Modulation of synaptic plasticity in area CA1 of rat hippocampus by purines and a putatively novel protein.

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Abstract.

1. The effects of adenosine agonists and antagonists were examined on population excitatory postsynaptic potential (population EPSP) slopes, population spike (PS) amplitudes and the relationship between the two i.e. EPSP-spike (E-S) coupling. Activation or blockade of adenosine A_{2A} receptors responses, evoked by stimulation in the stratum radiatum, had no effect on these parameters. However, activation of the adenosine A_1 receptor using N^6-cyclopentyladenosine (CPA) resulted in a significant decrease in both population EPSP and population spike.

2. When adenosine A_1 receptors were activated using CPA (50 nM) the decrease in population spike amplitude was greater than could be accounted for by the decrease in population EPSP, resulting in a dissociation in the EPSP-spike relationship as measured by a right-shift in the E-S curve or a decrease in the ratio PS/EPSP.

3. When adenosine A_1 and A_{2A} receptors were activated at the same time using CPA and 2-[p-(carboxyethyl)-phenylethylamino]-5'-N-ethylcarboxamidoadenosine (CGS 21680) respectively, the depression of the E-S relationship was significantly attenuated. This was due to a mainly postsynaptic effect whereby activation of A_{2A} receptors significantly attenuated the postsynaptic elevation of action potential threshold by adenosine A_1 receptor activation.

4. The use of a variety of signal transduction modulators suggested that adenylate cyclase, protein kinases or nitric oxide were not involved in the interaction between A_2 and A_1 receptors.

5. Blocking potassium channels with barium or glibenclamide reduced the inhibitory effects of CPA on population spike amplitude but not population EPSP slope and attenuated the inhibitory effects of adenosine A_1 receptor activation on E-S coupling in a similar manner to adenosine A_{2A} receptor activation. The increases in the ratio PS/EPSP seen when A_{2A} receptors were activated with CGS 21680 or when potassium channels were blocked with barium were not significantly different, suggesting that a change in postsynaptic potassium conductance may underlie the antagonistic effect of adenosine A_{2A} receptor activation on A_1-mediated responses.

6. Application of ATP 10 μM to hippocampal slices initially decreased population spike amplitude and then produced long-term potentiation (LTP). 2.5 μM ATP also resulted in LTP of responses although did not inhibit the potentials during perfusion.

7. The stable analogue of ATP, αβ-methyleneATP had no long-term effects on potentials. Blocking P2 receptors (with suramin) or N-methyl-D-aspartate (NMDA) receptors prevented the induction of LTP. When adenosine A_1 receptors were blocked or the slices superfused with adenosine deaminase no inhibition of responses was seen during

perfusion with ATP, nor was LTP induced. Adenosine itself did not induce LTP. These results suggest that induction of LTP following ATP perfusion requires the activation of both P2 and A_1 receptors.

8. During superfusion with ZM 241385, ATP 10 μM still induced LTP but no longer caused the inhibition of responses during perfusion.

9. Following electrically induced LTP (100 Hz for 1 second), perfusion of ATP caused no further potentiation of responses, suggesting that electrically and ATP induced LTP share common mechanisms.

10. Perfusion of a preparation of 5'adenylic acid deaminase (AMPase) from *Aspergillus* species for 10 min resulted in an initial increase in population spike amplitude which gave way to a decrease in potential size and EPSP slope that remained depressed for at least 30 min following washout of the enzyme, resulting in long-term depression (LTD). A decrease in EPSP-spike coupling was observed 30 min following washout of enzyme. Superfusion of AMPase on slices in which transmission had been potentiated using high frequency stimulation resulted in depotentiation of responses.

11. The LTD caused by AMPase could not be inhibited by the allosteric inhibitor of the enzyme 2,3-diphosphoglyceric acid. Further investigation also ruled out the involvement of nitric oxide, protein kinase, and

cyclooxygenase in the activity of the AMPase. The use of AMPase extracted from rabbit muscle resulted in no LTD of responses.

12. Analysis of the crude enzyme extract from *Aspergillus* revealed that the preparation was not pure. Separation of the various components in the crude preparation led to the discovery that the AMPase was not the active factor causing depression of evoked responses.

13. Further purification of the active protein and subsequent sequencing showed that the protein was similar to a β-glucosidase. However, perfusion of β-glucosidase extracted from almonds did not result in LTD of responses.

14. Overall, this thesis extends knowledge of how synaptic transmission can be modulated by purines, and raises the possibility that a new family of proteins, related structurally to glucosidases, may also affect synaptic plasticity.

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Abbreviations

A3	N-(2-aminoethyl)-5-chloro-1-napthalene sulphonamide
aCSF	artificial cerebrospinal fluid
AMP	adenosine monophosphate
AMPase	5'-adenylic acid deaminase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoazolepropionate
AP5	2-amino-5-phosphonopentanoic acid
AP5A	diadenosine pentaphosphate
AP6A	diadenosine hexaphosphate
ATP	adenosine triphosphate
8-Br cAMP	8-Bromoadenosine-3':5'-cyclic monophosphate
CSC	8-(3-chlorostyryl)caffeine
CGS 21680	2-[p-(carboxyethyl)-phenylethylamino]-5'-N-ethylcarboxamidoadenosine
CP 66713	4-amino-8-chloro-1-phenyl(1,2,4)-triazolo(4,3a)quinoxaline
CPA	N^6-cyclopentyladenosine
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
2,3-DPG	2,3-diphosphoglyceric acid
EPSP	excitatory postsynapitc potential
GF 109203	2-[1-(3-dimethylaminoproply)indol-3-yl]-3-(indol-3-yl)maleimide
H8	N-[2-methylamino)ethyl]-5-isoquinolinesulfonamide hydrochloride
L-NAME	N^G-nitro-L-arginine methyl ester
LTD	long-term depression
LTP	long-term potentiation

2 MeSATP	2-methylthioATP
7-NI	7-nitroindazole
NMDA	N-methyl-D-aspartate
PPADS	Pyridoxalphosphate-6-azo-phenyl-2',4'-disulphuric acid
8-PT	8-phenyltheophylline
SQ 22536	9-(tetrahydro-2-furanyl)-9H-purin-6-amine
ZM 241385	4-(2-[7-amino-2-{2-furyl}{1,2,4,}triazolo{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)phenol

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Declaration.

I declare that all the work in this thesis was carried out by myself except where referenced and that it has not been submitted for any previous higher degree.

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I am blessed to have a group of friends (which fortunately include my brothers and sisters) with big hearts and open minds. Words cannot adequately convey my appreciation for your support and love, especially over the past year- I will never forget it.

Finally, to my grandfather who died during the course of this work. If I can tackle life in the manner he did, I know I need not fear anything that lies ahead. I would like to dedicate this thesis to his memory.

Digging

by Seamus Heaney

Between my finger and my thumb
The squat pen rests; snug as a gun.

Under my window, a clean rasping sound
When the spade sinks into gravelly ground;
My father, digging. I look down

Till his straining rump among the flowerbeds
Bends low, comes up twenty years away
Stooping in rhythm through potato drills
Where he was digging.

The coarse boot nestled on the lug, the shaft
Against the inside knee was levered firmly.
He rooted out tall tops, buried the bright edge deep
To scatter new potatoes that we picked
Loving their cool hardness in our hands.

By God, the old man could handle a spade.
Just like his old man.

My grandfather cut more turf in a day
Than any other man on Toner's bog.
Once I carried him milk in a bottle
Corked sloppily with paper. He straightened up
To drink it then fell to right away

Nicking and slicing neatly, heaving sods
Over his shoulder, going down and down
For the good turf. Digging.

The cold smell of potato mould, the squelch and slap
Of soggy peat, the curt cuts of an edge
Through living roots awaken in my head.
But I've no spade to follow men like them.

Between my finger and my thumb
The squat pen rests.
I'll dig with it.

CHAPTER 1.

INTRODUCTION.

This thesis is concerned with the plasticity of area CA1 in the rat hippocampus, including the induction of long-term potentiation and long-term depression of evoked field potentials recorded extracellularly in the stratum pyramidale. Changes in synaptic efficiency utilising simultaneous recordings of population excitatory postsynaptic potentials (EPSP) and population spikes from the stratum radiatum and stratum pyramidale respectively will also be described in terms of changes in the EPSP-spike relationship.

I. The Hippocampus.

All the experiments undertaken in this study have been carried out in the rat hippocampal slice preparation. The following section will give an account of the properties of this preparation and provide the rationale for its choice in the investigations that follow.

Hippocampal anatomy.

In the brains of mammals the hippocampus is a bilateral curved structure found within the forebrain located beneath the posterior and temporal neocortex (O'Keefe & Nadel, 1978). In most species, including the human, the hippocampus appears as a ridge extending nearly the entire length of each lateral ventricle. The white outer coating (the *alveus*) is composed of myelinated fibres, most of which leave the region via a large efferent pathway, the fornix. The term hippocampus encompasses the hippocampus proper and *fascia denta* or dentate

gyrus. The hippocampal formation includes both these and also the surrounding cortical areas. Differences in cell morphology and fibre projections led to the division of the hippocampus proper into the *regio superior* and *regio inferior* (Cajal, 1911; Blackstad, 1956). The anatomist Lorente de Nó (1934) divided the hippocampus proper into four subfields using the Golgi method of staining. These subfields, termed *cornu ammonis* (CA) 1-4. Area CA1 corresponds to the *regio superior* while CA2 and CA3 correspond to the *regio inferior*. The CA4 region represents cells at the transition of the hippocampus and dentate gyrus.

There has been some question as to whether a CA2 region should be considered distinct from the adjacent CA1 and CA3 areas (e.g. Blackstad, 1956). However, ontogenetically the CA2 region develops two to three days earlier than CA1 and CA3 in the rat. It also shows a higher resistance to destruction by kainate administration than the adjacent CA areas implying that it has distinct properties relative to its neighbours. Ishizuka *et al.* (1990) using horseradish peroxidase and computer aided digitising support the existence of an anatomically distinct CA2 region in the rat.

Cajal (1968) divided the hippocampus proper into several layers. From the outer layer to the more medial these include the *alveus*, the *stratum oriens* which contains the basal dendrites of the pyramidal cells, the *stratum pyramidale* containing the pyramidal cell bodies, the *stratum radiatum* consisting mainly of proximal dendrites and their arborizations from the pyramidal cells, the *stratum lacunosum*, in which bundles of fibres are found of which some are from the CA3 region and also a number of cells of random organisation, and finally the

stratum moleculare. The dentate gyrus consists of closely packed cells (granule cells) which have dendritic brushes that extend to the outer molecular layer of the gyrus (Isaacson, 1987).

Neuronal circuitry in the CA1 area.

The main excitatory input to the CA1 area of the hippocampus is from axon collaterals of the CA3 pyramidal cells i.e. Schaffer collaterals. This is the last stage in the tri-synaptic pathway from the entorhinal cortex which synapses with the pyramidal cell apical dendrites in the *stratum radiatum* (Andersen *et al.*, 1966, 1971b). Neuronal outputs from the CA1 cells exit into the alveus and fornix to the lower brain areas and also project to the subicular region (Isaacson, 1987).

Inhibitory pathways also exist in the CA1 area. Both feedforward (occurring before activation of the pyramidal cells) and feedback (initiated by axon collaterals of the pyramidal cells that reach the interneurones) inhibitory pathways have been described. A type of hippocampal interneurone, the basket cell, found in the pyramidal layer and stratum oriens makes contact with numerous pyramidal cells to exert inhibitory influences. The basket cells mainly use the inhibitory transmitter γ-aminobutyric acid (GABA). Hippocampal interneurones are extremely sensitive to afferent stimulation and can be activated at lower intensities than those required to produce similar effects in pyramidal cells; it is on this basis that interneurones are thought to provide both feedforward and feedback inhibition.

Hippocampal slices.

Slices of mammalian CNS tissue were shown to be physiologically viable *in vitro* by Yamamoto & McIlwain (1966) using neocortical tissue. Since then, the slice preparation has been extensively employed in neurochemical and electrophysiological experiments and has virtually replaced the intact animal preparation for researchers interested in intracellular and membrane phenomena in the mammalian central nervous system. *In vitro/in vivo* studies by Skrede & Westguard (1971) comparing the effects of electrical stimulation revealed that the fundamental properties of the hippocampus such as inhibitory mechanisms, postactivation facilitation, frequency potentiation and post-tetanic potentiation are preserved *in vitro*. The lamellar structure of the hippocampus (Andersen *et al.*, 1971b), with its rather simple three-layered cortex, well defined input and output pathways make it an excellent candidate for a slice preparation (Schwartzkroin, 1987).

Advantages of the slice preparation include:

a. Technical simplicity compared with *in vivo* experiments.

b. Good control over the condition of the preparation. For example the ionic environment, pO_2 and temperature can be tightly controlled and manipulated. Also the variable effects of anaesthesia on the intact animal are not a problem in the slice preparation.

c. No pulsation. The slice tissue is stabilised in the recording chamber and is not subjected to movements caused by heart beat and respiration.

d. Improved visualisation of tissue. It is easy to visually guide recording and stimulating electrodes to their desired sites under low magnification

e. Direct access to the extracellular space. This means that drugs or putative transmitter substances can be easily tested by addition to the bathing medium.

f. Simplified preparation. Removing the tissue from the rest of the brain means it is no longer subject to neurohumoral effects or to inputs from distant brain regions.

The advantages of the slice preparation led increasing numbers to adopt this technique. However, as with most preparations the slice has drawbacks. In general, the limitations of the slice preparation stem from that fact that the tissue is not in a *normal* state. These limitations include:

a. Separation form normal inputs. Tonic excitatory and/or inhibitory influences, and long feedback loops are lost. Also local circuits orientated out of the plane of the slice may be interrupted.

b. The ionic environment does not mimic exactly the normal extracellular conditions *in vivo*.

c. The slicing process will damage some tissue. In general the slice preparation contains a region of intact tissue surrounded by two layers of damaged tissue (Bak *et al.*, 1980).

d. The tissue is subject to an anoxic period during preparation (Lipton and Whittingham, 1979).

II. Synaptic plasticity in the hippocampus.

Compelling evidence from lesion studies in higher primates, including humans, shows that the hippocampus is a critical component of a neural system that is required for the initial storage of long-term memory (Squire & Zola-Morgan, 1991). It has been postulated that long-lasting modifications in synaptic strength are required to accomplish this feat. Such modifications of synaptic strength include long-term potentiation (LTP) and long-term depression (LTD).

Long-term potentiation

Bliss & Lomo (1972, 1973) showed that brief high frequency stimulation of hippocampal pathways could evoke an increase in synaptic efficiency in the anaesthetised rabbit. This phenomenon was later described in the hippocampal slice preparation (Schwartzkroin & Webster, 1975; Alger & Tyler, 1976; Andersen *et al.*, 1977; Lynch *et al.*, 1977; Yamamoto & Chujo, 1978). Several properties of LTP make it an attractive cellular mechanism for memory formation (Bliss & Collingridge, 1993):

- Like memories, LTP can be formed rapidly and is strengthened by repetition.
- It exhibits input specificity i.e. it occurs at synapses stimulated by afferent activity but not at adjacent synapses on the same postsynaptic cell.
- LTP is associative i.e. temporally pairing activity in a weak input with activation of a strong input will result in LTP of the weak input.

It is well established that induction of LTP requires activation of postsynaptic N-methyl-D-aspartate (NMDA) receptors resulting in an increase in the intracellular calcium concentration, $[Ca^{2+}]_i$ (Collingridge & Lester, 1989). It has been suggested that during normal synaptic transmission, glutamate acts on both NMDA and AMPA receptors. Na^+ flows through α-amino-3-hydroxy-5-methyl-4-isoazolepropionate (AMPA) receptor channels but not NMDA receptor channels due to blockade by Mg^{2+}. Depolarisation of the postsynaptic cell during high frequency stimulation releases the Mg^{2+} blockade allowing Na^+ and Ca^{2+} to flow through the NMDA receptor channel. This increase in $[Ca^{2+}]_i$ is a necessary trigger for the events leading to LTP (Bliss & Collingridge, 1993). However, a threshold increase in $[Ca^{2+}]_i$ may not be the sole trigger for LTP. The degree of activation of NMDA receptors and the magnitude of increase in $[Ca^{2+}]_i$ within the spine may affect the stability and duration of LTP (Malenka & Nicholl, 1993). Afferent activity may also be essential (Kullman *et al.*, 1993). Also metabotropic glutamate receptors, which typically couple to phospoinositide turnover may need to be activated for LTP to occur (Bashir *et al.*, 1993).

Protein Kinases and signal transduction mechanisms in LTP.

The majority of work on the signal transduction processes for the conversion of the initial trigger signal for LTP into long-lasting modifications of protein has focused on the role of protein kinases, in particular Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), protein kinase C (PKC) and the tyrosine kinase, Fyn. Inhibitors of these enzymes have been shown to block LTP (Bliss &

Collingridge, 1993). However, gene-targeting approaches to produce "knockouts" of CaMKII, PKC or Fyn showed that induction of LTP was more difficult to elicit in these animals, but was not absent (Grant & Silva, 1994; Abeliovich *et al.*, 1993), suggesting the presence of compensatory redundant mechanisms in the LTP signalling cascade.

The role of purines in the expression of LTP.

Although the activation of NMDA receptors is an event considered important for the induction of LTP in the hippocampus, many other factors are thought to be involved in synaptic plasticity. For example, arachidonic acid caused a synaptic enhancement when accompanied by presynaptic activity (Williams *et al.*, 1989). Collins *et al.* (1995) also demonstrated an interaction between arachidonic acid and metabotropic glutamate receptors to produce an NMDA-indpendent LTP. Nitric oxide and carbon monoxide may also be involved in NMDA receptor-independent synaptic plasticity (Zhuo *et al.*, 1993) as well as platelet activating factor (Kato *et al.*, 1994) and interluckin-1β (Coogan & O'Connor, 1999). In addition, purinergic compounds have been shown to play an important role in long-term plasticity in the hippocampus. Activation of adenosine A_1 receptors has been shown to inhibit the induction of LTP (Arai *et al.*, 1990; de Mendonca & Ribeiro, 1990) perhaps due to its inhibitory effects on transmitter release, whereas adenosine A_2 receptors have been implicated in the induction (Kessey & Mogul, 1997; Kessey *et al.*, 1997) and facilitation of LTP (Sekino *et al.*, 1991; Forghani & Krnjevic, 1995). These receptors have been shown to signal via phospholipase C and therefore can increase the intracellular calcium concentration in the cell. Adenosine A_2 receptors can induce an NMDA receptor-

independent LTP by potentiating postsynaptic AMPA receptors in the CA1 area of rat hippocampus (Kessey & Mogul, 1997).

Exogenously applied adenosine 5'triphosphate (ATP) is able to induce long-lasting enhancement of the population spikes recorded from mouse (Wieraszko & Seyfried, 1989) and guinea-pig (Fujii *et al.*, 1995) hippocampal slices. The mechanism(s) by which ATP induces LTP in the hippocampus may involve activation of a subclass of ATP (P2) receptor, or hydrolysis by ecto-nucleotidase to adenosine. Alternatively, it may be possible that ecto-protein kinase, using extracellular ATP as a co-substrate, is involved in the underlying synaptic plasticity (Wieraszko & Ehrlich, 1994).

EPSP-spike Relationship.

In the first report of LTP in the hippocampus Bliss & Lomo (1973) showed a change in the population spike (PS) that could not be accounted for by the change in population excitatory postsynaptic potential (EPSP). By plotting PS latency (which varies inversely with PS amplitude), against EPSP amplitude, they found a downward shift following LTP induction. Andersen *et al.* (1980) plotted PS amplitude against EPSP slope and used the term EPSP-to-spike (E-S) potentiation to describe the upward shift following LTP.

The relationship between the slope of the population excitatory postsynaptic potential (EPSP) and the corresponding population spike (PS) amplitude at a given stimulus intensity gives an indication of the ability of the excitatory synaptic drive to induce an action potential in the postsynaptic cell. In other

words, the E-S relationship inclusively describes the input-output relationship for a population of neurones, including pre- and postsynaptic effects on the postsynaptic potential, network effects and action potential generation.

By varying stimulation intensity, it is possible to construct input-output graphs for PS amplitude or EPSP slope against intensity. From these curves it is then possible to plot an E-S curve by plotting the PS amplitude as a function of EPSP slope for the various stimulation intensities. A change in the E-S relationship indicates the probability of discharge of the postsynaptic cell is modified in response to an identical synaptic event. E-S potentiation occurs when neuronal populations produce more action potentials for a given amount of synaptic input. Conversely, E-S depression is observed when fewer action potentials are seen following a given synaptic input.

It has been suggested that changes in the E-S relationship are as important as LTP and LTD, in terms of information processing, as it provides the neuronal network with another variable, increasing the capacity for memory storage (Bernard & Wheal, 1995). The mechanisms of E-S modification are thought to be at least partly independent from those involved in LTP and LTD due to the fact that E-S potentiation can occur even when NMDA receptors (which, as already mentioned, are thought to be essential in both LTP and LTD) are blocked (Bernard & Wheal, 1995).

Long-term depression.

Long-term depression (LTD) was originally reported in the CA1 area of the hippocampus as a heterosynaptic correlate of homosynaptic LTP in this area (Lynch *et al.*, 1977). Later, heterosynaptic LTD was also seen in the dentate gyrus (Levy & Stewart, 1979; Abraham & Goddard, 1983; Levy & Stewart, 1983) and a homosynaptic form of LTD was found in the CA1 area of the hippocampus (Dudek & Bear, 1992; Mulkey & Malenka, 1992) and also in other brain regions (Linden, 1994). Surprisingly, LTD in the CA1 area of the hippocampus shares several features with LTP in that it is input specific, requires activation of NMDA type glutamate receptors and influx of Ca^{2+} through channels coupled to these receptors (Dudek & Bear, 1992; Mulkey & Malenka, 1992; Neveu & Zuker, 1996). This suggests that the same receptors but different efffectors are involved in these two types of plasticity. However, as with LTP forms of NMDA receptor-independent LTD have been described (Berretta & Cherubini, 1998; Tzounopoulos *et al.*, 1998).

Homosynaptic LTD in the CA1 area of the hippocampus is usually induced by prolonged low-frequency stimulation (LFS), the most common protocol used being 900 pulses at 1Hz. Unlike LTP, which can be induced robustly, and consistently using high-frequency stimulation, a reliable method for induction of LTD has proved more elusive. LTD can be elicited reliably only in slices from young rats up to six weeks-of-age (Dudek & Bear, 1992; Staubli & Ji, 1996). In fact, LTD expression in rats older than eight to ten weeks has very seldom been reported (Barnes, 1979; Heynen *et al.*, 1996). In contrast, LTP of a specific set of synapses can readily be reversed using the same stimulation paradigms as those used to induce LTD in naive (unpotentiated) slices (Baskys & Malenka,

1991). This reversal of LTP was first reported by Barrionuevo *et al.* (1980) and has been termed depotentiation (Fujii *et al.*, 1991). It is unclear whether LTD and depotentiation are the same process mediated by the same effector system (see e.g. Wagner & Alger, 1996).

The long-term plastic changes in synaptic function described above provide the best available model for the mechanism by which the brain stores information. The mechanisms underlying LTP and LTD provide the strongest clues for the experimental analysis of the physical substrate of memory. It is likely that these mechanisms are multifactorial, controlled by a network of interacting signalling cascades regulated at multiple levels.

III. Adenosine 5'triphosphate.

Adenosine triphosphate (ATP) is an ubiquitous intracellular energy source fundamentally involved in various metabolic cycles including energy metabolism and nucleic acid synthesis. The first investigators to recognise the potent extracellular effects of purine nucleotides were Drury & Szent-Gyorgyi (1929), with special reference to their actions in the mammalian heart. Following this report, further work with regard to the actions of ATP and its metabolite, adenosine, on the cardiovascular system was carried out as reviewed by Green & Stoner (1950).

An important advance was made by Holton (1959), who showed that antidromic stimulation of the great auricular nerve - which results in vasodilation of the rabbit ear vessels - was accompanied by ATP release. In the early sixties non-adrenergic, non-cholinergic (NANC) nerves were described in the gastrointestinal tract (Burnstock *et al.*, 1964). Burnstock (1972), using the criteria summarised by Eccles (1964) for the acceptance of putative neurotransmitters, proposed that the substance released in response to autonomic nerve stimulation was ATP and termed the nerves involved "purinergic". Supporting the idea that ATP was a neurotransmitter were the observations that ATP was synthesised, stored and released from non-adrenergic, non-cholinergic nerves supplying the intestine and that it mimicked the effects of nerve stimulation on these muscles (Burnstock *et al.*, 1970).

Evidence for pre-junctional purinergic receptors was obtained from experiments in the skeletal neuromuscular junction. In the isolated rat phrenic nerve - diaphragm preparation, adenosine and the adenine nucleotides decreased both the spontaneous and evoked release of acetylcholine from motor nerve terminals (Ginsborg & Hirst, 1972; Ginsborg *et al.*, 1973; Ribeiro & Walker, 1973). Noradrenaline release from sympathetic nerves was also shown to be inhibited by purine compounds in a wide variety of tissues (Hedqvist & Fredholm, 1976; Su, 1978). In 1979 it was shown that these prejunctional neuromodulatory effects of adenine nucleotides were mediated by receptors for adenosine, but not for ATP (De Mey *et al.*, 1979).

The demonstration that release of both [^{3}H]ATP and noradrenaline was blocked by guanethidine during stimulation of sympathetic nerves supplying guinea pig taenia coli gave the first indication that ATP may be released as a co-transmitter with noradrenaline (Su *et al.*, 1971). This concept was later extended to other tissues (Langer & Pinto, 1976; Burnstock, 1976, Burnstock, 1990).

ATP receptors.

In 1978 Burnstock proposed a basis for distinguishing two types of purinergic receptor namely the P_1 and P_2 purinoceptors.

This classification was based on four criteria:

1. The relative potencies of ATP, ADP, AMP and adenosine.
2. The selective actions of antagonists especially the methylxanthines.
3. The activation of adenylate cyclase by adenosine but not ATP.
4. The induction of prostaglandin synthesis by ATP but not by adenosine.

Thus, P_1 receptors are more selective for adenosine, act through adenylate cyclase and are antagonised by lower concentrations of methylxanthines than produce phosphodiesterase inhibition. P_2 receptors are more responsive to ATP and ADP than AMP and adenosine, are not antagonised by methylxanthines, do not act via adenylate cyclase and their occupation may lead to prostaglandin synthesis.

P2 receptors were further subdivided into P2X and P2Y receptor subtypes on the basis of pharmacological studies using isolated preparations from a number of species (Burnstock & Kennedy, 1985). In addition to this pharmacological distinction, it became clear that the subtypes also differed in their transduction mechanisms. P2X receptors are transmitter-gated ion channels whereas P2Y receptors are members of the G-protein coupled superfamily (Kennedy, 1990; Dubyak, 1991; Abbracchio & Burnstock, 1994, Fredholm *et al.*, 1994). During this time a number of other subtypes of P2 receptor were claimed, including an ADP selective P2T receptor responsible for platelet aggregation (Gordon, 1986), a P2Z receptor on mast cells (Gordon, 1986), a P2U receptor showing equipotency to ATP and UTP (O'Connor *et al.*, 1991), and a specific receptor for diadenosine polyphosphates, termed a P2D receptor (Pintor & Miras-Portugal, 1993). This method of nomenclature, described as a "random walk through the alphabet" was deemed unsatisfactory and a new method proposed by Abbracchio & Burnstock (1994).

P2X receptors.

The first P2X receptors were cloned in 1994 - $P2X_1$ from the rat vas deferens (Valera *et al.*, 1994) and $P2X_2$ from rat PC12 cells (Brake *et al.*, 1994). Since then the many genes that encode P2X subunits have been cloned from a variety of sources. Currently, seven distinct subunits are known to exist, and all can apparently form homomeric channels. However it is likely that in nature they exist as heteromeric assemblies.

P2X receptors have two hydrophobic transmembrane domains per subunit. intracellular N- and C- domains and a large extracellular loop which always contains 10 cystines (Brake *et al.*, 1994; North, 1996). The channel pore has been shown to be lined by residues from the second transmembrane domain (Rassendren *et al.*, 1997).

All P2X subunits form functional channels when expressed in expression systems such as the *Xenopus* oocyte or CHO cells. These presumably homomeric receptors show properties similar to P2X receptors identified in native cell types, suggesting that the native cell types may express homomeric P2X receptors as well, with subtypes $P2X_1$, $P2X_2$ and $P2X_3$ most likely to form homomers (Rae *et al.*, 1988). However, many native cell types express multiple P2X subunit mRNA transcripts, which is reflected at the protein level using detection by immunocytochemistry (Kidd *et al.*, 1995; Collo *et al.*, 1996; Vulchanova *et al.*, 1996). The possibility of heteropolymerisation between different P2X subunits therefore clearly exists. It has been shown that $P2X_2$ and $P2X_3$ subunits can form heteromeric channels (Lewis *et al.*, 1995; Radford *et al.*, 1997), as can $P2X_4$ and $P2X_6$ subunits (Le at al., 1998). The overlapping of P2X subunits in CNS neurones is suggestive of heteropolymerisation (Kidd *et al.*, 1995; Collo *et al.*, 1996; Vulchanova *et al.*, 1996; Vulchanova *et al.*, 1997) but functional evidence may have to wait until the discovery of potent subunit selective antagonists. A summary of the seven known P2X receptor subtypes is shown in table 1.

Receptor	Tissue (functional assay)	Agonist Profile	Suramin sensitive
$P2X_1$	Rat vas deferens	ATP = 2MeSATP = α,βMeATP	Yes
	Human HL60 cells	α,βMeATP ≥ ATP = 2MeSATP	Yes
$P2X_2$	Rat PC12 cells	ATP > 2MeSATP >> α,βMeATP	Yes
	Rat vagal preganglionic neurones	ATP >> α,βMeATP	Yes
$P2X_3$	Rat dorsal root ganglion	2MeSATP ≥ ATP ≥ α,βMeATP	Yes
$P2X_4$	Rat submandibular epithelial cells	ATP >> α,βMeATP	No
$P2X_5$	Rat trigeminal mesencephalic nucleus neurones	ATP >> α,βMeATP	Yes
$P2X_6$	Rat brain	ATP > 2MeSATP > ADP	No
$P2X_7$	Mouse microglila NTW-8 cells	BzATP > ATP ≥ 2MeSATP >> α,βMeATP	No

Table 1. Summary of known endogenous P2X receptor subtypes, their localisation and pharmacological characteristics.

A special feature of the $P2X_2$ receptor is its sensitivity to changes in pH, with maximum responses to ATP at pH 6.5 when expressed in oocytes (King *et al.*, 1996). The heteromeric $P2X_2/P2X_3$ receptor is also sensitive to changes in pH and it has been shown that various nociceptive substances potentiate ATP responses at this receptor (Wildman *et al.*, 1997). The $P2X_3$ receptor is expressed only in sensory neurones (Chen *et al.*, 1995), whereas the $P2X_4$ receptor is widely expressed in many central and peripheral tissues (Bo *et al.*, 1995; Buell *et al.*, 1996). It is interesting to note that the $P2X_4$ receptor in the rat is not sensitive to the classical ATP antagonists suramin, reactive blue 2 and pyridoxalphosphate-6-azo-phenyl-2',4'-disulphuric acid (PPADS), and indeed responses are even potentiated (Bo *et al.*, 1995). Human $P2X_4$ receptors seem to have a higher sensitivity to blockade by suramin and PPADS (Garcia-Guzman *et al.*, 1997). This along with the fact that the originally proposed P2X receptors selective agonist αβMeATP is not effective at all P2X subtypes (see table 1) raises the possibility that those receptors that are insensitive to αβMeATP and suramin may have been overlooked. It may also mean that novel responses mediated by P2X receptors remain to be discovered.

P2Y receptors.

P2Y receptors are G-protein coupled and typically have seven transmembrane domains which are well conserved, an extracellular N-terminus and an intracellular C-terminus which are more variable in structure. Binding sites have been identified on the 6^{th} and 7^{th} transmembrane domains (Barnard *et al.*, 1994).

To date, five mammalian P2Y receptors have been cloned and termed $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ receptors and a recently identified receptor that has been termed $P2Y_{11}$ (Webb *et al.*, 1993; Lustig *et al.*, 1993; Communi *et al.*, 1995; Nguyen *et al.*, 1995; Chang *et al.*, 1995; Communi *et al.*, 1997). All the mammalian P2Y receptors cloned to date activate phospholipase C. The $P2Y_{11}$ receptor has been shown to activate adenylate cyclase as well as phospholipase C (Communi *et al.*, 1997). It is likely that other members of the P2Y receptor family exist, such as the $P2Y_7$ receptor proposed following cloning from human erythroleukemia cells (Akbar *et al.*, 1996). However, it was later decided that this receptor should not be classified as a P2Y receptor as, although it has a 30% identity to the human $P2Y_6$ receptor, it's primary response is to leukotriene B4 (Yokomizo *et al.*, 1997). The p2y5 receptor (Webb *et al.*, 1996) was identified from chicken by a specific nucleotide binding series. It is designated in lower case because no functional response to nucleotides has been described.

The resolution of P2Y receptor subtypes is hampered due to the lack of subtype selective agonists and antagonists. In addition, the agonist selectivity of P2Y receptors is difficult to establish due to the presence of ecto-enzymes that metabolise and interconvert nucleotides (Zimmerman, 1996). The $P2Y_1$ receptor is the only receptor for which progress has been made in developing synthetic selective agonists and antagonists. 2-methylthioADP is an agonist with low nanomolar potency at the $P2Y_1$ receptor that does not activate the $P2Y_2$, $P2Y_4$ or $P2Y_6$ receptor. Suramin and reactive blue 2 exhibit low selectivity among P2 subtypes. Suramin is an antagonist at both $P2Y_1$ and $P2Y_2$ receptors (Charlton *et al.*, 1996). PPADS competitively antagonises the $P2Y_1$ and $P2Y_4$ receptors but is

inactive at the $P2Y_2$ receptor (Communi *et al.*, 1996; Charlton *et al.*, 1996). Adenosine 3',5'-bisphosphate has recently been introduced as a relatively high affinity $P2Y_1$ receptor antagonist that does not interact with the other P2Y receptors (Boyer *et al.*, 1996). A summary of the cloned mammalian P2Y receptors is given in table 2.

Receptor	**Localisation**	**Agonist profile**
$P2Y_1$	Brain, placenta, prostate and ovary	2MeSATP > ADP ≥ ATPγS > α,βMeATP
$P2Y_2$	Lung, bone, pituitary	UTP ≥ ATP >> 2MeSATP – α,βMeATP
$P2Y_4$	Placenta, brain.	UTP >> UDP > ATP
$P2Y_6$	Aorta smooth muscle, spleen, placenta	UDP >> UTP > ATP
$P2Y_{11}$	Spleen, granulocytes	ATP > 2MeSATP >> ADP

Table 2. Summary of known endogenous P2Y receptor subtypes, their localisation and pharmacological characteristics.

IV. Diadenosine polyphosphates.

Diadenosine polyphosphates, or adenine dinucleotides, consist of two adenosine molecules linked at their 5' region by a chain of 2-6 phosphate groups. The whole group is abbreviated to Ap_nA, where n indicates the number of phosphates in the connecting chain. The first demonstration that diadenosine polyphosphates could influence tissue function was shown in smooth muscle preparations (Stone, 1981a) where they induced contraction. The discovery of Ap_4A, Ap_5A, and Ap_6A in neurosecretory granules, co-stored with catecholamines or acetylcholine and ATP, enlarged their role to the nervous system (Pintor *et al.*, 1992,1992a). Upon release into the extracellular space, the dinucleotides may act as uncleaved molecules on a variety of target cells via purinergic receptors. However, they can also be cleaved by extracellular phosphodiesterases and ectoenzymes to ATP, ADP and finally adenosine. In the hippocampus, dinucleotides have been shown to depress postsynaptic field potentials and intracellularly recorded postsynaptic currents (Klishin *et al.*, 1994). However, increases in intracellular calcium levels have also been demonstrated in the hippocampus (Panchenko *et al.*, 1993), deermouse brain (Pintor, 1993), and midbrain synaptosomes (Pintor *et al.*, 1995).

Receptors for diadenosine polyphosphates.

Debate exists surrounding the receptors activated by diadenosine polyphosphates. Evidence exists for the activation of P2X (Hoyle *et al.*, 1989), P2Y (Castro *et al.*, 1990) and P1 receptors (Klishin *et al.*, 1994). The

dinucleotides may even act on a distinct subtype of P2 receptor. Displacement studies using [^{35}S]ADP$_4$A and competition studies looking at displacement of [^{35}S]ADP-β-S by adenosine polyphosphates in rat brain synaptic terminals found a binding profile which was distinct from previous P2 receptors and had a high affinity for diadenosine polyphosphates (Pintor *et al.*, 1993). This receptor was termed $P2_d$ or P4 (Pintor *et al.*, 1995), however it is now considered part of the P2Y receptor family although the receptor has not been cloned.

V. Adenosine.

In 1970, Sattin & Rall described the first evidence for the receptor-mediated effects of adenosine. Since then, adenosine has become established as an important neuromodulator in the peripheral and central nervous system. Adenosine can be synthesised intracellularly *de novo* and transported across the cell membrane to exert its extracellular effects or alternatively, adenosine can be formed from the metabolism of adenine nucleotides in the extracellular space (see Stone *et al.*, 1991 for review).

Adenosine Receptors

Adenosine acts on four well-defined G protein-coupled receptors. These are called A_1, A_{2A}, A_{2B} and A_3 receptors and have been collectively termed P1 receptors (Burnstock, 1978). The four receptors have been cloned from several mammalian species including man (see below). Although pharmacological data

exist suggesting the presence of undiscovered adenosine receptors (Cornfield *et al.*, 1992, Johansson *et al.*, 1993, Johansson & Fredholm, 1995, Cunha *et al.*, 1996, Lindstrom *et al.*, 1996), the cloning efforts of several laboratories have shown no evidence of this. One of the aberrant sites (a putative "A_x" receptor) was suggested to represent binding to a state of the A_{2A} receptor (Luthin & Linden, 1995). There is evidence in the case of the adenosine A_1 receptor that, as well as the traditional agonist site an allosteric site exists that regulates its agonist binding and biological effects (Bruns & Fergus, 1990; Bruns *et al.*, 1990; Mundumbi *et al.*, 1993; Leung *et al.*, 1995). Supporting this, the allosteric enhancer PD81723 has been shown to stabilise the adenosine A_1 receptors coupling to G-proteins.

Adenosine receptors were originally classified according to their actions on adenylate cyclase (Van Calker *et al.*, 1979), adenosine A_1 receptor activation inhibiting the enzyme to decrease cyclic AMP levels and adenosine A_{2A} receptor activation stimulating it. These effects on adenylate cyclase are mediated through the activation of G-proteins namely G_i or G_o for A_1 receptors (Munshi & Linden, 1989; Munshi *et al.*, 1991) and G_s for A_2 receptors (Morgan, 1991). This classification has been superseded since it has been shown that there is a plethora of effects mediated by both subtypes which are known not to involve adenylate cyclase. Below, the characteristics of the individual adenosine receptor subtypes will be discussed.

Adenosine A_1 receptors

Adenosine A_1 receptors have been cloned from a variety of mammals including man (Libert *et al.*, 1992; Townsend-Nicholson & Shrine, 1992), rat (Mahan *et al.*, 1991), mouse (Marquardt *et al.*, 1994), dog (Libert *et al.*, 1991), cow (Tucker *et al.*, 1992) and rabbit (Hill et al, 1997). Using Northern blot in the rat preparation Stehle *et al.* (1992) showed the receptors to be distributed in the following descending order of density:

Brain (cortex, hippocampus, cerebellum > ventral midbrain, pons, hypothalamus > striatum) = spinal cord > fat > testis > uterus > heart > kidney >> skeletal muscle, liver, intestine, stomach.

Their function in the body include depression of heart rate (Olsson & Pearson, 1990), inhibition of lipolysis (Fredholm, 1985), inhibition of neurotransmission in brain slices (Stone, 1991) and vas deferens (Hedqvist & Fredholm, 1976). Several selective agonists and antagonists are available for the A_1 receptor. These include the agonists, 2-chloro-N^6-cyclopentyladenosine (CCPA) > N^6-cyclopentyladenosine (CPA) > N^6-cyclohexyladenosine (CHA) = (R)-N^6-phenylisopropyladenosine (R-PIA) > (adenosine-5'-N-ethyluronamide (NECA). Antagonists for this receptor include the xanthine compounds 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and 8-phenyltheophylline (8-PT) and also the non-xanthine compound, WRC0571 (Martin *et al.*, 1996).

As previously mentioned, adenosine A_1 receptors can couple to all G_i and G_o proteins (Munshi & Linden, 1989; Munshi *et al.*, 1991) to inhibit adenylate

cyclase. However, activation of these receptors can also lead to phospholipase C and D activation (see Fredholm *et al.*, 1995), and protein kinase activation (Gerwins & Fredholm, 1995) opening of K^+ channels (which can inhibit neuronal firing) (Okada & Ozawa, 1980; Segal. 1982; Trussel & Jackson, 1985) and blockage of N-, P- and Q-type Ca^{2+} channels (to inhibit transmitter release) (Dolphin *et al.*, 1986; Schubert *et al.*, 1987).

Adenosine A_{2A} receptors

Adenosine A_{2A} receptors have been cloned from human, rat and mouse cells (Marquardt *et al.*, 1994; Chu *et al.*, 1996; Peterfreund *et al.*, 1996). In the brain, A_{2A} receptors are mainly localised in the striatum, however autoradiographic binding studies have shown they exist in the hippocampus as well (Cunha *et al.*, 1994a), usually closely associated with A_1 receptors. A_{2A} receptors have also been shown in platelets, neutrophil leukocytes, endothelium and lymphocytes (Ongini & Fredholm, 1996). They exert their effects through G_s and G_{15} stimulation of adenylate cyclase (Gudermann et al, 1997) and also through stimulation of phospholipase C. Thus, in cells that express abundant A_{2A} receptors it is reasonable to assume that adenosine, acting via this receptor, can increase intracellular concentrations of calcium ions $[Ca^{2+}]_i$.

In vivo, adenosine A_{2A} receptor functions include regulation of sensorimotor integration in the basal ganglia (Ferré et al, 1992), inhibition of platelet aggregation (Dionisotti *et al.*, 1992), inhibition of oxidative burst in polymorphonuclear leukocytes (Cronstein, 1994) and coronary vasodilation (Olsson & Pearson, 1990). Selective agonists and antagonists are available for

the adenosine A_{2A} receptor. The agonist 2-[p-(carboxyethyl)-phenylethylamino]-5'-N-ethylcarboxamidoadenosine (CGS 21680) is 70-140 fold selective for A_{2A} over A_1 receptors (Williams, 1991). Other A_{2A} receptor agonists include, adenosine-5'-N-ethyluronamide (NECA), and its derivatives APEC, PAPA-APEC and HE-NECA (Barrington *et al.*, 1989; Cristalli *et al.*, 1992), and several 2-alkoxyadenosines (Ukena *et al.*, 1991). Antagonists for A_{2A} receptors include 4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)phenol (ZM 241385) (Poucher *et al.*, 1995), 4-amino-8-chloro-1-phenyl(1,2,4)triazolo(4,3a)quinoxaline (CP 66713) and chlorostyryl-caffeine (CSC).

Adenosine A_{2B} receptors.

These receptors have been cloned from human, rat and mouse (Pierce *et al.*, 1992; Stehle *et al.*, 1992; Marquart *et al.*, 1994) but are less well characterised than the A_1 or A_{2A} receptors, mainly due to the lack of selective pharmacological tools available. No selective agonists are available, although alloxazine has been shown to be a slightly selective antagonist (Brackett & Daly, 1994). The transduction mechanisms of A_{2B} receptors include activation of adenylate cyclase and mobilisation of calcium ions via phospholipase C.

A_{2B} receptors are mainly found in the large intestine, bladder, lung and brain although PCR reveals low amounts in practically all cells. Their tissue functions include release of inflammatory mediators and relaxation of smooth muscle.

Adenosine A_3 receptors.

The latest adenosine receptor to be discovered is the A_3 receptor. It was first cloned from rat (Zhou *et al.*, 1992) and then later from sheep (Linden *et al.*, 1993) and man (Salvatore *et al.*, 1993). The cloned receptor from all three species was able to cause inhibition of adenylate cyclase, in addition A_3 receptors have been shown to activate phospholipase C (Ramkumar *et al.*, 1993) and a more sustained activation of phospholipase D (Ali *et al.*, 1996) leading to activation of protein kinase C.

In humans, A_3 receptors are found in the lungs, liver, heart and kidneys, with a lower density being found in the brain and testes (Linden, 1994). The concentration of endogenous adenosine required to activate A_3 receptors is higher than that required to activate either A_1 or A_{2A} receptors. High concentrations of adenosine occur during hypoxic stress and other cellular damage, so that the role of the A_3 receptor may be as an endogenous regulator under conditions of severe challenge.

Selective agonists and antagonists have become available which will help characterise these receptors further. The first agonist available was N^6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide (IB-MECA), which is approximately 50-fold selective in binding assays for rat A_3 versus either A_1 or A_{2A} receptors (Kim *et al.*, 1994). The 2-substituted analogue of this compound (2Cl-IB-MECA) is 2500- and 1400-fold selective for rat A_3 versus A_1 and A_{2A} receptors respectively (Jacobson, 1998). Antagonists for the A_3 receptor have been introduced only recently. The dihydropyridine and triazoquinazoline

derivatives, MRS 1191 and MRS 1220 have proved the most useful (Jiang *et al.*, 1996; Kim *et al.*, 1996), although significant species differences exist.

Interactions between adenosine receptors.

Many cells express more that one type of adenosine receptor. For example, in the hippocampus, adenosine A_{2A} receptor messenger RNA expression is mainly localised in the pyramidal and granular cells, the same regions that show adenosine A_1 receptor messenger RNA expression (Cunha *et al.*, 1994a). Adenosine A_1 and A_{2A} receptors have also been shown to be co-localised in the striatum and cortex. In these areas it has been shown that activation of adenosine A_{2A} receptors attenuates the inhibitory effects of adenosine A_1 receptor activation (Cunha *et al.*, 1994a, Dixon *et al.*, 1997, Lopes *et al.*, 1999). Adenosine A_3 receptors have also been shown to produce a desensitisation of A_1 receptors in the rat hippocampus through a mechanism involving protein kinase C (Dunwiddie *et al.*, 1997).

VI. 5' adenylic acid deaminase studies.

Adenylic acid deaminase (AMP aminohydrolase, AMP deaminase, AMPase, E.C.3.5.4.6.) catalyses the hydrolytic cleavage of adenosine monophosphate (AMP) to inosine monophosphate (IMP) and ammonia. It is found in a variety of eukaryotes, including mammals, birds, amphibians (Zielke & Suelter, 1971), plants (Turner & Turner, 1961) and yeast (Yoshino *et al.*, 1979). The enzyme

has been proposed to play a role in the regulation of adenylate energy metabolism (Yoshino & Murakami, 1981), in the control of the purine nucleotide cycle (Lowenstein, 1972) and in regulation of phosphofructokinase (Yoshino & Murakami, 1982).

Earlier studies in this laboratory involving the use of AMPase to inhibit the effects of AMP on epileptiform activity in hippocampal slices revealed a gradual decline in activity rate during washout of 0.2 U/ml AMPase. This depression of activity persisted for up to 90 min (Ross, 1997). Following this report of long-term depression of hippocampal firing, it was decided to investigate further the effects of AMPase on evoked responses in the CA1 area of rat hippocampus.

The effects of AMPase on hippocampal plasticity were of interest following reports that levels of the enzyme are elevated in the brains of Alzheimer's disease patients (Sims *et al.*, 1997), a condition intrinsically associated with learning and memory abnormalities. Further, activity of the enzyme increases with age (Zoref-Shani *et al.*, 1995). These observations provided a tantalising possibility that AMPase may act as a modulator of the mechanisms involved in learning and memory in the mammalian brain.

Chapter 5 describes the attempts to characterise the depressant effects of AMPase and the subsequent discovery that a protein contaminant of the enzyme preparation was, in fact, the active factor involved- not the AMPase molecule. Results from the purification, sequencing and subsequent attempts to identify this unknown protein are described.

CHAPTER 2.

MATERIALS AND METHODS.

I. Preparation of hippocampal slices.

Male Wistar rats (150-200g) were anaesthetised with intraperitoneal urethane (1.5g\kg). The animals were then killed by cervical dislocation, decapitated using a guillotine, and the brain rapidly removed to ice-cold artificial cerebrospinal fluid (aCSF). In some instances the animals were cooled on ice whilst breathing oxygen enriched air following administration of urethane, until rectal temperature reached 30 °C. This procedure was recommended by Newman *et al.*, (1992) to enhance the viability of slices. However, in these studies no obvious difference was observed between the viability of slices from cooled versus uncooled animals.

Following removal, the brain was placed in a filter paper lined petri dish and completely submerged in the ice-cold gassed aCSF. The cerebellum was removed and the cerebral hemispheres separated using a scalpel blade. The hippocampi were then dissected free of surrounding tissue using spatulas and cut transversely into slices 450 μm thick using a McIlwain tissue chopper. The slices were separated using sealed glass micropipettes and transferred to another petri dish where freshly gassed aCSF was added in sufficient quantity to just cover the slices. The dish was placed in an incubation chamber saturated with 95% O_2/5% CO_2 and the slices maintained at room temperature for at least one hour before being transferred to a recording chamber.

II. Composition of artificial cerebrospinal fluid (aCSF).

The composition of aCSF was (mmol.l^{-1}) KH_2PO_4 2.2, KCl 2, $NaHCO_3$ 25, NaCl 115, $CaCl_2$ 2.5 $MgSO_4$ 1.2 and glucose 10. It was gassed with a mixture of 95% O_2 and 5% CO_2 to yield a pH of 7.4. In experiments using barium, $MgCl_2$ was substituted for $MgSO_4$ in order to prevent precipitation of $BaSO_4$. This modification did not have any effect on slices compared with the standard aCSF solution.

III. Bath superfusion and application of drugs.

Following incubation, individual slices were transferred to a 1 ml recording chamber using a fine brush. Care was taken to minimise physical manipulation of the slices. The slice was placed on a fine wire mesh and held in place using a thin bar formed from a blunted seeker needle mounted on a micromanipulator. The slice was perfused constantly from a gravity feed through silicon tubing at a rate of 3 ml/min with gassed aCSF which had been heated in a thermostatically controlled water bath until the temperature in the bath was 30 ^{o}C. The flow rate and temperature were checked regularly to ensure consistency. Drugs were added to the perfusion medium and applied to the slices for a minimum of 10 min to ensure the attainment of equilibrium responses. To minimise organic growth in the recording chamber and in the silicon tubing, the apparatus was flushed with distilled water for at least one hour at the beginning and end of each day. In addition, the apparatus was dismantled weekly and the silicone tubing physically manipulated in order to dislodge any growth and both the tubing and recording

chamber were cleaned with dilute hydrochloric acid before being rinsed thoroughly with distilled water.

IV. Stimulating and recording arrangements.

Figure 2.1 is a representation of the hippocampal slice showing the main neuronal elements of the structure and the positioning of the recording and stimulating electrodes.

Stimulation.

The slices were orthodromically stimulated using a concentric bipolar electrode (Clark Electromedical Instruments Ltd.) placed in the stratum radiatum at the CA1/CA2 junction of the hippocampus. 1.0 Hz stimulation was used at the beginning of an experiment until the desired potential was found (population spike or EPSP), then stimulation was reduced to 20 s intervals in order to prevent the induction of long-term depression (LTD) in the slice. Reducing the stimulation frequency usually resulted in an initial depression of response which recovered to maximum amplitude within 20-30 min. For this reason slices were allowed to settle for at least 45 min before recordings of potentials were made. A submaximal response (70% of maximum PS amplitude) was used in all experiments.

Recording.

Recordings were made using borosilicate glass microelectrodes which were created using a Kopf vertical puller. The tips of the resulting electrodes were

broken back to 2-4 μm under low magnification using a glass probe. The electrodes were filled with sodium chloride 0.9% using a fine 36 gauge needle.

Simultaneous recordings of population spikes and population excitatory postsynaptic potentials (popEPSPs) were made from the stratum pyramidale and stratum radiatum of the CA1 area respectively. Only slices in which a population spike amplitude greater than 1 mV could be recorded were used in experiments.

Recorded signals were amplified (Neurolog), and monitored on a digital oscilloscope and on-line using Signal software (Cambridge Electronic Design, CED). In experiments where input/output relationships were examined, an average of five responses at a given stimulus intensity was calculated using CED software (Sigavg, Signal).

V. Data analysis and statistics.

Responses were quantified as the amplitude of the population spike (measured as the difference between peak negativity and the averaged values of the two peak positivities of the spike) and/or the slope of the negative arm of the population EPSP. For investigations of EPSP-spike coupling, input/output (I/O) curves were obtained by varying the stimulus intensity and plotting responses for population spike amplitude and population EPSP slope as a percentage of maximum control values against stimulation intensity. Changes in population EPSP slope and population spike amplitude were evaluated as differences in area under the curve

of their respective I/O curves before and after drug addition for individual slices and the mean and standard error of the mean calculated. From the I/O curves, EPSP-spike (E-S) curves could be constructed by expressing the population spike amplitude as a function of population EPSP slope. The effect of drugs on the E-S relationship could be evaluated by a shift in the E-S curve compared to control (a left shift indicates E-S potentiation whereas a right shift in the curve indicates E-S depression). To quantify shifts in the E-S relationship, the population spike amplitude seen at a population EPSP slope 70% of maximum was measured before and after drug addition for each slice and the values expressed as mean ± s.e.mean (Bernard and Wheal, 1995a,b). Alternatively, the ratio PS/EPSP was measured in each slice before and after drug addition to assess changes in EPSP-spike coupling. An increase in this ratio showed that E-S potentiation had occurred (Richter-Levin *et al.*, 1991).

In experiments measuring long-term effects of agents, the response observed 30 min after washout of drug was taken as the cut off point from where statistical comparisons were made (events were considered long-term if they lasted 30 min or more).

A paired Student's t-test was used to determine the degree of significance of responses before and after drug perfusion. Multiple comparisons involving more than two different sets of data were made using analysis of variance (ANOVA) followed by a Student-Newman-Keul's test. In the figures statistically significant differences between data are denoted with asterisks. $p < 0.05$ was taken to denote significance.

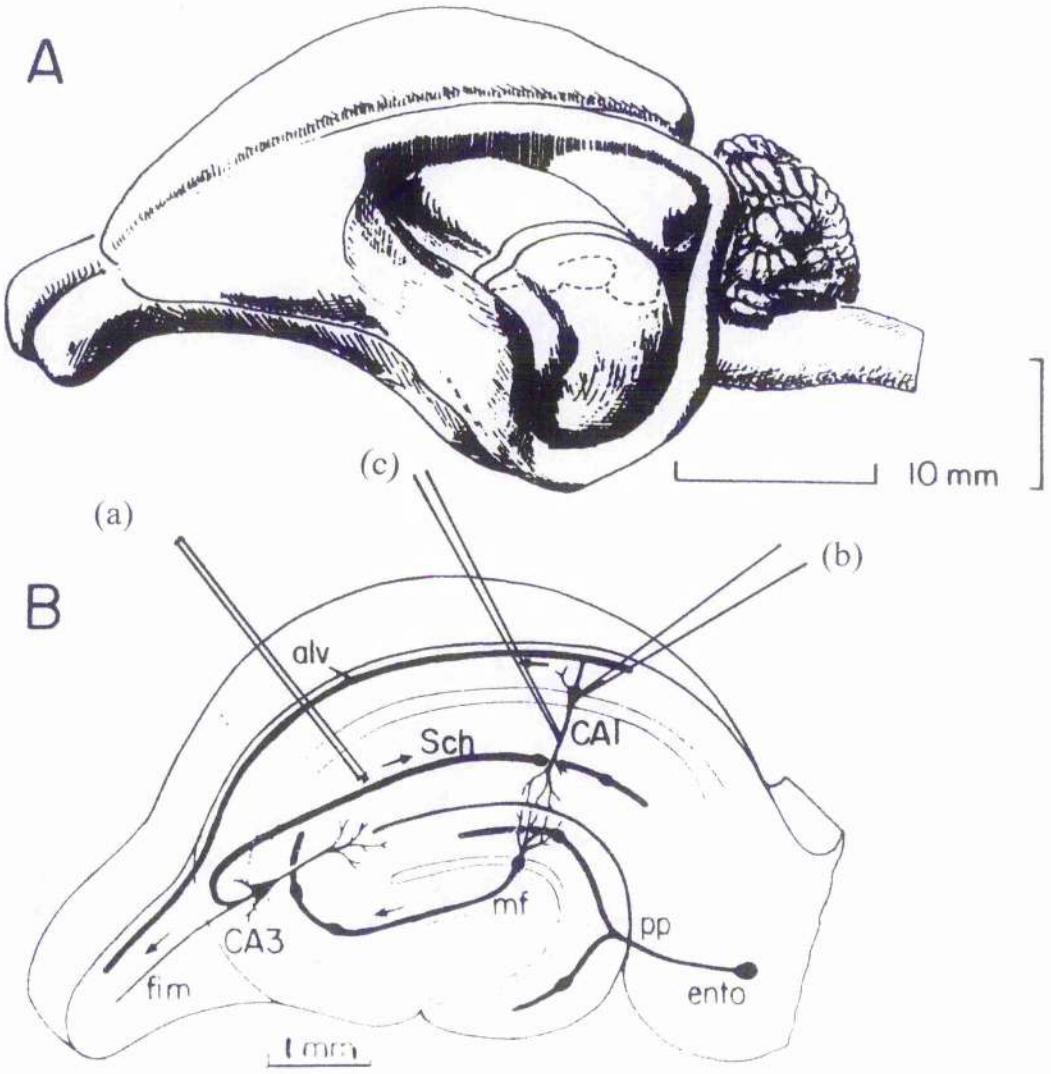

Figure 2.1 Diagram showing the organisation of a hippocampal slice. The stimulating electrode (a) was positioned in the stratum radiatum at the CA1/CA2 junction. Orthodromic population spikes and population excitatory postsynaptic potentials were recorded from the stratum pyramidale and stratum radiatum respectively using electrodes (b) and (c). (Adapted from Andersen *et al.*, 1971b)

VI. Protein purification techniques.

During the course of investigations into the effects of AMPase on hippocampal slices, it became apparent that the long-term depression of responses seen after removal of AMPase from slice preparations was not in fact due to an action of AMPase, but rather a contaminant in the preparation received from Sigma. It was decided to try and identify this element. The crude protein preparation was purified by means of fast performance liquid chromatograpic and gel filtration techniques by Prof. H.G. Nimmo, University of Glasgow (see appendix 1) and the purified protein sequenced by Dr. N. Morrice, University of Dundee (appendix 2).

VII. Drugs.

The majority of drugs were made up in distilled water to form a stock solution and then diluted to the required concentration in aCSF. N6-cyclopentyladenosine (CPA), 8-phenyltheophylline (8-PT) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), were dissolved in ethanol, 7-nitroindazole (7NI) and indomethacin were dissolved in methanol. Dimethylsulphoxide (DMSO) was used to dissolve 4-amino-8-chloro-1-phenyl(1,2,4)-triazolo(4,3a)quinoxaline (CP 66713), 4-(2-[7-amino-2-{2-furyl}{1,2,4,}triazolo{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)phenol (ZM 241385), forskolin, 2-[1-(3-dimethylaminoproply)indol-3-yl]-3-(indol-3-yl)maleimide (GF109203X), glibenclamide and 8-(3-chlorostyryl)caffeine (CSC) with the final concentration of DMSO never exceeding 0.1%. 5'adenylic acid

deaminase (AMPase) and adenosine deaminase were dissolved directly in aCSF with AMPase being filtered to remove residue.

Adenosine, adenosine triphosphate (ATP), adenosine deaminase, 5'adenylic acid deaminase (AMPase), N6-cyclopentyladenosine (CPA), 8-Bromoadenosine-3':5'-cyclic monophosphate (8Br cAMP), NG-nitro-L-arginine methyl ester (L-NAME), 7-nitroindazole (7NI) and forskolin were obtained from Sigma Chemical Co. Ltd.. 2-p-(2-carboxyethy)phenethylamino-5'-N-ethylcarboxamidoadenosine (CGS 21680), 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ 22536), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), and N-[2-methylamino)ethyl]-5-isoquinolinesulfonamide hydrochloride (H8) were obtained from Research Biochemicals International. 4-amino-8-chloro-1-phenyl(1,2,4)-triazolo(4,3a)quinoxaline (CP 66713) was obtained from Pfizer and 4-(2-[7-amino-2-{2-furyl}{1,2,4,}triazolo{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)phenol (ZM 241385) from Zeneca Pharmaceuticals. N-(2-aminoethyl)-5-chloro-1-napthalenesulphonamide (A3 HCl), and 2-[1-(3-dimethylaminoproply)indol-3-yl]-3-(indol-3-yl)maleimide (GF109203X) were obtained from Tocris Cookson.

CHAPTER 3.

INTERACTIONS BETWEEN ADENOSINE A_1 AND A_{2A} RECEPTOR SUBTYPES.

I. Introduction.

Adenosine is a neuromodulator with both presynaptic and postsynaptic effects in the mammalian CNS. It has been suggested that these effects are mediated by different mechanisms in view of, for example, their different sensitivities to agents such as pertussis toxin (Fredholm *et al.*, 1989; Hasuo *et al.*, 1992; Thompson *et al.*, 1992). Presynaptically, adenosine has been shown to inhibit the release of neurotransmitters such as acetylcholine (Spignoli *et al.*, 1984), dopamine (Michaelis *et al.*, 1979; Zetterstrom and Fillenz, 1990) serotonin (Feuerstein *et al.*, 1985) and glutamate (Fastbom and Fredholm, 1985). The mechanism of adenosine's presynaptic effects is not fully clear but it has been suggested that it causes an increase in potassium conductance which could hyperpolarize the axon terminal and so prevent transmitter release (Thompson *et al.*, 1992). Supporting this, it has been shown that K^+ channel blockers such as 4-aminopyridine can reduce the effects of adenosine (Stone, 1981). An indirect decrease of calcium currents or intracellular calcium levels by this adenosine activated potassium conductance has also been suggested as a mechanism of presynaptic inhibition (Dunwiddie and Haas, 1985; Michaelis *et al.*, 1988).

Alternatively, adenosine may exert its presynaptic effects by inhibiting calcium influx directly (Dolphin *et al.*, 1986; Schubert *et al.*, 1987).

Postsynaptically, the mechanism of action of adenosine is clearer. Several groups have suggested that adenosine causes an increase in outward potassium conductance leading to a hyperpolarisation of the postsynaptic membrane (Okada and Ozawa, 1980; Segal, 1982; Trussel and Jackson, 1985). This potassium conductance has been shown to be both calcium and voltage insensitive in CA1 neurones (Gerber *et al.*, 1989).

Earlier studies have suggested that the inhibitory presynaptic actions and postsynaptic hyperpolarising actions of adenosine are mediated by A_1 receptors (Dunwiddie and Fredholm, 1989; Alzheimer *et al.*, 1991; Ameri and Jurna, 1991; Lambert and Teyler, 1991; Hasuo *et al.*, 1992) which are present in high density in the hippocampus, especially in the CA1 region (Fastbom *et al.*, 1987). Electrophysiological (Sebastião and Ribeiro, 1992) and binding (Jarvis *et al.*, 1989; Cunha *et al.*, 1994a) studies have shown that adenosine A_{2A} receptors are also present in the hippocampus but conflicting reports exist as to the functional importance of these receptors and their ability to interact with A_1 receptors. Cunha *et al.*, (1994a) describe an attenuation of the inhibitory effects on population spike amplitude caused by activation of A_1 adenosine receptors with N^6-cyclopentyladenosine (CPA) in the presence of the selective A_{2A} adenosine receptor agonist 2-p-(2-carboxyethy)phenethylamino-5'-N-ethylcarboxamidoadenosine (CGS 21680) in the rat hippocampus. In contrast, Dunwiddie *et al.*, (1997) report that pre-treatment of hippocampal slices with

CGS 21680 had no effect on subsequent responses to adenosine. Recently, Dixon *et al.* (1997) have shown the appearance of a low-affinity binding site for the A_1 receptor agonist 2-chloro-N-6-cyclopentyladenosine (CCPA) in the rat striatum caused by incubation of striatal synaptosomes with CGS 21680.

Although the aforementioned pre- and postsynaptic effects of adenosine have been well characterised, little work has been carried out with regard to the effects of adenosine on the coupling between the two. This study investigates the effects of adenosine receptor agonists and antagonists on the population excitatory postsynaptic potential, the population spike, and the relationship between the two i.e. EPSP-spike (E-S) coupling. The population excitatory postsynaptic potential (popEPSP) gives primarily a measure of membrane potential changes generated by excitatory synapses on the apical dendrites of CA1 pyramidal neurones. This extracellularly recorded potential has been shown to be affected by adenosine in the same way as intracellularly recorded EPSPs from the same cells (Proctor and Dunwiddie, 1983). The population spike (PS) reflects the summated firing of CA1 pyramidal neurones (Andersen *et al.*, 1971) and gives a measure of the excitability of the postsynaptic neurone. E-S coupling gives an indication of the ability of a given level of synaptic depolarisation to induce the postsynaptic cell to fire an action potential. The three parameters measured can vary independently, and a description of all three is necessary to provide a full analysis of agents on neuronal function.

II. Results

Adenosine

A 10 min perfusion of adenosine 20 μM resulted in a decrease of both population spike (PS) amplitude and EPSP slope, but PS amplitude was reduced to a greater extent than EPSP slope. The relationship between stimulation strength and PS amplitude, EPSP slope and the corresponding E-S curve are summarised for a typical slice in Figure 3.1. Following the addition of adenosine in this slice the E-S relationship is shifted to the right as a result of the greater depression of PS amplitude compared with EPSP slope. From the population of slices examined, adenosine at 50 μM decreased both the population EPSP slope and PS amplitude significantly ($n = 5$, 40.9% ± 4.1, $p < 0.01$ and 65.5% ± 12.7, $p < 0.001$, respectively) (Figure 3.2a). At the lower concentration of 20μM, adenosine also decreased these potentials to a significant degree ($n = 5$, 28.6% ± 11.1, $p < 0.05$ and 48.8% ± 13.0, $p < 0.01$, respectively) (Figure 3.2a). In each case the PS amplitude was reduced by a greater extent than can be accounted for by the decrease in population EPSP slope, resulting in a shift in the E-S curve of 24.7% ± 10.2 ($p < 0.05$) with adenosine 20 μM and 43.5% ± 15.1 with adenosine 50 μM ($p < 0.05$) Figure 3.2b)

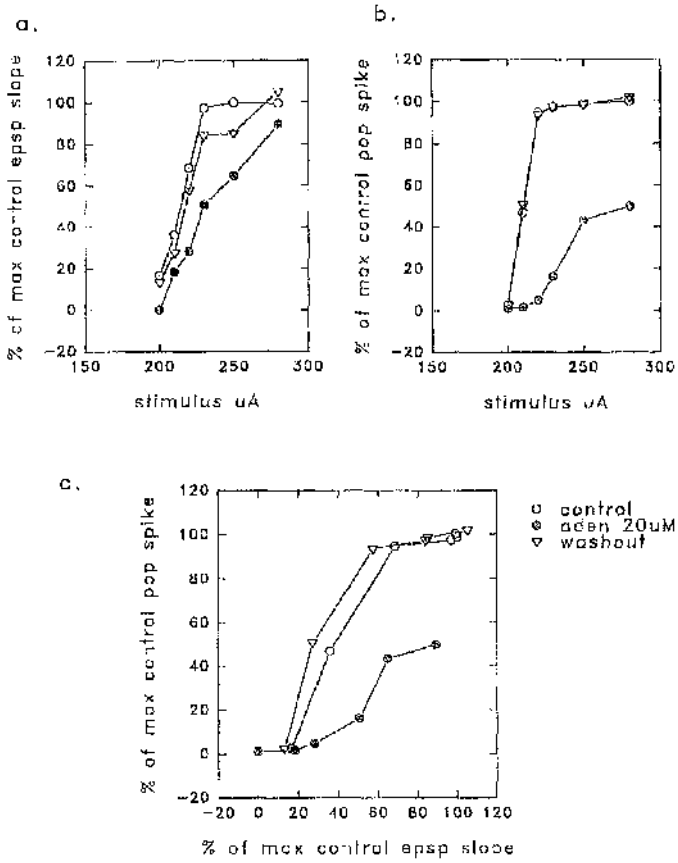

Figure 3.1. Graphs (a) and (b) show input/output curves for population spike amplitude and EPSP slope respectively for a single slice. In graph (c), population spike size is plotted as a function of EPSP slope showing the resulting EPSP-spike relationship. When adenosine 20μM was perfused, population spike amplitude was reduced by a greater extent than EPSP slope. This resulted in a change in the EPSP-spike relationship characterised by a right-shift from control values as seen in graph (c).

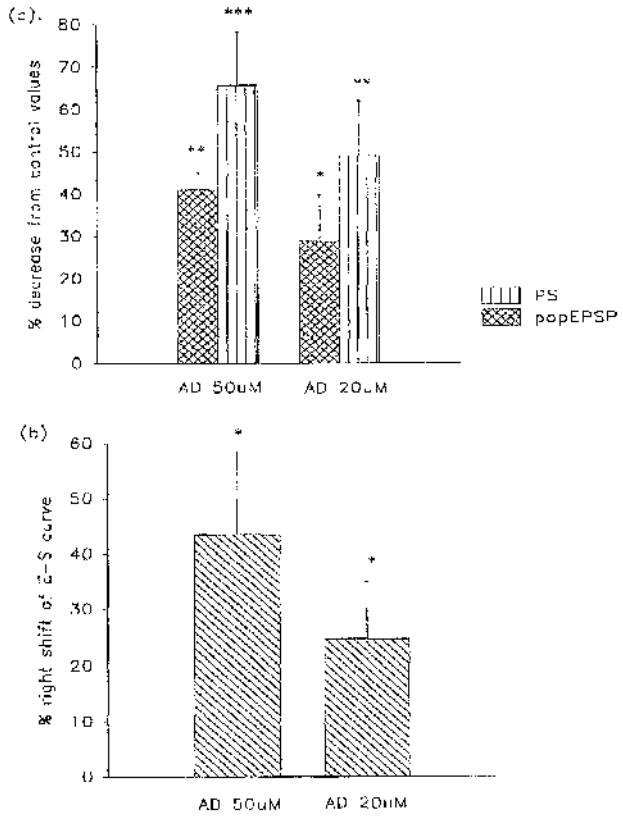

Figure 3.2. Pooled data from n=5 slices. Chart (a) shows the effects of adenosine (AD) 50μM and 20μM on population spike (PS) amplitude and population EPSP slope (popEPSP). It can be seen that perfusion of adenosine caused a concentration-dependent decrease in PS amplitude and EPSP slope. The decrease in PS amplitude was larger then could be accounted for by the decrease in EPSP slope resulting in a shift in the EPSP-spike relationship from control values (b). * $p < 0.05$, ** $p < 0.01$, *** $P < 0.001$.

Selective agonists and antagonists

Representative potentials showing the effect of the selective adenosine A_1 agonist N^6–cyclopentyladenosine (CPA) 50 nM on population spike and population EPSP are displayed in figure 3.3. CPA caused a significant decrease in both the population EPSP slope and PS amplitude (Figure 3.4, 3.5a). Input/output data quantify this decrease as 28.9% ± 7.9, n = 5, $p < 0.01$ for population EPSP slope and 83.9% ± 5.2, n = 5, $p < 0.001$ for PS amplitude. This resulted in a large shift to the right of the E-S curve of 81.8 % ± 5.2 ($p < 0.001$) (Figure 3.5b).

To ensure that CPA was exerting its effects on population EPSP slope as a result of decreased transmitter release and not due to an influence on presynaptic afferent excitability, the slope of the presynaptic volley was also measured in three experiments and was plotted as a function of stimulus intensity in the same manner as input/output curves (Figure 3.6). No change was observed in presynaptic afferent excitability with CPA, showing its effects are restricted to events occuring subsequent to the arrival of the fibre volley at the stratum radiatum axon terminals.

The selective adenosine A_{2A} agonist 2-[p-(carboxyethyl)-phenylethylamino]-5'-N-ethylcarboxamidoadenosine (CGS 21680) 30 nM (n = 5) and 100 nM (n = 4) had no significant effects on population EPSP slope, PS amplitude or the relationship between the two (Figure 3.5). Similarly the A_{2A} antagonists 4-(2-[7-amino-2-{2-furyl}{1,2,4,}triazolo{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)phenol (ZM 241385, 50 nM) and 4-amino-8-chloro-1-phenyl(1,2,4)-

triazolo(4,3a)quinoxaline (CP 66713, 10 μM) also showed no significant effects on the three parameters, although ZM 241385 showed a tendency to increase all three (Figure 3.5a,b).

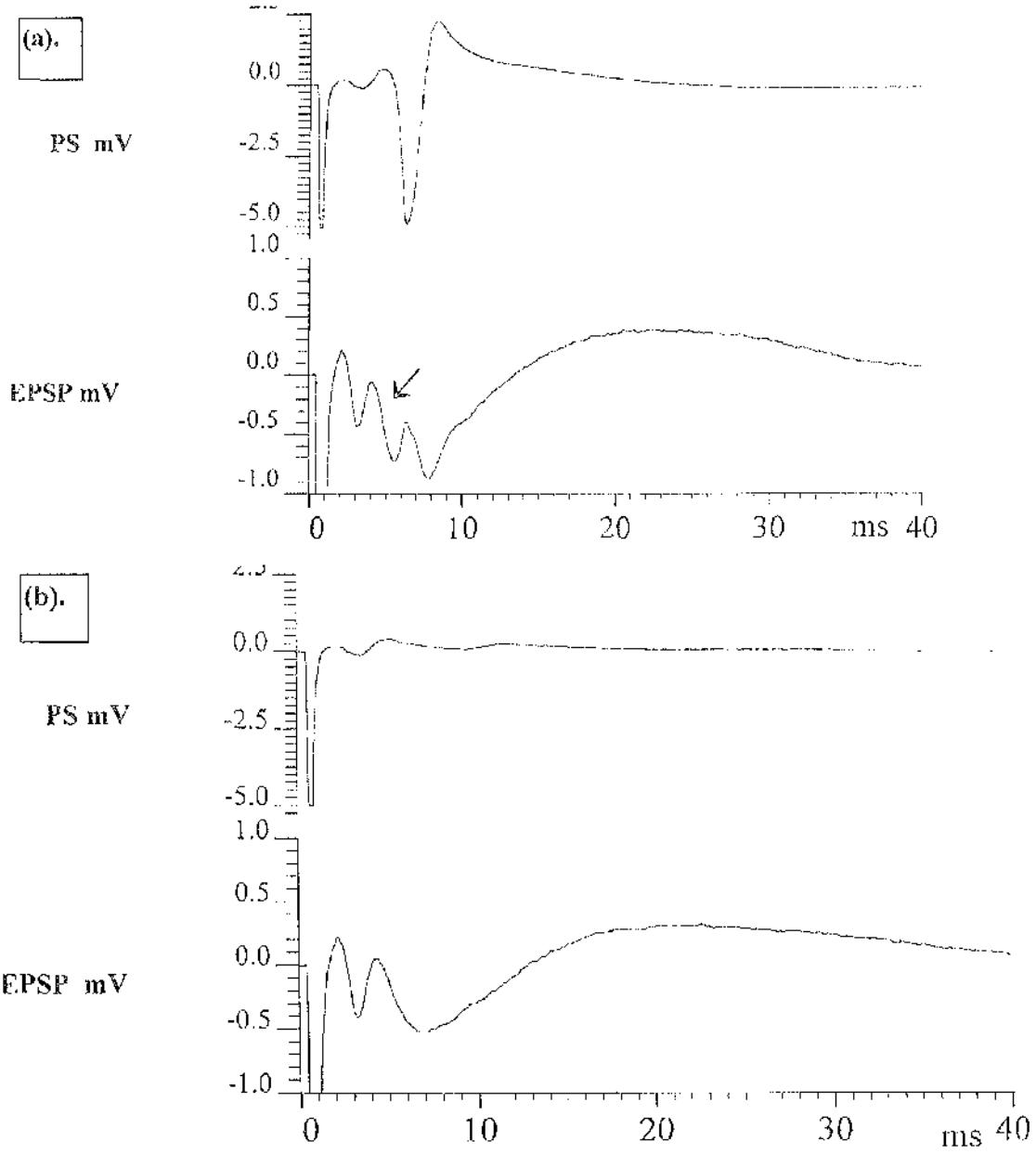

Figure 3.3. The effect of CPA 50 nM on population spike (PS) amplitude and EPSP slope (arrow). The traces show responses recorded from a single slice. It can be seen that a 10 min perfusion of 50 nM CPA decreased PS amplitude by a greater extent then EPSP slope.

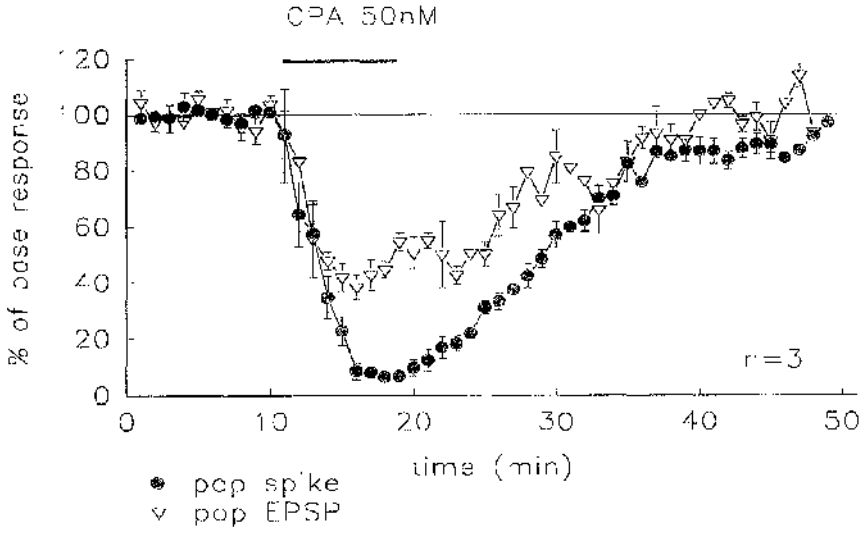

Figure 3.4. The effect of N^6-cyclopentyladenosine (CPA) on PS amplitude (filled circles) and EPSP slope (open triangles). 10 min perfusion of CPA 50 nM resulted in a greater inhibition of PS amplitude than EPSP slope, resulting in a dissociation of the EPSP-spike relationship which returned to control values following 30 min washout.

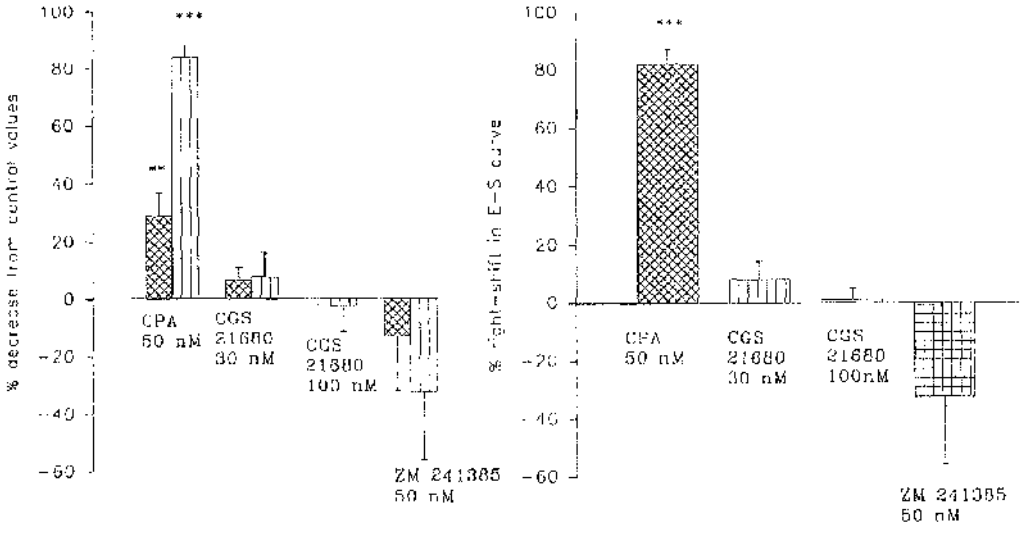

Figure 3.5 The effects of adenosine agonists and antagonists on input/output curves (a) and corresponding E-S curves (b). The A_1 agonist CPA decreased both EPSP slopes and PS amplitude, and shifted the E-S curve significantly to the right whereas A_{2A} receptor activation had no significant effects on any of the three parameters. ** $p < 0.01$, *** $p < 0.001$.

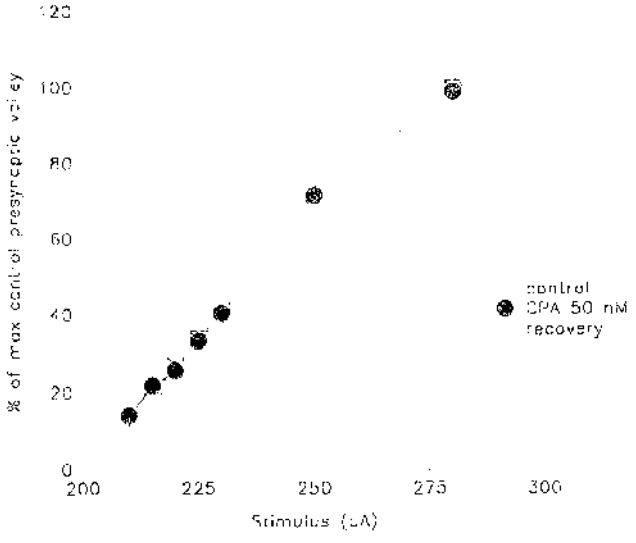

Figure 3.6 The effect of the selective adenosine A_1 agonist CPA on afferent fibre volley. The slope of the afferent fibre volley was plotted as a function of stimulus intensity. It can be seen that CPA had no effect on the afferent volley.

A_1/A_2 interactions

When adenosine A_1 and A_{2A} receptors were activated at the same time using CPA and CGS 21680 respectively, there was a reduction in the effects of CPA on population EPSP slope and PS amplitude. This can clearly be seen when Figure 3.7 is compared with Figure 3.3.

Perfusion of CGS 21680 30 nM for 10 min prior to and during CPA perfusion caused an attenuation in the reduction of PS amplitude seen with CPA alone, resulting in a reduction in the amount of E-S dissociation at a stimulus intensity 70% of maximum control (Figure 3.8).

Over a range of stimulation intensities, input/output data show CGS 21680 at both 30 nM and 100 nM caused a non-significant concentration related reduction in the effect of CPA 50 nM on PS amplitude (Figure 3.9a). Using 30 nM CGS 21680, the inhibition of PS amplitude was reduced from 83.9% ± 4.3 to 59.9% ± 6.5 ($p < 0.05$). The attenuation of the inhibition of PS amplitude by CPA resulted in a significant reduction in the effects of CPA on E-S coupling ($p < 0.05$ using CGS 21680 100 nM and $p < 0.01$ using CGS 21680 30 nM) (Figure 3.9b).

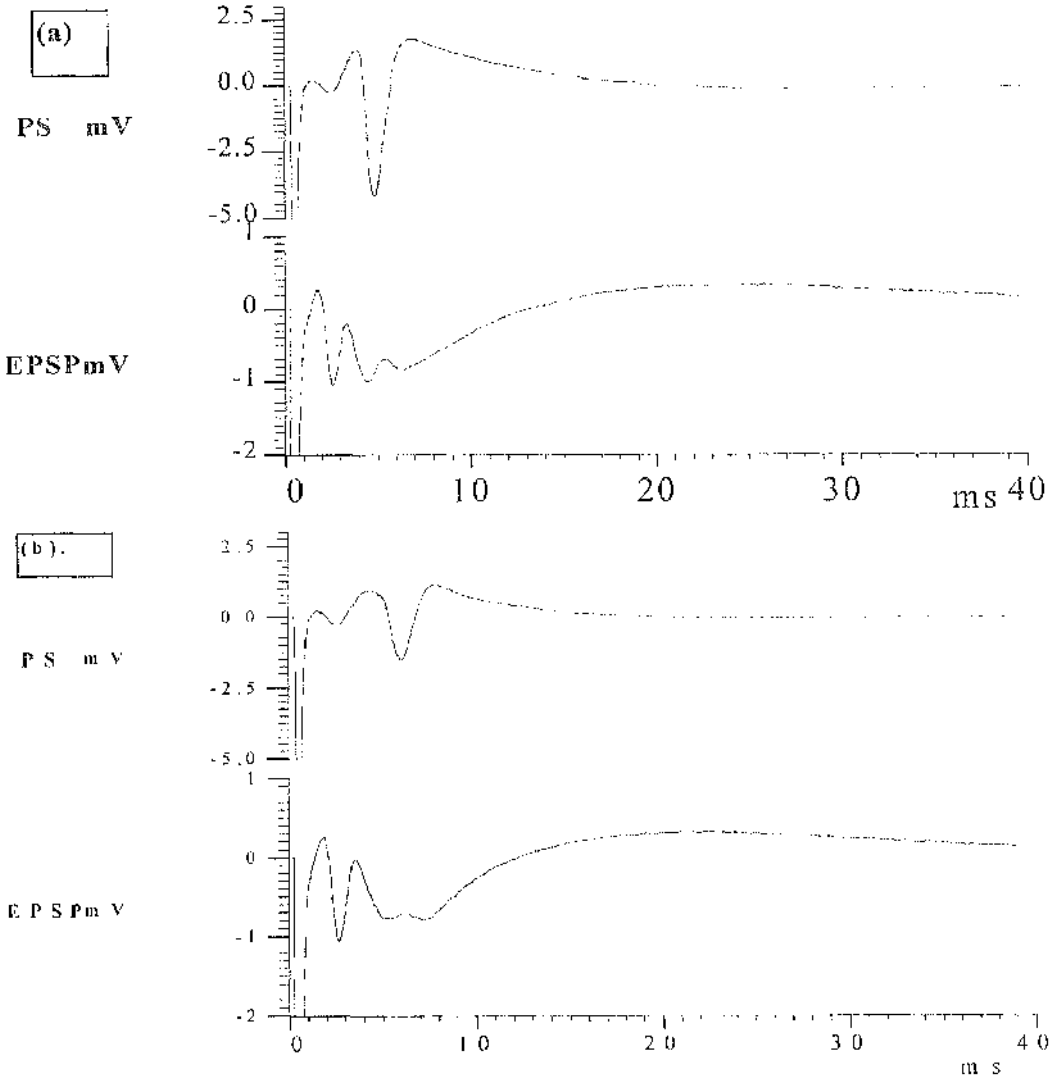

Figure 3.7. The effect of co-activation of adenosine A_1 and A_{2A} receptors with the selective A_1 and A_{2A} agonists CPA 50 nM and CGS 21680 30 nM. The inhibition of PS amplitude by CPA was attenuated when A_{2A} receptors were activated at the same time using CGS 21680.

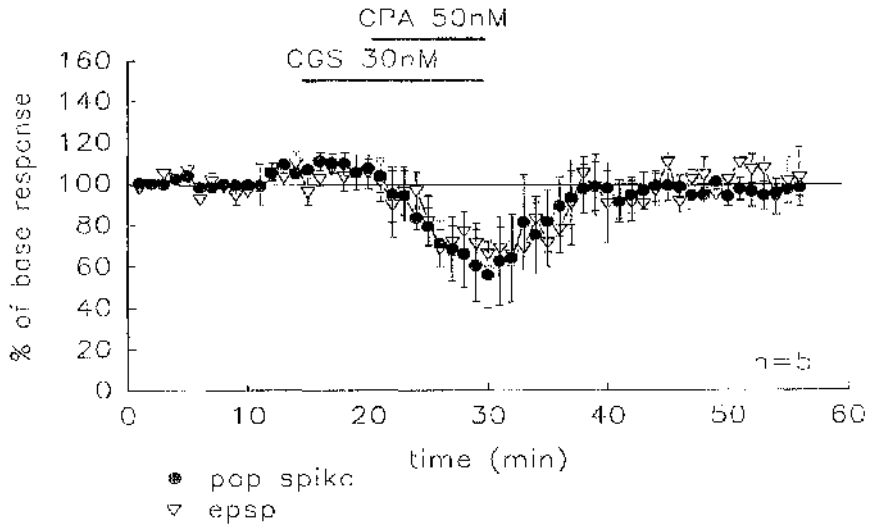

Figure 3.8. Activating adenosine A_{2A} receptors with CGS 21680 30 nM 10 min prior to and during activation of adenosine A_1 receptors using CPA 50 nM reduced the inhibitory effect of adenosine A_1 receptor activation alone and prevented the dissociation of the EPSP-spike relationship seen upon activation of adenosine A_1 receptors with CPA alone.

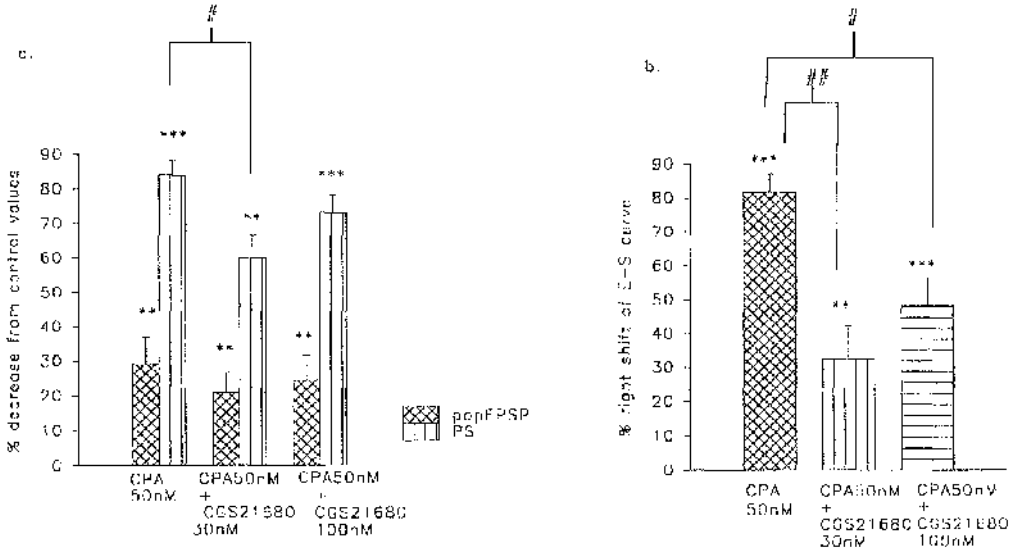

Figure 3.9. Pooled data (n=5) showing the effects of co-activation of A_1 and A_{2A} receptors with selective agonists. Addition of the adenosine A_{2A} receptor agonist CGS 21680 caused a significant reduction in the inhibition of population spike amplitude seen upon A_1 receptor activation alone (a). Addition of either 30 nM or 100 nM CGS 21680 resulted in a significant reduction in the right shift of the E-S curve seen upon A_1 receptor activation (b). ** $p<0.01$, *** $p<0.001$ compared with controls. # $p<0.05$, ## $p<0.01$ between columns.

Transduction modulators.

In an attempt to determine the mechanism by which A_{2A} receptor activation attenuated A_1-mediated responses, the role of various second messengers and nitric oxide was investigated. The membrane permeable analogue of cAMP: 8-Bromoadenosine-3':5'-cyclic monophosphate (8Br cAMP; 100μM, n=3) by itself decreased PS amplitudes but had no effect on the inhibition of popEPSP slope nor PS amplitude seen with CPA superfusion when used in combination with this agent. 8Br cAMP itself had no effects on the E-S curve and furthermore, had no significant effect on the magnitude of the shift obtained with CPA when the two compounds were added together (Table 3).

The adenylate cyclase activator forskolin (10μM, n=3) decreased both popEPSP slopes and PS amplitudes and caused a right-shift in the E-S curve (Table 3) but again no change was seen in CPA-mediated responses when in the presence of forskolin (Table 3). In contrast the adenylate cyclase inhibitor 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ 22536; 10μM, n=3) had no significant effects on I/O curves nor E-S coupling by itself, although when tested in combination with CPA it did not affect the inhibitory responses seen with CPA alone (Table 3). These results suggest that the adenylate cyclase/cyclic AMP system is not involved in the A_{2A} attenuation of A_1-mediated inhibitory responses.

To investigate the role of second messengers further downstream in the signal transduction process the effects of protein kinase inhibitors was investigated. The hydrochloride salt of the general protein kinase inhibitor N-(2-aminoethyl)-

5-chloro-1-napthalenesulphonamide (A3 HCl; 10μM, n=4) had no significant effect on popEPSP slopes, PS amplitudes nor the coupling between the two, nor did it affect the inhibition mediated by CPA when added in combination (Table 3, Figure 3.10). 2-[1-(3-dimethylaminoproply)indol-3-yl]-3-(indol-3-yl)maleimide (GF109203X; 100nM, n=5) is a very potent and selective inhibitor of protein kinase C (Toullec *et al.*, 1991). Addition of this compound to the superfusing medium resulted in a decrease of PS amplitude, but no resulting shift in E-S relationship (Table 3). GF 109203X also had no effects on the adenosine A_{2A}-receptor mediated attenuation of A_1 inhibition of responses (figure 3.12) Neither was the inhibitory effect of CPA affected by addition of this compound (Table 3). The protein kinase A inhibitor N-[2-methylamino)ethyl]-5-isoquinolinesulfonamide hydrochloride (H8; 30μM, n=4) had no significant effects on I/O curves or E-S coupling. Again this compound also had no effect on the inhibitory action of CPA (Table 1).

Nitric oxide.

To test whether nitric oxide was playing a part in the inhibition observed with A_1 receptor activation by CPA, the effects of nitric oxide synthase inhibitors were examined. Neither the competitive inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 20μM, n=4) nor the selective inhibitor of brain nitric oxide synthase 7-nitroindazole (7NI; 40μM, n=5) had any significant effects on popEPSP slopes, PS amplitudes or E-S coupling, nor did they have any effects on the responses to CPA (Table 3).

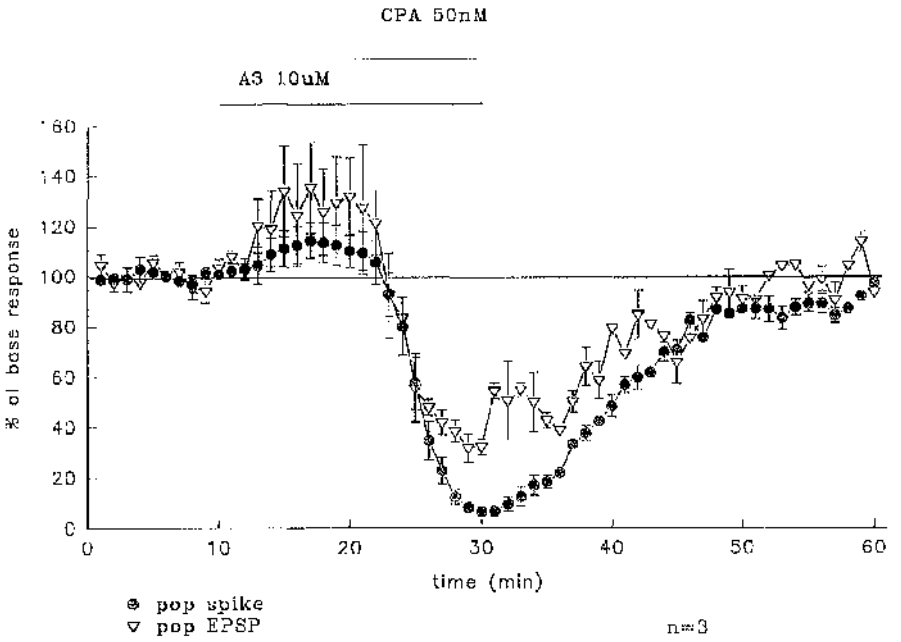

Figure 3.10. The effect of the general protein kinase inhibitor A3 10 μM on responses to CPA 50 nM

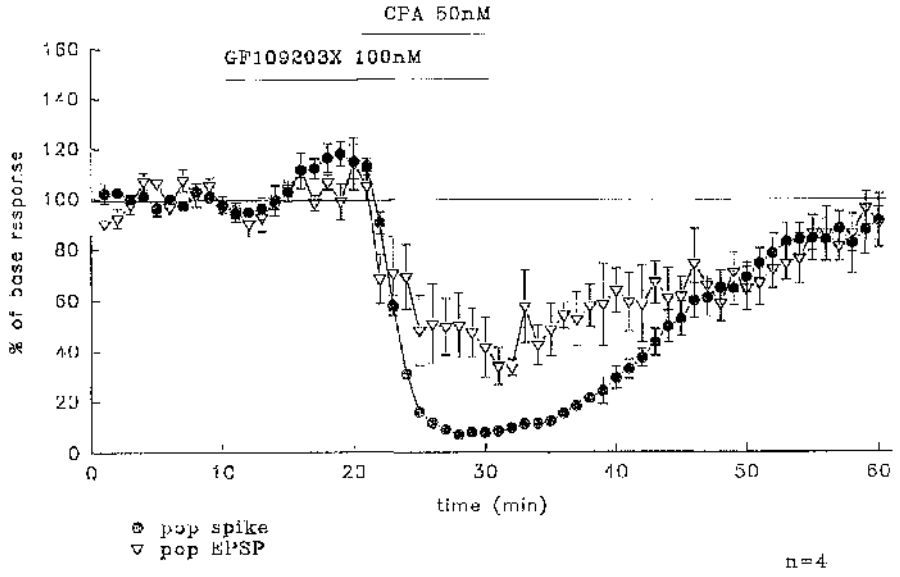

Figure 3.11. The effect of the selective protein kinase C inhibitor GF 109203X on responses to CPA 50 nM.

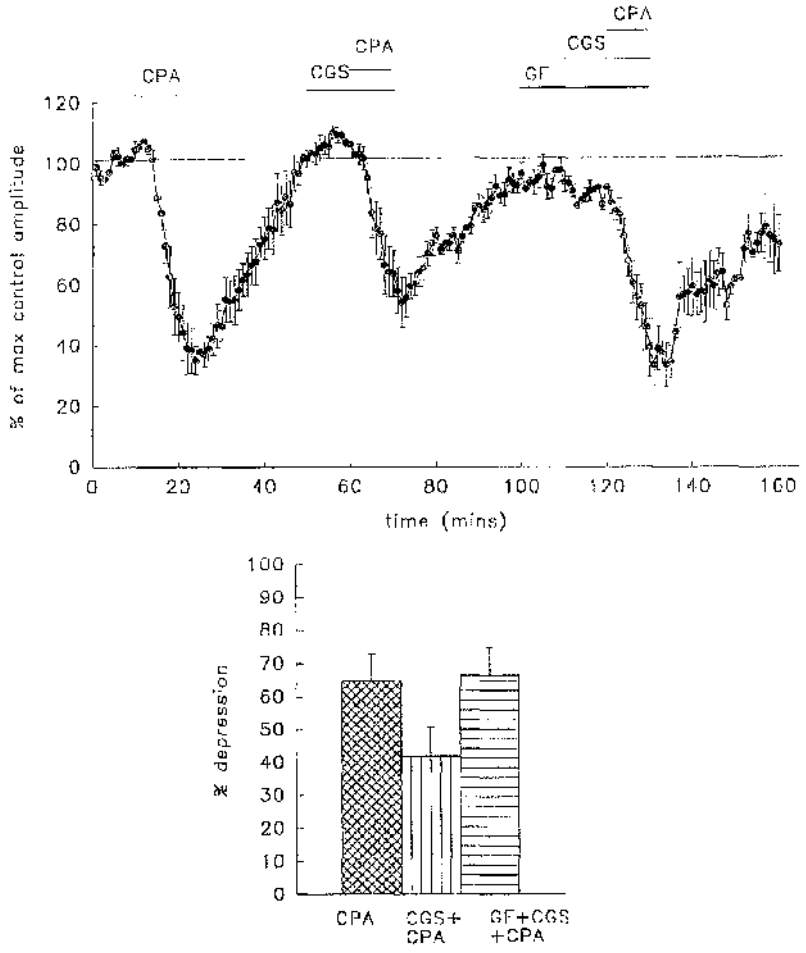

Figure 3.12. The effect of the protein kinase inhibitor GF 109203 on the inhibitory effects of adenosine A_{2A} receptor activation on A_1-mediated depression of PS amplitude.

Table 3. The effects of modulators of different transduction systems on population EPSP slopes (popEPSP), population spike (PS) amplitudes and E-S coupling used both alone and in combination with N^6-cyclopentyladenosine (CPA) 50 nM. Data for N^6-cyclopentyladenosine 50 nM are included for comparison. Statistics shown beside drugs used alone are relative to control values, whereas statistics displayed beside drugs when used in combination with N^6-cyclopentyladenosine 50 nM are relative to data for N^6-cyclopentyladenosine used by itself.

DRUG	n	popEPSP decrease %		PS decrease %		E-S right shift %	
CPA 50 nM	5	28.9±7.9	p<0.01	83.9±4.3	p<0.001	81.8±5.2	p<0.001
8Br cAMP 100 µM	3	35.4±16.5	n.s.	65.8±24.7	p<0.01	27.7±27.6	n.s.
8Br cAMP 100 µM+ CPA 50 nM	3	48.2±12.0	n.s.	90.7±6.6	n.s.	67.1+20.9	n.s.
forskolin 10 µM	3	59.4±5.5	p<0.001	81.9±3.5	p<0.001	59.4±7.2	p<0.01
forskolin 10 µM+CPA 50 nM	3	59.2±4.1	p<0.05	92.9±2.3	n.s.	90.1±2.2	n.s.
SQ22536 10 µM	3	-13.4±28.2	n.s.	-8.05±19.8	n.s.	-13.2±6.5	n.s.
SQ22536 10 µM + CPA 50 nM	3	30.95±14.7	n.s.	81.1±8.3	n.s.	50.7±26.7	n.s.
A3 HCl 10 µM	4	3.96±12.4	n.s.	4.3±12.8	n.s.	-19.6±12.4	n.s.
A3 Hcl 10 µM+ CPA 50 nM	4	37.1±8.0	n.s.	86.9±8.0	n.s.	62.975±19.1	n.s.
GF109203X 100 nM	5	19.9±14.2	n.s.	35.2±31.1	p<0.05	9.8±9.7	n.s.
GF109203X 100 nM+ CPA 50 nM	5	32.1±16.2	n.s.	95.3+4.1	n.s.	78.9±9.7	n s.
H8 30 µM	4	-12.7±24.3	n.s.	-31.7±18.5	n.s.	-30.1±32.4	n.s.
H8 30 µM +CPA 50 nM	4	26.1±14.2	n.s.	77.3±17.8	n.s.	63.9±23.4	n.s.
7-NI 40 µM	5	18.4±8.0	n.s.	15.9±13.8	n.s.	-2.0±10.0	n.s.
7-NI 40 µM+ CPA 50 nM	5	41.7±8.7	n.s.	80.5±14.1	n.s.	73.3±17.6	n.s.
L-NAME 20 µM	4	17.5±13.5	n.s.	25.1±24.7	n.s.	2.5±5.0	n.s.
L-NAME 20 µM+ CPA 50 nM	4	39.9±9.5	n.s.	91.7±4.3	n.s.	89.2±5.4	n.s.

Effect of blocking potassium channels.

Superfusion of the potassium channel blockers, glibenclamide 10 μM or barium 500μM to the slice 10min prior to, and during, CPA 50nM perfusion, attenuated the inhibitory effects of CPA on PS amplitude (Figure 3.13, 3.14), again resulting in a significant inhibition of the E-S dissociation seen with CPA alone. The changes in PS/popEPSP ratio seen when adenosine A_{2A} receptors were activated with CGS 21680 or when potassium channels were blocked with barium were not significantly different (Figure 3.15).

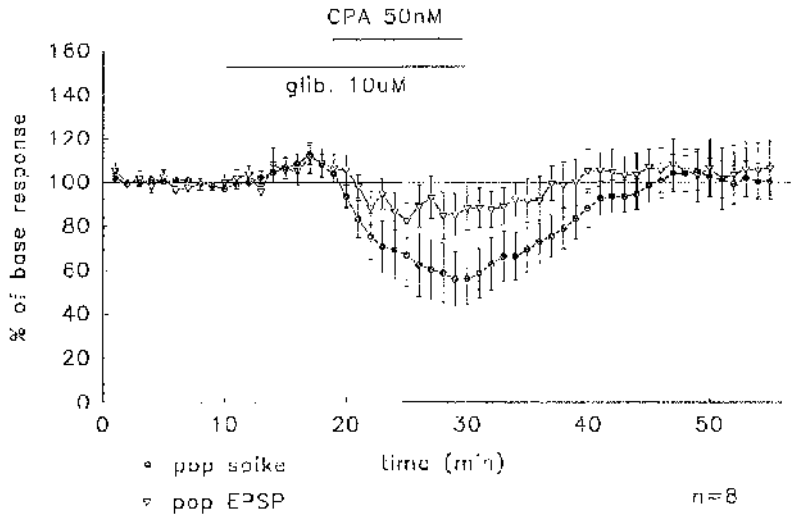

Figure 3.13. The effect of blocking potassium channels with glibenclamide 10 μM on responses to CPA.

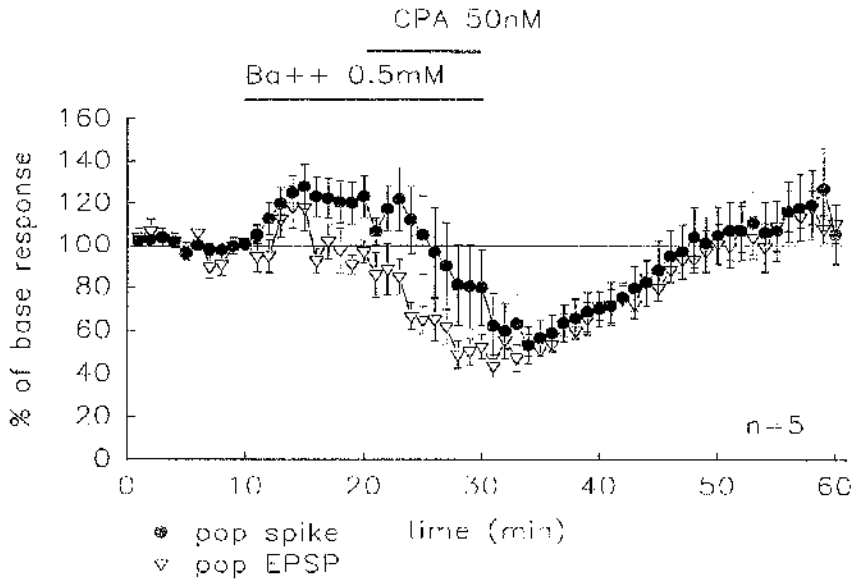

Figure 3.14 The effect of barium 0.5 mM on responses to CPA 50 nM.

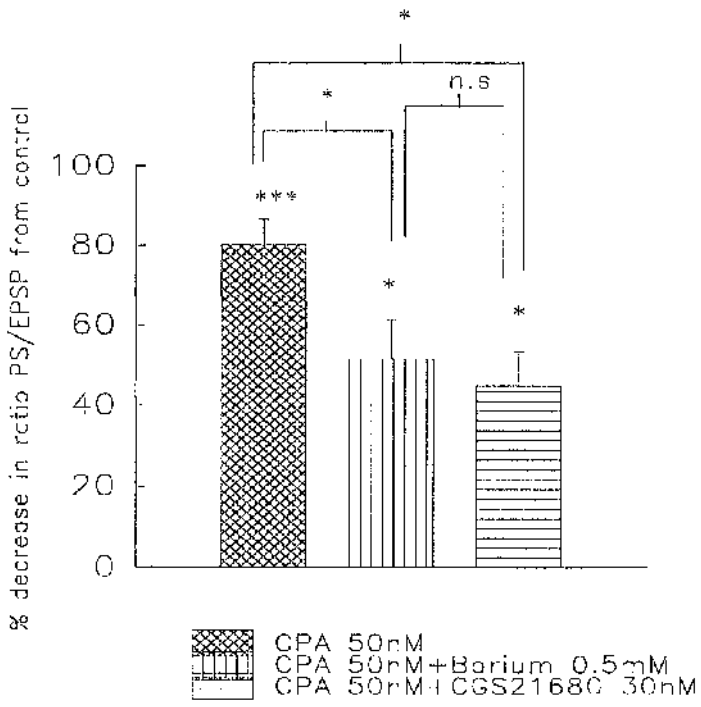

Figure 3.15. Comparison of the attenuation of CPA-mediated inhibitory effects on PS/EPSP ratio by CGS 21680 and barium.

III. Discussion.

The results reveal that adenosine reduces both the population EPSP slope and population spike amplitude in the CA1 area of rat hippocampus, showing that it has postsynaptic actions as well as presynaptic. Consistent with previous findings (Dunwiddie and Fredholm, 1989; Alzheimer *et al.*, 1991; Lambert and Teyler, 1991) these effects are mediated by adenosine A_1 receptors as shown by the fact that the selective A_1 agonist N^6-cyclopentyladenosine produced a similar depression of both potentials while the A_{2A} agonist CGS 21680 had no significant effects. N^6-cyclopentyladenosine caused a significantly greater shift in the E-S curve than adenosine, reflecting its greater effect on population spike amplitude combined with a lesser reduction in population EPSP slope compared with adenosine. This could possibly be due to a greater number of adenosine A_1 receptors at postsynaptic sites (Deckert and Jorgensen, 1988) or a greater proportion of A_{2A} receptors at postsynaptic sites which, when activated by adenosine cause an attenuation of the effects of A_1 receptor activation. Alternatively, this difference could be explained by a more efficient uptake system for adenosine operating at presynaptic compared with postsynaptic sites.

E-S dissociation has previously been described as a component of long term potentiation in the CA1 region of rat hippocampus (Andersen *et al.*, 1980; Bernard and Wheal, 1995a,b). Two mechanisms have been put forward to explain this E-S dissociation. The first is a change in inhibitory pathways (Chavez-Noriega *et al.*, 1989; Tomasulo *et al.*, 1991; Tomasulo and Ramirez, 1993) such as a modification of excitatory synapses on interneurones or changes

in inhibitory synapses. Secondly, E-S dissociation may occur via a postsynaptic mechanism which alters the firing threshold of the cells (Taube and Schwartzkroin, 1988; Hess and Gustafsson, 1990). It should be noted that these two mechanisms are not mutually exclusive. It has been suggested that adenosine modulates excitatory but not inhibitory synaptic transmission in the hippocampus (Lambert and Taylor, 1991; Yoon and Rothman, 1991; Brundege and Dunwiddie, 1996) and a decrease in transmitter release here may account for some of its actions on the E-S relationship. However, since the population spike amplitude, which reflects postsynaptic excitability, decreased more than could be accounted for by the decrease in transmitter release it would appear that postsynaptic mechanisms play the major role in the decrease in E-S coupling seen with adenosine A_1 receptor activation. This presumably results from the increased potassium conductance which increases the firing threshold of the cell (Segal, 1982; Trussell and Jackson, 1985; Thompson *et al.*, 1992). This conclusion is strongly supported by intracellular experiments, since N^6-cyclopentyladenosine proved able to inhibit spike initiation at a concentration of 100 nM which had little effect on resting potential (T.W. Stone, personal communication).

Conflicting evidence exists as to the ability of adenosine A_{2A} receptors to interact with A_1 responses (Cunha *et al.*, 1994a; Dixon *et al.*, 1997; Dunwiddie *et al.*, 1997). In this study we have seen a clear attenuation of the population spike response obtained following activation of adenosine A_1 receptors in the presence of the A_{2A} agonist CGS 21680, showing there is a cross-talk between the two classes of receptor. Adenosine A_{2A} receptor activation had no significant effect on the reduction of population EPSP slopes seen with A_1 receptor activation

suggesting that A_{2A} receptors interact with A_1 receptors selectively at postsynaptic sites. Previous reports suggest that adenosine A_{2A} receptors have an excitatory function in the cortex (O'Regan *et al.*, 1992), striatum (Kirkpatrick and Richardson, 1993) and hippocampus (Sebastião and Ribeiro, 1992), although Cunha *et al.* (1994a) suggest that this excitatory function is only apparent in the CA3 region and dentate gyrus. If this were the case here it would be expected that when both inhibitory A_1 and excitatory A_{2A} receptors are stimulated the net synaptic activity would be the result of the summation of the inhibitory versus excitatory mediated responses. However this seems an unlikely explanation since we saw no significant excitatory response with the 30 nM A_{2A} receptor selective concentration of CGS 21680 used alone, but an attenuation of A_1 receptor-mediated inhibitory responses in its presence. The selectivity of this interaction is well illustrated by intracellular results, since CGS 21680 was able to prevent the suppression of spike initiation by N^6-cyclopentyladenosine, in the absence of any significant changes of resting potential (T.W. Stone, personal communication).

Transduction systems.

The results obtained upon addition of agents to modify second messenger systems suggest that the effect of A_{2A} activation on A_1-mediated responses in the hippocampus is independent of cyclic AMP (cAMP) and protein kinases. No effect was found of adenylate cyclase activation with forskolin or inhibition with SQ 22536 on the inhibitory effects of N^6-cyclopentyladenosine. Previous investigators have also demonstrated a lack of relationship between cAMP levels and the electrophysiological effects of adenosine (Reddington and Schubert,

1979; Dunwiddie and Hoffer, 1980; Dunwiddie and Fredholm, 1984; Worley *et al.*, 1987) except in forskolin treated hippocampal slices (Fredholm *et al.*, 1983). Similarly, the general protein kinase inhibitor A3, the selective protein kinase A inhibitor H8 and the selective protein kinase C inhibitor GF 109203X did not modify sensitivity to adenosine A_1 receptor activation.

There is growing evidence for an interaction between adenosine and nitric oxide systems (Dirnagl *et al.*, 1994; McKie *et al.*, 1994; Peralta *et al.*, 1997). However, neither the competitive nitric oxide synthase inhibitor L-NAME nor the brain specific inhibitor 7NI showed any effects on the inhibition obtained upon addition of N6-cyclopentyladenosine, suggesting that nitric oxide does not play a significant role in the inhibition seen with A_1 activation.

In contrast, blockade of potassium channels with glibenclamide or barium attenuated the postsynaptic actions of adenosine A_1 receptor activation. It is well established that, postsynaptically, adenosine increases potassium conductance (Okada and Ozawa, 1980; Segal,1982; Trussel and Jackson, 1985), and it has been shown that barium will selectively block the postsynaptic hyperpolarising effects of adenosine (Haas and Greene, 1984; Gerber at al., 1989; Thompson *et al.*, 1992; Akhondzadeh and Stone, 1994). In this study we have shown that the E-S dissociation caused by adenosine A_1 receptor activation studied extracellularly, and the directly measured effect on spike threshold recorded intracellularly (T.W. Stone, personal communication), are also prevented by barium. The possibility exists that a similar suppression of a potassium current

may be the mechanism by which adenosine A_{2A} receptor activation causes inhibition of A_1 receptor-mediated changes of spike threshold. Since barium can block several potassium currents (Cook, 1988; Guatteo *et al.*,1998), it is not yet clear which of these might be involved in the A_1/A_{2A} receptor interaction. It is unlikely that the I_A current is involved, however, as Pan *et al.* (1995) have shown that, whereas barium blocks the postsynaptic hyperpolarisation induced by adenosine, it does not prevent adenosine activation of the A-current. It is possible that an ATP-sensitive potassium channel (K_{ATP}) is involved, since the sulphonylurea glibenclamide, which selectively blocks this type of K^+ channel, also attenuated the postsynaptic effects of CPA. K_{ATP} channel involvement in the postsynaptic effect of adenosine is consistent with a report that an inhibitory postsynaptic potential in spinal nociceptive neurones, apparently mediated by adenosine, was blocked by glibenclamide (Salter *et al.*, 1993). Adenosine induced hyperpolarisation has also been shown to be blocked by glibenclamide in the hippocampal preparation (Li and Henry, 1992).

A possible explanation for the observed $A_1\backslash A_2$ receptor interaction is that activation of the adenosine A_{2A} receptor binding site by CGS 21680 may lead to a conformational change in the A_1 receptor and result in a decrease in the affinity of the receptor binding site for the A_1 agonist. This process would not require a second messenger system. As previously mentioned, the A_1 receptor has an allosteric binding site (Bruns and Fergus, 1990; Bruns *et al.*, 1990; Mundumbi *et al.*, 1993; Leung *et al.*, 1995). CGS 21680 may act at this site to affect the ability of the A_1 receptor to interact with its agonist or with its ability to couple to G-

proteins. Autoradiographic studies show binding of [3H]CGS 21680 to be greatest in the stratum radiatum, and adenosine A_1 and A_{2A} receptors have previously been found together pre-synaptically at rat cholinergic nerve terminals (Correia-de-Sá *et al.*, 1991; Cunha *et al.*, 1994b). The close proximity of the two receptors may allow for this conformational interaction to occur. Dixon *et al.* (1997) reported the appearance of a low affinity binding site for the A_1 agonist 2-chloro-N-6-cyclopentyladenosine following incubation of striatal synaptosomes with CGS 21680. This was interpreted as desensitisation by the A_{2A} site of the A_1 receptor. Such an explanation could account for the present observations although the interaction reported by Dixon *et al.* (1997) was mediated by protein kinase C.

CHAPTER 4.

INDUCTION OF LTP IN THE CA1 AREA OF RAT HIPPOCAMPUS FOLLOWING ATP PERFUSION.

I. Introduction.

The role of ATP as a transmitter or co-transmitter at neuroeffector junctions in the periphery is well established (Hoyle and Burnstock, 1991). The first direct evidence that ATP acts as a fast transmitter between neurones came in 1992 when synaptic currents mediated by ATP were measured in neurones cultured from guinea pig coeliac ganglia (Evans *et al.*, 1992; Silinsky *et al.*, 1992). These currents could be blocked by suramin and mimicked by ATP and αβMeATP, suggesting the involvement of a P2 receptor. Since then ATP receptor mediated currents have also been reported in intact ganglia (Silinsky and Gerzanich, 1993).

In the CNS, ATP has also been reported to act as a transmitter. It has been shown to produce excitation of neurones in several regions of the CNS (Harms *et al.*, 1992, Sun *et al.*, 1992; Tschopl *et al.*, 1992; Frolich *et al.*, 1996). Edwards *et al.* (1992) provided the most direct evidence for the action of ATP as a fast neurotransmitter in the CNS. This group recorded evoked and miniature synaptic currents in the rat medial habenula and demonstrated that these currents were not affected by blockers of glutamate, γ- aminobutyric acid (GABA) or acetylcholine receptors, but were blocked

by suramin and the desensitising ATP analogue αβMeATP. ATP has also been shown to modulate potassium currents in various regions of the CNS, including rat striatum (Ikeuchi and Nishizaki, 1995), superior colliculus (Ikeuchi *et al.*, 1995), hippocampus (Ikeuchi *et al.*, 1995a) and nucleus tractus solitarii (Ueno *et al.*, 1992). Calcium currents have also been shown to be modulated by ATP. For example ATP causes a rapid increase in intracellular calcium ions in rat hypothalamic neurones (Chen *et al.*, 1994).

In the hippocampus the effects of ATP receptor activation remain unclear. A range of effects have been described - from inward currents and increased frequency of glutamatergic EPSPs (Inoue *et al.*, 1992) to no effects on orthodromic spikes (Stone and Cusack, 1989) to a decrease in extracellularly measured unit spikes (Di Cori and Henry, 1984). Several studies have shown that functional responses to adenine nucleotides on neuronal networks and synaptic transmission in the mammalian hippocampus are usually explained by metabolism to adenosine (Dunwiddie and Hoffer, 1980; Cusack and Stone, 1989; Cunha *et al.*, 1998). Receptors for ATP are present in the hippocampus as shown by binding studies (Michel and Humphrey, 1993; Bo and Burnstock, 1994; Balcar *et al.*, 1995); messenger RNA for several P2 receptor subtypes have also been identified in the hippocampus (Kidd *et al.*, 1995; Seguela *et al.*, 1996; Soto *et al.*, 1996). The demonstration of conductance changes on individual cells and coupling to second messenger pathways suggest that the visualised receptors are functionally coupled to channels or other transduction systems.

An additional piece of evidence for the role of ATP as a neurotransmitter is that it is released in response to tetanic (but not low frequency) stimulation (Wieraszko *et al.*, 1989). This is of interest as tetanic stimulation can induce long term potentiation (LTP) in the hippocampus (Bliss and Lomo, 1973) and, moreover, it has been reported that ATP can induce LTP in mouse and guinea pig hippocampus (Wieraszko and Seyfried, 1989; Fujii *et al.*, 1995; Chen *et al.*, 1996).

In light of these varied and inconsistent effects ATP is reported to have in the hippocampus, it is the aim of this study to characterise in greater detail, the effect of ATP on evoked potentials in the hippocampal slice preparation, using P2 purinoceptor agonists and antagonists.

II. Results.

Dependence on potential size.

Slices that exhibited a population spike of 1 mV or greater were used in the study. The induction of long-term potentiation (LTP) following perfusion with ATP seemed to depend on the initial size of the evoked population spike. When the maximum control population spike size exceeded 5 mV, no long-term potentiation was observed. However when the maximum control potentials were less than 5 mV in size, it was possible to evoke long-terms potentiation quite consistently following ATP perfusion. Figure 4.1 gives an example of such a response.

Long-term potentiation of evoked potentials following ATP perfusion.

When low concentrations of ATP, 2.5 μM, were superfused over the slices, no response was apparent during the period of application, but a consistent, highly reproducible increase of potential size developed on washout (Fig. 4.2). This increase of potential size developed over approximately 10 min to reach a plateau level which was maintained for up to at least 30 min after removal of ATP. As the concentration of ATP was increased to 10 μM, the synaptic potentials were reduced in size during ATP perfusion, but the development of long-term enhancement still occurred on washout (Fig. 4.3). The size of the ATP-induced increase of evoked potentials was concentration dependent, reaching a peak of 130.9 ± 4.6% at 25 min following perfusion of 2.5 μM (n=3) ATP and 146.4 ± 15.9% 13 min following perfusion of 10 μM ATP (n=5) compared with the control potential size.

Simultaneous measurements of the population spike and population EPSP revealed that the depression of spike size was greater than the decrease of EPSP size during the application of ATP, leading to a dissociation of the E-S relationship (i.e. E-S depression). However, the subsequent long-lasting potentiation following perfusion reached a similar magnitude for both components compared with control values (Fig. 4.3) restoring the E-S coupling. An alternative description of this result, however, would be that, compared with the sizes of the population spike and population EPSP during the application of ATP, the subsequent potentiation of population spike size amplitude was greater than that of the population EPSP.

When a higher concentration of 40 μM ATP was used, the inhibitory response was much larger, almost totally inhibiting the response in most slices, but this was not followed by any long-lasting potentiation of responses (Fig. 4.4).

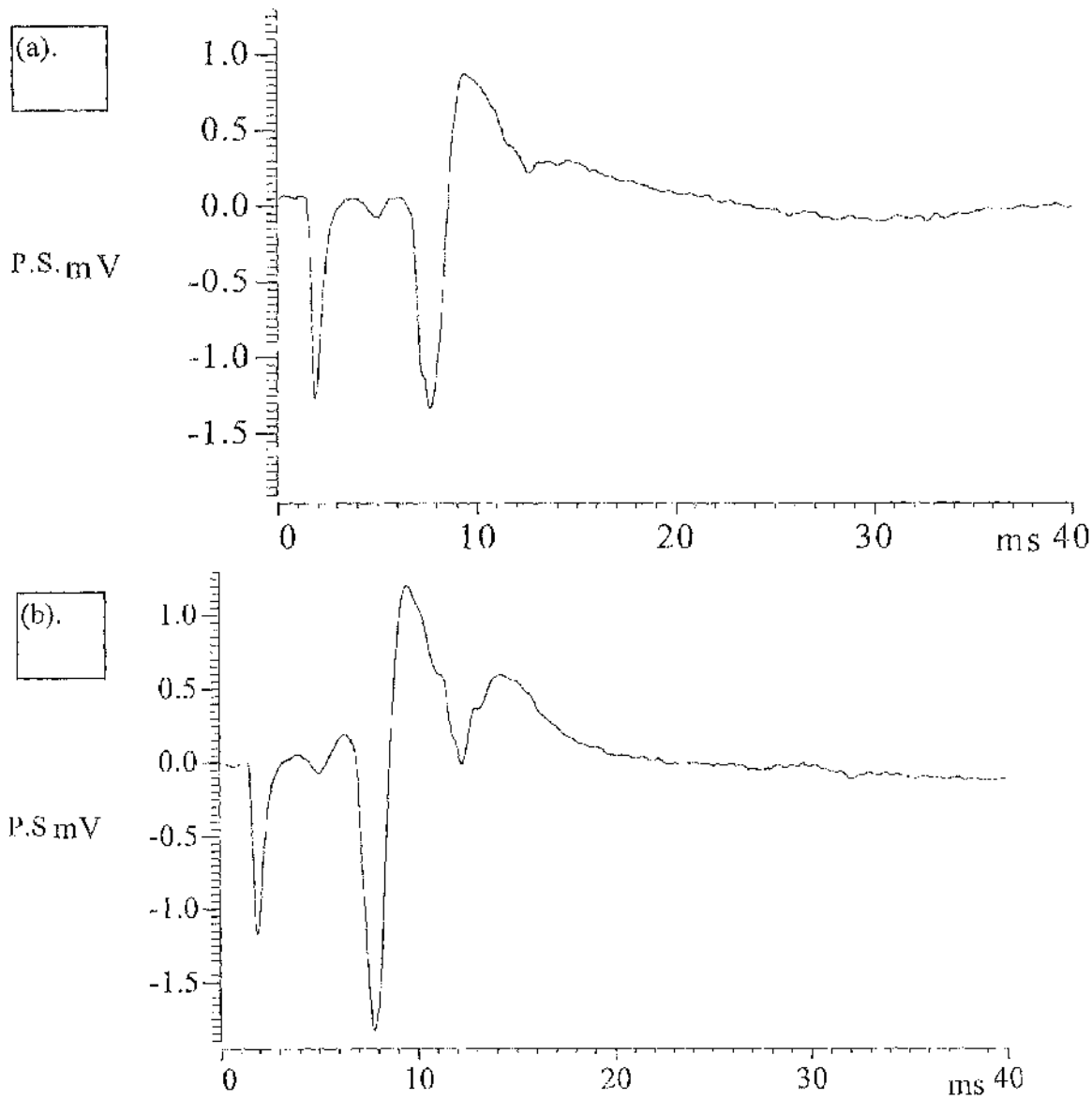

Figure 4.1. Raw data of a population spike recorded from the stratum pyramidale of a rat hippocampal slice. Trace (a) shows the control evoked response (measured at an amplitude 70% of maximum control size). 30 min following washout of a 10 min application of ATP the response is potentiated to 138% of control size (b).

[Initial control size = 2.2 mV; potentiated spike size = 3.04 mV]

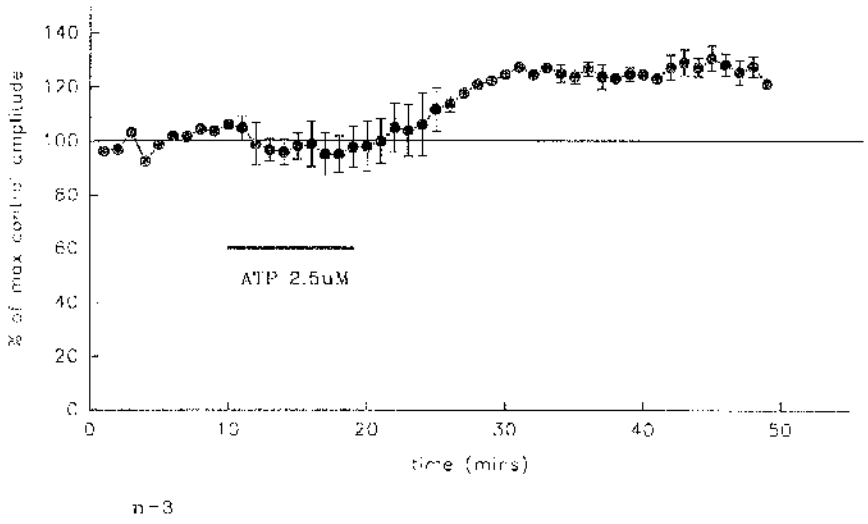

Figure 4.2. The effect of ATP 2.5 μM on population spike amplitude. During perfusion no effect on population spike amplitude was seen. However, 30 min following perfusion of ATP 2.5 μM responses were potentiated to 127.8±3.7% of control values (n=3, $p<0.05$).

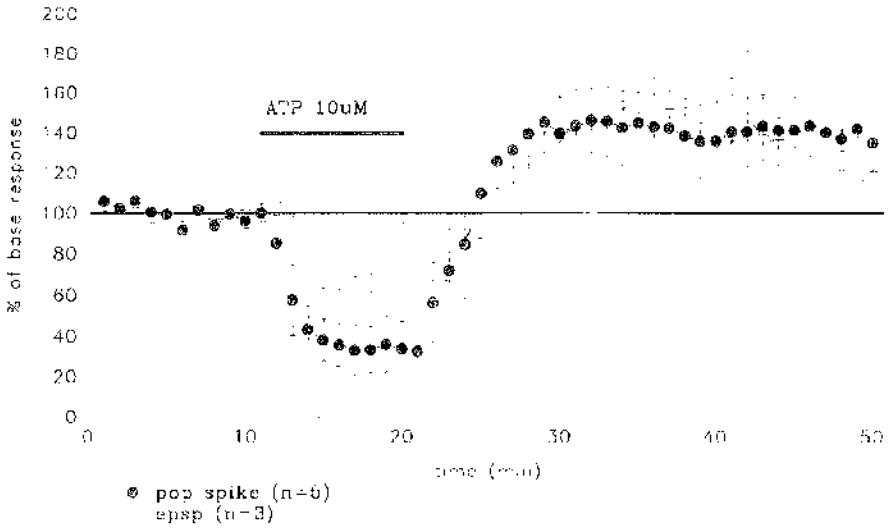

Figure 4.3. The effect of 10 min perfusion of ATP 10 μM on population spike (pop spike) amplitude (n=5) and EPSP slope (n=3). During perfusion, the population spike amplitude was reduced by a greater extent that the EPSP slope which caused a dissociation in the EPSP-spike relationship. Following perfusion of ATP, responses recovered to values greater than controls, resulting in long-term potentiation.

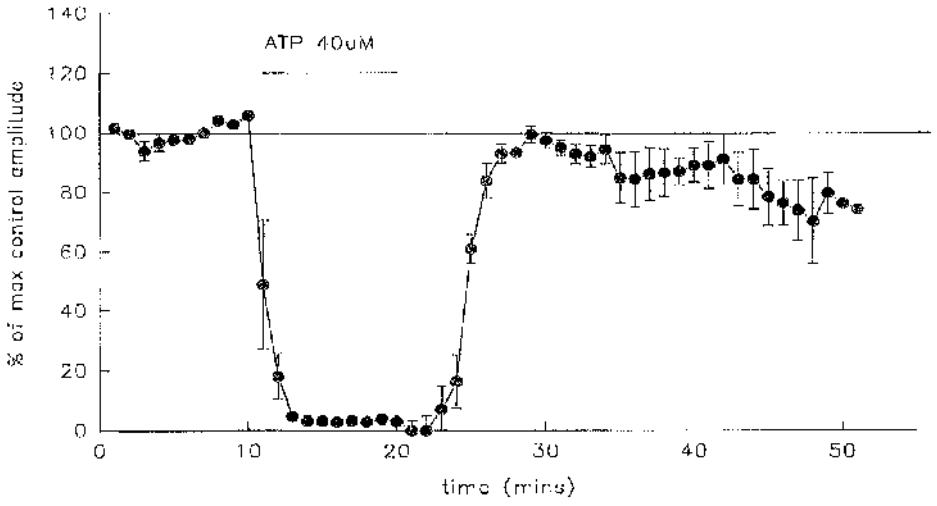

Figure 4.4. The effect of ATP 40 μM on population spike amplitude. During perfusion of 40 μM ATP population spikes were almost completely inhibited. No long-term potentiation was observed following washout of ATP, in fact population spike amplitude remained inhibited 30 min after ATP was removed from the system (n=3).

The effect of bath temperature on responses to ATP perfusion

Reducing the temperature in the recording chamber from 31 °C to 28 °C resulted in an attenuation of the inhibition of population spike amplitude during application of ATP 10 μM (Fig. 4.5). At 31 °C the population spike amplitude was reduced to 33.9 ± 13.7 % of control values while at 28 °C it was reduced to 54.5 ± 12.3 % of control values. At 28 °C no long-term potentiation of population spike amplitude was observed following washout of ATP.

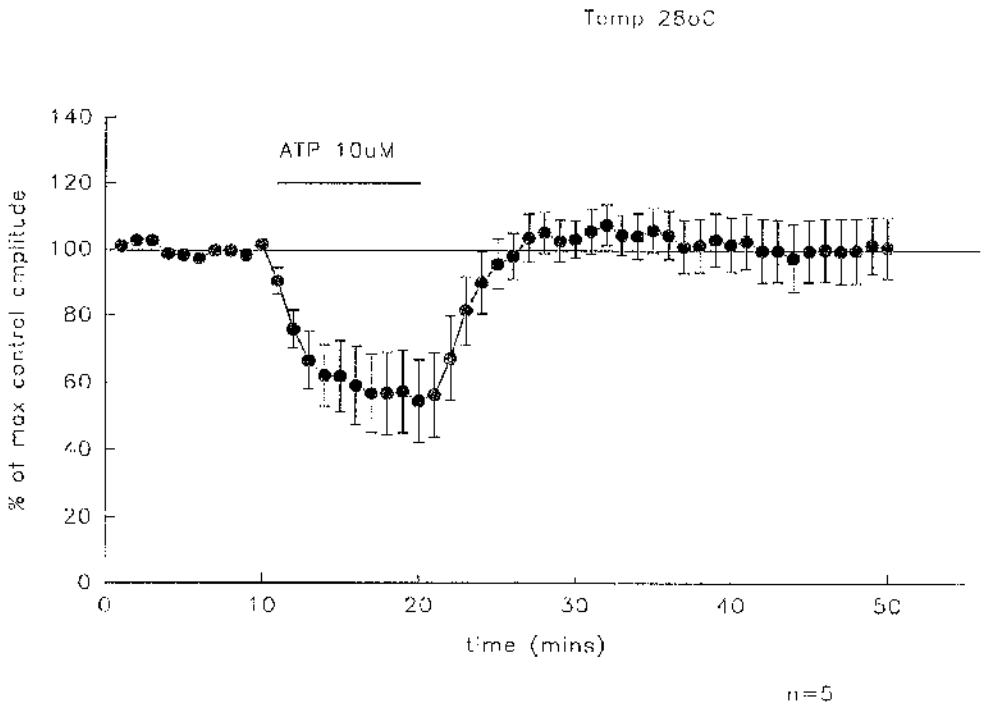

Figure 4.5. The effect of lowering the temperature in the perfusion chamber on responses to ATP. No long term effects on population spike amplitude were observed following perfusion of 10 μM ATP at 28 °C. During perfusion, less inhibition was seen at 28 °C than at 31 °C, however this was not significant.

The effect of ATP analogues on evoked potentials.

The stable analogue of ATP, αβmethyleneATP (αβMeATP) at 10 μM caused a slight decrease in population spike size during perfusion but had no long-term effects on either population spike size or population EPSP size on washout (Fig. 4.6). Another analogue of ATP, βγmethyleneATP (βγMeATP) at 10 μM, caused a significant depression of population spike amplitude during perfusion to 10.9 ± 0.2% of control ($p<0.001$), followed by a degree of excitation on washout (118.6 ± 10.2% at 5 min following washout (not significant). However, no long-lasting potentiation of response size was observed (Fig. 4.7). A 10 min application of 2MeSATP (10 μM, n=3) or 10 μM UTP (n=3) (Fig 4.8) also caused no significant long-term effects on evoked potentials.

When P2 and P1 receptors were activated at the same time using 10 μM αβMeATP and 50 μM or 10 μM adenosine, a significant reduction of population spike amplitude was observed during perfusion (to 12.9 ± 1.7% of control with 10 μM adenosine and 3.6 ± 0.9% of control with 50 μM adenosine; $p<0.001$ in both cases). On washout, both combinations resulted in an increase in response size, which was not significantly different from controls (Fig 4.9, 4.10). When 10 μM 2MeSATP and 10 μM adenosine was perfused, a significant inhibition of population spike amplitude was observed. On washout of drugs the responses returned to control levels. (Figure 4.11)

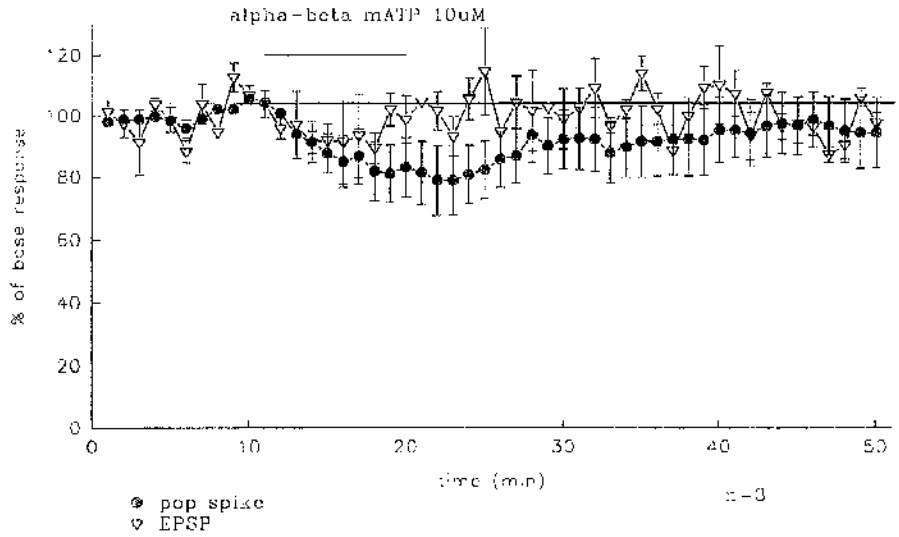

Figure 4.6. The effect of the stable analogue of ATP, α,β-methylene ATP (αβ-MeATP), on population spike amplitude and EPSP slope. Perfusion of 10 μM αβ-MeATP caused a decrease in PS amplitude to 81.6 ± 10.2 % of control (not significant) which recovered when it was washed out.

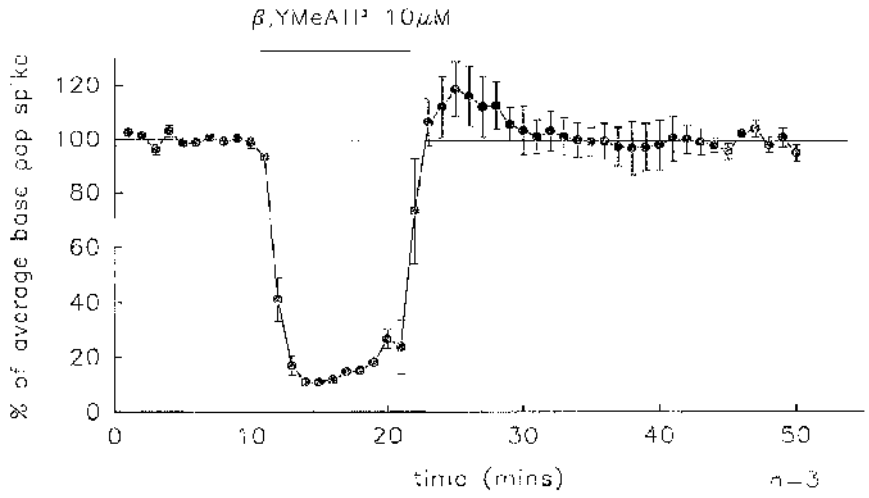

Figure 4.7. The analogue of ATP, β,γmethyleneATP (β,γMeATP) 10 μM, caused a significant reduction of population spike size during perfusion (to 10.9 ± 0.2% of control, $p<0.001$). No long-term effects were observed.

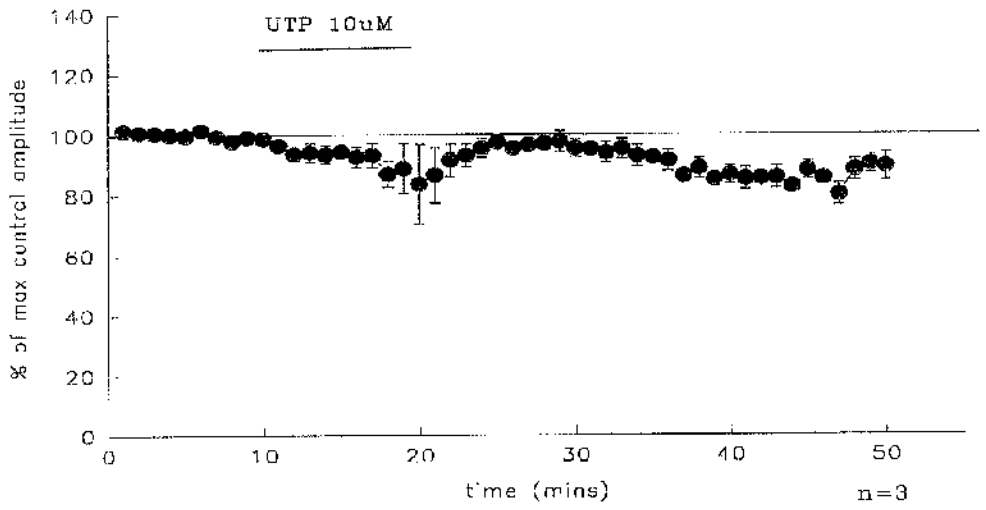

Figure 4.8. The effect of UTP 10μM on population spike amplitude.

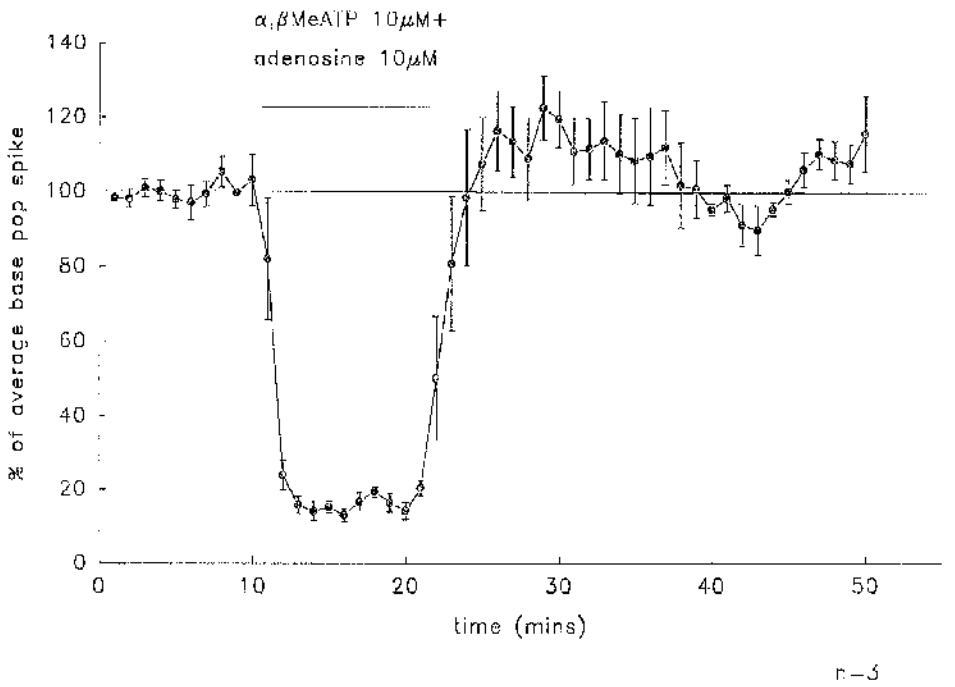

Figure 4.9. The effect of activating P1 and P2 receptors using αβMeATP 10 μM and adenosine 10 μM respectively. During perfusion of the two drugs, the population spike amplitude was significantly inhibited -presumably by adenosine acting on A_1 receptors. No significant long-term effects on population spike amplitude was observed.

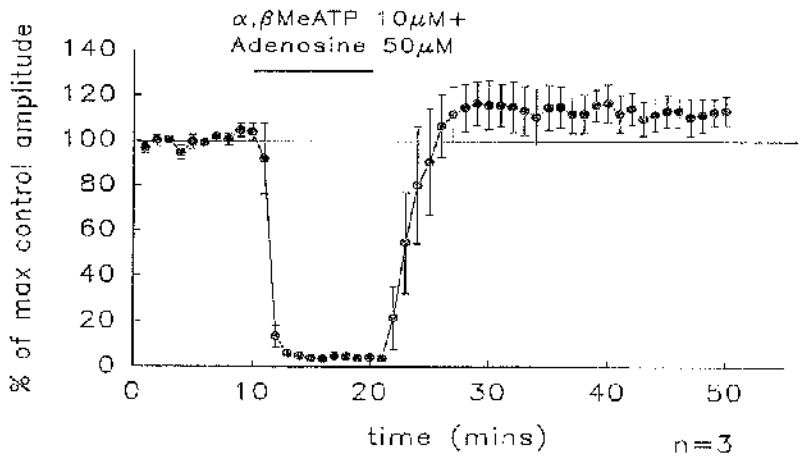

Figure 4.10. The effect of activating P1 and P2 receptors using αβMeATP 10 μM and adenosine 50 μM respectively. During perfusion, a significant inhibition of population spike amplitude was observed. Following washout, an increase in amplitude was observed, however this was not significant 30 min after perfusion of drugs.

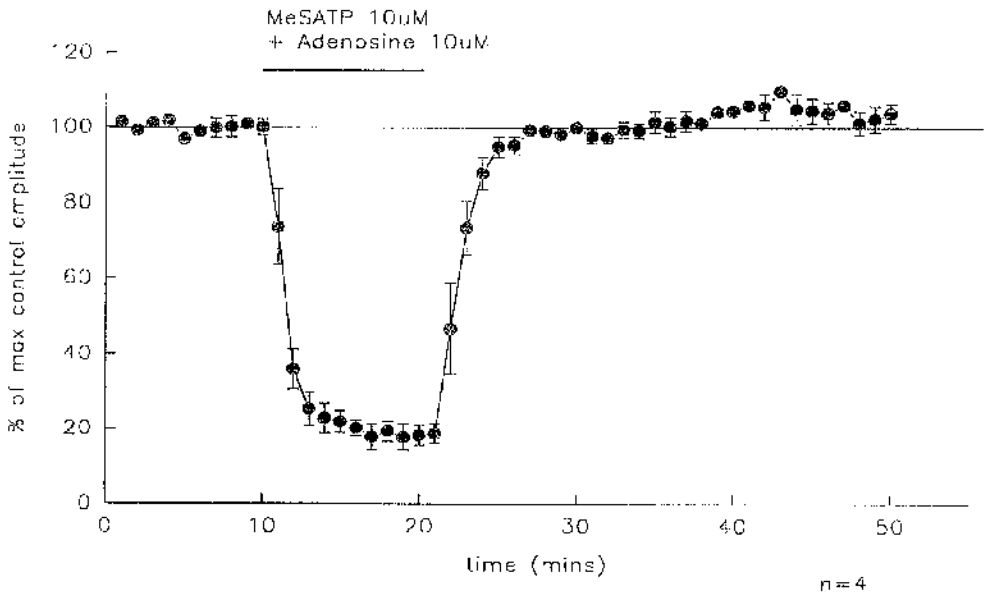

Figure 4.11. The effect of activating adenosine receptors and P2 receptors separately on population spike amplitude. The non-hydrolysable analogue of ATP, MeSATP and adenosine were used to activate P2 and adenosine receptors respectively. When the drugs were washed out of the system population spike amplitude returned to control values.

The effect of adenosine antagonists on responses to ATP

A concentration of 10 μM ATP was selected with which to examine in more detail the pharmacology of the potentiation seen following application of ATP to slices. The adenosine P1 receptor blocker 8-phenyltheophylline (8PT) (10 μM) and the selective adenosine A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (100nM) both abolished the inhibitory component of the response to ATP. Similarly, the long-term effects of ATP perfusion were abolished upon antagonism of P1 receptors (Figs. 4.12 , 4.13).

In the presence of the non-xanthine adenosine A_{2A} receptor antagonist, ZM 241385 (50 nM), perfusion of ATP 10 μM caused no inhibition of population spike amplitude or EPSP slope. In fact, potentiation of responses started during ATP perfusion. The responses remained potentiated 30 min following washout of ATP, with population spike amplitude reaching 124.5±7.0% ($p < 0.01$) of control value and EPSP slope reaching 125.7±5.4% ($p < 0.01$) of control value 30 min following washout (figure 4.14). In contrast, 8-(3-chlorostyryl)caffeine (CSC) another (xanthine) A_{2A} receptor antagonist, had no significant effect on the inhibition seen during ATP perfusion in the slices. However, no resulting LTP was observed on washout (figure 4.15).

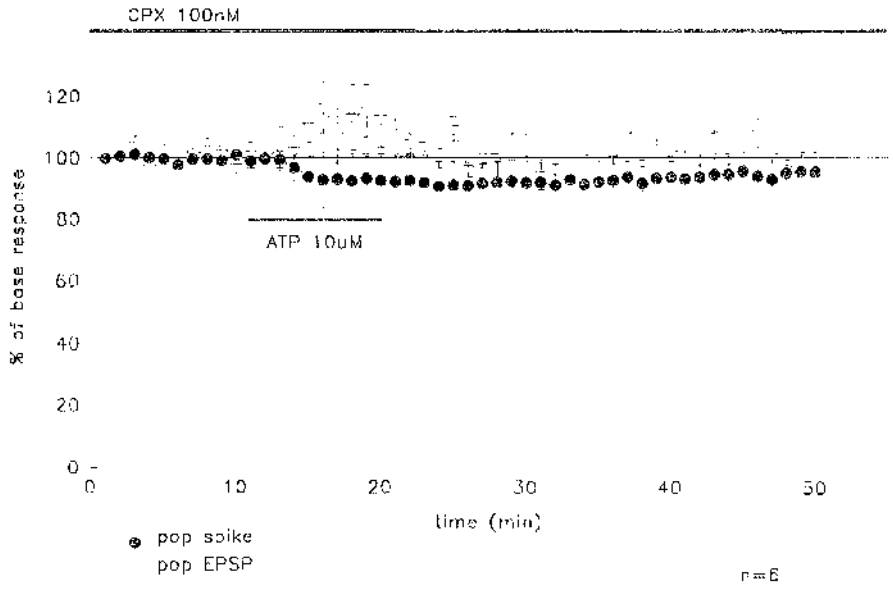

Figure 4.12. The effect of blocking adenosine A_1 receptors with 100 nM 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) on responses to ATP 10 μM perfusion. Both the inhibition during perfusion of ATP ($p < 0.01$), and the potentiation of responses ($p < 0.05$) following perfusion were significantly inhibited in the presence of DPCPX.

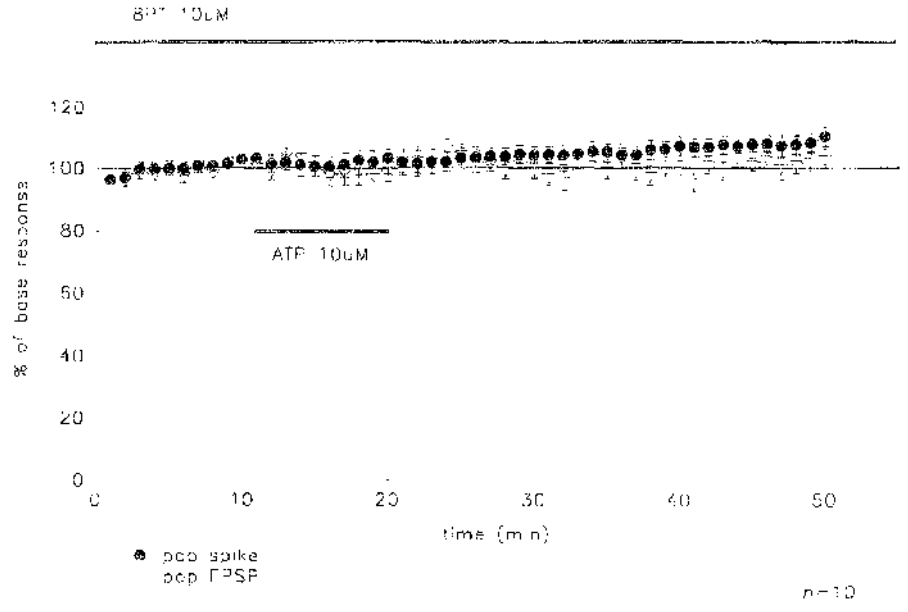

Figure 4.13. The effect of blocking adenosine receptors with 10 μM 8-phenyltheophylline (8-PT) on the actions of ATP 10 μM on population spike amplitude and EPSP slope. The inhibition during perfusion of ATP was significantly attenuated ($p < 0.001$) in the presence of 8-PT, as was the potentiation of responses following perfusion ($p < 0.05$).

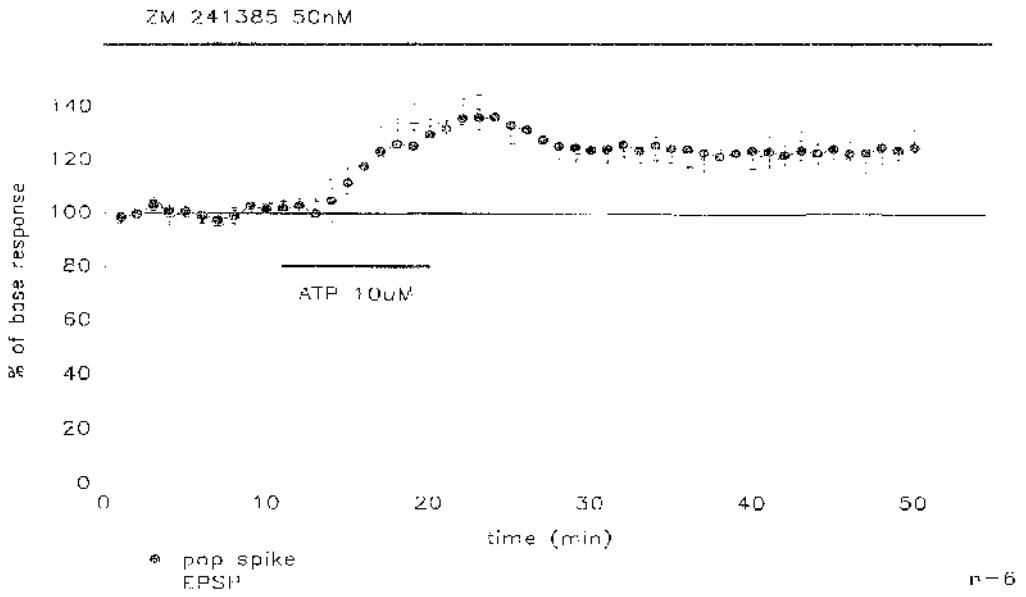

Figure 4.14. The effect of the A_{2A} antagonist 4-(2-[7-amino-2-{2-furyl}{1,2,4,}triazolo{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)phenol (ZM 241385) on ATP-induced responses.

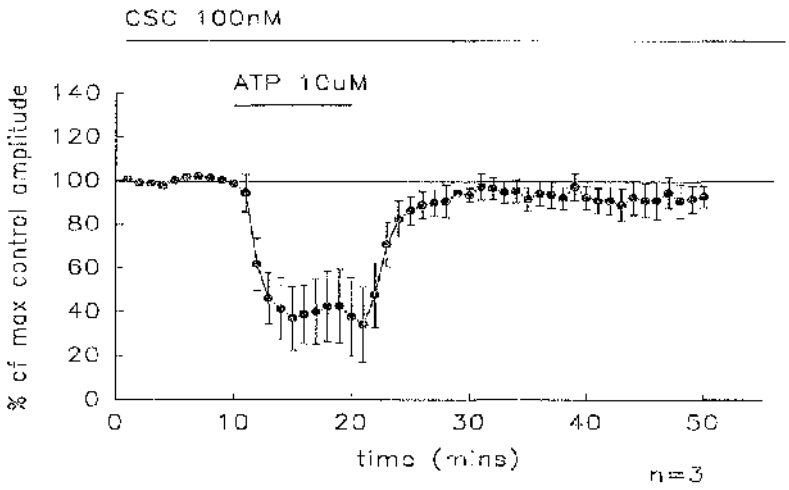

Figure 4.15. The effect of adenosine A_{2A} receptor antagonist CSC on responses to ATP.

The effect of adenosine deaminase on responses to ATP perfusion.

Superfusion with adenosine deaminase at a concentration of $0.2U.ml^{-1}$ reduced the size of the inhibitory component of the response to ATP. Following removal of the nucleotide, the potentials increased in size to a value greater than the control level to a maximum of 117.1 ± 4.1% ($p < 0.01$), but this was not maintained, declining to control values within approximately 20 min. (Fig. 4.16).

The effect of suramin on responses to ATP.

Suramin (50 μM) was perfused for 10 min before the addition of ATP to the slices and left in contact with the slices during the washout phase. In the presence of suramin, ATP depressed responses to 36.4 ± 10.8% of control after 10 min (not significantly different from ATP alone). However, the development of LTP during ATP washout was completely prevented (values after 30 min washout 99.1 ± 7.9%) (Fig. 4.17).

In order to determine whether suramin was affecting the induction or maintenance phases of long-term potentiation, this experiment was repeated with suramin being applied to slices immediately and 10 min after the application of ATP. Application of suramin immediately after ATP perfusion delayed the return of potential size to control values, but no long-term potentiation of the population spike was observed (figure 4.18). However if suramin was applied 10 min following the induction of LTP (i.e. 10 min following the washout of ATP), a depression of responses was observed which on washout returned to potentiated values (figure 4.19).

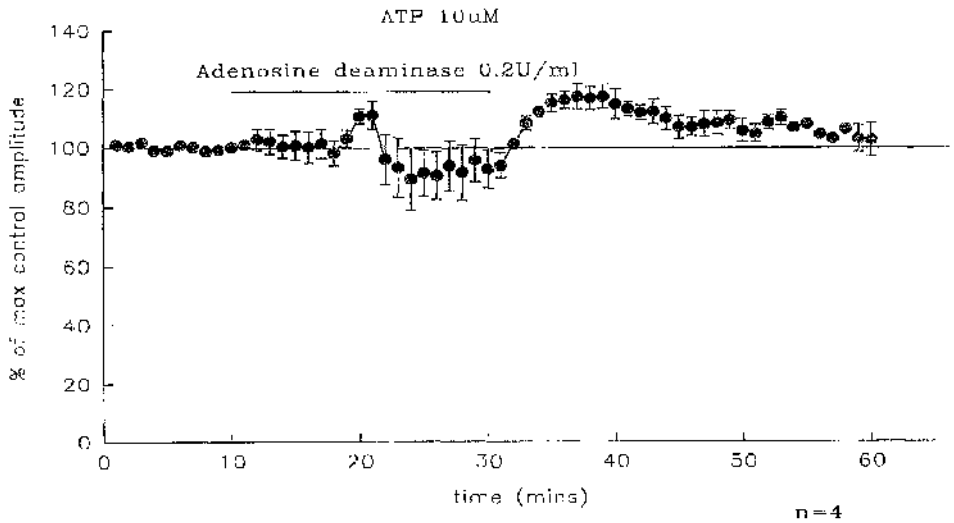

Figure 4.16. The effect of removing adenosine from the system using adenosine deaminase 0.2 U/ml on responses to ATP. No inhibition of population spike amplitude was observed during perfusion of 10 μM ATP. Following perfusion of ATP responses were potentiated for approximately 15 min. However, 30 min following washout of ATP responses were no longer potentiated and were not significantly different from control values.

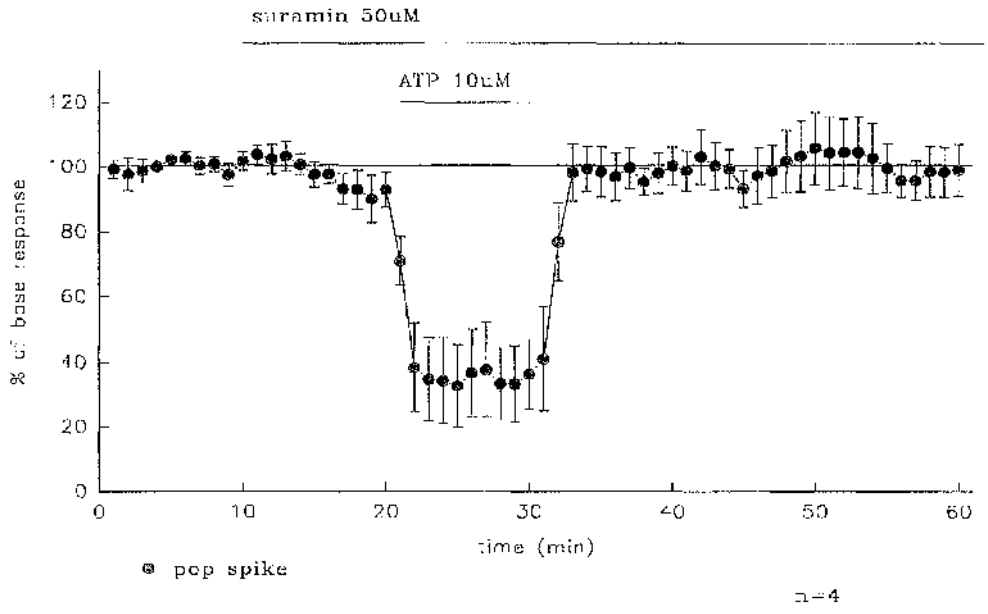

Figure 4.17. The effect of the P2 antagonist suramin 50 μM on responses to ATP 10 μM perfusion. Perfusion of ATP caused an inhibition of population spike amplitude which recovered to control values when ATP was removed from the system. No potentiation of responses was seen in the presence of suramin.

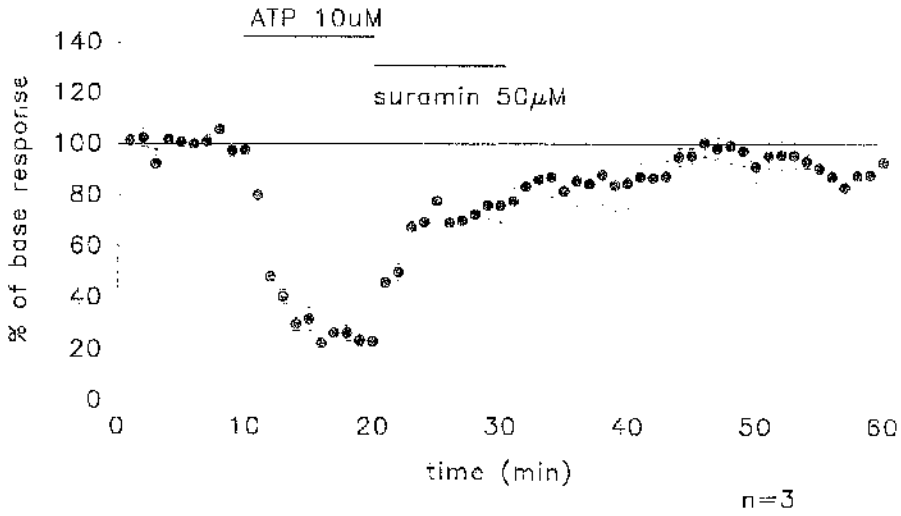

Figure 4.18. Perfusion of suramin 50 μM for 10 min immediately following ATP perfusion prevents the development of LTP. PS amplitude 30 min after washout was 93.1 ± 2.33 % (not significantly different from control).

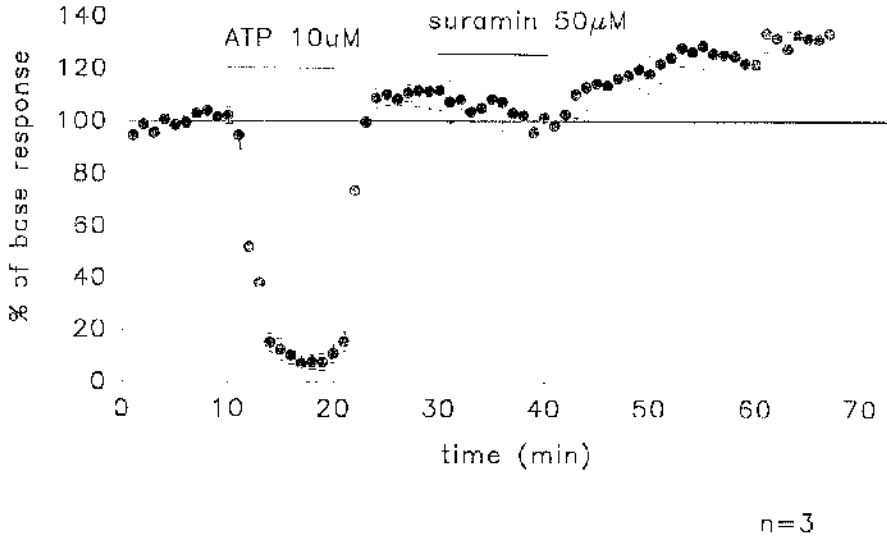

Figure 4.19. Perfusion of suramin 50 μM 10 min after ATP perfusion delayed the development of LTP. However, 30 min following washout PS amplitude was potentiated to 133.8 ± 6.8% of control ($p < 0.05$).

The effect of an N-methyl-D-aspartate (NMDA) antagonist on responses to ATP.

Inclusion of the NMDA antagonist 2-amino-5-phosphonopentanoic acid (AP5) (50 μM) for 10 min before and during the application of ATP did not change the inhibitory effect of ATP but prevented completely the development of LTP (Fig 4.20)

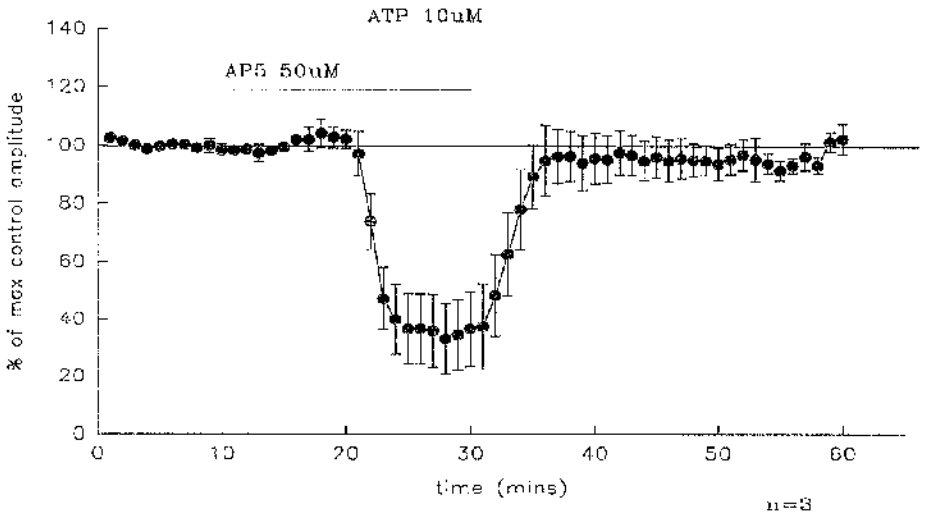

Figure 4.20. The effect of the NMDA antagonist, 2-amino-5-phosphonopentanoic acid (AP-5) 50 μM on responses to ATP 10 μM. When AP-5 was perfused for 10 min before and during ATP perfusion, no effect on the inhibition caused by ATP was observed, however following perfusion of ATP responses returned to control values with no potentiation of responses.

Occlusion of electrical- and ATP-induced long-term potentiation.

A set of experiments were performed in order to assess the relationship between the mechanisms of electrically-induced long-term potentiation and that produced by ATP. When LTP was induced electrically by stimulating the slices at high frequency (100 IIz for 1 second), an application of ATP for 10 min resulted in a decrease of response size during perfusion which recovered to potentiated levels following washout. No further potentiation of responses was observed following ATP perfusion (figure 4.21). Interestingly, the profile of inhibition seen during ATP perfusion when the slices were potentiated electrically differs from that seen in unpotentiated slices, in that the time taken to reach maximum depression is longer in potentiated slices.

In order to ensure that the LTP seen after high frequency stimulation was saturated, three consecutive bursts of 100 Hz for 1 second were delivered to the slices until no further potentiation of responses was observed. Slices were potentiated to 168.9 ± 12.2% of control value following the third burst. Application of ATP in this case again did not result in any further potentiation of responses (value following 30min washout, 158.8 ± 12.9%, figure 4.22).

When long-term potentiation was first induced using ATP, a burst of high frequency stimulation 30 min following washout resulted in a further potentiation of responses (figure 4.23) to an extent not significantly different than that seen when HFS was used first (figure 4.21).

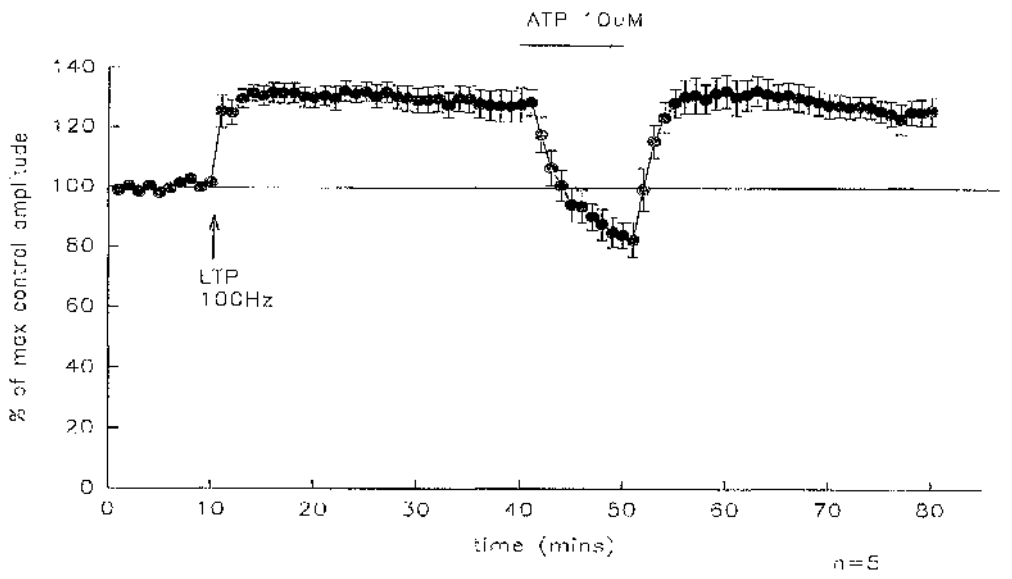

Figure 4.21. The effect of electrically inducing LTP before perfusion of 10 μM ATP. Application of ATP 30 min following induction of LTP using high-frequency stimulation resulted in a depression of evoked responses similar in magnitude to that seen in unpotentiated slices. On washout, population spike amplitude returned to potentiated levels with no further potentiation of responses.

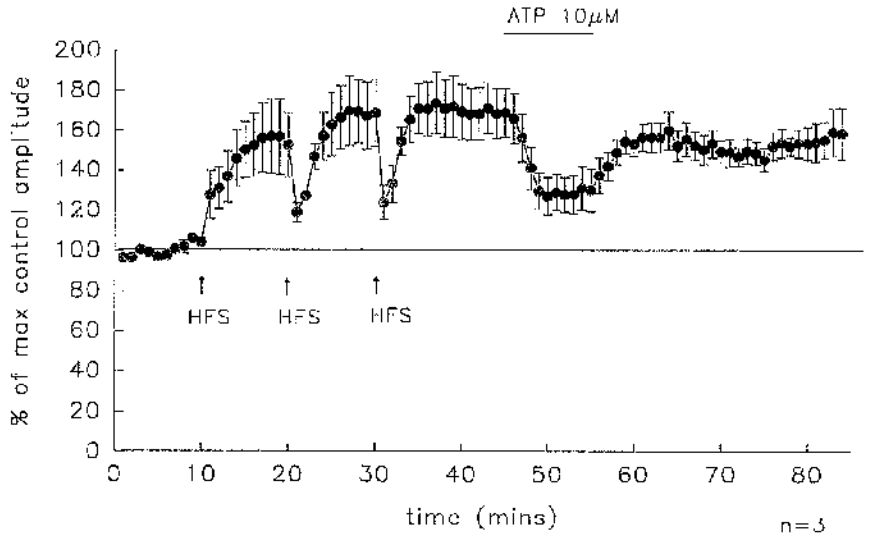

Figure 4.22. The effect of perfusion of ATP following saturation of LTP using consecutive bursts of high-frequency stimulation.

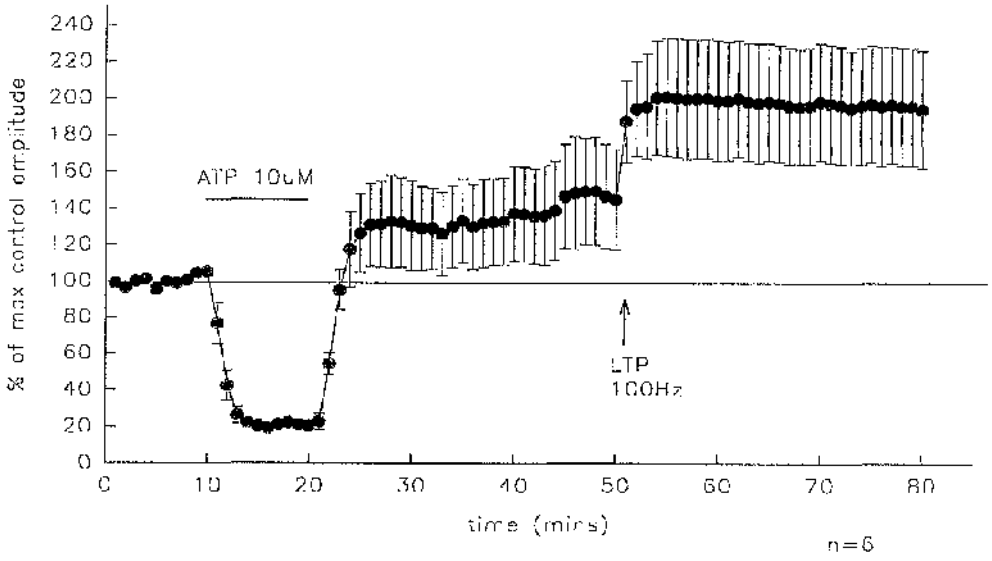

Figure 4.23. The effect of high-frequency stimulation on responses that have been potentiated following ATP application. Perfusion of 10 μM ATP resulted in a potentiation of population spike amplitude to 144.9 ± 27.5% of control levels following 30min washout. A high-frequency burst of stimulation further potentiated the responses to 194.3 ± 32.03% of control levels.

Repeated applications of ATP.

Attempts were made to saturate ATP-induced LTP in the same manner as saturation of electrically induced LTP, i.e. by repeated applications if the LTP-inducing stimulus. However, whereas repeated bursts of 100 Hz stimulation for 1 s resulted in a robust, saturable form of LTP (fig. 4.21), repeated applications of 10 μM ATP gave varied results. Figures 4.24 – 4.26 give examples of 3 experiments in which repeated applications of ATP were perfused over slice preparations.

In figure 4.24, the first application of ATP resulted in an approximately 50% potentiation of population spike amplitude. However, after a second application of ATP, responses were not potentiated further. In fact, the response fluctuates below control levels. A burst of HFS resulted in a very large potentiation of response size to approximately 200% of control level.

In the experiment shown in figure 4.25, responses remained potentiated after a second application of ATP. However, a third application resulted in responses taking longer to recover to control values and no potentiation. Again, when a burst of HFS was given, a potentiation of responses, greater than that following the two applications of ATP, was observed.

In figure 4.26, application of ATP resulted in a very large potentiation of spike amplitude, although the magnitude of potentiation was not consistent. Following another two applications of ATP, the responses were still potentiated to approximately the same level. Again, a burst of HFS further potentiated the responses.

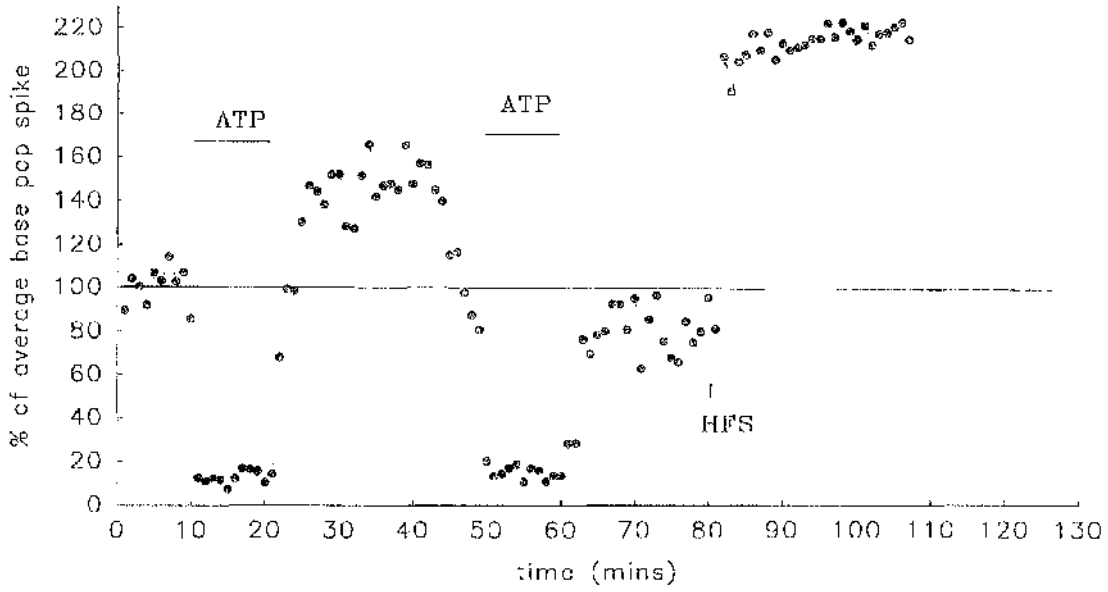

Figure 4.24. The effect of repeated applications of ATP on a slice preparation. Following the second application of ATP, the initial potentiation of population spike amplitude disappeared. A burst of HFS still produced LTP of responses.

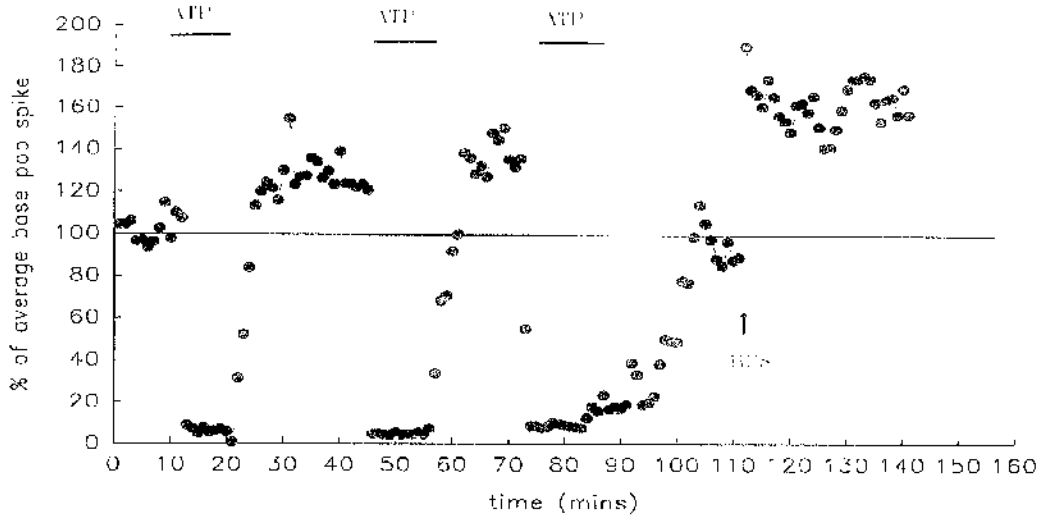

Figure 4.25. The effect of repeated applications of ATP on a slice preparation. Population spike amplitude remained potentiated following two successive applications of ATP. A third application removed the potentiation. A burst of HFS resulted in a higher degree of potentiation than following ATP perfusion.

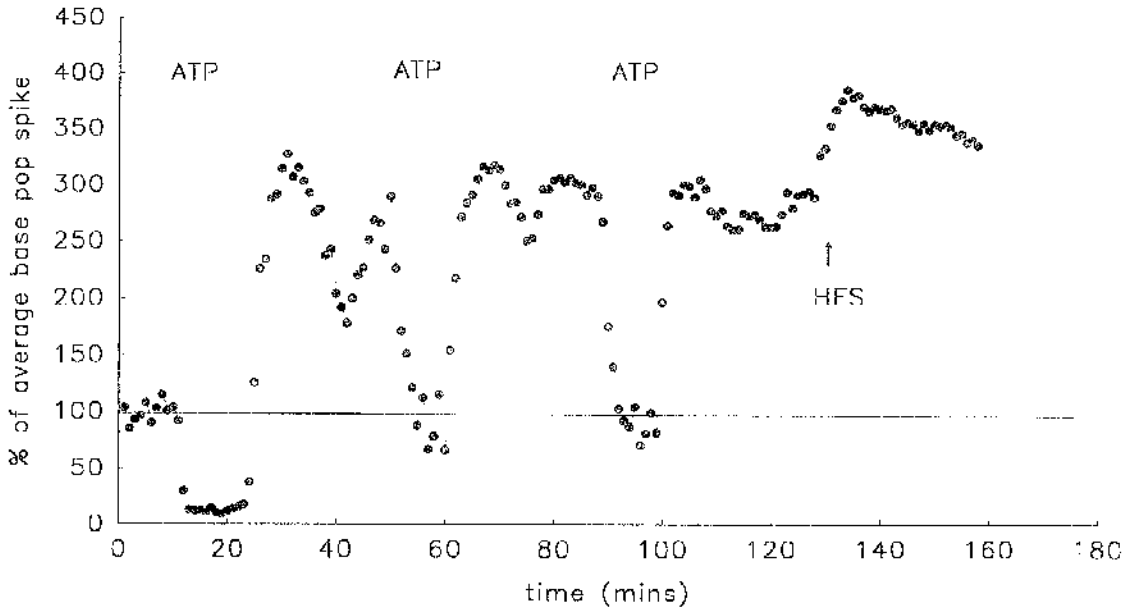

Figure 4.26. The effect of repeated applications of ATP on a slice preparation. Following three successive applications of ATP, population spike amplitude was greatly potentiated. A subsequent burst of HFS further potentiated the responses.

Diadenosine polyphosphates.

P1, P6-diadenosine hexaphosphate (Ap_6A) was applied at concentrations of 1 and 5 μM. At 5 μM, population spike amplitude was reduced to 30.7 ± 10.2 % of control values with a smaller decrease in the size of the population EPSP (62.6 ± 3.15% of control) during perfusion of Ap_6A (figure 4.27). A concentration of 1 μM was used in two slices. The results for an individual slice are shown in figure 4.28. In this case the population spike amplitude and EPSP slope was reduced by the same extent. In neither case was there evidence for a subsequent increase of potential amplitude and long-term potentiation (figure 4.27, figure 4.28).

Perfusion of 10 μM P1, P5-diadenosine pentaphosphate (Ap_5A) decreased population spike amplitude to 43.5 ± 10.9% ($p<0.001$) and EPSP slope to 81.8 ± 0.9% ($p<0.001$) of control. On washout, the responses increased with population spike reaching a maximum of 118.2 ± 5.8 % of control 15 min following washout ($p<0.05$) and EPSP slope reaching a maximum of 127.4 ± 19.5 % of control at 18 min. However, 30 min following washout the responses were not significantly different from controls (figure 4.29).

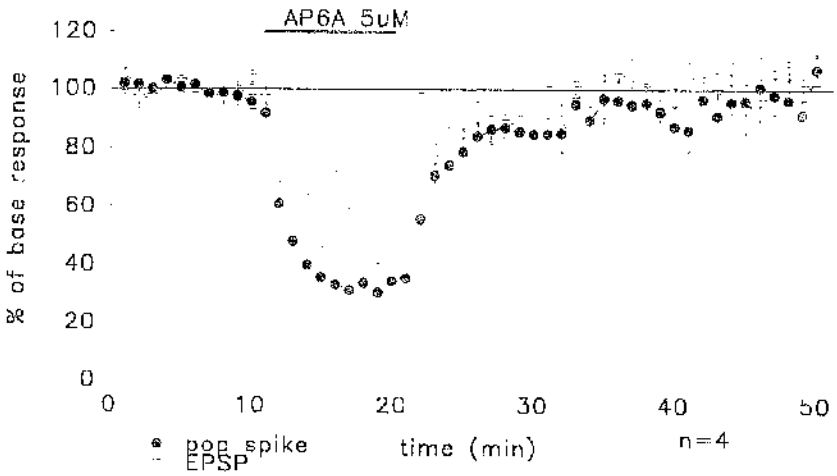

Figure 4.27. The effect of Ap_6A 5μM on population spike amplitude and EPSP slope.

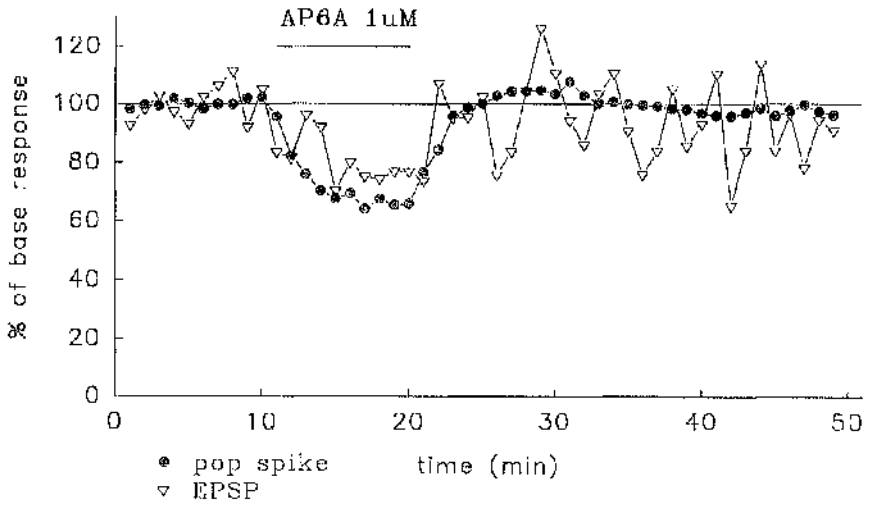

Figure 4.28. The effect of Ap_6A 1μM on population spike amplitude and EPSP slope in an individual slice.

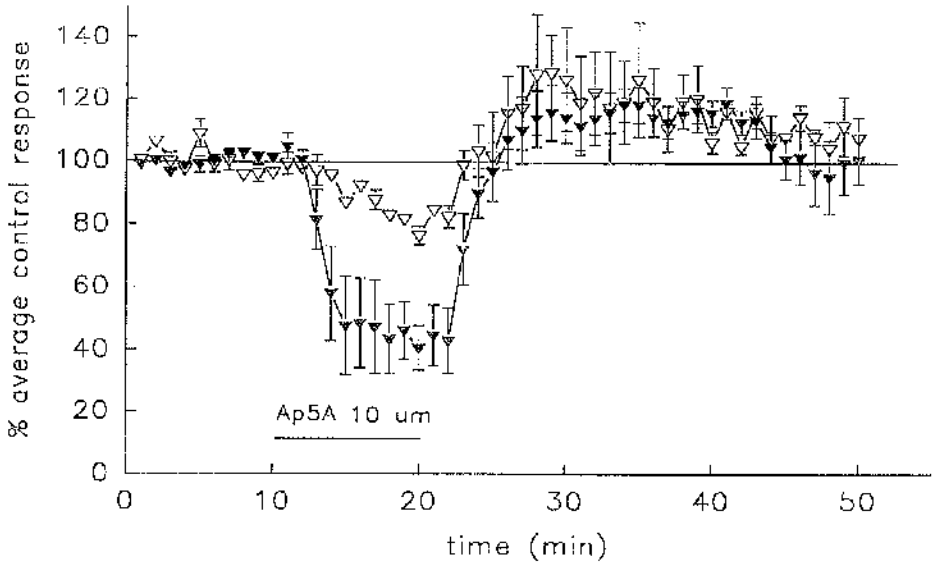

Figure 4.29. The effect of Ap_5A 10μM on population spike amplitude and EPSP slope.

III. Discussion.

The results support the view that ATP can induce a form of LTP in the hippocampal slice. The reason why LTP was only observed in slices with smaller starting potentials is not entirely clear, but may relate to the degree of enzymic activity available to metabolise the nucleotide to AMP and adenosine. Small starting spikes may be the result of the slices being in less good condition. Alternatively, the size of the spike may be a function of the number of available axons for activation by the stimulating electrode. This in turn will vary with the precise angle of cut of the slice and its position along the rostrocaudal axis of the hippocampus.

The inhibitory component of the response to ATP was blocked by the P1 receptor antagonist 8-phenyltheophylline (8PT) and the adenosine A_1 receptor selective compound DPCPX. This may mean that ATP is acting directly on the P1 receptor to cause the inhibitory effect, or more likely that it is being hydrolysed to adenosine since adenosine deaminase also prevented the inhibitory phase during ATP perfusion. This would be consistent with other investigators who showed that inhibition by extracellular ATP of hippocampal synaptic transmission required the localised extracellular catabolism by ecto-nucleotidases and channeling of the generated adenosine to inhibitory adenosine A_1 receptors (Cunha et al, 1998). Blocking P1 receptors with the antagonists also inhibited the induction of LTP following ATP perfusion, and adenosine deaminase prevented its maintenance, resulting in only a short term potentiation of responses. In addition the stable analogues of ATP, $\alpha\beta$-methyleneATP and 2MeSATP had no long term effects on the slices. Taken together,

these results suggest that the depressant action of ATP is due to its conversion to adenosine, but that the subsequent enhancement of potential size requires the activation of both P1 and P2 receptors. Activating both types of receptor using adenosine and 2MeSATP did not result in a long-term potentiation of responses. This is probably due to the fact that not all P2 receptor subtypes are activated by 2MeSATP.

As previously mentioned, the lack of selective P2 receptor blocking agents means that it is difficult to establish the exact role of P2 receptors in any biological response and the specific subtype involved. Nevertheless it is likely that the ability of suramin to block the induction of long-term potentiation by ATP represents blockade of a P2 receptor. The use of suramin, however, requires careful analysis as it has been shown that this compound has other effects that may complicate the picture. For example suramin has been shown to inhibit ecto-nucleotidases (Beukers *et al.*, 1995; Ziganshin *et al.*, 1995; Bultmann *et al.*, 1996; Ziganshin *et al.*, 1996) and even enhance the effects of ATP in some systems (Bailey and Hourani, 1995). Further, suramin has been shown to inhibit glutamate receptor channels in hippocampal neurones (Nakazawa *et al.*, 1995; Peoples and Lc, 1998). This should not have been the case in this study as the concentration of suramin used (50 μM), was lower than that seen to inhibit NMDA and non-NMDA type glutamate receptor channels and previous studies have confirmed that this is the case (Ross *et al.*, 1998). Interestingly, Wieraszko (1995) has demonstrated a suramin induced LTP in mouse hippocampal slices at concentrations of less than 30 μM and suggested that it may exert its action via a subpopulation of mouse P2 receptors or by decreasing the efficiency of inhibitory synapses.

The nature of the P2 receptor involved cannot be determined from this study. Localisation of ATP receptors by ligand binding techniques (Bo and Burnstock, 1994; Balcar *et al.*, 1995) and *in situ* hybridisation (Kidd *et al.*, 1995; Collo *et al.*, 1996) show a broad distribution of P2X receptors in the hippocampus. $P2X_4$ and $P2X_6$ ATP receptors are abundant in CA1-CA3 hippocampal pyramidal cells from adult rats (Collo *et al.*, 1996; Le *et al.*, 1998a), whereas in young animals $P2X_1$ and several isoforms of $P2X_2$ are also present (Kidd *et al.*, 1995; Simon et al, 1997, Kanjhan *et al.*, 1999). $P2X_6$ subunits do not form functional homomeric receptors. Homomeric assemblies of $P2X_4$ subunits respond poorly to αβmethyleneATP and are relatively insensitive to suramin. Combinations of $P2X_4$ and $P2X_6$ subunits, however have been shown recently to be sensitive to the agonist effects of αβmethyleneATP and blockade by suramin (Le *et al.*, 1998). The profile of the putative P2 receptor activated by ATP in this study is not consistent with any of these, and may indicate the involvement of a different heteromeric subunit combination.

The blockade of ATP-induced LTP by AP5 suggests similarities in the process of induction with LTP induced by electrical stimulation, since the latter is at least partly dependent upon the activation of NMDA receptors and can therefore be prevented by 2AP5 (Davies *et al.*, 1981). A necessary trigger for electrically induced LTP is the influx of calcium into the dendritic spine through NMDA receptors which have been relieved of their Mg^+ block following depolarisation (Collingridge *et al.*, 1988). In contrast, the current through ATP receptor channels is not blocked by Mg^+ and thus calcium dependent phenomena such as LTP could occur without prior depolarisation. In addition to this postsynaptic effect, the calcium permeability of ATP receptors

could result in presynaptic enhancement of transmitter release. ATP has been shown to elicit the release of glutamate in the hippocampus (Inoue *et al.*, 1992) and such an effect could account for the production of LTP.

The involvement of a common mechanism of ATP- and electrically-induced LTP is supported by the finding that the saturation of electrically induced LTP prevents the establishment of ATP-induced LTP. This in turn raises the question of whether electrically-induced LTP might involve the activation of ATP receptors. The fact that ATP is preferentially released upon high frequency stimulation (Cunha *et al.*, 1996a) also supports this idea. One difference that does occur between electrically- and ATP-induced LTP is that no EPSP-spike dissociation occurs upon ATP-induced LTP. However, this was only measured at one stimulation intensity (i.e. that giving a response 70% of maximum control). Several groups have reported a potentiation of the EPSP-spike relationship following electrically induced LTP (see for example Bernard and Wheal, 1995a,b) and the phenomenon has become an established feature of this form of LTP. A reason for this difference in ATP- versus electrically induced LTP may be that the LTP induced by ATP perfusion was not saturated. Whereas electrically induced LTP produced a robust LTP which could be saturated by repeated high frequency bursts until no further increase in response size was observed, it proved difficult to do the same using repeated applications of ATP. It may be the case that the LTP induced by ATP forms a component of that induced by high frequency stimulation i.e. ATP induces the synaptic component of LTP, reflected in the potentiation of the EPSP, resulting in an enhanced excitatory input to the neuronal pool and concomitantly increasing the number of synchronously discharging cells to produce a larger population spike, while high frequency stimulation leads to the

second component - EPSP-spike potentiation. It has been suggested that different mechanisms may underlie the two components of LTP. High frequency stimulation may induce potentiation of one component but not the other (Bliss and Lomo, 1973; Wilson *et al.*, 1981; Abraham *et al.*, 1985; Taube and Schwartzkroin, 1988). Also, a brief elevation in extracellular calcium, which mimics tetanus-induced LTP (Turner et al, 1982), does not produce EPSP-spike potentiation (Reyman *et al.*, 1986; Bliss *et al.*, 1987).

An alternative analysis of the lack of EPSP-spike potentiation 30 min following ATP application, however, would be that, compared with the sizes of the population spike and population EPSP during the application of ATP (i.e. the population spike was inhibited to a greater extent that the EPSP), the subsequent potentiation of population spike size amplitude was greater than that of the population EPSP, resulting in a potentiation of the EPSP-spike relationship albeit not from control values but from values following 10 min perfusion of ATP.

It has been suggested that ATP-induced LTP is due to ecto-protein kinase enzymes using ATP as a substrate, on the basis that, as confirmed here, αβMeATP does not mimic the LTP induced by ATP (Wieraszko and Erlich, 1994) and that an inhibitor of ecto-protein kinase, K-252b, which competes with ATP for the catalytic site of the kinase (Kase *et al.*, 1987), prevents the establishment of LTP by ATP (Fujii *et al.*, 1995). This kinase inhibitor has also been shown to block electrically induced LTP (Reymann *et al.*, 1990; Fujii *et al.*, 1995). Interestingly, the involvement of ecto-protein kinase in electrically induced LTP has been proposed by Chen *et al.* (1996) with the demonstration that LTP can be prevented by a monoclonal antibody to the

catalytic domain of protein kinase C. In other studies using "knockout" animals for various protein kinases electrically-induced LTP was more difficult to elicit but was not absent (Abeliovich *et al.*, 1993; Grant and Silva, 1994) suggesting compensatory or redundant mechanisms in the induction of LTP. These studies support the idea that ATP may contribute to electrically-induced LTP.

Dinucleotides

The adenine dinucleotides have been thought to act on ATP (P2) receptors since the original discovery of their actions on smooth muscle (Stone, 1981a). In the present study the most potent of these, Ap_6A, produced only inhibition of the slices, whereas Ap_5A caused an inhibition, followed by a rebound excitation of responses following washout which did not develop into a long-lasting potentiation. The lack of long-term effects following perfusion of diadenosine polyphosphates suggests that the receptors responsible for the ATP-induced LTP are not sensitive to dinucleotides.

As previously mentioned, dinucleotides have been suggested to act on P2 receptors, either as an uncleaved molecule, or due to enzymatic breakdown to ATP & ADP. Alternatively, evidence exists showing that the dinucleotides may act on a specific dinucleotide receptor which allows Ca^{2+} influx in the synaptic terminal. In this scenario, the dinucleotides may act as positive modulators of synaptic transmission. Ap_5A has been shown to elevate intrasynaptosomal calcium (Pintor *et al.*, 1995), an effect which may underlie the increase in response seen immediately following perfusion of Ap_5A in this study.

In summary, this study has shown for the first time, that in the rat hippocampus slice preparation, a form of long-term potentiation that can be induced by superfusion of

ATP. The phenomenon shows several similarities to electrically-induced LTP, and may involve the simultaneous activation of P1 and P2-purinoceptors. Another important finding of this study has been the novel action of ZM 241385 on the adenosine A_1 receptor-mediated inhibition of evoked potentials during ATP perfusion, an effect which warrants further investigation.

CHAPTER 5.

STUDIES USING 5'ADENYLIC ACID DEAMINASE (AMPASE).

I. Introduction.

As a consequence of the observation by Ross (1997) that, following a 10 min perfusion of adenylic acid deaminase (AMPase), a long lasting depression of responses was observed, it was decided to further investigate and characterise the effects of AMPase on hippocampal slices.

During the course of investigations it became apparent that the aminohydrolyase activity of the enzyme preparation was not responsible for the long-term depression (LTD) of evoked responses in the hippocampus. Further, the knowledge that extraction of many enzymes on an industrial scale can lead to a number of impurities being present, prompted the decision to purify the enzyme preparation in order to determine the active component involved.

The purification process led to the discovery that the factor causing long-term depression of evoked potentials in rat hippocampal slices was not in fact AMPase but an unknown protein.

The task in hand then became to identify the protein present in the crude enzyme preparation that caused the long-lasting depression of evoked potentials. This

entailed the scaling up of the purification process and subsequent sequencing of the purified protein (see appendix 1 and 2).

II. Results.

Perfusion of AMPase.

An example of the inhibition of responses seen 30 min after washout of a 10 min application of AMPase (E.C. 3.5.4.6. from *Aspergillus*) is illustrated for a single slice in the raw traces in figure 5.1. Figure 5.2 shows the time course of such an effect on population spike amplitude and population EPSP slope in 5 slices. Following the start of perfusion of AMPase, there is an initial increase in the size of CA1 orthodromic population spike amplitude which gives way to a decrease in potential size following removal of the enzyme. The inhibition of population spike amplitude and EPSP slope in the set of slices studied in figure 5.2 was 42.7 ± 13.4% ($p< 0.01$) and 59.3 ± 6.9% ($P < 0.01$) of initial control potential size respectively. The depression develops slowly, over a period of approximately 10-20 min, but persists for as long as recordings were made (up to 90 min).

The long –lasting depression effect of AMPase did not appear to be the result of a toxic effect on the slices for 2 reasons. Firstly, the neurones remained capable of returning to control responses when stimulation amplitude was increased following depression (figure 5.3). Secondly, long-term potentiation could still be induced by a standard tetanus of 100 Hz for 1 second (figure 5.3).

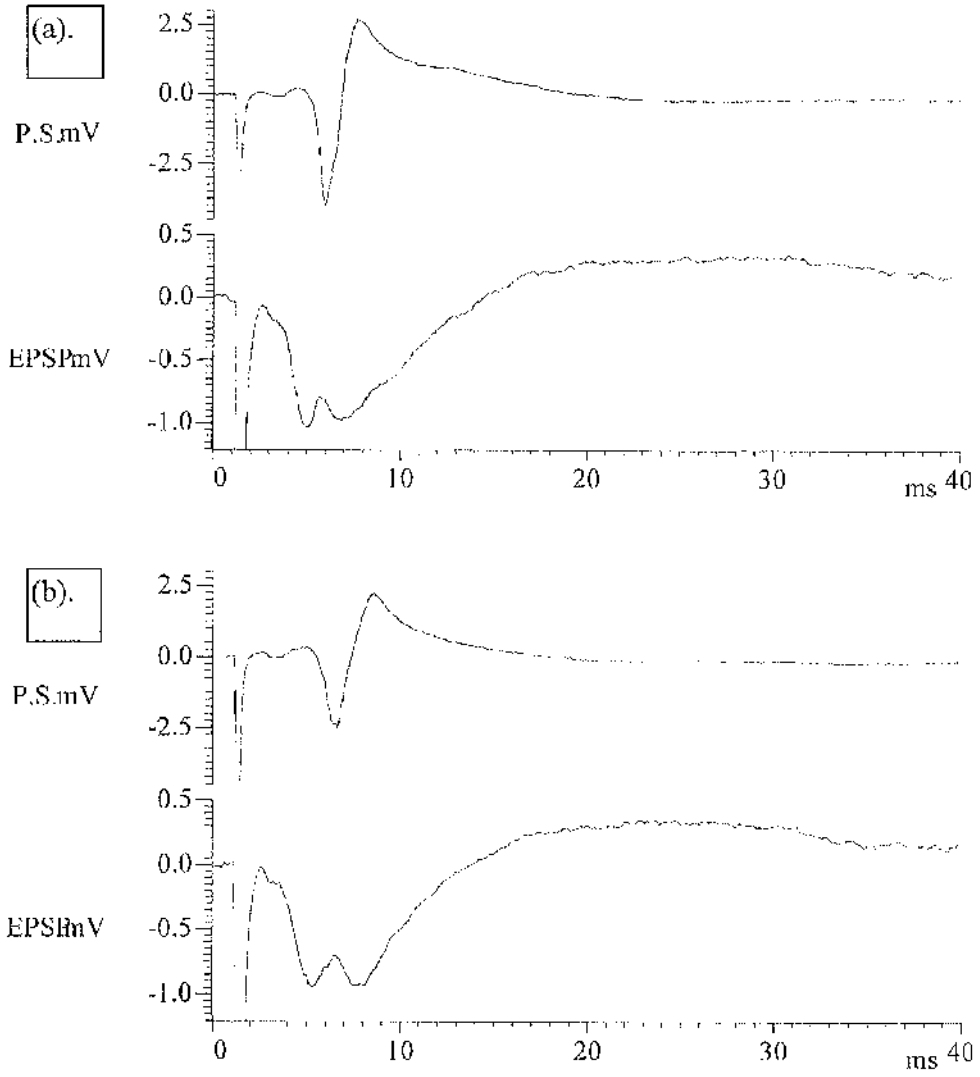

Figure 5.1 Raw traces from a single slice. (a) shows the population spike (PS) and population excitatory potential (EPSP) before the addition of AMPase to the perfusion medium. The traces in (b) show the same responses 30 min following perfusion of AMPase. In this slice, PS amplitude was reduced by 35%, whereas the EPSP slope was reduced by 19% 30 min following perfusion of 2 U/ml AMPase.

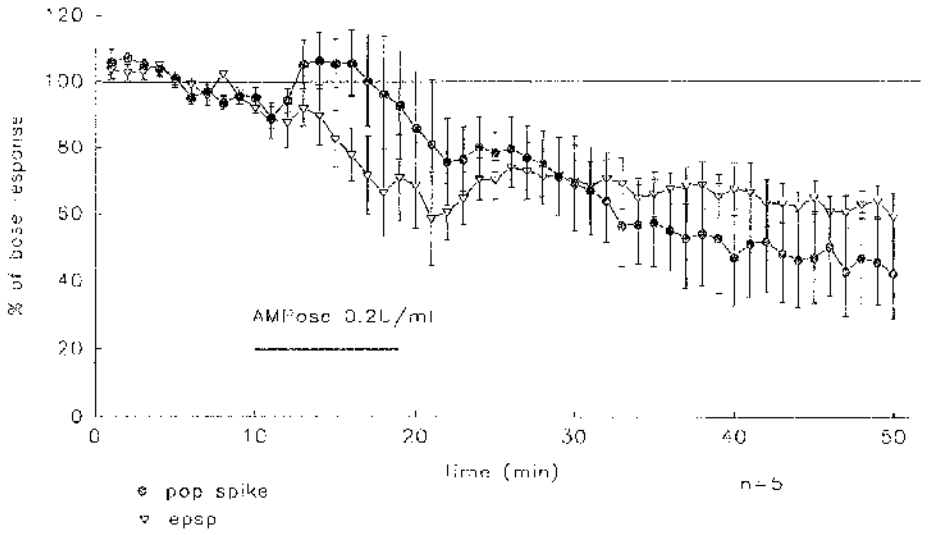

Figure 5.2. The effect of a 10 min application of AMPase on population spike amplitude (filled circles) and population EPSP slope (open triangles). During perfusion, an initial increase of population spike amplitude was observed, which started to decline approximately 7 min after the start of perfusion and continued to decline before reaching a plateau approximately 15 min after washout. The response remained depressed 30 min following washout. The population EPSP slope decreased during perfusion of AMPase but recovered to a still depressed level following washout. Again the depression of population EPSP slope was observed 30 in following washout of AMPase.

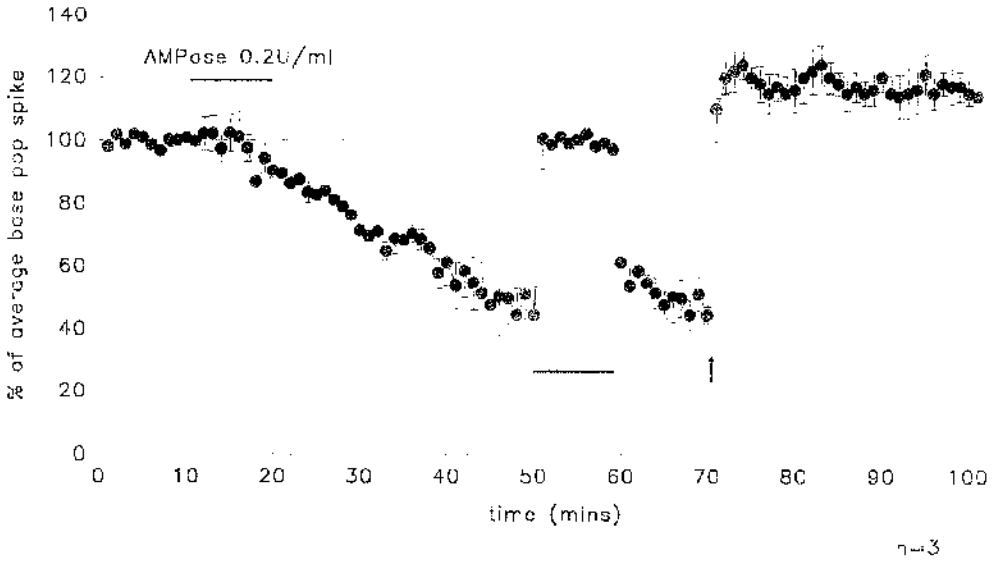

Figure 5.3. The perfusion of AMPase caused a long-term depression of 44.7 ± 8.9% ($p<0.01$) of control values. Responses could be returned to control values by increasing the stimulation amplitude (bar below record). Depressed responses could also be potentiated following a burst of high frequency stimulation (100 Hz for 1 second; arrow).

Effects of AMPase on EPSP-spike coupling.

When simultaneous measurements were made of the CA1 population EPSP and population spike, which primarily reflect transmitter release and postsynaptic excitability respectively, it was found that the population spike was depressed to a larger extent than the population EPSP (figure 5.4, 5.5a). The relationship between the two – the EPSP-spike coupling – showed a shift to the right (figure 5.4c) indicating that the AMPase was reducing the ability of EPSPs to induce a postsynaptic spike and implying that the enzyme was acting postsynaptically to modify spike threshold. Data from 5 slices show that the ratio PS/EPSP was significantly changed from control values 30 min after washout of AMPase confirming that population spike amplitude was decreased by a greater extent compared with EPSP slope (figure 5.5b).

The effect of AMPase from a different source.

The effect of AMPase extracted from rabbit muscle was tested on slices in order to determine if it was the *Aspergillus* form of the enzyme specifically that caused the long-term depression of evoked potentials. A 10 min perfusion of AMPase from rabbit muscle caused a biphasic response- initially increasing potentials to 115.0 ± 5.4% of control ($p<0.05$) which then started to decline after approximately 5 min (to 75.4 ± 17.9%, n.s.). On washout, the responses returned to values greater than control (117.0 ± 5.3 %; $p<0.01$) but then decreased and remained at control levels persistently. No long-term depression was thus observed with the rabbit form of AMPase (figure 5.6). Control for AMPase from rabbit was the vehicle it was prepared in i.e. 66% glycerol solution containing 1 mM mercaptoethanol and 0.33 mM KCl, pH 7.0.

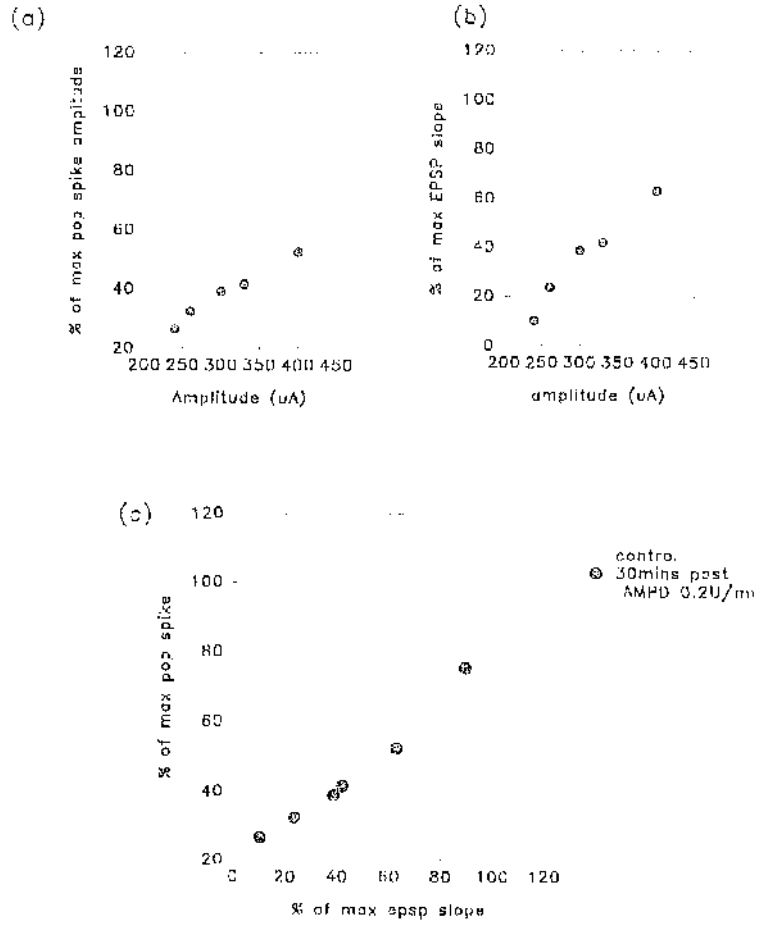

Figure 5.4. Input-output curves for a typical slice (and corresponding E-S curve) showing the effects on responses before (filled circles), and 30 min following (open triangles) AMPase perfusion. It can be seen that both population spike amplitude and population EPSP slope were depressed 30 min following washout of AMPase. Population spike amplitude (a) was depressed to a greater extent than population EPSP slope (b) resulting in a right-shift in the E-S curve (c).

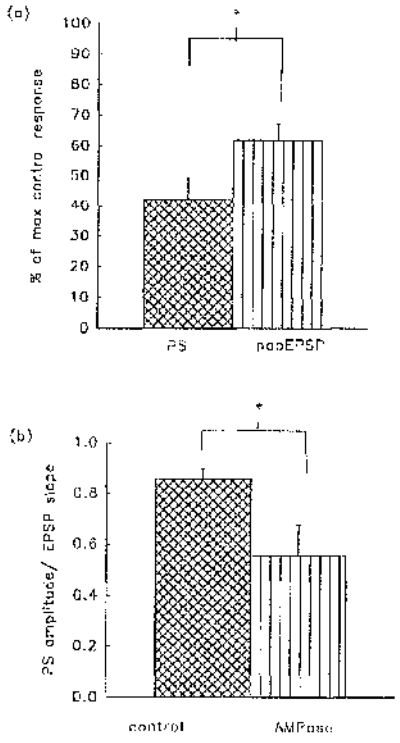

Figure 5.5. Pooled data from 5 slices, showing the decrease in population spike amplitude and population EPSP slope 30 min following washout of a 10 min perfusion of AMPase. Population spike amplitude was decreased to 42.1 ± 7.1% of control compared with 61.8 ± 5.3 % of population EPSP slope (a). When the ratio PS/EPSP was calculated before and 30 min following AMPase perfusion, it was observed that for the same population EPSP slope, a smaller population spike was seen, revealing a depression in the EPSP-spike relationship.

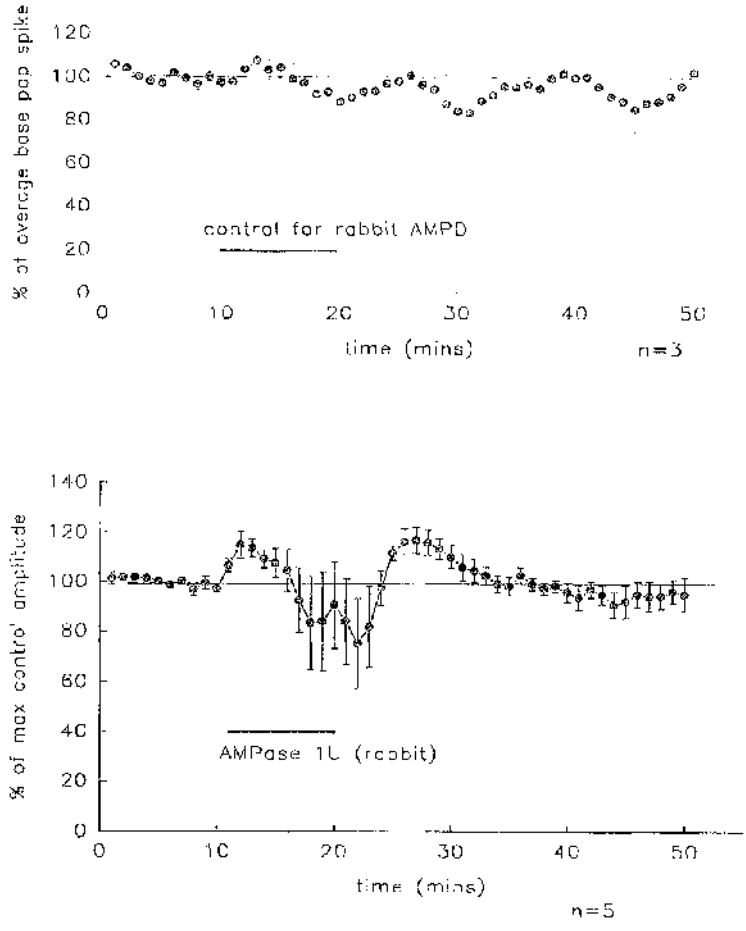

Figure 5.6. The effect of AMPase extracted from rabbit muscle on population spike amplitude. 10 min perfusion of 1U/ml AMPase caused an initial increase in population spike amplitude which started to decline during perfusion. On washout, there was a rebound excitation of population spike amplitude which returned to control values approximately 10 min following washout. No long-term effects on response size was observed.

The effect of urease and 5'nucleotidase on evoked potentials.

AMPase forms a member of the aminohydrolase superfamily related to urease. (Holm and Sander, 1997). It was decided therefore to examine the effects of another aminohydrolase, namely urease, on evoked potentials. Urease at a concentration of 1 and 2 U/ml had no significant effects on slices (figure 5.7, 5.8). 4 U/ml urease caused a depression of population spike amplitude during perfusion to 59.3 ±13.9% of control ($p<0.05$), but responses returned to control values on washout (figure 5.9).

Another enzyme involved in purine metabolism is 5' nucleotidase. This enzyme had an inhibitory action on evoked potentials during perfusion but had no long-term effects on population spike amplitude 30 min after washout (figure 5.10).

Perfusion of Aspergillus extract.

A culture of *Aspergillus nidulans* was grown and homogenised. The homogenate was suspended in aCSF and perfused over the slices. Figure 5.11 shows that during perfusion of the crude *Aspergillus* homogenate, an immediate depression of population spike to 35.2 ±1.3% ($p<0.01$) was observed. The responses were still depressed 30 min following washout of the homogenate to 74.5 ± 4.2% of control ($p<0.05$).

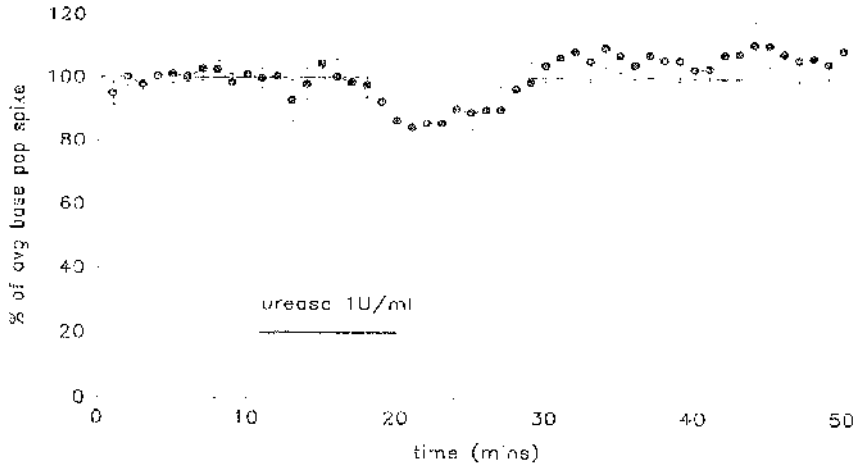

Figure 5.7. The effect of urease 1U/ml on population spike amplitude. No significant effects on response size were observed.

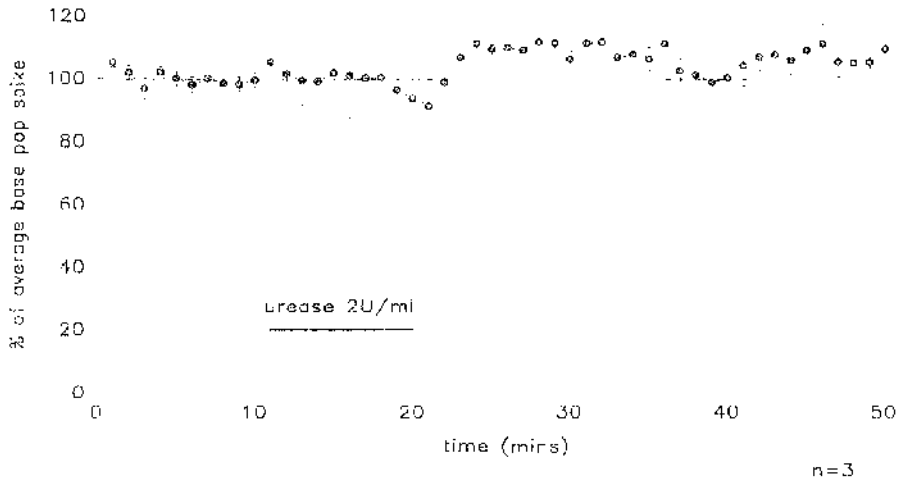

Figure 5.8. The effect of urease 2U/ml on population spike amplitude. Following washout a small potentiation of response size was observed, however, no significant long-term effects on population spike amplitude were observed.

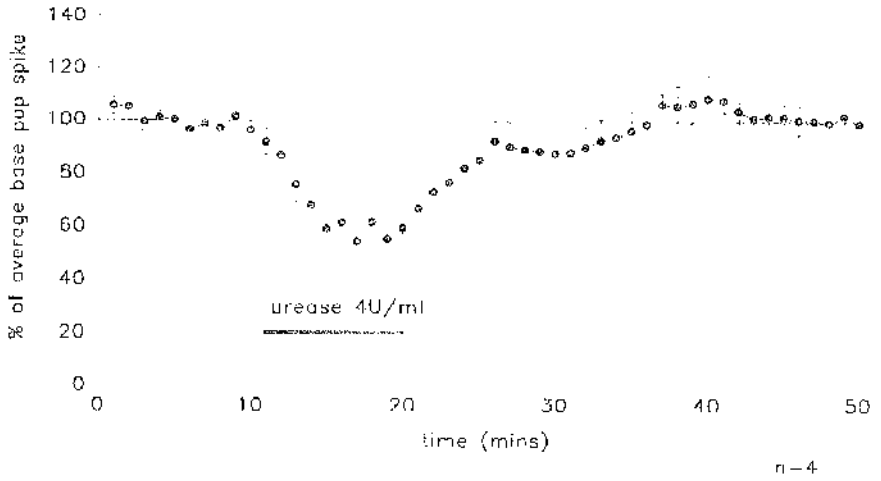

Figure 5.9. The effect of urease 4U/ml on population spike amplitude. During perfusion, population spike amplitude was significantly depressed. On washout, responses returned to control values.

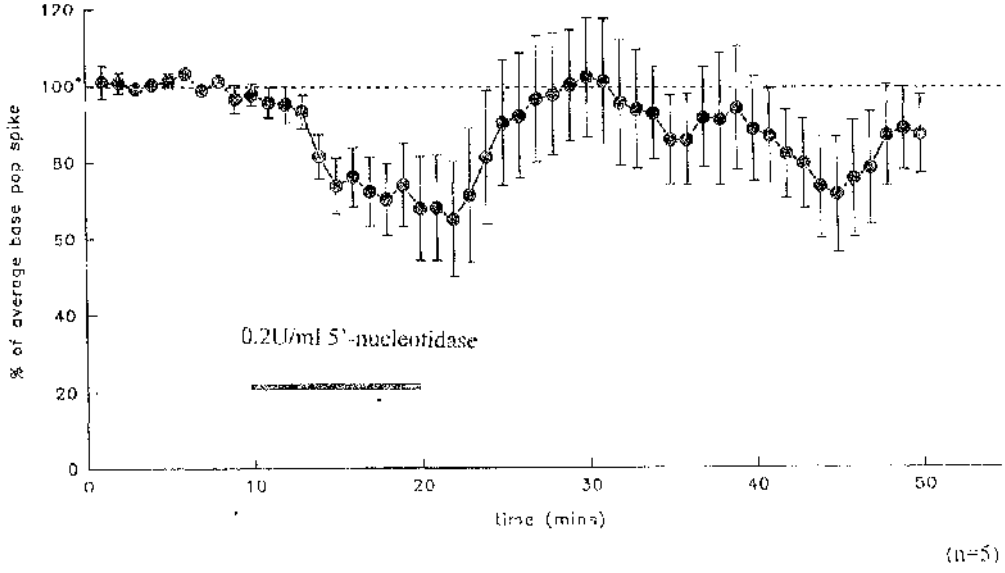

Figure 5.10 Effect of 5'nucleotidase 0.2U/ml on PS amplitude.

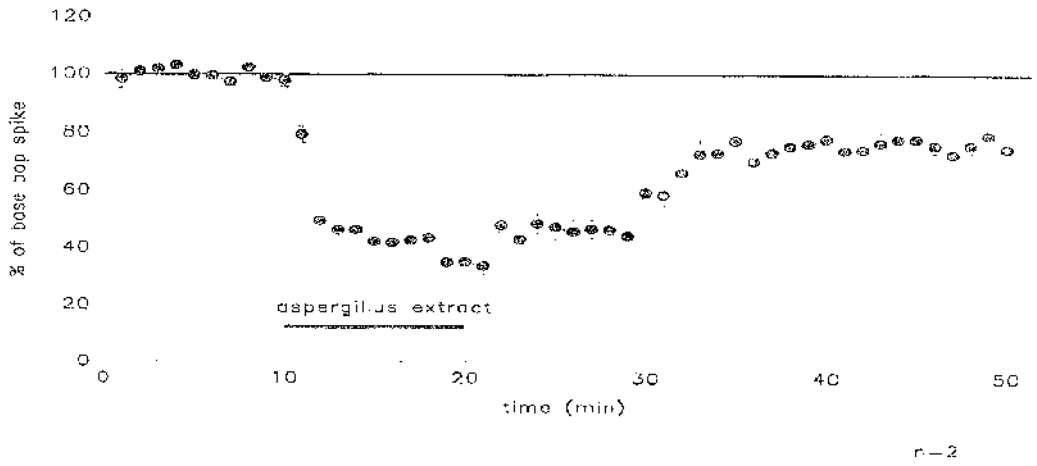

Figure 5.11. Perfusion of Aspergillus extract caused a significant decrease in population spike amplitude, which remained depressed 30 min following washout.

The effect of AMPase on potentiated slices.

Slices were potentiated using high frequency stimulation of 100 Hz for 1 second. Long-term potentiation was observed for 30 min before application of AMPase, at which time the population spike amplitude was potentiated to 147.4 ± 10.5% of control ($p<0.01$). Perfusion of AMPase caused a non-significant decrease of responses to 123.6 ± 25.0%, which recovered during washout before decreasing to unpotentiated levels (83.9 ± 8.6%, $p<0.01$ compared with potentiated values, not significantly different from control values), thus causing a long-term depression of the potentiatiated response (figure 5.12).

The effect of an inhibitor of AMPase.

The allosteric inhibitor of AMPase, 2,3-diphosphoglyceric acid (2,3-DPG) was used in an effort to block the actions of AMPase on evoked potentials.

2,3-DPG alone caused a significant depression (to 53.4 ± 9.7% of control, $p<0.01$) of population spike amplitude during perfusion which recovered to control values on washout (figure 5.13). When 2,3-DPG was perfused with AMPase no effects on either the initial increase of responses or the subsequent long-term depression following was observed. In the presence of 2,3-DPG, AMPase still depressed the population spike amplitude to 31.9 ± 5.0% of control ($p<0.001$) which is not significantly different from AMPase alone.

Figure 5.14 shows that AMPase does in fact inhibit the effects of AMP on evoked potentials. However when the allosteric inhibitor was used to block this inhibition the resulting long-term depression of responses was still observed.

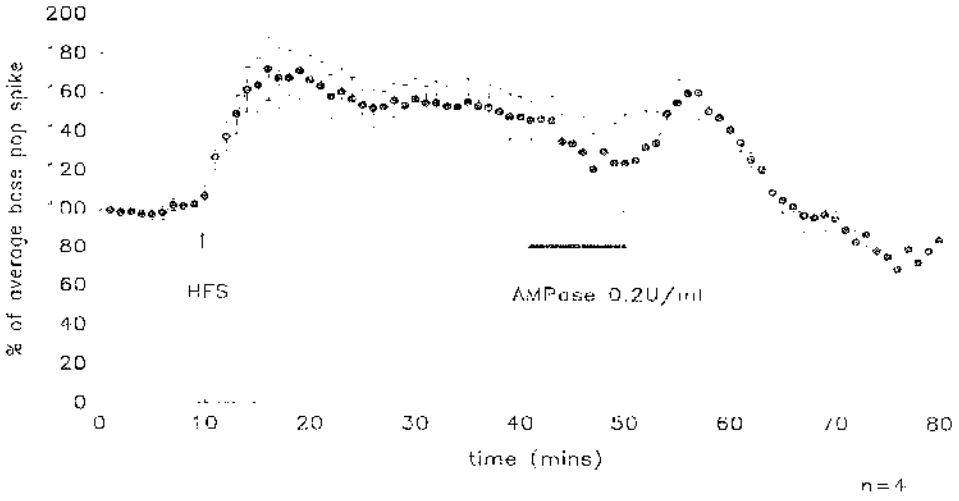

Figure 5.12. The effect of AMPase on potentiated responses. When slices were stimulated using high frequency stimulation (HFS, 100 Hz for 1 second), long-term potentiation (LTP) of responses was observed. Perfusion of AMPase 0.2U/ml 30 min following the induction of LTP resulted in a depression of responses.

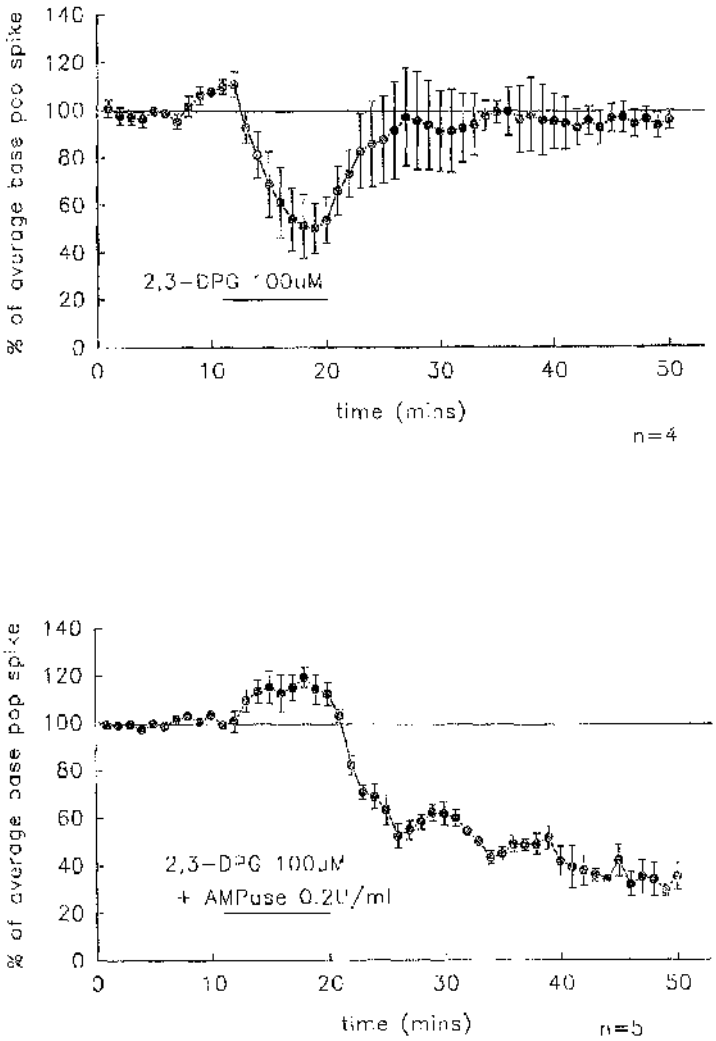

Figure 5.13. The effect of the allosteric inhibitor of AMPase, 2,3-diphosphoglyceric acid (2,3-DPG) on responses to AMPase. 2,3-DPG by itself caused a significant depression of population spike amplitude, however when in combination with AMPase, 2,3-DPG had no effects on either the initial potentiation of responses during perfusion, or the resulting depression seen on washout of AMPase.

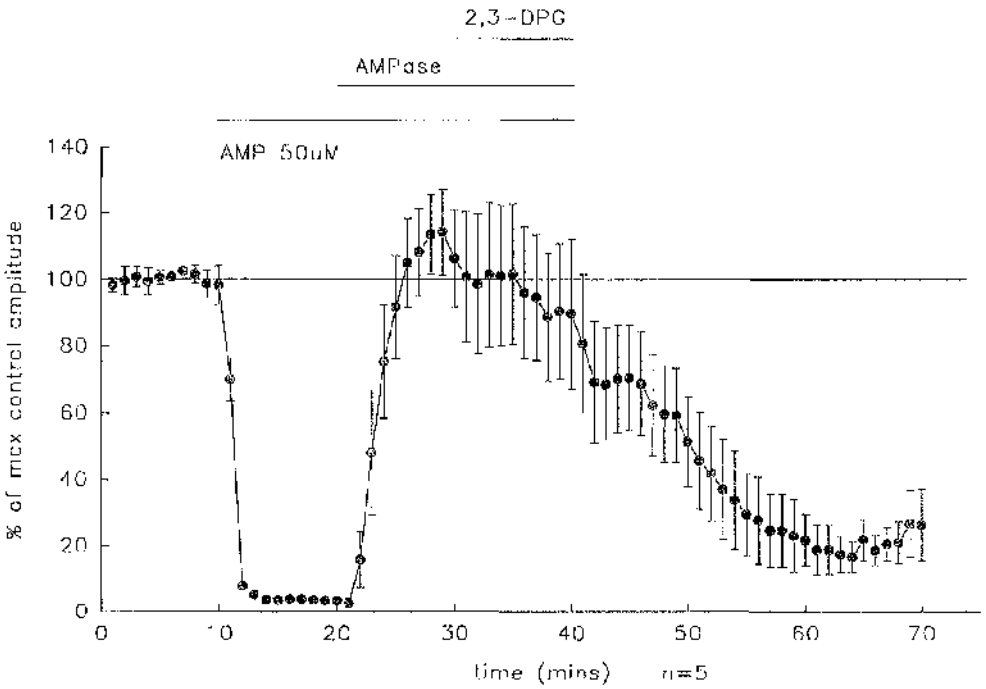

Figure 5.14. The effect of AMPase on responses to AMP and subsequent perfusion of the AMPase inhibitor 2,3-DPG.

AMPase effectively inhibited the effects of AMP 50 μM, however, 2,3-DPG did not lead to a return of the AMP-mediated inhibition of responses, nor did it prevent the long-term depression of responses following AMPase perfusion.

The effect of trypsin inhibitor on responses to AMPase.

Trypsin may be present in significant quantities in some enzyme preparations. To determine whether trypsin present in our AMPase preparation was responsible for the long-term depression of responses we perfused trypsin inhibitor along with AMPase.

Figure 5.15 shows that when trypsin inhibitor was perfused alone, a significant ($p<0.05$) depression of population spike amplitude was observed. When trypsin inhibitor was co-perfused with AMPase, no attenuation of the initial increase of responses, or the subsequent depression on washout was observed. The depression of responses when AMPase was co-perfused with trypsin inhibitor was 41.2 ± 8.9% of control values ($p<0.001$) which was not significantly different from the depression seen with AMPase alone.

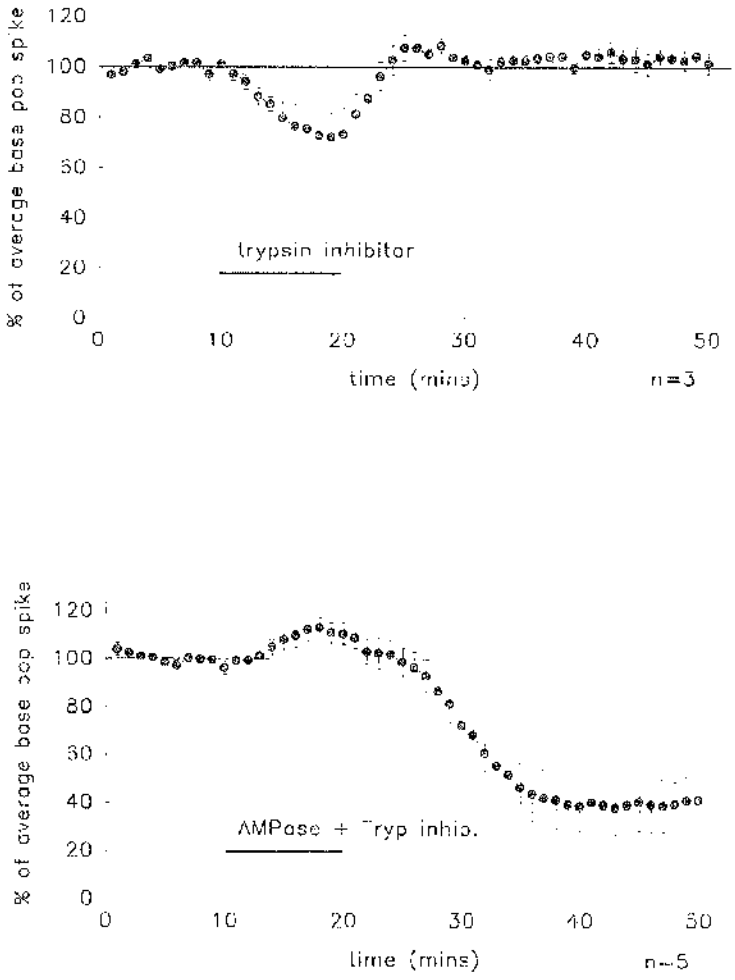

Figure 5.15. The effects of trypsin inhibitor on responses to AMPase.

Trypsin inhibitor caused a depression of population spike amplitude when perfused alone. However, when perfused in combination with AMPase, no effects on the initial potentiation of responses during AMPase perfusion, nor the subsequent long-term depression following washout were observed.

Other efforts to inhibit the activity of AMPase.

N-(2-aminoethyl)-5-chloro-1-napthalene sulphonamide (A3) a non-selective protein kinase inhibitor was co-perfused with AMPase, with no resulting modulation of the long-term depression observed (figure 5.16).

Indomethacin inhibits cyclo-oxygenase and hence metabolism of arachidonic acid. AMPase, in the presence of indomethacin (50 μM), continued to cause a resultant depression of population spike amplitude (figure 5.17).

N^G -nitro-L-arginine methyl ester (L-NAME), a competitive inhibitor of nitric oxide synthase (NOS) alone caused no significant effects on evoked potentials, nor did it affect the magnitude of depression seen 30 min following AMPase perfusion. However, an increase in potential size was observed immediately following washout of the agents. This recovery was short-lived, and responses began to decrease approximately 10-15 min after the start of washout and remained depressed for 30 min (figure 5.18). The brain specific NOS inhibitor, 7-nitroindazole (7-NI), in contrast, had no effects on the time course of inhibition following AMPase perfusion (figure 5.19).

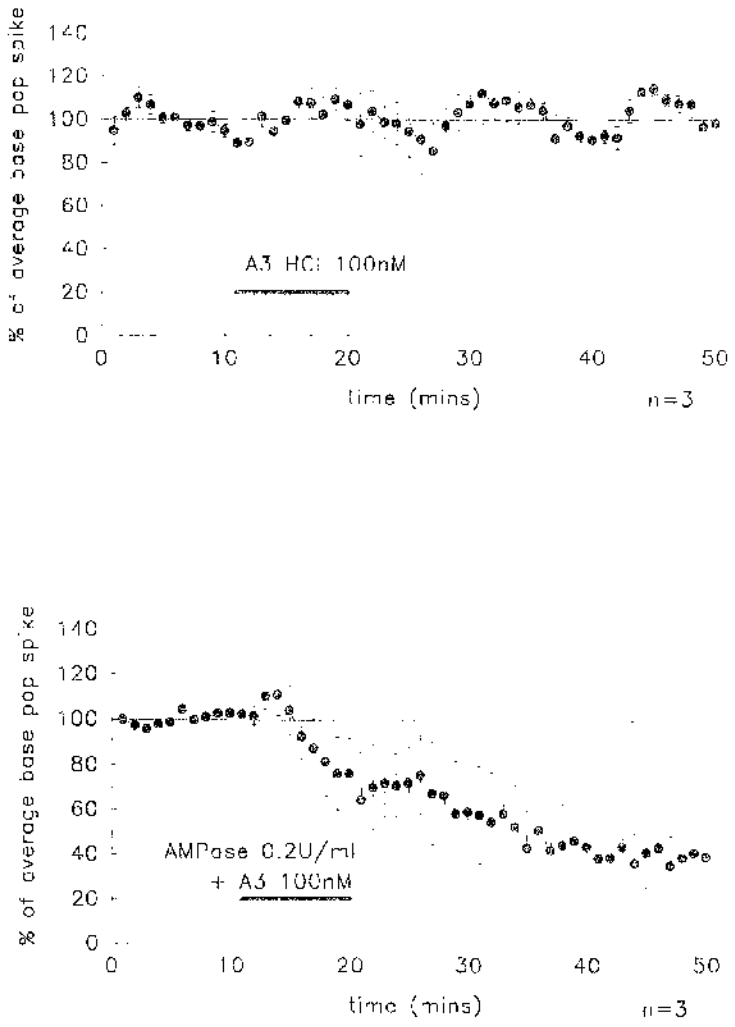

Figure 5.16. The effect of N-(2-aminoethyl)-5-chloro-1-napthalene sulphonamide hydrochloride (A3 HCl) a general protein kinase inhibitor by itself (a) and co-perfused with AMPase (b).

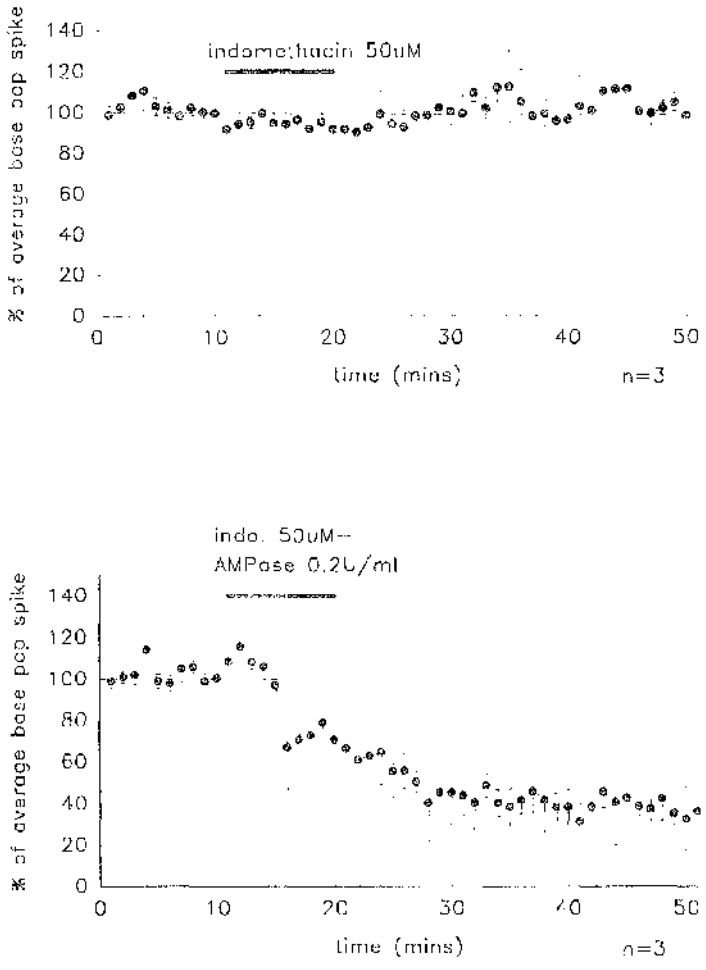

Figure 5.17. The effect of the cyclo-oxygenase inhibitor, indomethacin (50 μM) on responses to AMPase.

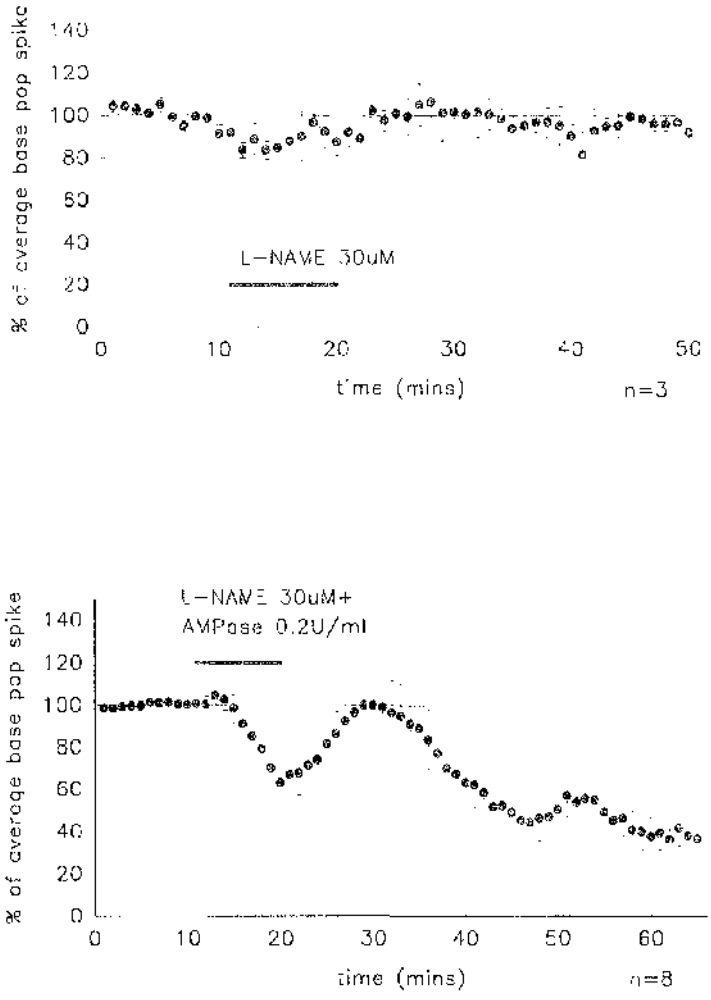

Figure 5.18. The effect of the nitric oxide synthase inhibitor N^G -nitro-L-arginine methyl ester (L-NAME) on responses to AMPase. L-NAME had no effects by itself (top) but inhibited the onset of long-term depression when co-perfused with AMPase.

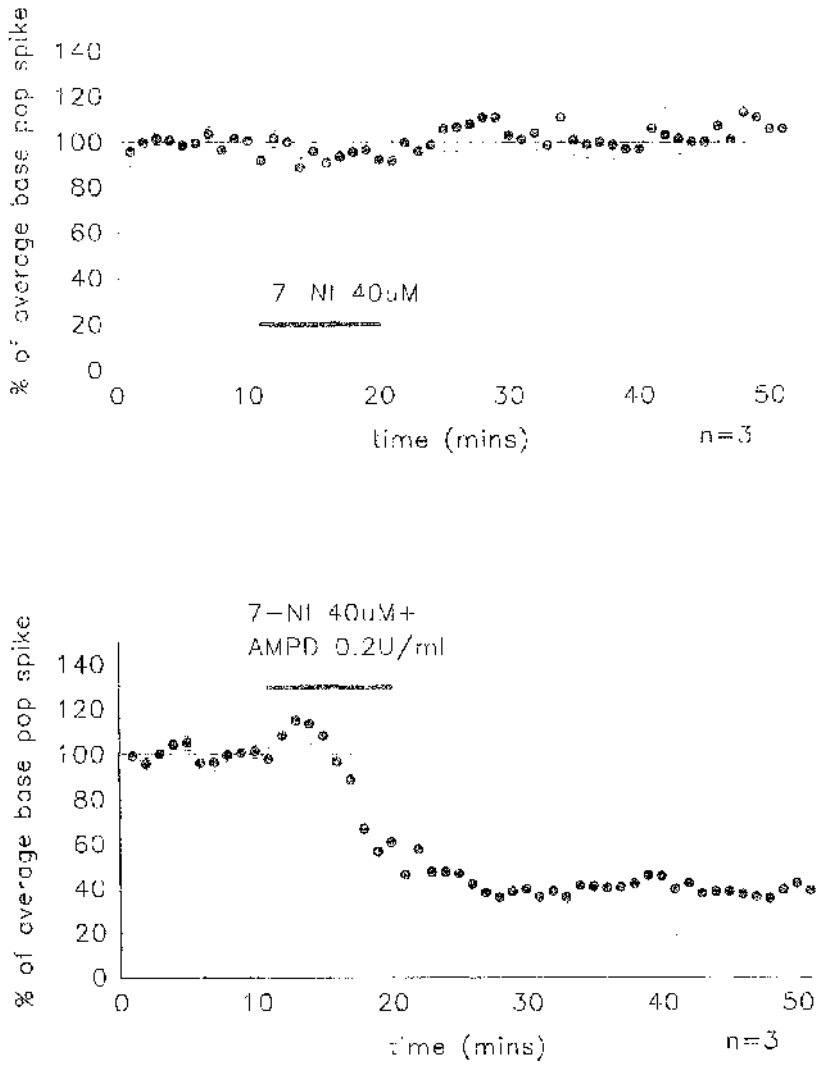

Figure 5.19. The effect of the specific brain nitric oxide synthase inhibitor, 7-nitroindazole (7-NI) on responses to AMPase.

Purification of the active component present in AMPase preparation.

It became increasingly apparent that the factor present in the enzyme preparation responsible for the long-lasting depression of evoked potentials may not be AMPase.

As a first step to determine how pure the preparation was, a SDS-PAGE gel was run to separate the various components present. Figure 5.20 shows the results from such a gel. It is obvious from the number of bands present that the enzyme preparation was quite "dirty".

A sample of the crude enzyme extract was passed through a FPLC column and gel filtration column (see appendix 1) and fractions collected every 90 seconds. Simultaneous UV spectrum analysis at 280nm of the fractions to determine which contained protein was of limited worth, as the crude extract was brown in colour and hence absorbed UV light at the same wavelength.

In order to obtain a greater quantity of material to assay, a precipitation step using ammonium sulphate was used before chromatography. Three concentration ranges were used to precipitate protein present in the crude preparation, i.e. 0-40%, 40-60%, and 60-80%. Of these, only protein that had precipitated in the 40-60% range caused long-term depression in the slice preparation (figure 5.21). The 40-60% sample was loaded onto the gel filtration column (Superose 12, Pharmacia) and fractions assayed on slices. Activity was seen in 3 of these fractions in increasing amounts (figures 5.22,5.23,5.24). The fractions were tested for the presence of AMPase using a biochemical assay (see appendix 1) and were also tested for depressant activity on slices. Interestingly,

the fractions that contained the AMPase activity were different from those that produced LTD in hippocampal slices (see figs 5.25, 5.22, 5.23, 5.24). Figure 5.26 shows a SDS-PAGE gel of the active fractions. It can be seen that a single protein band at approximately 90 kDa was present.

Identification of the active protein.

The fraction containing the purified active protein was sequenced using Edman degradation chemistry by Nick Morrice, MRC Protein Phosphorylation Unit, Department of Biochemistry, Medical Sciences Institute, University of Dundee, using an Applied Biosystems Procise sequencer (see appendix 2). The sequence obtained was:

VTNWDQAYTQAKTALDK

This 17 amino acid sequence was entered into a number of protein databases on 21st May 1999, including BLAST (http://www.blast.genome.ad.jp/), SwissProt (http://www.genome.ad.jp/dbget-bin/www_bfind?swissprot-today), and FASTA (http://www.fasta.genome.ad.jp/). No significant match with other known proteins was obtained. Further (internal) sequencing by tryptic digestion of the protein (Nick Morrice, University of Dundee) provided further information about the protein sequence (five extra sequences of varying lengths were obtained). These internal sequences were also entered into the databases (June, 1999). The searches revealed that the sequences had a similarity to a β-glucosidase molecule. The purified protein fraction when assayed biochemically (H.G. Nimmo, personal communication) showed some β-glucosidase activity. However, a

preparation of β-glucosidase, extracted from almonds when perfused over a slice in three different concentrations did not result in long-term depression of evoked responses (figure 5.27,5.28,5.29).

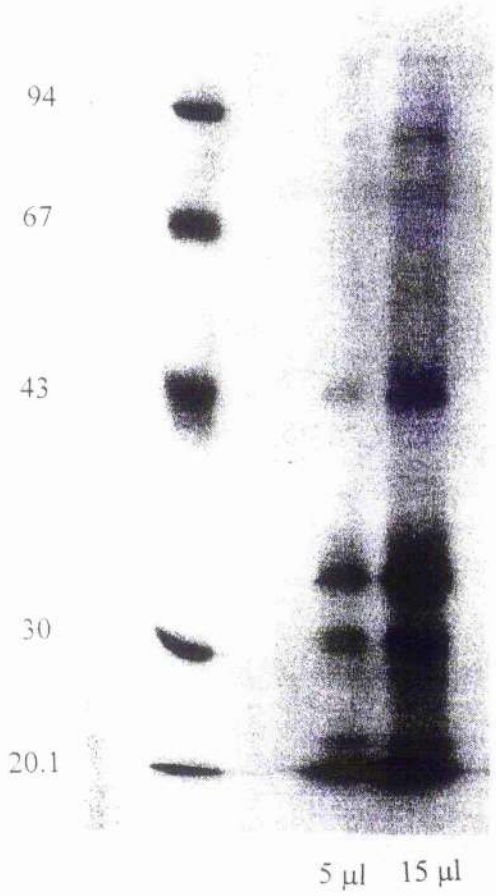

Figure 5.20 5 and 15 µl of a sample of AMPase which was prepared for chromatography (see appendix 1) was run on a 12.5% SDS-PAGE gel. A range of protein bands were apparent indicating that a variety of proteins were present in the crude sample.

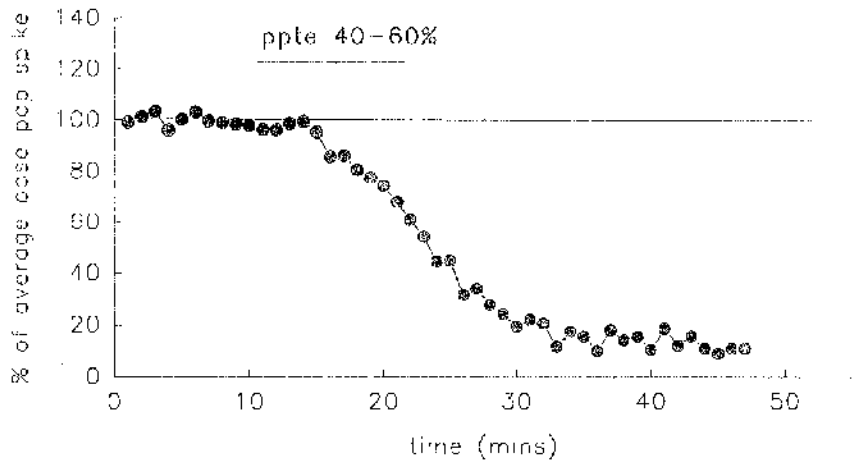

Figure 5.21. A protein in the crude AMPase extract which precipitated in 40-60% ammonium sulphate was responsible for long-term depression of population spike amplitude.

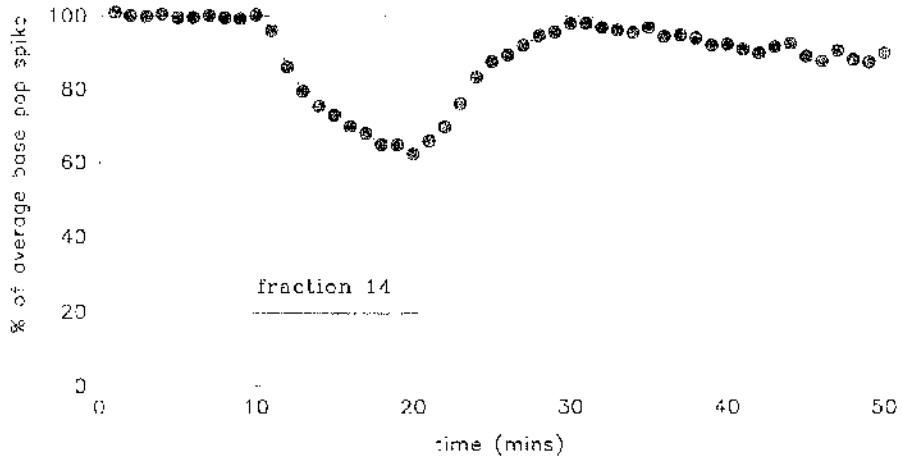

Figure 5.22. The effect of fraction 14 eluted from a chromatography column on population spike amplitude.

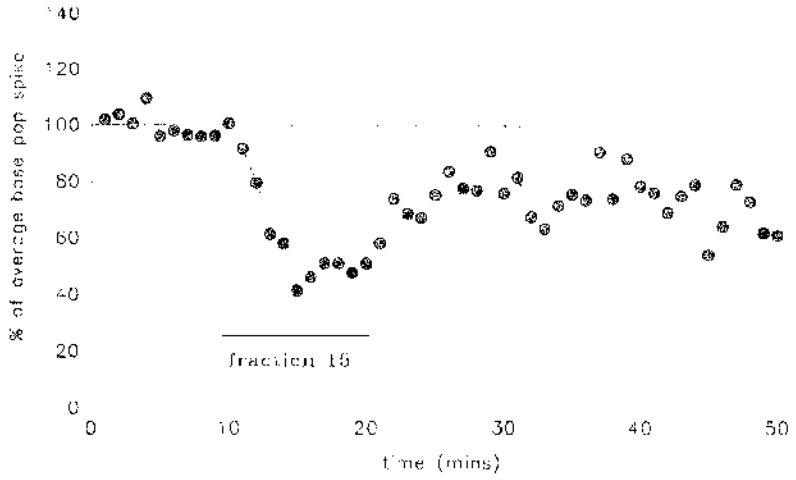

Figure 5.23. The effect of fraction 15 eluted from a chromatography column on population spike amplitude.

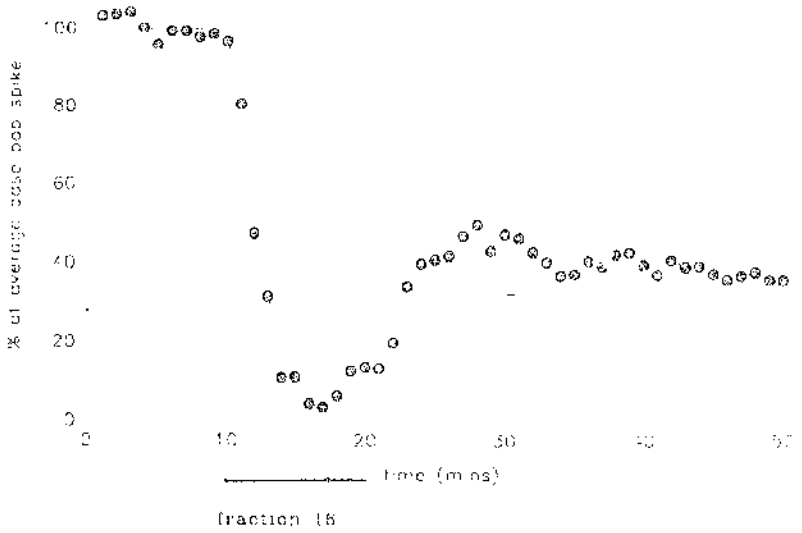

Figure 5.24. The effect of fraction 16 eluted from a FPLC column on population spike amplitude. More depression of responses was observed than in the previous two fractions, suggesting most of the active component was present in this fraction.

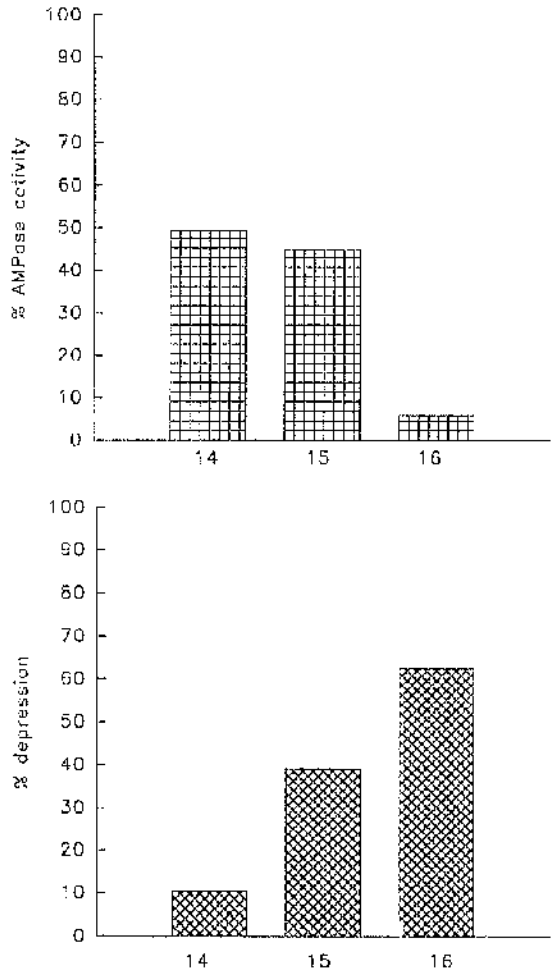

Figure 5.25. The AMPase activity of fractions 14, 15 and 16 when measured biochemically (see appendix 1) did not correlate with their depressant activity on population spike amplitude in the slice preparation.

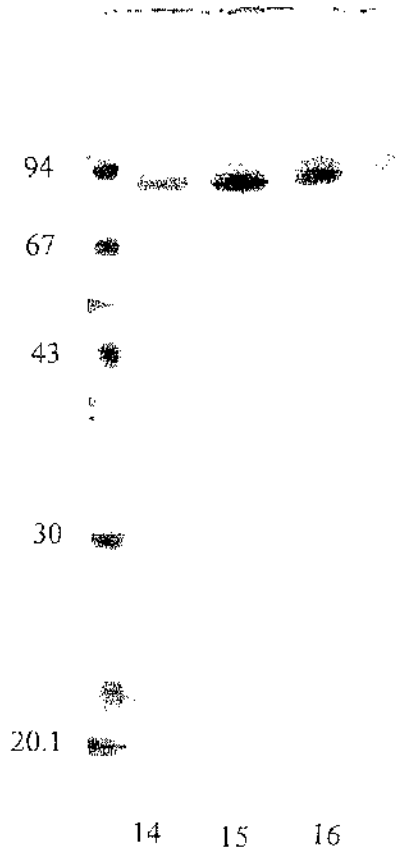

Figure 5.26 SDS-PAGE gel of fractions 14, 15 and 16 showing a single protein band present at approximately 90 kDa.

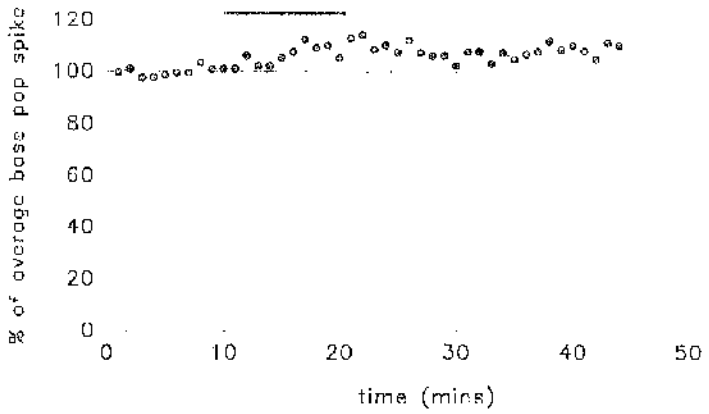

Figure 5.27 The effect of a 10 min perfusion (bar) of 0.25U/ml β-glucosidase from almonds on population spike amplitude.

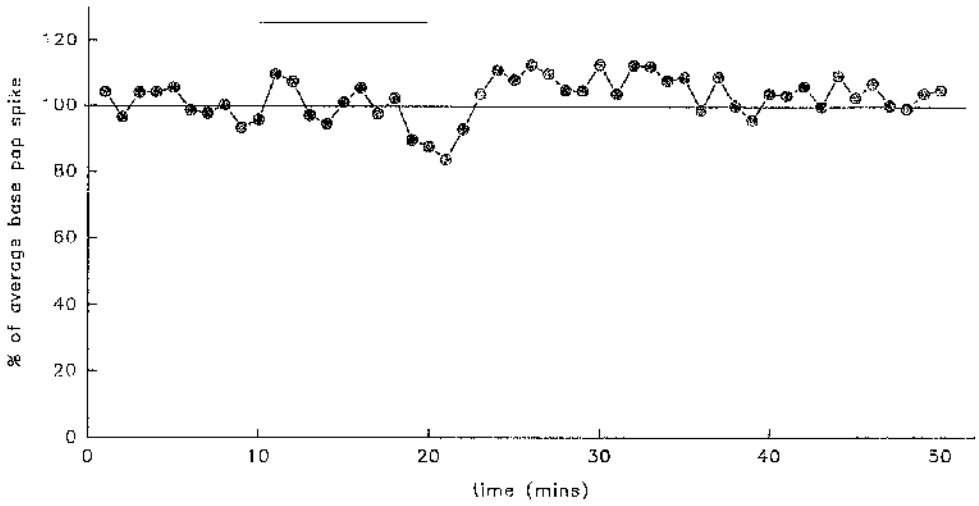

Figure 5.28. The effect of a 10 min perfusion (bar) of 1U/ml β-glucosidase from almonds on population spike amplitude.

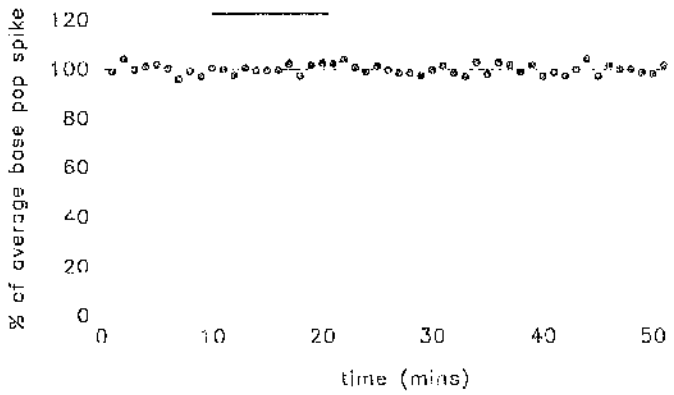

Figure 5.29 The effect of a 10 min perfusion (bar) of of 4U/ml β-glucosidase from almonds on population spike amplitude.

Inhibition of β-glucosidase.

Conduritol, an irreversible inhibitor of β-glucosidase, was incubated for at least one hour with a 40-60% ammonium sulphate precipitated fraction of the crude enzyme extract.

A biochemical assay (see appendix 1) showed that β-glucosidase activity in the preparation was inhibited by conduritol (H.G. Nimmo, personal communication). However, when perfused over a slice, LTD of population spike amplitude still occcured (Figure 5.31) to an extent not significantly different from the 40-60% fraction alone (figure 5.30).

During perfusion of the fraction which had been incubated with conduritol, population spike amplitude was reduced to 72.4 ± 4.4% of control ($p < 0.01$). This was significantly different from the results seen when the 40-60% fraction was perfused alone ($p<0.05$).

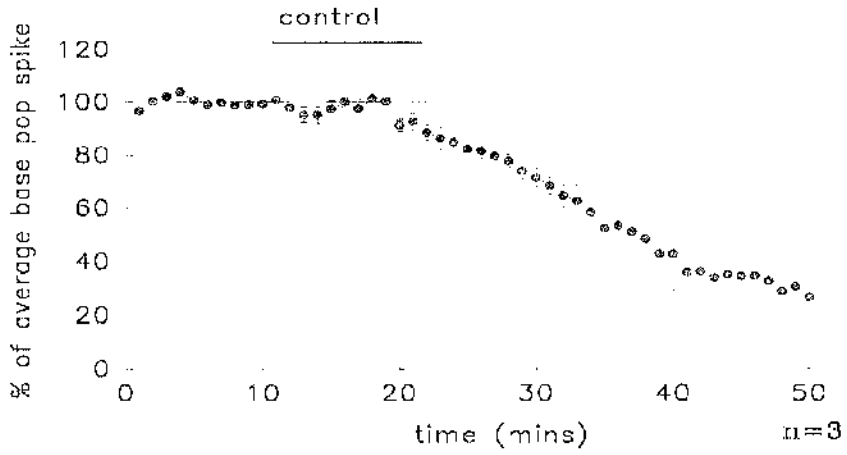

Figure 5.30. The effect of crude AMPase extract in which proteins were precipitated in 40-60% ammonium sulphate on population spike amplitude. Following 30 min washout of the extract population spike amplitude was 26.7 ± 9.32 % of control ($p < 0.01$).

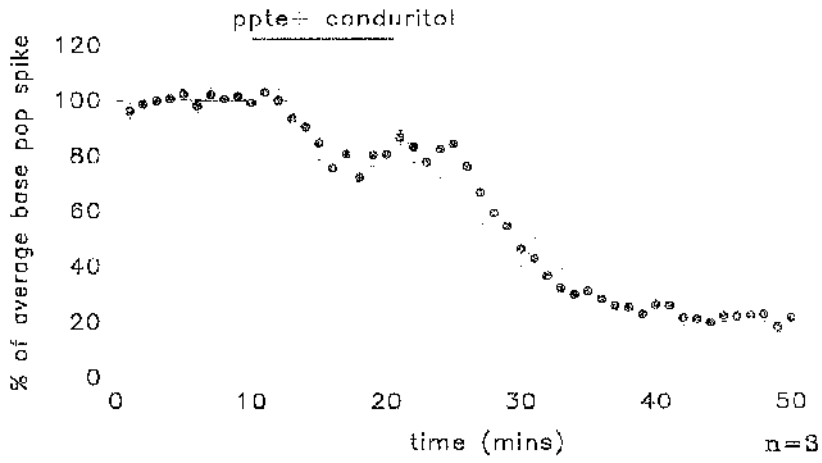

Figure 5.31. The effect of crude AMPase extract in which proteins were precipitated in 40-60% ammonium sulphate and incubated for at least 1 hour with conduritol to inhibit β-glucosidase activity. Following 30 min washout of the preparation population spike amplitude was depressed to 21.8 ± 2.8 % of control ($p<0.001$)

Brain homogenates.

It was decided to examine whether an endogenous factor present in the brain was responsible for long-term depression of evoked potentials. The brain of a rat, pig, and guinea-pig were homogenised and precipitated in the same manner as the AMPase extract (i.e. using ammonium sulphate at concentration ranges 0-40%, 40-60% and 60-80%). The re-suspended precipitates were then perfused over a rat hippocampal slice.

Neither rat or pig brain homogenates had any long-term effects on evoked potentials in any of the fractions tested. However, guinea-pig brain homogenate in which proteins had precipitated in the 40-60% ammonium sulphate range, caused a long lasting depression of population spike amplitude (figure 5.33, 5.34). Interestingly, this was the same range in which the crude AMPase extract caused LTD.

Figure 5.34 shows that the depression caused by guinea-pig homogenate precipitated in 40-60% ammonium sulphate - like that caused by AMPase perfusion (see fig 5.3) - could be reversed by increasing the stimulation amplitude. LTP could also be elicited in the slice using high frequency stimulation (100Hz, 1s).

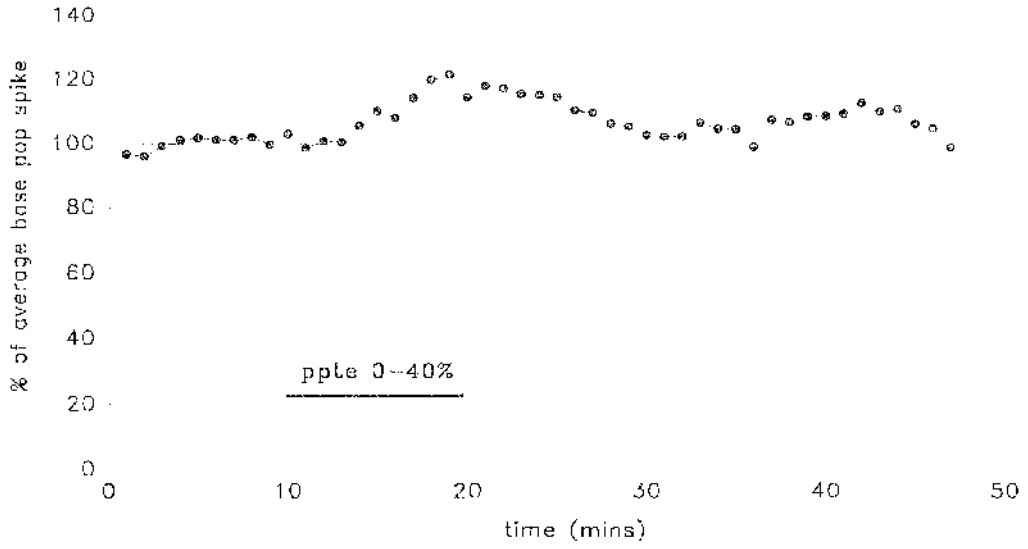

Figure 5.32. The effect of a perfusion of guinea-pig brain which had been homogenised and precipitated using 0-40% ammonium sulphate on population spike amplitude.

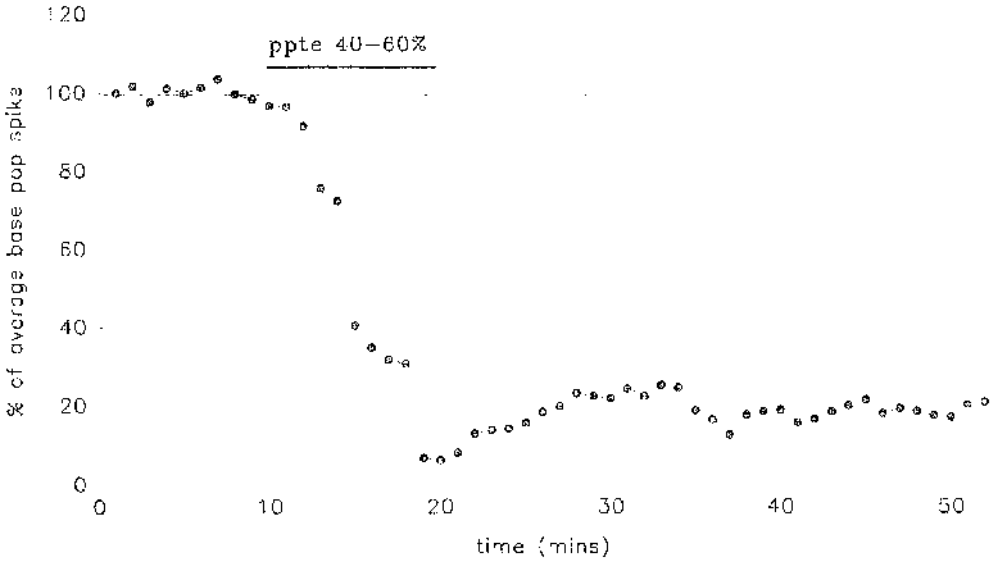

Figure 5.33. The effect of a perfusion of guinea-pig brain which had been homogenised and precipitated using 40-60% ammonium sulphate on population spike amplitude.

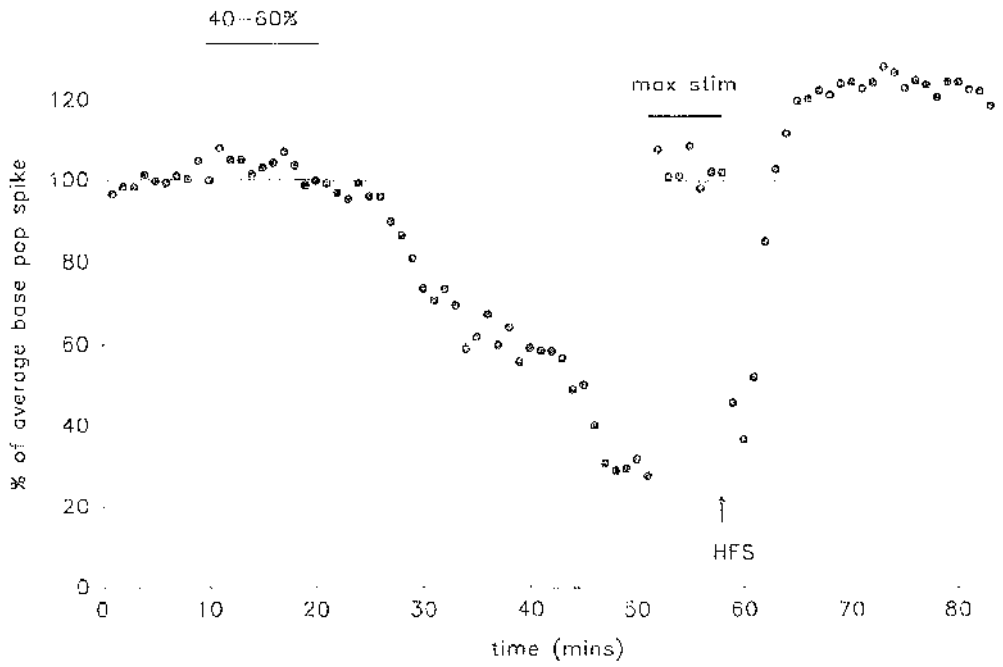

Figure 5.34. The depression caused by the 40-60% ammonium sulphate precipitation of guinea-pig brain could be reversed by increasing the stimulation amplitude (bar) or by high frequency stimulation (arrow).

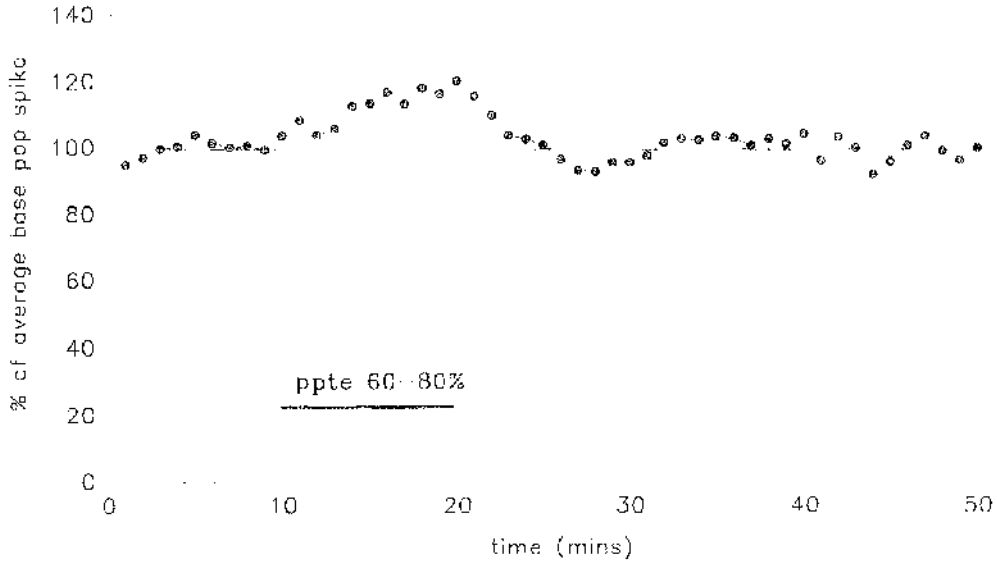

Figure 5.35 The effect of a perfusion of guinea-pig brain which had been homogenised and precipitated using 60-80% ammonium sulphate on population spike amplitude.

III. Discussion.

The results show that perfusion of a preparation of AMPase (E.C. 3.5.4.6.) from *Aspergillus* species resulted in a long-lasting depression of evoked potentials in the hippocampal slice. Population spike amplitude was depressed to a greater extent than could be accounted for by the depression in EPSP slope, resulting in a depression of the EPSP-spike relationship. Initially it was postulated that the effect of AMPase was a result of a property of the AMPase molecule other than its AMP metabolism, due to, for example, the fact that inhibiting the enzyme with 2,3-DPG did not affect the long-term depression seen following perfusion. Similar observations were made using coformycin (T.W. Stone, unpublished observations), a recognised inhibitor of AMPase (Dancer *et al.*, 1997). This compound also prevents the inhibitory effect of AMPase on AMP responses, indicating successful inactivation of the enzyme, but did not prevent the long-term depressant activity.

Contaminating ions present in the AMPase preparation were ruled out as mediators of the long-term effects by Ross (1997), who dialysed a solution of AMPase for four days. This treatment did not affect the enzymatic activity of AMPase or the LTD following perfusion. Further, boiling the enzyme preparation to denature the enzyme led to a removal of the long-term depression, suggesting that a protein (although not necessary the AMPase molecule) was responsible for the observed depression.

The fact that responses could be restored by increasing stimulation amplitude and potentiated using high frequency stimulation indicates that the cells were not damaged in any way; the behaviour of the enzyme preparation is reminiscent of that of a pharmacological competitive antagonist, against which increasing the dose of agonist is able to overcome the blockade of receptor.

AMPase may have caused depotentiation of responses which had been potentiated using high frequency stimulation, a feature common to stimulating at low frequencies which has also been shown to cause LTD in hippocampal slices, suggesting that electrically- and AMPase- induced LTD may share common mechanisms. Unfortunately, as reported by many other investigators, LTD could not be established electrically using low-frequency stimulation (LFS) in the set of slices investigated (Barrionuveo *et al.*, 1980; Fujii *et al.*, 1991; Bashir and Collingridge, 1994; Errington *et al.*, 1995; Wagner and Alger, 1996). Interestingly, with the exception of Errington *et al.*, depotentiation of responses could be demonstrated in the same preparations by these groups.

The mechanism of LTD in the slices following AMPase did not seem to involve protein kinase, cyclo-oxygenase or nitric oxide. Ca^{2+}/calmodulin-dependent kinase II (CaMKII) (Stanton and Gage, 1996) and protein kinase A (Brandon *et al.*, 1995) have both been implicated in long-term depression. However, use of the non-specific protein kinase inhibitor A3 had no effect on the depression following perfusion of the AMPase preparation. Indomethacin is an irreversible inhibitor of cyclo-oxygenase, the enzyme responsible for the metabolism of arachidonic acid, which is formed following activation of phospholipase A_2

(PLA_2). The final compounds derived from the metabolism of arachidonic acid are termed the eicosanoids and include the prostaglandins, thromboxanes, leukotrienes and lipoxins. An inhibitor of PLA_2, bromophenacylbromide has been shown to reduce or inhibit LTD in the hippocampus (Fitzpatrick and Baudry, 1994; Normandin *et al.*, 1996). Indomethacin did not affect evoked potentials alone, nor did it affect the LTD seen following perfusion of the AMPase preparation. Normandin *et al.* (1996), also demonstrated indomethacin did not reduce LTD in the hippocampus. They did, however, show that a lipoxygenase inhibitor partially blocked hippocampal LTD, implying that, although the PLA_2/arachidonic acid pathway involving cyclo-oxygenase is not involved in the LTD seen following AMPase perfusion, other mediators derived de novo from phospholipids may be implicated.

Whereas the involvement of nitric oxide (NO) in cerebellar long-term depression is well accepted, its role in hippocampal LTD is a matter of controversy. Izumi and Zorumski (1993) have reported that the nitric oxide synthase (NOS) inhibitors L-N(G)-monomethylarginine and L- N(G)-nitroarginine (L-NOArg) can block the induction of LTD following HFS in hippocampal slices. Similarly, Wu *et al.* (1997) showed that two novel NOS inhibitors, 1-(2-trifluromethylphenyl)imidazole and 3-bromo-7-nitro-indazole also blocked the induction of LTD in the hippocampus, an effect which could be prevented by pretreatment with L-arginine, the substrate amino acid used by NOS for nitric oxide production. In contrast, other groups have seen no effect of NOS inhibitors on hippocampal LTD (Cummings *et al.*, 1994; Malen and Chapman, 1997). In this study L-NAME delayed the onset of LTD in slices, suggesting that nitric oxide may play a role in the induction of this form of LTD.

AMPase extracted from rabbit muscle did not have the same responses on slices, suggesting that a specific *Aspergillus* form of the enzyme was responsible for the LTD. This is reinforced by the fact that a homogenate of cultured *Aspergillus nidulans* also induced LTD of responses.

Separation of the AMPase preparation extracted form *Aspergillus* species on a polyacrylamide gel showed that a variety of proteins were present. The observation that the protein responsible for the long-term depression of evoked responses was not the same as that causing the metabolism of AMP when this (now obviously crude) enzyme preparation was separated by FPLC confirmed that it was not the AMPase molecule that was responsible for the observed depression.

Further purification and sequencing of the unknown (90 kDa) protein revealed that it was similar to a β-glucosidase. This enzyme participtes in the removal of glucose residues from oligosaccharides. A fungal form of the enzyme may act extracellularly to digest complex external saccharides to allow the growth of hyphae.

As previously mentioned the β-glucosidase was 90 kDa in size. It is known that some large molecules can be released by neurones. Acetylcholinesterase, for example, can be released into the extracellular space and recent work has begun to reveal effects of this enzyme on neuronal growth and development, including non-enzymatic functions of the molecule (Sternfield *et al.*, 1998). Interestingly,

guinea-pig brain homogenate, when precipitated using ammonium sulphate showed a long lasting depression of responses at the same cut (40-60%) as that for the *Aspergillus* AMPase preparation. The long-term depression produced by the guinea-pig homogenate was similar in profile to that produced by the AMPase preparation, in that potentials could be restored to control values by increasing the stimulation amplitude, also LTP could be elicited using HFS. These results reveal the possibility that an endogenous factor present in the brain (at least of guinea-pig) may be responsible for long-term depression in the hippocampus and opens the question as to the mechanism of such an action.

If β-glucosidase is released by neurones it may act extracellularly (postsynaptically) to cleave glucose residues from receptors or change membrane properties of neurones, resulting in long-lasting depression of transmission. However, the results obtained with conduritol suggest that β-glucosidase activity is not involved in the induction of LTD, since the biochemical assay showed that β-glucosidase activity was inhibited in the presence of conduritol but slices still showed LTD following perfusion. Another factor suggesting that β-glucosidase activity is not necessary for the induction of LTD in the hippocampus is that the almond form of the enzyme, when perfused over a slice, did not result in LTD. However, it may be that an *Aspergillus* specific form of the enzyme is required to produce the effect. If it is not the enzymic properties of the β-glucosidase that causes the depression, it may be a consequence of its polypeptide structure acting on receptors to activate (or inhibit), yet to be defined, transduction mechanisms. It is important to note that the purified protein only had *limited* homology to the

β-glucosidase molecule and was not identical to the two *Aspergillus* forms of the enzyme in the database. It is a possibility that the depressant effects described in this study are due to a novel protein which possesses a similarity to β-glucosidase unconnected to its properties as a mediator of synaptic plasticity.

CHAPTER 6.

GENERAL DISCUSSION.

The initial intention of this study was to examine the adenosine (P1 receptor) and ATP (P2 receptor) effects on E-S coupling in the rat hippocampal slice. In fact, each section of the study led the work into slightly different avenues.

Purine studies.

The study of adenosine A_1 and A_2 receptor effects on E-S coupling revealed the A_1\A_2 interaction illustrated in Chapter 3. This interaction between adenosine A_1 and A_2 receptors could be of biological importance in situations in which the levels of endogenous adenosine rise substantially. The levels of adenosine in normal brain are in a concentration range of 1 to 10μM (Dunwiddie *et al.*, 1981; Fredholm *et al.*, 1984; Newman and McIlwain, 1977). The endogenous concentration normally seems able to activate A_1 receptors, as antagonists (e.g. caffeine, theophylline) have behavioural and electrophysiological effects which are normally attributed to inhibition of A_1 receptors with no significant correlation with other actions of these drugs such as phosphodiesterase antagonism (Dunwiddie *et al.*, 1981; Snyder *et al.*, 1981). In conditions where the levels of adenosine in the brain rise, for example in ischaemia, A_{2A} receptors may also be activated which would attenuate the inhibitory effects of A_1 receptor activation on transmitter release and postsynaptic membrane excitability, and may reduce the inhibitory and neuroprotective properties of A_1 receptor activation (MacGregor *et al.*, 1993).

The examination of the effects of ATP on evoked potentials revealed a LTP which became the focus of Chapter 4. As previously mentioned, high frequency stimulation of the type that induces LTP preferentially releases ATP. This in turn means that a higher proportion of the adenosine in the extracellular space following high frequency stimulation comes from metabolised ATP. It has been shown that adenosine formed from the metabolism of released adenine nucleotides preferentially activates excitatory (A_2) adenosine receptors in rat hippocampal synapses (Cunha *et al.*, 1996b). A_{2A} receptors have been shown to increase presynaptic fluxes (Mogul *et al.*, 1993) and also potentiate postsynaptic AMPA responses resulting in NMDA receptor-independent LTP (Kessey and Mogul, 1997). Indeed, adenosine A_2 receptors have been implicated in the development of LTP by a number of investigators. The A_{2A} antagonist CP 66713 has been shown to both inhibit the induction of, and facilitate the reversal of LTP (depotentiation) of evoked EPSPs (but not population spikes) in hippocampal CA1 neurons (Sekino *et al.*, 1991, Fujii *et al.*, 1992). Kessy et al (1997) provided evidence for the role of adenosine A_{2B} receptors in the induction of hippocampal LTP.

This evidence for the involvement of A_2 receptors in excitatory transmission provided the rationale for the use of the A_{2A} receptor antagonists ZM 241385 and CSC in this study. CSC had no effects on the adenosine A_1-receptor-mediated inhibition seen during ATP perfusion, but inhibited the following induction of LTP, supporting the involvement of A_{2A} receptor activation in LTP. Surprisingly, ZM 241385 prevented the inhibition produced during ATP perfusion - an effect ascribed to the activation of adenosine A_1 receptors by

adenosine released from the metabolism of ATP, without affecting the subsequent LTP, in fact the removal if the inhibitory effect of ATP exposed the induction of LTP almost immediately on perfusion of ATP. Why the supposedly A_1 receptor mediated inhibition of responses by ATP should be blocked by an A_{2A} receptor antagonist is unclear. Comparison of the binding characteristics of ZM 241385 in cortical and striatal membranes indicate a 500-1000 fold selectivity for A_{2A} receptors over A_1 receptors and no significant activity for adenosine A_3 receptors (Poucher *et al.*, 1995). The effect cannot be accounted for by the use of too high a concentration of ZM 241385 which also blocked A_1 receptors, since the more selective A_1 receptor antagonists reduced the size of the LTP. The previous chapter demonstrated an interaction between adenosine A_1 and A_{2A} receptors. An interaction between these receptors in this case seems unlikely given the evidence that activation of A_{2A} receptors causes a desensitisation-like effect on A_1 receptors (Cunha *et al.*, 1994a; Dixon *et al.*, 1997). An inhibition of A_1 effects by antagonism of A_{2A} receptors would involve the tonic enhancement by A_{2A} receptors of A_1 receptor inhibitory responses on neuronal excitability which would disappear upon A_{2A} receptor blockade, directly opposing these studies. An alternative explanation may be that adenosine A_{2A} receptors tonically increases the inhibitory drive in the hippocampus, which when blocked could raise the threshold for inhibition by A_1 receptor agonists. Activation of A_{2A} receptors have been shown to facilitate the release of GABA from rat synaptosomes (Cunha *et al.*, 1996c). The xanthine A_{2A} receptor antagonist chlorostyryl-caffeine did not produce the same effect on ATP induced inhibition of responses as ZM 241385, indicating that the effect is probably not attributable to the blockade of A_{2A} receptors. Adenosine A_{2B}

receptors have been implicated in LTP (Kessy *et al.*, 1997). Although the concentration of ZM 241385 used is selective for A_{2A} receptors, it has been shown that ZM 241385 has moderate affinity for human A_{2B} receptors in transfected in CHO cells (Ki 50 nM) (Ongini *et al.*, 1999).

A further mechanism by which ZM 241385 may block the inhibitory effects of ATP on evoked responses is that the structure of the molecule may affect the metabolism of ATP to adenosine, for example by blocking ecto-nucleotidases. Only a small amount of adenosine need be present for LTP induction to occur, as shown by the effects of 2.5 μM ATP. In this scenario, the adenosine released by the metabolism of ATP would not be sufficient to cause a significant reduction of response size, although subsequent LTP did occur. If ZM 241385 had an inhibitory effect on the breakdown of ATP, such that the concentration of adenosine produced was of the same order as that produced from 2.5 μM ATP, then its effects would be explained. At present, it is only possible to speculate that the effect of ZM 241385 is due to a novel action, by which it interferes with the processes associated with the P1 receptor-mediated depression of evoked potentials, but leaves unchanged more proximal steps in A_1 receptor activation needed for the ATP-induced establishment of LTP. The results also highlight the importance of taking these effects of ZM 241385 on adenosine A_1 receptors into consideration when interpreting studies concerned with its A_{2A} antagonism effects.

These studies re-affirm the position of purines as modulators of neuronal activity in the mammalian brain. Initially, the prospect of purines as messenger

molecules was greeted with scepticism. Many found it difficult to accept that compounds with the ubiquitous distribution and biochemical importance of adenosine and ATP could assume the equally fundamental but completely unrelated function of neurotransmitters. Indeed, it is possible that these compounds originated very early in the earth's development. It has been shown as early as the 1880's that the purine adenine can be synthesised in a single step from ammonia and hydrogen cyanide. As a consequence, it was proposed that purines may have been created in the "primordial soup" (warm pools of water, methane, carbon dioxide, ammonia and hydrogen) by the action of, for example, ultraviolet light, as has been demonstrated in the laboratory. The natural sequel to the formation of adenine would be the production of ATP and this provides a working hypothesis for the origin and evolution of the components basic to human life. In light of this, the argument against ATP and adenosine being neurotransmitters due to their ubiquity and biochemical importance can be turned on its head. If purine molecules are as old as life itself then it is reasonable to argue that during evolution they will become involved in virtually all aspects of cellular function. It should come as no surprise that they are involved in signalling between some of the most evolutionary advanced cell types, namely nerve cells.

Future work on the role of purines in neurotransmission, and in particular synaptic plasticity, may include the interaction of purines with other modulators of neuronal plasticity for example, arachidonic acid, nitric oxide, metabotropic glutamate receptors etc. Work could also include the interaction of purine molecules with tropic factors responsible for morphological changes in neuronal

networks leading to long-term changes in plasticity, for example the serine protease family (Turgeon and Houenou, 1997) or α-integrin (Grotewiel et al., 1998). Another interesting area of investigation would be the identification of changes in gene expression such as zif/268, c-fos, or Ca^{2+}/calmodulin protein kinase II (CAMKII) following induction of LTP by ATP.

Studies with 5' adenylic acid.

Previous work with ATP in this lab, together with a desire to determine whether the effects of AMPase were due to P_1 or P_2 receptors led into the entirely unexpected area of protein purification and the identification of a putatively novel modulator of hippocampal plasticity. Other groups have described apparent novel or unusual effects of enzyme preparations, which later have been found to be attributable to the presence of contaminants arising from the commercial extraction of the enzyme. For example, Sugumaran and Bolton (1998) have shown that the unusual oxidative conversion of 2,6-dimthoxyallyl phenol to its quinone methide, catalysed by commercial preparations of mushroom tyrosinase described by Krol and Bolton (1997) was, in fact, due to another enzyme - laccase – a contaminant in the commercial preparation. Korecka *et al.* (1999) proposed that the apparent inhibition of the enzyme inosine monophosphate dehydrogenase by mycophenolic acid glucuronide was attributable to the presence to trace amounts of contaminant mycophenolic acid. No information on the commercial large scale extraction of AMPase from *Aspergillus* species could be obtained from Sigma-Aldrich chemicals, as this was deemed classified. Therefore, potential opportunities for contamination in the commercial extraction could not be identified. However, the lyophilised powder

obtained from the company contained approximately 20% protein with the balance "primarily" diatomaceous earth, suggesting that the preparation was not entirely pure (a fact illustrated by the SDS-PAGE gel of the crude extract, figure 5.20). The protein involved in the LTD of potentials seems to originate from the *Aspergillus* species since a perfusion of *Aspergillus nidulans* homogenate resulted in the same effect on slices. The results may also be relevant to understanding the serious and often fatal neurological complications such as paralysis which accompany invasive cerebral aspergillosis (Pagano *et al.*, 1997; Harris, 1997; Guppy *et al.*, 1998). If the activity of the isolated protein can be modulated it may provide a basis for the treatment of a disease with a high mortality rate (50-100%) and difficult clinical detection. Further work is required to establish if there is a connection between the action of the isolated protein and the pathogenesis of aspergillosis, this may involve investigating its interactions with molecules released during infection e.g. the cytokines.

The study using AMPase extract described in chapter 5 raises the possibility that a novel protein has been identified with potent effects on hippocampal plasticity, which may provide a useful pharmacological tool in the continuing investigations into the neural basis of memory and learning. It is interesting that a homogenate of guinea-pig brain also resulted in a similar profile of depression as the AMPase contaminant. If the protein involved in the observed LTD can be released physiologically then interfering with its effects could result in an enhancement of hippocampal function which could be relevant to drug development. Further work is clearly merited on this putatively novel protein, including complete

structural identification by cloning and sequencing of the gene that codes for its expression.

In conclusion, the investigations described above illustrate the ability of hippocampal CA1 neurones to undergo long-lasting plastic changes and, in particular, highlights the ability of purinergic compounds to modulate synaptic transmission. This in turn raises the possibility that drugs which interfere with the mechanisms by which purines effect plasticity may be useful as modulators of memory and learning. This study also (unexpectedly) raises the possibility that a new family of proteins, related structurally to glucosidases, has been discovered in *Aspergillus* species (and perhaps also endogenously in guinea-pig brain) which may also affect synaptic plasticity. This discovery introduces a new member into the field of plasticity modulation, further complicating an already complex area, and highlights the intricacies surrounding the mechanisms of learning and memory.

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