STUDIES OF ADENOSINE AND GABA$_A$ RECEPTOR FUNCTIONS IN RAT HIPPOCAMPAL SLICES

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© Shahin Akhondzadeh Basti Pharm.D, Ph.D.

Department of Pharmacology,
Faculty of Medicine,
University of Glasgow,
Scotland,
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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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5. Induction of a novel form of long-term depression by muscimol

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Summary

Recent evidence has indicated that adenosine, in addition to potassium and calcium currents, may also affect chloride movement in hippocampal neurones. This project was undertaken to determine the possible role of adenosine on chloride channels and synaptic plasticity in comparison with a selective $GABA_A$ agonist, muscimol.

Extracellular recordings were made from the CA1 pyramidal cell layer of hippocampal slices in response to stimulation of Schaffer collateral fibres in stratum radiatum (0.01 Hz). Adenosine and muscimol induced concentration dependent reductions in the amplitude of orthodromically induced population potentials. In order to eliminate effects of these agents on potassium channels, experiments were performed in the presence of barium, 1mM (in some experiments in the presence of 1 mM tolbutamide). This concentration increased potential size, and reduced the inhibitory effect of adenosine on population spike size, but not synaptic potential size. This profile is consistent with the blockade of potassium channels associated only with the postsynaptic effects of adenosine. However, muscimol responses were unaffected. Adenosine potentiated the ability of muscimol to inhibit

evoked potentials in the absence or presence of barium. The potentiation was prevented by the A_1 selective antagonist 8-cyclopentyltheophylline. The effects of adenosine, as well as muscimol, were reduced by the chloride channel blocker DIDS, which also prevented the adenosine potentiation of muscimol. The results indicate the ability of adenosine to operate chloride channels in hippocampal neurones, and suggest a potentiative interaction between adenosine and muscimol which also involve chloride channels.

The second part of this study was to examine neurosteroids which have been reported to be positive modulators of the $GABA_A$ receptors. Alphaxalone and 5α-pregnan-3α-ol-20-one potentiated the inhibitory effect of muscimol on the population spike size at low concentrations (0.5 and 1 μM) that had no significant effect on the spike size by themselves. This profile is in agreement with other reports which have described the effect of these neurosteroids as barbiturate-like. Alphaxalone and 5α-pregnan-3α-ol-20-one also at low concentrations potentiated the inhibitory effect of adenosine alone and in the presence of barium 1 mM which blocked adenosine activated potassium channels. Alphaxalone failed to potentiate the inhibitory effect of adenosine in the presence of bicuculline 1 μM. It is

concluded that these neurosteroids enhanced the potentiative interaction between adenosine and muscimol in the presence of barium. The results indicate that adenosine's effects are normally enhanced by virtue of the potentiative interaction occurring with endogenous GABA. In addition to this, the results show the chloride channels activated by adenosine to be different from those operated by the $GABA_A$ receptor.

The third part of this project was to investigate the role of $GABA_A$ and adenosine receptors on long-term depression (LTD) and synaptic plasticity. Unlike long-term potentiation, LTD in the central nervous system remains poorly understood. Muscimol induced a time and concentration-dependent LTD in the amplitude of orthodromic potentials. Increasing the stimulation frequency from 0.01 Hz to 1 Hz for 10 seconds reversed the LTD induced by muscimol. Although adenosine decreased the spike size in a concentration-dependent manner, it failed to induce LTD. Muscimol also induced LTD in the absence of electrical stimulation.

Alphaxalone and 5α-pregnan-3α-ol-20-one at concentrations that did not have any effect themselves on the population spike (0.5 and 1 μM), potentiated the inhibitory effect of muscimol on the population spike

size. These neurosteroids at high concentrations (5 and 10 μM) decreased the spike size by themselves. On the other hand, at the low concentrations both steroids were able to potentiate the ability of muscimol to induce LTD. Moreover, muscimol 1 μM which is not able to induce LTD, alphaxalone and 5α-pregnan-3α-ol-20-one 1 μM maintained the LTD induced by muscimol 10 μM. Bicuculline 5 μM reversed the LTD induced by muscimol 10 μM. To examine the possible role of glutamate receptors in LTD induced by muscimol a number of NMDA and metabotropic receptors agonists and antagonists were used. The NMDA receptor antagonist 2-AP5, the NMDA/metabotropic antagonist 2-AP3 and selective metabotropic antagonist L(+)-AP3 failed to modify the LTD. Quisqualic acid and (1S,3R)-aminocyclopentane dicarboxylic acid (ACPD), a selective agonist at metabotropic receptors, did not induce LTD or short-term depression, whereas kynurenic acid prevented the reversal of the LTD obtained at 1 Hz. It is concluded that LTD can be induced by the selective activation of $GABA_A$ receptors and the lack of involvement of glutamate receptors in the protocol which is presented in this study confirms the unique role of classical $GABA_A$ receptors in the effect of muscimol and may indicate a novel type of long-lasting depression. Furthermore, the failure of adenosine to induce LTD taken together with

the earlier results, suggests that the adenosine activated chloride channel differs from the $GABA_A$ receptor chloride channel.

The last part of this study investigated the role of calcium in the induction of LTD and the mechanism of reversal of muscimol induced LTD. It was observed that the LTD can be obtained in the absence of calcium. In addition to this, the LTD was reversed by NMDA, kainic acid, high potassium medium, veratrine and calcium ionophore but not high calcium medium. Interestingly, high potassium medium in the absence of calcium reversed the LTD induced by muscimol 10 μM. The results suggest that this type of LTD can be induced in the absence of calcium and reversal of the LTD is due to a simple depolarization, as a result of sodium influx. These results emphasise the novelty of the muscimol-induced LTD, described here for the first time.

PUBLICATIONS

A. Papers:

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B. Abstracts:

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Abbreviations

ACPD	1-Aminocyclopentane-1,3-dicarboxylic acid
ACSF	Artificial cerebrospinal fluid
AD	Adenosine
AHP	Afterhyperpolarization
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	one-way analysis of variance
AP3	2-Amino-3-phosphonopropionic acid
AP4	2-Amino-4-phosphonobutyric
AP5	D(-)-2-amino-5-phosphonopentanoic acid
4-AP	4-Aminopyridine
APNEA	N^6-2-(4-aminophenyl)ethyladenosine
ATP	Adenosine triphosphate
BAC	Baclofen
CA1-4	Cornu Ammonis 1 to 4
cAMP	Cyclic adenosine monophosphate
CGS21680	2-[p-(2-Carboxyethyl)phenylethylamino]-5′-N-ethylcarboxamidoadenosine
CNS	Central nervous system
CPT	Cyclopentyltheophylline or 8-Cyclopentyl-1,3-dimethylxanthine
DIDS	4,4′-Diisothiocyanatostilbene-2,2′-disulphonic acid
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
EPSP	Excitatory postsynaptic potential
GABA	γ-Aminobutyric acid
GABA-T	GABA-aminotransferase
GAD	glutamic acid decarboxylase
I.P	Intraperitoneal
IPSP	Inhibitory postsynaptic potential
IP_3	inositol trisphosphate
K_{ATP}	ATP-sensitive potassium channels
LTD	Long-term depression

LTP	Long-term potentiation
min	Minute
n	Number
n.s.	Non-significant
NECA	5 N-ethylcarboxamidoadenosine
NMDA	N-methyl-D-aspartate
P.S.	Population spikes
PI	Phosphoinositide
R-PIA	R-N^6-Phenylisopropyladenosine
s.e.m	Standard error of the mean
S.S.	Secondary spikes
SSAD	succinic semialdehyde dehydrogenase
TEA	Tetraethylammonium

INTRODUCTION

1. Introduction

During the last 20 years, there has been intensive research into the neuromodulator adenosine. Adenosine receptors have been classified and their distribution and function thoroughly examined in the mammalian brain. Adenosine has been shown to modulate neuronal function in the central nervous system through a variety of receptor-mediated mechanisms including second messenger systems, ion fluxes and modulation of transmitter release. However, the mechanism of adenosine action on ion fluxes is still not well understood. This thesis will look at the possible role of adenosine on chloride channels in comparison with a selective $GABA_A$ agonist, muscimol and their role in synaptic plasticity.

1.1. Adenosine - an historical overview

The potent extracellular actions of adenosine and ATP were first reported by Dury and Szent-Györgyi (1929). In 1954 Holton and Holton reported that the ATP might represent the vasodilatory substance that was released upon antidromic stimulation of sensory nerves supplying the rabbit ear artery. The current interest in adenosine began with the realization of its potent inhibitory properties on

neurones. In 1970 Sattin and Rall showed that adenosine could stimulate cAMP formation in brain, while caffeine could antagonise this response. The inhibitory effect of adenosine in various regions of the brain was shown by Kostopoulos and Phillis (1977). Burnstock proposed a basis for distinguishing two types of purinoceptors, termed P_1 for the nucleoside receptor (e.g. adenosine) and P_2 for the nucleotide receptor (e.g. ATP)(Burnstock, 1978). This classification was mainly based on the relative potencies of ATP, ADP, and adenosine, the selective action of antagonists, in particular methylxanthines, and modulation of adenylate cyclase by adenosine, but not ATP. It subsequently became apparent that adenosine receptors could be classified into subtypes (A_1 and A_2) primarily characterized by their ability to affect adenylate cyclase activity (Van Calker et al., 1979). In addition to this, more recently, A_3 and A_4 receptor subtypes have also been identified by cloning (Zhou et al., 1992) and binding studies (Cronfield et al., 1992).

1.2 Biosynthesis, release and inactivation of adenosine

Adenosine is known to be released in a calcium-dependent fashion from nerve terminals following nerve stimulation (Stone, 1989). This could be derived from

intracellular adenosine formed by the action of cytosolic 5'-nucleotidase or S-adenosylhomocysteine hydrolase (Snyder, 1985). Adenosine can also be synthesized *de novo* from phosphoribosyl-1-pyrophosphate glutamine (Stone and Simmonds, 1991). Release of adenosine appears to be dependent on the metabolic status of the tissue, reflecting the role of adenosine in regulating energy homeostasis within the CNS (Meghji, 1991).

Electrical stimulation can release adenosine from brain slices (Sciotti et al., 1992). In addition to this, the concentration of adenosine can rise several hundred fold during metabolic seizures (During and Spencer, 1992), hypoxia (Rehncrona et al., 1978) or ischemia (Winn et al., 1981) and also by depolarisation with glutamate (Hoehn and White, 1990).

Adenosine is inactivated by adenosine uptake mechanisms and via break down to inosine by adenosine deaminase or phosphorylation to AMP by adenosine Kinase (Meghji, 1991,1993). It should be noted that the uptake system plays a key role in the inactivation of adenosine.

Although adenosine can be taken up by uptake mechanisms and released following depolarisation, unlike endocrine hormones it is not specifically produced by particular

cells or tissues, and unlike classical neurotransmitters it is not stored and released from particular neurones that form specific pathways.

1.3 Adenosine receptors

1.3.1 Classification and distribution

Adenosine receptors have been classified according to their action on adenylate cyclase (Van Calker et al., 1979), adenosine A_1-receptors having an inhibitory and A_{2a} stimulatory action. Although it has been shown that adenosine can act via cyclic adenosine monophosphate-independent mechanisms (Dunwiddie and Hoffer, 1980), the A_1/A_2-classification has been useful.

A_1-receptors have been thoroughly studied, since numerous ligands are available that have high affinity for this receptor and satisfactory specificity. A_1-receptors have been described as mediating the modulatory action of adenosine on neuronal activity, and autoradiographic studies have demonstrated the highly specific distribution of this receptor subtype. 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 and Synder, 1982; Erfurth and Reddington, 1986). The distinct distribution of A_1-receptors has been confirmed in studies of post mortem human brain tissue (Fastbom et al., 1987; Deckert et al., 1993). The results of Deckert and Jorgensen (1988), showing that adenosine A_1 receptors are located presynaptically and postsynaptically, are more consistent with the electrophysiological studies of Thompson et al., (1992) that adenosine acts at pre- and postsynaptic receptors which are pharmacologically indistinguishable.

Studying the A_2-receptor has been more difficult due to the lack of specific A_2 agonists and antagonists. In order to differentiate between the stimulatory effects of adenosine in striatum and cortex, a subclassification of A_2-receptors into A_{2a} and A_{2b} subtypes has been suggested. This classification is based on [^{3}H]NECA binding, high and low affinity respectively for A_{2a} and A_{2b} (Bruns at al., 1986). It should be noted that both these subtypes increase adenylate cyclase activity.

Autoradiographic techniques have so far only allowed the detection of the A_{2a} subtype, suggesting that these two subtypes exhibit different molecular properties in the

brain (Reddington and Lee, 1991). In rat (Reddington et al., 1986) and also in human brain (Martinez-Mir et al., 1991), A_{2a}-receptors have been described as being specifically located in striatum, nucleus accumbens and olfactory tubercle.

In addition to an action at extracellular adenosine receptors, adenosine can inhibit adenylate cyclase by interacting with an intracellular P-site, that appears to be located on the catalytic subunit of adenylate cyclase (Londos and Wolff. 1977). The P site has a low affinity for adenosine, in the high micromolar range, and is not blocked by adenosine receptor antagonists. Moreover, some adenosine derivatives, e.g. dideoxyadenosine, are selectively active at the P-site. The physiological role of the P-site is still unknown.

Recently, a third class of adenosine receptors (A_3) has been cloned from rat brain (Zhou et al., 1992).It has been shown that this A_3 receptor is coupled to a pertussis toxin-sensitive G protein. Furthermore, like A_1 receptors, A_3 receptors are coupled to both the inhibition of adenylate cyclase (Zohu et al., 1992) and the stimulation of inositol phosphate metabolism (Beaven et al., 1994). The A_3 receptors are insensitive to xanthines (Carruthers and Fozard, 1993).

Activation of A_3 receptors is associated with hypotensive, behavioral and cardioprotective effects and an anti inflammatory action (Fozard and Carruthers, 1993; Jacobson et al., 1993a,b; Liu et al., 1994).

1.3.2 Ligands for adenosine receptors

The chemical synthesis efforts made over several years have aimed to improve the potency and selectivity of adenosine analogs for either the A_1 or A_2 receptor (Williams et al., 1986; Williams and Cusack, 1990). There has been less success in developing A_{2b} and A_3 ligands compared with the A_1 receptor. Thus, the functional role of the A_{2b} and A_3 receptors is still largely unknown.

Most structural modifications were related to N^6-substituted adenosine derivatives which showed selectivity, high affinity and potency at A_1 receptor sites (Williams et al., 1986; Williams, 1987). Conversely, chemical substitution on C2 of either adenosine itself or 5'-N-ethylcarboxamidoadenosine (NECA) have led to a variety of compounds displaying A_{2a} agonist properties. Among them, the 2-alkynyladenosine and 2-alkynyl-NECA derivatives appear to be relatively potent at A_{2a} receptors and some of them show good A_{2a} vs A_1 selectivity. CGS 21680 has recently been

presented as a selective A_{2a} receptor agonist with 70-140 fold selectivity for this receptor in binding assays (Williams, 1991). To date for the A_3 receptor N^6-2-(4-aminophenyl)ethyladenosine (APNEA) is used as non-selective agonist (Fozard and Carruthers, 1993) and N^6-(3-iodobenzyl)5'-(N-methylcarbamoyl)adenosine (IB-MECA) as a selective agonist which is 50-fold selective in binding assays for rat brain A_3 vs either A_1 or A_{2a} receptors (Gallo-Rodriguez et al., 1994). The potency profile of these adenosine analogs at adenosine receptors in a variety of tissue is as follows:

A_1: CPA>R-PIA=CHA≥NECA>2CADO>S-PIA

A_{2a}: CGS21680=NECA>2CADO>R-PIA=CHA=CPA>S-PIA

A_{2b}: NECA>2CADO>R-PIA=CHA>S-PIA≥CGS21680

A_3: IB-MECA>APENEA>R-PIA=NECA>CGS21680 (Collis and Hourani, 1993; Dalziel and Westfall, 1994).

For investigating the functions of adenosine receptors antagonists have also been used. In addition to caffeine and theophylline, which have marked phosphodiesterase inhibitory activity, more selective and more potent adenosine receptor antagonists have been developed. For example, 8-phenyltheophylline is more potent than theophylline at blocking the A_1 receptor. Indeed,

theophylline and caffeine are not able to discriminate between A_1 and A_2 adenosine receptors (Daly et al., 1981; Fredholm and Persson, 1982). 8-cyclopentyl-1,3,dipropylxanthine (DPCPX) and cyclopentyltheophylline (CPT) show 740 and 130-fold selectivity for the A_1 receptor respectively (Williams, 1991). However, there is technical limitation to the use of DPCPX because of its low solubility.

8-(3-chlorostyryl) Caffeine (CSC) shows 520-fold selectivity for the A_{2a} receptor, whereas there is no selective A_{2b} adenosine receptor antagonist (Jacobson et al., 1993a). Fozard and Hanon have recently reported that 3-(3-Iodo-4-aminobenzyl)-8-(4-oxyacetate)-1-propylxanthine (BW-A522) blocks the adenosine A_3 receptor (Fozard and Hanon, 1994).

1.3.3. Functional effects of adenosine receptors

The A_1 and A_2 receptors mediate somewhat different effects in most tissues. The A_1 receptor has an inhibitory effect on nerve terminals and mediates negative inotropic effects on the heart. The A_2 receptor shows vasodilatory effects on vessels (Stone, 1991). There is debate concerning the inhibitory or stimulatory effect of A_2

receptors in CNS. The A_2 selective adenosine receptor agonist CGS21680 depressed the spontaneous, acetylcholine- and glutamate-evoked firing of rat cerebral sensorimotor cortical neurons (Phillis, 1990). In rat hippocampal slices, CGS21680 decreased the EPSP size in CA1. This A_2 agonist did not increase cAMP levels in the hippocampus but in the striatum it enhanced cAMP levels two fold. Therefore, it may act at an A_1 adenosine receptor in the hippocampus to show the inhibitory effect (Lupica et al., 1990). It has been shown that 2 μM NECA, which activates both A_1 and A_2 receptors, increased glutamate release slightly in pyramidal cells of hippocampal cultures (Prestwich et al., 1987). By intracellular recording in rat hippocampus, Ameri and Jurna (1991) also demonstrated that R-PIA and NECA at high concentration affected membrane properties and showed excitatory effects. Theophylline blocked this effect but DPCPX, an A_1 antagonist, was ineffective.

1.3.4. Presynaptic effects

A_1-receptors mediate both presynaptic and postsynaptic events in the CNS. Adenosine decreases the release of several neurotransmitters, including acetylcholine ((Dunèr-Engstrom and Fredholm, 1988; Fredholm, 1990a,b), dopamine

(Lupica et al., 1990), noradrenaline (Allgaier et al., 1987; Jonzon and Fredholm, 1984), serotonin (Feuerstein et al., 1988), GABA (Hollins and Stone, 1980) and glutamate (Dolphin and Archer, 1983; Prestwich et al., 1987). However, it should be noted that most reports show the inhibitory action of adenosine on excitatory rather inhibitory neurotransmitters (Hu and Fredholm , 1989). This may be consistent with the idea that adenosine receptors are located on excitatory terminals (Lambert and Teylor, 1991; Yoon and Rothman, 1991; Thompson et al., 1992).

Transmitter release is dependent on calcium influx into nerve terminals, and one possible mechanism of action of adenosine to inhibit transmitter release may be blocking of the calcium influx. In rat brain synaptosomal preparations, adenosine modulated calcium uptake by potassium (Wu et al., 1982) or veratridine-depolarized nerve terminals (Michaelis et al., 1988) through an action on extracellularly localized adenosine receptors. Adenosine analogues decreased calcium uptake across voltage sensitive calcium channels slightly in both synaptosomes and hippocampal slices (Bartrup, 1989) . By contrast, adenosine and adenosine analogs had no effect on calcium uptake by potassium-depolarisation (Barr et al., 1985; Garritsen et al., 1989) and veratridine stimulation (Garritsen et al.,

1989). The lack of effect of adenosine or its analogs in the Barr and Garritsen reports could be that adenosine receptors which control Ca^{2+} uptake are present only in a subset of the synaptosomes or adenosine affects the sensitivity for calcium rather than the calcium concentration in the neuronal cell. With ion sensitive microelectrodes adenosine decreased pre- and postsynaptic calcium signals of rat hippocampal cells in low calcium medium (Schubert et al., 1986). 4-aminopyridine could not prevent the inhibition of calcium influx by adenosine. Organic calcium blockers (verapamil and nifedipine) blocked postsynaptic calcium entry not presynaptic. Thus adenosine may depress calcium current at presynaptic nerve terminals by transient calcium channels (T or N)(Schubert et al., 1986). In another report Schubert (1988) showed that endogenous adenosine, via A_1 receptors, inhibits calcium influx. Silinsky (1986) proposed adenosine impairs transmitter secretion by reducing the affinity for calcium at a site beyond the external orifice of the calcium channel. Adenosine-evoked inhibition of calcium current may have resulted from a shunt of the calcium current secondary to the adenosine-evoked increase in potassium currents (Gerber et al., 1989 ; Greene and Haas, 1989).

1.3.5. Postsynaptic action

The anticonvulsant action of adenosine has been described as a postsynaptic event which is mediated by A_1 receptor. Postsynaptic mechanisms comprise activation of potassium channels and the control of calcium fluxes. However, the type of potassium channel activated by adenosine is still a matter of controversy.

Adenosine in cultured postganglionic neurones of avian ciliary ganglia under whole-cell voltage clamp blocked calcium-activated potassium channels (Bennett et al., 1991). Furthermore, adenosine hyperpolarised CA1 neurones and decreased input resistance in normal and low calcium medium. Adenosine also suppressed excitatory postsynaptic potentials (EPSP), by a presynaptic effect, without any effect on resting membrane potential (Segal , 1982). Okada and Ozawa (1981) also showed a hyperpolarisation action of adenosine in the hippocampus.

In single voltage clamp, adenosine evoked or increased three potassium conductances: 1)dependent on calcium and voltage, 2)dependent on calcium only and 3)insensitive to both and not inwardly rectifying (Haas and Greene, 1988). Adenosine at low concentration (10 μM) increased the after-

hyperpolarisation (AHP) induced by a burst of action potentials with no effect or little effect on the resting membrane potential (RMP). Increasing the concentration of adenosine to 50 μM often decreased AHP and input resistance. Tetraethylammonium (TEA), a blocker of delayed outward rectification, and 4AP (blocker of transient outward rectification) had no effect on adenosine action (Greene and Haas, 1985; Gerber et al., 1989). It has recently been reported that adenosine-induced hyperpolarisation was depressed by glibenclamide, a blocker of ATP-sensitive K^+ channels, in rat CA1 neurones (Li and Henry, 1992).

There is also growing evidence that adenosine, in addition to potassium and calcium currents, may also affect chloride movement via the A_1 receptor in hippocampal neurones (Mager et al., 1990,1995).

1.4. Phosphatidylinositol turnover

Stimulation of adenosine receptors primarily regulates the activity of adenyl cyclase, but adenosine also interferes with the hydrolysis of phosphoinositides (PIs). These compounds comprise one of the major transmembrane transducing systems in the mammalian brain. Unlike

acetylcholine, noradrenaline, serotonin and histamine, adenosine does not seem to have any direct effects on PI hydrolysis (El-Etr et al., 1989). However, it is involved in the regulation of the PI response to some of the transmitters such as histamine and noradrenaline. In the striatum, adenosine receptors potentiate the response to the muscarinic agonist carbachol (El-Etr et al., 1989). Histamine H_1-receptor-stimulated PI breakdown has been shown to be inhibited by adenosine in mouse (Kendall and Hill, 1988) and human cerebral cortex (Kendall and Firth, 1990) and in rat striatum (Petcoff and Cooper, 1987). In contrast, the histamine-stimulated PI turnover in guinea-pig cortex is increased by adenosine agonists (Hollingsworth et al., 1986).

Alexander and co-workers (Alexander et al., 1989) reported that the adenosine receptor mediating the modulation of histamine-stimulated PI hydrolysis is different to the receptor that stimulates cAMP formation. Inhibition of PI hydrolysis by adenosine seems to involve a direct inhibition of phospholipase C (Linden and Delahunty, 1989), the enzyme involved in the formation of the two second messengers of this transmembrane transducing system, inositol-1,4,5-trisphosphate and diacylglycerol.

1.5. Potassium Channels

Potassium channels are involved in a number of central nervous system processes including neuronal depolarisation and neurotransmitter release. It was originally hypothesized that hyperpolarisation induced by opening the potassium channels leads to suppression of neurotransmitter release by preventing the opening of voltage-dependent Ca^{2+} channels (Quast and Cook, 1989).

It has recently been reported that the adenosine potassium channel is ATP-sensitive type (Li and Henry, 1992). ATP-sensitive potassium (K_{ATP}) channels are from a group of potassium channels that occur in muscle tissue, in pancreatic β cells and in transporting and neuronal tissue (Nichols and Lederer, 1991). The ATP-dependent potassium channel is modulated by intracellular concentrations of ATP such that normal physiological concentrations of ATP inhibit the channel. However, if ATP levels decline the channel opens (Noma, 1983). Several other endogenous modulators of cellular K_{ATP} channel activity have been identified including the ATP/ADP ratio, certain nucleotide diphosphates, pH, lactate and G-protein kinase (Edwards and Weston, 1990). Several pharmacological agents have also been discovered that specifically modulate the K_{ATP} channel.

The potassium channel openers, which include nicorandil, levocromakalim, pinacidil and aprikalim activate the K_{ATP} channel (Edwards and Weston, 1990). The sulfonylurea agents glibenclamide and tolbutamide and the non-sulfonylurea compound sodium 5-hydroxydecanoate specifically block K_{ATP} channels (McCullough et al., 1991). Indeed, in pancreatic cells, where channel function is relatively well understood, depolarization induced by elevated ATP levels subsequent to glucose metabolism opens voltage-gated Ca^{2+} channels to release insulin.

1.6. Treatment perspectives of adenosine

The concentrations of adenosine in brain rise during hypoxia (Winn et al., 1981), ischaemia (Berne et al., 1974; Kleihues et al., 1974) and seizures (Winn et al., 1980). For example, after ischemia, adenosine levels in the millimolar range have been measured (Berne et al., 1974). In this view, it seems that adenosine may play a neuroprotective role in cerebrovascular diseases.

It has been shown that glutamate-gated cation channels play a key role in triggering ischaemia (Coyle and Puttfarcken, 1993). Several experimental data have shown that activation of A_1 adenosine receptors causes a marked

reduction in amount of excitatory transmitter release (Yoon and Rothmann, 1991). Thus, it may have a beneficial neuroprotective role (Rudolphi et al., 1992; Phillis et al., 1993). Adenosine can prevent seizures in vivo (Murray et al., 1985, 1993; Dragunow et al., 1985; Young and Dragunow, 1994) and in humans models (During and Spencer, 1992; Kostopoulos et al., 1989). Moreover, there are several reports that indicate adenosine antagonists can induce seizures (Skinner, 1990). Indeed, the convulsant action of theophylline and caffeine is used to facilitate electroconvulsive treatment in patients with psychiatric disorders (Hinkle et al., 1987).

A_1 antagonism may result in the release of multiple neurotransmitters, including acetylcholine and serotonin. Therefore, A1 antagonists might be useful in neurodegenerative cognitive disorders, such as Alzheimer's disease, that are associated with degeneration of cholinergic and serotonergic neurones. Recent biochemical studies have shown changes in adenosine receptors in the brains of patients with Alzheimer's disease (Jansen et al., 1990; Ikeda et al., 1993).

In the heart, in addition to being a potent vasodilator, adenosine slows heart rate (negative chronotropic effect), slows atrioventricular (AV) nodal

conduction (negative dromotropic effect), and antagonises the stimulatory effect (ie, inotropic and arrhythmogenic) of catecholamines (Belardinelli et al., 1989). These effects of adenosine, which increase the supply of oxygen and decrease cardiac work, are cardioprotective (Belardinell and Shryock, 1992). Consistent with this cardioprotective action, adenosine and agents that increase the interstitial concentration of adenosine such as nucleoside uptake blockers and adenosine deaminase or adenosine kinase inhibitors, have been shown to reduce myocardial cell damage and dysfunction during hypoxia and ischemia (Ely and Berne, 1992). Thus, modulation of adenosine metabolism and/or adenosine receptor activation may prove beneficial in the treatment of cardiac arrhythmias and ischemic heart disease. Dipyridamole, an adenosine uptake inhibitor, is used clinically in ischemic heart disease.

It should be noted that there is still a technical problem in the use of adenosine or adenosine analogs in the brain since adenosine crosses the blood-brain barrier poorly.

A possible strategy to solve this problem is to use an agonist which is able to pass the blood-brain barrier and an antagonist which is unable to pass the blood-barrier to minimise cardiovascular adverse effects of adenosine.

1.7. GABA as an inhibitory neurotransmitter in the brain

GABA is well known as the major inhibitory neurotransmitter in the mammalian brain. There is evidence to suggest that the nervous system is held in a state of perpetual restraint, by circuits which tonically inhibit pacemaker neurons and feedforward and/or feedback cell discharges, surround and dampen the output of spontaneously discharging neurones, and influence pre-synaptic inhibition and facilitation. The CNS operates by relaxing these restraints (i.e. disinhibition) via excitatory inputs. The majority of this inhibitory wiring is comprised of neurones which release GABA as their neurotransmitter.

GABA was first identified in the brain in 1950 (Awapara et al., 1950; Robert and Frankel, 1950) and shortly afterward its involvement in the mediation of seizure activity became apparent. Hayashi observed that direct application of GABA onto the motor cortex of the dog attenuated local epileptic discharge (Hayashi and Nagi, 1956; Hayashi, 1959) and Killam and Bain (1957) reported inhibition of GABA synthesis to be the principle mechanism of action of the convulsant hydrazides. Understanding of the synthesis (Weinstein et al, 1963) release (Jasper et al., 1965), postsynaptic effects (Krnjevic and Schwartz,

1987), specific antagonism (Galindo, 1969), and inactivation (Robert and Kuriyama, 1968) of GABA followed and precipitated its proposal as an inhibitory neurotransmitter in the mammalian brain.

GABA is formed, *de novo*, entirely from L-glutamic acid. This reaction is catalysed by the enzyme GAD (glutamate decarboxylase) in the presence of pyridoxal phosphate as a cofactor (Meldrum, 1957). GAD is exclusively localised to GABAergic neurones in the vertebrate CNS and can be employed as a definitive marker for these cells (Snead, 1983).

Following synaptic release, GABA exerts its actions via specific cell membrane receptors. At present three specific receptor sites for GABA have been identified in the mammalian brain and have been designated $GABA_A$, $GABA_B$ and $GABA_C$.

1.7.1. $GABA_A$ receptor

The $GABA_A$ receptor has been the subject of many studies (Olsen, 1982; Levitan et al., 1988; Olsen and Tobin, 1990) and is now well characterised. Extensive research has shown the $GABA_A$ receptor to be a macromolecular protein, composed

of five variable subunits, forming a chloride ion-selective channel and possessing additional binding sites for benzodiazepines (Ehlert, 1986), barbiturates (Willow and Johnston, 1983), picrotoxin (Olsen, 1981), penicillin (Twyman et al, 1992), and neurosteroids (Majewska et al, 1986). Binding of GABA to this receptor subtype leads to opening of the channel, chloride ion entry into the cell, hyperpolarisation, and inhibition of action potential generation (Enna and Gallagher, 1983). cDNA cloning has revealed the existence of a number of distinct subunit isoforms. Based on sequence similarity, five different $GABA_A$ receptor subunit families have been identified and have been named α, β, γ, δ, and ϱ subunits. Most of the families have multiple subtypes (α_{1-6}, β_{1-4}, γ_{1-3}, δ and ϱ_{1-2}), and the sequences within each subunit family show homology, with about 70-80% amino acid sequence identity. Although $GABA_A$ receptors *per se* are distributed widely, determination of the regional distribution of receptor subunits, by *in situ* hybridization, has confirmed that many of the subunits are highly localised within the brain (Macdonald, 1993) and may be responsible for the heterogeneity of $GABA_A$ receptors previously reported in binding studies (Olsen et al., 1984; McCabe et al., 1988).

1.7.2. $GABA_B$ receptor

It was reported that (-)baclofen decreases neurotransmitter releases in the CNS (Bowery et al., 1980). In 1981, it was established that this effect of (-)baclofen is exerted by activation of a bicuculline-insensitive and non-$GABA_A$ receptor (Hill and Bowery, 1981), and this receptor is termed the $GABA_B$ receptor. Although GABA and baclofen have been used as $GABA_B$ receptor agonists, studies on physiological and pharmacological roles of the $GABA_B$ receptor in the CNS have been delayed, mainly by the lack of selective and potent antagonists for this receptor. Recently, however, selective and potent agonists and antagonists for the $GABA_B$ receptor have been introduced. For example, 3-aminopropylphosphonoic acid (APPA) has been developed as a potent agonist at peripheral and central presynaptic $GABA_B$ receptors (Ong et al., 1990). On the other hand, phaclofen, 2-hydroxysaclofen and more recently CGP35348 have been introduced as selective antagonists for the $GABA_B$ receptor (Kerr et al., 1987, 1988; Curtis et al., 1988; Olpe et al., 1990).

In electrophysiological studies, it was demonstrated that activation of the $GABA_B$ receptor results in modulation of ion channels. Namely, a voltage-dependent Ca^{2+} channel

was inhibited by activation of the $GABA_B$ receptor (Holz et al, 1986). In addition, activation of the $GABA_B$ receptor induced an increase in K^+ conductance, which resulted in the occurrence of a slow IPSP (Newberry and Nicoll, 1984; Dutar and Nicoll, 1988). Modulation of these ion channels by the $GABA_B$ receptor was also known to be mediated by GTP-binding protein. Therefore, it seems likely that suppression of transmitter release by activation of the presynaptic $GABA_B$ receptor is caused by a decrease in Ca^{2+} conductance or an increase in K^+ conductance.

1.7.3. $GABA_C$ receptor

Recently, GABA-gated Cl^- channels with unusual pharmacology have been detected in the vertebrate retina, which do not fit into the conventional scheme of $GABA_A$ and $GABA_B$ receptors (Polezani et al., 1991; Feigenspan et al., 1993a,b; Quian and Dowling, 1993). It is very likely that these receptors, termed $GABA_C$ (Shimada et al., 1992), are composed of the ϱ_1 or ϱ_2 subunits (Cutting et al., 1991,1992). The novel bicuculline and baclofen-insensitive $GABA_C$ receptors are present on rat retinal bipolar cells (Feigenspan et al., 1993a,b) and on horizontal cells of white perch (Qian and Dowling, 1993). $GABA_C$ receptors may exert an important inhibitory role in the retinal circuitry.

1.7.4. Metabolism and uptake of GABA

Following receptor activation, GABA is removed from its site of action, in the synaptic cleft, by a high affinity uptake mechanism (Logan and Synder, 1971). This process, together with direct diffusion of GABA into postsynaptic nerve cell bodies (Hyden et al., 1986), appears to be one of the most significant mechanisms of GABA inactivation and can be observed in both neurones and glial cells (Iverson and Kelly, 1975). Upon cellular uptake, GABA is successively metabolised by the action of two mitochondrial enzymes. GABA-T (GABA transaminase) catalyses the conversion of GABA to succinic semialdehyde (SSAD) in the presence of a α-Ketoglutarate as cofactor and SSAD completes the metabolism to succinate in the presence of nicotinyl adenine dinucleotide (NAD; Meldrum, 1975).

1.7.5. Peripheral actions of GABA in vertebrates

The first observation of a peripheral action of GABA in a mammal was made by De Groat (1970) working on feline autonomic ganglia. By now, the idea of an exclusively central role of GABA has been superseded by many findings which show that enzymes for the synthesis and uptake of GABA, as well as GABA receptors (both $GABA_A$ and $GABA_B$), are

widely spread in the autonomic nervous system and in a number of neuroendocrine, endocrine and exocrine cells (Erdo and Wolff, 1990).

1.7.6. GABA as link between neuronal and hormonal control systems

A vast body of evidence suggests that GABA acts as an important bilateral link between the nervous system and the endocrine system. One aspect of this interaction is that various neuroendocrine and endocrine cells receive a GABAergic innervation and show responses mediated by $GABA_A$ and $GABA_B$ receptors (Randle and Renaud, 1987; Peters et al., 1988). On the other hand, certain steroid hormones and hormone metabolites are capable of potentiating the inhibitory actions of GABA through a modulatory effect on the $GABA_A$ receptor (Majewska et al, 1986; Twyman and Macdonald, 1992).

1.7.7. Neuroactive steroid action at the $GABA_A$ receptor

The $GABA_A$ receptor is a complex membrane-bound protein which is coupled to a Cl^- channel. Upon binding to the receptor, GABA facilitates the opening of the Cl^- channel resulting in hyperpolarisation of the neuron.

Therapeutically useful anticonvulsant, anxiolytics, and sedative-hypnotics such as the benzodiazepines and barbiturates are known to act through their binding sites on the $GABA_A$ receptor complex (Stephenson, 1988). These agents are positive $GABA_A$ allosteric modulators that increase the binding of GABA to the $GABA_A$ receptor. Conversely, negative $GABA_A$ receptor modulators such as β-carbolines that decrease the binding of GABA to the $GABA_A$ receptor, are anxiogenic or convulsants.

The discovery of neuroactive steroids and their distinct binding site on the $GABA_A$ receptor complex raises the possibility that this class of steroids may have therapeutic potential like other $GABA_A$ receptor ligands. Neuroactive steroids are found endogenously. The most potent naturally occurring neuroactive steroids are 3α-hydroxy-5α-pregnane-20-one (3α,5α-P) and 3α,21-hydroxy-5α-pregnane-20-one (5α-THDOC) which are metabolites of progesterone and deoxycorticosterone, respectively. These steroids are positive allosteric modulators of the $GABA_A$ receptor.

A second group of neuroactive steroids represented by pregnenolone and dehydroepiandrosterone and their sulfate metabolites have been shown to be negative modulators of

the $GABA_A$ receptor (Majewska and Schwartz, 1987). Collectively biochemical, pharmacological and physiological studies support the existence of a novel binding site for neuroactive steroids on the membrane-bound $GABA_A$ receptor complexes in the mammalian brain.

1.8. Chloride channels

Chloride is the most important physiological anion. Several pathways for chloride exchange between the extracellular and intracellular milieu exist, including Cl^- HCO_3^- exchange, $NaHCO_3$-HCl exchange, Na-K-2Cl cotransport, K-Cl and chloride channels (Palfrey and Rao, 1983; Warnock and Eveloff, 1989). Chloride channels in intracellular compartments are often found in conjunction with active cation transport systems where chloride appears as a counter ion to the transported cation. Plasma membrane chloride channels serve a variety of physiological roles. For excitable cells that change their membrane potential over wide ranges, activation of chloride channels stabilizes the membrane potential. This has an inhibitory effect on action potential generation because chloride equilibrium potential is close to resting membrane potential. The function of GABA and glycine receptors is based on this effect. In addition to this, in many

epithelia, chloride channels are essential for transport of salt (and water) across the cell layer (Hoffmann and Ussing, 1992). Ligand-gated chloride channels which are the most important class of chloride channels, are activated by the binding of an external messenger molecule. Typical representatives of this class are postsynaptic GABA and glycine-activated chloride channels. Structurally, these belong to a superfamily of direct transmitter-activated ion channels which also includes cation channels (Barnard et al, 1987).

Adenosine 3',5'-cyclic monophosphate (cAMP)-dependent chloride channels are activated by a rise in intracellular cAMP. The molecular mechanism is often phosphorylation by cAMP -dependent protein kinase A (PKA), but it cannot be excluded that other chloride channels may be directly activated by binding of cyclic nucleotides (Dhallan et al., 1990). An important example for activation by cAMP-dependent phosphorylation is the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel (Riordan et al., 1989), which also requires the intracellular binding and probably also hydrolysis of nucleotides for activity (Baukrowitz et al., 1994). The CFTR is involved in transport across many epithelia (Riordan et al., 1989).

Another important class of chloride channels is activated by elevated intracellular calcium (Marty et al., 1984). In many cases, it is not known whether calcium acts directly by binding to the channel protein, or via an interposed mechanism such as phosphorylation. To date, little biochemical information is available for calcium-dependent chloride channels. It should be noted that chloride channels largely have been ignored by many physiologists, but recently, interest in anion channels has increased significantly after the invention of the patch-clamp technique.

1.9. Glutamate as an excitatory neurotransmitter in the brain

L-glutamate is an endogenous excitatory amino acid neurotransmitter substance that contributes excitatory input into the majority of synapses in the central nervous system (Founum, 1984). During the intervening years a wealth of information pertaining to excitatory amino acids, and glutamate in particular, has been generated. Much is now known about the synthesis (Peng et al., 1991), release (Nicholls and Attwell, 1990), receptor-mediated effects (Monaghan et al., 1989) and inactivation (Schousboe et al., 1988) of neurotransmitter glutamate. Glutamate can be synthesised, *de novo*, by transamination of α-ketoglutarate catalysed by aspartate aminotransferase, by direct amination of α-ketoglutarate catalysed by glutamic acid dehydrogenase, and by the deamination of glutamine catalysed by glutaminase. All of these mechanisms have been implicated in the synthesis of neurotransmitter glutamate (Peng et al., 1991).

Astrocytes are believed to play the principal role here by synthesising and supplying the necessary precursors (Sonnewald et al., 1991). Synaptic release of glutamate is a calcium-dependent phenomenon, can be induced by

potassium, veratridine, or 4-AP depolarisation, and can be blocked by magnesium in a voltage sensitive manner (Horton, 1989; Nicholls and Attwell, 1990).

1.9.1. Glutamate receptor

Glutamate receptors are found throughout the mammalian brain, where they constitute the major excitatory transmitter system (Collingridge and Lester, 1989). The well-known glutamate receptors are ligand-gated ion channels (Monaghan et al., 1989) which are called ionotropic glutamate receptors and are permeable to cations. They have been categorised into three broad subtypes based upon pharmacological and electrophysiological features: α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors, Kainate (KA) receptors, and N-methyl-D-aspartate (NMDA) receptors (Nakanishi, 1992). Recently, however, a family of G protein-coupled glutamate receptors, which are also called metabotropic glutamate (Miller, 1991) or trans-1-aminocyclopentane-1,3-dicarboxylate (tACPD) receptors, was identified (Sugiyama et al., 1987; Collingridge and Lester, 1989). There are at least six subtypes of metabotropic glutamate receptor which are coupled to activation of phosphoinositide hydrolysis, and thus the mobilization of

intracellular calcium (Nakanishi, 1992; Tanabe et al., 1992). Most recently a 2-amino-4-phosphonobutyric acid (AP4) receptor has been reported as a new subtype of ionotropic glutamate receptor (Nakanishi, 1992). The AP4 receptor is less well characterised and is thought to be a presynaptic autoreceptor, providing a negative feedback mechanism to reduce further neuronal glutamate release (Scatton, 1993).

Inactivation of glutamate following its synaptic release occurs via a high affinity, sodium and energy-dependent uptake system present in both nerve endings and glia (Drejer et al., 1982). Recent evidence suggests that glial uptake is more important than neuronal uptake in the rapid removal of glutamate from the synaptic cleft (Schousboe et al., 1988). Autoradiographic measurement of glutamate uptake, and lesion studies of anatomically defined pathways, have facilitated identification of numerous glutamatergic pathways in the mammalian brain (Fagg and Foster, 1983). The majority of glutamate releasing pathways appear to be descending, originating in the neo and allo-cortex and innervating many sub-cortical regions and the spinal cord. Many cortico-cortical projections also employ glutamate as a neurotransmitter.

distinct allosteric sites for zinc ions, polyamines, phencyclidine (PCP and ketamine) and for glycine, which are closely associated with the ion channel of this complex (Lodge and Johnson, 1990).

1.9.3. Role of glutamate receptors in learning, memory and brain disorders

A generally accepted hypothesis in neurobiology has been that at the cellular level, memories are stored, at least in part, as long-term changes in the strength of synaptic transmission as a result of alterations in the efficacy of chemical synapses (Hebb, 1949). The glutamate receptor has become the focus of much work because of several experimental findings that indicate a central role for glutamate receptors in learning and memory (Bliss and Collingridge, 1993). The NMDA receptor has attracted much attention because its properties make it an ideal candidate for a receptor involved directly in the learning process. The NMDA receptor is the only ligand-gated ion channel whose probability of opening depends strongly upon the voltage across the membrane, under physiological conditions. This property permits this receptor to operate as a Hebbian molecule (Hebb, 1949).

A vast body of evidence suggests that glutamate receptors play a role in a number of cerebrovascular diseases (Appel, 1993), indeed, many neurological accidents involving strokes in which there is a loss of oxygen and glucose, or epilepsy, result in brain damage because of over-stimulation by glutamate. It has also been proposed that degenerative diseases such as Alzheimer's, Huntington's and amyotrophic lateral sclerosis (ALS) may involve neuronal cell death caused by over-activation of the glutamate receptor system (Coyle and Puttfarcken, 1993).

1.10. Hippocampus and memory system

Learning is a neural process which enables both human and animals to adapt to their environments by using experience to adjust their behaviour. The mechanisms of learning and memory have traditionally been interesting topics of study. It has been known for years that the hippocampal system plays a crucial role in learning and memory. The hippocampus is ideal for studies of memory and learning for two reasons. First, the hippocampus can serve as a model for cortical processing in general because of its regular and relatively simple cytoarchitecture. Second, the size and central location of the hippocampus indicates that is plays a prominent role in total brain function. Only recently has it become clear that only certain properties of memory processing depend on hippocampal system function and that these can be distinguished from other aspects of memory that do not. The earliest reports of amnesia emphasized the time limited role of the hippocampal region in memory, focusing on its critical function in consolidation processes that bridge between immediate memory and the long-term store (Scoville and Milner 1957). Amnesic patients and animals with hippocampal lesions show some disorders in temporary memory store (Rawlins 1985).

A generally accepted hypothesis in neurobiology has been that long-lasting activity-dependent changes in the efficacy of synaptic transmission in the mammalian brain are considered to be of fundamental importance for the development of neural circuitry and for the storage of information (Hebb, 1949; Nicol et al., 1988). The most compelling and reliable model for such changes has been long-term potentiation (LTP) and long-term depression (LTD) in the hippocampus.

1.10.1. Long-term potentiation

The storage of information in the hippocampus is believed to involve an increase in synaptic efficiency. This view was confirmed by the report of Bliss and Lomo (1972,1973) that brief, high-frequency activation of some hippocampal pathways could evoke an increase in the synaptic efficiency. This phenomenon is regarded as LTP (Bliss and Lomo, 1972, 1973). LTP has three properties which distinguish it from other forms of post-excitatory facilitation. Firstly, LTP has an extremely long duration and can last for days. However, it is necessary to emphasize that LTP is not permanent. Secondly, it enables long-lasting increments of synaptic efficiency to be produced by short, high-frequency stimulus trains and

finally, LTP seems to need a number of co-active convergent synapses to be activated (Lee, 1983). There is increasing evidence that this form of synaptic plasticity underlies some forms of memory. The rise in intracellular Ca^{2+} level that can occur by activation of glutamate receptors of the N-methyl-D-aspartate (NMDA) type (Collingridge and Lester, 1989) is crucial for induction of LTP. However, it should be stressed that there exist forms of LTP that are not dependent upon NMDA receptor activation (Johnston et al., 1992). It has been reported that LTP induction at mossy fibres synapses in area CA3 of hippocampus is independent of NMDA receptors (Johnston et al., 1992), whereas in the CA1 region, LTP is a result of Ca^{2+} influx through the NMDA receptor-associated ion channel (Collingridge et al., 1983; Kauer et al., 1988).

1.10.2. Long-term depression

The term, long-term depression (LTD) may apply to any form of long lasting depression in synaptic transmission. LTD has been described in several brain regions, including hippocampal region CA1 (Levy and Steward, 1979), the dentate gyrus, visual cortex (Artola and Singer, 1990), frontal cortex (Hirsch and Crepel, 1992) and cerebellum (Ito et al., 1982). Although LTD of evoked responses has

been reported in the hippocampal formation following a variety of stimulation procedures, most paradigms can be categorised into homosynaptic, heterosynaptic and associative LTD according to the level of presynaptic activity in the pathway undergoing LTD induction, as well as that in converging afferents (Braham and Srebro, 1987: Artola and Singer, 1993; Lovinger et al., 1993; Linden, 1994). The heterosynaptic LTD can be produced by presynaptic activation of different inputs on to the same postsynaptic cell but homosynaptic LTD can be induced by presynaptic activation of the same input on to the relevant neurone.

Associative LTD is a persistent decrease in synaptic strength that is produced while presynaptic activity occurs out of phase with strong postsynaptic activity, with the postsynaptic activity being driven by converging afferents. Owing to hyperpolarisation of the postsynaptic cell in the periods between strong postsynaptic activity, this results in presynaptic activity occurring in the absence of postsynaptic activity (Linden, 1994).

Several factors have been reported to trigger LTD, including the induction of LTP, depolarization of postsynaptic sites, activation of NMDA and non-NMDA receptors, activation of metabotropic glutamate receptors

and moderate postsynaptic Ca^{2+} influx (Kano and Kato, 1987: Aroniadou and Teyler, 1991: Yang et al., 1994). It has been reported that both homosynaptic and associative LTD are abolished by antagonists of NMDA receptors and Ca^{2+} chelators. In associative LTD, voltage-dependent Ca^{2+} channels have also been shown to be important for induction (Christie et al., 1994: Debanne and Thompson, 1994). Homosynaptic LTD has been found in a number of brain structures using one of two induction protocols: prolonged afferent stimulation at low frequency (1-5 Hz for 5-15 minutes) or stimulation at high frequency (50-100 Hz for 1-5 seconds)(Linden et al., 1994). Low frequency stimulation has been shown to induce homosynaptic LTD in the hippocampus but high frequency stimulation has been effective at striatal synapses (Braham and Serbro, 1987; Lovinger et al., 1992) . On the other hand, induction of heterosynaptic LTD requires a strong postsynaptic depolarization caused by synaptic activation. It should be noted that heterosynaptic depression in area CA1 has proved difficult to investigate, as several groups have failed to induce this type of LTD at all (Linden et al., 1994).

1.10.3. Calcium and neuronal plasticity

Intracellular calcium concentration has been proposed to play a pivotal role in neuronal plasticity (Malenka et al., 1989). Several families of Ca^{2+}-activated enzyme are known to be colocalized in synaptic structures. One type of protein kinase, Ca^{2+}/calmodulin-dependent (CaM) kinase, becomes activated in the presence of calmodulin and elevated intracellular Ca^{2+}. Recent progress in elucidating the mechanisms underlying synaptic plasticity indicates that both forms of plasticity require NMDA receptor-gated Ca^{2+} influx, whereas voltage-dependent Ca^{2+} influx can also contribute to the generation of LTD.

It has been reported that moderate elevation of intracellular Ca^{2+} triggers LTD, whereas higher concentrations are required to initiate LTP (Christie et al., 1994; Debanne and Thompson, 1994; Hirano et al., 1994).It appears as though the strength of a given synapse can be set between some minimum and maximum level by induction of LTD or LTP (Lisman, 1989). These two extremes seem to be determined by the level of phosphorylation of some as yet undefined substrate. Induction of LTD decreases their level of phosphorylation by triggering Ca^{2+} dependent protein phosphatase, while induction of LTP increases their

phosphorylation by triggering Ca^{2+}-dependent protein kinases. These kinases apparently require larger increases in intracellular Ca^{2+} for activation than do the phosphatases.

1.11. Anatomy of hippocampus

In the brains of mammals the hippocampus is a bilaterally represented structure that appears as a ridge extending into the lateral ventricle. The outer surface of the hippocampus is composed of myelinated fibers arising, in part, from cells of the hippocampus, most of which leave the region through a large efferent pathway, the fornix. The outer coating of the hippocampus is white due to the myelin coating of the axons of which it is made . The outer covering is called the alveus, and fibres in it give rise to the fimbria that, in turn , is continuous with the fornix (Isaacson, 1987). The hippocampal formation can be divided into the Ammon's horn, the dentate gyrus, and subiculum. Based on Golgi preparations, the anatomist Lorente de No divided Ammon's horn into four subfields: CA (Cornu Ammonis) 1 to CA4. More recent evidence strongly supports the view that the CA4 zone belongs to the dentate gyrus (Witter, 1989). From the outer layer to more medial, Ammon's horn contains alveus, and strata oriens,

pyramidale, radiatum, lacunosum and the molecular layer. The dentate gyrus consists of closely packed cells which are called granule cells. These cells have dendritic brushes that extend only in one direction- toward the outer, molecular layer of the gyrus (Isaacson, 1987). The distal part of CA1 borders the subiculum which is distally replaced by the pre- and para subiculum, and the adjacent entorhinal cortex ventrally and retrosplenial cortex dorsally (Witter, 1989; Isaacson, 1987).

1.11.1. Intrinsic connectivity of hippocampus

Neuronal input reaches the hippocampus from entorhinal regions, which send a digest or summary of what is occurring in the neocortex and consequently, the environment, to the hippocampal formation. The entorhinal cortex by the perforant pathway has a massive projection predominantly in the dentate gyrus. Projections to Ammon's horn also have been described (Witter, 1989). The granule cells of the dentate gyrus project through their mossy fibres to CA3. Pyramidal cells in CA3 give rise to collateralized axons of which the so-called Schaffer-collaterals provide the major input to CA1. From cells in the CA3 area axons leave the hippocampus via the fimbria and fornix, largely terminating in the lateral septal area.

In general, the Schaffer collaterals synapse in the stratum radiatum and stratum oriens with the dendrites of CA1 pyramidal cells. Finally, neuronal output from the CA1 cells exit into the alveus and subsequently into the fornix, also projecting in large part to the septal area and the subicular region (Isaacson, 1987, figure of hippocampus).

1.11.2. Hippocampal Slices

Since the demonstration that slices of mammalian CNS tissue were physiologically viable in vitro (Yamamoto and McIlwan, 1966), the hippocampal slice preparation (Skrede and Westgaard, 1971) has been extensively used in neurochemical and electrophysiological experiments. Several aspects of the slice preparation facilitate this type of analysis (Dunwiddie et al., 1983). The lamellar structure of hippocampus (Andersen et al., 1971), with its rather simple three-layered cortex, well-defined input and output pathways makes it a natural choice for a slice preparation (Schwartzkroin, 1987).

Compared with in vivo experiments, in vitro slice models, like hippocampal slices, have advantages:
-No interaction of substances with anaesthetics.

-From a single animal a large number of measurements can be performed simultaneously in different systems (measuring chambers) (saving of animals).
-Direct visualization of the measured or stimulated brain areas.
-Possibility of determining true dose-response curves with substance application in the bath.
-Possibility of substance application both in the bath and also locally with micropipette release.
-No indirect (peripheral) substance effects, only CNS substance action.
-Very stable intracellular measurement (no movement).
-Technical simplicity and multiplicity.
-Easy changing of extracellular fluid.

Brain slices, like all preparations, have disadvantages and limitations as follows:

-Blood-brain barrier no longer present (passive diffusion).
-The composition of the perfusion solution does not correspond to that of the natural extracellular medium.
-Disconnection of inhibitory and excitatory input and output pathways.
-Trauma and anoxic period of dissection.
-Limitation of tissue life-acute nature of the preparation (Schwartzkroin, 1981,1987; Clark and Wilson, 1992).

METHODS AND MATERIALS

2. Methods and materials

2.1. Preparation of slices

Male Wistar rats (170-200g) were anaesthetised with urethane (1.3g/Kg I.P.) and decapitated using a Guillotine. The scalps were removed and brains were rapidly taken out and put in ice-cold artificial cerebrospinal fluid (ACSF). The cerebellum was removed and the two cerebral hemispheres were separated with a surgical blade. The hippocampi were carefully pulled out with spatulas and put on the filter paper and cut transversely into 450 μM thick slice using a McIlwain tissue chopper. The slices were then transferred onto another filter paper in a petri dish containing ice-cold oxygenated ACSF and separated with glass seekers. The petri dish, with slices, was put into the incubation chamber containing an ACSF-saturated atmosphere of 95% O2 and 5% CO2 at room temperature for at least one hour before using. The fluid in the petri dish was adjusted to cover the slices (fiure 2.1.).

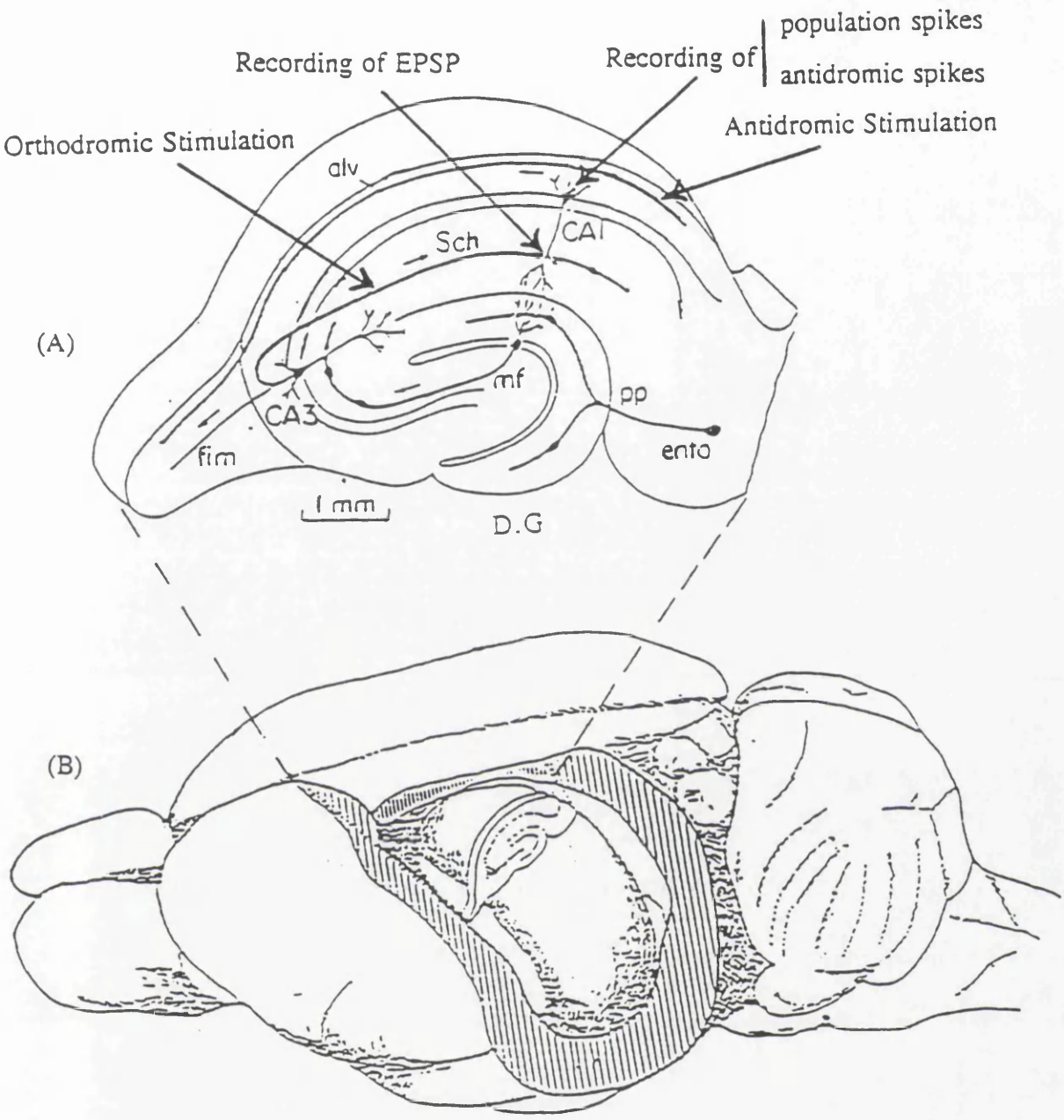

Figure 2.1. Anatomy of the hippocampal formation and the placement of the electrodes. (A). The hippocampal formation is enlarged to show the main neuronal elements of structure. (B). Lateral view of the rat brain with parietal and temporal neocortex to expose the hippocampal formation. after orthodromic stimulation of Schaffer collateral fibres in stratum radiatum near the border of CA2-CA3, recordings were made in the CA1 pyramidal cell layer or the stratum radiatum for population spikes (P.S.) or field EPSPs (fEPSP) respectively. Stimulation of the alveus was used to induce antidromic invasion of CA1 cells as shown in (A). Abbreviation for figure: alv.= alveus; D.G= dentate gyrus; ento.= entorhinal area; fim.= fimbria; mf.= mossy fibres; pp.= perforant path; sch.= Schaffer-collaterals; mm= millimeter. (Revised from Andersen et al., 1971).

2.2. Bathing medium

The formula of the bathing medium, artificial cerebrospinal fluid (ACSF), was as follows (mM): NaCl 115; KH_2PO_4 2.2; KCl 2.0; $MgCl_2$ 1.2; $CaCl_2$ 2.5; $NaHCO_3$ 25 and glucose 10.

In some antidromic experiments, calcium was omitted and $MgCl_2$ was raised from 1.2 to 4 mM (calcium-free high magnesium).

2.3. Bath superfusion and application of drugs

Individual slices were then transferred using a fine brush to a 1ml capacity recording chamber and superfused with ACSF at 30°C ± 0.5, gassed with O2/CO2 mixture to yield a pH of 7.4. The slices were completely submerged in the medium which was perfused continuously from a gravity feed at a rate of 4 ml/min.

In some experiments adenosine was applied locally to the somatic or dendritic region of pyramidal cells using pressure ejection from glass micropipettes (8-10 μM tip diameter), containing agent solution. In this set of experiments an attempt was made to maintain the fluid layer covering the slice as thin as possible.

2.4. Recording electrodes

The electrodes for recording were made from borosilicate glass tubing containing a glass capillary fibre for easy filling. The tubing, with characteristics of 1 mm internal diameter and 2 mm external (GC 200 F), was purchased from Clark Electromedical Instruments, U.K.. With a Kopf vertical electrode puller the electrodes were pulled and separated to yield two sharp tip electrodes. Under microscopic vision, the tips of the electrodes were broken with a glass probe to provide a tip diameter 2-4 μm. The electrodes were filled with 2 M sodium chloride via a syringe bearing a 0.2 μm syringe filter.

2.5. Recording and stimulation

After orthodromic stimulation of Schaffer collateral fibres in stratum radiatum near the border of CA2-CA3, recordings were made in the CA1 pyramidal cell layer or the stratum radiatum for population spikes or field EPSPs (fEPSPs) respectively. Stimulation of the alveus was used to induce antidromic invasion of CA1 cells by a bipolar electrode (figure 2.2.). Pulses of 0.1 ms duration and 100-500 μA amplitude were delivered every 100 seconds (0.01 Hz).

The slices were maintained in the perfusion chamber for 15 minutes before proceeding with any further experimentation. In long-term depression experiments, only slices exhibiting orthodromic potentials of between 9 and 12 mv were used for drug applications. Other slices were discarded.

For orthodromic fEPSPs the duration of the stimulus was decreased to 20-50 μs. Recordings were made with 2-4 μm diameter glass microelectrodes containing 2 M NaCl. The recorded signals were amplified by a Neurolog amplifier and displayed on a Gould storage oscilloscope and then plotted on a thermosensitive chart recorder.

The orthodromic population spikes (P.S.), fEPSPs and also secondary spikes (S.S.) after antidromic stimulation were reduced to 75% to avoid supramaximal stimulation.

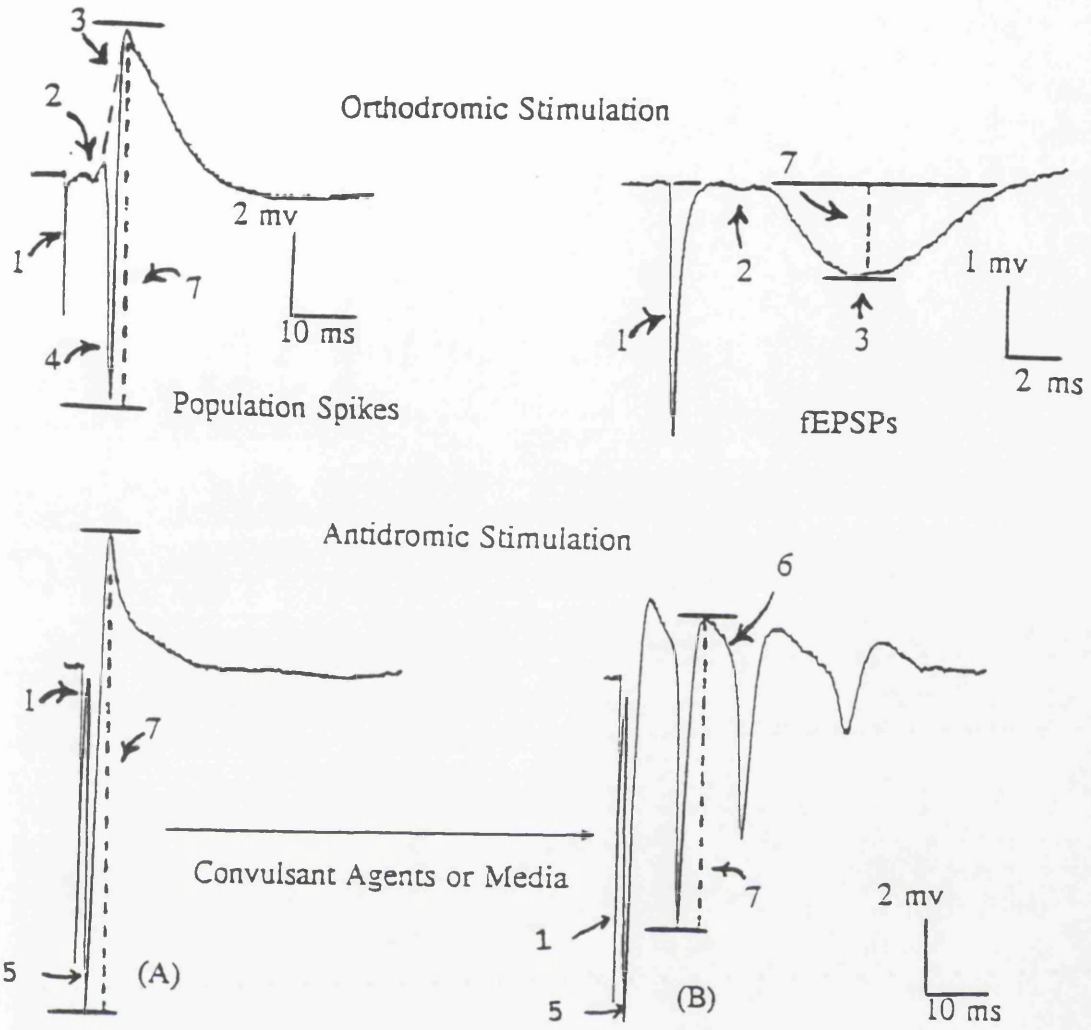

Figure 2.2. Demonstration and measurement of orthodromic and antidromic potentials evoked in the CA1 region. Antidromic potentials are demonstrated in (A) normal ACSF and in (B) the presence of convulsant agents or media. Explanations of each part of the evoked potentials are as follows:

1) Stimulus artifact;
2) Presynaptic fibres volley potential that reflects the compound action potential travelling in the stratum radiatum towards CA1;
3) The population excitatory postsynaptic potential or reflects the depolarization of dendrites by the synaptically released transmitter;
4) The population spike is a negative going spike-like potential by the pyramidal neurones once the EPSP has depolarized the cells to threshold
5) The first antidromic spikes which reflect the compound action potential from axon to soma and initial depolarisation of the soma;
6) Secondary spikes that reflect firing of cell bodies;
7) Amplitude of population spikes, fEPSP or secondary spikes.

2.6. Extracellular techniques

With extracellular measurements on individual neurones or neuron group it is possible to measure the spontaneous discharge rate of these cells. Optimum working conditions without external interference (e.g. mains and mains-borne signals) is achieved using a Faraday cage. If the excitatory fibres in the hippocampus are stimulated electrically and the evoked potentials measured in the region of the dendritic ending, the initial component of the response is a pre-synaptic fibres spike which is actually a summated action potential derived from axons and nerve endings. Directly following the fibre spike is the field EPSP (excitatory post-synaptic potential) response which is considered to mirror the population spike response measured in the cell body layer and is generated by the synchronous activation of a large neurone group.

If the axons of the neurons under investigation are stimulated electrically it is possible to measure the so-called antidromic population spike. It differs from the orthodromic population spike in that this response cannot be influenced by substances which have an effect on synaptic transmission. There are also many cases where the antidromic population spike is not modified by substances affecting the excitability of the neurones.

2.7. Analysis of drug effects

The size of the population spike following orthodromic stimulation and the size of the first and secondary spikes following antidromic stimulation were measured as the peak to peak amplitude. The size of fEPSPs was measured between the negative going peak and the base line of stimulation (figure 2.2.).

Results were considered as percentage of change of the potential size by drug compared with control size. The size of evoked potential in the absence of any agents is considered as zero percent change in potential size.

2.8. Statistical analysis

Results are presented as mean % ± s.e.m (standard error of the mean) for n experiments, and the statistical significance of any difference assessed by a paired Student's t-test. For multiple comparisons an analysis of variance (one way), ANOVA, with post test of Tukey-Kramer were used. Differences were considered significant with $P \leq 0.05$ or as otherwise mentioned.

2.8. Chemical agents

Constituents of ACSF were of Analar grade and purchased from BDH Chemicals Ltd. UK. Chemical agents generally were purchased from Sigma Chemical Co, UK, except for 8-cyclopentyl-1,3-dimethylxanthine (8-cyclopentyltheophylline; CPT), bicuculline methobromide, L(+)-2-amino-3-phosphonopropionic acid [L(+)-AP3], (±)-2-amino-5-phosphonopentanoic acid (2-AP5), (1S,3R) ACPD and quisqualic acid which were purchased from RBI. Veratrine was provided from B.D.H.

8-cyclopentylthyeophylline (CPT) was dissolved in sodium hydroxide. The buffering capacity of the ACSF ensured that the final pH of the perfusing medium was restored to pH 7.4.

Veratrine, calcium ionophore A23187, alphaxalone and 5α-pregnan-3α-ol-20-one were dissolved in ethanol. The concentration of ethanol in all experiments was less than 0.01 %v/v and ethanol at this dilution did not show any effect by itself. In other cases the vehicle was the superfusing medium such as ACSF or ACSF without calcium.

Interaction between adenosine and $GABA_A$ receptors on hippocampal neurones

3.1. INTRODUCTION

Adenosine is known to act as a general inhibitory modulator of synaptic transmission and neuronal activity in the peripheral and central nervous systems (Haas and Greene, 1988; Schubert, 1988; Stone, 1089; Stone and Simmonds, 1991). The nucleoside is able to inhibit activity via receptors located on both presynaptic and postsynaptic sites. At the former, adenosine reduces the release of a number of neurotransmitters including glutamate (Corradetti et al., 1984; Fastbom and Fredholm, 1985), acetylcholine (Spigonoli et al.,1984), 5-hydroxytryptamine (Feuerstein et al., 1985) and dopamine (Michaelis et al., 1979), an effect which is thought to be mediated either by a reduction of calcium influx into synaptic terminals or by a suppression of intracellular calcium availability to the exocytotic release process (Silinsky, 1986; Schubert, 1988).

The postsynaptic actions of adenosine include the ability to hyperpolarise neurones. In several studies evidence has been obtained that this is achieved primarily by the activation of potassium conductances, both in hippocampal and striatal neurones (Trussel and Jackson, 1987; Li and Henry, 1992; Thompson et al., 1992).

In the CNS, the type of potassium channel activated by adenosine is unclear. Barium can block the adenosine-activated potassium conductance in the rat hippocampus (Thompson and Gahwiler, 1992) but barium is a non-selective potassium channel and can block several K^+ currents such as I_K, I_{KATP} and I_M (Cook, 1988; Quayle et al., 1988). Most recently, it has been reported that adenosine-induced hyperpolarisation is depressed by glibenclamide, a blocker of ATP-sensitive K^+ channels, in rat CA1 neurones (Li and Henry, 1992).

GABA is one of the most important inhibitory neurotransmitters in the central nervous system (Krnjevic, 1974, 1987; Sivilotti and Nistri, 1991) and its receptors have been classified into two types. $GABA_A$ receptors are located predominantly on the postsynaptic membranes of supraspinal neurones, and usually mediate a bicuculline-sensitive hyperpolarisation resulting from an increase of chloride conductance (McCormic, 1989; Sivilotti and Nistri, 1991). The $GABA_B$ receptors, in contrast, are located pre-and postsynaptically and induce inhibition of transmitter release and hyperpolarisation mediated by potassium channels (Thompson and Gähwiler, 1992; Deisz et al., 1993).

Recent evidence has indicated that, besides acting on potassium currents, adenosine may also affect chloride movements in hippocampal neurones (Mager et al., 1990,1995). The present study was therefore designed to examine the effect of adenosine alone, and in combination with the $GABA_A$ agonist muscimol, on evoked potentials in rat hippocampal slices in the presence of barium and tolbutamide , a K_{ATP} blocker to remove the influence of adenosine or GABA effects mediated through potassium channels (Li and Henry et al., 1992; Thompson and Gähwiler, 1992; Thompson et al., 1992).

The objective of this part of the project was to determine whether adenosine had activity at chloride channels which would be apparent at the level of population potentials.

3.2. RESULTS

3.2.1. Effect of adenosine on orthodromic population spikes

Adenosine concentration-dependently abolished or reduced the amplitude of the population spike (P.S.) of orthodromic CA1 field potentials. A concentration-response curve for this effect is shown in figure 3.1. The depressant effect of adenosine was readily reversible and washed out in about 5 minutes. The threshold concentration for adenosine was 1 μM and 20 μM abolished the population spikes. Tachyphylaxis or refractoriness was not seen with repeated treatment of adenosine. Adenosine had no effect on the presynaptic fibre volley.

3.2.2. Effect of adenosine on orthodromic population spikes in the presence of barium 1 mM

In order to exclude the involvement of potassium channels, and to isolate possible effects on chloride channels, most experiments were performed in the presence of barium, 1 mM. In the presence of this concentration of barium, the concentration-response curve to adenosine was displaced to the right (figure 3.2.). Responses to adenosine at 2 μM were completely blocked by barium and this submaximal concentration was selected for use in the combination experiments described below.

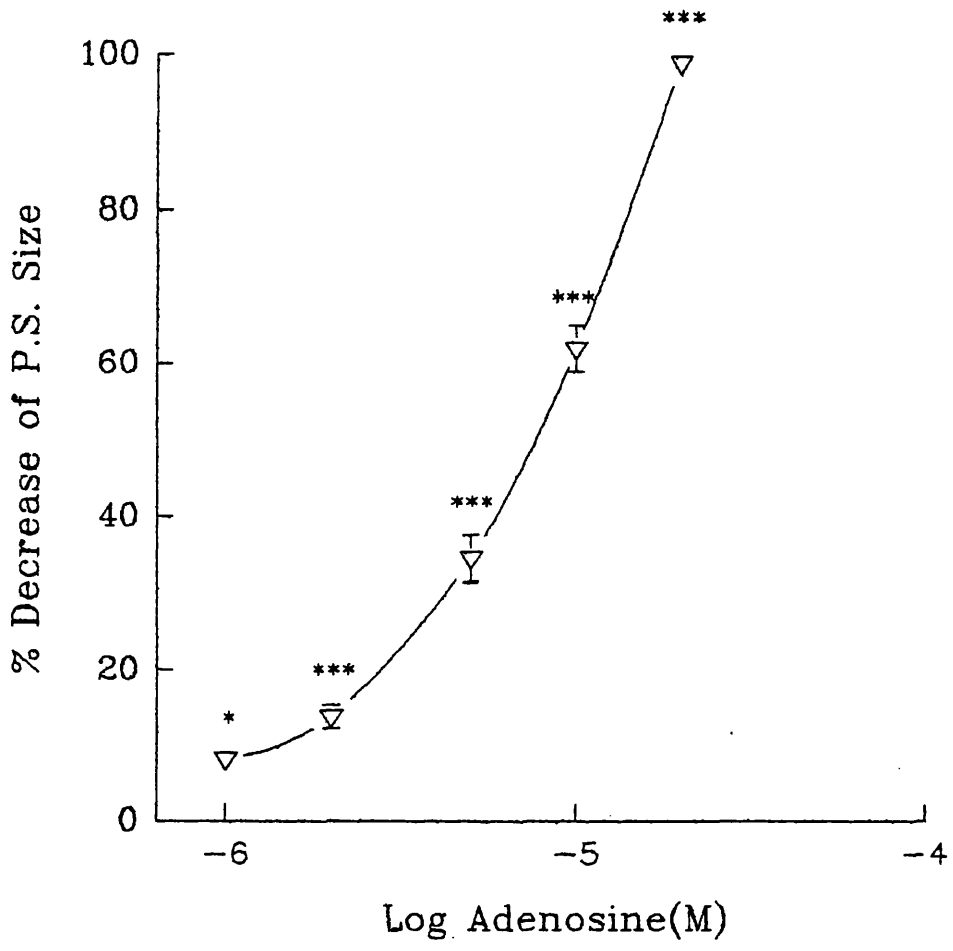

Figure 3.1. Concentration-response curve for the depression of orthodromically evoked CA1 population spike (P.S.) by adenosine. Each point represents the mean ± s.e.m. for n=6 experiments. A paired t test was employed to determine the significance level ($^{*}P \leq 0.05$; $^{***}P \leq 0.001$).

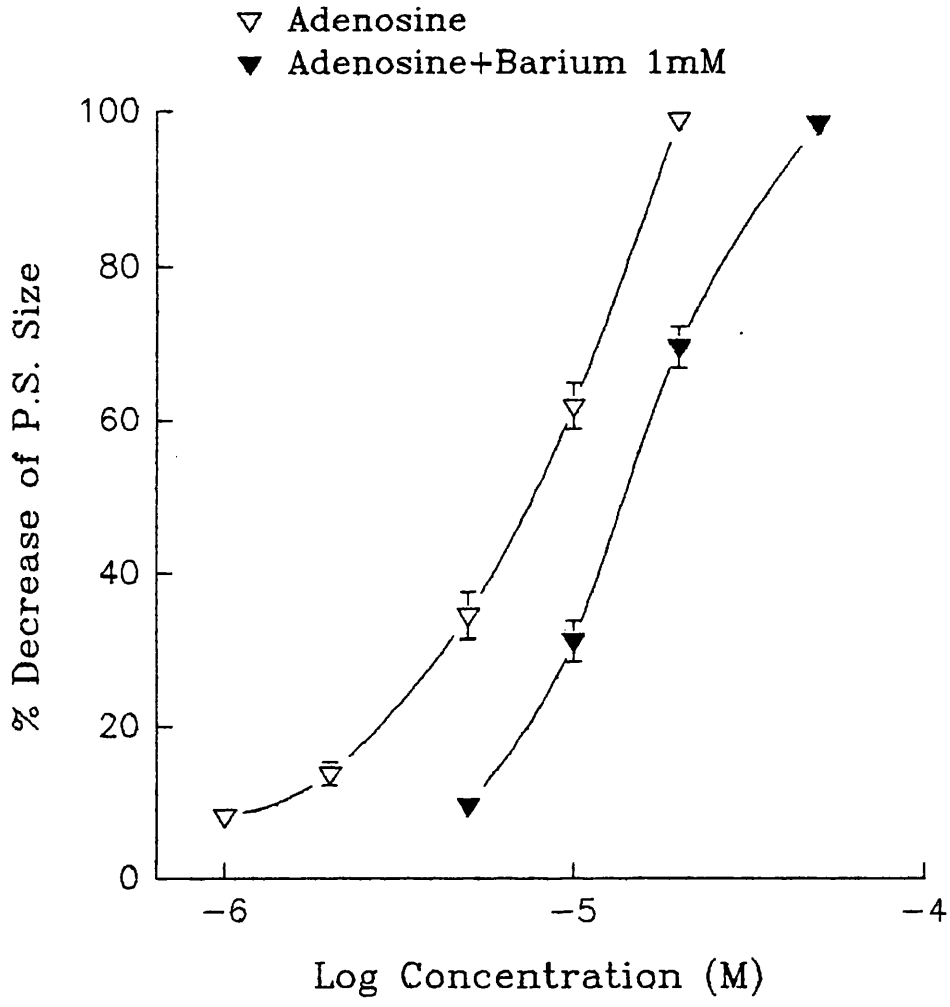

Figure 3.2. Concentration-response curves for the depression of orthodromically evoked CA1 population spike (P.S.) by adenosine (▽) and adenosine+barium 1 mM (▼). Each point represents the mean ± s.e.m. for n = 6 experiments.

3.2.3. Effect of barium and 8-cyclopentyl-1,3-dimethylxanthine (CPT) on orthodromic population spikes

The inclusion of barium itself in the perfusion medium increased the size of the population spikes by 27.36% ± 0.87 (mean ± s.e.m., $P \leq 0.001$, $n = 5$)(figure 3.3.). Superfusion of slices with 8-cyclopentyl-1,3-dimethylxanthine (CPT) at 100 nM, resulted in an increase of population potential by about 10% (figure 3.3.). Barium alone produced an increase of 27%, but the combined application of barium and CPT yielded an increase of only 9.56% ± 0.46, a change which was significantly different from control potentials ($P \leq 0.001$, $n = 5$) but not different from CPT alone.

3.2.4. Effect of baclofen and CPT on population spikes

Baclofen concentration-dependently decreased the size of population spikes evoked in the CA1 region by orthodromic stimulation (figure 3.4.). The maximum effect was observed at 4 μM which abolished the potential completely. The effects of this $GABA_B$ agonist were fully developed in less 10 minutes and recovery from a single concentration on washout occurred in a similar time.

Baclofen, like adenosine, had no effect on the presynaptic fibre volley. However, the potency of baclofen on reduction of orthodromic potentials was much greater than adenosine.

CPT, an adenosine A_1 receptor antagonist, decreased the inhibitory effect of baclofen 4 μM on population spikes to 72.75 ± 2.287 ($P \leq 0.001$, n=4)(figure 3.5).

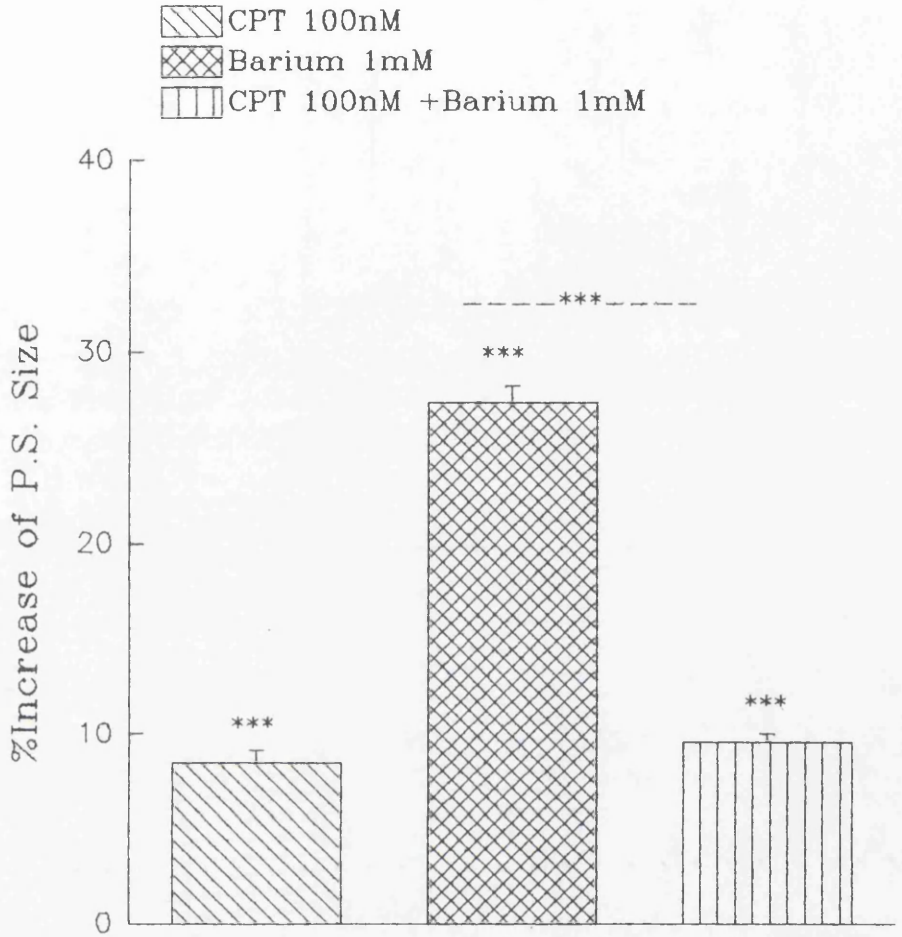

Figure 3.3. Increase of orthodromically evoked CA1 population spikes by CPT 100 nM, barium 1 mM and barium 1mM + CPT 100 nM. Each vertical bar represents the mean ± s.e.m. for n=5. A paired t test was employed to determine the significance level ($^{***}P \leq 0.001$). The horizontal line in this and subsequent figures indicates the level of significance between columns.

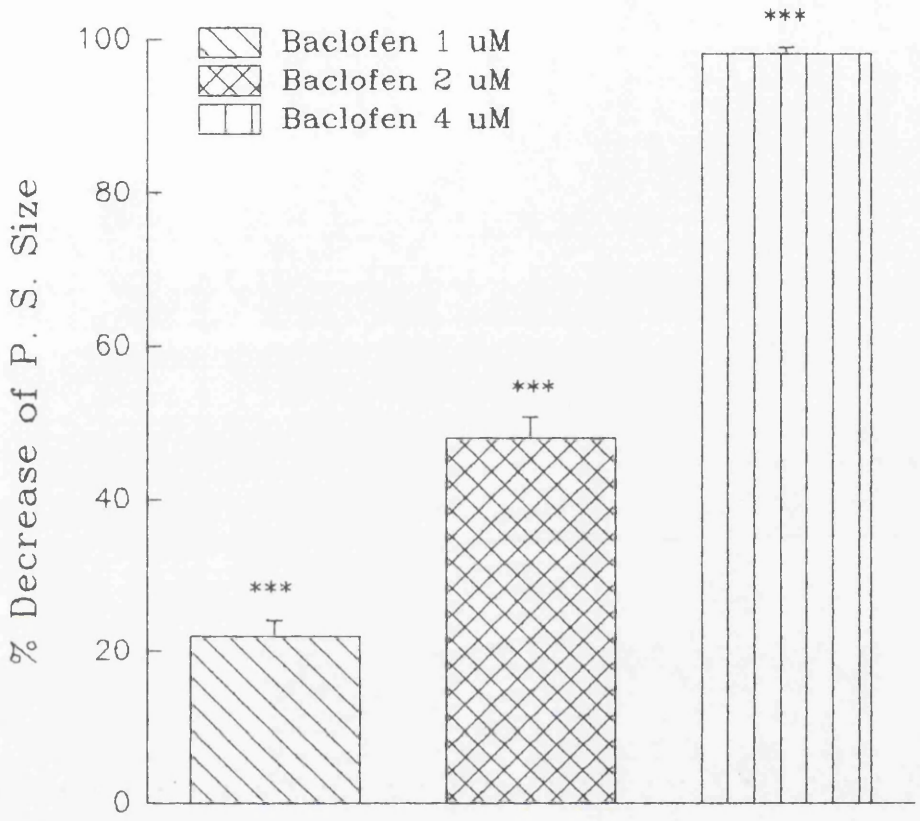

Figure 3.4. Depression of orthodromically evoked CA1 population spikes by baclofen. Each vertical bar represents the mean ± s.e.m. for n=4. A paired t test was employed to determine the significance level ($^{***}P \leq 0.001$).

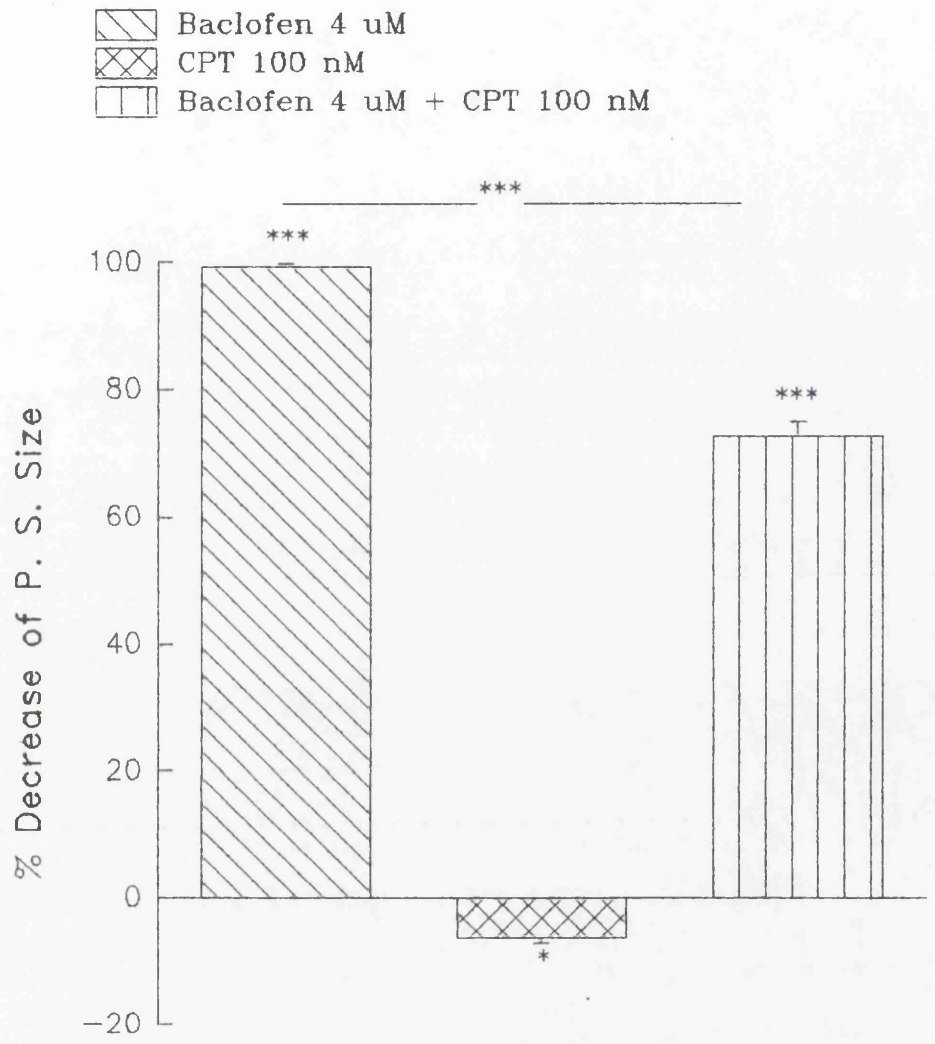

Figure 3.5. Depression of orthodromically evoked CA1 population spikes by baclofen 4 μM , CPT 100 nM and baclofen 4 μM + CPT 100 nM. Each vertical bar represents the mean ± s.e.m. for n=4. A paired t test was employed to determine the significance level ($^{*}P \leq 0.05$; $^{***}P \leq 0.001$).

3.2.5. Interaction of adenosine and barium on orthodromic field EPSPs

The population EPSP recorded in stratum radiatum was also reduced by adenosine. At 20 μM adenosine the potentials were reduced by 39.92% ± 2.77($P \leq 0.001$, $n = 5$) while in the presence of barium the reduction was 41% ± 3.85 (not different from adenosine alone (n=5) (figure 3.6.). Since barium itself tended to increase the size of the potential, the combination with adenosine was tested with and without any adjustment of the stimulation current to restore the potential size in barium to control size. In neither case did barium modify the sensitivity of the EPSP to adenosine (figure 3.6.).

3.2.6. Effect of muscimol on population spikes

Muscimol induced a concentration-dependent reduction in the amplitude of orthodromic population potentials. The maximum effect was seen at a concentration of 10 μM. The effects of muscimol were fully developed in less than 10 minutes and no desensitization was noted with different concentrations of muscimol(figure 3.7.).

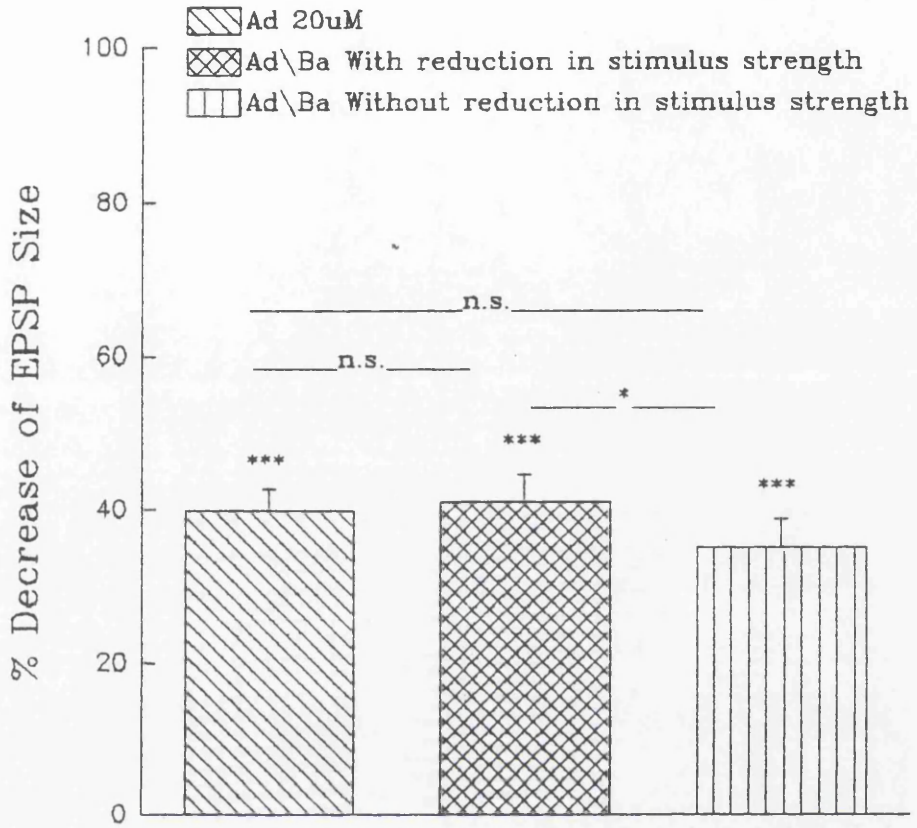

Figure 3.6. Depression of population EPSP size by adenosine 20 μM (Ad) and adenosine 20 μM + barium 1 mM. The second column indicates the effect of adenosine in the presence of barium when the stimulus strength was reduced to negate the increase of EPSP size produced by barium and thus restore the control potential size to that in the absence of barium. The third column indicates the adenosine response when the stimulus strength was not adjusted. Each vertical bar represents the mean ± s.e.m. for n = 5. A one-way analysis of variance test and Tukey-Kramer multiple comparisons test were employed to determine the significance level (*P≤0.05,***P≤0.001).

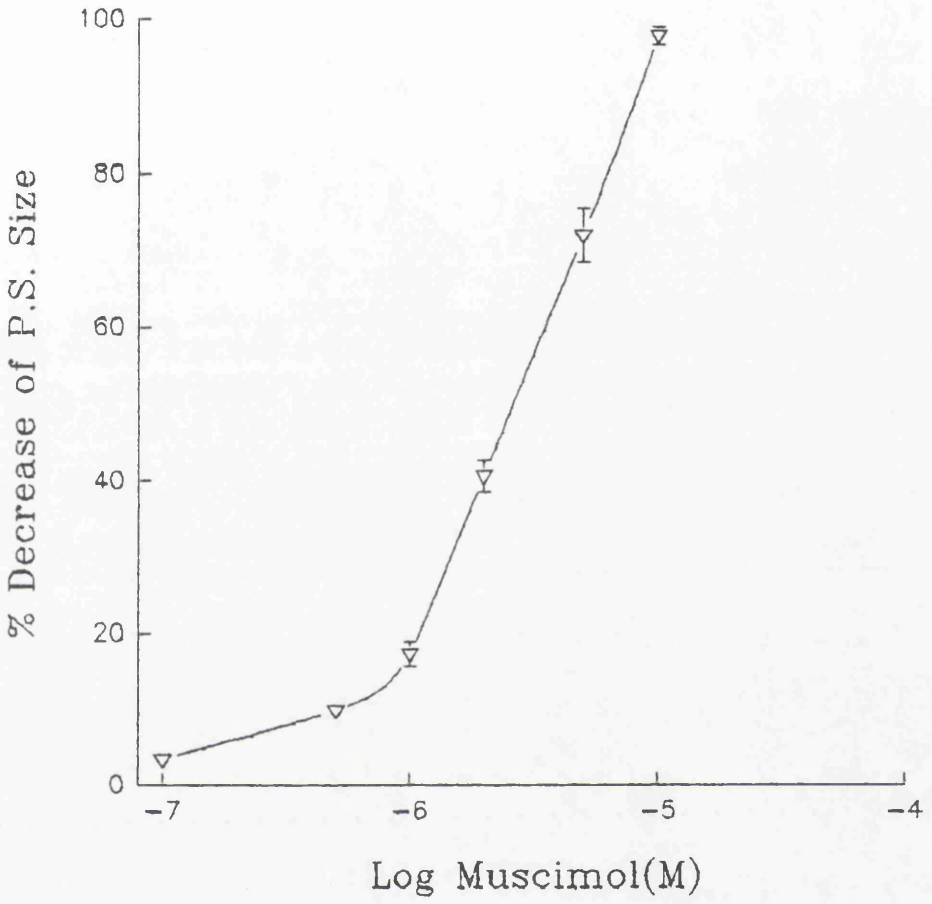

Figure 3.7. Concentration-response curve for the depression of orthodromically evoked CA1 population spikes (P.S.) by muscimol. Each point represents the mean ± s.e.m. for n=5.

3.2.7. Interaction of adenosine and muscimol on population spikes

A submaximal concentration of 1 μM was selected for combination experiments. When this concentration of muscimol was combined with a series of concentrations of adenosine (figure 3.8.), the depression of the population potentials in the presence of both agents was significantly greater ($P \leq 0.05$) at each adenosine concentration than the predicted additive response (figure 3.8.).

3.2.8. Effect of CPT on potentiative interaction between adenosine and muscimol in the presence of barium

It was found that CPT at 100 nM would antagonise completely responses to adenosine, 2 μM, without any effect upon responses to muscimol, 1 μM. Similarly, inclusion of barium at 1 mM blocked fully the response to adenosine but had no effect whatever on sensitivity to muscimol (figure 3.9.,3.10.). However, when adenosine, 2 μM was combined with muscimol, 1 μM in the presence of barium, a very marked potentiation was seen leading to a doubling of response size (figure 3.9.,3.10.). The additional inclusion of CPT then restored this response to that observed in the presence of muscimol alone, indicating that the potentiation involved the activation of adenosine receptors.

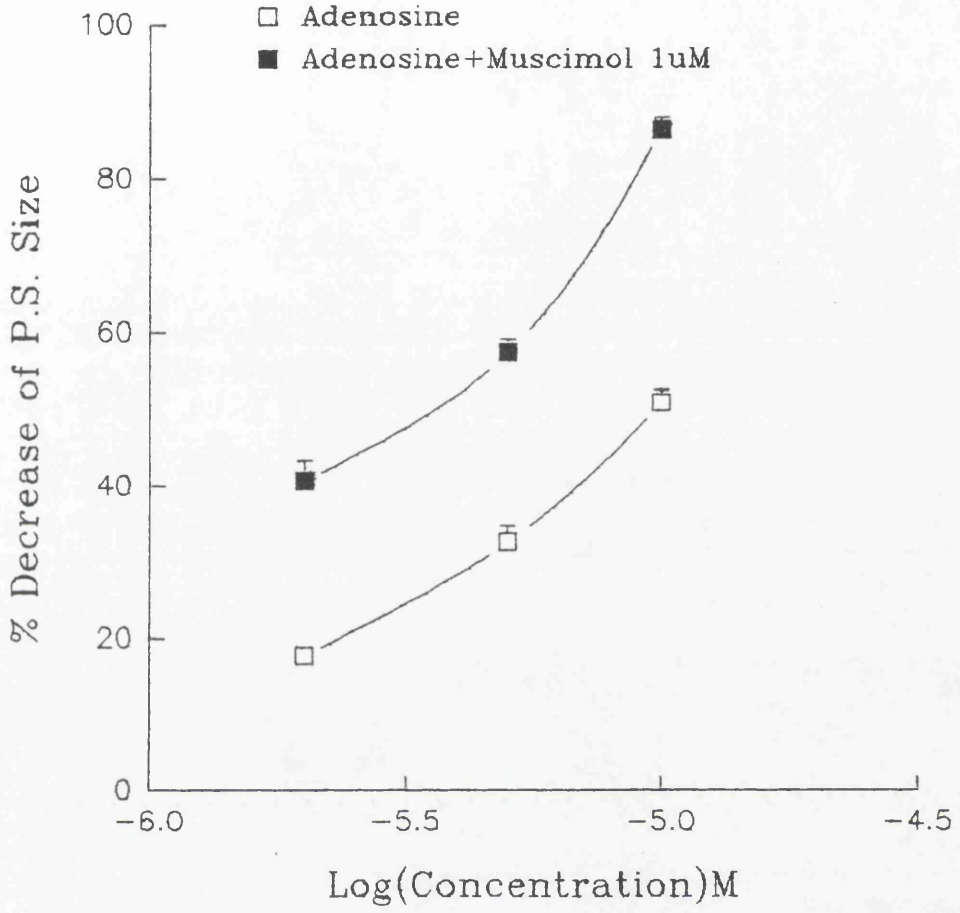

Figure 3.8. Concentration-response curves for the depression of orthodromically evoked CA1 population spikes (P.S.) by different concentrations of adenosine and adenosine + muscimol 1 μM. Each point represents the mean ± s.e.m. for n = 5.

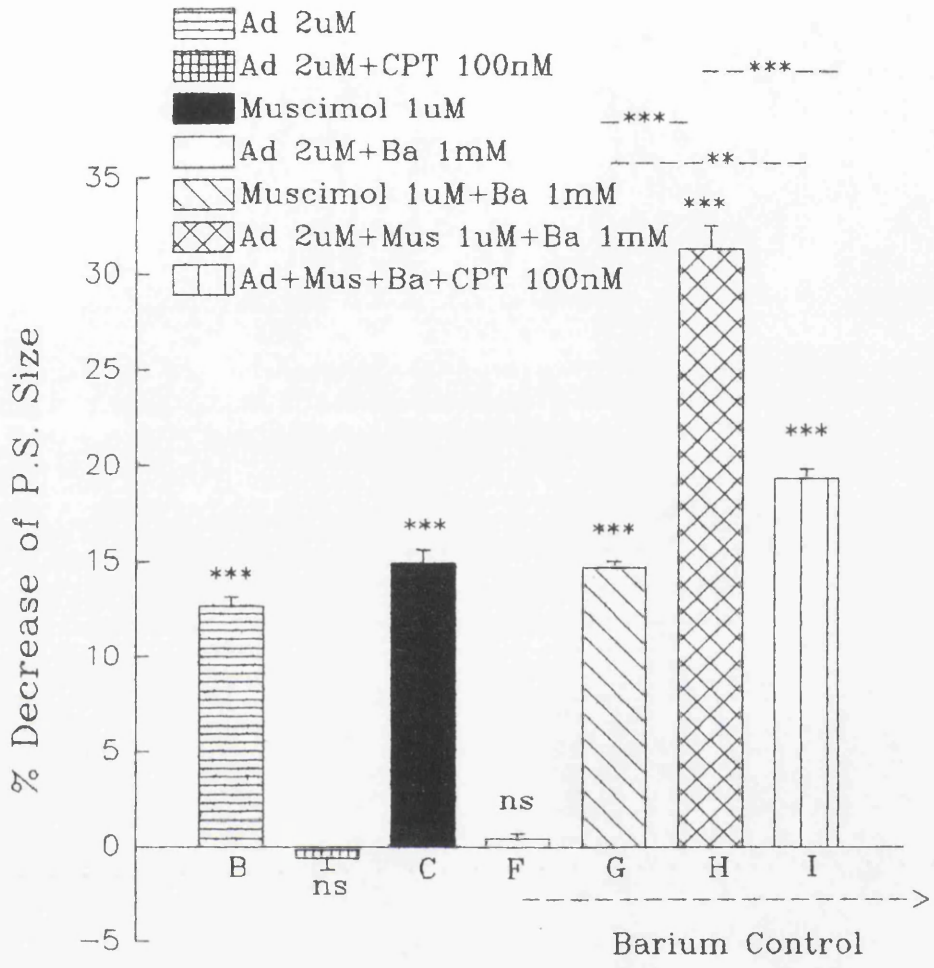

Figure 3.9 Histogram showing depression of orthodromically evoked CA1 population spikes by adenosine 2 μM (Ad), adenosine 2 μM + CPT 100 nM, muscimol 1 μM, adenosine 2 μM + Ba 1 mM, muscimol 1 μM + Ba 1 mM, adenosine 2 μM+muscimol 1 μM + Ba 1 mM and adenosine 2 μM + muscimol 1 μM + Ba 1 mM + CPT 100 nM. Each vertical bar represents the mean ± s.e.m. for n=5. A one-way analysis of variance test and Tukey-Kramer multiple comparisons test were employed to determine the significance level ($^{**}P \leq 0.01$; $^{***}P \leq 0.001$).

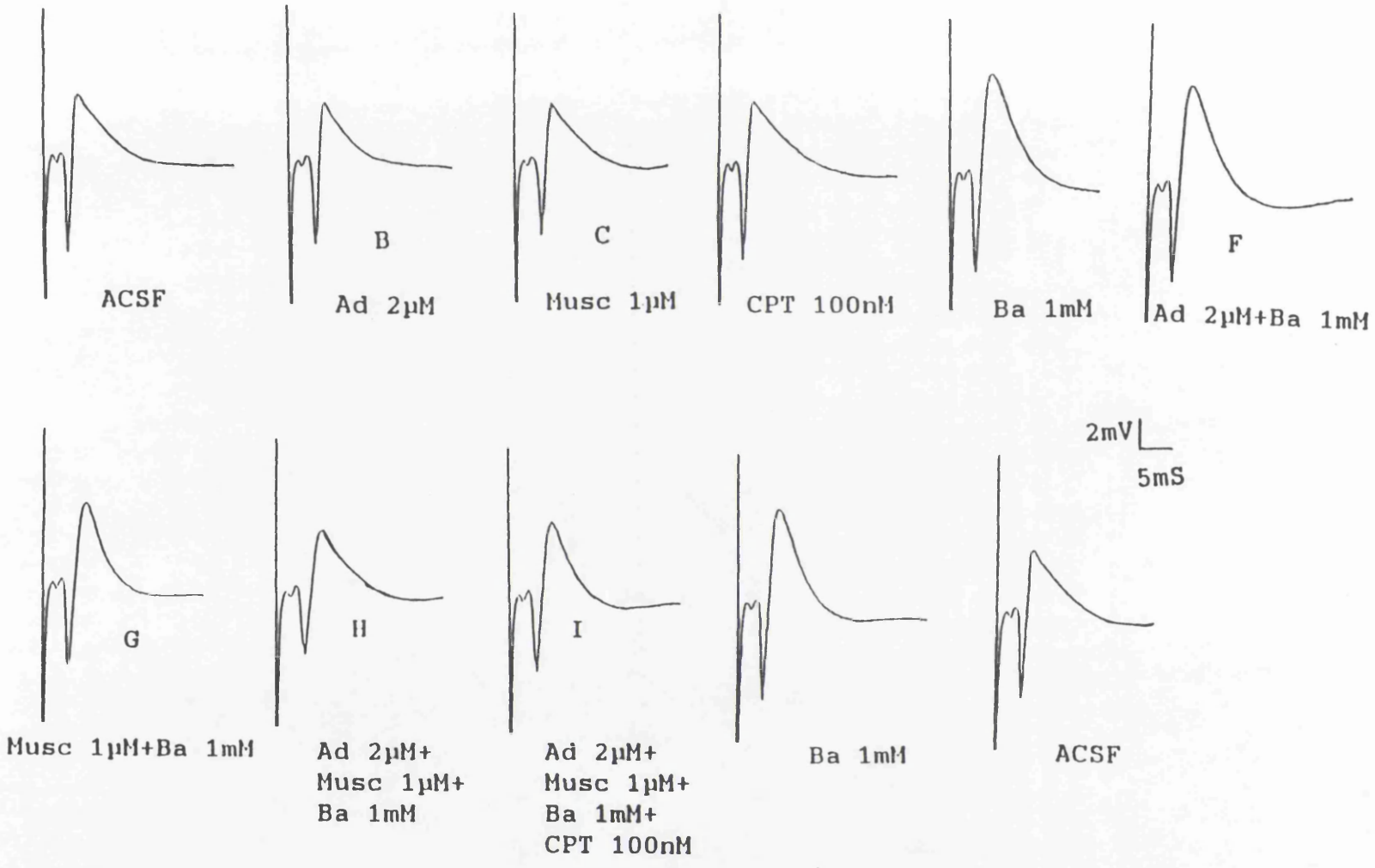

Figure 3.10. Sample records taken during experiments with combinations of agents used in Fig.3.9. The letters correspond to the columns illustrated in Fig.3.9.

3.2.9. Effect of adenosine and muscimol on orthodromic field EPSPs

In separate experiments the effect of combined applications of adenosine and muscimol were studied on the presynaptic terminals. Adenosine at 10 or 20 μM produced a submaximal depression of the amplitude of population EPSPs. Muscimol at 1 or 2 μM had no significant effect of its own on these potentials and did not modify the responses to adenosine (figure 3.11.).

3.2.10. Interaction of somatic or dendritic application of adenosine and muscimol in the presence of barium

Adenosine was effective in reducing orthodromic potentials either at the soma region or in the dendritic tree, whereas muscimol was only effective in the soma region at 1 and 2 μM concentrations. No significant interaction was observed between adenosine and muscimol in the dendritic area.

However, in the presence of barium 1 mM, a significant positive interaction between adenosine 2 μM and muscimol 1 μM was seen when they were applied in the somatic region (figure 3.12.,3.13.).

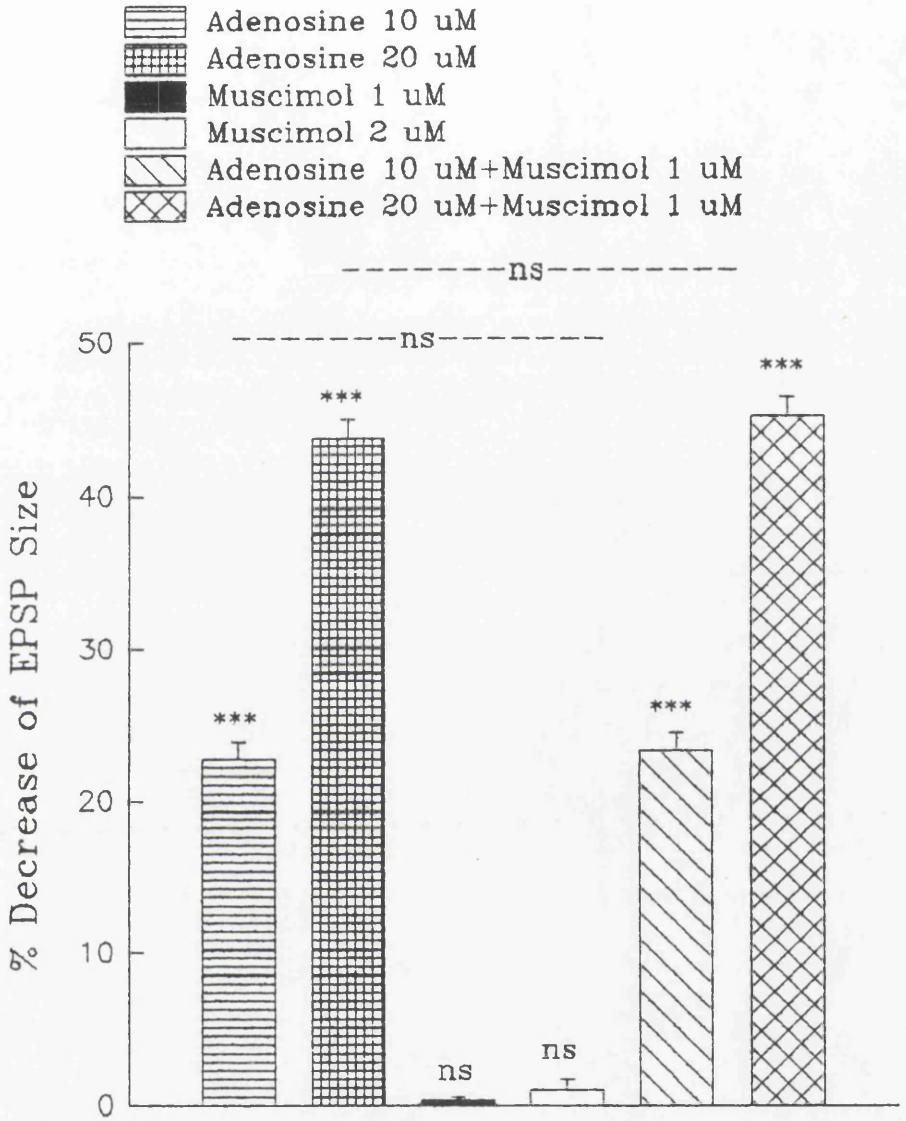

Figure 3.11. Depression of the field EPSP by adenosine 10 μM, adenosine 20 μM, muscimol 1 μM, muscimol 2 μM, adenosine 10 μM + muscimol 1 μM and adenosine 20 μM + muscimol 1 μM. Each vertical bar represents the mean ± s.e.m. for n = 4. A one-way analysis of variance test and Tuky-Kramer multiple comparisons test were employed to determine the significance level ($^{***}P \leq 0.001$).

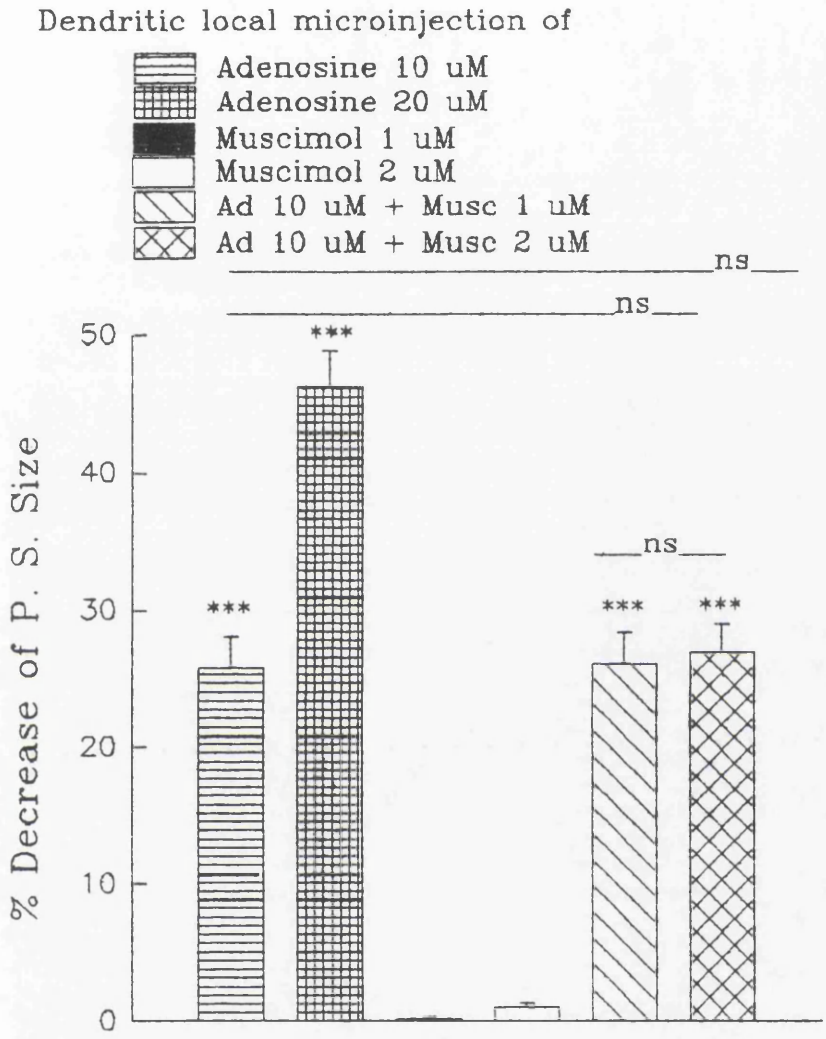

Figure 3.12. Histogram showing the effect of dendritic local microinjection of adenosine and muscimol on orthodromic population spikes. Each vertical bar represents the mean ± s.e.m. for n=4. A paired t test was employed to determine the significance level ($^{***}P \leq 0.001$).

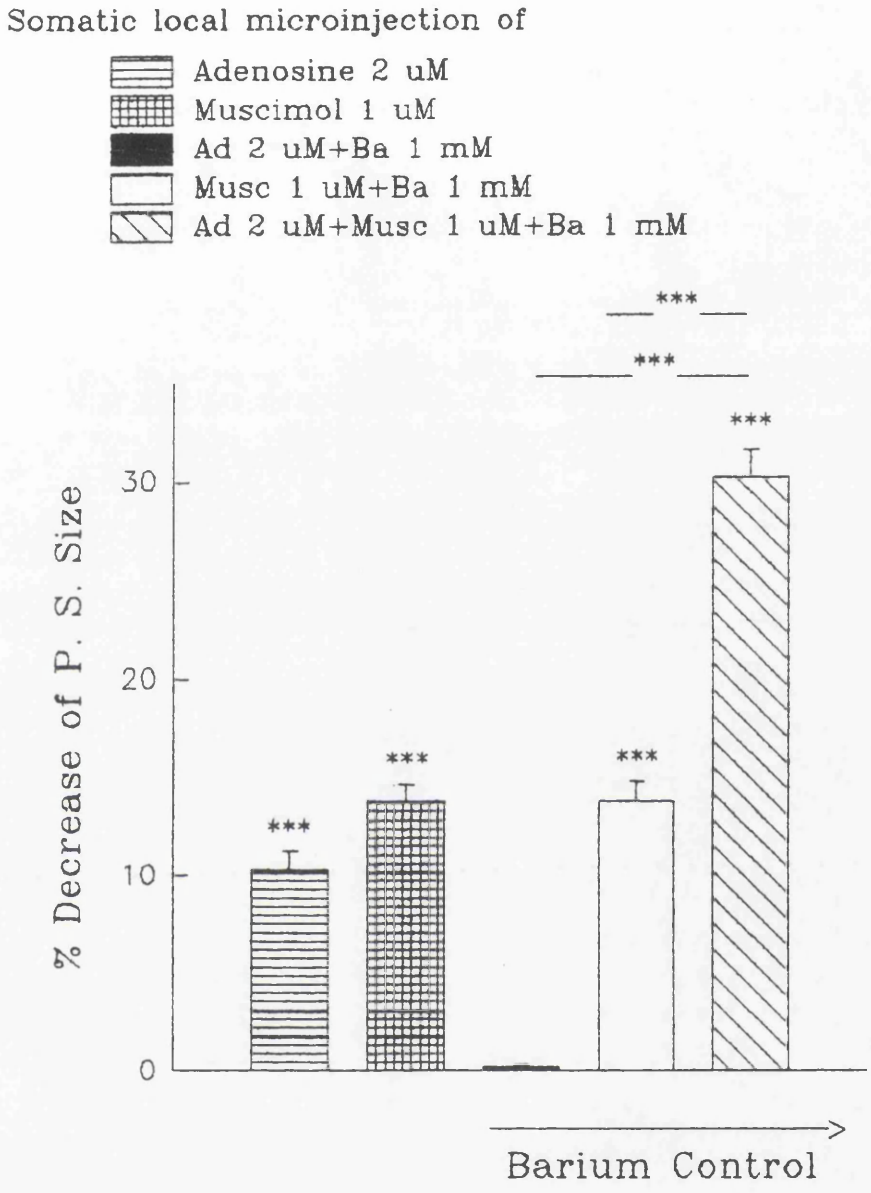

Figure 3.13. Histogram showing the effect of somatic local microinjection of adenosine and muscimol in the presence of barium 1 mM on orthodromic population spikes. Each vertical bar represents the mean ± s.e.m. for n=4. A paired t test was employed to determine the significance level ($^{***}P \leq 0.001$).

3.2.11. Effect of 4,4'-diisothiocyanotostilbene-2,2'disulfonic acid on potentiative interaction between adenosine and muscimol

During superfusion with the chloride channel blocker 4,4'-diisothiocyanatostilbene-2,2-disulfonic acid (DIDS) at 100 μM, population spike size was increased slightly, but significantly, by 6.28% ± 0.38 ($P \leq 0.001$, $n = 5$). In addition, DIDS proved able to reduce significantly the depressant effects of adenosine, 2 or 5 μM (figure 3.14.), and block the inhibitory effect of muscimol, 1 or 2 μM on population spike size (figure 3.15.).

In order to determine whether the action of adenosine could be accounted for entirely in terms of the blockade of barium-sensitive potassium current and DIDS-sensitive chloride channels, the effect of combining these was examined. At a concentration of 10 μM adenosine, which induced approximately 50 % inhibition of population potential size, the combination of barium 1 mM and DIDS 100 μM was significantly more effective than either agent alone in antagonising adenosine; adenosine responses were reduced by about 70 % compared with responses in barium alone (figure 3.16.). However, a residual sensitivity to adenosine was clearly present. When the concentration of

adenosine used was almost maximal, 20 μM, the additional presence of DIDS caused less inhibition, around 45 %, and the residual response to adenosine was correspondingly greater (figure 3.16.). The sequence of combinations reported above was therefore repeated on a different set of slices, but with the addition of a combination with DIDS. As above, barium blocked responses to adenosine, but not muscimol, and a potentiation was then obtained with adenosine and muscimol in the presence of barium, yielding here an inhibition of population potential of 30.85% ± 0.5. The addition of DIDS now abolished this potentiation and reduced the degree of inhibition of the combination by about 60 % (n=4) (figure 3.16.,3.17.,3.18.).

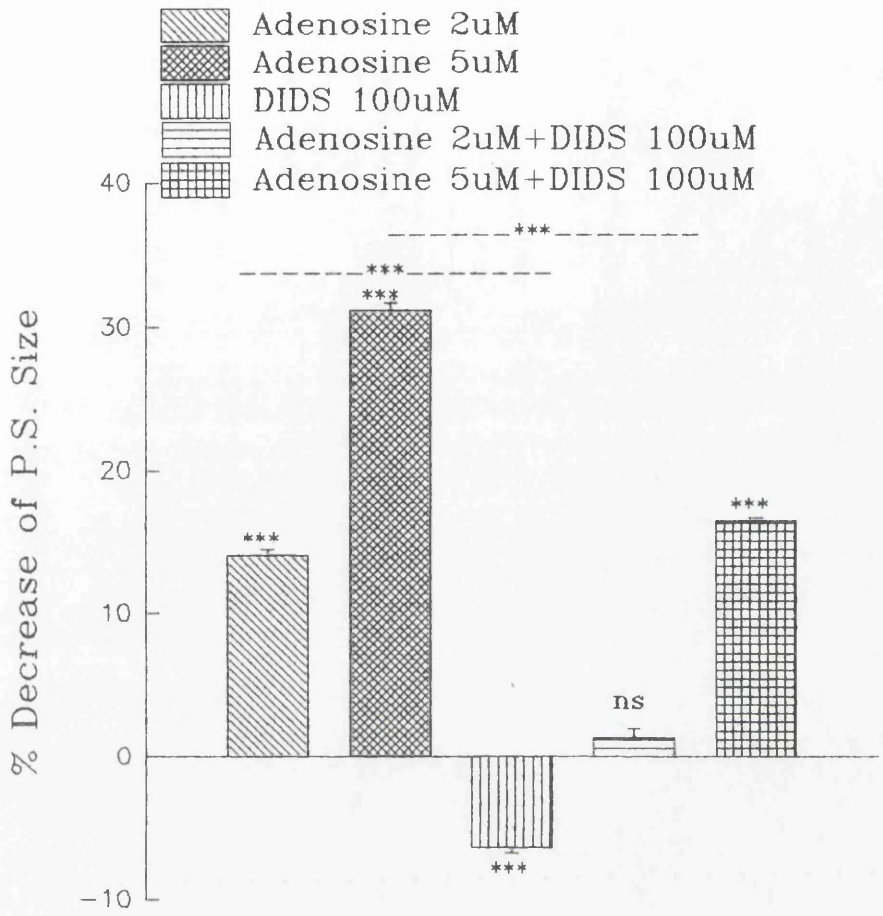

Figure 3.14. Depression of orthodromically evoked CA1 population spikes by different concentrations of adenosine alone and in the presence of DIDS 100 μM. Each vertical bar represents the mean ± s.e.m. for n = 4. A one-way analysis of variance test and Tuky-Kramer multiple comparisons test were employed to determine the significance level ($^{***}P \leq 0.001$).

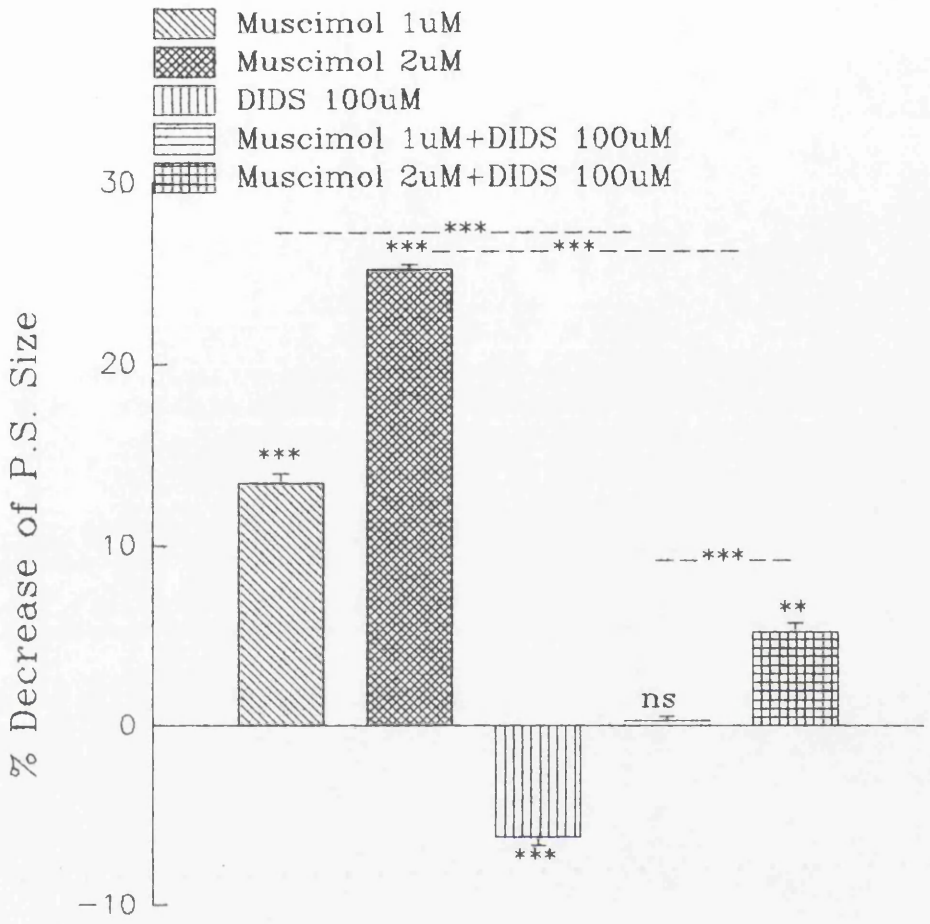

Figure 3.15. Depression of orthodromically evoked CA1 population spikes by different concentrations of muscimol alone and in the presence of DIDS 100 μM. Each vertical bar represents the mean ± s.e.m. for n = 4. A one-way analysis of variance test and Tuky-Kramer multiple comparisons test were employed to determine the significance level ($^{**}P \leq 0.01$; $^{***}P \leq 0.001$).

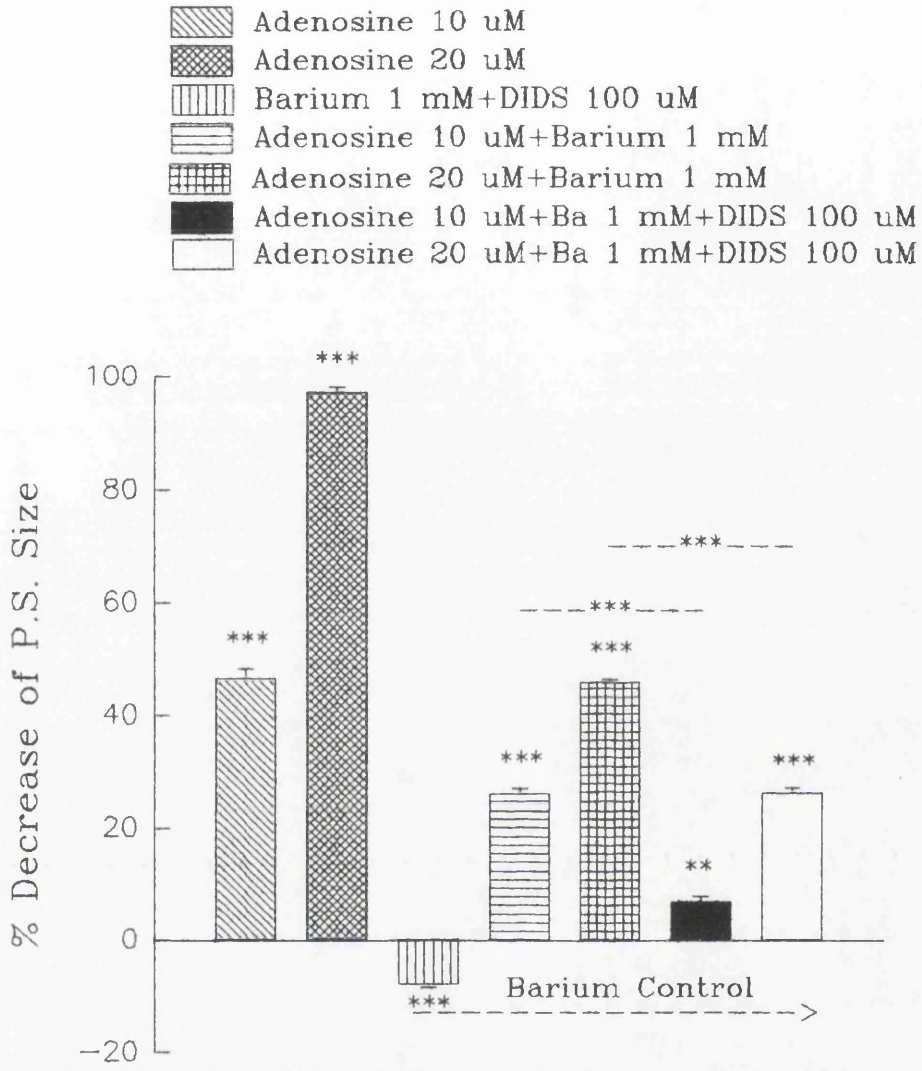

Figure 3.16. Histogram showing the depression of orthodromically evoked CA1 population spikes by adenosine, adenosine + barium and adenosine + barium + DIDS. Except for the first two columns, the changes of potential size were measured with respect to their amplitude in 1 mM barium. Each vertical bar represents the mean ± s.e.m. for n = 4. A one-way analysis of variance test and Tuky-Kramer multiple comparisons test were employed to determine the significance level ($^{***}P \leq 0.001$).

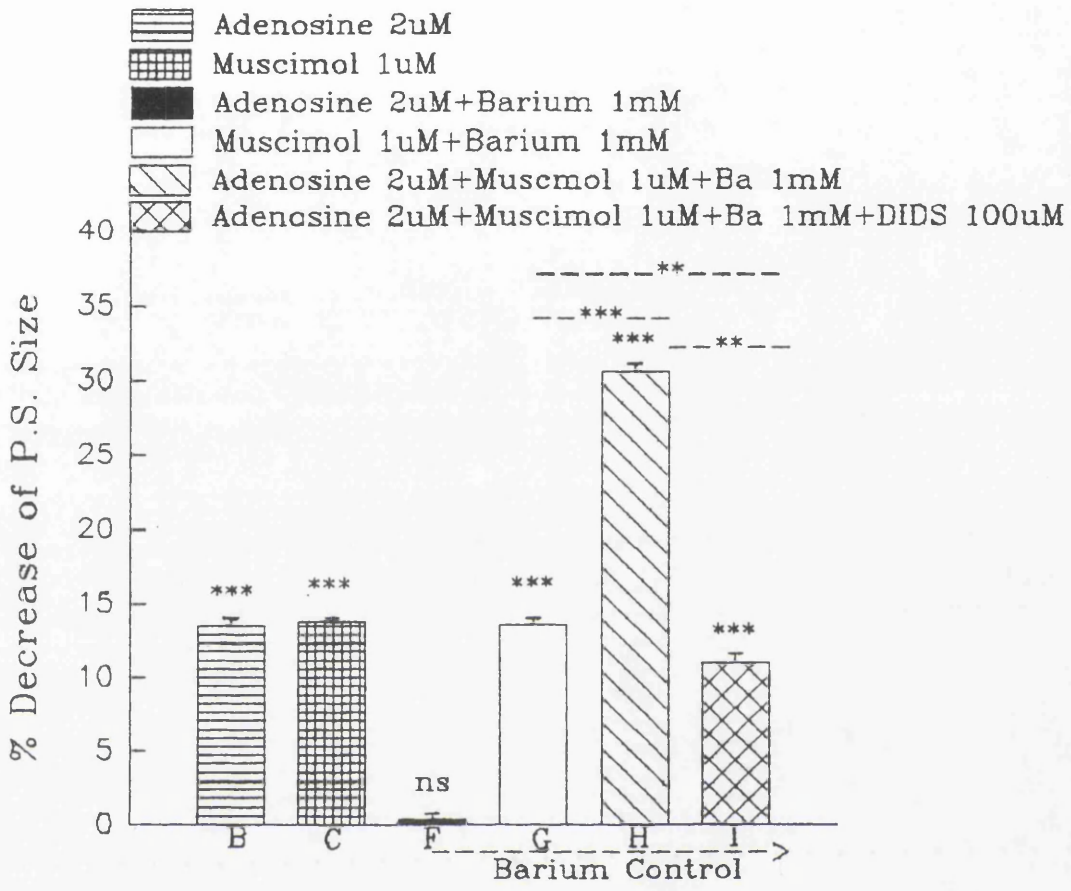

Figure 3.17. Depression of orthodromically evoked CA1 population spikes by adenosine 2 μM, muscimol 1 μM, adenosine 2 μM + Ba 1 mM, muscimol 1 μM + Ba 1 mM, adenosine 2 μM+muscimol 1 μM + Ba 1 mM and adenosine 2 μM + muscimol 1 μM + Ba 1 mM + DIDS 100 μM. The data are taken from a different set of experiments to those summarised in Fig.3.9. Except for the first 2 columns, the changes of potential size were measured with respect to their amplitude in 1 mM barium. Each vertical bar represents the mean ± s.e.m. for n = 4. The letters below the columns refer to the sample records illustrated in Fig.3.18. A one-way analysis of variance test and Tuky-Kramer multiple comparisons test were employed to determine the significance level ($^{**}P \leq 0.01$; $^{***}P \leq 0.001$).

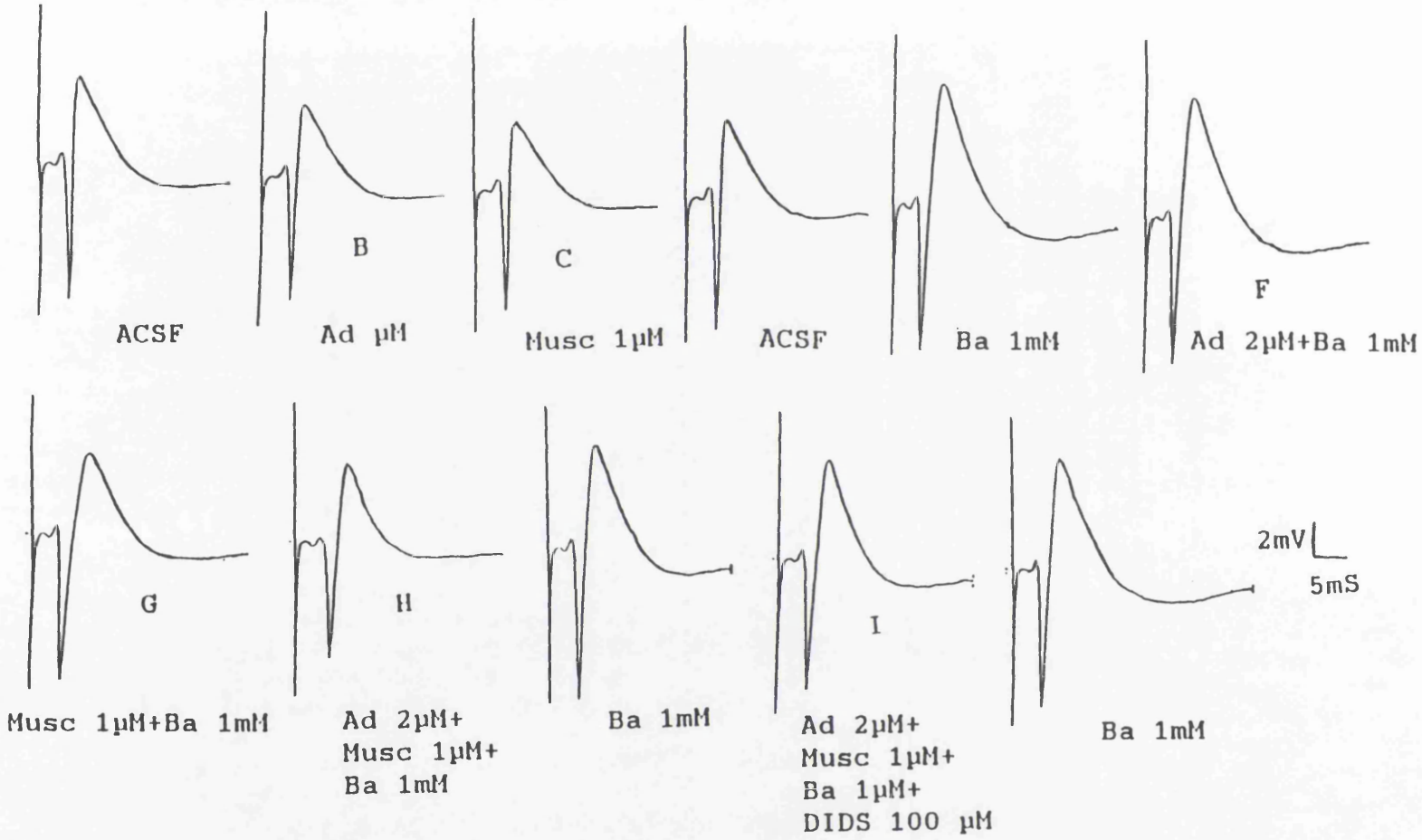

Figure 3.18. Sample records taken during experiments with combinations of agents used in Fig.3.17. The letters correspond to the columns illustrated in Fig.3.17.

3.2.12. Interaction of adenosine and tolbutamide on orthodromic population spikes

Adenosine at 2, 5 and 10 μM reduced the amplitude of orthodromic population spikes by 12.75% ± 1.11 ($P \leq 0.001$, n=4), 28.50% ± 1.44 ($P \leq 0.001$, n=4) and 55.25% ± 2.29 ($P \leq 0.001$, n=4) respectively. Tolbutamide 1 mM alone increased the population spikes by 12.13% ± 1.39 ($P \leq 0.01$, n=4). This effect was seen in less than 10 minutes. The inhibitory effect of adenosine 2 μM was completely blocked by tolbutamide 1 mM. In addition to this, tolbutamide 1 mM significantly decreased the effect of adenosine 5 and 10 μM to 22.38% ± 1.40 ($P \leq 0.05$, n=4) and 37.50% ± 2.60 ($P \leq 0.01$, n=4)(figure 3.19.).

3.2.13. Interaction of adenosine and tolbutamide on orthodromic field EPSPs

The inhibitory effect of adenosine was less on fEPSP size than population spike size. However, tolbutamide 1 mM did not affect this presynaptic effect of adenosine. Tolbutamide alone increased the size of field EPSP by 6.03% ± 1.61 ($P \leq 0.05$, n=4)(figure 3.20.).

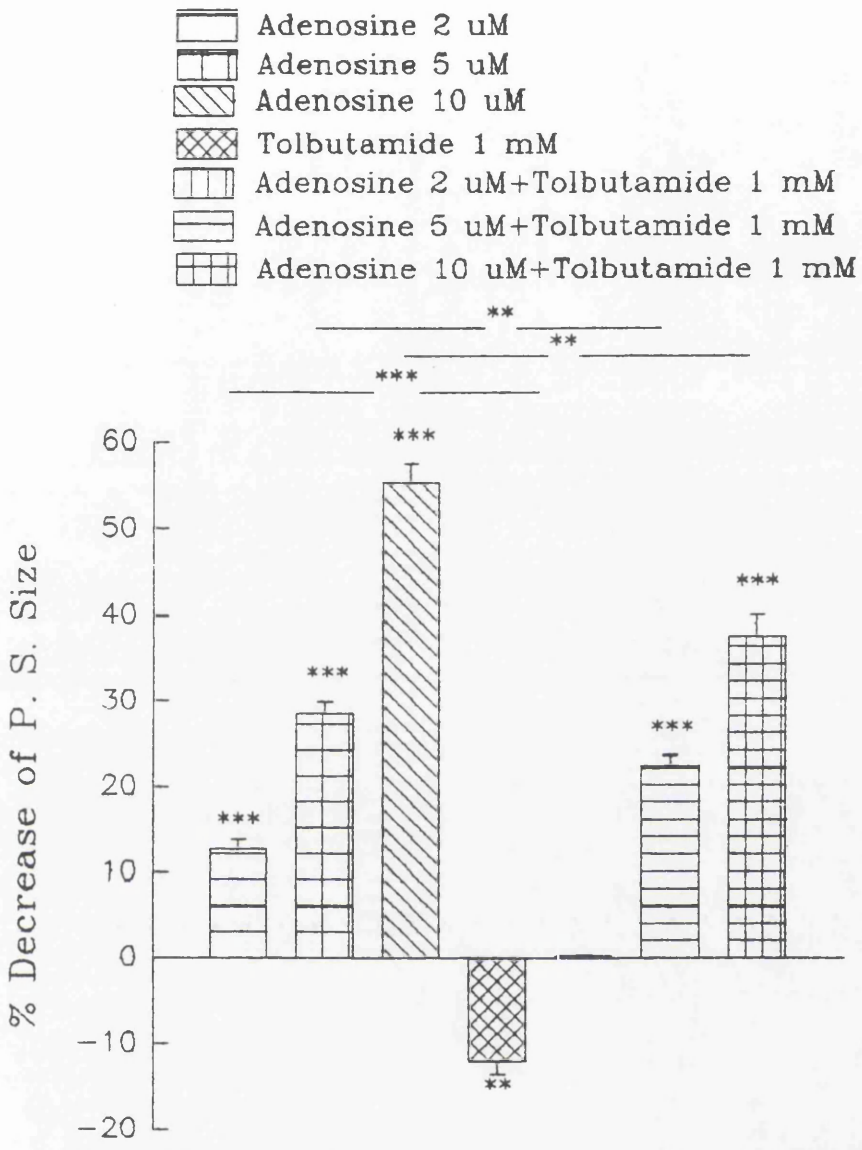

Figure 3.19. Histogram showing the effect of adenosine and tolbutamide on orthodromically evoked CA1 population spikes. Each vertical bar represents the mean ± s.e.m. for n = 4. A paired t test was employed to determine the significance level ($^{**}P \leq 0.01$; $^{***}P \leq 0.001$).

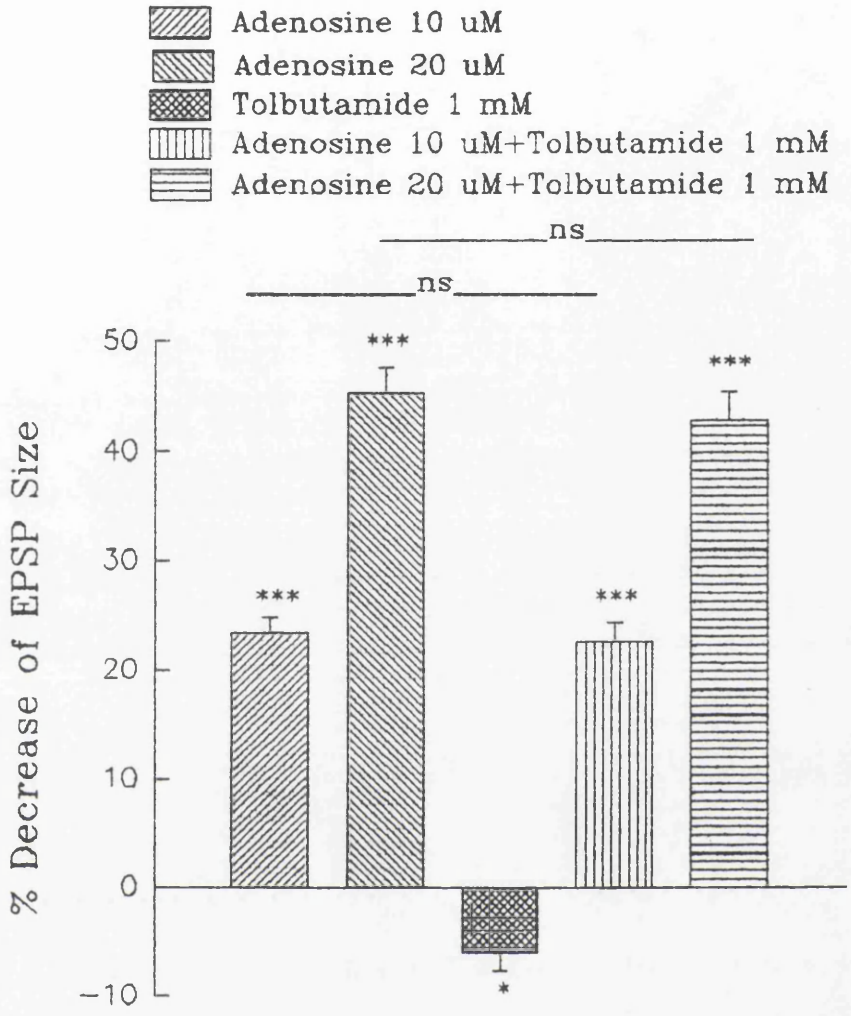

Figure 3.20. Histogram showing the effect of adenosine and tolbutamide on orthodromically evoked CA1 field EPSPs. Each vertical bar represents the mean ± s.e.m. for n = 4. A paired t test was employed to determine the significance level ($^{*}P \leq 0.05$; $^{***}P \leq 0.001$).

3.2.14. Interaction of adenosine and muscimol in the presence of tolbutamide 1 mM

Adenosine 2 μM and muscimol 1 μM reduced the amplitude of orthodromic population spikes by 12.75% ± 1.10 (P≤0.001, n=4) and 13.375% ± 1.02 (P≤0.001, n=4) respectively. In the presence of tolbutamide 1 mM, adenosine 2 μM had no significant effect on the population spike size, whereas muscimol reduced the spike size by 13.5% ± 0.64 (P≤0.001, n=4).

Combination of adenosine 2 μM and muscimol 1 μM in the presence of tolbutamide showed a potentiative interaction and reduced the spike size by 29.5% ± 1.848 (P≤0.001, n=4) (figure 3.21.).

3.2.15. Antidromic extracellular recording

It should be mentioned that the initial protocol to exclude potassium channels of adenosine and to show the possible residual effect of adenosine at the postsynaptic site, was to employ the antidromic extracellular technique. However, barium showed calcium-mimitic features on antidromic spikes and abolished them in less than three minutes. Because of this effect of barium in the antidromic

technique, the initial protocol was changed to the orthodromic technique.

3.2.16. Effect of adenosine on antidromic bursts induced in calcium-free medium with added magnesium (4 mM)

Adenosine concentration-dependently decreased or abolished secondary spikes in calcium-free high magnesium (4 mM) (figure 3.22.). Adenosine at a concentration of 20 μM, (the same concentration that abolished the orthodromic population spikes in normal ACSF) completely abolished the antidromic bursts in calcium-free medium high magnesium (figure 3.22.,3.23.).

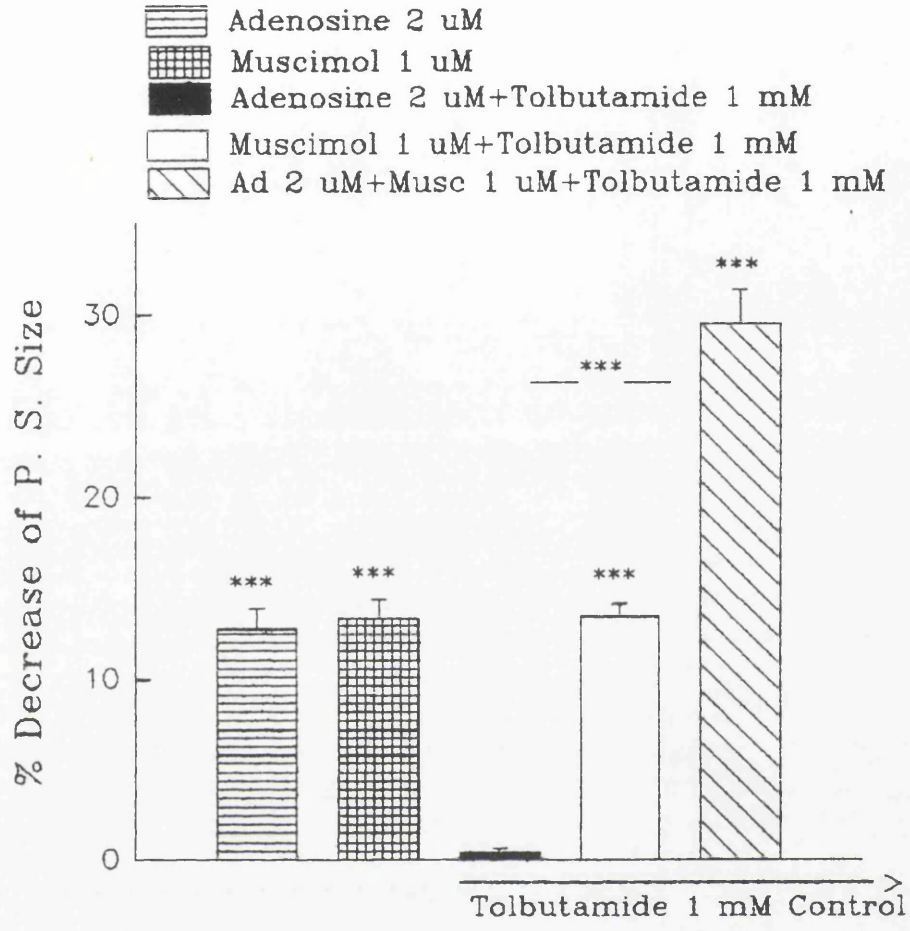

Figure 3.21. Depression of orthodromically evoked CA1 population spikes by adenosine 2 μM, muscimol 1 μM, adenosine 2 μM + tolbutamide 1 mM, muscimol 1 μM + tolbutamide 1 mM and adenosine 2 μM + muscimol 1 μM + tolbutamide 1 mM. Except for the first 2 columns, the changes of potential size were measured with respect to their amplitude in 1 mM tolbutamide. Each vertical bar represents the mean ± s.e.m. for n = 4. A one-way analysis of variance test and Tuky-Kramer multiple comparisons test were employed to determine the significance level ($^{***}P \leq 0.001$).

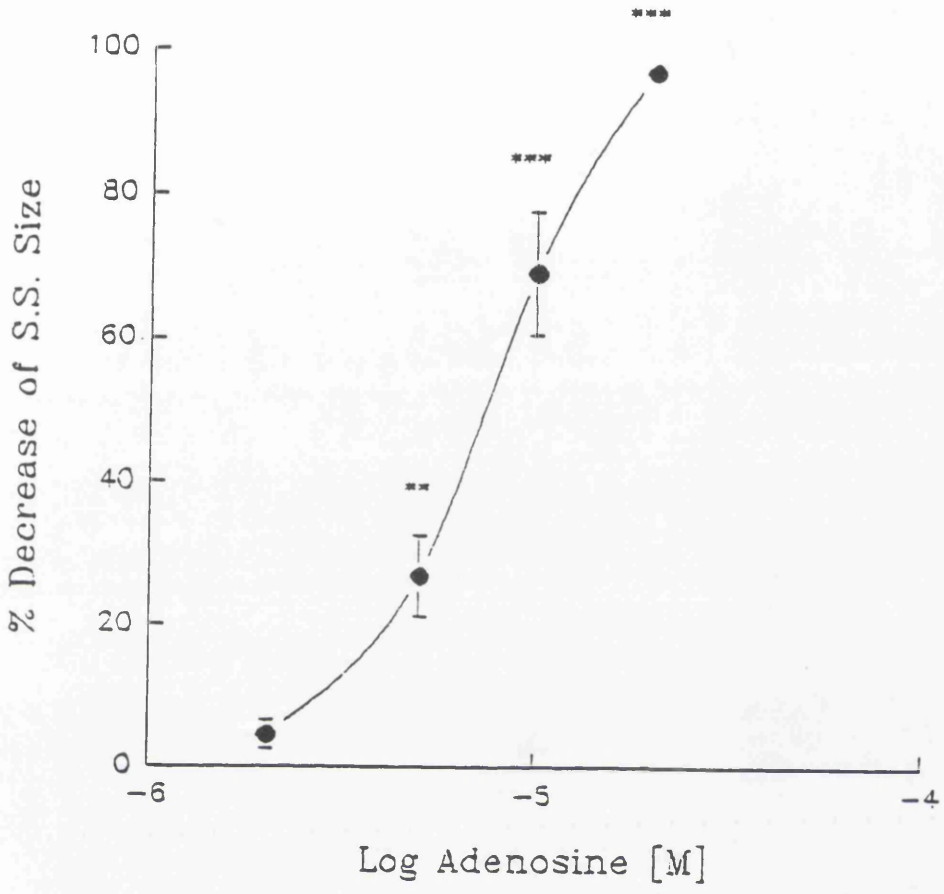

Figure 3.22. concentration-response curve for the effect of adenosine on antidromically induced secondary spikes (S.S.) in calcium-free media containing 4 mM magnesium. Each point represents the mean ± s.e.m. for n=4. A paired t test was employed to determine the significance level ($^{**}P \leq 0.01$; $^{***}P \leq 0.001$).

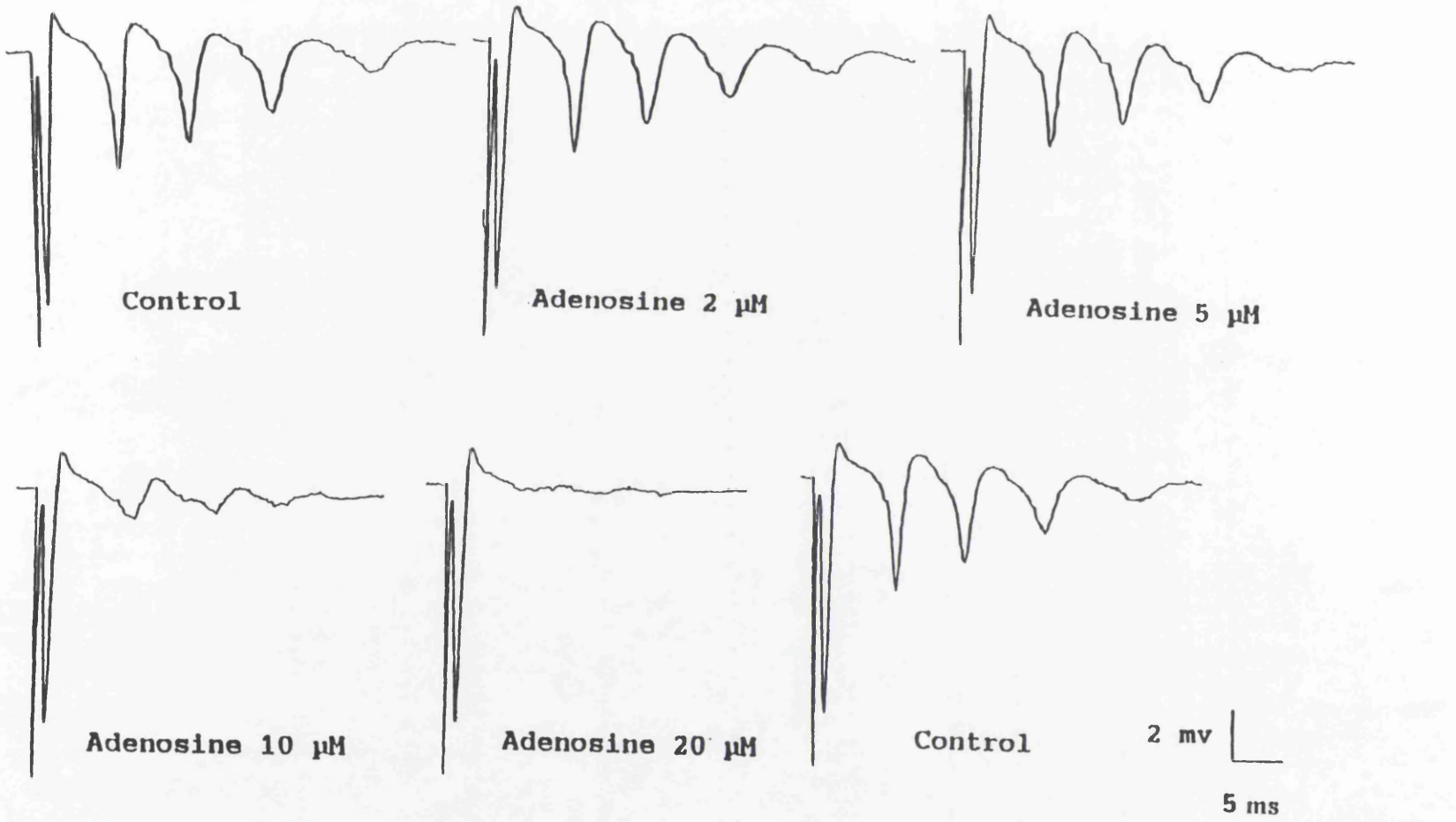

Figure 3.23. Sample records of antidromic potentials in CA1 following the different concentrations of adenosine.

3.3. DISCUSSION

3.3.1. Interaction of adenosine and muscimol in the presence of barium

The results indicate that barium at a concentration of 1 mM can block the inhibitory effect of adenosine on population spike potentials in the hippocampus, without altering inhibition of the population synaptic potential (EPSP) evoked by stimulation of the Schaffer collateral and commissural axons in stratum radiatum. This is entirely consistent with recent evidence from intracellular recordings that barium will block the postsynaptic, but not the presynaptic, effects of adenosine (Thompson and Gähwiler, 1992; Thompson et al., 1992).

The failure of barium to modify responses to muscimol emphasises the relative selectivity of barium for cation channels operated by adenosine, rather than channels associated with $GABA_A$ receptors.

When applied alone, barium also increased the amplitude of the orthodromically evoked population potentials, a finding which may reflect the blockade of potassium channels involved in transmitter mediated tonic inhibition of postsynaptic excitability. A contribution of endogenous

adenosine to this background inhibition was suggested by the finding that CPT also increased the population spikes (Bauman et al., 1992; Gribkoff and Bauman, 1992), an effect which probably results from the blockade of adenosine receptors at both presynaptic and postsynaptic sites. A component of the increased baseline size in barium could be due to a release of endogenous excitatory amino acids (McMahon and Nicholls, 1993).

The greater effect of barium (compared with CPT) on baseline potential size may reflect some blockade of channels associated with other tonically active receptors, such as $GABA_B$ or acetylcholine. Nevertheless, it might be expected that superfusion of slices with a mixture of barium and CPT would increase potential size to at least an additive degree. However, the combined effect was no greater than that obtained with CPT alone, and was substantially less than that obtained with barium alone but CPT proved able to decrease the inhibitory effect of baclofen as well. One possibility is that, CPT besides blocking the effect of adenosine, can block the other potassium channels. Thus, after application of CPT, there are no potassium channels to be affected by barium. Alternatively, since divalent cations are known to modulate binding to A_1 adenosine receptors, barium may be preventing the binding of CPT to receptors.

The major finding of this study was the observation that adenosine, in the presence of a sufficient concentration of barium to block fully responses to the purine alone at 2 μM, could markedly potentiate responses to muscimol, the combined response being approximately 100% greater than to muscimol alone.

The involvement of A_1 adenosine receptors in this potentiation was confirmed by the ability of CPT, a selective antagonist at A_1 receptors, to prevent the potentiation and restore the effect of the combination to that of muscimol alone. The implication of these findings is that there is a population of adenosine receptors which is able to potentiate the activation of $GABA_A$ receptors, but which is not coupled to barium-sensitive potassium channels. It should be emphasized that the potentiative interaction between adenosine and muscimol appears to be confined to the postsynaptic membrane. No interaction between adenosine and muscimol 1 and 2 μM was observed when these were tested on population EPSPs as a reflection of their activity at presynaptic receptors. Furthermore, microinjection experiments confirm that muscimol, at concentrations which were applied in this study, has no effect in the dendritic tree, whereas it has a significant inhibitory effect on the somatic region. This finding is

consistent with the previous experiment which indicates that the interaction of adenosine and muscimol occurs at the postsynaptic site.

The chloride channel blocker DIDS was able to reduce the inhibitory activity of muscimol, consistent with the generally accepted view that $GABA_A$ receptors induce hyperpolarisation primarily by opening chloride channels (Bormann, 1988). The same concentration of DIDS, however, also proved able to prevent the inhibitory activity of adenosine. This reduction of responses to adenosine by DIDS strongly supports the view that adenosine can exert part of its inhibitory activity on postsynaptic neurones by enhancing chloride fluxes. This idea arose initially from observations that chloride channel blockers could interfere with the change of calcium movement induced by purines or xanthines in hippocampal slices (Mager et al., 1990), although effects of purines on chloride movements have been reported in other tissues, such as airway epithelial cells (Rugolo et al., 1993).

When a combination of barium and DIDS was present the suppression of adenosine responses was, as expected, significantly greater than with either channel blocker alone. However, a residual response was seen with adenosine

which was proportionately greater at a higher concentration. This may indicate that higher concentrations can also activate populations of barium-insensitive or DIDS insensitive channels.

The results further indicate that the potassium-independent potentiation noted with the combination of adenosine and muscimol in the presence of barium is partly mediated by chloride channels, since the potentiated response was reduced by more than 60% following the addition of DIDS.

These data do not allow any definitive statement of the relationship between muscimol and adenosine activated chloride channels. However, since a combination of the two agents produced a potentiated effect on inhibition, it is possible that two distinct populations of channels are involved.

3.3.2. Interaction of adenosine and muscimol in the presence of tolbutamide

ATP-sensitive potassium channels constitute a new class of potassium channels that link membrane potentials to the bioenergetic status of the cell. An increase in the

intracellular concentration of ATP tends to close this channel and produce depolarisation (Bernardi and Lazdunski, 1993). In pancreatic cells, where channel function is relatively well understood, depolarisation induced by elevated ATP levels, subsequent to glucose metabolism, opens voltage-gated Ca^{2+} channels to release insulin. Sulphonylurea compounds, such as tolbutamide or glibenclamide, can block this potassium channel and trigger insulin release (Gopalakrishnan et al., 1993). The existence of sulphonylurea binding sites associated with ATP-sensitive K channels have been shown in the hippocampus and other parts of brain (Mourre et al., 1989).

Hypoglycaemia or hypoxia in CNS can activate K_{ATP} as a neuroprotective mechanism of neuronal cells. For example during anoxia, when cellular levels of ATP decline, these channels are activated and can block glutamate release (Zini et al., 1993).

The results indicate K_{ATP} channel involvement in the postsynaptic effect of adenosine and are consistent with a report that an inhibitory postsynaptic potential in spinal nociceptive neurones, apparently mediated by adenosine, was blocked by glibenclamide (Salter et al., 1993). The present result is also consistent with the block of adenosine-

induced hyperpolarisation by glibenclamide in the hippocampus (Li and Henry, 1992). The fact that tolbutamide had no effect on the presynaptic action of adenosine may indicate a totally different mechanism for the pre- and postsynaptic effects. The possible existence of different types of potassium channels activated by adenosine at pre- and postsynaptic sites cannot be excluded.

The results show that tolbutamide, at a concentration of 1 mM, like barium can block the inhibitory effect of adenosine 2 μM on population spike potentials, whereas it is not able to modify responses to muscimol, suggesting the selectivity of tolbutamide for adenosine-activated potassium channels, rather than channels associated with $GABA_A$ receptors.

Similar to the barium experiments, it was observed that adenosine, in the presence of a sufficient concentration of tolbutamide to block fully responses to purine alone at 2 μM, could markedly enhance responses to muscimol, the combined response being approximately 100% greater than to muscimol.

The interaction demonstrated here may help to explain the interactions which have been reported or postulated between purines and benzodiazepines in the CNS (Phillis, 1979; Phillis et al., 1979; Sierralta and Miranda, 1993). They may also explain the observation that the systemic administration of the A_1 selective agonist 2-chloro-N6-cyclopentyladenosine induces a 44% increase in the binding affinity of the chloride channel ligand t-butylbicyclophosphorothionate (TBPS) in mouse neocortex (Concas et al., 1993). This effect was prevented by 1,3-dipropyl-8-cyclopentylxanthine, supporting a role for A_1 receptors.

In summary, this study has revealed that the activation of DIDS-sensitive chloride channels on hippocampal CA1 pyramidal neurones contributes to the inhibitory effect of adenosine on population evoked potentials. This activity can lead to a potentiation of responses to $GABA_A$ receptor activation. Adenosine's activation of chloride channels may thus contribute both directly and indirectly to the overall control of neuronal excitability.

Potentiation by neurosteroids of muscimol/adenosine interaction in rat hippocampus

4.1. Introduction

GABA is an inhibitory neurotransmitter which is abundant in the vertebrate central nervous system (Sivilotti and Nistri, 1991; Veenmaan and Reiner, 1994). There is abundant evidence in favour of this hypothesis, including the presence of specialized neurons that synthesize, contain and release GABA; physiological actions of GABA mimic those of electrical activation of local circuit interneurons and inhibitory postsynaptic potentials (IPSPs) can be blocked by selective GABA antagonists (Iversen et al., 1971; Avoli, 1986; Connors et al., 1988).

At least two distinct GABA receptors have been identified, classified as $GABA_A$ and $GABA_B$ (Bormann, 1988). The $GABA_B$ receptor subtype is linked to K^+ or Ca^{2+} channels by second messenger systems which induce GTP binding proteins. The activation of $GABA_B$ receptors inhibits transmitter release presynaptically or hyperpolarises neurons, postsynaptically (Deisz et al; Kuriyma et al., 1993). Synaptically released GABA binds to $GABA_A$ receptors in the post synaptic membrane and causes an increase in Cl^- conductance and hyperpolarises the cells (Bormann et al., 1987).

The $GABA_A$ receptor complex consists of several polypeptide subunits which form a hetero-oligomeric chloride channel and provide binding sites at least for benzodiazepines, barbiturates, steroids and ethanol (Olsen and Tobin, 1990; Aguyo and Alurcon, 1993).

In 1941 Selye reported the anaesthetic and sedative properties of steroids like progesterone. There are both inhibitory and excitatory steroids that respectively, promote or inhibit GABA activity, at $GABA_A$ receptors. Specific brain neurosteroids which are synthesized in glial cells and some synthetic steroids allosterically modulate GABA gated Cl^- channels. These steroids potentiate both benzodiazepine and muscimol binding whereas, they inhibit the binding of the convulsant t-butylbicyclophosphorothionate (TBPS). It has been reported that alphaxalone and 5α-pregnan-3α-ol-20-one are positive modulators of the $GABA_A$ receptors (Harrison and Simmonds, 1984; Cottrell et al., 1987; Harrison et al., 1987; Peters et al., 1988; Turner and Simmonds, 1989). They have been shown to have two distinct action on the $GABA_A$ receptors. At low concentrations, they potentiate the $GABA_A$ action, while at higher concentrations they can directly open the chloride channel associated with the $GABA_A$ receptors (Majewska et al., 1986; Puia et al., 1991).

Adenosine is a potent inhibitor of neuronal activity in the central and peripheral nervous system (Dunwiddie, 1985; Stone, 1985; Nicoll et al., 1990). As a neuromodulator adenosine has a profound depressant action in the central nervous system. The mechanism of adenosine is not fully understood. However, the action of adenosine may be mediated pre- and postsynaptically through receptor mediated mechanisms including effects on second messenger systems, transmembrane ion fluxes and neurotransmitter release (Greene and Haas, 1991; Stone and Simmonds, 1991). Although the mechanism of presynaptic inhibition is not well known, adenosine may inhibit transmitter release by reducing voltage-dependent Ca^{2+} current, activating potassium current or by mechanisms downstream to Ca^{2+} influx (Greene and Haas, 1991; Thompson et al., 1992). At postsynaptic sites, adenosine and its analogs activate potassium currents (Trussel and Jackson, 1985; Gerber et al., 1989; Thompson et al., 1992).

Adenosine receptors have been categorized into three major subtypes (A_1, A_2, A_3), according to their effect on adenylate cyclase and on the basis of agonist structure-activity relations and the use of a range of xanthine and non-xanthine antagonists (Stone, 1985,1991; Vangalen et al., 1992; Gurden et al., 1993). However, some effects of

adenosine may not be mediated by changes in cyclic AMP levels (Scott and Dolphin, 1987).

Adenosine has been shown to have many effects on the CNS, including sedation, anti convulsant action and protection against ischemia (Greene and Haas, 1991). There is growing evidence that besides acting on potassium and calcium currents, adenosine may also affect chloride movement in hippocampus neurons (Mager et al., 1990,1995). In addition to this, several effects of benzodiazepines are antagonized by theophylline and caffeine which are adenosine receptor antagonists (Polc et al., 1981).

It was shown (chapter 3) that the effect of muscimol on population spike size was enhanced by adenosine while the adenosine activated potassium channels were blocked by barium 1 mM or tolbutamide 1 mM. Moreover, DIDS a chloride channels blocker (Miller and White, 1984) and CPT (cyclopenthyltheophylline) an adenosine A_1 receptor antagonist, decreased this potentiative interaction (Chapter 3).

In this part of the project, the effect of allosteric modulatory steroids was examined on the inhibitory effect of adenosine, muscimol and on the potentiative interaction between adenosine and muscimol.

4.2. Results

Of the various neurosteroids that are positive modulators of $GABA_A$ receptors, alphaxalone was selected as a synthetic neurosteroid and 5α-pregnan-3α-ol-20-one as an endogenous neurosteroid. Both agents have been shown to potentiate the inhibitory effect of $GABA_A$ at low concentrations while at high concentrations, like barbiturates, they are able to act directly on the $GABA_A$ chloride channel.

4.2.1. Effect of alphaxalone on orthodromic population spikes

At low concentrations of 0.5 and 1 μM alphaxalone had no significant effect on the orthodromic population potential size. Higher concentrations of 3, 5 and 10 μM, however, reduced spike size significantly by up to 16% (figure 4.1.).

4.2.2. Effect of 5α-pregnan-3α-ol-20-one on orthodromic population spikes

At a low concentration of 0.5 μM, 5α-pregnan-3α-ol-20-one had no effect on population spike size. Higher concentrations of 1, 5 and 10 μM reduced spike size significantly by up to 23% (figure 4.2).

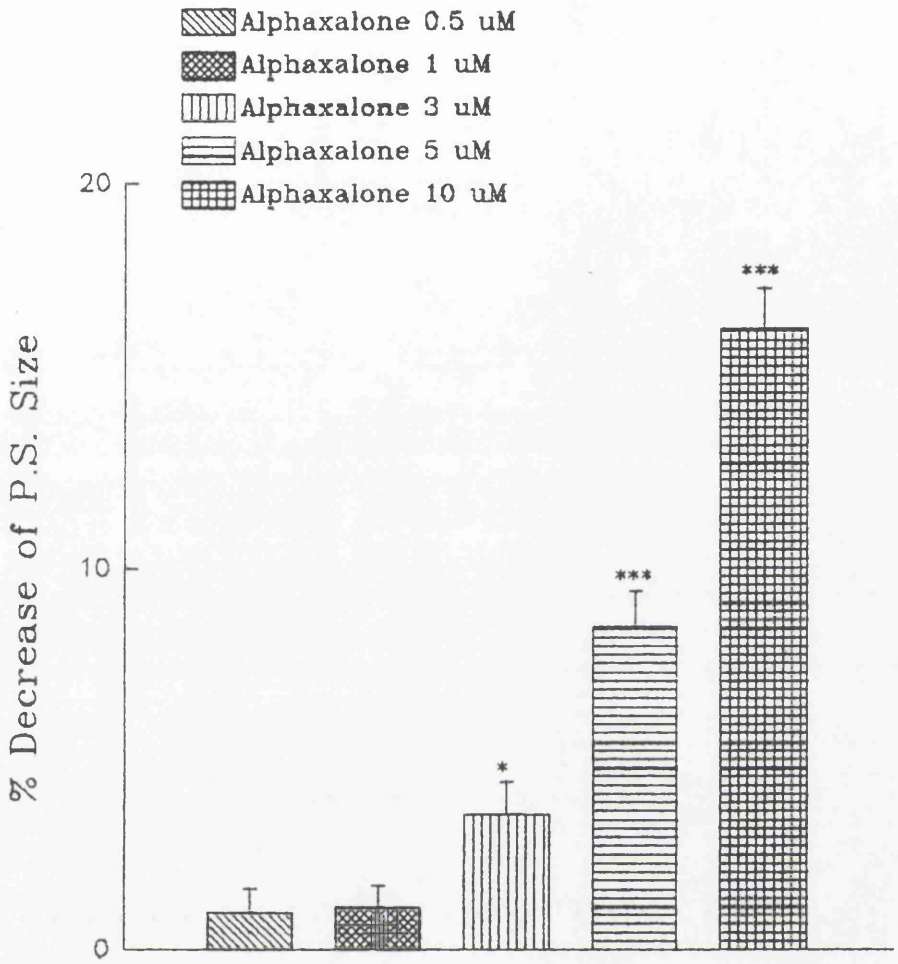

Figure 4.1. Depression of orthodromically evoked CA1 population spikes (P.S.) by alphaxalone. Each vertical bar represents the mean ± s.e.m. for n = 4 experiments. A paired Student's t-test was employed to determine the significance level ($^{*}P \leq 0.05$, $^{***}P \leq 0.001$).

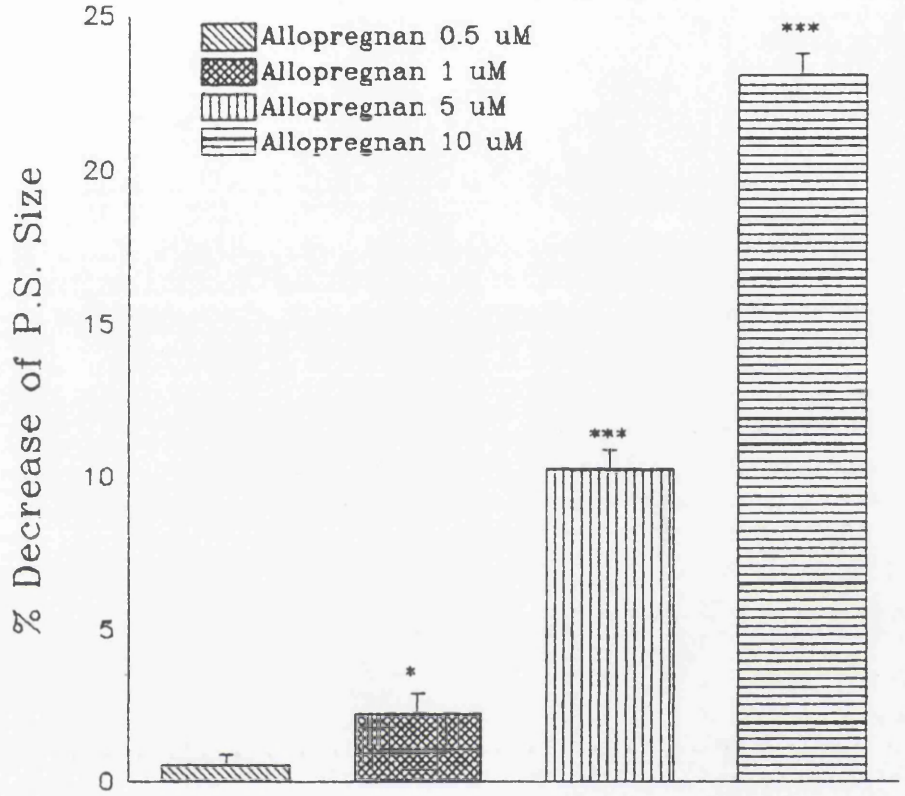

Figure 4.2. Depression of orthodromically evoked CA1 population spikes (P.S.) by 5α-pregnan-3α-ol-20-one (allopregnan). Each vertical bar represents the mean ± s.e.m. for n = 4 experiments. A paired Student's t-test was employed to determine the significance level ($^{*}P \leq 0.05$, $^{***}P \leq 0.001$).

4.2.3. Interaction of alphaxalone and muscimol on orthodromic population spikes

Muscimol 1 μM reduced spike size by 13.97% ± 0.65 ($P \leq 0.001$, $n = 4$). Alphaxalone 0.5 and 1 μM, concentrations that had no significant effect on the spike size, potentiated the inhibitory effect of muscimol to 20.82% and 32.50%. Although alphaxalone at 5 μM exerted a direct inhibition of spike size, it also potentiated the effect of muscimol significantly (figure 4.3.,4.4.).

4.2.4. Interaction of 5α-pregnan-3α-ol-20-one and muscimol on orthodromic population spikes

5α-pregnan-3α-ol-20-one 0.5 and 1 μM potentiated the inhibitory effect of muscimol to 23.50% and 37.18% (figure 4.5.).

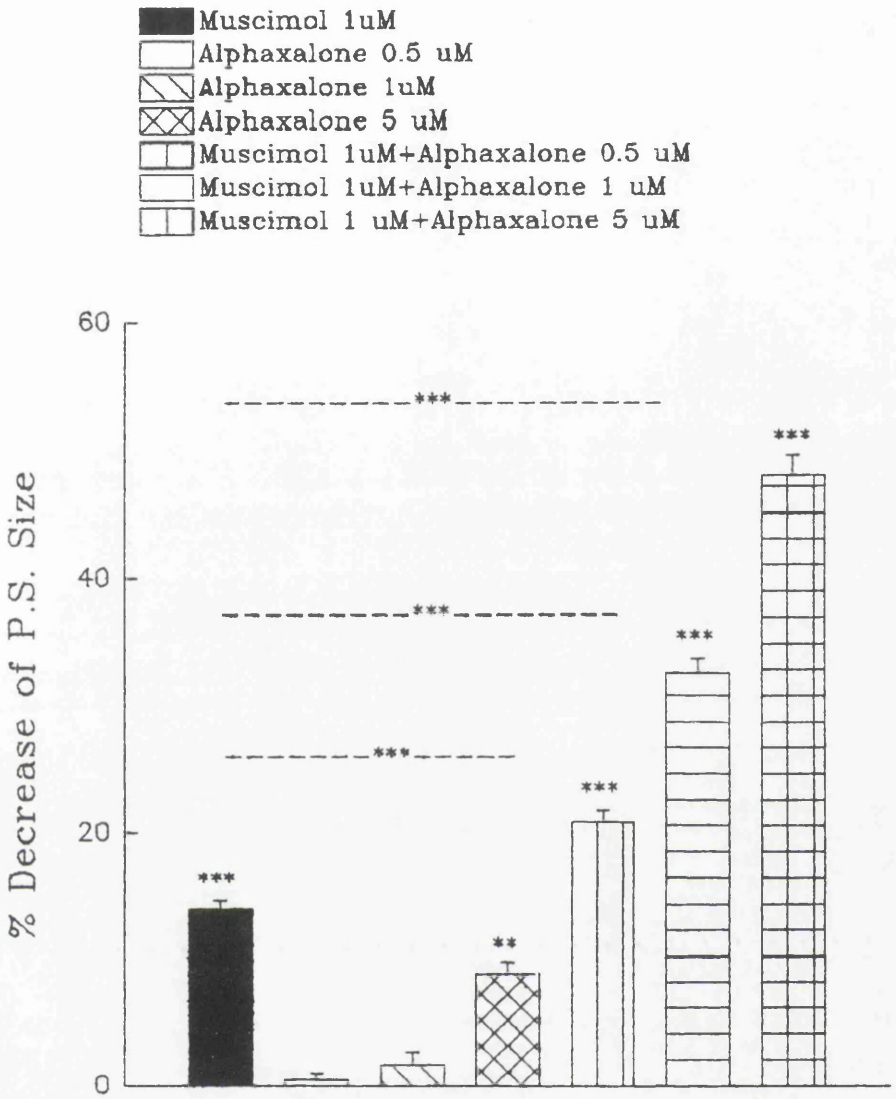

Figure 4.3. Depression of orthodromically evoked CA1 population spikes (P. S.) by muscimol 1 μM, alphaxalone and muscimol 1 μM + alphaxalone. Each vertical bar represents the mean ± s.e.m. for n = 4 experiments. A paired Student's t-test was employed to determine the significance level ($^{**}P \leq 0.01$, $^{***}P \leq 0.001$).

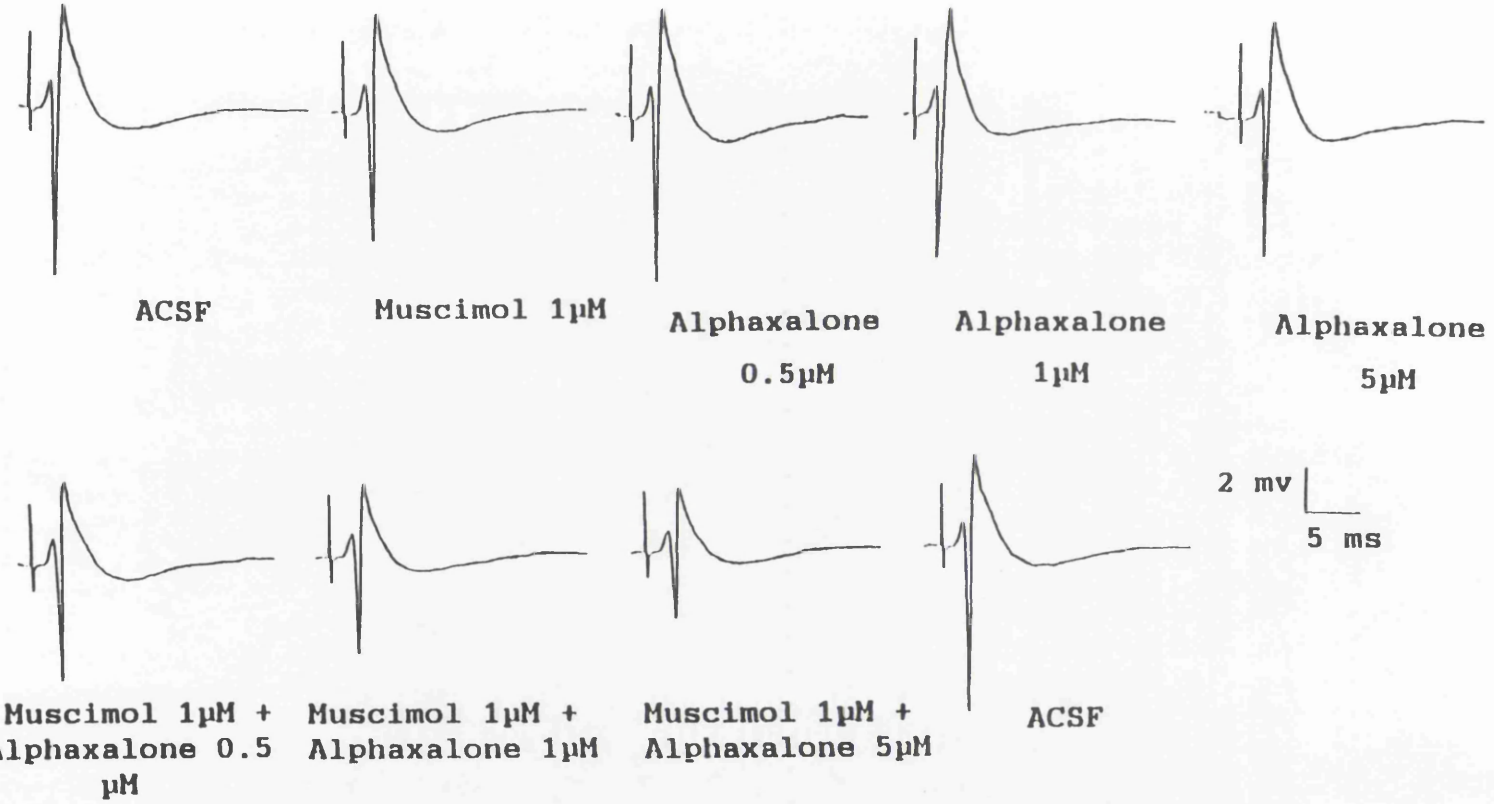

Figure 4.4. Sample records of orthodromic potentials CA1 taken during experiments with agents used in Fig.4.3.

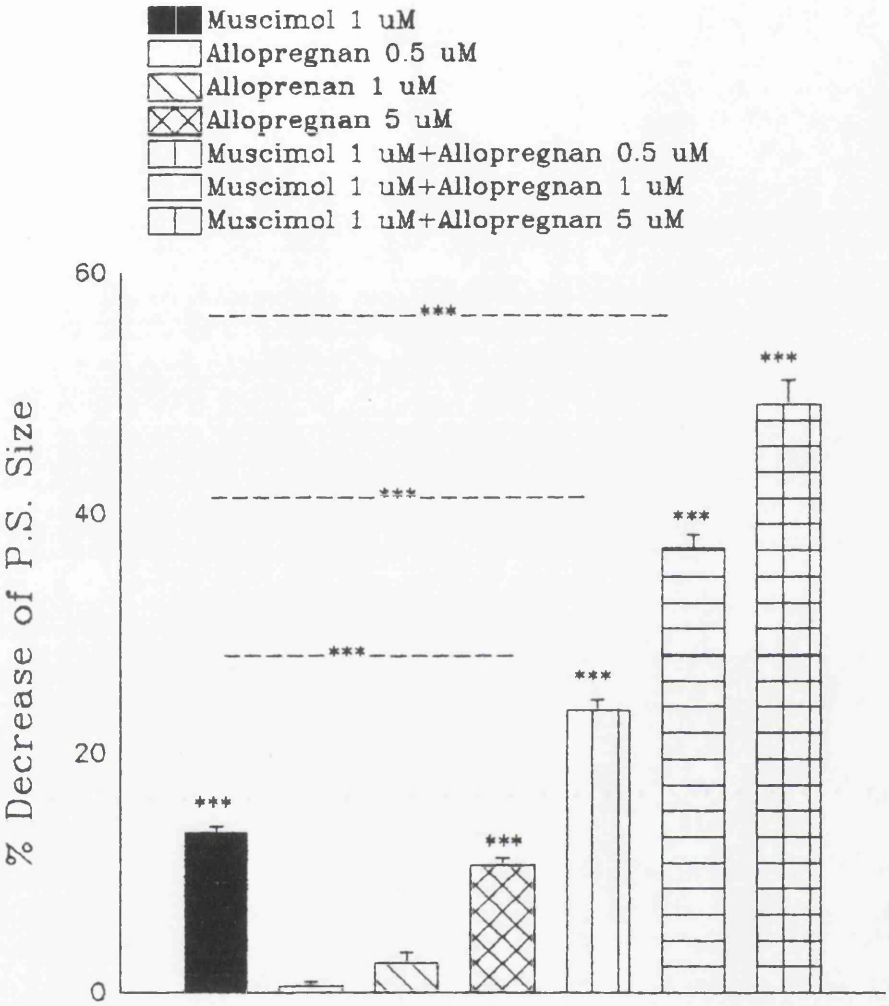

Figure 4.5. Depression of orthodromically evoked CA1 population spikes (P. S.) by muscimol 1 μM, 5α-pregnan-3α-ol-20-one (allopregnan) and muscimol 1 μM + allopregnan. Each vertical bar represents the mean ± s.e.m. for n = 4 experiments. A paired Student's t-test was employed to determine the significance level ($^{***}P \leq 0.001$).

4.2.5. Interaction of alphaxalone and adenosine on orthodromic population spikes

Adenosine 2 μM reduced the spike amplitude by 12.85% ± 0.53 ($P \leq 0.001$, n = 4). Alphaxalone at 1 μM, a concentration without any significant effect on the spike size potentiated the inhibitory effect of adenosine significantly. The combination of adenosine 2 μM and alphaxalone 5 μM reduced the spike size by 41.00% ± 1.29 ($P \leq 0.001$, n = 4) (figure 4.6.,4.7.).

In order to exclude the role of adenosine activated potassium channels in these interactions, these experiments were repeated in the presence of barium 1 mM. In the presence of barium, adenosine 2 μM became ineffective, whereas alphaxalone 5 μM still reduced the spike amplitude by 9% ± 0.37 ($P \leq 0.001$, n = 4). The combination of adenosine 2 μM with alphaxalone 1 and 5 μM in the presence of barium reduced the spike size by 5.90% and 20.13% respectively (figure 4.8.).

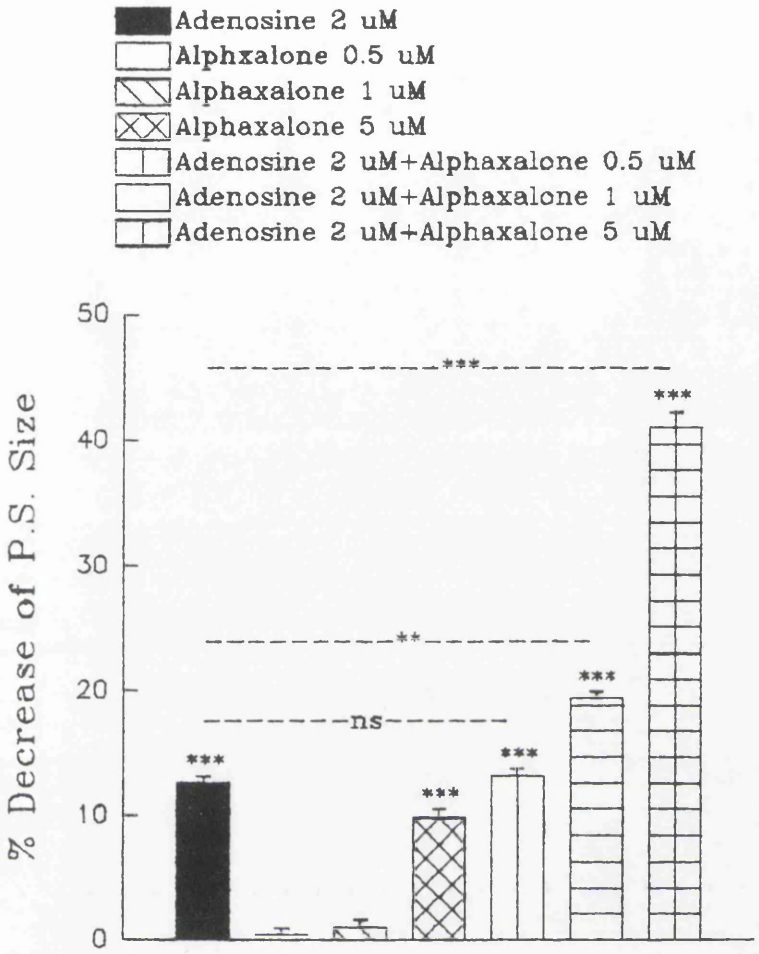

Figure 4.6. Depression of orthodromically evoked CA1 population spikes (P. S.) by adenosine 2 μM, alphaxalone and adenosine 2 μM + alphaxalone. Each vertical bar represents the mean ± s.e.m. for n = 4 experiments. A paired Student's t-test was employed to determine the significance level ($^{**}P \leq 0.01$, $^{***}P \leq 0.001$).

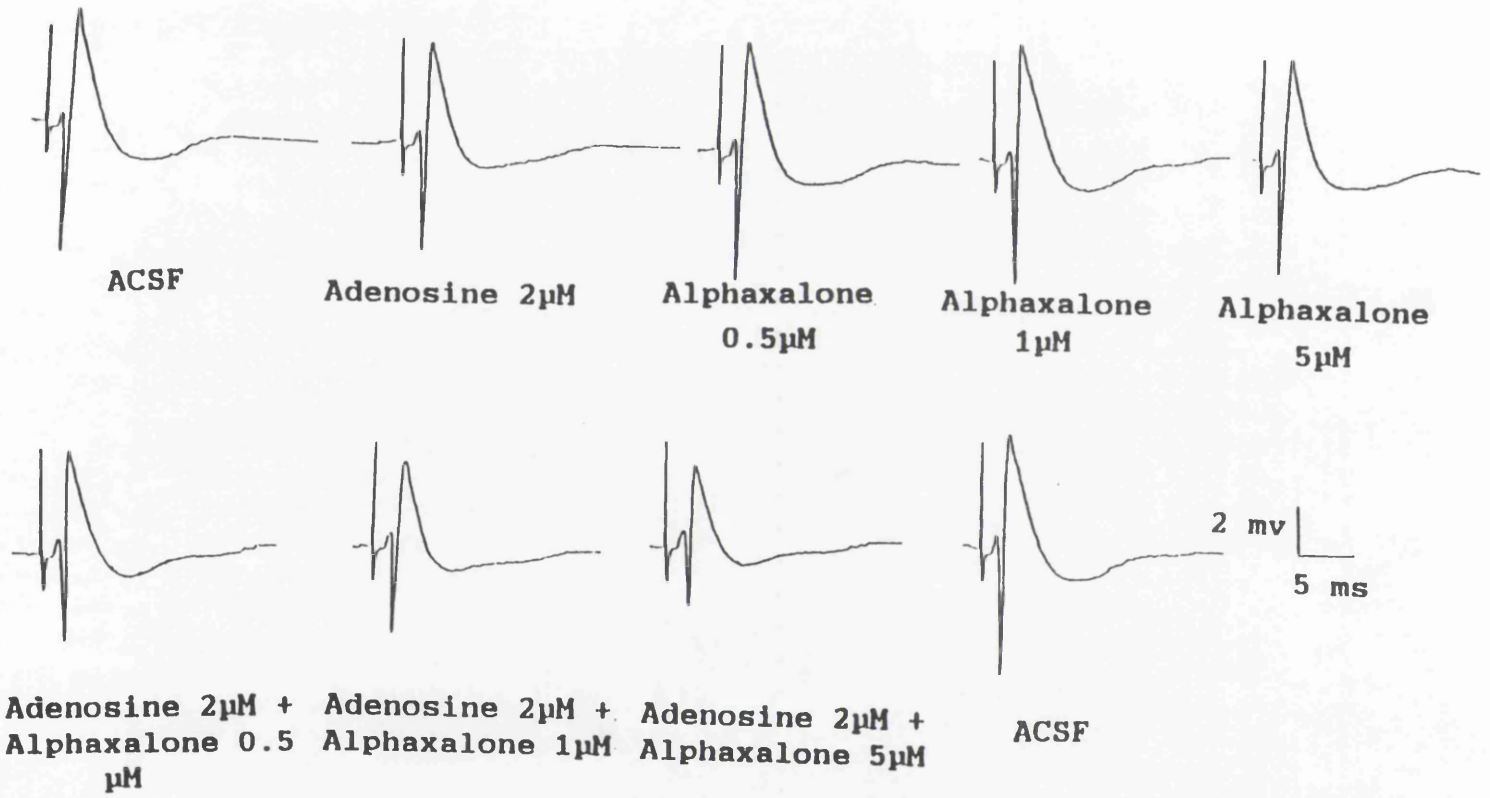

Figure 4.7. Sample records of orthodromic potentials CA1 taken during experiments with agents used in Fig.4.6.

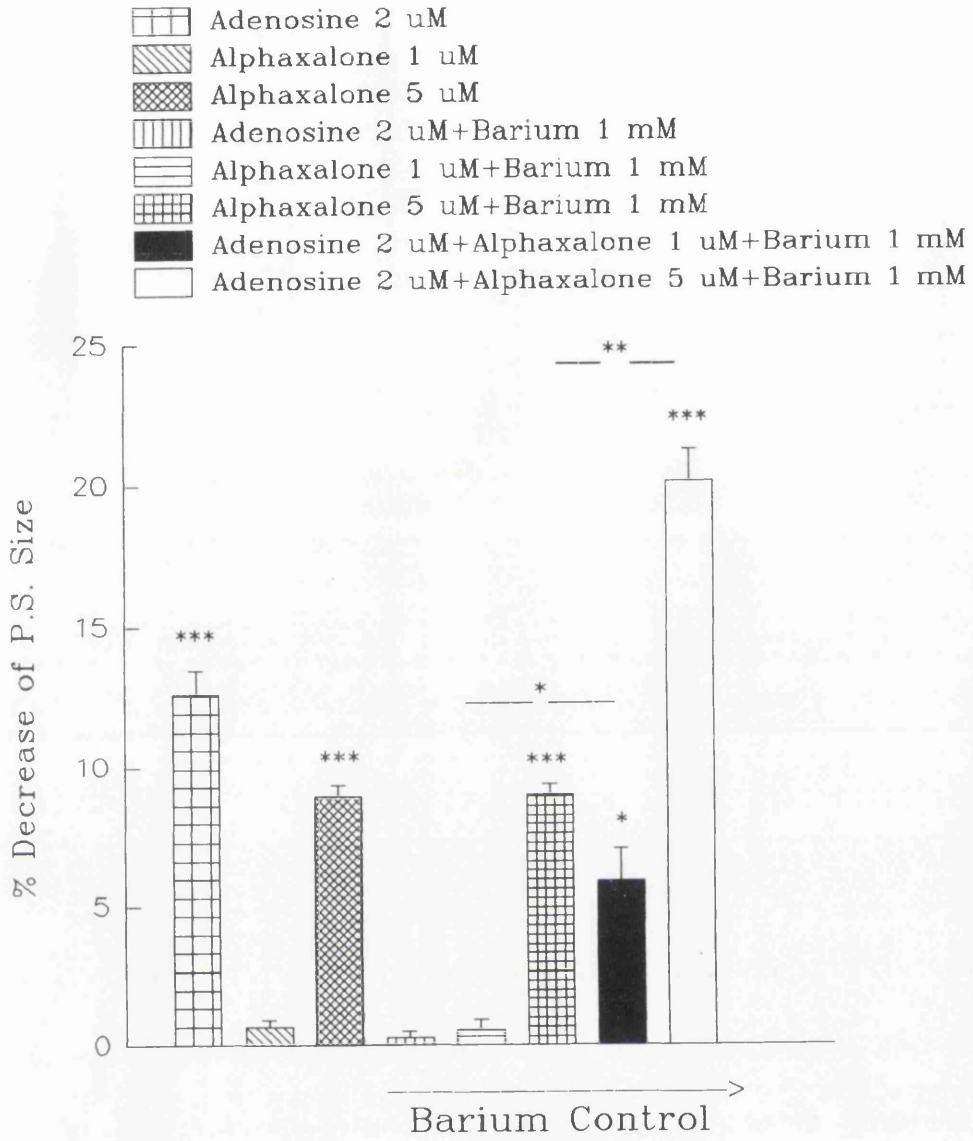

Figure 4.8. Depression of orthodromically evoked CA1 population spikes (P. S.) by adenosine 2 μM, alphaxalone, adenosine 2 μM + barium 1 mM, alphaxalone + barium 1 mM and adenosine 2 μM + alphaxalone + barium 1 mM. Each vertical bar represents the mean ± s.e.m. for n = 4 experiments. A one-way analysis of variance test and Tuky-Kramer multiple comparisons tast were employed to determine the significance level ($^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$).

4.2.6. Interaction of 5α-pregnan-3α-ol-20-one and adenosine on orthodromic population spikes

5α-pregnan-3α-ol-20-one 1 and 5 μM potentiated the inhibitory effect of adenosine 2 μM significantly (figure 4.9.). In the presence of barium 1 mM 5α-pregnan-3α-ol-20-one 5 μM reduced the population spike size by 10.47% (figure 4.10.). The combination of adenosine 2 μM with 5α-pregnan-3α-ol-20-one 1 and 5 μM in barium, reduced the spike amplitude by 6.10% and 23.20% respectively approximately double the response size expected from a purely additive interaction (figure 4.10.).

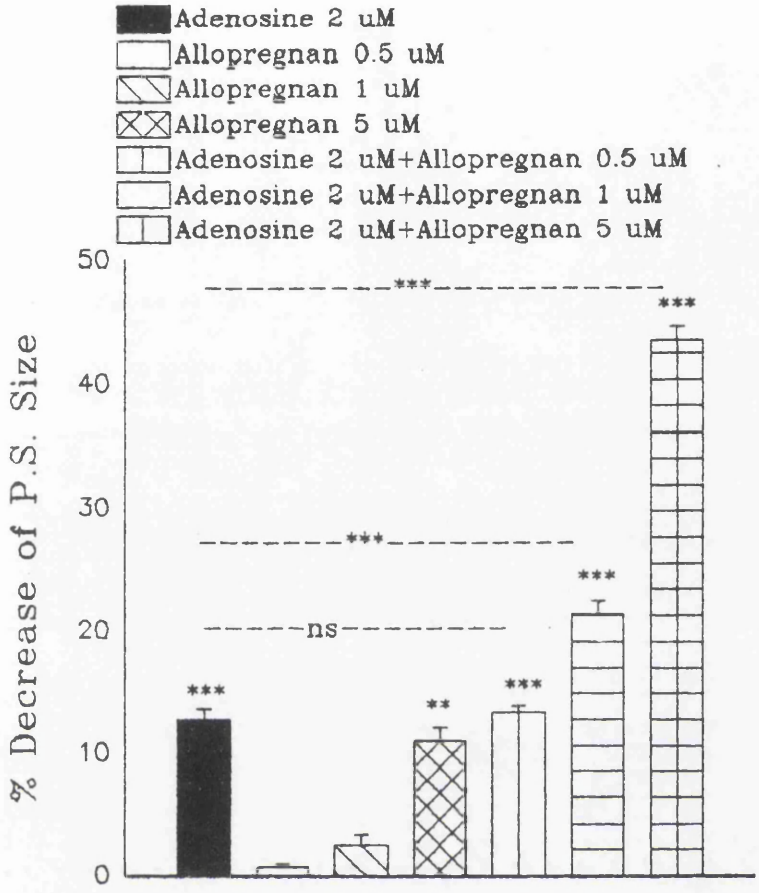

Figure 4.9. Depression of orthodromically evoked CA1 population spikes (P. S.) by adenosine 2 μM, 5α-pregnan-3α-ol-20-one (allopregnan) and adenosine 2 μM + allopregnan. Each vertical bar represents the mean ± s.e.m. for n = 4 experiments. A paired Student's t-test was employed to determine the significance level ($^{**}P \leq 0.01$, $^{***}P \leq 0.001$).

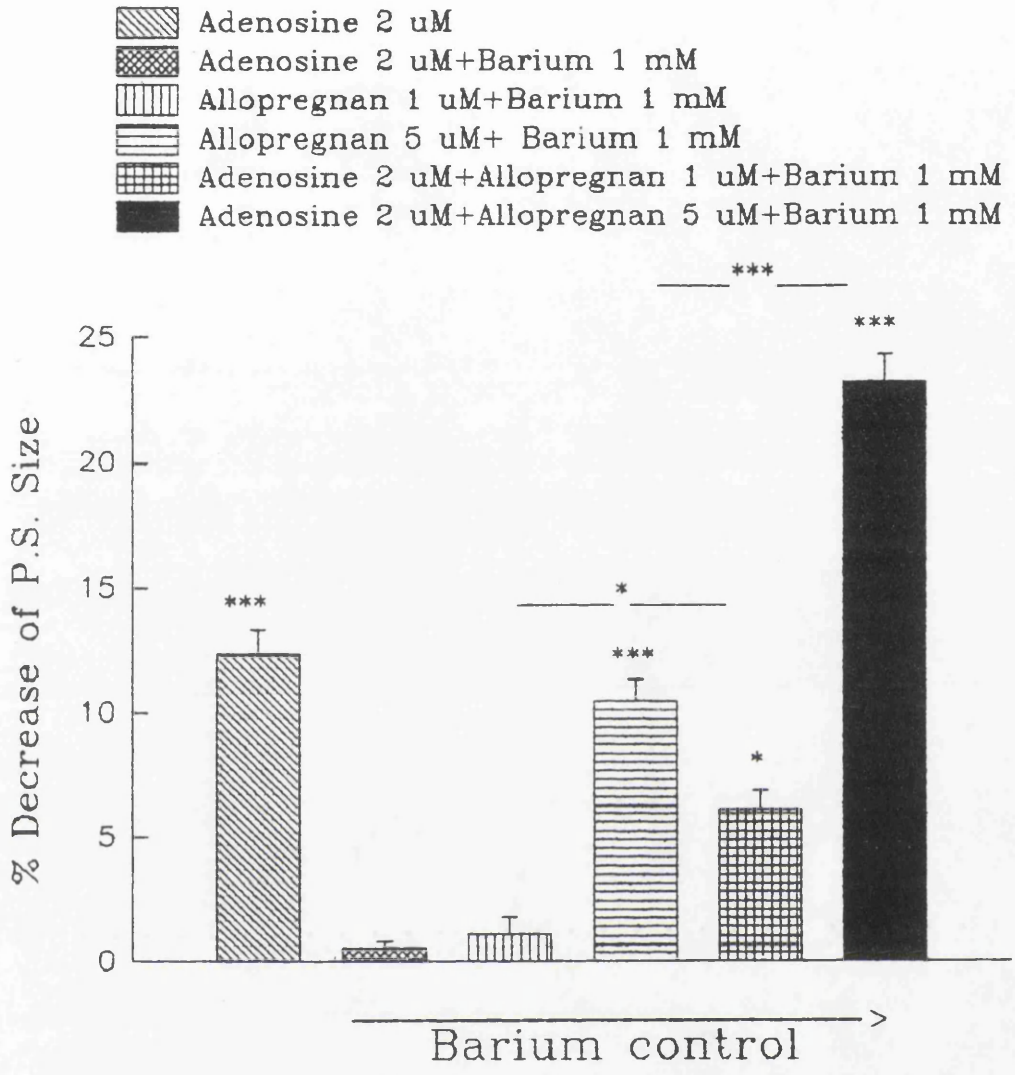

Figure 4.10. Depression of orthodromically evoked CA1 population spikes (P. S.) by adenosine 2 μM, 5α-pregnan-3α-ol-20-one (allopregnan), adenosine 2 μM + barium 1 mM, allopregnan + bairum 1 mM and adenosine 2 μM + allopregnan + barium 1 mM. Each vertical bar represents the mean ± s.e.m. for n = 4 experiments. A one-way analysis of variance test and Tuky-Kramer multiple comparisons test were employed to determine the significance level ($^{*}P \leq 0.05$, $^{***}P \leq 0.001$).

4.2.7. Effect of combination of adenosine 2 μM and muscimol 1 μM with alphaxalone in the presence of barium 1 mM

In barium, muscimol 1 μM reduced the spike size by 13.32% ± 0.71 (P≤0.001, n = 4) while adenosine 2 μM was ineffective. The combination of muscimol 1 μM with adenosine 2 μM reduced the spike size by 29.73% ± 0.83 (P≤0.001, n=4). This reduction was increased significantly in alphaxalone 1 and 5 μM to 39.00% ± 1.29 (P≤0.01, n=4) and 49.50% ± 1.04 (P≤0.001, n=4) (figure 4.11.).

4.2.8. Effect of combination of adenosine 2 μM and muscimol 1 μM with 5α-pregnan-3α-ol-20-one in the presence of barium 1 mM

The combination of adenosine 2 μM and muscimol 1 μM in barium reduced the spike size by 30.17% ± 0.98 (P≤0.001, n=4). This potentiative interaction was enhanced by 5α-pregnan-3α-ol-20-one 1 and 5 μM t0 40% ± 1.87 (P≤0.05, n=4) and 50.17% ± 3.02 (P≤ 0.01, n=4) respectively (figure 4.12.) .

To determine if these neurosteroids potentiate adenosine action directly or through the activating of endogenous GABA, the experiment with adenosine and 5α-

pregnan 3α-ol-20-one was performed in the presence of bicuculline 1 μM. Muscimol 1 μM has no effect in the presence of bicuculline 1 μM and bicuculline 1 μM increased the spike size by 11.75% ± 1.18 ($P \leq 0.001$, n=4). Agonist responses were therefore measured using the potential size in the presence of bicuculline as the control level. 5α-pregnan-3α-ol-20-one 1 and 5 μM did not modify the inhibitory effect of adenosine 5 μM in the presence of bicuculline 1 μM (figure 4.13.).

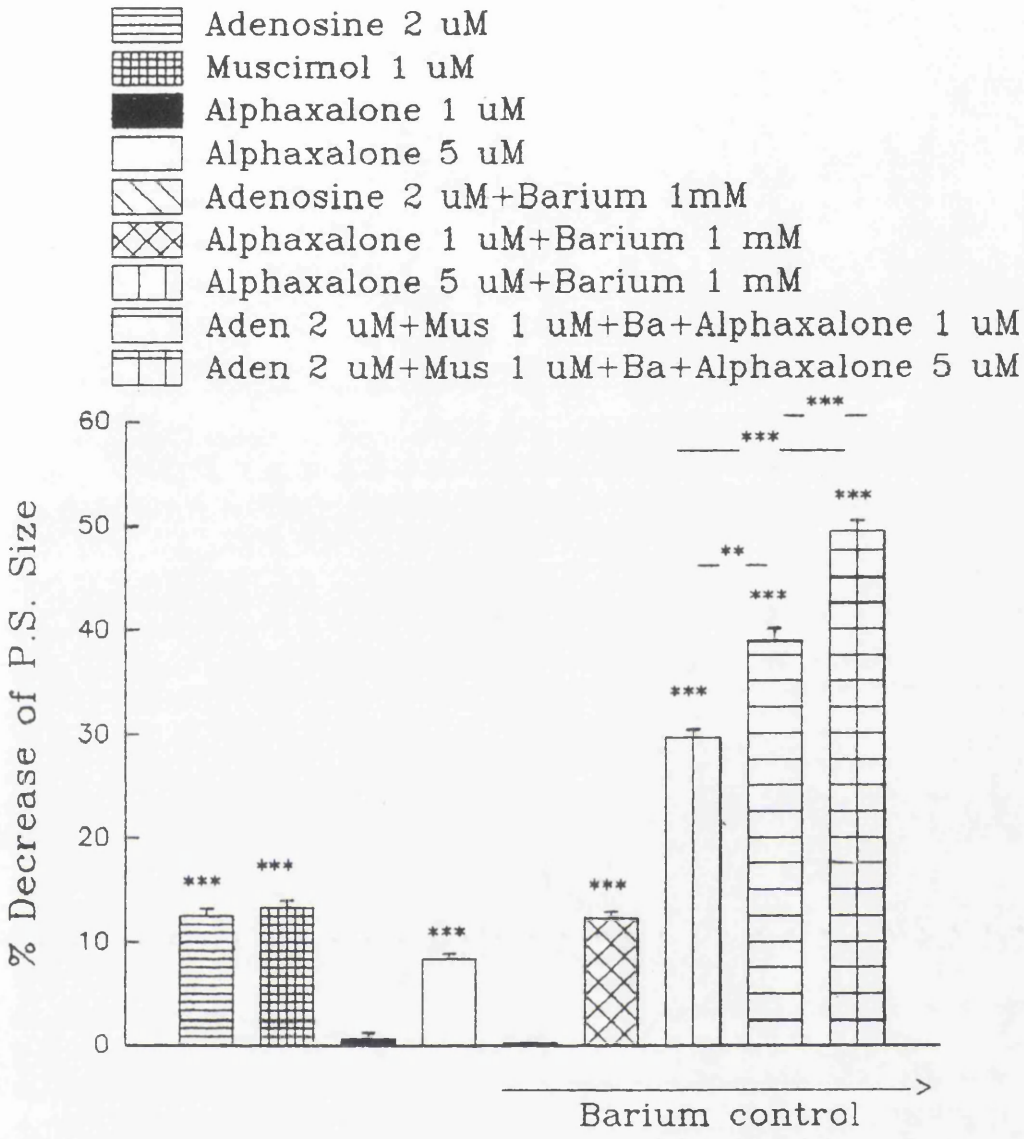

Figure 4.11. Depression of orthodromically evoked CA1 population spikes (P. S.) by adenosine 2 μM, muscimol 1 μM, alphaxalone, adenosine 2 μM + barium 1 mM, alphaxalone + barium, adenosine 2 μM + muscimol 1 μM + barium and adenosine 2 μM + muscimol 1 μM + alphaxalone + barium 1 mM. Each vertical bar represents the mean ± s.e.m. for n = 4 experiments. A one-way analysis of variance test and Tuky-Kramer multiple comparisons test were employed to determine the significance level ($^{**}P \leq 0.01$, $^{**}P \leq 0.001$).

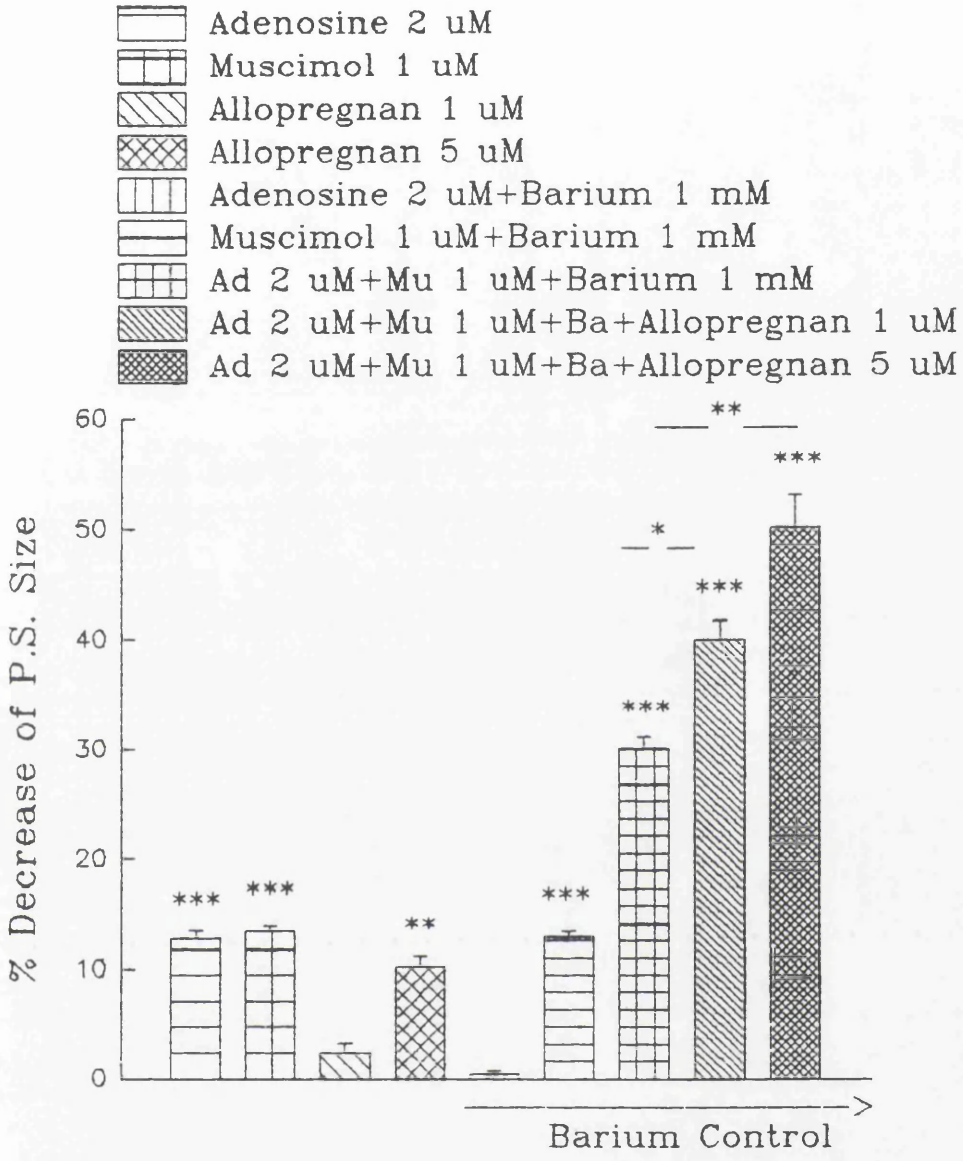

Figure 4.12. Depression of orthodromically evoked CA1 population spikes (P. S.) by adenosine 2 μM, muscimol 1 μM, 5α-pregnan-3α-ol-20-one (allopregnan), adenosine 2 μM + barium 1 mM, muscimol 1 μM + barium, adenosine 2 μM + muscimol 1 μM + bairum and adenosine 2 μM + muscimol 1 μM + allopregnan + barium 1 mM. Each vertical bar represents the mean ± s.e.m. for n = 4 experiments. A one-way analysis of variance test and Tuky-Kramer multiple comparisons tast were employed to determine the significance level ($^{*}P \leq 0.05$, $^{**}P \leq 0.01$).

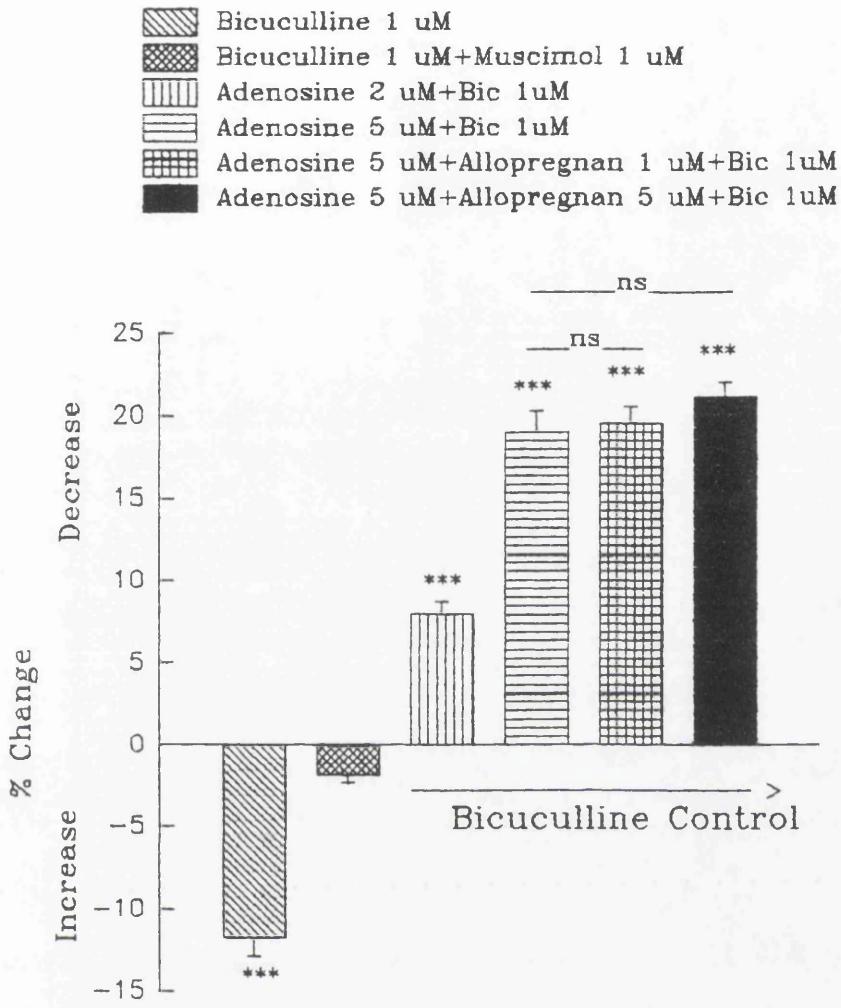

Figure 4.13. Depression of orthodromically evoked CA1 population spikes (P. S.) by adenosine 2, 5 μM, and adenosine 5 μM + allopregnan + bicuculline 1 μM. Each vertical bar represents the mean ± s.e.m. for n = 4 experiments. A one-way analysis of variance test and Tuky-Kramer comparisons tast were employed to determine the significance level ($^{***}P \leq 0.001$).

4.3. Discussion

A vast body of evidence shows that certain synthetic and endogenous steroids act as potent modulators at the $GABA_A$ receptor (Cottrell et al., 1987; Puia et al., 1991). The anaesthetic alphaxalone and some reduced metabolites of progesterone like 5α-pregnan-3α-ol-20-one potentiate GABA responses (Harrison and Simmonds, 1984; Harrison et al., 1987). In contrast, pregnenolone sulfate interacts with the $GABA_A$ receptor in an antagonistic manner (Majewska et al., 1986).

Several lines of evidence indicate that the alphaxalone and 5α-pregnan-3α-ol-20-one act in a barbiturate-like fashion at the $GABA_A$ receptors (Majewska et al., 1988). The results are in agreement with other reports using electrophysiological methods in the CA1 region of the hippocampus, and confirm that alphaxalone and 5α-pregnan-3α-ol-20-one, at low concentrations that have no significant effect on the population spike size, can potentiate the $GABA_A$ inhibitory effect. At higher concentrations they can act on the $GABA_A$ associated chloride channels directly.

The interesting finding of this study was interaction between the alphaxalone, 5α-pregnan-3α-ol-20-one and adenosine. It has previously been shown that, in addition to acting on potassium channels, adenosine can affect chloride channels (chapter 3). One possible explanation of the steroid potentiation of adenosine, therefore, could be that they are acting directly at adenosine-operated chloride channels.

To isolate the possible interaction of alphaxalone and 5α-pregnan-3α-ol-20-one with adenosine at the chloride channels, the adenosine activated potassium channels were blocked with barium 1 mM. It has been previously shown barium can block the adenosine potassium channel (Thompson et al., 1992 . Under these conditions, alphaxalone and 5α-pregnan-3α-ol-20-one still potentiated the inhibitory effect of adenosine although the effect was less marked than without barium, suggesting that some part of that interaction was due to the adenosine activated potassium channels activity.

To determine if these neurosteroids potentiate adenosine inhibitory effect directly or through the mediation of $GABA_A$ receptors, the $GABA_A$ receptor was blocked with bicuculline 1 μM. In the presence of

bicuculline, these neurosteroids failed to potentiate the adenosine effect on the population spike size. The results therefore suggest that these neurosteroids potentiate the adenosine indirectly by potentiating endogenous GABA at $GABA_A$ receptors. On the other hand, the results are consistent with previous reports that showed the chloride channels activated by adenosine to be different from those operated by $GABA_A$ receptors (Mager et al., 1990,1995).

Collectively, the results indicate that low endogenous concentrations of GABA and adenosine could potentiate each other sufficiently to exert a significant inhibition of neuronal activity in the CNS. Given the capacity of endogenous neurosteroids to potentiate $GABA_A$ receptor activation and thus apparently the GABA/adenosine interaction, it seems probable that the level of neuronal inhibition will be extremely sensitive to small variations in the concentrations of any of these agents.

In conclusion, the potentiation of the adenosine inhibitory effect in the presence of barium and enhancement of potentiative interaction of adenosine and muscimol by alphaxalone and 5α-pregnan-3α-ol-20-one, is a further confirmation for the point mentioned in chapter 3, that some part of the inhibitory effect of adenosine at

postsynaptic sites is due to acting on the chloride channels.

Induction of a novel form of hippocampal long-term depression by muscimol

5.1. Introduction

Synaptic efficacy plays an important role in some aspects of hippocampal memory storage (Nicol et al., 1988; Linden, 1994). According to Hebb's theory of learning, memory and learning are thought to be the processes of stabilising specific neuronal associations by strengthening or weakening synaptic transmission at specific synapses (Hebb, 1949).

As one example of synaptic strengthening, the mechanism of long-term potentiation (LTP) has been studied extensively (Bliss & Lomo, 1973; Bliss & Collingridge, 1993). Although both excitation and inhibition are important in controlling neuronal activity, long-term depression (LTD) remains poorly documented compared to LTP (Stanton & Sejnowski, 1989; Yang & Faber, 1991). Most of the work on LTP and LTD has been performed in the CA1 region of the hippocampus, cerebellar cortex, striatum, nucleus accumbens and neocortex (Levy & Steward, 1979; Ekerot & Kano, 1985; Ito, 1989; Kimura et al., 1989; Stanton & Sejnowski, 1989; Calabresi et al., 1992; Pennartz et al., 1993).

Glutamate is a major transmitter in the vertebrate central nervous system and its receptors have been categorized into two group: Ionotropic and Metabotropic. The ionotropic receptors contain integral, cation-specific ion channels, whereas the metabotropic are coupled to G-proteins and modulate the intracellular second messenger systems (Collingridge and Lester, 1989; Monaghan et al., 1989; Schoepp et al., 1990; Miller, 1991; Nakanishi, 1992). Metabotropic glutamate receptors have been reported to play a critical role in the modulation of different forms of neuronal activity in the hippocampus. It has been shown that trans-1-aminocyclopentyl-1,3-dicarboxylic acid (t-ACPD), selective agonist of metabotropic receptors depressed synaptic transmission in the hippocampus (Garaschuk et al., 1992; Goh & Musgave, 1993). Several studies have suggested the involvement of NMDA and metabotropic receptors or both in long-lasting changes of neuronal activity (LTP and LTD)(Kano and Kato, 1987; Aronidou and Teyler, 1991; Yang and Faber, 1991; Bashir & Collingridge, 1992; Dudek & Bear, 1992; Mulkey & Malenka, 1992; Yang et al., 1994).

A variety of different protocols can be used to induce LTD including those which involve homosynaptic or heterosynaptic pathways. When LTD occurs at inputs whose

activation contributes to the induction of modification, it is known as homosynaptic LTD and when it shows itself at inputs that had been inactive during induction it is referred as heterosynaptic LTD. There are two ways to induce homosynaptic LTD: a) With high frequency stimulation in the neocortex, striatum and nucleus accumbens (Lovinger et al., 1992; Walsh, 1993) b) With low frequency stimulation in the CA1 region of the hippocampus (Bramham & Serbro, 1987; Artola & Singer, 1993; Linden, 1994).

GABA, is a major inhibitory neurotransmitter in the vertebrate central nervous system, and its receptors have been classified into at least two types (Sivilotti & Nistri, 1991). $GABA_B$ receptors induce inhibition of transmitter release and hyperpolarization mediated by potassium channels, whereas $GABA_A$ receptors mediate a bicuculline-sensitive hyperpolarization. Binding to the chloride channel complex causes the opening of the channel and the influx of chloride ions into the neuron (Bormenn, 1989). The influx of chloride ion makes the membrane potential of the postsynaptic cell more negative and usually induces an inhibitory state. The $GABA_A$ receptor is believed to be a heterooligomer of 4-5 receptor subunits that makes a central ionic pore and contains certain binding sites at least for GABA, picrotoxin, barbiturates,

benzodiazepines and neurosteroids (Barnard et al., 1987; Delory & Olsen, 1992).

$GABA_A$ receptors mediate fast synaptic inhibition through the activation of receptor-linked chloride ionophores (Sivilotti & Nistri, 1991). Certain synthetic and endogenous steroids like alphaxalone and 5α-pregnan-3α-ol-20-one have been reported as positive modulators of $GABA_A$ receptor (Harrison & Simmonds, 1984; Turner & Simmonds, 1989). They have been shown to have two distinct action on the $GABA_A$ receptor. At low concentrations, they potentiate $GABA_A$ action, while at higher concentrations they can directly open the chloride channel associated with the $GABA_A$ receptor (Majewska et al., 1989; Puia et al., 1991). It has been reported that in the hippocampus low frequency stimulation of Schaffer collateral fibres combined with brief pulses of GABA induced a massive depression of Schaffer collateral-CA1 transmission (homosynaptic long-term depression) (Yang et al., 1994).

The purpose of this part of project was to improve the understanding of the role of $GABA_A$ receptors in LTD and synaptic plasticity in the hippocampus in comparison with adenosine, which has been well studied as an inhibitory neuromodulator (Stone, 1991), and to compare the receptors

involved with those defined in previous studies by examining the effect of the classical $GABA_A$ receptor modulating steroids. In addition, the second object of this study was to determine the possible involvement of metabotropic and NMDA receptors in the LTD induced by the protocol which is presented in this chapter.

5.2. Results

5.2.1. A new protocol for induction of LTD

Under normal experimental conditions, when the Schaffer collateral/ commissural fibres were stimulated at a frequency of 0.01 Hz, muscimol induced a concentration-dependent inhibition of the amplitude of the evoked population potential (figure 5.1.). The maximum effect of muscimol was obtained at 10 μM. When used at concentrations of 0.1 to 5 μM, perfused for 10 minutes, this inhibition was readily reversible, and upon washing the slices potential size was restored to control levels within 10 minutes. When tested at the higher concentration of 10 μM, however, a stable, long-lasting depression of the population potential was obtained. The potential remained depressed, with no sign of recovery, for at least 20 minutes after washing, the minimum period of washout investigated. This is illustrated in figure 5.2. Whenever tested, the depression was found to persist for at least 60 minutes after washout.

It was later realised that a similar long-lasting depression could be induced by using the lower concentrations of muscimol applied for longer periods. Thus, a concentrations of 5 μM could reliably induce long-lasting depression if applied for 20 minutes (figure 5.3.).

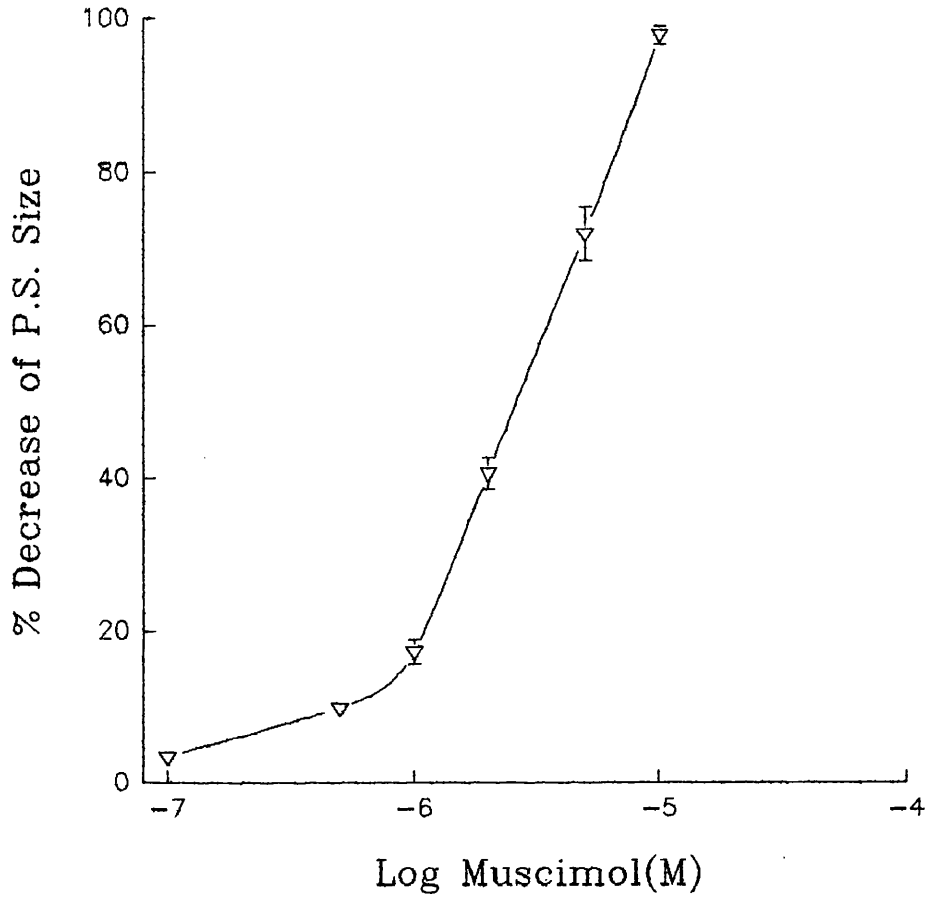

Figure 5.1. Concentration-response curves for the depression of orthodromically evoked CA1 population spikes (P.S.) by muscimol. Each point represents the mean ± s.e.m. for n = 5.

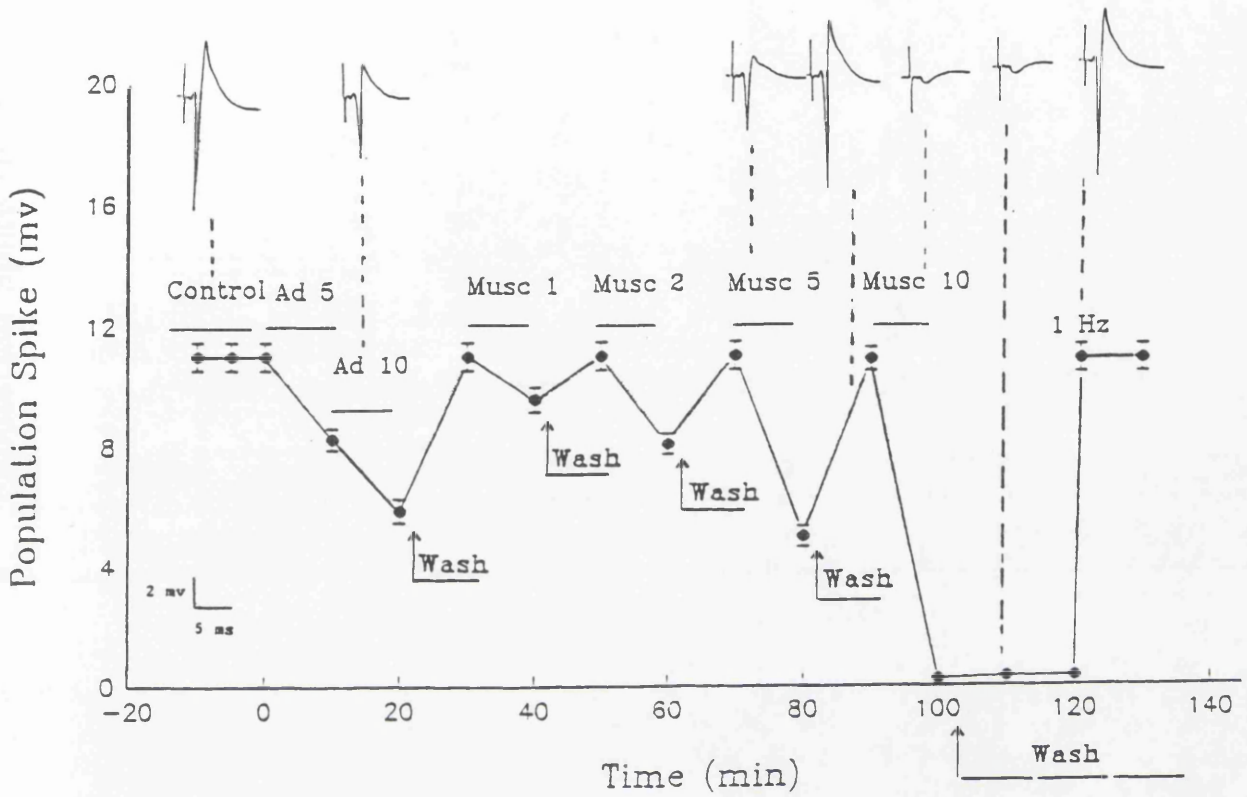

Figure 5.2. Time course of the effect of adenosine (Ad) and muscimol (Musc) on orthodromically evoked CA1 population spikes. The duration of treatment was 10 minutes. Each point represents the mean ± s.e.m. for n = 4 experiments. Sample records of orthodromic potentials are shown above the graph.

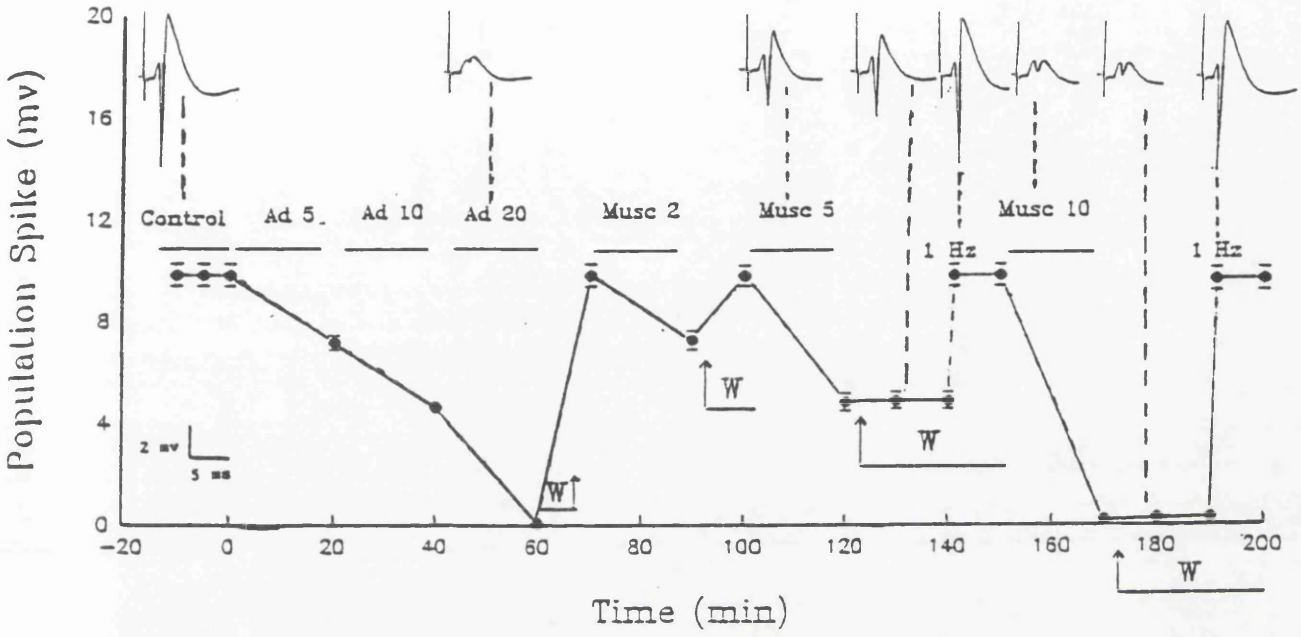

Figure 5.3. Time course of the effect of adenosine (Ad) and muscimol (Musc) on orthodromically evoked CA1 population spikes. The duration of treatment was 20 minutes. Each point represents the mean ± s.e.m. for n = 4 experiments. Sample records of orthodromic potentials are shown above the graph.

5.2.2. Reversal of LTD

Once long-term depression had been established, and the inability of washing to reverse it confirmed, the potentials could be restored to control size by delivering stimuli at a rate of 1 Hz for 10 seconds (figure 5.2.,5.3.,5.4.).

5.2.3. Adenosine and LTD

In order to draw a comparison with muscimol, a similar series of experiments was carried out using adenosine. At concentrations of 5, 10 and 20 μM, adenosine depressed the size of the population spikes, as reported on many previous occasions (by 24.88% ± 1.33, 48.75% ± 1.4 and 99.25% ± 0.48 respectively; mean ± s.e.m, n = 4, $P \leq 0.001$). However, adenosine failed to induce any sign of long-term depression; potentials always recovered to control levels within 5 minutes of washout (figure 5.2.,5.3.). Even at a concentration of 50 μM adenosine was not able to induce LTD (figure 5.5.).

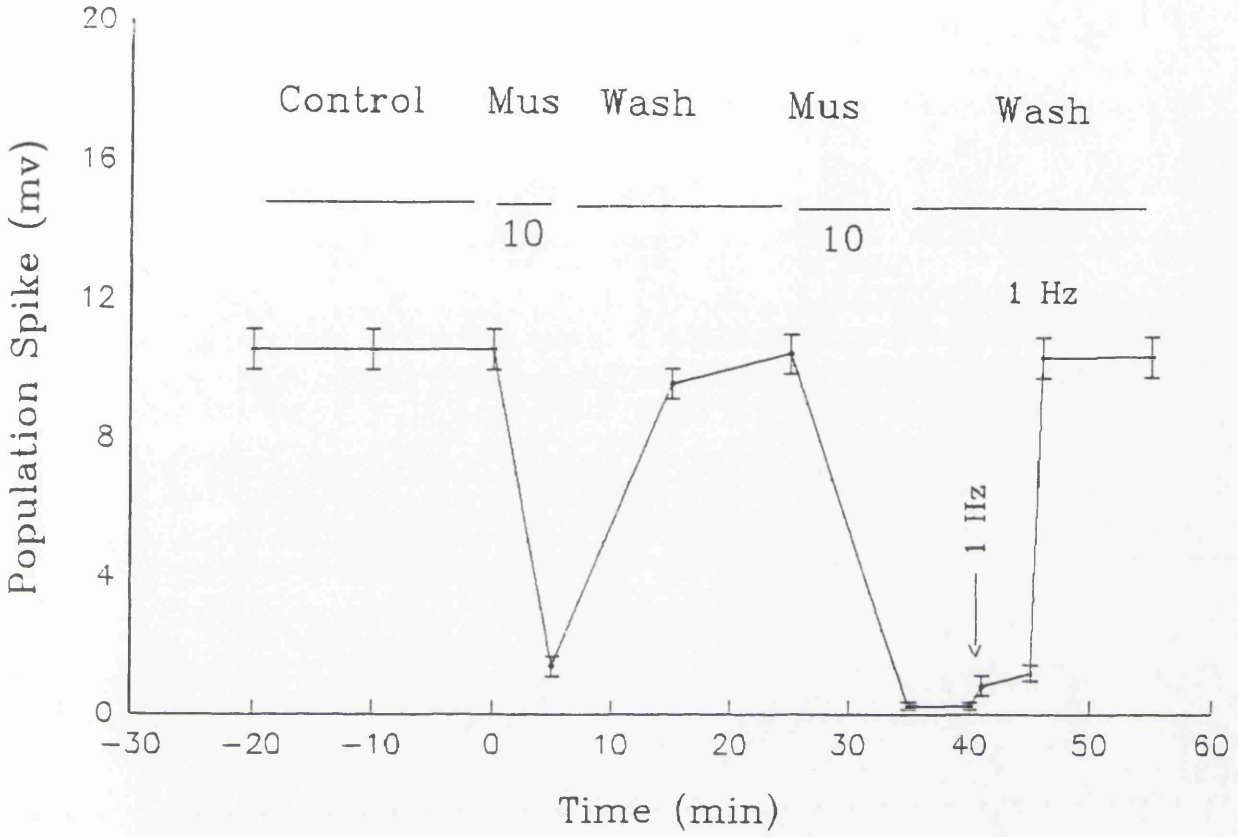

Figure 5.4. Time course of the effect of muscimol 10 μM (Mus) on orthodromically evoked CA1 population spikes. Each point represents the mean ± s.e.m. for n = 4 experiments.

5.2.4. Role of stimulation in induction of LTD

In view of the efficacy of 1 Hz stimulation to terminate LTD and restore potential size to control levels, experiments were also conducted to assess the importance of maintained stimulation for the induction of LTD by muscimol. In these experiments, the normal stimulation was turned off during the application of muscimol. This absence of synaptic activity did not modify the induction of LTD, as illustrated in figure 5.6. In order to confirm the involvement of $GABA_A$ receptors in the effect of muscimol, bicuculline methobromide was used as a receptor antagonist. When added to hippocampal slices, however, bicuculline can induce multiple epileptiform bursts of spikes which can complicate interpretation. In these experiments, therefore, LTD was induced by muscimol and bicuculline was then added into the perfusing medium. In each of 4 slices, bicuculline reversed the LTD within 2 minutes, and before the appearance of multiple population spikes (figure 5.7.).

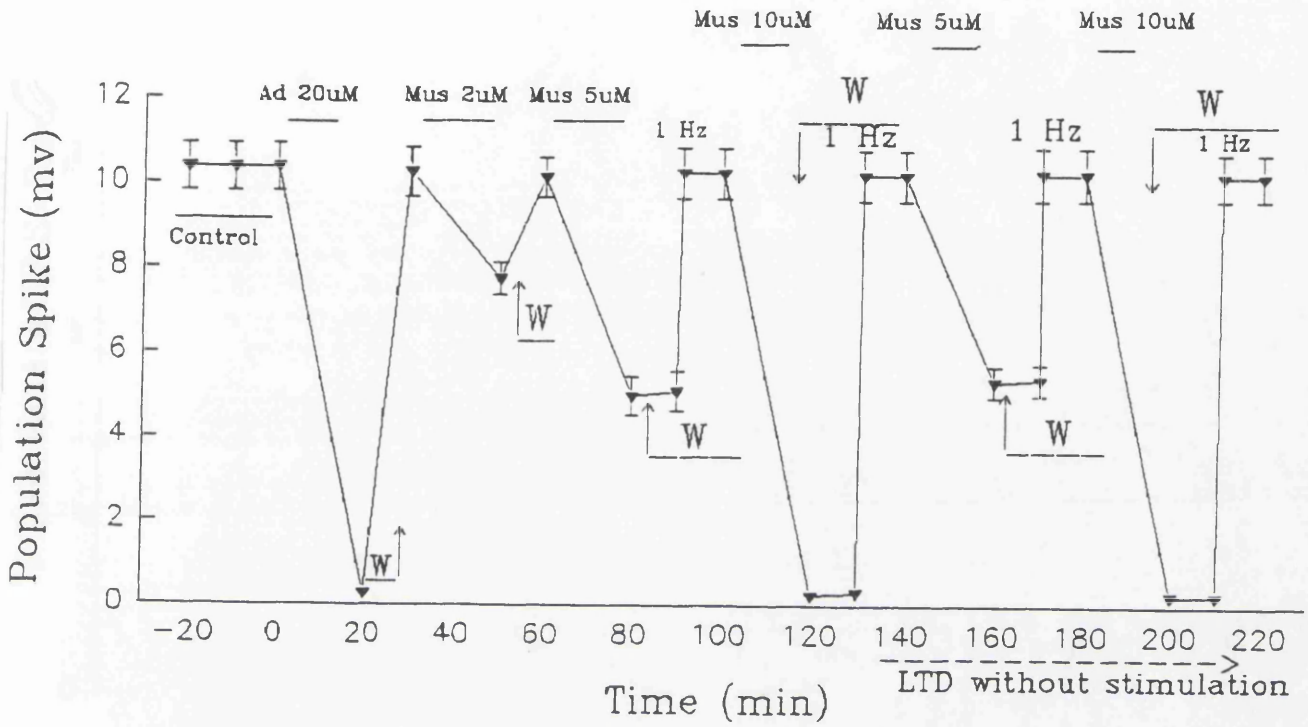

Figure 5.6. Time course of the effect of adenosine (Ad) and muscimol (Mus) on orthodromically evoked CA1 population spikes. The duration of treatment was 20 minutes. Second set of application of muscimol 5 and 10 uM has been carried on without stimulation. Each point represents the mean ± s.e.m. for n = 4 experiments.

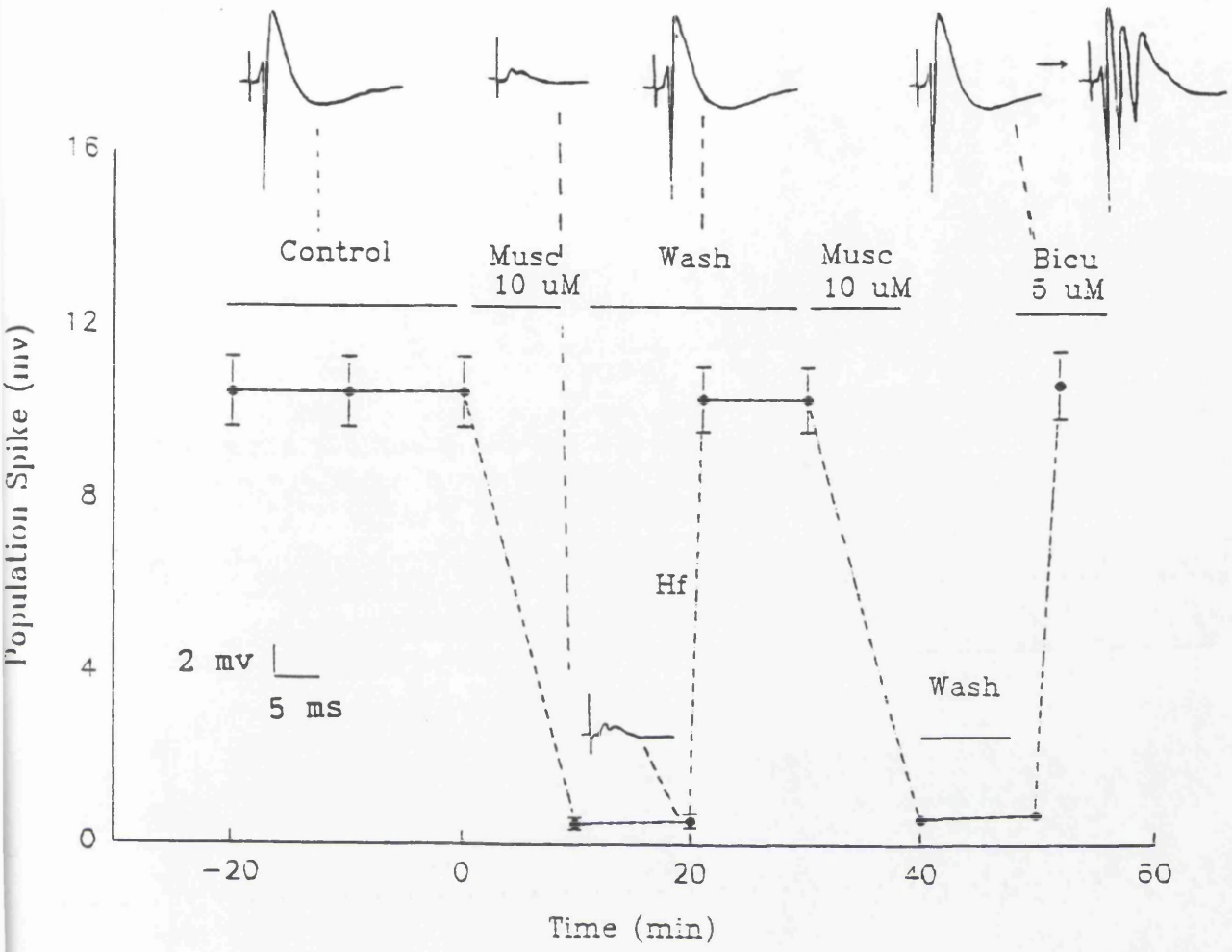

Figure 5.7. Time course of the effect of muscimol 10 μM (Musc) and bicuculline 5 μM (Bicu) on orthodromically evoked CA1 population spikes. Each point represents the mean ± s.e.m. for n = 4 experiments. Sample records of orthodromic potentials are shown above the graph.

5.2.5. Neurosteroids and LTD

In order to assess the role of conventional $GABA_A$ receptors in the induction of LTD, without the need for inducing the neuronal hyperexcitability and spontaneous epileptiform bursting which results from the use of bicuculline or picrotoxin, two neurosteroids were used in an attempt to enhance the effect of activating $GABA_A$ receptors.

The natural steroid, 5α-pregnan-3α-ol-20-one and the steroidal anaesthetic agent alphaxalone were superfused over the slices at concentrations of 0.5 to 10 μM. Neither compound had any effect itself on population potentials at the lower concentrations (0.5 -1 μM) but at 5 μM or above both were able to depress potential size (chapter 4). Even at the lower concentrations, however, both steroids proved able to potentiate the inhibitory activity of muscimol on population spikes; at 5 μM the effect of muscimol was increased almost 4-fold (chapter 4).

Both agents similarly potentiated the ability of muscimol to induce LTD. A normally ineffective concentration of 1 μM of the GABA agonist, applied for 10 minutes, for example, was able to induce LTD in the presence of only 1 μM

alphaxalone (figure 5.8.) or 5α-pregnan-3α-ol-20-one (figure 5.9.). As for mscimol aione, increasing stimulation frequency to 1 Hz for 10 seconds was sufficient to reverse the LTD and restore potentials to their control size.

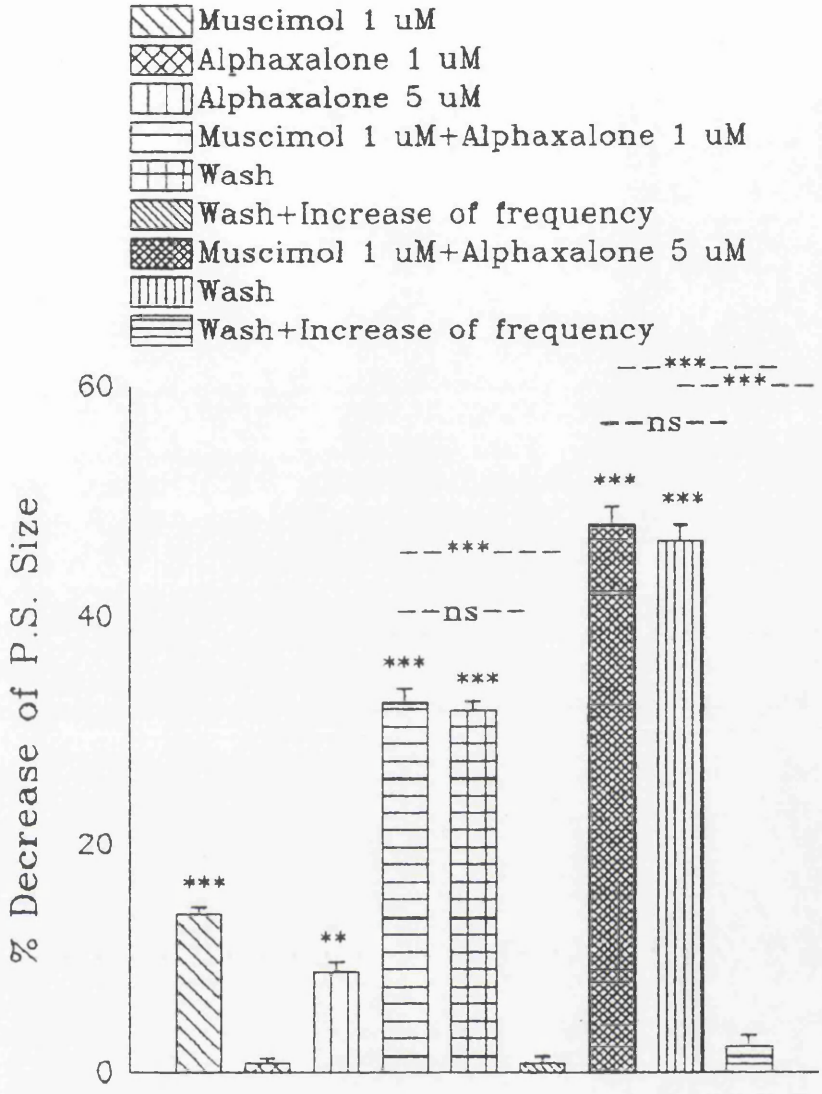

Figure 5.8. Depression of orthodromically evoked CA1 population spikes by muscimol, alphaxalone and muscimol + alphaxalone. Each vertical bar represents the mean ± s.e.m. for n=4 experiments. A paired Student's t test was employed to determine the significance level ($^{**}P \leq 0.01$; $^{***}P \leq 0.001$).

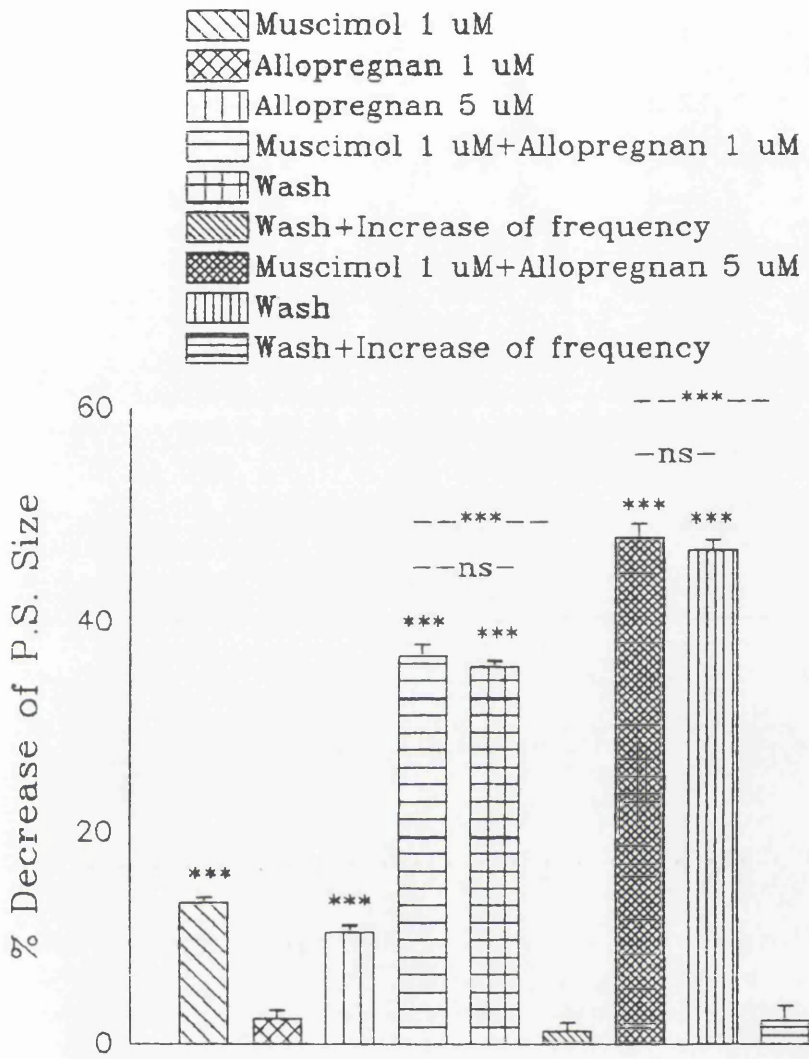

Figure 5.9. Depression of orthodromically evoked CA1 population spikes by muscimol 1 µM, 5α-Pregnan-3α-ol-20-one (allopregnan) and muscimol 1 µM + 5α-Pregnan-3α-ol-20-one. Each vertical bar represents the mean ± s.e.m. for n = 4 experiments. A paired Student's t test was employed to determine the significance level ($^{***}P \leq 0.001$).

5.2.6. Maintenance of LTD

When we induced LTD by application of muscimol 10 μM for 10 minutes, low concentrations of muscimol (0.5 and 1 μM), alphaxalone and 5α-pregnan-3α-ol-20-one 1 μM were superfused over the slices. Interestingly, muscimol 1 μM which is not able to induce LTD alone and positive modulators of $GABA_A$ like alphaxalone and 5α-pregnan-3α-ol-20-one in concentrations that can potentiate the inhibitory effect of muscimol, maintained the LTD induced by muscimol 10 uM. Moreover, the delivering of 1 Hz stimulation did not reverse the LTD in this series of experiments. Nevertheless, a lower concentration than 1 μM of muscimol was not able to maintain the LTD induced by muscimol 10 μM (figure 5.10.,5.11.,5.12.).

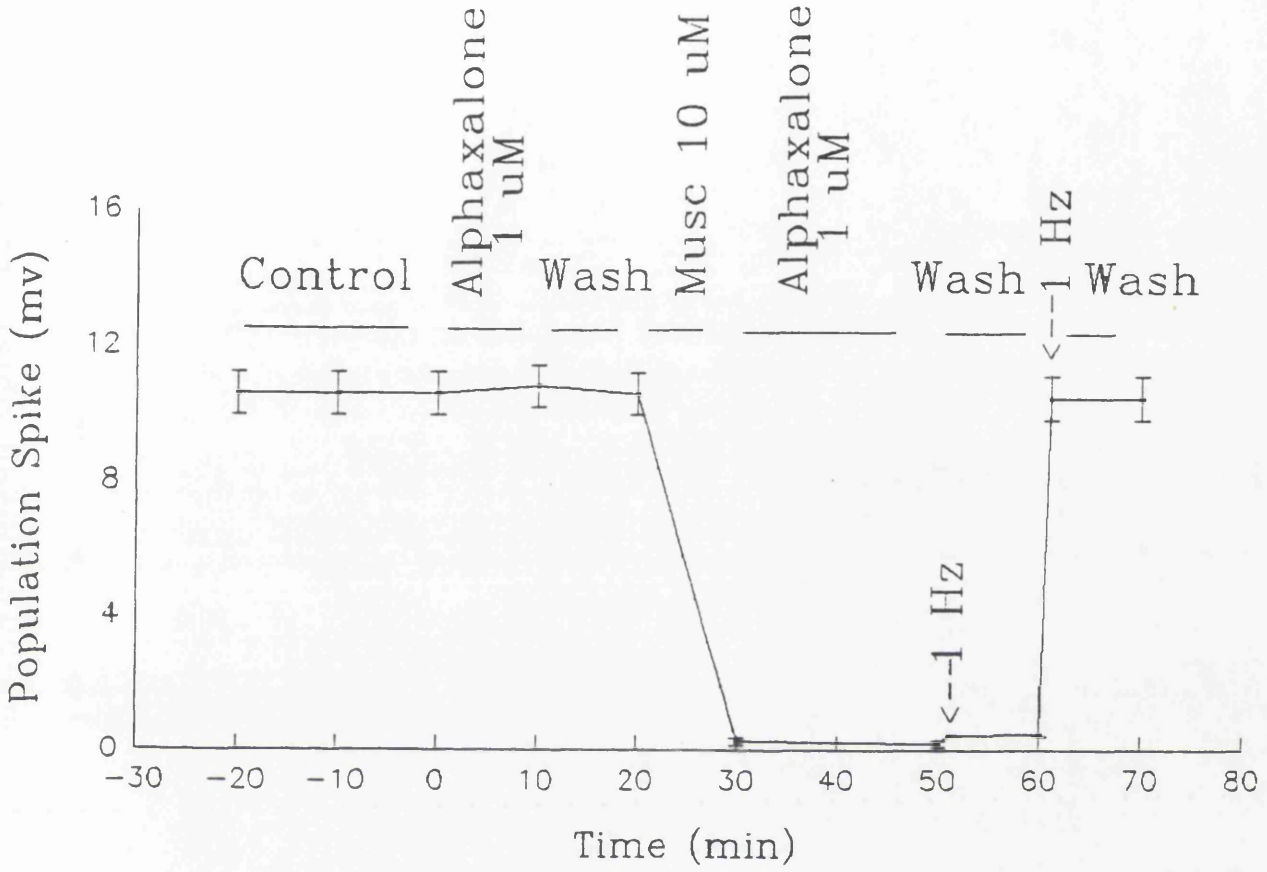

Figure 5.10. Time course effect of muscimol 10 μM (Musc) and alphaxalone on orthodromically evoked CA1 population spikes. Each point represents the mean ± s.e.m. for n=4 experiments.

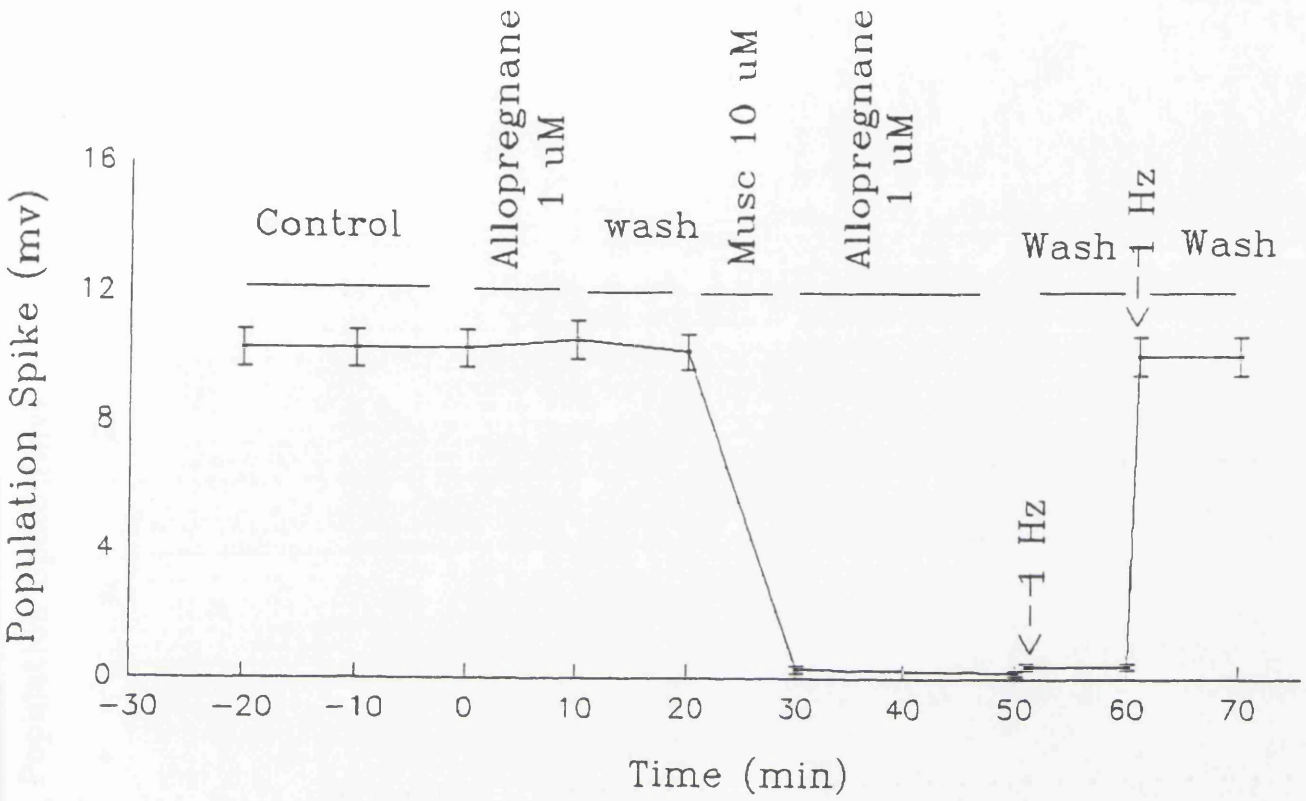

Figure 5.11. Time course effect of muscimol 10 μM (Musc) and 5α-pregnan-3α-ol-20-one (allopregnan) on orthodromically evoked CA1 population spikes. Each point represents the mean ± s.e.m. for n=4 experiments.

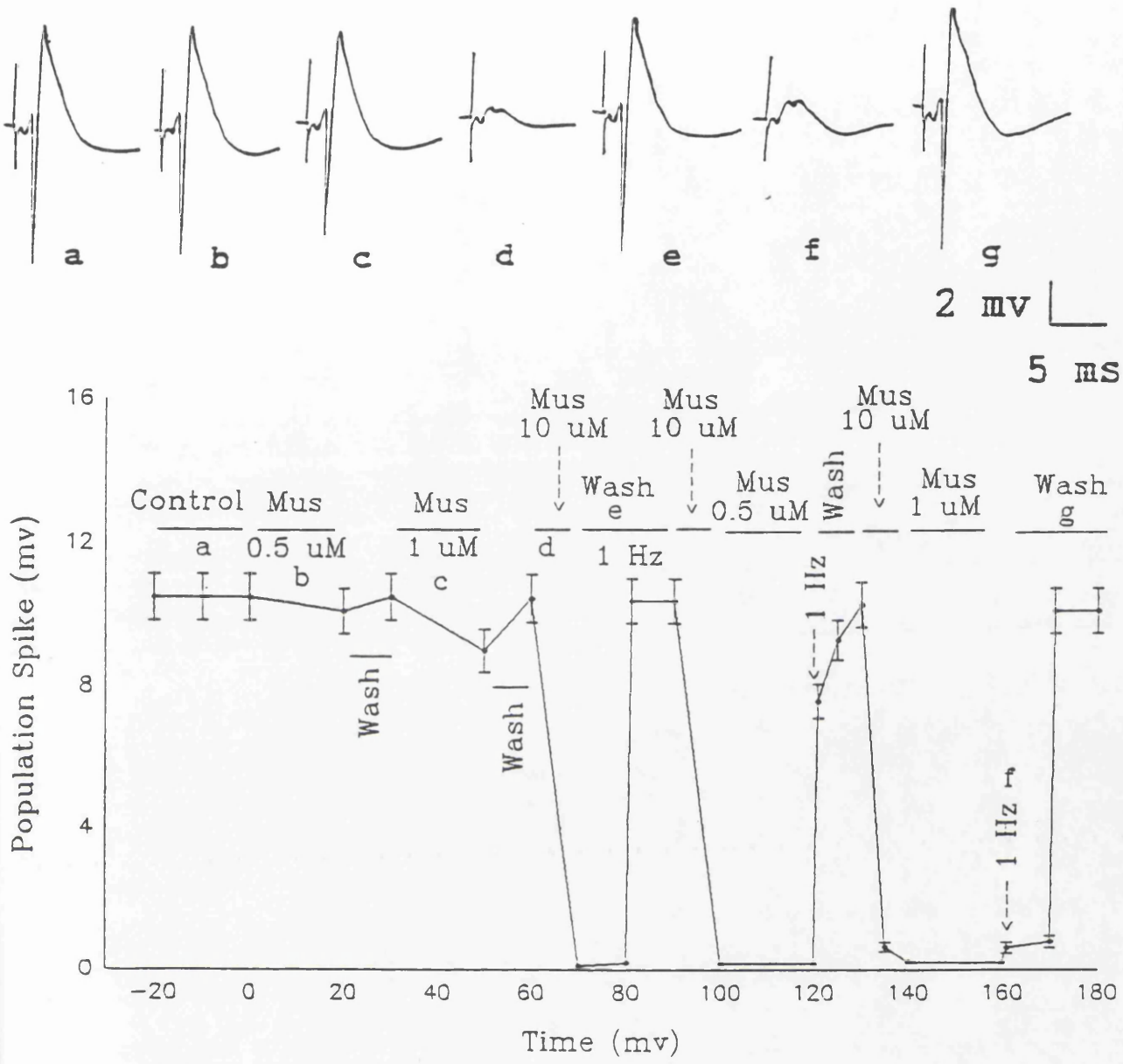

Figure 5.12. Time course effect of muscimol on orthodromically evoked CA1 population spikes. Each point represents the mean ± s.e.m. for n=4 experiments. Sample records of orthodromic potentials are shown above the graph.

5.2.7. Antagonists and agonists at glutamate receptors and LTD

To examine the possible involvement of glutamate receptors in the effect of muscimol, experiments were performed using the N-methyl-D-aspartate (NMDA) antagonist (±)-2-amino-5-phosphonopentanoic acid (2-AP5; 300 μM), the mixed metabotropic and NMDA receptor antagonist (±)-2-amino-3-phosphonopropionic acid (2-AP3; 200 μM) and the selective antagonist of metabotropic receptors L(+)-2-amino-3-phosphonopropionic acid [L(+)-AP3; 150 μM]. None of these compounds modified the ability of muscimol to induce LTD (figure 5.13.,5.14.,5.15.). Equally, treatment of the slices with the non selective metabotropic receptor agonist quisqualic acid at 10 and 50 μM and the selective metabotropic receptor agonist (1S,3R)-ACPD 100 μM did not induce LTD whereas both agents showed inhibitory effects on the population spike size (figure 5.16.,5.17.). In addition to this, kynurenic acid, a broad spectrum antagonist of NMDA and non NMDA receptors (100 and 200 μM) did not induce LTD but it prevented termination of LTD by stimulation at 1 Hz (figure 5.18.).

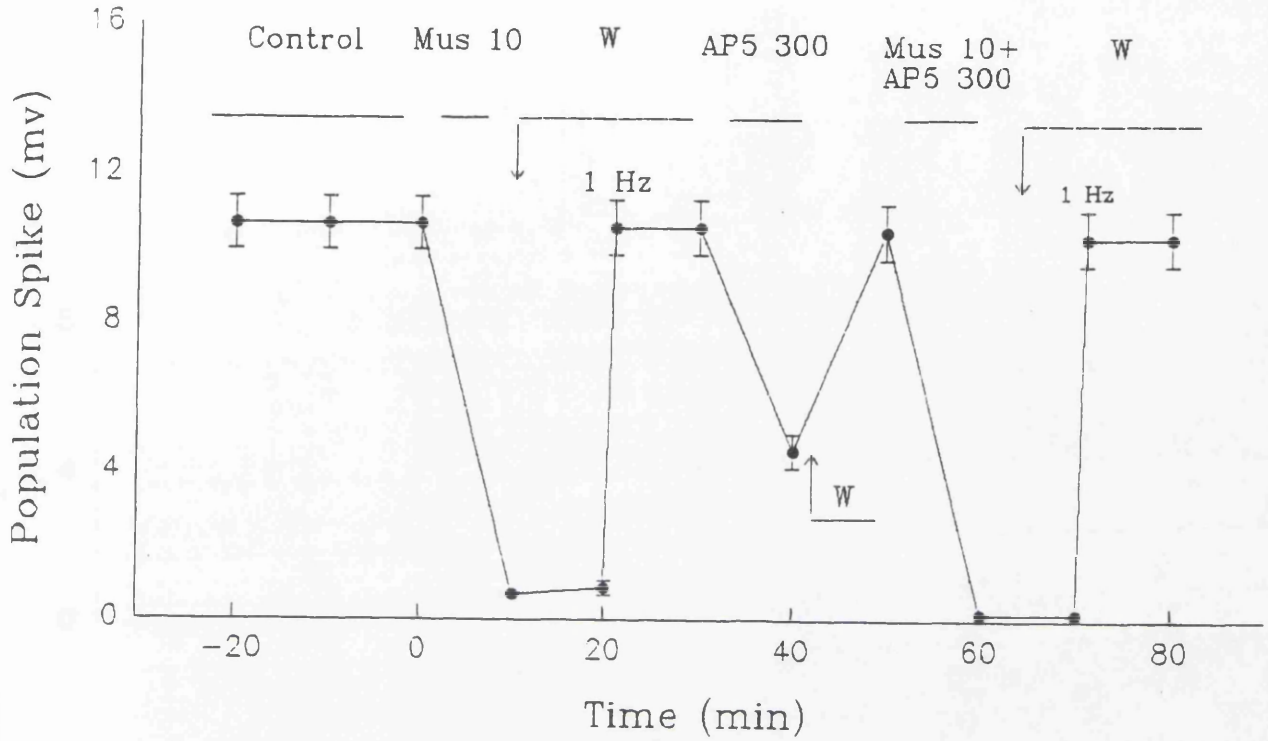

Figure 5.13. Time course of the effect of muscimol 10 μM (Mus) and (±)-2-Amino-5-phosphonopentanoic acid 300 μM (AP-5) on orthodromically evoked CA1 population spikes. Each point represents the mean ± s.e.m. for n = 4 experiments.

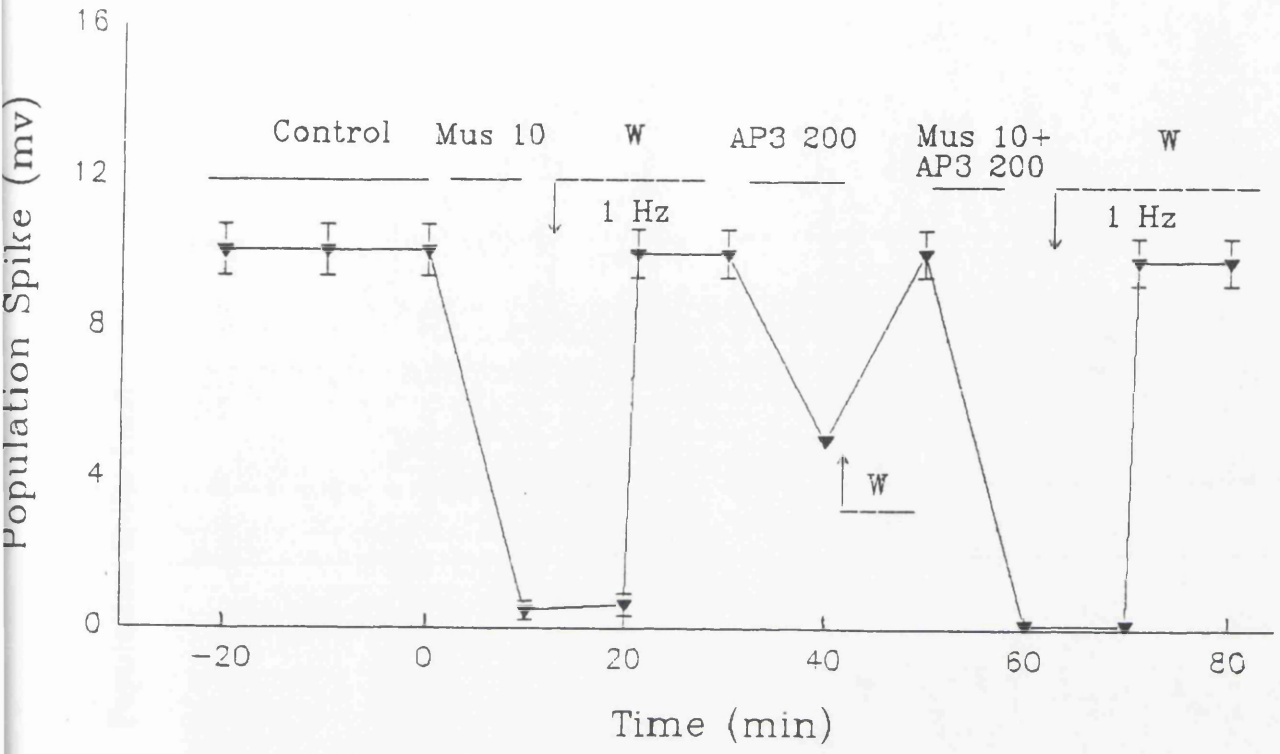

Figure 5.14. Time course of the effect of muscimol 10 μM (Mus) and (±)-2-Amino-3-phosphonopropionic acid 200 μM (AP-3) on orthodromically evoked CA1 population spikes. Each point represents the mean ± s.e.m. for n = 4 experiments.

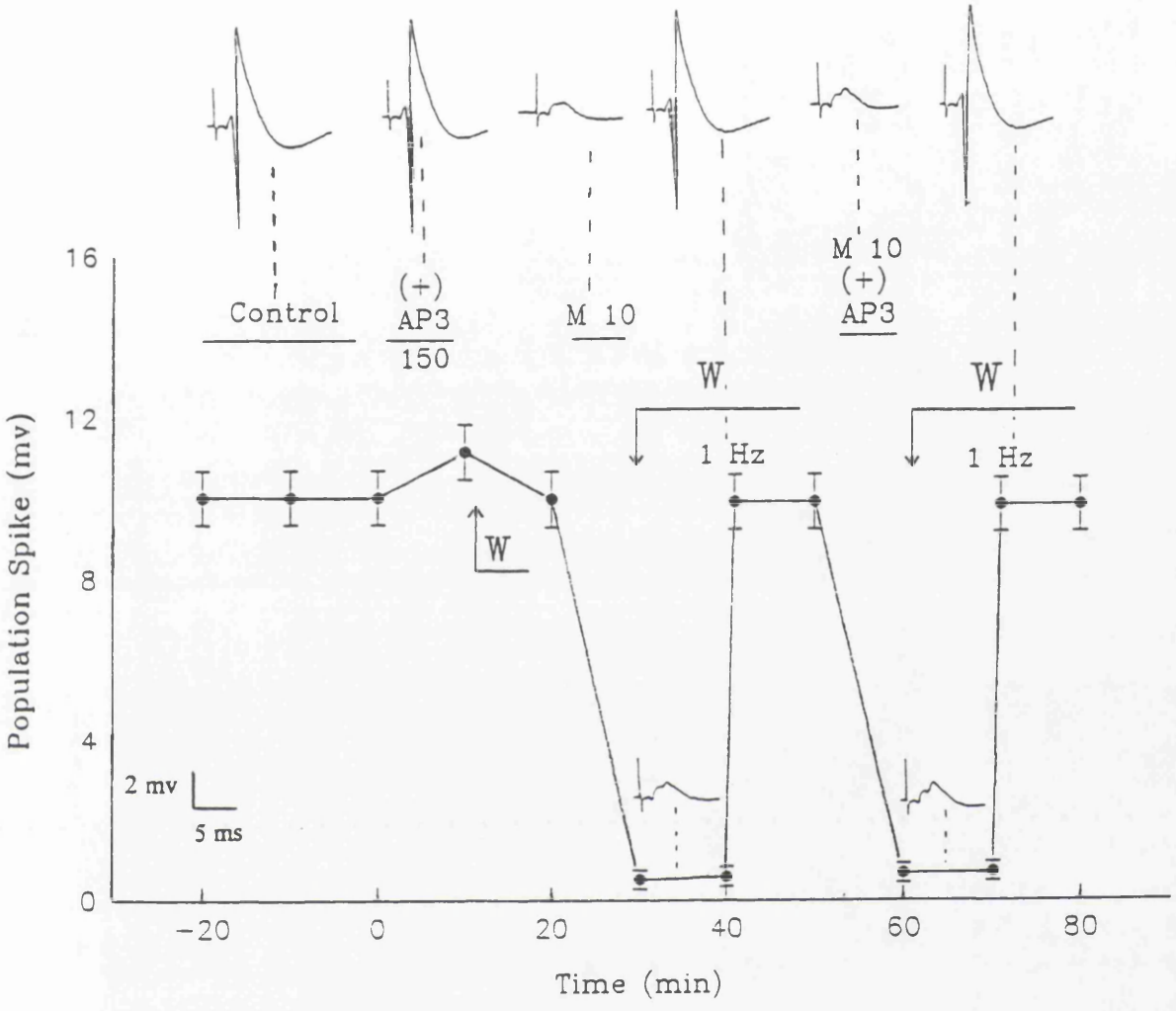

Figure 5.15. Time course of the effect of muscimol 10 μM (Mus) and L(+)-2-amino-3-phosphonopropionic acid 150 μM [L(+)-AP3] on orthodromically evoked CA1 population spikes. Each point represents the mean ± s.e.m. for n = 4 experiments. Sample records of orthodromic potentials are shown above the graph.

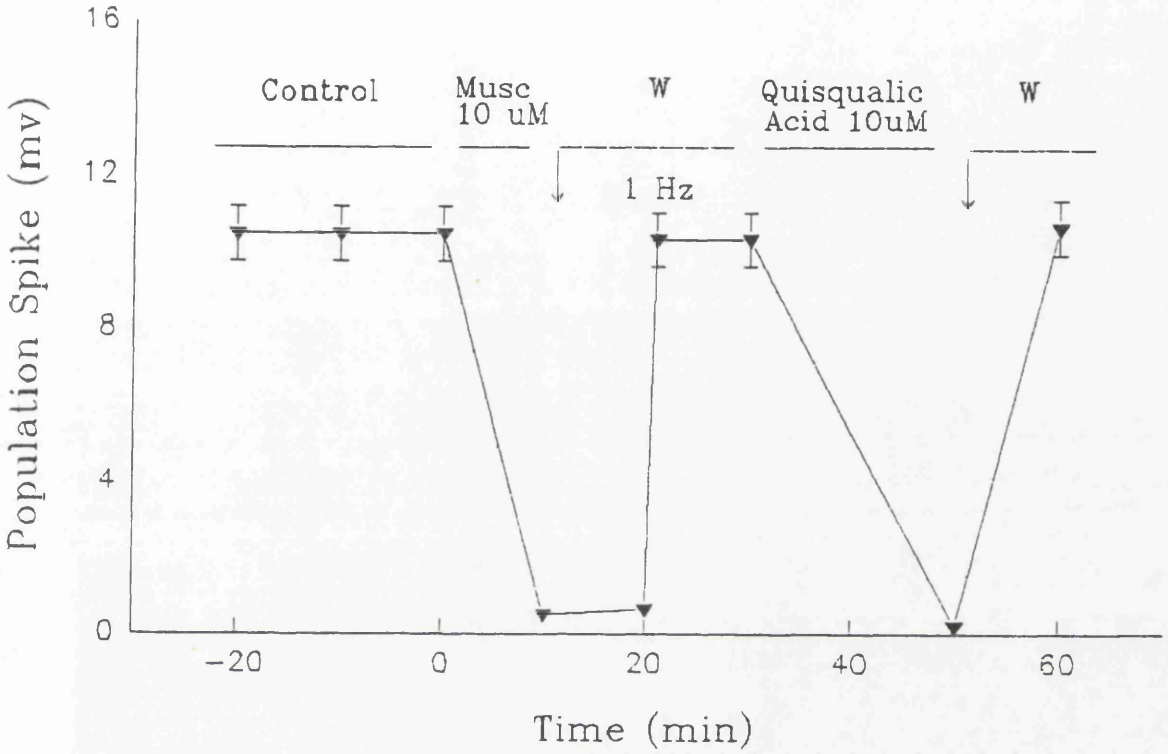

Figure 5.16. Time course of the effect of muscimol 10 µM (Musc) and quisqualic acid on orthodromically evoked CA1 population spikes. Each point represents the mean ± s.e.m. for n = 4 experiments.

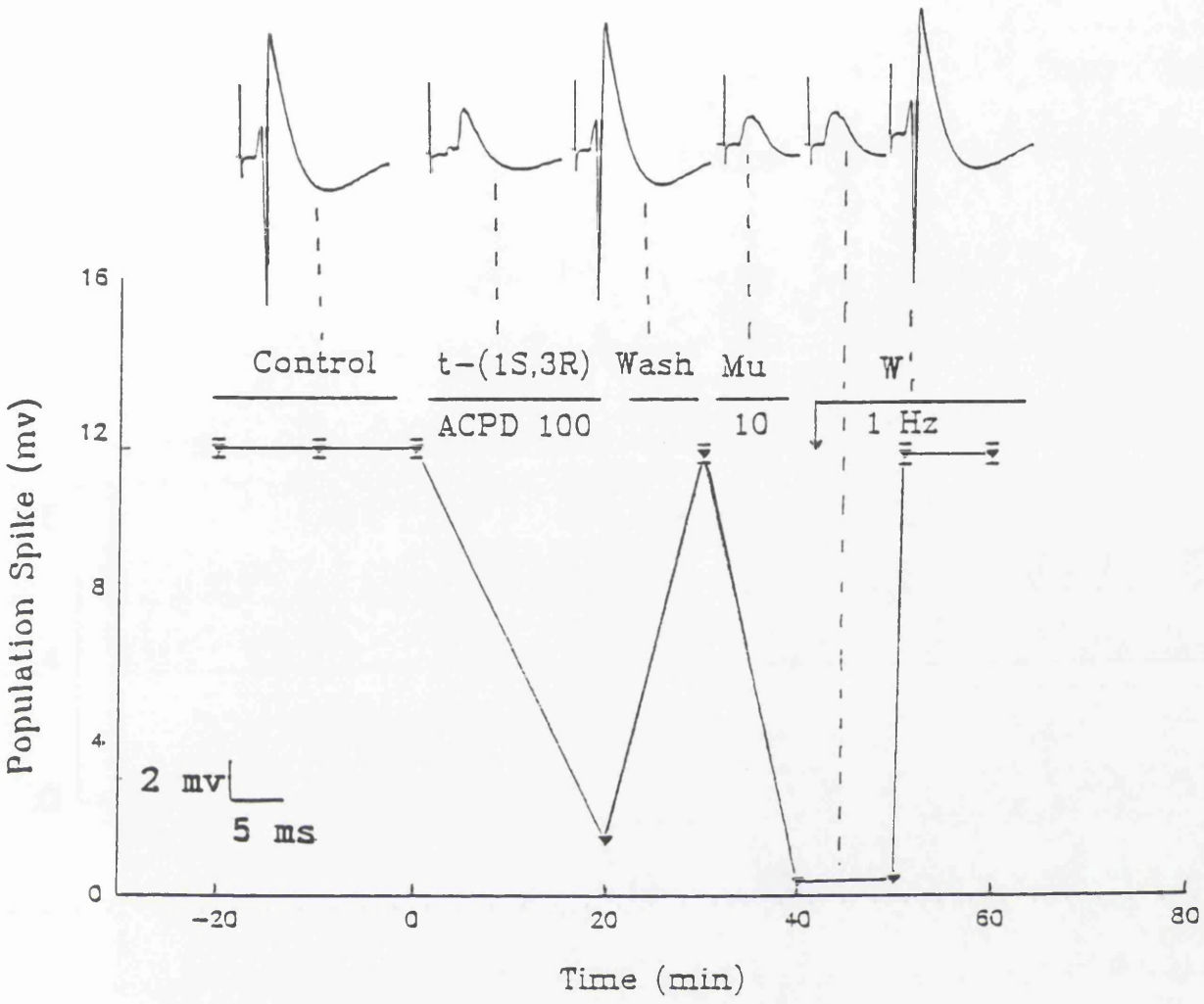

Figure 5.17. Time course of the effect of muscimol (Mu) 10 μM and t-(1S,3R)-ACPD 100 μM on orthodromically evoked CA1 population spikes. Each point represents the mean ± s.e.m. for n = 4 experiments. Sample records of orthodromic potentials are shown above the graph.

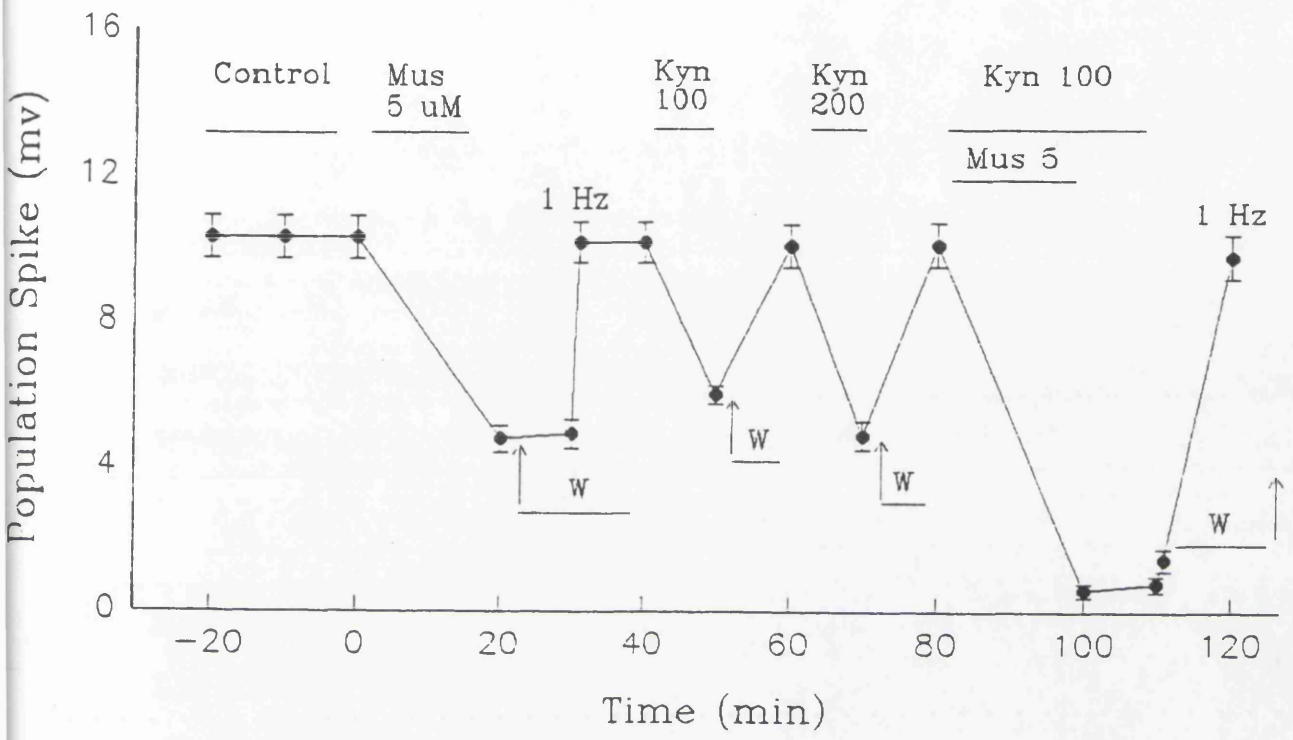

Figure 5.18. Time course of the effect of muscimol 5 μM (Mus) and kynurenic acid (Kyn) on orthodromically evoked CA1 population spikes. Each point represents the mean ± s.e.m. for n = 4 experiments.

5.3. Discussion.

Long-term depression is an activity dependent form of synaptic plasticity that is accepted as being important in learning and memory since at the cellular level, memories are believed to be stored as long-term modifications in the strength of synaptic transmission. The hippocampus is probably involved in processes such as learning and memory and hippocampal slices are a convenient model for investigating synaptic plasticity.

The present results demonstrate that the application of a selective $GABA_A$ agonist to hippocampal slices can induce LTD of synaptically-evoked population potentials when stimulation was effected at a low frequency of 0.01 Hz. Increasing stimulation frequency to 1 Hz abolished the LTD. This stands in contrast to the work of Yang et al (Yang et al., 1994) who observed that LTD could be induced while stimulating at 1 Hz, but is in agreement with the work of other groups which have indicated that low frequency stimulation is more effective in inducing LTD in the hippocampus than in neocortex, striatum or nucleus accumbens (Bramham & Srebro, 1987; Artola & Singer, 1993; Lovinger et al., 1993; Walsh, 1993; Linden, 1994). The most important difference between the protocol which is

presented by this thesis and previous protocols to induce LTD is the fact that the LTD is induced by using 0.01 Hz stimulation and, increasing stimulation to 1 Hz, reversed the LTD whereas other groups induced LTD by using 1-5 Hz stimulation and their low frequencies to induce LTD (1-5 Hz) is actually the frequency to reverse the LTD which is presented by this thesis (Braham and Srebro, 1987; Staulbi and Lynch, 1990). Indeed, in the present work it has been found that muscimol alone is effective in inducing LTD even in the absence of afferent stimulation. This would seem to rule out a possible mechanism for LTD which involves an interaction between $GABA_A$ receptors and a synaptically released agent or a second messenger molecule released from postsynaptic sites in response to a neurotransmitter.

In the present experiments the apparent desensitisation reported previously during prolonged applications of GABA or $GABA_A$ agonists was not observed (Forsch et al., 1992; Celentano & Wong, 1994).

It has also been found that the ability of muscimol to produce LTD is dependent on the concentration of muscimol used, and on the duration of application: lower concentrations can be effective if in contact with the slices for a longer period. This would be consistent with

the accumulation of a secondary compound postsynaptically or in the extracellular space, and which must attain a critical level in order to establish LTD.

The application of agonists at $GABA_B$ receptors has been found to induce LTD. Activation of this receptor population is believed to act via the opening of potassium channels (Deisz et al., 1993), whereas $GABA_A$ receptors are effective mainly as a result of operating chloride conductances (Sivilotti & Nistri, 1991). It is of particular interest, therefore, that adenosine, which is also capable of depressing neuronal excitability partly via the opening of chloride channels (Mager et al., 1990,1995), was not able to induce LTD. Adenosine proved ineffective at concentrations which were able to substantially inhibit normal orthodromic potentials. This may suggest that $GABA_A$ or $GABA_B$ receptor activation involves some form of transduction system which is not activated indiscriminately by any neuronal inhibitory agent. Furthermore, the results are consistent with previous reports that adenosine operated chloride channels differ from $GABA_A$ activated chloride channels (Mager et al., 1990,1995; Chapter 3 and 4 of this thesis).

The latter section of the present results represent an attempt to determine whether the $GABA_A$ receptors causing LTD show the same pharmacological properties as GABA receptors described previously on hippocampal neurones. Several neurosteroids which are synthesized in glial cells are known to modulate $GABA_A$ receptor properties via an allosteric site. These steroids at high concentrations, like barbiturates but unlike benzodiazepines, can open chloride channels. The steroidal anaesthetic alphaxalone, and the related compound 5-α-pregnan-3α-ol-20-one had no effect themselves upon population spike amplitude at low micromolar concentrations, whereas they were both able, at these same concentrations, to potentiate significantly the depressant activity of muscimol on evoked spikes. At the higher concentrations tested here, 5 and 10 uM, both steroids did have a direct depressant effect on evoked potentials, consistent with evidence that at these levels they can directly open chloride channels (Harrison & Simmonds, 1984; Turner & Simmonds, 1989; Puia et al., 1991). When examined on LTD, both the steroids were able to potentiate the effects of muscimol to the extent that concentrations which had been insufficient to induce LTD did so in the presence of the steroids. As with the LTD observed normally in response to muscimol alone, the LTD

produced by 1 uM muscimol plus steroids was also reversed by increasing the stimulation frequency to 1 Hz.

One of the interesting findings of this study was that the muscimol induced LTD showed features potentially important in a negative memory mechanism. Thus after application of 10 μM muscimol had induced the LTD, superfusion of either muscimol 1 μM or alphaxalone and 5α-pregnan-3α-ol-20-one 1 μM, which do not show any effect alone, were able to maintain the LTD. Moreover, delivering 1 Hz stimuli failed to restore the potentials to control size during the application of muscimol 1 μM and neurosteroids.

The significance of such a phenomenon for the whole animal are profound, since they imply that after a simple, brief exposure to a stimulus sufficient to raise inhibitory synaptic activity to a level which can induce a forgetting process, that loss of memory could be maintained by very low levels of tonic inhibition. Furthermore, and possibly even more significantly, the inhibition can be maintained by hormonal means-low levels of neurosteroids - as well as or instead of, synaptic inhibition.

The results thus greatly strengthen the view that forgetting is a physiological process amenable to subtle pharmacological manipulation, and raise the possibility that drugs capable of preventing forgetting may be as feasible as drugs facilitating learning.

The latter section of this study indicates that bicuculline is able to reverse the LTD induced by muscimol and our result is in contrast to the work of Yang et al (Yang et al., 1994) who reported bicuculline could not block LTD induced by muscimol. However, the frequency dependence of the LTD studied by Yang et al. (1994) differs from that of present study. This may suggest that different forms of LTD may occur in the hippocampus, involving distinct populations of receptors. Nevertheless, most recently, Thiels' et al reported a bicuculline-sensitive LTD that is in line with the result of this study (Thiels et al., 1994).

The excitatory amino acid glutamate is a major excitatory transmitter within the central nervous system (CNS)(Collingridge and Lester, 1989). The response to glutamate is mediated by activation of multiple glutamate receptor subtypes and leads to either an ionotropic or a metabotropic response. Of particular interest in recent

years has been the investigation of the role of glutamate in synaptic efficacy. Several groups have recently reported on factors affecting the induction of LTD, although no consensus has emerged as to the minimum conditions necessary or sufficient for the phenomenon. Some proposals include the involvement of NMDA receptors and glutamate metabotropic receptors (or both) in LTD (Kano and Kato, 1987; Aroniadou and Teyler, 1991; Yang and Faber, 1991; Yang et al., 1994). One objective of this study was to determine the possible involvement of glutamate receptors in the protocol which was presented by this thesis. Failure of this study to induce LTD or even short-term depression (STD) with quisqualic acid and (1S,3R)-ACPD argues against such a role in the hippocampus. Furthermore, the failure of the NMDA receptor antagonist 2-AP5, the mixed NMDA/metabotropic antagonist 2-AP3 and selective metabotropic antagonist L(+)-AP3 to modify LTD formation exclude a role of either of these receptor populations in $GABA_A$ induced LTD. It is postulated that the involvement of NMDA and metabotropic receptors in previous reports is due to the use of stimulation frequencies at least 100 times higher (1-5 Hz) than the frequency of stimulation which is used in this study (0.01 Hz) (Staubli and Lynch, 1990; Yang et al., 1994). On the other hand, kynurenic acid, a potent glutamate receptor antagonist, could prevent the reversal

of the LTD that is induced by increasing stimulation to 1 Hz. The results suggest that increased stimulation frequency reverses muscimol induced LTD as a result of the release of excitatory amino acids, such as glutamate.

In summary, the present study has presented a new protocol for inducing LTD through activation of $GABA_A$ receptors that can depress synaptic excitability in the hippocampal slice. In addition, it is reported that this ability is not shared by adenosine, but it is potentiated by neurosteroids known to enhance the binding and postsynaptic hyperpolarisation induced by classical $GABA_A$ receptors. Moreover, the lack of involvement of glutamate receptors in producing LTD, suggest that activation of $GABA_A$ receptors alone is sufficient to induce LTD and confirm the involvement of classical $GABA_A$ receptors in the protocol which has been presented by this thesis. This indicates that the phenomenon described in the present study represents a novel type of long-term synaptic inhibition.

Role of calcium in induction and reversal of muscimol-induced LTD

6.1. Introduction

It is believed that the hippocampus plays a prominent role in learning and memory processes (Eichenbaum et al., 1994). Long-term depression (LTD) is an activity dependent decrease in synaptic efficacy that, with its counterpart, long-term potentiation (LTP), has been considered to be critical for neuronal plasticity (Hebb, 1949; Nicol et al., 1988; Linden, 1994). Most of the work to date on LTD has been performed on the cerebellar cortex, nucleus accumbens, striatum, neocortex and CA1 region of the hippocampus (Levy and Steward, 1979; Ekerot and Kano, 1985; Ito, 1988; Kimura et al., 1989; Stanton and Sejnowski, 1989; Calabresi et al., 1992; Pennartz et al., 1993).

Several different protocols for the induction of LTD have been reported in the cerebellum and hippocampus which may be categorised as homosynaptic, heterosynaptic and associative LTD (Braham and srebro, 1987; Artola and Singer, 1993; Lovinger, 1993; Walsh, 1993; Linden, 1994). Although the mechanisms for the induction of LTP are fairly well established, the mechanisms underlying the induction of LTD have remained unresolved (Bliss and Lomo, 1973; Stanton and Sejnowski, 1989; Yang and Faber, 1991; Bliss and Collingridge, 1993).

Most excitatory synapses in the central nervous system use glutamate as a neurotransmitter. Glutamate receptors are classified in two general categories 1: Ionotropic receptors that are associated with ion channel activation and are affected by N-methyl-D-aspartate and kainic acid 2: metabotropic receptors that are coupled to G proteins and modulate the intracellular second messenger systems (Collingridge and Laster, 1989; Monaghan et al., 1989; Schoepp et al., 1990; Miller, 1991; Nakanishi, 1992).
NMDA and metabotropic glutamate receptors have been reported to play a critical role in the induction of different forms of LTD (Kano and Kato, 1987; Aroniadou and Teyler, 1991; Yang and Faber, 1991; Bashir and Collingridge, 1992; Dudek and Bear, 1992; Garaschuk et al., 1992; Mulkey and Malenka, 1992; Goh and Musgave, 1993; Yang et al., 1994). It has been shown that the various forms of plasticity (LTP and LTD) described to date need NMDA receptor-gated Ca^{2+} influx (Christi et al., 1994; Debanne and Thompson, 1994). This probably reflects the fact that the state of LTD or LTP depends on the level of phosphorylation of some undefined substrate. Small increases in intracellular Ca^{2+} decreases the level of phosphorylation by increasing Ca^{2+}-dependent protein phosphatase activity, whereas larger increases of calcium are associated with phosphorylation by enhancing Ca^{2+}

dependent protein Kinase: in general kinases need larger increases of Ca^{2+} for activation than phosphatases.Christi et al., 1994; Debanne and Thompson, 1994; Hirano et al., 1994).

γ-aminobutyric acid (GABA) is the most important inhibitory neurotransmitter in the CNS (Sivilloti and Nistri, 1991). Inhibitory processes are important for the stability and control of neuronal firing. These processes are mediated by GABA. Three types of pharmacologically and physiologically distinct GABA receptors have been reported. Activation of bicuculline-sensitive GABA type A ($GABA_A$) receptors induces the opening of ion channels permeable to Cl^-. The baclofen-sensitive GABA type B ($GABA_B$) receptors are coupled to K^+ or Ca^{2+} channels via G proteins. Most recently GABA type C ($GABA_C$) receptors have been described that couple to Cl^- channels but they are insensitive to bicuculline and baclofen (Barnard et al., 1987; Bormann, 1989; Sivilloti and Nistri, 1991; Deisz et al., 1993).

We have reported a novel type of LTD induced by muscimol (chapter 5), a $GABA_A$ agonist, that is reversed by the $GABA_A$ antagonist bicuculline and potentiated by neurosteroids such as alphaxalone and 5α-pregnan-3α-ol-20-one which are positive modulators of $GABA_A$ receptors

(Harrison and Simmonds, 1984; Magewska and Harrison, 1988; Peters et al., 1988; Turner and Simmonds, 1989; Puia et al., 1991). It has also been reported that NMDA, non NMDA and metabotropic glutamate receptors are not involved in the induction of this type of LTD (chapter 5). The activation of synapses is not necessary for the induction of this LTD since the long-lasting depression could be induced when there was no stimulation during perfusion of muscimol. This type of LTD is induced at low frequency (0.01 Hz) and is reversed by delivering stimulation at a frequency of 1 Hz for 10 seconds. Besides the lack of involvement of glutamate receptors, this reversal represents another major difference between muscimol-induced LTD and that reported previously, since other groups actually use stimulation at 1-5 Hz to induce glutamate-dependent LTD (Linden, 1994; Yang et al., 1994).

The aims of this part of project were twofold. The first objective was to investigate the possible role of calcium in the induction of glutamate independent LTD. The second objective was to determine the mechanism of reversal of the LTD by stimulation.

6.2. Results

6.2.1. Muscimol-induced LTD

Once stable hippocampal population potentials had been obtained at a frequency of 0.01 Hz, the addition of the $GABA_A$ agonist muscimol at concentration of 10 μM for 10 minutes induced a stable long-lasting depression of population potentials. The LTD induced by muscimol was concentration and time dependent as described in chapter 5 (figure 6.1.,6.2.,6.3.). There was no sign of recovery during washout with drug-free ACSF for at least 60 minutes. The activation of synapses was not necessary for the induction of LTD, as the depression could still be obtained when stimulation was turned off during the application of muscimol.

Once established, LTD persisted until the frequency of stimulation of the stratum radiatum was increased to 1 Hz for 10 seconds when potential size returned immediately to the control level (figure 6.1.,6.2.,6.3.).

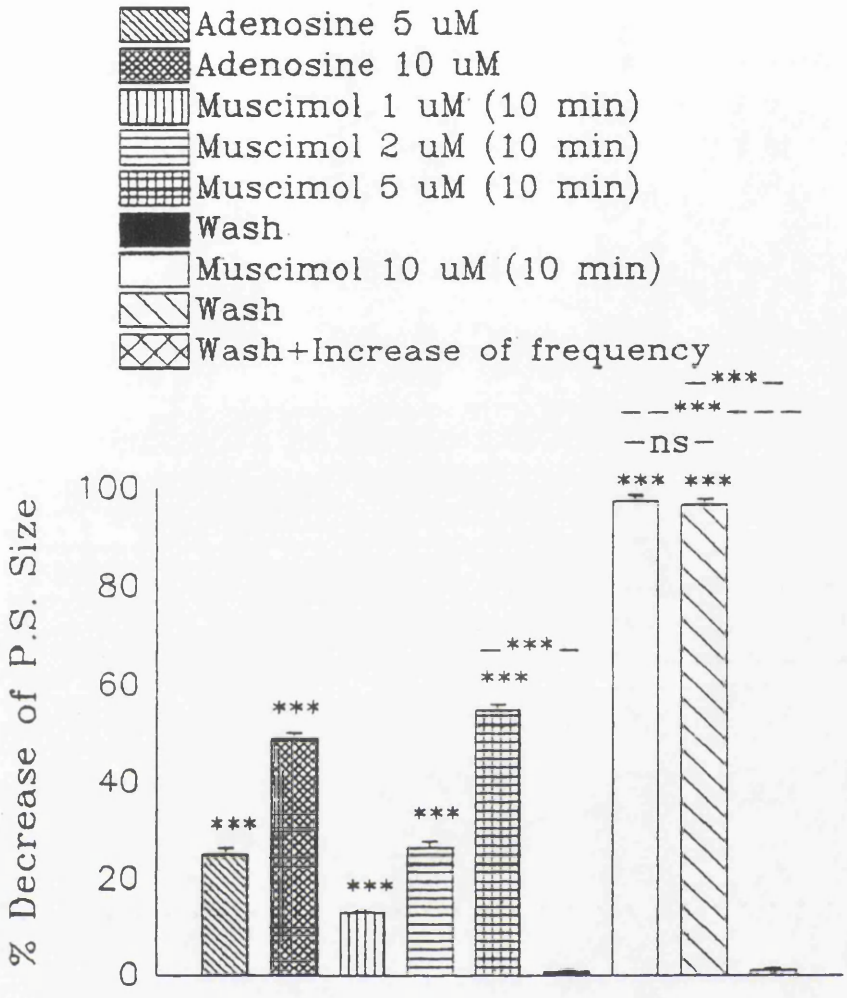

Figure 6.1. Depression of orthodromically evoked CA1 population spikes by adenosine and muscimol. The duration of treatment was 10 minutes. Treatment with adenosine or muscimol at 5 μM or less produced reversible depression of the population potentials. Muscimol at 10 μM produced long-lasting depression which was not reversed by washing but which was reversed by stimulation at 1 Hz. Each vertical bar represents the mean ± s.e.m. for n = 4 experiments. A paired Student's t test was employed to determine the significance level ($^{***}P \leq 0.001$).

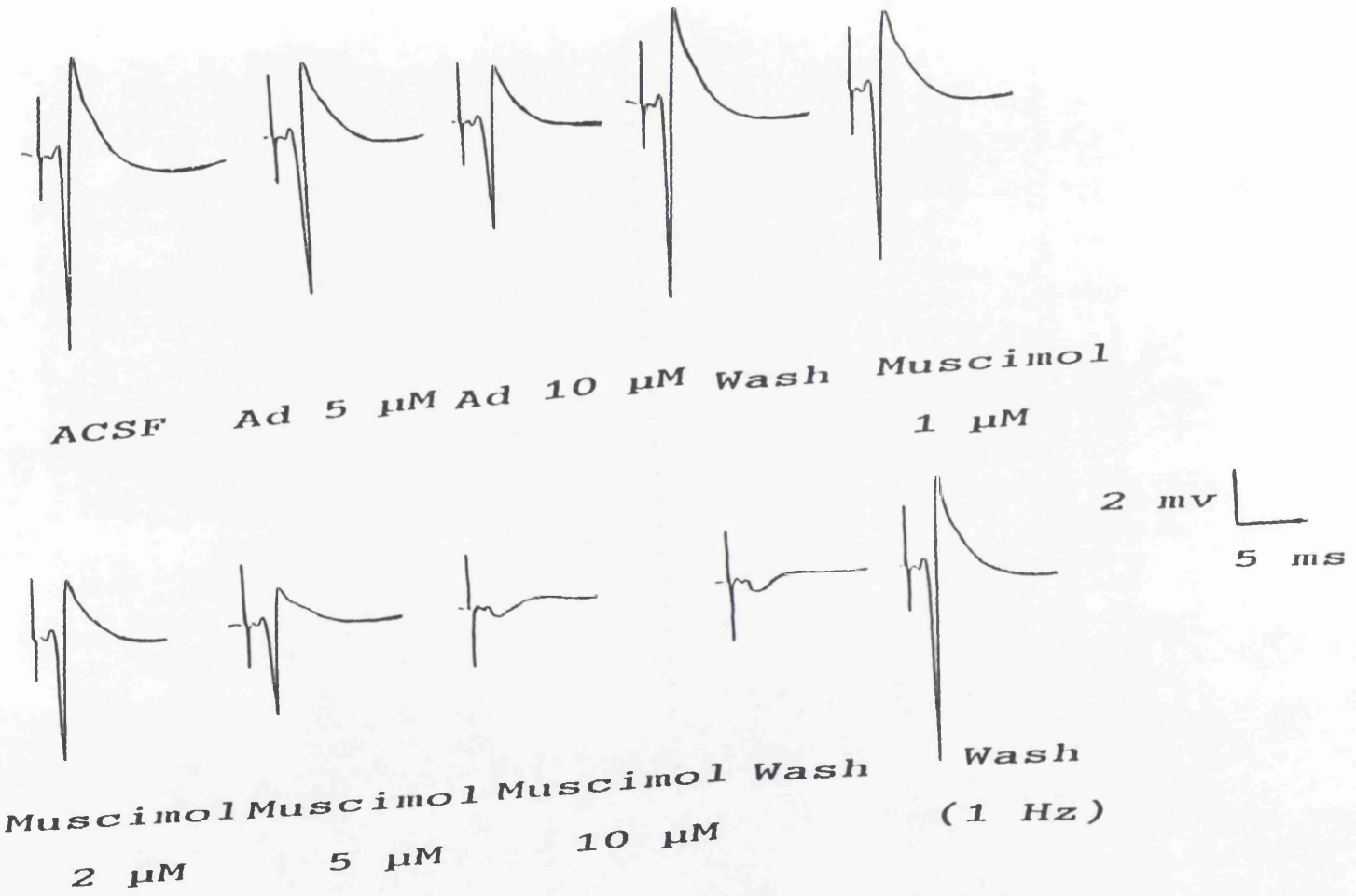

Figure 6.2. Sample records taken during experiments with agents used in Fig.6.1.

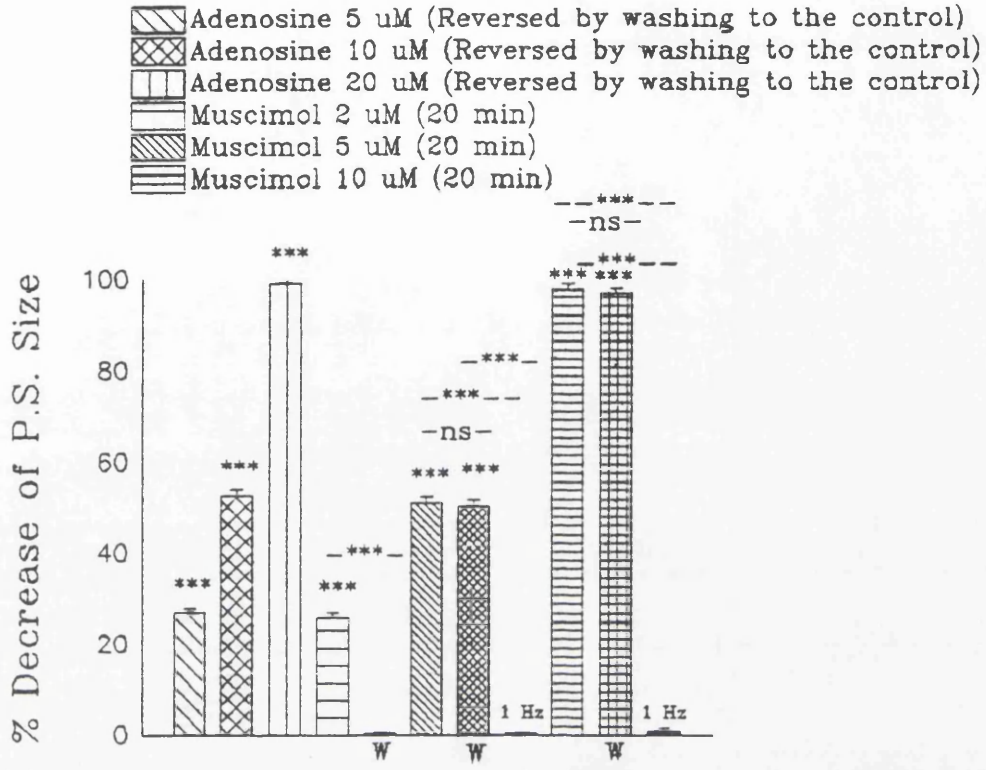

Figure 6.3. Depression of orthodromically evoked CA1 population spikes by adenosine and muscimol. The duration of treatment was 20 minutes. Each vertical bar represents the mean ± s.e.m. for n=4 experiments. A paired Student's t test was employed to determine the significance level. ($^{***}P \leq 0.001$).

6.2.2. Calcium-free medium and LTD

As expected from the synaptic nature of the evoked responses in CA1, superfusion with calcium-free medium led to a rapid loss of responses, but this recovered with a rapid time course when calcium was re-introduced (figure 6.4.). If slices were superfused with calcium-free medium until the CA1 evoked responses had disappeared and muscimol 10 μM was then perfused for 10 minutes, as in our earlier protocol, the re-introduction of calcium failed to restore potential size. This depression of potentials appeared to be exactly similar to that described earlier, since stimulation at a rate of 1 Hz for 10 seconds was sufficient to restore potential size to control levels (figure 6.4.).

6.2.3. The role of NMDA and non-NMDA receptor activation in the reversal of muscimol-induced LTD

In view of the efficacy of 1 Hz stimulation to terminate LTD and restore potential size to control levels, experiments were carried out to determine the possible involvement of glutamate receptors in the reversal of LTD induced by muscimol. Experiments were performed using Kainic acid 5 uM and NMDA 5 uM. At these concentrations both kainic acid (figure 6.5.) and NMDA (figure 6.6.)

induced a small increase in the size of the CA1 potentials when applied for 10 and 5 minutes respectively. Higher concentrations produced overdepolarisation and a loss of evoked responses. If LTD were induced by superfusion with muscimol at 10 μM for 10 minutes both amino acid receptor agonists proved able to reverse the depression (figure 6.5.,6.6.). Two additional points may be noted from the figures. Firstly kainate proved able to reverse muscimol-induced LTD more rapidly than did NMDA, although kainate alone produced a larger enhancement of normal evoked potential size than did NMDA. The more rapid reversal of LTD may therefore simply reflect a greater degree of depolarisation of the slices. Secondly, it should be noted that the prior superfusion with kainate or NMDA did not prevent or modify in any way the subsequent ability of muscimol to induce LTD.

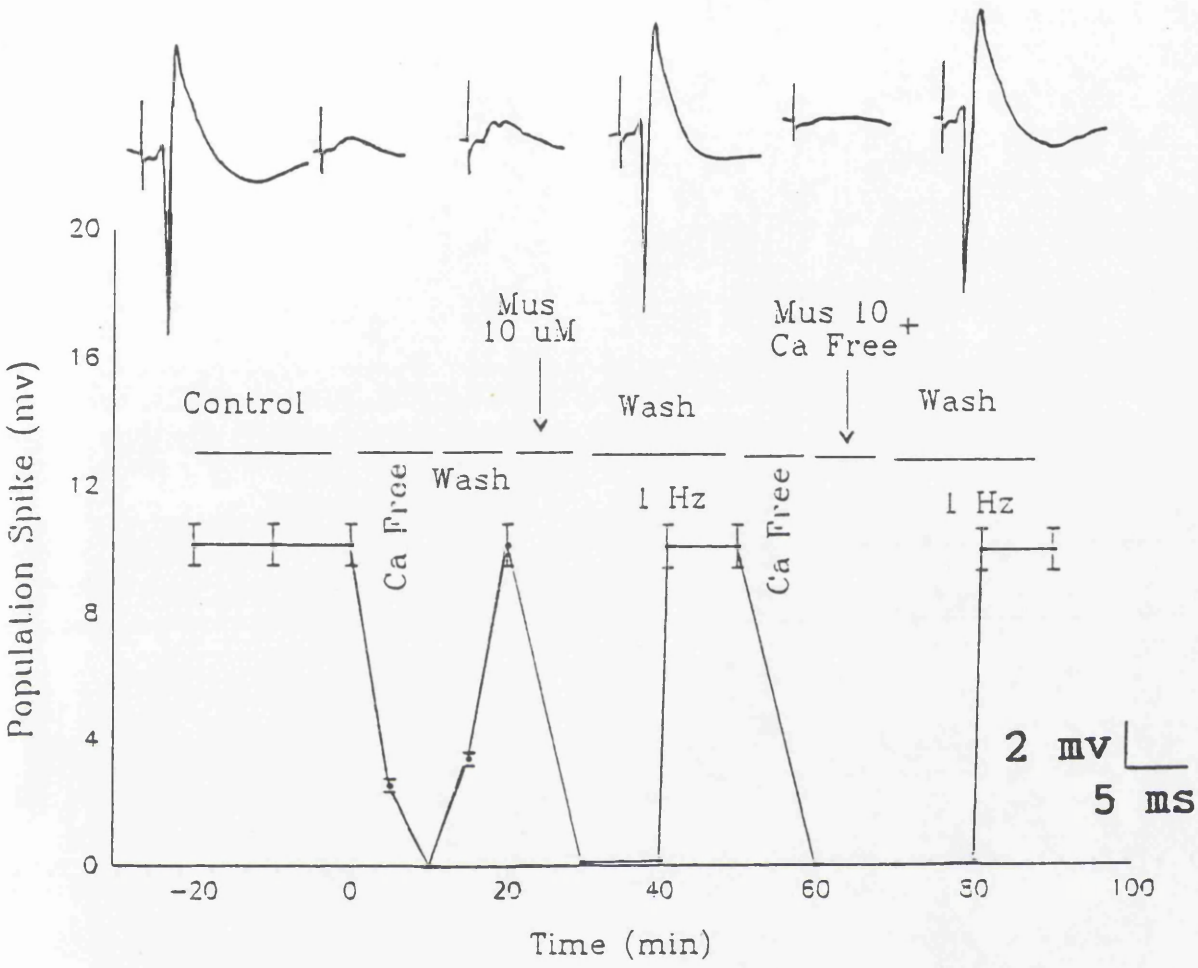

Figure 6.4. Time course of the effect of muscimol 10 μM (Mus) and free calcium medium on orthodromically evoked CA1 population spikes. Each point represents the mean ± s.e.m. for n = 4 experiments. Sample records of orthodromic potentials are shown above the graph.

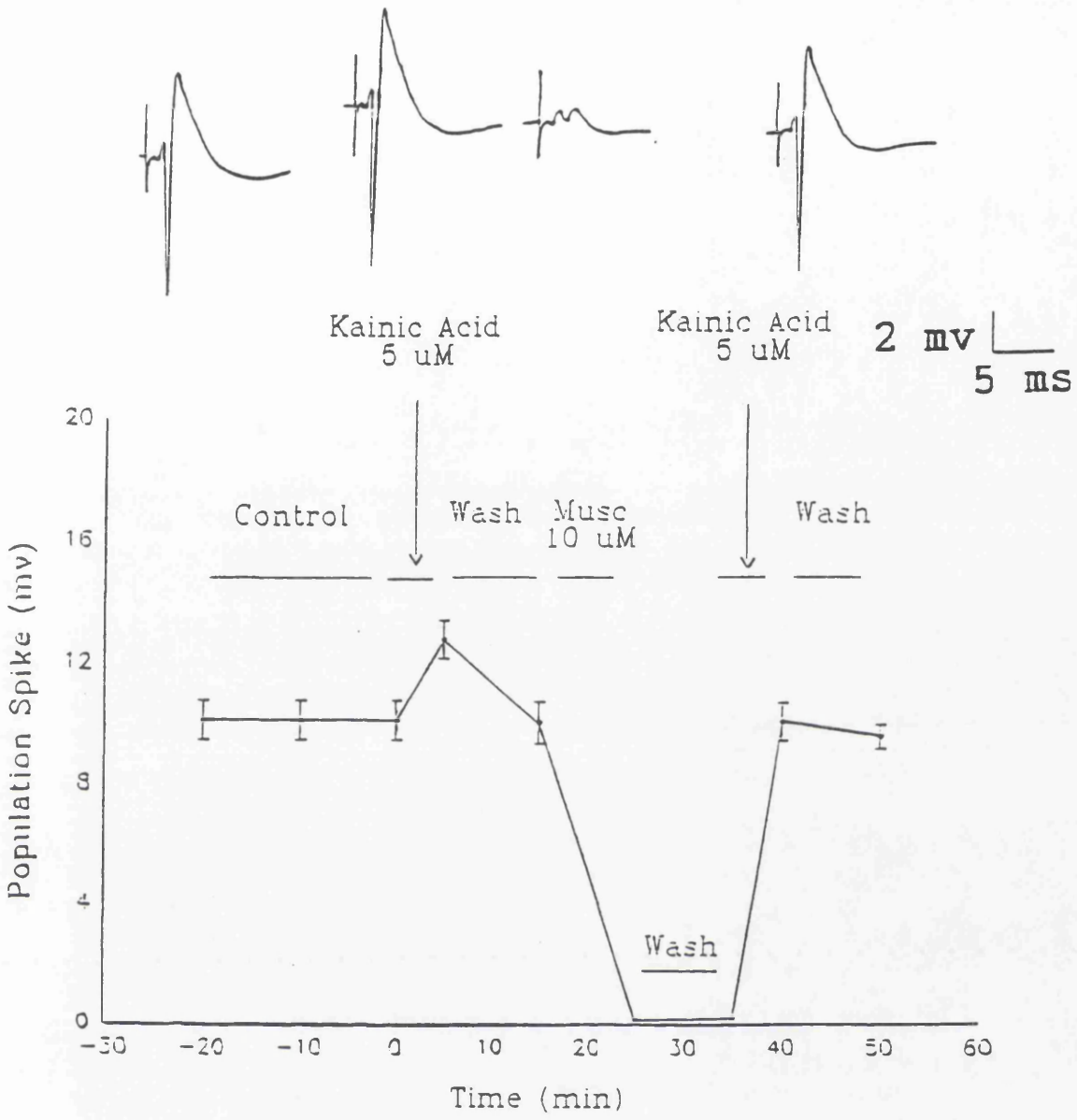

Figure 6.5. Time course of the effect of muscimol 10 μM (Mus) and kainic acid 5 μM on orthodromically evoked CA1 population spikes. Each point represents the mean ± s.e.m. for n = 4 experiments. Sample records of orthodromic potentials are shown above the graph.

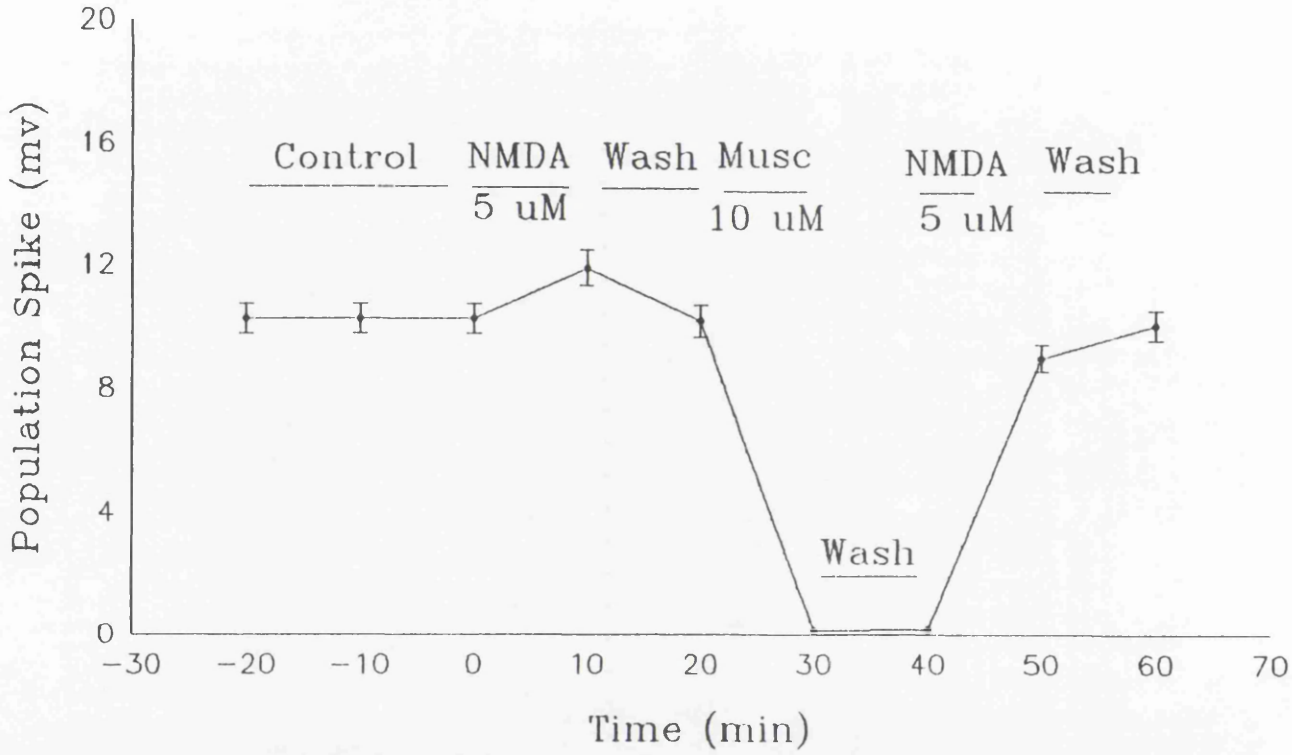

Figure 6.6. Time course of the effect of muscimol 10 μM (Mus) and NMDA 5 μM on orthodromically evoked CA1 population spikes. Each point represents the mean ± s.e.m. for n=4 experiments.

6.2.4. High potassium and veratrine and reversal of muscimol-induced LTD

In order to determine the mechanism by which muscimol-induced LTD was being reversed, slices were depolarised by superfusion with a medium containing a 5 mM increment of potassium chloride or with veratrine. In each slice examined, raised potassium was tested alone before muscimol application. Raising the potassium level by 5 mM itself increased field potential size (figure 6.7.). After washing to restore potential size to normal, LTD was induced by muscimol. This was reversed immediately by stimulation at 1 Hz or over the 5 minutes period of an application of raised potassium (figure 6.7.). Similarly, superfusion with veratrine at 4 μM also reversed muscimol-induced LTD, though the time course of this was slower than with potassium or excitatory amino acid agonists (figure 6.8.). Indeed full restoration of response size was not achieved until 20 minutes after beginning the washout of veratrine.

In a modification of the previous experiment, LTD was induced by muscimol but washout was performed using a calcium-free solution (figure 6.9.). The potassium concentration of the medium was then raised while still in calcium-free medium. When normal ACSF was introduced

subsequent to this, the LTD was found to be reversed, with evoked potentials regaining their original size within 10 minutes (figure 6.9.).

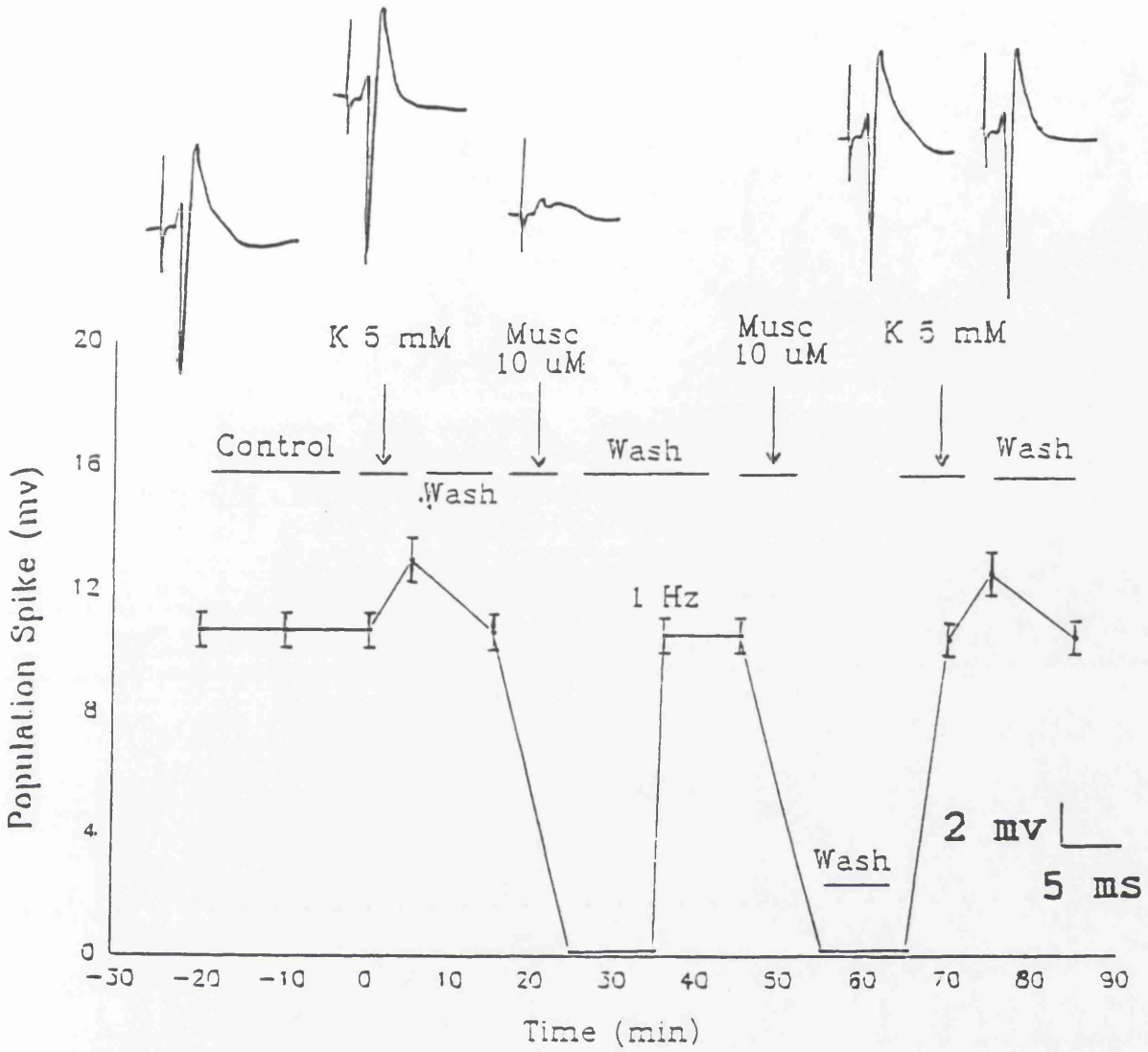

Figure 6.7. Time course of the effect of muscimol 10 μM (Mus) and high potassium medium (K 5 mM) on orthodromically evoked CA1 population spikes. Each point represents the mean ± s.e.m. for n = 4 experiments. Sample records of orthodromic potentials are shown above the graph.

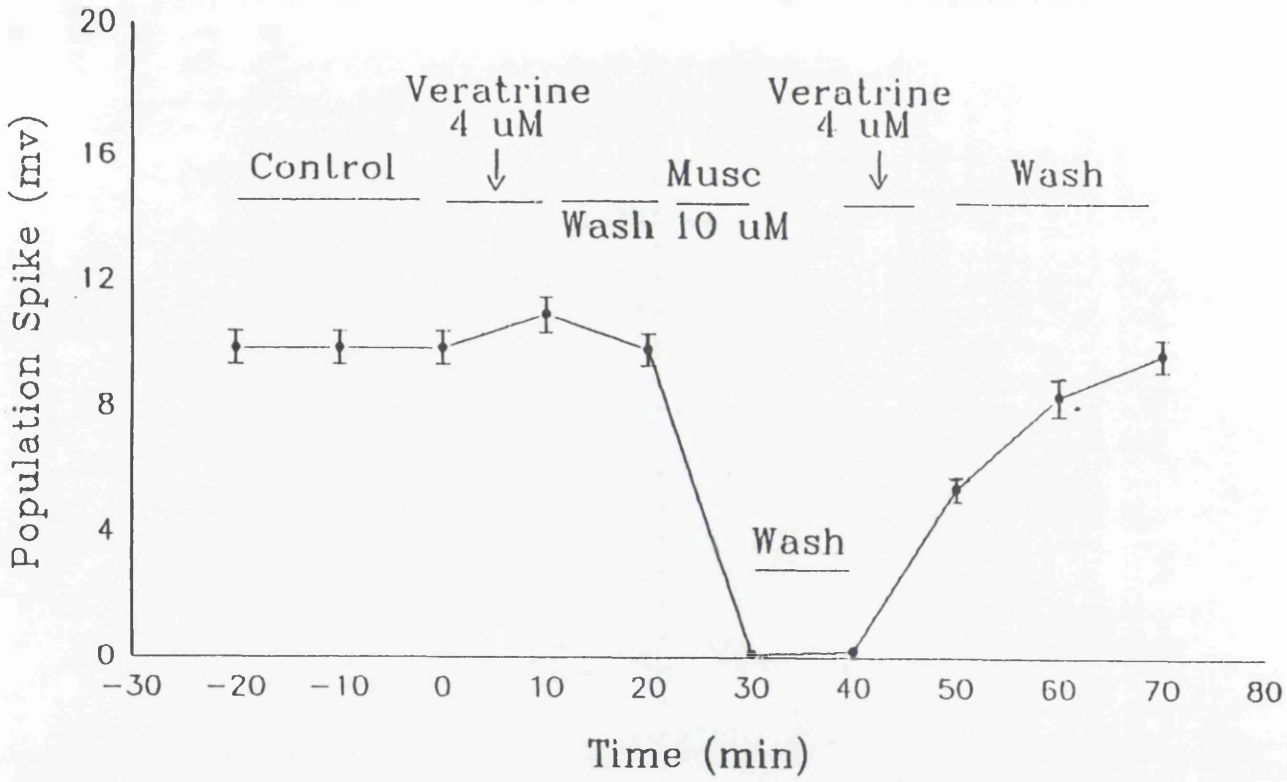

Figure 6.8. Time course of the effect of muscimol 10 μM (Mus) and veratrine 4 μM on orthodromically evoked CA1 population spikes. Each point represents the mean ± s.e.m. for n=4 experiments.

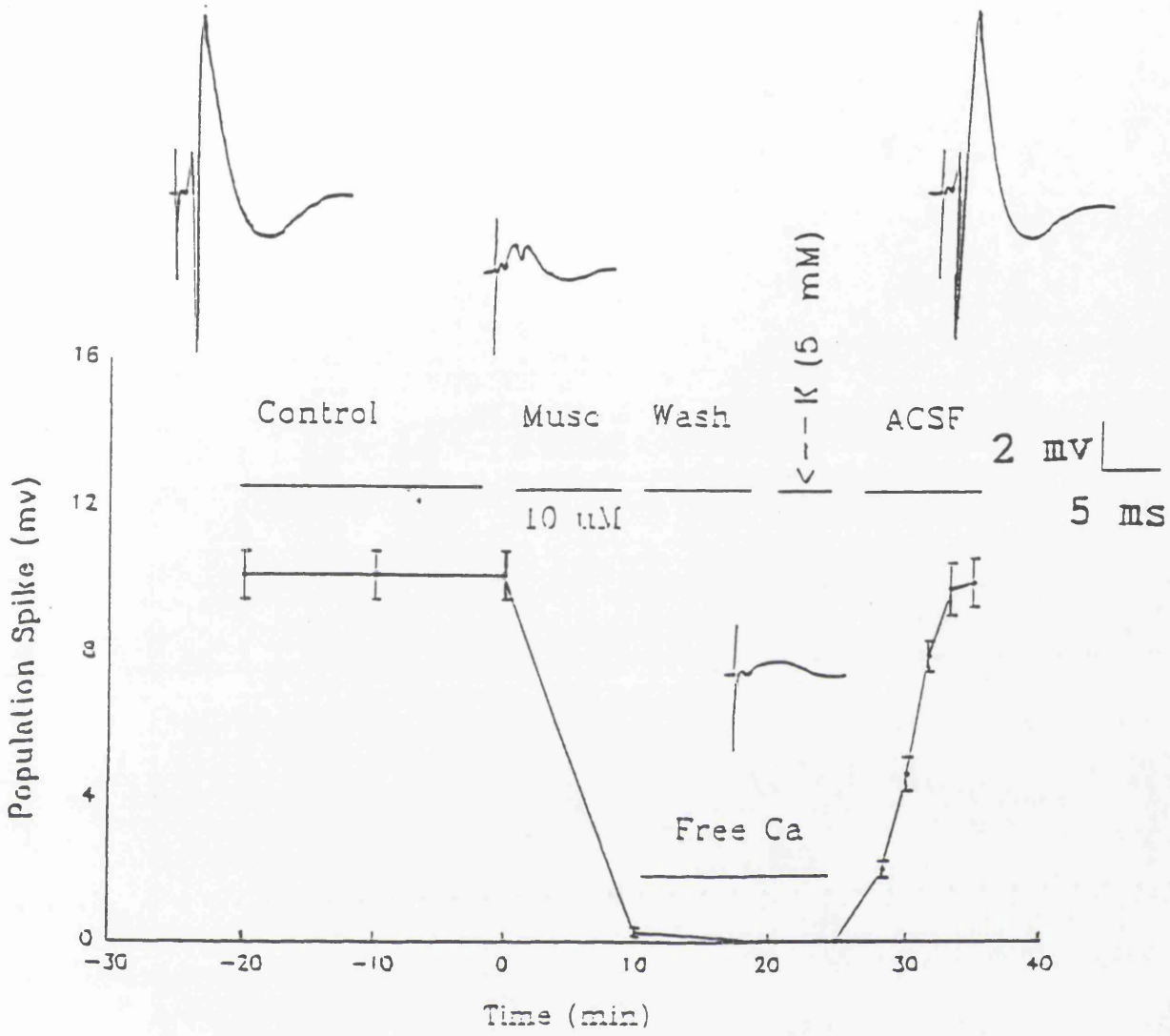

Figure 6.9. Time course of the effect of muscimol 10 μM (Mus), high potassium medium (K 5 mM) and free calcium medium on orthodromically evoked CA1 population spikes. Each point represents the mean ± s.e.m. for n = 4 experiments. Sample records of orthodromic potentials are shown above the graph.

6.2.5. High calcium or a calcium ionophore and reversal of muscimol-induced LTD

Raising the level of calcium in the superfusion medium to a total 10 mM increased the size of control evoked potentials to an extent comparable with elevated potassium, NMDA or kainic acid application, but did not prove able to reverse muscimol-induced LTD (figure 6.10.). Electrical stimlation of the stratum radiatum at 1 Hz however, produced its usual immediate reversal (figure 6.10.). On the other hand, superfusion with the calcium ionophore A23187 at 10 μM did reverse LTD, albeit with a relatively slow course similar to that observed with veratrine (figure 6.11.).

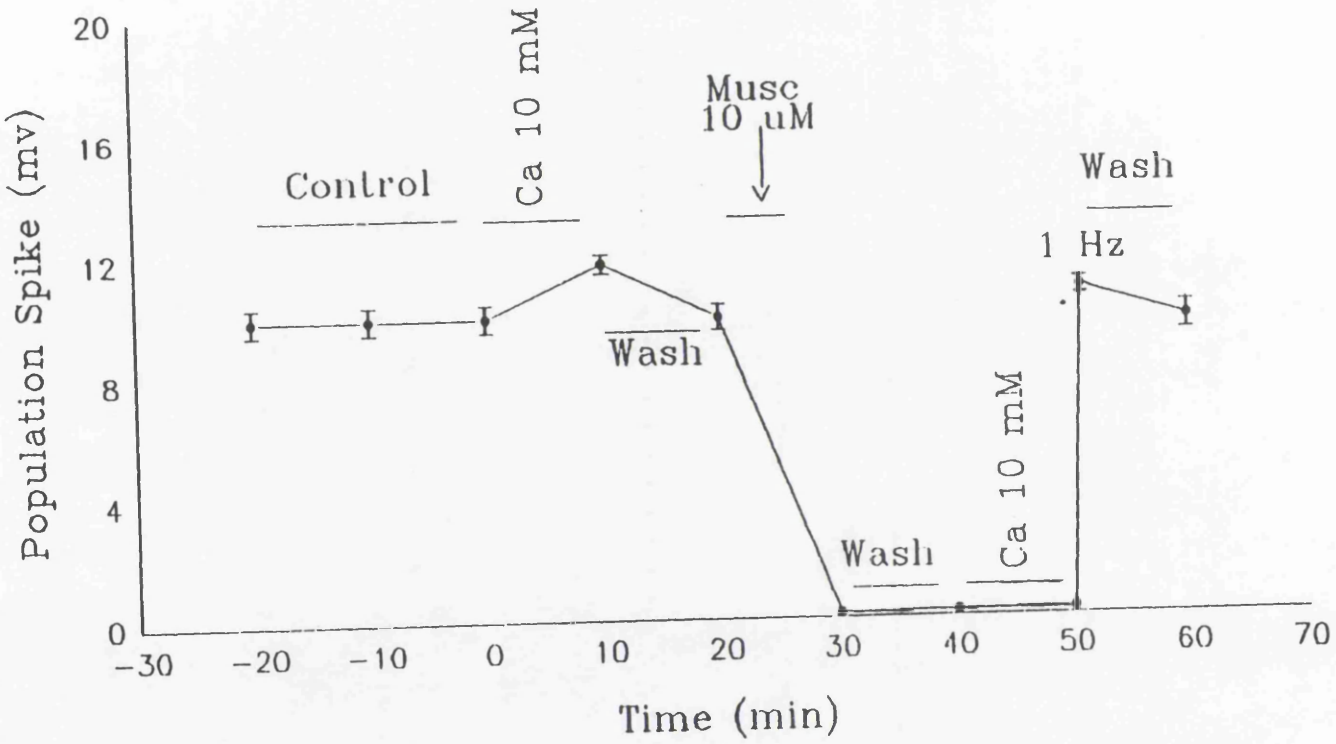

Figure 6.10. Time course of the effect of muscimol 10 μM (Mus) and high calcium medium (Ca 10 mM) on orthodromically evoked CA1 population spikes. Each point represents the mean ± s.e.m. for n=4 experiments.

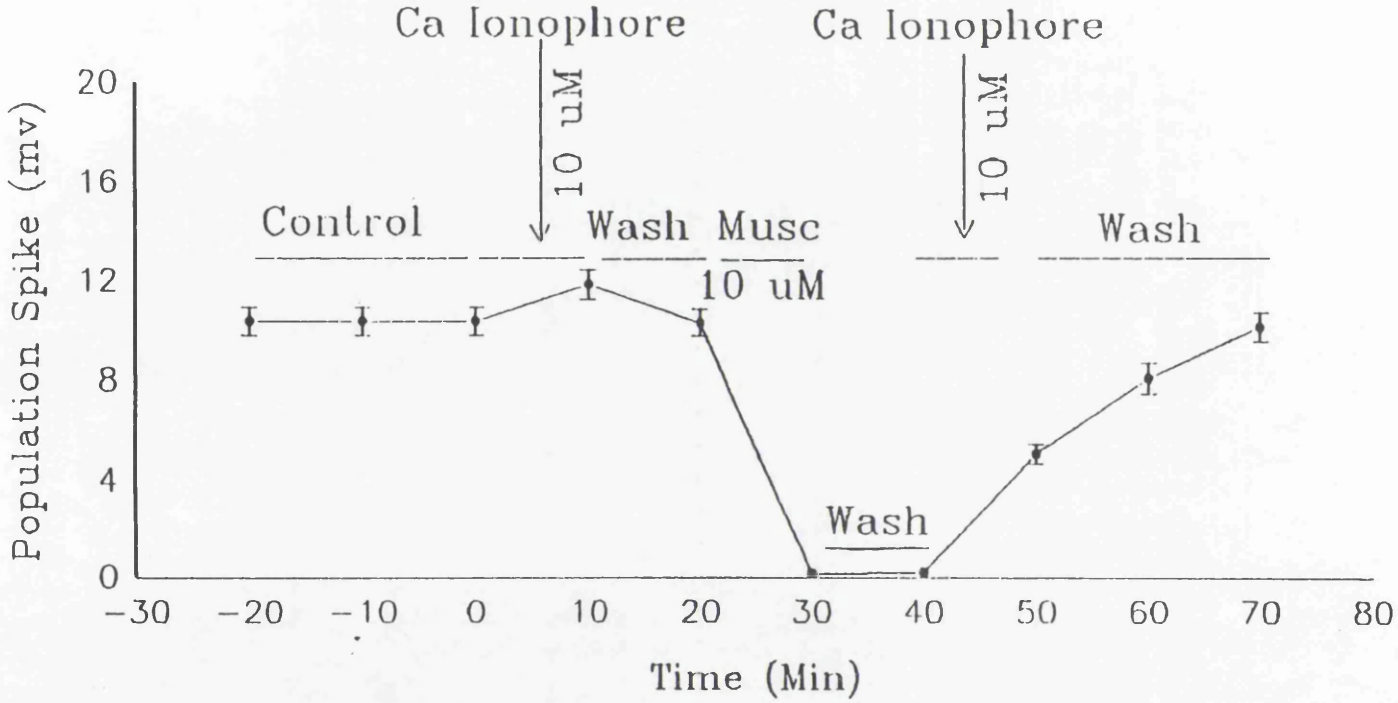

Figure 6.11. Time course of the effect of muscimol 10 μM (Mus) and calcium ionophore 10 μM on orthodromically evoked CA1 population spikes. Each point represents the mean ± s.e.m. for n=4 experiments.

6.3. Discussion

There is increasing interest in the phenomenom of LTD since, together with LTP, it represents a potential information storage mechanism in the hippocampus. A variety of protocols have been reported for producing LTD but these invariably seem to involve metabotropic or NMDA receptors in the induction of LTD (Aroniadou and Teyler, 1991; Yang and Faber, 1991; Lovinger et al., 1993). As it was described in chapter 5, there is a lack of involvement of either NMDA or metabotropic glutamate receptors in the induction of LTD by the protocol which is presented in this thesis, using the $GABA_A$ agonist muscimol. Another distinction between this protocol and other methods for producing LTD is that muscimol induced LTD can be demonstrated using a stimulation frequency of 0.01 Hz and indeed, even in the absence of stimulation. Other groups have induced LTD in the hippocampus by stimulating at a frequency of 1-5 Hz (Braham and Srebro, 1987; Staubli and Lynch, 1990; Yang et al., 1994), whereas muscimol-induced LTD is reversed at this frequency.

Several reports have confirmed the crucial role of calcium in induction of LTD or LTP (Hirano et al., 1994; yang et al., 1994). They have indicated that the state of

LTD or LTP depends on the balance between phosphorylation and dephosphorylation. Small amounts of calcium activate phosphatase enzymes and it has been shown that this leads to LTD, whereas the release of larger amount of calcium activates phosphorylation that can induce LTP (Christie et al., 1994; Debanne and Thompson, 1994; Hirano et al., 1994).

The object of this part of the project was to determine whether the novel form of muscimol-induced, glutamate-independent LTD was dependent upon a movement of calcium ions.

The results indicate that these mechanisms are unlikely to pertain to the muscimol-induced form of LTD, since the omission of calcium ions did not prevent the establishment of LTD by muscimol perfused during the calcium-free period. Taken together with previous evidence (chapter 5) for the lack of involvement of ionotropic or metabotropic glutamate receptors in muscimol LTD, this lack of dependence upon calcium emphasises further the novelty of this form of synaptic depression.

It was also of interest to examine the mechanisms of reversal of LTD since the frequency of orthodromic stimulation which reverses this depression (1 Hz) is similar to the frequency used by several other groups to induce (glutamate-dependent) LTD. Since it was shown that reversal of LTD can be prevented by the non-selective glutamate receptor antagonist kynurenic acid (chapter 5), it seemed likely that activation of NMDA or non-NMDA receptors would be implicated in the reversal. In fact, superfusion with either NMDA or kainic acid, at concentrations which had small excitatory effects on the pyramidal cells but which were below those needed to overdepolarise them, both induced reversal of LTD.

This lack of receptor selectivity for reversal, however, argued that a common feature, such as depolarisation *per se*, might be responsible for the reversal, rather than a receptor specific mechanism.

It is known that activation of both NMDA and kainate receptors is associated with the influx of calcium ions into neurones, but this is probably not the mechanism of LTD reversal here, since raising external calcium levels to 10 mM did not prove able to reverse LTD once established. The simple elevation of calcium levels does not, however,

entirely eliminate the possibility that reversal by the amino acid agonists is the result of depolarisation of the neurones which may (or may not) act via a larger, secondary depolarisation-induced release of intracellular calcium.

In order to test these possibilities, two other depolarising stimuli were examined, potassium and veratrine a sodium channels opener (Catteral, 1980; Leibowitz et al., 1986; Barnes and Hille, 1988). Both procedures should depolarise neurones with an influx of sodium ions, a consequent depolarisation-induced release of intracellular calcium, and an accompanying influx of calcium through voltage-dependent channels. Both stimuli were indeed able to reverse LTD, and subsequent testing of potassium in calcium-free medium was also able to do so. These results are thus consistent with the failure to reverse LTD by 10 mM calcium alone and reinforce the view that depolarisation induced calcium influx is not responsible for the reversal of LTD. Reversal could be obtained, however, by the calcium ionophore A23187, supporting the possibility that intracellular calcium is needed for the reversal of LTD.

Another possibility is that this effect of calcium ionophore is due to dephosphorylation of $GABA_A$ receptors that decreases $GABA_A$ ability to induce LTD. This suggestion

is in line with previous reports that indicate an increase of intracellular calcium decreases $GABA_A$ efficacy (Chen et al., 1990; Markram and Segal, 1991; Feigenspan and Bormann, 1994; Gyenes et al., 1994; Martina et al., 1994).

Overall the present data indicate that neither the induction of LTD, nor its reversal, require extracellular calcium. The reversal, however, may involve a rise of intracellular calcium levels. This is probably calcium released from intracellular storage sites in response to the sodium influx which accompanies the depolarisation of neurones, whether caused by glutamate receptor activation, high potassium or veratrine, but which can be mimicked by the action of calcium ionophore A23187.

General Discussion

7. General discussion

40 years have passed since Holton and Holton suggested that a purine compound could have a role in neurotransmission. Since then the role of purines in the brain has been well described and adenosine is now widely accepted as the major inhibitory neuromodulator in the central nervous system besides GABA. However, no adenosinergic drugs are used clinically for treatment of CNS disorders. One important limitation for the use of adenosine in the CNS is that we do not actually know the effect of adenosine on ion fluxes. Although the role of adenosine on potassium channels and potassium currents is relatively well described, the role of adenosine on calcium and, in particular, chloride channels is still a matter of controversy. By contrast, GABA, as an important and abundant inhibitory transmitter in the CNS, is well described. It has been shown that in the hippocampus, evoked fast inhibitory postsynaptic currents are mediated by a $GABA_A$ receptor-activated Cl^- conductance.

Chapters 3 and 4 of this thesis have looked at the possible role of adenosine on the chloride channels and the interaction between adenosine and muscimol as a global tonic inhibitory system in the hippocampus. The results

indicated that adenosine, besides affecting potassium and calcium channels, also acts on chloride channels. In addition to this, it has been shown that there is a tonic positive interaction between adenosine and the $GABA_A$ receptor at the level of chloride channels. It has been reported that impairment of fast inhibitory postsynaptic currents can lead to pathological conditions such as the generation of epileptic discharge and excitotoxity. Several agents which are used clinically such as anticonvulsant, antiepileptic, sedative/hypnotic and anxiolytic drugs, exert their effects at the $GABA_A$ receptor-chloride ionophore complex. Moreover, it has been reported that adenosine antagonists, such as caffeine or theophylline, can induce seizures and can block the behavioural effects of benzodiazepines. On the other hand, adenosine and its analogues can prevent seizures in vivo or in human models.

The Positive interaction between adenosine and the $GABA_A$ receptor at the level of chloride channels suggests that the idea of application of adenosine derivatives or in combination with a $GABA_A$ agonist or a positive modulator of $GABA_A$ receptors such as neurosteroids may be useful in the treatment of CNS disorders.

There is considerable evidence which suggests that the hippocampus plays a crucial role in certain forms of learning and memory. It has been reported that virtually all aspects of memory processing by the hippocampal system can be referred to its function as a temporary memory store. Moreover, hippocampal lesions induce retrograde and anterograde amnesia. In view of the predominant role of the hippocampus in learning and memory disorders and the increase of these in the last decades of life, several investigations have been centred on the hippocampal system and its pathophysiology.

Hippocampal synaptic plasticity (long-term potentiation and depression) involves long-lasting changes of the efficacy of information processing at the synaptic site. There is increasing interest in the phenomenon of LTD that, together with its allied phenomenon, LTP, is an important information storage mechanism in the CNS involving plastic changes of synaptic function lasting for several hours.

Chapters 5 and 6 of this thesis have been devoted to the role of adenosine and $GABA_A$ receptors in this synaptic plasticity. The results presented that this thesis described a novel type of LTD which is induced by muscimol, a $GABA_A$ agonist. The main point of novelty of muscimol

induced LTD is that the induction of muscimol induced LTD is calcium and glutamate independent. Furthermore, bicuculline as a $GABA_A$ antagonist reverses muscimol-induced LTD. On the other hand, neurosteroids which are positive modulators of $GABA_A$ receptors can potentiate the ability of muscimol in the induction of LTD. Another major finding of this thesis is that muscimol induced LTD can be produced in the absence of stimulation. As the classification of LTD is based on the stimulation of afferent pathways, this phenomenon emphasised the novelty of muscimol-induced LTD. Furthermore, a potentially important concept clinically rises from the maintenance of the LTD by neurosteroids. If neurosteroids are able to maintain LTD induced by a release of GABA that then triggers the forgetting process, then agents which decrease the effects of neurosteroids or negative modulators of $GABA_A$ receptors, may be able to reverse LTD to a normal state and thus help to reverse or prevent the forgetting process.

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