THE UNIVERSITY OF CALGARY

Characterization of the Striatal Metabotropic Glutamate Receptor In Vitro

by

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The undersigned certify that they have read, and recommend to the faculty of Graduate Studies for acceptance, a thesis titled **"Characterization of the Striatal Metabotropic Glutamate Receptor In Vitro"** submitted by **Bruce P. Symons** in partial fulfilment of the requirements for the degree of **Master of Science.**

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ABSTRACT

There are generally considered to be three subtypes of excitatory amino acid (EAA) receptors in the mammalian central nervous system activated by the endogenous ligand glutamate: the N-methyl-D-aspartic acid (NMDA) receptor, the kainic acid receptor, and the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor. A novel, fourth, "metabotropic" glutamate receptor coupled to phosphoinositide hydrolysis rather than an ion channel was first described in embryonic mouse striatal neurons in primary culture. Previous studies on this receptor have suggested that there may be heterogeneous mechanisms underlying its actions, including the existence of multiple subtypes. In this thesis, a detailed characterization was performed on the striatal metabotropic receptor *in vitro* in order to elucidate these heterogeneous mechanisms. This was accomplished by pulse-labelling striatal neurons with [^{3}H]-inositol, and assaying the accumulation of [^{3}H]-inositol monophosphate ([^{3}H]-InsP$_1$) in the presence 1mM Li^+. Agonists, antagonists and other pharmacologic agents were included with the assay, and the extracellular cationic milieu was selectively altered to ascertain its effect.

Quisqualic acid (Quis), ibotenic acid and trans-1-aminocyclopentyl-1,3-dicarboxylic acid (ACPD) were all effective metabotropic agonists. None of the agonists were additive with each other in evoking [^{3}H]-InsP$_1$ accumulation. 2-amino-3-phosphonopropionic acid (AP3) and 2-amino-4-phosphonobutyric acid (AP4) were found to be effacacious, non-competitive antagonists. This effect was most likely a

direct effect upon the metabotropic receptor itself, and not a secondary response to AP3 or AP4. 2-amino-5-phosphonovaleric acid (AP5), a NMDA receptor antagonist, was also found to attenuate ibotenate but not Quis-evoked [^{3}H]-InsP$_1$ accumulation. AP5 only had marginal effects on ACPD-evoked [^{3}H]-InsP$_1$ accumulation.

The [^{3}H]-InsP$_1$ accumulations evoked by all agonists were significantly inhibited by 1μM phorbol dibutyrate, a specific activator of protein kinase C. Furthermore, agonist-evoked [^{3}H]-InsP$_1$ accumulation was significantly attenuated in the absence of extracellular Mg^{2+}, and elevated in the absence of extracellular Na^+. This Na^+ enhancement was likely due to a reversal in tonic, basal Na^+/Ca^{2+} exchange, thereby increasing intracellular Ca^{2+}. [^{3}H]-InsP$_1$ accumulation was also significantly inhibited by the absence of extracellular Ca^{2+} in a dose-dependent manner, and Quis-evoked [^{3}H]-InsP$_1$ accumulation was slightly attenuated by pre-treatment with pertussis toxin, a specific inactivator of G-proteins. When combined, low extracellular Ca^{2+} and pertussis toxin inhibited [^{3}H]-InsP$_1$ accumulations evoked by all agonists nearly to baseline. This suggests that Ca^{2+} and G-protein activation is necessary and sufficient for EAA-evoked [^{3}H]-InsP$_1$ accumulation, and possibly reflects the existence of two phospholipase C isoforms selectively activated by Ca^{2+} and G-proteins.

These experiments revealed heterogenous mechanisms elicited by different EAA agonists. This suggests that glutamate-evoked phosphoinositide hydrolysis may be a complex series of interactions between glutamate receptor subtypes and metabotropic receptor subtypes that are simultaneously activated, and between multiple isoforms of phospholipase C that may exhibit substrate preference.

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LIST OF ABBREVIATIONS

ACPD	trans-1-aminocyclopentyl-1,3-dicarboxylic acid
AMNH	2-amino-3-[2-(3-hydroxy-5-methylisoxazol-4-yl)methyl-5-methyl-3-oxoisoxazolin-4-yl]propionic acid
AMOA	2-amino-3-[3-(carboxymethoxy)-5-methylisoxazol-4-yl]propionic acid
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
APB	see AP4
AP3	D,L-2-amino-3-phosphonopropionic acid
AP4	D,L-2-amino-4-phosphonobutyric acid
AP5	D,L-2-amino-5-phosphonovaleric acid
AP7	D,L-2-amino-7-phosphonoheptanoic acid
APV	see AP5
Asp	aspartic acid
BMAA	β-N-methylamino-L-alanine
CCh	carbamylcholine (carbachol)
CGS19755	cis-4-(phosphomethyl)-2-piperidine-carboxylic acid
CPP	3-(+)-2-(carboxypiperazin-4-yl)-propyl-1-phosphonic acid
CNQX	6-cyano-7-nitroquinoxaline-2,3,-dione
CNS	central nervous system
CTP	cytidine triphosphate
ddH_2O	deionized, double-distilled water
DG	1,2-diacylglycerol
DIV	days *in vitro*
DMEM/F12	Dulbecco's Modified minimal Essential Medium supplemented with F12 nutrient
DMSO	dimethylsulphoxide
DNQX	6,7-dinitroquinoxaline -2,3-dione
EAA	excitatory amino acid
EDTA	ethylenediaminetetracetic acid
EC_{50}	50% of the concentration required to achieve maximum stimulation (estimated)
EGTA	ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetracetic acid
EPSP	excitatory postsynaptic potential
E14	gestational/embryonic day 14
FBS	fetal bovine serum
5-HT	5-hydroxy-tryptamine (serotonin)
γDGG	γ-D-glutamylglycine
GAMS	α-D-glutamyl-aminomethylsulphonate
GDEE	L-glutamic acid-diethyl-ester
Glu	glutamic acid
G-protein	guanine nucleotide binding protein
GTP	guanidine triphosphate
$[^3H]$-$InsP_x$	tritiated inositol-x-polyphosphates
HA966	3-amino-1-hydroxypyrrolidine-2
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
Ibo	ibotenic acid

IC_{50}	50% of the concentration required to achieve maximum inhibition (estimated)
Ins	inositol
$InsP_1$	inositol-1-monophosphate
$InsP_x$	inositol-x-polyphosphates
JSTX	joro spider toxin
KA	kainic acid
KYN	kynurenic acid
L-SOP	L-serine-O-phosphate
LTD	long-term depression
LTP	long-term potentiation
mGluR	metabotropic glutamate receptor
MK801	(+)-5-methyl-10,11-dihydro-*5H*-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate
NE	norepinephrine
NMDA	N-methyl-D-aspartic acid
NMDG-Cl	N-methyl-D-glucamine chloride
PBS	phosphate buffered saline
PDBu	phorbol-12,13-dibutyrate
PCP	phencyclidine
PKC	protein kinase C
PLC	phospholipase C
PIP	phosphatidylinositol-4-monophosphate
PIP_2	phosphatidylinositol-4,5,-bisphosphate
PI-PLC	phosphatidylinositol-specific phospholipase C
PTX	pertussis toxin
Quis	quisqualic acid
TCP	1-(1-thienylcyclohexyl)piperidine

GENERAL INTRODUCTION

Thoughts are electrochemical signals. In its most elementary form, our consciousness, ideas, dreams, and sense of individuality arise from the network of ionic flow across the membranes of our brain cells. As you read this, positive signals generated by your brain cells are registering and processing these words, whereas inhibitory signals are filtering out extraneous distractions. The neurochemical responsible for the majority of these positive signals is the excitatory amino acid glutamate - it is the major excitatory neurotransmitter of the mammalian central nervous system. Therefore, it is of interest to understand how glutamate works.

For any cell to respond to an external stimulus, it must have three things: a mechanism to detect the stimulus (ie. a receptor), a method of relaying this recognition to the relevant parts of the cell (ie. intracellular messengers) and a mechanism to respond meaningfully to the stimulus (ie. an effector). For glutamate, there are several types of receptors that activate a number of intracellular messengers. The classical response of the neuronal cell is to generate an action potential: a sodium-based electric signal that propagates along the neuron to all other cells it contacts in the network of our brain. This electric signal also enables the flow of calcium ions into the cells via other glutamate receptor subtypes and channels, resulting in the activation of several intracellular enzymes.

Recently, a new type of glutamate receptor that does not behave in this classical

manner was discovered. This receptor does not directly result in an ionotropic electrical signal; rather, it causes a metabotropic response by causing the breakdown of membranous lipids into intracellular messengers. Therefore, this receptor does not function as a "classical" information relay, but rather serves to signal long-term changes within the neuron itself. This thesis is primarily concerned with the characterization of this novel, metabotropic glutamate receptor. The main questions to be answered by this thesis revolve around the pharmacology and modulation of this receptor - how does it work, and how does this relate to the function of other glutamate receptors? These questions, and the potential significance of the metabotropic receptor in the central nervous system, will be explored.

The introduction that follows is divided into two sections. The first section familiarizes the reader with the excitatory amino acids in general, and focuses on the structure, pharmacology and function of the metabotropic glutamate receptor in the central nervous system. Some of the mechanisms transducing the metabotropic glutamate signal into the cell, as well as their pharmacology, will be also be discussed to identify potential sites for regulation. This will give the reader adequate background to understand the questions being asked regarding this receptor. The second section deals with the striatal model of the metabotropic receptor. The striatum, the brain region wherein these questions will be answered, will be discussed on both ultrastructural and cellular levels. Finally, the advantages of the *in vitro* primary culture of striatal neurons will be introduced as the model of choice for characterizing the metabotropic glutamate receptor.

INTRODUCTION

SECTION ONE: METABOTROPIC GLUTAMATE RECEPTOR

1. DISCOVERY OF THE EXCITATORY AMINO ACIDS

In the early 1960s, Curtis and Watkins (1960; 1963) discovered that acidic amino acids can have a strong excitatory effect on mammalian spinal cord neurons. They erroneously concluded that this excitation was due to a general property of excitable membranes, and not to a *bona fide* receptor. Nevertheless, their initial work heralded research that culminated in the following discovery: despite the plethora of neurotransmitters and peptides available to the neuron, the simple amino acids L-glutamic acid (Glu) and L-aspartic acid (Asp) are the major excitatory neurotransmitters in the mammalian central nervous system - virtually every synapse expresses receptors that bind these excitatory amino acids (EAAs)(Robinson & Coyle, 1987).

1.1 Discovery of the EAA Receptor Types

In 1974, Duggan discovered differential responses to Glu and Asp in spinal interneurons, and proposed that there were two subtypes of the Glu receptor (Glu-R): a Glu-preferring subtype and an Asp-preferring subtype. The most generally

accepted nomenclature scheme involves 4 distinct types of Glu receptors in the mammalian brain. They are defined by their most specific agonists: N-methyl-D-aspartate (NMDA), kainic acid (KA), α-amino-3-hydroxy-5-methylisoxalzole-4-propionic acid (AMPA), and a fourth, inhibitory D,L-2-amino-2-phosphonobutyrate (AP4) receptor. The characteristics and known agonists and antagonists of these receptors are summarized on Table I hereafter.

The above 4 receptor subtypes are ionotropic Glu receptors - they either are or are coupled to ion channels. A novel, fifth type of Glu-R has been postulated to exist and is widely accepted to explain the generation of inositol phosphates (InsPs) upon stimulation (Sladeczek *et al.*, 1985). This receptor has been known variously as the metabotropic glutamate receptor, the metabotropic Quis receptor, the Q_x receptor, the Q_p receptor, and most recently as the ACPD receptor (Palmer *et al.*, 1989). For the purpose of clarity, I will be referring to this receptor as the metabotropic glutamate receptor (mGluR) to avoid the issue of most selective agonist. Thus, the glutamate receptor family appears to be analogous to the acetylcholine receptors, ie. there are ionotropic receptors (nicotinic) implicated in synaptic transmission as well as metabotropic (muscarinic) receptors implicated in the generation of second messengers. Each Glu-R subtype will be discussed briefly below.

1.2 The NMDA Receptor: Discovery, Structure and Pharmacology

The best characterized of all the Glu receptor subtypes is the NMDA receptor; much of its physiology and function has been elucidated. The initial work on the NMDA receptor was facilitated by the advent of the monoamino dicarboxylic

TABLE I: GLUTAMATE RECEPTOR SUBTYPES

NAME	AGONIST(S)	ANTAGONIST(S)	FUNCTIONS
NMDA	NMDA, L-Glu L-Asp, Glu	Receptor: D-AP5, CPP, CGS19755, HA966, CGP37849 Ionophore: PCP, MK801 Mg^{2+}, ketamine SKF10047, MNQX	receptor-ionophore complex; fluxes cations; voltage dependent Mg^{2+} blockade of channel; involved in LTP
	glycine allosteric co-agonist / modulator		
Kainate	L-domoate, L-Glu kainate, Quis	DNQX, CNQX γDGG, GAMS, GDEE JSTX, NBQX	ionophore that gates Na^+, K^+; involved in: synaptic transmission, excitotoxicity
AMPA	Quis, AMPA L-Glu, kainate	CNQX, DNQX γDGG, GAMS, GDEE JSTX	ionophore that gates Na^+, K^+ and Cl^-; functions as per kainate receptor plus fast EPSPs
AP4	L-AP4, L-Glu L-SOP	None	presynaptic inhibitory autoreceptor, blocks K^+ channels
Metabotropic	Quis, ACPD Ibo, L-Glu	AP3, AP4	coupled to PLC for phosphoinositide hydrolysis

Abbreviations: see List of Abbreviations, page xiii.

antagonists such as D-α-aminoadipate (Biscoe *et al.*, 1977), followed by the phosphono-substituted antagonists such as 2-amino-5-phosphonovalerate (AP5) (Davies *et al.*, 1982). These remarkably specific antagonists allowed selective targeting of the NMDA receptor separate from the KA/AMPA receptors. Physically, the NMDA receptor is actually a receptor-ionophore complex consisting of four discrete domains as follows:

(1) A L-Glu/L-Asp binding site. This is also the binding site for competitive antagonists such as AP5 and AP7 (2-amino-7-heptanoate)(Monaghan *et al.*, 1989).

(2) The glycine allosteric site. In 1987, Johnson and Ascher discovered that extremely low concentrations of glycine (EC_{50}=0.1 - 1μM) potentiate the excitatory effect of NMDA agonists, but had no intrinsic activity itself.

(3) The ion channel pore. The NMDA-R also contains a voltage-dependent ion channel that is permeable to Na^+, K^+, and Ca^{2+} at potentials between -30 to -20mV; thus, the channel is closed at normal resting potentials. Recently, antagonists such as 1-(1-phenylcyclohexyl)piperidine (PCP) and dizocilpine maleate (MK801) have been synthesized that block the ion pore. Mg^{2+} can voltage-dependently attenuate the PCP/MK801 blockade (Huettner & Bean, 1988), and is believed to act at a site inside the channel pore distinct from the PCP site.

(4) An inhibitory Zn^{2+} binding site. Peters *et al.* (1987) discovered that another divalent cation, Zn^{2+}, could also block NMDA responses. Unlike Mg^{2+}, however, this blockade is not voltage-dependent and is believed to act at a distinct site.

1.3 Purification and Cloning of the NMDA Receptor

Nakanishi and colleagues have isolated, cloned and sequenced the rat NMDA receptor (Moriyoshi *et al.*, 1991), and expressed it in *Xenopus* oocytes for pharmacologic and electrophysiologic characterization. The 4213bp cDNA clone yielded a single polypeptide of 938 amino acids ($M_r \approx 105,500$) with 4 transmembrane (TM) domains and a very large extracellular domain (540 and 110 amino acids at the amino and carboxy termini, respectively) with 10 potential glycosylation sites. This was somewhat unexpected, since previous studies have demonstrated that the NMDA-R was composed of 4 subunits (see Kumar *et al.* below). The second TM domain (TM2) was flanked by negatively charged residues, and is believed to be the channel pore. The NMDA-R clone bore an overall 22-26% homology to the KA/AMPA receptors (Moriyoshi *et al.*, 1991). A novel GluR, designated as GluRδ1, has recently been cloned in mice, and is believed to be the mouse NMDA-R (Yamazaki *et al.*, 1992), although it has not been expressed for characterization yet.

In addition, Kumar *et al.* purified 4 Glu binding proteins that were characterized as the NMDA receptor complex, and raised antibodies against some of them (Kumar *et al.*, 1991). The 70kD protein was cloned and yielded a 1.7kB cDNA that is believed to be the Glu-binding subunit of the NMDA-R (Kumar *et al.*, 1991). This cDNA clone yielded a deduced protein with 516 amino acids ($M_r \approx 57,020$) with 4 TM domains within a hydrophobic core, and 3 potential glycosylation sites. Although the TM2 domain was similar to other ligand-gated channels, it and TM1 contained positively-charged residues, whereas TM3 and TM4 contained negatively-

charged amino acids; this was speculated to confer the unique voltage sensitivity of the NMDA-R (Kumar *et al.*, 1991). However, the clone bore no significant homology to the published KA-R/AMPA-R or metabotropic Glu receptor sequences.

1.4 The Kainate/AMPA Receptors: Structure and Cloning

Although the KA/AMPA receptors were generally recognized to be functionally distinct subtypes, there has always been some controversy regarding this. Since the subunits to these receptors have been cloned both in rat (Hollman *et al.*, 1989; Keinänen *et al.*, 1990; Boulter *et al.*, 1990; Verdoorn *et al.*, 1991; Egebjerg *et al.*, 1991; Bettler *et al.*, 1992; Sommer *et al.*, 1992) and in mouse (Sakimura *et al.*, 1990, 1992), this has finally clarified the situation somewhat. From these data, it appears that the KA/AMPA receptors are a family of heteromers composed of 4 or 7 GluR subunits, designated as GluRs1-7 (Bettler *et al.*, 1992) or as GluRsA-D (Sommer *et al.*, 1991). These subunits can be further divided into 2 classes: GluR1-4 (or GluRA-C) and GluR5-7 (or GluRD). Compiling these data, the GluR subunits can be reconstructed as follows: an 862-900 residue polypeptide, with 5 TM domains. The TM5 domain near the NH_2 terminus appears to be a signal peptide, and the other 4 TM domains are in the carboxy-terminal half of the polypeptide (Boulter *et al.*, 1990). There are multiple Ca^{2+}-calmodulin-dependent kinase II and protein kinase C (PKC) phosphorylation consensus sequences on the intracellular loops (Keinänen *et al.*, 1990). In addition, the TM2 loop carries a net negative charge, and subsequent experiments using site directed mutagenesis at TM2 and exhaustive electrophysiology have identified it as the actual ion channel (Verdoorn *et al.*, 1991).

A specific site (the Q/R site) within the channel confers mono- versus divalent cation gating properties, depending on the presence of a glutamine or arginine residue at that site (Sommer *et al.*, 1992).

1.4.1 Functional Expression of GluR Clones: These subunits appear to conduct a greater net current when in dimers, and are usually expressed this way. The GluR-B subunit appears necessary to confer an AMPA-preference to any of the heteromeric combinations (Verdoorn *et al.*, 1991); thus, the GluR-B subunit may be seen as the AMPA binding receptor. Recently, Egebjerg *et al.* (1991) isolated a clone (GluR6) that had great affinity for KA and would appear to be the high-affinity KA-R found in the CNS. This was followed by Bettler *et al.* (1992) with GluR7, the low-affinity KA-R subunit, and by Sakimura *et al.* (1992) with the homologous gene in mouse. In addition, Heinemann and coworkers have recently managed to generate polyclonal antibodies to the GluR1 subunit, and have localized GluR1-immunoreactivity to postsynaptic densities in rat hippocampal and cerebellar membranes (Rogers *et al.*, 1991). Furthermore, these KA/AMPA subunits have been shown to be permeable to Ca^{2+}; when the GluR1 and GluR3 subunits are expressed together in *Xenopus* oocytes, they mediate an inward Ca^{2+} flux that was potentiated by cyclic AMP-dependent protein kinase A (Keller *et al.*, 1992),

1.4.2 Flip and Flop forms of the GluRs: Sommer *et al.* (1990) reported that the GluRA-D genes can each yield two slightly different mRNA transcripts, named 'flip' and 'flop'. Their original cloning was of the flop versions (Keinänen *et al.*, 1990).

The flip and flop sequences exist on adjacent exons separated by a 900 bp intron, and are believed to give rise to the alternative forms by differential transcription. These exons are 115bp segments that code for 38 amino acids within the conserved intracellular domain of the AMPA-R, just before TM4. These substitutions result in very conservative changes, and are largely silent with respect to the protein sequence. However, there is a tetrapeptide that always differs between the flip and flop forms. The physiologic function(s) of these forms is unknown, but it is known from *in situ* hybridizations in rats that the flop form is only expressed postnatally from postnatal day 6 (P6) onwards; expression of flip is unaffected by age (Monyer *et al.*, 1991). Although ligand affinity is NOT affected by flip versus flop forms, KA appears to activate larger currents through the flop forms, whereas the flip forms prefer AMPA and Glu (Sommer *et al.*, 1990). Furthermore, the presence of a GluRA subunit, which acts as the 'dominant' dimer, mediates a large, fast-desensitizing Glu current, whereas a GluRB subunit (also dominant) mediates a small but sustained Glu current (Sommer *et al.*, 1990)

1.5 Pharmacology of the Kainate/AMPA Receptors

These receptors mediate the traditional, fast glutamatergic synaptic transmission (Monaghan *et al.*, 1989) - ie. they are Na^+ and K^+ ion channels. Thus, they are responsible for voltage-independent, excitatory post-synaptic potentials arising from the release of L-Glu. Until recently, the pharmacology and physiology of these receptors has been difficult to study due to a lack of specific antagonists to distinguish between KA and AMPA binding sites. However, a new generation of

AMPA-based antagonists capable of distinguishing between KA and AMPA receptors [AMNH: (2-amino-3-[2-(3-hydroxy-5-methylisoxazol-4-yl)methyl-5-methyl-3-oxoisoxazolin-4-yl]propionic acid), and AMOA: (2-amino-3-[3-(carboxymethoxy)-5-methylisoxazol-4-yl]propionic acid)] has recently been developed (Frandsen *et al.*, 1990).

1.6 The AP4 Receptor: Pharmacology

Compared with the above receptors, relatively little is known about the inhibitory AP4-R. This is due to the fact that there are no known antagonists at this receptor, and the only specific agonists are L-AP4 and possibly serine-O-phosphate. It was first postulated to exist due to the antagonistic actions it had on glutamatergic synapses; it potently blocks synaptic transmission in many pathways and preparations (for review, see Monaghan *et al.*, 1989). However, since this antagonism is easily overcome by adding any of the ionotropic EAA agonists (eg. NMDA, KA, Quis, L-Glu, L-Asp), it was hypothesized to be acting at its own receptor (Evans *et al.*, 1982; Davies & Watkins, 1982). However, a retinal AP4 receptor that mimicks the effects of L-Glu by hyperpolarizing ON bipolar cells has also been discovered (for review, see Miller & Slaughter, 1986). The relationship between the retinal AP4-R and the CNS AP4-R is unclear at present.

Early radioligand binding studies indicated that L-AP4 bound to a presynaptic Cl^- dependent site that was not displaced by KA or NMDA, but was by Quis and L-glu (Fagg *et al.*, 1983; Monaghan *et al.*, 1983). There is much evidence, such as quantal

analysis of hippocampal synapses (Cotman *et al.*, 1986), that suggests that the AP4 receptor is presynaptic.

2. THE METABOTROPIC GLUTAMATE RECEPTOR

2.1 The Metabotropic Glutamate Receptor: Discovery and Cloning

The fact that Glu could stimulate phosphoinositide turnover was first reported by Weiss and coworkers in 1985 in primary cultures of striatal neurons (Sladeczek *et al.*, 1985). Subsequently, this finding was confirmed by Nicoletti and colleagues in rat hippocampal slices and primary cultures of cerebellar granule cells (Nicoletti *et al.*, 1986a-c), followed by Sugiyama *et al.* (1987) in *Xenopus* oocytes.

The metabotropic Glu receptor was recently cloned by two laboratories (Masu *et al.*, 1991; Houamed *et al.*, 1991), and designated as mGluR1. Both groups came up with the identical amino acid sequence - the 4282 base pair gene contains at least two introns (Houamed *et al.*, 1991), and a large open reading frame of 3597 base pairs that encodes for a 1199 residue polypeptide with a predicted mass of 133.2 kD. The receptor contains 3 putative structural domains: a 593-residue extracellular amino terminal domain, a central 239-amino acid core containing 7 transmembrane (TM) domains, and a 367 residue intracellular carboxy terminal domain (Houamed *et al.*, 1991).

The mGluR1 clone is considerably larger than other G-protein coupled receptors (O'Dowd *et al.*, 1989), and bears little homology to them (Masu *et al.*, 1991; Houamed *et al.*, 1991). This was a surprising result, as most G-protein coupled receptors belong to a well-conserved family of proteins (O'Dowd *et al.*, 1989). However, despite this apparent lack of homology, the following facts were deduced from its sequence: **(1)** the carboxy terminal domain has a 20 residue signal peptide similar to the sequences preceding the extracellular domains of glycosylated hormone receptors, **(2)** the carboxy terminal also bears weak homology to AMPA/KA receptors, **(3)** in the 7 TM domains, there is a truncated cytoplasmic loop between segments V and VI that is the putative interaction site for G-proteins, and **(4)** the large amino terminal bears 29% identity with the membraneous form of sea urchin guanylate cyclase (Masu *et al.*, 1991; Houamed *et al.*, 1991).

When expressed in *Xenopus* oocytes, the mGluR1 has the typical electrophysiological and pharmacological characteristics. Masu *et al.* (1991) also performed in situ hybridizations using mGluR antisense mRNA on rat hippocampal and cerebellar sections, and performed northern analyses using the same probe, and found surprisingly little mGluR mRNA in the adult rat striatum (Masu *et al.*, 1991).

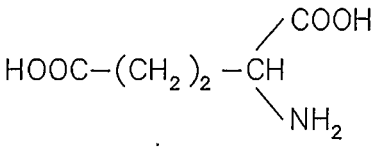
Using the mGluR1 as a probe, a family of 4 metabotropic receptors has very recently been cloned (mGluR1-mGluR4) from an adult rat brain library (Tanabe *et al.*, 1992). These 4 receptors have between 43-70% homology with each other, and mGluR2-4 are 3294, 3215 and 3704 bases long, respectively, translating into deduced amino

acid sequences of 872 (95.8 kD), 879 (99.0kD) and 912 (101.8 kD) residues each. All share 7 TM domains, a large extracellular amino terminus, a positively charged cytoplasmic domain near the TM domains, and completely conserved cysteine residues (Tanabe *et al.*, 1992). Expressing mGluR2-4 in *Xenopus* oocytes gave negative results in generating phosphoinositide hydrolysis and Ca^{2+} increases, whereas expressing mGluR2 in Chinese hamster ovary cells yielded modest increases in phosphoinositide hydrolysis, but significantly attenuated forskolin-stimulated cyclic AMP formation (Tanabe *et al.*, 1992). *In situ* hybridizations, however, revealed that mGluR1 and mGluR2 mRNA were localized to different neurons.

2.2 Agonist Pharmacology of the Metabotropic Glutamate Receptor

Several agonists have been identified at the metabotropic Glu receptor, and are summarized below in Fig. 1. Quisqualic acid, isolated from the seeds of the plant *Quisqualis fructus,* was the first potent agonist identified (Sladeczek *et al.*, 1985). Ibotenic acid, a rigid heterocyclic analog of glutamate isolated from the mushroom *Amanita strobiliformis,* was also found to stimulate phosphoinositide hydrolysis (Nicoletti *et al.*, 1986). Most recently, trans-ACPD (the rigid analog of Glu in its extended configuration) was shown to be a potent and selective metabotropic agonist; it did not have any activity at NMDA, KA or AMPA receptors (Palmer *et al.*, 1988). However, only 1S,3R-ACPD was found to be active; its stereoisomer 1R,3S-ACPD was a weak NMDA agonist (Irving *et al.*, 1990; Schoepp *et al.*, 1991). The endogenous ligand L-Glu appears to be a relatively weak agonist at the metabotropic Glu receptor *in vivo* due to avid reuptake mechanisms, but is a potent agonist in most *in vitro* preparations (eg. Sladeczek *et al.*, 1985).

FIGURE 1: EAA AGONIST STRUCTURES



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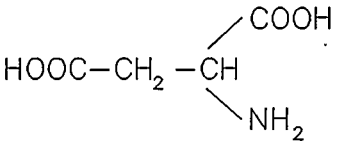

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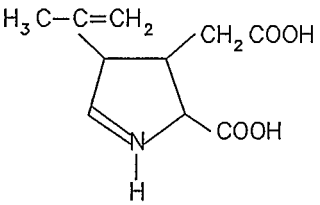
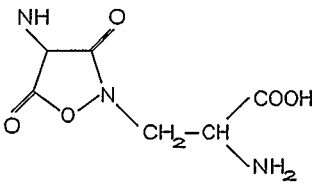
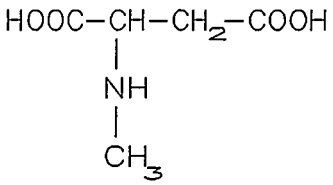


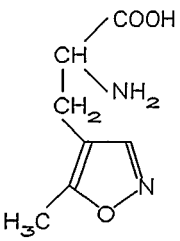
Kainate



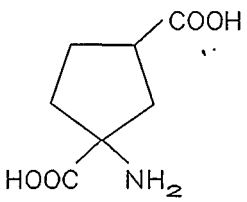
Quisqualate



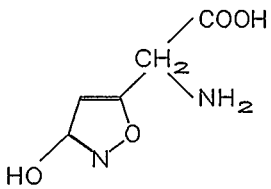
N-methyl-D-aspartate



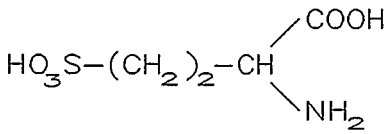
AMPA



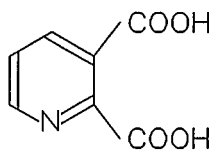
ACPD



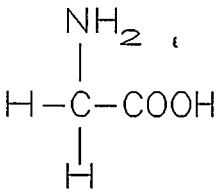
Ibotenate



L-homocysteate



Quinolinate



Glycine

Abbreviations: **ACPD**, trans-1-aminocyclopentyl-1,3-dicarboxylic acid; **AMPA**, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid

The rank order of agonist potency varies in various neuronal preparations. In striatal neurons in primary culture (Sladeczek *et al.*, 1985; Manzoni *et al.*, 1991) the order was Quis>Glu>Ibo>ACPD, whereas the rank efficacy was less clear (Quis≈Glu>Ibo>ACPD). In neocortical cultures, the order is Quis>Ibo>Glu (Patel *et al.*, 1990). In hippocampal slices, the order is Quis>Ibo≈ACPD>Glu (Schoepp & Johnson, 1989b; Schoepp *et al.*, 1991a). Other EAAs that have very weak or zero activity at the metabotropic Glu receptor include Asp, NMDA, KA, AMPA, L-homocysteate and taurine (Sladeczek *et al.*, 1985; Nicoletti *et al.*, 1986a,b; Sugiyama *et al.*, 1987; Palmer *et al.*, 1988; Doble & Perrier, 1989). Again, there are exceptions and slight variations according to the age of the neurons *in vivo*, brain region examined and preparation used.

The additivity of phosphoinositide hydrolysis evoked by these EAAs has also been investigated. The effects of Quis, Ibo and ACPD are not additive in primary cultures of striatal neurons (Manzoni *et al.*, 1991), neocortical cultures (Patel *et al.*, 1990), retinal slices (Osborne, 1990), cortical slices (Godfrey *et al.*, 1988). However, Quis and the muscarinic agonist carbachol (CCh) were additive in neocortical cultures (Patel *et al.*, 1990), and Quis and the α_1-adrenergic agonist norepinephrine (NE) were additive in primary cultures of striatal neurons and *Xenopus* oocytes injected with rat brain mRNA (Manzoni *et al.*, 1990a). Another important point about EAA-evoked phosphoinositide is that most agonists are not pure, specific agonists. Thus, Quis interacts strongly with the AMPA receptor, Ibo interacts strongly with the NMDA receptor (Doble & Perrier, 1989) and even ACPD may

interact somewhat at the NMDA receptor (Schoepp *et al.*, 1990a). This means that the net effect of Quis at evoking phosphoinositide hydrolysis is equal to the sum of its stimulatory effects at the metabotropic receptor plus its effects (stimulatory or inhibitory) at the AMPA receptor. The same logic applies to the effects of Ibo and ACPD. The relevance of these effects will be discussed later.

EAA stimulated phosphoinositide has been demonstrated in many neural preparations. A non-exhaustive list includes: **(1)** primary dissociated cell cultures from: mouse striatum (Sladeczek *et al.*, 1985), rat cerebellum (Nicoletti *et al.*, 1986a), rat neocortex (Patel *et al.*, 1990) and rabbit retina (Osborne, 1990), (2) brain slices from: rat hippocampus (Nicoletti *et al.*, 1986b; Schoepp & Johnson, 1988a,b), rat cortex (Godfrey *et al.*, 1988) and rabbit retina (Osborne, 1990), and **(3)** other preparations such as sympathetic ganglia (Bone & Michell, 1985), rat cortical astrocytes (Pearce *et al.*, 1986), rat brain synaptoneurosomes (Récasens *et al.*, 1987), and *Xenopus* oocytes injected with rat brain mRNA (Sugiyama *et al.*, 1987). L-Glu and Quis-evoked [^{3}H]-InsP$_1$ accumulation has also been demonstrated in slices from the occipital and temporal lobes, and cerebellar cortex of the human brain (Nicoletti *et al.*, 1989b).

The ability of the metabotropic Glu receptor to stimulate phosphoinositide hydrolysis appears to be a direct effect rather than an indirect one mediated by the release of other neurotransmitters. Weiss *et al.* (1988) report that inclusion of tetrodotoxin (a voltage dependent Na^+ channel blocker) and Godfrey *et al.* (1988) report that Cd^{2+}

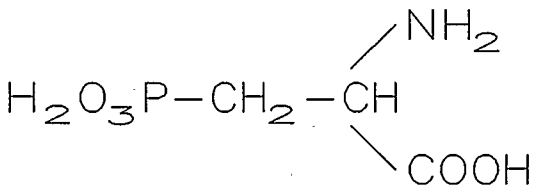
(a Ca^{2+} channel blocker) has no effect on Quis and Ibo stimulated phosphoinositide hydrolysis in primary cultures of striatal neurons and rat cortical slices, respectively. Similarly, blocking Ca^{2+} influx by verapamil, EGTA, or Cd^{2+} also have no effect on EAA-evoked phosphoinositide hydrolysis in rabbit retina (Osborne, 1990). In contrast, however, Baird and Nahorski (1990a) recently reported that the direct elevation of intracellular Ca^{2+} (via ionophores and Ca^{2+} channel agonists) increases InsP accumulation in rat cerebral cortical slices, and many investigators have also found similar results (Fisher & Agranoff, 1981; Cheuh & Gill, 1986; Nicoletti *et al.*, 1987b; Eberhard & Holz, 1988).

2.3 Antagonist Pharmacology of the Metabotropic Glutamate Receptor

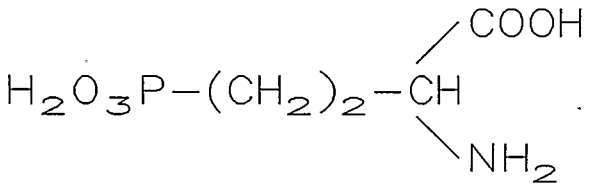
Most classic EAA antagonists are ineffective at attenuating EAA-evoked phosphoinositide hydrolysis. Of all the antagonists listed on Table I, only 2-amino-3-phosphonopropionate (AP3) and to a lesser extent AP4 have consistently demonstrated antagonistic properties against the metabotropic Glu receptor. The structural formulae of the EAA antagonists under discussion are summarized on Fig. 2 below.

2.3.1. AP3 Antagonism: Non-competitive AP3 antagonism has been demonstrated in rat hippocampal slices (Schoepp *et al.*, 1989b; Desai & Conn, 1990), although recently Manzoni *et al.* (1991) have reported competitive AP3 antagonism in primary cultures of striatal neurons. L-AP3 appears to be much more effective and potent in attenuating Glu, Quis and Ibo-evoked phosphoinositide hydrolysis in rat hippocampal slices than D-AP3 (Schoepp *et al.*, 1990b). Irving *et al.* (1990) report

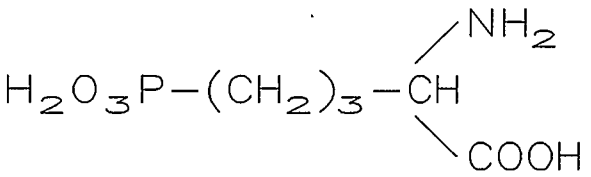
FIGURE 2: EAA ANTAGONIST STRUCTURES



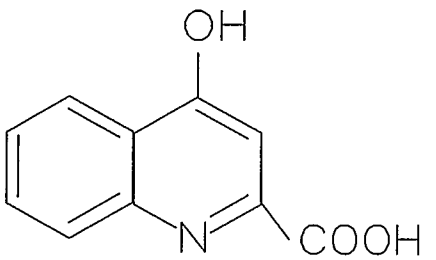
2-amino-3-phosphonopropionate (AP3)



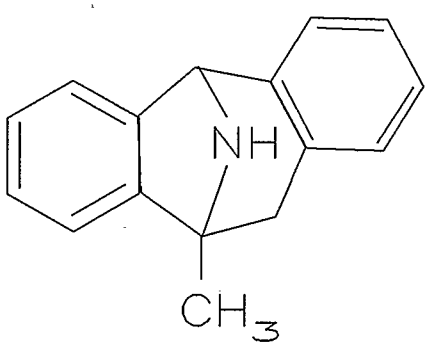
2-amino-4-phosphonobutyrate (AP4)



2-amino-5-phosphonovalerate (AP5)



kynurenic acid



dizocilpine maleate [(+)-5-methyl-10,11-dihydro-*5H*-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate]

that AP3 can block the ACPD-evoked increase in intracellular Ca^{2+} in rat cerebellar neurons. Not all of these studies are *in vitro* -if rats are given AP3 and then sacrificed, hippocampal slices from their brains have reduced EAA-evoked phosphoinositide hydrolysis (Schoepp *et al.*, 1991b). Finally, AP3 acts as a partial agonist at the metabotropic receptor; 1-2mM AP3 causes a 150-200% increase in basal phosphoinositide hydrolysis in primary cultures of striatal neurons (Manzoni *et al.*, 1991)and rat hippocampal slices (Schoepp & Johnson, 1989b).

2.3.2 AP4 Antagonism: L-AP4 antagonism has been observed in rat hippocampal slices (Nicoletti *et al.*, 1986c; Schoepp & Johnson, 1989b; Desai & Conn, 1990). However, no effect of L-AP4 was observed on EAA-evoked phosphoinositide hydrolysis in primary cultures of striatal neurons (Manzoni *et al.*, 1991), striatal slices (Doble & Perrier, 1989), cultured cerebellar granule cells (Nicoletti *et al.*, 1986a,b), neocortical cultures (Patel *et al.*, 1990), rabbit retinal slices (Osborne, 1990) and rat cortical slices (Godfrey *et al.*, 1988). However, L-AP4 was able to block L-Glu, Quis and Ibo-evoked increases in fura-2 imaged intracellular Ca^{2+} in rat cortical synaptosomes (Adamson *et al.*, 1991).

2.3.3 AP5 Antagonism: Although generally considered as a NMDA antagonist, there are reports of AP5 having an effect on the metabotropic response. L-AP5 attenuated L-Glu and veratridine but not Quis-evoked phosphoinositide hydrolysis in cultures of cerebellar granule cells (Nicoletti *et al.*, 1986b). Godfrey *et al.* (1988) reported that AP5 potentiates Ibo-evoked phosphoinositide hydrolysis, but has no

effect on Quis. However, others have reported no effect of AP5 on the metabotropic response in neonatal striatal slices (Doble & Perrier, 1989), rat neocortical cultures (Patel *et al.*, 1990) and rabbit retinal slices (Osborne, 1990). In addition, ACPD-evoked excitation in rat spinal cord slices *in vitro* was also D-AP5 sensitive (Magnuson *et al.*, 1988).

2.4 Functions of the Metabotropic Glutamate Receptor

The functions of the metabotropic Glu receptor are presently unclear. Nevertheless, enticing bits of evidence are now emerging. Anatomically, studies using [^{3}H]-Glu ligand binding and regional EAA-stimulated phosphoinositide hydrolysis have localized the metabotropic receptor to the telencephalic regions, such as the hippocampus, cortex, and striatum (Schoepp *et al.*, 1990; Young & Fagg, 1990). They are also localized in the cerebellum to Purkinje cells (Makowiec *et al.*, 1990) and granule cells (Irving *et al.*, 1990). A summary of the reported evidence supporting various functions of the metabotropic receptor is given hereafter:

(1) The most easily demonstrable functions of the metabotropic Glu receptor are electrophysiological. When Quis, Ibo and glutamate were tested on *Xenopus* oocytes injected with rat brain mRNA, oscillatory Cl^- currents were observed (Gundersen *et al.*, 1984). These oscillatory waves have since been the electrophysiologic hallmarks of metabotropic receptor activation. Injection of $InsP_3$ could mimic this, and it would cross-desensitize with L-Glu (Sugiyama *et al.*, 1987). Application of low concentrations of ACPD to hippocampal neurons (Stratton *et al.*, 1989) and spinal cord neurons (McLennan & Liu, 1982) increase their rate of firing.

The explanations for these observations are still somewhat speculative.

(2) Another set of electrophysiologic studies have demonstrated that a brief exposure of hippocampal and cortical slices to Quis and ACPD sensitizes them to AP4, and causes a 30 to 100-fold (Quis) and a 2 to 5-fold (ACPD) decrease in the EC_{50} required to depolarize the neurons (Whittemore & Cotman, 1991). However, since CCh and NE also caused a degree of sensitization similar to ACPD, it is likely that this is a result of the general activation of the phosphoinositide second messenger system, and not a direct result of the metabotropic Glu receptor.

(3) Long-term depression (LTD) is a related phenomenon to LTP, and occurs when heterosynaptic inputs converge onto a single neuron. If one of these inputs is weak whereas the other one is a strong, high-frequency input, then the weaker synapse undergoes LTD. LTD was first reported in the hippocampus (Lynch *et al.*, 1976), and the NMDA antagonist AP5 was found to be ineffective in attenuating LTD in rat hippocampal slices (Stanton & Sejnowski, 1989). However, the metabotropic antagonist AP3 was effective in blocking LTD in the same preparation (Stanton *et al.*, 1991). This suggests an involvement of the metabotropic Glu receptor in the modulation of synaptic inputs seen in LTD. One possible mechanism for this action was reported by Fagni *et al.* (1991), when they identified the activation of a large conductance Ca^{2+}-dependent K^+ channel in primary cultures of cerebellar granule cells treated with ACPD. Activation of this outward K^+ current would hyperpolarize the cell, and therefore render it less active. However, a recent report by Crepel *et al.* (1991) suggests that metabotropic Glu receptor activation does not underlie LTD in cerebellar slices.

(4) An enhancement of Ibo and ACPD stimulated phosphoinositide hydrolysis is seen 5 hours after the induction of LTP in rat hippocampal slices, but not two hours after induction (Aronica *et al.*, 1991). This enhancement can be blocked by NMDA antagonists during the tetanic stimulation. This is suggestive of a role in the late expression and maintenance of LTP for the metabotropic Glu receptor, probably via an intracellular [Ca^{2+}] increase leading to PKC activation. An *in vivo* correlate is that maze learning in rats results in increased Glu- and Ibo-evoked phosphoinositide hydrolysis from their hippocampal slices (Nicoletti *et al.*, 1988b).

(5) The metabotropic Glu receptor has also been implicated in excitotoxicity. In rats, hypoxic/ischemic brain damage (Chen *et al.*, 1988) and transient global ischemia (Seren *et al.*, 1989) result in enhanced Quis and Ibo stimulated InsP release in the damaged tissues, although no causal relationship has been established. In primary cultures of cortical neurons (Patel *et al.*, 1990) and hippocampal slices (Garthwaite & Garthwaite, 1989), exposing the neurons to Quis along with AMPA antagonists (eg. CNQX) failed to protect them against excitotoxic neurodegeneration. The metabotropic antagonist L-AP3 can protect rat hippocampal slice neurons in the CA1 region from hypoxic injury (Opitz & Reymann, 1991). However, a recent report demonstrated that concentrations of ACPD as high as 1mM failed to elicit any morphological or chemical signs of neurotoxicity in primary cultures of mouse cortical neurons, but did evoke significant increases in phosphoinositide hydrolysis and intracellular Ca^{2+} concentration (Koh *et al.*, 1991).

(6) Probably the most convincing role attributed to the metabotropic Glu receptor is its role in development and synaptic plasticity. Metabotropic responses to all the agonists are greatly enhanced in neonatal animals, and the response drops off towards adulthood. This has been demonstrated in primary cultures of striatal neurons, where the period of maximal Glu-evoked response at 7 days *in vitro* (DIV)(Weiss *et al.*, 1988) corresponds to the period of maximal synaptogenesis (Weiss *et al.*, 1986). This enhanced metabotropic response seen in neonatal tissues has also been tested *in vitro* with kindled rat hippocampal slices (Iadorola *et al.*, 1986), and *in vivo* in the kitten striate cortex by both agonist and AP3 (Dudek & Bear, 1989). Nicoletti *et al.* (1986c) also report that the magnitude of phosphoinositide hydrolysis evoked by agonists diminishes greatly after synaptogenesis, and lesioned glutamatergic pathways exhibit the neonatal, enhanced metabotropic response concomitant with re-afferentation (Nicoletti *et al.*, 1987a).

3. TRANSDUCTION OF THE METABOTROPIC GLUTAMATE RECEPTOR

Recall the three things necessary for a cell to respond to an external stimulus. The previous section has described the first step in the cellular response to glutamate - recognition by its receptor. This next section deals with the last two steps: the intracellular messenger that transduces the signal from the receptor into the cell, and the effectors.

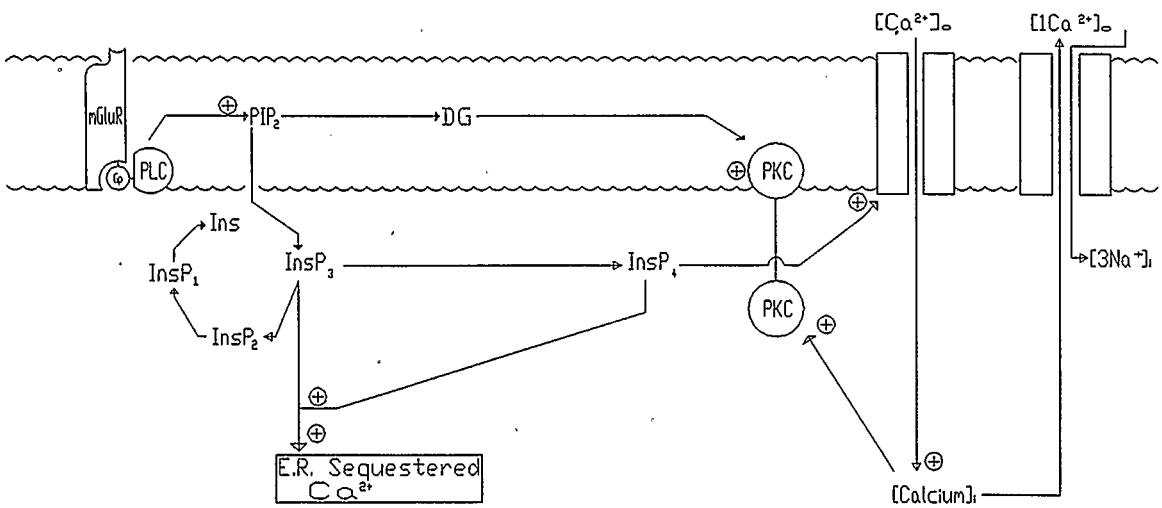
3.1 Discovery of the Phosphoinositide Signalling Pathway

The phosphoinositide signalling system was first discovered by Hokin and Hokin in 1953 when they noticed increased ^{32}P-labelling of phospholipids upon stimulation of pancreatic muscarinic receptors with acetylcholine. The formal link between receptor mediated phospholipid turnover and increases in intracellular Ca^{2+} was proposed in 1975 by Michell. This was quite an important idea, since the notion that a relatively minor phospholipid that was believed to function mainly for membrane structure could actually be an important metabolic intermediate was quite novel. Subsequently, Takai *et al.* (1979) demonstrated that PKC was activated by the 1,2-diacylglycerol (DG) formed from phosphoinositide hydrolysis. In 1981, Michell *et al.* identified the phospholipid as phosphatidylinositol-4,5-bisphosphate (PIP_2), and Berridge and Irvine (1984) identified d-myo-inositol-1,4,5,-trisphosphate ($Ins(1,4,5)P_3$) as the substance that released Ca^{2+} from the intracellular stores.

3.2 Sequence of Events Following Metabotropic Glu Receptor Activation

The sequence of events following metabotropic Glu receptor activation is briefly summarized as follows (see Fig. 3 below): The metabotropic Glu receptor is coupled via guanine nucleotide-binding proteins (G proteins) to a family of phospholipase C (PLC) enzymes, which catalyze the hydrolysis of membraneous PIP_2 into two second messengers: $Ins(1,4,5)P_3$) and a diacylglycerol (Fisher & Agranoff, 1987). The soluble $Ins(1,4,5)P_3$ diffuses from the plasma membrane and binds onto intracellular receptors that release sequestered Ca^{2+} from non-mitochondrial stores (Berridge, 1984). Meanwhile, the DG remains in the membrane and acts by stimulating the translocation of PKC from the membrane into the cytosol. PKC is also

FIGURE 3: DIAGRAMMATIC REPRESENTATION OF THE PHOSPHOINOSITIDE SIGNALLING CASCADE



Abbreviations: **DG**, 1,2-diacylglycerol; **E.R.**, endoplasmic reticulum; **G_p**, stimulatory GTP-binding protein; **Ins**, myo-inositol; **$InsP_1$**, inositol-1-monophosphate; **$InsP_2$**, inositol-1,4-bisphosphate; **$InsP_3$**, inositol-1,4,5-trisphosphate; **$InsP_4$**, inositol-1,4,5,6-tetrakisphosphate; **mGluR**, metabotropic glutamate receptor; **PIP_2**, phosphatidyl-inositol-4,5-bisphosphate; **PKC**, protein kinase C; **PLC**, phospholipase C

synergistically activated by the Ca^{2+} released by the InsP arm of the pathway.

This is, of course, a gross simplification of a very complex process. The key point is that activation of the metabotropic glutamate receptor eventually results in two 'effectors': an elevation in $[Ca^{2+}]_i$ and the activation of PKC. It is also obvious that such a complex cascade will have many points at which modulation could occur. Three potential sites of regulation, the G-protein coupling, the PLC coupling and PKC feedback, will be discussed below in sections 3.3 to 3.5

3.3 G-Protein Interaction

Upon ligand binding, the metabotropic Glu receptor undergoes a conformational change which activates the receptor. It then binds onto a G protein via a cytoplasmic loop on the transmembrane segments of the receptor. This binding in turn activates the G protein, and catalyzes the displacement of a GDP from the G protein. The protein then unfolds rapidly into an open configuration, and binds free GTP associated with the plasma membrane (Brandt & Ross, 1986). The metabotropic Glu receptor then diffuses from the activated GTP-G protein complex, and is capable of activating other G proteins to cause a 'cascading' effect. The activated GTP-G protein complex then dissociates into two subunits: a Gs(β,γ) subunit and an active Gs(α)-GTP subunit (Gilman, 1987). The activation is terminated by a 'timed' hydrolysis of GTP to GDP by the protein itself (Gilman, 1987).

The G protein itself is amenable to pharmacologic manipulation by pertussis toxin (PTX), the causative agent released by the pathogen *Bordetella pertussis*. PTX is a non-competitive antagonist that causes the ADP-ribosylation of the GTPase site of the G protein, thereby inactivating it (Gilman, 1987). PTX-sensitive metabotropic transduction evoked by metabotropic Glu receptor agonists has been demonstrated by Sugiyama *et al.* (1987) in mRNA injected *Xenopus* oocytes, Chuang and Dillon-Carter (1988) in NCB-20 neurohybrid cells, and Houamed *et al.* (1991) in *Xenopus* oocytes injected with the putative cDNA clone of the metabotropic receptor. The G protein activation process also has a micromolar requirement for Mg^{2+}. Although this requirement is not clearly understood, it is believed that the Mg^{2+} binds onto two distinct sites on the Gs(α) subunit and stabilizes the enzymatic activity. G protein involvement in phosphoinositide hydrolysis has also been suggested by numerous studies using non-hydrolyzable GTP analogs such as GTPγS to stimulate phosphoinositide hydrolysis (Cockcroft & Gomperts, 1985; Gonzales & Crews, 1985; Claro *et al.*, 1989; White & Scates, 1991), by using AlF_4^- to activate G proteins (Jope, 1988; Chandler & Crews, 1990). However, whether these G proteins are coupled to the metabotropic Glu receptor or to another receptor capable of inducing phosphoinositide hydrolysis (eg. the muscarinic receptor) is presently unknown.

3.4 Phospholipase C Interaction

Whilst it is activated, the Gs(α)-GTP subunit binds directly onto a polyphosphoinositide-specific PLC (Eberhard & Holz, 1988; Chandler & Crews, 1990). The PI specific PLC (PI-PLC) then catalyzes the hydrolysis of membraneous PIP_2 into $InsP_3$ and a DG. The exact DG released depends upon the fatty acid

composition of the original PIP_2. Recent evidence also suggests that Ca^{2+} may also directly activate PLC without a G-protein (for review, see Eberhard & Holz, 1988), or somehow facilitate PI-PLC activation (Patel *et al.*, 1991). This has been demonstrated in rat cerebral synaptoneurosomes (Chandler & Crews, 1990), rat cortical slices (Baird & Nahorski, 1990a), guinea pig cortical slices (Alexander *et al.*, 1990), bovine cerebellar membranes (Eberhard & Holz, 1988) and chicken brain membranes (Hofmann & Habermann, 1990). The best evidence comes from studies using Ca^{2+} ionophores. Kendall and Nahorski (1984) demonstrated that the production of InsP in rat cortical slices is increased by the Ca^{2+} ionophore A23187, and Fisher and Agranoff (1981) had similar findings in rat synaptosomes.

PI-PLC activation by GTPγS, CCh and NE in rat cortical slices is impervious to exogenous Li^+ (Etindi *et al.*, 1990). All these effects can be inhibited by reducing the extracellular Ca^{2+} concentration, or by including EGTA. It is believed that this Ca^{2+} preferring type of PLC is found predominantly on intracellular organelle membranes rather than the plasma membrane (Eberhard & Holz, 1988). This significance of this PI-PLC isoenzyme is not understood. However, the actual PLC enzyme itself consists of 4 isoforms: α, β_{1-2}, γ_{1-2}, and δ_{1-3}, which are the products of 4 discrete genes (Rhee et al., 1989). The 4 genes have been sequenced, and the corresponding amino acid sequences of the purified peptides have been largely elucidated (Rhee, 1991). All 4 have Ca^{2+}-dependent catalytic lipase activity (Rhee, 1991), but PTX-sensitive G-proteins specifically activate only the $\beta 1$ isozyme (Taylor et al., 1991).

3.5 Protein Kinase C Feedback Interaction

Both the DG and the Ca^{2+} released by metabotropic activation can induce translocation of PKC. This has been demonstrated in primary cultures of striatal neurons, and there is evidence of negative feedback by PKC on phosphoinositide metabolism (Weiss *et al.*, 1989; Manzoni *et al.*, 1990b). Potential feedback of PKC on the phosphoinositide cascade has been demonstrated by using tumorigenic phorbol esters, which potently enhance PKC activity by lowering its requirements for Ca^{2+} and lipid cofactors. This feedback has been demonstrated in primary cultures of striatal neurons stimulated by 10μM Quis (Manzoni *et al.*, 1990b, 1991), primary cultures of cerebellar granule cells (Canonico *et al.*, 1988), rat hippocampal slices (Stratton *et al.*, 1989) and rat cortical slices (Godfrey & Taghavi, 1990). Nicoletti and coworkers recently used another approach to this question by studying the desensitization of Glu- and Quis-evoked phosphoinositide hydrolysis caused by a 30-240 min. preexposure to Glu. They found that the PKC inhibitors sphingosine, polymyxin B and trisialogangliosides prevented this desensitization in cultured cerebellar granule cells (Catania *et al.*, 1991). In addition, the activation of PKC by Ca^{2+} has been suggested to involve calmodulin, thereby providing another potential site for regulation.

3.6 Effect of Extracellular Ca^{2+} on the Metabotropic Response

The inhibitory effect of removing extracellular Ca^{2+} cations on the metabotropic response has been reported previously in a number of preparations. This can be accomplished by simply not adding Ca^{2+} to the incubation buffer, or by chelating with

EGTA. This has been demonstrated in primary cultures of cortical neurons (Patel *et al.*, 1991), rat hippocampal slices (Palmer *et al.*, 1988) and guinea pig cortical slices (Alexander *et al.*, 1990). Direct stimulation of tritiated inositol polyphosphate ([^{3}H]-InsP_x) accumulation has been demonstrated with Ca^{2+} ionophores (Gonzales & Minor, 1989; Baird & Nahorski, 1990a). Guiramand *et al.* (1991b) studied the effects of Ca^{2+} on rat brain synaptoneurosomes with both agonist-evoked [^{3}H]-InsP_x accumulation and fura-2 imaging, using chelating agents, specific Ca^{2+} channel blockers, ionically modified buffers with Cd^{2+} and Ca^{2+} ionophores. They concluded that the Glu-evoked metabotropic response is dependent on extracellular Ca^{2+} entry via a Cd^{2+}-sensitive but channel blocker-insensitive mechanism.

It has been proposed that increased intracellular Ca^{2+} can directly activate PLC (for review, see Eberhard & Holz, 1988), and thereby enhance phosphoinositide hydrolysis. However, increased intracellular Ca^{2+} does not equate to increased phosphoinositide turnover, since all Glu-R subtypes (NMDA, KA, AMPA, ACPD) can increase intracellular Ca^{2+} as measured by fura-2 fluorescence (Holopainen *et al.*, 1991), but only the NMDA and ACPD receptors can cause any appreciable phosphoinositide turnover. Fowler and Tiger (1991) have suggested that the relative dependency on Ca^{2+} of the various agonists is a measure of the degree to which phosphoinositide hydrolysis is secondary to Ca^{2+} influx or a direct effect upon PLC for that agonist. Thus, for short incubation times (~30 sec.) there is an absolute requirement for Ca^{2+} for CCh-, Quis- and ACPD-evoked but not Glu-evoked responses (Alexander *et al.*, 1990). However, this Ca^{2+} requirement disappears for

CCh and Quis but not ACPD nor K^+ for long (>45 min.) incubations (Alexander *et al.*, 1990).

3.7 Effect of Extracellular Na^+ on the Metabotropic Response

An enhancement of EAA-evoked phosphoinositide hydrolysis caused by removal of extracellular Na^+ has been reported in primary cultures of cerebellar granule cells (Holopainen *et al.*, 1991), striatal slices (Doble & Perrier, 1989), rat cortical slices (Jope *et al.*, 1990) and rat brain membranes (Chandler & Crews, 1990; Guiramand *et al.*, 1991a). It is important to note that the inclusion of tetrodotoxin with the agonists does not enhance phosphoinositide hydrolysis as does Na^+ removal (Sladeczek *et al.*, 1985; Jope *et al.*, 1990; Guiramand *et al.*, 1991a); presumably, this indicates that the sodium effect is not mediated via voltage-sensitive sodium channels. However, various agents that increase intracellular Na^+ such as veratridine, batrachotoxin, joro spider toxin (all via voltage-gated Na^+ channels), monensin (a Na^+ ionophore) and ouabain (blocks Na^+/K^+-ATPase) all increase phosphoinositide hydrolysis in various neural preparations (Kendall & Nahorski, 1984; Gusovsky *et al.*, 1986; Hollingsworth *et al.*, 1986; Recasens *et al.*, 1987; Gusovsky & Daly, 1988b; Baird & Nahorski, 1990a).

Several investigators have proposed that this elevation of basal phosphoinositide hydrolysis in low Na^+ may be due to a reversal in the direction of the Na^+/Ca^{2+} exchanger, thereby increasing intracellular Ca^{2+} (Chandler & Crews, 1990; Jope *et al.*, 1990). This is supported by the fact that in synaptoneurosomes in nominally Na^+-

free medium, decreasing or increasing the extracellular Ca^{2+} results in dose-dependent decreases or increases, respectively, phosphoinositide hydrolysis (Chandler & Crews, 1990). In addition, intracellular Ca^{2+} levels are increased in fura-2 loaded synaptoneurosomes in Na^+-free medium (Daniell *et al.*, 1987; Chandler & Crews, 1990). Guiramand *et al.* (1991a) used amiloride and its derivatives, which are known to block both Na^+/Ca^{2+} and Na^+/H^+ exchangers, and found an elevation in basal but not Glu, CCh nor KCl-evoked [^{3}H]-$InsP_1$ accumulations in rat brain synaptoneurosomes. Similar results from Benuck *et al.* (1989) have implicated the Na^+/Ca^{2+} exchanger. There have also been suggestions that Na^+ acts upon G protein-PLC coupling to enhance phosphoinositide hydrolysis (Gusovsky & Daly, 1988b; Chandler & Crews, 1990). For example, the inhibitory G_i protein associated with cyclic AMP is reported to be Na^+-dependent (Jakobs & Wieland, 1989).

3.8 Effect of Extracellular Mg^{2+} on the Metabotropic Response

The role of extracellular Mg^{2+} in the metabotropic response is not clear. In cultures of cerebellar granule cells, Nicoletti *et al.* (1987b) found that removing Mg^{2+} from the extracellular buffer caused an increase in L-Glu and NMDA-evoked [^{3}H]-$InsP_1$ accumulation, but not Quis-evoked responses. O'Neill *et al.* (1991) also observed that Mg^{2+} inhibited [^{3}H]-PIP_2 hydrolysis in the post-mortem human brain. However, Doble and Perrier (1989) found that Mg^{2+} had negligible effects on NMDA and CCh-evoked responses in neonatal rat striatal slices. Despite these results, other investigators have found that Mg^{2+} stimulates PLC activity in rat cortical membranes (Litosch, 1987, 1989; Claro *et al.*, 1989). $Mg^{2+}$is also known to promote the activity

of many G proteins by activating and stabilizing the G_α subunit (for review, see Gilman 1987). And finally, the NMDA receptor exhibits a well-characterized, voltage-dependent Mg^{2+} blockade of the channel (for review, see Smart, 1989). With all these potential physiologic functions, interpretations of the action of Mg^{2+} is difficult.

SECTION TWO: THE STRIATAL MODEL OF THE METABOTROPIC RECEPTOR

4. INTRODUCTION TO THE STRIATUM

The brain region in which the metabotropic receptor will be studied is the striatum. Why is the striatum a good choice instead of, say, the cerebellum or hippocampus? Glutamate evoked phosphoinositide turnover has been reported in many brain regions including the cerebellum and hippocampus. The main criteria for selecting the striatum was that a good model system, namely the serum-free primary culture of embryonic mouse striatal neurons, was available and that the metabotropic response was originally discovered in this system. The neurons obtained from this system are homogeneous and highly differentiated, and thus represent a good substrate for the receptor. The advantages of this method will be discussed below.

The basal ganglia (BG) are generally considered to consist of the caudate nucleus, the putamen, and the external and internal globus pallidus (GPi and GPe). The caudate and putamen are collectively known as the striatum, and represent the input component of the BG. The GP is the output side of the BG. The BG are thought to function as a motor loop that processes information from the entire neocortex and returns it to the motor cortex via the substantia nigra (SN) and thalamic nuclei, thus acting as a "volume control" on movement by dominating the fine motor control from the lateral cerebellar lobes (McGeer & McGeer, 1987). However, connections

between the ventral striatum and the hypothalamus are thought to be more involved with behaviour and emotion than motor control (McGeer & McGeer, 1987).

4.1 Ultrastructural Organization of the Striatum

The organization of the BG can be considered on both the ultrastructural and cellular levels. Although the BG were traditionally thought to be a funnel for information from the neocortex, they are now believed to consist of parallel circuits, rather than a convergent funnel (Goldman-Rakic & Selemon, 1990).

4.2 Afferent Projections into the Striatum

Inputs into the striatum, largely from the neocortex, resolve it into three ultrastructural areas. All these cortical inputs are glutamatergic or aspartatergic (Carlsson & Carlsson, 1990). Columns of afferents segregate into contiguous longitudinal zones within the striatum. Within each zone, the afferents from distinct cortical areas terminate in a mosaic of non-overlapping, interdigitating domains of 350-900μM in diameter (Goldman-Rakic & Selemon, 1990).

4.3 Efferent Outputs from the Striatum

The output from the striatum works similarly to the input. In general, all projections from the striatum are inhibitory, GABAergic synapses. Subpopulations of these GABAergic neurons co-localize with substance P (Penny *et al.*, 1986), and with β-endorphin and met-enkephalin (Oertel *et al.*, 1983; Pasik *et al.*, 1987; Bolam & Smith, 1990). The GABA neurons colocalizing with substance P preferentially

project to the GPi, whereas GABA/enkephalin neurons preferentially project to the GPe; both types project equally to the SN (McGeer & McGeer, 1987). There are direct, striatal projections only to the GPe and SN; there are indirect, multisynaptic connections to the thalamus and subthalamic nuclei. Thus, the mapping is not somatotopic.

4.4 Cellular Organization and Composition of the Striatum

In its simplest from, the cellular composition of the striatum can be visualized as a three-dimensional mosaic of neurons. Since the primary cultures of striatal neurons used in these experiments are derived from dissociated whole striata, the composition of the cultures is likely to be very similar to the composition *in vivo*. The vast majority (95%) of neurons within the striatum are medium-sized and densely spined (Mettler, 1968), and were first identified by Ramón y Cajal in 1911. These are the input/output cells; each neuron receives multiple inputs and sends out multiple projections. Almost half (47%) of the spines on any one given neuron are heterosynaptic, ie. they receive more than one input (Freund *et al.*, 1984). Furthermore, all of the heterosynaptic spines on the striatal neurons have been shown to have dopaminergic input by dual labelling with antibodies to tyrosine hydroxylase and anterograde Golgi stains from the cortex and hippocampus (Freund *et al.*, 1984). Thus, dopamine modulates other inputs received by the same spine, and proximal-distal as well as modulatory processing can all occur on each neuron within the striatum. The remaining 5% of neurons in the striatum are mainly cholinergic interneurons that receive a tonic, excitatory glutamatergic input from the

cortex (Scatton, 1987).

4.5 Physiologic Functions of the Striatum *in vivo*

The striatum is important in orchestrating motor control. It does not, however, issue motor commands; rather, it specifies the memory, planning, combination, direction and sequence of movements (Goldman-Rakic & Selemon, 1990). It is involved in oculomotor and cognitive processing as well as skeletomuscular (Alexander & Crutcher, 1990). The various disorders of the BG are locomotory in nature, and can be divided into 2 broad categories: hypokinetic (eg. Parkinson's disease) and hyperkinetic (eg. hemiballismus, Huntington's chorea). Hypokinetic disorders are thought to be associated with excessive tonic and acute inhibitory output to the thalamus, whereas hyperkinetic movements are thought to be associated with insufficient output to the thalamus (DeLong, 1990).

Ionotropic Glu receptors in the striatum function to receive the motor input from the cortex, and cortical ablations will result in a loss of high-affinity Glu uptake in the striatum (McGeer *et al.*, 1977). Most ionotropic subtypes have been found in the striatum (see section 4.6 below). In rats, intrastriatal and intra-GP injections of NMDA induce ispsiversive rotations (Jenner *et al.*, 1981) and locomotor hyperactivity and dyskinesia (Kerwin *et al.*, 1980), respectively. Little is known about the physiology of striatal metabotropic receptors, save that intrastriatal injections of 1S,3R-ACPD induce contralateral rotation (Sacaan *et al.*, 1991).

4.6 Localization of Glutamate Receptors in the Striatum

Glutamate receptors in the striatum have been localized mainly by radiolabel binding. Endogenous EAA concentrations in the striatum vary between 2-14 μmoles/g tissue (McGeer & McGeer, 1987) and [^{3}H]-Glu binding is reported at 340 fmoles/mg protein in rat striatum (Yoneda *et al.*, 1991). NMDA receptors have been most recently localized with [^{3}H]-CPP, a specific antagonist, to postsynaptic terminals (Samuel *et al.*, 1990). [^{3}H]-KA (Monaghan & Cotman, 1982) and [^{3}H]-AMPA (Monaghan & Cotman, 1983) binding have also been demonstrated in the rat striatum, with a preponderance of [^{3}H]-KA binding sites (for review, see Monaghan *et al.*, 1989). Binding studies with [^{3}H]-AP3, a specific metabotropic Glu receptor antagonist, have not been performed for the striatum.

In situ hybridization studies with the recently cloned Glu receptors demonstrate a high concentration of GluRA flip and GluRC flip receptors in the striatum, whereas GluRB flip mRNA is high during development but declines in adulthood (Monyer *et al.*, 1991). Masu *et al.* (1991) reported very little metabotropic receptor mRNA in the adult rat striatum; no other studies have been performed on striatal neurons to date.

4.7 Pharmacology of the Striatal Metabotropic Glutamate Receptor

The pharmacology of the striatal metabotropic Glu receptor was performed both in slices (Doble & Perrier, 1989) and in primary cultures (Sladeczek *et al.*, 1985; Schmidt *et al.*, 1987; Weiss *et al.*, 1988; Weiss, 1989; Manzoni *et al.*, 1990a,b, 1991),

and was summarized in sections 2.2 and 2.3 above. Briefly, the rank order of agonist potency in striatal slices was Quis>Ibo>>L-Glu>L-Asp>AMPA>D-Glu>NMDA (Doble & Perrier, 1989), whereas the rank order in cultured striatal neurons was Quis>Glu>Ibo>ACPD (Sladeczek *et al.*, 1985; Manzoni *et al.*, 1991). Phosphoinositide hydrolysis in cultured striatal neurons was inhibited by 1mM AP3 and 0.1μM PDBu (Manzoni *et al.*, 1991), and striatal slices were attenuated and enhanced by extracellular Ca^{2+} and Na^{+} reductions, respectively (Doble & Perrier, 1989).

5. PRIMARY CULTURE OF STRIATAL NEURONS *IN VITRO*

5.1 Discovery of the Primary Culture of Striatal Neurons

In 1982, Prochiantz *et al.* developed a modification for the primary culture of embryonic mouse mesencephalon neurons. They needed a pure, glial-free culture, and had to preclude serum from their culture media since serum promotes glial proliferation. However, neurons do not survive in the absence of serum for more than 7-8 days (DiPorzio *et al.*, 1980). They resolved this problem by using media that had been previously conditioned by exposure to a monolayer of brain cells for 2 days. With this technique they were able to promote neuronal survival, maturation and differentiation whilst reducing the glial contamination to only 5% (Prochiantz *et al.*, 1982). This technique was subsequently improved upon by Weiss *et al.* (1986,

1988) by replacing the conditioned medium with a defined medium supplemented with insulin, transferrin, progesterone, putrescine and selenium. This technique also permitted the survival, maturation and differentiation of neurons whilst reducing the glial proliferation to only 7%.

5.2 Choice of Experimental Model for the Metabotropic Receptor

With any investigation, the model system of choice should be suited to the questions being asked. Since the study undertaken is to characterize the pharmacologic properties and modulation of the metabotropic Glu receptor, a model system on the cellular level (versus a cell-free biochemical preparation) is indicated. Ideally, the cellular pharmacology and kinetics of the metabotropic Glu receptor should be studied in a whole animal. This ensures that the receptor is always maintained and regulated in a physiological environment. The behavioral correlates of the metabotropic Glu receptor can also studied, and long-term chronic experiments could be performed. However, it is technically complex to keep an animal alive, possibly under anaesthesia, whilst perturbing it. Altering the ionic/pharmacologic milieu of the receptor in a living animal is not only difficult but ethically questionable, and there are numerous uncontrollable variables. The presence of multiple feedback systems, as well as the presence of glia, macrophages, etc. also confound any interpretation of data obtained *in vivo*. Finally, the pharmacologic doses of antagonists required (eg. NMDA antagonists) will cause an animal to convulse and possibly die.

Thus, a reduced preparation seems more amenable to the study of the metabotropic Glu receptor. Neuronal membranes represent the most reduced model available. Although binding studies are most easily performed on disrupted membranes, there is no cellular response to correlate any of these observations to and therefore agonists and antagonists cannot be distinguished. This can be overcome by using synaptoneurosomes (Hollingsworth *et al.*, 1986). Nevertheless, membrane preparations are very reduced and intracellular physiology cannot be studied; most of the intracellular machinery has been rendered inoperative. Striatal slices are a good potential model system. However, the limited lifespan and thickness of the slice means that labelling of membrane phosphoinositides by [^{3}H]-inositol to equilibrium is not feasible. Exogenously added agents, although acting faster than in a whole animal, are nevertheless slower than in a more reduced preparation. Furthermore, the slice is not a pure neuronal population; the presence of glia, possible endothelial cells, etc. can confound the interpretation of any results.

This leaves the preparation of interest: primary culture of striatal neurons. Since the neurons are intact, physiological interpretations of agonist/antagonist actions and intracellular mechanisms are possible. The culture preparation is relatively pure (>93% neuronal) and homogenous when compared to slices.

6. EXPERIMENTAL OBJECTIVES AND RATIONALE

6.1 Statement of Hypothesis

From the Introduction thus far, the reader ought to gain the impression that the metabotropic response evoked by the EAAs is heterogenous, and its pharmacology appears to vary between preparations. Some investigators have put forth the hypothesis that these heterogenous responses are due to the existence of several subtypes of metabotropic Glu receptor (for review, see Sladeczek *et al.*, 1988) that have differential temporal-spatial localizations on the basis of their pharmacology. The recent report by Tanabe *et al* (1992) of multiple clones of the metabotropic Glu receptor gives support to this argument. Therefore, the hypothesis to be tested in this thesis is: **The heterogeneous responses and pharmacology observed upon stimulation of the metabotropic glutamate receptor are primarily due to the existence of multiple subtypes of the metabotropic receptor that are differentially coupled to intracellular mechanisms.**

6.2 Experimental Objectives

Returning to the original questions posed in the opening paragraphs, these experiments will attempt to answer them by characterizing the pharmacology and modulation of the metabotropic glutamate receptor in primary cultures of striatal neurons. Although much is known about the importance of Glu and Asp receptor action in the CNS, especially their role in learning, memory and plasticity, there is a paucity of research into the metabotropic receptor. To this end, three series of

experiments have been devised. The first series of experiments deal with the initial set-up of the [^{3}H]-InsP$_1$ assay and optimization of various experimental and tissue culture parameters.

The second series of experiments deal with the pharmacology of the metabotropic Glu receptor, particularly with reference to the phosphono-substituted derivatives AP3, AP4 and AP5. The main objective of these studies was to identify if multiple subtypes of the metabotropic Glu receptor exist with respect to these antagonists. The secondary objective involves the antagonists themselves; since AP4 and AP5 are known to interact at other Glu-Rs, an examination of their effects on the metabotropic response should give some insight into the role of the metabotropic Glu receptor in the context of its ionotropic relatives.

The third series of experiments deal with the non-receptor modulation of the phosphoinositide hydrolysis cascade. The potential regulation points mentioned above, ie. the G protein interactions, PLC coupling, PKC feedback, and effects of extracellular ions, will all be examined. The main objective of this series of experiments was to characterize mechanisms which can modify the metabotropic response *in vitro*, and extrapolate them to potential *in vivo* processes which can affect the function of this receptor.

MATERIALS AND METHODS

1. MATERIALS USED

The tissue culture reagents Dulbecco's Modified Minimal Essential Medium (DMEM), F12 nutrient mixture, heat-inactivated fetal bovine serum (FBS), glutamine, and penicillin/streptomycin were obtained from GIBCO/BRL Canada, Burlington, Ont. Poly-L-ornithine hydrobromide, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), transferrin, insulin, putrescine, selenium chloride, progesterone, as well as 2-amino-3-phosphonopropionate (AP3), 2-amino-4-phosphonobutyrate (AP4), 2-amino-5-phosphonovalerate (AP5) and N-methyl-D-glucamine chloride (NMDG-Cl) were obtained from Sigma Chemicals, St. Louis, MO. Quisqualic acid and ibotenic acid were obtained from Cambridge Research Biochemicals, Cambridge, England. (±)trans-1-aminocyclopentyli-1,3-dicarboxylic acid (ACPD) was obtained from Tocris Neuramin, Bristol, England. All agonists/antagonists were routinely checked for pH and precipitation before use. Phorbol-12,13-dibutyrate (PDBu) and 4-α-PDBu were from Sigma. Pertussis toxin was obtained from List Biological Labs, Irvine, CA. Analytical grade anion exchange resin AG 1-X8 (100-200 mesh, formate form) was obtained from BioRad Canada, Mississauga, Ont. [^{3}H]-myo-inositol was obtained from New England Nuclear, Boston, MA. Universol scintillation cocktail was obtained from ICN Biomedicals, Irvine, CA. Twelve well, gamma-sterilized plastic tissue culture plates were obtained

from Costar, Cambridge, MA. Ten ml. plastic scintillation vials were obtained from Beckman Canada, Mississauga, Ont. All other reagents were of laboratory grade and obtained from either Sigma or BDH, Toronto, Ont.

2. EXPERIMENTAL TECHNIQUES

2.1 Cell Culture Method: Primary Culture of Striatal Neruons

The animals used were timed-pregnant CD_1 albino mice (Charles River Canada, Ste.-Constant, Que.) from 2-8 months old. At gestational day 14 (E14), the pregnant female mice were killed by cervical dislocation and the embryos carefully dissected out into sterile PBS buffer [composition (mM): NaCl, 137; KCl, 2.7; Na_2HPO_4, 8; K_2HPO_4, 1.5; pH 7.1-7.3 in superclean ddH_2O] supplemented with 0.6% glucose and 1 IU/ml penicillin/streptomycin. Using aseptic technique in a sterile tissue culture hood at room temperature, the brains were removed from the embryos under a dissecting microscope and rinsed in PBS. Two whole striata were micro-dissected from each brain and pooled.

The striata were mechanically dissociated by 25 triturations with a fire-polished Pasteur pipette, and were centrifuged at 2000*g* at room temperature. The supernatant was aspirated, and the pellet resuspended in about 4 mls of a 1:1 mixture of DMEM and F12 nutrient. A 0.1ml aliquot was used to count the viable cells on a haemocytometer by 0.4% trypan blue dye exclusion. The cell suspension

was diluted to a concentration of $1.0X10^6$ cells/ml in serum-free, culture medium. The culture medium consisted of DMEM/F12 supplemented with glucose (0.6%), glutamine (2mM), sodium bicarbonate (3mM) and HEPES buffer (5mM), and a defined hormone mixture consisting of insulin (25μg/ml), transferrin (100μg/ml), progesterone (20nM), putrescine (60μM) and selenium chloride (30nM).

The cells were then seeded onto gamma-sterilized 12-well tissue culture plates (The plates had been previously coated with 1.5μg/ml poly-L-ornithine for 1 hour at 37°C. The plates were rinsed with sterile, superclean ddH_2O, coated again with DMEM/F12 containing 10% FBS for 1 hour at 37°C. They were then aspirated, and rinsed successively with sterile water and PBS. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 until ready for use (usually at 14 DIV). Feeding of the cells was not necessary, nor was the inclusion of cytosine arabinoside necessary to inhibit glial proliferation.

2.2 Assay for the Accumulation of [^{3}H]-InsP_1 in Striatal Neurons

The InsP assay was performed as follows. Prior to beginning the experiment, the cells in each well were visually inspected through a phase-contrast microscope (Zeiss 43-9901, West Germany; magnification X100 and X200) to ensure the cells were healthy. In order to pulse-label the intracellular InsP pools, the culture medium was aspirated from the 12-well plates. The cells were washed twice with PBS (composition as above but including 0.5mM $MgCl_2$, 0.9mM $CaCl_2$ and 0.1% glucose) and incubated at 37°C with 2μCi/ml [^{3}H]-myo-inositol in 1ml/well of PBS for 3

hours. After the incubation, the cells were washed in PBS to remove trace radioactivity, and were incubated with 0.9ml. PBS containing 1mM LiCl for 10 min. Antagonists and PDBu, if any, were added in with the LiCl as noted above and adjusted to a final pH of 7.1-7.3.

Following this 10 min. equilibration, EAA agonists were added into each well (in 100μl aliquots for a final volume of 1ml/well) for 10 min. After this 10 min. stimulation period, the reaction was terminated by aspiration of the PBS and the addition of 1ml of ice cold methanol. From this point on, the entire procedure was carried out on ice. The cells were scraped by a rubber policeman, and the methanol aspirated into centrifuge tubes. The wells were rinsed again with 1ml of ice-cold ddH_2O, and this was added to the methanol for a final volume of 2mls. Cellular debris were sedimented by centrifugation at 5000*g* at 4°C for 20 min, and the supernatant retained to separate out the bulk [^{3}H]-InsP_1 formed.

2.3 Separation of Accumulated [^{3}H]-InsP_1 by Anion-Exchange Chromatography

A 1.4 ml aliquot of the supernatant was removed and diluted with 5ml of ice-cold ddH_2O, and the entire 6.4 ml applied to 0.6cc AG 1-X8 anion exchange columns. The columns had been previously treated with 0.1M formic acid/1M ammonium formate to regenerate them to their formate anion form. The columns were then rinsed successively with 1ml ddH_2O, 2mls 60mM sodium formate/5mM sodium tetraborate, and another 2mls of ddH_20. [^{3}H]-InsP_1 was finally eluted by 3mls of 0.1M formic acid/0.2M ammonium formate directly into 10ml scintillation vials. Six

mls of Universol scintillation cocktail was added to obtain a translucent gel, and the radioactivity determined by liquid scintillation spectrometry in a Beckman LS3801 scintillation counter. The percent ^{3}H counting efficiency of the Universol thixotropic gel at 50% aqueous content is 35%, and 60dpm were automatically subtracted by the scintillation counter as background radioactivity.

The concentrations of agonists and antagonists used are given in their final volume. During the equilibration period with LiCl, there is only 0.9ml volume per well, and thus the concentration of antagonist is actually 11% greater than listed until the agonists are added.

3. SPECIFIC EXPERIMENTAL MANIPULATIONS

3.1 Phorbol Ester Experiments

Phorbol-12,13-dibutyrate (PDBu) and 4-α-PDBu were prepared in 100% dimethyl sulphoxide as a $5X10^{-3}M$ stock, and were diluted in PBS to $10\mu M$ for use. A $100\mu l$ aliquot of PDBu, 4-α-PDBu or PBS containing 0.5% DMSO (vehicle) was included with the LiCl incubation step, and was allowed a 10 min. equilibration period prior to agonist addition.

3.2 Specific Extracellular Cation-Reduced Experiments

The Ca^{2+} and Mg^{2+} cation-reduced PBS buffers were made by simply excluding the

cations from the PBS recipe, since these cations are present at <1mM and would not significantly lower the osmolarity of the PBS. In the case of the low sodium PBS, the 137mM NaCl was isosmotically replaced by 137mM NMDG-Cl with 8mM Na_2HPO_4 remaining, and the pH adjusted to 7.1 to 7.4. In these experiments, the cells were radiolabelled in normal PBS. When the 1mM LiCl was added, it was in the specific cation-reduced buffer, and from then on the cells were treated in that buffer. The agonists were also prepared in that specific cation-reduced buffer and added in a 100μl aliquot as per the normal protocol.

3.3 Pertussis Toxin Experiments

For the pertussis toxin experiments, the cells were treated overnight (16-18 hours) to a final concentration of 0.1 μg/ml PTX or vehicle control in a 5μl aliquot added directly to the tissue culture medium. When the medium was aspirated for the experiment the following day, no further PTX was added. Experiments were then carried out as described above.

4. STATISTICAL ANALYSES

The results of the experiments were generally expressed as a percentage of the basal, unstimulated [^{3}H]-$InsP_1$ release in PBS ± the S.E.M. (n-1 weighting), instead of as raw dpm counts. This was done to standardize for variations in the density of the cultures from experiment to experiment. By expressing values as a percentage of

paired control experiments in the same culture, many of these variations could be eliminated. Also, variations from experiment to experiment that affected the basal level of radioactivity detected but not the fold stimulation (eg. [^{3}H]-Ins loading, bed capacity of the columns, etc.) could be accounted for by transforming the raw counts to a percentage frequency distribution. By analogous reasoning for the inhibitory dose response curves, the data were expressed as a percentage of the maximal stimulation without antagonist.

All graphs, histograms and regressions were computed and plotted by Sigmaplot (Version 3.1) software (Jandel Scientific, San Rafael, CA.). Statistical analyses were performed by Pharm/PCS (Version 4.1) pharmacologic calculation software (Tallarida & Murray, 1987; Springer-Verlag Press, New York, NY.) using Student's grouped or paired t-tests. ANOVA testing was not indicated, since these were not totally random samples. A *p*-value less than 0.05 was considered significant. The concentrations of agonist eliciting a half-maximal response on dose response curves (the EC_{50} value) were 'eyeballed' graphically by plotting the half-maximal response versus the curve and estimating the resulting concentration of agonist.

RESULTS

1. OPTIMIZATION OF EXPERIMENTAL PARAMETERS

This section deals with the optimization of experimental parameters used in this study. Although the culture conditions and [^{3}H]-InsP$_1$ assay have been previously established (Weiss *et al.*, 1989), it was necessary to re-establish these parameters in this laboratory. It was also necessary to perform a number of dose response curves to establish optimal agonist and antagonist concentrations for the pharmacologic experiments.

1.1 Age of Neurons in Culture

Table II shows that CCh-evoked [^{3}H]-InsP$_1$ accumulation approaches a maximum at 14 DIV, and does not appear to change significantly thereafter. Thus, it was determined that subsequent experiments would utilize cells at 14 ± 1 DIV of age, unless otherwise noted.

1.2 Agonist Stimulation Duration

We hypothesized that the shortest stimulation possible to get an appreciable signal would be the best, since there would be less interference from various non-specific effects such as receptor down-regulation, desensitization, internal feedback mechanisms within the InsP cascade, second messenger cross-talk, etc. In addition, the shortest possible stimulation period would also be the more physiologically

TABLE II: DEVELOPMENTAL PROFILE OF 1mM CARBACHOL EVOKED [^{3}H]-$InsP_1$ ACCUMULATION

AGE (DIV)	% STIMULATION
1	98.8
3	212.0
7	209.9
13	344.4
14	388.6
15	407.8
16	235.8

Primary cultures of striatal neurons of the ages indicated were stimulated for a 10 min. with 1mM CCh, and soluble [^{3}H]-$InsP_1$ mass-separated by anion exchange chromatography.

The data represent means from 1 experiment performed in triplicate, and are expressed as a percentage of the basal [^{3}H]-$InsP_1$ accumulation in the absence of agonists and antagonists in paired control trials. Basal scintillation counts averaged 154 dpm/well (1 DIV) to 1940.7 dpm/well (15 DIV).

relevant to the *in vivo* situation. This would also lessen the chance of cross-reactivity of Quis at the AMPA-R and Ibo at the NMDA-R (see Discussion). As seen from Fig. 4, a 5 min. incubation time yielded only a 194.0% and 182.2% increase in the basal [^{3}H]-InsP$_1$ accumulation for Quis and CCh, respectively. This was significantly less than the accumulations at all other time points for either agonist. However, by 10 min., the accumulation had increased to 390.9% and 342.2% of basal for Quis and CCh, respectively. These accumulations did not increase significantly beyond 10 min., up to the 30 min. time point. Thus, it was determined that subsequent experiments would utilize an incubation period of 10 min. with the agonists, unless otherwise indicated.

1.3 Carbachol Dose Response Curve

Many subsequent experiments have included carbachol in addition to the EAA agonists. CCh is an effective agonist for the muscarinic receptor coupled to phosphoinositide hydrolysis, and was included as a non-EAA control for responses unique to EAAs. Figure 5 shows a sample dose response curve for CCh from one such experiment. At saturating concentrations (1mM), CCh evoked a 324 % increase over the basal [^{3}H]-InsP$_1$ accumulation. Three such experiments were performed, with essentially the same results.

1.4 Effect of Li^+ on Carbachol-Evoked [^{3}H]-InsP$_1$ Accumulation

LiCl greatly enhances [^{3}H]-InsP$_1$ accumulation by blocking Ins-monophosphate- and Ins-polyphosphate phosphatases from dephosphorylating it to Ins (see Introduction).

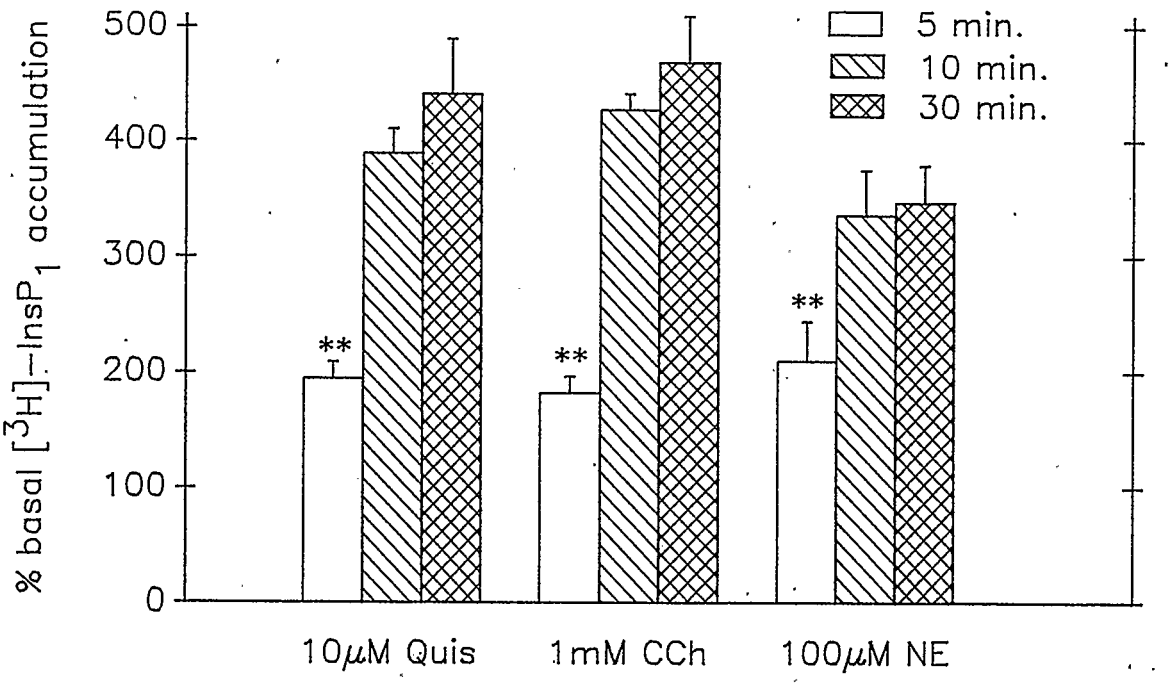


FIGURE 4. Effects of Agonist Incubation Time on [^{3}H]-InsP$_1$ Accumulation. Cultured striatal neurons were incubated with 1mM LiCl in PBS for 10 min., and were then stimulated for a further 5, 10, and 30 minutes with 10μM Quis, 1mM CCh and 100μM (-)arterenol (NE: norepinephrine).

The data represent means ± S.E.M. from 3 independent experiments in separate cultures, each performed in triplicate, and are expressed as a percentage of the basal [^{3}H]-InsP$_1$ accumulation in the absence of agonists and antagonists in paired control trials. Basal scintillation counts averaged 687.5 ± 44.6, 875.5 ± 99.7 and 1200.4 ± 190.5 dpm for 5, 10 and 30 min. trials, respectively.

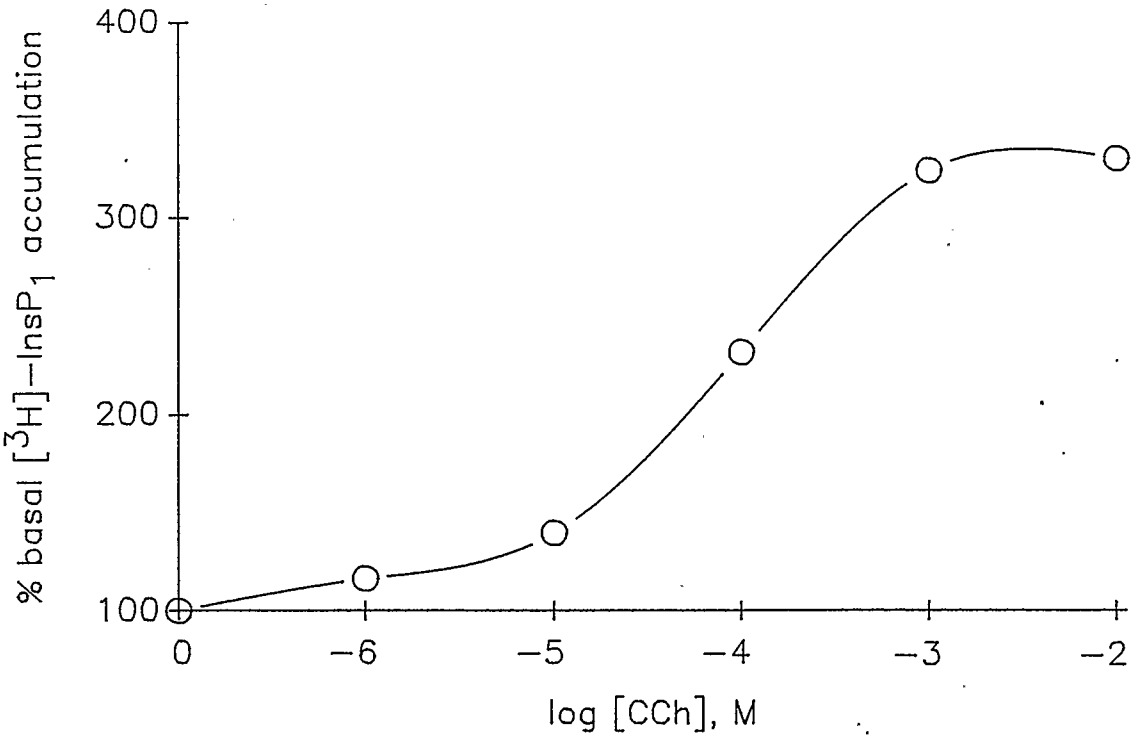


FIGURE 5. Carbachol Dose Response Curve. Cultured striatal neurons were incubated with 1mM LiCl in PBS for 10 min., and were then stimulated for a further 10 min. with varying concentrations of CCh.

The data represent means from one representative experiment performed in duplicate, and are expressed as a percentage of the basal [^{3}H]-InsP$_1$ accumulation in the absence of agonists and antagonists in paired control trials. Basal scintillation counts averaged 1069.3 ± 66.3 dpm.

As seen in Fig. 6, an extracellular Li^+ concentration of only 1mM already yields an appreciable increase in [^{3}H]-InsP_1 accumulation (181.8 ± 12.7% over control). Although higher concentrations of Li^+ did result in greater [^{3}H]-InsP_1 accumulation, it was determined that 1mM would suffice, and would serve to avoid any potential ionic side effects. Thus, all subsequent experiments utilized 1mM LiCl, unless otherwise noted.

2. RECEPTOR PHARMACOLOGY EXPERIMENTS

Previous studies have reported heterogenous responses for metabotropic receptor agonists in a variety of model systems, including primary cultures of striatal neurons (see Introduction). One possible explanation is that the agonists tested are acting at other EAA receptors (eg. at the NMDA-R) in addition to the metabotropic Glu receptor, thereby causing anomalous responses. An alternative explanation is that there may be subtypes of the metabotropic receptor coupled to different intracellular mechanisms for activating phosphoinositide hydrolysis that are selectively activated by different agonists. To discriminate between these possibilities, the following series of pharmacology experiments were performed. The operating hypothesis was that these experiments ought to identify if the agonists were acting via a common metabotropic receptor or via multiple subtypes.

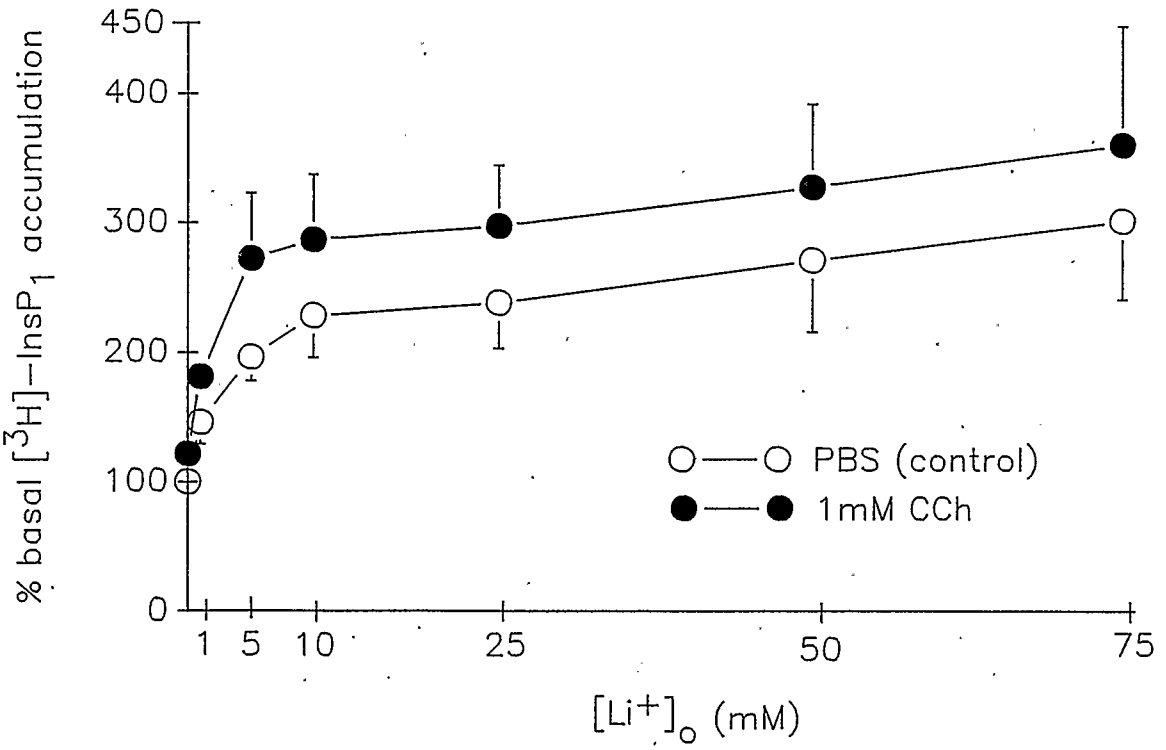


FIGURE 6. Effects of Extracellular Lithium on Carbachol-Evoked [^{3}H]-InsP$_1$ Accumulation. Cultured striatal neurons were incubated with varying concentrations of LiCl in PBS for 10 min., and were then stimulated for a further 10 min. with 1mM CCh.

The data represent means ± S.D. from 2 independent experiments in separate cultures performed in duplicate, and are expressed as a percentage of the basal [^{3}H]-InsP$_1$ accumulation in the absence of agonists and antagonists in paired control trials. Basal scintillation counts averaged 480.3 ± 51.3 dpm in the absence of Li^+ in PBS.

2.1 Agonist Dose Response Curves

In order to select the agonist concentrations used in subsequent experiments, dose response curves for the 3 EAA agonists were performed. Figure 7 shows dose response curves for the EAA agonists Quis, Ibo and ACPD (n=5). Quis was the most potent agonist, having an estimated EC_{50} of 0.2μM and a maximum effect of 362.6 ± 26.8 % over baseline at 1μM. In contrast, Ibo had an estimated EC_{50} of 4.4μM and a maximal effect of 305.1 ± 33.3 % at 10μM, whereas ACPD had an estimated EC_{50} of 31μM and a maximal effect of 306.7 ± 11.9 % at 100μM. Based upon these data, it was determined that subsequent experiments would utilize concentrations of 1μM, 10μM, and 100μM for Quis, Ibo and ACPD, respectively. These concentrations were saturating or near-saturating, and should yield robust responses.

2.2 Additivity Experiments

The simplest approach to determine whether the agonist action is mediated via the same receptor is to test the additivity of their responses. The prediction is that their effects on [^{3}H]-$InsP_1$ generation should be additive if the agonists act at different receptors, and non-additive if they act at the same receptors. Whether these receptors exist on all striatal neurons or on subpopulations *in vitro* will be addressed in the Discussion. As evident from Fig. 8, the effects of Quis, Ibo and ACPD on [^{3}H]-$InsP_1$ accumulation were not additive in any combination tested ($p > 0.05$). It could be argued, however, that the InsP cascade was already saturated by the concentration of EAAs used, and therefore no further additivity could be seen, ie. it is not the receptors but the other components of the cascade such as G proteins

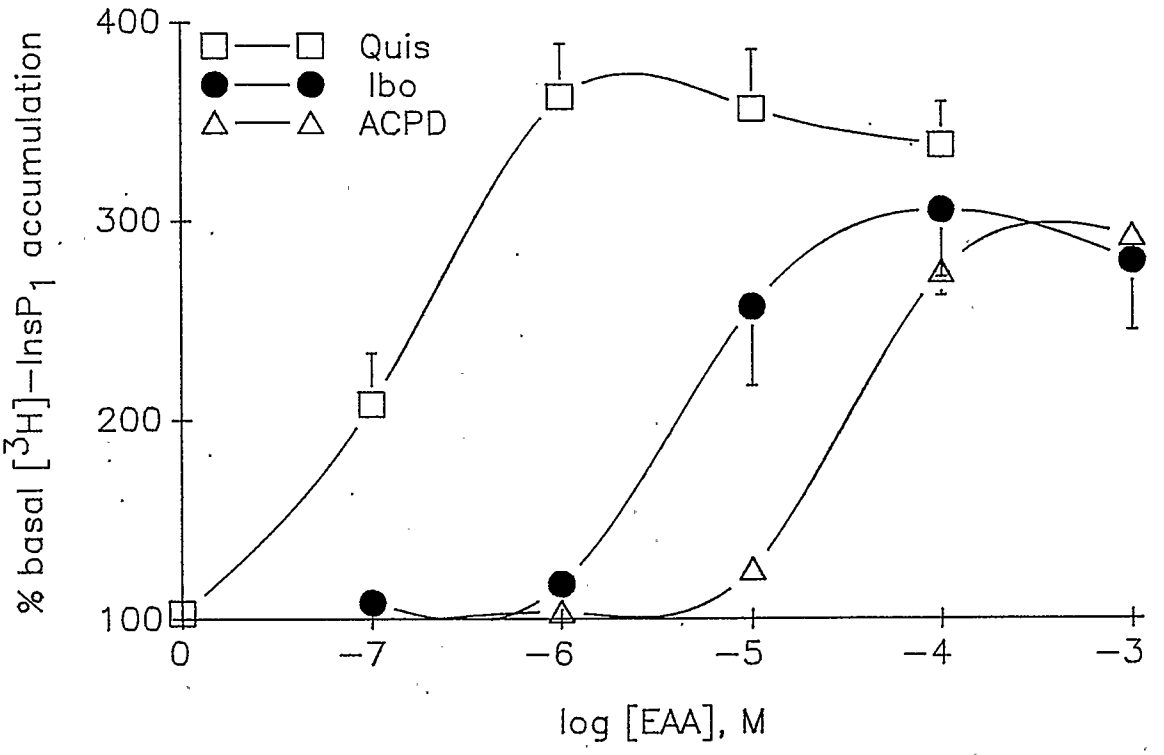


FIGURE 7. Excitatory Amino Acid Agonist Dose Response Curve. Cultured striatal neurons were incubated with 1mM LiCl in PBS for 10 min., and were then stimulated for a further 10 min. with varying concentrations of Quis, Ibo and ACPD.

The data represent means ± S.E.M. from 5 independent experiments performed in separate cultures in duplicate, and are expressed as a percentage of the basal [^{3}H]-$InsP_1$ accumulation in the absence of agonists and antagonists in paired control trials. Basal scintillation counts averaged 1069.3 ± 66.3 dpm. EC_{50} values were visually estimated to be 0.2μM, 4.4μM and 31μM, respectively, for Quis, Ibo and ACPD.

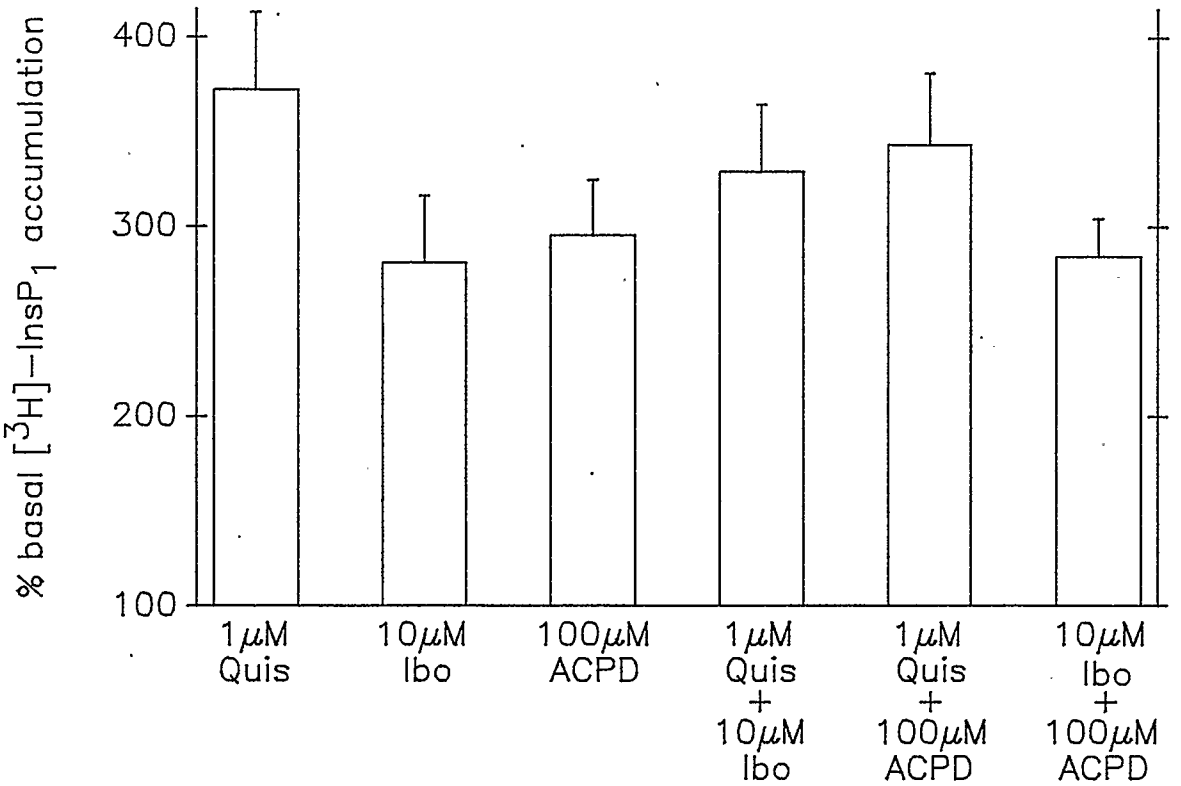


FIGURE 8. Excitatory Amino Acid Agonist Additivity Histograms. Cultured striatal neurons were incubated with 1mM LiCl in PBS for 10 min., and were then stimulated for a further 10 min. with 1μM Quis, 10μM Ibo and 100μM ACPD in the combinations listed.

The data represent means ± S.E.M. from 4-5 independent experiments performed in separate cultures in triplicate, and are expressed as a percentage of the basal [^{3}H]-$InsP_1$ accumulation in the absence of agonists and antagonists in paired control trials. Basal scintillation counts averaged 1081.2 ± 103.6 dpm.

None of the results were statistically different (Student's grouped t test).

or PLC that were being saturated. To discount this possibility, Table IV shows that the effect of Quis was additive with the effects of CCh and the adrenergic receptor agonist norepinephrine (NE). All of these agonists were additive; none of the double-agonist stimulations differed significantly from the theoretical additive value ($p > 0.05$). Thus, they lack of additivity amongst the EAA agonists is not due to saturation of the InsP cycle.

2.3 Antagonist Inhibitory Dose Response Curves in Fixed Concentrations of EAAs

Although the previous additivity experiments tested if the agonists were acting at a common receptor, they could not distinguish if the agonists were causing differential responses by acting at a common metabotropic receptor plus another EAA receptor, ie. the agonists were cross-reactive. Therefore, to elucidate this second possibility in cultured striatal neurons, inhibitory dose response curves were performed for the ω-phosphono-substituted EAA analogs AP3, AP4 and AP5. As previously outlined in the Introduction, AP3 and AP4 have been demonstrated to have inhibitory actions at the metabotropic receptor in various preparations, usually with a greater effect on neonatal tissues. AP5, a classic NMDA antagonist, was included to test for this putative cross-reactivity. If Quis, Ibo and ACPD were pure metabotropic agonists acting at a common receptor, the prediction would be that the inhibitory dose responses for AP3, AP4 and AP5 would be similar regardless of the agonist present. If the dose response curves were dissimilar, this would suggest that the agonists were acting via heterogenous mechanisms. Figures 9-11 are such inhibitory dose response curves for varying concentrations of AP3, AP4 and AP5 in the presence of 1μM Quis, 10μM Ibo and 100μM ACPD, respectively. It is interesting to note that at low

TABLE III: ADDITIVE EFFECTS OF EAA and non-EAA AGONISTS ON [^{3}H]-InsP$_1$ ACCUMULATION

AGONIST(S)	%BASAL [^{3}H]-InsP$_1$ ACCUMULATION	
1mM CCh	421.0 ± 6.6	
1μM Quis	421.7 ± 20.9	
100μM NE	415.0 ± 17.4	
1mM CCh+1μM Quis	662.2 ± 36.0	(742.7)
100μM NE+1μM Quis	756.5 ± 56.4	(736.7)
1mM CCh+100μM NE	695.4 ± 66.3	(736.0)

Cultured striatal neurons were incubated with 1mM LiCl in PBS for 10 min., and were then stimulated for a further 10 min. with 1μM Quis), 100μM (-)arterenol and 1mM CCh in the combinations listed.

The data represent means ± S.E.M. from 3 experiments performed in separate cultures in triplicate, and are expressed as a percentage of the basal [^{3}H]-InsP$_1$ accumulation in the absence of agonists and antagonists in paired control trials. The numbers in brackets represent the theoretical value predicted if the agonists were additive, with 100% basal subtracted. None of the actual experimental results differed significantly from the corresponding theoretical additive value ($p > 0.05$, Student's paired t test). Basal counts averaged 1081.2 ± 103.6 dpm.

concentrations (<3mM), AP3 and AP4 acted as partial agonists. This is not surprising, since they are phosphono-substituted derivatives of Glu and Asp, respectively, and suggests that they have their actions directly at the metabotropic receptor.

Figure 9 shows that AP3 is the most effective antagonist for Quis-evoked phosphoinositide hydrolysis; AP3 significantly attenuated the 1μM Quis-elicited [^{3}H]-$InsP_1$ accumulation at 1mM and 3mM ($p<0.01$). The maximal AP3 inhibition was achieved at 3mM, when [^{3}H]-$InsP_1$ accumulation was inhibited to 61.2 ± 3.3% of the Quis-evoked control response (defined as the 100% baseline). AP4 acted as a weaker antagonist, significantly attenuating the 1μM Quis-evoked response at concentrations > 1mM, with an observed maximal inhibition of 81.8 ± 6.6% at 3mM ($p<0.01$). AP5 was completely ineffective in attenuating the 1μM Quis-evoked response at concentrations up to 1mM.

Figure 10 shows that AP3 is also a potent antagonist for 10μM Ibo evoked [^{3}H]-$InsP_1$ release; at 3mM, it significantly ($p<0.01$) blocked the response to 67.5 ± 5.8% of the Ibo-evoked control response (defined as the 100% baseline). AP4 was also strongly antagonistic, achieving 67.4 ± 9% inhibition at 3mM ($p<0.01$). However, unlike the Quis response, AP5 was also remarkably effective upon Ibo. AP5 significantly attenuated the Ibo-evoked response at concentrations > 30μM, with a maximal inhibition to 74.5 ± 14.1% of control at 1mM ($p<0.01$).

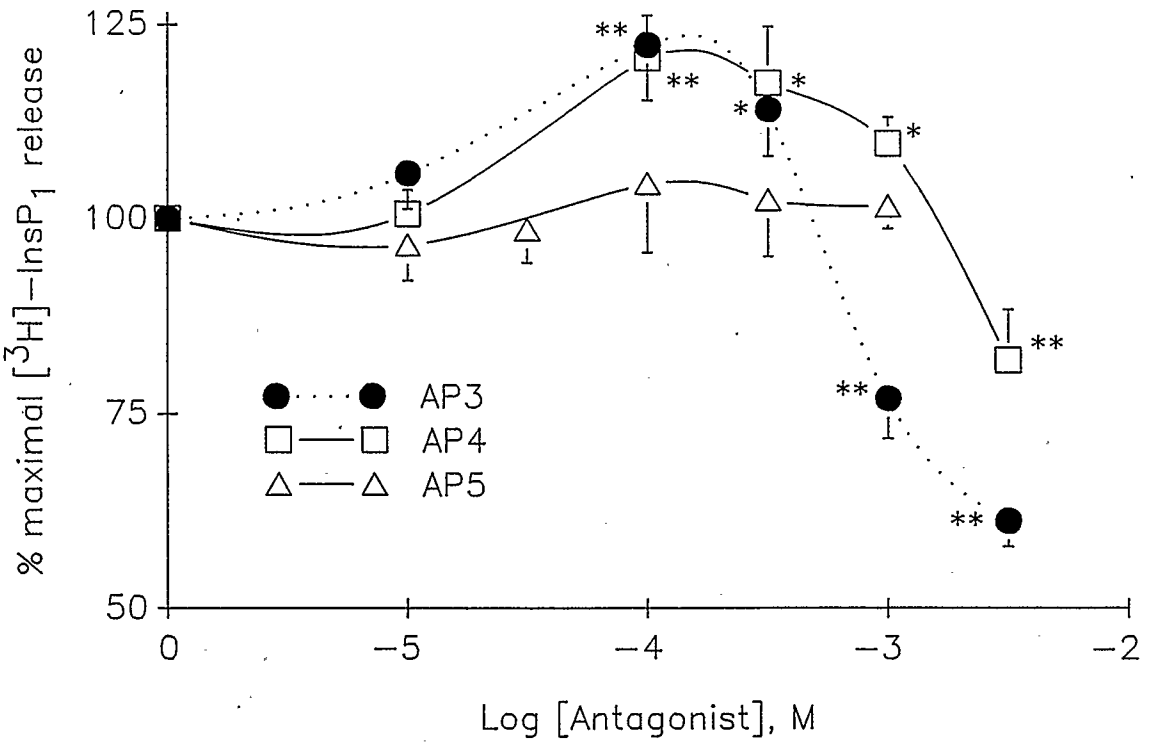


FIGURE 9. Excitatory Amino Acid Antagonist Inhibitory Dose Response Curves in the Presence of 1μM Quisqualate. Cultured striatal neurons were incubated with varying concentrations of D,L-2-amino-3-phosphonopropionate (AP3), L-2-amino-4-phosphonobutyrate (L-AP4) and 2-amino-5-phosphonovalerate (AP5) in the presence of 1mM LiCl in PBS for 10 min. as indicated above. The neurons were then stimulated for a further 10 min. with 1μM Quis.

The data represent means ± S.E.M. from 6-8 independent experiments performed in separate cultures in duplicate, and are expressed as a percentage of the maximal 1μM Quis-evoked [^{3}H]-InsP$_1$ accumulation in the absence of antagonists in paired control trials. Maximal Quis-evoked scintillation counts averaged 4057.8 ± 132.6 dpm.

*$p<0.05$, **$p<0.01$ compared to the 100% maximal stimulation (Student's grouped t test).

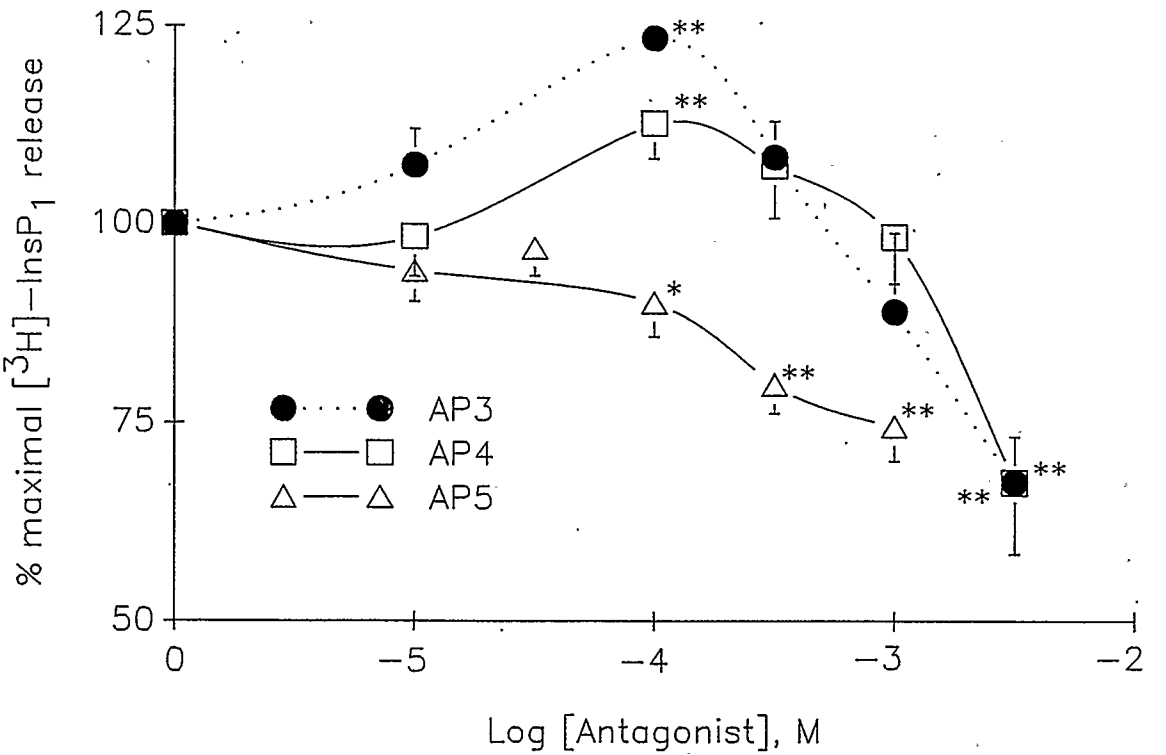


FIGURE 10. Excitatory Amino Acid Antagonist Inhibitory Dose Response Curves in the Presence of 10μM Ibotenate. Cultured striatal neurons were incubated with varying concentrations of D,L-2-amino-3-phosphonopropionate (AP3), L-2-amino-4-phosphonobutyrate (L-AP4) and 2-amino-5-phosphonovalerate (AP5) in the presence of 1mM LiCl in PBS for 10 min. as indicated above. The neurons were then stimulated for a further 10 min. with 10μM Ibo.

The data represent means ± S.E.M. from 6-8 independent experiments performed in separate cultures in duplicate, and are expressed as a percentage of the maximal 10μM Ibo-evoked [^{3}H]-InsP$_1$ accumulation in the absence of antagonists in paired control trials. Maximal Ibo-evoked scintillation counts averaged 2747.7 ± 83.6 dpm.

*$p<0.05$, **$p<0.01$ compared to the 100% maximal stimulation (Student's grouped t test).

Figure 11 demonstrates that AP3 and AP4 are almost equally potent at inhibiting 100μM ACPD-evoked [^{3}H]-InsP$_1$ accumulation. The maximal inhibitions achieved were 65.3 ± 7.1% of control for 3mM AP3 and 62.8 ± 5.8% of control for 3mM AP4 ($p<0.01$ for both). AP5 was a weak antagonist versus ACPD; it achieved significant inhibition at 0.3 - 1mM, blocking to a maximum of 84.7 ± 5.0% of the 100μM ACPD-evoked control response at 3mM ($p<0.01$). The profile of the AP5 effect are quite similar between Ibo and ACPD. Interestingly, low concentrations of AP3 and AP4 failed to act as partial agonists in the presence of 100μM ACPD. This observation will be discussed in greater detail later.

2.4 Agonist Dose Response Curves in Fixed Concentrations of EAA Antagonist

Figures 12-14 show dose response curves for Quis, Ibo and ACPD, respectively, in the presence of fixed concentrations of AP3, AP4 and AP5. These experiments were performed for the same reasons as the 3 previous inhibitory dose-response curves - to detect if cross-reactivity at other EAA receptors is the mechanism underlying the heterogenous actions of the agonists. In general, AP3 was a more effective antagonist than AP4 at equal concentrations. AP5 failed to have any significant effect at attenuating EAA-evoked [^{3}H]-InsP$_1$ accumulation, with the exception of Ibo at 1-100μM.

Figure 12a demonstrates that both 3mM AP3 and 3mM AP4 block Quis-evoked [^{3}H]-InsP$_1$ accumulation in a non-competitive fashion, although the AP4 inhibition was weaker and did not quite reach significance. 3mM AP3 in the absence of any agonist was able to elevate basal [^{3}H]-InsP$_1$ accumulation to 175.1 ± 24.1% ($p<0.01$).

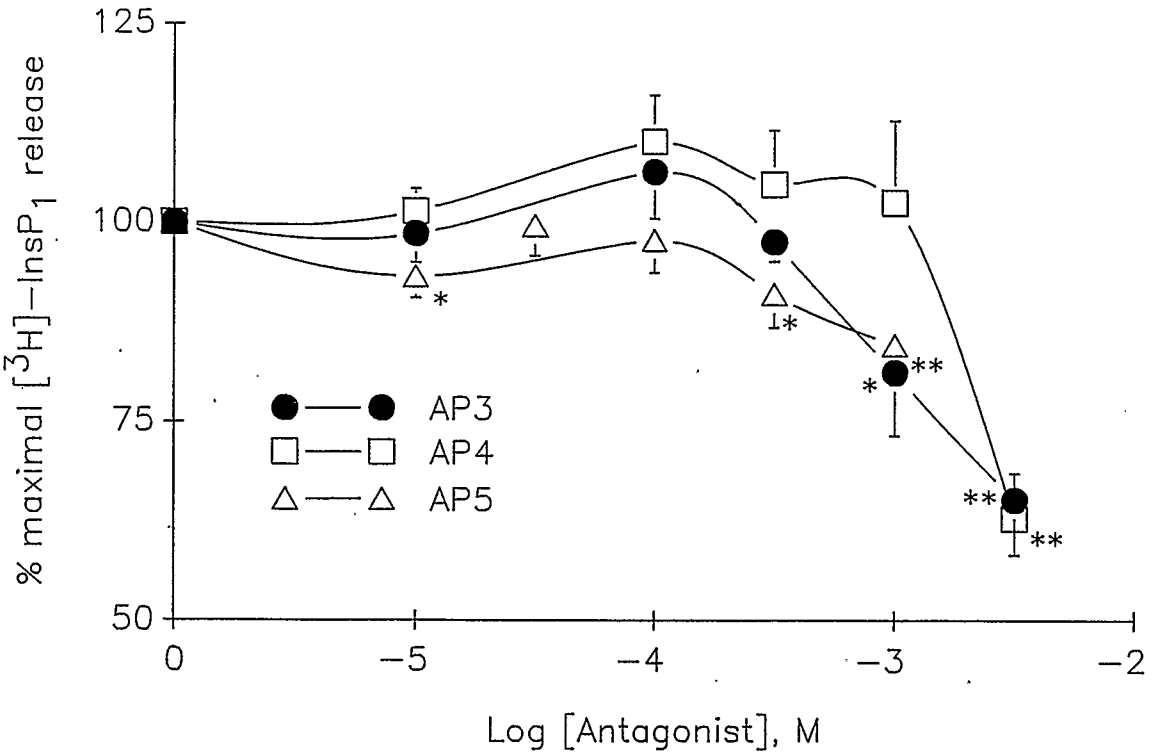


FIGURE 11. Excitatory Amino Acid Antagonist Inhibitory Dose Response Curves in the Presence of 100μM ACPD. Cultured striatal neurons were incubated with varying concentrations of AP3, L-AP4 and AP5 in the presence of 1mM LiCl in PBS for 10 min. as indicated above. The neurons were then stimulated for a further 10 min. with 100μM ACPD.

The data represent means ± S.E.M. from 6-8 independent experiments performed in separate cultures in duplicate, and are expressed as a percentage of the maximal 100μM ACPD-evoked [^{3}H]-InsP$_1$ accumulation in the absence of antagonists in paired control trials. Maximal ACPD-evoked scintillation counts averaged 3400.1 ± 171.3 dpm.

*$p<0.05$, **$p<0.01$ compared to the 100% maximal stimulation (Student's grouped t test).

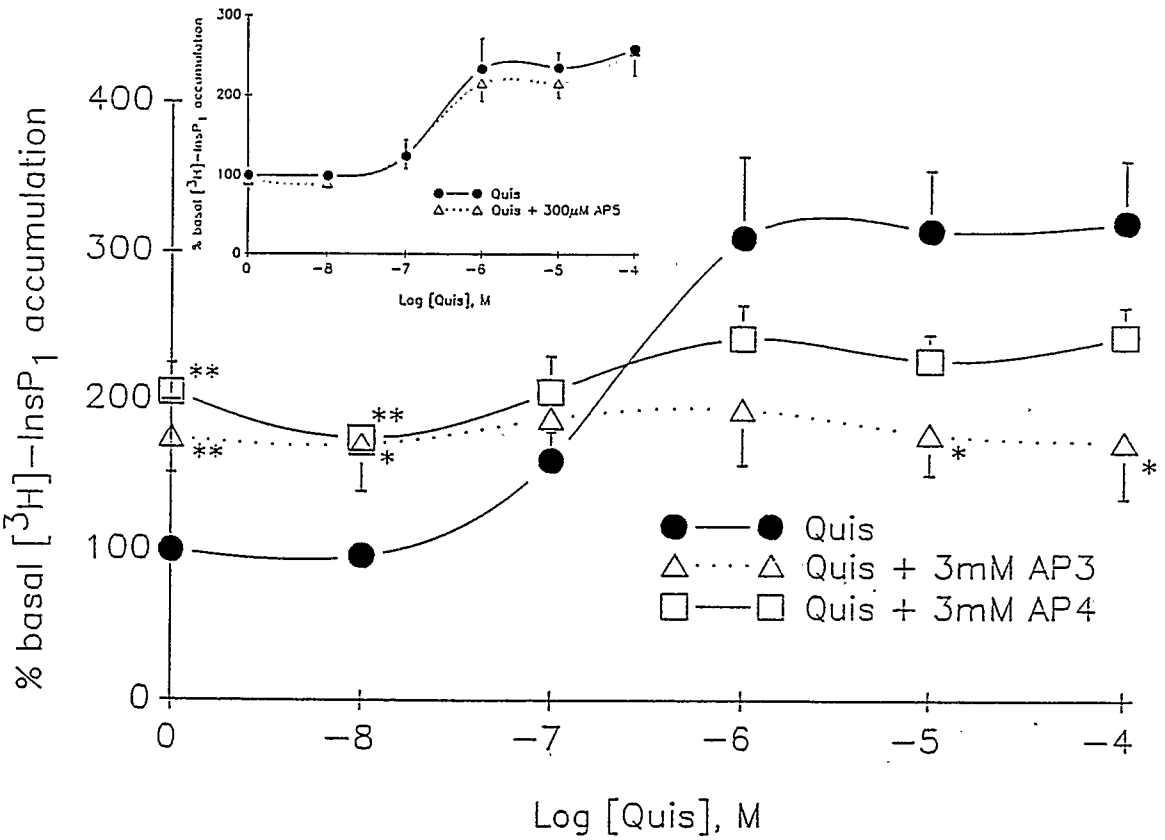


FIGURE 12. Quisqualate Dose Response Curves in Fixed Concentrations of EAA Antagonists. Cultured striatal neurons were incubated with 3mM AP3 or 3mM L-AP4 (Fig. 12a: main figure), or 300μM AP5) (Fig. 12b: insert) in the presence of 1mM LiCl in PBS for 10 min. The neurons were then stimulated for a further 10 min. with varying concentrations of Quis, as indicated.

The data represent means ± S.E.M. from 4 independent experiments performed in separate cultures in duplicate and are expressed as a percentage of the basal [^{3}H]-$InsP_1$ accumulation in the absence of agonists and antagonists in paired control trials. Basal scintillation counts averaged 1069.3 ± 66.3 dpm (Fig. 12a) and 875.5 ± 99.7 dpm (Fig. 12b).

*$p<0.05$, **$p<0.01$ compared with the control Quis curve for each concentration point (Student's grouped t test). The 300μM AP5 curve was not significantly different at any point (Fig. 12b). EC_{50} values were visually estimated to be 0.2μM (Fig. 12a) and 0.3 and 0.4μM in the absence or presence of 300μM AP5, respectively (Fig. 12b).

3mM AP3 had its greatest inhibitory effect on 100μM Quis, blocking from 320.8 ± 41.7% of basal (control) to 172.8 ± 38.3% of basal ($p<0.05$). 3mM AP4 in the absence of any agonist was able to elevate basal [^{3}H]-InsP$_1$ accumulation to 204.8 ± 19.5% ($p<0.01$ versus 100% baseline, but not significantly different from the 3mM AP3 alone-evoked increase). 3mM AP4 had its greatest inhibitory effect on 10μM Quis, blocking from 315.6 ± 40.9% of basal to 227.4 ± 17.9% ($p\approx0.1$). Figure 12b shows that 300μM AP5 did not have any significant effect on the Quis dose response curve at any agonist concentration ($p>0.05$). This finding agrees with the inhibitory dose-response curve previously shown (Fig. 9). The experiments testing AP3 and AP4 versus Quis were performed separately from those testing AP5 versus Quis. Thus, the experiments had different basal values and were plotted on separate graphs.

Figure 13 shows that 3mM AP3 and 3mM AP4 also blocked Ibo-evoked [^{3}H]-InsP$_1$ accumulation in a non-competitive fashion - this was not overcome even at 1mM Ibo ($p<0.01$ for AP3). 3mM AP3 in the absence of any agonist was able to elevate basal [^{3}H]-InsP$_1$ accumulation to 119.2 ± 5.0% ($p<0.01$). 3mM AP3 had its greatest inhibitory effect on 1mM Ibo, blocking from 273.1 ± 17.8% of basal (control) to 103.3 ± 2.6% ($p<0.01$). 3mM AP4 in the absence of any agonist was able to elevate basal [^{3}H]-InsP$_1$ accumulation to 157.1 ± 22.3% ($p<0.01$). 3mM AP4 had its greatest inhibitory effect on 100μM Ibo, blocking from 260.4 ± 21.4% of basal to 155.5 ± 31.9% ($p<0.05$). 300μM AP5 significantly attenuated phosphoinositide hydrolysis in the absence of any agonist (80.9 ± 6.9% of basal, $p<0.01$), and also at 10μM Ibo. This agrees with the previous inhibitory dose-response curve (Fig. 10), showing that

at concentrations > 100μM AP5 significantly blocked 10μM Ibo. However, this effect was competed out with increasing concentrations of Ibo. The estimated EC_{50} value for Ibo alone was 7.6μM, but increased to 19μM in the presence of 300μM AP5. Ibo was the only agonist whose EC_{50} was substantially altered by AP5; this will also be addressed later in the Discussion in section 2.3.3.

Figure 14 demonstrates that 3mM AP3 and 3mM AP4 also blocked ACPD-evoked [^{3}H]-$InsP_1$ accumulation in a non-competitive fashion. 3mM AP3 in the absence of any agonist was able to elevate basal (control) [^{3}H]-$InsP_1$ accumulation to 154.7 ± 21.5% of basal ($p < 0.05$). 3mM AP3 had its greatest inhibitory effect on 1mM ACPD, blocking it from 323.9 ± 16.8% of basal to 149.8 ± 35.1% ($p < 0.01$). 3mM AP4 in the absence of any agonist significantly elevated basal [^{3}H]-$InsP_1$ accumulation to 159.9 ± 29.5% of basal ($p < 0.05$). 3mM AP4 also had its greatest inhibitory effect on 1mM ACPD, blocking to 181.9 ± 30.5% ($p < 0.01$). 300μM AP5 slightly attenuated the ACPD-response at the agonist concentrations delineated, although it only approached significance at 100μM ACPD ($0.05 < p < 0.1$). This is in contrast to the inhibitory dose-response curve of AP5 on 100μM ACPD-evoked [^{3}H]-$InsP_1$ accumulation (Fig. 11), which showed that at concentrations above 300μM, AP5 significantly attenuated the actions of ACPD. This discrepancy will be addressed in the Discussion in section 2.3.3. The estimated EC_{50} values for ACPD and for ACPD plus 300μM AP5 were 35μM and 55μM, respectively.

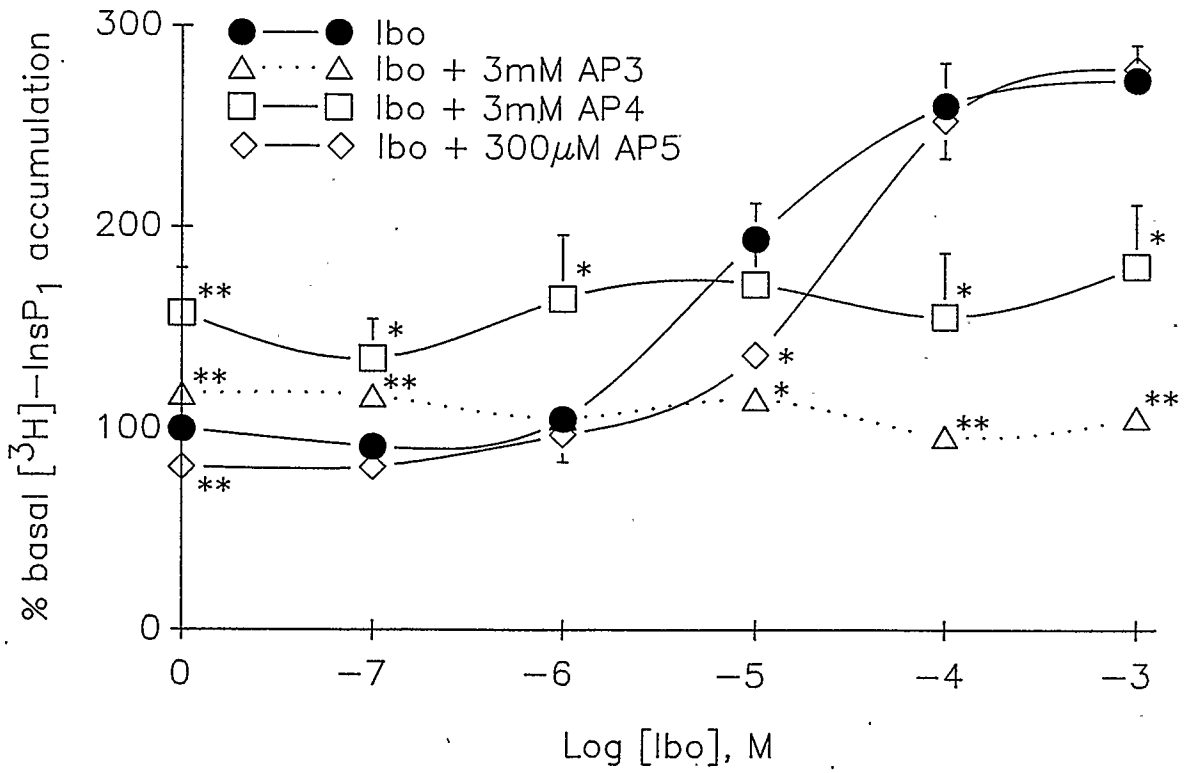


FIGURE 13. Ibotenate Dose Response Curves in Fixed Concentrations of EAA Antagonists. Cultured striatal neurons were incubated with 3mM AP3 or 3mM L-AP4 or 300μM AP5 in the presence of 1mM LiCl in PBS for 10 min. The neurons were then stimulated for a further 10 min. with varying concentrations of Ibo, as indicated.

The data represent means ± S.E.M. from 3-4 independent experiments performed in separate cultures in duplicate and are expressed as a percentage of the basal [^{3}H]-$InsP_1$ accumulation in the absence of agonists and antagonists in paired control trials. Basal scintillation counts averaged 1096.3 ± 66.3 dpm. EC_{50} values were visually estimated to be 8 and 19μM in the absence or presence of 300μM AP5, respectively.

*$p < 0.05$, **$p < 0.01$ compared with the control Ibo curve for each concentration point (Student's grouped t test).

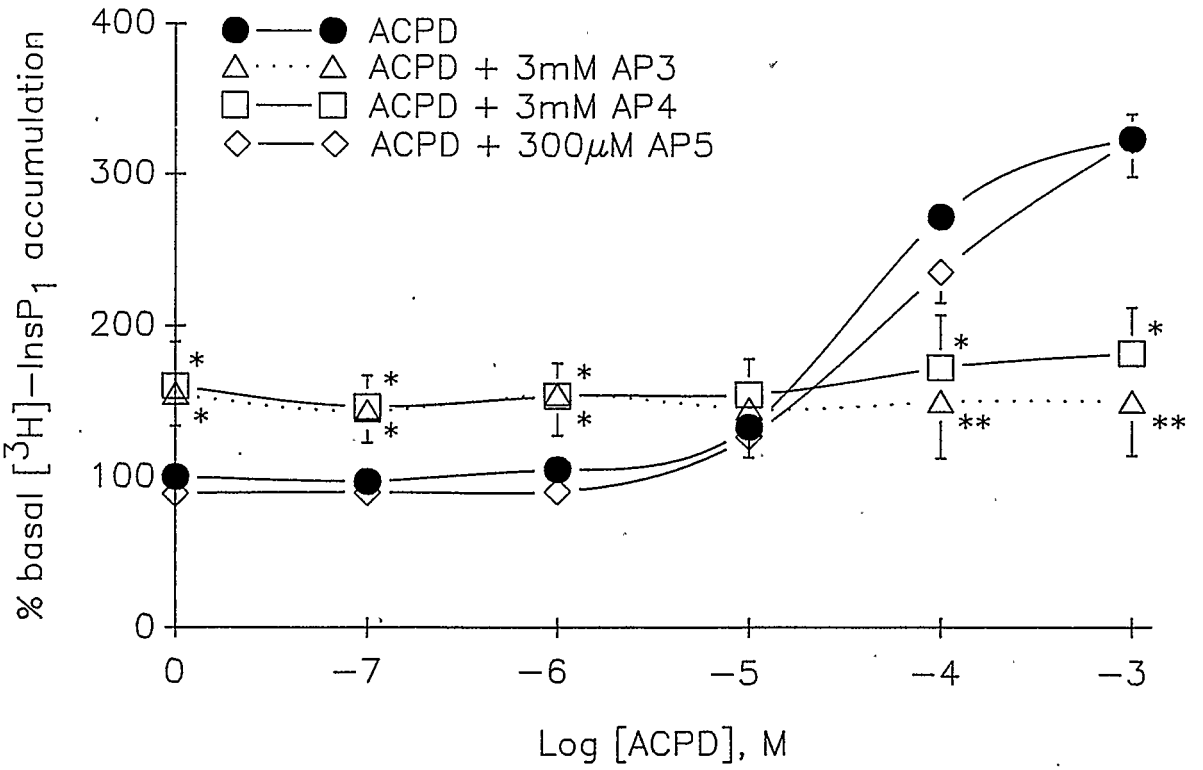


FIGURE 14. ACPD Dose Response Curves in Fixed Concentrations of EAA Antagonists. Cultured striatal neurons were incubated with 3mM AP3 or 3mM L-AP4 or 300μM AP5 in the presence of 1mM LiCl in PBS for 10 min. The neurons were then stimulated for a further 10 min. with varying concentrations of ACPD, as indicated.

The data represent means ± S.E.M. from 3-4 independent experiments performed in separate cultures in duplicate and are expressed as a percentage of the basal [^{3}H]-$InsP_1$ accumulation in the absence of agonists and antagonists in paired control trials. Basal scintillation counts averaged 1096.3 ± 66.3 dpm. EC_{50} values were visually estimated to be 35 and 55μM in the absence or presence of 300μM AP5, respectively.

*$p<0.05$, **$p<0.01$ compared with the control ACPD curve for each concentration point (Student's grouped t test).

2.5 MK801 Inhibitory Dose Response Curves

From Figures 10 and 11, it is apparent that at concentrations > 10μM, AP5 can attenuate 10μM Ibo and 100μM ACPD-evoked [^{3}H]-InsP$_1$ accumulations. However, this is not true of 1μM Quis-evoked [^{3}H]-InsP$_1$ accumulation (Figs. 9 and 12b). Since AP5 has been described as a specific antagonist of the NMDA receptor, we examined whether other NMDA antagonists might act in a similar fashion. Thus, an inhibitory dose response curve was performed for MK801 against 1μM Quis, 10μM Ibo and 100μM ACPD. As shown in Figure 15, MK801 was generally ineffective at antagonizing agonist evoked [^{3}H]-InsP$_1$ release; it was only significantly inhibitory at 10μM against 1μM Quis and 100μM ACPD ($p<0.01$ and 0.05, respectively).

3. MODULATION EXPERIMENTS

The previous pharmacology experiments attempted to identify differences between Quis, Ibo and ACPD-evoked [^{3}H]-InsP$_1$ accumulation at the receptor level. This series of experiments was designed to identify mechanisms that could modulate metabotropic receptor-mediated phosphoinositide hydrolysis cascade 'downstream' of the receptor.

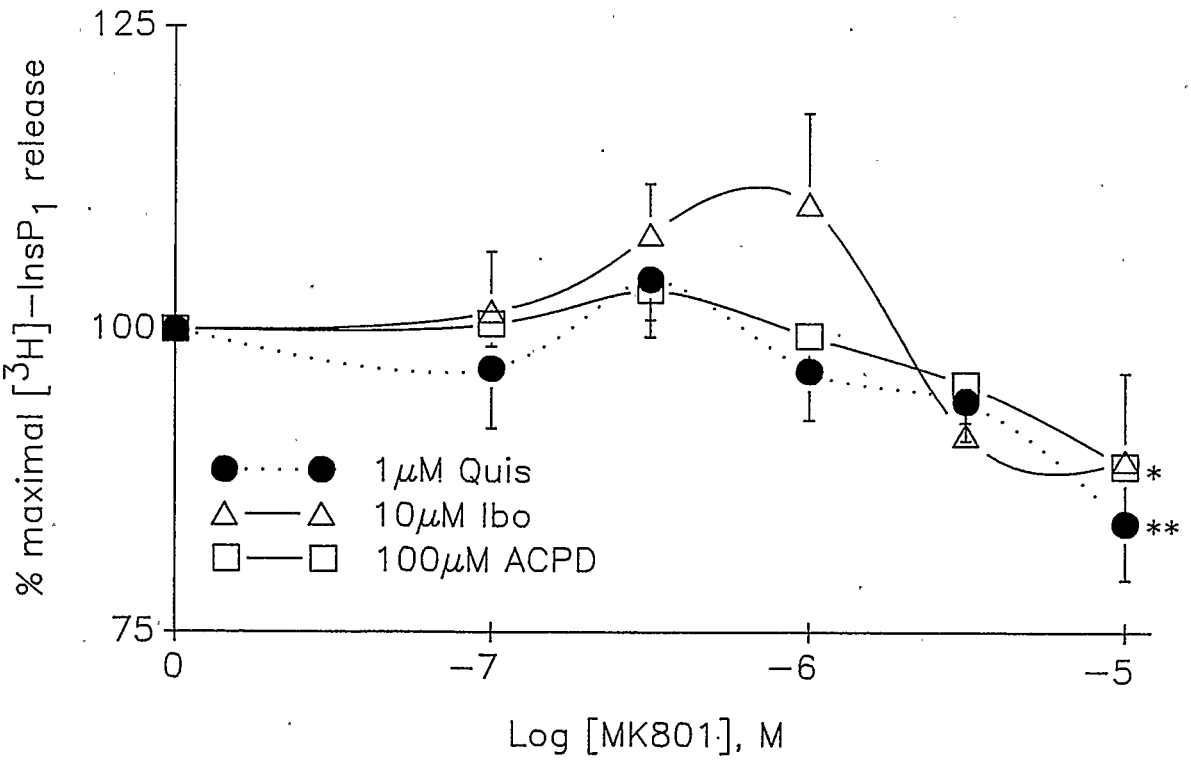


FIGURE 15. MK801 Antagonist Inhibitory Dose Response Curves in the Presence of Excitatory Amino Acid Agonists. Cultured striatal neurons were incubated with varying concentrations of dizocilpine maleate (MK801) and 1mM LiCl in PBS for 10 min. as indicated above. The neurons were then stimulated for a further 10 min. with 1μM Quis or 10μM Ibo or 100μM ACPD.

The data represent means ± S.E.M. from 5-7 independent experiments performed in separate cultures in duplicate, and are expressed as a percentage of the maximal [^{3}H]-InsP$_1$ accumulation evoked by that specific agonist in the absence of antagonists in paired control trials. The maximal agonist-evoked scintillation counts in the absence of MK801 averaged 4057.8 ± 132.6, 2747.7 ± 83.6 and 3400.1 ± 171.3 dpm for 1μM Quis, 10μM Ibo and 100μM ACPD, respectively.

*$p<0.05$, **$p<0.01$ compared to the 100% maximal stimulation (Student's grouped t test).

3.1 Effect of Phorbol Dibutyrate on Agonist-Evoked [^{3}H]-InsP$_1$ Accumulation

As previously outlined in the Introduction, PKC can feedback upon the phosphoinositide hydrolysis cascade and attenuate the agonist-evoked response. To test if PKC feedback acts differentially on Quis, Ibo and ACPD-evoked [^{3}H]-InsP$_1$ accumulation, the potent PKC activator phorbol-12,13-dibutyrate (PDBu) was included in incubation buffer with 1mM LiCl. Four-α-phorbol-12,13-dibutyrate (4-α-PDBu), the inactive congener of PDBu, was included as a control. As seen from Figure 16, 1μM PDBu caused a significant reduction in all agonist-evoked [^{3}H]-InsP$_1$ accumulations ($p < 0.01$ for all agonists); PDBu blocked 1μM Quis-evoked [^{3}H]-InsP$_1$ accumulation from 407.0 ± 35.2% to 168.4 ± 22.1%, 10μM Ibo-evoked responses from 367.9 ± 13.9% to 155.3 ± 6.1%, 100μM ACPD-evoked responses from 296.3 ± 12.2% to 117.0 ± 20.3%, and 1mM CCh-evoked responses from 292.4 ± 11.8% to 170.8 ± 11.3%. The inactive 4-α-PDBu did not significantly affect the metabotropic response of any agonist ($p > 0.05$). 1μM PDBu and 4-α-PDBu did not significantly alter baseline [^{3}H]-InsP$_1$ accumulation in the absence of agonists. PDBu also affected the CCh-evoked response, indicating that the feedback mechanism was not selective for the metabotropic receptor agonists, but probably targeted another component of the phosphoinositide cascade.

3.2 Effect of Extracellular Cations on Agonist-Evoked [^{3}H]-InsP$_1$ Accumulation

The effect of the extracellular ionic milieu on receptor-stimulated phosphoinositide hydrolysis was outlined in the Introduction. Of particular interest is the effect of extracellular Ca^{2+} and Na^+, since these cations have been demonstrated to inhibit and enhance, respectively, EAA-evoked phosphoinositide turnover in various

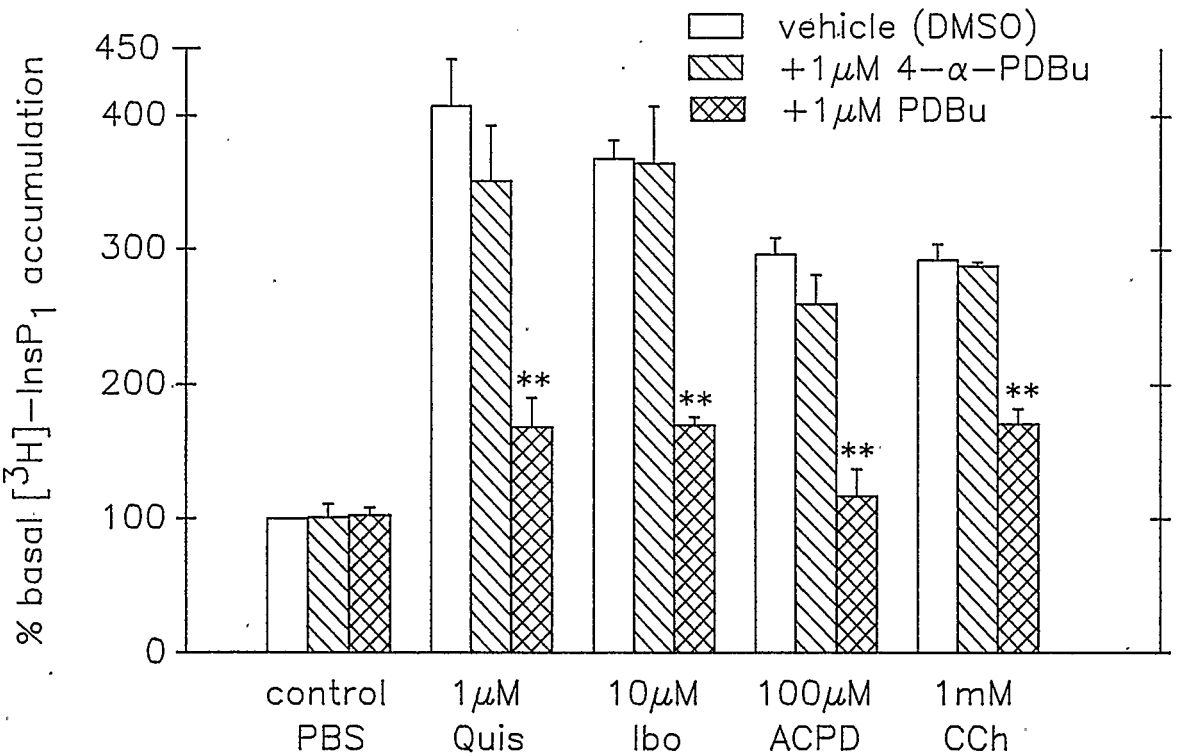


FIGURE 16. Effect of Phorbol Dibutyrate on Agonist-evoked [^{3}H]-InsP$_1$ Accumulation. Cultured striatal neurons were incubated with 1μM phorbol-12,13-dibutyrate (PDBu) or 1μM 4-α-phorbol-12,13-dibutyrate (4-α-PDBu) or dimethyl sulphoxide vehicle (DMSO) and 1mM LiCl in PBS for 10 min. as indicated above. The neurons were then stimulated for a further 10 min. with 1μM Quis or 10μM Ibo or 100μM ACPD or 1mM CCh.

The data represent means ± S.E.M. from 3-4 independent experiments performed in separate cultures in triplicate, and are expressed as a percentage of the basal, vehicle-treated [^{3}H]-InsP$_1$ accumulation in the absence of agonists, antagonists and PDBu in paired control trials. Basal scintillation counts averaged 1126.7 ± 53.9 dpm.

*$p<0.05$, **$p<0.01$ versus 100% baseline (Student's grouped t test).

the indicated cations with distilled water. In the case of Na^+, replacement of NaCl with NMDG-Cl reduced the $[Na^+]_o$ to 16mM whilst maintaining isotonic and isosmotic conditions (see Methods). The pH had to be re-adjusted to 7.2-7.4 when NMDG-Cl replacement was used. Again, CCh was included as a non-EAA control. However, since only 2-4 experiments were performed for CCh, the data did not always reach significance.

As seen from Figure 17, removing Ca^{2+} from the PBS caused an uniform, significant decrease in basal and all EAA-evoked [^{3}H]-$InsP_1$ accumulations. Omitting Ca^{2+} reduced the basal response from 100% to 81.1 4.9% ($p<0.01$), the 1μM Quis-evoked response from 300.7 21.6% to 183.8 9.9% ($p<0.01$), the 10μM Ibo-evoked response from 272.2 32.6% to 126.8 7.3% ($p<0.01$), and the 100μM ACPD-evoked response from 279.9 16.6% to 160.9 9.5% ($p<0.01$).

Reducing the extracellular Na^+ to 16mM had the opposite effect: it enhanced both basal and agonist-evoked [^{3}H]-$InsP_1$ accumulation. The basal response was elevated to 161.2 19.4% ($p<0.01$), the 1μM Quis-evoked response from 300.7 21.6% to 375.7 29.8% ($p<0.01$), the 10μM Ibo-evoked response from 272.2 32.6% to 326.8 4.3% ($p\approx0.05$, not significant), and the 100μM ACPD-evoked response from 279.9 16.6% to 353.8 26.8% ($p<0.05$). However, if the value of the basal increase (+61.2%) was subtracted from each of the agonist-evoked accumulations, they returned to approximately the untreated agonist level of [^{3}H]-$InsP_1$ accumulation.

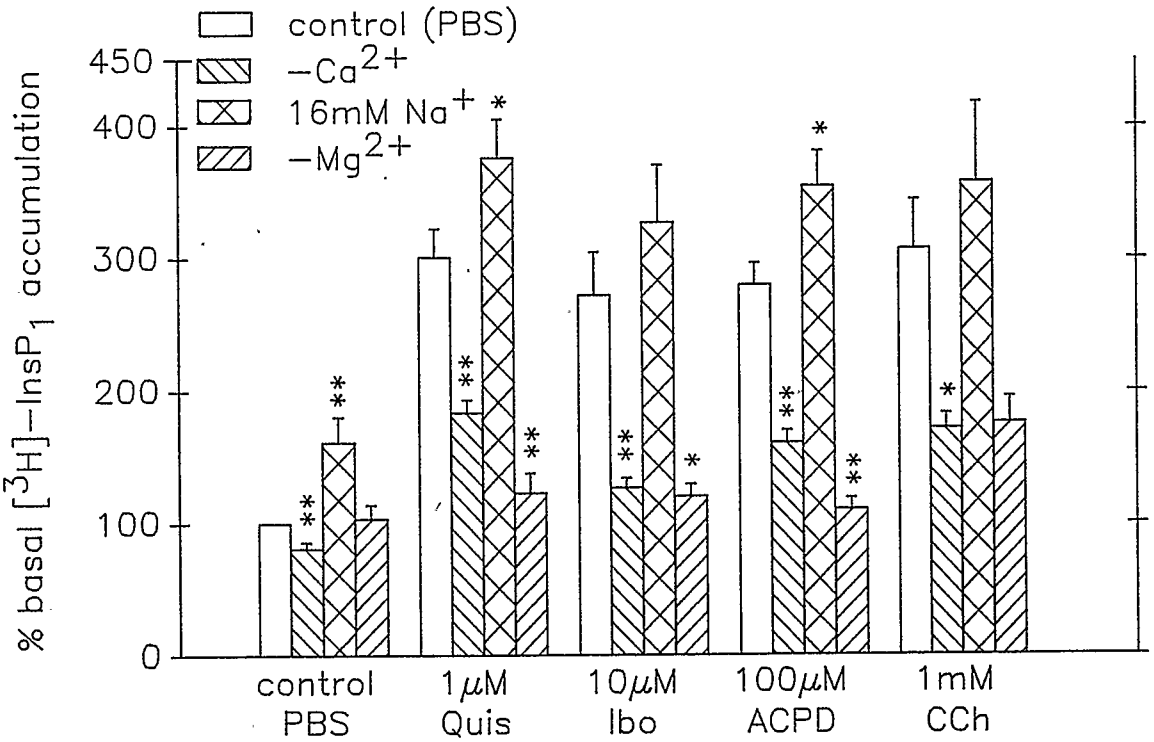


FIGURE 17. Effects of Extracellular Cation Reduction on Agonist-evoked [^{3}H]-$InsP_1$ Accumulation. Cultured striatal neurons were incubated with 10 1mM LiCl in normal or the appropriate cation-reduced PBS buffer for 10 min. The neurons were then stimulated for a further 10 min. with 1μM Quis or 10μM Ibo or 100μM ACPD or 1mM CCh, again in the appropriate cation-reduced PBS buffer.

The data represent means ± S.E.M. from 5-8 (Quis, Ibo, ACPD) or 2-4 (CCh) independent experiments performed in separate cultures in triplicate, and are expressed as a percentage of the basal [^{3}H]-$InsP_1$ accumulation in the absence of agonists and antagonists in paired control trials. Basal scintillation counts averaged 841.6 ± 17.6 dpm.

*$p < 0.05$, **$p < 0.01$ versus 100 % baseline in normal PBS (Student's grouped t test).

This will be discussed in detail subsequently in the Discussion. Removing Mg^{2+} did not alter the basal [^{3}H]-$InsP_1$ accumulation, but inhibited all agonist-evoked responses. The 1μM Quis-evoked response was inhibited from 300.7 21.6% to 122.9 14.9% ($p<0.01$), the 10μM Ibo-evoked response from 272.2 32.6% to 102.6 9.2% ($p<0.05$), and the 100μM ACPD-evoked response from 279.9 16.6% to 110.4 8.6% ($p<<0.01$).

3.3 Extracellular Ca^{2+} Dose Response Curve

Figure 17 demonstrated the inhibitory effect of lowering the $[Ca^{2+}]_o$, and it was thought that this merited a more careful examination using an extracellular Ca^{2+} dose response curve. This is shown in Fig. 18, which measured EAA-evoked [^{3}H]-$InsP_1$ accumulations against extracellular calcium concentrations of 0.0-0.9 mM. Both 1μM Quis and 10μM Ibo-evoked [^{3}H]-$InsP_1$ accumulations displayed a curvilinear dependence on extracellular Ca^{2+} that was best modelled by a second-order quadratic regression equation. There appeared to be a strong dependence of both agonist-evoked responses up to 0.2mM Ca^{2+}, which increased linearly thereafter.

3.4 Additive Effects of Pertussis Toxin and Extracellular Ca^{2+} Reduction on Agonist-evoked [^{3}H]-$InsP_1$ Accumulations

Finally, the additive effects of pertussis toxin (PTX) and extracellular Ca^{2+} on EAA-evoked [^{3}H]-$InsP_1$ release was examined. From Figs. 17 and 18, it was demonstrated that even in zero extracellular Ca^{2+} there was still some agonist-evoked [^{3}H]-$InsP_1$ accumulation that was not blocked. Thus, PTX was used to irreversibly block G

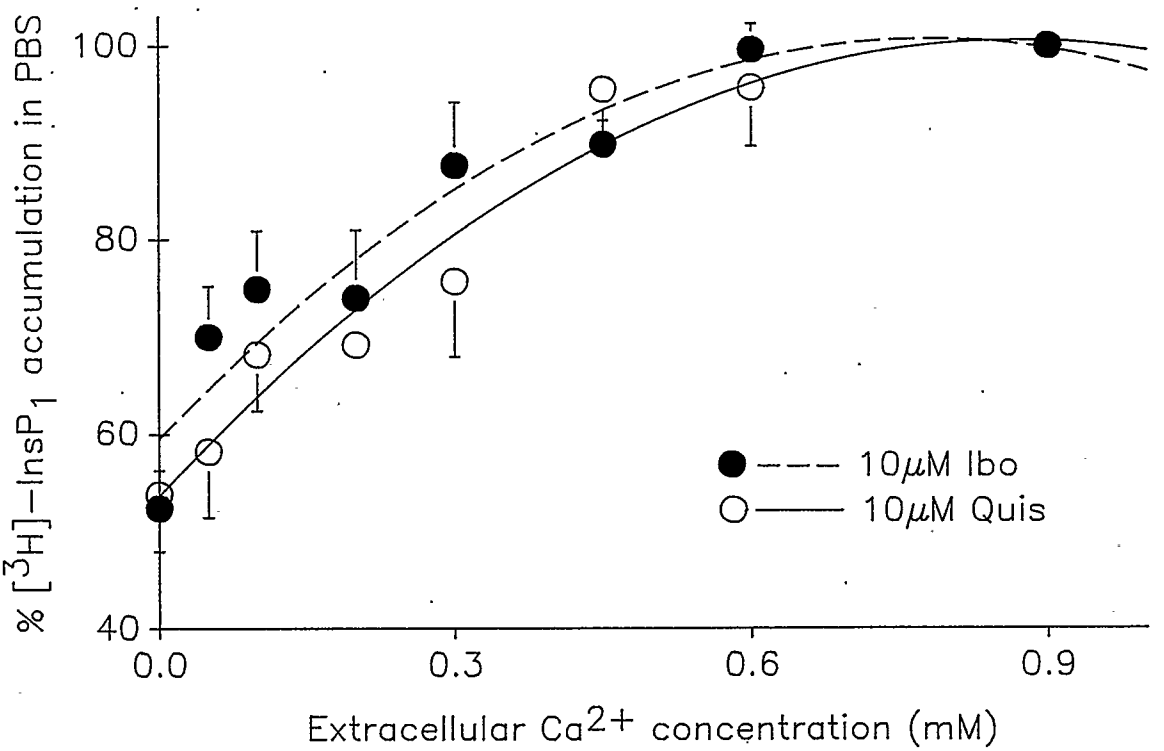


FIGURE 18. Effect of Extracellular Calcium Concentration on EAA-evoked [^{3}H]-$InsP_1$ Accumulation. Cultured striatal neurons were incubated with 10 1mM LiCl in PBS buffers with the indicated calcium concentrations for 10 min. The neurons were then stimulated for a further 10 min. with 1μM Quis or 10μM Ibo in the same calcium-reduced PBS buffer.

The data represent means ± S.E.M. from 3 independent experiments performed in separate cultures in duplicate, and are expressed as a percentage of the maximal [^{3}H]-$InsP_1$ accumulation evoked by that specific agonist in nominal calcium (0.9 mM) in paired control trials. Basal scintillation counts averaged 716.0 ± 14.2 dpm in zero extracellular calcium. The curves were fitted by second order quadratic equations derived from the data.

protein activation to see if this could account for the remaining [^{3}H]-$InsP_1$ accumulation. Figure 19 shows that PTX-mediated blockage of G proteins reduced the [^{3}H]-$InsP_1$ accumulation of all agonists, but was not as effective as calcium omission. PTX treatment (see Methods) did not affect the basal [^{3}H]-$InsP_1$ accumulation, but attenuated 1μM Quis-evoked responses from 337.9 40.6% to 253.0 21.6% ($p<0.05$, barely significant), 10μM Ibo-evoked responses from 302.6 41.2% to 271.4 36.7% ($p\approx0.15$) and 100μM ACPD-evoked responses from 316.2 29.1% to 283.5 29.5% ($p\approx0.4$). Neither Ibo nor ACPD were significantly affected by PTX.

Extracellular Ca^{2+} omission reduced basal [^{3}H]-$InsP_1$ accumulation from 100% to 78.2 11.4% ($p<0.05$), and attenuated 1μM Quis-evoked responses from 337.9 40.6% to 218.1 36.0% ($p<0.05$), 10μM Ibo-evoked responses from 302.6 41.2% to 156.5 26.6% ($p<0.05$) and 100μM ACPD-evoked responses from 316.2 29.1% to 173.5 12.2% ($p<0.05$).

Combining both PTX treatment with incubation in reduced-Ca^{2+} PBS was more effective than either treatment alone. In the absence of agonist, it reduced [^{3}H]-$InsP_1$ accumulation from 100% to 79.4 8.5% ($p<0.05$) and attenuated 1μM Quis-evoked responses from 337.9 40.6% to 167.4 19.7% ($p<0.01$), 10μM Ibo-evoked responses from 302.6 41.2% to 103.8 16.5% ($p<0.01$) and 100μM ACPD-evoked responses from 316.2 29.1% to 101.5 9.5% ($p<0.01$).

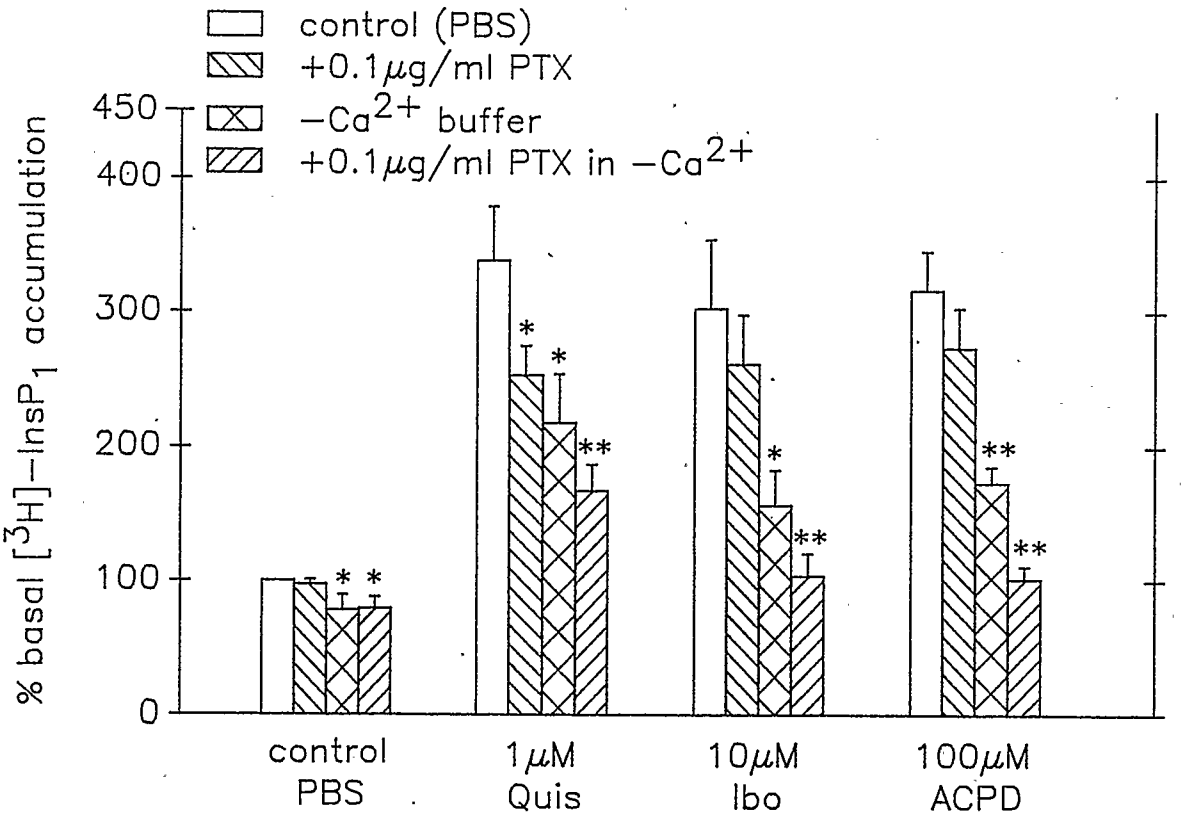


FIGURE 19. Additive Effects of Pertussis Toxin and Extracellular Calcium Reduction on Agonist-evoked [^{3}H]-InsP$_1$ Accumulation. Cultured striatal neurons were pretreated overnight (18 hours) with 0.1μg/ml. PTX or vehicle added directly to the culture medium. On the day of the experiment, the neurons were rinsed of excess PTX and were incubated with 1mM LiCl in normal or calcium-reduced PBS buffer for 10 min. The neurons were then stimulated for a further 10 min. with 1μM Quis or 10μM Ibo or 100μM ACPD, again in the appropriate PBS buffer.

The data represent means ± S.E.M. from 4-6 independent experiments performed in separate cultures in triplicate, and are expressed as a percentage of the basal, vehicle-treated [^{3}H]-InsP$_1$ accumulation in the absence of agonists, antagonists and PTX in paired control trials. Basal scintillation counts averaged 1086.5 ± 36.9 dpm.

*$p<0.05$, **$p<0.01$ versus 100% baseline (Student's grouped t test).

DISCUSSION

The Discussion will follow the same format as the Results, and will be divided into the three series of experiments. Each figure will be discussed separately, and a hypothetical model will be presented at the end of the Discussion to explain the observations.

1. OPTIMIZATION OF EXPERIMENTAL PARAMETERS

These experiments were performed to reestablish the culture and assay conditions previously described (Weiss *et al.*, 1986). Most of the parameters investigated did conform to those previously established - the only observation of interest worth discussing is the extracellular Li^+ dose-response curve (Fig. 6). LiCl inhibits the phosphoinositide cycle by blocking the recycling of $InsP_1$ to Ins via inositol monophosphatase (Berridge *et al.*, 1982). In this experiment, a linear increase in [^{3}H]-$InsP_1$ accumulation was seen with increasing concentrations of LiCl. It has been suggested that the inclusion of Li^+ beyond the 5 min. lag time (see Introduction) in the assay compromises normal cellular phosphoinositide metabolism, and that quantitative conclusions (such as those in this thesis) should be avoided (Nahorski & Challiss, 1991).

In addition to the Li^+ effect above, measuring mass levels of [^{3}H]-$InsP_1$ metabolite

accumulation "trapped" by Li^+ obviously masks subtle changes in Ins(poly)phosphate isomeric species, such as [^{3}H]-$InsP_3$ and [^{3}H]-$InsP_4$ (for review, see Fowler & Tiger, 1991). It is possible different receptor-mediated mechanisms causing phosphoinositide hydrolysis generate different $InsP_1$ species, which would have dramatically different effects *in vivo*. For example, whereas Ins(1,4,5)P_3 liberates Ca^{2+} from intracellular stores, Ins(1,3,4,5)P_4 promotes Ca^{2+} entry from the plasma membrane; this was first demonstrated by Irvine and Moor (1986) in sea urchin eggs.

2. RECEPTOR PHARMACOLOGY EXPERIMENTS

To uncover potential heterogeneous mechanisms underlying Glu-evoked phosphoinositide hydrolysis in striatal neurons, the pharmacology of the mGlu-R was examined first since it is the first step in metabotropic signal transduction. If a pharmacologically distinct receptor or receptor subtype(s) does exist, then the series of experiments outlined below ought to reveal them.

2.1 Agonist Dose-Response Curves

The rank order of potencies for the EAA agonists tested (Fig. 8) agreed with those in the literature (see Table IV below). The EC_{50} values obtained for Quis, Ibo and ACPD (0.2-0.3μM, 4.4-7.6μM and 31-35μM, respectively) are in agreement with the observations of Manzoni *et al.* (1990a,b; 1991) in a similar culture system; they reported EC_{50} values of 0.2-0.3μM, 6.7-9.7μM and 29μM, respectively, for Quis, Ibo and ACPD.

TABLE IV: PHARMACOLOGIC PARAMETERS OF EAA AGONISTS AND ANTAGONISTS IN NEURAL TISSUES

Preparation	EC_{50} (μM)	IC_{50} (μM)	Ref.
SLICES			
cerebral cortical slice:			
- 7 day old rats	Quis ≈ 2	L-AP3 on Quis=369 L-AP3 on Ibo=168	a
- 200-300g rats	Quis ≈ 20; Ibo ≈ 30		b
- adult guinea pig	Quis=10.9; ACPD ≈ 35-80	-	h
striatal slice:			
- 8-10 day old rats	Quis=0.26; Ibo=6.8 Glu=120; Asp=130; CCh=4.7	-	c
retinal slice:			
- adult rabbit	Quis=0.1; CCh=11.5	-	d
hippocampal slice:			
- 7 day old rats	Quis=2; Ibo=27 Glu=458	AP3 on Quis=369 AP4 on Quis=1915	e
- 10-13 day old rats	Quis ≈ 2		f
- 150-175g rats	1S,3R-ACPD=40 1R,3S-ACPD=n/a	AP3 on Ibo=120 AP4 on Ibo=160	m,n,s
- 35-40 day old rats	Ibo ≈ 200	-	g
- 30-75 day old rats	-	L-AP4 on Quis=74 L-AP4 on Ibo=750 L-AP4 on ACPD=630 L-AP4 on CCh=410	i
- 56-84 day old rats	Ibo=220; ACPD=60	-	j
SYNAPTONEUROSOMES			
rat forebrain:	Quis=0.12; Glu=23	-	k
rat cortex:	Glu ≈ 500	-	l
PRIMARY CULTURES			
mouse striatal neurons:	Quis=0.2; Glu=4 ACPD=9.7	-	o,p
rat cortical neurons:	Quis=0.033; Ibo=4; Glu=33		q
rat cerebellar granule cells:	Quis=50; Glu ≈ 10		r

TABLE IV cont'd...

Abbreviations: see List of Abbreviations, page xiii.

References: **a:** Schoepp *et al.*, 1990b; **b:** Godfrey *et al.*, 1988; **c:** Doble & Perrier, 1989; **d:** Osborne, 1990; **e:** Schoepp & Johnson, 1989a,b; **f:** Palmer *et al.*, 1988; **g:** Schoepp *et al.*, 1990b; **h:** Alexander *et al.*, 1990; **i:** Whittemore & Cotman, 1991; **j:** Desai & Conn, 1990; **k:** Ré casens *et al.*, 1987; **l:** Dudek *et al.*, 1989; **m:** Schoepp & Johnson, 1989a; **o:** Weiss *et al.*, 1989; **p:** Manzoni *et al.*, 1990a,b; **q:** Patel *et al.*, 1990; **r:** Nicoletti *et al.*, 1986a

Quis was by far the most potent and effective of the EAA agonists. It is important to note that Quis and Ibo are not pure, specific agonists. Thus, Quis interacts strongly with the AMPA receptor, and Ibo interacts strongly with the NMDA receptor (Doble & Perrier, 1989). Thus, the net effect of Quis at evoking [^{3}H]-InsP$_1$ accumulation can be viewed as being the sum of its stimulatory effects at the metabotropic Glu receptor plus its effects (stimulatory or inhibitory) at the AMPA receptor. The same logic applies to the effects of Ibo. Even the most selective agonist, ACPD, is not without effect at other EAA-Rs. For example, ACPD displaces NMDA-R binding (assayed by [^{3}H]-CGS19755) in rat brain synaptosomes (Schoepp *et al.*, 1990a). It is important to note that most of the experiments suggesting cross-reactivity were performed in brain slices with agonist stimulation durations ranging from 30 to 60 minutes (versus 10 min in this preparation). The significance of agonist stimulation duration will be discussed later in section 4.5 below.

Despite this potential cross-reactivity of the agonists, AMPA is not an agonist at the metabotropic receptor (Schoepp & Johnson, 1988a), and NMDA and KA are only able to marginally increase phosphoinositide hydrolysis in most preparations (Sladeczek *et al.*, 1985; Godfrey *et al.*, 1988; Doble & Perrier, 1989). These studies assaying [^{3}H]-InsP$_1$ accumulation have been replicated assaying intracellular Ca^{2+} with identical results (Murphy & Miller, 1988). In addition, in primary cultures of striatal neurons, concentrations of ACPD up to 1mM do not activate the ionotropic Glu-Rs (Manzoni *et al.*, 1990a). An earlier report from Weiss (1989) demonstrated

that two pharmacologically distinct Quis receptor systems are present on striatal neurons *in vitro*: the metabotropic receptor system, and a non-metabotropic system that exhibited the pharmacologic characteristics of the ionotropic AMPA receptor, but that was able to potentiate KCl- and veratridine-depolarization induced [^{3}H]-GABA release in striatal neurons. In addition, for 5 min. stimulations, neither 100μM AMPA nor 100μM NMDA significantly increased basal [^{3}H]-InsP$_1$ accumulation (Weiss, 1989). Thus, activation of the ionotropic Quis or NMDA receptors alone is not sufficient to evoke phosphoinositide hydrolysis.

2.2 Additivity of EAA Agonists

One of the strongest pieces of evidence that would suggest that Quis, Ibo and ACPD act via different metabotropic receptors is if their effects at evoking InsP$_1$ were additive, ie. they are acting at different receptors (or pharmacologically recognizable subtypes of a common receptor). The evidence from Fig. 9, however, suggests that Quis, Ibo and ACPD act at a common metabotropic receptor, since their effects on phosphoinositide hydrolysis were not additive. Similar results have been noted by Manzoni *et al.* (1990a, 1991); they found that Quis, Ibo and ACPD were not additive, but that NE and ACPD were. Patel *et al.* (1990) found that Quis and CCh were additive in primary cultures of rat cortical neurons, but that this did not quite reach the level of significance. This matches the data presented in Table IV. As mentioned before, saturation of the neuronal capacity to generate InsPs is not likely, since Table IV demonstrates that Quis was additive with CCh and NE.

Nevertheless, the data presented cannot preclude the existence of multiple receptor subtypes, since the culture is a heterogeneous mixture of neuronal types (see Introduction). It is possible that different populations of receptor (sub)types, with differing potencies and efficacies for each agonist, exist on different neuronal subtypes in culture, and that their existence was masked by the majority of GABAergic neurons. This will be discussed in greater detail in sections 4.4 and 4.5 below.

2.3 Agonist/Antagonist Pharmacology

Figures 10-12 show the inhibitory effects of AP3, AP4 and AP5, and Figs. 13-15 show the effects of these same antagonists on Quis, Ibo and ACPD-evoked [^{3}H]-InsP$_1$ accumulations. As summarized on Table IV, there is evidence for and against the results presented here.

2.3.1 The effect of AP3: AP3 was an effective, non-competitive antagonist of all EAAs tested. However, Manzoni *et al.* (1991) recently found AP3 to be a competitive antagonist in striatal neurons. However, the fact that AP3 acts as a non-competitive, irreversible metabotropic antagonist in other preparations is well established (see Table IV). It is also evident from Figs. 9-11 and 13-15 that AP3 acts as a classical partial agonist at the metabotropic Glu receptor; ie. it has weak intrinsic activity, but antagonizes the full agonists Quis, Ibo and ACPD. This is surprising, since AP3 is the β-phosphono-substituted derivative of Asp, not Glu. Therefore, one can hypothesize that AP3 inhibits phosphoinositide hydrolysis by

binding to the metabotropic Glu receptor with the resultant alteration of the Glu binding site. This is consistent with its non-competitive mode of antagonist action.

AP3 also inhibits ACPD-evoked Ca^{2+}-dependent Cl^- currents in *Xenopus* oocytes injected with rat brain mRNA (Watson *et al.*, 1990), Glu-evoked currents in *Xenopus* oocytes injected with the metabotropic Glu receptor clone (Houamed *et al.*, 1991), and blocks ACPD-evoked increases in intracellular Ca^{2+} in primary cultures of cerebellar neurons (Irving *et al.*, 1990). Finally, AP3 displaces [^{3}H]-Glu binding at metabotropic receptors (Cha *et al.*, 1990). All these lines of evidence, along with the fact that L-AP3 is more potent that its enantiomer D-AP3 (Schoepp *et al.* 1990b), strongly suggest that AP3 acts directly at the metabotropic Glu receptor rather than indirectly via a secondary mechanism. Furthermore, since AP3 is a non-competitive antagonist, this strongly suggests that it acts at an allosteric site on the receptor rather than at the agonist/ligand binding site. Masu *et al.* (1991) recently reported the existence of an unique region of their mGluR clone distinct from the agonist binding site, and suggested it as having a possible regulatory function. Therefore, it is possible that AP3 binds allosterically to this site.

The problem with a direct action at the metabotropic Glu receptor is that the relatively high doses of AP3 required to significantly block agonist-evoked [^{3}H]-$InsP_1$ accumulation (at least 1mM; see Figs. 9-11) suggest that this interaction is not very specific. Since there are no reports as to whether AP3 is taken up into neurons via an amino acid transporter, an intracellular effect of AP3 cannot be ruled out.

However, this is unlikely given the binding data above. An alternative explanation is that an effect of AP3 on Glu reuptake has also been suggested (Cross *et al.*, 1986). A very recent report from Ormandy (1992) demonstrated that L-aspartate-β-hydroxamate, a specific Glu uptake inhibitor, was able to potently attenuate Quis (IC_{50} 11μM) and Ibo, but not CCh nor K^+, evoked [^{3}H]-$InsP_1$ accumulation in rat hippocampal slices. Like AP3 and AP4, this inhibition was non-competitive. Since AP3 has been reported to displace D-[^{3}H]-Asp binding sites that correlated with Glu uptake (Cross *et al.*, 1986), the inhibitory effect of AP3 (and possibly AP4) on phosphoinositide hydrolysis may be somehow linked to Glu uptake. No mechanisms as to the effect of L-aspartate-β-hydroxamate on phosphoinositide hydrolysis have been suggested (Ormandy, 1992), and given the fact that Quis and Ibo are not subject to Glu reuptake, any putative mechanism must take this into account. However, the long (1 hr) agonist-stimulation period and the presence of glia in those experiments mean that a large number of cellular processes could be involved. Considering the lack of substantiating evidence for AP3 action on Glu uptake sites, it is perhaps more prudent to conclude that AP3 blocks phosphoinositide hydrolysis allosterically at the metabotropic receptor itself.

2.3.2 The effect of AP4: AP4 also appears to be a non-competitive antagonist of the EAAs tested, albeit less effective than AP3. AP4 was an antagonist against Quis-evoked phosphoinositide hydrolysis, but was better at inhibiting Ibo and ACPD (Figs. 11-12). However, AP4 was reported ineffective at blocking Quis, L-Glu and NMDA-evoked [^{3}H]-$InsP_1$ accumulation in rabbit retinal slices (Osborne, 1990) and

ineffective for L-glu and Ibo in primary cultures of rat cortical neurons (Patel *et al.*, 1990). These reports were, however, in adult neurons; L-AP4 has been reported effective upon neonatal hippocampal slices (Schoepp & Johnson, 1989b). Like AP3, AP4 also has partial agonist activity, probably because it is the γ-phosphono-substituted derivative of Glu. Therefore, the mechanism whereby AP4 inhibits EAA-evoked phosphoinositide hydrolysis is may be similar to the AP3 mechanism, ie. a direct interaction at the metabotropic Glu receptor itself.

However, this, is complicated by the fact that AP4 can act at its own presynaptic receptor. [^{3}H]-AP4 binding is also more prominent on neonatal neurons than adult neurons (Monaghan *et al.*, 1983), and thus creates a temporal window where AP4 could cross-react at multiple receptors. Therefore, it is possible that AP4 acts by binding to the AP4 receptor presynaptically, depressing excitatory currents within that cell, and inhibiting phosphoinositide hydrolysis. The role of depolarization and membrane voltage in [^{3}H]-InsP$_1$ accumulation will be discussed in greater detail in the section dealing with extracellular cations.

2.3.3 The effect of AP5: The effect of AP5 is much less clear than AP3 and AP4. As seen from Figs. 10-12, concentrations of AP5 $>10^{-4}$M are able to antagonize Ibo and ACPD evoked [^{3}H]-InsP$_1$ accumulation, but are ineffective against Quis. The discrepancy between the effects of AP5 on ACPD-evoked [^{3}H]-InsP$_1$ accumulation (see Figs. 11 vs. 14) is likely one of physiologic versus statistical significance. Although the effects of concentrations of AP5 $>300\mu$M in Fig. 11 are statistically

significant, the strongest blockade achieved at 1mM AP5 was only 15% of the maximal ACPD-evoked response, in contrast to the 38% blockade caused by AP3. Therefore, it is questionable whether the statistically significant effect of AP5 in Fig. 11 was physiologically relevant, or was a side-effect of pharmacologically high doses. Nevertheless, this AP5 effect on Ibo versus Quis is one of the strongest pieces of evidence suggesting multiple receptor-coupled mechanisms. Although it generally has no effect on the metabotropic response, AP5 has been reported to attenuate Glu but not Quis stimulated [^{3}H]-InsP$_1$ accumulation in primary cultures of granule cells (Nicoletti *et al.*, 1986a,b). To confuse matters, AP5 and MK801 have also both been reported to potentiate Ibo evoked-[^{3}H]-InsP$_1$ accumulation in rat cortical slices (Godfrey *et al.*, 1988).

One possible explanation for the heterogeneous effect of AP5 is that, since AP5 is a NMDA-R recognition site antagonist, it could be blocking NMDA-evoked Ca^{2+} entry, which in turn attenuates PLC activity (the effects of Ca^{2+} will be detailed below). This explains why AP5 was the most effective at attenuating Ibo, which is a NMDA agonist. The 1R,3S-ACPD stereoisomer also interacts at the NMDA-R (Schoepp *et al.*, 1991). Since the racemic mixture (±)-trans-ACPD was used in these experiments, it is possible AP5 exerted its effects via the NMDA-R. This is corroborated by the fact that AP5 did block ACPD-evoked responses weakly (Figs. 11 and 13). And since Quis has no activity at the NMDA-R, AP5 has no effect upon it (see Figs. 13a and b). Supporting this idea are the findings of Magnuson *et al.* (1989). They found that L-Glu and (1R,3s)-ACPD evoked AP5-sensitive Ca^{2+} spikes

in rat spinal cord slices, whereas (1S,3R)-ACPD and Quis were AP5-resistant. The problem with this mechanism is that co-activation of the NMDA-R with the metabotropic receptor is known to attenuate [^{3}H]-InsP$_1$ accumulation in a variety of preparations (Baudry *et al.*, 1986; Nicoletti *et al.*, 1986c; Palmer *et al.*, 1988), including primary cultures of striatal neurons (Schmidt *et al.*, 1987). In addition, Fig. 16 demonstrated that MK801 has negligible effects on [^{3}H]-InsP$_1$ accumulation until reaching 10μM. This would also suggest that AP5 is not acting specifically on the NMDA receptor. The definitive experiments to support this hypothesis would be replicating the antagonist inhibitory dose-response curves in the absence of Ca^{2+}, and using fura-2 fluorescence to see if AP5 does affect Ca^{2+} influx.

It is interesting that the experiments reporting an AP5 effect were conducted on purified cultures of neurons. Sladeczek *et al.* (1985) and Nicoletti *et al.* (1986a,b) used primary cultures, whereas Schoepp *et al.* and other investigators used brain slices. The presence of glia is definitely a confounding effect, as Glu, Quis, Ibo and GTP analogue-evoked [^{3}H]-InsP$_1$ accumulation has been demonstrated in astrocytes (Pearce *et al.*, 1986; Nicoletti *et al.*, 1990; Robertson *et al.*, 1990; Jensen & Chiu, 1991). Thus, it is impossible to distinguish between neuronal versus glial responses to EAA stimulation in such preparations. Furthermore, the homeostatic function of the glia may mask or prevent neuronal responses that could otherwise be detected *in vitro*. Given the current state of metabotropic agonist pharmacology, it is unjustified to conclude that AP5 affects ACPD-evoked [^{3}H]-InsP$_1$ accumulation in cultured striatal neurons, although it does affect the NMDA-preferring agonist Ibo.

3. MODULATION EXPERIMENTS

The modulation experiments were conducted to examine if there was differential regulation of EAA agonist-evoked [^{3}H]-InsP$_1$ accumulation with respect to PKC activation, the presence of extracellular cations and G protein function. If one or more of the agonists exhibited a distinct profile from the others that could be distinguished pharmacologically, this would suggest that there was a heterogeneous intracellular mechanism(s) underlying its effect.

3.1 Effect of Phorbol Dibutyrate [^{3}H]-InsP$_1$ Accumulation

Figure 17 clearly showed that 1μM PDBu, a potent activator of PKC, inhibited all agonist-evoked [^{3}H]-InsP$_1$ accumulations, whereas the inactive congener 4-α-PDBu was ineffective. Similar results have been previously reported in primary cultures of mouse striatal neurons (Weiss *et al.*, 1989; Manzoni *et al.*, 1990b) and rat cerebellar granule cells (Canonico *et al.*, 1988), rat cortical slices (Godfrey & Taghavi, 1990) and rat hippocampal slices (Schoepp & Johnson, 1988b). This effect of PDBu was reported to be reversed by PKC antagonists such as staurosporine (Manzoni *et al.*, 1990b; Schoepp & Johnson, 1988b; Godfrey & Taghavi, 1990). The data presented above suggest that the most likely mechanism for this inhibition is the phosphorylation of one or more proteins involved in the signal transduction cascade that results in negative feedback.

Supporting the above explanation, PKC has been implicated in the negative feedback

control of phosphoinositide hydrolysis in human platelets (Watson & Lapetina, 1985), and PKC can be translocated in striatal neurons by PDBu (Weiss *et al.*, 1989). Although it has been reported that the muscarinic receptor can be phosphorylated by PKC (Safran *et al.*, 1990), this has yet to be demonstrated in the context of PDBu inhibition of EAA-evoked [^{3}H]-InsP$_1$ accumulation. There have also been studies suggesting that G proteins may also be a target of PKC phosphorylation, thereby uncoupling the receptor-PLC interaction (Sagi-Eisenberg, 1989). It is interesting to note that downregulation of PKC in primary cultures of cerebellar granule cells does not enhance Glu-evoked [^{3}H]-InsP$_1$ accumulation, but does protect against excitotoxicity (Favaron *et al.*, 1990). Finally, desensitization of the Quis evoked response by a 30 min pre-exposure to Glu was attenuated by PKC inhibitors but not by arachidonate inhibitors in primary cultures of cerebellar granule cells, suggesting that desensitization of metabotropic receptors involves PKC but not arachidonic acid metabolism (Catania *et al.*, 1991).

3.2 Effects of Extracellular Cations on [^{3}H]-InsP$_1$ Accumulation

Figure 18 shows the effect of removing various cations from the incubation buffer on [^{3}H]-InsP$_1$ accumulation. The most obvious effect of cation removal is electrochemical; the neurons may become slightly depolarized with respect to their membrane potentials. Since depolarization with KCl is able to evoke [^{3}H]-InsP$_1$ accumulation in striatal neurons (Weiss *et al.*, 1988), this may elevate basal phosphoinositide hydrolysis. Inclusion of 1mM Ni^{2+}, which slightly hyperpolarizes neurons, caused a small but significant attenuation in basal and Glu, CCh and KCl

evoked [^{3}H]-InsP$_1$ accumulations in synaptoneurosomes (Guiramand *et al.*, 1991b). However, it is noteworthy that replacement of Na^+ by $NMDG^+$ does not cause any significant change in resting membrane potential in concentrations up to 125mM (Guiramand *et al.*, 1991a).

3.2.1 The effect of Ca^{2+}: Figures 18 and 19 demonstrate that in striatal neurons, both basal and agonist-evoked [^{3}H]-InsP$_1$ accumulations have a substantial requirement for Ca^{2+}. This observation has also been reported extensively in the literature (Gusovsky & Daly, 1988; Palmer *et al.*, 1988; Doble & Perrier, 1989; Alexander *et al.*, 1990; Chandler & Crews, 1990; Patel *et al.*, 1991; Guiramand *et al.*, 1991b; **but** see Baird *et al.*, 1989). Ca^{2+} is able to cause phosphoinositide hydrolysis directly; this has been demonstrated with various ionophores and channel agonists that elevate intracellular Ca^{2+} (Gonzales & Minor, 1989; Baird & Nahorski, 1990a; Guiramand *et al.*, 1991b). It has also been proposed that increased intracellular Ca^{2+} can directly activate some PLC isoforms (for review, see Eberhard & Holz, 1988), and thereby enhance phosphoinositide hydrolysis. Therefore, one likely mechanism whereby low extracellular Ca^{2+} decreases phosphoinositide hydrolysis is via an inhibition of PLC activity (thereby explaining a decrease in basal phosphoinositide hydrolysis) and a destabilization of the PLC-G protein-metabotropic Glu receptor complex (thereby decreasing agonist-evoked increases), as proposed by Patel *et al.* (1991).

Somewhat surprisingly, reducing the extracellular Ca^{2+} concentration in various

preparations either has no effect or slightly elevates intracellular Ca^{2+} levels (Murphy *et al.*, 1987), as assayed by both fura-2 fluorescence (Reynolds & Miller, 1989) and $^{45}Ca^{2+}$ efflux (Patel *et al.*, 1991). However, increased intracellular Ca^{2+} does not necessarily equate to increased phosphoinositide hydrolysis, since all Glu receptor subtypes (NMDA, KA, AMPA, ACPD) can cause increases in intracellular Ca^{2+} as measured by fura-2 fluorescence (Holopainen *et al.*, 1991), but only the NMDA and metabotropic Glu receptors can cause any appreciable [^{3}H]-$InsP_1$ accumulation. However, the caveat with regards to NMDA-evoked [^{3}H]-$InsP_1$ accumulation is that NMDA-R activation, especially in instances of long-duration agonist stimulation, leads to a variety of intracellular responses such as arachidonic acid release (Dumuis *et al.*, 1988) and tyrosine phosphorylation (for review, see Miller & Oliva, 1992) in addition to Ca^{2+} release. Therefore, caution is indicated in interpreting [^{3}H]-$InsP_1$ accumulation evoked by NMDA or NMDA agonists such as Ibo.

Fowler and Tiger (1991) have suggested that the relative Ca^{2+}-dependencies of each agonist are a measure of the degree to which Ca^{2+} directly affects PLC and therefore [^{3}H]-$InsP_1$ release, or Ca^{2+} influx is secondary to [^{3}H]-$InsP_1$ release. Thus, for short incubation times (~30 sec.), there is an absolute requirement for Ca^{2+} for CCh, Quis and ACPD but not Glu, since Glu can elevate intracellular Ca^{2+} independent of its metabotropic action (Alexander *et al.*, 1990). However, this Ca^{2+} requirement disappears for CCh and Quis but not ACPD nor K^+ for long (>45 min.) incubations (Alexander *et al.*, 1990). One obvious consequence of Ca^{2+}-stimulated phosphoinositide hydrolysis is that $Ins(1,4,5)P_3$ in turn releases more Ca^{2+}. This

positive feedback cycle is probably terminated by PKC mediated feedback (see above Discussion section 3.1 viz. phorbol dibutyrate).

3.2.2 The effect of Na^+: The reduction in external Na^+ from 137mM to 16mM resulted in an increase in basal and agonist-evoked [^{3}H]-$InsP_1$ accumulations. Similar observations have been made in striatal slices (Doble & Perrier, 1989), cerebral cortical slices (Jope *et al.*, 1990), synaptoneurosomes (Chandler & Crews, 1990; Guiramand *et al.*, 1991a) and primary cultures of granule cells (Holopainen *et al.*, 1991). The fact that reducing Na^+ causes a detectable but not statistically significant increase in CCh-evoked release has also been observed by other investigators (Jope *et al.* (1990); Chandler & Crews, 1990; Guiramand *et al.*, 1991a). In addition, Jope *et al.* (1990) report that reducing extracellular Na^+ to 5mM in cortical slices decreased the EC_{50} value for Quis-evoked [^{3}H]-$InsP_1$ accumulation by two orders of magnitude and increased its efficacy 6-fold. It is important to note that the inclusion of tetrodotoxin (TTX) with agonists does not enhance phosphoinositide hydrolysis as does Na^+ removal (Sladeczek *et al.*, 1985; Jope *et al.*, 1990; Guiramand *et al.*, 1991a); presumably, this indicates that the Na^+ enhancement is not mediated via voltage-sensitive sodium channels, nor through secondary neurotransmitter release. In addition, replacement of Na^+ by $NMDG^+$ in synaptoneurosomes does not significantly affect the membrane potential (Guiramand *et al.*, 1991a). However, various agents that increase intracellular Na^+ such as veratridine, batrachotoxin, JSTX (all via voltage-gated Na^+ channels), monensin (a Na^+ ionophore) and ouabain (blocks Na^+/K^+-ATPase) all increase phosphoinositide hydrolysis in various neural

preparations (Kendall & Nahorski, 1984; Gusovsky *et al.*, 1986; Hollingsworth *et al.*, 1986; Récasens *et al.*, 1987; Gusovsky & Daly, 1988; Tiger *et al.*, 1989; Baird & Nahorski, 1990a).

If the increase in basal [^{3}H]-InsP$_1$ accumulation (~60%) is subtracted from each agonist, then there are no significant increases in agonist-evoked [^{3}H]-InsP$_1$ accumulation - all are reduced essentially to the equivalent of agonist alone. Therefore, the mechanism(s) by which reduced extracellular Na^+ enhances phosphoinositide hydrolysis is not likely to be receptor-mediated, but more likely affects a tonically active, basal component of the signal transduction sequelae. Several investigators have proposed that the elevation of basal phosphoinositide hydrolysis in low Na^+ may be due to a reversal in the direction of the Na^+/Ca^{2+} exchanger, thereby causing increases in intracellular Ca^{2+} (Chandler & Crews, 1990; Jope *et al.*, 1990). This is supported by the fact that in synaptoneurosomes in nominally Na^+-free medium, decreasing or increasing the extracellular Ca^{2+} results in dose-dependent decreases or increases, respectively, of phosphoinositide hydrolysis (Chandler & Crews, 1990). The corollary of those experiments is that intracellular Ca^{2+} levels are increased in fura-2 loaded synaptoneurosomes in Na^+ free medium (Daniell *et al.*, 1987; Chandler & Crews, 1990; Guiramand *et al.* 1991b). Guiramand *et al.* (1991a) also used amiloride and its derivatives, which are known to block both Na^+/Ca^{2+} and Na^+/H^+ exchangers, and found an elevation in basal but not Glu, CCh or KCl-evoked [^{3}H]-InsP$_1$ accumulations in rat brain synaptoneurosomes. Similar results from Benuck *et al.* (1989) have also implicated the Na^+/Ca^{2+} exchanger.

There have also been suggestions that Na^+ acts upon G protein-PLC coupling to enhance phosphoinositide hydrolysis (Gusovsky & Daly, 1988; Chandler & Crews, 1990). For example, the inhibitory G_i protein associated with cyclic AMP is reported to be Na^+-dependent (Jakobs & Wieland, 1989; also see section 4.1 below). Either one of the mechanisms proposed above could satisfactorily explain the data observed.

3.2.3 The effect of Mg^{2+}: Reducing the extracellular Mg^{2+} concentration attenuated agonist-evoked [^{3}H]-$InsP_1$ accumulation, but did not affect the basal. Nicoletti *et al.* (1987) found an increase in L-Glu and NMDA-evoked responses, but not Quis-evoked responses in primary cultures of cerebellar granule cells in zero extracellular Mg^{2+}. O'Neill *et al.* (1991) observed that Mg^{2+} inhibited [^{3}H]-PIP_2 hydrolysis in the post-mortem human brain. However, Doble & Perrier (1989) found that Mg^{2+} had negligible effects on NMDA and CCh-evoked responses in neonatal rat striatal slices. Despite these results, other investigators have found that Mg^{2+} stimulates PLC activity in rat cortical membranes (Litosch, 1987, 1989; Claro *et al.*, 1989). Mg^{2+} is also known to promote the activity of many G proteins by activating and stabilizing the G_α subunit (for review, see Gilman 1987). Corroborating this, Llahi *et al.* (1992) recently found that Quis, GTPγS and Quis+GTPγS-evoked phosphoinositide turnover in rat cerebellar membranes was greatly attenuated in zero Mg^{2+}, and reached optimal levels at 10mM Mg^{2+}. And finally, the NMDA receptor exhibits a well-characterized, voltage-dependent Mg^{2+} blockade of the channel (for review, see Smart, 1989). With all these potential physiologic functions, interpretations of the

action of Mg^{2+} is difficult. One possible explanation is that upon receptor activation, the absence of Mg^{2+} impairs the ability of G proteins to couple the metabotropic receptor to PLC, and therefore results in an inhibition of the agonist-evoked response. Another explanation could be that the absence of extracellular Mg^{2+} is highly unfavourable for neuronal viability due to the absence of glia in this preparation, and damages the neuronal capacity for phosphoinositide release. However, both explanations are highly speculative and there is no evidence to support them.

3.3 Additive Effects of Pertussis Toxin and Extracellular Ca^{2+} Reduction on [^{3}H]-$InsP_1$ Accumulation

As seen in Fig. 20, 100ng/ml PTX pre-treatment was able to attenuate all agonist-evoked [^{3}H]-$InsP_1$ accumulations, although this did not quite reach significance for Ibo and ACPD. If these experiments were replicated in nominally Ca^{2+}-free buffer, the [^{3}H]-$InsP_1$ accumulation was attenuated to almost baseline. PTX ADP-ribosylates G-proteins, thereby inactivating them (Katada & Ui, 1982a,b). Similar observations regarding PTX have been made by Sugiyama *et al.* (1987) in mRNA injected *Xenopus* oocytes, Chuang and Dillon-Carter (1988) in NCB-20 neurohybrid cells, and Houamed *et al.* (1991) in *Xenopus* oocytes injected with the putative cDNA clone for the metabotropic receptor. G-protein involvement has also been suggested by numerous studies using non-hydrolyzable GTP analogues to stimulate phosphoinositide hydrolysis (Cockcroft & Gomperts, 1985; Gonzales & Crews, 1985; Claro *et al.*, 1989; White & Scates, 1991), by using F^- anions to activate G-proteins (Jope, 1988; Chandler & Crews, 1990), and by antibodies against the $G\alpha_q$ subunit

(Gutowski *et al.*, 1991). Llahi *et al.* (1992) recently found that Quis, Ibo, L-Glu and ACPD-evoked phosphoinositide hydrolysis in GTP-free rat cerebellar membranes was above basal, and could be further enhanced by GTPγS in a dose-dependent manner. This strongly suggests that there is a non G-protein-coupled mechanism to evoke phosphoinositide hydrolysis that is additive with the G-protein coupled mechanism.

The observation that PTX pretreatment and reducing Ca^{2+} were able to attenuate phosphoinositide hydrolysis almost to baseline suggests that there are two mechanisms present in striatal neurons: a PTX-sensitive, presumably G-protein mediated mechanism, and a second Ca^{2+}-sensitive mechanism. Some authors have suggested that this occurs at the level of receptor coupling to PLC, ie. that there are two isoforms of PLC: a G-protein dependent isoform, and a G-protein independent but Ca^{2+}-dependent isoform (Eberhard & Holz, 1988; Chandler & Crews, 1990). Two immunologically distinct forms of PLC that are differentially sensitive to Ca^{2+} have been isolated from bovine brain (Ryu *et al.*, 1987), and the hypothesis is that the G-protein isoform preferentially hydrolyses PIP_2 (leading to $Ins(1,4,5)P_3$ and DG, which activates PKC), whereas the Ca^{2+}-dependent isoform preferentially hydrolyses PIP (leading to $Ins(1,4)P_2$ and DG). Thus, G-protein route mobilizes Ca^{2+} and activates PKC, whereas the Ca^{2+} route preferentially activates PKC only. This is supported by evidence that stimulating $[^3H]$-$InsP_1$ accumulation with calcium ionophores versus CCh produces a disproportionately large amount of $[^3H]$-$InsP_2$ versus $[^3H]$-$InsP_3$ (Brammer *et al.*, 1988; Baird & Nahorski, 1990a). Kinetics of the

[^{3}H]-$InsP_2$ thus produced indicate that it does not come from the degradation of [^{3}H]-$InsP_3$ (Brammer & Weaver, 1989), suggesting that this might be mediated by preferential PLC hydrolysis. It is also theoretically possible that these two (or more) PLC isoforms may be coupled to different metabotropic Glu receptor subtypes.

4. MODELS OF EAA-EVOKED PHOSPHOINOSITIDE HYDROLYSIS IN STRIATAL NEURONS

4.1 Existence of two Metabotropic Receptor Subtypes

Many investigators have suggested the existence of two subtypes of the metabotropic Glu receptor: an Ibo/ACPD-preferring receptor, and a Quis-preferring receptor insensitive to all antagonists (Sladeczek *et al.*, 1988; Palmer *et al.*, 1988; Baird & Nahorski, 1990b). This hypothesis was largely based on the different profiles elicited by the above agonists. To define this more precisely, I propose that these subtypes are Glu-preferring and Asp-preferring/AP5 sensitive, respectively, since Quis and Ibo/ACPD are not endogenous substrates. However, other than the molecular cloning data, there is not much direct evidence (pharmacologic, electrophysiologic, immunocytochemical or histochemical) supporting this hypothesis.

The recent molecular work of Tanabe *et al.* (1992) yielded multiple clones of the metabotropic receptor, providing the genetic basis necessary for the existence of multiple sub-types. However, only their original mGluR1 clone (Masu *et al.*, 1991) was coupled to phosphoinositide hydrolysis; the mGluR2 clone attenuated forskolin

evoked cyclic AMP production (Tanabe *et al.*, 1992) in a manner similar to the recent report by Schoepp *et al.* (1992). The mGluR3 and mGluR4 clones were not successfully expressed in *Xenopus* for characterization. However, it was recently reported that ACPD inhibits forskolin-evoked [^{3}H]cAMP in 6 DIV cultured striatal neurons via a PTX-sensitive G protein (Manzoni *et al.*, 1992); this is probably the pharmacologic correlate of Tanabe's mGluR2 clone. Thus, metabotropic Glu receptors coupled to heterogeneous intracellular mechanisms do exist. Whether these receptors actually are those hypothesized in this thesis awaits further characterization. However, given the fact that at least 4 mGluR clones exist to constitute subunits for the metabotropic Glu receptor (it is unknown whether it exists as a monomer or heteromer) and that these subunits could potentially exist in flip/flop forms like the KA/AMPA receptors, it is quite likely that these receptor subtypes do exist. Whether they actually have differential agonist/antagonist responses and serve different functions *in vivo* remains to be elucidated.

4.2 Cross-reactivity of Metabotropic Agonists at other EAA Receptors

The alternative explanation is that the agonists are interacting with more than one EAA receptor, and generating differential metabotropic responses due to their co-activation. Since mouse striatal neurons *in vitro* do express functional NMDA (Weiss, 1990; Williams *et al.*, 1991) and AMPA receptors (Weiss, 1989; Tse *et al.*, 1991), cross-reactivity of Quis and Ibo is likely occur regardless of its effect on the metabotropic response. However, this cross-reactivity is greatly affected by the duration of agonist stimulation as discussed in section 4.5 below.

4.2.1 Cross-reactivity of Quis at the AMPA-R: Quis is well known to activate the AMPA-R, which depolarizes the neuron via a Na^+ influx (see Table I). This depolarization could also activate voltage-sensitive calcium channels (VSCCs), thereby allowing Ca^{2+} influx (Patel *et al.* 1991). The increased intracellular Na^+ could also activate Na^+/Ca^{2+} exchangers. The stimulatory effects of Ca^{2+} on phosphoinositide hydrolysis have already been discussed in section 3.2.1 above. However, recent studies suggest that if VSCCs are involved, then they are novel ones insensitive to classic organic and inorganic VSCC blockers against L, N, P and T type channels (Guiramand *et al.* 1991b). Supporting this idea of cross-reactivity, CNQX-sensitive Quis- and AMPA-evoked increases in intracellular fura-2 fluorescence have been observed that take less than 1 min. to occur (Manzoni *et al.* 1991). In addition, KCl or veratridine depolarization can cause TTX-resistant increases in intracellular Ca^{2+} (Gonzales & Minor, 1989). In guinea pig (Alexander *et al.*, 1990) and rat (Baird & Nahorski, 1990b) cortical slices, 1mM AMPA has been observed to cause a modest (200% basal at saturating concentrations) increase in phosphoinositide hydrolysis under both long and short agonist stimulation durations (45 min. and 5 min., respectively). In the 5 min. stimulation experiment, the AMPA-evoked [^{3}H]-$InsP_1$ accumulation was similar to that evoked by depolarization or Ca^{2+} ionophores (Baird & Nahorski, 1990b). In the 45 min. stimulation experiment, Quis and AMPA but not Glu nor ACPD-evoked [^{3}H]-$InsP_1$ accumulation was competitively inhibited by CNQX, indicating that Quis does act differently from other agonists. This is in contrast to cultured striatal neurons, where a 5 min. incubation with 100μM AMPA resulted in negligible [^{3}H]-$InsP_1$ accumulation (Weiss,

1989). The most likely explanation for this discrepancy is the agonist stimulation duration; if Quis is allowed to depolarize the neurons for an extended period, a number of indirect effects could occur. For example, joint activation of ionotropic AMPA and metabotropic Glu receptors (whether by Quis or by AMPA+ACPD, 15 min. duration) in cultured striatal neurons results in [^{3}H]arachidonic acid release (Dumuis *et al.*, 1990). In the present experiments, it is most likely that there was some contribution from AMPA receptor depolarization, since the 10 min. incubation was twice the duration of the previous experiments (Weiss, 1989). However, if Quis was acting partially at the AMPA-R, then it would likely be AP5-resistant as shown in the data (see Fig. 12a). Furthermore, AP3 and AP4 should only block the metabotropic receptor, and would not interfere with the putative AMPA-R contribution to Quis-evoked phosphoinositide hydrolysis (Fig. 12b).

The above argument presumes that the AMPA receptor, when activated by Quis, mediates a Na^+ influx that results in an increase intracellular Ca^{2+}. However, recent reports from Heinemann and coworkers (Hollmann *et al.*, 1991; Keller *et al.*, 1992) suggest that KA/AMPA receptor channels formed from the GluR1 and GluR3 subunits are also directly permeable to Ca^{2+}. This corresponds to the GluRA to GluRC subunits (Keinänen *et al.*, 1990), which also exhibit a preference for AMPA over KA. Thus, this may indicate the presence of an ionotropic AMPA receptor that is directly permeable to Ca^{2+} due to subunit configuration, which could account for the potency and efficacy of Quis as a metabotropic agonist (see section 3.2.1 above for the effects of Ca^{2+}).

One can also approach this from an ionotropic point of view. Under current clamp, Purkinje neurons exhibit a long-lasting, multiphasic response when stimulated by Quis or L-Glu (Yool *et al.*, 1992). This rapid, depolarization phase of this response can be mimicked by AMPA, and the long-lasting spiking can be mimicked by Ibo or ACPD (Yool *et al.*, 1992). It was therefore suggested that the "normal" effect of Quis (and the endogenous ligand L-Glu) under *in vivo*, physiologic conditions is to co-activate both ionotropic and metabotropic receptors (Yool *et al.*, 1992).

4.2.2. Cross-reactivity of Ibo at the NMDA-R: A similar argument could be applied to Ibo-evoked phosphoinositide hydrolysis, since Ibo is an NMDA agonist. Ibo could activate the NMDA-R, thereby allowing Ca^{2+} influx and enhancing PLC activity; Ibo-evoked increases in intracellular Ca^{2+} have been observed in striatal neurons that are MK801-sensitive but TTX-insensitive (Manzoni *et al.*, 1991). In addition, the depolarization caused by the NMDA-R may also be sufficient to activate VSCCs, and may thus allow an even greater Ca^{2+} influx (similar to the Quis depolarization above) that is otherwise insensitive to NMDA antagonists. Note, however, that the time course of agonist stimulation is a limiting factor (see section 4.5 below).

If Ibo were acting partially at the NMDA-R, it would likely be AP5-sensitive, as seen in Fig. 10 at AP5 concentrations $\geq$ 100μM. This AP5 effect was competitive, as seen in Fig. 13, and is therefore not likely to be acting at the metabotropic Glu receptor in a fashion similar to AP3 and AP4. However, the data from Fig. 15 suggest that the Ibo-evoked increase is not affected by up to 3μM MK801, and therefore argues

against this idea. The attenuation caused by extracellular Mg^{2+} removal (Fig. 17) is also not consistent with the known Mg^{2+}-induced blockade of NMDA-R. In addition, the fact that 300μM AP5 increased the EC_{50} for Ibo from 7.6 to 19μM also argues for a more direct effect at the metabotropic receptor itself.

4.2.3. Cross-reactivity of ACPD at the NMDA-R: ACPD has been demonstrated to be have some affinity at the NMDA-R. For example, the IC_{50} values for ACPD displacing [^{3}H]CGS-19755 binding were 421 and 547μM, respectively, for 1R,3S-ACPD and 1S,3R-ACPD (Schoepp *et al.*, 1991). In addition, ACPD-evoked excitation in rat spinal cord slices *in vitro* was also D-AP5 sensitive (Magnuson *et al.*, 1989). Given the fact that a racemic mixture of 1R,3S-and 1S,3R-ACPD was used in these experiments, and that it was usually at a pharmacologic dose of 100μM, this is not an unlikely proposition. The pharmacology of the ACPD-evoked phosphoinositide hydrolysis would therefore be expected to be similar to Ibo, except that it would be much less sensitive to AP5 (Figs. 11 and 14), and this is what was observed.

4.3 Differential Coupling of the Metabotropic Glu Receptor to G-Protein Preferring and Ca^{2+} Preferring Phospholipase C Isoforms

The argument for the existence of at least two PLC isoforms, differentially sensitive to PTX and reduced external Ca^{2+}, was largely presented in section 3.2.1 above. Although phosphatidylinositol-specific isoforms of PLC (PI-PLC) have been isolated from brain tissues (eg. δ-PI-PLC; Peterson *et al.*, 1991), it is not known whether these PI-PLC isoforms are coupled to G-proteins or are Ca^{2+}-sensitive, or even if they are

activated by the Glu metabotropic receptor. However, Xu and Nelsestuen (1992) recently characterized the bovine brain α-PI-PLC, and found that it did not bind Ca^{2+} at concentrations required to activate enzyme activity, either when associated with phospholipid vesicles or in solution. This isoform might correspond to the G-protein coupled, PTX-sensitive isoform of PI-PLC. Furthermore, PI-PLC may be subject to negative feedback control; using novel $InsP_3$ analogs, Hirata and colleagues recently demonstrated that one of the $InsP_3$ binding proteins purified from rat brain extracts was the δ isoform of PLC (Kanematsu *et al.*, 1992). Thus, the role of PI-PLC in regulating the metabotropic transduction pathway is becoming increasingly complex.

It is conceivable that the different agonists, such Quis versus Ibo/ACPD, activate different receptor subtypes that couple to different G-proteins, different PI-PLC, and therefore different transduction pathways. This is suggested by Fig. 19, since Quis-evoked [^{3}H]-$InsP_1$ accumulation was significantly affected by PTX pre-treatment, whereas Ibo and ACPD were less affected. These data suggest that Ibo/ACPD-evoked phosphoinositide hydrolysis may be more Ca^{2+}-preferring.

4.4 Existence of Multiple Neuronal Subpopulations *In Vitro*

The cellular composition of the striatum *in vivo* contains many neuronal types (see Introduction). The majority of these (95%) are medium-sized, densely spined GABAergic neurons with a few (2-3%) large, cholinergic interneurons (Mettler, 1968). Furthermore, some striatal GABAergic neurons colocalize with substance P or enkephalins (for review, see McGeer & McGeer, 1987). And although the

cultures were checked microscopically before use, the possibility of slight glial contamination does exist. It is therefore possible that the heterogenous responses observed were not due to different receptor subtypes, but rather due to different neuronal types in culture with different responses to the agonists, ie. different signal transduction coupling, such as through PLC isoforms. The presence of tachykinin or opiate receptors on subpopulations of neurons in culture may have a feedback or second messenger cross-talk effect. For example, Schoepp *et al.* (1992) found that ACPD inhibited forskolin-evoked cAMP production in rat hippocampal slices, but did not affect the basal cAMP level. Therefore, it is possible that the intrinsic neurochemical phenotype of the neurons *in vitro* may affect their response to metabotropic agonists and cause the heterogenous responses observed.

4.5 Time Course of Agonist Stimulation

The duration of EAA stimulation needs to be addressed, since this varies widely in the literature. The duration itself may be responsible for the heterogeneous responses observed, such as Quis-evoked depolarization at the AMPA-R (see section 2.1 above) and the cooperative release of [^{3}H]arachidonate by ACPD plus AMPA (Dumuis *et al.*, 1990). The clearest example of this is Glu itself. In isolated guinea pig hippocampal neurons, a brief (1-3 sec.) application of Glu results in a brief, transient elevation of intracellular Ca^{2+} that recovers to baseline rapidly upon washout (Connor *et al.*, 1988). However, a second repeat application of Glu (1-3 sec. again) resulted in a sustained (20-25 min.) elevation in intracellular Ca^{2+} (Connor *et al.*, 1988). In cultured rat cerebellar granule cells, a long (15 min.)

sec. again) resulted in a sustained (20-25 min.) elevation in intracellular Ca^{2+} (Connor *et al.*, 1988). In cultured rat cerebellar granule cells, a long (15 min.) application of 50μM Glu resulted in a sustained increase in $^{45}Ca^{2+}$ uptake 30 min. after removal of the agonist (Manev *et al.*, 1989). Another example of anomalous effects due to prolonged incubation is the effect of NMDA; 10μM NMDA inhibits Quis-evoked phosphoinositide hydrolysis in long (60 min.) incubations, possibly via PKC activation (Palmer *et al.*, 1988).

Therefore, interpretation of results from experiments with long agonist stimulation times should be made cautiously, since many potential confounding side effects exist, and these must be taken into consideration. Short stimulation durations, on the order of 5 min., are the easiest way to circumvent these problems. As indicated above, the intermediate stimulation durations used in these experiments probably invoke a number of undesirable side-effects, such as cross-reactivity at other EAA receptors.

CONCLUSIONS AND SUMMARY

1. RELATIONSHIP BETWEEN THE PHARMACOLOGY AND FUNCTIONS OF THE STRIATAL METABOTROPIC RECEPTOR

1.1 Major Findings of this Thesis

The major findings from the experiments performed in this thesis can be summarized as follows:

(i) Quis, Ibo and ACPD were all effective metabotropic agonists in striatal neurons.

(ii) AP3 and AP4 were non-competitive antagonists of the EAA-evoked [^{3}H]-$InsP_1$ accumulation, with AP3 being a more potent and efficacious antagonist. This effect was most likely a direct effect upon the metabotropic receptor, and not a secondary response to AP3 or AP4. AP5 appeared to selectively inhibit the Ibo-evoked response. This could be partially attributed to the cross-reactivity of Ibo at the NMDA-R during the 10 min. incubations rather than the existence of an Ibo-preferring subtype of receptor. However, the latter possibility cannot be precluded.

(iii) Both EAA-evoked and muscarinic [^{3}H]-$InsP_1$ accumulations were significantly inhibited by 1μM PDBu, presumably due to feedback by PKC upon some component of the signal transduction pathway not specific to EAA- or cholinergic-mediated phosphoinositide hydrolysis.

(iv) EAA-evoked [^{3}H]-$InsP_1$ accumulation was significantly inhibited by the absence of extracellular Ca^{2+}, and marginally attenuated by PTX pre-treatment.

When combined, the absence of Ca^{2+} and PTX inhibited EAA-evoked [^{3}H]-$InsP_1$ accumulation nearly to baseline. This suggests that the combination of extracellular Ca^{2+} and G-protein activation is both necessary and sufficient for EAA-evoked [^{3}H]-$InsP_1$ accumulation. This could possibly be mediated by two PI-PLC isoforms differentially activated by Ca^{2+} and G-proteins.

(v) Both EAA- and muscarinic-evoked metabotropic responses were significantly inhibited in the absence of extracellular Ca^{2+} and Mg^{2+}, and elevated in the absence of extracellular Na^+. The Ca^{2+} effect was explained above. The Mg^{2+} inhibition is difficult to interpret, given the multiplicity of physiologic effects attributed to Mg^{2+}. The Na^+ enhancement is likely due to a reversal in tonic, basal Na^+/Ca^{2+} exchange, thereby increasing intracellular Ca^{2+} and allowing activation of the Ca^{2+}-preferring PI-PLC isoform.

1.2 Interpretation of the Findings: An Answer to the Stated Hypothesis

A detailed characterization of the striatal metabotropic Glu receptor *in vitro* revealed heterogeneous effects elicited by different EAA agonists, supporting the original hypothesis of differential mechanisms mediating metabotropic glutamate receptor evoked phosphoinositide hydrolysis. These effects were probably due to a small degree of cross-reactivity of the agonists at other EAA receptors during the 10 min. incubation (eg. Quis at the AMPA-R and Ibo at the NMDA-R), as well as the existence of Quis and Ibo/ACPD-preferring metabotropic Glu receptor subtypes. Since structurally distinct mGluR subtypes have been demonstrated by molecular cloning (Tanabe *et al.*, 1992) and functionally distinct mGluR subtypes have been

demonstrated pharmacologically (Manzoni *et al.*, 1992), this is not an untenable supposition. Transduction of the metabotropic signal was found to be sensitive to PKC feedback, and was dependent on both extracellular Ca^{2+} and G-protein activation. This suggests differential activation of the metabotropic signal transduction pathways, possibly reflecting (1) the differing role of the metabotropic receptor in the neonatal versus adult striatum, and (2) the above mentioned mGluR subtypes being differentially coupled to the second messenger sequelae.

2. PROPOSED LINES OF RESEARCH AND FUTURE EXPERIMENTS

There is still much work to be done with the metabotropic glutamate receptor; many questions remain unanswered. With the cloning of the mGluR subtypes by Tanabe *et al.* (1992), the field has been opened to the tools of molecular biology. The following lines of inquiry are proposed to investigate the mGluR and resolve some issues that current pharmacologic tools cannot answer.

(i) A series of agonist stimulation time course experiments should be conducted to examine heterogeneous effects elicited by long incubations (eg. cross-activation of other EAA receptors), and to determine an ideal time frame for EAA stimulation that eliminates these extraneous factors.

(ii) The metabotropic glutamate receptor protein should be isolated, purified and reconstituted in *Xenopus* oocytes or in membrane vesicles. This can be accomplished by a number of biochemical methods. For example, using deduced

peptide sequences from the mGluR clones, antibodies could raised against the mGluR and used for immunoaffinity purification. Or a selective ligand (such as ACPD or another as it becomes available) could be immobilized on a column for affinity purification. This process is probably already underway in a number of laboratories. Once purified, the physical properties of the protein can be examined to determine if it exists as a monomer or as a homo/heterodimer, etc.

(iii) An exhaustive pharmacologic characterization needs to be carried on each purified mGluR clone, probably as expressed in *Xenopus* oocytes. A series of experiments similar to those in this thesis should be conducted to examine the pharmacology and regulation of each mGluR subtype. As more selective agonists and antagonists become available, they should also be used. High performance liquid chromatography is already in use to examine the inositol polyphosphate isomers and species evoked by the mGluR, and this technique should be extended to each mGluR clone. In addition, Ca^{2+} imaging techniques and fluorescent $InsP_3$ probes (as they become available) could also be used to analyze the intracellular transduction effects of each clone. Following this, the mGluR subtypes should be expressed in combination (assuming the that mGluR is not a monomer) to see if different subunit composition has differing pharmacology, different effects on signal transduction (eg. $InsP_x$ generation, PI-PLC/PKC activation, intracellular Ca^{2+}, etc.) and possibly different functions *in vitro*.

(iv) Using oligonucleotide probes deduced from the mGluR clones, *in situ* hybridization experiments using striatal neurons and glia of various ages from embryonic to senescent could be performed, along with indirect immuno-

cytochemistry to label the cells for other markers such as neurotransmitter, cytoskeletal components, etc. This ought to identify which types of cells express which receptors, and when. This could be further extended to examine cells in other preparations (acutely dissociated or in slices), as well as transformed neuronal lines. Other regions of the CNS could also be examined to determine if there is region-specific expression of metabotropic Glu receptor subtypes at various ages.

(v) Using the oligonucleotides in (iv) above, antisense mRNA experiments could be performed at various ages to determine the function(s) of the mGluR clones *in vitro* with respect to viability, excitotoxicity, phenotypic expression, etc. Other brain regions could also be subject to examination to determine the role of their endogenous metabotropic receptors.

(vi) The experiments listed in (iv) and (v) should be replicated *in vivo*, using an animal model such as rodents. In addition, various behavioural and pharmacokinetic studies could then be performed. These studies ought to elucidate more of the functions of the mGluR. In addition, the simultaneous manipulation of other EAA receptors (especially the NMDA-R and AMPA-R) should shed more light on the issue of cross-reactivity, and its role *in vivo* (if any).

(vii) Transgenic animals could be constructed containing a mGluR transgene. The gene could be directed under an inducible promoter (eg. heat-shock or lac-Z) to examine its effects on behaviour, or the mGluR promoter could be tagged with a marker gene (eg. β-galactosidase or luciferinase) to examine its distribution. Once stable, the transgenic lines could be subject to point mutations and deletions to determine which regions of the mGluR gene are necessary for function.

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