An Investigation of the Metabotropic Glutamate Receptor of Hippocampal Neurones with Flash Photolysis

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A thesis submitted for the degree of
Doctor of Philosophy in the University of London

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2002
Abstract

L-glutamate is an excitatory amino acid acting on ionotropic receptors, which mediate fast synaptic transmission, and metabotropic glutamate receptors (mGluRs), which are thought to modify excitability by modulating potassium channels and non-selective cation channels.

The aims of this project are to characterise the ionic basis of the mGluR response in hippocampal CA1 pyramidal cells, to identify channels that are responsible for the effect and to investigate underlying intracellular mechanisms.

Flash photolysis of ‘caged’ ligands is a powerful tool for time-resolved studies of the post-synaptic effects of neurotransmitters and the intracellular effects of caged second messengers. It can be used with whole-cell patch clamp and fluorescent calcium indicators to measure ionic currents and intracellular calcium concentrations ([Ca^{2+}]_i). The characterisation of a novel caged L-glutamate used in these experiments is described.

There are two components to the mGluR response; early, activating after about half a second, and late, activating after about a second. The two components have different reversal potentials, implying that there are two different channels, selective for different ions. There is a [Ca^{2+}]_i rise associated with the late mGluR response.

Ion substitution and pharmacological experiments identified the late channel as potassium-selective. The early component appeared to be a voltage-sensitive non-selective cation conductance. Antagonists acting selectively at different mGluR subtypes indicated that the late potassium channel is linked to activation of a Group I mGluR and the early channel to a Group II mGluR.

In many cells, Group I mGluRs activate the inositolphosphate (IP) intracellular pathway to increase [Ca^{2+}]_i, which activates calcium-sensitive potassium channels. Inositol 1,4,5-trisphosphate (IP_3), released by flash photolysis in pyramidal cells, resulted in a potassium current that resembled the late mGluR current in timecourse and amplitude. Thus, the photorelease of IP_3 mimics the late component of the mGluR response, both in [Ca^{2+}]_i increase and activation of the potassium conductance, indicating that the IP pathway mediates the late inhibitory response.
Acknowledgements

I thank my supervisor, Dr. David Ogden. He has been genuinely supportive, patient, tolerant and generous with his time. While his technical expertise provided an excellent grounding for my project, he bestowed me precious freedom to develop my own creativity and style, which I greatly appreciate.

Dr. Tom Carter, my second supervisor, has been consistently willing to help. Tom has always been keen to discuss my work and has offered valuable insights. Chris Magnus has been indispensable; eager to assist with technical problems, talk about scientific ideas and generally find things, order things and laugh about things.

I acknowledge Dr. Marco Canepari for his partnership during the cage characterisation experiments and for aiding my arduous introduction to Matlab. I am grateful to Drs. John Corrie, George Papageorgiou and David Trentham for their kind donation of the caged compounds. Thank you to Sarah Lilley, who taught me about master documents, and witnessed the thesis in its entirety for the first time!

Finally, I thank BG and PP for their support and empathy throughout a challenging experience, and TN for her sisterly love.
"...I seem to have been only like a boy playing on the sea-shore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me."

Isaac Newton.
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Chapter 1 – Introduction
Excitatory amino acids as neurotransmitters

Excitatory amino acids (EAAs) can activate neurones in the central nervous system (CNS). This was first shown by Japanese scientists studying epilepsy (Okamoto, 1951; Hayashi, 1953), who performed direct injections of glutamate and aspartate onto cortical neurones and then by Curtis and co-workers (1959), who demonstrated that spinal neurones respond to iontophoretic application of EAAs. The EAA L-glutamate became well established as an invertebrate peripheral neurotransmitter and has been studied in a number of invertebrate systems, for example the crayfish muscle (Takeuchi & Takeuchi, 1964).

In the central nervous system, the distributions of putative neurotransmitters were examined. It was found that in cat spinal cord, regions of high concentration coincided with areas of interneuronal excitatory release (Graham et al., 1967). L-glutamate and L-aspartate were suggested to be good candidates as endogenous neurotransmitters in the CNS, supported by pharmacological, binding and electrophysiological studies (Watkins & Evans, 1981). The EAAs appeared not only to mediate normal fast synaptic transmission along excitatory pathways (Fagg & Foster, 1983), but were also able to modify the efficiency of synaptic transmission.

Multiple receptor types

Curtis & Watkins (1961; 1963) tested analogues of glutamate on cat spinal and cortical neurones and found that N-methyl-D-aspartate (NMDA) was the most potent. Analogues of other acidic amino acids were also shown to have strong excitatory effects, indicating that there are four receptor populations which are sensitive to glutamate analogues (Foster & Fagg, 1984; Monaghan et al., 1989; Nicoll et al., 1990). These are NMDA receptors, quisqualate (Biscoe et al., 1975) or amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and
kainate receptors (Johnston et al., 1974).

Autoradiographical analysis was consistent with the pharmacological data. It revealed that \[^{3}H\]-L-glutamate (Monaghan et al., 1983), \[^{3}H\]-kainate (Simon et al., 1976) and \[^{3}H\]-AMPA (Honoré et al., 1982) binding sites were present throughout the brain (Fagg, 1985; Cotman et al., 1987).

**Ionotropic glutamate receptors**

A breakthrough in classifying EAA receptors came from the development of selective antagonists (Watkins et al., 1990). The first was D-α-aminoacidipate (DAA), an antagonist for NMDA-receptors (Biscoe et al., 1977). A more potent and selective NMDA-receptor antagonist, 2-amino-5-phosphonovaleric acid (AP5), was discovered by Davies and co-workers (1981). Davies & Watkins (1982) identified D-AP5 as the more potent and selective isomer of the drug (it was more effective at blocking synaptic activity).

Later, Davies et al. (1986) described the most potent NMDA-receptor antagonist yet, 3-((±)-2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP). The concentration of CPP required to inhibit a response by 50% was 5 times smaller than that of D-AP5, and the off-rate of CPP was approximately 18 times slower (Benveniste & Mayer, 1991).

Non-NMDA glutamate receptors were shown to be selectively blocked by quinoxalinediones, such as 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Honoré et al., 1988). However, CNQX has been shown, at high concentrations, to act at the NMDA-receptor, specifically at the site where glycine modulates the receptor (Mead & Stephens, 1999). A more potent non-NMDA antagonist is 6-nitro-7-sulphamoylbenzo[f] quinoxaline-2,3-dione (NBQX) (Yu & Miller, 1995).
There is homology both in structure and function of ionotropic glutamate receptors (reviewed by Bigge, 1999). AMPA receptor cDNAs were cloned in 1989 (Hollmann et al.) and NMDA-receptors cloned in 1991 (Moriyoshi et al.; reviewed by Nakanishi, 1992). The ionotropic receptors have subunits with four domains, M1 - M4. All domains except M2, which is a re-entrant membrane loop and lines the inner channel pore, are transmembrane segments.

The NMDA receptor has a high permeability to calcium, as well as to potassium and sodium, and is modulated by glycine. Non-NMDA receptors have a low permeability ratio of divalent to monovalent ions as a direct consequence of RNA editing on M2 on a particular AMPA-receptor subunit, GluR2 (or GluRB). A gene-specified glutamine (Q) codon is changed to an arginine (R) codon at a position known as the Q/R site, and this edited form of the receptor displays low divalent permeability (Burnashev et al., 1992).

In the region between transmembrane segments three and four of non-NMDA receptors, there is an alternative RNA splicing sequence called flip/flop. Flip and flop receptors have distinct kinetics and amplitudes of agonist-induced responses (Sommer et al., 1990). Receptors with flip occur during early development and desensitise slower (desensitisation is sensitive to cyclothiazide): flop is present in mature animals. Both kainate and AMPA-receptor subunits form heteromeric receptors with other members of their respective groups, but co-assembly of the two types does not occur.

**Electrophysiology of ionotropic glutamate receptors**

NMDA-receptor channels have been widely studied. In voltage clamp experiments, the current-voltage (I-V) relationship in response to NMDA was found to be highly non-linear: very little current flows at potentials of −70 mV and
below. By removing magnesium from the extracellular solution, this relationship can be converted to a linear one (Nowak et al. 1984; Mayer & Westbrook, 1984). This indicates that external magnesium, a pore-blocker, produces voltage dependence in a channel which itself has no intrinsic voltage dependence. NMDA-receptor channels are highly permeable to calcium; Mayer & Westbrook (1987) showed that the calcium:sodium permeability ratio was 10:6 (compared to a value of 0.15:1 for non-NMDA receptors). Mayer et al. (1987) correlated inward current responses to NMDA or glutamate (and not to kainate or quisqualate) with calcium transients that could be blocked by magnesium in a voltage-dependent manner.

In contrast to NMDA-receptor currents, the non-NMDA I-V relationships are linear over the range of −90 mV to +30 mV. The receptors that are unedited at the Q/R site, however, are sensitive to intracellular polyamines, such as spermine. Spermine confers inward rectification on calcium-permeable non-NMDA receptors (Kamboj et al., 1995).

The reversal potential for non-NMDA receptors is found to be close to zero, as expected for a channel with selectivity for monovalent cations (Colquhoun et al., 1992). The I-V plot for glutamate from −100 mV to +70 mV looks like a mixture of those of the NMDA and the non-NMDA, and antagonists can separate these responses (Mayer & Westbrook, 1984; Hestrin et al., 1990).

The timecourses and characteristics of non-NMDA and NMDA components of excitatory post-synaptic potentials (EPSPs) were initially described by Hestrin and co-workers (1990) and reviewed by Edmonds et al. (1995). The NMDA-receptor current rises slowly (within 20 ms) and decays slowly (40-200 ms). The AMPA-receptor response activates rapidly (rises within 1 ms) and decays rapidly (time constant of 1 ms) (Silver et al., 1992).

The factors determining the timecourses are not entirely clear. However, it is evident that the slow NMDA-receptor timecourse is determined by slow steps in channel kinetics (the NMDA-receptor has a very high affinity for glutamate) and not by agonist rebinding (Lester et al., 1990), diffusion or agonist uptake (Hestrin et al., 1990).
et al., 1990). It is not certain what shapes the decline of the non-NMDA receptor response, which could be simple deactivation (because the agonist disappears immediately by diffusion or uptake) or by desensitisation (the fading of a response by prolonged or repeated agonist application). Electrophysiologically, kainate receptors are characterised by a transient response to continuous kainate application, followed by complete desensitisation (reviewed by Feldmeyer & Cull-Candy, 1994).

The events at a central synapse are described in a review by Auger & Marty (2000). The degree of occupancy of the post-synaptic receptors, after glutamate has been released at a central synapse, has been speculated (Clements, 1996). A low affinity competitive antagonist was introduced and its displacement was measured. Using estimations of the $K_{\text{on}}$ and $K_{\text{off}}$ values for the antagonist, the concentration was approximated to 1-5 mM initially, but this was rapidly cleared so that after 500 µs the transmitter was fairly uniformly distributed at around 20 µM. It was estimated that glutamate saturated all NMDA receptors, but resulted in 60% occupancy of non-NMDA receptors, due to their lower affinity for glutamate.

**Metabotropic glutamate receptors**

The metabolism of inositol phospholipids (IPs) was coupled with EAA recognition sites in rat hippocampus (Nicoletti et al., 1986), when ibotenate, a structural analogue of glutamate, was shown to enhance the hydrolysis of membrane IPs. Soon after, rat brain mRNA was injected into *Xenopus* oocytes (Sugiyama et al., 1987) and a novel glutamate receptor was found. The receptor had a high affinity for quisqualate and directly activated inositol phosphate lipid metabolism and mobilisation of intracellular calcium. This response was blocked by pertussis toxin, implicating a guanosine 5'-triphosphate - binding protein (G-protein). Quisqualate and glutamate, but not kainate or NMDA, elicited the response and joro spidertoxin, which blocks some non-NMDA receptors, had no effect.
Murphy & Miller (1988) observed the mobilisation of calcium from stores by glutamate acting on a quisqualate-sensitive, but not AMPA-sensitive, receptor. This receptor was termed the metabotropic glutamate receptor (mGluR) and the order of potency (strongest first) of its agonists was quisqualate, glutamate, NMDA and kainate (Sladeczek et al., 1985; striatal neurones).

Intracellular calcium rises were also observed in response to mGluR agonists (Phenna et al., 1995) and stimulation (Miller et al., 1996) in hippocampal pyramidal cells. Agonists produced calcium concentration rises in the CA1 and CA3 and activation of a calcium-activated potassium conductance, inhibited by an mGluR antagonist (Jaffe & Brown, 1994; Bianchi et al., 1999).

The IP pathway has been studied extensively in non-neuronal systems, but its role in neurones remains unclear. The mystification arises because the process is slow compared to neurotransmission, so it cannot be directly responsible for the signalling itself, although it may be involved in the modulation of neuronal excitability. Ogden (1996) reviewed the other possible roles of IPs and calcium signalling in central neurones. It is likely that IPs and calcium are regulators of cellular actin assembly and disassembly; calcium being a releaser of filaments and IPs initiating actin polymerisation (reviewed by Janmey, 1994). It has been suggested that the IP mechanism may regulate intracellular membrane fusion (Sullivan et al., 1993) and also cause the formation of ER cisternal stacks (Takei et al., 1994). The pathway may also play a role in the secretion of neurotrophins, which modulate activity-dependent neuronal plasticity (Canossa et al., 2001).

The question can be raised as to why neurones need to release calcium from stores, instead of relying purely on the influx of calcium from the outside of the cell. The advantages of store release might be that it is localised within the cell and can also be regulated by second messenger systems.

Ross’s group (Nakamura et al., 1999; 2000) found that synaptic activation of mGluRs or application of selective mGluR agonists, paired with backpropagating action potentials, resulted in large supralinear wavelike increases in intracellular free
calcium concentrations ([Ca\(^{2+}\)]\(_{i}\)). As mGluR1 receptors have been known to
couple to the IP pathway, it was concluded that calcium release was synergistic
with the action potential-elicited [Ca\(^{2+}\)]\(_{i}\) increase.

mGluRs have been also found to be linked to other second messenger
pathways. In granule cells in the cerebellum, glutamate agonists increased cyclic
GMP production (Novelli et al., 1987) and in the hippocampus, they inhibited cyclic
AMP formation (Schoepp et al., 1992).

**Pharmacology and molecular characterisation of mGluRs**

The pharmacology and functions of mGluRs were reviewed by Conn & Pin
(1997). The first mGluR cDNA was cloned simultaneously by two groups (Masu et
al., 1991; Houamed et al., 1991). The amino acid sequence shares no homology
with any other G-protein coupled receptor. The receptors have been named
mGluR1 to 8 and were divided into three groups (Group I; mGluR1 and 5; Group
II, mGluR2 and 3; group III, mGluR4, 6, 7 and 8), based on their homology of
amino acid sequences and their transduction mechanisms (table 1). Seven other
genes and several splice variants were isolated.

Group I acts via the activation of phospholipase C (Masu et al., 1991; Abe et
al., 1992), with the hydrolysis of inositol-4,5-bisphosphate, the production of
inositol-1,4,5-trisphosphate (IP\(_3\)) and calcium release from intracellular stores.
Groups II and III act via inhibition of adenylyl cyclase. The pharmacology also
differs. 3,5-dihydroxyphenylglycine (DHPG) is an agonist for group I receptors,
6S-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid (LY354740) is an agonist
for group II receptors and L-amino-4-phosphonobutyrate (L-AP4) is for group III.
3R-1-amino-1,3-cyclopentanedicarboxylate (ACPD) is a non-selective agonist for
all groups. Phenylglycines as antagonists are described by Birse and others (1993),
a general one being α-methyl-4-carboxyphenylglycine (MCPG).
<table>
<thead>
<tr>
<th>Group</th>
<th>Location in Hippocampus</th>
<th>Effector</th>
<th>Selective Antagonist</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Post-synaptic</td>
<td>+PLC $G_q$</td>
<td>CpCCOEt</td>
</tr>
<tr>
<td>Group II R2, R3</td>
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<tr>
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<td>CPPG</td>
</tr>
</tbody>
</table>

*Table 1.1. Summary of the subtypes of metabotropic glutamate receptors (mGluRs). There are three groups (I, II and III) which are located differently in the hippocampus, and have different effector mechanisms and selective pharmacology.*
The structure of mGluRs was reviewed by Pin & Bockaert (1995). mGluRs have seven transmembrane segments, a large extracellular N-terminal domain containing a cysteine-rich region and a cytoplasmic –COOH domain. The second intracellular loop is thought to be involved in G-protein coupling.

**Distribution of mGluRs in the hippocampus**

mGluR5 mRNA is highly expressed in hippocampal pyramidal cells, localised in the neuropil of the CA1 region (Abe et al., 1992; Shigemoto et al., 1993). Immunohistochemical methods suggested that mGluR1a is not present in CA1 pyramidal cells, but is localised to other cell types in this area (Baude et al., 1993). However, *in situ* hybridisation studies revealed that mGluR1 is expressed at low levels in hippocampal pyramidal cells (Pin et al., 1992) and immunoreactivity for mGluR1b was found in hippocampal neurones (Blümke et al., 1996). Both Group III subtypes mGluR4 (Phillips et al., 1997) and mGluR7 (Bradley et al., 1996) are also expressed in CA1 pyramidal cells.

**Sub-cellular localisation of mGluRs**

Somogyi’s group used immunogold localisation in hippocampal pyramidal cells (Nusser et al., 1994) to show subsynaptic segregation of metabotropic and ionotropic glutamate receptors. Ionotropic receptors occupy the membrane opposite the release site in the main body of the synaptic junction, and the metabotropic receptors are located in the periphery of the same synapses. It was suggested that the spatial segregation of ionotropic and mGlu receptors permits the differential activation of these receptors according to the amount of glutamate released presynaptically. A high frequency of action potentials may be necessary to
evoke the mGluR-mediated part of post-synaptic responses, as has been suggested for long-term changes in synaptic efficacy.

The same group (Lujan et al., 1997) studied the localisation of specific mGluR subtypes, and concluded that mGluR5 (Group I) occurred at highest concentration in an annulus surrounding the edge of the post-synaptic membrane. Using immunohistochemistry, mGluR2 (Group II) was shown to be located both pre- and post-synaptically in the hippocampus (Neki et al., 1996). Group III subtypes, mGluR4a and 7, were also identified in the hippocampus (Bradley et al., 1996) with mGluR4 located both pre- and post-synaptically, while mGluR7 was largely presynaptic.

**mGluR pharmacology**

7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester (CpCCOEt) is a non-competitive mGluR1 antagonist. It inhibits receptor signalling without affecting glutamate binding (Litschig et al., 1999). Conversely, α-methyl-L-(2S,3S,4S)-α-(carboxycyclopropyl)glycine (MCCG) is a competitive antagonist for Group II mGluRs, but is not as selective and higher concentrations are required for block. (RS)-α-cyclopropyl-4-phosphonophenylglycine ((RS)-CPPG) is a Group III-selective blocker (Toms et al., 1996). A non-selective mGluR antagonist is α-methyl-4-carboxyphenylglycine (MCPG) (Birse et al., 1993).

**The regulation of ion channels and cell excitability by mGluRs**

The activation of post-synaptic mGluRs changes neuronal excitability by modulating voltage-dependent and voltage-independent channels (Conn & Pin,
1997), including potassium channels, non-selective cation channels and voltage-
dependent calcium channels. The effects of mGluR activation have been found to
be excitatory or inhibitory, and a variety of effector ion channels have been
identified.

**Excitatory effects of mGluRs activation**

*Reduction of a potassium conductance*

mGluRs may reduce outward potassium currents to increase cell excitability. Charpak and colleagues (1990) used voltage clamp and microfluorimetry in the
hippocampal slice to show that the glutamate agonist ACPD decreased calcium-
dependent potassium currents, associated with afterhyperpolarisation ($I_{AHP}$), and
voltage-gated, muscarine-sensitive potassium currents ($I_M$).

The background potassium 'leak' current ($I_{leak}$) was also reduced, resulting in a
net inward current, linear with membrane potential and with a reversal potential
close to that of potassium ions (Guérineau *et al.*, 1994). This was thought to be
mediated by G-proteins, as it was blocked by a stable GDP (guanosine 5'-
diphosphate) analogue (GDP$_{β}$S) and enhanced by a stable GTP (guanosine 5'-
triphosphate) analogue (GTP$_{γ}$S), loaded by passive diffusion from the patch
pipette.

A calcium-activated potassium conductance was inhibited in the dentate gyrus
(Abdul-Ghani *et al.*, 1996) by ACPD, a selective agonist at mGluRs. The slowly
inactivating voltage-dependent potassium current ($I_{K(slow)}$) also apparently
decreased by activating mGluRs in area CA3 of the hippocampus (Lüthi *et al*.,
1996). This group went on to find that ACPD caused an inward current, due to the
suppression of potassium current, that had negative slope conductance between
potentials of $-55$ mV and $-40$ mV (Lüthi *et al*., 1997). ACPD can also suppress
the voltage-activated potassium conductance in cultured pyramidal cells (Wu &
Barish, 1999).

*Activation of a cation conductance*

mGluRs increase excitability by activating an inwardly directed cation current.
In 1992, Zheng & Gallagher demonstrated that ACPD could induce, in rat
dorsolateral septal nucleus neurones, a slow after-depolarisation which was both
sodium- and calcium-dependent. The CAN (calcium-activated non-selective)
current (Caeser et al., 1993) could be induced by ACPD and was dependent on
external calcium. Crépel and others (1994) observed that ACPD produced a
current that had a V-shaped I-V relationship with two reversal potentials at −100
mV and −20 mV. The curve became linear and reversed at −20 mV when
potassium channel blockers were applied; this current represented the CAN
current.

There have been contradicting reports that this CAN current is mediated by G-
proteins (Congar et al., 1997); in contrast to Guérineau et al., (1995), and for it not
being activated by calcium (Miller et al., 1995; in contrast to Caeser et al., 1993).
There is the suggestion that G-proteins mediate inhibition of IAHP, but not the
inward cation current, which may occur via the activation of tyrosine kinases
(Heuss et al., 1999). Chuang and co-workers (2000) found a Group I-mediated
voltage-dependent cation current in pyramidal cells, and claimed that the IP
pathway was responsible for its generation (Chuang et al., 2001).

A sodium/calcium exchanger has been suggested to be responsible for the
mGluR-activated inward cation conductance, in the rat basolateral amygdala
(Keele et al., 1997), Purkinje neurones (Linden et al., 1994) and ventralmedial
hypothalamic neurones (Lee & Boden, 1997). Finally, voltage-gated calcium
channels (L-type) have been found to be directly activated by mGluRs, in
cerebellar granule cells (Chavis et al., 1996).
Inhibitory effects of mGluRs

Activation of potassium conductances

Activation of mGluRs elicits hyperpolarisation in pyramidal cells of the CA1 (Jaffe & Brown, 1994). In ventral midbrain dopamine neurones, Group I mGluRs, shown pharmacologically to couple via IP$_3$ production to internal calcium release, were reported to activate an apamin-sensitive small conductance calcium-activated potassium channel (SK$_{Ca}$) (Fiorillo & Williams, 1998). ACPD, a non-selective mGluR agonist, activated a large-conductance potassium channel (BK$_{Ca}$) in cultured cerebellar granule cells (Fagni et al., 1991), and intermediate channels (IK$_{Ca}$) in dissociated rat pyramidal cells (Shirasaki et al., 1994), although the mGluR subtype involved was uncertain.

Group II agonists activated large-conductance potassium channels (BK$_{Ca}$) in the rat basolateral amygdala (Holmes et al., 1996) and the G-protein inwardly rectifying potassium channel (GIRK) has been shown to be activated in the HVc of songbird forebrain (Dutar et al., 2000) and the rat cerebellum (Knoflach & Kemp, 1998).

Inhibition of a calcium conductance

In cultured hippocampal neurones, it was reported that mGlur agonists inhibited a voltage-dependent calcium conductance via a G-protein-coupled receptor (Lester & Jahr, 1990).
mGluRs and plasticity

The mGluRs have been proposed to play an important role in the changing of synaptic strength (reviewed by Anwyl, 1999). McGuinness et al. (1991) showed that ACPD, a non-selective mGluR agonist, caused an increase in long-term potentiation (LTP) in the hippocampus and suggested that this was a result of a rise in $[\text{Ca}^{2+}]$. It was also reported that the induction of LTP in the hippocampus was inhibited by MCPG, a non-selective mGluR antagonist (Bashir et al., 1993).

Conversely, mGluRs can be responsible for long-term depression (LTD) in the hippocampus. Oliet et al. (1997) induced LTD in CA1 pyramidal cells in the presence of NMDA-receptor antagonists. They proposed that this type of LTD required the activation of Group I mGluRs, protein kinase C (PKC) and T-type calcium channels and they support a model in which PKC and calcium act cooperatively to induce LTD.

The inositol phosphate (IP) signalling pathway

The activation of inositol phosphate metabolism as a signalling mechanism was discovered in 1953 by Hokin & Hokin who showed that the incorporation of $^{32}\text{P}$ into phospholipids in pancreas was stimulated by acetylcholine during the secretion of amylase. In 1975, Michell concluded that inositol phosphate metabolism was the mode of action of agonists that caused intracellular calcium changes.

Inositides are located in the plasma membrane and their metabolism results in calcium being released from the internal stores. The link between these two was an enigma until it was proposed that the connection was inositol-1,4,5-trisphosphate (IP$_3$) (Streb et al., 1983).
IP₃ is formed by metabolism of phosphatidylinositol-4,5-bisphosphate (PIP₂), situated in the plasma membrane. The agonist’s receptor is linked to a G-protein that stimulates the enzyme phospholipase C (PLC), a phosphodiesterase, which catalyses the reaction. PIP₂ breaks down into two products, IP₃ and diacylglycerol (DAG). GTP is required for agonist-stimulated PLC conversion of PIP₂ to DAG and IP₃ (Cockcroft et al., 1985; Smith et al., 1986).

DAG activates protein kinase C (PKC) (Nishizuka, 1988) while IP₃ releases calcium from internal stores (Berridge & Irvine, 1984). IP₃ releases calcium from the endoplasmic reticulum (ER), not mitochondria, and these stores are ATP-dependent (Burgess et al., 1984).

**IP₃ receptors**

*Cloning and purification*

The IP₃ receptor was first cloned in 1979 (Mikoshiba et al., 1979), who used antibodies against the protein, which they called P₄₀₀, and showed that it was located in the soma and dendrites of Purkinje cells. Spat and colleagues (1986) used ^3²P-IP₃ to show that IP₃ bound to a specific saturable site in permeabilised guinea pig hepatocytes and rabbit neutrophils. They found a close correlation between the ability of IP₃ to bind to its receptor and the release of calcium.

P₄₀₀, enriched in mouse Purkinje cells, was cloned (Furuichi et al., 1989). It had a relative mass of 250 kD and was synthesised in the cerebellum. Immunohistochemical study of the cerebellum showed that this protein was localised to dendrites, axons and cell bodies of Purkinje cells, on the plasma membrane, ER and post-synaptic density.
Supattapone and co-workers (1988b) also purified an IP$_3$-binding protein (Mr 260 kD) from the rat cerebellum. They proposed that this was the same protein as P$_{400}$ and identical to the IP$_3$ receptor protein, confirmed by a demonstration that the purified IP$_3$ receptor reacted with monoclonal antibodies against the P$_{400}$ protein (Maeda et al., 1990).

A mouse fibroblast L cell line that produced the P$_{400}$ protein was generated (Miyawaki et al., 1990), demonstrating that the protein is involved in the physiological calcium mobilisation within the cells. The receptor became known as the subtype 1 IP$_3$ receptor and was shown to be responsible for IP$_3$-induced calcium release in hamster eggs, using a monoclonal antibody to the IP$_3$ receptor which inhibited this response (Miyazaki et al., 1992).

**Reconstitution of IP$_3$-receptors**

Microsomes of aortic smooth muscle were studied in planar lipid bilayers, and IP$_3$ activated a calcium-permeable channel with 10 pS conductance (Ehrlich & Watras, 1988). Later, the purified IP$_3$-binding protein was reconstituted into lipid vesicles (Ferris et al., 1989). IP$_3$ stimulated calcium flux in the vesicles, indicating that the purified IP$_3$-binding protein was a physiological receptor responsible for calcium release.

The IP$_3$-binding protein contained both the IP$_3$-recognition site and the calcium channel. Further evidence for this was produced by reconstitution of the purified IP$_3$-receptor into a planar lipid bilayer (Maeda et al., 1991). In the presence of IP$_3$, there was a calcium conductance of 26 pS. The open probability of the channel was increased by micromolar ATP.
**Localisation**

The IP$_3$ receptor was localised in brain by autoradiographic techniques (Worley et al., 1987; 1989) using $^3$H-inositol 1,4,5-trisphosphate. The cerebellum contained the highest level of IP$_3$-binding sites in the brain, selectively localised to Purkinje cells. The second most dense levels of binding were in the hippocampus, where they were most concentrated in the CA1 region in all layers, suggesting that binding sites are present in both cell bodies and dendrites.

Electron microscopy (EM) studies on the cerebellum revealed a concentration of IP$_3$ receptors in sub-components of the ER. Two groups (Ross et al., 1989; Mignery et al., 1989) showed (by immunocytochemistry and EM) that the IP$_3$ receptor protein is present in all parts of the ER in the Purkinje cell, including those that extend into axon terminals and dendritic spines. Immuno-gold labelling (Satoh et al., 1990) exposed concentrations of IP$_3$ receptors in Purkinje cells. Immunohistochemical localisation of IP$_3$-receptors (Sharp et al., 1993) showed that in the hippocampus, pyramidal cells were labelled throughout, most intensely in the CA1.

**Molecular characterisation**

The structure of the IP$_3$-receptor is reviewed by Mikoshiba (1993). The purified IP$_3$-receptor corresponds to a single 260 kD band on SDS-PAGE analysis (Supattapone et al., 1988b). However, the native molecular weight of the IP$_3$-receptor is about one million Daltons, indicating that the receptor is a tetramer.

Mikoshiba's group (Maeda et al., 1991) confirmed the tetramer hypothesis by performing cross-linking experiments. They also carried out channel recordings of this protein in planar lipid bilayer experiments. They found that the channel binds...
adenine nucleotides and calmodulin, conducts calcium and sodium ions and that its sub-conductance state is regulated by ATP in the presence of IP$_3$. The IP$_3$ receptor is very similar to the ryanodine receptor of the sarcoplasmic reticulum in skeletal and cardiac muscle, showing greatest homology at the transmembrane regions near the C-terminal, which is likely to be the calcium-permeable pore (Furuichi et al., 1989; Mignery et al., 1990). Each of the four subunits binds a molecule of IP$_3$, as shown by Mignery & Südhof (1990) in deletion studies.

Mignery and co-workers (Mignery & Südhof, 1990; Mignery et al., 1990) studied the different regions of the IP$_3$-receptor protein structure, using the methods of overlapping cDNA clones, hydrophobicity analysis, transfection, protein synthesis and mutagenesis. They found that the transmembrane regions (probably eight in total), near the C-terminal, were necessary for forming tetramers. About 650 N-terminal amino acids were highly conserved; deletion of any small fragment within this region abolished IP$_3$-binding activity.

The amino acid sequence is conserved among different species. The IP$_3$-receptor sequence is conserved with less than 1% difference between rat and mouse. There is more than 90% conservation between mouse/rat and human.

**Heterogeneity**

The structure of subtype 2, which shares 69% homology with subtype 1, was determined in 1991 (Südhof et al.). The binding of IP$_3$ to the subtype 2 receptor is of a similar specificity but higher affinity. Maranto (1994) described the complete sequence of subtype 3. The presence of several types of IP$_3$-receptor raises the possibility that intracellular calcium signalling may involve multiple pathways with different regulatory properties, perhaps dependent on different IP$_3$-receptor transduction pathways.
There are also alternatively sliced transcripts encoding IP3 receptors (Danoff et al., 1991; Nakagawa et al., 1991; Ross et al., 1992). Using single cell reverse transcriptase polymerase chain reaction, in the hippocampus two splice variants were found to be present, in contrast to Purkinje cells in the cerebellum, which tested positive for one of the splice variants only (T. Capiod, C. Magnus & A. Sesay, unpublished data). This may be a factor in determining the sensitivity to IP3.

Regulation of receptor function

It has been shown that ATP enhances IP3-dependent calcium release (Maeda et al., 1991). ATP increases the open probability of the IP3 receptor channel in the presence of IP3. Protein kinase A (PKA) phosphorylates the receptor (Danoff et al., 1991) and decreases the effectiveness of IP3 in mediating 45Ca2+ flux in microsomes (Suttapone et al., 1988a).

The IP3 receptor is regulated by calcium. Coquil et al. (1999) characterised the calcium-dependent inhibition of [3H]IP3 binding to sheep cerebellar microsomes and concluded that IP3 and calcium each decrease the binding of the other. Recently (Miyakawa et al., 2001), it was shown that there is one key residue in the IP3 receptor sequence that is responsible for calcium-sensitivity. Substitution of this residue caused a ten-fold reduction in calcium-sensitivity, without affecting IP3 sensitivity, maximum rate of calcium release or ATP sensitivity.

Calcium inhibition of the IP3-receptor

Inhibition of the IP3-receptor produced by cytosolic free calcium concentration has been shown in many tissues (Ogden et al., 1990, guinea-pig hepatocytes;
Khodakhah & Ogden, 1995, Purkinje neurones; Carter & Ogden, 1997, endothelial cells). Parker & Ivorra (1990) found that, in Xenopus oocytes, IP$_3$-mediated calcium release was inhibited by raising [Ca$^{2+}$]$_i$. They claimed that the kinetics of this calcium inhibition were slow, occurring over several seconds, and suggested that the inhibition was a basis for oscillatory behaviour.

**Calcium stimulation of the IP$_3$-receptor**

Calcium can be stimulatory at low concentrations. Bezprozvanny and colleagues (1991) demonstrated a ‘bell-shaped curve’; the open probability of the IP$_3$-channel increased as the [Ca$^{2+}$]$_i$ increased from 10 nM to 250 nM, then decreased at concentrations greater than 250 nM. Iino & Endo (1992) found that when caged IP$_3$ was photolyzed at a calcium concentration near the peak of the bell-shaped curve (250 nM), the delay became shorter and the initial rate of calcium release and the rate of termination greater.

Experiments with D.M. nitrophen and the instant release of calcium showed that calcium immediately controls the rate of IP$_3$-evoked calcium release. Finch and colleagues (1991) found that the magnitude and timecourse of IP$_3$-induced $^{45}\text{Ca}^{2+}$ release were modulated by non-cooperative calcium binding. Ogden & Capiod (1997) noticed that the delay after the release of a low IP$_3$ concentration was reduced to zero by prior increase in [Ca$^{2+}$]$_i$.

Thus, studies of isolated reconstituted IP$_3$-receptors show that cytosolic calcium ions can both facilitate and inhibit. It is of interest to know how calcium influx in neuronal excitation interacts with IP$_3$-receptors in a physiological context.
Aims of the project

mGluRs have an important role in the modulation of neuronal excitability, and have been implicated in plasticity, regulating fast synaptic transmission and increasing or decreasing synaptic strength. However, their actions are varied and controversial. This project aims to investigate the results of activation of mGluRs. What are the ionic currents? What intracellular signalling cascades are deployed in generating them?

The technique of flash photolysis, which avoids delays associated with diffusion and eliminates presynaptic components, is used here to deliver L-glutamate to CA1 pyramidal neurones in slices. This is a novel method for probing the mGluR response, as previous studies have used agonists or synaptic stimulation protocols. Flash photolysis permits the accurate study of kinetics of the mGluR response, and the concentration of glutamate can be varied in a controlled manner.

There is a plethora of pharmacology available for use on the different subtypes of mGluR, and putative ion channels linked to their activation. In this project, mGluR currents are classified using both selective antagonists and electrophysiological methods, in ion substitution experiments.

The inositol phosphate pathway, an important intracellular second messenger system that is known to be linked to the activation of Group I mGluRs (which are located post-synaptically in pyramidal cells), is probed using caged IP₃. Does IP₃ result in a similar response to that obtained by the release of extracellular glutamate? Known concentrations of IP₃ are released and the kinetics of the currents are studied and compared to those of the mGluR response. In addition, the [Ca²⁺]ᵢ changes are measured, because calcium plays an important role in the IP₃ signalling cascade.
Chapter 2 – Methods
Preparation for Patch Clamp Measurements

Preparation of tissue for slicing

Male *Wistar* rats, 11-13 days old, were anaesthetised (Fluorethane) and decapitated. The brain was removed as quickly as possible to minimise damage from anoxia. It was placed in ice-cold low-calcium HEPES-buffered physiological salt solution (PSS) which contained (in mM): 140 NaCl; 4.7 KCl; 0.2 CaCl$_2$; 2.5 MgSO$_4$; 11 glucose; 10 HEPES; (pH 7.4 adjusted with NaOH; osmolality 300 milliosmolal) bubbled with O$_2$ and cooled for 3 minutes. The hemispheres were separated and each hemisphere cut as shown in fig. 2.1. The cut surface, parallel to the desired orientation of slices, was glued (cyanoacrylate glue) onto the slicing block. The regions CA1, CA3 and dentate gyrus (DG) were clearly visible as shown.

Slicing

A vibrating microtome sheer (Leica VT1000S) was used to cut brain slices of 250 µm thickness at a speed of 75 µm/s, frequency 80 Hz and amplitude of vibration in the sideways horizontal plane 500 µm. Slice thickness of 250 µm was optimal for cell survival and ease of visualisation when the slice was illuminated from below.

Incubation of slices

After slicing, the hippocampal slices were placed in a holding chamber of oxygenated PSS, which contained (in mM): 140 NaCl; 4.7 KCl; 2.5 CaCl$_2$; 1.2 MgSO$_4$; 11 glucose; 10 HEPES; (pH 7.4 adjusted with NaOH; osmolality 300
Fig. 2.1. Producing rat hippocampal slices. The whole rat brain (A) is sliced in two to divide the hemispheres. Each hemisphere is sliced (250 µm) at the orientation shown (B). Within each slice, the hippocampus is recognised by its characteristic structure (C).
milliosmolal) at 31°C for three-quarters of an hour and then at room temperature. The chamber consisted of a fine cotton mesh, on which the slices were placed, suspended in a beaker, with a mechanism for continual bubbling that circulated the solution (Edwards et al., 1989). Slices were transferred in and out of the chamber using the wide end of a Pasteur pipette.

**Mechanical fixation and perfusion**

One slice at a time was placed in a quartz coverslip-based recording chamber and held in place with a grid of flattened platinum wire with glued parallel glass fibres. The slice was perfused at room temperature with PSS, sometimes without MgSO₄, at 2 ml/min. The solution was removed with an aquarium pump connected to a collecting vessel via two bubble traps in the perfusion lines to prevent electrical interference. The perfusion was switched off during recording and hydrated O₂ was blown over the surface of the bath solution.

**Patch pipettes and internal solutions**

Patch pipettes were pulled (Narishige vertical puller) from borosilicate glass (Harvard apparatus, 1.5 mm outer diameter, 1.17 mm inner diameter, inner filament) and heat polished. In some experiments, the pipettes were coated with melted mineral oil/parafilm (40:60 mixture) as far as the shank (7 mm) to reduce the capacitance that was produced by immersion of the pipette in the chamber.

Three types of internal solution were used in patch clamp experiments; potassium gluconate (Kgluconate), potassium chloride (KCl) and caesium gluconate (Csgluconate), all pH 7.3 (adjusted with NaOH) and osmolality 300 milliosmolal (see table 2.1).

Pipettes had resistance of 3-5 MΩ. All experiments were carried out at room
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<th>K gluconate</th>
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<td>K gluconate</td>
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<td>Na-GTP</td>
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*Table 2.1. Compositions of internal solutions (K gluconate, KCl and Cs gluconate) used in patch clamp experiments (concentrations in mM).*
temperature (17-23°C). Calcium indicators (fluo-4 or fura-2FF) were included in the internal solution at 500 µM as appropriate. In experiments with photolysis of caged inositol-1,4,5-trisphosphate (IP$_3$), caged IP$_3$ was at a known concentration between 143 µM and 428 µM in the internal solution.

**Flash photolysis of caged compounds**

Flash photolysis of caged compounds (McCray & Trentham, 1989) is a powerful method used to study biological processes. Spatially uniform photolytic release of an active compound occurs with fast time resolution (within a millisecond), permitting the study of kinetics.

A useful caged neurotransmitter must be thermally stable (i.e. it must not hydrolyse in aqueous solution), fast and efficient in photorelease with respect to synaptic time scales and concentrations. The caged precursor, the photolytic intermediates and by-products, must be biologically inert and must have minimal interaction with the receptors, transporters and metabolism of the released neurotransmitter, nor should they interfere with other components of synaptic transmission at the concentrations used.

**Advantages of flash photolysis**

When compared with the alternative methods of applying a neurotransmitter to a cell, pressure injection or iontophoresis, the advantage with flash photolysis is that there are no delays due to diffusion to the site of action.

The advantage of flash photolysis over synaptic stimulation is that all pre-synaptic components of the process are eliminated with the appropriate antagonists present. Thereby, it is known that the response recorded is purely due to action on post-synaptic receptors on the cell that is being patch clamped.
In general, flash photolysis has the advantage that a known concentration of neurotransmitter is released.

**Disadvantages of flash photolysis**

When a flash lamp is used to deliver ultra-violet (UV) light through high magnification microscope optics then the spot on the microscope dish that is illuminated is approximately 200 µm in diameter. This is over 400 times the area of cell soma. The release of neurotransmitter over this large area will result in the activation of receptors over the entire cell that is being patch clamped, so responses may be larger than those obtained during synaptic stimulation experiments. In addition, the kinetics of the response are likely to be affected by the spatial distribution of synapses; those far from the pipette may contribute differently from those near, influencing the overall response.

The release of neurotransmitter over a large volume prevents its removal by diffusion from synaptic sites. Measurements show that the neurotransmitter remains in the dish for more than 10 s, and in the cerebellar slice the half time of L-glutamate due to uptake is about 200 ms (Canepari et al., 2001a). The large concentration of neurotransmitter present may cause desensitisation of receptors. The problem of widespread release may be remedied by uncaging of the neurotransmitter over a smaller area, using a laser as the source of UV light (a very expensive set-up).

Unless already tested and characterised, caged neurotransmitters or by-products may have uncharacterised antagonistic or toxic effects on the cell or receptors. The caged neurotransmitter may be unstable or sensitive to visible light, and might uncage very slowly before required. This may lead to the desensitisation of receptors and a decrease in response during flash photolysis experiments. Chapter 3 describes the characterisation of a novel caged compound that is used in experiments in subsequent chapters.
Caged compounds

The first photolabile compounds, used in photoisomerisation experiments to study the photoregulation of biological activity, were photosensitive azobenzenes (Kaufman et al., 1968) and Bis-Q (Lester et al., 1980).

Caged ATP (Kaplan et al., 1978) used 2-nitrobenzyl phosphate esters and 1-(2-nitrophenyl)ethyl phosphate esters as photolabile inactivating groups. Goldman et al. (1982) studied the mechanism of force transduction in muscle using the latter compound. In 1988, Walker et al. described the synthesis and mechanism of caged ATP derivatives and 3',5'-cyclic nucleotides. Since then, more 2-nitrobenzyl-derived caged compounds have been synthesised, including photolabile calcium chelators, caged inositol-1,4,5-trisphosphate (IP₃) (Walker et al., 1989) and caged diacylglycerol (DAG) (Huang et al., 1996).

Caged IP₃

The technique used to 'cage' ATP, with 2-nitrophenylethyl esters on the terminal phosphate (Walker et al., 1988) was applied by Walker et al. (1989) to synthesise 2-nitrophenylethyl phosphate esters of IP₃, substituted on the 1,4 or 5 phosphates (fig. 2.2A). The isomers were separated by anion exchange high pressure liquid chromatography and characterised using nuclear magnetic resonance. Their photochemical and physiological properties were evaluated. The rate of photolysis was determined spectrophotometrically from the decay of the activated aci-nitro intermediate and the formation of the byproduct, nitroso-acetophenone. The scheme of the photolytic reaction is shown in fig.2.2B.

At physiological ionic strength (0.2 M) and pH (pH 7.1) the P⁴ and P⁵ isomers
Fig. 2.2. Structure of caged IP$_3$ and its photolysis reaction. Structure (A) and photolysis of caged IP$_3$ (the P$^{51}$-(2-nitrophenyl)ethyl ester of IP$_3$) to release IP$_3$, H$^+$ and 2-nitrosoacetophenone (B).
photolysed with rates of 225 and 280 s\(^{-1}\) respectively, with half times near 3 ms. The quantum yields (ratio of molecules uncaged to photons absorbed) were 0.65 (Walker et al., 1989), similar to that of caged ATP which is 0.63 (McCray & Trentham, 1989).

The isomers were tested in smooth muscle for their calcium-releasing abilities in excitation contraction coupling (Walker et al., 1987). The P\(^1\) isomer (10 \(\mu\)M) resulted in maximal tension in permeabilised smooth muscle, indicating that calcium was released from stores. The P\(^4\) and P\(^5\) isomers did not release calcium at 50 \(\mu\)M, and did not inhibit calcium release caused by IP\(_3\). The three isomers were also tested for stability in the presence of enzymes that metabolise IP\(_3\) (Walker et al., 1989). P\(^4\) and P\(^5\) were resistant to hydrolysis by phosphatase and to phosphorylation by kinase. The P\(^5\) isomer is, however, an inhibitor of IP\(_3\) 3-kinase, which converts IP\(_3\) to IP\(_4\). The caged IP\(_3\) used in these experiments is a mixture of the P\(^1\) and P\(^5\) isomers.

Caged glutamate

Patchornik et al. (1970) first described the use of o-nitrobenzyl derivatives as photosensitive caging groups on peptides. Corrie et al. (1993) used a caged L-glutamate, N-1-(2-nitrophenyl)ethoxycarbonyl-L-glutamate, which produced post-synaptic activation when released rapidly in synaptic clefts in the stellate ganglion of the squid. Although stable, it was slow in photorelease, with a half time \(\geq\) 10 ms at pH 7. The quantum yield of 0.65, however, was relatively high.

\(\alpha\)-carboxy-2-nitrobenzyl-caged compounds were found to be faster in photorelease (half time of 21 \(\mu\)s; Wieboldt et al., 1994), but unstable in solution, with half time for hydrolysis of a few hours at pH 7 and room temperature. The \(p\)-hydroxyphenacyl esters of glutamate were found to undergo photorelease on a sub-
Fig. 2.3. Structure of NI-caged L-glutamate (A), MNI-caged L-glutamate (B) and NI-caged 5-(dihydroxyphosphoryloxy)-pentanoate (C).
Fig. 2.4. Photolysis reaction of Nl-caged glutamate to produce L-glutamate, a proton and a by-product, 5-methoxycarbonylmethyl-7-nitrosoindole.
μs time scale (rate constant $10^7 - 10^8$ s$^{-1}$ compared to 1-100 s$^{-1}$ for the o-nitrobenzyl analogues; Givens et al., 1997) but were inefficient.

Recently, 1-acyl-7-nitroindolinyl (NI) derivatives that release L-glutamate or other carboxylates have been described (fig. 2.3; Papageorgiou et al., 1999). They are highly resistant to spontaneous hydrolysis at physiological pH and room temperature. The half time of the photorelease process was previously estimated as ≤0.26 ms (pH 7, 20 °C) but more recent work shows that it is in the sub-μs (200 ns) time domain (P. Wan, J. Morrison and J. E. T. Corrie, unpublished data). The photolysis reaction takes place with 1:1 stoichiometry (fig. 2.4).

The NI-caged L-glutamate (fig. 2.3A) has been used to investigate the current generated by the activation of metabotropic glutamate receptors in cerebellar Purkinje neurones in slices (Canepari et al., 2001a). The control compound NI-caged phosphate (NI-caged 5-(dihydroxyphosphoryloxy)-pentanoate; fig. 2.3C), which has identical photochemistry but does not generate L-glutamate on photolysis, has been used to confirm that the intermediates and by-products of photolysis cause no biological effects.

Diffusion away from the illuminated spot has been measured, using HPTS (half time 40 seconds; Canepari et al., 2001a). The uptake of glutamate has also been measured. It disappears with a half time of 200 ms, as estimated by measuring AMPA-receptor-mediated currents in the presence of cyclothiazide (100 μM) to inhibit AMPA-receptor desensitisation in Purkinje cells (Canepari et al., 2001a).

A study of the 4-methoxy-7-nitroindolinyl (MNI) caged L-glutamate (fig. 2.3B) has shown that photorelease is 2.5-fold more efficient than from NI-caged compounds because of enhancements in both the absorption coefficient and the quantum yield (Papageorgiou and Corrie, 2000; Canepari et al., 2001a). The MNI-caged compounds have the same resistance to hydrolysis and the same photochemistry, so are expected also to have rapid photorelease.
Fluorescent calcium indicators

A fluorophore absorbs light at one wavelength and re-emits it at another. A fluorescent calcium indicator changes its fluorescent quantum efficiency (number of fluorescent photons emitted per photons absorbed) when the indicator binds calcium.

The temporal and spatial changes of intracellular free calcium \([\text{Ca}^{2+}]\), are important in many aspects of neuronal function. Fluorescent calcium indicators (Gryniewicz et al., 1985) are used for quantitative measurement of \([\text{Ca}^{2+}]\), in order to evaluate the role of calcium as an intracellular second messenger. Calcium indicators are introduced into the cytosol and the emitted fluorescence is measured with a photomultiplier tube. Changes in \([\text{Ca}^{2+}]\), can be detected with a millisecond time resolution.

Quin-2, an early calcium indicator, has limitations. Firstly, the excitation wavelength is in the UV range. UV excitation is not favourable because it is expensive to produce, potentially injurious to cells, can excite autofluorescence (for example, from pyridine nucleotides) and photolyses ‘caged’ compounds. Secondly, due to a low quantum efficiency and extinction coefficient (measure of photons absorbed), a large concentration of the indicator has to be used, resulting in extensive buffering of calcium in the cell. Thirdly, quin-2 is not sufficiently selective for calcium over magnesium.

High affinity indicators

A group of indicators were developed that are based on the calcium chelator bis-(O-aminophenoxy)-N,N,N',N'-tetraacetic acid (BAPTA), a relative of ethylene glycol bis (β-amo-no-ethylether)-N,N,N',N'-tetraacetic acid (EGTA) (Tsien, 1980) and are without some of the problems of quin-2. These dyes (for example, fluo-3
(Minta et al., 1989), Oregon Green and Calcium Green) have higher affinities than previous indicators, are more selective against competing cations, are brighter on calcium binding and do not require UV light for excitation. Upon binding of calcium, there is a rise in quantum efficiency without a shift in the absorption spectrum.

Fluo-4, described by Gee et al. (2000), was designed to quantify calcium concentrations in the 100 nM to 1 μM range. Its advantage over fluo-3 is that there is greater absorption near 488 nm, therefore it has brighter fluorescence emission and can be used at lower [Ca^{2+}]_i, buffering to a lesser extent.

The [Ca^{2+}]_i was calculated from the fluorescence F as:

\[ [\text{Ca}^{2+}]_i = \frac{K_{Ca}}{F_{\text{min}} - F} \left( F - F_{\text{max}} \right) \]

The derivation of this equation is given in Appendix 1.

High affinity indicators have large fluorescence changes near resting [Ca^{2+}]_i, which is similar in value to the \( K_{Ca} \). The concentration of free indicator is reduced significantly by calcium. In addition, the high affinity indicators produce attenuated and slow records with respect to [Ca^{2+}]_i changes. There is a saturating relationship between fluorescence and calcium, so that regions of high [Ca^{2+}]_i contribute to the fluorescence less than they should. This error is increased by the presence of regions of high local [Ca^{2+}]_i, occurring, for example, near to calcium flux sites early in the response.

Kinetic distortion is also produced by the slow equilibrium of calcium binding. The rate of calcium dissociation is slower from high affinity indicators.
(Hollingworth et al., 1992). The high affinity indicators will provide additional buffering and dissipate localised calcium transients adjacent to calcium channels. This will affect regulatory processes that are normally activated by high calcium concentrations.

The rate of change of \([\text{Ca}^{2+}]_i\) is underestimated by the rate of change of fluorescence, by an amount equal to \(\delta F \delta [\text{Ca}^{2+}]_i\):

\[
\frac{\delta F}{\delta t} \approx \frac{\delta [\text{Ca}^{2+}]_i}{\delta t} \frac{\delta F}{\delta [\text{Ca}^{2+}]_i}
\]

\(\delta F / \delta [\text{Ca}^{2+}]_i\) can be obtained by differentiating \([\text{Ca}^{2+}]_i\) with respect to F:

\[
\frac{\delta [\text{Ca}^{2+}]_i}{\delta F} = \left(1 + \frac{[\text{Ca}^{2+}]_i}{K_{Ca}}\right)^2
\]

so that \(\delta F / \delta t\) underestimates \(\delta [\text{Ca}^{2+}]_i / \delta t\) by 4-fold when \([\text{Ca}^{2+}]_i = K_{Ca}\), and is approximately linear for \(K_{Ca} > 10 [\text{Ca}^{2+}]_i\).

In the experiments described here, fluo-4 was used qualitatively but not quantitatively, due to its high affinity (345 nM).

**Low affinity indicators**

Low affinity indicators are suitable for measuring large \([\text{Ca}^{2+}]_i\) transients. Furaptra, which is based on the magnesium chelator o-aminophenol N,N,O-triacetate (APTRA), is structurally analogous to the calcium-chelator fura-2 and has a similar excitation shift on magnesium binding as well as calcium binding
(\(K_{\text{Ca}}(\text{Mg}^{2+}) = 1.5 \text{ mM; } K_{\text{Ca}}(\text{Ca}^{2+}) = 53 \text{ \mu M}\) (Raju et al., 1989). Furaptra binds calcium or magnesium with a 1:1 stoichiometry (Raju et al., 1989).

Furaptra produces minimal distortion of \([\text{Ca}^{2+}]_i\) transients in frog muscle (Konishi et al., 1991). In hepatocytes, Purkinje neurones and endothelial cells, furaptra provides better estimates of fast \([\text{Ca}^{2+}]_i\) signals when compared to fluo-3 (Ogden et al., 1995). Magnesium produces little interference with \([\text{Ca}^{2+}]_i\) signals.

Fura-2FF (2-[2(5-carboxyethyl-oxazole0]-5-[2-(2-

bis(carboxymethylmethyl)amino-5,6-difluorophenoxy)]ethoxy-6-

bis(carboxymethylmethyl)aminobenzofuran) (London et al., 1996) has the advantage over furaptra of a much lower affinity for magnesium.

Fura-2, furaptra and fura-2FF respond to calcium by shifting their excitation wavelengths much less than they shift the emission wavelengths, whilst still maintaining a high fluorescence (fig. 2.5). Indo-1 is the opposite; it emits near 400 nm when bound to calcium compared to 480 nm when free. These indicators may be used ratiometrically which means that a ratio of fluorescences at two wavelengths can signal calcium, having the advantage that other factors such as changes of detector efficiency, indicator concentration, optical pathlength are cancelled out.

The ratiometric method, however, has disadvantages. The dual-excitation source required is difficult to use and expensive and noisy signals give large errors in ratio values. In addition, the indicators require UV light for excitation when used ratiometrically, which gives rise to autofluorescence, photodamage in cells and photolyses 'caged' compounds.
Fig. 2.5. Excitation and emission spectra for fura-2FF at various [Ca\(^{2+}\)], measured in Ca\(^{2+}\) and Mg\(^{2+}\)-containing solutions using a spectrofluorimeter. [Ca\(^{2+}\)] was buffered between zero and 1.3 mM with 40 mM K-citrate. Free ion concentration estimates were taken from stability constants of Ca\(^{2+}\) and Mg\(^{2+}\) binding to citrate (K. Macleod; \(K_{Ca} = 2.99 \times 10^{-4}\); \(K_{Mg} = 4.22 \times 10^{-4}\) at 25°C, I=0.1M, pH=7.0). A. Excitation spectra at 510 nm emission in high and zero [Ca\(^{2+}\)]. At 420 nm (dashed line), the fluorescence intensity at zero [Ca\(^{2+}\)] is 20 fluorescence units; at high [Ca\(^{2+}\)] the fluorescence is near zero. B. Emission spectra at 420 nm excitation, which peaks at 510 nm (dashed line). Taken from R. Curran's Ph.D. thesis (1998).
Fig. 2.6. Schematic diagram of the slice microscope showing paths of illumination (brightfield), epifluorescence, emitted light and UV light from flash lamp.
The experimental set-up

A schematic diagram of the experimental set-up is shown in fig. 2.6. An upright microscope (Zeiss Axioskop) was used with brightfield optics. Light from a 100W bulb at the back of the microscope was transmitted via a 45° mirror to a substage condenser (silica condenser or ×40 objective N.A. 0.75) focused on the slice. The slice was viewed with a ×40 water-immersion achromat (N.A 0.75). Infra-red (IR) light was taken to a video CCD camera (Hamamatsu C2400) and contrast enhancement circuit allowed observation on a TV monitor.

Pipette current was recorded with an Axopatch 200A with an amplifier β = 1 headstage. The reference electrode comprised a Ag/AgCl pellet inside a syringe body connected to the recording chamber via a 150 mM NaCl salt bridge. Data were low-pass filtered at 2.5 kHz (3 dB), amplified by 10, digitised at 10 kHz by a CED (Cambridge Electronic Design) 1401-plus interface, and analysed in CED software (Spike 2).

Flash lamp

The light from a xenon flash lamp (Rapp OptoElektronik, Rapp & Güth, 1988; UG11 filter, Schott, bandwidth 290-370 nm) was focused through the condenser into the preparation through a quartz coverslip-based chamber.

Condensers and objectives were compared for their efficiency in transmitting near-UV (table 2.2). The silica condenser, chosen to be the condenser, maximised transmission of the flash lamp output. The ×40 N.A 0.75 objective was used as the condenser when the silica condenser was unavailable. The flash lamp illuminated a spot of 200 μm in diameter. The arc and optical train were aligned and focused by viewing the arc in continuous 'focus' mode, without the UG11 filter, with the objective set to view at the level of the surface of the preparation in
<table>
<thead>
<tr>
<th>Condenser</th>
<th>Fluorescence (Volts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silica</td>
<td>7.5</td>
</tr>
<tr>
<td>Zeiss DIC</td>
<td>3.0</td>
</tr>
<tr>
<td>Zeiss oil immersion 1.4</td>
<td>2.0</td>
</tr>
</tbody>
</table>

**Objective used as condenser**

<table>
<thead>
<tr>
<th>Objective Used as Condenser</th>
<th>Fluorescence (Volts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Russian 40x 0.75</td>
<td>6.8</td>
</tr>
<tr>
<td>Zeiss 40x 0.75 Neoflar</td>
<td>4.0</td>
</tr>
<tr>
<td>Zeiss 40x 0.75 Water ACROPLAN aperture removed, used without water</td>
<td>5.8</td>
</tr>
<tr>
<td>50x 1.00 W fluoreszenz</td>
<td>6.0</td>
</tr>
<tr>
<td>Zeiss 30x 0.65 APO</td>
<td>6.2</td>
</tr>
</tbody>
</table>

*Table 2.2. Comparison of the light transmission through a variety of condensers. When the silica condenser was unavailable, the Russian 40x 0.75 was used as substage condenser to maximise near-UV transmission.*
the chamber. After alignment, the image of the arc was focused more precisely via the condenser by maximising the output of a photodiode placed in one of the oculars.

The discharge voltage was set at 300V and the full capacitance was selected for all experiments, producing maximum flash lamp intensity. Lower intensities were obtained by neutral density filters placed in the condenser light path. An optical artefact on flash lamp discharge was removed by a UV-blocking filter and an IR-blocking filter in the emission path between the objective and the fluorescence-measuring apparatus.

**Photometry**

The epifluorescence was provided by a 100W quartz halogen lamp, run on a constant current power supply (Farnell). It passed through an excitation filter and was reflected by a dichroic mirror onto the slice. Emitted light passed through the dichroic mirror and was reflected to a photomultiplier tube via a near-IR dichroic long pass (700 nm).

The calcium indicators used in experiments were fura-2FF, for quantitative measurements, and fluo-4, for qualitative measurements. Fura-2FF was excited at 400-440 nm with emission at >510 nm. Fluorescein filters were used with fluo-4: excitation at 480 nm and emission at >530 nm.

Microspectrofluorimetry was from a region containing the soma and part of the apical dendrite, defined by a rectangular diaphragm in the image plane viewed by a TV camera. No attempts were made to calibrate the fluorescence in terms of \([\text{Ca}^{2+}]_i\); with fluo-4 because the signals were clearly attenuated by indicator saturation except near resting levels and there was distortion of kinetics.
To obtain a quantitative measure of \([Ca^{2+}]_i\), the low affinity dye fura-2FF was used with the methods described by Konishi et al. (1991). With the wavelengths used for excitation, the fluorescence of fura-2FF was quenched by high calcium to close to zero (fig. 2.5). The area to be viewed by the photomultiplier tube was set with a rectangular diaphragm and the shutter opened to record the intrinsic fluorescence (autofluorescence). This was recorded in the cell-attached mode before whole-cell recording began, and was used as an estimate of the fluorescence at saturating \([Ca^{2+}]_i\), \(F_{\text{max}}\). Following patch membrane rupture, the cell loaded with indicator to a steady level of fluorescence (8-10 minutes; fig. 2.7).

The \(K_{Ca}\) for fura-2FF was measured in the laboratories at N.I.M.R. by Rachel Curran (Ph.D., 1998). Calcium was buffered at different concentrations with potassium citrate buffer. The dissociation constants obtained were 29.8 \(\mu\text{M} \ (Ca^{2+})\) and 18.4 \(\text{mM} \ (Mg^{2+})\). The \(K_{Ca}\) value was not dissimilar from those measured elsewhere (Weiburg et al., 1997, \(K_{Ca} = 13 \ \mu\text{M}\); Dong et al., 1998, \(K_{Ca} = 25 \ \mu\text{M}\); Hajnoczky & Thomas, 1997, \(K_{Ca} = 35 \ \mu\text{M}\), and so was used in calculations here.

Accurate reporting of the kinetics of \([Ca^{2+}]_i\) changes requires the use of an indicator with a \(K_{Ca}\) that is high compared with the peak \([Ca^{2+}]_i\) levels. This is because the relationship between \(F\) and \([Ca^{2+}]_i\) is approximately linear at \([Ca^{2+}]_i\) levels much lower than the \(K_{Ca}\) (fig. 2.7).

At the resting \([Ca^{2+}]_i\) of approximately 40 nM present in pyramidal neurones (Helmchen et al., 1996; Maravall et al., 2000), fura-2FF has a fluorescence similar to that of zero \([Ca^{2+}]_i\). When the equation linking \(F\) and \([Ca^{2+}]_i\) is rearranged:

\[
F = \left( \frac{[Ca^{2+}]_i}{K_{Ca}} + 1 \right) = F_{\text{min}} - \frac{F_{\text{max}} - F_{\text{min}}}{K_{Ca}} [Ca^{2+}]_i
\]
Fluorescence (arbitrary units)

\[ F_{\text{max}} \leq F_{\text{resting}} \leq F_{\text{min}} \]

Cell loading with indicator

Fluorescence at resting \([\text{Ca}^{2+}]\)

Fig. 2.7. Fluorescence increase following whole-cell recording with fura-2FF (500 µM) in the internal solution. Excitation at 420 nm; emission at >510 nm. \(F_{\text{max}}\) at saturating \([\text{Ca}^{2+}]\) is the autofluorescence before whole-cell (cell-attached mode). The cell begins to load with indicator in the whole-cell mode and \(F_{\text{min}}\) is the fluorescence at resting \([\text{Ca}^{2+}]\) when the fluorescence has reached a steady state (after 8-10 minutes).
Fig. 2.8. Graph relating $F$ to $[Ca^{2+}]$ using the equation

$$[Ca^{2+}]_i = \frac{K_d (F_{\text{min}} - F)}{(F - F_{\text{max}})}$$

If the $[Ca^{2+}]_i$ levels to be measured are smaller than the $K_d$, accurate $F$ changes can be observed because the curve is steep here. In addition, the relationship is approximately linear, minimising underestimation of the flux (see explanation in text). These are reasons why low affinity (high $K_d$) indicators are good for recording the kinetics of $[Ca^{2+}]_i$ changes.
At resting $[\text{Ca}^{2+}]$:

\[
\frac{[\text{Ca}^{2+}]}{K_d} \approx \frac{1}{750}
\]

So:

\[
F \approx F_{\text{min}}
\]

This approximation allowed the resting fluorescence just prior to the flash to be used as an estimate of the fluorescence of calcium-free indicator, $F_{\text{min}}$.

Fura-2FF produced little calcium buffering (approximately 15 bound:free, see Appendix 2) relative to cell buffers (approximately 200:1 in hippocampal pyramidal neurones; Helmchen et al., 1996).

Data were recorded on a computer. Fluorescence data are shown integrated with a time constant of approximately 4 ms (The current-voltage converter contained a 10 MΩ resistor and a 400 pF capacitor). Analysis was with Matlab 5.1.

**Patch clamp recording from neurones in slices**

The patch clamp technique, developed by Neher & Sakmann (1976) and others (Hamill et al., 1981), has allowed successful studies of ionic currents and single-channel activity in CNS cells. It was originally used for cultured neurones or cells acutely dissociated enzymatically. However, improved optics in the experimental set-up and the process of 'cleaning' tissue away from it has permitted patch clamp in brain slices. Individual neurones can be visually identified in distinct regions of the hippocampus (fig. 2.9).
Fig. 2.9. Video image of a patched cell in region CA1 of a hippocampal slice.
Liquid junction potentials

Liquid junction potentials (LJPs) result from the different ionic mobilities and concentrations of different ionic species across a boundary of solutions. When two solutions of different compositions come in contact, a voltage develops to maintain the charge balance (Barry & Lynch, 1991; Neher, 1992).

When a pipette is in the chamber, the voltage is set to produce zero current, but includes a voltage equal to the LJP across the pipette. When a seal is made, the LJP disappears because there is no longer a solution interface. The error in the pipette voltage now has a magnitude equal to the LJP.

The LJP can be measured in voltage clamp or current clamp mode as follows (fig. 2.10). Firstly, there is extracellular solution in the chamber and internal solution in the pipette, generating the LJP that is to be measured (A). There is 3M KCl solution in the reference electrode, which is flowing, continuously renewing the junction. Potassium and chloride have very similar ionic mobilities so a concentrated solution of KCl here generates a negligible LJP between the reference electrode and the bath solution.

The voltage is adjusted to zero, concealing the LJP (B). This situation is analogous to the pipette being in the bath solution, before it is attached to the cell.

Next, the solution in the chamber is changed to internal solution, mimicking seal-formation (C). The LJP has disappeared but there is an error in the voltage measurement due to compensation for the LJP in the previous situation. The voltage reading for zero current now has a value that is equal and opposite to the LJP between the pipette and the bath solution. This reading is the error in the pipette voltage measurements.

The LJP was measured for the three different internal solutions: potassium gluconate (Kgluconate), potassium chloride (KCl) and caesium gluconate.
There is a LJP between the pipette and bath solution (−)

**Fig. 2.10.** Measuring junction potentials.  
A. External solution in the bath and internal solution in the pipette generated the LJP.  
B. The LJP was offset to zero.  
C. Substituting the bath solution for internal removed the LJP, leaving an error in the pipette voltage equal and opposite to the LJP.
(Csgluconate). The values for the errors obtained were: +12.6 mV, +3.3 mV and +9.8 mV respectively. These errors were taken into account (subtracted) when measuring the reversal potentials. Neher's values for the same solutions (except that glutamate took the place of gluconate, which has a similar mobility; Neher, 1992) were +10 mV, +3 mV and +11 mV respectively.

Series resistance

There is a resistance in series with the pipette and membrane \( (R_s) \), which is known from the reading on the patch clamp amplifier. When a current \( (I) \) flows across the membrane, this resistance causes a discrepancy between the pipette potential \( V_p \) (command voltage which is controlled by the clamping amplifier) and the true membrane potential \( V_m \). This error \( (V_p - V_m) \) is equal to \( I \times R_s \), so the larger the current, the larger the error. There are other sources of series resistance (for example between the reference electrode and the bath) but these are small compared to \( R_s \). When measuring reversal potentials, the standing current is used to calculate the error.

With commercial patch clamps, it is possible to partially compensate for \( R_s \) by adding a voltage signal, which is proportional to the membrane current, to the command voltage. Series resistance compensation was not used in these experiments. If \( R_s \) is known, the error can simply be subtracted from the command voltage to obtain the true potential. The series resistance in these experiments was 11.0 ± 4.1 MΩ (mean ± standard deviation). In experiments in Chapter 4, the late mGluR current reversed at -57 mV before LJP correction, at which there was no or very small standing current (near resting potential of cell), resulting in a very small error due to \( R_s \). The early mGluR current appeared to reverse at +20 mV, at which there was a large standing current of maximum amplitude 500 pA. The error was approximately +5 mV, and was used to correct measured values.
Drugs and reagents

Caged glutamate was synthesised by Drs. George Papageorgiou and John Corrie (Papageorgiou et al., 1999). Caged IP₃ (P⁴/P⁵ mixed) was supplied by Dr. D. R. Trentham. Fluo-4 came from Molecular Probes and fura-2FF from Tef Labs.

The ionotropic antagonists were 6-nitro-7-sulphamoylbenzo[f] quinoxaline-2,3-dione (NBQX), D- or DL- 2-amino-5-phosphonovaleric acid (AP5) and 3-((±)-2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) (all from Tocris). The potassium channel blockers were tetraethylammonium (TEA), apamin and iberiotoxin (all from Sigma). Metabotropic glutamate receptor antagonists were (S)-α-methyl-4-carboxyphenylglycine (MCPG), 7-(hydroxyimino)cycloprop[a]chromen-1a-carboxylate ethyl ester (CpCCOEt) and (2S,3S,4S)-2-methyl-2-(carboxycyclopropyl)glycine (MCCG) (all from Tocris). All reagents used to make solutions were AnalaR Grade from BDH. Tetrodotoxin (TTX) was from Tocris.

Calibration of photolysis

The aim is to estimate the proportion of cage compound converted to product with each pulse of the flash lamp. An assumption is made that on a single experimental set-up, the ratio between the extents of photolysis of any two different caged compounds is equal to this ratio on any other set-up. This is because the extent of photolysis depends on factors due to the experimental set-up, such as photon flux, pathlength and wavelength of light, and factors unique to the caged compound, namely the molar extinction coefficient and the quantum yield.

The molar extinction coefficient, ε, has units M⁻¹ cm⁻¹, is a property of the particular compound and depends on wavelength. The fraction of incident light absorbed depends on the thickness of the medium that is traversed. The law of absorption (Bouguer; Lambert) is described by the differential equation:
\[-dI/I = a \, dx\]

$I$ is the intensity of light at a distance $x$ from its entry into the medium, and $a$ is the \textit{extinction coefficient}. On integration with the boundary condition $I = I_0$ at $x = 0$;

\[I = I_0 \, e^{-Ex} \]

where $c$ is the molar concentration and $E$ is the \textit{extinction coefficient} per molar concentration per cm. This is Beer's Law. It is usually converted to log base 10 and the \textit{molar extinction coefficient}, $\varepsilon$, defined by:

\[I = I_0 \, 10^{-\varepsilon c x} \]

The \textit{quantum yield}, $Q$, has no units and is the ratio of molecules uncaged to photons absorbed. $Q$ is found by measuring the light absorbed and the concentrations of caged compound and product in a photolysis reaction.

The proportion photolysed depends on the product $\varepsilon \cdot Q$ and is often determined at 347 nm for comparison with known values of NPE-ATP. The methods for measuring the extents of photolysis in a particular set-up are as follows:

\textit{Caged ATP:} Photolysis of caged ATP followed by high pressure liquid chromatography separation and quantification of caged compounds and products (Walker \textit{et al.}, 1989).

\textit{Caged HPTS:} Photolysis of caged (8-(2-nitrophenylethyl)oxypyrene-1,3,6-trisulfonic acid trisodium salt) (cHPTS), which yields a fluorescent product, and the measurement of the resulting fluorescence (Jasuja \textit{et al.}, 1999). The extent of photolysis of this compound is the quickest and easiest to measure. This procedure was carried out as follows:
NPE-HPTS (cHPTS) is a photolabile derivative of the fluorescent pH indicator HPTS (8-hydropyrene-1,3,6-trisulfonic acid, trisodium salt {pyranine}) and is quenched (inactive) in its unphotolysed form.

\[
\text{NPE-HPTS} + \text{hv} \rightarrow \text{HPTS} + \text{2-nitrosoacetophenone} + \text{H}^+ 
\]

cHPTS was made up in 100 mM sodium borate pH 9. The fluorescent emission peak of HPTS depends on solution pH and, at pH 9, the change in fluorescence when cHPTS is photolysed is at its highest. The excitation wavelength used was 450 nm and the emission wavelength >510 nm.

The cHPTS in borate was mixed with liquid Sylgard resin in a ratio of 10:1 Sylgard:aqueous solution. Small drops (approximately 5-20 µl) were placed on coverslips, one on each, and left at 60°C to solidify. Vesicles of cHPTS solution in the Sylgard could then be visualised on the microscope. A 5 - 20 µm diameter vesicle was masked off and then flashed (300V) at about 5 second intervals while recording the fluorescence (via a photomultiplier tube), until the fluorescence signal reached a plateau, indicating that all the cHPTS had been photolysed. The fluorescence change was plotted against the number of flashes to give an exponential curve, from which the photolytic conversion per flash was estimated.

**Calculation of conversion per flash (\(\alpha\))**

For an irreversible reaction:

\[
\alpha \\
\text{Cage} \rightarrow \text{Product} \\
C \rightarrow P
\]

\(C = \text{concentration of cage;} \quad P = \text{concentration of product}\)

For 1 flash:

\[
\Delta P / C = \alpha
\]
Assume: closed volume uniformly irradiated.
\[ C + P = \text{constant} = C_0 \] initial concentration

The product has a fluorescence \( F \), measured in volts, which is proportional to its concentration:

\[ F = A \, P \]

A is constant

After a single flash:
\[ C_1 = C_0 - \alpha C_0 = C_0 (1-\alpha) \]
\[ P_1 = P_0 + \alpha C_0 \]

After second flash:
\[ C_2 = C_1 - \alpha C_1 = C_0 (1-\alpha)^2 \]
\[ P_2 = P_0 + (C_0 - C_2) \]
\[ = P_0 + C_0 (1 - (1-\alpha)^2) \]

After \( n \) flashes:
\[ C_n = C_{n-1} - \alpha C_{n-1} = C_0 (1-\alpha)^{n-1} - aC_0 (1-\alpha)^{n-1} \]
\[ = C_0 (1-\alpha)^{n-1} (1-\alpha) \]
\[ = C_0 (1-\alpha)^n \]
\[ P_n = P_0 + (C_0 - C_n) \]
\[ = P_0 + C_0 (1 - (1-\alpha)^n) \]

\[ F_0 = A \, P_0 \quad F_n = A \, P_n = A \{ P_0 + C_0 (1 - (1-\alpha)^n) \} \]

If \( \alpha \) is small (<0.1), as \( n \) approaches infinity, \( (1-\alpha)^n \approx e^{-\alpha n} \)
\[ F_n - F_0 = A \, C_0 (1 - e^{-\alpha n}) \]
With this approximation of the exponential, the estimate of $\alpha$ from exponential fits is too large by 1 % for $\alpha=0.01$, 2 % for $\alpha=0.05$, 5 % for $\alpha=0.1$ and 10 % for $\alpha=0.2$. For values of $\alpha>0.2$ a simple empirical correction $\alpha = \alpha_e - 0.35\alpha_e^{1.7}$ can be applied, where $\alpha_e$ is the estimate from least-squares fit by an exponential (Canepari et al., 2001b). This reduces the error in $\alpha_e$ due to the approximation to less than 5 % for $0.2<\alpha<0.9$. However, this correction was not applicable in experiments here because $\alpha$ for cHPTS was less than 0.2.

Exponential curves (figs. 2.11) were fitted to experimental values on two occasions during photolysis experiments. Calibrations of the photolytic efficiency of the set-up were made after the flash lamp bulb was changed or re-aligned to give estimations of $\alpha$ of 10.7 % and 14.3 % respectively.

Comparative extents of photolysis (from previous work) were as follows:

<table>
<thead>
<tr>
<th></th>
<th>NPE-ATP</th>
<th>(Jasuja et al., 1999)</th>
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<tbody>
<tr>
<td>NPE-HTPS :</td>
<td>10 %    : 50 %</td>
<td></td>
</tr>
<tr>
<td>NPE-ATP :</td>
<td>23 %    : 9.7 %</td>
<td></td>
</tr>
<tr>
<td>NI-Glutamate  :</td>
<td></td>
<td>(Papageorgiou et al., 1999)</td>
</tr>
</tbody>
</table>

These results were confirmed by measuring proton release to give extents of conversion (Canepari et al. 2001b). The extent of photolysis of NPE-IP$_3$ was approximately the same as that for NPE-ATP (Walker et al., 1989). MNI-Glutamate was approximately 2.5 times more efficient than NI-Glutamate (Canepari et al. 2001b).

Approximate ratios for photolytic efficiency were therefore:

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<tr>
<td>1             : 5 : 5 : 2 : 5</td>
<td></td>
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</tbody>
</table>
Fig. 2.11. Calculation of conversion per flash ($\alpha$). Vesicles of chHPTS (50 \(\mu\)M chHPTS in 100 mM borate pH 9) in 100 \(\mu\)M Sylgard were visualised on an upright microscope. A vesicle was masked off and flashed (300V) at approximately 5 second intervals while recording the fluorescence until the fluorescence signal reached a plateau, indicating that all the chHPTS had been photolyzed. The fluorescence change was plotted against the number of flashes to give an exponential curve, from which the photolytic conversion per flash ($\alpha$) was estimated. A. $\alpha = 10.7 \%$. B. $\alpha = 14.3 \%$. 
Chapter 3 - Characterisation of Ni- and MNI-caged glutamate
Introduction

To be useful, caged neurotransmitters need to satisfy a number of criteria. First, they must be thermally stable; second, fast and efficient in photorelease. Third, the caged precursor, the photolytic intermediates and by-products must be biologically inert and not interfere with synaptic transmission at concentrations used in an experiment.

The caged glutamates 7-nitroindolinyl-L-glutamate (NI-caged L-glutamate; Papageorgiou et al., 1999) and 4-methoxy-7-nitroindolinyl-L-glutamate (MNI-caged L-glutamate; Papageorgiou & Corrie, 2000) have been synthesised by John Corrie and George Papageorgiou at N.I.M.R. as novel, fast caged neurotransmitters. Table 3.1 summarises the chemical characteristics of the caged neurotransmitters.

The caged L-glutamates were evaluated pharmacologically using three methods. The results are published, bound in with the thesis (Canepari et al., 2001b). First, the effect of the caged compounds on responses to L-glutamate applied by iontophoresis was tested. Second, the concentration of cage present was varied whilst releasing the same concentration of L-glutamate. Third, the effect of the caged compounds on synaptic transmission was assessed. The caged compounds were also evaluated photochemically and the effects of photochemical intermediates tested with NI-caged-5-dihydroxyphosphoryloxypentanoate (caged NI-phosphate).
Table 3.1. Parameters of caged compounds NI L-glutamate, MNI L-glutamate and caged 1-(2-nitrophenyl)ethyl phosphate (NPE-phosphate). Q and ε were measured at a wavelength of 347 nm (Papageorgiou et al., 1999; Papageorgiou & Corrie, 2000).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NI</th>
<th>MNI</th>
<th>NPE-Phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Q</strong> <em>(quantum yield)</em></td>
<td>0.043</td>
<td>0.085</td>
<td>0.54</td>
</tr>
<tr>
<td><strong>ε</strong> <em>(extinction coefficient)</em> /M⁻¹ cm⁻¹</td>
<td>2720</td>
<td>4330</td>
<td>510</td>
</tr>
</tbody>
</table>
Whole-cell patch clamp recordings were made from hippocampal pyramidal neurones in culture and from cerebellar Purkinje neurones in sagittal slices from 20-day rats. Isolated hippocampal cells were prepared from the whole hippocampus (E15-17) which was incubated for 30 minutes at 37 °C in 250 U/ml papain in a calcium-free Earles salt solution containing 1 mg/ml cysteine. The cells were isolated by trituration and centrifugation after washing in a trypsin inhibitor and DNase solution, resuspended in L-glutamine-free culture medium and plated onto poly-D-lysine and laminin coated coverslips.

For slice preparation, 19-22 day old Wistar male rats were killed by cervical dislocation and the brain quickly removed from the skull and placed in ice-cold PSS. PSS contained (in mM): 135 NaCl, 3 KCl, 2 CaCl₂, 10 HEPES, 2 MgSO₄, 25 glucose, 1 NaHCO₃ (pH 7.3 adjusted with NaOH), and was bubbled with O₂ in the slice experiments. Parasaggital cerebellar slices (200 μm thick) were cut with a vibratome and kept at 32°C for 1 hour, then at room temperature (24 °C).

The experiments in cultured neurones were performed in the presence of 500 nM tetrodotoxin (TTX). AMPA-receptor mediated currents and NMDA-receptor currents were blocked by NBQX and D-AP5 respectively.

Whole-cell patch clamp recordings were carried out in voltage clamp and patch pipettes were pulled from pyrex glass tubing with filament 1.5 mm × 0.86 mm for culture experiments or 1.5 mm × 1.17 mm for slice experiments (Clarke Electromedical). When filled with intracellular solution based on potassium gluconate, pipettes had a resistance of 2-4 MΩ for Purkinje neurone experiments and 5-7 MΩ for cultured hippocampal neurones. In experiments with Purkinje neurones, N-(2,6-dimethylphenylcarbamoylmethyl) triethylammonium (QX314, 5 mM) was also included in the internal solution to block sodium action potentials.
Iontophoresis

Iontophoresis is a convenient way of applying small quantities of drug or neurotransmitter onto a cell. It is the electrophoretic migration of ions in a gradient of electric potential.

The drug or neurotransmitter, for example glutamate, is contained within an iontophoretic pipette and positioned close (about one cell soma diameter) away from a cell soma. A current source applied to the pipette provides a steady retaining potential (bias), which counteracts the net outward diffusion of glutamate (fig. 3.1A.). The ejecting potential is in the reverse direction to the bias and is applied in short pulses, in order to eject glutamate from the pipette (B).

The efflux \( q \) of glutamate depends on the current \( I \), charge on the ion \( z \), Faraday’s constant \( F \) and the transport number \( n \).

\[
q = - \frac{nI}{zF}
\]

\( n \) is empirically determined and expresses the fact that only part of the current is carried by ions of a given species. For example, during ejection of a cationic drug by outward current flow, anions from the external medium are simultaneously transported backwards into the pipette. Note that this framework is not entirely accurate. If \( I \) is zero, it would predict that \( q \) is also zero, which is not the case because there is a small amount of leakage due to diffusion.

An alternative method to iontophoresis is pressure injection through a micropipette. Quantities released are independent of electrochemical properties, so uncharged molecules can be ejected. However, the rate of ejection is strongly dependent on the physical dimensions of the microelectrode tip (proportional to the third power of the internal tip diameter) and so a given driving pressure may produce widely varying flow rates in different pipettes.
**Fig. 3.1.** The theory of iontophoresis. 

- **A.** The bias potential acts in the opposite direction to glutamate diffusion so glutamate is retained.
- **B.** The ejecting potential acts in the same direction as glutamate diffusion, so glutamate is released.
Fig. 3.2. Video image of the iontophoresis technique. A cultured pyramidal cell is patch clamped. Glutamate is released from the iontophoresis electrode in a pulsatile manner at a distance of approximately 20 μm from the cell.
During whole-cell patch clamp experiments, iontophoresis was used to release L-glutamate onto cultured hippocampal cells (fig. 3.2) in regular pulses, producing regular sub-maximal responses (increases in conductance).

The iontophoresis pipettes had tips approximately 1 μm in diameter, resistance of 10 MΩ and were filled with 50 mM L-glutamate solution, buffered to pH 7 (in 5 mM HEPES). There was a +10 to +50 pA bias and glutamate was ejected by -10 to -20 μA pulses at a frequency of 0.1 - 0.2 Hz.

Fig. 3.3 shows the effect of adding 500 μM NI-caged L-glutamate to the bath on currents evoked by iontophoresis in a hippocampal neurone, at a holding potential of -80 mV or -20 mV. No change in the amplitude or in the kinetics of the current was observed (n=2 cells). In all figure legends AMPA-receptor is abbreviated to AMPA-R and NMDA-receptor to NMDA-R.

Addition of 500 μM MNI-caged L-glutamate did not change the amplitude or the kinetics of AMPA-receptor or NMDA-receptor currents evoked by iontophoresis of L-glutamate at -80 mV and -20 mV holding potentials (n=2 cells, fig. 3.4). These results indicate that MNI-caged L-glutamate, like NI-caged L-glutamate, has no action at AMPA-receptors.

Photolysis of the control compound, caged NI-phosphate (Papageorgiou et al., 1999), releases an inert phosphorylated carboxylate species by the same photochemical reaction as release from NI-caged neurotransmitters. Fig. 3.5 shows the effect of photolysis of the control caged compound at 500 μM during currents activated in a hippocampal neurone at a holding potential of -80 mV or -20 mV by L-glutamate iontophoresis. The photochemical reactions did not modify the timecourse or amplitude of the currents, indicating that the intermediates and products of photolysis do not affect AMPA-receptor or NMDA-receptor responses (n=2 cells).
Fig. 3.3. Effect of NI-caged L-glutamate (NI-glutamate) on currents evoked by iontophoretic application of L-glutamate. (Top) AMPA-R-mediated currents evoked in a hippocampal neurone at -80 mV voltage clamp by iontophoretic application of L-glutamate, in control conditions (with 500 nM TTX) and after addition of 500 μM NI-glutamate. (Bottom) AMPA-R- and NMDA-R-mediated currents evoked in a hippocampal neurone at -20 mV voltage clamp by iontophoretic application of L-glutamate, in control conditions (with 500 nM TTX) and after addition of 500 μM NI-glutamate.
Fig. 3.4. Effect of MNI-caged L-glutamate on currents evoked by iontophoretic application of L-glutamate. (Top) AMPA-R-mediated currents evoked in a hippocampal neurone at -80 mV voltage clamp by iontophoretic application of L-glutamate, in control conditions (with 500 nM TTX) and after addition of 500 μM MNI-glutamate. (Bottom) NMDA-R- and AMPA-R-mediated currents evoked in a hippocampal neurone at -20 mV voltage clamp by iontophoretic application of L-glutamate, in control conditions (with 500 nM TTX) and after addition of 500 μM MNI-glutamate.
Control After photolysis of Nl-phosphate

Control After photolysis of Nl-phosphate

50 pA
400 ms

Fig. 3.5. Effect of the photolysis reaction on currents evoked by iontophoretic application of L-glutamate. (Top) AMPA-R-mediated currents evoked by iontophoretic application of L-glutamate in a hippocampal neurone at -80 mV voltage clamp, in control conditions (with 500 nM TTX) and with release of 150 μM 5-dihydroxyphosphoryloxypentanoate from Nl-caged dihydroxyphosphoryloxypentanoate (Nl-phosphate) at the arrow. (Bottom) NMDA-R- and AMPA-R-mediated currents evoked in a hippocampal neurone at -20 mV voltage clamp by iontophoretic application of L-glutamate, in control conditions (with 500 nM TTX) and with release of 150 μM 5-dihydroxyphosphoryloxypentanoate from Nl-phosphate at the arrow.
Flash photolysis

Slices were viewed with a Zeiss Axioskop 1FS (40× 0.75w Achroplan objective). Light was focused through the silica condenser (Reichert 0.9 NA) in these experiments. Transmission at 320 nm through 200 μm cerebellar slices from 20 day rats was measured as 0.45 in the molecular layer and 0.4 in the granule cell layer.

Flash lamp intensity was set to maximum, converting between 12 - 15 % of NI-caged compounds and 30 - 35 % of the MNI-caged L-glutamate. Lower intensities were obtained by using neutral density filters in the condenser light path.

In hippocampal neurones, L-glutamate activated both AMPA-receptor mediated currents and NMDA-receptor mediated currents. Fig. 3.6A shows currents evoked by photorelease of 70 μM L-glutamate in the presence of 2 mM magnesium at −80 mV (left hand panels) and −20 mV (right panels). The times of photolysis are indicated by the arrows. At −80 mV the glutamate-evoked current was blocked completely and reversibly by the AMPA-receptor antagonist NBQX at 100 μM. The NMDA-receptor antagonist D-AP5 (50 μM) had no additional effect. At −20 mV, in the presence of 100 μM NBQX, there was a residual glutamate-evoked current that was blocked reversibly by 50 μM D-AP5. It was due to NMDA-receptor activation and the relief of magnesium block at −20 mV relative to the strong magnesium block of NMDA-receptor seen at −80 mV (Ascher & Nowak, 1988).

The kinetics of the AMPA-receptor and NMDA-receptor-mediated currents evoked by 70 μM L-glutamate at −20 mV are shown in fig. 3.6B as the currents before and after addition of 100 μM NBQX. In 4 cells at −80 mV the AMPA-receptor-mediated current rose with half time of 0.59 ± 0.19 ms. NMDA-receptor-mediated currents at −20 mV (NBQX present) rose with half time 12.6 ± 1.0 ms (n=4). In hippocampal neurones, the decay of AMPA-receptor-mediated currents
Fig. 3.6. Pharmacological properties and kinetics of currents evoked by photolysis of Nl-caged L-glutamate. **A.** (Left panel) Voltage clamp currents evoked in a hippocampal neurone at -80 mV by the release of 70 μM L-glutamate from 500 μM Nl-glutamate, in control conditions (with 500 μM TTX), after addition of 100 μM NBQX, after addition of 50 μM D-AP5 and after washing out the two antagonists. (Right panel) currents evoked in a hippocampal neurone at -20 mV voltage clamp by 70 μM L-glutamate released from 500 μM Nl-glutamate, in control conditions, after addition of 100 μM NBQX, after addition of 50 μM D-AP5 and after washing out the two antagonists. **B.** AMPA-R-mediated current (left), and NMDA-R-mediated current (right; 100 μM NBQX), recorded in a hippocampal neurone at -80 mV shown on a fast time scale. The arrows indicate the times of flashes.
evoked by release of 70 μM L-glutamate at -80 mV was typically characterised by a fast phase due to receptor desensitisation (time constant 40 ± 20 ms, n=5, single exponential fit). The baseline current was restored slowly in more than 1 minute because of the persistence of L-glutamate in the photolyzed region.

The MNI-caged L-glutamate was investigated photochemically as one of a series of aromatic substitutions in Nl-caged compounds, and found to be approximately 2.5 times more efficient in releasing L-glutamate than Nl-caged L-glutamate (Papageorgiou & Corrie, 2000). L-Glutamate release from MNI-caged L-glutamate was also found to be more efficient when tested on hippocampal neurones in culture (fig. 3.7).

A straightforward test for effects of Nl-caged L-glutamate itself on AMPA-receptor and NMDA-receptor was to vary the concentration of cage present while releasing the same, sub-maximal concentration of L-glutamate. This was done by varying the intensity of the flash with neutral density filters at different cage concentrations to release the same L-glutamate concentration on the same cell.

Fig. 3.8 shows AMPA-receptor-mediated currents at -80 mV (A) and NMDA-receptor-mediated currents at -20 mV with 100 μM NBQX (B). The currents were evoked by the release of approximately 40 μM L-glutamate from 250 μM NI-caged L-glutamate, or from 1 mM NI-caged L-glutamate with the flash intensity reduced to 25% by a neutral density filter. The currents have the same amplitude.

MNI-glutamate was tested as an antagonist at glutamate receptors in the same way as NI-glutamate. The current evoked at 1 mM MNI-glutamate with intensity attenuated 4 fold was compared with that at 250 μM MNI-glutamate with no attenuation and gave a ratio of 1.2 ± 0.2 (standard deviation, n=4, fig. 3.9). The rise time had a half time of 0.78 ms (± 0.12, n=6) with a 1 ms photolysis pulse, similar to data with NI-caged L-glutamate.
Fig. 3.7. Comparison of the efficiency of Ni- and MNI-caged glutamate at a holding potential of -70 mV. A. The responses to Ni- and MNI-caged L-glutamate as the intensity of near-UV light is varied using neutral density filters in the light path. B. The peak current amplitudes plotted against light intensity for Ni- and MNI-caged glutamates.
Fig. 3.8. Effect of cage concentration at constant L-glutamate concentration. A. AMPA-R-mediated currents evoked in a hippocampal neurone at -80 mV by the release of 40 μM L-glutamate from 250 μM or 1 mM Ni-glutamate. B. NMDA-R-mediated in presence of 100 μM NBQX evoked in a different hippocampal neurone at -20 mV by the release of 40 μM L-glutamate from 250 μM or 1 mM Ni-glutamate. The arrows indicate the times of flashes.
Fig. 3.9. AMPA-R-mediated currents evoked in a hippocampal neurone at -80 mV by the release of 90 μM L-glutamate from MNI-glutamate in the presence of 250 μM or 1 mM MNI-glutamate.
Climbing fibre stimulation in cerebellar slices

The possibility that caged L-glutamate may interfere with synaptic mechanisms, particularly transmitter release, was tested at climbing fibre synapses in Purkinje neurones in cerebellar slices. This preparation is particularly suitable because each Purkinje neurone has one climbing fibre synapse and therefore a well-defined threshold for activation (Eccles et al., 1964; Konnerth et al., 1990). Furthermore, the climbing fibre synapse has a large number of active sites, reducing fluctuations in the synaptic responses resulting from the stochastic nature of the release process and shows a strong depression with repeated activation.

Climbing fibres in cerebellar slices were stimulated with constant current pulses of 50 μs duration delivered through a 5-10 μm tip glass pipette positioned on the surface of the slice in the granule cell layer. Climbing fibre responses were characterised by large excitatory postsynaptic currents (EPSCs) with a well-defined threshold, and intensity was adjusted to twice the threshold.

Experiments were made in the presence of 200 nM NBQX to reduce the amplitude of EPSCs. The stimulation protocol comprised a train of four pulses at 50 ms intervals delivered at 0.05 Hz in order also to test possible effects of the cage on the presynaptic depression of the climbing fibre synaptic responses (Silver et al., 1998). Data were filtered at 2 kHz (-3dB) digitised at 10 kHz on a CED 1401-plus with Spike 2 software and analysed in Matlab 5.1.

Fig. 3.10 shows ten consecutive recordings of the four EPSCs showing almost no fluctuations from their mean. Addition of 1 mM NI-caged L-glutamate did not change the amplitude of the EPSCs, the depression or the excitation threshold, indicating that it did not affect presynaptic processes. Also, photolysis of the control NI-caged-5-dihydroxyphosphoryloxypentanoate during periods of stimulation had no effect.
**Fig. 3.10.** Effect of Nl-caged L-glutamate on the currents evoked in a Purkinje neurone by climbing fibre stimulation. Ten superimposed recordings (A) and average (B) of 4 EPSCs at 50 ms interpulse intervals, evoked by the stimulation of the climbing fibre synapse, recorded in control conditions (top) and after addition of 1 mM Nl-glutamate (bottom).
Summary

The NI- and MNI- caged L-glutamate compounds were characterised for their efficiency of release and pharmacological properties. Photorelease from MNI-caged L-glutamate was approximately 2.5 times more efficient than NI-caged L-glutamate.

Neurotransmitters were released with half times that were fast enough to be useful on a synaptic time scale. Release of L-glutamate at 50-200 µM concentration with a 1 millisecond flash lamp pulse produced activation of AMPA-receptor-mediated currents with half rise time of less than a millisecond, and slower activation of NMDA-receptor-mediated currents, typically more than 10 ms. No evidence was found for interference by caged L-glutamate or the photochemical reaction compounds at 1 mM with glutamate receptor activation, synaptic transmission or excitability. NI- and MNI-caged L-glutamate compounds have proved useful tools for the investigation of synaptic processes.
Chapter 4 - The conductance change evoked by mGluR activation in CA1 pyramidal neurones
Introduction

The membrane conductance due to activation of mGluR by L-glutamate is difficult to identify when ionotropic NMDA-receptor and AMPA-receptor currents are also present. In the experiments described here, two methods were used to isolate the mGluR responses to L-glutamate.

First, the ionotropic currents were blocked pharmacologically using DL-AP5, D-AP5 or CPP (NMDA-receptor antagonists) and NBQX (AMPA-receptor antagonist). NBQX is a very effective blocker of AMPA-receptor currents, and 100 μM was sufficient to block the AMPA-receptor responses in these experiments. Conversely, the NMDA-receptor current was more difficult to block, and up to 1 mM DL-AP5, 400 μM D-AP5 or 200 μM CPP was required.

Second, the activation of mGluR was separated kinetically by rapid, millisecond, application of L-glutamate by flash photolysis of Nl-caged L-glutamate over the whole region of the neurone, with excitability suppressed by tetrodotoxin (TTX), cadmium and ionotropic antagonists. In this way, the timecourse and dependence on L-glutamate concentration of mGluR mediated events could be studied.

The caged Nl L-glutamate was used for experiments in this chapter. MNI L-glutamate was used in later experiments, and results with this compound are described in Chapter 6. The Russian 40× 0.75 objective was used as the condenser.

The concentration of glutamate released was calculated using measured parameters. First, the volume in the microscope chamber was 1 ml, resulting in a concentration of Nl-caged compound of 500 μM. Second, the efficiency of release was twice that of NPE-HPTS, the compound used in the calibration process (calculated from separate experiments; Jasuja et al., 1999; Papageorgiou et al., 1999; Canepari et al., 2001). Third, there was a 33 % extinction of UV light by the slice. This was measured with 316 nm (near-UV) light, and transmission through
the slice was obtained using a photomultiplier tube. Using the value of NPE-HPTS conversion efficiency (from calibration experiments) of 10.7% per pulse, the concentration of glutamate was calculated:

\[
\text{Concentration in bath} \times \text{slice extinction} \times \frac{\text{NPE - HPTS efficiency}}{\text{relative to NPE - HPTS}} = \text{final concentration}
\]

\[\alpha = \text{proportion released}\]

\[
500 \mu M \times 10.7\% \times \frac{1}{3} \times 2 = 36 \mu M
\]

Glutamate was released during a 1 millisecond flash, uniformly over an area of approximately 200 μm in diameter. It disappeared with a half time of 200 ms, as estimated in cerebellar slices by measuring AMPA-receptor-mediated currents in the presence of cyclothiazide (100 μM) to inhibit AMPA-receptor desensitisation in Purkinje cells (Canepari et al., 2001a).

In the absence of antagonists, photorelease of L-glutamate evoked a rapidly activating inward current, mainly due to ionotropic receptors, which was prolonged in comparison to synaptic release due to the large area over which it was photolysed. The inward current had an amplitude of several nA, activated during the 1 ms flash lamp pulse, as shown in fig. 4.1. The mGluR response was concealed when the ionotopic component was present.
Fig. 4.1. Current activated by L-glutamate release on a CA1 neurone in a hippocampal slice. Ionotropic and mGluR-mediated currents evoked in a hippocampal pyramidal neurone at -70 mV voltage clamp by release of 72 μM L-glutamate, by a 1 ms pulse at the time indicated by the arrow. Internal solution: Kgluconate. External solution: 200 nM TTX, 50 μM cadmium and 1.2 mM Mg$^{2+}$. Zero current indicated by dotted line. Caged compound is Nl L-glutamate. (Bottom) shorter time scale to show timecourse of activation of response.
Isolation of the mGluR response

The presence of a conductance change due to mGluR activation was shown in experiments in which the concentrations of ionotropic antagonists and L-glutamate were varied. It was to be expected that the concentrations of antagonists needed would be higher than those used in synaptic experiments because of the persistence of L-glutamate following photorelease, resulting in conditions approaching equilibrium rather than transient inhibition. Thus it was necessary to use a range of antagonist and L-glutamate concentrations to demonstrate adequate block of AMPA- and NMDA-receptors.

The results of an experiment in a CA1 pyramidal neurone to estimate the concentration of the NMDA antagonist DL-AP5 required in these conditions are shown in fig. 4.2. The AMPA-receptor currents are adequately blocked by 100 μM NBQX.

At 7 μM glutamate, only AP5-sensitive current was initiated. At a glutamate concentration of 36 μM, increasing AP5 from 100 μM (B) to 200 μM (A) decreased the current amplitude through NMDA-receptors to 21% of the initial peak. Increasing it to 400 μM decreased the peak to 7% of the original (G). At 800 μM, AP5 reduced the peak to 4% of its starting value (J). As the NMDA-receptor peak current decreased considerably after application of AP5, the later, mGluR, response remained approximately constant (133 ± 45 pA; mean ± standard deviation; n=3), seen in D, G, J as a noisy second peak during the decline of the NMDA-receptor current.

The block of the first peak, but not the second, by AP5, and the augmentation of both peaks by increasing glutamate concentration, seen in G & H, supported the idea that the first peak was NMDA-receptor-mediated and the late response was a result of activation of mGluRs. This hypothesis was strengthened by the block of the late response with selective mGluR antagonists (see later).
Fig. 4.2. Separation of NMDA-R and mGluR currents. NMDA-R currents (first peaks) and mGluR currents (second peaks) evoked in a CA1 hippocampal pyramidal neurone (-70 mV voltage clamp) by release of 7, 36 or 72 μM L-glutamate [glu], by a 1 ms pulse at times indicated by the arrows. Internal solution: Kgluconate. External solution: 100 μM NBQX, 200 nM TTX, 50 μM cadmium and zero Mg²⁺. Caged compound is NI L-glutamate. A. 7 μM glutamate, 100 μM DL-AP5; NMDA-R current only. B. 36 μM glutamate, 100 μM DL-AP5; mGluR current concealed by NMDA-R current. C. 7 μM glutamate, 200 μM DL-AP5; NMDA-R current only. D. 36 μM glutamate, 200 μM DL-AP5; NMDA-R current, mGluR current visible. E. 72 μM glutamate, 200 μM DL-AP5; mGluR current concealed by NMDA-R current. F. 7 μM glutamate, 400 μM DL-AP5; no current. G. 36 μM glutamate, 400 μM DL-AP5; NMDA-R current, mGluR current visible. H. 72 μM glutamate, 400 μM DL-AP5; NMDA-R current, mGluR current visible. I. 7 μM glutamate, 800 μM DL-AP5; no current. J. 36 μM glutamate, 800 μM DL-AP5; NMDA-R current, mGluR current visible.
D-AP5 is the active isomer and, when this was used alone, 400 µM was required to block the NMDA-receptor response. In subsequent experiments however, CPP was found to be more effective than D-AP5 (lower $K_d$; Davies et al., 1986; slower off-rate; Benveniste & Mayer, 1991) and 200 µM CPP was usually sufficient to block the NMDA-R response.

The effect of membrane potential on the mGluR response was investigated by holding the neurone at different potentials before and during photorelease of L-glutamate, as shown in fig. 4.3. Close examination of the mGluR response at different membrane holding potentials showed two components, one fast and one slow, hereafter referred to as the early component and the late component respectively. The early component activated after about half a second and the late component activated after a second.

The two components appeared to reverse at different membrane potentials, indicating that they were carried by different ions. Components occurred separately in some experiments, suggesting that there were separate intracellular pathways underlying each one. 84 cells were tested and the percentages of cells with mGluR responses were as follows: 45 % both components, 16 % late component only, 6 % early component only and 33 % no response to the release of 36 µM glutamate.

When the components occurred separately it was easier to identify the different reversal potentials, illustrated by data in figs. 4.4 & 4.5. The late component (fig. 4.4) appeared to reverse at -70 mV and the early component (fig. 4.5) at +5 mV. The amplitude of the early component appeared to be voltage-sensitive, having a maximum amplitude at potentials between -20 and -50 mV. The experimental reversal potentials were corrected for the liquid junction potential (LJP), which is +12.6 mV (see Methods, Chapter 2), and series resistance ($R_s$) error of +5 mV at the reversal potential of the early component.

The current-voltage data of the two components, plotted in figs. 4.6A & B, show that there were large variations in the amplitudes of peak currents from cell to
Fig. 4.3. Two components of the mGluR response. mGluR-mediated currents evoked in a CA1 hippocampal pyramidal neurone at -70, 50, -20, 0 and +20 mV voltage clamp by release of 36 μM L-glutamate, by 1 ms pulse at time indicated by the arrow. Internal solution: Kgluconate. External solution: 100 μM NBQX, 200 μM CPP, 200 nM TTX, 50 μM cadmium and zero Mg²⁺. Standing current values of -172, -90, +38, +158 and +278 pA are indicated. Caged compound is Nl L-glutamate.
Fig. 4.4. Late component of the mGluR response. mGluR-mediated currents evoked in a CA1 hippocampal pyramidal neurone at -70, -60, -30 and -20 mV voltage clamp by release of 36 μM L-glutamate, by 1 ms pulse at time indicated by the arrow. Internal solution: Kgluconate. External solution: 100 μM NBQX, 400 μM D-AP5, 200 nM TTX, 50 μM cadmium and zero Mg²⁺. Standing current values of -46, 0, +128 and +200 pA are indicated. Caged compound is NI L-glutamate.
Fig. 4.5. Early component of the mGluR response. mGluR-mediated currents (and residual ionotropic currents) evoked in a CA1 hippocampal pyramidal neurone at -70, -50, -20 and 0 mV voltage clamp by release of 36 μM L-glutamate, by 1 ms pulse at time indicated by the arrow. Internal solution: Kgluconate. External solution: 100 μM NBQX, 400 μM D-AP5, 200 nM TTX, 50 μM cadmium and zero Mg²⁺. Standing current values of -249, -49, +194, and +486 pA are indicated. Caged compound is NL L-glutamate.
Fig. 4.6. Summary of current-voltage relationships of the mGluR early (A) and late (B) components. Symbols correspond to individual cells. Cells displayed both components to varying extents. mGluR-mediated currents evoked in CA1 hippocampal pyramidal neurones by release of 36 μM L-glutamate, by 1 ms pulse. There is no correction for errors due to LJP or Rg. Internal solution: K gluconate. External solution: 100 μM NBQX, 200 μM CPP, 200 nM TTX, 50 μM cadmium and zero Mg$^{2+}$. Caged compound is NI L-glutamate.
Fig. 4.7. Summary of current-voltage relationships of the early mGluR component for cells that displayed both components to roughly the same extent. Symbols correspond to individual cells. mGluR-mediated currents evoked in CA1 hippocampal pyramidal neurones by release of 36 μM L-glutamate, by 1 ms pulse. There is no correction for errors due to LJP or R_s. Internal solution: Kgluconate. External solution: 100 μM NBQX, 200 μM CPP, 200 nM TTX, 50 μM cadmium and zero Mg^{2+}. Caged compound is Nl L-glutamate.
Fig. 4.8. Normalised current-voltage mean (± standard errors) relationships of the early (A) and late (B) mGluR components. Symbols correspond to individual cells. mGluR-mediated currents evoked in CA1 hippocampal pyramidal neurones by release of 36 μM L-glutamate, by 1 ms pulse. There is no correction for errors due to LJP or $R_s$. Internal solution: Kgluconate. External solution: 100 μM NBQX, 400 μM D-AP5, 200 nM TTX, 50 μM cadmium and zero Mg$^{2+}$. Currents are normalised to response at -70 mV (early component) or -20 mV (late component). Caged compound is Nl L-glutamate.
cell. Contributing to this variation is that the two components overlap in time at potentials positive to -70 mV and act in opposing directions. Consequently, the amplitude of one component influences the apparent amplitude of the other. Some cells lack one or other of the components or have a large difference in the amplitude of one component relative to the other.

Data from neurones showing both components with similar magnitudes at all holding potentials are plotted in fig. 4.7. These show less variation and after normalisation to -70 mV are plotted as mean ± standard error (n=6) in fig. 4.8A (early component) and B (late component). Data from the early component have a reversal potential of +7 mV (corrected for LJP and Rₛ) and the conductance shows a peak at approximately -30 mV (corrected for LJP). The late component (fig. 4.8B) has a reversal potential at -70 mV (corrected for LJP) similar to that seen in neurones showing the late component only as described above.

It can be concluded from the results that there are two different ion channels underlying the two mGluR components. The aim in subsequent experiments was to identify the ion species carried by the channels and identify the channels. Ion-substitution experiments were carried out to change ionic equilibrium potentials, with solution composition values given in table 4.1.

**Ion substitution experiments**

The reversal potentials of the two components give some indication of the ionic basis of the early and late currents. The first component reversed close to zero mV, midway between the equilibrium potentials for sodium or calcium, and potassium or chloride, indicating a high permeability to sodium and possibly other cations. The late component reversed close to the resting membrane potential, near the equilibrium potentials for potassium and chloride ions.
### Table 4.1

<table>
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<td>+72</td>
</tr>
<tr>
<td>$Cl^-$</td>
<td>151</td>
<td>5</td>
<td>-86</td>
</tr>
<tr>
<td>$Ca^{2+}$</td>
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<th>$E_R$ (mV)</th>
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<tr>
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<td>15</td>
<td>-28</td>
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<tr>
<td>$Na^+$</td>
<td>70.5</td>
<td>8</td>
<td>+55</td>
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<tr>
<td>$Cl^-$</td>
<td>151</td>
<td>140</td>
<td>-2</td>
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Nernst equation for calculating $E_R$ is:

$$E_R = \frac{RT}{zF} \ln \frac{[out]}{[in]}$$

Where $R$ is the gas constant, $T$ is the temperature in Kelvin, $z$ is the valency of the ion, $F$ is the Faraday constant, $[out]$ is the concentration of ion in the external solution and $[in]$ is the concentration of ion in the internal solution. $RT/F = 25.26$ at 20°C.
First, the external sodium concentration was changed to half of its original concentration, shifting the sodium equilibrium potential \( (E_{Na}) \) from +72 to +55 mV. Experiments were difficult to carry out because cells were not healthy in low sodium solutions. However, one experiment was performed, showing that the late component reversed around -60 mV (corrected for LJP), data in figs. 4.9 & 4.10. The early component is very small or non-existent in this cell. As the experimental reversal potential of the late component had not changed to a more negative potential, it was concluded that sodium was not an ion carried by this current.

The internal chloride ion concentration was changed to shift \( E_{Cl} \) from -86 mV to -2 mV by replacing gluconate ions. Fig. 4.11 shows the records from one of these experiments, in which only the late component was present, the significance of which is unknown. The current-voltage data plotted in fig. 4.12 show that the current reversal potential did not change from the control (the smaller LJP of 3.3 mV was taken into account), indicating that permeability to chloride ions did underlie the late component.

The contribution of potassium ions was tested by substituting most of the internal potassium with caesium ions, shifting \( E_{K} \) from -80 mV to -28 mV. This change of internal cation resulted in few experiments in which the late component was clearly seen. The more striking observation was a much enhanced amplitude of currents in the early component (fig. 4.13). The results are summarised in the current-voltage data plotted in fig. 4.14A & B. Thus, the absence of an outward current at positive potential with internal caesium indicates either that potassium carry the current, or that this component of the response is suppressed in caesium internal solution. The increased amplitude of the early component may be due to the fact that the potassium current was not opposing the fast component at potentials around -20 mV, because \( E_{K} \) changed, so it appeared larger. Alternatively, the opposing potassium currents (at more positive potentials) could have been reduced by caesium.
mGluR-mediated currents evoked in a CA1 hippocampal pyramidal neurone at -70, -50, -20 and 0 mV voltage clamp by release of 36 μM L-glutamate, by 1 ms pulse at time indicated by the arrow. Internal solution: Kgluconate. External solution: 100 μM NBQX, 200 μM CPP, 200 nM TTX, 50 μM cadmium and zero Mg²⁺. Standing current values of -450, -246, +140 and +534 pA are indicated. Caged compound is Ni L-glutamate.

Fig. 4.9. mGluR response with half sodium external solution.
Fig. 4.10. Current-voltage relationship of late component of the mGluR response with half sodium external solution, to show reversal potential. mGluR-mediated currents evoked in a CA1 hippocampal pyramidal neurone at -70, -50, and -20 mV voltage clamp by release of 36 μM L-glutamate, by 1 ms pulse at time indicated by the arrow. There is no correction for errors due to LJP or Rg. Internal solution: Kgluconate. External solution: 100 μM NBQX, 200 μM CPP, 200 nM TTX, 50 μM cadmium and zero Mg²⁺. Caged compound is NI L-glutamate.
Fig. 4.11. Effect of high chloride internal solution on the late component. mGluR-mediated currents evoked in a CA1 hippocampal pyramidal neurone at -80, -60, -30, 0 and +30 mV voltage clamp by release of 36 mM L-glutamate, by 1 ms pulse at time indicated by the arrow. Internal solution: KCl. External solution: 100 μM NBQX, 400 μM D-AP5, 200 nM TTX, 50 μM cadmium and zero Mg^{2+}. Standing current values of -106, -13, +126, +500 and +537 pA are indicated. Caged compound is NI L-glutamate.
Fig. 4.12. Current-voltage relationship of the late component for three cells with high chloride internal solution. Symbols correspond to individual cells. Control data from cell with Kgluconate internal solution also shown. mGluR-mediated currents evoked in CA1 hippocampal pyramidal neurones by release of 36 μM L-glutamate, by 1 ms pulse. There is no correction for errors due to LJP or $R_g$. Internal solution: KCl. External solution: 100 μM NBQX, 400 μM D-AP5, 200 nM TTX, 50 μM cadmium and zero Mg$^{2+}$. Caged compound is NI L-glutamate.
Fig. 4.13. Effect of substituting internal potassium with caesium on early and late components of the mGluR response. mGluR-mediated currents evoked in a CA1 hippocampal pyramidal neurone at -70, -50, -20, 0, and +20 mV voltage clamp by release of 36 μM L-glutamate, by 1 ms pulse at time indicated by the arrow. Internal solution: Csgluconate (15 mM potassium). External solution: 100 μM NBQX, 200 μM CPP, 200 nM TTX, 50 μM cadmium and zero Mg²⁺. Standing current values of, -148, -29, +34, +83 and +167 pA are indicated. Caged compound is NI L-glutamate.
Fig. 4.14. Summary of effect of internal potassium substitution by caesium on early and late components. (A) Early component: means (± standard deviations) in 6 cells. (B) Late component: one cell. mGluR-mediated currents evoked in CA1 hippocampal pyramidal neurones by release of 36 μM L-glutamate, by 1 ms pulse. There is no correction for errors due to LJP or Rg. Internal solution: Csgluconate (15 mM potassium). External solution: 100 μM NBQX, 200 μM CPP, 200 nM TTX, 50 μM cadmium and zero Mg²⁺. Caged compound is N1 L-glutamate.
**Ion channel pharmacology**

Having identified the late component as a potassium conductance, attempts were made to block this current using different potassium-channel antagonists. TEA is known to block calcium-activated potassium channels. 14 mM TEA blocked the late component of the mGluR response (fig. 4.15) in four experiments. Apamin, a blocker of small-conductance calcium-activated potassium channels, was tried in these cells (one experiment) and also blocked the potassium current. Iberiotoxin, which inhibits large-conductance calcium-activated potassium channels (100 nM) had no effect (two experiments). These results suggest that the channels are small-conductance calcium-activated potassium channels (SK$_{Ca}$).

**Pharmacological separation of mGlu receptors underlying the two components**

The two components of the mGluR response may be mediated by distinct intracellular pathways, perhaps activated by different subtypes of the mGluR receptor. With this in mind, selective antagonists for mGluR1 (CpCCOEt) and the Group II mGluRs (MCCG) were used in experiments to block one or the other of the two components. CpCCOEt is a selective, reversible antagonist and is used at a low concentration (20 μM). MCCG is used at the much higher concentration of 1 mM and is less selective.

CpCCOEt blocked the late component (the potassium conductance) (figs. 4.16 & 4.17) in nine experiments. MCCG blocked the early component (fig. 4.18) in five out of seven experiments. In another experiment, the two components were blocked sequentially with the two antagonists; first the early component, with MCCG, and second the late component, with CpCCOEt (fig. 4.19). In a separate experiment, both components were blocked by the non-selective mGluR antagonist MCPG (data not shown).
Fig. 4.15. Effect of TEA (14 mM) on mGluR current. mGluR-mediated currents evoked in a CA1 hippocampal pyramidal neurone at -50, -20 and 0 mV voltage clamp by release of 36 μM L-glutamate, by 1 ms pulse at time indicated by the arrow. Internal solution: Kgluconate. External solution: 100 μM NBQX, 200 μM CPP, 200 nM TTX, 50 μM cadmium and zero Mg²⁺. Black trace – control. Red trace – 14 mM TEA in external solution. Standing current values of 0, +118 and +233 pA are indicated. Caged compound is Nl L-glutamate.
Fig. 4.16. Effect of mGluR1-selective blocker CpCCOEt (20 μM) on mGluR current. mGluR-mediated currents evoked in a CA1 hippocampal pyramidal neurone at -20 mV voltage clamp by release of 36 μM L-glutamate, by 1 ms pulse at time indicated by arrow. Internal solution: Kgluconate. External solution: 100 μM NBQX, 200 μM CPP, 200 nM TTX, 50 μM cadmium and zero Mg²⁺. Black trace - control. Red trace - 20 μM CpCCOEt in external solution. Standing current value of +231 pA is indicated. Caged compound is NI L-glutamate.
Fig. 4.17. Effect of mGluR1-selective blocker CpCCOEt (20 μM) on mGluR current. mGluR-mediated currents evoked in a CA1 hippocampal pyramidal neurone at -70, -50, -20, 0 mV voltage clamp by release of 36 μM L-glutamate, by 1 ms pulse at time indicated by arrow. Internal solution: KCl. External solution: 100 μM NBQX, 200 μM CPP, 200 nM TTX, 50 μM cadmium and zero Mg²⁺. Black trace - control. Red trace - 20 μM CpCCOEt in external solution. Standing current values of -104, -5, +184 and +341 pA are indicated. Caged compound is NI L-glutamate.
Fig. 4.18. Effect of Group II mGluR-selective blocker MCCG (1 mM) on mGluR current. mGluR-mediated currents, and residual NMDA-receptor currents, evoked in a CA1 hippocampal pyramidal neurone at -20 mV voltage clamp by release of 36 μM L-glutamate, by 1 ms pulse at time indicated by the arrow. Internal solution: Kgluconate. External solution: 100 μM NBQX, 200 μM CPP, 200 nM TTX, 50 μM cadmium and zero Mg²⁺. Black trace - control. Red trace - 1 mM MCCG in external solution. Standing current value of +103 pA is indicated. Caged compound is Ni L-glutamate.
Fig. 4.19. Comparison of effects of mGluR1-selective blocker CpCCOEt and Group II mGluR-selective blocker MCCG on mGluR current. mGluR-mediated currents evoked in a hippocampal pyramidal neurone at 0 mV voltage clamp by release of 36 μM L-glutamate, by 1 ms pulse at time indicated by the arrow. Internal solution: K gluconate. External solution: 100 μM NBQX, 200 μM CPP, 200 nM TTX, 50 μM cadmium and zero Mg^{2+}. Black trace - control. Red trace - 1 mM MCCG in external solution. Green trace - 1 mM MCCG and 20 μM CpCCOEt in external solution. Standing current value of +145 pA is indicated. Caged compound is NL L-glutamate.
Summary

These results indicate that the early component of the mGluR response is due to a non-selective cation conductance and the late component is due to a potassium conductance. The slow potassium conductance is a consequence of the activation of mGluR1 receptors. These receptors are known to act via PI metabolism and calcium ion release from stores, which may in turn activate potassium channels. The early, sodium permeable excitatory conductance of the early component was insensitive to the Group I mGluR antagonist but blocked by a Group II antagonist. This mGluR activated conductance additionally showed a dependence on membrane potential, with increased conductance around -30 mV.

Thus, two mGluR receptor mediated pathways in CA1 pyramidal neurones are identified in the present experiments, mediating an early excitation and later inhibition. They are activated in 0.5 - 1 seconds by L-glutamate concentrations greater than about 36 μM. It is of interest to know how they are activated in a physiological context, and how they interact with ionotropic receptors and other second messenger responses.
Chapter 5 - Changes of $[\text{Ca}^{2+}]_i$ and membrane conductance evoked by photorelease of IP$_3$ from caged IP$_3$
Introduction

After a train of action potentials or a large depolarisation in hippocampal pyramidal neurones, there is a slowly decaying afterhyperpolarisation (AHP) that lasts several seconds. The after-hyperpolarisation current ($I_{AHP}$) is carried by potassium and is calcium-dependent (Hotson & Prince, 1980; Lancaster & Adams, 1986). An mGluR agonist was found to modulate the $I_{AHP}$ via a G-protein coupled, IP$_3$-mediated calcium release mechanism (Abdul-Ghani et al., 1996). In addition, calcium-activated calcium release, involving ryanodine receptors, was implicated in the generation of the $I_{AHP}$ (Sah & Maclachlan, 1991). There are fast and slow components to the $I_{AHP}$ and both play important roles in regulating the neuronal firing rate and controlling neuronal excitability.

Calcium-activated potassium channels were first studied in snail neurones (Meech et al., 1975), then chromaffin cells (Marty, 1981), hepatocytes (Burgess et al., 1981; Jenkinson et al., 1983) and muscle cells (Barrett et al., 1982). The channels were also found to underlie the AHP in rat hippocampal pyramidal cells (Lancaster & Adams, 1986). Varieties of calcium-activated potassium channels are reviewed by Latorre et al. (1989). Different types are responsible for the two components of the $I_{AHP}$ (Lancaster et al., 1991; Sah, 1996; Vergara et al., 1998).

The fast component of the $I_{AHP}$ is responsible for action potential repolarisation and mediated by large conductance (>100 pS) calcium-activated potassium channels (BK$_{Ca}$), blocked by tetraethylammonium (TEA), iberiotoxin and charybdotoxin. BK$_{Ca}$ channels do not inactivate, are voltage-sensitive, activated at potentials more positive than -40 mV (Storm, 1990). The modulation of these channels by neurotransmitters has not been reported.
The small-conductance (<10 pS) calcium-activated potassium channels (SK$_{Ca}$) mediate the slow component of the I$_{AHP}$, and are blocked by apamin, and by TEA, but at higher concentrations than are needed to block the BK$_{Ca}$ channel. The slow component is not voltage-sensitive, is activated slowly over more than a second and can be regulated by several transmitters.

As IP$_3$ has been implicated in the generation of calcium-activated potassium currents, the mechanism has been probed with caged IP$_3$. Caged IP$_3$ was used in the study of smooth muscle contraction (Walker et al., 1987) and later to probe calcium-activated potassium conductances in guinea-pig isolated hepatocytes (Ogden et al., 1990).

Photochemically generated intracellular IP$_3$ or free calcium activated the conductance (Ogden et al., 1990). Calcium release produced an immediate increase in membrane conductance that rose exponentially, consistent with a direct activation of calcium-dependent ion channels. However, IP$_3$ produced a rise in conductance after a brief delay, and hormonal activation had an even longer delay. The maximum conductance produced by IP$_3$ was similar in each cell to the peak recorded with hormonal stimulation, and could be evoked by IP$_3$ concentrations of 0.5 to 1 µM. There was also shown to be a negative interaction between calcium concentration and IP$_3$-evoked calcium release.

A metabolically stable analogue of IP$_3$ was shown to activate a potassium conductance in pyramidal cells of the rat hippocampal slice (McCarren et al., 1989). This conductance was blocked by intracellular calcium chelation, consistent with a role for mobilisation of calcium from intracellular stores.

The involvement of IP$_3$ in the modulation of AHP and excitability by neurotransmitters was studied recently by Ross & colleagues (Nakamura et al., 1999; 2000). They found that synaptic activation of mGluRs or application of mGluR1 agonists paired with backpropagating action potentials resulted in large (supralinear) wavelike increases in [Ca$^{2+}$]. As mGluR1 receptors have been
known to couple to the IP$_3$ pathway on other tissues, it was concluded that the calcium release caused by IP$_3$ is synergistic with the calcium increase due to the action potentials because of involvement of both IP$_3$ and ryanodine receptors.

IP$_3$ and calcium release has been studied in great detail in non-neuronal systems. However, the role of IP$_3$ in neurones remains elusive, and there has been relatively little conclusive research reported on the subject. The IP$_3$ pathway is a relatively slow mechanism compared to neurotransmission, so its role is probably in modifying the fast transmission, rather than being responsible for the signal itself.

**Photolytic application of IP$_3$ in CA1 pyramidal neurones**

Experiments were carried out to investigate the effects of photoreleasing IP$_3$ on [Ca$^{2+}$]$_i$ and membrane conductance. These were done, firstly, to see if the changes induced by IP$_3$ are similar to the changes evoked by Group I mGlur activation, which has been shown to act via IP$_3$ in some systems (Sugiyama et al., 1987). Secondly, the characteristics of IP$_3$ action in hippocampal CA1 pyramidal cells were compared with those in other neurones and peripheral autonomic tissues.

[Ca$^{2+}$]$_i$ was monitored with fura-2FF (see Methods, Chapter 2). The concentration of IP$_3$ released was calculated from the calibration with NPE-HPTS, making allowance for extinction by the slice. The efficiency of NPE-IP$_3$ photolysis has been shown to be similar to NPE-ATP (Walker et al., 1989). NPE-ATP is 5 times more efficient than NPE-HPTS. The flash lamp output was set to maximum and lower intensities achieved with calibrated neutral density (ND) filters to vary the concentration of IP$_3$ released.
The concentration of IP$_3$ released was calculated in a similar way to the concentration of glutamate in Chapter 4.

**Final concentration** =

\[
\text{Concentration in pipette} \times \text{slice extinction} \times \frac{\text{NPE - HPTS efficiency}}{\text{NPE - HPTS}} \times \frac{\text{efficiency relative to \%transmission}}{\text{ND filter}}
\]

\( \alpha = \text{proportion released} \)

The characteristics of the IP$_3$ evoked [Ca$^{2+}$]$_i$ and conductance change are illustrated by the records shown in fig. 5.1. Following a 1 ms flash, IP$_3$ is released uniformly in the cytosol with half time of 3 ms (Walker *et al.*, 1989). In the records shown, a rise of [Ca$^{2+}$]$_i$ and an outward current (at -20 mV clamp potential) occurred after a delay of approximately 300 ms. Both records rose to a peak in a further 200-300 ms before declining over 5-10 seconds.

The following parameters were measured for analysis in each record: the delays in both records; the peak [Ca$^{2+}$]$_i$ and current change; the maximum rate of rise of [Ca$^{2+}$]$_i$; the rise time for [Ca$^{2+}$]$_i$ to change from 10-90% of peak amplitude; and the initial rate of decline of free [Ca$^{2+}$]$_i$. 
Fig. 5.1. Change in conductance and \([\text{Ca}^{2+}]_i\), following release of IP$_3$. Current and \([\text{Ca}^{2+}]_i\) transient evoked in a CA1 hippocampal pyramidal neurone at -20 mV voltage clamp by release of 102 μM IP$_3$, by 1 ms pulse at time indicated by the arrow. Internal solution: Kgluconate, fura-2FF (500 μM). (Top trace) \([\text{Ca}^{2+}]_i\) transient. (Bottom trace) conductance. Parameters of the \([\text{Ca}^{2+}]_i\) trace that were measured are shown in red. Zero current indicated.
Fig. 5.2. Whole-cell current and [Ca^{2+}] following release of IP_{3}. Currents (A) and [Ca^{2+}] transients (B) evoked in a CA1 hippocampal pyramidal neurone at -90, -70, -50 and -20 mV voltage clamp by release of 102 μM IP_{3}, by 1 ms pulse at time indicated by the arrows. Internal solution: Kgluconate, fura-2FF (500 μM). Zero current relative to all current traces indicated.
Fig. 5.3. Peak current-voltage amplitudes and reversal potentials for IP$_3$-evoked conductances. Currents evoked in four CA1 hippocampal pyramidal neurones at -90, -70, -50 and -20 mV voltage clamp by release of 102 μM IP$_3$ (A; different symbols represent individual cells), and 102 μM IP$_3$ (n=4) or 26 μM IP$_3$ (n=3) (B), by 1 ms pulse. There is no correction for errors due to LJP or $R_e$. Internal solution: Kgluconate, fura-2FF (500 μM).
Ionic species carrying the IP₃ evoked current

The ions flowing during the conductance change were investigated by comparing current-voltage relations for the peak IP₃-evoked current with different ion substitutions, and from the effects of known channel pore blocking drugs. The dependence of the current and [Ca²⁺]ᵢ on clamp potential is shown for a CA1 neurone in fig. 5.2. It can be seen that the [Ca²⁺]ᵢ changes following release of 102 μM IP₃ are similar in amplitude and timecourse at each potential, whereas the current direction and amplitude depends on potential. In five cells tested the currents reversed at approximately -57 mV clamp potential, as shown in figs. 5.3A and B. Correction for the initial liquid junction potential gives a true reversal potential of -70 mV. This is close to $E_K$ and $E_{Cl}$, -80 mV, calculated from the ion concentrations in external and internal solutions, but very different from $E_{Na}$ and $E_{Ca}$.

Changing $E_{Cl}$ to close to zero by substitution of chloride ions for internal gluconate resulted in an outward current at -73 mV, after correction for LJP (fig. 5.4). The experimental reversal potential had therefore not changed, and chloride was not responsible for the IP₃-evoked current.

Changing $E_K$ to -28 mV by substitution of caesium ions for most of the internal potassium caused the current to reverse around this latter value, after correction for LJP (fig. 5.5), confirming that the current is indeed carried by potassium ions. The current amplitudes were smaller when caesium was used as an internal solution, probably due to the partial block of potassium channels by caesium ions. The current-voltage relationships of peak currents with the three different internal solutions are shown in fig. 5.6.
Fig. 5.4. Effect of high chloride internal on IP$_3$-evoked response at -70 mV. Currents and [Ca$^{2+}$]$_i$ transients evoked in a CA1 hippocampal pyramidal neurone at -70 mV voltage clamp by release of 102 μM IP$_3$, by 1 ms pulse at time indicated by the arrows. Internal solution: KCl, fura-2FF (500 μM). (Top trace) [Ca$^{2+}$]$_i$ transient. (Bottom trace) conductance. Zero current indicated. Correction for liquid junction potential smaller (3.3 mV), so current is outward at -70 mV holding potential.
Fig. 5.5. Effect of low potassium internal on IP₃-evoked response. Currents and [Ca²⁺] transients evoked in a CA1 hippocampal pyramidal neurone at -70, -50 and -30 mV voltage clamp by release of 34 μM IP₃, by 1 ms pulse at time indicated by the arrows. Internal solution: Cs gluconate (15 mM potassium), fura-2FF (500 μM). Zero current relative to current traces indicated.
Fig. 5.6. Current-voltage plot of IP$_3$-evoked peak current amplitude changes with different internal solutions and 500 μM fura-2FF. Currents evoked in three CA1 hippocampal pyramidal by release of 34 μM IP$_3$, by 1 ms pulse, with internal solutions K gluconate (green triangles), Cs gluconate (red circles) and KCl (black squares). There is no correction for errors due to LJP or $R_s$. 
Fig. 5.7. Effect of external TEA (14 mM) on IP$_3$ response. Current and 
$[\text{Ca}^{2+}]_i$, transient evoked in a hippocampal pyramidal neurone at $-70$ mV 
voltage clamp by release of 102 $\mu$M IP$_3$, by 1 ms pulse at time indicated 
by the arrow, before (black trace) and after (red trace) the application of 
14 mM TEA. Internal solution: Cs gluconate, fura-2FF (500 $\mu$M). Zero 
current indicated.
In fig. 5.5, the calcium signal is reduced after the third flash (-30 mV holding potential) in low potassium (Cs gluconate) internal solution. This may be due to impaired calcium release from stores. There is a K⁺/Ca²⁺ ion-exchange mechanism on a polyanionic matrix within the lumen of stores and two calcium-sensitive channels, one to import potassium into stores and another to release calcium into the cytosol (Nguyen et al., 1998). If the potassium concentration is low, such as in the caesium internal experiment, the ion-exchange process could be inhibited. Calcium would not be released from the matrix and free calcium in the store lumen would soon run out.

The pharmacology of the conductance was examined by applying TEA at 14 mM externally, which blocked the conductance increase without affecting the [Ca²⁺] change, as shown by the data in fig. 5.7 and in two other similar experiments. Also, the conductance was sensitive to apamin (100 nM) in two out of five experiments (data not shown).

The results are consistent with a mechanism in which IP₃ releases calcium ions from intracellular stores, which in turn activate potassium channels from the cytosolic side. The precise type of potassium channel has not been identified by the pharmacological investigation here, but shows no voltage dependence as expected for SKCa, but not BKCa types, and is sensitive to apamin and blocked by TEA at moderate concentration. Further characterisation of these channels, with more selective blockers, such as bis-quinolinyl and bis-quinolinium compounds based on dequalinium (Malik-Hall et al., 2000) is required to relate them to the components of the AHP.
Characteristics of the IP$_3$ response in CA1 pyramidal neurones

The proportion of cells responding and range of IP$_3$ concentrations effective were investigated. 43 neurones were tested and of these 58% responded. IP$_3$ could be released at concentrations up to 102 µM, limited by the stock concentrations of caged IP$_3$ available and attenuation of the flash lamp light in the slice. The smallest concentration found to produce a detectable response was 1 µM IP$_3$. This compares with 9 µM in cerebellar Purkinje neurones (Khodakhah & Ogden, 1993), 0.1 - 0.2 µM in vascular endothelium (Carter & Ogden, 1997) and guinea pig hepatocytes (Ogden et al., 1990) in the same conditions. Thus, CA1 pyramidal cells are more sensitive to IP$_3$ than Purkinje neurones, but both are much less sensitive than peripheral tissues.

The peak free calcium concentration depends on the rate of calcium flux from stores into the cytosol, the rate of calcium removal from the cytosol, the extent of calcium buffering and the duration of the flux. In pyramidal cells, the peak [Ca$^{2+}$]$_i$ increased with increasing IP$_3$ concentration but a large variability was found between individual neurones, shown by the variability of individual cells seen in fig. 5.8A. The range of peak [Ca$^{2+}$]$_i$ was 3.32 to 31.1 µM.

Normalisation of the peak [Ca$^{2+}$]$_i$ change to that at 51 µM IP$_3$ showed that peak [Ca$^{2+}$]$_i$ increased in the range 2 - 50 µM IP$_3$ and did not increase further at higher concentration fig. 5.8B. To avoid effects of cage depletion, the order of high and low concentrations released was varied from cell to cell.

Similarly, the peak conductance measured as the inward current at -70 mV, shown in fig. 5.9A, was evoked at IP$_3$ concentrations greater than 1 µM, and showed similar cell to cell variation as peak [Ca$^{2+}$]$_i$. On normalisation to 51 µM IP$_3$, shown in fig. 5.9B, reached a maximal level at approximately 51 µM IP$_3$. The 50% effective concentration was about 10 - 15 µM IP$_3$ for both peak [Ca$^{2+}$]$_i$ and current.
Fig. 5.8. Plot of peak [Ca^{2+}] amplitudes (A) and normalised peak [Ca^{2+}] amplitudes (B) with IP₃ concentration. Responses evoked in eight CA1 hippocampal pyramidal neurones at −70 mV voltage clamp by release of 102 μM IP₃, by 1 ms pulse. Internal solution: Kgluconate, fura-2FF (500 μM). In (B), [Ca^{2+}] peak value normalised to response at 51 μM [IP₃]. Different colour symbols represent individual cells.
Fig. 5.9. Plot of peak current amplitudes (A) and means (± standard errors) (B) with IP$_3$ concentration. Responses evoked in six CA1 hippocampal pyramidal neurones at -70 mV voltage clamp by release of 102 μM IP$_3$, by 1 ms pulse. Internal solution: Kgluconate, fura-2FF (500 μM). Different colour symbols in A represent individual cells.
Kinetics of the IP$_3$-evoked [Ca$^{2+}$]$_i$ increase

*Delays in [Ca$^{2+}$]$_i$ and conductance activation*

There are well-defined delays, range 80 to 3230 ms, between the flash and rise of [Ca$^{2+}$], or conductance, discernible despite the flash artefact (which has been minimised, see Methods, Chapter 2). The delays were found to be similar with fura-2FF and fluo-4, indicating that they do not arise from low sensitivity of fura-2FF. There is a large cell-to-cell variation in delays, shown in figs. 5.10A & B, plotted against IP$_3$ concentration. The delay decreased as IP$_3$ concentration increased in each cell to a minimum of 80 ms.

The delay in the conductance increase was found to be closely correlated to the delay before the [Ca$^{2+}$] transient (figs. 5.10 & 5.11). It was to be expected that the [Ca$^{2+}$] transient occurred prior to the conductance activation. However, the perfect correlation line (slope of 1 through the origin) indicated that, for long delays, current activation preceded that of the [Ca$^{2+}$] transient and, for short delays, [Ca$^{2+}$] activation precedes the current. The difference is small but could be because the delay in the [Ca$^{2+}$] transient was difficult to measure, especially at low fluxes (and therefore long delays), due to the noisy record.

Before describing the IP$_3$-evoked calcium flux from stores, it is necessary to consider the rate of calcium extrusion from the cell cytosol. The reason is that calcium flux is used as a measure of response to IP$_3$, representative of the number of open IP$_3$-receptor channels. It is important to know that the rate of extrusion is small relative to the flux.
Fig. 5.10. Plot of delays before $[\text{Ca}^{2+}]_j$, transient and conductance against $[\text{IP}_3]$. Currents and $[\text{Ca}^{2+}]_j$ transients, evoked in three CA1 hippocampal pyramidal neurones (A) and one cell that displayed particularly long delays (B), at -70 mV voltage clamp by release of 102 $\mu$M $\text{IP}_3$, by 1 ms pulse. Internal solution: Kgluconate, fura-2FF (500 $\mu$M).
Fig. 5.11. Correlation between delays in conductance and [Ca\(^{2+}\)] transient. Currents and [Ca\(^{2+}\)] transients evoked in CA1 hippocampal pyramidal neurones at -70 mV voltage clamp by release 102 μM IP\(_3\), by 1 ms pulse. Internal solution: K gluconate, fura-2FF (500 μM). Paired data of the cells are linearly correlated (R=0.95).
Rate of decline of free [Ca$^{2+}$],

The net rate of change of cytosolic free [Ca$^{2+}$], and the peak amplitude both depend on the rate at which calcium ions are removed from the cytosol, by sequestration or calcium pumps and transporters, as well as on rates of release into the cytosol by IP$_3$. Calcium removal is interesting for comparison with the rates of net increase of [Ca$^{2+}$], due to release from stores and can be measured as the maximal rate of decline of free [Ca$^{2+}$] following the peak. The maximum rate of decline occurs shortly after the peak [Ca$^{2+}$], and, because of the approximately exponential decline of [Ca$^{2+}$], back to basal levels, might be expected to depend on the [Ca$^{2+}$], available for sequestration or extrusion. The initial rates of decline (µM s$^{-1}$) are plotted with peak [Ca$^{2+}$], for individual cells in fig. 5.12. The rate of decline ranges from -1 to -8.5 µM s$^{-1}$, small values compared with the rates of rise of [Ca$^{2+}$], following IP$_3$ release reported below, and shows a correlation with peak [Ca$^{2+}$].

Net [Ca$^{2+}$], flux

The rate of change of [Ca$^{2+}$], (δ[Ca$^{2+}$], / δt) provides a measure of the net flux of calcium ions into unit volume of the cytosol, with units of moles per second per litre of cytosol. The flux is modified by calcium ion buffering in the cytosol. For this reason, the total calcium ion flux is underestimated by a factor that may be as large as 200-fold, taking account of fixed calcium buffer measurements in hippocampal pyramidal neurones (the [Ca$^{2+}$]-binding ratio of bound:free calcium is approximately 200; Helmchen et al., 1996).

Extrusion of calcium from the cytosol during the IP$_3$-evoked calcium flux is ignored because, as shown in fig. 5.12, the initial rate of decline of calcium concentration after the peak is small in comparison to the flux; averaging 10 ± 4% (mean ± standard deviation; n=57) of the preceding IP$_3$-evoked rise.
Fig. 5.12. Plot of initial rate of decay against peak $[\text{Ca}^{2+}]_{\text{peak}}$. Responses evoked in seven CA1 hippocampal pyramidal neurones at -70 mV voltage clamp by release of 102 $\mu$M IP$_3$, by 1 ms pulse. Internal solution: Kgluonate, fura-2FF (500 $\mu$M). Different colour symbols represent individual cells.
Fig. 5.13. [Ca^{2+}]_{i} transients in one cell at a high and low IP_{3} concentration, and in a different cell at the high IP_{3} concentration. [Ca^{2+}]_{i} transient evoked in a CA1 hippocampal pyramidal neurone at -70 mV voltage clamp by release of 102 μM IP_{3} (top trace) and 26 μM IP_{3} (middle trace), by 1 ms pulse at times indicated by the arrows. The bottom trace shows the calcium transient after release of 102 μM IP_{3} in a different neurone. Internal solution: Kgluconate, fura-2FF (500 μM). Red horizontal lines indicate 10-90 % rise times.
Fig. 5.14. Plot of peak free $[\text{Ca}^{2+}]$, flux (A) and normalised peak free $[\text{Ca}^{2+}]$, flux (B) against $[\text{IP}_3]$. Data from five CA1 hippocampal pyramidal neurones. Internal solution: K gluconate, fura-2FF (500 μM). In (B), the peak flux is normalised to the response at 51 μM $[\text{IP}_3]$. Different colour symbols represent individual cells.
Fig. 5.13 shows the difference of flux between two cells at the same IP$_3$ concentration (102 μM). Cell 1 displays a much larger flux than Cell 2, likely reflecting a higher density of IP$_3$ receptors. A lower concentration (26 μM) IP$_3$ in Cell 1 produces a smaller flux, due to a smaller number of IP$_3$ channels being activated. Thus, IP$_3$ concentration is not the only factor controlling [Ca$^{2+}$]$_i$ flux into the cytosol; the density of IP$_3$-receptors or other factors are also important.

The dependence of flux on IP$_3$ concentration within each cell, interpreted as an increased open probability of channels by IP$_3$, was maximal at about 50 μM [IP$_3$] or higher (fig. 5.14A). The cell-to-cell variability was reduced by normalisation of [Ca$^{2+}$]$_i$ flux to that at 51 μM IP$_3$ in fig. 5.14B, which shows a saturating dependence on IP$_3$ concentration with 50 % response at 10 – 20 μM IP$_3$.

### Analysis of dependence of calcium flux on IP$_3$ concentration

The IP$_3$ receptor comprises four subunits each with a binding region for IP$_3$. If binding to more than one site is needed for channel opening it might be expected that the slope of the concentration-flux relation will have a steep dependence on concentration. This can be quantified by fitting data with the equation

$$flux = \frac{flux_{max} \times [IP_3]^n}{(K)^n + [IP_3]^n}$$

where $K$ is the apparent equilibrium dissociation constant for each binding site and the Hill coefficient $n$ estimates the number of sites occupied for activation.

The normalised data are plotted in fig. 5.15 and the best fit least squares relation gives $K = 12 \pm 0.9$ μM and $n = 2.6 \pm 0.6$. Thus, in hippocampal pyramidal neurones the dependence of flux on IP$_3$ concentration is characterised by apparent
Fig. 5.15. Plot of means (± standard errors) of normalised [Ca\textsuperscript{2+}]\textsubscript{i} flux against [IP\textsubscript{3}]. Flux data from fig. 5.15 plotted on a log scale, and fitted by least squares with equation

$$flux = \frac{flux_{\text{max}} \times [IP_3]^n}{(K)^n + [IP_3]^n}$$

R=0.99. Hill coefficient (n) = 2.6. Affinity = 12 µM.
affinity 12 µM, lower than found for cerebellar Purkinje neurones (> 40 µM). It is, however, similar to that in midbrain dopamine neurones (10.6 µM; K. Khodakhah, personal communication) but much higher than in autonomic tissues (0.4 µM). A Hill coefficient of 2.6 is consistent with multiple IP$_3$ binding required for activation, but may also result from other mechanisms, for instance a requirement for calcium binding as well as IP$_3$ in activation (Bezprozvanny et al., 1991; Finch et al., 1991).

There have been reports of no or very little cooperativity in IP$_3$ activation of calcium release (Champeil et al., 1989; Finch et al., 1991; Iino & Endo, 1992; and Somlyo et al., 1992). In contrast, several groups have found evidence for cooperativity, with values of $n$ between 3 and 4: (Meyer et al., 1988; 1990; in permeabilised rat basophilic leukaemia cells; Carter & Ogden, 1997, in endothelial cells).

**Influence of voltage-gated calcium influx on IP$_3$-evoked release**

There is evidence that elevated [Ca$^{2+}$]$_i$ inhibits IP$_3$-evoked [Ca$^{2+}$]$_i$ release in Purkinje neurones, guinea-pig hepatocytes and pig aortic endothelial cells (Khodakhah & Ogden, 1995; Ogden & Capiod, 1997; Carter & Ogden, 1997; respectively). In each case, elevation of [Ca$^{2+}$]$_i$ prior to IP$_3$ release produced a large depression of flux from stores.

The inhibitory effect of [Ca$^{2+}$]$_i$ was tested in hippocampal pyramidal cells by raising the [Ca$^{2+}$]$_i$, by depolarisation with a voltage-step prior to photolytic release of IP$_3$. The duration of the depolarising pulse varied from 0.2 to 1.08 s and was delivered between 0.3 and 1.5 s before each flash. A control response to IP$_3$ was obtained subsequently without prior depolarisation. The rates of rise of [Ca$^{2+}$]$_i$ following photolytic release of the same IP$_3$ concentration were compared to assess the effect of calcium influx on IP$_3$-evoked efflux from stores. Fig. 5.16 shows partial inhibition of the [Ca$^{2+}$]$_i$ flux to IP$_3$ following depolarisation.
Fig. 5.16. Calcium inhibition of IP₃-evoked responses; a longer depolarisation produces complete inhibition of the response. Currents and [Ca^2+] transients evoked in CA1 hippocampal pyramidal neurones at -70 mV voltage clamp by 102 μM IP₃, by 1 ms pulse. Internal solution: Kgluconate, fura-2FF (500 μM). A. Partial inhibition. A depolarisation (duration 0.205 seconds) produced a conductance change (top trace) and a [Ca^2+] change (middle trace). The release of IP₃ at the arrow produced a small increase in [Ca^2+] compared to the control (bottom trace). B. Complete inhibition. A depolarisation (duration 1.08 seconds) produced a conductance change (top trace) and [Ca^2+] concentration change (middle trace). The release of IP₃ at the arrow produced no increase in [Ca^2+] compared to the control (bottom trace). Zero current indicated.
Fig. 5.17. Calcium inhibition of IP_3-evoked responses; a longer depolarisation and increased external [Ca^{2+}], produces complete inhibition of the response. Currents and [Ca^{2+}], transients evoked in CA1 hippocampal pyramidal neurones at -70 mV voltage clamp by 102 μM IP_3, by 1 ms pulse. Internal solution: Cs gluconate, fura-2FF (500 μM). A. Partial inhibition. A depolarisation (duration 0.200 seconds) produced a conductance change (top trace) and a calcium concentration change (middle trace). The release of IP_3 at the arrow produced a small increase in [Ca^{2+}], compared to the control (bottom trace). B. Complete inhibition. The [Ca^{2+}] external was raised from 2 to 5 mM. A depolarisation (duration 0.383 seconds) produced a conductance change (top trace) and [Ca^{2+}], change (middle trace). The release of IP_3 at the arrow produced no increase in [Ca^{2+}], compared to the control (bottom trace). Zero current indicated.
A longer depolarisation, resulting in more calcium influx into the cell, completely inhibited the response. A similar effect was caused by raising the external calcium concentration before depolarisation (fig. 5.17). In the latter experiment, caesium gluconate was used as the internal solution, potassium currents were blocked and the calcium currents are visible (upper traces).

The IP$_3$-evoked fluxes, at high [Ca$^{2+}$]$_i$, were normalised to the control response in each case. These results indicated that intracellular free calcium concentration as low as 2 μM could completely inhibit the response. Out of seventeen experiments, there were nine cases of total inhibition by calcium. In seven experiments, there was more than 60 % block and, in one experiment, there was just 20 % block. No potentiation of the IP$_3$ response by a prior rise in calcium was observed.

The results show complete inhibition of IP$_3$-evoked calcium release from stores by prior elevation of [Ca$^{2+}$]$_i$ by calcium influx through surface membrane calcium channels. The result agrees with the calcium inhibition demonstrated in Purkinje neurones (Khodakhah & Ogden, 1995) in autonomic tissues (Iino, 1990; Ogden et al., 1990; Carter & Ogden, 1997; Capiod & Ogden, 1997) in oocytes (Parker & Ivorra, 1990) and reconstituted IP$_3$ receptor systems (Bezprozvanny et al., 1991; Finch et al., 1991). As in Purkinje neurones, hepatocytes and endothelial cells with photorelease of IP$_3$, no facilitation of flux was seen even with small prior increases of [Ca$^{2+}$]$_i$ produced by membrane depolarisation. Thus, it is predicted that the physiological effects of strong membrane calcium influx during excitation would be to suppress IP$_3$-evoked calcium release.

Termination of the IP$_3$-evoked calcium flux

Several processes may be responsible for terminating the period of rapid IP$_3$-evoked calcium flux into the cytosol. The transient nature of the response could be due to desensitisation of IP$_3$-receptors, transient production of IP$_3$, calcium store
depletion, metabolism of IP$_3$, negative feedback by calcium or a combination of more than one of these factors.

**Desensitisation:** IP$_3$-receptors have been shown not to desensitise. When all [Ca$^{2+}$]$_i$ was buffered in the cytosol with fura-2, the response did not inactivate (Ogden & Capiod, 1997; Carter & Ogden, 1991). In addition, experiments have been carried out to show that $^{45}$Ca$^{2+}$ efflux was the same from both pre-stimulated and control cells, further ruling out an effect of desensitisation (Taylor & Potter, 1990).

**Metabolism of IP$_3$:** The rapid metabolism of IP$_3$ is not responsible, as demonstrated by experiments with caged thio-IP$_3$ in guinea-pig hepatocytes (Wootton et al., 1995) and stable IP$_3$ analogues (Muallem et al., 1989; Taylor & Potter, 1990). In addition, double-flash experiments with caged IP$_3$ were carried out here (two experiments). A second flash took place while the response to a first flash was declining (flashes were approximately one second apart). If the metabolism of IP$_3$ was the cause of response termination, then the two responses should have been equal or additive. They were not ([Ca$^{2+}$]$_i$ signals were 19.3 μM and 4.7 μM after the first flashes and zero after the second flashes), indicating that the raised [Ca$^{2+}$]$_i$ from the first response was having an inhibitory effect on the second IP$_3$ response.

**Effect of luminal [Ca$^{2+}$]:** It has been suggested that the intra-luminal [Ca$^{2+}$] may have some effect (Horne & Meyer, 1995). The release of $^{45}$Ca$^{2+}$ from permeabilised rat basophilic leukaemia cells was measured and it was found that, at low cytosolic [Ca$^{2+}$], IP$_3$-mediated release was proportional to the [Ca$^{2+}$] in the lumen. At high BAPTA concentrations and zero cytosolic [Ca$^{2+}$], IP$_3$-mediated release was inhibited. These results suggest that, if luminal [Ca$^{2+}$] is high, calcium leaks out of the lumen and ‘primes’ the IP$_3$-receptors, so that they are more sensitive to IP$_3$.

**All-or-none activation of calcium release:** There have been hypotheses put forward to suggest that the activation of calcium release from stores is luminal
Each releasable compartment could have its own threshold for [IP$_3$], at which it liberates all of its releasable load. Different compartments could have different sensitivities to IP$_3$, perhaps due to heterogeneity in the phosphorylation of IP$_3$-receptors or factors such as the protein ‘calmodulin’, which confers calcium-sensitivity to the IP$_3$-receptor (Danoff et al., 1988).

**Calcium inhibition:** Comparisons of the time course of Ca$^{2+}$ flux in response to cytosolic photorelease of IP$_3$ within and between different tissues have shown that the time taken to reach the peak [Ca$^{2+}$]$_i$ is generally shorter when the flux into the cytosol, measured as the rate of change of [Ca$^{2+}$]$_i$, is large. The records from CA1 pyramidal neurones shown in fig. 5.13 illustrate that data at different IP$_3$ concentrations in the same cell or the same IP$_3$ concentration in different cells appear to have this relation. This indicates that there is a process that terminates [Ca$^{2+}$]$_i$ flux in a way that depends on the magnitude of flux.

The process can be quantified by considering a ‘rate of termination’; this can be measured as the reciprocal of the 10-90% rise time. The flux, measured by rate of change of [Ca$^{2+}$]$_i$, varies widely from cell to cell in the same tissue at the same IP$_3$ concentration. It also varies with the IP$_3$ concentration, and varies between tissues. However, a comparison of the dependence of reciprocal rise time on rate of change of [Ca$^{2+}$]$_i$ showed a consistent, close to linear, relation over a wide range in three different tissues (Capiod & Ogden, 1997; Carter & Ogden, 1996). This relation was tested in CA1 neurones by plotting 1/(10-90% rise time) with δ[Ca$^{2+}$]/δ$t$ as shown in fig. 5.18.

The mechanism underlying the termination of IP$_3$-evoked efflux from stores may be related to the inhibition by high [Ca$^{2+}$]$_i$, shown here and reported elsewhere as discussed above. On a sub-micron spatial scale calcium ions accumulate adjacent to open IP$_3$ gated channels, reaching steady-state levels in a millisecond and proportional to the flux through open channels (Bezprozvanny & Ehrlich, 1994). This high local [Ca$^{2+}$]$_i$ may inhibit adjacent closed channels from further activation.
Fig. 5.18. The rate of termination of the [Ca^{2+}]_i transient is correlated with [Ca^{2+}]_i flux. Currents and [Ca^{2+}]_i transients evoked in CA1 hippocampal pyramidal neurones at -70 mV voltage clamp by release of 102 μM IP_3, by 1 ms pulse. Internal solution: Kgluconate, fura-2FF (500 μM). The rate of termination was represented by the reciprocal of 10-90 % of the rise time and was found to be roughly linearly correlated to [Ca^{2+}]_i flux (shown on a double-log-plot). R=0.42. Slope = 0.31±0.13.
Fig. 5.19. Rate of termination of \([\text{Ca}^{2+}]\) flux by calcium is reduced at high \([\text{IP}_3]\) concentration. Data from four neurones (different colour symbols). \([\text{Ca}^{2+}]\) signals evoked at -70 mV voltage clamp by 102 \(\mu\text{M}\) \([\text{IP}_3]\). Internal solution: Kgluconate, fura-2FF (500 \(\mu\text{M}\)). The rate of termination was represented by the reciprocal of 10-90 % of the rise time and was normalised to the calcium flux, thereby signifying the extent of inhibition by calcium. This was plotted against \([\text{IP}_3]\), to give an indication of the effect of \([\text{IP}_3]\) on calcium inhibition. As \([\text{IP}_3]\) increased, calcium became less effective at inhibiting the response, suggesting that there is some interaction between calcium and \([\text{IP}_3]\) at binding sites on the \([\text{IP}_3]\)-receptor.
Evidence favouring this mechanism in whole cell recording is the
demonstration of inhibition by prior elevation of [Ca$^{2+}$]. Also, calcium chelators
such as fura-2 at mM concentrations, which clamp [Ca$^{2+}$]$_i$ at resting levels, have
been shown to substantially prolong the IP$_3$ evoked flux. Furthermore, there is a
similarity in the rate of termination as a function of flux in different tissues with
widely differing calcium buffering capacity, indicating that the free [Ca$^{2+}$]$_i$ is the
important parameter (Ogden & Capiod, 1997; Carter & Ogden, 1991).

The calcium inhibition has been shown for the subtype 1 IP$_3$ receptor, however
there is evidence that the subtype 3 receptor stays open in the presence of
increasing [Ca$^{2+}$]$_i$ (Hagar et al., 1998). In this case store capacity for calcium ions
may be the terminating factor, and may contribute also to termination with the
subtype 1 receptor present in hippocampal neurones.

It has been shown for subtype 1 receptor that the calcium inhibition is less at
high than at low IP$_3$ concentrations. This was tested here by plotting the
dependence of rate of termination per unit flux, numerically $1/(\text{rise time} \times \text{flux})$,
with the IP$_3$ concentration, as shown in fig. 5.19. The data for four cells indicate
that at high IP$_3$ concentration the rate of termination is reduced at each level of
flux.

Summary

The proportion of CA1 neurones responding to IP$_3$ was 58% in apparently
good recordings. The sensitivity was 1 - 2 μM and the affinity $K = 12 \mu M$. After a
delay, there was a brief period of high [Ca$^{2+}$]$_i$ flux into the cytosol. This was
inhibited, but apparently not potentiated, by elevated [Ca$^{2+}$]$_i$. Calcium appears to
activate a potassium conductance, possibly due to small-conductance potassium
channels. These act to inhibit neuronal excitability, by polarising the membrane
towards $E_K$ and opposing excitation via fast transmission.
Chapter 6 - Changes of $[\text{Ca}^{2+}]_i$ evoked by mGluR activation
Introduction

The calcium-activated potassium conductance elicited by IP$_3$ in CA1 neurones appears to be similar to the late component of the mGluR response, which was shown to be due to a potassium conductance. The question to be answered is whether the mGluR potassium conductance is calcium-activated, indicating, if so, that it may be mediated by the IP$_3$-pathway. The IP$_3$-induced increase of [Ca$^{2+}$], has been studied and now needs to be compared to that evoked by mGluR activation following the release of glutamate.

In recombinant systems the Group I mGluR, shown here to mediate the late component of mGluR current in CA1 pyramidal neurones, acts via G-proteins to activate phospholipase C, producing IP$_3$ which in turn releases calcium from intracellular stores. The possibility that this activates the potassium conductance of the late component was tested by making measurements of the [Ca$^{2+}$], simultaneously with the mGluR current.

L-glutamate (47.5 μM) was released from MNI L-glutamate, on CA1 pyramidal cells in the slice. The ionotropic response was blocked with antagonists (100 μM NBQX, 200 μM CPP), leaving the two components of the mGluR response, and in some cells a residual ionotropic response. Experiments were done in the presence of tetrodotoxin (TTX) to block excitability. In contrast to experiments with caged glutamate in Chapter 4, no cadmium was applied to the external solution. This was because cadmium is know to bind to fura-2FF and modify its fluorescence (Molecular Probes, 2001).

The [Ca$^{2+}$], was recorded with the low affinity calcium indicator fura-2FF (see Methods, Chapter 2). Data of simultaneous membrane current and [Ca$^{2+}$], recorded at different potentials following release of 38 μM L-glutamate, are shown in fig. 6.1.

It can be seen in fig. 6.1 that there is a small, almost undetectable rise of [Ca$^{2+}$],
Fig. 6.1. Simultaneous recording of mGluR-mediated current and $[\text{Ca}^{2+}]_i$. mGluR-mediated currents (left panel) and $[\text{Ca}^{2+}]_i$ transients (right panel) evoked in a CA1 hippocampal pyramidal neurone at -90, -70, -50 and -20 mV voltage clamp by release of 47.5 μM L-glutamate, by 1 ms pulse at time indicated by the arrow. Internal solution: Kgluconate, fura-2FF (500 μM). External solution: 100 μM NBQX, 200 μM CPP, 200 nM TTX and zero Mg$^{2+}$. There is a residual ionotropic response. Zero current indicated. Caged compound is MNI L-glutamate.
during the residual ionotropic current at -50 to -90 mV. This may be an artefact and due to bleaching of the indicator by the flash, or it may result from calcium influx in residual NMDA-receptor channel current. There is no apparent increase associated in time with the early mGluR component at -50 mV and -70 mV, indicating that calcium influx in the early conductance is not large enough to be detected. Finally, there is a large rise of $[Ca^{2+}]_i$ coincident with the late component, whether outwardly (-20, -50 mV) or inwardly directed (-70, -90 mV). The analysis of the origin and role of this $[Ca^{2+}]_i$ change is given below.

The delayed rise and association with a potassium current indicate that the late $[Ca^{2+}]_i$ increase may be second messenger mediated and due to calcium release from internal stores. This was examined pharmacologically by use of blockers of potassium conductance and mGluR1 antagonists, and by comparing the properties of the $[Ca^{2+}]_i$ increase with those discussed in Chapter 5 due to photolytic IP3 release.

The records in fig. 6.2 show the effects of the potassium channel blocker TEA at 14 mM on the $[Ca^{2+}]_i$ change and the membrane current at -20 mV. The records due to release of 47.5 μM L-glutamate in 14 mM TEA (one experiment) had current reduced to 30 % of the amplitude of control but similar change of $[Ca^{2+}]_i$. The reversibility of the TEA block was not tested.

In one preliminary experiment, ibersotoxin, an inhibitor of large-conductance calcium activated potassium channels, did not block the potassium current. This is consistent with the channels being small-conductance calcium-activated potassium channels, as indicated by results described in Chapters 4 and 5.

The mGluR subtype underlying the response was tested with the selective Group I antagonist CpCCOEt at 20 μM. As seen in fig. 6.3, both the $[Ca^{2+}]_i$ change and the inward current at -50 mV were blocked by the Group I antagonist (three out of four experiments), in this case after a very long delay of 4.5 s. These results suggest that the likely mechanism is activation of calcium release from internal stores due to mGluR1
Fig. 6.2. TEA inhibits late mGluR current, but not the [Ca^{2+}] transient. mGluR-mediated conductance and [Ca^{2+}] transient evoked in a CA1 hippocampal pyramidal neurone at -20 mV voltage clamp by release of 47.5 μM L-glutamate, by 1 ms pulse at time indicated by the arrow. Internal solution: Kgluconate, fura-2FF (500 μM). External solution: 100 μM NBQX, 200 μM CPP, 200 nM TTX and zero Mg^{2+}. Black trace – control. Red trace – 14 mM TEA in the external solution. Standing current of +33 pA is indicated. Caged compound is MNI L-glutamate. [Ca^{2+}] trace is filtered at 25 Hz and sampled 1:10.
Fig. 6.3. mGluR1 antagonist CpCCOEt inhibits the mGluR-elicited conductance and $[\text{Ca}^{2+}]_i$ transient. mGluR-mediated conductance and $[\text{Ca}^{2+}]_i$ transient evoked in a CA1 hippocampal pyramidal neurone at -50 mV voltage clamp by release of 47.5 μM L-glutamate, by 1 ms pulse at time indicated by the arrow. Internal solution: Kgluconate, fura-2FF (500 μM). External solution: 100 μM NBQX, 200 μM CPP, 200 nM TTX and zero Mg²⁺. Black trace – control. Red trace – 20 μM CpCCOEt in the external solution. Standing current of -122 pA is indicated. Caged compound is MNI L-glutamate. $[\text{Ca}^{2+}]_i$ trace is filtered at 25 Hz and sampled 1:10.
and consequent activation of a calcium-activated potassium conductance. Both current
components and the \([\text{Ca}^{2+}]_i\) signal were blocked with 1 mM MCPG, a non-selective
mGluR antagonist (one experiment).

**Kinetics of the \([\text{Ca}^{2+}]_i\) transient**

To obtain further information about the mechanism of the mGluR1 response
seen here, the kinetics of the \([\text{Ca}^{2+}]_i\) change and conductance change were analysed
for comparison with each other and with the corresponding responses following
photorelease of IP$_3$ reported in Chapter 5. The response was analysed as three
components, a delay before detectable changes of \([\text{Ca}^{2+}]_i\), or potassium conductance
following glutamate release, the subsequent net flux of calcium into the cytosol and
the dependence of potassium current on \([\text{Ca}^{2+}]_i\), and finally the rate of decline of
\([\text{Ca}^{2+}]_i\), after the peak. The \([\text{Ca}^{2+}]_i\) peak varied from 4.39 to 44 \(\mu\text{M}\).

**Delays in \([\text{Ca}^{2+}]_i\), and conductance activation**

The delay between glutamate release (in each case 38 \(\mu\text{M}\) at -70 mV clamp
potential) and activation of the \([\text{Ca}^{2+}]_i\), or conductance increase was measured from
the flash to the first detectable change. There was a large variation in the delay
before a detectable \([\text{Ca}^{2+}]_i\) increase, from 434 to 7481 ms with mean 1260 ± 1450
ms (mean ± standard deviation; \(n=23\)). In the same experiments, the delays before
the conductance increase ranged from 140 to 6640 ms with mean 988 ± 1570 ms,
shorter than the delay in \([\text{Ca}^{2+}]_i\). A plot of the paired data of \([\text{Ca}^{2+}]_i\) delay and
conductance is shown in fig. 6.4. A linear correlation fitted to the paired data shows a
good correlation but with an intercept on the \([\text{Ca}^{2+}]_i\), axis of 272 ms, indicating that the
conductance rose before the \([\text{Ca}^{2+}]_i\).
Fig. 6.4. Comparison of delay in the late potassium current with the delay in the \([\text{Ca}^{2+}]\), transient. mGluR-mediated currents and \([\text{Ca}^{2+}]\), transients evoked in a CA1 hippocampal pyramidal neurone at -70 mV voltage clamp by release of 47.5 \(\mu\text{M}\) L-glutamate, by 1 ms pulse. Internal solution: Kgluconate, fura-2FF (500 \(\mu\text{M}\)). External solution: 100 \(\mu\text{M}\) NBQX, 200 \(\mu\text{M}\) CPP, 200 nM TTX and zero Mg\(^{2+}\). The delays were plotted against each other and are linearly correlated (R=0.99), with the \([\text{Ca}^{2+}]\), transient activating after the conductance (intercept on the \([\text{Ca}^{2+}]\), axis = 272 ms). Caged compound is MNI L-glutamate.
Comparison of the delays in mGluR $[\text{Ca}^{2+}]_i$ response with the IP$_3$-evoked responses shows first that the mean delays are longer for mGluR activation; 1260 ms in contrast to 988 ms for IP$_3$. This is likely to result from a greater number of reaction steps in calcium release via the mGluR1 pathway than with IP$_3$.

*Rate of decline of free $[\text{Ca}^{2+}]_i$*

The initial rate of decay of the calcium response showed a rough correlation with peak (figs. 6.5). This is consistent with the idea that calcium concentration in the cytosol increases, so does the driving force for calcium re-uptake into stores, therefore there is a higher rate and lower duration of uptake.

*Net $[\text{Ca}^{2+}]_i$ flux*

Fluxes ranged between 1.15 and 60.4 μM per second. Extrusion of calcium from the cytosol during the calcium flux is small in comparison; averaging 13 ± 8 % (mean ± standard deviation; n=36) of the preceding $[\text{Ca}^{2+}]_i$ rise. The values of flux were expected to be different to those obtained from the release of IP$_3$ because, as previously shown (fig. 5.14), flux depends on IP$_3$ concentration, which was unknown and probably different when glutamate was released extracellularly. In addition, the IP$_3$ concentration is probably localised when it is synthesised in the cell, and so local IP$_3$ concentration will vary after the release of glutamate, as opposed to a homogenous [IP$_3$] that occurs after the release of IP$_3$ from the caged compound.
Fig. 6.5. The initial rate of decay of [Ca$^{2+}$]$_i$ transient depends on the [Ca$^{2+}$]$_{peak}$ peak. mGluR-mediated currents and [Ca$^{2+}$]$_i$ transients evoked in a hippocampal pyramidal neurone at -70 mV voltage clamp by release of 47.5 µM L-glutamate, by 1 ms pulse. Internal solution: Kgluconate, fura-2FF (500 µM). External solution: 100 µM NBQX, 200 µM CPP, 200 nM TTX and zero Mg$^{2+}$. The initial rate of decay of the [Ca$^{2+}$]$_i$ transient was measured and plotted against the amplitude of the peak [Ca$^{2+}$]$_i$. R= 0.69. Caged compound is MNI L-glutamate.
The termination of calcium release correlates with flux

The rate of termination is related to flux (fig. 5.18) and so values varied accordingly. It was interesting to determine whether or not there was a similar relationship between rate of termination and flux (i.e. the same slope) during responses to release of IP$_3$ and glutamate. The same relationship would have suggested that the same process was occurring in each case, and that the local [Ca$^{2+}$]$_i$ was inhibiting the calcium flux through IP$_3$ channels.

If termination of calcium flux was produced by calcium binding to and inactivating channels, then the rate of termination should be linearly correlated to the local calcium concentration and hence to the flux produced locally by open channels. The rate of termination was represented by the reciprocal of the 10 - 90% rise time of each calcium response (range 159 to 2460 ms), and was found to have a roughly linear relationship with flux.

The relationship between rate of termination and flux for release of IP$_3$ and glutamate was plotted on the same double-log plot (fig. 6.6). The two parameters were found to be related by the same factor (slope = 0.1), indicating that the same process of calcium inhibition at the IP$_3$-receptors was occurring in each case. The values are generally smaller in the case of glutamate release, because the range of [IP$_3$] values were different, hence flux values were different.
Fig. 6.6. Plot of the rate of termination of the $[Ca^{2+}]$, transient against $[Ca^{2+}]$, flux for mGluR-elicited and IP$_3$-elicited $[Ca^{2+}]$, signals. mGluR- and IP$_3$-mediated currents and $[Ca^{2+}]$, transients evoked in hippocampal pyramidal neurones at -70 mV voltage clamp by release of 47.5 μM L-glutamate or 102 μM IP$_3$, respectively, by 1 ms pulse. Internal solution: Kgluconate, fura-2FF (500 μM). External solution (glutamate experiments): 100 μM NBQX, 200 μM CPP, 200 nM TTX and zero Mg$^{2+}$; caged compound is MNI L-glutamate. Rate of termination is represented by the reciprocal of 10-90 % of the rise time and is roughly linearly correlated with $[Ca^{2+}]$, flux with a slope of 0.1 (shown on a double-log-plot).
Summary

There is a $[\text{Ca}^{2+}]_{\text{i}}$ signal that corresponds with the late component of the mGluR response. The $[\text{Ca}^{2+}]_{\text{i}}$ transient activates after the early component, but before the late component, and the delays before $[\text{Ca}^{2+}]_{\text{i}}$ signal and late conductance are linearly correlated. This indicates that calcium release is associated with the late mGluR response, which is likely to be a calcium-activated potassium current.

Consistent with this hypothesis, while the late current is blocked by TEA, the $[\text{Ca}^{2+}]_{\text{i}}$ signal is not. Both the current and $[\text{Ca}^{2+}]_{\text{i}}$ transient are blocked by a mGluR Group I selective antagonist.

The study of the kinetics of the $[\text{Ca}^{2+}]_{\text{i}}$ signal associated with the late mGluR component reveals that the termination of the response has the same relationship to the $[\text{Ca}^{2+}]_{\text{i}}$ flux as IP$_3$-evoked $[\text{Ca}^{2+}]_{\text{i}}$ signals. This evidence, together with pharmacological experiments, suggests that that the late component of the mGluR response a calcium-activated potassium current, resulting from activation of Group I mGluRs and mediated by the IP$_3$ intracellular pathway.
Chapter 7 - Spatial imaging of $[\text{Ca}^{2+}]_i$ during the mGluR or IP$_3$ response
Introduction

Information about the spatial distribution of $[Ca^{2+}]$ during the response to mGluR activation or photolytic release of IP$_3$ was obtained by imaging with a cooled CCD camera. The high affinity calcium indicator fluo-4 was used, so that fluorescence changes due to calcium would be large and therefore easily observed. However, due to the limitations of high affinity indicators that were discussed earlier, the kinetics obtained from these experiments were distorted by indicator saturation, but the spatial information was of interest.

A hippocampal slice was perfused in the microscope chamber, as described previously, and a pyramidal cell in CA1 patch clamped. The external solution in these experiments was different in composition, with 3 mM potassium instead of 4.7. This small change shifted the potassium equilibrium potential so that the potassium current response to IP$_3$ and late component of the mGluR response were in the outward direction at a holding potential of -70 mV. When glutamate was released, the late current could therefore be distinguished from the early current.

The experiments with caged IP$_3$ used external solution that was buffered with carbonate, containing (in mM): 126 NaCl; 3 KCl; 2.5 CaCl$_2$; 1.2 MgSO$_4$; 15 glucose; 25 NaHCO$_3$ (pH 7.4; osmolality 300 milliosmolal), and continuously bubbled with 95% O$_2$; 5% CO$_2$. The external solution for the glutamate experiments was buffered with HEPES and contained (in mM): 135 NaCl; 3 KCl; 2.5 CaCl$_2$; 1.2 MgSO$_4$; 25 glucose; 10 HEPES; 2.5 NaHCO$_3$ (pH 7.4 adjusted with NaOH; osmolality 300 milliosmolal), bubbled with O$_2$.

Fluorescein filters with excitation at 480 nm (Polychrome II monochromator, Till Photonics) and emission at >530 nm were used. Images were acquired at a rate of 26 frames per second, by a Hamamatsu C4880-82 camera. Images were analysed using routines developed from Matlab 5.1 Image processing Toolbox.
For each image pixel, fluorescence signals were computed as ratios:

$$\frac{\Delta F(t)}{F(0)} = \frac{F(t) - F(0)}{F(0)}$$

In this expression, \(F(t)\) is fluorescence time \(t\) at following a stimulus that causes calcium elevation within the cell and \(F(0)\) is pre-stimulus fluorescence computed by averaging 4 images. Ratio magnitude was encoded by 8-bit pseudo-colour look-up tables to produce false-colour images. Regions of interest (ROI) were assigned to different regions of the neurone and the \(\Delta F/F(0)\) displayed as time records.

**Spatial imaging of \([Ca^{2+}]_i\)**

Fig. 7.1 shows the response to 34 \(\mu M\) IP\(_3\). The cell, including soma and large part of the apical dendrite, is displayed in the uppermost panel. The coloured squares mark areas (ROI) that have their corresponding fluorescence values represented in the top traces. The bottom trace shows the whole-cell current at \(-70\) mV, which interestingly exhibits two peaks, unusual for an IP\(_3\) response. The black triangles above the current trace mark time points at which the images in the bottom panel were taken. The \(\Delta F/F(0)\) due to \([Ca^{2+}]_i\) arose first in one end of the dendrite and migrated into the soma as a function of time. \(\Delta F/F(0)\) in the dendrite preceded the outward current.

The mGluR spatial \([Ca^{2+}]_i\) response is shown in fig. 7.2. Unfortunately, the \(\Delta F/F(0)\) and CCD was saturated in the soma of this cell, so the dendrite only is
Fig. 7.1. Spatial [Ca$^{2+}$] response to release of IP$_3$. A hippocampal pyramidal neurone was patch clamped; current and [Ca$^{2+}$] responses to release of 34 $\mu$M IP$_3$ were recorded (calcium indicator was 500 $\mu$M fluo-4). (Top panel) Neurone filled with indicator, showing soma and part of apical dendrite. Coloured squares mark regions of interest (ROI) that have their average fluorescences measured in the trace below. (Top trace) Fluorescence measurements in units of $\Delta F/F$ for regions from image above. (Bottom trace) Conductance change in response to IP$_3$ release on the same time scale as the fluorescence trace. Black triangles mark the points at which the images in the bottom panel were taken. Standing current is -360 pA. (Bottom panel) Images taken at times times indicated by the black triangles. Ratio magnitude is encoded by 8-bit pseudo-colour look-up tables to produce false-colour images with the scale -10 to +30 % as shown.
Fig. 7.2. Spatial \([\text{Ca}^{2+}]\) response to release of glutamate. A hippocampal pyramidal neurone was patch clamped; current and \([\text{Ca}^{2+}]\) responses to release of 46 \(\mu\text{M}\) glutamate were recorded (calcium indicator was 500 \(\mu\text{M}\) fluo-4). (Top panel) Neurone filled with indicator, showing part of apical dendrite only. Coloured squares mark regions of interest (ROI) that have their average fluorescences measured in the trace below. (Top trace) Fluorescence measurements in units of \(\Delta F/F\) for regions from image above. (Bottom trace) Conductance change in response to glutamate release on the same time scale as the fluorescence trace. Black triangles mark the points at which the images in the bottom panel were taken. Standing current is -276 pA. (Bottom panel) Images taken at times indicated by the black triangles. Ratio magnitude is encoded by 8-bit pseudo-colour look-up tables to produce false-colour images with the scale -20 to +60 \% as shown.
shown. $\Delta F/F(0)$ and $[Ca^{2+}]$ appeared to rise uniformly throughout the dendrite. $\Delta F/F(0)$ in the dendrite preceded the outward current, indicating that the late potassium current was activated by the rise in $[Ca^{2+}]_i$.

**Summary**

The $[Ca^{2+}]_i$ signal can be seen to arise in the dendrite before the soma in the response to IP$_3$. This may reflect differential distribution of IP$_3$-receptors. Alternatively, the surface (and therefore $[Ca^{2+}]_i$ flux) to volume ratio of the dendrite versus the soma may have an effect on the apparent magnitude of the signal. During the mGluR response, the $[Ca^{2+}]_i$ signal appeared to rise uniformly within the dendrite.
Chapter 8 – Discussion
**Introduction**

Although metabotropic glutamate receptors (mGluRs) have been shown to play a role in the regulation of neuronal excitability, the precise signalling mechanisms still require elucidation. mGluR-activated non-selective cation channels have been shown to increase excitability, but there is some dispute over whether channel activation is mediated by calcium, G-proteins or by protein tyrosine kinases. The inhibitory role of mGluRs is due to the activation of potassium channels, of which there are several types.

The mGluRs are thought to play an important role in the modification of synaptic strength (review by Anwyl, 1999). The mGluR-induced IP$_3$ intracellular signalling cascade has been shown to interact with backpropagating action potentials in hippocampal pyramidal cells, producing supralinear [Ca$^{2+}$]$_i$ signals (Nakamura et al., 1999; 2000). This pathway may have additional roles in central neurones (Ogden, 1996), including the regulation of cellular actin organisation, intracellular membrane fusion, formation of ER cisternal stacks and the secretion of neurotrophins, which in turn may regulate neuronal excitability.

The aim of this project was to investigate the role of mGluRs in CA1 pyramidal cells by identifying and characterising the currents and intracellular mechanisms that result from their activation. There are different subtypes of mGluR, which are known to act via distinct intracellular signalling cascades. The mGluR subtypes in the hippocampus are likely to be linked to more than one intracellular pathway, culminating in several possible ion conductances. In this project, two conductances were identified. One of these, a putative IP$_3$-mediated calcium-activated potassium conductance, was investigated further.
The technique of flash photolysis was used to deliver glutamate extracellularly to CA1 pyramidal cells in slices, and IP3 within the cytosol. Flash photolysis eliminated the diffusion barrier by releasing the neurotransmitter or messenger immediately at the site of action, with virtually no delay, and bypassed presynaptic processes. 7-Nitroindolinyl (NI) and 4-methoxy-7-nitroindolinyl (MNI) caged L-glutamates were first evaluated before use in experiments to investigate mGluR signalling in CA1 neurones.

**Characterisation of the caged L-glutamates**

The caged L-glutamate compounds were characterised for their efficiency of release and antagonistic properties. NI- and MNI-caged glutamates are thermally stable, fast and efficient sources of the free ligands. Photorelease from MNI-caged L-glutamate was approximately 2.5 times more efficient than NI-caged L-glutamate and was comparable to the release efficiency of NPE-ATP. NPE-caged phosphates, such as NPE-ATP, are currently among the most efficiently photolysed caged compounds in common use. The NI- and MNI-L-glutamates themselves were found to be pharmacologically inert in synaptic systems, proving to be useful tools in the investigation of synaptic processes.

Neurotransmitters were released from the nitroindolinyl cages with half times in the sub-μs (200 ns) time domain (P. Wan, J. Morrison and J. E. T. Corrie, unpublished data), which were fast enough to be useful on a synaptic time scale. There was 7% conversion of NI-glutamate and 24% conversion of MNI-glutamate with the flash lamp pulse in near-UV.
Release of L-glutamate at 50-200 μM concentration with a 1 millisecond flash lamp pulse produced activation of AMPA-receptor-mediated currents with half rise time of less than a millisecond, and slower activation of NMDA-receptor-mediated currents, typically more than 10 ms, resulting from the slower gating kinetics of this channel.

The physiological glutamate concentration in the synaptic cleft has not been measured directly but its time course and concentration has been modelled using a low affinity competitive antagonist (Clements, 1996). Clements measured the displacement of the antagonist using estimations of the on- and off-rates for the antagonist, and the initial concentration of glutamate immediately after synaptic release was estimated to be 1-5 mM. This high concentration, however, was rapidly cleared, so that after 500 μs the transmitter was fairly uniformly distributed at a concentration of approximately 20 μM.

Conversely, after the release of glutamate from caged compound, there was a uniform concentration of 30-40 μM over an area of about 200 μm in diameter. Consequently, glutamate took a longer time to clear and had more time to re-bind and activate receptors. It was found that glutamate disappeared with a half time of 200 ms, as estimated by measuring AMPA-receptor-mediated currents in the presence of cyclothiazide (100 μM) to inhibit AMPA-receptor desensitisation in Purkinje cells (Canepari et al., 2001a). As a result, higher concentrations of ionotropic antagonists were required for block than if synaptic stimulation elicited mGlur responses. The increased duration of glutamate presence at the receptors in the synapses resulted in an equilibrium between glutamate and antagonists. Sufficient antagonist concentration had to be applied to maximise the probability of antagonist molecules, not glutamate, re-binding to the receptors.
Isolation of the mGluR response

The mGluR response was separated from ionotropic components in two ways: temporally and pharmacologically. Pharmacological block of NMDA-receptor and AMPA-receptor responses using selective ionotropic antagonists allowed activation of the mGluR current to be detected. This approach revealed that activation of the mGluR current occurred with a delay of between half a second and one second after the ionotropic response.

There was a concern that the late component of the glutamate response was a delayed residual NMDA-receptor current, a result of antagonist molecules unbinding and glutamate taking its place. However, if this were the case, this late peak current would have decreased proportionally with increasing antagonist concentrations. In the experiment, the first peak declined progressively with increasing concentrations of antagonist, while the second peak remained relatively constant, indicating that it was not due to activation of NMDA-receptors. In addition, the same mGluR response kinetics were seen with either of two NMDA-receptor antagonists, CPP or AP5. The off-rates of these antagonists are very different (Benveniste & Mayer, 1991). If glutamate was simply rebinding to NMDA-receptors to cause the later peak, using an antagonist with a different off-rate would affect the timecourse of glutamate rebinding.

Investigation of the mGluR currents

Having isolated the mGluR response, it was investigated at different membrane holding potentials. The response was found to be clearly composed of two components, one activating approximately half a second after the flash, and the second
activating after a second. The two components appeared to be the result of two different conductances, perhaps mediated by different intracellular signalling pathways. In some cells, one or both components were absent.

There was a large variation in amplitude of responses, perhaps because cells differed in size; this was not easy to quantify as it was difficult to measure capacitance due to dendritic cable attenuation. In addition, whole-cell recording itself could have interfered with signalling pathways to affect currents. In either case, the apparent independence of the two components from each other offered evidence for the existence of two separate signalling systems, and multiple mGluR receptor subtypes, within the cell.

Ion substitution experiments revealed that the late component was a potassium current. When the late component was reduced using caesium block of potassium channels, the early component appeared to be considerably larger in magnitude. The two currents had opposite effects on excitability; at membrane potentials more positive than -70 mV they were opposite in polarity.

A likely candidate for the late component potassium conductance was the calcium-activated potassium channel, known to be responsible for after-hyperpolarisations in pyramidal cells. Pharmacology tentatively revealed the identity of this channel as a small-conductance potassium channel (SKCa), which has been reported to be modulated by neurotransmitters. Further work with more selective antagonists is needed to classify these channels further and to determine how they are involved in the regulation of excitability.

As the early component reversed around zero, it was likely to be a non-selective cation current, carrying sodium and potassium. The voltage-sensitivity of this current has been observed (Chuang et al., 2000). The mGluR response has been studied in cerebellar Purkinje cells with the same techniques used here (Canepari et al., 2001a).
Interestingly, the early, but not the late, component was found, and it did not display voltage sensitivity. It did, however, reach its peak in approximately 0.7 seconds, it reversed close to zero mV and was blocked by the non-selective mGluR antagonist MCPG and the Group I-selective antagonist CpCCOEt. A number of pore blockers were used without effect; divalent cations (magnesium, barium, cobalt, cadmium and galladium), a cyclic nucleotide channel antagonist and a purinoceptor antagonist. The polyamine spermine, however, did block the current at concentrations of 100 μM to 1 mM (Canepari & Ogden, Abstracts for the Society for Neuroscience, 2001).

The early component of the mGluR response is presently elusive, and could be probed with additional ion substitution experiments, for example, changing the external sodium or calcium concentration. Examining the $[Ca^{2+}]_i$ transients more closely with varied external $[Ca^{2+}]$ would reveal information about how external $[Ca^{2+}]$ is contributing to the calcium signal, in contrast to release from internal stores.

The mGluR dual current response in the hippocampus is possibly a result of activation of two mGluR subtypes. There is evidence in the hippocampus for subtypes mGluR1b and mGluR5 (both Group I) and Group II mGluRs post-synaptically. Antagonists for mGluR1 and Group II receptors were used to investigate the receptor subtypes responsible for the two components. It was concluded that mGluR1 receptors were involved in the generation of the mGluR response, in particular the potassium component. Group II receptors may well account for activation of the early component.

In Purkinje cells, there is an additional glutamate-elicited current that is not found in hippocampal pyramidal cells. This is the sodium-glutamate transporter current, which is responsible for electrogenic glutamate uptake (Otis & Kavanaugh, 2000) and is thought, in the cerebellum, to remove glutamate from the synaptic cleft within a few milliseconds (Auger & Attwell, 2000). This current is seen in Purkinje cells (Canepari et al., 2001a) in the presence of 1 mM NBQX, it is fast to activate (peaks in 7-8 ms), it
does not reverse, it is inwardly rectifying and it is blocked by threo-hydroxyaspartate (THA; competes with L-glutamate for transport). The reason for the apparent lack of this current in pyramidal cells is unknown, but implicates a role for glial cells, as opposed to pyramidal cells, in the uptake of glutamate.

Probing the inositolphosphate pathway

Having identified the slow component of the mGluR response as a calcium-activated potassium conductance, likely to be mediated by the inositolphosphate (IP) pathway because of Group I receptor activation, it was appropriate to probe this sequence of events. In addition, the ion substitution and pharmacology experiments revealed useful information about this component, so it seemed sensible to concentrate on further investigation of this pathway and the generation of the potassium current.

IP$_3$ was released from caged IP$_3$ in a fast and quantitative fashion. The responses to the release of IP$_3$ were compared to those obtained using caged glutamate. Intriguingly, the current elicited by IP$_3$ closely resembled the slow component of the mGluR response. Ion substitution experiments revealed that this, too, was a potassium current and pharmacological experiments showed that it shared the same identity as the mGluR channel responsible for the late component, the calcium-activated small conductance potassium channel (SK$_{Ca}$).

The sensitivity to IP$_3$ in hippocampal pyramidal cells appeared to be different to that in cerebellar Purkinje cells. This could be because the different subtypes or splice variants of receptor have varying IP$_3$ affinity. However, it has been found that affinities for IP$_3$ of IP$_3$-receptor subtypes vary to a small extent (Nerou et al., 2001)
and it is likely that receptor density is a factor in determining the sensitivities of
different brain regions. For example, a high density of IP$_3$-receptors is found in the
cerebellum (Worley et al., 1987; 1989), but Purkinje cells are less sensitive to IP$_3$ than
hippocampal pyramidal cells. The highly concentrated IP$_3$-receptors could act as a
'sink' to considerably reduce the apparent [IP$_3$] that the receptors are exposed to.

The release of IP$_3$ evoked fast-rising calcium transients which were a result of
calcium efflux into the cytosol. The [Ca$^{2+}$]$_i$ changes were measured, using fura-2FF, a
low-affinity indicator. The principal objective of these experiments was to compare
the [Ca$^{2+}$]$_i$ kinetics elicited by IP$_3$ to those obtained using caged glutamate. The delay
was expected to be different, due the extra steps in the pathway when glutamate is
released, compared to IP$_3$. If all other kinetics were the same, this would be evidence
consistent with the fact that the same mechanism was occurring in each case and that
the mGluR-induced calcium-activated potassium current is mediated by the IP$_3$-
pathway.

After the release of IP$_3$, the [Ca$^{2+}$]$_i$ signal consisted of a delay and then a steep rise
of [Ca$^{2+}$]$_i$, which terminated very abruptly. There was a minimal delay of 80 ms,
representing the minimum activation time of the receptor. Delays were highly
variable, but delay in the potassium current was linearly correlated to [Ca$^{2+}$]$_i$ transient
delay. This indicated that calcium released from stores activated the IP$_3$-elicited
potassium conductance. There was some evidence for cooperativity at the IP$_3$-
receptors (Hill coefficient of 2.6). This may be due to multiple IP$_3$-binding sites or the
situation may be more complex, involving interaction with calcium at the receptor
(Bezprozvanny et al., 1991; Finch et al., 1991).

The [Ca$^{2+}$]$_i$ peak of IP$_3$-elicited responses varied from cell to cell at each IP$_3$
concentration, indicating that IP$_3$ was not simply triggering the complete emptying of
stores. This evidence, in addition to the fast termination of the response observed,
suggested that there was an additional inactivation mechanism.
The rate of \([Ca^{2+}]\) release from stores is proportional to the number of open IP\(_3\) receptor channels within the cell. It was observed that flux was inversely proportional to the rise time of the response. As the reciprocal of the rise time gave a measure of the rate of termination of the response, the localised accumulation of \([Ca^{2+}]\) adjacent to channels (proportional to flux) was suggested to be contributing to the process of inactivation.

It was found that elevation of cytosolic \([Ca^{2+}]\) inhibited calcium release. This was shown by inducing calcium influx through voltage-gated channels in the plasma membrane by depolarisation prior to IP\(_3\) release. No evidence of stimulation by calcium was observed, but this effect has typically been seen at only very low calcium concentrations (less than 250 nM).

\([Ca^{2+}]\) signals, of a similar magnitude to those produced IP\(_3\), were also seen with the mGluR response. The \([Ca^{2+}]\) changes were activated after the early component and before the late component of conductance. It therefore appeared that IP\(_3\)-mediated release of calcium from stores was activating the calcium-activated potassium conductance (late component). The rate of termination of calcium release was related to the flux in the same way as the IP\(_3\)-mediated \([Ca^{2+}]\) signals. This indicated that the calcium inactivation process was occurring and that IP\(_3\)-receptor channels, sensitive to calcium, were involved.

The high affinity calcium indicator fluo-4 was used whilst imaging pyramidal cells following release of either IP\(_3\) or glutamate to provide information gained about the spatial distribution of \([Ca^{2+}]\). After the release of IP\(_3\), which elicited a double peak current response, the \([Ca^{2+}]\) signal appeared to initiate at one end of the apical dendrite and move towards the soma during the response. This could simply reflect the difference in surface (and therefore flux) to volume ratio of the cell regions; a particular flux in the dendrites may have appeared to represent a larger \([Ca^{2+}]\) signal than an identical flux in the soma.
However, the $[Ca^{2+}]_i$ signal appeared to move along the dendrite, so unless the depth of the dendrite varied along its length, this would indicate a non-uniform distribution of $IP_3$-receptors. After the release of glutamate however, there appeared to be a uniform $[Ca^{2+}]_i$ rise throughout the dendrite. In many experiments with L-glutamate, the soma fluorescence saturated that region of the CCD.

**Further experiments**

In addition to the future experiments already suggested, there are a number of experiments that remain to be done. The $IP_3$ pathway could be investigated further using photolabile calcium chelators (for example DM-nitrophen; Kaplan & Ellis-Davies, 1988). Although there has been substantial evidence in favour of the fact that the late component is mediated by the $IP_3$-pathway, a direct piece of linking evidence has not been produced in this project. In previous experiments, however, the $IP_3$-receptor blocker heparin has been shown to inhibit mGluR responses (Nakamura et al., 1999). In addition, Abdul-Ghani et al. (1996) reduced the mGluR response with neomycin, which inhibits phospholipase C (PLC) and ryanodine, which depletes calcium stores.

During caged glutamate experiments, pharmacology at the level of PLC (upstream of the production of $IP_3$) could be carried out. U-73122, a drug that is known to block the action of PLC, was used in the investigation of the mGluR current in Purkinje cells, but found to have no effect (Canepari et al., 2001a).
The role of backpropagating action potentials in the mGluR response need to be investigated, to follow on from experiments carried out by Nakamura et al. (1999; 2000). They suggested that IP$_3$ was involved in the synergistic response, and this could be directly tested using the release of IP$_3$ from caged IP$_3$.

The involvement of G-proteins in the mGluR response could be studied using compounds such as GTP$\gamma$S and GDP$\beta$S, which irreversibly activate and inhibit G-proteins respectively, as used by Abdul-Ghani et al. (1996). Intracellular mechanisms underlying the early current component could be probed using compounds such as caged cAMP and cAMP analogues, which are either stimulators or inhibitors of protein kinase A. The role of tyrosine kinases, as suggested by Heuss et al. (1999), could also be investigated using inhibitors.

Further experiments to investigate the mGluR response in neurones could involve the caged version of diacylglycerol (DAG), which is the accompanying product when is PIP$_2$ is hydrolysed. There is a possibility that DAG plays a role in the generation of the mGluR response. Scholz (1994) observed an increase in production of DAG after mGluR activation by selective agonists in cultured hippocampal pyramidal cells. The response was blocked by pertussis toxin, implicating a G-protein, and was thought to result in the activation of protein kinase C. Scholz suggested that phosphorylation by this enzyme played a role in plasticity and also in the development and maintenance of neurones.
Appendices
Appendix 1: Relationship between the fluorescence emitted from an indicator and the concentration of free ligand

A ligand, \( L \), binds with an indicator \( I \): \[ L + I \rightleftharpoons IL \]

The Law of Mass Action states that

\[ k_{+1} (L \cdot I) = k_{-1} IL \]

\( L, I \) and \( IL \) are the concentrations of free ligand, free indicator and bound indicator respectively.

\( K_a \) is the apparent equilibrium dissociation constant.

\[ K_a = \frac{k_{-1}}{k_{+1}} = \frac{L}{IL} \] \hspace{1cm} (I)

\( I_T = \) total indicator concentration \hspace{1cm} \( I_r = I + IL \)
$I_L^*$ and $I^*$ are the fractions of indicator in the bound and unbound state

$$I^* = \frac{I}{I_T} \quad \text{.............................................(2)}$$

$$IL^* = \frac{IL}{I_T} \quad \text{.............................................(3)}$$

And $K_d$ can be re-written in terms of $I^*$ and $IL^*$

$$K_d = \frac{L}{IL^*} \quad \text{.............................................(4)}$$

The fluorescence intensity $F$ is related to the indicator concentration $I$ by an efficiency term $S$ dependent on excitation intensity, extinction coefficient, pathlength, quantum yield and instrument efficiency (Gryniewicz et al., 1985).

In the unbound state ($I=I_T$):

$$S_\theta = \frac{F_\text{min}}{I_T} \quad \text{.............................................(5)}$$

In the bound state ($IL=I_T$):

$$S_L = \frac{F_\text{max}}{I_T} \quad \text{.............................................(6)}$$

$F_\text{min} = \text{fluorescence emitted by the indicator in the absence of ligand}$

$F_\text{max} = \text{fluorescence emitted by the indicator in the presence of saturating concentrations of the ligand}$
In the presence of non-saturating concentrations of the ligand, the fluorescence emitted by the dye is the sum of the products of the concentrations of the dye in the bound and unbound form with their respective S-factors:

\[ F = I \ S_0 + IL \ S_L \]

Substituting with (5) and (6) and using (2) and (3) gives:

\[ F = I^* \ F_{min} + IL^* \ F_{max} \] ............................(7)

Since

\[ I^* = 1 - IL^* \] .................................(8)

Equation (7) may be re-written and rearranged:

\[ F - F_{min} = IL^* \ (F_{max} - F_{min}) \] ............................(9)

And from equation (4)

\[ IL^* = \frac{L \ I^*}{K_d} \]

So

\[ F - F_{min} = \frac{-L \ I^* \ F_{min}}{K_d} + \frac{L \ I^* \ F_{max}}{K_d} \]
Which may be rearranged to

\[ L = \frac{K_d (F - F_{\text{min}})}{-I \cdot \frac{F_{\text{min}}}{F_{\text{max}}} + I \cdot \frac{F_{\text{max}}}{F_{\text{min}}}} \]

Using equation (8)

\[ L = \frac{K_d (F - F_{\text{min}})}{(I \cdot L \cdot F_{\text{min}}) - \frac{F_{\text{min}}}{F_{\text{max}}} + \frac{F_{\text{max}}}{F_{\text{min}}} - (I \cdot L \cdot F_{\text{max}})} \]

And (9) to get the final equation

\[ L = \frac{K_d (F - F_{\text{min}})}{F_{\text{max}} - F} \]

This equation permits the calculation of the concentration of free ligand \( L \) when the fluorescence emitted by the indicator at that concentration \( F \), \( F_{\text{min}} \), \( F_{\text{max}} \) and the \( K_d \) are known.

The assumptions made are that the indicator forms a 1:1 complex with the ligand and that the indicator is sufficiently dilute for the fluorescence intensity to be proportional to the concentration of fluorescent species. In addition, it is assumed that \( F \), \( F_{\text{min}} \) and \( F_{\text{max}} \) are determined at the same instrument sensitivity, optical pathlength and indicator concentration.
Appendix 2: Calcium buffering by calcium indicators

$[Ca]$ is the free calcium concentration; $[D]$ is the free dye concentration; $[DCa]$ is the bound dye concentration; $K_{Ca}$ is the dissociation constant

$$[Ca] + [D] \rightleftharpoons_{K_{Ca}} [DCa]$$

$$K_{Ca} = \frac{[Ca][D]}{[DCa]}$$

$$\text{fraction bound} = \frac{[DCa]}{[Ca]} = \frac{[D]}{K_{Ca}}$$

$[D]_{TOT}$ is the total dye concentration

$$[DCa] + [D] = [D]_{TOT}$$

Using $[D] = [D]_{TOT} - [DCa]$ to substitute for $[D]$:

$$\text{fraction bound} = \frac{[D]_{TOT} - [DCa]}{K_{Ca}}$$

$$[DCa] = \frac{[Ca][D]_{TOT}}{K_{Ca}} + [Ca]$$

$$\text{fraction bound} = \frac{[D]_{TOT}}{K_{Ca} + [Ca]}$$

At $[Ca] = 0.1$ mM, fluo-4 (500 mM; $K_{Ca} = 0.35$ mM) buffers calcium at a ratio of 2500 bound: 1 free. The ratio for fura-2FF (500 mM; $K_{Ca} = 29.8$ mM) is 15:1.
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Photochemical and pharmacological evaluation of 7-nitroindolinyl-and 4-methoxy-7-nitroindolinyl-amino acids as novel, fast caged neurotransmitters

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Received 23 May 2001; received in revised form 26 July 2001; accepted 27 July 2001

Abstract

Reagents capable of rapid and efficient release of neuroactive amino acids (L-glutamate, GABA and glycine) upon flash photolysis of thermally stable, inert precursors have been elusive. 7-Nitroindolinyl (NI)-caged and 4-methoxy-7-nitroindolinyl (MNI)-caged compounds that fulfil these criteria are evaluated here. These caged precursors are highly resistant to hydrolysis. Photolysis is fast (half time ≤ 0.26 ms) and the conversion achieved with a xenon flashlamp is about 15% for the NI-caged L-glutamate and about 35% for the MNI-caged L-glutamate. A procedure is described for calibration of photolysis in a microscope-based experimental apparatus. NI-caged L-glutamate itself showed no agonist or antagonist effects on AMPA and NMDA receptors in cultured neurones, and had no effect on climbing fibre activation of Purkinje neurones. A control compound with identical photochemistry that generated an inert phosphate upon photolysis was used to confirm that the intermediates and by-products of photolysis have no deleterious effects. MNI-caged L-glutamate is as stable and fast as NI-caged L-glutamate and similarly inert at glutamate receptors, but about 2.5 times more efficient. However, NI-caged GABA is an antagonist at GABA A receptors and NI-glycine an antagonist at glycine receptors. The results show the utility and limitations of these fast and stable caged neurotransmitters in the investigation of synaptic processes. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Caged neurotransmitters; Flash photolysis; Synaptic transmission

1. Introduction

Activation of synaptic transmission in the nervous system generally occurs on a sub-micron spatial scale with a sub-millisecond time scale and experimental approaches to synaptic function ideally require similar precision. A useful strategy for investigating the kinetics and distribution of synaptic processes is the photolytic release of neurotransmitters from caged precursors (Corrie and Trentham, 1993). This technique overcomes the problems of slow access and diffusional mixing, especially when working with brain slices or intact parts of the nervous system. It has been used in the nervous system to identify the neurotransmitter at the squid giant synapse (Corrie et al., 1993; Corrie and Trentham, 1993), to investigate the activation of postsynaptic receptors (Grewer, 1999; Grewer et al., 2000; Canepari et al., 2001b), and to map the connections between different regions in brain slices (Callaway et al., 1993; Kötter et al., 1998). However, despite the use of this technique in the last decade, development of stable caged neurotransmitters which are rapid and efficient in photorelease of neuroactive amino acids has posed difficulties, mainly in the development of stable precursors of amino acids.

A useful caged neurotransmitter must be thermally stable (i.e. it must not hydrolyse in aqueous solution), and fast and efficient in photorelease with respect to synaptic time scales and concentrations. Furthermore the caged precursor, the photolytic intermediates and by-products must be biologically inert and must have minimal interaction with the receptors, transporters and metabolism of the released neurotransmitter. Nor should they interfere with other components of synaptic
transmission at the concentrations used. Many of these criteria have not been satisfied by the caged compounds used in the past. Among the best reagents the \(\alpha\)-carboxy-2-nitrobenzyl-caged compounds have been found to be efficient \((Q > 0.1\) at 308 nm) and fast (half time of the order of \(\mu s\)) in photorelease, but quite unstable in solution, with half time for hydrolysis \((t_{1/2})\) of a few hours at pH 7 and room temperature (Wieboldt et al., 1994; Grewe and Corrie, 2000). \(p\)-Hydroxyphenacil esters of glutamate and GABA undergo photorelease on a sub-microsecond time scale (Givens et al., 1997) but are inefficient except under relatively short wavelength (308 nm) irradiation (Geibel et al., 2000). Recently, 1-acyl-7-nitroindoline (NI) derivatives that release L-glutamate or other carboxylates have been described (Papageorgiou et al., 1999). These reagents are highly resistant to spontaneous hydrolysis, which is negligible at physiological pH and have \(t_{1/2} > 6\) h for hydrolysis at pH 12 and 30°C. The half time of the photorelease process was previously estimated as \(\leq 0.26\) ms (pH 7, 20°C) but more recent work shows that the true half-time for photorelease is in the sub-\(\mu s\) time domain (P. Wan, J. Morrison and J.E.T. Corrie, unpublished data). The NI-caged L-glutamate has been used to investigate the current generated by the activation of metabotropic glutamate receptors in cerebellar Purkinje neurones in slices (Canepari et al., 2001a). The NI-caged compounds have the same resistance to hydrolysis and the same photochemistry, so are expected also to have rapid photorelease.

Characterisation of the photochemical and pharmacological properties of the NI-caged L-glutamate, GABA, glycine and an inert control compound, and of the MNI-caged L-glutamate are reported here. The structures of the NI-caged neurotransmitters and the MNI-caged L-glutamate are shown in Scheme 1. Methods to estimate the concentration of released products and to test the activation of ionotropic receptors by photorelease of neurotransmitters and the pharmacology of the caged precursors are described. The relative photorelease efficiencies of the NI-caged and MNI-caged neurotransmitters are also discussed. Part of this work has been presented in abstract form (Canepari et al., 2001a).

2. Methods

2.1. Cell preparation and external solutions

Patch clamp recordings were made from cerebellar granule cells, hippocampal pyramidal neurones or spinal cord neurones in culture, and from cerebellar Purkinje neurones in sagittal slices from 20 day rats. Cerebellar granule cell explant cultures were made from 250 to 500 \(\mu\)m diameter cerebellar fragments from 7-day-old male Wistar rats and cultured on coverslips previously coated with poly-D-lysine and laminin. Isolated hippocampal cells and spinal cord cells were prepared from papain-treated mechanically dissociated tissue with the same solutions, media and methods. Briefly, after dissection and cleaning, whole hippocampi or 0.5 mm thick slices of spinal cord from 15 to 17-day-old Wistar rat embryos were incubated for 30 min at 37°C in 250 U/ml papain in a calcium-free

![Scheme 1. Structures of NI-and MNI-caged compounds used in this work. (A) NI-caged L-glutamate; (B) NI-caged GABA; (C) NI-caged glycine; (D) NI-caged 5-(dihydroxyphosphoryloxy)pentanoate; (E) MNI-caged L-glutamate.](image-url)
Cells were isolated by trituration and centrifugation after washing in a trypsin inhibitor and DNase solution, resuspended in L-glutamine-free culture medium after washing in a trypsin inhibitor and DNase solution. For slice preparation, 19-22-day-old Wistar male rats were killed by cervical dislocation and the brain quickly removed from the skull and placed in ice-cold artificial cerebrospinal fluid solution (ACSF). ACSF contained (in mM), 135 NaCl, 3 KCl, 2 CaCl2, 10 HEPES, 2 MgSO4, 25 glucose, 1 NaHCO3 (pH 7.3 adjusted with NaOH), and was bubbled with O2 in the slice experiments. Parasagittal cerebellar slices (200 μm thick) were cut with a vibratome and kept at 32°C for 1 h, then at room temperature (24°C). All the experiments in cultured neurones were performed in the presence of 500 nM tetrodotoxin (TTX). Experiments at NMDA receptors were done with 50 μM glycine present in the external solution. AMPA receptor (AMPA) mediated currents, NMDA receptor (NMDAR) mediated currents, GABAAR receptor (GABAAR) mediated currents and glycine receptor (glyR) mediated currents were blocked by 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulphonamide (NBQX), 2-amino-5-phosphonopentanoic acid (AP5), bicuculline and strychnine respectively. All drugs were obtained from Sigma (Poole) or TOCRIS (Bristol, UK). 1-(2-nitrophenyl)ethyl ATP (NPE-ATP) and 1-(2-nitrophenyl)ethyl ether of pyrazine (NPE-HPTS) were gifts from Dr D.R. Trentham (National Institute for Medical Research). NI- and MNI-caged l-glutamate and NI-caged 5-(dihydroxyphosphoryloxy)pentanoate were from previous work (Papageorgiou et al., 1999, 2000). The caged GABA and glycine reagents were prepared as described below, analogously to the previous synthesis of NI-caged glutamate and related compounds. As shown in Scheme 1, the NI-caged GABA had an identical cage group to that in NI-caged glutamate but, for the caged glycine, the methyl ester in the side chain of the cage group was hydrolysed to the corresponding carboxylate. This was necessary to enable purification of the reagent. The nitroindoline caged compounds have identical photochemistry whether this side chain is present as the ester or the carboxylate (Papageorgiou et al., 1999). Caged compounds and drugs were applied in static 1 ml solution for 5–10 min prior to photolysis. In slice experiments, hydrated O2 was blown over the surface of the bath solution.

2.2. Caged GABA

A solution of crude methyl indoline-5-acetate (Papageorgiou et al., 1999; 291 mg, 1.52 mmol) and 1-hydroxybenzotriazole (540 mg, 4 mmol) under nitrogen in dry tetrahydrofuran (THF) (15 ml) was cooled to 0°C and treated with N-BOC-γ-aminobutyric acid (406 mg, 2 mmol), followed by dropwise addition of a solution of dicyclohexylcarbodiimide (433 mg, 2.1 mmol) in dry THF (5 ml). The mixture was stirred at 0°C for 1 h, then at room temperature overnight. The precipitate was filtered and washed with THF and the combined filtrates were evaporated. A solution of the residue in EtOAc was washed with dilute aqueous HCl, aqueous NaHCO3 and brine, dried and evaporated. Flash chromatography (EtOAc–hexanes (1:1)) gave methyl 1-[4-(tert-butoxycarbonylamino)butanoyl]-indoline-5-acetate (309 mg, 54%), mp 85–86°C (EtOAc–hexanes); 1H NMR (400 MHz, CDCl3): δ 8.15 (d, J 8 Hz, 1H, H-7), 7.11 (s, 1H, H-4), 7.08 (d, J 8 Hz, 1H, H-6), 4.78 (br s, 1H, NH), 4.04 (t, J 8.5 Hz, 2H, H-2), 3.68 (s, 3H, OMe), 3.57 (s, 2H, ArCH2), 3.23 (m, 2H, CH2NH), 3.18 (t, J 8.5 Hz, 2H, H-3), 2.46 (t, J 7 Hz, 2H, COCH3), 1.93 (quintet, J 6.7 Hz, 2H, CH2) and 1.42 (s, 9H, CMe3). Anal. Calcd for C20H22N2O2: C, 76.24; H, 7.50; N, 7.44. Found: C, 76.37; H, 7.63; N, 7.54.

A stirred solution of this compound (376 mg, 1 mmol) in trifluoroacetic acid (TFA) (5 ml) was treated with sodium nitrate (93 mg, 1.1 mmol) and the mixture was stirred for 4 h at room temperature, then evaporated under reduced pressure. The residue was dissolved in water (30 ml) and adjusted to pH 7 with 1 M NaOH. The solution was washed with ether, analysed by reverse-phase HPLC (mobile phase 25 mM Na phosphate, pH 6.0 + 75% MeOH at 1.5 ml/min) and quantified by UV absorption at 342 nm (819 pmol, 82%). HPLC showed a major and minor peak, tR 6.6 and 1.9 min, respectively. The minor peak was assumed to be the compound with a carboxylate in the side chain, as in the NI-caged glycine described below and as previously observed in related compounds (Papageorgiou et al., 1999). Part of the solution (containing 669 pmol) was lyophilised and purified by preparative HPLC (25 mM Na phosphate, pH 6.0, 2.5 ml/min). The column was first eluted with buffer for 1 h, then with water for 1 h and finally with 10 mM Na phosphate, pH 6.0 + 50% MeOH. Fractions eluted by the last of these solvents were analysed by reverse-phase HPLC as above. Two early fractions contained both the faster and slower eluting components (total 294 pmol) and were discarded. Subsequent fractions contained only the later-eluting (tR 6.6 min) component and were combined, quantified by UV absorption (383 μmol) and concentrated under reduced pressure to remove most of the methanol. The residue was diluted to ~ 20 ml and mixed for 20 min with Amberlite XAD-2 beads (Merck, Poole, 5 g). The beads were washed with water to remove inorganic salts, then extracted with MeOH (8 × 20 ml). The methanolic solution was quantified by UV absorption (269 μmol), evaporated and the residue, that contained methyl 1-(4-aminobutanoyl)-7-nitroindoline-5-acetate (NI-caged GABA), was dissolved in water and stored at −20°C; 1H NMR:...
Fig. 1. Photolysis calibration. (A) Increase in HPTS fluorescence by \( N = 11 \) flashes producing photolysis in vesicles containing NPE-HPTS or DMNB-HPTS solutions. Fluorescence (set initially at zero) has been normalised to the fluorescence (\( F_{\text{max}} \)) corresponding to complete photolysis. Least squares exponential fit to estimate the exponential coefficient \( \alpha \) in Eq. (1) gave \( \alpha = 0.112 \) for NPE-HPTS and \( \alpha = 0.053 \) for DMNB-HPTS. (B) Decrease in HPTS fluorescence excited at 450 nm due to proton release produced by \( N = 8 \) flashes of NPE-ATP. See text for composition of solution. Normalised fluorescence \( \left( F - F_{\text{inin}} \right) / \left( F_{\text{max}} - F_{\text{inin}} \right) \). Least squares fit of Eq. (1) gave \( \alpha = 0.59 \). (C) Decrease in HPTS fluorescence due to proton release produced by \( N = 8 \) flashes of NI-caged L-glutamate (NI-Glu) or MNI-caged L-glutamate (MNI-Glu). Composition of solutions given in text. Normalised fluorescence \( \left( F - F_{\text{inin}} \right) / \left( F_{\text{max}} - F_{\text{inin}} \right) \). Fit of Eq. (1) gave \( \alpha = 0.15 \) for NI-Glu and \( \alpha = 0.35 \) for MNI-Glu.

\[ \delta_1 \] (500 MHz, D$_2$O, acetone ref.) 7.64 (s, 1H, H-6), 7.55 (s, 1H, H-4), 4.32 (t, \( J \) 7.8 Hz, 2H, H-2), 3.82 (s, 2H, ArCH$_2$), 3.72 (s, 3H, OMe), 3.25 (t, \( J \) 7.8 Hz, 2H, H-3), 3.07 (t, \( J \) 7.8 Hz, 2H, COCH$_3$), and 2.02 (quintet, \( J \) 7.5 Hz, 2H, CH$_2$). FAB-MS: \( m/e \) (M + H)$^+$ Calcd for C$_{18}$H$_{24}$N$_2$O$_5$ + H: 322.1403. Found: 322.1413.

2.3. Caged glycine

A solution of crude methyl indoline-5-acetate (0.97 g, 5.1 mmol) in dry MeCN (30 ml) was treated with 4-dimethyaminopyridine (1.83 g, 15 mmol) and N-tert-BOC-glycine (1.00 g, 5.7 mmol), followed by 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.15 g, 6 mmol). The mixture was stirred at room temperature for 18 h, then evaporated and the residue was dissolved in EtOAc and washed successively with 0.5 M aqueous HCl, saturated aqueous NaHCO$_3$ and brine, dried and evaporated to give methyl 1-[2-( tert-butoxycarbonylamino)ethyl]indoline-5-acetate as white crystals (1.34 g, 76%), mp 154–155°C (EtOAc–hexanes); $^1$H NMR: \( \delta_1 \) (400 MHz, CDCl$_3$) 8.12 (d, \( J \) 8.2 Hz, 1H, H-7), 7.14 (s, 1H, H-4), 7.10 (d, \( J \) 8.2 Hz, 1H, H-6), 5.55 (br s, 1H, NH), 3.88–4.05 (m, 4H, NCOCH$_2$ and H-2), 3.69 (s, 3H, OMe), 3.59 (s, 2H, ArCH$_2$), 3.22 (t, \( J \) 8.4 Hz, 2H, H-3) and 1.46 (s, 9H, CMe$_3$). Anal. Calcd for C$_{18}$H$_{24}$N$_2$O$_5$: C, 62.05; H, 6.94; N, 8.01. A solution of the above compound (174 mg, 0.5 mmol) in a mixture MeOH (5 ml) and 0.5 M aqueous NaOH (2 ml, 1 mmol) was heated at 50°C for 3 h. The progress of the reaction was followed by TLC [EtOAc–hexanes (3:2)] and, when hydrolysis was complete, the solution was diluted with water, concentrated in vacuo and acidified to pH 3.8 with dilute HCl. The cloudy solution was extracted with EtOAc and the combined organic phases were washed with brine, dried and evaporated to give 1-[2-( tert-butoxycarbonylamino)ethyl]indoline-5-acetic acid as white crystals (125 mg, 75%), mp 156–157°C (EtOAc–hexanes); $^1$H NMR: \( \delta_1 \) (500 MHz, CDCl$_3$–DMSO–d$_6$) 8.09 (d, \( J \) 8.4 Hz, 1H, H-7), 7.16 (s, 1H, H-4), 7.10 (d, \( J \) 8.4 Hz, 1H, H-6), 5.75 (br s, 1H, NH), 3.98–4.05 (m, 4H, NCOCH$_2$ and H-2), 3.55 (s, 2H, ArCH$_2$), 3.23 (t, \( J \) 8.2 Hz, 2H, H-3) and 1.47 (s, 9H, CMe$_3$). Anal. Calcd for

Scheme 2. Photolysis reaction of NI-caged glutamate.
Fig. 2. Pharmacological properties and kinetics of currents evoked by photolysis of Nl-caged L-glutamate (NI-Glu). (A) (Left panel) Voltage clamp currents evoked in a hippocampal neuron at — 80 mV by the release of 70 μM L-glutamate from 500 μM NI-Glu, in control conditions (with 500 μM TTX), after addition of 100 μM NBQX, after addition of 50 μM AP5 and after washing out the two antagonists. (Right panel) currents evoked in a hippocampal neuron at — 20 mV VC by 70 μM L-glutamate released from 500 μM NI-Glu, in control conditions, after addition of 100 μM NBQX, after addition of 50 μM AP5 and after washing out the two antagonists. (B) AMPAR-mediated current (Left), and NMDAR-mediated current (Right; 100 μM NBQX), recorded in a hippocampal neuron at — 80 mV shown on a fast time scale. The arrows indicate the time of the flash.

C₁₇H₂₂N₂O₅: C, 61.07; H, 6.63; N, 8.38. Found: C, 61.16; H, 6.66; N, 8.32.

A solution of this material (138 mg, 0.41 mmol) in TFA (8 ml) was treated with NaNO₂ (39 mg, 0.45 mmol) and stirred at room temperature for 4 h. The solution was concentrated in vacuo and the residue was dissolved in water (25 ml), adjusted to pH 6.5 with 1 M aqueous NaOH and extracted with ether. The aqueous solution was passed through a 0.2 μm filter and analysed by reverse-phase HPLC (mobile phase 25 mM Na phosphate, pH 6.0 + 10% MeOH at 1.5 ml/min) to show one major peak, tR 4.0 min. The solution was diluted to 100 ml with 25 mM Na phosphate, pH 6.0 buffer and loaded onto a preparative reverse-phase HPLC column. The column was first washed with 25 mM Na phosphate, pH 6.0 for 1 h (all flow rates 2.0 ml/min), then the product was eluted with 25 mM Na phosphate, pH 6.0 + 10% MeOH. Fractions containing the product were analysed, combined and quantified (UV spectroscopy) to give a solution of 1-[2-(aminoethanoyl)-7-nitro]indoline-5-acetate (NI-caged glycine) (309 μmol, 75%). The solution was evaporated
and the residue was dissolved in water (80 ml) and desalted by re-application to the preparative reverse phase HPLC column. The column was first washed with water for 2 h, then the product was eluted with water-MeOH (2:1 v/v). Fractions containing the product were analysed, combined and quantified (UV spectroscopy) to give a solution of NI-caged glycine (258 pmol, 63%) that was concentrated and stored at -20°C; δH (500 MHz, D2O, acetone ref.) 7.60 (d, J 0.7 Hz, 1H, H-6), 7.53 (d, J 0.7 Hz, 1H, H-4), 4.25 (t, J 7.9 Hz, 2H, ArCH2), 3.59 (s, 2H, CH3NH+) and 3.27 (t, J 7.9 Hz, 2H, H-3).

2.4. Electrophysiology and data analysis

Whole cell patch clamp recordings were done in voltage clamp with an Axoclamp 2A or Axopatch 200A (Axon Instruments) and patch pipettes were pulled from pyrex glass tubing with filament 1.5 x 0.86 mm for culture experiments or 1.5 x 1.17 mm for slice experiments (Clarke Electromedical). When filled with intracellular solution based on K-gluconate (see below), pipettes had a resistance of 2–4 MΩ for Purkinje neurone experiments, of 5–7 MΩ for hippocampal neurones or spinal cord neurones and 8–10 MΩ for cerebellar granule cell experiments. Two types of internal solutions were used. A K-gluconate based solution, used for experiments with the NI-caged and MNI-caged L-glutamates, contained (in mM), 110 K-gluconate, 10 KCl, 4 MgCl2, 50 HEPES, 4 Na2ATP, 0.2 Na2GTP, 10 creatine phosphate (pH 7.3 adjusted with KOH). In experiments with Purkinje neurones, N-(2,6-dimethylphenylcarbamoylmethyl)triethylammonium (QX314, 5 mM) was also included in the internal solution to block sodium action potentials. A CsCl-based solution was used for experiments with Ni-caged GABA and glycine, and contained (in mM), 140 CsCl, 10 HEPES, 4 MgSO4, 4 Na2ATP, 0.2 Na2GTP, 0.5 EGTA (pH 7.3 adjusted with N-methyl-d-glucamine).

For iontophoretic application of neurotransmitters, 10 MΩ pipettes were filled with 50 mM solution, buffered to pH 7 for L-glutamate or to pH 10 for GABA and glycine, with +10 to +50 pA bias and ejected by −10 to −20 μA pulses at a frequency of 0.1–0.2 Hz.

For climbing fibre stimulation in cerebellar slices, constant current pulses of 50 μs duration were delivered through a glass pipette with 5–10 μm tip positioned on the surface of the slice in the granule cell layer. Climbing fibre response was characterised as a large excitatory postsynaptic current (EPSC) with a well-defined threshold (Eccles et al., 1964; Konnerth et al., 1990) and intensity was adjusted to 2 x threshold. Climbing fibre experiments were made in the presence of 200 nM NBQX to reduce the amplitude of EPSCs. The stimulation protocol comprised a train of four pulses at 50 ms intervals delivered at 0.05 Hz in order also to test possible effects of the cage on the presynaptic depression of the climbing fibre synaptic responses (Silver et al., 1998).

Data were filtered at 2 kHz (-3 dB) digitised at 10 kHz on a CED 1401+ with Spike 2 software and analysed in Matlab 5.1. All values reported are mean ± S.D. In analysis of iontophoretically-evoked current, populations of 10 consecutive amplitudes of evoked currents before and after addition of the caged neurotransmitter (or before and after photolyis of the control caged compound) were compared with a t-test. A t-test was also applied to the amplitudes of GABA$_A$-mediated miniature spontaneous synaptic currents before and after addition of Ni-caged GABA, Ni-caged glutamate or MNI-caged glutamate.

2.5. Flash photolysis

Slices were viewed with a Zeiss Axioskop 1FS (40 x 0.75w Achromplan objective) modified to permit light from a xenon flashlamp (Rapp OptoElektronik; Rapp

![Fig. 3. Effect of cage concentration at constant L-glutamate concentration. (A) AMPAR-mediated currents evoked in a hippocampal neurone at -80 mV by the release of 40 μM L-glutamate from 250 μM or 1 mM Ni-Glu. (B) NMDAR-mediated in presence of 100 μM NBQX evoked in a different hippocampal neurone at -20 mV by the release of 40 μM L-glutamate from 250 μM or 1 mM Ni-Glu. The arrows indicate the time of the flash.](image-url)
Fig. 4. Effect of Ni-caged L-glutamate (Ni-Glu) and of the photolysis reaction on currents evoked by iontophoretic application of L-glutamate. (A) (Top) AMPAR-mediated currents evoked in a hippocampal neurone at — 80 mV by iontophoretic application of L-glutamate, in control conditions (with 500 nM TTX) and after addition of 500 μM Ni-Glu. (Bottom) NMDAR-mediated currents evoked in a hippocampal neurone at — 20 mV by iontophoretic application of L-glutamate, in control conditions (with 500 nM TTX) and after addition of 500 μM Ni-Glu. (B) 150 μM 5-dihydroxyphosphoryloxypentanoate released from Ni-caged dihydroxyphosphoryloxypentanoate (Ni-phosph) at time indicated by the arrow during (Top) AMPAR-mediated currents evoked by iontophoretic application of L-glutamate in a hippocampal neurone at — 80 mV, in control conditions (with 500 nM TTX) and (Bottom) NMDAR-mediated currents evoked in a hippocampal neurone at — 20 mV by iontophoretic application of L-glutamate, in control conditions (with 500 nM TTX) and with release of 150 μM 5-dihydroxyphosphoryloxypentanoate from Ni-phosph at the arrow.

et al., 1988) filtered with a UG11 (Schott, bandwidth 290–370 nm) to be focused through a silica condenser (Reichert 0.9 NA) into the specimen through the base of the chamber, formed from a No. 1 coverslip (Khodakhah et al., 1995). The flashlamp illuminated a spot of 200 μm diameter and, after alignment, was focused into the specimen plane with the condenser by maximising the output of a photodiode placed in one of the oculars. With this optical arrangement neurotransmitter release was not localised and photolysis occurred in approximately 200 μm diameter region. Transmission at 320 nm through 200 μm slices from 20 day rats was measured as 0.45 in the molecular layer and 0.4 in the granule cell layer. This optical arrangement has an advantage over epi-illumination for extracellular cages of avoiding near-UV extinction in the cage solution between the slice and objective, calculated as 80% loss for 1 mM MNI cage with a 3 mm working distance objective. Flash lamp intensity was set to maximum, converting between 12 and 15% of Nl-caged compounds and 30–35% of the MNI-caged L-glutamate (see results). Lower intensities were obtained by using ND filters in the condenser light path.
3. Results

3.1. Experimental calibration of photolytic conversion/flash in an epi-fluorescence microscope

With an upright compound microscope and water immersion optics it is not possible to make direct chemical calibration of photolysis in the focal region by removing samples for HPLC. Instead fluorescence changes induced by photolysis can be used to estimate the conversion per flash in a particular microscope by two methods, either from the fluorescence generated on photolysis of a 'caged' fluorophore, or by monitoring fluorescence changes induced by protons released stoichiometrically in the photolysis reaction. In both methods the experiment was done in small (5–20 μm diameter) aqueous vesicles of the solution formed in Sylgard on a coverslip and placed in the experimental chamber. The aqueous solution was dispersed as small vesicles by stirring 10 μl into 100 μl Sylgard 184, dropping on a coverslip, allowing 30 min for air bubbles to come out, and curing at 60°C on a hotplate for 7 min, under a cover to retain warm air. Single aqueous vesicles were selected and viewed with brightfield or epi-illumination, and a diaphragm in an image plane was adjusted to include only the selected vesicle in the epifluorescent field. The results of calibration are generally compared with photolysis of NPE-ATP, for which the photochemical parameters are well defined (Walker et al., 1988).

3.1.1. Caged HPTS

The fluorophore HPTS (pyranine) is quenched by caging as its 1-(2-nitrophenyl)ethyl ether (Jasuja et al., 1999; NPE-HPTS) or as the commercially available 8-(4,5-dimethoxy-2-nitrobenzyl) ether (Molecular Probes, Eugene, OR; DMNB-HPTS) and both compounds generate free HPTS on photolysis. Either of the caged HPTS reagents were prepared as aqueous vesicles in Sylgard at 50 μM in 100 mM sodium borate pH 9. With epifluorescent observation, the fluorescence excited at 450 nm increased with each flashlamp pulse as free HPTS was generated, and was detected with a PMT (Hamamatsu R4220P) at >510 nm (FITC epifluorescence filter set). To obtain the photolytic conversion per flash, the fluorescence increase produced by identical photolysis pulses repeated at 10 s intervals was measured. Considering the simple photochemical process:

\[ \text{NPE-HPTS} + h\nu \rightarrow \text{HPTS} + \text{2-nitrosoacetophenone} + H^+ \]

the concentration of HPTS at equilibrium after the \( N \)th flash is given by:

\[ [\text{HPTS}] = [\text{HPTS}]_{\text{max}} [1 - (1 - \alpha)^N] \quad (1) \]

where \( \alpha \) is the photolytic conversion per UV flash. Fig. 1A shows an example of the calibration protocol of fluorescence change plotted against the number of flashes \( N \). With the approximation \((1 - \alpha)^N \approx \exp(-\alpha N)\)

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Fig. 5. Effect of NI-caged L-glutamate (NI-Glu) on the currents evoked in a Purkinje neurone by climbing fibre stimulation. Ten superimposed recordings (A) and average (B) of 4 EPSCs at 50 ms interpulse interval, evoked by the stimulation of the climbing fibre synapse, recorded in control conditions (Top) and after addition of 1 mM NI-Glu (Bottom).
applied to estimate $\alpha$ by least-squares fit of an exponential curve, conversions of NPE-HPTS and DMNB-HPTS were about 10–12% and 5–6% per flash, respectively. With this approximation of Eq. (1), the estimate of $\alpha$ from exponential fits is too large by 1% for $\alpha = 0.01$, 2% for $\alpha = 0.05$, 5% for $\alpha = 0.1$ and 10% for $\alpha = 0.2$. For values of $\alpha > 0.2$ a simple empirical correction $\alpha = \alpha_e - 0.35\alpha_e^2$ can be applied, where $\alpha_e$ is the estimate from least-squares fit by an exponential. This reduced the error in $\alpha_e$ due to the approximation to less than 5% for $0.2 < \alpha < 0.9$. The conversion of NPE-HPTS and DMNB-HPTS were 20 and 10% respectively of that found for NPE-ATP in the same microscope system (see below).

3.1.2. Calibration by proton release

The $H^+$ released stoichiometrically on photolysis of NPE-ATP ($P^3$-{1-(2-nitrophenyl)ethyl}-ATP; 0.1 mM) in the reaction:

$$\text{NPE-ATP} + h\nu \rightarrow \text{ATP} + 2\text{-nitrosoacetophenone} + H^+$$

was measured by the fluorescence at $>510$ nm of pyranine (HPTS, 1 mM; Aldrich) dispersed as small aqueous vesicles and excited alternately at 450 nm (pH sensitive) and 415 nm (isoemissive) wavelengths by means of a switching monochromator (Polychrome 2, TILL Photonics, Planegg, Germany). The solution was initially set at pH 7.5 and contained 5 mM reduced glutathione to prevent interference by the nitrosoacetophenone by-product and 4 mM MgSO$_4$ to minimise the effects of the phosphates on pH (see Walker et al., 1988). With epifluorescent observation, the fluorescence excited at 450 nm decreased with each flashlamp pulse as a result of net proton release during photolysis of NPE-ATP, and the fluorescence at the pH independent isoemissive excitation wavelength 415 nm served as a control for photobleaching. At pH $>8$ the HPTS showed complex fluorescence changes following a flash and calibrations were, therefore, made at pH 7.5.
A single flash results in the conversion of a proportion \( \alpha \) of the cage concentration \( C_0 \) initially present, generating \( \gamma = \alpha C_0 \) concentration of H\(^+\) in the closed vesicle volume. After \( N \) flashes the cage concentration remaining is \( C_N = C_0 (1 - \alpha)^N \) and the total concentration of H\(^+\) generated is:

\[
\gamma_N = C_0 (1 - (1 - \alpha)^N)
\]  

(2)

The increase in protonated HPTS concentration changes the fluorescence according to:

\[
\gamma = \text{HPTS}_{\text{total}} \left( \frac{1 - R(y)}{R_{\text{max}}} \right) + K \left( \frac{R_{\text{max}}}{R(y)} - 1 \right)
\]  

(3)

where \( R \) is the ratio of fluorescence excited at 450 nm \( (F_{450}) \) to that at 415 nm, \( R_{\text{max}} \) is the ratio at pH > 9, \( \text{HPTS}_{\text{total}} \) is the total HPTS concentration and \( K \) the protonation constant. In the conditions here \( \text{HPTS}_{\text{total}} = 1 \) mM and \( K \approx 10^{-7.4} \), and therefore, total proton release after \( N \) pulses is approximately:

\[
\gamma_N \approx \text{HPTS}_{\text{total}} \left( \frac{1 - R(y_N)}{R_{\text{max}}} \right)
\]

Furthermore, since fluorescence excited at 415 nm is independent of pH, if there is no bleaching \( \gamma_N \approx \text{HPTS}_{\text{total}} \left( 1 - \frac{R_{450}(N)}{R_{450\text{max}}} \right) \).

Equating the two expressions for \( \gamma_N \):

\[
\text{HPTS}_{\text{total}} \left( \frac{1 - R(y_N)}{R_{\text{max}}} \right) = C_0 (1 - (1 - \alpha)^N)
\]

(4)

and rearranging gives:

\[
(1 - \alpha)^N = 1 - \left( \frac{R(y_N)}{R_{\text{max}}} - 1 \right) \frac{\text{HPTS}_{\text{total}}}{C_0}
\]

(5)

For \( \alpha < 0.2 \), \( (1 - \alpha)^N \approx \exp(-\alpha N) \) and an estimate of \( \alpha \) can be obtained by non-linear least squares minimisation of \( 1 - \left( \frac{R(y_N)}{R_{\text{max}}} - 1 \right) \frac{\text{HPTS}_{\text{total}}}{C_0} \) as an exponential function of flash number \( N \). When \( \alpha > 0.2 \), the exponential evaluation of \( \alpha \) can be corrected empirically as described above, or a search routine employed directly to find the value of \( \alpha \) that minimises deviations from the \( N \) data points.

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**Fig. 7. Caged glycine.** (A) GlyR-mediated currents evoked in a spinal cord neurone at \(-70 \) mV by the release of 70 \( \mu \)M glycine from NI-Gly, in control conditions (with 500 nM TTX), after addition of 20 \( \mu \)M strychnine and after washing out the antagonist. (B) GlyR-mediated current recorded in a spinal cord neurone at \(-70 \) mV illustrated at a fast time scale. (C) GlyR-mediated currents evoked in spinal cord neurone at \(-70 \) mV by the release of 80 \( \mu \)M glycine from NI-Gly, in the presence of 250 \( \mu \)M or 1 mM NI-Gly. (D) GlyR-mediated currents evoked in a spinal cord neurone at \(-70 \) mV by iontophoretic application of glycine, in control conditions (with 500 \( \mu \)M TTX) and after addition of 500 \( \mu \)M NI-Gly. The arrows indicate the time of the flash.
Fig. 8. MNI-caged L-glutamate (MNI-Glu). (A) AMPAR-mediated currents evoked in a hippocampal neurone at \(-80\,\text{mV}\) by the release of 90 \(\mu\text{M}\) L-glutamate from MNI-Glu in the presence of 250 \(\mu\text{M}\) or 1 mM MNI-Glu. (B) (Top) AMPAR-mediated currents evoked in a hippocampal neurone at \(-80\,\text{mV}\) VC by iontophoretic application of L-glutamate, in control conditions (with 500 nM TTX) and after addition of 500 \(\mu\text{M}\) MNI-Glu. (Bottom) NMDAR-mediated currents evoked in a hippocampal neurone at \(-20\,\text{mV}\) VC by iontophoretic application of L-glutamate, in control conditions (with 500 nM TTX) and after addition of 500 \(\mu\text{M}\) MNI-Glu. The arrows indicate the time of the flash.

The calibration protocol is illustrated by Fig. 1. A single 1 ms flash (UG11 filter, 290–370 nm) produced 50–60% conversion of NPE-caged ATP (Fig. 1B). In the same conditions release of fluorophore from NPE-HPTS gave 10–12% conversion/flash and DMNBP-HPTS 5–6% conversion/flash (Fig. 1A). The extinction coefficient at 347 nm (\(e_{347}\)) and quantum yield \(\phi_{p}\) of NPE-ATP are 660 M\(^{-1}\)cm\(^{-1}\) and 0.63 respectively (Walker et al., 1988; McCray et al., 1989). If \(e_{347}\) and \(\phi_{p}\) are known the conversion of other caged compounds in the near-UV can be calculated for the same experimental conditions relative to NPE-ATP by comparing the product \(e\phi_{p}\) with that for NPE-ATP.

3.1.3. Direct calibration of the Nl-caged and MNI-caged L-glutamates

The Nl-and MNI-caged compounds also release H\(^+\) stoichiometrically on photolysis, as exemplified in Scheme 2 for Nl-caged glutamate (Papageorgiou et al., 1999, 2000). Direct assay of the H\(^+\) released by photolysis of 0.1 mM Nl-caged L-glutamate and MNI-caged L-glutamate in the presence of 1 mM HPTS is shown in Fig. 1C and gave values of 12–16% and 30–38%, respectively, for the conversion/flash in the experimental microscope with the same procedure used to calibrate with NPE-ATP. Papageorgiou et al. (2000) estimated a 2.2 fold greater conversion of MNI-than Nl-caged glutamate in cuvette experiments upon photolysing both compounds together and assaying the extent of reaction by HPLC.

3.2. Physiology and pharmacology of Nl-caged L-glutamate

The activation of ion channels by photoreleased L-glutamate from Nl-caged L-glutamate and the pharmacological effects of the caged precursors and photoproducts were tested in cerebellar granule cells in culture, hippocampal neurones in culture and in Purkinje neurones in slices. A single 1 ms flash (UG11 filter, 280–380 nm) produced approximately 15% conversion of Nl-caged L-glutamate in the experimental microscope. The neurotransmitter released in a region of 200 \(\mu\text{m}\) diameter diffuses away from the central region with half time approx 40 s as estimated by fluorescence measurements after uncaging NPE-HPTS (data not shown). In brain slices, photoreleased L-glutamate concentration falls with a half-time of 200 ms as estimated by measuring AMPA-mediated currents in the presence of cyclothiazide (100 \(\mu\text{M}\)) to inhibit AMPA receptor
desensitisation (data not shown; Canepari et al., 2001b).

In hippocampal neurones, L-glutamate activated both AMPA receptor (AMPAR) mediated currents and NMDA receptor (NMDAR) mediated currents. Fig. 2A shows currents evoked by photorelease of 70 μM L-glutamate in the presence of 2 mM Mg^{2+} and 50 μM glycine at −80 mV (left hand panels) and −20 mV (right panels). The times of photolysis are indicated by the arrows. At −80 mV the glutamate-evoked current was blocked completely and reversibly by the AMPAR antagonist AP5 (50 μM) had no additional effect. At −20 mV in the presence of 100 μM NBQX there was a residual glutamate-evoked current blocked reversibly by 50 μM AP5 due to NMDAR activation and the relief of Mg^{2+} block at −20 mV relative to the strong Mg^{2+} block of NMDAR seen at −80 mV (Ascher et al., 1988). The kinetics of AMPAR-mediated and NMDAR-mediated currents evoked by 70 μM L-glutamate at −20 mV are shown in Fig. 2B as the currents before and after addition of 100 μM NBQX. In four cells at −80 mV the AMPAR-mediated current rose with half rise time τ_{1/2} = 0.59 ± 0.19 ms comparable with data in the same conditions from cerebellar granule cells (τ_{1/2} (ms) = 0.74 ± 0.13, n = 8), and NMDAR-mediated currents at −20 mV with NBQX present rose with τ_{1/2} = 12.6 ± 1.0 ms (n = 4). In hippocampal neurones, the decay of AMPAR-mediated currents evoked by release of 70 μM L-glutamate at −80 mV was typically characterised by a fast phase due to receptor desensitisation (τ = 40 ± 20 ms, N = 5, single exponential fit), but the baseline current was restored slowly in more than 1 min because of the persistence of L-glutamate in the photolyzed region.

3.2.1. Tests for antagonist actions of Nl-caged L-glutamate

A straightforward test for effects of Nl-caged L-glutamate itself on AMPAR and NMDAR was to vary the concentration of cage present while releasing the same, sub-maximal concentration of L-glutamate. This was done by varying the intensity of the flash with neutral density filters at different cage concentrations to release the same L-glutamate concentration on the same cell. Fig. 3 shows AMPAR-mediated currents at −80 mV (A) and NMDAR-mediated currents at −20 mV with 100 μM NBQX (B) evoked by the release of approximately 40 μM L-glutamate from 250 μM Nl-caged L-glutamate, or from 1 mM Nl-caged L-glutamate with the flash intensity reduced to 25% by a neutral density filter. The currents have the same amplitude. In four cells tested for each type of response, the ratio of the current recorded at 250 μM Nl-caged L-glutamate and that recorded at 1 mM Nl-caged L-glutamate with 25% intensity was 1.1 ± 0.1 for AMPAR-mediated currents and 1.1 ± 0.3 for NMDAR-mediated currents, indicating that there is no antagonism due to the higher concentration of NI-cage present with the low intensity photolysis.

NI-caged L-glutamate was tested further by its effects on currents evoked by L-glutamate iontophoretically applied at AMPAR and NMDAR. Sub-maximal responses to iontophoretic pulses were elicited in the presence and absence of 500 μM NI-caged glutamate in hippocampal neurones and of 1 mM NI-caged L-glutamate in cerebellar granule cells. Fig. 4A shows the effect of adding 500 μM NI-caged L-glutamate on currents activated in a hippocampal neurone at −80 and −20 mV holding potential. No change in the amplitude or in the kinetics of the current was observed (n = 2 cells).

Photolysis of the control compound, NI-caged-5-dihydroxyphosphoryloxypentanoate (Scheme 1; Papa-georgiou et al., 1999) releases an inert phosphorylated carboxylate species by the same photochemical reaction as release from Nl-caged neurotransmitters. Fig. 4B shows the effect of photolysis of the control caged compound at 500 μM during currents activated in a hippocampal neurone at −80 and −20 mV holding potential by L-glutamate iontophoresis. The photochemical event did not modify the time course or amplitude of the currents, indicating that the intermediates and products of photolysis do not affect AMPAR or NMDAR responses (n = 2 cells). Similar results were also obtained in cultured cerebellar granule cells (n = 7, data not shown).

3.2.2. Tests for effects of Nl-caged L-glutamate on synaptic transmission

The possibility that Nl-caged L-glutamate may interfere with synaptic mechanisms, particularly transmitter release, was tested at climbing fibre synapses in Purkinje neurones in cerebellar slices. This preparation is particular suitable because each Purkinje neurone has one climbing fibre synapse and therefore, a well-defined threshold for activation (Eccles et al., 1964; Konnerth et al., 1990). Furthermore the climbing fibre synapse has a large number of active sites, reducing fluctuations in the synaptic responses resulting from the stochastic nature of the release process and shows a strong depression with repeated activation. Experiments were made with 200 nM NBQX present to reduce EPSC amplitudes. The protocol used for stimulation comprised four pulses at 50 ms interval delivered every 20 s. Fig. 5B shows ten consecutive recordings of the 4 EPSCs showing almost no fluctuations from their mean. Addition of 1 mM NI-caged L-glutamate did not change the amplitude of the EPSCs, the depression or the excitation threshold, indicating that it did not affect presynaptic processes. Also, photolysis of the control NI-caged-5-dihydroxyphosphoryloxypentanoate during periods of stimulation had no effect (data not shown).
3.3. NI-caged GABA and glycine

The release of GABA and glycine by photolysis of NI-caged compounds and the toxicities of the caged precursors were tested on cultured hippocampal neurones and cultured spinal cord neurones, respectively, with CsCl based internal solution.

3.3.1. Caged GABA

Photorelease of GABA (70 μM) on hippocampal neurones evoked a current mediated by GABA_A receptors (GABA_A) and reversibly blocked by 50 μM bicuculline (Fig. 6A). The τ_{1/2} of GABA_A-mediated currents evoked by 70 μM GABA released from 500 μM NI-caged GABA was 26.1 ± 20.3 ms (n = 8) (Fig. 6B).

The actions of NI-caged GABA on GABA_A-mediated currents were tested by comparing responses elicited by 40 μM GABA released from 250 μM or 1 mM NI-caged GABA (Fig. 6C). The ratio of GABA current at 1 mM to that at 0.25 mM NI-caged GABA was 0.44 ± 0.20 (S.D., n = 4). The inhibitory effect of 500 μM NI-caged GABA on responses to iontophoretic GABA application is shown in Fig. 6D, and in three cells showed significant depression by more than 30% of control (P < 0.01, n = 10 currents, t-test). Furthermore, spontaneous currents recorded in hippocampal neurones in the presence of NBQX (10 μM) and TTX (0.5 μM) were significantly (P < 0.001, t-test) reduced in amplitude by more than 50% in the presence of 500 μM NI-caged GABA (Fig. 6E). In contrast, addition of NI-caged glutamate or MNI-caged glutamate (1 mM) did not significantly change the amplitude of GABA_A-mediated spontaneous currents (data not shown). Thus, NI-caged GABA, but not NI- or MNI-caged glutamate, is an antagonist at GABA_A.

3.3.2. Caged glycine

Fig. 7 summarises similar experiments made with spinal neurones to test NI-caged glycine as a source of photoreleasable glycine. Photorelease of glycine (70 μM) evoked a Cl-current blocked reversibly by 20 μM strychnine (Fig. 7A). GlyR-mediated currents were activated with time course of τ_{1/2} 12.3 ms ± 4.3 (n = 6) in these conditions (Fig. 7B). The actions of NI-caged glycine as an antagonist were tested with release of 80 μM glycine from either 500 μM or 2 mM NI-glycine. A ratio of 0.32 ± 0.22 (n = 4) was found between GlyR-mediated currents at high 2 mM to low 0.5 mM NI-glycine. Furthermore, 500 μM NI-caged glycine was found significantly (P < 0.01, n = 10 currents, t-test) to inhibit currents evoked by iontophoretically applied glycine by more than 30% in two cells (Fig. 7C). Thus, NI-caged glycine is an antagonist at GlyR.

3.4. 4-Methoxy-7-nitroindolinyl-caged L-glutamate

The 4-methoxy-7-nitroindolinyl-caged L-glutamate (MNI-caged L-glutamate) was investigated photochemically as one of a series of aromatic substitutions in NI-caged compounds, and found to be 2–3 times more efficient in releasing L-glutamate than NI-caged L-glutamate (Papageorgiou et al., 2000). L-Glutamate release from MNI-caged L-glutamate was also found to be more efficient when tested on hippocampal neurones in culture. MNI-glutamate was tested as an antagonist at glutamate receptors in the same way as NI-glutamate. The current evoked at 1 mM MNI-glutamate with intensity attenuated 4 fold was compared with that at 250 μM MNI-glutamate with no attenuation and gave a ratio of 1.2 ± 0.2 (S.D., n = 4, Fig. 8A). The rise time had τ_{1/2} = 0.78 ms ± 0.12, n = 6) with a 1 ms photolysis pulse, similar to data with NI-caged L-glutamate. Addition of 500 μM MNI-caged L-glutamate did not change the amplitude or the kinetics of AMPAR or NMDAR currents evoked by iontophoresis of L-glutamate at −80 and −20 mV holding potentials (n = 2 cells, Fig. 8B). These results indicate that MNI-caged L-glutamate, like NI-caged L-glutamate, has no action at AMPAR and NMDAR.

4. Discussion

7-Nitroindolinyl and 4-methoxy-7-nitroindolinyl caged neurotransmitters are thermally stable, fast and efficient sources of free ligands. Photorelease from MNI-caged L-glutamate is about 2.5 times more efficient than NI-caged L-glutamate and is comparable to the release efficiency of NPE-ATP. NPE-caged phosphates, such as NPE-ATP, are among the most efficiently photolysed caged compounds in common use. Neurotransmitters are released from the nitroindolinyl cages with rates >3000 s⁻¹ fast enough to be useful on a synaptic time scale. Release of L-glutamate at 50–200 μM concentration with a 1 ms flashlamp pulse produced activation of AMPAR-mediated currents with half rise time <1 ms, and slower activation of NMDAR-mediated currents, typically more than 10 ms, resulting from the slower gating kinetics of this channel. No evidence was found for interference by NI-caged glutamate at 1 mM with glutamate receptor activation, synaptic transmission or excitability. Furthermore, the photochemical reaction did not interfere with these processes.

In contrast to the nitroindolinyl-caged L-glutamates, NI-caged GABA and glycine both showed interactions of the caged form with the target receptors, producing inhibition and slowing the rate of activation by released GABA or glycine, possibly due to dissociation of the caged compound from receptors. The results indicate
that NL-caged and MNI-caged L-glutamates will prove useful tools in the investigation of synaptic processes. NL-caged GABA and NI-caged glycine also have useful properties of stability, speed and efficiency when compared with available reagents, but are limited by the antagonist actions described here.

Acknowledgements

Supported by the MRC and Marie Curie Fellowship of the European Community (to MC, HPMP-CT-1999-00349). We thank Chris Magnus for cell culture and Dr Ranjit Munasinghe for recording NMR spectra. We thank the MRC Biomedical NMR Centre for access to facilities.

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