Receptor-Mediated Ca^{2+} Signalling in Cultured Rat Cerebellar Granule Cells

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Thesis submitted for the degree of Doctor of Philosophy

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November 1994

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Neurones possess many pathways which can mediate Ca^{2+} entry, and two mechanisms, involving inositol 1,4,5-trisphosphate ($InsP_3$) and ryanodine receptors, by which Ca^{2+} in intracellular stores can be released. Cultured cerebellar granule cells were utilized in this study as a model neuronal system for investigating the relationships between entry and store release, and between the different mechanisms of Ca^{2+} release.

Ca^{2+} entry mechanisms activated by K^+-depolarization and *N*-methyl-D-aspartate (NMDA) were studied using epifluorescence microscopy with the Ca^{2+}-binding dye fura 2, for $[Ca^{2+}]_i$ and Mn^{2+} quench studies. The voltage-operated Ca^{2+} channels involved in the K^+-evoked response were characterized, while the NMDA response was found to be mediated by the receptor channel and to display considerable variability between cell groups. Both agents were found to evoke significant Ca^{2+} release from stores, a phenomenon apparently dependent on Ca^{2+} entry. Interactions between $[Ca^{2+}]_i$ responses evoked by the metabotropic glutamate receptor agonist 1-aminocyclopentane-1S,3R-dicarboxylic acid (ACPD) and NMDA were studied and found to be complex, including a variable ACPD-evoked inhibition of NMDA $[Ca^{2+}]_i$ responses which was apparently mediated via protein kinase C activation.

Carbachol (CCh) acting on muscarinic M_3 receptors evoked a marked elevation of total inositol phosphates, $InsP_3$ mass and $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ response was inhibited by Ca^{2+} store-modulating agents. Both CCh- and NMDA-evoked $[Ca^{2+}]_i$ responses appeared to involve activation of ryanodine receptors, the muscarinic activation of ryanodine receptors probably occurring secondary to Ca^{2+} release via $InsP_3$ receptors. An apparent pharmacological differentiation of the primary stores involved in the two responses suggested that $InsP_3$ receptor-expressing stores may be physically different from ryanodine receptor-expressing stores in these neurones.

CCh, like ACPD, downregulated expression of $InsP_3$ receptors during chronic stimulation, as measured by Western blotting, leading to subsequently decreased responses to both CCh and ACPD, but was apparently without effect on directly ryanodine receptor-mediated responses. After chronic CCh pre-treatment, NMDA responses were unchanged and so may not require significant activation of $InsP_3$ receptors.

Thus several aspects of the complexity of neuronal Ca^{2+} signalling have been elucidated in this study, notably the activation of Ca^{2+} release by Ca^{2+} entry, the activation of both ryanodine and $InsP_3$ receptors by muscarinic stimulation, and the possible expression of at least two different Ca^{2+} stores. These findings may have important implications for our understanding of how neuronal signalling occurs *in vivo*.

Acknowledgements

Thanks are due to many past and present members of this Department for their assistance and advice over the past three years. In particular, the help of Jim, Dave L, Phil, Dave G, Richard, Steve S, Viral and John M is gratefully acknowledged. Particular thanks are due to my supervisors John Challiss and Steve Nahorski for helpful discussions and ideas, and thanks to John for his involvement in the $InsP_3$ mass and $InsP_3$ binding experiments contained in this Thesis. Also thanks to the members of Lab 324, Dave A, Karen, Raj and Alan, for making life more bearable. Raj's singing and musical taste were of great help in encouraging me to spend more time on the PTI than would otherwise have been humanly possible. Thanks to Marguerite for desk-tidying beyond the call of duty.

Also, to the people who have lived with me in Leicester, Cath, PJ, Neil, Colin, Paul and Karen, despite none of you reaching my high tidiness standards its been fun. Also thanks to my other drinking companions and friends, including Keena, Kath, Helen, Sue, Alison, Graham, Clare, Rob and Katie. Emma, I should have listened to you. Thanks to the Wellcome Trust for their generous financial support.

I am especially grateful to my parents for their great love and support over many years.

For my parents.

Figures

preceding text page

Tables

1. Introduction

1.1. Regulation of $[Ca^{2+}]_i$

Ca^{2+} is a cation with a small ionic radius and relatively high charge, allowing the formation of tight bonds between Ca^{2+} and a variety of proteins, and thus enabling it to play a key messenger role in cell signalling. Initial evidence for specific mechanisms facilitating Ca^{2+} entry into cells was obtained in the 1950s (Fatt and Ginsborg, 1958), and subsequent experiments demonstrated that Ca^{2+} could be actively transported from the cytoplasm into organelles (MacLennan, 1970), thereby establishing the existence of mechanisms for the regulation of the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$). It is now apparent that cells maintain a much lower concentration of free Ca^{2+} in the cytoplasm than in specialized Ca^{2+} stores found within organelles such as the endoplasmic reticulum, or in the extracellular environment, by means of a low resting Ca^{2+}-permeability of plasma and organellar membranes, active pumping mechanisms, and a large buffering capacity (see Carafoli, 1987). Thus even minor increases in the number of open Ca^{2+}-permeable channels in the plasma membrane or endoplasmic reticulum membrane can produce rapid and marked elevations of $[Ca^{2+}]_i$. Such elevations cause a range of effects on cell behaviour, over both the short and long term. Physiological consequences of elevated $[Ca^{2+}]_i$ include activation of enzymes including many kinases, initiation and maintenance of cellular plasticity, regulation of cell growth and development, and stimulation of hormone or transmitter release (Carafoli, 1987; Henzi and MacDermott, 1992; McCleskey, 1994). Ca^{2+} signalling in neurones is crucial to sensation, communication, and responsiveness in the peripheral and central nervous system, and involves Ca^{2+} entry, Ca^{2+} release from stores, or both mechanisms in combination, as discussed below.

1.2. Metabotropic receptor-mediated $[Ca^{2+}]_i$ elevation

1.2.1 Phosphoinositide signalling

In 1975, Michell hypothesized that agonist-evoked breakdown of membrane inositol phospholipids might release a soluble messenger molecule which mobilizes Ca^{2+} from stores inside cells. It was subsequently confirmed that inositol 1,4,5-trisphosphate ($InsP_3$), produced by the breakdown of phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$) by phosphoinositidase C (PIC), could do precisely this ($InsP_3$-induced Ca^{2+} release (IICR)) (Streb *et al.*, 1983), through an action on specific organellar receptors (Fig. 1.1A,B). These $InsP_3$ receptors ($InsP_3Rs$) have subsequently been purified and cloned (Supattapone *et al.*, 1988; Furuichi *et al.*, 1989), and the pathway of $PtdInsP_2$ metabolism is now known in considerable detail (Fig. 1.2). Recently it has been suggested that a second inositol polyphosphate metabolite, inositol 1,3,4,5-tetrakisphosphate ($InsP_4$)(see Fig. 1.2), may be able to release Ca^{2+} via $InsP_3Rs$ (Wilcox and Nahorski, 1994). PIC activation of $InsP_3$ production from $PtdInsP_2$ also causes synthesis of another important second messenger, diacylglycerol (DAG)(Fig. 1.2), which activates protein kinase C (PKC).

1.2.2. Muscarinic receptors

It has long been known that acetylcholine (ACh) can evoke both rapid depolarization, which can be replicated by nicotine, and a variety of slower-onset responses, which can be reproduced using muscarine. In 1986, cDNAs for 2 muscarinic receptor subtypes, now known as m1 and m2, were purified and cloned (Kubo *et al.*, 1986a,b). By low stringency screening with an oligonucleotide probe based on part of the m1 sequence, 3 further subtypes were identified, m3-m5 (Bonner *et al.*, 1987,1988). Muscarinic receptors possess homology with many other known receptors e.g. ~35% homology with α-adrenoceptors, and belong to the 7

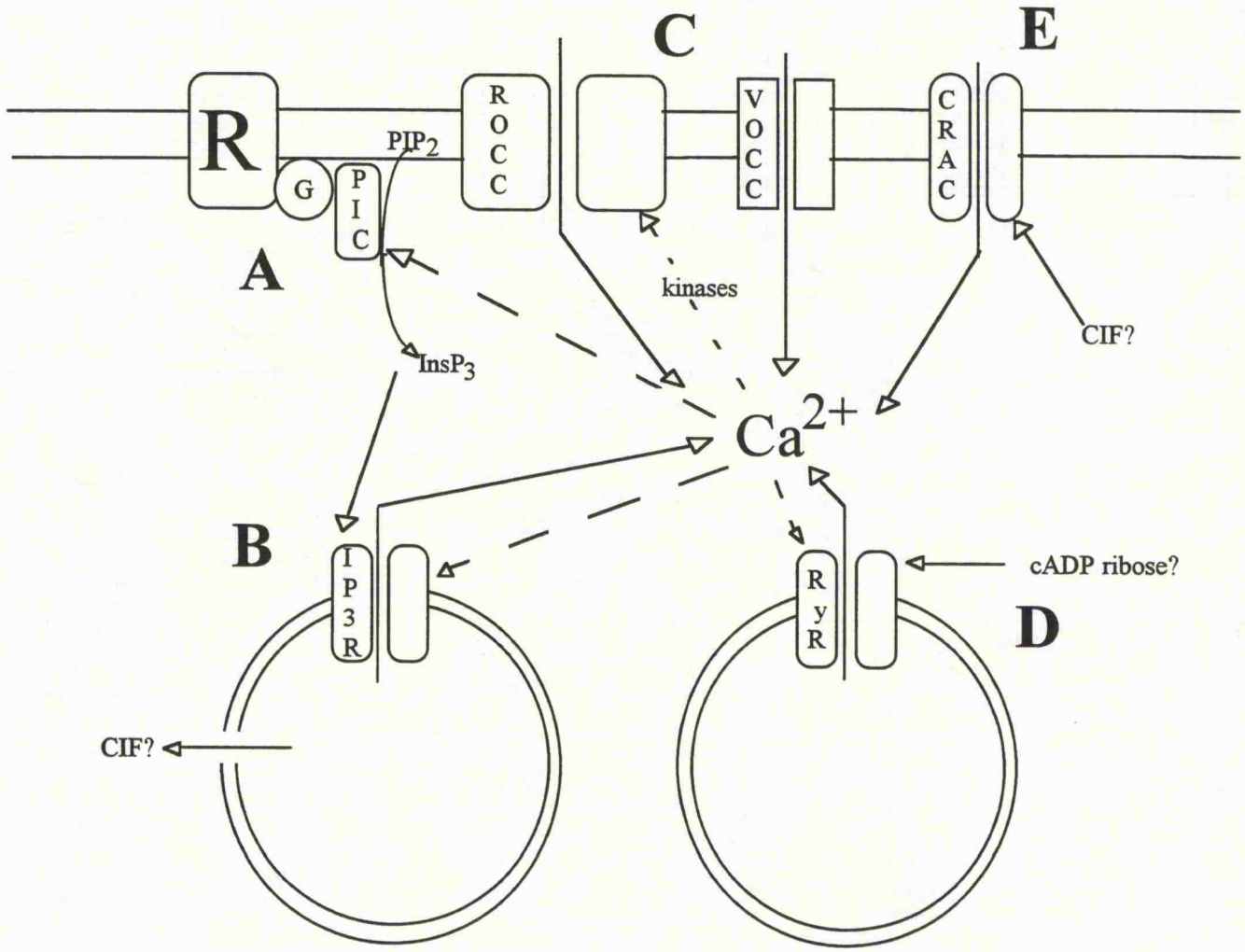

C
E
R
G
PIC
PIP2
ROCC
VOCC
CRAC
A
kinases
CIF?
InsP3
Ca2+
B
IP3R
RyR
cADP ribose?
D
CIF?

Fig. 1.1 General model of Ca^{2+} signalling mechanisms. *A)* Metabotropic receptors (R) can evoke elevation of $[Ca^{2+}]_i$ by activating phosphoinositidase C (PIC) via a G protein-mediated mechanism (G) to cause phosphodiesteric cleavage of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to yield the second messenger inositol 1,4,5-trisphosphate ($InsP_3$); Ca^{2+} can potentiate PIC activity. *B)* $InsP_3$ evokes Ca^{2+} release from stores via activation of specific organellar receptors at which Ca^{2+} can act as a co-agonist. Depletion of Ca^{2+} stores may cause release of an unidentified Ca^{2+} influx factor (CIF). *C)* Ca^{2+} entry into cells can occur via receptor-operated Ca^{2+} channels (ROCCs) i.e. Ca^{2+} permeant channels which are intrinsic to receptors, such that receptor activation results in a conformational change leading to Ca^{2+} entry. Also, membrane depolarization due to e.g. action potentials or Na^+ entry causes activation of voltage-operated Ca^{2+} channels (VOCCs), a parallel mechanism of Ca^{2+} entry. $[Ca^{2+}]_i$ elevation can feedback in a positive or negative manner on ROCCs and VOCCs by activation of enzymes, particularly kinases. *D)* Cytoplasmic Ca^{2+} can also evoke Ca^{2+} release from stores via another class of organellar release channels, ryanodine receptors (RyRs). cADP ribose has been postulated to act as a modulator or co-agonist of these receptors. *E)* Ca^{2+} store depletion causes activation of Ca^{2+} entry via another type of Ca^{2+}-permeant plasma membrane channel, Ca^{2+} release activated Ca^{2+} (CRAC) channels.

For initial ideas regarding this figure I am indebted to a scheme outlined by Dr. G. Willars (unpublished).

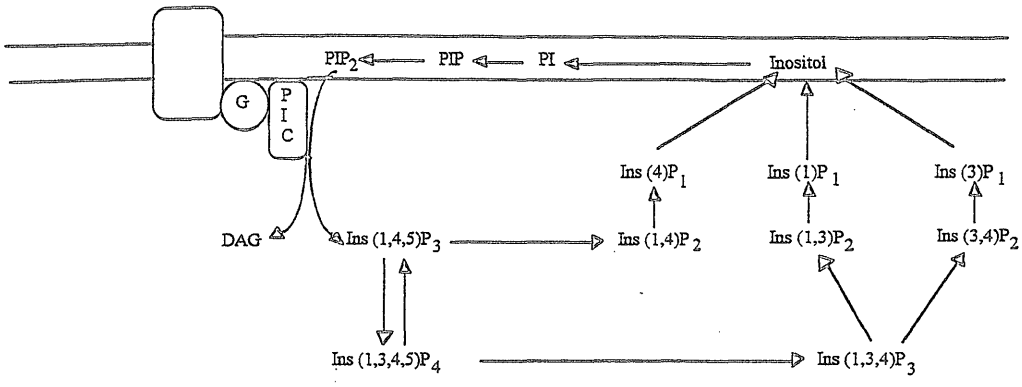


Fig. 1.2 Pathway of PIP_2 metabolism. On activation of receptors, PIP_2 is broken down by PIC to yield the second messengers DAG and Ins $(1,4,5)P_3$. Ins $(1,4,5)P_3$ is dephosphorylated to yield Ins $(1,4)P_2$ or phosphorylated to form Ins $(1,3,4,5)P_4$. Ins $(1,4)P_2$ is further dephosphorylated, eventually yielding inositol, which can be reincorporated into membrane phospholipids. Ins $(1,3,4,5)P_4$ can be converted into higher phosphates (not shown), or broken down via bisphosphates and monophosphates to eventually also yield inositol for reincorporation.

transmembrane domain superfamily of guanosine triphosphate (GTP) binding protein (G protein)-coupled receptors (Hulme *et al.*, 1990). Interaction between such receptors and G proteins causes guanosine diphosphate (GDP) / GTP exchange, which leads to the dissociation of heterotrimeric GDP-bound G proteins and the activation of GTP-bound G_{α} subunits (Rhee and Choi, 1992; Berridge, 1993). G_{α} initiates intracellular responses by interacting directly with ion channels or by altering the activity of effector enzymes (Rhee and Choi, 1992). Recombinant m2 and m4 receptors can inhibit adenylyl cyclase activity, whereas m1, m3 and m5 activate PIC to cleave $PtdInsP_2$ into $InsP_3$ and DAG, although a degree of receptor promiscuity has been reported at high expression levels (see Hulme *et al.*, 1990; Caulfield, 1993). Multiple receptor domains are involved in G protein coupling, but the third intracellular loop appears to control specificity of coupling (see Wess, 1993). The third intracellular loop of muscarinic receptors also possesses putative consensus sequences for phosphorylation, which may be important in agonist-evoked receptor desensitization, functionally mediated by an unidentified receptor kinase (Tobin *et al.*, 1992; Tobin and Nahorski, 1993; A. Tobin and S. Nahorski, unpublished). Muscarinic m1 or m3 receptor stimulation typically causes $G_{\alpha q}$ to bind to, and thus activate, $PIC_{\beta 1}$, the PIC subtype most selective for $PtdInsP_2$ (Rhee and Choi, 1992). $G_{\beta\gamma}$ subunits are now known to also mediate intracellular responses (see Caulfield, 1993).

Agonists for muscarinic receptors include the non-selective cholinergic agonist carbachol (CCh), and the putative muscarinic receptor-selective agent methacholine, while a variety of antagonists display partial selectivity for the different subtypes, such as pirenzepine at m1, methoctramine and AF-DX 116 at m2, and 4-diphenylacetoxy-*N*-methylpiperidine methoiodide (4-DAMP) at m3 (see Hulme *et al.*, 1990; Richards, 1991; Caulfield, 1993). Atropine remains widely used as a broad-spectrum muscarinic antagonist. Muscarinic receptors have a widespread distribution in brain as in many other tissues, mRNA for all 5 subtypes having been detected in brain, with differing but

overlapping distributions (see Caulfield, 1993). The phosphoinositide (PI)-linked subtypes M_1 and M_3 are predominantly, though not exclusively, localized in the forebrain (Hulme *et al.*, 1990; Caulfield, 1993). Muscarinic receptor-evoked inositol phosphate generation (Brown *et al.*, 1984; Xu and Chuang, 1987; Whitham *et al.*, 1991a) and $[Ca^{2+}]_i$ mobilization (Kudo *et al.*, 1988; Reynolds and Miller, 1989; Irving *et al.*, 1992a) have been demonstrated in several types of neurone. In the $[Ca^{2+}]_i$ studies, a response was present in either normal $[Ca^{2+}]$-containing or Ca^{2+}-free medium, suggesting that the overall $[Ca^{2+}]_i$ response results at least partly from release of intracellular stores.

1.2.3 Metabotropic glutamate receptors

Glutamate evokes many different responses in neurones, including effects which are central to neuronal plasticity e.g. long-term potentiation (LTP)(Bliss and Collingridge, 1993) and long-term depression (LTD)(Linden and Connor, 1993). The ability of glutamate to stimulate PI hydrolysis much more potently than ionotropic glutamate receptor-selective agonists was first demonstrated by Sladeczek and co-workers (1985). Glutamate is now known to act on several types of receptor, which are subdivided on the basis of response characteristics and selectivity of action of agonists into the ionotropic *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)/kainate receptors, and metabotropic receptors, for many of which 1-aminocyclopentane-1S,3R-dicarboxylic acid (ACPD) is a selective agonist.

Expression cloning of a rat cerebellar cDNA library led to the identification of a metabotropic glutamate receptor (mGluR), now known as mGluR1α, which could couple to PI turnover (Masu *et al.*, 1991; Houamed *et al.*, 1991). The sequence of this receptor was found to contain 7 putative transmembrane domains, like many other G

protein-coupled receptors, but lacked significant homology with them. From numerous studies using low stringency hybridization screening with mGluR1α probes, it has become apparent that many mGluRs exist, forming a new receptor family (see Hollman and Heinemann, 1994). Unusually, the second rather than third intracellular domain of mGluRs, along with a segment downstream of the seventh transmembrane domain, may be important for G protein coupling (Pin *et al.*, 1994). Within this family, subgroups can be identified with higher sequence homology, similar relative agonist potencies, and coupling to the same second messenger responses (Schoepp and Conn, 1993; Hollmann and Heinemann, 1994; Saugstad *et al.*, 1994). mGluRs 2 and 3 are potently activated by ACPD, but are relatively insensitive to quisqualate (Quis), whereas mGluRs 4, 6 and 7 are relatively insensitive to both ACPD and Quis, and are preferentially stimulated by L-1-amino-4-phosphobutanoic acid (L-AP4)(see Nakanishi, 1992; Schoepp and Conn, 1993; Westbrook, 1994). Both of these subgroups are coupled, at least in transfected cell lines (see Prezeau *et al.*, 1994), to inhibition of cAMP formation (Hollmann and Heinemann, 1994; Saugstad *et al.*, 1994). The subgroup of primary interest in the present study comprises the three splice variants of mGluR1 (α, β and c), and mGluR5, which are all coupled to PI hydrolysis. These subtypes are widely expressed in brain, with overlapping distributions (see Hollmann and Heinemann, 1994).

A weak partial agonist, 2-amino-3-phosphopropionate (L-AP3), has been reported to antagonise ACPD-evoked PI and $[Ca^{2+}]_i$ responses (see Irving *et al.*, 1990; Schoepp *et al.*, 1990), but use of this compound may be complicated by apparent agonist actions at other receptor types (Mistry *et al.*, 1994). Newer, more selective agonists and antagonists may help to distinguish between the effects of the various metabotropic receptor subtypes, including partial selectivity of (2S,3S,4S)-α-(carboxycyclopropyl)glycine (L-CCG-I) as an agonist of mGluR2 (Nakanishi, 1992), and of antagonists such as (+)α-methyl-4-carboxyphenylglycine ((+)MCPG) for

mGluR1 and 2 (Jane *et al.*, 1993; Hayashi *et al.*, 1994), α-methyl-2-amino-4-phosphopropionate (MAP4) for an unknown subtype of L-AP4 receptors (Jane *et al.*, 1994), and α-methyl-L-CCG-I for mGluR2 (Jane *et al.*, 1994). Two additional, as yet uncloned, mGluRs are reportedly expressed in astrocytes (Hollmann and Heinemann, 1994; Prezeau *et al.*, 1994), and other subtypes in neurones may also remain to be identified (Jane *et al.*, 1994; Hollmann and Heinemann, 1994).

1.3 Ionotropic mechanisms of $[Ca^{2+}]_i$ elevation

1.3.1 Voltage-operated Ca^{2+} channels

Membrane depolarization causes the opening of channels with a high selectivity for Ca^{2+} over other available cations, called voltage-operated Ca^{2+} channels (VOCCs), which permit Ca^{2+} flux down its concentration gradient and into the cell (Fig. 1.1C). This is typically evoked *in vivo* as a secondary consequence of Na^+ entry through other channels, such as are found intrinsic to some receptors, and experimentally by a range of methods including an increase in the extracellular potassium ion concentration ($[K^+]_o$). Different types of VOCC are now known: the low threshold voltage-activated T channels, and an ever-increasing array of high threshold voltage-activated subtypes. The best characterized of these pharmacologically are L channels, which are inhibited by verapamil and either inhibited or activated by various 1,4-dihydropyridines (DHPs); N channels, which are inhibited by the snail toxin ω-conotoxin GVIA; and P channels, which are inhibited by components of funnel web spider toxin which include ω-Aga-IVA (see Tsien and Tsien, 1990; Tsien *et al.*, 1991). These high voltage-activated subtypes have related but differing activation requirements, distributions within brain, and localizations within neurones, such that the subtype involved in a given neuronal response appears to depend heavily on the type of neurone and the experimental conditions used (McCleskey, 1994).

1.3.2 NMDA receptors

1.3.2.1 Structure

NMDA receptors (NMDARs), which are formed by the combination of several, probably five (see McBain and Mayer, 1994; Westbrook, 1994) subunits, possess an intrinsic ion channel permeant to Na^+, K^+ and Ca^{2+}. Thus when activated NMDAR channels permit direct entry of Ca^{2+} into neurones (Mayer *et al.*, 1987) and, by causing depolarization, can activate Ca^{2+} entry through VOCCs (Mayer and Miller, 1990)(see Fig. 1.1C). NMDAR-mediated Ca^{2+} entry is central to many forms of LTP (Bliss and Collingridge, 1993), and is an important cause of neurodegeneration both in culture and *in vivo* (Choi *et al.*, 1988; Finkbeiner and Stevens, 1988; Lysko *et al.*, 1989; Manev *et al.*, 1989; Michaels and Rothman, 1990; Frandsen and Schousboe, 1992; Hartley *et al.*, 1993). Expression cloning enabled the isolation of the first cDNA clone for a NMDAR subunit (Moriyoshi *et al.*, 1991), subsequently known as NMDAR1, which like many other ligand-gated ion channels may contain 4 putative transmembrane domains (Moriyoshi *et al.*, 1991; but see below). When expressed in *Xenopus* oocytes, NMDAR1 forms functional homomeric receptors possessing many properties which are highly characteristic of NMDARs *in vivo* (Moriyoshi *et al.*, 1991). 9 splice variants of NMDAR1 have been identified, as combinations of 3 independent splicing possibilities, which differ in agonist and antagonist potencies, size of currents through homomeric receptors, Zn^{2+} modulation and PKC modulation (see Hollmann and Heinemann, 1994; McBain and Mayer, 1994).

More recently, 4 cDNAs for other NMDARs (NMDAR2A-D) have been identified, with low homology to previously identified glutamate receptors (GluRs) e.g. 26-27% homology to NMDAR1, but with 42-56% homology to each other, thus constituting a new GluR gene subfamily (Hollmann and Heinemann, 1994). These encode larger proteins than NMDAR1, but in a similar manner include 4 putative

transmembrane domains. When transfected into oocytes, none of the NMDAR2s form homomers or form heteromers with only each other, but all can form heteromers with NMDAR1. These heteromeric receptors have currents ~100 fold larger than NMDAR1 homomers (Burnashev, 1993), and are thus more comparable to *in vivo* NMDAR channels. In addition, heteromeric NMDARs appear to be more readily expressed than NMDAR1 homomers (Nakanishi, 1992). The different subunit combinations vary in terms of channel conductance, open time, decay time and PKC modulation (Hollmann and Heinemann, 1994), indicating that the type or types of NMDAR expressed in a neurone will markedly affect the glutamate-evoked response. NMDAR1 mRNA is very widely expressed in the CNS, consistent with the apparent necessity of its expression for functional NMDA channels. There is, however, differential regulation of expression of the various splice variants, such that for instance in cerebellum, NMDAR1s with an N-terminal insertion are 5-fold more abundant than NMDAR1s lacking this insertion, whereas this prevalence is almost exactly reversed in the rest of brain. NMDAR2A mRNA is almost as widely expressed as NMDAR1, but the expression of mRNAs for the other NMDAR2 subunits is more localised: 2B is absent in thalamus, amygdala, hypothalamus, brainstem and cerebellum, 2D is prominent mainly in brainstem, cerebellum and olfactory bulb, and 2C is found almost exclusively in the granule cell layer of the cerebellum (Nakanishi, 1992; Monyer *et al.*, 1994; Hollmann and Heinemann, 1994).

1.3.2.2 Modulation

NMDARs are known to possess a variety of modulatory sites, for Mg^{2+}, glycine, Zn^{2+}, polyamines, Ca^{2+}, nitric oxide (NO), H^{+}, and redox (Wroblewski and Danysz, 1989; Sucher and Lipton, 1991; Legendre *et al.*, 1993; Lipton *et al.*, 1993; Pritchard *et al.*, 1994; McBain and Mayer, 1994)(Fig. 1.3). A number of potent and selective competitive and non-competitive antagonists have been developed, notably 2-

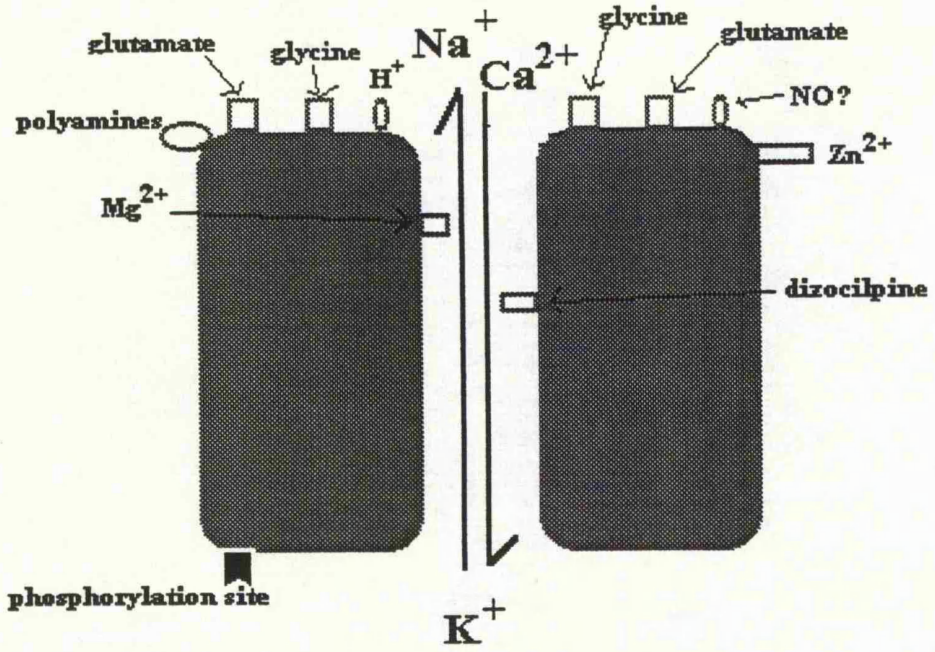


Fig. 1.3 Sites of activation and modulation of NMDA receptors. A wide variety of molecules can affect NMDAR activity, at extracellular, channel or intracellular sites, causing activation, potentiation or inhibition of responses. Most interactions and modifications occur at defined or undefined domains of NMDAR1. For clarity, the likely heteromeric sub-unit assembly of NMDARs is simplified in this figure.

amino-5-phosphonopentanoate (APV), a competitive antagonist at the glutamate binding site (Davies *et al.*, 1981), dizocilpine, which blocks the receptor channel (Wong *et al.*, 1986), and kynurenate derivatives such as 7-chlorokynurenate and 5,7-dichlorokynurenate (dcKyn), which are competitive antagonists at the glycine site (Baron *et al.*, 1990,1992).

Voltage-dependent Mg^{2+} block of NMDAR channels has been shown to be the mechanism by which NMDAR activity is affected by membrane potential (Nowak *et al.*, 1984; Mayer *et al.*, 1984; Mayer *et al.*, 1987), depolarization favouring removal of the impermeant cation Mg^{2+} from the NMDAR channel. An asparagine residue in the second putative transmembrane domain is involved in controlling the divalent cation permeability properties of NMDAR1 channels (Hollmann and Heinemann, 1994). An asparagine residue in the putative second transmembrane domain of NMDAR2s also modulates cation permeability, by strongly affecting Mg^{2+} though not Ca^{2+} permeability, unlike the equivalent NMDAR1 residue which affects Ca^{2+} permeability more than Mg^{2+} permeability (see Hollmann and Heinemann, 1994). In associative or co-operative LTP, a given synapse is potentiated if a presynaptic neurone releases sufficient glutamate at the same time as the postsynaptic dendrite is depolarized, by glutamatergic or other mechanisms. Thus depolarization-dependent Mg^{2+} block is a key reason for the ability of NMDARs to participate in LTP induction (Bliss and Collingridge, 1993).

The glycine site, which has been shown to reside on the same subunit as the glutamate recognition site, NMDAR1 (Moriyoshi *et al.*, 1991), is by far the best characterised of the non-glutamate binding sites shown in Fig. 1.3 (see Kemp and Leeson, 1993). Glycine appears to be a co-agonist rather than a modulator of the receptor, as occupation of the glycine binding site on NMDARs expressed in *Xenopus* oocytes has been found to be essential for receptor activation (Kleckner and

Dingledine, 1988). Several groups have suggested that antagonists of the glycine site may be useful clinically in a variety of conditions in which the NMDAR is implicated, as they possess the anticonvulsant and anxiolytic properties seen with other NMDA antagonists, but display a substantially different behavioural profile and may lack the side-effects of blockers of the NMDAR channel (Sheardown *et al.*, 1989; Singh *et al.*, 1990a,b; Kehne *et al.*, 1991). At least some of the effects of glycine can be ascribed to allosteric interactions between the glycine and glutamate binding sites (Monaghan *et al.*, 1988; Lester *et al.*, 1993; Grimwood *et al.*, 1993). It has been thought that *in vivo* the NMDAR glycine site may normally be fully saturated, as glycine is present at micromolar levels in cerebrospinal fluid (Ferraro and Hare, 1985). However, direct measurement of the synaptic concentration is not yet technically possible, and experiments such as the ionophoresis of NMDA with and without exogenous glycine in slice preparations (e.g. Garthwaite *et al.*, 1987) may be compromised by tissue damage and consequent release of glycine, thus rendering conclusions from such studies concerning normal glycine levels unreliable. There is evidence that at some cerebellar synapses the glycine site may not be normally saturated, as synaptic responses in cerebellar granule cells in slice preparations, mediated by NMDARs, were only seen during perfusion of exogenous glycine (D'Angelo *et al.*, 1990). Also, intracerebellar injection of the glycine site agonist D-serine potentiates the effect of endogenous glutamate on cerebellar cyclic guanosine monophosphate (cGMP) levels (Wood *et al.*, 1989). A high-affinity glycine uptake carrier is present in the cerebellum, mainly on Golgi cell axon terminals in the granule cell layer, which could regulate the concentration in the synaptic cleft (Wilkin *et al.*, 1981).

Putative consensus sites for PKC and Ca^{2+}/calmodulin-dependent protein kinase type II (CamK-II) phosphorylation were initially identified from the amino acid sequences of NMDAR1 and R2 subunits (Moriyoshi *et al.*, 1991; Nakanishi, 1992). CamK-II phosphorylation has been suggested to play a role in sustaining NMDA

channel currents (Sombati *et al.*, 1993), while PKC has been found to phosphorylate several loci on NMDAR1, most of which are within a single region in the C-terminus domain (Tingley *et al.*, 1993), with complex consequences on NMDAR-mediated responses. This region is an alternatively spliced exon, suggesting that the susceptibility of NMDARs to phosphorylation *in vivo* may vary greatly, depending on which variants are expressed. PKC phosphorylation of the C terminus implies an intracellular location for this region, necessitating revision of the 4 transmembrane domain model for NMDAR structure. Thus, either 5 such domains may be present, or possibly the fourth domain may be present in the membrane without completely traversing it (Tingley *et al.*, 1993). Stimulation of the NMDAR itself, via elevation of $[Ca^{2+}]_i$, can increase activity of Ca^{2+}-dependent kinases (Cambray-Deakin *et al.*, 1990; Favaron *et al.*, 1990; Balazs *et al.*, 1992)(Fig. 1.1C). In recombinant NMDARs expressed in *Xenopus* oocytes, PKC activation was found to potentiate NMDA responses, although this was highly dependent on the subunit combination and splice variant of the receptor expressed, some being markedly potentiated by the phorbol ester phorbol 12-myristate 13-acetate (PMA), but others being unaffected (Kutsuwada *et al.*, 1992; Durand *et al.*, 1993). However, the effects of PKC phosphorylation on NMDAR activity in neurones remains controversial. In a number of reports upregulation of activity of PKC has been shown to increase activity of NMDARs (Chen and Huang, 1992; Kelso et al, 1992; Anikstejn *et al.*, 1992; Durand *et al.*, 1993; Kitamura *et al.*, 1993; Murphy *et al.*, 1994). Other reports, however, have demonstrated an apparent inhibitory action of PKC-activating agents on NMDA-evoked $[Ca^{2+}]_i$ responses (Courtney and Nicholls, 1992; Snell *et al.*, 1994).

1.3.3 AMPA receptors

Addition of Quis or AMPA to many neuronal preparations evokes a membrane depolarization due to Na^+ entry, and consequently activation of Ca^{2+} entry via

VOCCs (Mayer and Miller, 1990). These responses were found to be competitively antagonized by various quinoxaline derivatives, including 6,7-nitro-quinoxaline-2,3-dione (DNQX) and 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX)(Drejer and Honore, 1988; Honore *et al.*, 1990). In 1989, Hollmann and co-workers isolated the first recombinant GluR subunit, subsequently known as GluR1, by expression cloning. From the sequence of that clone, standard homology screening and PCR screening identified 3 further related subunits, GluR2-4. These receptor subunits are all of similar size and display a high degree of homology (68-73%) (Hollmann and Heinemann, 1994). Although, like NMDARs, they possess little sequence homology with non-glutamatergic ligand-gated ion channels, it has been suggested that GluR1-4 have 4 transmembrane domains with extracellular N and C termini (see Hollmann and Heinemann, 1994). Other ionotropic GluR subunits are known, notably several (GluRs 5-7, KA1 and 2) which form receptors for which kainate is a relatively potent agonist. AMPA can evoke a current through some kainate receptor heteromers, but of much smaller amplitude than that gated by AMPA receptor (AMPAR) channels (Gallo et al., 1990; Wisden and Seeburg, 1993). Recent experiments, using a C-terminus-specific antibody (Ab), demonstrated no consequent detectable immunoreactivity in GluR6-expressing cells, but rather significant intracellular immunoreactivity, thus indicating that the C terminus may be intracellular and leading to a modified 5 transmembrane domain model for AMPA/kainate receptors (Taverna *et al.*, 1994). As kainate responses will not be examined in this Thesis, poorly AMPA-sensitive GluR subunits will not be reviewed further.

Each AMPAR subunit exists in two alternative forms ('flip' and 'flop'), alternative splicing leading to different sequences of a 38 amino acid region preceding the fourth putative transmembrane domain. A further degree of diversity in AMPARs is found, as GluR4 undergoes differential splicing at a site in the C terminus (Gallo *et al.*, 1992), although the functional importance of this is unknown. An AMPAR

variation with a more clearly defined function is the unusual RNA editing of GluR2, which converts an encoded glutamine residue in the second putative transmembrane domain into an arginine residue. AMPARs can all form homomers or heteromers when expressed in *Xenopus* oocytes, and GluR1 or GluR3 homomers and GluR1/GluR3 heteromers have been found to be permeant to Ca^{2+} (Hollmann *et al.*, 1991). Heteromeric combinations containing GluR2, however, exhibit no Ca^{2+} entry (Hollmann *et al.*, 1991). When GluR2 was mutated to express a glutamine at the edited site in the second transmembrane domain, as found for GluR1, 3 and 4 at the equivalent site, rather than an arginine residue, Ca^{2+}-permeant receptors containing GluR2 could be formed (Burnashev *et al.*, 1992). It has been hypothesised that GluR1-4 form pentameric receptors, with the second transmembrane domain of each subunit lining the channel, thus enabling this residue to affect divalent cation permeability (and also the rectification properties of the receptor). Most AMPARs in neurones have rectification and Ca^{2+}-permeability properties suggestive of the inclusion of GluR2 subunits (Wisden and Seeburg, 1993; Hollmann and Heinemann, 1994). However, recent studies have indicated that AMPARs expressed in at least some types of both astrocytes and neurones display significant Ca^{2+}-permeability (Linn and Christenson, 1992; Gibbons *et al.*, 1993; Schneggenburger *et al.*, 1993; Reichling and MacDermott, 1993; Brorson *et al.*, 1994; N. Hack and R. Balazs, unpublished).

AMPARs rapidly desensitize on addition of agonist, an adaptation which can be blocked by pre-treatment with the benzothiazidine cyclothiazide (Patneau *et al.*, 1993). GluR1-4 possess consensus sites for phosphorylation by CamK-II, PKC and probably protein kinase A (PKA), apparently enabling these kinases to increase current through AMPARs (McGlade-McCulloh *et al.*, 1993; Wang *et al.*, 1994a; Rosenmund *et al.*, 1994). GluR2 mRNA is nearly ubiquitously distributed in brain, while expression of the other AMPAR mRNAs varies greatly between different types of neurone (Hollmann and Heinemann, 1994).

1.3.4 Toxic effects of Ca^{2+} entry

In 1957, Lucas and Newhouse discovered that administration of monosodium glutamate to neonatal rats caused degeneration of neurones in the inner layer of the retina. Subsequently, accumulating evidence indicated that glutamate satisfied the criteria for classification as a neurotransmitter (see Fonnum, 1984). The ability of glutamate to evoke excitation and to cause toxicity led Olney (1978) to propose that, as well as its important signalling role, synaptically released glutamate could be a major cause of central nervous system (CNS) neurodegeneration, coining the term 'excitotoxicity'. Normally, transient elevations of $[Ca^{2+}]_i$ are buffered in neurones by binding to intracellular proteins, transportation across the plasma membrane by adenosine triphosphatases (ATPases) and Na^+/Ca^{2+} exchangers, and sequestration into organelles (Carafoli, 1987; Manev *et al.*, 1990). Decreased energy levels in e.g. stroke are likely to cause leakage of neurotransmitters and metabolites, including glutamate, out of neurones, and to inhibit the ability of neuronal pumps to successfully control $[Ca^{2+}]_i$ (Novelli *et al.*, 1988; Szatkowski and Attwell, 1994). Elevated $[Ca^{2+}]_i$ could increase neurotransmitter release and thus induce elevation of $[Ca^{2+}]_i$ levels in surrounding neurones. A causal role for elevated synaptic levels of glutamate evoking toxicity via increased $[Ca^{2+}]_i$ has been proposed in various acute neurodegenerative conditions, including stroke, status epilepticus, hypoglycaemic encephalopathy and perinatal hypoxia-ischaemia (see Wieloch *et al.*, 1985; Greenamyre *et al.*, 1987; Choi, 1988; Szatkowski and Attwell, 1994). Circumstantial evidence points to a role for glutamate toxicity in a wide variety of chronic neurodegenerative conditions as well, including Huntington's disease, Parkinson's disease, Alzheimer's disease, AIDS dementia, amyotrophic lateral sclerosis, and lathyrism (see Robinson and Coyle, 1987; Young *et al.*, 1988; Choi, 1988; Giulian *et al.*, 1990; Coyle and Puttfarcken, 1993).

It has been shown that agonists of non-NMDA ionotropic GluRs can mediate neurotoxic effects in culture (Patel *et al.*, 1990; Frandsen and Schousboe, 1992; Puttfarcken *et al.*, 1992; Brorson *et al.*, 1994) in slices (Garthwaite and Garthwaite, 1991) and *in vivo* (McDonald and Schoepp, 1992), but NMDARs appear to be the major mediators of glutamate-evoked toxicity under many conditions (Choi *et al.*, 1988; Finkbeiner and Stevens, 1988; Lysko *et al.*, 1989; Manev *et al.*, 1989; Michaels and Rothman, 1990; Hartley *et al.*, 1993). While activation of mGluRs alone evoked degeneration in some studies (Frandsen and Schousboe, 1992; McDonald *et al.*, 1993), mGluR agonists have been found to cause no significant toxic effects (McDonald and Schoepp, 1992), or indeed protect against the toxic effects of ionotropic GluR agonists (Koh *et al.*, 1991; Birrell *et al.*, 1993), in other studies. Thus, interactions between ionotropic and metabotropic GluRs are likely to lead to complex effects of glutamate on neuronal survival. A further layer of complexity is added by the report that NMDA can also promote neuroprotection under certain conditions (Marini and Paul, 1992).

Elevated $[Ca^{2+}]_i$ stimulates Ca^{2+}-dependent enzymes including proteases, kinases and nucleases, and their chronic activation under energy-deficient conditions is likely to contribute to Ca^{2+}-mediated toxicity (Manev *et al.*, 1989; Favaron *et al.*, 1990; Lee *et al.*, 1991; Meldrum, 1993). However, it is becoming increasingly apparent that perhaps the major cause of neurotoxicity both in many *in vitro* model systems and in various disease states is activation of enzymes which generate free radicals. Ca^{2+}-dependent activation of phospholipase A_2 generates arachidonic acid, the subsequent metabolic breakdown of which yields superoxide anions ($\cdot O_2^-$). Another Ca^{2+}-dependent enzyme, nitric oxide synthase (NOS), catalyses production of a free radical form of nitric oxide (NO$\cdot$) and subsequently, by the reaction of normal NO$\cdot$ with $\cdot O_2^-$, hydroxyl radicals ($\cdot$OH). Activation of proteases such as calpain I catalyses the conversion of xanthine dehydrogenase to xanthine oxidase, which then catalyses conversion of xanthine to uric acid with the consequent generation of

hydrogen peroxide (H_2O_2) and $\cdot O_2^-$ (Coyle and Puttfarcken, 1993). Evidence to date supports a central involvement of free radical generation in at least some degenerative conditions. 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine (MPTP), which causes severe neurodegeneration and a behavioural syndrome closely resembling Parkinson's disease, is accumulated in mitochondria in its active form MPP^+, where it inhibits complex 1, thereby generating free radicals (Tipton and Singer, 1993; Coyle and Puttfarcken, 1993). Transgenic mice over-expressing a form of the free radical scavenger superoxide dismutase (SOD) have been found to be resistant to the toxic effects of MPTP (Przedborski *et al.*, 1992). Mis-sense mutations in the gene encoding this same form of SOD, and consequent decreased SOD activity, have been found in a form of familial amyotrophic lateral sclerosis (Rosen *et al.*, 1993; Bowling *et al.*, 1993).

1.4 Ca^{2+} release from stores

1.4.1 $InsP_3$ receptors

$InsP_3Rs$ expressed in organellar membranes apparently function as homotetramers which form a central Ca^{2+}-permeant channel (Mignery and Sudhof, 1993). Three subtypes of $InsP_3R$ have now been identified (Furuichi *et al.*, 1994a). $InsP_3R1$ is the predominant subtype in the CNS, being ubiquitously expressed, with particularly high levels apparent in cerebellar Purkinje cells (Furuichi *et al.*, 1993). $InsP_3Rs$ 2 and 3 are also present in brain (Furuichi *et al.*, 1994a,b), although at much lower levels and according to some (Furuichi *et al.*, 1994b) but not all (Mignery and Sudhof, 1993) studies, with more discrete distributions. $InsP_3R$ subtypes may vary in their sensitivity to $InsP_3$ (Furuichi *et al.*, 1994a), but differences in their functional roles remain to be fully elucidated. $InsP_3R$ channel activity is highly sensitive to cytoplasmic $[Ca^{2+}]$ (Fig. 1.1B), in a 'bell-shaped' manner, such that peak $InsP_3R$ activity occurs at ~300 nM $[Ca^{2+}]$ (Bezprozvanny *et al.*, 1991; Finch *et al.*, 1991; Iino

and Endo, 1992). Low [Ca^{2+}]-activation and high [Ca^{2+}]-inactivation of $InsP_3Rs$ may play an important role in regenerative Ca^{2+} signals such as Ca^{2+} waves and oscillations (see Berridge, 1993; Pozzan *et al.*, 1994). Ca^{2+} modulation of $InsP_3R$ function in brain is complicated by the existence of calmedin, a protein which Ca^{2+}-dependently inhibits $InsP_3$ binding (see Pozzan *et al.*, 1994). $InsP_3R$ function is acutely regulated by a range of other effector mechanisms, including the kinases PKC, CamK-II and PKA, and the Ca^{2+}-activated protease calpain (Henzi and MacDermott, 1992; Magnusson *et al.*, 1993; Furuichi *et al.*, 1994a; Pozzan *et al.*, 1994). Also, adenosine triphosphate (ATP) enhances Ca^{2+} flux through the $InsP_3R$ channel, although whether this is a physiological mechanism of $InsP_3R$ regulation is uncertain, as the dynamic range of this effect occurs at below normal cytosolic ATP concentrations (see Henzi and MacDermott, 1992; Pozzan *et al.*, 1994).

Chronic activation of muscarinic receptors has been reported to cause downregulation of $InsP_3R$ expression (Wojcikiewicz *et al.*, 1992,1994a). In these studies, it was found that changes in type I $InsP_3R$ mRNA levels, in SH-SY5Y neuroblastoma cells, did not correlate with the kinetics of the CCh-evoked decrease in receptor expression, whereas CCh accelerated the loss of radioactivity from type I $InsP_3Rs$ immunoprecipitated from [^{35}S]-methionine-labelled cells, with a $t_{1/2}$ comparable to that of receptor downregulation (Wojcikiewicz *et al.*, 1994a). This was taken to indicate that prolonged muscarinic receptor activation causes accelerated $InsP_3R$ degradation. Again, the physiological relevance of this regulatory mechanism remains to be determined.

1.4.2 Ryanodine receptors

Many cell types possess a second mechanism by which Ca^{2+} can be released from intracellular stores. In addition to $InsP_3Rs$, they express subtypes of a family of

receptor/channel complexes structurally related to, but mechanistically very different from, $InsP_3Rs$, which are characterized by the ability of a plant alkaloid, ryanodine, to bind with nanomolar affinity, and therefore are known as ryanodine receptors (RyRs). Initially identified in skeletal and cardiac muscle, RyRs are now known to be present in many tissues, and mediate depolarization-activation, or more commonly Ca^{2+}-activation, of Ca^{2+} store release (see Meissner, 1994).

RyRs are formed from 560 kDa monomers, one of the largest proteins present within cells, which aggregate into functional tetrameric complexes with conductance 4-10 times that of $InsP_3Rs$ (Henzi and MacDermott, 1992). The skeletal muscle (or type I) RyR (Takeshima *et al.*, 1989) is found on the sarcoplasmic reticulum in close apposition to VOCCs on T-tubule infoldings of the plasma membrane (see Henzi and MacDermott, 1992). This co-localization of RyRs and VOCCs appears to allow depolarization of the plasma membrane to directly evoke Ca^{2+} release from the sarcoplasmic reticulum through the RyR channel. Cardiac muscle (or type II) RyRs (Nakai *et al.*, 1990), and the more recently identified brain (or type III) RyRs (Hakamata *et al.*, 1992), are activated by local accumulation of Ca^{2+}, thus allowing increased $[Ca^{2+}]_i$ to evoke further elevation of $[Ca^{2+}]_i$ by a mechanism known as Ca^{2+}-induced Ca^{2+} release (CICR)(Fig. 1.1D). There is 66-70% sequence homology between the 3 RyR subtypes (Meissner, 1994). Ryanodine has been reported to bind preferentially to the open conformation of RyRs, altering channel gating and locking the channel in an open, but low, conductance state (Nagasaki and Fleischer, 1989; Bezprozvanny *et al.*, 1991), or to abolish subsequent channel opening (McPherson *et al.*, 1991). The maintained open state would prevent store refilling, and may account for the use-dependence of ryanodine inhibition of CICR (Thayer *et al.*, 1988). RyR activity can be modified by a variety of agents other than ryanodine and Ca^{2+}, including the hydantoin derivative dantrolene, which inhibits ryanodine binding in muscle and blocks CICR in various preparations (Danko *et al.*, 1985; Thayer *et al.*,

1988; Ohta *et al.*, 1990; Nohmi et al., 1991; Lei *et al.*, 1992a; Segal and Manor, 1992; Frandsen and Shousboe, 1992), and caffeine, which both sensitizes RyRs to lower levels of $[Ca^{2+}]_i$ and increases the maximum rate of Ca^{2+} release (Kuba, 1980; Thayer *et al.*, 1988; Henzi and MacDermott, 1992; Friel and Tsien, 1992; Meissner, 1994). Caffeine may not, however, modulate type III RyRs (see Meissner *et al.*, 1994).

The presence of a caffeine-activated, dantrolene- and ryanodine-blockable, Ca^{2+} release mechanism has been demonstrated in a variety of neuronal types (Kuba, 1980; Thayer *et al.*, 1988; Murphy and Miller, 1989; Nohmi et al., 1991; Barish, 1991; Friel and Tsien, 1992; Pan *et al.*, 1994). The role of RyR-expressing Ca^{2+} stores in functional neuronal responses has, however, been less easy to determine (see e.g. Thayer *et al.*, 1988; Lipscombe *et al.*, 1988). Also, the subtype of RyR expressed in neurones has been controversial: while several studies have reported type II-like receptors (McPherson *et al.*, 1991), type II but not type I receptors (Lai *et al.*, 1992), or type I or type II depending on neuronal type (Kuwajima *et al.*, 1992), these investigations were performed before the identification of type III RyRs. Thus, some of the results may be compromised by cross-reactivity of the type I/II Abs used with type III receptors, making the expression and regional distribution of the different subtypes in brain uncertain (J. Mackrill, personal communication).

Endogenous modulators of RyR activity appear to include Mg^{2+}, adenine nucleotides such as ATP, calmodulin, and kinases, which may include PKA and protein kinase G (Meissner, 1994). Cyclic adenosine diphosphate-ribose (cADP-ribose) has been suggested to be an important endogenous activator of RyRs (see Galione, 1994)(Fig. 1.1D), as it is found in a wide variety of tissues, and addition of exogenous cADP-ribose evokes ryanodine-sensitive Ca^{2+} release from a variety of preparations, including neurones (Galione, 1994; Hua *et al.*, 1994). In pancreatic β cells, glucose stimulates cADP-ribose production (see Galione, 1994), thus potentially allowing this

molecule to act as a second messenger with a function paralleling that of $InsP_3$. However, the physiological relevance of cADP-ribose remains to be established. A recent study has reported a significant effect of exogenous cADP-ribose on type II RyR activity only in low-ATP conditions, with no cADP-ribose-evoked response in the presence of physiological ATP levels (Sitsapesan *et al.*, 1994), indicating that this molecule may simply substitute for ATP and so may be unlikely to have a second messenger role *in vivo*.

1.4.3 Store Ca^{2+}, cytoplasmic Ca^{2+} and extracellular Ca^{2+}

1.4.3.1 Sink or source

A variety of reports have suggested different interactions between Ca^{2+} entry and CICR depending on the refilling state of intracellular Ca^{2+} store, indicating that the Ca^{2+}-sensitive store may act as either a sink for cytoplasmic Ca^{2+} elevations, or as a source contributing to the elevation. In sympathetic neurones, Lipscombe and co-workers (1988) demonstrated that caffeine pre-treatment decreased $[Ca^{2+}]_i$ elevations evoked by subsequent depolarization. However, they noted that this could be explained either by the loss of normal depolarization-initiated CICR, or by caffeine-depleted stores having increased sequestration rates and thus buffering K^+-evoked Ca^{2+} entry more effectively. In further experiments, brief depolarizing pulses evoked $[Ca^{2+}]_i$ responses which outlasted Ca^{2+} channel inactivation, indicating that K^+ did activate CICR in these cells. However, after removal of a prolonged K^+ pulse, $[Ca^{2+}]_i$ decreased to a level which was above previous basal levels, but were reset to the initial basal after subsequent perfusion with caffeine. This was ascribed to a slow leak from stores which took up Ca^{2+} during depolarization, undetectable after uptake was reactivated by store depletion. In a subsequent study in the same neuronal preparation, K^+ depolarization-evoked $[Ca^{2+}]_i$ transients were also found to be diminished by pre-treatment with caffeine to deplete the RyR store (Friel and Tsien, 1992), but it was

further found that the inhibition was greatest in the time-period during which the caffeine-evoked response recovers i.e. during store refilling. Thus during this time-period depolarization-evoked Ca^{2+} entry leads to a significant amount of Ca^{2+} entering the store rather than remaining in the cytoplasm. However, perfusion of high-$[K^+]_o$ in the presence of low levels of caffeine evoked a faster-onset and greater $[Ca^{2+}]_i$ elevation than control $[K^+]_o$-stimulation, caffeine presumably sensitizing the CICR mechanism which is activated by depolarization under normal conditions (Friel and Tsien, 1992). In this cell type, then, Ca^{2+} appears to act as either a 'sink' or source for cytosolic responses, depending on the extent of store depletion and the nature of the depolarizing stimulus.

In PC12 pheochromocytoma cells, one agent has been found to activate CICR while another evokes store refilling. ATP responses, evoked via activation of P_{2Y} receptors, were greatly diminished by pre-treatment with a combination of caffeine and ryanodine to inhibit CICR, but this experimental condition had no effect on the response to K^+-depolarization. The recovery of the K^+-evoked $[Ca^{2+}]_i$ elevation, however, was inhibited by caffeine and ryanodine together, which prevent Ca^{2+} accumulation in RyR stores, and was increased by caffeine alone, which depleted the stores and allowed them to act as a sink for cytoplasmic Ca^{2+} (Barry and Cheek, 1994). Overall, it can be concluded that a complex relationship exists between Ca^{2+} entry and release, which depends on the nature of the stimulating agent, the cell type studied, and the refilling state of the RyR-expressing Ca^{2+} store.

1.4.3.2 Ca^{2+} release-activated Ca^{2+} entry

An involvement of Ca^{2+} entry in sustained $[Ca^{2+}]_i$ responses to PI-linked agonists has been widely reported (see Lambert and Nahorski, 1990), and proteins which bind $InsP_3$ and $InsP_4$ have been identified in plasma membranes (see Berridge,

1993; Putney and Bird, 1993). However, initial studies could not differentiate between a direct action of inositol polyphosphates on plasma membrane channels, and the 'capacitative entry' model (Putney, 1986; Putney and Bird, 1993) in which agonist-evoked store depletion leads to stimulation of Ca^{2+} entry. Thapsigargin is a sesquiterpene tumour promoter which inhibits the Ca^{2+}ATPase responsible for high-affinity sequestration of Ca^{2+} into intracellular stores (Thastrup *et al.*, 1990). In a variety of cell types it evokes an initial elevation of $[Ca^{2+}]_i$ which is independent of $[Ca^{2+}]_o$ (Thastrup *et al.*, 1990), and is apparently due to Ca^{2+} efflux from stores via $InsP_3R$ channels (Favre *et al.*, 1994), followed by a sustained elevation which is abolished in nominally Ca^{2+}-free medium (Thastrup *et al.*, 1990). Studies using this and similar agents have demonstrated that metabotropic receptor-evoked $[Ca^{2+}]_i$ plateau responses are undetectable during sustained $[Ca^{2+}]_i$ elevation due to the Ca^{2+}ATPase inhibitor. It is now widely accepted that depletion of stores, rather than production of a particular inositol polyphosphate, leads to the sustained $[Ca^{2+}]_i$ elevations evoked by PI-linked agonists as well as the sustained response evoked by direct store depletion using e.g. thapsigargin (see Putney and Bird, 1993). In both cases this phase of the response may be mediated by release from Ca^{2+} stores of a poorly characterized retrograde messenger, known as Ca^{2+} influx factor (CIF), which directly stimulates Ca^{2+} entry (Putney, 1993; Putney and Bird, 1993)(Fig. 1.1B,E). Ca^{2+} entry subsequent to store depletion occurs through a plasma membrane channel known as the Ca^{2+} release activated Ca^{2+} channel (CRAC channel) (Hoth and Penner, 1992; Penner *et al.*, 1993). However, a component of metabotropic responses due to direct inositol polyphosphate-activated Ca^{2+} entry cannot be completely excluded (see De Waard *et al.*, 1992; Berridge, 1993).

1.4.4 $InsP_3$- and Ca^{2+}-activated Ca^{2+} release

1.4.4.1 Ionotropic / metabotropic interactions

There are a number of mechanisms by which elevation of $[Ca^{2+}]_i$ can modulate the PI pathway. At least some isoforms of PIC are Ca^{2+}-sensitive, such that Ca^{2+} entry can directly activate or potentiate PI turnover (Eberhard and Holz, 1988; Cockcroft and Thomas, 1992; Rhee and Choi, 1992), enabling metabotropic agonists linked to $[Ca^{2+}]_i$ elevation to activate positive feedback on $InsP_3$ production, or enabling ionotropic agonists to either stimulate PI turnover directly or to potentiate metabotropic PI responses (Conn and Desai, 1991; Guiramand *et al.*, 1991)(Fig. 1.1A). In addition, as discussed above $InsP_3Rs$ display a 'bell-shaped' sensitivity to $[Ca^{2+}]_i$ (Bezprozvanny *et al.*, 1991; Finch *et al.*, 1991), such that Ca^{2+} can effectively function as a co-agonist of the receptor (Fig. 1.1B). This may enable released Ca^{2+} to feed back positively or negatively on at least two sites, causing potentiation or termination of the $InsP_3$-mediated response. Equally, activation of PI turnover can modify Ca^{2+} entry mechanisms, via either $InsP_3$ or DAG production (Kelso *et al.*, 1992; Markram and Segal, 1992)(e.g. Fig. 1.1C). Thus interactions between activation of metabotropic receptors, and of ionotropic receptors such as NMDARs, can occur at a variety of sites on the PI turnover and Ca^{2+} entry pathways, and it is not surprising to find that greatly differing modulatory effects have been reported using differing experimental protocols and preparations. These include CCh- or ACPD-evoked potentiation or inhibition of NMDAR responses (Courtney et al., 1990; Kelso *et al.*, 1992; Markram and Segal, 1992; Kinney and Slater, 1993; Harvey and Collingridge, 1993; Snell et al., 1994); NMDA-induced concentration-dependent potentiation or inhibition of metabotropic receptor-evoked PIC activity (Baudry et al, 1986; Schmidt *et al.*, 1987; Godfrey *et al.*, 1988; Morrisett *et al.*, 1990; Chen *et al.*, 1993; Challiss *et al.*, 1994a,b); NMDA-evoked modulation of mGluR expression during development (Aronica *et al.*, 1993); and ACPD-evoked potentiation of (McDonald and Schoepp,

1992) or ACPD- or CCh-evoked protection against (Koh *et al.*, 1991; Birrell *et al.*, 1993) NMDAR-mediated neurotoxicity.

1.4.4.2 Are $InsP_3$- and Ca^{2+}-sensitive stores different?

As $InsP_3$ evokes Ca^{2+} release and thus elevates $[Ca^{2+}]_i$, it is theoretically possible for the signal generated by this inositol polyphosphate to involve activation of further Ca^{2+} release, either by co-agonist action of Ca^{2+} on $InsP_3Rs$ (Bezprozvanny *et al.*, 1991; Finch *et al.*, 1991; Iino and Endo, 1992) or by Ca^{2+} activation of RyRs (see Meissner, 1994). Both of these CICR mechanisms may be involved in Ca^{2+} signalling responses, such as Ca^{2+} wave propagation, depending on the cell type studied (Berridge 1991, 1993; Amundson and Clapham, 1993). Berridge and co-workers (1991) suggested that $InsP_3$ generation causes a small Ca^{2+} release which is taken up into Ca^{2+}-sensitive stores. When these are filled, the remaining Ca^{2+} released by $InsP_3$ triggers a large release via activation of the CICR pool. Thus, CICR could cause oscillations in $[Ca^{2+}]_i$, and IICR could provide the steady elevation of $[Ca^{2+}]_i$ needed to load the CICR stores between each transient (Berridge, 1991; Dupont *et al.*, 1991).

While the above model assumes two separate $InsP_3$- and Ca^{2+}-sensitive Ca^{2+} pools, in reality the relationship between $InsP_3$- and Ca^{2+}-sensitive stores remains controversial, perhaps partly reflecting considerable heterogeneity between different cell types. In adrenal chromaffin cells, $InsP_3$- and Ca^{2+}-sensitive stores appear to exist as entirely separate entities: thus, $InsP_3$-evoked Ca^{2+} release from permeabilized chromaffin cells has no significant effect on subsequent responses to caffeine (Robinson and Burgoyne, 1991). However, ryanodine pre-treatment inhibited oscillations evoked by the PI-linked agonists bradykinin and histamine in these cells, indicative of functional interactions between the two pools (Malgaroli *et al.*, 1990). In

contrast, in PC12 cells, prior challenge with the PI-linked agonist bradykinin or with caffeine inhibits subsequent responses to each other, suggesting the existence of a single store sensitive to both agents (Zacchetti *et al.*, 1991). However, this finding was not reproduced in a different PC12 subclone, in which bradykinin inhibited subsequent responses to caffeine but caffeine did not inhibit subsequent bradykinin responses, results which are most consistent with a two pool model (Reber *et al.*, 1993). Ca^{2+} store-depleting agents have been widely utilized to investigate the relationship between $InsP_3$-sensitive and Ca^{2+}-sensitive stores, by possible selective depletion of one or other store. A variety of isoforms and splice variants of store Ca^{2+}ATPase are known, and several can co-exist within a given cell type (Papp *et al.*, 1991,1993; Pozzan *et al.*, 1994). Thapsigargin pre-treatment abolishes responses to $InsP_3$, but not to caffeine, in permeabilized adrenal chromaffin cells (Robinson and Burgoyne, 1991), consistent with the existence of two distinct non-overlapping pools in these cells. An action of thapsigargin on Ca^{2+} stores in addition to, and separate from, $InsP_3$-sensitive stores in rat liver microsomes was indicated by the finding that thapsigargin pre-treatment abolished $InsP_3$-evoked Ca^{2+} efflux, whereas $InsP_3$ pre-treatment decreased, but did not completely abolish, subsequent responses to thapsigargin (Thastrup *et al.*, 1990). The relationship between IICR and CICR stores may vary greatly in different types of neurone: in one study heterogeneity of Ca^{2+} stores was demonstrated in microsomes prepared from different brain regions: Ca^{2+} store sensitivity to $InsP_3$ varied from 51% in cerebellar microsomes to just 8% in olfactory bulb microsomes (Verma *et al.*, 1990). In cultured dorsal root ganglion neurones, caffeine evokes responses in most cell bodies but very few processes, whereas the PI-linked agonist bradykinin evokes responses in a proportion of both (Thayer *et al.*, 1988). Given that some processes respond to bradykinin but not to caffeine, and some cell bodies respond to caffeine but not to bradykinin, it appears that differentiable RyR- and $InsP_3$R-expressing pools may exist at least in this neuronal type.

1.5 Cerebellar granule cells

1.5.1 Physiology, maturation, and connections *in vivo*

Rat cerebellum contains ~1 x 10^8 granule cells, whilst human cerebellum contains ~1 x $10^{10\text{-}11}$, making cerebellar granule cells the most numerous cell type in the brain (Ito, 1984). These interneurones are generated after birth, mainly in the second post-natal week in rat (P8-14), in the external germinal layer, where they assume a bipolar morphology from which parallel fibres with neurophilic lamellipodia project. Granule cells migrate along Bergmann glia fibres through the molecular layer to the internal granular layer, a transition which takes ~3 days and occurs up to P21 (Burgoyne and Cambray-Deakin, 1988). Granule cells subsequently develop 2-7 dendrites of up to 30 μm in length (Palkovits *et al.*, 1972; Ito, 1984) and by P30 excitatory synaptic inputs from mossy fibres and inhibitory synaptic inputs from Golgi cells form (Ito, 1984; Burgoyne and Cambray-Deakin, 1988). Excitatory *en passant* synapses of parallel fibres onto the dendrites of basket cells, Golgi cells and particularly Purkinje cells also develop, during P15-21. Glutamate is released at these synapses, acting primarily on non-NMDA glutamatergic receptors on Purkinje cells (Elias *et al.*, 1993).

Cerebellar granule cells receive a simple monosynaptic input from mossy fibres, which originate mainly in nuclei in the brainstem and spinal cord. Each granule cell receives only 3-4 synaptic boutons, each to a different short dendrite (Palkovits *et al.*, 1972). It has been suggested that mossy fibres may use a variety of neurotransmitters, as circumstantial evidence suggests an involvement of both substance P and somatostatin in mossy fibre-granule cell transmission (Ito, 1984). High levels of ACh have been detected in mossy fibre terminals, as have the enzymes involved in ACh synthesis and degradation, and early post-natal granule cells in several cerebellar regions respond to exogenous muscarinic and nicotinic agonists (Rotter *et al.*, 1979;

Ito, 1984). However, spontaneous granule cell synaptic currents *in situ* appear to be predominantly mediated by NMDARs (D'Angelo *et al.*, 1990), and electrical stimulation of mossy fibres has been found to evoke granule cell responses via both NMDA and non-NMDA GluRs, depending on the frequency of stimulation (Garthwaite and Brodbelt, 1989), but not via any other type of receptor examined. Cerebellar granule cells may thus be activated primarily by glutamate released from mossy fibres, thereby receiving excitatory inputs from lower CNS centres which they then convey throughout the cerebellum via the parallel fibre network.

NMDAR activation may play an important role in granule cell migration during development, as granule cell movement in slices is inhibited by dizocilpine and APV, and potentiated by glycine or low levels of NMDA (Komura and Rakic, 1993). Also, NMDARs are present on granule cells during the migratory period at levels far in excess of those on granule cells of the adult cerebellum (Garthwaite *et al.*, 1987), and spontaneous NMDAR channel activity increases in proportion to the increasing rate of migration through the molecular layer (Rossi and Slater, 1993). NMDARs are known to interact with actin filaments (Rosenmund and Westbrook, 1994), making it possible that Ca^{2+} entry through NMDAR channels could initiate or maintain Ca^{2+}-dependent cytoskeletal events underlying movement, such as repeated actin filament assembly and disassembly (Burgoyne and Cambray-Deakin, 1988; Rossi and Slater, 1993). Other GluRs may also be of central importance in granule cell development: Quis-evoked inositol polyphosphate formation is maximal in cerebellum at P6, approximately the time at which many parallel fibres make contact with Purkinje cells (Palmer *et al.*, 1990).

In the cerebellar granule cell layer *in vivo*, expression of a wide variety of ionotropic GluR subunits has been reported, notably high levels of GluRs 2 ,4, and 6, KA2, and NMDARs 1, 2A, and 2C, as well as low levels of GluR5 (see Hollmann and

Heinemann, 1994). Before and during migration, granule cells possess NMDAR channels of 50 pS conductance, comparable to recombinant 1/2A or 1/2B channels. After P13 and increasingly in older animals, granule cells also express NMDAR channels of lower conductance (33 pS) and shorter open times, similar to the characteristics of recombinant 1/2C channels (Farrant *et al.*, 1994; see also Monyer *et al.*, 1994). Thus there appear to be developmental changes in NMDAR expression in granule cells. NMDAR2C subunits are the predominant NMDAR subunit expressed in adult cerebellum (Hollmann and Heinemann, 1994). mGluRs are also present in granule cells *in vivo*, mainly mGluR4, along with low expression of mGluR1 (Hollmann and Heinemann, 1994; but see Martin *et al.*, 1992), probably of the 1β form (Hampson *et al.*, 1993). Muscarinic receptors are present on granule cells *in vivo*, in a defined region of the cerebellum, the vestibulocerebellum, but only in early post-natal development. As these receptors disappear after P10 i.e. at approximately the stage when mossy fibre inputs develop (Rotter *et al.*, 1979), their functional relevance is uncertain. N-type VOCCs appear to be present on both granule cell bodies and parallel fibres (Maeda *et al.*, 1989), and it would be unexpected if near-ubiquitous (Tsien *et al.*, 1991) L-type channels were not also expressed. Lai *et al.* (1992) reported that ryanodine binding was very low in cerebellum, while Kuwajima *et al.* (1992) found that in *weaver* mutant rats which lack granule cells, cerebellar type II RyR levels, but not type I, were decreased. As indicated above (Section 1.4.2) the studies of RyR subtypes present in brain regions were performed primarily before the discovery of RyR3. Furuichi *et al.* (1993) reported detectable but very low expression of $InsP_3R1$ mRNA in the granule cell layer, but given the massive $InsP_3R$ expression in Purkinje cells it is unclear if this was expressed at least partly in granule cells or wholly in Purkinje cell axons. Sharp *et al.* (1993) have reported that $InsP_3R3$, but not $InsP_3R2$, is present at relatively high levels in cerebellar granule cell axons and cell bodies.

1.5.2 Culture conditions

Cultured cerebellar granule cells have been widely used as an *in vitro* model for the investigation of neuronal activity, as they can be grown to the relative exclusion of other cerebellar cell types. Following standard dissociation procedures (see Methods), a culture develops in which >95% of cells surviving are neuronal, >90% of these being granule cells (Balazs *et al.*, 1987). This level of homogeneity results because (i) much higher numbers of granule cells are present in the cerebellar cortex than of other cell types e.g. several hundred-fold more granule cells than Purkinje cells (Ito, 1984); (ii) cells which survive in culture are predominantly those still at the proliferative stage, before differentiation has occurred (Gallo *et al.*, 1986), and at P7-8, when the dissection is performed, most other cerebellar cells are highly differentiated: Golgi cell precursors and Purkinje cells stop dividing at embryonic day 14-17 (Ito, 1984); (iii) addition of a mitotic inhibitor, cytosine β,D-arabinofuranoside, prevents the development of significant numbers of astrocytes in the culture.

Depolarization stimulates granule cell maturation *in vitro*, denoted by the expression of amino acid carriers and VOCCs (Balazs *et al.*, 1988a), and the production of enzymes involved in glutamatergic neurotransmission (Moran and Patel, 1989a), leading to the development of stimulus-evoked glutamate release (Balazs *et al.*, 1988a). In the presence of serum, cultured granule cells mature to a stage at which they become dependent on excitatory environmental influences for survival (maturation is inhibited in the absence of serum (Balazs *et al.*, 1988a)), such that after 2-3 days in culture depolarization markedly increases the survival rate of granule cells *in vitro* (Gallo *et al.*, 1987). Depolarization has also been reported to significantly influence receptor expression on cultured cerebellar granule cells (Cox *et al.*, 1990; Van der Valk *et al.*, 1991; Condorelli *et al.*, 1993; Aronica *et al.*, 1993). The maturational effects of depolarization are inhibited by the DHP VOCC antagonist (-) PN 202 791,

and replicated by the VOCC channel agonist (+) PN 202 791 (Gallo *et al.*, 1987), NMDA (Balazs *et al.*, 1988b; Moran and Patel, 1989b) or the Ca^{2+} ionophore ionomycin (Pearson *et al.*, 1992), implicating Ca^{2+} entry as the mediator of these effects. This excitation is thought to mimic glutamatergic mossy fibre innervation, which may lead to the survival and maturation *in vivo* only of granule cells which have formed their proper synaptic connections (Balazs *et al.*, 1988a).

In culture, granule cells begin neurite outgrowth within hours, developing an extensive interconnecting network of fibres, and gradually migrate into cellular aggregations (Balazs *et al.*, 1987; see Figs. 2.1a,b,2.6b) whose size is dependent upon the density of cell plating. Neurite development can be abolished by the NMDA antagonist APV, implicating activation of NMDARs in this process, apparently via stimulation of PKC activity (Pearce *et al.*, 1987; Cambray-Deakin *et al.*, 1990).

1.5.3 Receptors expressed on granule cells *in vitro*

Cerebellar granule cells express a wide variety of ion channels and receptors in culture. These include histaminergic H_1 receptors, α_1 adrenoceptors and $5HT_2$ receptors (Dillon-Carter and Chuang, 1989); glycine receptors (Wahl *et al.*, 1994); $GABA_A$ and $GABA_B$ receptors (De Erausquin *et al.*, 1992; Mathews *et al.*, 1994); endothelin-1 receptors (Fukamauchi and Chuang, 1994); the membrane-spanning tyrosine kinase receptors trkB and trkC, which are the putative receptors for brain-derived neurotrophic factor (BDNF) and neurotrophin respectively, and the low-affinity neurotrophin receptor p75 (Lindholm *et al.*, 1993). While granule cells receive inhibitory GABAergic inputs from Golgi cells (Ito, 1984), it is by no means clear what the function of these other receptors would be, or whether the receptors present on cultured granule cells correspond to normal expression patterns *in vivo*.

The present experimental study has focussed on responses to activation of glutamatergic and cholinergic receptors, and the subtypes of these which are apparently expressed, and their relations to *in vivo* expression, are therefore of primary interest. The expression of a muscarinic receptor on cultured granule cells coupled to PI turnover was first demonstrated by Xu and Chuang (1987), subsequently identified as being the M_3 subtype (Whitham *et al.*, 1991a). A lower level of adenylyl cyclase-linked M_2 receptors is also expressed on these neurones (Xu and Wojcik, 1986; Whitham *et al.*, 1991a). This agrees with the reported presence of mRNA for M_2 and M_3 receptors, but not for M_1 or M_4, in cultured granule cells (Fukamauchi *et al.*, 1991). mRNAs for the two muscarinic receptor subtypes present have been reported to be expressed with somewhat differing temporal patterns (Fukamauchi *et al.*, 1991).

The existence of several types of GluR in cultured cerebellar granule cells is well established (Wroblewski et al., 1985; Nicoletti *et al.*, 1986; McCaslin and Morgan, 1987; Cull-Candy et al., 1988). At least one type of non-NMDA GluR coupled to PI turnover was initially identified from early granule cell studies (Nicoletti *et al.*, 1986). Molecular biological approaches have more recently identified the presence of an mGluR1 splice variant, apparently mGluR1α (Bessho *et al.*, 1993; Aronica *et al.*, 1993; Milani *et al.*, 1993), although the low expression of this subtype in cultured granule cells grown in 25 mM K^+ (Milani *et al.*, 1993) may suggest that glutamate- or ACPD-evoked PI responses are also partially mediated by other mGluR subtypes in these neurones. mGluR5 mRNA is present at very low (Santi *et al.*, 1994) or undetectable (Prezeau *et al.*, 1994) levels in cultured granule cells, and it appears unlikely that mGluR5-mediated PI signalling is important in this preparation: the $[Ca^{2+}]_i$ response to ACPD in granule cells is abolished by L-AP3 (Irving *et al.*, 1990), an antagonist to which mGluR5 is reportedly insensitive (Schoepp and Conn, 1993). The ability of NMDA in some studies to activate inositol phosphate formation in granule cells (Nicoletti *et al.*, 1986; Raulli *et al.*, 1991) has led one group to

hypothesize the existence of metabotropic NMDARs as well as ionotropic NMDARs in this preparation (Nicoletti *et al.*, 1986; Hynie *et al.*, 1989; Raulli *et al.*, 1991). At least one and probably two non-PI-coupled mGluR subtypes are also present on granule cells, possibly mGluR2 and mGluR4 (Chavis *et al.*, 1993; Prezeau *et al.*, 1994; see also Santi *et al.*, 1994).

NMDA2A and NMDA2B mRNAs, along with NMDAR1, although not the mRNAs for 2C or 2D, are present in cultured cerebellar granule cells (Bessho *et al.*, 1994), indicating the possible presence of more than one type of functional NMDAR in these neurones. In granule cells grown in 25 mM K^+, the concentration routinely used in the work described in this Thesis, NMDAR2A mRNA has been reported to be specifically upregulated, with a consequent increase in NMDAR expression levels (Bessho *et al.*, 1994). GluR1 is highly expressed in cultured cerebellar granule cells, unlike *in vivo*; also, lower levels of GluR2 and 3 have been detected, and GluR4 mRNA is present (expression of the GluR4 protein has not been examined)(Condorelli *et al.*, 1993). This is consistent with the report that more than one type of functional AMPAR appears to be present in cultured granule cells, as AMPA can activate a high conductance and a low conductance channel occurring in discrete patches (Wyllie *et al.*, 1993).

1.5.4 Ca^{2+} channels in granule cell plasma and organellar membranes

VOCC expression has also been widely investigated in cultured granule cells, with differing conclusions being drawn as to the types of channel present. This may at least partly be due to effects of the composition of the internal experimental solution on the ability to detect certain channel types (Pearson *et al.*, 1993). In at least some studies, up to 3 forms of L-type channel (Carboni *et al.*, 1985; Zhang *et al.*, 1993;

Forti and Pietrobon, 1993; Bossu *et al.*, 1994), and N-type (Zhang *et al.*, 1993; Bossu *et al.*, 1994), P-type (Pearson *et al.*, 1993; Zhang *et al.*, 1993), Q-type (Zhang *et al.*, 1993) and R-type (Zhang *et al.*, 1993) channels have been identified on cultured granule cells. Differences in pharmacological specificity from accepted VOCC classifications, and the ability of blockers of different channel types to inhibit the same currents in granule cells, have led to the suggestion that at least some of these channels may not truly belong in the assigned classes (De Waard *et al.*, 1991; Pocock *et al.*, 1993; Sutton *et al.*, 1993).

Initial evidence suggests that granule cells Ca^{2+} stores express both $InsP_3Rs$ and RyRs. PI-linked agonists elevate $[Ca^{2+}]_i$ in a partially or completely $[Ca^{2+}]_o$-independent manner (Irving *et al.*, 1992a) in granule cells, and in permeabilized granule cells $InsP_3$ mobilizes ~60% of $^{45}Ca^{2+}$ preloaded into stores (Whitham *et al.*, 1991b). Caffeine evoked no $^{45}Ca^{2+}$ release response in that study, making the nature of the remaining $InsP_3$-insensitive store uncertain (Whitham *et al.*, 1991b). However, significant caffeine-evoked $[Ca^{2+}]_i$ transients have been reported in other studies (Irving *et al.*, 1992b; Pearson *et al.*, 1992; Fohrmann *et al.*, 1993). The $InsP_3R$ and RyR subtypes present remain to be established, and the relationship between these stores in granule cells is uncertain (Whitham *et al.*, 1991b; Irving *et al.*, 1992b)(see Fig. 1.4).

1.6. Aims of this study

As indicated above, cerebellar granule cells possess a variety of mechanisms which can mediate $[Ca^{2+}]_i$ elevation, making them a useful model neuronal system for investigating questions relating to Ca^{2+} signalling and homeostasis. The current knowledge of Ca^{2+} signalling mechanisms in cultured granule cells is summarized in Fig. 1.4. In the light of the interactions which may occur between different Ca^{2+}

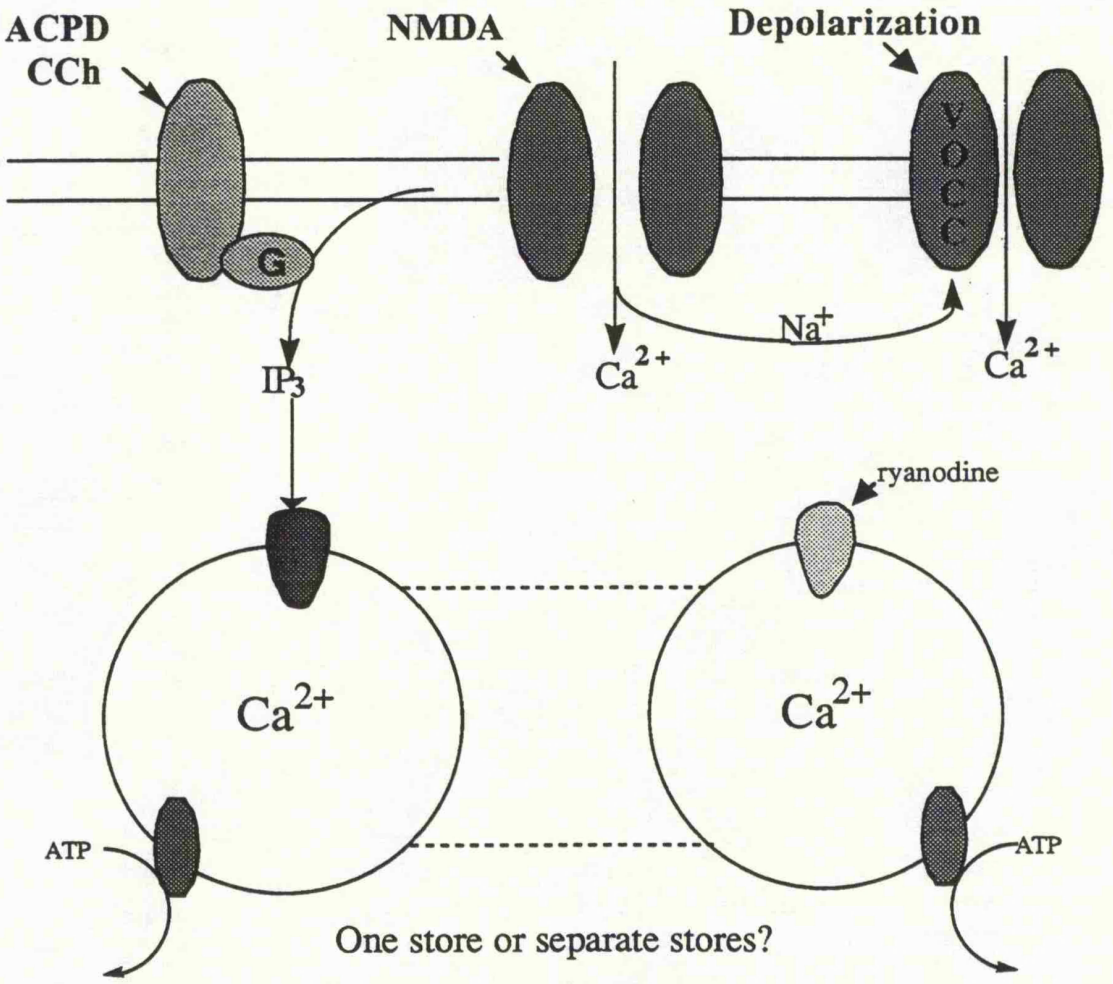


Fig. 1.4 Initial model of Ca^{2+} signalling in cerebellar granule cells. Included in this figure are the possible direct and indirect effects of NMDA on Ca^{2+} entry, the apparent effects of metabotropic agonists on Ca^{2+} release from stores, the uncertain role of RyRs in the system, and the uncertain relationship between $InsP_3$-sensitive and Ca^{2+}-sensitive stores.

signalling mechanisms (see Fig. 1.1), it is likely that the current knowledge of Ca^{2+} signalling in granule cells is deficient. This Thesis describes the results of studies undertaken in granule cells to address some of the possible weaknesses in the current knowledge of granule cell Ca^{2+} signalling. The major aims of the study were as follows:

(i) To characterize the $[Ca^{2+}]_i$ responses evoked by depolarization and glutamatergic and cholinergic receptor agonists in cultured cerebellar granule cells, by examining the mechanisms by which these agents elevate $[Ca^{2+}]_i$, with particular reference to the type of channels mediating Ca^{2+} entry and to the possible involvement of Ca^{2+} stores in these responses.

(i) To study effects on the NMDAR-mediated $[Ca^{2+}]_i$ responses caused by modulatory agents; also, to examine possible interactions between responses to agonists of different receptors, particularly of NMDARs and metabotropic receptors, in terms of potentiation or inhibition of inositol phosphate formation, and/or competitive or complementary effects on $[Ca^{2+}]_i$ elevation.

(iii) To investigate the characteristics and mechanisms of Ca^{2+} store release in cerebellar granule cells. This was to be done by investigation of the subtypes of $InsP_3$- and Ca^{2+}-induced Ca^{2+} release channel expressed; the effects of Ca^{2+}ATPase inhibitor-mediated store depletion and of agonist-evoked store release on Ca^{2+} entry and on $[Ca^{2+}]_i$, and by investigation of the relationship between $InsP_3$- and Ca^{2+}-sensitive stores in granule cells. The Ca^{2+} stores activated by ionotropic and metabotropic agonists were to be determined, by using agonists and store depleting agents to investigate whether more than one functional store in expressed, and by examination of possible consequences of modification of $InsP_3R$ and RyR expression due to prolonged ionotropic or metabotropic receptor stimulation.

2. Methods

2.1 Cerebellar granule cell culture

Cerebellar granule cells were prepared initially as described previously (Thangnipon *et al.*, 1983; Whitham *et al.*, 1991a), the method being adapted over time into the following protocol. Cerebella were dissected out from Wistar rats at P7-8, the meninges were removed as far as possible using forceps, and the cerebella cross-chopped using a McIlwain tissue chopper into 0.3 mm^2 blocks. The resultant tissue was subjected to mild trypsin digestion (250 μg trypsin / ml) in filter-sterilized Krebs-Henseleit buffer (KHB) (composition in mM: Na^+ 143.3, K^+ 5.9, Ca^{2+} 1.3, Mg^{2+} 1.2, Cl^- 125.9, $H_2PO_4^-$ 2.2, HCO_3^- 24.9, SO_4^{2-} 1.2, glucose 10; pH 7.4 (see Appendix I)) supplemented with bovine serum albumin (BSA)(0.3% (w/v)) and deoxyribonuclease I (DNase)(40 μg / ml) for 15 min at 37°C, in a shaking water bath. This incubation was terminated by addition of an equal volume of basal modified Eagle's medium (BME) containing 10% (v/v) foetal calf serum (FCS) (trypsin inhibitor being found to be unnecessary in addition), and centrifugation at 500g for 2-3 min at 15-20°C. The supernatant was discarded, and pelleted cells were triturated 15-20 times in KHB containing BSA and DNase using a 3 ml Pasteur pipette, then left for 1-2 min. Cells remaining in suspension were collected, and the trituration process was repeated twice with the remaining pellet. The total collected suspension was then re-centrifuged at 500g for 2-3 min, and suspended in BME containing 10% (v/v) FCS, 100 IU / ml penicillin, 100 mg / ml streptomycin, 50 mg / ml amphotericin B, and 2 mM glutamine. Unless otherwise stated, 25 mM KCl was also added to the medium, to improve cell survival.

8 or 24-well multidishes were precoated with poly-D-lysine (50 μg / ml) for at least 5 min, and washed with filter-sterilized KHB. Cells were plated out into the wells, at a density of ~3 x 10^6 cells / well for 8-well multidishes (4 ml of medium), or ~7.5 x 10^5 cells / well for 24 well multidishes (1.5 ml of medium). For fura 2 experiments,

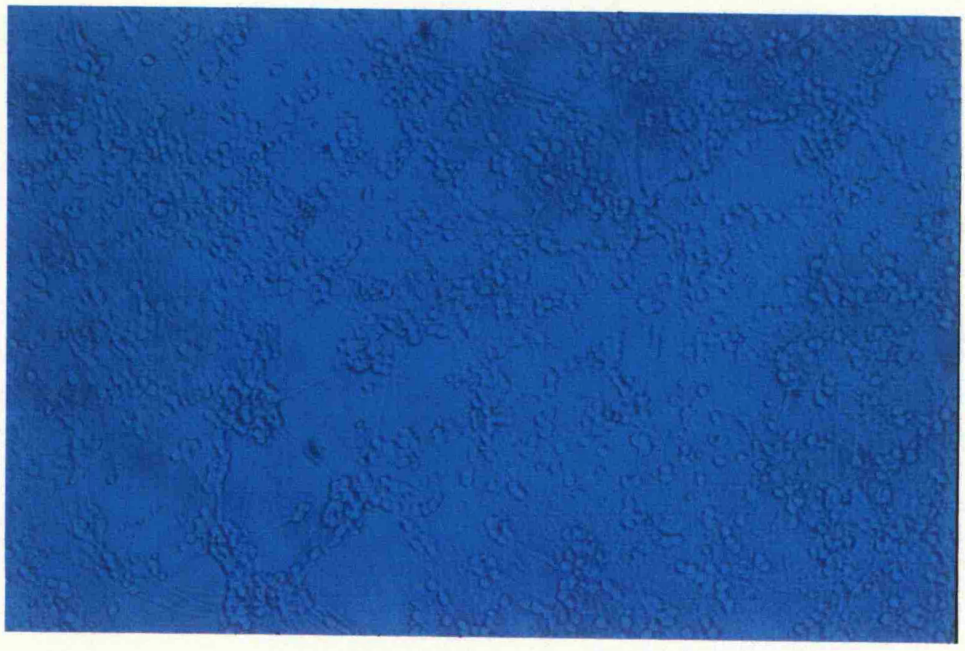


Fig. 2.1 ***a)*** **Cerebellar granule cells in culture.** Phase contrast image of 7 DIV cerebellar granule cells cultured on a glass coverslip (x20 Nikon Fluor Ph3DL objective, Nikon Diaphot-TMD microscope with fura 2 filter). Aggregations of cells are clearly visible, with many processes connecting different groups to each other.

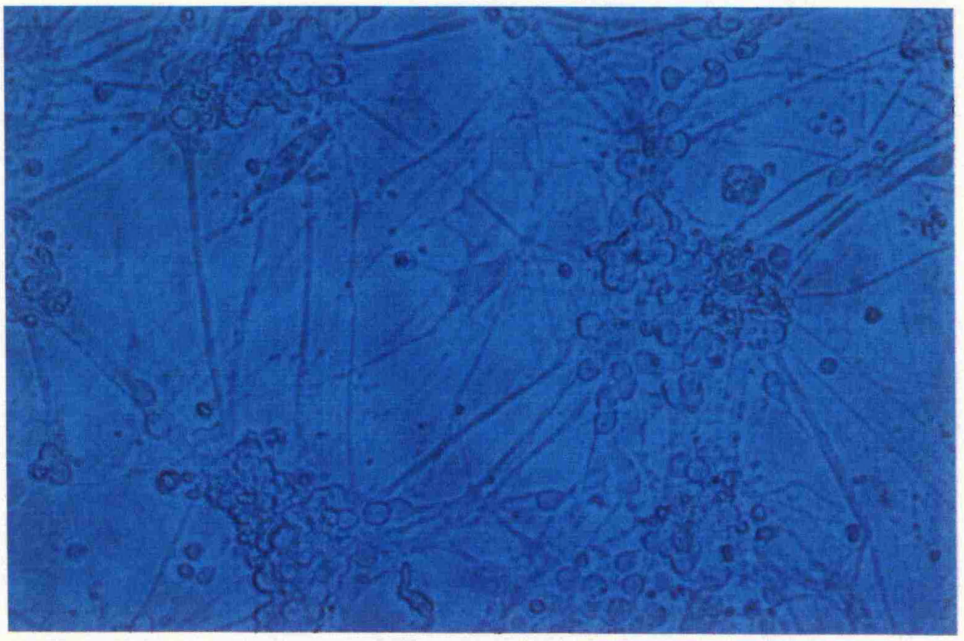


Fig. 2.1 *b)* Presence of non-granule cells in the cerebellar culture. Phase contrast image of 7 DIV cerebellar granule cells cultured on a glass coverslip (x40 Nikon Fluor Ph3DL objective, fura 2 filter). Among the aggregations of varying numbers of granule cells, several astrocytes can be seen, easily distinguishable by their much larger size and their flatter, less rounded shape. These made up a very small percentage of the cells surviving in culture, and were routinely avoided when selecting suitable groups of cells for $[Ca^{2+}]$ measurements.

glass coverslips were dipped in 70% (v/v) industrial methylated spirits and flamed, before being placed in 8-well plates. These were subsequently coated with poly-D-lysine as described above, cells plated out into these wells growing on the coverslip as on the well surface. After ~24 h in culture the medium was replaced, at which point the mitotic inhibitor cytosine β-D-arabinofuranoside (10 μM) was added to inhibit glial cell proliferation. Cultures were maintained in a humidified atmosphere of 5% CO_2 : 95% air at 37°C for up to 10 days *in vitro* (DIV). Neuronal aggregations were seen within 1-2 DIV, with many interconnecting processes developing over time (Fig. 2.1a). Very few, morphologically easily differentiable, glia survived (Fig. 2.1b).

2.2 Measurement of $[Ca^{2+}]_i$ in granule cells

2.2.1 Use of fura 2 for $[Ca^{2+}]_i$ experiments

Over recent years a series of dyes have been developed which alter their fluorescence on binding to Ca^{2+}, enabling their use as indicators of $[Ca^{2+}]_i$ levels in cells (Tsien, 1988). Fura 2 displays a biphasic change in fluorescence, increasing at short wavelength excitation (<360 nm) and decreasing at longer wavelengths (>360 nm)(Fig. 2.2). Fluorescence at 360 nm, the so-called isosbestic point, is Ca^{2+}-independent, a property which can be utilized for the assessment of increases in intracellular levels of other divalent cations e.g. Mn^{2+} (see Section 2.3). The major advantage of fura 2 over earlier Ca^{2+}-binding dyes is that whereas fluorescence measured at a given wavelength will be highly dependent on the amount of dye present within a cell, by measuring fura 2 emission consequent to excitation at both 340 and 380 nm, changes in the ratio of these two wavelengths will be independent of the dye concentration, and so responses can be fairly compared across different experiments. Fura 2 can be loaded into cells as its acetoxymethyl ester derivative fura 2-AM: this crosses the plasma membrane, whereupon the ester group is cleaved off by intracellular enzymes, leaving the largely membrane-impermeant dye locked within the cell.

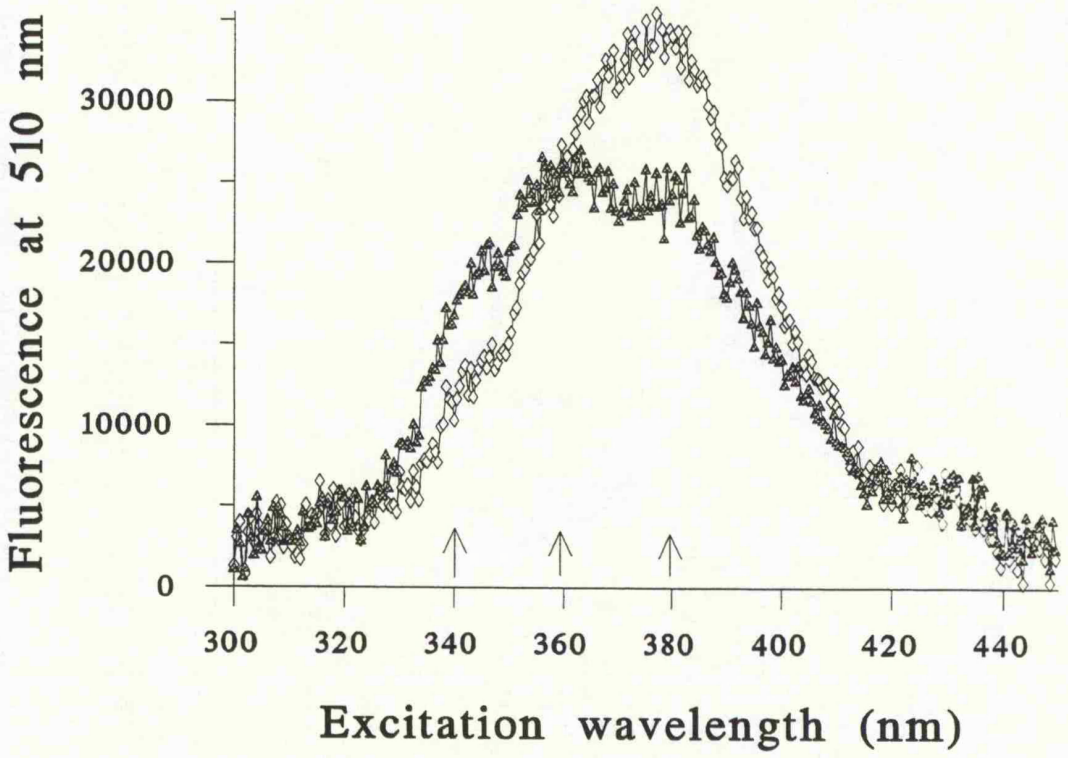


Fig. 2.2 Effect of Ca^{2+} binding on fura 2 fluorescence. Spectral scan of fura 2 free acid fluorescence at 510 nm emission wavelength in Ca^{2+}-free (◆) and 10 mM Ca^{2+}-containing (◊) KCl/K-3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer, with fluorescence from buffer without fura 2 subtracted. Ca^{2+} binding to fura 2 causes an increase in fluorescence at shorter excitation wavelengths, and a decrease at longer wavelengths, with no change at the isosbestic point. While the isosbestic point varies somewhat with experimental setup and recording apparatus, these scans indicate that, for experiments as performed in this Thesis on the PTI photometer system, it is close to 360 nm.

2.2.2 Population measurements

Initial experiments were performed using a Perkin Elmer LS 50 population fluorimeter. Cells grown on 11 x 22 mm coverslips were incubated with 2 μM fura 2-AM in KHB for 30 min, and then placed in a plastic cuvette at an angle of 60° to the incident light. Responses to the addition of depolarizing high-$[K^+]$ could be detected (Fig. 2.3), but these were not consistently found for differing coverslip populations, and responsiveness from individual coverslips was not maintained for long enough to allow more complex experiments to be performed. A major problem with population experiments was the fact that cerebellar granule cells do not grow to confluence, tending rather to aggregate together in 'clumps' which occupy only a fraction of the total surface area available (see Fig. 2.1a,b,2.6b). The incident light therefore largely refracted off the coverslip rather than the cells, making it difficult to obtain a sufficiently good signal : noise ratio for granule cell responses. Also, this type of aggregatory growth involves many of the cells growing partly or completely on top of others. This resulted in variable attachment to the poly-D-lysine-coated surface, making a portion of the cells prone to wash-off. The method of agonist addition, i.e. the injection of a relatively large volume of buffer containing the agonist into the cuvette, therefore tended in many experiments to remove a large percentage of the granule cells from the coverslip. This was probably the main reason for the inability to obtain reproducible or maintained responses.

2.2.3 Single cell and small group measurements

The availability of a Photon Technology International Deltascan D104C system made it possible to consider measuring $[Ca^{2+}]_i$ in individual cerebellar granule cells. Such experiments have many advantages over population measurements, including the potential to assess variability in response size and time-course between different cells, and whether a response is elicited from all or only a proportion of the population. This

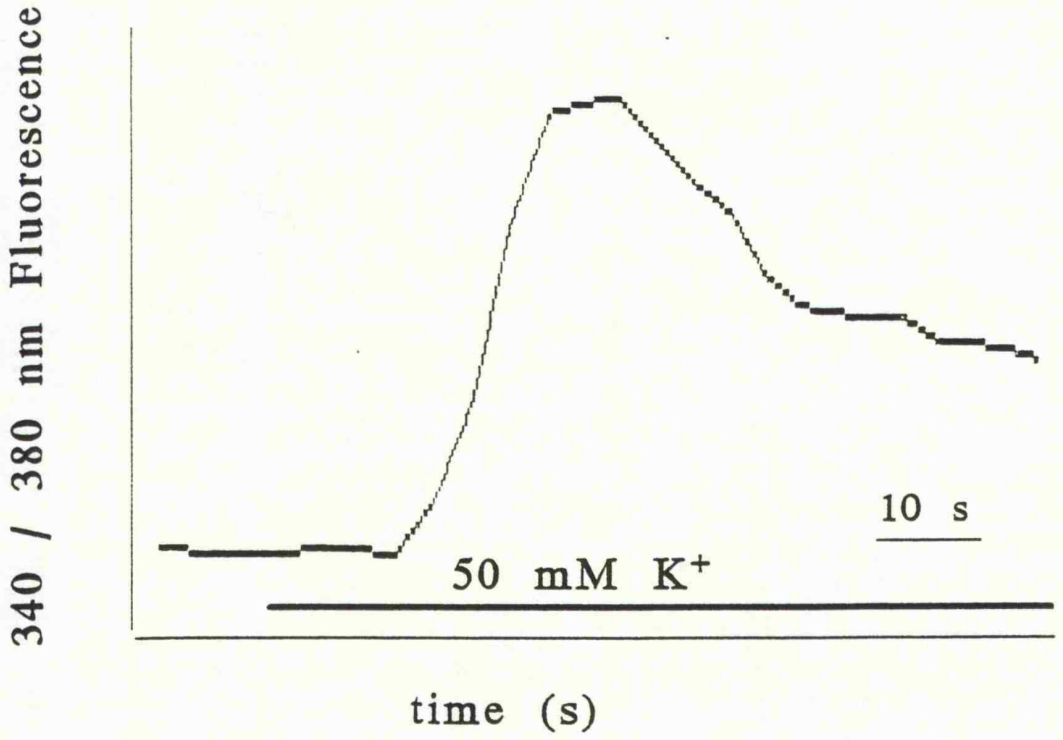


Fig. 2.3 Example trace of response to depolarization evoked in a population of granule cells. K^+ (50 mM) was added to the KHB in which a cover-slip of granule cells was held at the requisite angle, at 37°C in a Perkin-Elmer LS 50 population fluorimeter. The characteristic peak and plateau phases of the K^+-evoked response (see Chapter 3) were evident, although the response was relatively slow to develop. Few other such traces were obtained, and this population measurement approach was as a consequence not pursued further.

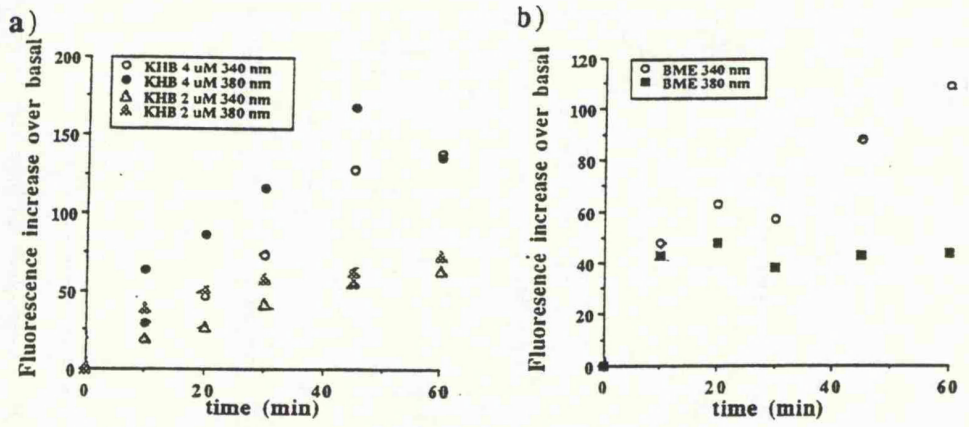


Fig. 2.4 Fluorescence of granule cell suspensions after fura 2 loading.

a) Loading of 2 μM fura 2-AM into granule cells in KHB resulted in an approximately linear increase in fluorescence up to 60 min, and the rate of loading was approximately doubled when 4 μM dye was used compared to 2 μM.

b) Loading of fura 2 into granule cells in BME resulted in a non-linear increase over time which appeared to be much greater for 340 nm excitation compared to 380 nm.

photometer system had previously been successfully used for measurements of $[Ca^{2+}]_i$ in single SH-SY5Y neuroblastoma cells (see Lambert and Nahorski, 1990). However, cerebellar granule cells are much smaller than SH-SY5Y neuroblastoma cells (~4 μm diameter (Ito, 1984)). Initial experiments indicated that a response could be detected from single granule cells, but that the background noise was large. A much higher and more consistent signal : noise ratio could be obtained by measuring $[Ca^{2+}]_i$ in groups of 5-8 granule cells, the size of a typical aggregation of cultured cerebellar granule cells at the density of plating used (see Figs. 2.1a,b,2.6b). As such groups were clearly the norm, measuring responses from isolated, single granule cells might result in inferences being drawn which were not true of typical, aggregated cells. For subsequent experiments, therefore, $[Ca^{2+}]_i$ changes were measured in groups of 5-8 cerebellar granule cells clustered together. The protocol for the determination of background fluorescence was as follows:

Cerebellar granule cells on a glass coverslip (22 x 22 mm) were transferred from culture medium to KHB, and the coverslip broken up using blunt forceps. A fragment was placed in an environmental chamber on the stage of a Nikon Diaphot-TMD inverted microscope. KHB was continually perfused into the chamber (0.4-0.7 ml) using a peristaltic pump at a flow rate of 1.0 ml / min, prewarmed to 37°C using a Pelletier system surrounding the environmental chamber. A region of coverslip containing 5-8 cerebellar granule cells was manoeuvred into the experimental field of view using x,y stage travel knobs. The field of view was defined by shutters fitted to the housing of a photon-counting photomultiplier tube connected to the microscope. The environmental chamber was illuminated using a 75W Xenon compact arc lamp, which provided wide beam illumination including ultraviolet. The light was directed via a reflective chopper wheel rotating at 100 Hz to two excitation monochromators set at wavelengths of 340 and 380 nm, the emergent light being subsequently combined into a beam which entered the rear port of the microscope. It was directed upward by a

dichroic mirror and was focussed onto the cells by the objective lens (x 40 magnification). Fluorescence emitted by the group of cells passed down through the objective, and via a filter fitted to the microscope was transmitted by the dichroic mirror into the photomultiplier tube and measured at an emission wavelength of 510 nm. The average fluorescence values of 6-9 groups of cells for each of the two excitation wavelengths were used as the background fluorescence, automatically subtracted from all experimental data obtained that day.

Previous fura 2 studies in cerebellar granule cells have used a wide variety of loading conditions:

	Fura 2-AM (μM)	Incubation medium	Incubation time (min)	Post-incubation time (min)	Temperature (°C)
Connor et al (1987)	4-6	CM (- serum)	30-45	90-180	36
Burgoyne *et al.* (1988)	2	BSS	40	60	20
Bouchelouche *et al.* (1989)	2	BSS	30	wash only	---
Holopainen *et al.* (1989)	4	BSS	30-40	wash only	37
Ciardo and Meldolesi (1991)	2	BSS	30-60	wash only	32
Courtney *et al.* (1992)	3	BSS + albumin	25-30	wash only	---
Irving *et al.* (1992a)	6	BSS	45	60	RT
Kiedrowski and Costa (1995)	4	CM	45	30	37

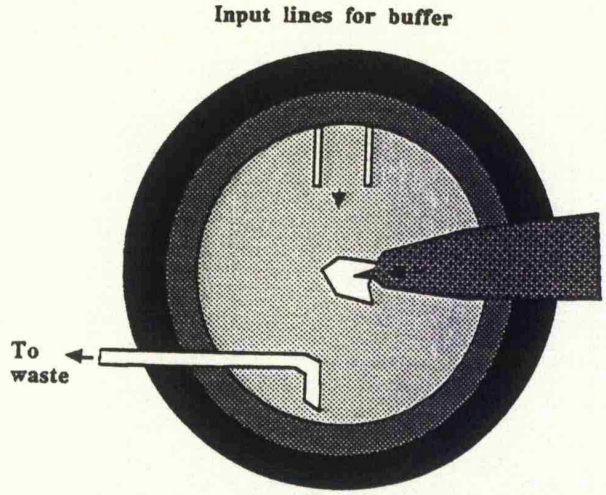
BSS = KHB-like balanced salt solution; CM = BME-like culture medium; --- = not detailed in methods; RT = room temperature.

It was necessary therefore in the first instance to determine optimal conditions for myself. Two variables were kept fixed at the conditions previously employed in this lab for other cell types, i.e. incubation at room temperature and a post-incubation period of 30 min, and all other factors were varied to examine their effects on loading and on the response to a given stimulus. To optimize loading conditions, cells were first incubated with 2 or 4 μM of fura 2-AM in either KHB or BME, for 0-60 min. Cells were then digested in 0.2% (v/v) Triton X-100, placed in a cuvette with KHB containing excess [Ca^{2+}], and fluorescence measured in the LS 50 fluorimeter. Dye loading increased approximately linearly over this time-period in KHB, and was approximately doubled in the higher fura 2 concentration (Fig. 2.4a). However, loading in BME was unsatisfactory, as the 340 / 380 nm ratio was highly time-dependent, due to the non-linearity of the fluorescence at 380 nm under these conditions (Fig. 2.4b). The consequences of loading conditions on detectable responsiveness were examined using the PTI system (methodology as described below) with 50 mM K^+ as the stimulus. Loading in KHB led to depolarization-evoked responses more than double those found after loading of the same fura 2-AM concentration in BME, confirming KHB as the preferred incubation medium. Loading of 4 μM fura 2-AM for ≥ 30 min, and loading of 2 μM fura 2-AM for 60 min, caused fluorescence to rise to > x5 over basal in virtually all cells, a level at which the possibility of significant Ca^{2+}-buffering by fura 2 arises. More moderate loading, with not significantly different responsiveness, was found with incubation of 2 μM fura 2-AM for 20-30 min. Consequently, all experiments were performed by incubating a coverslip of cerebellar granule cells in 2 μM fura 2-AM in KHB for 20 min at room temperature, washing, and leaving in KHB for at least 30 min to allow de-esterification to reach completion. Experiments were then performed as described below:

As before for background fluorescence determination, a fragment of the coverslip was placed in the environmental chamber, and a suitable group of cells

positioned within the field defined by the photomultiplier tube shutters. In early experiments a multibarrelled perfusion pipette was moved using approximate manual controls into the chamber, and a nearby group of cells selected for experimentation. The pipette was connected to a gravity feed system, with a 5 ml reservoir connected to each barrel, and with each line except the one containing the required agent being closed by butterfly clips. This system was functional, but made the choice of cells more limited and, as changing of lines had to be performed by switching butterfly clips in the dark, mistakes in carrying out the experimental protocol were not uncommon. This was subsequently upgraded to a system in which a suitable group of cells on the coverslip fragment could be freely selected and line-switching was much more reliable. In this setup, the multibarrelled pipette was held in a Narishige MO-203 micromanipulator and manoeuvred, using approximate manual controls and remote three dimensional hydraulic fine tuning, into close proximity to the chosen cells (Fig. 2.5)(see Langton, 1993). The pipette was connected via a diaphragm pump to a pressurized reservoir, such that the flow rate through the pipette could be controlled by altering the reservoir volume. Seven 2.5 ml reservoirs containing the experimental agents were held within a superfusion black box, each reservoir being connected to a barrel of the pipette. The black box ensured that only one line could flow at a time, the other lines being closed by miniature 12V solenoids and subminiature latching switches. Typically KHB in one reservoir was perfused down the pipette, and, after the presence of a stable baseline had been confirmed, the flow was changed via a switch box with LED display to that from a reservoir containing a receptor agonist or modulatory agent. Changes in the ratio of 510 nm fluorescence at 340 nm excitation to the fluorescence at 380 nm excitation were measured on-line by photon detection, and experimental traces collected using an IBM-compatible PC for analysis and storage on floppy disks.

a)



b)

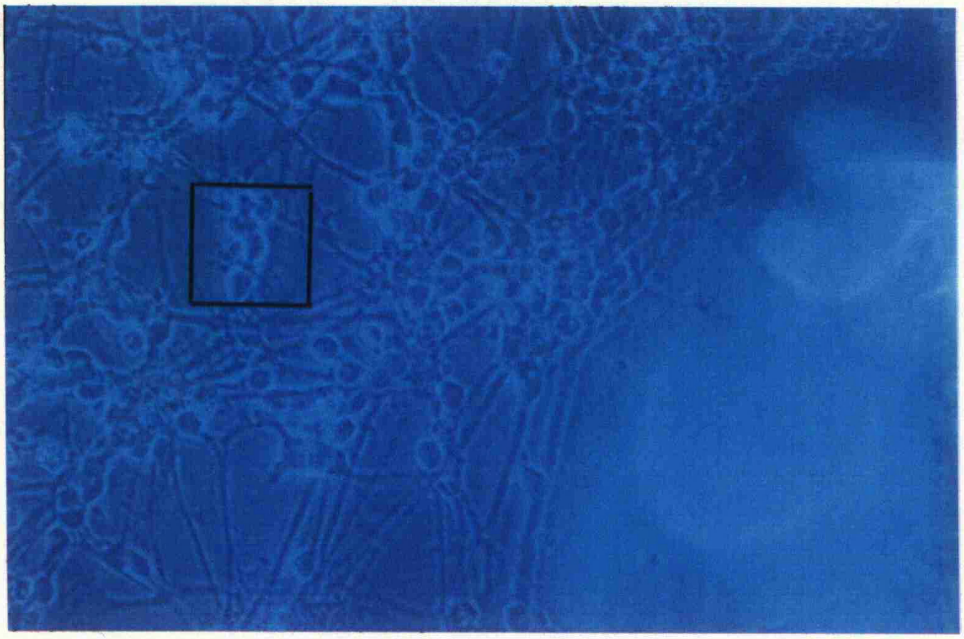


Fig. 2.5 [Ca^{2+}] measurement in cerebellar granule cells.

a) Arrangement of influx and efflux lines and multibarrelled pipette in the environmental chamber (18 mm diameter).

b) Phase contrast image of 7 DIV cerebellar granule cells cultured on a glass coverslip (x40 objective, fura 2 filter). A fragment of the coverslip has been placed in the environmental chamber for [Ca^{2+}] measurement. At the right hand side of the photograph the tip of the multibarelled pipette can be seen, marginally off the surface of the coverslip. The cells to be recorded from for this pipette position are as indicated by □.

Two kinds of multibarrelled pipette (see Langton, 1993) were made and used in these experiments, each having their own advantages and disadvantages. In both, polypropylene tubing was pulled over a low flame and cut in the middle, making two lines. Initially, pipettes were made from pulled multibarelled glass, tipped with thicker glass also pulled but which was cut using a diamond pencil, and fire-polished, to allow smooth and rapid flow. The pulled polypropylene lines were trimmed to a suitable length and fed into the barrels (initially 5 but later 7), beeswax being melted over the surface of the pipette and drawn inside each barrel to seal the lines in place. Beeswax was also melted over the broad end of the pipette and the connection of the pipette with the tip, to prevent leakage at either site. These pipettes had very little dead volume, allowing rapid changes between agents, but were difficult to make and very fragile.

The alternative method involved cutting a yellow (20-200 μl) Eppendorf tip at both ends to leave a ~1 cm section, and inserting the requisite number of polypropylene lines such that all protruded beyond the narrow end. The Eppendorf tip was then filled with Sylgard elastomer, and hung up to dry. A capping tip was made using a short section of pulled glass, flame-polished as before, and inserted into a cut end section of a different yellow tip. The initial tip containing the polypropylene lines was then cut back sufficiently to make a tight fit with the capping tip. These pipettes were more robust than the glass pipettes, but had a significantly greater dead volume, causing slower solution exchange and making them more prone to developing air bubbles. Glass pipettes were therefore used as far as was possible in this study, although in a significant number of experiments the latter type of tip was resorted to.

2.2.4 $[Ca^{2+}]_i$ calibration

340 / 380 nm ratio values were calibrated to changes in absolute $[Ca^{2+}]_i$ concentration using the equation of Grynkiewicz *et al.* (1985):

$$[Ca^{2+}] = K_D \times \frac{S_{f2}}{S_{b2}} \times \frac{R - R_{MIN}}{R_{MAX} - R}$$

where:

K_D = the apparent dissociation constant of fura 2 for Ca^{2+} at the experimental set temperature,

S_{f2} = the fluorescence at 380 nm of fura 2 free from Ca^{2+},

S_{b2} = the fluorescence at 380 nm of fura 2 saturated with $[Ca^{2+}]_i$,

R_{MIN} = the 340 / 380 nm ratio in minimal $[Ca^{2+}]_i$,

R_{MAX} = the 340 / 380 nm ratio in saturating $[Ca^{2+}]_i$,

and R = the given experimental 340 / 380 nm ratio.

The calibration was performed by measuring a steady-state basal ratio, stimulating with agonist then washing off, then causing maximal fura 2 fluorescence by adding the Ca^{2+} ionophore 4-bromo-A23187, which is highly effective at the experimental buffer pH of 7.4. First 5 μM and then 30 μM of the ionophore was added, and left sufficiently long for a steady plateau elevation to occur. 30 μM 4-bromo-A23187 was then perfused onto the cells in Ca^{2+}-free KHB containing ~1 mM EGTA, which reduced the fluorescence ratio to its minimum value by transporting much of the Ca^{2+} present out of the cells. This provided the R_{MAX} and R_{MIN} values, and from the 380 nm trace the values of S_{f2} and S_{b2} were also obtained. A revised value for the K_D of fura 2 at 37°C, 285 nM (Groden *et al.*, 1991), was used for calculation of $[Ca^{2+}]$. From this, the average basal $[Ca^{2+}]_i$ in cerebellar granule

cells was calculated to be 117 ± 14 nM. An calibrated example trace for NMDA is shown in Chapter 4.

2.3 Mn^{2+} quench

Fura 2 experiments measure changes in $[Ca^{2+}]_i$ which are a product of effects on a variety of mechanisms e.g. Ca^{2+} entry (Mayer *et al.*, 1987; Courtney *et al.*, 1990; Schneggenburger *et al.*, 1993), Ca^{2+}-induced Ca^{2+} release from intracellular stores (Lipscombe *et al.*, 1988), second messenger-induced Ca^{2+} release from stores (Murphy and Miller, 1989; Irving *et al.*, 1992a,b), and a decrease in Ca^{2+} extrusion (Arens *et al.*, 1992; Dumuis *et al.*, 1993; Khodorov *et al.*, 1993). It is therefore helpful to have complementary techniques by which these components of the response can be investigated individually. Mn^{2+} quench of fura 2 fluorescence has been widely used in non-excitable cells to examine divalent cation entry mechanisms, in isolation from other factors involved in the overall $[Ca^{2+}]_i$ response (Putney and Bird, 1993). Mn^{2+} is capable of entering cells via many types of cation channel, and binds to fura 2 with high affinity, quenching the fluorescence of the dye at all wavelengths at a rate proportional to its rate of entry into the cell (Hallam and Rink, 1985; Kwan and Putney, 1990). As there are no intracellular stores of Mn^{2+}, and Mn^{2+} entering cells may not accumulate within Ca^{2+} stores (Mertz *et al.*, 1990; but see Fasolato *et al.*, 1993), the rate of quench of fura 2 can be employed as an index of Ca^{2+}-permeant channel activation in isolation from other $[Ca^{2+}]_i$-elevating mechanisms. Little work has been done with this technique in neuronal preparations, but it appeared likely to be a useful method by which to identify the source of Ca^{2+} (i.e. extracellular or intracellular) causing changes in $[Ca^{2+}]_i$ seen in response to agonist addition.

Mn^{2+} is known to voltage-dependently block the passage of other divalent cations through brain VOCCs, but in the absence of a competing cation it passes

through such channels with an apparent conductance $^2/_3$ that of Ca^{2+} and with double the mean open time found for Ca^{2+} (Nelson, 1986). Experiments were thus performed in the absence of any divalent cations other than Mn^{2+}. The protocol adopted for Mn^{2+} quench experiments from small groups of granule cells was as described above for $[Ca^{2+}]_i$ measurement, except that the KHB perfused over granule cells during the experiments was nominally Ca^{2+}-free. 100 μM $MnCl_2$ was added under either control or stimulated conditions, and the fluorescence of fura 2 at 510 nm consequent to excitation wavelengths of 340 nm and 360 nm was measured, with fluorescence at 360 nm, the isosbestic point of fura 2, taken as an index of Ca^{2+}-independent, Mn^{2+}-dependent decreases in fluorescence.

2.4 Glutamate release

A variety of methods are available for measuring glutamate release, including high performance liquid chromatography (HPLC), release of [^{3}H]-D-aspartate, and release of [^{3}H]-glutamate after prelabelling with [^{3}H]-labelled glutamine, a metabolic precursor of glutamate. [^{3}H]-D-aspartate release has been demonstrated to not parallel glutamate release as measured by HPLC, whereas [^{3}H]-glutamine pre-incubation results in preferential labelling of readily releasable pools of glutamate (Levi *et al.*, 1991). Initial experiments using HPLC indicated that the amount of glutamate release from cerebellar granule cells was barely detectable, and co-eluted with a component of the experimental reaction cocktail. Parallel experiments using an adaptation of a method previously described for [^{3}H]-glutamine prelabelling (Huston *et al.*, 1990) proved more successful. In this protocol, cerebellar granule cells were gently washed in KHB, then incubated with 5 μCi [^{3}H]-glutamine in KHB for 1 h at 37°C. For static condition experiments, cells were grown in 24-well multiwells and after incubation with [^{3}H]-glutamine the wells gently washed 3 times in KHB. A series of 2 min incubations in KHB at room temperature (RT) were performed, with at set timepoints

the KHB containing 50 mM K^+. All fractions were retained and assayed as described below. For continuous perfusion experiments, cells were grown on cover-slips in 8-well multidishes. The cover-slips were gently washed x3 in KHB, then removed and attached to an experimental perfusion chamber with silicon grease (Fig. 2.6), and the chamber placed in a waterbath at 37°C. After a period of washthrough (~10 min), 2 min fractions were collected for analysis as before. KHB was perfused through the chamber at ~1 ml / min, and at set timepoints exchanged for high-[K^+] KHB. The time between solution exchange and collection of fractions was previously determined to minimise crossover between control and stimulated fractions. At the end of experiments, cells were homogenized in 1% (v/v) Triton X-100 and centrifuged, for analysis of total [^{3}H]-glutamate remaining.

[^{3}H]-glutamate was separated from [^{3}H]-glutamine using ion exchange chromatography. Columns were made from ~1.5 ml 50% (w/v) Bio-Rad AG 1 x 4 (400-800 mesh) slurry, washed with 10 ml 1M acetic acid (HAc) followed by 20 ml H_2O. Samples were pipetted onto the columns along with 10 mM standard glutamate and glutamine, which acted as carriers, 0.1% (v/v) Triton X-100, and in some experiments 1.5 nCi [^{14}C]-glutamate, which permitted correction for recovery. The columns were washed through with 6 ml 25 mM HAc, and glutamate eluted with 10 ml 0.5 M HAc into scintillation vials. A 2 ml fraction was retained in the vials, 18 ml Optiphase-X added, and the [^{3}H]-glutamate present determined by scintillation counting.

2.5 Total inositol phosphate measurement

Cerebellar granule cells were cultured in 24-well multidishes for this assay. Cells were preincubated with 4 μCi / ml [^{3}H]-*myo*-inositol in culture medium for 24 h, to allow the labelling of inositol lipids to reach equilibrium (Whitham, 1991 - Ph.D.

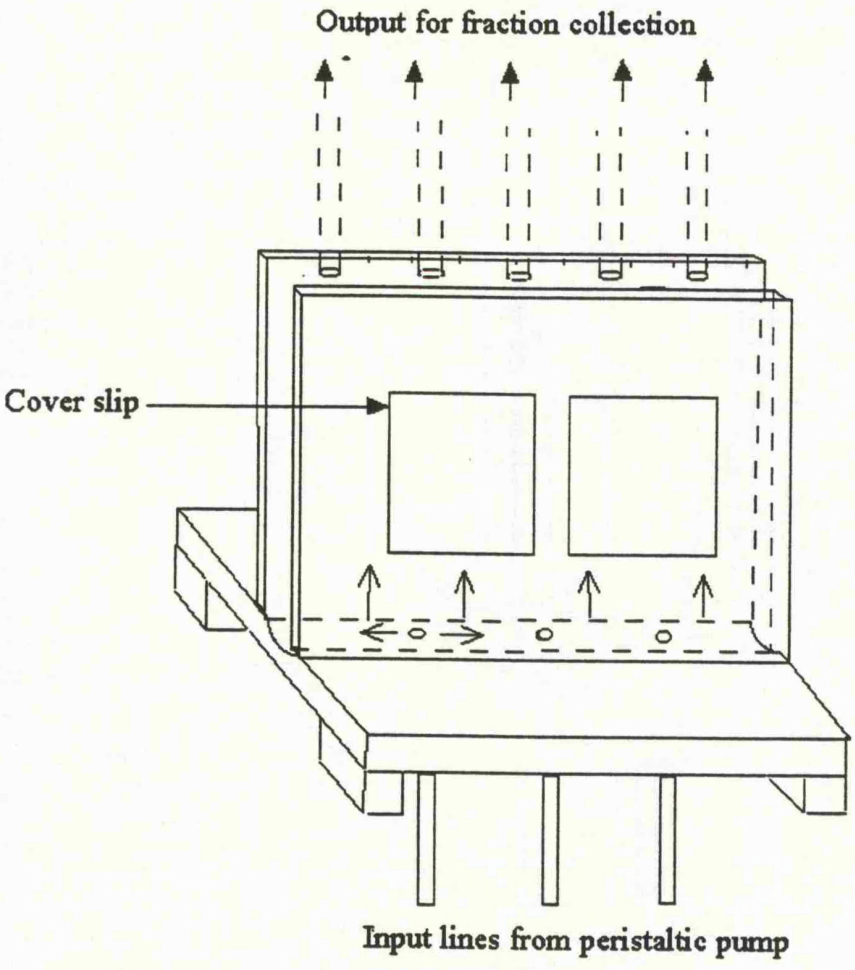


Fig. 2.6 Experimental chamber for continuous perfusion glutamate release experiments. The chamber was placed in a water bath at 37°C, with 3-6 input lines, carrying KHB or high K^+-KHB (prewarmed to 37°C) connected via a peristaltic pump, feeding into the shallow reservoir at the bottom of the chamber. This was sufficient to produce approximately linear flow upwards by capillary action between the two chamber sides, set 1 mm apart, and thus over the two coverslips of cells. 5 output lines at the top removed medium, at an identical rate to the input, via a second peristaltic pump into collection vials.

Thesis, University of Leicester). The culture medium was removed and the granule cells gently washed twice with KHB prewarmed to 37°C. Cells were then incubated in 250 µl KHB containing 5 mM Li^+ (to block degradation of inositol monophosphates and so enhance agonist-evoked responses), at 37°C. If pre-treatment with e.g. a Ca^{2+} store modulating agent or an antagonist was required, the agent was also added at this timepoint. After 15 min, the agonists to be investigated were pipetted into each well and incubated for 20 min. Agonist-evoked accumulation of total inositol phosphates ($[^3H]$-$InsP_X$) in cerebellar granule cells has previously been shown to be linear for > 30 min (Whitham *et al.*, 1991a). The incubation was terminated by addition of 250 µl 1M ice-cold trichloroacetic acid (TCA). The multidishes were placed on ice for ~15 min, then scraped to remove all cells. Granule cells suspended in TCA were transferred to Eppendorf tubes and pelleted at 12000g for 4 min. A 0.5 ml aliquot of each supernatant was transferred to a 10 ml opaque Sarstedt tube. 1.5 ml of water-saturated diethyl ether (wDE) was added, and the tube vortexed. After a short period of equilibration the top layer was removed from each vial. This process was repeated 2 more times to remove virtually all of the TCA present from the samples. Tubes were then left for several hours to allow any remaining wDE to evaporate off. 250 µl of each sample was transferred to an Eppendorf tube, and 50 µl 60 mM $NaHCO_3$ added. Example tubes were checked to ensure the pH was ~7, further $NaHCO_3$ being added if necessary. Samples were stored at 4°C until analysis.

$[^3H]$-$InsP_X$ in these samples was separated out by anion exchange chromatography, largely as previously described (Rooney and Nahorski, 1986). The anion exchange columns (0.75 ml chloride form Dowex 1 x 8 (100-200)) were prepared for use by adding to each 5 ml 2M HCl. This was allowed to drain through, and columns were subsequently washed with 20 ml H_2O. The experimental samples were added, and washed onto the columns with 10 ml H_2O, which carried through any excess $[^3H]$-inositol. After the columns had drained, 10 ml 25 mM ammonium formate

was added to elute off weakly bound glycerophosphoinositols. A collecting rack was then placed under the columns, and 10 ml 1M HCl added to each column to elute off inositol phosphates. 2 ml samples of eluant were retained, 10 ml Optiphase-X scintillant added, and the [^{3}H] present in each vial measured by scintillation counting. 10 ml 1M HCl was added to each column to remove any remaining sample components from the columns, and washed through with 10 ml H_2O. Columns could be used repeatedly if thoroughly regenerated and washed each time.

[^{3}H]-InsP_X was also measured in neonatal rat cortical slices, a preparation with marked differences in inositol phosphate signalling to granule cells. These experiments were performed as previously described (Challiss and Nahorski, 1993). Cerebral cortices were dissected out from P7-8 Wistar rats, and cross-chopped using a McIlwain tissue chopper into 0.35 mm^2 blocks. The resultant tissue was dispersed into KHB and left to settle, the KHB then being removed and replaced. This was repeated three times, and the slices were then incubated in KHB in a shaking water bath at 37^{o}C for 60 min. 3 times during this incubation the suspension was removed from the bath, left to settle, and the buffer replaced. At the end of this period, slices were left to settle again, and 25 µl aliquots pipetted into vials containing 250 µl KHB with 0.5 µCi [^{3}H]-inositol. These vials were incubated in the shaking water bath for 1 h at 37^{o}C, during which time they were purged four times with 95% O_2 : 5% CO_2. 5 mM Li^+ was then added to each vial, along with any Ca^{2+} store modulating agent or receptor antagonist required for the experiment, and left for 15 min. Agonists were then added to the vials, and incubated for 20 min. Agonist-evoked accumulation of ^{3}H-InsP_X in neonatal rat cortical slices has previously been shown to be linear over this time-period (Challiss *et al.*, 1994a). Incubations were terminated by the addition of an equal volume of 1M TCA, and vials placed on ice. Samples were intermittently vortexed for 20 min, then centrifuged at 4000g for 20 min at 4^{o}C. The supernatants were transferred to 10 ml opaque Sarstedt tubes for TCA extraction with wDE and scintillation counting as

above, the slices from representative vials being washed in 0.9% (w/v) NaCl and digested in 0.5M NaOH for subsequent protein measurement (Lowry *et al.*, 1951).

2.6 $InsP_3$ mass assay

Cerebellar granule cells were cultured in 24-well multidishes for this assay. At the appropriate timepoint, the culture medium was removed and the cells gently washed twice with KHB. They were then incubated at 37°C in 250 µl KHB containing the agonist of interest, and the experiment subsequently terminated by removal of KHB and addition of 250 µl ice-cold 0.5M TCA. The multiwells were placed on ice, then scraped to remove all cells from the dish. The cell suspension was collected in centrifuge tubes and spun at 12000g for ~4 min. TCA was extracted from the supernatant as described above, using 4 x 1 ml wDE. 100 µl of the remaining sample was treated with 40 µl 60 mM $NaHCO_3$ and 2.5 µl 0.5M EDTA, and stored at 4°C until use. The pellets from representative wells were washed in 0.9% (w/v) NaCl and digested in 0.5M NaOH for subsequent protein measurement (Lowry *et al.*, 1951).

$InsP_3$ mass was determined as previously described (Challiss *et al.*, 1990). 30 µl of sample was added to 30 µl buffer (100 mM Tris-HCl, 4 mM EDTA, pH 8.0) and 30 µl [^{3}H]-Ins(1,4,5)P_3 (~8000 dpm / assay) in vials on ice. 30 µl of previously prepared adrenal cortex binding protein (0.2-0.4 mg protein)(Challiss *et al.*, 1988) was then added to each, and the total volume incubated with intermittent vortexing for 30 min. In parallel, 30 µl samples of wDE-extracted TCA containing a range of concentrations of $InsP_3$ (0-12 pmol) was treated as for the experimental samples, to construct a standard curve. Vacuum manifolds were loaded with Whatman GF/B glass fibre filters and moistened with wash buffer (25 mM Tris-HCl, 5 mM $NaHCO_3$, 1 mM EDTA, pH 8.0). Bound and free [^{3}H]-Ins(1,4,5)P_3 were separated by adding 3 ml wash buffer to each sample and standard in turn and immediately pouring onto a filter.

This was rapidly followed by 2 further additions of 3 ml ice-cold wash buffer to each filter. The filter discs were subsequently placed in scintillation vials, 5 ml scintillant added, and the radioactivity present determined after >12 h extraction by scintillation counting.

2.7 Measurement of $InsP_3$ receptor expression levels

2.7.1 Western blotting for $InsP_3$ receptors

Cerebellar granule cells cultured in 8-well multidishes were treated overnight with agonist, added into the culture medium. Membranes were subsequently prepared from these and parallel untreated wells by scraping cells into ice-cold HEPES buffered saline (HBS) containing EGTA (155 mM NaCl, 10 mM HEPES, 0.7 mM EGTA; pH 7.4), and centrifugation at 500g for 3 min at 5°C. The supernatant was discarded, and the pellet resuspended in ice-cold Tris-EGTA solution (10 mM Tris, 1 mM EGTA; pH 7.5) which to inhibit protease activity was supplemented with dithriothreitol (1 mM), leupeptin (10 μM), pepstatin (10 μM) and phenylmethylsulphonylfluoride (100 μM) (homogenization buffer). The suspension was homogenized using an Ultra-Turrax (max speed, 15 s), and centrifuged at 38000g for 15 min at 5°C. Pelleted membranes were resuspended in a small volume of homogenization buffer, further disrupted with a manual homogenizer, and either assayed immediately or frozen (-20°C) until use. A sample was taken for protein estimation (Lowry *et al.*, 1951).

A Protean minigel was assembled containing a 6% (w/v) sodium dodecyl sulphate (SDS)-polyacrylamide separating gel with 5% (w/v) stacking gel. An equal volume of x2 sample buffer (2% (w/v) SDS, 2% (w/v) glycerol, 0.004% (w/v) bromophenol blue, 5% (w/v) 0.5 M Tris-HCl (pH 6.8)) was added to the membrane samples, which were then denatured by placing them in boiling water for ~3 min. High molecular weight (MW) markers were pipetted into one lane of the gel. Either 20 or

40 μg granule cell membrane protein was pipetted into each of the other lanes, and the minigel apparatus filled with running buffer (25 mM Tris-base, 250 mM glycine, 3.5 mM SDS). The proteins were separated by electrophoresis in the gel at 220 V for ~40 min.

Proteins were transferred onto nitrocellulose by semi-dry blotting. The gel was placed on top of a sheet of nitrocellulose and sandwiched with three pieces of filter paper soaked in blotting buffer (39 mM glycine, 48 mM Tris-base, 0.037% (w/v) SDS and 20% (v/v) methanol) on either side, and bubbles in the filter paper were removed by rolling with a glass pipette. Blotting was performed at 0.65 mA / cm^2 for >90 min. The area of the nitrocellulose sheet corresponding to the expected location of $InsP_3Rs$ (identified by the running of the prestained markers) was cut off and blocked for >60 min with gentle shaking in Tris buffered saline (20 mM Tris-base, 500 mM NaCl; TBS) containing 0.05% (v/v) tween 20 (TBS-T) and 5% (w/v) dried milk. A polyclonal Ab had previously been raised in rabbits against the C terminus of the rodent type I $InsP_3R$ by Dr. R. Wojcikiewicz, which has been shown to selectively bind to this receptor in neuroblastoma cells (Wojcikiewicz *et al.*, 1994a). The nitrocellulose was washed 3 times in TBS-T for ~5 min each, and incubated with the Ab (CT1), at 1 : 1000 dilution of crude serum in block buffer for >60 min. The primary Ab was then removed and the nitrocellulose given three further washes in TBS-T. This was followed by incubation in a goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) at a 1 : 500 dilution in block buffer for > 30 min. After three further washes in TBS-T and one in TBS, the nitrocellulose sheet was placed in reagents for electrochemical luminescence (ECL) analysis for 1 min. It was then drained, covered in clingfilm and exposed to autoradiographic film for 20-30 min, and developed. These exposure times for ECL reagents and film exposure were much longer than required for other cell types e.g. SH-SY5Y neuroblastomas (Wojcikiewicz *et al.*, 1992,1994a), indicative of low $InsP_3R$ expression levels in granule cells.

2.7.2 $InsP_3$ binding

While Western blotting provided a sensitive and readily detectable assay of type I InsP3R expression, the likely presence of several subtypes of $InsP_3R$ in neurones made interpretation of possible functional consequences of changes in type I expression in cerebellar granule cells difficult. For this reason, parallel experiments were performed using a binding assay which would measure expression of all membrane bound $InsP_3R$ subtypes. Cerebellar granule cells were treated and membranes prepared as described above for $InsP_3R$ Westerns. An $InsP_3$ binding assay was performed essentially as previously described (Challiss *et al.*, 1990). A range of concentrations of $InsP_3$ (0.12-12 pmol) were pipetted into vials, and 30 µl 100 mM Tris-EDTA buffer and 30 µl [^{3}H]-Ins(1,4,5)P_3 (~8000 dpm / assay) added. 30 µl samples of granule cell membranes or of previously prepared adrenal cortex binding protein standards (0.2-0.4 mg protein) were then added to each vial, and the total volume incubated with intermittent vortexing for 30 min. Bound and free [^{3}H]-Ins(1,4,5)P_3 were separated, and bound [^{3}H]-Ins(1,4,5)P_3 measured, using manifolds and scintillation counting as described for $InsP_3$ mass measurement.

2.8 Western blotting for ryanodine receptors

Membranes were prepared as described for $InsP_3R$ Westerns. The use of [^{3}H]-ryanodine in a binding assay for granule cells was attempted, but poor results were obtained. It was therefore decided to use the alternative SDS-polyacrylamide gel electrophoresis (SDS-PAGE) method for detection of RyRs, which as well as possibly greater sensitivity had the added advantage of permitting the determination of the subtype or subtypes of RyRs expressed in granule cells, given that, unlike for $InsP_3Rs$, Abs against all three subtypes of RyRs were available (a gift from Dr. A. Lai). In order to mobilize such a large MW molecule as the RyR by electrophoresis, 5% (w/v) gels

with 3% (w/v) stacking gels were used, with thicker spacers than needed for $InsP_3R$ Westerns to increase the stability of this less cross-linked gel. Preliminary experiments demonstrated that much larger amounts of membrane were needed than used for $InsP_3Rs$, in order for RyR expression in granule cells to be detectable. Therefore, up to 100 μg protein was run per lane, with denaturing being performed by boiling in x5 sample buffer to minimise the volume to be added (350 mM SDS, 1.53% (w/v) 2M Tris-HCl (pH 6.8), 25 mM DTT, 50% (w/v) glycerol, containing a small number of bromophenol blue crystals), for ~40 min at 220 V. Semi-dry blotting (in 150 mM glycine, 20 mM Tris-base and 0.037% (w/v) SDS) was performed at ~20 V for 3-4 hrs. Even at this voltage, Panceau S staining revealed that not all of the RyRs present in the gel crossed over into the nitrocellulose (J. Mackrill, personal communication), but sufficient did transfer to enable detection, if not precise determination of changes in expression levels. The nitrocellulose was washed three times in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4; PBS) containing 0.05% tween 20 (PBS-T), then blocked for >3 h in PBS containing 5% (w/v) milk. After three further washes in PBS-T, the nitrocellulose sheet was incubated with Abs raised in rabbits against RyR subtypes, at 1 : 500 dilution in block buffer. This was followed by 3 washes in PBS, and incubation with an anti-rabbit IgG conjugated with HRP at a 1 : 1000 dilution in block buffer for >60 min. ECL detection was performed as described above, with exposure to autoradiographic film (~30 min), and developing as before.

2.9 Western blotting for PKC isozymes

Membranes were prepared as for $InsP_3R$ Westerns, subsequently rehomogenized in an adjusted volume, and centrifuged at 4000g for 10 min. The supernatant was removed, TCA added (0.5 M final concentration), and the protein content subsequently pelleted by centrifugation. An equal volume of x2 sample buffer

was added, and this combination boiled for 7 min. The proteins were then separated by SDS-PAGE in a Protean minigel containing a 10% (w/v) running gel and 5% (w/v) stacking gel for 40 min at a constant current of 30 A. Proteins were transferred to nitrocellulose by incubation in a wet blotter containing transfer buffer (39 mM glycine, 48 mM Tris-base, 0.037% (w/v) SDS, 20% (v/v) methanol) for 1 h at 70 V. The nitrocellulose was blocked overnight in PBS (pH 7.4) containing 0.2% (v/v) tween 20 (PBS-T2) and 10% (w/v) powdered milk.

Samples were washed twice in PBS-T2, then in PBS-T2 containing 0.5 M NaCl for 15 min, then twice more in PBS-T2. Abs against protein kinase isozymes were incubated with the nitrocellulose strips at 1:3000 dilution (or 1:5000 for the PKC α Ab) in PBS-T2 containing 1% (w/v) powdered milk for 4 h. (PKCβ, γ, and δ Abs were a gift from Dr. P. Parker). The nitrocellulose was then washed as before, and incubated with HRP-conjugated anti-rabbit IgG (or anti-mouse for PKCα Ab) at 1:5000 dilution for 1 h. After a further washing as before, strips were incubated with ECL reagents for 1 min, wrapped in Saranwrap, exposed to autoradiographic film for 5-10 min, and developed.

2.10 Western blotting for Ca^{2+}ATPase isoforms

Membranes were prepared as described for $InsP_3R$ Westerns, that protocol being adapted for detection of a lower MW protein. Proteins were separated in an 8% (w/v) gel with 5% (w/v) stacking gel at 220 V for ~ 30 min, and transferred to nitrocellulose by semi-dry blotting as before. An Ab raised in mice which reportedly recognises both sarcoplasmic-endoplasmic reticulum Ca^{2+}ATPase (hereafter referred to as Ca^{2+}ATPase) 1 and 2 isoforms (a gift from Dr. F. Michelangeli) was used as the primary Ab, at 1 : 15 dilution of crude serum, and an HRP-conjugated anti-mouse IgG (a gift from Dr. A. Lai)(1 : 500 dilution) was used as the secondary Ab. A relatively

brief exposure to the autoradiographic film (~5 min) was sufficient to detect a band of the expected size, as determined by MW markers.

2.11 Statistical analysis

Results obtained under different experimental conditions were compared by analysis of variance or Student's t-test for paired or unpaired data, as appropriate, using Excel 4.0a (Microsoft, U.S.A.). Where indicated, means of all values obtained for each experimental condition were calculated for each day's experiments, and treated as single data points. For Mn^{2+} quench experiments, the decreases in fura 2 fluorescence at 360 nm were fitted by single exponential curves using regression analysis, and $t_{1/2}$ values calculated, utilizing the GraphPad Inplot program (GraphPad Software, U.S.A.). Results are presented as mean ± S.E. throughout.

3. K^+-depolarization responses

3.1 Characterization of responses evoked by K^+-depolarization

3.1.1 Use of K^+-depolarization as an experimental tool

The stimulation of Ca^{2+} entry across the plasma membrane is an important signal transduction mechanism for many receptor agonists, occurring via either non-selective cation-permeant channels, or relatively Ca^{2+}-specific channels such as VOCCs (see Tsien and Tsien, 1990). Ca^{2+} entry via VOCCs can be activated by membrane depolarization consequent to the activation of various types of receptor, particularly receptors containing intrinsic channels permeant to Na^+ such as glutamatergic NMDARs and AMPARs (see Mayer and Miller, 1990). Experimentally, increasing $[K^+]_o$ alters the ionic gradient across cell membranes, and would thus be expected to activate VOCCs and cause Ca^{2+} entry. While prolonged depolarization of neurones by increasing $[K^+]_o$ is not a physiological mechanism of influencing cell behaviour, it permits the investigation of fundamental mechanisms which occur as part of ongoing neuronal activity. Depolarization also allows the consequences of Ca^{2+} entry activation to be analysed without the difficulties of interpretation arising from the typically more complex and interconnected signalling pathways activated by ionotropic receptors. An initial series of experiments was therefore designed to investigate the effects of a simple stimulus, depolarization evoked by elevation of extracellular $[K^+]$, on $[Ca^{2+}]_i$ levels in cerebellar granule cells. It was envisaged that this might enable some of the $[Ca^{2+}]_i$-elevating mechanisms present in these neurones to be identified and defined, in advance of the investigation of more complex receptor-mediated responses. Experiments to determine the channels involved in depolarization-evoked entry, and the possible involvement of Ca^{2+} stores in the depolarization-evoked response, are detailed in this Chapter.

3.1.2 Effects of K^+-depolarization on $[Ca^{2+}]_i$

In cerebellar granule cells, increasing extracellular $[K^+]$ by 25 mM or 50 mM (over the 4.7 mM $[K^+]$ present in normal KHB) caused a biphasic elevation of $[Ca^{2+}]_i$, consisting of a rapid-onset peak elevation, which decayed within 30-60 s, and a steady-state plateau, which persisted throughout the stimulation period. The $[Ca^{2+}]_i$ elevation evoked by 50 mM K^+ was larger than that evoked by 25 mM, with the peak 340 / 380 nm ratio in some experiments reaching >10 (50 mM K^+: basal 340 / 380 nm ratio = 1.05 ± 0.05, peak elevation in 340 / 380 nm ratio over basal = 5.72 ± 0.49, plateau elevation over basal = 0.64 ± 0.06). As such large peak $[Ca^{2+}]_i$ increases may cause toxic effects in cells, and probably pass the point at which changes in 340 / 380 nm ratio are approximately linearly related to changes in $[Ca^{2+}]_i$ (cf Section 4.2.2), 25 mM $[K^+]_o$ increases were used in subsequent experiments. The peak and plateau phases of the response to 25 mM K^+ were temporally consistent, whereas the size of response displayed considerable variability between groups of cells, even from the same coverslip (25 mM K^+: basal ratio = 1.10 ± 0.02, peak elevation = 2.92 ± 0.07, plateau elevation = 0.90 ± 0.02). Both phases of the response were virtually absent in nominally Ca^{2+}-free KHB (Fig. 3.1), indicative of an absolute dependence on Ca^{2+} entry.

The ability of K^+-depolarization to increase $[Ca^{2+}]_i$ was examined in cells grown for various lengths of time *in vitro*. While differences were found between individual preparations, by analysing a large number of experiments, for all days examined (4-9 DIV) no significant correlation between the magnitude of either peak or plateau elevations evoked by 25 mM K^+ and DIV could be established (Fig. 3.2). It may be that other more complex factors, such as small differences in culture conditions e.g. perhaps between batches of FCS, have a more important influence on cell responsiveness than the age of the cells.

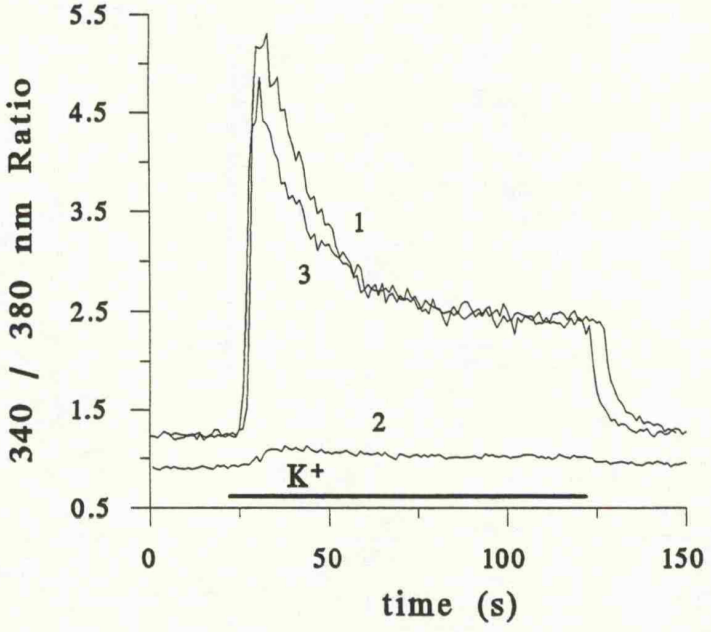


Fig. 3.1 Dependence of K^+-evoked $[Ca^{2+}]_i$ response on extracellular Ca^{2+}. 3 successive responses evoked by 25 mM K^+ in the same group of granule cells, at 5 min intervals. Elevation of $[Ca^{2+}]_i$ by 25 mM K^+ was characteristically biphasic (*1*), with a rapid-onset peak followed by a prolonged plateau response. In nominally Ca^{2+}-free buffer, there was virtually no response to a second challenge with 25 mM K^+ (*2*). The third response, after 5 min perfusion of normal $[Ca^{2+}]$-KHB, was not significantly different from the initial response (*3*), indicative of the high degree of reproducibility of response to K^+ in normal $[Ca^{2+}]_i$ within a given group of cells.

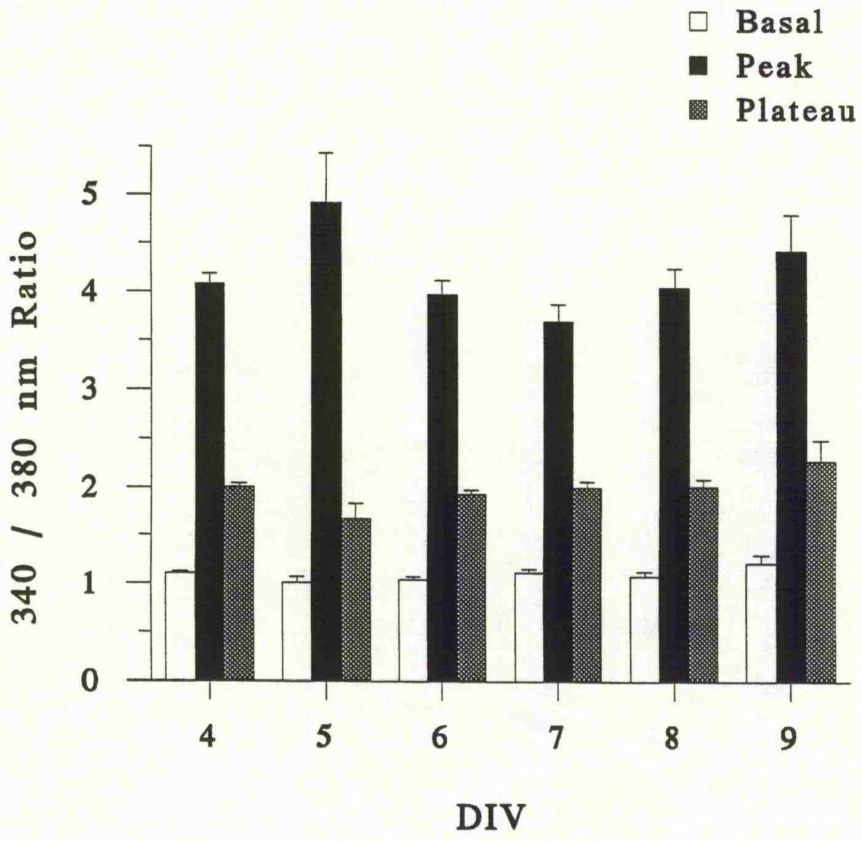


Fig. 3.2 Peak and plateau responses to K^+ compared to DIV. Meaned responses for 25 mM K^+ from many experiments on different days. No consistent significant trends were detected for either phase of the K^+-evoked $[Ca^{2+}]_i$ response from 3-10 DIV.

In contrast to previous reports suggesting a conditioning effect of an initial K^+ depolarization on the response to subsequent depolarization in granule cells (Connor *et al.*, 1987) or a gradual rundown of the effects of K^+ depolarization (Irving *et al.*, 1992a,b), the response to 1.5-3 min 25 mM K^+-depolarization at 5 min intervals was highly reproducible (S2/S1: peak = 91 ± 9%; plateau = 103 ± 6%)(Fig. 3.1). This enabled the response under a variety of experimental conditions to be compared to that evoked by an initial control stimulation within the same group of cells, an important method used throughout this study because of the variability in responses found between different groups.

3.2 Channels involved in depolarization-evoked Ca^{2+} entry

3.2.1 Effects of Ca^{2+} channel modulators on K^+-evoked $[Ca^{2+}]_i$ elevation

A wide variety of subtypes of VOCC have been detected in cerebellar granule cells (Carboni *et al.*, 1985; Carboni and Wojcik, 1988; De Waard *et al.*, 1991; Sutton *et al.*, 1993; Forti and Pietrobon, 1993; Zhang *et al.*, 1993; Bossu *et al.*, 1994), so it was of interest to determine which of these were the important mediators of Ca^{2+} entry under K^+-depolarizing conditions. The phenylalkylamine verapamil was not considered useful for identifying the channels involved in the depolarization-evoked response as it has been reported to block cerebellar granule cell VOCCs in a non-subtype-specific manner (Carboni and Wojcik, 1988). Consistent with this, in preliminary experiments in the present study its inhibitory effects on depolarization-evoked responses were found to be partially additive with those of DHPs (data not shown). Therefore other agents such as DHPs were utilised for more subtype-specific inhibition. The (+) and (-) enantiomers of PN 202 791, a DHP, have been reported to be respectively an agonist and an antagonist of L-type VOCCs (Hof et al, 1985). The plateau phase of the $[Ca^{2+}]_i$ response to K^+ was significantly decreased (51 ± 4%

inhibition (n=10)) by perfusion of 1 μM (-) PN 202 791, indicating a major involvement of L-type VOCCs in this sustained $[Ca^{2+}]_i$ elevation. The peak response after 5 min incubation with this enantiomer was not significantly different from the initial control peak response (8 ± 11% inhibition (n=5)), and so the peak phase appears to be predominantly or entirely due to Ca^{2+} entry via non-L-type channels (Fig. 3.3). Both phases of the K^+-evoked $[Ca^{2+}]_i$ response were potentiated by incubation with the (+) enantiomer of PN 202 791 (1 μM) (65 ± 13% increase in peak phase (n=5), 46 ± 9% increase in plateau (n=11))(Fig 3.3), consistent with an enantiomer-specific L channel effect of PN 202 791. The N-type Ca^{2+} channel antagonist ω-conotoxin GVIA (1 μM) also caused no inhibition of the K^+-evoked $[Ca^{2+}]_i$ elevation, and indeed in some cases caused an unexpected small potentiation of the K^+-evoked response (data not shown).

3.2.2 Effects of K^+-depolarization on glutamate release

Cerebellar granule cells *in vivo* respond to excitatory mossy fibre inputs by releasing glutamate at parallel fibre synapses with Purkinje cells and interneurones (Somogyi *et al.*, 1986; Elias *et al.*, 1993). As part of the present investigation of the effects of depolarization, the possibility that elevating $[K^+]_o$ would evoke glutamate release from cerebellar granule cells *in vitro* was investigated. Cells were preloaded with a $[^3H]$-labelled form of the metabolic precursor of glutamate, glutamine, for 1 h, which results in preferential labelling of readily releasable pools of glutamate (Levi *et al.*, 1991). Addition of 50 mM KCl for 2 or 6 min at room temperature caused an increase in $[^3H]$-glutamate release over control of 84 ± 11% (Fig. 3.4). After the cells were returned to KHB containing normal $[K^+]$, this progressively declined to near initial background levels in subsequent 2 min fractions. Rechallenge of granule cells with 50 mM K^+ caused a second release of $[^3H]$-glutamate, of comparable size to the

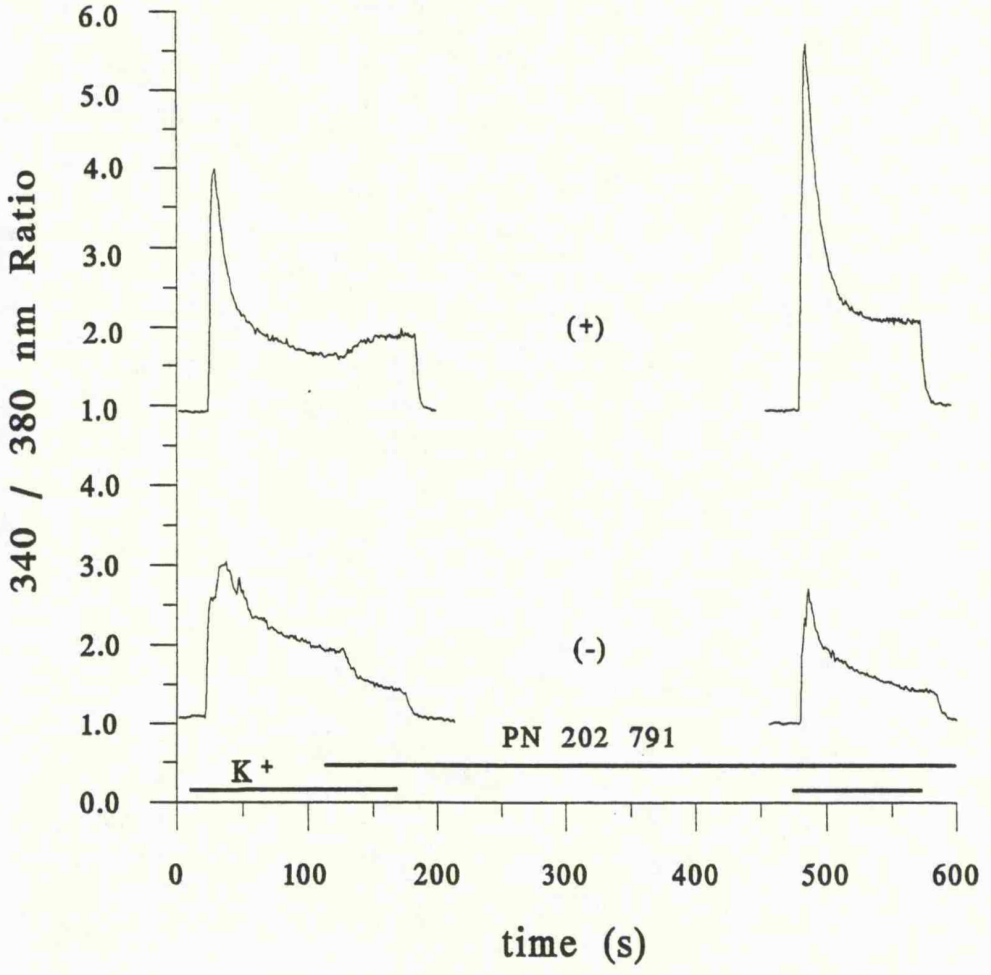


Fig. 3.3 Modulation of K^+-evoked $[Ca^{2+}]_i$ elevation by enantiomers of PN 202 791. Example traces typical of results from series of experiments performed on 3 different granule cell preparations. The (+) enantiomer trace has been shifted vertically on this graph for ease of comparison.

top trace: The (+) enantiomer of PN 202 791 (1 μM), an agonist of L-type VOCCs, increased both phases of the K^+-evoked $[Ca^{2+}]_i$ elevation.

bottom trace: The (-) enantiomer (1 μM), an antagonist of L-type VOCCs, rapidly decreased the plateau $[Ca^{2+}]_i$ response, but even after 5 min pre-incubation had no significant effect on the K^+-evoked peak response.

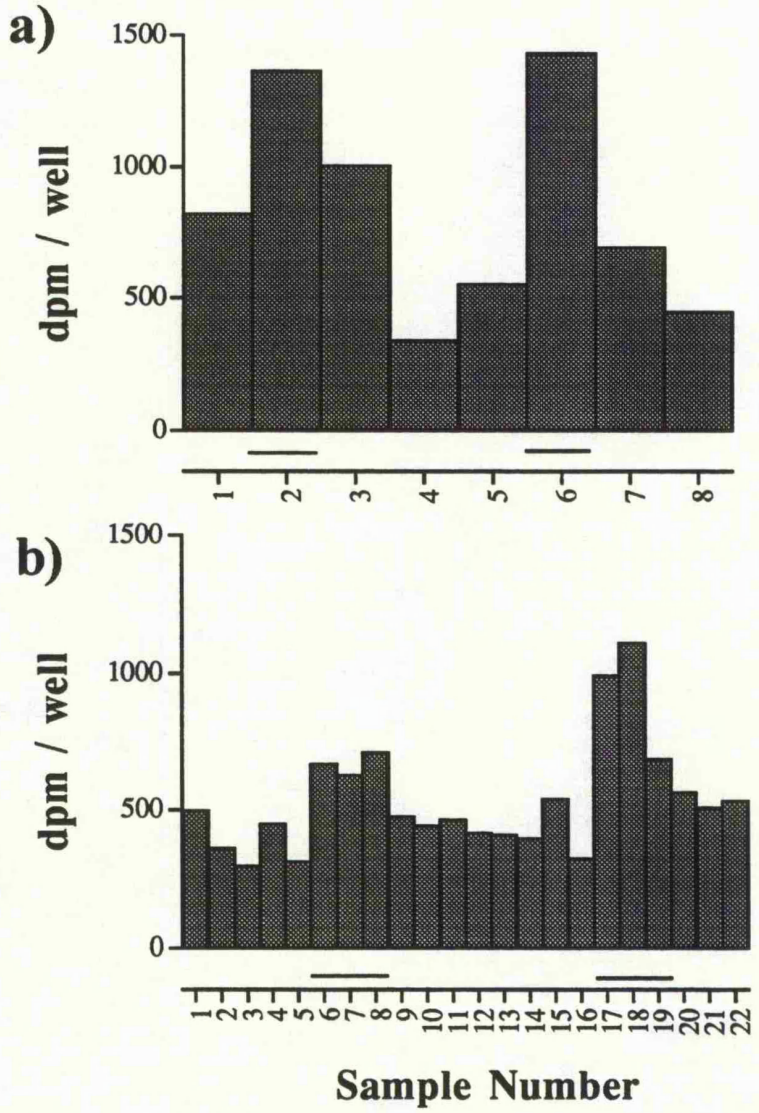


Fig. 3.4 K^+-evoked release of [^{3}H]-glutamate from cerebellar granule cells.

a) Meaned data from 2 experiments performed on granule cell preparations under static conditions. 2 successive stimulations with 50 mM K^+ (2 min) each evoked a marked elevation in [^{3}H]-glutamate release (8.2% of [^{3}H]-glutamate present was released during the experimental time-period). ***b)*** Meaned data from 2 experiments performed on granule cells under static conditions. 2 successive stimulations with 50 mM K^+ evoked similar [^{3}H]-glutamate release over 6 min stimulation periods, declining to basal release rapidly on return to normal K^+-KHB. During these longer experiments, 46.8% of [^{3}H]-glutamate present was released.

initial response (S2/S1 = 1.08 ± 0.16 (n=4)). Similar patterns of release were found for granule cells between 3 and 9 DIV.

While this protocol was sufficient to establish the existence of K^+-evoked glutamate release in cultured granule cells, it was considered helpful to subsequently examine the phenomenon under conditions more directly comparable with the method used for $[Ca^{2+}]_i$ measurements. A specially designed environmental chamber (see Methods) enabled the release of glutamate from cells grown on cover-slips to be examined under continuous perfusion conditions, with cells maintained at 37°C. The release of $[^3H]$-glutamate in 2 min fractions was, as under the initial experimental design, potentiated by elevation of $[K^+]_o$ (S1= 45% elevation, S2 = 154% elevation (n=1)).

As these findings indicate that 25 mM K^+ evoked release of significant levels of glutamate, for periods of ≥6 min, and a previous report had indicated that a major component of depolarization-evoked $^{45}Ca^{2+}$ uptake into cerebellar granule cells occurs through NMDA channels (Didier *et al.*, 1993), it therefore appeared possible that a significant component of K^+-evoked $[Ca^{2+}]_i$ elevations could occur secondary to glutamate release and activation of NMDARs. However, the $[Ca^{2+}]_i$ response to 25 mM K^+ was not inhibited by addition of the NMDA channel antagonist dizocilpine (1-5 μM) (Fig. 3.5), suggesting that depolarization-evoked Ca^{2+} entry occurs via VOCCs in granule cells, at least under the conditions used.

3.2.3 K^+-evoked stimulation of Mn^{2+} quench

To investigate the effect of depolarization on granule cell membrane permeability to cations more directly, the Mn^{2+} quench technique was utilized. Mn^{2+} quench of fura 2 fluorescence measures divalent cation entry mechanisms in isolation

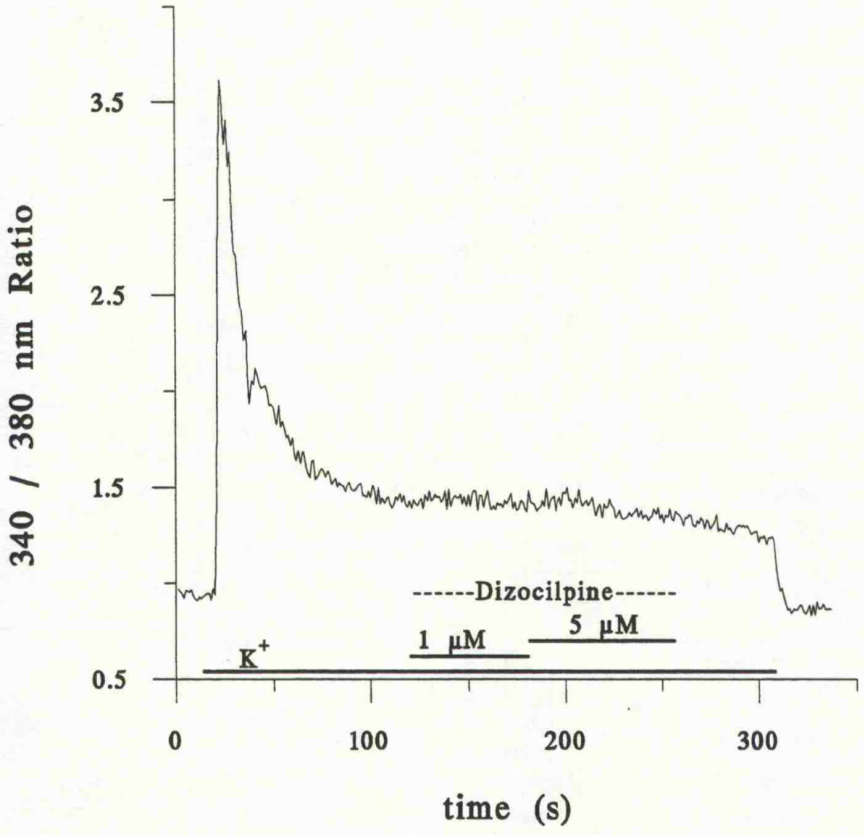


Fig. 3.5 Lack of effect of dizocilpine on the $[Ca^{2+}]_i$ elevation evoked by K^+-depolarization. Example trace typical of four series of experiments performed on different days. Addition of the NMDAR channel antagonist dizocilpine (1 µM, then 5 µM) had no significant effect on the response to 25 mM K^+.

from other factors involved in the overall $[Ca^{2+}]_i$ response (Putney and Bird, 1993). Mn^{2+} enters cells via many types of cation channel, and binds to fura 2 with high affinity, quenching the fluorescence of the dye at a rate proportional to its rate of entry into the cell (Hallam and Rink, 1985; Kwan and Putney, 1990). Perfusion of 0.1 mM Mn^{2+} onto cerebellar granule cells under unstimulated conditions caused a decrease in fura 2 fluorescence at 360 nm well fitted by a single exponential, which typically reached a plateau that was 5-10% of the initial fluorescence value (Fig. 3.6a). When 25 mM K^+ was added at the same time as Mn^{2+}, the rate of quench was increased 5-10 fold over basal quench (Fig. 3.6b, Table 3.1). This confirms that, despite the lack of

Condition	$t_{1/2}$ ± S.E. (s)
KCl Control	103.8 ± 29.4
KCl Plateau	36.6 ± 10.7*
KCl Peak	15.2 ± 3.1*#

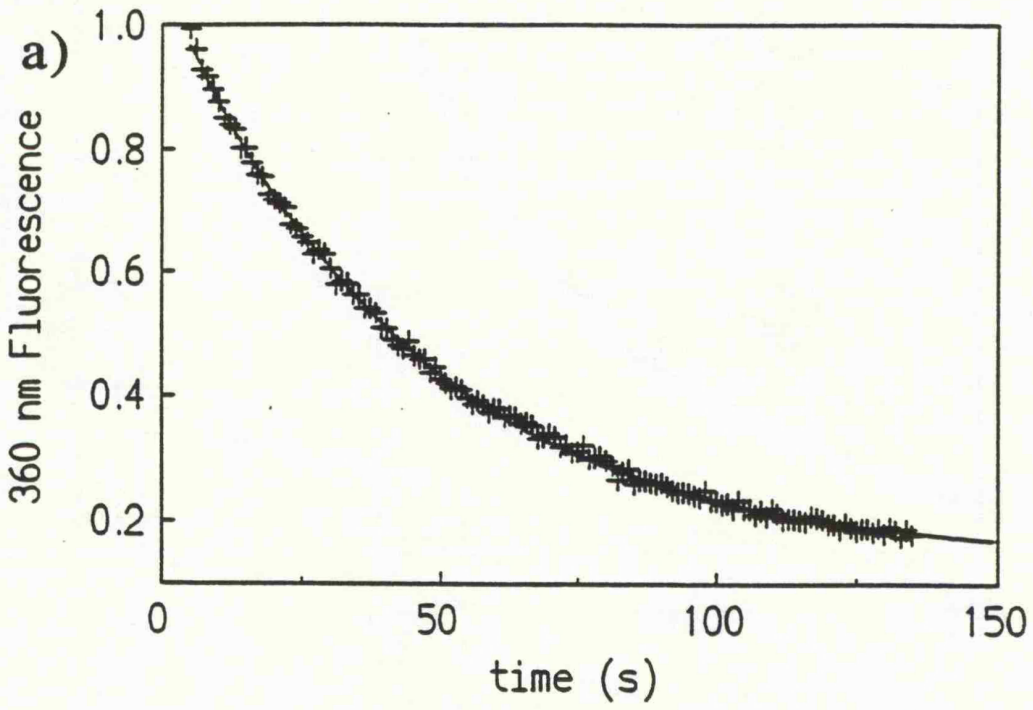
Table 3.1 Meaned results for K^+-evoked Mn^{2+} quench. Differences between groups were demonstrated by analysis of variance ($p < 0.005$). Data were subsequently analysed using Student's t-test. * = $t_{1/2}$ significantly lower than control ($p < 0.005$ for K^+ peak, $p < 0.01$ for K^+ plateau). # = $t_{1/2}$ significantly lower than plateau ($p < 0.05$ for K^+ peak) (n=6, with all data obtained on a given day meaned and treated as a single data-set.)

(-)PN 202 791- or ω-conotoxin-sensitivity of the peak $[Ca^{2+}]_i$ response described above, the K^+-evoked peak as well as plateau $[Ca^{2+}]_i$ phase involves Ca^{2+} entry, via unidentified channels. Addition of Mn^{2+} 100 s after the initiation of K^+-evoked depolarization evoked a significantly less marked increase in quench rate, although still elevated over control (Fig. 3.6b, Table 3.1). These data obtained using the Mn^{2+} quench technique clearly correlate well with the biphasic nature of the $[Ca^{2+}]_i$ elevation evoked by 25 mM K^+, with peak Mn^{2+} entry being greater than entry during the steady-state plateau, indicating that the Mn^{2+} quench technique may be of considerable use in studying entry mechanisms in granule cells.

3.3 Involvement of Ca^{2+} stores in K^+-evoked responses

3.3.1 Ca^{2+} stores in cerebellar granule cells

A variety of agents are now known which interact with specific sites on intracellular Ca^{2+} stores. Such interactions can cause (i) inhibition of Ca^{2+} release via $InsP_3R$ or RyR channels e.g. heparin acting on $InsP_3Rs$ (Henzi and MacDermott, 1992; Ji *et al.*, 1993), and either dantrolene (Danko *et al.*, 1985; Pessah *et al.*, 1986; Ohta *et al.*, 1990; Henzi and MacDermott, 1992; Nelson and Lin, 1993) or high concentrations of ryanodine (Rousseau *et al.*, 1987; Nagasaki and Fleischer, 1989; Bezprozvanny *et al.*, 1991; McPherson *et al.*, 1991) on RyRs; (ii) activation of these channels e.g. nanomolar ryanodine activating RyRs (see Berridge, 1993) or caffeine sensitizing RyRs (Kuba, 1980; Thayer *et al.*, 1988; Bezprozvanny *et al.*, 1991; Friel and Tsien, 1992); (iii) store depletion, by inhibiting the Ca^{2+}ATPase which causes sequestration of Ca^{2+} into stores, e.g. thapsigargin or 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone (BHQ) (Moore *et al.*, 1987; Thastrup *et al.*, 1990). The ability of several of these agents to activate or inhibit Ca^{2+} mobilization in cerebellar granule cells will be examined in detail throughout this Thesis. Initial studies focussed on the possible abilities of thapsigargin and caffeine to directly elevate $[Ca^{2+}]_i$ in granule


a)
360 nm Fluorescence
1.0
0.8
0.6
0.4
0.2
0
50
100
150
time (s)

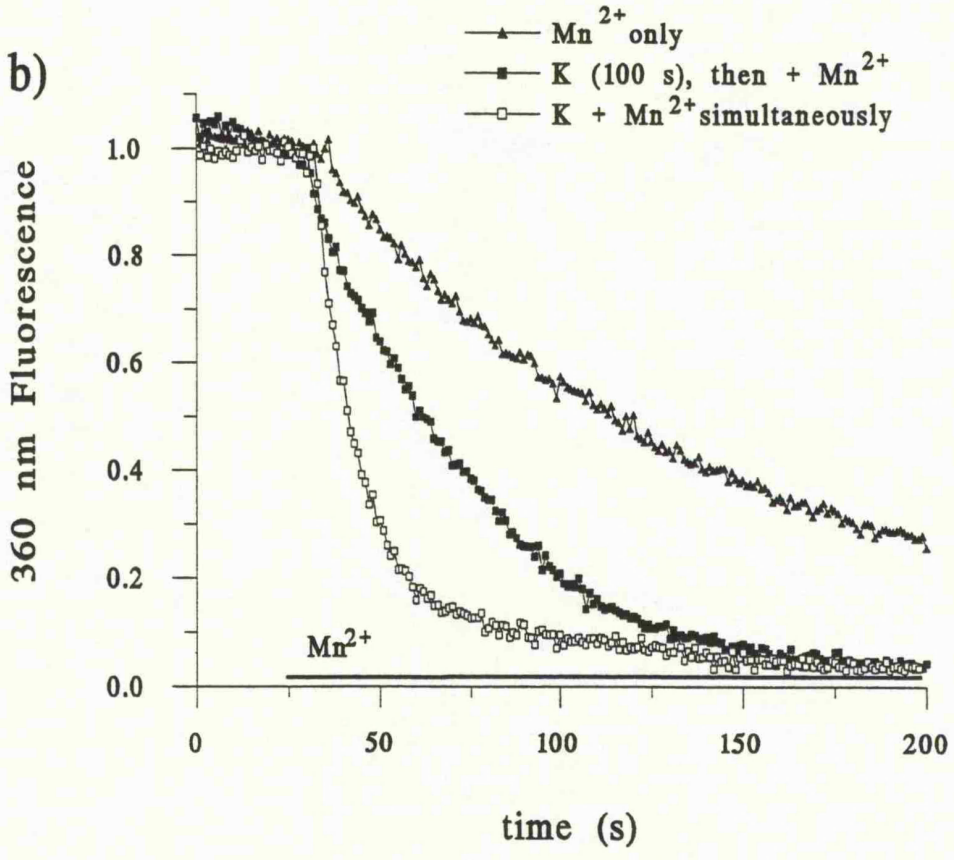

b)
Mn 2+ only
K (100 s), then + Mn 2+
K + Mn 2+ simultaneously
360 nm Fluorescence
1.0
0.8
0.6
0.4
0.2
0.0
Mn2+
0
50
100
150
200
time (s)

Fig. 3.6 The effects of K^+-depolarization on Mn^{2+} quench of fura 2 fluorescence.

a) Typical example trace for K^+-stimulated Mn^{2+} quench. Perfusion of 0.1 mM Mn^{2+} evoked a decrease in fura 2 fluorescence at 360 nm excitation, under both basal (not shown) and K^+-stimulated conditions in cerebellar granule cells, which could be well fitted by an exponential curve derived by regression analysis using the GraphPad program.

b) Example traces of normalized data from a series of experiments performed on the same day using cells from the same culture preparation, typical of 6 such series. Perfusion of 25 mM K^+ and 0.1 mM Mn^{2+} simultaneously markedly increased the rate of Mn^{2+} quench compared to Mn^{2+} alone. Mn^{2+} added 100 s after the initiation of K^+-depolarization i.e. during the plateau phase of the response (cf Fig. 3.1) evoked a more modest stimulation of quench than when added simultaneously with K^+.

cells. Thapsigargin (10 μM) perfused under normal-Ca^{2+} conditions often evoked a small transient peak elevation of $[Ca^{2+}]_i$, followed by a slow-onset, irregular but prolonged elevation (Fig. 3.7). In nominally Ca^{2+}-free medium, thapsigargin still often evoked a modest peak elevation, but did not activate a sustained response (Fig 3.7a). This is consistent with the peak phase corresponding to rapid Ca^{2+} store depletion, and the sustained phase being due to Ca^{2+} entry, presumably via a capacitative entry mechanism known to be activated by Ca^{2+} store release in a variety of cell types (Putney, 1993; Putney and Bird, 1993). The activation of cation entry by thapsigargin pre-treatment is addressed further in Chapter 4.

The ability of caffeine to increase $[Ca^{2+}]_i$ in cerebellar granule cells has been controversial, with responses evoked in all (depending on DIV)(Irving *et al.*, 1992b; Pearson *et al.*, 1992), some (Fohrmann *et al.*, 1993), or none (De Erausquin *et al.*, 1992; see also Whitham *et al.*, 1991b) of the cells examined. In the present study, perfusion of 50 mM caffeine evoked a marked though variable transient peak response from granule cell groups, with no sustained response (Fig 3.7b). In a small proportion of cells, virtually no response was found, but in most the peak elevation was marked, comparable in magnitude to some metabotropic agonist-evoked store release responses (see Chapter 5). The caffeine response tended to be greater at ~4 DIV than in older cultures (basal ratio = 0.93 ± 0.03, peak elevation at 3-5 DIV = 0.25 ± 0.03 (n=40); basal ratio = 0.73 ± 0.05, peak elevation at 6-9 DIV = 0.10 ± 0.03 (n=22)).

3.3.2 Inhibition of K^+-evoked $[Ca^{2+}]_i$ responses by store modulators

The ability of Ca^{2+} entry to release Ca^{2+} from stores via activation of RyRs has been reported in many non-neuronal preparations (see Henzi and MacDermott, 1992; Berridge, 1993). Involvement of stores in the K^+-evoked $[Ca^{2+}]_i$ elevation in

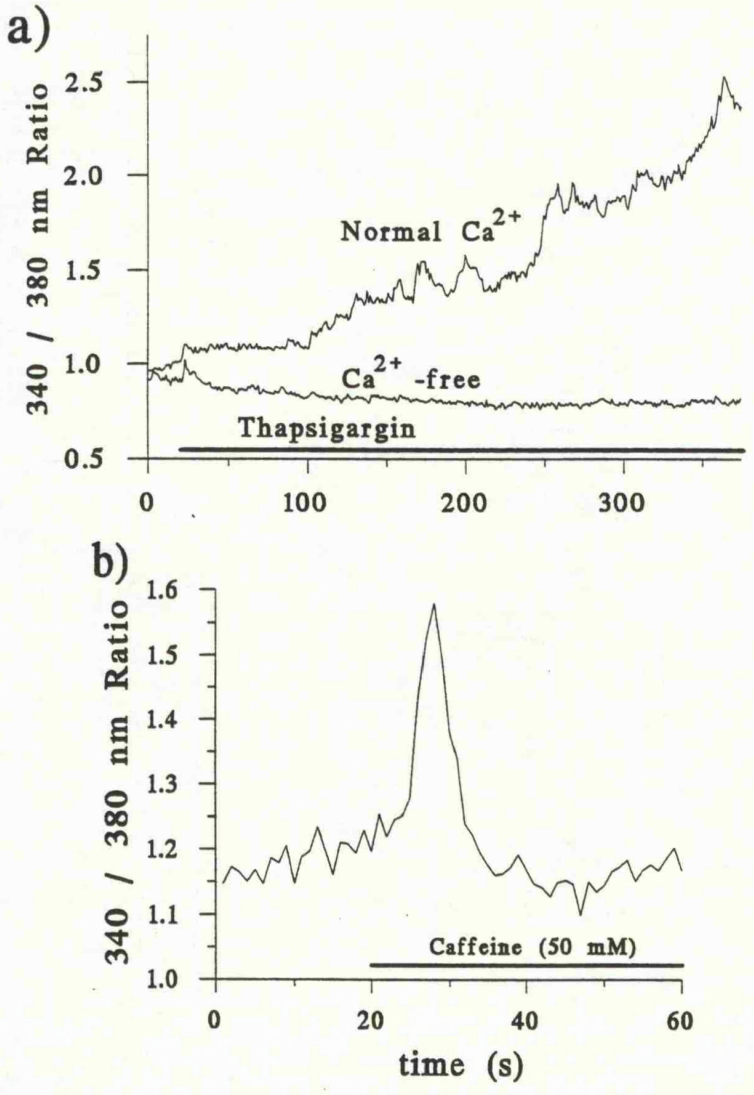


Fig. 3.7 ***a)*** **Effect of thapsigargin on $[Ca^{2+}]_i$, and dependence of response on $[Ca^{2+}]_o$.** Example traces of results from a series of experiments performed on the same day using cells from the same culture preparation. In the presence of normal (1.3 mM) Ca^{2+}-containing KHB, thapsigargin (10 μM) evoked a multiphasic elevation of $[Ca^{2+}]_i$ which persisted for several minutes. The rapid-onset peak phase was not found in all experiments, whereas the gradual-onset maintained elevation was variable in size but). consistently present. In nominally Ca^{2+}-free KHB, thapsigargin evoked a rapid-onset peak phase but no sustained elevation of $[Ca^{2+}]_i$.

b) **Effect of caffeine on $[Ca^{2+}]_i$.** Example trace typical of 6 series of experiments. 50 mM caffeine evoked a rapid-onset, transient peak $[Ca^{2+}]_i$ elevation, with no significant maintained response.

sympathetic neurones was suggested initially by the report that there was a non-linear relationship between depolarization-evoked Ca^{2+} plasma membrane current and $[Ca^{2+}]_i$ elevation (Kuba, 1980), and later by the findings that ryanodine could slow the onset of the depolarization-evoked response and that caffeine could accelerate it (Friel and Tsien, 1992). Having established that significant $[Ca^{2+}]_i$ responses can be evoked by store releasing agents in cerebellar granule cells, it therefore seemed important to investigate whether the $[Ca^{2+}]_i$ elevation evoked by K^+-depolarization in these neurones could be affected by inhibition of Ca^{2+} store release or by depletion of Ca^{2+} stores.

Dantrolene, which inhibits Ca^{2+} release in many cell types (see Henzi and MacDermott, 1992) via mechanisms including inhibition of RyRs (Pessah *et al.*, 1986; Nelson and Lin, 1993), was perfused onto granule cells during the plateau $[Ca^{2+}]_i$ elevation evoked by K^+. This agent dramatically and concentration-dependently reduced the K^+-evoked response, such that 10 μM dantrolene virtually abolished the plateau elevation (Fig 3.8a,b). While dantrolene has been clearly shown to inhibit opening of pig type I RyRs reconstituted into planar lipid bilayers from skeletal muscle (Nelson and Lin, 1993), the exact site mediating $[Ca^{2+}]_i$ response inhibition by dantrolene in neurones remains uncertain (Ohta *et al.*, 1990; Nohmi et al., 1991; Smith and Nahorski, 1993), and thus its specificity of action on stores was unclear (but see Section 4.6.2). Further studies were therefore undertaken using ryanodine and thapsigargin to confirm whether the marked effects of dantrolene might represent inhibition of depolarization-evoked Ca^{2+} store release. Micromolar ryanodine inhibits CICR by altering channel gating and stabilizing a low conductance open state (Rousseau *et al.*, 1987; Bezprozvanny *et al.*, 1991) or by preventing channel opening (McPherson *et al.*, 1991), whereas thapsigargin depletes Ca^{2+} stores by inhibition of Ca^{2+} uptake (Thastrup *et al.*, 1990). As the actions of these agents were relatively slow to develop, a protocol was adopted similar to that shown previously in this

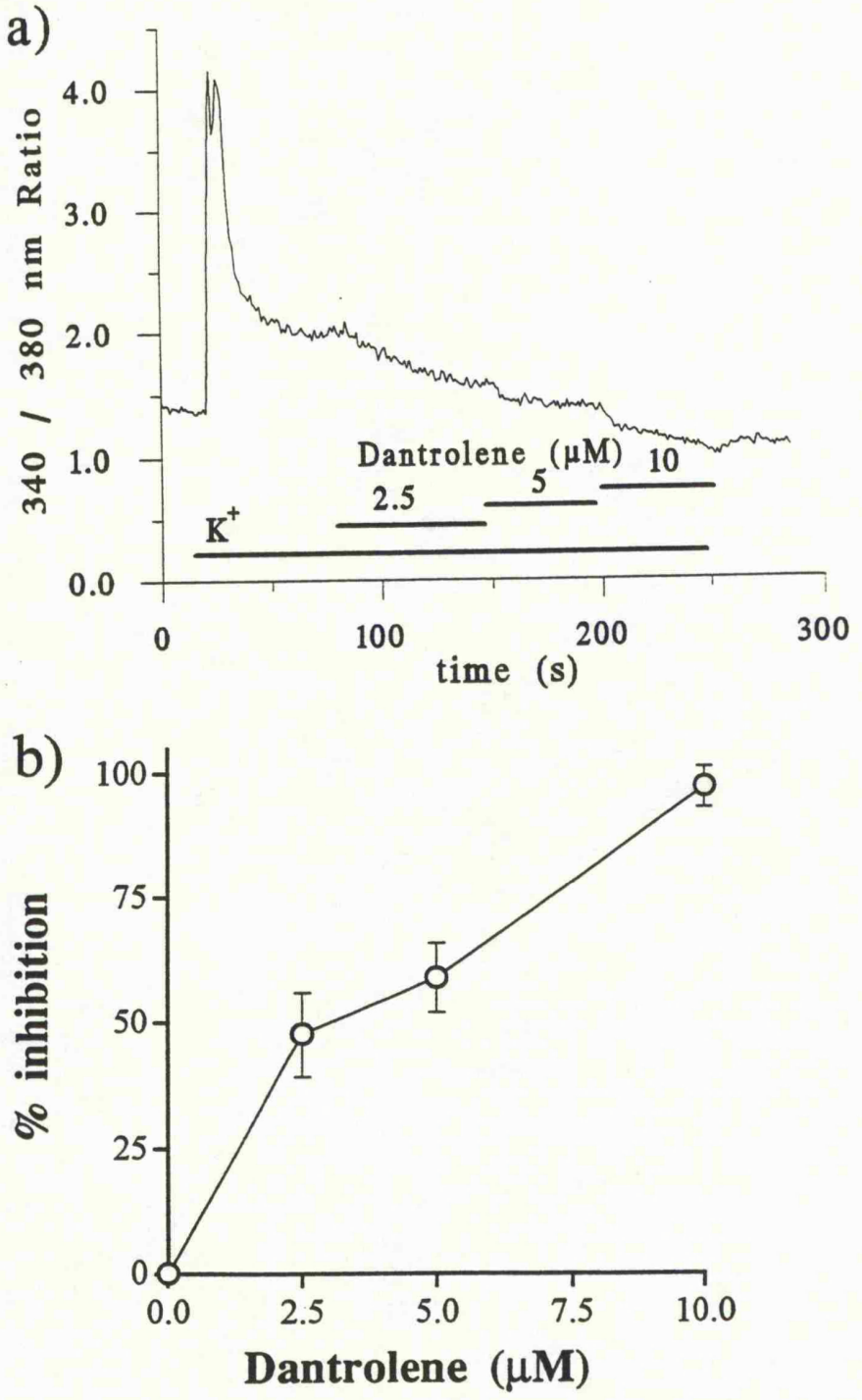


Fig. 3.8 Effect of dantrolene on K^+-evoked $[Ca^{2+}]_i$ responses. ***a)*** Example trace typical of four series of experiments. Addition of dantrolene during the maintained plateau phase of the response to K^+-depolarization caused a concentration-dependent decrease in the size of the plateau. ***b)*** Meaned results from 7-24 experiments for each concentration performed on at least three separate preparations. 10 μM dantrolene was sufficient to virtually abolish the K^+-evoked plateau.

Chapter for the experiments using PN 202 791. In order to minimise the length of time required to ensure their effects would be maximal, relatively high concentrations of these agents were used. Thapsigargin at a concentration found to evoke $[Ca^{2+}]_i$ responses (Fig. 3.7)(10 μM), or ryanodine at a release-inhibiting concentration (10 μM), were perfused over cells after an initial K^+-evoked control stimulation, and several subsequent stimulations were performed at 5 min intervals in their continuing presence. As shown in Fig. 3.9a,b, incubation with these agents had no consistent effect on the peak response to K^+, but both thapsigargin and ryanodine substantially reduced the K^+-evoked $[Ca^{2+}]_i$ plateau (see also Table 4.2). No greater inhibition was seen on subsequent responses to K^+ if longer periods of pre-treatment were employed. There was no significant additivity apparent in terms of the ability of these agents to decrease the K^+-evoked response (see Table 4.2).

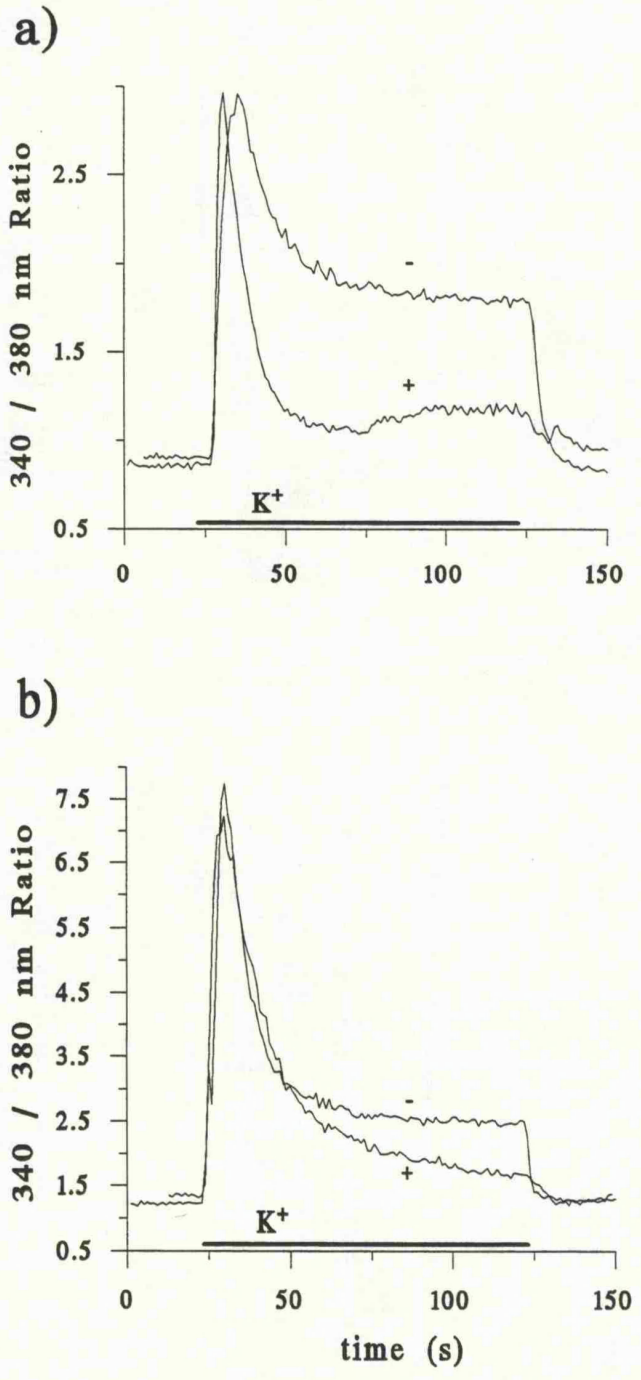


Fig. 3.9 Effects of thapsigargin and ryanodine on K^+-evoked $[Ca^{2+}]_i$ responses. Example traces typical of three series of several experiments performed on different days, often in parallel with the reproducibility experiments illustrated in Fig. 3.1. S1, and the second response in the presence of thapsigargin (10 μM)(***a***) or ryanodine (10 μM)(***b***)(S3) are shown. The peak response is unaffected by thapsigargin or ryanodine pretreatment, whereas the plateau phase is greatly inhibited by both agents.

3.4 Discussion

I

The present results demonstrate that K^+-depolarization of cultured cerebellar granule cells activates Ca^{2+} entry via several types of VOCC, which include:

(i) a rapidly activating, rapidly inactivating channel involved in the peak $[Ca^{2+}]_i$ elevation. This channel is permeant to Mn^{2+}, and is dizocilpine-, DHP- and ω conotoxin-insensitive. The lack of effect of (-) PN 202 791 on the peak elevation indicates that DHP-sensitive channels are not normally involved to a significant extent in this phase, in agreement with some previous studies (Courtney *et al.*, 1990; Pocock *et al.*, 1993). Other reports, employing larger depolarizing stimuli (~60 mM K^+) than used in the present study, found that the peak depolarization-evoked $[Ca^{2+}]_i$ response in granule cells was partially DHP-sensitive (Carboni and Wojcik, 1988; Ciardo and Meldolesi, 1991). The present results show that L-type VOCCs can be recruited to join the unidentified peak phase channels by addition of the L-channel agonist (+) PN 202 791, consistent with a possible recruitment of this subtype under favourable conditions, and perhaps in agreement with the involvement of L-type channels in the peak response to greater depolarization in those previous reports (Carboni and Wojcik, 1988; Ciardo and Meldolesi, 1991). However, L-type VOCCs are clearly not significantly involved in the peak phase of the response to moderate (25 mM K^+) depolarization alone, under present experimental conditions.

(ii) a non-inactivating channel involved in the plateau $[Ca^{2+}]_i$ elevation which is ω-conotoxin-insensitive but is inhibited by DHPs i.e. an L-type channel. The ability of (+) PN 202 791 to potentiate the plateau phase indicates that these L-type channels are not normally maximally activated by 25 mM K^+.

(iii) a non-inactivating channel involved in the plateau $[Ca^{2+}]$ elevation which is dizocilpine-, ω conotoxin- and DHP-insensitive.

At least some of the channels involved in the plateau phase i.e. (ii) and/or (iii), like those characterized in (i), appear to be Mn^{2+}-permeant.

Increasing $[K^+]_o$ markedly elevated the release of [^{3}H]-glutamate from cerebellar granule cells over control, in agreement with previous reports (e.g. Levi *et al.*, 1982,1991; Huston *et al.*, 1990). Ca^{2+} entry evoked by K^+ is believed to be the trigger for glutamate release, although other activators of Ca^{2+} entry such as NMDA do not stimulate release to a comparable extent in these neurones (Huston *et al.*, 1990). Dizocilpine had no effect on either phase of the K^+-evoked $[Ca^{2+}]_i$ elevation. It therefore appears that despite the clearly demonstrated effects of K^+-depolarization on glutamate release under similar experimental conditions, glutamate release does not result in significant activation of NMDARs in $[Ca^{2+}]_i$ experiments. This finding is consistent with most (Courtney *et al.*, 1990; Parks *et al.*, 1991; Pocock *et al.*, 1993) but not all (Didier *et al.*, 1993) previous studies. The most likely explanation for the difference between the present findings and that earlier study, and for the apparent contradiction inherent in finding K^+-evoked glutamate release but no K^+-evoked NMDAR activation, is that this is a consequence of the use of a continuous perfusion system and multibarrelled perfusion pipette. Continuous perfusion may lead to increased transmitter release in both $[Ca^{2+}]_i$ and glutamate experiments, but the relatively rapid perfusion rate (1.0 ml / min) and direct application of agonist onto cells in the field of view in $[Ca^{2+}]_i$ experiments would be expected to decrease the local accumulation of released transmitters, thus minimising their effects on the recorded cells (see also Chapter 4 Discussion). The reproducibility of repeated transmitter release over time is in agreement with the near-unity of the S2/S1 ratio for K^+-evoked $[Ca^{2+}]_i$ responses in the present study, and is indicative of no significant loss of neuronal viability during even prolonged experimental time periods.

Conventional fura 2 fluorescence techniques measure $[Ca^{2+}]_i$ changes which are the product of a variety of mechanisms, and it is helpful to have complementary techniques by which components of a response can be investigated individually. Mn^{2+} quench of fura 2 fluorescence has been widely used in inexcitable cells (see Putney and Bird, 1993) to examine divalent cation entry, but little work has previously been done utilizing this technique in neuronal preparations. In this study cerebellar granule cells have been found to have higher permeability to Mn^{2+} under control conditions than has been reported for most (see Putney and Bird, 1993 for references) though not all (Kwan and Putney, 1990) inexcitable cell types, a finding which is at least partly due to entry through VOCCs (see Section 4.5.2). This may make it difficult to detect the effects of agents which evoke small stimulations of divalent cation entry on top of the relatively high rate of background quench (see Chapters 4 and 5). K^+-depolarization, however, is a potent elevator of $[Ca^{2+}]_i$, and greatly increased the rate of Mn^{2+} quench. Mn^{2+} is known to voltage-dependently block the passage of other divalent cations through brain VOCCs, but on its own it passes through such channels with an apparent conductance $^2/_3$ that of Ca^{2+} and with doubled open times (Nelson, 1986). Differences in Mn^{2+} entry rates between the peak and plateau phases of the K^+-evoked response in granule cells were found, which correlated well with the pattern of $[Ca^{2+}]_i$ changes seen. This finding, together with the absence of a $[Ca^{2+}]_i$ response in nominally Ca^{2+}-free conditions, indicates an involvement of Ca^{2+} entry in the K^+-evoked response throughout the stimulation period. The present results demonstrate that Mn^{2+} quench can be used in cultured neurones to investigate divalent cation entry, and the regulation of entry, such as mediated here by temporal changes in VOCC activity during K^+-depolarization. The ability to investigate entry in isolation from other $[Ca^{2+}]_i$-elevating mechanisms, and, unlike for other entry-measuring techniques, the ability to do so under conditions directly paralleling those used for conventional fura 2-based $[Ca^{2+}]_i$ studies, make Mn^{2+} quench a useful correlate to

$[Ca^{2+}]_i$ measurement. Further use of the Mn^{2+} quench technique to examine activation and modulation of divalent cation entry is described in Chapters 4 and 5.

III

Thapsigargin caused a marked, biphasic $[Ca^{2+}]_i$ elevation in granule cells, presumably as a consequence of inhibition of the store Ca^{2+}ATPase and subsequent store leakage and depletion. The prolonged, irregular response to thapsigargin found in the presence of normal Ca^{2+} was absent in nominally Ca^{2+}-free conditions, indicating that this agent may elevate $[Ca^{2+}]_i$ largely as a consequence of Ca^{2+} entry, probably via CRAC channels (see Putney and Bird, 1993; Penner *et al.*, 1993). While this mechanism of activation of Ca^{2+} entry has been partially characterized in non-excitable preparations, the present study provides the first indication that Ca^{2+} influx can be induced by store depletion in a neuronal preparation. Further findings concerning this mechanism are reported in Chapter 4.

The characteristics and functions of neuronal RyRs have been subject to considerable controversy. Whitham *et al.* (1991b) reported that $InsP_3$ evoked release of ~65% of $^{45}Ca^{2+}$ preloaded into stores in permeabilized cerebellar granule cells, but as 10 mM caffeine evoked no significant $^{45}Ca^{2+}$ release in this preparation, the nature of this $InsP_3$-insensitive store was not determined. Using fura 2 fluorimetry, the present study has shown that in cerebellar granule cells caffeine can elevate $[Ca^{2+}]_i$, indicating that these neurones apparently express RyRs as well as $InsP_3$Rs (see also Chapters 5-7). This is in agreement with at least one previous study (Irving *et al.*, 1992b). However, another report on granule cells indicated that dantrolene-sensitive Ca^{2+} release could be evoked in granule cells by the combined action of elevated K^+ with caffeine or with the $GABA_B$ receptor agonist baclofen, but not by any of these agents alone (De Erausquin *et al.*, 1992). This would indicate an unexpected degree of

voltage-dependence of the action of caffeine on Ca^{2+} stores, which has not been reproduced in this or other (Irving et al., 1992b) reports. The findings of the present study may provide clues as to the subtype of RyR expressed in granule cells, as it has been reported that both type I and II RyRs are caffeine-sensitive, but that type III may lack this mechanism of activation (Meissner, 1994), although this difference in caffeine sensitivity between subtypes remains to be confirmed. Heterogeneity in responses to caffeine in the present study may reflect variations either in the size of the caffeine-sensitive pool between cells or in the state of refilling of Ca^{2+} stores (see also Chapter 5).

A number of studies using cultured peripheral and spinal neurones have indicated that depolarization can activate Ca^{2+} release via caffeine-sensitive intracellular stores (Kuba, 1980; Thayer *et al.*, 1988; Lipscombe *et al.*, 1988; Hernandez-Cruz *et al.*, 1990; Barish, 1991; Holliday *et al.*, 1991; Kuba *et al.*, 1992). The involvement of CICR in depolarization-evoked responses varies between different methodologies employed to evoke depolarization. Kuba *et al.* (1992) reported that depolarization of similar length of time to action potentials did not activate CICR in sympathetic ganglion cells, but that more prolonged depolarization (>100 ms) evoked significant Ca^{2+} release. They hypothesized that CICR may therefore be prominent in response to action potentials in this neuronal type only during simultaneous membrane depolarization evoked by slow-acting transmitters, which might indicate that CICR can therefore play an important role in synaptic integration. In a more recent study, long-duration depolarizing pulses evoked more prominent CICR than brief pulses in cerebellar Purkinje cells (Llano *et al.*, 1994). The extent as well as the time period of depolarization may also affect the degree of CICR evoked: Lipscombe *et al.* (1988) demonstrated a much greater inhibition by pre-treatment with caffeine of the $[Ca^{2+}]_i$ response to 30 mM K^+ (58%) in sympathetic neurones than of the response to 60 mM K^+ (19%). In contrast, Barish (1991) reported in spinal neurones that caffeine had a

greater inhibitory effect on the response to 50 mM K^+ (56%) than on the response to 20 mM K^+ (37%), and ryanodine in the same preparation significantly inhibited the peak elevation evoked by 50 mM K^+ (by 42%) but not that evoked by 20 mM K^+. Further, different neuronal types may display different degrees of depolarization-evoked Ca^{2+} release. In cultured spinal and dorsal root ganglion neurones, a much greater proportion of the high depolarization-evoked response appears to be due to Ca^{2+} stores than described above in sympathetic neurones. Both nuclear and cytosolic $[Ca^{2+}]_i$ elevations in these preparations evoked by elevated $[K^+]_o$ (50-100 mM) were virtually abolished by prior store depletion (Holliday *et al.*, 1991; Kocsis *et al.*, 1994), responses being dependent on Ca^{2+} entry but triggered even in very low $[Ca^{2+}]_o$. In contrast, depolarization has been reported to not significantly activate CICR in cultured septal neurones (Bleakman *et al.*, 1993). The present results using thapsigargin, ryanodine and dantrolene demonstrate that Ca^{2+} store release in cerebellar granule cells can similarly be activated by membrane depolarization, the major component of the plateau phase of 25 mM K^+-evoked $[Ca^{2+}]_i$ elevation apparently being due to release from these stores. Indeed, the results suggest that the role of Ca^{2+} influx in the plateau K^+-evoked response in granule cells may largely be confined to a triggering of release, rather than directly causing significant elevation of $[Ca^{2+}]_i$ as detectable by fura 2, as reported for cultured spinal and dorsal root ganglion neurones (Holliday *et al.*, 1991; Kocsis *et al.*, 1994).

As K^+-evoked responses were largely inhibited in nominally Ca^{2+}-free medium, Ca^{2+} release in granule cells also appears to be triggered by and dependent upon Ca^{2+} entry across the plasma membrane, indicative of CICR. The response inhibition by ryanodine and dantrolene suggest that the mechanism of CICR is predominantly Ca^{2+} entry-evoked activation of RyRs. While the reports detailed above have identified CICR in cultured peripheral and spinal neurones, the present study is the first to report evidence of depolarization-evoked CICR in brain-derived

neurones. These results contrast with a previous study in granule cells performed at room temperature (Irving *et al.*, 1992b), in which neither thapsigargin nor ryanodine were found to influence K^+-evoked responses. It is possible that the contribution of store release was masked by the rundown in response to K^+ in their preparations, or that release is more marked at 37°C than it is at room temperature. In the present study the peak response to K^+ was not significantly affected by thapsigargin or ryanodine, and so may be due directly to Ca^{2+} influx (or possibly to release from stores unaffected by these agents).

An increasing body of evidence indicates that CICR may be of central importance in various physiological events in neurones. Depletion of stores by incubation of newly dissociated dorsal root ganglion neurones with thapsigargin for 20 min greatly inhibited subsequent neurite outgrowth, implicating Ca^{2+} release in the initiation of neuritogenesis (Kocsis *et al.*, 1994). This may occur via CICR effects on gene expression, as depolarization-evoked Ca^{2+} mobilization has been found to be more prominent in the nucleus than in the cytoplasm during neurite generation in these neurones (Kocsis *et al.*, 1994; but see Al-Mohanna *et al.*, 1994a,b, and Rand *et al.*, 1994). Incubation with a store depleting agent for 6 h affected the subsequent neurite outgrowth in spinal neurones as well, although in these cells such treatment led to development of longer rather than shorter neurites than found for control neurones (Holliday *et al.*, 1991). GABA immunoreactivity was lower 12 h after store depletion than in control spinal neurones (Holliday *et al.*, 1991), indicating that other aspects of neuronal differentiation and maturation are also dependent on Ca^{2+} stores. It is therefore possible that excitatory inputs *in vivo* may markedly affect neuronal development via CICR-dependent processes.

Quantal release of ACh at a buccal ganglion synapse of *Aplysia* was potentiated by BHQ (Fossier *et al.*, 1992), and caffeine has been reported to activate

release of [^{3}H]-dopamine preloaded into PC12 cells via RyR stimulation (Avidor *et al.*, 1994), indicating that activation of Ca^{2+} store release can cause secretion at least under certain circumstances (see also Brosius *et al.*, 1992). However, the physiological role of synaptic Ca^{2+} stores may be to buffer excess Ca^{2+} entry rather than to participate in the activation of transmitter release (Fossier *et al.*, 1992).

LTD in cerebellar Purkinje cells is evoked by the conjunction of a complex action potential with parallel fibre stimulation (Linden and Connor, 1993). Long-duration depolarizing pulses evoked more prominent CICR than brief pulses in cultured Purkinje neurones (Llano *et al.*, 1994). Llano *et al.* suggested that the excitatory input from glutamatergic parallel fibres *in vivo* would depolarize the Purkinje cell plasma membrane sufficiently to allow an action potential to activate CICR, and that the subsequently increased $[Ca^{2+}]_i$ elevation may play an important role in LTD development (see also Konnerth, 1993). IICR may also play a role in LTD (Konnerth, 1993), consistent with the central involvement of mGluRs in LTD (see Linden and Connor, 1993; Hartell, 1994; Conquet et al., 1994). Whether IICR would be partially or wholly complementary to CICR depends on the relationship between $InsP_3$-sensitive and Ca^{2+}-sensitive pools in neurones (see Chapter 5).

A more complete characterization of the mechanisms of Ca^{2+} release in granule cells, and of the stores involved, is detailed in subsequent Chapters.

4. NMDA responses

4.1 Investigation of NMDA-evoked $[Ca^{2+}]_i$ elevation

Cell signalling pathways activated by the ionotropic glutamate agonist NMDA are a major focus of interest in the present study. In this Chapter, a series of experiments to characterize NMDA-evoked $[Ca^{2+}]_i$ elevations, and to determine the mechanisms involved in this response, are described. These experiments focussed initially on the importance of addition of exogenous glycine for the detection of maximal NMDA responses, and characterization of the relationship between plating density and the necessity for addition of glycine. The effect of another modulatory agent, nitric oxide, on NMDA-evoked responses was also investigated. Further experiments were performed to illuminate the differences and possible similarities between NMDA-evoked responses and the effects of K^+-depolarization on $[Ca^{2+}]_i$ detailed in the preceding Chapter. In particular, Ca^{2+} entry mechanisms activated by NMDA were examined, using an NMDAR channel blocker and VOCC agonists and antagonists, with both $[Ca^{2+}]_i$ and Mn^{2+} quench experiments. In the light of the finding of CICR activation by depolarization described in Chapter 3, the possible involvement of intracellular stores in the NMDA-evoked response was investigated, using the same store modulatory agents. The NMDA response characterization in this Chapter underlies many of the experiments involving NMDA and metabotropic agonists detailed in subsequent Chapters.

4.2 Sensitivity of NMDA-evoked response to glycine

4.2.1 Effect of exogenous glycine on response

The $[Ca^{2+}]_i$ response of cerebellar granule cells to perfusion of NMDA consisted of a long-lasting monophasic plateau (Fig. 4.1), which increased with NMDA concentration (Fig. 4.2), sometimes preceded by a rapid-onset, short-lasting peak elevation. The effect of exogenous glycine on the NMDA response was examined

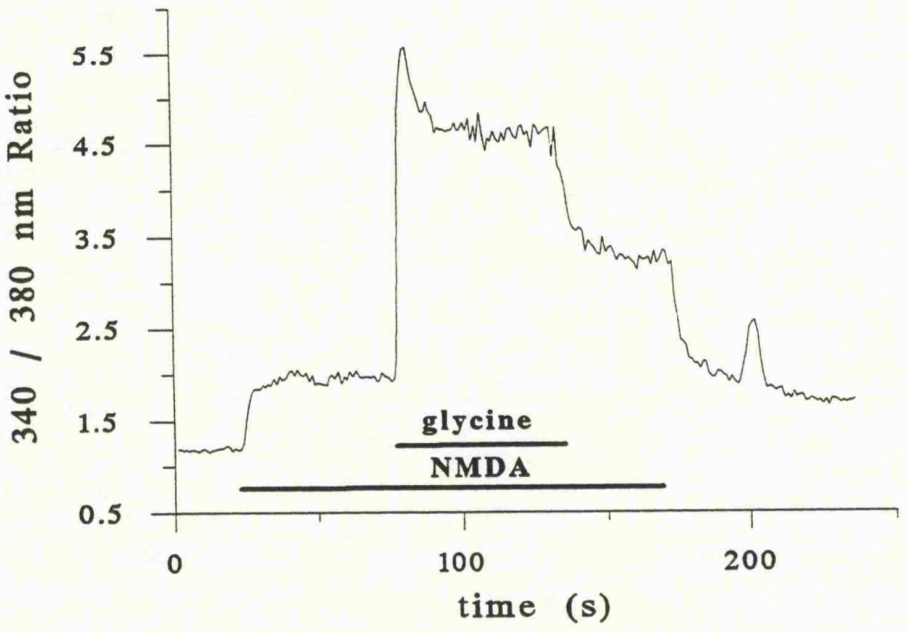


Fig. 4.1 Response to NMDA in the absence and presence of exogenous glycine. Example trace for 800 μM NMDA, with co-perfusion of 10 μM glycine as indicated. A peak response was seen after but not before addition of glycine, and glycine markedly increased the plateau response. (The 'blip' at ~200 s was a rarely found artefact of the method of agent addition used.)

by adding 10 μM glycine, sufficient to fully saturate the glycine site on the receptor (Kleckner and Dingledine, 1988; Baron *et al.*, 1990), after the plateau was established. A large, though variable, potentiation of the response was found, and for all concentrations of NMDA tested the response in the presence of glycine was consistently biphasic i.e. a rapid transient increase in $[Ca^{2+}]_i$ almost always preceded the sustained plateau (Fig. 4.1). The potentiation was partially, though not completely, removed when perfusion of glycine was terminated. Glycine did not significantly alter the 340 / 380 nm ratio on its own (data not shown). While the size of response to NMDA was greatly increased by exogenous glycine, there was no marked shift in the NMDA concentration-response curve (Fig. 4.2).

4.2.2 Effect of glycine site antagonists

5,7-Dichlorokynurenic acid (dcKyn), a potent anticonvulsant, is reportedly a specific competitive antagonist of the strychnine-insensitive glycine site (Baron *et al.*, 1990,1992). When added during the plateau phase of the response to 200 μM NMDA, in the absence of exogenous glycine, dcKyn concentration-dependently decreased the $[Ca^{2+}]_i$ elevation, with an IC_{50} of 8 nM (Fig. 4.3a). This indicates that dcKyn is a more potent functional antagonist of the strychnine-insensitive glycine site than was suggested by its ability to inhibit [^{3}H]-glycine binding (K_i = 70 nM, Baron *et al.*, 1992). Submicromolar concentrations of dcKyn completely abolished the response to NMDA (inhibition by 100 nM dcKyn = 98 ± 3%). Comparable effects were seen using the AMPAR antagonist CNQX, which is also active at the glycine site (Sheardown *et al.*, 1989). While a complete concentration-inhibition curve was not constructed for this agent, ~50% inhibition was induced by 4 μM CNQX.

The antagonism by dcKyn of the NMDA-evoked $[Ca^{2+}]_i$ elevation was overcome by addition of exogenous glycine in a concentration-dependent manner (Fig.

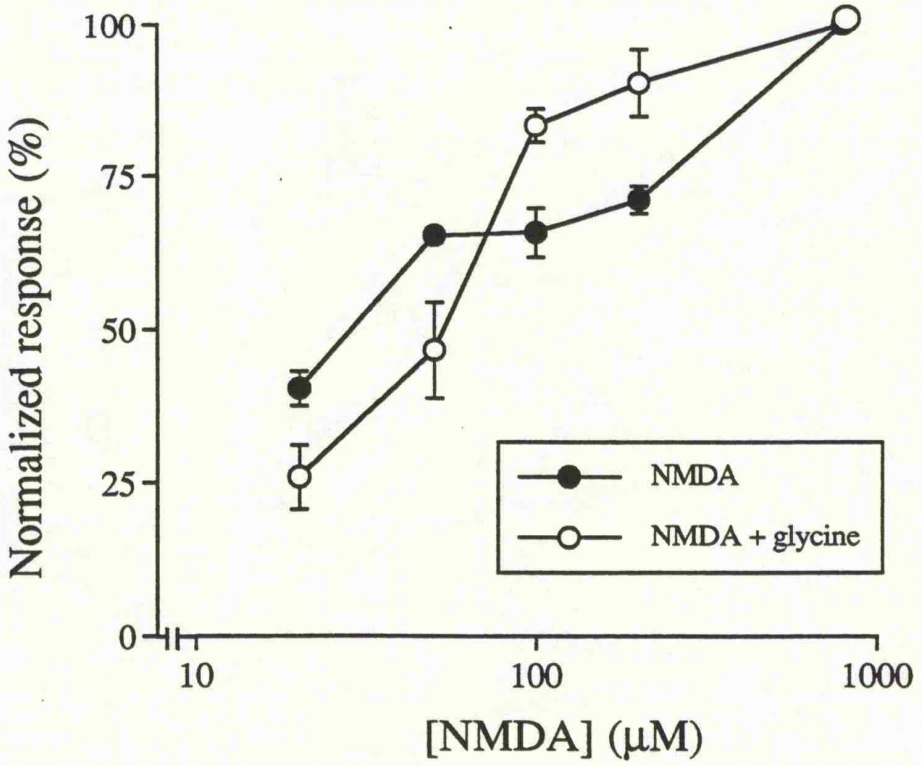


Fig. 4.2 Concentration-response curves for NMDA in the absence and presence of exogenous glycine. Meaned plateau data from cumulative addition experiments performed at 5-6 DIV. Values are presented as percentages of the response to 800 μM NMDA, or 800 μM NMDA in the presence of 10 μM glycine, respectively, allowing direct comparison of the curves despite the very different maximum values. Although glycine markedly increased the plateau level for all concentrations tested (Fig. 4.1), the concentration-response curve was not markedly affected.

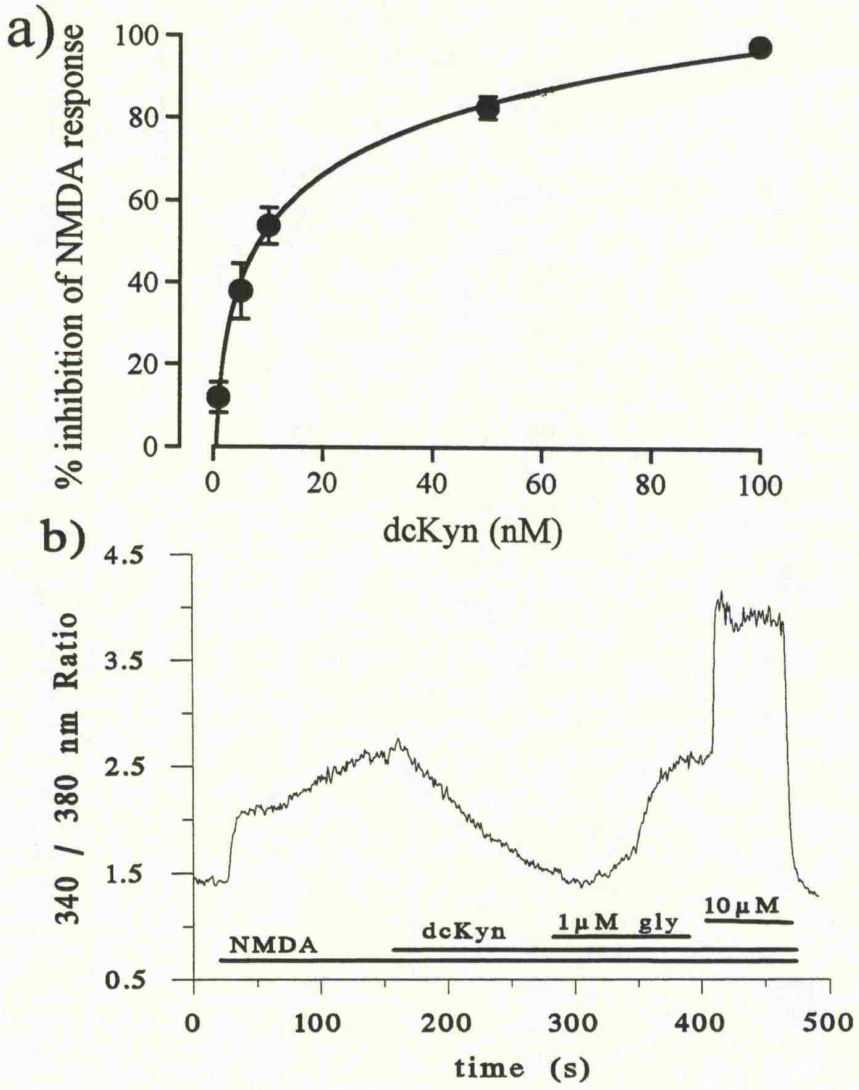


Fig. 4.3 ***a)*** **Concentration-inhibition curve for dcKyn on the NMDA-evoked response.** dcKyn was perfused in increasing concentration during the plateau response to 200 µM NMDA in the absence of exogenous glycine. The NMDA response was attenuated in a concentration-dependent manner, and at ≥100 nM dcKyn it was completely abolished.

b) **Ability of glycine to overcome the inhibitory effect of dcKyn.** Example trace demonstrating the gradual-onset inhibition of the response to 200 µM NMDA caused by 100 nM dcKyn, and the concentration-dependent manner by which exogenous glycine can overcome the inhibition, and potentiate the NMDA response as in the absence of antagonist (Fig. 4.1).

4.3b). At high concentrations of glycine, the potentiation of the NMDA response found in the presence of dcKyn was similar to that previously described in its absence.

4.2.3 Importance of plating density for glycine responsiveness

To examine whether NMDA response potentiation by exogenous glycine was dependent on the density at which cells were plated, granule cells cultured from single cerebellar preparations were plated out into 8-well plates, each well differing only in the granule cell plating density. The effects of stimulation by 200 μM NMDA alone, and 200 μM NMDA in the presence of 10 μM exogenous glycine, were examined on groups of cells from these cultures. An inverse correlation was found between plating density and potentiation by exogenous glycine, with cells plated at high density having a significantly greater responsiveness to NMDA compared to cells plated at 'normal' density, without a correspondingly increased responsiveness to NMDA in the presence of glycine (Fig. 4.4). This is consistent with the existence of a leakage or exocytotic mechanism for glycine in cultured granule cells, such that at higher density of plating, sufficient glycine was released by surrounding cells to cause significantly greater activation of glycine binding sites, and thus increased NMDAR-mediated responses, than in low density cultures.

All subsequent NMDA experiments in this Thesis were performed in the presence of 10 μM exogenous glycine.

4.3 Characterization of $[Ca^{2+}]_i$ responses to NMDA

4.3.1 NMDA-evoked Ca^{2+} entry

Addition of the NMDA channel antagonist dizocilpine during the plateau phase of the $[Ca^{2+}]_i$ response to 200 μM NMDA caused a concentration-dependent

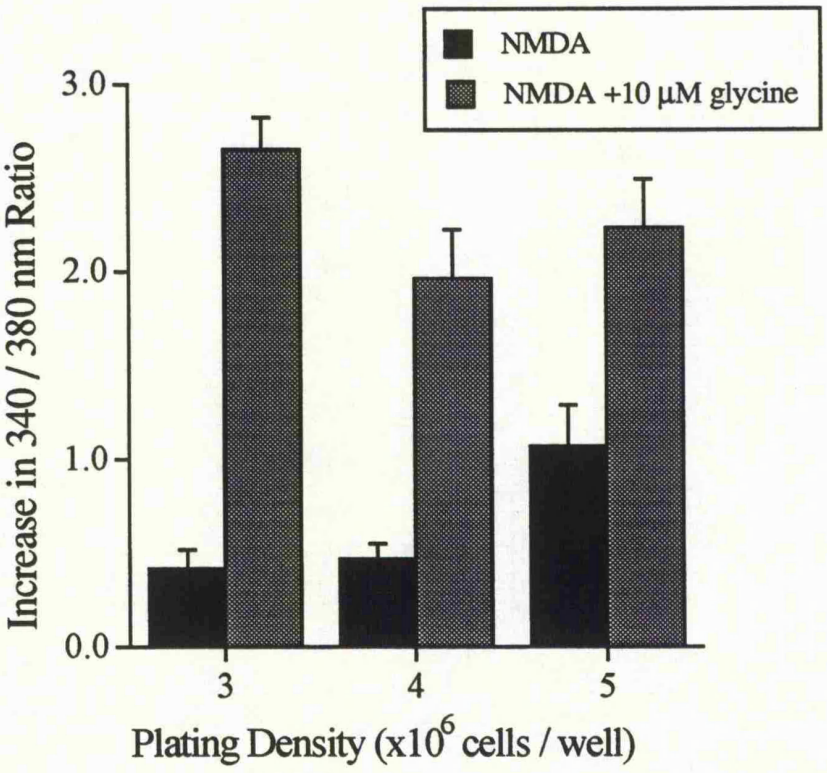


Fig. 4.4 Dependence of the glycine potentiation of the NMDA response on plating density. Results from a series of experiments performed on a representative single day, with an experimental protocol as in Fig. 4.1. Comparison of the response to 200 μM NMDA with and without 10 μM glycine from coverslips plated at differing cell densities. Increasing plating density caused increasing response to NMDA alone, but no significant change in the response to NMDA in the presence of 10 μM glycine.

decrease, such that 10 μM dizocilpine completely abolished the response (101 ± 4% inhibition). The NMDA-evoked $[Ca^{2+}]_i$ elevation was markedly reduced in nominally Ca^{2+}-free medium: the peak was greatly (though variably) reduced, and the plateau was virtually abolished (Fig. 4.5). A previous study in granule cells in the presence of Ca^{2+}-chelating agents has reported no NMDA response in very low $[Ca^{2+}]_o$ (Parks *et al.*, 1991). Taken together with the ability of dizocilpine and dcKyn to abolish the response in the present study, it would therefore appear that the NMDA response is dependent on Ca^{2+} entry, as previously reported in striatal neurones (Murphy *et al.*, 1987).

As found for K^+-evoked responses, NMDA-evoked responses were highly reproducible at 5 min intervals (S2/S1: peak = 108 ± 9%; plateau 96 ± 4%). This enabled experiments to be designed as in Chapter 3, with the effects of agents on the NMDA-evoked response being detected by comparison with control responses from the same group of granule cells. However, while responses within a given group were reproducible, for groups even from the same coverslip there appeared to be no consistent temporal relationship between peak and plateau phases of the response, and the relationship between peak and plateau sizes was highly variable (Fig. 4.6). While a clear separation of the two phases was often detected (top two traces), this was rarely as prolonged as shown in top left trace. Typically, the two phases were sufficiently closely related temporally that no decrease in 340 / 380 nm ratio, or only a partial decrease, in response level was detected between peak and plateau phases (bottom two traces).

4.3.2 Response size and its dependence on days *in vitro*

The ability of NMDA to increase $[Ca^{2+}]_i$ was examined in cells cultured for various lengths of time *in vitro*. Significant responses to NMDA were observed as

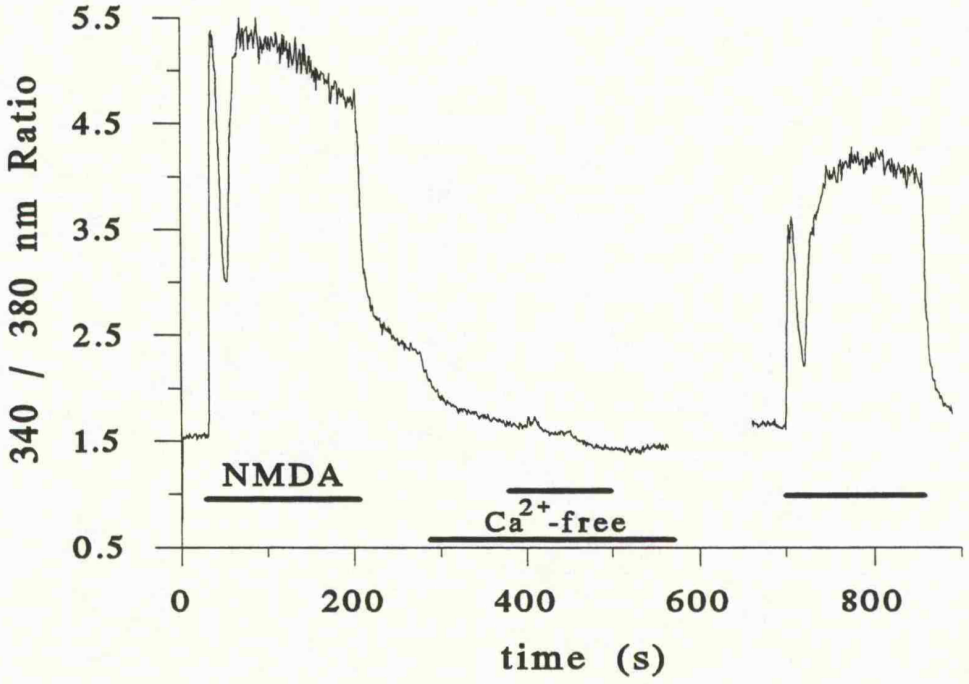


Fig. 4.5 Dependence of NMDA-evoked response on $[Ca^{2+}]_o$. Example responses from a single group of cells. NMDA evoked a significant initial peak and plateau response, both of which were markedly reduced, indeed virtually absent in this example, after 5 min in Ca^{2+}-free KHB. The response recovered to near initial levels after 5 min in normal $[Ca^{2+}]$. In all cases the plateau phase was virtually abolished, although a residual small peak was often observed in nominally Ca^{2+}-free KHB.

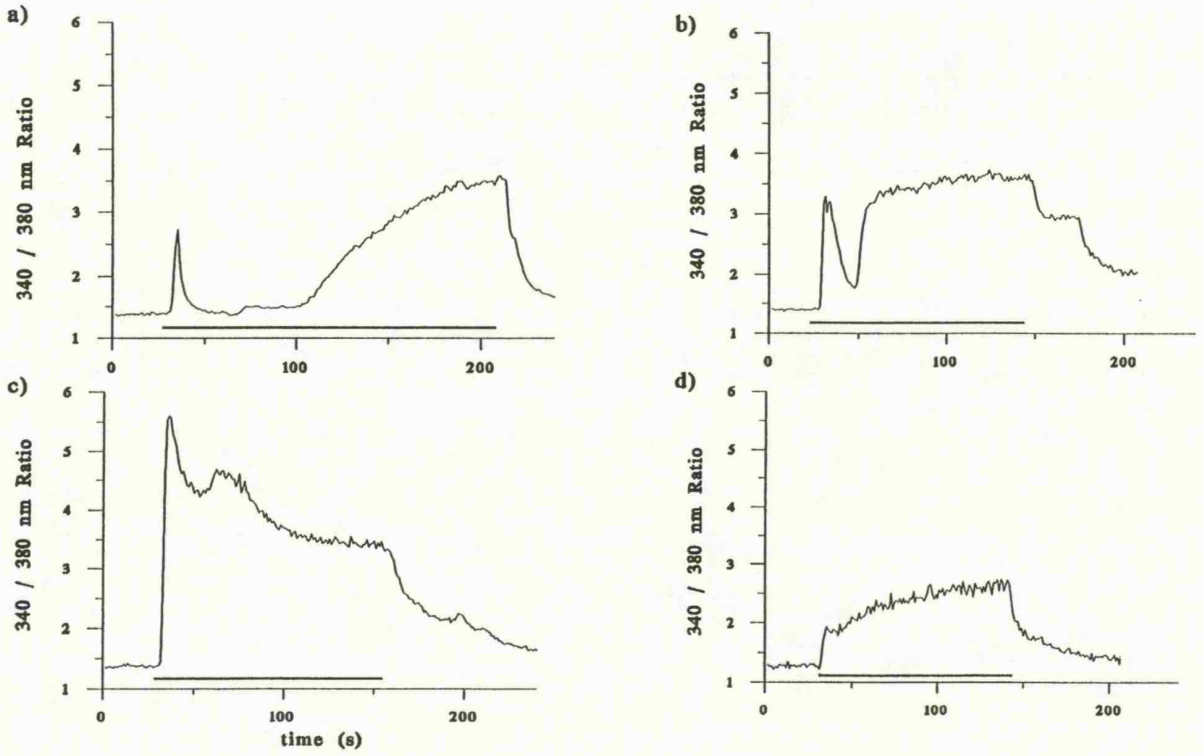


Fig. 4.6 Variability in the size and timecourse of the NMDA-evoked response. Example traces of responses to 200 μM NMDA from groups of cells from different culture preparations (10 μM glycine present throughout these and subsequent example traces). A rapid-onset, transient peak phase and a plateau that persists throughout the stimulation are apparent in all these traces, but the temporal relationship between the two phases was highly variable. The sizes of the peak and plateau also displayed considerable heterogeneity.

early as 1 DIV. Differences between individual preparations were much more marked than differences due to time in culture, and indeed over all experiments, no significant relationship between either peak or plateau elevations was detected with respect to age of cultures, for 3-10 DIV (Fig. 4.7). Experiments were not generally performed on cultures older than these, but in a small number of experiments an apparent decline in responsiveness was detected at >12 DIV (not shown). This is broadly the same pattern previously described for K-depolarization, confirming that simply the age of the cells has less influence on cell responsiveness than other, complex factors controlling variability such as small differences in culture conditions e.g. perhaps differences between batches of FCS.

Analysing NMDA responses evoked at 3-10 DIV, it was found that from a basal 340 / 380 nm ratio of 1.15 ± 0.02, 200 μM NMDA in the presence of 10 μM glycine evoked a peak increase of 2.03 ± 0.13, and a plateau increase of 1.99 ± 0.11 (n=72). A small number of NMDA experiments were performed in parallel with the calibration of $[Ca^{2+}]$ (see Methods), and an example trace shown in Fig. 4.8 demonstrates a quantitation of the change in $[Ca^{2+}]_i$ which NMDA evokes in cerebellar granule cells.

4.3.3 Effects of Ca^{2+} channel agonists and antagonists on response

As NMDAR activation results in not only Ca^{2+} entry but also entry of Na^+, addition of NMDA would be expected to depolarize neurones and increase the open probability of VOCCs (see Mayer and Miller, 1990). Indeed, a previous report has suggested that the peak phase of the $[Ca^{2+}]_i$ response to NMDA in granule cells is entirely due to Ca^{2+} entry through L-type Ca^{2+} channels (Courtney *et al.*, 1990). However, this possibility was not actually fully tested in that paper, and the subsequent report that at least some DHPs may interact directly with NMDARs (Skeen *et al.*,

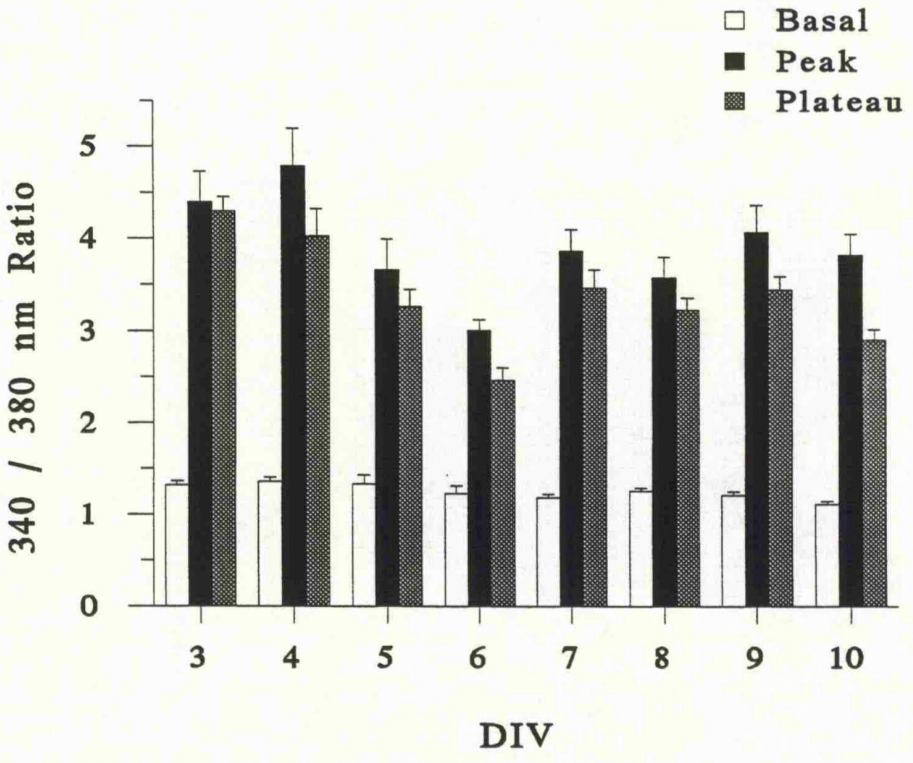


Fig. 4.7 Peak and plateau responses to NMDA compared to DIV. Meaned responses for 200 μM NMDA from many experiments on different days. No consistent significant trends were detected for either phase of the NMDA-evoked $[Ca^{2+}]_i$ response from 3-10 DIV, although smaller numbers of experiments at e.g. 1 or 14 DIV suggested that the responses at these ages may be smaller (not shown).

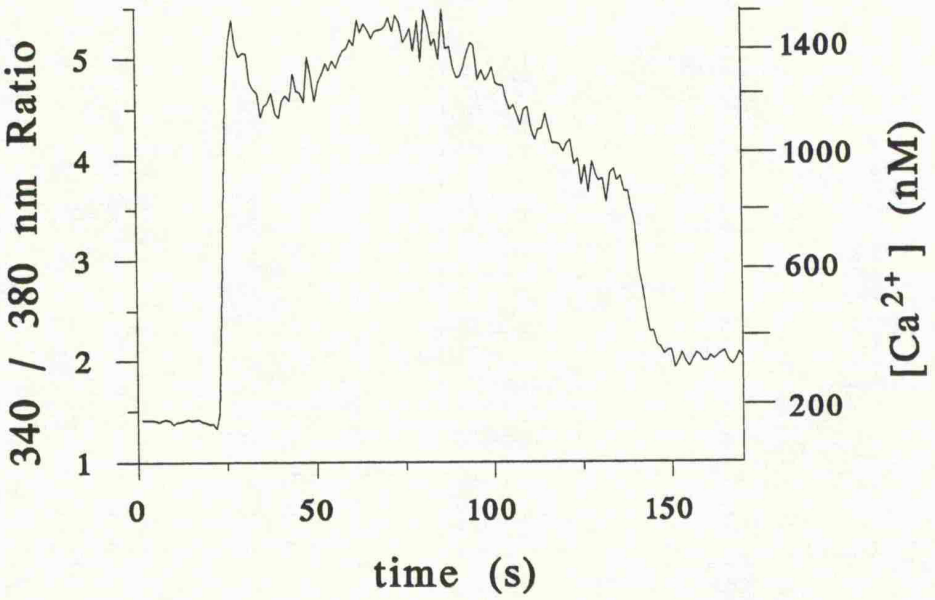


Fig. 4.8 Quantification of the $[Ca^{2+}]_i$ elevation evoked by NMDA. Example response to 200 μM NMDA, performed before calibration of $[Ca^{2+}]_i$ as described in Chapter 2. Note that, as measured by ratio, this is towards the upper end of the range of observed responses evoked by perfusion of NMDA.

1993; but see Section 4.5.2) appears to complicate or invalidate the conclusion drawn by Courtney and co-workers (1990). The putative involvement of channels other than the integral NMDAR channel in the $[Ca^{2+}]_i$ elevation evoked by NMDA was therefore examined in the present study. Enantiomers of PN 202 791, previously found to modify depolarization-evoked Ca^{2+} entry (see Chapter 3), were perfused over granule cells, and the NMDA response after pre-treatment compared to an initial control response. 1 μM (+) PN 202 791 potentiated both phases of the NMDA response (increase in peak response = 27 ± 12%, increase in plateau response = 25 ± 2%), but at the same concentration the (-) enantiomer had no significant effect on the NMDA-evoked peak and caused only a small (7 ± 3%) inhibition of the plateau (Fig. 4.9). 1 μM ω-conotoxin did not inhibit either phase of the response, and indeed as seen for K^+-evoked responses occasionally caused a small potentiation of the response. The lack of VOCC involvement in the NMDA response might be explained by physical separation between VOCCs and NMDARs e.g. the main expression of VOCCs being on processes or at terminals and the NMDA response being evoked from the cell body. However, the experiments using (+) PN 202 791 indicate that L-type VOCCs are present in a suitable location such that they can be recruited to the NMDA response under L channel agonist-facilitated conditions.

4.4 NMDA and AMPA

4.4.1 Effects of AMPA on $[Ca^{2+}]_i$ and Mn^{2+} quench

At least some types of AMPAR channels have been reported to be Ca^{2+}-permeant (Hollmann *et al.*, 1991; Linn and Christenson, 1992; Gibbons *et al.*, 1993; Schneggenburger *et al.*, 1993; Reichling and MacDermott, 1993; Brorson *et al.*, 1994; N. Hack and R. Balazs, unpublished), and Na^+ entry via these channels would be likely to depolarize cells and, as hypothesized for NMDA channels, activate VOCCs (Mayer and Miller, 1990; Reichling and MacDermott, 1993). The ability of AMPA to increase

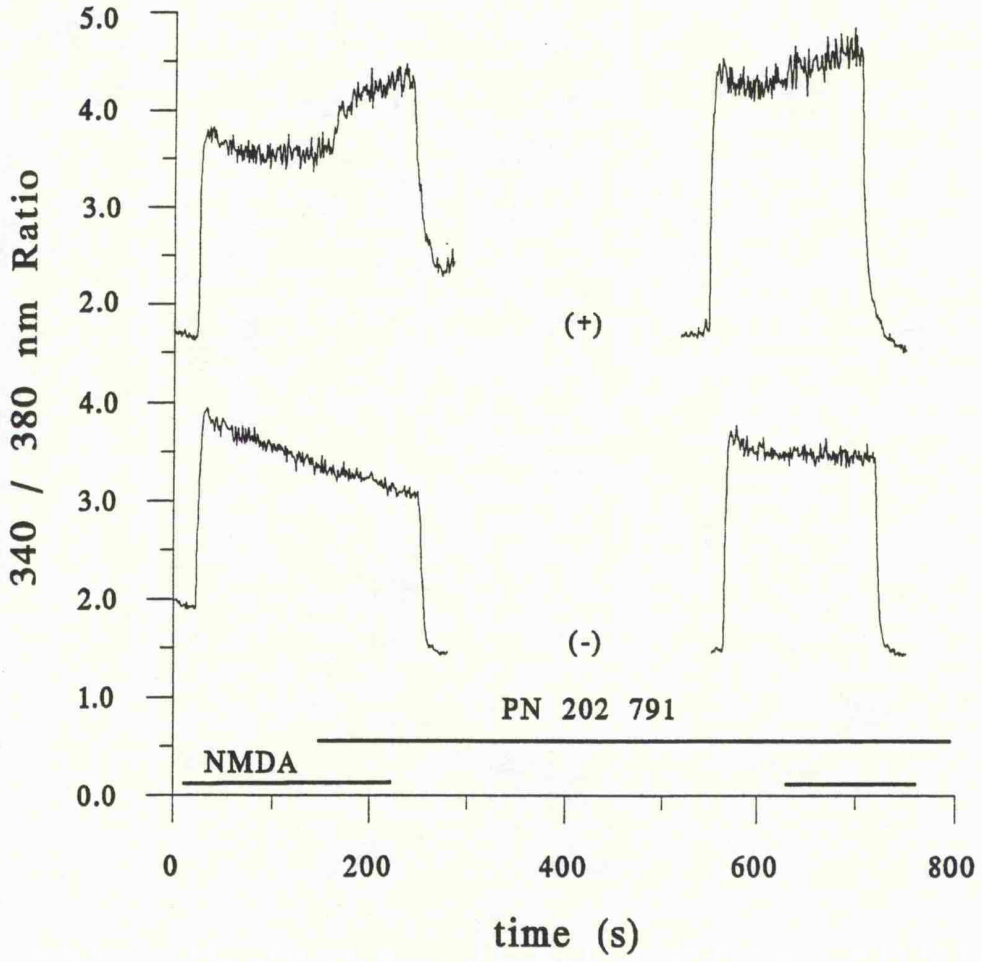


Fig. 4.9 Effects of the isomers of PN 202 791 on the $[Ca^{2+}]_i$ response to NMDA. Example traces from different groups of cells from the same coverslip, with the (+) isomer trace shifted vertically for ease of comparison.

top trace: 1 μM of (+) PN 202 791 elevated the plateau phase rapidly, and increased the peak response to a subsequent NMDA challenge compared to the initial control response.

bottom trace: 1 μM of the (-) isomer had no significant effect on the subsequent peak response to NMDA. While in this example trace no inhibition of the plateau can be detected either, over a number of experiments a very small but significant inhibitory effect of the DHP on the plateau was found.

$[Ca^{2+}]_i$ in cerebellar granule cells was therefore examined. Despite the two potential mechanisms of action described above, AMPA does not appear to be a potent or efficacious activator of Ca^{2+} entry into cerebellar granule cells: perfusion of a range of concentrations of AMPA (5-500 μM) evoked only small, monophasic elevations of $[Ca^{2+}]_i$ in these cells (Fig. 4.10). This elevation typically persisted throughout the time period of stimulation, but attempts to construct stepwise concentration-response curves were largely confounded by the insufficient experimental window which the response provided, and probably also by desensitization of AMPARs over the experimental time period (Sharp *et al.*, 1994). Receptor desensitization is also a likely explanation of the small $[Ca^{2+}]_i$ response which the agonist evoked. Apparently maximal effects were produced with 50-200 μM AMPA (increase over basal ratio 0.23 ± 0.04 (n=34)), and concentrations of such magnitude were used in all subsequent experiments.

Further support for the lack of marked AMPA-evoked Ca^{2+} entry into granule cells was provided by the finding that 200 μM AMPA did not increase the rate of Mn^{2+} quench above control levels (control $t_{1/2}$ = 82.2 ± 9.5s, AMPA $t_{1/2}$ = 71.4 ± 14.0s)(Fig. 4.11). The absence of AMPA stimulation of Mn^{2+} quench suggests that either the divalent cation-permeant channel activation evoked by AMPA is too small to be detected by this technique, or that the channels activated are impermeant to Mn^{2+}. The ability of AMPA to evoke Ca^{2+} entry in some neuronal systems secondary to Na^+ entry through the AMPA channel is well established (Mayer and Miller, 1990), but as stated above more recent reports from several groups have indicated that some AMPA channels are directly permeable to Ca^{2+} (Hollmann *et al.*, 1991; Linn and Christenson, 1992; Gibbons *et al.*, 1993; Schneggenburger *et al.*, 1993; Reichling and MacDermott, 1993; Brorson *et al.*, 1994). The expression of such receptors in cultured cerebellar granule cells has been suggested (N. Hack and R. Balazs, unpublished), but remains controversial, as in another study L-type VOCC inhibitors virtually abolished the

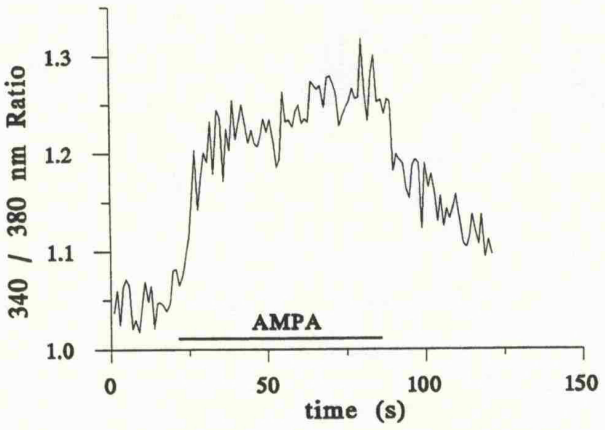


Fig. 4.10 $[Ca^{2+}]_i$ response to perfusion with AMPA. Example response to 200 μM AMPA. No peak elevation was evoked, and only a small plateau (cf scale for NMDA traces).

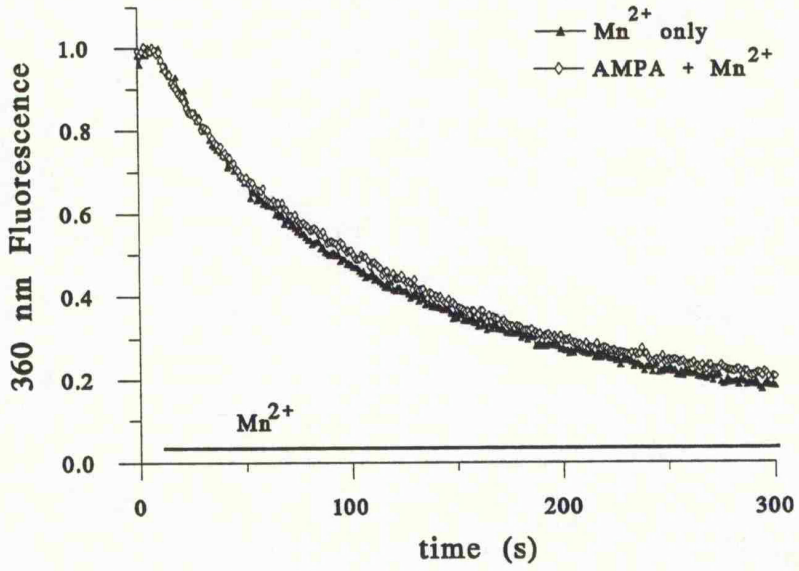


Fig. 4.11 Lack of effect of AMPA on Mn^{2+} quench. Example traces of normalized data from a series of experiments for control quench and quench during perfusion of 200 μM AMPA, performed on the same day using cells from the same coverslip, typical of three such series. Addition of AMPA at the same time as 0.1 mM Mn^{2+} did not significantly increase the rate of quench of fura 2 fluorescence.

AMPA-evoked response in cerebellar granule cells (Rodriguez *et al.*, 1991). While cyclothiazide may reveal aspects of AMPAR-mediated activity not otherwise seen, experiments throughout the present study were conducted primarily in order to study events which may occur consequent to physiological stimulation. There is currently no evidence of an endogenous ligand for the cyclothiazide site (M. Mayer, personal communication).

4.4.2 Possible interaction between NMDA and AMPA receptors

AMPARs and NMDARs provide parallel, though temporally differing, mechanisms by which glutamate can evoke Ca^{2+} entry into neurones. As many types of interaction between GluRs have previously been reported (e.g. Koh *et al.*, 1991; Kelso *et al.*, 1992; McDonald and Schoepp, 1992; Courtney and Nicholls, 1992), including a permissive effect of AMPAR activation on NMDARs in Mg^{2+}-containing medium (Raiteri et al, 1992), the possibility that either pre- or co-incubation of NMDA would increase the AMPA-evoked response in granule cells was investigated using a range of concentrations of NMDA and AMPA. Addition of AMPA (50-500 μM) during perfusion of NMDA evoked a $[Ca^{2+}]_i$ elevation on top of the NMDA-evoked plateau (Fig. 4.12), of comparable temporal profile and similar magnitude to that evoked by an initial perfusion of AMPA alone. No potentiation of the AMPA response subsequent to NMDA challenge was apparent either (data not shown). This absence of interaction between AMPAR and NMDAR mechanisms of $[Ca^{2+}]_i$ elevation in Mg^{2+}-free KHB is consistent with an inability of either receptor to activate enzymes which modulate activity of the other, e.g. PKC or CamK-II for NMDARs (Moriyoshi *et al.*, 1991; Durand *et al.*, 1993; Kitamura *et al.*, 1993; see Chapter 5) or protein kinase A for AMPARs (Rosenmund *et al.*, 1994), although AMPAR currents have been reported to be potentiated by PKC and CamK-II (McGlade-McCulloh *et al.*, 1993; Wang *et al.*, 1994a). The possibility of permissive effects of AMPA-evoked

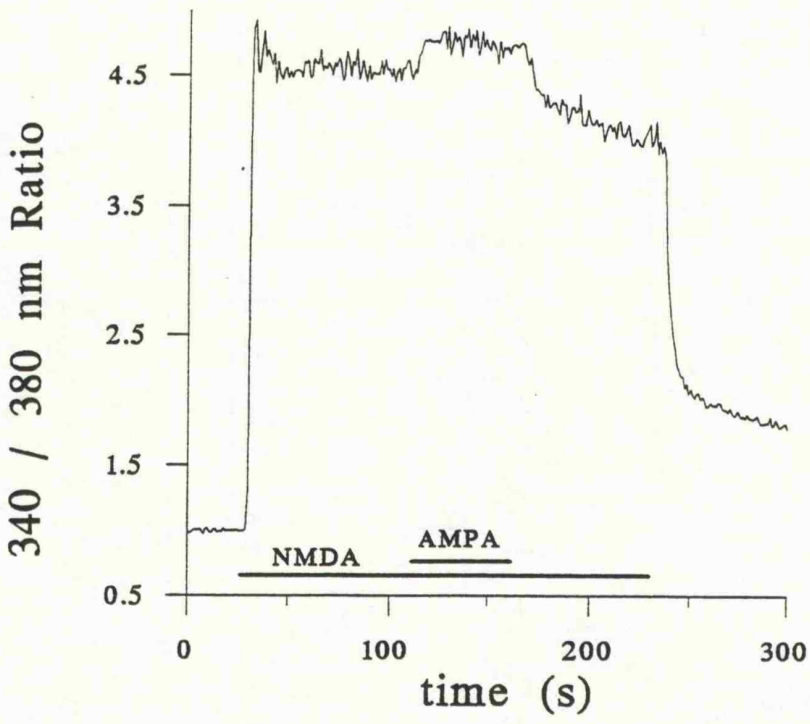


Fig. 4.12 Additivity of effect of AMPA and NMDA on $[Ca^{2+}]_i$. Addition of 200 μM AMPA on top of the plateau response to 200 μM NMDA caused an approximately additive effect on $[Ca^{2+}]_i$, with no marked interactions occurring between the two responses.

depolarization on NMDA responses in Mg^{2+}-containing KHB in this preparation remains to be addressed.

4.5 NMDA-evoked Mn^{2+} quench

4.5.1 Characterization of the response

The efficacy of NMDA as an activator of divalent cation entry into granule cells having been established from $[Ca^{2+}]_i$ experiments, more direct information on the mechanisms and characteristics of this entry was sought, using the Mn^{2+} quench technique. Mn^{2+} permeability through NMDAR channels is considerably lower than the permeability for Ca^{2+}, due to its partial channel blocking action (Ascher and Nowak, 1988; Iino *et al.*, 1990), so it was important initially to establish if Mn^{2+} quench could be used to assess NMDA channel activation. Addition of NMDA (200 μM) caused a marked increase in the rate of Mn^{2+} quench in cerebellar granule cells compared to control (Table 4.1, Fig. 4.13). In order to investigate whether the Mn^{2+} entry activated by NMDA occurred through NMDAR channels, dizocilpine (10 μM) was perfused onto granule cells either before or during NMDA-evoked Mn^{2+} quench. As illustrated in Fig. 4.13, this resulted in a rapid decrease in the rate of quench, reducing it to a rate not significantly greater than basal quench. These results demonstrate that NMDA-evoked Mn^{2+} entry occurs via NMDA channels, and does so sufficiently rapidly to markedly increase the rate of Mn^{2+} quench of fura 2 fluorescence, thus providing an experimental window in which to examine modulation of NMDA channel activation. Given the variability in the relationship between peak and plateau phases of the NMDA-evoked $[Ca^{2+}]_i$ elevation (see Section 4.3.1), it appeared possible that modulation of NMDA channel activity may be occurring temporally, during NMDA challenge. The increase in quench rate evoked by NMDA perfused simultaneously with Mn^{2+} was compared to the quench rate for Mn^{2+} perfused 100 s after the initiation of stimulation with NMDA, i.e. during the NMDA

Condition	$t_{1/2}$ ± S.E. (s)
KCl Control	103.8 ± 29.4
NMDA Control	104.0 ± 37.7
KCl Plateau	36.6 ± 10.7*
NMDA Plateau	26.7 ± 7.4 *
KCl Peak	15.2 ± 3.1 *#
NMDA Peak	12.2 ± 3.0 *#

Table 4.1 Meaned results for K^+- and NMDA-evoked Mn^{2+} quench. Differences between groups were demonstrated by analysis of variance ($p < 0.005$ for K^+, $p < 0.005$ for NMDA). Data were subsequently analysed using Student's t-test. * = $t_{1/2}$ significantly lower than control ($p < 0.005$ for K^+ peak, $p < 0.001$ for NMDA peak, $p < 0.01$ for K^+ plateau, $p < 0.05$ for NMDA plateau). # = $t_{1/2}$ significantly lower than plateau ($p < 0.05$ for K^+ peak, $p < 0.05$ for NMDA peak) (n=6, with all data obtained on a given day meaned and treated as a single data-set.)

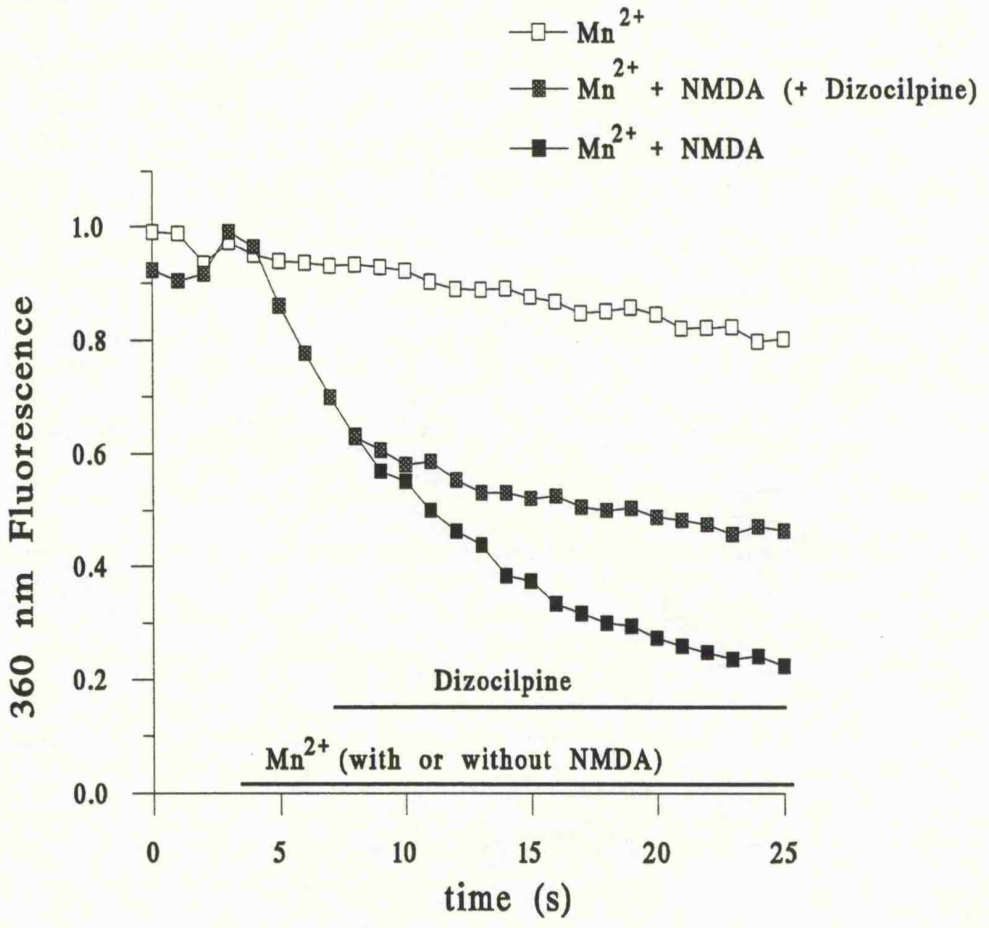


Fig. 4.13 Effect of NMDA on the rate of Mn^{2+} quench. Example traces of normalized data from a series of experiments for control quench and quench during perfusion of 200 μM NMDA, performed on the same day using cells from the same coverslip. NMDA markedly increased the rate of quench compared to control. Dizocilpine (10 μM), added either at the same time as NMDA (not shown) or during the NMDA-evoked response, reduced the rate of quench to a level not significantly greater than control, demonstrating that the response to NMDA involved cation entry through the integral NMDA channel.

$[Ca^{2+}]_i$ plateau phase. The peak quench rate was found to be significantly faster than the plateau quench rate (Table 4.1).

4.5.2 Effect of nitrendipine on Mn^{2+} quench

As mentioned above, it has previously been reported that nitrendipine, a DHP L-type VOCC antagonist (Bean, 1985) can modify NMDAR activity, by interacting directly with the NMDA channel (Skeen *et al.*, 1993). However, in other reports DHPs including nitrendipine have been shown to have little or no effect on NMDAR-mediated responses (Holopainen *et al.*, 1989; Manev *et al.*, 1989; Parks *et al.*, 1991; Segal and Manor, 1992; Alford *et al.*, 1993). In the present study little inhibition of the NMDA-evoked $[Ca^{2+}]_i$ response was found after addition of (-) PN 202 791 (see Section 4.3.3). The ability of nitrendipine to affect NMDA channel permeability was therefore examined, by pretreating cerebellar granule cells with 2 μM nitrendipine for 2 min, then adding either Mn^{2+} alone or NMDA and Mn^{2+} simultaneously, and comparing the rates of quench with those evoked in control cells from the same coverslip.

Nitrendipine increased the $t_{1/2}$ for Mn^{2+} quench under control conditions (control $t_{1/2}$ = 66.5 ± 27.9 s, nitrendipine $t_{1/2}$ = 99.0 ± 36.8; $p < 0.05$), an elevation which despite the variation in control quench from day to day was consistently ~1.5-fold (Fig. 4.14). This finding indicates that granule cells are already partially depolarized in the absence of exogenous stimuli, such that entry via VOCCs accounts for part of the control Mn^{2+} quench. As NMDA potently stimulated the rate of quench, the relatively small inhibitory effect of nitrendipine on background quench would not be expected to greatly alter the NMDA-evoked response. Nitrendipine pre-treatment had no significant effect on NMDA-evoked Mn^{2+} quench (NMDA $t_{1/2}$ = 13.7 ± 6.1 s, NMDA + nitrendipine $t_{1/2}$ = 17.6 ± 11.2 s)(Fig. 4.15). This result,

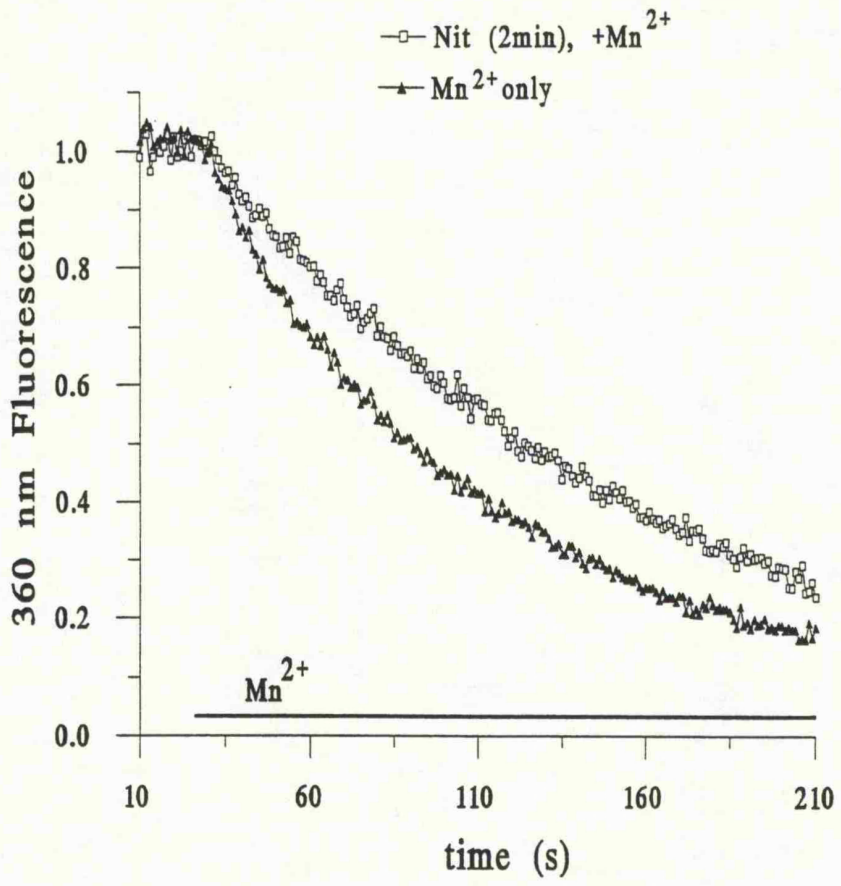


Fig. 4.14 Involvement of L-type VOCCs in control quench. Example traces of normalized data from a series of experiments for control quench and quench during perfusion of nitrendipine, performed on the same day using cells from the same coverslip, typical of three such series. 2 min preincubation with nitrendipine (2 μM) significantly increased the $t_{1/2}$ of Mn^{2+} quench under control conditions by ~1.5-fold ($p<0.05$).

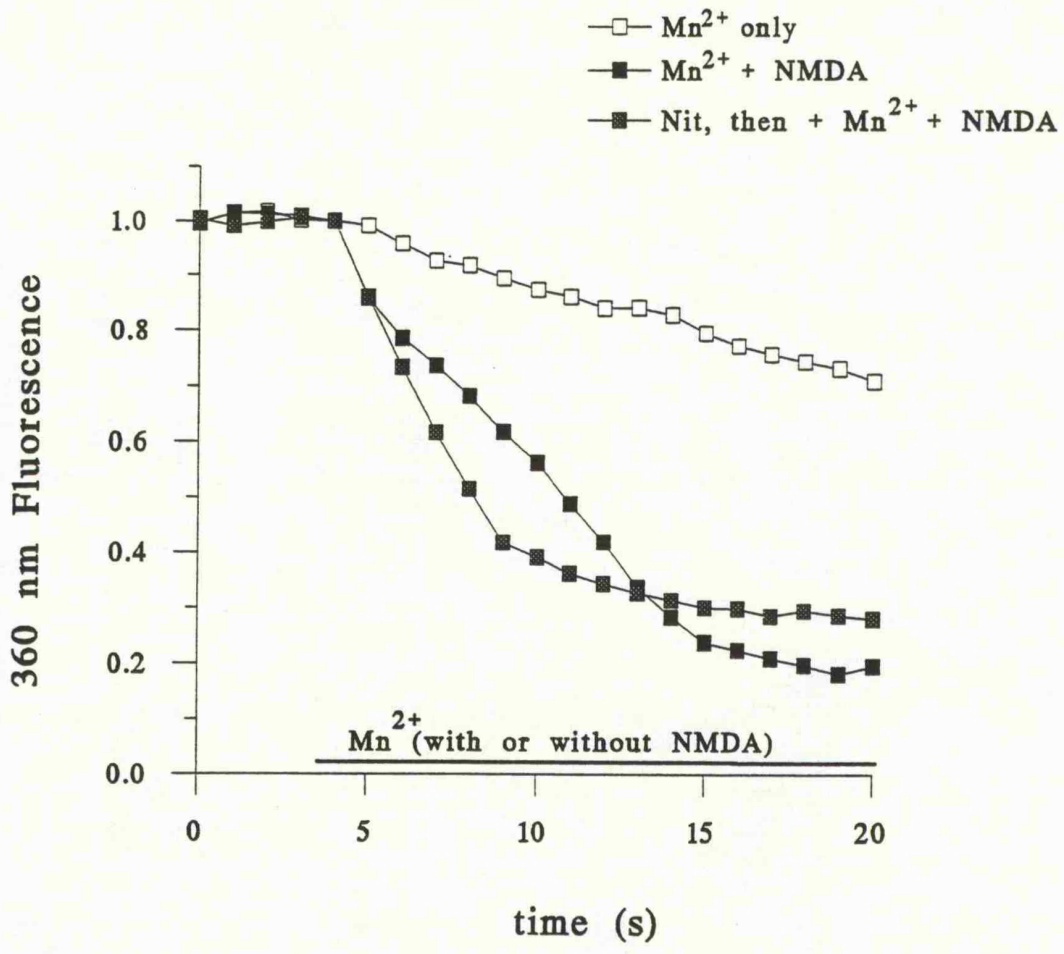


Fig. 4.15 Lack of involvement of L-type VOCCs in NMDA-evoked quench. Example traces of normalized data from a series of experiments for control quench and quench during perfusion of 200 μM NMDA, performed on the same day using cells from the same coverslip, typical of three such series. 2 min preincubation with nitrendipine (2 μM) had no significant effect on the $t_{1/2}$ of Mn^{2+} quench evoked by 200 μM NMDA. Apparent differences between the 2 traces shown here are no greater than the degree of variability encountered for a single given experimental condition, even for cells from the same coverslip.

alongside the $[Ca^{2+}]_i$ experiments using PN 202 791, appears to confirm a lack of L-type VOCC involvement in NMDA-evoked responses in cerebellar granule cells, and also demonstrates an apparent absence of effect of nitrendipine on NMDAR channels, at least under these experimental conditions.

4.6 Involvement of stores in NMDA response

4.6.1 Effects of store modulators on response

As demonstrated in Chapter 3, Ca^{2+} entering across the plasma membrane through VOCCs can activate Ca^{2+} release from intracellular stores in cultured cerebellar granule cells. It was therefore of interest to establish whether Ca^{2+} entry via the NMDAR channel evoked a comparable Ca^{2+} release response, or whether the $[Ca^{2+}]_i$ changes evoked by NMDA were solely due to Ca^{2+} entry. Experiments to examine this were designed, initially using dantrolene, the agent which apparently inhibited K^+-evoked CICR most effectively. Dantrolene perfused onto granule cells after the NMDA-evoked plateau was established concentration-dependently reduced the $[Ca^{2+}]_i$ elevation. As shown in Fig. 4.16, 30 μM dantrolene was sufficient to reduced the NMDA response by ~80%. Given the uncertainty over the site of action of dantrolene (but see Section 4.5.2), further experiments were performed using thapsigargin and ryanodine. As NMDA responses were similarly reproducible at 5 min intervals (Fig. 4.17a; see Section 4.3.1), an identical protocol was adopted as for the K^+-depolarization experiments (see Chapter 3).

Pre-treatment with thapsigargin (10 μM) for 10 min significantly reduced the $[Ca^{2+}]_i$ response to NMDA, affecting both peak and plateau phases to a similar degree (Fig. 4.17b, Table 4.2). Ryanodine (10 μM) also reduced both phases of the NMDA response (Fig. 4.17c, Table 4.2), in a partially use-dependent manner. Thus, the inhibition by ryanodine was greater for a second NMDA challenge 10 min after the

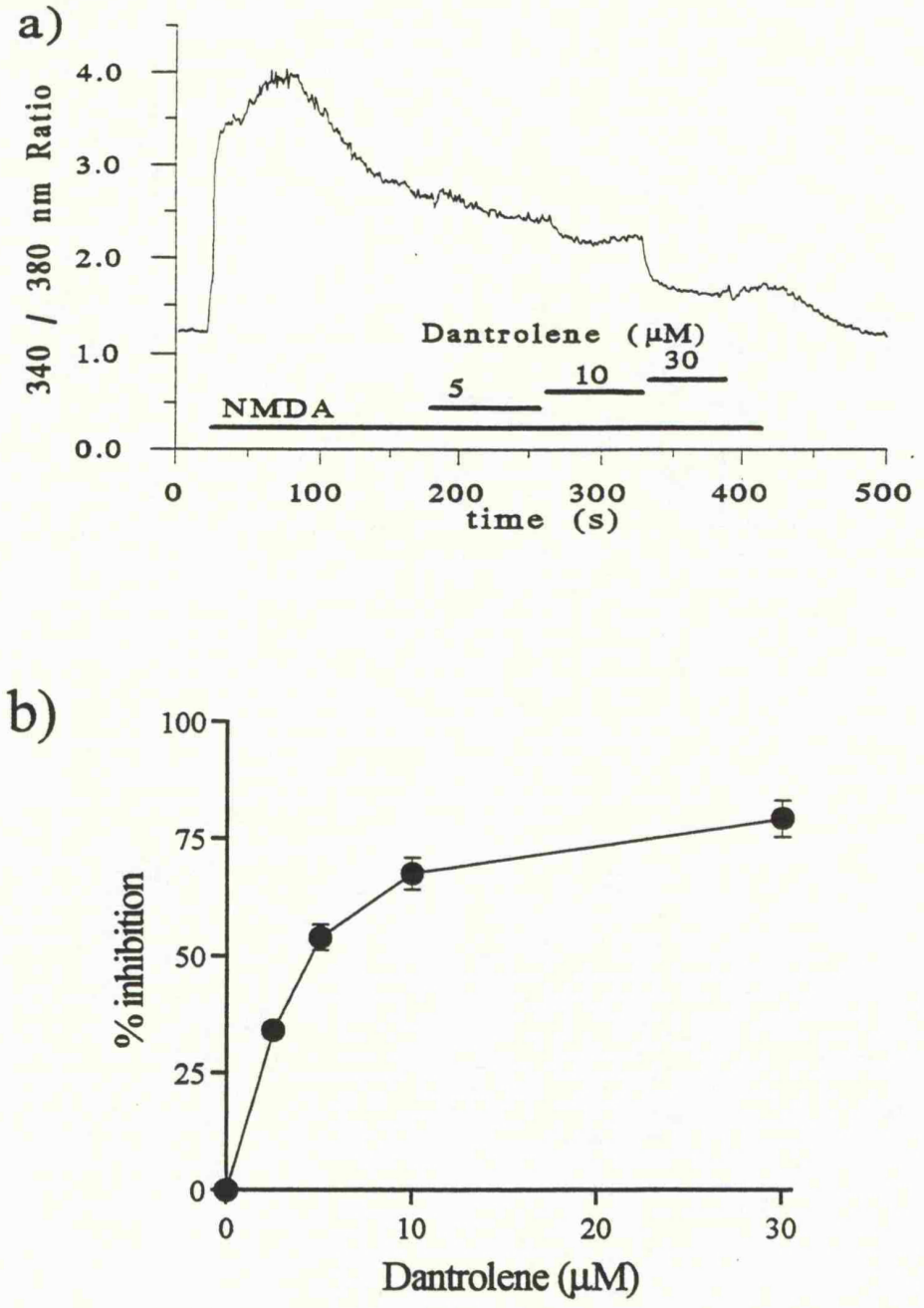


Fig. 4.16 Inhibition of the plateau $[Ca^{2+}]_i$ responses to NMDA by dantrolene. ***a)*** Example trace typical of the results from four series of experiments. Increasing concentrations of dantrolene added in a stepwise manner after the development of a steady plateau response to NMDA (200 μM) caused a concentration-dependent decrease in the size of the plateau. ***b)*** Meaned results from 7-24 experiments for each concentration performed on four separate preparations. 10 μM dantrolene was sufficient to virtually abolish the K^+-evoked plateau.

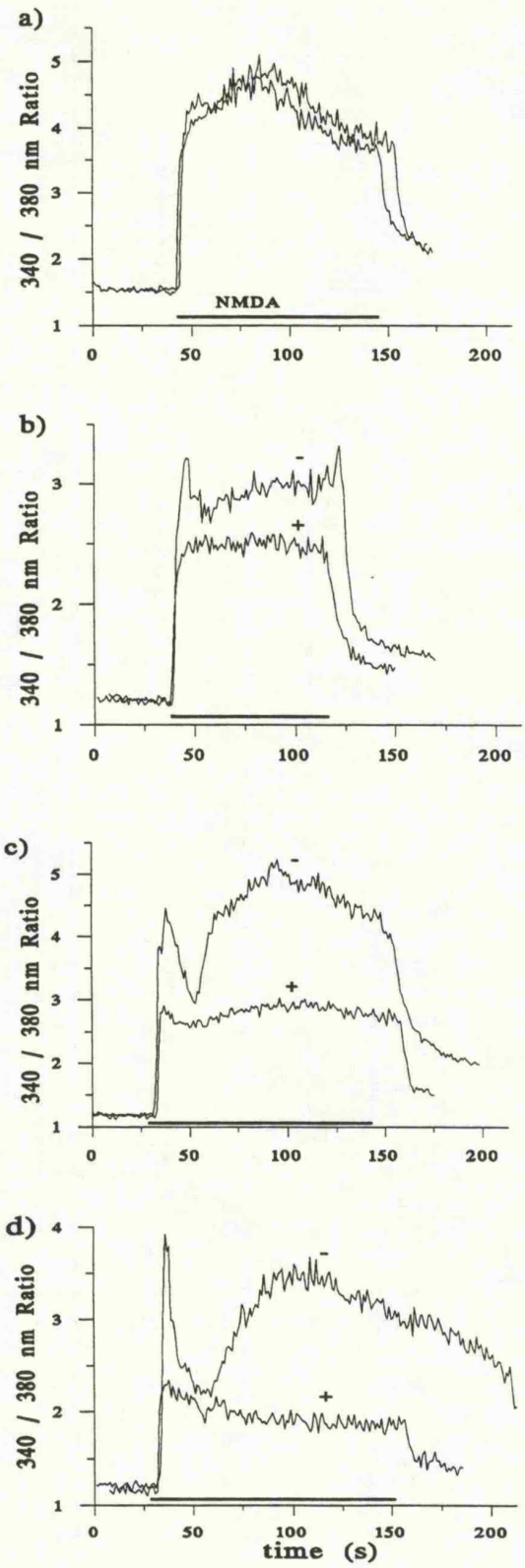

a)
340 / 380 nm Ratio
NMDA
b)
340 / 380 nm Ratio
-
+
c)
340 / 380 nm Ratio
-
+
d)
340 / 380 nm Ratio
-
+
time (s)

Fig. 4.17 Inhibition of the $[Ca^{2+}]_i$ responses to NMDA by other store modulators. ***a)*** Responses to 200 μM NMDA from a given group of cells were highly reproducible with 5 min intervals between challenges. ***b,c)*** Initial response to NMDA (-) and the second response during incubation with 10 μM ryanodine or thapsigargin respectively (+). The store modulators decreased both peak and plateau phases of the NMDA-evoked response. ***d)*** Incubation with ryanodine and thapsigargin in combination caused a greater inhibition of the plateau response than either agent on its own.

control pulse than for a first challenge at 5 min in the same cells, or a first challenge at 10 min in parallel experiments ($p < 0.02$, Student's t-test). The effects of these agents were not greater on subsequent NMDA responses and were not significantly increased by longer incubation periods (data not shown).

Condition		% inhibition of response		
		Thapsigargin	Ryanodine	Combined
25 mM K^+	Plateau	66 ± 2	69 ± 3	73 ± 3
200 μM	Peak	48 ± 4	42 ± 2	64 ± 6 *
NMDA	Plateau	43 ± 2	38 ± 3	69 ± 3 #

Table 4.2 Meaned results for inhibition of responses to NMDA and K^+ by thapsigargin and ryanodine. Results shown as mean ± S.E. (n = 7-14). One group of cells was unaffected by thapsigargin, and one group was unaffected by ryanodine: these values are not included in the data shown here. Peak responses to K^+ were unaffected by either agent, and so are not included in the table. * = greater than ryanodine alone ($p < 0.01$); # = greater than either ryanodine or thapsigargin alone ($p < 0.001$).

As there has been some controversy as to whether cerebellar granule cells contain more than one type of Ca^{2+} pool, or a single Ca^{2+} pool accessible to both $InsP_3$- and Ca^{2+}-evoked mechanisms of release (Whitham *et al.*, 1991b; Irving *et al.*, 1992b), further studies were undertaken to determine if the effects of thapsigargin and ryanodine were greater when perfused over granule cells together compared to the effects of either agent alone. A significantly greater inhibition of both phases of the NMDA response was found compared to the effects of ryanodine alone, and a significantly greater inhibition of the NMDA-evoked plateau was found compared to the effects of thapsigargin alone (Fig. 4.17d, Table 4.2).

4.6.2 Effects of thapsigargin and dantrolene on Mn^{2+} quench

While the activity of dantrolene, thapsigargin and ryanodine at the level of Ca^{2+} stores is well established, the specificity of these agents has been questioned. For example, it has been shown in adrenal glomerulosa cells and GH_3 cells that thapsigargin not only inhibits the Ca^{2+}ATPase of intracellular stores, but also inhibits some Ca^{2+}-permeant plasma membrane channels (Rossier *et al.*, 1993; Nelson *et al.*, 1994). Dantrolene inhibits ryanodine binding to its receptor in muscle (Pessah *et al.*, 1986), but the extent of this effect is controversial (El-Hayek *et al.*, 1992). Dantrolene also interacts with VOCCs at higher concentrations (El-Hayek *et al.*, 1992), and its exact site of action in neuronal preparations remains unclear (Smith and Nahorski, 1993). It was therefore important to establish whether the above effects of these agents on $[Ca^{2+}]_i$ signalling in the present study were due to inhibition of store release or to modification of plasma membrane permeability to Ca^{2+}. For this reason the possibility that either thapsigargin or dantrolene could inhibit NMDA- or K^+-evoked Mn^{2+} quench of fura 2 fluorescence in granule cells was examined.

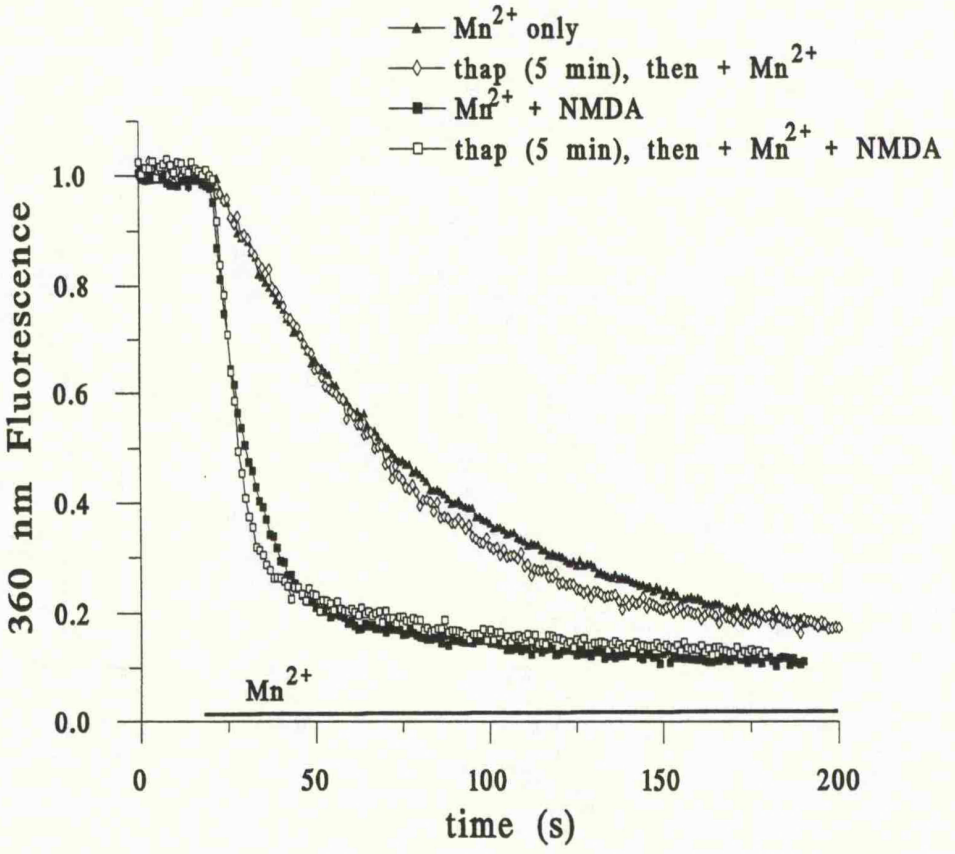


Fig. 4.18 Lack of effect of thapsigargin on control and NMDA-evoked Mn^{2+} quench. 5 min incubation with 10 μM thapsigargin did not significantly affect the rate of Mn^{2+} quench under control conditions or the rate of quench stimulated by 200 μM NMDA.

Condition	$t_{1/2}$ ± S.E. (s)
Control	76.7 ± 32.3
Thapsigargin	46.1 ± 6.8
Control	56.2 ± 9.4
Dantrolene	73.3 ± 6.1
NMDA peak	11.4 ± 0.5
NMDA + Thap	9.9 ± 1.1
NMDA peak	14.6 ± 5.9
NMDA + Dant	19.6 ± 10.6
KCl plateau	36.5 ± 17.2
KCl + Thap	28.5 ± 23.0
KCl plateau	20.6 ± 5.3
KCl + Dant	21.1 ± 1.5

Table 4.3 Effect of store modulators on Mn^{2+} quench under control and stimulated conditions. No significant effects were found for either thapsigargin (10 μM) or dantrolene (30 μM) under any of the above conditions.

Pre-treatment with dantrolene (30 μM) for 2 min had no significant effect on Mn^{2+} quench under control or 200 μM NMDA- or 25 mM K^+-evoked conditions (Table 4.3). Pre-incubation with thapsigargin (10 μM) for 5 min also did not affect

Mn^{2+} quench under control or NMDA- or K^{+}-stimulated conditions (Fig. 4.18, Table 4.3). These results suggest that the effects of these agents are indeed due to actions on intracellular stores, not on Ca^{2+} entry. It is notable that even under control conditions, in which the store-depleting actions of thapsigargin appear to lead to capacitative Ca^{2+} entry (see Chapter 3), Mn^{2+} quench is unaffected, which might indicate that the CRAC current activated by store depletion may be too small to be detected by Mn^{2+} quench, but given the marked $[Ca^{2+}]_i$ elevation which thapsigargin evokes (see Fig. 3.7), and the ~80-fold higher affinity of Mn^{2+} compared to Ca^{2+} for fura 2 (Kwan and Putney, 1990), such that Mn^{2+} quench of fura 2 is likely to be a sensitive indicator of divalent cation entry even through poorly Mn^{2+}-permeant channels, this seems unlikely. It seems more probable that thapsigargin-evoked Ca^{2+} entry may occur via Mn^{2+}-impermeant channels in granule cells. Indeed, CRAC channels appear to have low Mn^{2+}-permeability in many (Kwan and Putney, 1990; Fasalato *et al.*, 1993; Putney and Bird, 1993; Kass et al., 1994), though probably not all (Zhang and Melvin, 1993), cell types. Indeed, it appears that because of its low CRAC channel permeability Mn^{2+} can cause a block of entry of other divalent cations in some cell types (see Penner *et al.*, 1993), as also found for the entry of other cations through VOCCs (Nelson, 1986) and NMDAR channels (Ascher and Nowak, 1988).

4.7 Role of nitric oxide in modulating NMDA response

It is now well established that NMDA, by Ca^{2+} entry-mediated activation of nitric oxide synthase (NOS), can evoke production of nitric oxide (NO)(Lei *et al.*, 1992b; Hoyt *et al.*, 1992; Vige *et al.*, 1993; Dawson *et al.*, 1993; Fujisawa *et al.*, 1993; Lipton *et al.*, 1993; Coyle and Puttfarcken, 1993; Fagni *et al.*, 1993; Schuman and Madison, 1994). This molecule has been reported to mediate NMDAR-evoked neurotoxicity both *in vitro* and *in vivo* (Vige *et al.*, 1993; Dawson *et al.*, 1993; Fujisawa *et al.*, 1993). However, the mechanisms of action (Lei *et al.*, 1992b; Hoyt *et*

al., 1992; Lipton *et al.*, 1993; Fagni *et al.*, 1993) and overall consequences of NO production evoked by NMDA (Schuman and Madison, 1994) remain highly controversial. As cerebellar granule cells have been reported to contain very high levels of NOS (Garthwaite, 1991), it appeared possible that synthesis of this molecule could play a role in modulating NMDA-evoked $[Ca^{2+}]_i$ elevations. This was investigated using L-arginine (L-Arg), the metabolic precursor of NO, and N^{ω}-nitro-L-arginine (NARG), an inhibitor of NOS. The response to NMDA after incubation with 100 μM NARG for 5 min was compared to an initial NMDA-evoked response, and to repeated NMDA challenges in parallel cell groups. In these parallel experiments, the S2/S1 ratio for control plateau responses was 0.91 ± 0.02 (slightly lower than for a larger series of experiments reported in Section 4.2.1). The ratio of NMDA response during perfusion of NARG to initial NMDA response was 1.09 ± 0.09 (Fig. 4.19), a significantly greater response ratio than for repeated control NMDA perfusion ($p < 0.05$). When NARG was added during the sustained phase of NMDA-evoked $[Ca^{2+}]_i$ responses, it elevated the plateau in 6 out of 7 experiments (Fig. 4.19). There was no consistent effect of NARG on NMDA-evoked peak responses (Fig. 4.19). After pre-treatment for >30 min with 1 mM L-Arg, the NMDA response was not potentiated by NARG as before: the ratio of the NMDA response in the presence of NARG to the initial NMDA response was 0.77 ± 0.07. L-Arg pre-treatment itself for 5 or 30 min did not significantly affect NMDA-evoked responses, although 5 min pre-treatment tended to cause elevated basal 340 / 380 nm ratios (initial ratio = 1.29, ratio after 5 min L-Arg = 1.76 (n=2)).

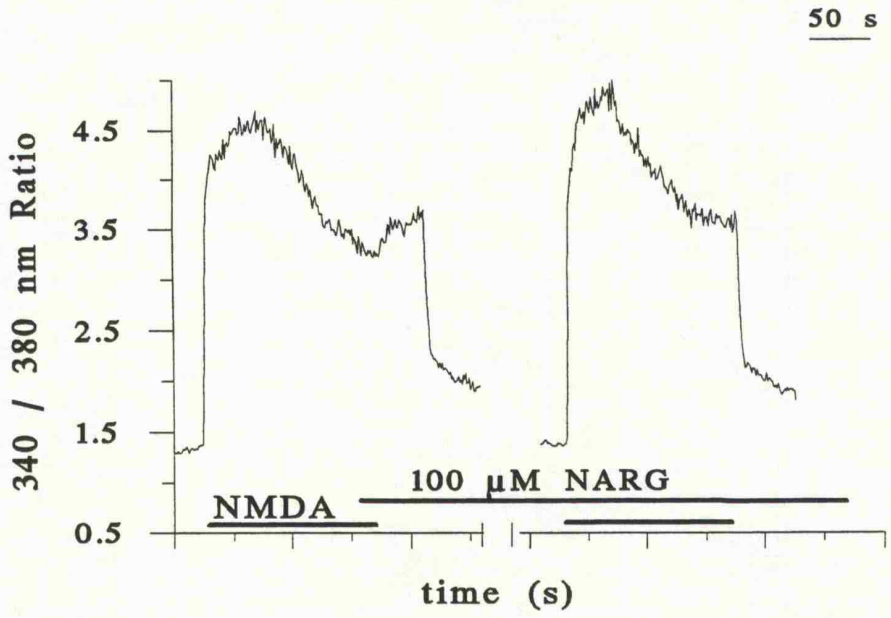


Fig. 4.19 Potentiation of NMDA-evoked $[Ca^{2+}]_i$ response by NARG. 100 µM NARG rapidly increased the $[Ca^{2+}]i$ plateau elevation evoked by 200 µM NMDA, an effect which persisted for the plateau response to a second NMDA challenge after a 5 min interval.

4.8 Discussion

II

Cerebellar granule cells responded to addition of NMDA by a moderate elevation of $[Ca^{2+}]_i$. This elevation was greatly, though variably, potentiated by addition of exogenous glycine, indicating that the coagonist site is not saturated by endogenous levels of glycine. This is in contrast to several previous reports (Courtney *et al.*, 1990; Holopainen *et al.*, 1990; Ciardo and Meldolesi, 1991), but in agreement with other $[Ca^{2+}]_i$ (Parks *et al.*, 1991; Baron *et al.*, 1992) and electrophysiological (Van der Valk *et al.*, 1991) findings. The variability in the effect of exogenous glycine within cultures plated at 3 x 10^6 cells per well may be due to the characteristic aggregations of cells found in cerebellar granule cell cultures (see Fig. 2.1a,b). The apparent discrepancy between our findings on the effect of glycine and those of some of the aforementioned reports can be explained by the experiments demonstrating the importance of plating density on the potentiation induced by exogenous glycine. The response to NMDA alone was significantly higher in cultures plated more densely than in our standard culture conditions. This was not due to increased maximal responsiveness to NMDA, as there was no significant change in the response to NMDA in the presence of saturating exogenous glycine with changing density. The increased $[Ca^{2+}]_i$ elevation evoked by application of NMDA alone is likely to be due to a higher mean concentration of glycine in the more densely plated cultures. Although the different methods of plating used make direct comparisons of densities difficult, it seems likely that where other groups have demonstrated little or no potentiation by exogenous glycine (Courtney *et al.*, 1990; Holopainen *et al.*, 1990; Ciardo and Meldolesi, 1991), they were employing more densely plated cultures than ourselves.

Whilst ligand binding studies have demonstrated complex interactions between the glycine and NMDA binding sites (Monaghan *et al.*, 1988; Grimwood *et al.*, 1993), the glycine potentiation was not accompanied by any dramatic shift in the concentration-response curve for NMDA, in accordance with previous functional reports (Johnson and Ascher, 1987; Parks *et al.*, 1991).

All groups of cultured cerebellar granule cells tested displayed a robust $[Ca^{2+}]_i$ response to addition of NMDA in the presence of glycine, consisting of distinct peak and plateau phases, but significant variability between groups was found with respect to the magnitude and pattern of the $[Ca^{2+}]_i$ elevation. Peak and plateau sizes varied in an apparently unconnected manner, and the temporal relationship between these two phases also displayed considerable heterogeneity. The biphasic nature of the response found is in agreement with some previous reports (Burgoyne *et al.*, 1988; Courtney *et al.*, 1990), but several groups have reported only a long-lasting plateau response (Holopainen *et al.*, 1989; Parks *et al.*, 1991; Baron *et al.*, 1992; Irving *et al.*, 1992a). This difference does not appear to correspond to any consistent pattern of method of agonist application, presence or absence of exogenous glycine, temperature at which experiments were performed, or number of cells used per experiment.

Significant differences in Mn^{2+} quench rate were evoked during the two phases of the NMDA response despite the overall $[Ca^{2+}]_i$ elevations being similar. These results suggest different mechanisms of $[Ca^{2+}]_i$ elevation may be involved in the two phases of the NMDA response. However, the suggestion by Courtney *et al.* (1990) that the peak phase may be due to activation of L-type VOCCs is not supported by the present study, as the peak was not inhibited by an L-type, nor an N-type, VOCC antagonist, and an L-type antagonist did not inhibit the peak phase of NMDA-evoked Mn^{2+} quench. These possible differences in mechanisms involved in the two phases require further investigation, particularly with respect to the potential

involvement of Na^+/Ca^{2+} exchangers in plasma (Kiedrowski *et al.*, 1994) and mitochondrial (see Friel and Tsien, 1994; L. Kiedrowski and E. Costa, submitted) membranes in the NMDA-evoked response.

The ability of nitrendipine to inhibit basal quench is consistent with the results of a previous study in which Ni^+ inhibited the relatively high basal quench in lacrimal acinar cells (Kwan and Putney, 1990), and given the lack of spontaneous activity under basal conditions in the present study (see Chapter 5) it is likely that the high affinity and small volume of cerebellar granule cells results in little Mn^{2+} entry being needed for significant basal or stimulated quench to occur. This sensitivity, alongside the convenience and reliability of Mn^{2+} quench as an experimental technique, indicates that this technique will prove of significant utility for future neuronal studies. L-type VOCCs may be activated by the release of neurotransmitters from surrounding cells causing a partial depolarization, despite the continuous perfusion system employed being likely to decrease the accumulation of such transmitters compared to static systems such as have been previously employed (e.g. Didier *et al.*, 1993)(see also Chapter 3 Discussion). Detectable, and variable, effects of endogenously released glycine from granule cells can be detected in the present system (see Section 4.2). Spontaneous activity has been previously noted in granule cells under Mg^{2+}-free conditions, due to activation of NMDARs presumably by released glutamate (Ciardo and Meldolesi, 1991; Lawrie *et al.*, 1993). This oscillatory behaviour may be dependent on culture conditions, however, such that it is present in cells grown in low K^+-containing culture medium but not in cells grown in high K^+ such as in the present study (see Chapter 5; Lawrie *et al.*, 1993). Also, dizocilpine inhibited NMDA-evoked Mn^{2+} quench, but did not reduce the rate to below background, suggesting that Mn^{2+} entry under control conditions is unlikely to be occurring as a result of endogenously released glutamate or glycine stimulating NMDARs. Nevertheless, the nitrendipine findings suggest that other transmitters may be released into the medium, in sufficient

quantity to influence the membrane potential of surrounding cells. The possible influence of other factors, such as long-term effects of culturing in 25 mM K^+, on tonic VOCC activity have yet to be established.

The glycine site on the NMDAR is much better characterized than the site (or sites (Fagni *et al.*, 1993)) of action of NO. The results of the present study demonstrate a small but potentially important NO inhibition of NMDA-evoked responses, possibly mediated by a free radical form of this molecule interacting directly with the NMDAR (Lipton *et al.*, 1993). These findings are in broad agreement with recent reports suggesting that NO can directly inhibit NMDAR activity (Manzoni *et al.*, 1992a; Lei *et al.*, 1992b; Hoyt *et al.*, 1992; Manzoni and Bockaert, 1993). If NMDARs in granule cells are tonically inhibited by NO, then a decrease in production of this unstable molecule by NARG inhibition of NOS would be expected to rapidly alleviate the inhibition, resulting in larger NMDA-evoked responses. The presence of high levels of L-Arg may cause sufficient NO production to maintain NMDAR inhibition even in the presence of NARG, explaining the lack of effect of NARG after pre-treatment with 1 mM L-Arg. The apparent lack of an inhibitory effect of exogenous L-Arg may suggest that endogenous L-Arg levels are sufficient to saturate NOS, such that tonic NO modulation of NMDARs in granule cells approaches maximal levels. A second possible cause of response inhibition, NO· free radical generation and subsequent granule cell neurotoxicity, did not apparently occur consequent to even prolonged treatment with L-Arg, probably because of the relative insensitivity of cerebellar granule cells to toxic effects of this molecule (although not to the effects of other free radicals) (Lafon-Cazal *et al.*, 1993). The hypothetical usefulness of agents which increase NO production for e.g. stroke therapy, to decrease NMDA-evoked Ca^{2+} entry, would clearly be dependent on NO-mediated NMDAR inhibition being submaximal under pathological conditions. The efficacy of such an intervention is further questioned by the direct toxic effects which NO-derived free

radicals produce (see Coyle and Puttfarcken, 1993). It appears likely, therefore, that a much better understanding of the complex actions of NO will be required before beneficial NO-elevating or -decreasing therapeutic approaches can be reliably designed.

III

Dantrolene and thapsigargin inhibited both K^+- (Chapter 3) and NMDA-evoked $[Ca^{2+}]_i$ responses in the present study. As neither agent affected the Mn^{2+} quench activation by K^+ or NMDA, their effects can confidently be ascribed to modulation of intracellular Ca^{2+} stores. Thus, the inhibition evoked by modulators of Ca^{2+} stores of the NMDA-evoked $[Ca^{2+}]_i$ response apparently demonstrates that Ca^{2+} entry through NMDAR channels, as well as through VOCCs (see Section 3.3.2), activates Ca^{2+} release from intracellular stores in cerebellar granule cells. As NMDA (and K^+) causes only a very modest stimulation on inositol phosphate production (see Section 6.3.1), it appears unlikely that this finding could be the result of release of an endogenous agonist and the subsequent mobilization of Ca^{2+} stores via PI-linked receptor activation. Rather, the present data are consistent with NMDA- (and K^+-)evoked Ca^{2+} entry sensitizing Ca^{2+} release mechanisms directly.

It is now accepted that InsP3Rs may be activated by resting or only slightly raised $InsP_3$ levels during even a moderate increase in $[Ca^{2+}]_i$ (see Berridge, 1993), so it is possible that NMDA-evoked CICR occurs partly via $InsP_3$Rs as well as RyRs. Irving and co-workers (1992b) have previously suggested that the entire Ca^{2+} pool in cerebellar granule cells i.e. including both $InsP_3$- and ryanodine-sensitive Ca^{2+} stores is a single physical or at least functional entity, as caffeine stimulation prevents subsequent responses to the $InsP_3$-linked agonist ACh and vice versa, and in at least some cells ryanodine completely inhibits responses to ACh and ACPD. However, the

partial additivity of the inhibitory effects of thapsigargin and ryanodine on the NMDA-evoked response is difficult to explain in terms of a single pool model, as the concentration of thapsigargin used in the present study would have been sufficient to elicit maximal release from any thapsigargin-sensitive stores, and the addition of ryanodine would not, in this single pool model, be expected to evoke a detectable additional inhibitory effect. The present results are better explained by a model in which ryanodine and thapsigargin affect two overlapping, but non-identical, Ca^{2+} pools. Indeed, the presence of an $InsP_3$-insensitive store has previously been established in permeabilized cerebellar granule cells (Whitham *et al.*, 1991b), which could broadly correspond to the ryanodine-sensitive pool demonstrated here in intact cells. It is interesting that the maximal effect of dantrolene was similar to that of thapsigargin and ryanodine combined, suggesting that dantrolene may have actions at an unidentified site differing from, or in addition to, RyRs.

Taken together with recent findings in other cultured neurones (e.g. Segal and Manor, 1992; Frandsen and Schousboe, 1992; Alford *et al.*, 1993), the present results indicate that CICR may be a major component of the NMDAR response in many neuronal types, and thus is likely to be centrally involved in NMDAR-mediated phenomena such as excitotoxicity and LTP. Dantrolene has been reported to block LTP induction, for which NMDAR activation is essential (see Bliss and Collingridge, 1993), in a slice preparation of the hippocampal CA1 region (Obenaus *et al.*, 1989). Similarly, thapsigargin blocks induction of LTP, but has no effect on its expression if administered 30 min after induction (Harvey and Collingridge, 1992). Consistent with these reports, NMDA-evoked $[Ca^{2+}]_i$ elevations in cultured hippocampal neurones were markedly decreased (67%) by pre-treatment with dantrolene (Segal and Manor, 1992), and the tetanus-evoked peak dendritic $[Ca^{2+}]_i$ transient in voltage-clamped hippocampal neurones was inhibited ~65% by incubation with ryanodine or thapsigargin (Alford *et al.*, 1993). Co-incubation with caffeine has been reported to

potentiate responses evoked by low concentrations of NMDA in cultured hippocampal neurones, possibly by a sensitization of NMDA-evoked CICR (Segal, 1992). However, caffeine pre-treatment has been found to inhibit post-tetanic potentiation in hippocampal slices, but had no effect on the induction of LTP (Lee *et al.*, 1987). A significant involvement of intracellular stores in the $[Ca^{2+}]_i$ response to NMDA has also been reported in cortical neurones (90% inhibition by dantrolene: Frandsen and Schousboe, 1992; 51% inhibition by ionomycin, 56% by dantrolene: Lei *et al.*, 1992a), retinal ganglion neurones (30% inhibition by dantrolene: Lei *et al.*, 1992a), cone horizontal cells (supralinearity of $[Ca^{2+}]_i$ response compared to NMDAR channel current: Linn and Christenson, 1992) and hypothalamic neurones (~100% inhibition by BHQ: Dayanithi *et al.*, 1993), besides the present findings in cerebellar granule cells. Initial observations suggest that significant NMDA-evoked CICR may also occur *in vivo* (Lazarewicz *et al.*, 1994). Various physiological consequences of NMDAR activation besides LTP are also decreased by inhibition of CICR: NMDAR-mediated activation of a Ca^{2+}-sensitive K^+ channel and subsequent hyperpolarization of cortical neurones is blocked by treatment with the inhibitor of store release procaine (Higashi *et al.*, 1991), NMDA-evoked taurine release from hippocampal slices is decreased by 55% after dantrolene pre-treatment (Menendez et al., 1993), and NMDA-evoked elevation of phosphate-activated glutaminase activity in cultured cerebellar granule cells, an indicator of NMDA-evoked neuronal differentiation and maturation, is inhibited by incubation with dantrolene (30%) or ryanodine (60%) (Moran and Olvera, 1993). Pathophysiological consequences of NMDA also appear to involve CICR: NMDA-evoked neurotoxicity is decreased by incubation with dantrolene in cultured cortical (100% inhibition: Frandsen and Schousboe, 1992) and retinal ganglion neurones (75% inhibition: Lei *et al.*, 1992a).

It is notable when comparing the above studies that the different agents used appear to vary significantly in their effectiveness depending on both the type of

neurone and the type of response examined. This may be related to the apparent differences in the extent of the $InsP_3$-insensitive component of intracellular stores which have been found in different brain regions (Verma *et al.*, 1990). Also, the sensitivity of stores in different brain regions to a given modulatory agent may vary: NMDA-evoked $[Ca^{2+}]_i$ responses in hypothalamic neurones were abolished by BHQ (Dayanithi *et al.*, 1993), but were unaffected by pre-treatment with thapsigargin (G. Dayanithi, personal communication), whereas the effectiveness of these agents was reversed in the present study (see Chapter 5). As most studies have used only one of the pharmacological agents available, there is a possibility that a lack of complete effectiveness of a single agent may have underestimated the actual involvement of CICR in the NMDA-evoked response, a suggestion consistent with the partial additivity of effect of thapsigargin and ryanodine in the present study (see also Gagov *et al.*, 1993).

An alternative explanation of the differential effects of store modulators in this and other studies is that pharmacologically differentiable stores may exist within the same neurone, which by virtue of their physical location or release properties may be preferentially involved in some tasks rather than others. Attempts to characterize the Ca^{2+} stores present in granule cells in greater detail will be described in subsequent Chapters 5 and 7. Also, several series of experiments designed to address the possibility that NMDA-activated CICR may involve InsP3Rs were performed, which are also detailed in Chapters 5-7.

5. Metabotropic responses

5.1 Effects of PI-linked agonists on $[Ca^{2+}]_i$

5.1.1 Importance of metabotropic $[Ca^{2+}]_i$ responses

Metabotropic receptor agonists, like ionotropic agents, evoke many of their effects consequent to elevation of $[Ca^{2+}]_i$. mGluRs have been found to be involved in important physiological and pathological events including LTP (Bortolotto and Collingridge, 1993; Otani *et al.*, 1993; Bortolotto *et al.*, 1994), LTD (Linden and Connor, 1993; Hartell, 1994; Conquet et al., 1994), neurotoxicity (McDonald and Schoepp, 1992; Frandsen and Schousboe, 1992; McDonald *et al.*, 1993) and neuroprotection (Koh *et al.*, 1991; Birrell *et al.*, 1993). Therefore, the pathways by which metabotropic agonist-evoked $[Ca^{2+}]_i$ elevation occurs are of considerable interest. In addition, as both synaptic plasticity (Bliss and Collingridge, 1993; Alford *et al.*, 1993; Perkel *et al.*, 1993; Frenguelli *et al.*, 1993; Linden and Connor, 1993) and excitotoxicity (Choi *et al.*, 1988; Finkbeiner and Stevens, 1988; Lysko *et al.*, 1989; Manev *et al.*, 1989; Michaels and Rothman, 1990; Patel *et al.*, 1990; Koh *et al.*, 1991; Garthwaite and Garthwaite, 1991; McDonald and Schoepp, 1992; Frandsen and Schousboe, 1992; Puttfarcken *et al.*, 1992; Birrell *et al.*, 1993; Hartley *et al.*, 1993; Brorson *et al.*, 1994) also involve $[Ca^{2+}]_i$ elevations mediated by ionotropic GluRs, interactions between metabotropic and ionotropic mechanisms of $[Ca^{2+}]_i$ elevation are likely to play important roles in the integration of glutamatergic signals, and so the nature of these interactions is also of significant interest. In this Chapter, $[Ca^{2+}]_i$ responses to the metabotropic agonists CCh, ACPD, and Quis, which each activate receptors coupled via G-proteins to PIC activation and the consequent production of the second messenger $InsP_3$, are characterized, and the possible involvement of Ca^{2+} entry and Ca^{2+} store mobilization in the responses to one such agonist, CCh, are investigated. Also, the nature of the interactive effects of metabotropic receptor-mediated responses with NMDAR-evoked $[Ca^{2+}]_i$ elevations are investigated, as are putative mechanisms which may be responsible for such modulatory effects.

5.1.2 Characterization of metabotropic $[Ca^{2+}]_i$ responses

An initial series of experiments was performed to address the abilities of CCh, 1S,3R-ACPD (ACPD), and Quis, to mobilize Ca^{2+} in cultured cerebellar granule cells. Each evoked a characteristic rapid-onset peak elevation of $[Ca^{2+}]_i$, which decayed within ~40 s to a much smaller or negligible plateau (Figs. 5.1,5.3,5.4,5.7). The selective, but possibly partial, mGluR agonist ACPD was on average less potent as an elevator of $[Ca^{2+}]_i$ in granule cells than Quis (basal ratio = 1.26 ± 0.04, Quis peak = 0.60 ± 0.08, plateau = 0.15 ± 0.01 (n=49); basal ratio = 1.23 ± 0.08, ACPD peak = 0.37 ± 0.09, plateau = 0.10 ± 0.03 (n=47))(Table 5.1). Quis possesses agonist activity at AMPARs as well as mGluRs. It seemed likely, therefore, that at least a component of the Quis-evoked plateau response would be due to AMPAR activation. However, the small monophasic nature of the AMPAR-mediated $[Ca^{2+}]_i$ response in granule cells (see Section 4.4) permitted the use of Quis as an mGluR agonist, particularly for investigation of peak $[Ca^{2+}]_i$ elevations: perfusion of the AMPAR antagonist CNQX (0.1-4 μM) over granule cells either before or during a Quis-evoked response had a (partial) inhibitory effect in only 1 out of 9 experiments (data not shown). The responses to both ACPD and Quis varied greatly, even between cells from the same preparation (Fig. 5.1).

Like ACPD and Quis, CCh (1 mM) increased $[Ca^{2+}]_i$ in the majority of cell groups examined (basal ratio = 1.22 μ± 0.05, CCh peak = 0.52 ± 0.06, plateau = 0.06 ± 0.01 (n=117)). On the basis of antagonist potencies, CCh-evoked $[Ca^{2+}]_i$ elevations in granule cells have been previously demonstrated to occur via M_3 receptors, and not the co-expressed M_2 subtype (Fohrmann *et al.*, 1993), as expected from normal second messenger coupling for these subtypes. Compared to other M_3 muscarinic receptor-expressing preparations (Lambert and Nahorski, 1990; Forsythe *et al.*, 1992; Tobin *et al.*, 1992), activation of M_3 receptors in cerebellar granule cells evoked only

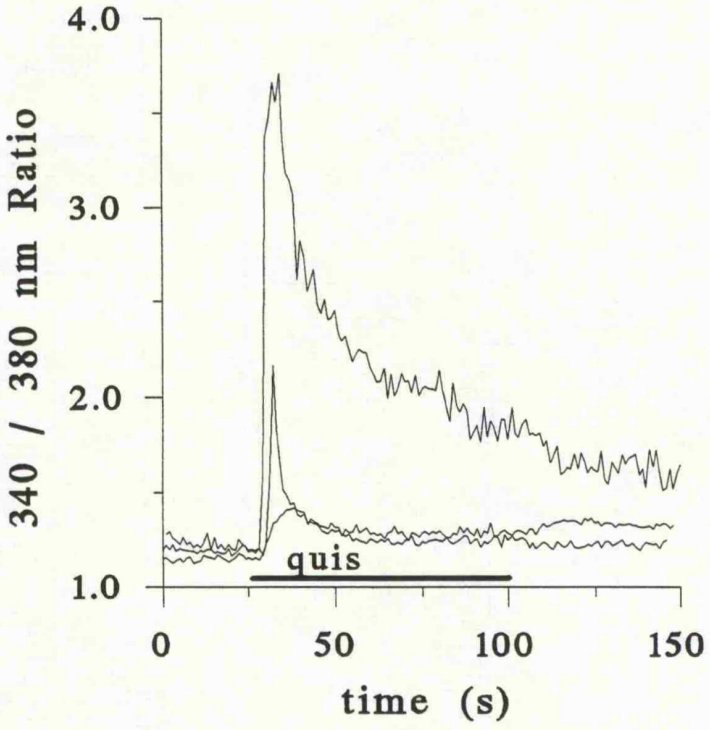


Fig. 5.1 Variability in $[Ca^{2+}]_i$ responses to mGluR stimulation. Example traces of $[Ca^{2+}]_i$ responses to 10 μM Quis from different groups of cells from the same coverslip. A rapid-onset peak phase was typically followed by a much smaller plateau elevation, but both peak and plateau responses varied greatly in size.

a very small sustained plateau elevation of $[Ca^{2+}]_i$ in the present investigation, despite a similar rapid peak $[Ca^{2+}]_i$ response. This could be due to low Ca^{2+} entry evoked by CCh, or to Ca^{2+} buffering e.g. pumping of Ca^{2+} out of the cell or into intracellular organelles (Carafoli, 1987). While CCh is a broad-spectrum cholinergic agonist, there appear to be no detectable nicotinic cholinergic receptors in granule cells on which it could act (Ciardo and Meldolesi, 1991; Irving *et al.*, 1992a; D. Gray, personal communication).

5.1.3 Dependence of response on KCl content of medium

It has previously been reported that the level of K^+ in culture medium greatly influences the expression of GluRs. However, these reports have been highly contradictory in their conclusions: in one study it was reported that mGluR-mediated $[Ca^{2+}]_i$ responses are present in granule cells cultured in 5-10 mM K^+ but not in 25 mM K^+ (Zhao and Peng, 1993), whereas in another study mGluR $[Ca^{2+}]_i$ responses were found to be greater in cells grown in culture medium containing 25 mM K^+ than in cells grown in 5 mM K^+ (Irving, 1991 - Ph.D. Thesis, University of Bristol). In a third report, $InsP_X$ responses to Quis were reportedly unchanged by differing K^+ levels in the medium (Aronica *et al.*, 1993). The developmental profile, although not the maximal amplitude, of CCh responses has also been reported to be affected by the level of K^+ in the medium (Irving, 1991 - Ph.D. Thesis, University of Bristol; Aronica *et al.*, 1993). An initial comparative study was therefore undertaken to examine metabotropic responses in cells cultured in parallel in either 10 or 25 mM K^+. Responses to ACPD, Quis and CCh were found to be present under both conditions, and no significant differences in the magnitude of any these responses was detectable in 10 mM K^+ cells, compared to the responses in 25 mM K^+ cells detailed in the previous Section (data not shown). The only consistent difference due to the differing levels of $[K^+]_o$ was that cells grown in 10 mM K^+ often displayed large and transient spontaneous

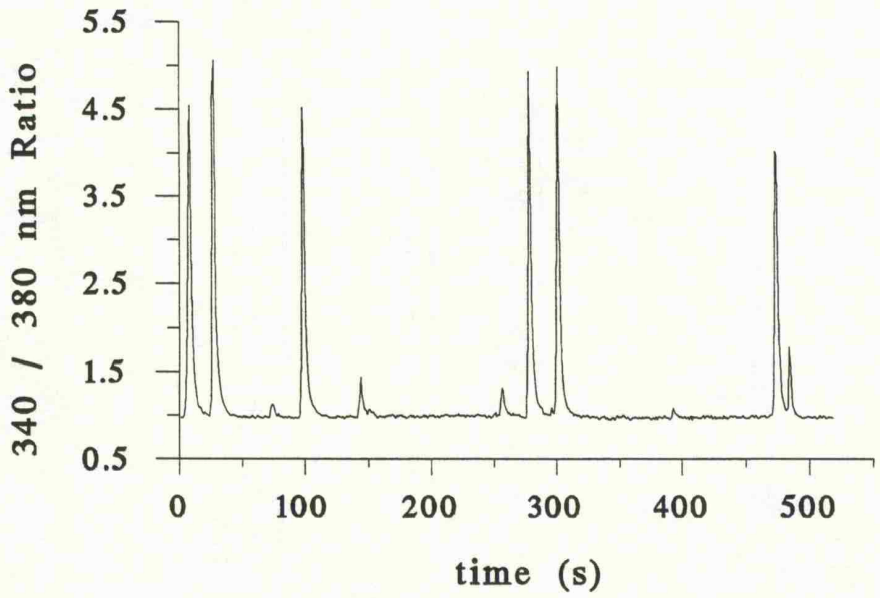


Fig. 5.2 Spontaneous $[Ca^{2+}]_i$ oscillations in granule cells grown in 10 mM K^+. Example trace. In most groups of cells which had been grown in low K^+, transient $[Ca^{2+}]_i$ elevations were found throughout the time period of recording. The size of the transients varied from a given cell group over time, and the pattern of oscillation was not consistent between different groups.

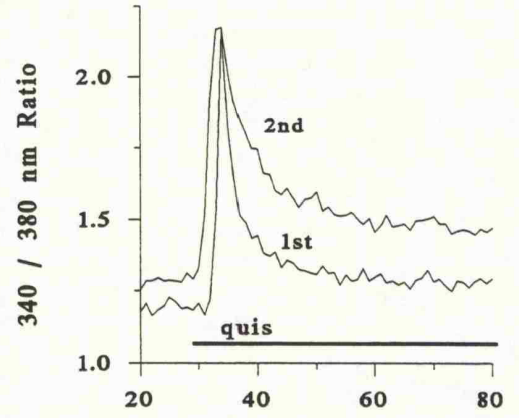
oscillations (Fig. 5.2). To maintain consistency with ionotropic experiments reported in previous Chapters, therefore, it was decided to perform all subsequent metabotropic experiments in this study on granule cells cultured in 25 mM K^+.

5.1.4 Putative refilling effect of depolarization

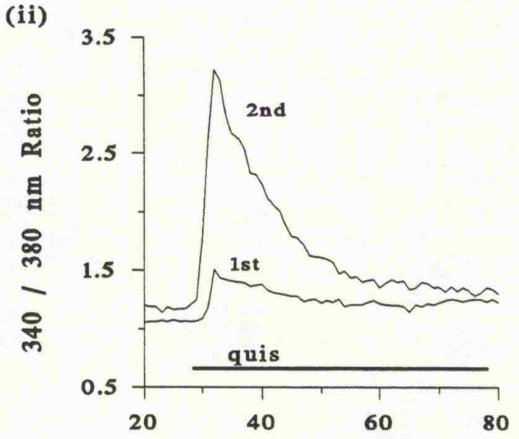
As indicated above, $[Ca^{2+}]_i$ elevations evoked by the mGluR agonists Quis (10 μM) (Fig. 5.1,5.3a) and ACPD (50-200 μM) (not shown) varied greatly in size between different groups of granule cells, even from the same coverslip. This is in accordance with a previous report (Irving *et al.*, 1990), although unlike that study which suggested that ~50% of granule cells lacked any mGluR-mediated response, in the present investigation $[Ca^{2+}]_i$ elevations were evoked by these agonists from virtually all cell groups studied. Pre-depolarization with 25 mM KCl has previously been reported to refill Ca^{2+} stores in neurones, including cerebellar granule cells, and so permit reproducible mGluR responses to be obtained from a given cell group (Murphy and Miller, 1989; Courtney *et al.*, 1990; Irving *et al.*, 1992a). In the present study, however, such a protocol did not promote either larger or more consistent responses to metabotropic agonists. Whilst in some groups of cells the $[Ca^{2+}]_i$ response to ACPD or Quis was markedly greater after depolarization, in others the reverse was true (Fig. 5.3b). A similar variation in response size from groups of cells was found after perfusion with KHB without depolarization (Fig. 5.3a,b).

Responses to CCh were typically highly reproducible within a given cell group, with 5 min intervals between stimulations (see Figs. 5.7,5.9). Agonist-evoked desensitization of muscarinic receptors in transfected CHO cells has previously been reported to cause CCh responses to take up to 15 min to fully recover (Tobin *et al.*, 1992), but a high degree of reproducibility of muscarinic responses has been previously

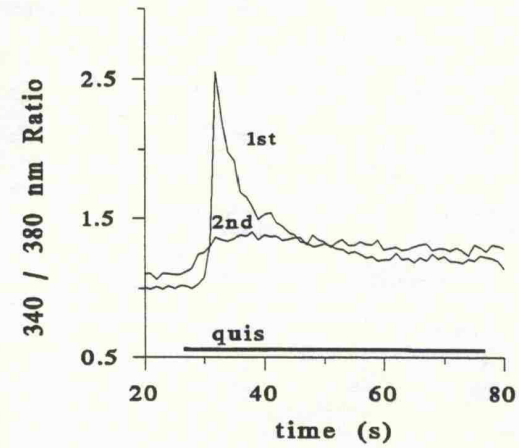
a) (i)


340 / 380 nm Ratio
2.0
1.5
1.0
2nd
1st
quis
20
40
60
80

(ii)


340 / 380 nm Ratio
3.5
2.5
1.5
0.5
2nd
1st
quis
20
40
60
80

(iii)


340 / 380 nm Ratio
2.5
1.5
0.5
1st
2nd
quis
20
40
60
80
time (s)

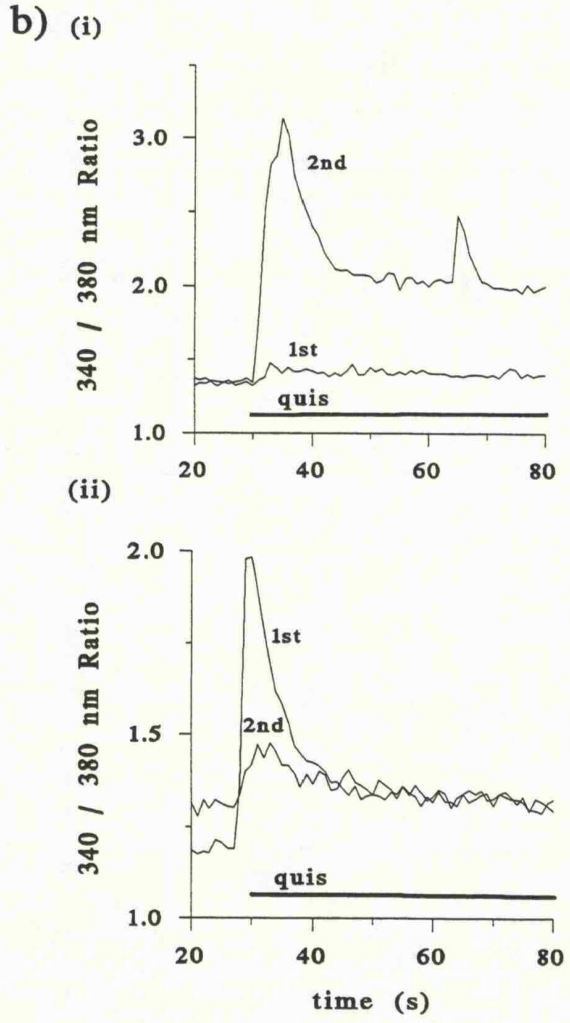


Fig. 5.3 Lack of reproducibility of responsiveness to mGluR activation.

Example traces of successive responses to 10 μM Quis at 5 min intervals from groups of granule cells.

a) Responses to Quis after 5 min perfusion with buffer containing normal $[K^+]$ were similar ***(i)***, much greater ***(ii)*** or much smaller ***(iii)*** than the initial response.

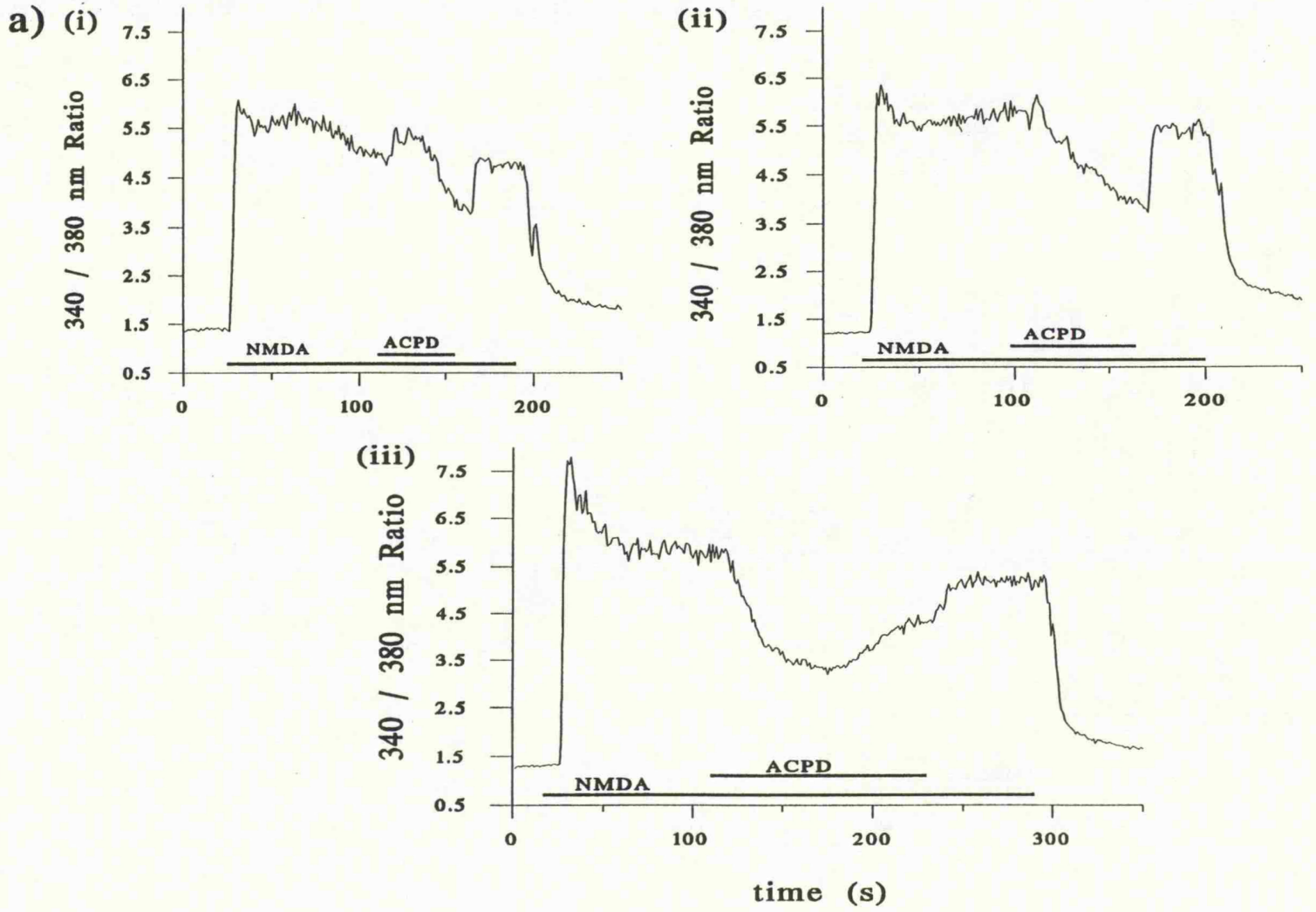
b) Pre-depolarization of cerebellar granule cells with 25 mM KCl for 1-2 min did not consistently increase the magnitude or consistency of Quis-evoked $[Ca^{2+}]_i$ elevations. While in some cases the Quis response after depolarization was much greater than the initial response ***(i)***, in others little change was seen (not shown) or a much smaller response found after depolarization ***(ii)***.

reported in granule cells using a very similar temporal protocol to that employed in the present study (Irving *et al.*, 1992a).

5.2 mGluR / NMDAR interactions

5.2.1 Effects of mGluR activation on NMDA-evoked response

To examine the possible interactions between $[Ca^{2+}]_i$ elevations evoked by NMDA and agonists of mGluRs in granule cells, ACPD (50-200 μM) or Quis (10 μM) was added during the plateau phase of the NMDA-evoked $[Ca^{2+}]_i$ response. As shown in Fig. 5.4, a variety of effects on the NMDA-evoked elevation were produced by metabotropic agonists using this experimental protocol. In some cases ACPD or Quis evoked peak $[Ca^{2+}]_i$ elevations, such as described above for perfusion of these agonists under control conditions, on top of the NMDA-evoked response (ACPD peak elevation = 0.38 ± 0.05 (n=14); Quis peak = 0.75 ± 0.15 (n=17))(Fig. 5.4a(i),(ii))(Table 5.1). In other experiments, addition of these metabotropic agonists caused a marked though variable inhibition of the NMDA $[Ca^{2+}]_i$ plateau, the average effects of the two agonists being very similar (ACPD-evoked inhibition = 39 ± 7% of NMDA response (10 out of 23 experiments); Quis-evoked inhibition = 49 ± 7% (13 out of 24 experiments)(Fig. 5.4a,b)(Table 5.1). These responses were not mutually exclusive, as in some experiments an mGluR agonist-evoked peak elevation was followed by an inhibition of the NMDA response (ACPD $[Ca^{2+}]_i$ elevation only in 11, inhibition only in 7, elevation followed by inhibition in 3, elevation then cells lost in 1, no ACPD effect in 2, out of 24 experiments; Quis $[Ca^{2+}]_i$ elevation only in 10, inhibition only in 7, elevation followed by inhibition in 6, elevation then cells lost in 1, no Quis effect in 1, out of 25 experiments)(Fig. 5.4a(i),(ii)). Inhibition of the NMDA-evoked response due to metabotropic stimulation was not consistently a gradual decline, as shown in Fig. 5.4a(i),(ii): on other occasions it rapidly reached its maximal effect, and then remained steady or returned towards NMDA plateau level during


a)
(i)
(ii)
(iii)
340 / 380 nm Ratio
7.5
6.5
5.5
4.5
3.5
2.5
1.5
0.5
NMDA
ACPD
0
100
200
300
time (s)

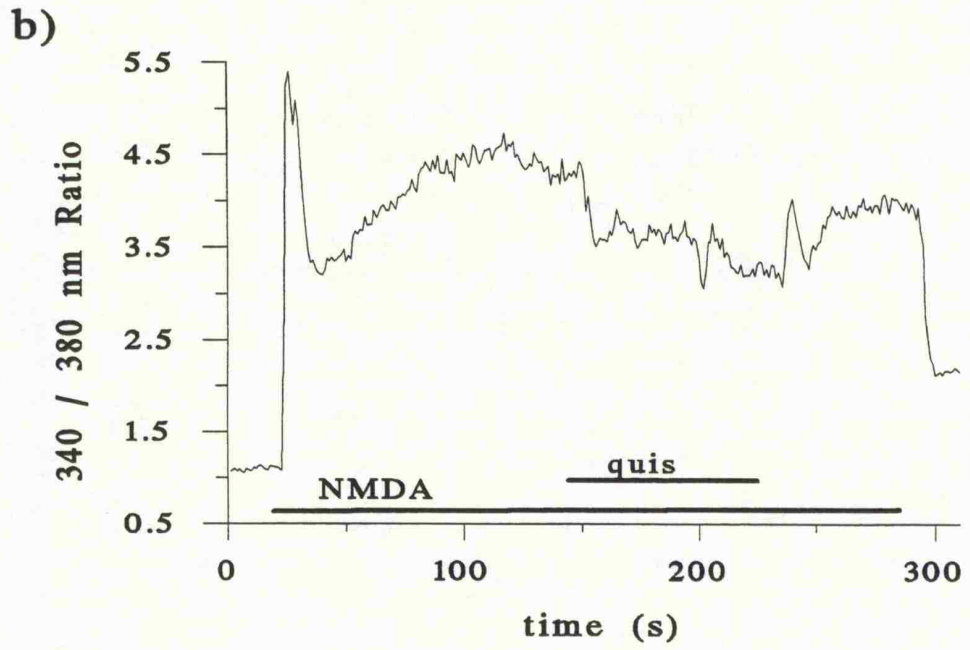


Fig. 5.4 Effects of mGluR agonists on the $[Ca^{2+}]_i$ response to NMDA.

a) Example traces of experiments performed on 3 groups of cells from the same coverslip. ACPD (200 μM), added after the plateau phase of the response to NMDA was established evoked a peak response in some experiments ***(i,ii)***, sometimes followed by a plateau elevation ***(i)***. However in many experiments it also evoked a marked inhibition of the NMDA plateau ***(i,ii,iii)***, which was completely and rapidly reversible when perfusion of the metabotropic agonist was ceased.

b) A rapid-onset, uneven and reversible inhibition of the response to NMDA was also evoked by Quis (10 μM) in some experiments, although peak and plateau elevations on top of the NMDA plateau were evoked in some cases (not shown).

continued perfusion of the metabotropic agonist (Fig. 5.4a(iii),b). The inhibition was abolished when perfusion of the mGluR agonist was terminated (Fig. 5.4a,b), the NMDA plateau subsequently recovering to a level not significantly different to that found before addition of the metabotropic stimulus.

Agonist	$[Ca^{2+}]_i$ increase over basal	$[Ca^{2+}]_i$ increase on top of NMDA plateau	% of groups displaying increase over NMDA	% decrease in NMDA response	% of groups displaying NMDA inhibition
ACPD	0.37 ± 0.09	0.38 ± 0.05	63	39 ± 7	43
ACPD in glycine-free	-----	0.39 ± 0.09	65	45 ± 8	50
Quis	0.60 ± 0.08	0.75 ± 0.15	68	49 ± 7	54

Table 5.1 Effects of mGluR agonists on $[Ca^{2+}]_i$ and on NMDA-evoked responses. The $[Ca^{2+}]_i$ elevations evoked by ACPD or Quis were not significantly different in the presence or absence of NMDA. ACPD and Quis caused similar, marked inhibitions of NMDA plateau responses in a similar proportion of experiments. This was unaffected by the presence or absence of exogenous glycine.

5.2.1 Effects of NMDAR activation on mGluR-evoked response

Co-incubation with NMDA potentiates PI turnover evoked by metabotropic agonists in cerebellar granule cells (Chapter 6). However, there appears to be no consequent effect of NMDA on the $[Ca^{2+}]_i$ elevations evoked by such agonists. In cells in which responses to ACPD or Quis were evoked before and during NMDA stimulation, no marked difference in the metabotropic response was found during the NMDA-evoked elevation, in those experiments where a mGluR-mediated peak during the NMDA response was seen, compared to the initial metabotropic challenge (Table 5.1). This may well be because the $InsP_3$ production induced by mGluR agonists without co-addition of NMDA is sufficient to evoke maximal Ca^{2+} release from $InsP_3$-sensitive stores, consistent with the reports in other preparations which indicate that the EC_{50} for $[Ca^{2+}]_i$ elevation by PI-linked agonists is markedly lower than for the EC_{50} for $InsP_3$ production by the same agonists (Thompson *et al.*, 1991; G. Willars and S. Nahorski, in preparation). However, depolarization has been reported to potentiate $[Ca^{2+}]_i$ responses concurrently evoked by CCh in cerebellar granule cells (del Rio *et al.*, 1994). It is clear that the variability in mGluR responsiveness from granule cell groups over time described above would make any change in mGluR agonist-evoked $[Ca^{2+}]_i$ response size due to co-incubation with NMDA difficult to detect. Also, the relationship between 340 / 380 nm ratio and $[Ca^{2+}]_i$ may not closely approximate linearity for ACPD or Quis responses obtained on top of large NMDA plateaus (see Fig. 4.8), making comparison using ratios only of mGluR responses on top of NMDA stimulations with responses in otherwise unstimulated cells problematical.

5.3 Comparison with PKC-mediated effects on NMDA response

A number of studies have indicated that consensus sequences for PKC and CamK-II are present on NMDARs (Moriyoshi *et al.*, 1991; Durand *et al.*, 1993; Kitamura *et al.*, 1993). Indeed, NMDAR phosphorylation may play an important role in LTP (see Roche *et al.*, 1994). There are at least eight known isoforms of PKC, all of which, except for the constitutively active ζ isoform, are activated by a range of effectors which include DAG, and of which three, PKCα, β and γ, are Ca^{2+} dependent (see Hug and Sarre, 1993). Many receptors can thus cause upregulation of the activity of PKC, including receptors coupled to PIC and consequent production of DAG. Important influences on NMDAR-mediated responses induced by PI-linked receptors have been reported to be mediated by activation of kinases (Chen and Huang, 1992; Kelso et al, 1992; Courtney and Nicholls, 1992; Aniksztejn *et al.*, 1992; Lopez-Molina *et al.*, 1993; Snell *et al.*, 1994; Wang and Salter, 1994). However, the nature of the NMDAR modulation has been the subject of apparently contradictory reports. In a number of studies, upregulation of the activity of PKC (Chen and Huang, 1992; Kelso et al, 1992; Aniksztejn *et al.*, 1992; Durand *et al.*, 1993; Kitamura *et al.*, 1993) or inhibition of phosphatases (Wang *et al.*, 1994b; Wang and Salter, 1994; Lieberman and Mody, 1994) has been shown to increase activity of NMDARs, while inhibition or downregulation of PKC was found to decrease NMDAR currents (Favaron *et al.*, 1990; Lopez-Molina *et al.*, 1993). At least some of the upregulation of NMDAR activity has been ascribed to PKC phosphorylation of an NMDA channel domain (Kitamura *et al.*, 1993). In contrast, other reports have demonstrated an apparent inhibitory action of PKC-activating agents, and of metabotropic agonists, on NMDA-evoked $[Ca^{2+}]_i$ responses (Courtney and Nicholls, 1992; Snell *et al.*, 1994). The possible involvement of PKC in mGluR-mediated modulation of NMDA responses in cerebellar granule cells was therefore investigated, using agents which either upregulate or inhibit PKC activity.

5.3.1 Expression of PKC isozymes in cultured granule cells

All isoforms of PKC have been identified in brain tissue, but their expression pattern in particular neuronal types remains less well defined (see Hug and Sarre, 1993). Initially, then, the presence of PKC isozymes in cerebellar granule cells was investigated in granule cell membranes, using isozyme-specific Abs. Western blotting demonstrated the presence of a single band recognized by a monoclonal PKCα Ab, of approximately the expected MW (~90 kDa) for this subtype, whereas no detectable levels of β,γ or δ were found (Fig. 5.5). This is not necessarily inconsistent with previous reports which suggested that PKCβ is present in cultured (Favaron *et al.*, 1990) and *in situ* (Huang *et al.*, 1988) cerebellar granule cells, as it is possible that PKC isozymes present predominantly in the cytosolic fraction would not have been detected using this protocol. It also remains possible that isozymes other than the 4 investigated here are also present, either in granule cell cytosol or in membranes.

5.3.2 Effects of PKC modulators on NMDA-evoked response

To investigate the possible involvement of PKC in the mGluR-mediated modulation of NMDA responses detailed above, the effects of ACPD and Quis were compared to those of a phorbol ester, PMA, which is known to activate PKC (see Wilkinson and Hallam, 1994). Phorbol esters are thought to mimic the mechanism of activation of DAG even in the absence of Ca^{2+} and, because of their persistence in the plasma membrane, can evoke prolonged stimulation (see Hug and Sarre, 1993). The effect of 100 nM PMA on NMDA-evoked responses was found to be variable: in some experiments PMA evoked a marked decrease in NMDA-evoked $[Ca^{2+}]_i$ plateau elevation, comparable to the inhibition evoked by mGluR agonists in many experiments (36 ± 5% PMA inhibition of NMDA response (n=13))(Fig. 5.6(i,iii))(cf Fig. 5.4a,b). This effect was largely reversed on removal of PMA. In contrast, however, in many

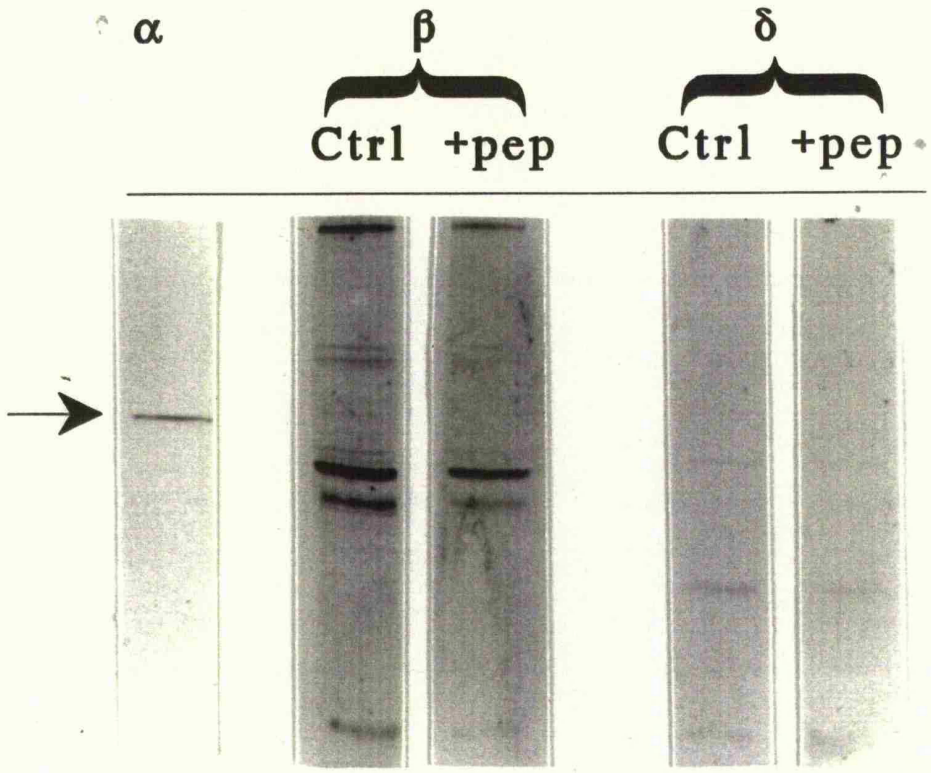


Fig. 5.5 Presence of PKC isozymes in cerebellar granule cells. Western blot of granule cell membranes with specific Abs for several PKC isozymes. A single band was detected using a monoclonal PKCα Ab, whereas all bands detected using polyclonal β or δ Abs (Ctrl) were still present when Ab and peptide were incubated together with the nitrocellulose (+pep), indicative of non-specific binding to non-PKC proteins. Thus, only PKCα can be identified in granule cell membranes. No specific band was detected for PKCγ either (not shown).

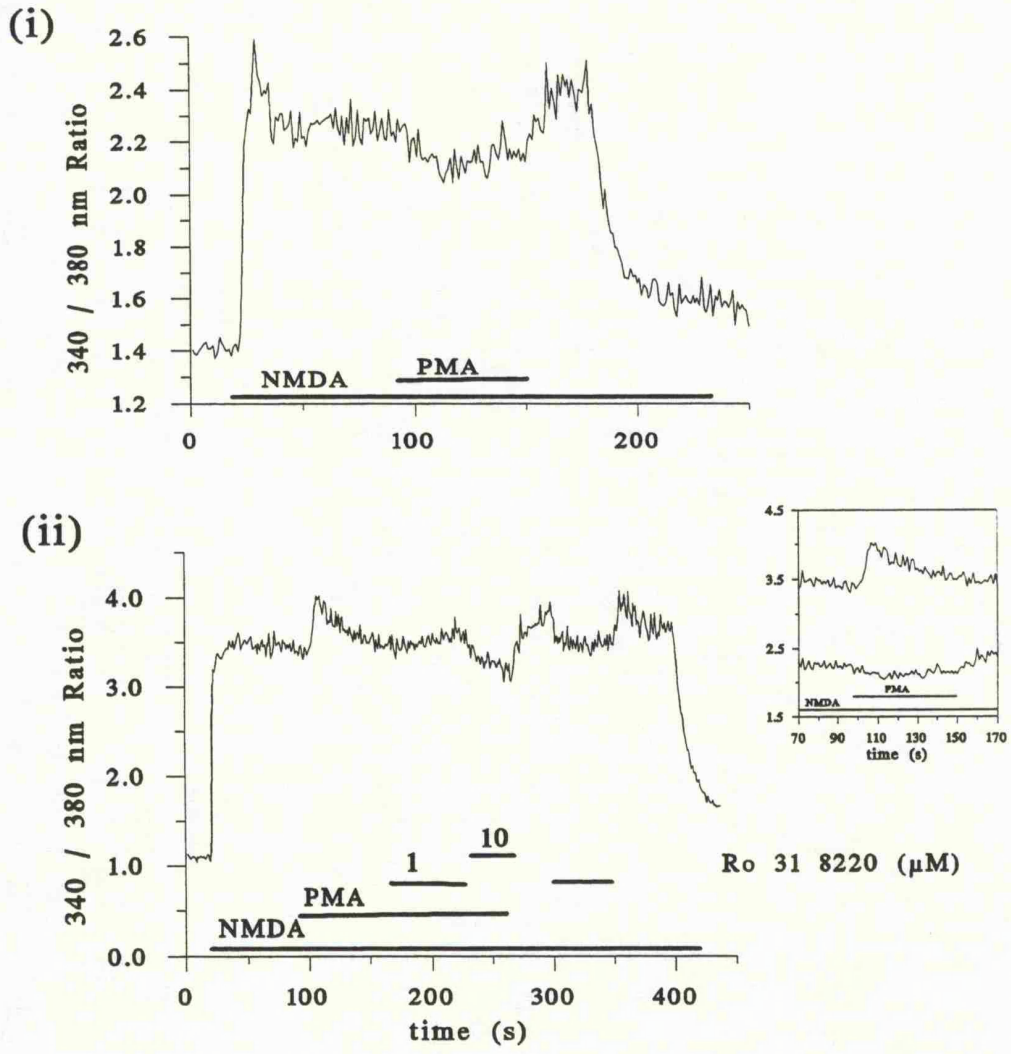


Fig. 5.6 Effects of PMA and Ro 31 8220 on NMDA responses.

Example traces of responses to 200 μM NMDA and the variable modification of this response by 100 nM PMA and 1-10 μM Ro 31 8220. In ***(i)***, PMA caused a rapid-onset decrease in the NMDA plateau elevation, similar to the effect of mGluR agonists in many experiments (cf Fig. 5.4a,b). This effect was largely reversed on removal of PMA. In ***(ii)***, however, PMA evoked a marked elevation of the NMDA plateau, the contrast with the previous response being highlighted in the inset to this figure. The effect of PMA was abolished by 10 μM Ro 31 8220 in this experiment, while1 μM Ro 31 8220 by itself caused an inhibition of the NMDA response similar to the effect of PMA in ***(i)***.

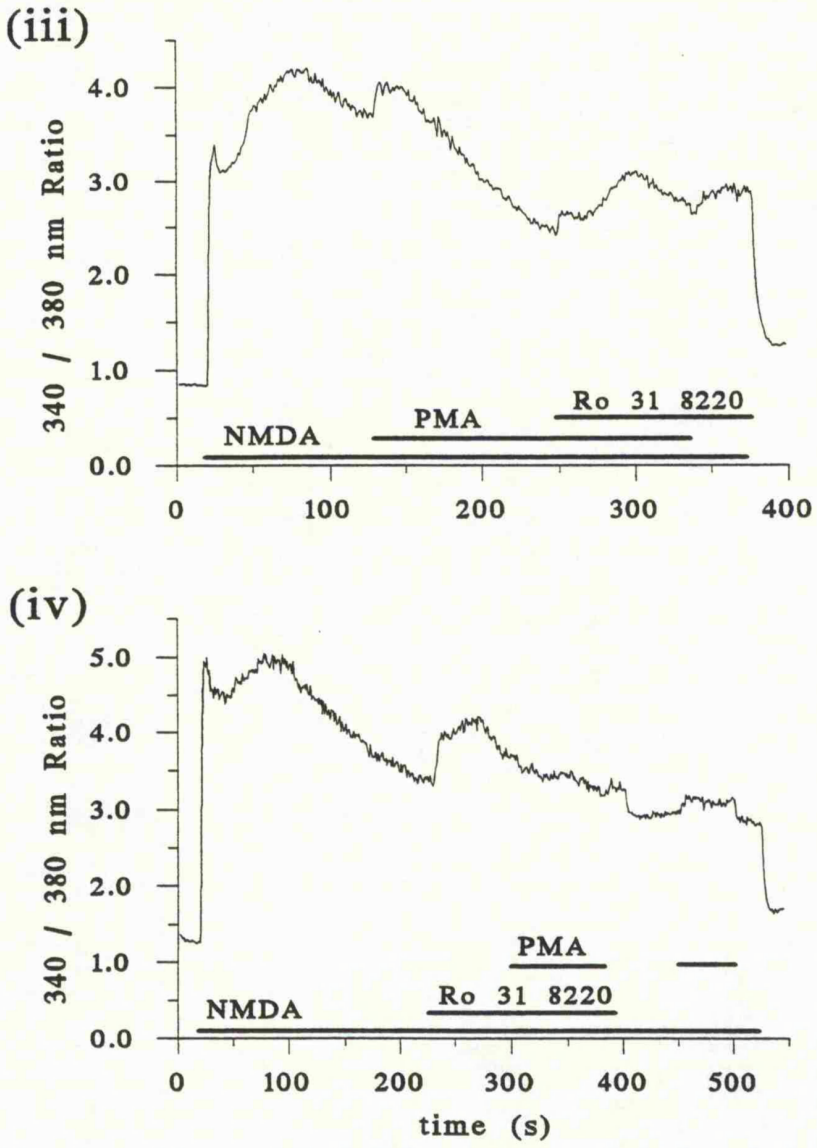


Fig. 5.6 (cont.) In ***(iii)***, PMA evoked a moderate increase in the NMDA plateau, followed by an apparent inhibition of the response, although the slow onset of this makes it difficult to distinguish from a possible time-dependent decline in the NMDA plateau. In this example, the PMA-induced decrease is partially abolished by co-incubation with 1 μM Ro 31 8220. In ***(iv)***, the ability of 1 μM Ro 31 8220 to cause an increase in the NMDA plateau is demonstrated. 100 nM PMA had little or no effect during the presence of the PKC inhibitor, but caused a small potentiation of the NMDA response by itself after removal of Ro 31 8220.

experiments PMA caused a moderate but significant increase in the NMDA response (340 / 380 nm ratio elevation = 0.32 ± 0.04, corresponding to 25 ± 4% increase over NMDA plateau (n=18))(Fig. 5.6(ii,iv)). In a small number of experiments, PMA evoked a significant, transient elevation on top of the NMDA plateau, followed by an inhibition of the NMDA response (Fig. 5.6(iv))(PMA $[Ca^{2+}]_i$ elevation only in 13, inhibition only in 8, elevation followed by inhibition in 5, elevation then cells lost in 1, no PMA effect in 4, out of 31 experiments).

To clarify whether these contradictory stimulatory and inhibitory effects of PMA were both PKC-linked, a further series of experiments was performed using the PKC inhibitor Ro 31 8220, known to act potently on PKCα as well as on other isozymes (see Wilkinson and Hallam, 1994). By itself, Ro 31 8220 caused either increase (340 / 380 nm ratio elevation = 0.28 ± 0.07, corresponding to 21 ± 5% increase over NMDA plateau (n=10)) or decrease (21 ± 3% inhibition (n=4)) in NMDA-evoked responses (Fig. 5.6(ii,iv)), or both a transient elevation followed by inhibition (Ro 31 8220 $[Ca^{2+}]_i$ elevation only in 7, inhibition only in 1, elevation followed by inhibition in 3, out of 11 experiments), thus paralleling rather than contrasting with the effects of PMA. The potentiatory and inhibitory effects of PMA were partially or completely reversed by removal of PMA or addition of Ro 31 8220 in many, although not all, experiments (Fig. 5.6(ii,iii,iv)), while the effects of Ro 31 8220 were decreased upon its removal (Fig. 5.6(ii,iv)). However, it was not always possible to dissociate with certainty the direct $[Ca^{2+}]_i$-elevating or -inhibiting effects of Ro 31 8220 alone from its reversal of PMA-evoked PKC activation. The failure of Ro 31 8220 pre-treatment to abolish the inhibitory effects of PMA, or of ACPD, on NMDA responses in some experiments remains to be fully explained. It would have been interesting to examine the possibility of additivity of the effects of PMA and mGluR agonists in inhibiting NMDA responses. However, it has recently been reported that acute pre-treatment with PMA markedly reduces glutamate or Quis-evoked $InsP_X$

production in cerebellar granule cells, apparently via PKC phosphorylation of mGluRs or an associated protein (Paulsen *et al.*, 1994), which would wholly compromise the interpretation of results from such experiments.

It has previously been suggested that PKC activation causes an inhibition of NMDAR activity in granule cells, which is due to an alteration in agonist affinity, and is highly dependent on the level of glycine present (Snell *et al.*, 1994). Identical experiments were performed with ACPD and NMDA as shown above (Fig. 5.4a), but without the inclusion of exogenous glycine. It has been shown in the present study that glycine endogenously released into the experimental medium is insufficient to cause saturation of its site on the NMDAR (Chapter 4). In the absence of exogenous glycine, a similarly mixed pattern of potentiatory and inhibitory effects of ACPD on the NMDA response was found as under normal, saturating glycine, conditions. The ACPD-evoked inhibition of the NMDA-evoked response was of frequency and magnitude not significantly different to that detailed above for ACPD on NMDA responses in the presence of 10 μM glycine (ACPD peak 0.39 ± 0.09 (n=12), ACPD-evoked inhibition 45 ± 8% (n=9))(Table 5.1)(ACPD $[Ca^{2+}]_i$ elevation only in 8, inhibition only in 6, elevation followed by inhibition in 2, elevation then cells lost in 1, out of 17 experiments).

5.4 Mechanisms of CCh-evoked $[Ca^{2+}]_i$ elevation

Further experiments were performed to characterize the mechanisms involved in metabotropic agonist-evoked $[Ca^{2+}]_i$ elevations in cultured granule cells. Given its greater consistency of response over time than mGluR agonists, CCh was adopted as the agent of choice for these studies, which necessitated repeated stimulations. The possible involvement of Ca^{2+} entry and Ca^{2+} release from stores in the CCh-evoked $[Ca^{2+}]_i$ elevation was investigated, and the nature of the Ca^{2+} store release

mechanisms activated by CCh was examined in detail, using several different store modulatory agents and experimental protocols.

5.4.1 Effects of store modulators on response

The CCh-evoked $[Ca^{2+}]_i$ response was in many cases abolished by pre-incubation with thapsigargin (10 μM) for 5-10 min (4 out of 8 experiments), consistent with this response being due to Ca^{2+} release from intracellular stores, presumably triggered by formation of the second messenger $InsP_3$ (see Chapter 6). As Ca^{2+} release from stores may activate CICR via $InsP_3Rs$ and RyRs, it appeared possible that a component of the response to CCh could be due to Ca^{2+} release via RyRs as well as $InsP_3Rs$. This was examined using the agents employed in Chapters 3 and 4 to inhibit RyR function, dantrolene and ryanodine. Pre-incubation with ryanodine (10 μM) suppressed the CCh-evoked response (response abolished in 4 experiments, reduced in 1, unaffected in 3)(Fig. 5.7a), as to a similar extent did dantrolene (25 μM)(Fig 5.7b)(response abolished in 2 experiments, reduced in 1, unaffected in 1).

In a further series of experiments, a different protocol was used in order to assess the possible involvement of RyR-expressing stores in CCh-evoked responses. In PC12 cells, Zacchetti *et al.* (1991) showed that prior treatment with either the PI-linked agonist bradykinin or caffeine inhibited subsequent responses to the other, to a degree closely related to the amplitude of the response to the initial agent. Using a similar protocol, granule cells were challenged with CCh (1 mM) and caffeine (50 mM), with a brief (30 s) interval between perfusion of the two agents, and the ability of challenge with one agent to occlude a subsequent response to the other was assessed. Pre-treatment with either CCh or caffeine typically evoked a marked and easily detectable response when perfused first in these studies, but a much reduced caffeine response was detectable after prior stimulation with CCh (Fig. 5.8a)(caffeine

response after CCh challenge = 27 ± 6% of control). An initial challenge with caffeine reduced the magnitude of subsequent responses to CCh (Fig. 5.8b(i,ii)), although the CCh response remained of significant size (CCh response after caffeine challenge = 45 ± 19% of control). In a further series of experiments, caffeine challenges were repeated until no response to caffeine could be detected, and CCh was then perfused over the cells. Using this protocol, a small CCh response was still evoked (Fig. 5.8b(ii),c)(CCh response after caffeine response abolished = 31% of control (n=2 series of experiments)). Prior challenge with CCh, or repeated challenges with caffeine, caused an additional, unexpected effect on subsequent responses to caffeine: in the absence of a large peak $[Ca^{2+}]_i$ elevation, a rapid-onset decrease in $[Ca^{2+}]_i$ was often revealed (Fig. 5.8a,b(ii)). Indeed, in some experiments, addition of caffeine to naive cells also evoked a decrease in the basal 340 / 380 nm ratio detectable both before and after the transient $[Ca^{2+}]_i$ elevation (bottom trace, Fig. 5.8b(i)).

Caution must be exercised in the interpretation of such results, as caffeine inhibits $InsP_3$-evoked Ca^{2+} transients in permeabilized hepatocytes, which are thought to lack RyRs, leading to the suggestion that caffeine was acting in that study by direct inhibition of $InsP_3Rs$ (Missiaen *et al.*, 1992). A similar, non-RyR-specific action has been suggested to contribute to caffeine-evoked inhibition of ACh and ACPD responses in granule cells, though apparently not to a major extent (Irving *et al.*, 1992b). In the present study, moderate responses were often evoked by the second agent added, either CCh or caffeine, but subsequent re-challenge with the initial agent after the second response often also evoked a peak in those experiments, indicative of a relative resistance to depletion of the Ca^{2+} pool in some granule cells (Fig. 5.8c). Indeed, in the experiments described above in which caffeine responses were repeated until no response remained, this protocol required 2-6 caffeine challenges depending on the cell group being studied. These experiments, interesting by themselves in terms of granule cell heterogeneity, also provide evidence that the inhibitory effect of caffeine

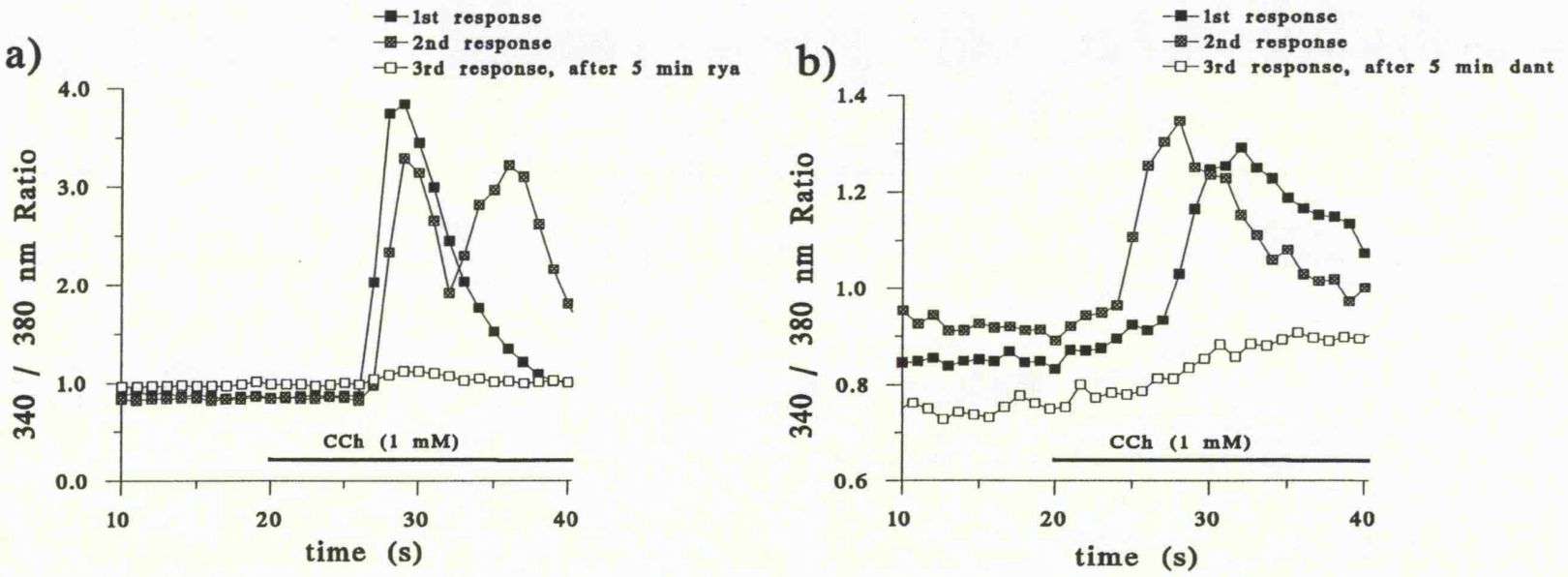


Fig. 5.7 Effects of RyR modulating agents on CCh-evoked $[Ca^{2+}]_i$ responses.

a) Example traces of successive responses to CCh (1 mM) at 5 min intervals from a single group of cells. Traces have been shifted vertically for ease of comparison. The CCh response in some granule cell groups was highly reproducible using a 5 min interval stimulation protocol. Pre-incubation with ryanodine (10 μM) markedly inhibited the CCh-evoked $[Ca^{2+}]_i$ peak.

b) Example traces of successive responses to CCh (1 mM) at 5 min intervals from a single group of cells. Traces have been shifted vertically for ease of comparison. The large response to CCh (1 mM) in this group was again highly reproducible using a 5 min interval stimulation protocol. Pre-incubation with dantrolene (25 μM) inhibited the CCh-evoked $[Ca^{2+}]_i$ peak, an effect which was at least partially removed by 10 min wash-out (not shown).

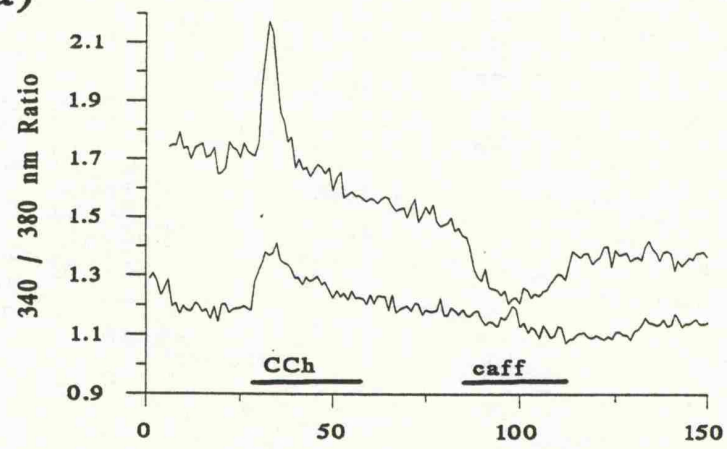
on the CCh response is unlikely to be due to an effect at the level of $InsP_3R$ activation, as the CCh response was typically not greatly inhibited by caffeine only when the RyR-mediated caffeine $[Ca^{2+}]_i$ response remained partially intact.

Taken together, the inhibitory effect of caffeine stimulation, and of pre-treatment with ryanodine or dantrolene, on CCh-evoked $[Ca^{2+}]_i$ elevations, suggest that Ca^{2+} stores expressing RyRs, and indeed CICR via these receptors, may be involved in M_3 muscarinic receptor-mediated $[Ca^{2+}]_i$ elevation. This CICR may be triggered not by Ca^{2+} entry across the plasma membrane (see below), but by Ca^{2+} release from an $InsP_3$-sensitive store. Thus, caffeine depletes the stores responsible for most though not all of the CCh-evoked response, and dantrolene and ryanodine inhibit the main mechanism of CCh-evoked Ca^{2+} release. An alternative, though perhaps less likely, explanation is that CCh-evoked $InsP_3$ production can release Ca^{2+} by IICR from stores which also express RyRs, such that caffeine can deplete the same stores as CCh activates, and that dantrolene and ryanodine inhibit CCh responses due to an interaction between RyRs and $InsP_3Rs$ which controls $InsP_3R$-mediated Ca^{2+} release.

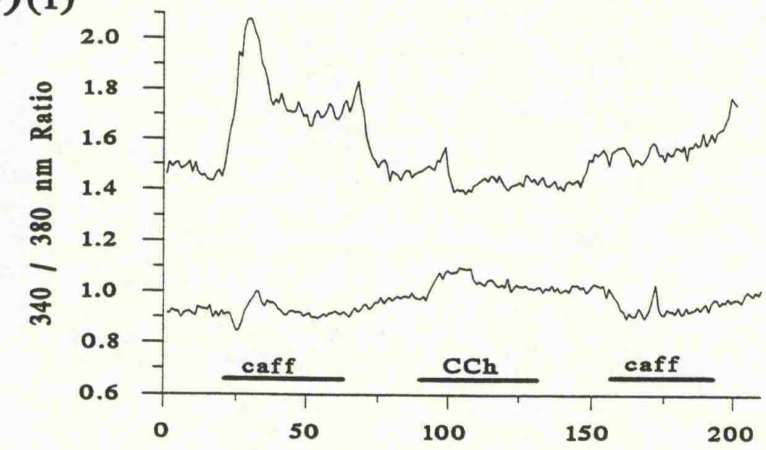
5.4.2 Effect of CCh on Mn^{2+} quench

The possibility that a component of the CCh-evoked $[Ca^{2+}]_i$ response was due to Ca^{2+} entry was examined using the Mn^{2+} quench technique. 1 mM CCh did not significantly alter the rate of quench compared to control (control $t_{1/2} = 63.9 \pm 26.5$ s, CCh $t_{1/2} = 68.1 \pm 14.3$ s)(Fig 5.8). This agrees well with the above data implicating store release as the major mechanism involved in the response to CCh, and suggests that in granule cells, CCh does not significantly alter membrane permeability to divalent cations either by directly evoking entry, or by evoking entry secondary to store depletion. However, the possibility of capacitative Ca^{2+} entry due to store depletion occurring due to CCh challenge via Mn^{2+}-impermeant channels, or at too

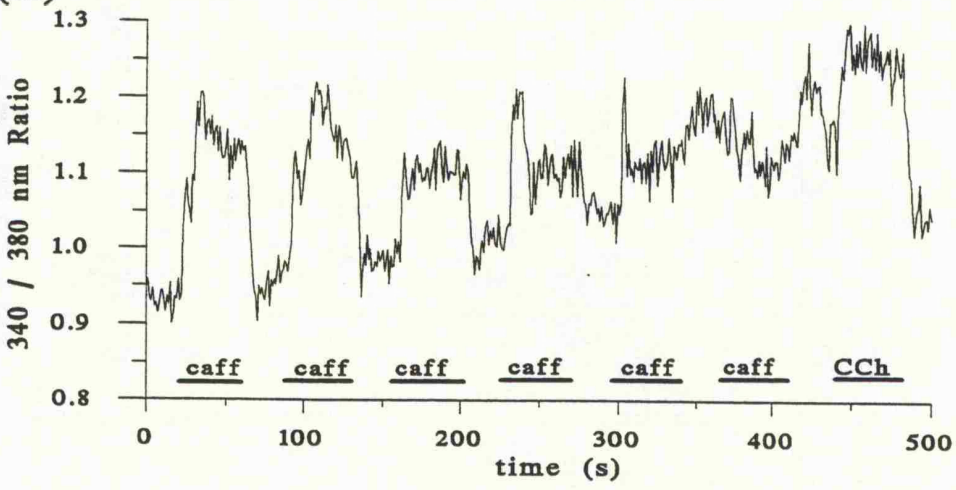
a)


340 / 380 nm Ratio
2.1
1.9
1.7
1.5
1.3
1.1
0.9
CCh
caff
0
50
100
150

b)(i)


340 / 380 nm Ratio
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
caff
CCh
caff
0
50
100
150
200

(ii)


340 / 380 nm Ratio
1.3
1.2
1.1
1.0
0.9
0.8
caff
caff
caff
caff
caff
caff
CCh
0
100
200
300
400
500
time (s)

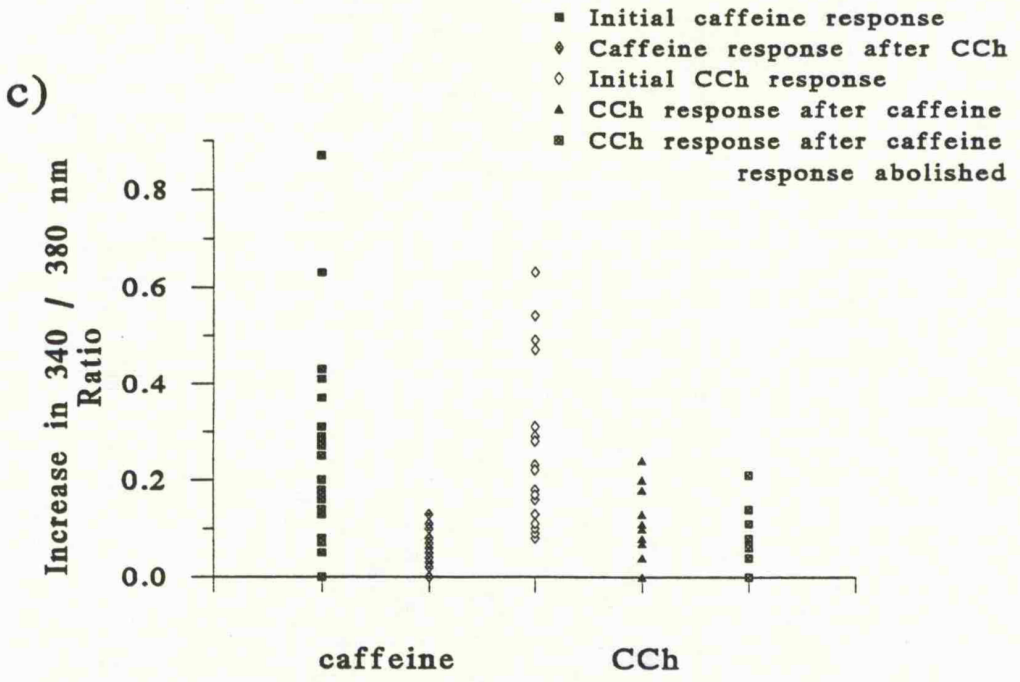


Fig. 5.8 Effect of CCh or caffeine pre-stimulation on subsequent responses to each other. Example traces typical of results obtained from at least 3 independent series of experiments.

a) CCh (1 mM) evokes a large peak response, whereas addition of caffeine (50 mM) 30 s after removal of CCh evokes virtually no response.

b) Caffeine (50 mM) evokes a moderate or large peak response, whereas addition of CCh (1 mM) 30 s after removal of caffeine evokes either no response, or a response reduced compared to control (cf ***a***).

c) Scatter graph showing the results of all experiments performed. Caffeine responses after pre-stimulation with CCh are markedly reduced compared to responses in naive cells; CCh responses are reduced by caffeine pre-stimulation, but to a lesser degree. CCh still evoked a residual response after the caffeine response had been completely abolished due to repeated caffeine challenge at 30 s intervals.

low a rate to be detected on top of the high basal quench, cannot be completely excluded (see also Chapters 3 and 4).

5.5 Do CCh and NMDA access the same Ca^{2+} stores?

The co-existence of more than one isoform of store Ca^{2+}ATPase in the same cell has been demonstrated within a variety of preparations (see Pozzan *et al.*, 1994); for instance, adrenal chromaffin cells have been reported to possess distinct caffeine-sensitive and $InsP_3$-sensitive stores which apparently correlate with the distribution of two Ca^{2+}ATPases with differing MWs (Burgoyne *et al.*, 1989). Two Ca^{2+}ATPases have also been identified in platelets (Papp *et al.*, 1991). It has been demonstrated in platelets that low concentrations of thapsigargin inhibit one isoform of Ca^{2+}ATPase, and higher concentrations of thapsigargin (Thastrup *et al.*, 1990) inhibit both isoforms present, whereas the structurally unrelated Ca^{2+}ATPase inhibitor BHQ (Moore *et al.*, 1987) affects only the relatively thapsigargin-insensitive form (Papp *et al.*, 1991,1993). In platelet membrane vesicles BHQ preferentially inhibits Ca^{2+} sequestration into $InsP_3$-sensitive stores, and low thapsigargin preferentially inhibits sequestration into $InsP_3$-insensitive stores (Papp *et al.*, 1993), so different Ca^{2+}ATPases may label the different pools, a low thapsigargin-sensitive Ca^{2+}ATPase being found on IICR pools, and a BHQ-sensitive (and high thapsigargin-sensitive) Ca^{2+}ATPase being found only on the CICR pool. The possibility of an analogous discriminatory action of thapsigargin and BHQ on different Ca^{2+} pools in neurones was suggested by the finding that not all $^{45}Ca^{2+}$ loaded into brain microsome Ca^{2+} stores is thapsigargin-sensitive (Verma *et al.*, 1990), suggesting that more than one type of Ca^{2+}ATPase may be present on brain Ca^{2+} stores. Also, BHQ but not thapsigargin has been found to inhibit NMDA responses in hypothalamic neurones (Dayanithi *et al.*, 1993; G. Dayanithi, personal communication). A Ca^{2+} store in at least one type of central neurone has been found to be BHQ-insensitive (Lambert *et al.*, 1994). Experiments

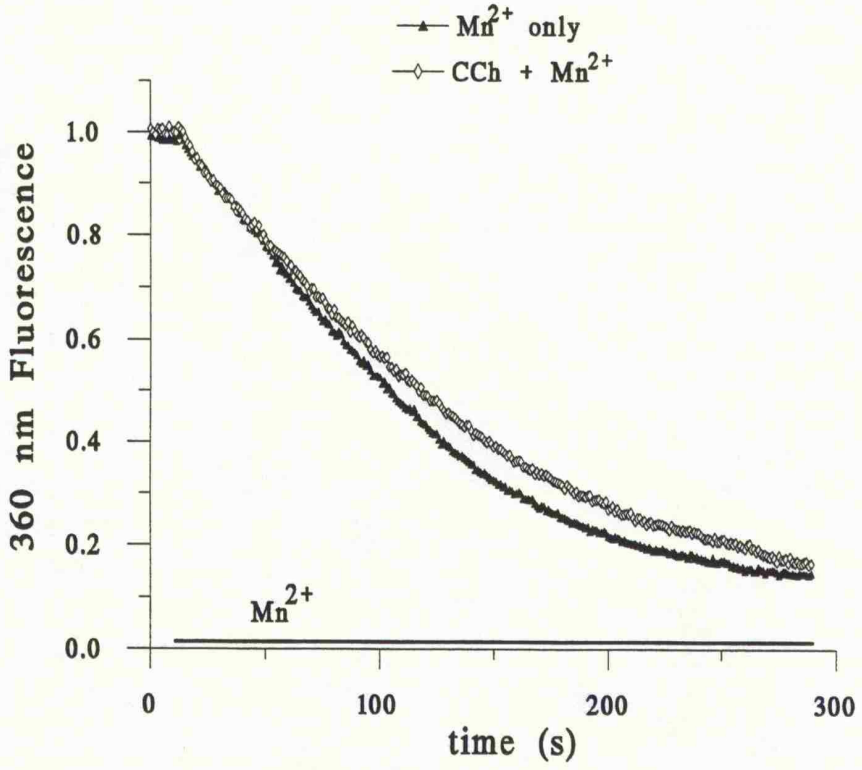


Fig. 5.9 Lack of stimulation of Mn^{2+} quench by CCh. Example traces from experiments representative of 3 series performed on separate granule cell preparations. There is no significant difference between the control rate of Mn^{2+} quench, and the rate of quench evoked by the simultaneous addition of Mn^{2+} with 1 mM CCh.

were therefore performed in cerebellar granule cells to examine if BHQ could differentiate between CICR- and IICR-sensitive pools, both processes having been found to be inhibited by thapsigargin (see above and Chapter 4).

In an initial study, Western blotting of granule cell membranes was performed with an Ab which recognizes both type 1 and type 2 Ca^{2+}ATPases. A single band was detected in granule cell membranes which co-localized with a clear band from skeletal muscle microsomes run in a parallel lane, probably corresponding to granule cell Ca^{2+}ATPase(s). This alone does not provide clear information as to the number of Ca^{2+}ATPase isoforms present, however, as in platelets the two isoforms present are of very similar MW (Papp *et al.*, 1991).

Subsequent experiments investigated the consequences of pre-incubation with BHQ on $[Ca^{2+}]_i$ responses previously found to be sensitive to thapsigargin pre-treatment. As for thapsigargin (see above), 5-10 min pre-incubation with BHQ (25 μM) inhibited $[Ca^{2+}]_i$ responses to CCh (1 mM)(Fig. 5.10a,b)(response abolished in 6 experiments, reduced in the other 3 experiments performed). However, in parallel studies BHQ pre-treatment was found to have no effect on at least the sustained phase of the NMDA-evoked $[Ca^{2+}]_i$ elevation (NMDA response after BHQ pre-treatment = 108 ± 8% of control response (n=24))(Fig. 5.10b,c). An apparent inhibition, or slowing of the rate of rise, of the NMDA peak response was seen in 12 out of 24 experiments, suggesting that a component of the NMDA-evoked peak may be inhibited by BHQ pre-treatment (Fig. 5.10c). Clearly, however, the CCh-evoked response is much more sensitive to BHQ inhibition than the NMDA-evoked response, and the sustained phase of the NMDA response is entirely BHQ-insensitive. An inhibitory effect of dantrolene (25 μM) was demonstrated on some of the NMDA responses unaffected by BHQ in these experiments, confirming that the lack of BHQ effect was not due to a lack of involvement of CICR (Fig. 5.10d). In this small series

of experiments, dantrolene was found to affect on the peak as well as the plateau of the NMDA-evoked response (~63% inhibition), an action not directly tested in the experiments reported in the previous Chapter. This is consistent with the inhibitory actions of thapsigargin and ryanodine on both phases of the NMDA response (Chapter 4).

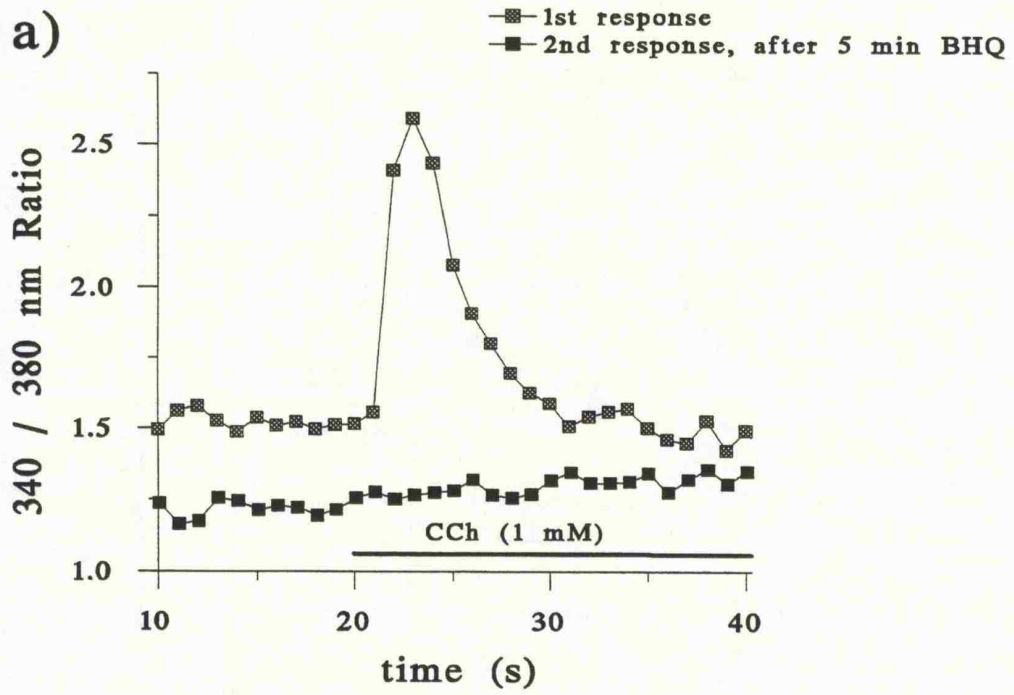
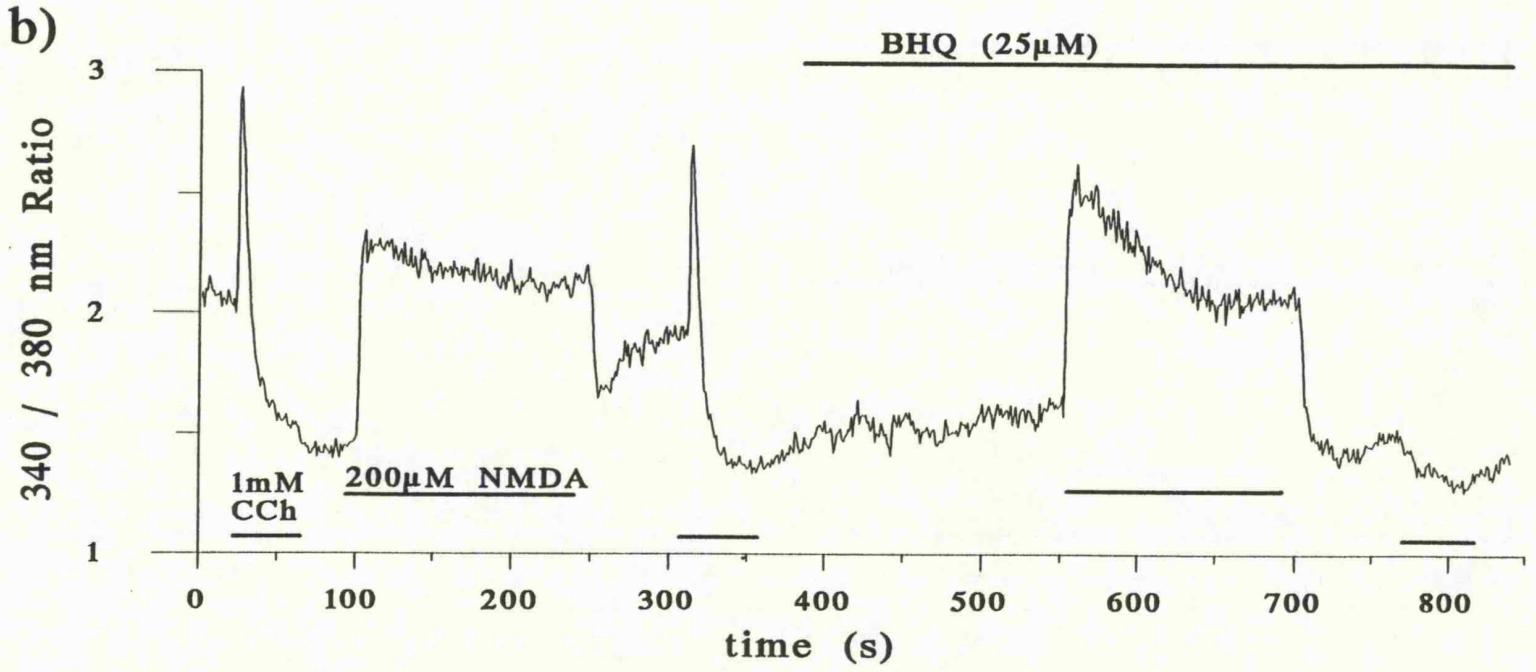
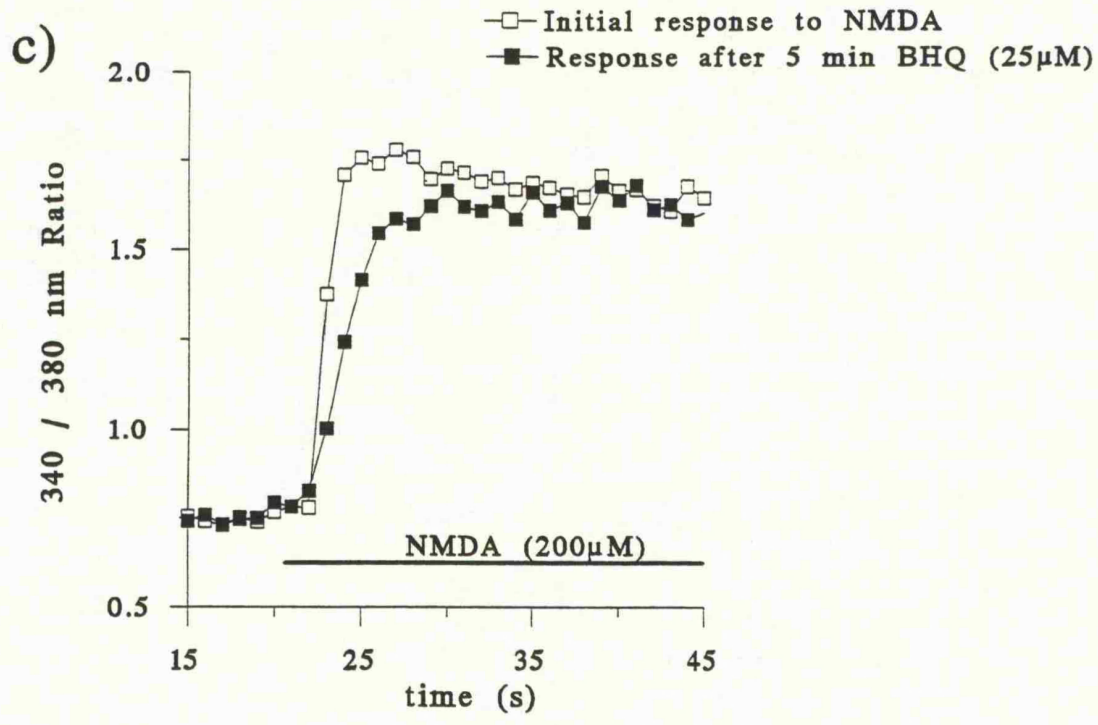


Fig. 5.10 Effect of BHQ on CCh- and NMDA-evoked $[Ca^{2+}]_i$ responses.

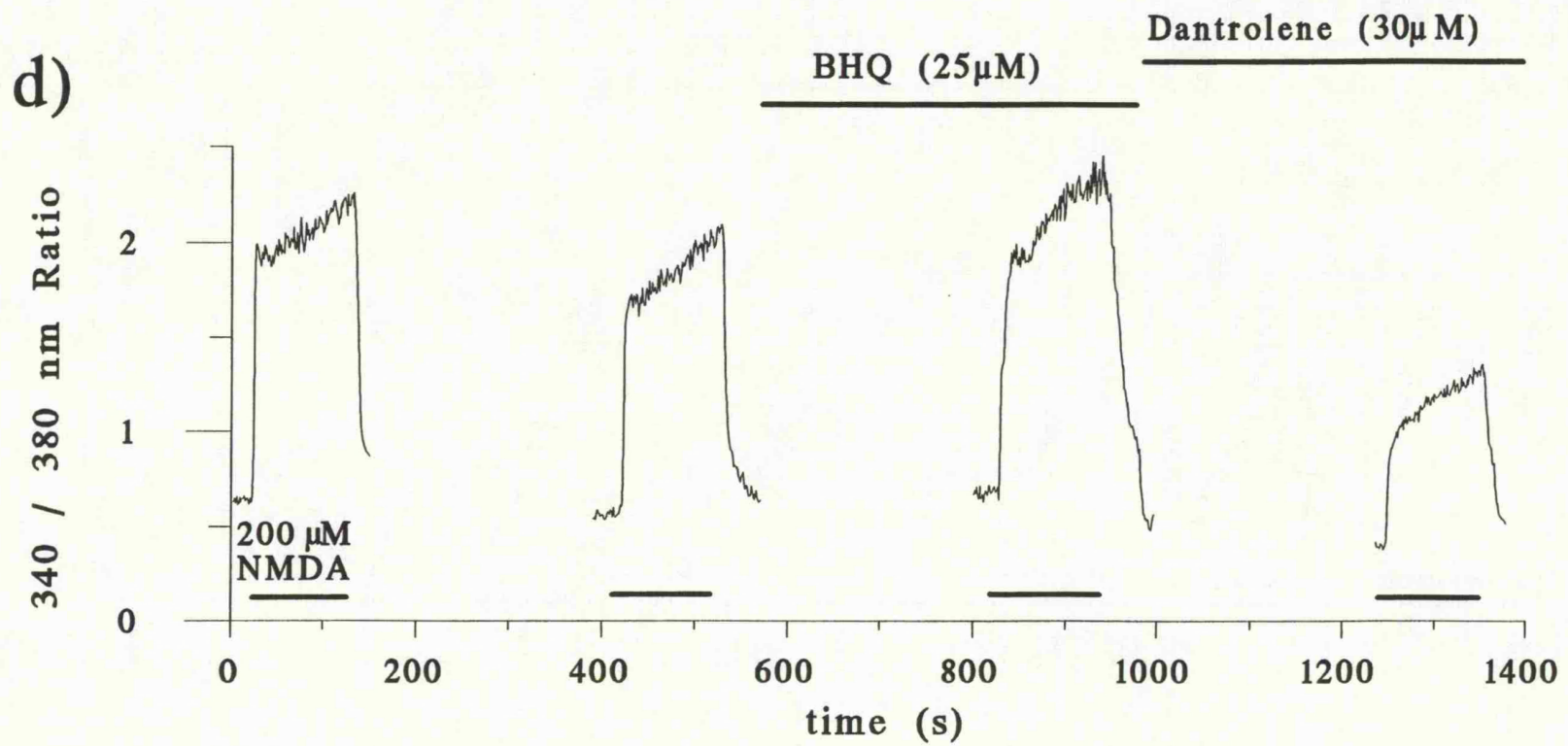
a) Example traces of successive responses to CCh (1 mM) at a 5 min interval from a single group of cells. The peak response evoked by CCh initially was not reproduced after incubation with BHQ (25 μM).



b) Example trace. The response to CCh (1 mM) is reproducible at a 5 min interval between challenges, regardless of the intervening NMDA (200 µM) response. The CCh response was abolished by incubation with BHQ (25 µM). The magnitudes of neither the peak nor the plateau phase of the NMDA response, however, were significantly affected by 5 min BHQ.



c) Example traces of successive responses to NMDA (200 μM) at a 5 min interval from a single group of cells. An effect of BHQ on the peak NMDA $[Ca^{2+}]_i$ response was found in 12 out of 24 experiments with differing cell groups. After incubation with BHQ (25 μM), the peak component of the NMDA response was inhibited, or temporally retarded such that it became indistinguishable from the plateau phase.



d) Example traces of successive responses to NMDA (200 µM) from a single group of cells. The magnitude of the NMDA response was reproducible at 5 min intervals and was not significantly affected by 5 min pretreatment with BHQ (25 µM). However, 5 min incubation with dantrolene (25 µM) markedly depressed both the peak and plateau phases of the response.

5.6 Discussion

I

The results presented in this Chapter have demonstrated that a rapid-onset $[Ca^{2+}]_i$ elevation is evoked by the muscarinic receptor agonist CCh, and the mGluR agonists ACPD and Quis, in cerebellar granule cells. The responses of different groups of granule cells to these agonists varied greatly. However, the finding that these agonists evoked a response in virtually all groups tested indicates that at least some degree of mGluR and M_3 muscarinic receptor expression appears to be present on most granule cells. As only 5% of granule cells cultured in 25 mM KCl were found to express mGluR1α at 7-8 DIV (Milani *et al.*, 1993), it is possible that other PI-linked mGluR subtypes, i.e. mGluR5 or other splice-variants of mGluR1, are also present on these cells. An alternative explanation would be that the apparent discrepancy between the findings of the present study and those of Milani and co-workers is due to the majority of experiments in the present study being conducted at earlier DIV than in the mGluR1α expression study, at which time-points a higher proportion of granule cells may express mGluR1α. However, in an least one other study, ACPD evoked $[Ca^{2+}]_i$ responses in nearly all granule cells examined at 11-12 DIV (Manzoni *et al.*, 1992b). Also, these receptors were found to differ pharmacologically from those expressed at 2-3 DIV, which provides further evidence for more than one PI-linked mGluR being expressed in granule cells, although the temporal overlap of expression of these putative different subtypes / splice variants is uncertain. Some evidence suggests that a multiplicity of mGluR subtypes are co-expressed on cerebellar granule cells (Fagni *et al.* 1993; Prezeau *et al.*, 1994; Santi *et al.*, 1994), but at least some of these are non-PI-linked subtypes: mRNA for mGluRs 2, 3 and 4 is present in cultured granule cells (Prezeau *et al.*, 1994; Santi *et al.*, 1994), and mGluR inhibition of L-type VOCCs on granule cells can be evoked either by ACPD or the mGluR2/3 subtype-preferring agonist L-CCG-I (Fagni *et al.*, 1993). Very low, but significant, levels of mGluR5

mRNA have been reported in granule cells maintained in 25 mM K^+ (Santi *et al.*, 1994), although this report remains controversial (Prezeau *et al.*, 1994), while the possible expression of mGluR1 splice variants other than 1α has yet to be established.

The responses to mGluR agonists evoked in a single group of cells typically varied greatly during the time-course of a given experiment. K^+-depolarization has been previously reported to refill neuronal Ca^{2+} stores (Murphy and Miller, 1989), with stores acting as a sink for depolarization-activated entry. It is interesting to note that in the present study, depolarization with 25 mM KCl did not stabilize or increase mGluR-mediated $[Ca^{2+}]_i$ responses, and indeed K^+-depolarization has been found to deplete Ca^{2+} stores via CICR (Chapter 3), stores thus acting as a source contributing to the K^+-evoked response. The variability in the mGluR-mediated response over time, either with or without pre-depolarization, suggests either that the coupling of receptor-effector mechanisms is not stable, or that the extent of store repletion is not constant in cerebellar granule cells. The changes cannot be ascribed to loss of cell viability over time, as responses to NMDA and K^+-depolarization remained highly reproducible when an identical protocol was followed (see Chapters 3 and 4), and metabotropic responses did not consistently decline, but indeed often increased, during the timecourse of experiments.

III

Activation of receptors coupled via a G protein to PIC and production of $InsP_3$ and DAG has been shown to affect responses mediated by NMDARs using various experimental protocols in many neuronal types (Koh *et al.*, 1991; Courtney and Nicholls, 1992; Kelso *et al.*, 1992; Markram and Segal, 1992; McDonald and Schoepp, 1992; Kinney and Slater, 1993; Harvey and Collingridge, 1993; Birrell *et al.*, 1993). A variety of possible effector mechanisms have been proposed to mediate these

interactions. The present results indicate at least one mechanism by which such effects can occur in granule cells, namely metabotropic agonist modulation of NMDAR function by activation of PKC. On the basis of the present results, this effect may occur via the PKCα isozyme. However, the additional presence of PKCβ in granule cells has been suggested on the basis of previous work (Favaron *et al.*, 1990; see also Huang *et al.*, 1988), and the possible presence of all known PKC isozymes has not been fully investigated in these neurones.

In this present study, an attempt has been made to investigate and compare the consequences of agonist activity at metabotropic receptors, and of direct modulation of PKC activity, on NMDA-evoked elevations of $[Ca^{2+}]_i$ in cultured cerebellar granule cells, in an attempt to understand why confusion may exist in the literature as to what the likely effects of PKC activation would be on NMDAR-mediated responses *in vivo*. The consequences of PKC activation on NMDAR-evoked $[Ca^{2+}]_i$ responses appear more complex than was suggested previously in granule cells, in a study which involved several million cells per experiment (Courtney and Nicholls, 1992), as the effects of mGluR agonists on the $[Ca^{2+}]_i$ response to NMDA displayed marked heterogeneity in the present work. Both potentiation and inhibition of the NMDA response were evoked by ACPD or Quis, effects which were not related in any simple manner to DIV. This variation in metabotropic modulation of NMDAR activity was apparent between groups of granule cells even from the same coverslip population. Courtney and Nicholls (1992) suggested that variations in the modulatory effects of metabotropic stimuli were solely due to mGluR-evoked $[Ca^{2+}]_i$ responses obscuring inhibitory effects of PKC activation on NMDAR responses, and demonstrated that any additivity of NMDA and ACPD on $[Ca^{2+}]_i$ was abolished by depletion of intracellular Ca^{2+} stores. However, the present study has shown that depletion of Ca^{2+} stores would have complex effects on NMDAR / mGluR interactions, as the NMDA-evoked response would also be diminished (see Chapter 4). Also, such a protocol would not

enable determination of the likely overall effect of ionotropic and metabotropic receptor activation in normal synaptic processing. The present results demonstrate that the effects of the PKC activator PMA on the NMDA-evoked $[Ca^{2+}]_i$ elevation also display considerable heterogeneity between cell groups, either potentiating or inhibiting the response to NMDA in an unpredictable fashion. Similarly, the PKC inhibitor Ro 31 8220 either inhibited or potentiated NMDA responses, depending on the cell group examined. These unexpected findings indicate that simply the combination of an additivity of $[Ca^{2+}]_i$ responses evoked by metabotropic agonists and NMDA, and an inhibitory action on the NMDA response evoked by metabotropic agonists and mediated by PKC, does not fully explain the mixed effects of ACPD and Quis on NMDAR responses. Rather, at least part of the complexity of interaction seen between mGluR and NMDAR responses in granule cells appears to be due to differing effects of PKC in different granule cell groups. Variations in the levels of other cellular components may influence the ability of metabotropic agonists to modulate the NMDA response: it has previously been reported that mGluR-evoked activation of PKC is highly dependent on the level of arachidonic acid present (Coffey *et al.*, 1993). Further, mGluR-mediated release of glycine and of excitatory amino acids (Collins, 1993) may contribute to contradictory effects of mGluR agonists, depending on the preparation used, the experimental protocol adopted, and the local environment of the neurones studied. Other effector mechanisms activated by metabotropic stimulation (see e.g. Markram and Segal, 1992; Harvey and Collingridge, 1993) may also contribute to interactions with NMDARs. A further complication is the ability of both NMDARs (Favaron *et al.*, 1990) and mGluRs to activate PKC, and the apparent ability of PKC to modulate mGluRs (Paulsen *et al.*, 1994) as well as NMDARs. The results of these interactions are therefore likely to differ depending on the degree of stimulation, and the spatial relationship between the receptors and PKC, in a given type of neurone.

In granule cells in which the phorbol ester PMA evoked a potentiation of the NMDA-evoked response in this study, this potentiation was much smaller than has been reported in other cell types (Chen and Huang, 1992; Kitamura *et al.*, 1993; Durand *et al.*, 1993). It has previously been suggested that in striatal neurones PKC activity may be essential for NMDA-evoked $[Ca^{2+}]_i$ responses (Murphy *et al.*, 1994), such that an endogenous level of PKC activation modulates NMDAR function. The potentiating effect of PKC on NMDA responses is highly dependent on the NMDAR subunit combination and splice variants expressed, some being markedly potentiated by PMA whilst others are unaffected (Kutsuwada *et al.*, 1992; Durand *et al.*, 1993). However, no cloned form of NMDAR has yet been shown to be susceptible to PKC-mediated inhibition, a mechanism which appeared to occur in cerebellar granule cells in the present study and in previous reports (Courtney and Nicholls, 1992; Snell *et al.*, 1994). Possible explanations of the inhibition observed in this and other studies include: (i) the action of a different isozyme of PKC in cerebellar granule cells from those present in other neurones and in the above recombinant studies in *Xenopus* oocytes; (ii) NMDARs in granule cells consisting of a combination of subunits differing from the subunit combinations tested in these above studies; (iii) PKC phosphorylation of a protein linked to but separate from the NMDAR itself, which is found in cerebellar granule cells but not in *Xenopus* oocytes.

The current findings, alongside previous work (Courtney and Nicholls, 1992; Snell *et al.*, 1994), confirm that PKC can cause inhibition of NMDA-evoked responses in cerebellar granule cells. PKC-mediated potentiation of NMDA responses has been reported only in other types of neurone (Chen and Huang, 1992; Kitamura *et al.*, 1993; Durand *et al.*, 1993), where the complement of PKC isozymes and/or the subtypes of NMDAR expressed may well be different to those present in granule cells (Monyer *et al.*, 1994). It has previously been demonstrated that the NMDARs found in cerebellar granule cells differ pharmacologically from those in cortical neurones (Priestley and

Kemp, 1994). The consequences of endogenous PKC activation on NMDAR function in granule cells, which might be expected to contrast with the findings of Murphy and co-workers (1994) in striatal neurones, remains to be clearly established, but preliminary experiments in the present study found no clear effect of 24 h PMA pre-incubation on granule cell NMDA responses (data not shown).

Dependence of the inhibitory effect of PKC in granule cells on the presence of insufficient glycine to saturate its NMDAR binding site has been suggested in one report (Snell *et al.*,1994), but not in another (Courtney and Nicholls, 1992) or in the present study. Whether a modulatory effect which was not present at saturating glycine levels would be of physiological relevance is uncertain (although see Chapter 4). It is interesting to note that a discrepancy also exists within the literature concerning potentiatory effects of PKC on NMDAR responses, as one report has ascribed this effect to a PKC-mediated relief of Mg^{2+} block of NMDA channels (Chen and Huang, 1992), whereas other studies have reported PKC-mediated NMDAR potentiation in Mg^{2+}-free conditions (Anijkstejn *et al.*, 1992; Kelso *et al.*, 1992; Murphy *et al.*, 1994). It may therefore be that differing mechanisms are involved in PKC-NMDAR interactions in both different preparations and different experimental conditions.

In view of the variation of the PKC modulation between cell groups reported here, it is further of interest that two different types of NMDAR2 subunits, 2A and 2B, are present in cultured cerebellar granule cells (Bessho *et al.*, 1994), indicating the possible presence of more than one type of functional NMDAR in these neurones. Striatal neurones possess several pharmacologically distinguishable types of NMDAR (Nankai *et al.*, 1994), and as NMDA responses from individual striatal neurones display marked and important differences (Weiss *et al.*, 1993), there is reason to suggest that the complement of NMDARs may differ from neurone to neurone in that brain region. It is tempting to speculate on the distribution of NMDAR subtypes in

cerebellar granule cells, and how this might relate to the variable consequences of PKC activation on the NMDA-evoked response in different groups of cells.

The ability in some experiments of removal of PMA or Ro 31 8220 to partially or completely abolish the effects which had been evoked by their addition was unexpected, as the actions of these agents on PKC have been widely reported to be irreversible. The specificity of action of some PKC-modulating agents has recently been questioned (Wilkinson and Hallam, 1994); indeed, several PKC antagonists have been reported to interact directly with NMDARs (Amador and Dani, 1991; Lopez-Molina *et al.*, 1993). However, no reports have suggested direct effects of the agents used in this study, PMA and Ro 31 8220, on NMDARs. Also, as the effects of PMA in the present study so closely parallel those of the physiological activators of PKC i.e. mGluR agonists, whereas no comparable effects were evoked by AMPA (see Chapter 4), which does not activate DAG production, it is difficult to convincingly ascribe any of the effects of PMA and Ro 31 8220 shown here to a non-PKC mechanism. The possibility that Ro 31 8220, a strongly coloured agent, exerted direct Ca^{2+}-independent effects on fluorescence remains to be completely ruled out, but no effect on the basal 340 / 380 nm ratio was caused by this agent alone (not shown).

III

The effects of CCh acting at the M_3 subtype of muscarinic cholinoceptor were qualitatively similar to the $[Ca^{2+}]_i$ responses evoked by ACPD and Quis, consisting of a rapid peak and a negligible plateau elevation. The greater reproducibility of the response to CCh enabled reliable investigation of the effects of store modulators on this response. Thapsigargin markedly decreased the $[Ca^{2+}]_i$ elevation evoked by CCh in many (~50%) cell groups tested, indicative of a major involvement of Ca^{2+} store release in muscarinic responses. Consistent with this, CCh was found not to increase

significantly the rate of Mn^{2+} quench, suggesting that this agonist may not markedly elevate Ca^{2+} entry into granule cells. In many experiments caffeine pre-treatment abolished subsequent responses to CCh, which appears to indicate that both RyR activation and CCh release Ca^{2+} primarily from a common Ca^{2+} pool. This alone does not necessarily demonstrate that the CCh-evoked response is simply due to $InsP_3R$ activation of a pool which also expresses RyRs. Pre-incubation with ryanodine and dantrolene were often also found to inhibit the response to CCh, demonstrating that activation of RyRs, as well as of $InsP_3Rs$, appears to be an important mechanism in CCh-evoked $[Ca^{2+}]_i$ elevation. Indeed, it is interesting to note that a single challenge with CCh caused significant inhibition of a subsequent response to caffeine, whereas repeated additions of caffeine were required to inhibit CCh responses, possibly indicative of a greater ability of CCh to deplete RyR-expressing stores than of caffeine. In the light of these findings, a more plausible mechanism of CCh-evoked $[Ca^{2+}]_i$ elevation than simply $InsP_3R$ activation would be a small Ca^{2+} release activated directly by $InsP_3$ either from a portion of the RyR pool or possibly from a different Ca^{2+} pool, which then evokes the major component of the $[Ca^{2+}]_i$ response by CICR via RyRs (see Fig. 5.11). The inhibitory effect of ryanodine on CCh-evoked responses did not display clear use-dependence, unlike in a previous report in which no inhibition of an initial CCh response in the presence of ryanodine was observed, but a marked reduction was found in the response to further CCh challenges (Irving *et al.*, 1992b). While perhaps surprising, the present finding may be consistent with the only partial use-dependence of its inhibitory effect on NMDA responses in this study (Chapter 4). This may be a consequence of the relatively high ryanodine concentration used.

The decrease often found in basal 340 / 380 nm ratio in response to perfusion of caffeine, after Ca^{2+} store depletion by prior caffeine or CCh challenge, remains to be fully explained. This phenomenon may be related to complete emptying of the caffeine-sensitive store enhancing Ca^{2+} uptake from the cytoplasm. Such a mechanism

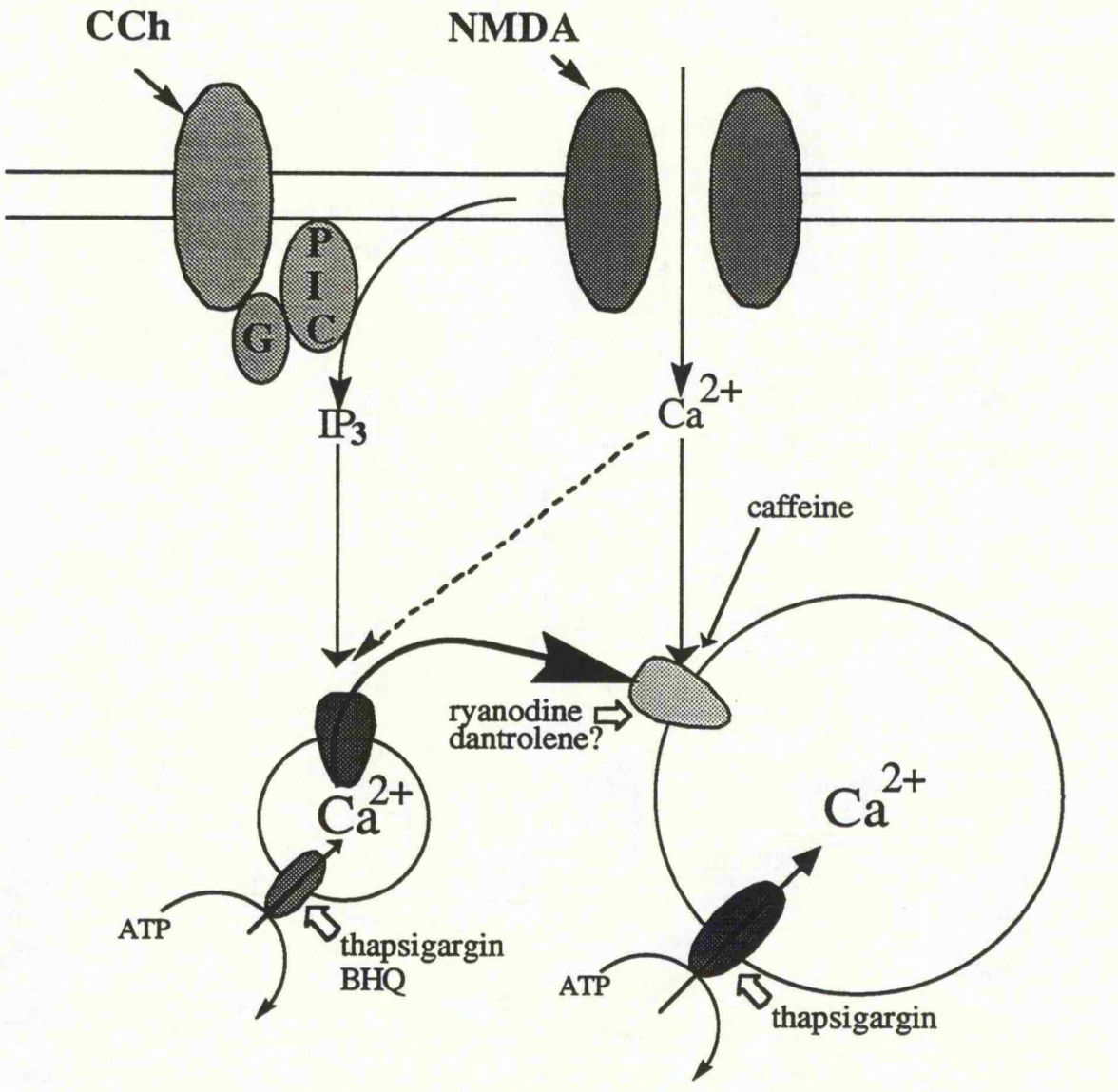


Fig. 5.11 Model for Ca^{2+} release mechanisms which may be activated by CCh and NMDA in cultured cerebellar granule cells. CCh-evoked generation of $InsP_3$ causes Ca^{2+} release from a small $InsP_3R$-expressing pool, which then triggers more marked release via CICR from a large Ca^{2+} pool expressing RyRs. Rapid depletion of the $InsP_3$ pool would cause the CCh-evoked $[Ca^{2+}]_i$ elevation to be of short duration. NMDA-evoked Ca^{2+} entry evokes a high level of Ca^{2+} release via RyR CICR. NMDA-evoked Ca^{2+} entry may also evoke Ca^{2+} release via a sensitization of $InsP_3$ receptors.

has been previously suggested as the explanation for the ability of caffeine to abolish depolarization-evoked plateau responses in PC12 cells (Barry and Cheek, 1994). It is interesting to note that in the present study, caffeine-evoked Ca^{2+} transients were sometimes evoked concurrently with a decrease in the basal 340 / 380 nm ratio, suggesting a degree of complexity in the ability of RyR-expressing stores to act as a sink and a source for Ca^{2+} in neurones. A direct effect of caffeine on fura 2 fluorescence also remains a possible explanation.

The dependence of the CCh-evoked peak elevation of $[Ca^{2+}]_i$ on Ca^{2+} entry differs between neuronal preparations, with the response in some neuronal types being markedly depressed in Ca^{2+}-free medium (Kudo *et al.*, 1988; Reynolds and Miller, 1989). However, the lack of Mn^{2+} quench evoked during the CCh-evoked peak $[Ca^{2+}]_i$ response is in agreement with previous studies demonstrating no involvement of Ca^{2+} entry in muscarinic receptor-mediated peak $[Ca^{2+}]_i$ elevations in cerebellar granule cells (Irving *et al.*, 1992a; Fohrmann *et al.*, 1993). A CCh-evoked plateau response apparently mediated by Ca^{2+} entry has been found in previous reports in this cell type from another laboratory (Courtney *et al.*, 1990; del Rio *et al.*, 1994), whereas a negligible sustained elevation was found in the present study. CCh has been found to evoke no Mn^{2+} quench in lacrimal or parotid acinar cells despite marked muscarinic agonist-evoked Ca^{2+} entry, consistent with activation of Ca^{2+} entry via Mn^{2+}-impermeant, possibly CRAC channels (Merritt and Hallam, 1988; Kwan and Putney, 1990; but see Mertz *et al.*, 1990). In sublingual mucous acini, addition of CCh evoked an elevation of $[Ca^{2+}]_i$ and of the rate of Mn^{2+} quench, effects which were both inhibited by pre-treatment with 10 μM dantrolene (Zhang and Melvin, 1993). This was not due to a direct interaction of dantrolene with divalent cation entry pathways, as the agent had no inhibitory effect if perfused after stores were already depleted by CCh. That report used a very different protocol to the one adopted in the present study, as cells were pre-treated with CCh for 5 min, then with CCh and atropine for 5 min,

before Mn^{2+} was added. As well as ensuring that only store depletion-activated cation entry, not receptor-operated entry, was measured, this protocol allowed a much longer time for CCh to evoke store depletion. It might be valuable in future experiments to investigate whether prolonged rather than acute perfusion of CCh in granule cells could activate significant Mn^{2+} entry. However, the lack of Mn^{2+} quench in response to thapsigargin may indicate that store depletion-activated Ca^{2+} entry occurs in granule cells via Mn^{2+}-impermeant channels (see Chapter 4 Discussion). High basal quench may make activation of a small amount of influx difficult to detect, but may also be consistent with high sensitivity to activation of Mn^{2+}-permeant pathways (see Chapter 4 Discussion).

The involvement of stores in both mGluR- and muscarinic receptor-mediated elevation of $[Ca^{2+}]_i$ in cerebellar granule cells has previously been demonstrated by experiments using store-modulatory agents, notably thapsigargin, which was found to abolish responses to agonists of these receptors (Irving *et al.*, 1992a,b; Fohrmann *et al.*, 1993), broadly consistent with the findings of the present report. The involvement of RyRs in M_3-mediated $[Ca^{2+}]_i$ responses in granule cells is more controversial, with one study finding that pre-treatment with dantrolene had no effect on subsequent CCh-evoked responses (Fohrmann *et al.*, 1993). Irving and co-workers (1992b), however, reported that caffeine stimulation abolished subsequent responses evoked by ACh or ACPD, with reciprocal inhibitory effects on caffeine responses evoked by the metabotropic agonists. Also in that study, ryanodine was found to use-dependently inhibit responses to caffeine, ACPD and ACh, although the inhibition of ACPD and ACh responses varied from cell to cell. More recently, mGluR-mediated formation of NO_2^-, a product of NO· metabolism, in cerebellar synaptosomes has been found to be abolished by dantrolene pre-treatment (Gorbunov and Esposito, 1994). The present study has demonstrated that an inhibition of the $[Ca^{2+}]_i$ response evoked by CCh in cultured cerebellar granule cells in many, though not all, experiments, is caused by pre-

treatment with thapsigargin, BHQ, dantrolene or ryanodine. It is not clear why the effects of these agents were not found in all experiments. Possible explanations of this include a variable involvement of stores in the responses of different neurones, or differences in the pharmacological sensitivity of stores between neurones. Given the current paucity of clear information concerning the sites and mechanisms of action of dantrolene (see Pessah *et al.*, 1986; Ohta *et al.*, 1990; El-Hayek *et al.*, 1992; Smith and Nahorski, 1993), it remains difficult to exclude the possibility that it acts to inhibit metabotropic receptor-mediated Ca^{2+} mobilization in this and previous (Mine *et al.*, 1987; Frandsen and Schousboe, 1992; Zhang and Melvin, 1993) studies by means of an inhibition of $InsP_3Rs$ rather than by acting solely on RyRs. It was therefore important to be able to confirm the involvement of RyRs in the CCh-evoked response by other means, and the present results using the more specific agent ryanodine provide good evidence on their own, as well as in combination with the dantrolene findings, that muscarinic receptors evoke Ca^{2+} mobilization in granule cells by mechanisms which include RyR activation. Further experiments to clarify the mechanism of action of dantrolene in neuronal preparations would clearly be helpful in interpreting the results of studies such as the present one.

The $InsP_3$-sensitive Ca^{2+} pool in granule cells has previously been reported to be ~60% of the total granule cell Ca^{2+} pool (Whitham *et al.*, 1991b), but whether this was entirely direct $InsP_3$-evoked release, or partly occurred as a consequence of secondary CICR, is uncertain in the light of the present findings. The $InsP_3$-insensitive component of the stores in that study might correspond to stores expressing only RyRs not $InsP_3Rs$. However, permeabilization may cause disruption of store integrity (see Putney and Bird, 1993), thus making conclusions as to the degree of store insensitivity to $InsP_3$, or to caffeine, potentially unreliable. $^{45}Ca^{2+}$ release in that granule cell study required relatively high levels of $InsP_3$ (Whitham *et al.*, 1991b). It is therefore possible that a significant degree of complexity exists in cerebellar granule cells Ca^{2+} stores,

such that $InsP_3$- and Ca^{2+}-sensitive stores partially overlap but that some are more readily released by physiological levels of Ca^{2+} than by physiological levels of $InsP_3$. The bell-shaped sensitivity of $InsP_3$Rs to $[Ca^{2+}]_i$ (Bezprozvanny *et al.*, 1991; Finch *et al.*, 1991) may complicate further the interpretation of Ca^{2+}- and $InsP_3$-sensitive stores, as a significant degree of positive and negative feed-back and feed-forward may occur between the two release mechanisms.

Importantly, while thapsigargin, ryanodine and dantrolene have been found to inhibit both CCh- and NMDA-evoked Ca^{2+} release in the present study, BHQ apparently differentiates between the two responses, inhibiting IICR but not CICR. It is therefore possible that the directly $InsP_3$-sensitive pool is physically different from the CICR pool, possessing a Ca^{2+}ATPase sensitive to thapsigargin and BHQ, whereas the Ca^{2+}ATPase on the Ca^{2+}-sensitive pool may be thapsigargin-blockable but insensitive to BHQ. Clearly, complexity exists in granule cell CICR and IICR stores, and one possible model to explain the results of the present study, and of previous investigations such as Irving *et al.* (1992b), is shown in Fig. 5.11. According to this model, CCh-evoked generation of $InsP_3$ causes Ca^{2+} release from a small $InsP_3$R-expressing pool, which then triggers greater release via CICR from a large Ca^{2+} pool expressing RyRs. Rapid depletion of the $InsP_3$R-expressing pool would cause CCh-evoked $[Ca^{2+}]_i$ elevation to be of short duration. NMDA-evoked Ca^{2+} entry evokes a high degree of Ca^{2+} release via RyR-mediated CICR. As NMDAR cation entry persists for several minutes during sustained agonist treatment (see Section 4.5.1), release from the RyR-expressing pool could be involved throughout the NMDA plateau elevation. NMDA-evoked Ca^{2+} entry may also evoke Ca^{2+} release via a sensitization of $InsP_3$Rs: this possibility is addressed in Chapter 7. However, it must be borne in mind that studies such as the present one, and those using permeabilized cell preparations, need to be cross-compared, and perhaps more specific pharmacological tools developed, before a convincing and reliable model for the co-expression of

different Ca^{2+} stores, and the nature of interactions between IICR and CICR, can truly be developed. The involvement of spatially differing stores may, in the future, best be analysed using highly focal stimulation and recording techniques (see Malinow *et al.*, 1994), which might allow separation of entry, initial store activation and involvement of secondary store release.

Pharmacological interventions in phenomena which involve interactions between NMDARs and metabotropic receptors, notably neurotoxicity, may need to be re-evaluated in the light of the apparent ability of both receptors to mediate Ca^{2+} store mobilization. For instance, ACPD-induced limbic seizures and consequent brain injury can be inhibited by pre-treatment with dantrolene (Tizzano *et al.*, 1993; McDonald *et al.*, 1993), as can both Quis- and NMDA-evoked $[Ca^{2+}]_i$ elevations and neurotoxicity in cultured cortical neurones (Frandsen and Schousboe, 1992). (Given the lack of effect of dantrolene on AMPA responses in this preparation, these Quis results can be ascribed to inhibition of mGluR-mediated responses.) It may be that for pathological conditions which involve toxicity via both ionotropic and metabotropic pathways, blocking Ca^{2+} store release e.g. by inhibition of RyR activity would have the advantages of treatment with a broad-spectrum GluR antagonist, while having the additional benefit of leaving at least some important glutamate signalling mechanisms i.e. Ca^{2+} entry, DAG production, and directly $InsP_3$-evoked Ca^{2+} release, intact. Little work has been done on possible therapeutic uses of store-modulatory agents to date. In other situations it appears that mGluRs mediate neuroprotection (Koh *et al.*, 1991; Birrell *et al.*, 1993), although as it has yet to be determined whether this occurs via PI-linked or adenylyl cyclase-linked subtypes, it may be that inhibition of Ca^{2+} release would contribute to rather than inhibit neuroprotection in these situations also.

Dantrolene and thapsigargin have both been reported to inhibit induction of LTP (Obenaus *et al.*, 1989; Harvey and Collingridge, 1992; Bortolotto and

Collingridge, 1993), whereas thapsigargin was without effect on LTP expression if added 30 min after induction (Harvey and Collingridge, 1992). It is now known that mGluR activation is necessary for induction of several forms of LTP (Bortolotto and Collingridge, 1993; Otani *et al.*, 1993; Bortolotto *et al.*, 1994), so these store depletion results might be interpreted as inhibition of the functional consequences of mGluR activation. However, pre-incubation with thapsigargin or ryanodine caused a ~70% inhibition of the dendritic Ca^{2+} transient evoked by tetanic stimulation (Alford *et al.*, 1993), whereas the mGluR antagonist (+)-α-methyl-4-carboxyphenylglycine (MCPG) caused only a 20-30% inhibition of this signal (Frenguelli *et al.*, 1993), consistent with the involvement in tetanic responses of Ca^{2+} mobilized by mechanisms not limited to mGluR activation. This is likely to be NMDAR-mediated, given that APV inhibits tetanus-evoked $[Ca^{2+}]_i$ transients by ~85% (Perkel *et al.*, 1993; Frenguelli *et al.*, 1993), although other ionotropic GluRs present can also activate CICR in some cells (Kocsis et al., 1993). The presence of stores differentially activated by $InsP_3$ and Ca^{2+}, such as have been suggested in granule cells in the present study, may contribute to synaptic integration in LTP and other complex responses. Indeed, an apparent activation of CICR subsequent to IICR by mGluRs in cultured hippocampal neurones, measured in terms of induction of an intermediate-conductance $I_{K(Ca)}$, has recently been reported (Shirasaki *et al.*, 1994). The involvement of two related, but distinct, Ca^{2+} release mechanisms may be of importance to a variety of responses evoked by glutamate, ACh and indeed other transmitters, and a greater knowledge of the characteristics and roles of these stores could provide valuable insights into key physiological and pathological phenomena.

6. Inositol phosphates

6.1 Inositol phosphate turnover in granule cells

The conclusions of the previous Chapter concerning interactions between ionotropic and metabotropic signalling focussed upon mGluR-mediated modulation of NMDARs, and agonist actions on identical or differing Ca^{2+} stores. A third important site of potential interaction between these signalling mechanisms is PIC, an enzyme central to metabotropic PI and $[Ca^{2+}]_i$ responses, some isoforms of which are sensitive to Ca^{2+} (Eberhard and Holz, 1988; Fisher *et al.*, 1989; Lambert *et al.*, 1991; Cockcroft and Thomas, 1992; Rhee and Choi, 1992; Wojcikiewicz *et al.*, 1994b). Elevations of $[Ca^{2+}]_i$ activated by Ca^{2+} entry or release from stores could, by acting on PIC, activate or potentiate activation of $InsP_X$ production and thus increase IICR. As both NMDA and CCh release Ca^{2+} from stores in cerebellar granule cells (see Chapters 4 and 5), a component of the actions of both these agents on $InsP_X$ levels could be due to Ca^{2+} release feeding back on PI turnover. It was therefore important in the present study (i) to characterize the $InsP_3$ and $InsP_X$ responses to metabotropic agonists, particularly CCh, and ionotropic agents, particularly NMDA; (ii) to examine the possible interactions between these PIC-activating mechanisms; (iii) to investigate the effects of pre-incubation with store modulatory agents on the $InsP_X$ responses to ionotropic and metabotropic agonists. The series of experiments reported in this Chapter also act as control experiments for Chapter 5, as it was necessary to demonstrate whether the effects of presumed store modulators on CCh responses described in that Chapter could actually be due to effects on PI turnover.

6.2 $InsP_3$ mass accumulation

Cerebellar granule cells were incubated with a given agonist in KHB for pre-determined periods of time, the incubation terminated using TCA, and cells scraped, centrifuged and the $InsP_3$ levels present analysed using a radioreceptor assay (see

Methods). Incubation of cultured cerebellar granule cells with CCh (1 mM) evoked a biphasic timecourse of elevated $InsP_3$ accumulation. A ~3 fold peak increase in $InsP_3$ over control occurred at ~10 s after initiation of receptor stimulation, and subsequently decayed within 1 min to a smaller (≤2 fold), approximately steady-state plateau (Fig. 6.1). Muscarinic receptor-mediated $InsP_3$ production has been previously described in cerebellar granule cells (Whitham *et al.*, 1991a), with a similar degree of CCh-evoked peak and plateau elevation of $InsP_3$ levels as found in the present study, but in that report significantly increased $InsP_3$ accumulation was described only for agonist incubation periods of <5 min. A more extensive analysis of the second phase of $InsP_3$ accumulation in this study revealed the plateau phase to be highly persistent, being significantly higher than control for all incubation times up to 40 min ($p < 0.05$, Student's t-test). A similar response size and temporal profile was found for muscarinic receptor activation of $InsP_3$ accumulation at either 2-3 or 7-8 DIV. In contrast, neither ACPD (200 μM) nor NMDA (200 μM) evoked detectable elevation of $InsP_3$ mass at any time-point examined (data not shown).

6.3 Total inositol phosphate accumulation

6.3.1 $InsP_X$ accumulation evoked by ionotropic agents

Cells were equilibrated with [^{3}H]-*myo*-inositol for 48 h, pre-incubated with Li^+, then agonists added to wells for 20 min incubation periods. Incubations were terminated using TCA, and the $InsP_X$ levels present analysed by anion exchange chromatography (see Methods). NMDA, the most potent elevator of $[Ca^{2+}]_i$ in granule cells which has been used in this study, evoked a modest increase in $InsP_X$ levels over basal (0.6 ± 0.2 fold for 300 μM NMDA) in the presence of Li^+ (see Figs. 6.2,6.3b), which was abolished by co-incubation with 10 μM dizocilpine (data not shown). The mechanism involved in this elevation is uncertain (see Nicoletti *et al.*, 1986; Raulli et al., 1991), but probably involves a small direct Ca^{2+} activation of PIC

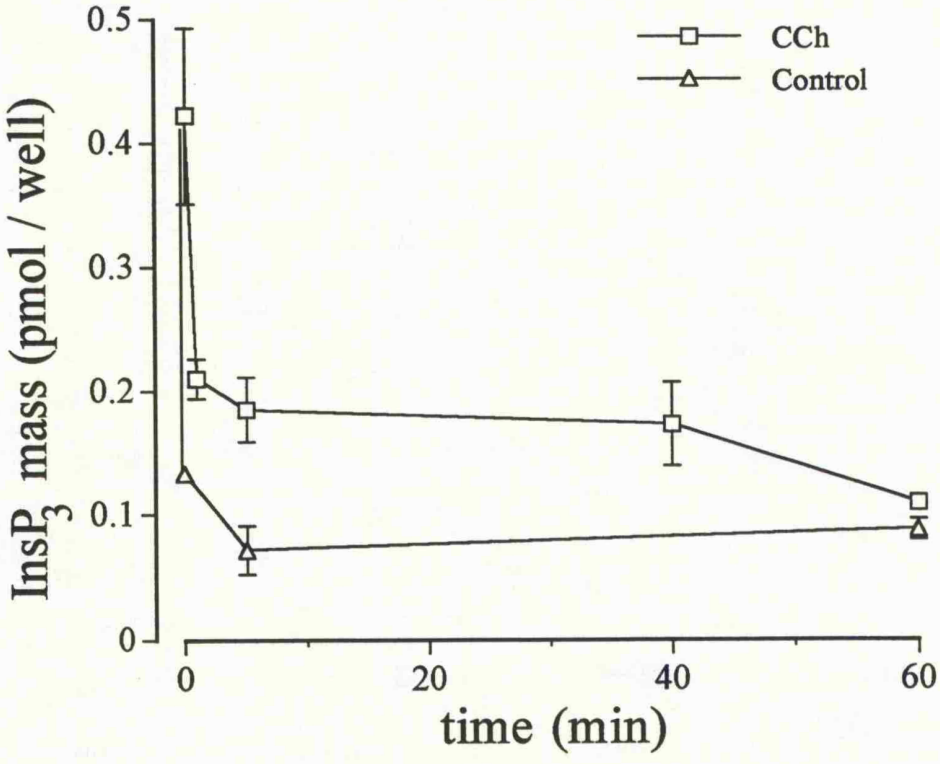


Fig. 6.1 $InsP_3$ mass accumulation evoked by CCh in cerebellar granule cells. Results from an example experiment performed in triplicate, typical of 3 experiments performed on different culture preparations. Incubation of cerebellar granule cells with 1 mM CCh evoked a peak accumulation of $InsP_3$ mass at ~10 s, which rapidly decayed within 1 min to a secondary plateau phase. Matched control cells from the same preparation incubated with KHB displayed little change in $InsP_3$ mass during the experimental time period. Analysing all experiments, the plateau remained significantly above matched controls at all timepoints examined up to 40 min.

occurring even in the absence of synchronous metabotropic receptor-G protein activation (Diamant and Atlas, 1989; Baird and Nahorski, 1991). AMPA (300 μM), a much less potent activator of Ca^{2+} entry in these neurones (see Section 4.4.1), evoked a comparable degree of $InsP_X$ accumulation to NMDA (1.3 ± 0.8 fold). The effects of 300 μM AMPA and 300 μM NMDA in combination were not significantly greater than either agent on its own (Fig. 6.2). 25 or 50 mM K^+ also caused a modest elevation in $InsP_X$ levels (0.6 fold for 50 mM (n=2)), with little additivity apparent for K^+ with either AMPA or NMDA (Fig. 6.2). Thus there may be a low maximal PIC activation which can be directly evoked by Ca^{2+} entry, larger increases in PIC activity requiring G protein-mediated activation (see below).

6.3.2 $InsP_X$ accumulation evoked by metabotropic agents

In preliminary experiments, ACPD (300 μM) evoked an elevation of $InsP_X$ levels of 1.7 fold increase over basal, whereas CCh (1 mM), in accordance with its much greater effect on $InsP_3$ mass accumulation than ACPD (see above; also Whitham et al., 1991 - Ph.D. Thesis, University of Leicester), caused an increase in $InsP_X$ levels of 5.9 ± 0.8 fold. CCh was therefore the metabotropic agonist of choice for subsequent experiments. Interactions between CCh responses and ionotropic effects on $InsP_X$ were subsequently examined in cerebellar granule cells and, for comparative purposes, in neonatal cortical slices as well, in which, on the basis of previous studies, larger metabotropic $InsP_X$ responses might be expected.

6.3.3 CCh and NMDA effects on $InsP_X$ in neonatal cortical slices and cerebellar granule cells

CCh (1 mM) markedly elevated total inositol phosphate accumulation in neonatal cortical slices, evoking a 22.7 ± 4.3 fold increase over 20 min incubation,

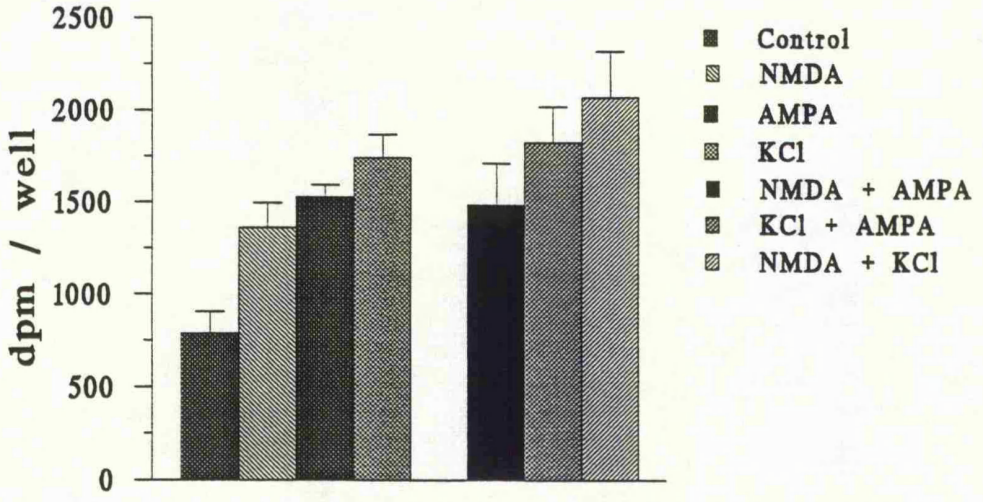


Fig. 6.2 Effects of ionotropic agents on $InsP_X$ accumulation in granule cells. 20 min incubation with NMDA (300 μM), AMPA (300 μM), and K^+ (50 mM) each evoked a relatively small increase in $InsP_X$ over basal levels. When these agents were added in various combinations, little additivity of effect was found, indicating that the maximal stimulation of PIC activity by ionotropic agents alone is small.

which was indeed much larger than the CCh response found in granule cells. Co-addition of a low concentration of NMDA (10 μM) potentiated CCh-evoked $InsP_X$ accumulation, by 50 ± 5%, an effect which was presumably to be due to Ca^{2+} potentiation of CCh-evoked PIC activity (Fig. 6.3a). Co-application of a high concentration of NMDA (100 μM), however, which by itself caused significant inositol phosphate accumulation over 20 min (3.1 ± 0.8 fold over basal), greatly inhibited the large CCh-evoked response (70 ± 4% inhibition)(Fig. 6.3a). These findings are in good agreement with previous reports on the interactive effects of metabotropic and ionotropic agonists on $InsP_X$ accumulation in this tissue (Challiss *et al.*, 1994a,b). In those previous reports, the inhibitory effect of high NMDA on metabotropic receptor-mediated $InsP_X$ accumulations was ascribed to a toxic effect on cortical slice viability, as the inhibition was blocked by prior addition of dizocilpine, but not affected by addition of dizocilpine 10 min after the start of incubation with NMDA, and NMDA inhibited not only metabotropic responses but also the incorporation of [^{3}H]-inositol into membrane phospholipids.

In cerebellar granule cells, co-application of NMDA with CCh potentiated the $InsP_X$ accumulation evoked by 1 mM CCh in a concentration-dependent manner, such that a maximal concentration of NMDA with CCh evoked a response 3.1 ± 0.9 times that seen for CCh alone (Fig. 6.3b). This potentiation of the CCh-evoked response was abolished by dizocilpine (10 μM)(data not shown). No inhibition of the $InsP_X$ response to CCh was evoked by high NMDA in this preparation, although a 'top-over' effect was found, such that the response to CCh + 300 μM NMDA was consistently slightly lower than the response to CCh + 100 μM NMDA (see Fig. 6.3b). In one preliminary experiment NMDA (200 μM) was found to potentiate the CCh-evoked $InsP_3$ response as well, increasing both peak and plateau phases (data not shown). In preliminary experiments NMDA also potentiated the $InsP_X$ response evoked by ACPD, but neither 50 mM K^+ nor 300 μM AMPA, despite similar direct effects on $InsP_X$ levels as

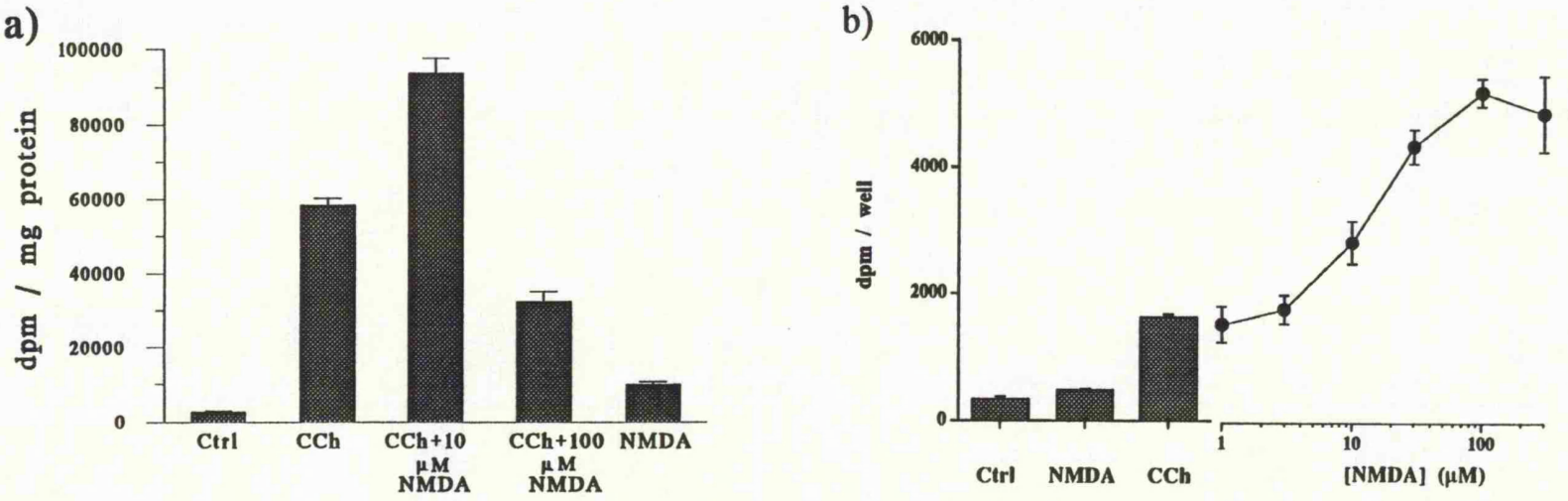


Fig. 6.3 ***a)*** **Elevation of $InsP_X$ by CCh and NMDA in neonatal cortical slices.** Results from an example experiment performed in triplicate, typical of 4 experiments performed on different slice preparations. CCh (1 mM) evoked a large increase in $InsP_X$ levels over 20 min incubation, whereas NMDA (100 μM) had a much smaller though significant stimulatory effect. NMDA either potentiated or inhibited the CCh-evoked response, in a concentration-dependent manner.

b) **NMDA potentiation of CCh $InsP_X$ accumulation in cerebellar granule cells.** Results from a typical example experiment performed in triplicate. NMDA (300 μM) had only a slight effect on $InsP_X$ levels over 20 min incubation, whereas CCh (1 mM) evoked a much greater accumulation. NMDA concentration-dependently potentiated the CCh-evoked response, the combined effect of CCh + NMDA being much greater than the summation of their individual responses.

NMDA, were likewise able to markedly potentiate CCh-evoked $InsP_X$ accumulation (data not shown).

6.4 Effect of store modulators on $InsP_X$ responses

Some isoforms of PIC are known to be Ca^{2+}-sensitive (Eberhard and Holz, 1988; Fisher *et al.*, 1989; Lambert *et al.*, 1991; Cockcroft and Thomas, 1992; Rhee and Choi, 1992; Wojcikiewicz *et al.*, 1994b), including presumably the isoform affected by NMDA in the current study. It has been shown that both CCh and NMDA evoke Ca^{2+} release from intracellular stores in cerebellar granule cells (Chapters 4 and 5). It therefore appeared possible that a component of the CCh-evoked $InsP_X$ response was due to a positive feedback of released Ca^{2+} inducing a facilitation of PIC activity, and concomitantly, that the activation of $InsP_X$ accumulation and potentiation of the CCh response evoked by NMDA was to some degree mediated by Ca^{2+} released from stores activating PIC, as well as by Ca^{2+} entry. This was investigated in a series of experiments in granule cells, using the same protocol as before. $InsP_X$ responses after pre-treatment of granule cells with thapsigargin, ryanodine, dantrolene or caffeine were compared to responses in untreated wells in the same multidish. The possibility that CCh responses, low concentrations of NMDA-evoked potentiation or the inhibitory, toxic effect of high [NMDA] on the $InsP_X$ response to CCh in neonatal cortical slices could be reduced by store modulators was similarly examined.

In neonatal cortical slices, pre-treatment with dantrolene, with thapsigargin, or in preliminary experiments with ryanodine, each had no effect on basal, CCh-evoked or CCh and low concentrations of NMDA-evoked $InsP_X$ formation (Table 6.1; Fig. 6.4a). Interestingly, high [NMDA]-evoked inhibition of the CCh response was also unaffected by pre-treatment with any of these agents (Table 6.1; Fig. 6.4a). Pre-treatment for ~10 min with ryanodine (10 μM) had no significant effect on basal

Experimental condition	% of control	Control response (fold over basal)	Response with agent (fold over basal)
Dantrolene on Basal	110 (n=2)	1.0	1.1
Thapsigargin on Basal	110 (n=2)	1.0	1.1
Dantrolene on CCh	103 ± 10	24.5 ± 5.5	25.3 ± 5.5
Thapsigargin on CCh	119 ± 13	18.6 ± 1.9	21.5 ± 6.6
Dantrolene on CCh + 10 μM NMDA	94 ± 5	36.5 ± 7.6	33.5 ± 5.2
Thapsigargin on CCh + 10 μM NMDA	94 ± 2	27.9 ± 4.0	26.3 ± 3.7
Dantrolene on CCh + 100 μM NMDA	104 ± 8	13.3 ± 3.5	13.5 ± 2.8
Thapsigargin on CCh + 100 μM NMDA	115 ± 5	8.7 ± 1.9	9.8 ± 1.8
Dantrolene on NMDA	100 (n=2)	4.5	4.4
Thapsigargin on NMDA	128 (n=2)	2.9	3.8

Table 6.1 Lack of effect of store modulators on $InsP_X$ formation in neonatal cortical slices. Meaned results from a series of experiments, with the response in the presence of store modulators compared to the control responses in slices from the same preparation. Pre-treatment with dantrolene (30 μM) or thapsigargin (10 μM) did not inhibit either basal $InsP_X$ levels, or $InsP_X$ accumulation stimulated by 1 mM CCh, 100 μM NMDA, or CCh + 10 μM NMDA. Additionally, neither agent significantly inhibited inhibition of CCh-evoked $InsP_X$ accumulation evoked by 100 μM NMDA. Pre-treatment with ryanodine (10 μM) was similarly without effect.

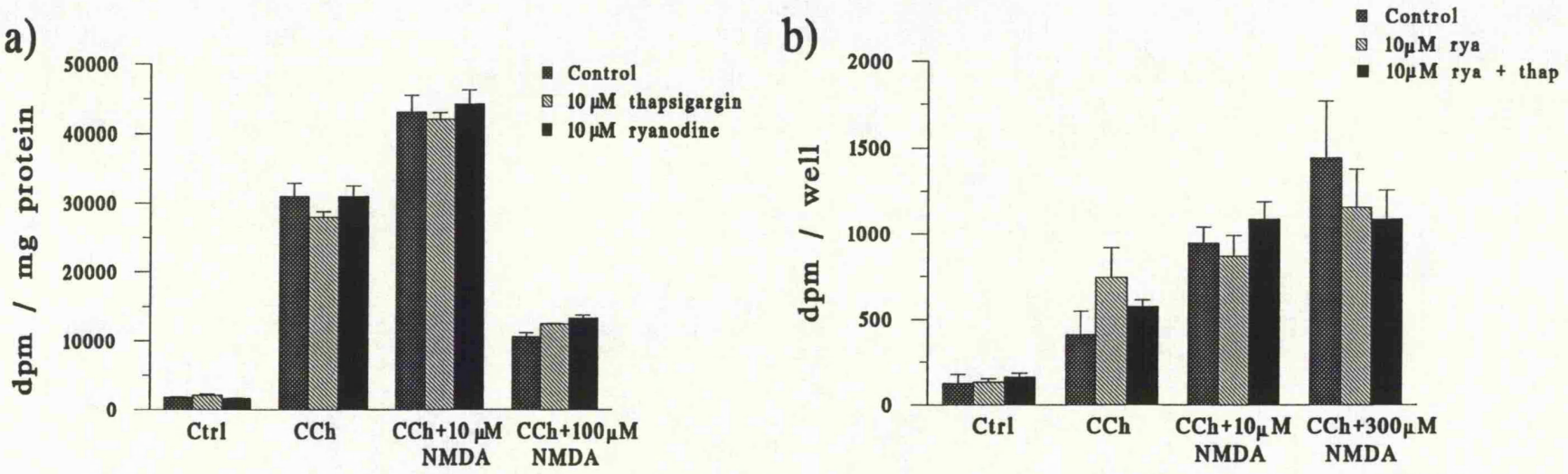


Fig. 6.4 ***a)*** **Lack of effect of ryanodine and thapsigargin on cortical slice $InsP_X$ accumulation.** Results from a typical example experiment performed in triplicate. Neither thapsigargin nor ryanodine significantly affected basal, CCh-evoked or NMDA-potentiated $InsP_X$ accumulation. In addition, neither affected the inhibition of the response to CCh induced by 100 μM NMDA.

b) **Lack of effect of ryanodine and thapsigargin on granule cell $InsP_X$ accumulation.** Results from a typical example experiment performed in triplicate. Pre- and co-incubation with ryanodine (10 μM) or ryanodine plus thapsigargin (each 10 μM) did not significantly affect basal, CCh-evoked or NMDA-potentiated $InsP_X$ accumulation.

$InsP_X$ levels in cerebellar granule cells (Table 6.2, Fig. 6.4b). Ryanodine also had no effect on CCh-evoked $InsP_X$ formation, or on NMDA-evoked potentiation of this response (Table 6.2, Fig. 6.4b). Dantrolene (30 μM) had no effect on any of these experimental conditions (Table 6.2).

In an additional series of experiments, ~10 min pre-treatment of cerebellar granule cells with the Ca^{2+} store releasing agent caffeine (10-50 mM) was found to abolish subsequent responses to a variety of agonists including CCh and NMDA (data not shown). However, the lack of specificity of this effect, abolishing responses to e.g. AMPA as well as store releasing agents, suggests that this may be unrelated to Ca^{2+} store effects. It has previously been reported that caffeine partially inhibits bradykinin-evoked PIC activation in PC12 cells (Zacchetti *et al.*, 1991). In one experiment in the present study, basal $InsP_X$ levels were markedly decreased by caffeine, such that the possibility of a toxic or PIC-inhibiting effect of prolonged caffeine treatment on cerebellar granule cells might be worth investigating in future experiments.

Experimental condition	% of control	Control response (fold over basal)	Response with agent (fold over basal)
Dantrolene on Basal	98 (n=2)	1.00	0.98
Ryanodine on Basal	110 (n=2)	1.00	110
Dantrolene on CCh	70 ± 14	4.93 ± 0.90	4.43 ± 1.05
Ryanodine on CCh	111 ± 45	6.67 ± 1.12	4.69 ± 1.48
Dantrolene on CCh + NMDA	90 ± 8	8.92 ± 1.92	8.29 ± 2.28
Ryanodine on CCh + NMDA	92 ± 9	9.35 ± 1.49	8.83 ± 1.94
Dantrolene on NMDA	100 (n=1)	1.11	1.11
Ryanodine on NMDA	not tested		

Table 6.2 Lack of effect of store modulators on $InsP_X$ formation in granule cells. Meaned results from a series of experiments, with the response in the presence of store modulators compared to the control responses in parallel wells from the same multidish. Pre-treatment with dantrolene (30 μM) or ryanodine (10 μM) did not inhibit either basal $InsP_X$ levels or $InsP_X$ accumulation stimulated by CCh, NMDA or CCh + NMDA. Pre-treatment with thapsigargin (10 μM) was similarly without effect. Percentages of control are presented as mean of mean changes over matched control from all individual experiments, whereas control and modified response columns are presented as meaned values of all experiments.

6.5 Discussion

A number of mechanisms can mediate adaptation to chronic agonist stimulation within the PI signalling system, including receptor phosphorylation, receptor internalization, G protein uncoupling and changes in transcription rate (see Wojcikiewicz *et al.*, 1993; Maloteaux and Hermans, 1994), the combined effect being a downregulation of PI turnover. At least one such mechanism may be responsible for the alteration in rate of $InsP_3$ production after 5-10 s of receptor activation, which results in biphasic $InsP_3$ accumulation as found in this and previous studies (see Wojcikiewicz *et al.*, 1993). Despite such time-dependent downregulatory mechanisms, the $InsP_x$ accumulation data in this study are, nevertheless, consistent with significantly elevated PIC activity over at least 20 min in granule cells stimulated with metabotropic agonists, and, less efficaciously, by ionotropic agents. No significant increase in $InsP_3$ accumulation was evoked by NMDA, consistent with a previous report in adult rat cortical slices which demonstrated that ionotropic agents predominantly elevate $InsP_1$ and $InsP_2$, with little effect on $InsP_3$ or $InsP_4$ levels (Baird and Nahorski, 1991). The ability of CCh to sustain $InsP_3$ accumulation in granule cells for at least 40 min indicates that desensitization mechanisms are unable to cause complete inhibition of $InsP_3$ accumulation in these neurones during the maintained presence of agonist even over a prolonged time period. Investigations into possible consequences of maintained $InsP_3$ production in granule cells are detailed in Chapter 7.

There was a marked difference in metabotropic / ionotropic interactions on $InsP_x$ levels in cerebellar granule cells compared to neonatal cortical slices, NMDA potentiating CCh-evoked responses in granule cells, but inhibiting CCh-evoked responses at high concentration in slices. NMDA-evoked inhibition of metabotropic $InsP_x$ formation in neonatal or adult brain slices has been widely reported (Baudry *et al.*, 1986; Schmidt *et al.*, 1987; Godfrey *et al.*, 1988; Morrisett *et al.*, 1990; Baird and

Nahorski, 1991; Challiss *et al.*, 1994a,b). The present findings may indicate a relative resistance of granule cells to acute NMDA-evoked neurotoxicity. It is possible that damage and anoxia during preparation of slices, and the prolonged incubation periods necessary for these experiments (see Methods), partially compromise neuronal viability, making this tissue more susceptible to NMDA neurotoxicity than granule cells. Given their prolonged depolarization due to the presence of 25 mM K^+ in the culture medium, cerebellar granule cells are likely to have elevated activity of metabolic processes which control the toxic effects of Ca^{2+} entry, as reported recently for spinal neurones cultured under similar depolarizing conditions (Tymianski *et al.*, 1994). It should be noted, however, that NMDA does cause neurodegeneration in cultured granule cells, although only after prolonged post-incubation periods in the present study (data not shown). The ability of Ca^{2+} entry activated by depolarization or ionotropic receptor agonists to activate $InsP_X$ formation directly, and to potentiate receptor-G protein-mediated formation, has been previously demonstrated in various neuronal preparations (Diamant and Atlas, 1989; Schwartz and Atlas, 1989; Baird and Nahorski, 1990,1991; Conn and Desai, 1991; Challiss and Nahorski 1994a,b; del Rio *et al.*, 1994), and the small direct effect of NMDA on $InsP_X$ in the present study is in agreement with such reports. However, several reports in granule cells have demonstrated a large NMDA-evoked $InsP_X$ response which has been attributed to a hypothetical metabotropic NMDAR (Nicoletti *et al.*, 1986; Hynie *et al.*, 1989; Raulli *et al.*, 1991). In the present study, the NMDA response is of similar magnitude to the response to other ionotropic agents, and is largely non-additive with them. This appears consistent with a mechanism of action restricted to Ca^{2+} sensitization, rather than G protein-mediated activation, of PIC. Some isoforms of PIC, activated by G_α subunits, have been reported to be sensitive to elevation of $[Ca^{2+}]_i$ (Eberhard and Holz, 1988; Fisher *et al.*, 1989; Lambert *et al.*, 1991; Cockcroft and Thomas, 1992; Rhee and Choi, 1992; Wojcikiewicz *et al.*, 1994b). As well as Ca^{2+}-sensitization of PIC by other agonists coupled to Ca^{2+} entry, a component of CCh-evoked $InsP_X$

accumulation has been reported to be activated by CCh-evoked Ca^{2+} entry in hippocampal and neuroblastoma preparations (Palazzi *et al.*, 1991; Wojcikiewicz *et al.*, 1994b). However, as an apparent absence of muscarinic receptor-evoked divalent cation entry has been demonstrated in granule cells in this study (see Chapter 5), consistent with the results of a previous study in these neurones (Irving *et al.*, 1992a), Ca^{2+} entry is unlikely to be an important mechanism mediating CCh responses in granule cells. The possible involvement of Ca^{2+} entry in CCh-evoked $InsP_3$ formation remains to be fully investigated in this preparation, although preliminary experiments indicate that CCh responses in nominally Ca^{2+}-free medium may not markedly differ from responses in normal Ca^{2+}-KHB in these neurones (D. Gray, personal communication). It is interesting to compare the ability of NMDA to potentiate CCh-evoked $InsP_X$ responses in granule cells with its lack of effect on CCh-evoked $[Ca^{2+}]_i$ elevations (Irving *et al.*, 1992a); the reason for this difference is unclear, although possibly indicative of maximal $InsP_3R$ activation by CCh-evoked $InsP_3$ production. Other evidence from the present study indicates that activation of virtually all $InsP_3Rs$ present may be necessary for maximal responses to metabotropic agonists (see Chapter 7), such that potentiation of $InsP_3$ formation by NMDA would not cause any increase in Ca^{2+} release.

The toxic effect of a high concentration of NMDA in cortical slices has, like the potentiation of the CCh-evoked response by a low concentration of NMDA, been attributed to the consequences of Ca^{2+} entry (Challiss *et al.*, 1994a,b). Experiments with thapsigargin, ryanodine and dantrolene in the present study indicated that there is little apparent involvement of Ca^{2+} release in CCh-, NMDA- or CCh and NMDA-evoked PIC activation in either cortical slice or granule cell preparations. This is in agreement with a recent study which found that pre-treatment with dantrolene in sublingual mucous acini inhibited CCh-evoked $[Ca^{2+}]_i$ elevation without significantly affecting CCh-evoked $InsP_3$ or $InsP_4$ production (Zhang and Melvin, 1993), and with

a study in SH-SY5Y neuroblastoma cells which reported no effect of thapsigargin alone on CCh-evoked $InsP_3$ production (Wojcikiewicz *et al.*, 1994b). As Wojcikiewicz and co-workers (1994b) reported an inhibitory effect of EGTA or the VOCC blocker Ni^{2+} on the plateau phase of CCh-evoked $InsP_3$ production in SH-SY5Y cells, and of EGTA on both peak and plateau phases in CHO cells expressing m3 receptors (CHO-m3 cells), a dependence of $InsP_3$ production on Ca^{2+} entry, but not on release from stores, might be concluded. However, that study also reported that while pre-treatment with thapsigargin, or with EGTA, did not affect CCh-evoked peak $InsP_3$ formation, pre-treatment with thapsigargin and EGTA in combination significantly decreased both peak and plateau production of $InsP_3$ (Wojcikiewicz *et al.*, 1994b). From this it was concluded that Ca^{2+} release from stores did contribute to PIC activation. Incubation with EGTA and thapsigargin may be expected to have severe and complex effects on cellular Ca^{2+} homeostasis, and it is difficult to conclude with certainty that their effect on $InsP_3$ production was due to loss of PIC activation evoked by a combination of Ca^{2+} entry and store release, particularly as the effect of EGTA plus thapsigargin in SH-SY5Y cells was reproduced by EGTA alone in CHO-m3 cells. The lack of effect of thapsigargin in that study, dantrolene in the study by Zhang and Melvin (1993), and of thapsigargin, ryanodine, thapsigargin plus ryanodine, and dantrolene in the present study, suggest rather that Ca^{2+} release from stores is not necessary for CCh-evoked stimulation of PIC. In addition, the present results demonstrate that Ca^{2+} entry is sufficient to explain modulation of CCh-evoked $InsP_x$ formation by NMDA. It must be noted, however, that the low [^{3}H]-labelling of granule cells compared to cortical slices contributed to large standard errors, making it difficult to rule out small modulatory effects of the agents used. Future experiments examining in greater detail the effect of store modulatory agents on the concentration-response relationships for CCh, and CCh in the presence of NMDA, might be helpful in fully establishing the presence or absence of effects of activation or inhibition of Ca^{2+} store release on $InsP_x$ production in granule cells.

A more recent study in this lab using SH-SY5Y neuroblastoma cells (G. Willars and S. Nahorski, in preparation) has provided possibly clearer evidence than that of Wojcikiewicz *et al.* (1994b) that release of Ca^{2+} from intracellular stores may have an important role in PIC stimulation, under some circumstances. Pre-stimulation with CCh was found to inhibit responses to another PI-linked agonist, bradykinin, perfused during the continued presence of CCh. The bradykinin $[Ca^{2+}]_i$ response recovered within <2 min after addition of atropine, but no recovery occurred after atropine when the experiment was conducted in very low $[Ca^{2+}]_o$. This is consistent with a necessity for Ca^{2+} entry to refill stores depleted by CCh in order for bradykinin to evoke a $[Ca^{2+}]_i$ response. The bradykinin-evoked $InsP_3$ response, as well as the bradykinin $[Ca^{2+}]_i$ response, was inhibited by CCh and recovered after atropine only in normal $[Ca^{2+}]_o$. It therefore appears that replete stores are necessary for significant bradykinin stimulation of PIC. Further studies are clearly needed to establish more convincingly the importance of entry and store release mechanisms in metabotropic receptor stimulation of PI turnover.

Given the contradictory evidence detailed above concerning the effect of release of Ca^{2+} stores on PIC stimulation, perhaps the most important conclusion which can be confidently drawn from the present results is that the various store modulatory agents used in Chapter 5 are unlikely to significantly inhibit CCh-evoked $[Ca^{2+}]_i$ responses by acting at the level of PI turnover. Activity of several of these modulatory agents at sites other than store release has been postulated in previous studies (Nohmi et al., 1991; El-Hayek *et al.*, 1992; Vercesi *et al.*, 1993; Rossier *et al.*, 1993; Nelson *et al.*, 1994). In combination with the lack of an inhibitory effect of thapsigargin or dantrolene on the rate of basal or stimulated Mn^{2+} quench (see Chapter 4), the present results suggest that the conclusions drawn in previous Chapters do not appear to be compromised by non-specific effects of these agents on sites differing from, or in addition to, Ca^{2+} stores in cerebellar granule cells. Future

experiments to examine the possible effects of BHQ on Mn^{2+} quench and $InsP_X$ production would be helpful in establishing the specificity of this agent for Ca^{2+} stores.

In the present study also, no involvement of Ca^{2+} released from stores was found in the acute NMDA-evoked toxicity apparently responsible for inhibition of CCh-evoked $InsP_X$ accumulation in cortical slices. Interestingly, chronic neurodegeneration in cultured cortical neurones, evoked by 9 h exposure to NMDA, has been found to be markedly inhibited by dantrolene pre-treatment (Frandsen and Shousboe, 1992). These two findings together may implicate NMDA-evoked CICR in later, but not early, phases of excitotoxicity. A variety of mechanisms are known to be involved in excitotoxic cell death (see Randall and Thayer, 1992; Tymianski *et al.*, 1993; Coyle and Puttfarcken, 1993; Szatkowski and Attwell, 1994; Pinelis *et al.*, 1994), and further experiments are required to address this possible differentiation of mechanisms involved in differing stages of neurodegeneration more directly.

7. InsP3 and Ryanodine receptors

7.1 Ca^{2+} release channels in cerebellar granule cells

The activation of Ca^{2+} store release by elevated $[Ca^{2+}]_i$ or $InsP_3$ has been central to the studies detailed in previous Chapters. The results described are consistent with the expression of at least one subtype of both $InsP_3Rs$ and RyRs in granule cells, but the characteristics of these intracellular receptors in this preparation have not previously been closely examined. Also, the ability of agonists to modulate, as well as stimulate, activity of $InsP_3Rs$ and RyRs in granule cells remains unknown. It was therefore important to investigate more directly the characteristics of the Ca^{2+} release channels present in these neurones, and the effects of cell-surface receptor stimulation on their expression and activation. In this Chapter, experiments designed to examine $InsP_3R$ expression, and the effects on this of chronic treatment with ionotropic and metabotropic agonists, using Western blotting for a specific subtype, and a radioligand binding assay, are reported. Subsequent epifluorescence $[Ca^{2+}]_i$ experiments in $InsP_3R$ downregulated cells, performed in order to determine whether the extent of $InsP_3R$ expression was important for Ca^{2+} signalling responses to ionotropic and metabotropic agonists, are also presented. Finally RyR subtype expression, and effects of agonists on expression and on functional RyR activity, examined in a parallel manner using Western blotting and $[Ca^{2+}]_i$ measurements, are included in this Chapter.

7.2 $InsP_3$ receptor expression

7.2.1 Consequences of maintained $InsP_3$ production

Evidence from the present study (Chapters 5 and 6) and previous reports (Irving *et al.*, 1990,1992a,b; Courtney *et al.*, 1990; Whitham *et al.*, 1991a,b; Fohrmann *et al.*, 1993) has demonstrated that, as for PI-linked receptors in many other cell types (see Berridge, 1993), agonists of muscarinic receptors and mGluRs in

cultured cerebellar granule cells are capable of evoking rapid and transient Ca^{2+} release from intracellular stores, via formation of the second messenger $InsP_3$. However, compared to other M_3 receptor-expressing preparations e.g. SH-SY5Y neuroblastoma cells (Forsythe *et al.*, 1992), or CHO-m3 cells (Tobin *et al.*, 1992), muscarinic stimulation of cerebellar granule cells evokes only a very small sustained plateau elevation of $[Ca^{2+}]_i$, despite a similar rapid peak $[Ca^{2+}]_i$ response (see Sections 5.1.2,5.4). The present study has demonstrated that muscarinic receptors evoke not only rapid peak production of $InsP_3$ in granule cells, but also a prolonged plateau phase (see Fig. 6.1), but the lack of a parallel sustained $[Ca^{2+}]_i$ elevation makes the physiological role of this sustained $InsP_3$ production uncertain.

Little is known of the consequences of prolonged exposure to metabotropic agonists on PI and Ca^{2+} signalling mechanisms in neurones. However, it is possible that maintained accumulation of $InsP_3$ has effects on neuronal responses other than a simple activation of Ca^{2+} release. A number of possible sites are present within the PI signalling system where adaptation to chronic stimulation may occur (Wojcikiewicz *et al.*, 1993; Maloteaux and Hermans, 1994), one such locus being an alteration in the expression of $InsP_3Rs$ on intracellular stores. $InsP_3R$ expression can be downregulated by chronic activation of muscarinic M_3 receptors in the SH-SY5Y neuroblastoma cell line (Wojcikiewicz and Nahorski, 1991; Wojcikiewicz *et al.*, 1992,1994a). It was therefore of interest to investigate whether such regulation also occurs in non-transformed primary cultures of granule cells, in response to activation of muscarinic, metabotropic glutamate, or indeed ionotropic receptor activation. Firstly, preliminary experiments were performed to investigate normal $InsP_3R$ expression in granule cells.

7.2.2 Expression of $InsP_3$ receptors in neonatal cerebellum

In a preliminary experiment using a binding assay for [^{3}H]-$InsP_3$ (see Methods), the level of $InsP_3$Rs in cerebellum from P8 Wistar rats was compared to the level in cerebellum of adults of the same strain. No significant differences in the K_D values were found, but the B_{max} values of the two preparations were markedly different, with much smaller expression levels of $InsP_3$Rs being found in neonatal cerebellum compared to adult (neonatal cerebellum B_{max} = 1.4 pmol / mg protein, K_D = 40 μM; adult cerebellum: B_{max} = 7.3 pmol / mg protein, K_D = 28 μM). It is likely that most cerebellar $InsP_3$Rs are expressed in cerebellar Purkinje cells (Furuichi *et al.*, 1989; Sharp *et al.*, 1993). However, the difference between neonatal and adult $InsP_3$R levels suggests that $InsP_3$R expression is developmentally regulated, such that levels in various types of neurones cultured from neonatal rats, including granule cells, might be expected, like neonatal cerebellum, to be similarly low.

7.2.3 Expression of type I $InsP_3$ receptors in cerebellar granule cells

Type I $InsP_3$R expression in cultured cerebellar granule cells was measured by Western blotting of granule cell membranes with a polyclonal Ab (CT1) raised in rabbits against a 12 amino acid sequence at the C terminus of the rodent type I $InsP_3$R (Wojcikiewicz *et al.*, 1994a). The experimental procedure required to obtain detectable $InsP_3$R immunoreactivity with these membranes involved exposure times for ECL reagents and for autoradiographic film (see Methods) which were longer than required for SH-SY5Y cells (Wojcikiewicz *et al.*, 1994a), indicative of the predicted low $InsP_3$R expression levels in granule cells. The CT1 Ab detected a single band in cerebellar granule cell membranes, at an approximate size of 260 kDa (Fig. 7.1). This is smaller than the MW of the rodent $InsP_3$R estimated from its cDNA sequence (313

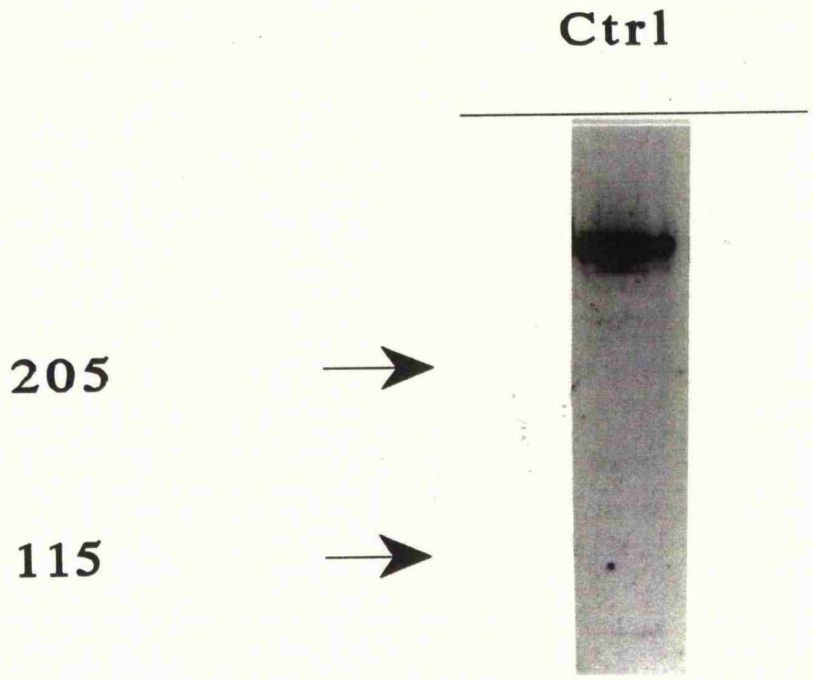


Fig. 7.1 Expression of type I $InsP_3Rs$ in cultured cerebellar granule cells. Example Western blot for type I $InsP_3Rs$ in 7-8 DIV cerebellar granule cell membranes (40 μg protein per lane). A polyclonal antibody raised against rodent type I $InsP_3Rs$ detected a single band in SDS-PAGE experiments with membranes prepared from cerebellar granule cells. By comparison with high MW markers, this was estimated to be ~260 kDa.

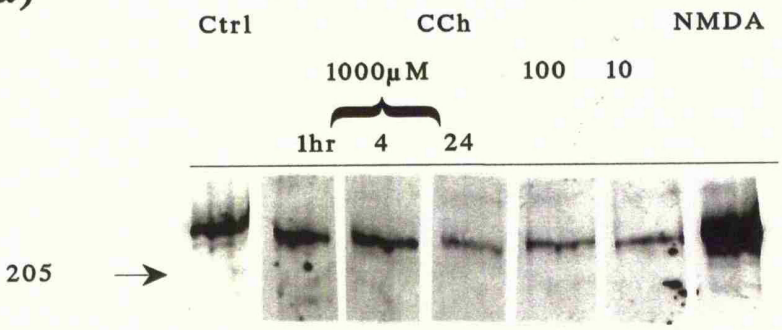
kDa, Furuichi *et al.*, 1989), a difference which may be explained by non-linearity of movement in the gel of large MW proteins, or by post-translational modification of the receptor. A similar MW estimation has been previously made from SDS-PAGE analysis for $InsP_3Rs$ in other preparations (Supattapone *et al.*, 1988; Furuichi *et al.*, 1989; Wojcikiewicz *et al.*, 1992).

7.3 Effect of metabotropic agonists on $InsP_3$ receptor expression

7.3.1 M_3 muscarinic receptor-mediated $InsP_3$ receptor downregulation

The effect of maintained $InsP_3$ production by CCh on $InsP_3R$ expression in granule cells was investigated, by incubation of cells with varying concentrations of CCh in the culture medium. As shown in Fig. 7.2a, >50% downregulation of type I $InsP_3R$ expression in cerebellar granule cells occurred within 1 h of addition of a maximally effective concentration of CCh (1 mM). Over longer time periods of incubation, downregulation was found to continue (Fig. 7.2a), apparently at a slower rate, such that as determined by densitometry, 1 mM CCh caused a downregulation of granule cell $InsP_3R$ expression over 24 h corresponding to a ~85% decrease in the band detected by CT1. This downregulatory effect was similar whether experiments were performed at 2-3 or 7-8 DIV. By analysis of the results of several experiments, the concentration of CCh which caused a half-maximal decrease in $InsP_3R$ levels after 24 h exposure was estimated to be ≤ 1 μM, much lower than the EC_{50} for CCh-stimulated $InsP_X$ accumulation in cerebellar granule cells (38 μM, Whitham *et al.*, 1991a). Both the $t_{1/2}$ and half-maximal agonist concentration for type I $InsP_3R$ downregulation in granule cells were, however, closely comparable to the characteristics of CCh-evoked type I $InsP_3R$ downregulation found in SH-SY5Y cells (Wojcikiewicz *et al.*, 1992,1994a). In one experiment, pre-treatment with thapsigargin (10 μM) did not inhibit CCh-evoked downregulation in granule cells (data not shown),

a)


Ctrl
CCh
NMDA
1000µM
100
10
1hr
4
24
205

b)

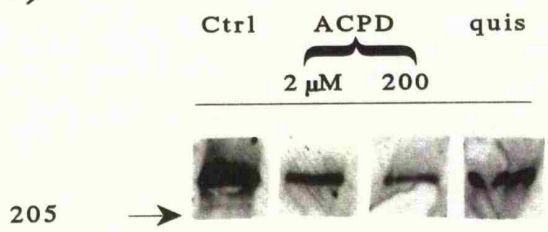

Ctrl
ACPD
quis
2 µM
200
205

Fig. 7.2 Effects of chronic activation of cell surface receptors on type I $InsP_3R$ expression. Example Western blots for chronic incubation with agonists (24 h unless otherwise stated) in culture medium. In all experiments shown, 40 μg of membrane protein was added to each lane.

a) Incubation with CCh caused a decrease in expression of type I $InsP_3Rs$ in granule cells which was both time- and concentration-dependent. 24 h incubation with 1 mM CCh evoked ~85% downregulation of type I $InsP_3R$ expression, as determined by densitometry. 1 mM CCh evoked ~50% downregulation after 1 h incubation, while over a number of experiments it was found that a marked decrease was evoked by submicromolar concentrations. Incubation with 200 μM NMDA in some experiments evoked an apparent increase in $InsP_3R$ expression.

b) 24 h incubation with the mGluR agonists ACPD (200 μM) and Quis (300 μM) at 2-3 DIV caused a concentration-dependent decrease in type I $InsP_3R$ levels. 200 μM ACPD evoked ~86% downregulation, as assessed by densitometry. 100-300 μM Quis evoked ~64% downregulation.

in contrast to the results of a more extensive investigation (Wojcikiewicz *et al.*, 1994a).

Expression in cerebellar granule cells of the other $InsP_3R$ subtypes now described (Mikoshiba *et al.*, 1993; Furuichi et al., 1994a) remains to be determined, so the effect of muscarinic receptor activation on overall $InsP_3R$ expression was measured using [^{3}H]-$InsP_3$ binding to granule cell membranes. The [^{3}H]-$InsP_3$ binding characteristics under control conditions (B_{max} = 863 ± 112 fmol / mg protein, K_D = 35 nM) were similar to the results of a previous study (Whitham *et al.*, 1991b). 24 h incubation with CCh (1 mM) was found to significantly affect $InsP_3$ binding, with a marked decrease in the B_{max} following CCh treatment compared to control cells (64 ± 6%), without a significant alteration of the K_D (Fig. 7.3). However, compared to the decrease in type I receptor immunoreactivity, total $InsP_3$ binding does not appear to be as greatly affected by chronic CCh treatment, which may indicate that other $InsP_3R$ subtypes are present in cerebellar granule cells, and are downregulated by CCh to a lesser extent than type I $InsP_3Rs$.

7.3.2 mGluR-mediated $InsP_3$ receptor downregulation

ACPD (200 μM) evoked a similar decrease (~86%) in granule cell type I $InsP_3R$ expression to CCh over a 24 h incubation period, at 2-3 DIV (Fig. 7.2b). Quis (100-300 μM) also downregulated expression (Fig. 7.2b), although to a smaller (~64%) and apparently more variable extent than ACPD. The findings with these agonists contrast with a previous study, which found that non-muscarinic PI-linked receptors did not downregulate type I receptor expression in a variety of cell lines (Wojcikiewicz *et al.*, 1994a). Brief (5 min) exposure to CCh did not cause downregulation in SH-SY5Y cells harvested 6 h later (Wojcikiewicz *et al.*, 1992), suggesting that $InsP_3$ production needs to be sustained over extended time periods for

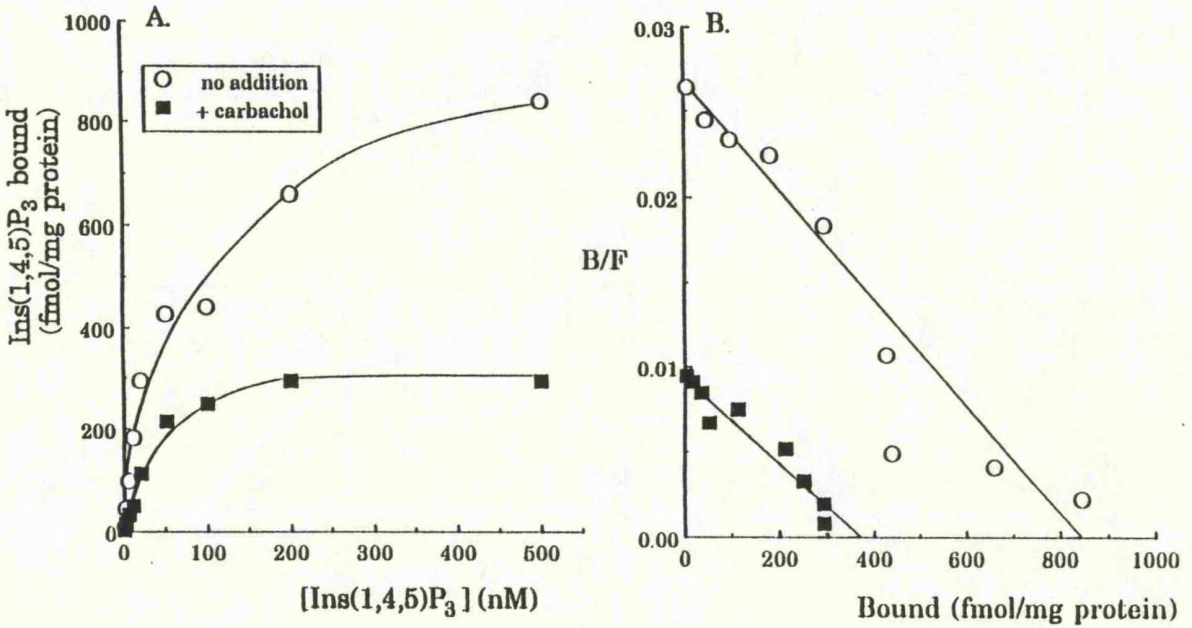


Fig. 7.3 Decrease in [^{3}H]-Ins(1,4,5)P_3 binding after incubation with CCh. Results are from a representative experiment performed in duplicate, similar results being obtained from 2 other experiments. Non-specific binding was defined as the binding remaining in the presence of 10 μM $InsP_3$, and was subtracted to yield the specific binding component shown. 24 h incubation with CCh (1 mM) markedly decreased [^{3}H]-Ins(1,4,5)P_3 binding to cerebellar granule cell membranes **(A)**, but had no significant effect on the K_D (35 nM), as shown by Scatchard analysis **(B)**. Over three independent experiments, CCh was found to decrease B_{max} values by 64 ± 6% (Control = 863 ± 112, +CCh = 299 ± 37 fmol / mg protein ($p < 0.01$)). (B/F is the ratio of bound to free [^{3}H]-$InsP_3$.)

$InsP_3R$ downregulation to occur. Maximally effective concentrations of ACPD caused a negligible increase in $InsP_3$ levels in granule cells as detected by mass assay, unlike the marked elevation evoked by CCh (see Section 6.2). However, $InsP_3Rs$ are activated in a positively co-operative manner by $InsP_3$ with released Ca^{2+} acting as a co-agonist at $InsP_3Rs$ (Bezprozvanny *et al.*, 1991; Finch *et al.*, 1991; Iino and Endo, 1992). Given also the low levels of CCh required to elicit significant downregulation, it appears likely that a very small, sustained accumulation of $InsP_3$ accompanied by a small, sustained release of Ca^{2+} may be sufficient to downregulate $InsP_3R$ expression in cerebellar granule cells.

7.4 Effect of NMDA on $InsP_3$ receptor expression

Experiments in the present study using ryanodine and dantrolene have indicated that the Ca^{2+} stores activated by NMDA appear to at least include a pool expressing RyRs (see Chapter 4). However, as it is now accepted that, by increased cytoplasmic Ca^{2+} levels, $InsP_3Rs$ can be sensitized to activation by resting or only slightly elevated levels of $InsP_3$ (Bezprozvanny *et al.*, 1991; Iino and Endo, 1992; see also Berridge, 1993), it appeared possible that at least some of the CICR evoked by NMDA in the present study could occur via $InsP_3R$ channels (see also Finch *et al.*, 1991). If Ca^{2+} release via $InsP_3R$ channels were to occur consequent to NMDA stimulation, chronic incubation with NMDA might lead to a similar pattern of downregulation as described above for agonists which elevate $InsP_3$ levels. As NMDA-evoked elevation of $[Ca^{2+}]_i$ would affect $InsP_3Rs$ in a different manner to PI-linked agonists, however, it is not certain whether activation of $InsP_3Rs$ in a manner independent of $InsP_3$ generation would be expected to downregulate expression. 24 h incubation with 200 μM NMDA in the presence of 10 μM glycine was found to reduce expression of type I InsP3Rs compared to control in only 1 out of 4 experiments. In the other 3 experiments, NMDA pre-treatment unexpectedly upregulated receptor levels (Fig. 7.2a), to a

variable extent (22-80% increase over control). There was no clear relationship between the consequences of NMDA treatment on $InsP_3R$ expression and DIV, and the mechanism and physiological importance of this upregulatory effect remains uncertain. Thus, further experiments to investigate the possible ability of NMDA to activate Ca^{2+} release via $InsP_3R$ channels were required (see below).

7.5 $[Ca^{2+}]_i$ responses in $InsP_3$ receptor-downregulated cells

It has previously been reported that chronic incubation of cerebellar granule cells with 100 μM CCh inhibits the maximal inositol phosphate response to subsequent CCh stimulation, in a time-dependent manner, such that 18 h incubation causes a decrease in response of ≥60% (Xu and Chuang, 1987; Dillon-Carter and Chuang, 1989). The decrease in inositol phosphate response evoked by 2 h incubation with CCh was not paralleled by a loss of muscarinic binding (Xu and Chuang, 1987), and was therefore ascribed to uncoupling of the muscarinic receptor from its G protein or of the G protein from PIC. However, longer muscarinic receptor activation did lead to decreased receptor expression. 18 h incubation with 100 μM CCh decreased binding of the muscarinic antagonist [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) to granule cell membranes by 60%, closely paralleling the inositol phosphate response inhibition, and 24 h incubation decreased [^{3}H]-QNB binding by 75% (Xu and Chuang, 1987; Fukamauchi *et al.*, 1991). This decrease appears to be largely, although not exclusively, due to an acceleration of muscarinic receptor degradation (Fukamauchi *et al.*, 1991). Interestingly, CCh pre-incubation has been found to be without effect on the inositol phosphate response evoked by other PI-linked agonists, indicating that M_3 receptors mediate homologous, but not heterologous, desensitization in these neurones (Dillon-Carter and Chuang, 1989). Given the $InsP_3R$ downregulation reported above, it was therefore of considerable interest in the present study to determine whether CCh pre-treatment would affect agonist-evoked $[Ca^{2+}]_i$ responses. The consequences of

CCh pre-treatment in cerebellar granule cells on responses to a range of agents was examined, notably CCh itself, an agonist of another PI-linked receptor (ACPD), ionotropic (NMDA) stimulation, and direct RyR stimulation (by caffeine). Any effect of CCh pre-treatment on subsequent CCh responses may be expected to be due to a combination of M_3 receptor downregulation, uncoupling of M_3 receptors from PIC activation, and downregulation of $InsP_3R$ expression. However, any effect of CCh pre-treatment on $[Ca^{2+}]_i$ responses evoked by ACPD, which on the basis of the previous findings (Dillon-Carter and Chuang, 1989) will have intact PI responses under these conditions, may be ascribed mainly or solely to the decrease in $InsP_3R$ expression.

Granule cells were pre-treated with CCh in culture medium as before, and subsequently loaded with fura 2-AM in KHB in the absence of CCh. Cells loaded with fura 2-AM in the presence of CCh and left for >30 min post-incubation in CCh-free KHB had very similar responses to the findings described below, consistent with the reported slow rate of $InsP_3R$ turnover ($t_{1/2}$ > 8h, Wojcikiewicz *et al.*, 1994a). The basal 340 / 380 nm ratio in CCh-pre-treated cerebellar granule cells was not significantly different to that in control cells (control ratio = 0.97 ± 0.02 (n=61), CCh-pre-treated ratio = 1.06 ± 0.04 (n=56)). CCh (1 mM) evoked a peak rise in $[Ca^{2+}]_i$ in granule cells pre-treated for 24 h which was greatly reduced compared to control cells from the same preparation (Table 7.1). Peak responses to ACPD (200 μM) in CCh-pre-treated cells were also greatly reduced compared to control cells (Table 7.1; Fig. 7.4a).

CCh-evoked $[Ca^{2+}]_i$ responses appear to involve activation of RyRs (Chapter 5), probably consequent to Ca^{2+} release via $InsP_3Rs$. However, $[Ca^{2+}]_i$ responses to caffeine (50 mM) were unaffected by chronic CCh pre-treatment (Table 7.1; Fig. 7.4), indicative of an apparent lack of downregulation of RyR expression or function by prolonged CCh treatment, in contrast to the effect on $InsP_3Rs$. As shown in Chapter 4,

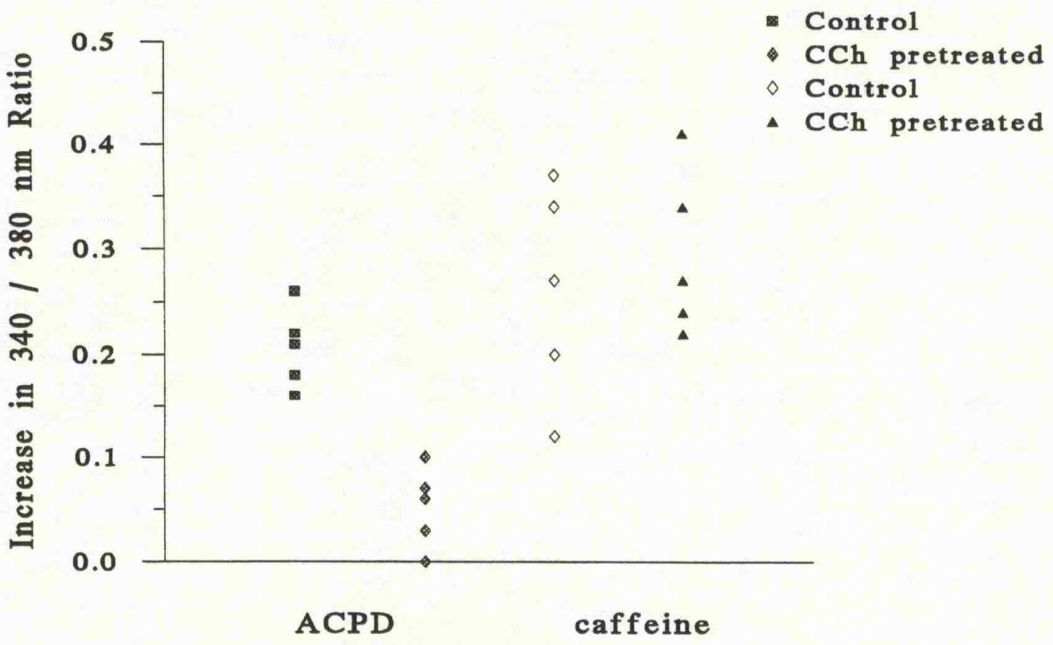


Fig. 7.4 Effects of $InsP_3R$ downregulation on Ca^{2+} signalling in cerebellar granule cells. Results of a single series of experiments for challenge with ACPD (200 μM) or caffeine (50 mM), using groups of granule cells subjected to 24 h CCh (1 mM) pre-incubation, compared to other groups from the same culture preparation not pre-treated with CCh. CCh pre-treatment markedly decreased ACPD-evoked $[Ca^{2+}]_i$ responses, but had no significant effect on responses to caffeine.

the $[Ca^{2+}]_i$ response to NMDA in cerebellar granule cells involves a significant component due to CICR. The NMDA-evoked response is partially ryanodine-sensitive (see Chapter 4), but as discussed above (see Section 7.4) it was possible that NMDA-evoked CICR could also occur partially via $InsP_3Rs$. To investigate this possibility, the NMDA response was therefore also examined in cells pre-treated with CCh for 24 h. The response to NMDA (200 μM) after CCh-evoked $InsP_3R$ downregulation was not significantly different to that in control cells, in terms of either peak or plateau response size (Table 7.1).

Agent	Peak $[Ca^{2+}]_i$ Response (% of control)
CCh	23 ± 14
ACPD	27 ± 1
caffeine	110 ± 13
NMDA peak	97 ± 8
NMDA plateau	97 ± 13

Table 7.1 Effects of $InsP_3R$ downregulation on Ca^{2+} signalling in cerebellar granule cells. Mean results from at least 3 series of experiments from different culture preparations, with meaned values from each day treated as a single data-set. Effects of 24 h incubation with CCh (1 mM) on subsequent responses evoked by CCh (1 mM), ACPD (200 μM), caffeine (50 mM) and NMDA (200 μM).

7.6 RyR expression

The expression of RyR subtypes was examined in cerebellar granule cells using Abs specifically targeted against the known RyR subtypes. In preliminary experiments, type II RyR Ab reacted with a high MW band in brain microsomes and, weakly, in membranes prepared from whole cerebellum, while the type III Ab detected a band in membranes from whole cerebellum (data not shown). Western blotting with 40 μg cerebellar granule cell membrane protein per lane, the same amount as used for $InsP_3R$ determination, yielded little if any immunoreactivity to the Abs tested. With 100 μg membrane protein per lane, however, a faint high MW band was detected using the type II RyR Ab (Fig. 7.5). No similar band was detected using the type III Ab even at this protein concentration (Fig. 7.5). In parallel studies, no band was detected for granule cell membranes using a type I Ab (J. Mackrill, personal communication). Low RyR expression in cerebellar granule cells was not unexpected, as [^{3}H]-ryanodine binding has been found to be >10 fold lower in cerebellum than in the rest of brain (Lai *et al.*, 1992).

7.7 RyR downregulation

Initial experiments were performed by pretreating granule cells with NMDA (200 μM) for 24 h, and subsequently comparing type II RyR expression to membranes derived from cells in parallel wells. The results of these experiments (not shown) suggested that NMDA may decrease RyR expression, in a dizocilpine-blockable manner. However, the low level of expression detected made such studies inherently prone to error and misinterpretation. Also, Panceau S staining revealed that not all of the RyRs present in the gel crossed over into the nitrocellulose using the present protocol (J. Mackrill, personal communication), thus making determination of changes in expression levels by this method highly problematical. Subsequent experiments were therefore performed using a decrease in caffeine-evoked $[Ca^{2+}]_i$ elevation as the

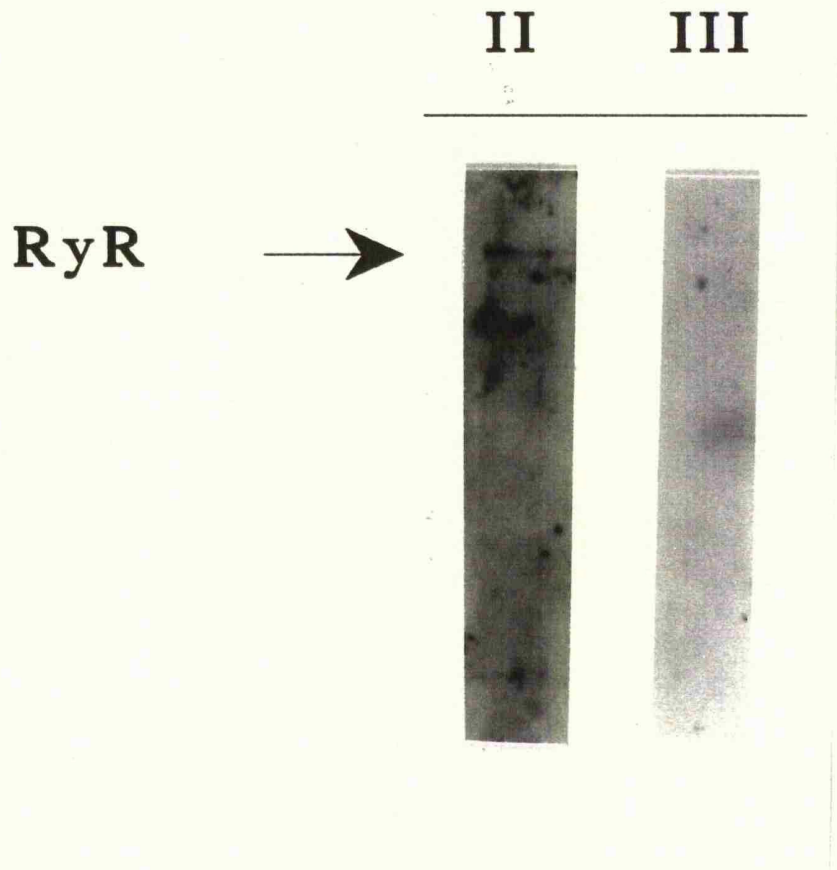


Fig. 7.5 Expression of type II but not type III RyRs in cerebellar granule cells. Western blot of granule cell membranes (100 μg per lane) with subtype-specific RyR Abs. Low but detectable expression of a high MW protein was detected with the type II Ab, but no band was found with the type III Ab.

measure of RyR downregulation or desensitization. 24 h pre-treatment with 200 μM NMDA was found to have little effect on the basal 340 / 380 nm ratio (control ratio = 0.88, NMDA-pre-treated ratio = 0.92 (n=2 series of experiments)), consistent with an effect not mediated via excitotoxicity (see also Discussion). However, in a small number of experiments chronic NMDA pre-treatment decreased the subsequent response to NMDA compared to the response in control cells from a parallel well (NMDA peak response in pre-treated cells = 53.3% of control, plateau elevation = 54.9% of control). This may be partly due to a downregulation or desensitization of NMDARs, but an effect via alteration of RyR expression or function was also considered possible. To examine this, the effect of NMDA pre-treatment on responses to caffeine was also examined. The $[Ca^{2+}]_i$ elevation evoked by 50 mM caffeine appeared reduced by this treatment, compared to the response from cells from a parallel well (Fig. 7.6). However, the average caffeine response in this series of experiments was small, as the experiments were performed at 6-9 DIV, which made it impossible to determine the degree of response change with great accuracy.

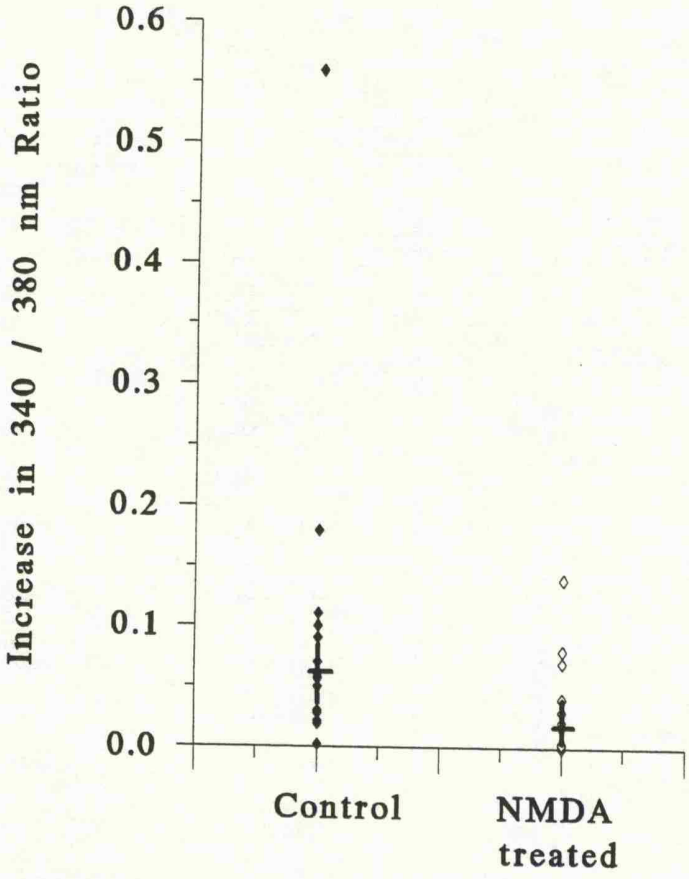


Fig. 7.6 Effect of NMDA on $[Ca^{2+}]_i$ responses evoked by caffeine. Scatter-graph of results of experiments performed on three separate granule cell preparations. NMDA pretreatment appeared to evoke a moderate decrease in the response to 50 mM caffeine.

7.8 Discussion

Prolonged stimulation of cell-surface receptors can lead to a desensitization of signal transduction by a variety of processes, such as phosphorylation, internalization, uncoupling and changes in transcription rate (see Wojcikiewicz *et al.*, 1993; Maloteaux and Hermans, 1994). While activation of M_3 receptors in cerebellar granule cells evokes only a very small sustained plateau elevation of $[Ca^{2+}]_i$ (Chapter 5), the results in the present Chapter demonstrate that there is at least one functional consequence of maintained muscarinic receptor-mediated activation of $InsP_3$ production, a downregulation of $InsP_3R$ levels. Thus, downregulation of PI signal transduction by prolonged agonist stimulation occurs by a plurality of mechanisms in granule cells, as CCh pre-treatment also decreases muscarinic receptor binding (Xu and Chuang, 1987), and uncouples muscarinic receptors from inositol phosphate production (Xu and Chuang 1987; Dillon-Carter and Chuang, 1989). Agonist-evoked $InsP_3R$ downregulation has previously been described in a transformed neuroblastoma cell line (Wojcikiewicz and Nahorski, 1991; Wojcikiewicz *et al.*, 1992,1994a), and the present study demonstrates that this also occurs within a more physiologically relevant system, primary cultured neurones. A previous study (Wojcikiewicz *et al.*, 1994a) indicated that M_3-mediated downregulation in SH-SY5Y neuroblastoma cells is due to the accelerated proteolytic degradation of $InsP_3Rs$. Changes in type I $InsP_3R$ mRNA levels did not correlate with the kinetics of the CCh-evoked decrease in receptor expression, whereas CCh accelerated the loss of radioactivity from type I $InsP_3Rs$ immunoprecipitated from [^{35}S]-methionine-labelled SH-SY5Y cells, with a $t_{1/2}$ comparable to that of receptor downregulation. Further work is necessary to demonstrate whether a similar mechanism is involved in $InsP_3R$ downregulation in cerebellar granule cells.

Wojcikiewicz *et al* (1994a) also reported that non-muscarinic PI-linked receptors did not downregulate type I receptor expression, in a variety of cell types. The present findings show that downregulation of $InsP_3Rs$ in cultured granule cells is not a phenomenon specifically associated with activation of muscarinic receptors, but rather may be a common mechanism of action of PI-linked receptors which evoke prolonged stimulation of $InsP_3$ production. Whilst the physiological importance of such a decrease in the number of $InsP_3Rs$ remains uncertain, the sensitivity of $InsP_3Rs$ to low levels of agonist suggests that prolonged 'tonic' release of transmitters such as ACh or glutamate *in vivo* might be able to control $InsP_3R$ expression in target neurones. It is therefore also possible that glutamate present in components of the culture medium, or released into it by granule cells, could have contributed to the relatively low $InsP_3R$ expression levels detected in this study.

Prolonged stimulation of glutamatergic receptors is widely believed to be involved in several neurodegenerative conditions (Wieloch *et al.*, 1985; Greenamyre *et al.*, 1987; Robinson and Coyle, 1987; Choi, 1988; Young *et al.*, 1988; Giulian *et al.*, 1990; Coyle and Puttfarcken, 1993; Szatkowski and Attwell, 1994), and a decrease in $InsP_3R$ expression evoked by glutamate under such conditions could play a role in mGluR-mediated neurotoxicity. Alzheimer's disease is one of the many conditions in which glutamate-evoked toxicity has been implicated (see Robinson and Coyle, 1987; Choi, 1988). Intriguingly, a marked and specific loss of $[^3H]$-$InsP_3$ binding has recently been found in the post-mortem brains of Alzheimer's disease patients, with an 86% loss in cerebellum compared to age-matched controls (Garlind *et al.*, 1994). An involvement of muscarinic mechanisms in Alzheimer's disease has also been widely suggested in the light of loss of cholinergic projections from the basal forebrain to the cortex and hippocampus, and region-specific changes in expression of muscarinic receptors, which are characteristically found in this condition (see McKinney and Coyle, 1991; Aubert *et al.*, 1992). Thus, downregulation of $InsP_3R$ expression in

Alzheimer's disease could occur as a consequence of either of the mechanisms, chronic muscarinic or mGluR activation, demonstrated in this Chapter.

Pre-incubation with NMDA, unlike metabotropic agonists, did not downregulate $InsP_3R$ expression, but rather in some cases appeared to increase expression levels over control. One possible explanation for this finding would be that the increase in receptor expression occurred as a consequence of NMDA-evoked neurotoxicity. It should be noted, however, that in the present study addition of NMDA into the culture medium of granule cells evoked little cell damage, although significant cell death (assessed by lactate dehydrogenase release or decrease in [^{3}H]-ouabain binding (see Choi *et al.*, 1988; Markwell *et al.*, 1991)) was apparent 24 h after even relatively brief periods of incubation with NMDA in high-Ca^{2+} (2.6 mM) KHB (data not shown). This may be because [Ca^{2+}] in culture medium (~1.3 mM) is insufficient to mediate a severe toxic insult, or because the toxicity experiments were performed at DIV before excitotoxic mechanisms had fully developed. Either way, neurotoxicity is unlikely to be a major mediator of NMDA-evoked responses under the conditions used for these $InsP_3R$ expression experiments. An alternative explanation is that, as $InsP_3Rs$ display a bell-shaped concentration-response curve to cytoplasmic $[Ca^{2+}]_i$ (Bezprozvanny *et al.*, 1991; Finch *et al.*, 1991), NMDA-evoked $[Ca^{2+}]_i$ elevation does indeed modulate $InsP_3Rs$, but serves to decrease rather than increase $InsP_3R$ activation, by elevating $[Ca^{2+}]_i$ beyond the concentration range at which Ca^{2+} activates $InsP_3Rs$, and into the inhibitory range. It could thus remove ongoing downregulation evoked by $InsP_3R$ activation by unstimulated levels of $InsP_3$, and so increase $InsP_3R$ expression levels over control. The upregulatory effect of NMDA in the present study was evoked in culture medium containing 25 mM K^+, and it is possible that this depolarizing stimulus itself modulates $InsP_3R$ expression in a manner analogous to NMDA, by evoking a maintained entry of Ca^{2+} (thus perhaps counteracting the hypothesized downregulatory action of released glutamate mediated

by mGluRs). It would therefore be of considerable interest to compare $InsP_3R$ expression in granule cells cultured under 25mM K^+-depolarizing conditions with its expression in granule cells maintained in 5 mM K^+. Alterations in $InsP_3R$ levels between cells grown in these different conditions could account for some of the reported differences in responsiveness between cells cultured under these differing conditions (see Cox *et al.*, 1990; Irving, 1991 - Ph.D. Thesis, University of Bristol; Zhao and Peng, 1993). Changes in RyR expression could also partially mediate these differences (see below).

The effects of $InsP_3R$ downregulation by chronic muscarinic receptor activation on Ca^{2+} signalling in cultured cerebellar granule cells provides further information on the mechanisms of action of CCh and other agonists on Ca^{2+} stores in these neurones. Subsequent CCh-evoked $[Ca^{2+}]_i$ responses were greatly inhibited by CCh pre-treatment, a not unexpected result given the decreases in M_3 receptor expression and muscarinic receptor-stimulated inositol phosphate production, as well as $InsP_3R$ expression, which have been shown to be induced by chronic muscarinic receptor stimulation in granule cells (Xu and Chuang 1987; Dillon-Carter and Chuang, 1989). Indeed, given the multiplicity of downregulatory consequences of chronic CCh pre-treatment (see also Maloteaux and Hermans, 1994), it is perhaps surprising that any CCh-evoked $[Ca^{2+}]_i$ response can be detected. The present findings are in broad agreement with a previous study in granule cells, which reported that 12 h pre-treatment with 100 μM CCh caused a complete loss of response to subsequent addition of CCh in 50% of cells, and a ~65% decrease in the $[Ca^{2+}]_i$ response in the remaining cells (Fohrmann *et al.*, 1993).

CCh-evoked $InsP_3R$ downregulation as determined by $[^3H]$-$InsP_3$ binding appeared greater than as determined by type I $InsP_3R$ Western blotting. This may be due either to the methodological differences themselves, or to the detection by $[^3H]$-

$InsP_3$ binding but not by Western blotting of other $InsP_3R$ subtypes. The present study thus does not advance speculation on the roles of different $InsP_3R$ subtypes in metabotropic responses, as several $InsP_3R$ subtypes may be present in granule cells, and may be at least to some extent downregulated by CCh pre-treatment. Thus the $InsP_3Rs$ remaining after CCh pre-treatment could be of more than one subtype. Also, the percentage change in mGluR-mediated $[Ca^{2+}]_i$ responses after CCh pre-treatment is similar to the percentage decrease in total $InsP_3R$ expression (73% decrease in ACPD-evoked $[Ca^{2+}]_i$ response, 64% decrease in $[^3H]$-$InsP_3$ binding), thus providing no evidence of differential sensitivity to $InsP_3$ of a downregulation-resistant proportion or subpopulation of $InsP_3Rs$ in these cells. It has previously been shown that CCh only directly causes homologous, not heterologous, desensitization of receptor expression and PI responses in granule cells (Dillon-Carter and Chuang, 1989). The present study demonstrates that via an action not on cell-surface receptor expression or receptor-effector coupling, but rather downstream of PI turnover, by downregulating $InsP_3R$ expression, CCh pre-treatment markedly inhibits $[Ca^{2+}]_i$ responses evoked via another PI-linked receptor, mGluR. This effect may thus be considered an indirect heterologous desensitization of mGluR-mediated responses.

The marked inhibitory effect of CCh-evoked $InsP_3R$ downregulation on the $[Ca^{2+}]_i$ response to ACPD demonstrates that a significant level of $InsP_3R$ expression is needed to maintain normal metabotropic signalling. Thus, few if any 'spare' $InsP_3Rs$ may be present, such that agonist-evoked modulation of $InsP_3R$ expression levels *in vivo* could have significant effects on physiological Ca^{2+} signalling. Significant, but more moderate, decreases in maximal $InsP_3$-evoked $^{45}Ca^{2+}$ release have been reported in permeabilized SH-SY5Y cells (48%), and CHO cells expressing m1 (16%) or m3 muscarinic receptors (30%), after prolonged CCh treatment (Wojcikiewicz and Nahorski, 1993). $InsP_3R$ expression was downregulated in those cells to a similar extent as found for granule cells in the present study, so the lesser effect of CCh pre-

treatment on Ca^{2+} mobilization especially in CHO cells is perhaps indicative of a greater degree of $InsP_3R$ reserve in CHO cells than in cerebellar granule cells. However, it should be noted methodological differences, notably the possible loss of store integrity due to cell permeabilization in $^{45}Ca^{2+}$ studies (see Putney and Bird, 1993), might also account for such differences.

III

The present study has provided evidence for the expression of type II RyRs in cultured cerebellar granule cells. Type I RyRs are associated with Ca^{2+} mobilization activated directly by membrane depolarization, whereas types II and III appear to be activated by locally elevated $[Ca^{2+}]$ (see Meissner, 1994). In a study of RyR expression in brain by Lai and co-workers (1992), immunoreactivity against a type II Ab was found for microsomes made from whole brain minus cerebellum, but was undetectable for cerebellar microsomes. Kuwajima et al (1992), however, detected light immunohistochemical staining of granule cells *in situ* with a type I/II pan Ab, and noted that Western blots of rat cerebellar membranes derived from a mutant strain lacking granule cells had less reactivity against a type II RyR Ab, but not against a type I RyR Ab, than control cerebellar membranes, consistent with type II RyR expression in granule cells *in vivo*. The caffeine-evoked elevation of $[Ca^{2+}]_i$ reported in Chapter 3 and the present Chapter is also consistent with expression of type I or type II RyRs in cultured granule cells (see Meissner, 1994), while the apparent involvement of RyRs in CICR mechanisms in these cells (see Chapters 4 and 5) is consistent with expression of type II or type III receptors. It must be noted, however, that low expression levels of type I or type III RyRs could still be present in cultured granule cells, as there is no guarantee that the Abs used in the present study have equal efficacy in detecting receptor subtypes. Indeed, lower background reactivity for the type III Ab appeared to be detected compared to type II reactivity in the results of the present study. While

early studies on RyR expression in granule cells *in situ* suggested that type II receptors were present, the subsequent discovery of type III RyRs has made interpretation of such studies problematical. However, a recent report has confirmed that type II RyRs appear to be the predominant subtype present in the adult granule cell layer *in vivo*, with no type I present, and that low levels of type III are found in some but not all cells in this layer (Furuichi *et al.*, 1994b). Thus, the RyR subtype identified in cultured granule cells may also be the predominant form *in vivo*. CICR activation described in the present investigation may thus reflect mechanisms which could occur in granule cells *in vivo*, given that in the granule cell layer NMDARs (Garthwaite and Brodbelt, 1989; Farrant *et al.*, 1994), VOCCs (Maeda *et al.*, 1989) and type II RyRs (Kuwajima *et al.*, 1992; Furuichi *et al.*, 1994b) are expressed.

Muscarinic $[Ca^{2+}]_i$ elevations in granule cells appear to involve CICR via RyR activation (Chapter 5; Irving et al., 1992b). Unfortunately, Western blotting cannot with confidence be utilized to investigate the effect of CCh on RyR expression, as the RyR expression levels appear very low. For this reason, the effect of CCh on RyRs was measured by comparison of the size of caffeine-evoked $[Ca^{2+}]_i$ transients in chronically CCh pre-treated or control cells. On this basis, prolonged muscarinic receptor activation appears not to affect RyR function. Chronic CCh pre-treatment also did not affect subsequent NMDA-evoked $[Ca^{2+}]_i$ responses in these neurones. Given that $InsP_3Rs$, but not apparently RyRs, are downregulated by CCh pre-treatment, this suggests that activation of $InsP_3Rs$ may not contribute significantly to NMDA-evoked elevation of $[Ca^{2+}]_i$ in cerebellar granule cells. This is consistent with the finding that chronic NMDA pre-treatment did not downregulate type I $InsP_3R$ expression, but rather apparently caused an upregulation via an undetermined mechanism. NMDA-evoked CICR may, therefore, occur solely via type II RyRs in granule cells.

The preliminary findings in the present investigation of apparent downregulation of RyR function by chronic incubation with NMDA indicates another possible mechanism by which agonists, or culture conditions, may modulate neuronal function. It has been suggested that culturing spinal neurones in the presence of 25 mM K^+ decreases CICR compared to control, resulting in decreased NMDA-evoked neurotoxicity without affecting NMDAR channel currents (Tymianski *et al.*, 1994). This finding may imply a Ca^{2+} entry-mediated downregulation of RyR activity in spinal neurones, consistent with indications from the present work. Thus NMDA treatment might increase $InsP_3R$ expression and decrease RyR function, which could have important consequences for cellular Ca^{2+} signalling. However, a study of NMDA and K^+ effects on RyR responsiveness at a developmental stage at which more prominent caffeine responses are present will be necessary to determine the validity of the above supposition.

8. Conclusions and future directions

8.1 Main conclusions

8.1.1 Mechanisms of $[Ca^{2+}]_i$ elevation by NMDA and K^+

The present study has significantly increased knowledge of the mechanisms involved in Ca^{2+} signalling in cultured cerebellar granule cells, the importance of which could be widespread as common $[Ca^{2+}]_i$-elevating mechanisms may be central to signal transduction in a wide variety of neurones. Using epifluorescence microscopy with fura 2-loaded granule cells to measure $[Ca^{2+}]_i$, two different ionotropic agents, K^+-depolarization and NMDA, have been found to activate CICR, a phenomenon which appears to account for much of the elevation of $[Ca^{2+}]_i$ which these agents evoke. K^+-depolarization of cultured cerebellar granule cells activates Ca^{2+} entry via several types of VOCC but not NMDAR channels, whereas NMDA activates entry via NMDAR channels with minimal stimulation of VOCCs. However, these independent pathways mediating Ca^{2+} entry converge at the Ca^{2+} store level, being sensitive to inhibition by the same agents, thapsigargin, ryanodine and dantrolene. Partial additivity of the inhibitory effects of thapsigargin and ryanodine was found with respect to the NMDA-evoked response, which would be difficult to explain in terms of activation of a single homogenous Ca^{2+} pool (see below).

Also in the present study, Mn^{2+} quench of fura 2 fluorescence has been demonstrated to be a convenient, reliable and reproducible method of assessing divalent cation-permeant channel activation in cultured granule cells. This technique lacks the ambiguity of interpretation possible from conventional fluorescence-based techniques which may measure the consequences of a variety of mechanisms involved in $[Ca^{2+}]_i$ elevation. While it is arguable that small effects on membrane Ca^{2+} permeability, such as those which are possibly associated with the activation of AMPA or muscarinic receptors, or of direct Ca^{2+} store depletion, in cerebellar granule cells, may not be easily detected using this method, it is clearly a useful method by which to

assess changes in membrane divalent cation permeability which are large and sustained. In the present study, complexity in the mechanisms of Ca^{2+} entry evoked by NMDA was suggested, as NMDA-evoked peak and plateau $[Ca^{2+}]_i$ elevations were found to be of similar size, but differences in the rates of Mn^{2+} quench were detected which correlated temporally with these two similar magnitude phases of the NMDA response. The reason for this difference between the NMDA response as measured by $[Ca^{2+}]_i$ and by Mn^{2+} quench remains to be fully established. Possibilities include differential store involvement in the two phases, not proven in the present study, or time-dependent activation of other mechanisms such as uptake into mitochondria or accelerated Ca^{2+} extrusion. The Mn^{2+} quench technique has also assisted in establishing the specificity of action of the store-modulating agents thapsigargin and dantrolene, which as they inhibit NMDA $[Ca^{2+}]_i$ responses, without affecting NMDA-evoked Mn^{2+} quench, were concluded to be acting intracellularly, presumably at the Ca^{2+} store level. It is now possible to envisage many further uses for the Mn^{2+} quench technique in neuronal preparations.

8.1.2 Ca^{2+} stores activated by CCh

Another important finding of this study is that the muscarinic receptor agonist CCh appears to activate both IICR and apparently CICR in granule cells. Thus, ryanodine, as well as the store-depleting agents thapsigargin and BHQ, abolishes metabotropic responses, as does caffeine activation of RyRs. The ability of caffeine or CCh pre-treatment to inhibit subsequent responses to each other indicates that the majority of the signal evoked by both agents was due to activation of release from a common Ca^{2+} pool. The relationship between RyR- and $InsP_3R$-expressing pools remains to be fully elucidated in this preparation. However, BHQ markedly inhibited CCh responses without significantly affecting the maintained phase of NMDA responses, whereas thapsigargin inhibited both responses. Thus, CCh may activate

CICR secondary to depletion of a separate $InsP_3R$-expressing pool, the CICR pool probably being identical to that activated by ionotropic stimulation. A model for Ca^{2+} stores and their release by ionotropic and metabotropic agonists in cerebellar granule cells has been proposed as a consequence of several series of experiments in the present study (see Fig. 5.11). This model hypothesizes the existence of two distinct $InsP_3R$ and RyR-expressing pools in granule cells, which are distinguishable on the basis of the pharmacological sensitivity of their Ca^{2+}ATPases. The evidence for this model is substantial but by no means compelling, and several other explanations for the present results can be envisaged. Indeed, the model does not account for all findings made in the present study, notably partial additivity of the effects of thapsigargin and ryanodine on NMDA responses, and it has been deliberately proposed as the simplest working model, for guidance, rather than as a prescriptive or dogmatic conclusion to the present study of Ca^{2+} mobilization. The key purpose of developing such a model is that it can be experimentally tested in a variety of ways, and subsequently modified or abandoned in favour of a more convincing model.

8.1.3 Modulation of $InsP_3R$ and RyR expression

Maintained stimulation with CCh or the metabotropic glutamate agonists Quis and ACPD was found to downregulate $InsP_3R$ expression in granule cells, with further experiments demonstrating that CCh-evoked downregulation has interesting and important consequences for Ca^{2+} signalling. Thus, chronic CCh pre-treatment decreased subsequent $[Ca^{2+}]_i$ responses to both CCh and ACPD, indicating that agonist-evoked downregulation of $InsP_3Rs$ may be sufficient to decrease Ca^{2+} mobilization evoked by PI-linked agonists to a similar extent as the degree of $InsP_3R$ downregulation, without any detectable effect on caffeine or NMDA, RyR-mediated, responses. The apparently parallel relationship between $InsP_3R$ expression and $InsP_3$-mediated $[Ca^{2+}]_i$ mobilization provides initial information concerning the degree of

$InsP_3R$ 'reserve' in granule cells. In contrast to the effects of maintained metabotropic stimulation, chronic NMDA pre-treatment was found in this study to cause an apparent increase in $InsP_3R$ expression, and a possible decrease in RyR function as measured by caffeine responsiveness. These effects were unexpected and novel, with potentially at least as much importance as the metabotropic agonist-evoked $InsP_3R$ downregulation described above.

8.2 Future experimental approaches

8.2.1 Functions of NMDA- and K^+-evoked Ca^{2+} store release

NMDAR activation and depolarization play important roles in neuronal signalling in physiological and pathological situations, and so the mechanisms by which their responses are mediated are of considerable interest. The nature of the Ca^{2+} stores activated by these agents, although investigated in some depth throughout this Thesis, remains only partially characterized (see below). The relevance of the present findings for K^+- or NMDA-evoked functional responses, and neurotoxicity, in granule cells, will therefore be important to investigate. Many established techniques could be utilized to investigate more fully whether release of Ca^{2+} stores is central to ionotropic responses and toxicity in cerebellar granule cells, as might be predicted from the results of the present study. In particular [^{3}H]-glutamate release, demonstrated in this and previous studies to be a convenient and reliable method by which to assess release of the main granule cell neurotransmitter, glutamate (Chapter 3; Huston *et al.*, 1990; Levi *et al.*, 1991), could be used to investigate the possible involvement of release of Ca^{2+} stores in depolarization-activated secretion. The ability of Ca^{2+} directly released from stores by BHQ or caffeine to activate transmitter release has been previously established in other preparations (Fossier *et al.*, 1992; Avidor *et al.*, 1994; see also Brosius *et al.*, 1992), but it remains uncertain whether Ca^{2+} store release is functionally involved in secretion of transmitter stimulated either by depolarization or

by receptor activation. Also, as both depolarization and NMDA stimulate granule cell maturation and differentiation (Balazs *et al.*, 1988a,b; Moran and Patel, 1989a,b), the use of markers of cell differentiation such as phosphate-activated glutaminase and aspartate aminotransferase (Moran and Olvera, 1993), or assessment of the extent of neurite outgrowth (Pearce *et al.*, 1987; Cambray-Deakin *et al.*, 1990; Holliday *et al.*, 1991; Kocsis *et al.*, 1994; Zimprich *et al.*, 1994) may provide important information concerning the relative importance of Ca^{2+} entry and release from stores in K^+- or NMDA-stimulated granule cell development. Thirdly, Ca^{2+} stores have been implicated in NMDA-evoked neurotoxicity in other neuronal preparations (Lei *et al.*, 1992a; Frandsen and Schousboe, 1992), but as Ca^{2+} stores may vary in properties and importance between different neuronal types (see Discussion to Chapters 3 and 4), the use of markers of cell death such as lactate dehydrogenase release (see Choi *et al.*, 1988) or decrease in [^{3}H]-ouabain binding (Markwell *et al.*, 1991) may provide important information concerning the role of Ca^{2+} release in NMDA-evoked neurotoxicity in granule cells.

8.2.2 Mechanisms of NMDA-evoked $[Ca^{2+}]_i$ elevation

Further experiments will be necessary to fully explain the differences between NMDA responses determined by $[Ca^{2+}]_i$ and Mn^{2+} quench techniques described in Section 8.1.1. Specifically, effects on NMDA responses of agents which interfere with mitochondrial membrane potential, such as antimycin A1 and carbonyl cyanide m-chlorophenyl-hydrazone (L. Kiedrowski and E. Costa, submitted); inhibitors of Na^+/Ca^{2+} exchange e.g. bepridil, or 5-(*N*-4-chlorobenzyl)-2,4-dimethyl-benzamil (Dumuis *et al.*, 1993; Kiedrowski *et al.*, 1994); replacement of extracellular Na^+ with cations unable to substitute for Na^+ in Na^+/Ca^{2+} exchange e.g. tetramethylammonium or Li^+ (Dumuis *et al.*, 1993; Khodorov *et al.*, 1993); or vanadate which blocks the Ca^{2+}/Mg^{2+} ATPase (Arens *et al.*, 1992), should be examined, in order to more fully

reveal the mechanisms involved in elevation of $[Ca^{2+}]_i$ by NMDA in cerebellar granule cells, and any subsequent homeostatic mechanisms which may become activated to modulate this response. In this and indeed other future studies, the utilization of the Mn^{2+} quench technique, in parallel with conventional fura 2 epifluorescence microscopy, would enable the mechanism of action of $[Ca^{2+}]_i$-elevating agents to be more rapidly and reliably assessed, and the relationship between store release, entry and extrusion to be more fully explored than would be possible using $[Ca^{2+}]_i$ measurement alone.

8.2.3 Characterization of cerebellar granule cell Ca^{2+} stores

As present knowledge of neuronal Ca^{2+} stores is distinctly limited, the design and implementation of a series of experiments to test the present hypothesis concerning the characteristics of granule cell Ca^{2+} stores (Fig. 5.11) may provide a framework by which the stores present in at least one simple neuronal system, granule cells, may be examined. Various modulators of Ca^{2+} stores not utilized in the present study, such as the Ca^{2+}ATPase inhibitors cyclopiazonic acid (Seidler *et al.*, 1989) and bis-phenol (G.R. Brown, S.L. Benyon, C.J. Kirk, M. Wictome, J.M. East, A.G. Lee and F. Michelangeli, submitted), the $InsP_3R$ blocker heparin (see Ji *et al.*, 1993), and nonhydrolyzable $InsP_3$ analogues, might prove useful in future $[Ca^{2+}]_i$ studies in further differentiating between these stores on a pharmacological basis. Measurement of $^{45}Ca^{2+}$ release from permeabilized cells provides the opportunity to study Ca^{2+} mobilization more directly, such that the mobilizing effects of e.g. thapsigargin and BHQ, or CCh and NMDA, as well as the other store-modulating agents used in the present study or mentioned above, could be directly compared. Pre-treatment with thapsigargin, BHQ, cyclopiazonic acid or bis-phenol to partially or fully deplete stores, followed by stimulation with agonists, would provide information, in both $[Ca^{2+}]_i$ and $^{45}Ca^{2+}$ release experiments, concerning the ability of agonists to act on different Ca^{2+}

stores, and concerning the specificity of action of Ca^{2+}ATPase inhibitors on distinguishable Ca^{2+} pools.

8.2.4 Modulation of $InsP_3$ and RyR levels - mechanisms and consequences

Results of the present study, notably a ~73% decrease in the $[Ca^{2+}]_i$ elevation evoked by ACPD after chronic CCh treatment, provide important evidence for physiological consequences of agonist-evoked $InsP_3R$ downregulation. However, the use of maximal $InsP_3R$ downregulation, and of a single, maximal, concentration of ACPD, limit the conclusions which can be drawn from this study. Future experiments might therefore be helpful, in which partially as well as maximally $InsP_3R$-downregulated granule cells are subsequently exposed to a range of ACPD concentrations, in order to investigate whether $InsP_3R$ downregulation causes a rightward shift in EC_{50} for $[Ca^{2+}]_i$ elevation evoked by the mGluR agonist, before the decrease in maximal responsiveness noted in the present study. This would demonstrate whether a significant $InsP_3R$ 'reserve' exists in granule cells, which may not have been detectable in the present comparison of control and downregulated cells because of the very large downregulation evoked by CCh. Given the inherent difficulties in performing a concentration-response experiment for ACPD and $[Ca^{2+}]_i$ in granule cells, because of poor reproducibility of response within a given cell group, and variability in response between different cell groups, population experiments might need to be performed, with different cover-slips of cells being exposed to a single concentration and results gathered together to form a concentration-response curve. Using such a protocol, responses in control, partially $InsP_3R$-downregulated and maximally $InsP_3R$-downregulated cells could be compared. Population $[Ca^{2+}]_i$ experiments did not, however, prove successful in the present study. Alternatively, the approach used by Wojcikiewicz et al (1994a) in permeabilized CHO cells expressing

transfected muscarinic receptors could be utilized in granule cells. In that study, $InsP_3$-evoked $^{45}Ca^{2+}$ mobilization in control and $InsP_3R$-downregulated cells were compared, with both a shift in EC_{50} and partial decrease in maximal response being found, indicative of a considerable $InsP_3R$ 'reserve' in these cells. From the much greater inhibition of maximal responsiveness to ACPD found in the present study than of $InsP_3$ responses in CHO-m3 cells as reported by Wojcikiewicz *et al.* (1994a), it might be expected that a greater decrease in $InsP_3$-evoked maximal $^{45}Ca^{2+}$ release would also be found in granule cells. It would be of considerable interest to establish whether a shift in EC_{50} could also be detected, given the apparent proportionality of $InsP_3R$ downregulation and ACPD response inhibition reported in the present study, which might suggest that no such change would be expected. Previous results (Fohrmann *et al.*, 1993) suggest that metabotropic $[Ca^{2+}]_i$ responses in single granule cells are not 'all-or-none' in nature, so if such permeabilized cell experiments also proved problematical ($^{45}Ca^{2+}$ release from granule cells is technically difficult to measure), large numbers of small group $[Ca^{2+}]_i$ experiments with meaned results could be performed for control, partially $InsP_3R$-downregulated and maximally $InsP_3R$-downregulated cells, this possibly proving the less direct and more tedious, but nevertheless more successful, experimental approach.

From results in the present study, NMDA-evoked modulation of $InsP_3R$ and RyR expression has been indicated. Future experiments should address the mechanisms by which these NMDA-evoked modulatory processes may occur. The mechanisms of CCh-evoked $InsP_3R$ downregulation have been carefully analysed using a variety of techniques, notably store depletion by thapsigargin before agonist treatment, addition of antagonist after brief agonist exposure, possible agonist-evoked changes in receptor mRNA, and loss of incorporated [^{35}S]-methionine from immunoprecipitated receptors (Wojcikiewicz *et al.*, 1992,1994a). These techniques could be utilized to investigate the mechanisms by which NMDA may affect receptor expression in granule cells, as

well as to confirm whether the mechanisms activated by CCh in granule cells parallel those described in SH-SY5Y cells. Also, parallel experiments should be performed to address the possible effects of chronic depolarization on $InsP_3R$ and RyR expression in these neurones, and the mechanisms by which any such changes in expression levels occur. It is possible that some of the complex effects of prolonged incubation with NMDA or K^+ on granule cell survival and receptor-mediated responses (Gallo *et al.*, 1987; Balazs *et al.*, 1988a,b; Moran and Patel, 1989a,b; Cox *et al.*, 1990; Irving *et al.*, 1991 - Ph.D. Thesis, University of Bristol; Zhao and Peng, 1993) could be related to effects of these agents on $InsP_3R$ and RyR expression. The consequences of alteration in store expression on granule cell development, and on responses to agonist addition, could be examined, using the approaches described above for investigating store involvement under normal conditions (Section 8.2.1). The complexity of effect of NMDA and K^+-depolarization on granule cell survival, and on plasma membrane receptor expression, may make identification of the mechanisms of $InsP_3R$ and RyR regulation difficult. However, agonist or depolarization-evoked regulation of Ca^{2+} release mechanisms may have important consequences for normal or pathological signalling processes, and should be pursued in this, or other, preparations.

8.3 Usefulness of granule cells as a neuronal model

In the present study as in previous reports, cultured cerebellar granule cells have proven a useful and reliable model neuronal system in which to investigate $[Ca^{2+}]_i$ regulation and elevation. They possess a major advantage over other preparations, such as cerebellar Purkinje or hippocampal cultures, of high homogeneity, such that protocols requiring population measurements, such as $InsP_X$ or $InsP_3$ assays, and Western blotting for proteins in cell membranes, can be performed in parallel with $[Ca^{2+}]_i$ assessment. While it is arguable that $[Ca^{2+}]_i$ measurement in single cells or small groups of hippocampal or cerebellar Purkinje cells, in which

phenomena such as LTD or LTP occur *in situ*, may be of more direct interest than $[Ca^{2+}]_i$ experiments in granule cells, the inability to obtain pure cultures limits possible studies requiring other techniques. Thus for instance, the series of experiments performed in the present study to cross-compare the time-course of $InsP_3$ production with $InsP_3R$ downregulation, and subsequently $InsP_3R$ downregulation with $[Ca^{2+}]_i$ signalling, would be difficult if not impossible in other cultured neuronal preparations.

However, it is becoming increasingly apparent that a degree of heterogeneity exists in receptor expression in cultured granule cells, which may partially compromise the conclusions drawn from such comparisons. Thus, many but not all granule cells may possess muscarinic receptors or mGluRs (Irving *et al.*, 1990,1992a,b; Manzoni *et al.*, 1992b; Fohrmann *et al.*, 1993; Milani *et al.*, 1993), making the consequences of chronic stimulation of $InsP_3R$ expression probably highly variable between cells. Also, distinct differences in response characteristics in experiments performed in different laboratories have been reported. For instance, Irving (1991 - Ph.D. Thesis, University of Bristol) found that the NMDA $[Ca^{2+}]_i$ response had disappeared by 9 DIV, in contrast to studies such as that of Courtney *et al.* (1990), who reported that the response was approximately maximal at this time-point. In the present report, over a large number of experiments there were no significant changes in response to NMDA between 3-9 DIV (Chapter 4). The reasons for such differences between laboratories remain to be fully explained, but the high sensitivity of granule cells to culture conditions (Cox *et al.*, 1990; Schramm *et al.*, 1990; Van der Valk *et al.*, 1991; Condorelli *et al.*, 1993; Aronica *et al.*, 1993; Lawrie *et al.*, 1993) may indicate that small variations in conditions used in different laboratories may make comparisons of results highly problematic, as the cells may possess differing properties in different laboratories.

In conclusion it appears, therefore, that cerebellar granule cells remain the preparation of choice for studies such as the present one, in which several different techniques besides $[Ca^{2+}]_i$ measurement are involved, despite being less than the ideal homogenous preparation. The ongoing development of transformed cells transfected with glutamate and muscarinic receptors provides a complementary approach to work in primary cultured neurones, but, as suggested by the PKC series of experiments in Chapter 5, conclusions from such preparations may be hampered by differences in intracellular components compared to neuronal preparations. It must be hoped that truly homogenous, neuronal-like, cells can be developed in the future, which will lack the difficulties associated with granule cells and other currently available model systems.

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Publications arising from this Thesis

Papers

Simpson, P.B., Challiss, R.A.J. and Nahorski, S.R. (1993) Involvement of intracellular stores in the Ca^{2+} responses to *N*-methyl-D-aspartate and depolarization in cerebellar granule cells. *J. Neurochem.* **61**, 760-763.

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Review

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Abstracts

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Appendix I - Preparation of KHB

To make up 1l of buffer:

Stock Components	ml
0.9% (w/v) NaCl / 10 mM HEPES, pH 7.45	778
1.15% (w/v) KCl	31
3.82% (w/v) $MgSO_4.2H_2O$	8
2.11% (w/v) KH_2PO_4	8
1.3% (w/v) $NaHCO_3$	163
0.11M $CaCl_2$ (added last)	12
10 mM glucose	1.8g

Equilibrated with 95% CO_2 : 5% O_2 at 37°C.

Appendix II - List of reagents

From Sigma Chemical Company Limited, Poole, Dorset, U.K.

Acrylamide (C_3H_3NO)

Ammonium formate ($CH_2O_2.NH_3$)

Anti-rabbit IgG-horseradish peroxidase

L-Arginine ($C_6H_{14}N_4O_2.HCl$) (L-Arg)

Bovine serum albumin (BSA)

Calcium chloride dihydrate ($CaCl_2.H_2O$)

Carbamylcholine chloride (CCh)

Cytosine β-D-arabinofuranoside

Deoxyribonuclease I (DNase)

Dantrolene sodium ($C_{14}H_{10}N_4O_5Na$)

Dimethylsulphoxide (DMSO)

DL-Dithiothreitol

Dizocilpine

Ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid ($C_{14}H_{24}N_2O_{10}$) (EGTA)

Folin and Ciocalteu's phenol reagent

Fura 2-acetoxymethyl ester (Fura 2-AM)

Glycerol ($C_3H_8O_3$)

N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] ($C_8H_{18}N_2O_4S$) (HEPES)

High molecular weight protein standards

Leupeptin

N-Methyl-D-aspartate (NMDA)

3-(*N*-morpholino)propanesulfonic acid (MOPS)

Nitrendipine

N^{ω}-Nitro-L-Arginine (NARG)

Pepstatin

Phenylmethylsulphonylfluoride

Phorbol 12-myristate 13-acetate (PMA)

Poly-D-lysine

Quisqualate (Quis)

Sodium dodecyl sulphate (SDS)

Tris [hydroxymethyl]aminomethane hydrochloride (Tris-HCl)

Triton X-100 (octylphenoxypolyethoxyethanol)

Trypsin

Tween-20 (polyoxyethylenesorbitan monolaurate)

From Fisons Scientific Equipment, Loughborough, Leics., U.K.

Acetic acid (HAc)

Caffeine ($C_8H_{10}N_4O_2$)

Copper sulphate ($CuSO_4.5H_2O$)

Diethyl ether (DE)

Glycine

D-glucose

Hydrochloric acid (HCl)

Magnesium sulphate ($MgSO_4.7H_2O$)

Manganese chloride ($MnCl_2$)

Potassium chloride (KCl)

Potassium dihydrogen orthophosphate (KH_2PO_4)

Potassium sodium tartrate ($KNaC_4H_4O_6.4H_2O$)

Sodium carbonate (Na_2CO_3)

Sodium chloride (NaCl)

Sodium hydrogen carbonate ($NaHCO_3$)

Sodium hydrogen orthophosphate dihydrate ($Na_2HPO_4.2H_2O$)

Sodium hydroxide (NaOH)

Trichloroacetic acid ($CCl_3.COOH$) (TCA)

Tris-(hydroxymethyl)-methylamine ($NH_2.C(CH_2OH)_3$) (Tris base)

From BDH Limited, Poole, Dorset, U.K.

Bromophenol blue

Ethylenediaminetetraacetic acid ($[CH_2N(CH_2.COOH)_2]_2$) (EDTA)

Lithium chloride (LiCl)

Magnesium chloride hexahydrate ($MgCl_2.6H_2O$)

Sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4.2H_2O$)

From Tocris Neuroamin, Bristol, U.K.

1-Aminocyclopentane-1S,3R-dicarboxylic acid (ACPD)

α-Amino-hydroxyisoxazole propionate (AMPA)

6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX)

5,7-Dichlorokynurenic acid (dcKyn)

From Amersham International PLC, Aylesbury, Bucks., U.K.

ECL reagents

L-[^{14}C]-Glutamate

L-[^{3}H]-Glutamine

[^{3}H]-*myo*-Inositol

D-*myo*-[^{3}H]-inositol 1,4,5-trisphosphate ([^{3}H]-$InsP_3$)

From Calbiochem.Novabiochem Limited, Nottingham, U.K.

Thapsigargin

4-Bromo-A23187

Fura 2 free acid

From Research Biochemicals International - Semet Technical (U.K.) Limited, St. Albans, Herts., U.K.

ω-Conotoxin GVIA

Ryanodine

Other items

Basal modified Eagle's medium and supplements - **GIBCO.BRL, Paisley, Scotland, U.K.**

2,5-Di(*tert*-butyl)-1,4-benzohydroquinone (BHQ) - **Fluka Chemika, Germany**

D-*myo*-Inositol 1,4,5-trisphosphate ($InsP_3$) - **University of Rhode Island Foundation Chemistry Group, U.S.A.**

PKCα antibody - **Upstate Biotechnology Inc., U.S.A.**

Gifts

Ca^{2+}ATPase antibody - Dr. F. Michelangeli, University of Birmingham.

PKCβ, γ and δ antibodies - Dr. P. Parker, Cancer Research Institute, London.

RyR antibodies - Dr. A. Lai, Mill Hill, London.

Anti-mouse IgG-horseradish peroxidase - Dr. A. Lai, Mill Hill, London.

PN 202 791 - Sandoz, Basle, Switzerland.

Ro 31 8220 - Roche Products Limited, Welwyn Garden City, Herts., U.K.

Appendix III - List of abbreviations

Ab	Antibody
ACh	Acetylcholine
ACPD	1-Aminocyclopentane-1S,3R-dicarboxylic acid
AMPA	α-Amino-hydroxyisoxazole propionate
AMPAR	AMPA receptor
APV	2-Amino-5-phosphonopentanoate
ATP	Adenosine 5'-triphosphate
BHQ	2,5-Di(*tert*-butyl)-1,4-benzohydroquinone
B_{max}	Maximal specific binding capacity
BSA	Bovine serum albumin
Ca^{2+}ATPase	Sarcoplasmic-endoplasmic reticulum Ca^{2+}ATPase
$[Ca^{2+}]_i$	Cytoplasmic Ca^{2+} concentration
CamK-II	Ca^{2+}/calmodulin-dependent protein kinase type II
CCh	Carbamylcholine chloride
cDNA	Cloned deoxyribonucleic acid
CHO cells	Chinese Hamster Ovary cells
CHO-m3 cells	CHO cells expressing muscarinic m3 receptors
CICR	Ca^{2+}-induced Ca^{2+} release
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione
CRAC channel	Ca^{2+} release activated Ca^{2+} channel
DAG	*sn*-1,2-Diacylglycerol
dcKyn	5,7-Dichlorokynurenic acid
DHP	1,4-Dihydropyridine
DIV	Days *in vitro*
DMSO	Dimethylsulphoxide
DNase	Deoxyribonuclease I

dpm	Disintegrations per minute
EC_{50}	The concentration of an agonist which produces 50% of the maximum possible response for that agonist
ECL	Electrochemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid
FCS	Foetal calf serum
Fura 2-AM	Fura 2-acetoxymethyl ester
GluR	Glutamate receptor
G protein	Guanine nucleotide binding protein
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
IC_{50}	The concentration of a competing ligand which displaces 50% of the specific binding of another ligand
IICR	$InsP_3$-induced Ca^{2+} release
$InsP_3$	D-*myo*-Inositol 1,4,5-trisphosphate
$InsP_3R$	Inositol 1,4,5-trisphosphate receptor
$InsP_X$	Total inositol phosphates
K_D	Equilibrium dissociation constant
KHB	Krebs-Henseleit buffer
L-Arg	L-Arginine
L-CCG-I	(2S,3S,4S)-α-(carboxycyclopropyl)glycine
LTD	Long-term depression
LTP	Long-term potentiation
mGluR	Metabotropic glutamate receptor
mRNA	Messenger ribonucleic acid
MW	Molecular weight

NARG	N^{ω}-Nitro-L-Arginine
NMDA	*N*-Methyl-D-aspartate
NMDAR	*N*-Methyl-D-aspartate receptor
PI	Phosphoinositide
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIC	Phosphoinositidase C
P*n*	Post-natal day *n*
PKA	Protein kinase A
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
RyR	Ryanodine receptor
Quis	Quisqualate
$t_{1/2}$	Time taken to decay to half the amount present initially
TCA	Trichloroacetic acid
VOCC	Voltage-operated Ca^{2+} channel
wDE	Water-saturated diethyl ether