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ASPECTS OF PURINE RECEPTOR FUNCTION IN HIPPOCAMPAL SLICES

by

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ABSTRACT

This study is concerned with the effect of adenosine and ATP in the CA1 area of hippocampus and their interaction with muscarinic and N-methyl-D-aspartate (NMDA) receptors. Single and paired-pulse stimuli have been applied to the Schaffer collateral- and comissural fibers and recordings were made by a glass microelectrode containing 0.9 % NaCl from stratum pyramidale.

The paired-pulse paradigm includes paired-pulse inhibition (PPI) and paired-pulse facilitation (PPF) which provide a more accurate indication of presynaptic events than the study of single population spikes and postsynaptic potentials. This technique has been employed to distinguish presynaptic events from postsynaptic. In paired-pulse experiments, the stimulus intensity of the conditioning pulse (first pulse) and testing pulse (second pulse) stimuli were always the same. The nature of the paired-pulse interaction in the absence of exogenous agents was dependent on the interstimulus intervals between two pairs of stimuli. A biphasic pattern has been observed with inhibition at shorter interstimulus intervals (10ms) and facilitation at longer intervals (20 & 50ms). However in a few slices no facilitation was observed at 20ms.

The effect of ATP and its stable analogus on single and paired-pulse responses has been examined. ATP and βγ-methyleneATP reproduced the effect of adenosine, reducing the paired-pulse inhibition at shorter interstimulus intervals and increasing facilitation at longer stimulus intervals. Their effects were mostly prevented by application of 8-phenyltheophylline (8PT), an A_1 receptor antagonist, and adenosine deaminase indicating their mediation by adenosine. The effect of βγ-methyleneATP was also significantly reduced by suramin 50μM suggesting the possible activation of P_2 receptors.

It has unexpectedly been found that the qualitative nature of the response to ATP and non-hydrolysable analogue βγ-imidoATP was dependent upon the population spike size.

When both nucleotides were applied to hippocampal slices having ppoulation sikes with amplitude≥5mV, population spikes were depresed but a significant fade in response has been observed during 10 minutes of perfusion. Suramin prevented the fade, while αβ-methylene ADP, an inhibitor of 5′-nucleotidase, enhanced the fade supporting the involvement of P_2 receptors through the release of endogenous adenosine. It has been concluded that in slices with large population spikes, a part of the inhibitory effect of nucleotides is produced by adenosine generated from the breakdown of nucleotides but another part may result from endogenous adenosine which is released following activation of P_2 receptors.

The second part of this thesis was to examine the interaction between adenosine and muscarinic receptors. The effect of the adenosine A_1 receptor agonist cyclopentyl adenosine (CPA) and the M_2 muscarinic receptor agonist oxotremorine-M was examined. Both compounds reduced the paired-pulse inhibition and increased the paired-pulse facilitation confirming the ability of both compounds to depress synaptic transmission at presynaptic sites. When combination of both at a range of concentration has been tested on evoked excitatory postsynaptic potentials (EPSPs), it has been found that the combined effect was significantly less than the additive responses.

In order to understand the common mechanism by which cyclopentyl adenosine (CPA) and oxotremorine-M depress transmitter release, the modulation of presynaptic K^+ conductances has been examined. Both inclusion of high potassium and application of 4-aminopyridine (4AP) prevented the effect of both agents on the paired-pulse interaction, suggesting that both agents inhibit synaptic transmission through the potassium channels located on the presynaptic nerve terminals.

The last part of this study was to investigate the interaction between adenosine and NMDA receptors activated directly by applied NMDA or indirectly using glycine, applying electrical long term potentiation (LTP) or removing Mg^{++} from media. The results showed that NMDA receptor activation significantly suppressed the single and paired-pulse responses to adenosine. However, the NMDA receptor antagonist 2-amino-5-phosphono-pentanoic acid (AP5) restored some of the effects of adenosine on paired-pulse interaction. The suppressant effect of NMDA was still seen in slices superfused

with bicuculline, 30μM. The inhibitory effect of adenosine on population spikes or the reduction in paired-pulse inhibition were suppressed when slices were superfused with magnesium-free media. Neither α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) nor kainate produced a suppression of adenosine responses.

Activating NMDA receptors following induction of electrical LTP or application of glycine also significantly reduced the effects of adenosine on population spike and paired-pulse interactions which could be antagonized by AP5. In the present experiments NMDA did not modify the inhibitory responses of the the adenosine A_1 agonist CPA but increased the excitatory effect of the A_{2A} receptor agonist 2-(2-carboxyethyl)phenylethylamino)-5'-N-ethylcarboxamidoadenosine (CGS21680) which was prevented by (4-(2-[7-amino(2furyl)[1,2,4]-triazolo[2,3-a][1,3,5]triazin- 5-ylamino]ethyl) phenol (ZM241835).

Overall, the results from this chapter have clearly shown the existence of both adenosine and NMDA receptors on presynaptic nerve terminals where NMDA receptor activation can modulate the effect of adenosine. It seems that an increase in the excitatory action of A_{2A} receptors is more relevant than a depression of A_1 receptor function.

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Contents

List of figures

Abbreviations

ACSF	Artificial cerebrospinal fluid
ADA	Adenosine deamianase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid
ANOVA	One-way analysis of variance
4-AP	4-Aminopyridine
2-AP5	2-amino-5-phosphonopentanoic acid
AMP	Adenosine monophosphate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
αβ-mATP	Alpha-beta-methyleneATP
βγ-mATP	Beta-gama-methyleneATP
CA1, CA2 , CA3	Cornu Ammonis 1, 2, 3
cAMP	Cyclic adenosine monophosphate
CCPA	2-chloro-N^6-cyclopentyl adenosine
CGS21680	2-[p-(2-carboxyethyl) phenylethylamino]-5' -N-ethylcarboxamidoadenosine
CHA	N^6-cyclohexyladenosine
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	Central nervous system
CPA	N^6-Cyclopentyladenosine
CPT	8-cyclopentyl-1,3-dimethylxanthine
CSC	8-(3-chlorostyryl) caffeine
DAG	Diacylglycerol
DCKA	Dichloro Kynurenic acid (DCKA)
DMSO	Dimethyl sulfoxide
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
EGTA	Ethylen glycol-bis (β-aminoethylether) N, N, N, N-tetraacetic acid
EPSP	Excitatory post synaptic potential

GABA	γ-Aminobutyric acid
GAD	Glutamate acid decarboxylase
IB-MECA	N^6-(3-iodobenzyl)5'-(N-methylcarbamoyl)adenosine
IMP	Inosine monophosphate
IPSP	Inhibitory post synaptic potential
IP_3	Inositol triphosphate
LC	Locus coeruleus
LTD	Long-term depression
LTP	Long-term potentiation
MAChR	Muscarinic Acetylcholine receptor
MCPG	α-methyl-4-carboxyphenyl glycine
mGluR	metabotropic glutamate receptor
NAChr	Nicotinic acetylcholine receptor
NECA	N-ethylcarboxamidoadenosine
NMDA	N-methyl D-aspartate
n.s.	non-significant
NPY	Neuropeptide Y
PIA	Phenyl-isopropyl-adenosine
PIP2	Phosphatidylinositol-4,5-bisphosphate
PPADS	Pyridoxalphosphate-6-azophenyl-2', 4'-disulphonic acid
PPD	Paired-Pulse Depression
PPF	Paired-Pulse Facilitation
P.S.	Population spike
8-PT	8-Phenyltheophylline
SAH	S-adenosylhomocysteine
S. E. M.	Standard Error of the Mean
STD	Short-Term Depression
STP	Short-Term Potentiation
TEA	Tetraethyl ammonium
UTP	Uridine triphosphate
ZM241385	(4-(2-[7-amino-2-(2-furyl)[1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl) phenol

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1: INTRODUCTION

Adenosine: an historical perspective

Adenosine is an important endogenous modulator that has been shown to play a role in the regulation of physiological activity in several organs and tissues. It has also been proposed as an important modulator of transmission at many synapses in the nervous system and its ability to depress transmitter release as well as postsynaptic responses in many systems is well established. In the central nervous system adenosine has a marked depressant effect upon excitatory synaptic transmission but it is unclear whether these depressant effects are induced by diminishing transmitter release, reducing postsynaptic sensitivity to the transmitter, other postsynaptic actions of adenosine or a combination of pre-and post synaptic actions.

Adenosine has been shown to exert a number of physiological effects on the various tissues. The first demonstration which showed that adenosine is active was made by Drury and Szent-Györgyi in 1929. They reported that extracts from heart muscle, brain, kidney and spleen had pronounced effects on the cardiovascular system. In 1959 Holton showed that during antidromic stimulation of sensory nerves ATP was released in sufficient quantities to produce vasodilatation of rabbit ear arteries and in 1963 Berne suggested that adenosine can be the regulator of coronary blood flow during reactive hyperaemia.

It was showed by Sattin and Rall (1970) that adenosine was involved in transduction mechanisms. They showed that adenosine could stimulate cyclic adenosine monophosphate (cAMP) formation in brain, while caffeine could antagonise this response. Kostopoulos and Phillis (1977) demonstrated the depressive effect of adenosine in different areas of brain. It has been hypothesised that adenosine acts as a local hormone that modulates the activity of tissues near its site of release although there is a lack of evidence that adenosine acts as a classical neurotransmitter (Arch & Newsholme, 1978b). Burnstock (1978) proposed two major types of purinergic receptors: P_1 purinoreceptors that are selective for adenosine and act via the adenylate

cyclase pathway, and are antagonised by methylxanthines, and P_2 receptors, which are selective for nucleotides.

Formation, release and inactivation of adenosine

Formation

The de novo pathway of adenosine production starts with 5-phosphoribosyl-1-pyrophosphate. By using 5 molecules of ATP (adenosine triphosphate) inosine monophosphate (IMP) is formed. IMP can be converted into adenine nucleotide derivatives (AMP). Adenosine can be formed by dephosphorylation of AMP by 5'nucleotidase. The conversion of hypoxanthine to IMP by the enzyme hypoxanthine-guanine phosphoribosyltransferase is a metabolic source of adenosine. The second metabolic source of adenosine is the hydrolysis of S-adenosylhomocysteine (SAH) to adenosine and homocysteine (Stone & Simmonds, 1991).

Release

Adenosine is released in a calcium-dependent manner from nerve terminals following nerve stimulation (Meghji, 1991). Adenosine may be produced intracellularly following special conditions such as ischemia and hypoxia as a result of an imbalance between energy demand and energy supply. Therefore adenosine levels can be regulated by the balance of energy supply and demand (Stone et al, 1989).

It does not seem that adenosine is sequestered in nerve terminals and released in the manner of classical neurotransmitters. The possible sources for extracellular adenosine could be from catabolism of nucleotides which are released into the extracellular space by a non-synaptic mechanism (Stone et al ., 1991; Brundege & Dunwiddie, 1997).

Chemical agents such as veratridine or excitatory amino acids can release adenosine from brain slices (Pedata et al., 1991). In addition, electrical stimulation can also release adenosine from the brain (Sciotti, 1993). Extracellular brain adenosine levels increase during various pathological conditions such as ischemia, hypoxia and systemic hypotension (Bender et al., 1981). ATP is stored with acetylcholine and noradrenaline in

cholinergic and sympathetic nerves respectively (Silinsky, 1975; Lew & White, 1987; Von Kugelgen & Strake, 1990; Zimmermann, 1994a). There is some evidence that ATP which is subsequently converted to adenosine by ectonucleotidases could be released as a cotransmitter with acetylcholine or noradrenaline.

Breakdown and Removal

Adenosine is taken up into cells and appears to be converted back into ATP intracellularly (Bender et al., 1981). Uptake of adenosine occurs via nucleoside transporters which work by a facilitated diffusion mechanism and are bidirectional. Adenosine uptake into the cells only occurs if the intracellular concentration of adenosine is kept below the extracellular concentration. Adenosine is inactivated either via the breakdown to inosine by cytosolic adenosine deaminase and subsequently hypoxanthine and xanthine or conversion to adenosine monophosphate by phosphorylation by cytosolic adenosine kinase (Arch & Newsholme, 1979; Meghji, 1991). In addition, adenosine can be inactivated extracellularly without any need to be taken up because of the presence of adenosine deaminase in an extracellular membrane-associated form (Meghji, 1991). It seems that phosphorylation occurs at low adenosine concentrations whereas the deamination pathway is important at higher adenosine concentrations (Meghji, 1991).

Adenosine receptors

Classification

In 1978,Burnstock introduced a classification of purinoreceptors into $P_1$and P_2 types. At the P_1-purinoreceptors, adenosine acts with greater potency than ATP and the response is inhibited by theophylline. At the P_2 purinoreceptors, however, ATP is more potent than adenosine and not antagonised by theophylline. Therefore receptors were classified as P_1 and P_2 receptors with their preference for binding either adenosine or adenine nucleotides respectively. P_1 receptors were classified as A_1 and A_2 subtypes based on the ability to depress and stimulate cyclic adenosine monophosphate (cAMP) accumulation

respectively (Burnstock, 1978; Van Calker et al., 1979). Adenosine A_1 receptors are best distinguished by 2-chloro-cyclopentyl adenosine (CCPA) binding (Bailey & Hourani, 1990) whereas A_2 receptors can be selectively identified by 2[p-(2-Carboxyethyl) phenylethylamino]-5'-N-ethylcarboxamidoadenosine (CGS 21680) (Bailey & Hourani, 1992). Biochemical and genetic studies now confirm the subdivision of adenosine receptors into A_1 and A_2 subtypes. Various molecular probes were used to isolate and identify the receptors. On the basis of these studies it has been shown that both receptors (A_1 & A_2) are glycoprotein but with different masses (Collis & Hourani, 1993). A_2 receptors were subsequently subdivided into A_{2A} and A_{2B} receptors on the basis of the presence of a low affinity and high affinity adenosine sensitive site for A_{2B} and A_{2A} respectively (Collis & Hourani, 1993). It is now accepted that adenosine receptors are divisible into four subtypes named A_1, A_{2A}, A_{2B} and A_3 (Olah & Stiles, 1995).

Distribution

At present the four subtypes of adenosine receptors have been identified and cloned. All of these receptors belong to the G-protein coupled receptor superfamily (Palmer & Stiles, 1995). A_1 receptors are the most widespread adenosine receptors in the nervous system and bind adenosine with high affinity. A_1 receptors are located throughout the brain, but in the rat brain, areas of high concentration of A_1 binding sites include the molecular layer of the cerebellum, dendritic zones of hippocampus, the medial and lateral nuclei of the thalamus, the lateral septum and medial geniculate body (Lewis et al., 1981; Goodman & Snyder, 1982; Fastbom et al., 1987).

A_2 receptors have been further subdivided into A_{2A} and A_{2B} subtypes. It has been demonstrated by ligand binding and molecular genetic techniques that A_{2A} receptors are concentrated in dopamine receptor rich areas in the brain including caudate-putamen, nucleus accumbens and olfactory tubercle (Schiffmann et al., 1991; Stehle, 1992), though they occur in hippocampus, cerebral cortex but in a lesser extent (Ongini & Fredholm, 1996; Sebastiao & Riberio, 1996). Adenosine A_{2A} receptors are also found in several peripheral tissues including liver, kidney and on the several blood vessels, platelets and polymorphonuclear leukocytes (Ongini & Fredholm, 1996). A_{2B} receptors

have also been identified in glial and in neuronal cells, probably with a widespread distribution in the brain (Sebastiao & Ribeiro, 1996). It has been shown that binding sites for [H^3] CGS 21680, a radioligand with high affinity and selectivity for A_{2A} receptor subtypes are localised in the caudate nucleus, globus pallidus and putamen of human brain (Latini et al., 1996).

Ligand binding studies

Agonist radioligands

The first successful binding studies were performed with the radiolabeled N^6 - substituted adenosine dervivatives [H^3]CHA and [H^3]Phenylisopropyl adenosine (PIA) (Williams & Jacobson, 1990). Three compounds have been synthesised which are more potent and more selective than the original compounds. N^6-cyclopentyladenosine (CPA), 2-chloro-N^6-cyclopentyladenosine (CCPA) and S-N^6-(2-endo-norbornyl)adenosine have been reported to have higher potency and selectivity for A_1 adenosine receptors than R-PIA (Moos et al., 1985). [H^3]CCPA has been introduced as a highly selective radioligand for labelling A_1 receptors.

There has been less success in identifying and developing A_2 receptor ligands. Until a few years ago, only a few selective agonists for A_2 adenosine receptors had been demonstrated, the only ligand identified for studying A_2 receptors being N-ethylcarboxamidoadenosine with a relatively high affinity for A_1 receptors and therefore not A_2 selective. A considerable improvement was obtained by using the agonists in the presence of a high concentration of R-PIA. More recently the highly A_2 selective agonist radioligand [H^3]CGS 21680 has been introduced (Reddington and Lee, 1991). CGS21680 has 140 fold selectivity for A_{2A} versus A_1 receptors (Ralevic & Burnstock, 1998).

At present N^6 -(3-iodobenzyl)5'-(N-methylcarbamoyl) adenosine (IB-MECA) is the most useful agent for activation of the A_3 receptors. It is an A_3 selective agonist which is 50-fold selective in binding assays for rat brain A_3 Vs either A_1 or A_{2A} receptors (Gallo-Radriguez et al., 1994).

Antagonist radioligands

Several adenosine antagonists have also been radioactively labelled and used for identification of receptors. 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) is a specific antagonist ligand for A_1 receptors with an A_1 selectivity of 740 fold Vs A_2 receptor in rat brain membranes (Ralevic & Burnstock, 1998). DPCPX is now widely used as the preferred radioligand for the study of A_1 adenosine receptors because it has a low proportion of non-specific binding (Schwabe, 1991). 8-(3-chlorostyryl) caffeine (CSC) shows 5620-fold selectivity for A_2 receptors but there is not any selective A_{2B} receptor antagonist (Jacobson et al., 1993).

Peripheral actions of adenosine

Adenosine receptors are present on most cells and organs in the mammalian body. It seems that adenosine participates in numerous processes underlying normal tissue function.

Adenosine has significant effects on cardiovascular physiology. It reduces heart rate by a negative chronotropic effect on cardiac pacemakers. It also displays a negative inotropic effect by shortening the duration of the cardiac action potential and by reducing the force of contraction of arterial muscle (Pelleg, 1993). However, excitatory effects of adenosine on cardiac ventricular automaticity have been reported in patients given adenosine and in animal studies [in *vitro* and *in vivo*] (Hernandez & Ribeiro, 1996).

With the exception of kidney and placenta adenosine has a potent vasodilatory effect on all vascular beds. It reduces the peripheral vascular resistance via activation of A_2 receptors (Olsson, 1981). Blood cell functions are affected by physiological concentrations of adenosine. Platelet aggregation may be inhibited by adenosine via A_2 receptor activation. Some functions of neutrophils are inhibited by adenosine. Adenosine inhibits phagocytosis, generation of toxic /oxygen metabolites and white cell adhesion (Cronstein, 1994).

Central actions of adenosine

Adenosine has been known as a potent and effective modulator of the central nervous system. In 1974 Phillis et al observed that using a large number of purines by microiontophoresis could depress the firing rate of neurones in the cerebral cortex of rats. Adenine derivatives were the most potent compounds among them. Later other investigators showed that adenosine and related agents have a common depressant action in other regions of the central nervous system including olfactory bulb, striatum, hippocampus and thalamus (Kostopoulos & Phillis, 1977). In the central nervous system adenosine has presynaptic, postsynaptic or non-synaptic actions.

Presynaptic actions

It is widely accepted that adenosine inhibits the release of several neurotransmitters including acetylcholine, glutamate, noradrenaline, dopamine and 5-hydroxytryptamine (Brundege & Dunwiddie, 1997). It is also accepted that adenosine inhibits transmitter release by receptors that are pharmacologically similar to A_1 receptors. However some evidence exists that A_2 receptor activation enhances transmitter release (Fredholm & Dunwiddie, 1988). Decreasing the turnover of neurotransmitters by adenosine and related analogues and increasing them by adenosine receptor antagonists is widely accepted but the mechanism of this inhibitory effect of adenosine is not completely clear. Inhibition of neurotransmitter release appears to be a presynaptic effect because (1) adenosine decreases the amplitude of excitatory postsynaptic potentials without changing the resting membrane potential or the input resistance of postsynaptic neurones (2) sensitivity of postsynaptic neurones is not decreased significantly (3) adenosine decreased the quantal content of evoked release of neurotransmitter more efficiently than spontaneous release (Ribeiro, 1995). In the case of the hippocampus, the depression of synaptic responses strongly appears to be primarily presynaptic, suggesting that adenosine can suppress the release of excitatory transmitters at Schaffer and commissural synapses as much as it does in other preparations.

The release of excitatory neurotransmitters appears to be more strongly affected than that of inhibitory neurotransmitters. It has been shown that GABAergic neurones are not sensitive to the release-inhibiting features of adenosine receptors in the hippocampus,(Lambert & Teyler, 1991; Thompson et al., 1992) suggesting that the role of A_1 receptors in the hippocampus is to inhibit excitatory neurotransmitters (Brundege & Dunwiddie, 1997). A large proportion of the adenosine receptors are located not in cell bodies or dendrites but in terminal regions (Fredholm et al., 1996). In conclusion it seems that adenosine inhibits neurotransmitter release by activating inhibitory receptors (A_1 family) which are located on nerve terminals. A_1 receptors are coupled to members of the G protein family which then inhibit adenylate cyclase and result in decreased intracellular cAMP accumulation, activate potassium channels and inactivate some types of voltage dependent Ca^{2+}-channels and calcium channel activated phospholipase C (Brundege & Dunwiddie, 1997).

However, it is not clear yet if adenosine acts independently on each of these mechanisms or in combination. One possible mechanism of adenosine to inhibit transmitter release may be inhibition of calcium entry into the nerve terminal (Schubert et al., 1986). Another mechanism could be the ability of adenosine to activate a potassium conductance in the presynaptic terminal, which results in decreasing the action potential and voltage dependent calcium conductance. In addition adenosine may inhibit neurotransmitter release directly in the presynaptic terminal (Brundege & Dunwiddie, 1997). Silinsky proposed that adenosine impairs transmitter secretion by reducing the affinity for calcium at a site beyond the external orifice of the calcium channels (Silinsky, 1986).

Excitatory actions of adenosine receptors on neurotransmitter release in the central and peripheral nervous system have been reported. A high concentration of nonselective or mixed adenosine agonists increases transmitter release rather than inhibiting it. The excitatory action of adenosine on neurotransmitter release is mediated by A_2 receptors. By using A_1 selective antagonists it has been observed that NECA (N-ethylcarboxamidoadenosine), a non selective adenosine receptor agonist induces an excitatory action on neurotransmission (Latini et al, 1996).

Postsynaptic actions

Several groups concluded that postsynaptic receptor effects of adenosine are mediated by potassium channels (Trussell & Jackson, 1987; Thompson et al., 1992) whereas control of calcium fluxes could be another postsynaptic effect of adenosine.

In hippocampal neurons, activation of A_1 receptors causes a hyperpolarization of the cell membrane with a decrease in the membrane resistance. This effect has been shown to be mediated by activation of a postsynaptic potassium conductance (Dunwiddie & Fredholm, 1989). In a set of experiments Trussell and Jackson (1987) showed that activation of potassium channels in postsynaptic neurones from rat striatum is induced by adenosine. Postsynaptic actions of adenosine result in a further reduction in the magnitude of the excitatory postsynaptic potential (EPSP) which appear to reflect the activation of post synaptic K^+ currents.

Activation of A_1 receptors also inhibits calcium current in a wide variety of neurones (Umemiya & Berger, 1994). It seems that all of these postsynaptic events are mediated by a pertussis toxin sensitive G-protein (Mynlieff & Beam, 1994).

Post- receptor mechanism of adenosine

Intracellular responses to agonist stimulation will be different according to the cell type and post receptor mechanisms.

G-protein

G-proteins consist of a heterotrimeric compound of alpha, beta and gamma subunits. The alpha subunit directly regulates adenylate cyclase and other enzymes and effectors. The other parts are required for efficient coupling of the alpha subunit to the receptor and influence the rate of GDP dissociation (Johnson, 1990). A GTP binding protein transduces signals in to the effector systems such as an ion channel, adenylate cyclase and phospholipase C. It seems that all of the A_1 family of adenosine receptors in the

hippocampus are linked to G-proteins. Adenosine receptors appear to be linked to many effectors via various classes of G-proteins (Fredholm et al., 1989).

Adenylate cyclase and cyclic AMP system

In 1970 Sattin and Rall suggested that some actions of adenosine could be related to modulation of adenylate cyclase activity. These authors showed that adenosine increases cyclic AMP accumulation in cortical slices (Sattin & Rall, 1970). A biphasic effect of adenosine on cAMP accumulation has been reported. In low micromolar concentration adenyl cyclase is stimulated and in higher micromolar concentration it is inhibited by adenosine (Haslam & Lynham, 1972). These biphasic concentration-dependent effects of adenosine are suppressed by A_1 and A_2 antagonists such as caffeine and other methylxanthines. Inhibition of adenylate cyclase resulted in decreasing intracellular cAMP levels which in turn induced some physiological responses to A_1 receptor stimulation. Two good examples of this mechanism are the A_1-mediated lipolytic effect in adipocytes and the negative inotropic effect in ventricular myocytes (Green, 1991).

Diacylglycerol/phosphatidyl inositol (PI)

Many actions of several neurotransmitters including acetylcholine,5-hydroxytryptamine and noradrenaline are mediated by second messengers named inositol 1,4,5,-trisphosphate (IP_3) and diacylglycerol (DAG) which are produced from the breakdown of the phospholipid, phosphatidylinositol 4,5,bisphosphate via the action of the enzyme phospholipase C. It does not seem that adenosine has a direct effect on phosphatidylinositol turnover. However some evidence exists that adenosine is involved in the modulation of phosphatidyl inositol (PI) responses to other neurotransmitters (Morgan, 1991).

Neurotransmission in hippocampus

A large number of putative neurotransmitters and neuromodulators have been studied in the hippocampus. They can be divided in two main categories: excitatory and inhibitory neurotransmitters.

Excitatory neurotransmitters

Glutamate

The most important excitatory neurotransmitter in the hippocampus is glutamate. Glutamate is the precursor for the equally important neurotransmitter GABA as well.
The first demonstration was carried out during the years 1974-1976 that the excitatory pathways of mammalian CNS release glutamate and/or asparate (Clark & Collins, 1976). There is now a general agreement that glutamate is the major excitatory neurotransmitter in the hippocampus. The glutamate function in the brain is mediated by two main types of receptors, ionotropic and metabotropic.
Ionotropic glutamate receptors fall in to three categories, (a): N-methyl-D-aspartate (NMDA) receptor, (b): α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors and (c): kainate receptors.

Most excitatory postsynaptic currents (EPSP's) are generated through activation of both AMPA and NMDA receptors (McBain & Mayer, 1994). AMPA and kainate receptors mediate fast excitatory postsynaptic potentials (fast EPSPs) whereas the NMDA component is much slower and smaller. NMDA, AMPA and kainate receptors are present at high concentration in the hippocampus (Cotman et al., 1987), although the combination of those receptors in the different synapses and pathways are varied. It has been reported that fewer NMDA receptors exist at mossy fibre synapses (Monaghan et al., 1983) whereas a report suggested the presence of pure NMDA receptors but no detectable AMPA receptors in a proportion of excitatory synapses on CA1 pyramidal synapses (Isaac et al., 1995).

The highest concentration of NMDA receptors in the whole brain is present in the dendritic area of Schaffer collaterals and the perforant pathway synapses. The density of binding sites in mossy fibre synapses is much lower relatively (Brown & Zador, 1990). Biophysical studies revealed that ion channels gated by non-NMDA glutamate receptors are highly selective for Na^+ and K^+(Macdermott et al., 1986; Jahr & Stevens, 1987), whereas the NMDA receptor channel is permeable to Ca^{++} as well as Na^+ and K^+(Jahr & Stevens, 1987; Mayer & Westbrook, 1987).

Under physiological conditions (in the presence of Mg^{++}) the NMDA group contribute little to synaptic transmission because the associated ion channel is blocked in a voltage dependent manner by magnesium ions (Nowak et al., 1984; Mayer et al., 1984). The Ca^{++} permeability in non-NMDA receptors is about 100 times less than for NMDA receptors, except for particular subtypes of the non-NMDA receptor (Ogura et al., 1990; Iino et al., 1990). The non-NMDA currents show little voltage dependency and can be blocked by the selective antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX).

Metabotropic glutamate (mGluRs) differ from ionotropic NMDA and non-NMDA glutamate receptors because they mediate their action through intermediary G-proteins (Miller 1991). These G-protein coupled receptors can control ion channels or intracellular messenger systems.

Phospholipase C is activated by class 1 (mGluR) which results in the formation of diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). Activation of some metabotropic glutamate receptors results in modulation of adenylate cyclase and subsequent reduction in cAMP level (Saugstad et al., 1997).

The presence of several subtypes of mGlu receptors in the hippocampus of the adult rat has been shown through Northern blotting, in situ hybridisation and immunohistchemistry studies (School & Kimblberg, 1999) and physiological effects of a number of mGluRs in the hippocampus of neonatal and adult rats has been reported (Ross et al., 2000).

Acetylcholine

Acetylcholine (ACh) is the second important excitatory neurotransmitter that modulates neurotransmission in the hippocampus. Cholinergic neurones arise from the medial septum-diagonal band complex (MSDB) of the basal forebrain which is known as a septohippocampal pathway and synapse on the excitatory and local inhibitory interneurones in the hippocampal formation (Jones & Yakel, 1997). The hippocampal formation receives the septal innervation through three routes: the fimbria, the dorsal fornix and the supracallosal striae.

Using choline acetyltransferase (chAT) immunocytochemistry showed the distribution of cholinergic neurons in the hippocampus. Cholinergic cells are localised within the subiculum. In the area CA1 of Ammon's horn and in dentate gyrus most of these cells are located in the stratum lacunosum moleculare and in the border of the stratum radiatum and the stratum lacunosum moleculare. The molecular layer of the dentate gyrus also contains some of the cholinergic neurones. The existence of cholinergic nerve terminals was shown in the hippocampus under the light and electron microscopes (Martínez-murillo & Rodrigo, 1997).

Acetylcholine (ACh) receptors in the hippocampus

The effect of acetylcholine is mediated by two different classes of receptors named nicotinic and muscarinic receptors.

Nicotinic receptors

The nicotine ACh receptor is a pentameric protein, consisting of two α subunits, a β, a γ and a δ subunits and belongs to the ligand gated ion channel receptors (ionotropic receptors).

Modulation of neurotransmitter release by nAchRs has been reported recently. Glutamate release from the terminals of mossy fibres of granule cells in the dentate gyrus can be modulated by nicotinic receptors, which are present on the presynaptic nerve terminal (Gray et al., 1996). Relatively little binding of nAChRs in the hippocampus has been expressed in the human brain with exception of the stratum lacunosum moleculare in CA2-CA3 and to a lesser extent in supragranular and subgranular areas of the dentate.

It seems that nAChR mediate postsynaptic nicotinic responses widely in the nervous system due to the presence of functional nAChRs on cell bodies of many neurones (Brown et al., 1983). Recently, Jones and Yakel (1997) using patch clamp methods demonstrated the effect of functional nAChRs on interneurons in the hippocampus. Activation of these receptors can modulate the neuronal excitability. It has been shown that nicotinic binding sites are reduced in neuropathological conditions such as Alzheimer's and Parkinson's diseases (Nordberg, 1994).

The nicotinic excitation is characterised by a rapid depolarisation of postsynaptic neurones, which result from the opening of a non-specific cationic channel associated with the receptor complex.

Muscarinic receptors

Five genes encoding muscarinic receptors have been cloned from hippocampal formation (m1-m5) and all of their associated mRNA are present in the hippocampal formation (Bonner et al., 1988). Immunocytochemistry studies and using of specific antibodies showed the presence of four receptor proteins (M_1-M_4) in hippocampus but the m_5 has not been detected (Levey et al., 1991; Hersch et al., 1994). Autoradiographic studies also showed the distribution of mAChR subtypes in different parts of the brain. The hippocampus is enriched in the pirenzepine sensitive receptor (M_1). The abundance of M_3 in the hippocampus seems to be similar to the M_1 (Quirion et al., 1993). M_3 receptor binding sites are concentrated in dentate gyrus and CA1 pyramidal cells. However M_1 is distributed throughout the whole hippocampus (Dutar et al., 1995). Muscarinic receptors are present presynaptically and postsynaptically in the hippocampus (William & Johnston., 1993).

It has been shown that a subset of M_2 muscarinic receptors is located on cholinergic axon terminals in hippocampus. The recent anatomical lesioning studies by Levey et al (1995) approved the existence of M_2 muscarinic autoreceptors on cholinergic septo-hippocampal terminals.

Mechanisms of the effect of ACh in the hippocampus

Presynaptic inhibition of neurotransmitter release is the most important mechanism by which muscarinic agents can inhibit the synaptic responses.

Hounsgaard (1978) presented the first clear evidence for the presynaptic effect of muscarinic receptors in hippocampal slices. He showed that application of cholinergic agonists depressed the EPSPs evoked from CA1 pyramidal neurons indicating the inhibition of release of the excitatory neurotransmitter glutamate from nerve terminals (Hounsgaard, 1978), although facilitation of neurotransmitter release by nicotinic agents has been demonstrated. Ionophoresis application of ACh on the dendrites of a pyramidal neuron near synaptic sites inhibited the synaptic responses suggesting the presynaptic effect of Ach, but it has the opposite action if iontophoresed on the soma (Valentino & Dingledine., 1981).

The existence of presynaptic muscarinic receptors on interneurons has also been reported; activation of these receptors can increase the excitability of the interneuron and increase the GABA release which in turn can modulate synaptic transmission in hippocampus (Pitler and Alger, 1992).

Muscarinic cholinergic responses

The muscarinic effects can be divided into muscarinic excitatory and muscarinic inhibitory actions. It is a general agreement that most of the excitatory responses to cholinergic agents are mediated by suppression of potassium conductances.

These responses are summarised as follows.

1-Muscarinic depolarisation of neurones with characteristics of long-term duration, slow onset and an increase in input resistance. The depolarisation effect can enhance the excitatory post-synaptic potentials and initiate action potential firing by bringing the membrane potential closer to the threshold. The depolarising effects of cholinergic agents in brain were initially reported by Krnjevic et al (1971) following iontophoretic application of ACh or its stable analogue carbachol on to cat neocortical neurons *in vivo*. Subsequently, the strong depolarising effects of ACh and the other analogues was demonstrated on slice preparations by using extra- and intracellular recording and patch-clamp analysis. The results from several experiments showed that the effect of cholinergic agonists is due to a decrease in potassium conductances. The effect is blocked by a non-selective muscarinic antagonist atropine and is antagonised by a M_1 receptor selective antagonist pirenzepine.

2: The afterhyperpolarisation (AHP) which is normally observed following a series of action potentials has a long duration of decay from tens to a few hundred milliseconds and is due to a calcium-dependent potassium conductance. It is blocked by muscarinic activity which facilitates the ability of excitatory inputs to bring the cell to the threshold. Several attempts have been made to determine the muscarinic receptor subtype, which is responsible for AHP suppression. Dutar and Nicoll (1988) reported that the blockade by low concentration of carbachol (1μM) of AHP is completely preventable by pirenzepine.

3: Blockade of voltage and time dependent potassium current, called M current. The M-current hyperpolarises the cell membrane when the neuron is depolarised toward the threshold for action potential firing. I_M is inhibited by muscarinic receptors and can contribute to the enhancement of neuronal responses to depolarising synaptic events (Marrion, 1997).

Several studies have found that gallamine was effective to produce an almost total antagonism of blockade of the M current, indicating the involvement of the M_2 receptor subtype (Dutar & Nicoll., 1988). Although most of the postsynaptic excitatory effect of muscarinic agents are mediated by modulation of potassium conductances, some other mechanisms have been suggested, such as blockade of voltage- independent Cl conductances.

Activation of non-selective cationic conductances (Brown and Selyanko, 1985) and increasing the intracellular free Ca^{++} (Kudo et al., 1988) in different nerve tissues are also mechanisms by which the muscarinic agents can increase excitability of the cells.

Presynaptic depression of EPSPs is sensitive to gallamine and therefore is probably mediated by m_2 receptors (Dutar & Nicoll, 1988). One possible mechanism responsible for the presynaptic inhibitory effect of muscarinic agents is the blockade of voltage activated Ca^{++} channels in the presynaptic nerve terminal and subsequent reduction in Ca^{++}influx through these channels (Anwyl, 1991).

Inhibitory Neurotransmitters in Hippocampus

GABA

γ-Aminobutyric acid (GABA) is known as one of the major inhibitory neurotransmitters in the brain (Roberts et al., 1976). GABA is essential for the overall balance between neuronal excitation and inhibition.

GABA receptors are classified into ionotropic and metabotropic. The ionotropic receptor ($GABA_A$) is a ligand- gated anion-selective channel with a pentameric complex of structurally homologous subunits (Smith & Olsen, 1995; Sieghart ., 1995).

$GABA_A$ receptors are the targets for many important neuroactive drugs, including benzodiazepines, barbiturates, steroids and general anaesthetics (Macdonald & Olsen, 1994).

$GABA_A$ receptors are coupled to a fast chloride conductance and activation of these receptors by GABA results in an increase in neuronal membrane conductance for Cl^- and subsequent hyperpolarisation (Macdonald & Twyman, 1992). The effect of depolarising stimuli is suppressed following a decrease in input resistance in post synaptic cells induced by excitation of the $GABA_A$ receptor subtype (Bormann, 1988).

The second type of GABA receptor is the $GABA_B$ receptor which belongs to the metabotropic receptors. They are insensitive to bicuculline and activated by the selective

agonist baclofen. Postsynaptic effects of $GABA_B$ receptor activation is the consequence of activating potassium conductances and is mediated through a GTP-binding protein. $GABA_B$ receptor activation inhibited the release of several neurotransmitters including glutamate and GABA from nerve terminals.

Reduction in Ca^{++} conductances could be one of the possible mechanisms by which GABA can suppress the transmitter release (Deisz, 1997).

Glycine

Glycine is considered as inhibitory transmitter in the brain stem but its role as classical neurotransmitter in hippocampus is quite limited. However it has attracted considerable attention as a possible modulatory site for NMDA-receptor mediated synaptic events (Forsythe et al., 1988). The potentiation of N-methyl D-aspartate (NMDA) receptor-mediated responses by glycine has been shown in cultured brain neurons (Thomson, 1989).

Glycine has been defined as a positive modulator of NMDA responses and its effects are not blocked by strychnine and can be antagonised by 7-chlorokynurenic acid and Indole-2-carboxylic acid (Kemp et al., 1988; Izumi et al., 1990).

ATP

Drury and Szent-Gyorgyi (1929) first reported the potent extracellular effects of purine nucleosides and nucleotides on the mammalian heart.

Holton (1959) presented the first evidence indicating a transmitter role for ATP in the nervous system by showing the release of ATP during antidromic stimulation of sensory nerves. Later the nonadrenergic noncholinergic (NANC) nature of the nervous innervation of the gut and bladder was proposed (Burnstock et al., 1970; 1972). Burnstock in 1972 proposed the purinergic hypothesis, suggesting that the main substance released from NANC nerves, at least in the intestine and bladder was the purine nucleotide ATP, and the corresponding nerves were called purinergic (Burnstock et al., 1972; Burnstock, 1972).

Recently a role for purinergic neurotransmission in the central nervous system has also been revealed (Edwards et al., 1992; Zimmermann, 1994). ATP is currently recognised as a neurotransmitter or cotransmitter in several tissues (Hoyle & Burnstock, 1991; Von Kügelgen & Strake 1991; Zimmermann, 1994a).

Storage and release of ATP

ATP, which is continuously produced by mitochondria through oxidative phosphorylation of glucose, can be stored in all types of synaptic vesicles. Furthermore ATP is present in various amounts not only in the synaptic vesicles but also in the cytoplasm of every living cell and can be released by glial cells as well (Vizi et al., 1999). It has been shown that ATP is coreleased with other neurotransmitters including noradrenaline and acetylcholine, and other neuroactive compounds (Burnstock 1997). The cytosolic concentration of ATP has been reported to be greater than 5mM (Gordon, 1958). Many studies have demonstrated the release of ATP from peripheral and central nervous system. In 1962 Abood et al showed that ATP was released when a frog nerve-muscle preparation was stimulated electrically. By using a luciferin-luciferase assay the corelease of ATP with ACh has been demonstrated following activation of rat nerve innervating the diaphragm (Silinsky, 1975). There is some evidence that ATP is released from the sympathetic nerves innervating the vas deferens and certain blood vessels (Kirkpatrick & Burnstock, 1987, Vizi et al., 1992; Todorov et al., 1996). Some studies indicated that ATP may not be released from noradrenergic vesicles but released from separate populations of synaptic vesicles within noradrenergic or cholinergic nerves. The release of ATP as a co-transmitter with ACh from parasympathetic nerve terminals supplying bladder and striated muscle has been proposed. ATP stored with noradrenaline in the sympathetic branch of autonomic nervous system (Zimmerman, 1994a) can be released following nerve stimulation (Lew & White, 1987; Von Kügelgen & Strake, 1991b).

Following stimulation with KCl the release of ATP from brain synaptosomal preparations has been demonstrated (White 1978). K evoked release of ATP from the dorsal horn of spinal cord (Salter & Henry, 1985) and both K and veratridine evoked release from primary cultures of striatum has been shown (Zhang et al., 1988). ATP is

also released in a Ca^{++} dependent manner from the medial habenula (Sperlagh et al., 1995). In a recent study using patch clamp it has been shown that ATP and glutamate are released from separate populations of neurones in medial habenula, indicating they are not co-released (Robertson & Edwards, 1998). The release of ATP from hippocampus was Ca^{++} dependent and was not changed by glutamate receptor antagonists indicating the presynaptic nature of release (Wieraszko et al 1989).

In hippocampus Wieraszko et al (1989) showed the release of ATP from the Schaffer collateral and comissural pathway of CA1 area in hippocampal slices. This release was not evoked by glutamate implying that ATP may be stored with glutamate in synaptic vesicles so they may be released together, ATP is not only released by neuronal cells but also released from non-neuronal cells including glial cells (Wieraszko & Seyfried, 1989; Cunha, 1997).

P_2 Purinoceptors

Burnstock (1976) originally identified the P_2 purinoceptor, which mediates the effects of extracellular ATP. He classified purinoceptors into two types, P_1 and P_2 on the bases of some characteristic as follows (Burnstock, 1978). The P_1 purinoreceptor is more sensitive to adenosine and AMP than ADP and ATP (adenosine>AMP>ADP>ATP) whereas P_2 purinoreceptors are more sensitive to ATP and ADP than AMP and adenosine (ATP>ADP>AMP>adenosine).

P_1 purinoreceptors are competitively antagonised by methylxanthine derivatives such as theophylline and caffeine but P_2 purinoreceptors are not antagonised by methylxanthine compounds.

Activation of P_1 purinoreceptors by their agonists resulted in positive or negative changes in the level of intracellular cAMP through the activation or inhibition of the adenylate cyclase pathway but P_2 purinoreceptors can not modulate the intracellular cAMP or associated enzymatic pathway.

P_2 receptor activation by ATP can stimulate the synthesis and release of prostaglandins (Needleman et al., 1974) but adenosine can not. It has been shown that phosphorous-modified analogues were unable to stimulate the prostaglandin synthetase and it appears that these characteristics may be confined to ATP itself.

P_2 purinoreceptors were divided in two major divisions P_{2X} and P_{2Y} (Burnstock & Kennedy, 1985). Subsequently in 1986 P_{2T} receptors and P_{2Z} were distinguished which are selective for ADP on platelets and macrophages respectively (Gordon, 1986). A fifth class, P_{2U} purinoreceptors, has been introduced on the basis that this receptor recognises pyrimidine derivatives such as UTP (Uridine Tri Phosphate) as well as ATP (O'Conner et al., 1991). Current nomenclature and classification of P_2 purinoceptors has been shown in table 1 (Fredholm et al., 1994).

P_{2X} receptors are ligand -gated ion channel receptors and P_{2Y} receptors are G-protein coupled receptors (Dubyak 1991, Abbracchio & Burnstock, 1994). At present seven p_{2X} subtypes and eight P_{2Y} receptor subtypes have been identified (Fredholm et al 1997). Because of the sensitivity of a number of these subclasses to pyrimidines as well as purines it was suggested to use the term P_2 receptor rather than P_2 purinoreceptor (Fredholm et al., 1996).

P_{2X} Receptors

P_{2X} receptors are ligand-gated ion channels that open an intrinsic ion channel pore when challenged with extracellular ATP. The channel forming ATP receptors in the rat and human are encoded by seven identical genes named $P2X_1$-$P2X_7$. Activation of P_{2X} receptors by agonists open a non-selective channel for monovalent cations and shows an appreciable permeability to Ca^{++}(Rogers & Dani, 1995; Garcia-Guzman et al., 1997b). In situ hybridisation showed that the main P_{2X} receptor subtypes, expressed in adult rat brain, are $P2X_4$ and $P2X_6$ (Collo et al., 1996), although some P_{2X} mRNA levels (i.e-$P2X_2$) are decreased in adulthood compared with the neonatal period (Collo et al., 1995; Kidd et al., 1995). Moderate to high levels of $P2X_4$ mRNA are expressed in the pyramidal layers of the CA1 and CA3 regions and also mossy cells of the hilar region as well as dentate granule cells (Buell et al., 1996b; Soto et al., 1996; Garcia-Guzman et al., 1997b). The presence of the $P2X_2$ receptor subtype has been detected in the CA1-CA3 regions of the hippocampus, dentate gyrus and striatum by Northern blotting and in situ hybridisation (Kidd et al., 1995), although Collo et al (1996) found no signs of $P2X_2$ receptors in hippocampus or cerebellum.

The $P2X_3$ receptor is selectively localised to sensory pathways in trigeminal, nodose and dorsal ganglia (Chen et al., 1995; Lewis et al., 1995). The hyperalgesic actions of ADP, produced by the peripheral administration of ATP (Collier et al., 1966). could be attributed to the activation of $P2X_3$ receptors by this nucleotide.

In rat vas deferens, activation of $P2X_1$ receptors elicited rapid and transient contraction. In situ hybridisation showed the existence of $P2X_1$ subtypes in CA1-CA3 region of the hippocampus, cerebellum, striatum and dentate gyrus (Kidd et al., 1995). This mRNA was found in the rat brain associated with $P2X_1$ subtypes (Collo et al., 1996). The $P2X_7$ receptor is a functional receptor in mast cells, platelets, macrophages and lymphocytes. The presence of $P2X_7$ in the superior cervical ganglion and spinal cord has been demonstrated (Surprenant et al., 1996). The $P2X_5$ receptor was isolated from rat coeliac ganglia (Collo et al., 1996), but no labelling was found in the brain through in situ hybridisation. However mRNA for $P2X_6$ receptor was expressed heavily in the CNS with strong hybridisation in hippocampal formation.

P_{2Y} receptors

An adenine nucleotide selective $P2Y_1$ subtype has been defined in primary astrocytes (Neary et al., 1988; Ciccarelli et al., 1994). It has been revealed that $P2Y_1$ receptor subtypes are expressed in brain (Illes et.al, 1996) and sensory neurones (Nakamora and Strittmater, 1996). P_{2U} receptors renamed as $P2Y_2$ subtypes are also identified in immortalised astrocytes (Wu & Sun., 1997). Pharmacological profiles showed the existence of $P2Y_1$ as well as $P2Y_2$ subtypes in dorsal horn of the spinal cord (Ho et al, 1995). A $P2Y_1$ receptor was cloned from chick embryonic whole brain. Using Northern hybridisation analysis P_{2Y} receptors have been detected in brain, and mRNA for the $P2Y_1$ receptor subtype was detected in mammalian brain (Tokuyama et al., 1995). The high abundance of the avian $P2Y_1$ transcript had been detected in chick brain by in situ hybridisation (Webb et al, 1998). mRNA for the $P2Y_2$ receptor subtype was also sequenced in mouse (Lusting et al., 1993) and human brain (Parr et al., 1994). mRNA for $P2Y_3$/$P2Y_6$ receptor subtypes is also found in chick brain and spinal cord (Barnard et al., 1997). P_{2Y} purinoreceptors acts through G-protein coupled receptors and resulted in activation of phospholipase C and subsequent increase in degeneration of the

phospholipid phosphatidylinositol- 4,5-bisphosphate (PIP_2) and elevated the generation of IP_3 (Webb et al., 1993; Parr et al., 1994). IP_3 formation induces the release of Ca^{++} from intracellular storage sites causing a rise in the cytoplasmic free Ca^{++} ion concentration. Other transduction mechanisms can be modulated by the activation of P_{2Y} receptors. The diacylglycerol (DAG) produced from the breakdown of PIP_2 can activate the protein kinase C which in turn can phosphorylate some intracellular proteins by which a variety of effects on cellular function can be induced (Boarder et al., 1995). Modulation of adenylate cyclase (Boyer et al., 1993; Berti-mattera et al., 1996) and phospholipase A_2 has been proposed as well (Stella et al., 1997).

Table 1: Nomenclature and Classification of P2 Purinoceptors

Name	P_{2X}	P_{2Y}	P_{2U}	P_{2T}	P_{2Z}	P_{2D}
Effector type	Intrinsic ion channel Na^+, K^+, Ca^{++}	G-protein-coupled σIP3/Ca^{++}/ DAG;τcAMP, σphospholipase A2 (τ k^+conductance	G-protein coupled IP3/Ca^{++}/ DAG; Ca^{++}, Cl^- and K^+ currents	G-protein coupled IP_3/Ca^{2+}/ DAG; cAMP	Non-selective pore Na^+, K^+, Ca^{++}	G-protein coupled σCa^{2+}
Agonists	αßmeATP> γmeATP>ATP>ADP>2meATP>> UTP	2mesATP> ATP=ADP >>αßmeATP>>UTP	UTP>ATP =ATPγS>> 2meATP=α ß-meATP	2-substituted ADP>ADP	ATP^{4-}	AP_4A> ADPßS> AMPPNP> AP_5A>aßmeATP>2-meSATP
Antagonists	Desensitisation by αß-meATP ANAPP3 Suramin PPADS	Suramin	Not known	ATP Suramin FPL 66069		Not known
Radioligands	[^{3}]D-αß-meATP	[35S} ADDBS		B[^{32}P]2-meSADP, [^{35}SATPas		[^{3}H]AP_4A
Distribution	Smooth muscles, brain, heart, spleen	Wide distribution	Wide distribution. Found in cultured cells and in vascular muscle	Platelets	Mast cells, macrophags ,vasdeferens	Chromaffin cells, rat brain synaptosom

(Fredholm et al .,1994)

ATP functions

The role of ATP as a neurotransmitter or cotransmitter in various tissues including smooth muscles (Burnstock & Kennedy, 1985), peripheral neurones (Bean & Friel, 1990) and central nervous system (Jahar & Jessel, 1983; Edwards et al., 1992; Wieraszko et al., 1989; Wieraszko, 1996) has been revealed.

It has generally been recognised that ATP and several neurotransmitters such as noradrenaline, acetylcholine, glutamate and some neuroactive compounds such as substance P and nitric oxide are co-localised and can be released from several vesicles. ATP can modulate synaptic transmission via different ways.

1: ATP can directly induce fast synaptic transmission by activation of P_2 purinoreceptors

2: ATP can modulate the action of primary transmitters, when receptors are co-localised on the postsynaptic receptors.

3:After degradation to adenosine by nucleotidase and subsequent activation of adenosine receptors

The effects of ATP in the peripheral nervous system

In rat sensory neurones ATP increased a cationic current, which is carried by sodium, calcium and potassium ions. In bullfrog sympathetic ganglion cells ATP depolarised neurones and decreased the membrane conductances by suppression of the M currents (Akasu et al., 1983). Blockade of calcium sensitive potassium currents in guinea-pig myenteric neurones has been demonstrated (Harms et al., 1992). ATP induces excitatory effects produced by an inward current in a subset of neurones, dissociated from sensory neurons of rats, cats and bullfrog (Krishtal et al., 1983 ; Bean et al, 1990). Fast excitatory postsynaptic potentials evoked from the coeliac ganglion were mimicked by ATP and blocked by the P_2 purinoreceptor antagonist suramin (Evans et al., 1992). In parasympathetic nerve neurotransmission to the urinary bladder, ATP and acetylcholine act as cotransmitters. The initial and fast excitatory potentials are mediated by ATP via the activation of P_{2X} purinoreceptors and the slow potentials via muscarinic receptors acting through the G-protein coupled system (Hoyle & Burnstock, 1985).

ATP functions in the central nervous system

It is generally accepted that ATP might act as a fast excitatory neurotransmitter in the CNS. ATP activates depolarising inward currents in neurones of spinal cord (Jahr & Jessel, 1983), spinal ganglia (Krishtal et al., 1983) and cerebral cortex (Sun et al., 1992). The first evidence that ATP has a role in synaptic transmission in the CNS was obtained from a whole cell patch-clamp study in brain slices taken from the medial habenula (Edwards et al., 1992). Fast synaptic currents recorded in these cells were resistant to blockers of glutamate, GABA, and nicotinic receptors but prevented by P_2 receptor antagonists. The effect of ATP as a fast excitatory neurotransmitter is mediated by ligand-activated cationic P_{2X} type ATP receptors (Surprenant et al., 1995). ATP increases the firing rate of neurones in the locus coeruleus (LC) in the presence of DPCPX, an A_1 receptor antagonist, indicating an excitatory P_2 effect. More stable analogues of ATP such as 2-methylthio-ATP and α, ß-meATP facilitated the firing of LC neurones (Tschöpl et al., 1992; Fröhlich et al., 1996) and those effects were prevented by the P_2 receptor antagonists suramin, reactive blue 2 (RB_2) and pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) (Tschöpl et al., 1992; Fröhlich et al., 1996; Nieber et al., 1997). Application of tetrodotoxin that abolishes all synaptic inputs does not affect the responses to αß-meATP which probably acts directly on the locus coeruleus neurones. Inhibition of resting K^+ conductances and/or opening of Ca^{++} selective cationic channels could be the possible mechanism by which nucleotides facilitate the firing of LC cells (Illes & Nörenberg, 1993; Shen & North, 1993). Microdialysis experiments showed that ATP increased the release of dopamine from the rat striatum (Zhang et al., 1995) which in turn has an important role in regulating the behavioural response to environmental stimuli (Mogenson et al., 1993). ATP caused an increase in the concentration of intracellular calcium, which was sensitive to suramin (Chen et al., 1994). ATP may be released with glutamate from terminals of dorsal root ganglia neurones and can activate the P_{2X} receptors and stimulate further glutamate release (Gu & MacDermott, 1997).

ATP effects in the hippocampus

Application of exogenous ATP stimulates glutamate release from cultured hippocampal neurones, and the released glutamate can facilitate rapid depolarisation currents (Inoue et al., 1992). Although the expression of P_{2X} subunits in the hippocampus has been shown, the presence of ATP elicited inward currents has been observed only in cultured hippocampal neurones (Inoue et al., 1992; Balachandran & Bennett, 1996). ATP also induces the release of glutamate from presynaptic nreve terminals in hippocampus (Motin & Bennett, 1995). Using whole cell patch clamp recording showed that about 20% of the excitatory post-synaptic currents at CA1 neurones is induced by purinergic transmission activating several subtypes of P_{2X} receptors (Illes & Zimmermann, 1999). Electrical stimulation of hippocampal slices induces the release of ATP from Schaffer collateral and comissural afferents, which can produce long term potentiation and exogenous ATP is able to induce a long-lasting enhancement of population spikes recorded from rat, mouse and guinea pig hippocampal slices (Wieraszko & Seyfried, 1989; Nishimura et al., 1990; O'Kane & Stone, 2000).

In contrast to those excitatory effects of ATP in hippocampus the inhibitory effect of ATP have also been reported. By using electrophysiological methods and Ca^{++} imaging (Koizumi & Inoue, 1997) it has been demonstrated that ATP inhibits the release of glutamate from cultured rat hippocampus. The release of noradrenaline from rat hippocampus was inhibited when ATP was applied externally (Koch et al., 1997). Therefore it seems that ATP may play an excitatory or inhibitory role in hippocampus depending on the site of the effect and receptor subtypes. The mechanism of inhibitory effects of ATP could be attributed to the inhibition of presynaptic L type voltage gated Ca^{++} channels (VGCCS) and subsequent inhibition of transmitter release. Similar effects have been reported in PC12 cells (Nakazawa & Inoue, 1992). Increasing the release of inhibitory neurotransmitters GABA and glycine could be the other mechanism by which ATP induced an inhibitory effect. Some reports also suggest no presence of P_{2X} and P_{2Y} receptors for adenine nucleotides on rat hippocampal CA1 pyramidal cells at the

Schaffer collateral and comissural terminals in stratum radiatum and ruled out any pure functional effect of adenine nucleotides (Stone & Cusack, 1989).

Plasticity in the hippocampus

According to the duration of onset, development and duration, synaptic plasticity can be divided in two categories:

Short term plasticities with a duration range from milliseconds to several seconds and have been defined as follows

1: Facilitation that is known as paired-pulse facilitation

2: Augmentation is also a kind of facilitation but lasting for several seconds.

3: Post tetanic potentiation:

When repetitive stimuli were applied to the presynaptic fibres at high frequency for a short period a transient increase in postsynaptic responses was observed which lasted for several minutes after the train. This phenomenon was called post-tetanic potentiation (Magleby & Zengal, 1975)

4: Depression: Because of a decrease in sensitivity of postsynaptic receptors to neurotransmitters, depression can occur for a very short time scale (Stevens & Wang, 1995; Wang & Kelly, 1996).

Long-Term Plasticities

Long-term plasticity has now been observed in different parts of the central nervous system but they are extensively studied in hippocampus because of their possible contribution to learning and memory.

They can be classified as short term potentiation (STP), long term potentiation (LTP), short term depression (STD) and long term depression (LTD).

Long term potentiation is a lasting increase in synaptic efficacy that follows brief- high frequency tetanization of monosynaptic excitatory pathways. LTP was first found in the dentate gyrus of hippocampus (Bliss & Lømo, 1973) and later in other regions of the brain.

LTP has also been demonstrated and recorded from CA3 and CA1 areas following application of high frequency stimuli to mossy fibres and Schaffer collateral pathways respectively (Schwartzkroin & Wester, 1975; Alger & Teyler, 1976).

LTP properties in hippocampus

LTP is distinguished by three basic properties from the other postsynaptic facilitations. –

1- Duration: LTP can exist for many hours, days or weeks after induction (Douglas & Goddard., 1975). However it is not permanent.

2- Cooperativity: The afferent fibers can co-operate some how in induction of LTP, since activation of a few afferent fibres can not trigger LTP. The membrane must be adequately depolarised to remove the Mg^{++} from NMDA channels and induce subsequent LTP (McNaughton et al., 1978; Levy & Steward, 1979; Gustafsson & Wigström, 1988).

3:Input specificity: LTP is confined to those synapses that receive the high frequency stimulation, a phenomenon referred to as input specificity (Dunwiddie & Lynch, 1978; Andersen et al., 1980)

4: Associativity: Associativity refers to the fact that sufficient depolarisation to induce LTP can be provided by activation of different sets of pathways at the same time (McNaughton et al., 1978; Levy & Steward, 1979; Madison et al., 1991). For analytical purpose LTP can be differentiated into two phases: Induction and maintenance. The period of high frequency stimulation is called the induction phase, which takes several seconds. The maintenance phase is much longer than the induction phase and it can persist for even several days after induction.

Involvement of both NMDA and non-NMDA glutamate receptors in the induction phase of LTP has been reported. It is a general agreement that a sufficient increase in intracellular calcium is necessary for induction of LTP, because it was found that the induction of LTP in CA1 pyramidal neurons could be blocked by the intracellular injection of the Ca^{++} chelator EGTA (Lynch et al., 1983). At resting membrane potentials, the NMDA associated ion channels are blocked by a physiological concentration of Mg^{++} (Nowak., 1984 & Mayer et al., 1984). Depolarisation of the cell

membrane can remove Mg from the channels, and now activation of those channels by glutamate can induce LTP following the influx of Ca^{++}.

The role of NMDA receptors in induction of LTP was supported by application of NMDA receptor antagonists such as 2-amino-5- Phosphonopentanoate (AP5) and MK-801 which prevented the generation of LTP in the Schaffer collateral pathway (Collingridge et al., 1983; Teyler & Discenna, 1987), although NMDA by itself could not induce persistent LTP (Collingridge et al., 1983).

It has also been reported that an antagonist of the allosteric glycine site of the NMDA receptor (7-Chlorokynurenic acid) and cycloleucine block the induction of LTP in CA1 of the hippocampus (Bashir et al., 1990 ; Izumii et al., 1990). All together the above reports indicate that the NMDA receptor has an essential and important role in the induction of LTP.

Some reports favour the involvement of non-NMDA and metabotropic glutamate receptors in induction of LTP.

It has been reported that the AMPA receptor antagonist 6,7-dinitro-quinoxaline-2, 3-dione (DNQX) is able to prevent the induction of LTP following high frequency stimulation of Schaffer collateral fibres indicating involvement of AMPA receptors in induction of LTP (Muller et al; 1988)

It is likely that metabotropic glutamate receptor has an important role in modulation, induction or maintenance of LTP. Involvement of mGlu receptors in the induction of LTP is supported by using a metabotropic glutamate receptor antagonist MCPG (α-methyl-4-carboxyphenyl glycine) which can block the induction of LTP in pyramidal neurones (Bashir et al 1993; Bortolotto et., 1994).

Maintenance of LTP

The site and nature of the alterations that are responsible for the maintenance of LTP is still a matter of debate.

It has been suggested that coexistence of pre and post-synaptic mechanisms is necessary for LTP (Kullmann & Nicoll, 1992; Larkman et al., 1992). Pre-synaptic modifications such as an increase in the amount of L-glutamate released from nerve terminals, post-synaptic alterations including changes in the number of receptors or modification in

receptor sensitivity, extrasynaptic modulation, i.e alteration in neurotransmitter uptake by glial cells and morphological changes are the possible mechanisms which may be involved in the maintenance of LTP.

It was proposed that some membrane permeable molecules (retrograde messengers) might diffuse across the synaptic cleft and enhance transmitter release. Several candidates have been reported to play a role as a retrograde messenger including arachidonic acid, nitric oxide (NO) and platelet activating factor (Bliss & Collingridge, 1993; Medina & Izquierdo, 1995; Williams, 1996).

Long-Term Depression

Long term depression in hippocampus is a form of long-lasting decrease in synaptic transmission following some types of electrical stimulation. Expression of LTD can last from 30 minutes to several hours.

LTD can be categorised as two main types; heterosynaptic and homosynaptic. Heterosynaptic LTD was first reported by Levy and Steward in 1979, and can be produced by pre-synaptic activation of different inputs on the same post-synaptic cells and only the strength of passive synapses is depressed.

Monosynaptic LTD can be induced by pre-synaptic activation of the same input on the target neuron (Dudek & Bear, 1992). In the case of homosynaptic LTD, only the strength of the active synapses is depressed.

Homosynaptic LTD can be induced by low frequency stimulation while high frequency stimulation is required for induction of hetero-synaptic LTD.

Paired-Pulse Interactions

Synaptic transmission can be modulated in different ways. Two types of this modulation which have been described in the mammalian brain, including hippocampus, are paired-pulse facilitation and paired-pulse depression.

Paired-pulse facilitation

Facilitation was first described in the frog neuromuscular junction (Castillo & Katz, 1954) and can be defined as an increase in the response to the test stimulus (second stimulus) compared to the response to the conditioning stimulus (first one) when both stimuli are delivered with identical strength. Several mechanisms underlying paired-pulse facilitation have been proposed. The best known mechanism is the residual Ca^{++} hypothesis (Katz & Miledi, 1968). When the first action potential is terminated a small amount of Ca^{++} (residual) which is produced by the first stimulus can remain in the nerve terminals. This residual Ca^{++} is too small to trigger the release of neurotransmitters but can add to the Ca^{++} which enters the second stimulus and results in a higher concentration of Ca^{++} and subsequent increase in the probability of transmitter release (Zucker, 1989; Hess & Kuhnt, 1992; Wu & Saggau, 1994; Debanne et al., 1996).The Ca^{++} concentration in the pre-synaptic nerve terminal affects the probability of neurotransmitter release. It is worth to mention that the arrival of an action potential in presynaptic nerve terminals does not result in neurotransmitter release at every time but the probability of synaptic release can be modulated. The residual Ca^{++} can increase this probability (Katz & Miledi, 1968) by increasing the fusion of synaptic vesicles with the presynaptic membrane and raising the number of quanta released by an action potential. A linear relationship was found between the Ca^{++} level in the presynaptic nerve terminals and paired-pulse facilitation in CA3-CA1 synapses (Wu & Saggau, 1994). When presynaptic Ca^{++} influx was manipulated by changing the $[Mg^{++}]$ / $[Ca^{++}]$ ratio (Creager et al., 1980; Manabe et al., 1993) or applying adenosine (Lupica et al., 1992; Higgins & Stone 1995) or its selective agonist cyclopentyladenosine (Nikbakht & Stone, 1999), paired-pulse facilitation (PPF) was changed indicating the

presynaptic origin of PPF. Both increasing the $[Mg^{++}]/[Ca^{++}]$ ratio and using adenosine decrease the probability of release of neurotransmitter during the first action potential and increase the probability of release by the second action potential resulting in higher responses. In contrast, lowering the concentration ratio of $[Mg^{++}]/ [Ca^{++}]$ decreases the PPF in hippocampus (Creager et al., 1980; Manabe et al., 1993). A similar effect has been reported in the neuromuscular junction (Mallart & Martin, 1968). A concomitant reduction in GABAergic inhibition has been suggested as one of the possible mechanisms causing PPF in hippocampus (Nathan et al, 1990; Nathan & Lambert, 1991; Brucato et al., 1992; Kahl & Cotman, 1993). Increasing GABA release by activation of interneurons could have a negative feedback on its own release by stimulating the $GABA_B$ autoreceptors located on the presynaptic nerve terminal. It has been shown in CA1 of hippocampus that a reduction in depression by activating $GABA_B$ autoreceptors has an important role in PPF (Nathan et al., 1990). In perforant path synapses baclofen (a $GABA_B$ agonist) increased PPF (Rich-Bennett et al., 1993). In a recent study using extracellular recording, the persistence of facilitation has been revealed under conditions in which $GABA_A$ inhibition was markedly reduced by application of the $GABA_A$ antagonist bicuculline (Higgins & Stone, 1993). In contrast some reports suggested that changes in PPF can not be attributed to changes in inhibition by GABA agonists (Schulz et al., 1994), supporting the residual calcium hypothesis rather than concomitant inhibitory interneurons underlying the mechanism of PPF.

Paired-pulse depression

When two stimulii with identical strength are delivered in rapid succession, the response to the second stimulus is weaker and the amplitude of the EPSP and/ or population spike is shorter, a phenomenon called paired-pulse depression (PPD). PPD was first reported for monosynaptic inhibitory postsynaptic currents (Davies et al., 1990).

Several mechanisms may cause PPD including desensitisation of postsynaptic receptors, depletion of presynaptic vesicles or other presynaptic mechanisms contributing to the depression of vesicle release.

Numerous studies showed that synaptic depression is sensitive to release probability. When release probability is elevated (i.e by decreasing the [Mg/Ca] ratio) paired-pulse depression is obtained (Debanne et al., 1996). However, when release probability is decreased, synaptic depression is reduced or may be converted to PPF (Zucker, 1989; Dobrunz & Stevens, 1997). The mechanism responsible for decreasing release probability could be depletion of vesicles (Thies, 1965; Kusano & Landau, 1975; Stevens & Wang., 1994; Debanne et al., 1996; Dittman & Regehr, 1997) especially those primed and ready to release (Bittner &Holz, 1992). In the hippocampal slice preparation, paired-pulse inhibition has been demonstrated at interstimulus intervals less than 40ms and in the present experiments at less than 20ms while paired-pulse facilitation is observed at longer time intervals (Creager et al., 1980; Dunwiddie et al., 1980; Lynch et al., 1983, Higgins & Stone 1995, Nikbakht & Stone, 1999). In a very recent study in end bulb synapses it has been shown that the level of presynaptic Ca^{++} entry produced by the first stimulus and Ca^{++} binding to a molecular site, intimately is involved in modulation of synaptic release but not vesicle depletion or postsynaptic desensitisation (Bellingham & Walmsley, 1999).

On the other hand activation of inhibitory interneurones releasing GABA is also suggested to be one of the mechanisms by which the PPD may be mediated. The similarity between the time course of paired-pulse inhibition and $GABA_A$ mediated fast inhibitory postsynaptic potentials implies a role for GABA inducing paired-pulse inhibition (Davies et al., 1990; Higgins & Stone, 1995)

Hippocampus anatomy

The hippocampal formation including the dentate gyrus, the hippocampus, the subiculum, presubiculum, parasubiculum and the entorhinal cortex is located within the limbic system beneath the neocortex. The hippocampus itself has a bilateral curved structure that appears as a ridge extending into the lateral ventricle. The unusual shape of the human hippocampus resembles that of a sea horse (in Greek hippo means "horse" and Kampus means "seamonster").

Ramon Cajal (1911) divided the hippocampus in two major regions called regio superior with larger cells and closer to the dentate gyrus and regio inferior with smaller cells and further from dentate gyrus. Later in 1934 the anatomist Lorente de No' divided the pyramidal cell layer of hippocampus into 4 CA (Cornu ammonis) sectors (CA1 to CA4). CA4 is no longer used because it referred to the region occupied by the polymorphic layer of the dentate gyrus (Witter, 1989). In the Lorente de No classification, CA1 is equivalent to regio superior and CA2 and CA3 fields are equivalent to the regio inferior. CA1 and CA3 have different patterns of innervation, and are separated by a narrow transitional zone, CA2. The functional significance of CA2 is not clear.

The dentate gyrus contains round, tightly packed neurons called granule cells. It is not continuous with the CA layer, but bends around it. The dentate gyrus consist of three layers: the granule layer, which is the principal layer, the molecular layer, (located above the granule cell layer) and a polymorphic cell layer below the granule cell layer (Cajal 1968).

In all CA fields the most superficial layer is the alveus which contains a thin sheet of outgoing and incoming fibres. Below this is the stratum oriens, which contains the basal dendrites of the pyramidal cells. The pyramidal cell layer is the next layer. The cell bodies are clearly visible under a microscope as a dark band. Below this is the stratum radiatum consisting mainly of the apical dendrites of the pyramidal cells and the Schaffer collaterals, which are collateral branches from axons of pyramidal cells in the CA3 region.

The inner layer is the stratum lacunosum moleculare in which bundles of fibres are found of which some are from the CA3 region and also a number of cells of random organisation.

The functional organisation of the hippocampus has traditionally been described in term of the trisynaptic circuit (Andersen et al., 1971).

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 (Fig. 2. 1.).

The fibres 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 trisynaptic pathway with the dendrites of granule cells in the dentate gyrus.

The axons of these granule cells are called mossy fibres because of the peculiar appearance of their synaptic terminals. They leave the dentate gyrus and terminate on the proximal dendrites of the CA3 pyramidal cells.

CA3 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 connection with dendrites in area CA1. Axons from CA1 pyramidal nerouns project heavily to neurons in the subicular complex. There is also a major projection from subicular complex to enthorhinal cortex to close the circuit.

Aim

This work to be described was performed to investigate effects of adenosine and ATP receptors in the hippocampus, and their interactions with transmitter receptor notably those for acetylcholine and NMDA. Emphasis has been placed on the examination of these effects and interactions on presynaptic terminals, using paired-pulse experiments.

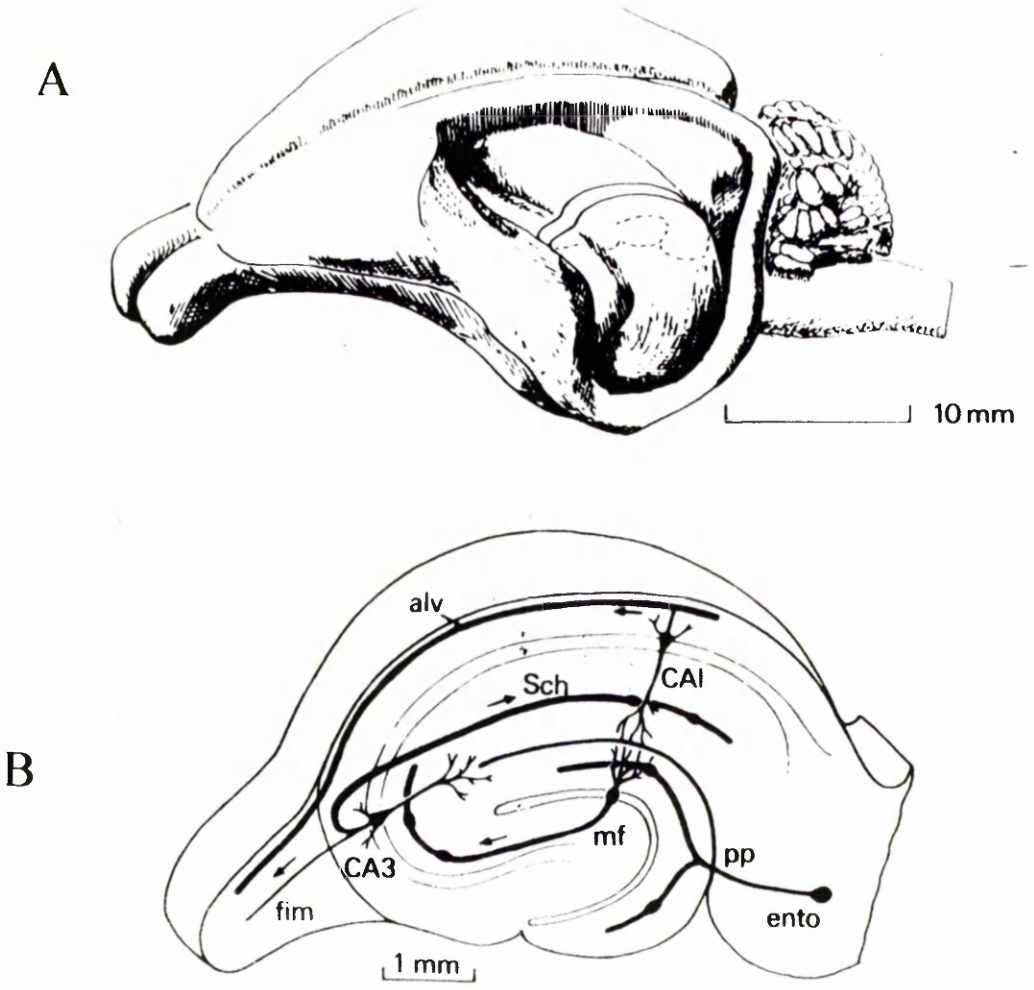

Figure 2. 1. A: Lateral view of the rat brain with parietal and temporal neocortex to expose the hippocampal formation.

B: A diagram of a hippocampal slice in which alv: alveus, fim: fimbria, ento: entorhinal cortex, pp: perforant path, sch: Schaffer collateral and mf: mossy fibers. (Adapted from Anderson et al., 1971).

2. MATERIALS AND METHODS

Preparation of slices

Male Wistar rats weighing 150-200 g were anaesthetised with intraperitoneally injected urethane (25% solution, 1.3 g/kg i.p) and decapitated using a guillotine. The brain was rapidly taken out and put in ice-cold and oxygenated artificial cerebro-spinal fluid (ACSF), which had been saturated with 95 percent of oxygen and 5% of carbon dioxide. The cerebellum was removed and the two hemispherewere seperated using a scalpel blade. Both hippocampi were dissected out from the hemispheres using microspatulas and put on the filter paper. Transverse slices (450mM thick) were prepared using a McIlwain tissue chopper. Individual slices were then seperated using a pair of fine glass seekers. The slices were maintained at room temperature on a filter paper in a petri dish containing a small amount of fresh, well-gassed ACSF. The petri dish was put in an interface holding chamber in a water vapour atmosphere saturated with 5% CO_2 in oxygen.

Media

The composition of the bathing medium (ACSF) was as follows (mM) NaCl 115; $NaHCO_3$ 25; KCl 2; KH_2PO_4 2.2; $CaCl_2$ 2.5; $MgSO_4$ 1.2; glucose 10; saturated with a mixture of 95% oxygen-5% carbon dioxide. In some experiments, magnesium-free ACSF was prepared by omitting $MgSO_4$ from normal ACSF.
In some experiments the concentration of potassium was raised to 4mM.

The recording chamber and bath superfusion

After a period of at least one hour of incubation, individual slices were transferred to a 5 ml capacity 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 kept submerged with a thin metal bar and heated using a thermostatically controlled water bath to yield a temperature between 28° and 30°C. A digital thermometer was used to measure the recording chamber temperature. The pH of the superfusate solution was maintained at 7.4. Drugs were normally applied to the perfusion medium for a minimum of 10min.

Stimulating and recording

A concentric bipolar stimulating electrode (Clark Electromedical, Reading, U.K) was placed in the stratum radiatum near the commissural border of CA1/CA2 for orthodromic activation of pyramidal cells via the Schaffer collateral and commissural pathways. Stimulation was by square wave constant-current pulses of 100-300ms duration and up to 1mA amplitude, which were delivered through the same electrode. The stimulus strength was set to gave a submaximal population potential size of about 70% of maximum.

For paired-pulse recording, the intensity of the conditioning pulse (first pulse) and the testing pulse (second pulse) were always the same. In paired pulse experiments the interstimulus intervals at 10, 20 and 50 ms were examined.

Extracellular population spike potentials and population excitatory postsynaptic potentials (EPSPs) were recorded from the CA1 pyramidal cell layer or the stratum radiatum respectively. Recording electrodes were made from fibre glass capillaries with an outside diameter of 2mm (Clark Electromedical Instruments. U. K.), pulled on a Kopf vertical electrode puller. The tip was bumped backto 2-4 μm under low-magnification using a glass probe. The electrodes were filled with sodium chloride (2M) using a fine needle. A microscope was used for electrode placement in the slices.

Data analysis

Slices were used when the maximum population spike size was 3mV. Measuring any change in the size of the population spike or population EPSP was used to assess the effect of each drug. The size of the population spike, in mV, was measured as a difference between the peak of the positive-going synaptic potential and the peak of the negative going population spike. When the EPSP was measured, the size was measured as the slope of negative going potential. Results were considered as the percentage of change of the spike size by drug compared with the control size. The control size was calculated as the mean of 10 responses taken during 10 minutes before drug perfusion. The drug effect were taken as the mean of 3 final observations of drug perfusion period. When paired-pulse interactions were examined, inhibition or facilitation was expressed as the percentage change in the response to the second stimulus (R_2) compared with the first one. For the analysis of drug effect, the mean was taken of paired-pulse changes obtained in two control tests (before drug application) and two tests made at the end of ten minute period of drug perfusion.

Statistical test

Results are given as mean ± standard error of mean (s.e.m) for n slices. Statistical significance was determined using paired or unpaired Student's t test as appropriate. For comparison between two sets of data from the same slices, a paired t-test was used but when different slices were examined a non-paired t-test was used. Analysis of variance (ANOVA) followed by a Student-Newman-Keul's test was used for comparison when more than two different sets of data were used. The significant level was set at $P<0.05$ and denoted with a $^{*, \#}$ or $^{\&}$ in the figures.

Chemical agents and drugs

Analar grade compounds (ACSFconstituents) were dissolved in distilled water to prepare fresh ACSF of the composition given above. Most of the drugs were dissolved in distilled water to obtain a stock solution. Glycine, adenosine deaminase were dissolved directly in ACSF to give the desired concentration. AP5 and 8PTwere firstly dissolved in NaOH then distilled water was added to give a stock solution. DMSO (Dimethyl sulphoxide) was used as the vehicle for 2,4-dichloro kynurenic acid, CPA and ZM.241385. The final concentration of vehicle was never more than 0.1%. DMSO by itself at a concentration up to 0.1% had no effect on the slices.

Analar grade compounds were obtained from BDH Chemicals LTD in UK.

The majority of drugs was purchased from Sigma Chemical Company, UK

2AP5, 8-phenyltheophylline, cyclopentyladenosine (CPA) were from Research Biochemicals Incorporated (RBI). Suramin was from Bayer.

3. RESULTS

The effect of purines on population spikes

Comparison between the effect of adenosine and ATP on orthodromic population spikes

In normal ACSF both adenosine and ATP in a concentration dependent manner inhibited or abolished the amplitude of orthodromic population spikes evoked from CA1 fields in hippocampal slices. Concentration -response curves for these effects are demonstrated in figure 3.1 and 3. 2. The depressant effects of these two agents was reversible and washed out in 5 to 10 minutes. ATP was less potent than adenosine; the EC_{50} for adenosine was 9.5 mM, where as the EC_{50} for ATP was 19mM.

Comparison between the effect of ATP and its more stable analogues on orthodromic population spikes

The effect of ATP, and its more stable analogues βγ-methylene-ATP which is an ATP analogue substituted in the γ position and αβ-methyleneATP, an ATP analogue substituted in the α position were examined and compared on orthodromic evoked CA1 population spikes as illustrated in figure 3. 3. ATP decreased the size of population spikes by 28.1% ± 8.58 ($P<0.05$, n=4) at 10mM and by 70. 67% ± 11.3 at 50mM. Application of βγ-methyleneATP had no effect at 2mM (depression at 5.7% ± 3.0, n=4) but depressed the potentials significantly by 42.4% ± 7.2 at 10mM ($P<0.01$; n=4). αβ-methyleneATP was used at 10mM but did not induce any significant effect on the size of the population spikes. The inhibitory effect of all nucleotides mentioned above was reversed by reinfusion of normal ACSF in 10 to 15 minutes.

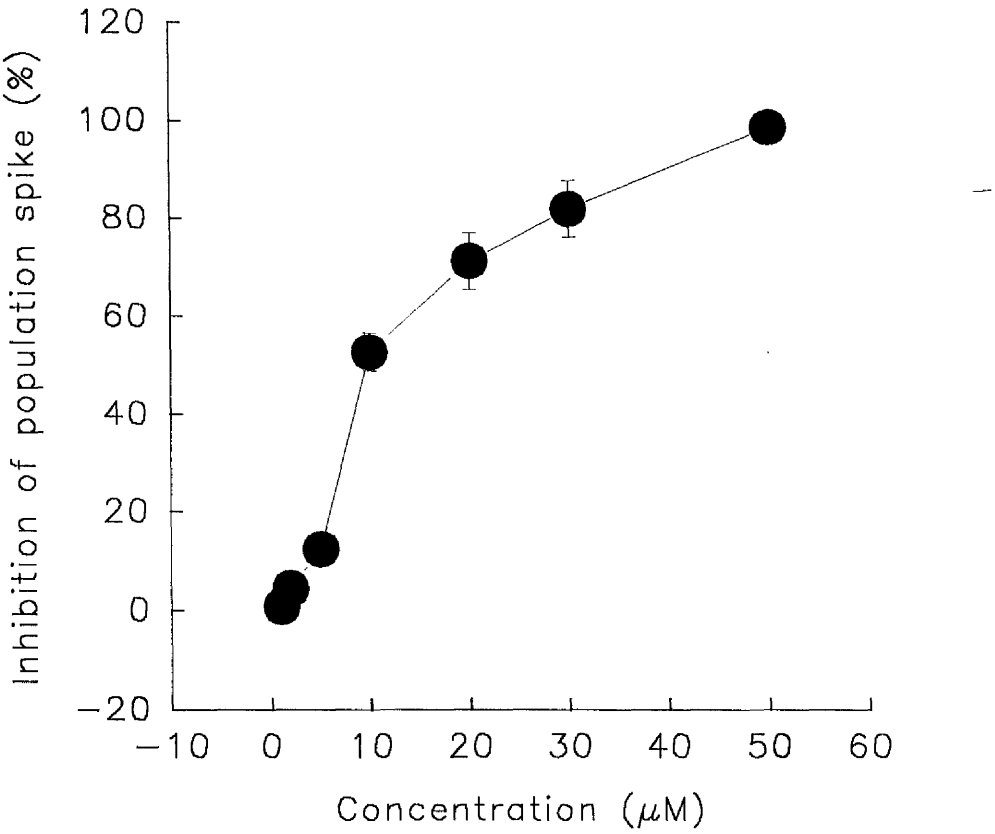

Figure 3. 1. Concentration–resonse curve for the depression effect of adenosine on the size of population spikes evoked from the CA1 region of hippccampal slices. All stimuli were orthodromic and stimulus strength was adjusted to 70% of maximum. Each vertical bar represents the mean ± S. E. M for n=5 slices.

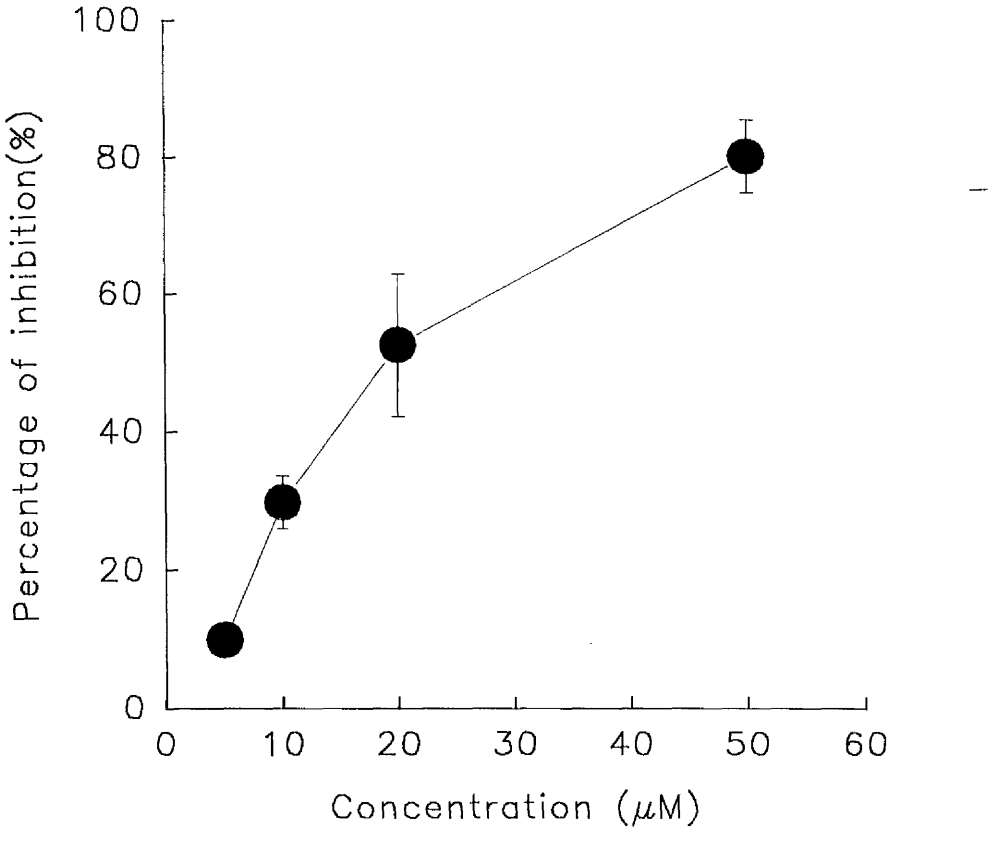

Figure 3. 2. Concentration response curve for the inhibitory effect of ATP on orthodromic population spikes evoked from the CA1 area in hippocampal slices. The stimulus strength was 70% of maximum. Each point represents the mean ± S. E. M for n=6 slices.

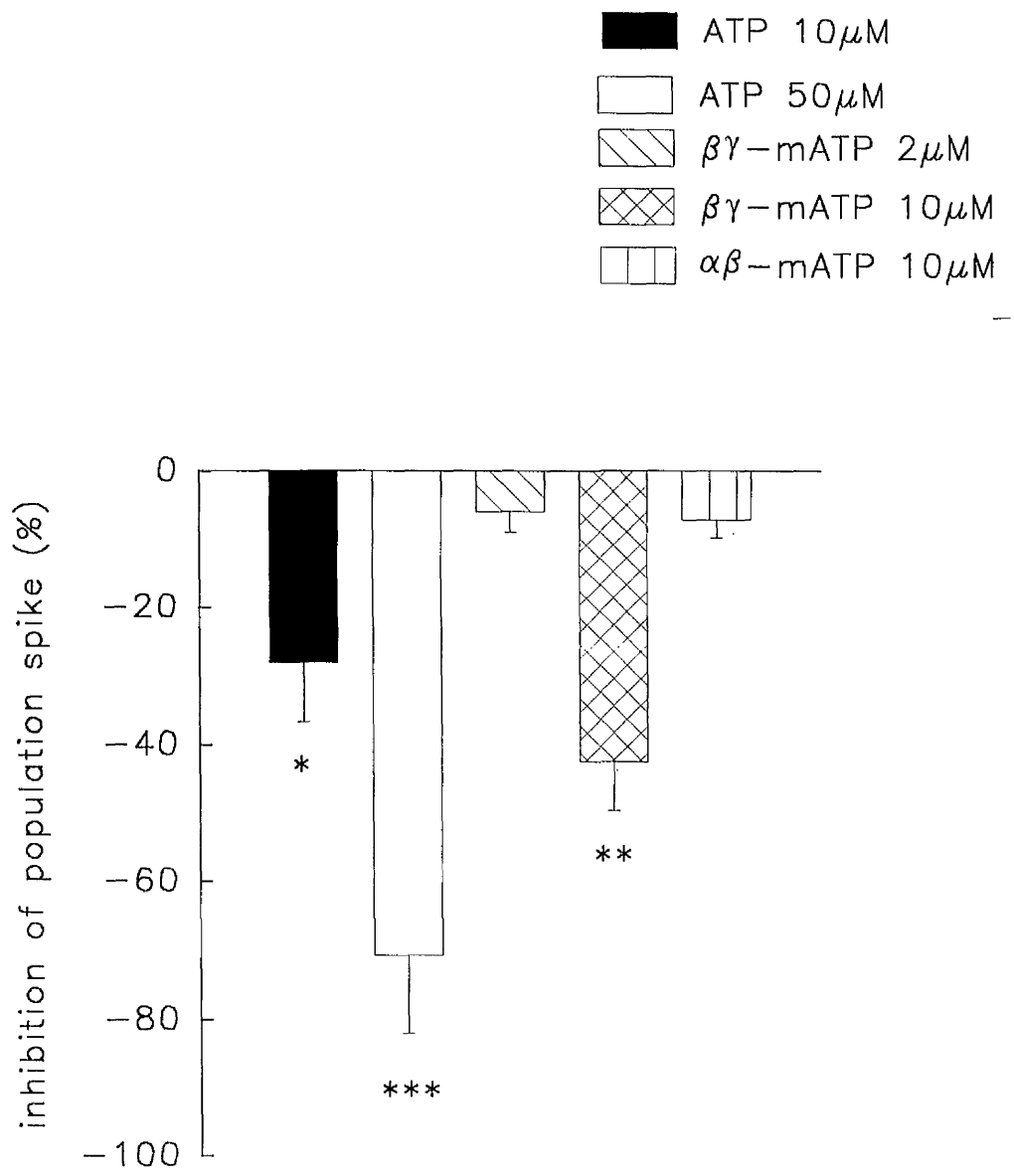

Figure 3. 3. The inhibitory effect of ATP, $\beta\gamma$−mATP and $\alpha\beta$−mATP on orthodromic population spikes. Each vertical bar shows mean ± S. E. M for n=4 experiments.
* P<0.05, ** P<0.01, *** P<0.001 (relative to control size).

The effect of combination of purines with 8PT on CA1 evoked population spikes

In order to determine whether the inhibitory effects of nucleotides are mainly mediated by activation of P_1 receptors by adenosine generated through the extracellular catabolism of nucleotides those experiments were repeated in the presence of 8-phenyltheophylline, (8PT) a P_1 purine receptor antagonist. 8PT was superfused 10 minutes prior and during the superfusion of purines. 8PT up to 50μM by itself had no significant effect on the size of the population spikes (Fig 3. 4). In order to control the efficacy of 8PT, the effect of 8PT first was examined on the inhibitory responses induced by adenosine 30μM. 8PT 50μM completely blocked the depressant effect of adenosine 30μM (Fig. 3. 4). When 8PT 50μM was superfused with ATP 50μM, the inhibitory effect of ATP was abolished (Fig 3. 5). The depressant effect of βγ-methyleneATP 10μM was also markedly reduced, although a small but still significant inhibitory effect remained even in the presence of 8PT (Fig. 3. 6).

The effect of combination of nucleotides with adenosine deaminase on CA1 evoked population spikes

The next step was to determine whether adenine nucleotides must be converted to adenosine before they can activate P_1 receptors. To examine this hypothesis both ATP and βγ-methyleneATP were reapplied in the presence of adenosine deaminase 0.2 unit/ml. Adenosine deaminase by itself does not have any significant effect on the amplitude of population spikes. It was first superfused 10 minutes prior and during the application of adenosine, and under these conditions the inhibitory effect of adenosine was completely prevented (Fig. 3. 7). Adenosine deaminase was able to abolish the inhibitory effect of βγ-methyleneATP on the amplitude of population spikes as is shown in Fig 3. 8. ATP at 50μM tended to decrease the amplitude of population spikes even in the presence of adenosine deaminase but this did not reach a significant level (Fig. 3. 9).

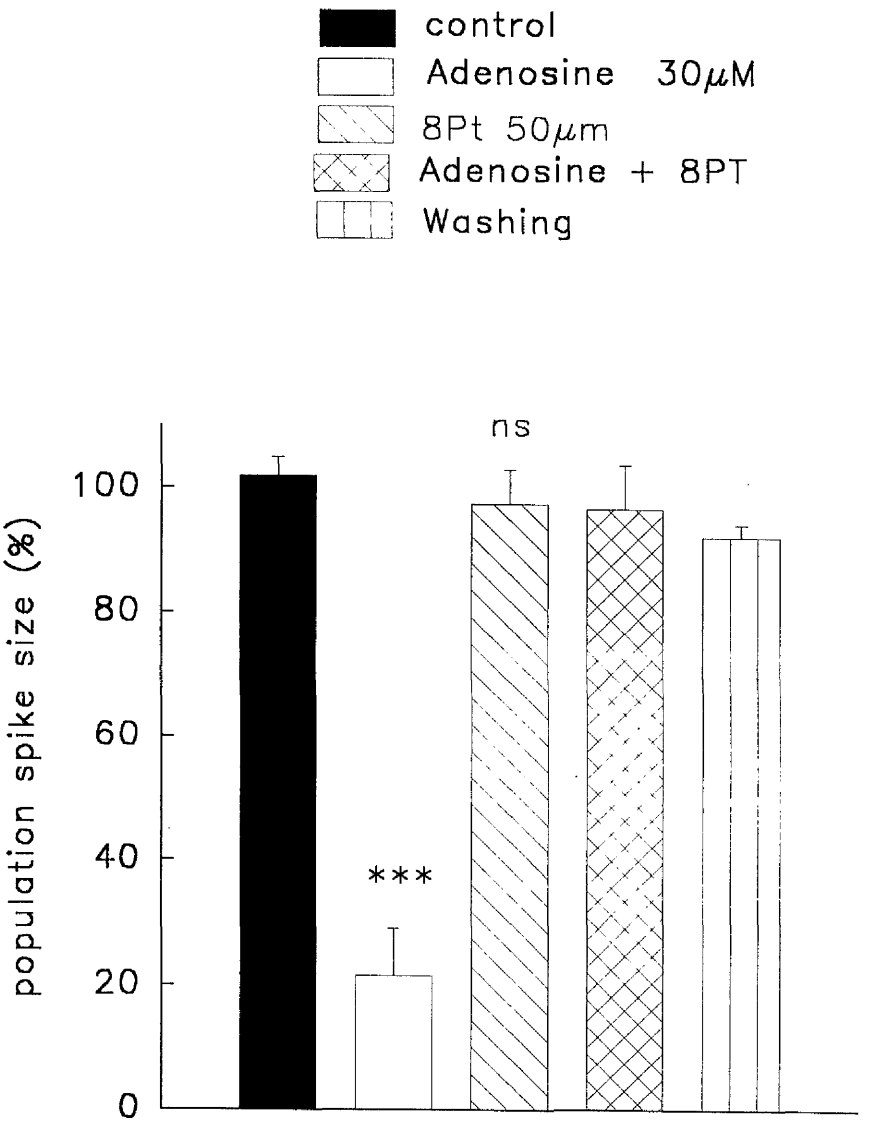

Figure 3. 4. The effect of adenosine 30μM alone and in combination with 8PT 50μM on population spike size. Each vertical bar shows mean ± S. E. M for n=4 experiments. *** P<0.001

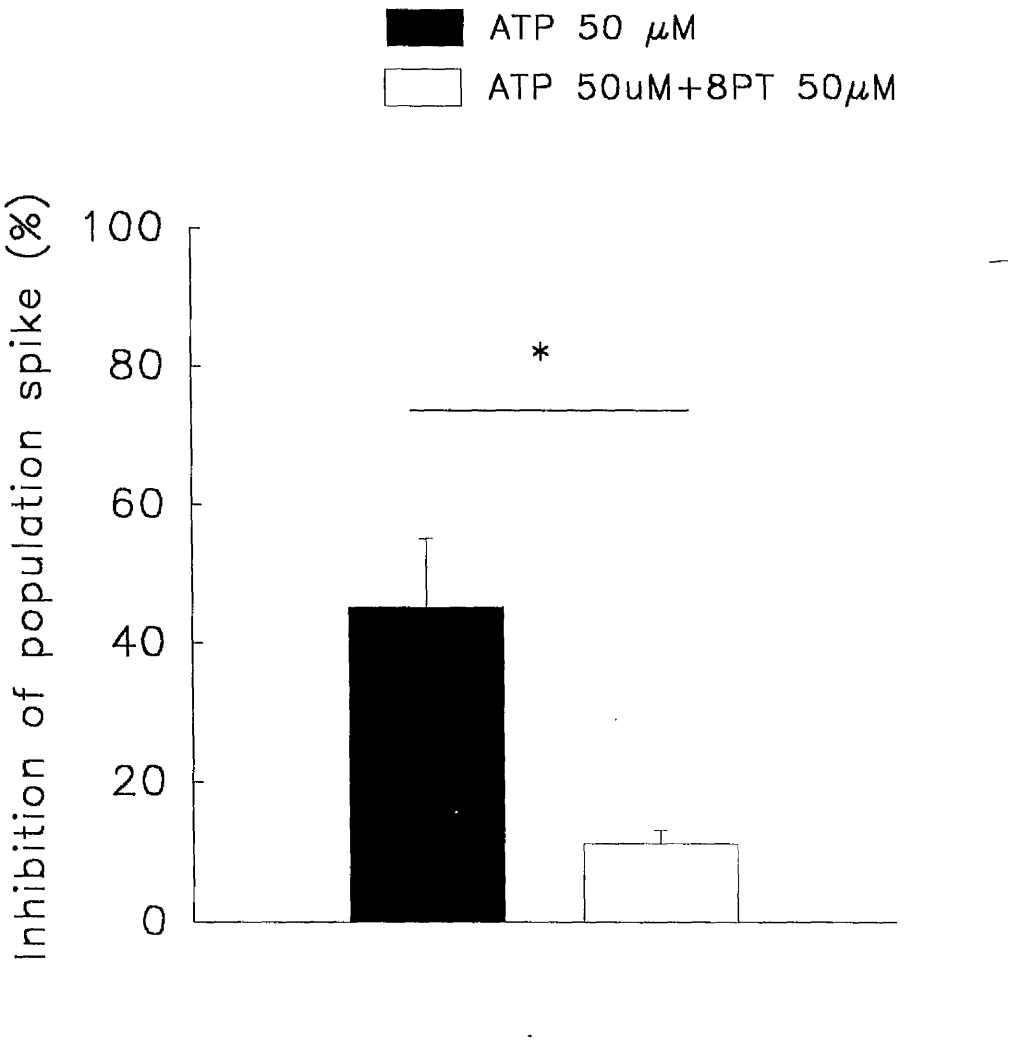

Figure 3. 5. Histogram showing the effect of ATP alone and in combination with 8PT on orthodromic population spikes evoked from the CA1 region in the hippocampus. Each vertical bar shows the mean ± S. E. M for n=5 slices. * P<0.05

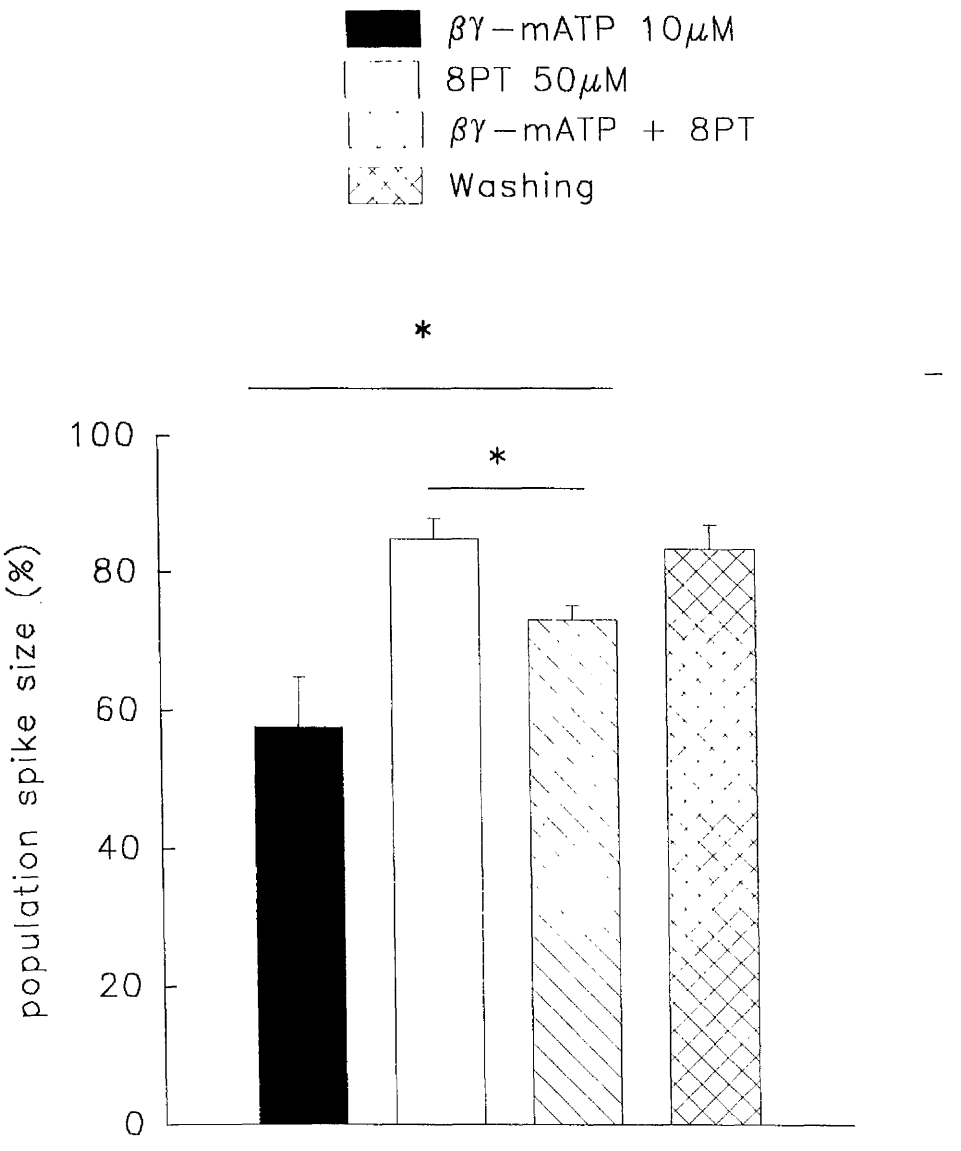

Figure 3, 6. Histogram showing the effect of βγ−mATP 10 μM alone and in the presence of 8PT 50μM on orthodromic evoked CA1 population spikes. Each vertical bar shows mean ± S. E. M for n=4 experiments. * P<0.05

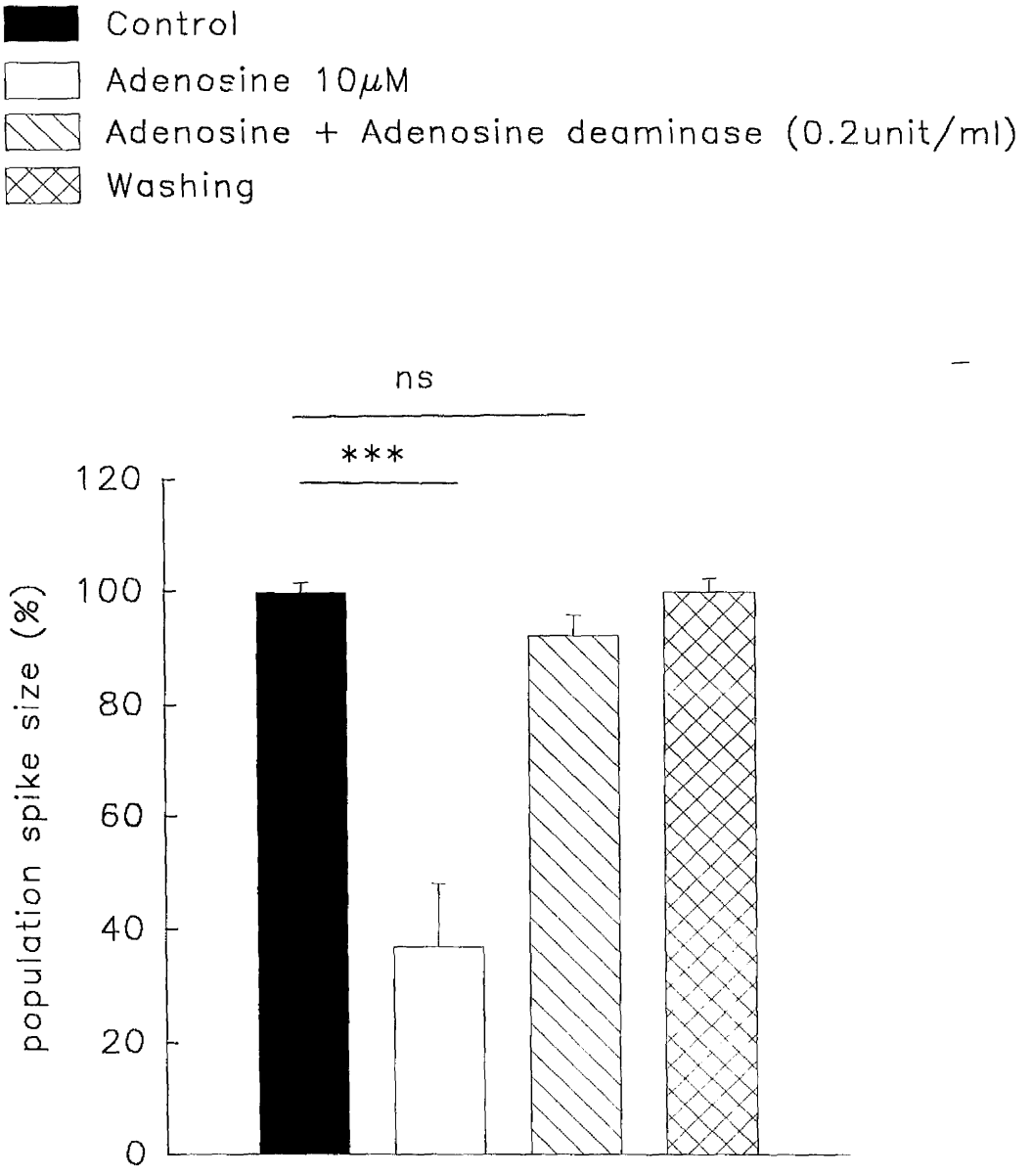

Figure 3. 7. Histogram showing the effect of adenosine 10μM alone and in combination with adenosine deaminase 0.2 unit/ml on population spikes. Each vertical bar shows the mean ± S. E. M for n=3 slices.

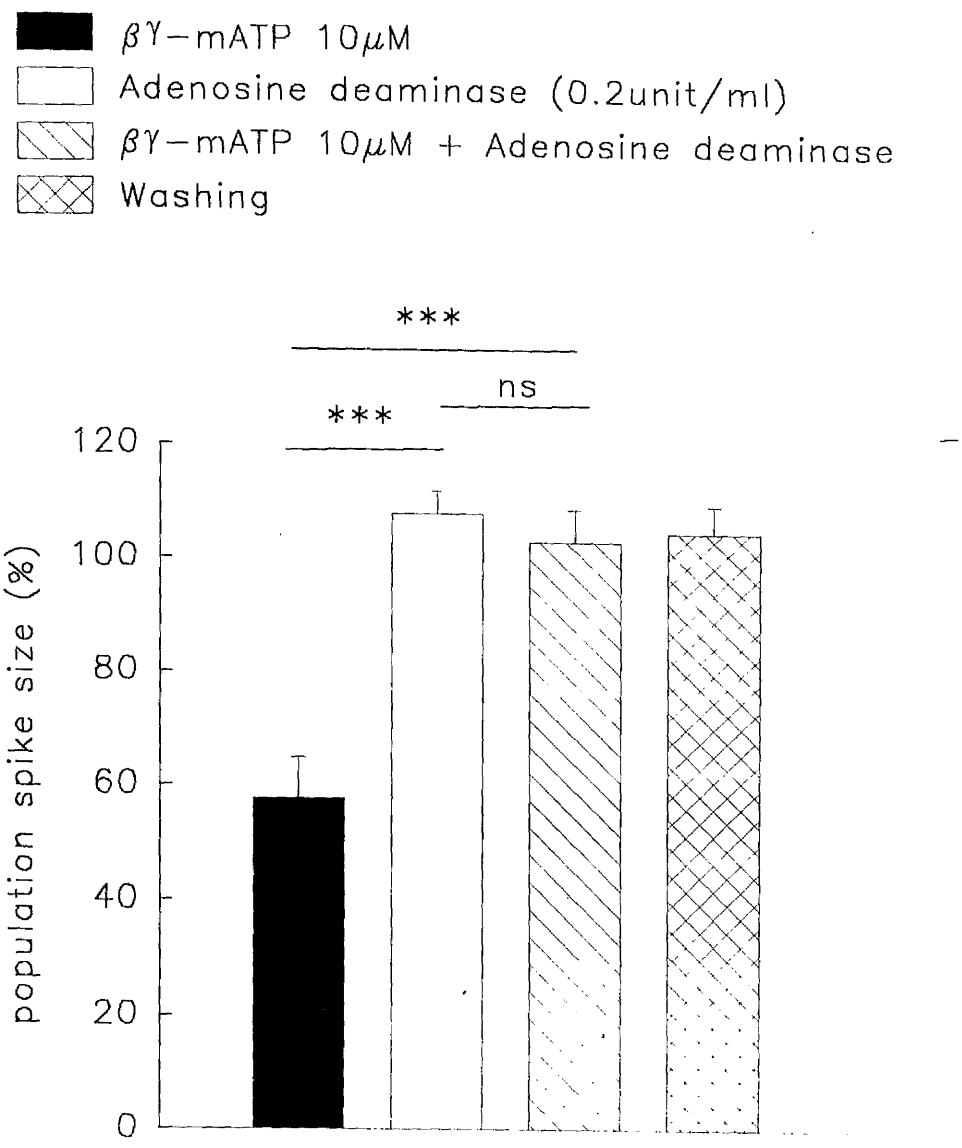

Figure 3. 8. Histogram showing the effect of βγ−mATP 10 μM in the presence of adenosine deaminase 0.2 unit/ml on the population spikes. Each vertical bar shows the mean ± S. E. M for n=5 experiments. ** P<0.01, *** P<0..001

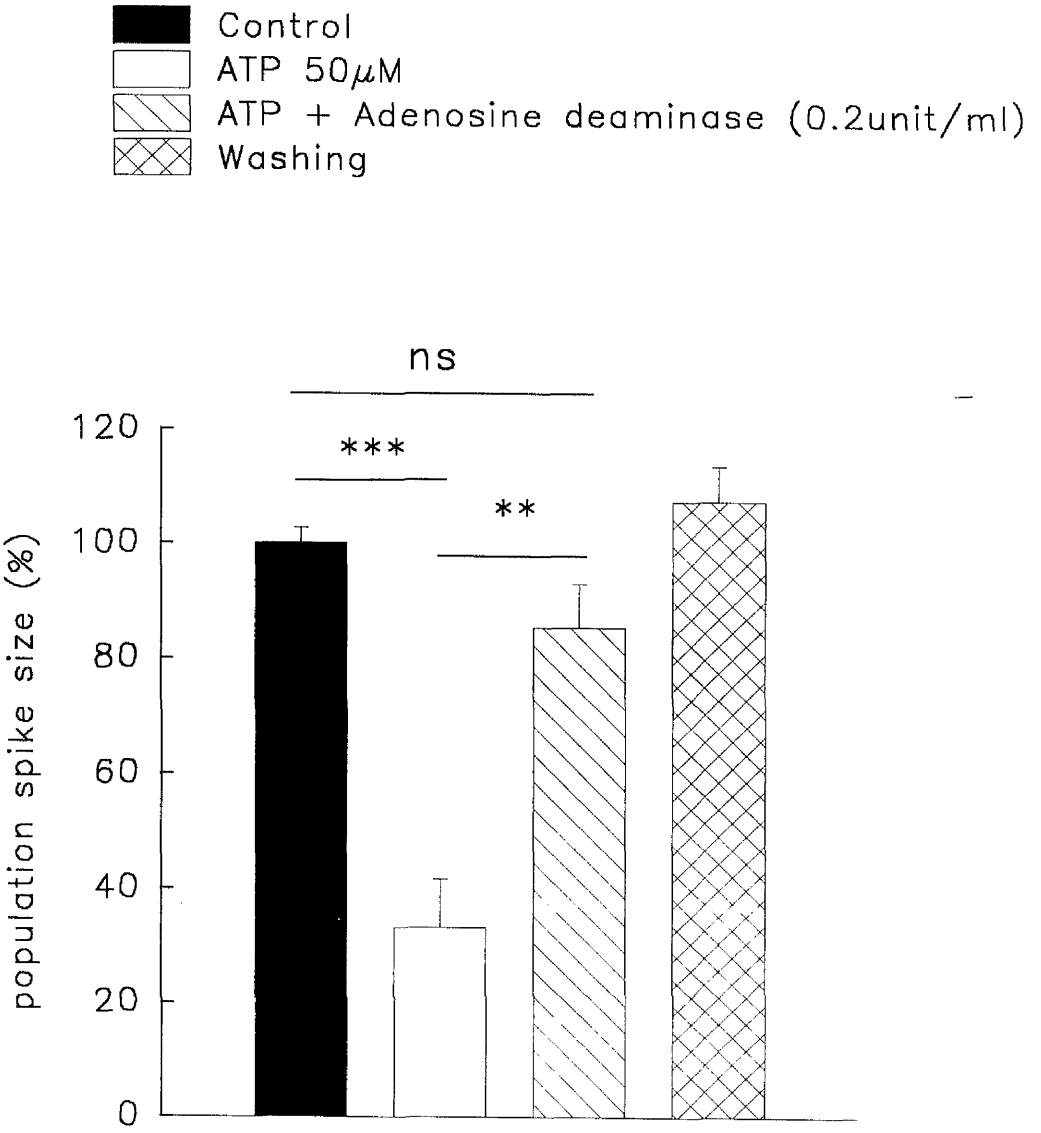

Figure 3. 9. Histogram showing the effect of ATP 50μM alone and in combination with adenosine deaminase 0.2 unit/ml on population spikes. Each vertical bar shows the mean ± S. E. M for n=3 slices.

The effect of combination of suramin and ATP on CA1 evoked population spikes

The possible involvement of P_2 receptor activation in inhibitory responses can not be simply ruled out. In a series of experiments suramin, a P_2 receptor antagonist, was added to the perfusion medium. A concentration of 50μM was selected for use in experiments with ATP. Suramin was left in contact with slices 10 minutes before and during addition of the nucleotide. Suramin significantly reduced the inhibitory effect of ATP 50μM (Fig. 3. 10).

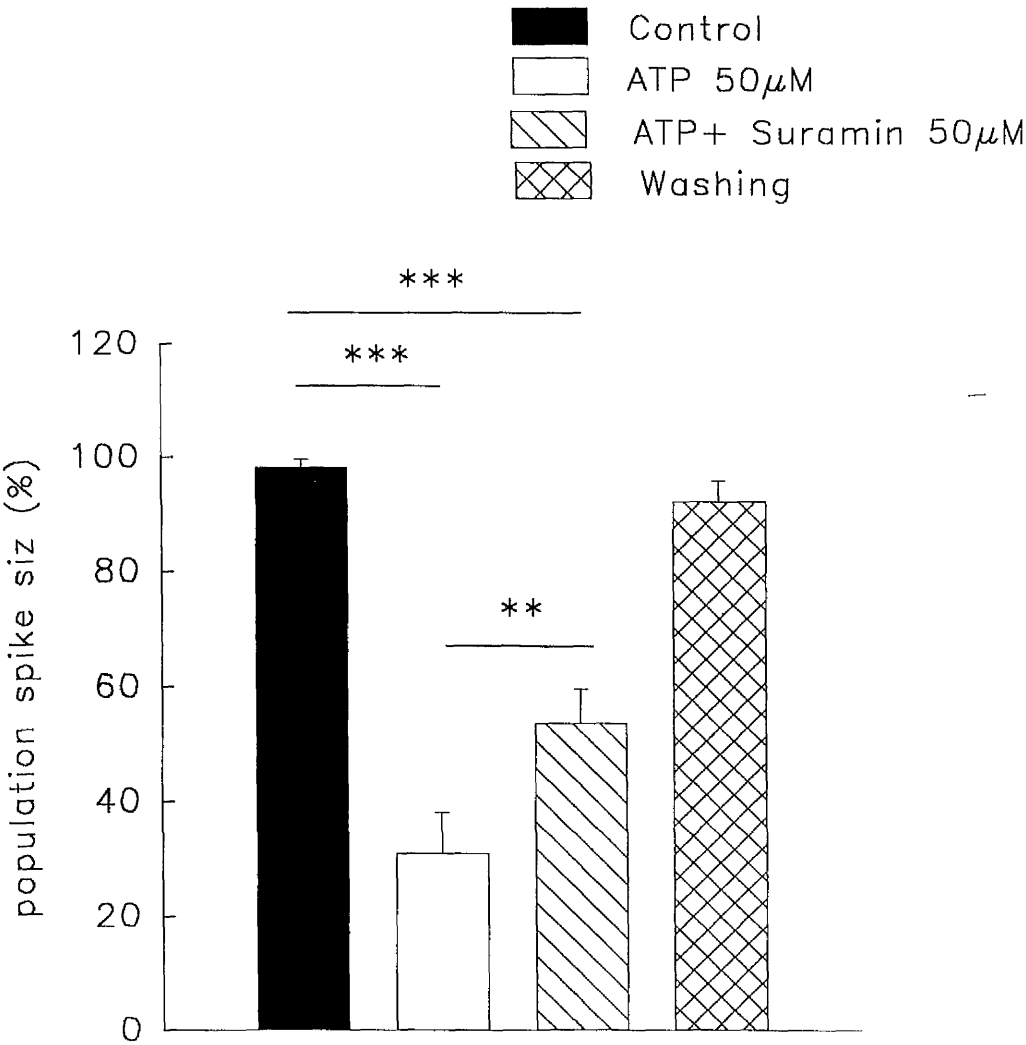

Figure 3. 10. Histogram showing the effect of ATP 50μM alone and in combination with suramin 50μM on CA1 population spikes evoked in hippocampal slices. Each vertical bar shows mean ± S. E. M for n=4 slices. ** P<0.01, *** P<0.001

Effects of purines on paired- pulse interaction

The profile of paired-pulse responses obtained with stimuli delivered at interpulse intervals of 10, 20 and 50ms was studied first in the absence of added agents in 5 slices (Fig 3. 11). For paired- pulse responses, the intensity of the conditioning pulse (first pulse) and testing pulse (second pulse) were adjusted to 70% of maximum. All examined slices showed paired-pulse inhibition at the shortest interval (10ms). As interstimulus interval was increased inhibition decreased and paired pulse facilitation was observed in all slices at the longest interstimulus intervals (50ms). The response at interpulse intervals of 20ms was variable between the slices. 3 slices showed a degree of facilitation at this interval. The protocol was therefore adopted of studying control and test responses to each pairing of agonist and antagonist agents in the same slices.

The effect of adenosine on paired-pulse interaction

Adenosine at 10μM significantly enhanced paired-pulse facilitation at 20 and 50ms compared with control ACSF (Fig. 3. 12) but did not change paired-pulse inhibition at 10ms. The effect of adenosine on paired-pulse interaction completely reversed when adenosine was washed from the recording chamber.

The effect of adenosine on paired-pulse interaction in the presence of 8PT

When adenosine was superfused in the presence of 8PT, adenosine was not able to induce any significant change in the paired-pulse ratio compared with control at any tested interstimulus intervals as illustrated in figure 3. 13.

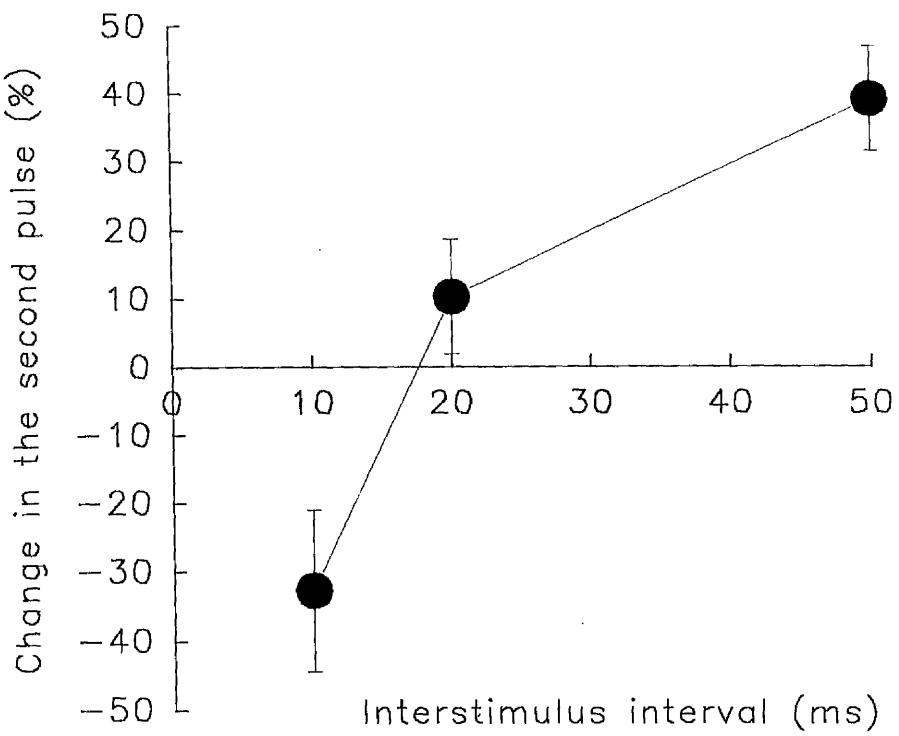

Figure 3. 11. The effect of different interstimulus intervals on interaction between pairs of the evoked orthodromic potentials in the absence of exogenous agents. The initial stimulus strength was 70% of maximum. The points shows the mean ± S. E. M for n=5 slices.

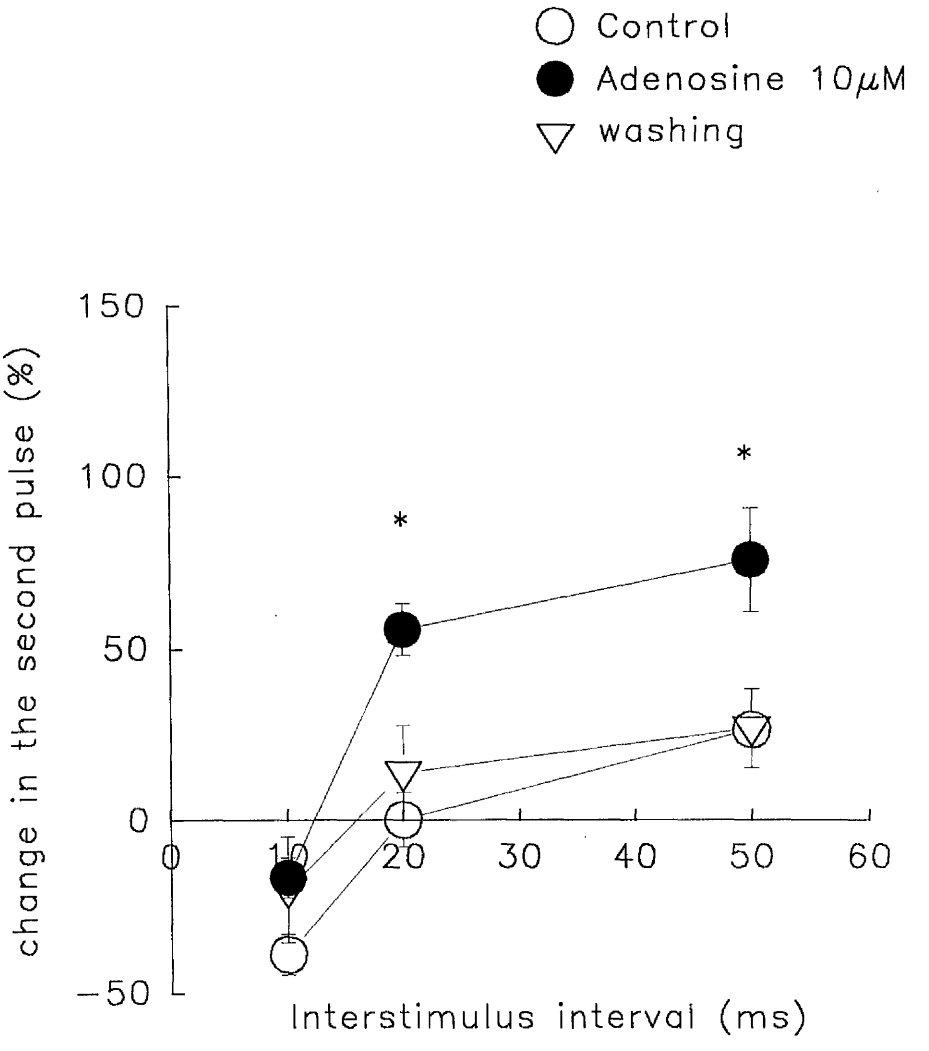

Figure 3. 12 Modulation of orthodromic evoked paired-pulse population spikes by adenosine 10μM at interpulse intervals at 10, 20 and 50ms. Each point shows mean ± S. E. M for n=4 slices. * $P<0.05$

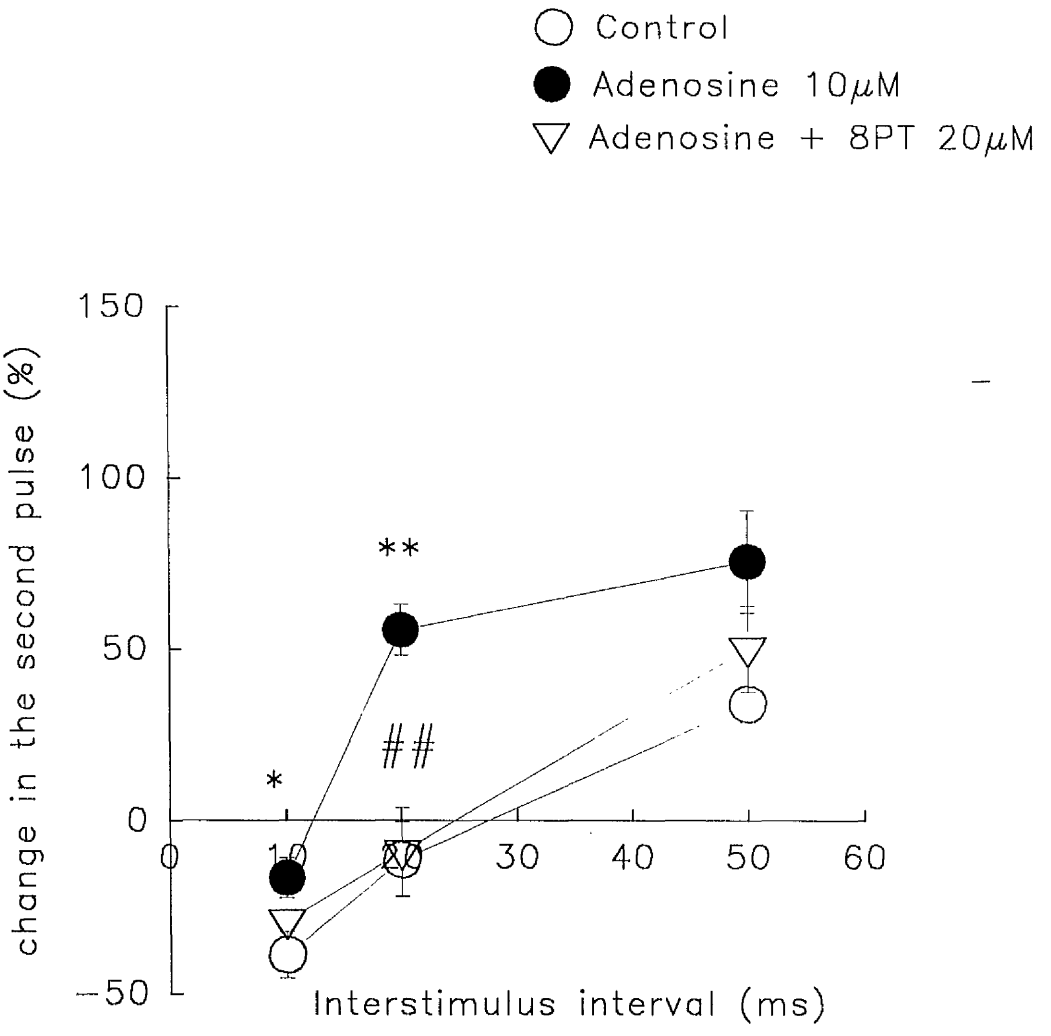

Figure 3. 13. Modulation of orthodromic evoked paired-pulse population spikes by adenosine 10μM alone and in the presence of 8PT 20μM. Each point shows mean ± S. E. M for n=4 experiments. * P<0.05, ** P<0.01 for difference between control and adenosine . ## P<0.01 for difference between adenosine and adenosine + 8PT.

The effect of P_2 receptor agonists on paired-pulse interaction

ATP: ATP at 50μM was superfused for 10 minutes. ATP produced a larger but qualitatively similar change of paired-pulse profile compared with adenosine. The effect of ATP was to reduce paired-pulse inhibition at interstimulus intervals of 10ms and increase facilitation at 20 and 50ms (Fig. 3. 14). All of the above effects were reversible after 10 minutes of washing.

βγ-methyleneATP

The effect of βγ-methyleneATP as a more stable analogue of ATP was also examined on the paired-pulse population spikes. Application of βγ-methyleneATP 10μM yielded a similar result as was observed with ATP with an apparent conversion from paired-pulse inhibition to paired-pulse facilitation at shorter innterpulse intervals and marked increase in facilitation at longer intervals. (122.25% ± 48.84 and 113.925 ± 42.7%) at 20ms and 50ms respectively (Figs. 3. 15A & 3. 15B).

The effect of this nucleotide has also been tested on EPSPs evoked from CA1 hippocampal slices. βγ-methylene ATP reduced the amount of inhibition and increased facilitation significantly (Fig. 3. 15C).

αβ-methyleneATP

αβ-methyleneATP at 10μM was superfused for 10 minutes, but no significant change in the paired-pulse inhibition or facilitation was observed compared with the control ACSF magnitude (Fig. 3. 16).

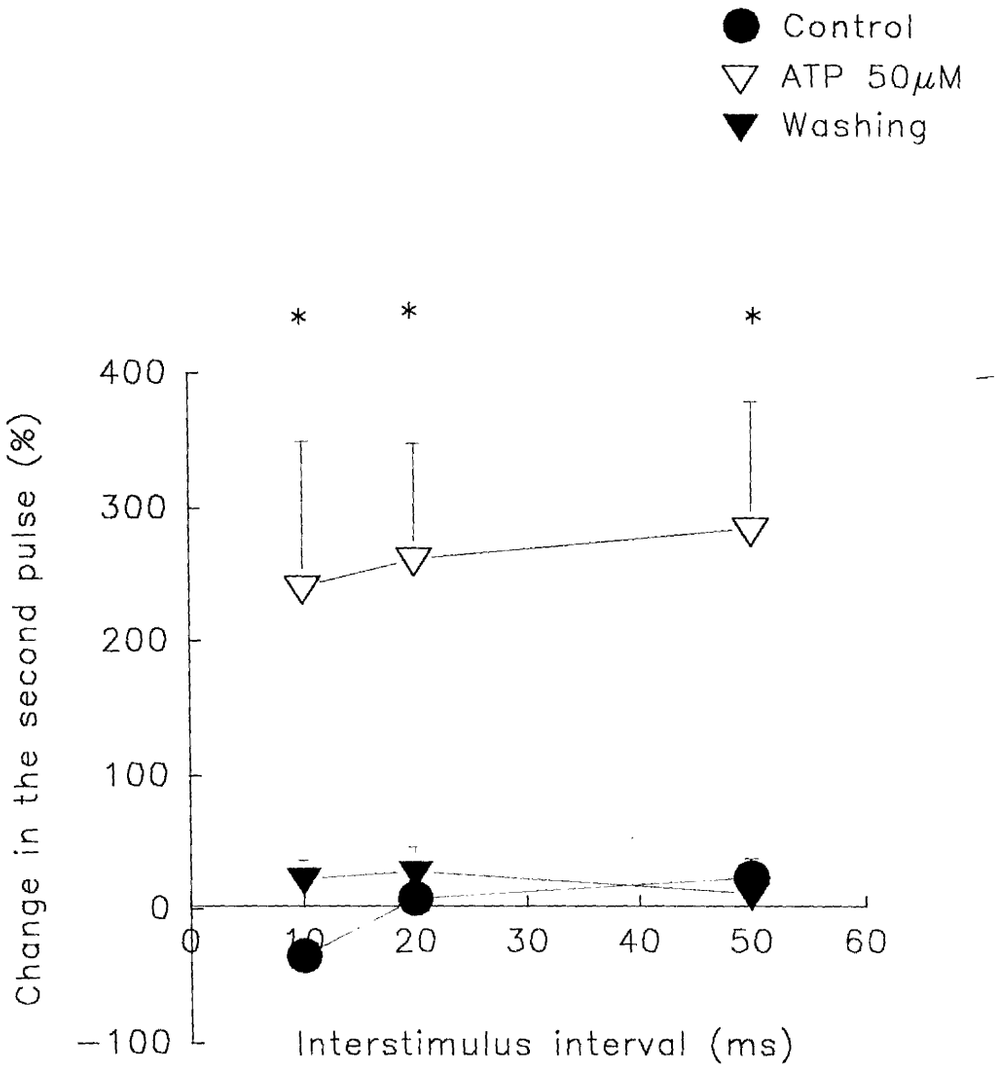

Figure 3. 14. The effect of ATP 50μM on paired−pulse interaction **at different interstimulus intervals (10, 20 and 50ms).** Each point indicates mean ± S. E. M for n=4 slices. * P<0.05

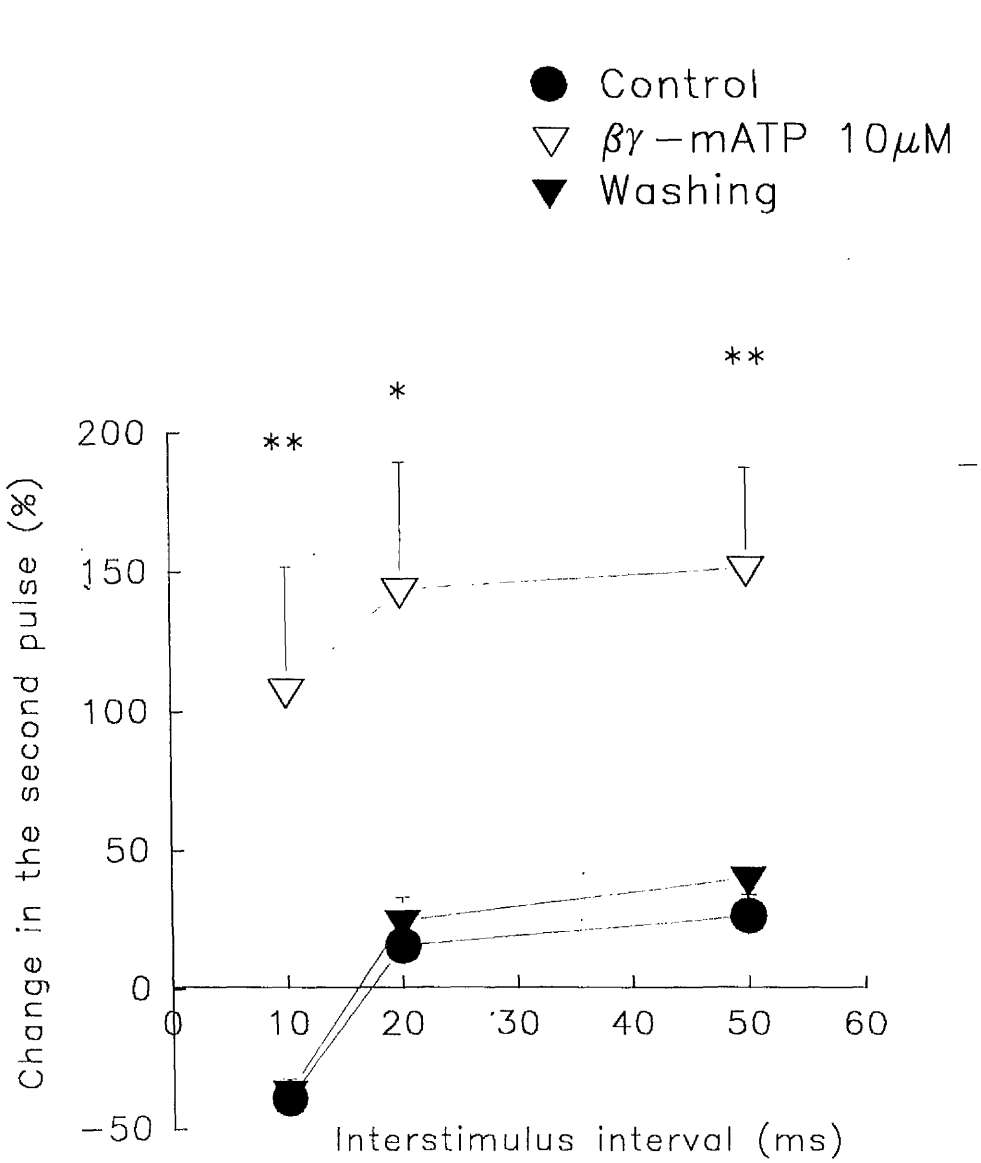

Figure 3. 15A. The effect of βγ−mATP 10μM on paired−pulse interaction at different interpulse intervals (10, 20 and 50ms). Each point shows mean ± S. E. M for n=5 slices. * P<0.05 ** P<0.01

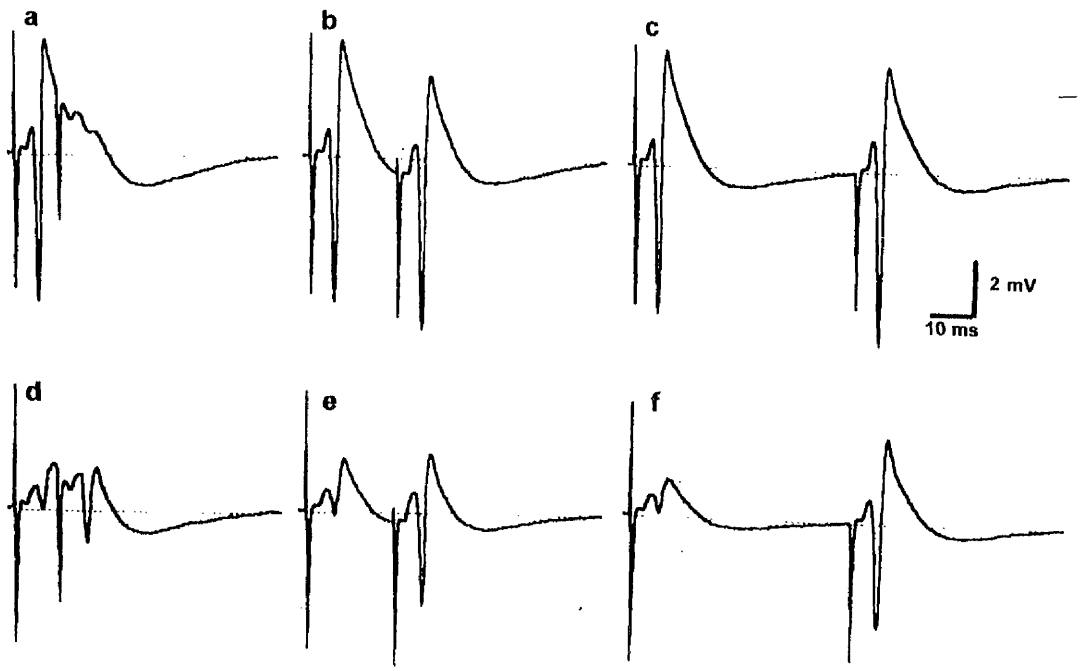

Figure 3. 15B. Representative records of potentials obtained using the paired-pulse paradigm. Records (a-c) illustrate control potentials obtained at interstimulus intervals of 10, 20 and 50 ms, respectively. Records (d-f) illustrate potentials obtained in the presence of βγ-methylene ATP, 10 μM. The nucleotide depresses the first response of each pair but produces marked facilitation of the second especially at the longer interstimulus intervals.

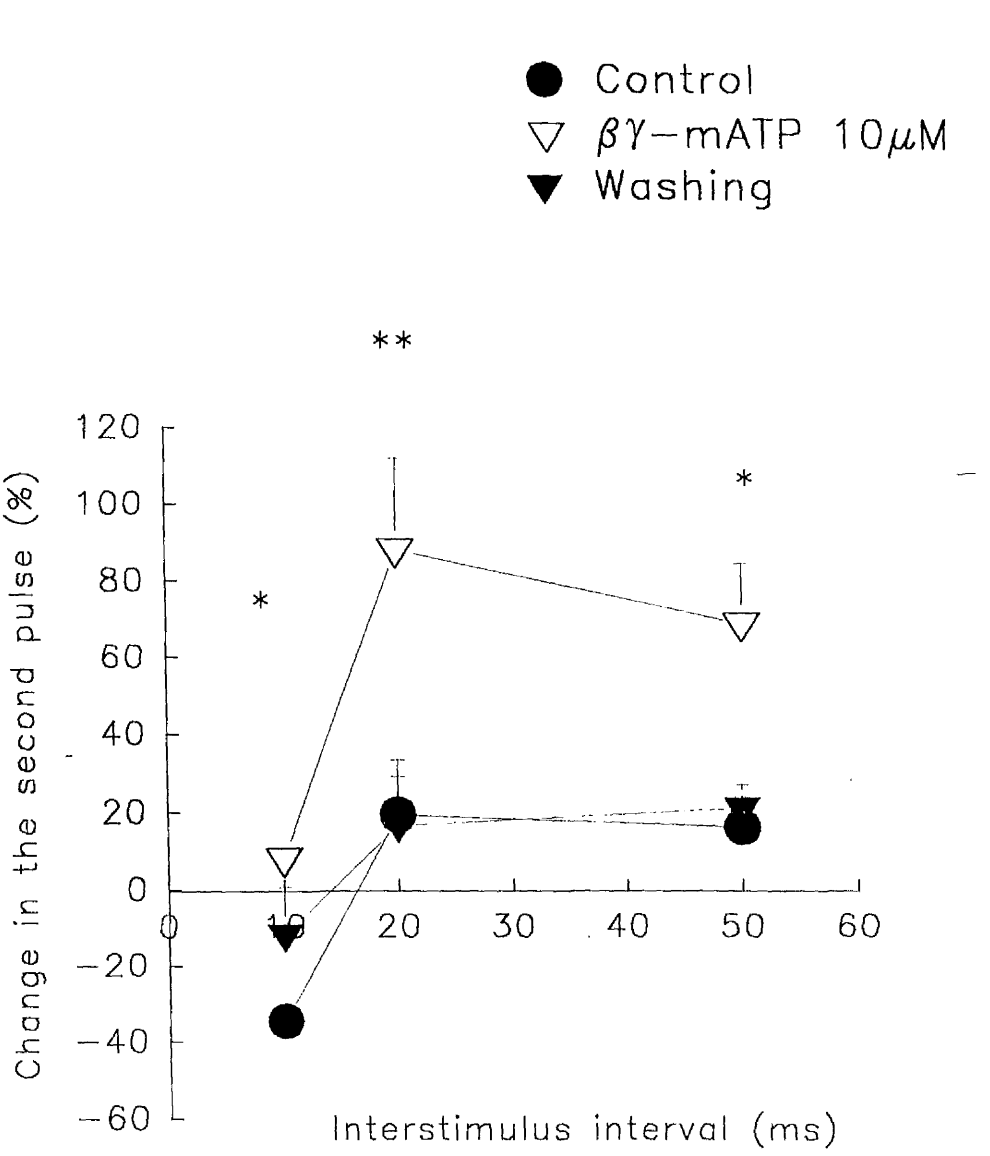

Figure 3. 15C. The effect of βγ−mATP 10μM on paired−pulse EPSPs at different interpulse intervals (10, 20 and 50ms). Each point shows mean ± S. E. M for n=5 slices. * P<0.05 ** P<0.01

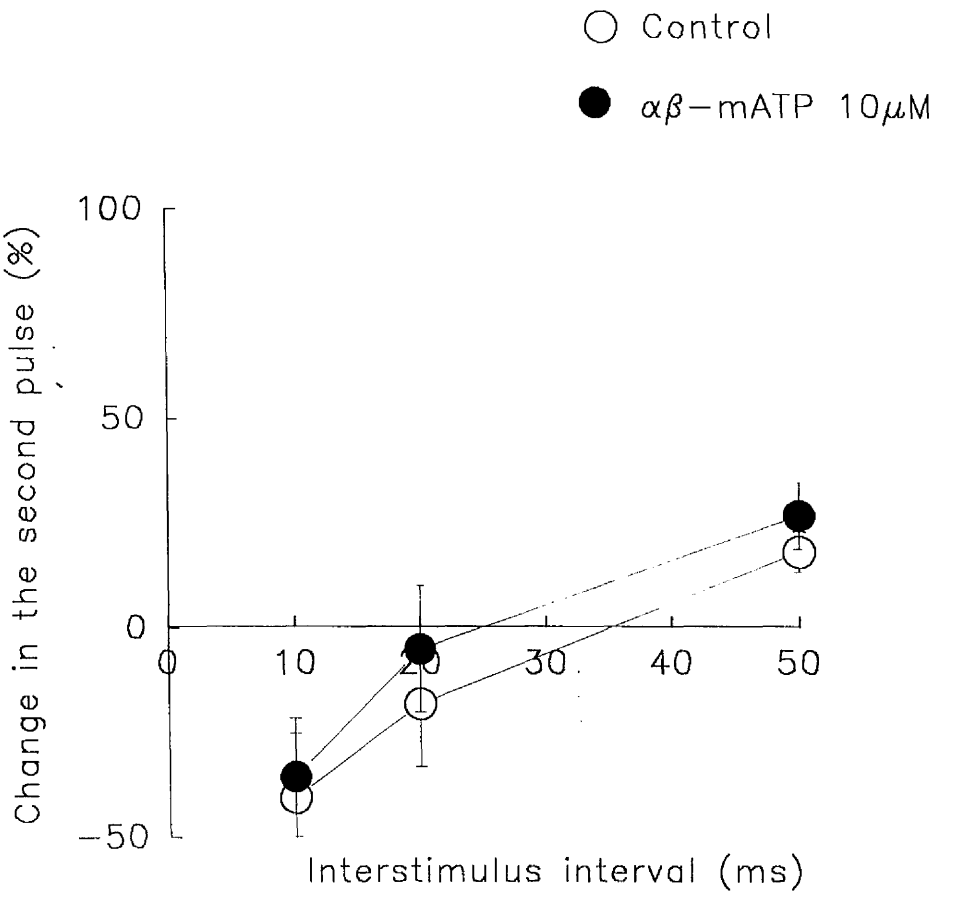

Figure 3. 16. Graph shows that no significant change in the paired−pulse responses has been observed folowing application of αβ−mATP 10μM.

The effect of ATP in the presence of P_1 receptor antagonists

8PT

In order to determine whether the effect of ATP on the paired-pulse paradigm resulted from the activation of P_1 receptors or not, another set of experiments was performed to examine the effect of ATP in the presence of 8PT. 8PT50 μM completely abolished the responses to ATP at the smaller paired-pulse intervals and reduced facilitation at longer intervals, although a small but significant increase in facilitation remained at 50ms (fig 3. 17).

Adenosine deaminase

That the effect of ATP depended mainly on the formation of extracellular adenosine was also supported by a separate set of experiments in which ATP was superfused in a medium containing adenosine deaminase 0.2 unit/ml. Adenosine deaminase was able to prevent the effect of ATP 50μM on paired-pulse interaction at all examined interstimulus intervals (Fig. 3. 18).

The effect of P_1 receptor antagonists on paired-pulse responses to βγ-methyleneATP

8PT

The effect of βγ-methylene ATP 10μM on paired-pulse population spikes was re-examined when 8PT at 20μM was present throughout the experiments. 8PT prevented the effect of βγ-methyleneATP on paired-pulse inhibition and decrease paired-pulse facilitation, induced by the nucleotide. However a small significant effect remained at 50ms (Fig. 3. 19).

Adenosine deaminase

Similar experiments were conducted using βγ-methyleneATP 10μM in combination with adenosine deaminase at 0.2 unit/ml. Adenosine deaminase blocked the modulation of paired-pulse interaction produced by βγ-methyleneATP (Fig 3. 20).

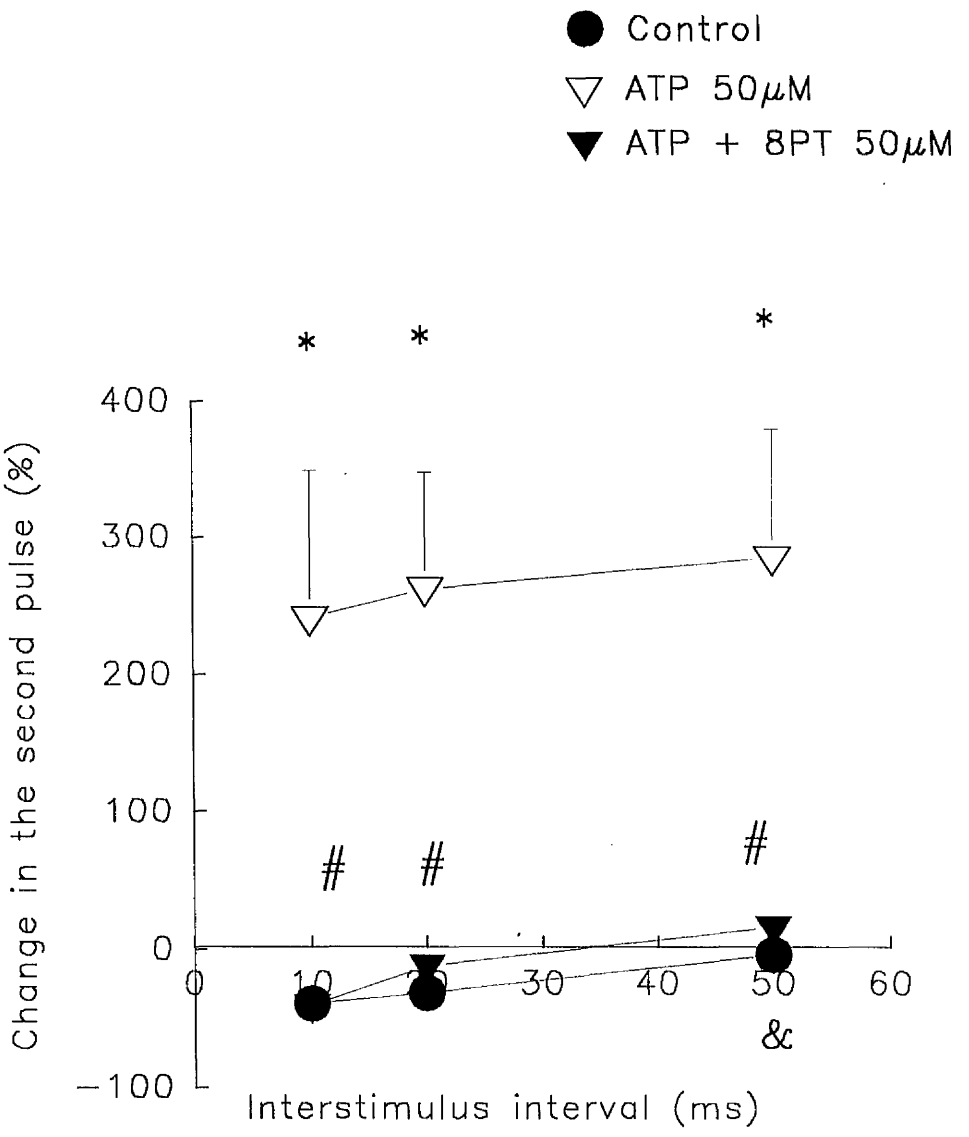

Figure 3. 17. The effect of ATP 50μM alone and in the presence of 8PT 50μM on the paired-pulse interaction between orthodromic potentials at the interstimulus intervals of 10, 20 and 50ms. Each point shows mean + S. E. M for n=4 slices. * $P<0.05$ for difference between control and ATP. # $P<0.05$ for difference between ATP and ATP + 8PT. & $P<0.05$ for difference between control and ATP + 8PT.

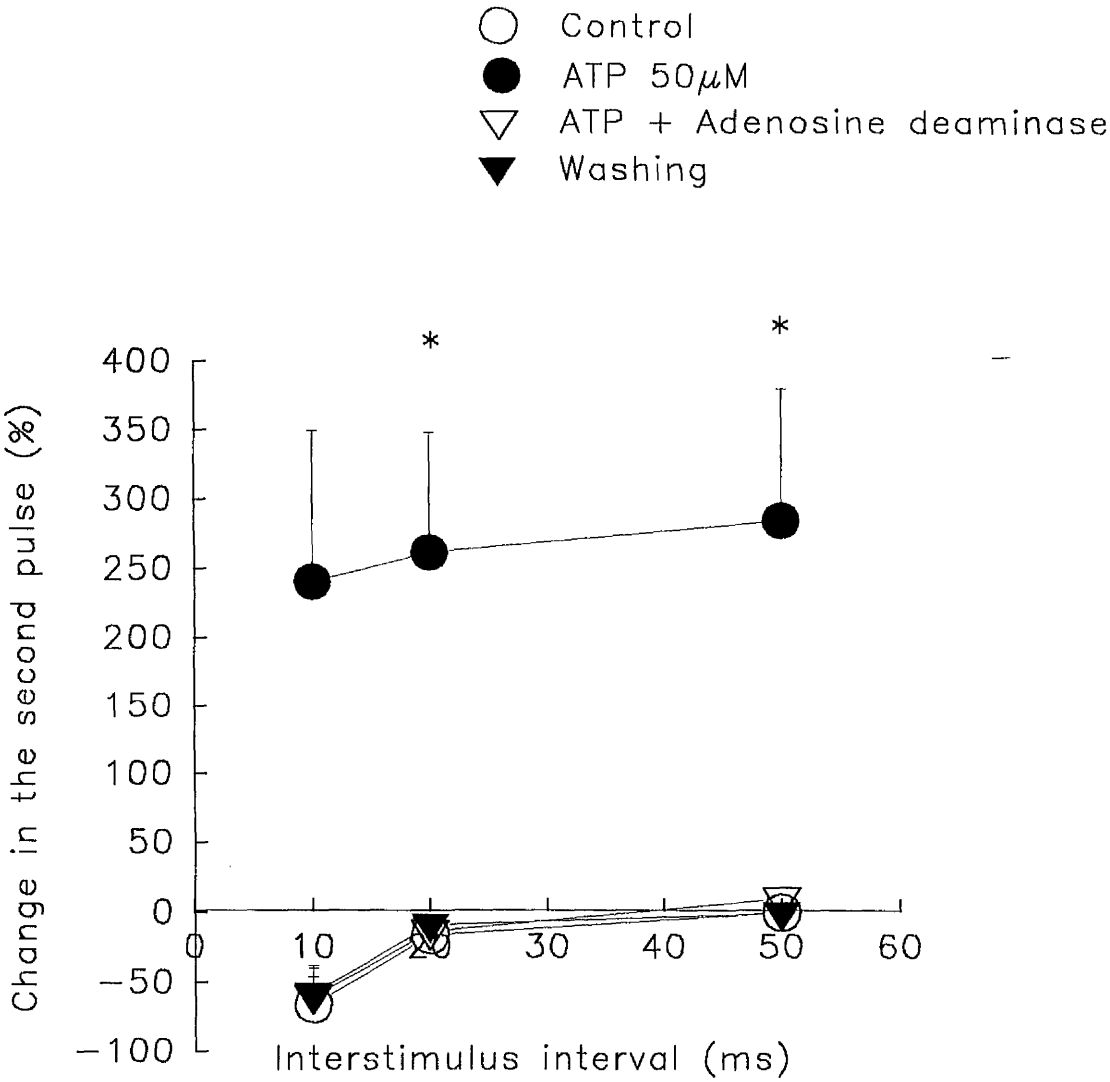

Figure 3. 18. The effect of ATP 50μM alone and in combination with adenosine deanminase 0,2 unit/ml on paired−pulse interactions between CA1 potentials evoked from hippocampal slices at interstimulus intervals at 10, 20 and 50ms. Each point shows mean + S. E. M for n=3 experiments. * P<0.05.

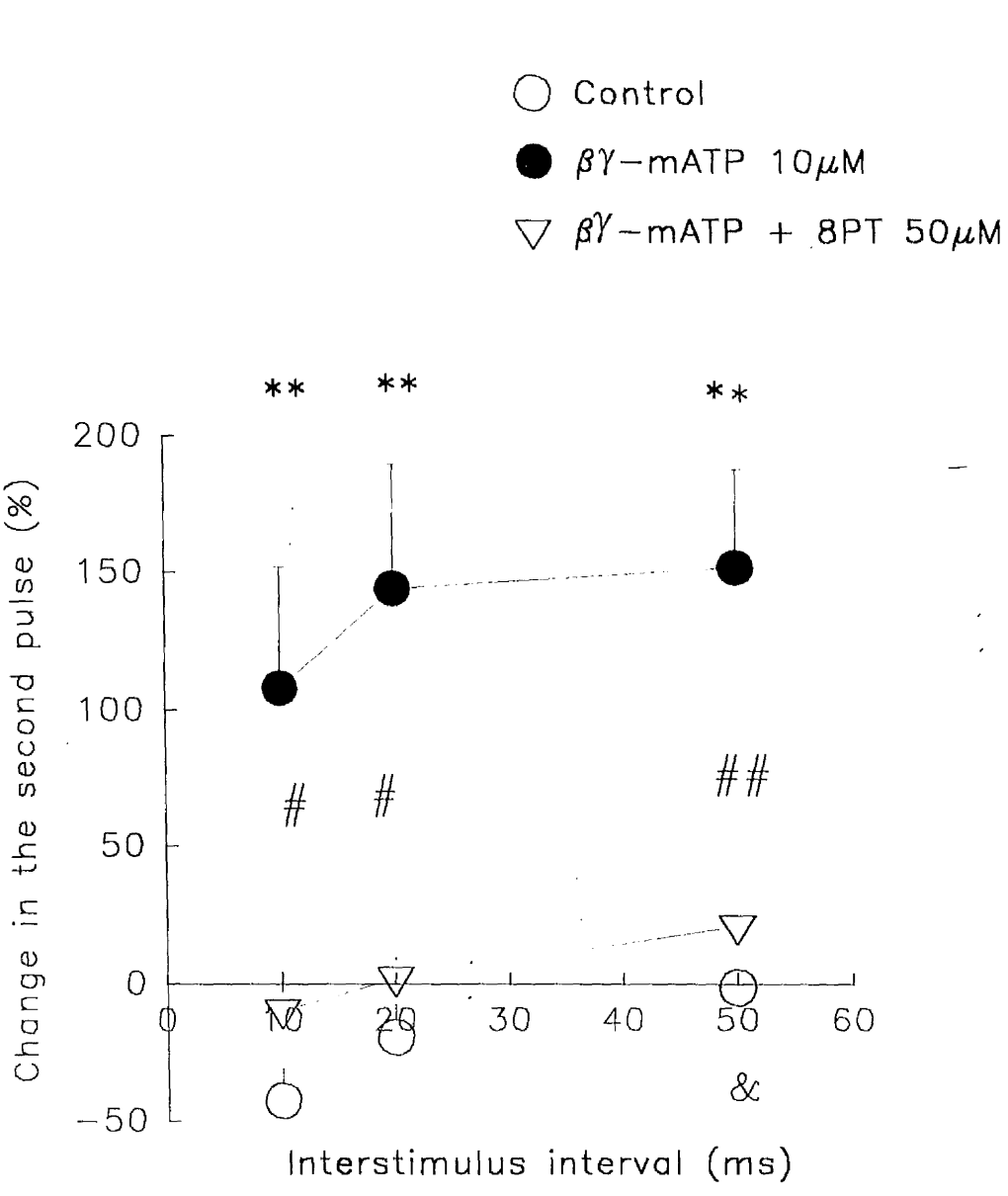

Figure 3. 19. The effect of βγ−mATP 10μM alone and in the presence of 8PT 50μM on paired−pulse population spikes at interstimulus intervals of 10, 20 and 50ms. Each vertical bar shows mean ± S. E. M for n=5 slices. ** P<0.01 for difference between control and βγ−mATP. # P<0.05, ## P<0.01 for difference between βγ−mATP and βγ−mATP + 8PT. & P<0.05 for difference between control and βγ−mATP..

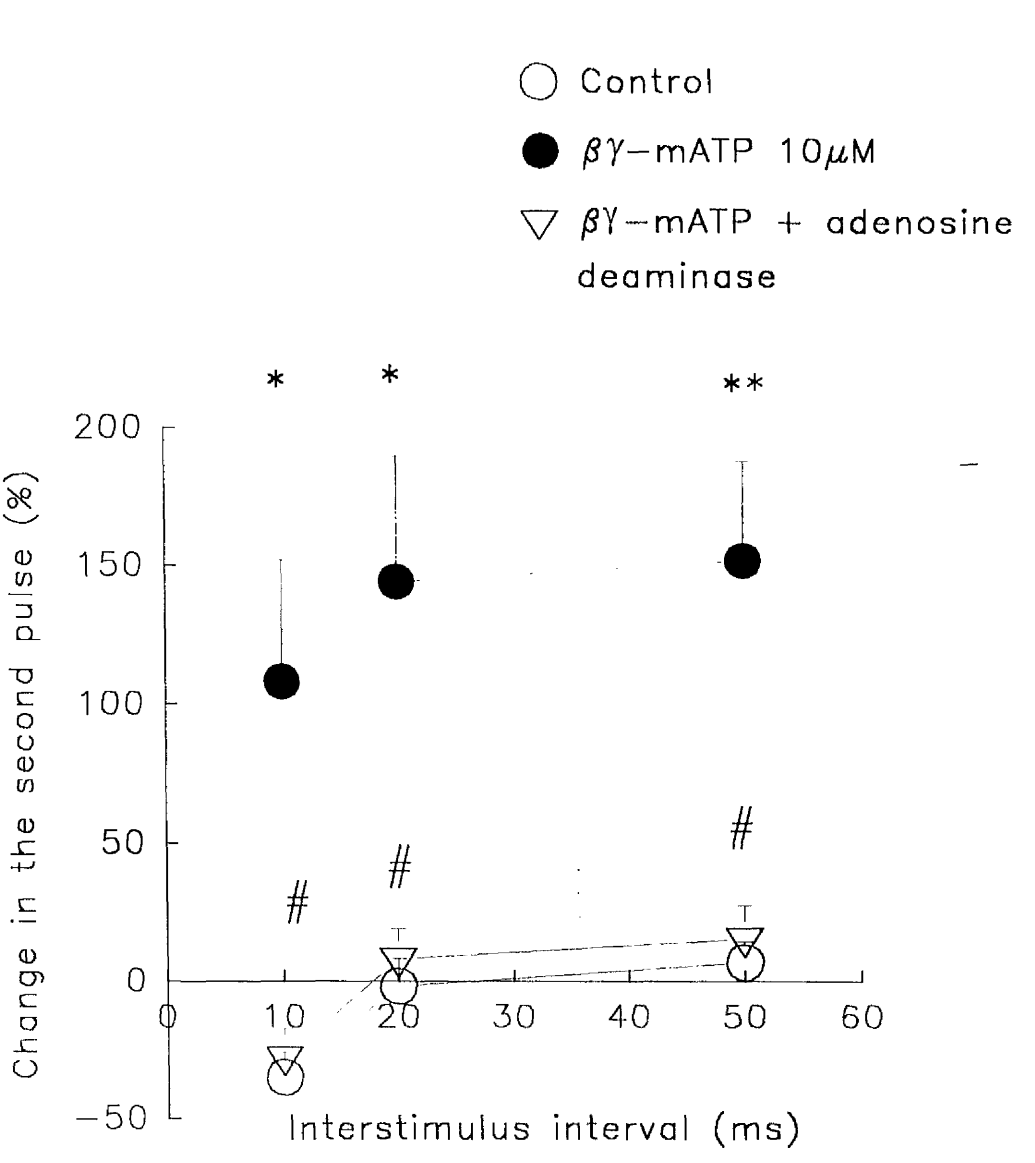

Figure 3. 20. THe effect of βγ−mATP 10μM alone and in the presence of adenosine deaminase 0 02 unit/ml on paired−pulse population spikes. Each point shows mean ± S. E. M for n=5 slices. * P<0.05, ** P<0.01 for difference between control and βγ−mATP. # P<0.05 for difference between βγ−mATP and βγ−mATP and βγ−mATP + adenosine deaminase. No significant difference has been observed between control and βγ−mATP + adenosine deaminase.

The effect of a P_2 receptor antagonist, suramin, on paired-pulse responses to nucleotides

In order to determine any involvement of P_2 receptor activation in paired-pulse responses to ATP and βγ-methyleneATP the effect of both nucleotides was re-examined when suramin 50μM was superfused 10 minutes prior and during the application of nucleotides. Suramin significantly decreased both the reduction in inhibition and elevation in facilitation produced by βγ- methyleneATP to the level that responses were not significantly different from that recorded in the control condition (Fig. 3. 21). Suramin was unable to induce any marked change in paired-pulse responses to ATP (Fig. 3. 22).

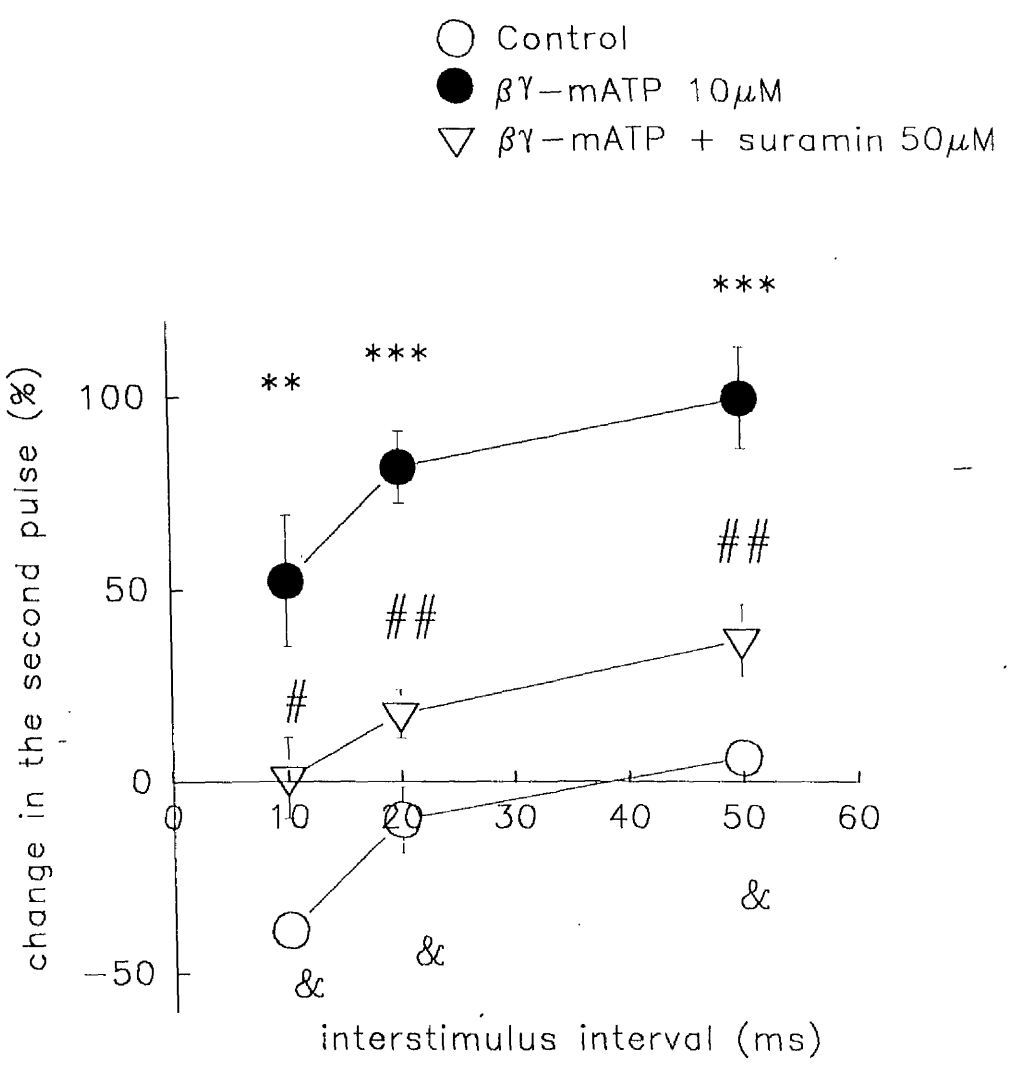

Figure. 3. 21. The effect of βγ−mATP 10μM alone in the presence of suramin 50μM on paired−pulse population spikes. Each point shows mean + S. E. M for n=4 slices. ** P<0.01, *** P<0.001 for difference between control and βγ−mATP. # P<0.05, ## P<0.01 for difference between βγ−mATP and βγ−mATP + suramin. & P<0.05 for difference between controol and βγ−mATP + Suramin.

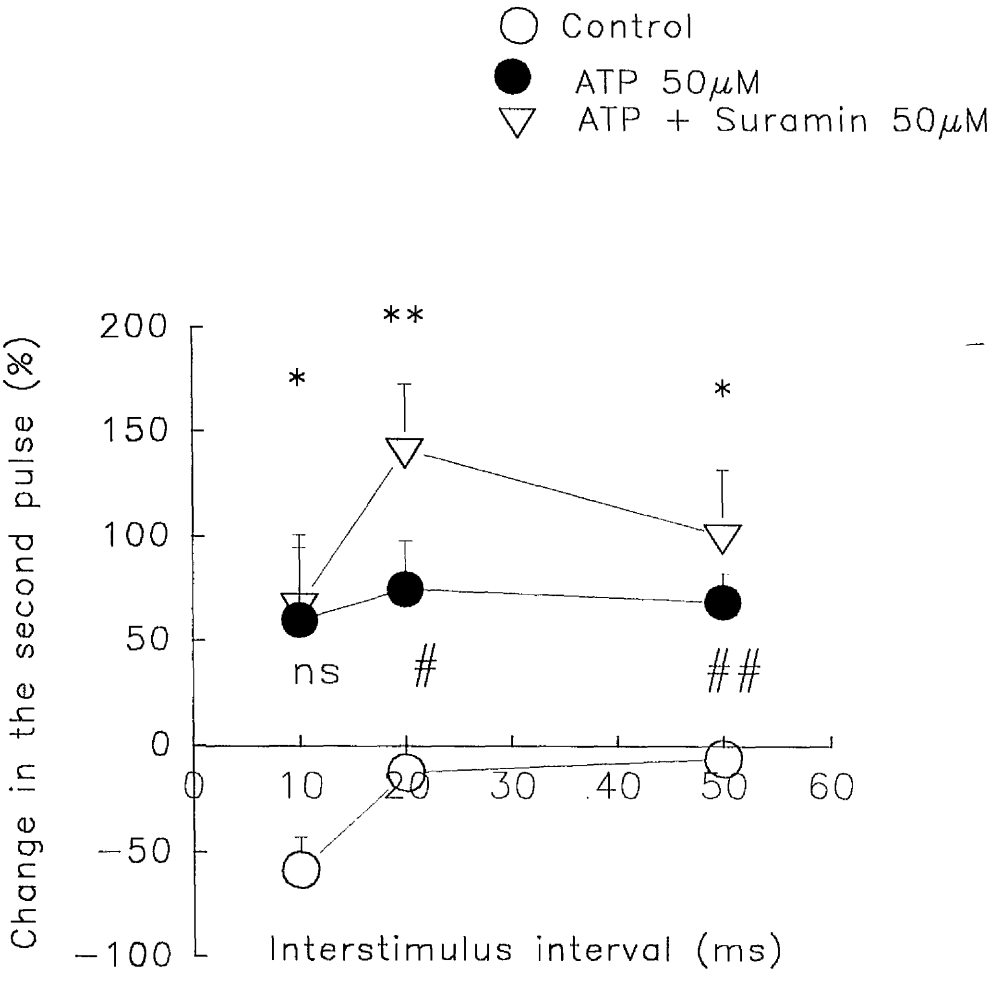

Figure 3. 22. The effect of ATP 50μM alone and in the presence of suramin 50μM on paired−pulse interaction between the orthodromic potentials evoked in hippocampal slices. Each point shows mean ± S. E. M for n=4 slices.
* P<0.05, ** P<0.01 for difference between control and ATP.
# P<0.05, ## P<0.01 for difference between ATP and ATP + suramin. No significant difference has been observed between ATP and ATP + suramin.

Fade in responses to nucleotides ATP and βγ-imidoATP when the amplitude of population spikes was greater than 5mv

The qualitative nature of the responses to ATP and βγ-imidoATP on orthodromic potentials was dependent upon the initial size, subject to the criterion noted earlier that only slices exhibiting population spikes of 3mv or greater were used. All slices showed a depression of the evoked potentials during superfusion with these nucleotides, but when the maximum control population spike size exceeded 5mV, inhibition was not maintained throughout the application of nucleotides as it was in the slices with a smaller spike size. The greatest inhibition of population spikes, which was normally obtained in the initial 3 to 4 minutes of drug application was compared with the end of 10 minutes perfusion.

Fade in responses to ATP

When ATP was tested at concentrations up to 50μM in slices with population spike size >5mV, ATP induced an initial inhibition of spike size which was not maintained throughout the 10 minutes of application. The extent of the inhibition during the perfusion of ATP 50μM declined such that in some slices, the population spike size had almost recovered to control level by the end of the 10 minutes application. In different slices the amount of fade varied from about 30% to 70% of population spike size. The mean population spike size increased from 32.9% ± 10.6 to 83.6 %± 4.13 of control size ($P<0.05$, n=5) (Fig. 3.23).

No fade in responses to adenosine

In order to understand whether the fade in response is a result of adenosine receptor activation by adenosine generated from hydrolysis of ATP, the experiments were repeated with adenosine in another 4 slices with population spike size more than 5mv. Adenosine at 30μM induced an inhibitory effect which remained unchanged during the experiment (Fig. 3. 24). Therefore the profile of responses to ATP was clearly distinct from the pure maintained depression observed with adenosine.

Fade in responses to βγ-imidoATP

A set of experiments was carried out to understand whether the apparent desensitisation which was observed in response to ATP was related to the metabolic stability of ATP. The more stable analogue βγ-imidoATP 30μM was tested on the evoked population spike with amplitude more than 5mV. An apparent desensitisation was still seen similar to that observed with ATP (Fig. 3. 25).

No fade in responses to αβ- methyleneATP

Another stable analogue of ATP, αβ-methyleneATP, at 10μM was also tested on large population spikes evoked from 4 slices but induced no change in the size of the evoked potentials (Fig. 3. 26).

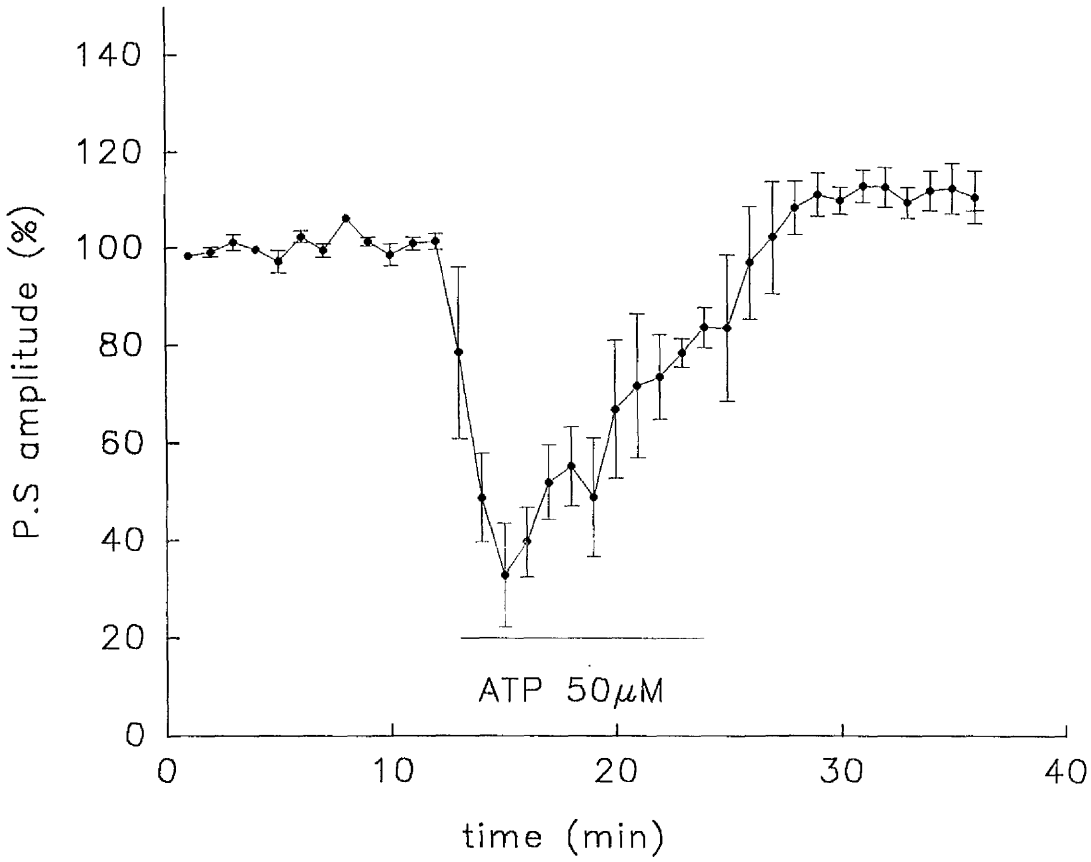

Figure 3. 23. Changes in the population spike potentials during superfusion of ATP 50μM. All slices showed initial population spikes >5mV in size. Responses to ATP showed a significant fade in the degree of inhibition. Points are shown as mean ± S. E. M for n=3 slices.

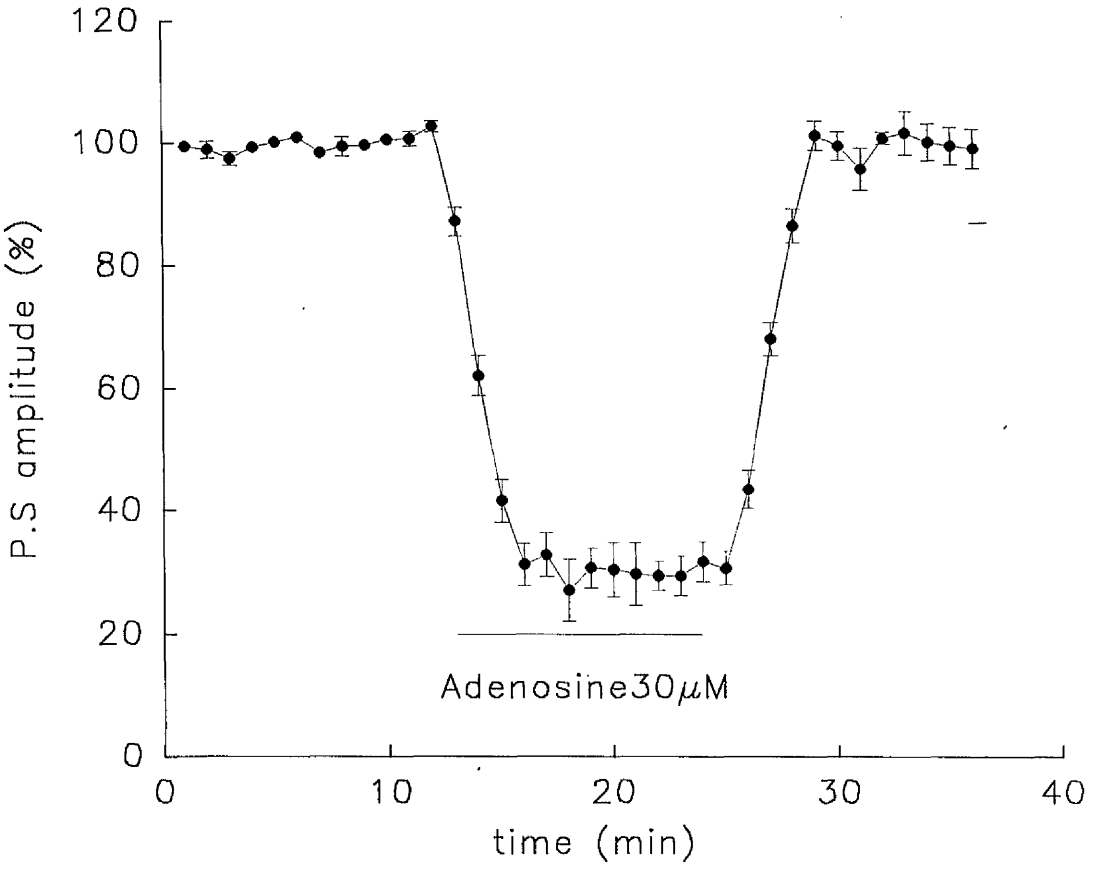

Figure 3. 24. Changes in the CA1 population spike size during the perfusion of adenosine 30μM. The amplitude was adjusted to 70% of maximum. Each vertical bar shows mean ± S.E.M for n=4 slices.

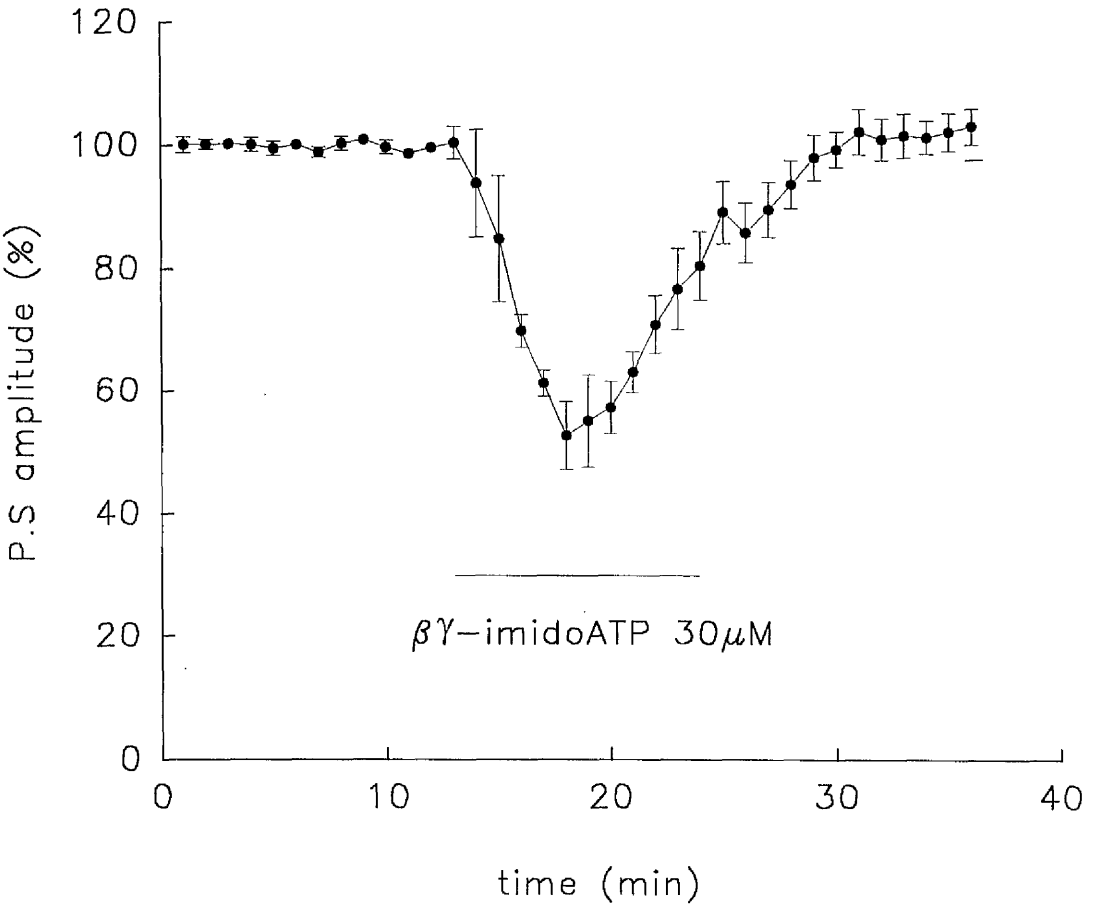

Figure 3. 25. Graph showing the effect of $\beta\gamma-$ imidoATP 30μM on the evoked CA1 population spikes. The fade in responses during superfusion of $\beta\gamma-$ imidoATP was considered as very significant. Each point shows mean ± S. E. M for n=4 slices.

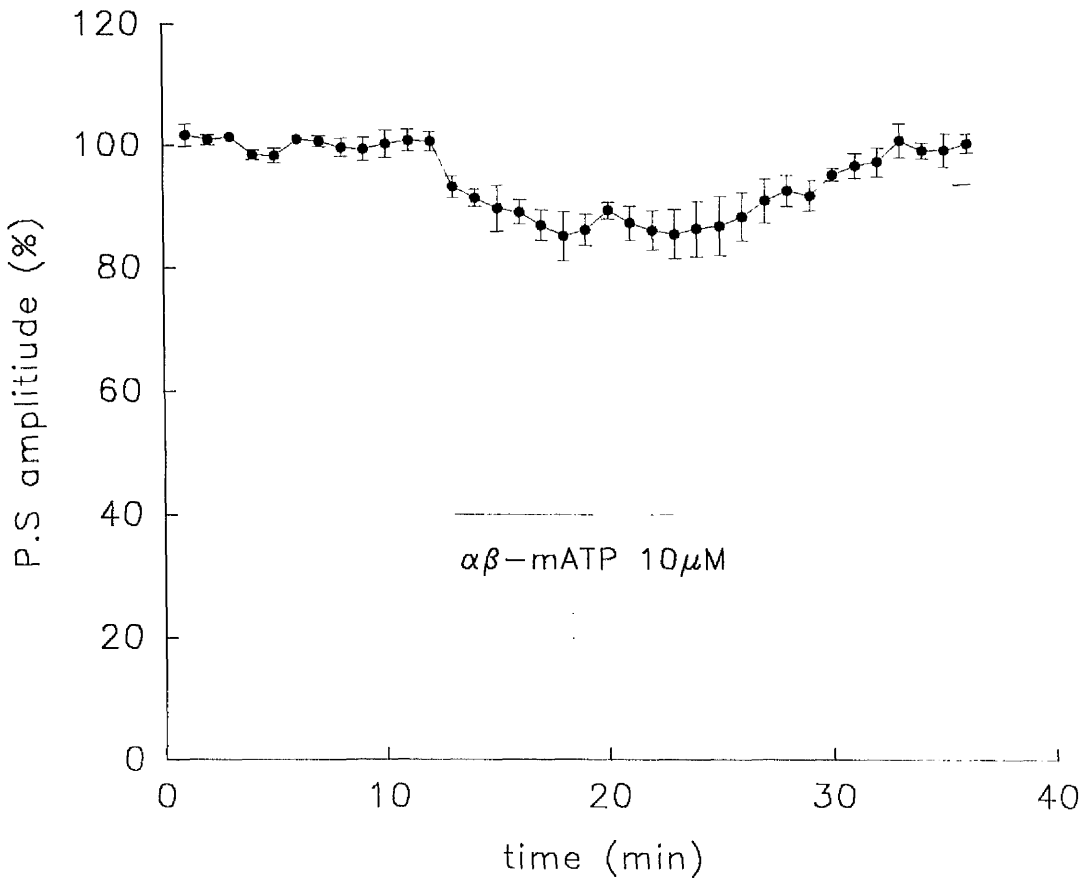

Figure 3. 26. Graph showing the effect of $\alpha\beta$−mATP 10μM on population spikes. No fade in responses has been observed during 10 minutes of perfusion. All slices showed initial population spikes >5 mV in size. Points are shown as meam ± S. E. M for n=5 slices.

Combination effect of βγ-imidoATP and adenosine antagonists on desensitisation

The effect of βγ-imidoATP was re-examined on similar slices when 8PT 10μM was present in the recording chamber from 10 minutes prior and during superfusion of application of nucleotides. 8PT prevented the depressant effect βγ-imidoATP 30μM completely (Figs. 3. 27).

Adenosine deaminase

In a similar manner adenosine deaminase 0.2 unit/ml also completely abolished the depression effect of βγ-imidoATP (Figs. 3. 28). In the presence of this enzyme the initial inhibition was completely blocked.

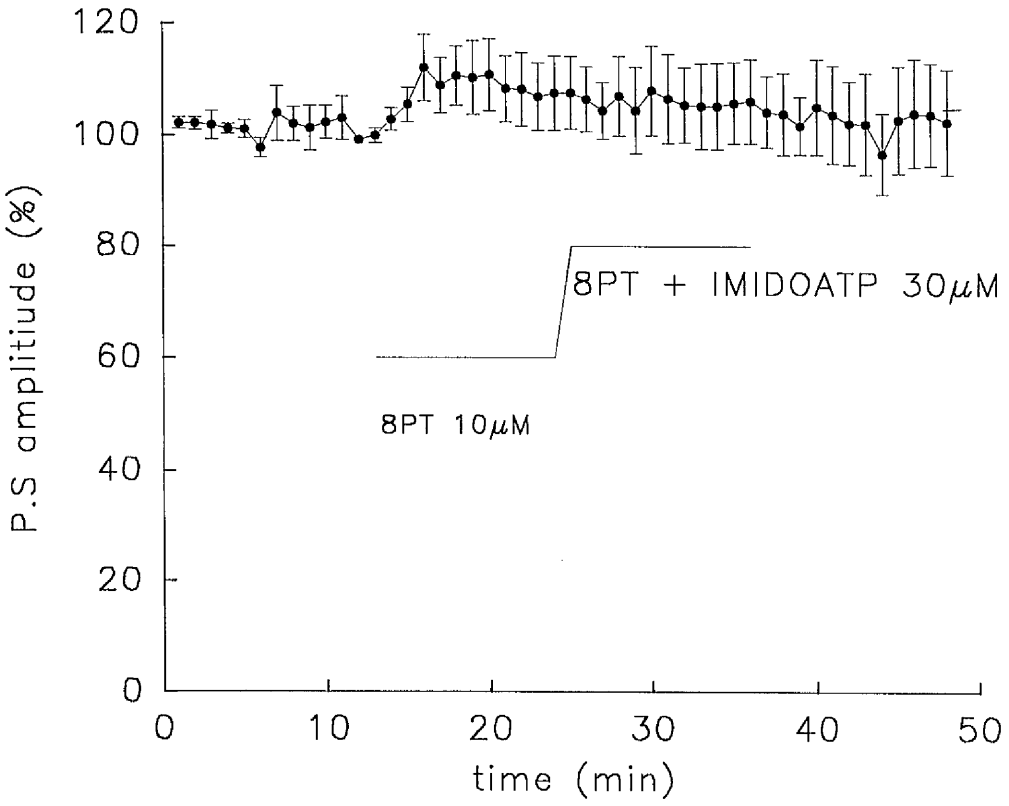

Figure 3. 27. Graph showing the population spike size when $\beta\gamma$ −imidoATP 30μM was superfused in the presence of 8PT 10μM. 8PT prevented both the inhibition and fade in responses produced by this nucleotide. All slices showed initial population spikes>5mV in size. Points are shown as mean ± S. E. M for n=4 slices.

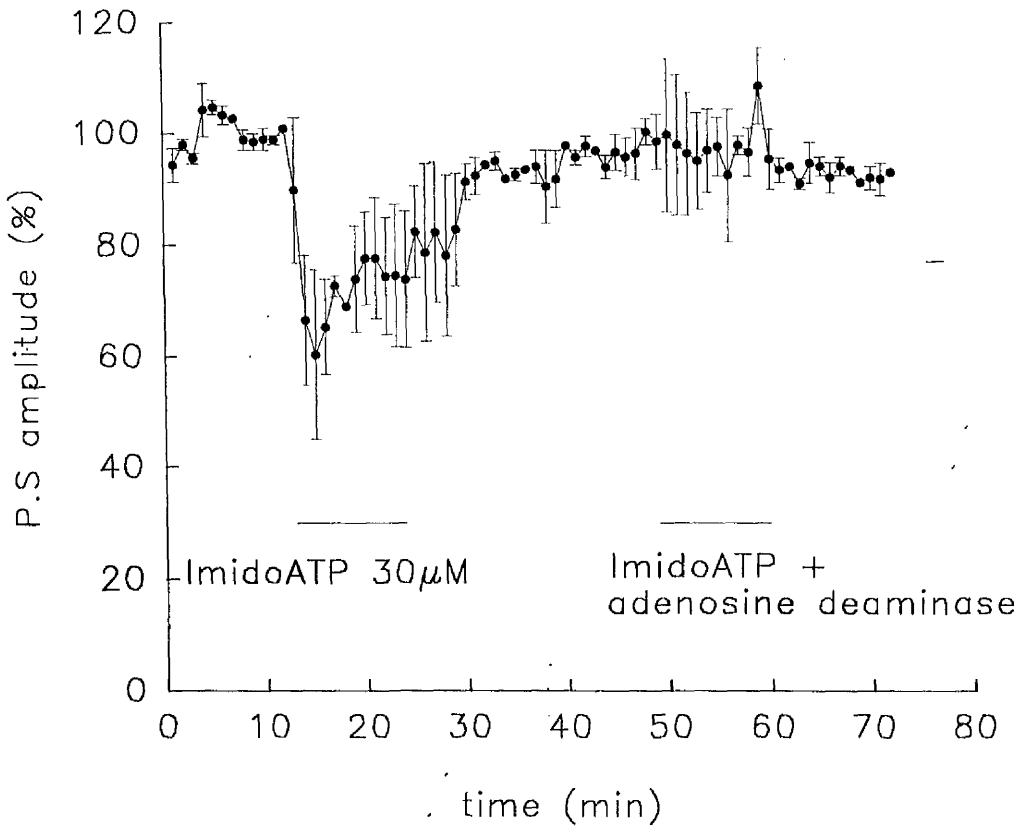

Figure 3. 28. Graph showing changes in the population spike potentials during superfusion of $\beta\gamma$-imidoATP 30μM alone and in the presence of adenosine deaminase 0.2 unit/ml. The modulation in the population spikes amplitude by $\beta\gamma$-imidoATP was prevented by adenosine deaminase. All slices showed initial population spikes > 5mV in size. Points are shown as mean ± S. E. M for n=4 slices.

The effect of suramin on responses to nucleotides

Co-application of suramin at 50μM, reduced the overall extent of the depression produced by ATP 50μM or βγ-imidoATP 30μM, but with an associated change of desensitisation profile (Figs. 3. 29 and 3. 30). In the presence of suramin the initial maximal depression produced by both nucleotides was preferentially suppressed, sueh that the remaining smaller responses showed no apparent desensitisation, whether the agonist used was βγ-imidoATP or ATP itself. When suramin was removed from the medium in the same slices, fade in responses re-appeared (Fig. 3. 30).

The effect of adenosine in combination with suramin

In another set of experiment, suramin was applied with adenosine 30μM. Suramin induced no significant change in the extent or the time course of the inhibitory responses to adenosine (Fig. 3. 31).

The effect of ATP on desensitisation in the presence of αβ-methyleneADP

In order to examine whether the apparent desensitisation produced by ATP but not by adenosine could be attributed to the conversion of ATP to adenosine, another set of experiments was performed in which αβ-methyleneADP, an inhibitor of 5′ nucleotidase, was added to the bath medium 10 minutes before and during the application of ATP. In the presence of nucleotidase inhibitor the fade of the nucleotide-induced inhibition was enhanced. This could be demonstrated more clearly by using a lower concentration of ATP, 20μM, than used in most experiments. The loss of inhibition during the control application of ATP at this concentration was then limited to 18.93 % of the maximum response, fading from 67.45% ± 15.4 inhibition to 48.52 ± 13 (Fig. 3. 32). In the presence of αβ-methyleneADP, the inhibition of the population spike declined more quickly, fading to a significantly larger degree over the 10 minutes application period, from 75.16% ± 9.61 inhibition of the control potential size to 29.74% ± 5.1 after 10 minutes (a fade of 45.42%, n=4, $p<0.01$).

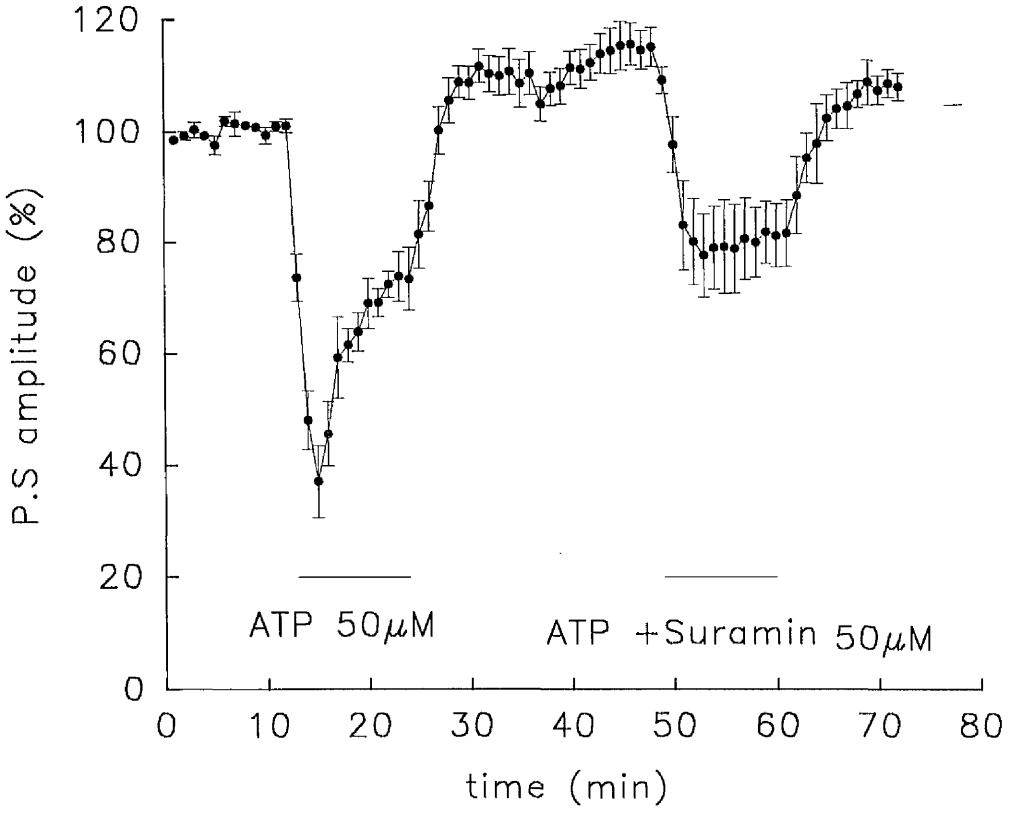

Figure 3. 29. The graph showing the size of CA1 population spike amplitude in the presence of ATP 50μM alone and when cosuperfused with suramin 50μM. Very significant fade in responses was observed during the superfusion of ATP. However suramin 50μM prevented the initial peak of inhibition and subsequent fade of responses. All slices showed initial population size>5mV. Points are shown as mean ± S. E. M for n=4 slices.

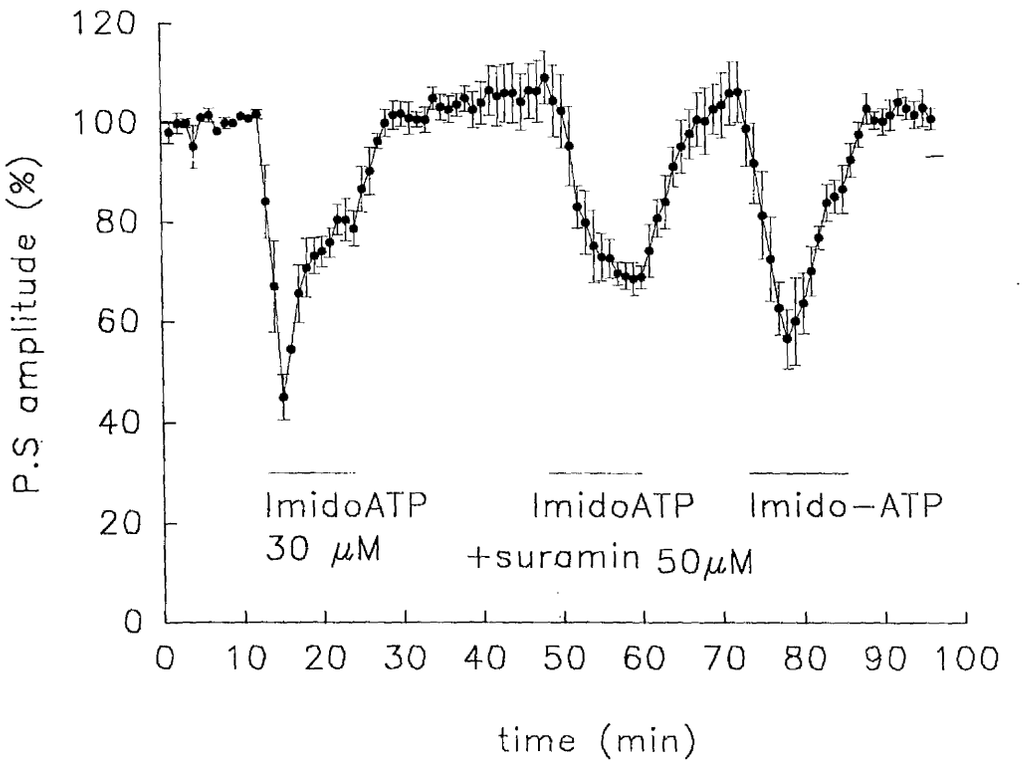

Figure 3. 30. Changes in the population spike potential during superfusion of $\beta\gamma$−imidoATP (30μM) either alone or in the presence of suramin (50μM). All slices showed initial population spikes>5mV in size. Suramin prevents the initial peak of inhibition and the subsequent fade of the response, but these re−appeared after removal of suramin. Points are shown as mean ± S. E. M for n=4 slices.

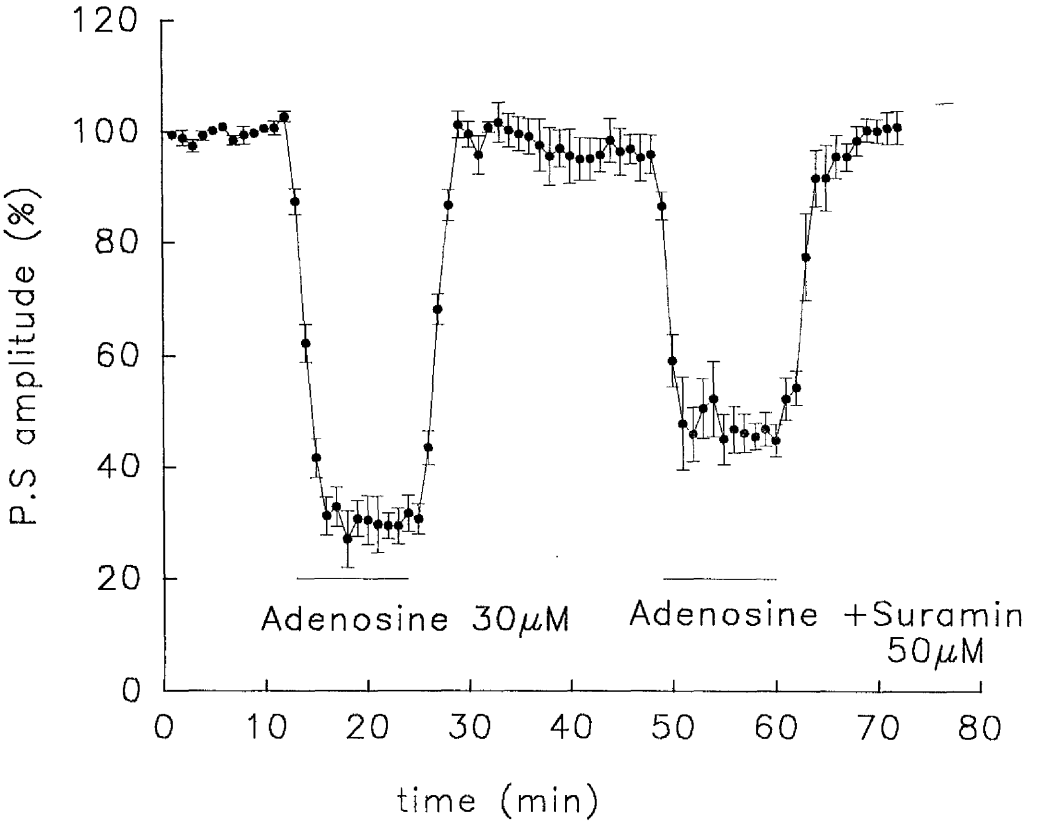

Figure 3. 31. The effect of adenosine 30μM alone and in the presence of suramin 50μM on CA1 population spikes evoked in hippocampal slices. No significant difference was observed in the responses to adenosine after perfusion of suramin. Each point shows mean ± S. E. M for n=4 slices.

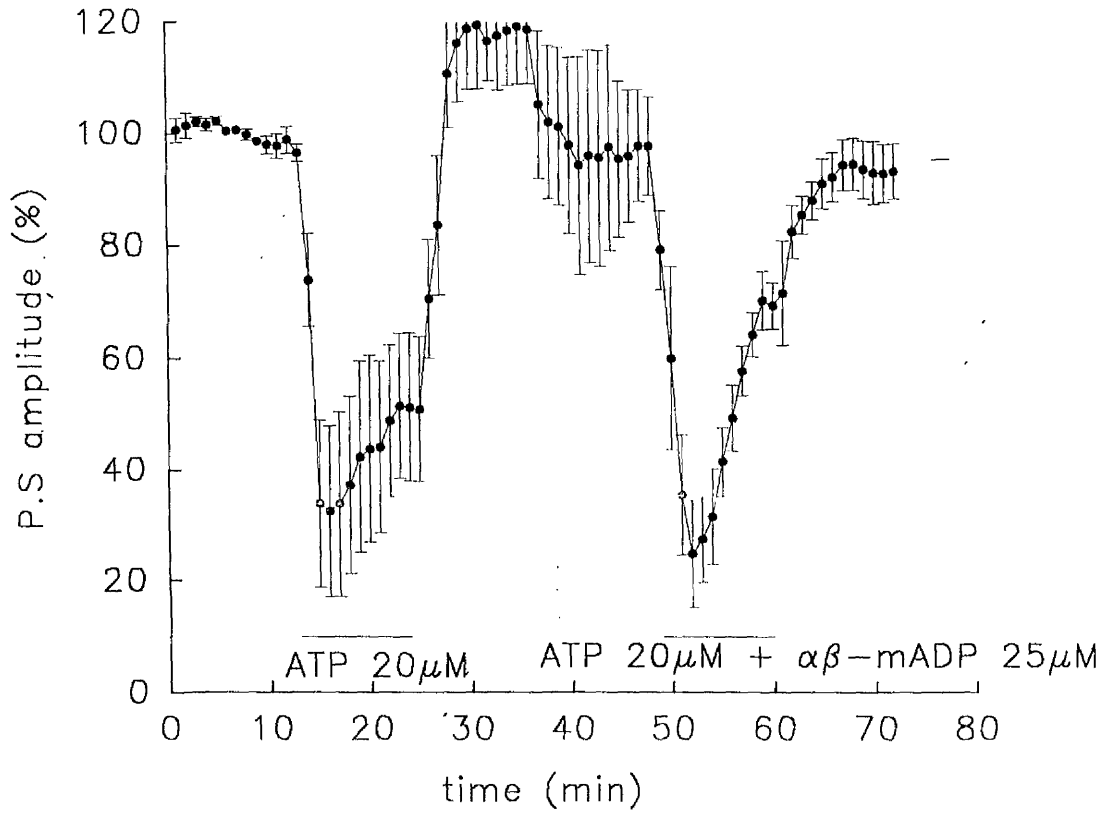

Figure 3. 32. Changes in the population spike potentials when ATP 20μM was superfused alone and in the presence of $\alpha\beta$−mADP 25μM. All slices showed initial population spikes>5mV in size. $\alpha\beta$−mADP tends to increase the initial peak of inhibition and enhances the subsequent fade of responses. Points are shown as mean ± S. E. M for n=4 slices.

Effect of oxotremorine-M on orthodromic single population spikes

To assess the effect of an M_2 receptor selective agonist on orthodromically evoked population spikes in the hippocampus different concentrations of oxotremorine-M were used. In a concentration-dependent manner oxotremorine-M reduced or abolished the amplitude of the population spikes (Fig. 3. 33). The potentials completely disappeared when 200nM of oxotremorine-M were superfused. The threshold concentration for oxotremorine-M was 20nM. The effect of oxotremorine-M was reversible and the slices fully recovered after 20 minutes of washing. The effect of this drug commenced after 6 min of perfusion and fully developed in 10 minutes. No effect on the presynaptic volley was seen during perfusion.

Additive effect of combinations of adenosine and oxotremorine-M

In order to define any interaction between oxotremorine-M and adenosine a set of experiments was organised. The effects of adenosine (5 and 10 μM), oxotremorine-M (50,100 nM) and combinations of both were examined on population spikes evoked from the CA1 area. Firstly, adenosine was used and the percentage of inhibition was measured. After washing and full recovery, oxotremorine-M was superfused and its effect was measured. The same slice was washed again and when properly recovered a combination of agonists was superfused and the percentage of inhibition was calculated. The results indicated that combinations of these drugs produced no significant degree of potentiation or occlusion (Figs 3. 34 - 3. 37).

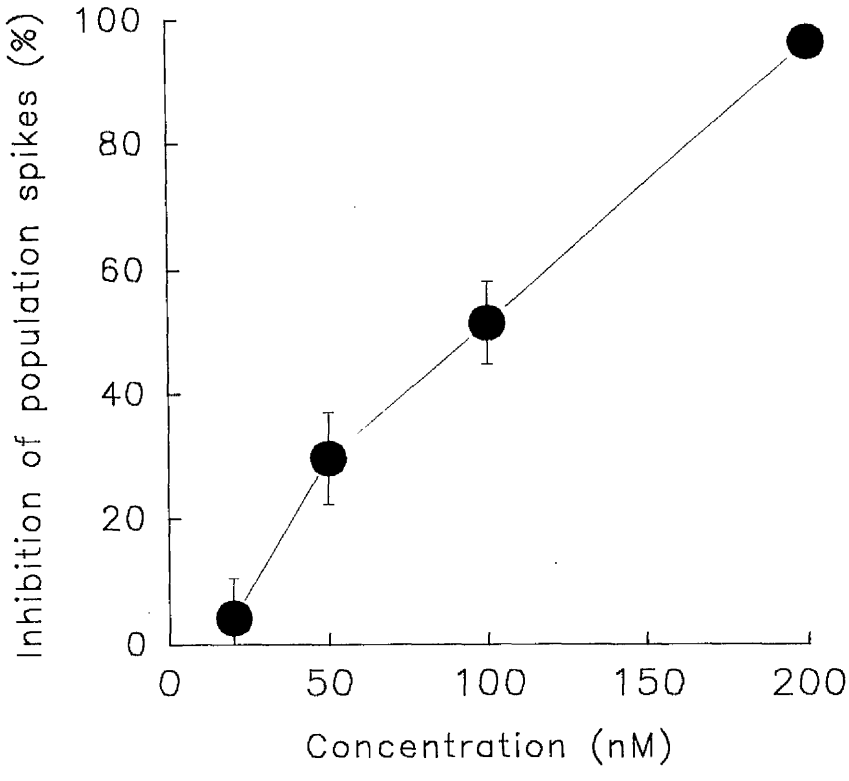

Figure 3. 33. Concentration–response curve for the depression effect of oxotremorine–M on the size of population spikes evoked from hippocampal slices. Each point represents the mean ± S. E. M for n=5 slices.

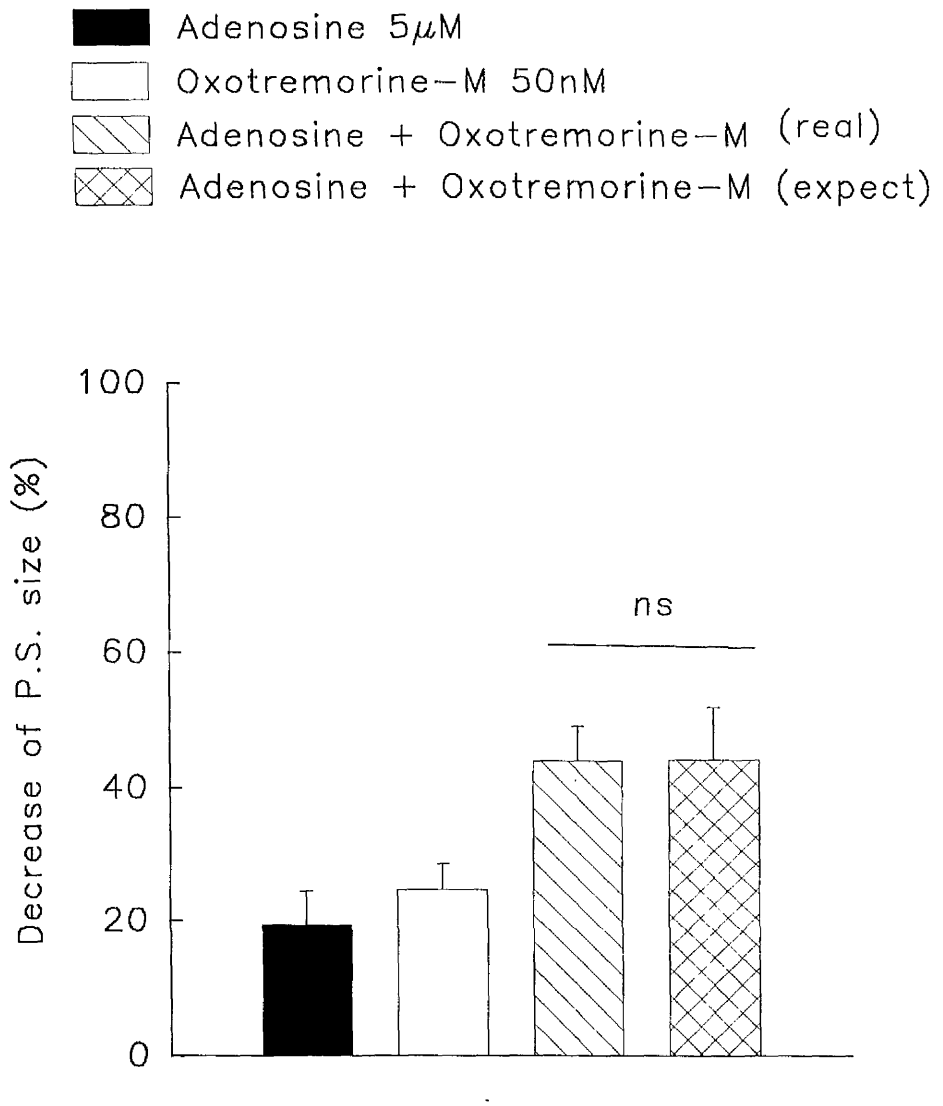

Figure 3. 34. Histogram showimg depression of orthodromic evoked CA1 population spikes by adenosine 5μM, oxotremorine–M 50nM and adenosine + oxotremorine–M. The final column indicates the expected effect if responses to the two agents had been additive. Each vertical bar shows mean ± S. E. M for n=3 slices.

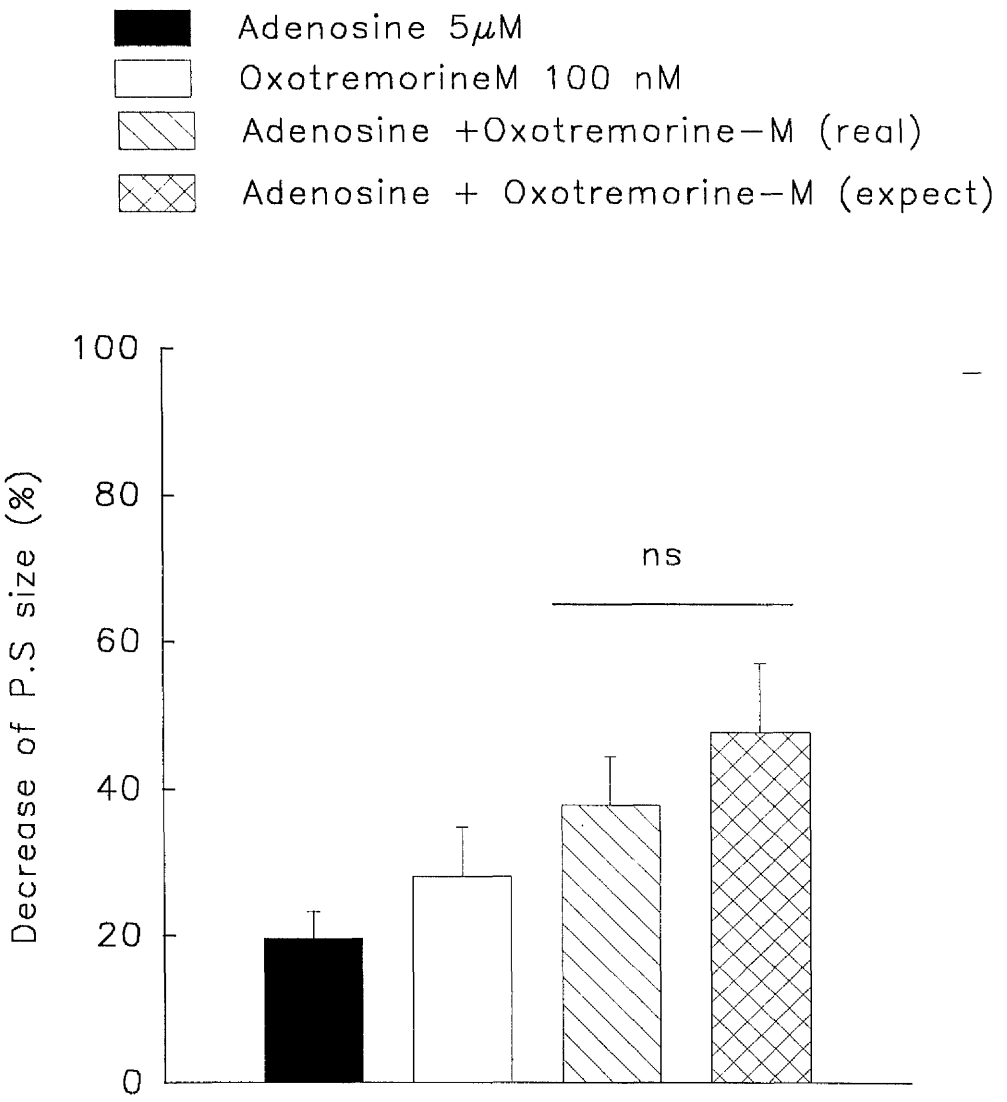

Figure 3. 35. Histogram showing the depression effect of adenosine 5μM, oxotremorine–M 100nM and adenosine + oxotremorine–M. The final column indicates the expected effect if responses to the two agents had been additive. Each vertical bar shows mean ± S. E. M for n=7 experiments.

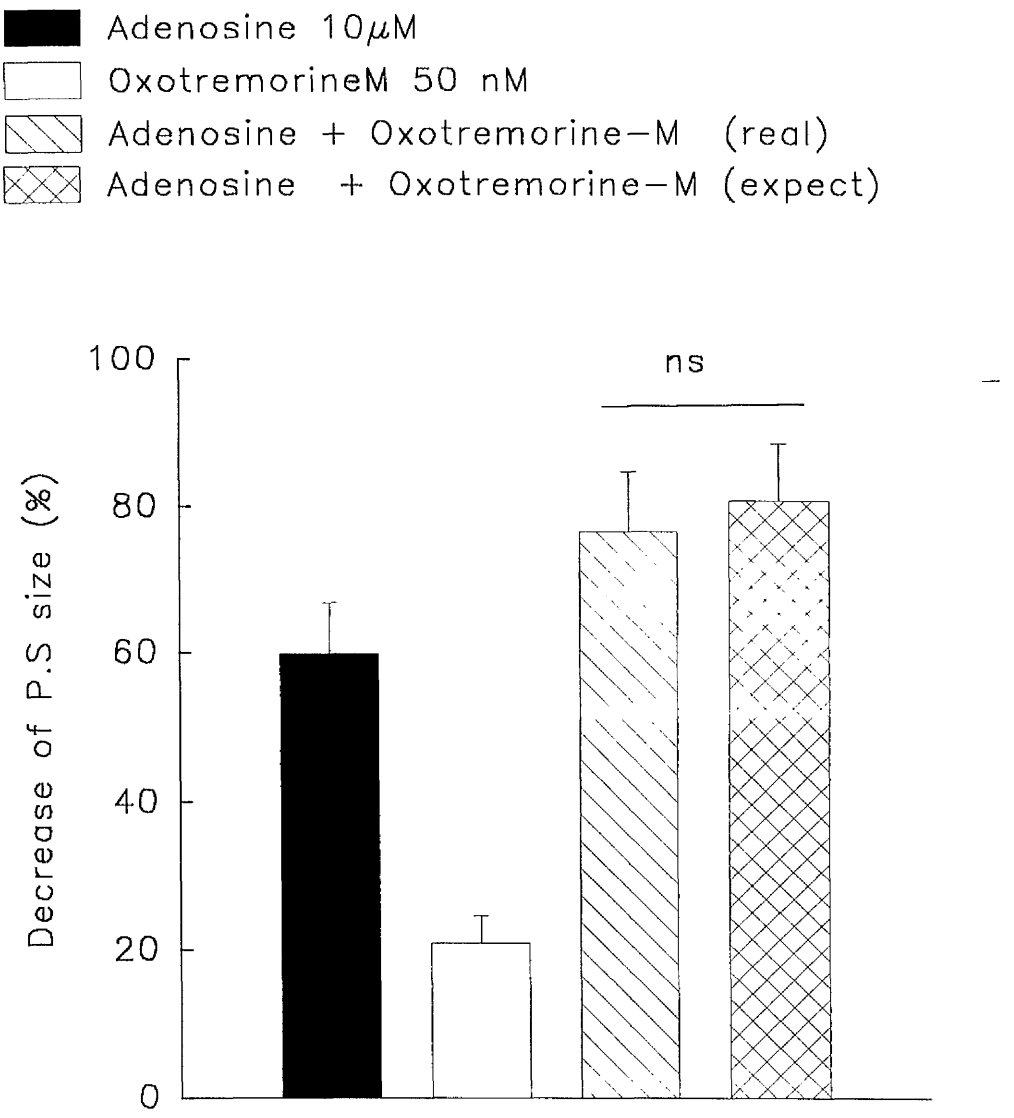

Figure 3. 36. Depression of orthodromic evoked population spikes by adenosine 10μM, oxotremorine–M 50nM and adenosine + oxotremorine–M. The final column indicates the predicted effect if responses to the two agents had been additive. Each vertical bar shows mean ± S. E. M for n=5 experiments.

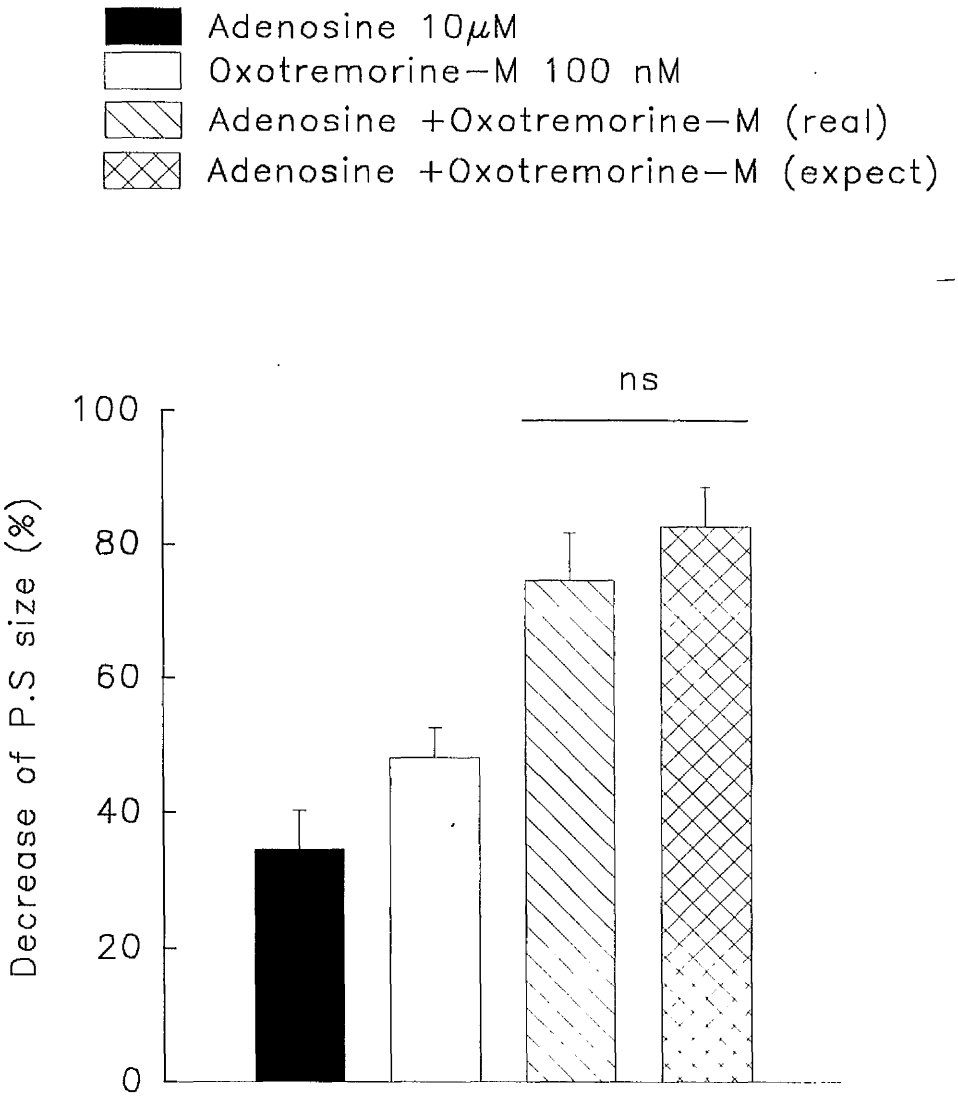

Figure 3. 37. Depression of orthodromic evoked CA1 population spikes by adenosine 10μM, oxotremorine–M 100nM and adenosine + oxotremorine–M. The final column indicates the expected effect if responses to the two agents had been additive. Each vertical bar shows mean ± S. E. M for n=5 experiments.

Receptor selectivity

Since adenosine is able to activate A_1 and A_2 receptors, further experiments were designed to examine selective agonists of these receptors, and to test EPSPs as well as population spikes.

The effect of CPA on single EPSPs

The effect of CPA 20nM was examined on EPSPs evoked from the CA1 area of hippocampal slices. CPA reduced the slope of EPSPs very significantly by 70.95% ± 5.14 after 10 minutes of perfusion. The effect of CPA was reversed after 20-25 minutes of washing (Fig. 3. 38).

The effect of oxotremorine-M 50nm on single EPSPs

In 7 slices the effect of oxotremorine-M 100nM was investigated. Oxotremorine-M depressed the slope of EPSPs to a level (42.52% ± 9.5) which was significantly different from the control (Fig. 3. 39).

Occlusive effect of CPA and oxotremorine-M on EPSPs evoked from CA1 in hippocampus

When combinations of CPA and oxotremorine-M were tested at a range of concentrations, the decrease in the slope and amplitude of the population EPSPs were as shown in Figs. 3. 40 - 3. 43. The columns marked "expect" were calculated by adding the individual responses to CPA and oxotremorine-M, the difference between the real and expected effect being tested using a paired t test. With a combination of 10 nM CPA and 30nM oxotremorine-M, or 20 nM CPA and 100 nM oxotremorine-M the combined effect was substantially and highly significantly less than the calculated additive responses.

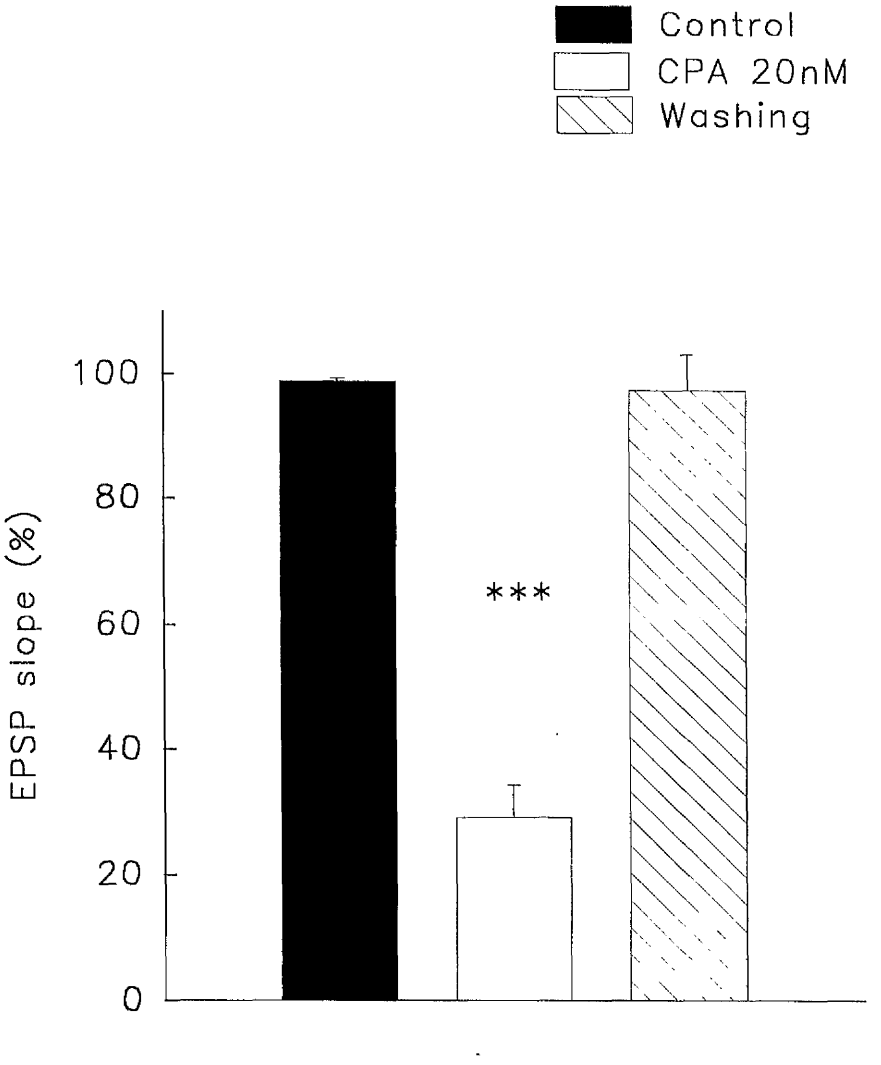

Figure 3. 38. Histogram showing the inhibitory effect of CPA 20 nM on CA1 EPSPs. Stimulus amplitude was 70% of maximum. Each vertical bar shows mean ± S.E.M for n=4 slices. *** P<0.001

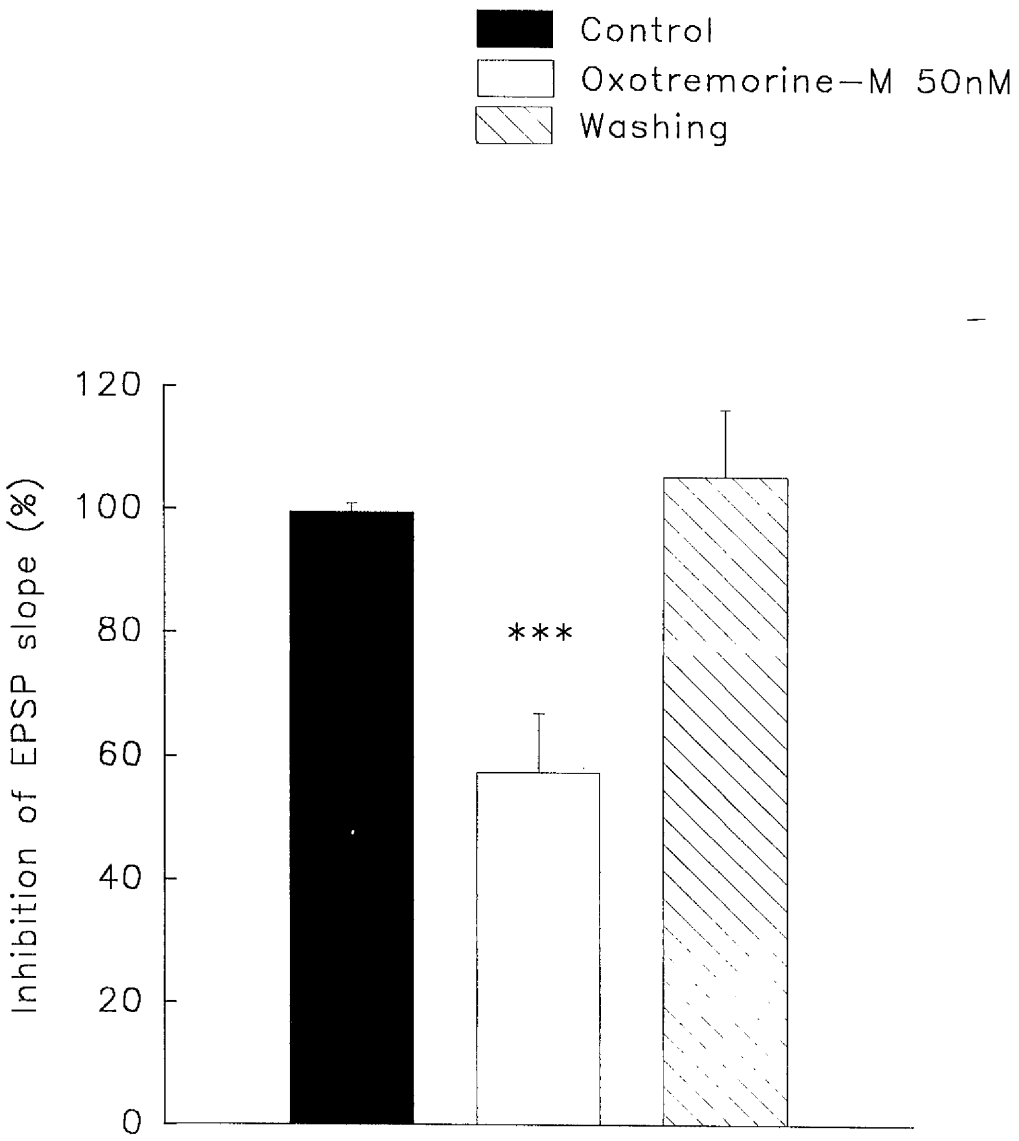

Figure 3. 39. Histogram showing the effect of oxotremorine–M 50nM on evoked CA1 EPSPs. Each vertical bar shows mean ± S. E. M for n=7 slices. *** P<0.001

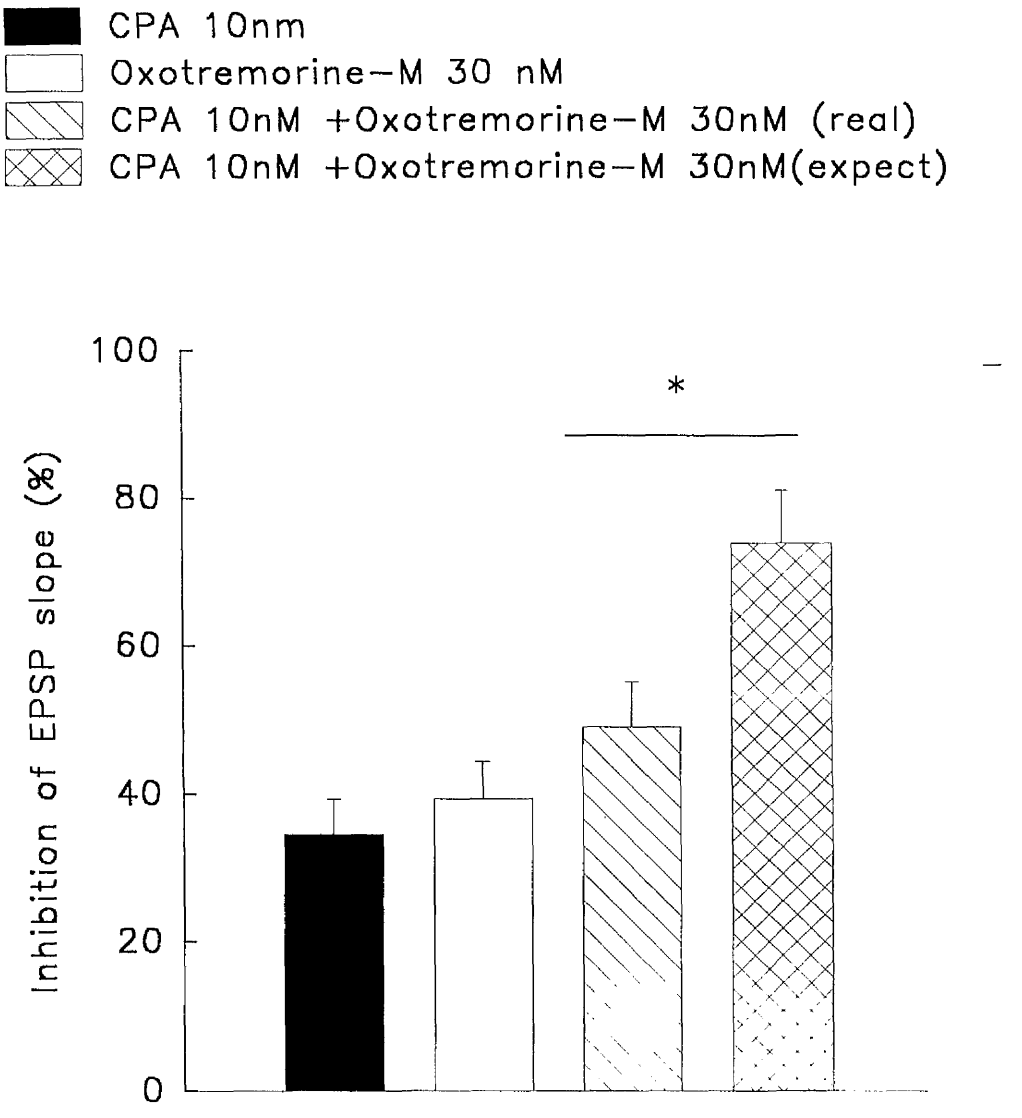

Figure 3. 40. The effect of CPA 10nM, oxotremorine–M 30nM and CPA + oxotremorine–M on EPSPs evoked from CA1. The final column indicates the expected effect if responses to the two agents had been additive. Each vertical bar shows mean ± S. E. M for n=5 slices. * $P<0.05$ compared with the expected additive effect.

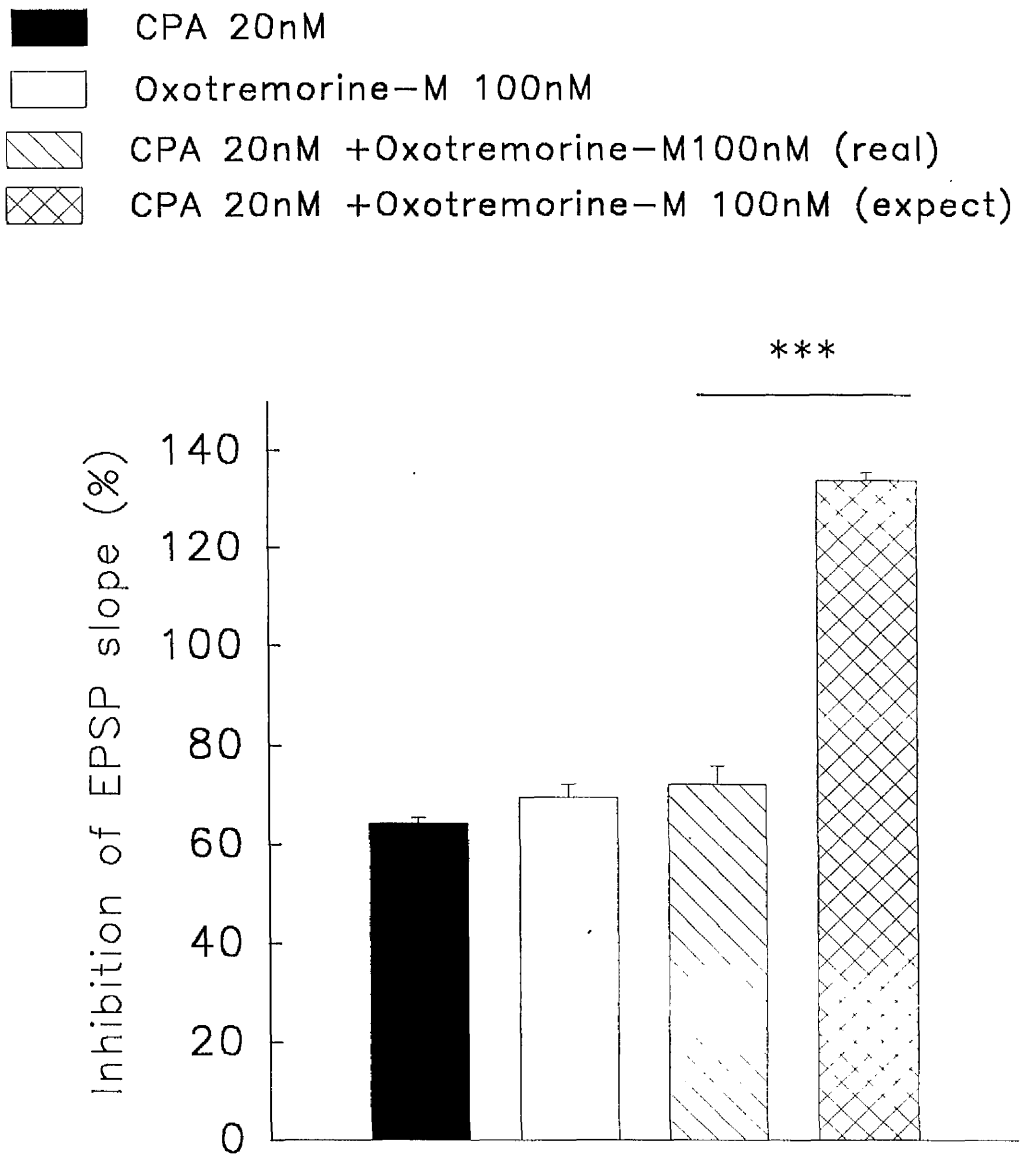

Figure 3. 41. Histogram showing the depression effect of CPA 20nM, oxotremorine–M 100nM and CPA + oxotremorine–M on evoked CA1 EPSPs. The final column indicates the expected effect if responses to the two agents had been additive. Each vertical bar shows mean ± S. E. M for n=4 slices. *** P<0.001 compared with the expected additive effect.

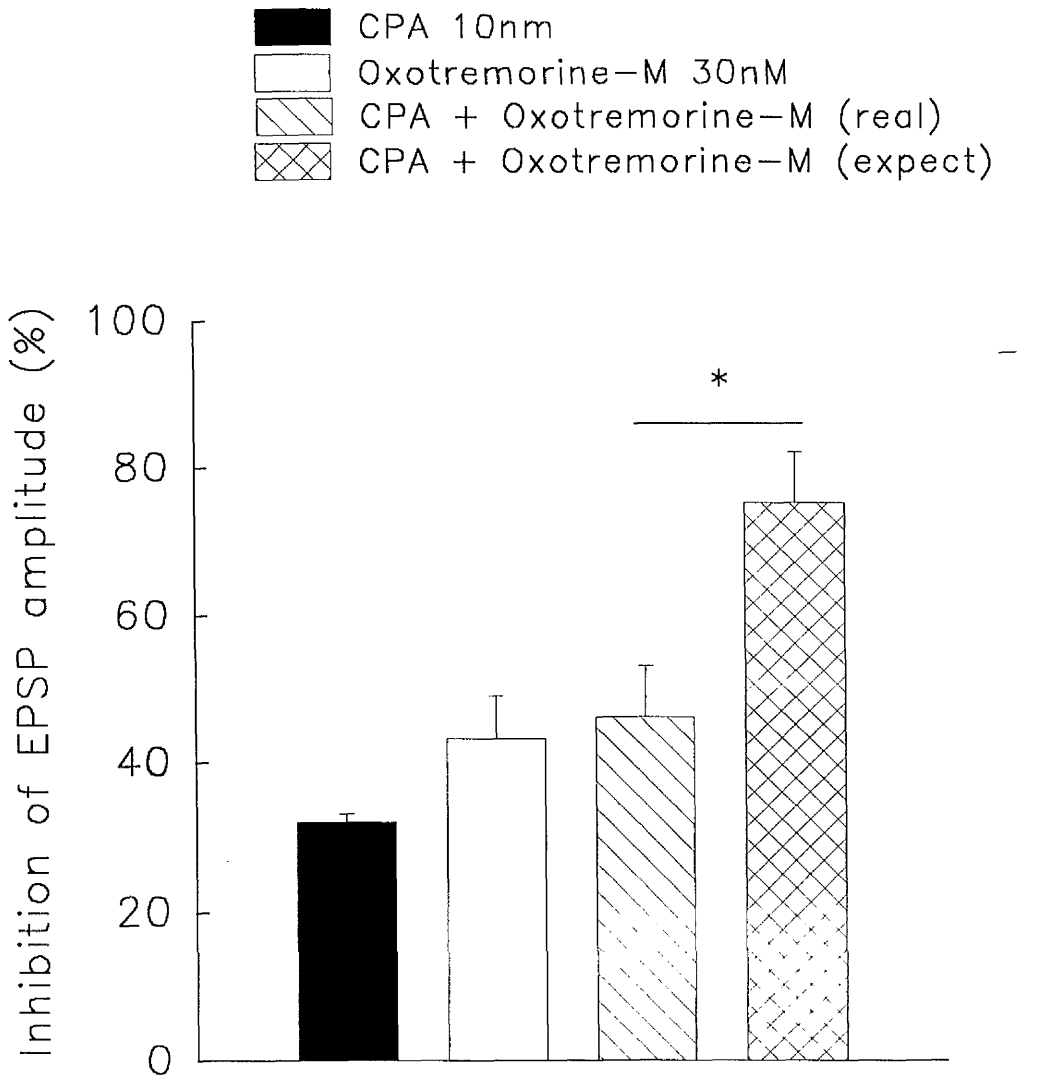

Figure 3. 42. The depression effect of CPA 10nM, oxotremorine–M 30nM and CPA + oxotremorine–M on evoked CA1 EPSP amplitude. The last column indicates the expected effect if responses to the two agents had been additive. Each vertical bar shows mean ± S. E. M for n=3 slices. * $P<0.05$ compared with the expected additive effect.

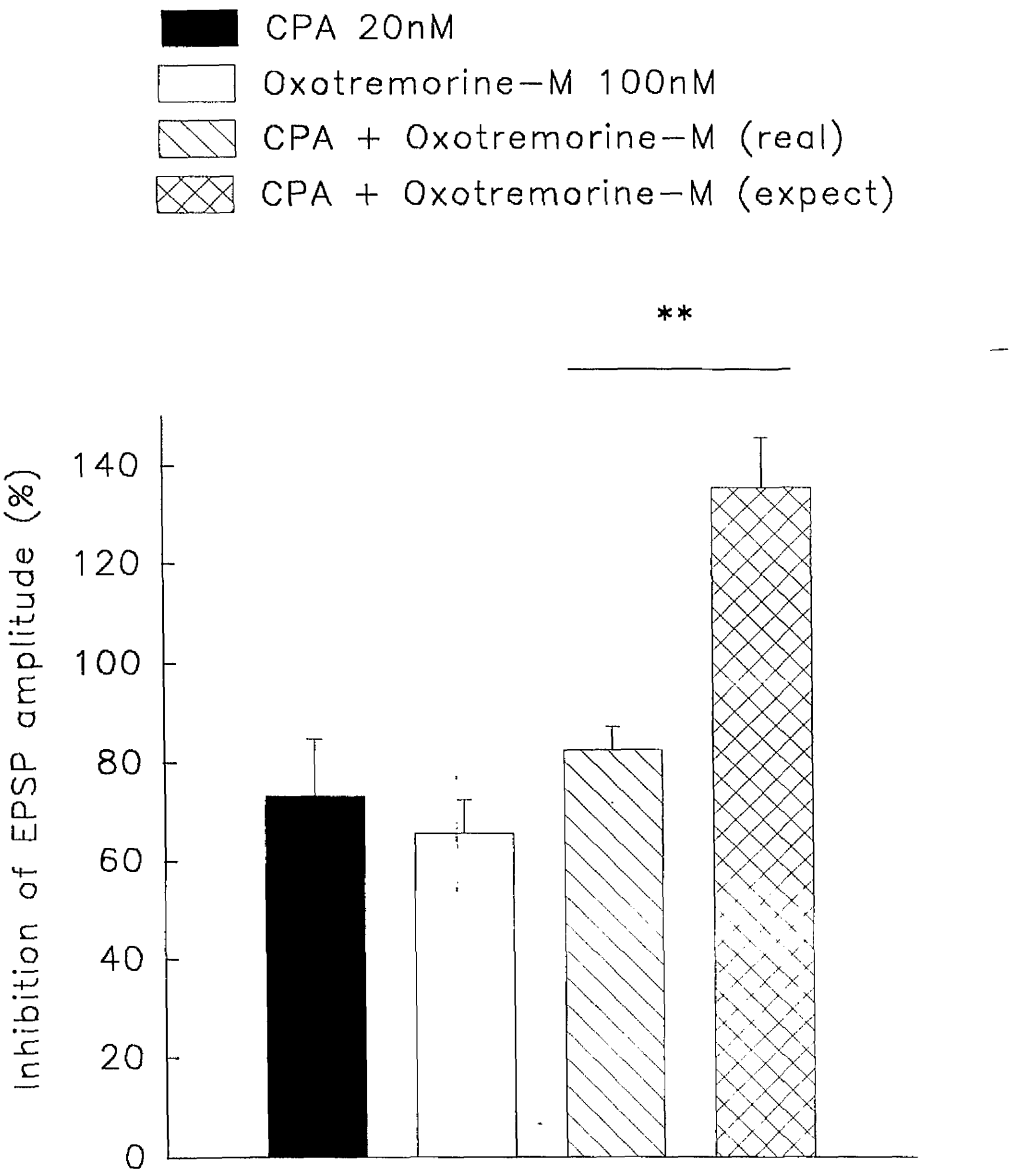

Figure 3. 43. The depression effect of CPA 20nM, oxotremorine–M 100nM and CPA + oxotremorine–M on evoked CA1 EPSP amplitude. The last column indicates the expected effect if responses to the two agents had been additive. Each vertical bar shows mean ± S. E. M for n=3 slices. ** $P<0.01$ compared with the expected additive effect.

The effect of CPA on paired-pulse population spikes

To investigate the effect of an adenosine A_1 selective agonist on paired pulse EPSPs and population spikes a set of experiments was done with cyclopentyladenosine (CPA). Before adding drugs all slices showed a small paired pulse inhibition at 10 ms interstimulus interval. However no paired-pulse inhibition or facilitation was observed at 20 and 50ms in control responses. When CPA (20nM) was superfused extremely significant paired pulse facilitation was observed at all examined intervals (Fig 3. 44). Wash out of CPA took 20min to obtain a stable recovery.

The effect of CPA on paired-pulse EPSPs

The effect of CPA on paired-pulse EPSPs recorded from the dendrite layer of CA1 hippocampal slices was also examined in similar experiments. Control responses showed a small inhibition at 10ms and significant facilitation at 20 and 50 ms. Perfusion of CPA 20nM induced an apparent conversion from paired-pulse inhibition to facilitation at 10ms and a significant increase of the magnitude of facilitation at 20 and 50ms (Fig. 3. 45).

The effect of CGS 21680 on paired-pulse population spikes

The effect of an A_2 selective agonist, CGS 21680, was examined on paired pulse interaction in five slices of hippocampus. Under control conditions all of the slices showed facilitation at interstimulus intervals at 20 ms and 50ms. However at 10ms 2 slices showed facilitation while 3 showed paired pulse inhibition. When CGS21680 30nM was superfused, the secondary pulse was significantly depressed at interstimulus interval of 10 ms but no significant change was observed at interstimulus intervals of 20 and 50ms (Fig. 3. 46).

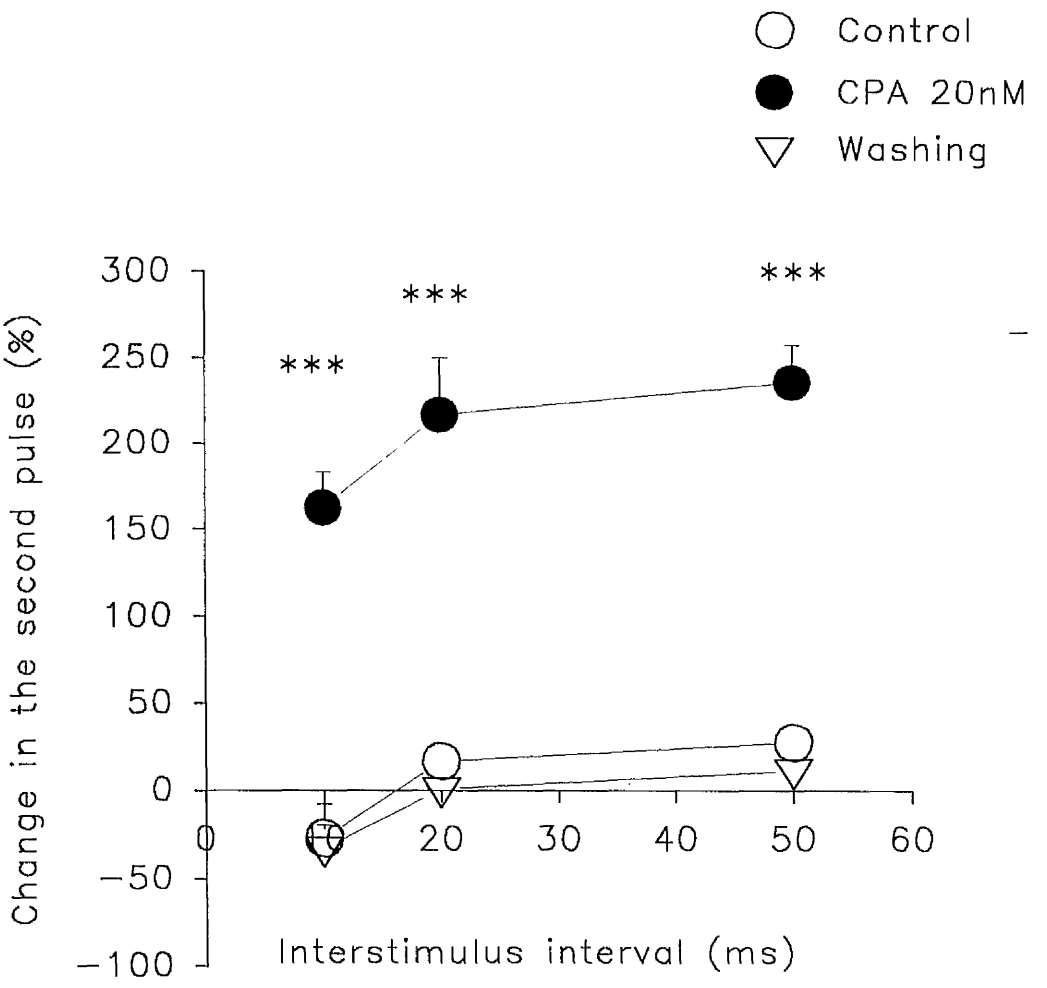

Figure 3. 44. The effect of CPA 20nM on paired−pulse population spikes evoked from the CA1 area of hippocampal slices at different interpulse intervals (10, 20 and 50ms). All stimuli were orthodromic. Each point shows mean ± S. E. M for n=3 slices. *** P<0.001

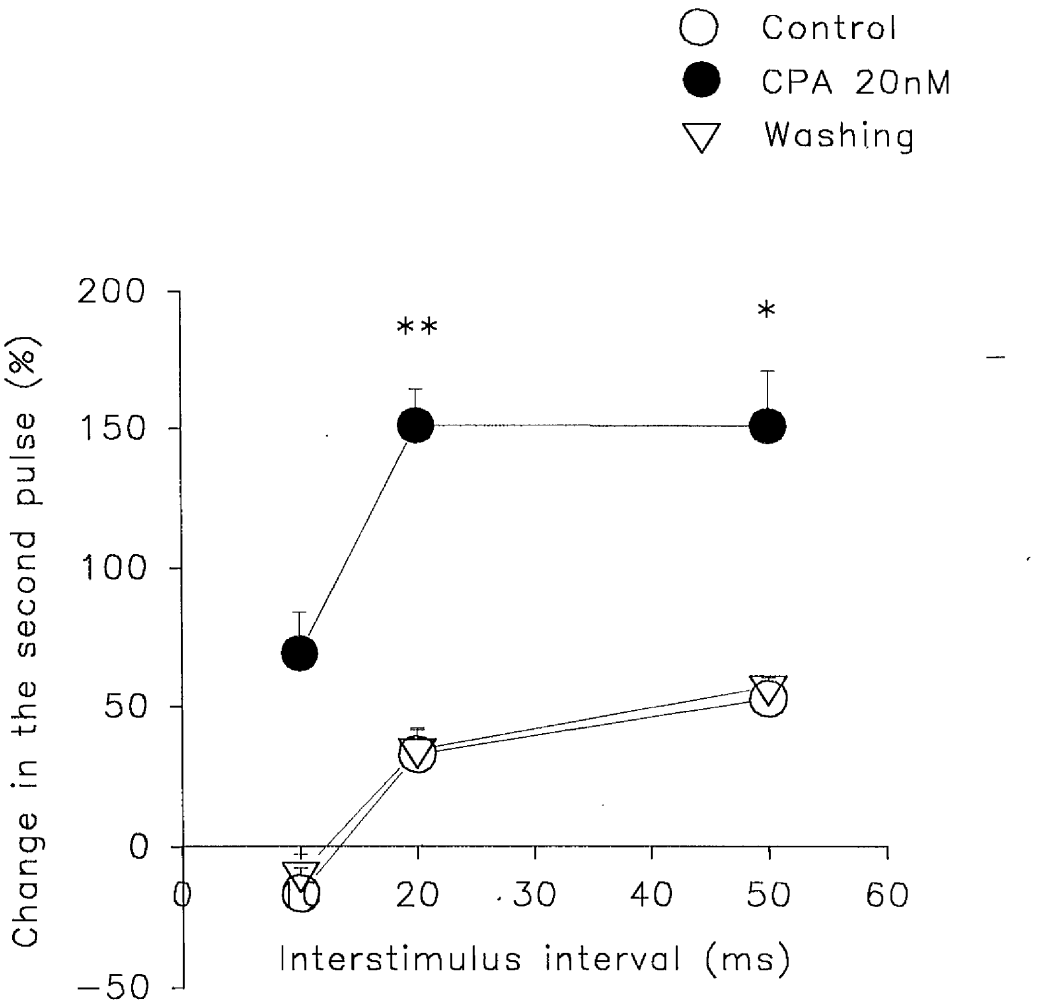

Figure 3. 45. The effect of CPA 20nM on paired−pulse EPSPs evoked from the CA1 area of hippocampal slices in normal ACSF. The interstimulus intervals of 10, 20 and 50ms were tested. Each point shows mean ± S. E. M for n=4 slices. * P,0.05, ** P<0.01

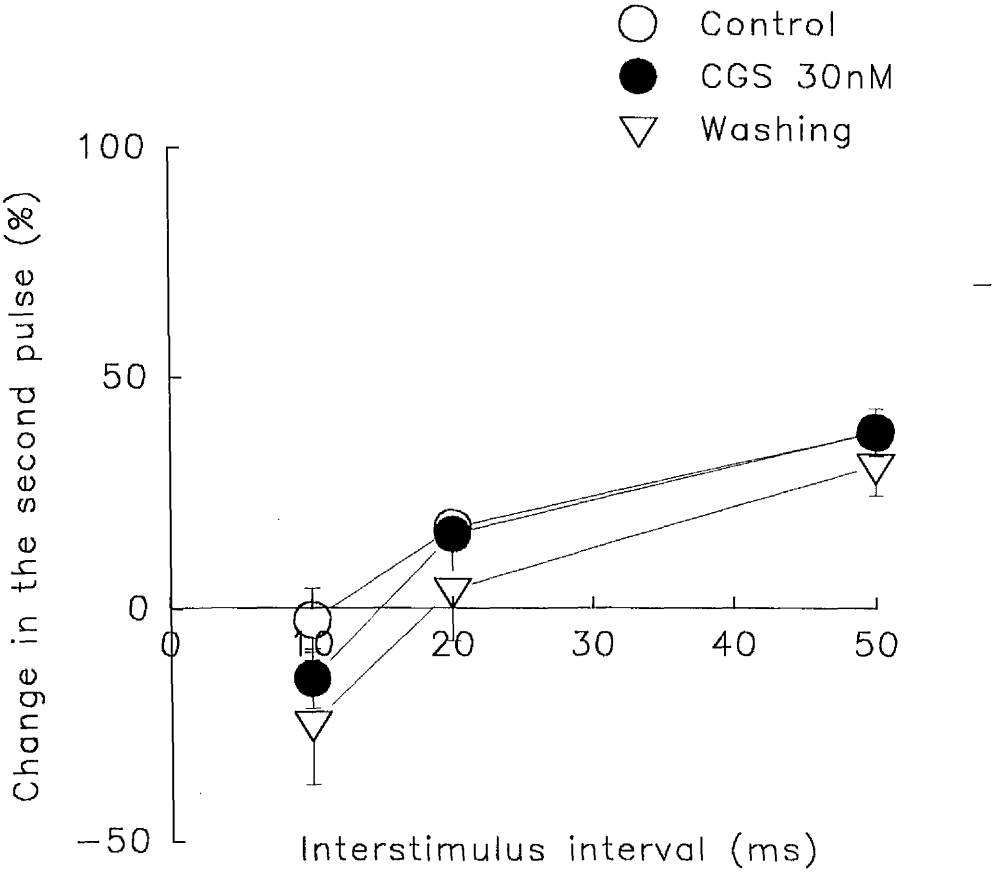

Figure 3. 46. The effect of CGS 21680 on paired−pulse population spikes evoked from hippocampal slices at interpuls intervals of 10, 20 and 50ms. Each vertical bar shows mean ± S. E. M for n=6 experiments.

Effect of oxotremorine-M on paired pulse population spikes

Some experiments were done in order to see the effect of oxotremorine-M on paired pulse population spikes in the hippocampal slices. Before perfusion of drug all of the slices showed significant inhibition at an interstimulus interval of 10 ms and a small facilitation at 20ms and 50ms. When the oxotremorine-M (100nM) was used, obvious reduction in inhibition at 10ms and significant facilitation at 20 ms and 50 ms was seen (Fig. 3. 47), indicating the presence and activation of muscarinic m_2 receptors on the presynaptic sites in hippocampus.

Effect of oxotremorine-M on paired-pulse EPSPs

The modulation of the paired-pulse paradigm by oxotremorine-M was further supported when oxotremorine-M 100nM changed the paired-pulse ratio compared with control. In this series of experiments control potentials showed a small facilitation in all tested interstimulus intervals. Application of oxotremorine-M 100nM induced a significant facilitation at all intervals (Fig. 3. 48).

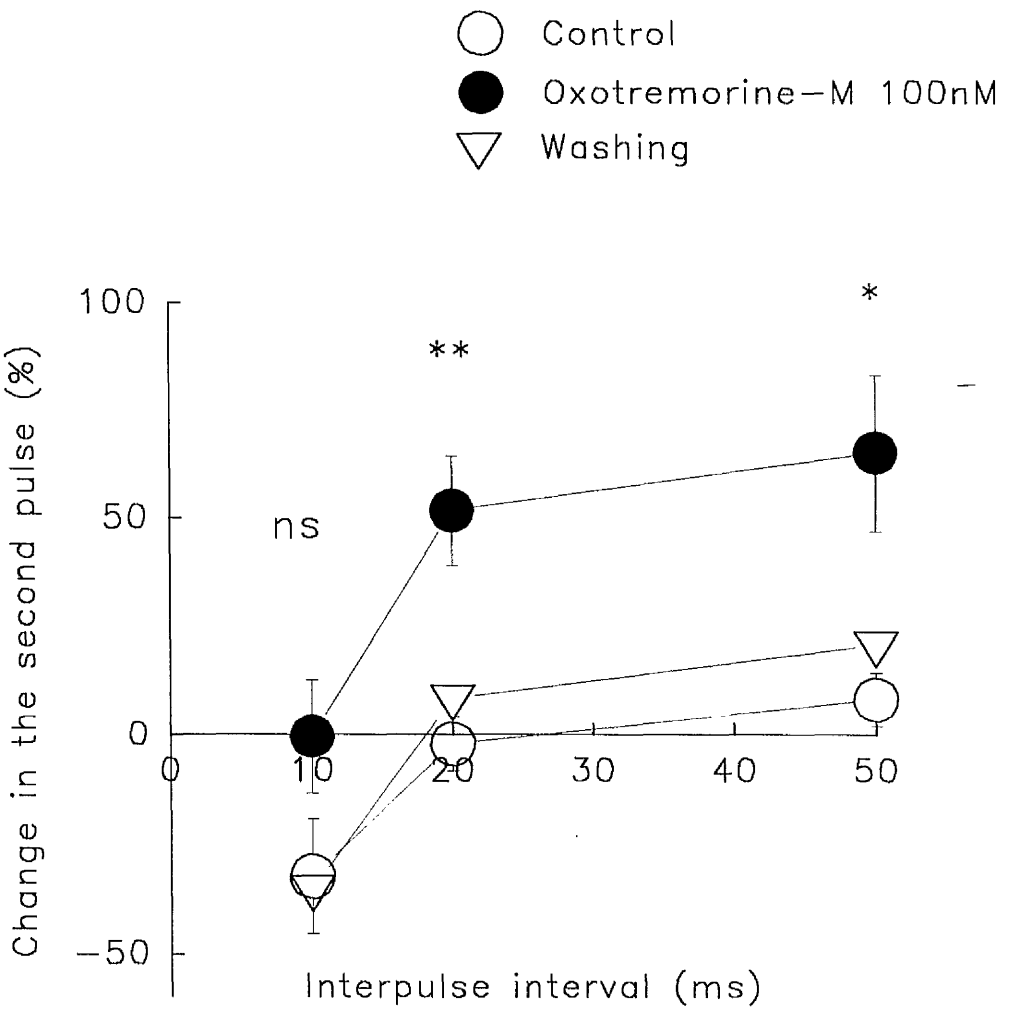

Figure 3. 47. The effect of oxotremorine−M 100nM on paired−pulse population spikes evoked from hippocampal slices. Interpulse intervals of 10, 20 and 50ms were tested. Points are shown as mean ± S. E. M for n=5 slices.
* P<0.05, ** P<0.01

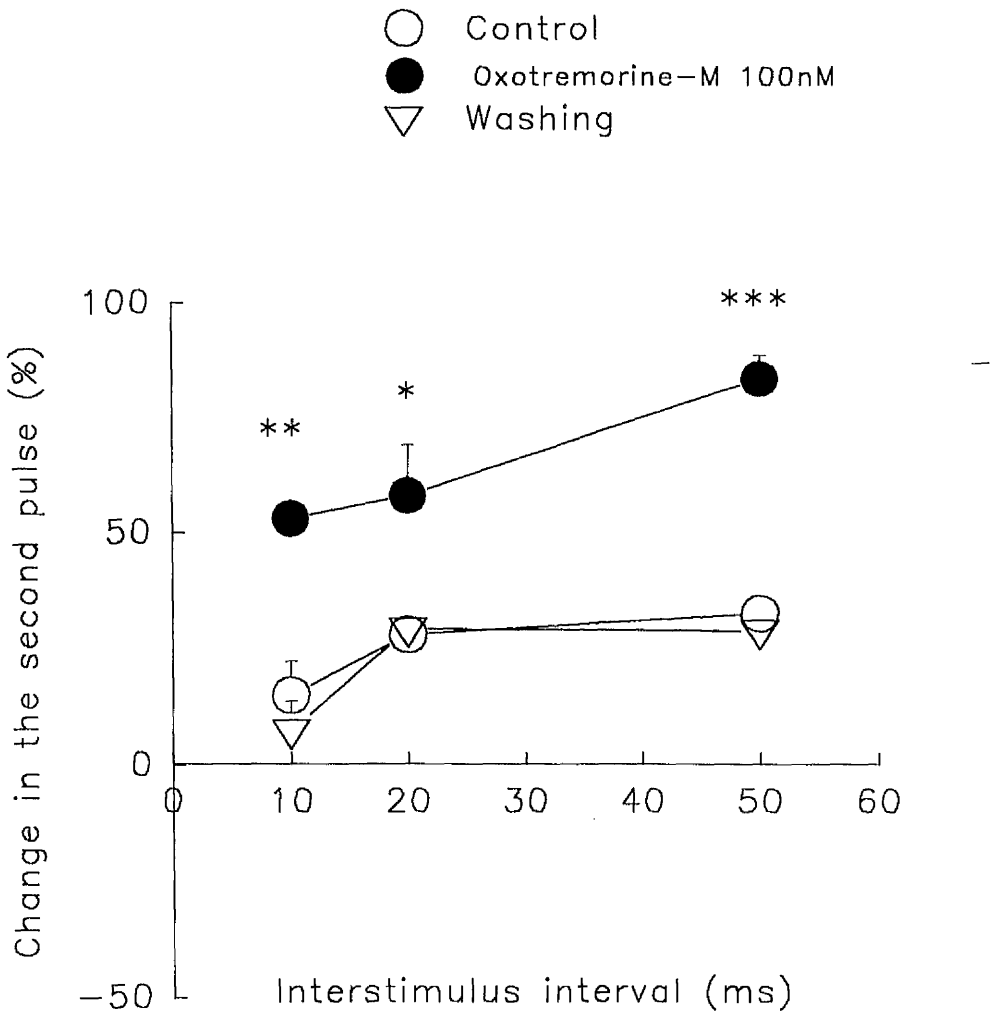

Figure 3. 48. The effect of oxotremorine–M 100nM on paired–pulse EPSPs evoked from CA1 in hippocampal slices at different interpulse intervals. Each point shows mean ± S. E. M for n=4 slices. * P<0.05, ** P<0.01, ***, P<0.001

The effect of high potassium and 4-aminopyridine (4AP) on responses to CPA and oxotremorine-M

In the present studies it was shown that the combined effect of CPA and oxotremorine-M on EPSPs was significantly less than the calculated additive responses. In order to determine whether presynaptic K conductances represent a common target of adenosine receptor agonists responsible for the occlusive behaviour the effect of high potassium and 4AP on responses to CPA and oxotremorine-M was examined.

The effect of elevated potassium on paired-pulse responses to CPA

As illustrated before CPA 20nM reversed the short latency inhibition into facilitation and increased the degree of facilitation at 20 and 50ms in both population spike and EPSP potentials (Figs. 3. 44 and 3. 45). In a new line of experiments the effect of CPA 20 nM on paired-pulse responses was re-examined when the level of extracellular KCl was raised to 4.4mM. In elevated potassium solution the effect of CPA on paired-pulse population spikes was greatly reduced (Fig. 3. 49). A small facilitation of population spikes remained but paired-pulse changes of the EPSPs were completely prevented (Fig. 3. 50).

The effect of high potassium on paired-pulse responses to oxotremorine-M

Similar experiments were performed to test the effect of raising potassium on paired-pulse responses to oxotremorine-M. In normal ACSF superfusion of oxotremorine-M induced an obvious reduction in paired-pulse inhibition and increased paired-pulse facilitation of fEPSP and population spikes (Figs. 3. 46 & 3. 47). In high K^+ solution these effects of oxotremorine-M were completely prevented on population spikes (Fig. 3. 51) and on EPSPs (Fig. 3. 52).

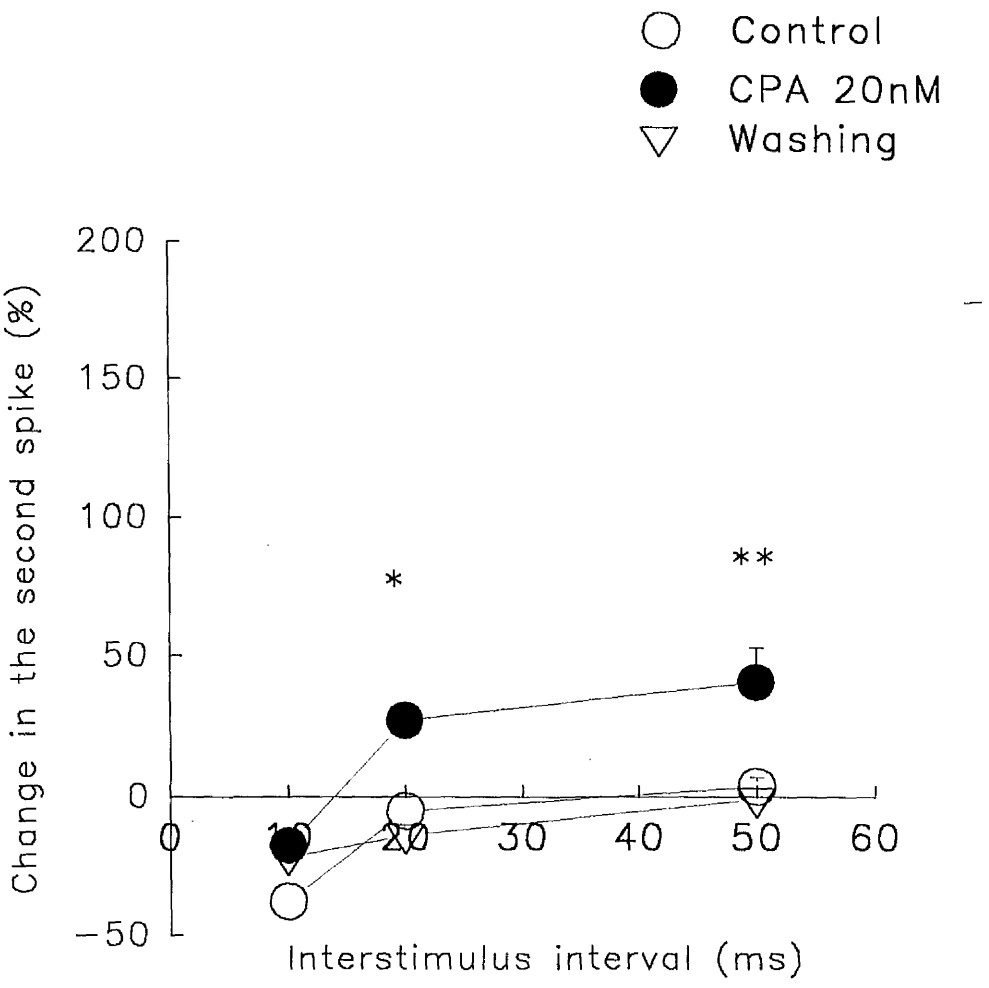

Figure 3. 49. The effect of CPA 20nM on paired−pulse population spikes evoked from CA1 hippocampal slices in high potassium ACSF. The interstimulus intervals at 10, 20 and 50ms were tested. Each point shows mean ± S. E. M for n=3 slices. * P<0.05, ** P<0.01

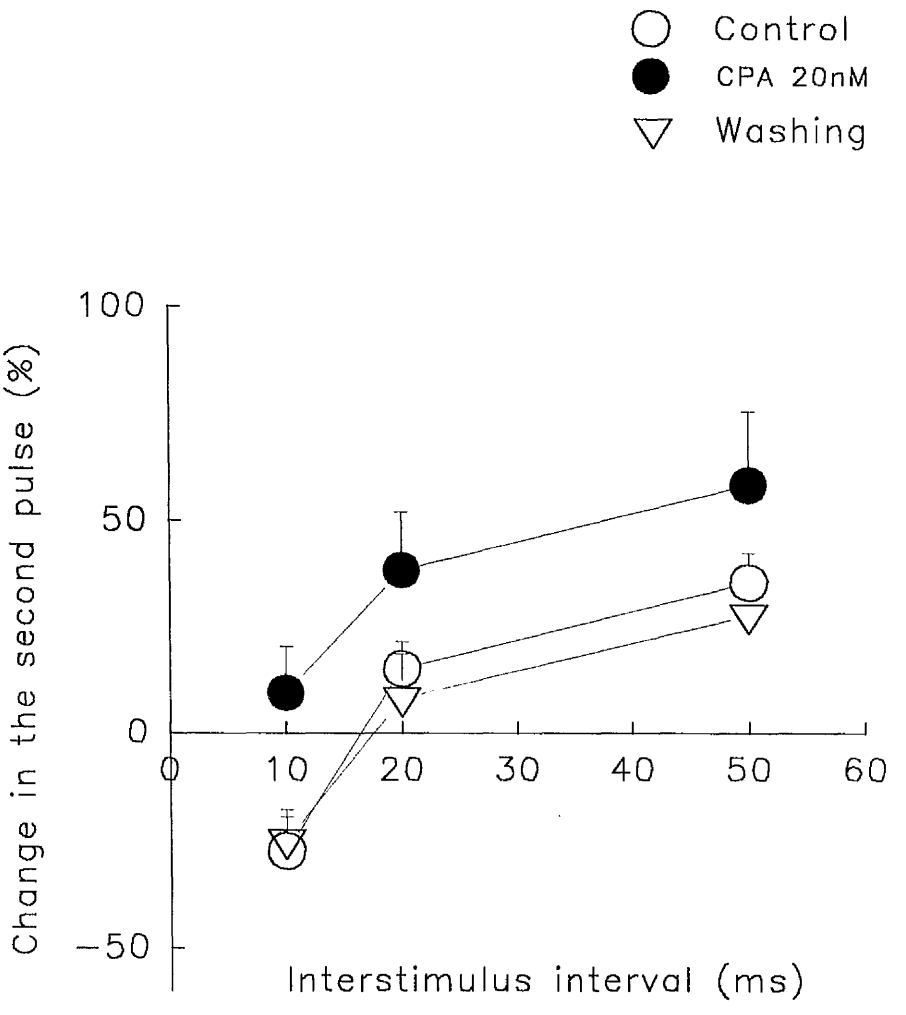

Figure 3. 50. The effect of CPA 20nM on orthodromic evoked CA1 paired-pulse EPSPs in high potassium ACSF. The interpulse intervals of 10, 20 and 50ms were tested. The stimulus strength was submaximal (70%). Each vertical bar shows mean + S.E.M for n=4 slices. There is no significant difference between control and CPA.

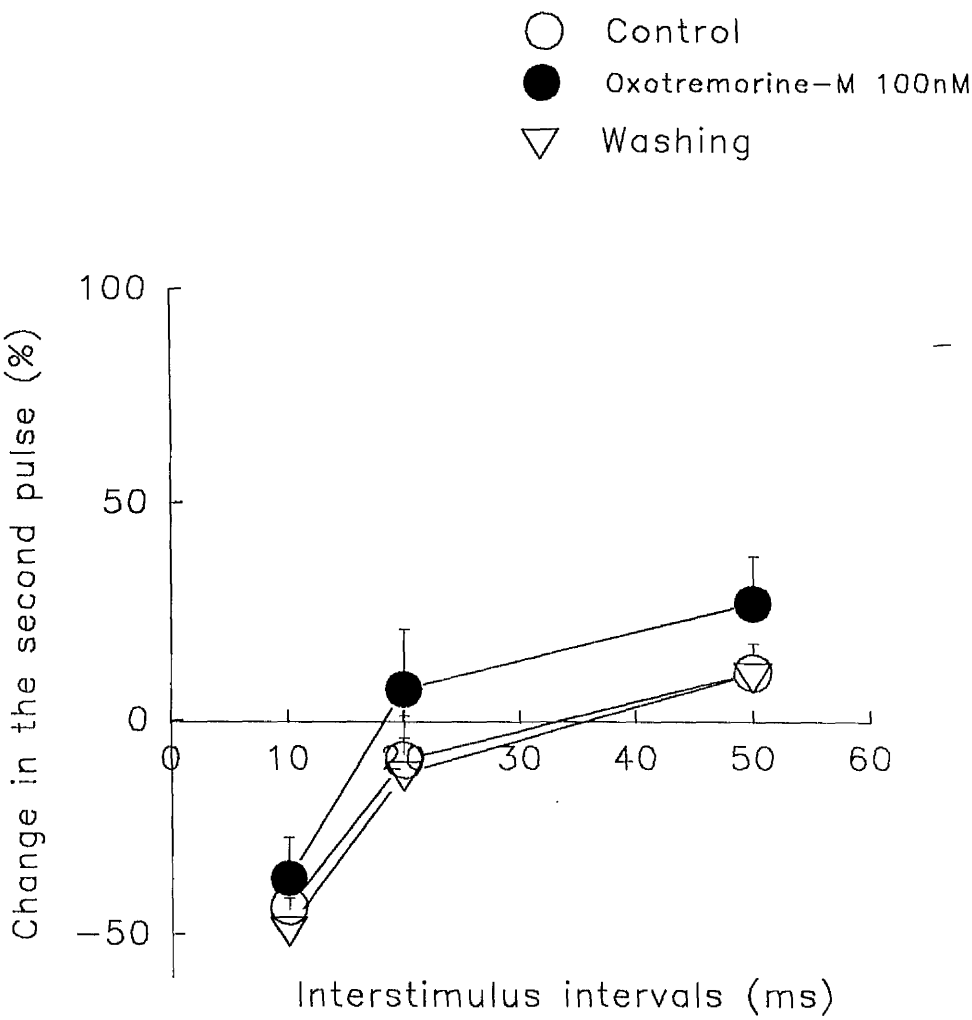

Figure 3. 51. The effect of high potassium ACSF on paired-pulse changes of the population spikes by oxotremorine-M 100nM in hippocampal slices. Both conditioning and test stimuli were orthodromic and stimulus strength was 70% of maximum. Interpulse intervals of 10, 20 and 50ms were tested. Points are shown as mean ± S. E. M for n=7 slices. There is no significant difference between control and oxotremorine-M.

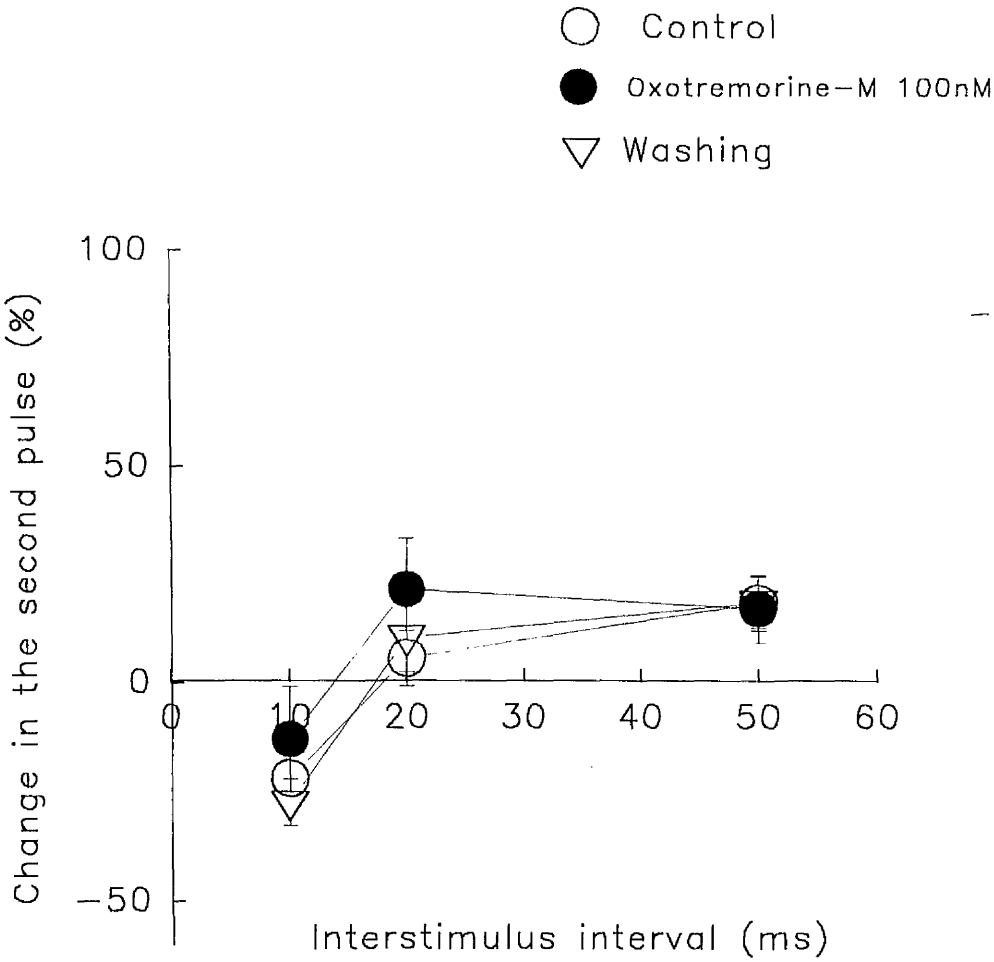

Figure 3. 52. The inhibitory effect of high potassium ACSF on paired-pulse changes of the EPSPs by oxotremorine-M 100nM at interstimulus intervals of 10, 20 and 50ms. All stimuli were orthodromic and evoked from CA1 in hippocampal slices. Each point shows mean ± S. E. M for n=4 slices.

The effect 4-aminopyridine on paired-pulse responses to CPA and oxotremorine-M

In order to confirm that the above modulation of responses to CPA and oxotremorine-M by elevated potassium was caused by changes of potassium conductances, the experiments were performed in the presence of 4-aminopyridine 50μM. 4-aminopyridine was present in the solution throughout the experiments. 4-aminopyridine alone increased the amount of inhibition at 10ms and decreased the facilitation at 20ms and 50ms. In the presence of 4-aminopyridine neither CPA nor oxotremorine-M were able to change the extent of paired-pulse inhibition or facilitation of population spikes (Figs.3. 53 & 3. 54).

The effect of 4-aminopyridine on single responses to CPA and oxotremorine-M

The effect of CPA 20nM and oxotremorine-M was also studied on single population spikes. The depressant effect of both agents on the population spike size was also prevented by adding 4-aminopyridine in the superfused solution (Figs. 3. 55 & 3. 56).

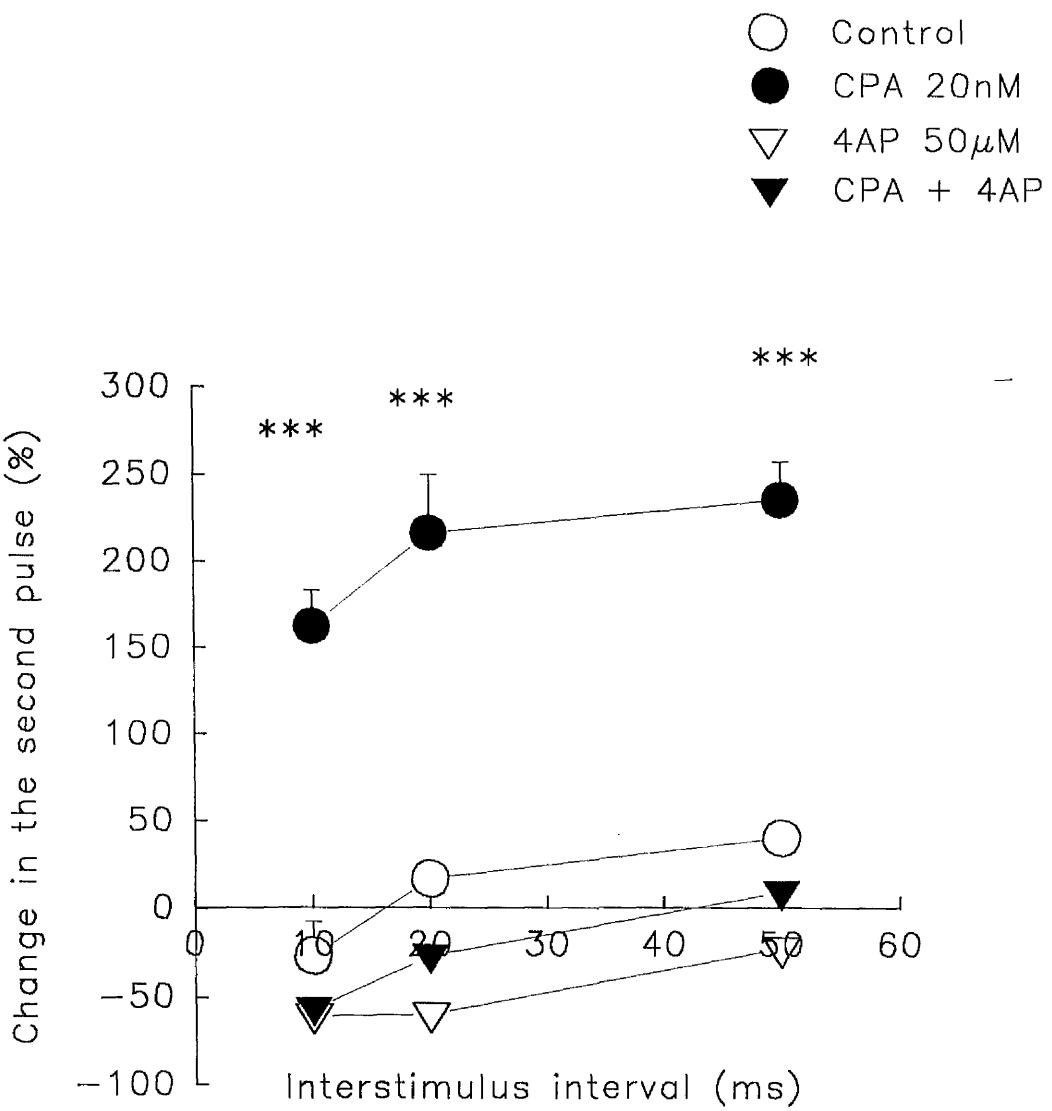

Figure 3. 53. The effect of CPA 20nM alone and in the presence of 4AP 50μM on paired−pulse population spikes evoked from CA1 hippocampal slices. The interpulse intervals ot 10, 20 and 50ms were tested. Each point shows mean ± S. E. M for n=3 slices. *** P<0.001 for difference between CPA and CPA + 4AP. There was no significant difference between CPA + 4AP and 4AP.

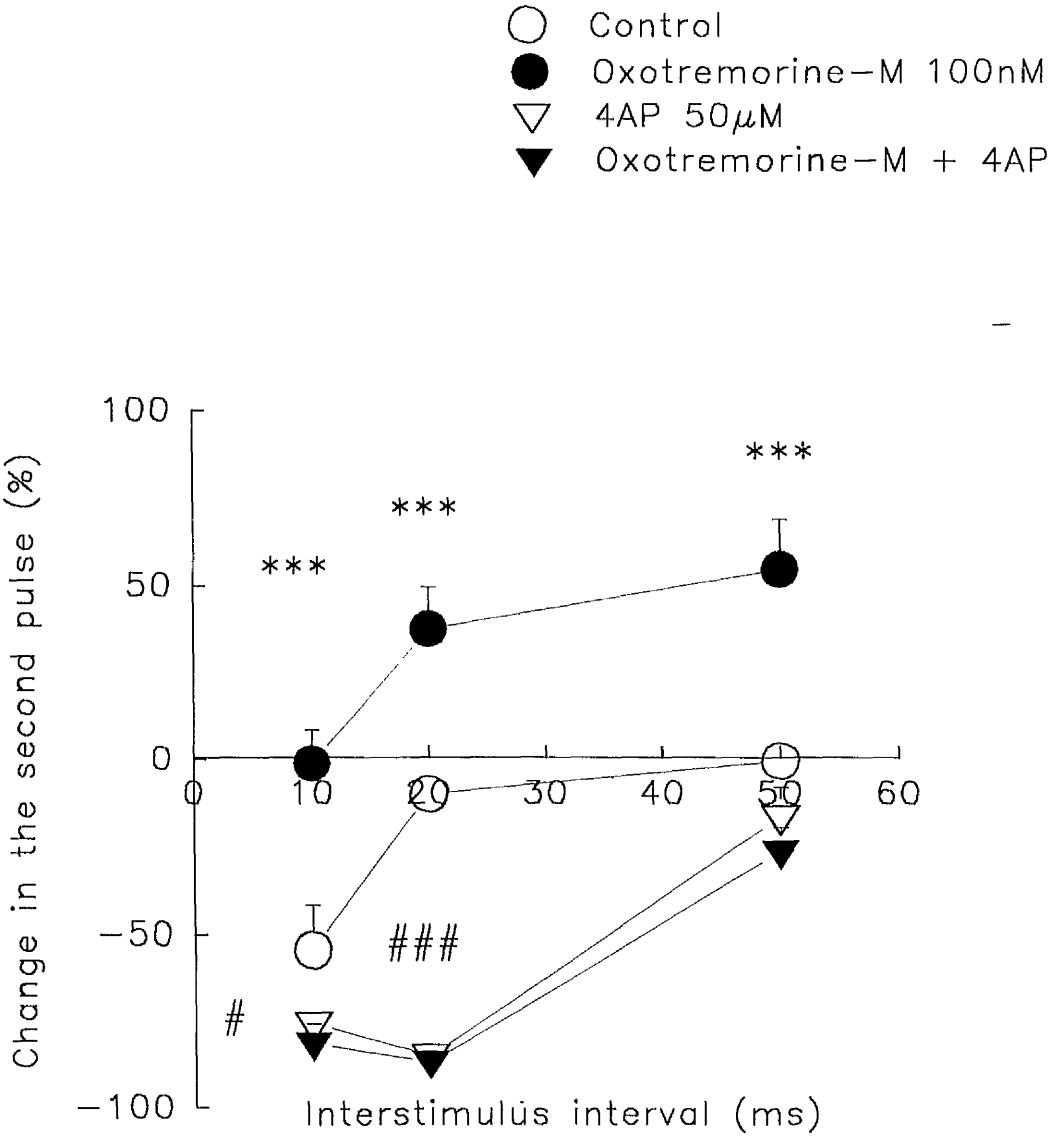

Figure 3. 54. The effect of oxotremorine−M 100nM, 4AP 50μM and oxotremorine−M + 4AP on orthodromic paired−pulse evoked EPSPs in hippocampal slices. Each point shows mean ± S. E. M for n=5 slices. *** P<0.001for difference between oxotremorineM and oxotremorine−M + 4AP. P<0.05, ### P<0.001 for difference between control and oxotremorine−M + 4AP. There was no difference between 4AP and oxotremorine−M + 4AP.

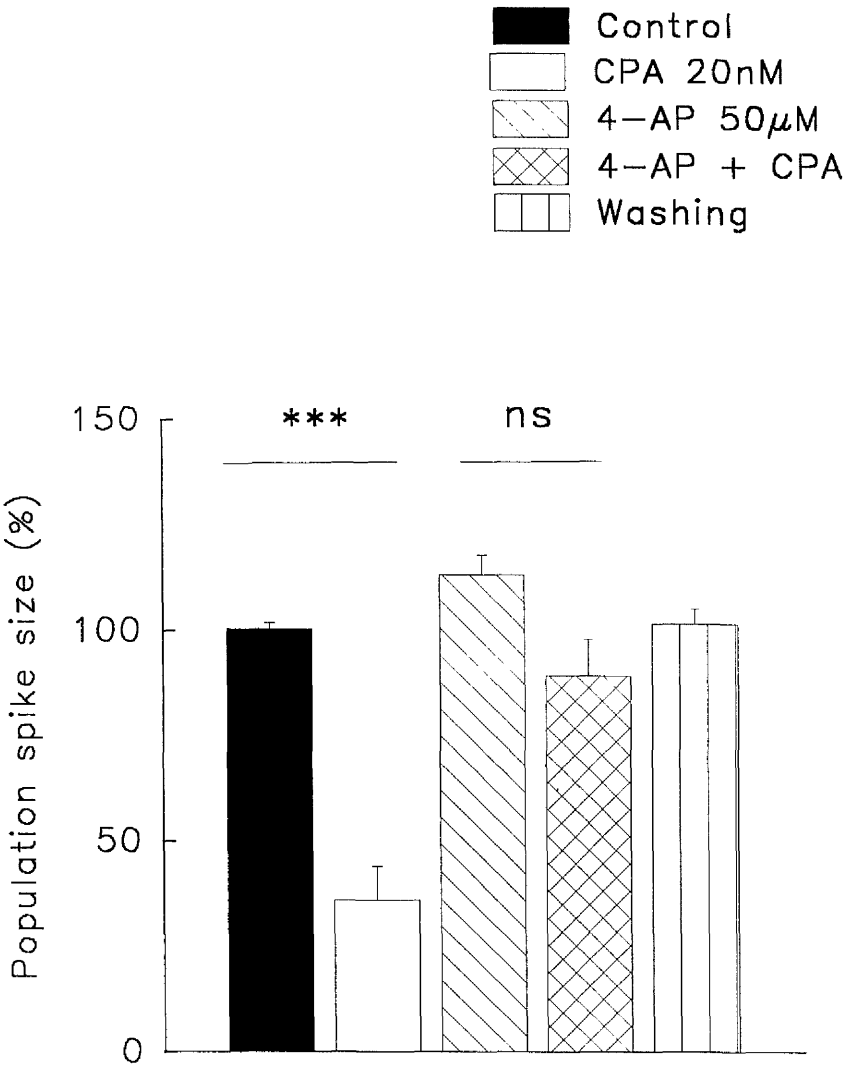

Figure 3. 55. Histogram showing the effect of CPA 20nM alone and in the presence of 4AP 50μM on the size of population spikes evoked in hippocampal slices. Each vertical bar shows mean ± S. E. M for n=5 experiments. *** P<0.001

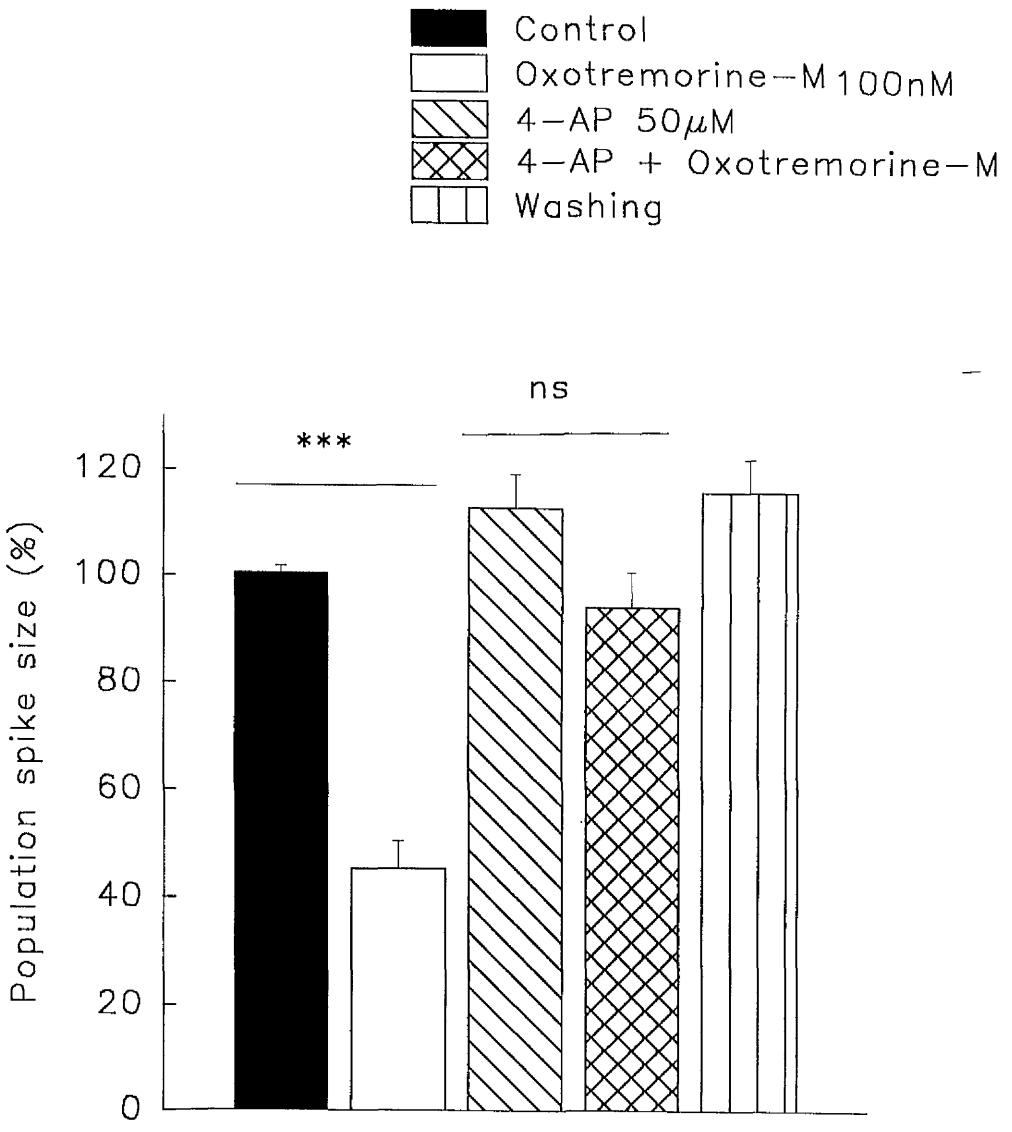

Figure 3. 56. Histogram showing the effect of oxotremorine–M 100nM alone and in the presence of 4AP on the size of single population spikes evoked in hippocampal slices. Each vertical bar shows mean ± S. E. M for n=4 slices. *** $P<0.001$, ns, $P>0.05$

The effect of NMDA receptor activation on the presynaptic responses to adenosine in hippocampus

The concentration-dependent effect of N-methyl-D-aspartate (NMDA) on population spikes

The effect of NMDA on orthodromic evoked population spikes was investigated using hippocampal slices. NMDA was perfused for 10 minutes, and the effect was concentration dependent. NMDA 4μM did not induce any change in the size of population spike potentials (Fig. 3. 57) whereas at 6μM it gave a small depression of population spike size (reducing by 20.51% ± 6.86 of control size) with a subsequent short lasting enhancement during the washing period (Fig. 3. 58). The depression effect of NMDA 10 μM was greater, but with no consistent increase in the size of the population spikes after 20 minutes of washing (Fig. 3. 59).

The effect of NMDA on paired-pulse population spikes

The effect of NMDA at 4 and 10 μM was also tested on the paired-pulse population spikes at interpulse intervals at 10, 20 and 50ms. NMDA 4μM did not modify the paired-pulse ratio at any of the tested intervals (Fig. 3. 60). When NMDA at 10 μM was applied, a small facilitation was seen at interval of 50ms. The effect of NMDA was reversible after washing (Fig. 3. 61).

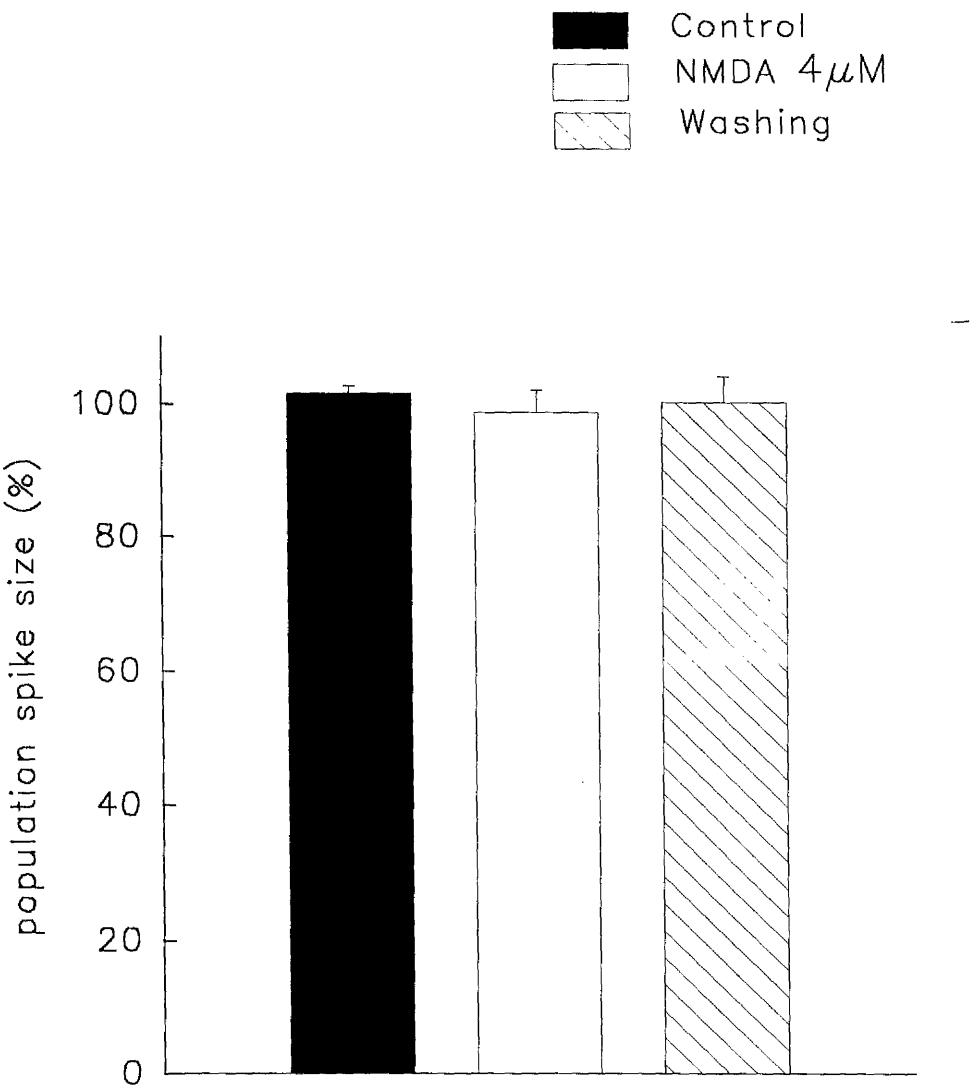

Figure 3. 57. Histogram showing the population spike size in the presence of NMDA 4μM. Orthodromic evoked population spikes were recorded from the CA1 region. Each vertical bar shows mean ± S. E. M for n=5 slices.

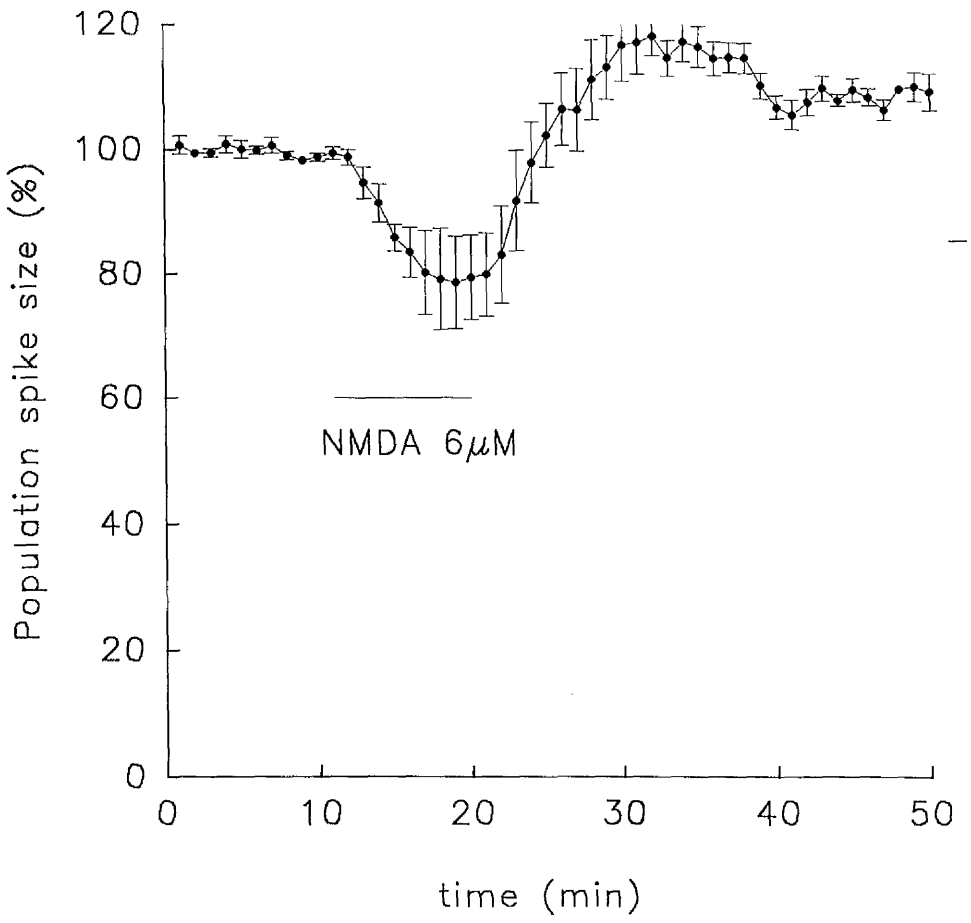

Figure 3. 58. Population spikes were recorded from CA1 in hippocampal slices. Application of NMDA 6μM induced short-term potentiation. Each point shows mean ± S. E. M for n=4 slices.

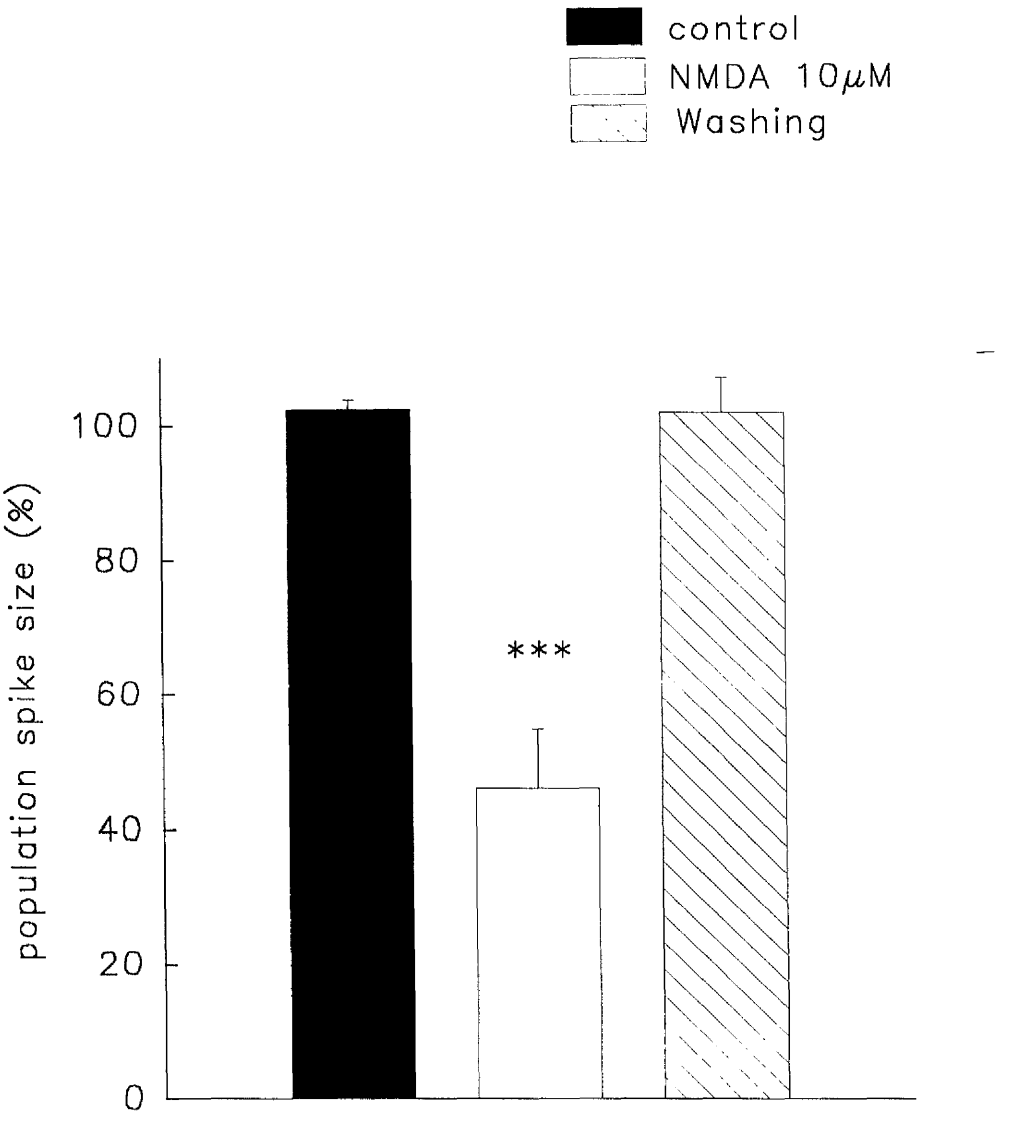

Figure 3. 59, Histogram showing the depression effect of NMDA 10μM on orthodromic evoked population spikes. Each vertical bar shows mean ± S. E. M for n=4 slices. *** P<0.001

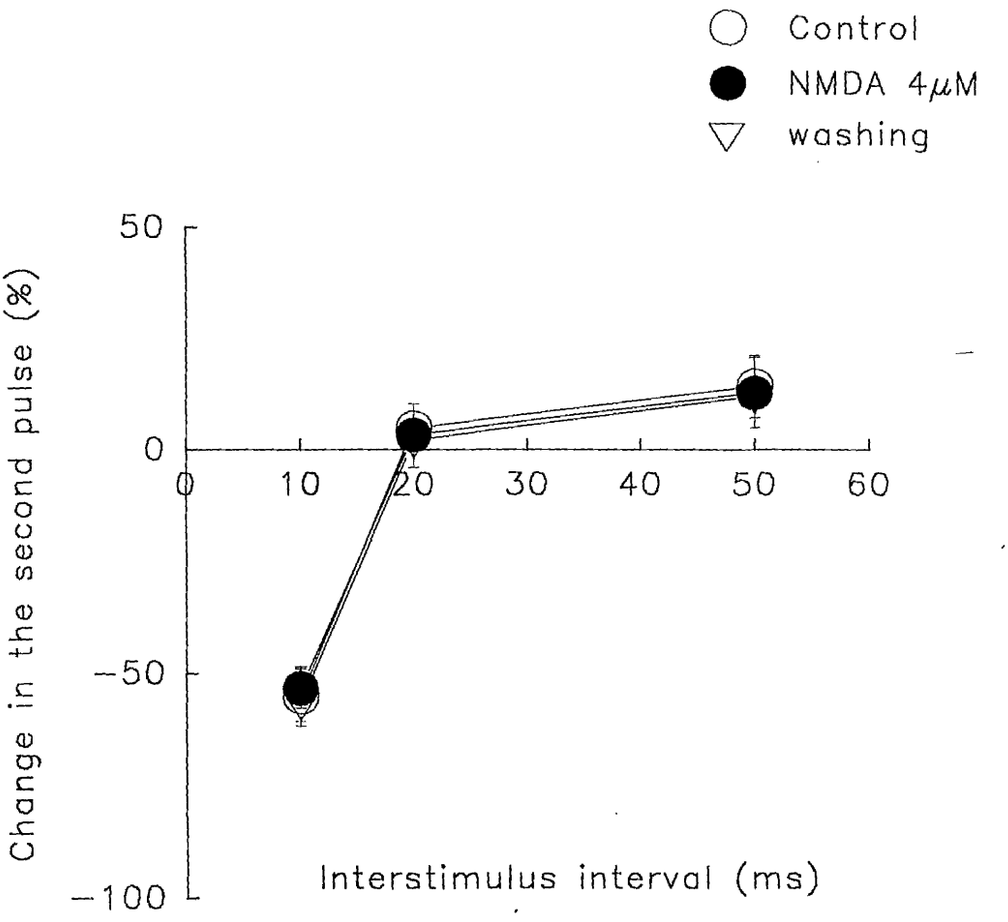

Figure 3. 60. Orthodromic paired−pulse population spikes were recorded from CA1. NMDA 4μM did not induce any significant change in the paired−pulse ratio. The interpulse intervals at 10, 20 and 50ms were tested. Each point shows mean ± S. E. M for n=5 slices.

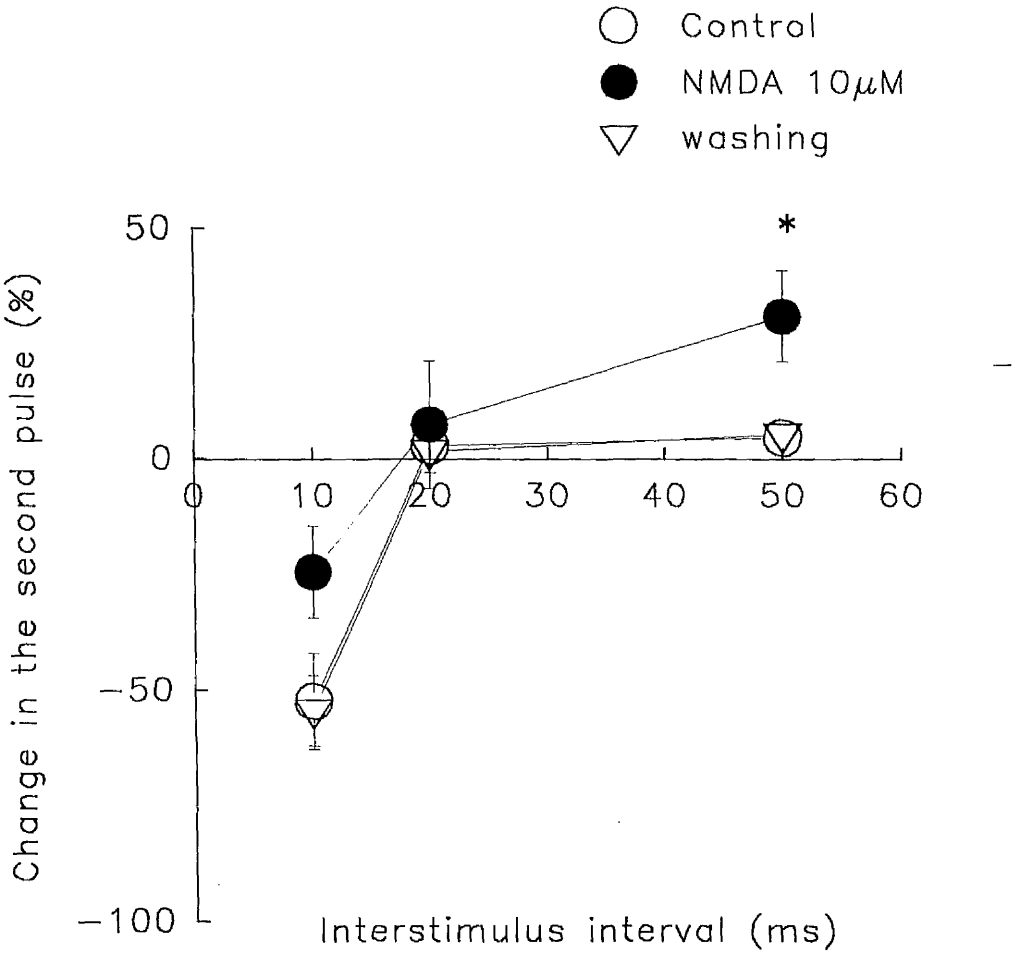

Figure 3. 61. The effect of NMDA 10μM on paired-pulse population spikes evoked from CA1 at interstimulus intervals at 10, 20 and 50ms. Each point shows mean ± S. E. M for n=4 slices. * $P<0.05$.

The combination effect of adenosine and NMDA on single and paired-pulse population spikes

Following a report that application of NMDA induced inactivation of adenosine sensitivity, expriments were performed using a combination of adenosine and NMDA. The paired-pulse method was also used to define the site of this interaction as presynaptic or postsynaptic. Adenosine originally depressed the single population spikes size by 69.3% ± 4.69 of the control size (Fig.3. 62), but when a subtreshold concentration of NMDA (4μM), which had no effect on its own on the population spike was superfused for 10 minute before and during reapplication of adenosine, the inhibitory responses to adenosine were significantly reduced to the level 44. 93 % ± 4. 68 of the control size (Fig. 3. 62).

The paired-pulse responses to the combination of adenosine and NMDA were determined as well. The interstimulus intervals of 10, 20 and 50ms were tested. NMDA at 4μM did not modify the paired pulse interaction. Superfusion of adenosine 10μM alone converted the inhibition to facilitation and produced a marked increase in facilitation at 20 and 50ms. When NMDA at 4μM was added, the effect of adenosine was reduced towards the control level (Fig. 3. 63).

The effect of combination of NMDA and adenosine on single and paired-pulse responses when NMDA receptors are blocked

To block the NMDA receptors, 2AP5 50μM was used and under this, the experiments with combination of NMDA and adenosine have been repeated. The inclusion of 2AP5 50μM prevented the suppression of adenosine responses by NMDA and restored the adenosine effect on both single and paired-pulse responses to the control levels (Figs. 3.64 & 3.65).

The combination effect of adenosine and NMDA on single and paired-pulse EPSPs

In order to confirm the presynaptic site of the interaction between adenosine and NMDA, the effect of adenosine was examined on the slope of EPSPs when a subthreshold concentration of NMDA was present. In a similar manner but to a lesser extent than the population spikes modulation, NMDA suppressed the responses to adenosine and prevented some of the effect of adenosine on single and paired-pulse EPSPs evoked from stratum radiatum (Figs. 3. 66A, 3. 66B & 3. 67).

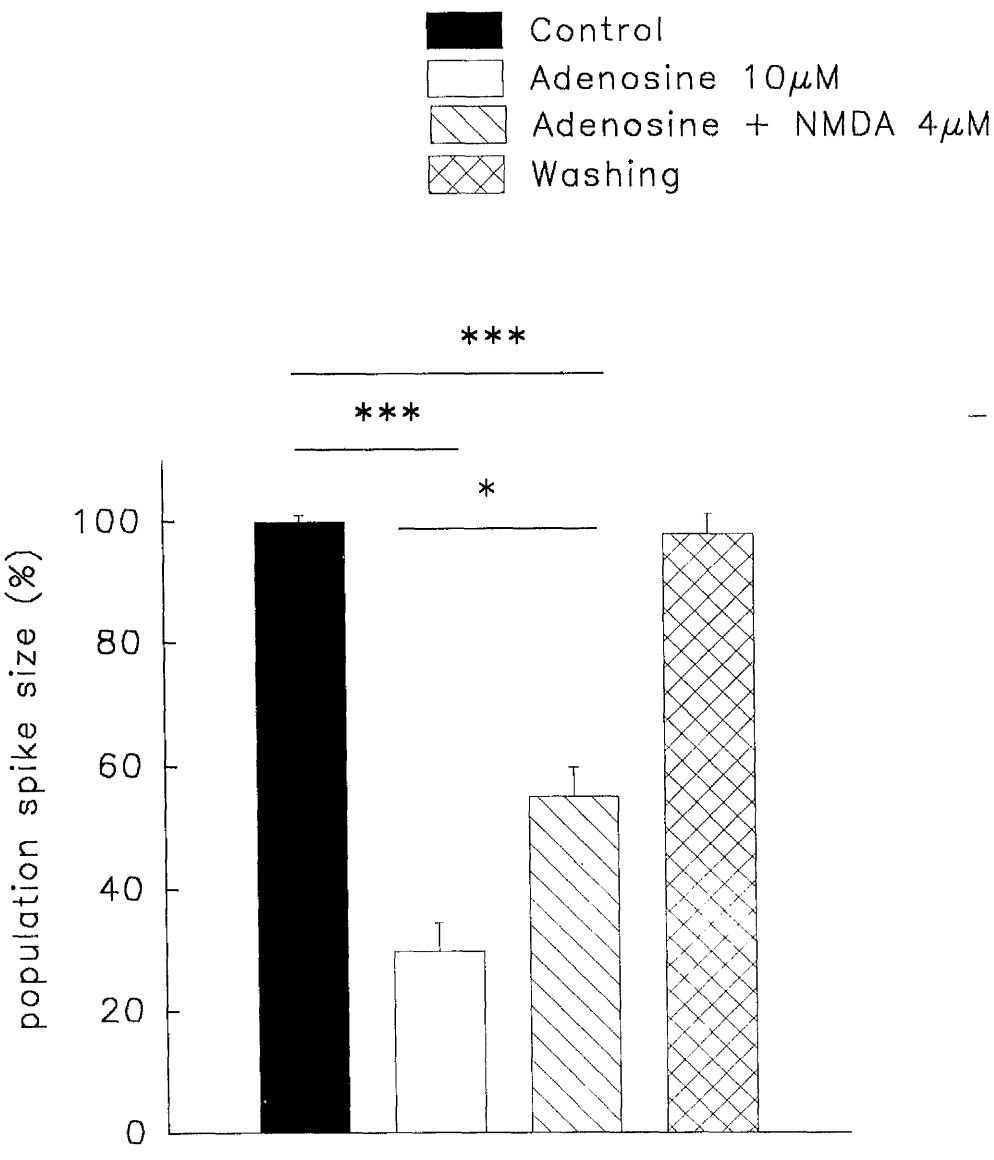

Figure 3. 62. Histogram showing the effect of adenosine 10μM alone and in the presence of NMDA 4μM on evoked CA1 population spikes. Each vertical bar shows mean ± S. E. M for n=4 slices. * $P<0.05$, *** $P<0.001$.

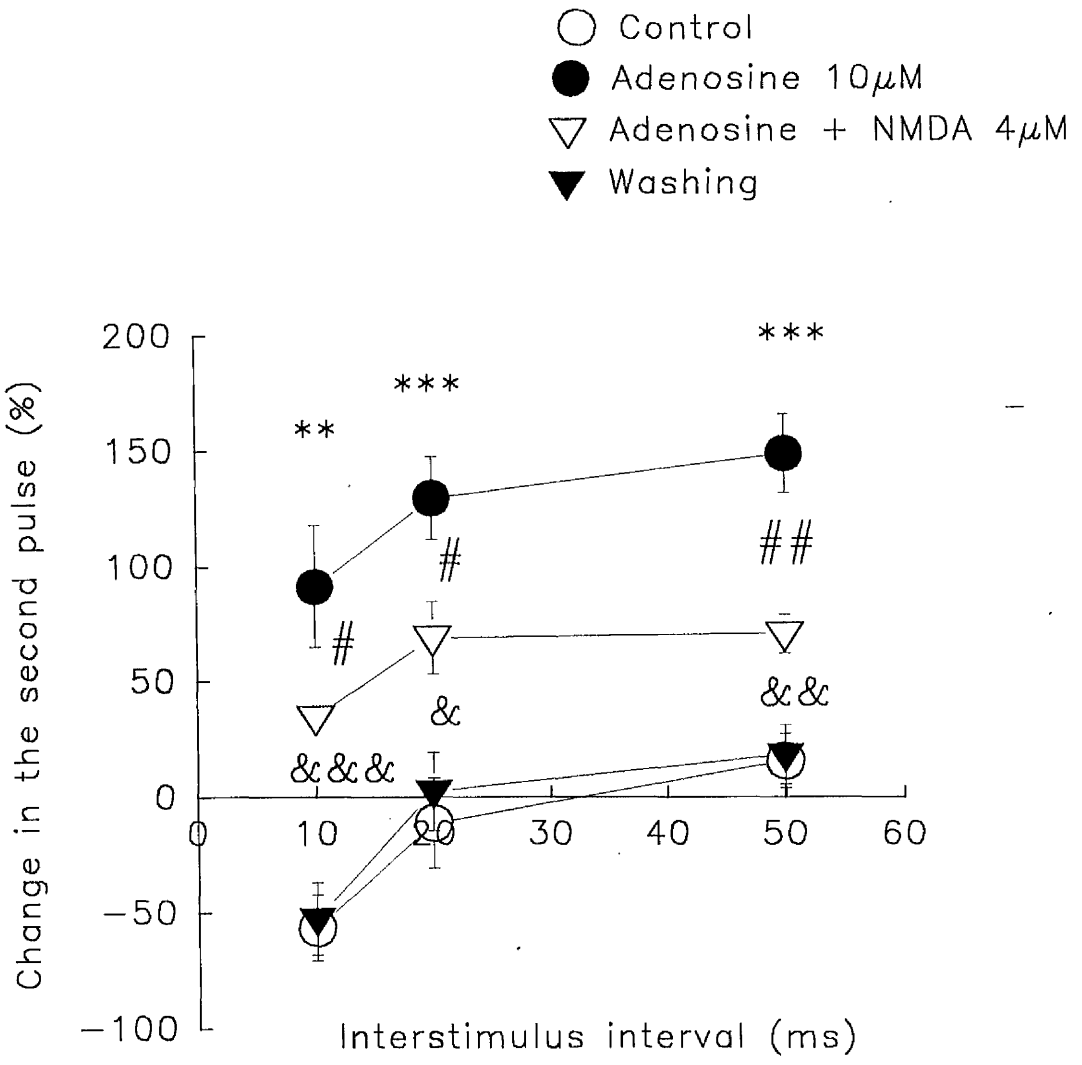

Figure 3. 63. The effect of adenosine 10μM alone and in presence of NMDA 4μM on paired−pulse population spikes evoked from CA1 at interstimulus intervals of 10, 20 and 50ms. Points are shown as mean ± S. E. M for n=5 slices. ** P<0.01, *** P<0.001 for difference between control and adenosine 10μM. & P<0.05, && P<0.01, &&& P<0.001 for difference between control and adenosine + NMDA.
# P<0.05, ## P<0.01 for difference between adenosine and adenosine + NMDA.

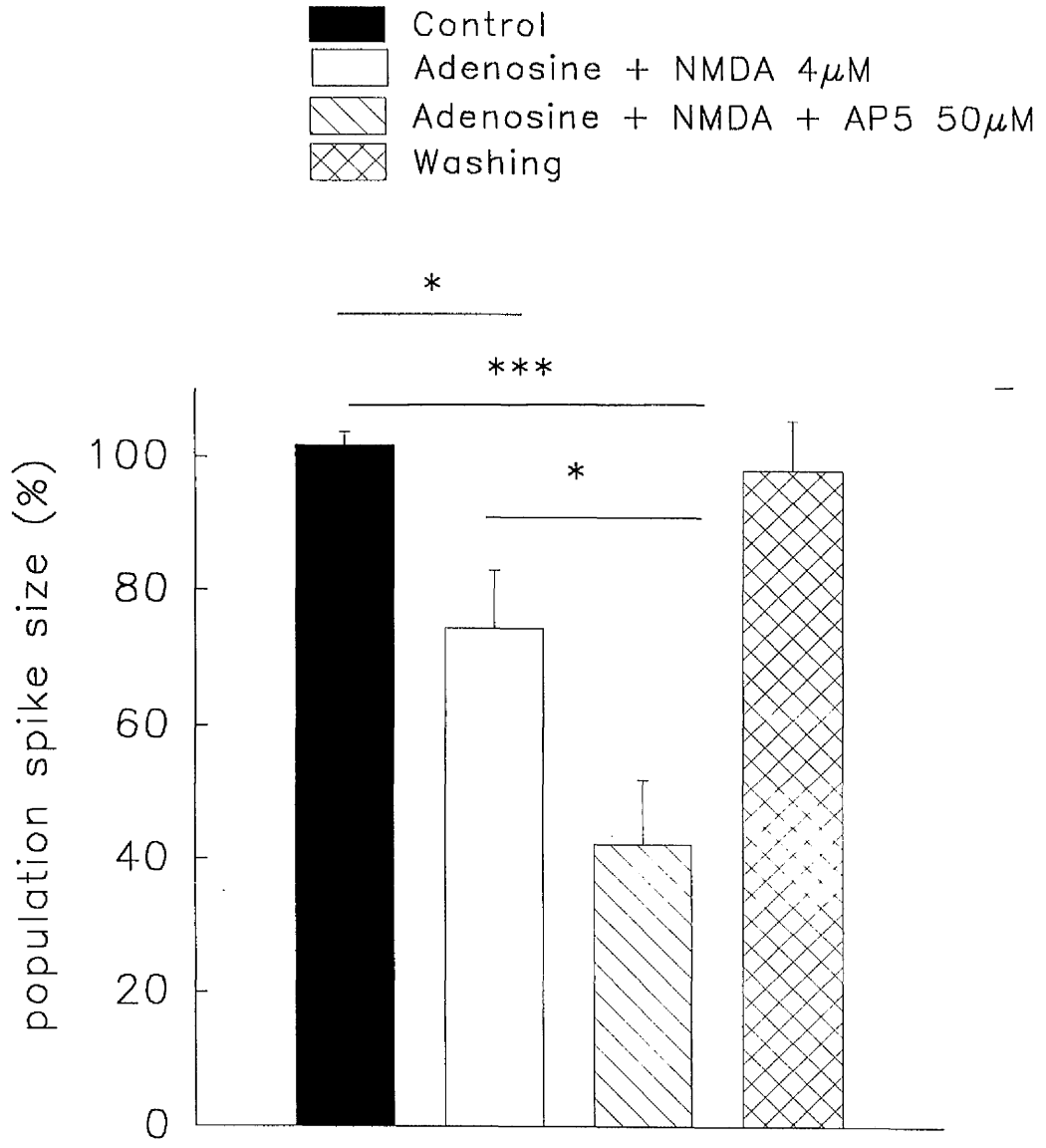

Figure 3. 64. Histogram showing the prevention of the effect of NMDA on adenosine responses by AP5 50μM. Each vertical bar shows mean±S. E. M for n=4 slices. * P<0.05, *** P<0.001

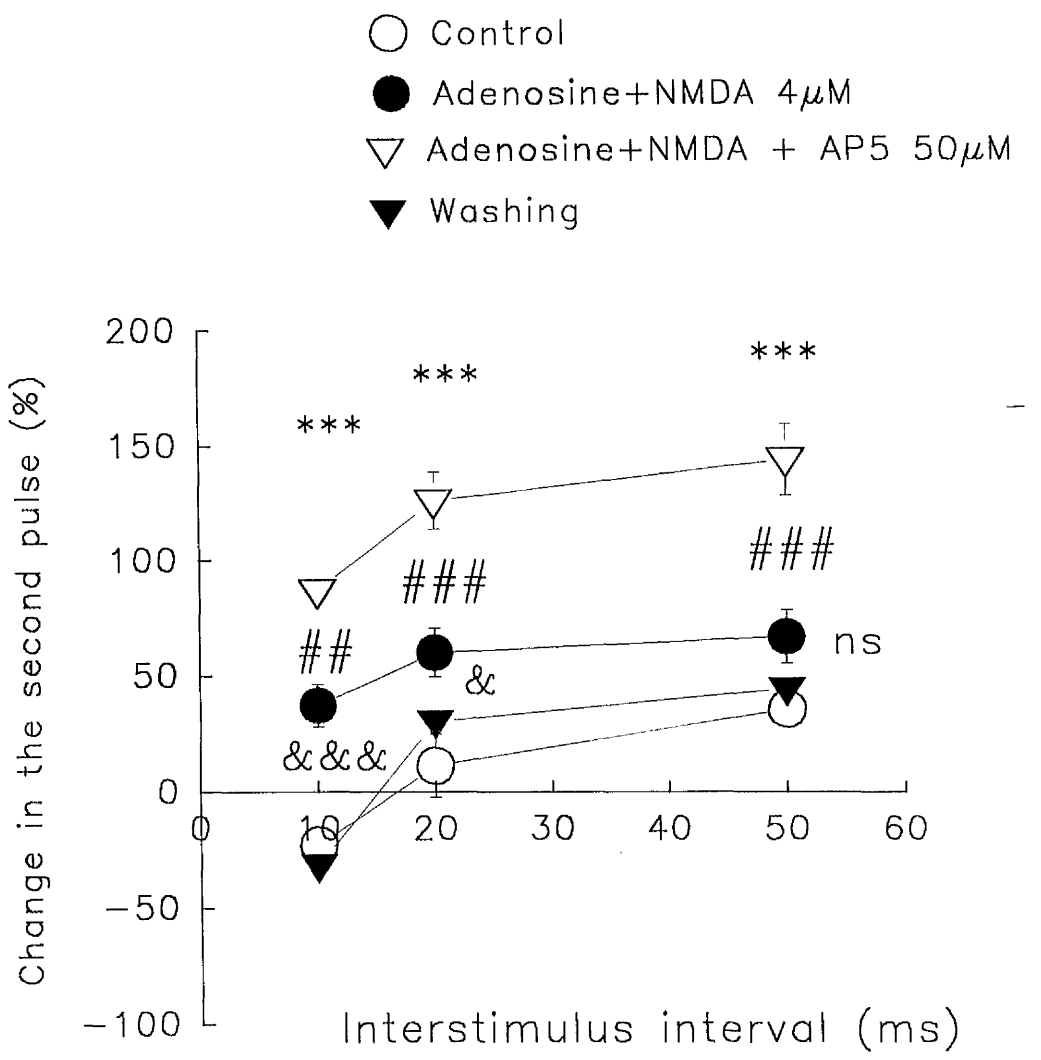

Figure 3. 65. Graph showing the prevention of NMDA effect on paired-pulse responses to adenosine. Interstimulus intervals at 10, 20 and 50ms have been examined. Points are shown as mean + S. E. M for n=4 slices. *** $P<0.001$ for difference between control and adenosine + NMDA + AP5. ## $P<0.01$, ### $P<0.001$ for difference between adenosine + NMDA and adenosine + NMDA + AP5. & $P<0.05$, &&& $P<0.001$ for difference between control and adenosine + NMDA.

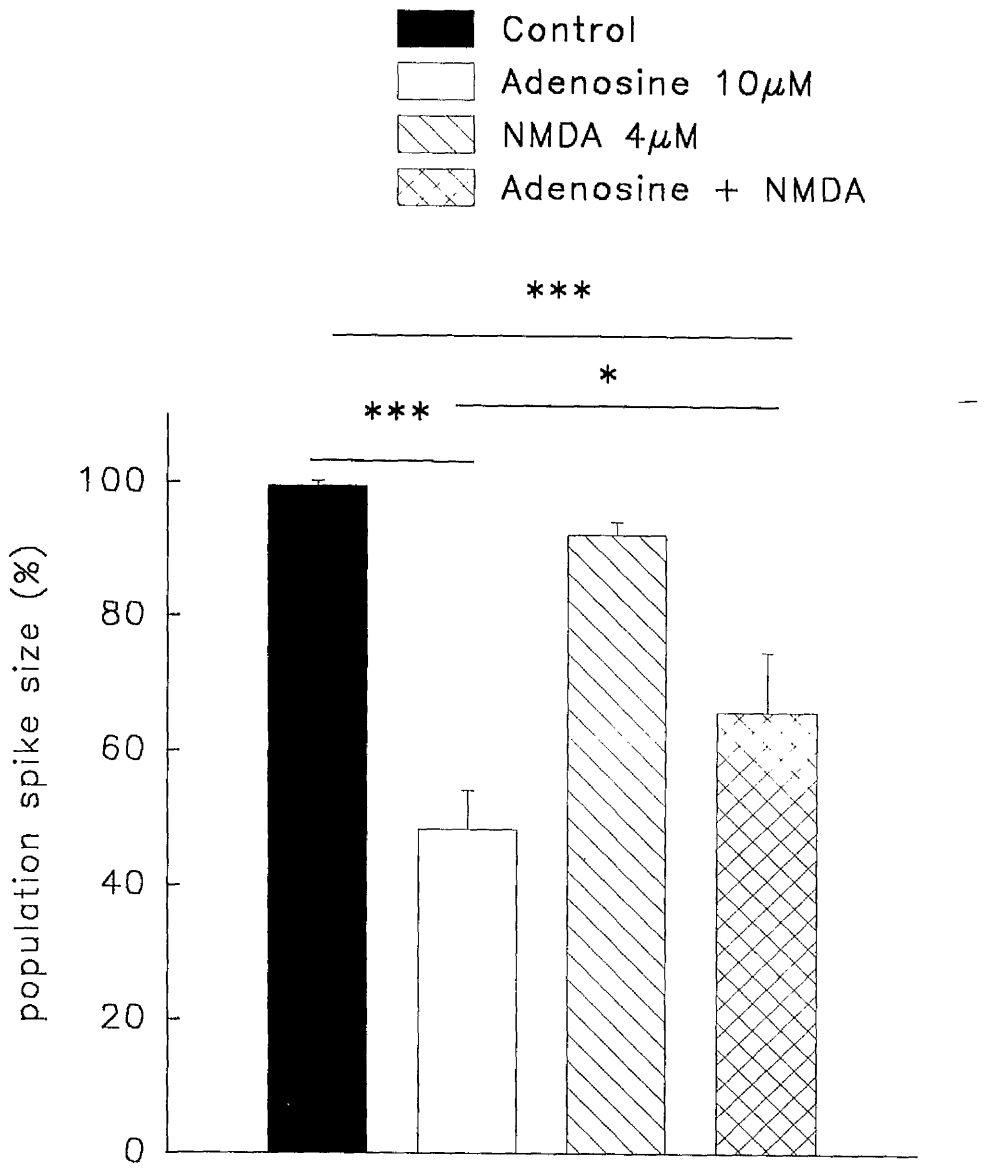

Figure 3. 66A. Histogram showing the effect of adenosine 10μM alone and in the presence of NMDA 4μM on EPSPs evoked from CA1 hippocampal slices. Each vertical bar shows mean ± S. E. M for n=5 slices. * $P<0.05$, *** $P<0.001$.

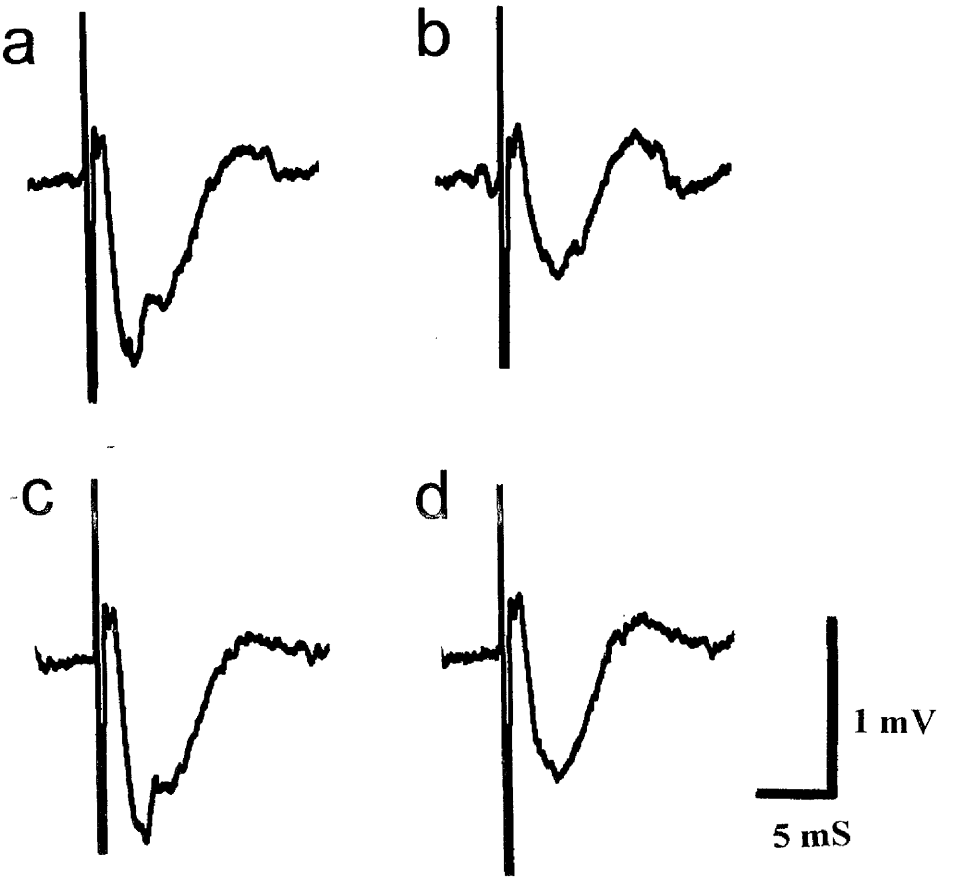

Figure 3. 66B. Representative records of field EPSPs showing the effect of denosine alone and in the presence of NMDA 4μM. Record (a) is a control EPSP and (b) illustrates the effect of adenosine 10μM . Record (c) is a control response taken in the presence of NMDA 4μM, and (d) shows the reduced response to adenosine in the presence of NMDA.

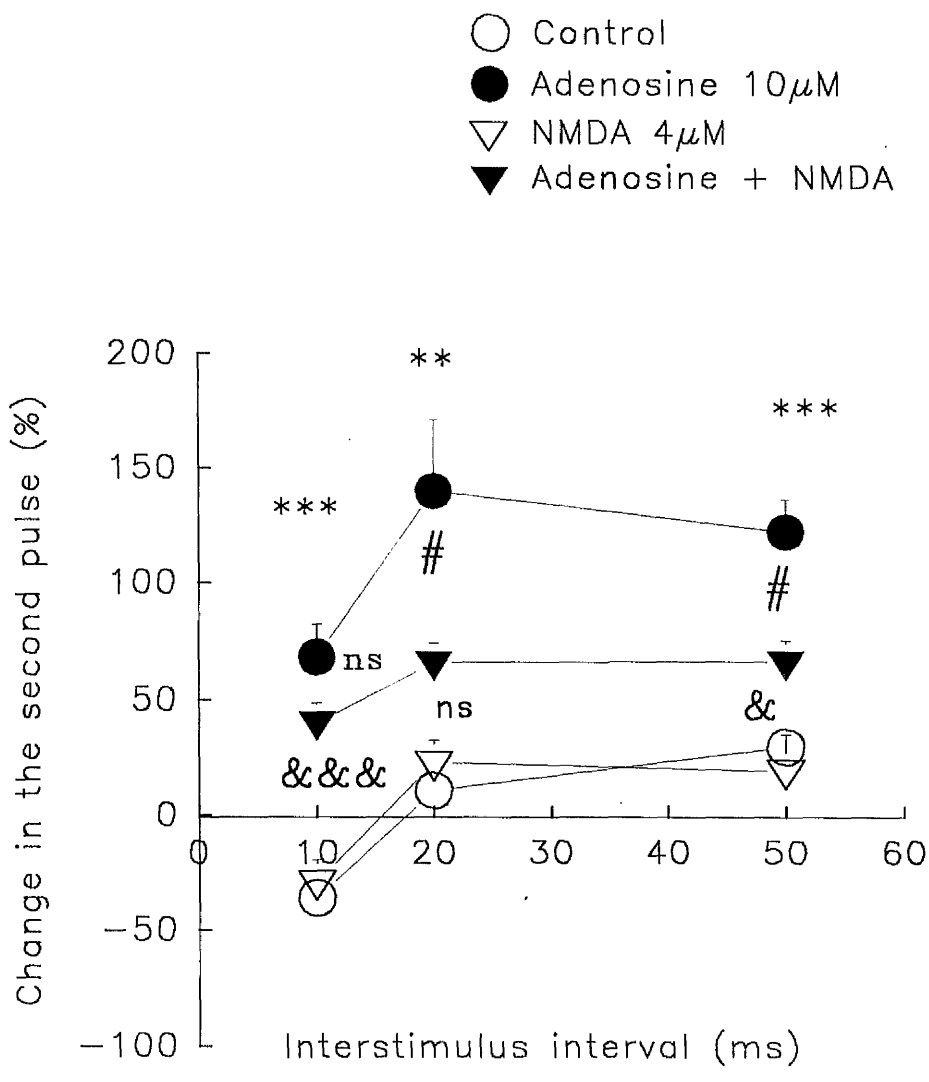

Figure 3. 67. The effect of adenosine alone and in the presence of NMDA on paired-pulse EPSPs. Each vertical bar Shows mean ± S. E. M for n=5 slices. ** P<0.01, *** P< 0.001 for differrence berween control and adenosine. # P<.05 for difference between adenosine and adenosine +NMDA. & P<0.05, &&& P<0.001 for difference between control and adenosine + NMDA.

The combined effect of adenosine and AMPA on single and paired-pulse responses

In order to understand whether the other subtypes of glutamate receptors can also affect the responses to adenosine, the experiments were repeated in the presence of AMPA 100nM. AMPA by itself did not change the population spike size. AMPA also was unable to change significantly the effect of adenosine 10μM on single pulses or paired pulses (Figs. 3. 68 & 3. 69).

The combination effect of adenosine and kainic acid on single and paired-pulse resoponses

Similar experiments were performed with a subthreshold concentration of kainic acid and adenosine 10uM in order to examine the specificity of the interaction with NMDA. However, when examined on population spikes, kainic acid did not induce any change on either single or paired-pulse responses to adenosine (Figs. 3. 70 & 3. 71).

The combined effect of NMDA and adenosine when the $GABA_A$ receptor antagonist bicuculline was superfused

In order to determine the possible involvement of interneuron activation in the interaction between adenosine and NMDA, in a series of 4 slices the supressant effect of NMDA receptor activation on adenosine responses has been re-examined when a $GABA_A$ receptor antagonist bicuculline 30μM was also superfused. Most of the slices exposed to bicuculline developed or produced spontaneous bursts and secondary spikes. Because of contamination of the second pulse with secondary spikes, it was impossible to measure the paired-pulse changes at shorter interstimulus interval (10ms) correctly. Instead longer interpulse intervals (100ms) were examined in these experiments. Bicuculline was unable to change the modulation of adenosine responses by NMDA and the suppressant effect of NMDA receptor activation on adenosine was still seen in the presence of bicuculline. This ruled out the involvement of interneurons and subsequent $GABA_A$ receptor activation (Figs. 3. 72 & 3. 73).

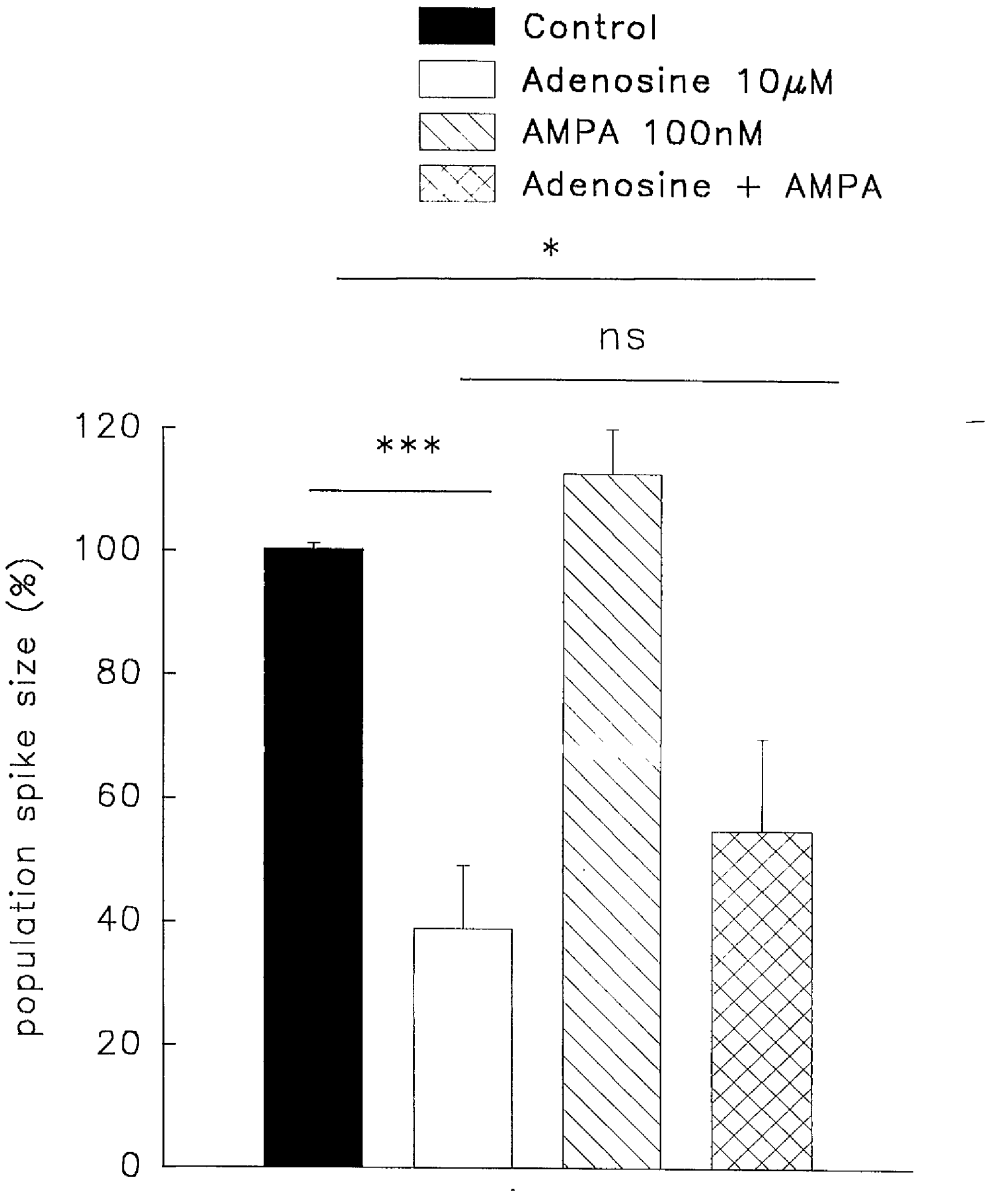

Figure 3. 68. Histogram showing the effect of adenosine 10μM alone and in the presence of AMPA 100nM on evoked CA1 population spikes. Each vertical bar shows mean + S. E. M for n=4 slices. * $P<0.05$, *** $P<0.001$.

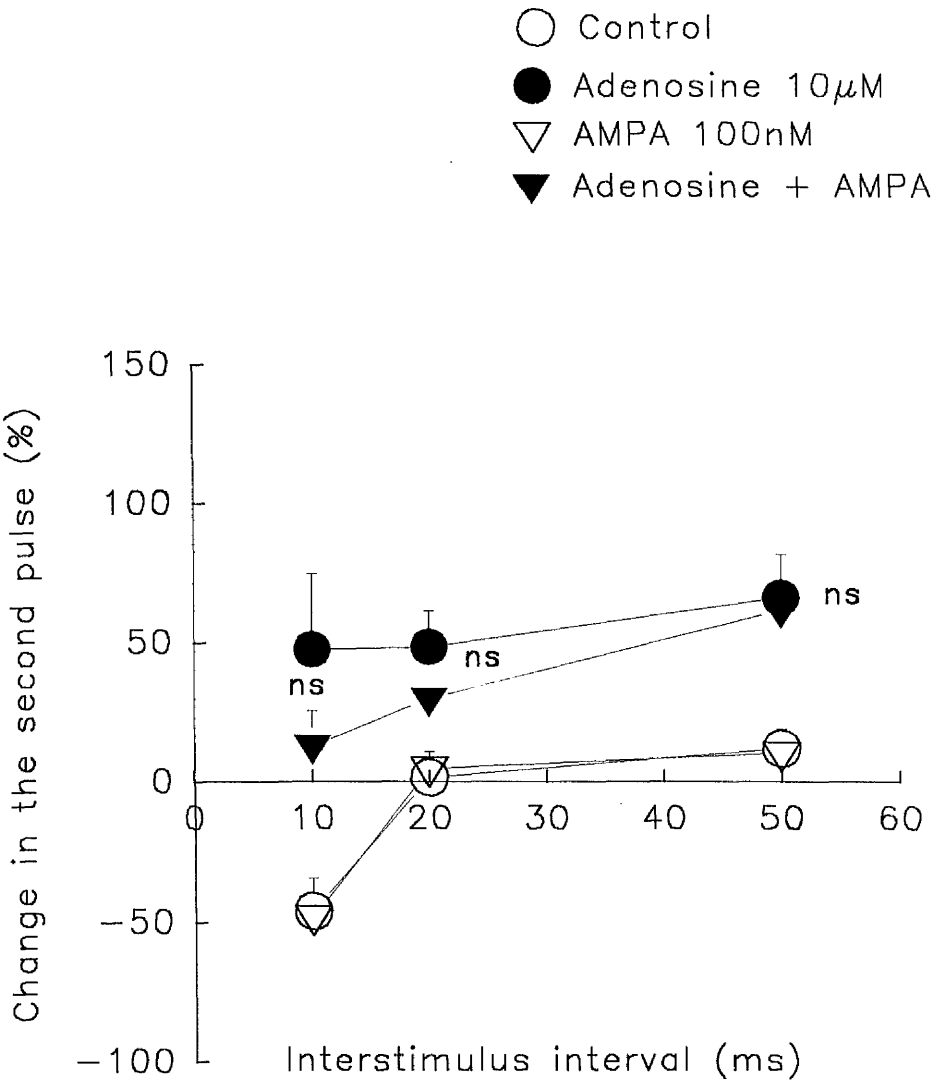

Figure 3. 69. The effect of adenosine alone and in the presence of AMPA 100nM on paired−pulse population spikes evoked from CA1 . Points are shown as mean ± S. E. M for n=3 experiments. No difference was observed between adenosine and adenosine + AMPA.

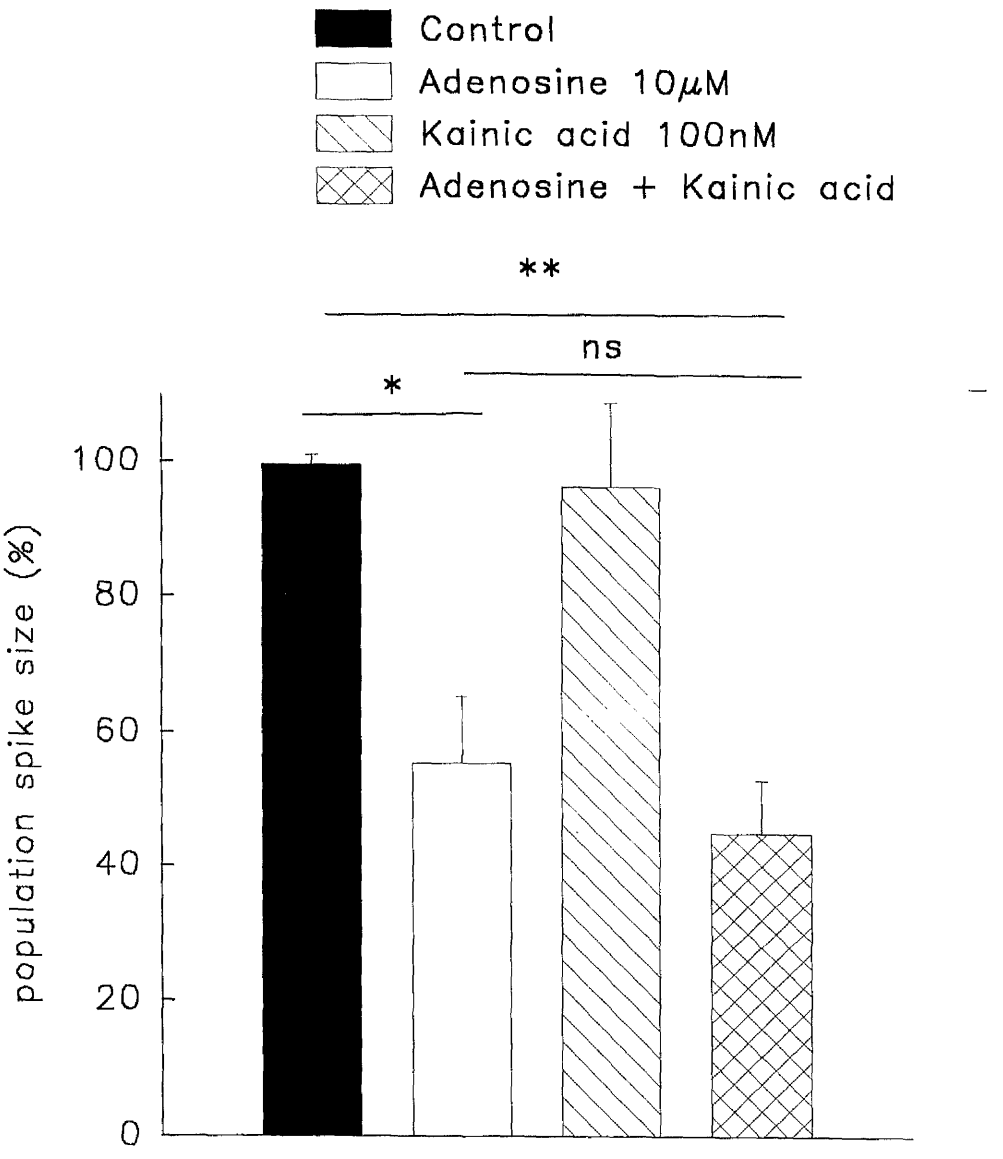

Figure 3. 70. Histogram showing the effect of adenosine 10μM alone and in the presence of kainic acid 100nM on evoked CA1 population spikes. Each vertical bar shows the mean ± S. E. M for n=3 slices. * $P<0.05$, ** $P<0.01$.

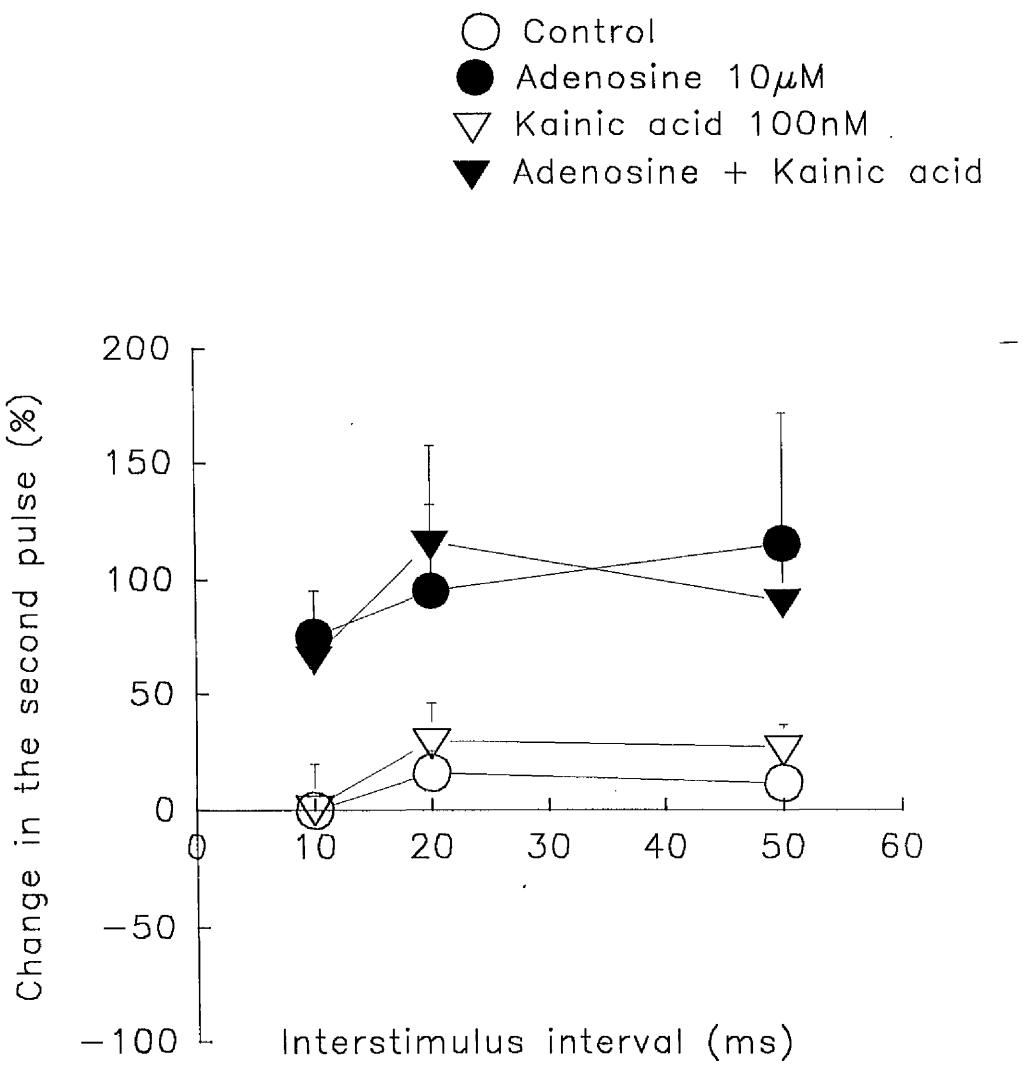

Figure 3. 71. The effect of adenosine alone and in the presence of kainic acid on paired−pulse population spikes evoked from CA1. Each vertical bar shows mean ± S. E. M for n=3 slices.

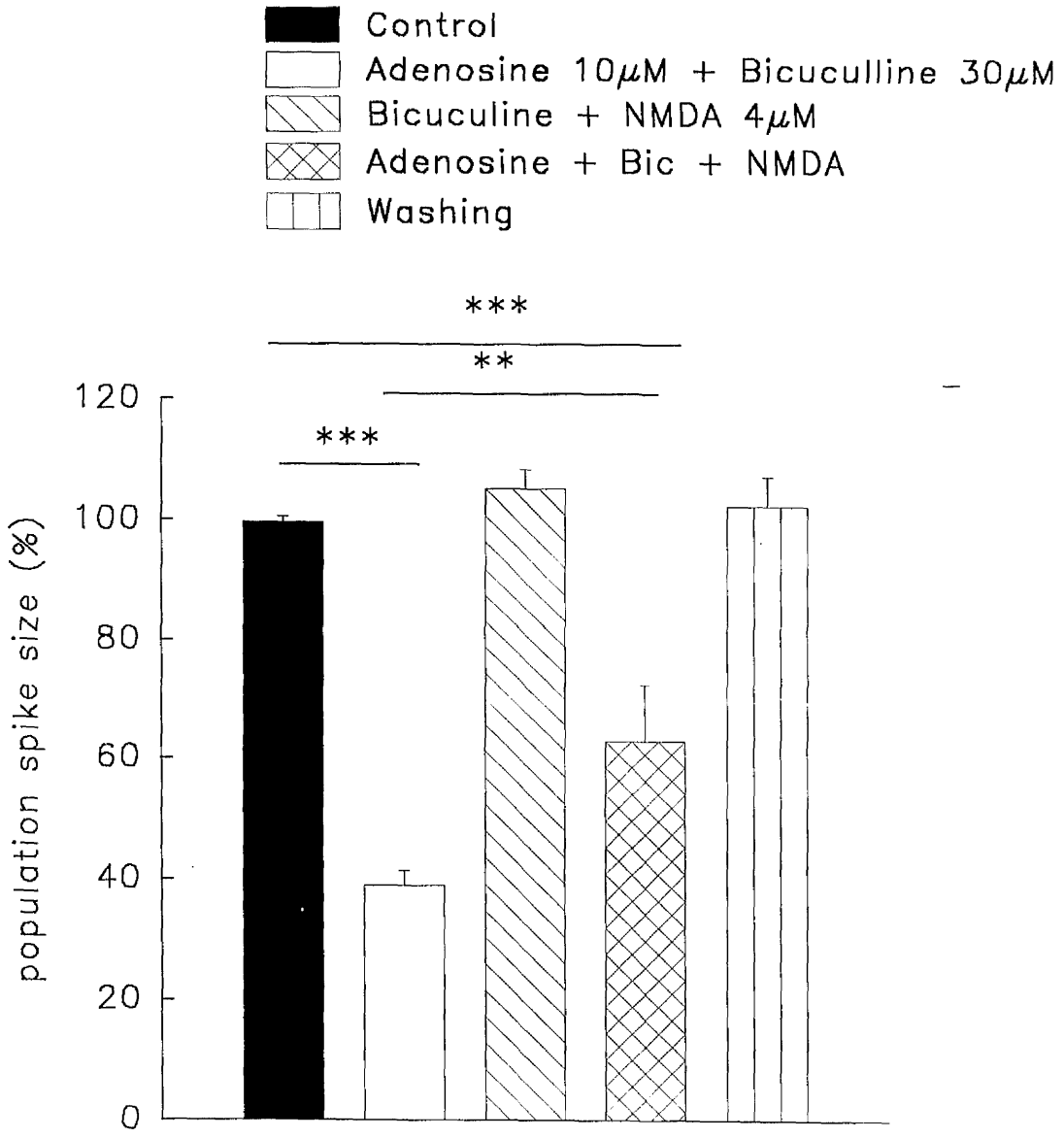

Figure 3. 72, Histogram showing that depressant effect of NMDA on adenosine responses was still seen in the presence of bicuculline 30μM. Each vertical bar shows mean ± S. E. M for n=4 slices. ** P<0.01, *** P<0.001.

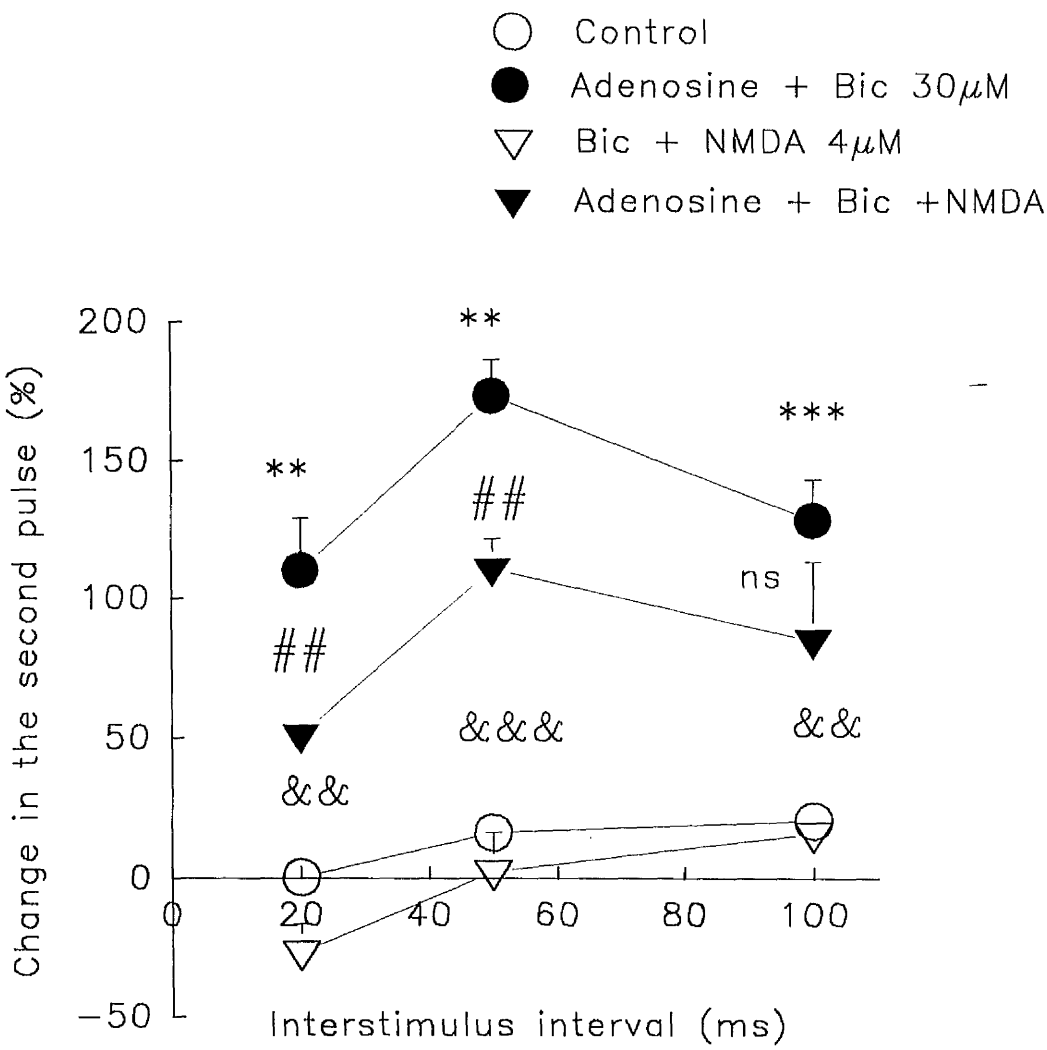

Figure 3. 73. The effect of NMDA 4μM on paired−pulse responses to adenosine in the presence of bicuculline 30μM. Each vertical bar shows mean ± S. E. M for n=4 slices. ** P<0.01, *** P<0.001 for difference between control and adenosine + bicuculline. ## P<0.01 for difference between adenosine + bicuculline and adenosine + bicuculline + NMDA. && P<0.01, &&& P<0.001 for difference between control and adenosine + bicuculline + NMDA.

The effect of magnesium free ACSF on paired-pulse responses to adenosine

A simple method for studying NMDA receptor-mediated synaptic potentials is to perfuse theslices with Mg^{++}-free ACSF to remove the blockade of NMDA associated channels. In setof experiments paired-pulse responses to adenosine 10μM in Mg^{++}free-ACSF were compared to the responses in normal ACSF. Magnesium-free ACSF was perfused for 20 minutes before the application of adenosine. Omission of Mg^{++} from ACSF completely prevented the effect of adenosine on paired-pulse interaction at all tested intervals (Fig. 3. 74).

Adenosine receptor subtype involved in interaction with NMDA

In order to determine whether the suppression activity of NMDA receptors was directed selectively towards the A_1 or A_{2A} receptors, the effect of the A_1 receptor agonist cyclopentyladenosine (CPA) and the A_{2A} receptor agonist (CGS21680) were studied.

CPA: CPA at 20nM alone depressed the population spike size by 44.83% ± 8.03 but the inhibitory effect of CPA was not modified when applied in the presence of NMDA 4μM (Fig. 3. 75).

CGS 21680: Similar experiments were performed using a combination of CGS 21680 at a concentration of 30nM and NMDA 4μM. CGS 21680 was able to increase the size of population spike size significantly in the presence of NMDA 4μM (increase by 15.8% ± 1.45). But when NMDA 4μM was removed, reapplication of CGS 21680 did not modify the population spike size, indicating that NMDA could increase the excitatory responses to CGS 21680 (Figs. 3. 76A & 3.76B). CGS 21680 was also unable to change the paired-pulse ratio by itself, but when reintroduced in the presence of NMDA 4μM, more inhibition at 10 ms was seen compared with control, but no change at longer intervals (Fig 3. 77).

ZM241385: In order to confirm that the increase in the size of population spikes which is produced in the simultaneous presence of CGS 21680 and NMDA is mediated by

activation of A_{2A} receptors, the effect of combination of CGS21680 and NMDA was retested when A_{2A} receptor antagonist ZM241385 at 100nM was present throughout the experiments. Application of ZM241385 prevented of the increase in the size of population spikes, supporting modification of A_{2A} receptor activation by subthreshold concentration of NMDA (Figs. 3.78 & 3. 79).

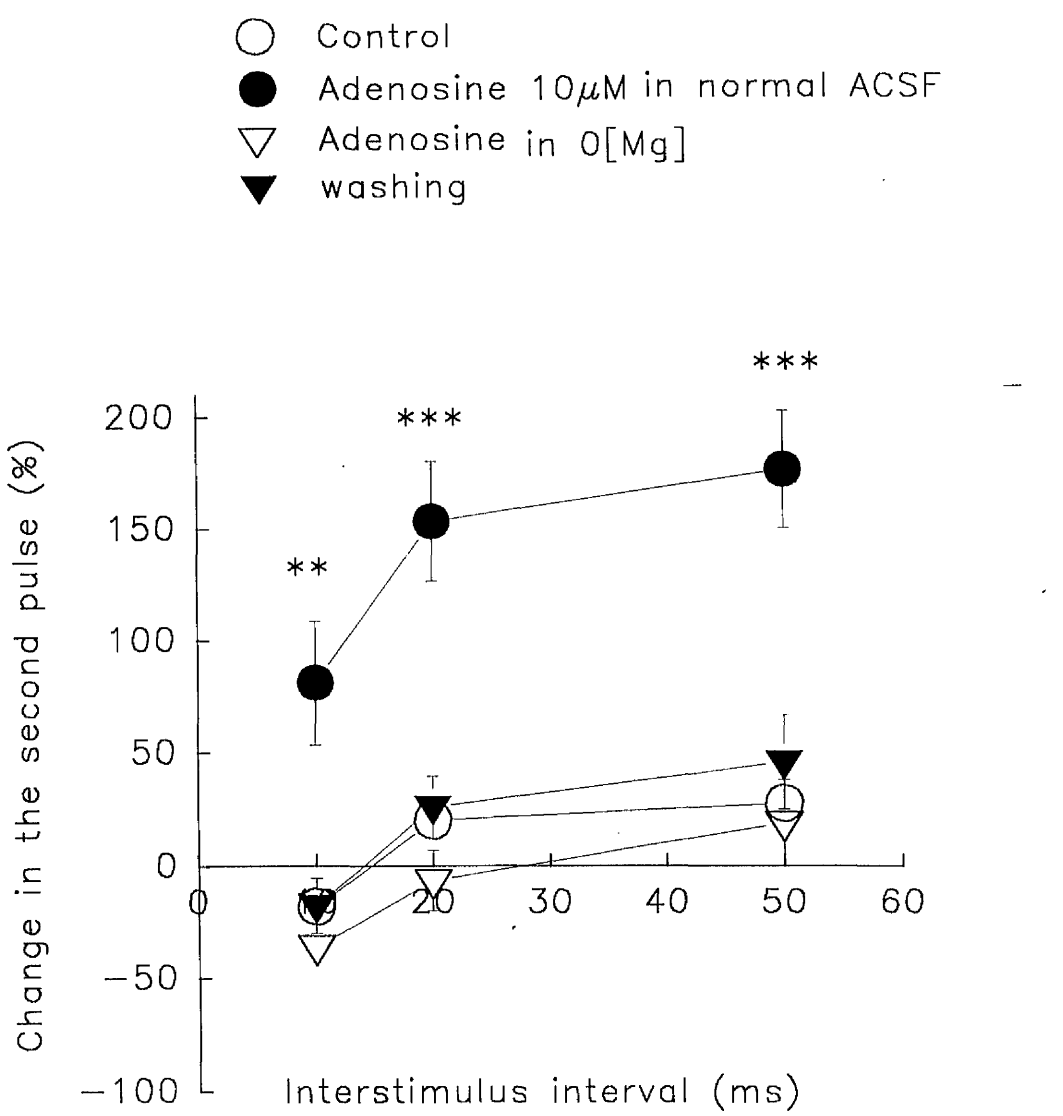

Figure 3. 74. Comparison between the effect of adenosine 10μM on paired−pulse population spikes evoked from CA1 in hippocampal slices in normal ACSF and in magnesium−free media. Each point shows mean ± S. E. M for n=3 slices. * P<0.01, *** P<0.001. There was no significant difference between control and adenosine in zero magnesium.

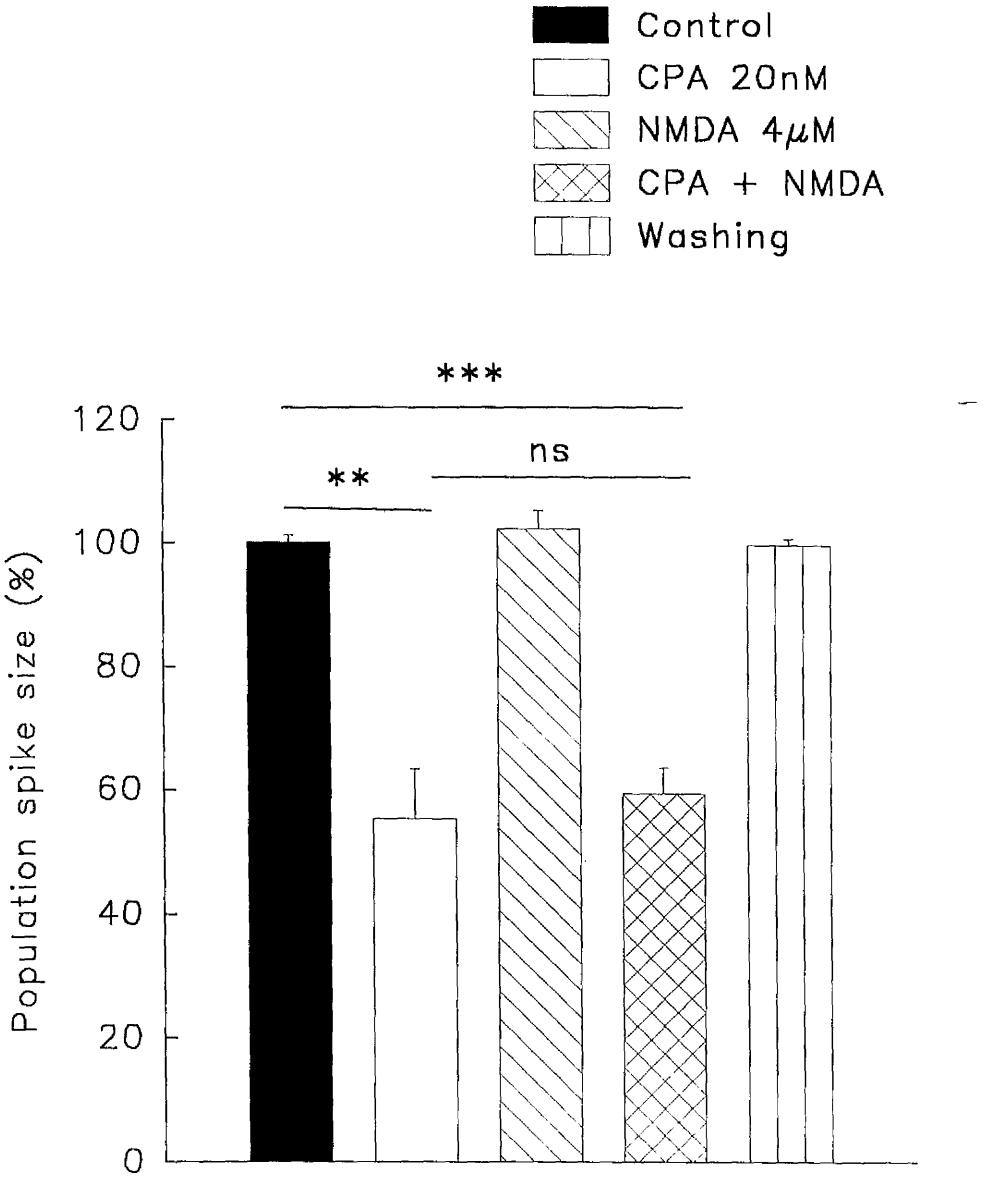

Figure 3. 75. Histogram showing the effect of cyclopentyladenosine 20nM (CPA) alone and in combination with NMDA 4μM on population spike size. Each vertical bar shows mean ± S. E. M for n=4 slices. ** P<0.01, *** P<0.001.

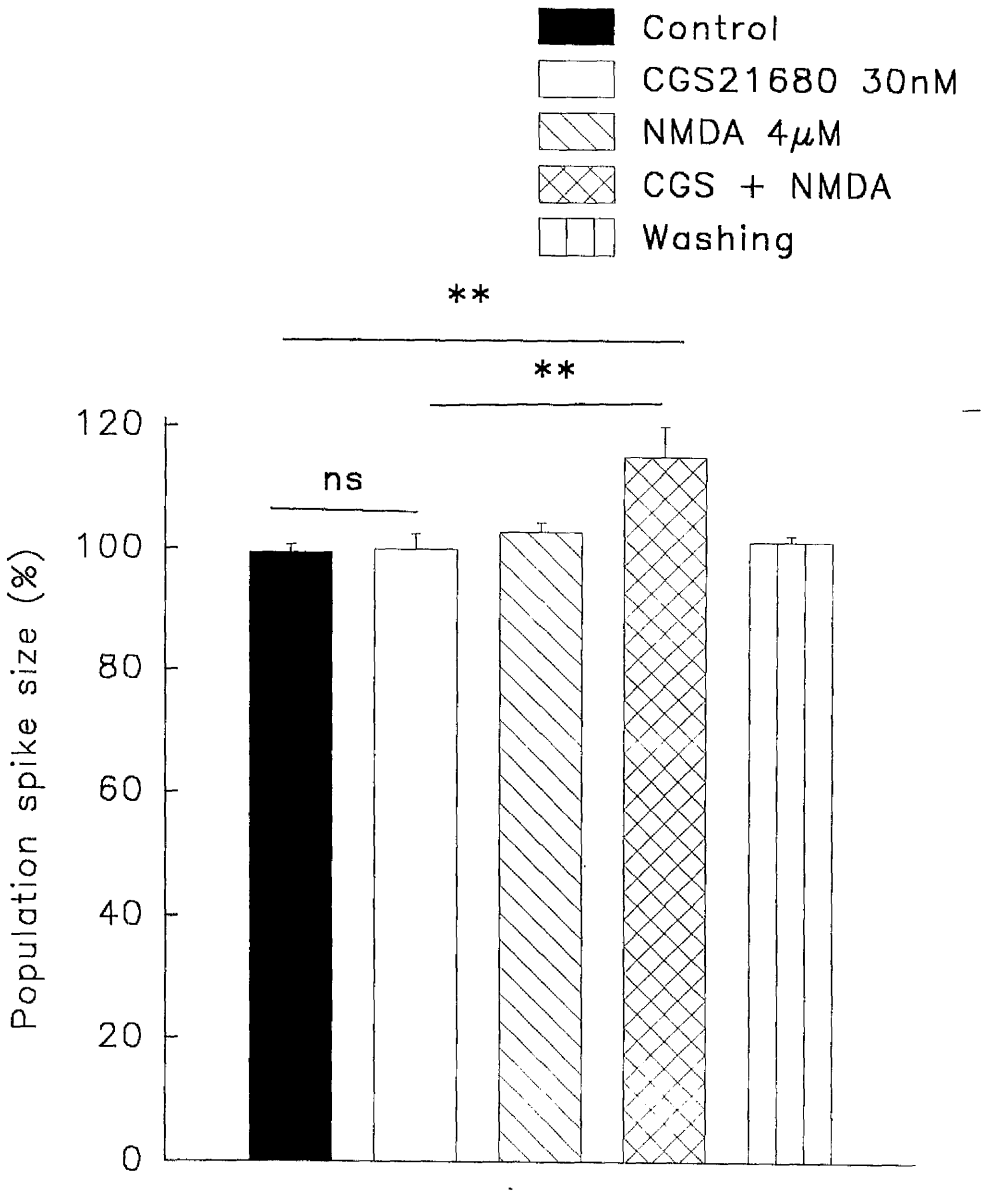

Figure 3. 76A. Histogram showing the effect of CGS21680 30nM alone and in combination with NMDA 4μM on the size of population spikes. Each vertical bar shows mean ± S. E. M for n=4 slices. ** P<0.01.

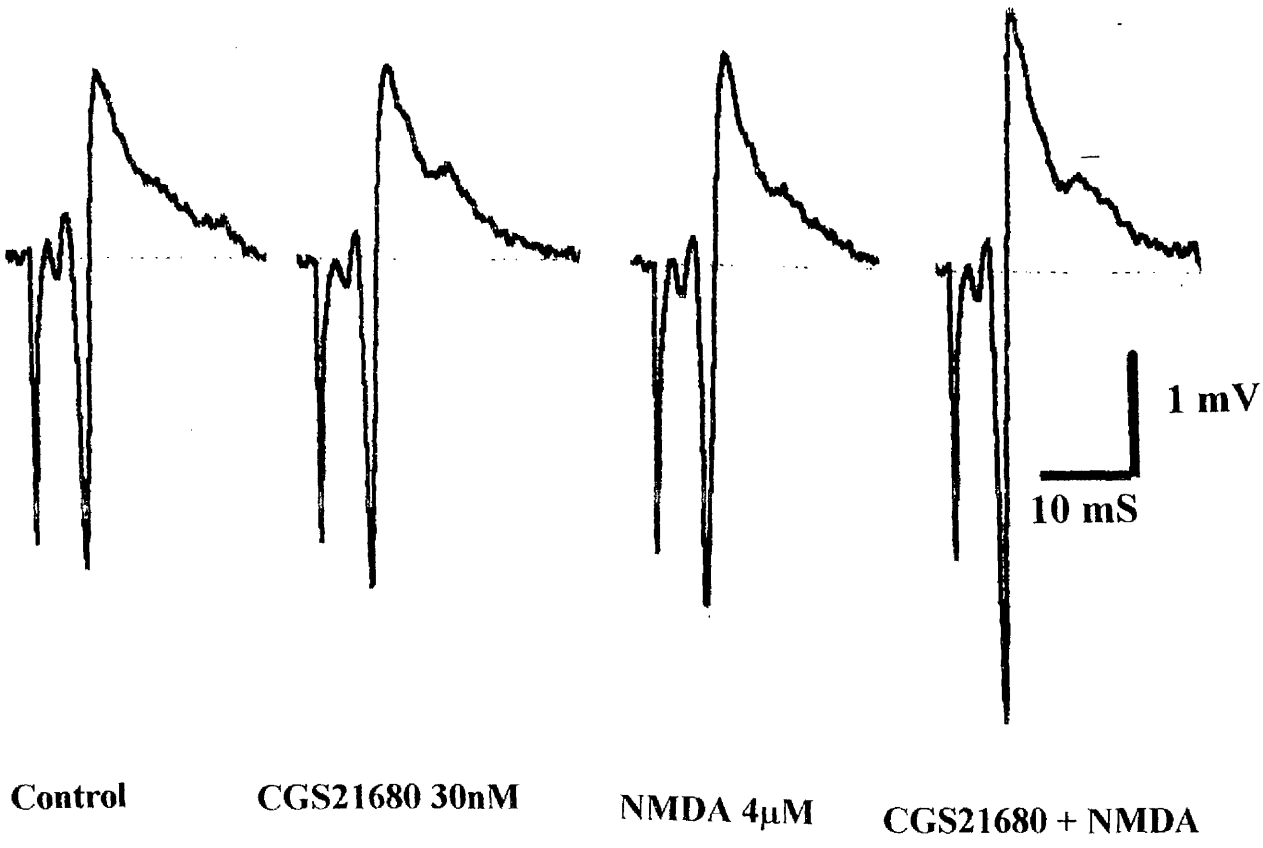

Figure 3. 76B. Sample records of orthodromic evoked potentials showing the effect of CGS21680 30nM alone and in the presence of NMDA 4μM.

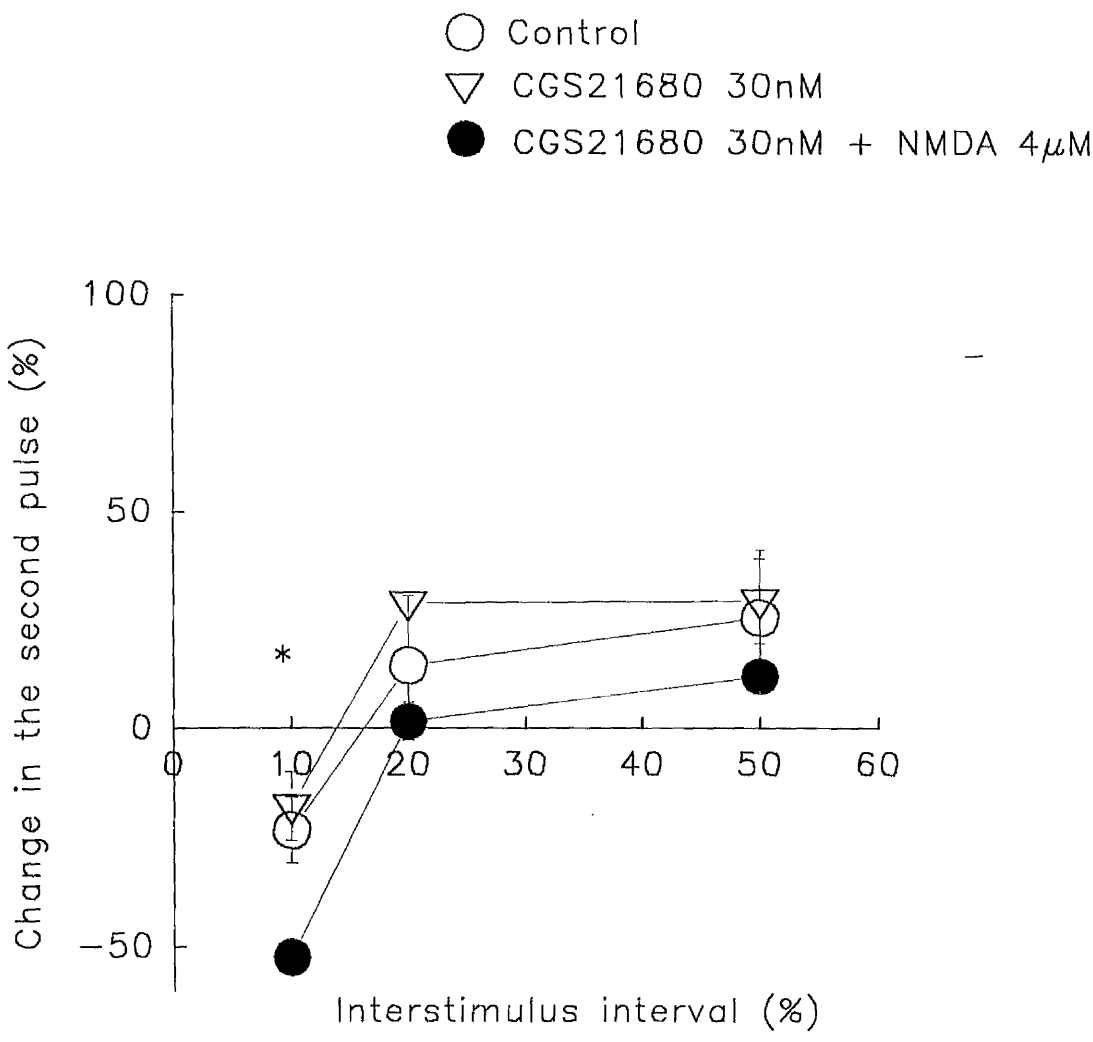

Figure 3. 77. The effect of CGS21680 3onM alone and in the presence of NMDA 4μM on the paired−pulse population spikes evoked from CA1 hippocampal slices * P<0.05 for difference between CGS + NMDA and CGS. at different interstimulus intervals (10, 20 and 50ms). Each vertical bar shows mean ± S.E.M for n=3 slices.

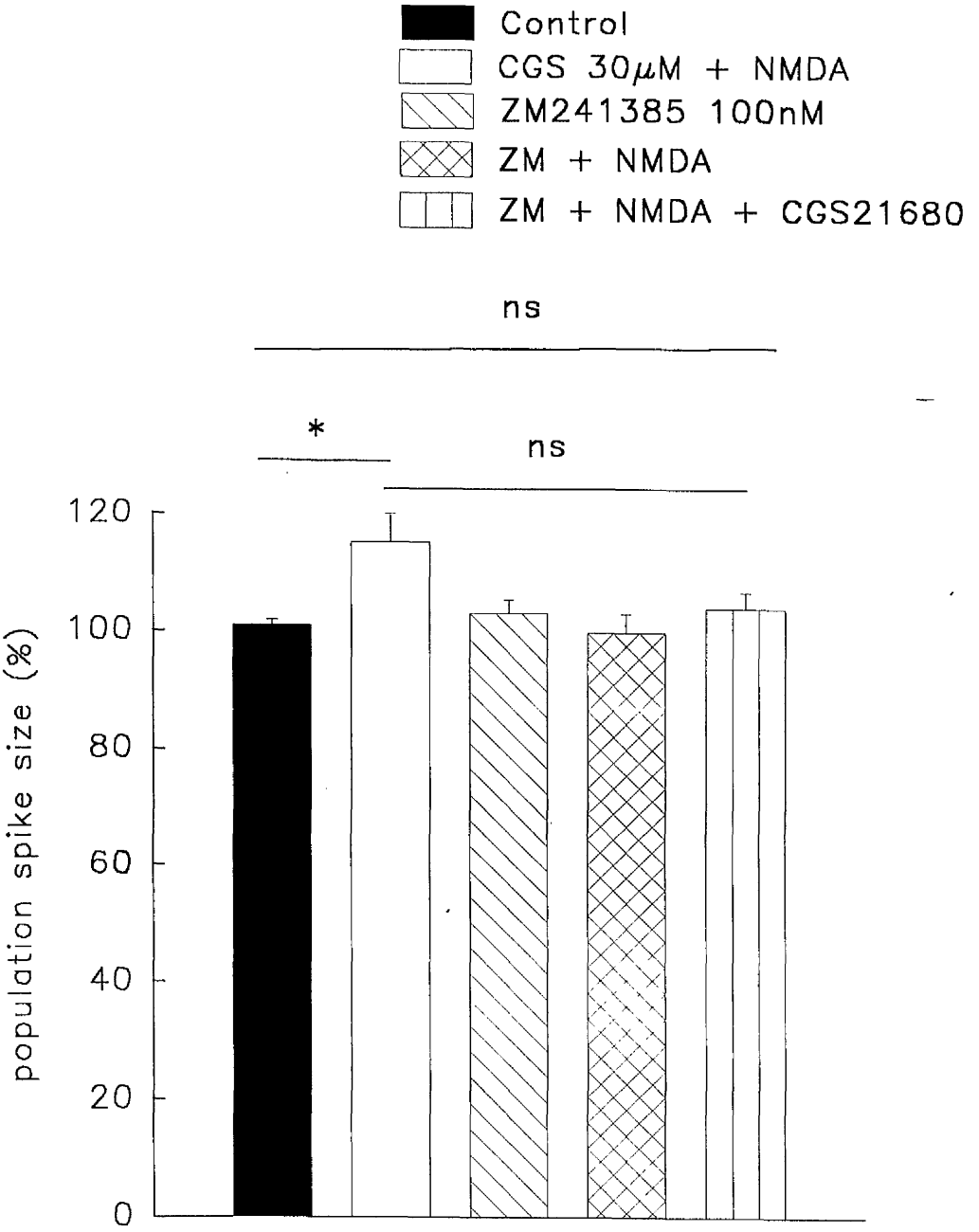

Figure 3. 78, Histogram showing the blockade of excitatory effect of CGS21680 in the presence of NMDA 4μM by ZM241385 100nM. Each vetrical bar shows mean ± S. E. M for n=4 slices. * P<0.05.

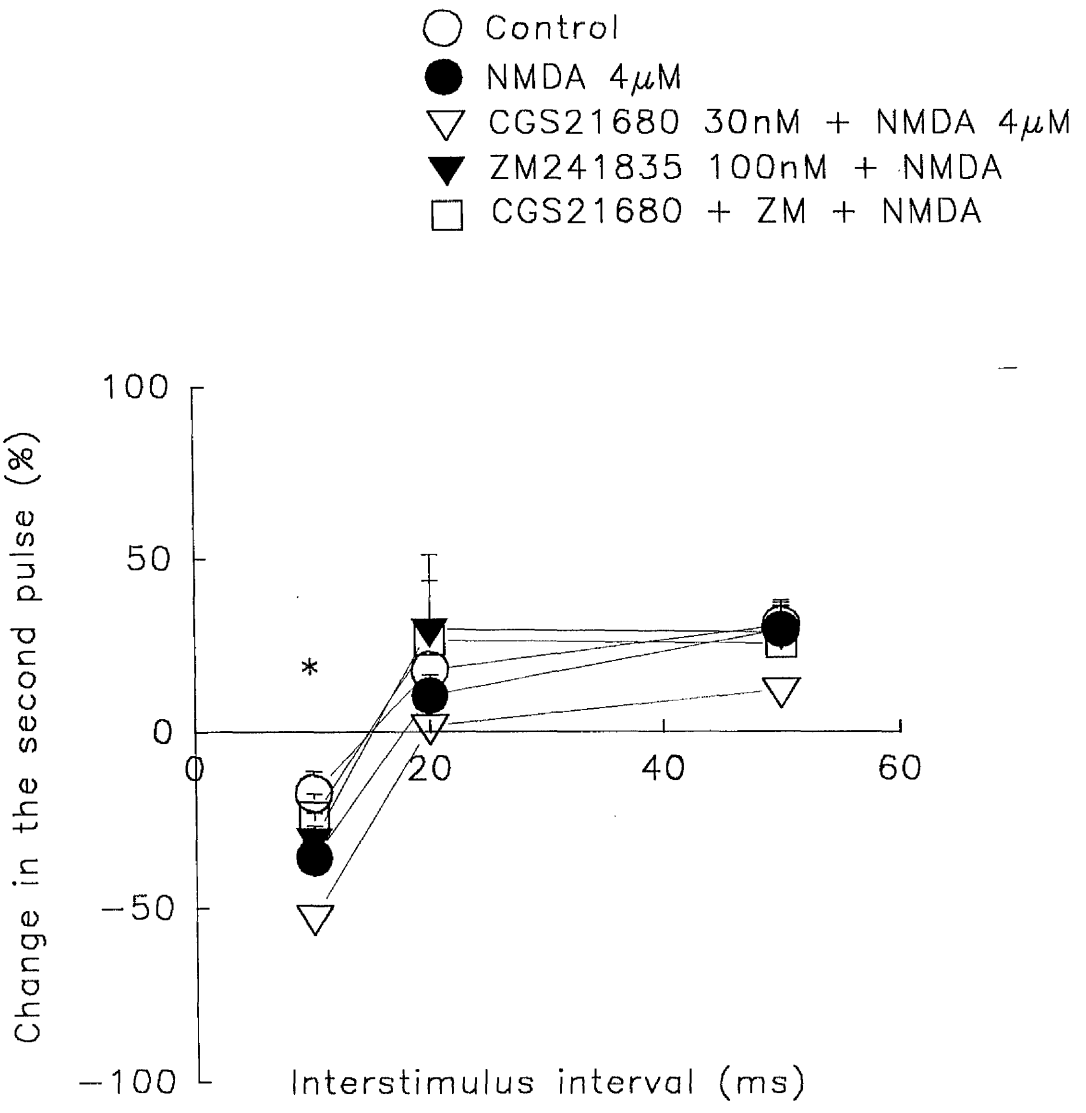

Figure 3. 79. Graph shows comparison between the effect of simultaneous application of CGS21680 30 nM and NMDA 4μM in the presence and absence of A2A antagonist ZM241835 100nM. Paired−pulse interactions at 10, 20 and 50ms has been examined. * $P<0.05$ for difference between CGS + NMDA and CGS + NMDA + ZM. No significant difference was observed at intervals of 20 and 50ms.

Activation of NMDA receptors by induction of electrical LTP and subsequent interaction with adenosine

Another alternative way to activate NMDA receptors is induction of electrical LTP by tetanic stimulation (100 impulses at 100 Hz) (Fig 3. 80), and which may be more physiologically relevant than application of NMDA itself. The effect of adenosine on population spike size before and after the establishment of long-term potentiation was compared. Adenosine depressed the population spike size by 54.88% ± 5.4 of the control size before the induction of LTP, whereas after establishment of electrical LTP it depressed the spike size significantly less (32.13% ± 3. 94) (Figs. 3. 81A & 3.81B). The effect of adenosine on paired-pulse interactions was compared before and 10 minutes after establishment of electrical potentiation. Following the induction of LTP, the effect of adenosine on paired-pulse responses was significantly reduced (Fig. 3. 82).

The effect of stimulus strength on paired-pulse responses to adenosine

In order to control for the fact that LTP, by definition, involves an increase in population spike size, experiments were also performed to assess the effect of increasing the stimulus strength from the submaximal level (producing a spike size approximately 70% of the maximum amplitude), to a level producing a maximal spike size. The manoeuvre did not modify significantly the effect of adenosine on paired-pulse interaction, as illustrated in Fig. 3. 83. confirming that the effect of LTP was independent of the increase in population spike amplitude.

The effect of baclofen alone and in the presence on NMDA on single and paired-pulse population spikes

In order to determine the specifity of interaction between adenosine and NMDA, the interaction between the $GABA_B$ receptor agonist baclofen which is known to suppress transmitter release and NMDA was examined. Baclofen at 2μM substantially depressed spike size and reduced paired-pulse inhibition and increased facilitation. In contrast to

adenosine, co-superfusion of NMDA 4μM neither changed the single nor paired-pulse responses to baclofen 2μM (Figs. 3. 84 & 3. 85).

Comparision between the effect of baclofen before and after the establishment of electircal LTP

Similar experiments were performed to examine the effect of baclofen 2μM ~~on~~ population spike size after and before the induction and establishment of electrical LTP and to compare the effect of baclofen with adenosine. Baclofen depressed the population spike size by 76.8% ± 5.2 of control size which was not significantly different from the depressant effect of baclofen after the establishment of LTP (depression of 66.7% ± 7. 93 of control size) (Fig. 3. 86).

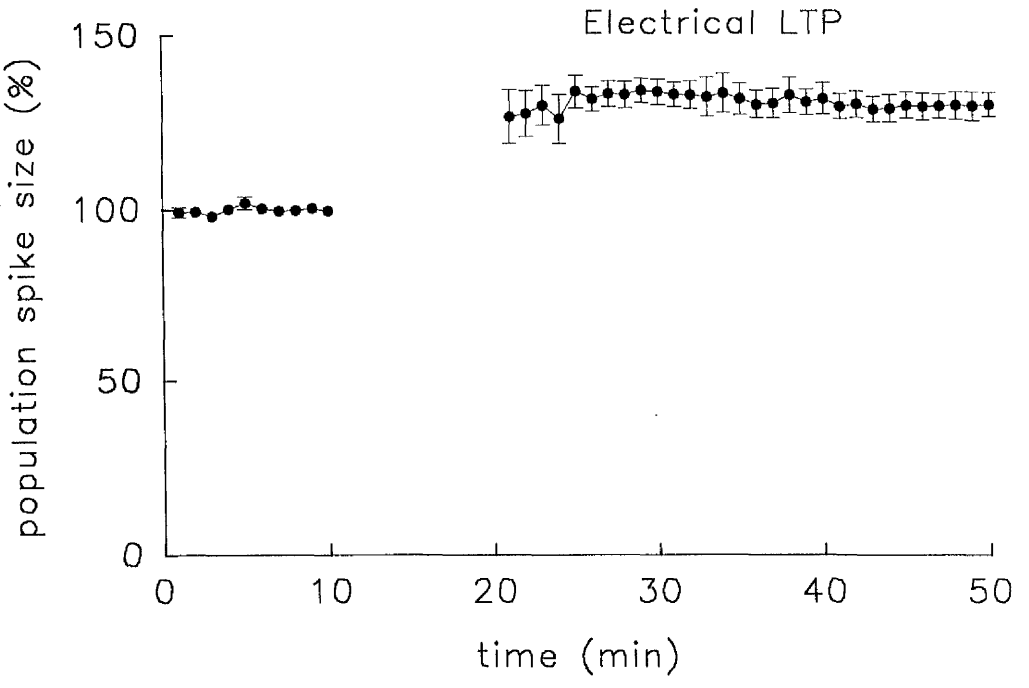

Figure 3. 80. Graph showing the enhancement of the size of evoked CA1 population spikes after the induction of electrical LTP. Points are shown as mean ± S. E. M for n=5 slices.

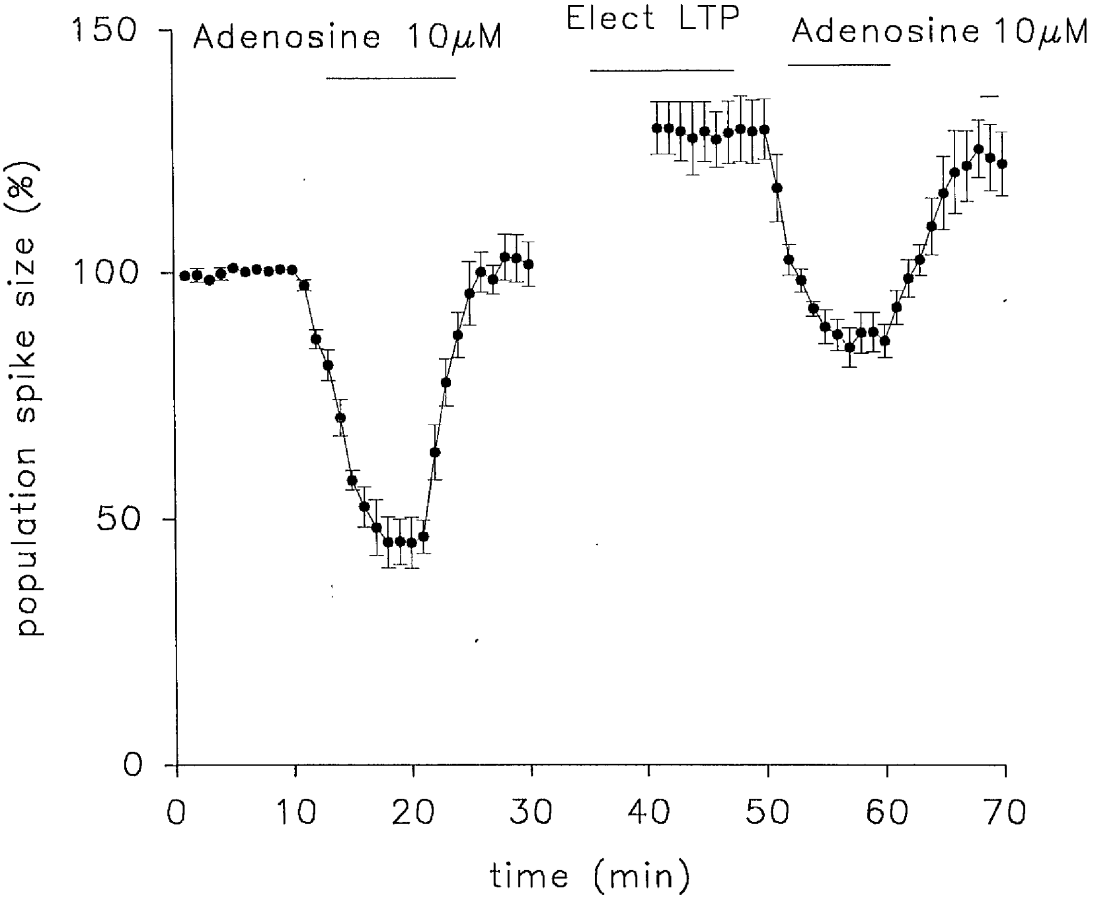

Figure 3. 81A. Comparision between the inhibitory effect of adenosine 10μM before and after induction of electrical LTP. The difference was significant. Each point shows mean ± S. E. M for n=5 slices.

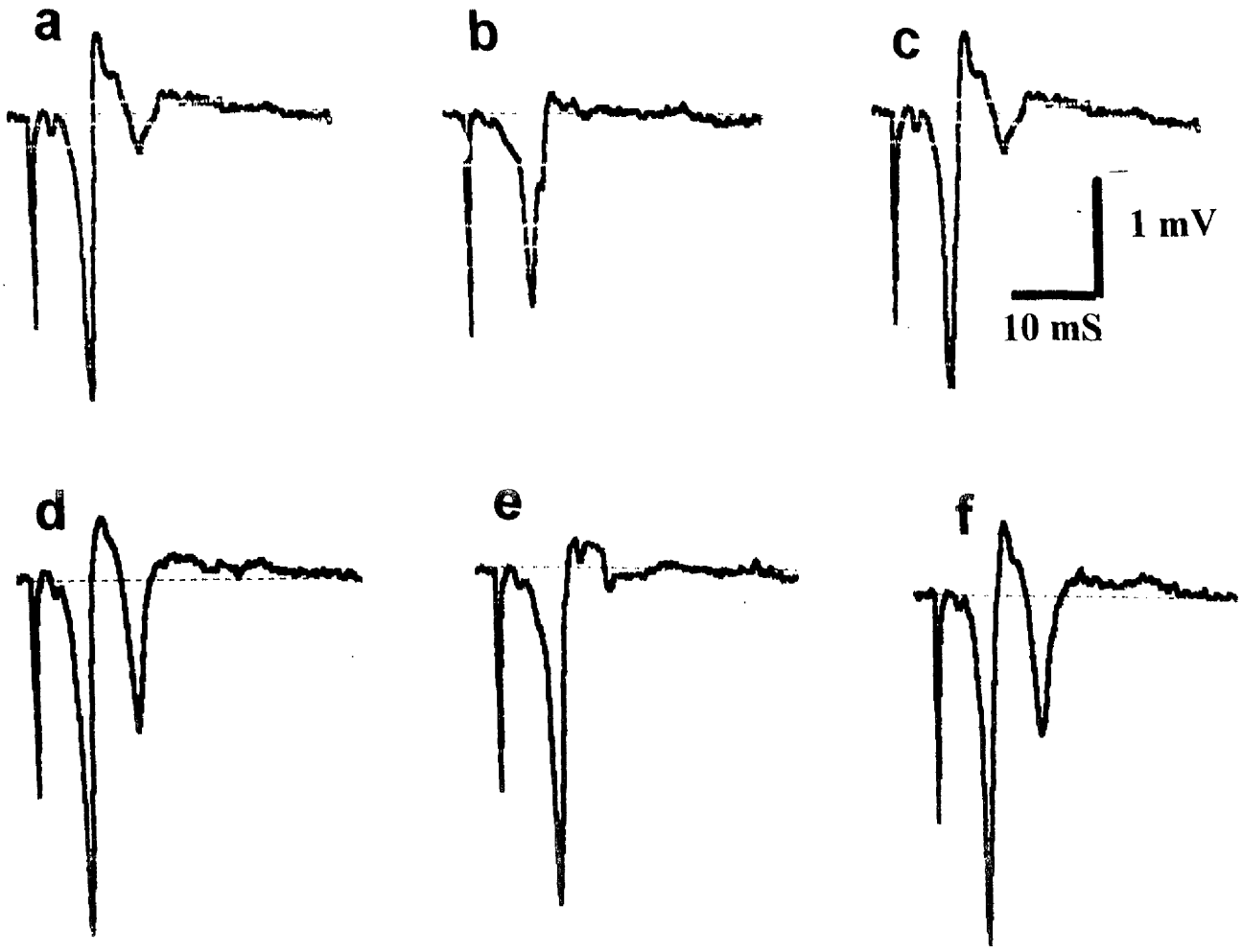

Figure 3. 81B. Sample records of orthodromic evoked potentials showing the effect of adenosine 10μM before and after establishment of electrical LTP. The letters a, b and c illustrate control , adenosine 10μM and washing respectively before electrical LTP and d, e and f show control, adenosine and washing after electrical LTP. This record is not a typical record used in these experiments, the slices without secondary spikes has normaly been used throughout this work.

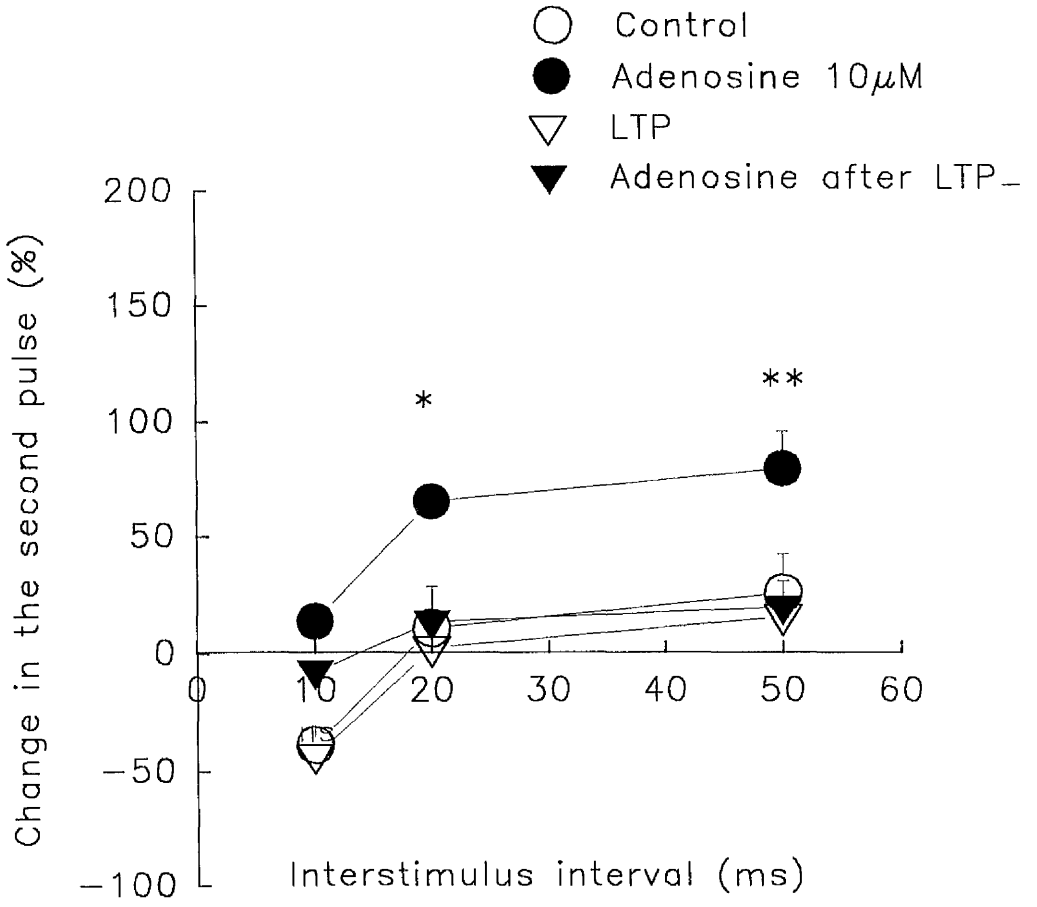

Figure 3. 82. Comparison between the effect of adenosine before and 10 minutes after the establishment of long-term potentiation. Each vertical bar shows mean ± S. E. M for n=3 slices. * P<0,05, ** P<0.01

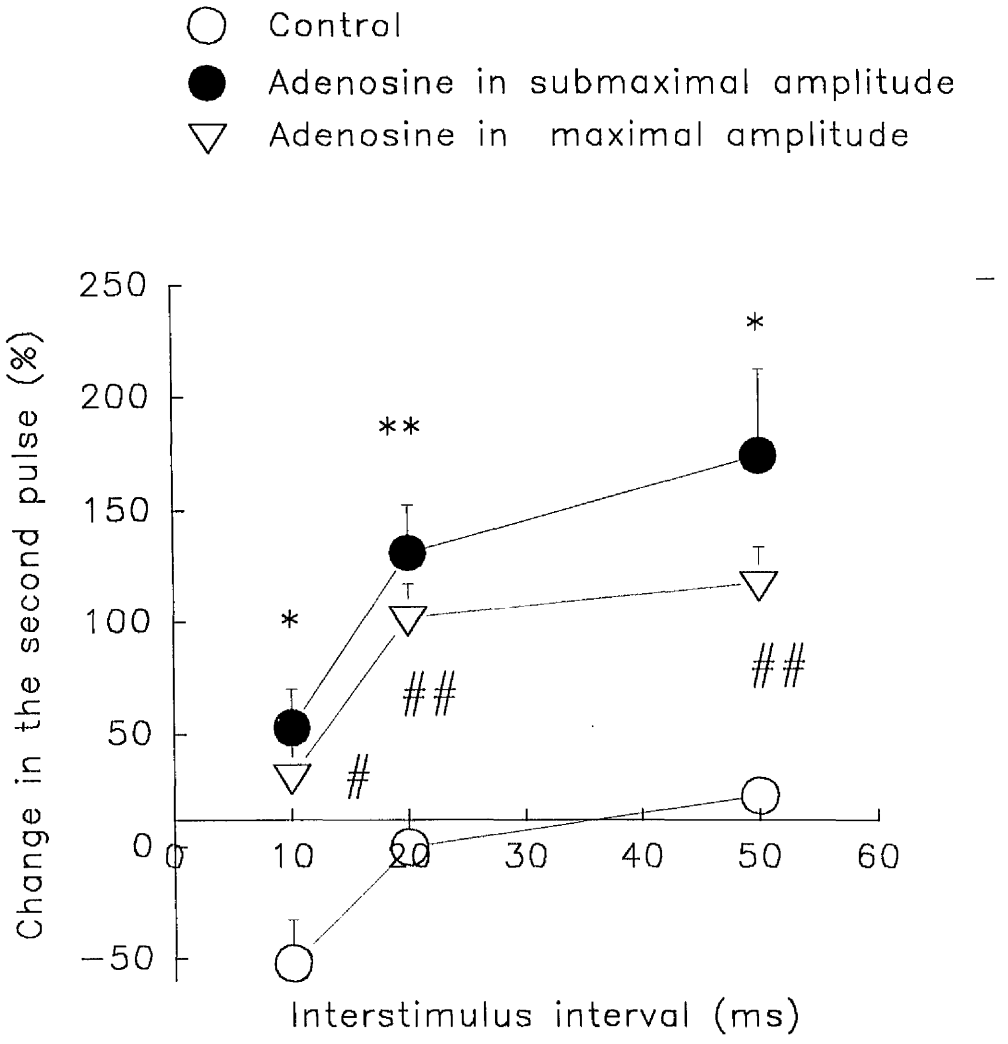

Figure 3. B3. Comparison between the effect of adenosine 10μM on paired−pulse interaction using submaximal (70%) and maximum stimulus amplitude. Each vertical bar shows mean ± S. E. M for n=3 slices. * P<0.05, ** P<0.01 for difference between control and adenosine in submaximal amplitude. # P<0.05, ## P<0.01 for difference between control and adenosine in maximal amplitude. There is no significant difference between the effect of adenosine in submaximal and maximal amplitude.

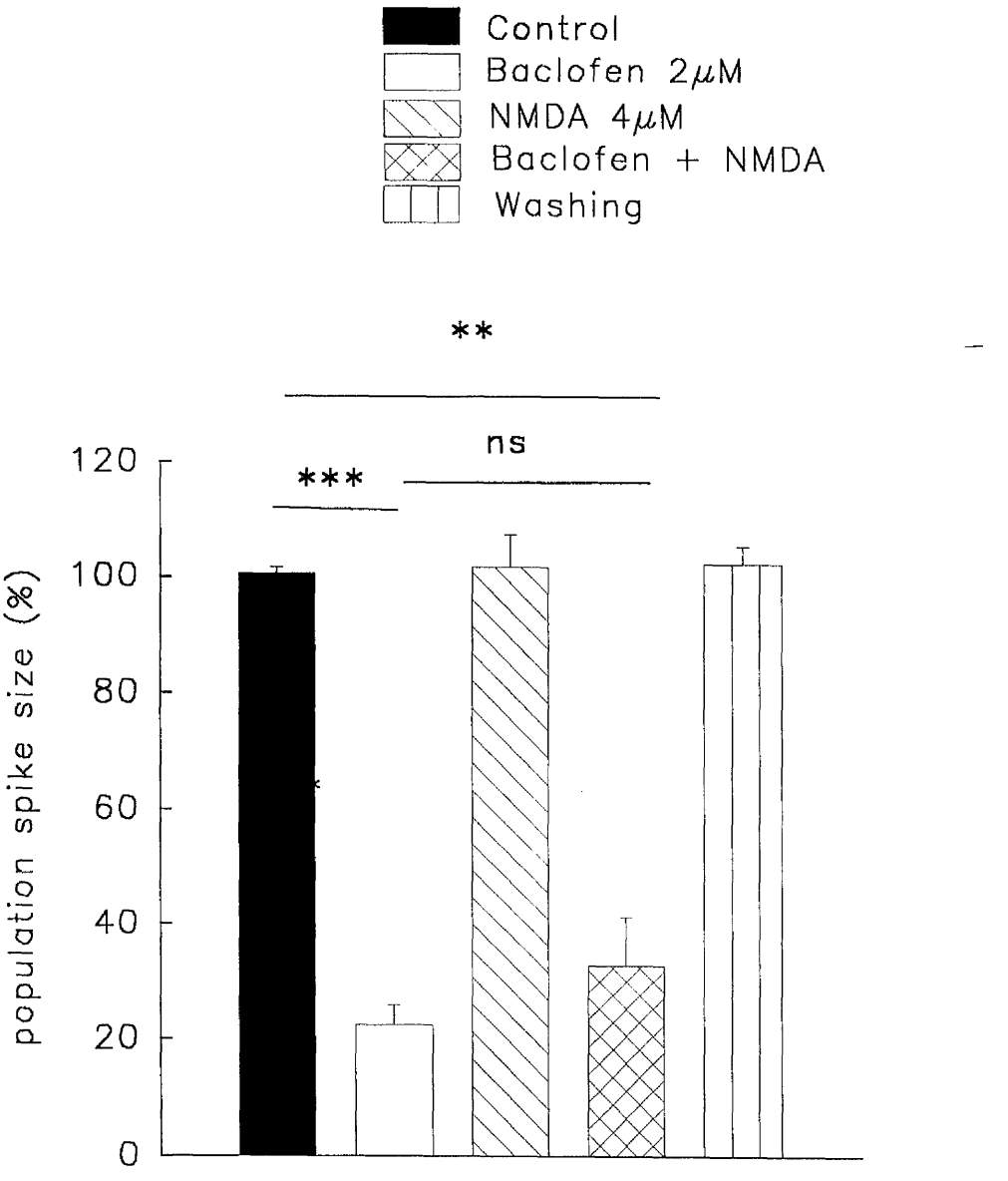

Figure 3. 84. The effect of baclofen 2μM alone and in the presence of NMDA 4μM on CA1 population spikes. Each vertical bar shows mean ± S. E. M for n=3 slices. ** P<0.05, *** P<0.001.

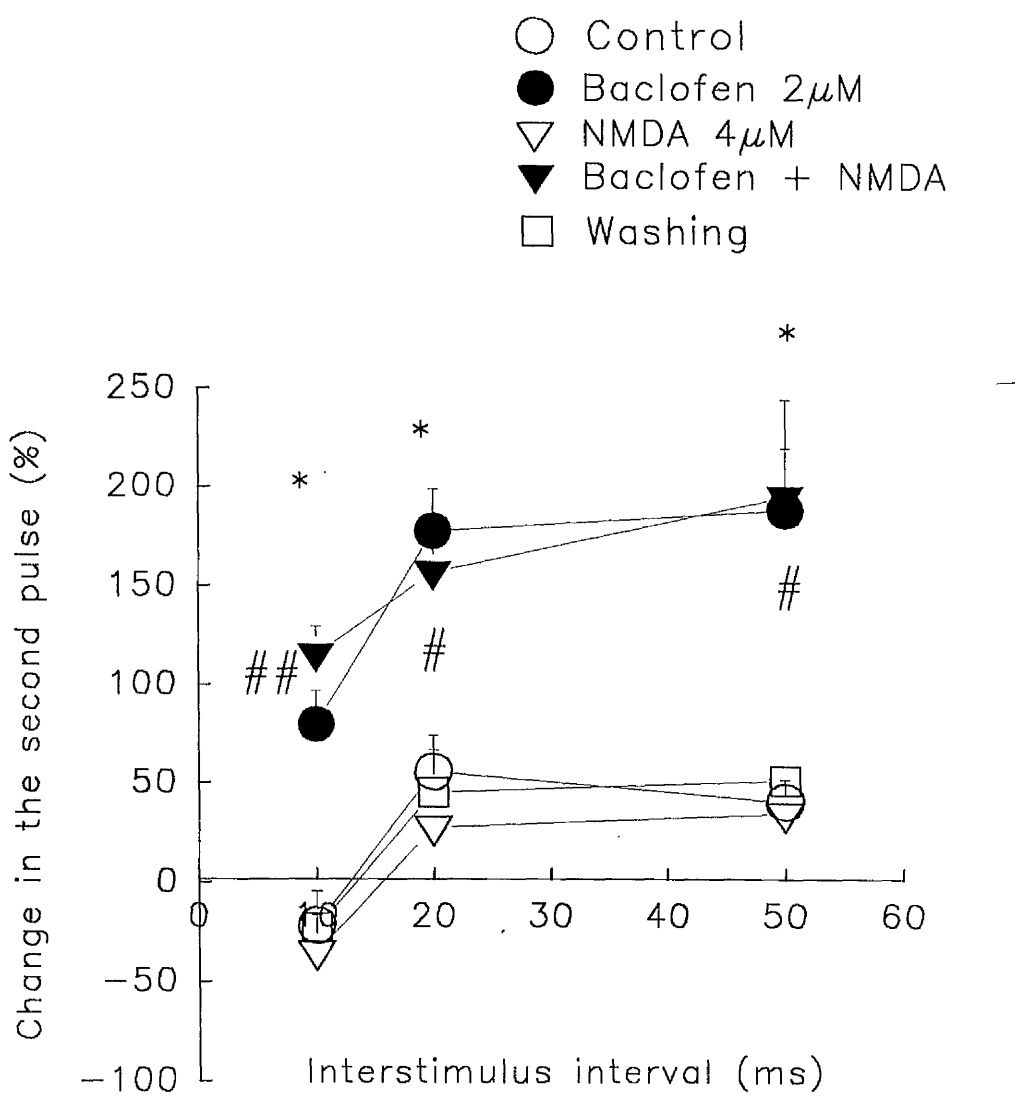

Figure 3. 85. The effect of baclofen 2μM alone and in combination with NMDA 4μM on paired-pulse population spikes evoked from CA1. Each vertical bar shows mean ± S. E. M for n=3 slice. * P<0.05 for difference between control and baclofen. # P<0.05, ## P<0.01 for difference between control and baclofen + NMDA.There is no difference between responses to baclofen and baclofen + NMDA.

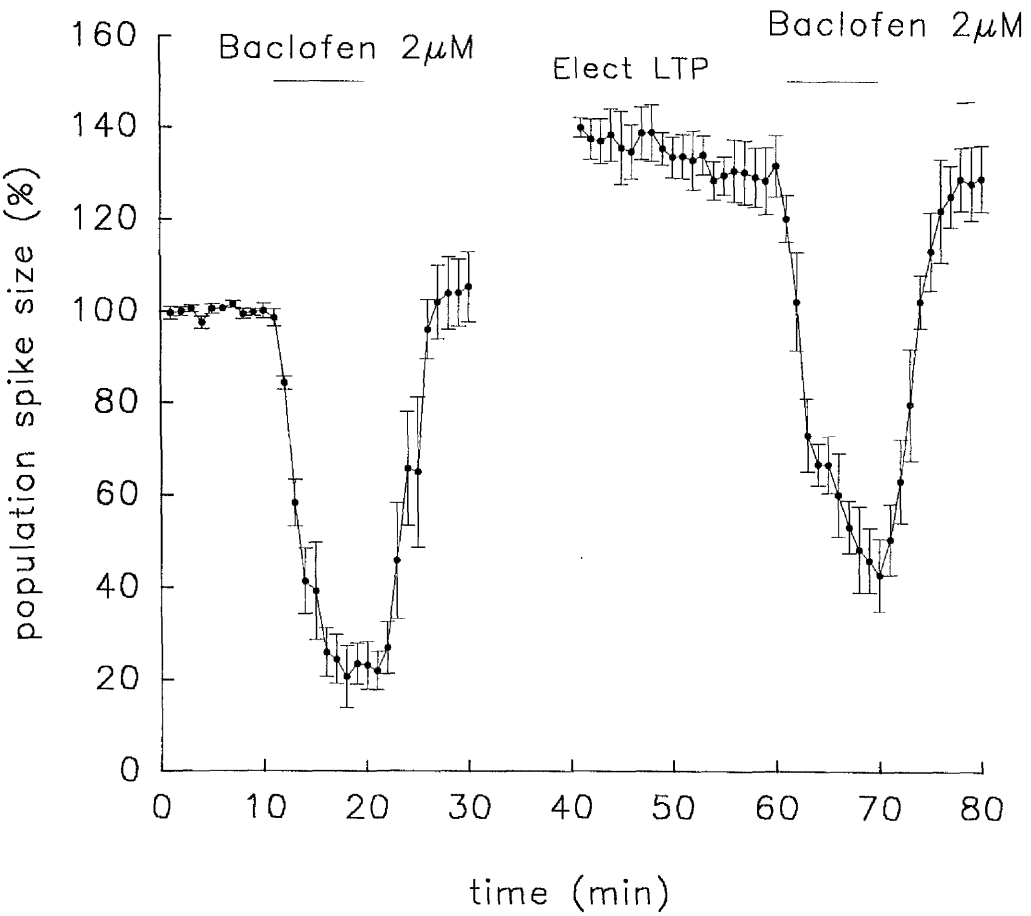

Figure 3. 86. Graph showing the effect of baclofen 2μM before and after induction of electrical LTP on the size of population spikes evoked from CA1 in hippocampal slices. Points are shown as mean ± S. E. M for n=4 slices. The difference was not significant.

Induction and establishment of LTP by application of glycine

Glycine can potentiate the NMDA receptor activation by acting at a strychnine insensitive receptor site. Therefore a separate set of experiments were performed using glycine to activate strychnine-insensitive allosteric glycine sites on the NMDA receptors. Glycine was used at concentrations of 1 and 5mM. At 5mM glycine induced a large long lasting enhancement of the population spike size (Figs. 3. 87A & 3.87B). Inducion and establishment of long term potentiation was also obtained by application of glycine at 1mM (Fig. 3. 88) The amount of LTP attained a level of 21.6% ± 5.51 greater than control amplitude.

The combination effect of glycine and glycine receptor antagonist 7-chlorokynurenic acid on long-term potentiation

To test whether antagonism of the glycine site would prevent the induction of long term potentiation, the experiments were performed using glycine 1mM in the presence of 5, 7-dichloro kynurenic acid (DCKA) 20μM. DCKA was applied 10 minutes prior and during the application of glycine 1mM. Under these condition no significant LTP was observed during 30 minutes of washing as illustrated in Fig. 3. 89.

The effect of glycine and subsequent LTP on responses to adenosine

The responses to adenosine were compared before the application of glycine, in the presence of glycine and after subsequent establishment of LTP. The simultaneous superfusion of glycine 1mM with adenosine did not induce any change in the responses to adenosine in normal ACSF. When the subsequent glycine-induced LTP had become established, however, the response to adenosine was depressed such that the population spike was only reduced by 43.32% ± 9.25 of the control size whereas before perfusion of glycine, application of adenosine reduced the population spike size by 64.67% ± 8.5 of control ($p<0.05$) (Fig. 3.90).

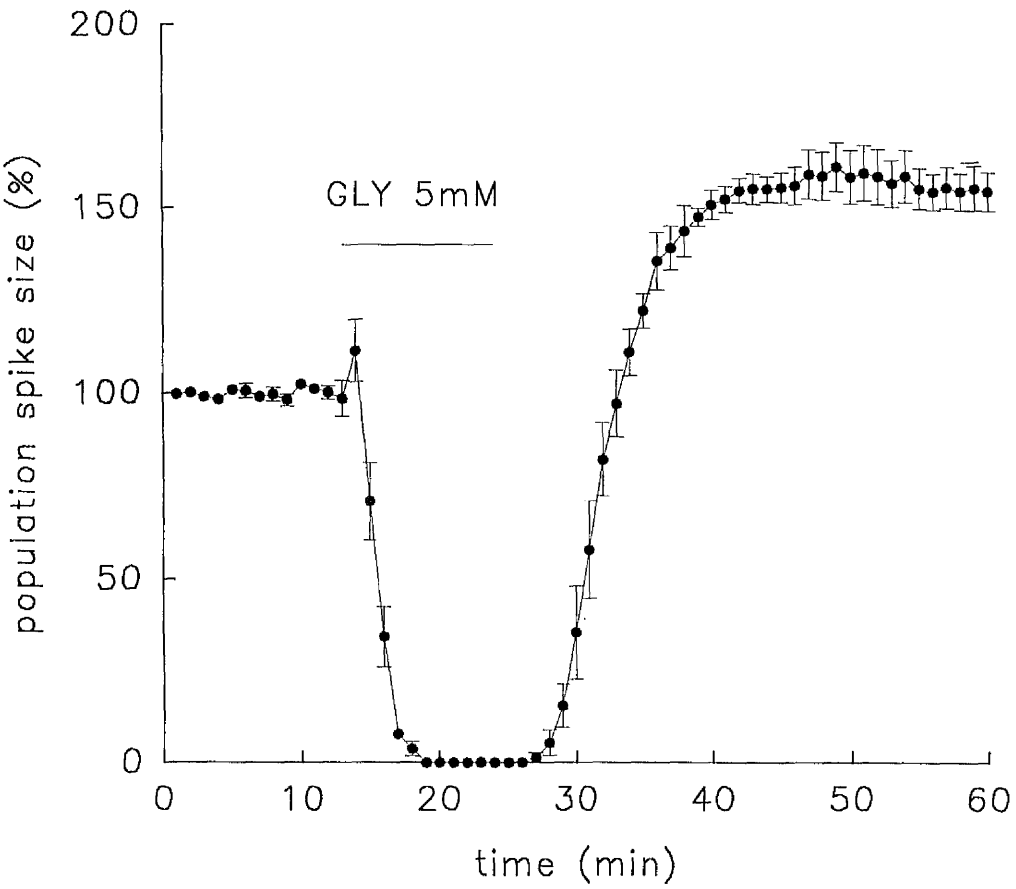

Figure 3. 87A. Time course of the effect of glycine 5mM on orthodromic evoked CA1 population spikes. Points are are shown as mean ± S. E. M for n=5 experiments.

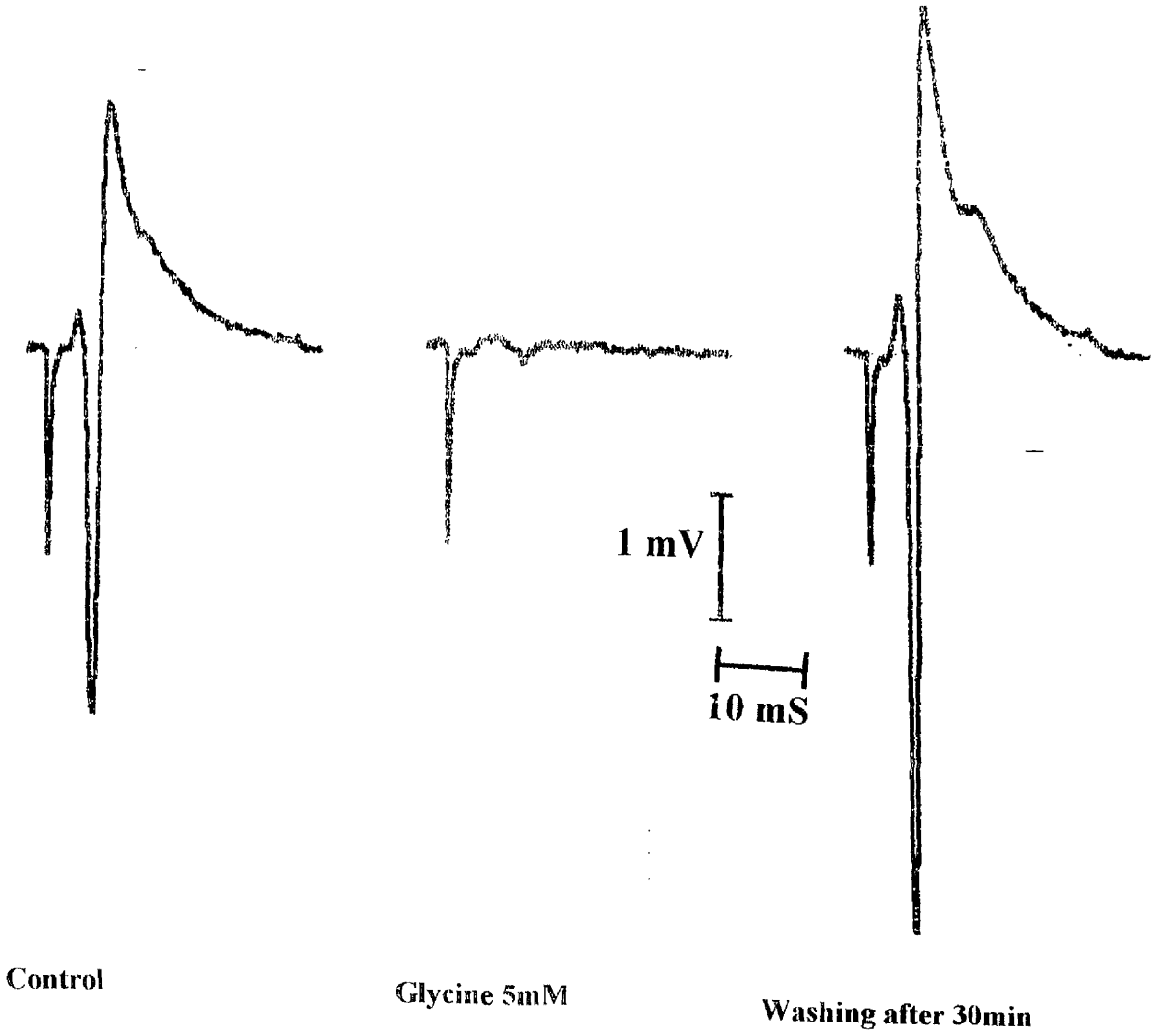

Figure 3. 87B. Sample records showing the long-term potentiation of orthodromic population spikes following application of glycine 5mM.

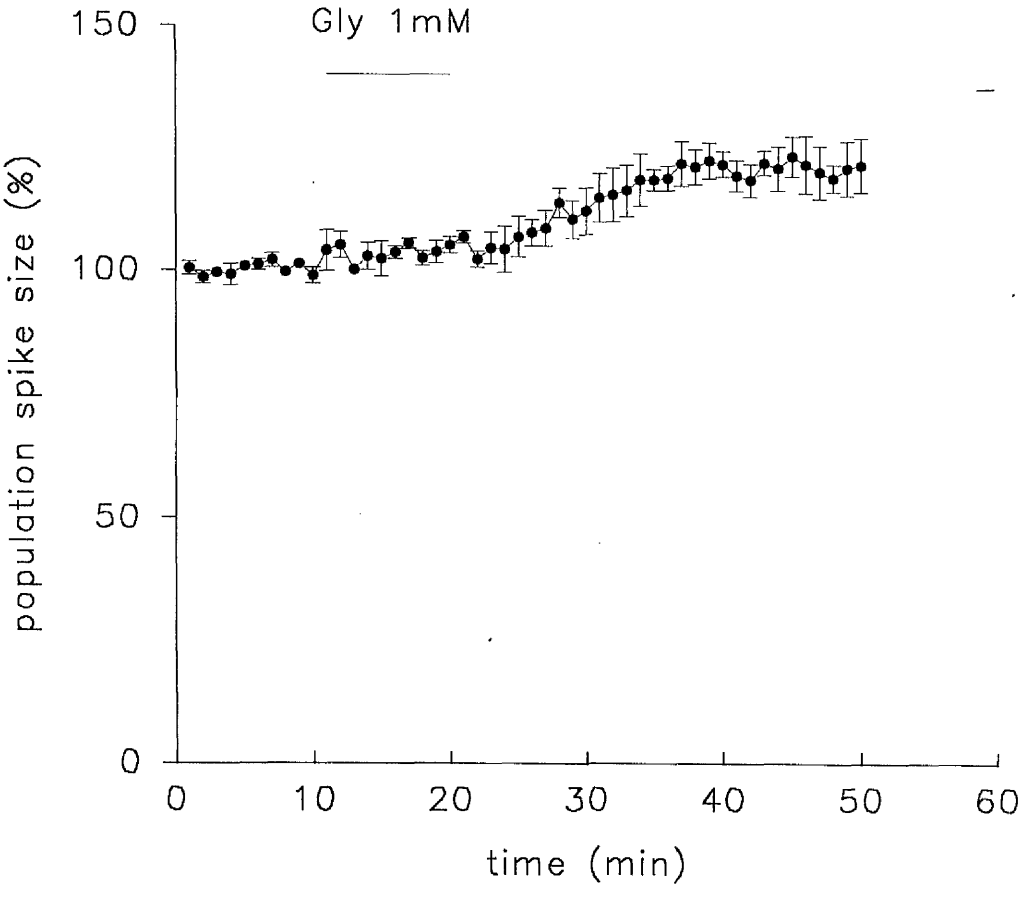

Figure 3. 88. Demonstration of long-lasting enhancement of evoked CA1 population spikes by application of glycine 1mM. Points are shown as mean ± S. E. M for n=3 slices.

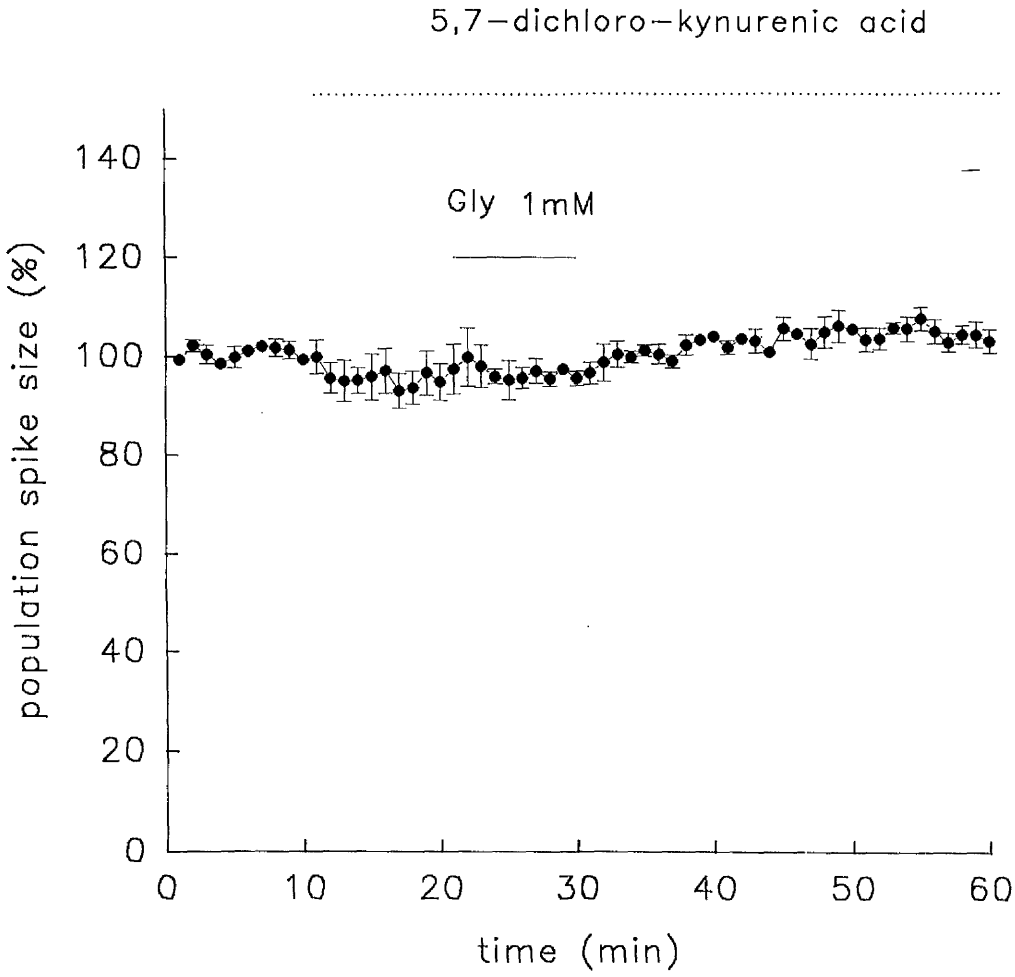

Figure 3. 89. Demonstration that 5,7−dichloro−kynurenic acid at 20μM prevented the long lasting potentiation effect of glycine 1mM on the size of CA1 evoked population spikes. Points are shown as mean ± S. E. M for n=3 slices.

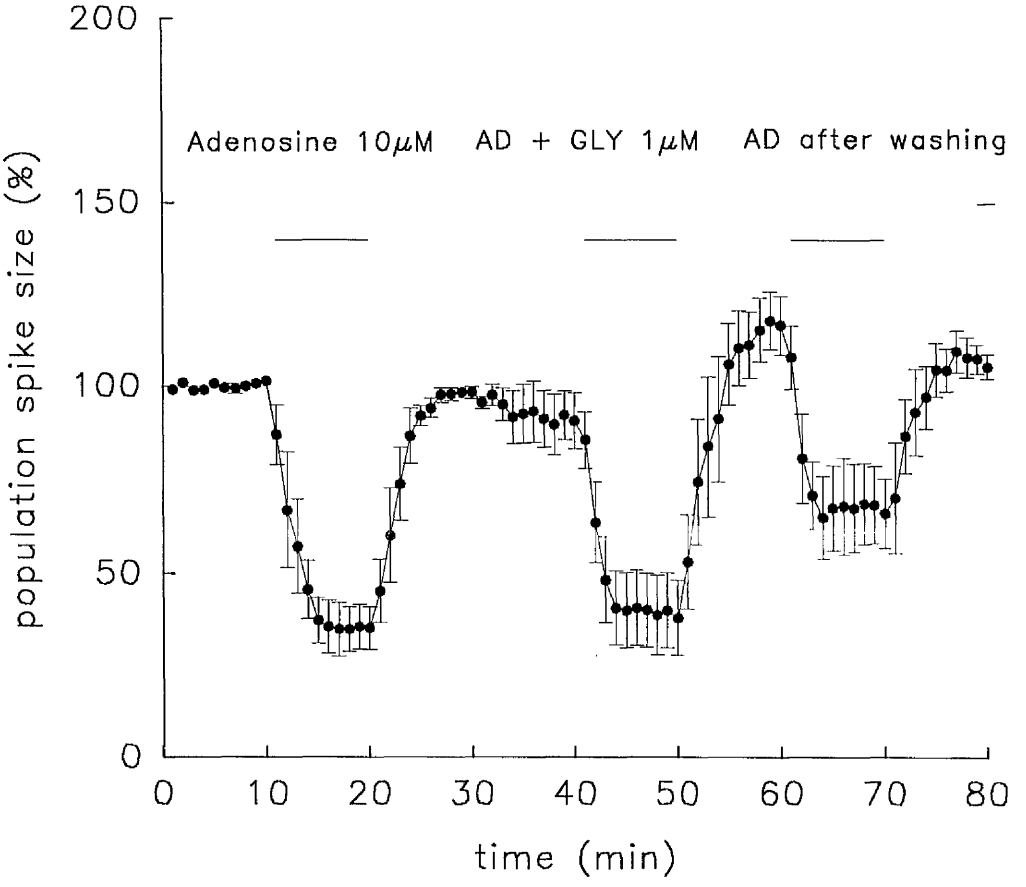

Figure 3. 90. Demonstration of the effect of adenosine alone, in the presence of glycine 1mM and after removal of glycine on the evoked population spikes. Significant difference was observed between the inhibitory effect of adenosine before the application of glycine and after that. Each point shows mean ± S. E. M for n=4 slices.

4: DISCUSSION

Use of hippocampal slices

Since the first demonstration that slices of mammalian CNS could be maintained *in vitro* and were physiologicaly viable (Yamamoto & McIlwain, 1966) the *in vitro* slice preparation has been widely used. Later, it was shown that the hippocampal slice is a useful brain stucture from which electrical activity could be recorded over a long time course (hours), (Bliss & Richard, 1971; Skrede & Westgaard, 1971). Inhibitory mechanisms, frequency potentiation and posttetanic potentiation are well preserved in the slice (Skrede & Westgaard., 1971).

Compared with *in vivo* expriments the advantages of slice preparations are as follows.

1-Certain main afferent and intrinsic pathways can be easily identified and activated selectively

2-Because of the absence of respiratory and cardiac activity in the slice preparation there is no pulsation causing interference with recording .

3-No problem with anesthesia or blood presure maintenance.

4-It is possible to change drug concentration of the bathing medium easily (Schwartzkroin, 1981; Clarck & Wilson, 1992). Therefore the effect of changing ionic composition can be easily studied.

5-Possibility of application of drugs with out the restriction of the blood-brain barrier

6-Possibility of local application of drugs by microiontophoresis.

There are also some disadvantages and limitations in brain slice preparations.

1- Loss of some inhibitory and excitatory input and output pathways. Furthermore particular afferent pathways such as cholinergic , serotonergic and noradrenergic can not be stimulated in hippocampal slices *in vitro* (Dunwiddie et al., 1983).

2- GABAergic inhibitory neurons which are located longitudinally in hippocampal slices are cut when the slice is cut transversely.

3- the Trauma and anoxic period of dissection

4- The ionic environment does not mimic exactly normal extracellular conditions *in vivo* and could influence responses.

Paired-pulse interaction as an indicator to distinguish pre-synaptic from post-synaptic events

Despite intensive investigation, the pre- and/or post-synaptic locus underlying paired-pulse interactions is not entirely clear. In several studies paired-pulse facilitation (PPF) was not altered by post-synaptic events. Schulz et al (1994) showed that the increase in PPF which occured shortly after high frequency stimulation of hippocampal slices was not changed following application of CNQX which was able to return the EPSP slope to baseline. Changing the membrane potential also does not affect the ratio of paired pulse facilitation mediated by AMPA receptors (Clark et al., 1994). It has been revealed that paired-pulse facilitation induced homosynaptically and the change in the test response did not result from the changes in post-synaptic properties such as input resistance (Creager et al., 1980). Using voltage-clamp experiments to examine paired-pulse interaction of pure NMDA responses, it has been demonstrated that paired-pulse facilitation of EPSPs was voltage dependent with maximum facilitation at hyperpolarised stimulus levels and little facilitation at depolarised membrane potential, implying a post- synaptic origin of paired-pulse facilitation (Clark et al., 1994).

Using cultured hippocampal neurones it has been shown that the frequency and amplitude of miniature potentiation was not changed following an IPSP indicating that postsynaptic mechanisms could not be underlying the PPD of IPSP currents. Application of different $GABA_B$ receptor antagonists did not modify the PPD of IPSP in hippocampal neurones in culture (Wilcox & Dichter, 1994).

The other hypothesis underlying paired-pulse facilitation was that the number of synapses which is activated by the second pulse is larger than the first one resulting in a greater test response. No change in the fibre volley as indicator of presynaptic fibre potential was observed during facilitation or inhibition of both EPSP and population spikes (Lømo, 1971b; Higgins & Stone, 1995; Nikbakht & Stone, 2000). Therefore it can be concluded that facilitation is not related to the number of presynaptic fibers activated by the second pulse. In a recent study at climbing fibre Purkinje synapses it has

been demonstrated that changing the number of stimulated climbing fibres does not change the amount of PPD (Hashimoto & Kano, 1998). In an individual synapse the level of PPF was associated with synaptic release. It is possible that where presynaptic Ca^{++} is adequate to produce release and hence an EPSP for the conditioning response it is also sufficient to cause facilitation. Conversely when Ca^{++} influx is inadequate to produce release and an EPSP, it may follow that it is inadequate to produce facilitation (Chen et al., 1996).

On the other hand some evidence shows more directly that paired-pulse facilitation and paired-pulse inhibition are presynaptic in nature. Changing the calcium /magnesium ratio modified the paired-pulse depression or facilitation in both peripheral and central nervous system (Thies, 1965; Hashimoto & Kano, 1998) as we demonstrated here by omitting Mg^{2+} from the medium. Lowering the Ca^{2+} resulted in lesser Ca^{++} influx and subsequent reduction in transmitter release but the amount of PPD decreased. Decreasing the Mg^{2+} concentration resulted in greater influx of Ca^{2+} and induced more PPD. In hippocampal neurones, lowering Ca^{2+} (or high Mg^{2+}) increased and high Ca^{2+} (or low Mg^{2+}) decreased PPF (Leung & Fu, 1994). It seems that a direct relationship exists between the amount of facilitation and initial release probabilities. The release probabilities appears to depend on the size of a readily releasable pool. Synapses behave as if they have a readily releasable pool of a small size that can be depleted, and the synapse's release probability is partly determined by the size of this pool. If PPF resulted from residual Ca^{2+} in the presynaptic nerve terminal it can be concluded that there is less residual Ca^{2+} in solution containing elevated $[Mg^{2+}]/[Ca^{2+}]$ compared with reduced $[Mg^{2+}]/[Ca^{2+}]$ ratio. In the neuromuscular junction it has been shown that the amount of PPD is also positively correlated with the quantal content of the first response suggesting that there is a decrease in the number of quanta available to be released by the second action potentials. However, the depletion is limited to a subset of vesicles having higher release probabilities so that a given terminal is less likely to respond if there has been a successful fusion of synaptic vesicles with the presynaptic membrane in response to the first action potentials (Debanne et al., 1996). Furthermore, using fluorescent imaging of Ca^{2+} in the presynaptic terminal zone an increase of Ca^{2+} with the second of a pair of afferent pulses has been demonstrated (Hess & Kuhant, 1992).

In contrast, in another study in CA1 hippocampal slices using single synapses it was concluded that the paired-pulse facilitation was not dependent on the release machinery because failure or success of release does not make any difference to paired-pulse facilitation (Stevens & Wang, 1995). But overall most of the evidence is in favour of a direct relationship between Ca^{2+} influx and release machinery with paired-pulse changes.

In hippocampus manipulation of various presynaptic targets affects the amount of PPF, adenosine and baclofen, each of which has been known as an inhibitor of neurotransmitter release could increase the amount of paired-pulse facilitation and reduce depression by activating adenosine A_1 and $GABA_B$ receptors respectively, indicating that paired-pulse interaction is due to the presynaptic changes (Dunwiddie & Haas, 1985; Davies et al., 1990; Nathan et al., 1990; Higgins & Stone, 1995; Nikbakht & Stone, 2000).

Activation of presynaptic metabotropic glutamate receptors modulates paired-pulse responses. In Calyx of Held synapses in rat brain slices activation of metabotropic receptors inhibit the transmitter release through a reduction in the presynaptic Ca^{2+} currents (Takahashi., 1996).

In the present experiments the interstimulus intervals of 10, 20 and 50 ms have been examined. PPF was greater for intervals of 50 and 20 than for the shorter interval of 10ms. One explanation may be that the absolute level of the intracellular $[Ca^{2+}]$ achieved by the second action potential is less for the short intervals supporting the dependency of paired-pulse depression and facilitation on the Ca^{++} hypothesis and subsequent neurotransmitter release.

However in general, measurement of paired-pulse ratio in experiments performed with extracellular stimulation in the hippocampus should be interpreted with caution, because the responses will reflect the mean of the simultaneous occurrence of PPF and PPD in different synapses. Debanne et al (1996) has demonstrated that PPF and PPD occured randomly in given synapses under their control. If fewer synapses undergo PPD, the mean paired-pulse ratio from many synapses will be increased.

There is increasing interest in the phenomenon of paired-pulse facilitation and depression as an increase and decrease in synaptic output. Involvement of PPF in the

pathological process of seizure susceptibility has been suggested (Zhao & Leung, 1993) because in the hippocampal slices prepared from previously partially kindled animals, a persistent increase in paired-pulse ratio of the EPSP has been observed. It seems that a normal PPF may be necessary for hippocampal function. Kindled rats had a deficiency in retention of a radial arm maze task (|Leung & Shen, 1991) and mutant mice without the α-calcium-calmodulin kinase II showed lower PPF, lower long-term potentiation and deficient spatial learning (Silver et al., 1992).

Effects of nucleotides on single and paired-pulse responses

The ability of adenosine to decrease the size of single orthodromic potentials and to depress the paired-pulse inhibition and increase paired-pulse facilitation is well documented. In the present experiments these effects of adenosine are also reconfirmed. The synaptic adenosine that mediates the inhibitory effects on neuronal excitability and transmitter release may come from a variety of sources. The most obvious source is degradation of ATP and the other nucleotides to adenosine by ectonucleotidases. In some instances it has been shown that the majority of ATP is metabolised in biological systems within 30s (Green al, 1995). This could result in build up of the adenosine concentration in the synaptic region.

The other possibility could be direct activation of hippocampal P_2 receptor by nucleotides. Indeed, P_2 receptors are expressed in the hippocampus (Kidd et al , 1995; Collo et al., 1996). The possibility of direct action of nucleotides could be examined by application of non-hydrolysable analogues such as βγ-methyleneATP or inhibition by recognised P_2 receptor antagonists. Although the effect of ATP on synaptic transmission is not completely prevented by pharmacological tools interfering with adenosine neuromodulation (Cunha et al., 1996), P_2 receptor antagonists are able to produce effects in hippocampal preparations (Motin & Bennett, 1995; Wieraszko, 1995). Wieraszko (1996) showed that P_2 receptors may have a physiological role in modulating synaptic transmission. However the present results do not show any inhibition of synaptic transmission as a result of direct activation of P_2 receptors because of their prevention by P_1 receptor antagonists. However the frequency of stimulation under

which these experiments were carried out was low while high frequency stimulation was applied in the previous experiments.

Most of the evidence presented here is consistent with the view that nucleotides exert their presynaptic effects to modify paired-pulse inhibition via the activation of adenosine (P_1) receptors. The inhibitory effect of ATP and its more stable analogue βγ-methylene ATP on single spikes and changes in paired-pulse responses was mostly or totally prevented by application of 8PT , a P_1 receptor antagonist, and adenosine deaminase which converts adenosine to inactive compounds and abolished the effect of endogenous and/or added adenosine. These results suggest that conversion of nucleotides to adenosine is an obligatory step for most of the inhibitory effects, because they must be converted to adenosine before they can act on presynaptic adenosine receptors. Application of βγ-methylene ATP induced a small but significant inhibitory effect on both single and paired pulse responses even in the presence of 8PT, although in the presence of adenosine deaminase it was unable to modify the responses.

Suramin, a P_2 receptor antagonist at 50μM prevented the changes induced by βγ-methylene ATP such that paired-pulse inhibition was no longer significantly different from control in all tested interstimulus intervals. This concentration of suramin should block P_2 receptors but would have relatively little effect on nucleotidase activity, for which suramin has an IC50 of at least 100μM in many preparations (van Rhee et al., 1994; Ziganshin et al., 1995; Bultmann et al., 1996; Ziganshin et al., 1996). The ability of suramin to modify the effect of nucleotides further supports this idea that the extracellular catabolism of ATP and γ-substituted ATP analogous into adenosine is mainly responsible for the inhibitory effect, but the possibility of a role for P_2 receptors can also not be excluded. Moreover, P_2 receptors that are insensitive to the known P_2 receptor antagonists are also recognised (Buell et al., 1996).

Another hypothesis, suggests that nucleotides act directly on P_1 receptors without being first metabolised to adenosine. This view was supported by the suppression of the presynaptic effect of ATPγS, a slowly hydrolysable analogue of ATP by an adenosine antagonist. Blockade of spontaneous epileptiform bursts evoked by magnesium-free

ACSF in the CA3 region of the rat by both adenosine and ATP was prevented in the presence of 8-cyclopentyl-1,3-dimethylxanthine (CPT) but the effect of ATP was resistant to adenosine deaminase indicating a direct activation of P_1 receptors by ATP (Ross et al., 1998). It was recently reported that adenine nucleotides may act directly on P_1 receptors in the heart (Hoyle et al., 1996), although binding studies showed that γ–substituted analogues are not effective displacers of adenosine A_1 receptor binding. (Williams & Braunwalder, 1986).

One resolution of this problem could be that nucleotides activate a P_2 receptor, which then promotes the release of endogenous adenosine within the slices. Such a mechanism was proposed by Stone (1981) to explain some effects of nucleotides on smooth muscle tissue and confirmed directly by showing the release by nucleotides of radio-labelled adenosine in these preparations. The possibility of such a mechanism represents an additional complication in the interpretation of nucleotide effect in the nervous system. This problem is compounded by the complexity of P_2 receptor subtypes and their sensitivity to agonists.

The other objective of this study was to determine the effect of the nucleotides on presynaptic or postsynaptic sites by using paired-pulse phenomena. The results demonstrated here clearly showed that both ATP and βγ-methylene ATP reproduced the effects of adenosine and reduced the amount of inhibition at a 10ms stimulus interval and increased facilitation at 20 and 50ms suggesting the presynaptic site of these responses. αβ-MethyleneATP which is another stable analogue of ATP induce no effect either on single or on paired-pulse responses in this study. Many P_2 receptor subunits, for example such as $P2X_3$ and $P2X_4$ subunits found in the CNS have little sensitivity to αβ-methyleneATP, possibly explaining the absence of response to the latter nucleotide in this study.

In summary the results presented in this section show the presynaptic inhibitory effects of nucleotides were mostly suppressed by P_1 receptor and partly by P_2 receptor antagonists, which could be accounted for by the induction or release of endogenous adenosine. This theory may help to describe the apparent discripancy by Cunha et al

(1998) that the original concentration of substituted analogues of ATP including βγ-methyleneATP, βγ-imidoATP and ATPγS were not changed significantly, showing that these nucleotides were not significantly metabolised. Their inhibitory effects were partly blocked by adenosine deaminase, a P_1 receptor antagonist and αβ-methylATP. These results therefore could be accounted for if activation of a P_2 receptor triggers the release of endogenous adenosine from the slice. Some adenosine was detected by Cunha et al (1998) in the presence of nucleotides and it is likely that the concept of channelling– remains correct in principle but modified in the sense that the local generation of adenosine is due to activation of P_2 receptor at or in the vicinity of axon terminals.

Complex responses to ATP: fade due to P2-nucleotidase inhibition and receptor mediated adenosine release

An unexpected finding was during the comparison of hippocampal responses to adenosine and ATP when it become clear that the response profiles were different in slices with large population spikes. It was revealed that the qualitative nature of the responses to ATP was dependent on the size of the orthodromic field potential as described in the result section. When the maximum control population spikes exceeded 5mV, the initial inhibition exhibited a fade with time, suggesting a form of desensitisation. The responses to the ATP and during 10 minutes of application can be divided in two components.

1: The initial inhibitory responses, which reached their maximum after 3 to 4 minutes of superfusion

2: The fade in responses, which has been seen after that initial inhibition and was observed with both ATP and βγ-imidoATP

Adenosine showed inhibition but no fade was seen during application of this nucleoside. The inhibitory responses to ATP and βγ-imidoATP were inhibited by the P_1 receptor antagonist 8-phenyltheophylline and by superfusion with adenosine deaminase. Blockade of nucleotide responses by P_1 antagonists and more specially adenosine deaminase supported the view which was suggested by previous authors (Cunha et al 1998; Dunwiddie et al., 1997; Nikbakht & Stone, 2000) that nucleotides must be converted to adenosine to induce their inhibitory effect. However, if in these

experiments nucleotides had to be broken down to adenosine in order to inhibit the synaptic potentials, more resistant compound such as βγ-imidoATP should be less effective than the parent compounds. This difference was not observed in these experiments. The potency of βγ-imidoATP was approximately the same as adenosine, which seems surprising for a P_1 receptor. This is characterised by the potency order adenosine>AMP>ADP>ATP (Kennedy, 1990).

The fade in responses which was observed with both ATP and βγ-imidoATP could not be attributed to the P_1 receptor activity. Firstly, the fade in responses was not observed in response to adenosine. Secondly, suramin as a P_2 receptor antagonist was able to prevent the desensitisation component of the nucleotide responses. Suramin did not induce any effect on responses to adenosine implying that the P_1 receptor was not affected by suramin. Moreover suramin blocked some of the initial inhibitory responses to nucleotides raising the question, why, if adenosine is responsible for the nucleotide-induced inhibition, does suramin modify the response profile?

These findings indicated that the nucleotide responses are more complex than can be simply explained by adenosine formation. It was considered that direct activation of P_2 receptors might be responsible for the fade in responses. Indeed, ATP function as a fast excitatory neurotransmitter in the central nervous system has been recognised (Edwards, et al., 1992). ATP can also produce excitation of neurones in several regions of the CNS (Harms et al., 1992; Sun et al, 1992; Tschopl et al., 1992; Frohlich et al., 1996). A subpopulation of rat dorsal horn cells was also depolarised by ATP by activating sodium conductances (Jahr & Jessel, 1983. The demonstration of conductance changes on individual cells and coupling to second messenger pathways suggests that visualised receptors are functionally coupled to channels or other transduction pathways. Excitatory effects of ATP can be mediated by activation of both potassium (Tschopl et al., 1992; Nakazawa & Inoue, 1994; Ikeuchi & Nishizaki, 1995; Dave& Mogul, 1996; Robertson et al., 1996) and calcium (Chen et al., 1994; Koizumi & Inoue, 1997) conductances. In hippocampus, the existance of P_2 receptors has been clearly delineated (Michel & Humphrey, 1993; Balcar et al., 1995). In a very recent study using intracellular recording evidence was provided that nucleotides can directly inhibit

glutamate synaptic release in the CA1 axon terminal of the hippocampus (Mendoz-Fernandez et al., 2000).

A possible role for nucleotidase

The ability of αβ-methyleneADP, an inhibitor of 5′nucleotidase, to increase the amount of fade obtained suggests that the fade could be due to inhibition of conversion of ATP to adenosine. EctoATPases or ectonucleotidases, which were firstly described by Engelhardt (1957), are now recognised as major players in the extracellular metabolism of adenine nucleotides (Zimmermann, 1996) rapidly converting ATP into 5′AMP and ultimately adenosine (Dunwiddie et al., 1997; Cunha et al., 1998). These ectonucleotidase activities are variably expressed on the cell surface of different tissues and cells (Zimmermann, 1992). Those enzymes have been found on the cell membrane of hippocampal cells and the metabolites of ATP produced by activation of ectonucleotidase have been detected in the different brain regions including hippocampus (Cunha et al 1992; Cunha et al., 1994b).

The metabolism of ATP by ectonucleotidases in the neurones of different areas of the brain including hippocampus and in the synaptosomes from a number of brain regions including the cortex (Lin & Way, 1982) and the hippocampus (Nagy et al., 1986) has been reported. However they are different from intracellular ectoenzynmes such as Na^{+}/K ATPase, mitochondrial ATPase and Ca^{++}-transport ATPase (Nagy et al., 1986). It was proposed that when ATP or βγ-imidoATP are added to the slices they are initially hydrolysed by ectonucleotidases to adenosine, which then mediate the inhibition of the evoked potentials.

However, most nucleotides which act as substrate for nucleotidases, including ATP itself, are able to produce feed-forward inhibition of those enzymes (Gorden et al., 1986). This will attenuate the production of adenosine and thus limit activation of adenosine receptors most of which are inhibitory in the hippocampus. Thus, as the level of mucleotide in the slice increases during its superfusion, it will increasingly inhibit its own hydrolysis, resulting in the apparent 'desensitisation', or fade, reported here. It

should be noted that a similar loss of sensitivity to nucleotides has been seen by some previous authors, although without explanation (Kilshin et al., 1994).

It is proposed that the slices with large (>5mV) initial population spikes used in the present study are in better metabolic condition than others, and posses substantially higher levels of nucleotidase activity which would allow this mechanism to operate. The absence of any effect of αβ-methyleneATP in this study is consistent with this explanation, since this nucleotide is said not to be susceptible to metabolism by nucleotidases (Picher et al., 1996). This mechanism may also account for the observation that responses to 50 and 20 μM ATP were often comparable in peak size although the former showed more rapid fade, presumably because of the greater and more rapid inhibition of nucleotidase activity.

However, the ATP responses in the presence of αβ-methyleneADP reach an initial peak, which is at least as large as the initial peak of control responses (Fig. 3. 32) and is certainly not decreased significantly as would be expected on the above proposal. It is possible, therefore, that the initial phase of the nucleotide responses is due to a different mechanism. Since suramin, at a concentration of only 50μM, prevents the initial phase of the responses, this initial component of inhibition may be due to the activation of a P_2 receptor. This would leave a need to reconcile the activation of a P_2 receptor with the earlier evidence that the nucleotide responses are entirely prevented by adenosine antagonists or deaminase. As mentioned earlier, the resolution of this paradox may be that P_2 receptor activation causes the release of adenosine in the slices. A similar mechanism was invoked by Stone (1981) to explain certain features of nucleotide actions in smooth muscles, in which it was shown directly that nucleotides could promote the release of radiolabelled adenosine from tissues.

In summary, the result of this section are consistent with a two-component explanation of the inhibitory effects of ATP and βγ-imidoATP involving their metabolism to adenosine by nucleotidase, with an initial component due to a P_2-receptor mediated release of adenosine.

Occlusive response to adenosine A_1 receptors and muscarinic M_2 receptors

The main finding of this part was that:

1: The combined effects of adenosine A_1 receptors and muscarinic M_2 receptors on neurotransmitter release from stratum radiatum terminals are mutually occlusive.

2: Both muscarinic M, receptors and adenosine A

receptors are located presynaptically.

Presynaptic inhibition mediated by muscarinic receptors has been found in the hippocampus. (Hounsgaard, 1978; Valentino & Dingledine, 1981). Adenosine receptors are found in abundance both pre and postsynaptically and able to modulate neuronal activity in the central nervous system (Stone et al., 1991; Stone & Simmonds, 1991). Both adenosine (Lupica et al., 1992) and acetylcholine ACh (Psarropoulou et al., 1998) act on presynaptic receptors to regulate glutamate release from synaptic terminals including those of the CA1 Schaffer collateral and commissural axons. Adenosine modulates the neuronal sensitivity to ACh. Adenosine can enhance or depress sensitivity to ACh. A negative interaction between M_1 mAChR and adenosine A_1 receptor in the superior cervical ganglion has been reported (Connolly & Stone, 1995). Adenosine A_1 receptors are able to suppress the protein kinase-C mediated stimulatory effect of muscarinic receptors on glutamate release in hippocampal neurones which involve M_1 receptors (Bouron & Reuter, 1997). In Helix central neurones adenosine and its analogues have biphasic actions, enhancing acetylcholine release at higher concentration but depressing them at lower concentration (Brook & Stone, 1988). Both M_1 and M_2 receptors have been suggested to be involved in the regulation of ACh release. However the major muscarinic receptor responsible for cholinergic inhibition of transmitter release is the M_2 subtype. Pohorecki et al., (1988) showed that atropine but not pirenzepine is able to enhance the evoked release of ACh from hippocampal slices, and suggested the autoreceptor in this preparation may be classified as M_2. In the periphery there is evidence that the regulation of potassium conductances by M_2 muscarinic receptors is modulated by adenosine (Brandts et al., 1997).

In the present work when combinations of CPA and oxotremorine-M were tested at a range of concentration on EPSPs, the combined effect was substantially and significantly less than the calculated additive responses. However when the population spike size was tested the combined actual responses were not significantly different from the calculated response. Moreover when combinations of adenosine and oxotremorine-M were examined on population spikes, no significant degree of potentiation or occlusion was observed. Since the EPSP is a better reflection of presynaptic events and population spikes are more probably related to the postsynaptic site, it may be concluded that these interactions occurred at presynaptic sites but not postsynaptic. Experiments using the paired-pulse paradigm presented here allowed confirmation of the above results. Both CPA and oxotremorine-M reduce the paired-pulse inhibition and enhanced paired-pulse facilitation indicating the presence of both A_1 and M_2 receptors on presynaptic terminals and suppression of transmitter release from presynaptic terminals.

It has been reported that ACh depressed synaptic transmission induced in CA1 by stimulation of Schaffer collaterals (Dutar & Nicoll, 1988; Hounsgaard, 1978) and in CA3 by stimulation of the mossy fibres (Williams & Johnston, 1993). Application of ACh on the dendrites of CA1 neurones in hippocampus decreased the amplitude of population spike potentials suggesting that this is a presynaptic effect (Hounsgaard, 1978). It is generally recognised that of the five known subtypes of muscarinic receptors, the M subtype is mainly responsible for postsynaptic depolarisation, whereas the M_2 receptors are primarily responsible for the presynaptic inhibition of neurotransmitter release. Indeed, the release of ACh itself from cholinergic terminals is modulated by M_2, but not by M_1 agonist and antagonists (Meyer & Otero, 1985).

At the neuromuscular junction, activation of presynaptic muscarinic receptors depresses the amplitude of the end-plate potentials, due to a reduction in the number of vesicles released without any change in the postsynaptic sensitivity of the end-plate to ACh (Arenson, 1989). The evidence for presynaptic modulation of transmitter release arises both from electrophysiological studies (Dutar & Nicoll, 1988), as well as the direct measurement of transmitter release (Richards, 1990). However some muscarinic

agonists such as carbachol have stimulatory effects on synaptic transmission in the hippocampus, which are mediated by M_2 receptors located postsynaptically. In the presence of pirenzepine carbachol increased the amplitude of population spikes probably by activation of M_2 muscarinic receptor at postsynaptic sites (Kojima & Onodera, 1998). Conversely, M_1 receptors are able to inhibit transmitter release from the Schaffer collateral / commisural pathway in the hippocampus (Richards, 1990). The muscarinic depression of the synaptically evoked potentials in olfactory cortex slices has been attributed to the activation of presynaptic M_1 receptors (Williams & Constantin, 1988).

Oxotremorine-M has been used as a selective and strong agonist at M_2 receptors (Richards, 1990) to activate selectively the M_2 receptors and to exclude the activation of the other subtypes of muscarinic receptors. On the other hand CPA as a selective adenosine receptor agonist was used to inhibit synaptic transmission. The present results show the ability of A_1 adenosine receptors to depress synaptic transmission at the CA3 to CA1 synapses by a presynaptic mechanism and that the activation of muscarinic M_2 receptors has a similar presynaptic effect.

The present result is different from Worley and colleagues (1987) who observed that oxotremorine-M would prevent the inhibitory effect of adenosine on orthodromically induced synaptic potentials and adenosine induced CA1 hyperpolarisation. However the concentration of oxotremorine-M used was 20μM, 200 times higher than used in the present experiments, yet there was little inhibitory effect at this concentration on the population spikes. The potency of oxotremorine-M obtained here with an IC50 of around 122nM is more typical of M_2 receptor studies. For example, McKinney et al reported an EC50 of 430nM for the inhibition of acetylcholine release, and Auerbach & Segal (1996) observed depression of synaptic transmission at concentrations around 1μM. Although the interactions between adenosine and muscarinic agonists which involve M_1 or M_3 receptors have been described in the other studies (Brook & Stone, 1988; Connolly & Stone, 1995), the present results show that the modulatory effects of adenosine receptors are also exhibited at presynaptic M_2 receptors. This would be consistent with evidence from a mutually occlusive interaction between A_1 and M_2 receptors on cardiac muscle (Brandts et al., 1997). Very recently it has been

demonstrated that muscarinic M_2 receptors only contribute to depression of synaptic transmission during hypoxia when adenosine A_1 receptors are blocked, suggesting a kind of occlusion between these two receptors (Coelho et al., 2000).

The role of potassium conductances in occlusive responses to CPA and oxotremorine-M

Occlusive and nonadditive suppression of transmitter release by CPA and oxotremorine-M suggest that both compounds are acting via a common mechanism. One of the possible mechanisms by which these two receptors exhibit the occlusion could be a potassium conductance. Both receptors are known to modulate potassium currents postsynaptically including the M-current, a leak current and inwardly rectifying K currents (Egan & North, 1986; McCormic & Prince, 1986; Christie & North, 1988; Gerber et al., 1991; Pan & Williams, 1994; Moises & Womble, 1995).

Raising extracellular potassium from 2 to 4mM suppressed the responses to both CPA and oxotremorine-M, suggesting that both receptor types are operating by increasing potassium conductances. Paired-pulse experiments demonstrated here support the view that those potassium channels that are blocked by CPA and oxotremorine-M are located at the axon terminals because raising the potassium concentration prevented changes induced by those agents in normal ACSF. These effects are also supported by the effect of 4-aminopyridine (4AP). 4AP is an aminopyridine which is used as a blocker of several membrane potassium channels (Meves & Pichon, 1977., Perkins & Stone, 1980., Cook & Quast, 1990). It has been shown that 4AP is able to facilitate synaptic transmission and cause seizures in the hippocampus and other cortical regions (Buckle & Haas, 1982; Galvan et al, 1982). However the mechanism of the effect of 4AP could be broadening the presynaptic action potential and subsequently increasing the Ca^{++} influx (Haas et al., 1983). 4AP facilitates transmitter release in the periphery (Thesleff, 1980) and in the hippocampus reduces the paired-pulse facilitation of the EPSP. 4AP by itself was able to reduces the paired-pulse facilitation and increased inhibition by increasing the conditioning pulse compared with control. The present results show that 4AP is able to prevent the presynaptic inhibitory effects of both adenosine and

oxotremorine-M because neither of the agonists used here was able to change significantly the paired pulse ratio compared with control. Perkins & Stone (1980) suggested that an increase in K conductances might mediate adenosine action and Scholfied & Steel (1988) proposed that an aminopyridine-sensitive potassium conductance in nerve terminals of olfactory cortex are responsible for the effect of adenosine.

The present results suggest that presynaptic K conductances represent a common target of adenosine and muscarinic receptor agonists responsible for occlusive behaviour. There is some evidence that muscarinic activation of K conductances and subsequent hyperpolarisation is mediated by the M_2 receptor subtype (Dutar & Nicoll, 1988). Adenosine is also able to modulate synaptic transmission by acting at presynaptic A_1 receptors, though the ionic mechanisms remain unclear (Dunwiddie & Haas, 1985; Fredholm & Dunwiddie, 1988; Thompson et al., 1992). Previous work has suggested that the suppression of transmitter release is mediated by a reduction of calcium influx (Schubert & Mitzdrof, 1979 ; Ribeiro et al., 1979) or the calcium availability to the release process (Hounsgaard, 1978). The blockade of voltage dependent calcium channels by adenosine and muscarinic receptors exhibits occlusion on presynaptic calcium influx (Qian & Saggau, 1997) and might, therefore, underly their occlusive interaction on transmitter release. There are also some reports that activation of muscarinic M_2 receptors and adenosine A_1 receptors may be linked to the transduction systems, such as inhibition of adenylate cyclase (Van Calker et al., 1979; Vickroy & Cadman, 1989). However several groups have reported that presynaptic cholinomimetic effects in the hippocampus are not mediated by a suppression of calcium channels (Scanziani et al., 1995), so that potassium conductances may be more relevant.

Taken together, these finding suggest that the occlusive interaction of A_1 and M_2 receptors on transmitter release may stem from a common action on presynaptic potassium conductances. A similar convergence was reported by McCormick and Williamson (1989) on postsynaptic sites. These effects could be secondary to the reported effects on calcium conductances or the calcium changes could be secondary to the changes of potassium movements altering the polarisation state of the presynaptic

terminals. Halliwell & Scholfield (1984) showed that when K conductances were blocked by application of 4-AP, tetraethyl ammonium (TEA) or barium, adenosine had no effect on transmitter release whereas Ca^{++} action potentials in the soma were still adenosine sensitive, suggesting that adenosine blocked these Ca action potential directly. In one study, the effect of 2-chloroadenosine, neuropeptideY (NPY) and baclofen have been compared on the EPSPs evoked from hippocampal slices in the presence of 4AP and low calcium medium. 4AP reduced the inhibitory effect of all compounds on EPSP slope. Using low calcium medium restored the effect of NPY and baclofen but not 2-chloroadenosine. The authors concluded that both NPY Y2 and $GABA_B$ receptors inhibit transmitter release by a calcium dependent mechanism but A_1 adenosine receptors may operate on a different presynaptic mechanism (Klapstein & Colmers, 1992).

Interaction between adenosine and NMDA receptors

The strategy underlying the experiments in this chapter was to study the interaction between adenosine and NMDA receptors. The present experiments mainly focused on presynaptic sites by using the paired-pulse paradigm which is modified by presynaptic modulation that changes the probability of transmitter release and is accepted as providing a more accurate indicator of presynaptic events than the study of population spikes and post-synaptic events (Hess et al., 1987; Wilcox & Dichter, 1994; Wu & Saggau, 1994; Hashimoto & Kano, 1998). Paired-pulse inhibition at interpulse intervals of around 10ms reflects 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, on the other hand, at longer interpulse intervals, results from residual intraterminal calcium which increases transmitter release (Hess et al., 1987; Wu & Saggau, 1994; Debanne et al., 1996; Kleschevnikov et al., 1997).

Different approaches were used to activate the NMDA receptors under which the effect of adenosine on single and paired-pulse population spikes was studied. The influences of these approaches on adenosine responses are discussed under the appropriate heading in the following sections.

Decreased sensitivity to adenosine by direct application of NMDA

In accord with many previous studies, adenosine at 10μM depressed the population spike amplitude. This effect was prevented by superfusing the slices with NMDA at 4μM, confirming the results of our original study (Bartrup & Stone, 1990). The original studies on adenosine sensitivity and magnesium removal (Bartrup & stone, 1988 & 1990) were performed using population spike potentials, changes of which are normally considered to involve changes of postsynaptic excitability. A subsequent study of single cell responses to the activation of P_1 adenosine receptors showed a depression of firing which was less apparent in the presence of NMDA than in the presence of quisqualic acid or

acetylcholine (Bartrup et al., 1991). Although interpreted as consistent with a postsynaptic locus for the interaction between NMDA and adenosine receptors, it is difficult to be certain of the site of action of agents applied by microiontophoresis (Stone, 1985), and attempts to do so by, for example, lowering extracellular calcium, complicate interpretation by modifying neuronal excitability and receptor function.

Several possibilities may account for the interaction between adenosine and NMDA.

a) Activation of postsynaptic NMDA receptors that are in abundance in the postsynaptic sites (Fagg et al., 1986) reduces the adenosine sensitivity simply by reducing hyperpolarisation of the cell membrane which is induced by adenosine via potassium or chloride conductances.

b) The second possibility could be modulation of presynaptic adenosine receptors by postsynaptic NMDA receptors through retrograde messenger compounds. Following activation of postsynaptic NMDA receptors retrograde messenger agents such as arachidonic acid or platelet activating factors or gaseous molecules including carbon monoxide (CO) and nitric oxide (NO) cross the synaptic cleft and induce a series of undefined process to increase transmitter release (Williams, 1996) which can in turn reduce the effect of adenosine.

c) Finally a pure presynaptic mechanism could be underlying the interaction between these two receptors. This mechanism could be more relevant because if two receptor types are located close to each other for example on the presynaptic nerve terminals, it is more probable that they may interact with each other. There is already ample evidence for the existence of presynaptic glutamate receptors (Forsythe & Clements, 1990) and especially presynaptic NMDA receptors (Fink et al., 1990; Martin et al., 1991; Overton & Clark, 1991; Cai et al., 1991; Kato et al., 1999) on terminals in the hippocampus and other regions of CNS.

It is a general agreement that most of the adenosine receptors are present on the presynaptic nerve terminals in the hippocampus and they are substantially responsible for modulation of the other neurotransmitters.

The present data show that the presynaptic adenosine and NMDA receptor populations can interact in such a way that NMDA receptor activation suppresses the inhibitory effects of adenosine on transmitter release assessed using paired-pulse interactions. This occurs at levels of NMDA receptor activation, which are not themeselves sufficient to

alter paired-pulse inhibition and strongly suggests that the primary site of the interaction is presynaptic. These effects of NMDA were prevented by co-application of AP5.

Kato., et al (1999) showed that N6cyclohexyladenosine, an adenosine agonist, changed the PF without changing the baseline responses. They also demonstrated that adenosine receptor antagonists and increasing the extracelullar concentration of Ca^{++} was able to block the LTP inhibition mediated by low concentration of NMDA. Chernevskaya et al (1991) noted that NMDA receptor agonists inhibited the neurotransmitter release by depression of the N-type Ca^{++} channel. This depression was blocked by 2-AP5, indicating the involvement of presynaptic NMDA receptors in synaptic transmission.

The NMDA mediated suppression of presynaptic adenosine responses may occur at NMDA gated calcium channels or at the NMDA receptor itself. The present study demonstrates that inclusion of AP5 prevented the suppression of adenosine responses by NMDA suggesting that the interaction of adenosine and NMDA occurred at the receptors.

Moreover, the inability of AMPA and kainic acid to modulate single and paired-pulse responses provided further evidence for the specificity NMDA receptor interactions with adenosine.

The effect of electrical LTP on responses to adenosine

Another protocol, which was used to activate NMDA receptors, was the induction of electrical LTP. This protocol may be more physiologically relevant than slice perfusion with NMDA itself. After induction of LTP, which is known to involve the activation of NMDA receptors by synaptically released glutamate adenosine responses were reduced. Electrical LTP also significantly reduced the effects of adenosine in reduction of paired-pulse inhibition or enhancement of paired-pulse facilitation. Increasing the stimulus strength from approximately 70% of maximum to a level producing maximum spike size did not affect the responses to adenosine. This confirmed that the effect of LTP was not due to the increase in population spikes. Although most evidence favours a postsynaptic site for the expression of LTP by activating the postsynaptically located NMDA receptor complex, presynaptic effects can not entirely excluded. Several groups have reported a decrease in synaptic failures during LTP which can be interpreted as a change in the

probability of neurotransmitter release and could be due to a purely presynaptic modification (Kullmann & Nicoll, 1992; Stevens & Wang, 1994; Nicoll & Malenka, 1999).

Alternatively, retrograde messenger agents which can be produced following NMDA activation by electrical LTP may have a role in the modification of adenosine responses to paired-pulse interactions. Kleschevnikov et al., (1997) described a positive correlation between PPF and the early phase of LTP in CA1 hippocampal slices. They concluded that early phase of LTP resulted from a mixture of presynaptic and postsynaptic changes by an increase in the probability of transmitter release and increase in the number of postsynaptic binding sites respectively. It has also been reported earlier that the maintenance phase of LTP and PPF induce the same changes in quantal content due to a presynaptic mechanism in guinea-pig hippocampal slices (Kuhnt & Voronin, 1994). An *in vivo* study on the synapses of the perforant path to dentate gyrus granule cells demonstrated a parallel increase in paired pulse ratio indicating involvement of presynaptic changes in the maintenance of LTP (Christie et al., 1994).

Role of magnesium ions in adenosine responses

When magnesium was omitted from the perfusion solution, a dramatic decrease in potency of adenosine in the depression of population spikes has been observed, such that the adenosine responses were not significantly different from control. Similar results have been reported by previous authors (Bartrup & Stone, 1988). The paired-pulse responses also showed that adenosine was no longer able to induce any significant change in the paired-pulse ratio compared with control.

Zero Mg^{++} may influence the inhibitory action of adenosine in several ways. It is generally believed that the NMDA receptor is coupled to voltage sensitive conductances and that the voltage sensitivity of NMDA channels is regulated by Mg^{++} in a negative manner (MacDonald et al., 1982; Mayer et al., 1984). An increase in the inward ionic currents through the ionic channels linked to the NMDA receptors result in generation of excitability (Mayer et al., 1984). Results from the paired-pulse tests presented here show that zero Mg^{++} may affect some presynaptic targets at and prevent the effect of adenosine. A reduction in sensitivity to adenosine may be due in part to the activation of

presynaptic NMDA receptors following withdrawal of magnesium. Stratton et al (1988) carried out similar experiments in the dentate gyrus and showed a decrease in adenosine responses in zero magnesium media. Following the reintroduction of magnesium but in the presence of AP5, adenosine responses were not restored, indicating the point that NMDA channel activation is necessary for suppression of adenosine responses.

One may simply argue that reduction in sensitivity to adenosine in f Mg^{++} free media resulted from an increase in excitability, since Mg^{++} and Ca^{++} are physiological antagonists and any decrease in extracellular concentration of one results in an effective increase in concentration of the second one and greater influx into the cell. In zero Mg^{++} more Ca^{++} enters the cell generating more depolarisation which can counteract the hyperpolarisation effect of adenosine. Moreover, activation of postsynaptic CA3 pyramidal cells in the hippocampus could elevate the amount of neurotransmitter release in the synaptic region between CA3 and CA1 cells. However at the presynaptic locus, Smith & Dunwiddie (1993) suggested that decreasing Mg^{++} concentration resulted in a larger amount of Ca^{++} entering the nerve terminals, thus facilitating subsequent transmitter release and reducing the presynaptic effect of adenosine.

Some evidence shows that it is unlikely that an increase in excitability accounted for a dramatic decrease in adenosine potency in Mg^{++} free media. Adenosine applied at very high concentrations in experiments performed in zero Mg^{++} ACSF still was not able to abolish the population spikes completely (Bartrup & Stone, 1988). In another experiment the potency of adenosine in reducing the epileptiform activity induced by different technique was compared. When zero Mg^{++} was used, a greater amount of adenosine was required to reduce epileptiform activities to the similar extent than those situations in which bicuculline or penicillin was applied (Lee et al., 1984; Jansuz & Berman, 1993).

A specific interaction between adenosine and NMDA receptors could also be possible at presynaptic nerve terminals. The presynaptic effects of adenosine through the presynaptic Ca^{++} channels could be compensated by opening another route of Ca^{++} via activation of NMDA channels following removal of Mg^{++}. Yeung et al (1985) reported that magnesium ion is necessary for the effect of adenosine through adenosine A_1 subtypes and subsequent presynaptic inhibition of synaptic transmission. From a radioligand binding study it has been revealed that following reduction of Mg^{++}

concentration to 0.5mM the binding of hydroxyphenyl-isopropyladenosine to rat brain A_1 receptors is reduced by 45% (Ströher et al., 1989). A few studies suggested a non-specific enhancement of adenosine effect by bivalent cations. In those studies Ca^{++} but also Mg^{++}, Mn, Co or barium were proposed as ions which are able to facilitate transmitter release (Yeung et al., 1985; Smith & Dunwiddie, 1993). Both Ca^{++} and Mg^{++} have been proposed as the necessary ions to obtain the maximal binding of A_1 agonists to their receptors (Yeung et al., 1987; Traversa et al., 1990).

The effect of glycine on responses to adenosine

In contrast to Mg^{++} , glycine is known as a positive modulator of NMDA receptor channels. The effect of glycine as another alternative method of activating NMDA receptors was also examined. Although the endogenous levels of glycine are believed to be adequate to saturate the strychnine-resistant binding site on the NMDA receptor, the application of exogenous glycine can enhance the activation of NMDA receptors (Minota et al., 1989), and this may be sufficient to induce or facilitate LTP in regions such as hippocampus (Shahi et al., 1993) and superior colliculus (Abe et al., 1990; Platt et al., 1998). The highest density of strychnine-resistant glycine receptors in the rat brain has been found in the CA1 region of hippocampus (Bristow et al., 1986). The generation of LTP by glycine and some structurally related amino acids in rat hippocampal slices have been compared. The results showed that the ability of those amino acids to facilitate LTP is consistent with their rank order of potency to stimulate glycine modulatory sites (Watanabe et al., 1992).

Since exogenous glycine induces LTP in different areas of the brain as demonstrated by previous authors (see above) and the present study, it is assumed that the physiological concentration of glycine is at least too low near the NMDA receptor complex. It is likely that some uptake system may regulate the concentration of glycine at submaximal levels in the cell or in particular near NMDA receptor binding sites. The existence of high - affinity glycine uptake molecules has been reported in the axons of Golgi cells in both hippocampus and neocortex (Pycock & Kerwin, 1981; D'Angelo et al., 1990).

Glycine-induced LTP in organotypic cultures is also due to activation of NMDA receptors, as it can be prevented selectively by NMDA antagonists (Tauck & Ashbeck, 1990; Newell et al., 1997). Although some of these effects may be triggered by the activation of NMDA receptors, such changes may be expressed by mechanisms, which involve associated changes of AMPA receptor function (Shahi et al., 1993). The ability of 1 and 5mM glycine in the present study to induce LTP which was prevented by 20μM dichlorokynurenate confirms the involvement of the glycine site of the NMDA receptor; since dichlorokynurenate has high selectivity for the site (Stone, 2000). The mechanistic similarity between electrically and glycine-induced LTP was further suggested by the occlusion of electrically induced LTP at a population spike size generated by saturating the LTP process.

Adenosine receptors involved in the interaction with NMDA

One explanation for some of the earlier data of Bartrup & Stone (1990) was proposed by Smith and Dunwiddie (1993), who argued that the effects of magnesium removal could not simply reflect the altered balance between calcium and magnesium in determining the amounts of transmitter release and thus account for the loss of sensitivity to adenosine. However parallel and consistent reduction in the potency of adenosine following application of different techniques able to activate of NMDA receptors namely, direct application of NMDA itself, induction of electrical LTP, withdrawal of Mg^{++} and using exogenous glycine, indicates that the above reason can not represent the whole explanation and that amino acid receptors probably contribute to the phenomenon. Of course it is still possible that the activation of NMDA receptors changes sensitivity to adenosine by way of an alteration of intracellular calcium levels or availability to the transduction mechanism.

The specificity of the interaction between NMDA or LTP and adenosine is indicated by the lack of any interaction with the $GABA_B$ receptor agonist baclofen which, like adenosine, is known to suppress transmitter release at low concentrations.

Finally, one of the most surprising observations of this study arose from an examination of adenosine receptor selective agonist, with the finding that, contrary to expectation, NMDA did not modify the inhibitory effects of cyclopentyladenosine, excluding an

interaction between NMDA receptors and A_1 receptors as an explanation of the NMDA-induced loss of sensitivity to adenosine. Rather, the combination of a subthreshold concentration (4μM) of NMDA and CGS21680 at a concentration known to be selective for the activation of A_{2A} receptors and which alone had no effect on the slices, together produced a significant increase of spike size.

The different behaviour of NMDA towards A_1 and A_2 receptor activation implies that the apparent loss of sensitivity to adenosine produced by the presence of NMDA is not due to an antagonism of those adenosine actions responsible for decreasing transmitter release, but reflects a masking of the A_1-mediated inhibition by an enhancement of A_{2A} receptor function. Activation of the A_1 receptor subtype leads to the suppression of transmitter release, especially of glutamate, acetylcholine, norepinephrine and 5-hydroxytryptamine (Corradetti et al., 1984; Spignoli et al., 1984; Jonzon & Fredholm, 1984; Feuerstein et al., 1985; Prince & Stevens, 1992), probably by increasing potassium conductances or inhibiting calcium movements or availability (Haas & Greene, 1984; Trussel & Jackson, 1985). The A_{2A} receptor population has been reported to enhance transmitter release (Correia-de-Sa et al., 1991; Sebastiao & Ribeiro, 1992; Kirkpatrick & Richardson, 1993; Latini et al., 1996; Cunha et al., 1997).

It is tempting to question whether the apparently small (but significant) increase of potential size which develops in the simultaneous presence of CGS21680 and NMDA is sufficient to account for the much more dramatic loss of adenosine sensitivity reported in this and earlier papers. At present it is difficult to resolve this question, but it may be that receptor interactions are relevant. For example, it is now clear that the activation of A_{2A} receptors is able to suppress the effects of A_1 receptor activation (Cunha et al., 1994; O'Kane & Stone, 1998; Latini et al., 1999). The interaction between CGS21680 and NMDA is, therefore, likely to lead to an indirect decrease of A_1 receptor effects as well as producing a direct increase of excitability, and resulting in a further decrease of adenosine sensitivity.

This result appears particularly surprising in view of demonstrations that the activation of A_{2A} receptors can suppress neuronal responses to NMDA in slices and patch-clamp experiments (De Mendonca et al., 1995; Norenberg et al., 1997; Wirkner et al., 2000). It should be emphasised, however, that the interactions described in the present study involved a concentration of NMDA which was not active when tested alone. It therefore

seems that the simultaneous activation of A_{2A} and NMDA receptors at low (subthreshold) concentrations produces an increase of glutamate release and neuronal excitability, whereas their combined activation at higher concentrations which are themselves depolarising - results in antagonism.

Overall, therefore, NMDA receptor activation seems able to modify selectively the presynaptic responses to activation of A_{2A} adenosine receptors, leading to the masking of adenosine's inhibitory activity on transmitter release. The physiological significanee of this is potentially interesting. Craig and White (1992) have proposed that adenosine A_1 receptors present a barrier to the actions of NMDA receptors which must be overcome if the full effects of NMDA receptor activation are to be observed in phenomena such as LTP. The present work suggest that part of the mechanism of overcoming this barrier may be that, under conditions in which the amount of adenosine released by neurons and glia is greatly increased so that the relatively low affinity A_{2A} receptors are activated, the inhibitory A_1 receptors effects are overcome. Such a sequence provides at least one rationale for the otherwise curious co-existence of inhibitory A_1 and facilitatory A_{2A} receptors on the same population of glutamatergic terminals, and is consistent with earlier proposals that A_{2A} receptor activation can suppress responses mediated by A_1 receptors (Cunha et al., 1994; O'Kane & Stone, 1998; Latini et al, 1999).

GENERAL DISCUSSION

The original objective of this project was to explore the interaction between P_1 and P_2 purine receptors and muscarinic receptors for acetylcholine and between adenosine and NMDA receptors. The first study undertaken was of presynaptic adenosine A_1 receptors and muscarinic M_2 receptors. The discovery that selective agonists at these sites produced a less than additive, or occlusive interaction may have significance for the physiological control of transmitter release. Several authors have shown that for example, A_1 receptor antagonists increase transmitter release, implying some resting basal activity of endogenous adenosine.

The first compound, which has been shown to function as a neurotransmitter in the central nervous system, was acetylcholine. In summary acetylcholine influences the neuronal activity via both ionotropic (nicotinic) and metabotropic muscarinic receptors result in increasing excitability, depolarisation of neurones and initiating or inhibition of action potentials. Among the several neurotransmitter systems that have been shown to be modulated in Alzheimer's disease, the most clearly implicated changes are in the cholinergic system. On the bases of pharmacological studies (antagonist blockade) and lesion studies the role of cholinergic systems in memory and learning has been established. AFDX-116, a muscarinic M_2 receptor antagonist, improves memory at eight-arm radial maze tasks in a time dependent manner. Therefore, antagonism of M_2 receptors might also be useful in treatment of memory impairment. Occlusive responses to combinations of CPA and oxotremorine-M could result from an antagonist effect on M_2 receptors imposed by the adenosine agonist. Modulation of ACh release by adenosine receptor subtypes could be used as a therapeutic objective. For example in some types of cognitive deficits enhancing ACh release can be useful. It is established that activation of A_1 receptor subtypes inhibits the release of ACh and activation of A_2 receptors enhances the release of this transmitter. In a recent investigation in synaptosomes prepared from electric organs the stimulatory effect of adenosine on ACh release has been attributed to A_{2B} receptor subtypes. The interaction was purely presynaptic because the preparation was free from post-synaptic membrane. Similarly, the present experiments using paired-pulse phenomena support on interaction at

presynaptic sites. Simultaneous activation of both receptors could be important in the alteration of neuronal damage in stroke or other cerebrovascular disorders including hypoxia or ischemia. It has recently reported that M_2 muscarinic receptors depressed synaptic transmission following hypoxia when adenosine A_1 receptors were blocked.

Blockade of the effect of the adenosine agonist (CPA) and oxotremorine-M by 4-aminopyridine and raising the extracellular potassium suggest that activation of muscarinic and adenosine receptors converge at some points in the functional pathway to the K channel located presynaptically. However we can not exclude the other common pathways for example inhibition of adenylate cyclase and the mechanism of interaction has yet to be explored in detail.

Both adenosine A_1 and A_2 receptors co-exist in the same nerve terminal and their net effect depends on the activation of each receptor in every area. The inactivity of adenosine itself in these experiments can be attributed to the compensation of modulation of muscarinic effects produced via activation of each adenosine receptor subtype by the other one.

Similar thoughts apply to the interaction between NMDA receptors and adenosine receptors. Glutamate is the major excitatory transmitter in the hippocampus and the other regions of CNS. NMDA receptors are considered to be important in different behavioural processes and cognitive functions including anxiety, depression, schizophrenia, psychomotor stimulation, learning and memory and sensitization or tolerance to the behavioural effects of drugs. According to the present data, activation of NMDA receptors would have the effect not only of directly depolarising neurones, but also of suppressing the presynaptic inhibitory tone mediated by adenosine. This double action would be expected to greatly enhance the excitatory effect of NMDA receptor activation and may account for the ability of NMDA receptors to mediate phenomena such as epileptiform bursting. Interaction between these two receptors is of potentially great importance in studies of the mechanisms controlling neuronal excitability. The effect of NMDA receptor activity on adenosine sensitivity was also supported by the fact that 2AP5 restored responsiveness to adenosine. The other alternative ways to activate the NMDA receptor complex, namely application of co-agonist, glycine, or induction of

electrical LTP mimicked the effect of NMDA itself on adenosine responses. The site of the interaction between adenosine and NMDA remained unclear in the Bartrup & Stone (1991) experiments, but the paired-pulse experiments carried out in the present work favoured a presynaptic site for this interaction and suppression of adenosine sensitivity could result from an interplay between those receptors at presynaptic nerve terminals. The inability of bicuculline to prevent the suppression effect of NMDA on adenosine responses ruled out the interference of interneurone activation and GABA receptor modulation on this interaction. The mechanism by which NMDA receptors could reduce the effect of purines is not entirely clear. The increase in synaptic efficacy and LTP following activation of NMDA receptors could result from the suppression of endogenous adenosine. Simultaneous activation of A_{2A} and NMDA receptors by co-superfusion of both agonists can increase neurotransmitter release and could trigger LTP. In the presence of NMDA, CGS 21680 significantly increased the population spike size, an effect which prevented by an A_{2A} antagonist, ZM241385, while did not have any marked effect by itself.

The effectiveness of NMDA receptor activation in inducing seizures or kindling could be also attributed to suppression of inhibitory tone mediated by endogenous adenosine. It is interesting that the enhancement of A_1 receptor activity by administration of selective agonists is able to suppress both epileptiform activity and the cell damage mediated by NMDA receptors. Conversely, inhibition of A_1 receptors following activation of A_{2A} receptors in the presence of NMDA could result in suppressing the neuroprotective role of adenosine.

The field of receptor-receptor interactions is becoming more and more important, and the present work adds to the growing list of examples in which adenosine receptors may be implicated. It seems clear that adenosine A_2 receptors can modulate sensitivity of striatal neurones to dopamine receptor agonists, and there are recent reports of interactions between adenosine and peptides (Ribeiro, 1999). The present results emphasise that adenosine receptors, playing a key and widespread role in the control of neuronal excitability, are a major target for receptor-receptor interactions.

As a prelude to study the interactions between nucleotides and acetylcholine receptors, an analysis was performed of the effects of ATP and stable analogues on population spikes and paired-pulse interactions. It was from this work that three new discoveries were made. Firstly, there appears to be little evidence for a direct effect of nucleotides on presynaptic receptors in the hippocampus. This is surprising in view of the evidence from radiolabelling studies for the presence of P_2 receptors on these terminals. However, as discussed above, the experiments performed here suggest a resolution of this paradox with the conclusion that P_2 receptors may trigger the release of adenosine which then mediates the inhibitory effect of nucleotides on terminals. Not only does this explain the present results, but it also accounts for the presence of adenosine in brain slices stimulated by nucleotides even in the absence of nucleotide breakdown (Cunha et al., 1998).

There is a general principle to be learned from these results, that the presence of receptors for, and responses to an agent, does not necessarily indicate that the observed effects are the direct result of receptor stimulation and could, as in this case, be due to their mediation by a second compound. Such secondary agents might be referred to as 'intermediate messengers' to distinguish them from the 'second messengers' such as cyclic AMP and diacylglycerol.

In testing the effects of nucleotides it also became clear that in slices with population spikes greater than 5mV at least two components could be distinguished in the time course of the depression of spike size. This eventually led us to the conclusion that nucleotidases were able to rapidly metabolise nucleotides to adenosine, which then mediated the inhibitory responses. The physiological relevance of this observation is not clear. However, the phenomenon is an example of a self-regulation that might be important for cell survival. Extensive cell damage will cause the release of large amounts of nucleotides into the extracellular space. As adenosine is generated, the stimulation of adenosine receptors could produce a profound depression of neuronal activity that may depress life support systems such as respiration. Perhaps the feedforward inhibition of nucleotidases leading to the 'fade' of inhibition seen here, represents a mechanism for limiting the generation of disastrous levels of adenosine.

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