3R-ACPD-stimulated [³H]-InsP₁ accumulation (30 μM and 1 mM) in the presence of nominal, 0.5, 0.9, 1.3, 2.5 and 4 mM Ca²⁺ (n = 4). Increasing [Ca²⁺]₀ over the range stated again had no effect on basal [³H]-InsP₁ accumulation. [³H]-InsP₁ accumulation in response to stimulation with a maximal concentration of 1S,3R-ACPD (1 mM) increased as [Ca²⁺]₀ was increased across the nominally Ca²⁺-free to 0.9 mM range (nominally Ca²⁺-free; 560 ± 115, to 0.5 mM; 4682 ± 235, (p<0.05) to 0.9 mM; 7585 ± 357 d.p.m. well⁻¹ (p<0.05)). Increasing [Ca²⁺]₀ above 0.9 mM caused no further significant increases in [³H]-InsP₁ accumulation in response to stimulation with 1 mM 1S,3R-ACPD. A similar trend was seen upon stimulation with a sub-maximal concentration of 1S,3R-ACPD (30 μM) with a significant increase (p<0.05) in [³H]-InsP₁ accumulation being observed as [Ca²⁺]₀ was increased from nominally Ca²⁺-free to 0.5 mM, and 0.5 mM to 1.3 mM.

Increasing [Ca²⁺]₀ over the range nominally Ca²⁺-free to 1.3 mM, resulted in graded increases in agonist-stimulated [³H]-InsP₁ accumulation via mGluR₁α inducibly expressed in a CHO cell background, following challenge with sub-maximal and maximal concentrations of both quisqualate and 1S,3R-ACPD. These data suggest that varying [Ca²⁺]₀ has a modulatory effect on agonist-stimulated phosphoinositide signalling via mGluR₁α in CHO-lac-hmGluR₁α cells, as well as in BHK-mGluR₁α cells.
Fig. 4.2 [Ca$^{2+}$]$_e$-dependency of basal (■) and 1S,3R-ACPD-stimulated (30 μM; ▼, 1 mM; ◆) [³H]-InsP$_1$ accumulations in CHO-lac-hmGluR1α cells. CHO cells inducibly expressing mGluR1α were incubated with 2.5 μCi ml$^{-1}$ [³H]-inositol for 48 h, mGluR1α expression was induced with 100 μM IPTG for the last 20 h. Cells were then washed with KHB and incubated with KHB + 3 U ml$^{-1}$ GPT and 5 mM pyruvate 15 min prior to addition of LiCl to a final concentration of 10 mM for a further 15 min prior to challenge with the indicated concentrations of 1S,3R-ACPD for 15 min. [³H]-InsP$_1$ data are expressed as d.p.m. well$^{-1}$. Data are shown as means ± standard error for 4 separate experiments.
4.2 Investigations into the effect of varying $[\text{Ca}^{2+}]_e$ on phosphoinositide signalling in CHO cells stably expressing the M3-muscarinic receptor.

In order to determine whether the modulatory effect of $[\text{Ca}^{2+}]_e$ was receptor (mGluR1α) or cell-type (CHO) selective, the effect of varying $[\text{Ca}^{2+}]_e$ on the PLC coupled M3-muscarinic acetylcholine receptor stably expressed in a CHO cell background was investigated.

4.2.1 $[\text{Ca}^{2+}]_e$-dependence of basal and agonist-stimulated $[^3\text{H}]-\text{InsP}_1$ accumulation in CHO-m3 cells.

The influence of $[\text{Ca}^{2+}]_e$ on the coupling of the M3-muscarinic receptor to PLC in CHO cells was studied by measuring $[^3\text{H}]-\text{InsP}_1$ accumulation in the presence of 10 mM LiCl in cells which had been incubated with 2.5 $\mu$Ci ml$^{-1}$ $[^3\text{H}]-\text{inositol}$ for 48 h. Data were analysed using one-way ANOVA followed by a Duncan's multiple range test.

Fig. 4.3. shows basal and methacholine-stimulated $[^3\text{H}]-\text{InsP}_1$ accumulation (0.1 $\mu$M, 1 $\mu$M and 100 $\mu$M) in the presence of nominal, 0.5, 0.9, 1.3, 2.5 and 4 mM Ca$^{2+}$ (n = 4). Increasing $[\text{Ca}^{2+}]_e$ over the range stated had no effect on basal $[^3\text{H}]-\text{InsP}_1$ accumulation. $[^3\text{H}]-\text{InsP}_1$ accumulation in response to stimulation with a maximal concentration of methacholine (100 $\mu$M) was unaffected as $[\text{Ca}^{2+}]_e$ was increased across the nominally Ca$^{2+}$-free to 4 mM range. Upon stimulation with sub-maximal concentrations of methacholine (0.1 $\mu$M and 1 $\mu$M) $[^3\text{H}]-\text{InsP}_1$ accumulation was reduced under nominally Ca$^{2+}$-free conditions ($p<0.05$), however increasing Ca$^{2+}$ from 0.5 mM to 4 mM had no effect on $[^3\text{H}]-\text{InsP}_1$ accumulation.
Fig. 4.4. shows basal and arecoline-stimulated $[^3H]$-InsP$_1$ accumulation (3 μM, 10 μM and 100 μM) in the presence of nominal, 0.5, 0.9, 1.3, 2.5 and 4 mM Ca$^{2+}$ (n = 5). Increasing [Ca$^{2+}$]$_e$ over the range stated had no effect on basal $[^3H]$-InsP$_1$ accumulation. Upon stimulation with both maximal (100 μM) and sub-maximal concentrations of arecoline (3 μM and 10 μM) $[^3H]$-InsP$_1$ accumulation was reduced under nominally Ca$^{2+}$-free conditions (p<0.05), however increasing Ca$^{2+}$ from 0.5 mM to 4 mM had no effect on $[^3H]$-InsP$_1$ accumulation.

Thus, under nominally Ca$^{2+}$-free conditions $[^3H]$-InsP$_1$ accumulation in response to agonist-stimulation of the M$_3$-muscarinic receptor can be reduced. However, in contrast to the CHO-lac-hmGluR1α cells increasing Ca$^{2+}$ above 0.5 mM has no effect on phosphoinositide signalling via the M$_3$-muscarinic receptor. These data suggest that the modulatory effect of Ca$^{2+}$ is selective to signalling via mGluR1α rather than the CHO cell-type.

As stated in Chapter 3 the modulatory effects of Ca$^{2+}$ on signalling via mGluR1α could be due to Ca$^{2+}$ interacting directly with the receptor or at some intracellular site in the signal transduction pathway downstream of the receptor, via Ca$^{2+}$ entry into the cell. The remainder of this Chapter concentrates on investigations to distinguish between these possibilities.
Fig. 4.3 [Ca\textsuperscript{2+}]_e-dependency of basal (■) and methacholine-stimulated (0.1 μM; O, 1 μM; ▼, 100 μM; ♦) \([^{3}\text{H}]\)-InsP\textsubscript{1} accumulations in CHO-m3 cells. CHO cells stably expressing the M\textsubscript{3}-muscarinic receptor were incubated with 2.5 μCi ml\textsuperscript{-1} \([^{3}\text{H}]\)-inositol for 48 h. Cells were then washed with KHB and incubated with LiCl to a final concentration of 10 mM for a further 15 min prior to challenge with the indicated concentrations of methacholine for 15 min. \([^{3}\text{H}]\)-InsP\textsubscript{1} data are expressed as d.p.m. well\textsuperscript{-1} and are shown as means ± standard error for 4 separate experiments.
Fig. 4.4 $[\text{Ca}^{2+}]_e$-dependency of basal (■) and arecoline-stimulated (3 μM; O, 10 μM; ▼, 100 μM; ◆) $[^3\text{H}]$-InsP$_1$ accumulations in CHO-m3 cells. CHO cells stably expressing the M$_3$-muscarinic receptor were incubated with 2.5 μCi ml$^{-1}$ $[^3\text{H}]$-inositol for 48 h. Cells were then washed with KHB and incubated with LiCl to a final concentration of 10 mM for a further 15 min prior to challenge with the indicated concentrations of arecoline for 15 min. $[^3\text{H}]$-InsP$_1$ data are expressed as d.p.m. well$^{-1}$ and are shown as means ± standard error for 5 separate experiments.
4.3 Membrane phospholipid measurements in CHO-lac-hmGluR1α cells
following agonist stimulation of mGluR1α inducibly expressed in CHO cells.

Incubation of CHO-lac-hmGluR1α cells with 2.5 μCi ml⁻¹ [³H]-inositol for 48 h prior to experimentation results in the incorporation of [³H]-inositol into the different components of the phosphoinositide cycle, including the membrane located phosphoinositides phosphatidylinositol (PI), phosphatidylinositol 4-monophosphate (PIP) and PIP₂. Fig. 4.5 shows total membrane inositol phospholipids measured under basal conditions and following challenge with 30 μM quisqualate in the presence of different [Ca²⁺]₀ i.e. nominally Ca²⁺ free, 0.5 mM and 1.3 mM. No significant difference was seen in membrane phospholipid levels between different [Ca²⁺]₀, following incubation of the cells under basal conditions (nominally Ca²⁺ free; 30039 ± 3123, 0.5 mM; 36995 ± 4150, 1.3 mM; 37302 ± 3304 d.p.m. well⁻¹, p>0.05) or challenge with 30 μM quisqualate (nominally Ca²⁺ free; 32919 ± 1855, 0.5 mM; 27325 ± 2183, 1.3 mM; 25498 ± 3394 d.p.m. well⁻¹, p>0.05). Data were analysed using one-way ANOVA followed by a Duncan's multiple range test.

These data suggest that the modulatory effects of Ca²⁺₀ on [³H]-InsP₁ accumulation in CHO-lac-hmGluR1α cells are not due to changes in the levels of membrane phospholipids in the presence of different [Ca²⁺]₀.

4.4 Intracellular Ca²⁺ measurements in CHO-lac-hmGluR1α cells following agonist-stimulation of mGluR1α.

It has previously been shown that [Ca²⁺]₀ elevation in CHO-lac-hmGluR1α cells following stimulation with a maximal concentration of quisqualate (20 μM) follows a biphasic pattern, with a rapid peak of [Ca²⁺]₀ elevation followed by a sustained phase
Fig. 4.5 [Ca\textsuperscript{2+}]_e-dependency of total membrane inositol phospholipid labelling in control (unshaded bar) and quisqualate-stimulated (shaded bar) CHO-lac-hmGluR1α cells. CHO cells inducibly expressing mGluR1α were incubated with 2.5 μCi ml\textsuperscript{-1} [\textsuperscript{3}H]-inositol for 48 h, mGluR1α expression was induced with 100 μM IPTG for the last 20 h. Cells were then washed with KHB and incubated with KHB + 3 U ml\textsuperscript{-1} GPT and 5 mM pyruvate 15 min prior to addition of LiCl to a final concentration of 10 mM for a further 15 min prior to challenge with vehicle / quisqualate for 15 min. Total inositol membrane phospholipids were measured as described in Methods (Section 2.6). Data are expressed as d.p.m. well\textsuperscript{-1} and are shown as means ± standard error for 3 separate experiments.
of \([\text{Ca}^{2+}]_i\) elevation (Hermans et al, 1998b). Such biphasic \([\text{Ca}^{2+}]_i\) elevations have been observed in many cell types (Putney and Bird, 1993), for example; in response to muscarinic receptor agonists in SH-SY5Y cells, the initial peak phase resulting from release of \(\text{Ca}^{2+}\) from intracellular stores and the sustained phase due to \(\text{Ca}^{2+}\) entry into the cell (Lambert and Nahorski; 1990, Murphy et al, 1991). These experiments have examined the influence of varying \([\text{Ca}^{2+}]_e\) on the peak and sustained phases of \([\text{Ca}^{2+}]_i\) elevation observed in CHO-lac-hmGluR1\(\alpha\) cells. The Photon Technology Deltascan International system was used to measure \([\text{Ca}^{2+}]_i\) elevation in single CHO-lac-hmGluR1\(\alpha\) cells cultured overnight in the presence of 100 \(\mu\text{M}\) IPTG to allow receptor expression, and incubated in presence of 2 \(\mu\text{M}\) fura 2-AM for 2 h prior to use. Statistical analysis was performed using a Kruskal-Wallis test followed by a Dunn's post test.

4.4.1 Reproducibility of \([\text{Ca}^{2+}]_i\) elevation following repeated stimulation of mGluR1\(\alpha\) with quisqualate.

In order to examine the effect of \([\text{Ca}^{2+}]_e\) on the peak phase \([\text{Ca}^{2+}]_i\) elevation it was necessary to establish that peak \([\text{Ca}^{2+}]_i\) elevation was reproducible following repeated stimulation of mGluR1\(\alpha\) with quisqualate at both a maximal and sub-maximal concentration (to be determined from concentration-effect curves), thus any differences in the height of the peak \([\text{Ca}^{2+}]_i\) elevation observed in the presence of different \([\text{Ca}^{2+}]_e\) can be attributed to varying \([\text{Ca}^{2+}]_e\). Initially CHO-lac-hmGluR1\(\alpha\) cells were repeatedly challenged with 30 \(\mu\text{M}\) quisqualate (known to be maximal for \([^{3}\text{H}]\)-InsP\(_{1}\) accumulation) for 2 min at 10 min intervals.
Fig. 4.6A shows peak \([\text{Ca}^{2+}]_i\) elevation in response to repeated challenge with 30 μM quisqualate for 2 min at 10 min intervals, in single CHO-lac-hmGluR1α cells. Statistical analysis revealed there to be no significant decrease in the height of the peak \([\text{Ca}^{2+}]_i\) elevation (stimulation number 2; 102 ± 2.67, 3; 104 ± 2.81, 4; 103 ± 1.05, 5; 104 ± 1.05, 6; 104 ± 1.60, 7; 104 ± 1.29 % of the height of the first peak of \([\text{Ca}^{2+}]_i\) elevation) throughout 7 successive challenges of mGluR1α with a maximal concentration of quisqualate (30 μM). Fig. 4.6B shows a representative trace of the above data.

Fig. 4.7A shows a concentration-effect curve for the height of peak \([\text{Ca}^{2+}]_i\) elevation following challenge of single CHO-lac-hmGluR1α cells with quisqualate, at the indicated concentrations for 2 min at 10 min intervals. The log EC\(_{50}\) (M) value for the quisqualate-stimulated peak \([\text{Ca}^{2+}]_i\) elevation was -6.9 ± 0.1 (0.12 μM). Maximal peak \([\text{Ca}^{2+}]_i\) elevation was reached upon stimulation with 1 μM quisqualate. Thus, quisqualate challenge results in a concentration-dependent increase in the height of peak \([\text{Ca}^{2+}]_i\) elevation in single CHO-lac-hmGluR1α cells. Fig. 4.7B shows a representative trace from the above data.

It was not possible to obtain a reproducible response to 0.1 μM quisqualate (which approximates the EC\(_{50}\) value for quisqualate-stimulated peak \([\text{Ca}^{2+}]_i\) elevation in single CHO-lac-hmGluR1α cells). This could be due to single CHO-lac-hmGluR1α cells having different sensitivities to quisqualate, a phenomenon demonstrated in other cell types, e.g. single HeLa cells exhibit different sensitivities to histamine (Bootman et al, 1992). The lowest concentration of quisqualate to evoke a repeatable response was 0.7 μM. Fig. 4.8A shows peak \([\text{Ca}^{2+}]_i\) elevation in response
to repeated challenge with 0.7 μM quisqualate for 2 min at 10 min intervals, in single
CHO-lac-hmGluR1α cells. Statistical analysis revealed there to be no significant
decrease in the height of the peak [Ca²⁺]ᵢ elevation (stimulation number 2; 100 ±
2.46, 3; 102 ± 2.25, 4; 104 ± 0.87, 5; 101 ± 2.14, 6; 103 ± 2.06 % of the height of the
first peak of [Ca²⁺]ᵢ elevation in response to challenge with 0.7 μM quisqualate)
throughout 6 successive challenges of mGluR1α with a sub-maximal (0.7 μM)
concentration of quisqualate. Fig. 4.8B shows a representative trace from these
data.

These results demonstrate that single CHO-lac-hmGluR1α cells can be repeatedly
challenged with quisqualate, at both a sub-maximal (0.7 μM) and supra-maximal (30
μM) concentrations of quisqualate with no decline in the height of peak [Ca²⁺]ᵢ
elevation. Following challenge with quisqualate for 2 min, an interval of 10 min in
the presence of 1.3 mM Ca²⁺ before successive challenge is sufficient for full
recovery from the previous stimulation. Therefore any differences in peak height
observed in the presence of different [Ca²⁺]ₑ following challenge with both a sub-
maximal (0.7 μM) and maximal (3 μM) concentration of quisqualate can be attributed
to the modulatory effect of [Ca²⁺]ₑ on signalling via mGluR1α.

4.4.2 Changes in peak [Ca²⁺]ᵢ elevation following stimulation of mGluR1α with
quisqualate in the presence of different [Ca²⁺]ₑ.

The effect of varying [Ca²⁺]ₑ on the height of peak [Ca²⁺]ᵢ elevation following
challenge with both a maximal (3 μM) and sub-maximal (0.7 μM) concentration of
quisqualate has been investigated. Single CHO-lac-hmGluR1α cells were perfused
Fig. 4.6 A: Peak $[\text{Ca}^{2+}]_i$ elevation in response to repeated challenge with 30 μM quisqualate for 2 min at 10 min intervals, in single CHO-lac-hmGluR1α cells. CHO cells inducibly expressing mGluR1α were cultured overnight in the presence of 100 μM IPTG to allow receptor expression. Cells were washed with KHB and loaded with KHB + 2 μM fura 2-AM for 2 h prior to use. Loading and loaded cells were incubated in the presence of 3 U ml$^{-1}$ GPT and 5 mM pyruvate. Peak $[\text{Ca}^{2+}]_i$ elevation data are expressed as a percentage of the peak $[\text{Ca}^{2+}]_i$ elevation in response to the first quisqualate stimulation. Data are shown as means ± standard error for 4-7 separate experiments. B: A representative trace of peak $[\text{Ca}^{2+}]_i$ elevation in response to repeated challenge with 30 μM quisqualate (→) for 2 min at 10 min intervals, in a single CHO-lac-hmGluR1α cell.
Fig. 4.7 A: Concentration-dependency of peak \([Ca^{2+}]_i\) elevation in response to challenge with quisqualate for 2 min at 10 min intervals, in single CHO-lac-hmGluR1α cells. CHO cells inducibly expressing mGluR1α were cultured overnight in the presence of 100 μM IPTG to allow receptor expression. Cells were washed with KHB and loaded with KHB + 2 μM fura-2-AM for 2 h prior to use. Loading and loaded cells were incubated in the presence of 3 U ml⁻¹ GPT and 5 mM pyruvate. Peak \([Ca^{2+}]_i\) elevation data are expressed as a percentage of the peak \([Ca^{2+}]_i\) elevation in response to challenge with 30 μM quisqualate. Data are shown as means ± standard error for at least 3 separate experiments. B: A representative trace of the concentration-dependency of peak \([Ca^{2+}]_i\) elevation in response to challenge with the indicated concentration of quisqualate (—) for 2 min at 10 min intervals, in a single CHO-lac-hmGluR1α cell.
Fig. 4.8  A: Peak $[\text{Ca}^{2+}]_i$ elevation in response to repeated challenge with 0.7 μM quisqualate for 2 min at 10 min intervals, in single CHO-lac-hmGluR1α cells. CHO cells inducibly expressing mGluR1α were cultured overnight in the presence of 100 μM IPTG to allow receptor expression. Cells were washed with KHB and loaded with KHB + 2 μM fura 2-AM for 2 h prior to use. Loading and loaded cells were incubated in the presence of 3 U ml⁻¹ GPT and 5 mM pyruvate. Peak $[\text{Ca}^{2+}]_i$ elevation data are expressed as a percentage of the peak $[\text{Ca}^{2+}]_i$ elevation in response to the first 0.7 μM quisqualate stimulation. Data are shown as means ± standard error for 4 separate experiments. B: A representative trace of peak $[\text{Ca}^{2+}]_i$ elevation in response to repeated challenge with 0.7 μM quisqualate (—) for 2 min at 10 min intervals, in a single CHO-lac-hmGluR1α cell.
with KHB of the appropriate [Ca\(^{2+}\)]\(_e\) for 2 min prior to and subsequent to challenge with quisqualate. Between successive agonist challenges cells were perfused with KHB + 1.3 mM Ca\(^{2+}\) for 10 min to allow recovery from the previous agonist challenge.

Fig. 4.9A shows peak [Ca\(^{2+}\)]\(_j\) elevation in response to challenge with 3 \(\mu\)M quisqualate for 2 min in the presence of different [Ca\(^{2+}\)]\(_e\). Statistical analysis revealed there to be no significant decrease in the height of the peak [Ca\(^{2+}\)]\(_j\) elevation upon challenge with 3 \(\mu\)M quisqualate in the presence of different [Ca\(^{2+}\)]\(_e\) (0.5 mM; 99 ± 1.5, nominally Ca\(^{2+}\)-free; 98 ± 1.2, 4 mM; 99 ± 1.0 % of the height of the peak of [Ca\(^{2+}\)]\(_j\) elevation in the presence of 1.3 mM Ca\(^{2+}\)\(_e\)). Fig. 4.9B shows a representative trace from this data.

Fig. 4.10A shows peak [Ca\(^{2+}\)]\(_j\) elevation in response to challenge with 0.7 \(\mu\)M quisqualate for 2 min in the presence of different [Ca\(^{2+}\)]\(_e\). Although a slight, significant increase in the height of peak [Ca\(^{2+}\)]\(_j\) elevation (p<0.05) is observed in the presence of 4 mM Ca\(^{2+}\)\(_e\) compared to in the presence of 0.5 mM Ca\(^{2+}\)\(_e\) and nominally Ca\(^{2+}\)-free buffer, increasing [Ca\(^{2+}\)]\(_e\) over a range does not result in a graded increase in the height of peak [Ca\(^{2+}\)]\(_j\) elevation. No significant difference is revealed between the height of the peak of [Ca\(^{2+}\)]\(_j\) elevation in the presence of different [Ca\(^{2+}\)]\(_e\) compared to that seen in the presence of 1.3 mM Ca\(^{2+}\)\(_e\) (0.5 mM; 93 ± 1.7, nominally Ca\(^{2+}\)-free; 93 ± 2.5, 4 mM; 108 ± 4.8 % of the height of the peak of [Ca\(^{2+}\)]\(_j\) elevation in the presence of 1.3 mM Ca\(^{2+}\)\(_e\)). Fig. 4.10B shows a representative trace from this data.
Fig. 4.9  A: Peak [Ca\(^{2+}\)]\text{\textsubscript{j}} elevation in response to repeated challenge with 3 \(\mu\text{M}\) quisqualate for 2 min in the presence of different [Ca\(^{2+}\)]\text{\textsubscript{e}} in single CHO-lac-hmGluR1\(\alpha\) cells. Cells were perfused with KHB of different [Ca\(^{2+}\)] for 2 min prior to and 2 min subsequent to stimulation with quisqualate in KHB of the appropriate [Ca\(^{2+}\)], between these conditions cells were perfused with KHB at 1.3 mM Ca\(^{2+}\) for 10 min. CHO cells inducibly expressing mGluR1\(\alpha\) were prepared as described previously. Peak [Ca\(^{2+}\)]\text{\textsubscript{j}} elevation data are expressed as a percentage of the peak [Ca\(^{2+}\)]\text{\textsubscript{j}} elevation in response to stimulation with 3 \(\mu\text{M}\) quisqualate in the presence of 1.3 mM Ca\(^{2+}\). Data are shown as means ± standard error for 4 separate experiments. B: A representative trace of peak [Ca\(^{2+}\)]\text{\textsubscript{j}} elevation in response to repeated challenge with 3 \(\mu\text{M}\) quisqualate (---) for 2 min in the presence of different [Ca\(^{2+}\)]\text{\textsubscript{e}} (nominally Ca\(^{2+}\)-free; - - - - -, 0.5 mM; - - - -, 4 mM; - - - -) in a single CHO-lac-hmGluR1\(\alpha\) cell.
Fig. 4.10  A: Peak $[\text{Ca}^{2+}]_i$ elevation in response to repeated challenge with 0.7 $\mu$M quisqualate for 2 min in the presence of different $[\text{Ca}^{2+}]_e$ in single CHO-lac-hmGluR1\(\alpha\) cells. Cells were perfused with KHB of different $[\text{Ca}^{2+}]$ for 2 min prior to and 2 min subsequent to stimulation with quisqualate in KHB of the appropriate $[\text{Ca}^{2+}]$, between these conditions cells were perfused with KHB at 1.3 mM $[\text{Ca}^{2+}]$ for 10 min. CHO cells inducibly expressing mGluR1\(\alpha\) were prepared as described previously. Peak $[\text{Ca}^{2+}]_i$ elevation data are expressed as a percentage of the peak $[\text{Ca}^{2+}]_i$ elevation in response to stimulation with 0.7 $\mu$M quisqualate in the presence of 1.3 mM $[\text{Ca}^{2+}]_e$. Data are shown as means ± standard error for 7 separate experiments. B: A representative trace of peak $[\text{Ca}^{2+}]_i$ elevation in response to repeated challenge with 0.7 $\mu$M quisqualate (—) for 2 min in the presence of different $[\text{Ca}^{2+}]_e$ (nominally $\text{Ca}^{2+}$-free; - - - - - - - , 0.5 mM; — — — , 4 mM; ———) in a single CHO-lac-hmGluR1\(\alpha\) cell.
Thus, varying $[\text{Ca}^{2+}]_e$ does not result in the peak height of $[\text{Ca}^{2+}]_i$ elevation being graded in proportion to $[\text{Ca}^{2+}]_e$ following challenge with either a maximal (3 $\mu$M) or sub-maximal (0.7 $\mu$M) concentration of quisqualate.

4.4.3 Changes in the plateau phase of $[\text{Ca}^{2+}]_i$ elevation following stimulation of mGluR1α with quisqualate in the presence of different $[\text{Ca}^{2+}]_e$.

Having established the height of peak $[\text{Ca}^{2+}]_i$ elevation in response to challenge with quisqualate to be unaffected by varying $[\text{Ca}^{2+}]_e$, the effect of varying $[\text{Ca}^{2+}]_e$ on the plateau phase of $[\text{Ca}^{2+}]_i$ elevation following challenge with a sub-maximal (0.7 $\mu$M) and maximal (3 $\mu$M) concentration of quisqualate was also investigated. Investigation of the plateau required challenge with quisqualate for a longer time period, thus complications could arise if the recovery of the response from the previous challenge was incomplete. However, if the height of peak $[\text{Ca}^{2+}]_i$ elevation is reproducible despite the longer challenge, this indicates full recovery of the response from the previous challenge. Single CHO-lac-hmGluR1α cells were challenged with quisqualate in KHB of the appropriate Ca$^{2+}$ concentration for 5 min to allow observation of the plateau phase. Measurements were taken at the point at which perfusion with quisqualate ceased.

Fig. 4.11 shows the height of the plateau phase of $[\text{Ca}^{2+}]_i$ elevation following challenge with 3 $\mu$M quisqualate in the presence of different $[\text{Ca}^{2+}]_e$ (A) and a representative trace showing that the height of peak $[\text{Ca}^{2+}]_i$ elevation is unaffected despite the longer 5 min challenge with quisqualate (B). The height of the plateau phase of $[\text{Ca}^{2+}]_i$ elevation increased in a graded manner as $[\text{Ca}^{2+}]_e$ increased (1.3
mM; 32.0 ± 7.4, 0.5 mM; 13.5 ± 4.0, nominally Ca²⁺-free; -7.7 ± 1.8, 4 mM; 39.6 ± 4.0 % above or below basal [Ca²⁺]). Fig. 4.11B shows a representative trace from these data.

Fig. 4.12 shows the height of the plateau phase of [Ca²⁺] elevation following challenge with 0.7 μM quisqualate in the presence of different [Ca²⁺]e (A) and a representative trace showing that the height of peak [Ca²⁺] elevation is unaffected despite the 5 min agonist challenge (B). The height of the plateau phase of [Ca²⁺] elevation increased in a graded manner as [Ca²⁺]e increased (1.3 mM; 30.6 ± 6.2, 0.5 mM; 11.4 ± 2.1, nominally Ca²⁺-free; -4.7 ± 5.0, 4 mM; 45.2 ± 16.5 % above or below basal [Ca²⁺]). Fig. 4.12B shows a representative trace from these data.

Thus, the extent of plateau phase [Ca²⁺] elevation increased in a graded manner as [Ca²⁺]e was increased following challenge with both a sub-maximal (0.7 μM) and maximal (3 μM) concentrations of quisqualate.

4.5 Intracellular Ca²⁺ measurements in CHO-m3 cells following agonist stimulation of the M₃-muscarinic receptor.

The difference in the height of the plateau phase of [Ca²⁺] elevation could be a result of the modulatory effect of [Ca²⁺]e on signalling via mGluR1α or it could be a phenomenon of the CHO cell type, resulting from [Ca²⁺]e affecting the degree of Ca²⁺ entry into the cell. Thus, the above experiments were repeated using single CHO-m3 cells to investigate the selectivity of the effect of varying [Ca²⁺]e on the height of the plateau phase of [Ca²⁺] elevation in CHO-lac-hmGluR1α cells.
Fig. 4.11 A: Sustained \([\text{Ca}^{2+}]_i\) elevation in response to repeated challenge with 3 \(\mu\)M quisqualate for 5 min in the presence of different \([\text{Ca}^{2+}]_e\) in single CHO-lac-hmGluR1\(\alpha\) cells. Cells were perfused with KHB of different \([\text{Ca}^{2+}]_e\) for 2 min prior to and 2 min subsequent to stimulation with quisqualate in KHB of the appropriate \([\text{Ca}^{2+}]_e\), between these conditions cells were perfused with KHB at 1.3 mM \(\text{Ca}^{2+}\) for 15 min. CHO cells inducibly expressing mGluR1\(\alpha\) were prepared as described previously. Sustained \([\text{Ca}^{2+}]_i\) elevation data are expressed as a percentage of basal \([\text{Ca}^{2+}]_i\) in the presence of 1.3 mM \(\text{Ca}^{2+}\). Data are shown as means ± standard error for 3 separate experiments. B: A representative trace of sustained \([\text{Ca}^{2+}]_i\) elevation in response to repeated challenge with 3 \(\mu\)M quisqualate (---) for 5 min in the presence of different \([\text{Ca}^{2+}]_e\) (nominally \(\text{Ca}^{2+}\)-free; - - - - - - 0.5 mM; - - - - - - 4 mM; --- ---) in a single CHO-lac-hmGluR1\(\alpha\) cell. Measurements were taken at the point at which perfusion with quisqualate ceased.
Fig. 4.12 A: Sustained [Ca\textsuperscript{2+}]\textsubscript{i} elevation in response to repeated challenge with 0.7 μM quisqualate for 5 min in the presence of different [Ca\textsuperscript{2+}]\textsubscript{e} in single CHO-lac-hmGluR1α cells. Cells were perfused with KHB of different [Ca\textsuperscript{2+}] for 2 min prior to and 2 min subsequent to stimulation with quisqualate in KHB of the appropriate [Ca\textsuperscript{2+}], between these conditions cells were perfused with KHB at 1.3 mM Ca\textsuperscript{2+} for 15 min. CHO cells inducibly expressing mGluR1α were prepared as described previously. Sustained [Ca\textsuperscript{2+}]\textsubscript{i} elevation data are expressed as a percentage of basal [Ca\textsuperscript{2+}]\textsubscript{i} in the presence of 1.3 mM Ca\textsuperscript{2+}. Data are shown as means ± standard error for 3 separate experiments. B: A representative trace of sustained [Ca\textsuperscript{2+}]\textsubscript{i} elevation in response to repeated challenge with 0.7 μM quisqualate (——) for 5 min in the presence of different [Ca\textsuperscript{2+}]\textsubscript{e} (nominally Ca\textsuperscript{2+}-free; - - - - - - , 0.5 mM; - - — — , 4 mM; ———) in a single CHO-lac-hmGluR1α cell.
4.5.1 Reproducibility of \([\text{Ca}^{2+}]_j\) elevation following repeated stimulation of the M₃-muscarinic receptor with methacholine.

The reproducibility of the height of peak \([\text{Ca}^{2+}]_j\) elevation following repeated stimulation of the M₃-muscarinic receptor with methacholine at both a maximal and sub-maximal concentration (to be determined from concentration-effect curve) was established by repeated challenge of CHO-m3 cells with methacholine. Initially CHO-m3 cells were repeatedly challenged with 100 µM methacholine (known to be maximal for \([^3\text{H}]\text{-InsP}_1\) accumulation) for 2 min at 10 min intervals.

Fig. 4.13A shows peak \([\text{Ca}^{2+}]_j\) elevation in response to repeated challenge with 100 µM methacholine in single CHO-m3 cells. The height of peak \([\text{Ca}^{2+}]_j\) elevation was unaffected \((p>0.05)\) throughout 6 successive challenges with a maximal concentration of methacholine (100 µM). Fig. 4.13B shows a representative trace from these data.

Fig. 4.14 shows a concentration-effect curve for the height of peak \([\text{Ca}^{2+}]_j\) elevation following challenge of single CHO-m3 cells with methacholine at the indicated concentrations for 2 min at 10 min intervals. The log EC₅₀ (M) value for the methacholine-stimulated peak \([\text{Ca}^{2+}]_j\) elevation was -6.9 ± 0.3 (0.12 µM). Maximal peak \([\text{Ca}^{2+}]_j\) elevation was reached upon stimulation with 10 µM methacholine. Thus methacholine challenge results in a dose-dependent increase in the height of peak \([\text{Ca}^{2+}]_j\) elevation in single CHO-m3 cells. Fig. 4.14B shows a representative trace from these data.
Fig. 4.13  A: Peak $[\text{Ca}^{2+}]_i$ elevation in response to repeated challenge with 100 $\mu$M methacholine for 2 min at 10 min intervals, in single CHO-m3 cells. Cells were washed with KHB and loaded with KHB + 2 $\mu$M fura 2-AM for 2h prior to use. Peak $[\text{Ca}^{2+}]_i$ elevation data are expressed as a percentage of peak $[\text{Ca}^{2+}]_i$ elevation in response to the first stimulation with 100 $\mu$M methacholine. Data are shown as means ± standard error for 4 separate experiments. B: A representative trace of peak $[\text{Ca}^{2+}]_i$ elevation in response to repeated challenge with 100 $\mu$M methacholine (—) for 2 min at 10 min intervals, in a single CHO-m3 cell.
Fig. 4.14 A: Concentration-dependency of peak $[Ca^{2+}]_i$ elevation in response to challenge with methacholine for 2 min at 10 min intervals, in single CHO-m3 cells. Cells were washed with KHB and loaded with KHB + 2 μM fura 2-AM for 2h prior to use. Peak $[Ca^{2+}]_i$ elevation data are expressed as a percentage of peak $[Ca^{2+}]_i$ elevation in response to challenge with 100 μM methacholine. Data are shown as means ± standard error for 3 separate experiments. B: A representative trace of the concentration-dependency of peak $[Ca^{2+}]_i$ elevation in response to challenge with the indicated concentrations of methacholine (—) for 2 min at 10 min intervals, in a single CHO-m3 cell.
The lowest concentration of methacholine to give a repeatable response was 0.3 μM. Fig. 4.15A shows the height of peak $[\text{Ca}^{2+}]_i$ elevation in response to repeated challenge with 0.3 μM methacholine for 2 min at 10 min intervals, in single CHO-m3 cells. The peak height of $[\text{Ca}^{2+}]_i$ elevation was unaffected ($p>0.05$) throughout 4 successive challenges with a sub-maximal concentration of methacholine. Fig. 4.15B shows a representative trace from these data.

Thus these results demonstrate that single CHO-m3 cells can be repeatedly challenged with methacholine, at both a sub-maximal (0.3 μM) and supra-maximal (100 μM) concentration of methacholine with no decline in the height of peak $[\text{Ca}^{2+}]_i$ elevation. Following challenge with methacholine for 2 min, an interval of 10 min in the presence of 1.3 mM $\text{Ca}^{2+}$ before successive agonist-challenge is sufficient for full recovery from the previous challenge.

4.5.2 Changes in peak $[\text{Ca}^{2+}]_i$ elevation following stimulation of the M₃-muscarinic receptor with methacholine in the presence of different $[\text{Ca}^{2+}]_o$.

The effect of varying $[\text{Ca}^{2+}]_o$ on the height of peak $[\text{Ca}^{2+}]_i$ elevation following challenge with both a maximal (10 μM) and sub-maximal (0.3 μM) concentration of methacholine was then investigated. Cells were perfused with KHB of the appropriate $[\text{Ca}^{2+}]_o$ for 2 min prior to and subsequent to challenge with methacholine.

Fig. 4.16A shows peak $[\text{Ca}^{2+}]_i$ elevation in response to challenge with 10 μM methacholine for 2 min in the presence of different $[\text{Ca}^{2+}]_o$. The height of peak $[\text{Ca}^{2+}]_i$ elevation was unaffected as $[\text{Ca}^{2+}]_o$ was increased upon stimulation with 10 μM.
Fig. 4.15  A: Peak \([\text{Ca}^{2+}]_i\) elevation in response to repeated challenge with 0.3 \(\mu\text{M}\) methacholine for 2 min at 10 min intervals, in single CHO-m3 cells. Cells were washed with KHB and loaded with KHB + 2 \(\mu\text{M}\) fura 2-AM for 2h prior to use. Peak \([\text{Ca}^{2+}]_i\) elevation data are expressed as a percentage of peak \([\text{Ca}^{2+}]_i\) elevation in response to the first stimulation with 0.3 \(\mu\text{M}\) methacholine. Data are shown as means ± standard error for 4 separate experiments. B: A representative trace of peak \([\text{Ca}^{2+}]_i\) elevation in response to repeated challenge with 0.3 \(\mu\text{M}\) methacholine (—) for 2 min at 10 min intervals, in a single CHO-m3 cell.
Fig. 4.16 A: Peak $[\text{Ca}^{2+}]_i$ elevation in response to repeated challenge with 10 $\mu$M methacholine for 2 min in the presence of different $[\text{Ca}^{2+}]_e$ in single CHO-m3 cells. Cells were perfused with KHB of different $[\text{Ca}^{2+}]_e$ for 2 min prior to and 2 min subsequent to stimulation with quisqualate in KHB of the appropriate $[\text{Ca}^{2+}]_e$, between these conditions cells were perfused with KHB at 1.3 mM Ca$^{2+}$ for 10 min. Peak $[\text{Ca}^{2+}]_e$ elevation data are expressed as a percentage of the peak $[\text{Ca}^{2+}]_e$ elevation in response to stimulation with 10 $\mu$M quisqualate in the presence of 1.3 mM Ca$^{2+}$. Data are shown as means ± standard error for 3 separate experiments. B: A representative trace of peak $[\text{Ca}^{2+}]_i$ elevation in response to repeated challenge with 10 $\mu$M methacholine (—) for 2 min in the presence of different $[\text{Ca}^{2+}]_e$ (nominally Ca$^{2+}$-free; - - - -, 0.5 mM; - - - - , 4 mM; ———) in a single CHO-m3 cell.
methacholine in the presence of different [Ca$^{2+}$]$_{o}$ (nominally Ca$^{2+}$-free; 99 ± 0.6, 0.5 mM; 93 ± 1.9, 4 mM; 94 ± 2.0 % of peak [Ca$^{2+}$]$_{i}$ elevation in the presence of 1.3 mM [Ca$^{2+}$]$_{o}$, p>0.05). Fig. 4.16B shows a representative trace from these data.

Fig. 4.17A shows peak [Ca$^{2+}$]$_{i}$ elevation in response to challenge with 0.3 μM methacholine for 2 min in the presence of different [Ca$^{2+}$]$_{o}$. The height of peak [Ca$^{2+}$]$_{i}$ elevation was unaffected upon stimulation with 0.3 μM methacholine in the presence of different [Ca$^{2+}$]$_{o}$ (nominally Ca$^{2+}$-free; 99 ± 1.8, 0.5 mM; 102 ± 4.7, 4 mM; 110 ± 9.0 % of peak [Ca$^{2+}$]$_{i}$ elevation in the presence of 1.3 mM [Ca$^{2+}$]$_{o}$, p>0.05). Fig. 4.17B shows a representative trace from these data.

Thus, following a 10 min recovery period in KFIB and 1.3 mM Ca$^{2+}$, the peak [Ca$^{2+}$]$_{i}$ elevation upon stimulation with either a maximal (10 μM) or sub-maximal (0.3 μM) concentration of methacholine is unaffected by varying [Ca$^{2+}$]$_{o}$.

4.5.3 Changes in the plateau phase of [Ca$^{2+}$]$_{i}$ elevation following stimulation of the M$_{3}$-muscarinic receptor with methacholine in the presence of different [Ca$^{2+}$]$_{o}$.

Having established that the height of peak [Ca$^{2+}$]$_{i}$ elevation in response to challenge with methacholine is unaffected by varying [Ca$^{2+}$]$_{o}$, the effect of varying [Ca$^{2+}$]$_{o}$ on the plateau phase of [Ca$^{2+}$]$_{i}$ elevation could be investigated. As for CHO-lac-hmGluR1α cells investigation of the plateau requires challenge with methacholine for 5 min, thus complications could arise with the recovery of the response from the previous challenge, however, as in CHO-lac-hmGluR1α cells, if the height of peak [Ca$^{2+}$]$_{i}$
Fig. 4.17 A: Peak [Ca^{2+}]_i elevation in response to repeated challenge with 0.3 μM methacholine for 2 min in the presence of different [Ca^{2+}]_e in single CHO-m3 cells. Cells were perfused with KHB of different [Ca^{2+}] for 2 min prior to and 2 min subsequent to stimulation with quisqualate in KHB of the appropriate [Ca^{2+}], between these conditions cells were perfused with KHB at 1.3 mM Ca^{2+} for 10 min. Peak [Ca^{2+}]_i elevation data are expressed as a percentage of the peak [Ca^{2+}]_i elevation in response to stimulation with 0.3μM quisqualate in the presence of 1.3 mM Ca^{2+}. Data are shown as means ± standard error for 3 separate experiments. B: A representative trace of peak [Ca^{2+}]_i elevation in response to repeated challenge with 0.3 μM methacholine (---) for 2 min in the presence of different [Ca^{2+}]_e (nominally Ca^{2+}-free; - - - - -, 0.5 mM; - - - - , 4 mM; ———) in a single CHO-m3 cell.
elevation is unaffected despite the longer stimulation, this indicates full recovery of the response from the previous challenge. The cells were stimulated with methacholine in KHB of the appropriate Ca\(^{2+}\) concentration for 5 min to allow observation of the plateau phase. Measurements were taken at the point at which perfusion with methacholine ceased.

Fig. 4.18 shows the height of the plateau phase of [Ca\(^{2+}\)]\(_i\) elevation following challenge with 10 \(\mu\)M methacholine in the presence of different [Ca\(^{2+}\)]\(_e\) (A) and a representative trace showing that the height of peak [Ca\(^{2+}\)]\(_i\) elevation is unaffected despite the 5 min agonist challenge (B). The height of the plateau phase of [Ca\(^{2+}\)]\(_i\) elevation increased in a graded manner as the [Ca\(^{2+}\)]\(_e\) increased (1.3 mM; 76.7 ± 20.4, 0.5 mM; 20.9 ± 7.0, nominally Ca\(^{2+}\)-free; 10.6 ± 7.6, 4 mM; 100.3 ± 9.0 % above or below basal [Ca\(^{2+}\)]\(_i\)). Fig. 4.18B shows a representative trace from these data.

Fig. 4.19 shows the height of the plateau phase of [Ca\(^{2+}\)]\(_i\) elevation following challenge with 0.3 \(\mu\)M methacholine in the presence of different [Ca\(^{2+}\)]\(_e\) (A) and a representative trace showing that the peak height is unaffected despite the 5 min agonist challenge (B). The height of the plateau phase of [Ca\(^{2+}\)]\(_i\) elevation increased in a graded manner as the [Ca\(^{2+}\)]\(_e\) increased, as for stimulation with 10 \(\mu\)M methacholine (1.3 mM; 90.7 ± 12.2, 0.5 mM; 37.3 ± 14.6, nominally Ca\(^{2+}\)-free; 17.4 ± 15.6, 4 mM; 102.1 ± 13.8 % above or below basal [Ca\(^{2+}\)]\(_i\)). Fig. 4.19B shows a representative trace from these data.
Fig. 4.18 A: Sustained [Ca^{2+}]_{i} elevation in response to repeated challenge with 10 μM methacholine for 5 min in the presence of different [Ca^{2+}]_{e} in single CHO-m3 cells. Cells were perfused with KHB of different [Ca^{2+}] for 2 min prior to and 2 min subsequent to stimulation with quisqualate in KHB of the appropriate [Ca^{2+}], between these conditions cells were perfused with KHB at 1.3 mM Ca^{2+} for 15 min. Sustained [Ca^{2+}]_{i} elevation data are expressed as a percentage of basal [Ca^{2+}]_{i} in the presence of 1.3 mM Ca^{2+}. Data are shown as means ± standard error for 3 separate experiments. B: A representative trace of sustained [Ca^{2+}]_{i} elevation in response to repeated challenge with 10 μM methacholine (—) for 5 min in the presence of different [Ca^{2+}]_{e} (nominally Ca^{2+}-free; ————, 0.5 mM; ————, 4 mM; ————) in a single CHO-m3 cell. Measurements were taken at the point at which perfusion with methacholine ceased.
Fig. 4.19 A: Sustained [Ca$^{2+}$]$_i$ elevation in response to repeated challenge with 0.3 μM methacholine for 5 min in the presence of different [Ca$^{2+}$]$_e$ in single CHO-m3 cells. Cells were perfused with KHB of different [Ca$^{2+}$] for 2 min prior to and 2 min subsequent to stimulation with quisqualate in KHB of the appropriate [Ca$^{2+}$], between these conditions cells were perfused with KHB at 1.3 mM Ca$^{2+}$ for 15 min. Sustained [Ca$^{2+}$]$_i$ elevation data are expressed as a percentage of basal [Ca$^{2+}$]$_i$ in the presence of 1.3 mM Ca$^{2+}$. Data are shown as means ± standard error for 3 separate experiments. B: A representative trace of sustained [Ca$^{2+}$]$_i$ elevation in response to repeated challenge with 0.3 μM methacholine (—) for 5 min in the presence of different [Ca$^{2+}$]$_e$ (nominally Ca$^{2+}$-free; - - - - - , 0.5 mM; — — — , 4 mM; ————) in a single CHO-m3 cell.
These data show that the extent of plateau phase \([\text{Ca}^{2+}]_i\) elevation increases in a graded manner as the \([\text{Ca}^{2+}]_e\) increases following challenge with both a maximal and sub-maximal concentration of methacholine.

Thus, in both CHO-lac-hmGluR1\(\alpha\) and CHO-m3 cells the height of the plateau phase of \([\text{Ca}^{2+}]_i\) elevation increases in a graded manner with the increase in \([\text{Ca}^{2+}]_e\), suggesting that this is a phenomenon of the CHO cell-type, and not selective to signalling via mGluR1\(\alpha\). Therefore, these data suggest that the increase in the height of the plateau phase of \([\text{Ca}^{2+}]_i\) elevation with increasing \([\text{Ca}^{2+}]_e\) in CHO-lac-hmGluR1\(\alpha\) cells cannot conclusively be associated with the modulatory effect of \([\text{Ca}^{2+}]_e\) on \([^3\text{H}]-\text{InsP}_1\) accumulation observed in these cells.

4.6 Investigations to determine the importance of calmodulin (CaM) and protein kinase C (PKC) in the modulation of signalling via mGluR1\(\alpha\) by \([\text{Ca}^{2+}]_e\).

The group 1 mGluRs have a number of serine and threonine residues in their C-termini, which are putative PKC phosphorylation sites, and may be involved in the regulation of receptor activity (Minakami \textit{et al}, 1997). Interestingly, both PKC and CaM have been shown to bind to the same sites in the C-terminus of mGluR5. PKC phosphorylation prevents CaM binding, and CaM binding prevents phosphorylation by PKC. Thus, the binding of CaM and PKC phosphorylation are mutually antagonistic. This binding of CaM to mGluR5 was shown to be abolished by the removal of \(\text{Ca}^{2+}_e\), and is therefore \(\text{Ca}^{2+}\)-dependent. Comparison of the sequences of the C-termini of mGluR1\(\alpha\) and mGluR5 provides evidence that there are sites homologous to the CaM binding sites of mGluR5 in mGluR1\(\alpha\), therefore it is possible
that the mutually antagonistic binding of CaM and PKC is a feature of mGluR1α as well (Minakami et al, 1997). If this is so it is possible that the binding of CaM to mGluR1α increases in a graded manner with increasing [Ca\(^{2+}\)]\(_e\) resulting in modulation of \([^{3}H] \text{InsP}_1\) accumulation, either independently or by affecting the degree of phosphorylation of mGluR1α by PKC.

4.6.1 Comparison of the effects of a PKC activator (PdBu) and a PKC inhibitor (Ro-31-8220) on the concentration-dependence of agonist-stimulated \([^{3}H] \text{InsP}_1\) accumulation in CHO-lac-hmGluR1α and CHO-m3 cells.

Before the importance of PKC phosphorylation of mGluR1α on the modulatory effect of Ca\(^{2+}\)_e on agonist-stimulated \([^{3}H] \text{InsP}_1\) accumulation via mGluR1α could be established, the effect of PdBu and Ro-31-8220 on the concentration-dependence of agonist-stimulated \([^{3}H] \text{InsP}_1\) accumulation in CHO-lac-hmGluR1α cells needed to be compared to that seen in CHO-m3 cells. Data were analysed using one-way ANOVA followed by Duncan’s multiple range test.

Fig. 4.20 shows basal \([^{3}H] \text{InsP}_1\) accumulation and concentration-dependent increases in \([^{3}H] \text{InsP}_1\) accumulation in response to stimulation of the M3-muscarinic receptor with methacholine under control conditions and following treatment with 1 μM PdBu (a PKC activator) or 10 μM Ro-31-8220 (a PKC inhibitor). Previous studies have shown that treatment with PdBu results in a decrease in inositol phosphate accumulation in response to stimulation of muscarinic receptors with methacholine (Labarca et al, 1984; Lai et al, 1988; Willars et al, 1996). In common with these observations, a slight, not significant, decrease in \([^{3}H] \text{InsP}_1\) accumulation

124
was seen following PdBu treatment, in particular following challenge with 0.1 μM methacholine (control; 15048 ± 4668, PdBu treated; 6948 ± 3644 d.p.m. well⁻¹). The effects of Ro-31-8220 on inositol phosphate accumulation following stimulation of muscarinic receptors is complicated by its antagonist activity at muscarinic receptors (Willars et al., 1996), however following treatment with Ro-31-8220, there was a significant increase in [³H]-InsP₁ accumulation following challenge with 0.1 μM methacholine (control; 15048 ± 4668, Ro-31-8220; 29112 ± 3613 d.p.m. well⁻¹, p<0.05).

Fig. 4.21 shows basal [³H]-InsP₁ accumulation and concentration-dependent increases in [³H]-InsP₁ accumulation in response to stimulation of mGluR₁α with quisqualate under control conditions and following treatment with 1 μM PdBu and 10 μM Ro-31-8220, in the presence of 1.3 mM Ca²⁺. This study supports the observations from CHO-m3 cells in that PdBu treatment resulted in a slight decrease in [³H]-InsP₁ accumulation at sub-maximal concentrations of quisqualate (e.g. 0.3 μM quisqualate, control; 5237 ± 2171, PdBu; 1400 ± 304), however treatment with Ro-31-8220 has no effect on quisqualate-stimulated [³H]-InsP₁ accumulation.

4.6.2 Effect of a PKC activator (PdBu) and PKC inhibitors on [³H]-InsP₁ accumulation in the presence of different [Ca²⁺]ₑ in CHO-lac-hmGluR₁α cells.

As CaM binding has been shown to be inhibited by the phosphorylation of mGluR5 by PKC, the influence of PKC on the modulation of [³H]-InsP₁ accumulation by [Ca²⁺]ₑ in CHO-lac-hmGluR₁α cells was examined with the use of PKC inhibitors and activators.
Fig. 4.20 Basal and methacholine-stimulated $[^3]$H-InsP$_1$ accumulations in CHO-m3 cells under control conditions (O) and following treatment with PdBu (■) or Ro-31-8220 (♦). CHO cells stably expressing the M$_3$-muscarinic receptor were incubated with 2.5 µCi ml$^{-1}$ $[^3]$H-inositol for 48 h. The cells were then washed with KHB and incubated with KHB for 20 min prior to addition of LiCl to a final concentration of 10 mM for a further 30 min prior to challenge with the indicated concentrations of methacholine for 15 min. Treatment with 10 µM Ro-31-8220 occurred 5 min before LiCl addition; treatment with 1 µM PdBu occurred 5 min before challenge with methacholine. $[^3]$H-InsP$_1$ data are expressed as d.p.m. well$^{-1}$ and are shown as means ± standard error for 3 separate experiments.
Fig. 4.21 Basal and quisqualate-stimulated \(^{3}H\)-InsP\(_1\) accumulations in CHO-lac-hmGluR1\(\alpha\) cells under control conditions (O) and following treatment with PdBu (■) or Ro-31-8220 (♦"). CHO cells inducibly expressing mGluR1\(\alpha\) were incubated with 2.5 \(\mu\)Ci ml\(^{-1}\) \(^{3}H\)-inositol for 48 h, mGluR1\(\alpha\) expression was induced with 100 \(\mu\)M IPTG for the last 20 h. The cells were then washed with KHB and incubated with KHB + 3 U ml\(^{-1}\) GPT and 5 mM pyruvate for 20 min prior to addition of LiCl to a final concentration of 10 mM for a further 30 min prior to challenge with the indicated concentrations of quisqualate for 15 min. Treatment with 10 \(\mu\)M Ro-31-8220 occurred 5 min before LiCl addition; treatment with 1 \(\mu\)M PdBu occurred 5 min before challenge with quisqualate. \(^{3}H\)-InsP\(_1\) data are expressed as d.p.m. well\(^{-1}\) and are shown as means ± standard error for 3 separate experiments.
Fig. 4.22 shows the $[\text{Ca}^{2+}]_e$-dependency of $[^3\text{H}]\text{InsP}_1$ accumulation following challenge with 30 $\mu$M quisqualate under control conditions and following treatment with 1 $\mu$M PdBu. PdBu was shown to have no effect on basal $[^3\text{H}]\text{InsP}_1$ accumulation per se. PdBu treatment resulted in a significant decrease in quisqualate-stimulated $[^3\text{H}]\text{InsP}_1$ accumulation in the presence of 0.5 mM and 4 mM $\text{Ca}^{2+}$: $0.5$ mM $\text{Ca}^{2+}$; - PdBu; 4990 ± 437, + PdBu; 3201 ± 535 (p<0.05), 4 mM $\text{Ca}^{2+}$; - PdBu; 10576 ± 66, + PdBu; 8914 ± 875 d.p.m. well$^{-1}$, (p<0.05)), however $[^3\text{H}]\text{InsP}_1$ accumulation still increased in a graded manner as the $[\text{Ca}^{2+}]_e$ was increased from nominally $\text{Ca}^{2+}$-free to 1.3 mM.

Figs. 4.23-4.25 show the $[\text{Ca}^{2+}]_e$-dependency of $[^3\text{H}]\text{InsP}_1$ accumulation following challenge with quisqualate under control conditions and following treatment with 10 $\mu$M Ro-31-8220, 10 $\mu$M bisindolylmaleimide I and 10 $\mu$M Gö 6976, respectively. The inhibitors themselves were shown to have no significant effect on basal $[^3\text{H}]\text{InsP}_1$ accumulation per se. No significant effects on $[^3\text{H}]\text{InsP}_1$ accumulation were seen following treatment with any of these PKC inhibitors, $[^3\text{H}]\text{InsP}_1$ accumulation still increasing in a graded manner as the $[\text{Ca}^{2+}]_e$ was increased from nominally $\text{Ca}^{2+}$-free to 1.3 mM.

Thus, treatment of CHO-lac-hmGluR1$\alpha$ cells with a PKC activator and several PKC inhibitors has little or no effect on the modulation of $[^3\text{H}]\text{InsP}_1$ accumulation by $[\text{Ca}^{2+}]_e$. Therefore, these data suggest that phosphorylation of mGluR1$\alpha$ by PKC may not be involved in the modulation of signalling via mGluR1$\alpha$ by $[\text{Ca}^{2+}]_e$. 

126
Fig. 4.22 [Ca\textsuperscript{2+}]_o-dependency of quisqualate-stimulated [\textsuperscript{3}H]-InsP\textsubscript{1} accumulation in CHO-lac-hmGluR1\alpha cells following treatment with PdBu (vehicle; ■, PdBu; ▼, vehicle + quisqualate; ◆, PdBu + quisqualate; O). CHO cells inducibly expressing mGluR1\alpha were incubated with 2.5 μCi ml\textsuperscript{-1} [\textsuperscript{3}H]-inositol for 48 h, mGluR1\alpha expression was induced with 100 μM IPTG for the last 20 h. The cells were then washed with KHB and incubated with KHB + 3 U ml\textsuperscript{-1} GPT and 5 mM pyruvate for 20 min prior to addition of LiCl to a final concentration of 10 mM for a further 30 min prior to challenge with the indicated concentrations of quisqualate for 15 min. Treatment with 1 μM PdBu occurred 5 min before challenge with quisqualate. [\textsuperscript{3}H]-InsP\textsubscript{1} data are expressed as d.p.m. well\textsuperscript{-1} and are shown as means ± standard error for 3 separate experiments.
Fig. 4.23 \([\text{Ca}^{2+}]_e\)-dependency of quisqualate-stimulated \([\text{H}]\)-\(\text{InsP}_1\) accumulation in CHO-lac-hmGluR1\(\alpha\) cells following treatment with Ro-31-8220 (vehicle; ■, Ro-31-8220; V, vehicle + quisqualate; ♦, Ro-31-8220 + quisqualate; ○). CHO cells inducibly expressing mGluR1\(\alpha\) were incubated with 2.5 µCi ml\(^{-1}\) [\(\text{H}\)]-inositol for 48 h, mGluR1\(\alpha\) expression was induced with 100 µM IPTG for the last 20 h. The cells were then washed with KHB and incubated with KHB + 3 U ml\(^{-1}\) GPT and 5 mM pyruvate for 20 min prior to addition of LiCl to a final concentration of 10 mM for a further 30 min prior to challenge with the indicated concentrations of quisqualate for 15 min. Treatment with 10 µM Ro-31-8220 occurred 5 min before LiCl addition. [\(\text{H}\)]-\(\text{InsP}_1\) data are expressed as d.p.m. well\(^{-1}\) and are shown as means ± standard error for 3 separate experiments.
Fig. 4.24 [Ca^{2+}]_e-dependency of quisqualate-stimulated [^{3}H]-InsP_1 accumulation in CHO-lac-hmGluR1α cells following treatment with bisindolylmaleimide I (vehicle; ■, bisindolylmaleimide I; ▼, vehicle + quisqualate; ◆, bisindolylmaleimide I + quisqualate; ○). CHO cells inducibly expressing mGluR1α were incubated with 2.5 μCi ml^{-1} [^{3}H]-inositol for 48 h, mGluR1α expression was induced with 100 μM IPTG for the last 20 h. The cells were then washed with KHB and incubated with KHB + 3 U ml^{-1} GPT and 5 mM pyruvate for 20 min prior to addition of LiCl to a final concentration of 10 mM for a further 30 min prior to challenge with the indicated concentrations of quisqualate for 15 min. Treatment with 10 μM bisindolylmaleimide I occurred 5 min before LiCl addition. [^{3}H]-InsP_1 data are expressed as d.p.m. well^{-1} and are shown as means ± standard error for 3 separate experiments.
Fig. 4.25  $[\text{Ca}^2+]_e$-dependency of quisqualate-stimulated $[^{3}\text{H}]$-InsP$_1$ accumulation in CHO-lac-hmGluR1α cells following treatment with Gö 6976 (vehicle; ■, Gö 6976; ▼, vehicle + quisqualate; ♦, Gö 6976 + quisqualate; ○). CHO cells inducibly expressing mGluR1α were incubated with 2.5 μCi ml$^{-1}$ $[^{3}\text{H}]$-inositol for 48 h, mGluR1α expression was induced with 100 μM IPTG for the last 20 h. The cells were then washed with KHB and incubated with KHB + 3 U ml$^{-1}$ GPT and 5 mM pyruvate for 20 min prior to addition of LiCl to a final concentration of 10 mM for a further 30 min prior to challenge with the indicated concentrations of quisqualate for 15 min. Treatment with 10 μM Gö 6976 occurred 5 min before LiCl addition. $[^{3}\text{H}]$-InsP$_1$ data are expressed as d.p.m. well$^{-1}$ and are shown as means ± standard error for 3 separate experiments.
4.6.3 Effect of CaM antagonists on $[^3\text{H}]$-InsP$_1$ accumulation in the presence of different [Ca$^{2+}$]$_e$ in CHO-lac-hmGluR1$\alpha$ cells.

If CaM binds to mGluR1$\alpha$ it is possible that this binding increases in a graded manner with increasing [Ca$^{2+}$]$_e$ resulting in modulation of $[^3\text{H}]$-InsP$_1$ accumulation. To test this hypothesis the dependence of $[^3\text{H}]$-InsP$_1$ accumulation on [Ca$^{2+}$]$_e$ was investigated in CHO-lac-hmGluR1$\alpha$ cells following treatment with CaM antagonists. Data were analysed using one-way ANOVA followed by Duncan’s multiple range test.

Two CaM antagonists have been used, W-7 and W-13 which displace $[^3\text{H}]$-W-7 binding from CaM with IC$_{50}$s of 31 $\mu$M and 55 $\mu$M respectively (Hidaka et al, 1981; Chafouleas et al, 1982).

Fig. 4.26A shows the effect of W-7 on basal $[^3\text{H}]$-InsP$_1$ accumulation in the presence of different [Ca$^{2+}$]$_e$. W-7 did not cause any consistent changes in $[^3\text{H}]$-InsP$_1$ accumulation compared to control. Fig. 4.26B shows the [Ca$^{2+}$]$_e$-dependency of quisqualate-stimulated $[^3\text{H}]$-InsP$_1$ accumulation in CHO-lac-hmGluR1$\alpha$ cells following treatment with the CaM antagonist W-7. At all concentrations of W-7 below 100 $\mu$M the graded increase in $[^3\text{H}]$-InsP$_1$ accumulation as [Ca$^{2+}$]$_e$ is increased was still evident (e.g. at 30 $\mu$M W-7, nominally Ca$^{2+}$-free; 380 ± 35 to 0.5 mM; 3078 ± 501 (p<0.05), to 1.3 mM; 5819 ± 200 (p<0.05), to 4 mM Ca$^{2+}$; 8283 ± 428 d.p.m. well$^{-1}$ (p<0.05)), even though the degree of $[^3\text{H}]$-InsP$_1$ accumulation decreased at concentrations of W-7 higher than 10 $\mu$M. At a concentration as high as 100 $\mu$M there is the possibility of non-specific cell toxic effects of the compound.
Fig. 4.26  A: Effect of the CaM antagonist W-7 on $[^3$H]-InsP$_1$ accumulation per se (nominally Ca$^{2+}$-free; clear bar, 0.5 mM; lightly shaded bar, 1.3 mM; heavily shaded bar, 4 mM; filled bar). B: [Ca$^{2+}$]$_e$-dependency of quisqualate-stimulated $[^3$H]-InsP$_1$ accumulations in CHO-lac-hmGluR1α cells following treatment with W-7 (nominally Ca$^{2+}$-free; O, 0.5 mM; ■, 1.3 mM; V, 4 mM; ♦). CHO cells inducibly expressing mGluR1α were incubated with 2.5 μCi ml$^{-1}$ $[^3$H]-inositol for 48 h, mGluR1α expression was induced with 100 μM IPTG for the last 20 h. The cells were then washed with KHB and incubated with KHB + 3 U ml$^{-1}$ GPT and 5 mM pyruvate for 20 min prior to addition of LiCl to a final concentration of 10 mM for a further 30 min before challenge with 30 μM quisqualate for 15 min. Treatment with W-7 at the indicated concentrations occurred 5 min before LiCl addition. $[^3$H]-InsP$_1$ data are expressed as d.p.m. mg$^{-1}$ protein and are shown as mean ± standard error for 3 separate experiments.
To test whether the decrease in \( [^3\text{H}]\)-InsP\(_1\) accumulation as the concentration of W-7 is increased is actually due to the antagonism of CaM by W-7, rather than a non-specific cell-toxic effect, CHO-lac-hmGluR1\(\alpha\) cells were treated with the same concentrations of a non-active analogue W-5, and \( [^3\text{H}]\)-InsP\(_1\) accumulation following challenge with 30 \( \mu \text{M} \) quisqualate measured. Fig. 4.27 shows the \([\text{Ca}^{2+}]_e\) dependency of quisqualate-stimulated \( [^3\text{H}]\)-InsP\(_1\) accumulation in CHO-lac-hmGluR1\(\alpha\) cells following treatment with the W-7 analogue W-5. Even at the highest concentration of W-5 used (100 \( \mu \text{M} \)), there was no decrease in the \( [^3\text{H}]\)-InsP\(_1\) accumulation compared to control at any \([\text{Ca}^{2+}]_e\) (e.g. 1.3 mM \( \text{Ca}^{2+}\); control; 9657 \( \pm \) 326, 100 \( \mu \text{M} \) W-5; 10653 \( \pm \) 319 d.p.m. well\(^{-1}\), \( p>0.05 \)). These data provide some support for the assertion that the results described above can be attributed to the antagonism of CaM by W-7.

Fig. 4.28A shows the effect of an additional CaM antagonist; W-13, on basal \( [^3\text{H}]\)-InsP\(_1\) accumulation in the presence of different \([\text{Ca}^{2+}]_e\). W-13, at either 50 or 100 \( \mu \text{M} \), has no significant effect on basal \( [^3\text{H}]\)-InsP\(_1\) accumulation at any \([\text{Ca}^{2+}]_e\) studied. Fig. 4.28B shows the \([\text{Ca}^{2+}]_e\) dependency of quisqualate-stimulated \( [^3\text{H}]\)-InsP\(_1\) accumulation in CHO-lac-hmGluR1\(\alpha\) cells following treatment with the CaM antagonist W-13. At all concentrations of W-13 below 300 \( \mu \text{M} \) the graded increase in \( [^3\text{H}]\)-InsP\(_1\) accumulation as \([\text{Ca}^{2+}]_e\) is increased was still evident even though the degree of \( [^3\text{H}]\)-InsP\(_1\) accumulation decreased as the concentration of W-13 was increased. At a concentration as high as 300 \( \mu \text{M} \), as for W-7, there is the possibility of non-specific cell-toxic effects.
Fig. 4.27 [Ca\textsuperscript{2+}]\textsubscript{i}-dependency of quisqualate-stimulated \[^{3}\text{H}\text{-}]\text{-InsP}_1 accumulation in CHO-lac-hmGluR1\alpha cells following treatment with the inactive calmodulin antagonist W-5 (nominally Ca\textsuperscript{2+}-free; O, 0.5 mM; ■, 1.3 mM; ▼, 4 mM; ♦). CHO cells inducibly expressing mGluR1\alpha were incubated with 2.5 \(\mu\text{Ci m}^{-1}\) \[^{3}\text{H}\text{-}]\text{-inositol for 48 h, mGluR1}\alpha expression was induced with 100 \(\mu\text{M IPTG for the last 20 h. The cells were then washed with KHB and incubated with KHB + 3 U m}^{-1}\text{GPT and 5 mM pyruvate for 20 min prior to addition of LiCl to a final concentration of 10 mM for a further 30 min before challenge with 30 \(\mu\text{M quisqualate for 15 min. Treatment with W-5 at the indicated concentrations occurred 5 min before LiCl addition.}[^{3}\text{H}\text{-}]\text{-InsP}_1 data are expressed as d.p.m. mg}^{-1}\text{ protein and are shown as mean ± standard error for 3 separate experiments.}
**Fig. 4.28** A: Effect of the CaM antagonist W-13 on $[^3H]$-InsP$_1$ accumulation per se (nominally Ca$^{2+}$-free; clear bar, 0.5 mM; lightly shaded bar, 1.3 mM; heavily shaded bar, 4 mM; filled bar). B: [Ca$^{2+}$]$_e$-dependency of quisqualate-stimulated $[^3H]$-InsP$_1$ accumulations in CHO-lac-hmGluR1$\alpha$ cells following treatment with W-13 (nominally Ca$^{2+}$-free; O, 0.5 mM; ■, 1.3 mM; V, 4 mM; ♦). CHO cells inducibly expressing mGluR1$\alpha$ were incubated with 2.5 $\mu$Ci ml$^{-1}$ $[^3H]$-inositol for 48 h, mGluR1$\alpha$ expression was induced with 100 $\mu$M IPTG for the last 20 h. The cells were then washed with KHB and incubated with KHB + 3 U ml$^{-1}$ GPT and 5 mM pyruvate for 20 min prior to addition of LiCl to a final concentration of 10 mM for a further 30 min before challenge with 30 $\mu$M quisqualate for 15 min. Treatment with W-13 at the indicated concentrations occurred 5 min before LiCl addition. $[^3H]$-InsP$_1$ data are expressed as d.p.m. mg$^{-1}$ protein and are shown as mean ± standard error for 3 separate experiments.
Taken together, these data suggest that CaM binding to the receptor may be important for agonist-stimulated signalling via mGluR1α but that it is not essential for the modulatory effect of Ca\(^{2+}\) on agonist-stimulated \[^3H\]-InsP\(_1\) accumulation via mGluR1α.

4.7 Discussion.

When expressed in *Xenopus* oocytes, mGluR1α has been shown to be activated by both glutamate and Ca\(^{2+}\) (Kubokawa *et al.*, 1996). Our investigations have extended these observations demonstrating that Ca\(^{2+}\) modulates agonist-stimulated phosphoinositide signalling via mGluR1α expressed in BHK cells, thus as [Ca\(^{2+}\)]\(_e\) is increased over the range; nominally Ca\(^{2+}\)-free to 1.3 mM, \[^3H\]-InsP\(_1\) accumulation in response to agonist-stimulation is enhanced with an increase in the response to a maximal concentration of agonist and a decrease in the EC\(_{50}\) value for agonist-stimulated \[^3H\]-InsP\(_1\) accumulation (Saunders *et al.*, 1998). Thus, Ca\(^{2+}\) can act as an agonist of rat mGluR1α (Kubokawa *et al.*, 1996), or as a modulator of agonist-stimulated signalling via mGluR1α (Saunders *et al.*, 1998), suggesting that mGluR1α may contain a Ca\(^{2+}\) binding site within its extracellular domain.

Although the studies described in Chapter 3 provide strong evidence to suggest that the modulatory effect of Ca\(^{2+}\) is indeed selective to signalling via mGluR1α rather than a general phenomenon of GPCR expression in BHK cells, studies to elucidate the site of action of Ca\(^{2+}\) were inconclusive. It is possible that the modulatory effect of Ca\(^{2+}\) could be due to graded activation of Ca\(^{2+}\)-sensitive PLCs or graded influx through voltage-operated Ca\(^{2+}\) channels. Thus, the ability of Ca\(^{2+}\) to modulate
signalling via mGluR1α in an additional mammalian cell line; CHO-lac-hmGluR1α cells (Hermans et al, 1998b), has been investigated, with the aim of determining the site at which Ca²⁺ has its modulatory effects.

The influence of [Ca²⁺]ₐ on the coupling of mGluR1α to PLC in an inducible CHO cell system was studied by measuring [³H]-InsP₁ accumulation in the presence of 10 mM LiCl. Increasing [Ca²⁺]ₐ over the range nominally Ca²⁺-free to 1.3 mM, had a graded modulatory effect on agonist-stimulated phosphoinositide signalling via mGluR1α, in response to stimulation with both sub-maximal and maximal concentrations of both quisqualate, a full agonist of the receptor and 1S,3R-ACPD, a partial agonist of the receptor. Although [³H]-InsP₁ accumulation decreased under nominally Ca²⁺-free conditions, increasing [Ca²⁺]ₐ above 0.5 mM had no effect on signalling via the M₃-muscarinic receptor. These differential effects of varying [Ca²⁺]ₐ on phosphoinositide signalling via two different receptors expressed in a common cell background again strongly suggest that such modulation is receptor- (mGluR1α) rather than cell-type selective.

Although these data contradict observations which suggest a role for Ca²⁺ as an agonist of mGluR1α *per se*, they support a role for Ca²⁺ as a modulator of agonist-stimulated signalling via mGluR1α in common with our previous studies in BHK cells expressing mGluR1α (Saunders et al, 1998). Serine 166 located in the extracellular domain of rat mGluR1α has been proposed to be critical for the Ca²⁺ sensitivity of the receptor expressed in *Xenopus* oocytes, providing evidence that Ca²⁺ interacts directly with the receptor to have its modulatory effect (Kubo et al, 1998). Subsequent studies in this Chapter have investigated the site at which [Ca²⁺]ₐ
modulates agonist-stimulated phosphoinositide signalling via human mGluR1α inducibly expressed in a mammalian CHO cell line.

Incubation of cells with [3H]-inositol prior to experimentation results in its incorporation into the different components of the phosphoinositide cycle, including the membrane located phosphoinositides PI, PIP and PIP2. If varying [Ca2+]o resulted in a decrease in levels of these membrane phospholipids this could explain the decrease in agonist-stimulated [3H]-InsP1 accumulation as [Ca2+]o is decreased, as the generation of inositol phosphates following activation of PLC would be limited by the availability of PIP2. No significant difference was seen in membrane phospholipid levels between different [Ca2+]o, following incubation of the cells under basal conditions or challenge with 30 μM quisqualate. This suggests that changes in the levels of membrane phospholipids in the presence of different [Ca2+]o are not responsible for the modulatory effects of Ca2+ on 3[H]-InsP1 accumulation in CHO-lac-hmGluR1α cells.

Following agonist activation of the group 1 mGluRs, and the hydrolysis of PIP2, in addition to the production of Ins(1,4,5)P3 and the resultant increase in [Ca2+]i elevation, diacylglycerol is produced resulting in the stimulation of PKC. A number of serine and threonine residues, thought to constitute PKC phosphorylation sites, are present in the C-termini of the group 1 mGluRs. A number of studies suggest modulation of group 1 mGluRs following PKC activation (Aramori and Nakanishi, 1992; Thomsen et al, 1993; Alaluf et al, 1995b; Kawabata et al, 1996) and recent studies demonstrate mediation of mGluR5 desensitisation by PKC (Gereau and Heinemann, 1998). PKC and CaM bind to the same sites in the C-terminus of
mGluR5; PKC phosphorylation preventing CaM binding to mGluR5, and CaM binding preventing phosphorylation of mGluR5 by PKC. Thus, the binding of CaM and PKC phosphorylation are mutually antagonistic (Minakami et al 1997). The binding of CaM to mGluR5 was also shown to be Ca^{2+} dependent. It is proposed that the different temporal characteristics of [Ca^{2+}] elevation, (and the resultant Ca^{2+}-CaM complexes) and PKC activation may influence the extent of phosphorylation of mGluR5 thus regulating receptor responses (Minakami et al, 1997). Such mutual antagonism by PKC and CaM appears not to be unique to mGluR5, there is a growing body of evidence to suggest that PKC and CaM share target sites in a number of substrates, e.g. mGluR7 (Nakajima et al, 1999), neurogranin and neuromodulin, MARCKS (myristoylated alanine-rich C-kinase substrate) protein, the NMDA receptor NR1 subunit, and the plasma membrane Ca^{2+} pump (Chakravarthy et al, 1999). Thus, such a mechanism has been implicated in a number of cellular responses, and adds a temporal aspect to cellular signalling (Chakravarthy et al, 1999).

Comparison of the sequences of the C-termini of mGluR1α and mGluR5 provides evidence that there are sites homologous to the CaM binding sites of mGluR5 in mGluR1α (Minakami et al, 1997). Indeed, CaM has been shown to bind to the C-terminus of mGluR1α (Nakajima et al, 1999). Therefore, it is possible that CaM and PKC also bind to mGluR1α in a mutually antagonistic manner. It should be considered that the observations of Minakami et al, were gained using molecular biological techniques and their observations have not been confirmed in a heterologous expression system. This study has investigated the general influence of CaM and PKC on the activity of mGluR1α expressed in a heterologous system.
and, in addition, the possibility that the degree of CaM binding increases in a graded manner with the \([\text{Ca}^{2+}]_e\) and could be responsible for the increase in \([\text{H}]\text{-InsP}_1\) accumulation observed as \([\text{Ca}^{2+}]_e\) increases. This could be possible due to either; an increase in \([\text{Ca}^{2+}]_e\) resulting in increased amounts of \(\text{Ca}^{2+}\) binding to CaM and subsequently increasing amounts of CaM binding to mGluR1\(\alpha\), or by different amounts of \(\text{Ca}^{2+}\) binding to a \(\text{Ca}^{2+}\)-binding site in the extracellular domain of mGluR1\(\alpha\) causing a conformational change in the receptor resulting in more CaM binding to mGluR1\(\alpha\). If the binding of CaM blocks phosphorylation by PKC, this would result in a decrease in phosphorylation of mGluR1\(\alpha\) by PKC as \([\text{Ca}^{2+}]_e\) increases. This could therefore affect the degree of modulation of mGluR1\(\alpha\) by PKC phosphorylation resulting in a graded increase in \([\text{H}]\text{-InsP}_1\) accumulation as \([\text{Ca}^{2+}]_e\) increases. If the binding of CaM and PKC are not mutually antagonistic it is still possible that CaM could have an independent effect on the activity of mGluR1\(\alpha\) such that a graded increase in CaM binding to mGluR1\(\alpha\) results in a graded increase in agonist-stimulated \([\text{H}]\text{-InsP}_1\) accumulation as \([\text{Ca}^{2+}]_e\) increases. Thus, the influence of PKC and CaM on \([\text{H}]\text{-InsP}_1\) accumulation following agonist-stimulation of mGluR1\(\alpha\) in the presence of different \([\text{Ca}^{2+}]_e\) have been investigated.

If the degree of PKC phosphorylation of mGluR1\(\alpha\), due to binding of different amounts of CaM to mGluR1\(\alpha\) is responsible for the increase in \([\text{H}]\text{-InsP}_1\) accumulation as \([\text{Ca}^{2+}]_e\) increases, then the pattern of agonist-stimulated \([\text{H}]\text{-InsP}_1\) accumulation in the presence of different \([\text{Ca}^{2+}]_e\) would be expected to change with the activation or inhibition of PKC prior to agonist activation. For example, if PKC was activated before agonist-stimulation of mGluR1\(\alpha\) an abolition of, or decrease in \([\text{H}]\text{-InsP}_1\) accumulation may be indicative of an involvement of PKC and CaM in the
modulation of mGluR1α by Ca²⁺, the prior activation of PKC preventing binding of CaM to mGluR1α, this would be equivalent to the [³H]-InsP₁ accumulation in the absence of Ca²⁺ where CaM cannot bind to the receptor. If PKC activation is inhibited prior to agonist stimulation and thus mGluR1α is not phosphorylated, this would be equivalent to conditions where the binding of CaM is preventing PKC phosphorylation of mGluR1α, and thus modulation of the response, so it may be expected that [³H]-InsP₁ accumulation would be equivalent to that seen at higher [Ca²⁺], [³H]-InsP₁ accumulation no longer being graded with increasing [Ca²⁺]. If PKC and/or CaM are shown to influence mGluR1α the involvement of the carboxy-terminal binding sites of these compounds could be tested by repeating the experiments using mGluR1β which has a shorter carboxy-terminal tail lacking these sites.

The influence of PKC on the modulation of [³H]-InsP₁ accumulation by [Ca²⁺] in CHO-lac-hmGluR1α cells was examined with the use of PKC inhibitors and a PKC activator. As a control to verify the activities of PdBu and Ro-31-8220, CHO-m3 cells were treated with PdBu and Ro-31-8220 prior to activation of the M₃-muscarinic receptor and measurement of [³H]-InsP₁ accumulation. Treatment of CHO-m3 cells with PdBu resulted in a slight but not significant decrease in [³H]-InsP₁ accumulation compared to control, and treatment with Ro-31-8220 resulted in a slight increase in [³H]-InsP₁ accumulation compared to control at sub-maximal concentrations of methacholine. In common with the CHO-m3 cells, treatment of CHO-lac-hmGluR1α cells with PdBu resulted in a slight decrease in [³H]-InsP₁ accumulation compared to control, however treatment with Ro-31-8220 had no effect on [³H]-InsP₁ accumulation.
The $[\text{Ca}^{2+}]_e$ dependency of $[^3\text{H}]$-InsP$_1$ accumulation following challenge with quisqualate under control conditions and following treatment with a PKC activator, PdBu, showed that although PdBu treatment resulted in a decrease in $[^3\text{H}]$-InsP$_1$ accumulation in the presence of 0.5 mM $\text{Ca}^{2+}$, $[^3\text{H}]$-InsP$_1$ accumulation still increased in a graded manner as the $[\text{Ca}^{2+}]_e$ was increased from nominally $\text{Ca}^{2+}$-free to 1.3 mM. No significant effects on $[^3\text{H}]$-InsP$_1$ accumulation were seen following treatment with any of the PKC inhibitors, $[^3\text{H}]$-InsP$_1$ accumulation still increasing in a graded manner as the $[\text{Ca}^{2+}]_e$ was increased from nominally $\text{Ca}^{2+}$-free to 1.3 mM. Thus, treatment of CHO-lac-hmGluR1α cells with a PKC activator and several PKC inhibitors has no effect on the modulation of $[^3\text{H}]$-InsP$_1$ accumulation by $[\text{Ca}^{2+}]_e$. Therefore, these data suggest that phosphorylation of mGluR1α by PKC is not involved in the modulation of signalling via mGluR1α by $[\text{Ca}^{2+}]_e$.

However it should be pointed out that the effects of treatment with PKC activators and inhibitors were very small and these data should not be taken as conclusive evidence that PKC does not modulate signalling via mGluR1α. The conditions of experimentation, e.g. time of treatment, concentration of drugs, may not have been optimum for PKC activation and inhibition.

As the data regarding regulation of mGluR1α by PKC are inconclusive the hypothesis of the involvement of CaM in the modulation of agonist-stimulated $[^3\text{H}]$-InsP$_1$ accumulation by $[\text{Ca}^{2+}]_e$ was tested, before further work was carried out to determine the best way of measuring the influence of PKC on mGluR1α. CHO-lac-
hmGluR1α cells were treated with CaM antagonists to test the above hypothesis that CaM binding to mGluR1α may increase as [Ca²⁺]ᵢ increases, resulting in an increase in [³H]-InsP₁ accumulation. Thus, a change in the pattern of agonist-stimulated [³H]-InsP₁ accumulation in the presence of different [Ca²⁺]ᵢ following treatment with a CaM antagonist may be indicative of an involvement of CaM in the Ca²⁺-sensitivity of agonist-stimulated phosphoinositide signalling via mGluR1α. For example, no [³H]-InsP₁ accumulation may be seen at any [Ca²⁺]ᵢ, as antagonism of CaM binding is equivalent to the abolition of CaM binding in the absence of Ca²⁺ᵢ.

At all concentrations of W-7 and W-13 below 100 µM and 300 µM, respectively, the modulatory effect of increasing [Ca²⁺]ᵢ on [³H]-InsP₁ accumulation was still evident, although the response declined at all [Ca²⁺]ᵢ as the concentration of the CaM antagonists was increased. To test whether the decrease in [³H]-InsP₁ accumulation as the concentration of W-7 was increased was due to the antagonism of CaM by W-7, rather than simply a non-specific toxic effect, CHO-lac-hmGluR1α cells were treated with the same concentrations of a non-active analogue of W-7: W-5, and [³H]-InsP₁ accumulation following challenge with 30 µM quisqualate measured. No decrease in the [³H]-InsP₁ accumulation was seen compared to control for W-5 concentrations up to 100 µM, thus the results described above can be attributed to the effect of W-7 on CaM binding. These data suggest that CaM binding to mGluR1α may be important for agonist-stimulated signalling via the receptor, resulting in a decrease in [³H]-InsP₁ accumulation following treatment with W-7 and W-13. However, the graded increase in [³H]-InsP₁ accumulation is observed at all but the highest concentrations of W-7 and W-13 used as [Ca²⁺]ᵢ is increased. It therefore appears that the modulatory effect of Ca²⁺ᵢ on agonist-stimulated [³H]-InsP₁
accumulation is not mediated by Ca^{2+}-sensitive binding of CaM to mGluR1α. As CaM serves a number of functions in the cell the importance of CaM binding to mGluR1α for agonist-stimulated signalling via the receptor needs to be investigated further, (e.g. by the use of an antibody raised against, or the introduction of mutations within, the CaM binding sites of mGluR1α).

The characteristics of [Ca^{2+}]_i elevation were also investigated with the aim of determining the site at which Ca^{2+} modulates agonist-stimulated signalling via mGluR1α. [Ca^{2+}]_i elevation in CHO-lac-hmGluR1α cells in response to a maximal concentration of agonist follows a biphasic pattern, with a peak of [Ca^{2+}]_i elevation followed by a sustained phase of [Ca^{2+}]_i elevation (Hermans et al, 1998b). The influence of [Ca^{2+}]_o on these peak and sustained phases of [Ca^{2+}]_i elevation has been investigated. As a number of factors could affect the reproducibility of the peak [Ca^{2+}]_i elevation, for example; desensitisation of mGluR1α following agonist challenge, insufficient time for intracellular Ca^{2+} stores to have refilled, or IP_{3}R desensitisation or inactivation, it was necessary to establish that peak [Ca^{2+}]_i elevation was reproducible following repeated stimulation of mGluR1α with quisqualate. Having established the peak height of [Ca^{2+}]_i elevation was reproducible using a protocol of repeated challenge with 30 μM quisqualate for 2 min, with a 10 min interval between successive stimulations, it was necessary to repeat this observation upon challenge with a sub-maximal dose of quisqualate and therefore to produce a concentration-effect curve for [Ca^{2+}]_i elevation following challenge with quisqualate in single CHO-lac-hmGluR1α cells. These experiments were necessary for several reasons; the EC_{50} for quisqualate-stimulated peak [Ca^{2+}]_i elevation lies to the left of that for Ins \((1,4,5)P_3\) mass production, the relative EC_{50}
values are; 0.1 μM, and 1 μM (Hermans et al, 1998b). Therefore, only a small amount of Ins(1,4,5)P₃ production is required to produce maximal [Ca²⁺]ᵢ elevation following challenge of CHO-lac-hmGluR1α cells with quisqualate. Thus, it is possible that a supra-maximal concentration of quisqualate could result in a reproducible peak of [Ca²⁺]ᵢ elevation under conditions where a sub-maximal concentration of quisqualate would not; for example if some mGlu1α receptors remain desensitised from the previous challenge with quisqualate, there may be enough functional receptors to cause maximal and reproducible release from stores when stimulated with a supra-maximal concentration of quisqualate, but not a sub-maximal concentration of quisqualate. Also, if the intracellular Ca²⁺ stores have not refilled properly, but the previous stimulation did not completely empty the stores, a supra-maximal concentration of quisqualate may be able to release the remaining Ca²⁺ whereas a sub-maximal concentration may not. If this is the case then the concentration-effect curves for quisqualate-stimulated [Ca²⁺]ᵢ elevation could be misleading, thus, this experiment will also confirm the validity of this approach. However, the peak height of [Ca²⁺]ᵢ elevation is reproducible upon repeated stimulation of mGluR1α with a sub-maximal (0.7 μM) concentration of quisqualate, which also confirms that quisqualate evokes a dose-dependent increase in the height of peak [Ca²⁺]ᵢ elevation.

Thus, following challenge with both maximal and sub-maximal concentrations of quisqualate for 2 min, an interval of 10 min in the presence of 1.3 mM Ca²⁺ before successive challenge is sufficient for full recovery from the previous stimulation. These data allowed the investigation of the effect of varying [Ca²⁺]ᵢ on the peak height of [Ca²⁺]ᵢ elevation following challenge of mGluR1α with both maximal and
sub-maximal concentrations of quisqualate, as changes in the peak height of \([Ca^{2+}]_i\) elevation could be attributed to the modulatory effect of \([Ca^{2+}]_o\) on signalling via mGluR1α. Cells were perfused with KHB of the appropriate \([Ca^{2+}]_o\) for 2 min prior to and subsequent to challenge with quisqualate to ensure the correct bath concentration of \(Ca^{2+}\) prior to challenge with quisqualate and that the \([Ca^{2+}]_i\) elevation is allowed to reach its peak in the appropriate \([Ca^{2+}]_o\). Following stimulation of the cells with either a maximal or sub-maximal concentration of quisqualate in the presence of different \([Ca^{2+}]_o\) the height of peak \([Ca^{2+}]_i\) elevation was not graded in proportion to \([Ca^{2+}]_o\). Had the heights of peak \([Ca^{2+}]_i\) elevation increased in response to challenge of mGluR1α with quisqualate as \([Ca^{2+}]_o\) increased then I could have investigated the dependence of this variation on \(Ca^{2+}\) entry into the cell by using \(Ca^{2+}\) channel blockers. If the \(Ca^{2+}\) channel blockers had resulted in the abolition of a difference in the heights of peak \([Ca^{2+}]_i\) elevation in the presence of different \([Ca^{2+}]_o\) then this would be indicative of \(Ca^{2+}\) entering the cell to bring about its modulatory effect on agonist-stimulated signalling via mGluR1α, if not it would have indicated that \(Ca^{2+}\) bound to the receptor itself to have its modulatory effects.

The dependence of the plateau phase of \([Ca^{2+}]_i\) elevation on \([Ca^{2+}]_o\) was then investigated. The height of the plateau phase was shown to increase in a graded manner as the \([Ca^{2+}]_o\) increased, following challenge of mGluR1α with both maximal and sub-maximal concentrations of quisqualate. This difference in the plateau phase of \([Ca^{2+}]_i\) elevation could be selective to signalling via mGluR1α or a general phenomenon of the CHO cell type, resulting from \([Ca^{2+}]_o\) affecting the degree of \(Ca^{2+}\) entry into the cell following agonist-stimulation of mGluR1α. Such \(Ca^{2+}\) entry has
been shown to be necessary for the sustained phase of \([\text{Ca}^{2+}]_j\) elevation observed in SH-SY5Y cells (Lambert and Nahorski, 1990). It is possible that this variation in the plateau phase with \([\text{Ca}^{2+}]_e\) could affect the activity of \(\text{Ca}^{2+}\)-dependent PLCs in a graded manner. This may explain the effect of \([\text{Ca}^{2+}]_e\) on agonist-stimulated \(^{3}\text{H}\)\-InsP\(_1\) accumulation in CHO-lac-hmGluR1\(\alpha\) cells, as a \(\text{Ca}^{2+}\)-mediated activation of PLC could feed-forward to affect the degree of PIP\(_2\) hydrolysis in a graded manner and thus \(^{3}\text{H}\)\-InsP\(_1\) accumulation in the presence of different \([\text{Ca}^{2+}]_e\). Thus, the above series of experiments were repeated using single CHO-m3 cells to investigate the selectivity of the effect of varying \([\text{Ca}^{2+}]_e\) on the height of the plateau phase of \([\text{Ca}^{2+}]_j\) elevation in CHO-lac-hmGluR1\(\alpha\) cells.

As for the CHO-lac-hmGluR1\(\alpha\) cells, the CHO-m3 cells could be repeatedly challenged with both a sub-maximal and maximal concentration of methacholine with a reproducible height of peak \([\text{Ca}^{2+}]_j\) elevation. In common with CHO-lac-hmGluR1\(\alpha\) cells the height of peak \([\text{Ca}^{2+}]_j\) elevation did not increase in a graded manner as \([\text{Ca}^{2+}]_e\) was increased in CHO-m3 cells. However, as for CHO-lac-hmGluR1\(\alpha\) cells the plateau phase of \([\text{Ca}^{2+}]_j\) elevation did increase in a graded manner as the \([\text{Ca}^{2+}]_e\) was increased. As this is a common phenomenon in both CHO-lac-hmGluR1\(\alpha\) cells and CHO-m3 cells, these results suggest that the graded increase in the plateau phase is unlikely to be responsible for the modulatory effect of \(\text{Ca}^{2+}\) on \(^{3}\text{H}\)\-InsP\(_1\) accumulation in CHO-lac-hmGluR1\(\alpha\) cells via feed-forward onto \(\text{Ca}^{2+}\)-sensitive PLCs. However, it is possible that differential localisation of mGluR1\(\alpha\) and the M\(_3\)-muscarinic receptor in the cell may allow mGluR1\(\alpha\), but not the M\(_3\)-muscarinic receptor, to access \(\text{Ca}^{2+}\)-sensitive PLC isoforms (PLC\(_5\)) resulting in a graded \(^{3}\text{H}\)\-InsP\(_1\) accumulation. It seems unlikely that such highly structured organisation

140
occurs as the necessary scaffold proteins may not be present in CHO cells. This possibility could be examined by measuring translocation of specific PLC isoforms, e.g. PLCδ, as a measure of PLC activation following agonist-stimulation of GPCRs. It is likely that either another Ca²⁺-sensitive intracellular element is involved or the modulatory effect of [Ca²⁺]₀ on [³H]-InsP₁ accumulation is indeed due to Ca²⁺ binding to mGluR1α, as supported by the observations that serine 166 of the amino acid sequence of rat mGluR1α is essential for the Ca²⁺ sensitivity of the receptor expressed in Xenopus oocytes (Kubo et al, 1998).

To summarise, these studies have demonstrated that varying [Ca²⁺]₀ from nominally Ca²⁺-free to 1.3 mM results in an increase in agonist-stimulated [³H]-InsP₁ accumulation via mGluR1α, as [Ca²⁺]₀ is increased in CHO-lac-hmGluR1α cells. In contrast, increasing [Ca²⁺]₀ above 0.5 mM has no effect on agonist-stimulated [³H]-InsP₁ accumulation via the M₃-muscarinic receptor in CHO-m3 cells. Therefore, it appears that the modulatory effect of [Ca²⁺]₀ on phosphoinositide-signalling in CHO-lac-hmGluR1α cells is selective to signalling via mGluR1α. Studies of the influence of varying [Ca²⁺]₀ on membrane phospholipid levels and manipulations of the levels of PKC and CaM in the cells provide evidence against their involvement in the modulation of phosphoinositide signalling via mGluR1α by [Ca²⁺]₀. Studies of [Ca²⁺]₀ elevation have been unsuccessful in determining the site of action of Ca²⁺, although they do provide evidence against [Ca²⁺]₀ modulating agonist-stimulated [³H]-InsP₁ accumulation via a graded activation of Ca²⁺ sensitive PLC.
Although these studies do not precisely define the site of action of Ca\textsuperscript{2+}, studies have shown that the serine 166 residue of the amino acid sequence of rat mGluR1\textalpha{} is essential for the Ca\textsuperscript{2+} to behave as an agonist of mGluR1\textalpha{} upon expression of mGluR1\textalpha{} in *Xenopus* oocytes (Kubo et al, 1998). The importance of this residue for the modulation by Ca\textsuperscript{2+} of agonist-stimulated phosphoinositide signalling via mGluR1\textalpha{} expressed in a mammalian model system is the theme of the next Chapter.
The Ca²⁺-sensitivity of mGluR1α has been demonstrated in a number of model systems. Initially Ca²⁺ₐ was shown to act as an agonist of rat mGluR1α per se, upon expression of mGluR1α in Xenopus oocytes (Kubokawa et al, 1996). The initial observations in Chapter 3 addressed the question of the ability of Ca²⁺ to modulate signalling via rat mGluR1α expressed in a mammalian baby hamster kidney cell-line, and demonstrated a modulation of agonist-stimulated phosphoinositide signalling via mGluR1α by varying [Ca²⁺]ₐ in the mM range (Saunders et al, 1998). The studies in Chapter 4 also provided support for a role for Ca²⁺ₐ as a modulator of agonist-stimulated signalling via mGluR1α. Varying [Ca²⁺]ₐ in a physiological range resulted in the modulation of agonist-stimulated phosphoinositide signalling via human mGluR1α inducibly expressed in a Chinese hamster ovary cell-line. This Ca²⁺ sensitivity of mGluR1α could be due to Ca²⁺ binding to a Ca²⁺-binding site located in the extracellular domain of mGluR1α, or conceivably to an intracellular element of the signal transduction pathway downstream of mGluR1α provided a suitable pathway of Ca²⁺ entry were present. Although the site at which [Ca²⁺]ₐ influences signalling via mGluR1α has been investigated in both Chapters 3 and 4, such investigations have not produced conclusive evidence to suggest the precise site of modulation of agonist-stimulated phosphoinositide signalling via mGluR1α by Ca²⁺.

However, studies of the Ca²⁺-sensitivity of mGluR1α expressed in Xenopus oocytes have demonstrated that the serine 166 residue of the amino acid sequence of rat mGluR1α is critical for the Ca²⁺ₐ sensitivity of mGluR1α, providing evidence that
Ca\(^{2+}\)_e interacts directly with an extracellular Ca\(^{2+}\)-binding site in mGluR1\(\alpha\) to have its stimulatory effect (Kubo et al., 1998). The importance of this residue for the Ca\(^{2+}\)-sensitivity of rat mGluR1\(\alpha\) expressed in a mammalian model system is addressed in this Chapter.

### 5.1 The pmGR1 plasmid.

The cDNAs for mGluR1\(\alpha\) and S\(^{166D}\)-mGluR1\(\alpha\), in the pmGR1 plasmid (Masu et al., 1991, adapted to contain an Eco RV site for construction of chimera and mutant cDNAs) were kindly provided by Prof. Y. Kubo (see Kubo et al., 1998). To test the viability of the provided plasmids both pmGR1 and pmGR1-S166D were transformed into E. Coli, purified using large scale plasmid purification techniques and restriction endonuclease digestion with Eco RV and/or Eco RI performed. For both plasmids Eco RI digestion produced 2 DNA fragments, of 720 bp and 6880 bp, and Eco RI and Eco RV digestion produced 3 DNA fragments of 720 bp, 1410 bp and 5470 bp. Thus, the expected fragment sizes were produced and the DNA was considered suitable for future cloning. Figure 5.1 shows a map of the pmGR1 plasmid with the restriction endonuclease sites denoted.

### 5.2 Isolation of the cDNA for mGluR1\(\alpha\) and S\(^{166D}\)-mGluR1\(\alpha\) from pmGR1 and pmGR1-S166D.

To enable comparison of the effects of varying [Ca\(^{2+}\)]\(_e\) on signalling via wild-type mGluR1\(\alpha\) and S\(^{166D}\)-mGluR1\(\alpha\) expressed in a mammalian system it was necessary to isolate the cDNAs for mGluR1\(\alpha\) and S\(^{166D}\)-mGluR1\(\alpha\) from the pmGR1 plasmid by restriction endonuclease digestion and insert them into a suitable plasmid for transfection into a mammalian cell-line. The G418 resistance-conferring plasmid; pcDNA 3 was selected as a suitable plasmid for the insertion of the cDNAs for mGluR1\(\alpha\) and S\(^{166D}\)-mGluR1\(\alpha\). The insertion of the cDNAs into a plasmid such as
Fig. 5.1 Map of the 7.6 kb pmGRI plasmid. The map shows the relative location of the restriction endonuclease sites. The DNA between the Eco RI site at 0 bp and the Not I site at 4280 bp, corresponds to the DNA, including the coding sequence of mGluR1α, inserted into the bluescript II KS+ plasmid (Masu et al., 1991). The map also shows the Eco RV restriction endonuclease site engineered into the mGluR1α sequence to allow construction of chimera, and the site of the S166D mutation (Kubo et al., 1998). All numbers represent base pairs (bp). ATG and TAG represent the start and stop codons, respectively, for mGluR1α and S166D-mGluR1α.
pcDNA 3 is necessary as pmGR1 is a plasmid used to produce RNA in bacteria, which can then be isolated and injected into *Xenopus* oocytes. This type of plasmid construct does not allow expression in a mammalian cell-line, because the promoter and enhancer are not suitable, i.e. they are encoded by a plasmid for bacteria, whereas pcDNA 3 encodes a promoter and enhancer for mammalian cell expression. Digestion of pmGR1 and pmGR1-S166D at the *Sac II*, *Not I* and *Sca I* restriction endonuclease sites was recommended for isolation of the 3950 bp fragment containing the cDNA for mGluR1α and ^S166D^-mGluR1α. However, restriction endonuclease digestion at the amino-terminal *Sac II* restriction endonuclease site, with *Sac II* was unsuccessful. Examination of the sequence of pmGR1 and pmGR1-S166D revealed that there are no other restriction endonuclease sites located outside the coding sequence for the mGluR1α and ^S166D^-mGluR1α which correspond to the restriction endonuclease sites found in pcDNA 3. A restriction endonuclease site for *Bam HI* is found in pcDNA 3 but not in pmGR1 and pmGR1-S166D. A section of DNA was therefore engineered to include a *Bam HI* restriction endonuclease site followed by the sequence for the amino-terminal region of mGluR1α and ^S166D^-mGluR1α up to the *Eco RI* restriction endonuclease site, to enable insertion of the full coding sequence of mGluR1α and ^S166D^-mGluR1α into pcDNA 3.

5.3 Engineering of DNA with a *Bam HI* restriction endonuclease site followed by the sequence for the amino-terminal region of mGluR1α and ^S166D^-mGluR1α.

Following construction of suitable primers, the section of DNA containing a *Bam HI* site followed by the sequence for the amino-terminal region of mGluR1α was produced using PCR (see figure 5.2 A). Restriction endonuclease digestion with *Bam HI* and *Eco RI* and gel purification techniques were employed to isolate the required 420 bp fragment (see figure 5.2 B). In order to make a large scale preparation of this section of DNA, it was necessary to insert this amino-terminal fragment into the pSK+ plasmid. The pSK+ is a multiple cloning site, multiple copy
plasmid, allowing production of more DNA than the pcDNA 3 plasmid. The pSK+ plasmid is digested with the same restriction endonucleases. The engineered DNA containing the Bam HI restriction endonuclease site followed by the amino-terminal sequence of mGluR1α up to the Eco RI restriction endonuclease site was then ligated into the pSK+ plasmid, which contains complementary sites for ligation due to digestion with the same restriction endonucleases (see figure 5.2 C). The DNA was then transformed into E. Coli, 18 potential recombinant colonies were picked and PCR used to test whether the amino-terminal fragment of mGluR1α, containing the Bam HI site had undergone successful ligation into the pSK+ plasmid. Of the 18 colonies picked 17 screened positive for successful ligation, and a single colony, no 11, was chosen for future use and a large scale plasmid purification performed.

Restriction endonuclease digestion was carried out using Eco RI and Bam HI or Kpn I. The fragments obtained were of the predicted sizes, for Eco RI and Bam HI, 3000 bp and 500 bp, for Kpn 1, 3400 and 180 bp, as shown in figure 5.3. This confirms insertion of the engineered DNA with Bam HI restriction endonuclease site and the sequence for the amino-terminal region of mGluR1α into the pSK+ plasmid. This Bam HI / amino-terminal fragment could now be isolated from the pSK+ plasmid and used to reconstruct the full length DNA for both mGluR1α and \( S^{166D} \)-mGluR1α.

5.4 Isolation of the 3560 bp region of both mGluR1α and \( S^{166D} \)-mGluR1α from pmGR1 and pmGR1-S166D between the Eco RI and Not I restriction endonuclease sites.

It was then necessary to prepare the rest of the mGluR1α and \( S^{166D} \)-mGluR1α sequences for addition of the engineered amino-terminal fragment. This required isolation of the mGluR1α and \( S^{166D} \)-mGluR1α sequences between the Eco RI and Not I restriction endonuclease sites from the pmGR1 and pmGR1-S166D plasmids and ligation into pcDNA 3. Restriction endonuclease sites are found for Eco RI at 720 bp and Not I at 4280 bp within the section of DNA containing the mGluR1α and \( S^{166D} \)-mGluR1α sequences inserted into bluescript II KS+ (see figure 5.4 A). The pmGR1
Fig. 5.2  A: Map showing the mGluR1α insert DNA. P1 and P2 are the primers used to re-engineer part of the insert to include a Bam HI site and the N-terminal sequence of mGluR1α up to the Eco RI site. B: The DNA product of PCR amplification using the P1 and P2 primers, subsequent to restriction endonuclease digestion with Bam HI and Eco RI. C: Insertion of the Bam HI/N-terminal fragment obtained in B into the pSK+ plasmid, following restriction endonuclease digestion of the pSK+ plasmid with Bam HI and Eco RI. ATG represents the start codon for mGluR1α.
Fig. 5.3 Test digest of the clone #11 construct of the Bam HI/N-terminal ligation into the pSK+ plasmid. A: Restriction endonuclease digestion with Eco RI and Bam HI. Fragments of 3000 bp and 500 bp are obtained as expected. B: Restriction endonuclease digestion with Kpn I. Fragments of 3400 bp and 180 bp are obtained as expected.
and pmGR1-S166D plasmids containing the coding sequence for mGluR1α and S166D-mGluR1α were cut with Eco RI /Not I, and in addition Pvu I (to digest the bluescript KSII+ fragment, making isolation of the required 3560 bp fragments easier) to isolate the fragment of 3560 bp prior to insertion into pcDNA 3 (see figure 5.4 B). pcDNA 3 was also cut with Eco RI and Not I to give a linear plasmid of 5400 bp with compatible sites for ligation of the 3560 bp fragment of mGluR1α and S166D-mGluR1α. As restriction endonuclease digestion resulted in fragments of the appropriate sizes, the DNA was then isolated by gel purification. The 3560 bp fragments of mGluR1α and S166D-mGluR1α were then ligated with the 5400 bp fragment of pcDNA 3 (see figure 5.4 C). The DNA was then transformed into E. Coli and 18 potential recombinant colonies picked and screened using PCR. Of the recombinant colonies obtained no 4 (mGluR1α) and no 15 (S166D-mGluR1α) were chosen for future use and large scale plasmid purification of each DNA performed. A series of different restriction endonuclease digestions were then performed to test that fragments of the expected sizes could be obtained. Each construct was digested with Eco RI and Xba, Hind III, and Eco RI and Eco RV and expected fragment sizes of 5400 and 3500 bp, and 660 bp and 120 bp, and 1410 and 7490 bp, respectively, were obtained in all cases, as shown in figure 5.5. So the 3560 bp fragments of mGluR1α and S166D-mGluR1α between 720 to 4280 bp had been successfully ligated into pcDNA 3.

5.5 Ligation of the amino-terminal fragment of mGluR1α into pcDNA 3 containing the 3560 bp fragments of mGluR1α or S166D-mGluR1α.

Both the pcDNA 3 plasmid containing the 3560 bp region of either mGluR1α or S166D-mGluR1α sequence, and the pSK+ plasmid containing the Bam HI amino-terminal region of mGluR1α, were digested with Bam HI and Eco RI (see figure 5.6 A and B). The fragment sizes obtained were as expected, 420 bp for the amino-terminal region and 8900 bp for both mGluR1α and S166D-mGluR1α in the pcDNA 3 plasmid. The fragments were then gel-purified. Ligation of the amino-terminal region of mGluR1α
Fig. 5.4 A: Map showing the mGluR1α insert DNA, and the site at which the S166D mutation is located. B: The DNA fragment produced from restriction endonuclease digestion of pmGR1 and pmGR1-S166D with Eco RI and Not I, to obtain the 3560 fragments of both mGluR1α and S166D-mGluR1α for independent ligation into pcDNA 3. C: Insertion of the 3560 fragment (with and without the S166D mutation) obtained in B into the pcDNA 3 plasmid, following restriction endonuclease digestion of the pcDNA 3 plasmid with Eco RI and Not I. All numbers represent base pairs (bp). ATG and TAG represent the start and stop codons, respectively, for mGluR1α and S166D-mGluR1α.
Fig. 5.5 Test digests of clone #4 (pcDNA 3 mGluR1α) and 15 (pcDNA 3 S166D-mGluR1α) constructs of the ligation of the 3560 bp regions of mGluR1α and S166D-mGluR1α into the pcDNA 3 plasmid. A and C: Restriction endonuclease digestion of clone #4 and clone #15, respectively, with Eco RI and Xba I. In both cases fragments of 5400 bp and 3500 bp were obtained as expected. B and D: Restriction endonuclease digestion of clone #4 and clone #15, respectively with Hind III. In both cases fragments of 660 bp and 120 bp are obtained as expected. E and F: Restriction endonuclease digestion of clone #4 and clone #15, respectively, with Eco RI and Eco RV. In both cases fragments of 1410 bp and 7490 bp were obtained as expected.
Fig. 5.6  A: Bam HI/N-terminal fragment obtained following restriction endonuclease digestion of the Bam HI / N-terminal pSK+ plasmid with Bam HI and Eco RI. B: pcDNA 3 mGluR1α and pcDNA 3 $^{S166D}$-mGluR1α fragments obtained following restriction endonuclease digestion with Bam HI and Eco RI. C: Ligation of the fragments shown in A and B to create a plasmids containing the full length coding sequences for either mGluR1α or $^{S166D}$-mGluR1α between the Bam HI and Not I restriction endonuclease sites. ATG and TAG represent the start and stop codons, respectively, for mGluR1α and $^{S166D}$-mGluR1α.
with the 3560 bp fragment of both mGluR1α and S166D-mGluR1α in pcDNA 3 was then performed (see figure 5.6 C). The DNA was then transformed into E. Coli, and 18 potential recombinant colonies picked and screened using PCR. Of the positive recombinant colonies no 4 (mGluR1α) and no 14 (S166D-mGluR1α) were chosen for further use. The potentially full-length DNA for each of mGluR1α and S166D-mGluR1α in the pcDNA 3 plasmid was then subjected to digestion with a number of restriction endonucleases, Bam HI and Not I, Bam HI and Eco RI, and Eco RI and Xho I, and fragments of 3980 bp and 5400 bp, 520 bp and 8860 bp and 3470 bp and 6210 bp obtained as expected, as shown in figure 5.7. This confirmed that full length cDNA sequences for both mGluR1α and S166D-mGluR1α have been successfully cloned into pcDNA 3.

5.6 Transient transfection of the pcDNA 3 mGluR1α and pcDNA 3 S166D-mGluR1α into COS 7 cells.

Expression of the mGluR1α and S166D-mGluR1α proteins from the new constructs was tested by transient transfection into COS 7 cells. Expression of the receptors was detected using an anti-mGluR1α antibody, by Western blot immunodetection of total protein extracts. Wild-type mGluR1α was successfully expressed, however the S166D-mGluR1α mutant was not expressed as shown in figure 5.8. Sequencing (performed by P.N.A.C.L.) of the wild-type mGluR1α and mutant S166D-mGluR1α plasmids showed that both receptors have the correct sequence.

5.7 Introduction of the S166D mutation into pcDNA 3 containing the cDNA for mGluR1α.

A second approach was taken to produce a vector containing the cDNA for S166D-mGluR1α, which could be successfully expressed in a mammalian cell-line.
Fig. 5.7 Test digests of clone #4 (pcDNA 3 mGluR1α) and 14 (pcDNA 3 S166D-mGluR1α) constructs of the ligation of the pcDNA 3 mGluR1α and pcDNA 3 S166D-mGluR1α with the Bam HI / N-terminal fragment. A: Restriction endonuclease digestion of clone #4 with Bam HI and Not I, fragments of 3980 bp and 5400 bp were obtained as expected. Lane D was misloaded. B and E: Restriction endonuclease digestion of clone #4 and clone #14, respectively with Bam HI and Eco RI. In both cases fragments of 520 bp and 8860 bp are obtained as expected. C and F: Restriction endonuclease digestion of clone #4 and clone #14, respectively, with Bam HI and Xba I. In both cases fragments of 3470 bp and 6210 bp were obtained as expected.
Fig. 5.8 Transient transfection of pcDNA 3 mGluR1α and pcDNA 3 $^{S166D}$ mGluR1α into COS 7 cells. Expression of the receptors was detected using an anti-mGluR1α antibody against the C-terminal of mGluR1α. A: No DNA, B: pcDNA 3, C: pcDNA 3 mGluR1α and D: pcDNA 3 $^{S166D}$-mGluR1α. mGluR1α expression is detected as a band of poor resolution due to overloading of a highly expressed protein. No corresponding band is seen for $^{S166D}$-mGluR1α expression.
Having established the viability of the pcDNA 3 mGluR1α plasmid, this plasmid could be used to produce a pcDNA 3 plasmid containing the cDNA for S166D-mGluR1α. The original mutant construct pmGR1-S166D was digested with Eco RI and Eco RV to obtain the 1410 bp fragment (containing the S166D mutation) required, and isolated using gel purification (see figure 5.9 A). The fragment was then ligated with the Eco RI / Eco RV digested 7970 bp section from pcDNA 3 mGluR1α (see figure 5.9 B and C). This DNA was then transformed into E. Coli, 20 colonies picked and screened using PCR. Introduction of the S166D mutation into the mGluR1α sequence and thus the pcDNA 3 plasmid was tested by sequencing 10 of the potential recombinant colonies. This sequencing was unsuccessful, so the pcDNA 3 mGluR1α plasmid and one of potential pcDNA 3 S166D-mGluR1α plasmids were purified (using the large scale Qiagen Plasmid Maxi-Kit 500) to increase the purity of the DNA. The sequencing of the Qiagen prepared constructs confirmed that the pcDNA 3 mGluR1α plasmid had the correct sequence and that the ligation of the 1410 bp fragment of S166D-mGluR1α with the 7970 fragment of pcDNA 3 mGluR1α had been successful. The plasmids were then transformed into E. Coli to produce glycerol stocks. The pcDNA 3 mGluR1α and pcDNA 3 S166D-mGluR1α plasmids were then subjected to digestion with Bam HI and Not I, and Eco RI and Eco RV and fragments of 3980 bp and 5400 bp, and 1410 bp and 7970 bp obtained as expected, confirming that both plasmids contain the expected DNA, as shown in figure 5.10.

5.8 Transient transfection of pcDNA 3 mGluR1α and pcDNA 3 S166D-mGluR1α into HEK cells.

The ability of cells to express both the mGluR1α and S166D-mGluR1α pcDNA 3 proteins was tested by calcium phosphate transfection (Sambrook et al, 1989) into HEK cells. Both plasmids were expressed as demonstrated by Western blot immunodetection of total protein extracts, see figure 5.11.
Fig. 5.9  A: S166D-containing fragment obtained following restriction endonuclease digestion of the pmGR1-S166D plasmid Eco RI and Eco RV. B: pcDNA 3 mGluR1α fragment obtained following restriction endonuclease digestion with Eco RI and Eco RV. C: Ligation of the fragments shown in A and B to create a plasmid containing the full length coding sequence for S166D-mGluR1α. ATG and TAG represent the start and stop codons, respectively, for mGluR1α and ^{S166D}-mGluR1α.
Fig. 5.10 Test digests of pcDNA 3 mGluR1α and pcDNA 3 S166D-mGluR1α plasmid constructs. A and C: Restriction endonuclease digestion of pcDNA 3 mGluR1α and pcDNA 3 S166D-mGluR1α, respectively, with Bam HI and Not I. In both cases fragments of 3980 bp and 5400 bp were obtained as expected. B and D: Restriction endonuclease digestion of pcDNA 3 mGluR1α and pcDNA 3 S166D-mGluR1α, respectively with Eco RI and Eco RV. In both cases fragments of 1410 bp and 7970 bp are obtained as expected.
Fig. 5.11  Transient transfection of pcDNA 3 mGluR1α and pcDNA 3 S166D-mGluR1α into HEK cells. Expression of the receptors was detected using an anti-mGluR1α antibody against the C-terminal of mGluR1α. A: mGluR1α, B: S166D-mGluR1α. In both cases expression of the receptors is detected as a band at approximately 145 kDa.
Following successful introduction of the S166D mutation into mGluR1α cDNA in the pcDNA 3 vector, and successful transient expression of both mGluR1α and S\textsuperscript{166D}-mGluR1α, it was concluded that the cDNAs for both mGluR1α and S\textsuperscript{166D}-mGluR1α were now contained in a plasmid suitable for stable transfection into CHO cells.

5.9 Stable transfection of wild-type mGluR1α and S\textsuperscript{166D}-mGluR1α cDNA into CHO cells.

Using calcium phosphate precipitation stable transfection of mGluR1α and S\textsuperscript{166D}-mGluR1α into CHO cells was attempted with the aim of comparing the effect of varying \([\text{Ca}^{2+}]_e\) on signalling via both the wild-type and mutant receptors.

For both stable transfections, i.e. mGluR1α and S\textsuperscript{166D}-mGluR1α, into CHO cells, 24 clones resistant to G-418 selection were tested for mGluR1α or S\textsuperscript{166D}-mGluR1α expression, by measuring \([^3\text{H}]-\text{InsP}_1\) accumulation following agonist challenge of the receptors and by immunodetection of receptor expression using a carboxy-terminal mGluR1α antibody.

The efficiency of coupling of mGluR1α or S\textsuperscript{166D}-mGluR1α to PLC in CHO cells was studied by measuring \([^3\text{H}]-\text{InsP}_1\) accumulation in the presence of 10 mM LiCl in cells which had been incubated with 1 μCi ml\(^{-1}\) \([^3\text{H}]-\text{inositol}\) for 48 h. For each clone \([^3\text{H}]-\text{InsP}_1\) accumulation was measured under basal conditions and following challenge with 1 μM and 30 μM quisqualate (N.B. the estimated EC\(_{50}\) values of the glutamate-stimulated Ca\(^{2+}\)-dependent Cl\(^-\)-current has been shown to be unaffected by the introduction of the S166D mutation in \textit{Xenopus} oocytes (Kubo \textit{et al}, 1998), thus the same concentrations of quisqualate were used for screening the stable
transfectants). None of the clones tested demonstrated any coupling of mGluR1α or S166D-mGluR1α to PLC.

Following SDS-PAGE, and Western blotting of lysates from each of the clones, immunodetection techniques were employed to determine whether the clones were expressing mGluR1α or S166D-mGluR1α. In both cases, very few clones screened positive for receptor expression (not shown).

As the stable transfections proved unsuccessful another approach was taken to examine the effect of varying [Ca²⁺]o on signalling via both the wild-type mGluR1α and the S166D-mGluR1α mutant receptor.

5.10 Transient transfection of wild-type mGluR1α and S166D-mGluR1α cDNA into CHO cells.

Initially a period of 24 h was used between addition of 300 ng cDNA well⁻¹ to cells, using FuGene-6, and the measurement of receptor expression by immunodetection techniques and the coupling of the receptors to PLC by the measurement of [³H]-InsP₁ accumulations. Neither mGluR1α or S166D-mGluR1α expression was identified using Western blot immunodetection of total protein extracts, in addition no increase in [³H]-InsP₁ accumulation above basal was observed following challenge of mGluR1α or S166D-mGluR1α transiently transfected cells. Thus, 24 h may not be a suitable period of time to enable mGluR1α or S166D-mGluR1α expression.
The interval of 24 h was then extended to 72 h. A small amount of receptor expression was observed by Western blot immunodetection of total protein extracts, as shown in figure 5.12, but no substantial coupling to PLC was observed for either mGluR1α or S166D-mGluR1α. However, a small increase (~2 fold) between basal [3H]-InsP₁ accumulation and [3H]-InsP₁ accumulation following challenge with 30 μM quisqualate was observed for both mGluR1α and S166D-mGluR1α compared to the vector-control transfection (pcDNA 3), see figure 5.13.

Thus, it was considered possible that the application of 300 ng cDNA well⁻¹ to the cells may not be sufficient for receptor expression and coupling to the phosphoinositide signal transduction pathway. 600 ng well⁻¹ and 900 ng well⁻¹ of mGluR1α and S166D-mGluR1α DNA were then applied to cells for 72 h, using FuGene-6, and receptor expression tested as above. Expression of both receptors was observed using Western blot immunodetection of total protein extracts, as shown in figure 5.14. In addition, both receptors were shown to couple to PLC, resulting in a concentration-dependent increase in [³H]-InsP₁ accumulation following challenge with quisqualate, as shown in figure 5.15. Application of 600 ng cDNA well⁻¹ results in a 3.3 fold (mGluR1α) and a 6.3 fold (S166D-mGluR1α) increase in [³H]-InsP₁ accumulation following challenge with 30 μM quisqualate. This [³H]-InsP₁ accumulation increases further with the application of 900 ng cDNA well⁻¹, to a 5.4 fold (mGluR1α) and a 8.0 fold (S166D-mGluR1α) increase in [³H]-InsP₁ accumulation following challenge with 30 μM quisqualate. The EC₅₀ value for the quisqualate-stimulated [³H]-InsP₁ accumulation following application of 900 ng wild-type mGluR1α cDNA well⁻¹ is 0.45 μM which corresponds closely with values reported for mGluR1α expressed in different cell types (i.e. 1.95 μM in BHK cells and 1.6 μM in
Fig. 5.12 Transient transfection of 300 ng well\(^{-1}\) pcDNA 3, pcDNA 3 mGluR1\(\alpha\) and pcDNA 3 \(^{S166D}\)-mGluR1\(\alpha\) into HEK cells, with Fugene-6. Expression of the receptors was detected using an anti-mGluR1\(\alpha\) antibody against the C-terminal of mGluR1\(\alpha\). A: pcDNA 3, B: mGluR1\(\alpha\), C: \(^{S166D}\)-mGluR1\(\alpha\).
Fig. 5.13 Basal and quisqualate-stimulated $[^{3}H]$-InsP$_1$ accumulation in CHO cells transiently transfected with 300 ng well$^{-1}$ vector control (clear bar), wild-type mGluR1α (shaded bar) or $^{S166D}$-mGluR1α mutant (filled bar) cDNA. CHO cells were incubated with 1 μCi ml$^{-1}$ $[^{3}H]$-inositol for 48 h prior to experimentation. Cells were incubated with 3 U ml$^{-1}$ GPT and 5 mM pyruvate for 20 min prior to addition of LiCl to a final concentration of 10 mM for a further 15 min before challenge with the indicated concentrations of quisqualate for 30 min. $[^{3}H]$-InsP$_1$ data are expressed as d.p.m. well$^{-1}$, from a single experiment.
Fig. 5.14 Transient transfection of pcDNA 3, pcDNA 3 mGluR1α and pcDNA 3 S^{166D} mGluR1α into HEK cells, with FuGene-6. Expression of the receptors was detected using an anti-mGluR1α antibody against the C-terminal of mGluR1α. A: pcDNA 3, B: mGluR1α, C: S^{166D} mGluR1α following application of 600 ng DNA well⁻¹. D: pcDNA 3, E: mGluR1α, F: S^{166D} mGluR1α following application of 900 ng DNA well⁻¹. Expression of mGluR1α and S^{166D} mGluR1α is detectable as a band at approximately 145 kDa. No band is seen at 145 kDa in lanes A and D (pcDNA 3) as expected.
CHO cells). The introduction of the S166D-mutation causes a slight increase in the EC\textsubscript{50} value for quisqualate-stimulated [\textsuperscript{3}H]-InsP\textsubscript{1} accumulation, to 1.4 \mu M (see figure 5.15).

5.11 Effect of varying [Ca\textsuperscript{2+}]\textsubscript{e} on quisqualate-stimulated [\textsuperscript{3}H]-InsP\textsubscript{1} accumulation in CHO cells transiently transfected with mGluR1\alpha or S\textsuperscript{166D}-mGluR1\alpha.

900 ng cDNA well\textsuperscript{-1} was applied to cells for 72 h, using FuGene-6, to investigate the effect of varying [Ca\textsuperscript{2+}]\textsubscript{e} on signalling via wild-type mGluR1\alpha compared to S\textsuperscript{166D}-mGluR1\alpha. Figure 5.16 shows the quisqualate concentration-dependence of [\textsuperscript{3}H]-InsP\textsubscript{1} accumulation in the presence of different [Ca\textsuperscript{2+}]\textsubscript{e} in CHO cells transiently transfected with (A) mGluR1\alpha or (B) S\textsuperscript{166D}-mGluR1\alpha. In cells transfected with wild-type mGluR1\alpha there was a noticeable graded decrease in the EC\textsubscript{50} for quisqualate-stimulated [\textsuperscript{3}H]-InsP\textsubscript{1} accumulation as the [Ca\textsuperscript{2+}]\textsubscript{e} is increased (nominally Ca\textsuperscript{2+}-free; 0.9 \mu M, 0.5 mM Ca\textsuperscript{2+}; 0.6 \mu M, 1.3 mM Ca\textsuperscript{2+}; 0.1 \mu M, 4 mM Ca\textsuperscript{2+}; < at 1.3 mM, N.B. the lowest concentration of quisqualate used at 4 mM Ca\textsuperscript{2+} evokes a near maximal response, thus, an accurate EC\textsubscript{50} value cannot be obtained from this curve), this graded decrease was not seen in the cells transfected with S\textsuperscript{166D}-mGluR1\alpha (nominally Ca\textsuperscript{2+}-free; 10.5 \mu M, 0.5 mM Ca\textsuperscript{2+}; 3.6 \mu M, 1.3 mM Ca\textsuperscript{2+}; 5.6 \mu M, 4 mM Ca\textsuperscript{2+}; 3.0 \mu M). Thus, these initial data provide some evidence to support the observation in Xenopus oocytes that the S166 residue of mGluR1\alpha is essential for the Ca\textsuperscript{2+} sensitivity of the receptor.
Fig. 5.15 Concentration-dependency of quisqualate-stimulated $[^{3}\text{H}]-\text{InsP}_1$ accumulation in CHO cells transiently transfected with A: 600 ng well$^{-1}$, B: 900 ng well$^{-1}$ wild-type mGluR1α (■) or $^{\text{S166D}}$mGluR1α mutant (▼) cDNA. CHO cells were incubated with 1 μCi ml$^{-1}$ $[^{3}\text{H}]$-inositol for 48 h prior to experimentation. Cells were incubated with 3 U ml$^{-1}$ GPT and 5 mM pyruvate for 20 min prior to addition of LiCl to a final concentration of 10 mM for a further 15 min before challenge with the indicated concentrations of quisqualate for 30 min. $[^{3}\text{H}]-\text{InsP}_1$ data are expressed as d.p.m. well$^{-1}$, from a single experiment.
Fig. 5.16 Concentration-dependency of quisqualate-stimulated \( [^3\text{H}]\text{-InsP}_1 \) accumulation in CHO cells transiently transfected with 900 ng well\(^{-1}\) wild-type mGluR1\(\alpha\) (A) or \(^{S166D}\)-mGluR1\(\alpha\) mutant (B) cDNA, in the presence of different [Ca\(^{2+}\)]\(_e\) (nominally Ca\(^{2+}\)-free; O, 0.5 mM; ■, 1.3 mM; ▼, 4 mM; ♦). CHO cells were incubated with 1 \( \mu \text{Ci ml}^{-1} \) \( [^3\text{H}]\)-inositol for 48 h prior to experimentation. Cells were incubated with 3 U ml\(^{-1}\) GPT and 5 mM pyruvate for 20 min prior to addition of LiCl to a final concentration of 10 mM for a further 15 min before challenge with the indicated concentrations of quisqualate for 30 min. \( [^3\text{H}]\text{-InsP}_1 \) data are expressed as d.p.m. well\(^{-1}\), from a single experiment.
5.12 Discussion.

Ca\(^{2+}\) has been shown to act as an agonist of rat mGluR1\(\alpha\) per se, upon expression of mGluR1\(\alpha\) in *Xenopus* oocytes (Kubokawa *et al*, 1996), and a modulator of agonist-stimulated phosphoinositide signalling via rat mGluR1\(\alpha\), stably expressed in BHK cells (Saunders *et al*, 1998) and via human mGluR1\(\alpha\) inducibly expressed in CHO cells. Investigations in both Chapters 3 and 4, provide no conclusive evidence as to the precise site of modulation of agonist-stimulated phosphoinositide signalling via mGluR1\(\alpha\) by Ca\(^{2+}\). However, studies of the Ca\(^{2+}\)-sensitivity of mGluR1\(\alpha\) expressed in *Xenopus* oocytes have demonstrated that the serine 166 residue of the amino acid sequence of rat mGluR1\(\alpha\) is critical for the Ca\(^{2+}\) sensitivity of mGluR1\(\alpha\), providing evidence that there is a Ca\(^{2+}\)-binding site located in the extracellular domain of mGluR1\(\alpha\) (Kubo *et al*, 1998). The importance of this residue for the Ca\(^{2+}\)-sensitivity of agonist-stimulated phosphoinositide signalling via rat mGluR1\(\alpha\) expressed in a mammalian model system was addressed in this Chapter.

The viability of the cDNAs for mGluR1\(\alpha\) and \(^{\text{S166D}}\)-mGluR1\(\alpha\), in the pmGR1 plasmid was initially confirmed by restriction endonuclease digestion with *Eco RV* and/or *Eco RI*. The expected fragment sizes were obtained and the DNA was considered suitable for future manipulation. The cDNAs for mGluR1\(\alpha\) and \(^{\text{S166D}}\)-mGluR1\(\alpha\) were then isolated from the pmGR1 plasmid by restriction endonuclease digestion and inserted into a suitable plasmid; pcDNA 3, for transfection into a mammalian cell-line. This involved re-engineering the amino-terminal regions of mGluR1\(\alpha\) and \(^{\text{S166D}}\)-mGluR1\(\alpha\) to contain a *Bam HI* restriction endonuclease site, as digestion with the only suitable restriction endonuclease Sac II was unsuccessful. The engineered amino-terminal fragment of mGluR1\(\alpha\) containing the *Bam HI* site was then ligated with the pSK+ plasmid. The rest of the coding region (720 to 4280 bp) of the
mGluR1α and S166D-mGluR1α sequences were then isolated from the pmGR1 and pmGR1-S166D plasmids and ligated with a 5400 bp fragment of pcDNA 3. Both the pcDNA 3 plasmid containing this 720 to 4280 bp region of either the mGluR1α or S166D-mGluR1α sequence, and the pSK+ plasmid containing the amino-terminal region of mGluR1α, were then digested with Bam HI and Eco RI. Full length sequences for both mGluR1α and S166D-mGluR1α in the pcDNA 3 plasmid were then obtained by ligation of the amino-terminal region of mGluR1α with the 720 to 4280 bp of either mGluR1α of S166D-mGluR1α, respectively.

Upon transient transfection into COS 7 cells, wild-type mGluR1α was successfully expressed, however the S166D-mGluR1α mutant was not. Thus, having established the viability of the pcDNA 3 mGluR1α plasmid, this plasmid could be used to produce a pcDNA 3 plasmid containing the cDNA for S166D-mGluR1α. The mGluR1α and pcDNA 3 S166D-mGluR1α plasmids were digested to yield fragments of 1410 and 7970 bp. The 1410 bp fragment from pcDNA 3 S166D-mGluR1α, supposedly containing the mutation, was ligated with the 7970 bp fragment from pcDNA 3 mGluR1α. However sequencing of the ligated construct showed that it did not contain the S166D mutation.

The original mutant construct pmGR1-S166D was then digested to obtain the 1410 bp fragment (containing the S166D mutation), which was then ligated with the already prepared 7970 bp fragment from pcDNA 3 mGluR1α. Sequencing confirmed that the pcDNA 3 mGluR1α plasmid had the correct sequence and that the ligation of the 1410 bp fragment of S166D-mGluR1α with the 7970 fragment of pcDNA 3 mGluR1α had been successful.

Upon transient transfection into HEK cells both mGluR1α and S166D-mGluR1α plasmids were expressed. Thus, attempts were made to express the cDNAs for both mGluR1α and S166D-mGluR1α by stable transfection into CHO cells.
Of 24 clones resistant to G-418 tested for mGluR1α or S166D-mGluR1α expression, none showed an increase in [3H]-InsP₃ accumulation following agonist challenge of mGluR1α or S166D-mGluR1α, thus successful coupling of mGluR1α or S166D-mGluR1α to PLC did not occur. In addition, very few clones screening positive for receptor expression by immunodetection of mGluR1α and S166D-mGluR1α expression. Thus stable expression of mGluR1α and S166D-mGluR1α had been unsuccessful.

Another approach was taken to examine the effect of varying [Ca²⁺]₀ on signalling via both the wild-type mGluR1α and S166D-mGluR1α mutant receptors. The pcDNA 3 mGluR1α and S166D-mGluR1α plasmids were transiently transfected into CHO cells using FuGene. The calcium phosphate method was not used as incubation of the cells with CaCl₂ prior to an investigation of the effects of varying [Ca²⁺]₀ on receptor-mediated signalling could cause complications. As pcDNA 3 contains viral sequences which allow high levels of protein expression in mammalian cells, a period of 24 h to 72 h between application of DNA and measurement of the product of expression, i.e. mGluR1α, is generally considered suitable. A period of at least 24 h is also considered necessary as it allows recovery of the cells from the transfection process and accumulation of protein in the cell. Following investigation of a number of conditions, the optimum conditions for transient transfection of mGluR1α and S166D-mGluR1α into CHO cells were considered to be 900 ng cDNA well⁻¹ applied to cells for 72 h. Investigation of the effect of varying [Ca²⁺]₀ on signalling via wild-type mGluR1α compared to S166D-mGluR1α, revealed a graded decrease in the EC₅₀ for quisqualate-stimulated [3H]-InsP₃ accumulation as [Ca²⁺]₀ is increased in cells transfected with wild-type mGluR1α, which was not observed in cells transfected with S166D-mGluR1α. Thus, these initial data provide some evidence to support the
observation in *Xenopus* oocytes that the S166 residue of mGluR1α is essential for the Ca^{2+} sensitivity of the receptor.

However, the results obtained from transient transfections are inconsistent, the transfection efficiency varying from day-to-day, thus due to time limitations this work has not been continued further. It is possible that the conditions for transfection are still not optimal. Therefore, any future work carried out on this system should involve a detailed study of the conditions used. It is possible that the concentration of DNA applied to the cells is not saturating, thus, if the transfection efficiency is good then 900 ng cDNA well^{-1} appears to be suitable, whereas if the transfection efficiency is poor then receptor number may be limiting, reflected by poor [^{3}H]-InsP_{1} accumulation following agonist-stimulation of mGluR1α or S^{166D}-mGluR1α. Thus, transient transfection is a relatively crude technique and may not be suitable for such sensitive measurements. Stable transfection of the pcDNA 3 mGluR1α and S^{166D}-mGluR1α plasmids into another cell-line is another possibility for future work.
The research presented in this Thesis has investigated the modulation of mGluR1α signalling by PTx and $[Ca^{2+}]_o$. PTx-treatment is confirmed to enhance agonist-stimulated phosphoinositide hydrolysis in BHK-mGluR1α cells, via an immediate effect on the potency of agonist activation of mGluR1α (specific to mGluR1α), and an effect on the response to a maximal concentration of agonist (which may be a general phenomenon of the BHK cell type), evident at longer time points (Hermans et al, submitted). The level of expression and sub-cellular distribution of mGluR1α, different G-protein subtypes present in the cell and PLC-β-isoform expression could influence the extent to which $G_{9/0}$ protein activation can limit the stimulatory effect of $G_{q/11}$ protein activation on PLC activity and thus the net PLC activity in different neuronal populations. Thus, the ability of mGluR1α to activate both $G_{q/11}$ and $G_{9/0}$ proteins resulting in the dual regulation of PLC activity could have implications for the diversity of patterns of intracellular signalling via $[Ca^{2+}]_i$ elevation and protein kinase activation following mGluR1α activation (Hermans et al, submitted). This could have implications for key roles suggested for mGluR1α in a number of physiological and pathophysiological processes.

The majority of this study has focused on the modulation of signalling via mGluR1α by $Ca^{2+}_o$. It has been demonstrated that $Ca^{2+}_o$ modulates agonist-stimulated phosphoinositide signalling via mGluR1α expressed in both BHK and CHO cells, in a receptor-selective manner. $[Ca^{2+}]_i$ elevation provides another index by which to
measure receptor activation. Peak [Ca\(^{2+}\)]\(_i\), elevation following challenge of mGluR1\(\alpha\) with quisqualate was shown to be unaffected, as [Ca\(^{2+}\)]\(_e\) was increased. This may be explained by the EC\(_{50}\) for quisqualate-stimulated peak [Ca\(^{2+}\)]\(_i\) elevation lying to the left of that for Ins(1,4,5)P\(_3\) mass production, thus a small amount of Ins(1,4,5)P\(_3\) can result in maximal [Ca\(^{2+}\)]\(_i\) elevation via IP\(_3\)R activation. Thus, variations in the levels of Ins(1,4,5)P\(_3\) mass production are unlikely to affect [Ca\(^{2+}\)]\(_e\). A graded increase in the plateau phase of [Ca\(^{2+}\)]\(_i\) elevation was observed as [Ca\(^{2+}\)]\(_e\) was increased, following challenge of mGluR1\(\alpha\) with quisqualate. This graded capacitative Ca\(^{2+}\) entry could affect the activity of the more Ca\(^{2+}\)-sensitive PLC\(\delta\) isoforms in a graded manner and may explain the effect of [Ca\(^{2+}\)]\(_e\) on agonist-stimulated \[^{3}\text{H}\]-InsP\(_1\) accumulation in CHO-lac-hmGluR1\(\alpha\) cells. Increasing activation of PLC could feed-forward to affect the degree of PIP\(_2\) hydrolysis in a graded manner and thus \[^{3}\text{H}\]-InsP\(_1\) accumulation in the presence of different [Ca\(^{2+}\)]\(_e\). However, this appears not to be so, as the height of the plateau phase of [Ca\(^{2+}\)]\(_i\) elevation also increased in a graded manner as the [Ca\(^{2+}\)]\(_e\) was increased in CHO-m3 cells and differential localisation of the receptors allowing access to different isoforms of PLC would appear unlikely. Thus, these data indicate that Ca\(^{2+}\) is either modulating another Ca\(^{2+}\)-sensitive intracellular element or binding directly to mGluR1\(\alpha\). An investigation of the effect of varying [Ca\(^{2+}\)]\(_e\) on Ins(1,4,5)P\(_3\) mass production would have determined whether [Ca\(^{2+}\)]\(_e\) had an immediate effect on phosphoinositide hydrolysis, and thus, would have helped distinguish between these possibilities. However, measurement of Ins(1,4,5)P\(_3\) mass production to investigate the effect of varying [Ca\(^{2+}\)]\(_e\) was not possible as agonist-stimulated increases in Ins(1,4,5)P\(_3\) in these cells were small and variable and thus, there was insufficient dynamic range to allow a detailed quantitative investigation. Further studies to try to elucidate the site of
action of Ca\textsuperscript{2+} show that varying [Ca\textsuperscript{2+}]\textsubscript{e} has no effect on membrane phospholipid levels and manipulations of PKC and CaM levels in the cells suggest these proteins are not involved. However, the data obtained using CaM antagonists suggest CaM may be required for efficient agonist-stimulated phosphoinositol signalling via mGluR1\textalpha. Several observations lend support for an extracellularly located Ca\textsuperscript{2+} binding site in mGluR1\textalpha; the limitation of the modulatory effect of Ca\textsuperscript{2+} in BHK-\textsuperscript{S165A}-mGluR1\textalpha compared to BHK-mGluR1\textalpha cells and initial observations of a graded decrease in the EC\textsubscript{50} for quisqualate-stimulated [\textsuperscript{3}H]-InsP\textsubscript{1} accumulation as [Ca\textsuperscript{2+}]\textsubscript{e} is increased in transiently transfected CHO-mGluR1\textalpha cells, but not CHO-\textsuperscript{S166D}-mGluR1\textalpha cells. However due to time limitations these latter investigations have not been continued further. Suggestions for further work were presented in Chapter 5.

With the development of a ligand binding assay for a truncated, soluble, hexahistidine tagged receptor, comprising only the extracellular domain of mGluR1 (Okamoto et al, 1998), the influence of varying [Ca\textsuperscript{2+}]\textsubscript{e} on ligand binding to such a receptor could be examined in future experiments.

In addition to modulating InsP\textsubscript{1} accumulation, it is also possible that varying [Ca\textsuperscript{2+}]\textsubscript{e} could influence the other branch of the phosphoinositide signalling pathway, that of DAG production and the resultant PKC activation. A number of the key roles of mGluR1\textalpha are suggested to be mediated via PKC-mediated protein phosphorylation. Thus, it would be interesting to study the effects of varying [Ca\textsuperscript{2+}]\textsubscript{e} on PKC activation, as this could have implications for the involvement of mGluR1\textalpha in a number of physiological and pathophysiological processes.
mGluR1α stimulation also results in the activation of a number of signal transduction pathways in addition to the phosphoinositide pathway, in both heterologous expression systems and native preparations, e.g. stimulation of AC (Aramori and Nakanishi, 1992; Pickering et al., 1993; Thomsen et al., 1996) and the phosphorylation and activation of ERKs (Ferraguti et al., 1999). These studies could therefore be expanded to encompass investigation into the effect of varying [Ca^{2+}]_o on these additional mGluR1α-coupled pathways.

The use of model cell systems provides a means to study the properties of different subtypes of GPCRs in isolation, without the complications of interactions with other receptors or pathways and considerations of the specificity of agonists and other pharmaceutical agents employed. However, it is also important to consider that the characteristics of heterologous cell systems, such as G-protein profiles may differ from those surrounding GPCRs in their native environment and could affect the properties of the receptor. Thus, with the development of selective agonists and antagonists for a number of different mGluR subtypes, i.e. the group I mGluR-selective agonist 3,5-DHPG (Schoepp et al., 1994; Brabet et al., 1995) and the mGluR1α-selective antagonist CPCCOEt (Hermans et al., 1998a; Litschig et al., 1999), the influence of varying [Ca^{2+}]_o on mGluR1α in native systems could provide an avenue for further study.

Studies in *Xenopus* oocytes have shown that mGluR1α can be activated by both Ca^{2+} and glutamate (Kubokawa et al., 1996). In this present study Ca^{2+}_o has been demonstrated to have a modulatory effect on agonist-stimulated phosphoinositide signalling via rat and human mGluR1α expressed in BHK and CHO cells,
respectively. Thus, Ca\(^{2+}\) can behave as both an agonist of mGluR1\(\alpha\) (Kubokawa et al, 1996) and as a modulator of agonist-stimulated signalling via mGluR1\(\alpha\) (Saunders et al, 1998). In the experiments in which Ca\(^{2+}\) is reported to activate mGluR1\(\alpha\) alone, Ca\(^{2+}\) may also be behaving as a modulator of signalling, allowing the receptor to be activated by glutamate present in the extracellular medium at a concentration which is too low to activate the receptor alone.

The studies of Kubokawa et al (1996) in which Ca\(^{2+}\), and other polyvalent cations, known to be agonists of the CaSR (Brown et al, 1993), behave as agonists of mGluR1\(\alpha\) suggest that mGluR1\(\alpha\) may contain Ca\(^{2+}\) binding sites within its extracellular domain. Initially it was hypothesised that such binding sites may consist of glutamic acid and aspartic acid doublets and triplets located in the extracellular domains of both mGluR1\(\alpha\) and its salmon homologue, sBimR, and proposed as putative Ca\(^{2+}\) binding sites in the CaSR (Houamed et al, 1991; Brown et al, 1993; Kubokawa et al, 1996). However, studies leading on from the observations of Kubokawa et al (1996) have shown that mutation of a single amino acid residue, serine 166, is sufficient to dramatically reduce the sensitivity of mGluR1\(\alpha\) to Ca\(^{2+}\) (Kubo et al, 1998). This Ca\(^{2+}\) sensitivity was shared by mGluR3 and 5, and serine 152, homologous to serine 166 of mGluR1\(\alpha\), was shown to be essential for the Ca\(^{2+}\) sensitivity of mGluR3 (Kubo et al, 1998). Alignment of the sequences (see figure 6.1) of mGluRs shows that a serine is present in equivalent positions in all but mGluR2, which was shown not to be Ca\(^{2+}\)-sensitive (Kubo et al, 1998, Galvez et al, 2000). Thus, it will be interesting to see if this residue is ubiquitous in conferring Ca\(^{2+}\) sensitivity to mGluRs. Although a serine is conserved in both GABA\(_B\) receptor sub-types (serine 248 in GABA\(_B\)-R1\(\alpha\) and serine 137 in GABA\(_B\)-R2) at the site
**Fig. 6.1** Alignment of the periplasmic binding proteins LIVPB and LBP, mGluRs, bovine CaSR (BoPCaR1), and GABA<sub>B</sub>R1 and 2 (rat-BR1 and 2), in the ligand binding domain. Residues in italics are involved in ligand binding. Residues in bold have been demonstrated (mGluR<sub>1α</sub> or mGluR3, CaSR and GABA<sub>B</sub>R1) to be, or align with residues, responsible for the Ca<sup>2+</sup>-sensing ability of mGluRs, CaSR and GABA<sub>B</sub>R1. The numbers above the sequences correspond to the positions of the residues in LIVPB, rat mGluR<sub>1α</sub>, bovine CaSR and rat GABA<sub>B</sub>R1. Adapted from Galvez et al (2000).
corresponding to serine 166 in mGluR1α the ability of Ca\textsuperscript{2+} to modulate the GABA response was unaffected when these residues were mutated (Wise et al, 1999).

The alignment of the extracellular domains of mGluR1α and LIVBP demonstrated that serine 165 and threonine 188 of mGluR1α aligned with residues of LIVBP important for ligand binding; serine 79 and threonine 102 (O’Hara et al, 1993). Accordingly, serine 165 and threonine 188 were shown to be involved in glutamate binding (O’Hara et al, 1993). Interestingly, the serine 166 residue responsible for the Ca\textsuperscript{2+}-sensitivity of mGluR1α (Kubo et al, 1998) is in close proximity to these residues. Indeed, the serine 165 residue, in addition to being important for high affinity agonist binding also appears to be important for the modulatory effect of Ca\textsuperscript{2+} on the coupling efficiency of, but not the agonist potency at, mGluR1α (see Section 3.3.4). In addition, serine 165 and threonine 188 of mGluR1α align with serine 147 and serine 170 of the CaSR and serine 246 and serine 269 of GABA\textsubscript{A}R1, which are also important for ligand binding in their respective receptors (Bräuner-Osborne et al, 1999, Galvez et al, 1999). Serine 269 of GABA\textsubscript{A}R1 has also been shown to be responsible for the Ca\textsuperscript{2+}-sensitivity of the receptor (Galvez et al, 2000).

Thus, the residues highlighted in Figure 6.1 play critical roles in the ligand binding and/or Ca\textsuperscript{2+} modulation of the family 3 GPCRs.

Due to the diverse roles of mGluRs in glutamatergic signalling in the central nervous system, the Ca\textsuperscript{2+}-sensitivity of the mGluRs (except mGluR2) may have some physiological or pathophysiological relevance. Glutamate has been reported to reach concentrations of several millimolar in the synaptic cleft, due to the extremely small volume of the cleft; ~ 2 attolitres (Bergles et al, 1999). Thus, it is conceivable
that the local concentration of Ca\(^{2+}\) in the synaptic cleft could change dramatically upon increased postsynaptic Ca\(^{2+}\)-influx, through NMDA receptors and perhaps voltage dependent Ca\(^{2+}\)-channels, following excitation of the postsynaptic cell, due to removal of Ca\(^{2+}\) from such a small volume. Indeed, the \([\text{Ca}^{2+}]_e\) has been shown to decrease transiently by \(\sim 30\%\) during excitatory activity (Heinemann et al, 1990; Knjjevic et al, 1982). Under pathophysiological conditions \([\text{Ca}^{2+}]_e\) has been demonstrated to decrease to as little as \(\sim 100\ \mu\text{M}\) (Hansen and Zeuthen, 1981; Pumain et al, 1983, Siesjo et al, 1989). In our studies decreasing \([\text{Ca}^{2+}]_e\) from 1.3 mM to 0.5 mM results in a 20-30 % decrease in the response of mGluR1\(\alpha\) to a maximal concentration of quisqualate, which can reach 70-90 % as \([\text{Ca}^{2+}]_e\) is decreased to micromolar levels. Thus, the Ca\(^{2+}\) sensitivity of mGluRs 1\(\alpha\), 3 and 5 (Kubokawa et al, 1996; Kubo et al, 1998; Saunders et al, 1998) could have implications for the involvement of mGluRs in a number of physiological, and especially pathophysiological, processes as \([\text{Ca}^{2+}]_e\) decreases to a greater extent under such conditions.

mGluR5 has been implicated as an autoreceptor involved in the potentiation (Herrero et al, 1992; Herrero et al, 1998; Rodriguez-Moreno et al, 1998; Sistiago et al, 1998) of glutamate release from the presynaptic terminal. The Ca\(^{2+}\)-sensing ability of the receptor could contribute to limiting the extent of this potentiation, as the resultant activation of NMDA receptors and subsequent Ca\(^{2+}\) entry into the postsynaptic cell result in decreased \([\text{Ca}^{2+}]_e\) in the synapse. The resultant decrease in the activation of mGluR5 may limit the potentiation of glutamate release by mGluR5.
The $Ca^{2+}$ sensing ability of mGluR1$\alpha$ may have implications for memory processes. Kubokawa et al propose that neurones which express mGluR1$\alpha$ may be continually activated by $Ca^{2+}$ within a physiological range. This in turn leads to Ins(1,4,5)P$_3$ production and a subsequent increase in $[Ca^{2+}]_i$. It is possible that following the induction of LTD or LTP, such continual activation of mGluR1$\alpha$ by $Ca^{2+}$ could aid the maintenance and stabilisation of such long-term synaptic changes (Kubokawa et al, 1996).

The ability of postsynaptic neurons to 'sense' $[Ca^{2+}]_e$ may also be important for the induction of LTP. During the induction of LTP, depolarisation of CA1 neurones occurs such that the voltage-dependent Mg$^{2+}$ block of the NMDA receptor is relieved, and activation of voltage-dependent $Ca^{2+}$ channels occurs. Subsequently $Ca^{2+}$ influx into the cell occurs, contributing to the induction of LTP. A novel $Ca^{2+}$-sensing cation channel characterised by Xiong et al may then be activated by the accompanying decrease in $[Ca^{2+}]_e$, to cause further depolarisation and amplification of the excitatory signal (Xiong et al, 1997). In addition, this decrease in $Ca^{2+}$ could be important for temporal aspects of LTP processes and ensuring that the degree of $Ca^{2+}$ entry into the cell during such glutamatergic transmission does not become neurotoxic, by decreasing the activity of mGluR1$\alpha$ and thus the enhanced NMDA receptor activation mediated by mGluR1$\alpha$ (Fitzjohn et al, 1996; Pisani et al, 1997a; Ugolini et al, 1997) and the degree of $Ca^{2+}$ influx into the postsynaptic cell.

By a similar mechanism the novel cation channel may act to synchronise and amplify the excitatory activity which occurs during seizure, and may thus be involved in the epileptogenic-like neural activity seen when hippocampal slices are bathed in
solutions containing low concentrations of Ca\textsuperscript{2+} (Jefferys and Haas, 1982; Xiong \textit{et al}, 1997). The group I mGluRs have also been implicated in increasing epileptogenic activity and the resultant neurodegeneration (McDonald \textit{et al}, 1993; Tizzano \textit{et al}, 1993; 1995; Camon \textit{et al}, 1998, Keele \textit{et al}, 1999; Merlin, 1999). [Ca\textsuperscript{2+}]\textsubscript{e} has been shown to decrease in the kindling model of epilepsy (Pumain \textit{et al}, 1983; Wadman \textit{et al}, 1985). It is possible that the decrease in group I mGluR activity mediated by such a decrease in [Ca\textsuperscript{2+}]\textsubscript{e} may limit the degree of neurodegeneration seen under such conditions.

Glutamate is neurotoxic in high concentrations and is responsible for the cell death and damage which occurs during ischaemia, hypoglycaemia and anoxia (Choi, 1988). Initial cell death occurs following NMDA receptor activation, allowing Ca\textsuperscript{2+} overload of cells under conditions where glutamate is present in excess causing excessive stimulation of NMDA receptors. However, most cell death is delayed occurring up to 24 hours after ischaemia, hypoglycaemia and anoxia. This is believed to be due to glutamate-induced glutamate release (Monyer \textit{et al}, 1992). Several groups have reported a rapid decrease in extracellular Ca\textsuperscript{2+} concentration during ischaemia, from 1.3 mM to ~ 100 \textmu M over a period of seconds (Hansen and Zeuthen, 1981; Siesjo \textit{et al}, 1989). Group I mGluRs are implicated in both enhancing (Bruno \textit{et al}, 1995a; Buisson and Choi, 1995; Orlando \textit{et al}, 1995; Strasser \textit{et al}, 1998) and limiting (Pizzi \textit{et al}, 1996; Montoliu \textit{et al}, 1997; Schroder \textit{et al}, 1999) NMDA-mediated neurotoxicity. Thus, the Ca\textsuperscript{2+} sensitivity of mGluR1\textalpha has implications for the degree of cell death which occurs under such conditions, the decrease in activity of mGluR1\textalpha seen as [Ca\textsuperscript{2+}]\textsubscript{e} decreases could either be limiting the enhancement of neurotoxicity mediated by group I mGluRs, or decreasing the
degree of neuroprotection provided by group I mGluR activation. The decreased activity of mGluR1α in the presence of low \([\text{Ca}^{2+}]_e\) could also be important in limiting the cell death which occurs due to glutamate-induced glutamate release, via a decrease in the potentiation of the NMDA receptor by mGluR1α (Fitzjohn et al., 1996; Pisani et al., 1997a; Ugolini et al., 1997) and limitation of \([\text{Ca}^{2+}]_e\) elevation mediated by mGluR1α activation. The decrease in the potentiation of mGluR5-mediated presynaptic glutamate release as proposed earlier could also be involved in limiting cell death under such conditions.

The group I mGluRs are well documented to be concentrated in a perisynaptic annulus around synaptic junctions, but not within the main body of the synapse where iGluRs are concentrated (Baude et al., 1993; Nusser et al., 1994; Lujan et al., 1996). This could explain the involvement of mGluRs in excitation evoked by high frequency stimulation in contrast to low frequency stimulation. Under high frequency stimulation the glutamate released is proposed to reach a high enough concentration to diffuse to and activate the perisynaptic mGluRs (Baude et al., 1993). It is proposed that such organisation has developed to influence the mGluR/iGluR ratio. An increase in the iGluR/mGluR ratio may be seen when the size of the synapse increases, as a result of an increase in the region occupied by iGluRs, where the postsynaptic membrane specialisation maintains a regular edge. If the synapse increases its size by producing an irregular edge (a perforated synapse) there could be an increase in the mGluR/iGluR ratio (Lujan et al., 1997). This has implications for the roles of mGluRs, and the consequences of mGluR modulation by decreasing \([\text{Ca}^{2+}]_e\), at different types of synapses, under physiological and pathophysiological conditions.
Thus, evidence suggests that Ca\textsuperscript{2+}-sensitivity may be a ubiquitous property of the family 3 GPCRs. A single serine residue, corresponding to serine 166 of mGluR1\textalpha, conserved in all mGluRs except mGluR2, has been proposed to be essential for Ca\textsuperscript{2+} to behave as an 'agonist' of mGluR1\textalpha and 3. In order to determine whether this residue is important for the modulation of agonist-stimulated phosphoinositide signalling via mGluR1\textalpha, further investigations using the wild-type mGluR1\textalpha and mutant S\textsuperscript{166D}-mGluR1\textalpha plasmids developed in Chapter 5 are necessary. Such Ca\textsuperscript{2+}-sensitivity may have implications for the mGluRs, and other members of the family 3 GPCRs, in a number of processes within the central nervous system.


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