On The Role Of Arachidonic Acid

In Long-term Potentiation

by

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Abbreviations:

AA: Arachidonic acid
AACOCF₃: Arachidonyl trifluoromethyl ketone
ACPD: Aminocyclopentane (1S-3R)dicarboxylate
aCSF: Artificial cerebro-spinal fluid
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxaline propionic acid
AP5: Amino-phosphonopentanoic acid
BPB: Bromophenacyl bromide
BSA: Bovine serum albumin
C20:2: Eicosadienoic acid
C20:5: Eicosapentaenoic acid
[Ca²⁺]: Intracellular calcium ion concentration
CCD: Charge-coupled device
CNS: Central nervous system
DAG: Diacylglycerol
DAG-L: Diacylglycerol lipase
EPSC: Excitatory postsynaptic current
mini-EPSC: Spontaneous miniature excitatory postsynaptic current
EPSP: Excitatory postsynaptic potential
IP₃: Inositol trisphosphate
IPSC: Inhibitory postsynaptic current
IPSP: Inhibitory postsynaptic potential
LTP: Long-term potentiation
NMDA: N-methyl-D-aspartate
NO: Nitric oxide
NOS: Nitric oxide synthase
PC: Phosphatidylcholine
PC-PLC: Phosphatidylcholine specific phospholipase C
PLA₂: Phospholipase A₂
cPLA₂: Cytosolic Phospholipase A₂
sPLA₂: Secretory Phospholipase A₂
PMT: Photo-multiplier tube
uv: Ultra-violet light
VDCCs: Voltage dependent calcium channels
Abstract

Arachidonic acid (AA) is a putative retrograde messenger in hippocampal LTP. There are several enzymatic routes by which AA can be mobilised from membrane phospholipids: via secretory PLA$_2$ (sPLA$_2$), via cytoplasmic PLA$_2$, and via the sequential actions of PLC and DAG lipase. Antagonists of sPLA$_2$ have been shown to block the induction of LTP, but the role of AA mobilised via other routes has not been investigated.

In this thesis, I present data suggesting a role for each of these pathways in the induction and/or expression of LTP in area CA1 of the hippocampal slice. I have confirmed that perfusion with the sPLA$_2$ antagonist BPB blocks the induction but not the expression of LTP. Inhibition of either cPLA$_2$ by AACOCF$_3$, or of DAG-lipase by RHC80267, results in a blockade of both the induction and the expression of LTP. D609, an inhibitor of phosphatidyl-choline-specific-PLC, also blocks the expression of LTP. D609 injected into CA1 pyramidal cells blocked both the induction of LTP, and the expression of pre-existing LTP, suggesting that the source of AA required for both induction and expression of LTP is at least partly postsynaptic. The selectivity and efficacy of the drugs used were validated by measuring their effects on basal release of radiolabelled AA from hippocampal tissue.

As AA was found to be required for the induction of LTP, I have investigated possible interaction between AA and glutamate receptors in cultured hippocampal neurones. AA was found to enhance the $[Ca^{2+}]_i$ transient induced by iontophoretic application of NMDA, but not those induced by AMPA.

To act as a retrograde messenger, AA must be capable of enhancing the release of neurotransmitter. To assess this, I have recorded the frequency of
spontaneous mini-EPSCs and IPSCs in cultured hippocampal neurones. Mini-EPSCs show an increase in frequency following the application of AA, whereas the frequency of IPSCs is unchanged. The modal amplitude of mini-EPSCs is unchanged by AA.

I propose a two-phase model for LTP, in which the sPLA₂ pathway is utilised in the induction of LTP, possibly involving enhanced entry of calcium into the postsynaptic cell via the NMDA receptor. The maintenance of LTP requires the persistent mobilisation of AA from the cPLA₂ and DAG pathways; it is during this later phase that AA may act as a retrograde messenger, by enhancing excitatory neurotransmitter release.
Introduction

1.1: Basic principles of neuroscience

1.1.1: The neurone

Early studies of the brain suggested that the brain was formed from a reticulate network of fibres, and what we now know as neurones were believed to be nodes upon this network. This interpretation was challenged in the mid 1880s by His on the basis of studies of embryonic development, and Forel on the basis of the selectivity with which neurones atrophy. Independently, Ramon y Cajal had reached the same conclusions from intense microscopic examination of neural tissue stained with the Golgi technique (from Eccles 1964). At much the same time, Sherrington, arguing from the physiological properties of the reflex arc, argued that signal transmission required 'a surface of separation between neurone and neurone'. This, he christened 'the synapse' (Sherrington 1906)

1.2: The synapse

The synapse is the point at which the axon of one cell comes into contact with the dendrite (axo-dendritic), soma (axo-somatic), or axon (axo-axonic) of another cell. Some of the characteristic differences between synaptic transmission and nerve propagation, as set out by Sherrington (1906) are as follows: "Slower speed ... "less close correspondence between the moment of cessation of stimulus and appearance of end-effect... "less close correspondence between the grading of intensity of the stimulus and the grading of intensity of the end effect... "considerable
resistance to passage of a single nerve impulse, easily forced by a succession of impulses (temporal summation)... irreversibility of direction... fatigability...
susceptibility to anaesthetics.”

The mode of transmission is now known to be chemical (Dale and Feldberg 1934). The chemical neurotransmitter is stored in synaptic vesicles at the axon terminal. There are two classes of synaptic contact, based on their effects upon the postsynaptic membrane potential; excitatory, tending to lead to the firing of an action potential, and inhibitory, hyperpolarising the cell away from the firing threshold. On electrical stimulation the synaptic vesicles fuse with the terminal membrane on the presynaptic side of the synapse, and release their contents into the synaptic cleft. Diffusion across the narrow cleft is rapid, and when molecules of neurotransmitter reach the postsynaptic membrane, they bind to specific receptors. On binding an excitatory agonist, these receptors open, allowing Na⁺ ions to enter the postsynaptic compartment, causing depolarisation. A depolarisation of sufficient magnitude will lead to an action potential being generated in the postsynaptic neurone. Inhibitory agonists bind to different receptors, and make the membrane potential more negative, for example by allowing Cl⁻ ions to enter the cell, in the case of GABAₐ receptors.

The principles of synaptic transmission are similar in the neuromuscular junction, where they were first described (del Castillo and Katz 1954) and in the central nervous system (CNS). A major difference in the CNS, is that neurotransmission is thought to involve the release of only one vesicle at a time, per release site (Bekkers et al. 1990). As a result, synapses have a certain probability of releasing neurotransmitter (glutamate in the case of excitatory connections in the hippocampus) when stimulated by an action potential. This will be discussed in section 1.5.1. Although we understand the general principles of chemical
neurotransmission, the details contain a wealth of complexity which include the mechanisms of synaptic plasticity with which this study is concerned.

1.3 Glutamate receptors

As the properties of glutamate receptors have considerable significance for the mechanisms of LTP I shall briefly review their general properties and distribution within the hippocampus. Broadly, there are two classes of receptor; those which are ligand gated ion channels (ionotropic receptors) and those which are linked to second messenger systems (metabotropic receptors). The ionotropic glutamate receptors are divided on the basis of their sensitivity to various agonists, into NMDA and non-NMDA (or AMPA-kainate) receptors (Hammond 1996). These two groups have very different properties; NMDA receptors are Ca\(^{2+}\) permeant, and show a strong membrane-potential dependence to their activation, which is due to a voltage dependent block of the channel by Mg\(^{2+}\) ions. AMPA-kainate receptors are relatively impermeable to Ca\(^{2+}\), and show no such voltage dependence in the presence of external Mg\(^{2+}\) ions. Both NMDA and non NMDA ionotropic receptors are present in the rat hippocampus (Collingridge et al 1983).

Metabotropic glutamate receptors (mGluRs) are a younger class, the first demonstration of their existence being in 1987 (Sugiyama et al 1987). They are now classified on the basis of sequence homology and second messenger coupling into three groups (Pin and Duvoisin 1995). Group I receptors are coupled to increased phosphoinositide turnover, and include type 1 and 5 mGluRs. Groups II and III negatively couple with adenylyl cyclase; group II consists of mGluRs 2 and 3, and group III consists of mGluRs 4a & 4b, 6, 7 and 8. Full characterisation of these receptors is incomplete, due to a scarcity of specific pharmacological agonists and
antagonists, and additional complexities introduced by various splice-variants. In some cases, *in situ*-hybridization and immunolocalization have been used to examine the localisation of these receptors is the brain. Of the Group I mGluRs, type 1 shows strong staining in the pyramidal layer of areas CA2-4 of the hippocampus, with somewhat weaker staining in neurones of area CA1 (Shigemoto *et al* 1992). A later study by Kerner *et al* (1997) indicates that both mGluR 1 and 5 are present in identified neurones within the hippocampus, and a study using anti-sense RNA has also indicated the presence of mGluR 5 in area CA1 (Dorri *et al* 1997). Type 2 of mGluR group II, is expressed very weakly in the hippocampus (Ohishi *et al* 1993). Of the group III mGluRs, type 4a is highly expressed throughout the hippocampus, whereas mGluR 7 is only weakly expressed, and then primarily in *stratum radiatum* (Bradley *et al* 1996). (1S-3R) ACPD, an agonist used in this study, is a potent activator of group I mGluRs, and, rather more weakly, will also stimulate group II.

1.2: Structures within the brain have specific roles

In order to identify roles for structures within the body, the simplest method is to identify examples of regional damage, and find out what deficits in function are associated with that particular form of damage. In neuroscience, these localised injuries have generally been due to strokes, industrial accidents, and warfare. In the mid-nineteenth century, a French physician, Dax, published a little noticed paper on the correlation he had observed in his patients between strokes afflicting the right side of the body, and speech deficits. A few years later, Broca presented data from autopsies on a number of speech-afflicted stroke victims, showing again that speech deficits were again associated with damage to the left hemisphere of the brain (from
Springer and Deutch 1989). At much the same time, Harlow published a paper describing the curious case of one Phineas Gage. Gage was a railway engineer, who was involved in an accident which resulted in an iron bar being driven upwards through his head. Although he survived for another twelve and a half years, his behaviour and character had changed dramatically; he became socially disinhibited and swore vociferously. In the words of his acquaintances (as reported by Harlow) it was as if he were ‘no longer Gage’ (from Nathan 1988). On his death it was found that most of his frontal lobes had been destroyed by the passage of the bar. These two sets of data finally destroyed the pre-existing dogma that the brain functioned as an indivisible unit. The demonstration that individual regions of the brain had specific functions opened the way for modern neuroanatomy.

1.2.2: The hippocampus is involved in learning

In the study of the mechanisms of learning and memory one particular region of the brain has received the most attention; the hippocampus. The most striking demonstration of the crucial role this part of the brain plays in memory was provided by the unfortunate case of patient H. M. After undergoing bilateral removal of the hippocampus, a treatment intended to treat his epilepsy, he lost the ability to remember recent events, and was also unable to form new memories. His other cognitive powers however, were unaffected (Scoville and Milner 1957). In practice this means that he can practice a skill, such as a playing table tennis, and improve at it without being aware that he has ever played it before. This introduces the important distinction between procedural, and declarative memory. Essentially this is the difference between knowing how to do something, and knowing that something is the case, a distinction initially proposed by the philosopher Gilbert Ryle (1949). Patient
H. M.'s case has demonstrated its structural importance in terms of learning and remembering. A more recent case is that of R. B., who suffered memory impairment following global ischaemia; post-mortem analysis revealed a bilateral lesion of the CA1 sub field of the hippocampus (Zola-Morgan et al. 1986). Once again the hippocampus had been implicated in memory. Subsequent experiments have been carried out, particularly in rats, in order to assess the role of the hippocampal formation in learning. To do this it was necessary to develop a learning task where procedural and declarative memories can be dissociated. Spatial memory has become the favoured subject, as this appears to be wholly declarative. Rats lacking the hippocampus are deficient in spatial memory. A popular test for spatial learning deficiencies is the Morris water maze (Morris et al. 1986), which consists of a tank filled with water clouded by milk powder. Hidden beneath the surface is a platform which enables the rat to find its way out of the water. Repeated trials enable the rat to find the platform by its location relative to various spatial clues which are hung around the bath. How good the rat is at learning this task, is assessed on the basis of how long it takes for the animal to find its way to the platform (if the animal is learning, then this should get faster with repeated trials). A second measure is gained by removing the platform, and then observing the swimming pattern of the rat. If the rat has learnt to associate a particular region with the platform, it will spend longer swimming around in that sector, than it will in other areas of the bath.

1.3: Neuronal correlates

The hippocampus then, is a brain structure with a clear role in the formation and retrieval of declarative memory. Are there any neuronal correlates to this?
1.3.1: ‘Place’ cells

One of the more suggestive experimental findings to have linked the electrophysiology of the hippocampus with spatial learning, is the discovery of ‘place cells.’ O'Keefe and Dostrovsky (1971) recorded activity in the rat hippocampus using a double electrode. They found that as the animal explored its environment, cells would become tuned to specific regions within that environment. These cells would remain fixed to those regions until either the animal was placed in a new environment, or the old one was changed so radically that the rat behaved as though it was in a new environment (rapid exploration). Later work with more sophisticated multi-electrode recording techniques has refined the characteristics of these cells further. McNaughton et al. (1983) have demonstrated that these cells can show selectivity for direction, and speed of movement causes a change in the frequency of unit firing. From these and other findings, (O'Keefe, 1976; O'Keefe and Nadel, 1978; Wilson and McNaughton, 1993), it has been proposed that the rat establishes dynamic maps of its surroundings. Further support for a link between place cells and spatial learning comes from recent data using region specific gene knockout technology. McHugh et al. (1996) have examined place cell activity in a mouse where the NR1 subunit of the NMDA-receptor (see section 1.4.3) has been selectively deleted. These mice show a deficit in spatial learning (Tsien et al. 1996), and although they possess some place cell activities, these show less directional and spatial specificity than do their wild-type litter mates.
1.3.2: Synaptic plasticity in the hippocampus

One of the reasons that the hippocampus has been so extensively studied is that it has well defined synaptic circuitry which can be thought of as a simple, excitatory, tri-synaptic loop. Projections from the entorhinal cortex make synapses to the granule cells of the dentate gyrus. These project mossy fibres to the giant pyramidal cells of area CA3, which in turn make projections to the pyramidal cells of area CA1 (see figure 1.1). Using extracellular recording and stimulating techniques it is possible to stimulate known afferent fibres and record the resultant responses from populations of known synapses and cell bodies. It was using this approach that Bliss and Lomo discovered the phenomenon now known as long-term potentiation (LTP) (Bliss and Lømo 1973). Stimulating the perforant path and recording in the dentate gyrus in anaesthetised rabbit, they found that if they applied a high frequency train of stimuli to the pathway, then subsequent shocks to that pathway would give rise to an enhanced response in the postsynaptic cell population. This enhancement, or potentiation, was found to be very long lasting, persisting for hours in the anaesthetised animal (Bliss and Lømo 1973) or even weeks in the intact animal (Bliss and Gardner-Medwin 1973).

1.4: Long-term potentiation

1.4.1: Characteristics of LTP

With the techniques described, the characteristics of LTP which make it such a promising model of the synaptic basis of learning and memory have been formalised (Bliss and Collingridge 1993). LTP cannot necessarily be induced by repetitive stimulation of a weak input, there is a threshold of activation which must be
**Figure 1.1:** A diagram of a transverse-section through the hippocampus. The fundamental trisynaptic loop is evident. Areas CA1 and CA3 are as marked, DG refers to Dentate Gyrus. Approximate positions of stimulating electrodes for two pathway recording in area CA1 are shown.
overcome by activation of multiple inputs; this is the criterion of *cooperativity* (McNaughton *et al.* 1978). Secondly, for weak inputs to become potentiated, their activation must be paired with strong inputs; this is the criterion of *associativity* (McNaughton *et al.* 1978; Levy and Steward 1979). Thirdly, LTP can be induced by strong stimulation of one pathway, but other pathways which are not active whilst this conditioning occurs, do not show LTP. This is the criterion of *input specificity* (Lynch *et al.* 1977). LTP is a synapse-specific potentiation.

1.4.2: Postsynaptic induction

Experiments have shown that postsynaptically loaded EGTA, a calcium chelator, prevents the induction of LTP (Lynch *et al.* 1983). This requirement for postsynaptic calcium has been found to be mediated by postsynaptic N-methyl D-aspartate receptors (NMDA-R).

The characteristics of LTP induction have been well explained by the involvement of the NMDA receptor. Experimentally this involvement was established by the observation that 2-amino-5-phosphonovalerate (AP5), an antagonist at the NMDA sensitive glutamate receptor, blocks the induction of LTP (Collingridge *et al.* 1983). The NMDA receptor is blocked by magnesium ions at normal resting membrane potentials. When the cell is depolarised, however, this blockade is lifted, allowing the channel to open in response to glutamate and to admit calcium ions. In order for the NMDA receptor to respond to synaptic transmission the cell must then be depolarised; this can happen if there are multiple steps of glutamate release, so that as the cell is depolarised from the first load of glutamate, a second wave arrives to find the NMDA receptor relieved of its magnesium block and able to open. The phenomena of cooperativity, associativity and selectivity can be explained by this
notion of a voltage dependent receptor which triggers a specific postsynaptic response, and acts as a detector of near simultaneous pre- and postsynaptic activity.

Cooperativity, the requirement for sufficient activation in order to induce LTP, and associativity, the fact that activation of multiple independent inputs can perform this function, are now thought to be due to the depolarisation necessary to relieve the magnesium blockade of the NMDA-R. A unitary synaptic event does not seem to depolarise the postsynaptic site sufficiently to relieve the magnesium block. In order to do this, the region must be subject to greater depolarisation. This can be artificial, as is the case in certain LTP induction paradigms where the postsynaptic cell is clamped at a heavily depolarised potential (for example +10mV (Bolshakov and Siegelbaum 1995)), or ‘natural’. In the latter case this can be through a few afferent fibres stimulated at a high frequency - depolarisation can build if each new stimulus arrives before the previous one has had time to decay, and in this case, only the pathway which received the high frequency stimulation will be potentiated. Alternatively, sufficient depolarisation can come through relatively low frequency stimulation of one pathway, temporally coupled with a high frequency train in a second pathway, so that the depolarisation evoked by each overlaps and heightens the overall depolarisation. In this case both pathways undergo potentiation, despite the fact that only one received a high frequency train.

Synapse specificity, one of the key characteristics for LTP as a model for learning processes, also appears to be based upon the properties of the NMDA-R. Only those inputs active at the time of LTP induction participate in potentiation. This is because the NMDA-R, in the presence of physiological concentrations of magnesium, operates as a Hebbian coincidence detector, because recent (or convergent) activity, in the form of depolarisation, is necessary in order to unmask the
NMDEA response. Since it is the unmasking of the NMDA response, and consequent entry of calcium into the postsynaptic compartment which induces LTP, potentiation is dependent on near simultaneous activity in the pre- and postsynaptic cell. Synapses where the presynaptic side is silent at this time (most of them) do not meet this requirement for simultaneous activity, and so do not participate in LTP.

The NMDA receptor is thus central to our understanding of LTP, at least in the synapses linking the entorhinal cortex to the dentate gyrus, and the synapses linking the CA3 region to the CA1 region. The mossy fibre inputs to area CA3 exhibit a different form of LTP which is NMDA receptor independent and has very different mechanisms underlying its plasticity.

1.4.3: LTP and learning

As yet there has been neither conclusive proof nor disproof of the hypothesis that LTP, or some process very much like it, underlies the acquisition of information through the hippocampus. (Morris et al. 1986) have shown that perfusion of the NMDA-R antagonist AP5 into the hippocampus impairs the ability of rats to learn a water-maze task. Doubt was shed on this conclusion by the recent report that in thy1+ mice, no LTP was seen in the dentate gyrus in vivo (Nosten-Bertrand et al. 1996); these mice showed no learning impairment. This suggested that LTP in the Dentate Gyrus might not be necessary for learning, but since it was a regional specific deficit, CA1 LTP was unaffected, and could perhaps compensate. This has further been weakened by the report that in recordings made from freely moving Thy1+ mice, LTP is only slightly impaired (Errington et al. 1997).

Perhaps the strongest correlation between LTP and spatial learning comes from the series of experiments, already discussed, examining a CA1 specific
knockout of the NMDA NR1 subunit. This abolishes NMDA mediated neurotransmission in CA1, and hence LTP, but does not cause any other detectable abnormalities in synaptic function (Tsien et al. 1996). These mice show deficits in behavioural learning, and abnormal place cell function (McHugh et al. 1996).

Interpretation of perfusion of AP5 and other NMDA antagonists is complicated by the important role NMDA-R mediated transmission plays in the neocortex. No such criticism can be levelled at a cell type specific knockout. Thus, the data is in favour of the hypothesis that LTP, or something much like it, underlies the acquisition of spatial information in the rat and mouse.

1.5: Mechanisms of LTP

Although the importance of the NMDA receptor is widely accepted, the later steps in the signalling cascade which leads to LTP are poorly understood. An important distinction to make is that between the induction of LTP, which occurs rapidly at the time of the high frequency train, and the subsequent expression of potentiation. The expression of LTP is the observed increase in the magnitude of the excitatory postsynaptic potential (EPSP), which might be maintained by one or more of several different proposed mechanisms, the roles of which are unclear at present.

1.5.1: Presynaptic or postsynaptic?

As LTP is a synaptic phenomenon, factors acting either pre- or postsynaptically are potential mediators of this synaptic plasticity. On the presynaptic side, EPSPs can be enhanced by release of more neurotransmitter. This can come
about in several different ways, involving quantal size, and probability of release, - all models derived from Katz's original work on the neuromuscular junction (del Castillo and Katz, 1954). Quantal size could change, so that every synaptic vesicle would release more glutamate into the synaptic cleft. Alternatively, presynaptic terminals could show multi-quantal release, so where one vesicle was released before the conditioning stimulus, two might be released after, once again increasing the postsynaptic response. For either of these to be relevant, the postsynaptic receptors must not be saturated by normal (unpotentiated) synaptic transmission. At present this is not yet resolved (Bliss and Collingridge 1993; Bekkers 1994). Generally receptor saturation has been assessed through quantal analysis. This technique strives to detect the release of individual quanta (measured postsynaptically) evoked by minimal stimulation (intended to evoked responses in only a few fibres). The amplitudes of the responses to these are plotted as a frequency histogram. If the responses are quantal, then the histograms will show a 'peaky' distribution. This can then be interpreted on the basis of the width of the peaks, which is an indicator of the quantal variability. A major factor in this variability is interpreted to be differential activation of postsynaptic receptors. If such an amplitude histogram shows very tight clean peaks, it is interpreted as indicating postsynaptic receptor saturation. A similar form of analysis can be used on spontaneous mini-EPSCs (thought to be responses generated by the random release of individual quanta). When such quantal-analyses have been performed, (Edwards et al. 1990; Larkman et al. 1991; Foster and McNaughton 1991; Larkman et al. 1992), they have supported a model of synaptic function with receptor saturation, as have studies on mini-EPSCs (Tang et al. 1994). However, these studies are somewhat indirect, being somatic measurements of an electrically distant phenomenon, and so more direct evidence is still needed. An alternative technique,
involving modelling the time course of neurotransmitter in the synaptic cleft, has suggested that NMDA-Rs are saturated, whereas AMPA-Rs are not. (Clements et al. 1992) More direct techniques are now being developed, involving imaging (Liu and Tsien 1995; Emptage et al. 1997) and directly recording from synapses (Forti et al. 1997). These throw doubt on the interpretation of the quantal-analysis studies, as they show considerable quantal variance and suggest that postsynaptic receptors are not saturated.

The favoured theory on how presynaptic release might vary in synaptic plasticity, is a change in release probability. Bolshakov and Siegelbaum (1995) working in slices, and Murthy et al (1997) in cultured cells have recently shown that the majority of synapses on hippocampal neurones have a low (<0.5) probability of release. The induction of LTP might be sufficient to convert this to a higher probability. In cases where multiple sites are activated by neurotransmission (such as stimulation of afferent bundles, as occurs in conventional extracellular recording) this will be seen as an increase in the evoked EPSP, since this is the sum of many unitary responses.

Two main postsynaptic models have been proposed as mechanisms for LTP. The size of the measured postsynaptic response could be increased by changes in the postsynaptic receptors. This could involve a change in affinity for glutamate, a change in the probability of channel opening when ligand is bound, or a change in the channel conductance. The second possibility is that the synapse itself might change in some way. One suggestion has been the conversion of 'silent' NMDA only synapses to conventional synapses containing both NMDA and AMPA receptors. In this case, before the induction of LTP there are only a few synapses actively participating in neurotransmission, while after, there are many (Edwards, 1991; Isaac et al. 1995). A
more dramatic extension of this, is the suggestion that substantial morphological change may occur following LTP, such as bifurcation of dendritic spines so that where before there was one, stimulation can cause a second to emerge (Geinisman et al. 1991). Morphological change on this sort of scale should be properly thought of as 'trans-synaptic' rather than merely postsynaptic, since the presynaptic terminal must undergo a similar process in parallel. Such a process is likely to be slow, however, and to involve protein synthesis. The protein synthesis-dependent component of LTP (termed 'late-LTP') is believed to be the period from about three hours following the induction of potentiation (Frey et al. 1988). Since the work described in this thesis is concerned only with the first 1-3 hours following potentiation, it seems unlikely that large scale morphological changes need be considered.

1.5.3: The expression of LTP - postsynaptic mechanisms

A postsynaptic locus for the induction of LTP is attractive because it has the virtue of simplicity. The NMDA-R ensures synapse specificity, and the rise in [Ca^{2+}], believed to be the crucial first step in the induction of LTP occurs postsynaptically. Why then, need one look to the presynaptic terminal for the expression of LTP?

As has already been discussed, quantal analysis is a popular means of analysing pre- and postsynaptic components of synaptic transmission. When applied to LTP, this has again led to inconsistent results. Many studies have suggested a presynaptic locus (Bekkers and Stevens 1990; Malinow and Tsien 1990; Stevens and Wang 1994), but other studies have shown either a postsynaptic locus (Foster and McNaughton 1991) or a mixture of apparently pre- and postsynaptic mechanisms ((Larkman et al. 1992; Kullmann and Nicoll 1992; Oliet et al. 1996).
The simplest model for a postsynaptic mechanism is neurotransmitter receptor modification. Early evidence for this came from studies showing increased responsiveness to glutamatergic agonists following the induction of LTP. (Davies et al. 1989). Since elevated postsynaptic kinase activities are required for LTP (Malenka et al. 1989; Wang and Feng 1992; O'Dell et al. 1991), the glutamate receptors would be likely candidates for phosphorylation. Recently, there has been a report from Barria et al. (1997) that the AMPA receptor is phosphorylated following LTP. The authors also show that currents through recombinant AMPA receptors, expressed in *Xenopus* oocytes, are enhanced when the receptors are phosphorylated on this site.

Another model for the postsynaptic expression of LTP is the so-called 'silent synapse' model. This is a favoured mechanism by which analyses showing apparent increases in the probability of release (a presynaptic component) can be reconciled with a wholly postsynaptic interpretation. A 'silent' synapse is one where only NMDA-Rs are expressed, and as a result does not participate in normal neurotransmission. When the postsynaptic cell becomes depolarised however, (as in the induction of LTP), the NMDA-R can allow calcium to enter the cell in a normal manner. This LTP is then expressed by the insertion of AMPA-Rs into the postsynaptic membrane. Kullman (1994) has gained indirect evidence supporting this from comparison between NMDA and AMPA EPSCs, which suggested that many more synapses contain NMDA-Rs than AMPA receptors, and that this discrepancy was reduced following the induction of LTP. Liao et al. (1995), and Isaac et al. (1995) have demonstrated the existence of NMDA-R only synapses, and shown that an LTP-like phenomenon can be induced in these synapses, which is expressed by the appearance of functional AMPA receptors. For this to be a dominant mechanism for
the expression of LTP, it is necessary that a very large proportion of synapses be silent. Durand et al. (1996) has recently reported that in preparations obtained from very young animals, synapses are primarily NMDA only ('silent') and that these can be induced to form functional synapses by an LTP-like pairing paradigm. By the age of approximately fifteen days postnatal, however, functional, AMPA-incorporating synapses are dominant.

1.5.4: The expression of LTP - retrograde messengers

Despite the postsynaptic locus of the induction of LTP, there are many reports suggesting that the maintenance of LTP is, associated with increased glutamate release (initially Dolphin et al. 1982, later studies include; Bliss et al. 1986; Bliss et al. 1990; Galley et al. 1993). Other evidence includes quantal analysis (Bekkers and Stevens 1990; Malinow and Tsien 1990; Stevens and Wang 1994; Larkman et al. 1992; Kullmann and Nicoll 1992; Oliet, Malenka et al. 1996; Malinow and Tsien 1990), paired recordings eliciting single synapse responses (Bolshakov and Siegelbaum 1995), and combined imaging and recording (Malgaroli and Tsien 1992; Malgaroli et al. 1995). However, it is not immediately clear how blockade of a postsynaptic element (the NMDA receptor) can prevent the presynaptic expression of LTP. In order to reconcile these points it has been proposed that there might be some retrograde messenger, produced in the postsynaptic cell but able to diffuse across the synapse to directly or indirectly facilitate release of neurotransmitter (Bliss et al. 1986). Such a molecule would have to be small, so capable of rapid diffusion, and be soluble in both lipid and aqueous phase.
1.6: Retrograde messengers.

The two most prominent candidates for a role as a retrograde messenger at hippocampal synapses are arachidonic acid (AA) and Nitric Oxide (NO).

1.6.1: Nitric oxide

Nitric oxide, (NO) is a candidate retrograde messenger which has received a great deal of attention in recent years. It is produced in the hippocampus in response to NMDA-R stimulation (East and Garthwaite 1991), by the enzyme nitric-oxide-synthase (NOS). Inhibition of this enzyme has been shown to prevent the induction of LTP (Bohme et al. 1991; O'Dell et al. 1991; Schuman and Madison 1991; Haley et al 1992; Arrancio et al 1996). Additional experiments have demonstrated that if preparations are perfused with compounds capable of sequestering NO, such as Haemoglobin (O'Dell et al. 1991; Schuman and Madison 1991; Haley et al 1992) or Oxy-myoglobin (Arrancio et al 1996), the induction of LTP is prevented, suggesting that the extracellular diffusion of NO is a necessary part of the induction of LTP. Further supporting a role for NO in LTP, are experiments where exogenous NO has been applied, either directly bubbled into solution (O'Dell et al. 1991), applied via NO donors (Bon et al 1992; Zhou et al 1993) or via caged NO (Arrancio et al 1996), resulting in a form of potentiation.

The interpretation of these experiments has become more controversial however, after a number of other reports. The blockade of LTP by NOS inhibitors has not always been replicated (Kato and Zorumsky 1993; Cummings et al 1994; Kirkwood and Bear 1994) and in one case has been found to be dependent on both the age of the animals, and the temperature at which the experiments were carried out.
(Williams et al. 1993); in mature animals, and at temperatures above ambient, NOS inhibitors do not block the induction of LTP. Inhibition of NOS also has no effect on LTP in vivo (Bannerman et al. 1994a) or spatial learning (Bannerman et al. 1994b). A further indication of the inconsistency of the evidence for the involvement of NOS in LTP are experiments where the genes expressing two major forms of NOS have been knocked out. Knockout of either eNOS or nNOS alone (endothelial and neuronal forms of the enzyme respectively) does not effect LTP (Son et al 1996 and O’Dell et al 1994), and while a double knockout reduces the magnitude of LTP, it does not abolish it completely (Son et al 1996)

The plausibility of NO as a retrograde messenger has been reduced further by the observation that it can actually block the induction of LTP, either when stimulated by activation of NMDA-Rs prior to the induction of LTP (Izumi et al. 1992), or when photolytically released (Murphy et al. 1994). These results can be explained by an interaction between NO and the NMDA-R (Lipton and Kater 1989). For the reasons outlined above, although NO must remain a candidate for some sort of role in LTP, it is not unreasonable to examine other potential candidates for the role of retrograde messenger.

1.6.2: Arachidonic acid

Arachidonic acid is a fatty acid which is involved in many processes within the body. It is derived from the essential fatty acid linolenate, and in common with other polyunsaturated fatty acids, plays an important role in the regulation of membrane fluidity. Many of the metabolites of arachidonic acid also play important roles in a number of processes, the best characterised being those associated with inflammation, platelet aggregation, and the immune system (Stryer 1988, Rang et al.
1995). Biologically important pathways for the metabolism of AA include the lipoxygenase pathways (producing leukotrienes), cyclo-oxygenase pathways (a target of aspirin, and source of both prostaglandins and thromboxanes). Another recently described role for AA is as precursor for Anandamide, and endogenous ligand for the cannabinoid receptor (Devane et al., 1992).

In 1987 it was demonstrated that lipoxygenase metabolites of arachidonic acid presynaptically enhance neurotransmitter release in Aplysia (Piomelli et al. 1987). As arachidonic acid is a relatively small molecule capable of diffusing through aqueous and membrane fractions, such as those which separate the two sides of the synapse (see figure 1.2 for the structure of AA, and two related fatty acids), it was suggested that arachidonic acid might be capable of acting as a retrograde messenger at hippocampal synapses. Experiments using inhibitors of phospholipase A$_2$ (PLA$_2$) (Williams and Bliss 1988; Lynch et al. 1989; Massicotte et al. 1990) have been consistent with this, blocking the induction but not the expression of LTP. Further reports (Lynch et al. 1989; Bliss et al. 1990; Lynch and Voss 1994) have demonstrated a prolonged increase in levels of free arachidonic acid following the induction of LTP. Additionally, Williams et al (1989) have demonstrated that perfusion of arachidonic acid, when coupled with a subthreshold tetanic protocol, induces a slow onset potentiation of evoked EPSPs. Biochemical support for a role for AA as a retrograde messenger includes reports that co-application of (1S,3R) ACPD and arachidonate result in increased release of glutamate from synaptosomes (Lynch and Voss 1990; Herrero et al. 1992; Lynch and Voss 1994). In hippocampal slice, coapplication of arachidonic acid and (1S 3R) ACPD leads to an LTP-like potentiation. (Collins et al. 1995).
Figure 1.2: The structure of arachidonic acid, and that of two closely related lipids (used as controls in this study), differing only in the number of carbon-carbon double bonds, eicosadienoic acid (C20:2) and eicosapentaenoic acid (C20:5).
Arachidonic Acid
C20:4

Eicosapentaenoic Acid
C20:5

Eicosadienoic Acid
C20:2
1.6.3: Unanswered questions for AA as a retrograde messenger

In the light of these results it is puzzling that blockade of PLA₂ blocks only the induction of LTP. Why should levels of arachidonic acid remain elevated after LTP if it is required only for a brief period during the induction of LTP? Release of arachidonic acid in a short burst will not be sufficient to result in a sustained elevation of AA levels, because AA will be subject to diffusion away from the synapse, and the normal processes of fatty acid turnover. A prolonged change in fatty acid metabolism, either enhanced mobilisation or reduced levels of metabolism must be postulated to explain the observed increase in AA levels. This prolonged change in AA metabolism must be through a route which is insensitive to those inhibitors which have been previously employed.

The discrepancy can be resolved by the proposal that enzymes other than PLA₂ are responsible for the mobilisation of arachidonic acid following the induction of LTP. Figure 1.3 shows the pathways for the mobilisation of arachidonic acid from a typical phospholipid. This can be a direct process, through PLA₂ activities, or more indirectly via diacylglycerol (DAG). The sequential action of phospholipase D and phosphatidic phosphohydrolase, or phospholipase C, gives rise to DAG. DAG is then deacylated by the sequential action of DAG lipase and monoacylglycerol lipase to give glycerol and free fatty acid. The situation is complicated by the existence of different forms of many of these enzymes. Previous experiments have been concerned with the small secretory PLA₂ (sPLA₂), as this was the only type of PLA₂ known to exist in brain. Recently a novel, high molecular weight PLA₂ was cloned (Clark et al. 1991), and this has been designated the cytosolic phospholipase A₂ (cPLA₂). The cPLA₂ enzyme possesses no structural or sequence homology with sPLA₂ and demonstrates a substrate preference for phospholipids containing arachidonic acid at
Figure 1.3: Scheme for the mobilisation of AA from phosphatidylcholine (PC), a common membrane phospholipid. sPLA₂ and cPLA₂ will each liberate AA directly from its favoured position on carbon 2 of the glycerol backbone (sn-2 position). Alternatively, PC-specific phospholipase C (PC-PLC) can remove the choline headgroup from PC, to give rise to diacylglycerol (DAG). This in turn can be sequentially hydrolysed by DAG-lipase and monoacylglycerol lipase (MAG-lipase) to release AA. In this route, DAG-lipase is the rate-limiting step.
the sn-2 position (Hanel et al. 1993). This cPLA₂ can be selectively inhibited by the trifluoromethyl ketone of arachidonic acid (AACOCF₃) (Street et al. 1993).

Another route for the mobilisation of arachidonic acid from phospholipids is through the sequential action of diacylglycerol (DAG) lipase and monoacylglycerol lipase, which releases arachidonic acid from diacylglycerol, a well characterised second messenger. Diacylglycerol can be generated from many phospholipids, either as part of phosphatidylinositol metabolism, when it is released together with inositol trisphosphate (IP₃), and from phosphatidylcholine via a specific phospholipase C (PC-PLC). DAG lipase can be inhibited by the compound RHC80267 (Sutherland and Amin 1982), and PC-PLC is inhibited by the compound D609 (Schutze et al. 1992).

1.7: This study.

Here I present data which suggests a biphasic role for arachidonic acid in LTP. I have confirmed the observation that bromophenacyl bromide (BPB), an inhibitor of sPLA₂, blocks the induction of LTP without affecting pre-established LTP (Massicotte, Oliver et al. 1990). I also show that inhibition of any of DAG lipase, PC-PLC or cPLA₂ blocks both the induction and the expression of LTP. Additionally, I have demonstrated that DAG lipase and cPLA₂ operate through complementary pathways; the effects of the two inhibitors on the expression of LTP are additive. In LTP it appears that three pathways of arachidonic acid mobilisation are triggered. The sPLA₂ is activated only transiently during the earliest phase of LTP, whereas the activity of cPLA₂ and PC-PLC/DAG lipase is necessary for the continued expression of LTP. By injecting an inhibitor of PC-PLC directly into the postsynaptic cell, I have shown that PC-PLC activity is also required specifically in the postsynaptic cell.
To further characterise possible mechanisms by which AA may influence LTP, I have also examined its possible pre- and postsynaptic actions. AA alone can induce a small, transient elevation in $[\text{Ca}^{2+}]_i$, and can markedly enhance $[\text{Ca}^{2+}]_i$ transients induced by NMDA. AA also causes an increase in the frequency of spontaneous miniature EPSCs, suggesting that it is able to facilitate neurotransmitter release at excitatory synapses between hippocampal cells. The data are consistent with a role for arachidonic acid as a facilitator of LTP and as a retrograde messenger over a prolonged time period.
Methods

2.1: Electrophysiology

2.1.1: The in vitro slice preparation

The in vitro slice preparation, originally described by (Li and McIlwain 1957) and refined for the hippocampus by (Skrede and Westgaard 1971), provides a convenient model for studying the electrical properties of neurones in an environment not dissimilar to their native one (Richards 1981). In essence, the technique involves cutting thin (200-400μm) slices along a known orientation, from the brain structure of interest, and then maintaining the slice in a chamber where it is supplied with oxygen, and bathed in a solution with similar chemical composition to cerebral spinal fluid (CSF), known as artificial CSF or aCSF. The key advantages of the technique lie in the accessibility of cells, and the ease of manipulation of their external environment (aCSF composition). Because the slices are cut through a defined plane, much of the local circuitry of laminated structures such as the hippocampus remains intact. Only those connections nearly perpendicular to the plane of section are lost.

The hippocampus is a brain structure well suited to the slice technique, since it possesses a well ordered structure, easily seen when cut in the transverse plane (see fig 1.1). The connections within the hippocampus can be simplistically defined as a loop; projections from the entorhinal cortex synapse onto the granule cells of the Dentate Gyrus, which in turn connect to the pyramidal cells of area CA3. These project to the contralateral hippocampus part of the commissural fibres, and also to the CA1 pyramidal cells of the ipsilateral hippocampus (the Schaffer collateral...
fibres). CA1 neurones send axons to both the subiculum and back to the entorhinal cortex.

There are broadly two types of chamber for maintaining brain slices in a healthy state, the interface chamber, and the submerged chamber. The interface chamber maintains the slice at the interface between oxygenated aCSF flowing at a low rate (about 100μl/min), and an atmosphere saturated with moist O₂ gas. The submerged chamber, on the other hand, maintains the slice beneath the surface of oxygenated aCSF, which is then passed through at a much higher rate (2-3 ml/min) to maintain oxygen tension in the tissue. The two designs have different strengths and weaknesses. Submerged chambers are felt by many to keep slices in good health for longer time periods (>6 hours), but the potentials recorded are inevitably reduced by the shunting of the signal current into the surrounding fluid. Also, much larger volumes of fluid are used, and this means that drug costs can become very expensive. Interface chambers can maintain slices in good health for up to about 6 hours (possibly longer), give larger potentials, and use much less in the way of aCSF and so reduce the cost of pharmacological studies. A schematic of the chamber design used in the experiments performed in this study is shown in fig 2.1.

Transverse hippocampal slices were prepared from young male Sprague-Dawley rats (5-7 weeks old, 70-140g in weight). Animals were killed by stunning followed by cervical dislocation and decapitation. The brain was rapidly removed and the hippocampus dissected out and placed in cold (0-4°C) oxygenated (95% O₂, 5% CO₂) artificial cerebrospinal fluid (aCSF) of the following composition (in mM): NaCl 120, KCl 3, MgSO₄ 2, CaCl₂ 2, NaH₂PO₄ 1.2, NaHCO₃ 23, glucose 11.
Figure 2.1: Diagram of an interface-type slice chamber, as used in this study. Warmed, oxygenated aCSF is perfused onto an insert of lens-tissue, upon which a brain slice sits. This is partially enclosed in an atmosphere of warmed, moist oxygen/carbon dioxide (95/5%).
An interface slice chamber

- Vent to allow moist atmosphere to reach slice
- Thermometer Controller - maintains water bath at 30 degrees
- Temperature probe
- Heat exchanger to warm ACSF before it reaches the slice
- Slice sits on lens tissue perfused with ACSF
- Prism to direct light up through slice
- ACSF outflow
- Fibre optic from cold light source
- Oxygenated ACSF
- 95% O₂, 5% CO₂
- Sintered glass bubbler
- Heated water bath to maintain warm, damp, oxygenated atmosphere.
- Heater for water bath
Transverse slices (nominal thickness of 420 μm) were cut with a McIlwain tissue chopper, the CA3 sub field was excised, and the resulting mini slice transferred directly to an interface-type recording chamber (P.E. Scientific System Design, U. K.) maintained at 30 ± 0.3°C and perfused with oxygenated aCSF at a flow rate of 100 μl/min or to a holding chamber containing oxygenated aCSF at room temperature (22-24°C).

2.1.2: Field potential recording

Field potential recording relies on extracellular electrodes for the stimulation of bundles of afferent fibres, and additional recording electrodes, also extracellular, for the detection of the voltage changes caused by large numbers of cells reacting in near synchrony. To do this there must be a sufficient gap between the stimulating and recording electrodes to prevent direct stimulation of the cell population being examined. The shape of the evoked field potentials is also an indicator of slice health. A healthy slice shows a large dendritic field potential (>1mV) with only moderate population spike invasion (i.e. is not hyper-excitabale), and the ratio of the magnitudes of the presynaptic fibre volley to evoked EPSP should be greater than 3:1.

Field EPSPs were monitored in the stratum radiatum of area CA1 using extracellular glass microelectrodes filled with a solution of 0.5M sodium acetate and 2% Pontamine sky blue (impedance 8-12MΩ). EPSPs were amplified, digitised at 10 kHz using a laboratory interface (National Instruments Lab NB board) and then displayed and stored on a Macintosh Iicx computer which was also used for subsequent off-line analysis of the EPSP slope (software: A/Dvance, Fine Scientific Tools, U.S.A.). EPSPs were evoked by alternate stimulation of two independent
pathways at 0.066Hz. Two monopolar tungsten electrodes (0.005 inches tip diameter; A-M system Inc, U.S.A.) were placed either side of the recording microelectrode, to stimulate two populations of convergent but non-overlapping fibres of the Schaffer collateral-commissural pathway. The independence of the two pathways was assessed for every experiment as follows. Each pathway was stimulated once, then both pathways were stimulated together. If the stimulating electrodes were activating most fibres in common, then stimulation of the fibres either side of the recording site simultaneously would have led to a decreased response (due to destructive interference between the two waves of activation). If, on the other hand, the fibres being activated were independent of each other, the response would be larger than each alone, as seen. A post-hoc demonstration of independence is the ability to support LTP in a pathway specific manner, which was seen in every case.

The timing and duration of test stimuli (pulse width 20ms, intensity adjusted in the range 50-200 µA) were under computer control. The test shock was set at an intensity that evoked a response with an initial EPSP slope that was approximately 33% of the maximum slope. In all experiments, the protocol used to induce LTP was 20 shocks at 100Hz repeated six times with an inter-train interval of 3 seconds. Drugs were introduced into the system by perfusion; the source was switched from a reservoir containing aCSF only, to one containing the drug in aCSF. The ‘dead-time’ from this transfer was 5 minutes.

2.1.3: Intracellular recordings

Unlike field potential recording, intracellular recording is specifically from a single cell, not a population. The activation of the inputs onto the cell however, is still achieved using an extracellular electrode; as a result, large, easily resolved EPSPs can
be produced. In bridge balance mode, recordings are made at the resting membrane potential of the cell.

Cells were impaled with sharp electrodes pulled on a P2000 laser puller (Sutter instruments) and were filled with 4M potassium acetate. Impedances lay in the range 70-90MΩ. The procedure adopted was to advance the electrode tip slowly down through the CA1 cell body layer in hippocampal slices (prepared as described above), pulsing current steps at 0.5-1Hz, controlled by the amplifier, an Axoclamp 2B (Axon Instruments). The resultant voltage steps were displayed on a Gould digital triggering oscilloscope. When the electrode neared a cell-soma, a sag was observed in the voltage response. At this point a gentle 'zap' was applied to the electrode (brief capacitance ringing), causing the electrode tip to vibrate, in order to help it penetrate the cell membrane. Once inside the cell, hyperpolarising current was applied to assist the cell in re-sealing its membrane, and re-establishing a stable membrane potential. Input resistance and membrane potential were used as indicators of cell health. All cells included in this study had input resistances between 25 and 40MΩ, and membrane potentials in the range of 63-70mV. Cells which did not meet these criteria within five minutes of penetration, were deemed damaged and recording was terminated.

One or two pathways were stimulated at a frequency of 0.05Hz (for each pathway) during normal recording, with the strength of the test shock adjusted to evoke an EPSP with an amplitude halfway to firing threshold. Input resistance was either measured at the beginning and end of an experiment, or (in most cases) repeatedly throughout the experiment at the same frequency as the test shock. Only those cells showing less than 5% change in input resistance and membrane potential
over the course of the experiment were included in the final analyses. The same protocol as used previously was used to induce LTP in these experiments.

2.1.4: Whole cell recording

In order to record the pA currents caused by activation of single postsynaptic sites in response to the spontaneous release of single synaptic vesicles, I adopted the whole cell voltage clamp recording technique pioneered by Hammill et al. (1981) and refined by Marty and Neher (1983). To allow patching under visual guidance, cells were mounted in a glass bottomed incubation chamber on an inverted microscope (Zeiss Axiovert 25), and the final approach viewed with VAREL optics on a 40x objective.

The extracellular bathing solution was (in mM) NaCl 145, KCl 3, CaCl$_2$ 2, MgCl$_2$ 2, Glucose, 11, HEPES 10, buffered to pH 7.4. In most experiments this solution included 1μM Tetrodotoxin (TTX). Pipettes (5-7 MΩ) were pulled on a two-step Narishige puller. Pipette tips were coated with wax containing 1:1 (by weight) paraffin: mineral oil, in order to reduce noise due to pipette tip capacitance.

The internal solution was (mM) K-gluconate 153, Na$_2$ATP 3, MgSO$_4$ 3, HEPES 8, pH 7.3. Patch seal resistances were between 2 and 10 GΩ, and zero current potentials (resting membrane potential) were more negative than -65mV. Cells were clamped at -70mV for the duration of recording. The signal was amplified by an axon instruments Axopatch 1D amplifier, low-pass filtered at 2-5KHz, and acquired continuously to DAT tape (Sony Corporation). Subsequent computer acquisition was at 10-20 kHz through a CED 1401 interface using PAT software (John Dempster,
University of Strathclyde). One second portions of data were analysed blind (i.e. with no knowledge of where the data lay in the time course of the experiment) and mini-frequency assessed by eye. Subsequently, the amplitudes of the mini-EPSCs were measured and plotted as a frequency amplitude histogram.

2.2: Fluorescence measurements

2.2.1: Principles of fluorescence

The measurement of calcium concentration by fluorescence is a powerful technique which allows dynamic signals to be resolved without the need for physical contact with the cell being studied. This reduces the risk of cell pathology influencing results. The physical principle underlying fluorescence is the inter conversion of photons and energy. When light of a particular wavelength is absorbed by a dye, the energy of the photons can convert electrons from one energy level, to another, higher one. This excited state tends to be unstable, and after a short amount of time (μs to s) the electron decays back to its previous, unexcited state. As it does this, it releases the energy which maintained it at the higher state in the form of another photon, of slightly less energy than the first (longer wavelength). This is known as the ‘Stokes-Einstein shift’. Fluorescent molecules thus have two spectra; excitation spectra, which shows the wavelength of light at which they most readily absorb light, and emission spectra which show for a particular excitation wavelength, the amount of light emitted at each wavelength.
Figure 2.2: Chemical structure of the ratiometric calcium-sensing dye Fura-2, together with low and high calcium excitation spectra.
Fura - 2

Emmission: 510 nm

![Fura-2 molecule structure](image)

Graph showing emission intensity vs. wavelength for 1mM Calcium and Zero Calcium conditions.

- **1mM Calcium**: Intensity increases with wavelength, peaking around 350 nm.
- **Zero Calcium**: Intensity decreases with wavelength, showing a different profile from 1mM Calcium.
Indicator dyes are more complex than the simple energy conversion shown above. In addition to the chemical moiety which provides the fluorescence (usually a complex organic group), they possess closely linked binding domains for the substrate which they are intended to monitor (see figure 2.2 for structure of fura-2, the dye used in this study). When they bind this substrate, either the excitation spectrum or the emission spectrum is altered. In simple terms, on binding substrate the dyes either brighten or dim. Calcium dyes which show these properties include Fluo-3 (brightens on binding calcium) and Furaptra (quenched by calcium). This sort of dye is termed non-ratiometric. The fluorescence is dependent on both substrate concentration, and dye concentration (ionic environment also has an impact, but can usually be disregarded). Since dye concentration has a direct effect on fluorescence, photo-bleaching (destruction of the dye by photon-induced damage) and loading differences will present difficulties in the interpretation of data. Ratiometric dyes are used to overcome this difficulty. These dyes have two peaks in either their excitation or emission spectra. Substrate binding causes a shift at both of these peaks; one becoming larger, the other smaller. In the case of either ratiometric excitation or emission dyes, it is the ratio between these peaks which is used as the basis of measurement. This ratio value is independent of dye concentration, and so removes one of the major source of artefact associated with fluorescence measurement. Indo-1 is an example of a ratiometric emission dye, which is to say that it is excited at a single wavelength, but the emission is measured at two separate wavelengths. Fura-2, the dye used in this study, is a ratiometric excitation dye, which is excited alternately at two different wavelengths (340nm and 380nm) and the emission measured at one (510nm) (Gryniewicz et al. 1985).
In order to visualise fluorescence, an appropriate microscope is required. Fluorescence microscopes operate in a mode known as 'Epi-fluorescence'. The objective lens on the microscope is used both for excitation and for detection of the signal. The two light paths are kept separate by a dichroic mirror which is chosen so that the wavelength at which it begins to reflect lies midway between the excitation and emission wavelengths. A schematic of the arrangement used in this study is shown in figure 2.3. A xenon lamp provides the ultraviolet (uv) light necessary for excitation of fura-2. As explained above, fura-2 is a dual excitation dye, and to be able to excite at two different wavelengths it is necessary to have either two different tuned light sources (e.g. laser lines), or a broad spectrum lamp, filtered alternately by two different narrow band pass filters. The latter is the approach used in these experiments, since there is no need for a coherent light source. The time taken for the filter wheel to change position is one of the practical limitations of this approach.

Two different detection systems were used in the experiments presented here. Initially a single cell photon counting system was used, but in later experiments a slow-scan, cooled, charge-coupled device (CCD) camera was used. I will briefly describe the principles and advantages of each system.

2.2.2: Photon Counting

Single-cell photon counting relies on a photo multiplier tube (PMT) to detect photons emitted from the fluorophore. These are restricted to those emitted from the cell or region of interest by a resizable iris which can be positioned under visual guidance (a TV camera is fed with the same light path as the PMT). The rest of the set-up is as shown in figure 2.3 - see above). PMTs are very sensitive devices for detecting photons at relatively high efficiencies (up to about 12%). No spatial
Figure 2.3: The experimental setup used for fluorescence measurement. Cells were grown on glass coverslips which could be mounted on the stage of an inverted microscope as shown. Fura-2 loaded cells were excited by excitation at 340 and 380nm, and the light emitted above 480nm collected by a photomultiplier tube or cooled CCD camera.
Figure 2.4: The photomultiplier tube. This is a device used to amplify single photon signals so that they can be detected by conventional electronics. Collected light incident on the phosphor coated screen can 'dislodge' electrons. These then interact with a series of high voltage cathodes which form the 'dynode chain'. At each step a single electron is amplified by a fixed factor. This eventually gives rise to a high order of amplification, which can be detected by additional devices (overall a PMT is <12% efficient, and weakest at detecting longer wavelength light). In photon-counting mode, each incident photon gives rise to a signal which is registered as a small current. A threshold discriminator identifies this against the noise, and a digital counter sums these events per time period (in the case of these experiments, those periods of excitation at 340nm and 380nm).
The Photomultiplier Tube (PMT)

Photons enter the PMT and are absorbed by the phosphor screen, releasing electrons. These electrons are then accelerated by a.high voltage, causing further cathodes to emit secondary electrons. This process continues along a dynode chain, amplifying the signal. The amplified signal is then measured by anode and displayed on a computer for further analysis.

Photon counting:

A photon enters the PMT, triggering an electronic event. The signal is then passed through a threshold discriminator to determine if it is significant enough to be counted, and finally, it is counted by a digital counter. The count is then transmitted to a computer for analysis.
information is retained, however, as photon detection results in a series of current pulses, the frequency of which is proportional to the light intensity. Figure 2.4 shows the principle by which PMTs operate. Incoming photons hit the phosphor-coated screen, and knock out electrons. These then hit a series of highly charged cathodes, known as the dynode chain. Each time an electron strikes a cathode, 3-4 electrons are released to interact with the next. The signal is thus exponentially (but uniformly) amplified as it progresses along the dynode chain, until from a single incident photon, about a million electrons have been generated. The current pulse as this reaches the anode is then detected by a threshold discriminator (to eliminate artefactual blips) and converted into a square current pulse. A digital counter records these, and feeds the information into a computer for storage. This method has very low computing overheads, and so a 20 MHz IBM-clone 80286-based computer is completely adequate for storage and analysis of this type of data. This method is sensitive and highly affordable, but as already mentioned, does not discriminate spatial information within the region of interest set by the iris. The set-up used in this study is illustrated in figure 2.3. The specimen was mounted on a Nikon Diaphot microscope with a 40X fluorescence objective, operating in epi-fluorescence mode.

A filter wheel containing 340nm and 380nm excitation filters allowed dual excitation measurement of fura-2 illuminated by a xenon ultraviolet lamp through a 4% transmittance neutral density filter. Emitted light was collected by a bialkali end-window photo-multiplier tube operating in photon counting mode. Data acquisition and control of the filter wheel was carried out by an IBM-based 80286 computer running COUNT (Newcastle Photometric Systems). Files were output as ASCII files and analysed using Microcal's ORIGIN v3.5 software. Cells were loaded with fura-2 by the AM-ester technique. This makes the dyes cell permeant by the
Figure 2.5: The basic principle underlying the CCD camera. CCDs are solid state devices made from a sandwich of silicon and silicon dioxide. The conducting silicon is etched into regions which demarcate pixels. Each pixel is controlled by three elements attached to separate voltage lines (the clock lines). It is these which define the potential 'wells' which are the focal point of each pixel (how these operate, and how they are read out is explained in figure 2.7). The layer of silicon on the top surface of the CCD chip prevents short wavelengths (uv and shorter) from penetrating the device. Longer wavelengths will penetrate, however, and where absorbed, they excite the SiO$_2$ matrix to create an electron and a hole. The potentials applied to the clock lines hold the electrons in the local potential well. In this way, each pixel element accumulates charge as it absorbs light. So-called 'dark' noise is due to the a similar process involving infra red radiation. Since this is generated within the device, it cannot be shielded out; instead, cooling is used to minimise this radiation.
Figure 2.6: Readout from a CCD camera. Potential wells are formed by creating a region of negative charge, surrounded by opposite polarity, and these accumulate charge in the manner described previously. In order to read a complex array of pixels, CCD cameras take the data line by line and pixel by pixel. The clock lines are used to move the potential wells along the line, so that at the end of the line it receives each group of electrons in sequence (thus it reads pixel N+2, then N+1, then N). Having completed one line, the camera moves onto the next. This laborious process is the reason for the relatively slow readout time of CCD cameras, though this can be speeded up by use of additional readout buses.
Direction of charge transfer
substitution of large ester groups onto the dye. Although non-fluorescent in this form, the dye is readily taken up into cells. Once inside a cell, esterases remove the substituents, and the dye is once again fully fluorescent.

The incubation medium was the same as that used for whole-cell recording, with the exception that Mg$^{2+}$ was not present in the medium. NMDA (10 mM) was applied iontophoretically (150 µA, 1-2 seconds) from a pipette driven by a Neurophore iontophoresis unit (Medical Instruments Corporation, New York). When not stimulated, the agonist was prevented from leaking from the pipette by application of a small holding current (10 µA). Other drugs were bath applied. All experiments were carried out at room temperature (23-28°C)

2.2.3: Cooled Slow-scan CCD imaging

In order to gain spatial information from fluorescent probes, there are a number of techniques which can be employed. Confocal laser scanning microscopy gives very good spatial and temporal resolution at the cost of great expense and large amounts of photo-damage due to the lower sensitivity of detection. Solid state video cameras (CCDs) also give good temporal resolution, but have very poor sensitivity, and have relatively low image resolution. Over the last few years a variant of this CCD technology has been developed, primarily for astronomy, where efficiencies of detection are all important. These slow-scan devices can integrate photon collection over long periods, in a manner analogous to long exposures on photographic film but with much higher sensitivity. The drawback of this method is that there is a certain level of 'dark noise' much of which is due to infrared photons generated within the
camera itself. To reduce this, slow-scan CCDs are often cooled, since thermal noise is halved by each reduction in temperature by 7-8°C.

CCD cameras operate in a very different manner to PMTs. As solid state devices, the magnitude of charge carried by a single electron is sufficient to be detected. The fundamental principle of operation is illustrated in figures 2.5 and 2.6. The camera is composed of multiple potential wells which operate as photo-diodes. The incoming photons free electrons in the SiO₂ which are accumulated in the potential wells. After a certain recording time, the charge in the wells is read out row by row. Each well corresponds to a pixel, and the final image is produced by the balance of intensities in the pixels. Each pixel is capable of a fixed dynamic range. 8 bit cameras (such as those in video cameras) can respond to 2⁸ or 256 levels of grey scale.

CCD cameras differ widely in both grey scale and spatial resolution (number of pixels), and to improve either requires a trade-off in speed. Readout speed is dependent on the bit depth of each pixel and the total number of pixels; 400 pixels take longer to read than 200. In order to improve readout with a high resolution camera, pixel binning can be employed. This can be done in hardware, or post-hoc in software; however software binning will only help improve signal to noise. Hardware binning will improve data transfer since it is equivalent to making each pixel larger, and reducing the overall number of pixels. Delineating a region of interest (ROI) is another technique to speed data throughput. This tells the camera to disregard those pixels outside the area, again reducing image size, and the consequent strain on the data bus.

Imaging experiments were carried out on a similar system to the one used for single-cell photon-counting. The microscope was a Nikon Diaphot with a 0.8 NA 40X
fluorescence objective. Imaging was carried out with a 20% transmittance neutral density filter. The camera (Digital Pixel, Brighton), an 800x1200 12 bit device and the filter wheel (Sutter Instruments) were controlled by Acquisition Manager software supplied by Kinetic Imaging (Liverpool) running on an Intel Pentium (150 MHz) with a 512Mb DAMM drive for fast data acquisition. Subsequent analysis was in Lucida v3.51 (also Kinetic Imaging) and Microcal ORIGIN. Solutions were as described for the single-cell photon counting, with the substitution of AMPA for NMDA in some experiments. All experiments were carried out at ambient temperature (23-28°C).

2.3: Cultured hippocampal cells

Hippocampi were removed from 1-4 day postnatal rats. These were incubated for 20-30 minutes in a trypsin-versine mix. Following washing in serum containing DMEM, the cells were triturated in flamed pipettes of decreasing tip diameter. Cells were then plated at a density of 40,000 per cover slip (in the case of the fluorescent work) or 400,000 per 55 mm dish (for whole-cell recording). Good expression of functional NMDA-Rs can be difficult to achieve in cultured cells, and so slightly different approaches were made in culturing for the two systems. The cells for fluorescent work, where good NMDA-R expression was essential (that being the object of the study), were plated as a small drop onto poly-L-lysine coated cover slips. These were then left for 2 hours to settle, at which point a defined medium containing 1% serum was added (SF1C - see appendix) to fill the culture dish. This medium was used to assist NMDA-R expression, and to minimise glial growth, to provide clear fields for imaging. The cells for whole-cell recording were dropped in suspension into medium already covering poly-L-lysine coated coverslips. In this case, the medium
consisted of 10% fetal calf serum, 10% Ham’s F12 nutrient mix with glutamine. The culture dishes contained a layer of ‘feeder’ cells - largely glia - which had been grown to confluence some weeks earlier. The purpose of these was to condition the medium to make it more capable of sustaining a healthy neuronal population than such a simple medium would ordinarily be.

2.4: Biochemical Methods

Enzymatic activities were assessed through mobilisation of $[1^{14}\text{C}]$ arachidonate from either 1-stearoyl 2-$[1^{14}\text{C}]$arachidonyl-$sn$ - glycerophosphocholine, or 1-stearoyl-2-$[1^{14}\text{C}]$arachidonyl-$sn$ - glycerol (both 50mCi/mmol, Amersham U.K.), by a method modified from Clements, Bliss et al. (1991). Animals (Sprague-Dawley rats, 150-170g in weight) were killed by stunning followed by cervical dislocation and decapitation. The brain was rapidly removed and the hippocampus dissected out and placed in cold (0-4°C) oxygenated (95% O$_2$, 5% CO$_2$) aCSF. A crude microsomal fraction including plasma membrane was prepared from pooled hippocampal tissue by homogenisation of tissue with 30 strokes of a Dounce type homogeniser at 4°C, in Tris buffer (pH 7.4) containing 50mM leupeptin, 2.5mM microcystein, 100mM PMSF, 80mM molybdate 2mM pyrophosphate and 2mM MgSO$_4$. The resulting homogenate was spun down at 3000 rpm for 5 min to remove nuclear debris and unbroken cells. The homogenate was left on ice for 1 hour for full lysis of microsomes.
Each sample contained approximately 300mg of protein, together with 1kBq of radiolabelled substrate suspended as vesicles in Tris buffer containing 1% fatty acid free BSA (Sigma). Samples were prepared in duplicate, vortex-mixed and incubated for 1 hour in a shaking incubator at 37°C. The reaction was stopped by addition of chloroform-methanol-acetic acid at a ratio of 4:2:1. Separation of the organic and aqueous phases was facilitated by the addition of 1.2M KCl, and separated by centrifugation after vortex-mixing. The organic phase was removed and dried down under nitrogen, then resuspended in 20ml chloroform and spotted onto thin layer silica gel plates (Merck, U. K.). Plates were run using solvent mixtures of chloroform-methanol-acetic acid-water (5:10:3:3) for phosphatidyl choline based assay or hexane-diethyl ether-acetic acid (105:45:5) for diacylglycerol based assay. Spots containing [1-^14C] arachidonate were visualised by autoradiography. Plates were exposed for 24-36 hours to Fuji X-ray film before development. Samples were identified by their mobility relative to authentic standards (Amersham U. K.) and removed separately from the plates. Scintillant was added to each sample before counting in a Beckman LS 5000CE scintillation counter. The protein content of each preparation was assayed by the method of (Lowry, Roseborough et al. 1951) and was typically 11mg/ml. Enzymatic activities were expressed as nmol arachidonic acid mobilised per mg of protein.

2.5: Drug handling and sources.

All drug solutions were made up freshly each day. Bromophenacyl bromide (Sigma, U. K.) was made up in aCSF at 50μM. Arachidonyl triflourmethyl ketone
(AACOCl) (LC Laboratories, U. K.), RHC80267 (Affiniti U. K.), and D609 (LC Laboratories) were made up from stock solution in aCSF containing 1% DMSO to a concentration of 50µM. CNQX (Tocris Cookson, Bristol) was made up to 10µM in aCSF and sonicated for 1-2min to get it into free solution. AP5 (Tocris-Cookson, Bristol) was stored as a stock solution (1mM) at -20°C and made up to 25µM shortly before use. L-Nitro Arginine (Sigma, U.K.) was made up in Na-Locke to a final concentration of 40µM, and then added to the incubation chamber to give a final concentration of 10µM. Arachidonic Acid, eicosidienoic acid and eicosapentaenoic acid were all supplied as 0.3M stocks in ethanol (Cayman Chemicals, supplied by LC Laboratories, U.K.). Tetrodotoxin (Sigma U.K.) was stored at 1mM at -20°C and made up to 1µM in bathing solution Aqueous solutions were made up shortly before each application and kept on ice until ~1min before application when they were allowed to warm to room temperature.
Assaying AA mobilisation from phospholipids

3.1: Testing the effectiveness of the inhibitors used

In any experimental series involving the use of inhibitors, there are two particular worries. The first is whether the inhibitors are effective in blocking the appropriate activity in the tissue of choice. Do AACOCF and BPB block PLA₂ activity in hippocampal tissue? Does RHC80267 block DAG lipase activity in this preparation? The second worry is one of specificity. Where two different inhibitors present similar pharmacological profiles, are they doing this through specific actions on the chosen enzymatic target, or are they in fact inhibiting a different enzyme? Ideally this can be largely excluded by the use of several chemically unrelated compounds which inhibit a particular target enzyme. There is shortage of suitable inhibitors available for AA metabolism, however, and so that approach was not available.

To address these issues of selectivity and potency we have performed biochemical assays on a crude microsomal extract prepared from hippocampal tissue. These assays have investigated the endogenous activities involved in cleavage of the arachidonate-binding sn-2 bond in phosphatidyl choline, and diacylglycerol. The calcium dependence of these activities has also been investigated.
3.2: Mobilisation of [1-\(^{14}\)C]arachidonic acid from DAG is calcium independent and inhibited only by RHC80267.

Mobilisation of [1-\(^{14}\)C] arachidonic acid from diacylglycerol was calculated as nmol arachidonic acid released per mg protein, with values from a blank subtracted. Values plotted in figure 3.1 represent the average of 4 experiments performed in duplicate. The extent of arachidonic acid mobilisation in the presence or absence of inhibitors was independent of calcium concentration. In all cases 50\(\mu\)M RHC80267 greatly reduced arachidonic acid mobilisation from diacylglycerol \((p<0.05)\), typically inhibiting 73\% of arachidonic acid release. The presence of either 50\(\mu\)M BPB or AACOCF\(_3\), or both together, caused no significant reduction in the mobilisation of arachidonic acid from diacylglycerol at any of the calcium concentrations shown.

3.3: Mobilisation of [1-\(^{14}\)C] arachidonic acid from phosphatidyl choline is a calcium dependent process, due mainly to the activities of sPLA\(_2\) and cPLA\(_2\).

The extent of arachidonic acid mobilisation from phosphatidyl choline was also measured as nmol arachidonic acid mobilised from the parent lipid, per mg of protein. Values plotted in figure 3.2 are the average of seven experiments performed in duplicate. The investigation of release of arachidonic acid bound in phosphatidyl choline showed a very different profile. Here a marked calcium dependence was observed, with very low levels of activity observed in the presence of 3mM EGTA (effectively zero calcium). At this concentration none of the inhibitors were effective
**Figure 3.1:** Mobilisation of AA from DAG expressed as nmol [1-\(^{14}\)C]AA mobilised per \(\mu\)g of protein (n=4, performed in duplicate). The release of radiolabelled AA is independent of the calcium concentration, and inhibited only by RHC80267 (an inhibitor of DAG-lipase).
Figure 3.2: Mobilisation of AA from PC expressed as nmol [1-\textsuperscript{14}C]AA mobilised per \( \mu \text{g} \) of protein (n=7, performed in duplicate). Release of AA showed marked calcium dependence, and was differentially inhibited by blockers of sPLA\(_2\) (BPB) and cPLA\(_2\) (AACOCF\(_3\)). In the presence of 3mM EGTA, phospholipid hydrolysis was at basal levels and all inhibitors were ineffective at further reducing the liberation of radiolabelled AA. At a calcium concentration of 100\( \mu \text{M} \), the primary enzymatic activity was that of cPLA\(_2\). Increasing the calcium concentration further (to 1mM) provided a much greater degree of activation of sPLA\(_2\).
The diagram shows the effect of various compounds and conditions on the production of [AA*].

- **RHC80267**
- **BPB**
- **AACOCF<sub>3</sub>**
- **AACOCF<sub>3</sub> + BPB**
- **Control**

These are measured in nmol [AA*]/ng. The X-axis represents the concentration of calcium ([Ca<sup>2+</sup>]), and the Y-axis represents the concentration in mM 3mM EGTA 100 µM.
at reducing the mobilisation of arachidonic acid from phosphatidyl choline. When the
calcium concentration was increased to 100µM, the effect of RHC80267 was not
significant. BPB showed some effect at this calcium concentration (48 ±4.4%
inhibition, p<0.001), but the effect of AACOCF₃ was most marked (76 ±8.1%
inhibition, p<0.001), as would be predicted by the greater sensitivity of cPLA₂ to
calcium. Co-incubation of the reaction mix with BPB and AACOCF₃ (in four
experiments) showed no further inhibition than that seen with AACOCF₃ alone (78
±6.5% inhibition).

On increasing the calcium concentration further, to 3mM, the degree of
arachidonic acid mobilisation from phosphatidyl choline increased. All inhibitors
were significantly different from control, with BPB (63.8 ± 5.5%, p<0.001) and
AACOCF₃ 56.3 ± 4.8%, p<0.001) being most effective. Co-incubation of the reaction
mix with the two inhibitors showed a greater effect than either inhibitor alone (86
±9.0%, p<0.01).

3.4: RHC80267 partially inhibits arachidonic acid mobilisation through
its actions on a phosphatidyl-choline-specific-phospholipase-C-dependent route.

A difficulty of experiments assessing mobilisation of arachidonic acid from a
lipid such as phosphatidyl choline, is that there are many routes of possible
metabolism. In order to assess the contribution of the sequential action of
phosphatidyl choline specific phospholipase C (PC-PLC), DAG lipase and
monoacyl-glycerol lipase, we have used the specific inhibitor of PC-PLC, D609.
Figure 3.3 shows that the proportion of arachidonic acid mobilisation inhibited by
RHC80267 (34 ±6.8%) is equal to that shown by D609 (32 ±8.3), and showed no
Figure 3.3: Mobilisation of AA from PC expressed as nmol [1-14C]AA mobilised per μg of protein (n=4, performed in duplicate). Comparison of PC-PLC activity to the RHC80267-sensitive activity found in this system. Inhibition of PC-PLC by D609 gave a similar pattern of response to that seen with RHC80267 (a DAG-lipase inhibitor). When the two drugs were co-applied, no further inhibition was observed, suggesting that the two drugs work on the same pathway of AA mobilisation.
further inhibition on coapplication of the two drugs (35 ±5.8%). This result is good
evidence that D609 and RHC80267 act on the same pathway of AA mobilisation.

3.5: Conclusions.

These results demonstrate that the hippocampus does possess those routes for
AA mobilisation which have been described in other tissues (see figure 1.3 for a
scheme of AA release from phosphatidyl choline). The drugs have also been shown to
be effective at the concentrations used, in blocking these activities. Finally, there
seems to be very little non-specific activity of any of the inhibitors (e.g., BPB does
not inhibit DAG-lipase). As a result, the compounds seem to be suitable for the
applications to which they are put in the later chapters of this thesis.
Does AA play a part in long-term potentiation?

4.1: Is LTP dependent on the mobilisation of AA from phospholipids?

One of the simplest predictions one might make if AA is a necessary factor in LTP, is that inhibition of the various enzymatic pathways by which it is generated should effect either the induction of LTP, or its subsequent expression. The *in vitro* slice preparation is a good tool for this, since it allows accurate recording from sub regions of the hippocampus, and good control over the chemical environment in which the slice rests. The pathways which have been studied are those mentioned in the previous chapter: cPLA$_2$, sPLA$_2$, DAG-lipase, and PC-PLC.

4.1.1: Experimental design

In order to investigate the dependence of LTP on pathways of AA mobilisation, I adopted a two-pathway design. A stimulating electrode was placed either side of the recording electrode in the *stratum radiatum* of sub region CA1. In this way independent bundles of fibres could be stimulated, leading in turn to the activation of two independent populations of synapses in the area of the recording electrode. The independence of these pathways was checked by looking at the interactions between the two; where a stimulus is given to each pathway simultaneously, the response should be larger than that seen with stimulation of either pathway alone. A post-hoc demonstration of independence is also the ability of each pathway to undergo LTP without effecting the other. This approach enabled me to
investigate drug effects on baseline and potentiated responses at the same time, within the same slice.

4.1.2: The magnitude of LTP is consistent between two pathways

As shown in figure 4.1, LTP can be independently induced in two alternately stimulated pathways within the same slice. The two pathways, P1 and P2 were stimulated at test shock intensity for 30 minutes. At this point, a high frequency train of pulses at 150% test shock intensity (see methods) was applied to pathway P1. This resulted in a potentiation of that pathway, but not of pathway P2, to which no high frequency train had been applied. Forty five minutes after the induction of LTP in pathway P1, an equivalent high frequency stimulus was applied to pathway P2. This induced LTP in pathway P2 without affecting pathway P1. Additionally, the magnitude of the LTP expressed in each pathway was comparable (170.1 ± 13.37% in pathway P1, compared to 159.1 ± 12.87% in P2, n.s.).

4.1.3: Secretory PLA₂ is a necessary factor in the induction of LTP

Perfusion of 50μM bromophenacyl bromide (BPB), an inhibitor of sPLA₂, had no effect on baseline synaptic transmission, nor on potentiated responses when perfused into the system 15 minutes following the induction of LTP in pathway P1 (figure 4.2a). As previously reported by Massicotte et al. (1990), however, when a high frequency train normally sufficient to induce LTP was applied in the presence of BPB, LTP was blocked (figure 4.2b). A small transient potentiation was observed, which declined to baseline within 10-20 min.
Figure 4.1: LTP of equivalent magnitude can be independently induced in each pathway by tetanic stimulation (6 trains of 20 stimuli at 100Hz, given 3 seconds apart). Following a 30 minute control period, a high frequency train was applied to pathway P1. The two pathways were then monitored for a period of 45 minutes, and then a high frequency train was applied to pathway P2. The two pathways were then monitored for a further hour.

A: Representative potentials from each pathway (each is the average of five consecutive responses) taken from the control period 10 minutes prior to the induction of LTP in that pathway. There is no change in the afferent volley of the response, but the EPSP slope is markedly enhanced.

B: Percent change in the slope of the field EPSP is displayed as a function of time for pathways P1 (filled circles) and P2 (open circles).
Control 20 min post tetanus

Control 20 min post tetanus 2mV

A

P1

P2

20 min post tetanus

B

EPSP slope (% change)

0 30 60 90 120 150 Time (min)

0 50 100 150

-50

• P1

○ P2
**Figure 4.2:** (a) Pooled data showing a two-pathway control experiment. When a high frequency train is applied to pathway P1 (filled circles) at the point indicated by the filled triangle, the slope of the fEPSPs becomes potentiated, while the responses of an independent, but convergent, pathway (P2, open circles) are unaffected. Application of the same high frequency train to pathway P2, 45 minutes later, induces LTP in this pathway also (n=8).

(b) The same protocol was used as in figure 2(a), but with 50mM BPB (an inhibitor of cPLA₂) applied (solid bar) to the slices 15 minutes after the induction of LTP in pathway P1. While BPB has no effect on LTP which has been established before exposure to the drug, or on baseline responses, it nevertheless blocks the induction of LTP (n=8).

(c) and (d) The blockade of the induction of LTP by BPB is not due to a direct action on the NMDA-receptor. (c) Individual responses taken from a single experiment, showing the initial response in normal aCSF, then the NMDA-receptor-mediated-response following 90 minutes incubation in 10mM CNQX dissolved in aCSF containing no added Mg²⁺. The final two responses show the effect of 50mM BPB, and then 20mM AP5. (d) BPB has no effect on the slope of NMDA-receptor-mediated EPSPs, whereas 20mM AP5 rapidly blocks the response (n=5).
**Graphs and Figures**

**Graph a:**
- **Y-axis:** EPSP Slope (%)
- **X-axis:** Time (min)
- **Legend:**
  - Control P1
  - Control P2

**Graph b:**
- **Y-axis:** EPSP Slope (%)
- **X-axis:** Time (min)
- **Legend:**
  - BPB P1
  - BPB P2

**Graph c:**
- **Legend:**
  - NMDA-R
  - + BPB
  - + AP5
- **Time Scale:** 10ms
- **Amplitude Scale:** 1mV

**Graph d:**
- **Y-axis:** EPSP Slope (%)
- **X-axis:** Time (min)
- **Legend:**
  - NMDA
  - AP5
  - BPB
4.1.4: Is the action of BPB on the induction of LTP due to an impairment of NMDA-receptor function?

To investigate whether the blockade of LTP by BPB is due to interference with the function of the NMDA receptor, I isolated the NMDA receptor-mediated response pharmacologically. This was achieved using field potential recording, again in area CA1. 10μM CNQX was perfused into the system via aCSF containing zero-added Mg\(^{2+}\). Figure 4.2d shows the course of onset (blockade of the AMPA-receptor-mediated response) and the slow recovery of synaptic transmission due to washout of Mg\(^{2+}\), and the resultant uncovering of NMDA-receptor-mediated potentials. The stimulus intensity was slightly increased to give responses of 1-2mV height, but this was still insufficient to give rise to potentials showing bursts of population spikes. These responses were then stable for as long as the experiment lasted. Addition of BPB to the perfusion medium at the same concentration as used previously (50μM) had no effect on the profile or the magnitude of the responses. However the responses were completely abolished by 20μM D-AP5, a specific NMDA-receptor antagonist.

4.1.5: Continued mobilisation of arachidonic acid is required for the expression of LTP.

The inhibitors of cPLA\(_2\) and DAG-lipase, AACOCF\(_3\) and RHC80267 are rather insoluble in aqueous solution. To overcome this problem, we applied both drugs in aCSF containing 1% DMSO, and so it was important to ascertain that this had no effect on the ability of evoked responses to undergo and support LTP. Although DMSO causes an increase in the magnitude of field responses, it neither prevents the induction of LTP independently in each pathway, nor impairs the
Figure 4.3: (a) Two pathway control in the presence of 1% DMSO throughout. High frequency trains were applied to pathway P1 (at the filled triangle) and to pathway P2 (at the open triangle). In both cases LTP was successfully induced (n=8).

(b) Inhibition of DAG-lipase by 50μM RHC80267 (applied for the period shown by the solid bar) had no effect on baseline responses, but caused a decline in pathway P1 where LTP had been pre-established. The presence of this drug also blocked the induction of LTP in pathway P2 (n=9). DMSO was present throughout.

(c) In the continuous presence of 1% DMSO, 50μM AACOCF₃ (an inhibitor of cPLA₂) was applied (solid bar) to slices between the application of high frequency trains to each pathway. No effect was seen on baseline responses, but in pathway P1, in which LTP had previously been induced (solid triangle), drug application caused a decline in responses, eventually to baseline. In the presence of AACOCF₃, it proved impossible to induce LTP (n=9).

(d) PC-PLC, a possible source of the DAG for DAG-lipase, was inhibited using 50μM D609 dissolved in DMSO (applied as shown by the solid bar). Baseline responses showed no effect on perfusion of the drug, but effects on pre-established LTP and the induction of LTP were consistent with those seen with the DAG-lipase inhibitor (n=7).
expression of that potentiation, at least for the first hour and three quarters (figure 4.3a).

Perfusion of 50μM AACOCF₃ (fig 4.3b), an inhibitor of cPLA₂, had no effect on baseline responses. The potentiated pathway, however, declined steadily after application of the drug to a value of 109.9 ± 9.3% at the end of the experiment, not significantly different from the pre-tetanus value. A high-frequency train, ordinarily sufficient to induce LTP, failed to do so in the presence of AACOCF₃. A small initial potentiation was observed, but this declined to baseline (105.7 ± 5.2%) within the space of one hour. These values were not different from pre-tetanus values, but were significantly lower than corresponding values for the relevant pathways in the control experiments (p<0.05 for pathway P1, p<0.001 for pathway P2, n=9).

Inhibition of DAG-lipase by 50μM RHC80267 had no effect on baseline synaptic transmission (figure 4.3c). When applied to a pathway where LTP had previously been induced, the drug caused a slow but steady decline which reduced the potentiation to 111.0 ± 8.8% by the end of the experiment. This was not significantly different from the pre-tetanic control period, and was significantly lower than in control experiments. Attempts to induce LTP in the presence of RHC80267 were unsuccessful. A small potentiation was observed but in all cases it declined to baseline (103.3 ± 8.5%) within one hour. Again, this was significantly less than comparable values of potentiation in control slices (p < 0.001). Taken together, these results suggest that a persistent supply of AA derived from cPLA₂ and DAG is required for the maintenance of LTP.
4.1.6: *Phosphatidyl choline is the likely source of DAG in the expression of LTP.*

Several different forms of phospholipase C (PLC) have in common an ability to hydrolyse phosphoinositides, to produce IP₃ and DAG. However, such activity is generally only transient, lasting at most 10-15 minutes. For this reason, we have chosen to investigate phosphocholine-specific phospholipase C (PC-PLC), an enzyme which has been shown to be activated for much longer time periods than the more conventional PLC activities.

D609, an inhibitor of PC-PLC, was dissolved in DMSO to give a final concentration of 50µM in 0.25% DMSO, and perfused into hippocampal slices (fig 4.3d). The presence of D609 did not affect baseline responses, but LTP which had been previously established in pathway P1 slowly declined, reaching baseline (100.5 ± 6.5%) by the end of the experiment. Application of a high-frequency train, normally sufficient to induce LTP, proved ineffective in the presence of D609, the value of the EPSP at 1 hr being 92.9 ± 3.6%. These values were not different from pre-tetanus values, but were significantly lower than corresponding values for the relevant pathways in the control experiments (p<0.05 for pathway P1, p<0.01 for pathway P2, n=7 and 9 for D609 and control groups, respectively).

4.1.7: *AACOCF₃ and RHC80267 block LTP more than additively 1 hour after the induction of LTP*

An unanswered question from the above experiments, is ‘for how long are these enzymatic activities essential for LTP?’ In order to address this, I have studied the effects of AACOCF₃ and RHC80267 on LTP when applied one hour following the induction of LTP. In a two pathway control experiment (figure 4.1), a high frequency train was applied to pathway P1 after a thirty minute control period. The two
Figure 4.4: LTP remains sensitive to RHC80267 and AACOCF$_3$ one hour following induction.

Top left. Two pathway control in the presence of 1% DMSO. High frequency trains were applied to pathway P1 at the point indicated by the filled triangle; pathway P2 received only test shocks. LTP was sustained for 2 hours with no decline (n=5).

Bottom left. Single pathway experiment showing a decline in the magnitude of LTP following the perfusion of 50 μM RHC80267 into the recording chamber one hour after the induction of LTP. High frequency trains were applied to pathway P1 at the point indicated by the filled triangle. Drug addition is indicated by the grey bar (n=7).

Bottom right. Single pathway experiment showing a decline in the magnitude of LTP following the perfusion of 50 μM AACOCF$_3$ into the recording chamber one hour after the induction of LTP. High frequency trains were applied to pathway P1 at the point indicated by the filled triangle. Drug addition is indicated by the open bar. (n=7)

Top right. Two pathway experiment indicating the effect of coapplication of AACOCF$_3$ and RHC80267. High frequency trains were applied to pathway P1 at the point indicated by the filled triangle; pathway P2 received only test shocks. One hour following the induction of LTP, the two drugs were perfused into the recording chamber (addition of the drugs is indicated by the two bars). Responses, monitored for a further hour, showed a rapid decline back to pre-tetanic values, but did not fall below those values. (n=6)
pathways were then monitored for a further 2 hours. A stable LTP was obtained in pathway P1 (155.13 ± 11.28%) and a stable baseline was seen in P2 (99.15 ± 4.78%).

Where RHC80267 and AACOCF₃ were applied 1 hour following the induction of LTP, the magnitude of LTP seen at the end of the experiment was much reduced (129.79 ± 8.02%, 125.73 ± 4.93% for RHC80267 and AACOCF₃ respectively.) When the two drugs were co-applied at the 1 hour time point, the potentiated pathway (P1) showed a much greater degree of decline (96.78 ± 4.55%) which was not reflected by a drop in the control pathway (93.27 ± 7.14%).

4.1.8: Conclusions

The results presented in this chapter lead to several conclusions. AA is released by (at least) three pathways during LTP, and these can be grouped into two types on the basis of when they become essential for LTP. Secretory PLA₂ activity is required for the induction of LTP, but not for its subsequent expression. Clearly AA release here is acting as some kind of ‘switch’, but what it might be acting upon is hard to say. A first guess might be the NMDA-receptor, but if so, it is stimulated release which is important, not tonic release, as inhibition of this activity has no effect on NMDA-receptor mediated potentials. Interactions between AA, [Ca²⁺], and the NMDA receptor will be addressed in more detail in chapter 6.

The second group of AA-mobilising enzymes includes cytoplasmic PLA₂, and the PC-PLC/DAG-lipase pathway. Inhibition of these enzymes acts on the expression of LTP. It is hard to assess whether the drugs have any impact on the induction event of LTP itself (the elevated [Ca²⁺], due to depolarisation and entry of Ca²⁺ through the NMDA-receptor) since there is insufficient time resolution to distinguish between this
and early events in the expression of LTP, such as for instance the generation of a retrograde messenger. Experiments such as those carried out by Lynch et al (1989) which revealed a long term elevation of AA following the induction of LTP have always been hard to reconcile with the reports in the literature that blockade of PLA₂ results in a block of the induction of LTP, whilst having no effect on pre-established LTP. By considering other enzymes, which can be blocked by different drugs, it is now possible to resolve this. The data in this chapter suggests that sustained mobilisation of AA is essential for the expression of LTP, and these results are consistent with the idea that AA might act as a retrograde messenger in LTP.

4.2: Is the postsynaptic mobilisation of AA necessary for the induction and expression of LTP?

The data presented in the first part of this chapter suggest that the mobilisation of AA from phospholipids is an essential mechanism for the expression of LTP. A retrograde messenger has additional requirements; it must be produced postsynaptically, and act presynaptically. Proving the site of action is difficult, but one way in which the site of production can be investigated is the injection of inhibitors specifically into the postsynaptic cell, and attempting to induce LTP in the synapses on that cell. This was done using sharp microelectrodes to penetrate single CA1 pyramidal cells, record their membrane potential (including synaptically evoked events) in bridge balance mode, and inject an agent specifically into that cell. Other cells will not be affected. Any effects on the induction or expression of LTP will be due solely to the postsynaptic action of the drug.
4.2.1: Experimental design (i)

Two protocols were adopted to address the dependence of the induction and expression of LTP on postsynaptic PC-PLC activity. The first protocol addressed the role of postsynaptically generated AA in LTP without distinguishing between induction and expression. To do this, a simple experimental design was adopted. Following the successful impalement of a CA1 pyramidal cell, judged by membrane potential and input resistance (see methods), hyperpolarising iontophoretic current was applied for 15 minutes to inject the compound (D609 - chosen as it exists in a charged form) into the cell. A single electrode was used to stimulate the Schaffer collateral/ commissural pathway and the resulting EPSPs in the target cell were recorded. The input resistance of each cell was monitored throughout the experiment.

4.2.2: Postsynaptic mobilisation of AA is required during the induction and expression of LTP

In control experiments where no drug was present in the electrode tip, application of a high frequency stimulus after a 10 minute control period, resulted in a potentiation of evoked EPSP amplitude of 168.0 ± 18.8% (n=8) - see figure 4.4. When D609 was present in the electrode tip, and injected by iontophoresis prior to the start of recording, the high-frequency train which reliably induced LTP in control conditions, now failed to produce potentiation (99.4 ± 7.99% measured 40 min after the tetanus, n=7, p<0.001 compared to control experiments)
Figure 4.5: (a) Control experiment with responses recorded intracellularly. 2.5% DMSO was present in the recording pipette. At the point indicated by the filled triangle, a high frequency train was applied, resulting in the induction of LTP (n=8).
(b) With 500mM D609 present in the pipette tip, and injected by iontophoresis prior to recording, a high frequency train proved unable to induce LTP (n=7).
(c) A hyper-polarising pulse (0.3nA for 20 minutes) was applied 10 minutes after the induction of LTP. This increased the size of evoked potentials (removed from record) but did not destabilise the LTP (n=4).
(d) With 500mM D609 present in the pipette tip, recording was rapidly initiated following impalement. A high frequency train was applied to pathway P1 as indicated by the filled triangle, resulting in LTP. 10 minutes following the induction of LTP, a hyper-polarising pulse (0.3nA for 20 minutes) was applied in order to inject the drug into the cell. Following the return to resting membrane potential, the control, untetanised pathway showed no change in the amplitude of the response, whereas the pathway in which LTP had been induced showed a decline down to baseline (n=6).
4.2.3: *Experimental design (ii)*

The postsynaptic involvement of PC-PLC in the expression of LTP was investigated using a second two-pathway protocol. Following successful impalement of a CA1 pyramidal cell, two independent inputs were stimulated alternately. After a 10 minute control period, a standard tetanus was applied to pathway P1. Following the successful induction of LTP in this pathway synaptic potentials were monitored for 10 minutes, to check for stability. Recording in cells failing to show LTP, due to a presumed leakage of D609 into the cell, was terminated. At this point, 0.3nA hyperpolarising current was injected for 10 minutes, in order to drive the drug into the cell. The current was then returned to zero and responses monitored for a further 20 minutes.

4.2.4: **AA mobilisation is required for the expression of LTP 10 minutes after its successful induction.**

Pathway P1, to which the high frequency had been applied, showed a significant potentiation (178.0 ± 16.2%, measured 10 min after the tetanus) which was not seen in pathway P2 (101.5 ± 7.6%), to which no tetanus had been applied. Ten minutes after the induction of LTP in pathway P1, 0.3nA of hyperpolarising current was applied to the cell for 10 minutes. This caused a slight increase in the amplitude of evoked EPSPs in both pathways due to the increase in the electrochemical driving force across the cell membrane. When the current was removed, the potentiated pathway (P1) was observed to have declined back to baseline values (107.1 ± 18.2%, p<0.05 when compared to values at corresponding times in the control experiments; see below), whereas the control pathway (P2), had not changed (98.3 ± 8.6%, n=6). In a series of control experiments in which the
recording electrode was not loaded with D609, the potentiation of evoked EPSPs induced by a high frequency stimulation (202.6 ± 25.4%) was not altered after application of a ten minute hyperpolarising current (196.9 ± 26.5%, n=4).

4.2.5: Conclusions

The requirement of postsynaptic generation is a necessary characteristic for a retrograde messenger. Messengers which fail to show this may still be important in the mechanism by which LTP is induced and maintained, but they cannot be the much sought after retrograde messenger. The data presented here indicate that not only is AA required for the induction and expression of LTP, but that this production takes place postsynaptically. Furthermore the expression of LTP requires AA, as suggested by the earlier field recording experiments, to be mobilised even 10-20 minutes after LTP has been successfully established. These results are consistent with the retrograde messenger hypothesis.

4.3: Does AA enhance presynaptic release of neurotransmitter?

LTP is thought to be maintained, at least in part, by an increase in glutamate release (see introduction) mediated by a retrograde messenger such as AA. Since the number of synapses which participate in potentiation will necessarily be a small subset of all those present, such increases are hard to measure biochemically, and even when they are measured, it is hard to distinguish between metabolic and synaptic pools of glutamate. Another way to assess transmitter release is by measuring the frequency of spontaneous ‘mini’ EPSCs - postsynaptic depolarizations due to the spontaneous release of synaptic vesicles. This spontaneous activity is
independent of presynaptic action potentials, and is largely due to the spontaneous opening of presynaptic Ca^{2+} channels triggering release.

This series of experiments was performed on cultured hippocampal cells (rather than in slice) for two reasons. Detection of mini-EPSCs requires good seals, in order to get the noise sufficiently low to be able to resolve them. Patching in acute adult slice is difficult since the pyramidal cells are covered by layers of debris and glia which make obtaining a good seal very difficult; this is not the case in culture. The second reason again relates to the accessibility of the cells (though in this case it is an issue for the drug rather than the electrode). AA is a hydrophobic molecule with a tendency to partition into the lipid phase. When applied to a slice this would result in a situation where the top and bottom of the slice would see the full concentration of AA, but those cells in the middle (which is where recording is normally done) will see far less. In order to have any certain knowledge of the concentration of AA at the recording site, a system with good accessibility must be employed.

4.3.1: Spontaneous EPSCs versus mini-EPSCs

Even when unstimulated, some cells will generate action potentials, and so while recording from a cell, there are occasional EPSCs and IPSCs which can be detected. These have amplitudes in the range of 20-100pA (see figure 4.6). Bath application of TTX, however, abolishes these EPSCs, leaving only the much smaller unquantal mini-EPSCs, which have a median amplitude of about 5pA (see histogram fig 4.8). This discrepancy in amplitudes is likely to reflect the hyper-connectivity between cell in culture. In intact tissue, it is likely that connections are more discrete.
4.3.2: The frequency of mini-EPSCs, but not mini-IPSCs increases on perfusion with arachidonic acid.

In a series of four experiments, cells were recorded from in a medium containing 1μM TTX. After a 15 minute control period, AA was added to the chamber to a final concentration of 3μM. Recording then continued for an additional 15 minutes. Four fixed time points were taken from each of these periods, and digitised. They were then analysed blind, to eliminate any interpretational bias. Following the addition of AA, the frequency of spontaneous mini-EPSCs increased to 138 ± 3% (p<0.01 when compared to values before the addition of AA; see figure 4.7). This was not the case with IPSCs, which showed no significant change.

4.3.4: The observed increase in mini-EPSC frequency is not due to a change in the amplitude distribution of spontaneous events.

An observed increase in frequency can be easily interpreted in three ways. It could reflect a change in the probability of release in most synapses; a facilitation of multivesicular release; or an increase in postsynaptic receptor sensitivity. These last two could be seen as an increase in frequency as it is likely that there are events which were too small to resolve in this system. In order to differentiate between these models, I have performed a limited analysis of the distribution of amplitudes detected (see figure 4.8). Those amplitudes below 3pA were omitted as there was insufficient signal to noise resolution to allow an accurate measurement. A 30 second period was analysed before and after the addition of AA, and plotted against frequency. Although there is an increase in frequency at most amplitudes, the relative distribution of amplitudes remains constant, and when fitted to a Gaussian, the peak amplitude
Figure 4.6: (a) Spontaneous events recorded from a cultured hippocampal neurone using whole-cell voltage clamp. An example EPSC and IPSC is shown; these are of much greater amplitude than would be expected of single synapse responses.
(b) Largest response seen in the same cell after the addition of 1μM TTX
(c) Responses of a more typical size seen in 1μM TTX.
shows no shift. This pattern of distribution is incompatible with either an increase in multiquantal release or an increase in postsynaptic sensitivity, as both models would predict a shift of peak amplitude in the direction of higher currents.

4.3.4: Conclusions

These experiments were intended to investigate whether AA was capable of acting as a retrograde messenger at excitatory synapses in the hippocampus. They demonstrate that AA causes an apparent increase in the frequency of spontaneous mini-EPSCs, which is not associated with any change in their amplitude. This suggests that AA can indeed act to facilitate glutamate release at a physiologically relevant concentration (3μM).

The mechanism by which AA exerts this effect is unclear. Possible mechanisms would include membrane fluidity changes, modulation of the secretory apparatus, and enhancement of calcium influx. Fluidity changes seem an unlikely mechanism for this effect, since IPSCs are not affected. The most likely possibility is that AA interacts specifically with some part of the exocytotic pathway which is in some way distinct in excitatory nerve terminals.
Figure 4.7: (a) Pooled data from 4 experiments showing the time course of AA on the frequency of spontaneous mini EPSCs and IPSCs in the presence of 1µM TTX. 1 minute periods were analysed at the times shown. Values are normalised to the mean of the first four points. Following the addition of AA, the frequency of mini-EPSCs was increased, whereas the frequency of spontaneous IPSCs was unchanged. P<0.01 where indicated by double asterisks.

(b) When grouped on the basis of being before, or after, the addition of AA, a marked increase in mini-EPSC, but not IPSC, frequency can be seen. These groups are significantly different (p<0.001).
Figure 4.8: The frequency distributions of mini-EPSC amplitudes for periods from two experiments are shown. Solid bars represent number of minis of a given amplitude recorded in a 30 second period, open bars the amplitude distribution observed following addition of AA. Minis of less than 4pA were disregarded, as being too poorly resolved above noise to allow a meaningful measurement of amplitude. In both experiments, the frequency of mini-EPSCs was increased following addition of AA, whereas the modal amplitude (around 5pA) was unaffected.
Interactions between AA and postsynaptic $[\text{Ca}^{2+}]_i$

transients in cultured hippocampal cells

5.1: Introduction

In the previous chapter, data was presented which indicated that AA might have a specific role in the induction of LTP. Candidate mechanisms for this include interactions with the NMDA-receptor (to increase the entry of calcium through the channel), changes in calcium buffering (so that elevations are sustained for longer), and direct action of AA to cause release of calcium from internal stores. These are all ways by which $[\text{Ca}^{2+}]_i$ can be raised sufficiently to activate the long term changes necessary for LTP, such as activation of protein synthesis, gene transcription, and even the generation of a possible retrograde messenger.

To address these issues two fluorescence-based techniques have been used. In both cases the dye of choice was Fura-2, a ratiometric calcium sensing dye. An AM-ester form of the dye was used to allow loading of all cells on each cover slip. Two related techniques were used in these experiments: single cell photon counting and CCD imaging (see methods). Single-cell photon counting is a technique where a photo-multiplier tube is used to detect the arrival of photons from dye excited by a uv light source in a conventional fluorescence microscope. This light is restricted to that coming from a specific area of interest within the visible field (in this case, individual cells), by an adjustable aperture. In the other technique a CCD camera is used in place of the photomultiplier tube, allowing fluorescence from all cells in the field to be collected. This approach allows regional differences within cells (for example,
neurites versus somata) to be examined. All data was collected from hippocampal cells which had been in culture for 5-7 days.

5.2: Characterising \([\text{Ca}^{2+}]_i\) transients elicited by iontophoretic application of NMDA and AMPA

\([\text{Ca}^{2+}]_i\) transients were elicited in cultured hippocampal cells by brief pulses of the subtype-specific ionotropic-glutamate-receptor agonists AMPA and NMDA. The amplitudes of these responses were kept small (see methods) and high resting ratios, or the inability of cells to recover, was regarded as an indication of poor cell health. This discrimination was intended to minimise dye saturation and cytotoxic effects. Responses were assessed on the integrated area of the response.

5.2.1: NMDA responses show a slow rundown

NMDA was iontophoretically applied for 1 second from a pipette positioned about 10 μm from the soma. Driving current was applied to the pipette every thirty seconds. The \([\text{Ca}^{2+}]_i\) transients elicited in this way were rapid (<4 seconds to peak) and typically returned to baseline in about 30 seconds (see figure 5.1 for a typical series of responses recorded with the single-cell photon counting apparatus). The decay phase of the response could not be fitted to a simple exponential function. Although initially stable, responses showed a decremental decline over time, to 87.52 ± 23.03 on the eighth application, as shown in figure 5.5.
**Figure 5.1:** The effect of iontophoretically applied NMDA on $[\text{Ca}^{2+}]$, in hippocampal neurones maintained in culture. A representative experiment is shown. Eight 1 second pulses of NMDA were applied at the times indicated by the arrows. The responses were measured by calculating the area of each peak, above baseline. Although initially consistent, the responses show a slow rundown.
5.2.2: *NMDA rundown is enhanced in the absence of ultraviolet irradiation, an effect which is blocked by the nitric oxide synthase inhibitor L-nitro arginine*

In order to determine if this slow rundown of the NMDA-induced response was related to toxic effects of the ultraviolet (uv) irradiation, a slight modification of the protocol was adopted. After three stimuli had been applied, and the calcium signals had declined back to baseline, the irradiating light was blocked out with a shutter for 100 seconds (t=200 to 300 seconds). Thirty seconds after the light was restored (t=330) the stimulation resumed. Surprisingly, the responses now showed a much greater degree of rundown, the final response being 68.2 ± 7.8% of the average of the first three (n=10, p<0.05, see figure 5.2). Addition of 25μM L-nitro arginine (L-NA), an inhibitor of nitric-oxide synthase (NOS) abolished this effect (-6.4 ± 6.5%, n=9, figure 5.2).

5.2.3: *The majority of the calcium entry due to activation of the NMDA-receptor enters by voltage-dependent calcium channels*

In a separate series of experiments, the role of voltage-dependent calcium channels (VDCCs) in the generation of NMDA induced [Ca\(^{2+}\)] transients was assessed. In control experiments it was found that with very brief (300ms) applications of NMDA, the second of two responses 90 seconds (60 image pairs) apart showed modest desensitisation (77.0 ± 11.3%). Cd\(^{2+}\) is a non-subtype specific blocker of VDCCs (Bean 1989), which does not block the NMDA receptor ((Medina et al. 1994)). When added to the bath at a concentration of 10μM, Cd\(^{2+}\) blocked 68.1 ± 10.4% of the calcium response due to activation of NMDA receptors (figure 5.3). A slow (>60s before onset) rise in fluorescence was observed with Cd\(^{2+}\).
Figure 5.2: The effects of the nitric oxide synthase inhibitor L-Nitroarginine (L-NA) on $[\text{Ca}^{2+}]$, transients evoked by iontophoretic application of NMDA. Seven pulses of NMDA were applied, and the area of the responses were measured. After the third application of NMDA, the irradiating uv light was blanked off for 100 seconds. On resuming the repeated challenges with NMDA, the responses were found to show an enhanced rundown (open circles). The addition of L-NA at the beginning of the ‘dark period’, as indicated by the shaded bar, prevented this rundown (filled circles). Average of 9 experiments normalised to the first three responses.
Figure 5.3: The effect of 10μM Cd$^{2+}$ on NMDA- and AMPA-evoked [Ca$^{2+}$], transients. AMPA induced responses were abolished by Cd$^{2+}$, whereas NMDA-induced responses were reduced, but not blocked.
which is likely to be due to entry of the ion into the cell, and binding to Fura-2. This was accompanied by universal quenching of the 380nm fluorescence, but not 340nm - thus leading to an increase in ratio.

5.2.4: All entry of calcium due to activation of AMPA-receptors is blocked by Cd2+

With the same experimental protocol as described above, except with AMPA in the iontophoresis pipette, [Ca2+]i transients were monitored before and after the addition of 10μM Cd2+. A one second iontophoretic application of AMPA induced rapid (<2 seconds to peak), calcium transients which returned rapidly to baseline. Responses showed a modest desensitisation (89.9 ± 8.82) on the second application. Addition of 10μM Cd2+ completely abolished the effects of subsequent application of AMPA on [Ca2+]i, (94.43 ± 8.89% blockade, n=12 for AMPA alone, n=10 for AMPA + Cd2+; the second responses were significantly different, p<0.01) (see figure 5.3).

5.2.5: Conclusions

The aim of the experiments described so far in this chapter has been to characterise the transient elevation of [Ca2+]i elicited by iontophoretic application of the glutamatergic agonists AMPA and NMDA. There are two possible ways for activation of ionotropic receptors to lead to [Ca2+]i transients. Both receptors are highly permeant to Na+ when activated, and so their activation leads to postsynaptic depolarisation. This can activate VDCCs (Hille 1992), which will then allow Ca2+ into the cell. An alternative route of entry is through the receptor channel itself. NMDA receptors (Mayer and Westbrook 1987), also allow Ca2+ to pass enter the
cell. It has been estimated (Garuschuk et al. 1996) that 12% of the current through the NMDA receptor channel is carried by Ca$^{2+}$.

Activation of both ionotropic glutamate receptors leads to rapid [Ca$^{2+}$]i transients, which show consistent speed and decay rates. Both show some degree of rundown. Blanking off the uv irradiation caused a marked decline in the NMDA-induced response (an increase in this rundown) which was blocked by L-NA, an inhibitor of NOS. This suggests that activation of NOS by calcium, and the subsequent release of NO (which is very unstable in the presence of uv light), is responsible for the rundown of NMDA induced responses. Other groups have reported interactions between NO and the NMDA receptor (Lipton et al. 1993) and [Ca$^{2+}$], mediated NMDA rundown (Medina, Filippova et al. 1994); and photolytic release of caged NO causes a marked decline in NMDA-receptor mediated EPSPs (Murphy, Williams et al. 1994). The results presented here show a link between these various observations, and provide a mechanism for the known, Ca$^{2+}$-sensitive desensitisation of NMDA-receptor mediated responses.

The dependence of AMPA and NMDA-induced [Ca$^{2+}$]i transients on the entry of Ca$^{2+}$ through VDCCs was investigated using the non-subtype specific VDCC blocker, Cd$^{2+}$. The response elicited by AMPA was completely blocked by the addition of Cd$^{2+}$, whereas about 30% of the response induced by NMDA proved insensitive to this concentration of Cd$^{2+}$. AMPA receptors have different subunit compositions in different cells, and this makes a great difference to their function. The calcium permeability of AMPA receptors is largely regulated by GluR2 (Hollmann et al. 1991), which prevents calcium from entering. Since the AMPA receptors activated in this study show little calcium permeability, this indicates that the AMPA receptors present in these cultured cells incorporate the GluR2 subunit.
Equally, the effects of Cd²⁺ indicate that while some of the response induced by NMDA is due to direct Ca²⁺ entry through the NMDA receptor channel, most is due to the activation of VDCCs by depolarisation. Clearly, the balance between these two routes will be dependent on the magnitude of the NMDA response.

5.3: Investigating the effects of AA upon NMDA and AMPA-induced [Ca²⁺]ᵢ transients

Having established the normal pattern of NMDA- and AMPA-induced [Ca²⁺]ᵢ transients, it was then possible to examine the effect of AA on such responses. The protocol was much the same as that used for application of L-NA when the responses were being characterised. In order to test the specificity of the effect of AA, two other fatty acids closely related to AA were also used. These were eicosadienoic acid (C20:2) and eicosapentaenoic acid (C20:5). These fatty acids have the same length hydrocarbon backbone, and differ only in the number of *cis* carbon-carbon double bonds. AA possesses four double bonds, at positions 5, 8, 11, and 14. The C20:2 used had double bonds at 11 and 14, and the C20:5 has double bonds at positions 5, 8, 11, 14, and 17 (see fig 1.2). As a result these two fatty acids will share some of the structure of AA, and as one is less saturated than AA, and one more saturated, they will also stand either side of AA in their ability to fluidize membranes.

5.3.1: AA enhances NMDA-induced [Ca²⁺]ᵢ transients.

Cells loaded with fura-2-AM ester were repeatedly stimulated with NMDA applied by iontophoretic injection. Eight stimuli were given in all; three prior to the application of AA, and five following. The three stimuli before application of AA
were stable, and of consistent profile with those which had been previously characterised. In some cases (8 out of 14 cells) application of AA induced a small calcium response; this will be discussed in a later section.

Following application of AA, the integrated area of the first NMDA application was increased in size in 11 out of 14 cells. Pooling all 14 cells, gave an NMDA-induced response of 132.0 $\pm$ 12.8% when compared to control values. This was significantly greater than that seen in the absence of AA (-4.0 $\pm$ 5.3% see figure 5.5). The enhancement was due both to an increase in the peak height of the response and the area (see figure 5.4), although most of the increase in area was due to a reduction in the rate of decline of the response. The subsequent applications of NMDA elicited responses with declining magnitude; none of the four were significantly different from control responses in either height or area.

5.3.2: The increase in NMDA-induced responses is a specific response to AA, rather than an effect of general addition of fatty acids.

In order to assess the specificity of the interaction between AA and the NMDA-receptor, two other 20-carbon fatty acids were used, eicosadienoic acid, and eicosapentaenoic acid. The area of the first response following application of eicosadienoic acid showed no change from control (89.3% $\pm$ 8.2% n=7; see figure 5.6) and subsequent responses showed a similar profile of slow decline to that seen in control experiments (see figure 5.7). Eicosapentaenoic acid also had little effect on the area of responses (-7.8 $\pm$ 5.4%, n=7) other than a gradual decline.
Figure 5.4: The effect of 2μM AA on [Ca^{2+}], transients evoked by iontophoretic application of NMDA. (a) Enlarged [Ca^{2+}], transients evoked by iontophoretic application of NMDA, comparing the last response before the addition of AA, to the first following that addition. The response shows an increase in height and a slower decay from peak; both are factors in the increase in measured area.
(b) An individual experiment showing the effect of AA on [Ca^{2+}], transients evoked by iontophoretic application of NMDA. NMDA was applied at the times indicated by the arrows and the open bar indicates the period of application of AA.
Figure 5.5: The averaged response of hippocampal neurons to iontophoretic application of NMDA and its modulation by 2µM AA. (see fig 5.4). AA application is indicated by the shaded bar. Each point is the mean of 14 observations ± s.e.m.; *p<0.05, **p<0.01 by t-test. Lower panel shows change in baseline ratio over time (initial baseline ratio subtracted from ratio before each stimulus) for those experiments where AA was added after the third stimulus.
Figure 5.6: Neither eicosadienoic acid (C20:2) nor eicosapentaenoic acid (C20:5) significantly effect $[\text{Ca}^{2+}]_{i}$ transients evoked by iontophoretic application of NMDA.

(a) Data are the normalised mean of 7 experiments. Eight pulses of NMDA were applied as in previous experiments. After the third response, 2µM C20:2 was added to the recording chamber. Unlike AA, this had no potentiating effect.

(b) An equivalent series of experiments to those in (a), substituting C20:5 for C20:2; again data are a mean of 7 experiments.
Figure 5.7: Summary histogram comparing the effect of 2μM AA on [Ca^{2+}], transients evoked by iontophoretic application of NMDA, with the lack of apparent effect seen on application of c20:2 and C20:5.
5.3.3: Transient elevations of $[Ca^{2+}]_i$ due to application of AMPA are unaffected by AA

As more than half of the NMDA-induced $[Ca^{2+}]_i$ transient is due to entry of Calcium through VDCCs (see section 5.1), it was of interest to find the component enhanced by AA. As AMPA-induced $[Ca^{2+}]_i$ transients are mediated almost entirely by VDCCs, the effects of AA on these were assessed; this also allowed me to examine the glutamate receptor specificity of the effect. In a separate series of experiments, using CCD imaging, AMPA was iontophoretically applied before and after the addition of AA. Addition of 2μM AA caused a slight further decline in the response from 89.9 ± 8.1% (n=12), see previous section, to 80.6 ± 8.2% (n=9) which was not significant (see figure 5.8)

5.3.4: AA induces a transient increase in $[Ca^{2+}]_i$

In the experiments discussed in the previous section, AA was applied to the cells between applications of NMDA or AMPA. In 8/14 of experiments with photon counting, AA induced a short lived elevation in $[Ca^{2+}]_i$. Figure 5.9 shows pooled data from those cells which responded to AA with a calcium transient. Higher concentrations of AA appeared to give rise to a greater $[Ca^{2+}]_i$ transient, but also showed signs of cytotoxicity; this was not investigated further.

5.3.5: Conclusions

Entry of calcium into the postsynaptic cell as result of NMDA-receptor activation is the central event in the induction of LTP. Since AA has also been shown to be necessary for the induction of LTP (see previous chapter) one mechanism for
**Figure 5.8:** 2μM AA has no effect on [Ca\(^{2+}\)]\(i\) transients evoked by iontophoretic application of AMPA. [Ca\(^{2+}\)]\(i\) transients before and after the addition of AA were compared. In contrast to the result seen with NMDA, there was no enhancement of the response, rather a small depression was observed, but this was not significant.
NMDA AMPA

% change

50 25 0 -25

(D D) c (D ü 0 ')

126
Figure 5.9: The effect of 2μM AA on [Ca\(^{2+}\)]. In some of those experiments where AA was added to the recording chamber, it elicited an elevation in [Ca\(^{2+}\)]. Results for those cells that responded to the addition of AA are shown (8/14=57%). AA was added for the period indicated by the hatched bar (n=8).
this would be an interaction with the NMDA receptor such as that seen by Miller et al (1992) cerebellar granule cells. Since it is the calcium which appears to be important, rather than simply the current passing through the receptor channel, it seemed worthwhile to investigate the impact of AA on NMDA-induced [Ca^{2+}], transients in cultured hippocampal neurones.

2μM AA exerts a potentiating action on the NMDA-induced entry of calcium, and this is not seen on application of either eicosapentaenoic acid, or eicosadienoic acid, two fatty acids which are very closely related to AA. [Ca^{2+}], transients due to application of AMPA did not show a similar enhancement on application of AA, indicating that the effect is specific to the NMDA-receptor. Since all the calcium which enters the cell as a result of AMPA-receptor activation seems to be due to VDCCs (see previous section) the enhancement of NMDA-induced responses cannot be due to an action of AA on N or L-type VDCCs, which are both blocked by Cd^{2+} (Bean, 1989). It seems likely then that the mechanism by which AA enhances NMDA-induced calcium entry is due to a specific interaction with the NMDA-receptor complex, increasing the current passing through the receptor in a non-ion specific manner. The greater depolarisation due to this enhanced current will activate more VDCCs and consequently more calcium entry. The increased calcium entry is thus due to an increase in the amount entering both through VDCCs and through the NMDA-receptor.
5.4: mGluR activation and interactions with the NMDA-receptor.

In considering ways in which AA might interact with the induction of LTP, means of stimulation of AA mobilisation must also be considered. Tonic release of AA continuously enhancing the NMDA response was ruled out by the observation that perfusion with BPB, at a concentration sufficient to block LTP, had no effect on NMDA-receptor mediated EPSPs (see chapter 4). A logical place to look for an AA mobilising signal would then be the metabotropic glutamate receptors, which have been implicated in LTP (Bashir et al 1993). Secretory PLA$_2$ has been shown to be activated by DAG (Kolesnick & Paley 1987), which is produced by activation of Group I mGluRs. To investigate this as a possibility, NMDA-induced [Ca$^{2+}$]$_i$ transients before and after application of ACPD (an agonist at Group I and V mGluRs) were monitored, as described previously.

5.4.1: ACPD induces a dose-dependent elevation in [Ca$^{2+}$]$_i$

Three concentrations of $\text{(1S-3R)}$ ACPD were used in this series of experiments, since the responses to this agonist tend to show wide variance; some cells responding strongly, whilst others fail to do so even with very high agonist concentrations (Mathie and Richards 1997). The concentrations used were 25, 50 and 100µM, and were applied between iontophoretic applications of NMDA. 100µM ACPD caused a large response in all cells (see figure 5.10). This showed a fast peak followed by a slow decline to approximately half peak, and a subsequent plateau phase in many of the cells (7 out of 11 cells). This profile was consistent with that seen in cortical cells (Mathie and Richards 1997). 50µM ACPD elicited a more mixed
Figure 5.10: Averaged traces for the effects of (1S-3R) ACPD (25, 50 and 100μM) on [Ca$^{2+}$], and on transients evoked by iontophoretic application of NMDA. 25μM elicits a slight, transient elevation in [Ca$^{2+}$], which rapidly returns to values from shortly before the addition. 50μM evokes a larger elevation in [Ca$^{2+}$], which appears not to quite return to baseline levels. 100μM elicits a large elevation in [Ca$^{2+}$], which decays to a plateau level, which is still elevated relative to the original resting value at the end of the experiment.
response. Some cells showed the peak/plateau (4 out of 10 cells), some responded, but failed to maintain a plateau (3 out of 10 cells), even though they appeared to possess some $[Ca^{2+}]_i$ oscillations, and some showed no response (3 out of 10 cells).

25µM ACPD produced a response which was weaker still. No cells showed a plateau phase, although 5 out of 12 cells showed small $[Ca^{2+}]_i$ transients in response to the agonist.

5.4.2: ACPD can modulate NMDA-induced $[Ca^{2+}]_i$ transients

In the above experiments, NMDA was applied before and after the challenge with ACPD. Responses prior to application of ACPD were comparable to those used in other experiments. As before, data are expressed as percent of pre-treated response.

100µM ACPD proved to be hard to work with for these experiments since the decay phase of the ACPD stimulation was still occurring when NMDA was being applied. Extrapolated baselines however, gave some measure of the NMDA-induced response. In these experiments treatment with ACPD reduced the second response to 59.9 ± 14.4% (n=10). There appeared to be two populations even within these experiments; one centred about 40%, and one around 110% (see figure 5.11)

Reduction of the concentration of ACPD to 50µM made the data easier to analyse. Overall, the NMDA response showed no increase (106.3 ± 11.86%, n=10). The data again showed signs of containing two populations, and when split around 100%, formed two groups of 5, which showed much tighter standard deviations (76.2 ± 6.8%, and 136.6 ± 11.55%). These two groups were significantly different (p<0.05). When plotted against the magnitude of $[Ca^{2+}]_i$ responses induced by ACPD, there proved to be no correlation. 25µM ACPD showed a similar sort of pattern of
Figure 5.11: Breakdown of the $[\text{Ca}^{2+}]_i$ transients evoked by iontophoretic application of NMDA, after application of (1S,3R) ACPD. The distribution of responses seen at all 3 concentrations of ACPD used (25, 50 and 100µM) are suggestive of multiple populations; perhaps one enhanced, and the other depressed. Data were first normalised to values prior to application of ACPD.
responses; an overall effect of 93 ± 13.3% (n=11) hid (at least) two apparent populations. When split about 100%, they gave very different values with reduced standard deviations (69.9 ± 6.8%, n=8, and 155.3 ± 13.3%, n=3, see fig 5.11). Again these were statistically different (p<0.001).

5.4.3: Conclusions

The conclusions which can be derived from this work fall into two categories: direct consequences for [Ca^{2+}]i on activation of mGluRs, and indirect consequences, through modulation of NMDA-induced [Ca^{2+}]i. It is well known that group I mGluRs are coupled to PI-PLC, so that activation of these receptors will release IP3 and DAG. IP3 releases calcium from internal stores through activation of the IP3 receptor. The results described here are consistent with those seen in cortical cells; at 100μM, ACPD activates the vast majority of neurones, and in most cases gives rise to a long, plateau phase of [Ca^{2+}]i elevation (Mathie & Richards, 1997). Reducing the agonist concentration reduces the magnitude of the response, and the probability of any cell responding. The dose response data are consistent with those seen in cortical cells.

The interaction between mGluR activation and NMDA-induced [Ca^{2+}]i transients appears to be as complex, and mediated by a different route. Multiple populations of interaction appear to be present, both enhancing and diminishing the response. This may suggest an involvement of NOS. As seen earlier in this chapter, [Ca^{2+}]i dependent activation of NOS seems to cause a rundown of the NMDA response, just as AA causes an increase. At 100μM ACPD, the dominant population of responses is depressed. At 50μM, the responses seem to split evenly into two
groups; slightly depressed, and enhanced. 25μM ACPD also showed an apparent grouping into two, again, slightly depressed, and enhanced.

Analysis of data which contains sub-populations is always problematical, but in this case the sub-groupings are very believable, particularly as no such grouping is seen in control experiments (see section characterising the responses). The simplest explanation for the subgroups is activation of both NOS and sPLA₂. NOS is activated by the [Ca^{2+}] transient elicited by (1S-3R) ACPD, whereas the enhancement of NMDA responses appears to be independent of this. This might mean that the enhancement relies on a different pathway to PI-PLC, or simply that activation of sPLA₂ by DAG simply has a very different affinity to that shown by the IP₃ receptor for IP₃.
General Discussion

6.1: Overall summary

Does arachidonic acid have a role in the potentiation of synapses in area CA1?

The aim of these experiments was to address the discrepancy between the prolonged increase in levels of AA observed following the induction of LTP (Bliss, Errington et al. 1990) and those experiments showing that blockade of AA mobilisation by BPB prevents the induction but not the expression of LTP. I have shown that the release of AA in LTP is catalysed by three distinct enzymes, in a manner which appears to be biphasic. The induction of LTP is blocked by application of BPB, an sPLA₂ inhibitor. Pre-existing LTP is not, however, affected by BPB. Thus during the first few minutes of LTP, sPLA₂ activity is crucial, but its role is limited to some period shorter than the first fifteen minutes. AA retains its importance in the later phases of LTP, but is released through a different route. The expression of this later phase of LTP is vulnerable to the inhibition of the AA-mobilizing enzymes cPLA₂, DAG lipase and PC-PLC. For PC-PLC at least, this activity takes place in the postsynaptic cell.

Does arachidonic acid act at presynaptically or postsynaptically?

Although there is no direct evidence for a postsynaptic role for AA, the data presented here suggests that this is likely, at least under certain circumstances. AA is capable of transiently elevating [Ca^{2+}], directly (which could occur either pre- or postsynaptically), but is also capable of enhancing the [Ca^{2+}], transient due to
stimulation by NMDA. In the absence of identified presynaptic NMDA-Rs in the hippocampus, this would appear to be a solely postsynaptic action.

In order to examine possible presynaptic actions of AA, I have recorded the whole-cell currents generated by spontaneous release of synaptic vesicles (mini-EPSCs). The frequency at which this occurs is a wholly presynaptic measure, whereas analysis of the amplitude of mini-EPSCs is more complex, since it is being detected by potentially heterogeneous receptor sites. On the basis of frequency analysis, AA increases random release of synaptic vesicles; this suggests that AA is capable of acting as a retrograde messenger.

*Antagonistic effects of NO on NMDA-receptors*

Nitric oxide is a compound which has been the subject of great interest as an inter-neuronal (Garthwaite 1993), and potential retrograde messenger (Schuman and Madison 1991). Although the present study does not address this issue directly, it does provide evidence that NO, generated as a result of calcium activation of NOS, will reduce further entry of calcium via the NMDA receptor. This is consistent with some other reports, showing that NO can block the induction of LTP (Izumi, Clifford et al. 1992), and that photolytic application of NO depresses NMDA-mediated field-potential EPSPs.

6.2: Are the drugs used selective?

The interpretation of these results depends on the selectivity of the drugs used. On the basis of the biochemical experiments presented here, I can argue that only RHC80267 affects DAG lipase activity. All three drugs inhibit AA mobilisation.
from phosphatidyl choline, but it is likely that the action of RHC80267 is on residual DAG lipase activity, which will release AA from any DAG produced by PC-PLC activity. This argument is strongly supported by the demonstration that the effect of D609, a PC-PLC inhibitor, is both equivalent to, and occluded by RHC80267. Thus the two enzymes are blocking different points on the PC-PLC/DAG lipase pathway, and do not affect either sPLA$_2$ or cPLA$_2$. The pharmacological profiles of BPB and AACOCF$_3$ are sufficiently different with respect to their effects on both LTP, and AA mobilisation from phosphatidyl choline, to indicate that there is no common action of these two inhibitors.

The rationale for investigating the calcium dependency of arachidonate mobilisation from phosphatidyl choline lies in the fact that sPLA$_2$ and cPLA$_2$, whilst both being calcium sensitive, show very different affinities for calcium. The amino acid sequence of cPLA$_2$ contains a calcium binding motif which possesses high homology with the equivalent domain in PKC (Clark, Lin et al. 1991). Like PKC, the role of this calcium binding motif is to cause a translocation of the enzyme from the cytoplasm to the plasma and ER membranes, where its substrates are to be found. This has been demonstrated for cPLA$_2$ by (Huang et al. 1994). The result of this is that cPLA$_2$ possesses a high affinity for, and sensitivity to calcium, and so at the concentration of 100mM used, cPLA$_2$ will have been subject to its maximal activation by calcium. In contrast to the role calcium plays in cPLA$_2$, it act as an obligate cofactor in the reaction catalysed by sPLA$_2$ (Yu et al. 1993) and maximal activity is not reached until the calcium concentration reaches around 1-2mM. Whilst it did not prove possible to entirely separate the two types of PLA$_2$ activity, the results show that BPB shows little activity against cPLA$_2$ and AACOCF$_3$ shows little inhibition of
sPLA₂ activity. This is in keeping with results reported by Street et al (Street, Lin et al. 1993).

An additional area where drug specificity must be considered carefully is interactions between the compounds, and receptors such as the NMDA-R which are essential for LTP. Since BPB was the only drug for which this could be plausible mechanism (it being the only compound to block the induction of LTP whilst not affecting expression), I have examined the effect of 50μM BPB on pharmacologically isolated NMDA-mediated field potentials. This showed no sign of any interaction.

6.3: Interactions between AA and [Ca²⁺]ᵢ

In chapter five of this work, I present data indicating that AA can participate in the transient elevation of [Ca²⁺]ᵢ, both directly, and by enhancing calcium entry mediated by the NMDA-R. Calcium entry due to activation of the NMDA-R is believed to be the essential first step in the induction of LTP, and so an enhancement of this might well alter the ability of a synapse to become potentiated. This may explain the role of sPLA₂ in the induction of LTP, a topic which will be discussed more fully in section 6.5.

The observation that AA alone can raise [Ca²⁺]ᵢ in at least a subset of cells may also have some bearing on the role of AA in the induction of LTP. It is not yet clear what profile of elevation of [Ca²⁺]ᵢ is necessary for the induction of LTP. Since the small [Ca²⁺]ᵢ transients elicited by normal (non-NMDA-dependent) synaptic transmission are insufficient to induce LTP, it seems that some form of coding of the
calcium response is necessary. This might be either the amplitude or the duration of the response, or even both (frequency seems unlikely given the variety of stimulus regimes capable of inducing LTP). Adding an additional route of elevating $[\text{Ca}^{2+}]_i$ would perhaps make LTP 'easier' to induce. In other words, a conditioning stimulus which ordinarily would fail to induce LTP, would, with an additional source of calcium, become sufficient.

**6.4: NMDA-receptor rundown and NO**

On repeated application of NMDA to cultured hippocampal neurones, the resultant calcium transients show a decline. Previous studies have shown that elevation of $[\text{Ca}^{2+}]_i$ will inactivate the NMDA channel (Legondre et al. 1993), and (Medina, Filippova et al. 1994) have shown that prior application of kainate will inactivate NMDA-currents, in a Cd$^{2+}$, and BAPTA sensitive manner, which would implicate entry of calcium through VDCCs. It seems likely that the data presented in chapter five reflects a similar $[\text{Ca}^{2+}]_i$-dependent inactivation. A possible mechanism for this would be the $[\text{Ca}^{2+}]_i$-stimulated activation of NOS. NO has been shown to depress NMDA mediated currents (Lipton et al. 1993; Murphy et al. 1994). This has been given further support by the observation that inhibition of NOS by L-NA prevents this $[\text{Ca}^{2+}]_i$-dependent inactivation. Although this provides further evidence for NO as a signalling molecule in neurones, it seems inconsistent with a role for NO as a retrograde messenger in LTP, since it would tend to prevent the induction of LTP, rather than facilitate it. It seems then that AA and NO have antagonistic effects at the NMDA receptor. A simple scheme for this is shown in figure 6.1.
Figure 6.1: A simple scheme for the modulation of NMDA-induced 
$[Ca^{2+}]_i$ transients in hippocampal neurones. $Ca^{2+}$ influx through the NMDA channel can activate both phospholipase A$_2$ (PLA$_2$) and nitric oxide synthase (NOS). The resulting generation of NO leads to a rundown of the $[Ca^{2+}]_i$ transient evoked by NMDA. Activation of PLA$_2$ generates AA which acts in an antagonistic way, enhancing the NMDA-evoked $[Ca^{2+}]_i$ transient.
6.5: The role of AA in the induction of LTP

To a certain extent, the puzzle of the role of AA in LTP is not how it might act to support the expression of LTP - this role is that proposed for a retrograde messenger more than a decade ago (Bliss et al. 1986) - but why sPLA₂ activity is necessary for the induction of LTP, and not later.

Activation of NMDA-receptors has been demonstrated to be the central step in the induction of LTP (Collingridge et al. 1983), presumably due to the entry of calcium into the postsynaptic cell. In order to explain how activation of sPLA₂ might fit in with this, there are broadly two ways one might go. If AA acts as a retrograde messenger, then it might be that an initial surge of AA is required at the induction of LTP in order to raise AA levels to that sufficient for the expression of LTP. The other enzymes which have been implicated in the production of AA for LTP would then merely have to maintain these levels against diffusion and re-incorporation into cell membranes. This seems unlikely, as one would then predict that if LTP was blocked by an sPLA₂ inhibitor, it would show a very slow onset potentiation instead of the normal abrupt enhancement of synaptic strength. This was not seen.

An alternative explanation is that AA might actually participate in the postsynaptic induction process itself. It has been shown that NMDA-receptor currents are potentiated by AA (Miller et al., 1992), and here I present data showing a corresponding increase in NMDA-induced [Ca^{2+}]\textsubscript{i} transients. (O'Dell, Hawkins et al. 1991) have shown that application of AA can convert a subthreshold tetanus to one sufficient for the induction of LTP, and that this process is NMDA-receptor dependent. This suggests that interactions between AA and the NMDA-R are functionally significant, at least in the \textit{in vitro} slice preparation.
To allow an interaction between AA and the NMDA receptor, however, AA mobilisation must be rapidly stimulated. A possible route might be by DAG (Kolesnick and Paley 1987) generated by activation of metabotropic glutamate receptors, which have also been linked with LTP (Bashir et al. 1993). While this could explain a role for AA in LTP induced by gentle, patterned stimulation which often takes tens of seconds (see methods) or longer with some 'theta-burst' type stimulations, Okada et al. (1989) showed that LTP induced by 100Hz for 1 second was also sensitive to inhibition of sPLA$_2$ by BPB. This seems too short a time window for activation of a multistage enzymatic pathway, and I have shown that there is no sign that sPLA$_2$ mediates tonic production of AA - which would be rapidly down regulated by sPLA$_2$ inhibitors - in order to enhance NMDA-R responses. Two additional possibilities present themselves; AA is known to raise [Ca$^{2+}$], in certain cell-types (Murthy et al. 1995; Tsunoda et al. 1996), including the hippocampus (see section 5.3.4), and AA and DAG have been shown to synergistically activate PKC in the hippocampus (Bramham et al. 1994).

6.6: A possible link between AA and a [Ca$^{2+}$]$_i$ threshold for the induction of LTP

Calcium is a well characterised signal for all sorts of cellular processes, but its very ubiquity raises additional difficulties; how can the cell distinguish between one sort of [Ca$^{2+}$]$_i$ transient and another? Evidence has been accumulating that these [Ca$^{2+}$]$_i$ signals are coded by their temporal and spatial characteristics, as well as by amplitude (see Berridge (1997) for a brief recent review). The requirement for AA in the induction of LTP is consistent with there being a level above which [Ca$^{2+}$]$_i$ must
be raised for the induction of LTP, which might include a minimum length of time for that elevation to be sustained; a 'Calcium threshold'.

There are three ways by which $[\text{Ca}^{2+}]_i$ can be raised in neurones; depolarisation will activate voltage-dependent calcium channels (VDCCs), NMDA-receptor activation (coupled with depolarisation to remove the $\text{Mg}^{2+}$ block) also allows calcium influx, and calcium can be released from internal stores. We have seen no evidence that AA affects depolarisation induced calcium transients (induced by AMPA - see section 5.2) but, as has already been discussed, AA does enhance NMDA-receptor mediated currents, and can elevate $[\text{Ca}^{2+}]_i$, directly. These factors suggest that the requirement for AA in the induction of LTP (initially via an sPLA$_2$-dependent route) may be a consequence of a fundamental dependence of the induction of LTP on levels of postsynaptic $[\text{Ca}^{2+}]_i$.

6.7: Could arachidonic acid be a retrograde messenger in LTP?

Since the first reports consistent with a role for arachidonic acid in LTP were published (Lynch et al. 1988; Williams and Bliss 1988), few details of the mechanism of action of arachidonic acid in this system have been established. Equally, the mechanism by which arachidonate mobilisation is stimulated has not been clearly demonstrated. At the time of the earlier work on PLA$_2$ activity and LTP, the novel cPLA$_2$ had not yet been described and DAG lipase activity was not investigated. These problems made interpretation of the data difficult.

Additional support for a possible involvement of arachidonate in LTP came from reports that stimulation of the NMDA receptor leads to a calcium dependent
release of arachidonate from striatal neurones (Dumuis, Sebben et al. 1988),
cerebellar granule neurones (Lazarewicz, et al. 1988; Lazarewicz, et al. 1990) and
hippocampal neurones (Sanfeliu, et al. 1990; Pellerin and Wolfe 1991). Additionally,
it has been shown that joint stimulation of NMDA and quisqualate sensitive
metabotropic glutamate receptors causes much greater release of arachidonate
(Dumuis, et al. 1990) than either agonist alone.

(Williams, et al. 1989) have shown that application of arachidonate to an in
vitro hippocampal slice, induces an activity dependent slow onset potentiation of the
EPSP, which was insensitive to the NMDA receptor antagonist, AP5. However,
(O'Dell et al. 1991) failed to reproduce this, observing instead a rapid activity
dependent potentiation, which was AP5 sensitive, suggesting that it is mediated
through the interaction between arachidonic acid and NMDA receptors. Despite this,
there have been reports that coapplication of (1S,3R) ACPD and arachidonate result
in increased release of glutamate from synaptosomes (Lynch and Voss 1990; Herrero
et al. 1992; Lynch and Voss 1994). In hippocampal slice, coapplication of
arachidonic acid and (1S 3R) ACPD leads to an LTP-like potentiation. (Collins et al.
1995) These findings suggest that arachidonic acid could be responsible for inducing
and maintaining a synapse specific increase in stimulated glutamate release.

Analysis of the effect of AA on the frequency of spontaneous miniature
EPSCs reported here, supports the notion that AA might act to stimulate transmitter
release. It is thought that most of the spontaneous vesicle fusion events that occur at
synapses are due to the spontaneous opening of presynaptic calcium channels. In
order to enhance this, there are many possible mechanisms. AA might act by
changing the fluidity of the terminal and vesicle membranes, so that it is more
energetically favourable for fusion to occur. This would be a likely mechanism were
it not for the fact that the frequency of mini-IPSCs was unaffected by AA. Two
dominant alternatives exist; that AA in some way enhances entry of calcium through
the presynaptic calcium channels, or that AA might change the sensitivity of the
calcium sensor which stimulates exocytosis. Although the insensitivity of mini-IPSCs
to AA argues against a direct interaction with presynaptic calcium channels, it does
not rule out less direct means, such as activation of a kinase or phosphatase cascade.
One possible route might be Protein Kinase C (PKC), which has been implicated in
both pre- and postsynaptic components of LTP (Malinow et al. 1989; Huang et al.
1992). PKC is known to be synergistically activated by DAG and AA (Lester et al.
1991; Chen and Murakami 1992), and this synergism has been shown to play a role in
LTP (Bramham, Alkon et al. 1994). A way in which this synergism might act
presynaptically has been suggested by the finding that AA induces PKC-mediated
phosphorylation of GAP-43, a protein associated with synaptic growth (Schaechter
and Benowitz 1993).

I have suggested that a change in the sensitivity of the presynaptic calcium
sensor might also account for the observed increase in mini-EPSCs. It is unclear
whether exocytosis can be triggered by the brief elevation of $[\text{Ca}^{2+}]$, seen in the
immediate vicinity of an open calcium channel; in some synapses, such as the squid
giant axon, introduction of slow calcium chelators such as EGTA has little impact on
synaptic transmission, suggesting that the sensor is located very close to the opening
calcium channels (Adler et al. 1991). In contrast Borst and Sakmann (1996) have
shown in the rat medial nucleus of the trapezoid body, that relatively low
concentrations of EGTA can impair transmission. These conflicts make it difficult to
assess the extent to which the kinetics of calcium binding to the presynaptic sensor
might affect transmitter release. A candidate for the role of calcium sensor has been
identified however, synaptotagmin is a synaptic vesicle associated protein which 
undergoes a conformational change on binding calcium (Brose et al. 1992). This 
protein is only able to bind calcium when incorporated into a vesicle surface; 
phospholipids are essential for this. It seems possible that AA could thus influence the 
functioning of synaptotagmin by changing the environment within which it sits. A 
demonstration that AA can enhance transmitter release in intact, functioning, 
excitatory synapses derived from the rat hippocampus, thus opens more questions 
than it answers.

6.8: How might arachidonic acid mobilisation be stimulated in LTP?

In order to fit my results in with what is already known about LTP, I propose 
the following model for the mechanism of induction and maintenance of the first two 
to three hours of LTP (figure 6.2).

Entry of calcium into the postsynaptic cell via the NMDA receptor has been 
inferred to be the central step in the induction of LTP (Collingridge et al. 1983). 
However, there has been a lot of recent interest in the involvement of mGluRs in LTP 
(Bashir et al. 1993; Riedel et al. 1994; Collins et al. 1995), but this is not 
uncontroversial (Chinestra et al. 1993; Brown et al. 1994; Manzoni et al. 1994; Selig 
et al. 1995; Thomas and O'Dell 1995). The model which I would like to propose to 
explain the results presented here is one which invokes co-activation of postsynaptic 
mGluRs and NMDA receptors. This co-activation is required for the elevation of 
$[Ca^{2+}]$, above a specific threshold; be it triggered by the amplitude of the
Figure 6.2: Scheme for the role of AA in the induction and expression of LTP. Activation of metabotropic glutamate receptors may lead to activation of sPLA$_2$, and so to generation of AA. AA could act in the induction of LTP in two ways; enhancing the entry of Ca$^{2+}$ due to activation of the NMDA-receptor, or interacting with IP$_3$-mediated Ca$^{2+}$ release. The elevated [Ca$^{2+}$], generated from this stage is the critical step for the induction of LTP, leading to activation of the enzymes and kinases responsible for the subsequent expression of LTP. AA generated from cPLA$_2$, and from the PC-PLC/DAG-lipase pathway is involved in the expression of LTP, potentially as a retrograde messenger.
Induction and Expression

Presynaptic facilitation

mGluR → sPLA2 → AA → DAG → NMDAR → Ca^{2+} → IP_3

PI-PLC → DAG-lipase → DAG → PC-PLC → Kinases

Induction
signal or its duration. Other means of elevating \([Ca^{2+}]\), to a similar level or duration may bypass the need for this dual activation.

It has been demonstrated that activation of type I mGluRs activates PI metabolism, generating \(IP_3\) and DAG (for a recent review see Pin and Bockaert (1995)) DAG is known to activate sPLA\(_2\) (Kolesnick and Paley 1987), and as already discussed, arachidonate exerts a potentiating effect on the NMDA receptor. Thus the induction of LTP may rely on arachidonate production, stimulated through mGluRs, feeding back upon the NMDA receptor to enhance the entry of calcium into the postsynaptic compartment. This enhanced entry of calcium would be then above the critical threshold for the induction of LTP. As a result, a number of kinases would be activated, leading to the activation of cPLA\(_2\) (possibly by MAP kinase; Lin et al. (1993) and PC-PLC (Schutze, Potthoff et al. 1992) (producing DAG from which DAG lipase can produce further arachidonic acid) which produces the 'steady phase' of arachidonate production. The arachidonic acid produced at this slightly later phase, we would suggest, is that which may be considered to act as a retrograde messenger, acting co-operatively with glutamate feedback to enhance transmitter release. This is a refinement of the hypothesis proposed by Lynch et al. (1988), where they proposed that PLA\(_2\) might produce arachidonic acid at the induction of LTP, and that further arachidonic acid could be produced by glutamate stimulation of PI turnover followed by DAG lipase activity.

In the past it has been suggested that the finding by O'Dell et al. (1991), that although AA coupled with low frequency stimulation can lead to an LTP-like phenomenon, this enhancement is still sensitive to the NMDA-R antagonist AP5, is incompatible with the notion that AA might act as a retrograde messenger. With this revised model for the actions of arachidonic acid in LTP, the results of O'Dell et al
can be re-examined. I would suggest that the AP5 sensitivity of the effect resulted from insufficient levels of arachidonic acid being present at the synapse (possibly as a result of the experiment being carried out in brain-slice). Consequently, the main action of arachidonic acid was to potentiate the NMDA receptor, and enhance the potency of a low frequency train to the extent that it was capable of inducing LTP. Subsequent to this event, it would seem that the normal routes of arachidonic acid mobilisation would be stimulated, and LTP would be maintained in a normal cPLA$_2$ and DAG lipase-dependent fashion.

6.9: In Conclusion.

The results presented here are consistent with a role for arachidonic acid as a facilitator of the induction of LTP, and a retrograde messenger in the maintenance of LTP. They resolve the discrepancy between the prolonged increase in arachidonic acid concentration observed following LTP, and the observation that BPB and other drugs are ineffective at blocking LTP after the induction step. I have proposed a model where arachidonic acid derived from different sources has different temporal roles in LTP.
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Appendix

The composition of SF1C medium

SF1C medium is a “serum-free” defined medium of the following composition:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum essential medium (MEM)</td>
<td>45 ml</td>
</tr>
<tr>
<td>Dulbecco’s modification of MEM</td>
<td>40 ml</td>
</tr>
<tr>
<td>Ham’s F12 medium</td>
<td>10 ml</td>
</tr>
<tr>
<td>Glutamine (200 mM)</td>
<td>0.25 ml</td>
</tr>
<tr>
<td>Bovine serum albumin (Fatty-acid free) (10%)</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Fetal bovine serum</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Catalase</td>
<td>50 μl</td>
</tr>
<tr>
<td>Insulin/transferrin/selenium</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>5FU/U mixture</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Di Porzio mixture</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

2 ml of standard penicillin-streptomycin solution is added to each 100 ml of SF1C medium before use.

*5FU/U mixture* is a mitotic inhibitor. To make 1 ml of 1000 x stock:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-fluoro-2-deoxyuridine</td>
<td>6.7 mg</td>
</tr>
<tr>
<td>Uridine</td>
<td>16.5 mg</td>
</tr>
</tbody>
</table>

Use at 0.1 ml per 100 ml of medium.
**Di Porzio mixture.**

Stock solutions:

- **Progesterone** (water soluble form) \(1.25 \text{ mg.ml}^{-1}\) 12.5 mg in 10 ml water
- **Hydrocortisone** (water soluble form) \(2.00 \text{ mg.ml}^{-1}\) 20 mg in 10 ml water
- **Triiodothyronine** \(1.00 \text{ mg.ml}^{-1}\) 5 mg in 5 ml 20 mM NaOH
- **Putrescine** \(20.0 \text{ mg.ml}^{-1}\) 100 mg in 5 ml Hanks’ solution
- **Superoxide dismutase** 20,000 units.ml\(^{-1}\) 18 mg in 3.6 ml Hanks’ soln.
- **Transferrin** \(20 \text{ mg.ml}^{-1}\) 100 mg in 5 ml Hanks’ solution.

To make 10 x 1 ml aliquots of the mixture:

- Progesterone 0.05 ml
- Hydrocortisone 0.02 ml.
- Triiodothyronine 0.02 ml.
- Putrescine 0.12 ml.
- Superoxide dismutase 1.0 ml.
- Transferrin 5.0 ml.
- Hanks’ balanced salt solution 3.79 ml.

All items are available from Sigma, Gibco, Calbiochem and other suppliers of tissue culture materials.