INTRACELLULAR MODULATION
OF SINGLE NMDA RECEPTOR CHANNELS
IN THE RAT HIPPOCAMPUS

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“If we knew what it was we were doing, it would not be called research, would it?”

Albert Einstein

“Science is always wrong. It never solves a problem without creating ten more.”

George Bernard Shaw
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The NMDA receptor is subject to a range of intrinsic modulatory systems. By investigating these pathways it is possible to accumulate knowledge concerning potential therapeutic targets. The NMDA receptor plays a key role in many aspects of brain physiology and pathology, therefore information concerning the control of NMDA receptor activity could prove very useful. Certain second messenger systems including protein kinase C, calcineurin and calmodulin in addition to calcium and the cytoskeletal protein alpha-actinin-2, have been shown to alter NMDA receptor activity. The aim of this study was to investigate the molecular mechanisms of this modulation by means of single channel analysis of native NMDA receptors in hippocampal slices. Outside-out patches were taken from the dentate gyrus cell layer of twelve day-old rats. Steady-state channel activations produced by NMDA (0.1 - 10 μM) and glycine (10 μM) were studied over a range of holding potentials (-80 to -30 mV).

Exposure of the intracellular surface of outside-out patches to the PKC and calcineurin inhibitors, calphostin-C (n = 4) and cyclosporin-A (n = 5), did not affect single NMDAR channel characteristics. However application of 1.66 nM active calcineurin (n = 7) to outside-out patches caused a 14 % reduction in NMDA receptor mean open time at -60 mV. As calmodulin, a cofactor for calcineurin, was present in these experiments, the ability of calmodulin to change NMDA receptor behaviour was also tested. With the same concentration of calmodulin (12 nM) in the absence of calcineurin, mean open time was reduced by 50 % at -60 mV. Therefore inhibition of the NMDA receptor by calmodulin and possibly calcineurin was mediated by a reduction in channel open time. This was concluded to be through an allosteric mechanism rather than channel block, as receptor shut times remained unaffected.
The duration of groups of channel openings designated bursts, clusters and superclusters were also reduced in the presence of 12 nM calmodulin (n = 8). The mean total open time and charge passed by bursts, clusters and superclusters were also reduced but the average open probability within these events was unchanged. The observed effects on superclusters suggest that calmodulin inhibits the macroscopic NMDA-mediated current and hence has implications for the synaptic current and integration of synaptic activity.

To distinguish between the actions of calmodulin at its two binding sites on the NMDA receptor NR1 subunit (C0 and C1 regions), two concentrations of calmodulin were investigated. At 12 nM active calmodulin, the high affinity binding site, the C1 region, is expected to be predominantly occupied, whereas at 800 nM active calmodulin it is expected that both sites would be saturated. At the high calmodulin concentration, a reduction in single NMDA channel mean open time was less obvious than with the low concentration, 29 % as compared to 50 %. This was fully reversed by antagonising calmodulin binding at its low affinity C0 site, using the C0 binding cytoskeletal protein alpha-actinin. In addition to verifying the competition between calmodulin and alpha-actinin for the C0 region, these results may imply that the two calmodulin binding sites exhibit negative co-operativity.
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LIST OF ABBREVIATIONS

AIDS: Acquired Immunodeficiency syndrome
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP: Action potential
CA1: Cornu Ammon region 1
CONSAM: Continuous sampling program
DAG: Diacylglycerol
EGTA: Ethylene Glycol-bis(β-aminoethyl Ether) N,N,N',N'-Tetraacetic acid
EKDIST: Single channel distribution fitting program
EPSC: Excitatory post-synaptic current
HEK 293 cells: Human embryonic kidney 293 cells
HEPES: N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
LTD: Long term depression
LTP: Long term potentiation
mGluR: Metabotropic glutamate receptor
mRNA: Messenger ribonucleic acid
NMDA: N-Methyl-D-Aspartic acid
NMDAR: N-Methyl-D-Aspartic receptor
NR1: N-Methyl-D-Aspartic receptor subunit 1
NR2A: N-Methyl-D-Aspartic receptor subunit 2A
NR2B: N-Methyl-D-Aspartic receptor subunit 2B
NR2C: N-Methyl-D-Aspartic receptor subunit 2C
NR2D: N-Methyl-D-Aspartic receptor subunit 2D
NR3: N-Methyl-D-Aspartic receptor subunit 3
PKA: Protein kinase A
PKC: Protein kinase C
P_{open}: Channel open probability
PP2B: Protein phosphatase 2B, calcineurin
pS: picosiemen
PSD-95: Post-synaptic density protein 95
SAP-102: Synaptic associated protein 102
SCAN: Single channel analysis program
$t_{\text{crit}}$: Critical time

$T_i$: Rise time

$\tau$: Time constant
CHAPTER 1

INTRODUCTION

The past forty years has witnessed the identification of the NMDA receptor and assessment of its contribution to excitatory synaptic transmission within the mammalian central nervous system (for review see Ozawa et al., 1998). Meanwhile the latter ten years have provided a wealth of information concerning the intracellular modulation of NMDA receptor activity. Within a single neuron there is a whole universe of organised biochemical reactions responsible for fine-tuning synaptic responses. Within the postsynaptic cell phosphorylation and dephosphorylation by protein kinases and phosphatases can regulate NMDA receptor activity. More recent advances include the discovery of NMDA receptor inhibition by calmodulin and the dissociation of cytoskeletal elements such as alpha-actinin (for review see Yamakura & Shimoji, 1999).

The majority of this type of research, however, has been performed on recombinant receptors, leaving insubstantial information concerning native NMDA receptor modulation. In this thesis I have focused on the intracellular modulation of native NMDA receptors from rat hippocampal slices. By using the single channel patch-clamp technique, the mechanism of this modulation was investigated under conditions that allowed fine control over both the extracellular and intracellular environment of the receptor. This permitted the exact molecular consequence of each individual modulatory factor to be determined.

1.1 The NMDA receptor

The NMDA receptor belongs to the family of glutamate-sensitive receptors, including those of ionotropic (NMDA, AMPA and Kainate) and metabotropic origin (mGluR). It is quite distinct in terms of pharmacology and physiology and due to its high calcium permeability (MacDermott et al., 1996; Mayer & Westbrook, 1987; Ascher & Nowak, 1988; Schneggenburger et al., 1993), has a significant role in the biochemical integration of synaptic activity with the neuron (Lisman, 1989), in addition to synaptic plasticity (Bliss & Collingridge, 1993; Bear & Malenka, 1994; Bliss & Collingridge, 1995) and is believed to be involved in memory, learning (Collingridge, 1987; Martin et al., 2000) and neuronal development (Fox et al., 1992; Komuro & Rakic, 1993). In addition, excessive NMDA receptor activation is thought
to be involved in various neurodegenerative diseases such as Alzheimer's, Parkinson's and AIDS dementia, and in neuronal cell death resulting from anoxic ischaemia, stroke and epilepsy (for review see Choi, 1992; Lipton & Rosenberg, 1994; Lancelot & Beal, 1998). The NMDA receptor mediates prolonged excitatory postsynaptic potentials (Dale & Roberts, 1985; Forsythe & Westbrook, 1988) and exhibits slow activation-deactivation kinetics (Lester et al., 1990), compared to the non-NMDA (AMPA and kainate) receptor types (Colquhoun et al., 1992). Co-activation with glycine is necessary for NMDA receptor activity (Johnson & Ascher, 1987; Kleckner & Dingledine, 1988), and the channel is blocked by Mg^{2+} ions under physiological conditions, therefore creating a highly voltage-dependent macroscopic conductance (Nowak et al., 1984; Mayer et al., 1984).

1.2 NMDA receptor subunits

To date six NMDA receptor subunits have been identified. The NR1 subunit which carries the glycine binding site (Hirai et al., 1996) was discovered first (Moriyoshi et al., 1991) and subsequently NR2A, NR2B, NR2C, NR2D and NR3A subunits (the latter is also known as chi-1 or NMDAR-1) have been identified (Monyer et al., 1992; Ishii et al., 1993; Ciabarra et al., 1995, Sucher et al., 1995), which are responsible for binding glutamate (Laube et al., 1997; Anson et al., 1998; 2000). The NR1 subunit is subject to alternative splicing at both amino and carboxy terminals producing nine splice variants, eight of which are functional when co-expressed with NR2 subunits in oocytes (for review see Hollman & Heinemann, 1994). The terminology of the NR1 splice variants is illustrated in Figure 1.1.

![FIGURE 1.1 Alternative splicing of NMDA receptor subunits.](image)

FIGURE 1.1 Alternative splicing of NMDA receptor subunits. The different NR1 subunit splice variants arise from alternative splicing of the exons 5, 21, and 22, giving rise to the cassettes N1, C1, C2, and C2' (with permission from Dingledine et al., 1999).
1.3 Membrane topology of the NMDA receptor subunits

Originally, the membrane topology of the NMDA receptor subunits was presumed to resemble the nAChR, with each subunit consisting of four transmembrane regions TM1-4. However by analogy with AMPA receptors on the basis of analyses of their N-glycosylation sites (Hollmann et al., 1994) and following cysteine scanning experiments on NR2A (Kuner et al., 1996a) a membrane topology of three transmembrane regions is now accepted (Figure 1.2). The originally defined TM2 forms a re-entrant loop (M2) instead of crossing the cell membrane and comprises part of the ion channel pore (Kuner et al., 1996a).

FIGURE 1.2 Representation of the proposed structure of the NMDA receptor channel NR1 and NR2 subunits. Top, amino acid sequences of segment M2 of five NMDA receptor channel subunits, critical residues conferring calcium and magnesium selectivity and sensitivity are indicated (N and N+1). Bottom, the three transmembrane segment topology model. The amino acid residues at the N and N+1 sites of the NMDA receptor channel subunits are indicated as filled and open circles, respectively. The binding sites for glutamate (Glu) and glycine (Gly) are shown as hatched circles, and the alternatively spliced cassettes (N1, C1 and C2) of the NR1-1 subunit are represented (with permission from Yamakura & Shimoji, 1999).

1.4 Stoichiometry of the NMDA receptor

NMDA receptors are known to be heteromeric with a combination of NR1 plus an NR2 subunit being required for normal function (Monyer et al., 1992). However, the exact subunit composition and stoichiometry of native receptors remains elusive (for review see Sucher et al. 1996). Recombinant receptors containing NR1 with only one type of NR2 subunit show distinct similarities to the functional properties of native receptors (Stern et al. 1992; Wyllie et al. 1996). Subsequently, the
possibility that more than one type of NR2 subunit can be contained in a single receptor has come under scrutiny (Sheng et al., 1994; Chazot & Stephenson, 1997; Dunah et al., 1998; Hawkins et al., 1999; Cheffings & Colquhoun, 2000). In terms of stoichiometry, evidence tends towards a tetrameric composition for the NMDA receptor (Behe et al., 1995; Laube et al., 1998; Rosenmund et al., 1998) but the possibility of a pentamer (Premkumar & Auerbach, 1997) has not been ruled out.

1.5 Subunit-related physiology of recombinant NMDA receptors

NR1 and NR2 subunits contribute particular characteristics to NMDA receptor function and also contain specific sites for modulation by phosphorylation, polyamines, zinc, protons, redox agents and nitric oxide (for reviews see McBain & Mayer, 1994; Ozawa et al., 1998; Dingledine et al., 1999; Yamakura & Shimoji, 1999). Two molecules of NMDA/glutamate and glycine are necessary to activate ion channel gating (Benveniste et al., 1990; Benveniste & Mayer, 1991; Clements & Westbrook, 1991). Electrophysiological experiments suggest the binding site for glycine is located on the NR1 subunit (Hirai et al., 1996; Laube et al., 1998) and the glutamate binding site on the NR2 subunit (Laube et al., 1998; 1997; Anson et al., 1998; Anson et al., 2000). At low glycine concentrations, NMDA-mediated whole-cell currents undergo a rapid reduction in amplitude over the course of agonist application, (Mayer et al., 1989; Benveniste et al., 1990; Vyklicky et al., 1990). This glycine-dependent desensitisation is virtually absent when glycine is present in saturating amounts (Vyklicky et al., 1990), the corresponding concentration of which is believed to be present endogenously (Ferraro & Hare, 1985).

Unique NMDA receptor characteristics such as high calcium permeability and voltage-dependent block by magnesium are conferred by critical asparagine residues (Figure 1.2) in the pore forming M2 region of both NR1 and NR2 subunits (Burnashev et al., 1992; Sakurada et al., 1993; Wollmuth et al., 1998a; Wollmuth et al., 1998b; Wollmuth & Sakmann, 1998). NR2 subunits are the main determinants of NMDA receptor functional diversity (Monyer et al., 1992; 1994, for review see Yamakura & Shimji, 1999). This includes varying magnesium sensitivity (Monyer et al., 1992; Monyer et al., 1994; Kuner & Schoepfer, 1996b), agonist and antagonist sensitivity (Mishina et al., 1993; Williams, 1993; Laurie & Seeburg, 1994a; Mori & Mishina, 1995), single channel properties (Stern et al., 1992 and Wyllie et al., 1996; Hawkins et al., 1999; Cheffings & Colquhoun, 2000), activation and deactivation kinetics (Monyer et al., 1992, 1994; Wyllie et al., 1998) and cytoskeletal attachment (Sheng & Pak, 2000). NR1 subunit splice variants also contribute to variation in
receptor physiology. For example, inclusion of the N1 cassette affects NMDA receptor activation/deactivation kinetics (Rumbaugh et al., 2000), whereas the C1 cassette is subject to phosphorylation/dephosphorylation by kinases/protein phosphatases (Chen & Huang, 1991; 1992) and binds calmodulin and alpha-actinin (Ehlers et al., 1996; Wyszynski et al., 1997), factors thought to be involved in the modulation of receptor activity (Ehlers et al., 1996; Zhang et al., 1998; Krupp et al., 1999).

1.6 Interaction of the NMDA receptor with the cytoskeleton

Intensive research into cytoskeletal proteins, which interact with the NMDA receptor (Figure 1.3), has brought about the discovery of the PSD-95 family which includes: PSD-95/SAP90, chapsyn-110/PSD-93, SAP102 and SAP97 (for review see Kennedy, 1997). These proteins form a substantial element of the postsynaptic density at excitatory synapses and they interact with each other and the C-terminal of the NR2 subunit, via ‘PDZ’ domains (Kornau et al., 1997). Found on the C-termini of interactive proteins, ‘PDZ’ domains are amino acid sequences, of approximately 90 residues, that are necessary in the specific assembly and regulation of protein signalling complexes at synaptic junctions (for review see Garner et al., 2000). Rao et al. (1998) suggest that these PSD-95 proteins form a scaffold at developing synapses awaiting NMDA receptor attachment (for review see Dingledine et al., 1999; Sheng & Pak, 2000).

FIGURE 1.3 NMDA receptor interactions with the cytoskeleton and postsynaptic density. NR1 binding proteins are also included such as α-actinin, calmodulin (CaM), yotiao, NF-L (neurofilament-L) and NR2 binding protein CAMKII, (Sheng & Pak, 2000; with permission from the Annual Review of Physiology, Volume 62, © 2000, by Annual Reviews www.AnnualReviews.org).
Alpha-actinin, a member of the spectrin superfamily, is also enriched in the postsynaptic density. It binds to the C0 region of the NR1 C-terminal, linking the NMDA receptor to the actin cytoskeleton (Rao et al., 1998), and specifically the isoform alpha-actinin-2 has been shown to compete with calmodulin at this site (Wyszynski et al., 1997). Alpha-actinin-2 arrives at dendritic spines later in development, anchoring and clustering NMDA receptor channels at synapses (Wyszynski et al., 1998).

Brain spectrin also binds to the NMDA receptor via the NR1, NR2A and NR2B subunits, providing cross-linking to the actin cytoskeleton (Wechsler & Teichberg, 1998). Its interaction with the NR2B subunit is antagonised by tyrosine phosphorylation and calcium, whereas binding to the NR1 subunit is inhibited by PKA/PKC phosphorylation and calmodulin. Two proteins yotiao and neurofilament L have been found to interact with the alternatively spliced C1 exon on the NR1 subunit (Lin et al., 1998; Ehlers et al., 1998). The exact functions of these proteins are unclear. It has been speculated that neurofilament-L provides anchorage or localisation for the NMDA receptor, whereas yotiao binds both PKA and protein phosphatase 1, possibly facilitating targeting of these enzymes to their substrates.

1.7 Single channel properties of recombinant and native NMDA receptors

The expression of recombinant receptors in Xenopus oocytes and various cell lines has given a great deal of information concerning NMDA single channel properties (Stern et al., 1992, 1994; Behe et al., 1995; Wyllie et al., 1996, Anson et al., 2000; Cheffings & Colquhoun, 2000). By investigating recombinant single channel characteristics, the subunit properties of native receptors can be inferred (Farrant et al., 1994; Momiyama et al., 1996; Momiyama, 2000; Misra et al., 2000). The NR2 subunit confers a great deal of functional variation in recombinant receptors: most obvious is the appearance of "high" and "low" conductance channels (Table 1.1). Generally "high conductance" channels are though to be generated by NR2A or NR2B-containing receptors whereas the "low conductance" channels are generated by NR2C or NR2D-containing receptors (Cull-Candy et al., 1998).

Shut time distributions have only been described for recombinant NR1/NR2A and NR1/NR2D receptors (Wyllie et al., 1998). These are compared with single channel shut time distributions from new-born rat hippocampal granule cells in Table 1.2. The NMDA receptor exists in a minimum of five or six distinct shut states, thus due to this complexity it is difficult to draw direct comparisons between
TABLE 1.1 *Comparison of recombinant and native NMDA receptor single channel conductances*

<table>
<thead>
<tr>
<th>Subunit composition/region</th>
<th>Main conductance level (pS)</th>
<th>Subconductance level (pS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIGH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR1/NR2A&lt;sup&gt;1&lt;/sup&gt;</td>
<td>50.1</td>
<td>38.3</td>
</tr>
<tr>
<td>NR1/NR2B&lt;sup&gt;1&lt;/sup&gt;</td>
<td>50.9</td>
<td>38.7</td>
</tr>
<tr>
<td>Dentate Gyrus&lt;sup&gt;2&lt;/sup&gt;</td>
<td>55</td>
<td>40</td>
</tr>
<tr>
<td>LOW</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR1/NR2C&lt;sup&gt;3&lt;/sup&gt;</td>
<td>30.6</td>
<td>17.9</td>
</tr>
<tr>
<td>NR1/NR2D&lt;sup&gt;3&lt;/sup&gt;</td>
<td>35.0</td>
<td>17.0</td>
</tr>
<tr>
<td>Cerebellar Purkinje cells&lt;sup&gt;4&lt;/sup&gt;</td>
<td>38</td>
<td>18</td>
</tr>
</tbody>
</table>

<sup>1</sup> Stern et al. (1992), <sup>2</sup> Strecker et al. (1994), <sup>3</sup> Wyllie et al. (1996), <sup>4</sup> Momiyama et al. (1996)
**TABLE 1.2 Comparison of recombinant and native NMDA receptor single channel shut time distributions**

<table>
<thead>
<tr>
<th>Subunit composition/region</th>
<th>$\tau_1$ (μs)</th>
<th>$\tau_2$ (ms)</th>
<th>$\tau_3$ (ms)</th>
<th>$\tau_4$ (ms)</th>
<th>$\tau_5$ (ms)</th>
<th>$\tau_6$ (ms)</th>
<th>Mean (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR1/NR2A$^1$</td>
<td>45 ± 8</td>
<td>0.498 ± 0.08</td>
<td>4.86 ± 0.78</td>
<td>28.9 ± 4.6</td>
<td>--</td>
<td>7472 ± 1750</td>
<td>1222 ± 575</td>
</tr>
<tr>
<td></td>
<td>(40 ± 3 %)</td>
<td>(22 ± 2 %)</td>
<td>(17 ± 2 %)</td>
<td>(8 ± 1 %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR1/NR2D$^1$</td>
<td>55 ± 7</td>
<td>0.374 ± 0.028</td>
<td>6.51 ± 0.7</td>
<td>38.8 ± 5.4</td>
<td>221 ± 34</td>
<td>5061 ± 343</td>
<td>128 ± 36</td>
</tr>
<tr>
<td></td>
<td>(42 ± 5 %)</td>
<td>(19 ± 2 %)</td>
<td>(9 ± 1 %)</td>
<td>(16 ± 3 %)</td>
<td>(12 ± 2 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dentate gyrus$^2$</td>
<td>23 ± 4</td>
<td>0.3 ± 0.08</td>
<td>0.8 ± 0.05</td>
<td>7 ± 1</td>
<td>394 ± 297</td>
<td>910 ± 622</td>
<td>91 ± 61</td>
</tr>
<tr>
<td></td>
<td>(63 ± 7 %)</td>
<td>(9 ± 2 %)</td>
<td>(9 ± 6 %)</td>
<td>(4 ± 1 %)</td>
<td>(6 ± 0.4 %)</td>
<td>(8 ± 2 %)</td>
<td></td>
</tr>
</tbody>
</table>

recombinant and native channels. Despite this, shut time distributions contain additional information concerning the identification of groups of channel openings, which will be discussed further.

Open time distributions for NR1/NR2A, NR1/NR2B, NR1/NR2C and NR1/NR2D recombinant receptors indicate that "high-" and "low-conductance" channels exhibit different open states. The "high conductance" channels have three exponential components, compared to two for the "low conductance" channels, and "high conductance" channels have a longer mean open time. Comparisons with open time distributions from single channel recordings from P0 dentate gyrus hippocampal slices and P0-P8 Purkinje cells from cerebellar slices are presented in Table 1.3. Information conveyed in the open time distributions indicates that the NMDA receptor channel exists in two or three individual open time states and this preference is determined by the receptor subunit composition.

1.8 NMDA receptor-mediated synaptic current

NMDA receptor-mediated excitatory postsynaptic potential's (EPSC’s) have a slow rise-time and decay time-course in comparison with the fast non-NMDA receptor EPSC (Hestrin et al., 1990a; Lester et al., 1990; Keller et al., 1991). In hippocampal neurons, the NMDA receptor EPSC has a rise time of approximately 10 ms and a decay phase which can be fitted by double exponentials, with approximate time constants of 60 ms and 250-500 ms, for the fast and slow component, respectively (Lester et al., 1990).

The prolonged NMDA receptor EPSC results from channels flickering between the open and closed state whilst glutamate is still bound. The long decay time-course of the NMDA receptor EPSC is therefore determined by channel kinetics (Hestrin et al., 1990b; Lester et al., 1990) and not agonist diffusion as previously speculated. Accordingly, brief pulses of glutamate to outside-out patches from hippocampal neurons were shown to elicit currents with a similar rise-time and decay time course to the synaptic NMDA EPSC (Lester et al., 1990).

It should, theoretically, be possible to predict the decay time-course of the NMDA receptor synaptic current from the single channel kinetics. However to achieve this, both the duration of the first latencies to channel opening in response to rapid agonist delivery and the duration of the single channel activation need to be measured. Measurement of the first latency (the time taken for the channel to open following agonist application) is technically difficult owing to the possibility of low-concentration
**TABLE 1.3 Comparison of recombinant and native NMDA receptor single channel open time distributions**

<table>
<thead>
<tr>
<th></th>
<th>$\tau_1$ (ms)</th>
<th>$\tau_2$ (ms)</th>
<th>$\tau_3$ (ms)</th>
<th>Mean (ms)</th>
<th>Voltage (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR1/NR2A$^1$</td>
<td>67 ± 1</td>
<td>1.64 ± 0.4</td>
<td>4.27 ± 0.7</td>
<td>1.96</td>
<td>-60</td>
</tr>
<tr>
<td></td>
<td>(30 ± 5 %)</td>
<td>(40 ± 7 %)</td>
<td>(30 ± 4 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR1/NR2B$^2$</td>
<td>183 ± 300</td>
<td>1.83 ± 0.3</td>
<td>4.99 ± 0.1</td>
<td>2.59</td>
<td>-60</td>
</tr>
<tr>
<td></td>
<td>(23 ± 5 %)</td>
<td>(41 ± 7 %)</td>
<td>(36 ± 8 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dentate Gyrus$^3$</td>
<td>65 ± 3</td>
<td>0.73 ± 0.1</td>
<td>4.4 ± 0.4</td>
<td>2.94</td>
<td>-60</td>
</tr>
<tr>
<td></td>
<td>(7 ± 5 %)</td>
<td>(31 ± 3 %)</td>
<td>(62 ± 1 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR1/NR2C$^{1,2}$</td>
<td>490 ± 100</td>
<td>1.19 ± 0.1</td>
<td>--</td>
<td>0.84</td>
<td>-60</td>
</tr>
<tr>
<td></td>
<td>(50 ± 8 %)</td>
<td>(50 ± 8 %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR1/NR2D$^2$</td>
<td>74 ± 100</td>
<td>1.54 ± 0.1</td>
<td>--</td>
<td>0.94</td>
<td>-100</td>
</tr>
<tr>
<td></td>
<td>(28 ± 2 %)</td>
<td>(72 ± 3 %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebellar</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.84</td>
<td>-60</td>
</tr>
<tr>
<td>Purkinje cells$^4$</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td></td>
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agonist contamination of control solutions generating spurious long latencies (Edmonds & Colquhoun, 1992). However, most indirect estimations of the channel first latencies suggest that they are short relative to the duration of the receptor activation (Jahr, 1992; Benveniste & Mayer, 1995). In steady-state single channel recordings, groups of openings can be separated into “activations”, defined as the sequence of channel openings and closures that occur between agonist binding and dissociation. Ensemble currents from the alignment of these “activations” have been shown to exhibit a similar time constants describing the decay time course to those resulting from the decay time-course of a current elicited by a brief exposure to glutamate (Wyllie et al., 1998).

1.9 Distribution of NMDA receptor subunits

The NR1 subunit is present in all areas of the developing and adult rat brain (Moriyoshi et al., 1991; Monyer et al., 1994, Benke et al., 1995; Goebel and Poosch, 1999), consistent with the need for at least one NR1 subunit to create a functional NMDA channel (Monyer et al., 1992). Splice variants of the NR1 subunit exhibit regional and developmental variation in expression (Laurie & Seeburg, 1994b). NR1-1a and 2a isoforms occur most commonly in the cortex and hippocampus, whereas NR1-2a and 4b isoforms are most readily found in the cerebellum and brainstem.

NR2 and NR3 subunit expression changes during development and is most probably related to maturation of the nervous system. At birth only NR2B, NR2D and NR3 subunits are prevalent (Monyer et al., 1994; Ciabarra et al., 1995; Sucher et al., 1995). NR2B is widely distributed throughout the brain at this time but becomes progressively restricted to the forebrain during the next twelve days (Wenzel et al., 1997). The NR2D and NR3 subunits are found in the diencephalon and the brainstem at birth but again expression decreases dramatically during the following two weeks (Wenzel et al., 1996). Conversely, NR2A and NR2C subunit expression increases after birth, with NR2C expression becoming particularly evident in the cerebellum and NR2A in hippocampus and cortex.

In adult rat brain NR2A is the most widely distributed NR2 subunit, predominating in the cortex, hippocampus, striatum and cerebellum, whereas the NR2B subunit is now mainly expressed in the forebrain, cortex and hippocampus. NR2C is almost exclusively found in the cerebellum, and the NR2D subunit at very low levels in the thalamus and brainstem (Goebel & Poosch, 1999). Adult rat brain NR3
subunit distribution remains similar to the newborn, but expression levels are drastically lower (Ciabarra et al., 1995; Sucher et al., 1995).

1.10 Intracellular modulation of the NMDA receptor

Intracellular mechanisms involved in NMDA receptor modulation can be put into three loose categories; (1) phosphorylation by protein kinases, (2) dephosphorylation by protein phosphatases, and (3) calcium-dependent inactivation by calmodulin or cytoskeletal dissociation. The balance between NMDA receptor potentiation and inhibition, determined by these processes, is thought to underlie regulation of the strength of NMDA synaptic currents (Soderling & Derkach, 2000).

1.10.1 NMDA receptor modulation by protein kinases

PKC, a calcium- and diacylglycerol (DAG)-activated serine/threonine kinase, has been suggested to potentiate NMDA currents following phorbol ester application in rat dorsal horn neurones (Gerber et al., 1989), dentate gyrus (Obokata et al., 1997) and CA1 neurons (Lozovaya & Klee, 1995) of rat hippocampal slices, and cultured trigeminal neurones (Chen & Huang, 1991, 1992), in addition to recombinant receptors expressed in Xenopus oocytes (Durand et al., 1993; Zheng et al., 1997, 1999; Logan et al., 1999). Suggestions for a mechanism of action include; increase in NMDA channel open probability (Chen & Huang, 1992; Xiong et al., 1998), reduction in magnesium block (Chen & Huang, 1992), calcium amplification (Zheng et al., 1997), activation of tyrosine kinases (Lu et al., 1999; Grosshans & Browning, 2001) and increased receptor trafficking (Lan et al., 2001) although these explanations are subject to debate (Wagner & Leonard, 1996; Xiong et al., 1998; Logan et al., 1999).

The calcium-dependence of PKC is contributed by a single calcium binding site, with a dissociation constant of 700 nM (Mosior & Epand, 1994) located on the regulatory domain. Calcium binding is responsible for initiating PKC translocation to the cell membrane where it exerts its enzymatic actions. Various phosphorylation sites for PKC have been found on the NR1, NR2A and NR2B subunits of the NMDA receptor (Tingley et al., 1997; Leonard & Hell, 1997). Critical residues are contained in the C1 exon cassette of the NR1 subunit C-terminus, ser890 and ser896 (Tingley et al., 1997).

The exact relationship between PKC phosphorylation and NMDA receptor potentiation is unclear. Recombinant receptors that lack the C1 exon have been shown to exhibit a higher degree of potentiation in response to phorbol application, than those
containing the C1 exon (Durand et al., 1993; Zheng et al., 1997, 1999; Logan et al., 1999). In addition, receptors containing point mutations of the critical serine residues were potentiated to a greater extent by PKC (Zheng et al., 1997; 1999).

Almost certainly, potentiation of NMDA-mediated currents by PKC is dictated by the absence or presence of the C1 exon (Logan et al., 1999) and is influenced by the associated NR2 subunit (Xiong et al., 1998; Logan et al., 1999). However there appears to be a negative relationship between phosphorylation and the physiological activity of PKC. It is possible that the C1 exon contains inhibitory PKC elements which, once removed, allow the full extent of PKC potentiation to occur.

Much less is understood about the effects of PKA on NMDA receptor activity. Due to its co-localisation with calcineurin, a protein phosphatase, by anchoring proteins at the synaptic density (Coghlan et al., 1995), the actions of PKA and calcineurin are thought to compliment each other concerning NMDA receptor regulation. Accordingly, Raman et al. (1996) found that inhibition of PKA prevented recovery of NMDA receptors from calcineurin-mediated inhibition induced by synaptic activity. Similarly, elevation of PKA activity overcame the ability of calcineurin to depress the amplitude of NMDA EPSC's. Stimulation of PKA or intracellular application of its catalytic subunit has also been shown to enhance NMDA whole-cell currents (Cerne et al., 1993). The NR1, NR2A and NR2B subunits also contain phosphorylation sites for PKA that are distinct from those for PKC (Tingley et al., 1997; Leonard & Hell, 1997).

Similarly, the calcium/calmodulin-dependent protein kinase II (CaMKII) has been shown to bind the NR2A and NR2B subunits of the NMDA receptor (Omkumar et al., 1996; Gardoni et al., 1999; Strack et al., 1998, 2000). Although at present, no functional modulation of NMDA receptor activity by CaMKII has been observed.

Wang & Salter (1994) first demonstrated protein tyrosine kinase (PTK) modulation of the NMDA receptor, in spinal dorsal horn neurons. Inclusion of Src PTKs potentiated NMDA receptor-mediated whole-cell currents, whilst conversely PTK inhibitors depressed NMDA receptor-mediated whole-cell currents. Activation of endogenous Src kinases was found to increase single channel open probability and mean open time, and alter shut time kinetics; producing a higher proportion of short shut states and a decrease in the proportion of long shut states, resulting in altered burst, cluster and supercluster duration (Yu et al., 1997).

NMDA receptor-mediated EPSC's were also enhanced by endogenous Src kinases (Yu et al., 1997), which has been related to the induction of LTP in the
hippocampus (Lu et al., 1998). Phosphorylation sites for PTKs have been found on NR2A and NR2B subunits (Lau & Huganir, 1995), although only recombinant NR1/NR2A receptors appeared to exhibit any functional modulation by Src PTKs (Kohr & Seeburg, 1996). Potentiation of recombinant NR1/NR2A NMDA receptor function by Src PTKs is thought to be mediated by a reduction in zinc-dependent receptor inhibition (Zheng et al., 1998). However, Src PTK potentiation of native NMDA receptors in hippocampal or dorsal horn neurons remained unaffected by the extracellular zinc concentration (Xiong et al., 1999).

1.10.2 NMDA receptor modulation by protein phosphatases

The most studied phosphatase involved in NMDA receptor regulation is the serine/threonine phosphatase calcineurin, alternatively known as protein phosphatase 2B. As a very highly conserved protein, even in evolutionary remote species it is uniquely the only protein phosphatase which is dependent upon both calmodulin and calcium for activation (Stewart et al., 1982) (for general reviews see Klee et al., 1988; Yakel, 1997; Aramburu et al., 2000; Rusnak & Mertz, 2000).

Calcineurin exists in a heterodimeric formation consisting of a catalytic and calmodulin-binding subunit, calcineurin A, and a calcium-binding regulatory subunit, calcineurin B. It is this latter domain, calcineurin B, which is responsible for the highly specific activity of this enzyme (Milan et al., 1994). Calcineurin A alone has very little activity, for it exists in a state of autoinhibition due to an inhibitory domain contained within its structure (Hashimoto et al., 1990). Upon binding of calmodulin and calcium this inhibitory domain is displaced, allowing calcineurin to become active.

Calcineurin exhibits one binding site for calmodulin, with a very high affinity, in the order of 0.1 nM (Hubbard & Klee, 1987), and four binding sites for calcium (Klee et al., 1979), one high affinity site, approximately 24 nM and three of a lower affinity approximately 15 μM (Kakalis et al., 1995). It has been observed that occupancy of at least two calcium sites is required for calcineurin activity (Stemmer & Klee, 1994). There is a substantial amount of positive co-operativity displayed by the simultaneous binding of calcium and calmodulin, whereby binding of calcium increases the affinity of calcineurin for calmodulin and vica-versa. If calmodulin is present in equimolar concentrations to calcium, calcineurin activity increases more than 20-fold. This suggests that less calcium is necessary to cause the same level of calcineurin activity if the concentration of calmodulin is increased and similarly less calmodulin is needed if the concentration of calcium is increased (Stemmer & Klee, 1994). These conjoint
attributes enable small increases in cell calcium to be accentuated and consequently evoke a greater response from calcineurin.

Calcineurin was originally discovered owing to its involvement in T-cell responses, and is the target of the immunosuppressant drug cyclosporin-A (Clipstone & Crabtree, 1992). Since then it has since been discovered in abundance in the brain (Klee et al., 1979). At the cellular level this extends to the cell body, PSD, dendrites, axons, spines and presynaptic terminals, where 50% resides in the cytoplasm and 50% is membrane bound (Klee et al., 1988). Consistent with this localisation, calcineurin has been shown to regulate presynaptic release of glutamate and GABA (Nichols et al., 1994; Sihra et al., 1995; Stelzer, 1992), NMDA receptor function (Lieberman & Mody, 1994), amphibian M currents (Marrion, 1998), L-type calcium channels (Armstrong, 1989; Lai et al., 1993) and stimulation of nitric oxide synthase (NOS) during glutamate excitotoxicity (Snyder & Sabatini, 1995).

Lieberman & Mody (1994) were the first to investigate the actions of calcineurin on NMDA receptors. Single channel openings were recorded from cell-attached patches, taken from dissociated rat hippocampal granule cells. Upon application of okadaic acid, a non-selective phosphatase inhibitor, and FK506, a selective calcineurin inhibitor, channel open time increased and the duration of bursts, clusters and superclusters were prolonged. This resulted in an overall increase in channel open probability. Alternatively, direct application of calcineurin and half-saturated calcium/calmodulin to the cytoplasmic surface of inside-out patches reduced single channel open time.

Involvement of calcineurin in NMDA receptor inactivation was also highlighted by Tong & Jahr (1994). They found the decay of NMDA receptor-mediated currents from outside-out patches, elicited by glutamate concentration jumps, was calcium-dependent. Involvement of calcineurin, a calcium dependent protein, was verified by use of a calcineurin inhibitory peptide, which caused the decay of NMDA-mediated currents to become prolonged. Following on from this work, the same group (Tong et al., 1995) demonstrated that inhibition of calcineurin had a similar effect on the decay of NMDA-mediated EPSCs.

Although phosphatases 1 and 2A are involved in AMPA channel modulation, it is not so clear whether this also extends to the NMDA receptor (Figurov et al., 1993). Whilst Lieberman & Mody (1994) concluded that PP1 and PP2A were not involved in modulation of the NMDA receptor, Wang et al. (1994) found the very opposite. Inhibition of PP1 and PP2A in perforated-patched recordings, from cultured
hippocampal neurons, increased NMDA channel activity. In accord with this, application of constitutively active PP1 and PP2A reduced the NMDA channel mean open time, open probability and opening frequency in inside-out patches. The effects of PP1 and PP2A on NMDA receptor channel activity were less obvious in concentration jump experiments, on outside-out patches from cultured hippocampal cells, although a small component of the NMDA mediated current decay was seen to be reduced by inhibitors of PP1 and PP2A (Tong & Jahr, 1994).

Finally protein tyrosine phosphatases (PTPs) have been shown to inhibit NMDA receptor activity. In addition to their work on PTKs, Wang & Salter (1994) demonstrated that whole-cell NMDA currents are potentiated by intracellular application of PTP inhibitors, in spinal dorsal horn neurons. A more qualitative investigation revealed that PTPs reduce single channel open probability, and PTP inhibitors increase single channel activity (Wang et al., 1996). Conversely, though, Tong & Jahr (1994) and Tong et al. (1995) found PTP inhibition had no effect on macroscopic NMDA currents.

1.10.3 Calcium-dependent inactivation

Calcium-dependent inactivation is apparent during NMDA receptor-mediated whole-cell recordings as a decrease in current over time, during agonist exposure for several seconds. It is likely to represent a combination of inhibitory processes, although it is not affected by kinase activity or prevented by phosphatase inhibitors (Mayer et al., 1989; Clark et al., 1990; Legendre et al., 1993; Rosenmund & Westbrook, 1993a; Vyklicky, 1993; Krupp et al., 1996; Medina et al., 1996). The process is NR2 subunit specific, with NR1/NR2A and NR1/NR2C channels showing a similar degree of inactivation, whereas NR1/NR2B and NR1/NR2D channels exhibit little or no inactivation (Medina et al., 1995; Krupp et al., 1996). This could mean there really is no calcium-dependent inactivation with NR2B or NR2D subunits, or it could mean these receptors inactivate too fast for the process to be evident from whole-cell recordings.

Most evidence points to an intracellular site of action for calcium, near to the inner mouth of the channel (Legendre et al., 1993; Vyklicky, 1993). Whether calcium interacts directly with the NMDA receptor-channel is not known. Evidence to suggest this is not the case is exemplified by the inability to produce calcium-dependent inactivation in excised patches (Rosenmund & Westbrook, 1993a; Medina et al., 1996).

Rosenmund & Westbrook (1993a) found that application of calcium to the intracellular surface of inside-out patches had no effect on single NMDA channel
characteristics. Meanwhile, Medina et al. (1996) found that upon initial application of calcium to inside-out patches, the open probability of the NMDA receptor-channel was decreased. Further applications had no effect, which would suggest that some cytosolic or cytoskeletal factor necessary for this process is lost, or washed out. Accordingly, examination of NMDA channel characteristics in cell-attached recordings, which allows the intracellular environment to remain intact, revealed that open probability was reduced and remained so, regardless of subsequent increases in intracellular calcium (Legendre et al., 1993).

In addition to calcium-dependent inactivation, NMDA receptor rundown occurs after prolonged agonist exposure. In whole cell recordings, rundown manifests as a decrease in peak current with successive agonist application (Sather et al., 1990). Similar to inactivation, rundown is dependent upon the intracellular calcium concentration (Rosenmund & Westbrook, 1993b). Rundown was prevented by the presence of ATP and phalloidin (Medina et al., 1996; Rosenmund & Westbrook, 1993a, 19993b). According to Rosenmund & Westbrook (1993a) this prevents NMDA receptor rundown by maintaining the structural integrity of the actin cytoskeleton.

The subsequent scheme proposed by Rosenmund & Westbrook (1993a), to explain calcium-dependent inactivation and rundown of whole-cell currents, suggests that NMDA receptor-channels are attached to the actin cytoskeleton by a calcium-sensitive regulatory protein. When the NMDA receptor-channel is activated calcium influx causes dissociation of the regulatory protein from the channel, resulting in inactivation. If prolonged agonist exposure ensues, the rise in intracellular calcium causes depolymerisation of the actin filaments and rundown of the NMDA receptor-channel occurs.

Although follow-up studies by Medina et al. (1996) were found to disagree with the progressive relationship between inactivation and rundown, both agreed that a cytosolic protein was involved in calcium-dependent inactivation. The question concerning the identity of this diffusiable intracellular factor still remains unanswered.

1.10.3.1 Calmodulin-dependent inactivation

Calmodulin, to state a colloquialism, 'has its finger in every pie'. As a small ubiquitous protein found in most eukaryotic organisms, with a high degree of homology and cross-reactivity from bovine brain to cotton seed (Wallace & Cheung, 1979), its acknowledged functions are ever increasing. Calmodulin has been described
as an internal calcium receptor, for many actions of calcium are transduced through this small cytosolic protein (for reviews see Klee & Vanaman, 1982; Means et al., 1982).

Upon binding calcium the 16,700 kDa protein, calmodulin forms a dumbbell shaped molecule revealing two hydrophobic patches (James et al., 1995). These areas are exposed to bind specific substrates. The N- and C-terminal lobes both bind two calcium ions, each exhibiting a different dissociation constant. Binding of calcium ions to the C-terminal brings about a massive conformational change that allows substrate binding (James et al., 1995). From this it was concluded that at least two binding sites must be occupied for calmodulin to become active. The dissociation constants for these calcium binding sites range from 10 nM to 1000 nM (Haiech et al., 1981) and exhibit a high degree of positive co-operativity, whereby binding of the first molecule of calcium will increase the affinity for the second.

Calmodulin is certainly abundant throughout the brain, especially in the cerebral cortex, caudate nucleus and hippocampus, and less so in the hypothalamus, medulla, posterior pituitary and the anterior pituitary (Klee & Vanaman, 1982). As previously explained some unidentified cytosolic factor appears to be involved in calcium-dependent inactivation of NMDA receptor-mediated whole-cell currents. Thus the possibility that this factor is calmodulin has come under scrutiny. Calmodulin has certainly been implicated in calcium-dependent modulation of various other ion channels including the olfactory cyclic nucleotide-gated channel (Liu et al., 1994), calcium-activated potassium channels and L-type calcium channels (For review see Levitan, 1999; Ehlers & Augustine, 1999).

Calmodulin binds to the NR1 subunit of the NMDA receptor, in a calcium-dependent manner (Hisatsune et al., 1997), at two discrete regions of the C-terminus (Ehlers et al., 1996). One region lies within the C0 region (aa K839-Q863), common to all NR1 splice variants. The second region is contained within the alternatively spliced C1 exon (aa K875-K898). The C0 region displays a lower affinity for calmodulin, with a dissociation constant ($K_d$) of approximately 87 nM, when compared to a $K_d$ of approximately 4 nM for the binding site contained in the C1 exon (Ehlers et al., 1996).

These estimates of calmodulin's affinity for the NMDA receptor channel were made using synthetic fusion proteins of the NR1a and NR1c splice variant subunits, the former of which contained both C1 and C0 calmodulin binding sites whereas the latter contained only the C0 calmodulin binding region. The change in emission fluorescence spectra of dansyl-calmodulin when bound to these fusion proteins was measured and results presented as a Scatchard plot to estimate calmodulin binding affinity for both
Scatchard relationship. The insets show saturation plots of the fraction of bound dansyl-CaM versus free NR1a-Cterm (B, inset) or free NR1c-Cterm (C, inset) fusion protein. Data from two experiments are shown in each case (with permission from Ehlers et al., 1996).

sites (Figure 1.4). In affirmation of reliability, the estimated affinity of calmodulin binding to the C0 region of both NR1a and NR1c subunit fusion proteins was consistent between experiments, giving a value of 72.9 nM for NR1a fusion proteins and 87.7 nM for NR1c fusion proteins.

Functional studies further supported evidence generated from binding studies concerning the involvement of calmodulin in NMDA receptor inhibition. Calmodulin was found to decrease open probability and reduce the mean open time of recombinant NMDA receptor-channels (NR1/NR2A) in inside-out patches, in a calcium dependent manner (Ehlers et al., 1996; Hisatsune et al., 1997). Small active concentrations of calmodulin (2 nM) were sufficient to maximally inhibit NMDA channel activity (Ehlers et al., 1996). NMDA receptors containing the C1 exon were 4-
fold more susceptible to calmodulin, measured as a reduction in charge passed through the open channel. Subsequently, Zhang et al. (1998) found that deletion of the C0 region impaired the ability of calmodulin to reduce single channel open probability. Concentrations of calmodulin used were at least 10-fold higher than that needed to produce maximal inhibition in NR1a subunit containing NMDA receptors (Ehlers et al., 1996).

\[ \text{FIGURE 1.5 Functional analysis of the sensitivity of NMDA receptor channels to calmodulin.} \]

The total electrical charge passing through open NMDA channels during calcium-calmodulin perfusion is normalised to the control level and plotted against the concentration of calmodulin. Structures of the COOH-terminal domains of the five different NR1 constructs are shown on the right. C1 and C2 indicate exon cassettes; C2' indicates the alternative COOH-terminus generated by removal of the C2 exon cassette (with permission from Ehlers et al., 1996).

The results of Ehlers et al. (1996), describing the functional inhibition of recombinant NMDA receptors by calmodulin, have some surprising characteristics. Fitting the inhibition curves with the Hill equation suggests a very steep Hill coefficient for this inhibition, implying more than two molecules of calmodulin bind to the NMDA receptor. In addition, maximal inhibition of NR1a subunit containing receptors was observed at a calmodulin concentration of 2 nM, whereas binding studies indicated a dissociation constant of 4 nM for calmodulin occupancy of the C1 region (Figure 1.5). It is plausible that calmodulin has a higher affinity for the whole receptor in comparison to NR1 subunit C-terminal fusion proteins such as those used in the binding study. This suggests that calmodulin exhibits efficacy when bound to
the NMDA receptor, possibly resulting from a conformational change in receptor structure. However, the data from this functional study are not as quantitatively convincing as results from the binding studies and thus the affinity constants generated from calmodulin binding to the NMDA receptor fusion proteins have generally been accepted.

With the knowledge that PKC phosphorylates residues contained within the C1 exon of the NR1 subunit and this regions importance in the determination of PKC potentiation of the NMDA receptor, Hisatsune et al. (1997) investigated the relationship between calmodulin and PKC. Accordingly, these two proteins were found to compete for substrate on the NR1 subunit; phosphorylation of the NR1 subunit by PKC, inhibited calmodulin binding. This is interesting considering that calmodulin has been shown to inhibit the NMDA receptor via binding to the C1 exon and the C1 exon exerts some inhibitory influence over PKC potentiation. From these early studies, the idea became apparent that the C1 exon plays some role in NMDA receptor modulation, perhaps serving as a convergent site for several modulatory mechanisms.

However, when the effect of calmodulin was measured on NMDA-mediated whole-cell currents, the presence of the C1 exon did not appear to be necessary for calcium-dependent inactivation (Zhang et al., 1998; Rafiki et al., 1997; Krupp et al., 1999). Only whole-cell currents from recombinant NMDA receptors that contained deletions or point mutations in the C0 region of the NR1 subunit displayed a reduced form of calcium-dependent inactivation.

Whether calmodulin is solely responsible for calcium-dependent inactivation is under dispute, as evidence is conflicting. Only Zhang et al. (1998) found whole-cell dialysis with a calmodulin binding domain protein reduced calcium-dependent inactivation in whole-cell recordings from recombinant NMDA receptor (NR1/NR2A) cell lines, whereas research by Krupp et al. (1996, 1999) indicates the opposite. Additional studies showed that calcium-dependent inactivation was not affected by the presence of calmidazolium, an inhibitor of calmodulin, in whole cell currents from hippocampal cultured neurons and recombinant cell lines (Legendre et al, 1993; Krupp et al., 1999).

This lead to the suspicion that perhaps other as yet unidentified intracellular components were also involved in calcium-dependent inactivation. Following on from the work of Rosenmund & Westbrook (1993a), cytoskeletal attachment became a prime candidate. The cytoskeletal protein alpha-actinin-2 had been shown to bind to the
NMDA receptor and compete with calmodulin for occupation of the C0 region of the NR1 subunit (Wyszynski et al., 1997). This seemed a good place to start.

1.10.3.2 Cytoskeletal-dependent inactivation

Involvement of alpha-actinin, a member of the spectrin family, has been well documented in calcium-dependent inactivation of the NMDA receptor. Building upon the scheme by Rosenmund & Westbrook (1993a) it was suggested that alpha-actinin could be the 'regulatory protein' attaching the NMDA receptor to the actin cytoskeleton. It was possible that dissociation of the NMDA receptor from the cytoskeleton was in fact responsible for calcium-dependent inactivation, mediated by displacement of alpha-actinin due to competitive binding of calmodulin at the C0 region.

Zhang et al. (1998) first illustrated the involvement of alpha-actinin in calcium-dependent inactivation by over-expressing dominant negative truncated alpha-actinin-2 constructs in HEK 293 cells. Each construct was designed to compete with and dissociate endogenous alpha-actinin-2 from the cytoskeleton or the NMDA receptor NR1 subunit C-terminus. This resulted in a reduction of calcium-dependent inactivation of recombinant NMDA-receptor mediated whole-cell currents, presumably because inactivation would be complete under these conditions and therefore insensitive to further rises in intracellular calcium. Thus it was proposed that calmodulin causes calcium-dependent inactivation by competing with alpha-actinin-2 for occupancy of the NMDA receptor and hence causing dissociation of the receptor from the cytoskeleton, which leads to inactivation.

Krupp et al. (1999) further demonstrated the necessity of bound alpha-actinin, and not just the alpha-actinin-2 isoform, for the functional integrity of the NMDA receptor. Over-expression of calcium-insensitive (skeletal and smooth muscle) alpha-actinin constructs in HEK293 cells abolished calcium-dependent inactivation of NMDA receptor-mediated whole cell currents, whereas calcium-sensitive (non-muscle) alpha-actinin constructs maintained calcium-dependent NMDA receptor inactivation. Subsequently, competition between calcium-insensitive alpha-actinin and calmodulin in intact cells was illustrated by including calmodulin in the whole-cell pipette and showing restoration of calcium-dependent inactivation.

Thus Krupp et al. (1999) suggested that calcium-dependent inactivation, resulting from displacement of the NMDA receptor from alpha-actinin and the cytoskeleton, could occur by two pathways (Figure 1.6).
1. Receptor opens and leads to calcium influx. Calcium binds to calmodulin, which displaces alpha-actinin, the NMDA receptor is detached and inactivation ensues.
2. Calcium binds to alpha-actinin and displaces binding to the C0 region, again NMDA detachment and inactivation ensues.

The latter pathway would be dependent on whether the calcium-sensitive or insensitive isoform of alpha-actinin is expressed, as both are present in the brain (Waites et al., 1992; Wyszynski et al., 1997).

![Diagram of NMDA receptor inactivation](image)

**FIGURE 1.6** A molecular model for NMDA receptor inactivation by calmodulin and alpha-actinin. Calmodulin (CaM) dependent and independent mechanisms are illustrated. Calcium-dependent NMDA receptor inactivation is indicated by the receptor moving into a low open probability (low Po) state (with permission from Krupp et al., 1999).

Other cross-linking elements have been shown to attach NMDA receptors to the cytoskeleton including neuronal intermediate filaments (Ehlers et al., 1998) and brain spectrin (Wechsler & Teichberg, 1998). Despite not having been studied in the context of calcium-dependent inactivation, these proteins interact with the high affinity calmodulin binding site, in the C1 exon of the NR1 C-terminal. Brain spectrin has been shown to compete with calmodulin at this site (Wechsler & Teichberg, 1998) and therefore could be involved in NMDA receptor modulation. Phosphorylation of the C1 exon by PKA and PKC also inhibits spectrin-NR1 interactions, supporting the idea that
the C1 exon is a key site in NMDA receptor modulation through the interplay of calmodulin or phosphatase inactivation and kinase potentiation.

1.11 Aims of Experiments

Primarily, the aim of this study was to analyse and verify the actions of certain intracellular modulatory proteins on native NMDA receptor function. These included: protein kinase C, calcineurin, calmodulin, alpha-actinin and calcium. Secondly, the possible interactions or influences these modulatory systems may exert upon each other were evaluated, for example:

1. To determine the relationship between calmodulin and calcineurin in NMDA receptor inhibition, given that calmodulin is a co-factor for activation of calcineurin.
2. To elucidate the contribution of each calmodulin binding site to NMDA receptor inhibition.
3. To determine if alpha-actinin can alter NMDA receptor inhibition by calmodulin.
CHAPTER 2
MATERIALS AND METHODS

2.1 Solutions

2.1.1 Slicing solution

A modified Krebs solution (Edwards et al., 1989) with the following composition was used for slicing (in mM): NaCl, 125; KCl, 2.5; CaCl₂, 1.0; MgCl₂, 4.0; NaH₂PO₄, 1.25; NaHCO₃, 24; glucose, 25; pH 7.4.

2.1.2 External solution for recording

The composition of the external solution used for recording was similar to that of the slicing solution but with no added Mg²⁺ (Nowak et al., 1984). The free Mg²⁺ concentration in this solution has been estimated to be around 4 μM (Gibb & Colquhoun, 1992). The slicing and external solution for recording were continuously gassed with a mixture of O₂ (95%) and CO₂ (5%) (BOC Gases, Manchester, UK).

2.1.3 Internal solution for recording

In preliminary experiments, control internal solution consisted of (in mM): CsCl, 140; NaCl, 10 mM; HEPES, 10 mM; EGTA, 10 mM and neutralised to pH 7.3 with NaOH. Subsequently, in order to eliminate contaminant chloride currents at the recording potential used, patch pipettes were filled with a low chloride (10 mM) control internal solution in the majority of experiments. The composition of this control internal solution was (in mM): NaCl, 10; EGTA, 10; HEPES, 10; D-gluconic acid (sodium salt), 140; pH: 7.3. A final pH of 7.3 was obtained only after 38 - 40 mM of NaOH was added, giving a final theoretical Na⁺ concentration of 188 - 190 mM. Other internal solutions contained either an additional: 200nM cyclosporin-A or 200nM calphostin-C (also contained in the recording solution); 12nM free calcium, 12nM calcium/calmodulin active complex plus 12 nM calcium or 1.66nM calcineurin/calmodulin/calcium active complex plus 12 nM active calmodulin plus 12 nM calcium; 800nM free calcium, 800nM calcium/calmodulin active complex plus 800 nM calcium or 2.5μM alpha actinin plus of 800nM calcium/calmodulin active complex plus 800 nM calcium. These solutions were stored frozen in aliquots.
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TABLE 2.1 Summary of solutions used in each experimental group, amounting to eight treated groups and six control groups.
2.1.4 Determination of pipette solution calcium concentration

In order to include the correct amount of calcium in the pipette solution to activate an exact concentration of calmodulin (12nM or 800nM), an analysis of calcium-calmodulin binding was performed. The dissociation constant for each of the four calcium binding sites (C1, C2, C3 and C4) on calmodulin was provided by Haiech et al. (1981) and for calmodulin to become activated two or more calcium binding sites must be occupied (James et al., 1995). Thus the probability of more than one calcium ion binding to calmodulin was estimated (see appendix). The free calcium concentration corresponding to a 1 % probability of two or more calcium binding sites being occupied was selected: thus from a 1.2 µM or 80 µM solution of calmodulin, 12nM and 800nM would be activated, respectively. From the work by Ehlers et al. (1996) 12nM activated calmodulin is sufficient to ensure 75% occupancy of the C1 region on the NR1 C-terminus but only 12% of C0, whereas 800nM will ensure 90% occupancy of the C0 region and 99.5% of the C1 (see appendix). From the value for \([Ca^{2+}]_{\text{free}}\) predicted from this analysis, \([Ca^{2+}]_{\text{total}}\) (calcium concentration in the presence of 10mM EGTA) was calculated using the program ALEX by Michael Vivaudou which is based on that described by Fabiato (1988). Determination of active calcium/calmodulin/calcineurin was calculated in a similar fashion using the affinity of calmodulin for calcineurin from Hubbard & Klee (1987) and the affinity for calcium for calcineurin from Kakalis et al. (1995). \([Ca^{2+}]_{\text{total}}\) was also measured using a calcium electrode. On a semi-logarithmic scale, a linear relationship exists between the millivolt calcium electrode readout and the molarity of the calcium solution. Thus the calcium concentration of the sample solution was determined by reference to stock calcium solutions of known molarity.

2.2 Drugs and chemicals

NaCl, NaOH, NaH₂PO₄, NaHCO₃, KCl, CaCl₂, MgCl₂ and glucose were purchased from BDH (Poole, England). HEPES (N-[2-Hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]), EGTA (Ethylene Glycol-bis(β-aminoethyl Ether) N,N,N’,N’-Tetraacetic Acid) and D-gluconic acid (2,3,4,5,6-Pentahydroxycaproic acid, sodium salt) were purchased from Sigma (St. Louis, MO, USA). Glutamate (L-glutamic acid) and NMDA (N-Methyl-D-Aspartic acid) were purchased from Tocris (Bristol, UK). Cyclosporin-A was an in-house gift from Dr Assem. Calphostin-C, calmodulin and calcineurin were purchased from Sigma (St. Louis, MO, USA). Alpha-actinin purified
from rabbit skeletal muscle was purchased from Cytoskeleton via Totam Biologicals Ltd (Northampton, UK).

2.3 Drug application

Before drug application, control recordings were made in the absence of agonists or antagonists. All solutions were superfused into the recording chamber by gravity. The exchange between solutions was made by manually switching a two way tap. Each outside-out patch was exposed to a constant low concentration of NMDA (0.1 - 10 μM) for periods of time that varied from 1.5 to 40 minutes in the presence of a saturating concentration of glycine (10 μM).

2.4 Brain slice preparation

Hippocampal slices were prepared as described elsewhere (Edwards et al., 1989; Gibb & Edwards, 1994). P12 (postnatal day 12) Sprague-Dawley rats were killed by decapitation using a pair of surgical scissors (RS6930, Roboz, Germany). The head was immediately submerged in a 100 ml plastic weighing boat (Fisher Scientific, Loughborough, UK) containing ice-cold oxygenated slicing solution. The skin was cut along the midline with a pair of small scissors. The skull was cut along the midline with a pair of fine curved forceps. The brain was exposed, cut along the midline with a scalpel (N° 11, Swann-Morton, Sheffield, UK) and dissected out using a fine spatula. Immediately, both brain halves were transferred to a 100 ml plastic weighing boat previously filled with ice-cold oxygenated slicing solution and continuously bubbled with a mixture of O₂ (95%) and CO₂ (5%). Both brain halves were allowed to cool down for 3-5 minutes for transverse hippocampal slices, a flat surface was cut across the dorsal side of both brain halves with a scalpel. This surface was glued, using cyanoacrylate adhesive (RS Components, Corby, UK), to the tissue block of the specimen bath of a vibroslicer (Vibroslice 752, Campden Instruments LTD, Loughborough, UK) with the temporal part of the hippocampus perpendicular to the cutting edge of the blade. Immediately, the specimen bath was filled with oxygenated ice-cold slicing solution until the tissue was completely covered. Slices were cut using carbon steel blades (Campden Instruments LTD, Loughborough, UK) at a thickness of 250 μm. Long hypodermic needles (Monoject, Ballymoney, UK) were used to dissect out the hippocampal formation from the rest of the brain slice. Slices were transferred into an incubation chamber, which was
continuously bubbled with a mixture of O$_2$ (95%) and CO$_2$ (5%) (Edwards & Konnerth, 1992) at room temperature (22°C), using a Pasteur pipette (John Poulten Ltd., Barking, UK) cut and fired polished to a tip opening of 3-5 mm across. Each slice was transferred in the same way to a recording chamber fitted to the stage of an upright microscope (Axioscope, Zeiss, Jena, Germany).

2.5 Cell visualisation and identification

The cell bodies of individual neurons in brain slices were visualised under Nomarski differential interference contrast optics (Yamamoto, 1975; Takahashi, 1978; Edwards et al., 1989) using an upright microscope (Axioskop, Zeiss, Oberkochen, Germany) with an Achromat 40X water immersion objective with a numerical aperture of 0.75 and a working distance of 1.6 mm at a total magnification of 600X. Visualisation was carried out on a monochrome video monitor (VM-902K, Hitachi-Denshi, Tokyo, Japan) connected to CCD monochrome camera (RS Components, Corby, UK) mounted on top of the microscope trinocular head. Slice health was visually checked before patching and the presence of a considerable proportion of neurons with a smooth surface readily evident on the surface of the slice was used as an indicator of a good healthy slice. Individual healthy cells were identified by the smooth appearance of their surface. Granule cells in the granule cell layer of the dentate gyrus were identified by their size and morphology and position in the hippocampus.

2.6 Patch pipette fabrication

Patch pipettes were made in a vertical pipette puller (L/M-3P-A, List-Medical, Darmstadt, Germany or MF-83, Narishige, Tokyo, Japan) from thick-walled aluminosilicate glass capillaries containing internal filament (SM150F-7.5, outer diameter 1.5 mm, inner diameter 0.80 mm, Clark Electromedical, Reading, UK). They were coated with an insulating silicone resin (Sylgard 184®, Dow Corning, Midland, MI, USA) under a dissecting microscope using a metal or glass hook. Coating started from about 100 μm away from the pipette tip to a distance of several millimeters up to the pipette shoulder and it was subsequently cured by moving the coated pipette through a heated coil connected to a variable voltage source. The tip of the pipette was cleaned, smoothed and reduced by fire polishing on a microforge (MF-83, Narishige, Tokyo, Japan). Pipettes were back-filled with the internal solution by using a thin plastic pipette tip connected to a 0.2 μm pore size syringe filter (Minisart-RC4, Sartorius, Surrey, UK,)
attached to a 1 ml sterile syringe (Plastipak, Becton-Dickinson, Spain). After back-filling, air bubbles remaining in the pipette were removed by tapping the pipette with finger. Internal solution level in the pipette was kept to a minimum to prevent electrical interference produced by solution creeping up the electrode or going into the suction line.

2.7 Patching procedure

Patch pipettes were positioned on an electrode holder connected to the head-stage of a patch-clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA, USA). Positive pressure was applied through a piece of tubing (Portex Ltd, Hythe, UK) connected to the back of the electrode holder to generate a tiny stream of internal solution at the tip of the pipette that prevented the accumulation of debris at the tip and the mixing of the internal solution inside the pipette with the external solution in the recording chamber. The patch pipette was then lowered into the recording chamber. Once in the solution, the pipette resistance was measured by passing a 5 mV square pulse through the input using the patch clamp amplifier. Patch pipettes usually had a final resistance of 20 - 30 MΩ when filled with recording internal solution. Positioning of the patch pipette was carried out under the optic field of the microscope using a micromanipulator (Melles Griot, Cambridge, UK) and under visual control the patch pipette was lowered further down until visual contact with the slice was made. At that moment, a piezo-electric device (Melles-Griot, Cambridge, UK) connected to the micromanipulator was used to approach the cell with the patch pipette. Contact between the patch pipette and the cell was visually confirmed by the formation of a characteristic dimple on the cell surface. Immediately, the positive pressure was released and a high resistance, less than 10 gigahm seal was formed between the cell membrane and the tip of the electrode. The membrane under the patch pipette was held at -60 mV and suction was applied through the tubing connected to the back of the electrode holder to break the membrane and gain electrical access to the cell interior (Hamill et al., 1981). After the whole cell configuration was obtained the patch electrode was very slowly withdrawn away from the cell using the piezo-electric manipulator until an outside out patch was obtained.

Electrical noise was reduced by bringing the patch pipette towards the surface of the bath leaving only its tip in the solution. The patch pipette was also brought toward the inlet of the recording chamber to improve contact between the patch and the
incoming solutions. Before recording was attempted, the noise level was checked and an RMS noise level below 0.300 pA at a bandwidth of 5 kHz (Butterworth filter) was considered acceptable for recording. The presence of spontaneous single channel openings was also checked and patches with spontaneous single channel activity were discarded.

2.8 Data acquisition and analysis

2.8.1 Detection and recording of single channel currents

Steady state single channel activity produced by a constant low concentration of NMDA (0.1-10 μM) and a constant saturating concentration of glycine (10 μM) was monitored using a patch-clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA, USA) in the voltage clamp configuration. Signals were filtered at a 10kHz bandwidth and recorded using a modified Digital Audio Tape recorder (DTR 1202, BioLogic, Claix, France). Data were stored on magnetic tapes (Maxell, DAT 120) for off line digitisation and analysis. Current-voltage relationships were built by holding the patches for 1.5-3 minutes at potentials between -80 and -20 mV. All recordings were performed at room temperature (22°C).

2.8.2 Record digitisation

After experiments were carried out, tapes were played back and the signals were filtered at a cut-off frequency of 2 kHz using a Bessel type low pass filter (-3dB, 8-pole) to reduce background noise. The filtered signal was then digitised at 10 times the cut-off frequency of the low-pass filter (20 kHz) using an analogue-to-digital converter (CED 1401plus, Cambridge Electronics Design Ltd, Cambridge, UK) and stored on the hard disc of a 486D computer (Dell Computer Corporation, Berkshire, UK) using a computer program for continuous sampling (CONSAM) designed by D. Colquhoun.

2.8.3 Detection and measurement of events in the digitised record

Each digitised record was then scanned and transitions were detected, measured and fitted using an interactive computer program (SCAN) that carried out direct fitting of each event time-course based on the step response of the recording system (Colquhoun & Sigworth, 1995). Briefly, the digitised recording was displayed and scanned by scrolling it across the computer screen under visual inspection. Events were
detected after crossing a threshold placed close to the baseline. Once it was decided that
the event could be fitted, the program made initial guesses for the positions of all the
transitions and amplitudes and performed a least-squares fit on the basis of these
guesses; finally, it displayed the fitted curve superimposed on the digitised event. If the
step-response function fitted poorly, the fit was adjusted to obtain the fit that best
described the event. Fits were stored as a list of values with the amplitude and duration
of each open period and the duration of each closed period. For incompletely resolved
openings SCAN constrained their amplitude to be the same as that of the closest
opening longer than 2 filter rise-times if such an opening was present in the region of
trace being fitted, if on the contrary, there were no openings with such characteristics in
the section of trace being fitted, they were fitted as openings to the mean amplitude
level. After the record was fitted, a data file containing all the values describing the
lifetime and amplitude of all single channel events present in the record was created
and stored as a computer file which was later used during the analysis of the single-
channel data.

2.9 Display and analysis of single-channel data

Display and analysis of single-channel data was done using EKDIST, a computer
program designed by D. Colquhoun. A fixed resolution of open times and closed times
was imposed that gave a false event rate less than or equal to $10^{-8}$ events per second
(Colquhoun & Sigworth, 1995). This was usually between 100 µs and 332 µs for both
open and closed times. Before distributions were built, the data was checked for
stability by building stability plots for amplitudes, open times, shut times and $P_{open}$
(Weiss & Magleby, 1989).

2.9.1 Stability plots

2.9.1.1 Stability plots for amplitudes

Stability plots for amplitudes were built by plotting the individual single-
channel current amplitude measurements of each opening against the interval number
in which the opening was detected. The amplitude of each single opening longer than 2
filter rise-times was plotted. Each data point on the plot represented a single
observation independent of its duration.
2.9.1.2 Stability plots for open times, shut times and $P_{\text{open}}$

Under steady state conditions of temperature, voltage and agonist concentration, the kinetic behaviour of NMDA receptor-channels can be described by a Markov process in which the probability of channels existing in a given kinetic state (open or closed) does not change with time (Gibb & Colquhoun, 1992). Before analysing distributions of open and closed times, the stability of the channel activity was assessed by building stability plots which allowed detection of changes in shut times, open times and probability of being open ($P_{\text{open}}$) that could undermine the analysis of dwell times and amplitude distributions. Stability plots for open and shut time intervals were constructed by calculating a moving average of a certain number of consecutive open or shut time intervals with an overlap of a half and plotting this average against the interval number at the centre of the averaged values. Stability plots for open probability ($P_{\text{open}}$) were constructed by calculating a $P_{\text{open}}$ value for each set of open and shut times as total open time over total length.

Once the stability of the record was confirmed values were sorted into bins and used in the construction of frequency distribution histograms.

2.9.2 Distribution of fitted amplitudes

The amplitude of channel openings can be measured accurately only if the duration is at least twice the rise-time ($T_r$) of the recording system (Colquhoun & Sigworth, 1995). Frequency distribution histograms containing individual open-channel amplitudes longer than 2 filter rise-times were constructed and fitted with the sum of two Gaussian components with their standard deviations constrained to be the same. The relative area occupied by each Gaussian component represents the relative frequency of events to each particular amplitude level rather than the relative time spent at each level. Each single channel opening longer than 2 filter rise-times represented one observation independent of its duration.

2.9.3 Current-voltage relationship plots

To calculate the slope conductance of the channels, single channel currents were recorded for 1.5-3 minutes at holding potentials between -80 and -30 mV. For each holding potential, an amplitude histogram was built and fitted with Gaussian components to estimate the mean current amplitude that was plotted against the
holding potential. Linear regressions fitted through the points gave the slope conductance of each unitary current.

2.9.4 Distribution of open times and shut times

Because the duration of closed and open time intervals varied from tens of microseconds to tens of seconds, frequency distribution histograms were constructed using a logarithmic transformation of the abscissa (McManus et al., 1987; Sigworth & Sine, 1987) and a square root transformation of the ordinate (Sigworth & Sine, 1987). Distributions were fitted using the maximum likelihood method with probability density functions that were the sum of one or more exponential components (Colquhoun & Sigworth, 1995).

2.9.5 Bursts

Bursts were defined as groups of openings separated by shuttings of duration less than a critical shut time or \( t_{\text{crit}} \) which was calculated from the fitted parameters of the distribution of shut times such that the 2\(^{nd}\) and 3\(^{rd}\) briefest exponential components of the shut time distribution were classified as 'gaps within bursts'. Critical shut time or \( t_{\text{crit}} \) values were found by numerical solution by the bisection method using EKDIST. Critical shut time or \( t_{\text{crit}} \) values were calculated using three different criteria:

Colquhoun & Sakmann (1985):

A \( t_{\text{crit}} \) value was calculated so that equal percentages of short and long shuttings were misclassified. Values for \( t_{\text{crit}} \) were calculated by solving:

\[
e^{-t_{\text{crit}}/\tau_{\text{fast}}} = 1 - e^{-t_{\text{crit}}/\tau_{\text{slow}}}
\]


A \( t_{\text{crit}} \) value was calculated so that equal number of short and long shuttings were misclassified. Values for \( t_{\text{crit}} \) were calculated by solving:

\[
a_{\text{fast}}e^{-t_{\text{crit}}/\tau_{\text{fast}}} = a_{\text{slow}}(1 - e^{-t_{\text{crit}}/\tau_{\text{slow}}})
\]

Jackson et al. (1983):

A \( t_{\text{crit}} \) value was calculated so that the total number of events that were misclassified was minimised. Values for \( t_{\text{crit}} \) were calculated by solving:
\[
(a_{\text{fast}}/\tau_{\text{fast}})e^{-t_{\text{crit}}/\tau_{\text{fast}}} = (a_{\text{slow}}/\tau_{\text{fast}})e^{-t_{\text{crit}}/\tau_{\text{slow}}}
\]

Where \(a_{\text{fast}}\) and \(a_{\text{slow}}\) are the areas of the two exponential components (\(\tau_{\text{fast}}\) and \(\tau_{\text{slow}}\)) between which the \(t_{\text{crit}}\) value must lie. Values of \(t_{\text{crit}}\) obtained using the criterion proposed by Colquhoun & Sakmann (1985) were usually used. Only when calculation of \(t_{\text{crit}}\) values using this criterion failed were alternative \(t_{\text{crit}}\) values used, calculated using one of the two other different criteria.

2.9.5.1 Distribution of burst lengths

Values for \(t_{\text{crit}}\) were calculated between the 3\textsuperscript{rd} and 4\textsuperscript{th} exponential components of the distribution of shut times. The definition of bursts was imperfect because an unambiguous classification between shuttings 'within bursts' and 'between bursts' could not be achieved because the difference between the exponential components of the distribution of shut times was not big enough.

2.9.5.2 Distribution of total open time per burst

The distribution of the total open time per burst is much simpler than the distribution of burst lengths because the number of exponential components is expected to be equal to the number of open states while the effect that the presence of incompletely resolved events may have on the determination of the number of open states is very much reduced. It is also less sensitive to missed events than the distribution of open times.

2.9.5.3 Burst \(P_{\text{open}}\)

The burst \(P_{\text{open}}\) or fraction of time during which the channel dwells in any open state during a burst of openings was calculated for each experiment by dividing the mean total open time per burst by the mean burst length.

2.9.6 Clusters

Activation of a single NMDA receptor produces clusters of channel openings (Gibb & Colquhoun, 1991). Clusters of openings were defined as groups of openings separated by shut times shorter than a critical length or \(t_{\text{crit}}\). For each experiment, a \(t_{\text{crit}}\) was calculated from the fitted parameters of the shut time distribution, assuming the 4\textsuperscript{th} exponential component of the shut time distribution as being "within-clusters" and the
5th component as being "between-clusters". In each case, $t_{\text{crit}}$ values were calculated so as to make the percentage of long shut times that were misclassified as "within-clusters" equal to the percentage of short shut times that were misclassified as "between-clusters" (Colquhoun & Sigworth, 1995). Cluster length, total open time per cluster and cluster $P_{\text{open}}$ were calculated in the same way as for bursts.

2.9.7 Super-clusters

Super-clusters were defined as groups of openings separated by gaps underlying the first five components of the shut time distribution (Gibb & Colquhoun, 1992). Super-cluster length, total open time per super-cluster and super-cluster $P_{\text{open}}$ were calculated in the same way as in bursts and clusters.

2.9.8 Alignment of activations

Clusters and super-clusters of openings separated by the same $t_{\text{crit}}$ value used to build distributions of clusters and super-clusters were aligned with the start of the first opening of each cluster or super-cluster occurring simultaneously. Sections of the data record of a fixed length and containing only one activation (cluster or super-cluster) were visually checked and stored for subsequent averaging. Those containing simultaneous openings (doubles) were discarded. Ensemble averages resulting from these alignments were constructed and the time-course of their decays fitted with a sum of exponentials.

2.9.9 Statistics

Unless otherwise indicated, data were tested using a two-sample t-test, assuming unequal variance, to the 5 % level ($P < 0.05$). All results were tested and checked using the in-house computer program RANTEST designed by D. Colquhoun. RANTEST avoids assuming patch data are normally distributed, by repeatedly and randomly assigning all observations into two data sets, calculating the difference in mean of the two data sets and then compiling histograms from this information. This and not the t-distribution is then used to compare the actual difference in mean of the two data groups. Probability, and hence statistical significance, of the two data sets originating from the same population is then evaluated.

Differences in the slope and elevation of regression lines of two data sets, defining the relationship of each data set with holding potential, for example single
channel conductance, was tested for significance using analysis of covariance, COVAR, to the 5% level (P < 0.05) (Zar, 1999).
CHAPTER 3

EFFECTS OF THE PHOSPHATASE INHIBITOR CYCLOSPORIN-A AND PROTEIN KINASE C INHIBITOR CALPHOSTIN-C ON SINGLE NMDA CHANNEL PROPERTIES

3.1 SUMMARY

i. NMDA receptor single channel currents were recorded from dentate gyrus granule cells in P12 rat hippocampal slices in order to determine whether the protein phosphatase 2B inhibitor cyclosporin-A and the PKC inhibitor calphostin-C alter single NMDA receptor channel characteristics in outside-out patches.

ii. Either 200nM cyclosporin-A or 200nM calphostin-C were applied in the bath and internal pipette solution. Single channel activity in response to 0.1 - 10 \( \mu \text{M} \) NMDA and 1 - 10 \( \mu \text{M} \) glycine was compared to control recordings.

iii. Single NMDA channel amplitudes contained a mixture of high and low conductance levels in both treated and control groups. High conductance amplitudes gave a slope conductance of 53 pS in control recordings, while this was 55 pS and 53 pS for cyclosporin-A and calphostin-C treated groups, respectively. For the low conductance channel amplitudes, control recordings gave a slope conductance of 39 pS and for cyclosporin-A and calphostin-C treated groups 45 pS or 39 pS, respectively. Single channel conductance remained unaffected in the presence of calphostin-C. Cyclosporin-A increased the low conductance level amplitude by 6 pS but not significantly.

iv. NMDA channel shut time distributions were best fitted with the sum of three to five exponential components, depending upon patch activity. The mean shut times for all five components and for the first two components (within burst shut times) were calculated to be 352 ± 91 ms and 0.74 ± 0.1 ms for control, 802 ± 204 ms and 0.95 ± 0.3 for cyclosporin-A and 679 ± 313 and 0.96 ± 0.2 for calphostin-C treated patches. Cyclosporin-A was found to significantly increase mean shut time and calphostin-C significantly increased the weighted mean shut time within a burst. The voltage-
dependence of mean shut time and weighted mean shut time within a burst remained unaffected.

v. NMDA channel open time distributions were best fitted with the sum of two or three exponential components in both control and treated groups. For control recordings this gave a mean open time of $3.34 \pm 0.42$ ms at a holding potential of $-60$ mV, whilst cyclosporin-A and calphostin-C treated patches gave a mean open time of $3.25 \pm 0.45$ ms and $3.92 \pm 0.33$ ms, respectively. Thus, neither cyclosporin-A nor calphostin-C significantly altered single NMDA channel mean open time recorded in outside-out patches.

vi. The NMDA channel mean open time is normally voltage-dependent, increasing e-fold for every $54$ mV depolarisation in control recordings. In the presence of cyclosporin-A and calphostin-C the voltage-dependence was e-fold for every $43$ mV and $40$ mV respectively. Thus cyclosporin-A and calphostin-C did not significantly alter the voltage-dependence of the channel mean open times.

vii. At a holding potential of $-60$ mV the open probability was $0.0196 \pm 0.0086$ for control, $0.0069 \pm 0.0031$ for cyclosporin-A and $0.0186 \pm 0.0111$ for calphostin-C treated patches. Over all the membrane potentials recorded, the presence of cyclosporin-A or calphostin-C was not found to significantly alter the activation of single NMDA receptors in granule cell outside-out patches.

viii. To improve the quality of further patch recordings the CsCl-based pipette solution used in these experiments was replaced by a Na-gluconate solution. The effects of this replacement are considered here by comparing channel conductance, open times and shut times, for these two recording conditions. In general the results from CsCl based and Na-gluconate based experiments were similar except for a slight but not significant increase in the voltage-dependency of NMDA receptor channel mean open time for Na-gluconate patches, presumed to be an increase in sensitivity to contaminant magnesium in the recording solutions. Mean open time increased e-fold for every $34$ mV depolarisation in Na-gluconate patches, in comparison to $53$ mV in CsCl patches.
3.2 INTRODUCTION

Cyclosporin-A is a selective inhibitor of calcineurin (protein phosphatase 2B). It was originally developed as an immunosuppressant drug for use in tissue transplantation (Pedersen et al., 1995), as calcineurin is involved in a T-cell signal transduction pathway which leads to the expression of interleukin 3 (Nair et al., 1994). Calcineurin was also found to be present in the central nervous system (Klee et al., 1979) and since then cyclosporin-A has also become a useful tool with which to investigate the role of calcineurin in the central nervous system (Klee et al., 1988; Lieberman & Mody, 1994; Tong & Jahr, 1994).

Calphostin C is a selective inhibitor of PKC (Kobayashi et al., 1989). With potent cytotoxic, antitumour activity it has been a candidate in cancer therapy and has also served to decipher pathways that involve PKC due to its specificity for this enzyme.

Activators of PKC increase the macroscopic NMDA current (Chen & Huang, 1992; Zheng et al., 1997) and it has been suggested that PKC is involved in modulation of NMDA currents during LTP, a form of synaptic plasticity (for review, see Ben-Ari et al., 1992). The molecular basis of these actions of PKC are yet unresolved and under contention. Some studies have suggested an increase in NMDA open probability (Xiong et al., 1998) and decrease in magnesium sensitivity (Chen & Huang, 1992) as a mechanism of action, although the latter is under debate (Wagner & Leonard, 1996; Xiong et al., 1998).

PKC phosphorylates the NR1 C-terminus of the NMDA receptor channel in a conserved region of the C1 exon (Tingley et al., 1997). Whether phosphorylation of this site directly results in potentiation of NMDA receptor function is unclear. Studies have shown that potentiation of NMDA receptor mediated currents by PKC displays an unusual relationship with the C1 exon and other phosphorylation sites on the NR2 subunit (Durand et al., 1993; Sigel et al., 1994; Zheng et al., 1999).

Calcineurin reduces NMDA receptor channel activity in cell-attached and inside-out patches (Lieberman & Mody 1994), presumably by receptor dephosphorylation or a related pathway, although this has not been directly demonstrated. Inhibition of calcineurin with selective agents increases NMDA receptor channel open probability, mean open time, burst, cluster and supercluster length (Lieberman & Mody 1994) measured in cell-attached patches, reduces NMDA synaptic current desensitisation (Tong et al., 1995), prevents the onset of LTD (Ramakers et al., 2000) and induces synaptic potentiation (Wang & Kelly, 1997).
Additionally, application of calcineurin to inside-out patches has been shown to cause a reduction in NMDA single channel mean open time (Lieberman & Mody 1994).

The aim of these experiments was to further assess the effect of calcineurin and PKC on native NMDA receptor single channel activity. Outside-out patches taken from granule cells of the dentate gyrus of P12 rats. NMDA channel openings were unequivocally identified by agonist application. Considering slice and pipette solutions were both treated with inhibitor, either cyclosporin-A or calphostin-C, the effect should be a shift in the phosphorylation state of the NMDA receptor channel compared with control recordings. This may give us an indication as to whether this balance between phosphorylation and dephosphorylation is responsible for the previously reported effects of calcineurin and PKC on NMDA receptor single channel activity.

3.3 RESULTS: Comparison of single NMDA channel properties in control recordings and in the presence of cyclosporin-A or calphostin-C

3.3.1. Single NMDA channel recordings

Figure 3.1 illustrates recordings of NMDA receptor single channel currents in control, cyclosporin-A and calphostin-C treated patches. In each experiment the NMDA concentration was chosen to evoke a relatively low level of channel activity. In control patches the open probability \( P_{\text{open}} \) ranged between 0.0091 and 0.0196 for NMDA concentrations between 0.1 and 10 \( \mu \text{M} \). Glycine was co-administered at a saturating concentration of 10 \( \mu \text{M} \). As expected channel amplitude increased with more negative holding potentials (from -30 mV to -80 mV) allowing calculation of the characteristic single channel conductance from the relation between single channel current and membrane potential.

3.3.2 Amplitude stability plots

To ensure that single channel amplitudes remained stable throughout the duration of the patch recording, indicating long-term patch viability, amplitude stability plots were made (Figure 3.2A-C). Each example presented in figure 3.2 of control (A), cyclosporin-A (B) and calphostin-C (C) treated patches contains a dense zone around 3.2 pA indicating the high conductance level and another more diffuse
FIGURE 3.1 NMDA receptor single channel currents from control, cyclosporin-A and calphostin-C treated patches. Downward deflections in base line indicate single channel openings in the presence of 0.1 - 10μM NMDA and 1 - 10 μM glycine, recorded from outside-out patches taken from dentate gyrus, hippocampal granule cells. Patches were recorded at holding potentials between -30 and -80 mV in control bath and pipette solutions or with the inclusion of cyclosporin-A or calphostin-C. Currents were low-pass filtered at 2 kHz (-3dp, 8 pole Bessel filter). Trace recordings are 162 ms long.
FIGURE 3.2 Amplitude stability plots for (A) control, (B) 200 nM cyclosporin-A and (C) 200 nM calphostin-C treated patches. NMDA receptor-mediated openings recorded from outside-out patches excised from hippocampal dentate gyrus cells. Patches were held at -60 mV and exposed to 0.1 - 10 μM NMDA and 1 - 10 μM glycine. Stability plots contain (A) 229, (B) 290 and (C) 193 plotted amplitudes, for amplitudes longer than two filter rise-times, respectively.
zone around 2.6 pA representing the low conductance level. These two components will be referred to as the main and subconductance level.

**TABLE 3.1 Comparison of single channel current amplitudes at -60 mV**

<table>
<thead>
<tr>
<th></th>
<th>1st Gaussian</th>
<th>2nd Gaussian</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amplitude</td>
<td>Area</td>
</tr>
<tr>
<td></td>
<td>(pA)</td>
<td>(%)</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>2.35 ± 0.12</td>
<td>17 ± 3.1</td>
</tr>
<tr>
<td>(n = 9 patches)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cyclosporin-A</strong></td>
<td>2.61 ± 0.07</td>
<td>16 ± 1.5</td>
</tr>
<tr>
<td>(n = 5 patches)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Calphostin-C</strong></td>
<td>2.50 ± 0.20</td>
<td>19 ± 7.4</td>
</tr>
<tr>
<td>(n = 4 patches)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The third smallest component (1.51 ± 0.5 pA) contributed 7 ± 6 % of control openings (n = 6); (1.74 ± 0.12 pA) 3 ± 1 % in cyclosporin-A (n = 3) and (1.66 ± 0.33 pA) 4 ± 1 % in calphostin-C (n = 3) treated patch recordings.

### 3.3.3 Distributions of NMDA receptor single channel current amplitudes

Frequency distributions were compiled containing the amplitudes of all openings that were greater than two filter rise-times in duration. In control and both cyclosporin-A and calphostin-C treated groups the resulting distributions were most often best fitted by the sum of two (or occasionally three) Gaussian components. For control, cyclosporin-A and calphostin-C treated patches (figure 3.3A - C) the relative area associated with the 1st (smaller amplitude) Gaussian component was substantially and consistently smaller than that of the 2nd Gaussian component. Mean amplitude and mean relative area of the first and second component and the 1st/2nd Gaussian component area ratio were compared for recordings made at -60 mV (Table 3.1). These results show that cyclosporin-A or calphostin-C did not change the single channel amplitude of NMDA currents or the proportion of sublevel to main level openings. In cyclosporin-A treated patches the 1st/2nd Gaussian components area ratio is reduced but not significantly so. The third component, seen occasionally, was smaller in amplitude and gave a slope conductance of 19.7 ± 1.6 pS (n = 6) for control, 28.5 ± 4.5 pS for cyclosporin-A (n = 4) and 28.8 ± 3.2 pS for calphostin-C (n = 3) treated patches.
FIGURE 3.3 Amplitude histograms from (A) control, (B) 200 nM cyclosporin-A and (C) 200 nM calphostin-C treated patches. Distributions of channel amplitudes were fitted with the sum of either two or three Gaussian components for amplitudes longer than two filter rise-times. Mean amplitude, standard deviation and relative area are shown in the insets. Channel openings recorded in response to 0.1 – 10 μM NDMA and 1 – 10 μM glycine applied to outside-out patches taken from hippocampal dentate gyrus granule cells, held at -60 mV.

(A) Control

(B) Cyclosporin-A

(C) Calphostin-C
3.3.4 Effects on single channel conductance

The slope conductance of single NMDA channel currents was estimated from the relationship between membrane potential and single channel amplitude (I/V plot) for all patches giving the following results for main and subconductance respectively, for control: 54.5 ± 3.1 pS and 40.6 ± 4.0 pS; cyclosporin-A: 54.8 ± 1.6 and 44.4 ± 2.3 pS; calphostin-C: 55.8 ± 3.8 pS and 42.5 ± 3.2 pS. In addition, control, cyclosporin-A and calphostin-C channel amplitude data were all fitted simultaneously with linear regression that was constrained and weighted to converge at the same point (reversal potential) on the x-axis (Figures 3.4A and B). It was assumed that all groups would have the same reversal potential as the pipette and bath solutions were the same for each group of experiments. In this instance the reversal potential was found to be -0.46 mV. The constrained slope conductance for control, cyclosporin-A and calphostin-C main and sub conductance channel amplitudes (± the standard deviation of parameter estimates) are presented in Table 3.2 below. These data show that cyclosporin-A and calphostin-C have no effect on the main conductance level in comparison to control. Cyclosporin A appears to have increased the slope conductance of the subconductance level slightly, although not significantly so.

<table>
<thead>
<tr>
<th></th>
<th>Main conductance</th>
<th>Sub conductance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(pS)</td>
<td>(pS)</td>
</tr>
<tr>
<td>Control (n = 9)</td>
<td>53.4 ± 1.1</td>
<td>39.3 ± 1.1</td>
</tr>
<tr>
<td>Cyclosporin-A</td>
<td>54.6 ± 1.1</td>
<td>45.6 ± 1.3</td>
</tr>
<tr>
<td>(n = 5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calphostin-C</td>
<td>53.1 ± 1.4</td>
<td>38.7 ± 1.4</td>
</tr>
<tr>
<td>(n = 4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.3.5 Stability plots for shut times, open times and $P_{\text{open}}$

To ensure no time dependent changes in average channel kinetic activity were present during patch recordings, stability plots of shut times, open times and $P_{\text{open}}$ were made. Groups of 20 – 50 measurements of shut times, open times and $P_{\text{open}}$ were averaged and plotted as a function of interval number for control
FIGURE 3.4 Single channel conductance of NMDA receptors measured from (A) control and 200 nM cyclosporin-A or (B) control and 200 nM calphostin-C treated patches. Plots showing current-voltage relationships for single channel currents activated by 0.1 - 10 μM NMDA and 1 - 10 μM glycine. Each point represents the mean single-channel current amplitude identified by fitting Gaussian components to the amplitude histograms at holding potentials between -30 and -80 mV (9 patches for control, 5 patches for cyclosporin-A and 4 patches for calphostin-C). Channel slope conductance was estimated by linear regression (solid line for control and dashed line for treated groups), slope conductances are presented in the inset. The main and subconductance for all plots were simultaneously constrained to give a common reversal potential of -0.46 mV.
FIGURE 3.5 Stability plot analysis of shut times, open times and $P_{\text{open}}$ during activation of NMDA receptors in (A) control, (B) 200 nM cyclosporin-A and (C) 200 nM calphostin-C treated patches. Plots show a running average of shut times (top), open times (middle) and $P_{\text{open}}$ (bottom) for recording of duration 177 - 305 s at -60 mV. Bins represent the average of 10 - 20 intervals with increments of 5 - 10 intervals between averages. Horizontal broken lines show the average of each parameter for the whole recording. The overall mean shut time, mean open time, $P_{\text{open}}$ and mean opening frequency was 704.8 ms, 4.05 ms, 0.00572 and 1.41 openings/sec for control; 707.1 ms, 3.10 ms, 0.00436 and 1.41 openings/sec for cyclosporin-A and 952.1 ms, 4.03 ms, 0.00422 and 1.05 openings/sec for calphostin-C. A resolution of 165 μs was imposed on both openings and shuttings which gave a false event rate of $1 \times 10^4$, at this holding potential (-60 mV).
cyclosporin-A and calphostin-C treated patches (Figure 3.5A-C). Mean open time remained relatively constant in all recordings whereas mean shut time fluctuated depending upon bursts of channel activity also reflected in the open probability.

3.3.6 Shut times distributions

In the majority of patches shut time distributions were best fitted with the sum of five exponential components. However, in some patches where channel activity was low, or at low membrane potentials where the resolution does not allow detection of some components, four or three exponential components were used to fit the data (Figure 3.6A-C). Time constants and associated area of the exponential components for shut times at a holding potential of -60 mV are compared between control, cyclosporin-A and calphostin-C treated patches in Table 3.3. Mean shut time (Figure 3.7A and B) and the weighted mean shut time of the first two components (Figure 3.8A and B) were also compared between groups. Cyclosporin-A was found to significantly increase mean shut time and calphostin-C significantly increased the weighted mean shut time within a burst.

**TABLE 3.4 Comparison of open times at -60 mV from control, 200 nM cyclosporin-A and 200 nM calphostin-C treated patches**

<table>
<thead>
<tr>
<th></th>
<th>$\tau_1$ (ms)</th>
<th>$\tau_2$ (ms)</th>
<th>$\tau_3$ (ms)</th>
<th>Mean (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.37 ± 0.1</td>
<td>1.47 ± 0.3</td>
<td>5.33 ± 0.5</td>
<td>3.34 ± 0.4</td>
</tr>
<tr>
<td>(n = 9)</td>
<td>(26 ± 8 %)</td>
<td>(20 ± 6 %)</td>
<td>(54 ± 7 %)</td>
<td></td>
</tr>
<tr>
<td>Cyclosporin-A</td>
<td>0.29 ± 0.07</td>
<td>2.01 ± 0.6</td>
<td>6.14 ± 0.5</td>
<td>3.26 ± 0.5</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>(32 ± 10 %)</td>
<td>(23 ± 10 %)</td>
<td>(45 ± 6 %)</td>
<td></td>
</tr>
<tr>
<td>Calphostin-C</td>
<td>0.53 ± 0.2</td>
<td>4.16 ± 0.5</td>
<td>6.13 ± 0.7</td>
<td>3.92 ± 0.3</td>
</tr>
<tr>
<td>(n = 4)</td>
<td>(32 ± 7 %)</td>
<td>(13 ± 8 %)</td>
<td>(55 ± 8 %)</td>
<td></td>
</tr>
</tbody>
</table>

3.3.7 Open times distributions

Open time distributions from control, cyclosporin-A and calphostin-C treated patches were fitted with the sum of three (or in a few cases two) exponential components (Figure 3.9A-C). The exponential time constants and associated areas
FIGURE 3.6 Distribution of shut times for (A) control, (B) 200 nM cyclosporin-A and (C) 200 nM calphostin-C treated patches. In these distributions shut time intervals ranging from 0.165 to 8845 ms were best fitted with the sum of five (and sometimes four) exponential components. Time constants and associated areas are shown in the inset. Predicted shut time means (and observed and predicted true number of observations) were 597 ms (76 and 89.1) in control, 852 (176 and 180.09) in cyclosporin-A and 860 (174 and 201) for calphostin-C treated patches, held at -60 mV. Mean shut time found to have significantly increased in the presence of cyclosporin-A, at all membrane potentials recorded, by analysis of COVAR (P < 0.05).
TABLE 3.3 Comparison of shut times between control, 200 nM cyclosporin-A and 200 nM calphostin-C treated patches at -60 mV. Shown are the mean ± SEM for time constants and (in brackets) relative area of each exponential component of the shut time distributions.

<table>
<thead>
<tr>
<th></th>
<th>$\tau_1$ (µs)</th>
<th>$\tau_2$ (ms)</th>
<th>$\tau_3$ (ms)</th>
<th>$\tau_4$ (ms)</th>
<th>$\tau_5$ (ms)</th>
<th>Mean (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>131 ± 16</td>
<td>0.96 ± 0.14</td>
<td>9.85 ± 1.43</td>
<td>207 ± 78</td>
<td>1341 ± 629</td>
<td>352 ± 91.1</td>
</tr>
<tr>
<td>(n = 9)</td>
<td>(11 ± 5 %)</td>
<td>(32 ± 4 %)</td>
<td>(9 ± 1 %)</td>
<td>(18 ± 5 %)</td>
<td>(31 ± 5 %)</td>
<td></td>
</tr>
<tr>
<td>Cyclosporin-A</td>
<td>423 ± 78</td>
<td>2.98 ± 1.3</td>
<td>24.6 ± 5.5</td>
<td>183 ± 36</td>
<td>1286 ± 327</td>
<td>802 ± 204</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>(17 ± 4 %)</td>
<td>(9 ± 4 %)</td>
<td>(7 ± 3 %)</td>
<td>(5 ± 3 %)</td>
<td>(61 ± 2 %)</td>
<td></td>
</tr>
<tr>
<td>Calphostin-C</td>
<td>530 ± 110</td>
<td>3.12 ± 0.75</td>
<td>13.3 ± 1.2</td>
<td>150 ± 37</td>
<td>2222 ± 577</td>
<td>679 ± 313</td>
</tr>
<tr>
<td>(n = 4)</td>
<td>(32 ± 6 %)</td>
<td>(11 ± 5 %)</td>
<td>(13 ± 8 %)</td>
<td>(17 ± 8 %)</td>
<td>(26 ± 12 %)</td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 3.7 Comparison of mean shut time for (A) control and 200 nM cyclosporin-A and (B) control and 200 nM calphostin-C treated patches. Each point represents the mean shut time of all patches, at that membrane potential, calculated from the five (or four) exponential components fitted to the shut time distributions. Data points were fitted by linear regression to investigate any voltage-dependence of the channel shut times (solid line for control and dashed lines for treated groups). Mean shut time was found to have significantly increased in the presence of cyclosporin-A, at all membrane potentials recorded, by analysis of COVAR (P < 0.05).
FIGURE 3.8 Comparison of the weighted mean of the first two shut time components for (A) control and 200 nM cyclosporin-A and (B) control and 200 nM calphostin-C treated data. Each point represents the mean shut time of all patches, at that membrane potential, calculated from the first two exponential components fitted to the shut time distributions. Data points were fitted by linear regression to investigate any voltage-dependence of the channel shut times (solid line for control and dashed lines for treated groups). Weighted mean shut time within burst was significantly increased in the presence of calphostin-C, at all membrane potentials recorded, by analysis of COVAR (P < 0.05)
FIGURE 3.9 Comparison of distributions for all individual open times in (A) control, (B) 200 nM cyclosporin-A and (C) 200 nM calphostin-C treated patches. In these distributions open time intervals ranging from 0.165 to 37.28 ms were best fitted with the sum of three exponential components, time constants and associated areas are inset. Predicted mean open time (and observed and predicted observations) were 3.28 ms (131 and 165) in control, 2.01 (209 and 327) in cyclosporin-A and 2.84 (173 and 238) for calphostin-C treated patches, held at -60 mV.
FIGURE 3.10 Comparison of mean open time correlated to membrane potential for (A) control and 200 nM cyclosporin-A and (B) control and 200 nM calphostin-C treated patches. Each point represents the mean open time of all patches, at that membrane potential, calculated from the three (or two) exponential components fitted to the open time distributions. The data were fit using linear regression (solid line for control and dashed lines for treated groups) to allow estimation of the voltage-dependence of the open times. In control this was 54 mV depolarisation for an e-fold change in mean open time, 43 mV in cyclosporin-A and 40 mV in calphostin-C treated patches.
FIGURE 3.11 Comparison of channel mean open probability between (A) control and 200 nM cyclosporin-A and (B) control and 200 nM calphostin-C treated patches. Each point corresponds to the mean open probability for all patches at any given membrane potential, calculated from the mean open and shut times. The data were fit by linear regression (solid line for control and dashed lines for treated groups) to allow estimation of any voltage-dependence of $P_{\text{open}}$.

(A) &bullet; Control (n = 9)
&square; Cyclosporin A (n = 5)

(B) &bullet; Control (n = 9 patches)
&square; Calphostin-C (n = 4 patches)
(mean ± S.E.M.) for each component and overall mean open time, at a holding potential of -60 mV, are presented in Table 3.4. No significant difference was observed in the open time distribution parameters or mean open time between control and treated groups except for an increase in the time constant for the second component for calphostin-C data.

3.3.8 Dependence of mean open time on membrane potential

NMDA channel mean open time in control and in the presence of cyclosporin-A or calphostin-C is presented as a function of membrane potential in Figure 3.10A and B. In control, cyclosporin-A and calphostin-C patches the voltage-dependence was e-fold for every 54 mV, 43 mV and 40 mV depolarisation, respectively. Neither cyclosporin-A nor calphostin-C produced any significant change in the voltage-dependence of the channel open time. Although cyclosporin-A had an effect on mean shut time and calphostin-C increased the weighted mean shut time within a burst, calculated $P_{\text{open}}$ did not significantly change between control and treated groups at any of the voltages recorded (Figure 3.10A-C).

3.4 RESULTS: Comparison of NMDA receptor single channel properties recorded using pipette solutions based on CsCl or Na-gluconate

3.4.1 Single channel recordings

NMDA (0.1 - 10 μM) and glycine (10 μM) were applied to outside-out patches taken from hippocampal dentate gyrus granules cells in hippocampal slices from P12 rats. Pipettes contained either CsCl or Na-gluconate based pipette solution. No obvious qualitative differences were apparent in the single channel recordings obtained under these conditions as illustrated in Figures 3.11A and B, although the Na-gluconate patch has more NMDA receptor activity than the CsCl patch presumably due to a greater channel population in this example.

3.4.2 Comparison of amplitude stability plots

Long-term stability of single channel amplitudes recorded using Na-gluconate or CsCl-based pipette solution is compared in Figure 3.13A and B. Inclusion of Na-gluconate did not affect patch stability. In both cases a dense band can be seen approximating to -3.5 pA and a more diffuse band at the -2.5 pA level. This indicates the presence of both a main and subconductance level in each group.
FIGURE 3.12 NMDA receptor single channel currents from patches formed in the presence of either CsCl or Na-gluconate based pipette solution. Downward deflections in base line indicate single channel openings in the presence of 0.1 - 10μM NMDA and 1 - 10 μM glycine, recorded from outside-out patches taken from dentate gyrus, hippocampal granule cells. Patches were recorded at holding potentials between -30 and -80 mV. Pipette electrodes contained either CsCl or Na-gluconate based internal solution. Currents were low-pass filtered at 2 kHz (-3dp, 8 pole Bessel filter). Trace recordings are 162 ms long.

<table>
<thead>
<tr>
<th>Voltage (mV)</th>
<th>CsCl</th>
<th>Na-gluconate</th>
</tr>
</thead>
<tbody>
<tr>
<td>-30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-80</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 pA  100 ms
FIGURE 3.13 Amplitude stability plots for control patches using either (A) CsCl or (B) Na-gluconate based pipette solution. NMDA receptor-mediated openings recorded from outside-out patches excised from hippocampal dentate gyrus cells. Patches were held at -60 mV and exposed to 0.1 - 10 μM NMDA and 1 - 10 μM glycine. Stability plots contain (A) 229 and (B) 1511 plotted amplitudes for amplitudes longer than two filter rise-times.
3.4.3 Comparison of single channel current amplitudes

Amplitude distributions for both CsCl and Na-gluconate patches were fitted with two (and in some cases three) Gaussian components (Figure 3.14A and B). Table 3.5 shows mean amplitudes, associated area and the area ratio for the 1st and 2nd Gaussian component from patches recorded at -60 mV. Neither the amplitude of the main conductance level nor the amplitude of the subconductance level, were significantly different in the presence of Na-gluconate. The 1st/2nd Gaussian ratio was reduced in the presence of Na-gluconate but not significantly so. Additionally a third smaller component was seen in six CsCl and all Na-gluconate patches.

<table>
<thead>
<tr>
<th>TABLE 3.5 Comparison of amplitude distribution parameters at -60 mV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1st Gaussian</strong></td>
</tr>
<tr>
<td>Amplitude</td>
</tr>
<tr>
<td>(pA)</td>
</tr>
<tr>
<td>CsCl * (n = 9 patches)</td>
</tr>
<tr>
<td>Na-gluconate * (n = 6 patches)</td>
</tr>
</tbody>
</table>

* Third amplitude component 1.51 ± 0.15 pA contributed 7 ± 6 % of total channel openings in CsCl (n = 6) and for Na-gluconate patches the third components 1.65 ± 1.14 contributed 2 ± 0.4 % (n = 6).

Each point in Figure 3.15A indicates the relative area associated with either the 1st or 2nd Gaussian component from both CsCl and Na-gluconate treated channel amplitude distributions. Filled circles for CsCl and open circles for Na-gluconate. Two distinct and similarly spread levels are observed for CsCl and Na-gluconate patches. The smaller component approximating to 15 % of all openings represents the relative area for the sub conductance level and the higher level approximating to 85 % represents the relative area of the main conductance level. This indicates that the proportion of low : high conductance channels is not affected by changing the pipette solution from a CsCl based to a Na-gluconate based solution.
FIGURE 3.14 Amplitude histograms from control patches using either (A) CsCl or (B) Na-gluconate based pipette solution. Distributions of channel amplitudes were fitted with the sum of either two or three Gaussian components for amplitudes longer than two filter rise-times. Mean amplitude, standard deviation and relative area for each Gaussian component are inset, for patches held at -60 mV.
3.4.5 Comparison of single channel conductance

The slope conductance of single NMDA channel currents was estimated for CsCl and Na-gluconate patches to be 54.4 ± 1.6 pS and 49.0 ± 1.3 pS for the main conductance level; 40.0 ± 1.4 pS and 39.7 ± 1.9 pS for the sub conductance level respectively. The third amplitude component found in six of nine CsCl and all Na-gluconate patch recordings gave a slope conductance of 19.7 ± 1.9 pS and 29.3 ± 4.1 pS.

Single NMDA channel amplitude was plotted against membrane potential to construct I/V plots from which channel conductance can be estimated for both low and high conductance levels for both CsCl and Na-gluconate patches (Figure 3.15B). Linear regression was performed on each group individually, with low and high conductance levels constrained to regress to the same reversal potential. The resulting slope conductances and reversal potentials are presented in Table 3.6. It is evident from Figure 3.15B that although both groups have a similar slope conductance for both low and high conductance levels, the reversal potential is different.

<table>
<thead>
<tr>
<th></th>
<th>Main conductance (pS)</th>
<th>Subconductance (pS)</th>
<th>Reversal potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsCl (n = 9)</td>
<td>54.4 ± 1.6</td>
<td>40.0 ± 1.4</td>
<td>-1.41 ± 1.4</td>
</tr>
<tr>
<td>Na-gluconate (n = 6)</td>
<td>50.6 ± 1.2</td>
<td>37.6 ± 0.9</td>
<td>6.83 ± 1.3</td>
</tr>
</tbody>
</table>

3.4.5 Comparison of stability plots for shut times, open times and $P_{open}$

To ensure average kinetic behaviour did not change over time and was not altered by the presence of Na-gluconate in comparison to CsCl, stability plots for shut times, open times and $P_{open}$ were compiled for each patch analysed. As illustrated in Figure 3.16A and B, Na-gluconate does not affect patch stability. Mean open time remains constant, while in this example, mean shut time is shorter in the Na-gluconate patch, which is also reflected in $P_{open}$. This is probably due to greater channel activity, as a result of a greater receptor population because both patches were recorded at 100 nM NMDA and 10 μM glycine.
FIGURE 3.15 (A) Relative area associated with the 1st and 2nd Gaussian amplitude components and (B) relationship between holding potential and NMDA channel amplitude for patches using either CsCl or Na-gluconate based pipette solution. (A) Relative percentage of main and sub level openings, estimated from Gaussian components fitted to amplitude distributions in CsCl and Na-gluconate patches. (B) Single channel conductance of NMDA receptors estimated from CsCl and Na-gluconate patches. The data were fit by linear regression to give the slope conductances inset (straight line for CsCl and dashed line for Na-gluconate patches. 9 patches for CsCl and 6 patches for Na-gluconate).

(A)  
- CsCl (n = 9 patches)  
- Na-gluconate (n = 6 patches)

(B) Membrane Potential (mV)

- CsCl 52.9 pS  
- Na-gluconate 50.6 pS  
- CsCl 38.8 pS  
- Na-gluconate 37.6 pS
FIGURE 3.16 Stability plot analysis of shut times, open times and $P_{open}$ during activation of NMDA receptors in patches using either (A) CsCl or (B) Na-gluconate based pipette solution. Plots show a running average of shut times (top), open times (middle) and $P_{open}$ (bottom) during 177 - 215 s held at -60 mV. Bins represent the average of 10 - 75 intervals with increments of 5 - 37 intervals between averages. Horizontal broken lines represent the average values for the whole recording. The overall mean shut time, mean open time, $P_{open}$ and mean opening frequency were 704.8 ms, 4.05 ms, 0.00572 and 1.41 openings/sec (CsCl) and 86.0 ms, 3.97 ms, 0.0439 and 11.18 openings/sec (Na-gluconate). Both openings and shuttings analysed at a resolution of 165 μs which gave a false event rate of 1 x 10^{-5}, at this holding potential.

(A) CsCl

(B) Na-gluconate
3.4.7 Comparison of shut time distributions

The majority of shut time distributions were best fitted with the sum of five exponential components (Figure 3.17A and B). Time constants and associated areas of the exponential components for shut times from CsCl and Na-gluconate patches are compared at a holding potential of -60 mV in Table 3.7. These values are very similar; the time constants do not appear to differ between groups although the associated relative areas vary slightly.

The comparison of mean shut time over various holding potentials indicates no significant difference between the values for CsCl and Na-gluconate patches (Figure 18A). The mean shut time of the first two components is also very similar between groups over various holding potentials (Figure 18B). Na-gluconate was found to significantly increase the weighted mean shut time within a burst.

### Table 3.8 Comparison of individual open time distribution parameters and voltage dependence of the open times for CsCl and Na-gluconate patches

<table>
<thead>
<tr>
<th>Potential</th>
<th>Component</th>
<th>CsCl</th>
<th>Na-gluconate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \tau_1 )</td>
<td>( \tau_2 )</td>
<td>( \tau_3 )</td>
</tr>
<tr>
<td>-30 mV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CsCl</td>
<td>0.32 ± 0.01</td>
<td>3.56 ± 0.3</td>
<td>7.41 ± 0.9</td>
</tr>
<tr>
<td>(n = 9)</td>
<td>(23 ± 6%)</td>
<td>(13 ± 7%)</td>
<td>(64 ± 7%)</td>
</tr>
<tr>
<td>Na-gluconate</td>
<td>0.47 ± 0.1</td>
<td>2.63 ± 0.6</td>
<td>7.79 ± 1.3</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(11 ± 5%)</td>
<td>(22 ± 8%)</td>
<td>(67 ± 7%)</td>
</tr>
<tr>
<td>-80 mV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CsCl</td>
<td>0.22 ± 0.1</td>
<td>0.87 ± 0.2</td>
<td>3.70 ± 0.8</td>
</tr>
<tr>
<td>(n = 9)</td>
<td>(21 ± 11%)</td>
<td>(31 ± 13%)</td>
<td>(48 ± 10%)</td>
</tr>
<tr>
<td>Na-gluconate</td>
<td>0.23 ± 0.06</td>
<td>1.15 ± 0.2</td>
<td>1.87 ± 0.5</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(33 ± 14%)</td>
<td>(14 ± 10%)</td>
<td>(51 ± 14%)</td>
</tr>
</tbody>
</table>

3.4.7 Comparison of open time distributions

Open time distributions for all CsCl and Na-gluconate patches were best fitted with the sum of three (or in a few cases two) exponential components (Figure 3.19A and B). Comparing time constants for NMDA open time exponential components at -30 mV and -80 mV in Table 3.8, it is possible to see the effect of membrane potential on open time parameters. At -30 mV the time constants and associated areas (in brackets) are relatively similar to CsCl patches, which is reflected in the data illustrated in Figure 3.20A. At -80 mV the first time constant (\( \tau_1 \)) does not
FIGURE 3.17 Distribution of shut times for patches recorded in the presence of (A) CsCl and (B) Na-gluconate based pipette solution. In these distributions shut time intervals ranging from 0.165 to 7652 ms were best fitted with the sum of five exponential components, time constants and associated areas are inset. Predicted shut time means (and observed and predicted number of observations were 597 ms (76 and 89.1) for CsCl (n = 6); 74.3 (1199 and 13464) for Na-gluconate (n = 9) treated patches, at -60 mV.

(A) CsCl

(B) Na-gluconate
TABLE 3.7 Comparison of shut times between CsCl and Na-gluconate patches at -60 mV. Shown are the mean ± SEM for time constants and (in brackets) relative area of each exponential component of the shut time distributions.

<table>
<thead>
<tr>
<th></th>
<th>$\tau_1$ (μs)</th>
<th>$\tau_2$ (ms)</th>
<th>$\tau_3$ (ms)</th>
<th>$\tau_4$ (ms)</th>
<th>$\tau_5$ (ms)</th>
<th>Mean (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsCl</td>
<td>131 ± 16</td>
<td>0.96 ± 0.14</td>
<td>9.85 ± 1.43</td>
<td>207 ± 78</td>
<td>1341 ± 629</td>
<td>352 ± 91.1</td>
</tr>
<tr>
<td>(n = 9)</td>
<td>(11 ± 5 %)</td>
<td>(32 ± 4 %)</td>
<td>(9 ± 1 %)</td>
<td>(18 ± 5 %)</td>
<td>(31 ± 5 %)</td>
<td></td>
</tr>
<tr>
<td>Na-gluconate</td>
<td>345 ± 94</td>
<td>1.17 ± 0.16</td>
<td>37.8 ± 5.7</td>
<td>602 ± 244</td>
<td>1931 ± 982</td>
<td>386 ± 123</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(21 ± 3 %)</td>
<td>(38 ± 6 %)</td>
<td>(23 ± 4 %)</td>
<td>(6 ± 3 %)</td>
<td>(28 ± 7 %)</td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 3.18 Comparison of (A) overall mean shut time and (B) mean shut time within a burst for patches recorded in the presence of either CsCl or Na-gluconate based pipette solution. (A) Each point represents the mean shut time of all patches or (B) weighted mean shut time of first two shut time components, at each membrane potential, calculated from the five exponential components fitted to the shut time distributions. The data were fitted by linear regression to investigate the voltage dependence of the shut times (straight line for CsCl and dashed lines for Na-gluconate).
differ between Na-gluconate and CsCl patches. The third open time component ($\tau_3$), however, is shorter in Na-gluconate patches resulting in a reduction of mean open time. The second time constant has increased in the presence of Na-gluconate at more negative potentials. This may have resulted from the increased proximity of the second and third time constant and thus made true determination of the second time constant more difficult. Despite these trends there was not a significant difference in the mean open time in the presence of Na-gluconate when compared to CsCl treated patches at -80 mV.

### 3.4.8 Comparison of mean open time and correlation with membrane potential

The relationship between NMDA channel mean open time and holding potential in the presence of CsCl and Na-gluconate internal solution is shown in Figure 3.20A. There is a steeper relationship between the log of mean open time and membrane potential in the presence of Na-gluconate than CsCl. The voltage-dependence of the channel open time was not found to be significantly different between the two recording conditions, though, being e-fold for 54 mV in CsCl patches and e-fold for 34 mV in Na-gluconate patches.

$P_{\text{open}}$ was calculated from open and shut times (Figure 3.20B). For Na-gluconate patches $P_{\text{open}}$ was more dependent on membrane potential when compared to the $P_{\text{open}}$ for CsCl patches. This effect was not found to be statistically significant but probably reflects the increased voltage dependence of the NMDA receptor channel mean open time seen in Na-gluconate patches.

### 3.5 DISCUSSION: Effects of cyclosporin-A and calphostin-C

Results obtained by Lieberman & Mody (1994) and Chen & Huang (1992) are presented in Table 3.9 with the results obtained in this thesis. Experimental protocol, inhibitor used, single channel conductance, mean shut time, mean open time and $P_{\text{open}}$ are compared and discussed below.

#### 3.5.1 Single channel conductance

Two conductance levels were observed in all patches analysed regardless of the presence of cyclosporin-A and calphostin-C. In some patches a third conductance level was also evident. The high conductance level was 53 pS for control data, 55 pS for cyclosporin A and 53 pS for calphostin-C treated patches and 39 pS, 46 pS and 39
FIGURE 3.19 Comparison of distributions for all individual open times in patches recorded with (A) CsCl or (B) Na-gluconate based pipette solution. In these distributions open time intervals ranging from 0.165 to 37.28 ms were best fitted with the sum of three exponential components, time constants and associated areas are inset. Predicted mean open time (and observed and predicted observations) were 3.28 ms (131 and 165) for CsCl and 3.47 (1196 and 1352) for Na-gluconate patches, at -60 mV.
FIGURE 3.20 Comparison of (A) mean open time and (B) mean open probability correlated to membrane potential for patches recorded in the presence of CsCl and Na-gluconate based pipette solution. (A) Each point represents the mean open time of all patches, at each membrane potential, calculated from the three (or occasionally two) exponential components fitted to the open time distributions. (B) Each point corresponds to the mean open probability for all patches at each membrane potential, calculated from the mean open and shut times. The data were fit by linear regression (solid line for CsCl and dashed lines for Na-gluconate patches) to examine the voltage-dependence of the mean open time and open probability. Mean open time increased e-fold for every 54 mV depolarisation in CsCl patches compared to 34 mV for Na-gluconate patches.
pS respectively for the low conductance level. These values are similar to a previously published figure of 56 pS for single NMDA channel conductance in hippocampal granule cells (Strecker et al., 1994). The results suggest that neither cyclosporin-A or calphostin-C had any effect on the NMDA receptor single channel conductance, in agreement with the findings of Lieberman & Mody (1994). However the subconductance level appears to have increased in the presence of cyclosporin-A although not significantly. This has not been observed before and could be related to inhibition of calcineurin but, because little charge is passed by this conductance level (approximately 6.7% of total), this effect would be expected to make very little difference to the macroscopic NMDA synaptic current, approximately 0.6%.

The presence of a third smaller conductance level found here has not been reported before in the hippocampus, where the NMDA receptor is predominantly composed of the NR1/NR2A or NR2B subunits giving rise to an approximate 50/40 pS conductance channel (Stern et al., 1992). The third conductance level could arise from a contaminant channel, as it is not present in all the patches analysed, but considering the low level of detection of the third conductance level in the patches analysed here it could just have a low probability occurrence. There is not enough evidence from this study, though, to substantiate the presence of a novel conductance level.

3.5.2 Shut times

The mean shut time was calculated as one indicator of possible effects of cyclosporin-A and calphostin-C on the gating of the NMDA receptor channel. At a holding potential of -60 mV mean shut times were 352 ± 91.1 ms, 802.4 ± 203.6 ms and 678.7 ± 312.5 ms for control, cyclosporin-A and calphostin-C treated patches, respectively. These values are associated with a large standard error that indicates a large variation between individual patches.

Cyclosporin-A significantly increased mean NMDA channel shut time, which would suggest that calcineurin decreases mean shut time. This contradicts the results indicated by Lieberman & Mody (1994) who found no change of NMDA receptor mean shut time by okadaic acid, a calcineurin inhibitor. Mean shut time is not an entirely reliable measurement though; the fifth time constant tends to vary between patches because it is dependent upon the number of channels in a patch and to some degree agonist concentration. It is not possible to accurately determine how many
### TABLE 3.9 Comparison of the results obtained during this thesis with previously published work, all at -60 mV

<table>
<thead>
<tr>
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<tbody>
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</tr>
<tr>
<td>Experimental</td>
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</tr>
<tr>
<td>Protocol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Okadaic acid</td>
<td>Cyclosporin-A</td>
<td>PKC</td>
<td>Calphostin-C</td>
</tr>
<tr>
<td></td>
<td>Cell-attached</td>
<td>Outside-out patch</td>
<td>Outside-out patch</td>
<td>Outside-out patch</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
<td>Control</td>
<td>Treated</td>
</tr>
<tr>
<td>Conductance (pS)</td>
<td>~50</td>
<td>~50</td>
<td>53.4</td>
<td>54.6</td>
</tr>
<tr>
<td>Mean shut time (ms)</td>
<td>120.4 ± 47.5</td>
<td>149.8 ± 70.4</td>
<td>352.2 ± 91.1</td>
<td>802.4 ± 204</td>
</tr>
<tr>
<td>Mean open time (ms)</td>
<td>1.27 ± 0.23</td>
<td>2.04 ± 0.49*</td>
<td>3.34 ± 0.42</td>
<td>3.26 ± 0.45</td>
</tr>
<tr>
<td>( P_{\text{open}} \times 10^4 )</td>
<td>33.9 ± 14.7</td>
<td>30.1 ± 8.0</td>
<td>196 ± 86</td>
<td>69 ± 31</td>
</tr>
</tbody>
</table>

* Statistical significance with Student's t-test (P > 0.5) Chen & Huang (1992) and (P > 0.05) Lieberman & Mody (1994)
channels are present in a patch and therefore it is not possible to correct for this source of variability.

A similar increase in mean shut time is also seen with calphostin-C but this was not found to be significant. If mean shut time were increased by calphostin-C it would suggest that PKC reduces NMDA mean shut time, which could plausibly contribute to an increase in NMDA receptor activity, as seen with PKC (Chen & Huang, 1992; Xiong et al., 1998). However, in the outside-out patch studies here there is no clear indication of any effect of PKC inhibitors.

In order to determine if cyclosporin-A or calphostin-C selectively affected the short gaps within NMDA activations, the weighted means of the shut times underlying the first two components were compared. The time constants of the first two components describe the shut times within short bursts of openings. The group of channel openings defined as a burst (Colquhoun & Hawkes, 1982) and the duration of the shut times between channel openings within a burst can be compared with a greater degree of accuracy; these are more consistent between patches since they do not depend on the number of channels in the patch. The receptor population within a patch and agonist concentration does not affect these parameters because they reflect a single NMDA receptor flickering in and out of an activated state whilst the agonist remains bound.

The weighted mean of the first two shut time components for control, cyclosporin-A and calphostin-C treated patches at a holding potential of -60 mV was 0.74 ± 0.1 ms, 1.05 ± 0.3 ms and 0.96 ± 0.18 ms, respectively. Only calphostin-C significantly increased the mean shut time within a burst when compared to control recordings but only by a small margin. Thus I conclude that the presence of cyclosporin-A or calphostin-C does not greatly affect the mean shut time or mean shut time within a burst. Additionally, the voltage-dependence of these two measurements remained unchanged in the presence of cyclosporin-A and calphostin-C.

3.5.3 Open times

The open time distribution exponential time constants and relative areas for control, cyclosporin-A and calphostin-C treated patches indicate no change in the kinetics of single NMDA channel openings. The mean open time for control (3.34 ± 0.4 ms), cyclosporin-A (3.26 ± 0.45 ms) and calphostin-C (3.92 ± 0.33 ms) treated patches also show no significant difference between treated and control groups at a
holding potential of -60 mV. The voltage dependence of NMDA channel mean open time also remains similar to control in the presence of both cyclosporin-A and calphostin-C, indicated by a similar increase in membrane potential every e-fold increase in mean open time.

These results differ from previous work by Lieberman & Mody (1994) who found inhibition of calcineurin significantly altered NMDA channel mean open time (Table 3.9). Additionally, the lack of effect on the voltage sensitivity of the NMDA channel mean open time is not consistent with the results of Chen & Huang (1992). They postulated that PKC activation reduced the voltage-dependency of the NMDA receptor channel by reducing sensitivity to magnesium ions present in the recording solution.

Although, theoretically, it is not completely accurate to compare the channel open probability between patches, as each will have a different receptor population, as an approximate indication as to overall channel activity the open probability was calculated. This is a constraint of using the outside-out patch-clamp technique. For control, cyclosporin-A and calphostin-C treated patches the open probability at -60 mV is 0.0198 ± 0.009, 0.0069 ± 0.003 and 0.019 ± 0.01, respectively. These measurements did not differ significantly over the complete range of holding potentials recorded.

From these lines of investigation I conclude that neither cyclosporin-A nor calphostin-C altered single NMDA receptor channel open time or open probability in outside-out patches. This contrasts with previous reports using cell-attached or inside-out patch recordings (Chen & Huang, 1992; Lieberman & Mody 1994; Xiong et al., 1998). Possible reasons for this discrepancy lie, most probably, in experimental protocol. Lieberman & Mody (1994) used a cell-attached configuration for their experiments with calcineurin inhibitors. Thus there may be a cytosolic factor necessary to cause the increase in mean open time and open probability observed in their study. This is also relevant to the control values for NMDA mean open time and mean shut time observed by Lieberman & Mody (1994), which were smaller than those found here. This difference has been observed before by Gibb & Colquhoun (1992) who found NMDA receptor channel mean open time was 0.72 ms for cell-attached patches and 1.52 ms for inside-out patches. The difference could be due to the presence of regulatory systems in the intact cell but not the excised patch.

Counter to this argument, though, the experiments in this thesis were conducted with cyclosporin-A present in the recording solution. Considering this
compound is membrane permeable, an effect on mean open time and open probability should have been observed if calcineurin is responsible for reducing NMDA receptor channel activity, provided NMDA receptors are not altered by formation of the outside-out patch.

Experiments by Chen & Huang (1992) were conducted on outside-out patches but the increase in open probability was a result of PKC being applied directly to the patch in the internal pipette solution. Similarly Xiong et al. (1998) applied a constitutively active fragment of PKC to the intracellular surface of inside-out patches, from cultured hippocampal neurones. This would suggest that in the experiments reported here, PKC is either not present in the patch after excision from the cell, or is not active under these conditions and thus cannot be inhibited. Although, again calphostin-C was present in the recording solution and should have entered the cell inhibiting protein kinase activity before patch excision.

3.5.4 Conclusions

These results indicate that cyclosporin-A and calphostin-C have no significant effect on single NMDA receptor channel behaviour except for a small effect on overall mean shut time and mean shut time within a burst.

3.6 DISCUSSION: Comparison of control recordings using CsCl and Na-gluconate based internal pipette solutions

3.6.1 Single channel conductance

Using a Na-gluconate based pipette solution, NMDA channel openings exhibited two conductance levels, a main conductance level of 51 pS and a sub conductance of 38 pS. These measurements are very similar to previous single channel research performed using Na-gluconate as the internal pipette solution (Pina-Crespo, 1998). Single NMDA channel conductance with CsCl pipette solution gave 53 pS and 39 pS for the main and sub conductance levels, respectively. These values are comparable to previously published values from hippocampal granule cells, for example Strecker et al. (1994) who found a 56 pS channel conductance.

NMDA channel slope conductance was not affected by Na-gluconate but the reversal potential differed substantially from -1.41 mV for CsCl patches to 6.83 mV in Na-gluconate patches. This is to be expected according to Eisenman’s sequence for equilibrium ion exchange (Eisenman, 1962). The NMDA receptor is less permeable to
Na\(^{+}\) than Cs\(^{+}\) (Dani, 1986) and the relative permeability to monovalent ions is consistent with the NMDA channel being a fairly weak-field-strength site. Thus the outward movement of Na\(^{+}\) ions is likely to be less than Cs\(^{+}\) at any particular voltage making the reversal potential more positive for Na\(^{+}\)-gluconate-based pipette solutions.

### 3.6.2 Shut times

The individual time constants for the shut time exponential components do not vary greatly which suggest that channel shut time kinetics are not affected. The fourth component \((\tau_4)\) varies the greatest but this may result from the fact that the fourth component is usually the hardest to detect and therefore is not present in some shut time distributions. Mean shut time and the weighted mean of shut times underlying the first two components were not significantly different in the presence of Na-gluconate compared with CsCl pipette solution.

### 3.6.3 Open times

There is a trend to suggest that use of Na-gluconate as the pipette solution changes NMDA receptor open time kinetics. The time constants for the open time distribution exponential components change in the presence of Na-gluconate, and mean open time is reduced in a voltage-dependent manner, when compared with recordings made with a CsCl-based pipette solution. No difference is apparent between CsCl and Na-gluconate patches at a holding potential of -30 mV. At -80 mV though, the mean open time of Na-gluconate patches appears reduced in comparison with CsCl patches, although this did not test significant.

This voltage-dependent effect of Na-gluconate on NMDA mean open time could be linked to an effect on magnesium block. Some residual magnesium will be present in the recording solution, since no magnesium buffers are available that do not also buffer essential calcium ions. NMDA receptor channel mean open time usually exhibits some voltage dependence as seen in the CsCl experiments. There is a general trend to suggest that Na-gluconate enhances this voltage dependence, increasing e-fold every 34 mV instead of every 54 mV, for Na-gluconate and CsCl respectively, but again this was not statistically significant.

The main differences between the Na-gluconate and CsCl internal pipette solutions include the presence of a higher concentration of sodium ions and no caesium ions in the Na-gluconate solution and a small proportion of sodium ions but
high proportion of caesium ions in the CsCl solution. This can be viewed as a decrease in caesium ions, in the Na-gluconate solutions, by replacement with sodium.

Antonov & Johnson (1999) reported on the ability of monovalent cations to influence the channel blocking effects of magnesium. They report that decreasing the internal caesium concentration increased the magnesium block of the NMDA receptor channel. This was explained by the presence of two monovalent ion binding sites at the external entrance to the NMDA channel (Antonov et al., 1998). When these sites are occupied with either sodium or caesium, external magnesium cannot reach its blocking site, which is located further down in the channel pore (Wollmuth et al., 1998).

Caesium has a higher affinity for these monovalent binding sites, almost tenfold more than sodium. Thus when the internal caesium ion concentration is reduced magnesium is more able to block the channel pore. Due to the lower affinity of sodium for these binding sites, exchanging internal caesium for sodium will not cause an equal extent of magnesium block prevention. This would be the case for the experiments with Na-gluconate described here. Magnesium would more readily block the NMDA channel, resulting in a decrease of NMDA receptor channel mean open time, which was observed.

Open probability was not significantly altered by the presence of Na-gluconate although there appears to be a trend to suggest $P_{\text{open}}$ is also voltage dependent. This is a reflection of the voltage sensitivity of the mean open time.

3.6.4 Conclusions

These results so far justify the use of Na-gluconate as an alternative control pipette solution. The only difference found with Na-gluconate compared with CsCl was a shift in reversal potential and an increase in the voltage dependency of NMDA receptor channel mean open time, although not significantly so, which is assumed to be due to an increase in magnesium sensitivity. Apart from this, NMDA channel recordings were generally found to more stable and associated with less noise when using Na-gluconate rather than CsCl as the pipette solution.
CHAPTER 4

EFFECTS OF CALMODULIN AND PROTEIN PHOSPHATASE 2B, CALCINEURIN ON NMDA RECEPTOR SINGLE CHANNEL PROPERTIES

4.1 SUMMARY

i. The effects of calmodulin and protein phosphatase 2B, calcineurin, on the properties of single NMDA channel openings, in comparison with an appropriate calcium control, were investigated in outside-out patches from P12 rat hippocampal granule cells.

ii. Since calmodulin requires calcium for activation and calcineurin requires both calcium and calmodulin for activity, the effects of application of 12 nM free calcium to the intracellular surface of the patch were investigated and the results compared with data recorded using control Na-gluconate based pipette solution.

iii. The amplitude of single channel openings evoked by 0.1 - 10 μM NMDA and 1 - 10 μM glycine exhibited a main and sub conductance level. In control recordings the slope conductance was 50.6 pS and 37.6 pS and in the presence of 12 nM calcium 53.2 pS and 39.3 pS, for main and subconductance levels respectively. No significant difference in single channel slope conductance was observed between groups, although a negative shift in the extrapolated reversal potential was apparent in the presence of calcium.

iv. Shut time distributions were best fitted with the sum of four or five exponential components. The weighted mean shut times for all five components and for the first two components (within-burst shut times) were calculated at an open and shut time resolution of 110 μs. The presence of 12 nM intracellular calcium did not significantly affect NMDA channel mean shut time but reduced mean shut time within a burst. At a holding potential of -60 mV the mean shut time of all five components was 326 ± 104 ms for control and 227 ± 57 ms for calcium treated patches. The mean shut time within a burst was 0.84 ± 0.12 ms for control, 0.66 ± 0.08 ms and for calcium treated patches.
v. Single NMDA channel open time distributions were best fitted with the sum of three exponential components. 12 nM calcium decreased the voltage dependence of the mean open time, although not significantly. At a holding potential of -30 mV there was no difference between control and calcium treated patches, whilst at -80 mV control recordings gave a mean open time of 0.90 ± 0.2 ms and calcium treated patches 1.56 ± 0.4 ms. In control experiments mean open time increased e-fold for every 54 mV depolarisation, and e-fold for every 41 mV for calcium treated patches.

vi. Additional experiments were performed using 12 nM active calmodulin or 1.66 nM active calcineurin plus 12 nM active calmodulin in the pipette solution. Single channel activity was compared with experiments where 12 nM free calcium was contained in the pipette solution.

vii. Slope conductance was not significantly changed in the presence of 12 nM active calmodulin nor 1.66 nM active calcineurin plus 12 nM active calmodulin. In 12 nM calcium recordings the slope conductance was 53.2 pS and 39.3 pS, 48.1 pS and 36.5 pS in the presence of 12 nM active calmodulin and finally 53.9 pS and 40.4 pS in the presence of 1.66 nM active calcineurin plus 12 nM active calmodulin, for main and subconductance levels respectively.

viii. The presence of intracellular 12 nM active calmodulin or 1.66 nM active calcineurin plus 12 nM active calmodulin did not affect NMDA channel shut times. At a holding potential of -60 mV the mean shut time of all five components was 227 ± 57 ms in 12 nM calcium, 308 ± 95 ms for calmodulin and 363 ± 94 ms for calcineurin treated patches. The mean shut time within a burst was 0.66 ± 0.08 ms for calcium, 0.76 ± 0.13 ms for calmodulin and 0.69 ± 0.09 ms for calcineurin treated patches.

ix. At a holding potential of -60 mV mean open time was 2.29 ± 0.4 ms, 1.14 ± 0.2 ms and 1.98 ± 0.3 ms for 12 nM calcium, 12 nM active calmodulin and 1.66 nM active calcineurin plus 12 nM active calmodulin, treated patches. Thus calmodulin significantly reduced the mean open time in comparison to calcium. Calcineurin was found to reverse this effect although not totally as mean open
time was still significantly reduced when compared with calcium treated patch data.

x. The voltage dependence of the NMDA receptor mean open time was not significantly changed by the presence of calmodulin or calcineurin in comparison with calcium treated patches. Mean open time increased e-fold for every 41 mV, 42 mV and 47 mV depolarisation for calcium, calmodulin and calcineurin treated patches, respectively.

xi. These results therefore suggest that 12 nM calcium does not significantly affect NMDA receptor channel characteristics except for a decrease in the mean shut time within a burst. 12 nM calmodulin and 1.66 nM calcineurin plus 12 nM calmodulin had no effect on NMDA receptor channel behaviour except for a significant reduction in mean open time in comparison to 12 nM calcium. 1.66 nM calcineurin plus 12 nM calmodulin did not reduce mean open time to the same extent as 12 nM calmodulin alone, suggesting that calcineurin antagonises the effects of calmodulin to some extent.

4.2 INTRODUCTION

Calmodulin and calcineurin, protein phosphate 2B, are involved in the regulation of NMDA receptor activity (Ehlers et al., 1996; Liebermann & Mody, 1994). Both proteins are also involved in the regulation of many other cellular processes including the modulation of various other ion channels (for review see Klee & Vandaman, 1982; Klee et al., 1988; Aramburu et al., 2000). The relatively isolated environment of the excised patch makes it a good experimental model with which to examine the direct effects of calmodulin and calcineurin on single NMDA receptor channel activity because the composition of the solutions on each side of the patch membrane can be precisely controlled.

Calmodulin is necessary for the activation of calcineurin (Hubbard & Klee, 1987) and both are dependent upon calcium (Babu et al., 1985; Kakalis et al., 1995; Stemmer & Klee, 1994). Therefore it has been difficult in previous studies to dissect out which protein is (or indeed whether both are) responsible for direct modulation of the NMDA receptor channel.

To complicate matters, calcium itself has also been reported to cause inactivation of the NMDA receptor channel (Legendre et al., 1993; Vyklicky, 1993;
Rosenmund and Westbrook, 1993b; Rosenmund and Westbrook, 1995; Medina et al., 1996). In excised patch recordings calcium dependent inactivation was found to disappear after 3-5 minutes (Medina et al., 1996). It was suggested that a cytosolic protein, dependent on calcium for activation, was in fact responsible for calcium-induced NMDA receptor inactivation and that this was gradually washed out of the excised patch preparation.

These preliminary studies first led to speculation concerning the involvement of calmodulin and calcineurin, as they are two major neuronal calcium binding proteins. In 1994 Leibermann & Mody found that direct application of calcineurin to the intracellular surface of an inside-out patch resulted in a decrease in the mean open time, burst duration, cluster duration and overall $P_{\text{open}}$ of the NMDA receptor channel, whereas calcineurin antagonist studies with okadaic acid showed the reverse in cell-attached patches. Other studies have shown a decrease in synaptic desensitisation (Tong et al., 1995) and an increase in the induction of synaptic potentiation in hippocampal slices following application of a calcineurin antagonist (Wang & Kelly, 1997).

Ehlers et al. (1996) first reported that calmodulin reduces the mean open time and open probability of recombinant receptors recorded from inside-out patches. Other studies on recombinant NMDA receptors following deletion of calmodulin binding sequences from the NR1 subunit carboxy tail, have shown calcium dependent inactivation to be absent in whole-cell recordings (Zhang et al., 1998).

The aim of the experiments described in this chapter was to investigate the effects of calmodulin and calcineurin on native NMDA receptor single channel characteristics. Because active calmodulin will also be present in the calcineurin pipette solution, the calmodulin experiments also serve as a control for the calcineurin experiments. In addition, both of these experimental groups were compared with an appropriate calcium control, in order to assess any possible effects on NMDA receptor channel behaviour by calcium alone. Comparing the effects of both calmodulin and calcineurin under the same experimental conditions allows the effect of each individual protein to be assessed.

Active calmodulin was buffered to 12 nM, which made the active calcineurin concentration 1.66 nM as predicted from the dissociation constant of calmodulin for calcineurin (Hubbard & Klee, 1987). This means calmodulin will predominantly occupy its high affinity site, the C1 exon (predicted occupancy 75 %), as compared to its low affinity site in the C0 region (predicted occupancy 12 %), both of which are
present in the NMDA receptor C-terminus (Ehlers et al., 1996). The C1 exon is a substrate for phosphorylation by PKA (amino acid S897) and PKC (amino acids S890 and S896) (Tingley et al., 1997) which makes it a suitable region for dephosphorylation by calcineurin (Donella-Deana et al., 1994) although no direct evidence is available. This suggests that calmodulin and calcineurin may compete with each other for occupancy of the NMDA receptor C-terminal C1 exon, as would presumably occur under physiological conditions.

Calmodulin is thought to directly bind to the NMDA receptor C-terminus (Ehlers et al., 1996) whereas the actions of calcineurin are presumably via dephosphorylation of the receptor. Given that a certain proportion of NMDA receptors will be phosphorylated at any given time, at the point of patch excision we will gain a snapshot of receptors at the physiological level of phosphorylation. With the presence of calcineurin in the patch pipette, in the absence of protein kinases or ATP, the NMDA receptor channel should tend towards a dephosphorylated state.

4.3 RESULTS: Comparison of control and calcium treated patches

4.3.1 Properties of single NMDA receptor channel activations

Figure 4.1 contains representations of single channel recordings from a control and calcium (12 nM) treated patch. Recordings were made at membrane potentials between -30 and -80 mV in the presence of 0.1 - 10 μM NMDA and 10 μM glycine. No obvious differences are apparent between control and calcium treated patches except that the calcium treated patch contains slightly fewer channel openings. Both show evidence of occasional sublevel openings.

4.3.2 Amplitude stability plots

In control and calcium treated patches the single channel amplitude remained consistent throughout the duration of the recording. An example of each is given in Figure 4.2 A and B. Both control (A) and calcium (B) treated patches exhibit a main single channel current of approximately -3.5 pA and indicate the presence of a subconductance level at approximately -2.5 pA. This is more clearly depicted in the calcium treated patch.

4.3.3 Distributions of NMDA receptor single channel current amplitudes

Single channel amplitude distributions for control (A) and calcium (B) treated patches are illustrated in Figure 4.3. Channel amplitude distributions were fitted with
FIGURE 4.1 NMDA receptor single channel currents from control and calcium treated patches. Downward deflections in baseline indicate single channel openings in the presence of 0.1 - 10μM NMDA and 1 - 10 μM glycine, recorded from outside-out patches taken from dentate gyrus, hippocampal granule cells. Patches were recorded at holding potentials between -30 and -80 mV with a control intracellular pipette solution or in the presence of an addition of 12 nM free calcium. Currents were low-pass filtered at 2 kHz (-3dB, 8 pole Bessel filter). Trace recordings are 162 ms long.
FIGURE 4.2 Amplitude stability plots for (A) control and (B) 12 nM calcium treated patches. NMDA receptor-mediated openings recorded from outside-out patches excised from hippocampal dentate gyrus cells. Patches were held at -60 mV and exposed to 0.1 - 10 μM NMDA and 1 - 10 μM glycine. Stability plots contain (A) 216 and (B) 1047 plotted amplitudes, for amplitudes longer than two filter rise-times, respectively.
FIGURE 4.3 Amplitude histograms from (A) control and (C) 12 nM calcium treated patches. Distributions of channel amplitudes were fitted with the sum of two Gaussian components for amplitudes longer than two filter rise-times. Mean amplitude, standard deviation and relative area are shown in the insets. Channel openings recorded in response to 0.1 - 10 μM NDMA and 1 - 10 μM glycine applied to outside-out patches taken from hippocampal dentate gyrus granule cells, held at -60 mV.

(A) Control

(B) Calcium
the sum of two (or occasionally three) Gaussian components. Two conductance levels are apparent with relative areas of 87 % and 13 % for main and subconductance, respectively. Mean amplitude and mean relative area of the first and second component and the $1^{st}/2^{nd}$ Gaussian component area ratio were compared for recordings made at -60 mV (Table 4.1). These results show that the presence of calcium did not affect single channel amplitude or the proportion of main to subconductance openings at -60 mV. A third, smaller, amplitude component was seen in five of six control patches and three of six calcium treated patches. Additionally, two out of the three calcium patches displaying a third component also displayed a fourth smaller amplitude component.

### TABLE 4.1 Comparison of single channel current amplitudes from control and 12 nM calcium treated patches, at -60 mV

<table>
<thead>
<tr>
<th></th>
<th>1$^{st}$ Gaussian</th>
<th>2$^{nd}$ Gaussian</th>
<th>1$^{st}$/2$^{nd}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amplitude (pA)</td>
<td>Area (%)</td>
<td>Amplitude (pA)</td>
</tr>
<tr>
<td>Control*</td>
<td>2.49 ± 0.05</td>
<td>12 ± 1.0</td>
<td>3.32 ± 0.08</td>
</tr>
<tr>
<td>(n = 6 patches)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium*</td>
<td>2.41 ± 0.06</td>
<td>12 ± 0.8</td>
<td>3.30 ± 0.06</td>
</tr>
<tr>
<td>(n = 6 patches)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The third (smallest) component in control patches gave a mean amplitude of $1.65 \pm 0.14$ pA and contributed $1.6 \pm 0.4$ % of total channel openings (n = 6) at -60 mV. A third component was seen in three calcium treated patches at -60 mV and gave a mean amplitude of $1.51 \pm 0.09$ pA with a relative area of $1.4 \pm 0.1$ %.

### 4.3.4 Single channel conductance

Mean single channel amplitude for the 1$^{st}$ and 2$^{nd}$ Gaussian component was plotted against membrane potential for both control (A) and calcium (B) treated patches to give the single channel slope conductance (Figure 4.4). Mean single channel amplitudes were fitted simultaneously with a linear regression and weighted to converge at the same reversal potential, for control and calcium treated patches independently. The constrained slope conductance ($\pm$ standard deviation of the parameter estimates) for main and subconductance levels from control and calcium treated patches are presented in Table 4.2. There is a slight but not
FIGURE 4.4 Single channel conductance of NMDA receptors measured from control and 12 nM calcium treated patches. Plots showing current-voltage relationships for single channel currents activated by 0.1 – 10 μM NMDA and 1 – 10 μM glycine. Each point represents the mean single-channel current amplitude identified by fitting Gaussian components to the amplitude histograms at holding potentials between -30 and -80 mV (6 patches for control and 6 patches for calcium). Channel slope conductance was estimated by linear regression (solid line for control and dashed line for treated groups), slope conductances are presented in the inset. The main and subconductance for each plot were simultaneously constrained to give a common reversal potential of 6.83 mV and 1.93 mV for control and calcium patch recordings, respectively.

Membrane Potential (mV)

-90 -80 -70 -60 -50 -40 -30 -20 -10 0 10

Amplitude (pA)

-5 -4 -3 -2 -1 0

- Control 50.6 pS
- Control 37.6 pS
- Calcium 53.2 pS
- Calcium 39.3 pS
significant increase in both the main and subconductance levels in the presence of calcium compared to the control patches and a shift in the extrapolated reversal potential towards 0 mV. Otherwise it appears that the presence of 12 nM calcium does not alter the single channel conductance. The unconstrained slope conductance for control patches was 48.6 ± 1.3 pS and 41.1 ± 1.2 pS and for calcium treated patches 52.0 ± 2.7 pS and 42.5 ± 2.5 pS for the main and subconductance level respectively. A third smaller amplitude component seen in all control patches and three calcium treated patches gave a slope conductance of 29.3 ± 4.1 pS (n = 6) and 36.9 ± 4.5 pS (n = 3), respectively. Additionally, one of the calcium patches exhibited a fourth amplitude component that had a slope conductance of 17.1 pS (n = 1).

**TABLE 4.2** Slope conductance for single channel current amplitudes from control and 12 nM calcium treated patches

<table>
<thead>
<tr>
<th></th>
<th>Main conductance (pS)</th>
<th>Subconductance (pS)</th>
<th>Reversal potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>50.6 ± 1.2</td>
<td>37.6 ± 0.9</td>
<td>6.83 ± 1.3</td>
</tr>
<tr>
<td><em>(n = 6)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Calcium</strong></td>
<td>53.2 ± 1.4</td>
<td>39.3 ± 1.1</td>
<td>1.93 ± 1.2</td>
</tr>
<tr>
<td><em>(n = 6)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.3.5 Stability plots for shut times, open times and \( P_{\text{open}} \)

Figure 4.5 illustrates the stability of mean shut time, mean open time and \( P_{\text{open}} \) during control (A) and calcium (B) treated patch recordings. These stability plots allow assessment of any time-dependent changes in average kinetic activity. For the examples shown in Figure 4.5 mean open time remains fairly consistent, whereas mean shut time tends to vary in keeping with the stochastic properties of single channel openings. This is also reflected in the mean \( P_{\text{open}} \). This analysis indicates that patch recordings remained stable in the presence of 12 nM calcium in the intracellular (pipette) solution.

4.3.6 Shut time distributions

Shut time distributions were best fitted with the sum of five exponential components, or occasionally four in the case of some recordings where channel activity
FIGURE 4.5 Stability plot analysis of shut times, open times and \( P_{\text{open}} \) during activation of NMDA receptors in (A) control and (B) 12 nM calcium treated patches. Plots show a running average of shut times (top), open times (middle) and \( P_{\text{open}} \) (bottom) for recording of duration 274 and 264 s at -60 mV. Bins represent the average of 20 and 60 intervals with increments of 10 and 30 intervals between averages for control and calcium, respectively. Horizontal broken lines show the average of each parameter for the whole recording. The overall mean shut time, mean open time, \( P_{\text{open}} \) and mean opening frequency was 683.9 ms, 2.21 ms, 0.00323 and 1.46 openings/sec for control and 143.1 ms, 3.74 ms, 0.0255 and 6.82 openings/sec for calcium. A resolution of 110 \( \mu \)s was imposed on both openings and shuttings which gave a false event rate of \( 1 \times 10^8 \), at this holding potential.
FIGURE 4.6 Distribution of shut times for (A) control and (B) 12 nM calcium treated patches. In these distributions shut time intervals ranging from 0.11 to 9258 ms were best fitted with the sum of five exponential components. Time constants and associated areas are shown in the inset. Predicted shut time means (and observed and predicted true number of observations) were 689 ms (215 and 231) in control and for calcium treated patches 126 ms (945 and 1116).
### TABLE 4.3 Comparison of shut times between control and 12 nM calcium treated patches at -60 mV

<table>
<thead>
<tr>
<th></th>
<th>$\tau_1$ (μs)</th>
<th>$\tau_2$ (ms)</th>
<th>$\tau_3$ (ms)</th>
<th>$\tau_4$ (ms)</th>
<th>$\tau_5$ (ms)</th>
<th>Mean (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>362 ± 91 (26 ± 3 %)</td>
<td>1.25 ± 0.14 (33 ± 6 %)</td>
<td>23.5 ± 3.3 (7 ± 2 %)</td>
<td>205 ± 30 (10 ± 6 %)</td>
<td>1191 ± 271 (24 ± 6 %)</td>
<td>325 ± 104</td>
</tr>
<tr>
<td>Calcium</td>
<td>210 ± 70 (25 ± 4 %)</td>
<td>1.07 ± 0.14 (30 ± 4 %)</td>
<td>18.5 ± 2.7 (12 ± 2 %)</td>
<td>226 ± 36 (10 ± 3 %)</td>
<td>970 ± 318 (23 ± 6 %)</td>
<td>227 ± 57</td>
</tr>
</tbody>
</table>
was low. Examples of shut time distributions from a control (A) and calcium (B) treated patch are presented in Figure 4.6. Time constants and associated area (mean ± S.E.M.) for all control and calcium treated patches are compared in Table 4.3. It is evident that the presence of 12 nM intracellular calcium does not affect the mean time constants or areas associated with the five shut time components.

The mean shut time and weighted mean shut time of the first two exponential components of the shut time distribution were compared between groups and at each membrane potential recorded, in Figure 4.7A and B. There is no difference in overall mean shut time but calcium significantly reduced the weighted mean shut time of the first two components at membrane potentials between -30 and -80 mV. Effectively, this means that calcium reduces the mean shut time within a burst.

**TABLE 4.4 Comparison of individual open time distribution parameters and voltage dependence from control and 12 nM calcium treated patches**

<table>
<thead>
<tr>
<th></th>
<th>( \tau_1 ) (ms)</th>
<th>( \tau_2 ) (ms)</th>
<th>( \tau_3 ) (ms)</th>
<th>Mean (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-30 mV*</td>
<td>Control</td>
<td>0.14 ± 0.06</td>
<td>2.54 ± 0.5</td>
<td>7.79 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>(n = 6)</td>
<td>(16 ± 4 %)</td>
<td>(29 ± 6 %)</td>
<td>(55 ± 4 %)</td>
</tr>
<tr>
<td></td>
<td>Calcium</td>
<td>0.23 ± 0.1</td>
<td>2.21 ± 0.6</td>
<td>9.71 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>(n = 6)</td>
<td>(26 ± 3 %)</td>
<td>(30 ± 7 %)</td>
<td>(44 ± 8 %)</td>
</tr>
<tr>
<td>-80 mV*</td>
<td>Control</td>
<td>0.04 ± 0.01</td>
<td>0.54 ± 0.2</td>
<td>2.05 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>(n = 6)</td>
<td>(23 ± 5 %)</td>
<td>(46 ± 15 %)</td>
<td>(31 ± 11 %)</td>
</tr>
<tr>
<td></td>
<td>Calcium</td>
<td>0.07 ± 0.01</td>
<td>1.18 ± 0.3</td>
<td>2.59 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>(n = 6)</td>
<td>(28 ± 5 %)</td>
<td>(20 ± 11 %)</td>
<td>(52 ± 14 %)</td>
</tr>
</tbody>
</table>

* Resolution was set to 170 \( \mu s \) for -30 mV recordings and 80 \( \mu s \) for -80 mV recordings

**4.3.7 Open time distributions**

For control and calcium treated patches, open time distributions were best fitted with the sum of three exponential components. Examples of control (A) and calcium (B) treated patch open time distributions at -60 mV are presented in Figure 4.8. Time constants and associated area (mean ± SEM) for all control and calcium treated patches at -30 mV and -80 mV are compared in Table 4.4. At -30 mV mean time constants and associated area are similar between control and calcium, but not at -80
FIGURE 4.7 (A) Comparison of mean shut time for control and 12 nM calcium and (B) comparison of the weighted mean of the first two shut time components for control and 12 nM calcium treated patches. Each point represents the mean shut time of all patches, at that membrane potential, calculated from the five exponential components fitted to the shut time distributions. Data points were fitted by linear regression to investigate any voltage-dependence of the channel shut times (solid line for control and dashed lines for treated groups). Calcium significantly decreased mean shut time within a burst, at all membrane potentials recorded, by analysis of COVAR ($P < 0.05$).
FIGURE 4.8 Comparison of distributions for all individual open times in (A) control and (B) 12 nM calcium treated patches. In these distributions open time intervals ranging from 0.11 to 32.3 ms were best fitted with the sum of three exponential components, time constants and associated areas are inset. Predicted mean open time (and observed and predicted observations) were 1.40 ms (214 and 338) in control and 2.41 (944 and 1467) for calcium treated patches.
Figure 4.9 Comparison of mean open time correlated to membrane potential for control and 12 nM calcium treated patches. Each point represents the mean open time of all patches, at that membrane potential, calculated from the three exponential components fitted to the open time distributions. The data were fit using linear regression (solid line for control and dashed lines for treated groups) to allow estimation of the voltage-dependence of the open times. In control this was 54 mV depolarisation for an e-fold change in mean open time and 41 mV in calcium treated patches.
mV. The second mean time constant has increased in the presence of calcium at -80 mV and associated area has decreased relative to the control patches. Additionally, the area associated with the third mean time constant has increased giving an overall increase in mean open time, although not significantly so.

4.3.8 Dependence of mean open time on membrane potential

Single NMDA channel mean open time, from control and calcium treated patches, was compared at membrane potentials between -30 and -80 mV (Figure 4.9). 12 nM intracellular calcium appears to reduce the voltage sensitivity of mean open time. At -30 mV no difference in mean open time was observed between control and calcium treated patches but at more negative potentials, for example at -80 mV, there was an increase, although not significant, in mean open time in comparison to control. The voltage-dependence was e-fold for every 54 mV in control patches and 41 mV in calcium treated patches, but when the slope of the two regression populations were compared no significant difference was found.

4.4 RESULTS: Effects of calmodulin and calcineurin on single channel activity in comparison with calcium treated patches

4.4.1 Comparison of NMDA receptor single channel activity

Figure 4.10 illustrates examples of single NMDA channel openings from 12 nM free calcium, 12 nM active calmodulin and 1.66 nM active calcineurin plus 12 nM active calmodulin treated patches. All three patches exhibit the presence of a subconductance level. It is also evident that channel openings in the calmodulin patch appear shorter in duration when compared to the calcium and calcineurin patches.

4.4.2 Comparison of amplitude stability plots

To ensure channel amplitude remained consistent during patch recordings, amplitude stability plots were compiled. In Figure 4.11 there are three amplitude stability plots from calcium (A), calmodulin (B) and calcineurin (C) treated patches. In these particular examples the denser area, indicative of the main conductance level, approximates to -3.5 pA and the more diffuse area directly above represents the subconductance level, approximately -2.5 pA. This is most clearly defined in the calcium patch owing to the greater number of transitions analysed, indicated on the X-
FIGURE 4.10 NMDA receptor single channel currents from calcium, calmodulin and calcineurin plus calmodulin treated patches. Downward deflections in baseline indicate single channel openings in the presence of 0.1 - 10μM NMDA and 1 - 10 μM glycine, recorded from outside-out patches taken from dentate gyrus, hippocampal granule cells. Patches were recorded at holding potentials between -30 and -80 mV with an intracellular pipette solution containing either 12nM free calcium, 12 nM active calmodulin or 1.67 nM active calcineurin plus 12nM active calmodulin. Currents were low-pass filtered at 2 kHz (-3dB, 8 pole Bessel filter). Trace recordings are 162 ms long.
FIGURE 4.11 Amplitude stability plots for (A) 12 nM calcium (B) 12 nM calmodulin or (C) 1.66 nM calcineurin plus calmodulin treated patches. NMDA receptor-mediated openings recorded from outside-out patches excised from hippocampal dentate gyrus cells. Patches were held at -60 mV and exposed to 0.1 - 10 μM NMDA and 1 - 10 μM glycine. Stability plots contain (A) 1047, (B) 907 and (C) 617 plotted amplitudes, for amplitudes longer than two filter rise-times.

(A) Calcium

(B) Calmodulin

(C) Calcineurin
axis by the interval number, either because of a longer recording or greater channel activity.

**TABLE 4.5 Comparison of single channel current amplitudes at -60 mV from 12 calcium, 12 nM calmodulin and 1.66 nM calcineurin treated patches**

<table>
<thead>
<tr>
<th></th>
<th>1&lt;sup&gt;st&lt;/sup&gt; Gaussian</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; Gaussian</th>
<th>1&lt;sup&gt;st&lt;/sup&gt;/2&lt;sup&gt;nd&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amplitude (pA)</td>
<td>Area (%)</td>
<td>Amplitude (pA)</td>
</tr>
<tr>
<td><strong>Calcium</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 6 patches)</td>
<td>2.41 ± 0.06</td>
<td>12 ± 0.8</td>
<td>3.30 ± 0.06</td>
</tr>
<tr>
<td><strong>Calmodulin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 7 patches)</td>
<td>2.61 ± 0.15</td>
<td>23 ± 7.9</td>
<td>3.41 ± 0.16</td>
</tr>
<tr>
<td><strong>Calcineurin + Calmodulin</strong></td>
<td>2.49 ± 0.10</td>
<td>14 ± 3.3</td>
<td>3.36 ± 0.12</td>
</tr>
</tbody>
</table>

* A third component was seen in three calcium treated patches at -60 mV giving a mean amplitude of 1.51 ± 0.09 pA with a relative area of 1.4 ± 0.1%. A third component was seen in every calmodulin patch at -60 mV giving a mean amplitude of 1.75 ± 0.18 pA and relative area 12 ± 5%. Only one calcineurin treated patch contained a third amplitude component of 2.2 pA with an associated area of 7.9% at -60 mV. Additionally a fourth component was seen in three calmodulin patches (1.36 ± 0.24 pA (6 ± 3%)) and one calcineurin patch (1.53 pA (32%)) at -60 mV. One calcium treated patch exhibited a fourth component but not at -60 mV.

**4.4.3 Single channel current amplitudes**

Histograms containing single channel openings greater than two filter rise-times in duration were best fitted with the sum of two (and in some cases three or four) Gaussian components. Examples of this from a calcium (A), a calmodulin (B) and a calcineurin plus calmodulin (C) treated patch are displayed in Figure 4.12. In all groups a main and subconductance level is evident. The mean amplitude and associated area for all calcium, calmodulin and calcineurin treated patches at a holding potential of -60 mV are presented in Table 4.5. Whilst the mean amplitude for the 1<sup>st</sup> and 2<sup>nd</sup> Gaussian components are similar when compared between groups, calmodulin and calcineurin plus calmodulin treated patches appear to exhibit a greater proportion of sublevel openings, as indicated by the 1<sup>st</sup>/2<sup>nd</sup> area ratio.

In all calmodulin patches a third smaller amplitude component was apparent, three of which also exhibited a fourth amplitude component. A third and fourth
FIGURE 4.12 Amplitude histograms from (A) 12 nM calcium, (B) 12 nM calmodulin and (C) 1.66 nM calcineurin plus calmodulin treated patches. Distributions of channel amplitudes were fitted with the sum of either two or three Gaussian components for amplitudes longer than two filter rise-times. Mean amplitude, standard deviation and relative area for each Gaussian component are inset, for patches held at -60 mV.
component was detected in one calcineurin treated patch and one calcium treated patch contained a third component but no fourth component.

4.4.4 Comparison of single channel conductance

The mean single channel amplitude for the 1st and 2nd Gaussian components for all calcium, calmodulin and calcineurin plus calmodulin treated patches were plotted against membrane potential to give the slope conductance for the main and subconductance levels. Linear regression was performed on each group individually constraining the main and subconductance level in each case to the same reversal potential. Figure 4.13A compares calcium and calmodulin treated patches, Figure 4.13B compares calcium and calcineurin plus calmodulin treated patches and Figure 4.13C compares calmodulin and calcineurin plus calmodulin treated patches. The single channel slope conductance, main and subconductance for each group (± the standard deviation of parameter estimates), is presented in Table 4.6. Slope conductance for calcium and calcineurin plus calmodulin treated patches gave similar values for both the main and subconductance level but calmodulin appears to have reduced both conductance levels in comparison to calcium but not significantly so.

The reversal potentials for channel recordings in the presence of calcium, calmodulin and calcineurin plus calmodulin, as predicted by simultaneous linear regression of the main and subconductance levels, are also presented in Table 4.6. Whilst calcium and calcineurin plus calmodulin treated patch recordings have a similar reversal potential, in the calmodulin treated patches there appears to have been a shift to a more positive reversal potential.

| TABLE 4.6 Slope conductance for single channel current amplitudes |
|------------------|------------------|------------------|
|                  | Main conductance (pS) | Subconductance (pS) | Reversal potential (mV) |
| Calcium (n = 6)  | 53.2 ± 1.4         | 39.3 ± 1.1         | 1.9 ± 1.2                |
| Calmodulin (n = 7)| 48.1 ± 2.8         | 36.5 ± 2.1         | 10.7 ± 3.4               |
| Calcineurin + Calmodulin (n = 7) | 53.9 ± 1.8 | 40.4 ± 1.4 | 3.36 ± 1.8               |
FIGURE 4.13 Relationship between holding potential and channel amplitude for (A) 12 nM calcium and 12 nM calmodulin, (B) 12 nM calcium and 1.66 nM calcineurin plus calmodulin or (C) 12 nM calmodulin and 1.66 nM calcineurin plus calmodulin treated patches. The data were fit by linear regression to give the slope conductances inset (6 patches for calcium, 7 patches for calmodulin and 7 patches for calcineurin plus calmodulin). The main and subconductance for each plot were simultaneously constrained to give a common reversal potential of 1.93 mV for calcium patch, 10.7 mV for calmodulin and 3.36 mV for calcineurin plus calmodulin treated patch recordings.
Unconstrained slope conductance gave the following values for calcium 52.0 ± 2.7 pS and 42.5 ± 2.5 pS; for calmodulin 48.7 ± 2.1 pS and 35.8 ± 1.9 pS, finally for calcineurin 52.0 ± 1.0 pS and 42.9 ± 1.2 pS, main and subconductance levels respectively. The subconductance present in calmodulin patches was significantly reduced when compared with calcium treated patches. In addition the slope conductance of the third and fourth amplitude components observed in the calcium treated patches were 36.9 ± 4.5 pS (n = 4) and 17 pS (n = 1); for calmodulin treated patches 24.6 ± 3.5 pS (n = 7) and 15.3 ± 3.5 pS (n = 3) and for calcineurin plus calmodulin treated patches 15.0 pS (n = 1) and 3.0 pS (n = 1), respectively.

4.4.5 Comparison of stability plots for shut times, open times and $P_{\text{open}}$

Figure 4.14 represents the mean shut time, open time and $P_{\text{open}}$ of a patch over the time course of the recording. Examples from a calcium (A), calmodulin (B) and calcineurin plus calmodulin (C) treated patch are illustrated. Patch activity was found to be stable in all recordings and thus appears unaffected by the presence of calmodulin or of calcineurin plus calmodulin.

4.4.6 Comparison of shut time distributions

Histograms containing all shut times were best fitted with the sum of five (or in some cases four) exponential components. Figure 4.15 shows examples of shut time distributions for calcium (A), calmodulin (B) and calcineurin plus calmodulin (C) treated patches. Table 4.7 shows the five mean time constants and associated areas from all calcium, calmodulin and calcineurin plus calmodulin treated patches at -60 mV. No major differences in mean shut time distribution parameters are apparent between groups at this membrane potential.

To see if this was also evident at membrane potentials between -30 mV and -80 mV, mean shut time was plotted against membrane potential for all groups, in Figure 4.16A. Calmodulin does not appear to alter mean shut time in comparison to the calcium data. Calcineurin plus calmodulin significantly increased the mean shut time in comparison to calcium and calmodulin data. As previously mentioned, results from mean shut times can sometimes be misleading as it is impossible to match channel activity in different outside-out patches owing to different receptor populations in each patch.

When the weighted mean shut time of the first two components was compared at all membrane potentials for each group, as presented in Figure 4.16B, there was no
FIGURE 4.14 Stability plot analysis of shut times, open times and $P_{\text{open}}$ during activation of NMDA receptors in (A) 12 nM calcium, (B) 12 nM calmodulin or (C) 1.66 nM calcineurin plus calmodulin treated patches. Plots show a running average of shut times (top), open times (middle) and $P_{\text{open}}$ (bottom) during 228 - 327 s held at -60 mV. Bins represent the average of 30 – 70 intervals with increments of 15 – 35 intervals between averages. Horizontal broken lines represent the average values for the whole recording. The overall mean shut time, mean open time, $P_{\text{open}}$ and mean opening frequency were 143.1 ms, 3.74 ms, 0.0255 and 6.82 openings/sec for calcium, 403.3 ms, 0.997 ms, 0.00247 and 2.47 openings/sec for calmodulin and 225.7 ms, 3.15 ms, 0.0138 and 4.38 openings/sec for calcineurin plus calmodulin. Both openings and shuttings analysed at a resolution of 110 µs which gave a false event rate of $1 \times 10^6$, at this holding potential.
FIGURE 4.15 Distribution of shut times for (A) 12 nM calcium, (B) 12 nM calmodulin and (C) 1.66 nM calcineurin plus calmodulin treated patches. In these distributions shut time intervals ranging from 0.11 to 7553 ms were best fitted with the sum of five exponential components, time constants and associated areas are inset. Predicted shut time means (and observed and predicted number of observations) were 126 ms (945 and 1116) in calcium; 74.3 (420 and 453) in calmodulin and 214 (543 and 581) in calcineurin plus calmodulin treated patches.
TABLE 4.7 Comparison of shut times between 12 nM calcium, 12 nM calmodulin and 1.66 nM calcineurin treated patches at -60 mV

<table>
<thead>
<tr>
<th></th>
<th>$\tau_1$ ((\mu)s)</th>
<th>$\tau_2$ (ms)</th>
<th>$\tau_3$ (ms)</th>
<th>$\tau_4$ (ms)</th>
<th>$\tau_5$ (ms)</th>
<th>Mean (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>210 ± 70</td>
<td>1.07 ± 0.14</td>
<td>18.5 ± 2.7</td>
<td>226 ± 36</td>
<td>970 ± 318</td>
<td>227 ± 57</td>
</tr>
<tr>
<td></td>
<td>(25 ± 4 %)</td>
<td>(30 ± 4 %)</td>
<td>(12 ± 2 %)</td>
<td>(10 ± 3 %)</td>
<td>(23 ± 6 %)</td>
<td></td>
</tr>
<tr>
<td>Calmodulin</td>
<td>282 ± 96</td>
<td>1.21 ± 0.28</td>
<td>12.9 ± 2.3</td>
<td>225 ± 100</td>
<td>1167 ± 528</td>
<td>308 ± 95</td>
</tr>
<tr>
<td></td>
<td>(15 ± 2 %)</td>
<td>(26 ± 6 %)</td>
<td>(15 ± 3 %)</td>
<td>(16 ± 5 %)</td>
<td>(28 ± 3 %)</td>
<td></td>
</tr>
<tr>
<td>Calcineurin</td>
<td>249 ± 101</td>
<td>0.97 ± 0.09</td>
<td>17.0 ± 3.6</td>
<td>253 ± 64</td>
<td>1027 ± 155</td>
<td>363 ± 94</td>
</tr>
<tr>
<td></td>
<td>(19 ± 2 %)</td>
<td>(33 ± 6 %)</td>
<td>(9 ± 1 %)</td>
<td>(6 ± 3 %)</td>
<td>(33 ± 6 %)</td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 4.16 Comparison of (A) overall mean shut time and (B) mean shut time within a burst for 12 nM calcium, 12 nM calmodulin and 1.66 nM calcineurin plus calmodulin treated patches. (A) Each point represents the mean shut time of all patches or (B) weighted mean shut time of first two shut time components, at each membrane potential, calculated from the five exponential components fitted to the shut time distributions. The data were fitted by linear regression to investigate the voltage dependence of the shut times (solid line for calcium, short dashed lines for calmodulin and long dashed line for calcineurin plus calmodulin). Calcineurin plus calmodulin significantly increased mean shut time, at all membrane potentials recorded, by analysis of COVAR ($P < 0.05$).

(A) ● Calcium (n = 6 patches)  
□ Calmodulin (n = 7 patches)  
△ Calcineurin (n = 7 patches)

(B) ● Calcium (n = 6 patches)  
□ Calmodulin (n = 7 patches)  
△ Calcineurin (n = 7 patches)
significant difference between calcium, calmodulin and calcineurin plus calmodulin treated patches. The points lie almost exactly on top of each other suggesting that mean shut time within a burst of channel openings remains unaffected by the presence of calmodulin and calcineurin plus calmodulin.

**4.4.7 Comparison of open time distributions**

Open time distributions for all calcium, calmodulin and calcineurin plus calmodulin treated patches were best fitted with the sum of three exponential components. Figure 4.17 contains examples of open time distributions for calcium (A), calmodulin (B) and calcineurin plus calmodulin (C) treated patches at -60 mV. It is immediately evident that the open time distribution has changed in the presence of calmodulin. Openings appear to have become much shorter, with a higher relative percentage of openings underlying the shorter exponential components of the distribution.

Table 4.8 shows the mean open time constants and associated area for all patches in each group at -60 mV. The impression gained from the open time distributions is again reflected in the mean time constants for all calmodulin patches. The time constant for the third (slowest) component was reduced in the presence of calmodulin indicating channel openings have become shorter. The area associated with the third component was reduced and the area associated with the first component was increased in comparison with calcium patch data, suggesting that channel openings are more likely to be of a shorter duration in the presence of calmodulin. The most obvious and pronounced effect overall was a reduction in mean open time which tested significant in comparison to calcium patches.

When the effect of calcineurin plus calmodulin was compared with calcium patch data it was apparent that the area associated with the first component had increased and the area associated with the second component decreased. Thus there is an increased occurrence of short openings and fewer medium duration openings. The mean open time was also found to be reduced in the presence of calcineurin plus calmodulin when compared with calcium patch data, but not significantly so at this membrane potential.

Comparing calcineurin with calmodulin data, the first component is very similar in both groups, although the area associated with the second component is reduced in the presence of calcineurin plus calmodulin and the third component is
FIGURE 4.17 Comparison of distributions for all individual open times in (A) 12 nM calcium, (B) 12 nM calmodulin and (C) 1.66 nM calcineurin plus calmodulin treated patches. In these distributions open time intervals ranging from 0.11 to 32.3 ms were best fitted with the sum of three exponential components, time constants and associated areas are inset. Predicted mean open time (and observed and predicted observations) were 2.41 (944 and 1467) for calcium, 0.613 (419 and 715) for calmodulin and 2.23 (542 and 769) for calcineurin plus calmodulin treated patches.

(A) Calcium

(B) Calmodulin

(C) Calcineurin
increased. The mean open time was found to have significantly increased in the presence of calcineurin plus calmodulin when compared with calmodulin patch data.

**TABLE 4.8 Comparison of open times at -60 mV in 12 nM calcium, 12 nM calmodulin and 1.66 nM calcineurin plus calmodulin treated patches**

<table>
<thead>
<tr>
<th></th>
<th>(\tau_1) (ms)</th>
<th>(\tau_2) (ms)</th>
<th>(\tau_3) (ms)</th>
<th>Mean (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>0.07 ± 0.01</td>
<td>1.09 ± 0.2</td>
<td>3.80 ± 0.5</td>
<td>2.29 ± 0.4 (^d)</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(19 ± 4 %) (^a, b)</td>
<td>(34 ± 10 %) (^c)</td>
<td>(48 ± 10 %) (^c)</td>
<td></td>
</tr>
<tr>
<td>Calmodulin</td>
<td>0.06 ± 0.01</td>
<td>1.21 ± 0.3</td>
<td>2.97 ± 0.7</td>
<td>1.14 ± 0.2 (^{d,e})</td>
</tr>
<tr>
<td>(n = 7)</td>
<td>(35 ± 6 %) (^a)</td>
<td>(34 ± 6 %) (^c)</td>
<td>(31 ± 7 %) (^c)</td>
<td></td>
</tr>
<tr>
<td>Calcineurin + Calmodulin</td>
<td>0.06 ± 0.004</td>
<td>1.41 ± 0.3</td>
<td>3.42 ± 0.7</td>
<td>1.98 ± 0.3 (^e)</td>
</tr>
<tr>
<td>(n = 7)</td>
<td>(30 ± 5 %) (^b)</td>
<td>(17 ± 6 %) (^c)</td>
<td>(53 ± 5 %) (^c)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a, b, c, d, e\) Denote statistical significance \((P < 0.05)\)

**4.4.8 Mean open time and correlation with membrane potential**

Figure 4.18 illustrates the effects of calmodulin and calcineurin plus calmodulin on mean open time in comparison with calcium treated patches at all membrane potentials recorded. It appears that calmodulin significantly reduces mean open time at all potentials when compared with calcium treated patches. The dependence of mean open time on membrane potential was not affected by calmodulin, being e-fold for every 41 mV in calcium treated patches and 42 mV for calmodulin treated patches.

For calcineurin plus calmodulin treated patches again a significant reduction in mean open time was observed at all potentials in comparison with calcium treated patches, and a significant increase in mean open time in comparison to calmodulin patch data. No change in the membrane dependence of mean open time occurred in the presence of calcineurin plus calmodulin when compared with calcium or calmodulin patch data, giving an e-fold increase for every 47 mV for calcineurin treated patches, 41 mV and 42 mV for calcium and calmodulin treated patches, respectively.
FIGURE 4.18 Comparison of mean open time correlated to membrane potential for 12 nM calcium, 12 nM calmodulin and 1.66 nM calcineurin plus calmodulin treated patches. Each point represents the mean open time of all patches, at that membrane potential, calculated from the three exponential components fitted to the open time distributions. The data were fit using linear regression to allow estimation of the voltage-dependence of the open times (slope line for control, short dashed lines for calmodulin and long dashed lined for calcineurin plus calmodulin). In calcium this was 41 mV depolarisation for an e-fold change in mean open time, 42 mV in calmodulin and 47 mV in calcineurin plus calmodulin treated patches. By analysis of COVAR, calmodulin and calcineurin plus calmodulin decreased mean open time in comparison to calcium patch data, whereas calcineurin plus calmodulin increased mean open time in comparison to calmodulin patch data, at all membrane potentials recorded (P < 0.05).

- Calcium (n = 6 patches)
- Calmodulin (n = 7 patches)
- Calcineurin (n = 7 patches)
4.5 DISCUSSION: Comparison of calcium treated patch data with control

4.5.1 Single channel conductance

Channel recordings made in the presence of control (Na-gluconate based) and calcium containing (12 nM free) pipette solution exhibit two conductance levels. The main and subconductance levels were 51 pS and 38 pS for control recordings and 53 pS and 39 pS for calcium treated patch recordings. These values agree with previous studies that used a Na-gluconate based pipette solution (Pina-Crespo, 1998). Whilst intracellular calcium did not appear to affect single channel conductance, the reversal potential was reduced from 6.83 mV in control recordings to 1.93 mV in the presence of 12 nM calcium. As the NMDA receptor channel is permeable to calcium, this effect could be due to the addition of 12 nM intracellular calcium, which according to the Nerst equation would predict a shift to a more negative reversal potential. However, quantitatively, since the extracellular calcium concentration is 1 mM, a change of 12 nM in the intracellular calcium concentration could not produce this magnitude of shift in reversal potential. A more likely explanation therefore is that the pipette solution in the control and calmodulin experiments was slightly more dilute compared to the calcium and calcineurin experiments. The volume of NaOH added to neutralise the pipette solution can vary, which would account for this source of error.

The presence of a third and fourth component in some of the control and calcium treated patches is unusual. This may represent some contaminant channel in the recordings; alternatively the NMDA channel may have a third and perhaps fourth sublevel component. A Na-gluconate based pipette solution was used instead of CsCl to eliminate chloride channel contamination, and appeared to reduce the appearance of non-NMDA channel openings. Considering the mass of channels present in the dentate gyrus it is entirely plausible that some spontaneous unidentified channel openings will occur during the patch recording, which is on average 20 minutes. The standard error associated with the third and fourth amplitude components and slope conductance is quite large, which suggests the channel openings are not all of the same phenotype, substantiating the idea of spontaneous contaminant channels.

Alternatively, the large standard error could result from only a small proportion of these channel openings being present in a patch recording, as this makes the mean amplitude harder to discern. Also, considering there is a third amplitude component apparent in most patches, it is possible that the NMDA channel is exhibiting a third conductance level that is present only very occasionally.
Unfortunately it is not possible to substantiate this idea from the results presented in this thesis; a much more detailed study would be necessary. The fourth component is present in an even smaller proportion and is therefore harder to account for. The presence of NR2D containing NMDA receptors in the dentate gyrus at this age (P12) would account for a third and fourth amplitude component, but apart from histochemical evidence dispelling this idea (Wenzel et al., 1996), the mean slope conductance for the third and fourth component does not match the accepted values for a NR2D containing receptor channel exactly (35 pS and 15 pS).

4.5.2 Shut times

The mean time constants and associated area of the exponential components described in the shut time distributions were extremely consistent between control and calcium treated patch recordings at -60mV. Despite this it would appear that 12 nM calcium significantly reduces the mean shut time within a burst but is not reflected in the overall mean shut time. No detailed examination concerning the effects of calcium on single channel shut time kinetics has been performed before. In order to review this effect of 12 nM calcium on shut times it would be necessary to perform burst analysis.

4.5.3 Open times

Whilst the time constants of the open time distributions and their associated areas are not altered in the presence of calcium at -30 mV, when open times in the presence of calcium are compared with control recordings at -80 mV, certain differences are apparent. Channel openings are longer in the presence of calcium, indicated by an increase in the second and third time constant. A greater relative percentage of channels remain open for longer, indicated by the decrease in associated area for the second component and an increase in the associated area of the third component. Despite these trends mean open time was not found to significantly increase in the presence of calcium. Again no reference can be made to previous studies, as the effect of a low nanomolar intracellular calcium concentration has not been investigated on NMDA single channel open time components.

Although there is a trend to suggest that the voltage-dependence of the mean open time was reduced in the presence of 12 nM calcium when compared with control, this was not significant. Previous studies investigating the effect of calcium on single NMDA receptor channels have reported either no effect (Rosenmund & Westbrook, 1993b) or a decrease in open probability which disappears after a certain time (Medina
et al., 1996), thought to be indicative of a vital cellular protein being washed out. Neither of these studies involved examining mean open time over a range of membrane potentials.

Calcium may reduce the mean open time by interaction with an intracellular region of the NMDA receptor channel but considering the effect is voltage dependent it may, more likely, cause a change in the magnesium sensitivity of the NMDA receptor. The voltage dependence of single channel mean open time is thought to result from magnesium contamination of the recording solution (Gibb & Colquhoun, 1992). Buffers such as EDTA cannot be used as they also buffer calcium to a greater extent than magnesium. A reduction in external calcium concentration not only cause patch instability but will change the single channel conductance (Gibb & Colquhoun, 1992). It could be that calcium, being a divalent ion, interacts with the magnesium binding site within the channel, thereby competing with and reducing magnesium-dependent channel block.

4.5.4 Conclusion

The presence of 12 nM calcium has no effect on NMDA single channel conductance but shifts the reversal potential to a more negative value. The mean shut time within a burst is reduced and it appears that the voltage dependence of the mean open time is reduced, although not significantly. This 12 nM calcium containing pipette solution thus characterised was used as the control with which to assess any additional effects of calmodulin and calcineurin plus calmodulin on single NMDA channel activity.

4.6 DISCUSSION: Effects of calmodulin and calcineurin

4.6.1 Single channel conductance

Channel openings in both calmodulin and calcineurin plus calmodulin treated patches exhibit two conductance levels, as seen with the calcium treated patches. These had slope conductances of 53 pS and 38 pS for the main and subconductance levels in calcium treated patches, 48 pS and 37 pS for calmodulin and 54 pS and 40 pS in calcineurin plus calmodulin treated patches. The reversal potential was 1.9 mV for calcium, 10.7 mV for calmodulin and 3.4 mV for calcineurin plus calmodulin treated patches. Whilst the slope conductance and reversal potential from calcium and calcineurin treated patches is similar, the slope conductance in the presence of
calmodulin appears to be reduced although this did not prove to be statistically significant.

The reversal potential is more positive in the presence of calmodulin; this is surprising considering that all three pipette solution have the same ionic composition. The calcineurin pipette solution will also contain the same amount of active calmodulin; therefore a direct affect of calmodulin appears unlikely. Possibly the detection of up to four amplitude components in calmodulin treated patches has made determination of each component more difficult. This is perhaps also indicated by the larger standard error associated with the reversal potentials of calmodulin treated patches. Additionally, it should be remembered that these reversal potentials are extrapolations from experimental data. For a more exact measurement, the range of membrane potential studied should also include positive values, but this was not a principal goal of this study.

The detection of a third and fourth amplitude component in some of the patches is again unusual. Ideas to account for the occurrence of these two sublevels, described in the first part of this discussion, comparing control and calcium treated patches, also apply here.

4.6.2 Shut times

The mean time constants for shut time components and their associated areas and the mean shut time within a burst remained unchanged in the presence of calmodulin and calcineurin plus calmodulin when compared with calcium treated patches. Only calcineurin plus calmodulin increased mean shut time but as stated this is not a reliable measurement. No previous study has reviewed the effect of calmodulin on NMDA receptor channel shut times. Leibermann & Mody (1994) reviewed the effects of calcineurin inhibition in cell-attached patches and found no change in overall mean shut time. No reports have reviewed the effect of calcineurin on NMDA receptor channel shut time when applied directly to the patch. Nor has the effect of calmodulin or calcineurin been reviewed in respect to shut times within a burst.

4.6.3 Open times

Mean time constants and associated areas for open time distributions were altered in the presence of calmodulin, which resulted in an overall significant reduction in the mean open time, when compared with calcium. This was apparent at all membrane potentials between -30 mV and -80 mV. The voltage dependence of mean
open time was not altered by the presence of calmodulin when compared with calcium treated patch data.

This supports the work of Ehlers et al. (1996) who observed a 47% reduction in mean open time in the presence of 2.5 nM calmodulin and 50 μM calcium in inside-out patches held at -60 mV. Here we see a 50% reduction in mean open time in the presence of 12nM active calmodulin at -60 mV.

I propose this effect on NMDA receptor channel mean open time is a result of calmodulin predominantly binding to the C1 exon in the NR1 C-terminus. At this concentration (12 nM) calmodulin will preferentially occupy this high affinity site (predicted occupancy 75%) rather than the low affinity site (predicted occupancy 12%) in the neighbouring C0 region.

Calcineurin plus calmodulin does not affect the mean time constants for the open time components, but appears to alter the area associated with the first and second component as if to increase the relative percentage of short openings. This translates as a significant decrease in NMDA receptor channel mean open time when compared with calcium treated patch data, over all membrane potentials recorded. Again the voltage dependence of mean open time was unaffected by calcineurin plus calmodulin in comparison with calcium treated patch data.

This agrees with the work of Liebermann & Mody (1994) who reported a 28% reduction in mean open time in the presence of 40 μg calcineurin, 20 μM calmodulin and 40 μM calcium. The reduction in mean open time by calcineurin in comparison with calcium patch data was not as pronounced although it still proved significant. The active concentration of calcineurin used in this series of experiments (1.66 nM) was much less, in order to force calmodulin and calcineurin to compete or interact during binding to the C1 exon. This was intended to prevent the masking by any possible actions of calmodulin binding to the C0 region such as those implicated in calcium-dependent inactivation of NMDA receptor-mediated whole-cell currents (Zhang et al., 1998; Krupp et al., 1999).

It is perhaps surprising that NMDA receptor channel mean open time increased in the presence of calcineurin plus calmodulin when compared with calmodulin. It may have been expected that the presence of calcineurin would reduce mean open time even further than just calmodulin alone. Considering that previous studies found calcineurin reduced NMDA receptor channel mean open time (Liebermann & Mody, 1994), if calcineurin did inhibit NMDA receptor activity through a calmodulin-independent mechanism, this should have been the case.
Experiments by Liebermann & Mody (1994) could have been misleading though, because active calmodulin was present in their calcineurin solution, so it is impossible to say whether calcineurin or calmodulin was directly responsible for the effect on mean open time reported by Liebermann & Mody (1994), as no calmodulin or calcium controls were provided.

One simple interpretation of these results is that calcineurin competes with calmodulin for binding to the C1 exon. Although the presence of calcineurin still significantly reduced mean open time in comparison with calcium patch data, this may have resulted from partial antagonism of the effect of calmodulin on mean open time. Alternatively, calcineurin is acting as a partial agonist in terms of reducing NMDA channel mean open time.

Considering that calcineurin was present at a small concentration, relative to calmodulin, its affinity for the C1 exon must be greater than calmodulin. The dissociation constant for calmodulin binding to the C1 exon has been estimated to be 4 nM (Ehlers et al., 1996). Unfortunately there is no published value for the affinity of calcineurin for the NMDA receptor with which to compare. Thus it can be implied that although calmodulin negatively modulates NMDA receptor activity, its actions are kept in check by calcineurin. It could be that the active concentrations of each protein and their activation/inactivation kinetics, when intracellular calcium levels rise, are the deciding factors in determining the overall modulation of NMDA receptor channel behaviour.

4.6.4 Conclusions

Calmodulin changes the reversal potential of single channel openings and significantly reduces mean open time. Calcineurin reverses the effect of calmodulin on mean open time and may itself have a slight effect on mean open time. The latter effect implies that calmodulin and calcineurin may compete for binding to the C1 exon, indicating that the relationship between the two proteins and their effect on NMDA receptor function is more complicated than previously thought.
CHAPTER 6

OCCUPATION OF BOTH CALMODULIN BINDING SITES: EFFECTS ON SINGLE NMDA RECEPTOR CHANNEL ACTIVITY AND ANTAGONISM WITH ALPHA-ACTININ

6.1 SUMMARY

i. The NMDA receptor contains two binding sites for calmodulin on the NR1 C-terminus, namely the C1 and C0 region. Previously it has been shown that NMDA receptor mean open time is reduced by occupation of the C1 region. Here we investigate how calmodulin alters NMDA receptor behaviour when bound to the C0 region.

ii. Channel openings were recorded in the presence of either 800 nM free calcium, 800 nM free calcium plus 800 nM active calmodulin or 800 nM free calcium plus 800 nM active calmodulin plus 2.5 µM alpha-actinin, at the intracellular membrane surface. At this calmodulin concentration the high affinity C1 and low affinity C0 binding sites are expected to be occupied. In the presence of alpha-actinin, calmodulin binding to the C0 region is predicted to be reduced by approximately 80%. Due to antagonism by alpha-actinin of calmodulin binding to the C0 region, the actions of calmodulin can be assessed at this site.

iii. Single NMDA channel openings were observed after bath application of 0.1 - 10 µM NMDA and 10 µM glycine to outside-out patches from the dentate gyrus hippocampal cell layer, in P12 rats. Single channel amplitude was not significantly different between treated groups, giving a main level slope conductance of 60.6 ± 1.9 pS for calcium, 53.4 ± 1.9 pS for calmodulin and 59.0 ± 1.9 pS for alpha-actinin treated patches. The sublevel amplitude gave a slope conductance of 46.3 ± 1.4 pS for calcium, 39.4 ± 1.5 pS for calmodulin and 44.9 ± 1.4 pS for alpha-actinin treated patches. Although not significantly different the results suggest that 800 nM calmodulin may affect single channel conductance.

iv. Shut time distributions were best fitted with the sum of five exponential components for all treated groups. Time constants and relative areas were consistent between groups indicating that NMDA receptor shut time is not
altered by 800 nM calmodulin or the additional presence of 2.5 μM alpha-actinin. Mean shut time was not significantly different between groups, being 211 ± 53 ms for calcium, 291 ± 89 ms for calmodulin and 321 ± 103 ms for alpha-actinin treated patches, at -60 mV. Similarly mean shut time within a burst was not significantly different between groups; for calcium, calmodulin and alpha-actinin treated patches at -60 mV this was 0.539 ± 0.08 ms, 0.564 ± 0.10 ms and 0.687 ± 0.08 ms, respectively.

v. NMDA receptor channel mean open time for calcium, calmodulin and alpha-actinin patch data were 4.14 ± 0.6 ms, 2.93 ± 0.4 ms and 1.84 ± 0.4 ms, respectively at -60 mV. Thus calmodulin reduced mean open time in comparison with control and the presence of alpha-actinin reduced mean open time in comparison with calcium and calmodulin patch data.

vi. Mean open time was significantly reduced by calmodulin and alpha-actinin in comparison with calcium patch data at all membrane potentials recorded. In addition, alpha-actinin significantly reduced the mean open time in comparison with calmodulin patch data, at all membrane potentials recorded. The voltage-dependence of the mean open time was not altered by the presence of calmodulin nor alpha-actinin in comparison with calcium patch data. Mean open time increased e-fold for every 48 mV for calcium, 47 mV for calmodulin and 45 mV for alpha-actinin patch data.

vii. Values for NMDA receptor mean open time from 800 nM calcium, 800 nM calmodulin and 2.5 μM alpha-actinin treated patches were compared with values in the presence of 12 nM calcium and 12 nM calmodulin. 800 nM free calcium was found to significantly increase NMDA receptor mean open time in comparison with 12 nM free calcium.

viii. 800 nM active calmodulin did not reduce mean open time to the same extent as 12 nM active calmodulin. The percentage decrease in mean open time produced by 12 nM calmodulin was approximately 50 % over all membrane potentials recorded. For 800 nM calcium and calmodulin it was approximately 30 %, whereas between 2.5 μM alpha-actinin and 800 nM calcium it was approximately 50 %. These results suggest that the C1 and C0 calmodulin
binding sites both contribute to a decrease in NMDA receptor mean open time but that perhaps some form of negative co-operativity exists between the two regions. Alternatively, perhaps alpha-actinin can also reduce mean open time by binding to the C0 region.

6.2 INTRODUCTION

The C-terminus of the NMDA receptor channel contains two calmodulin binding sites, one of high affinity (4 nM) contained in the C1 exon and another of low affinity (87 nM) contained in the C0 region of the NR1 subunit (Ehlers et al., 1996). Previous studies suggest that occupancy of the low affinity site (C0) by calmodulin and not the C1 high affinity site, is responsible for calcium-dependent NMDA receptor inactivation in whole-cell recordings (Rafiki et al., 1997; Zhang et al., 1998, Krupp et al., 1999). Deletions or point mutations in the C0 region were shown to reduce calcium-dependent inactivation in whole-cell currents. In outside-out patches, though, results from this study (chapter 4) have shown that occupancy of the high affinity site (C1) by calmodulin is responsible for a reduction in NMDA single channel mean open time, a possible mechanism behind calcium-dependent NMDA receptor inactivation. It was, therefore, of interest to investigate the effect of calmodulin on single channel activity under conditions where calmodulin is expected to occupy the low affinity site, which might explain the findings of Rafiki et al. (1997), Zhang et al. (1998) and Krupp et al. (1999).

A higher concentration of calmodulin (800 nM) was used in these experiments, which was predicted to result in around 99 % and 90 % occupancy of the high (C1) and low (C0) affinity sites, respectively. In order to verify the effects of calmodulin resulting from occupancy of the low affinity (C0) region, experiments were also performed in the presence of 2.5 μM alpha-actinin, a C0 region binding protein.

Alpha-actinin-2 is a member of the spectrin family, responsible for cross-linking synaptic NMDA receptors to the actin cytoskeleton (Wyszynski et al., 1997). Alpha-actinin-2 binds to the NMDA receptor via the C0 region of the NR1 subunit and competes with calmodulin at this site (Wyszynski et al., 1997) contributing to calcium-dependent NMDA receptor inactivation (Zhang et al., 1998; Krupp et al., 1999). The concentration of alpha-actinin present in these experiments, purified from rabbit skeletal muscle, containing both 2 and 3 isoforms with a dissociation constant of 48 nM (Krupp et al., 1999), would be predicted (assuming simple competitive antagonism) to antagonise calmodulin at the C0 low affinity site, reducing its occupancy from 90 % to
Binding of alpha-actinin-2 to the NR1 subunit and competition with calmodulin was unaffected in splice variants that did not contain the C1 exon (Wyszynski et al., 1997) thus presumably leaving occupancy of this site by calmodulin unaltered in these experiments.

The aim of these experiments was to investigate the effect of 800 nM active calmodulin on single NMDA receptor channel behaviour, a concentration predicted to occupy both high (C1) and low (C0) affinity binding sites on the NR1 subunit carboxy tail. Control experiments were performed in the presence of 800 nM free intracellular calcium. Further experiments included 2.5 μM alpha-actinin in the presence of 800 nM calmodulin to antagonise the effects of calmodulin on NMDA receptor behaviour, attributed to occupancy of the C0 region. Finally, these results were compared to findings from Chapter 4 in which 12 nM active calmodulin was used, allowing only the high affinity C1 region to be occupied. It was hoped that the individual contribution of each calmodulin binding site to NMDA receptor inhibition could be further evaluated, by observing any changes in single channel characteristics.

6.3 RESULTS: Comparison of high calcium, high calmodulin and alpha-actinin treated patch recordings

6.3.1 Single NMDA receptor channel recordings

Channel openings resulted from application of 0.1 - 10 μM NMDA and 10 μM glycine to outside-out patches from dentate gyrus cells in hippocampal slices. Figure 6.1 contains examples of channel traces from patches treated with 800 nM calcium, 800 nM calmodulin plus 800 nM calcium and 2.5 μM alpha-actinin plus 800 nM calmodulin and 800 nM calcium. Quantitatively, the channel behaviour is similar in each recording solution with at least two conductance levels observed in all groups at holding potentials between -30 and -80 mV.

6.3.2 Amplitude stability plots

Channel amplitude remained stable in the presence of 800 nM intracellular calcium throughout the duration of the recordings (Figure 6.2A). This was also true for patch recordings in the presence of 800 nM active calmodulin and 2.5 μM alpha-actinin plus 800 nM active calmodulin (Figure 6.2B and C).
FIGURE 6.1 Examples of NMDA receptor-channel openings from calcium, calmodulin and alpha-actinin plus calmodulin treated patches. Downward deflections in base line indicate single channel openings in the presence of 0.1 - 10µM NMDA and 1 - 10 µM glycine, recorded from outside-out patches taken from dentate gyrus, hippocampal granule cells. Patches were recorded at holding potentials between -30 and -80 mV with an intracellular pipette solution containing either 800 nM free calcium (labelled as calcium), 800 nM active calmodulin in the presence of 800 nM calcium (labelled as calmodulin) or 2.5 µM alpha-actinin in the presence of 800 nM active calmodulin and 800 nM calcium (labelled alpha-actinin). Currents were low-pass filtered at 2 kHz (-3dB, 8 pole Bessel filter). Each trace is 162 ms long.

<table>
<thead>
<tr>
<th>Voltage (mV)</th>
<th>Calcium</th>
<th>Calmodulin</th>
<th>Alpha-actinin</th>
</tr>
</thead>
<tbody>
<tr>
<td>-30 mV</td>
<td><img src="image" alt="Trace" /></td>
<td><img src="image" alt="Trace" /></td>
<td><img src="image" alt="Trace" /></td>
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<tr>
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<td><img src="image" alt="Trace" /></td>
<td><img src="image" alt="Trace" /></td>
<td><img src="image" alt="Trace" /></td>
</tr>
<tr>
<td>-70 mV</td>
<td><img src="image" alt="Trace" /></td>
<td><img src="image" alt="Trace" /></td>
<td><img src="image" alt="Trace" /></td>
</tr>
<tr>
<td>-80 mV</td>
<td><img src="image" alt="Trace" /></td>
<td><img src="image" alt="Trace" /></td>
<td><img src="image" alt="Trace" /></td>
</tr>
</tbody>
</table>

1 pA
100 ms
FIGURE 6.2 Amplitude stability plots for (A) 800 nM free calcium (B) 800 nM active calmodulin and (C) 2.5 μM alpha-actinin plus 800 nM active calmodulin treated patches. NMDA receptor-mediated openings recorded from outside-out patches excised from hippocampal dentate gyrus cells. Patches were held at -60 mV and exposed to 0.1 - 10 μM NMDA and 10 μM glycine. Stability plots contain (A) 1910, (B) 1245 and (C) 899 amplitudes measured from openings longer than two filter rise-times (332 μs).
6.3.3 Distributions of NMDA receptor single channel current amplitudes

Frequency histograms of single channel amplitude were best fitted with the sum of three (or sometimes two) Gaussian components for all groups (Figure 6.3A, B and C). A main high probability component, a second smaller amplitude or sublevel component of a lower probability, and a third very small and less frequent 2nd sublevel were observed. The third smallest component was noted in all but one alpha-actinin treated patch but not necessarily at all membrane potentials recorded. Mean amplitude and mean relative area of the first and second component and the \( \frac{1^{st}}{2^{nd}} \) Gaussian component area ratio were compared for recordings made at -60 mV (Table 6.1). Information concerning the third component is given in the table footnote.

<table>
<thead>
<tr>
<th>TABLE 6.1 Comparison of single channel current amplitudes at -60 mV from 800 nM calcium and 800 nM calmodulin and 2.5 μM alpha-actinin treated patches</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st Gaussian</td>
</tr>
<tr>
<td>Component</td>
</tr>
<tr>
<td>Calcium* (n = 6 patches)</td>
</tr>
<tr>
<td>Calmodulin* (n = 7 patches)</td>
</tr>
<tr>
<td>Alpha-actinin* (n = 6 patches)</td>
</tr>
</tbody>
</table>

* The third smallest component was seen in all high calcium treated patches and gave a mean amplitude of 1.64 ± 0.18 pA with 1.4 ± 0.5 % relative area. A third component was seen in every high calmodulin patch giving a mean amplitude of 1.55 ± 0.09 pA and relative area 2.1 ± 0.5 %. Five out of six alpha-actinin treated patches exhibited a third component with a mean amplitude of 1.8 ± 0.1 pA with an associated area of 1.1 ± 0.4 %.

6.3.4 Single channel conductance

Mean channel amplitude for the first and second amplitude component was plotted against holding potential to determine slope conductance. For calcium, calmodulin and alpha-actinin plus calmodulin treated patches the following values were obtained; 57.2 ± 2.7 pS and 44.7 ± 3.1 pS, 53.6 ± 2.3 pS and 43.3 ± 2.3 pS, 55.5 ± 1.5 pS and 48.0 ± 1.5 pS for the main and subconductance levels, respectively. The slope conductance for the third smaller amplitude component was also estimated giving the
FIGURE 6.3 Amplitude histograms from (A) 800 nM calcium, (B) 800 nM calmodulin plus 800 nM calcium and (C) 2.5 μM alpha-actinin plus 800 nM calmodulin and 800 nM calcium treated patches. Distributions of channel amplitudes were fitted with the sum of three Gaussian components for amplitudes longer than two filter rise-times (332 μs). Mean amplitude, standard deviation and relative area for each Gaussian component are inset, for patches held at ~60 mV.

(A) High Calcium

(B) High Calmodulin

(C) Alpha-actinin
following value for calcium $33.4 \pm 5.4$ pS, calmodulin $31.8 \pm 3.8$ pS and alpha-actinin plus calmodulin $34.8 \pm 0.5$ pS.

In addition, weighted linear regression was performed on each group individually constraining the main and conductance level in each case to have the same reversal potential. Assuming that both main and subconductance levels should give the same reversal potential, this transformation also allowed results to be extrapolated back to the x-axis in order to estimate the reversal potential. Main and sublevel slope conductance and reversal potential for each group are presented in Table 6.2. The relationship between single channel amplitude and holding potential is compared between calcium and calmodulin treated patches in Figure 6.4A, between calcium and alpha-actinin plus calmodulin in Figure 6.4B and between calmodulin and alpha-actinin plus calmodulin in Figure 6.4C.

It is evident that single channel slope conductance and reversal potential is very similar between calcium and alpha-actinin plus calmodulin patch data. However, calmodulin patch data gave a smaller slope conductance for both main and sublevel, and also a more positive reversal potential. These differences in slope conductance were not statistically significant, though.

<table>
<thead>
<tr>
<th>Table 6.2 Slope conductance for single channel current amplitudes from 800 nM calcium and 800 nM calmodulin and 2.5 μM alpha-actinin treated patches</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Main conductance</strong></td>
</tr>
<tr>
<td>(pS)</td>
</tr>
<tr>
<td>Calcium</td>
</tr>
<tr>
<td>(n = 6)</td>
</tr>
<tr>
<td>Calmodulin</td>
</tr>
<tr>
<td>(n = 7)</td>
</tr>
<tr>
<td>Alpha-actinin</td>
</tr>
<tr>
<td>(n = 6)</td>
</tr>
</tbody>
</table>

6.3.5 Comparison of stability plots for shut times, open times and $P_{\text{open}}$

Patch activity was recorded under steady-state conditions. Stability plots for all groups containing average data concerning shut time, open time and $P_{\text{open}}$ were reviewed to ensure single channel activity was consistent during patch recordings (Figure 6.5A, B and C).
FIGURE 6.4 Relationship between holding potential and channel amplitude for (A) 800 nM calcium and 800 nM calmodulin, (B) 800 nM calcium and 2.5 μM alpha-actinin plus 800 nM calmodulin or (C) 800 nM calmodulin and 2.5 μM alpha-actinin plus 800 nM calmodulin treated patches. The data were fit by linear regression to give the slope conductances inset (6 patches for calcium, 7 patches for calmodulin and 6 patches for alpha-actinin plus calmodulin). The main and subconductance I-V relation for each pipette solution were simultaneously constrained to give a common reversal potential. These were -0.13 mV for calcium, 3.10 mV for calmodulin and 0.53 mV for alpha-actinin plus calmodulin treated patch recordings.
FIGURE 6.5 Stability plot analysis of shut times, open times and $P_{\text{open}}$ during activation of NMDA receptors in (A) 800 nM calcium, (B) 800 nM calmodulin or (C) 2.5 μM alpha-actinin plus 800 nM calmodulin treated patches. Plots show a running average of shut times (top), open times (middle) and $P_{\text{open}}$ (bottom) during 250 - 340 s held at -60 mV. Bins represent the average of 50 - 60 observations with increments of 25 - 30 observations between averages. Horizontal broken lines represent the average values for the whole recording. The overall mean shut time, mean open time, $P_{\text{open}}$ and mean opening frequency were 162.5 ms, 4.60 ms, 0.0276 and 6.01 openings/sec for calcium, 145.2 ms, 3.42 ms, 0.0230 and 6.75 openings/sec for calmodulin and 181.4 ms, 2.52 ms, 0.0137 and 5.44 openings/sec for alpha-actinin plus calmodulin. Both openings and shuttings were analysed at a resolution of 110 μs which gave a false event rate of $1 \times 10^4$, at this holding potential.
6.3.6 Comparison of shut time distributions

Shut times distributions were best fitted with the sum of five exponential components. The sixth component described in the previous chapter was also evident in some distributions but five components were found to describe the data adequately in all cases (Figure 6.6A, B and C). Mean time constants, associated area and mean shut time for all five components at -60 mV were collated for all calcium, calmodulin and alpha-actinin plus calmodulin treated patch data and are presented in Table 6.3. Shut time components are very consistent between groups. This is reflected in the mean shut time; when compared over all the membrane potentials recorded (Figure 6.7A) there is little variation between groups. To illustrate this, at a holding potential of -60 mV, mean shut time is 211 ± 52.8 ms, 291 ± 89.5 ms and 321 ± 103 ms for calcium, calmodulin and alpha-actinin plus calmodulin treated patch data, respectively. The mean shut time within a burst (Figure 6.7B), calculated as the weighted mean of the first two shut time components, again does not vary greatly between groups. At a holding potential of -60 mV, mean shut time within a burst is 0.539 ± 0.08 ms, 0.564 ± 0.10 ms and 0.687 ± 0.08 ms for calcium, calmodulin and alpha-actinin plus calmodulin, respectively. In agreement with these observations, the elevation and slope of these lines in Figure 6.7A and B, depicting the relationship between mean shut time or mean burst shut time and membrane potential, were not statistically different between groups (analysis of covariance P > 0.05). This suggests that neither 800 nM calmodulin nor alpha-actinin plus 800 nM calmodulin alter NMDA receptor shut times or the voltage dependence of the shut time.

Mean shut time and mean burst shut time from 800 nM free calcium and 800 nM active calmodulin treated patch data were also compared to 12 nM free calcium and 12 nM active calmodulin patch data. It is evident from Figure 6.8A and B that an approximate 70-fold increase in free calcium and active calmodulin concentration does not affect NMDA receptor channel shut time. There was no statistically significant difference between any of the groups.

6.3.7 Comparison of open time distributions

Open time distributions were best fitted with the sum of three exponential components. It is apparent from Figure 6.9A, B and C that the presence of 800 nM calmodulin shifts the open time distribution to the left to an overall shorter open time state, relative to the 800 nM calcium data. Application of alpha-actinin plus 800 nM
FIGURE 6.6 Distribution of shut times for (A) 800 nM calcium, (B) 800 nM calmodulin and (C) 2.5 μM alpha-actinin plus 800 nM calmodulin treated patches. In these distributions shut time intervals ranging from 0.11 to 3541 ms were best fitted with the sum of five exponential components, time constants and associated areas are inset. Predicted mean shut times (and observed and predicted number of observations) were 103 ms (1022 and 1213) in calcium; 129 ms (880 and 963) in calmodulin and 157 ms (733 and 812) in alpha-actinin plus calmodulin treated patches.

(A) 800 nM Calcium

(B) 800 nM Calmodulin

(C) 2.5 μM Alpha-actinin
TABLE 6.3 Comparison of shut times distributions between 800 nM calcium, 800 nM calmodulin and alpha-actinin plus 800 nM calmodulin treated patches at -60 mV

<table>
<thead>
<tr>
<th></th>
<th>$\tau_1$ ((\mu s))</th>
<th>$\tau_2$ (ms)</th>
<th>$\tau_3$ (ms)</th>
<th>$\tau_4$ (ms)</th>
<th>$\tau_5$ (ms)</th>
<th>Mean (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>157 ± 60</td>
<td>1.03 ± 0.24</td>
<td>12.7 ± 2.8</td>
<td>316 ± 156</td>
<td>897 ± 290</td>
<td>211 ± 53</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(26 ± 5 %)</td>
<td>(26 ± 2 %)</td>
<td>(13 ± 3 %)</td>
<td>(12 ± 2 %)</td>
<td>(24 ± 3 %)</td>
<td></td>
</tr>
<tr>
<td>Calmodulin</td>
<td>237 ± 65</td>
<td>0.99 ± 0.07</td>
<td>13.5 ± 4.2</td>
<td>184 ± 66</td>
<td>1037 ± 413</td>
<td>291 ± 89</td>
</tr>
<tr>
<td>(n = 7)</td>
<td>(24 ± 3 %)</td>
<td>(23 ± 5 %)</td>
<td>(12 ± 3 %)</td>
<td>(14 ± 3 %)</td>
<td>(27 ± 5 %)</td>
<td></td>
</tr>
<tr>
<td>Alpha-actinin</td>
<td>227 ± 36</td>
<td>1.06 ± 0.16</td>
<td>21.0 ± 6.9</td>
<td>280 ± 91</td>
<td>772 ± 258</td>
<td>321 ± 103</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(17 ± 4 %)</td>
<td>(25 ± 5 %)</td>
<td>(12 ± 2 %)</td>
<td>(10 ± 4 %)</td>
<td>(36 ± 3 %)</td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 6.7 Comparison of (A) overall mean shut time and (B) mean shut time within a burst for 800 nM calcium, 800 nM calmodulin and 2.5 µM alpha-actinin plus 800 nM calmodulin treated patches. (A) Each point represents the mean shut time of all patches or (B) weighted mean shut time of first two shut time components, at each membrane potential, calculated from the five exponential components fitted to the shut time distributions. The data were fitted by linear regression to investigate the voltage dependence of the shut times (solid line for calcium, short dashed lines for calmodulin and long dashed line for alpha-actinin plus calmodulin).
FIGURE 6.8 Comparison of (A) overall mean shut time and (B) mean shut time within a burst for 12 nM calcium, 800 nM calcium, 12 nM calmodulin and 800 nM calmodulin treated patches. (A) Each point represents the mean shut time of all patches or (B) weighted mean shut time of first two shut time components, at each membrane potential, calculated from the five exponential components fitted to the shut time distributions. The data were fitted by linear regression to investigate the voltage dependence of the shut times (solid line for 12 nM calcium, short dashed lines for 12 nM calmodulin, long dashed line for 800 nM calcium and dot-dash line 800 nM calmodulin).
calmodulin also shifts the open time distribution to the left and to a greater extent than just 800 nM calmodulin alone.

Upon examination of the individual mean time constants, relative areas and mean open time presented in Table 6.4, it is apparent that both 800 nM calmodulin and alpha-actinin plus 800 nM calmodulin treated patch data exhibit a shorter mean open time, in comparison to 800 nM calcium patch data. For calmodulin treated patches this translates as a slight decrease in the time constant for the first component ($\tau_1$), an increase in the area associated with the first component and a decrease in the area associated with the second component. Additionally, the mean time constant of the third component has been reduced. For alpha-actinin patch data the area associated with the second component has increased and the time constant for the third component has decreased.

Comparing 800 nM calmodulin with alpha-actinin plus 800 nM calmodulin patch data it is apparent that the mean open time of the later group is shorter. The area associated with the second component has increased and the area associated with the third component has decreased, which would explain this outcome. Any significant difference according to an unpaired t-test ($P < 0.05$) found between groups is highlighted in Table 6.4.

**TABLE 6.4 Comparison of individual open time components from 800 nM calcium, 800 nM calmodulin and 2.5 µM alpha-actinin treated patches at -60 mV**

<table>
<thead>
<tr>
<th></th>
<th>$\tau_1$ (ms)</th>
<th>$\tau_2$ (ms)</th>
<th>$\tau_3$ (ms)</th>
<th>Mean (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>0.14 ± 0.03</td>
<td>2.07 ± 0.7</td>
<td>6.26 ± 1.0*</td>
<td>4.14 ± 0.6*</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(17 ± 4 %)</td>
<td>(24 ± 6 %)</td>
<td>(59 ± 7 %)</td>
<td></td>
</tr>
<tr>
<td>Calmodulin</td>
<td>0.09 ± 0.02</td>
<td>1.63 ± 0.6</td>
<td>4.61 ± 0.5</td>
<td>2.93 ± 0.4</td>
</tr>
<tr>
<td>(n = 7)</td>
<td>(31 ± 4 %)</td>
<td>(9 ± 4 %)</td>
<td>(60 ± 5 %)</td>
<td></td>
</tr>
<tr>
<td>Alpha-actinin</td>
<td>0.13 ± 0.03</td>
<td>1.35 ± 0.3</td>
<td>3.77 ± 0.4*</td>
<td>1.84 ± 0.4*</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(21 ± 3 %)</td>
<td>(45 ± 13 %)</td>
<td>(34 ± 12 %)</td>
<td></td>
</tr>
</tbody>
</table>

* All denote statistical significance according to an unpaired t-test ($P < 0.05$)
FIGURE 6.9 Comparison of distributions for all individual open times in (A) 800 nM calcium, (B) 800 nM calmodulin and (C) 2.5 μM alpha-actinin plus 800 nM calmodulin treated patches. In these distributions open time intervals ranging from 0.11 to 40 ms were fitted with the sum of three exponential components (time constants and associated areas are inset). Predicted mean open time (and observed and predicted number of observations) were 4.15 ms (1021 and 1143) for calcium, 2.78 ms (879 and 1097) for calmodulin and 1.48 ms (732 and 1245) for alpha-actinin plus calmodulin treated patches.
6.3.8 Comparison of mean open time and correlation with membrane potential

To evaluate the effects of 800 nM calcium, 800 nM calmodulin and 2.5 μM alpha-actinin plus 800 nM calmodulin over all membrane potentials recorded, mean open time was plotted against membrane potential for each treated group (Figure 6.10). In comparison to 800 nM calcium, the presence of 800 nM calmodulin reduces mean open time at all potentials. The elevation of the line, depicting the relationship between mean open time and membrane potential for calmodulin, was significantly lower than that for calcium. The gradients of the lines were not significantly different between the calcium and calmodulin groups, suggesting that the voltage dependency of mean open time remains unaltered by calmodulin. For calcium treated patches mean open time increased e-fold for every 48 mV depolarisation and for calmodulin every 47 mV. Mean open time in the presence of alpha-actinin plus calmodulin was also significantly different from that of the calcium treated group at all membrane potentials. Again, though, the dependency of mean open time on membrane potential remained unchanged by the presence of alpha-actinin in comparison with high calcium, increasing e-fold for every 45 mV depolarisation.

Mean open time in the presence of alpha-actinin plus calmodulin was also significantly different to the calmodulin group at all potentials. It appears that the addition of alpha-actinin decreases mean open time to a greater extent than just calmodulin alone. Voltage dependence of mean open time was not significantly affected by alpha-actinin in comparison with the calmodulin treated group data.

6.3.9 Comparison of mean open time in the presence of 12 nM and 800 nM calcium, calmodulin and also 2.5 μM alpha-actinin

The voltage-dependence of the mean open time in the presence of 12 nM and 800 nM intracellular free calcium and 12 nM and 800 nM active calmodulin is illustrated in Figure 6.11A. The elevation of the regression line for 800 nM calcium is significantly higher than that of the 12 nM calcium treated group, although the slope of the regression lines are the same (also presented in Figure 6.11B for clarity). This suggests that an increase in intracellular free calcium increases NMDA receptor channel mean open time, although the voltage dependence remains the same. The same effect can be seen between 12 nM and 800 nM calmodulin (Figure 6.11C), suggesting that 800 nM active calmodulin increases NMDA receptor channel mean open time in comparison with a smaller 12 nM concentration of calmodulin. This result at first seems paradoxical. A possible explanation for this will be discussed below. The
FIGURE 6.10 Comparison of mean open time correlated to membrane potential for 800 nM calcium, 800 nM calmodulin and 2.5 μM alpha-actinin plus 800 nM calmodulin treated patches. Each point represents the mean open time of all patches, at that membrane potential, calculated from the three exponential components fitted to the open time distributions. The data were fit using linear regression to allow estimation of the voltage-dependence of the open times (solid line for calcium, short dashed lines for calmodulin and long dashed lines for alpha-actinin plus calmodulin). For calcium treated patches there was a 48 mV depolarisation for an e-fold change in mean open time, 47 mV in calmodulin and 45 mV in alpha-actinin plus calmodulin treated patches. By analysis of COVAR, calmodulin and calmodulin plus alpha-actinin were shown to significantly reduce mean open time in comparison to calcium patch data, whereas alpha-actinin plus calmodulin significantly reduced mean open time when compared to calmodulin alone, at all membrane potentials recorded, (P < 0.05).
FIGURE 6.11 Comparison of mean open time correlated to membrane potential for (A) 12 nM and 800 nM calcium and calmodulin treated patches, further separated into (B) 12 nM and 800 nM calcium treated patches and (C) 12 nM and 800 nM calmodulin treated patches, for clarity. Each point represents the mean open time of all patches, at that membrane potential, calculated from the three exponential components fitted to the open time distributions. The data were fit using linear regression.

(A) 12 nM Calcium (n = 6 patches)
    □ 12 nM Calmodulin (n = 7 patches)
    ▲ 800 nM Calcium (n = 6 patches)
    ◇ 800 nM Calmodulin (n = 7 patches)

(B) 12 nM Calcium (n = 6 patches)
    ▲ 800 nM Calcium (n = 6 patches)

(C) □ 12 nM Calmodulin (n = 7 patches)
    ◇ 800 nM Calmodulin (n = 7 patches)
FIGURE 6.12 (A) Mean channel open time in the presence of 12 nM and 800 nM calmodulin and 2.5 μM alpha-actinin plus 800 nM calmodulin is expressed as a percentage of control and plotted against membrane potential. (B) Absolute values for mean open time plotted against membrane potential. Mean open time is calculated from the three exponential components fitted to the open time distributions. The data were fit using linear regression.

(A)  
- • 12 nM Calmodulin (n = 7 patches)
- □ 800 nM Calmodulin (n = 7 patches)
- △ 2.5 mM Alpha-actinin (n = 6 patches)

(B)  
- • 12 nM Calmodulin (n = 7 patches)
- □ 800 nM Calmodulin (n = 7 patches)
- △ 2.5 mM Alpha-actinin (n = 6 patches)
gradients of the regression lines are similar, indicating the dependence of mean open time on membrane potential is not changed by an increase in the active intracellular calmodulin concentration.

Overall, both concentrations of calmodulin reduce mean open time in comparison with their respective control calcium concentration. However, 12 nM calmodulin produces a greater percentage reduction in mean open time (50 %) to a greater extent than 800 nM calmodulin (30 %). This is shown in Figure 6.12A where mean open time is expressed as a percentage of control. Mean open time in the presence of 12 nM calmodulin is expressed as a proportion of mean open time in the presence of 12 nM calcium whereas alpha-actinin and 800 nM calmodulin patch data are expressed as a proportion of mean open time in the presence of 800 nM calcium. From this transformation it is evident that 12 nM calmodulin and 2.5 µM alpha-actinin reduce mean open time to a greater extent than 800 nM calmodulin. Alpha-actinin appears to be returning mean open time to levels observed with 12 nM calmodulin (predominate C1 occupation) by antagonising the actions of 800 nM calmodulin at the C0 site, leaving calmodulin bound only to the C1 region. Absolute values for mean open time in the presence of 12 nM calmodulin, 800 nM calmodulin and 2.5 µM alpha-actinin are presented in Figure 6.12B.

6.4 DISCUSSION: Effects of calmodulin at both NMDA binding sites and antagonism by alpha-actinin

To summarise the results from this chapter, 800 nM calcium, 800 nM calmodulin plus 800 nM calcium and 2.5 µM alpha-actinin plus 800 nM calmodulin plus 800 nM calcium did not affect single channel conductance or NMDA shut times. Open time components and mean open time were changed in the presence of calmodulin and alpha-actinin plus calmodulin in comparison with calcium patch data.

Mean open time was maximally reduced in the presence of 2.5 µM alpha-actinin plus 800 nM calmodulin. 800 nM calmodulin also reduced mean open time but not to the same extent as 2.5 µM alpha-actinin plus 800 nM calmodulin. Mean open time in the presence of 800 nM calcium, 800 nM calmodulin or 2.5 µM alpha-actinin plus 800 nM calmodulin was compared with data from patches treated with 12 nM calcium or 12 nM calmodulin. A rise in the intracellular calcium concentration from 12 nM to 800 nM increased mean open time. Similarly, a rise in the intracellular calmodulin concentration from 12 nM to 800 nM also increased mean open time but appeared to be reversed by the presence of alpha-actinin. Implications are discussed below.
6.4.1 Single channel conductance

Slope conductance was not significantly changed in the presence of 800 nM calmodulin or 2.5 μM alpha-actinin plus 800 nM calmodulin, when compared with 800 nM calcium. Values for main and subconductance levels were 61 pS and 46 pS for calcium treated patches, 53 pS and 39 pS for calmodulin treated patches and 59 pS and 45 pS for alpha-actinin plus calmodulin. There does appear to be a slight trend to suggest that this 800 nM calmodulin reduces single NMDA channel slope conductance in comparison with 800 nM calcium patch data, for both the main and sublevel. This is also reversed by the presence of alpha-actinin. Assuming that alpha-actinin is acting as an antagonist of calmodulin at the C0 region of the NR1 C-terminus, this might be a genuine action of calmodulin on single channel conductance, which should be investigated further.

The reversal potential is shifted slightly positive in the presence of 800 nM calmodulin, which is again reversed by the presence of alpha-actinin. Values are -0.13 mV for calcium, 3.1 mV for calmodulin and 0.53 mV for alpha-actinin plus calmodulin.

Ehlers et al. (1996) found a main and subconductance of 56 pS and 42 pS from single channel experiments on recombinant NR1a-NR2A receptors, which is similar to values found in this study and in previous studies of recombinant NR1a/NR2A receptors (Stern et al., 1992). They did not report a change in single channel conductance after application of 2 - 250 nM calmodulin. Additionally, they noted the occasional presence of a 21 pS conductance level, which is consistent with data presented in this thesis. The slope conductance for this extra sublevel was 33 pS for calcium, 32 pS for calmodulin and 35 pS for alpha-actinin plus calmodulin treated patches. Although these values are higher than that reported by Ehlers et al. (1996), this discrepancy may arise from the very low occurrence of this sublevel, which would also explain its lack of recognition. The presence of NR2D containing NMDA receptors would account for the appearance of this third amplitude component. Although histochemical studies have shown that at P12, the age of rats used in this study, the NR2D subunit is no longer expressed (Wenzel et al., 1996). Additionally, a 15 pS sublevel would also be expected as the subconductance for NR2D containing receptors. Further experiments would be necessary to clarify this issue.

6.4.2 Shut times

800 nM calmodulin does not affect the NMDA receptor channel shut time distributions, mean shut time or mean shut time within a burst. This is consistent with
results obtained with a low 12 nM concentration of calmodulin. Due the lack of a selective effect on any of the shut time exponential components it would seem that calmodulin does not inactivate the NMDA receptor by a blocking mechanism such as the ball and chain theory, in agreement with Krupp et al. (1999).

There are no previous studies with which to compare these results. Some comparisons can be made with Lieberman & Mody (1994) who applied 20 μM calmodulin and 40 μM calcium with 0.5 μM calcineurin to the intracellular surface of inside-out patches. A high concentration of active calmodulin would be present in these experiments, as the respective proportions of calcium, calmodulin and calcineurin would suggest. No change in NMDA shut time distributions or mean shut time was observed, supporting the observations found here, although the comparison is complicated by the presence of calcineurin in Lieberman & Mody’s study.

Shut time distributions were also unaffected by the presence of alpha-actinin. Alpha-actinin is thought to be involved in calcium-dependent inactivation in whole-cell currents (Zhang et al., 1998; Krupp et al., 1999). No previous studies, though, have investigated the effects of alpha-actinin on single channel mechanisms with which to compare the results presented here. Firstly, it can be concluded from these experiments that if alpha-actinin has a direct effect on NMDA receptor activity it is not via modulation of shut times. Secondly, this absence of effect on shut times encourages the theory that alpha-actinin interacts with the binding of calmodulin to the C0 region (Wyszynski, et al., 1997; Zhang et al., 1998) as reflected in the effect of calmodulin on NMDA mean open time, discussed below.

6.4.3 Open times

800 nM intracellular calmodulin significantly reduces NMDA receptor mean open time in comparison with 800 nM intracellular free calcium. This is attributed to calmodulin occupying, according to dissociation constants (Ehlers et al., 1996), both the C0 (90 %) and C1 (99 %) region of the NR1 C-terminus. In order to determine the contribution of calmodulin occupying the C0 region to this reduction in mean open time, experiments were performed using alpha-actinin to antagonise the actions of calmodulin at the C0 region. At the concentrations of alpha-actinin (2.5 μM) and calmodulin (800 nM) used, occupation of the C0 region was predicted to be reduced to 16 %, assuming simple competitive antagonism between the two proteins, whilst leaving occupation of the C1 region by calmodulin unaffected. If alpha-actinin did
interact with the C1 region it would still only, theoretically, reduce occupation of the C1 region by calmodulin to 91%.

Results obtained in the presence of alpha-actinin were surprising for alpha-actinin reduced mean open time further than levels seen in the presence of 800 nM calmodulin. In the alpha-actinin internal solution both 800 nM free calcium and 800 nM active calmodulin were present; therefore the additional reduction in mean open time must be attributed to the presence of alpha-actinin. This could indicate that alpha-actinin reduces NMDA receptor channel mean open time but this contradicts the work of Krupp et al. (1999) who found that calcium-dependent inactivation of the NMDA receptor was prevented in the presence of alpha-actinin.

In order to understand these results more clearly, experiments in the presence of 800 nM calcium and 800 nM calmodulin were compared with previous experiments where a lower concentration of calcium (12 nM) and calmodulin was used (12 nM). At 12 nM, calmodulin should predominantly occupy the C1 (75%) over the C0 (12%) region. Therefore these results suggest that the effects of calmodulin on NMDA receptor inactivation at this concentration results from predominant occupation of the C1 region.

Primarily, it was observed that 800 nM calcium increases mean open time in comparison with 12 nM calcium. This finding must be reconciled with previous studies on calcium-dependent NMDA receptor inactivation, that found calcium reduces NMDA receptor activity (Legendre et al., 1993; Vycklicky, 1993; Medina et al., 1996). A possible explanation for this finding is the difference in experimental protocol. These studies investigated calcium-dependent NMDA receptor inactivation in whole-cell recordings, cell attached recordings or in autaptic EPSCs. In all these experimental configurations, endogenous calcium-dependent intracellular components will be present, such as calcineurin and calmodulin which are both known to modulate NMDA receptor activity (Lieberman & Mody, 1994; Ehlers et al., 1996; Zhang et al., 1998; Krupp et al., 1999). It is highly likely that these cellular constituents are in fact responsible for the reduction in activity and not calcium alone. Medina et al. (1996) commented on the fact that calcium-dependent inactivation disappeared in whole cell recordings after 2-5 minutes, which they suggested was due to washout of some responsible calcium-dependent factor.

Owing to the relatively simple environment of the patch most intracellular components, if not membrane bound, are probably lost upon patch excision. Therefore any effects of calcium observed here are presumably due to either a direct action of
calcium or activation of membrane bound calcium-dependent components. However, other studies have investigated the effects of calcium on inside-out patches (Vyckicky, 1993; Rosenmund and Westbrook, 1993a; Medina et al., 1996). Rosenmund and Westbrook (1993a) concluded that calcium does not alter NMDA receptor activity, Medina et al. (1996) observed a transient reduction in activity, which disappeared after 3-5 minutes. Only Vyckicky (1993) found a sustained reduction in NMDA receptor activity, in the presence of calcium.

It is possible to explain these inconsistencies by looking at the different calcium concentrations and time scales employed in each study. Rosenmund and Westbrook (1993a) and Medina et al. (1996) used an intracellular calcium concentration of 100 µM - 2 mM, whereas here the highest calcium concentration employed was 800 nM. Perhaps this increase in NMDA receptor mean open time is only obvious at nanomolar calcium concentrations, with further rises in intracellular calcium approaching saturation of this mechanism.

Medina et al. (1996) acknowledged that the transient reduction in NMDA receptor activity was probably due to washout of a calcium-dependent factor. This is also probably an explanation for the results observed by Vyckicky (1993) but owing to the short time-scale of their experiments the inactivation was not seen to disappear. The manner and longer time scale in which outside-out patches are obtained reduces the chances of intracellular constituents being retained on patch excision, which may be the reason why a transient reduction in receptor activity was not observed in this study. In addition, previous studies only observed the effect of intracellular calcium on NMDA receptor activity at one membrane potential. In this thesis, outside-out patches were routinely recorded at potentials between -30 mV and -80 mV to give a full correlation of the effects of calcium on mean open time with holding potential, resulting in a more in-depth analysis. In addition, the cited studies used an automated single channel analysis program, which would not provide as much detail or accuracy as the method employed here.

The increase in NMDA receptor mean open time seen here may involve the activation of membrane bound calcium-dependent enzymes. These would be retained in the excised patch and may be responsive to the higher 800 nM intracellular calcium concentration used in these experiments. PKC is a likely candidate; it is membrane bound (Parker, 1992), activated by calcium and has already been shown to potentiate NMDA responses (Chen & Huang, 1992; Zheng et al., 1997), although this has been
shown to involve a decrease in mean shut time (Xiong et al., 1998) rather than an increase in mean open time as seen here.

Alternatively, PKC phosphorylates residues in the C1 exon, which could have reduced the inhibitory effect of calmodulin at this region on NMDA receptor activity. Although previous experiments with the PKC inhibitor calphostin-C concluded that PKC was not present or active in the excised patch, inclusion of 800 nM intracellular calcium may have initiated translocation of PKC to the cell membrane. A higher proportion of PKC would therefore be retained in the excised patch allowing subsequent PKC activation. Given that PKC binds calcium with an affinity of 700 nM (Mosior & Epand, 1994) at 800 nM free calcium, 53 % occupancy of PKC would be observed compared with 2 % in the presence of 12 nM calcium.

Alternatively, some yet undiscovered calcium-dependent protein or direct action of calcium in the channel pore, such as “calcium amplification” reported by Zheng et al. (1997), is responsible for this result (see Discussion 7.2.5). In any case, the results here imply that nanomolar increases in intracellular calcium are able to increase NMDA receptor mean open time. Whether this is through a direct or indirect mechanism will require further investigation.

800 nM calmodulin increased mean open time in comparison with 12 nM calmodulin, which again was surprising. If calmodulin occupation of the C1 region reduces mean open time, it might have been expected that calmodulin occupation of the C1 and C0 region would either give the same result or further reduce mean open time. Considering that the calcium concentrations for each set of experiments are different, it is not strictly correct to directly compare the results from these two calmodulin concentrations. Therefore, to make this comparison the mean open time of treated group data were expressed as a proportion of control values. From Figure 6.11, 800 nM calmodulin on average reduced the mean open time by approximately 30 % in comparison with 800 nM calcium and 12 nM calmodulin reduced mean open time by approximately 50 % in comparison with 12 nM calcium, over all membrane potentials recorded. Thus it is evident that 12 nM calmodulin reduces NMDA receptor mean open time further than 800 nM calmodulin.

It is possible that occupation of the C1 region by calmodulin has a more profound effect on mean open time than when both the C0 and C1 regions are occupied. Although, speculatively, this could be complicated by the preferential activation of PKC under high calcium conditions, competing with calmodulin at the C1 region in the presence of 800 nM calcium. More plausibly, the level of NMDA receptor
inhibition observed when calmodulin binds to the C1 region, in comparison with the C1 and C0 region, could mean that some form of negative co-operativity exists between the two calmodulin binding sites. This idea is supported by the alpha-actinin experiments were NMDA receptor mean open time was reduced by 50%, a proportion similar to that achieved by 12 nM calmodulin, presumably through antagonism of calmodulin at the C0 region.

This system could provide a robust physiological feedback mechanism dependent on calcium influx through NMDA receptor channels to control receptor activity. Given that the affinity of calmodulin for the C1 region is 4 nM, the resting calcium concentration of the cell is approximately ~100 nM, and the intracellular calmodulin concentration is approximately ~10 μM (Vincenzi and Hinds, 1980), at rest the NMDA receptor should be tonically inhibited by calmodulin occupying the C1 region. This is validated by Ehlers et al. (1996) who showed that 2 nM active calmodulin was found to maximally inhibit recombinant NMDA receptor activity, at least when measured simply as the change in electrical charge passed. When intracellular calcium levels are raised during neuronal excitation, more calmodulin will be activated allowing occupation of the C0 region. Instead of increasing the level of NMDA receptor inhibition, a reduction in inhibition would be predicted to occur from our results. Therefore in essence, instead of the receptor changing from a state of overactivity to one of progressive inhibition by calmodulin, the receptor starts off maximally inhibited and then is moved to a less inhibited state with increasing calmodulin concentrations. This would allow transduction of larger calcium signals in tandem with the extent of NMDA receptor activation whilst keeping the receptor under constant control.

To explain the implications of both calcium and calmodulin experiments, I propose the following: nanomolar increases in the intracellular calcium concentration facilitate NMDA receptor activity by increasing the open time. Equally, intracellular calcium will activate endogenous calmodulin, which will overall reduce NMDA receptor activity by shortening receptor mean open time and the duration of receptor activations. The opposing forces of calcium and calmodulin are maintained in equilibrium so that NMDA receptor activity is not shut down completely like a switch but its activity is dampened by calmodulin. This way a strong NMDA receptor mediated signal can be quickly transmitted but the receptor is still kept in check by calmodulin and thus prevented from entering a state that could cause excitotoxicity.
6.4.4 Summary

The results of this study suggest that a rise in intracellular calcium can increase NMDA receptor mean open time, possibly via a direct action or through activation of a calcium-dependent second messenger. Calmodulin overall reduces NMDA receptor mean open time but inhibition is maximal at low calmodulin concentrations, when only the high affinity site, C1 is expected to be occupied. Subsequent occupation of the low affinity C0 region by calmodulin results in a slight disinhibition regarding mean open time, suggesting some form of negative co-operativity exists between the two sites.
CHAPTER 7

DISCUSSION

The encompassing aim of this thesis was to investigate the various mechanisms of native NMDA receptor intracellular modulation. This can be either via potentiation or inhibition, although this study has been primarily concerned with NMDA receptor inhibition. Considering the involvement of the NMDA receptor in excitotoxicity and related afflictions, there are, potentially, various pharmacological and therapeutic implications for NMDA receptor inhibition.

It was important to verify the outcome of previous research on recombinant receptors with effects seen here on the native NMDA channel, for it is necessary to corroborate research performed on recombinant receptors with a naturally occurring system. Equally examining single channel characteristics has allowed the molecular mechanisms of native NMDA receptor modulation to be assessed. Furthermore, channel kinetics can be used to predict consequences for the NMDA receptor-mediated EPSC, in response to application of individual modulatory proteins. During whole-cell recordings the actions of discrete proteins could become camouflaged or obscured by other cellular constituents present. Although the latter is set in a more physiological context, it is necessary to break these pathways down in order to gain full understanding of these modulatory systems.

7.1 Intracellular modulation of the NMDA receptor in outside-out patches from rat hippocampus

This study is a detailed examination of intracellular modulation of the native NMDA receptor by PKC, calcineurin, calmodulin, alpha-actinin and calcium. This chapter will first address the overall implications of the results obtained with each discrete modulatory system examined in this study. Secondly, the interaction and overlap of these modulatory systems within the synapse and predictions for the NMDA macroscopic current will be discussed with respect to findings here and previously published observations.
7.2 Summary of experiments with cyclosporin-A, calphostin-C, calcineurin, PKC, calmodulin, alpha-actinin and calcium

To summarise the findings of this thesis:

- Inhibition of endogenous calcineurin and PKC, with cyclosporin-A and calphostin-C, did not affect native NMDA single channel characteristics in outside-out patches.
- Application of calmodulin or calcineurin plus calmodulin was found to reduce single channel mean open time. This was concluded to be a direct effect of calmodulin rather than calcineurin.
- Increasing the intracellular calcium concentration increased NMDA receptor mean open time.
- Calmodulin reduced mean open time at low concentrations, predicted to predominantly occupy the C1 region. Surprisingly, at high concentrations, predicted to occupy both the C1 and C0 region, the reduction in mean open time was less.
- The effects of calmodulin binding to the C0 region on NMDA receptor activity were antagonised to the levels seen with predominant occupancy of the C1 region by the presence of alpha-actinin, suggesting that the two binding sites exhibit negative co-operativity in terms of their effect on single channel open time.
- Calmodulin binding to the C1 region significantly reduced burst, cluster and supercluster length, total open time per burst, cluster and supercluster and charge passed per burst, cluster and supercluster. However, the open probability of bursts, clusters and superclusters was not significantly affect by calmodulin.

7.2.1 Calphostin-C and cyclosporin-A

Results obtained from outside-out patches in the presence of PKC and calcineurin inhibitors, calphostin-C and cyclosporin-A, did not indicate any change in single channel activity. If PKC and calcineurin are responsible for single NMDA receptor modulation, as shown by previous studies (Chen & Huang, 1992; Xiong et al., 1998; Lieberman & Mody, 1994), the results presented here appear unusual. Considering that calphostin-C and cyclosporin-A were present in the recording solution in addition to the pipette solution, the ratio of NMDA receptor phosphorylation to dephosphorylation in the cell would expected to be altered in the brain slice before and during patch formation. This is of course assuming receptor phosphorylation / dephosphorylation is responsible for NMDA receptor potentiation.
inhibition by PKC and calcineurin, of which there is no definite evidence (Sigel et al., 1994; Zheng et al., 1999).

To explain these findings it is possible that patch excision causes an over-riding force that prevents the effects of these inhibitors from being observed. For example, during procurement of an outside-out patch, some intracellular constituents vital to the functioning of phosphatases and kinases, such as ATP, calmodulin and calcium, could be lost. This would feasibly alter the balance between phosphorylation and dephosphorylation, disguising the consequences of previous kinase and phosphatase inhibition. From these studies it was decided that direct application of the protein(s) rather than inhibitor was a preferable means to evaluate regulation of single NMDA channel currents, in outside-out patches.

Regarding these preliminary studies with PKC and calcineurin inhibitors it was presumed that there is no natural activity of PKC and calcineurin in the excised patch, under these conditions. PKC activity was unlikely in the absence of ATP and DAG, and in the presence of buffered low calcium. Likewise calcineurin is probably inactive at a sub-nanomolar calcium concentrations. These considerations were taken into account whilst analysing the results of subsequent experiments. For example, during direct application of calcineurin or calmodulin to the intracellular surface of the patch it was assumed that no endogenous actions of PKC or calcineurin were present to complicate or confuse results.

Although, in general, calphostin-C and cyclosporin-A did not alter conventional NMDA channel characteristics, there was some indication that calphostin-C increased NMDA receptor mean shut time within a burst. This is consistent with the ability of PKC to potentiate NMDA receptor currents by decreasing mean shut time and therefore increasing open probability, and agrees with the results of Xiong et al. (1998) and Chen & Huang (1992). It is not possible, though, to draw any definite conclusions from the results of this study. Further experiments using lengthy but detailed burst analysis would perhaps clarify this issue.

7.2.2 Calcineurin or calmodulin?

One of the main aims of this thesis was to evaluate inhibitory modulatory processes, particularly the relationship between calcineurin and calmodulin and the role of each protein in NMDA receptor inhibition. After the NMDA receptor was first shown to be inhibited by calcineurin (Liebermann & Mody, 1994), it came to light that calmodulin, a cofactor for calcineurin activation, also had modulatory properties
(Ehlers et al., 1996). Therefore, the question arose, which protein is actually responsible for NMDA receptor inhibition or, alternatively, is it a combination of both?

In this study, the presence of either calmodulin or calcineurin plus calmodulin reduced mean open time relative to control, whilst leaving other single channel characteristics unaffected. Surprisingly, calmodulin alone reduced mean open time to a greater extent than calcineurin plus calmodulin. In answer to the above question, the results here suggest that calmodulin alone, rather than calcineurin, is predominantly responsible for reducing NMDA receptor mean open time.

Calmodulin binds to the C1 and C0 region of the NR1 subunit C-terminus (Ehlers et al., 1996). Phosphorylation sites are contained in the C1 but not the C0 region, so it is possible that calcineurin and calmodulin will compete for physiological activity through occupancy of the C1 region. Considering that both calmodulin and calcineurin only affected single channel mean open time, the possibility that their actions are mediated by a common mechanism can be entertained. Given that mean open time was reduced to a greater extent in the presence of calmodulin alone compared with calcineurin, it can be proposed that calcineurin either antagonises calmodulin at the C1 region or acts as a partial agonist at the C1 region. The same active concentration of calmodulin was present in the calcineurin experiments; therefore, some property of calcineurin must have prevented the full extent of NMDA receptor inhibition, as seen with calmodulin alone.

The most likely explanation is that calcineurin does not directly affect NMDA receptor activity at the single channel level but incidentally antagonises the actions of calmodulin. This is consistent with the inability of the calcineurin inhibitor cyclosporin-A to affect single NMDA channel characteristics in inside-out patches. However, under normal cellular conditions where a proportion of NMDA receptors would possibly exist in a phosphorylated state due the presence of PKC or PKA, dephosphorylation of the C1 exon by calcineurin would allow calmodulin access to its binding site. Calcineurin did not affect any single channel characteristics other than mean open time, in agreement with previous findings (Leibermann & Mody, 1994).

Although the active concentration of calcineurin was low (1.66 nM) in these experiments, the affinity of calcineurin for its substrate is thought to be very high (Klee et al., 1988). Unfortunately there is no direct measurement of the affinity of calcineurin for the NMDA receptor, but as an indication the affinity of calcineurin for active calmodulin is very high ($K_d = 0.1$ nM) (Hubbard & Klee, 1987).
7.2.3 Calmodulin occupation of the C1 versus C0 region

It was important to assess the contribution of each calmodulin binding site, C0 and C1, in NMDA receptor inactivation. There is confusion in the literature as to whether the actions of calmodulin are exerted through occupation of the C1 or C0 region. Owing to the different affinities of these calmodulin binding sites, the NMDA receptor could either be under tonic inhibition by calmodulin or would be inhibited predominantly when the intracellular calcium concentration rises.

This study addressed this question by assessing the effects of calmodulin on single NMDA channel characteristics when bound predominantly to the C1 region, and comparing results with those obtained when calmodulin occupies both C1 and C0 regions. In order to verify the effects of calmodulin resulting from occupancy of the C0 region, further studies included alpha-actinin to antagonise calmodulin occupancy at this site. If the mechanism by which calmodulin alters NMDA receptor behaviour is different when bound to either the C1 or C0 region, we may understand why the two calmodulin binding regions were shown to be related to either single channel inhibition or whole-cell current inactivation.

The results of this study were rather surprising. In terms of single channel mechanism, calmodulin only affected mean open time, regardless of whether it was predicted to be bound to the C1 or C0 region or both. Thus calmodulin binding at either site may inactivate NMDA receptors via a common mechanism but the two binding sites may not contribute equally. When calmodulin was expected to be bound to the C1 region, mean open time was reduced to a greater extent than when calmodulin was bound to both C1 and C0 regions. However, when calmodulin binding at the C0 region was antagonised with alpha-actinin, mean open time was similar to that seen during calmodulin occupancy of only the C1 region. Thus it appears that a negative co-operativity may exist between the two calmodulin binding sites.

These results suggest that the NMDA receptor will be maximally inhibited by calmodulin occupying the C1 region. Instead of increasing NMDA receptor inhibition, progressive disinhibition occurs as the calcium concentration is raised during neuronal excitation and activated calmodulin begins to occupy the C0 region. According to this scheme, instead of allowing the NMDA receptor to approach a level of activity which could compromise the cell’s viability, inhibition is constantly present. This suggests an interesting mechanism, where the inhibitory factor modulates its own level of efficiency to allow an appropriate response to the synaptic input.
The results described in this thesis detailing the effect of calmodulin at the C1 and C0 exon are not entirely consistent with the work of other groups. Originally, Ehlers et al. (1996) showed that in excised patches low concentrations of calmodulin, such as 4 nM, are sufficient to maximally inhibit recombinant NMDA receptor activity, by reducing open time and open probability. Consistent with the results here this would presumably be due to occupation of the C1 region as it has the highest affinity for calmodulin (Kd = 4 nM) compared with the C0 region (Kd = 87 nM) (Ehlers et al., 1996). However, Zhang et al. (1998) found that calmodulin failed to affect the open probability of recombinant NMDA receptors containing mutations in the C0 region, in inside-out patches. Therefore it was suggested that only the C0 region is involved in NMDA receptor inhibition. This was supported by Krupp et al. (1999) who found that recombinant receptors containing the NR1-4a splice variant, which does not contain the C1 exon, were inactivated in response to application of calmodulin to inside-out patches.

With respect to the findings of both Zhang et al. (1998) and Krupp et al. (1999) it is possible that if binding of calmodulin to the C1 and C0 regions exhibits cooperativity, mutations in the C0 region could alter the ability of the C1 region to cause receptor inhibition. In addition, although NMDA receptor inhibition is evident in the absence of the NR1 subunit C1 exon, this does not mean that receptor inhibition would not be evident and possibly more so if the C1 region was present. In whole-cell recordings where the number of receptors is unknown it is difficult to make an absolute measurement of receptor inhibition. It would be necessary to perform more single channel experiments, possibly antagonising calmodulin binding to the C1 region of native receptors, before the results of Zhang et al. (1998) and Krupp et al. (1999) are fully substantiated.

In past literature more attention has been given to the idea that calmodulin occupation of the C0 region is involved in calcium-dependent inactivation of NMDA-mediated whole-cell currents (Rafiki et al., 1997; Zhang et al., 1998; Krupp et al., 1999) and therefore studies have not pursued results at the single channel level. These studies all agree that the C1 region is not involved in this phenomenon and put emphasis on the C0 region. This appears to contradict the results obtained from this single channel study but there are two reasons to explain this inconsistency.

First of all there is conflicting evidence as to whether calmodulin is solely responsible for calcium-dependent NMDA receptor inactivation. Experiments performed by Zhang et al. (1998) found that calcium-dependent inactivation of whole-
cell currents could be prevented by inclusion of a calmodulin binding peptide, designed to antagonise calmodulin binding to the receptor. However, a reduction in inactivation was determined as a decrease in the extent of observed inactivation during agonist application rather than an increase in the steady-state current, which according to Krupp et al. (1999) is a more appropriate measure (since extent of inactivation measured depends on accurately measuring the peak of the current). Conforming to this criterion, the same experiments performed by Krupp et al. (1999) showed that the presence of the calmodulin binding peptide did not decrease steady-state inactivation in whole-cell currents. Previous studies with whole-cell currents have also found the presence of calmodulin inhibitors unable to reverse the effects of calcium-dependent inactivation (Legendre et al., 1993; Rosenmund & Westbrook, 1993a; Krupp et al., 1996).

Secondly, the effects of calmodulin on NMDA receptor mean open time through occupation of the C1 and C0 region reported here might not be involved in the calcium-dependent NMDA receptor inactivation seen in whole-cell currents. Or it simply may not be appropriate to make direct extrapolations between single channel and whole-cell data. For instance, depending on the time course of the change in calcium concentration in microdomains under the receptor, the change in NMDA receptor mean open time by calmodulin may achieve steady state relatively quickly and therefore inactivation due to this mechanism may not be resolvable on the timescale of whole-cell recordings.

7.2.4 Involvement of alpha-actinin and interaction with the cytoskeleton

Although the physiological role of alpha-actinin has not been assessed in this study, there are some conclusions that can be drawn concerning the relationship of alpha-actinin and calmodulin with respect to the NMDA receptor. In light of the finding that in biochemical experiments calmodulin and alpha-actinin-2 compete for binding to the C0 region of the NR1 subunit (Wyszynski et al., 1997), alpha-actinin (purified from rabbit skeletal muscle) was utilised as an antagonist for calmodulin in this study. The rabbit skeletal muscle preparation contains calcium-insensitive alpha-actinin subtypes 2 and 3, where alpha-actinin-2 exhibits binding to the NMDA receptor unlike alpha-actinin-3 (Wyszynski et al., 1997).

Indeed in this study, alpha-actinin reversed the effect on NMDA receptor mean open time produced by calmodulin binding to the C0 and C1 region, towards that seen when calmodulin binding is predominantly to the C1 exon. Alpha-actinin had no other effect on single NMDA channel characteristics, which concurs with the findings of
Wyszynski et al. (1997) that alpha-actinin-2 and calmodulin interact via the NR1 subunit C0 region.

Building on the idea that calcium-dependent inactivation is not solely dependent upon activation of calmodulin, alpha-actinin-2 and other calcium-sensitive non-muscle alpha-actinin isoforms were found to play a substantial role by Zhang et al. (1998) and Krupp et al. (1999). It was proposed that calcium-dependent inactivation of NMDA receptor-mediated whole-cell currents results from dissociation of the receptor from alpha-actinin and the cytoskeleton. This is in part mediated by calmodulin displacing alpha-actinin at the C0 region and also calcium binding to alpha-actinin and promoting detachment from the C0 region. It can be predicted that under natural resting conditions, the C0 region is occupied by alpha-actinin, for its affinity is twice that of calmodulin (Kd = 48 nM, Krupp et al., 1999; 87 nM, Ehlers et al., 1996, respectively). Additionally, Wyszynski et al. (1997) reported that 100 nM calcium was insufficient to antagonise alpha-actinin-2 binding to the NR1 subunit in-vitro, suggesting that alpha-actinin-2 is bound to the NMDA receptor at resting intracellular calcium concentrations.

In addition to dissociation of the NMDA receptor from the cytoskeleton, receptor internalisation could also provide the basis of calcium-dependent inactivation. It has previously been shown that disruption of actin filaments releases NMDA receptor clusters from their postsynaptic sites and selectively reduces the activity of synaptically activated NMDA receptors (Allison et al., 1998; Sattler et al., 2000). Receptor localisation was similarly disrupted when C-terminal segments containing the C0 region were co-expressed in recombinant NMDA receptor cell lines (Matsude & Hirai, 1999). Thus it was suggested that this region of the NMDA receptor is involved in association to the actin cytoskeleton, via alpha-actinin-2. Curiously, disruption of the actin cytoskeleton was found to provide neuroprotection against excitotoxicity (Sattler et al., 2000), possibly by calcium-dependent NMDA receptor inactivation or internalisation.

With this in mind it is possible to readdress inconsistencies previously mentioned between the effects of calmodulin on NMDA channel open time and calcium-dependent inactivation of whole cell currents. It is possible that the latter process involves receptor inhibition and/or receptor internalisation through dissociation from the cytoskeleton. Receptor internalisation would also account for the reduction in the steady-state whole cell currents seen after calcium-dependent inactivation. Therefore the reduction in NMDA receptor mean open time by
calmodulin investigated in this study may not be a mechanism directly involved with the onset of calmodulin and calcium-dependent inactivation of whole-cell currents, but nevertheless may be just as important in regulating NMDA receptor function in the brain.

7.2.5 Calcium-dependent modulation

In contrast to previous reports, in this study it was found that increasing the intracellular calcium concentration increases NMDA receptor activity by increasing mean open time. In whole-cell or cell-attached experiments a calcium-dependent reduction in NMDA receptor activity is clearly observed (Legendre et al., 1993; Vyklicky, 1993; Medina et al., 1996). It is highly likely that this is due to the activation of inhibitory calcium-dependent processes, dependent on the intracellular environment of the cell being intact. Applying calcium directly to the intracellular surface of the patch, as performed in this study, allows a direct test of whether calcium itself can affect NMDA receptor activity.

Although the effect of calcium on inside-out patches has been reported, in general it was concluded that calcium has no direct effect on NMDA receptor activity (Rosenmund and Westbrook, 1993a; Medina et al., 1996). Sometimes a transient decrease in NMDA receptor activity was initially observed but this was not sustained. This could be due to the manner in which inside-out patches are formed in comparison to outside-out patches, which could allow some inhibitory processes to be retained initially but subsequently washed-out. These previous studies (Rosenmund and Westbrook, 1993a; Vyklicky, 1993; Medina et al., 1996) did not perform such an in-depth quantitative analysis as that employed in the present study. Only one membrane potential was studied and the single channel analysis programs used would not provide as much detail as the method employed here.

Further experiments would be necessary to assess whether calcium increases mean open time by a direct mechanism or by activation of otherwise dormant calcium-dependent processes. In terms of a direct effect of calcium perhaps a similar mechanism to that observed by Zheng et al. (1997) might apply. It was suggested that PKC potentiation of NMDA receptor activity was due to calcium-dependent current amplification. This was suggested to involve calcium binding to a regulatory domain in the channel pore and required a rise in the intracellular free calcium.

Alternatively, the calcium concentration (800 nM) used in this study could possibly have induced translocation of PKC to the cell membrane during the whole-cell
recording phase of forming an outside-out patch (Mosior & Epand, 1994) which would therefore be retained in the excised patch. Activation of PKC could have increased mean open time, observed as an increase in channel open probability in other studies (Chen & Huang, 1992), although constitutively active PKC has been shown to preferentially increase mean shut time rather than increase open time (Xiong et al., 1998). Equally, preferential activation of PKC in experiments using 800 nM calcium could have caused PKC to compete with calmodulin for occupancy of the C1 exon and thus increase channel mean open time by preventing the actions of inhibitory calmodulin.

7.2.6 Conclusions

To unify results from all calmodulin, alpha-actinin and calcium experiments in this study and with previous research, I propose the following: A rise in intracellular calcium increases NMDA receptor mean open time, by some yet undetermined means. Activation of calmodulin inhibits single NMDA channel activity by reducing mean open time, as seen in excised patch recordings. Although there is less receptor inhibition when both calmodulin binding sites are occupied, as opposed to occupancy of mainly the high affinity C1 region, there is still a total reduction in NMDA receptor mean open time relative to that in the presence of calcium alone. Thus, as previous stated, calmodulin serves to dampen the NMDA receptor response whilst still allowing the receptor activity to be modulated.

Additionally, in the intact cell the rise in the intracellular calcium concentration and concomitant calmodulin activation will displace alpha-actinin from the NR1 subunit C0 region, resulting in either calcium-dependent NMDA receptor inactivation or internalisation. These two pathways may serve to allow regulation of synaptic currents in a manner analogous to a volume control, while constantly maintaining inhibitory control (calmodulin/alpha-actinin) and potentially signal extinction (via receptor internalisation).

7.2.7 Future Experiments

The results of this thesis have raised a number of interesting questions that will require further experiments to determine:

- Investigate the direct effects of PKC on NMDA receptor modulation by calmodulin and alpha-actinin.
• Investigate the properties of bursts, clusters and superclusters in the presence of PKC.

• Antagonism of calmodulin at the C1 region by NF-1 or brain spectrin (unfortunately neither of these are commercially available at present), to see what actions of calmodulin can be directly attributable to occupation of the C0 region.

• Imaging NMDA receptor clustering in the cellular membrane in the presence of calmodulin would help elucidate whether calmodulin contributes to receptor internalisation in response to cytoskeletal dissociation.

• Control experiments could have been performed in the presence of a calmodulin inhibitor to assess whether there was any endogenous calmodulin activity in the excised patch.

• If a way could be found to control the intracellular active calmodulin concentration, it would have been interesting to perform these experiments again in the cell-attached configuration and compare the results with those from excised outside-out patches. It may be directly possible to ascertain the function and necessity of certain intracellular constituents lost in excised patch formation.

• Investigate whether a higher concentration of calcineurin would expose any interaction with calmodulin binding to the C0 region.

• Investigate the properties of bursts, clusters and superclusters in the presence of 800 nM calmodulin and compare these results with those obtained with 12 nM calmodulin. Given that that both concentrations of calmodulin reduced mean open time it may be that the same trends in bursts, clusters and superclusters seen in the presence of 12 nM calmodulin would also be seen with 800 nM calmodulin but to a lesser extent.

• Investigate whether there is an effect of calmodulin on block of NMDA receptor by magnesium.

• Investigate the effect of a broader spectrum of calcium concentrations on NMDA receptor function in outside-out patches. Follow up the effect of 12 nM calcium on mean shut time within a burst by performing burst analysis.

7.3 The big picture

Although examination of single channel characteristics provides a very detailed and precise way to investigate modulation of the receptor, it is still of interest to consider how these results may relate to the role of NMDA receptors within the brain.
7.3.1 Implications for interactive modulation of the synaptic NMDA receptor

The overview emerging from past and present observations is that under normal resting physiological conditions the synaptic NMDA receptor is bound to alpha-actinin via the C0 region of the NR1 subunit. This is assumed because alpha-actinin has an affinity for the C0 region twice that of calmodulin, although their effective active concentrations at the postsynaptic density should also be taken into account. No other cytoskeletal elements or modulatory proteins have been discovered that compete for binding to the C0 region. Therefore the modulatory balance of NMDA receptors attributed to this region is relatively simple. As the intracellular calcium concentration rises, the active calmodulin concentration also increases. Calmodulin in turn competes with alpha-actinin for occupation of the C0 region and calcium binding to alpha-actinin results in calcium-dependent NMDA receptor inactivation and/or internalisation by cytoskeletal dissociation, as suggested in whole-cell studies by Krupp et al. (1999) and also indicated by Matsuda & Hirai (1999) and Allison et al. (1998).

Interactions at the C1 region are far more complex because the number of modulatory proteins currently known to associate with this region include: calmodulin, calcineurin, PKC, PKA, brain spectrin, NF-L and yotiao. It is tempting to suggest that the synaptic NMDA receptor is under maximal tonic inhibition by calmodulin binding to the C1 exon, owing to its high affinity for this region. One possible challenge to this idea is that there may be cytoskeletal attachment of the NMDA receptor via the C1 region by yotiao, NF-L and brain spectrin (Lin et al., 1998; Ehlers et al., 1998; Wechsler & Teichberg, 1998). Although neither yotiao nor NF-L has been shown to compete with calmodulin for binding to the C1 region, brain spectrin has (Wechsler & Teichberg, 1998). Unfortunately, the dissociation constant of brain spectrin for the C1 region is not known and in comparison the affinity of calmodulin for the C1 region is very high \( K_d = 4 \text{ nM}, \) Ehlers et al., 1996). It is likely that a proportion of NR1 subunits will either be bound to calmodulin or brain spectrin at any one time but it is impossible to predict what that proportion will be, without detailed knowledge of the concentration and affinities of each protein.

If the C1 region is phosphorylated by PKC or possibly PKA, calmodulin will not be able to bind (Hisatsune et al., 1997). Little information is available about the affinity of PKA and PKC for the NMDA receptor but PKC needs to bind calcium for activation \( (K_d = 700 \text{ nM}, \) Mosior & Epand, 1994). Given the high affinity of calmodulin for the C1 region \( (K_d = 4 \text{ nM}, \) Ehlers et al., 1996), it may be that PKC will be unable to
compete with calmodulin under resting conditions. Depending upon the relative active concentrations of each at the inner surface of the membrane during a synaptic current, though, PKC could compete with calmodulin for occupancy of the C1 region. Once phosphorylated, calmodulin will not be able to rebind until the C1 region is dephosphorylated (Hisatsune et al., 1997), possibly by calcineurin, which infers that receptor dephosphorylation could be the key regulatory step in calmodulin-mediated inhibition of the NMDA channel. This may explain, in part, the reported involvement of calcineurin in synaptic desensitisation (Tong et al., 1995), plasticity (Tomizawa et al., 1996; Wang & Kelly, 1996a) and transmission (Wang & Kelly, 1996b). If calcineurin activity is prevented through selective antagonism, the NMDA receptor will remain phosphorylated rendering calmodulin unable to bind and exert its inhibitory influence.

Additionally, the C1 region has been implicated in the clustering and association of NMDA receptor with the plasma membrane (Ehlers et al., 1995). Absence of the C1 region or phosphorylation by PKC causes disruption of these clusters and hence disperses the NMDA receptors in the cell cytoplasm (Ehlers et al., 1995; Tingley et al., 1997). Therefore prevention of PKC phosphorylation by calmodulin binding to the C1 region may provide a means to localise or maintain NMDA receptors at the postsynaptic membrane, with implications for receptor density at synaptic sites. This interaction between calmodulin and PKC may underlie a novel mechanism concerned with the regulation of NMDA receptors in the postsynaptic membrane, which requires further investigation.

In light of reports concerning PKC potentiation of NMDA receptor-mediated currents, mediated by phorbol ester application (Chen & Huang, 1991; 1992), it appears contradictory that phosphorylation by PKC should reduce receptor clustering and association with the plasma membrane. Actual surface expression, though, is dependent upon the NR2 subunit (McLhinney et al., 1996; Okabe et al., 1999) and therefore maybe more complicated than simple receptor clustering mediated by the C1 exon. The C1 exon maybe involved in receptor subunit assembly (seen as clustering) rather than surface expression, although this has, so far, not been determined experimentally. Furthermore, PKC potentiation of NMDA receptor-mediated currents has recently been shown to involve increases in NMDA receptor surface expression via up-regulation of receptor-containing vesicle trafficking, in addition to changes in channel gating (Lan et al 2001).

The balance of these process, and possibly others, will concurrently contribute to synaptic strength through modulation of NMDA receptor activity and receptor
density. The NMDA receptor is involved in the induction of synaptic potentiation (LTP) or depression (LTD), owing to its high calcium permeability (Lynch et al., 1983). The magnitude of intracellular calcium transients, generated mainly through the NMDA receptor and voltage-gated calcium channels, is thought to determine the balance between LTP and LTD but exactly how remains elusive.

The differential activation of cellular second messenger systems by calcium has been a popular theory; Lisman (1989) first proposed a scheme to suggest this could be the case. High levels of calcium were suggested to preferentially activate CaMKII, which facilitates LTP by phosphorylating non-NMDA receptors, whereas low calcium levels mainly activate calcineurin and protein phosphatase-1 (PP1) which were suggested to result in LTD. In addition, because PP1 inhibits CAMKII, and thus keeps the level of LTP activity in check by a negative feedback mechanism, if intracellular calcium reached a certain critical level PP1 would become inactivated leaving the activity of CaMKII to dominate. Thus there is theoretically a calcium threshold that if passed will allow the memory of synaptic activity to be retained.

A similar mechanism could plausibly account for modulation of NMDA receptor activity. A threshold concentration of calcium may determine whether potentiation by PKC or inhibition by calcineurin, calmodulin and alpha-actinin is prevalent. If the increase in intracellular calcium surpassed this threshold, calcineurin or PKC would become inactivated, depending upon which had faster inactivation kinetics. This would, in one case, leave the majority of NMDA receptors phosphorylated and unable to bind inhibitory calmodulin, producing a larger and more prolonged EPSC. In the other case, activation of calcineurin and calmodulin would dominate, overcoming phosphorylation by PKC, creating detachment from the cytoskeleton and thus reducing NMDA receptor activity.

7.3.2 Implications for the NMDA receptor-mediated excitatory postsynaptic current

It is possible to make some predictions concerning the time course of synaptic EPSC's from single channel kinetics by measuring single channel activations that constitute superclusters (Wyllie et al., 1998). Superclusters are described as the series of openings and closures that occur whilst agonist is bound (Gibb & Colquhoun, 1992). The lengths of these activations are related to the duration of the NMDA receptor-mediated excitatory postsynaptic current, since agonist molecules do not rebind to the receptor during a synaptic current (Lester et al., 1990; Hestrin et al., 1990b). Therefore, channel openings during an activation, or supercluster, will be responsible for the
charge passed during the postsynaptic response of a single receptor. As calcium contributes around 16% of the NMDA channel current (Schneggenburger, 1996), any alterations in the NMDA channel open time or activation length will affect calcium-dependent processes such as synaptic plasticity, as discussed above. In addition, if the time course of the NMDA EPSC is altered there will be implications for the role of the NMDA receptor as a coincidence detector and its involvement in synaptic integration.

In the experiments described here, 12 nM active calmodulin reduces NMDA receptor mean open time by 50%. This results in a reduction of burst cluster and supercluster length, total open time and total charged passed per burst, cluster and supercluster, all attributable to the effect of calmodulin on open time since no other single channel characteristic, such as shut time or conductance, was affected by calmodulin.

When macroscopic averaged currents were generated by the alignment of superclusters, the decay time course of the ensemble current was dramatically shortened by calmodulin. This indicates that calmodulin has the ability to potentially reduce the NMDA-mediated EPSC, as predicted from the increased rate of decay of ensemble currents and reduction of total charge passed during the ensemble when compared with control. This would be expected to affect the role of the NMDA receptor in the computational aspects of neuronal function. Weakening of the NMDA EPSC by calmodulin could reduce the critical time period for temporal and spatial summation of synaptic inputs and their transmission to the axon hillock. This would, theoretically, alter the ability of two or more synchronised EPSCs to initiate action potentials (APs). Additionally, in the knowledge that APs propagate back into the dendritic tree (Stuart & Sakmann, 1994), weakening of the NMDA EPSC by calmodulin could reduce the interaction of pre and postsynaptic activity and thus alter integration of synaptic activity. It has been shown that to strengthen the association of presynaptically evoked EPSCs and postsynaptic APs in the dendritic tree, the two inputs must coincide within 100 ms in the neocortex (Markram et al., 1997). Considering that calmodulin increases the decay of the EPSC, the window for this form of neuronal plasticity could be reduced or in a different light perhaps simply fine-tuned.

7.4 Concluding remarks

Although this thesis has been primarily concerned with modulation of the NMDA receptor at the single channel level, it is possible to see there are at least three
tiers of physiological consequences. At first NMDA channel activity is altered, perhaps by a simple reduction in open time in the case of calmodulin and calcineurin. A change in calcium influx through the receptor will then modify the interaction of other calcium-dependent second messenger proteins, with possible consequences for all systems in the cell that are influenced by intracellular calcium including synaptic plasticity. Ultimately, alterations in the macroscopic NMDA current will occur and reorganisation of computational neuronal circuitry may follow. Although it is acceptable to speculate on, or predict the possible consequences of receptor inhibition on synaptic transmission, it would be necessary to experimentally validate these suppositions. Perhaps only then may the full potential of intrinsic receptor channel modulatory systems, such as those discussed in this thesis, be fully understood.
APPENDIX

In these experiments calmodulin was in excess and the buffered calcium concentration was used as the limiting factor in setting the active calmodulin concentration. In order to work out the calcium concentration needed to activate a certain amount of calmodulin the following was determined.

Calmodulin has four calcium binding sites thus (James et al., 1995):

\[
\begin{align*}
K_1 & \quad K_2 & \quad K_3 & \quad K_4 \\
C_0 & \Leftrightarrow & C_1 & \Leftrightarrow & C_2 & \Leftrightarrow & C_3 & \Leftrightarrow & C_4
\end{align*}
\]

(1)

\(K_e\) = Dissociation equilibrium constant

\(C_x\) = Calcium binding site

Where:

\[
P_{C0} + P_{C1} + P_{C2} + P_{C3} + P_{C4} = 1
\]

(2)

\(P_{Cx}\) = Proportion of binding site bound by calcium

Therefore at equilibrium:

\[
P_{C0} \cdot [Ca^{2+}] \cdot k_{+1} = P_{C1} \cdot k_{-1}
\]

\(k_{+1}\) = Association rate constant

\(k_{-1}\) = Dissociation rate constant

\(k_{+1}/k_{-1} = K_1\) = Dissociation equilibrium constant

\([Ca^{2+}]\) = calcium concentration

combining rate constants

\[
P_{C0} \cdot [Ca^{2+}] = P_{C1} \cdot K_1
\]

and rearranging

\[
P_{C0} = \frac{K_1 \cdot P_{C1}}{[Ca^{2+}]}
\]
likewise

\[
P_{C1} = \frac{K_2}{[Ca^{2+}]} \cdot P_{C2}
\]

\[
P_{C2} = \frac{K_3}{[Ca^{2+}]} \cdot P_{C3}
\]

\[
P_{C3} = \frac{K_4}{[Ca^{2+}]} \cdot P_{C4}
\]

If calmodulin is active only when two or more binding sites are occupied by calcium (James et al., 1995) then:

\[
P\text{ (active)} = P_{C2} + P_{C3} + P_{C4}
\]

To express equation (2) in terms if \(P_{C2}, P_{C3}\) and \(P_{C4}\):

For \(P_{C2}\):

\[
P_{C0} = \frac{K_1 \cdot K_2}{[Ca^{2+}]^2} \cdot P_{C2}
\]

\[
P_{C1} = \frac{K_2}{[Ca^{2+}]} \cdot P_{C2}
\]

\[
P_{C3} = \frac{[Ca^{2+}]}{K_3} \cdot P_{C2}
\]

\[
P_{C4} = \frac{[Ca^{2+}]^2}{K_3 \cdot K_4} \cdot P_{C2}
\]

\[
P_{C2} \left\{ \frac{K_1 \cdot K_2}{[Ca^{2+}]^2} + \frac{K_2}{[Ca^{2+}]} + 1 + \frac{[Ca^{2+}]}{K_3} + \frac{[Ca^{2+}]^2}{K_3 \cdot K_4} \right\} = 1
\]

\[
\left\{ \frac{K_1 \cdot K_2}{[Ca^{2+}]^2} + \frac{K_2}{[Ca^{2+}]} + 1 + \frac{[Ca^{2+}]}{K_3} + \frac{[Ca^{2+}]^2}{K_3 \cdot K_4} \right\} = \frac{1}{P_{C2}}
\]
accordingly

\[
\frac{1}{\{ K_1 \cdot K_2 \cdot K_3 + K_2 \cdot K_3 + K_3 + 1 + [Ca^{2+}] \}} = P_{C_3}
\]

\[
\frac{1}{\{ K_1 \cdot K_2 \cdot K_3 \cdot K_4 + K_2 \cdot K_3 \cdot K_4 + K_3 \cdot K_4 + K_4 + 1 \}} = P_{C_4}
\]

If we know the values for \(K_1, K_2, K_3\) and \(K_4\), and calcium concentration, the variable in this instance, we can work out \(P_{C_2}, P_{C_3}\) and \(P_{C_4}\), and add them together to give the proportion of active calmodulin.

For our calculations \(K_1, K_2, K_3\) and \(K_4\) were taken from Haiech et al. (1981) where:

\(- K_1 = 67 \text{ nM}
\]
\(- K_2 = 170 \text{ nM}
\]
\(- K_3 = 600 \text{ nM}
\]
\(- K_4 = 900 \text{ nM}
\]

According to these calculations a concentration of 12 nM free calcium would result in 1.02 % of calmodulin containing two or more occupied calcium binding sites. Thus the total calmodulin concentration used in these experiments was 1.2 \(\mu\)M, where 1.02 % would give rise to a final active calmodulin concentration of 12.24 nM. According to Ehlers et al. (1996) this would result in 75 % occupancy of the NR1 subunit C1 region, calmodulin binding site.

Similarly using the same protocol but different affinity constants for calcium binding to calcineurin (Kakalis et al., 1995):

\(- K_1 = 24 \text{ nM}
\]
\(- K_2 = 15 \mu\text{M}
\]
\(- K_3 = 15 \mu\text{M}
\]
\(- K_4 = 15 \mu\text{M}
\]
12 nM free calcium would result in 0.668 % of the total calcineurin* having more than two occupied calcium binding sites. Considering the total concentration of calcineurin was 0.25 μM this would result in 1.67 nM active calcineurin. Calmodulin is also necessary for calcineurin activity but with an affinity of 0.1 nM, calmodulin binding of calcineurin should be almost saturated resulting in a final concentration of 1.66 nM active calcineurin.

Occupancy of the NMDA NR1 subunit calmodulin binding sites, the C0 and C1 regions, were predicted by analogy with the Hill-Langmuir equation:

\[
P_{\text{CaMR}} = \frac{[\text{CaM}]}{K + [\text{CaM}]}
\]

(Hill-Langmuir equation)

where

\[
\begin{align*}
\text{CaM} + \text{R} &\rightleftharpoons \text{CaMR} \\
& \quad \quad k_{+1} \\
& \quad \quad k_{-1}
\end{align*}
\]

\(P_{\text{CaMR}}\) = Proportion of calmodulin receptor complex

\(\text{CaM}\) = Calmodulin

\([\text{CaM}]\) = Calmodulin concentration

\(\text{R}\) = Receptor

\(k_{+1}\) = Association rate constant

\(k_{-1}\) = Dissociation rate constant

\(K\) = Dissociation equilibrium constant

Applying the Hill-Langmuir equation to the concentration of active calmodulin used in our experiments and assuming that calmodulin has an affinity of 4 nM for the NMDA receptor C1 region, as determined by Ehlers et al. (1996)

\[
P_{\text{C1}} = \frac{12 \text{ nM}}{4 \text{ nM} + 12 \text{ nM}}
\]

\(P_{\text{C1}} = 0.75\)
Whereas occupancy of the C0 region, with an affinity of 87 nM for calmodulin (Ehlers et al., 1996), would be

\[ P_{C0} = \frac{12 \text{ nM}}{87 \text{ nM} + 12 \text{ nM}} \]
\[ P_{C0} = 0.121 \]

For 800 nM active calmodulin, occupancy of the C1 and C0 regions were estimated in the same manner.

\[ P_{C1} = \frac{800 \text{ nM}}{4 \text{ nM} + 800 \text{ nM}} \]
\[ P_{C1} = 0.995 \]

\[ P_{C0} = \frac{800 \text{ nM}}{87 \text{ nM} + 800 \text{ nM}} \]
\[ P_{C0} = 0.902 \]

It is also possible to work out percentage occupancy of ligand in the presence of a competing ligand (B)

\[ L + R \rightleftharpoons LR \]
\[ B + R \rightleftharpoons BR \]

L = Ligand
B = Competing ligand
LR = Ligand receptor complex
BR = Competing ligand receptor complex
We have

\[ [L] [R] = K_L \cdot [LR] \]
\[ [B] [R] = K_B \cdot [BR] \]

\[ [L] = \text{Concentration of ligand} \]
\[ [B] = \text{Concentration of competing ligand} \]
\[ K_L = \text{Ligand dissociation equilibrium constant} \]
\[ K_B = \text{Competing ligand dissociation equilibrium constant} \]
\[ [LR] = \text{Concentration of ligand receptor complex} \]
\[ [BR] = \text{Concentration of competing ligand receptor complex} \]

expressing in proportions

\[ [L] \cdot P_R = K_L \cdot P_{LR} \]
\[ [B] \cdot P_R = K_B \cdot P_{BR} \]

Again working on the assumption that

\[ P_R + P_{LR} + P_{BR} = 1 \]

Expressing in terms of \( P_{AR} \)

\[ \frac{K_L}{[L]} P_{LR} + \frac{P_{LR}}{K_B} + \frac{[B]}{[L]} P_{LR} = 1 \]

collecting terms and rearranging

\[ P_{LR} = \frac{[L]}{K_L (1 + [B]) + [L]} \]  \hspace{1cm} (5) \]

We can apply equation 5 to our antagonist studies with calmodulin and alpha-actinin, where alpha-actinin is the competing ligand and calmodulin is the ligand. Using alpha-actinin at a concentration of 2.5 \( \mu \text{M} \) and assuming an affinity for the C0 region of the
NR1 subunit of 48 nM (K₉) (Krupp et al., 1999), in the presence of alpha-actinin the occupancy of the C0 region by calmodulin will be:

\[
P_{\text{CO}} = \frac{800 \text{ nM}}{87 \text{ nM} \left(1 + 2500 \text{ nM}\right) + 800 \text{ nM}}
\]

\[
P_{\text{CO}} = 0.159
\]

Hence the occupancy of the C0 region by calmodulin is predicted to be reduced from 90 % to 16 % in the presence of 2.5 μM alpha-actinin.

Similarly we can calculate how much the occupancy of the C0 region by alpha-actinin would be affected by the presence of 800 nM active calmodulin by reversing the notation. Now calmodulin is the competing ligand and alpha-actinin is the ligand

\[
P_{\text{CO/alpha-actinin}} = \frac{2500 \text{ nM}}{48 \text{ nM} + 2500 \text{ nM}}
\]

\[
P_{\text{CO/alpha-actinin}} = 0.981
\]

In the presence of calmodulin

\[
P_{\text{CO/alpha-actinin}} = \frac{2500 \text{ nM}}{48 \text{ nM} \left(1 + 800 \text{ nM}\right) + 2500 \text{ nM}}
\]

\[
P_{\text{CI}} = 0.836
\]

Therefore occupancy of the C0 region by alpha-actinin would be far less affected by the presence of 800 nM active calmodulin, with occupancy of the C0 region being reduced by 14 % compared to 84 % for calmodulin in the presence of 2.5 μM alpha-actinin.
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“A scientist is a blind man in a dark room looking for a black cat that isn’t there.”

Sir Karl Popper