Modulation of adenosine responses in CA1 area of rat hippocampus

by

Ali Shahraki

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Division of Neuroscience and Biomedical Systems, University of Glasgow

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Abstract

In various conditions like hypoxia, ischemia and brain injury, the extracellular levels of the important neuromodulator, adenosine are increased. This study has considered the effects of adenosine in the CA1 area of hippocampus and its interactions with metabotropic glutamate receptors, nitric oxide and free radicals. Single and paired-pulse stimuli have been applied to the Schaffer collateral fibers in stratum radiatum and extracellular recordings were made from the CA1 pyramidal cell layer of hippocampal slices. We have examined excitatory postsynaptic potentials and paired-pulse interactions to obtain more information about the site and mechanism of these interactions.

The results show suppression of adenosine sensitivity by metabotropic glutamate receptors, explained by a selectively reduced responsiveness to A_1 receptor stimulation, which does not involve any facilitation of A_{2A} adenosine receptors, since it can be obtained in the absence of endogenous adenosine and is not prevented by the A_{2A} receptor blocker ZM241385.

The glutamate receptors involved are of the group I class since the suppression of adenosine sensitivity is produced by ACPD and the group I selective compound DHPG. Furthermore, the effects of DHPG could be prevented by LY367385, a selective antagonist at the $mGlu_{1a}$ subtype of group I receptors. The selective antagonist at $mGlu_5$ receptors, SIB1893, did not prevent the suppression of adenosine sensitivity by DHPG. Blockade of the DHPG/adenosine interaction was also obtained by superfusion with the protein kinase C inhibitor chelerythrine.

Since the suppression of adenosine responses by metabotropic receptor agonists was seen in the paired-pulse paradigm, we conclude that the observed interactions occur at the level of the presynaptic terminals. The interaction with adenosine receptors is not specific, but applies also to a suppression of responses mediated by the $GABA_B$ receptor agonist baclofen.

In conclusion, activation of the $mGlu_{1a}$ subtype of receptor can suppress responses mediated via adenosine A1 receptors, probably by activating protein kinase C. Since the changes induced by metabotropic glutamate receptor agonists last for at least 60 minutes, the data also imply that these interactions could play an important role in changes of synaptic function long after even transient increases of glutamate release in the CNS.

The second part of this study investigated the interaction between adenosine and nitric oxide. Activation of NMDA receptors has been shown to suppress neuronal responses to adenosine in hippocampal slices. Since NMDA receptor activation is known to lead to the generation of nitric oxide (NO), we have now examined whether NO is able to modify neuronal responses to adenosine and mediate the actions of NMDA.

The superfusion of the NO donor, S-nitroso-N-acetylpenicillamine (SNAP) induced a long-lasting potentiation of fEPSP slope and reduced responses to adenosine. The guanylate cyclase inhibitor ODQ prevented the inhibitory effects of SNAP on adenosine responses and also prevented the SNAP-induced LTP, suggesting that the action of NO is mediated through cyclic GMP.

The third part of this study was to examine the effects of another free radical species, superoxide on adenosine responses and fEPSPs to determine whether the

suppression effects on adenosine were specific for NO. Superfusion of a xanthine / xanthine oxidase mixture (X/XO) induced LTP and significantly suppressed responses to adenosine. ODQ and superoxide dismutase (SOD) prevented the inhibitory effects of X/XO on adenosine responses and their induction of LTP, suggesting that oxygen free radicals are involved, probably due to formation of peroxynitrite. The effects of ODQ and SOD on the interaction between adenosine and NMDA indicate that cGMP does not mediate this interaction but oxygen free radicals might contribute. Paired-pulse interactions showed that the suppression of inhibitory effects of adenosine by NO and X/XO are happening presynaptically. Overall we have demonstrated several factors which can modify adenosine sensitivity in the hippocampus. These interactions may contribute to the physiological and pathological regulation of neuronal excitability and plasticity.

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Abbreviations

ACPD	(1S, 3R)-1-aminocyclopentane-1,3-dicarboxylic acid
ACSF	Artificial cerebrospinal fluid
ADA	Adenosine deamianase
Aden	Adenosine
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid
ANOVA	One-way analysis of variance
2AP5	D-2-amino-5-phosphono-pentanoic acid
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CGS21680	2-[p-(2-carboxyethyl) phenylethylamino]-5' -N-ethylcarboxamideoadenosine
CHA	N^6-cyclohexyladenosine
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	Central nervous system
CPA	8-cyclopentyladenosine
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
DHPG -	(RS)-3,5-dihydroxyphenylglycine
fEPSPs	Field excitatory postsynaptic potentials
GABA	γ-Aminobutyric acid
GAD	Glutamate acid decarboxylase
GF 109203X	2-[1-(3-Dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl)maleimide
IB-MECA	N^6 - (3-iodobenzyl) 5'-(N-methylcarbamoyl) adenosine
IMP	Inosine monophosphate
IP_3	Inositol trisphosphate

IPSP	Inhibitory postsynaptic potential
Hb	Haemoglobin
KT5720	(9R,10S,12S)-2,3,9,10,11,12-hexahydro-10hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-di-indolol[1,2,3-fg:3,2,1-kl]pyrrolo[3,4][1,6]benzodiazocine-10-carboxylic acid hexyl ester
L-NAME	N^G-Nitro-L-arginine methyl ester hydrochloride
LTD	long-term depression
LTP	long-term potentiation
LY367385	(S)-(+)-α-amino-4-carboxy-2-methylbenzeneacetic acid
MGluR	metabotropic glutamate receptor
NO	Nitric Oxide
ns	Non-significant
NMDA	N-methyl-D-aspartate
ODQ	1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one
PPD	Paired-Pulse Depression
PPF	Paired-Pulse Facilitation
P.S.	Population spike
SAH	S-adenosylhomocysteine
SC-9	5-chloro-N-(6-phenylhexyl)-1-naphthalenesulphonamide
S. E. M.	Standard Error of the Mean
SIB1893	2-methyl-6-(2-phenylethenyl)pyridine
SNAP	S-nitroso-N-acetylpenicillamine
SOD	Superoxide dismutase
STD	Short-Term Depression
STP	Short term potentiation
X	Xanthine
XO	Xanthine oxidase
ZM241385	4-(2-[7-amino-2-(2furyl)[1,2,4]triazolo[2,3a][1,3,5]triazin-5-ylamino]ethyl)phenol

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1. Introduction

There are several experimental and pathological situations, such as hypoxia or ischemia, in which there is increased release of glutamate from cells. In many of these circumstances, there is also an increase in the extracellular levels of adenosine (Stone & Simmonds, 1991; Latini & Pedata, 2001) which can protect neurons from damage. Interactions between adenosine receptors and ionotropic excitatory amino-acid transmission (via NMDA receptors) have previously been reported from our laboratory (Bartrup & Stone, 1988, 1990; Nikbakht & Stone, 2001), and there have been reports of interactions between adenosine and mGluRs (de Mendonca & Ribeiro, 1997; Budd & Nicholls, 1995). It is not clear, however, whether the reported interactions are specific for adenosine receptors, and whether they occur primarily at presynaptic or postsynaptic sites. Since these interactions may reduce the protective effect of adenosine, we have now extended the earlier work by studying paired-pulse interactions, comparing the effects of adenosine and the $GABA_B$ receptors agonist baclofen, identifying the nature of the adenosine and glutamate receptors involved, and examining the time course of the interactions.

Adenosine has two parallel modulatory roles in the CNS, acting as a homoeostatic modulator and also as a neuromodulator at the synaptic level. Adenosine is known as a substantial metabolite in neurons and other mammalian cell types, participating in the synthesis of nucleic acids, amino acid metabolism and modulation of cellular metabolic status (Stone, 1985). Considering the homeostatic role of adenosine related to the control of cellular metabolism, adenosine has been called a "local hormone" (Arch & Newsholme, 1978) and

"retaliatory metabolite" (Newby, 1984) to summarise the role of adenosine in stressful conditions. In some circumstances the intracellular concentration of adenosine increases from the nanomolar range to micromolar concentrations and adenosine is released to the extracellular medium to maintain the cell metabolism of neighbouring cells (Meghji, 1991).

Adenosine also acts as a neuromodulator in the nervous system. Adenosine modulates the release of neurotransmitters, the postsynaptic responses to transmitters, and the action of other receptor systems. Four adenosine receptors (A_1, A_{2a}, A_{2b}, A_3) belonging to the family of G protein-coupled receptors are known so far and have been cloned and pharmacologically characterized.

There are several experimental and pathological situations, such as hypoxia or ischaemia, in which there is increased release of glutamate from cells. In many of these same circumstances there is also an increase in the extracellular levels of adenosine (Stone & Simmonds, 1991; Latini & Pedata, 2001). Interactions between adenosine receptors and ionotropic excitatory amino acid transmission (via NMDA receptors) have previously been reported from our laboratory (Bartrup & Stone, 1988, 1990; Nikbakht & Stone, 2001), and there have been reports of interactions between adenosine and metabotropic glutamate receptors (de Mendonca & Ribeiro, 1997; Budd & Nicholls, 1995). It is not clear, however, whether the reported interactions are specific for adenosine receptors, and whether they occur primarily at presynaptic or postsynaptic sites. We have now, therefore, extended the earlier work by studying paired-pulse interactions, comparing the effects of adenosine and the $GABA_B$ receptor agonist baclofen, identifying the

nature of the adenosine and glutamate receptors involved, and examining the time course of the interactions.

Adenosine

An historical prespective

The first report about the potent action of adenosine on the cardiovascular system was published more than 70 years ago (Drury & Szent-gyorgi, 1929). In the 1950s further evaluation on the effects of adenosine on mammalian cellular function was done (Green & Stoner, 1950; Winbury et al., 1953; Wolf & Berne, 1956). The hypotensive effects of adenosine were reconfirmed, as was the ability of the purine to modulate pulmonary and CNS function.

Sattin and Rall (1970) discovered the effects of adenosine on the accumulation of cyclic AMP (cAMP) in cortical slices together with the observations that adenosine is released from stimulated neuronal slices. In 1972 the modulatory function of adenosine on neuromuscular transmission was shown by Ginsborg and Hirst. In 1978, Burnstock suggested a division of receptors for adenosine and ATP into P_1 and P_2-purinoceptor subtypes (Burnstock, 1978). This classification was based on four criteria: the relative potencies of ATP, ADP, AMP and adenosine, the selective actions of antagonists, the activation of adenylate cyclase by adenosine but not by ATP, and the induction of prostaglandin (PG) synthesis by ATP but not by adenosine. Thus this classification was proposed: P1 purinoceptors are more responsive to adenosine and AMP than to ATP and ADP, methylxanthines such as theophylline and caffeine are selective antagonists for P_1 receptors while P_2 purinoceptors are more responsive to nucleotides (such as ATP

and ADP) than to adenosine and AMP, and are not antagonised by methylxanthines.

Formation, release and inactivation of adenosine

Adenosine can either be synthesised intracellularly either de novo or retrieved by salvage and transported across the membrane, or can result from the metabolism of ATP. 5´-phosphoribosyl-1-amine (PRA) is the first product in the de novo pathway of adenosine formation. The amino acids glutamine, glycine and aspartic acid present nitrogen and carbon atoms for this pathway. In a subsequent reaction the parent purine nucleotide, inosine-5-monophosphate (IMP) is formed by utilising 5 molecules of ATP. Once IMP is formed it can be converted to the adenosine nucleotide derivative, AMP. Then cytosolic 5-nucleotidase acts on AMP and converts it to adenosine by dephosphorylation (Stone & Simmonds, 1991; Meghji, 1991). Adenosine formation from the salvage pathway occurs with transmethylation of S-adenosylmethionine (SAM) that forms S-adenosylhomocysteine (SAH). SAH is metabolized further by SAH-hydrolase to adenosine and homocysteine (Ueland, 1982). Recycling of hypoxanthine to IMP by the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) is another salvage route for adenosine production (Stone & Simmonds, 1991).

There is no evidence that adenosine is stored in synaptic vesicles and released from nerves similar to the other neurotransmitters (White & Hoehn; 1991). Much of the released adenosine arises from the intracellular adenosine that can pass bidirectionally via a facilitated-diffusional transporter. Adenosine accumulates during ischemia, hypoxia, head injury, and seizures (Hagberg et. al., 1987; Phillis,

1990; Bell et. al., 1998). Increased adenosine formation is due to the imbalance between energy supply and demand (Bardenheuer & Schrader, 1986). Potassium, veratridine and electrical stimulation have been shown to release adenosine both invivo and invitro from various brain regions (White & Hoehn, 1991). Furthermore, excitatory amino acids can also induce adenosine release from brain slices (Latini & Pedata, 2001). It seems that the adenosine released during excitatory amino acid (EAA) activation will provide much protection against EAA mediated excitotoxicity in the CNS.

Some of the adenosine is produced from the extracellular metabolism of released adenine nucleotides, in particular ATP (Wieraszko et al., 1989; Cunha et. al., 1996). ATP is co-stored with either noradrenaline in the sympathetic nerves or acetylcholine (Ach) in cholinergic synaptosomes (Morel & Meunier, 1981;White, 1988). This ATP could provide a source of adenosine by the action of ectonucleotidase.

Adenosine can be inactivated by uptake into neurons and neighbouring cells by a facilitated diffusion through the nucleoside transporters. This process is regulated by the concentration gradient for adenosine. After being taken up, it may be phosphorylated by adenosine kinase or deaminated by adenosine deaminase. In general, adenosine is phosphorylated at low adenosine concentrations and deaminated at high adenosine concentrations (Arch & Newsholme, 1978; Meghji, 1991). Adenosine deaminase also exists in an extracellular form. Therefore, adenosine may be deaminated extracellularly without prior need to be taken up.

Adenosine receptors

Classification

Adenosine receptors are members of the G protein-coupled receptor family and mediate the different physiological effects of adenosine. The broadest classification of adenosine receptors was proposed by Burnstock (1978). Based on this classification purine receptors were divided into P_1 receptor subtypes preferring nucleosides such as adenosine and P_2 receptor subtypes preferring adenine nucleotides such as ATP and ADP. The P_1 receptor was subdivided into A_1 and A_2 subtypes according to whether they increased or decreased adenylate cyclase activity. Activation of A_1 adenosine receptors inhibited adenylate cyclase and adenosine 3,5´cyclic monophosphate (cyclic AMP) accumulation, while activation of A_2 adenosine receptors stimulated such activity (Van Calker, et. al., 1979). A_1 receptors are preferentially activated by N^6-substituted adenosine analogues such as L-N^6-phenylisopropyladenosine (L-PIA), whereas A_2 receptors have preference for 5´- substituted compounds such as 5´-N-ethyl-carboxamidoadenosine (NECA) (Burnstock, 1989). In general A_1 receptors are sensitive to low concentrations of adenosine derivatives in the nanomolar range, while micromolar concentrations are required for A_2 receptor activation (Reddington & Lee, 1991).

A_2 adenosine receptors have been subdivided into two subtypes, A_{2a} with relatively high affinity for adenosine and A_{2b} with lower affinity to adenosine.

In the early 1990s a new subtype of adenosine receptor, the A_3 receptor was cloned and characterized (Meyerhof, et al., 1991; Zhou, et al., 1992). 2-chloro-N^6-(3-iodobenzyl)-5´-N-methylcarboxamidoadenosine (L-IB-MECA) is the selective

agonist for A_3 adenosine receptors with activation of this receptor mediating the inhibition of adenylate cyclase (Abbracchio et al., 1998).

Distribution

Information about the distribution of adenosine receptors has been revealed from the studies of biochemical and physiological roles of adenosine and radioligand binding studies. Radioligand binding studies have shown that A_1 receptors predominantly existed in the molecular layer of the cerebellum and in the CA1 and CA3 regions of hippocampus (Goodman & Snyder, 1982; Lee & Reddington, 1986). Moderate A_1 receptor levels are found in the thalamus, caudate-putamen, septum, and cerebral cortex. Consistent with radioligand binding studies, Northern blot analysis of mRNA from various tissues has shown A_1 adenosine receptor message in kidney, heart, testis and epididymal fat (Stehle et al., 1992).

Studies on A_2 adenosine receptors have detected that both A_{2a} and A_{2b} receptors are expressed in brain. A_{2a} receptor expression is limited to striatum, nucleus accumbens and olfactory tubercle whereas A_{2b} receptor mRNA is distributed throughout the brain (Stehle et al., 1992).

The more sensitive RT-PCR technique (reverse transcription polymerase chain reaction) has shown that A_{2a} receptor mRNA can be detected in all brain regions (Dixon, et al., 1996). There are high level of A_{2a} receptors in striatum and nucleus accumbens, and lower level in the cortex, amygdala, olfactory tubercles, hippocampus, hypothalamus, thalamus, and cerebellum. For example hippocampus contains approximately 25% of the striatal density of A_{2a} receptors (Cunha, et al., 1994a).

Northern blotting experiments have shown reasonable expression of the A_{2a} receptors in heart, lung, thymus and epididymal fat, whereas A_{2b} receptor message is demonstrated in the caecum, lung, intestine, urinary bladder, with lower distribution in lung (Stehle et al., 1992).

The expression of A_{2a} receptors with other neurotransmitter binding sites has been found in some areas. For instance co-localization of A_{2a} receptors and dopamine D_2 receptors occur on striatopallidal neurons on the same cell and they can influence each other's function (Johansson, et al., 1997).

Ligand binding Studies

Agonist radioligands

[^{3}H] Adenosine and [^{3}H]2-choloroadenosine were the early radioligand compounds that were used to identify adenosine receptors by radioligand binding technology (Schutz & Burgger, 1982). Due to some difficulties such as degradation by tissue enzymes and unstability, these ligands have been replaced with the N^6-substituted ligands, N^6-cyclohexyladenosine (CHA) and R-N^6-phenylisopropyl adenosine (R-PIA) (Williams, 1987). Both ligands have been widely used for several years as A_1-selective agonists. More selective N^6-substituted agonists, including N^6-cyclopentyladenosine (CPA) and 5´-chloro-N^6-(2-endo-norbornyl) adenosine (CENBA) have been synthesized (Linden, 1991). CPA, CENBA and 2-chlorocyclopentyladenosine (CCPA) are the most potent and selective agonists for the A_1 adenosine receptor (Miller & Hsu, 1992).

The A_2 receptor has been further subdivided into A_{2a} and A_{2b} subclasses. Due to its high affinity for A_{2a} receptor, 5´-N-ethylcarboxamidoadenosine (NECA) has

been used as a radioligand for A_{2a} receptors. However, NECA also binds with high affinity to A_1 receptors, and to achieve selectivity for A_{2a} receptors in NECA binding assays, it is necessary to block A_1 receptors by addition of a highly selective A_1 agonist such as CPA or the antagonist (Williams, 1987). 2-[P-(2-carboxyethyl)phenylethylamino]-5-N-ethylcarboxamidoadenosine (CGS 21680), a new ligand with high affinity and selectivity for A_{2a} over A_1 receptors has been developed. CGS 21680 and similar radioligands can directly label A_{2a} receptor in rat brain without the need to block binding activity at the A_1 receptor (Jarvis et al., 1989). Useful radioligands for the A_{2b} receptors have not yet been developed.

Changes at two sites in adenosine structure at the N^6- and 5´- positions are required to obtain high potency agonist for A_3 adenosine receptors. N^6-(3-iodobenzyl)-adenosine-5´-N-methyl-uronamide (IB-MECA) was the first highly potent and selective A_3 receptor agonist both in vivo and in vitro (Von Lubitz, et al., 1994; Stambaugh, et al., 1997; Jacobson, et al., 1997). It is 50-fold selective in binding assays for rat A_3 vs A_1 or A_{2a} receptors.

Substituion of both N^6- and 5´ positions in adenosine increased the A_3 receptor affinity and selectivity. Therefore, 2-chloro-N^6-(3-iodobenzyl)-adenosine- 5´-N-methyluronamide (2CI-IB-MECA) is 1400-fold selective for rat A_3 vs either A_1 and A_{2a} receptors, respectively (Jacobson, 1998).

Antagonist radioligands

8-phenylxanthines were developed as potent A_1-selective antagonists. Problems with some antagonists were their poor aqueous solubilities and variable activities as cyclic nucleotide phosphodiesterase inhibitors. Replacing the phenyl with

cyclopentyl on the 8-position of xanthine results in compounds with improved affinity, aqueous solubility and selectivity for A_1 receptors. 1,3-Dipropyl-8-cyclopentyl xanthine (DPCPX) is a useful compound as a highly selective A_1 antagonist (Liden, 1991). A keto derivative of DPCPX, KFM19 has been reported to have increased aqueous solubility (Ijzerman & Van Galen, 1990).

The most potent A_2 receptor antagonist 9-chloro-2-(2-furanyl)[1,2,4]triazolo[1,5-c]quinazolin-5-amine (CGS 15943) is a nonxanthine antagonist, originally synthesized as a benzodiazepine antagonist. CP-66 713 is the only compound that combines high potency and greater than 10-fold selectivity for A_2 receptors (Sarges et al., 1990).

Attempts to find selective xanthine-based antagonists for A_3 receptors failed because xanthine tended to bind only weakly to A_3 receptors. Different molecules were adopted in the design of selective A_3 receptor antagonists. For example, 3-ethyl5-benzyl-2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS-1191) a trisubstituted 1,4-dihydro-6-phenylpyridine analogue, has been found to inhibit radioligand binding at the human and rat A_3 receptors. In addition, MRS-1191 antagonised the effects of the A_3 receptor-selective agonist IB-MECA on inhibition of adenylate cyclase (Jacobson et al., 1997; Jacobson, 1998).

Naturally phenolic derivatives and other compounds such as triazolonaphthyridine, thiazolopyrimidine and a derivative of the triazoloquinazoline CGS 15943 have also been reported as high-affinity A_3 receptor selective antagonists (Jacobson et al., 1996; Kim et al., 1996).

Peripheral actions of adenosine

Investigations on several organ systems have revealed that many physiological processes are modulated by extracellular adenosine acting at A_1 receptors. These include the inhibition of lipolysis and stimulation of glucose uptake in adipocytes and modulation of neurotransmitter release in the central nervous system (Olsson & Pearson, 1990).

The cardiovascular effects of adenosine have been intensively studied, including negative inotropy and chronotropy that are mediated by the A_1 receptor subtypes. A_{2a} receptor activation in vascular beds initiates smooth muscle relaxation, resulting in vasodilation and drop in blood pressure (Olsson & Pearson, 1990). Activation of A_{2a} receptors on platelet membranes elevates intracellular cAMP levels, resulting in the inhibition of platelet aggregation (Palmer & Stiles, 1995). Adenosine is able to inhibit neutrophil activation, subsequently decreasing neutrophil adhesion to endothelial cells and preventing capillary plugging (Miller & Hsu, 1992).

Exogenous adenosine affects all aspects of renal function such as renal blood flow, glomerular filtration rate, renin secretion and transmitter release from renal efferent nerves. Since many of these effects are antagonized by alkylxanthines and mimicked by adenosine analogs acting as adenosine receptor agonists, they are produced by adenosine receptors (Churchill & Bindani, 1990). The capability of adenosine to induce bronchoconstriction has been demonstrated in the respiratory system (Churchill & Bindani, 1990).

Central actions of adenosine

Adenosine is a modulator that has generally inhibitory effects on neuronal activity. Activation of adenosine receptor by adenosine in brain tissue leads to inhibitory effects that seem to be mediated by both adenosine A_1 and A_{2a} receptors. The involvement of adenosine in diverse neural phenomena, include regulation of sleep, neuroprotection, regulation of seizure susceptibility, locomotor effects, analgesia, mediation of the effects of ethanol and chronic drug use has been demonstrated (Schwarz-Bloom et al., 1999; Fiorillo & Williams, 2000; Mendelson, 2000; Dunwiddie & Masino, 2001).

Different adenosine receptors may be involved in the neuroprotective function. At least three cellular mechanisms are mediated via A_1 receptors, including inhibition of transmitter release (in particular glutamate), hyperpolarization of neurons, and directly inhibition of certain kinds of Ca^{2+} channels. These mechanisms could reduce excitotoxicity by decreasing Ca^{2+} entry, which is thought to be a key step in excitotoxic damage. Also by reducing metabolic rate, adenosine helps to preserve ATP stores that are essential for pumping the Ca^{2+} out of the cell (Dunwiddie & Masino, 2001).

Presynaptic effects of adenosine

Under most experimental conditions, the effect of adenosine or adenosine receptor agonists is an inhibition of neuronal activity mediated by A_1 receptor activation. A_1 receptors are linked to inhibition of the release of the most classical neurotransmitters, including glutamate, gamma-amino butyric acid (GABA), norepinephrine, acetylcholine, 5-hydroxytryptamine (5-HT), and dopamine

(Dunwiddie, 1985; Greene & Hass, 1991). Inhibitory effects are generally seen on excitatory glutamergic systems. Inhibitory modulation of inhibitory systems (e.g. GABA) is less frequently observed, therefore the net effect of adenosine receptor activation in almost all regions of the brain is to reduce excitability. It was recognised that the inhibitory action of adenosine in the CNS predominantly results from its presynaptic action (Lupica, et al., 1992). This inhibitory modulation of transmitter release depends on a direct inhibition, via G-proteins, of mainly N-type calcium channels, although this is still the subject of debate (Wu & Saggau, 1994a; Ribeiro, 1995). Further inhibitory mechanisms of adenosine effect seem to operate in motor neurons. Silinsky et al. (1989) have shown that intracellular calcium buffers do not prevent the inhibitory effects of adenosine on acetylcholine release (Silinsky, 1989). This and other studies suggest that adenosine inhibits transmitter release from motor nerves by reducing the responsiveness of some component of the secretory apparatus to Ca^{2+}. Adenosine by inhibiting phospholipase, could inhibit neurotransmitter release by reducing intraneuronal Ca^{2+} mobilization and by attenuating the activation of PKC.

It is also possible that adenosine modulates potassium currents in presynaptic terminals. In support of this possibility, it has been demonstrated that blockers of potassium channels (e.g. 4-aminopyridine) are able to prevent the inhibitory effects of adenosine on transmitter release in peripheral sympathetic nerves (Stone, 1981a) and in glutamate-releasing fibers in the hippocampus (Scholfield & Steel, 1988).

Adenosine receptors may also enhance neurotransmitter release, but these actions are less common than the inhibition of transmitter release (Cunha et al., 1994b).

The development of A_{2a} receptor agonists and antagonists has provided tools to find the role of A_{2a} receptors in the control of neurotransmitter release in different brain regions as well as in the peripheral nervous system. In striatal cholinergic nerve terminals, at the neuromuscular junction and in hippocampus, GABAergic nerve terminals, modification of neurotransmitter release via A_{2a} receptors is decreased by inhibitors of adenylate cyclase (Cunha & Ribeiro, 2000a; Gubitz et al., 1996). Protein kinase C (PKC) or phospholipase inhibitors are able to inhibit the effects of A_{2a} receptors, whereas protein kinase A (PKA) inhibitors slightly inhibit or do not change the effects of A_{2a} receptor activation. Further investigation has demonstrated that activation of A_{2a} receptors triggers cAMP accumulation but a parallel signaling system via PKC is also activated by presynaptic A2a receptor activation (Cunha & Ribeiro, 2000b).

A_{2a} receptors can also modify neurotransmitter release via P-type calcium channels where its activity seems to be modified by both PKC and PKA. However, it is not clear whether both pathways can modulate this same target or just P-type calcium channels are the main target for PKC pathway (Cunha, 2001).

Postsynaptic effects of adenosine

Postsynaptic mechanisms comprise activation of K^+ channels and the control of Ca^{2+} fluxes (Fredholm & Dunwiddie, 1988; Allgaier et al., 1991). Postsynaptic inhibition is mediated via activation of A_1 adenosine receptors and an increase in K^+ conductance which seems to be mediated via ATP- sensitive K^+ (KATP) channels (Li & Henry, 1992).

Pertussis toxin (PTX) is known to ADP-ribosylate and inactivate several types of G-proteins. Postsynaptic effects of adenosine could be blocked by PTX treatment in vivo (which caused adenylate cyclase inhibition, increased K^+ conductance) while presynaptic effects have not been affected by PTX (Gilman, 1987).

Post-receptor mechanisms

A cascade of intracellular events occurs after stimulation of adenosine receptors that transduce extracellular signals to effector responses such as ion channels, adenylate cyclase and phospholipase C via G-proteins.

G-proteins

Intermediatory guanine nucleotide-binding proteins (G proteins) can modulate ion channels directly via physical interactions between G-protein subunits and the channel protein, or indirectly via second messengers and protein kinases. G proteins are not a single protein but consist of a heterotrimeric protein with alpha, beta and gamma subunits (Dascal, 2001). The α-subunit directly regulates adenylyl cyclase and other enzymes and effectors. The β/γ subunit complex is required for efficient coupling of the α-subunit to receptors, and influences the rate of GDP dissociation from the GTP/ GDP binding site. The number of heterotrimeric G protein subunits identified in the sequence of human genome study consists of 7 alph, 5 beta and 14 Gamma subunits (Albert & Robillard, 2002).

Many neurotransmitter receptors act via G-proteins including adenosine receptors. A_1 receptor-mediated responses are coupled via pertussis toxin sensitive G-protein

to different effectors including adenylate cyclase, phospholipase C, Ca^{2+} channels, Cl^- channels and K^+ channels (Dascal, 2001).

Adenylate cyclase and the cyclic AMP system

Adenylyl cyclase (AC) converts ATP to cyclic AMP. This enzyme is regulated by hormones, neurotransmitters and other regulatory molecules through its interaction with G-proteins and receptors. A total of nine isoforms of adenylyl cyclase have been cloned in mammals and they are numbered from AC1-AC9 (Hanoune, et al., 1997).

Adenosine A_1 and A_2 receptors were initially characterized on the basis of their effect on adenylyl cyclase and production of cAMP. Adenosine A_2 receptor agonists increase the rate of ATP metabolism to cAMP by enhancing the activity of adenylyl cyclase through its action with stimulatory G protein. Conversely, adenosine A_1 receptor agonists inhibit the rate of ATP metabolism to cAMP by attenuating the activity of adenylyl cyclase, through its action with the inhibitory G protein.

A_1 and A_2 adenosine antagonists including caffeine and other methylxanthines, block both the stimulatory and inhibitory actions of adenosine on adenylyl cyclase (Sattin & Rall, 1970; Hanoune, et al., 1997).

Diacylglycerol/ phosphatidyl inositol

Inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) are generated from the breakdown of the phospholipid, phosphatidylinositol-4,5-bisphosphate (PIP2) due to action of the enzyme phospholipase C. These compounds act as second messengers mediating the effects of many neurotransmitters including acetylcholine, 5-hydroxy tryptamine and noradrenaline and form the

internalization of the extracellular signal. Adenosine does not seem to have any direct effect on phosphatidylinositol turnover. However, adenosine and several analogues have been reported to mediate the regulation of phosphatidylinositol (PI) responses to other neurotransmitters such as histamine and noradrenaline (Morgan, 1991).

Glutamate receptors

Glutamate receptors (GluRs) mediate most of the excitatory neurotransmission in the mammalian central nervous system (CNS). They also participate in most aspects of normal brain function including cognition, memory, and learning (Headley & Grillner, 1990). Glutamate and related excitatory amino acids are toxic to central neurons. Excessive activation of GluRs during stress to the brain, such as ischemia, head trauma and epileptic seizure lead to the death of central neurons. The glutamate neurotoxicity may also be involved in the producing of various neurodegenerative disease (Choi & Rothman, 1990). Therefore, GluRs participate in both the physiology and pathology of brain functions.

The GluRs have been classified into two major classes, ionotropic and metabotropic receptors. The ionotropic receptors (iGluRs) contain cation-specific ion channels, and include three subclasses of receptor: α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), kainate (KA) and N-methyl-D-aspartate (NMDA) receptor channels. The metabotropic glutamate receptors (mGluRs) are coupled to GTP-binding proteins (G-proteins) and modulate the production of intracellular messengers (Ozawa, et al., 1998).

Ionotropic glutamate receptors

AMPA receptors

Glutamate activates three major types of ionotropic receptor namely, AMPA, KA, and NMDA receptors. Since neither agonists nor antagonists could distinguish between AMPA and KA receptors, they were often, collectively referred to as non NMDA receptors. These AMPA receptors were initially called quisqualate receptors. Since quisqualate could also act on mGLUR and AMPA affected ionotropic receptors, they were more specifically renamed AMPA receptors. Mammals express four AMPA receptor subunits GLUR1-GLUR4, that are distributed throughout the CNS (Danysz et al, 1995).

The AMPA receptor has at least three separate binding sites at which agonists or antagonists can act: glutamate binding, desensitization and intra-ion channel binding sites. AMPA receptor channels are permeable only to Na^+ and K^+ and almost impermeable to Ca^{2+} in central neurons. However, it was found that AMPA receptors displayed a substantial permeability to Ca^{2+} and a strong inward rectification in cultured rat hippocampal neurons. In contrast, AMPA receptor displayed a slight outward rectification and low permeability to Ca^{2+} in certain neurons like xenopus oocytes (Ozawa et al., 1998).

Kainate receptors

Kainate is a potent agonist of the AMPA receptor, but it also activates a distinct class of iGluRs, i.e. Kainate-preferring receptors (KA). A family of KA receptors has been cloned and five subunits, termed GluR5, GluR6, GluR7, KA1, and KA2 have been identified. KA receptors on the presynaptic terminals at hippocampal CA_1 synapses negatively regulate the synaptic release of glutamate, but KA

enhances transmitter release from synaptosomes prepared from the hippocampal CA_3 region. Therefore, modulating actions of presynaptic KA receptors may vary from synapse to synapse (Ozawa, et al., 1998).

NMDA receptors

These receptors are coupled to high conductance cationic channels permeable to K^+, Na^+ and Ca^{2+} and are modulated positively by polyamines (spermine and spermidine) and glycine, which binds to a specific, strychnine-insensitive site. The NMDA channels are blocked in a use and voltage dependent manner by Mg^{2+}. This means that NMDA receptors are only activated following depolarisation of the postsynaptic membrane by, for example, AMPA receptor activation, which relieves their voltage – dependent blockade by Mg^{2+}. This biophysical property and the high Ca^{2+} permeability of NMDA receptors make them inherently suitable for their role in mediating synaptic plasticity such as that underlying learning processes. Similar to Mg^{2+}, uncompetitive NMDA receptor antagonists such as ketamine, dextromethorphan, memantine, phencyclidine and MK-801 block the NMDA channel in the open site (Collingridge & Watkins, 1994).

NMDA receptors also exist in multiple forms, probably as pentameric assemblies, which have different physiological and pharmacological properties and are distributed throughout the brain. So far two major subunits NMDAR1 and NMDAR2, have been cloned from the rat CNS. Alternative splicing has revealed eight functional isoforms for the NMDAR1 subfamily (NMDAR1A-

NMDAR1H). The NMDAR2 subfamily consists of four individual subunits, NMDAR2A-NMDAR2D (Collingridge & Watkins, 1994).

Investigations have shown that the NMDA response is potentiated by glycine in cell cultures of central neurons. Later, it was revealed that glycine is not only a strong potentiator of the NMDA receptor but is also required to open the NMDA receptor channel, so that glycine has a co-agonist role for this receptor. It has been proven that the NMDA receptor response in culture and in brain slice preparations are due to existing endogenous glycine (Vyklicky et al., 1990).

The NMDA receptor is characterized with three properties: (a) It remains blocked at resting potentials by Mg^{2+}. After depolarisation ionic currents occur through the receptor. (b) Considerable amounts of extracellular Ca^{2+} enter the intracellular space during the opening of the receptor (c) NMDA receptor-mediated neurotransmission occurs slowly and lasts for a long period. These properties make the NMDA receptor a molecular apparatus that can identify the coincidence of the presynaptic depolarization and induce in the postsynaptic cell an adequate amount of the second messenger ion Ca^{2+} that will initiate synaptic plasticity (Kaczmarek et al., 1997). There is much evidence to suggest that AMPA and NMDA receptors are colocalized and activated by the transmitter glutamate, released into the synaptic cleft. Therefore, it seems that the AMPA receptor has a low affinity and becomes unbound quickly after the clearance of the transmitter, while the NMDA receptor has a greater affinity, causing a long binding (Ozawa et al., 1998).

Metabotropic glutamate receptors

The mGluRs represent a large family of G protein-coupled excitatory amino acid (EAA) receptors that activate multiple second messenger systems leading to various signal transduction processes and are distinct from the iGluRs that form ion channels. The mGluRs can be classified into three groups based on their sequence homology, transduction mechanism and pharmacology (Nakanishi, 1994; Conn & pin, 1997). Group I mGluR receptors ($mGluR_1$ and $mGluR_5$) stimulate phospholipase C, resulting in an increase in phosphoinositide turnover and the subsequent activation of protein kinase C and Ca^{2+} release from internal stores. Group II ($mGluR_2$ and $mGluR_3$) and III ($mGluR_{4,6,7,8}$) receptors inhibit adenylyl cyclase, leading to the reduction of cAMP formation in the cell (Baskys, 1992; Conn & Pin 1997).

The mGluRs play an important role in the regulation of synaptic transmission via their modulation of ion channels and ionotropic glutamate receptors in diverse neuronal cell types (Pin & Duvoisin, 1995; Conn & Pin, 1997). For example activation of mGluRs induces an inward current that is possibly mediated via sodium channels in rat dopamine mesencephalic neurons and decreases currents through voltage-dependent calcium channels in rat hippocampal (Swartz & Bean, 1992) and striatal neurons (Stefanic et al., 1994). Different subtypes of potassium channels may also be modulated by mGluRs in several types of neuronal cells (Conn & Pin, 1997). Activation of mGluRs has been shown to potentiate AMPA-mediated responses in neurons of the tractus solitarius nucleus (Glaum & Miller, 1993) and NMDA-mediated currents in hippocampal neurons (Aniksztejn et al., 1992; Behnisch & Reymann, 1993; Fitzjohn et al., 1996) but to attenuate NMDA-

mediated responses in neostriatal neurons (Colwell & Levine, 1999). These results indicate that the activation of mGluRs has diverse effects on the modulation of iGluR as well as of ion channels in different cell populations, and thus may play a unique role in regulating synaptic transmission in different brain regions (Conn & Pin 1997; Glaum & Miller, 1993; Colwell & Levine, 1999).

The mGluRs also have a role in various forms of synaptic plasticity. It has been reported that the induction of long-term potentiation (LTP) in the hippocampus requires synaptic activation of mGluRs (Bashir et al., 1993; Riedel et al., 1995), and hippocampal LTP is greatly reduced in mGluR1 knock-out mice (Aiba et al., 1994). MGluRs are also involved in the induction of long-term depression (LTD) at parallel fiber-Purkinje cell synapses in the cerebellum (Linden & Connor, 1993; Hartell, 1994). Activation of group I mGluRs with the selective agonist (RS)-3,5-dihydroxyphenylglycine (DHPG) induces LTD in the CA1 region of hippocampal slices in Mg^{2+} free medium or during blockade of GABA-mediated inhibition (Palmer et al., 1997).

Group I metabotropic glutamate receptors

Group I includes $mGluR_1$ and $mGluR_5$ which are positively coupled to PLC and lead to an increase in diacylglycerol (DAG) and inositol trisphosphate (IP_3) and in some cases to an activation of adenyl cyclase (Conn, et al., 1997).

Over recent years the group I mGluRs have been extensively studied in experimental animals, leading to an appreciation of their great importance in the CNS. Structures including the hippocampus, cortex, thalamus and cerebellum and involvement of these receptors in a variety of disorders including epilepsy,

ischemia, pain and neurodegenerative diseases is beginning to emerge. Cloned mGluRs expressed in heterologous mammalian systems have been used to characterize the activity of selective compounds on mGluR subtypes. Quisqualate is the most potent agonist of these receptors followed by ibotenate and glutamate. The group I mGluR specific agonists are 3,5-dihydroxyphenylglycine (DHPG), 3-hydroxyphenylglycine (3-HPG) and trans-azetidine-2,4-dicarboxylate (t-ADA), which are devoid of activity in other mGluR groups. A new phenylglycine derivative, (RS)-Z-chloro-5-hydroxyphenylglycine (CHPG), has been recently reported to activate only $mGluR_5$, but not $mGluR_1$ in transfected cells. This is the first compound available to distinguish pharmacologically the two members of the group I, although it is not very potent (Bordi & Ugolini, 1999).

Distribution of group I mGluRs in the mammalian brain

Studies of the distribution of the mRNA for $mGluR_1$ by in situ hybridization revealed an abundant expression of this receptor in hippocampal neurons and cerebellar Purkinje cells. In the cerebellum, $mGluR_1$ is distributed in cells and dendrites of Purkinje, stellate and some Golgi cells. No $mGluR_5$ is found in the cerebellum, except a small amount in the granule cell layer but not in the Purkinje cells. Other areas of expression of $mGluR_1$ are the olfactory bulb, the amygdala, the thalamus and the basal ganglia. The presence of this receptor in these area has inspired study of the potential role of group I mGluRs in extrapyramidal motor diseases like Huntington`s chorea or Parkinson's disease (Bordi & Ugolini, 1999; Rouse et al., 2000).

In the hippocampus, $mGluR_1$ are concentrated in the postsynaptic membrane at the periphery of synaptic junctions, but ionotropic glutamate receptors are concentrated within the synaptic junction. Because of the special segregation of ionotropic receptors and mGluRs, it has been suggested that they may respond differently to glutamate stimulation; the ionotropic receptors respond to glutamate under normal presynaptic stimulation, whereas the perisynaptic $mGluR_1$ is involved in excitatory responses evoked only by strong presynaptic stimulation. The postsynaptic metabotropic receptors situated at the periphery would appear to have a role of delayed activation (Conn, et al., 1997).

The hippocampus of the rat and the human contains many neurons that express $mGluR_5$. In the CA_1 region of the rat $mGluR_5$ is present in both pre and postsynaptic membranes. Most CA_3 pyramidal and granule cells express both $mGluR_5$ and $mGluR_1$ (Ozawa, et al., 1998). Immunocytochemistry studies have shown while $mGluR_5$ is the most abundant group I mGluR in CA_1 pyramidal cell, these cells express $mGluR_1$ mRNA as well (Berthele et al., 1998; Ferraguti et al, 1998). Therefore, it is possible that both $mGluR_1$ and $mGluR_5$ are involved in regulating CA_1 pyramidal cell function.

Neurophysiological role of group I mGluRs

Substances that activate group I mGluRs cause neuronal depolarization and excitation while antagonists of these receptors inhibit depolarization and excitation. In addition, activation of these receptors can modulate synaptic transmission by regulating GABA release or by interfering with iGluRs. Recent

evidence has also shown the importance of group I mGluRs in modulating other brain neurotransmitter systems.

Neuronal excitability

Application of the broad spectrum mGluR agonist ACPD produces various physiological effects, both inhibitory and excitatory. Group I mGluRs seem to involve primarily postsynaptic excitatory effects. In the CA_1 area of the hippocampus, activation of mGluRs with ACPD leads to depolarization, increase of input resistance, and reduction in spike frequency adaptation (Desai et al., 1992; Mannaioni et al., 1999). The increase in neuronal excitability caused by activation of group I mGluRs is produced primarily by modulation of potassium channels. ACPD induces net inward currents by inhibiting K^+ conductances in neurons in hippocampus, amygdala, and hypothalamus. This effect is blocked by group I mGluR selective antagonists (Gereau & Conn, 1995). In the CA_3 region of the hippocampus the reduction of K^+ conductance in response to mGluR activation is not mediated by PKC or PKA and it exhibits a voltage sensitivity which could be important in physiological processes such as long lasting changes in cellular excitability or persistent modification of synaptic efficacy (Bordi & Ugolini, 1999).

Activation of group I mGluRs can also modulate currents through N-type, L-type and other voltage dependent calcium channels. Quisqualate inhibits N-type Ca^{2+} channels in hippocampus and cortex, where it is mediated by G-protein and does not involve protein kinases, but it facilitates L-type channel function in the

granule cells of the cerebellum via G-protein mechanism not sensitive to pertussis toxin (Bordi & Ugolini, 1999).

Modulation of synaptic transmission

Activation of presynaptic mGluRs causes a widespread reduction of glutamatergic transmission in the CNS. This action is determined almost exclusively by presynaptic group II and group III mGluRs. Reduction of glutamatergic transmission attributed to group I mGluRs has been observed in the hippocampus, at the level of the synapses between Schaffer collaterals and CA_1 pyramidal cells (Gereau & Conn, 1995; Conn & Pinn, 1997). In this region inhibition of fEPSPs is induced by DHPG or quisqualate and is accompanied by an increase in paired-pulse facilitation, a common and reliable test of presynaptic mechanisms. $MGluR_5$ probably mediates this effect because there is immunochemical evidence for the presynaptic expression of $mGluR_5$ in CA_1 neurons (Gereau & Conn, 1995; Romano et al., 1995).

Modulation of neurotransmitters

It is now becoming evident that mGluRs regulate different neurotransmitters in a number of brain structures. Activation of mGluRs reduces GABAergic synaptic transmission in the hippocampus, the striatum, the thalamus, and the olfactory bulb. The identity of the mGluR group responsible for this effect is not certain, but evidence for a presynaptic activation of group II or III receptors is emerging.

In the hippocampus, GABAergic interneurons can be activated by mGluR either pre or postsynaptically. In the CA_3 region activation of group I mGluRs,

presumably located on the somato-dendritic membrane of GABA interneurons, enhances excitability. In contrast, group II mGluRs are located on the inhibitory terminals and reduce GABA release. In the CA_1 region, group I mGluRs are involved in modulating transmission from GABAergic interneurons onto pyramidal cells. Analysis of spontaneous inhibitory postsynaptic currents (IPSCs) suggests that these receptors are located presynaptically (Vezina & Kim, 1999).

In the hippocampus DHPG (specific group I mGluR agonist) is able to attenuate the inhibitory effects of adenosine A_1 receptor activation and this attenuation occurs via a PKC-dependent mechanism. This interaction may be relevant to the pathology that occurs after hypoxia, where adenosine is an endogenous protective substance (Ozawa, et al., 1998).

In cultures of astrocytes, $mGluR_5$ is associated with the regulation of β-adrenergic receptor function. The agonist DHPG, but not agonists for group II or III mGluRs, potentiates cAMP accumulation induced by β-adrenergic stimulation. This effect is independent of intracellular Ca^{2+} or the PKC pathway, but can contribute to the opening of Ca^{2+} channels and modulate neuronal activity by a feedback process to neurons (Balazs, et al., 1997).

Activation of mGluRs has been shown to modulate NMDA and AMPA receptor-mediated membrane currents in a number of brain areas and cell types (Colwell & Levine, 1999; Fitzjohn et al., 1996; Colwell & Levine, 1999). Potentiation of NMDA responses by ACPD was reported in the CA_1 region of the hippocampus, cerebellum, neocortex, striatum and spinal cord. In some studies, however, ACPD attenuates NMDA responses and this discrepancy can be explained by the NMDA receptor composition in different cell types (Shen et al., 1995).

ACPD also potentiates AMPA receptors in spinal cord and visual cortex but not in the hippocampus. The potentiation of excitatory responses is explained by an mGluR-mediated depolarization that increases cell excitability. Inhibition of K^+ currents is implicated in these mGluR-induced depolarizations, resulting in an increase in cell input resistance. PKC, which is triggered by activation of group I mGluRs, has been proposed to mediate the potentiation of iGluR responses (Bordi & Ugolini, 1999).

Group II metabotropic glutamate receptors

Group II mGluRs consist of $mGluR_2$ and $mGluR_3$ and are negatively coupled with the adenylyl cyclase system. Group II mGluRs are potently activated by (25,1`R, 2`R,3`R)-2-(2,3-dicarboxy cyclopropyl) glycine (DCG-IV) (Pin & Duvoisin, 1995).

Most of the physiological roles and pathological implications of group II receptor subtypes have been associated with their presynaptic localization. Indeed, group II mGluRs inhibit transmitter release from cortico-striatal terminals, a pathway that affects the functions of a large percentage of caudatal neurons (Ozawa, et al.,1998).

Group II agonists are also thought to exert a role in protection against glutamate excitotoxic neuronal cell death. A possible mechanism for group II agonist-mediated neuroprotection involves the release of trophic factors such as transforming growth factor β (TFG-β), a known endogenous neuroprotective agent upon stimulation of mGluR3 subtypes localized in astrocytes (Pellicciari & Costantino, 1999).

Group III metabotropic glutamate receptors

Group III mGluRs consist of $mGluR_{4,6,7,8}$ that are negatively coupled with the adenylyl cyclase system. The major agonist of this group is a phosphonate analogue of glutamate, L-2-amino-4-phosphonobutyric acid (L-AP4) (Pin & Duvoisin, 1995). Group II and III mGluRs are coupled to the inhibition of adenylyl cyclase. Group II show strong inhibition of forskolin-induced cAMP formation. All these effects of group II and III are strongly inhibited by PTX, suggesting that the G-proteins involved in the coupling are of the Gi family.

Group III mGluRs participate in the modulation of glutamate release and the prevention of excitotoxicity in different cell types (Gereau & Conn, 1995). A potential neuroprotective role of group III receptors has been postulated in cortical cultures, although the involvement of specific subtypes still need clarification. Recently mice lacking $mGluR_4$ showed altered spatial learning and memory, indicating a role for the presynaptically expressed $mGluR_4$ in the process of learning (Romano, et al., 1995).

Metabolism of glutamate

Overactivation of glutamate receptors is harmful and glutamate is toxic in high concentrations. To reduce damage Glutamate is removed from the extracellular space, to reduce its action on glutamate receptors which exist on most of the cellular elements (dendrites, nerve terminals, neuronal cell bodies & glial cells). There is no evidence for any extracellular enzyme that can metabolise glutamate. The rapid route to remove glutamate from extracellular space is cellular uptake. Simple diffusion is also a substantial way for glutamate removal from synaptic

cleft. After being taken up into astrocytes, glutamate may be metabolised via two different pathways: (a) it may be converted to glutamine in an ATP-dependent route and released to the extracellular fluid. Glutamine is taken up via glutamine transporters by neurons which is reconverted to glutamate and is reused as transmitter (the glutamate-glutamine cycle)(Fonnum 1993; Laake et al., 1995; Danbolt, 2001). (b) It may be metabolised to α-ketoglutarate by deamination or transamination. α-ketoglutarate may be converted to succinate, fumarate and malate in the tricarboxylic acid cycle. Malate may be further metabolised in the tricarboxylic acid cycle, or it may be decarboxylated to pyruvate and lactate which has been shown in vitro (Mckenna et al., 1996; Danbolt, 2001). Lactate is transported from astrocytes to extracellular fluid that can enter neurons.

GABA receptors

γ-aminobutyric acid is the most widely distributed inhibitory neurotransmitter in the central nervous system and is released via many cells, particularly by interneurons. GABA receptors are divided into two classes: ionotropic receptors (e.g. $GABA_A$, $GABA_C$) and metabotropic receptors (e.g. $GABA_B$). The $GABA_A$ receptors directly act on a chloride ionophore and are composed of modulatory binding sites for benzodiazepines, barbiturates and neurosteroids. Structurally $GABA_C$ receptors seem to be very similar to $GABA_A$ receptors. The $GABA_A$ responses are blocked competitively by bicuculline and non-competitively by picrotoxinin. $GABA_C$ receptors are activated selectively by cis-4-aminocrotonic acid (CACA) and are blocked competitively by [(1,2,5,6-tetrahydropyridine-4-yl) methylphosphonic acid] (TPMPA) and non-competitively by picrotoxinin.

$GABA_C$ receptors do not respond to bicuculline, $GABA_A$ modulatory drugs and baclofen (Chebib & Johnston, 1999; Bormann, 2000).

$GABA_B$ receptors are coupled to Ca^{2+} and K^+ channels via G- proteins. These receptors are activated by β-chlorophenyl GABA (baclofen) and are resistant to drugs that modulate $GABA_A$ receptors (Bowery et al., 2000). $GABA_B$ receptors are present at both postsynaptic and presynaptic sites. Activation of $GABA_B$ receptors in the CA_3 area of the hippocampus activates postsynaptic K^+ conductances via pertussis toxin-sensitive G-proteins (Thompson & Gahwiler, 1992). In addition to their postsynaptic actions $GABA_B$ receptors are involved in the presynaptic inhibition of neurotransmitter release. In the hippocampus, $GABA_B$ receptors mediate inhibition of both GABAergic and glutamatergic transmission. It seems that both N-type and non N-type calcium channels contribute to the action of $GABA_B$ receptors on inhibitory transmission (Doze et al., 1995; Bonanno et al., 1997).

Nitric oxide

Activation of NMDA receptors suppresses neuronal responses to adenosine in hippocampal slices. Since NMDA receptor activation leads to the generation of nitric oxide (NO) and superoxide, we have considered whether these can modify neuronal responses to adenosine and mediate the actions of NMDA. Nitric oxide (NO) is formed from the amino acid arginine by the enzyme nitric oxide synthase (NOS). Since NO is a labile free radical, it is not synthesized in advance and stored in synaptic vesicles as are other known neurotransmitters. Therefore, it must be synthesized on demand and acts immediately. Its effects are brief because

NO is a highly reactive free radical that remains for less than 10 seconds before it reacts with oxygen and water to form inactive nitrates and nitrites (Zhang and Snyder, 1995). NO plays a role in long term potentiation. In the CA1 region of the hippocampus the induction of most forms of LTP by high frequency stimulation is dependent on Ca^{2+} entry into the post synaptic neuron by NMDA receptor activation (Pellmar & lipinski, 1992) and partly causes an increased in presynaptic release (Malinow, 1991; Bolshalcov & Siegelbaum, 1995). NO may act as a retrograde messenger in LTP. It activates soluble guanylyl cyclase (SGC) that generates the intracellular second messenger cyclic GMP (cGMP). Interestingly, tetanic stimulation of hippocampal slices results in large rises in cGMP that are blocked by NOS inhibitors (Chetkovich et al., 1993; Lu et al., 1999). Cytosolic cGMP acts on the activity of various receptor proteins, including the cGMP-dependent protein kinase (cGK). There is some evidence that indicate cGK plays a role in the induction of LTP such as: cGK inhibitors block LTP, and cGK activators can facilitate LTP in response to rather weak tetanic stimulation (Zhuo et al, 1994; Arancio et al., 1995). However, studies on mice lacking the genes for cGK1, cGK2 or both have not shown any changes in LTP although LTP could be reduced by using NOS inhibitors and NMDA antagonists, respectively (Kleppisch et al., 1999).

Protein kinase C (PKC) is also one of the necessary biochemical steps for the induction of LTP (Malinow et al., 1989). An additional mechanism that might participate in the activation of PKC during the induction of LTP is an oxidative mechanism consisting of reactive oxygen species (ROS) such as superoxide anion (O_2^{-o}). The level of superoxide increases during the induction of LTP (Bindokase

et al., 1996; Klann et al., 1998; Knapp & Klann, 2002). It has been observed that NMDA receptor activation in the CA1 region of hippocampal slices causes increased production of superoxide and with increasing superoxide levels, it has been shown to increase PKC activity (Palumbo et al., 1992). Furthermore, recent studies show that superoxide dismutase (SOD), which removes the superoxide, impaired LTP (Gahtan et al., 1998; Thiels et al., 2000). Another interesting target for NO in LTP may be a cytosolic ADP-ribosyltransferase (ADPPT) (Brune and Lapetina, 1989). An NO-stimulated ADPRT may ribosylate a GTP binding protein or other enzyme, which alters ion channel activity or changes the Ca^{2+} sensitivity of the neurotransmitter release process such that more neurotransmitter is released during LTP (Schuman & Madison, 1994). Certain studies have shown that LTP can be prevented by extracellular application of ADP-ribosyltransferase inhibitors (Schuman et al., 1992). ADP-ribosylation is occluded partly in hippocampal slices by LTP induced by tetanic stimulation but is not reproduced by cGMP (Williams et al., 1992; Duman et al., 1993).

Synaptic plasticity in hippocampus

The neurophysiological basis of learning and memory is thought to involve changes in the efficiency of synapses between neurons in a network. Two models of activity-dependent synaptic plasticity are long-term potentiation (LTP) and long-term depression (LTD).

Long-term potentiation

The phenomenon of long-term potentiation of synaptic transmission consists of potentiation of the size of synaptic potentials for periods in excess of one hour. LTP has three properties; input specificity, cooperativity and associativity. It seems that the strengthening of the synapses is controlled by the coincident activity of both pre and postsynaptic neurons. Collingridge et al., (1988) showed that NMDA receptors are essential for the induction of LTP. It was shown that during high frequency stimulaion the evoked EPSPs were summated, and the Mg^{2+} block of the NMDA receptor channel was removed. Since the activated NMDA receptors are permeable to Ca^{2+} (Ascher & Nowak, 1988) and LTP induction could be blocked by intracellular injection of the Ca^{2+} chelator EGTA (Lynch et al., 1983), it was assumed that the Ca^{2+} entry through the NMDA receptor was the trigger for LTP. Activation of mGluRs result in an increase in NMDA receptor-mediated currents and it was suggested that activation on mGluRs might reduce the threshold for inducing LTP (Ben-Ari et al., 1992).

Long-term depression

Because of the short history of LTD (Lynch et al., 1977) not much is known about the mechanisms involved in this form of synaptic plasticity. LTD was reversibly blocked by the NMDA receptor antagonist D-AP5, indicating that LTD is NMDA receptor–dependent and suggesting that Ca^{2+} entry through this receptor is necessary for its induction (Dudek & Bear, 1992). It was demonstrated that LTD is saturable, repeated low frequency stimulation (1 Hz) caused a decrease that saturated at 50% (Dudek & Bear, 1992). It has also been revealed that synapses

depressed by low frequency stimulation can be potentiated by high frequency stimulation and vice versa (Ngezahayo et al., 1997).

Paired-pulse interactions

Synaptic transmission in the mammalian brain can be regulated in different ways. Two examples of this regulation are short-term plasticity of synapses consisting of paired-pulse facilitation (PPF) and paired-pulse depression (PPD).

Paired-pulse facilitation

Paired-pulse facilitation (PPF) is a form of short term plasticity in which the synaptic response to the test pulse (second stimulus) given rapidly after conditioning pulse (first stimulus) is enhanced in comparison to the conditioning pulse. The best known hypothesis to explain the mechanism of PPF is the presynaptic accumulation of Ca^{2+} after an initial stimulation. Indeed, the first pulse induces a small Ca^{2+} influx that cannot trigger transmitter release itself, but remains in the terminal for several hundred milliseconds. This residual Ca^{2+} combined with Ca^{2+} entering during the second pulse leads to increasing transmitter release (Katz & Miledi, 1968; Zucker, 1989; Thomson, 2000). The Ca^{2+} concentration in the presynaptic nerve terminal affects the probability of neurotransmitter release. Different studies have suggested that the second of two paired action potentials at the neuromuscular junction induces release with higher probability than the first one as result of a small but lasting increasing of the intracellular Ca^{2+} concentration in the axon terminal. The residual Ca^{2+} can enhance this probability by increasing the fusion of synaptic vesicles with the

presynaptic membrane and enhancing the number of quanta released by an action potential (Katz & Miledi, 1968; Debanne et al., 1996). Neuromodulators or physiological processes that affect transmitter release probability affect paired-pulse ratio. Increasing the external Mg^{2+}/Ca^{2+} ratio (Davies and Collingridge, 1993; Lambert & Wilson, 1994; Wilcox & Dichter, 1994) and applying adenosine (Lupica et al., 1992; Higgins & Stone, 1995) decrease the probability of release of neurotransmitter during the first action potential and increase the probability of release by the second action potential causing higher responses. In the hippocampus, PPF of excitatory synaptic potentials in areas CA1 and CA3 is observed when large numbers of axon are stimulated (Creager et al., 1980; Manabe et al., 1993), whereas under condition in which the release probability is elevated by decreasing the Mg^{2+}/Ca^{2+} ratio the PPF is reduced (Nathan et al., 1990; Kahle & Cotman, 1993).

Paired-pulse depression

When two action potentials of similar amplitude are elicited in the presynaptic cell, the amplitude of the second EPSP and/or population spike is smaller than the first one, a phenomenon called paired-pulse depression (PPD).

The mechanism underlying the PPD of inhibitory synaptic transmission is unknown. One hypothesis is activation of GABAB autoreceptors (Deisz & Prince, 1989), but studies of Wilcox and Dichter (1994) has shown that GABAB receptor antagonists did not attenuate PPD of unitary IPSPs in pairs of cultured hippocampal neurons. An alternative presynaptic mechanism for PPD is a transient decrease in the quantal content caused by depletion of the readily

releasable vesicle pool by the first stimulus (Stevens and Wang, 1995; Debanne et al., 1996).

Postsynaptic mechanisms such as desensitisation of $GABA_A$ receptors (Alger, 1991) and a reduced driving force due to intracellular accumulation of Cl^- and/or extracellular accumulation of K^+ (McCarren and Alger, 1985) might also explain the PPD.

In hippocampal slices paired-pulse inhibition has been reported at interstimulus intervals less than 40 ms and in the present experiments at less than 20 ms while PPF is observed at longer time intervals (Higgins & Stone, 1995; Lynch et al. 1983; Nikbakht & Stone 2000).

Anatomy of hippocampus

For three reasons, the hippocampus is one of the most studied areas of central nervous system. First, it has an easily identifiable structure at the gross and histological levels. Secondly, it has been recognized that hippocampus plays a basic role in learning and memory. Patients who have had bilateral hippocampal removal have suffered from a permanent loss of encoding new information into long-term memory. Thirdly, the hippocampus is very susceptible to seizure and is very vulnerable to the effects of ischemia and anoxia (Johnston & Amaral, 1998).

In the brains of mammals the hippocampus is a bilaterally represented structure that appears as a ridge extending into the lateral ventricle. The outer surface of the hippocampus is composed of myelinated fibers and seems white and is called the alveus. The hippocampal formation can be divided into the Ammon's horn, the dentate gyrus, subiculum and the entorhinal cortex. Based on Golgi preparations,

the anatomist Lorente de No divided Ammon's horn into four subfields: (Cornu Ammonis) CA_1 to CA_4. CA4 is no longer used because it referred to the region occupied by the polymorphic layer of the dentate gyrus. CA_1 is equivalent to region superior and CA_2 and CA_3 fields are equivalent to the region inferior. CA_1 and CA_3 are separated by a narrow transitional zone, CA_2.

The dentate gyrus contains round, tightly packed neurons called granule cells. The dentate gyrus consists of three layers: the granule layer, which is the principal layer, the molecular layer, (above the granule layer) and a polymorphic cell layer below the granule cell layer (Isaacson, 1987).

In all CA fields below the alveus is the stratum oriens, which contains the basal dendrites of the pyramidal cells. The cell bodies are clearly visible under a microscope as a dark band. Below this, is the stratum radiatum consisting of the apical dendrites of the pyramidal cells and the Schaffer collaterals, which are collateral branches from axons of pyramidal cells in the CA_3 region.

The functional organisation of the hippocampus has been described in terms of a trisynaptic circuit. Information that flows from the neocortex into and out of the hippocampus travels in a unidirectional manner through this trisynaptic pathway. The entorhinal cortex is considered to be the starting point of the circuit. The fibers of the perforant pathway arise from pyramidal cells of entorhinal cortex. They pass through the adjoining subicular complex and terminate in the molecular layer of the dentate gyrus to form the first synapse in the synaptic pathway with the dendrites of granule cells in the dentate gyrus. The axons of these granule cells are called mossy fibres.

They leave the dentate gyrus and terminate on the proximal dendrites of the CA_3 pyramidal cells. CA_3 pyramidal cells produce branched axons and send their axons out of the hippocampus in the fornix. A branch of this axon "the Schaffer collateral" forms strong synaptic connections with dendrites in area CA_1. Axons from CA_1 pyramidal neurons project heavily to neurons in the subicular complex. There is also a major projection from subicular complex to entorhinal cortex to close the circuit (Witter, 1989).

Aim

The aim of this work was to obtain more information about the site and mechanism of interactions between mGluRs and adenosine receptors in the CA1 area of rat hippocampal slices and to identify the nature of the adenosine and mGluRs involved. Then we have examined whether the effects of $GABA_B$ receptor-mediated responses were also modulated by activation of mGluRs. The time course of the interactions was investigated and a paired-pulse paradigm was used to examine the actions of compounds on presynaptic terminals.

The activation of NMDA receptors can lead to the generation of NO. We have therefore examined whether NO is able to modify neuronal responses to adenosine, since such an action could contribute to the modulation of LTP and to the suppression of adenosine responses by activation of NMDA receptors. For comparison the actions of another free radical species, superoxide were investigated. These aims are represented in figure 1.1 which summarise a hypothetical scheme which this thesis will address.

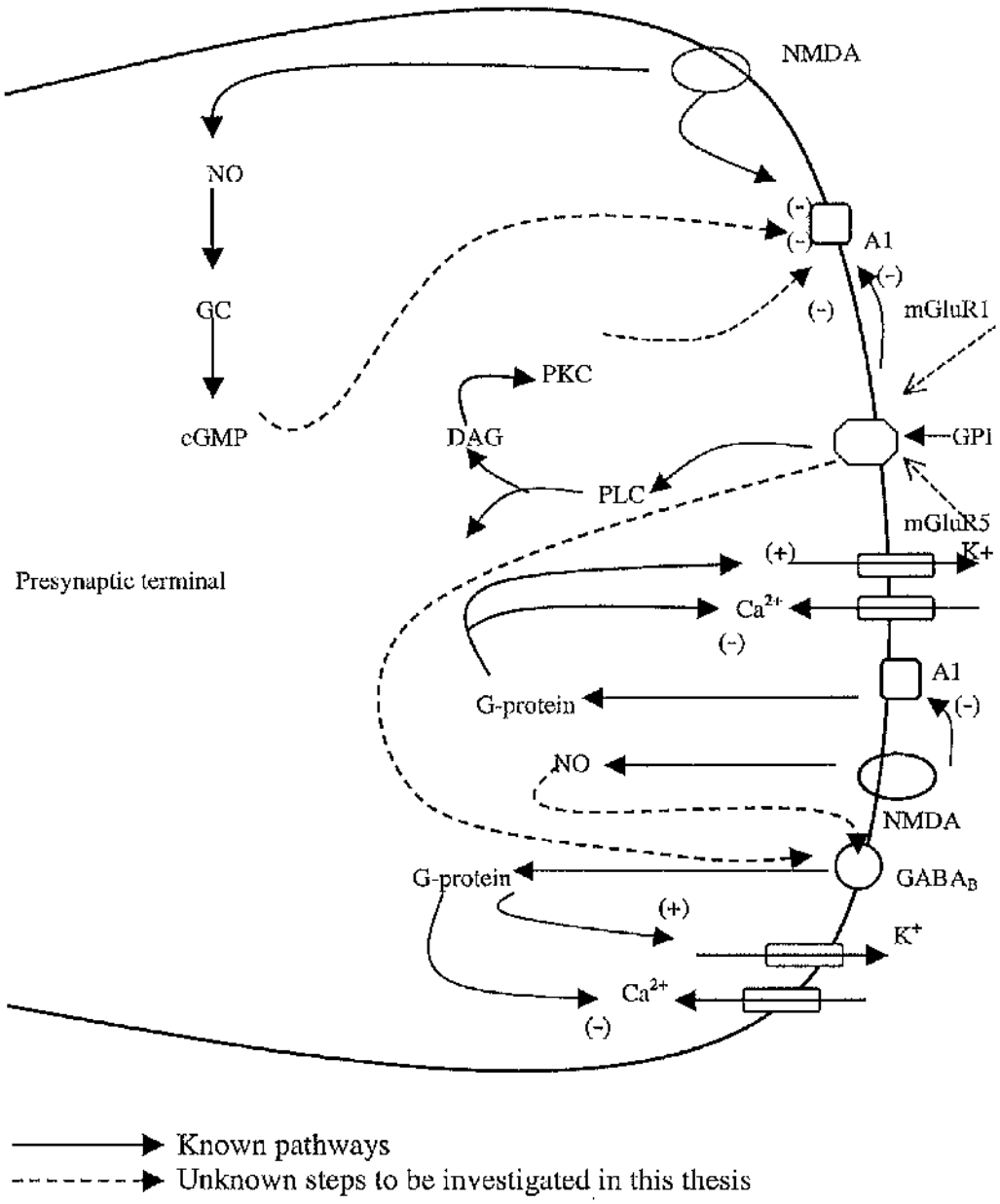

Figure.1.1. This figure illustrates the known pathways and unknown steps to be investigated in this thesis including the nature of the metabotropic glutamate receptor (mGluR) modulating adenosine responses, the role of phospholipase C (PLC) and the role of NO in modulating adenosine and $GABA_B$ responses by NMDA receptors.

2. Material and methods

Hippocampal slices

In vitro slices of mammalian CNS tissue were shown to be physiologically viable for many hours by Yamamoto and McIlwain (1966). Since then the slice preparation has been extensively used for the study of synaptic transmission and electrophysiological experiments in the mammalian central nervous system. Yamamoto (1972) developed slices of hippocampus that showed good electrical activity following intracellular recording (Yamamoto, 1972; Kerkut & Wheal, 1981).

Advantages of the in vitro slice preparation over in vivo techniques include:

1- Technical simplicity: it is relatively easy to record via extracellular, intracellular and whole-cell patchclamp compared with in vivo experiments.

2- Control over the condition of the preparation. For example there is no blood pressure to monitor and no heart rate to stabilize. Also the variable effects of anaesthesia on the intact animal are not a problem in the slice preparation.

3- Improved visualization of tissue. The stimulating and recording electrodes can be positioned at the desired site under low magnification.

4- Direct access to extracellular space. The ionic composition of the extracellular environment can easily be manipulated.

5- Known concentrations of drugs can be applied to the synapse by addition to the bathing solution.

The advantage of the slice preparation led increasing numbers of neuroscientists to adopt this technique. However, there are also some disadvantages with brain slice preparations including:

1- Slices are separated from their normal sensory input and have no motor output. Long feedback loops and some excitatory and inhibitory pathways are lost. Some afferents such as noradrenaline, serotonin and acetylcholine cannot be activated in hippocampal slices in vitro (Dunwiddie et al., 1983).
2- Inevitably, some tissue will be damaged by the slicing process.
3- The tissue is subject to an anoxic period during preparation (Lipton & Whittingham, 1979).
4- The ionic environment does not mimic exactly the normal extracellular conditions in vivo.

Preparation of hippocampal slices

Male Wistar rats (140-200g) were anaesthetised with an intraperitoneal injection of urethane solution (1.3 $g.Kg^{-1}$). The animals were then killed by cervical dislocation, decapitated using a guillotine, and the brain rapidly removed to ice-cold and oxygenated artificial cerebrospinal fluid (ACSF). The cerebellum was removed and the two cerebral hemispheres were separated with a scalpel blade. The hippocampi were then dissected free of surrounding tissue using spatulas and cut transversely into slices 450μm thick using a McIlwain tissue chopper. The slices were maintained at room temperature on a filter paper in a petridish containing a small amount of fresh gassed ACSF. The petridish was kept in an

incubation chamber at room temperature in an atmosphere of 95% O_2 and 5% CO_2 bubbled through water for at least 1 hour prior to recording.

Composition of ACSF

The composition of the bathing medium, ACSF was (in mM): KH_2PO_4 2.2, KCl 2, $NaHCO_3$ 25, NaCl 115, $CaCl_2$ 2.5, $MgSO_4$ 1.2, and glucose10. It was gassed with a mixture of 95% O_2 and 5% CO_2 to yield a pH of 7.4.

Bath superfusion and application of drugs

Following incubation individual slices were transferred to a 1 ml recording chamber using a fine brush and continuously superfused with fresh ACSF by gravity at a rate of 3-4 ml/min. The slices were maintained submerged with a thin metal bar and bubbled with 95% O_2 and 5% CO_2 and heated using a thermostatically controlled water bath to yield a temperature between 28°C and 30°C. Drugs were added to the perfusion medium and applied to slices for a minimum of 10 minutes.

Stimulating and recording

The slices were orthodromically stimulated using a concentric bipolar electrode (Clark Electromedical Instruments Ltd) positioned in the stratum radiatum at the CA_1/CA_2 junction for orthodromic activation of pyramidal cells. Pulses of 300μs duration were delivered at 0.05 Hz. Evoked responses were amplified (X1000, Neurolog) and filtered from DC to a high frequency of 1 KHz. A PC computer was used to digitise, analyse and store the responses via a CED (Cambridge

Electronic Design) micro 1401 interface and signal software (CED). Single glass microelectrodes were used for recording that filled with 1M NaCl with a tip diameter approximately 2-4 μm and resistance approximately 2-5 MΩ.

Paired stimuli were delivered through the same electrode. Extracellular population spike potentials and population excitatory postsynaptic potentials (EPSP) were recorded from the CA_1 pyramidal cell layer or the stratum radiatum respectively. Slices with P.S.<3mV amplitude or fEPSPs<1mV were not studied. Slices were allowed to settle for 1 hour, after which recording were made for 20 minutes within 15% of the starting potential and only if the potential remained stable during that 20 minutes was the experiment continued.

Data analysis

Responses were quantified as the amplitude of the population spike in mV (difference between the peak of the positive-going synaptic potential and the peak of the negative going population spike) and / or the slope of the negative arm of the population EPSP.

Results were considered as the percentage of the spike size in the presence of drug compared with control size. The control was calculated as the mean of the 6 observations immediately preceding start of drug perfusion expressed as a percentage of the mean of 30 observations recorded over the previous 10 minutes. The effect of added agents was taken as the mean of the final 6 observations made during the 10 minutes period of perfusion. When paired-pulse interactions were examined, inhibition or facilitation was expressed as the percentage change in the second response of a pair compared with the first response.

Statistical test

Results are presented as mean ± standard error of mean (s.e.m) for n experiments, and the statistical significance of any difference assessed by a paired or unpaired Student's t-test. The significance of the difference between more than two means obtained in the same slices was calculated with repeated measure ANOVA followed by the Student-Newman-Keuls test. P values less than 0.05 were considered statistically significant.

Chemical agents and drugs

Constituents of ACSF were of Analar grade and dissolved in distilled water to prepare fresh ACSF. Baclofen, (RS)-3,5-dihydroxyphenylglycine (DHPG), and chelerythrine were dissolved in distilled water to obtain stock solution. Adenosine deaminase was dissolved directly in ACSF to give the desired concentration. Trans-(±)-1-amino-(1S, 3R)-cyclopentanedicarboxylic acid (ACPD), kynurenic acid, xanthine, xanthine oxidase, and (S)-(+)-α-amino-4-carboxy-2-methylbenzeneacetic acid (LY367385) were firstly dissolved in 100mM NaOH then distilled water was added to give a stock solution. Dimethyl sulphoxide (DMSO) was used to dissolve 2-methyl-6-(2-phenylethenyl)pyridine (SIB), D-2-amino-5-phosphono-pentanoic acid (2AP5), 8-cyclopentyladenosine (CPA), 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3a][1,3,5] triazin-5-ylamino]ethyl)phenol (ZM241385), S-Nitroso-N-acetylpenicillamine (SNAP), 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), 2-[1-(3-Dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl)maleimide (Gf 109203X), and (9R,10S,12S0-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-di-indolo[1,2,3-fg:3′,2′,1′-Kl]pyrrolo[3,4-1][1,6]benzodiazocine-10-

carboxylic acid, hexyl ester (KT 5720) with the final concentration of DMSO never exceeding 0.1%. 5-chloro-N-(6-phenylhexyl)-1-naphthalenesulfonamide (SC-9) was dissolved in ethanol. The final concentration of ethanol in perfusing solution was less than 0.1%. The solvents DMSO and ethanol were tested at their final concentration that had no effect on the potential size.

A majority of drugs were obtained from Tocris Cookson Ltd. Analar grade compounds were obtained from BDH chemicals Ltd. UK. Adenosine deaminase, xanthine, xanthine oxidase, and haemoglobin were obtained from Sigma chemical Co. Ltd. ZM241385 and CPA were purchased from Research Biochemicals International. A summary of drug solutions is given in table 2.1.

Drug	Source	Solvent	Stock solution	Used concentration
ACPD	Tocris	NaOH 100mM	100mM	20,50 and 100μM
ADA type VII	Sigma	ACSF	0.1IU.ml^{-1}	0.1IU.ml^{-1}
Adenosine	Sigma	H_2O	10mM	1,2,5,10,20,30 and 50μM
Antifoam A	Sigma	ACSF	50PPM	50PPM
DL-AP5	Sigma	NaOH 100mM	50mM	50μM
Baclofen	Sigma	H_2O	5mM	2μM
Chelerythrine Chloride	Tocris	H_2O	10mM	5μM
CPA	RBI	DMSO	10mM / 20μM	20nM
DHPG	Tocris	H_2O	10mM	10μM
GF109203X	Tocris	DMSO	5mM / 100μM	100nM
Hb	Sigma	ACSF	50μM	50μM
KT5720	Tocris	DMSO	0.2mM	100nM
LY367385	Tocris	NaOH 100mM	100mM	100μM
L-NAME	Tocris	H_2O	100mM	100μM
NMDA	Sigma	H_2O	10mM	4μM
ODQ	Tocris	DMSO	10mM	10μM
SC-9	Tocris	Ethanol	10mM	40μM
SIB1893	Tocris	DMSO	20mM	40μM
SNAP	Tocris	DMSO	100mM	100μM
SOD	Sigma	ACSF	120Uml^{-1}	120U.ml^{-1}
Xanthine	Sigma	NaOH 100mM	100mM	1000μM
Xanthine oxidase	Sigma	ACSF	0.02U.ml^{-1}	0.02U.ml^{-1}
ZM241385	RBI	DMSO	10mM / 50μM	50nM

Table.2.1. Showing the compounds used for experiments with their sources, solvents, stock solutions and used concentrations.

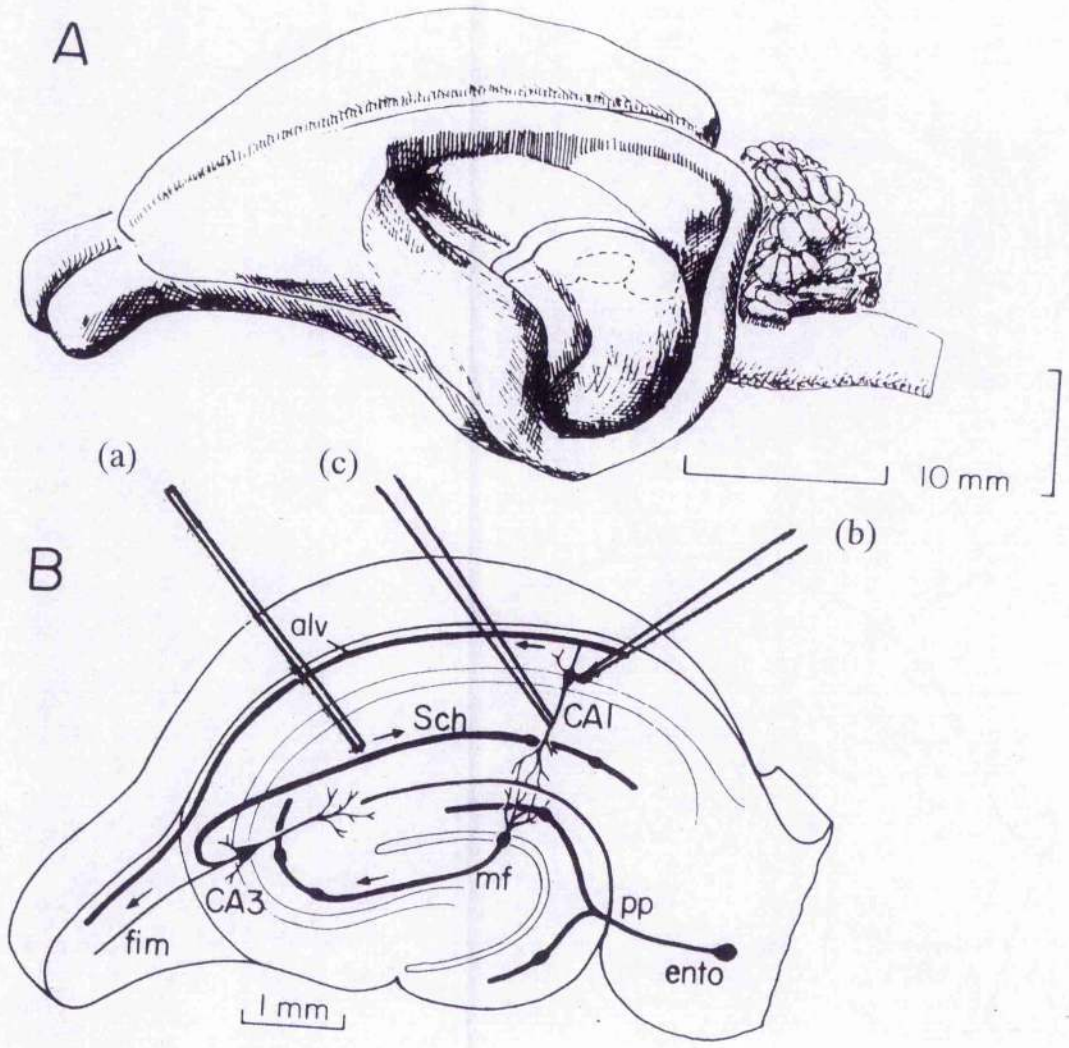

Figure2.1. Diagram showing anatomy of the hippocampal formation and the placement of the electrodes. (a) The stimulating electrode was positioned in the stratum radiatum at the CA_1/CA_2 junction. Orthodromic population spikes and excitatory postsynaptic potentials were recorded from the stratum pyramidal and stratum radiatum respectively using electrodes (b) and (c). (adapted from Andersen et al., 1971).

3. Results

Interactions between adenosine and metabotropic glutamate receptors

The effect of adenosine on orthodromically evoked population spikes

In normal ACSF, adenosine concentration–dependently decreased or abolished the amplitude of orthodromic popoulation spikes evoked from CA1 regions in hippocampal slices. Concentration response curve for these effects are shown in Figure 3.1. The depressant effect of adenosine was reversible and washed out in 5 to 10 minutes.

The concentration – dependent effect of ACPD on population spikes

The effect of ACPD on orthodromic evoked population spikes was investigated in hippocampal slices. ACPD was perfused for 10 minutes, and the effect was concentration dependent. 20μM ACPD did not induce any change in the size of population spike potentials (Fig. 3.2), whereas at 50μM induced a depression of 38.18% ± 4.43 (Fig. 3.3) and at 100μM it induced a substantial depression (reducing by 88.2% ± 1.9), and its effect reversed to normal during 10-15 minutes washing (Fig. 3.4).

The effect of ACPD at 20, 50 and 100 μM was also tested on the paired-pulse population spikes at 10, 20 and 50 ms interstimulus intervals. ACPD 20 μM did not modify the paired-pulse ratio at any of the tested intervals (Fig. 3.5). Application of ACPD at a concentration of 50μM produced a significant facilitation at 20 ms (Fig. 3.6) and at 100μM induced a small facilitation but not significant at 10, 20 and 50 interstimulus intervals (Fig. 3.7).

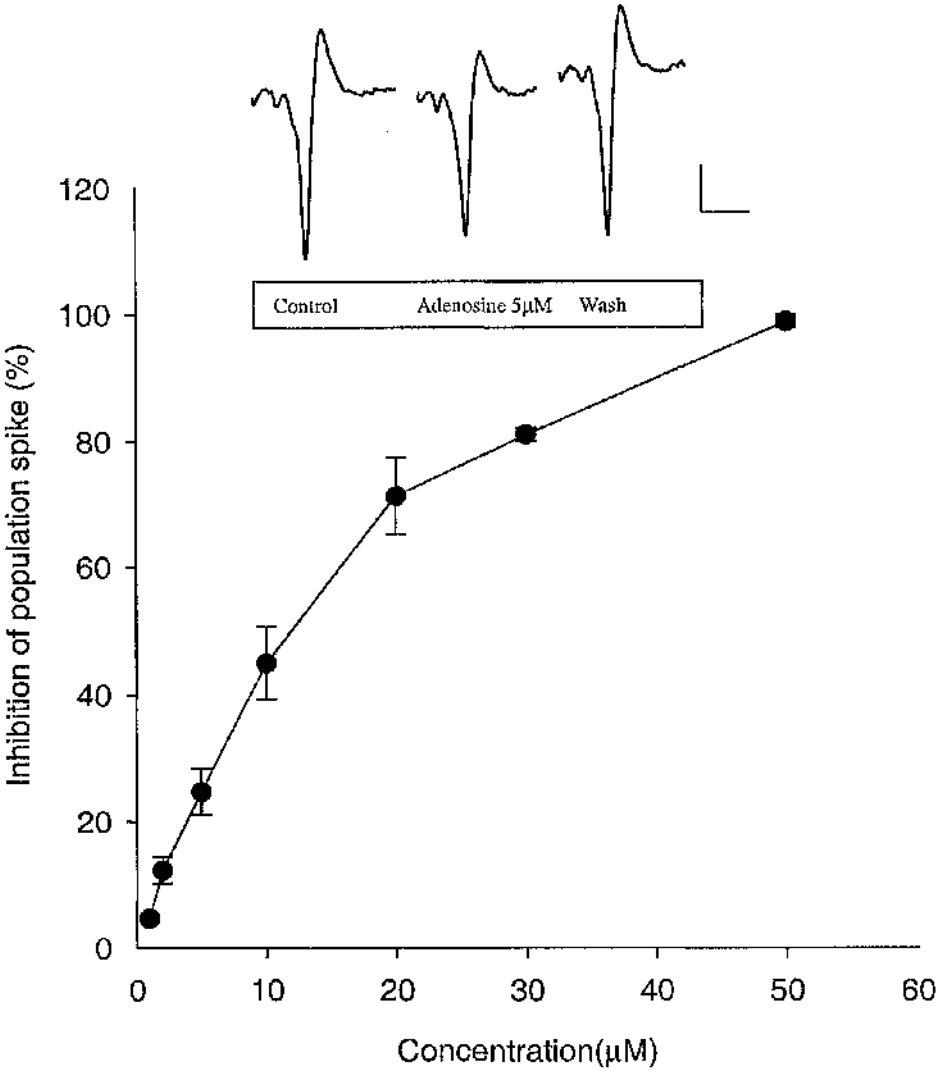

Figure 3.1. Concentration response curve for the inhibitory effect of adenosine on orthodromic population spikes evoked from the CA1 area in hippocampal slices. The stimulus strength was adjusted to 70% of maximum. Each point presents the mean ± S.E.M for n = 5 slices. Sample records represent Control, adenosine 5μ, and wash: Calibration 1mV, 10ms.

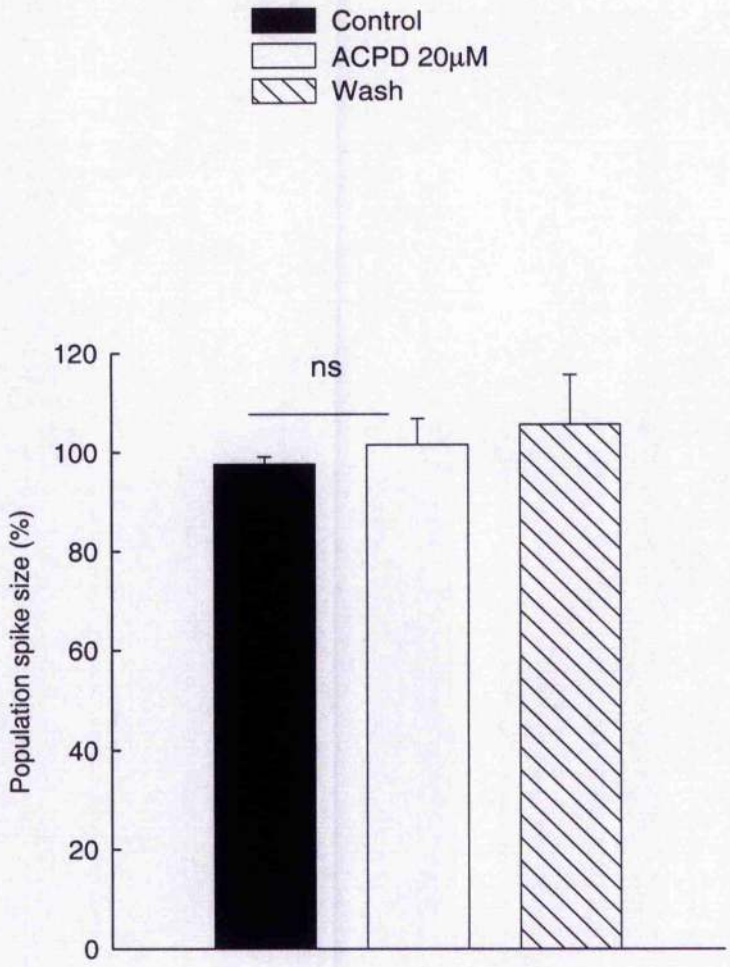

Figure 3.2. Histogram showing the effect of ACPD 20μM on orthodromic population spikes evoked from CA_1 region. Each vertical bar shows mean ± S.E.M for n = 5 slices.

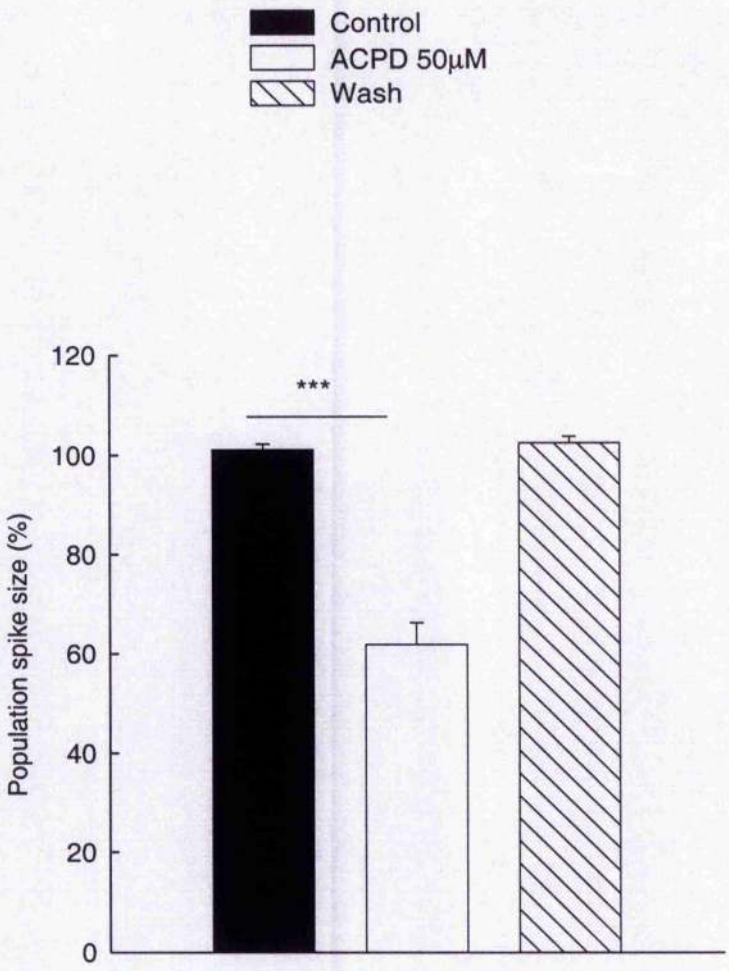

Figure 3.3. Histogram showing the effect of ACPD 50μM on orthodromic population spikes evoked from CA_1 region. Each vertical bar shows mean ± S.E.M for n = 6 slices. *** P<0.001

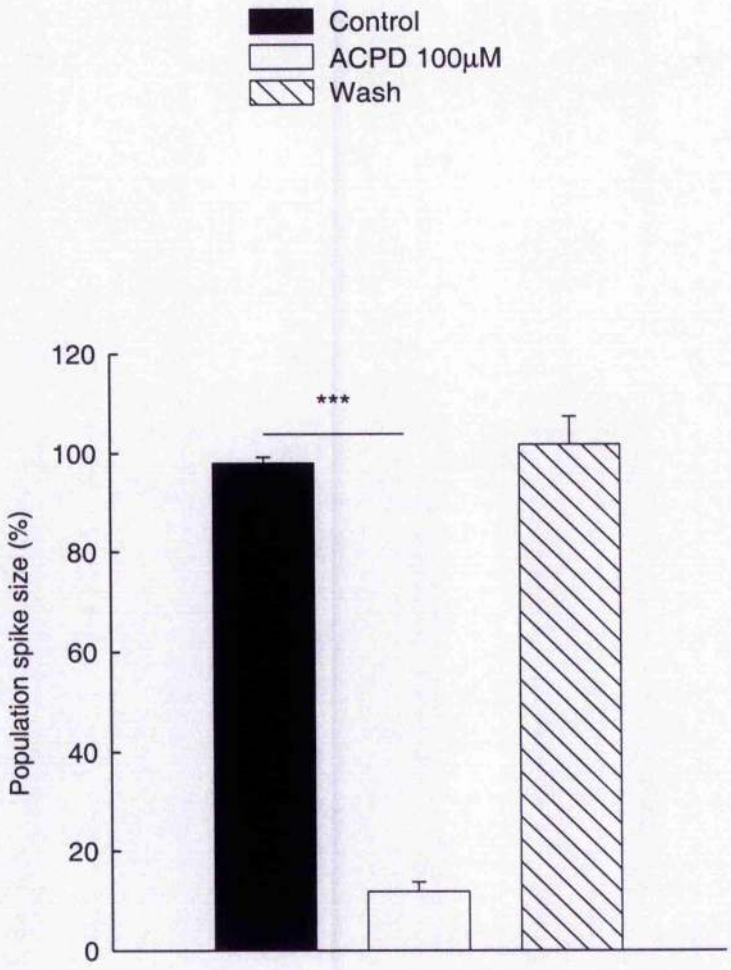

Figure 3.4. Histogram showing the suppression effect of ACPD 100μM on orthodromic population spikes evoked from CA_1 region. Each vertical bar shows mean ± S.E.M for n = 5 slices. *** P<0.001

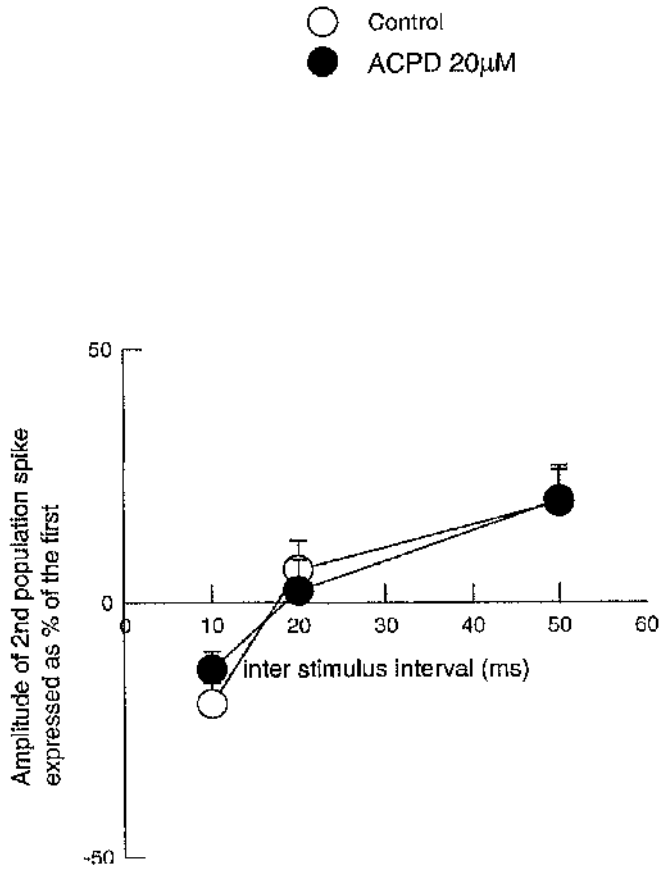

Figure 3.5. Graph showing the effect of ACPD 20μM on orthodromic paired–pulse population spikes recorded from CA_1. Data are shown for interstimulus intervals of 10, 20 and 50 ms. ACPD 20μM did not induce any significant change in the paired – pulse ratio. Each point shows the mean ± S.E.M for n = 5 slices.

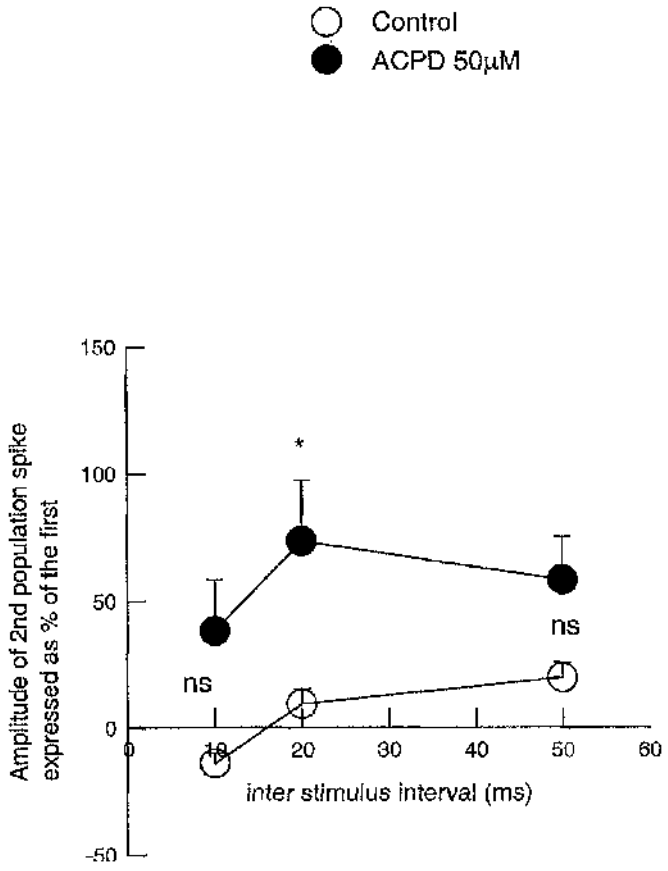

Figure 3.6. The effect of ACPD 50μM on paired – pulse population spikes evoked from CA_1 at interstimulus intervals of 10, 20 and 50 ms. Each point shows mean ± S.E.M for n = 6 slices. * $P<0.05$

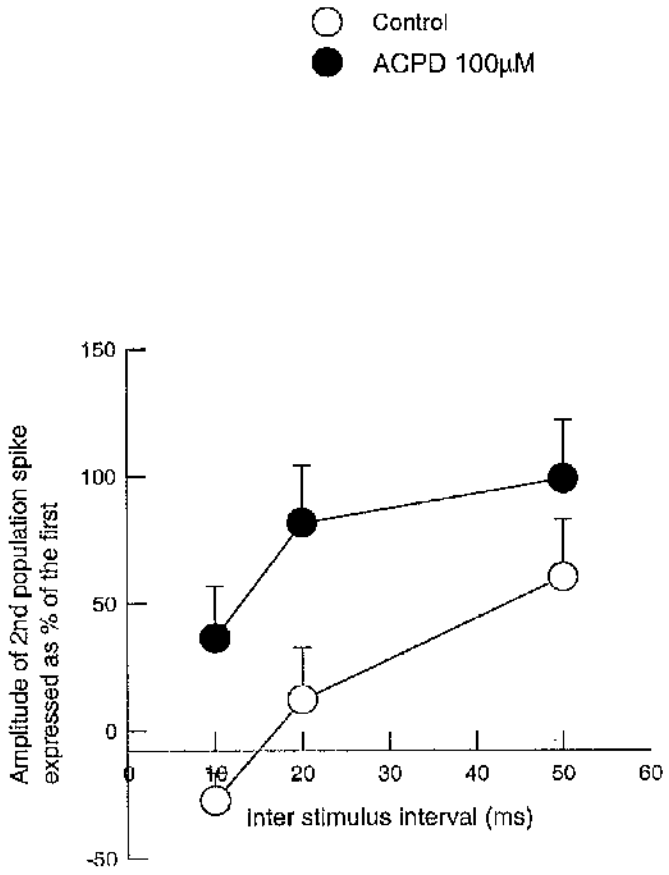

Figure 3.7. The effect of ACPD 100μM on paired – pulse population spikes evoked from CA_1 at interstimulus intervals of 10, 20 and 50 ms. Each point shows mean ± S.E.M for n = 5 slices.

The Interaction between adenosine and ACPD on single excitatory postsynaptic potentials

To investigate the effects of metabotropic glutamate receptors on adenosine responses a broad spectrum mGluRs agonist ACPD was used. A concentration of 10 μM adenosine was chosen for most of these experiments, as it produced an approximately 50% inhibition of fEPSPs slope, from which increases or decreases could readily be observed and quantified.

In order to determine whether responses to adenosine due to receptor engagement decrease in a serial application, a series of adenosine responses was obtained before ACPD perfusion (Fig. 3.8). As Figure 3.8 demonstrates there were no significant differences between consecutive adenosine responses. The last adenosine effect before ACPD was chosen to compare adenosine responses before ACPD with adenosine responses after ACPD application. Adenosine 10 μM depressed single EPSPs by 59.35% ± 3.39 ($p < 0.001$; $n = 4$) relative to the initial control size. When applied alone, the non-selective mGluR agonist ACPD (100 μM) reduced the fEPSPs slope by 80.83% ± 0.59 ($p < 0.001$; $n = 4$) (Fig.3.8).

When a series of adenosine responses was obtained, and an application of ACPD made after the first of these, it was found that the subsequent responses were reduced significantly in size. fEPSPs slope was depressed by only 38.7% ± 5.59 by an application of adenosine 20 minutes after the ACPD perfusion ($p < 0.01$ compared with the initial adenosine response, $n = 4$). Adenosine responses obtained 40 and 60 minutes after the ACPD application remained significantly smaller than the initial response ($p < 0.05$)(Fig. 3.8).

Paired-pulse interactions

In the paired-pulse paradigm, interstimulus intervals of 10, 20 and 50 ms were tested which have previously been shown to generate paired-pulse inhibition (at 10 ms) and later paired-pulse potentiation (20 and 50 ms) (Higgins & Stone, 1996, Nikbakht & Stone 2001). Superfusion of adenosine alone converted the inhibition to facilitation at 10 ms and produced a marked increase in facilitation at 20 and 50 ms (Fig. 3.9). All these changes were highly significant ($p < 0.001$; $n = 4$). Applied alone, ACPD tended to produce a similar shift but this did not reach statistical significance. When adenosine was tested 20 minutes following a 10 minutes application of ACPD, its effects were again reduced significantly (Fig. 3.9; $p < 0.05$ compared with the initial adenosine response; $n = 4$). Subsequent responses to adenosine gradually returned towards the original size over a 60 minutes period as in the study of EPSP slope size described above (Fig. 3.9).

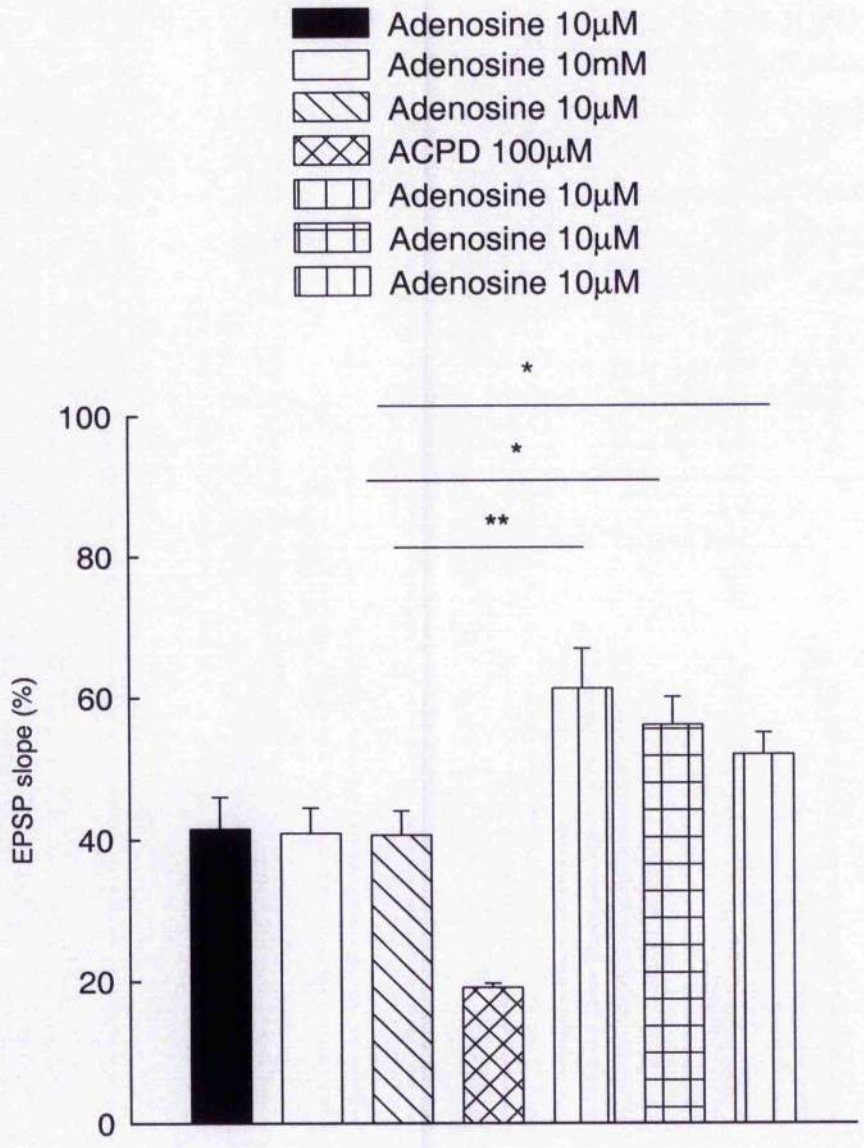

Figure. 3.8A. Histogram showing the interaction between adenosine 10μM and ACPD 100μM on the fEPSPs slope in hippocampal slices. The columns summarise, respectively, the inhibitory effect of a series of three control applications of adenosine alone, the effect of ACPD alone, and three reduced responses to adenosine observed 20, 40 and 60 minutes after the application of ACPD. The columns indicate the mean ± S.E.M for n = 4 slices. Statistically significant differences between columns are indicated as * $P<0.05$, ** $P<0.01$.

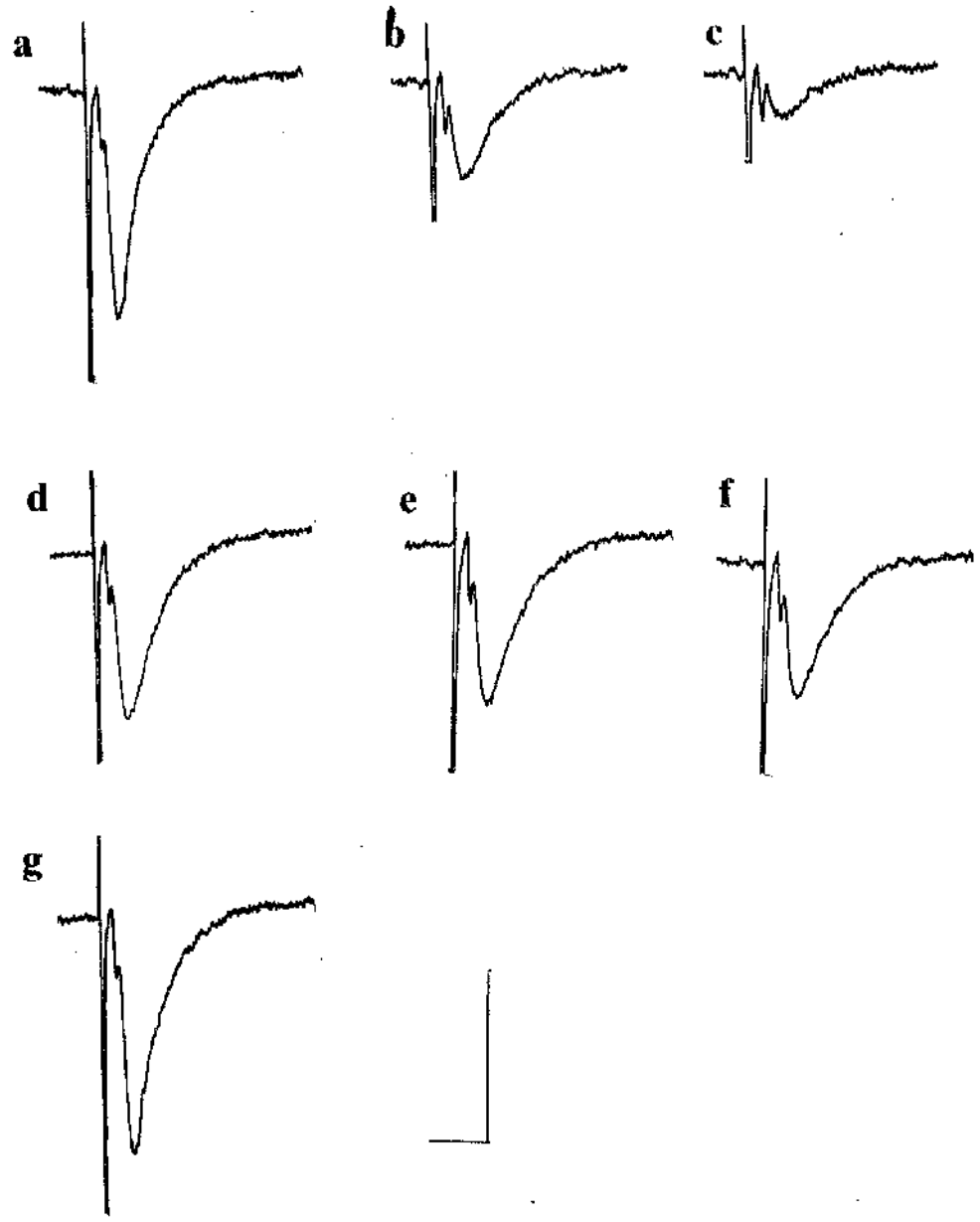

Figure 3.8B. Sample records of the orthodromic evoked fEPSPs representing the effect of adenosine before and after the superfusion of ACPD. The letters a, b and c illustrate control, adenosine 10μM before ACPD and the effect of ACPD respectively and d, e and f showing the effect of adenosine 20, 40 and 60 minutes after ACPD application and g shows washing after the last adenosine application. Calibration: 1mV and 10ms

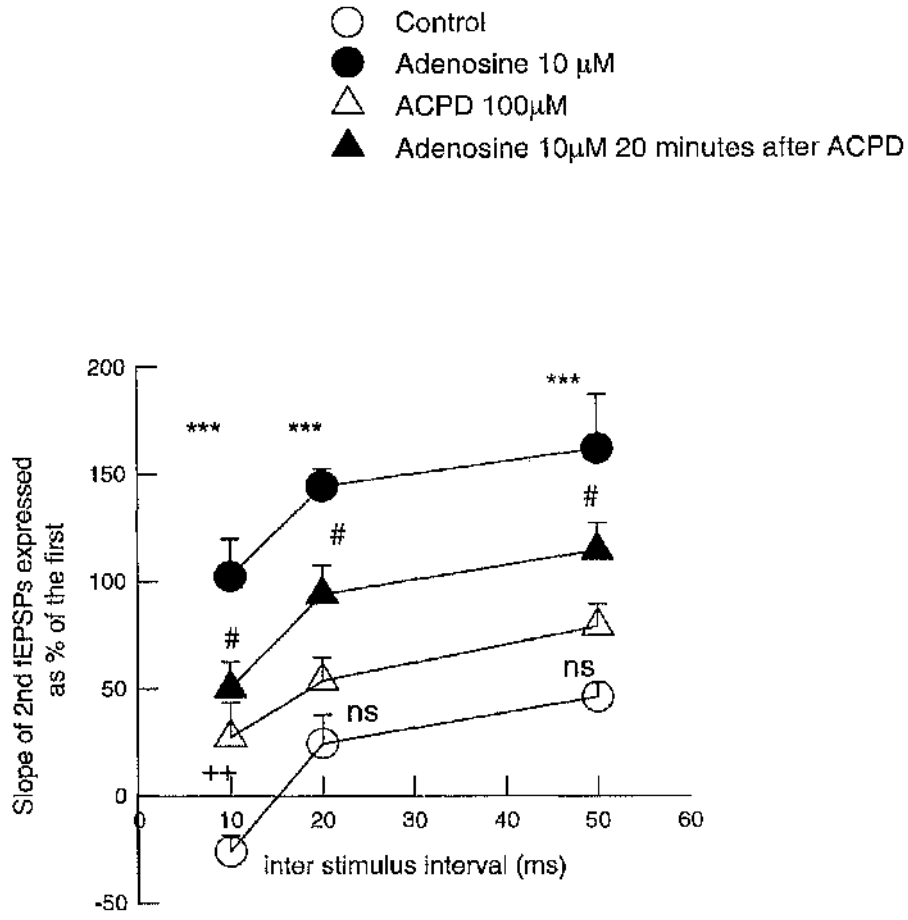

Figure 3.9. Graph showing the changes of paired-pulse response of fEPSPs slope (the % change in the second of a pair of responses relative to the first) for interstimulus intervals of 10, 20 and 50 ms. Each point represents mean ± S.E.M for n = 4 slices. Only the first adenosine response after ACPD, obtained at 20 minutes, is shown for clarity. *** P<0.001 for difference between control and adenosine before ACPD; # P<0.05 between adenosine responses before and after ACPD; ++ P<0.01 between control and ACPD 100μM.

Interaction between ACPD and baclofen

In order to determine whether this lasting depression of sensitivity was specific for adenosine, similar experiments were performed using ACPD 100 μM and baclofen 2 μM. Baclofen initially depressed the single EPSP slope by 59.86% ± 2.64 ($p < 0.001$; $n = 4$) of the basal fEPSPs whereas, after perfusing ACPD, baclofen reduced the EPSP slope by only 41.66% ± 3.57 ($p < 0.01$ compared with the initial baclofen response, $n = 4$), 43.61% ± 1.69 and 47.57% ± 3.42 after 20, 40 and 60 minutes (Fig. 3.10). ACPD also reduced significantly the decrease in paired-pulse inhibition and later paired-pulse facilitation produced by baclofen (Fig. 3.11).

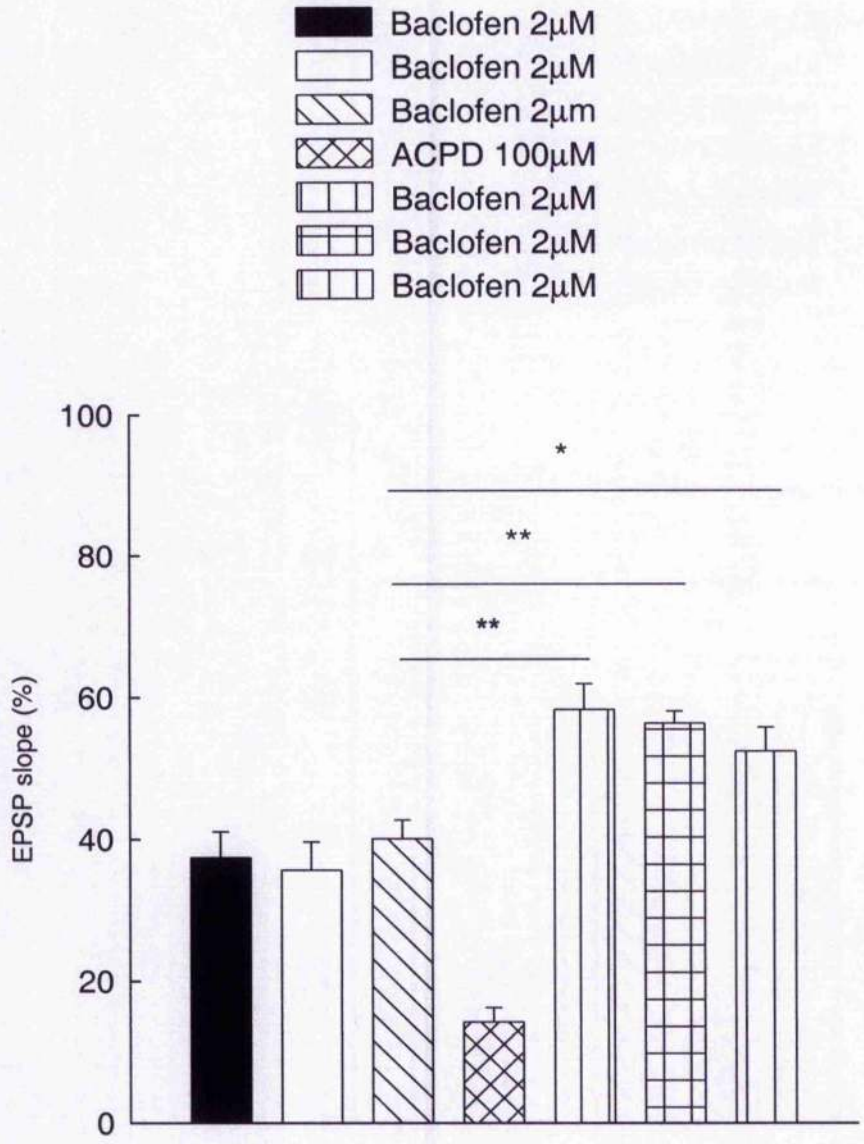

Figure 3.10. Histogram showing the interaction between baclofen (2μM) and ACPD 100μM on the fEPSPs slope in hippocampal slices. The columns summarise, respectively, the inhibitory effect of a series of three control applications of baclofen alone, the effect of ACPD alone, and three reduced responses to baclofen observed 20, 40 and 60 minutes after the application of ACPD. The columns indicate the mean ± S.E.M for n = 4 slices. Statistically significant differences between columns are indicated as * $P<0.05$, ** $P<0.01$.

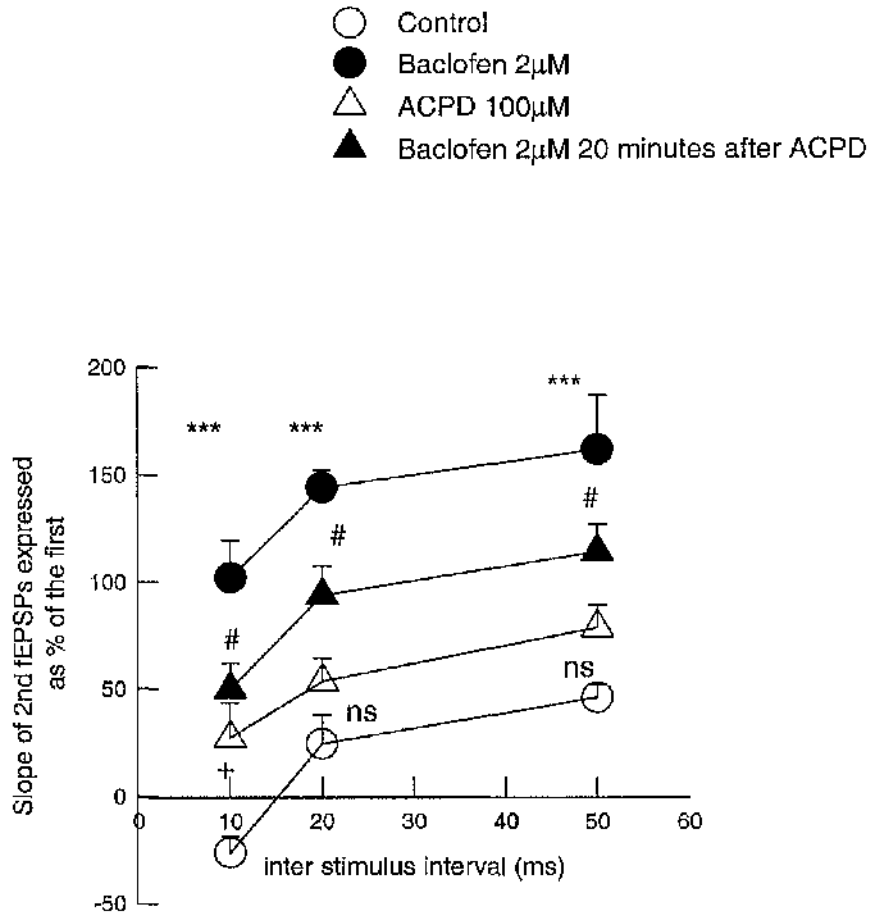

Figure 3.11. Graph showing the changes of paired–pulse response of fEPSPs slope (the % change in the second of a pair of responses relative to the first) for interstimulus intervals of 10, 20 and 50 ms. Each point represents mean ± S.E.M for n = 4 slices. Only the first baclofen response after ACPD, obtained at 20 minutes, is shown for clarity. *** P<0.001 for difference between control and baclofen responses before ACPD; # P<0.05 between baclofen responses before and after ACPD; + P<0.05 between control and ACPD 100μM.

Identification of metabotropic glutamate receptors

In order to determine whether group I mGluRs were responsible for the effect of ACPD, the selective agonist DHPG was used. Before DHPG, adenosine 10 μM depressed EPSP size by 58.74% ± 3.32 of the basal level ($p < 0.01$; $n = 5$) in this series of experiments. DHPG (10 μM) alone decreased the EPSP slope by 40.18% ± 4.29 ($p < 0.001$; $n = 5$). After perfusing DHPG 10 μM, the responses to adenosine were reduced significantly after 20 and 40 minutes respectively (Fig.3.12).

Adenosine at 10 μM removed any early paired-pulse inhibition and enhanced later facilitation ($p < 0.001$; $n = 5$). DHPG had no significant effect alone, but it reduced the effect of adenosine at both the 10 and 20 ms stimulus intervals (Fig. 3.13). As in the earlier studies above, sensitivity to adenosine gradually recovered so that, after 60 minutes, responses were not different from the original size.

Interaction between baclofen and DHPG on single and paired – pulse excitatory postsynaptic potentials

In order to determine whether the effects of group I mGluRs agonist is specific for adenosine, interactions between DHPG and $GABA_B$ agonist baclofen were examined. Baclofen (2 μM) depressed the single EPSP slope by 69.11% ± 3.37 of the basal potential ($p < 0.001$; $n = 4$) whereas at 20 and 40 minutes after perfusion with DHPG, it reduced EPSP slope significantly less (Fig.3.14). In the paired-pulse experiments, DHPG tended to reduce the effect of baclofen, though this did not reach significance (Fig. 3.15).

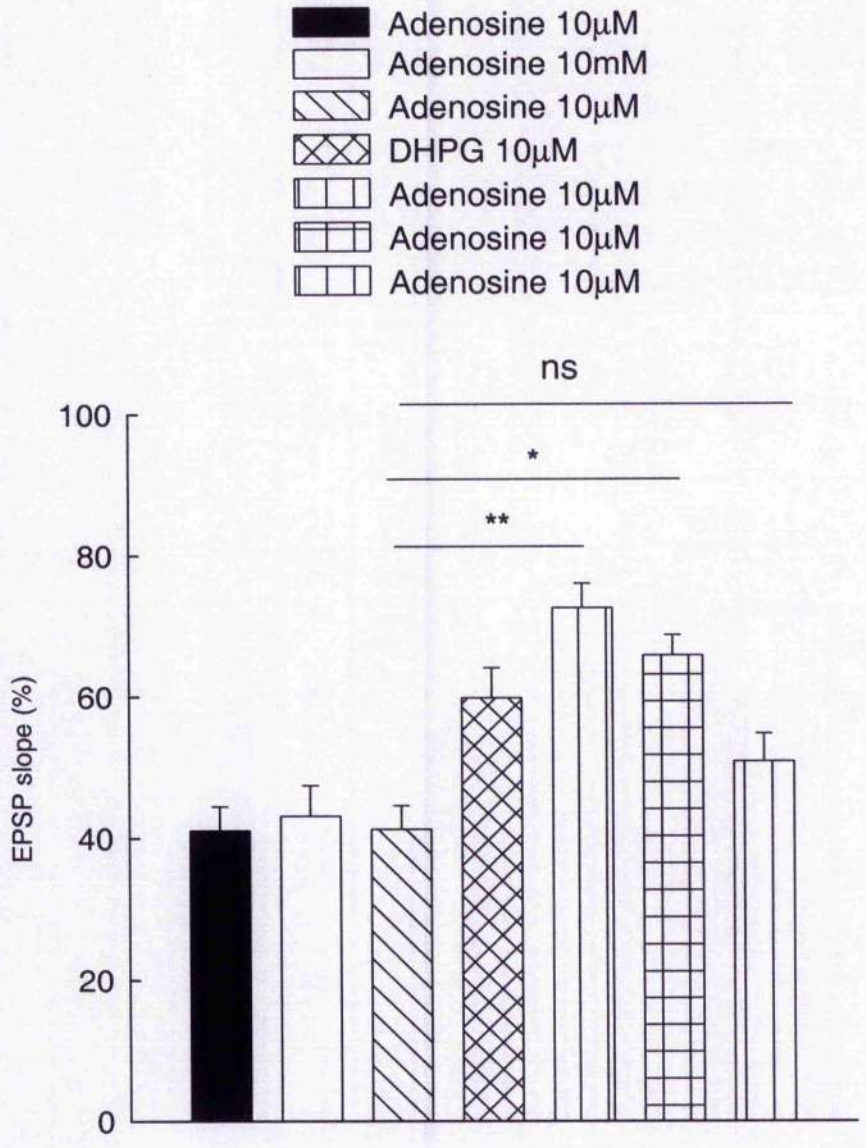

Figure. 3.12A. Histogram showing the interaction between adenosine 10μM and DHPG 10μM on the fEPSPs slope in hippocampal slices. The columns summarise, respectively, the inhibitory effect of a series of three control applications of adenosine alone, the effect of DHPG alone, and three reduced responses to adenosine observed 20, 40 and 60 minutes after the application of DHPG. The columns indicate the mean ± S.E.M for n = 4 slices. Statistically significant differences between columns are indicated as * $P<0.05$, ** $P<0.01$.

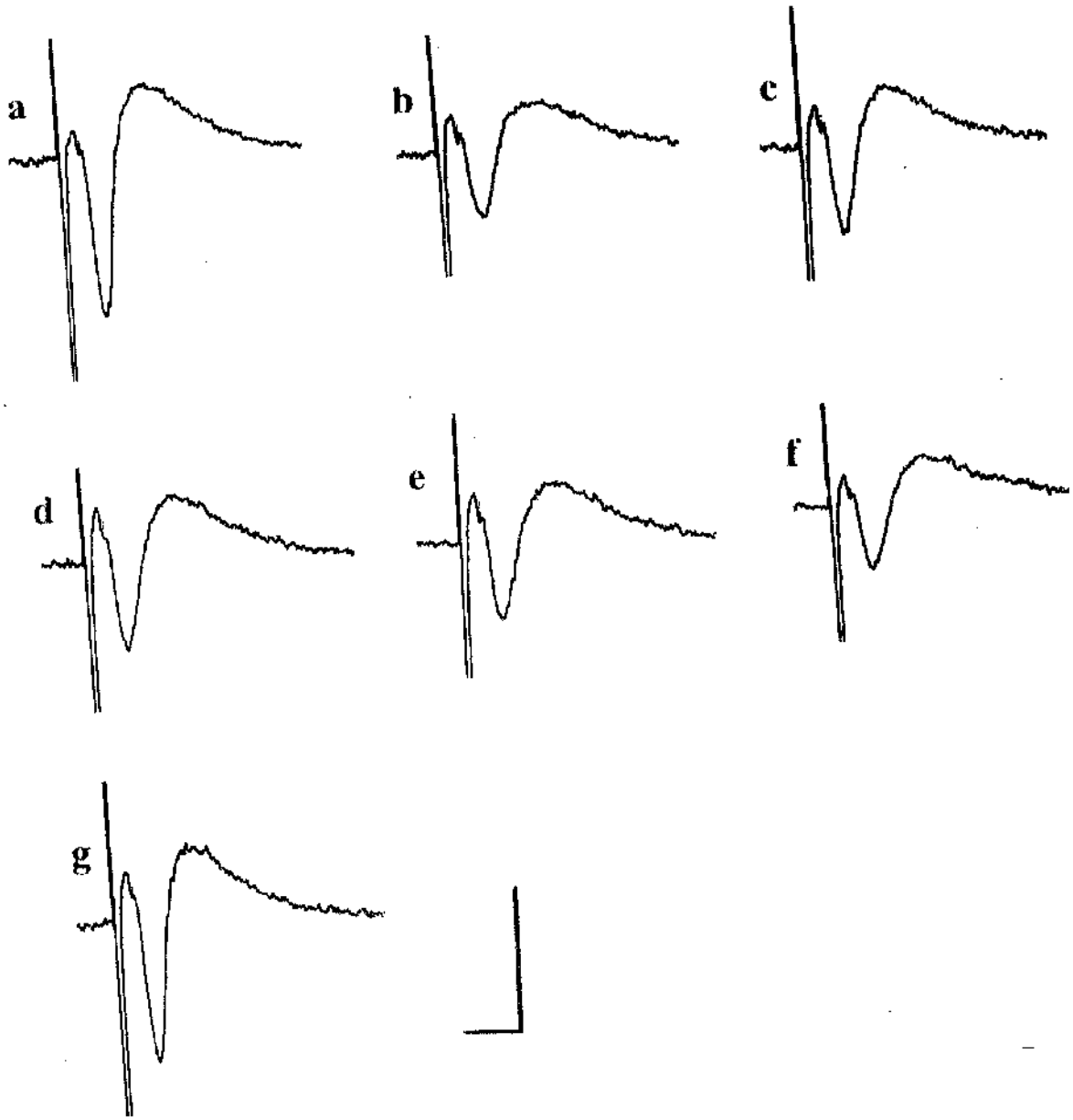

Figure 3.12B. Sample records of the orthodromic evoked population EPSP showing the effect of adenosine before and after the superfusion of DHPG. The letters a, b and c illustrate control, adenosine 10μM before DHPG, and the effect of DHPG respectively and d, e and f showing the effect of adenosine 20, 40 and 60 minutes after DHPG application and g shows washing after the last adenosine application. Calibration: 1mV and 10ms.

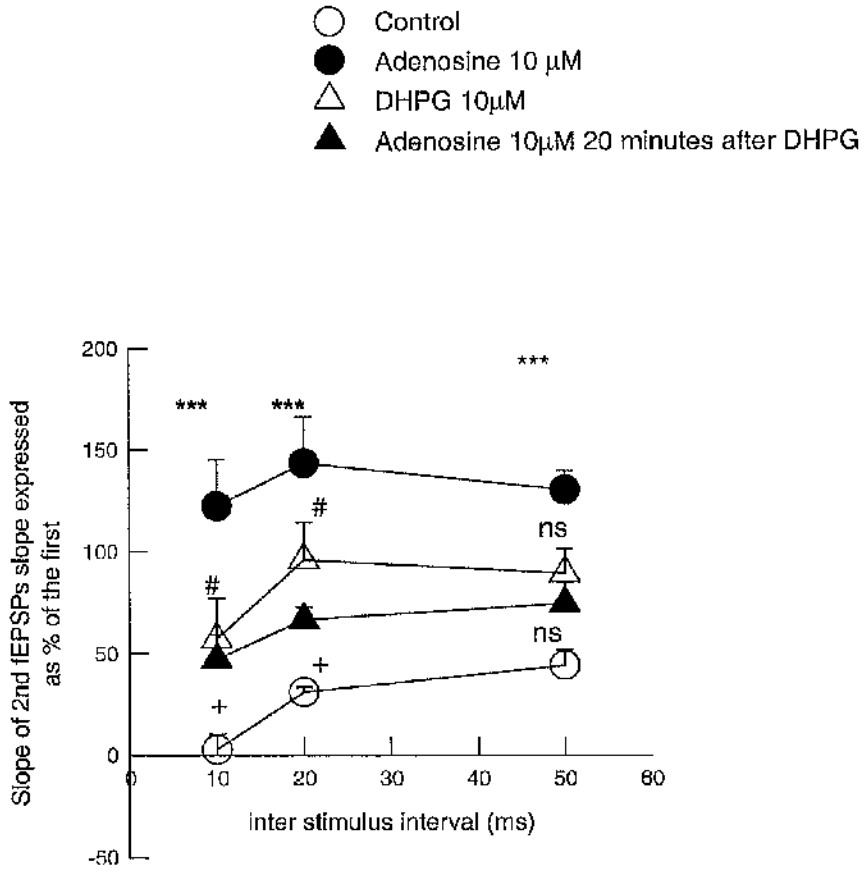

Figure 3.13A. Graph showing the changes of paired – pulse response of fEPSPs slope (the % change in the second of a pair of responses relative to the first)) for interstimulus intervals of 10, 20 and 50 ms. Each point represents mean ± S.E.M for n = 4 slices. Only the first adenosine response after DHPG, obtained at 20 minutes, is shown for clarity. *** P<0.001 for difference between control and adenosine before DHPG; # P<0.05 between adenosine responses before and after DHPG; + P<0.01 between control and DHPG 100µM

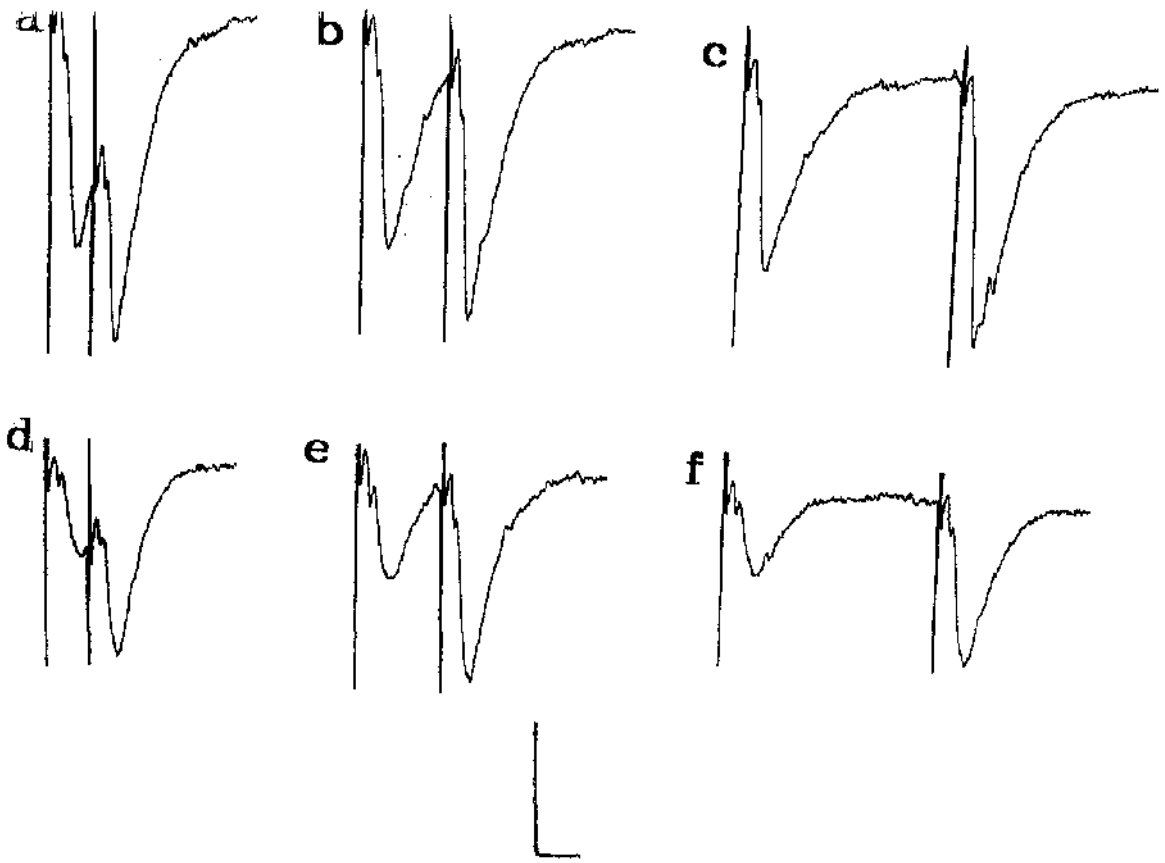

Figure 3. 13B. Records of the population fEPSPs during paired-pulse interactions, showing control responses at interstimulus intervals of (a) 10ms, (b) 20ms, (c) 50ms. The lower traces (d-F) illustrate the effect of adenosine, 10μM at the same stimulus intervals. Calibrations: 1mV and 10ms

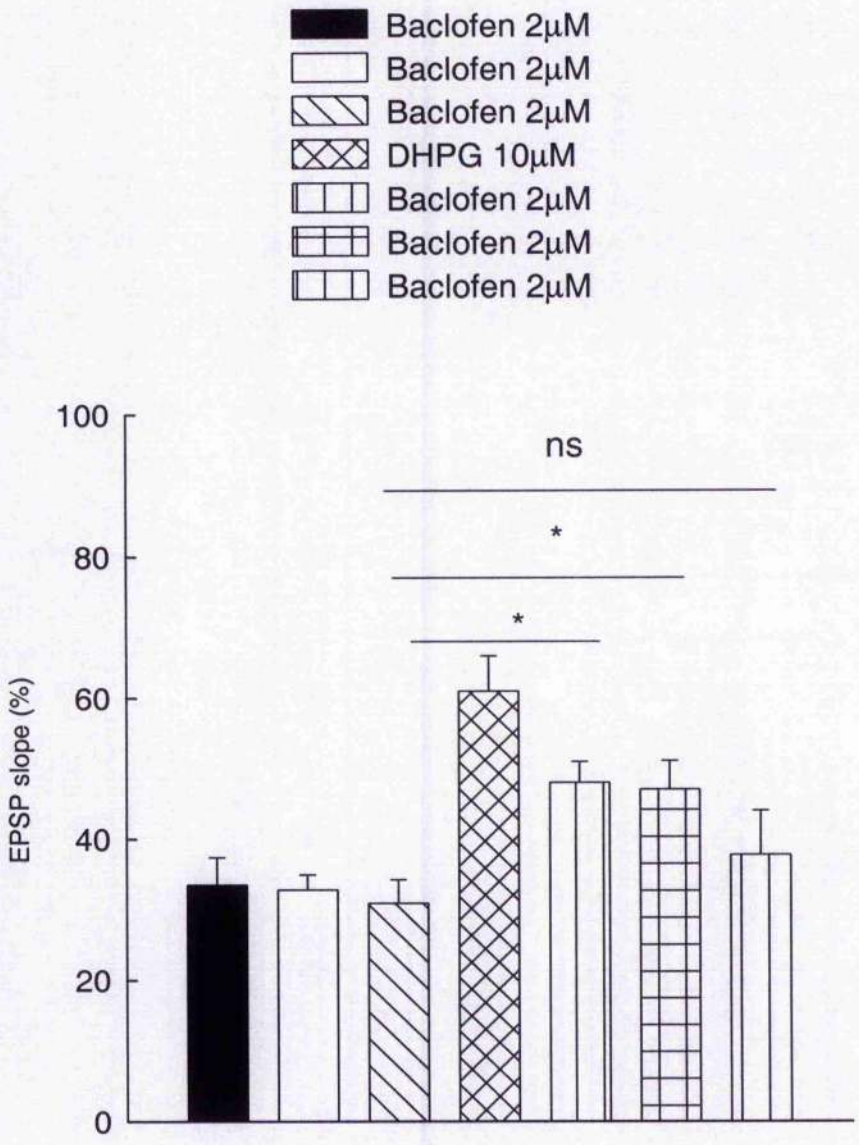

Figure 3.14. Histogram showing the interaction between baclofen 2μM and DHPG 10μM on the fEPSPs slope in hippocampal slices. The columns summarise, respectively, the inhibitory effect of a series of three control applications of baclofen alone, the effect of DHPG alone, and three reduced responses to baclofen observed 20, 40 and 60 minutes after the application of DHPG. The columns indicate the mean ± S.E.M for n = 4 slices. Statistically significant differences between columns are indicated as * $P<0.05$.

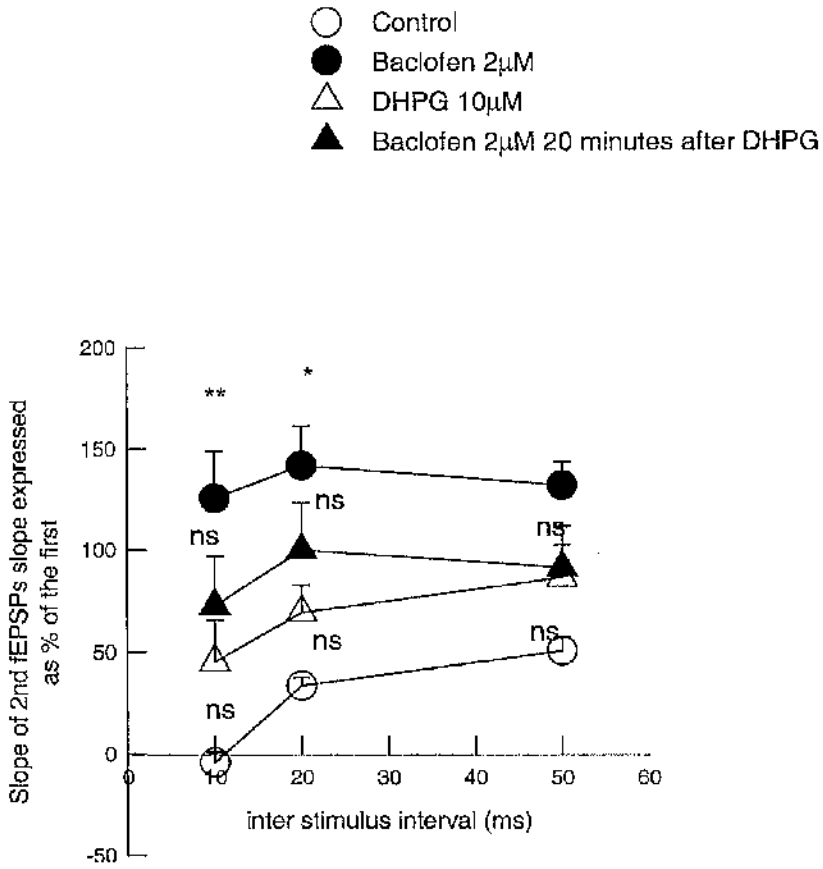

Figure 3.15. Modulation of orthodromic evoked fEPSPs by baclofen 2μM, DHPG 10μM and baclofen 20 minutes after DHPG application. Each point represents mean ± S.E.M for n = 4 slices. * $P<0.05$ and ** $P<0.01$ for difference between control and baclofen.

Interaction between adenosine and DHPG on single and paired – pulse excitatory postsynaptic potentials in the presence of receptor sub – type antagonists

Two selective antagonists were used to assess the involvement of subtypes of the group I mGluRs. (S)-(+)-α-amino-4-carboxy-2-methylbenzeneacetic acid (LY367385), an antagonist at $mGlu_{1a}$ receptors was superfused at 100 μM as described by Mannaioni et al., 2001. This compound had no effect itself on the baseline size of the EPSP slope, and did not modify sensitivity to adenosine. It did, however, prevent the direct inhibitory activity of DHPG itself on EPSP slope (Fig. 3.16) and on paired-pulse interactions (Fig 3.17). It also prevented completely the depression by DHPG of adenosine sensitivity, so that the effect of adenosine on EPSP slope and paired-pulse interaction was not different from that recorded before the superfusion of DHPG (Figs. 3.16, 3.17).

The $mGlu_5$ antagonist 2-methyl-6-(2-phenylethenyl)pyridine (SIB1893) at 40 μM did not prevent the reduction of adenosine sensitivity by DHPG, either on EPSP slope(Fig. 3.18) or paired-pulse interactions (Fig. 3.19).

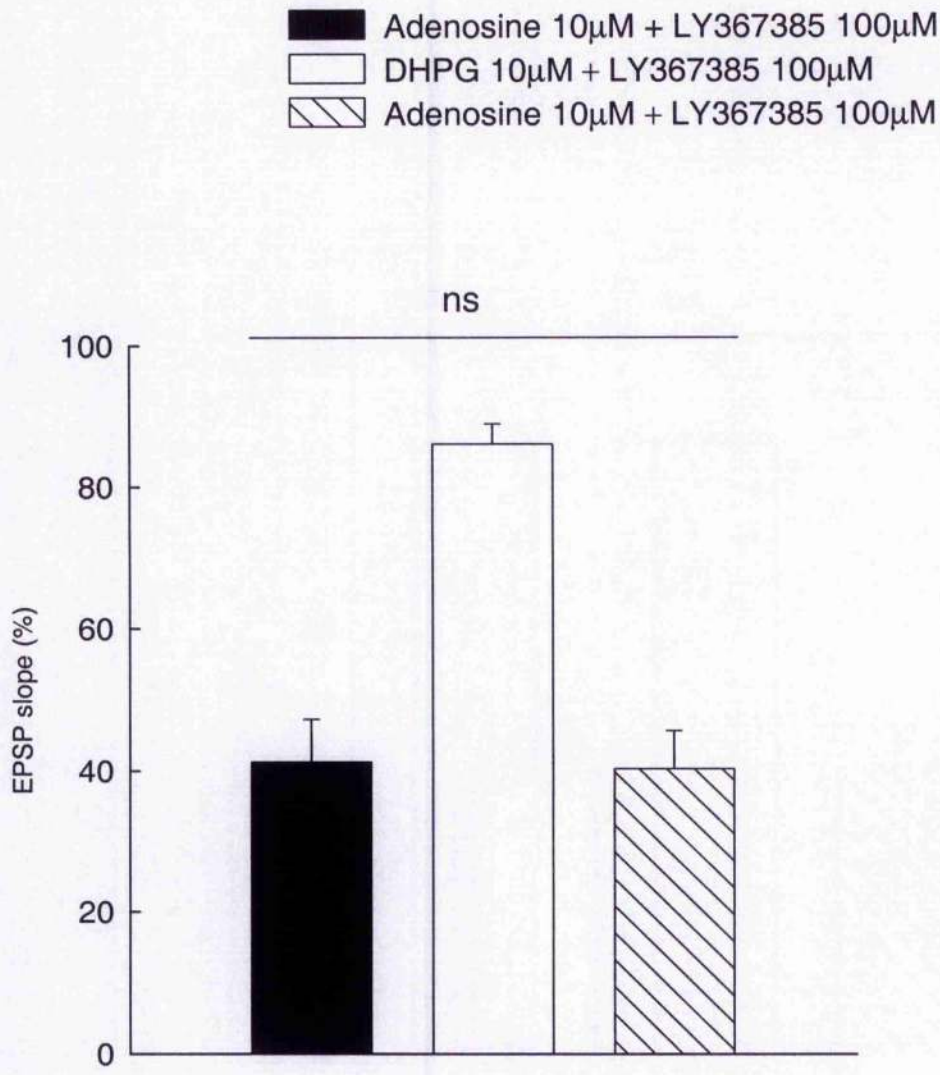

Figure 3.16. Histogram showing the interaction between adenosine 10μM and DHPG 10μM in the presence of (S)-(+)-α-amino-4-carboxy-2-methylbenzeneacetic acid (LY367385) 100μM, an antagonist at $mGlu_{1a}$ receptors, on the fEPSPs slope in hippocampal slices. Each vertical bar represents mean ± S.E.M for n = 4 slices.

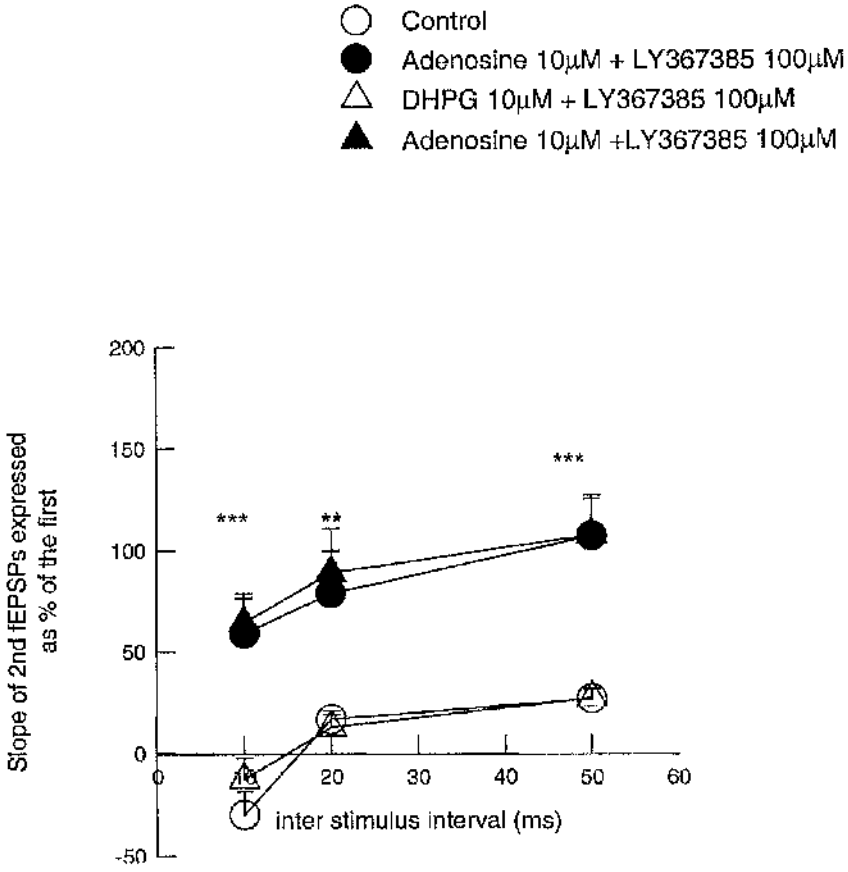

Figure 3.17. Graph showing the interaction between adenosine 10μM and DHPG 10μM in the presence of LY367385 100μM on the paired–pulse responses of fEPSPs in CA_1 area of hippocampus for interstimulus intervals of 10, 20 and 50 ms. Each point represents mean ± S.E.M for n = 4 slices. *** $P<0.001$ and ** $P<0.01$ between control and adenosine in the presence of LY367385 before and after DHPG.

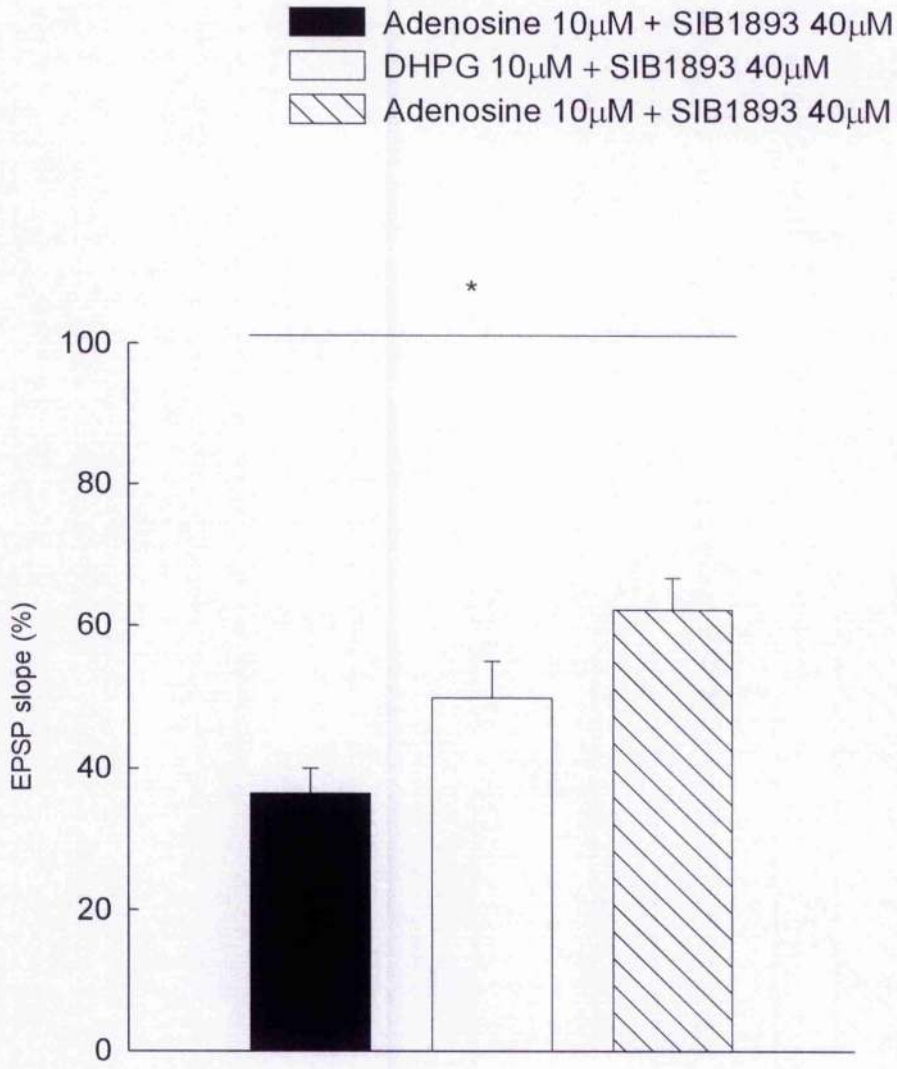

Figure 3.18. Histogram showing the effect of adenosine 10μM and DHPG 10μM in the presence of the $mGlu_5$ antagonist 2-methyl-6-(2-phenylethenyl)pyridine (SIB1893) at 40 μM on fEPSPs evoked from the CA1 region of hippocampal slices. Each vertical bar represents mean ± S.E.M for n = 4 slices. * $P<0.05$

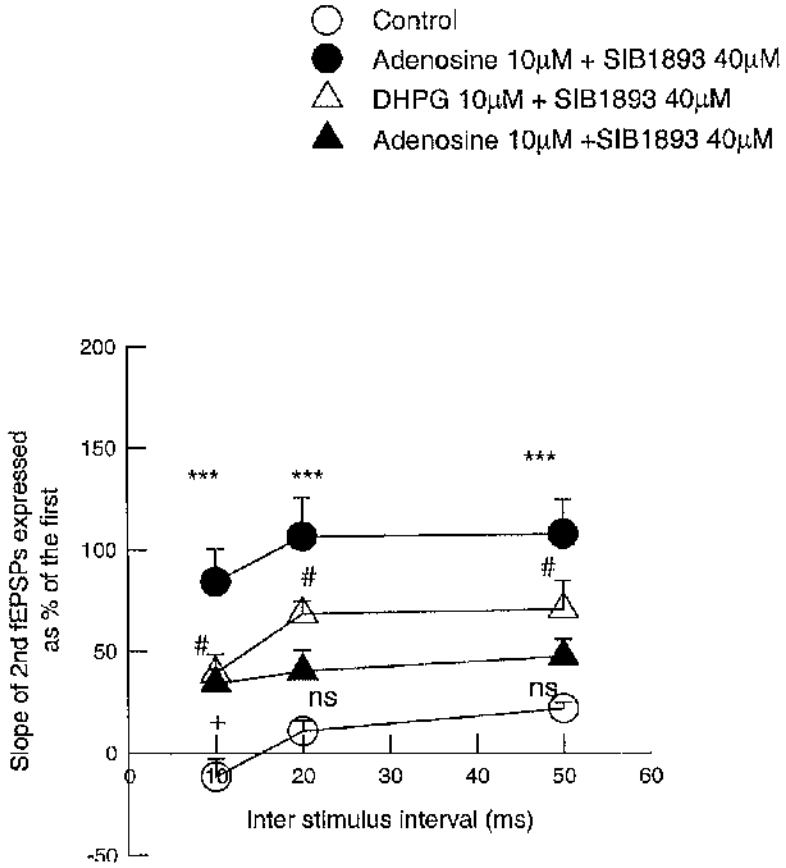

Figure 3.19. Modulation of orthodromic evoked paired-pulse fEPSPs by adenosine 10μM and DHPG 10μM in the presence of The $mGlu_5$ antagonist 2-methyl-6-(2-phenylethenyl)pyridine (SIB1893) at 40 μM. Each point represents mean ± S.E.M for n = 4 slices. *** $P<0.05$ for difference between control and adenosine in the presence of SIB1893. # $P<0.05$ for difference between adenosine before and after DHPG in the presence of SIB1893. + $P<0.05$ between control and DHPG in the presence of SIB1893.

Interaction between adenosine and DHPG on single and paired – pulse excitatory postsynaptic potentials in the presence of NMDA antagonist.

In order to examine the possible involvement of NMDA receptors in the interactions between adenosine and metabotropic agonists, the experiments were repeated in the presence of D-2-amino-5-phosphono-pentanoic acid, 50 μM (2AP5). This compound prevented the suppression of adenosine responses after perfusion with DHPG, assessed both as the change in EPSP slope (Fig. 3.20) and as the change of paired-pulse inhibition (Fig. 3.21).

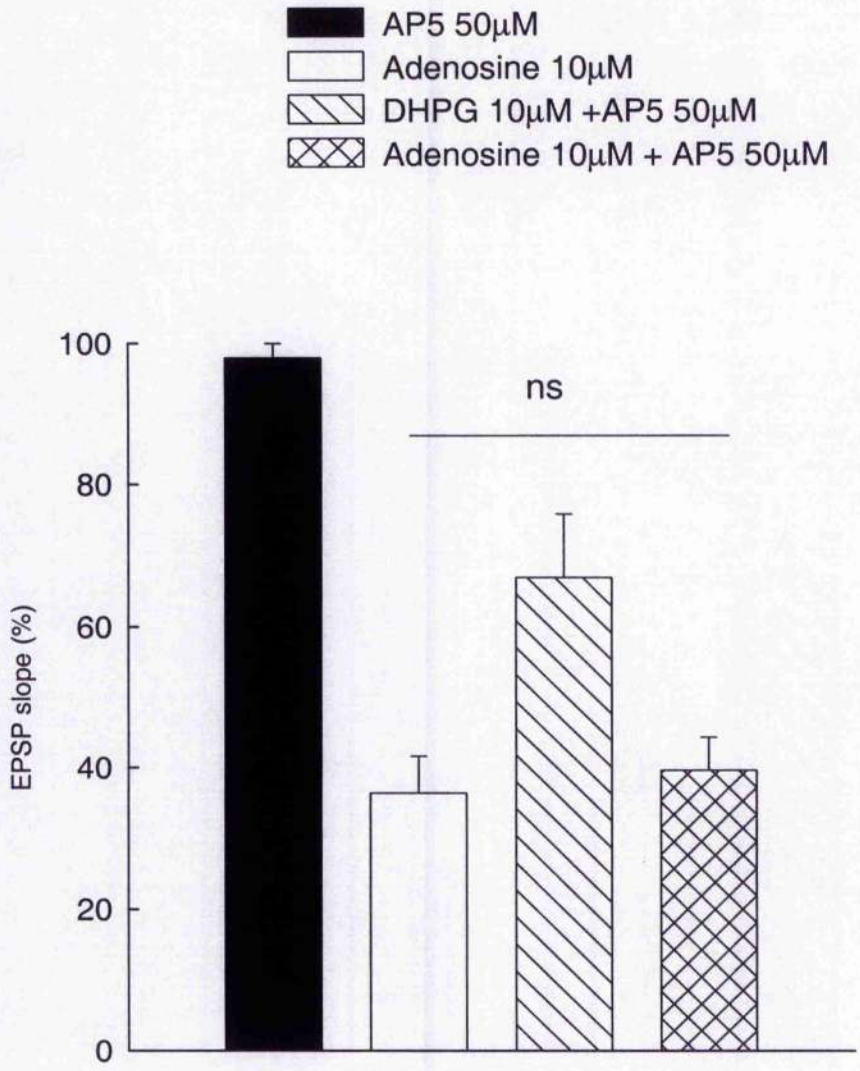

Figure 3.20. Histogram showing the effect of adenosine 10μM and DHPG 10μM in the presence of D-2-amino-5-phosphono-pentanoic acid (2AP5) 50μM on fEPSPs from the CA1 region of hippocampal slices. Each vertical bar represents mean ± S.E.M for n = 4 slices. The columns summarise, respectively, the lack of effect of DHPG in the presence of 2AP5 and the normal response to adenosine observed 20 minutes after the application of DHPG.

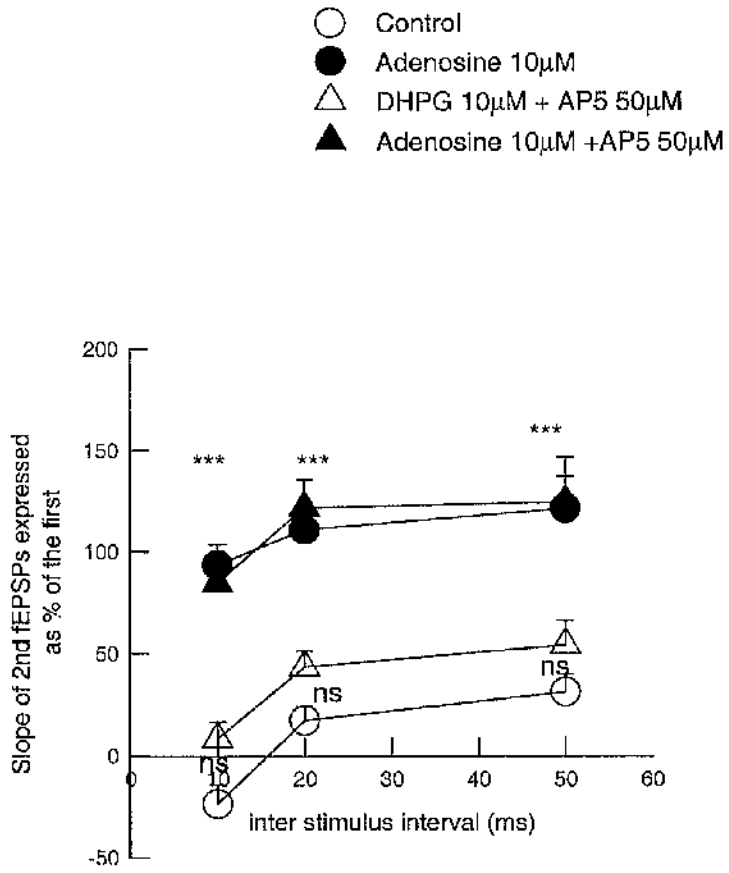

Figure 3.21. Modulation of orthodromic evoked paired-pulse fEPSPs by adenosine 10μM and DHPG 10μM in the presence of 2AP5 50μM. Each point represents mean ± S.E.M for n = 4 slices. *** P<0.05 for difference between control and adenosine in the presence of 2AP5 before and after DHPG 10μM application.

Involvement of protein kinase C

The protein kinase C inhibitors chelerythrine and 2-[1-(3-dimethylaminopropyl) indol-3-yl]-3-(indol-3-yl) malcimide (GF109203X) were used to assess the involvement of protein kinase C in the effects of the mGluR ligands. Both were applied for 10 minutes before, during and following the application of DHPG. At a concentration of 5 μM, chelerythrine had no significant effect of its own but did prevent the suppression of adenosine responses by DHPG on the single EPSP slope (Fig. 3.22). In addition, chelerythrine prevented the changes of paired-pulse interactions (Fig. 3.23), such that these remained at the initial control size for up to 60 minutes after the application of DHPG.

At 100nM GF109203X (as described by Toullect et al., with IC_{50} from 5 to 70 nM) similarly had no significant effect alone or on the responses to adenosine, but did prevent the suppression of adenosine responses by DHPG on the single EPSP slope (Fig.3.24). Furthermore, GF109203X prevented the changes by DHPG of adenosine effects on paired-pulse interactions (Fig. 3.25), such that these remained at the initial control size for up to 60 minutes after the application of DHPG.

In contrast, the protein kinase A inhibitor (9R, 10S, 12S)-2,3,9,10,11,12-hexahydro-10hydroxy-9-methyl-1-oxo-9, 12-epoxy-1H-di-indolo [1,2,3-fg:3´,2´,1´-kl] pyrrolo[3,4-l][1,6]benzo-diazocine-10-carboxylic acid hexyl ester (KT5720) 100nM did not interfere with the interaction between DHPG and adenosine on both single(Fig. 3.26) and paired-pulse interaction (Fig. 3.27).

Application of protein kinase C activator

As a further test of the possible role of PKC, the PKC activator 5-chloro-N-(6-phenylhexyl)-1-naphthalenesulfonamide (SC-9) 10μM (as described by Ito et al., K_m value of PKC activated was 5.8μM, so we selected 10μM for better responses) was superfused for 10 minutes before, during and after a test application of adenosine. This resulted in a significant reduction in the size of response to adenosine, examined on a single EPSP slope (Fig.3.28) or on paired-pulse interactions (Fig. 3.29).

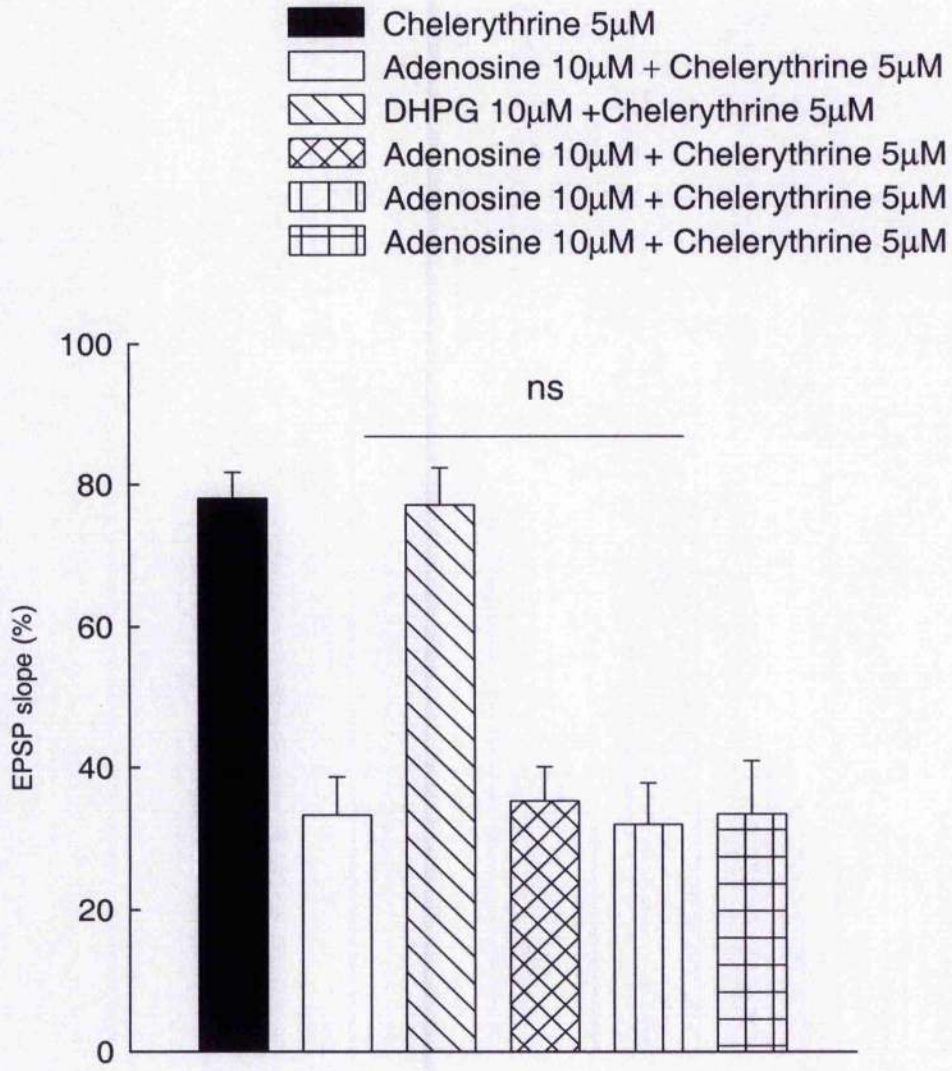

Figure 3.22. Histogram showing the effect of adenosine 10μM and DHPG 10μM in the presence of chelerythrine 5μM on fEPSPs evoked from the CA_1 region of hippocampal slices. Each vertical bar represents mean ± S.E.M for n = 4 slices.

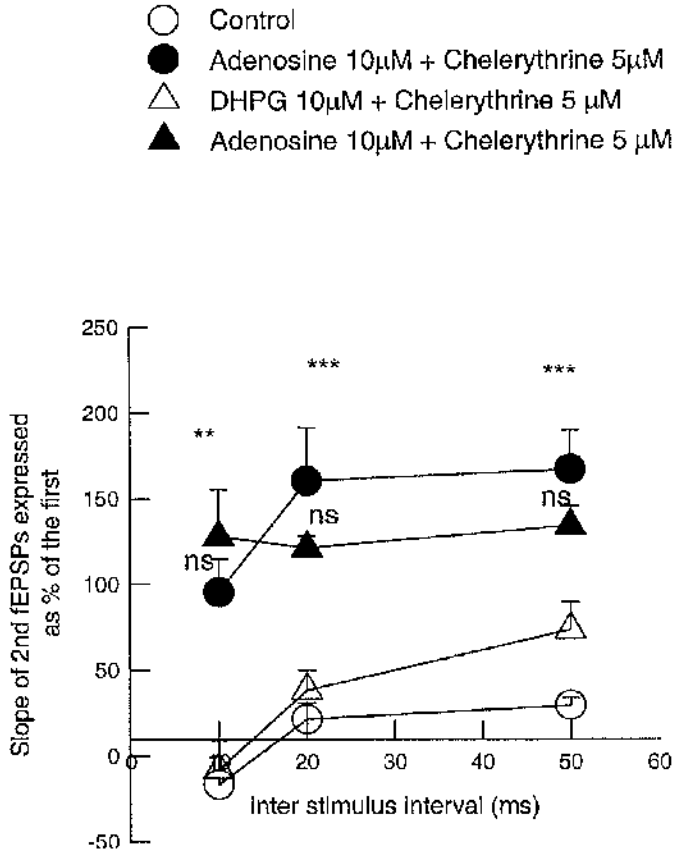

Figure 3.23. Graph showing the interaction between adenosine 10μM and DHPG 10μM in the presence of the PKC inhibitor chelerythrine 5μM on the paired–pulse fEPSPs recorded from CA_1 for interstimulus intervals of 10, 20 and 50 ms. Each point represents mean ± S.E.M for n = 4 slices. ** P<0.01, *** P<0.001 and between control and adenosine in the presence of chelerythrine before DHPG.

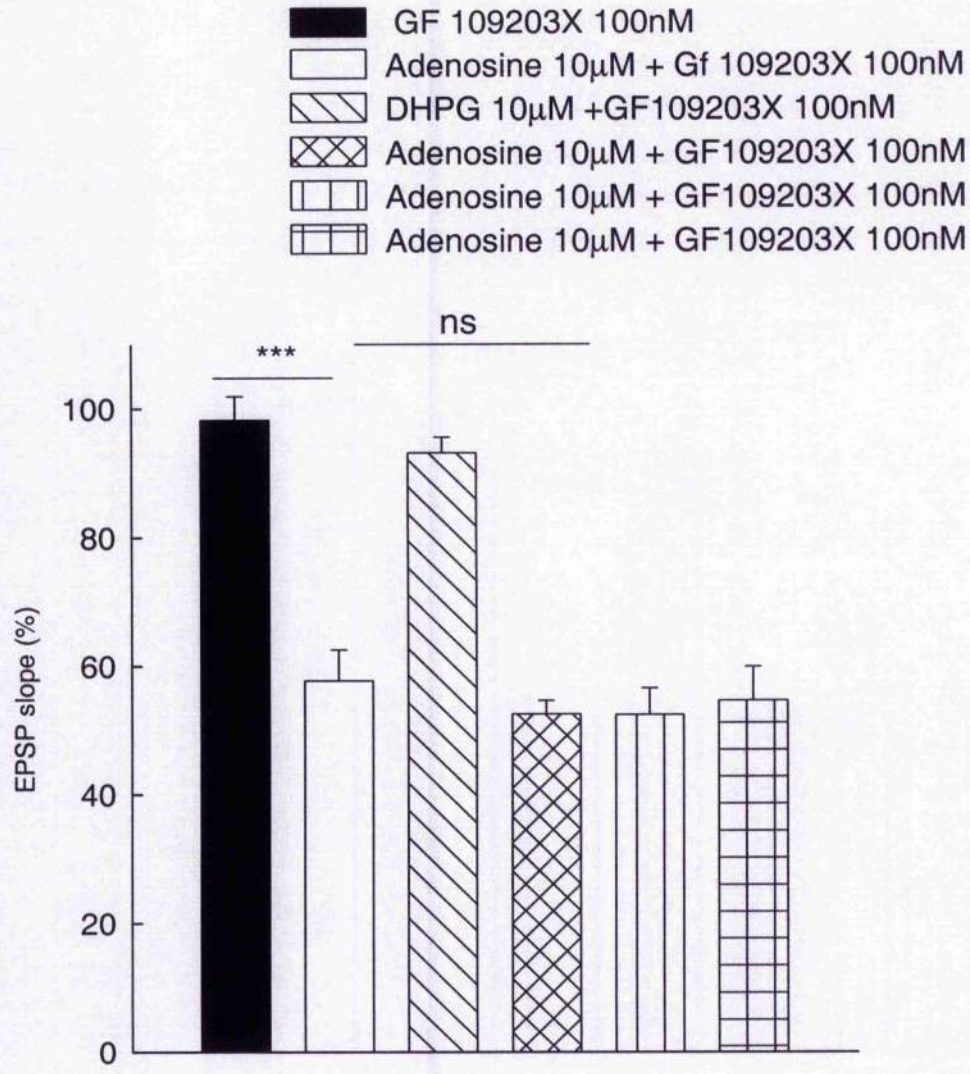

Figure 3.24. Histogram showing the influence of GF 109203X (100nM) on the interaction between adenosine 10μM and DHPG 10μM on the fEPSPs slope in hippocampal slices. Normal responses to adenosine were observed 20, 40 and 60 minutes after the application of DHPG + GF 109203X. Each vertical bar represents mean ± S.E.M for n = 4 slices. *** $P<0.001$.

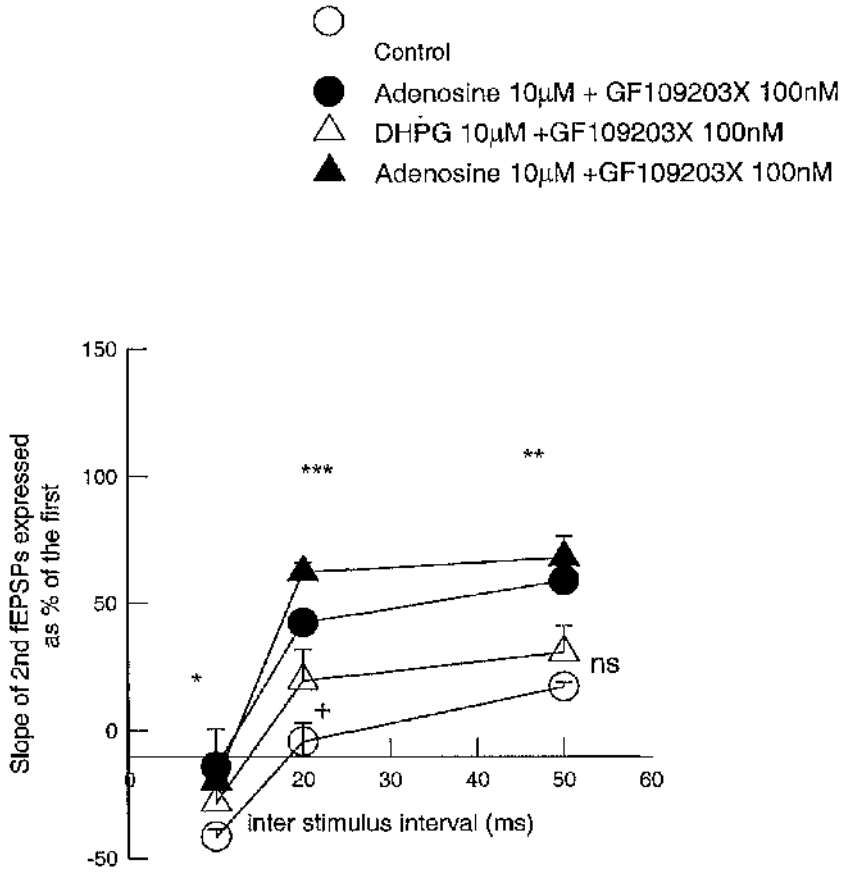

Figure 3.25. Modulation of orthodromic evoked paired-pulse fEPSPs by adenosine 10μM and DHPG 10μM in the presence of PKC inhibitor GF109203X 100nM. Each point represents mean ± S.E.M for n = 4 slices. Only the first adenosine response after DHPG, obtained at 20 minutes, is shown for clarity. * P<0.05, ** P<0.01, *** P<0.001 for difference between controls and adenosine before DHPG. + P<0.05 between controls and DHPG.

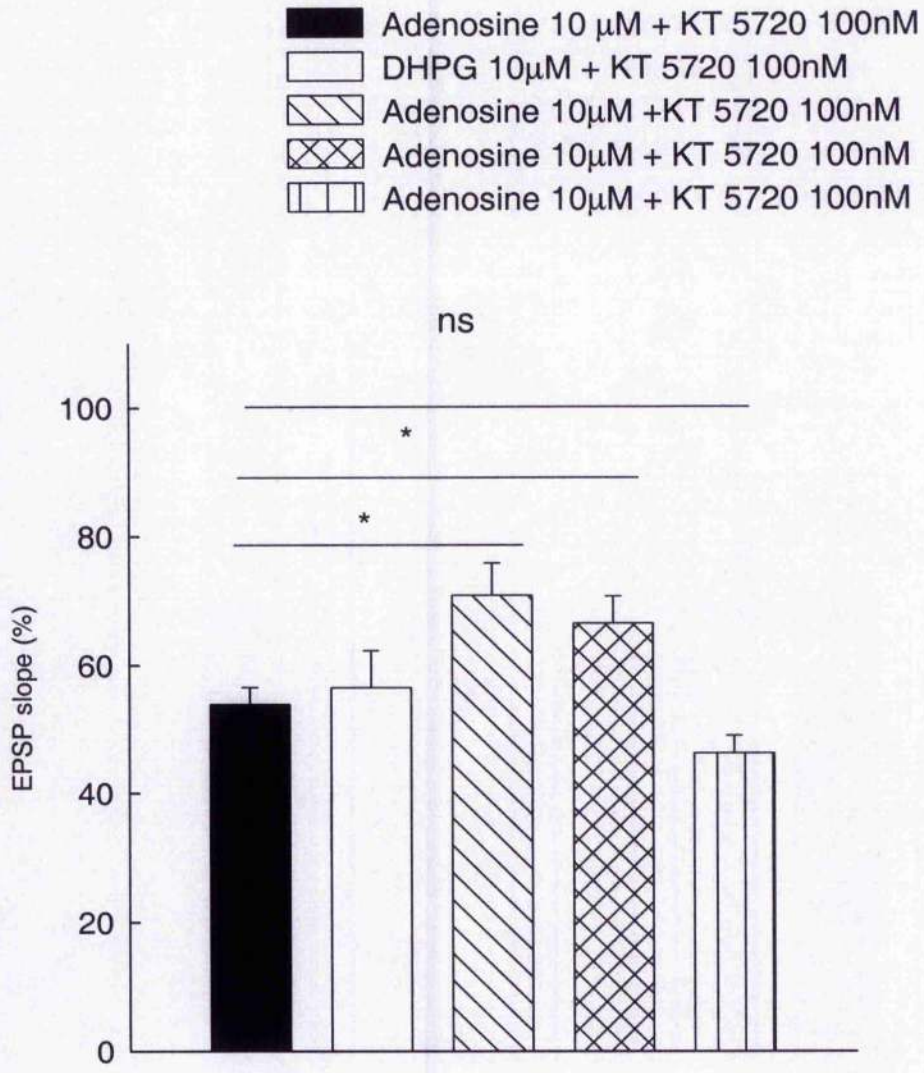

Figure 3.26. Histogram showing the influence of PKA inhibitor KT5720 100nM on the interaction between adenosine 10μM and DHPG 10μM on fEPSPs slope in hippocampal slices. The columns indicate the mean ± S.E.M for n = 4 slices. * P<0.05

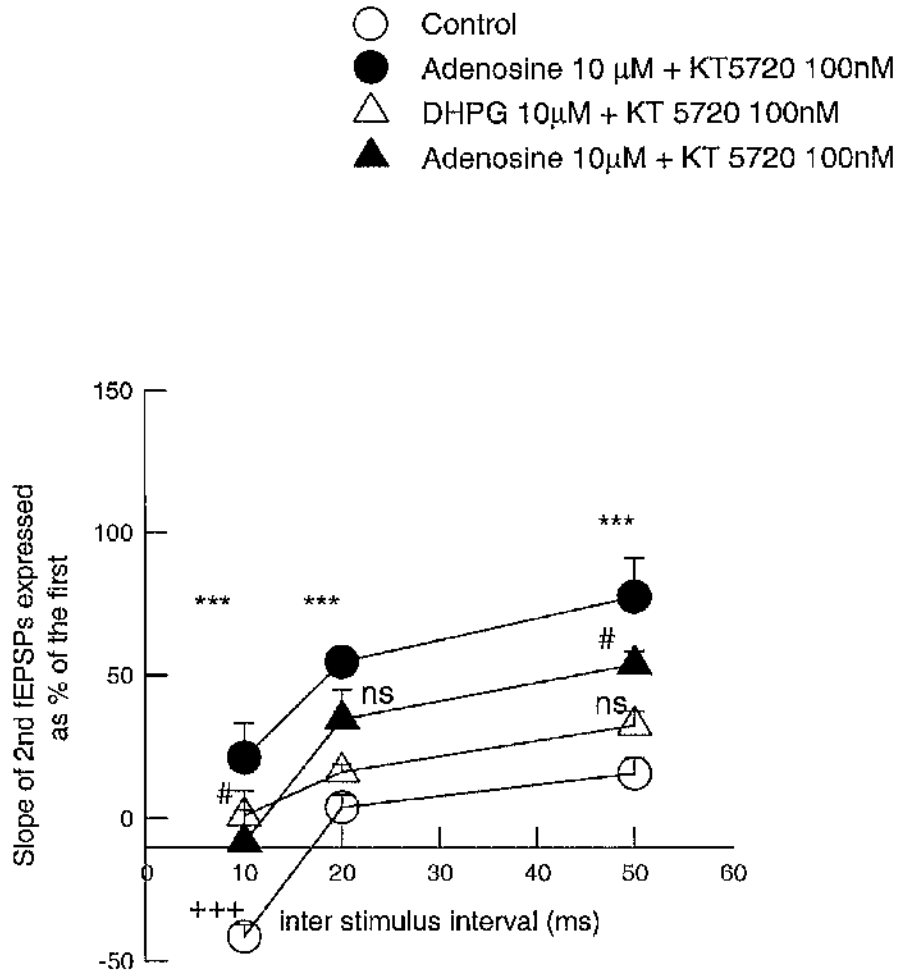

Figure 3.27. Modulation of orthodromic evoked paired-pulse fEPSPs by adenosine 10μM and DHPG 10μM in the presence of PKA inhibitor KT5720 100 nM. Each point represents mean ± S.E.M for n = 4 slices. Only the first adenosine response after DHPG, obtained at 20 minutes, is shown for clarity. *** P<0.001 for difference between controls and adenosine before DHPG. # P<0.05 between adenosine before and after DHPG in the presence of KT5720. +++ P<0.001 between controls and DHPG + KT5720.

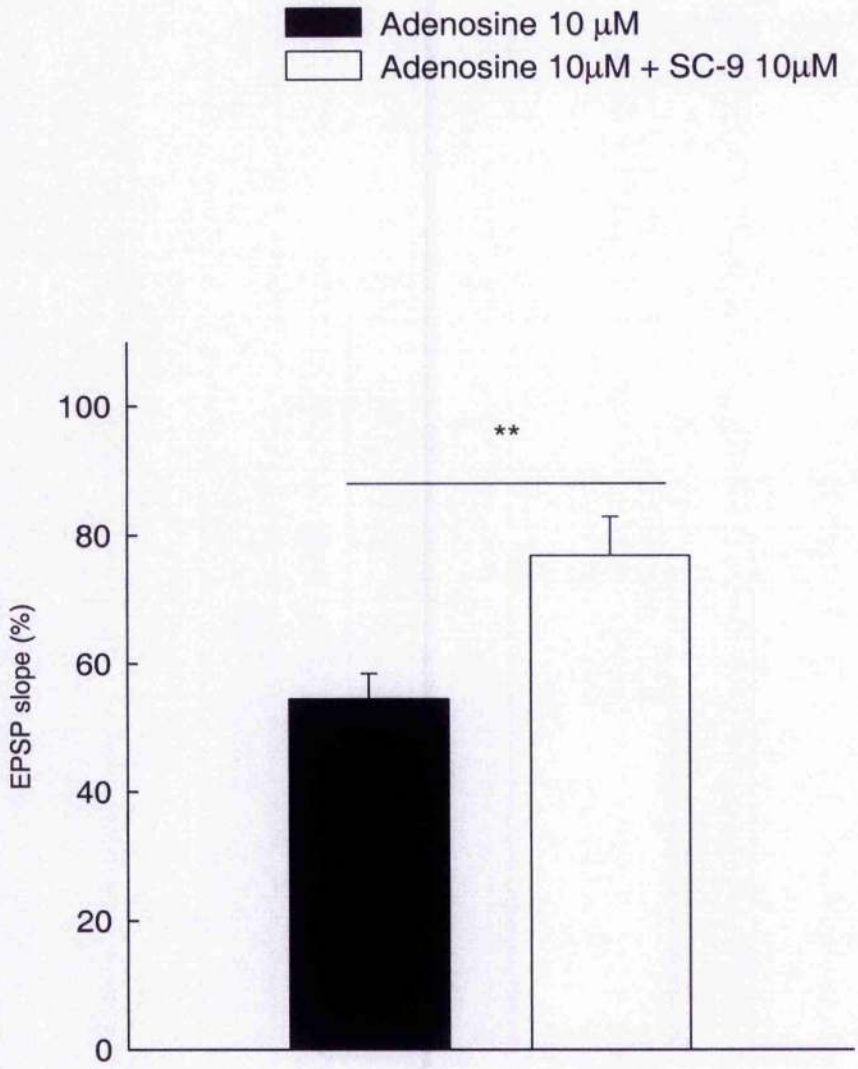

Figure 3.28. Histogram showing the influence of PKC activator 5-chloro-N-(6-phenylhexyl)-1-naphthalenesulfonamide (SC-9) 10μM on the effects of adenosine 10μM on the fEPSPs slope in hippocampal slices. The columns indicate the mean ± S.E.M for n = 4 slices. ** $P<0.01$

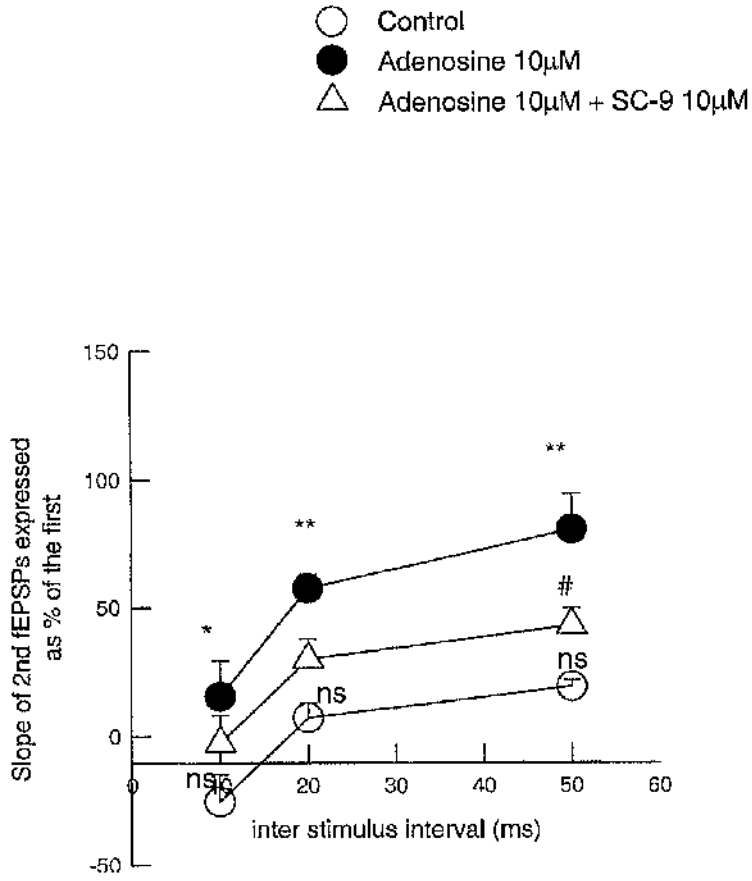

Figure 3.29. Modulation of orthodromic evoked paired-pulse fEPSPs by adenosine 10μM and adenosine + SC-9 10μM (PKC activator). Each point represents mean ± S.E.M for n = 4 slices. * P<0.05, ** P<0.01 for difference between controls and adenosine before SC-9. # P<0.05 between adenosine before and after SC-9.

Identification of adenosine receptors

In order to determine whether the effect of mGluR activation on adenosine responses was mediated via an action on A_1 adenosine receptors, experiments were performed in which slices were superfused with adenosine deaminase ($0.1U.ml^{-1}$). Adenosine deaminase was included to remove endogenous adenosine from both A1 and A2 adenosine receptors and thus ensure that receptors were activated only by experimentally receptor-selective compounds. A_1 receptors were activated using the selective agonist 8-cyclopentyladenosine (CPA). When superfused alone, CPA 20 nM significantly depressed the EPSP slope (Fig. 3.30) and enhanced paired-pulse facilitation (Fig. 3.31) as has been reported in previous studies (Nikbakht and Stone, 2001). Following the application of DHPG (10 μM), the response to CPA was reduced significantly on EPSP slope ($p < 0.05$, $n = 4$)(Fig. 3.30) and paired–pulse responses (Fig. 3.31). The ability of the deaminase activity to remove adenosine was confirmed by showing its prevention of the responses to adenosine in separate experiments (Fig. 3.32 & Fig. 3.33).

The possible involvement of adenosine A_{2A} receptors was then examined by including the selective antagonist ZM241385. When superfused at a concentration of 50 nM, which should be selective for the A_{2A} receptor subtype, this agent had no effect of its own on potential size and did not modify the depression of EPSP slope (Fig. 3.34) or paired-pulse inhibition induced by adenosine or DHPG (Fig. 3.35). Following the application of DHPG, however, adenosine responses were still reduced compared with their initial level, indicating that ZM241385 had not modified the antagonistic effect of DHPG.

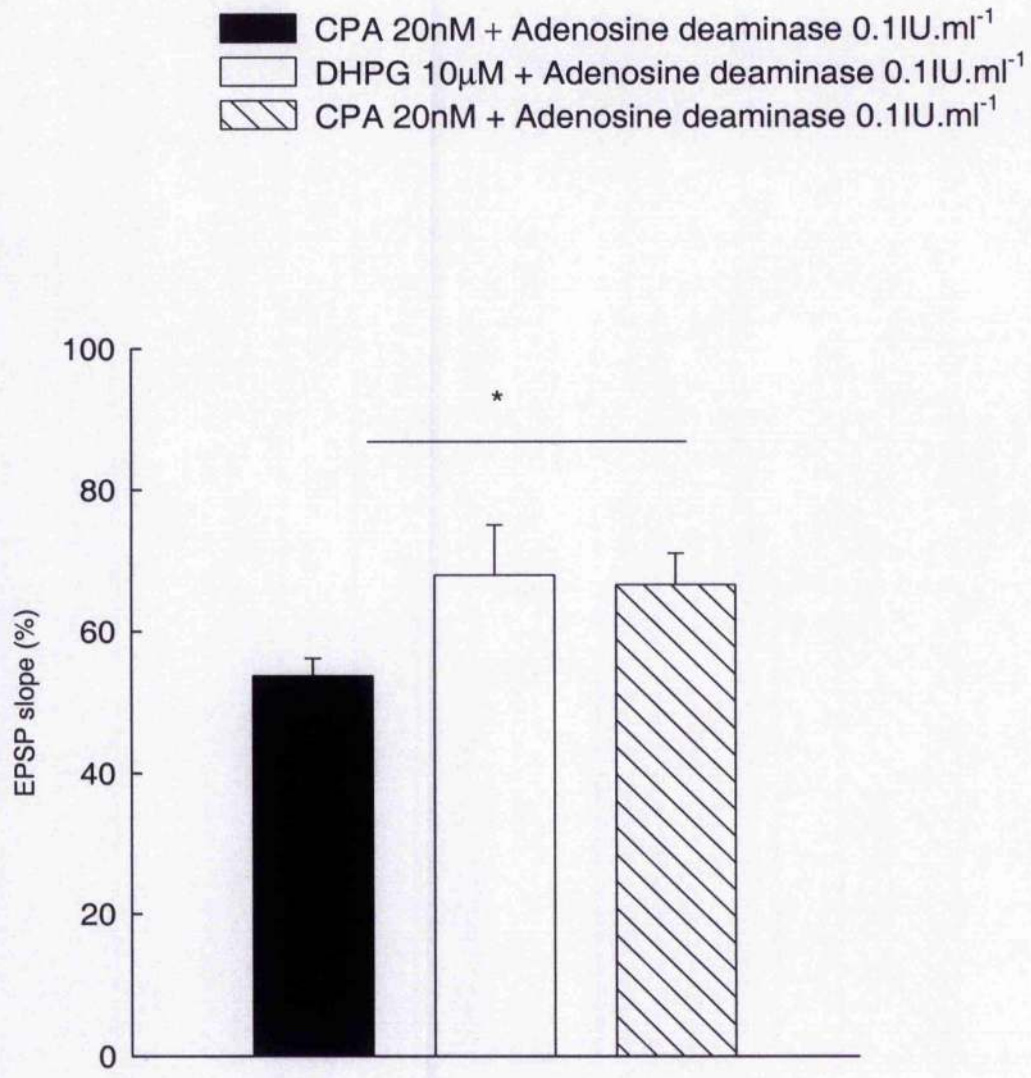

Figure 3.30. Histogram showing the effect of CPA 20nM and DHPG 10μM in the presence of adenosine deaminase 0.1 $IU.ml^{-1}$ on fEPSPs evoked from the CA1 region of hippocampal slices. Each vertical bar represents mean ± S.E.M for n = 4 slices. * $P<0.05$

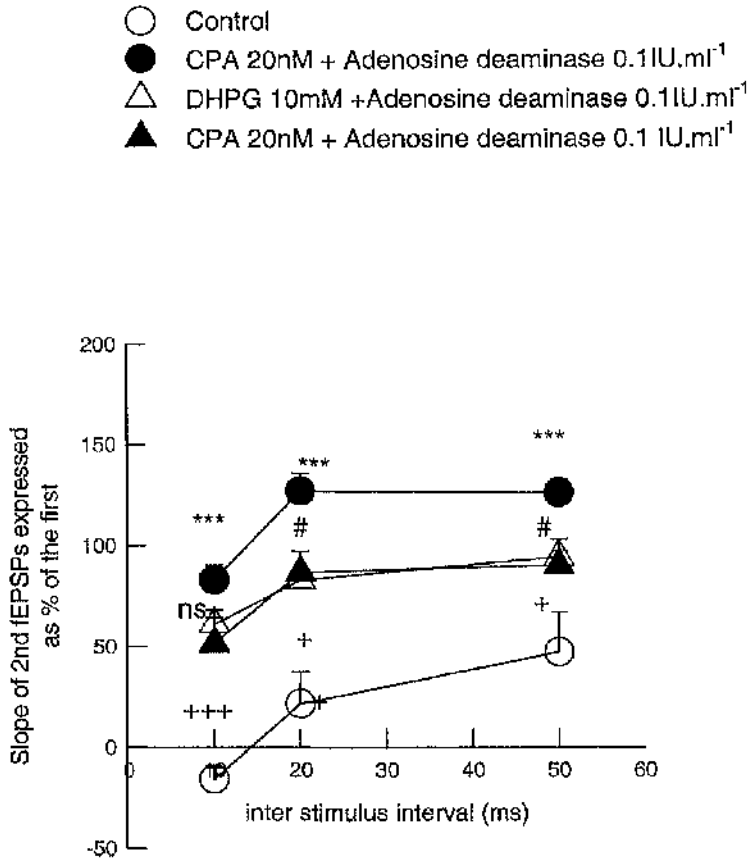

Fig. 3.31. Modulation of orthodromic evoked paired-pulse fEPSPs by CPA 20nM and DHPG 10μM in the presence of adenosine deaminase 0.1 $IU.ml^{-1}$. Each point represents mean ± S.E.M for n = 4 slices. *** P<0.05 for difference between control and CPA before DHPG. # P<0.05 for difference between CPA before and after DHPG. + P<0.05, ++ P<0.01 for difference between control and DHPG.

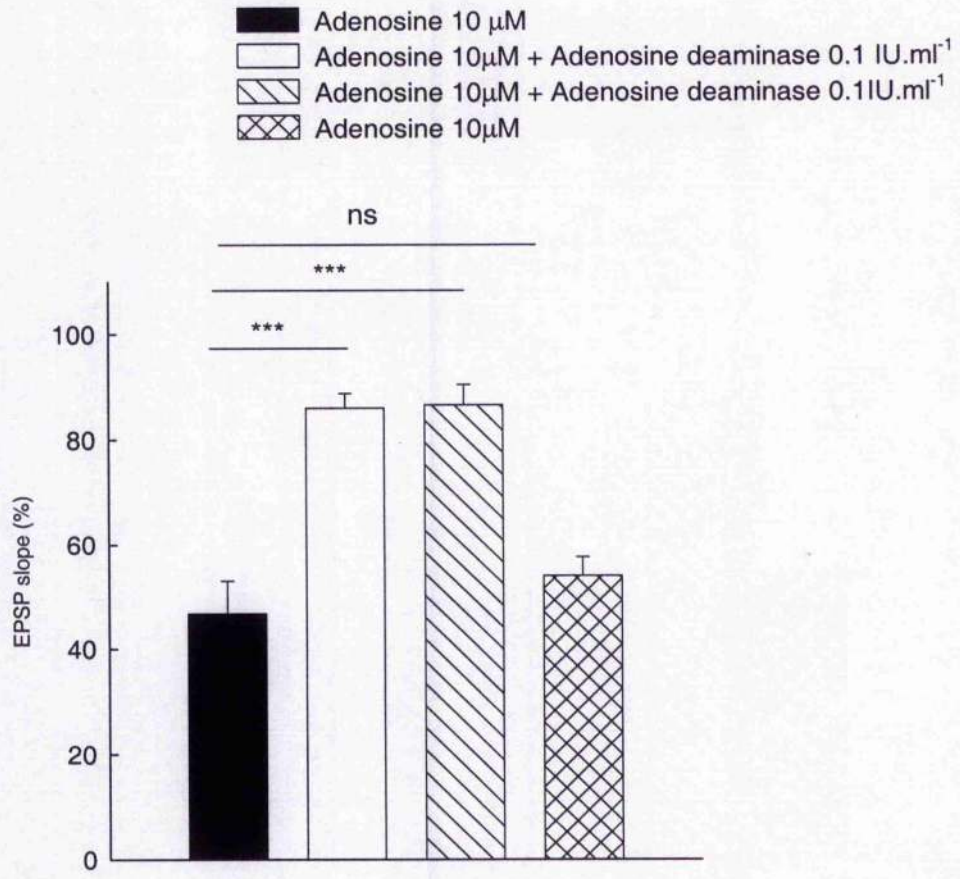

Figure 3.32. Histogram showing responses to adenosine 10μM and the prevention of these responses in the presence of adenosine deaminase 0.1IU.mll^{-1} on fEPSPs evoked from the CA1 region of hippocampal slices. Adenosine deaminase present during the second and third applications. Each vertical bar represents mean ± S.E.M for n = 4 slices. *** P<0.001

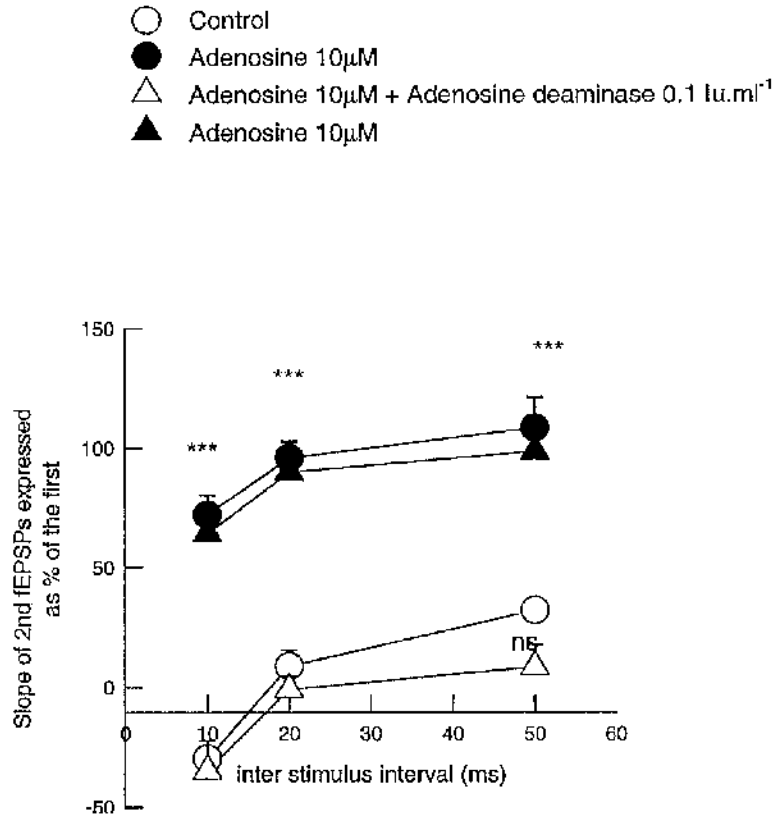

Figure 3.33. Modulation of orthodromic evoked paired-pulse fEPSPs by adenosine 10μM in the presence of adenosine deaminase 0.1 $IU.ml^{-1}$. Data are shown for interstimulus intervals of 10, 20 and 50 ms in control slices and the effect of adenosine before, during and after superfusion with ADA. Each point represents mean ± S.E.M for n = 4 slices.

*** $P<0.001$.

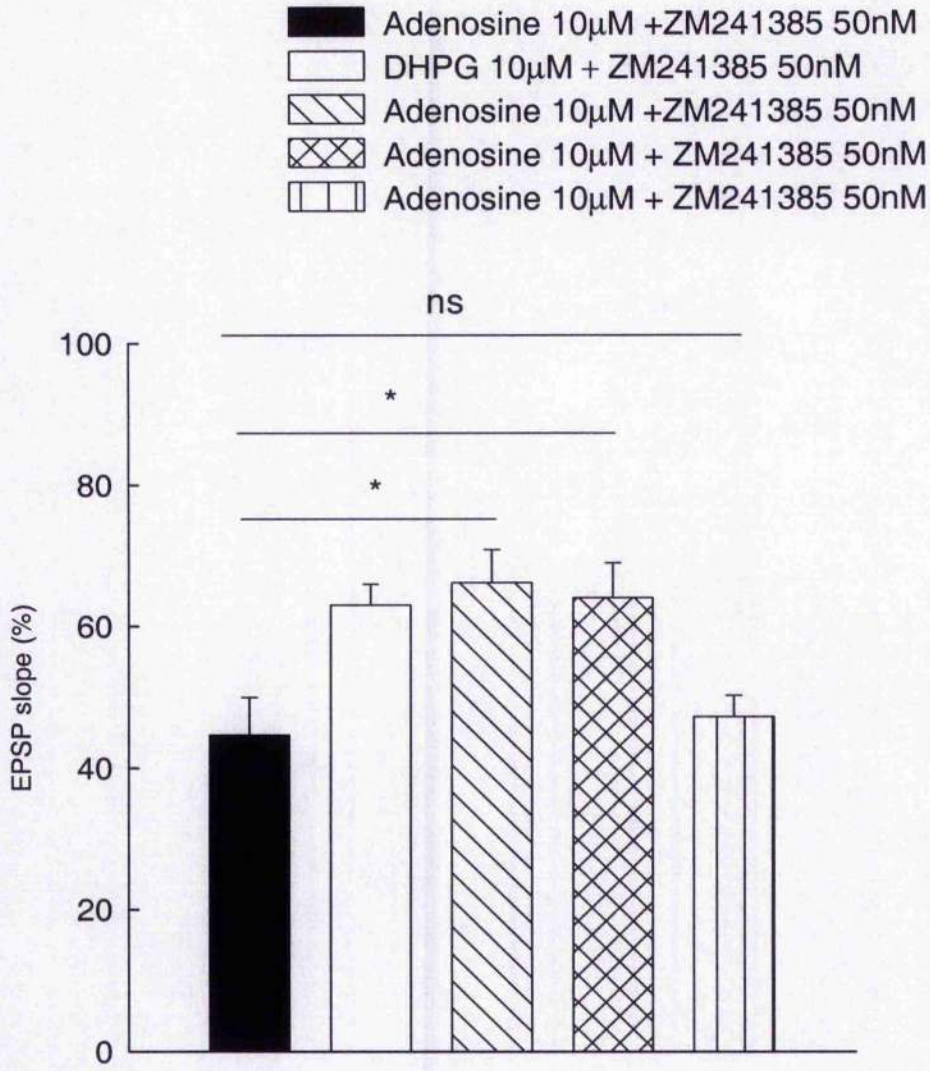

Figure 3.34. Histogram showing the interaction between adenosine 10μM and DHPG 10μM in the presence of ZM241385 50nM on fEPSPs evoked from the CA1 region of hippocampal slices. Each vertical bar represents mean ± S.E.M for n = 4 slices. * $P<0.05$

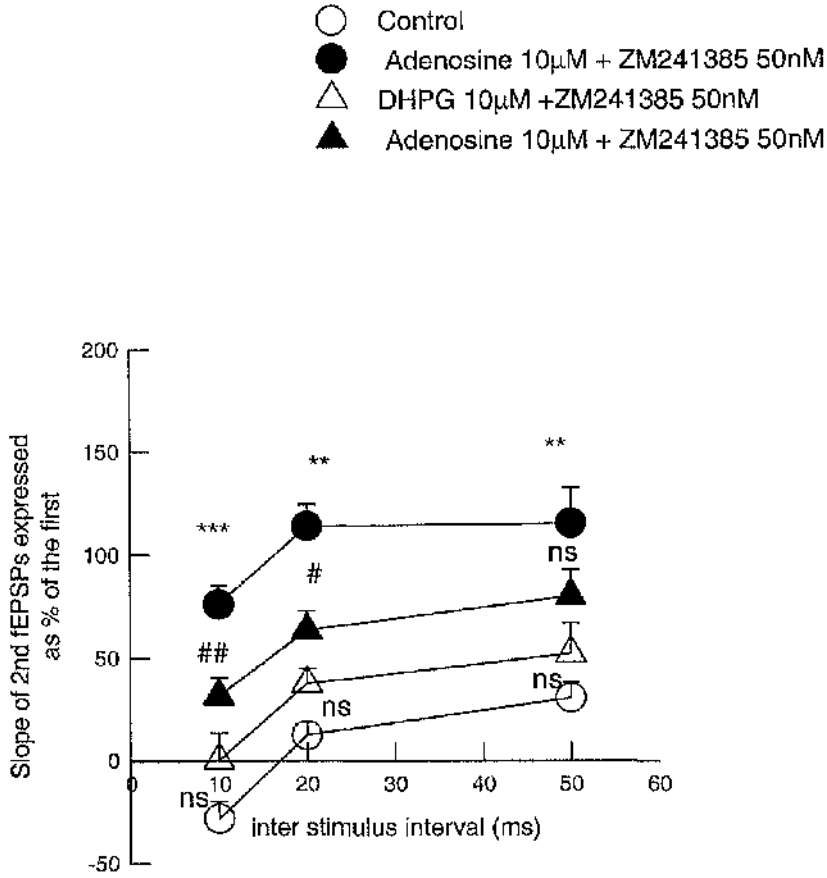

Fig. 3.35. Modulation of orthodromic evoked paired-pulse fEPSPs by adenosine 10μM and DHPG 10μM in the presence of ZM241385 50nM. Only the first adenosine response after DHPG, obtained at 20 minutes, is shown for clarity. Each point represents mean ± S.E.M for n = 4 slices. *** P<0.001 and ** P<0.01 between controls and adenosine before DHPG in the presence of ZM241385. # P<0.05 and ## P<0.01 between adenosine before and after DHPG in the presence of ZM241385.

Nitric oxide suppression of presynaptic responses to adenosine

Interaction between adenosine and SNAP on evoked excitatory post synaptic potentials in hippocampal slices

The activation of NMDA receptors can suppress the inhibitory action of adenosine on synaptic transmission in hippocampal slices (Bartrup & Stone, 1988, 1990; Nikbakht & Stone, 2001). We have now explored the possible involvement of NO in this interaction. A concentration of 10μM adenosine was selected for these experiments as previous work has shown that this consistently results in approximately 60% depression of evoked potentials that allows an increase or decrease of response slope to be detected. Adenosine 10μM depressed single EPSPs by 60.14% ± 2.58 ($p < 0.001$; n = 5) of the initial control size. When SNAP was superfused at a concentration of 10μM, no significant change was observed either in baseline EPSP slope or in the responses to two applications of adenosine obtained 10 mins apart, which reduced fEPSPs slope by 55.9% ± 5.69 and 53.24% ± 4.3 (n = 3)(Fig. 3.36).

In contrast, the superfusion of SNAP at a higher concentration of 100μM induced a long-lasting potentiation of fEPSPs slope which attained a level of 141% ± 10.98 (n = 4) compared with the baseline slope (NO-LTP). Before the application of SNAP, adenosine reduced fEPSP slope by 54.46% ± 5.6 (n = 4) of the control size, whereas during the application of SNAP two adenosine responses obtained 10 mins apart were significantly smaller, with reductions of 30.5% ± 3.29 and 20.6% ± 2.02 (n = 4) respectively (Fig. 3.37a & 3.37b).

Paired-pulse interactions

An interstimulus intervals of 10, 20, 50 ms, Paired-pulse stimulation induced the pattern of paired-pulse inhibition at 10 ms and facilitation at 20 and 50 ms reported previously (Higgins and Stone, 1996; Nikbakht and Stone, 2000). Adenosine alone and with SNAP 10μM changed the inhibitory component to facilitation at 10 ms interstimulus interval and increased the facilitation at 20 and 50 ms interstimulus intervals (Fig. 3.38). Meanwhile, when perfused with SNAP 100μM, this concentration was sufficient to reduce significantly the effects of adenosine on paired-pulse phenomena (Fig. 3.39). Induction of NO-LTP after perfusion of SNAP 100μM modified the paired-pulse interactions at 10 and 20 ms interstimulus intervals.

Effect of EPSP size on adenosine sensitivity

In order to determine whether the reduction of adenosine response is due to LTP or NO, the stimulus was reduced to restore potential size to control level after the induction of LTP by NO. The reduction of adenosine response (41.47% ± 4.79, $P<0.05$ n=3) was still observed on single and paired-pulses EPSP (Fig. 3.40 & Fig. 3.41). Adenosine alone decreased the fEPSPs slope by 57.94 ± 5.98 (n=3).

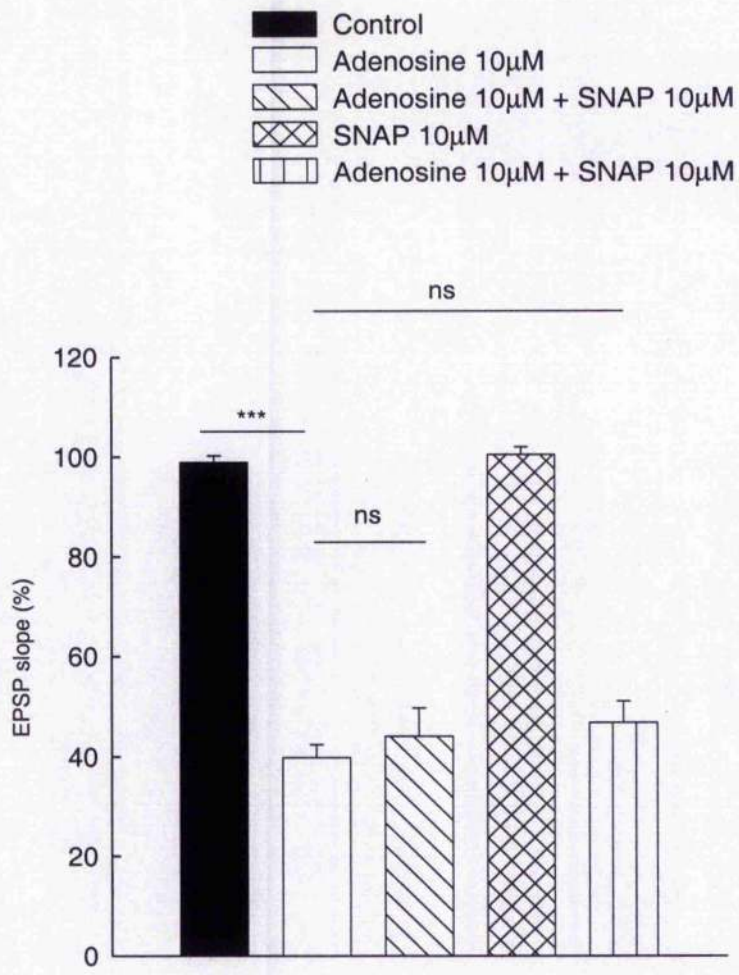

Figure 3.36. Histogram showing the interaction between adenosine 10μM and SNAP 10μM on fEPSPs evoked from the CA1 region of hippocampal slices. Each vertical bar represents mean ± S.E.M for n = 3 slices. *** $P<0.001$

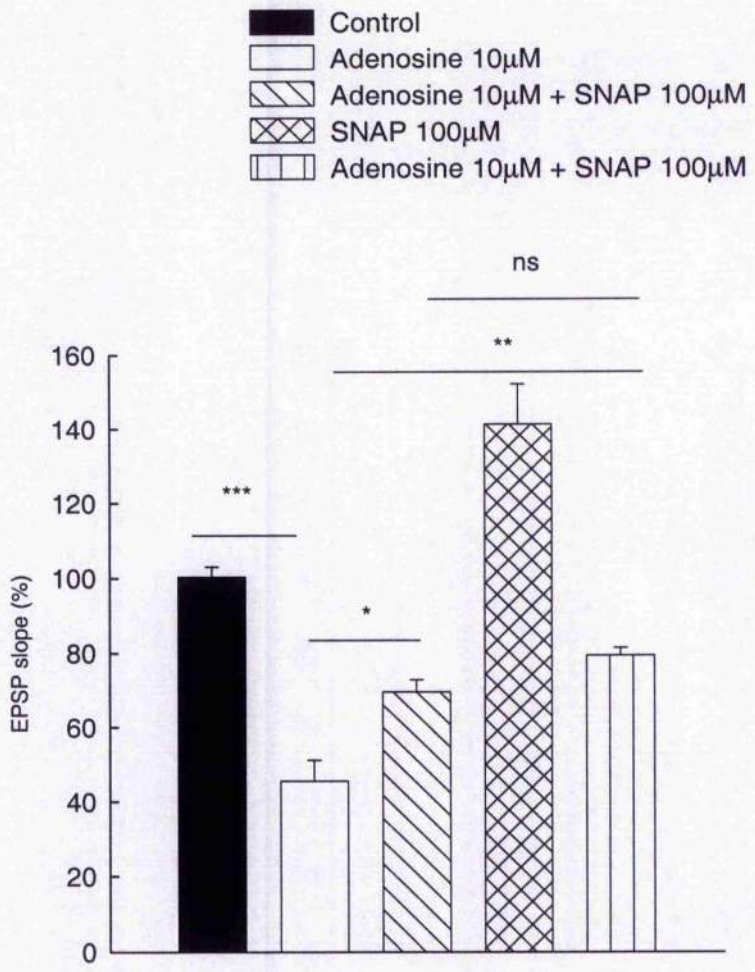

Figure 3.37a. Histogram showing the interaction between adenosine 10μM and SNAP100μM on fEPSPs evoked from the CA1 region of hippocampal slices. Each vertical bar represents mean ± S.E.M for n = 4 slices. * P<0.05, ** P<0.01, *** P<0.001

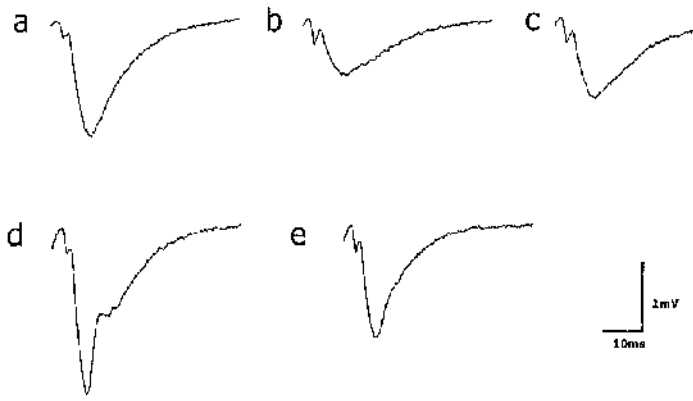

Fig. 3.37b. The original records of orthodromic evoked excitatory postsynaptic potentials illustrate the interaction between adenosine and SNAP. (a) is a control EPSPs, (b) the effect of adenosine 10μM, record (c) shows the reduced response to adenosine in the presence of SNAP 100μM (first response), (d) is taken in the presence of SNAP 100μM alone, and (e) the reduced effect of adenosine in the presence of SNAP 100μM (second response).

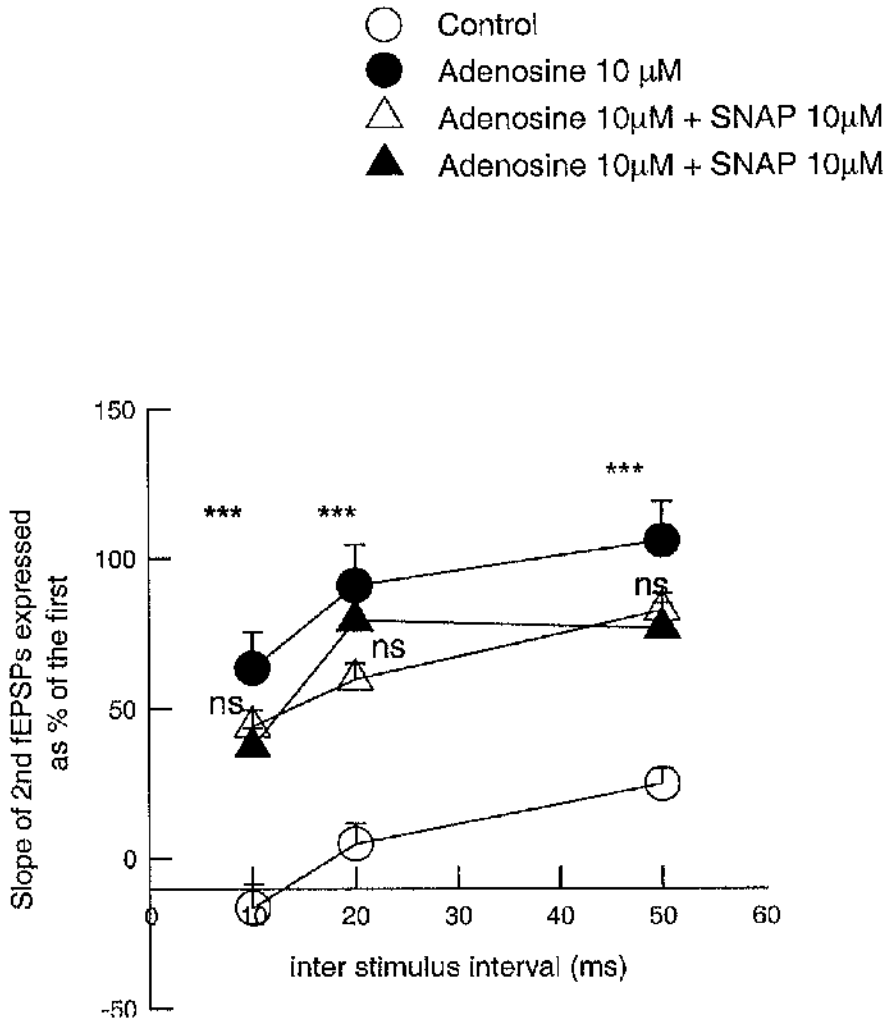

Figure 3.38. Modulation of orthodromic evoked paired-pulse fEPSPs by adenosine 10μM in the presence of SNAP 10μM. Data are shown for interstimulus intervals of 10, 20 and 50 ms in control slices and the effect of adenosine alone, and Adenosine + SNAP. Each point represents mean ± S.E.M for n = 3 slices. *** P<0.001

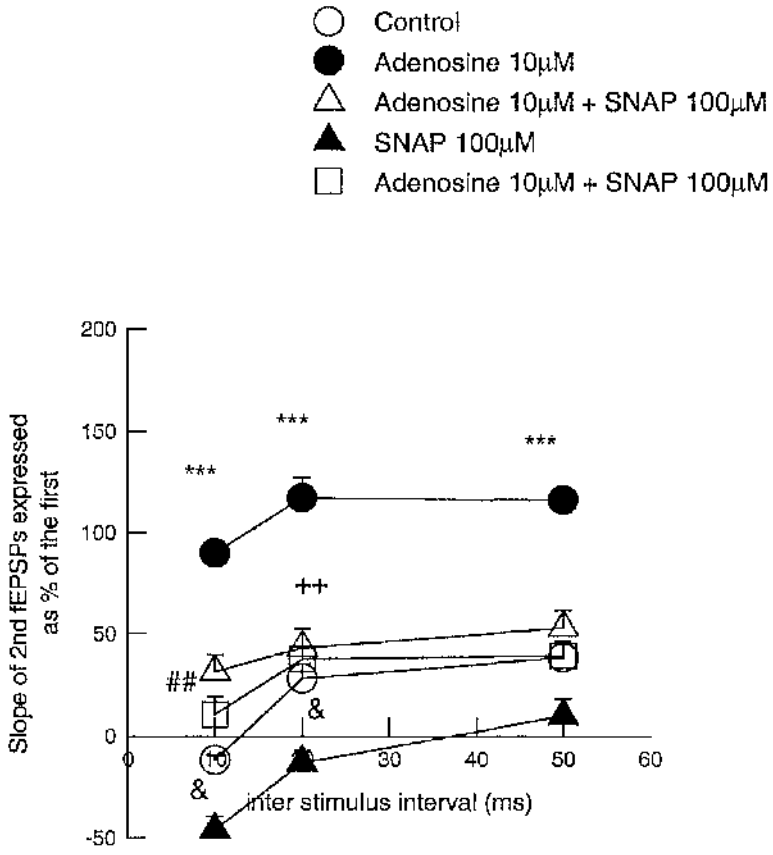

Figure 3.39. Modulation of orthodromic evoked paired-pulse fEPSPs by adenosine 10μM in the presence of SNAP 100μM. Data are shown for interstimulus intervals of 10, 20 and 50 ms in control slices and the effect of adenosine alone, SNAP alone and Adenosine + SNAP. Each point represents mean ± S.E.M for n = 4 slices. *** P<0.001 between adenosine and control, ++ P<0.01 between adenosine and adenosine + SNAP, ## between adenosine and adenosine +SNAP, & P<0.05 between control and SNAP.

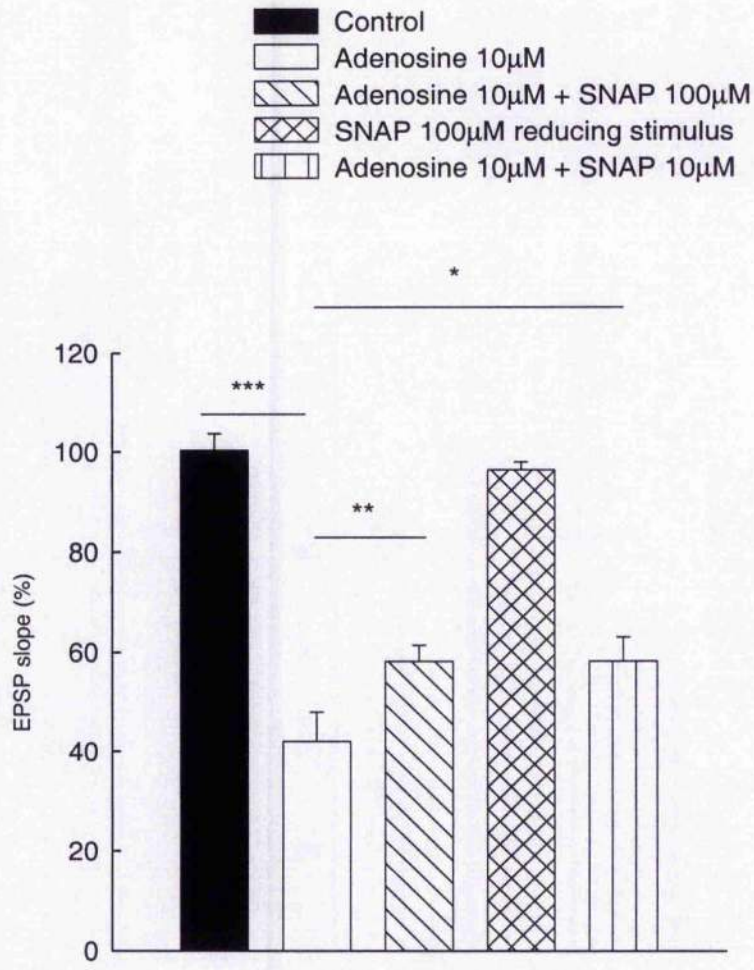

Figure 3.40. . Histogram showing the interaction between adenosine 10μM and SNAP 100μM on fEPSPs evoked from the CA1 region of hippocampal slices with stimulus strength adjusted to restore potentials to control size. Each vertical bar represents mean ± S.E.M for n = 3 slices.

* P<0.05, ** P<0.01, *** P<0.001

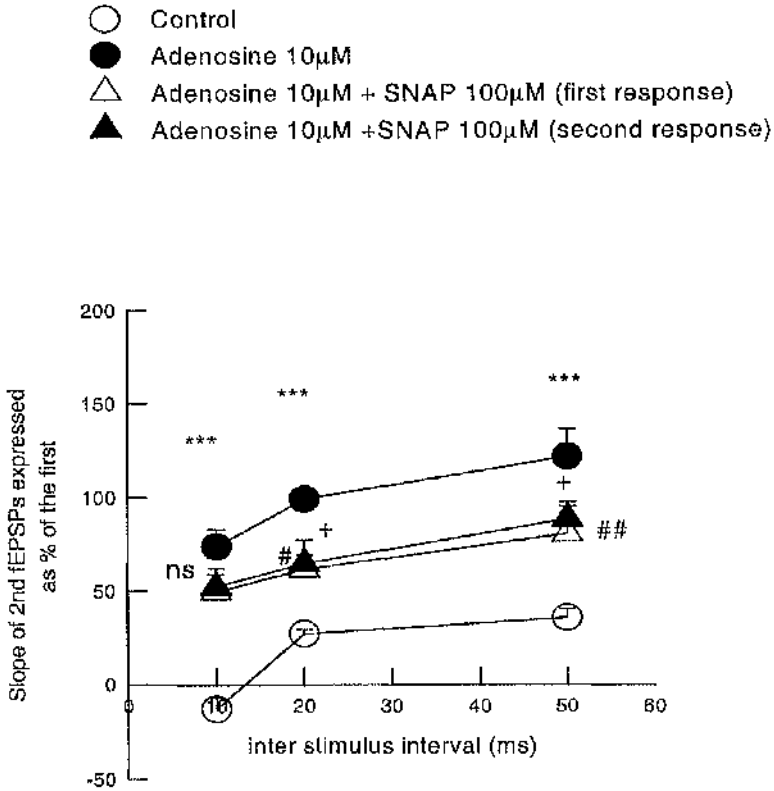

Figure 3.41. Modulation of orthodromic evoked paired-pulse fEPSPs by adenosine 10μM alone, adenosine + SNAP 100μM and adenosine + SNAP 100μM after reducing stimulus strength to restore potentiated EPSP size to control level. Data are shown for interstimulus intervals of 10, 20 and 50 ms in control slices and the effect of adenosine alone, and Adenosine + SNAP, and adenosine +SNAP after reducing stimulus. Each point represents mean ± S.E.M for n = 3 slices. *** P<0.001 between adenosine and control, + P<0.05 between adenosine and adenosine + SNAP, # P<0.05 between adenosine and adenosine + SNAP after reducing stimulus, ## P<0.01 between adenosine and adenosine + SNAP after reducing stimulus.

Role of adenosine blockade in SNAP-induced LTP

In order to determine whether the induction of LTP with SNAP was itself partly due to the reduction of adenosine sensitivity, adenosine deaminase was applied at 0.1 $IU.ml^{-1}$. Adenosine deaminase at this concentration has already been shown sufficient to remove adenosine added to the slices. Adenosine deaminase itself had no effect on EPSP slope. When the slices were also perfused with SNAP at 100μM for 10 min, SNAP induced a long-term potentiation of EPSP slope which attained a maximum level of 165.3% ± 6.6 (n = 4) of baseline, and remained elevated at 141.55% ± 5.35 of baseline after 40 min of washing with ACSF containing adenosine deaminase alone (Fig. 3.42). These values were not significantly different from those obtained in response to SNAP in the absence of adenosine deaminase.

Paired-pulse interactions

In the control state a small degree of paired-pulse depression was observed at the 10 ms interval, with potentiation of the second spike at the longer intervals. The effect of SNAP 100μM in the presence of adenosine deaminase 0.1 $IU.ml^{-1}$ was to induce a significant inhibition at 10, 20, 50 ms intervals. Paired-pulse inhibiton at the 10 ms interval was still significant after 10 min. of washing (Fig. 3.43).

The effects of 2AP5 on NO-LTP

We then examined whether the LTP induced by SNAP involved the activation of NMDA receptors, since NMDA receptors have previously been shown to reduce sensitivity to adenosine (Nikbakht and Stone 2001). Superfusion with 2-amino-5-

phosphono-pentanoic acid (2AP5) at 50mM did not prevent the SNAP-induced increase of fEPSP slope which attained a level of 140.87% ± 10.21 (n = 4) of the control potential size after 40 min. Similarly, 2AP5 did not prevent the reduction of adenosine response by NO (24.53% ± 9.08 and 31.43% ± 5.44, n = 4, $P < 0.05$) compared with the control (adenosine plus AP5) response size of 53.75% ± 5.56, (n = 4) (Fig. 3.44).

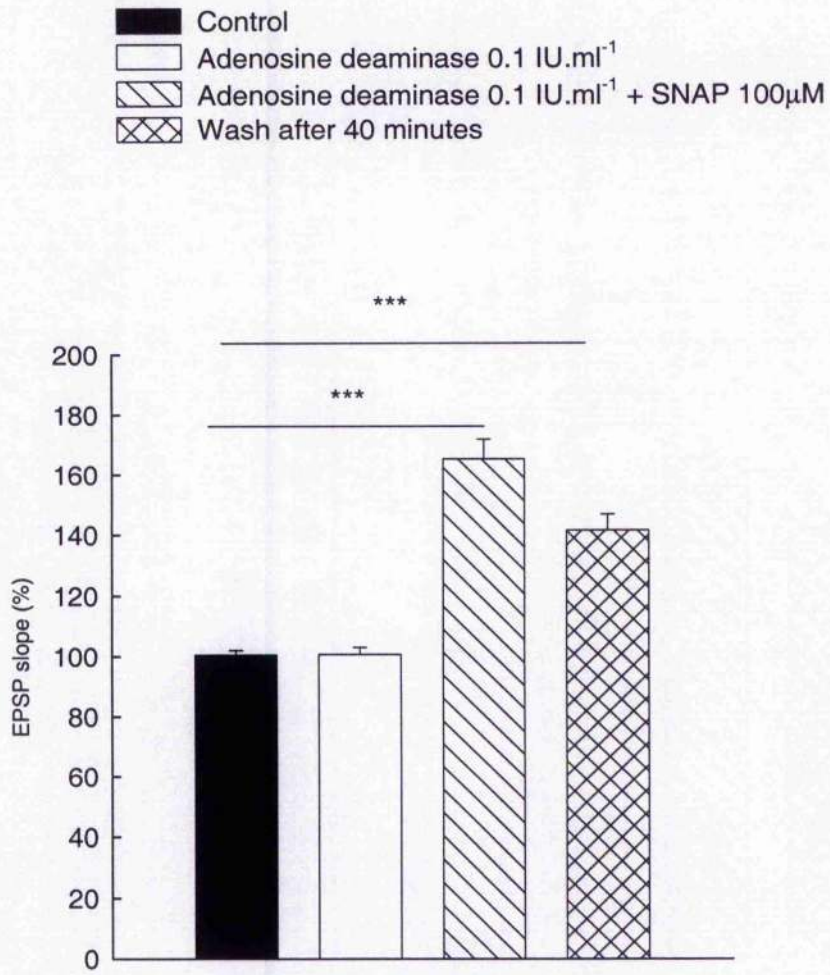

Figure 3.42. Histogram showing the effect of SNAP 100μM in the presence of adenosine deaminase 0.1IU.ml^{-1} on fEPSPs evoked from the CA1 region of hippocampal slices. Each vertical bar represents mean ± S.E.M for n = 4 slices. *** P<0.001

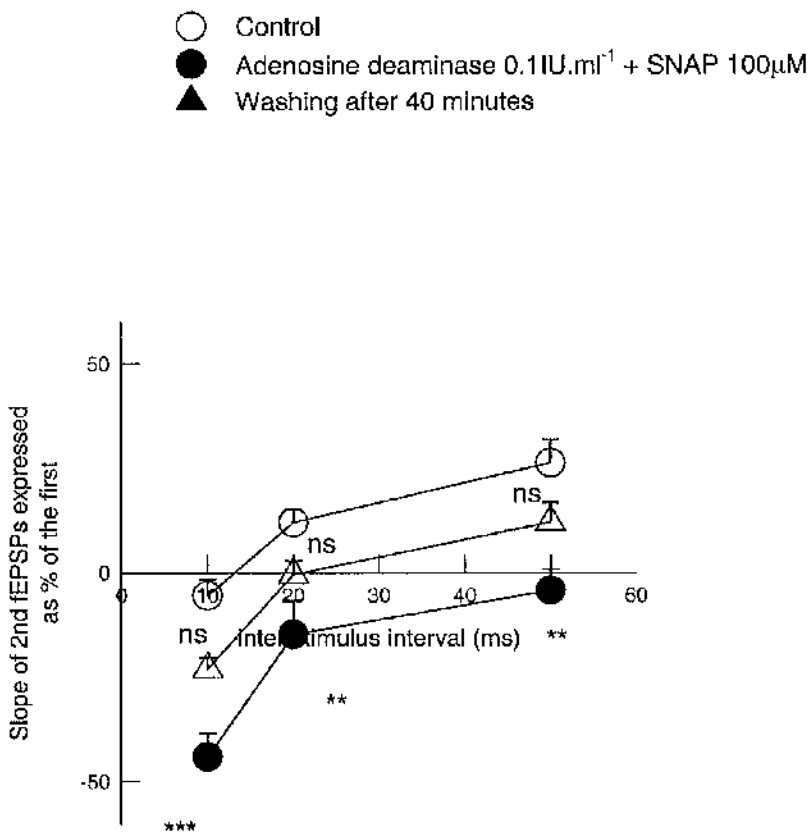

Figure 3.43. Modulation of orthodromic evoked paired-pulse fEPSPs by SNAP 100µM in the presence of adenosine deaminase 0.1IU.ml^{-1}. Each point represents mean ± S.E.M for n = 4 slices. *** P<0.001, ** P<0.01 between control and SNAP + ADA, # P<0.05 between control and washing after 10 min.

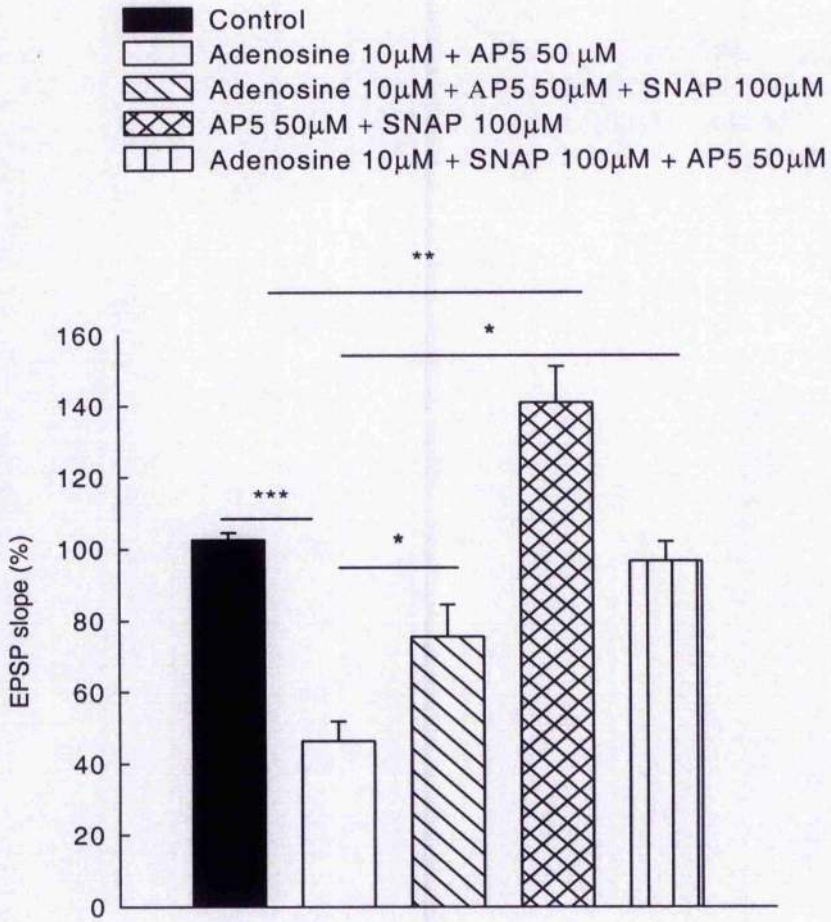

Figure 3.44. Histogram showing the effect of AP5 50μM on NO-LTP and interactions between adenosine and SNAP 100μM on fEPSPs evoked from the CA1 region of hippocampal slices. Each vertical bar represents mean ± S.E.M for n = 4 slices. * P<0.05, ** P<0.01, *** P<0.001

Interaction between SNAP and baclofen

In order to determine the specifity of interaction between adenosine and NO, the interaction between the $GABA_B$ agonist baclofen and NO was investigated.

The $GABA_B$ receptor agonist baclofen at 2μM depressed the fEPSP slope by 65.88% ± 4.06 (n = 5). During the first few minutes of perfusion with SNAP at 100μM, the depression by baclofen remained unchanged at 63.0% ± 4.70 of baseline slope. A subsequent application of baclofen 10 mins later, however, was significantly reduced to 40.9% ± 8.62 (n = 5) (Fig. 3.45).

Paired-pulse interaction

In the paired-pulse paradigm, superfusion of baclofen 2μM alone reversed the inhibition to facilitation at 10 ms and increased significantly the facilitation at 20 and 50 ms. SNAP at 100μM had no effect on paired-pulse interactions and did not modify the size of the first response to baclofen. At the 10 and 20 ms interstimulus intervals, a second response to baclofen obtained 10 mins later was significantly smaller than the controls (Fig. 3.46).

Effect of EPSP size on baclofen sensitivity

In order to determine whether the reduction of baclofen response is due to LTP or NO, the stimulus was changed to restore potential size to control level after induction of NO-LTP. No significant change was observed in baseline fEPSP slope and response between the first baclofen application (66.1% ± 3.48, n=4) and baclofen after SNAP and reduced stimulus strength in single fEPSP (63.12% ± 2.59, n=4) (Fig. 3.47) and paired-pulse interactions (Fig. 3.48).

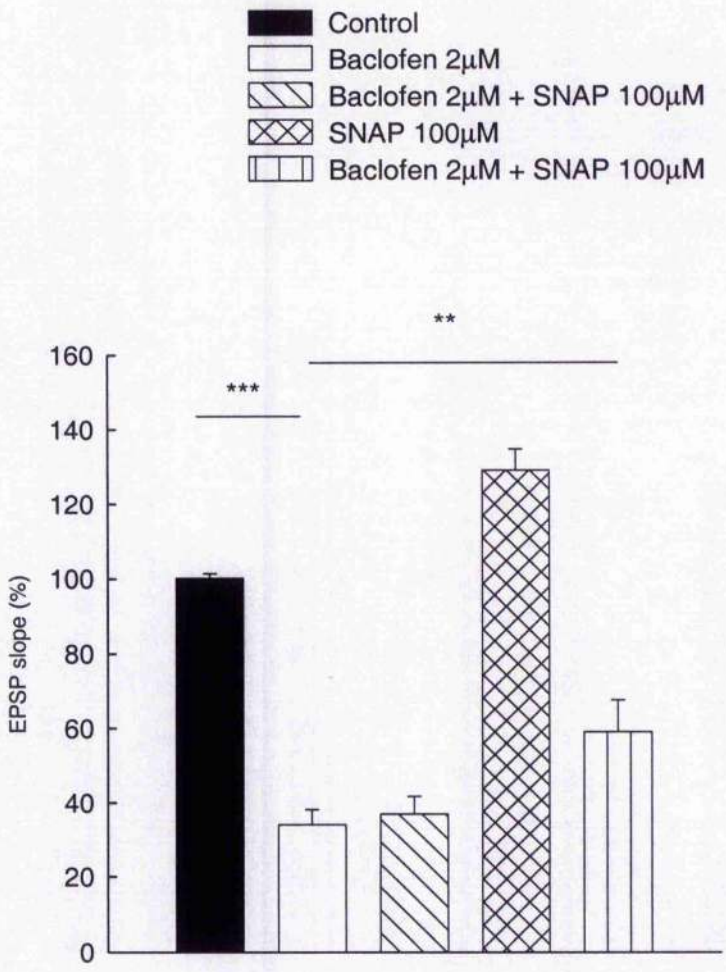

Figure 3.45. Histogram showing the interaction between baclofen 2μM and SNAP 100μM on fEPSPs evoked from the CA1 region of hippocampal slices. Each vertical bar represents mean ± S.E.M for n = 4 slices. ** P<0.01, *** P<0.001

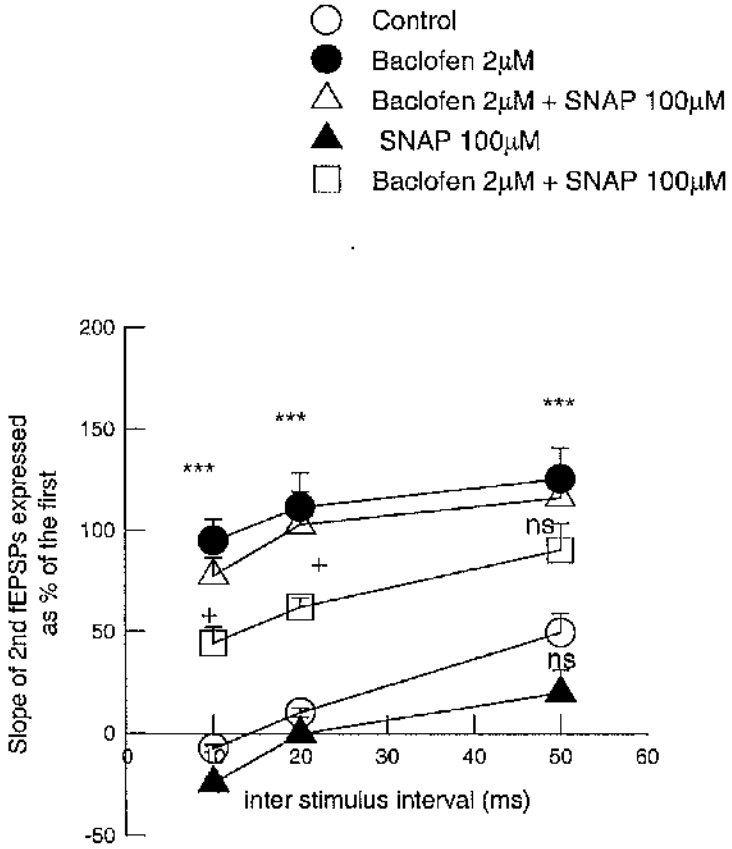

Figure 3.46. Modulation of orthodromic evoked paired-pulse fEPSPs by baclofen 2μM and SNAP 100μM. Each point represents mean ± S.E.M for n = 4 slices. *** P<0.001 control and baclofen and also between control and baclofen + SNAP before inducing LTP. + P<0.05 between baclofen and baclofen + SNAP after inducing LTP.

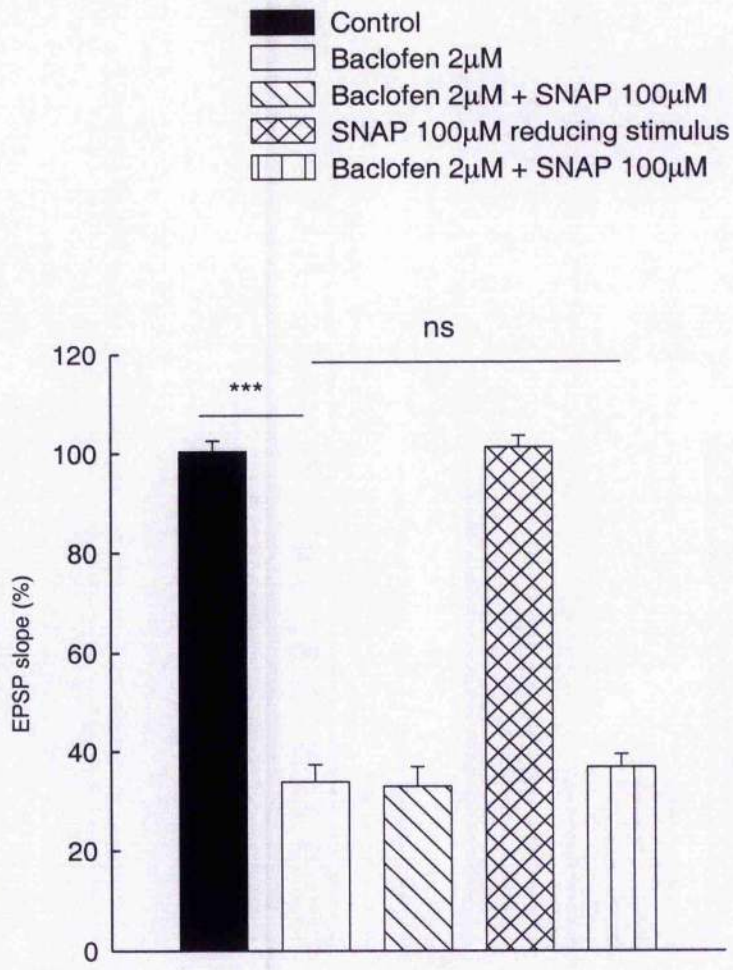

Figure 3.47. Histogram showing the interaction between baclofen 2μM and SNAP100μM after reducing the stimulus for restoring the potential size to control levels on fEPSPs evoked from the CA1 region of hippocampal slices. Each vertical bar represents mean ± S.E.M for n = 4 slices. *** $P<0.001$

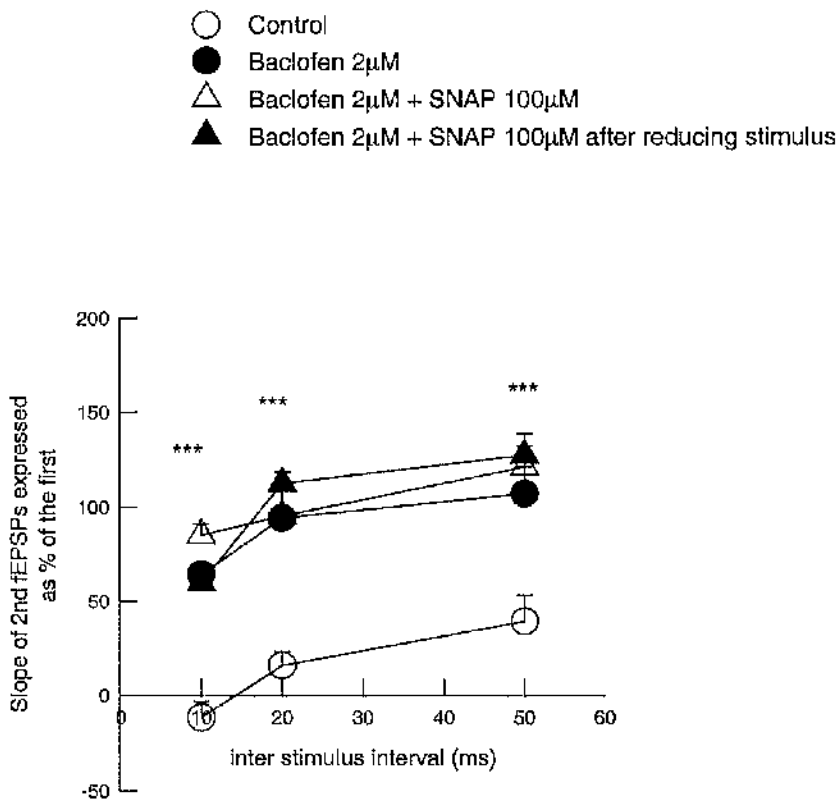

Figure 3.48. Modulation of orthodromic evoked paired-pulse fEPSPs by baclofen 2μM and SNAP 100μM after reducing the stimulus strength to restore the potential response size to control levels. Each point represents mean ± S.E.M for n = 4 slices. *** P<0.001 control and baclofen and also between control and baclofen + SNAP.

The effect of guanylyl cyclase inhibitor on interaction between adenosine and SNAP

The guanylyl cyclase inhibitor ODQ 10μM (as showed with Boulton et al., 1995) was applied to determine whether the effects of NO were mediated via the activation of guanylyl cyclase.

The effect of SNAP 100μM on adenosine responses was blocked by application of the guanylyl cyclase inhibitor ODQ 10 μM.The first response to adenosine alone was 58.71% ± 2.23, n=5 and the first and second adenosine responses to SNAP in the presence of ODQ were (49.38 ± 3.41 n=5) and (51.3 ± 3.03 n=5) (Fig.3.49).

Involvement of adenosine A1 receptors

Although the presynaptic inhibitory effects of adenosine are generally considered to be mediated by A1 receptors, we have also examined the interaction between NO and the A1 receptor selective agonist, N6-cyclopentyladenosine (CPA). At 20nM, CPA significantly suppressed the fEPSP slope (40.90% ± 4.82 n = 5, $P < 0.001$), but this effect was substantially and significantly reduced in the presence of SNAP 100μM (Fig. 3.50). Wash out of CPA took 20-25 min to obtain a stable recovery.

Alternative free radicals

Since NO is a free radical, and NMDA receptor activation is known to result in the generation of free radicals such as superoxide in addition to NO, we considered the possibility that its effects might be non-specific and shared by any free radical species producing oxidative damage to the adenosine A1 receptor. We

therefore turned to an examination of the superoxide generating system of xanthine plus xanthine oxidase (X/XO).

Interaction between adenosine and xanthine/xanthine oxidase on single pulse and paired-pulse excitatory postsynaptic potentials

The activation of NMDA receptors can generate superoxide anions (Palumbo et al., 1992). We have, therefore, examined whether superoxide could modify adenosine responses. The superfusion of xanthine (100μM) / xanthine oxidase (0.02U.ml^{-1}) (X/XO) during the application of adenosine resulted in a significant suppression of the responses to adenosine (32.6% ± 3.02, n=5, P<0.05) while adenosine alone depressed the EPSPs slope by 48.68% ± 4.12 (n=5) of the control size. Washing with X/XO after application of adenosine and X/XO induced a long-term potentiation of EPSP slope which attained a level of 126.2% ± 6.17 (n=5) after 10min of washing relative to the control EPSP slope. The effects of adenosine in the presence of X/XO 100μM/0.02U.ml^{-1} were depressed after induction of NO-LTP (28.46% ± 4.15, n=5, P<0.001) using single pulses and paired-pulses EPSP.

Effect of EPSP size on adenosine sensitivity

In order to determine whether the reduction of adenosine response is due to LTP or X/XO, the stimulus was changed to the control level after induction of LTP. Reduction of adenosine responses was still observed (25.6% ± 5.91, P<0.001, n=5) whereas adenosine alone decreased the EPSP slope by 48.86% ± 4.53 n=5

(Fig. 3.51). The reduction of adenosine effect on paired-pulse activity was also still observed (Fig. 3.52).

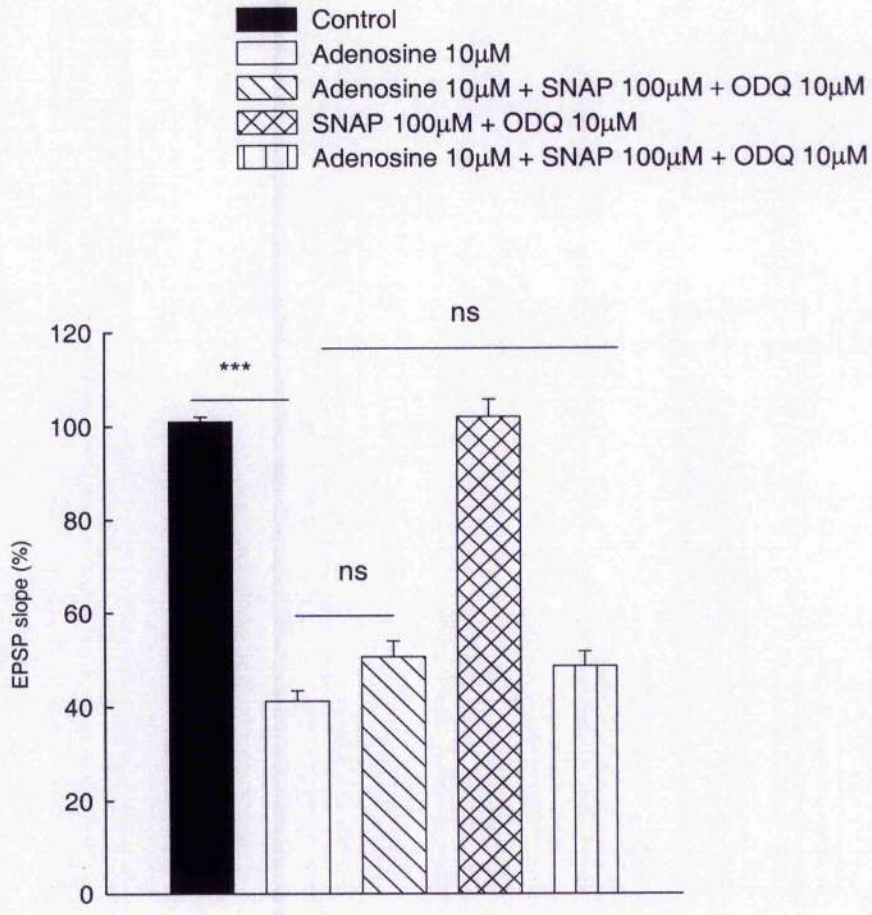

Figure 3.49. Histogram showing the interaction between adenosine 10μM and SNAP100μM in the presence of ODQ 10μM on fEPSPs evoked from the CA1 region of hippocampal slices. Each vertical bar represents mean ± S.E.M for n = 4 slices. *** P<0.001

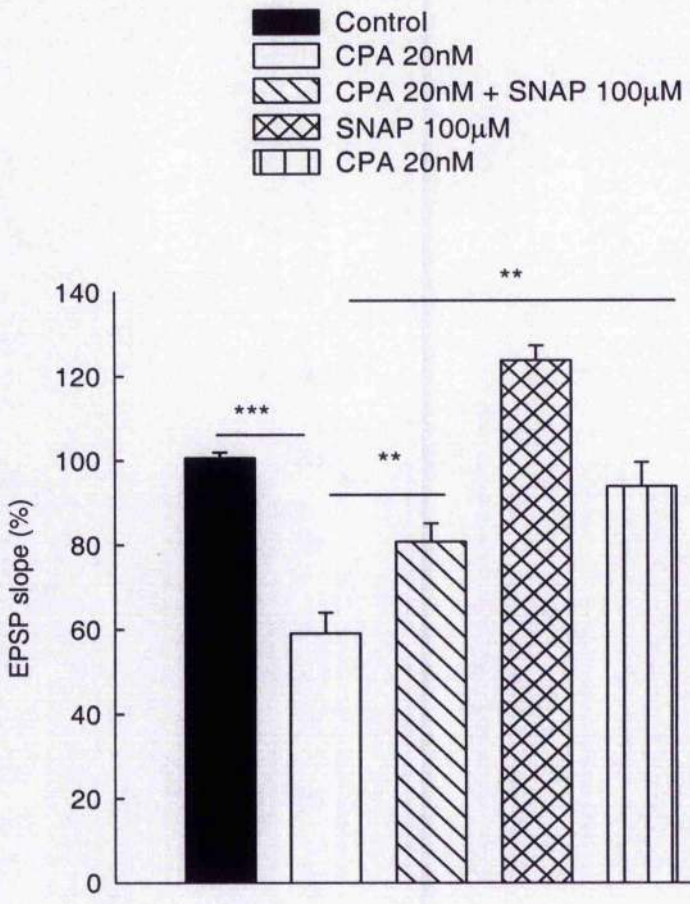

Figure 3.50. Histogram showing the interaction between the A1 receptor selective agonist CPA 20nM and SNAP100μM on fEPSPs evoked from the CA1 region of hippocampal slices. Each vertical bar represents mean ± S.E.M for n = 5 slices. ** P<0.01, *** P<0.001

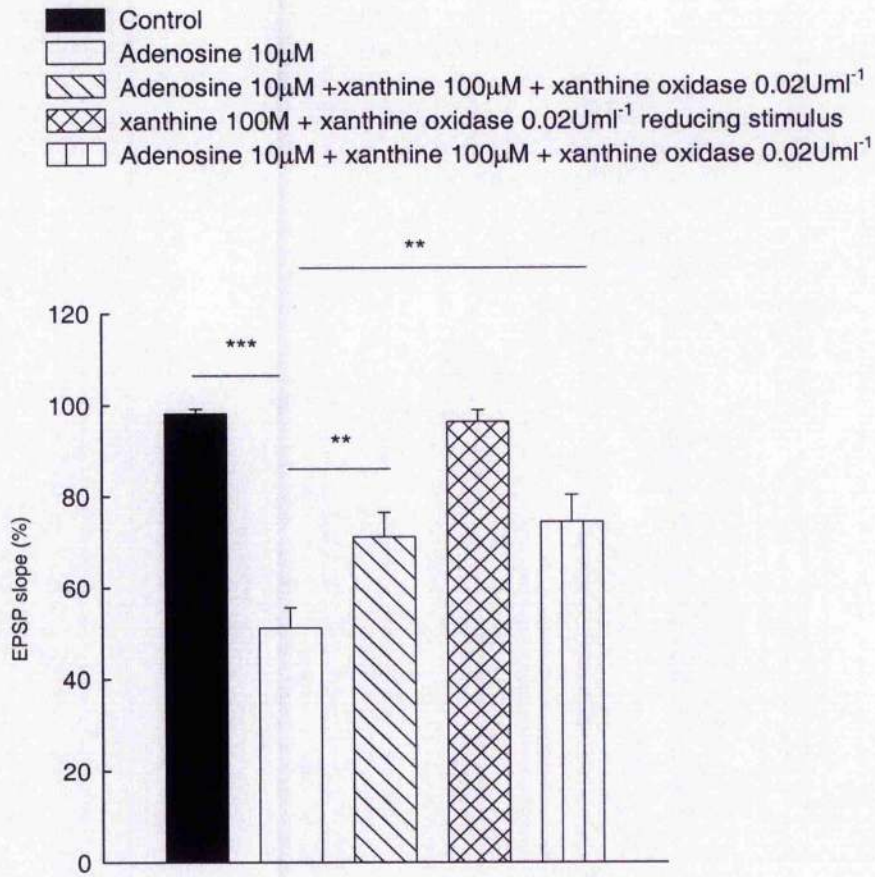

Figure 3.51. Histogram showing the interaction between adenosine 10μM and xanthine 100μM/xanthine oxidase 0.02Uml^{-1} after reducing stimulus strength to restore the potential responses size to control level on fEPSPs evoked from the CA1 region of hippocampal slices. Each vertical bar represents mean ± S.E.M for n = 5 slices. ** P<0.01, *** P<0.001

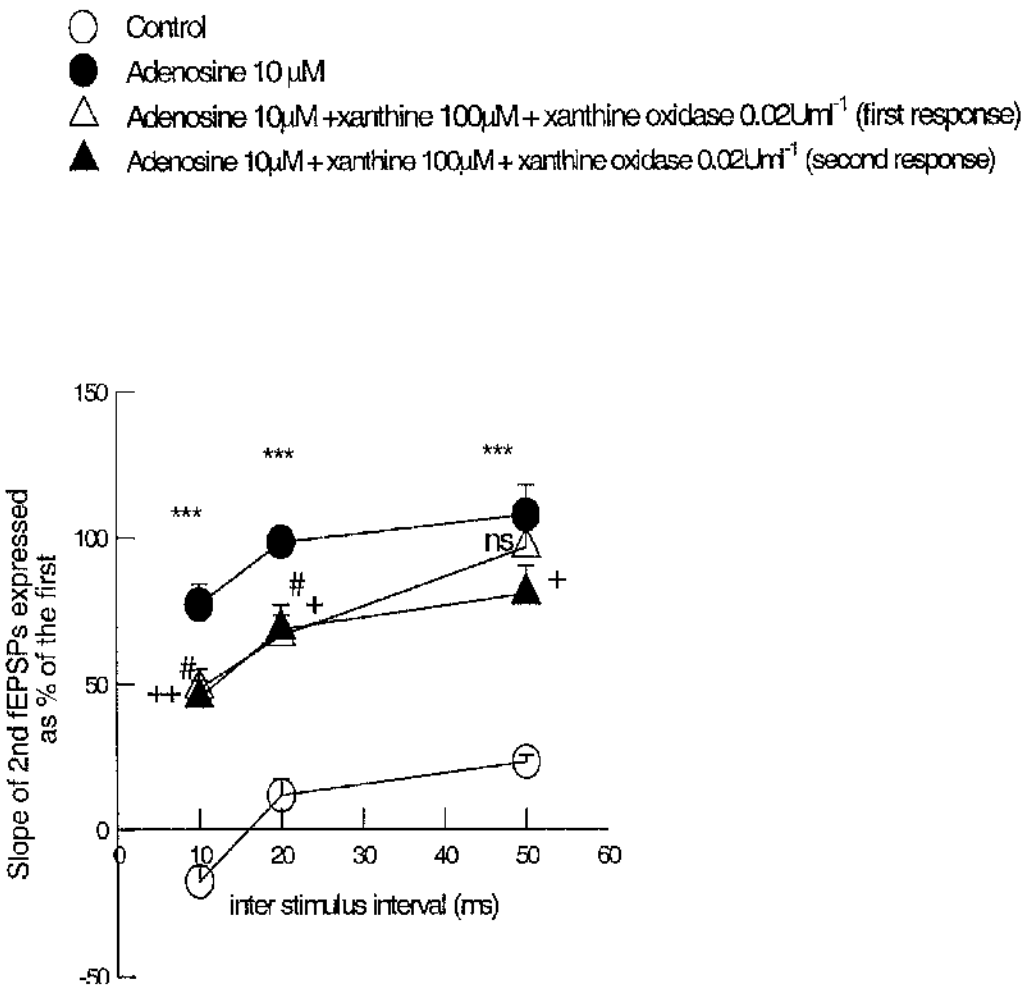

Figure 3.52. Modulation of orthodromic evoked paired-pulse fEPSPs by adenosine 10µM and xanthine100µM/xanthine oxidase 0.02Uml^{-1} with stimulus strength adjusted to restore potentials to control size. Each point represents mean ± S.E.M for n = 4 slices. *** P<0.001 control and adenosine, # P<0.05 between adenosine alone and adenosine +X/XO (first response), + P<0.05, ++ P<0.01 between adenosine and adenosine + X/XO (second response).

Application of Xanthine and xanthine oxidase separately

In order to determine which compound was involved in the X/XO effects xanthine and xanthine oxidase were applied separately. Xanthine 100μM alone could induce LTP in the slices (125.4% ± 4.87, n=4). Adenosine alone reduced EPSP slope by 43.25% ± 5.1, n=4 and reduced EPSP slope by 17.48% ± 4.87, n= 4 (P<0.001) and 14.15% ± 5.09, n=4 (P<0.001) in the presence of xanthine 100μM. After changing stimulus to restore the potential size to the control level application of xanthine 100μM reduced response to adenosine significantly by 24.07% ± 5.9, n=3, P<0.001 and 22.47% ± 4.97, n=3, P<0.001 compared to the control level (Fig. 3.53). The effect of adenosine on paired-pulses was also reduced significantly in the presence of xanthine after reducing stimulus strength (Fig. 3.54).

Adenosine response on single pulses and paired-pulses in the presence of xanthine oxidase 0.02 U/ml were also reduced significantly (20.14% ± 7.03, n=3, p<0.05) compared with adenosine alone that reduced EPSP slope by (48.04% ± 5.9, n=3). The first adenosine response did not change in the presence of xanthine oxidase (45.77% ± 6.34, n=3). Xanthine oxidase could not induce LTP in the slices (Fig. 3.55 & Fig. 3.56).

Role of guanylyl cyclase inhibitor and super oxide dismutase

In order to determine whether guanylyl cyclase was involved in the effects of X/XO, the experiments with X/XO were repeated in the presence of the guanylyl cyclase inhibitor, ODQ.

Interestingly, the guanylyl cyclase inhibitor ODQ 10μM could prevent the inhibitory effects of X/XO on adenosine response (46.73% ± 5.33, n=4) and adenosine alone then reduced EPSP slope by 52.95% ± 3.56, n=4 (Fig.3.57).

Application of superoxide dismutase (SOD) 120 $U.ml^{-1}$ (as shown with Klann et al., 1998) could prevent the inhibitory effect of xanthine/xanthine oxidase on the adenosine response. Adenosine alone suppressed EPSP slope by 52.95% ± 7.33, n=4 and it was 49.5% ± 6.99, n=4 and 51.5% ± 9.07 in the presence of xanthine/xanthine oxidase and SOD (Fig. 3.58).

SOD also prevents the induction of LTP and reduction of the inhibitory effect of xanthine on adenosine response. Adenosine alone suppressed EPSP slope by 52.19% ± 6.51, n=3 and in the presence of xanthine and SOD by 46.9% ± 4.56, n=3 and 42.19% ± 6.16, n=3.

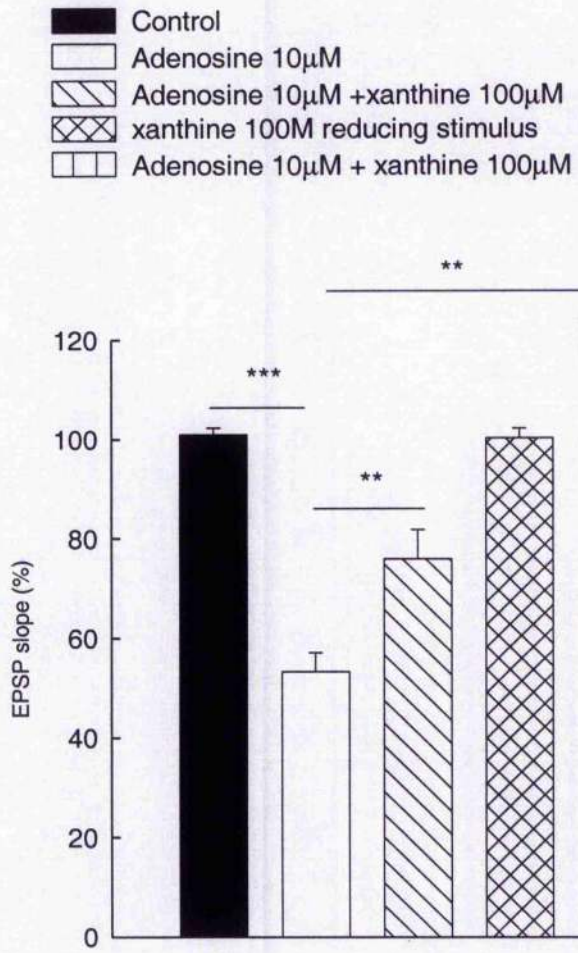

Figure 3.53. Histogram showing the interaction between adenosine 10μM and X100μM on fEPSPs evoked from the CA1 region of hippocampal slices. Each vertical bar represents mean ± S.E.M for n = 3 slices. ** P<0.01, *** P<0.001,

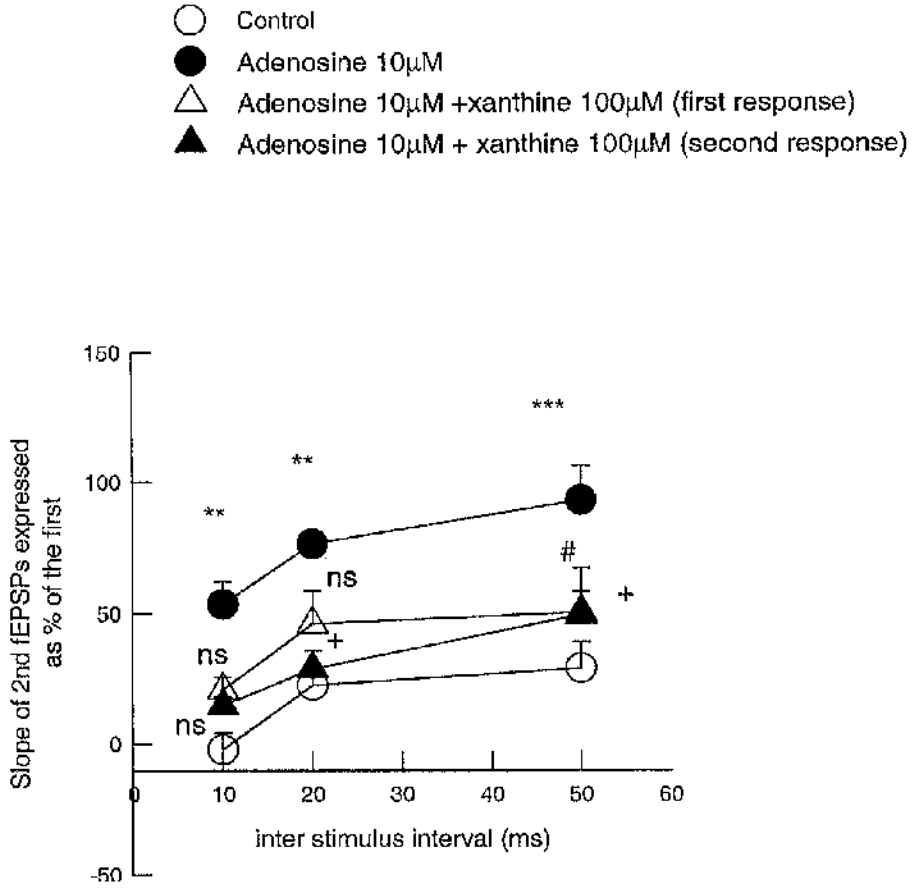

Figure 3.54. Modulation of orthodromic evoked paired-pulse fEPSPs by adenosine 10μM and xanthine 100μM after reducing the stimulus to restore the potential size to control level. Each point represents mean ± S.E.M for n = 3 slices. ** P<0.01 and *** P<0.001 control and adenosine, # P<0.05 between adenosine and adenosine + xanthine (first response), + P<0.05 between adenosine and adenosine + xanthine (second response).

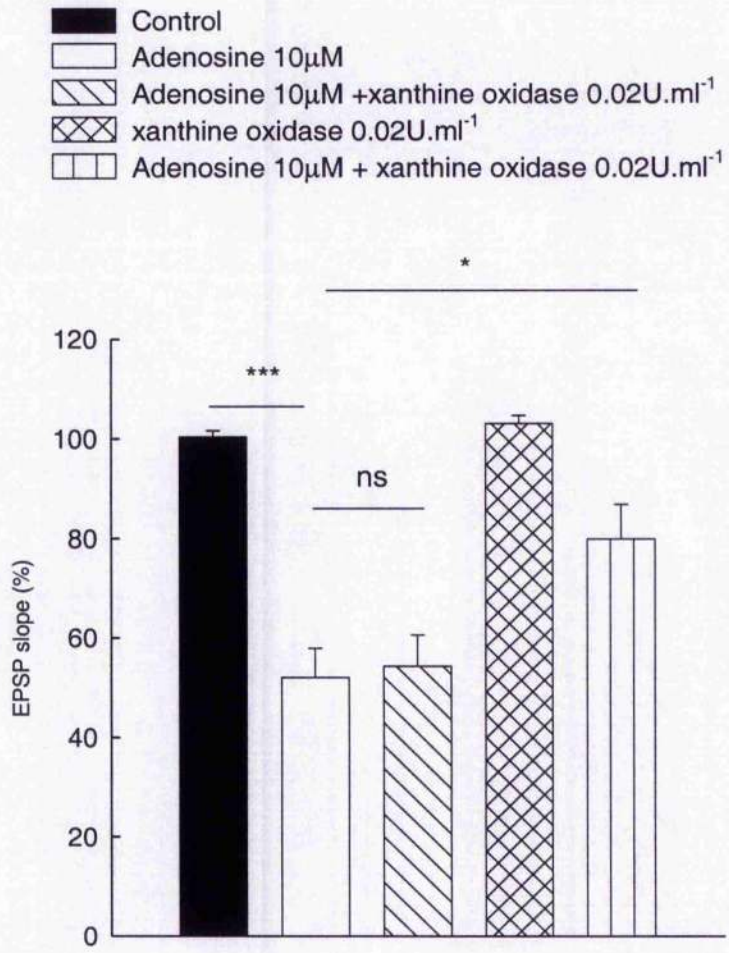

Figure 3.55. Histogram showing the interaction between adenosine 10μM and XO 0.02U.ml^{-1} on fEPSPs evoked from the CA1 region of hippocampal slices at normal stimulus strength. Each vertical bar represents mean ± S.E.M for n = 4 slices. * P<0.05, *** P<0.001,

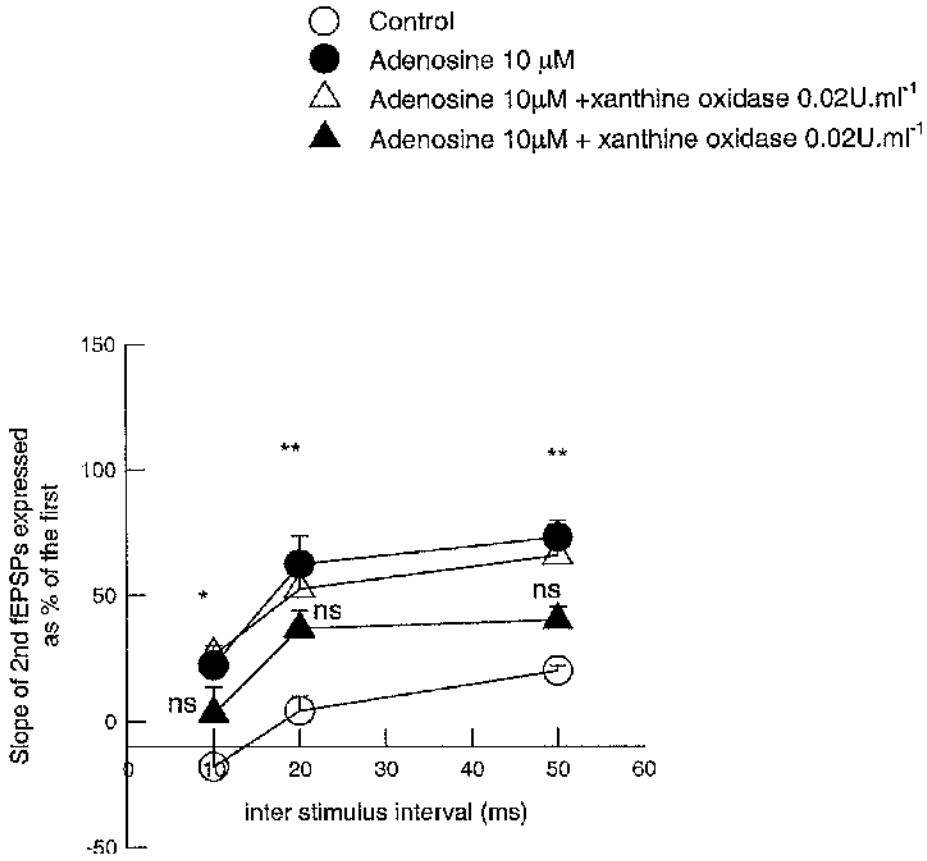

Figure 3.56. Modulation of orthodromic evoked paired-pulse fEPSPs by adenosine 10μM and XO 0.02U.ml^{-1} at normal stimulus strength. Each point represents mean ± S.E.M for n = 4 slices. * P<0.05, ** P<0.01 between control and adenosine.

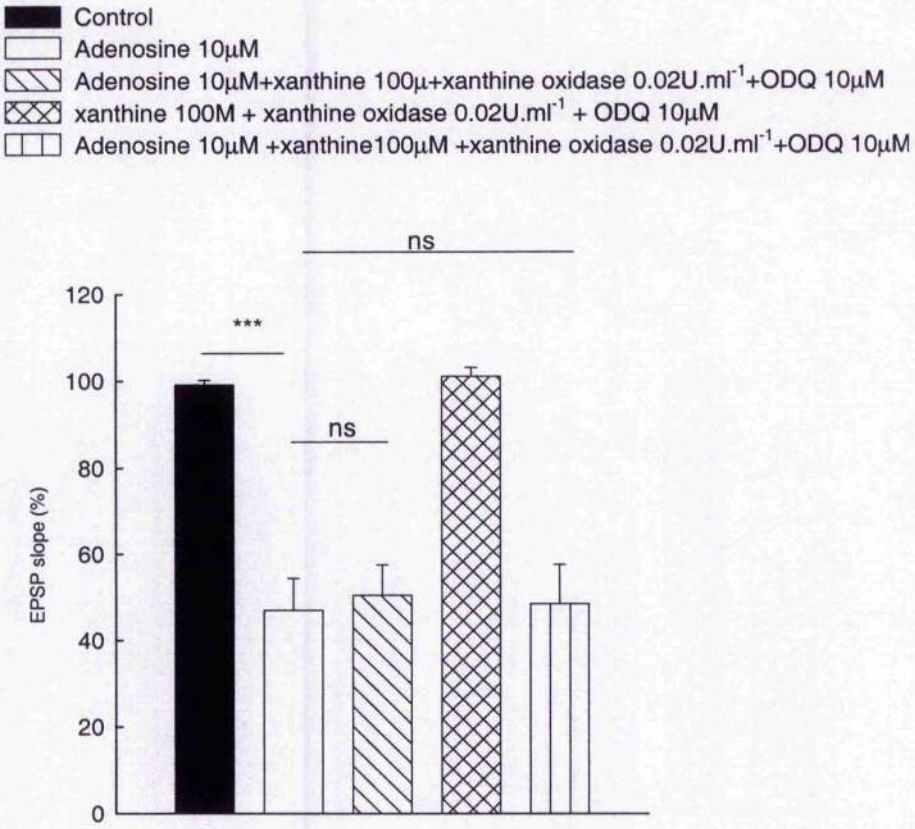

Figure 3.57. Histogram showing the interaction between adenosine 10μM and X100μM/XO 0.02U.ml^{-1} in the presence of ODQ 10μM on fEPSPs evoked from the CA1 region of hippocampal slices at normal stimulus strength. Each vertical bar represents mean ± S.E.M for n = 4 slices. *** P<0.001,

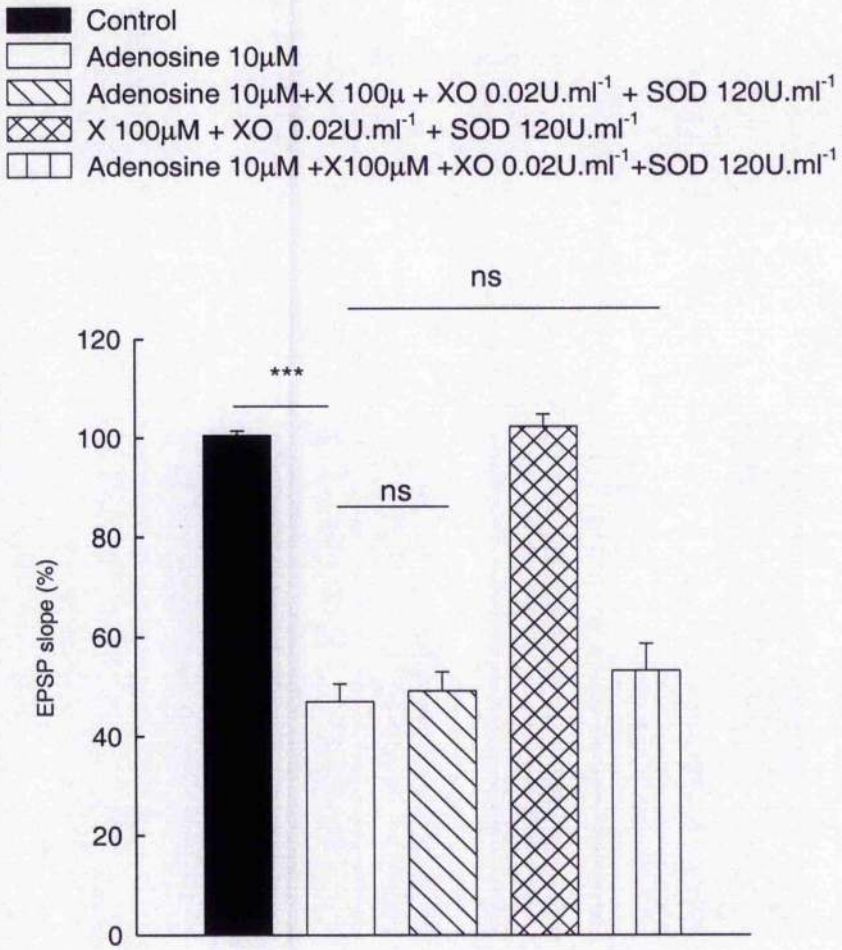

Figure 3.58. Histogram showing the interaction between adenosine 10μM and X100μM/XO 0.02Uml^{-1} in the presence of SOD 120Uml^{-1} on fEPSPs evoked from the CA1 region of hippocampal slices at normal stimulus strength. Each vertical bar represents mean ± S.E.M for n = 4 slices. *** P<0.001

Does superoxide act via the generation of NO?

It was of some surprise that the effects of superoxide produced by the X/XO mixture were prevented by ODQ, since there is little evidence that superoxide works via the activation of adenylate cyclase. We therefore assessed whether superoxide might act to suppress adenosine receptors indirectly, via the generation of NO.

The nitric oxide synthase inhibitor

When slices were superfused with the nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), the X/XO mixture was still able to inhibit responses to adenosine (Fig. 3.59). We also tested haemoglobin at 50μM, since this is well established as a scavenger of NO in solution. When superfused over the slices, haemoglobin prevented the reduction of adenosine responses by X/XO and also prevented the induction of LTP by X/XO (Fig. 3.60). Due to the formation of foam following perfusion of Hb, the antifoaming agent antifoam A was included at 50p.p.m during these experiments. This agent had no effect by itself on fEPSP slope.

NMDA receptors

Having established that NO and superoxide could both suppress sensitivity to adenosine selectively and via the activation of guanylate cyclase, we tested SOD and the guanylate cyclase inhibitor ODQ against the ability of NMDA to reduce adenosine responses. The superfusion of NMDA at 4μM for 10 min had no significant effect itself on fEPSP size. When NMDA was applied before and during the application of adenosine 10μM there was a significant suppression of the responses to adenosine (34.25% ± 3.65, $n = 6$, $P < 0.05$, compared with

adenosine alone 54.43% ± 3.8, n = 6). The superfusion of ODQ at 10μM did not prevent the suppression of inhibitory adenosine responses by NMDA, which remained significantly different from the responses to adenosine alone, but not different from responses to adenosine in the presence of NMDA (Fig. 3.61) indicating that NO did not mediated the suppression effects of adenosine by NMDA receptors. SOD at 120U.ml^{-1} had an intermediate, ambiguous effect. There was no significant difference between the size of adenosine responses in the presence of NMDA and in the presence of NMDA plus SOD, suggesting a lack of effect. On the other hand, the adenosine responses in NMDA plus SOD were not significantly different from the control responses to adenosine alone (Fig. 3.62).

A diagram summarising all the results relating to the interaction between adenosine, NO and free radicals is given in figure 3.65.

Electrically-induced LTP

For comparison with the effects of exogenous NMDA, we also examined the effects of electrically induced LTP using a 1s tetanus at 100Hz. Twenty minutes after electrical stimulation, LTP reached a level of 127.34% ± 5.13, (n = 5, P < 0.01). Adenosine induced a reduction of 54.92% ± 6.93 of fEPSP slope before induction of LTP, but 25 min after inducing LTP and adjusting stimulus strength to restore the potentiated responses to control size, adenosine reduced the responses by only 31.19% ± 5.81 (n = 5, P < 0.01) (Fig. 3.6). L-NAME was tested in a series of slices in which the electrically-induced LTP reached 126.76% ± 9.53 (n = 4, P < 0.05). L-NAME itself at 100μM had no effect on fEPSP slope, but it prevented the reduction of adenosine responses by electrically-induced LTP (Fig. 3.64).

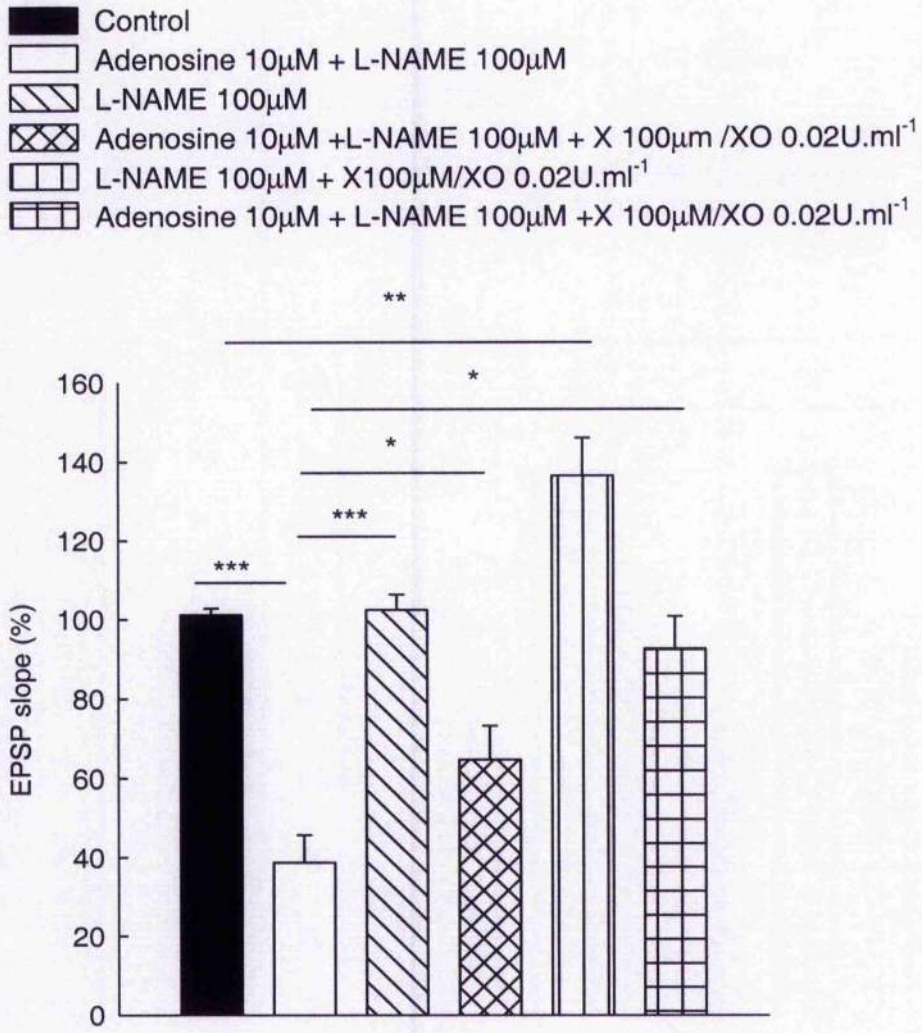

Figure 3.59. Histogram showing the interaction between adenosine 10μM and X100μM/XO 0.02Uml^{-1} in the presence of L-NAME 100μM on fEPSPs evoked from the CA1 region of hippocampal slices at normal stimulus strength. Each vertical bar represents mean ± S.E.M for n = 4 slices. *P<0.05, ** P<0.01, *** P<0.001

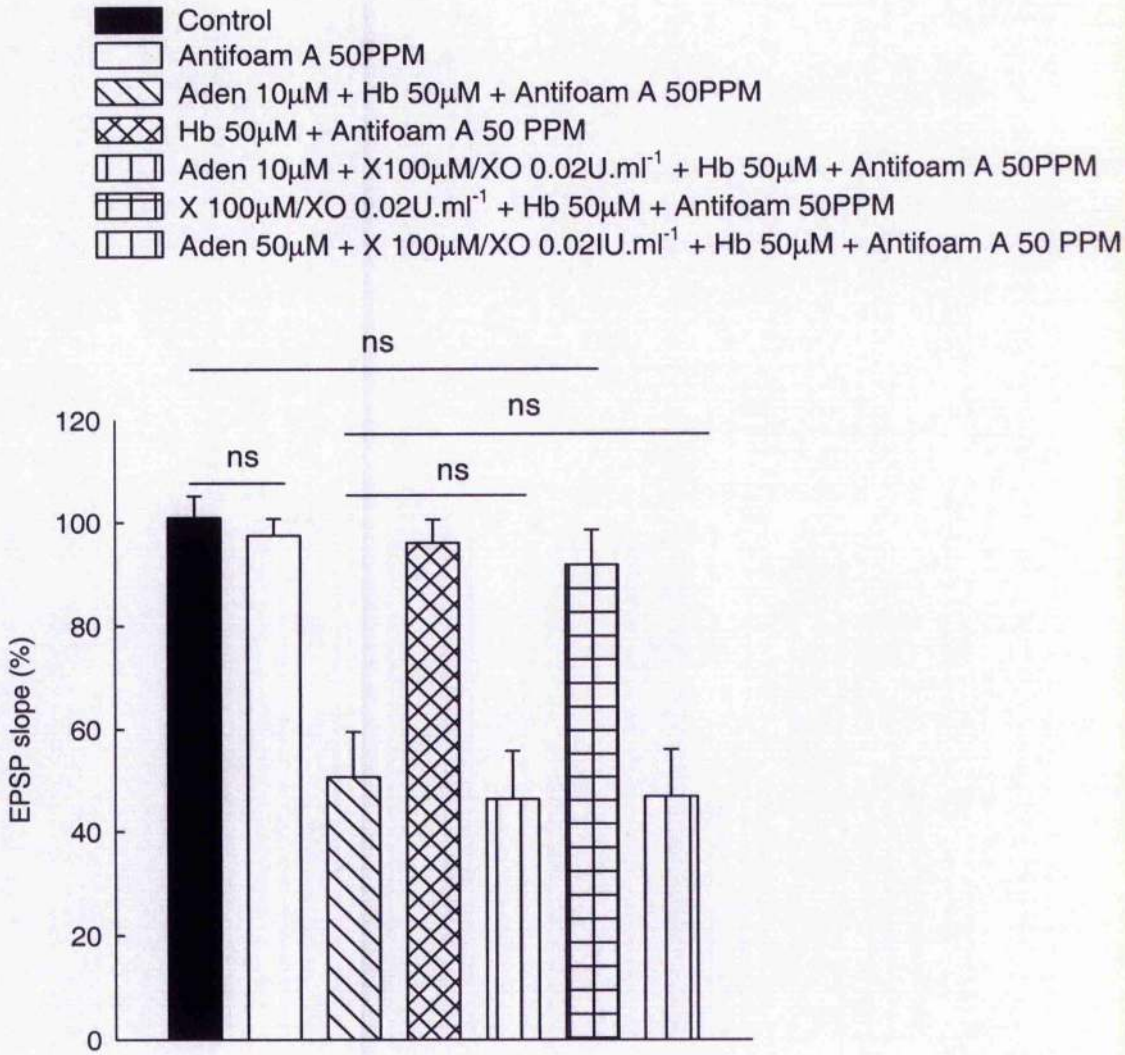

Figure 3.60. Histogram showing the interaction between adenosine 10μM and X 100μM/XO 0.02Uml^{-1} in the presence of haemoglobin 50μM on fEPSPs evoked from the CA1 region of hippocampal slices at normal stimulus strength. Each vertical bar represents mean ± S.E.M for n = 4 slices.

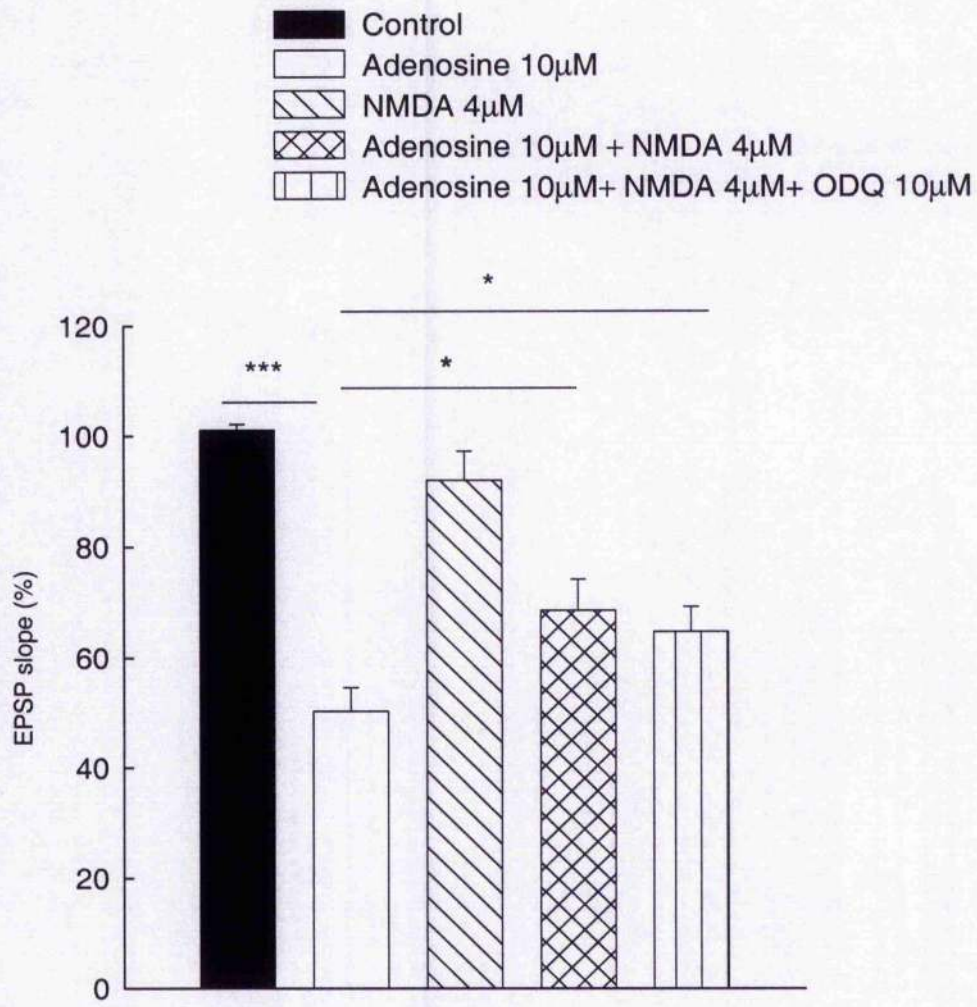

Figure 3.61. Histogram showing the interaction between adenosine 10μM and NMDA 4μM in the presence of guanylyl cyclase inhibitor ODQ 10μM on fEPSPs evoked from the CA1 region of hippocampal slices at normal stimulus strength. Each vertical bar represents mean ± S.E.M for n = 4 slices. *P<0.05, *** P<0.001

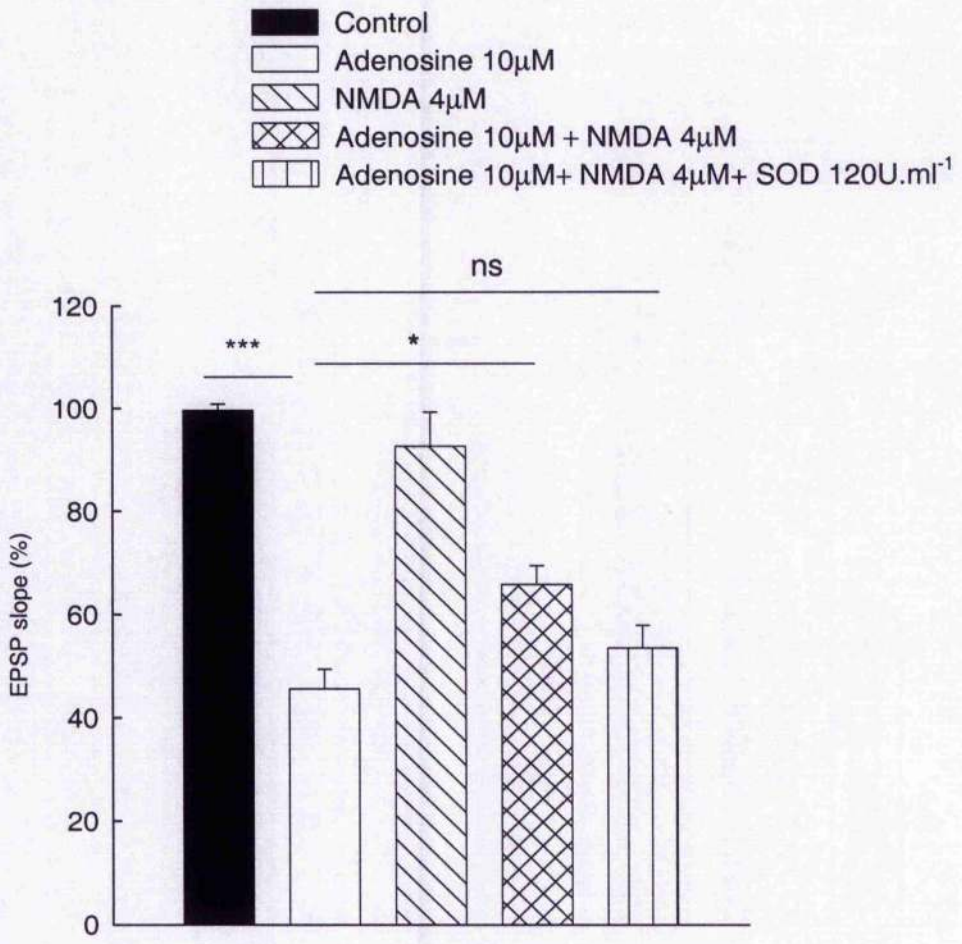

Figure 3.62. Histogram showing the interaction between adenosine 10μM and NMDA 4μM in the presence of SOD 120U.ml^{-1} on fEPSPs evoked from the CA1 region of hippocampal slices at normal stimulus strength. Each vertical bar represents mean ± S.E.M for n = 4 slices. *P<0.05, *** P<0.001

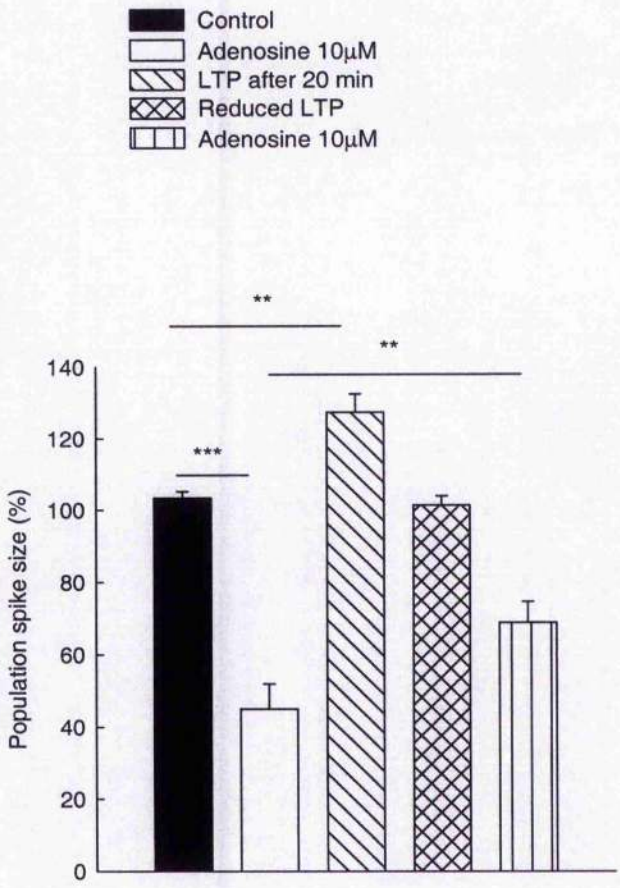

Figure 3.63. Histogram showing the effects of adenosine 10μM after reducing the stimulus to control level following induction of electrical LTP on population spike evoked from the CA1 region of hippocampal slices at normal stimulus strength. Each vertical bar represents mean ± S.E.M for n = 4 slices. ** P<0.01, *** P<0.001

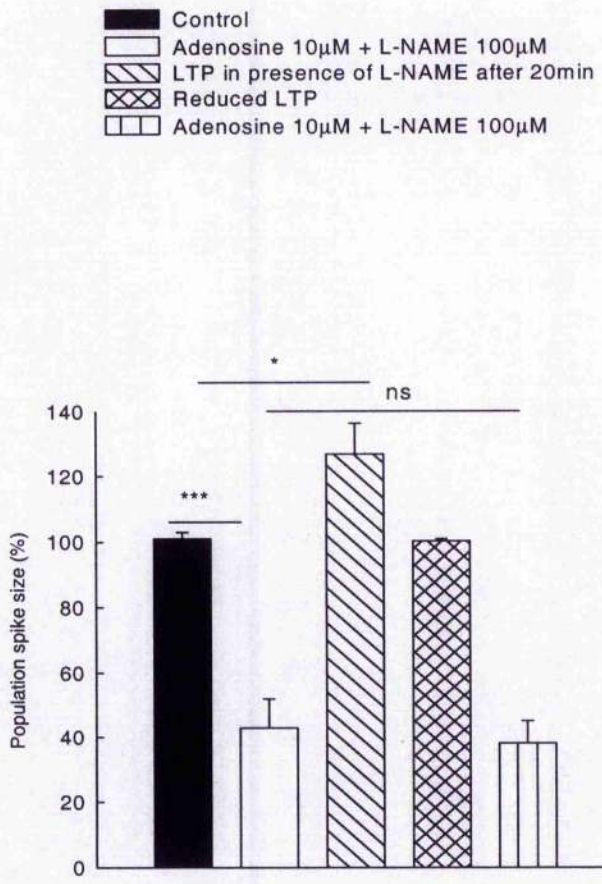

Figure 3.64. Histogram showing the effects of adenosine 10μM after reducing the stimulus to control level following induction of electrical LTP in the presence of L-NAME 100μM on population spike evoked from the CA1 region of hippocampal slices at normal stimulus strength. Each vertical bar represents mean ± S.E.M for n = 4 slices. * $P<0.05$, *** $P<0.001$

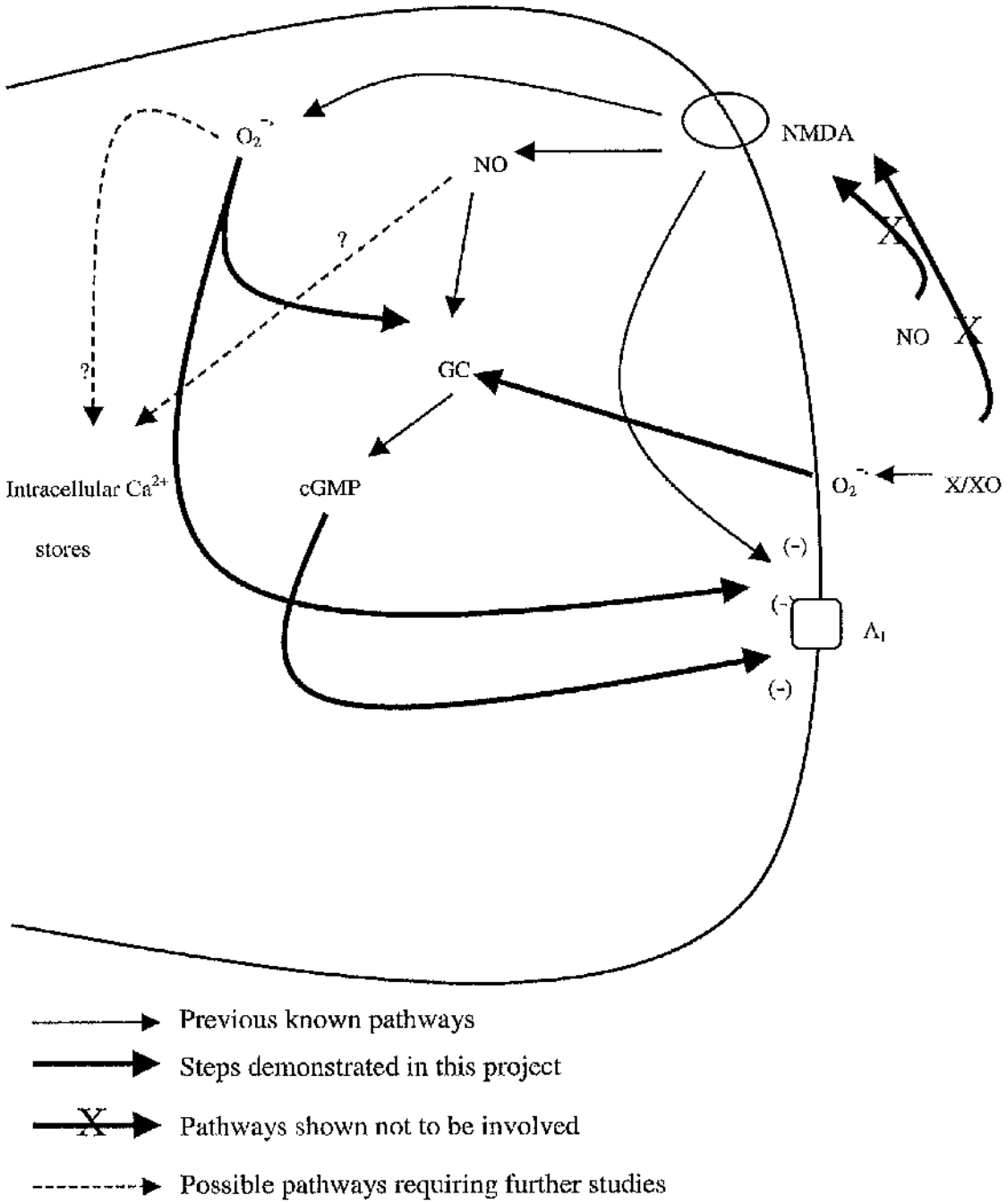

Figure 3.65. This diagram illustrates the pathways investigated in this study on the interaction between adenosine and NO, and adenosine and other free radicals (superoxide). The possible mechanism of the effects of NO and superoxide on suppression of adenosine effects by NMDA is also investigated.

4. Discussion

Interactions between adenosine and metabotropic glutamate receptors

Glutamate has been recognized as the major excitatory neurotransmitter within the mammalian CNS playing critical roles in brain function (Conn & Pin, 1997; Nakanishi et al., 1998). As result of its excitatory actions, glutamate can also modulate the release of various other neurotransmitters from synaptic terminals. The synaptic functions of glutamate are mediated through both ionotropic receptors and G protein-coupled metabotropic glutamate receptors. This study has considered the roles of metabotropic glutamate receptors in the modulation of adenosine receptors.

It is now well established that adenosine acts on presynaptic A_1 receptors to depress neurotransmitter release, and that this action underlies its ability to depress transmission in the Schaffer-collateral to CA1 synapses. Several previous studies have examined interactions between the ionotropic N-methyl-D-aspartate (NMDA) receptors and adenosine receptors (Bartrup & Stone, 1990; de Mendonca et al. 1995; Norenberg et al. 1997; Nikbakht & Stone, 2001), but interactions with metabotropic glutamate receptors have received relatively limited attention. Activation of mGluR2 receptors reduces the release of adenosine from brain slices (Casabona et al. 1994), and the non-selective agonist ACPD has been shown to inhibit the reduction by adenosine of glutamate release from cortical synaptosomes (Budd & Nicholls, 1995). Metabotropic glutamate receptors can also potentiate the activation of adenylate cyclase by adenosine A_{2A} receptors (Alexander et al. 1992; Winder & Conn, 1993; Schoepp & Johnson,

1993). In a previous electrophysiological study it was noted that DHPG could depress the inhibitory activity of CPA in hippocampal slices (de Mendonca & Ribeiro, 1997), but the site and mechanism of action were not investigated in detail.

Clarification of the adenosine receptor

In the present study we have confirmed that ACPD and the group I agonist DHPG depress the inhibitory effects of adenosine and CPA (de Mendonca & Ribeiro, 1997). In principle, however, this apparent antagonism could result from an inhibition of the inhibitory A_1 receptor responses, or a facilitation of the excitatory adenosine A_{2A} receptor responses generated by endogenous levels of adenosine. Such a facilitation has been demonstrated in other studies: the unilateral activation of striatal mGluRs by ACPD produces contralateral rotation which appears to be mediated in part by the activation of adenosine A_{2A} receptors since agonists enhance, and selective antagonists suppress, the turning (Kearney & Albin, 1995). A synergistic interaction has also been described between adenosine A_{2A} receptors and metabotropic glutamate receptors in elevating cyclic AMP levels (Alexander et al. 1992; Winder & Conn, 1993; Schoepp & Johnson, 1993). The previous electrophysiological study of de Mendonca and Ribeiro (1997) did not clearly distinguish between these mechanisms, as endogenous adenosine was present in the slices which could have allowed either action to be superimposed on any direct interaction between metabotropic glutamate agonists and A_1 receptors. In the present work, we have removed endogenous adenosine with adenosine deaminase, allowing us to conclude that the interaction is between group I

glutamate receptors and adenosine A_1 receptors. We have also obtained further evidence in support of this conclusion, with the demonstration that the A_{2A} receptor blocker ZM241385 did not modify the ability of DHPG to suppress adenosine sensitivity.

The effects of mGluR I agonists on hippocampal slices

Activation of group I mGluRs induces acute depression of fEPSP slope and LTD at CA3-CA1 synapses of the rat hippocampus. Whole-cell patch clamp (Manzoni & Bockaert, 1995) and analysis of paired-pulse facilitation experiments (Gereau & Conn, 1995; Faas et al., 2002) have suggested a presynaptic calcium reduction for these effects. The specific mGluR1 antagonist LY367385 suppressed acute depression of transmission induced by DHPG (Faas et al., 2002; Shahraki & Stone, 2002) whereas mGluR5 antagonist MPEP had no effect on EPSP depression induced by DHPG but completely prevented DHPG-induced LTD (Mannaioni et al., 2001). These results indicate that the depression of transmitter caused by DHPG is dependent on mGluR1 activation whereas for mGluR-LTD activation of mGluR5 is necessary in CA1 region of hippocampus

Three possible mechanisms might be involved in reduction of presynaptic calcium transients by group I mGluR activation: a) Direct blockade of voltage dependent calcium channels that might be mediated by reduced conductance, probability or open time, or number of active channels. b) Regulation of the ionic currents in the presynaptic action potential, causing a reduction in voltage dependent calcium channel activation. c) Decreased calcium release from intracellular stores (Faas et al., 2002). d) Decreased sensitivity to calcium of the release process.

Based on the results of Faas et al., (2002) two distinct presynaptic mechanisms may be involved in DHPG- induced depression. The first one is reversible and involves mGLUR1 activation which reduces the stimulation-induced calcium transients. The second one mediates mGLUR-LTD by mGLUR5 activation which is based on downstream inhibition of presynaptic calcium influx.

Clarification of the metabotropic glutamate receptor

In the present work, both ACPD and DHPG themselves produced a significant depression of synaptic potentials. Inhibitory effects of ACPD on synaptic transmission have been reported in several brain regions and across species (Krieger et al., 1996; King & Liu, 1996), including inhibition of synaptic transmission at the Schaffer collateral-CA1 synapse (Gereau & Conn, 1995).

Both subtypes of group I receptor ($mGluR_1$ and $mGluR_5$) are known to be present in the hippocampal CA1 region. Many effects of group I agonists have been attributed to the $mGluR_5$ subtype, largely because of its presence in higher abundance in the CA1 region (Lujan et al., 1996). Immunocytochemical studies have revealed that group I mGluRs are located primarily on the postsynaptic sites, rather than presynaptically in the CA1 area (Lujan et al., 1996; Shigemoto et al. 1997). However, functional evidence has indicated the presence of presynaptic group I mGluRs which increase intraterminal calcium levels and promote transmitter release (Croucher et al., 2001; Schwartz & Alford, 2000). This paradox may be due to the postsynaptic receptor causing release of a retrograde messenger (Levenes et al., 2001). While the use of selective ligands makes it clear that group I receptors are responsible for the interaction with adenosine (de

Mendonca & Ribeiro, 1997; Shahraki & Stone 2002), we have now extended this observation by showing that the effects of DHPG upon adenosine sensitivity could be prevented by LY367385, an antagonist selective for the $mGlu_{1a}$ subtype (Mannaioni et al., 2001), but not by SIB1893 which is selective for the $mGlu_5$ subtype (Varney et al., 1999). We conclude that it is primarily the $mGlu_{1a}$ subtype which mediates the suppression of adenosine sensitivity, and our confidence in this conclusion is strengthened by our selection of drug concentrations. At the concentrations used here, both LY367385 and SIB1893 have been shown to produce a greater than 50% blockade of responses mediated by $mGlu_{1a}$ and $mGlu_5$ receptors in other experimental paradigms (Mannaioni et al., 2001; Varney et al. 1999).

Group I mGLUR selective agonists can activate phospholipase C and induce phosphoinositide hydrolysis, so producing inositol-1,4,5-trisphosphate (IP3) as well as diacylglycerol, which in turn activates protein kinase C. Activation of PKC could suppress the presynaptic inhibitory effects of adenosine in the hippocampus (Thompson et al., 1992) and the neuromuscular junction (Sebastiao and Ribeiro, 1990). PKC is also able to decrease the inhibitory effect of the selective A1 receptor agonist CPA on glutamate release in cerebrocortical synaptosomes (Budd & Nicholls, 1995).

Time course

In addition, it is apparent from the present data that the interaction between DHPG and adenosine receptors substantially outlasts the application of the glutamate receptor agonist. The reduction of adenosine sensitivity is apparent up to at least

60 minutes after a 10 minute application of ACPD or DHPG, even though baseline potential size is restored to control levels within minutes of the agonist application and remains so for at least 10 minutes until the next application of adenosine or CPA. This is consistent with biochemical studies that have reported effects of group I metabotropic receptor stimulation lasting more than 10 minutes (Budd & Nicholls, 1995) or up to 40 minutes (Alexander et al., 1992), and with a report of increased excitability of spinal neurons, and NMDA sensitivity lasting up to 60 minutes (Budai & Larson, 1998). These observations emphasise that even transient increases in the extracellular levels of glutamate could produce long-lasting changes of neuronal excitability and synaptic transmission.

The long time course of action of ACPD and DHPG would also be consistent with the apparent mediation of the metabotropic receptor effects by protein kinase C (Shabraki & Stone 2003; de Mendonca & Ribeiro, 1997). A role for protein kinase C would be entirely consistent with previous demonstrations that the activation of protein kinase C is able to decrease the presynaptic inhibitory effects of adenosine in cerebrocortical synaptosomes (Budd & Nicholls, 1995), the hippocampus (Thompson et al., 1992) and neuromuscular junction (Sebastiao et al., 1990).

Role of NMDA receptors

There are several indications that the activation of group I mGluRs can facilitate the activation of NMDA receptors (Fitzjohn et al., 1996; Awad et al., 2000; Salt & Binns, 2000; Attucci et al. 2001), probably mediated by protein kinase C (Pisani et al. 1997; Liao et al., 2001). Since it has been shown that activation of NMDA

receptors can reduce the inhibitory activity of adenosine (Bartrup & Stone, 1990; Nikbakht & Stone 2001), it was possible that the metabotropic receptor-mediated reduction of adenosine sensitivity was mediated indirectly by a facilitation of NMDA receptor activity. The results do indeed indicate that the mGluR / adenosine interaction could be prevented in the presence of the NMDA antagonist 2AP5, making this a likely explanation.

Paired-pulse interaction, presynaptic mechanism and non-selectivity

Due to the small size of most nerve terminals, direct functional studies of synapses are difficult, so indirect methods can help researchers to study the control of neurotransmitter release. Paired-pulse stimulation is one of these methods that two stimuli are delivered at a short interval and a difference between the amplitudes of the first and the second responses is used to calculate a paired-pulse ratio (PPR). PPR is affected by neuromodulators or physiological conditions that change transmitter release probability. The following mechanisms affect the amplitude of an EPSP: a) the number of neurotransmitter release sites b) the probability of neurotransmitter release c) amplitude of the postsynaptic unit response at each release site. Paired-pulse facilitation is an inverse function of the initial probability of release related to the first pulse. If initial probability of release is high, indicating most terminals will release neurotransmitter in response to the first pulse, there will be little residual calcium, the probability of neurotransmitter release in response to the second stimulus will be reduced and PPF ratio will be small. In contrast, when the probability of neurotransmitter release is very low, few terminals will release in response to the first stimulus, and

the enhanced residual calcium causes release of many more terminals in response to the second stimulus and PPF ratio will be high (Santschi & Stanton, 2003). These explanations indicate that paired-pulse interaction is a presynaptic phenomenon rather than postsynaptic. The experiments of Debanne et al. (1996) revealed that paired-pulse interaction is not a postsynaptic mediated process because PPR remained unchanged following application of 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX) or receptor desensitisation with aniracetam.

When inhibitory synapses are activated by paired stimulation paired-pulse depression occurs because less transmitter is released by the second stimulus due to activation of $GABA_B$ autoreceptors in the CA1 area (Davies et al., 1990) or because of transmitter depletion resulting from the first stimulus, as it has been observed in CA3 cells in the slice preparation (Lambert & Wilson, 1994) and at the inhibitory synapses in tissue culture (Wilcox & Dichter, 1994).

Depletion of the presynaptic readily releasable pool of synaptic vesicles has been proposed as the major component of PPD (Oleskevich et al., 2000; Meyer et al., 2001). However, the role of other mechanisms such as presynaptic calcium channel inactivation (Gingrich & Byrne, 1985), negative feedback through inhibitory autoreceptors (Forsythe & Clements, 1990) or receptor desensitisation (Otis et al., 1996; Neher & Sakaba, 2001) cannot be excluded. Inactivation of presynaptic voltage-dependent sodium channels which may affect coupling of action potentials to transmitter release was recently proposed that contribute to PPD (Brody & Yue, 2000). He et al. (2001) showed that sodium channel inactivation was associated with EPSc depression under depolarising physiological or pathophysiological conditions.

An important finding in the present study is that the depressant effect of group I agonists is not selective for adenosine A_1 receptors, but is also exerted against the $GABA_B$ receptor agonist, baclofen. This raises the possibility that the group I effects may not result from interactions at the receptor level, but are due to generalised changes in presynaptic terminal excitability or release processes. Such a mechanism would be consistent with the mediation of these effects by protein kinase C, which would be expected to modify transmitter release more generally than in response to only a single species of presynaptic receptor. Indeed, although relatively little work has been performed on functional aspects of group I metabotropic receptors on synaptic terminals, Schwartz & Alford (2000) and Croucher et al. (2001) have concluded that group I receptors on presynaptic terminals can increase glutamate release. The effects of group I agonists may not, therefore, indicate a selective interaction between metabotropic glutamate receptors and adenosine receptors, but may simply be the result of a non-specific antagonism of any agent with a tendency to suppress transmitter release. It should be emphasised, however, that the interaction with baclofen was less clear than with adenosine. Firstly, the responses to baclofen always recovered to control levels before 60 minutes following the glutamate receptor agonist and, secondly, the tendency to depress baclofen's actions on paired-pulse phenomena often did not reach statistical significance.

The preceding discussion is based on the assumption that the interactions between the group I agonists and adenosine or baclofen are mediated at a common, presumably presynaptic site. Our use of paired-pulse interactions confirms that this is so. Particularly when studied on EPSPs, the paired-pulse paradigm

represents a valuable means of assessing the actions of compounds on presynaptic terminals. The paired-pulse inhibition obtained at interpulse intervals of 10 ms results from the depletion of transmitter from presynaptic stores (Burke & Hablitz, 1994; Wilcox & Dichter, 1994; Hashimoto & Kano, 1998), and is reduced by agents or procedures which decrease transmitter release. Paired-pulse facilitation, observed at longer interpulse intervals, is due to the residual intraterminal Ca^{2+} which increases transmitter release (Hess et al., 1987; Wu & Saggau, 1994b; Debanne et al., 1996; Kleschevnikov et al., 1997). The fact that the metabotropic glutamate receptor agonists can suppress the effects of adenosine in this system provides a strong argument that the interactions are occurring at presynaptic terminals. The present data do not, however, address the question as to whether the interaction between group I receptors and adenosine is a direct or indirect action. There is clear evidence that the activation of group I metabotropic receptors at postsynaptic sites can lead to the release of retrograde messenger that is responsible for at least part of the presynaptic effects of agonists (Levenes et al. 2001; Maejima et al., 2001; Watabe et al., 2002). Only when there is a clear identification of the nature of such a messenger can its role in the depression of adenosine sensitivity be investigated further.

The activation of mGluRs can facilitate long-term potentiation, whereas antagonists inhibit it (Bortolotto et al. 1994; Petrozzino & Connor, 1994; Izumi et al. 2000). Cohen & Abraham (1996) reported that a single, 10 minute duration application of ACPD could produce a long-lasting enhancement of stimulation-induced LTP. The authors showed that this enhancement was not dependent on the activation of NMDA receptors, but did appear to involve group 1 mGluRs.

Since the blockade of adenosine A1 receptors by conventional antagonists is well known to facilitate LTP (de Mendonca & Ribeiro, 1994), it may be that the suppression of adenosine sensitivity could contribute to the reduction by ACPD of LTP threshold.

Nitric oxide suppression of presynaptic responses to adenosine

The paired-pulse paradigm provides a valuable means of assessing the actions of compounds on presynaptic terminals. Paired-pulse inhibition at interpulse intervals of 10 ms reflects the depletion of transmitter from presynaptic stores (Burke & Hablitz, 1994; Wilcox & Dichter, 1994; Hashimoto & Kano, 1998), and is reduced by agents or procedures which decrease transmitter release (such as adenosine). Paired-pulse facilitation, observed at longer interpulse intervals around 20-50ms, is due to the residual intraterminal Ca^{2+} remaining after the first pulse and which facilitates subsequent transmitter release (Hess et al., 1987; Wu & Saggau, 1994; Debanne et al., 1996; Kleschevnikov et al., 1997). The present work has shown that the inhibitory actions of NO and superoxide on adenosine responses are reflected not only in changes of the fEPSP slope, but also in changes of these paired-pulse phenomena, strongly suggesting that the interactions between ROS and adenosine occur primarily at the presynaptic terminals. This is entirely in accord with a wealth of evidence that adenosine A1 receptor-mediated actions are almost exclusively presynaptic in nature, including a recent study in which A1 receptors were deleted selectively from CA3 or CA1 neurons (Scammell et al. 2003).

Adenosine and NO

Adenosine is an endogenous purine that has an important role in the regulation of neuronal excitability and synaptic transmission in the hippocampus and which is able to modulate synaptic plasticity. Adenosine or its analogues inhibit LTP and LTD in hippocampal slices via adenosine A1 receptors (de Mendonca & Ribeiro,

1990, 2000; Arai et al., 1990; Kemp & Bashir, 1997; de Mendonca et al., 1997; Stone et al. 2003), partly by depressing transmitter release and partly by a direct inhibitory action on NMDA receptors (de Mendonca et al., 1995). The modulation of adenosine sensitivity is, therefore, a key factor in the control of network excitability in the hippocampus and elsewhere.

The activation of NMDA receptors can suppress the inhibitory action of adenosine on synaptic transmission in hippocampal slices (Bartrup & Stone, 1988, 1990; Nikbakht & Stone, 2001), and we have now explored the possible involvement of NO in this interaction. The observation that administration of the NO donor SNAP to hippocampal slices resulted in the induction of LTP is consistent with previous reports of the induction or facilitation of LTP (Zhou et al., 1994; Malen & Chapman, 1997; Bon & Garthwaite, 2001) and of the involvement of NO in electrically-induced LTP (Doyle et al. 1996). In addition, SNAP reduced the size of the inhibitory responses to adenosine. Even when the NO-induced LTP effect was cancelled by a compensatory reduction of stimulus strength, which reduced the increased potential size to that at the start of the experiment, adenosine responses remained suppressed. It could be argued that a reduction of stimulus strength is only a limited control procedure, since the biochemical consequences of inducing LTP may still be active. However, this procedure does at least allow a distinction between the effects of NO on sensitivity to adenosine and to baclofen, responses to the latter being unaffected by SNAP after this adjustment of stimulus strength, and indicating, whatever the underlying mechanisms, some specificity in the interaction between NO and adenosine responses.

The NO-induced LTP is not attributable to its inhibition of adenosine sensitivity since adenosine deaminase did not produce LTP in these slices, and even in its presence, NO was still capable of generating normal LTP. Neither is the NO-induced LTP dependent on NMDA receptors, since it was not impaired by 2AP5 as reported by Zhou et al., (1994) and Malen and Chapman (1997). Similarly, NMDA receptor activation does not appear to be instrumental in the inhibition by NO of responses to adenosine.

Superoxide

The activation of NMDA receptors can generate superoxide anions (Palumbo et al., 1992). Superoxide anions are also generated during the induction of LTP (Bindokas et al., 1996; Klann et al., 1998; Knapp & Klann, 2002), and their application to hippocampal slices induces LTP (Knapp & Klann, 2002). Conversely, the removal of superoxide by SOD impairs the induction of LTP (Gahtan et al., 1998; Thiels et al., 2000). Indeed, the fact that lipoxygenase activity can generate superoxide (Kukreja et al., 1986) and its inhibition can prevent LTP has led some to postulate a physiological role for superoxide in LTP (Lynch et al., 1989; Klann, 1998). We have, therefore, examined whether superoxide could modify adenosine responses.

Application of X/XO - a known source of superoxide - could induce LTP of evoked fEPSPs. Confirmation for the involvement of superoxide was provided by the blockade of the LTP by SOD. Superoxide was also able to suppress the inhibitory activity of adenosine. Interestingly, both actions were prevented, as was the activity of NO, by the guanylate cyclase inhibitor, ODQ, suggesting that the

actions of NO and superoxide are both mediated through cyclic GMP. Cyclic GMP activates cyclic GMP-dependent protein kinase C that has been proposed to play a role in induction of LTP by NO (Hawkins, et al., 1998; Calabresi, et al., 2000).

Since there is little direct evidence in the literature for an action of superoxide on guanylate cyclase, we considered whether this anion could induce the production of NO as a mediator of its effects on the cyclase. The NO-scavenger haemoglobin did prevent the actions of X/XO, both on LTP and the suppression of adenosine responses, but the NO synthase inhibitor L-NAME had no effect. These data may indicate that superoxide facilitates the formation of NO via the non-enzymic route described by Zweier et al. (1995) or that Hb interacts directly with X/XO (Sadrzadeh et al., 1984).

It was also of interest that xanthine itself could generate LTP and block the effects of adenosine. This did not appear to be a simple blockade of adenosine receptors, as it could be prevented by SOD. It is likely, therefore, that xanthine interacts with endogenous XO within the slices to generate ROS.

It has been suggested that free radicals can induce the release of excitatory amino acids in hippocampal slices (Pellegrini-Giampietro et al. 1988; Dawson et al., 1993; Gilman et al. 1994). The ability of ROS to suppress the inhibitory effects of adenosine could contribute significantly to this action by blocking presynaptic inhibitory adenosine receptors on glutamate-releasing terminals. Conversely, an enhancement of glutamate release, acting via NMDA receptors might contribute to the suppression of the inhibitory effects of adenosine by SNAP or X/XO (Nikbakht & Stone 2001).

Since the hypothesis which triggered this study was that the inhibition of adenosine responses by NMDA was mediated by NO, we performed a more direct test of the hypothesis using SOD and ODQ. We were able to confirm our earlier report that NMDA does indeed depress the inhibitory activity of adenosine but, whereas the inhibitory action of NMDA could be prevented by SOD, it was not convincingly prevented by guanylate cyclase inhibition which blocked the effects of both NO and X/XO. This could indicate that a different species of free radical, other than superoxide or NO, mediates the effect of NMDA on adenosine responses, perhaps acting independently of guanylate cyclase, or acting at a point between receptor activation and the enzyme. In contrast, the reduction of adenosine responses which follows the induction of LTP by electrical stimulation was prevented by L-NAME, implying that NO mediates this effect.

In summary, these data indicate that radical species such as NO and superoxide can suppress the inhibitory activity of adenosine at presynaptic sites, although neither of these radical species can fully account for the reduction of adenosine responses by NMDA. In contrast, electrically-induced LTP reduces sensitivity to adenosine via the generation of NO. The suppression of presynaptic adenosine responses may contribute to the injurious effects of free radicals in the CNS.

5. General discussion

Adenosine can inhibit or enhance neuronal responses by operating G-protein coupled receptors. Interactions between adenosine receptors and other G-protein coupled receptors and ionotropic receptors might be involved in a fine-tuning of neuronal activity. The main purpose of this study was to explore the interactions between adenosine and metabotropic glutamate receptors, nitric oxide and free radicals in the CA1 region of the hippocampus. We have extended earlier studies of the interactions between metabotropic glutamate receptors and adenosine receptors (de Mendonca & Ribeiro, 1997) to show, firstly, that the suppression of adenosine sensitivity is explained by a selectively reduced responsiveness to A_1 receptor stimulation, seen even in the absence of endogenous adenosine, and does not involve any facilitation of A_{2A} adenosine receptors. In addition we have shown that this effect can be localised to presynaptic terminals. However, the present data do not address the question as to whether the interaction between group I mGLURs and adenosine is a direct or indirect action. There is clear evidence that activation of group I metabotropic receptors at postsynaptic sites can lead to the release of a retrograde messenger that is responsible for at least part of the presynaptic effects of agonists (Levenes et al., 2001; Maejima et al., 2001; Watabe et al., 2002). Figure 5.1 summarises all results obtained in this thesis and presents them in the form of a hypothetical scheme. Only when there is clear identification of the nature of such a messenger can its role in the depression of adenosine sensitivity be investigated further. The interaction is not specific for adenosine receptors, but applies also to a suppression of responses mediated by $GABA_B$ receptors. The glutamate receptor involved appears to be the $mGlu_{1a}$

subtype and the action of the metabotropic receptor is mediated indirectly by activation or facilitation of NMDA receptors. The long duration of the changes induced by metabotropic glutamate receptor agonists implies that these interactions could play an important role in changes of synaptic function long after even transient increases of glutamate release in the CNS.

Reduction of the inhibitory effects of adenosine by activation of group I mGLURs might be involved in the pathophysiological situations such as hypoxia and ischemia that exhibit considerable release of both excitatory amino acids and adenosine. It seems that the excessive release of glutamate would control the neuroprotective properties of adenosine. These observations would be helpful for designing viable therapeutic strategies for treatment of neurological disorders. Group I metabotropic glutamate receptors antagonists or adenosine A1 receptor agonists attenuate the neuronal damage following brain injury, hypoxia or ischemia (Mukhin, et al., 1996).

Activation of NMDA receptors also suppresses neuronal responses to adenosine in hippocampal slices (Nikbakht & Stone, 2001). Since NMDA receptor activation leads to the generation of nitric oxide and superoxide, we have examined whether these can modify neuronal responses to adenosine and mediate the actions of NMDA. Nitric oxide is a physiological messenger in the central nervous system that is produced by activation of nitric oxide synthase. Pathological conditions such as hypoxia and ischemia leads to generation of NO and excessive NO becomes neurotoxic (Dawson et al., 1991). Nitric oxide is able to increase cellular calcium influx by opening the NMDA associated calcium channel, or augment excitatory amino acids by stimulating the release of both

glutamate and aspartate and inhibiting glutamate uptake. A further mechanism by which excess NO formation may lead to neuronal death is energy depletion due to mitochondrial dysfunction, such that it is not able to produce ATP. The mechanism of this dysfunction is not fully revealed but it has been suggested lack of mitochondrial respiration, swelling and increase in lipolysis of the mitochondrial membrane may be involved in this condition (Bolanos et al., 1997). An additional indirect mechanism by which NO may cause damage is depletion of the toxic molecule scavenger glutathione, through the formation of intracellular S-nitrosoglutathione.

Our findings indicate that radical species such as NO and superoxide can suppress the inhibitory activity of adenosine at presynaptic sites, although neither of these radical species can fully account for the reduction of adenosine responses by NMDA. In contrast, electrically induced LTP reduces sensitivity to adenosine via the generation of NO. The suppression of presynaptic adenosine responses may contribute to the injurious effects of free radicals in the CNS. This may represent one mechanism by which adenosine exerts its neuroprotective effect. The effects of adenosine on neuroprotection are exerted not only by promoting vasodilatation but possibly also by its neuromodulatory activities. Adenosine inhibits release of neurotransmitters presynaptically by inhibiting adenylate cyclase, opening K^+ channels and closing Ca^{2+} channels. Synaptic transmission is under control of endogenous extracellular adenosine with pre and postsynaptic interactions with other neuromodulators. The most frequent consequence of this interplay is neuronal protection through A1 receptor activation, but in some cases, A_{2A}-

mediated increases in neuronal activity may be harmful and therefore A_{2A} antagonism may be needed.

Application of X/XO - a known source of superoxide - could induce LTP of evoked fEPSPs and blockade of LTP with SOD supports its involvement in LTP. Superoxide was also able to suppress the inhibitory effects of adenosine. Interestingly, the guanylate cyclase inhibitor, ODQ, prevented both actions of X/XO and the activity of NO, suggesting that the actions of NO and superoxide are both mediated through cyclic GMP. Cyclic GMP activates cyclic GMP-dependent protein kinase C that has been proposed to play a role in induction of LTP by NO (Monfort, et al., 2002).

The NO-scavenger haemoglobin did prevent the actions of X/XO, both on LTP and the suppression of adenosine responses, while, the NO synthase inhibitor L-NAME had no effect. We assume that superoxide facilitates the formation of NO via the non-enzymic route (Zweier et al., 1995).

Overall this work has shown that presynaptic adenosine responses can be modulated by glutamate receptors and free radicals in way which may have important physiological and pharmacological relevance.

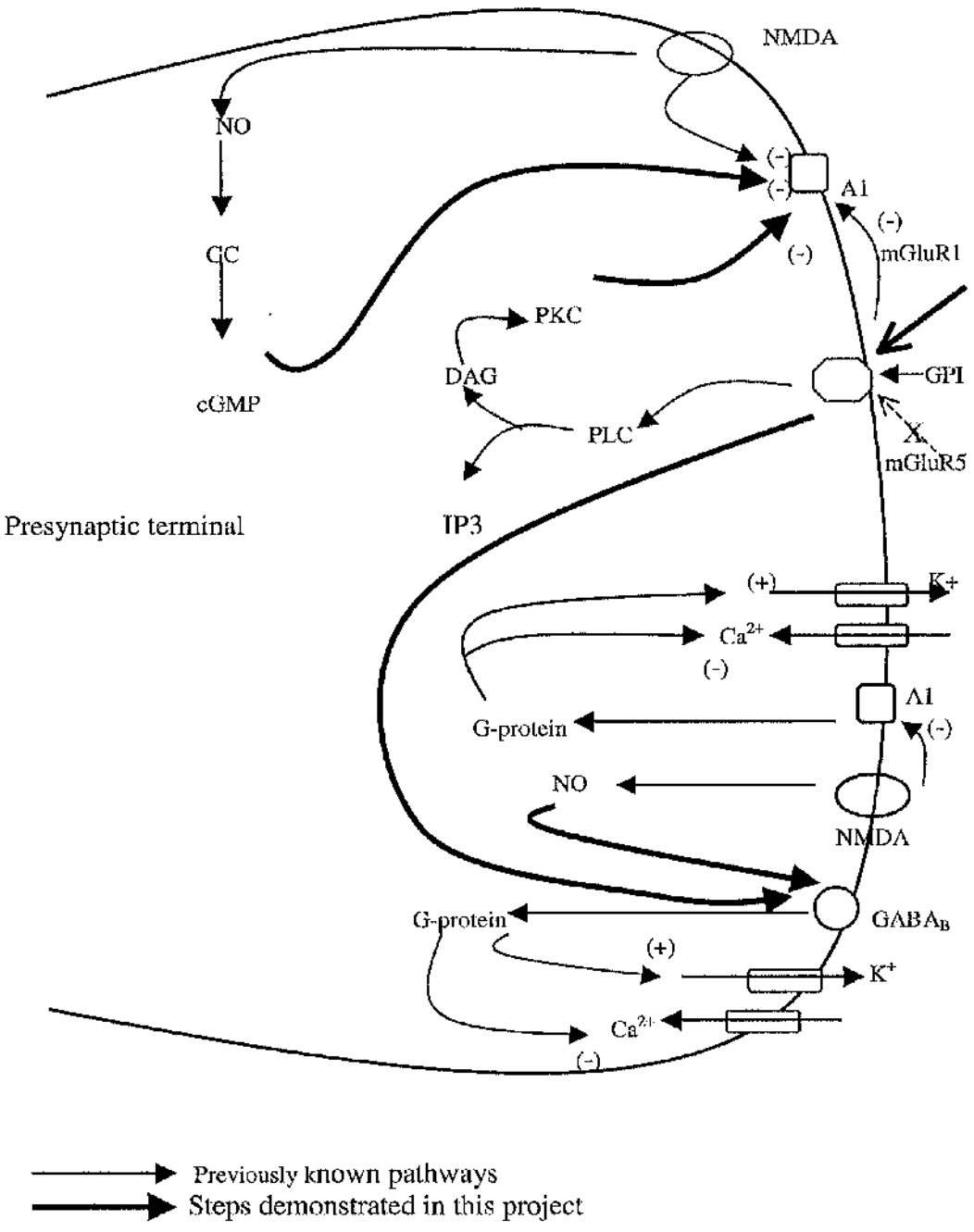

Figure. 5.1. This diagram represents the unknown pathways that have been found in this study on the interaction between adenosine and mGluRs, and interactions between adenosine and NO and free radicals in CA_3-CA_1 synapses in hippocampal slices.

Future work

A numbers of suggestions for further experiments are proposed as follows:

a) Further evaluation of interaction between adenosine and NMDA receptors to clarify which NMDA subunits are involved in the reduction of adenosine responses.

b) Investigating the interaction between adenosine and group II and III mGluRs.

c) Investigating site of possible interactions between adenosine and group II and III mGluRs using paired-pulse experiments and recording of spontaneous miniature EPSPs using intracellular or patch clamp methods.

d) Further studies to investigate the effects of xanthine that itself induced LTP and reduction of adenosine responses, using application of xanthine oxidase inhibitors. Such results would show whether endogenous xanthine oxidase is responsible for the effects of xanthine or some other systems may be involved.

e) Searching for the pathways that produce enzyme-independent formation of NO.

f) Investigate the kinds of protein kinases that might be involved in the effects of NO and X/XO.

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