SENSITIVITY TO ADENOSINE OF HIPPOCAMPAL PYRAMIDAL NEURONES IN CALCIUM-FREE MEDIUM

A thesis submitted for the degree of Doctor of Philosophy in the University of Glasgow by

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August 1994.

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Acknowledgements

I should like to thank Professor Trevor Stone for his supervision during my thesis and encouragement specially during the first dark year of electrophysiology. I also wish to express my appreciation to him for his extreme patience and help specially during the preparation of this thesis.

Thanks to Ministry of Health of I.R. of Iran and Mashhad Faculty of Pharmacy to give me this opportunity to continue my education.

Thanks to Duncan for providing varying degrees of humour and help.

Thanks to other Ph.D. students (Mike, Alex, Hillary and Shahin) for an interesting company at all times.

Thanks to my wife for her encouragement and patience to be alone with my daughter at house during weekend and other days which I had experiments.

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SUMMARY

Biochemical studies have shown that divalent cations modulate adenosine receptor binding. In this electrophysiological study the effect of calcium on the postsynaptic sensitivity to adenosine was investigated.

Extracellular recordings were made in the CA1 pyramidal cell layer of rat hippocampal slices following orthodromic stimulation of Schaffer collateral fibres in the stratum radiatum or antidromic stimulation of the alveus. Field excitatory postsynaptic potentials (fEPSPs) were recorded in stratum radiatum. In antidromic stimulation experiments, $CaCl_2$ was omitted (calcium-free medium) or reduced to 0.24 mM (low calcium medium) and in some experiments $MgSO_4$ was increased to 2 mM or 4 mM.

Adenosine and baclofen induced a concentration-dependent reduction in the amplitude of orthodromic potentials with maximum effects at 20 and 5 μM, respectively.

In nominally Ca^{2+} free medium, multiple population spikes were obtained in response to antidromic stimulation. Adenosine had little effect on reducing the secondary spike amplitude. Kynurenic acid, an excitatory amino acid antagonist, at high concentration had no effect on secondary spikes in calcium-free or low calcium medium.

2-Chloroadenosine (1-500 μM) and R-PIA (50 μM), which are not substrates for either the nucleoside transporters or adenosine deaminase were inactive in the absence of calcium. S-(2-Hydroxy-5 nitrobenzyl)-6-thioinosine, an

adenosine uptake blocker, at a concentration of 100 μM had no effect on secondary potential size and did not restore adenosine sensitivity in calcium free medium.

Sensitivity to adenosine in calcium-free medium was restored by 240 μM calcium medium or by raising magnesium (0.8-2.8 mM). Raising the divalent cations concentration increased the inhibitory effect of adenosine and desensitisation was seen. Thapsigargin, which discharges intracellular calcium stores, at 1 μM had no significant effect on the bursts and did not change the effect of 0.5 mM adenosine in calcium free medium.

Unlike adenosine, baclofen concentration-dependently reduced the secondary spike size in calcium free medium and at maximum effect (0.5 mM) or above no sign of recovery was observed during maintained superfusion for up to 45 minutes.

The activity of adenosine was restored in the presence of the stabilizer agents procaine or carbamazepine, known inhibitors of sodium channels. The $GABA_B$ agonist baclofen did not restore sensitivity to adenosine.

The xanthines theophylline and cyclopentyltheophylline, the latter of which is selective for A_1 purine receptors, depressed the excitability of hippocampal pyramidal neurones in calcium-free media. Chelating residual calcium with EGTA reduced excitability which was additive with the xanthine effect, while 100 μM calcium depressed the response to theophylline. The inhibition by xanthines was prevented by adenosine, which

had no effect by itself, but was not reproduced or modified by adenosine deaminase. The xanthine effects were also prevented by baclofen and carbamazepine.

Tolbutamide 1 mM blocked the inhibitory effect of adenosine on the size of orthodromic population spikes but had no effect on the inhibitory action of adenosine on field EPSPs. Tolbutamide did not change the inhibitory action of baclofen on the population spikes or antidromic secondary spikes induced in calcium-free media with high magnesium but dramatically blocked the effect of adenosine in the calcium-free media. Levcromakalim 100 μM potentiated the inhibitory effect of adenosine, but not baclofen, on orthodromic population spikes.

The results of this study showed that the mechanisms of postsynaptic activity of adenosine and baclofen are different and at postsynaptic, but not presynaptic, sites adenosine may activate an ATP-(tolbutamide or levcromakalim) sensitive potassium channel. Loss of postsynaptic sensitivity to adenosine in calcium-free solution may result from increased sodium conductances. A common feature of adenosine, baclofen and carbamazepine which may account for their antagonism of the xanthines is the blockade of calcium fluxes. It is proposed that in the presence of low external concentrations of calcium xanthines can reduce excitability by promoting the mobilisation and trans-membrane movement of residual calcium in the medium or neuronal membranes.

PUBLICATIONS

A. Papers:

1. HOSSEINZADEH, H. & STONE, T.W. (1994). The effect of calcium removal on the suppression by adenosine of epileptiform activity in the hippocampus: demonstration of desensitization. *Br. J. Pharmacol.*, 112, 316-322.

2. HOSSEINZADEH, H. & STONE, T.W. (1994). Mechanism of the hippocampal loss of adenosine sensitivity in calcium-free media. *Brain Res.*, In press.

3. HOSSEINZADEH, H. & STONE, T.W. (1994). A paradoxical inhibitory effect of xanthines on hippocampal excitability in calcium-free media. *Brain Res.*, In press.

4. HOSSEINZADEH, H. & STONE, T.W. (1994). Tolbutamide blocks the postsynaptic effect of adenosine on hippocampal CA1 neurones but not its presynaptic effects or responses to baclofen. *Brain Res.*, Submitted.

B. Review:

5. HIGGINS, M.J., HOSSEINZADEH, H., MACGREGOR, D.G., OGILVY, H. & STONE, T.W. (1994). Release and actions of adenosine in the central nervous system. *Pharm. World Sci.*, 16, 62-68.

C. Abstracts:

6‡. HOSSEINZADEH, H. & STONE, T.W. (1993). Apparent desensitization to adenosine of hippocampal pyramidal cells. *Br. J. Pharmacol.*, **109**, 75P.

7. HOSSEINZADEH, H. & STONE, T.W. (1994). Modulation of postsynaptic adenosine sensitivity in calcium-free media in rat hippocampal slices. *Br. J. Pharmacol.*, **112**, 324P.

8. HOSSEINZADEH, H. & STONE, T.W. (1994). On the mechanism of theophylline suppression of antidromic population spikes in rat hippocampus. *Br. J. Pharmacol.*, In press.

9. HOSSEINZADEH, H. & STONE, T.W. (1994). Effects of tolbutamide on responses to adenosine and baclofen in hippocampal slices. *Br. J. Pharmacol.*, Submitted.

‡This abstract partially was presented in meeting of "Scottish Neuroscience Group" , University of Edinburgh, 15th September, 1993 as "Loss of adenosine sensitivity postsynaptically on hippocampal pyramidal cells in calcium-free medium."

Abbreviations

ACSF	Artificial cerebrospinal fluid
AD	Adenosine
ADA	Adenosine deaminase
ADP	Adenosine diphosphate
AHP	Afterhyperpolarization
AMP	Adenosine monophosphate
ANOVA	one-way analysis of variance
4-AP	4-Aminopyridine
APNEA	N^6-2-(4-aminophenyl)ethyladenosine
ATP	Adenosine triphosphate
BAC	Baclofen
BK_{Ca}	Big (high) conductance calcium-activated potassium channel
BTX	Batrachotoxin
BW-A522	3-(3-iodo-4-aminobenzyl)-8-(4-oxyacetate)-1-proylxanthine
CA1-4	Cornu Ammonis 1 to 4
2CADO	2-chloroadenosine
cAMP	Cyclic adenosine monophosphate
CBZ	Carbamazepine
CGS21680	2-[p-(2-Carboxyethyl)phenylethylamino]-5'-N-ethylcarboxamidoadenosine
CHA	N^6-Cyclohexyladenosine
CNS	Central nervous system
CPA	N^6-Cyclopentyladenosine
CPT	Cyclopentyltheophylline or 8-Cyclopentyl-1,3-dimethylxanthine
CSC	8-(3-Chlorostyryl)caffeine
DIDS	4,4'-Diisothiocyano-2,2'-disulphonic stilbene acid
DMSO	Dimethyl sulfoxide
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
DPX	1,3-Diethyl-8-phenylxanthine
DTX	Dendrotoxin
EGTA	Ethylen glycol-bis (β-aminoethylether) N,N,N',N'-tetraacetic acid
E_K	Equilibrium potential
EPSP	Excitatory postsynaptic potential
F.S	First spikes
G	Guanosine
GABA	γ-Aminobutyric acid
GTP	Guanine triphosphate
HNBTI	S-(2-Hydroxy-5-nitrobenzyl)-6-thioinosine
HVA	High-voltage activated
I	Current
I.P.	Intraperitoneal
IK_{Ca}	Medium (intermediate) conductance calcium-activated potassium channel
IPSP	Inhibitory postsynaptic potential
K_A	Fast transient potassium channel
K_{ATP}	ATP-sensitive potassium channels
K_{Ca}	Calcium-dependent (activated) potassium channels
K_D	Slowly inactivating potassium channel

K_M	M-current
K_{Na}	Sodium-activated potassium channels
K_V	Delayed rectifier potassium channel
K_{Vol}	Cell-volume-sensitive potassium channel
LCRK	Levcromakalim
LTD	Long-term depression
LTP	Long-term potentiation
LVA	Low-voltage activated
MCD	Mast cell degranulating peptide
min	Minute
n	Number
n.s.	Non-significant
NDGA	Nordihydroguaiaretic acid
NECA	5 N-ethylcarboxamidoadenosine
NPY	Neuropeptide Y
P.S.	Population spikes
PD81,723	2-Amino-4,5-dimethyl-3-thienyl)-[3(trifluoromethyl)-phenyl]methanone
R-PIA	R-N^6-Phenylisopropyladenosine
PTX	Pertussis toxin
s.e.m	Standard error of the mean
S.S.	Secondary spikes
SK_{Ca}	Slow (small) conductance calcium-activated potassium channel
TEA	Tetraethylammonium
TLB	Tolbutamide

INTRODUCTION

INTRODUCTION

Adenosine has effects on almost all kinds of mammalian tissue including the central nervous system. As a neuromodulator adenosine has a profound depressant action in the central nervous system. The mechanism of adenosine action is not fully understood. Its action may be mediated pre- and postsynaptically through receptor-mediated mechanisms including effects on second messenger systems, transmembrane ion fluxes and neurotransmitter release.

1. HISTORICAL PERSPECTIVE OF ADENOSINE

A definite action of adenosine was first demonstrated by Drury and Szent-Györgyi (1929). They showed that adenosine in extracts of heart muscle and other tissues such as brain have a specific action upon the heart. Sattin and Rall (1970) later reported an effect of adenosine on cAMP accumulation in the CNS. Phillis et al., (1975) showed that adenosine and several adenine nucleotides depressed the excitability of cerebral cortical neurones of rat in vivo. The depressive effect of adenosine in different areas of brain was later demonstrated by Kostopoulos and Phillis (1977). In 1978 Burnstock proposed the terms P_1 for the nucleoside receptors like adenosine and P_2 for the nucleotide receptors like ATP (Burnstock, 1990). The adenosine receptors were divided by Van Calker et al., (1979) into A_1 and A_2 based on the stimulation or inhibition

of cAMP. The 1980s witnessed the more detailed characterization of adenosine receptors. These years were also spent elucidating the second messengers by which adenosine may act to elicit its array of biological responses. The 1990s will lead this field into the realm of molecular pharmacology, with determination of the amino acid sequences for adenosine receptors and ultimately the genes coding for these receptors (Cushing & Mustafa, 1991).

2. BIOSYNTHESIS, RELEASE AND INACTIVATION OF ADENOSINE

The two likeliest sources of adenosine are the dephosphorylation of 5′-AMP by 5-nucleotidase and the action of S-adenosylhomocysteine hydrolase upon S-adenosylhomocysteine (Snyder, 1985). Adenosine also as a nucleoside of adenine can be synthesized de novo from 5-phosphoribosyl-1-pyrophosphate and glutamine (Stone & Simmonds, 1991).

Adenosine levels in the brain are regulated by the balance of energy supply and demand (Meghji, 1991). There is equilibrium between the cytoplasmic concentrations of ATP, ADP and AMP, and because the ATP concentration in resting cells is much more than the AMP concentration, a very small percentage fall in ATP concentration can increase cytoplasmic AMP concentration substantially, with the formation of adenosine (Fredholm, 1987; Stone et al., 1990).

The concentration of adenosine is increased several times during seizures (During & Spencer, 1992), hypoxia or ischaemia (Fowler 1990, 1993a,b; Gribkoff and Bauman, 1992). Chemical agents such as excitatory amino acids or veratridine, or electrical brain stimulation can also release adenosine (Pedata et al., 1991; Sciotti et al., 1993). Potassium can also evoke a calcium-dependent extrasynaptosomal accumulation of endogenous adenosine (MacDonald & White, 1985).

Adenosine can be inactivated by uptake into neurones and neighbouring cells through a nucleoside transporter by a facilitated diffusion process which is largely regulated by the concentration gradient for adenosine. It is also inactivated either by phosphorylation to AMP by adenosine kinase or deamination to inosine by adenosine deaminase (Meghji, 1991,1993). Nucleoside transport inhibitors such as dipyridamole or nitrobenzyl-thioinosine enhance the effect of adenosine (Greene & Haas, 1989).

In contrast to the sites and mechanisms of adenosine actions, the sites and mechanisms of adenosine formation and subsequent release are still subject to much argument and controversy (Stone et al., 1990). Although released extracellular ATP is broken down to adenosine by ecto-nucleotidases, blocking of this enzyme was found to be without effect on the basal or evoked release of adenosine from rat hippocampal slices (Lloyd et al., 1993) or

embryonic chick neurones and glia in cell culture (Meghji et al., 1989). It seems adenosine is formed predominantly intracellularly and released to the extracellular space.

Although adenosine can be taken up by synaptosomes and released following depolarization, there is no evidence that adenosine is stored in synaptic vesicles and no clear cut adenosinergic pathways have been established in the brain (Browning, 1992). Adenosine is also poorly released by potassium from preparations in comparison with neurotransmitters and there is difference in time course of release between adenosine and neurotransmitters (Stone et al., 1990). Therefore the term neuromodulator may be better used for adenosine.

3. ADENOSINE RECEPTORS

A. CLASSIFICATION

The finding of Sattin and Rall (1970) that methylxanthines, theophylline and caffeine, can block effects of adenosine was the first evidence for specific adenosine receptors. In 1978 Burnstock divided purine receptors into P_1 for the nucleoside receptor and P_2 for the nucleotide receptors (Burnstock, 1990). The P_1 receptors are most readily characterised as sites at which xanthines act as competitive antagonists and adenosine has higher affinity than ATP (Stone, 1989, 1991).

P_1 receptors are further subdivided to A_1 and A_2 based on the inhibition or stimulation of adenylate cyclase respectively. Adenosine has nanomolar affinity for A_1 receptors and micromolar affinity for A_2 receptors (Van Calker et al., 1979). In another study P_1 receptors were subdivided to Ri and Ra for A_1 and A_2 respectively, the subscripts of which refer to the inhibition(i) and activation(a) of adenylate cyclase activity. R refers to the ribose group of adenosine which is necessary for agonist activity (Londos & Wolff, 1977; Londos et al., 1980). Because some physiological effects of adenosine are not mediated via a cAMP-dependent mechanism (Dunwiddie and Hoffer, 1980), the general use of the A_1/A_2 nomenclature which does not inherently imply any activation or inhibition for adenylate cyclase was recommended (Stone, 1985). Based on [^{3}H]NECA binding, adenosine A_2 receptors are also further divided into A_{2a} (high affinity) and A_{2b} (low affinity) receptors (Bruns et al., 1986). Both these subtypes increase adenylate cyclase activity.

There is also a P-site which mediates inhibition of adenylate cyclase and requires integrity of the purine ring for activity but can tolerate compounds with a modified ribose moiety (Londos & Wolff, 1977). Inhibition of adenylyl cyclase activity by the P-site action of adenosine apparently involves a direct effect on the catalytic subunit of adenylyl cyclase. This effect of adenosine requires higher adenosine concentrations than needed for A_1

adenosine receptor-mediated adenylyl cyclase inhibition and is not blocked by adenosine receptor antagonists. Furthermore, some adenosine derivatives, e.g. dideoxyadenosine, are specifically active at this P-site. Inhibition of adenylyl cyclase via the P-site is most efficient when the enzyme is activated (Reithmann et al., 1990). The physiological role of this inhibitory site is still unknown, but recent data indicate that the P-site may be a physiological target for 3′-AMP and related nucleotides (Schwabe et al., 1993).

Besides extracellular (A_1 and A_2 receptors) and intracellular (P-site) adenosine receptors, recently Zhou et al., (1992) reported the cloning, expression and functional aspects of a new adenosine receptor, which they called the A_3 receptor. They showed this receptor coupled to a pertussis toxin-sensitive G protein, and can inhibit adenylate cyclase. This receptor is different from an earlier proposed A_3 site which reported by Ribeiro and Sebastião (1986). The first A_3 receptor was based on pharmacological tools. The non-selective adenosine receptor agonist 5′-N-ethylcarboxamidoadenosine (NECA) is equipotent to the nominally selective A_1 receptor ligands cyclohexyladenosine (CHA) and R-N^6-phenylisopropyladenosine (R-PIA), and more potent than 2-chloroadenosine. This receptor was not linked to adenylyl cyclase but involved inhibition of Ca^{2+} influx and/or mobilization. The major difference between this receptor and the subsequently

cloned receptor is the fact that the latter receptor is not sensitive to alkylxanthine antagonists (Carruthers & Fozard, 1993a).

B. DISTRIBUTION

Adenosine A_1 Receptor

A quantitative autoradiographic study in the human brain showed that Adenosine A_1 receptors were heterogeneously distributed throughout the brain and essentially localized to the gray matter The highest receptor densities were found in the stratum oriens, pyramidale and radiatum of the hippocampus. High densities were also found in the cerebral cortex and the striatum. The hypothalamus had low receptor densities (Fastbom et al., 1987). Ex vivo autoradiographic distribution of [^{3}H]DPCPX in brain also showed high levels of adenosine A_1 receptor in tissues like cerebellum and hippocampus and a lower density in brain stem or hypothalamus (Bisserbe et al., 1992).

Combining binding and autoradiographic procedures with different lesion methods such as transient ischemia, chemical agents (kainic acid or colchicine) and mechanical lesions were used to clarify the exact site of adenosine receptors, pre- or postsynaptically (Deckert & Jorgensen, 1988; Dragunow et al., 1988; Onodera & Kogure, 1988). Using lesioning techniques and autoradiographic methods in rat

hippocampus it was demonstrated that adenosine A_1 receptors were localized on the terminals of the perforant path (Dragunow et al., 1988). With similar techniques Onodera and Kogure (1988) showed adenosine A_1 receptors in the CA1 and CA3 subfields of rat hippocampus, located predominantly on intrinsic pyramidal cells. The results of Deckert and Jorgensen (1988) showing that adenosine A_1 receptors are located presynaptically and postsynaptically are more consistent with the electrophysiological study of Thompson et al., (1992) that adenosine acts at pre- and postsynaptic receptors which are pharmacologically indistinguishable.

Adenosine A_2 Receptors

With the autoradiographic study of distribution of [^{3}H]CGS21680, which binds more to A_{2a} adenosine receptors, it was shown that this receptor is highly concentrated in striatum, nucleus accumbens and olfactory tubercle of rat brain. Lower levels of binding were also found in the globus pallidus. No significant amounts of specific ligand binding were observed in any other brain region (Jarvis & Williams, 1989). A similar result was also reported in human and rat brain by binding assay. Low binding of the A_2 agonist was found in cerebellum and hippocampus (Wan et al., 1990). A_{2b} adenosine receptors are distributed widely throughout brain tissue (Bruns et al., 1986).

Adenosine A_3 Receptor

There are limited studies about adenosine A_3 receptors. There was relatively low expression of this novel receptor in the central nervous system but high expression was observed in testis (Zhou et al., 1992).

C. LIGANDS FOR ADENOSINE RECEPTORS

Many agonist ligands for the adenosine A_1 receptor have been investigated over the past 30 years. These include the N6-subsituted analogues cyclohexyladenosine (CHA), cyclopentyladenosine (CPA), phenylisopropyladenosine (PIA) and 2-chloroadenosine (2CADO, Williams et al., 1986; Williams, 1987; Williams and Cusack, 1990).

There has been less success in developing A_2 adenosine ligands. For a period of time, the 5-substituted adenosine analogue, 5'-N-ethylcarboxamidoadenosine (NECA) was used to define tissue responses mediated by A_2 receptor activation. However, this ligand is non-selective and approximately equipotent at both A_1 and A_2 receptors. A recently produced compound, CGS21680 is 70-140 fold selective on A_{2a} receptors in binding assays (Williams , 1991). At present for the A_3 receptor N^6-2-(4-aminophenyl)ethyladenosine (APNEA) is the most useful agent for activation of this receptor (Carruthers & Fozard, 1993b & c; Fozard & Carruthers, 1993a).

The agonist potency orders for different adenosine receptors are:

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: APNEA>R-PIA=NECA>CGS21680 (Collis & Hourani, 1993).

The most famous antagonists at adenosine receptors are theophylline and caffeine which share a xanthine structure. These agents can not discriminate between A_1 and A_2 adenosine receptors (Daly et al., 1981; Fredholm & Persson 1982; Stone and Simmonds, 1991). 8-phenyl substituted xanthine molecules like 8-phenyltheophylline show more selectivity for A_1 receptors. Alterations in the substituents at the 1- and 3- positions alter both the activity and pharmacological selectivity of the xanthines. 1,3-Diethyl-8-phenylxanthine(DPX) is a potent A_1 antagonist with 18-fold selectivity. The cyclopentyl xanthine, 8-cyclopentyl-1,3,-dipropyl xanthine (CPX or DPCPX) has subnanomolar affinity for the A_1 receptor and is 740-fold selective. Cyclopentyltheophylline (CPT) is 130-fold selective for the A_1 receptor (Williams, 1991). CPT is 40 times more soluble than DPCPX (Bruns et al., 1987).

There is not any selective A_{2b} adenosine receptor ligand available. There are a few A_{2a} antagonists such as 8-(3-Chlorostyryl)caffeine (CSC) which is 520-fold selective for this receptor (Jacobson et al., 1993a).

Recently, an A_3 receptor antagonist, 3-(3-iodo-4-aminobenzyl)-8-(4-oxyacetate)-1-propylxanthine (BW-A522) has been introduced which blocked adenosine A_3 receptor-mediated hypotensive responses in the rat (Fozard & Hannon, 1994).

D. FUNCTIONAL EFFECTS OF ADENOSINE RECEPTORS

Generally adenosine has depressant actions in the hippocampus (Dunwiddie & Hoffer, 1980; Dunwiddie et al., 1991; Lee & Schubert, 1982; Schubert & Mitzdorf, 1979), cerebral cortical neurones (Phillis et al., 1975), olfactory cortex (Collins & Anson, 1985; Motley & Collins,1983; Scholfield , 1987), and different areas of brain (Kostopoulos and Phillis 1977). There are a few reports such as those of Okada and his colleagues which suggest that adenosine has excitatory effects in guinea pig hippocampal slices with concentrations of 1 μM or below (Nishimumura et al., 1992; Okada, 1991; Okada et al., 1992).

The A_1 and A_2 receptors mediate somewhat different effects in most tissues. A_1 adenosine receptors have inhibitory action (Alzheimer et al., 1991; Dunwiddie et al., 1984; Sebastião et al., 1990). In guinea pig hippocampal slices, the highly adenosine A_1 receptor selective antagonist, DPCPX, produced parallel, rightward shifts of the concentration-response curve for CPA-induced

decreases in orthodromically evoked population spikes of CA1 neurones (Alzheimer et al., 1991).

An increasing volume of data indicates that excitatory actions of adenosine, such as depolarization and an enhancement of transmitter release, can be mediated by A_2 receptors (Ameri & Jurna, 1991). There are some reports that A_2 receptor agonists have inhibitory effects. 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 this agonist may act at an A_1 adenosine receptor in hippocampus to show the inhibitory effect (Lupica et al., 1990). Sebastião and Ribeiro (1992) showed that nonomolar concentrations of CGS21680 reversibly increased in a concentration-dependent manner the amplitude of orthodromically-evoked population spikes recorded from the CA1 pyramidal cell layer of rat hippocampal slices. Thus it seems the inhibitory effect of this agent may be related to actions on A_1 receptors at high concentration. By intracellular recording in rat hippocampus, Ameri and Jurna (1991) demonstrated that R-PIA and NECA at high concentration affected membrane properties and showed excitatory effects. Theophylline, non-selective for adenosine receptors, blocked this effect but DPCPX, an A_1 selective antagonist, was ineffective.

E. ALLOSTERIC ENHANCERS

Recently, a new class of compound has been shown to enhance adenosine receptor binding. These compounds originated from a series of 2-mino-3-benzoyl-thiophenes. PD81,723 enhanced the inhibitory effect of exogenously applied adenosine, with no effect alone, in hippocampal brain slices (Janusz et al., 1991). In another study, this group showed that in low magnesium-induced bursting, which induces adenosine release, this agent alone reduced the duration of epileptiform bursting (Janusz & Berman, 1993).

4. PRESYNAPTIC ACTIONS OF ADENOSINE

There is abundant documentation that adenosine diminishes transmitter release via an action at the presynaptic terminal. Adenosine or adenosine analogues inhibit release of glutamate (Fastbom & Fredholm, 1985; Burke & Nadler, 1988), acetylcholine (Fredholm, 1990; Jackisch et al., 1984; Kirkpatrick & Richardson, 1993), noradrenaline (Allgaier et al., 1987; Jonzon and Fredholm, 1984), dopamine (Jin et al., 1993; Michaelis et al., 1979), GABA (Hollins and Stone, 1980) and serotonin (Feuerstein et al., 1988) in the central nervous system. Adenosine also peripherally inhibits release of neurotransmitters such as acetylcholine (Nagano et al., 1992).

The potency of adenosine on inhibition of neurotransmitter release in the different areas of brain and for each transmitter is different. For example adenosine depressed GABA release about 35 percent in cerebral cortex only with high concentration, 1 mM (Hollins and Stone ,1980). Attempts by Fredholm and Jonzon (unpublished data in Dunèr-Engström and Fredholm, 1988) to demonstrate that adenosine analogues inhibit GABA release were unsuccessful. The effect of adenosine on noradrenaline release is also much less (Hu and Fredholm , 1989). It seems adenosine is a more potent inhibitor of release of excitatory than inhibitory neurotransmitters. This may be consistent with the idea adenosine receptor are located on excitatory terminals (Lambert and Teyler, 1991; Thompson et al., 1992; Yoon and Rothman, 1991).

The mechanism of inhibitory effect of adenosine on transmitter release is not completely clear. Transmitter release is largely dependent on calcium influx into nerve terminals and the mobilization of intracellular calcium (Sihra & Nichols, 1993; Zucker, 1993). Therefore one possible mechanism of adenosine to inhibit transmitter release may be blocking of the calcium influx or an effect on the calcium sensitivity of the release process.

Inhibitory effect(s) of adenosine on Ca^{2+} influx are controversial. There are some positive effects of adenosine on the uptake of labelled ^{45}Ca into synaptosomes. In rat

brain synaptosomal preparations, adenosine modulated calcium uptake by potassium depolarized nerve terminals (Ribeiro et al., 1979; Wu et al., 1982). Adenosine analogues decreased calcium uptake across voltage sensitive calcium channels slightly in both synaptosomes and hippocampal slices (Bartrup, 1989). By contrast, other laboratories reported adenosine and adenosine analogues had no effect on calcium uptake by potassium or veratridine depolarization (Barr, 1985; Garritsen et al., 1989; Michaelis et al., 1988).

2-Chloroadenosine decreased calcium currents in cultured rat hippocampal pyramidal or dorsal root ganglion neurones under whole-cell voltage clamp (Dolphin et al., 1986; Scholz & Miller, 1991a). The effect of adenosine analogues was relatively weak in these experiments. In acutely isolated pyramidal neurones from the CA3 region the calcium channels which were blocked by activation by adenosine A_1 agonists were of the N-type (Mogul et al., 1993). Scanziani et al., (1992) reported that in the presence of cadmium, which did not change either frequency or amplitude of miniature synaptic currents, adenosine and baclofen decreased the frequency of miniature synaptic currents of organotypic hippocampal slice cultures. They concluded that the reduction in synaptic strength was not by reduction of postsynaptic sensitivity to neurotransmitter and that the reduction of presynaptic glutamate release by adenosine and baclofen was not

mediated by inhibition of a Cd^{2+}-sensitive presynaptic Ca^{2+} current.

With ion sensitive microelectrodes it was shown that adenosine decreased pre- and postsynaptic calcium signals of rat hippocampal cells in low calcium medium (Schubert et al., 1986). In another report Schubert (1988) demonstrated that endogenous adenosine also, via A_1 receptors, inhibits calcium influx in the synaptic and pyramidal cell soma layer in the CA1 area of rat hippocampal slices. However they did not clearly indicate into which compartment, neuron or glia, calcium moves.

Silinsky (1986) proposed adenosine impairs transmitter secretion by reducing the affinity for calcium at a site beyond the external orifice of the calcium channel. He speculated on possibilities such as that adenosine may reduce a Ca^{2+}-dependent docking of synaptic vesicles or reduce the affinity of calcium binding protein for alkaline earth cations. Then Ca^{2+} would not be as effective in reducing the electrostatic energy barrier between terminal membrane and the vesicles containing neurotransmitters and transmitter release would be impaired.

Another possibility for inhibition of calcium current is 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).

The reasons for the difference of results might be related to different nerve terminals, species, or age, but it is also possible that different mechanisms co-exist in one and the same nerve terminal and that the relative importance of these mechanisms will vary depending on , for example, how the transmitter release is induced (Fredholm & Dunwiddie, 1988).

5. POSTSYNAPTIC ACTIONS OF ADENOSINE

There is a relatively large amount of evidence that adenosine hyperpolarizes postsynaptic cells via potassium channels. Adenosine hyperpolarized CA1 neurones of rat hippocampus and decreased input resistance in normal and low calcium medium when synaptic activity was blocked. Adenosine also suppressed excitatory postsynaptic potentials (EPSP), by a presynaptic effect, without any effect on resting membrane potential (Segal , 1982). Okada and Ozawa (1980) also showed a hyperpolarizing action of adenosine in guinea pig hippocampal slices.

In more precise experiments, using the patch clamp technique, Trussell and Jackson (1985) also confirmed that adenosine can activate potassium channels in postsynaptic neurones from rat striatum. The same group also showed this effect in hippocampus and implicated a G-protein in the activation of potassium channel (Trussell & Jackson, 1987). 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-hyperpolarization (AHP) induced by a burst of action potentials with no effect or little effect on the resting membrane potential. Increasing concentrations of adenosine to 50 μM often decreased AHP and input resistance and induced direct hyperpolarization. Tetraethylamonium (TEA), a blocker of delayed outward rectification, and 4-AP (blocker of transient outward rectification) had no effect on adenosine action (Greene and Haas, 1985; Gerber et al., 1989). Recently Li and Henry (1992) suggested that adenosine induces opening of potassium channels in the postsynaptic membrane of CA1 rat neurones, including K_{ATP} channels. Glibenclamide, a blocker of K_{ATP}, reversibly depressed the 2-chloroadenosine-induced hyperpolarization and the increase in membrane conductance.

These inhibitory postsynaptic action(s) may be mediated by A_1 adenosine receptors (Alzheimer et al., 1989b, 1993; Dunwiddie & Fredholm, 1989).

Besides acting on potassium, adenosine via an A_1 adenosine receptor and pertussis-sensitive effect can induced a steady-state inward current by a voltage-dependent chloride conductance in cultured hippocampal neurones. This current was blocked by application of DIDS,

a putative Cl^- channel blocker (Mager et al., 1990). However other groups, examining reversal potentials, have reported postsynaptic effects of adenosine in hippocampal neurones which involve a chloride-insensitive hyperpolarization (Gerber et al., 1989; Okada & Ozawa, 1980; Segal, 1982; Trussell & Jackson, 1985).

6. POST-RECEPTOR MECHANISM OF ADENOSINE

A. G PROTEIN

GTP-binding proteins are closely related proteins which transduce extracellular signals into effector responses such as ion channels, adenylate cyclase and phospholipase C (Dunlap et al., 1987).

In whole-cell patch-clamp, adenosine evoked an outward potassium current in cultured mouse hippocampus and striatum and in low-resistance patch electrodes lost its action. GTP in the patch electrode filling solution restored the adenosine effect. Thus a G protein is involved in the coupling between the adenosine receptors and a potassium channel (Trussel and Jackson, 1987). This potassium channel mediates postsynaptic effects of adenosine and is sensitive to barium and coupled to a pertussis toxin-sensitive GTP binding protein (Thompson et al., 1992).

The involvement of pertussis toxin-sensitive G proteins in the inhibitory effects of adenosine on neurotransmission is stiil a matter of controversy. N-Ethylmaleimide, a G protein inactivator, decreased adenosine inhibition of the release of acetylcholine (Fredholm, 1990) and noradrenaline (Allgair et al., 1987) evoked by electrical pulses in hippocampus slices. Pertussis toxin also reversed adenosine inhibition of neuronal glutamate release from cerebellar neurones maintained in primary culture (Dolphin & Prestwich, 1985). Using whole-cell patch-clamp techniques, the effect of 2-chloroadenosine on calcium current was abolished by pre-incubation of cultured cells with pertussis toxin (Scholtz & Miller, 1991a). However, in area CA3 of organotypic hippocampal slice cultures, Thompson et al., (1992) showed that the effect of adenosine on field excitatory postsynaptic potentials (fEPSPs) was not sensitive to the toxin. The adenosine-mediated hyperpolarization and decreased input resistance as well as the adenosine-mediated inhibition of low calcium-induced bursting in pyramidal CA1 neurones were virtually abolished by injection of pertussis toxin intraventricularly close to the hippocampus 48-60 hour before experiment. The inhibitory effect of R-PIA on tritiated noradrenaline and acetylcholine release evoked by field stimulation in hippocampus slices was affected hardly or not at all by the toxin treatment. PTX pretreatment also had no effect on adenosine inhibitory effects on evoked potentials in the

CA1 region. This strongly suggests that closely similar A_1 receptors might be coupled to G proteins that differ in their sensitivity to PTX treatment (Fredholm et al., 1989).

They are some reports of direct coupling of adenosine receptors to G proteins. Marala and Mustafa (1993) demonstrated the coupling of A_2-adenosine receptors to G_s protein in bovine striatum using a combination of techniques including agonist-induced tritiated GDP release and the blockage of the agonist-stimulated adenylate cyclase activity by Gsα antibody.

Interactions of adenosine receptors with different effectors such as ion channels or adenylate cyclase via G proteins is not clear. The more probable interaction is that adenosine act at one type of receptor, which couples with different G proteins, each of which interacts with a different effector (Fredholm and Dunwiddie, 1988; Dunwiddie & Fredholm, 1989).

B. CYCLIC AMP

Although the original classification of adenosine receptors was based on the changing cAMP level (Londos et al., 1980; Van Calker et al., 1979) the functional role of this second messenger in relation to adenosine in CNS is not clear.

Raising [cAMP]i either with bath applied forskolin or 8-bromo cAMP did not change the adenosine-evoked potassium outward current in the hippocampus and striatum and as mentioned above the potassium channel was coupled via a G protein (Trussel and Jackson, 1987). In rat hippocampal slices, application of $PbCl_2$, which has a disruptive effect on adenylate cyclase, had no significant effect on depressant responses to adenosine. Isoprenaline, which increases the cyclic AMP levels, had no effect on the amplitude of adenosine-mediated depressant responses, whereas noradrenaline potentiated very modest inhibitory effects of adenosine. Thus, adenosine-mediated cyclic AMP accumulation can be either inhibited or facilitated without markedly affecting the electrophysiological responses to adenosine; this suggests that accumulation of cyclic AMP is not directly involved in such responses(Dunwiddie & Fredholm, 1985).

Some physiological responses to A_1 receptor stimulation are mediated by the inhibition of adenylate cyclase and subsequent lowering in intracellular cAMP level. The A_1 receptor-mediated antilipolytic effect in adipocytes and the negative inotropic effect in ventricular myocytes are two good examples of this mechanism (Green, 1991).

C. PHOSPHATIDYLINOSITOL TURNOVER AND CALCIUM MOBILIZATION

Adenosine can modulate (decrease or increase) phospholipase activity depending upon the animal species, the tissue and the nature of activation of this enzyme. Adenosine inhibited inositol phospholipid hydrolysis elicited in rat cortical slices by mM histamine concentrations. This modulation was selective for histamine; adenosine has no effect on either basal or carbachol-, glutamate-, quisqulate- and noradrenaline-stimulated inositol phosphate generation. The rank order of potency of adenosine agonists and inhibition of the adenosine effect by DPCPX, an A_1 receptor antagonist, indicated the involvement of A_1 receptors (Paoletti et al., 1992). Worley et al., (1987) also showed a cholinergic agonist and a phorbol ester could block the inhibitory action of baclofen and adenosine in rat hippocampal slice preparations via actions on the phosphatidylinositol system.

In peripheral tissue, for example a smooth muscle cell line, activation of adenosine A_1 receptors causes an increase in intracellular levels of inositol 1,4,5-trisphosphate and intracellular free calcium in a time- and concentration-dependent manner. The effect of adenosine was by a pertussis toxin-sensitive G protein (Dickenson & Hill, 1993; Gerwins & Fredholm, 1992a, b).

D. ARACHIDONIC ACID

The inhibitory actions of adenosine on hippocampus responses were unaffected by a phospholipase inhibitor (p-bromophenacyl bromide), lipoxygenase inhibitor (nordihydroguaiaretic acid=NDGA) and a cyclo-oxygenase inhibitor, indomethacin (Dunwiddie et al., 1991). The lipoxygenase inhibitor, NDGA, also failed to antagonize adenosine inhibition of the release of acetylcholine evoked by electrical pulses (Fredholm, 1990).

7. THERAPEUTIC ASPECTS OF ADENOSINE

In the CNS, the potential therapeutic uses of adenosine consists of: anti-convulsant (Dragunow, 1988), anti-ischaemic (Kolvenbach, 1993; Mori et al., 1992; Rudolphi et al., 1992), neuroprotective (MacGregor & Stone, 1993a, b), Parkinson's disease (Lau & Mouradian, 1993), antipsychotic, anxiolytic, sedative, analgesic and Alzheimer's disease (Daval, 1991; Williams, 1990, 1993; Williams & Cusack, 1990). The failure of existing entities and the perceived disadvantages of classical medicinal chemical approaches for adenosine receptor drugs has led a number of laboratories to produce prodrugs, and indirect adenosine agonists such as adenosine uptake blocker and allosteric enhancers (Jacobson et al., 1991). The anticonvulsant action of adenosine will be explained in more detail relative to other potential therapeutic effects of adenosine here because of the relation to this study.

Adenosine can prevent seizures in vivo (Dragunow et al., 1985; Murray et al., 1985, 1993; Whitcomb et al., 1990; Young & Dragunow, 1994), in slices in vitro (Ault & Wang, 1986), and in human models (During & Spencer, 1992; Kostopoulos et al., 1989). It is effective in chemical seizure models such as bicuculline (Ault and Wang , 1986; Thompson et al., 1992), high potassium and/or penicillin (Dunwiddie , 1980; Frank et al., 1988) low calcium (Lee et al., 1984; Dunwiddie and Fredholm 1989), kainate, picrotoxin (Williams, 1987), pentylentetrazole (Murray et al., 1985), strychnine; as well as electrically evoked and audiogenic seizure models (Dragunow, 1988).

In cultured rat hippocampal slices, adenosine at very low doses (30 nM-1 μM) decreased the frequency or blocked spontaneous epileptiform bursting produced by bicuculline. However, much higher concentrations of adenosine (50 μM) were required to fully block stimulus-evoked events (Thompson et al., 1992).

There is a belief that endogenous adenosine also exerts an anticonvulsant action. Adenosine antagonists like theophylline or adenosine deaminase increased burst frequency. Thus an endogenous release of adenosine may contribute to the termination of epileptic bursts. (Dragunow et al., 1985; Dunwiddie, 1980; Murray et al., 1993). In guinea pig hippocampal slices, a transient and selective block of A_1 receptors by DPCPX initiates, probably

by modulation of a second-messenger system, a self-sustaining process leading to persistent epileptiform activity in area CA3 (Alzheimer et al., 1989b, 1993). However, in *cultured* rat hippocampal slices, Thompson et al., (1992) could not show any tonic effect of adenosine. DPCPX had no effect on the amplitude of control synaptic responses and only when there were bursts by another agent, bicuculline, did this A_1 antagonist increase burst frequency.

8. CALCIUM

Ions and other constituents of brain slice bathing medium, ACSF (Artificial Cerebrospinal Fluid), have been designed to maintain metabolic activity and electrophysiological function of slices. Any change of this environment may dramatically alter the excitability of the slice. Calcium is one of these ions which is particularly relevant to the present thesis.

9. FUNCTIONAL ROLES OF CALCIUM

A. NEUROTRANSMISSION

One of the main roles of calcium in neurones is to support neurotransmission and neurotransmitter release. Depolarization of the nerve terminal leads to the influx of calcium through voltage-sensitive Ca^{2+}-channels, which trigger the release of neurotransmitter by exocytosis of intraterminal vesicles in which the neurotransmitter is stored (Sihra & Nichols, 1993). Calcium not only triggers exocytosis, but seems to mobilize secretory granules to release sites (Zucker, 1993).

B. CALCIUM-DEPENDENT ION CHANNELS

Calcium can activate different ion channels. Three broad categories of Ca^{2+}-dependent channels can be

distinguished on the basis of ion selectivity: those selective for K^+, for monovalent cations, or for chloride (Cl^-) ions (Marty, 1989). The first group (calcium-activated K^+ channels), which have been more extensively studied (Blatz & Magleby, 1987; Leinders et al., 1992; Meech, 1978; Schwartz & Passow, 1983), will be discussedunder the potassium channel classification. Calcium can activate channels which are not selective for cations (calcium-activated non-specific cation channels) and which appear to be a unique class of channels with an important role in excitation-secretion coupling and in determining neuron firing patterns (Partridge & Swandulla, 1988). Calcium-activated Cl^- channels with low conductance have been reported in hippocampus and some other neurones. In sensory and spinal neurones this channel can generate depolarizing after-potentials but its function in hippocampus is not clear (Brown et al., 1990a).

C. STABILIZING ACTION

The stabilizing action of calcium emerged from the fact that removal of calcium caused an increase in sodium and potassium conductance and increased excitability (Frankenhaeuser & Hodgkin, 1957). Calcium can organize cationic lipids, within the external layer of the membrane, into laminar micellae (closed form). These laminar micellae may be shifted into globular ones possessing anionic properties by potassium (open form). Modifications in the

membrane selective permeability depend on the structural state of the membrane and may therefor rely on the ratio of K^+/Ca^{2+} in the extracellular space (Agrigoroaei & Neacşu, 1990). Divalent cations including calcium have large effects on the delayed rectifier potassium channels. These ions may stabilize a closed conformation of the channel and their absence may remove the selectivity of the channel (Begenisich, 1988).

Omitting calcium from the extracellular medium removes the stabilizing action of calcium on the cell membrane and bursts of action potentials appear (Agopyan & Avoli, 1988; Haas & Jefferys, 1984). The mechanisms involved in this kind of burst will be discussed in more detail in the discussion section.

D. LTP AND LTD

Calcium is involved in long lasting phenomena including long-term potentiation (LTP) or depression (LTD) in synaptic plasticity. LTP and LTD, which have been more studied in the hippocampus and cerebellum respectively, have a key role in memory. LTD has an important role in memory elements of cerebellar motor learning. Besides glutamate receptors, calcium is involved in the molecular mechanisms of LTP and LTD. Injection of the calcium chelator, EGTA, into dendrites of hippocampal pyramidal cells or Purkinje cell dendrites in cerebellar slice preparations effectively abolished LTP and LTD respectively (Ito, 1989).

E. NEUROTOXICITY

The cytosolic concentration of Ca^{2+} plays a critical role in neuronal death during anoxic episodes, or during sustained exposure to glutamate. In cell cultures of rat hippocampus, Ogura et al., (1988) have shown that there is a correlation between the extent of neuronal loss and intracellular calcium elevated by glutamate. A sustained increase of cytosolic Ca^{2+} concentration, is due at least in part to a suppression of the Ca^{2+} extrusion from the cell (in particular via Na^{+}/Ca^{2+} exchange) rather than from a persistent increase in Ca^{2+} permeability of the neuronal membrane (Khodorovet al., 1993). Depletion of intracellular stores of Ca^{2+} or blocking of internal calcium stores revealed that at least 50% $[Ca^{2+}]_i$ may be involved in NMDA receptor-mediated neurotoxicity in cultured rat cortical and retinal ganglion cells (Lei et al., 1992).

F. CALCIUM TARGETS

In neurons, there are two major classes of Ca^{2+} targets in membranes and three in the cytosol. In the membrane, calcium stimulates Ca^{2+}-dependent channels and two important families of membrane phospholipases, which are activated by a rise in $[Ca^{2+}]_i$; phospholipase C, which hydrolyses phosphatidylinositol phosphates (PIPs), and phospholipase A_2, which cleaves fatty acids, including arachidonic acid, from the glycerolipid backbone. The three major cytosolic

targets include protein kinase C, c lpain (a Ca^{2+}-dependent protease) and calmodulin (Kennedy, 1989).

10. CALCIUM CHANNELS

Calcium channels play a major role in neuronal function, in part, because of the important second messenger actions exerted by free intracellular Ca^{2+}. Calcium channels in the surface membrane at least can be divided into voltage-dependent Ca^{2+}-selective channels and ligand-gated Ca^{2+}-selective channels (Faingold, 1992).

Voltage-gated (dependent) Ca^{2+} channels provide an essential connection between transient changes in membrane potential and a variety of cellular responses. These ion channels electrophysiologically have been divided to: L, T, N and P-type (Krizanova et al., 1993).

L-type (long-lasting) or high-voltage activated (HVA) channels are high activation , slow inactivation and sensitive to calcium antagonists (1,4-dihydropyridines such as nifedipine, phenylalkylamines such as verapamil and benzothiazepines such as diltiazem). L-type Ca^{2+} channels can thus be distinguished from T-, N- and P-type Ca^{2+} channels, which are generally resistant to calcium antagonists. They are found in all excitable tissues and in many non-excitable cells. L-type calcium channels are the major pathway for voltage-gated Ca^{2+} entry in heart and

smooth muscle, and they help control transmitter release from endocrine cells and some neuronal preparations (Krizanova et al., 1993; Tsien et al., 1991).

T-type (transient) Ca^{2+} channels are also known as low-voltage activated (LVA) calcium channels and can be opened by small depolarization from relatively negative holding potentials and are rapidly inactivated. There are few, or no, specific blockers of T-type channel and the lack of high-affinity probes has hindered structural definition. These calcium channels are found in a wide variety of excitable and non-excitable cells. The most prominent functions of T-type channels are to support pacemaker activity or Ca^{2+} entry at negative membrane potentials. In neurones they may be responsible for burst firing (Spedding & Paoletti, 1992; Tsien et al., 1991). In sensory neurones, they are sensitive to Ni^{2+}, but less sensitive to cadmium and are resistant to calcium antagonist; in dissociated rat CA1 neurones low concentrations of the dihyropyridine nicardipine blocked T-type channels.

N-type (neuronal) Ca^{2+} channels are 'high-voltage activated' and differ pharmacologically from L-type channels in being resistant to calcium antagonists and largely blocked by ω-conotoxin. They are much restricted to neurones and have a major role in mediating transmitter release (Tsien et al., 1991).

P-type (Purkinje) channels are a novel class of high-voltage activated Ca^{2+} channels that are particularly prominent in cerebellar Purkinje cells. This kind of calcium channel is resistant to calcium antagonists and ω-conotoxin but sensitive to spider web venom Aga IV (Tsien et al., 1991).

Receptor systems may be directly linked to channels with some selectivity for Ca^{2+} under certain conditions. In these systems receptor activation rather than voltage-dependent activation is the prime trigger, although there may be some modulation by voltage. The NMDA-gated channel is an example of this kind of calcium channel (Spedding & Paoletti, 1992).

11. CALCIUM RECOVERY (FADE OF CALCIUM)

Intracellular homeostasis of calcium plays an important role in cell functioning and survival. Cytosolic free Ca^{2+} is very low in relation to outside. In CA3 pyramidal cells at the holding membrane potential of -50 mv, the intracellular concentration of this ion is about 30 nM, which increased with reduction of the membrane potential to more positive values (Knöpfel et al., 1990).

In general several mechanisms may contribute to the whole process of $[Ca^{2+}]_i$ recovery: 1) calcium binding by cytoplasmic proteins and other ligands, representing fast

calcium buffers; 2) calcium redistribution in the cytoplasm by simple diffusion; 3) Na^{+}/Ca^{2+} exchange; 4) calcium pump; 5) calcium uptake within the cell by mitochondria or 6) by endoplasmic reticulum (Kostyuk et al., 1989).

12. POTASSIUM CHANNELS

Potassium ions are selectively concentrated in the interior of cells, and the concentrations of potassium in the extra- and intracellular fluid are approximately 4 and 150 mM, respectively. Potassium ions play a dominant role in controlling the resting membrane potential in most excitable cells and maintain the transmembrane voltage positive to the potassium equilibrium potential (E_K) of about -90 mv. During depolarization, sodium ion influx causes the transmembrane potential to become more positive relative to E_K; repolarization causes the cell to return toward E_K (become more negative) and is mediated in large part by the efflux of potassium ions down their concentration and electrical gradients (Robertson & Steinberg, 1990).

The main function of open potassium channels is to stabilize the membrane potential. These channels generally lower the excitability of the cell when they are open (Hille, 1984). Agents that block potassium channels tends to produce membrane depolarization; i.e, they shift the transmembrane potential in a positive direction away from E_K. Compounds which open potassium channels tend to produce membrane hyperpolarization and shift the resting membrane potential toward E_K (Blatz & Magleby, 1987; Robertson & Steinberg, 1990). Such an effect would oppose the opening of voltage-operated calcium (or sodium) channels which

require depolarization above a certain threshold membrane potential for activation (Cook, 1988).

13. CLASSIFICATION OF POTASSIUM CHANNELS

Potassium channels are ubiquitous in eukaryotic cells and exhibit more diverse characteristics than channels for other ions. Over a dozen types of potassium channels have already been identified using variations on the patch clamp technique (Faingold, 1992).

Potassium channels are regulated through voltage, calcium, G protein and ligands. These ion channels can be classified according to functional properties of ion selectivity and conductance, gating behaviour, pharmacology, and regulation (Moczydlowski et al., 1988). Here potassium channels in hippocampus are categorized as voltage-sensitive (gated) calcium-independent potassium channels, calcium-sensitive (dependent) potassium channels, receptor coupled (opening) potassium channels and miscellaneous potassium channels.

A. VOLTAGE-SENSITIVE CALCIUM-INDEPENDENT POTASSIUM CHANNELS

Voltage-gated potassium channels terminate the action potential, repolarize the neuron, set its resting potential, and regulate neurotransmitter release from the presynaptic terminal (Rehm & Tempel, 1991). This group can

be divided at leastintofour types: delayed rectifier, slow delayed rectifier, A-channel and inward rectifier potassium channel.

The first potassium channel which was described is the delayed rectifier potassium channel(K_V), that activates rapidly on depolarization and inactivates slowly. This is the classic potassium conductance of Hodgkin and Huxley that is responsible for the repolarization phase of the action potential (Halliwell, 1990; Faingold , 1992). This current is blocked by tetraethylammonium (TEA), (partly by 5mM, completely by 25-30 mM) and is little affected by 0.01-5 mM 4-aminopyridine. Modulation by transmitter substances has not been reported for K_V (Rutecki et al., 1990; Storm, 1990).

The fast transient potassium channel (K_A) is activated much more rapidly upon depolarization than the delayed rectifier and is also inactivated faster than K_V (Rogawski, 1985; Halliwell, 1990). K_A is involved in spike repolarization and reduces the interspike interval to space successive action potentials (Hille, 1984). This current is blocked by 1-5 mM 4-aminopyridine (4-AP) and dendrotoxin, a toxin from snake venom, (DTX, 50-300 nM) in the hippocampus (Storm, 1990).

K_D or the slowly inactivating potassium channel was recently shown in rat hippocampal slices (Storm, 1988). In

contrast to K_A, this channel is much more sensitive to 4-aminopyridine (30 μM). It activates at potentials that are more negative than the spike threshold and inactivates very slowly. K_D can cause a several seconds long delay in the onset of firing in response to long-lasting depolarizing stimuli. K_D seems fast enough to participate in spike repolarization and the presynaptic spike broadening due to block of K_D may mediate the increase in transmitter release which is seen with small doses of 4-AP (Storm, 1988, 1990).

Inward rectifier potassium channels are activated by hyperpolarization, rather than depolarization, and they can pass larger K^+ currents into the cell than outwards. Because of this behaviour, which is opposite to the classical delayed rectifier currents, this type of channel is often known as the "anomalous rectifier" (Harvey, 1993).

B. CALCIUM-DEPENDENT POTASSIUM-CURRENTS

The existence of calcium mediated increases in potassium-conductance was shown in both CA1 and CA3 regions of guinea-pig (Schwartzkroin and Stafstorm, 1980) and rat hippocampus (Alger and Nicoll, 1980). Calcium-dependent (activated) potassium channels K_{Ca} which are common in excitable and secretory cells, are dependent on the free intracellular Ca^{2+} concentration which may or may not be voltage-gated. An increase in the intracellular calcium leads to an increase in the opening of these channels

(Faingold, 1992). These channels have been categorized into at least three subdivision as a big (high) conductance calcium-activated potassium channel or fast calcium-activated potassium channel (BK_{Ca}), medium (intermediate) conductance calcium-activated potassium channel (IK_{Ca}) and slow (small) conductance calcium-activated potassium channel, SK_{Ca} (Blatz and Magleby, 1987; Storm, 1988; Halliwell, 1990).

The BK_{Ca} repolarizes the action potential and the SK_{Ca} reduces action potential discharge rate (accommodation or adaptation) that typically occurs during a long duration depolarization (Nicoll, 1988). The BK_{Ca} channel is very voltage-dependent and is blocked by charybdotoxin and TEA(Goh et al., 1992; Goldstein & Miller, 1993). The SK_{Ca} channel has little or no voltage-dependency but higher than BK_{Ca} is calcium dependent and is resistant to TEA (Lancaster & Adams, 1986; Rudy, 1988). Apamin can block SK_{Ca} in different tissues such as neocortex (Szente et al., 1988), neuroblastoma and rat muscle cell (Lazdunski et al., 1985), sympathetic neurones (Kawai and Watanabe, 1986) and ganglion cells (Nishimura et al., 1988; Goh and Pennefether, 1987). The slow AHP is also decreased or abolished by acetylcholine, histamine, serotonin, noradrenaline (Nicoll, 1988; McCormick & Williamson, 1989; Bijak et al., 1991) Adenosine at less than 50 μM potentiated the slow AHP in rat hippocampus slices (Greene and Haas, 1985).

C. RECEPTOR-OPERATED POTASSIUM CURRENTS

Several neurotransmitters modify neuronal excitability by modulating the function of K^+ channels. Potassium channel regulation by these neurotransmitters differs from the action of neurotransmitters such as acetylcholine on nicotinic receptors. Here the channel is a separate molecule from the receptor and the effects on potassium channels are mediated via second messengers activated as a result of neurotransmitter-receptor interaction (Rudy, 1988). There are at least three receptor-coupled channels: M-current, atrial muscarinic-activated potassium channel (K_{ACH}) and 5-HT-inactivated channel ($K_{5\text{-}HT}$). Here the M-current, which is more important in neurones will be explained.

The M-current (K_M) was so called because, in the original tests on frog ganglion cells, it was inhibited by muscarinic acetylcholine receptor agonists, such as the alkaloid muscarine (Brown, 1988). The M-current is a slow, "non-inactivating" voltage-dependent potassium current and activates close to rest or at hyperpolarized potentials (Rudy, 1988). In hippocampal pyramidal cells, K_M underlies an early phase of spike frequency adaptation, and it seems to be the main factor in the medium AHP which follows a single spike or spike burst (Storm, 1990). Besides acetylcholine, K_M may be modulated or blocked by serotonin (McCormick & Williamson, 1989), 1 mM Ba^{2+}, or 5-10 mM TEA,

but not by 0.1-1mM 4-AP. Somatostatin, on the other hand, seems to enhance K_M in rat CA1 cells (Brown, 1988; Storm , 1990).

D. MISCELLANEOUS POTASSIUM CHANNELS

There are some other potassium channels such as ATP-sensitive potassium channels (K_{ATP}), sodium-activated potassium channels (K_{Na}) and cell-volume-sensitive potassium channels (K_{Vol}) which could not completely be categorized in the above classification.

ATP-sensitive potassium channels constitute a new class of potassium channels that link membrane potential variations to the bioenergetic situation of the cell. An increase of internal concentration of ATP tends to close this channel and produce a depolarization (Bernardi & Lazdunski, 1993). 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. Sulfonylurea compounds such as glibenclamide can block this potassium channel and trigger insulin release (Gopalakrishnan et al., 1993). The existence of sulfonylurea binding sites associated with ATP-sensitive K^+ channels have been shown in the hippocampus and other parts of brain (Mourre et al., 1989, 1991; Ohno-Shosaku & Yamamoto, 1992). 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 block glutamate release (Miller, 1990; Tromba et al., 1992, Zini et al., 1993).

14. HIPPOCAMPAL SLICES

The in vitro slice model has been widely used over the past several years. In vitro brain slices have considerable value as pharmacological tools with which to study the physiological actions of neurotransmitters and drugs on the central nervous system. Several aspects of the slice preparation facilitate this type of analysis (Dunwiddie et al., 1983). The lamellar structure of hippocampus (Andersen, 1971), with its rather simple three-layered cortex, well-defined input and output pathways (Schwartzkroin,1987) and its suitability for evoking large extracellular potentials (Dunwiddie et al., 1983) makes it a natural choice for a slice preparation.

A. 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 fibres arising, in part, from cells of the hippocampus, most of which leave the region through a large efferent pathway, the fornix. 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).

B. INTERNAL STRUCTURE

The hippocampal formation can be divided into the Ammon's horn, the dentate gyrus, and subiculum. Based on the 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).

C. INTRINSIC CONNECTIVITY OF HIPPOCAMPUS

Neuronal input reaches the hippocampus from entorhinal regions, which sends a digest or summary of what is occurring in the neocortex and consequently, the environment, to the hippocampal formation. The entorhinal cortex via 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 (Skrede & Westgaard, 1971; Isaacson, 1987).

D. FUNCTION OF HIPPOCAMPUS

Since the late 1950s, it has been popular to associate the hippocampus with some form of memory. This is due in large part to the recent memory deficiencies of Dr. Williams Scoville's patient, H.M., who demonstrated such impairment after bilateral removal of the hippocampal formation and its associated medial temporal lobe structures (Isaacson, 1987). This patient had a profound impairment in new learning, but he had intact access to representations of remote memory (Eichenbaum et al., 1992). Long-term potentiation which is believed to have a role in memory can be efficiently induced in hippocampus. Using

specific gene deletions that eliminate hippocampal LTP, impairment of hippocampal-dependent learning was demonstrated (Eichenbaum & Otto, 1993).

15. ADVANTAGES AND DISADVANTAGES OF BRAIN SLICES

In vitro slice models, like hippocampal slices, have advantages versus in vivo models:

-Technical simplicity and multiplicity: recordings can be obtained using extracellular, intracellular and whole-cell patch-clamp techniques (Henderson, 1992);
-Stability of model: There is no pulsation or respiration which may interfere with recording;
-No systemic metabolism and biotransformation. This is a good point for a new chemical agent to be closely examined;
-Improved visualization of tissue;
-Easy changing of extracellular fluid.(Clark and Wilson, 1992; Schwartzkroin 1981,1987).

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

-Loss of inputs: Some afferents such as noradrenaline, serotonin and acetylcholine cannot be activated in hippocampal slices in vitro (Dunwiddie et al., 1983);
-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

A. PREPARATION OF SLICES

Male Wistar rats (170-210 g) were anaesthetized with urethane (1.3 g/kg, I.P.) and decapitated using a guillotine. The scalps were removed and brains were rapidly taken out and put in ice-cold and oxygenated 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 slices 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% O_2 and 5% CO_2 at room temperature for at least one hour before using. The fluid in the petri dish was adjusted to just cover the slices.

B. BATHING MEDIUM

The formula of the bathing medium, artificial cerebrospinal fluid (ACSF), was as follows: (mM): NaCl 115, KH_2PO_4 2.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 10.

In some antidromic experiments, calcium was omitted (calcium-free medium) or reduced to 0.1-0.24 mM (low calcium

medium). Analysis of the nominally calcium-free solution by flame ionisation spectroscopy revealed a residual level of 12 μM calcium.

In some antidromic experiments, calcium was omitted and $MgSO_4$ was raised from 1.2 to 2 or 4 mM (calcium-free high magnesium).

In high potassium experiments, potassium was raised to 8.5 mM and sodium chloride was reduced accordingly.

C. BATH SUPERFUSION AND APPLICATION OF DRUGS

Individual slices were transferred using a brush to a one ml capacity recording chamber and superfused with ACSF at 30°C ± 0.5, gassed with the O_2/CO_2 mixture to yield a pH of 7.4. The slices were kept submerged in medium with a metal holder and the medium was perfused continuously from a gravity feed or a pump 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 a solution of 20 μM adenosine in ACSF for orthodromic stimulation or 10 mM in calcium-free or low calcium media for antidromic stimulation. In this set of experiments an attempt was made to maintain the fluid layer covering the slice as thin as possible.

D. RECORDING ELECTRODS

The electrodes for recording were made from borosilicate glass tubing containing a glass capillary fibre for easy filling. The tubing with characteristic 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.

E. 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 2.1.). Pulses of 0.1 ms duration and 100-500 μA amplitude were delivered every one minute. For orthodromic fEPSPs the duration of 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

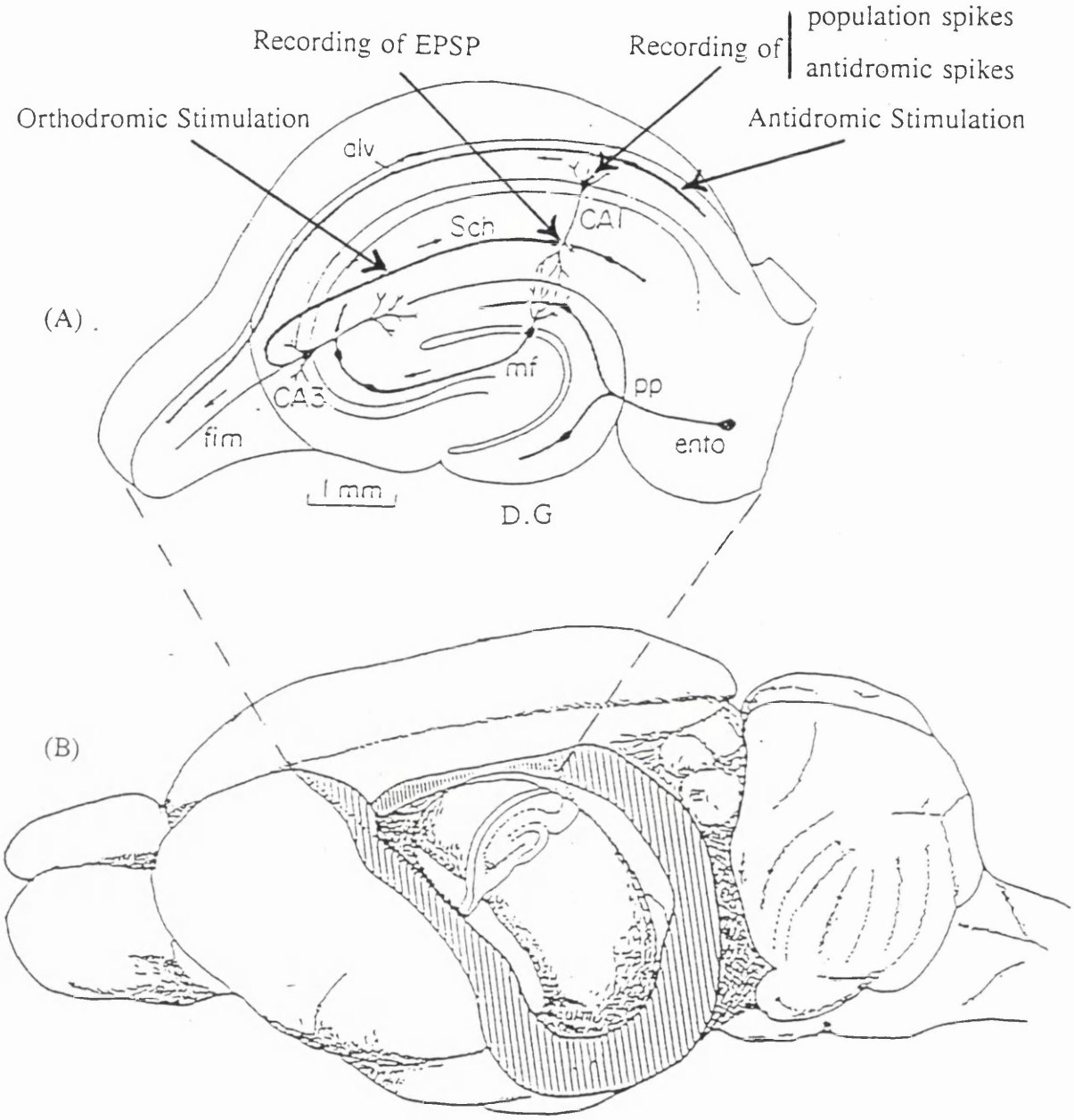

Figure 2.1. Anatomy of the hippocampal formation and the placement of the electrodes. (A). The hippocampal formation is enlarged to show the main neuronal elements of structure. (B). Lateral view of the rat brain with parietal and temporal neocortex to expose the hippocampal formation. After orthodromic 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 (fEPSPs) 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= millimetre. (Revised from Andersen et al., 1971)

Gould storage oscilloscope and then plotted on a thermosensitive chart recorder. Spontaneous activity was recorded on a Grass polygraph (model 79 D).

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

F. ANALYSIS OF DRUG EFFECTS

The size of the population spike following orthodromic stimulation and the first antidromic spike and secondary spike 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.).

The number of bursts of spontaneous activity was counted for 10 minutes and reported as the burst frequency per minute.

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

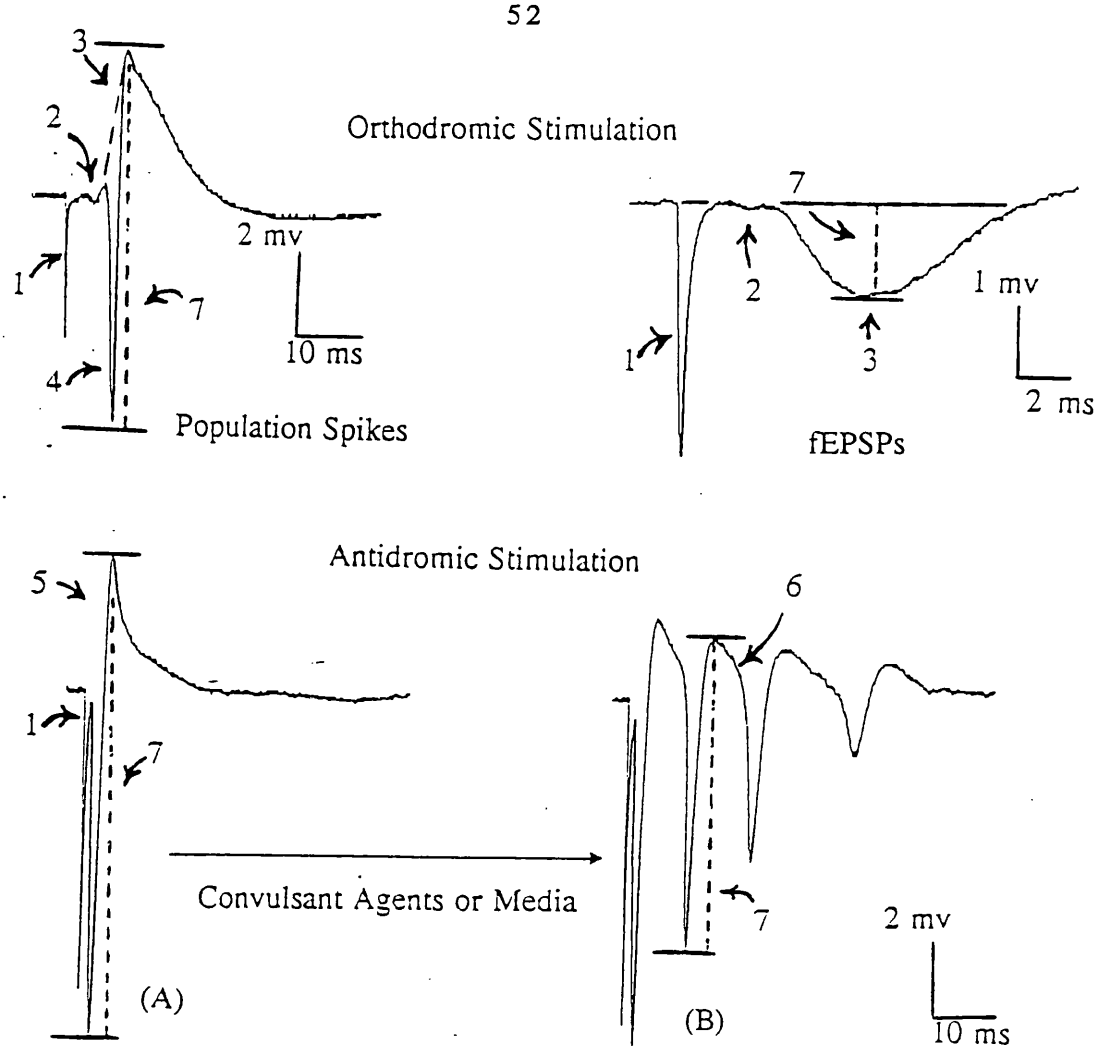

Figure 2.2. Demonstration and measurement of orthodromic and antidromic potentials evoked in the CA1 region. Antidromic potentials are demonstrated in (A) normal ACSF and in (B) the presence of convulsant agents or media. Explanations of each part of the evoked potentials are as follows:
1) Stimulus artifact;
2) Presynaptic fiber volley potential that reflects the compound action potential travelling in the stratum radiatum towards CA1;
3) The population excitatory postsynaptic potential or population EPSP is a slow positive going potential which reflects the depolarization of dendrites by the synaptically released transmitter;
4) The population spike is a negative going spike-like potential which reflects the production of the action potentials 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;
6) Secondary spikes that reflect firing of cell bodies;
7) Amplitude of population spikes, fEPSPs or secondary spikes.

G. 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 or unpaired 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.

H. 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 N^6-cyclopentyladenosine (CPA) which was purchased from Research Biochemicals Inc. (Semat Industries, UK) and levcromakalim (BRL 38227) which was provided from SmithKline Beecham pharmaceuticals. PD81723 was a gift from Parke-Davis.

Dimethyl sulfoxide (DMSO) was used as the vehicle for carbamazepine, CPA, levcromakalim, PD81723, (R-)-N^6-phenylisopropyladenosine (R-PIA), thapsigargin and tolbutamide. Control experiments showed that DMSO itself had no effect on the slice at concentrations up to 0.1%.

8-cyclopentylthyeophylline (CPT) and theophylline were dissolved in sodium hydroxide (1 M). The buffering capacity of the

ACSF ensured that the final pH of the perfusing medium was restored to pH 7.4.

Adenosine deaminase (ADA) from calf intestinal mucosa, type VII, lyophilized powder was used.

In other cases the vehicle was the superfusing medium such as ACSF or ACSF without calcium.

RESULTS

3. RESULTS

SECTION I: COMPARISON OF ADENOSINE ACTIONS ON ORTHODROMIC AND ANTIDROMIC RESPONCES OF CA1 NEURONES

3.1. EFFECT OF CALCIUM ON ADENOSINE INHIBITORY ACTIONS

3.1.1. EFFECT OF ADENOSINE ON ORTHODROMIC POPULATION SPIKES

In normal ACSF (2.5 mM calcium), adenosine concentration-dependently abolished or reduced the amplitude of the population spike (P.S.) of orthodromic CA1 field potentials. A cumulative 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 (figure 3.2.).

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 fiber volley.

The population spikes evoked by orthodromic stimulation were completely abolished by changing from normal ACSF to nominally calcium-free or low calcium (0.24 mM) medium. This process took about 10 minutes. The presynaptic volley did not change in calcium-free or low calcium medium (n=3).

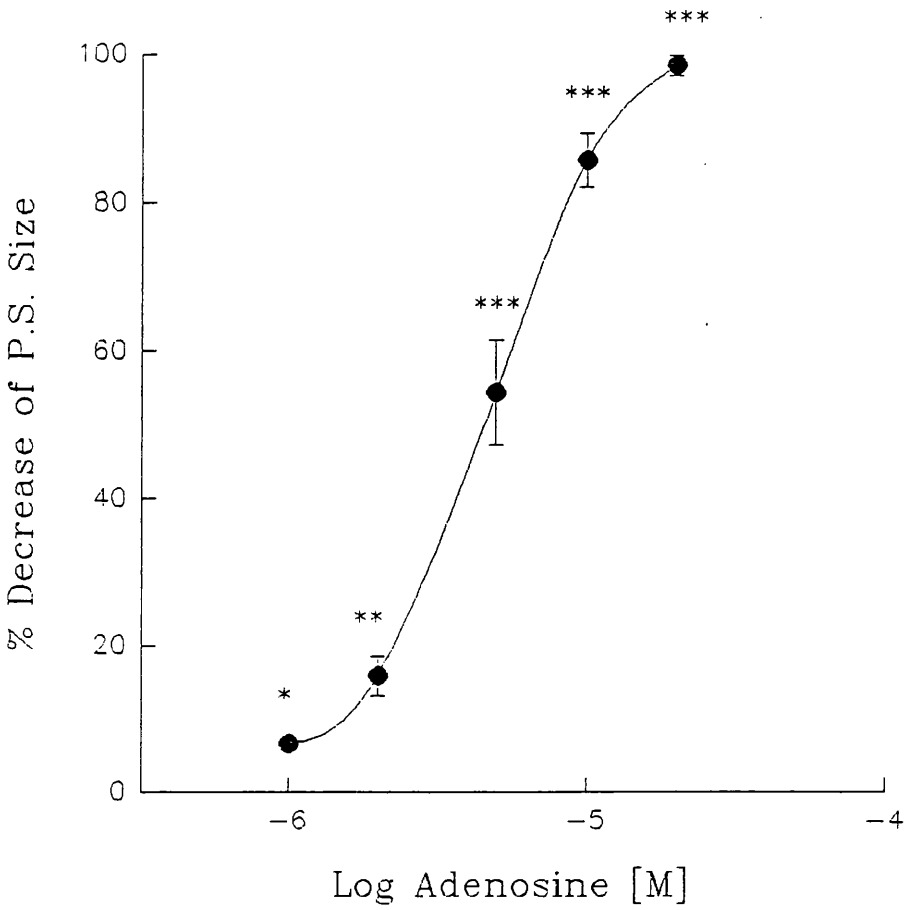

Figure 3.1. Cumulative concentration-response curve for the depression of orthodromically evoked CA1 population spikes (P.S.) by adenosine. Each point represents the mean ± s.e.m. for n=6 experiments. A paired *t* test was employed to determine the significance level(*=P<0.05; **=P<0.01; ***=P<0.001).

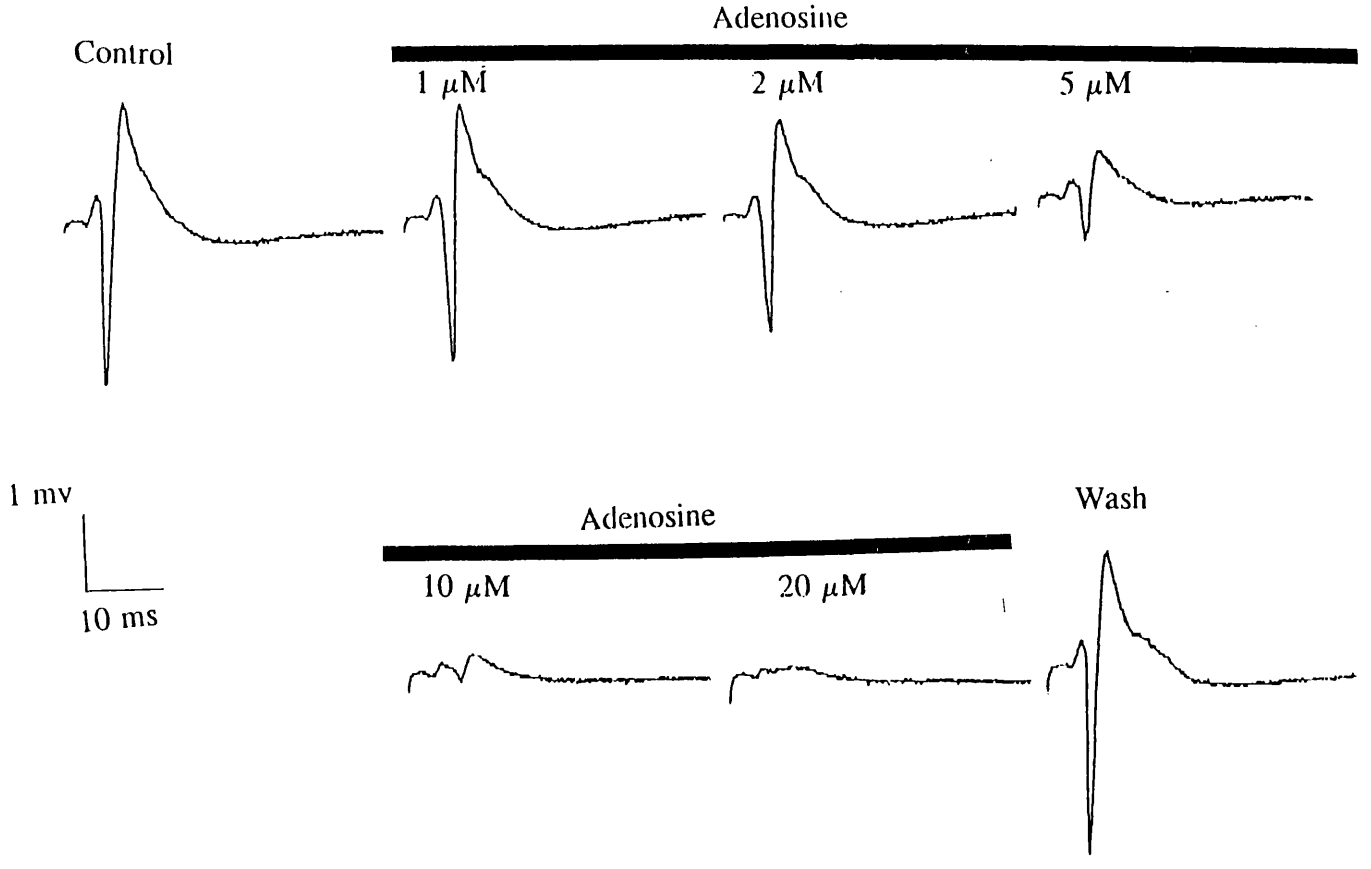

Figure 3.2. Sample records of orthodromic potentials in CA1 before, during and 10 minutes following the cumulative addition of 1-20 μM adenosine. In this and all subsequent records the stimulus artifact is seen at the beginnig of each trace.

3.1.2. EFFECT OF CALCIUM-FREE MEDIUM ON ANTIDROMIC POTENTIAL

In nominally calcium-free medium, bursts of multiple population spikes (secondary spikes) were obtained in response to antidromic stimulation. These became apparent 3-10 minutes after beginning calcium-free perfusion, with stable, maximum amplitude spikes being obtained in less than 30 minutes. These stable bursts usually consisted of 2-3 afterdischarges (see figure 3.6.).

The effect of calcium-free medium on slices was reversible and with reinfusion of normal ACSF medium, antidromically-induced responses fully recovered and epileptiform discharges disappeared in about 5 minutes. Reperfusion of the slice with calcium-free medium then quickly induced bursts and stable bursts appeared in less than 10-15 minutes. This shorthening of burst development latency was also seen in slices superfused with high potassium (8.5 mM in normal ACSF) and washed out for 10 minutes.

In order to ensure testing in the absence of synaptic transmission, drugs were applied to the medium after 45 minutes perfusion with calcium-free medium.

3.1.3. EFFECT OF ADENOSINE ON ANTIDROMIC BURSTS INDUCED IN CALCIUM-FREE MEDIUM

In nominally calcium-free medium, a wide range of adenosine concentration, 0.020-5 mM, was used. Adenosine was perfused for 10 minutes and for each slice only one concentration of adenosine was used to prevent any desensitization.

In contrast to the very sensitive effect of adenosine in reducing orthodromic population spike size in normal ACSF, adenosine was almost ineffective in suppression of epileptiform burst discharges, sensitivity almost being abolished in calcium-free media except for varible and small responses at high concentrations (figure 3.3.). Adenosine had no significant effect on the first antidromic spike amplitude.

To find the reason for the loss of adenosine postsynaptic sensitivity in calcium-free medium different posssibilities were considered such as:

1) Decrease of receptor binding of adenosine,
2) Increase of metabolism and uptake of adenosine,
3) Increase of overall excitability,
4) Loss of ionic conductances,
5) Interaction between A_2 and A_1 receptors
6) Desensitization to adenosine

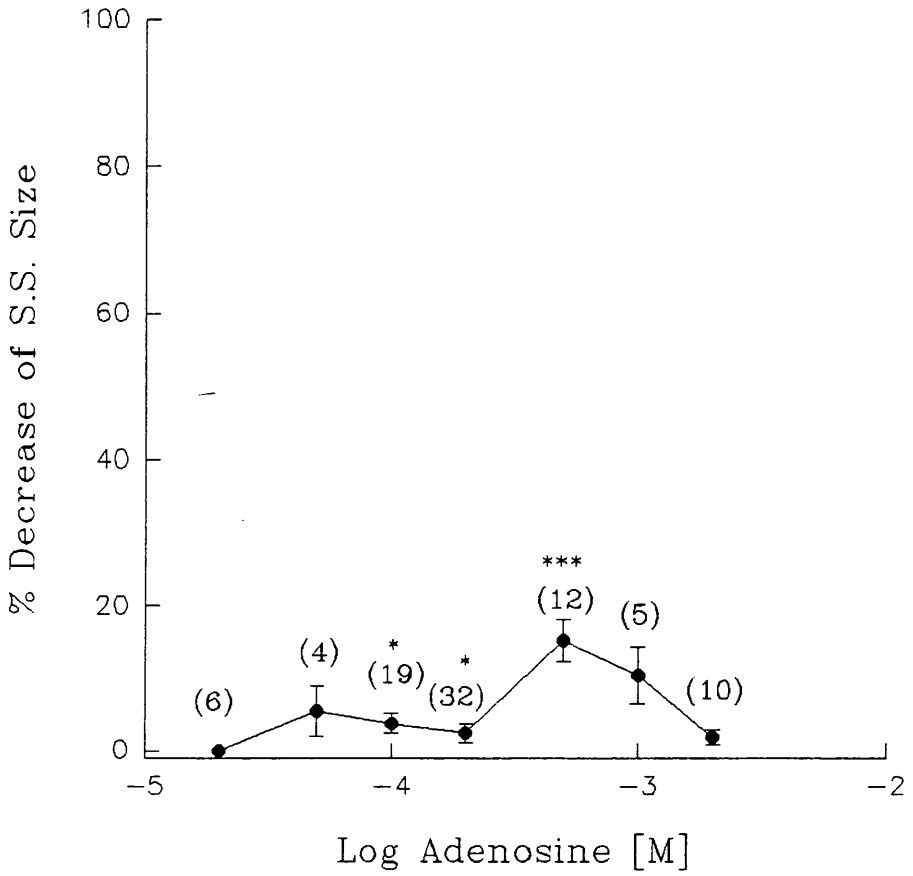

Figure 3.3. Concentration-response curve for the depression of antidromically evoked CA1 secondary spikes (S.S) by adenosine in calcium free medium. Each point represents the mean ± s.e.m. for n as mentioned in the parenthesis. A paired Student's *t* test was employed to determine the significance level (*=P<0.05; ***=P<0.001).

3.1.4. EFFECT OF ADENOSINE ON ANTIDROMIC BURSTS INDUCED IN LOW CALCIUM MEDIUM (0.24 mM)

In low calcium medium (0.24 mM), a secondary spike developed in response to the antidromic stimulus within 5-15 minutes. The stable burst took more than 30 minutes to develop in some slices. In low calcium more secondary spikes (3-6) apeared than in calcium-free medium.

Changing a calcium-free medium to a low calcium medium initially caused a decrease of secondary spike size but after about 20 minutes the amplitude returned to that in calcium-free medium (n=3). The effect of low calcium on the slices was reversible and following reinfusion of normal calcium medium, antidromically elicited responses recovered fully.

Raising the concentration of calcium to 0.24 mM also increased the sensitivity to adenosine concentration-dependently (figure 3.4.). For example, adenosine at 100 μM reduced the secondary spike size by 55.02 ± 3.17% (n=6, $P<0.001$).

Because of desensitization to adenosine in low calcium media, each concentration of adenosine was tested on only one slice. This phenomenon will be explained more in section 3.1.6.

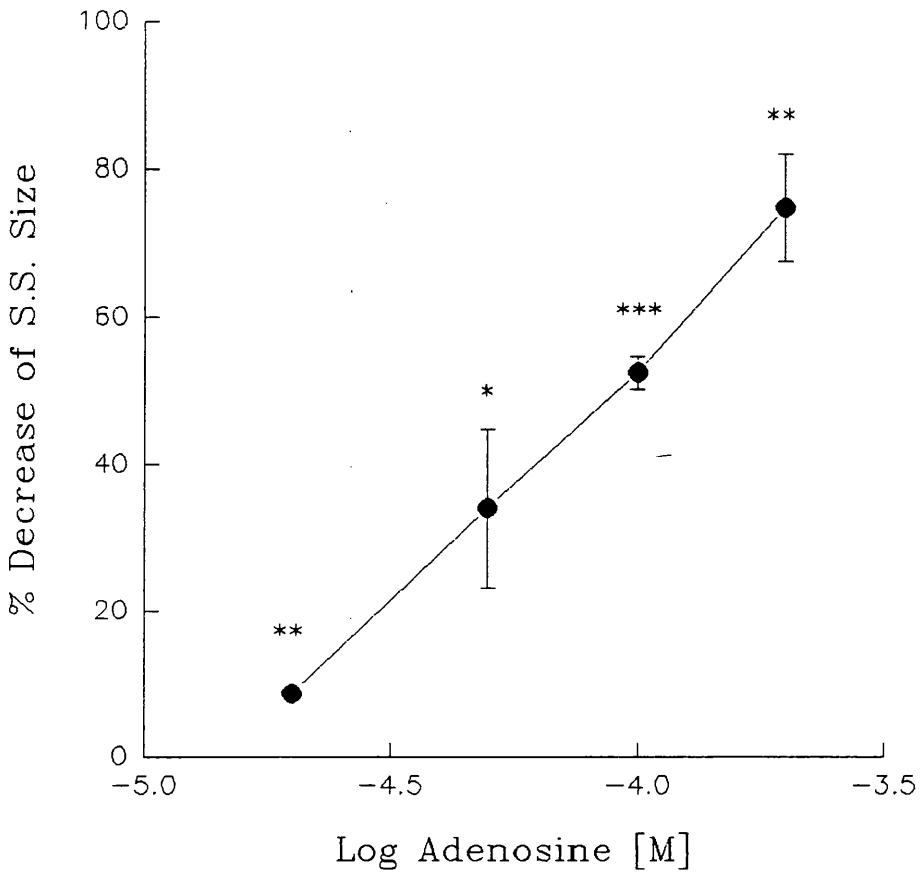

Figure 3.4. Concentration-response curve for the effect of adenosine on antidromically induced secondary spike (S.S.) size in low calcium media (0.24 mM). Each point represents the mean ± s.e.m. for n≥3 . A paired *t* test was employed to determine the significance level(*=P<0.05; **=P<0.01; ***=P<0.001).

3.1.5. EFFECT OF ADENOSINE ON ANTIDROMIC BURSTS INDUCED IN CALCIUM-FREE MEDIUM WITH ADDED MAGNESIUM (0.8 OR 2.8 mM)

As in calcium-free and low calcium solutions, bursts of action potentials appeared in calcium-free media in the presence of added 0.8 or 2.8 mM magnesium (total of 2 and 4 mM respectively), although in this medium more time was needed to reveal the first secondary spike. In calcium-free medium plus 4 mM magnesium in some slices it took 60 minutes for the first secondary spike to appear and more than 90-120 minutes for stable bursts to develop.

After switching from calcium-free medium to the medium with total 2 mM magnesium the first secondary spike size decreased (n=4), although after 20-30 minutes no significant difference between amplitude in the two media was seen. In two slices the secondary spikes even increased in size (30.77% and 17.65%).

Adding 2.8 mM magnesium to calcium-free medium in which antidromic bursts were established compeletely abolished secondary spikes at first, but after about 45 minutes more a secondary spike appeared again.

Raising the magnesium concentration by 0.8 mM increased the inhibitory effect of adenosine after 10 minutes superfusion (figure 3.5.). For example, adenosine at 200 μM reduced the secondary spike size by 90.42 ± 5.42% ($P<0.01$, n=4, figures 3.5. and 3.6.).

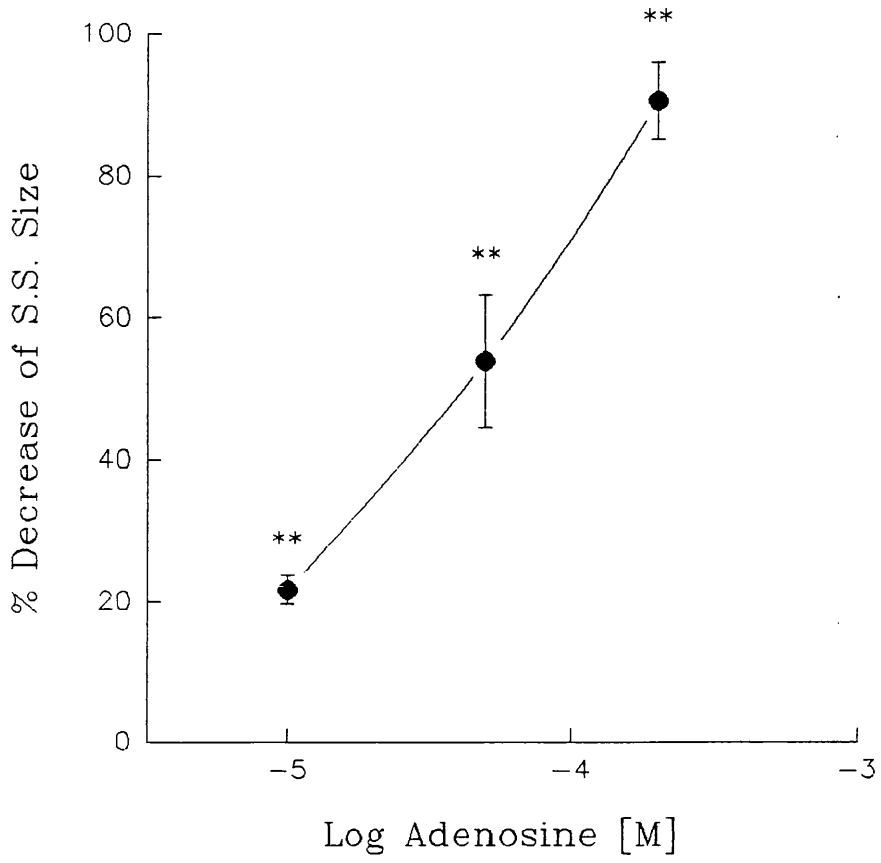

Figure 3.5. Concentration-response curve for the effect of adenosine on antidromically induced secondary spike (S.S.) size in calcium-free media plus 0.8 mM magnesium (total of 2 mM magnesium). Each point represents the mean ± s.e.m. for n≥3. A paired *t* test was employed to determine the significance level (**=P<0.01).

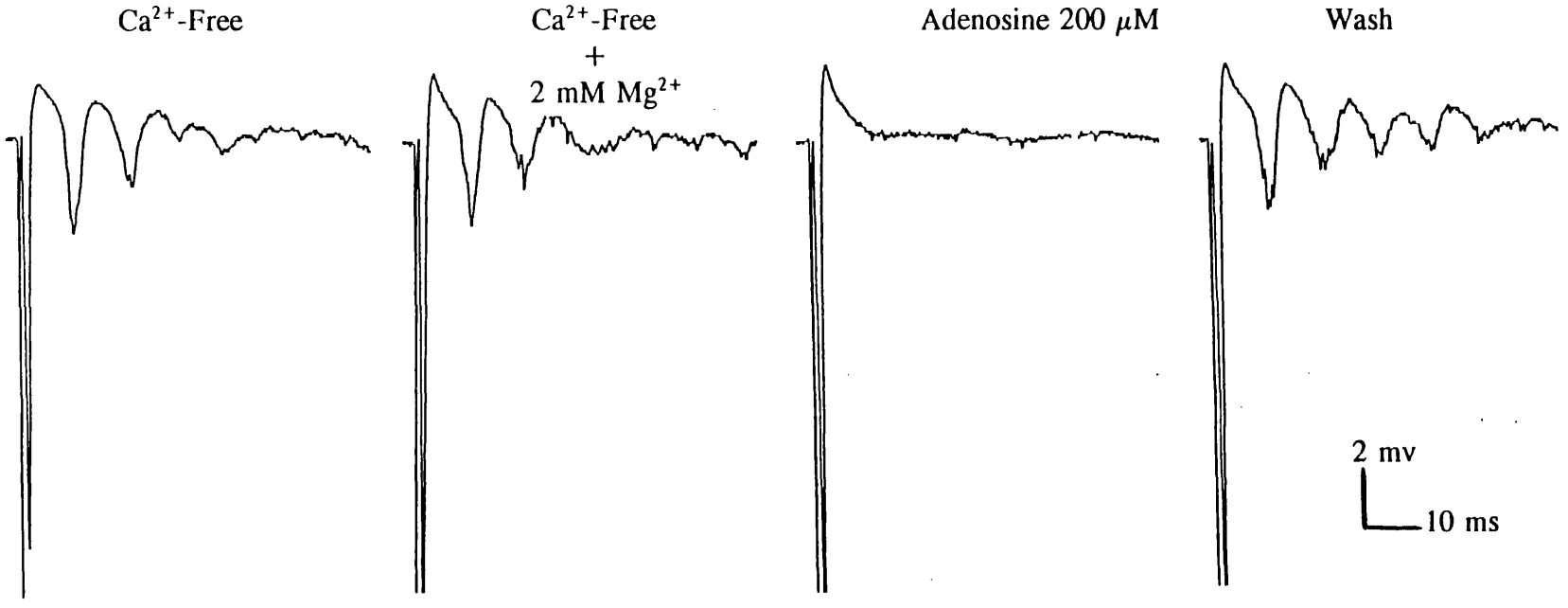

Figure 3.6. Sample records of antidromic potentials in CA1 and calcium-free medium with switching to calcium-free medium plus 2mM magnesium (30 minutes) before, during and 15 minutes following perfusion of 200 μM adenosine.

Adenosine concentration-dependently and effectively decreased or abolished secondary spikes in calcium-free high magnesium (4mM) (figure 3.7.). 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 (4 mM magnesium).

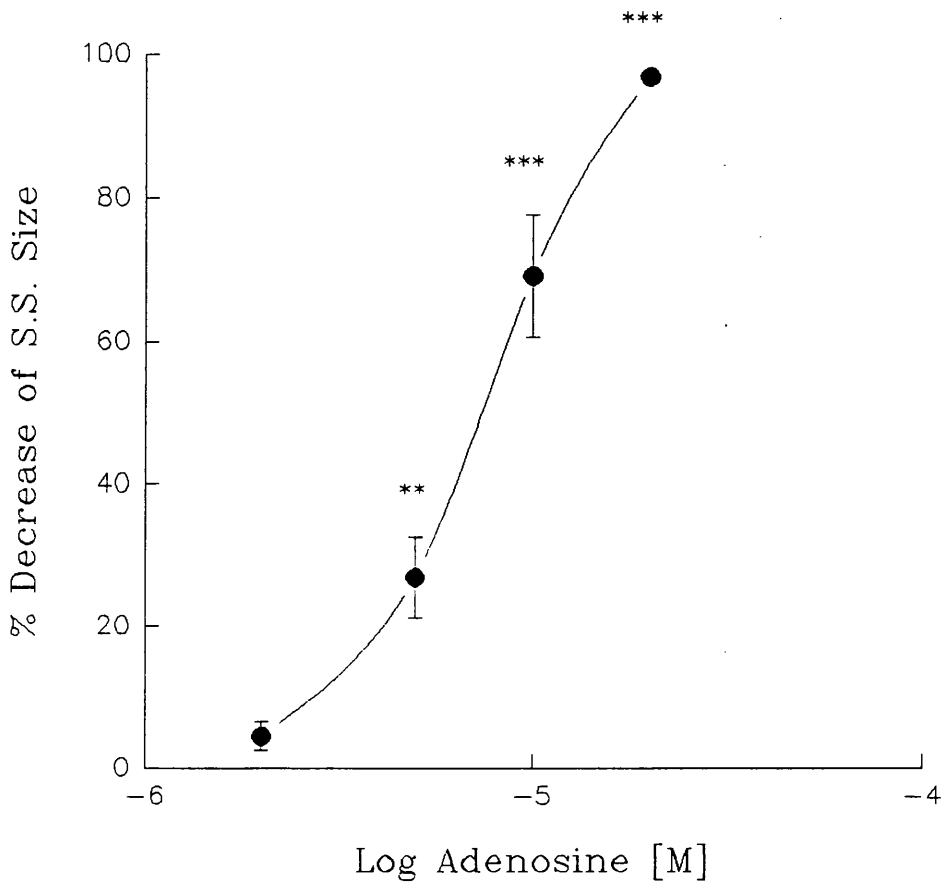

Figure 3.7. Cumulative concentration-response curve for the effect of adenosine on antidromically induced secondary spikes (S.S.) in calcium-free media containing 4 mM magnesium. Each point represents the mean ± s.e.m. for n=8. A paired *t* test was employed to determine the significance level (**=P<0.01; ***=P<0.001).

3.1.6. DESENSITIZATION TO ADENOSINE IN LOW CALCIUM MEDIUM

Raising the divalent cation concentrations in calcium-free medium increased adenosine postsynaptic sensitivity as just described. This was accompanied by desensitization to adenosine (figure 3.8.).

Desensitization was normally apparent after the previous exposure of slices to several application of adenosine, although exposure to low concentrations was sufficient to enable subsequent desensitization to higher concentrations. For example exposure of naive slices to 100 μM adenosine caused inhibition of secondary antidromic spikes by 55.02 ± 3.17% (n=6) after 10 min. If slices were previously exposed for periods of 10 minutes to 20 μM and 50 μM adenosine with washing between each period to regain control response size, then the inhibition by 100 μM adenosine was reduced to 34.9 ± 1.63% ($P<0.001$, n=4). Similarly, responses to 2 mM adenosine were reduced from 92.19 ± 3.62% in naive slices to 33.75 ± 2.30% following pretreatment with lower concentrations of adenosine ($P<0.01$, n=3).

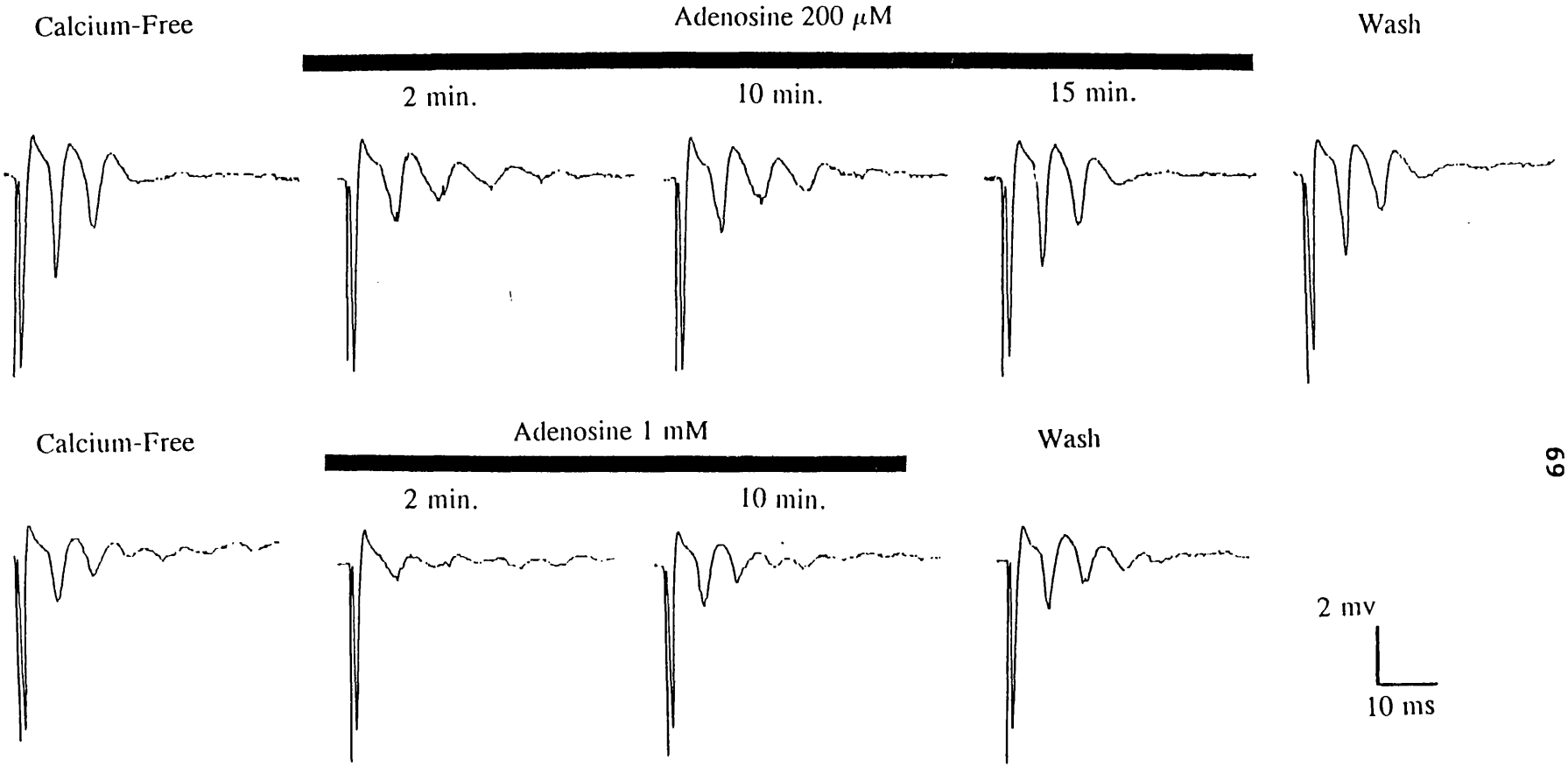

Figure 3.8. Records of evoked burst activity showing desensitization to the depressant effect of adenosine at 200 μM and 1 mM in 2 different slices perfused with low calcium medium. Adenosine 500 μM and repetitive concentrations of 20, 50, 100 μM adenosine perfused before perfusion of adenosine 1 mM and 200 μM respectively (not shown).

3.1.7. EFFECT OF SOMATIC OR DENDRITIC APPLICATION OF ADENOSINE ON ANTIDROMIC BURSTS INDUCED IN CALCIUM-FREE OR LOW CALCIUM MEDIUM

To assess whether desensitization to adenosine was occurring at the somatic or dendritic sites, adenosine was applied locally in these regions.

When applied locally from micropipettes containing 10 mM adenosine, the purine was not able to depress antidromically induced bursts in calcium-free medium, whether applied into the somatic or dendritic regions. However, in low calcium media adenosine did decrease the secondary spikes when applied either to the cell bodies or dendrites (figure 3.9.). The effect of adenosine was desensitized after second and third applications to the dendritic site (table 3.1.).

Table 3.1. Effect of somatic or dendritic adenosine on antidromic secondary spikes in low calcium media.

	Somatic	Dendritic
First application	30.60 ± 5.27%	40.22 ± 7.03 %
Second application	32.35 ± 4.79	24.52 ± 7.58‡
Third application	30.27 ± 5.25	20.50 ± 9.11‡
n=	(6)	(6)

‡Significantly different from first application, $P<0.05$, ANOVA and Tuky-Kramer post test.

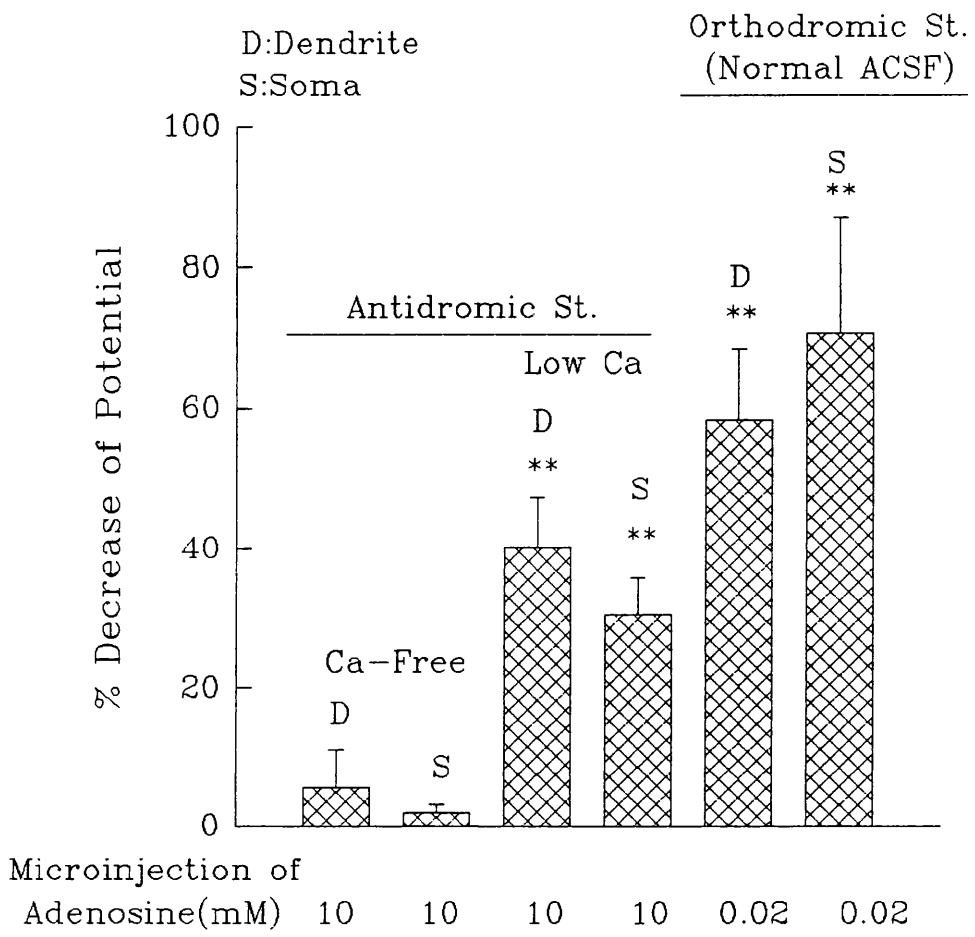

Figure 3.9. Histogram showing the effect of somatic or dendritic local microinjection of adenosine on orthodromic population spikes in normal ACSF or antidromic secondary spikes in calcium-free or low calcium (0.24 mM) medium. Each vertical bar represents the mean ± s.e.m. for n≥3. A paired t test was employed to determine the significance level(**=P<0.01).

3.1.8. EFFECT OF SOMATIC OR DENDRITIC APPLICATION OF ADENOSINE ON ORTHODROMIC POPULATION SPIKES

In normal ACSF, when applied locally, adenosine was effective in reducing orthodromic potentials either at the soma region, 70.6% ± 16.4 inhibition ($P<0.01$, $n=3$) or in the dendritic tree, 58.2% ± 10.1 ($P<0.01$, $n=5$, figure 3.9.). For these effects the concentration of adenosine in the microinjection pipette was reduced to 20 μM.

3.1.9. PROLONGED SUPERFUSION OF ADENOSINE IN NORMAL ACSF

After perfusion of adenosine 10 μM for 10 minutes the orthodromic population spikes wre reduced by 39.71% ± 5.62 ($n=4$, $P<0.01$). No sign of desensitization was seen after superfusing adenosine for 60 minutes in normal ACSF (figure 3.10.).

At high concentration, adenosine (2 mM) completely abolished orthodromic population spikes without any significant effect on the presynaptic volley ($n=3$). After 60 minutes there was no sign of recovery of population spikes. Washout of the high concentration of adenosine took more than 30 minutes in order to restore the field potential.

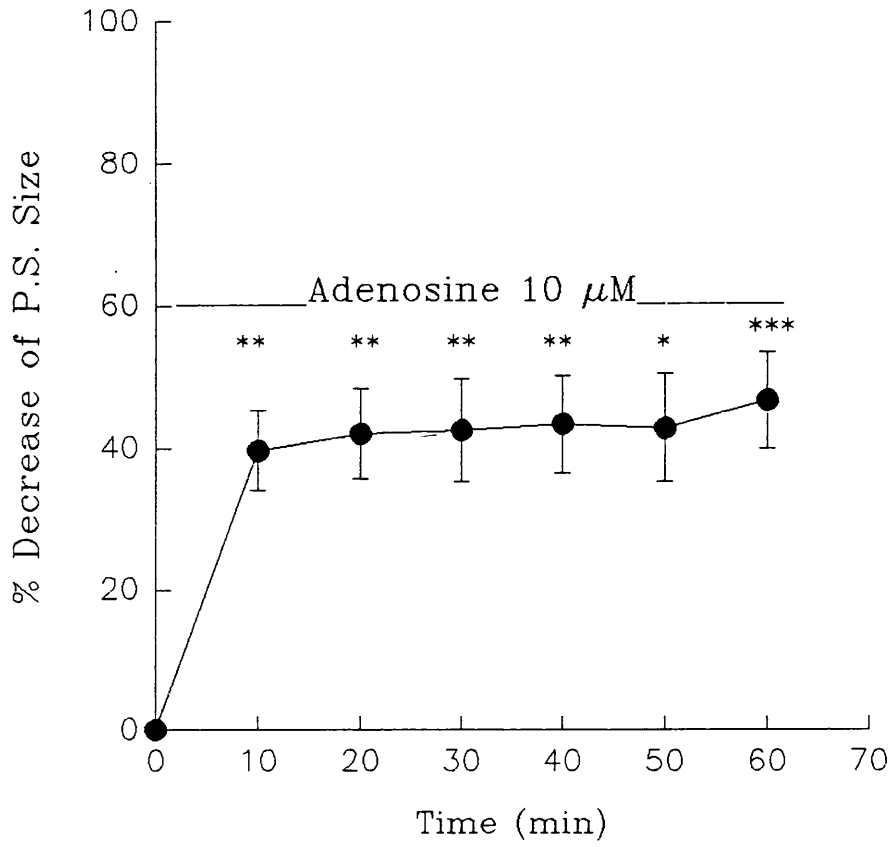

Figure 3.10. Prolonged effect of adenosine 10 μM on orthodromic population spikes (P.S.). Responses to adenosine did not desensitize during 60 minutes superfusion in normal ACSF. Each point represents the mean ± s.e.m. for n=4. A one-way analysis of variance (ANOVA) with post Tukey-Kramer multiple comparisons test was employed to determine the significance level (*=P<0.05; **=P<0.01; ***=P<0.001).

3.1.10. EFFECT OF PD81723, AN ALLOSTERIC ADENOSINE BINDING ENHANCER, ON THE INHIBITORY EFFECT OF ADENOSINE ON ORTHODROMIC POPULATION SPIKES

Bath application of PD81723 10 and 30 μM for 30 minutes alone had no significant effect on orthodromic population spikes in separate slices. At a concentration of 30 μM, PD81723 significantly increased the inhibitory effect of adenosine 5 μM (figure 3.11.).

3.1.11. INTERACTION OF PD81723 AND ADENOSINE ON ANTIDROMIC BURSTS INDUCED IN CALCIUM-FREE MEDIUM

In calcium-free media PD81723 10 and 30 μM had no significant effect on burst activity in separate slices. This agent also did not enhance activity of adenosine 100 μM in calcium-free media (figure 3.12.).

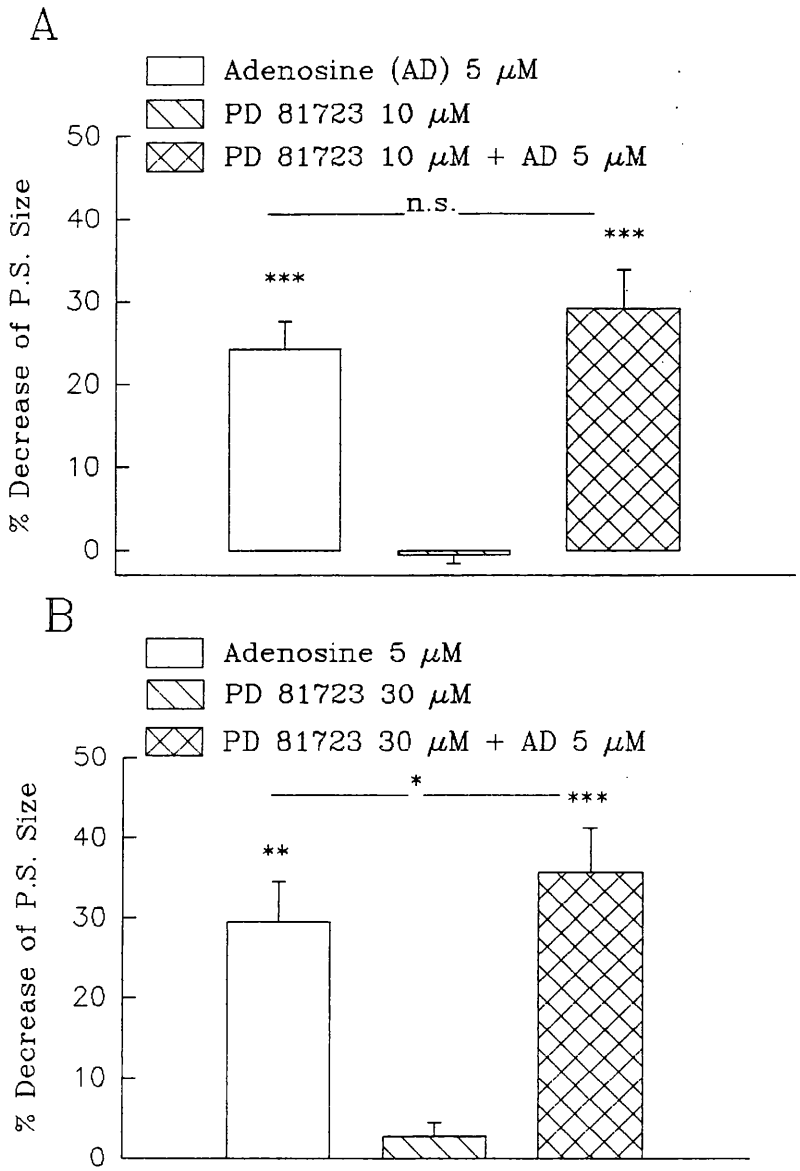

Figure 3.11. Histograms showing interaction of adenosine and PD81723 A) 10 μM B)30 μM on orthodromically evoked CA1 population spikes in normal ACSF. Each vertical bar represents the mean ± s.e.m. for n=8 and 5 for graphs A and B respectively. A paired *t* test was employed to determine the significance level (*=P<0.05; **=P<0.01; ***=P<0.001).

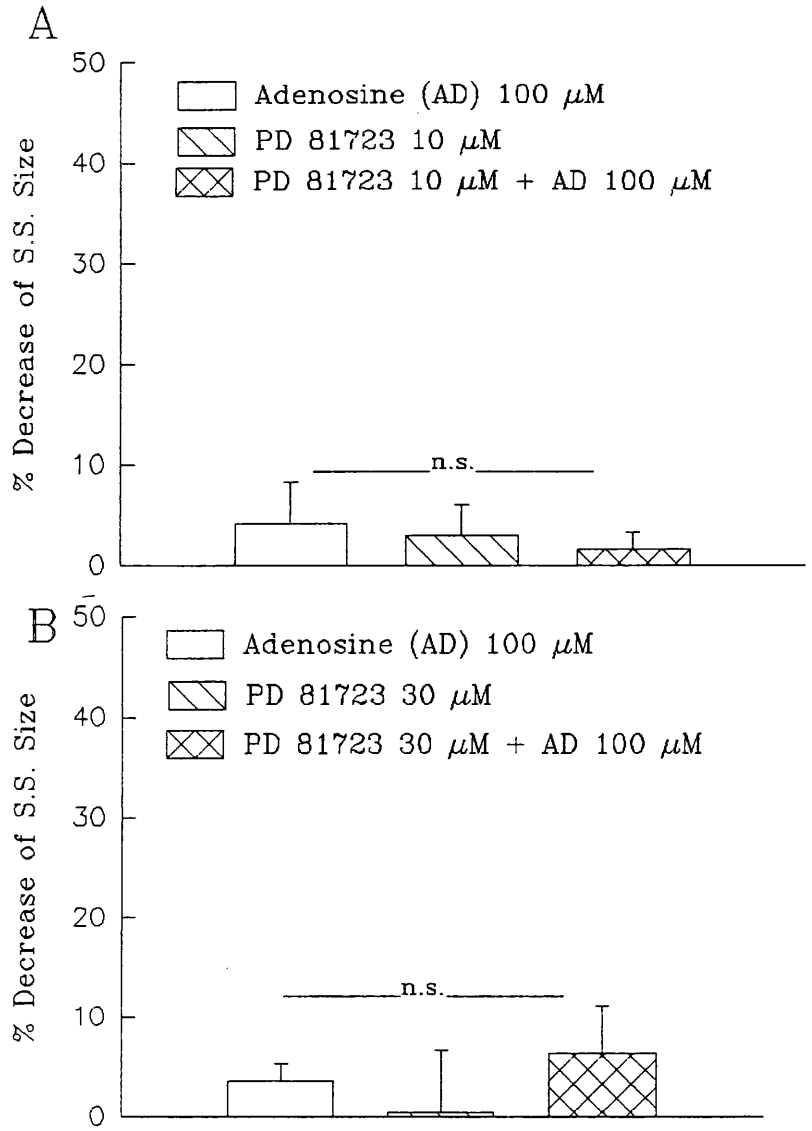

Figure 3.12. Histograms showing interaction of adenosine and PD81723 A) 10 μM B)30 μM on antidromically evoked CA1 secondary spikes in calcium-free medium. Each vertical bar represents the mean ± s.e.m. for n=3 and 5 for graphs A and B respectively. A paired *t* test was employed to determine the significance level. n.s.=non-significant.

3.1.12. ROLE OF HYPEREXCITABILITY IN CALCIUM FREE-MEDIUM ON THE LOSS OF ADENOSINE SENSITIVITY

Since the removal of calcium increased excitability of cells and induced epileptiform activity, then one possibility to be considered was that adenosine lost its efficacy as a result of the overall increase in excitability. To test this the following experiments were done to decrease the excitability:

1) Decrease of evoked secondary spike size more than fifty percent,
2) Study of the postsynaptic effect of adenosine in another model of in vitro bursting,
3) Decrease the excitability using baclofen,
4) Using isolated CA1 to prevent any spontaneous activity.

3.1.12.1. EFFECT OF STIMULUS STRENGTH ON THE LOSS OF ADENOSINE SENSITIVITY IN CALCIUM-FREE MEDIUM

In calcium-free medium, adenosine 200 μM still had no significant effect even when stimulus strength was reduced so that the amplitude of the control secondary spike was less than 50% of its maximum (n=3). As mentioned in Methods, stimulus strength was reduced in all experiments to submaximal levels so as to yield a spike size of approximately 75% of maximum.

3.1.12.2 EFFECT OF ADENOSINE ON ANTIDROMIC BURSTS INDUCED IN HIGH POTASSIUM MEDIUM

With antidromic stimulation in the presence of high potassium (8.5 mM) and normal calcium media, the bursts began to develop within about 15 minutes and became stable after more than 30 minutes. Adenosine decreased the secondary spikes concentration-dependently and reversibly (figure 3.13.).

In high potassium media, a release of excitatory amino acid occurs, and adenosine may suppress this kind of burst by reducing excitatory amino acid release presynaptically. For this reason kynurenic acid, an antagonist of excitatory amino acids, was used.

Kynurenic acid, 1 mM, abolished the antidromic secondary spikes in less than 3 minutes in the high potassium medium (n=3). Raising potassium levels to further induce bursts by direct depolarization of the cells was also unsuccessful. Kynurenic acid 1 mM still suppress the burst at higher concentration of potassium, 10 mM. At the concentration of 12 and 17 mM potassium alone or with kynurenic acid the first and secondary spikes abolished reversibly in about 2 minutes and did not recover for 30 minutes superfusion of high potassium.

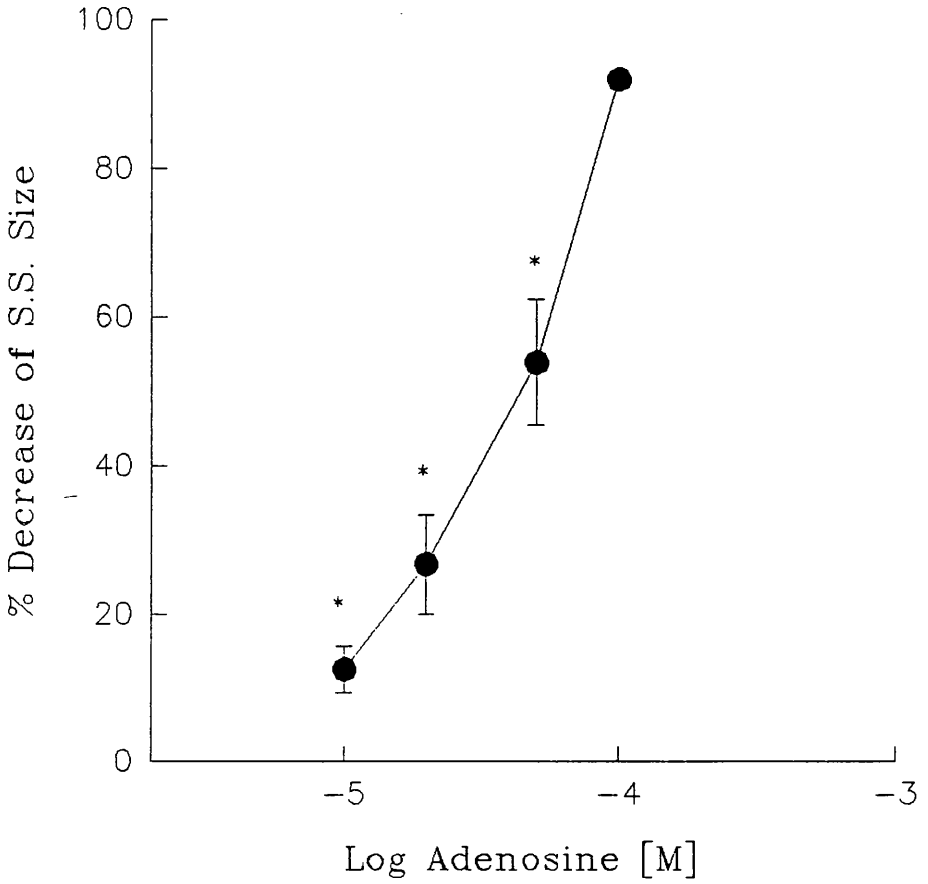

Figure 3.13. Cumulative concentration-response curve for the depression of antidromically evoked CA1 secondary spikes (S.S.) by adenosine in high potassium (8.5 mM) medium. Each point represents the mean ± s.e.m. for n=3 experiments except for adenosine 100 μM the mean for n=2. A paired t test was employed to determine the significance level(*=P<0.05).

3.1.12.3 DEPOLARIZATION OF PYRAMIDAL CELLS BY VERATRIDINE

The use of veratridine, a sodium channel activator, for depolarization of pyramidal cells was also unsuccessful. In fact this agent time and concentration dependently abolished the first spikes. At 1 and 5 μM veratridine abolished the antidromic first spike in normal ACSF after about 30 and 15 minutes respectively. Washout of veratridine took a long time but at 30 minutes partial recovery was seen.

3.1.12.4. INHIBITORY EFFECT OF BACLOFEN

3.1.12.4.1. EFFECT OF BACLOFEN ON ORTHODROMIC POPULATION SPIKES

Baclofen concentration-dependently decreased the size of population spikes evoked in the CA1 area by orthodromic stimulation (figure 3.14.). The maximum effect was seen at 5 μM which abolished the potential completely (n=3). 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.

When a series of different concentrations of baclofen was used on the same slice, full recovery from the depressant effect of baclofen took more than 15 minutes and in three slices about 30 minutes.

Baclofen, like adenosine, had no effect on the presynaptic fiber volley and no desensitization was seen with cumulative application of different baclofen concentrations.

The potency of baclofen on reduction of orthodromic potentials was much greater than adenosine. Comparing the effect of three concentrations of 1, 2, 5 μM these two inhibitors were significantly different at the level of $P<0.001$ (n=6, unpaired Student's t-test).

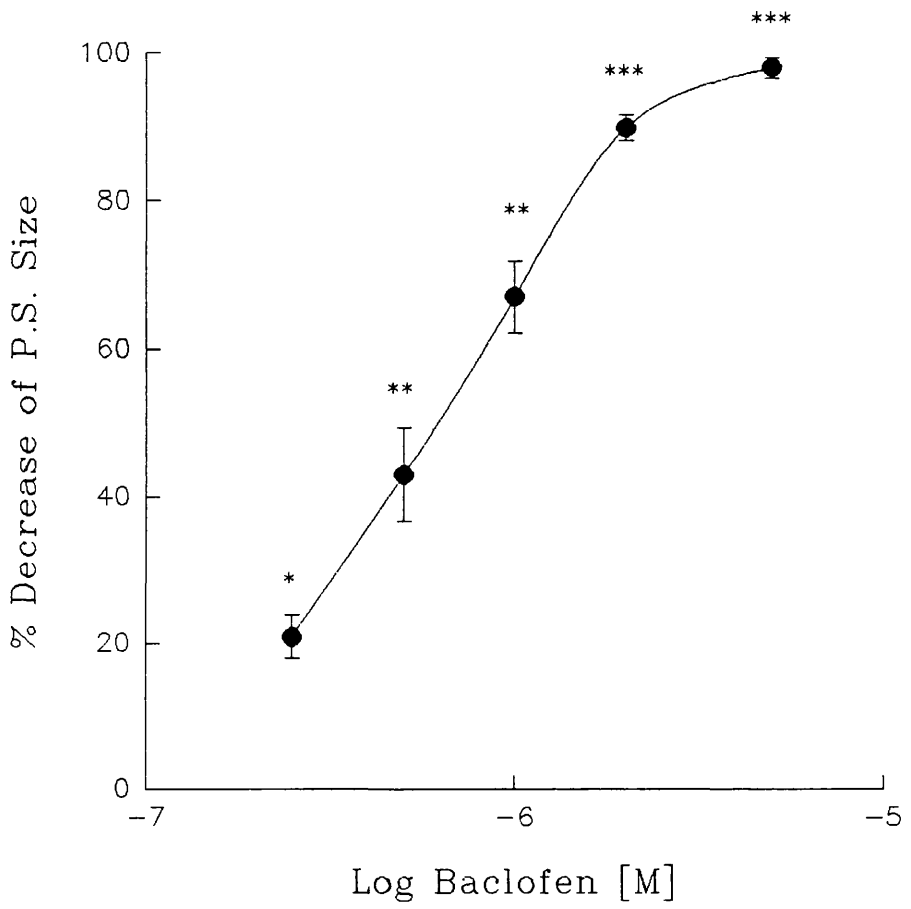

Figure 3.14. Cumulative concentration-response curve for the depression of orthodromically evoked CA1 population spikes (P.S.) by baclofen. Each point represents the mean ± s.e.m. for n=6 experiments. A paired *t* test was employed to determine the significance level(*=P<0.05; **=P<0.01; ***=P<0.001).

3.1.12.4.2. EFFECT OF BACLOFEN IN CALCIUM-FREE MEDIUM

3.1.12.4.2.1. EFFECT OF BACLOFEN ON ANTIDROMIC INDUCED BURSTS IN CALCIUM-FREE MEDIUM

Unlike adenosine, which practically had no effect in calcium-free medium, baclofen continued to reduce secondary spike size in a concentration-dependent fashion even in calcium-free medium (figure 3.15.) although its potency was reduced. For example at 20 μM baclofen reduced the secondary spike amplitude by 48.48% ± 5.65 ($P<0.001$, $n=21$), although it had no effect on the first antidromic spike.

Because desensitization occured to baclofen in some slices, each concentration was tested on one one slice. The maximum effect of baclofen was seen with 500 μM. Washout of this concentration took more than 15 minutes.

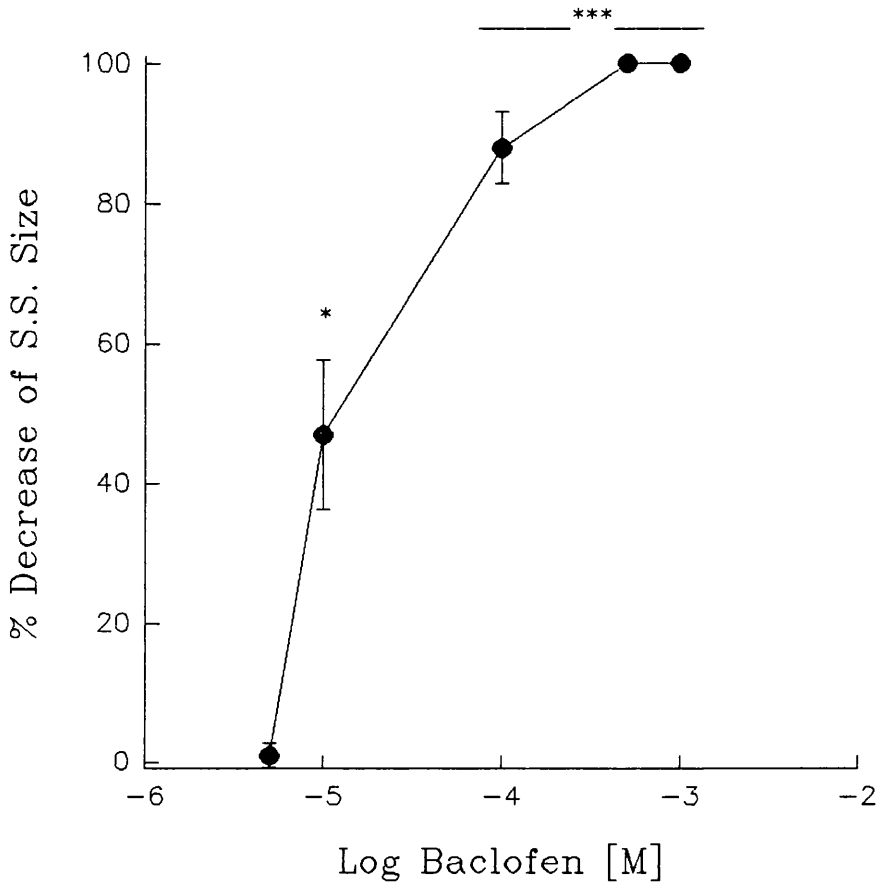

Figure 3.15. Concentration-response curve for the depression of antidromically evoked CA1 secondary spikes (S.S.) by baclofen in calcium free medium. Each point represents the mean ± s.e.m. for n≥3. A paired Student's *t* test was employed to determine the significance level (*=P<0.05; ***=P<0.001).

3.1.12.4.2.2. INTERACTION OF ADENOSINE AND BACLOFEN ON ANTIDROMIC BURSTS INDUCED IN CALCIUM-FREE MEDIUM

In order to study the role of overexcitability in calcium free-medium on the loss of adenosine postsynaptic sensitivity, combination of adenosine and baclofen was used. For each concentration of adenosine only one slice was used.

Adenosine alone at concentrations of 50, 100, 200 μM had no effect on secondary spikes size. Even in the presence of baclofen, 20 μM, which decreasesthe secondary spike about 50%, different concentrations of adenosine up to 200 μm did not decrease the antidromic potential size more than baclofen alone, in other words baclofen did not restore the inhibitory effect of adenosine (figure 3.16.).

In four experiments, responses to baclofen showed evidence of desensitization, and were excluded from the results.

After the presence of adenosine, 2 mM, for 10 minutes (which had no effect on secondary spikes) the addition of a just-maximal concentration of baclofen (500 μM) was still able to abolish the secondary evoked potential with no recovery during 30 minutes superfusion (n=4).

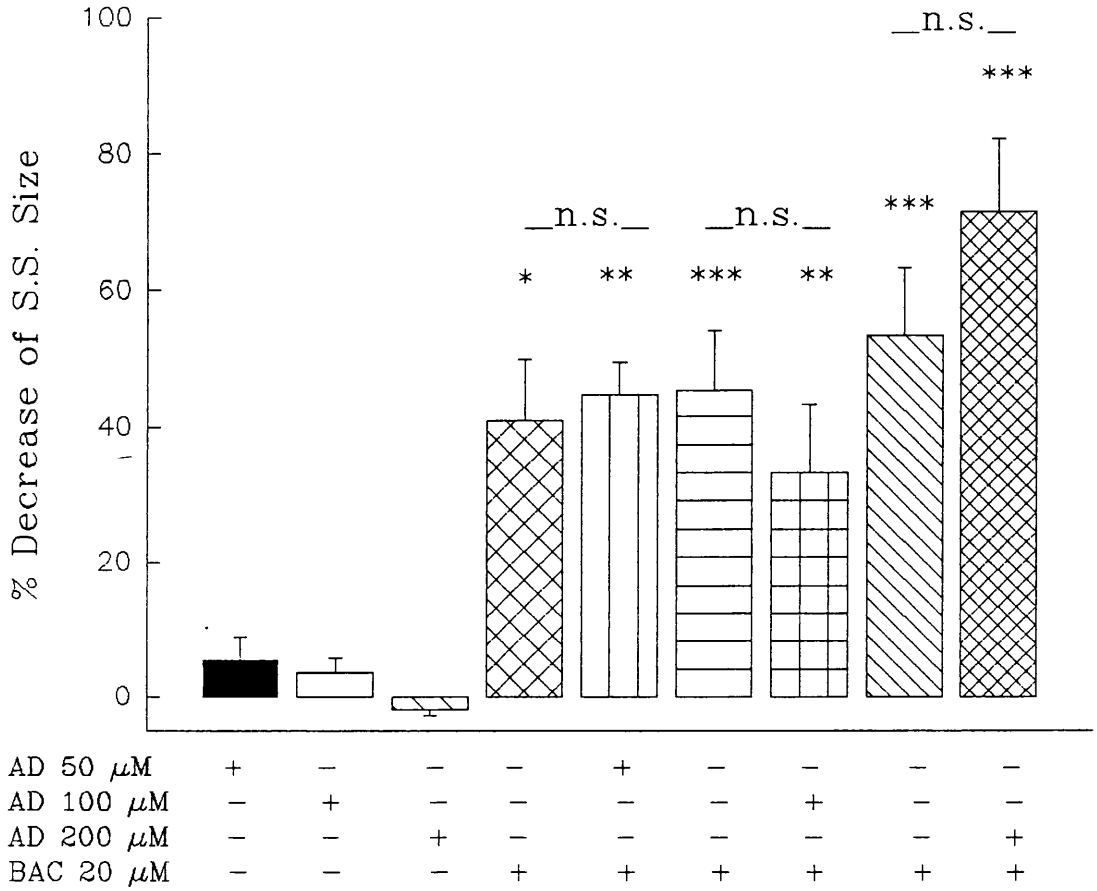

Figure 3.16. Histogram showing effect of adenosine and baclofen on antidromically evoked CA1 secondary spikes (S.S.) in calcium-free medium. Each vertical bar represents the mean ± s.e.m. for n=4-10. A paired *t* test was employed to determine the significance level (*=P<0.05; **=P<0.01; ***=P<0.001). n.s.=non-significant.

3.1.12.5. EFFECT OF ADENOSINE ON ISOLATED CA1 ANTIDROMIC BURSTS IN CALCIUM-FREE MEDIA

Although in calcium-free media, no spontaneous "epileptiform" activity was noted, the possibility was considered that small amplitude spontaneous activity could have contributed to the loss of adenosine sensitivity in calcium-free media. Experiments were therefore performed in which the connection between CA1 and CA3 was cut using a surgical blade.

In these isolated CA1 slices, adenosine 200 μM still had no significant effect on secondary spikes size (n=4, 2.79% ± 2.16, P=0.286, n.s.).

3.1.13. INHIBITORY EFFECT OF KYNURENIC ACID

3.1.13.1. EFFECT OF KYNURENIC ACID ON ORTHODROMIC POPULATION SPIKES

In normal ACSF, kynurenic acid, which blocks excitatory amino acid receptors, reduced or abolished, concentration-dependently orthodromic population spikes evoked in CA1 (figure 3.17.). Kynurenic acid was applied for 10 minutes. Onset of the effect and washout of kynurenic acid took about 5 minutes.

3.1.13.2. EFFECT OF KYNURENIC ACID ON ANTIDROMIC BURSTS INDUCED IN CALCIUM-FREE MEDIUM

Kynurenic acid at concentrations of 1 and 5 mM had no significant effect on first or secondary spike size in calcium-free or low calcium medium (figure 3.17.).

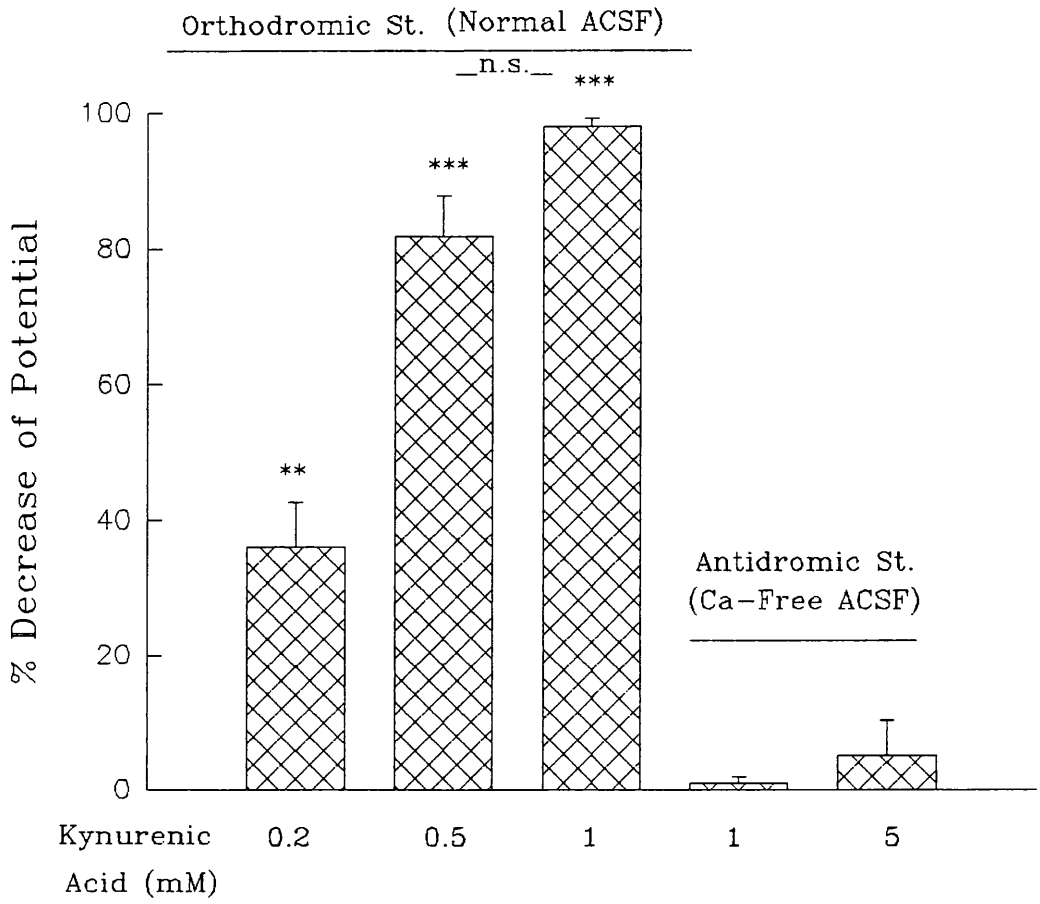

Figure 3.17. Histogram showing the effect of kynurenic acid on orthodromically or antidromically evoked CA1 population spikes or secondary spikes (S.S.) respectively in normal ACSF or calcium-free media. Kynurenic acid failed to decrease S.S. size. Each vertical bar represents the mean ± s.e.m. for n=6 and n=3 with orthodromic or antidromic stimulation respectively. A paired t test was employed to determine the significance level (**=P<0.01; ***=P<0.001). St.=stimulation.

3.1.14. ROLE OF METABOLISM OR UPTAKE OF ADENOSINE IN CALCIUM-FREE MEDIUM ON THE LOSS OF ADENOSINE SENSITIVITY

3.1.14.1. INHIBITORY EFFECT OF 2-CHLOROADENOSINE

3.1.14.1.1. EFFECT OF 2-CHLOROADENOSINE ON ORTHODROMIC POPULATION SPIKES

In normal ACSF, the adenosine analogue, 2-chloroadenosine effectivly decreased or abolished the population spike evoked in CA1 by orthodromic stimulation (figure 3.18.). 2-Chloroadenosine had no effect on the presynaptic volley. The analogue of adenosine was applied for 15 minutes.

Its action was not as quick as adenosine but it washed out in less than 10 minutes.

3.1.14.1.2. EFFECT OF 2-CHLOROADENOSINE ON ANTIDROMIC BURSTS INDUCED IN CALCIUM-FREE MEDIUM

Even at high concentration, 2-Chloroadenosine, which is not degradable or subject to uptake processes, had no significant effect on secondary spikes size evoked in CA1 area in calcium-free medium. It also had no effect on the first antidromic spike (figure 3.18.).

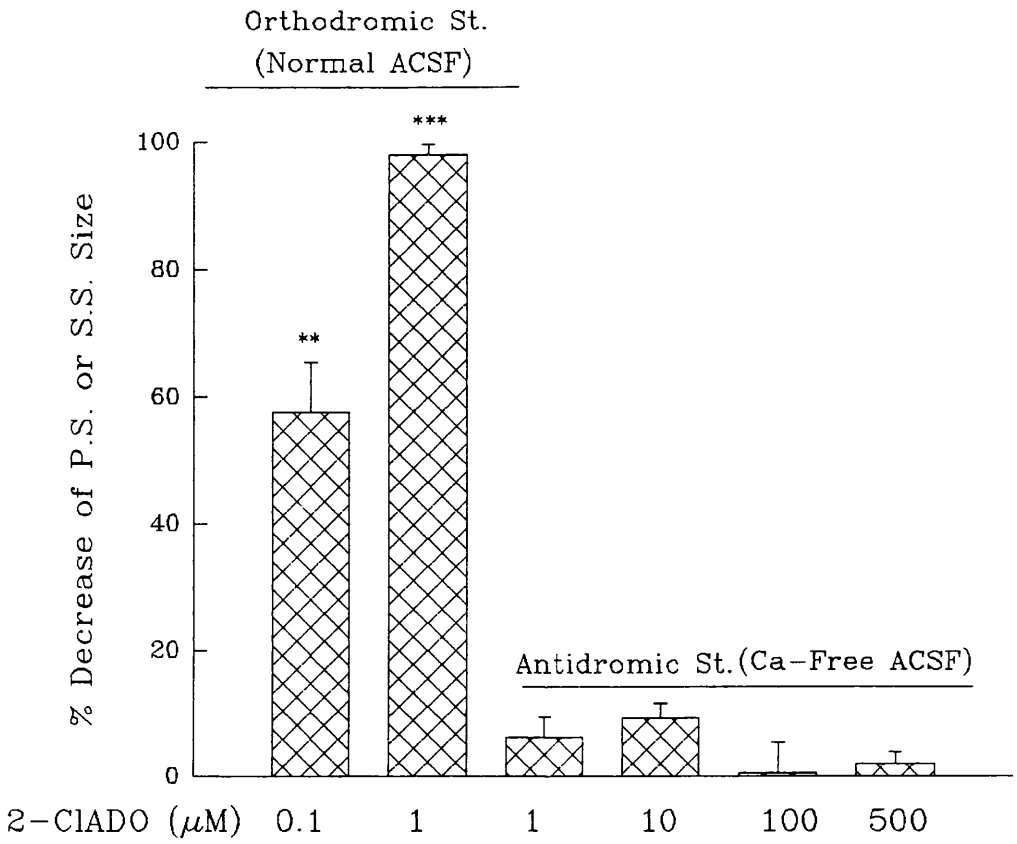

Figure 3.18. Histogram showing effect of 2-chloroadenosine (2ClADO) on orthodromically or antidromically evoked CA1 population spike or secondary spikes respectively in normal ACSF or calcium-free media. Each vertical bar represents the mean ± s.e.m. for n=5 and n=3 with orthodromic or antidromic stimulation respectively. A paired t test was employed to determine the significance level(**=P<0.01; ***=P<0.001). St.=Stimulation.

3.1.14.2. INHIBITORY EFFECT OF (R-)-N^6-Phenylisopropyladenosine (R-PIA)

3.1.14.2.1. EFFECT OF R-PIA ON ORTHODROMIC POPULATION SPIKES

In normal ACSF, R-PIA decreased or abolished the orthodromic population spike evoked in CA1. It had no effect on the presynaptic volley. R-PIA was applied for 50 minutes for 10 nM and 30 minutes for 50 nM. The response onset and washout were slow and concentration dependent (figure 3.19.). The full response to 10 and 50 nM of R-PIA appeared after about 45 and 15 minutes respectively.

3.1.14.2.2. EFFECT OF R-PIA ON ANTIDROMIC BURSTS INDUCED IN CALCIUM-FREE MEDIUM

In calcium-free media, R-PIA with high concentration had no significant effect on first or secondary antidromic spikes (figure 3.19.). R-PIA was applied 30 minutes.

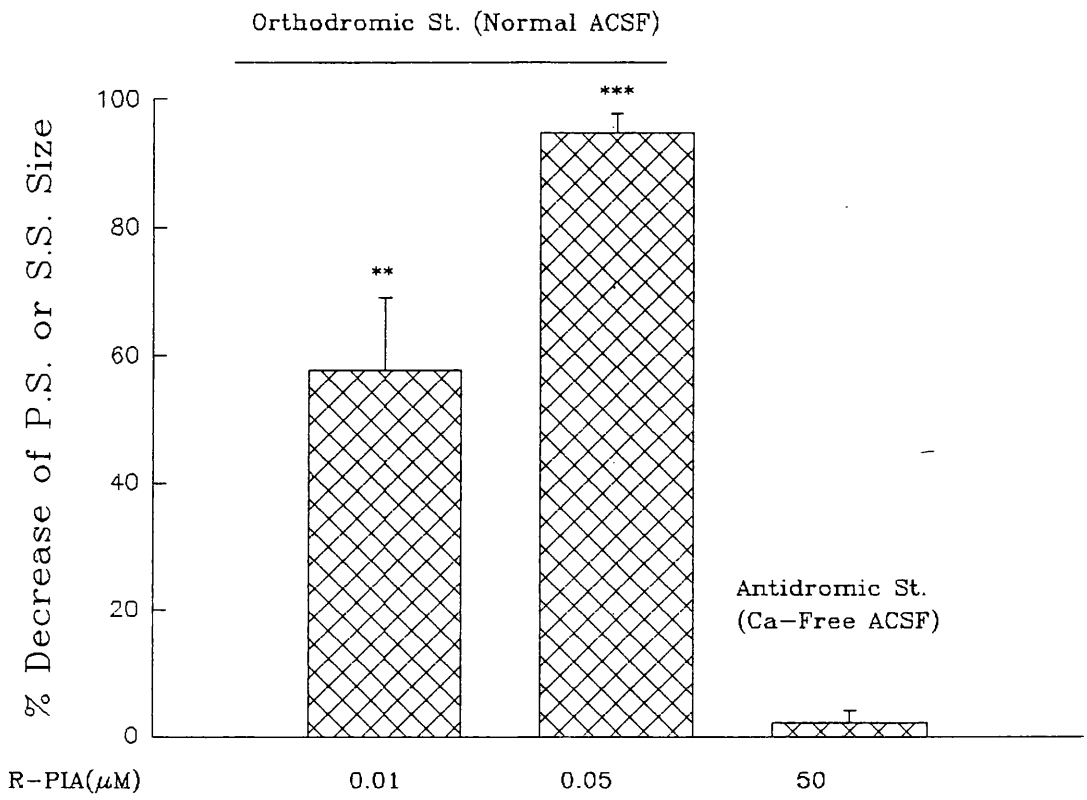

Figure 3.19. Histogram showing effect of (R-)-N-^{6}Phenylisopropyladenosine(R-PIA) on orthodromically or antidromically evoked CA1 population spikes (P.S.) or secondary spikes (S.S.) respectively in normal ACSF or calcium-free media. Each vertical bar represents the mean ± s.e.m. for n=5 and n=3 with orthodromic or antidromic stimulation respectively. A paired t test was employed to determine the significance level(**=P<0.01; ***=P<0.001). St.=stimulation.

3.1.14.3. EFFECT OF S-(2-HYDROXY-5-NITROBENZYL)-6-THIOINOSINE (HNBTI) ON ADENOSINE SENSITIVITY

3.1.14.3.1 EFFECT OF HNBTI ON ORTHODROMIC POPULATION SPIKES

Superfusion of HNBTI 100 μM alone for 30 minutes had no significant effect on orthodromic population spikes. Coadministration of HNBTI 100 μM potentiated the inhibitory effect of adenosine 5 μM (figure 3.20.).

3.1.14.3.2. EFFECT OF S-(2-HYDROXY-5-NITROBENZYL)-6-THIOINOSINE (HNBTI) ON ADENOSINE SENSITIVITY OF ANTIDROMIC BURSTS INDUCED IN CALCIUM-FREE MEDIUM

HNBTI had no significant effect on the bursts when superfused alone for 30 minutes at 100 μM. Similarly HNBTI proved unable to elicit a response when applied together with a high concentration of adenosine (2 mM) in calcium-free medium (figure 3.21.).

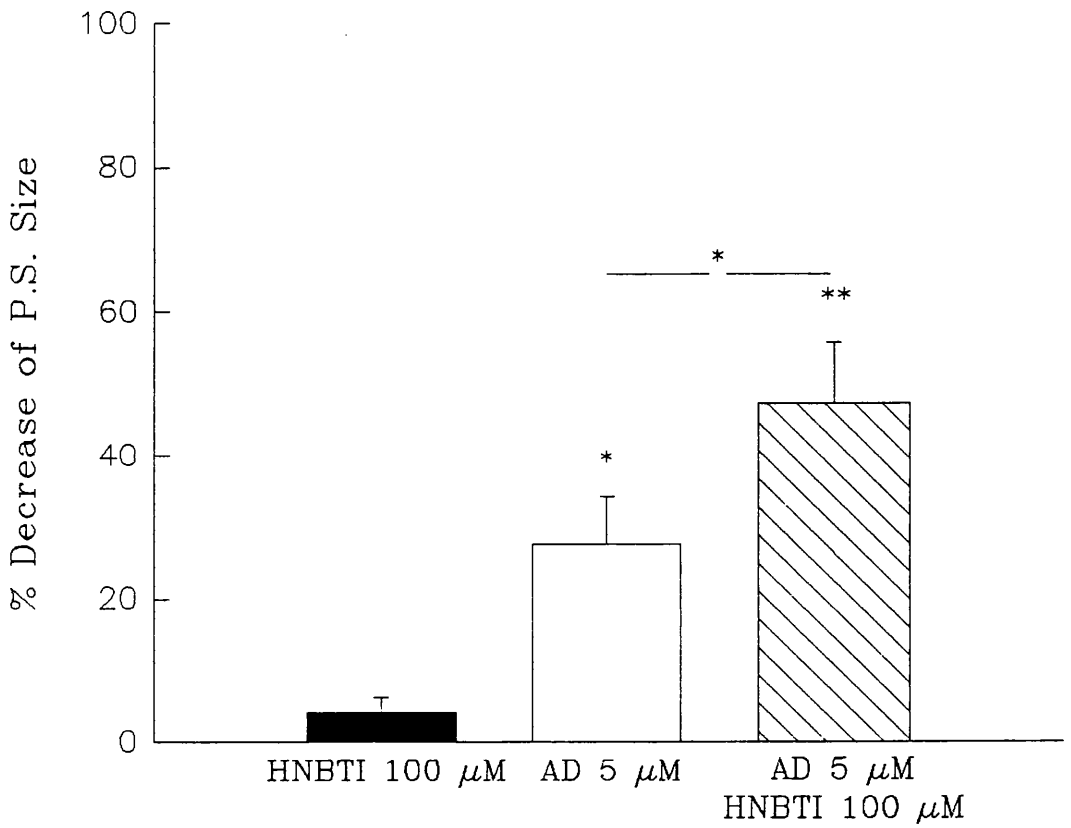

Figure 3.20. Histogram showing the effect of S-(2-Hydroxy-5-nitrobenzyl)-6-thioinosine (HNBTI) and adenosine (AD) on orthodromically evoked CA1 population spikes (P.S.) in normal ACSF. Each vertical bar represents the mean ± s.e.m. for n=5. A paired t test was employed to determine the significance level(*=P<0.05; **=P<0.01).

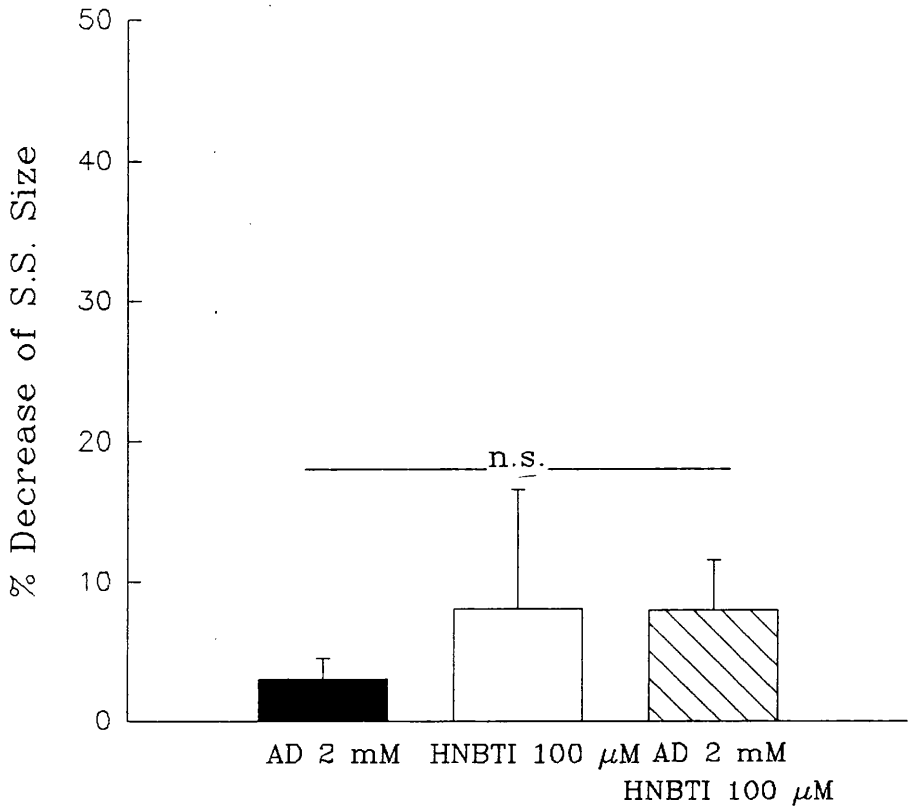

Figure 3.21. Histogram showing the effect of S-(2-Hydroxy-5-nitrobenzyl)-6-thioinosine (HNBTI) and adenosine (AD) on antidromically evoked CA1 secondary spikes (S.S.) in calcium-free medium. Each vertical bar represents the mean ± s.e.m. for n=3. A paired t test was employed to determine the significance level. n.s.=non-significant.

3.1.15. EFFECT OF THAPSIGARGIN ON RESTORATION OF ADENOSINE INHIBITORY EFFECT IN CALCIUM-FREE MEDIUM

Since postsynaptic adenosine sensitivity was restored in a low amount calcium, thapsigargin was used to clarify the site of calcium action.

Thapsigargin, which discharges intracellular calcium stores by inhibition of the endoplasmic reticulum calcium ATPase, had no significant effect on the antidromic evoked secondary spike size when applied alone at a concentration of 1 μM, or when superfused with adenosine 0.5 mM for 10 minutes in calcium-free medium (figure 3.22.).

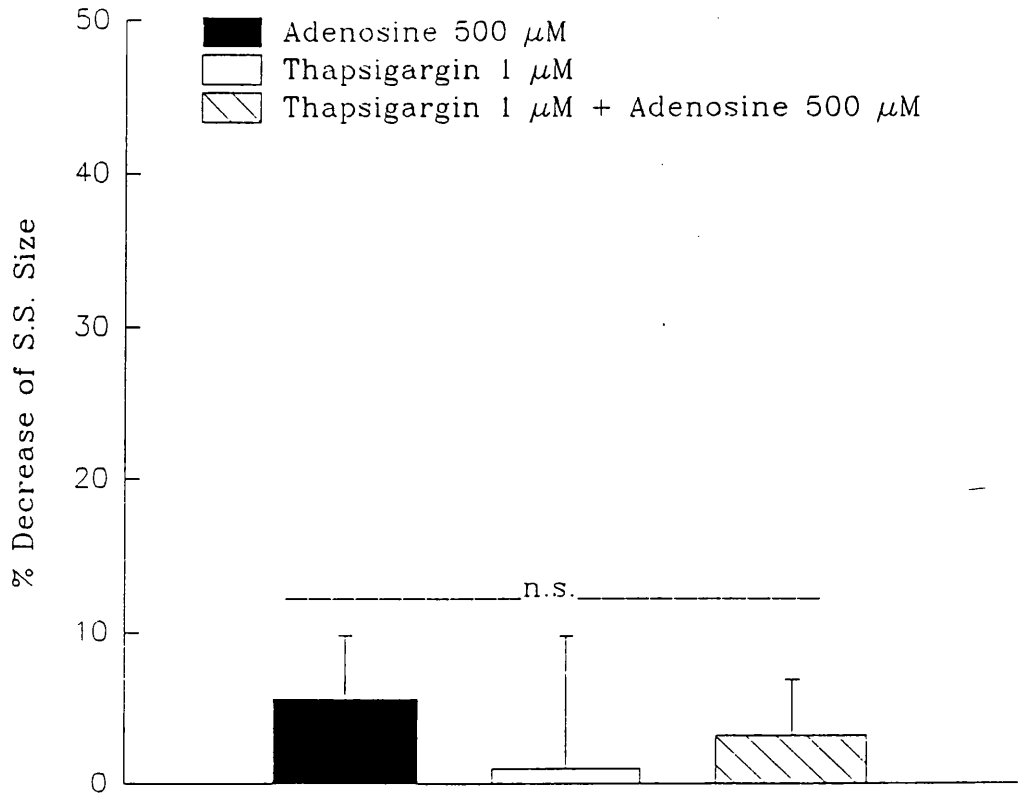

Figure 3.22. Histogram showing the effect of thapsigargin and adenosine on antidromically evoked CA1 secondary spikes (S.S.) in calcium-free medium. Each vertical bar represents the mean ± s.e.m. for n=3. A paired t test was employed to determine the significance level. n.s.=non-significant.

3.1.16. INHIBITORY EFFECT OF N^6-CYCLOPENTYLADENOSINE (CPA)

Although adenosine A_1 receptor generally have inhibitory effects, adenosine A_2 receptors can depolarize the cells and show excitatory effects. For this reason the highly selective A_1 adenosine receptor agonist, CPA, was tested.

3.1.16.1. EFFECT OF CPA ON ORTHODROMIC POPULATION SPIKES

CPA induced a concentration-dependent reduction in the amplitude of orthodromic population potentials (figure 3.23.). The maximum effect was seen at about 30 nM. The A_1 agonist had no effects on presynaptic fiber volley. CPA was applied for 25 minutes, since CPA required 15-20 minutes both to achieve a stable response size, and for recovery after washout.

3.1.16.2. EFFECT OF CPA ON ANTIDROMIC BURSTS INDUCED IN CALCIUM-FREE MEDIUM

In nominally calcium-free medium CPA at concentrations of 100-200 nM had no significant effect on antidromic first and secondary spike size (figure 3.23.).

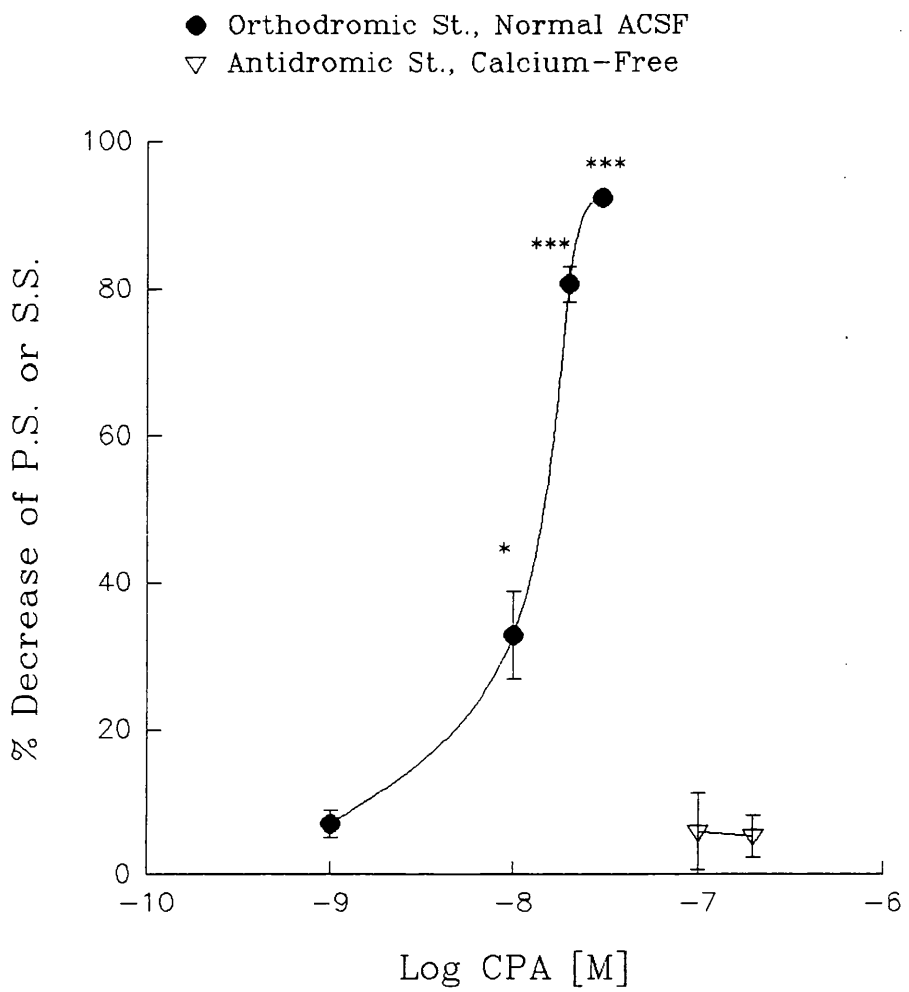

Figure 3.23. Cumulative concentration-response curve of N^6-cyclopentyladenosine (CPA) on orthodromically evoked CA1 population spikes (P.S.) in normal ACSF and on antidromically evoked CA1 secondary spikes (S.S.) in calcium-free medium. Each point represents the mean ± s.e.m. for n=3 for CPA. A paired *t* test was employed to determine the significance level (*=P<0.05; ***=P<0.001). St.=Stimulation.

3.1.17. EFFECTS OF STABILIZER AGENTS ON RESTORATION OF ADENOSINE POSTSYNAPTIC SENSITIVITY IN CALCIUM-FREE MEDIUM

Because omitting calcium destabilizes the cell membrane, two stabilizer agents, procaine and carbamazepine were examined on responses to adenosine.

3.1.17.1. EFFECT OF PROCAINE ON POSTSYNAPTIC SENSITIVITY TO ADENOSINE IN CALCIUM-FREE MEDIUM

Procaine, at 100 μM decreased reversibly the amplitude of both first and secondary antidromic spikes (12.04% ± 1.95, $P<0.01$, $n=4$ and 17.26% ± 4.014, $P<0.05$, $n=4$) respectively. A higher concentration of 500 μM procaine completely abolished both first and secondary spikes. The effect of procaine became stable after about 5-10 minutes. When combined with adenosine, procaine was superfused for 15 minutes before addition of the purine. Although 100 μM adenosine had no effect alone on the antidromic potential, its combination with procaine 100 μM decreased secondary spikes (figure 3.24.).

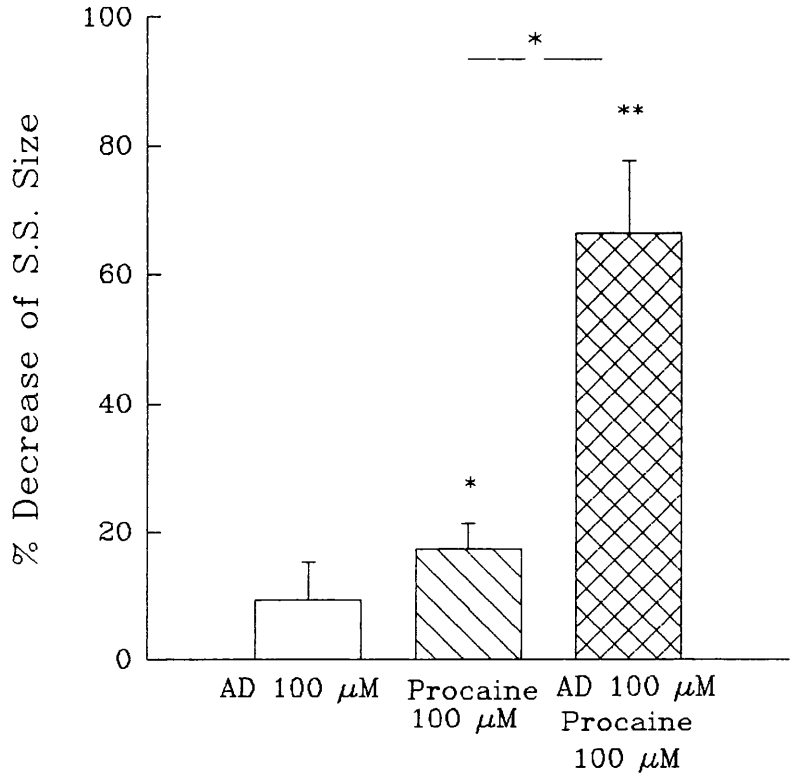

Figure 3.24. Histogram showing the effect of adenosine (AD) and procaine on antidromically evoked CA1 secondary spikes (S.S.) in calcium-free medium. Each vertical bar represents the mean ± s.e.m. for n=4. A paired *t* test was employed to determine the significance level (*=P<0.05; **=P<0.01).

3.1.17.2.1. EFFECT OF CARBAMAZEPINE ON POSTSYNAPTIC SENITIVITY OF ADENOSINE IN CALCIUM-FREE MEDIUM

In nominally calcium-free medium, carbamazepine, at 50 or 100 μM, concentration-dependently decreased the first and secondary antidromic spike size. The effect of carbamazepine became prominent and stable after about 10 minutes. Complete washout of carbamazepine took more than 30 minutes.

Carbamazepine was applied 15 minutes before superfusion of adenosine. Carbamazepine restored the effect of adenosine sensitivity in calcium-free medium (figure 3.25. and 3.26.).

3.1.17.2.2. INTERACTION OF CARBAMAZEPINE AND BACLOFEN ON BURSTS INDUCED IN CALCIUM-FREE MEDIUM

Carbamazepine at concentration of 50 μM did not increase the inhibitory effect of 20 μM baclofen (figure 3.27).

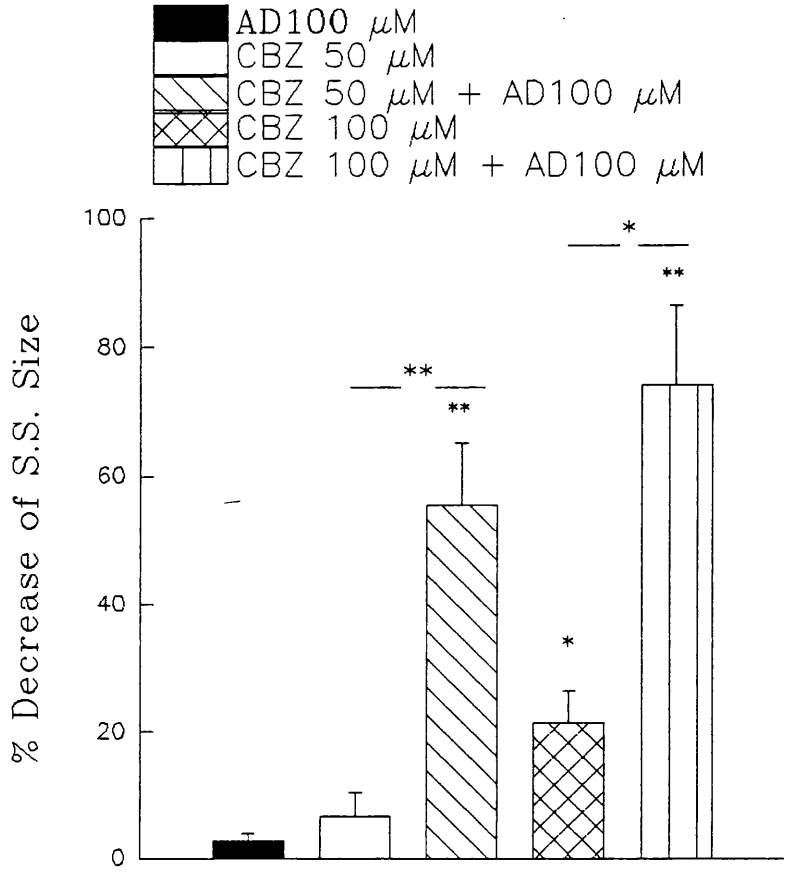

Figure 3.25. Histogram showing the effect of adenosine (AD) and carbamazepine (CBZ) on antidromically evoked CA1 secondary spikes (S.S.) in calcium-free medium. Each vertical bar represents the mean ± s.e.m. for n≥4. A paired *t* test was employed to determine the significance level (*=P<0.05; **=P<0.01).

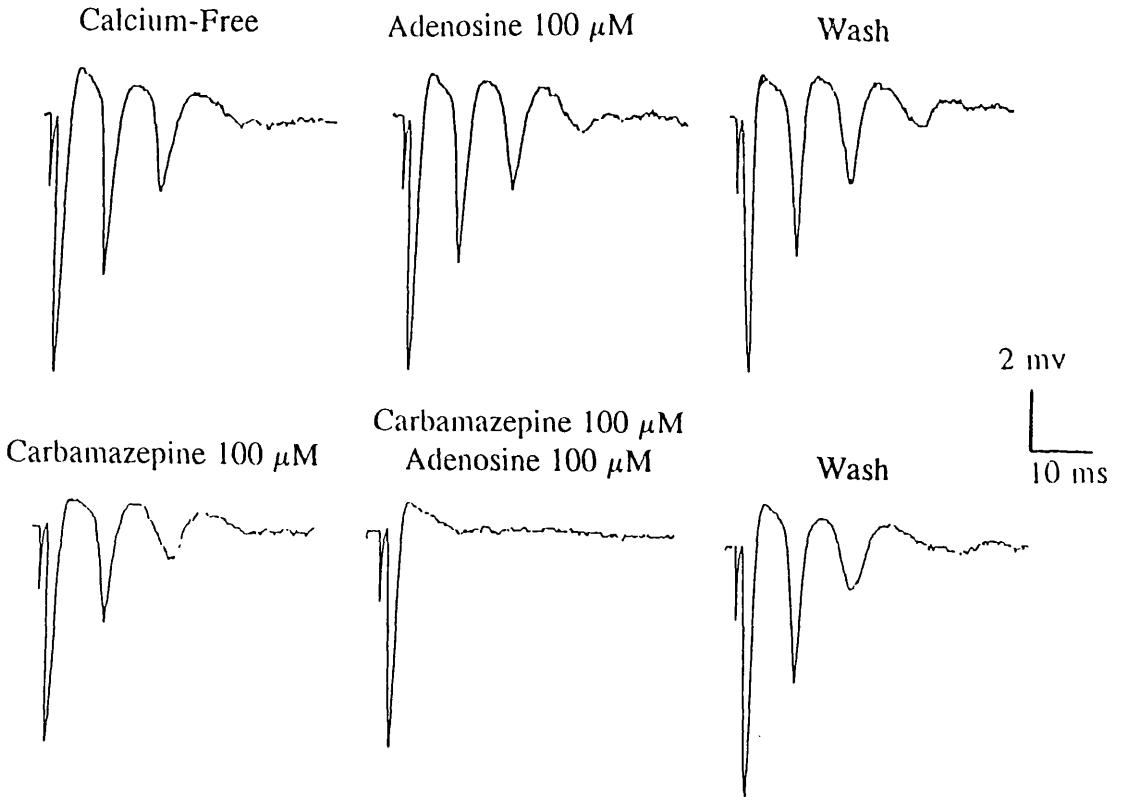

Figure 3.26. Sample records of evoked burst activity and the effect of adenosine and carbamazepine on secondary spike size. Carbamazepine restored adenosine sensitivity in calcium-free medium.

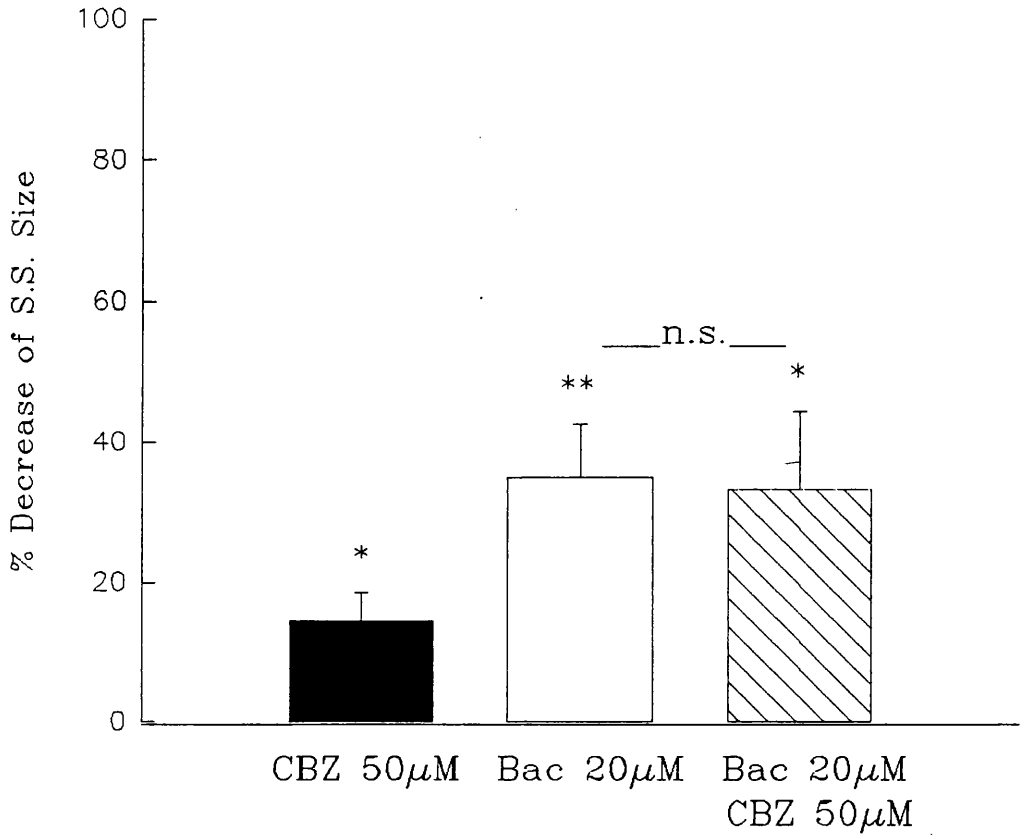

Figure 3.27. Histogram showing the effect of baclofen (Bac) and carbamazepine (CBZ) on antidromically evoked CA1 secondary spikes (S.S.) in calcium-free medium. Each vertical bar represents the mean ± s.e.m. for n=4. A paired *t* test was employed to determine the significance level (*=P<0.05; **=P<0.01). n.s.=non-significant.

3.1.18. EFFECT OF METHYLXANTHINES IN CALCIUM-FREE MEDIUM

3.1.18.1.1. EFFECT OF ADENOSINE AND THEOPHYLLINE ON ORTHODROMIC POTENTIALS

Adenosine at 50 μM abolished completely the orthodromic population spikes. Theophylline, a non-selective adenosine receptor antagonist, at a concentration of 50 μM alone increased population spikes by 21.17% ± 6.24 ($P<0.05$, n=4). The effect of theophylline began after about 3-5 minutes and recovery on washout took more than 15 minutes. Theophylline did not induce secondary spikes in any of the slices. In the presence of theophylline 50 μM, adenosine now reduced the population spikes by 69.93% ± 3.25 ($P<0.01$, n=4, figure 3.28).

3.1.18.1.2. EFFECT OF ADENOSINE AND THEOPHYLLINE ON ANTIDROMIC BURSTS INDUCED IN CALCIUM-FREE MEDIUM

In calcium-free medium theophylline, 50 μM unexpectedly decreased the size of the first antidromic spike size by 29.96% ± 5.67 and secondary spike size by 50.61% ± 6.79 ($P<0.001$, n=14). In 4 slices washout of theophylline 50 μM even for 30 minutes did not restore the size of secondary spikes to control. In four other slices the first and secondary spike dramatically decreased within 2-3 minutes and then they were increased but the size did not recover to control. Adenosine at 200 μM had no effect itself in calcium-free medium but prevented the inhibitory effect of the xanthines (figures 3.29).

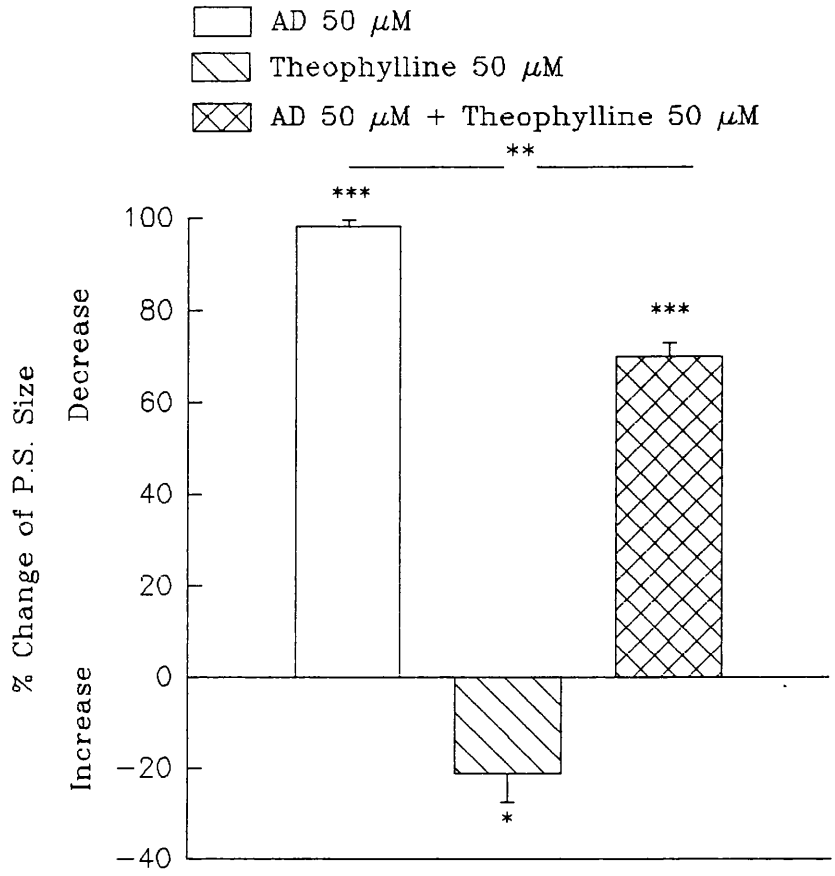

Figure 3.28. Histogram showing the effects of adenosine (AD) and theophylline on orthodromically evoked CA1 population spikes (P.S.) in normal ACSF. Each vertical bar represents the mean ± s.e.m. for n=4. A paired *t* test was employed to determine the significance level (*=P<0.05; **=P<0.01; ***=P<0.001).

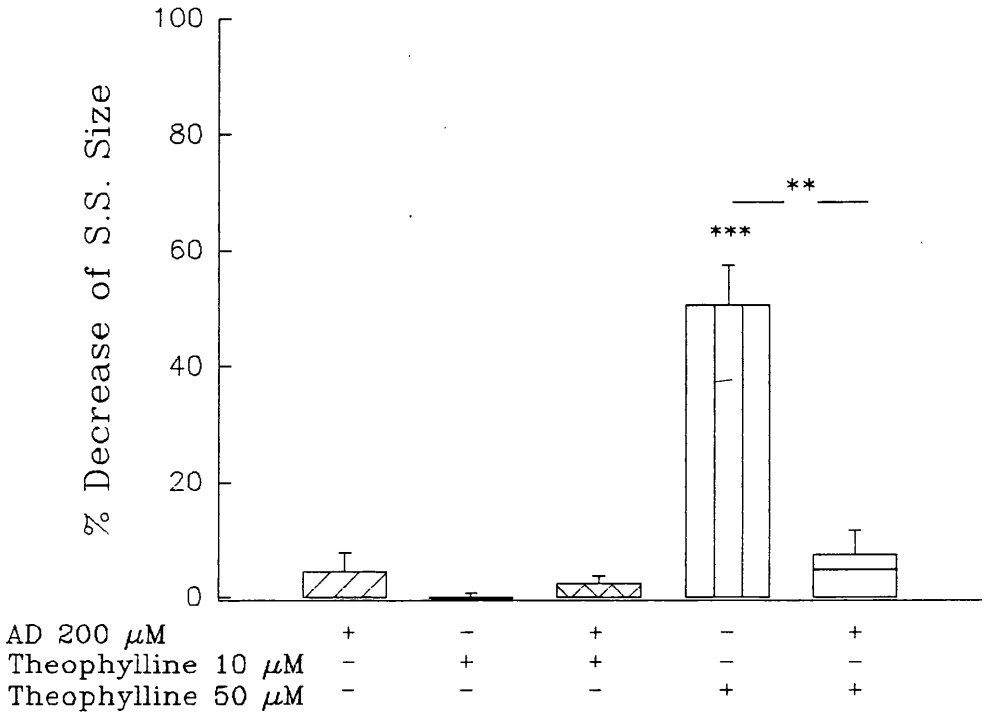

Figure 3.29. Histogram showing the effects of adenosine (AD) and theophylline on antidromically evoked CA1 secondary spikes (S.S.) in calcium-free medium. Each vertical bar represents the mean ± s.e.m. for n=4. A paired *t* test was employed to determine the significance level (**=P<0.01; ***=P<0.001).

3.1.18.1.3 EFFECT OF THEOPHYLLINE ON ANTIDROMIC FIELD ACTION POTENTIAL IN NORMAL ACSF

In normal calcium-containing ACSF, theophylline, 50 μM slightly increased the size of the first antidromic spike by 5.64% ± 2.01 ($P<0.05$, n=6)

3.1.18.1.4. INTERACTION OF THEOPHYLLINE AND BICUCULLINE ON ANTIDROMIC FIELD ACTION POTENTIAL IN NORMAL ACSF

In order to study the effect of theophylline on secondary spikes in the presence of calcium, bicuculline, a $GABA_A$ antagonist was used for induction of bursts.

Bicuculline 10 μM quickly induced secondary spikes in less than three minutes, and after about 5-10 minutes stable bursts with 3-6 secondary spike were stablished. Washout of bicuculline took more than 30 minutes.

Theophylline 50 μM did not decrease the first (0.65% ± 1.4, P=0.66, n.s.) or secondary (0.67% ± 1.99, P=0.74, n.s.) antidromic spikes induced by bicuculline (n=6). The methylxanthine also induced spontanous activity in 3-5 minutes (4.14 ± 1.01 spikes/minute, n=6, $P<0.01$).

3.1.18.2.1. EFFECT OF ADENOSINE AND 8-CYCLOPENTYL-1,3-DIMETHYLXANTHINE (CPT) ON ORTHODROMIC POTENTIAL

CPT, an A1 receptor antagonist, at a concentration of 100 nM antagonized responses to adenosine 50 μM, which now reduced the population spike by only 22.77% ± 3.22 ($P<0.001$, n=6, figure 3.30.). The effect of CPT was slower in onset than theophylline, taking about 10 minutes, and recovery on washout took more than 30 minutes.

3.1.18.2.2. EFFECT OF ADENOSINE AND CPT ON ANTIDROMIC STIMULATION INDUCED IN CALCIUM-FREE MEDIUM

In calcium-free medium CPT 100 nM decreased the size of the first antidromic spike size by 17.91% ±3.73 ($P<0.05$, n=4) and secondary spike size by 50.56% ± 11.45 ($P<0.05$, n=4, figure 3.31.).

Adenosine at 200 μM had no effect itself in calcium - free medium but prevented the inhibitory effect of the xanthine (figure 3.31.).

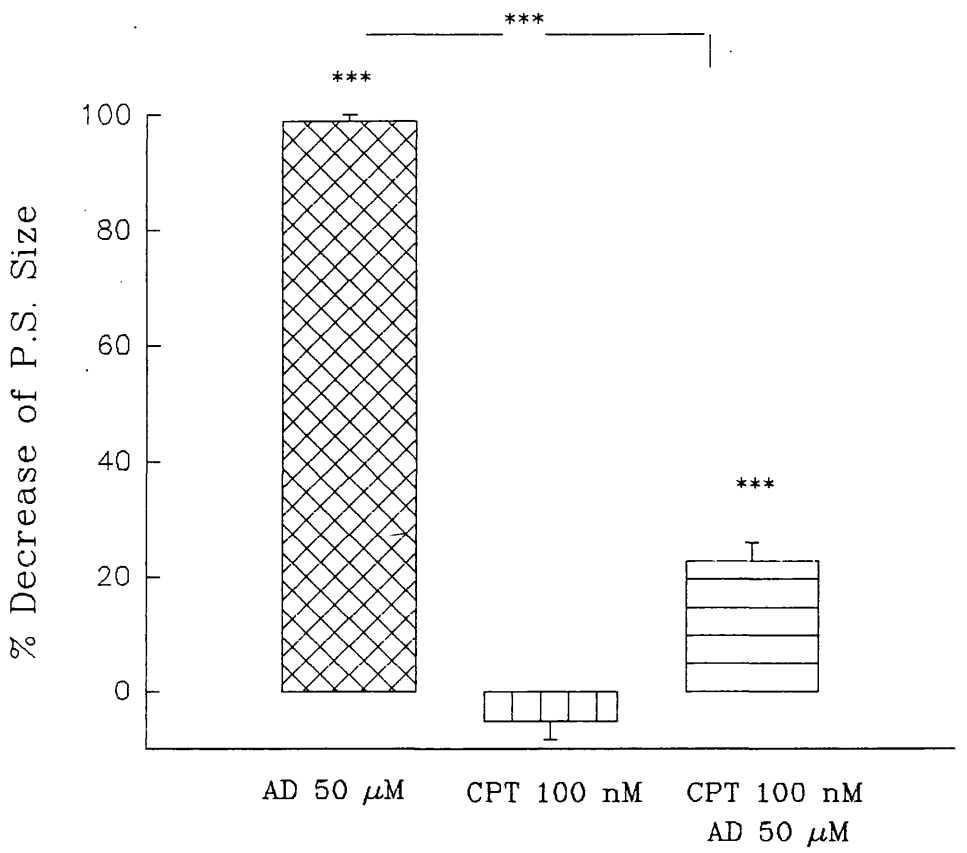

Figure 3.30. Histogram showing the effects of adenosine (AD) and 8-cyclopentyl-1,3-dimethylxanthine (CPT) on orthodromically evoked CA1 population spikes (P.S.) in normal ACSF. Each vertical bar represents the mean ± s.e.m. for n=6. A paired *t* test was employed to determine the significance level (***=P<0.001).

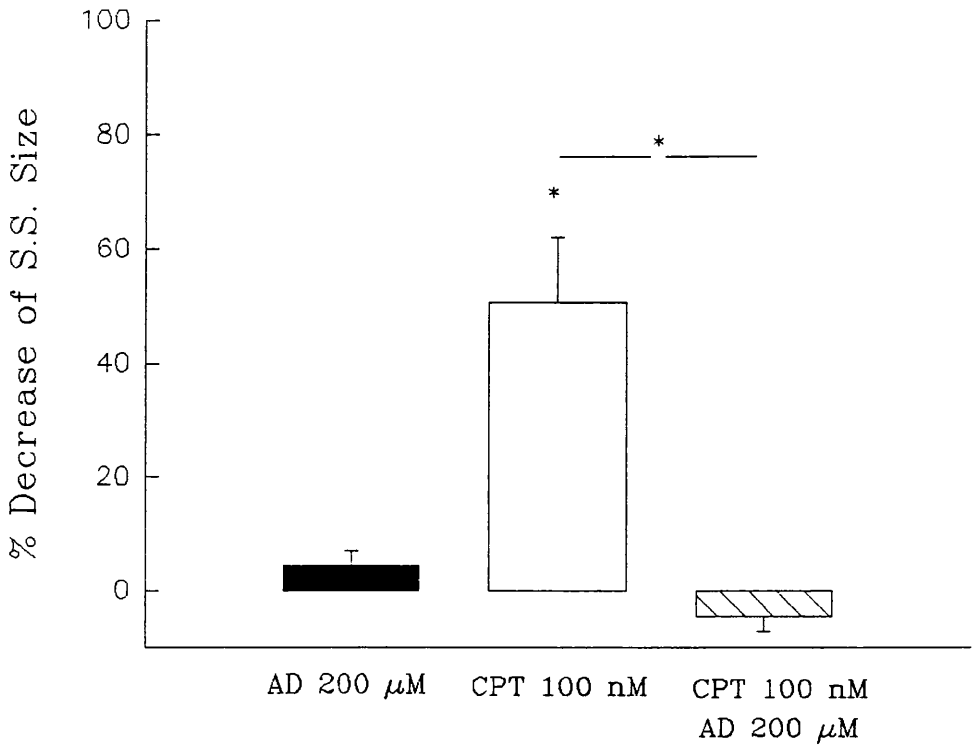

Figure 3.31. Histogram showing the effects of adenosine (AD) and 8-cyclopentyl-1,3-dimethylxanthine (CPT) on antidromically evoked CA1 secondary spikes (S.S.) in calcium-free medium. Each vertical bar represents the mean ± s.e.m. for n=4. A paired *t* test was employed to determine the significance level (*=P<0.05).

3.1.18.3. MECHANISM(S) OF PARADOXICAL EFFECT OF XANTHINES IN CALCIUM-FREE MEDIUM

As mentioned above theophylline showed two opposite effects, an expected excitatory one when examined with orthodromic stimulation in normal ACSF, but an inhibitory one when tested on antidromic responses to antidromic stimulation in calcium-free medium. We therefore attempted to examine the reasons for this differenc.

3.1.18.3.1. EFFECT OF THEOPHYLLINE ON ANTIDROMIC BURSTS IN LOW CALCIUM MEDIUM (0.1 mM)

Adding 0.1 mM calcium had no significant effect on secondary spike size established in calcium-free medium. However, at this concentration calcium reduced the inhibitory effect of theophylline to 16.3% ± 1.49 ($P<0.001$, $n=5$, figure 3.32). Calcium did slightly but significantly increase the size of the primary antidromic spike and again abolished the inhibitory of theophylline (figure 3.33.).

3.1.18.3.2. EFFECT OF CPT ON ANTIDROMIC BURSTS IN CALCIUM-FREE MEDIUM PLUS 1 mM EGTA

Adding 1 mM EGTA to calcium-free medium decreased secondary spike size significantly by 14.82% ± 4.69 ($P<0.05$, $n=7$) and also reduced bursts with four or three spikes down to two spikes. CPT 100 nM decreased secondary spike size by 29.79% ± 2.52 ($P<0.001$, $n=5$) in calcium-free medium. In calcium-free media with 1 mM EGTA, CPT decreased secondary spike size by 45.60% ± 5.63 ($P<0.01$, $n=7$) which was significant when compared to EGTA alone in calcium-free media (figure 3.34.).

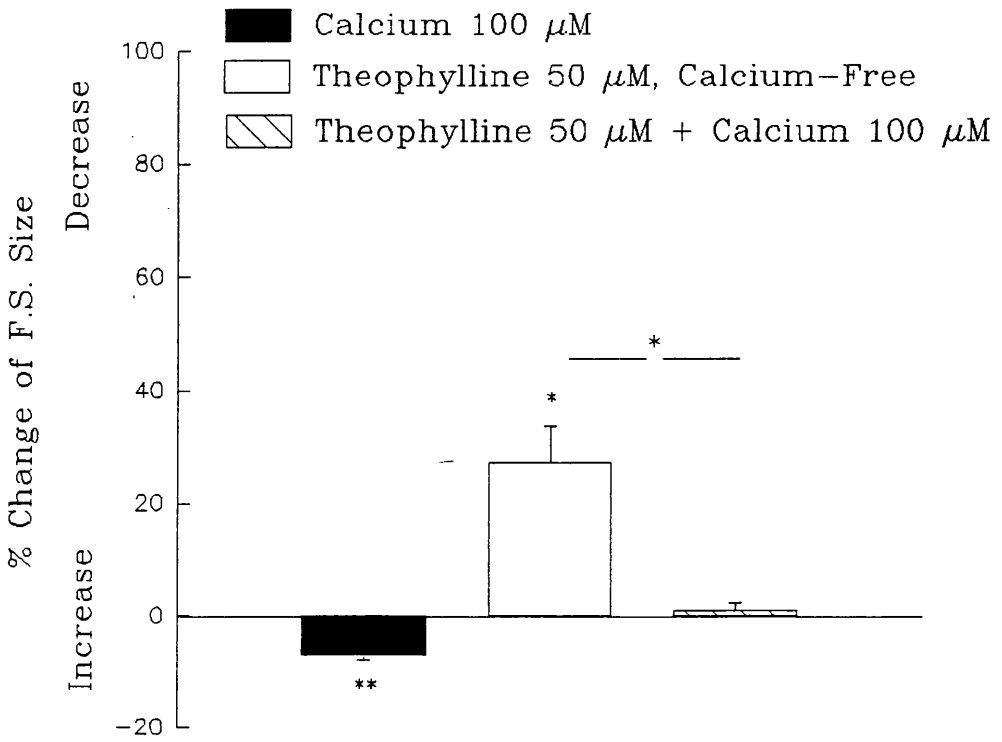

Figure 3.32. Histogram showing the effect of theophylline on antidromically evoked CA1 first spike (F.S.) size in calcium-free medium or 100 μM calcium. Each vertical bar represents the mean ± s.e.m. for n=5. A paired *t* test was employed to determine the significance level (*=P<0.05; **=P<0.01).

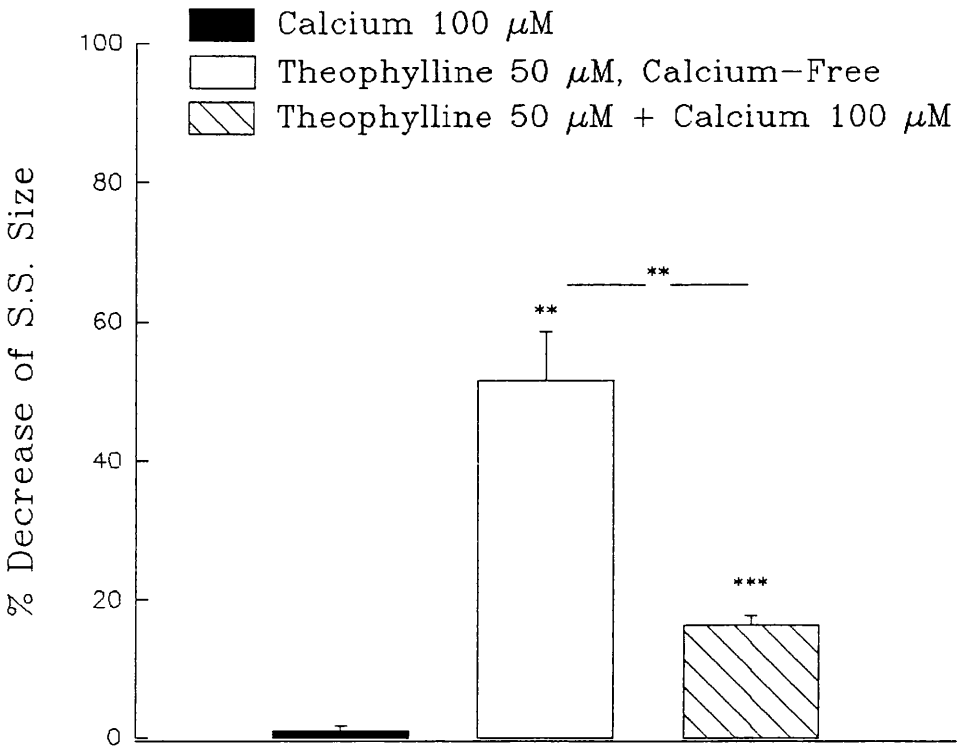

Figure 3.33. Histogram showing the effect of theophylline on antidromically evoked CA1 scondary spike (S.S.) size in calcium-free medium or 100 μM calcium. Each vertical bar represents the mean ± s.e.m. for n=5. A paired *t* test was employed to determine the significance level (**=P<0.01; ***=P<0.001).

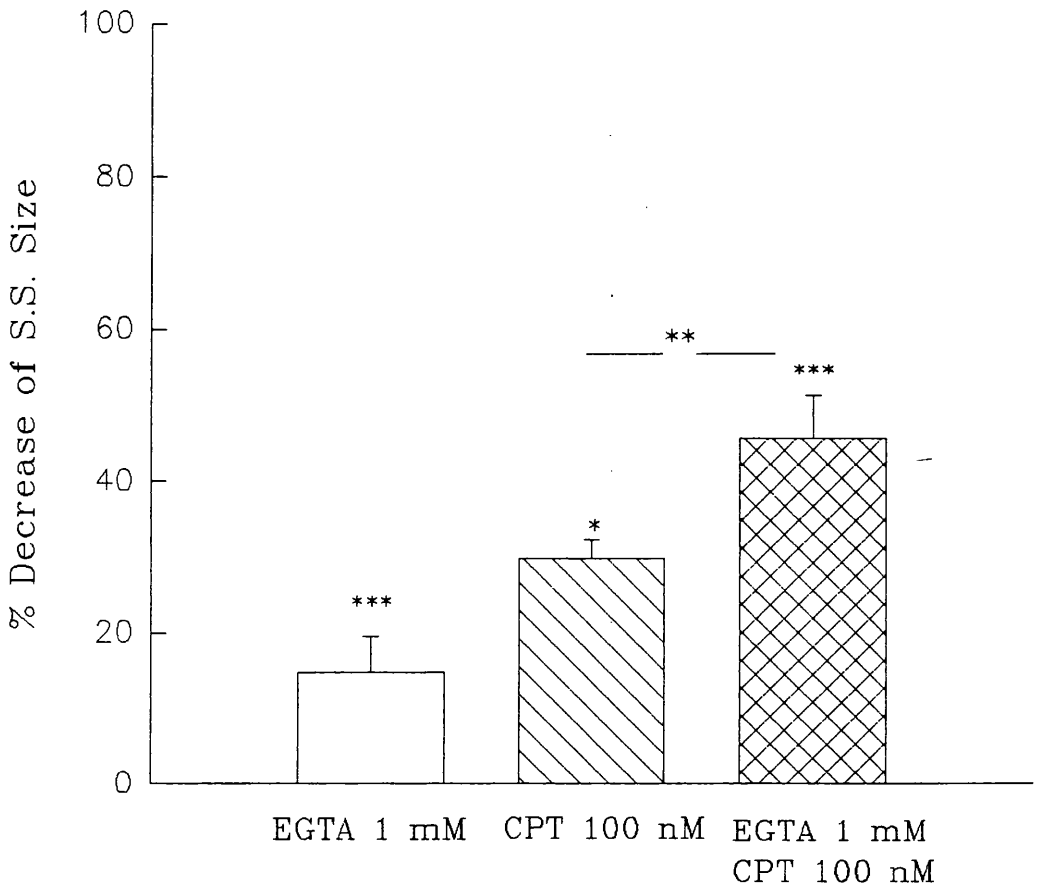

Figure 3.34. Histogram showing the effects of 8-cyclopentyl-1,3-dimethylxanthine (CPT) on antidromically evoked CA1 secondary spikes (S.S.) in calcium-free medium and calcium-free media plus 1 mM EGTA. Each vertical bar represents the mean ± s.e.m. for n=5-7. A paired and unpaired *t* test was employed to determine the significance level (*=P<0.05; **=P<0.01, ***=P≤0.001).

3.1.18.3.3. INTERACTION OF BACLOFEN AND THEOPHYLLINE ON ANTIDROMIC BURSTS INDUCED IN CALCIUM-FREE MEDIUM

Baclofen, a $GABA_B$ agonist, at 5 μM had no effect on the size of secondary spikes but prevented the inhibitory action of theophylline on secondary spike size ($P<0.05$, n=6, figures 3.35. and 3.36.).

3.1.18.3.4.1. INTERACTION OF CARBAMAZEPINE AND THEOPHYLLINE ON ANTIDROMIC BURSTS INDUCED IN CALCIUM-FREE MEDIUM

Carbamazepine, 50 μM decreased slightly the size of the first and secondary spikes by 8.49% ± 2.22 ($P<0.05$, n=6) and 17.66% ± 4.87 ($P<0.05$, n=6) respectively. At this concentration carbamazepine completely blocked the depression induced by theophylline, 50 μM in calcium-free medium (figure 3.37.).

3.1.18.3.4.2. EFFECT OF CARBAMAZEPINE ON THE EXCITATORY EFFECT OF THEOPHYLLINE ON ORTHODROMIC SPIKES IN NORMAL ACSF

The excitatory activity of theophylline, 50 μM was still seen in the presence of carbamazepine, 50 μM and was not significantly different from that seen with theophylline alone ($P=0.568$, n=6)

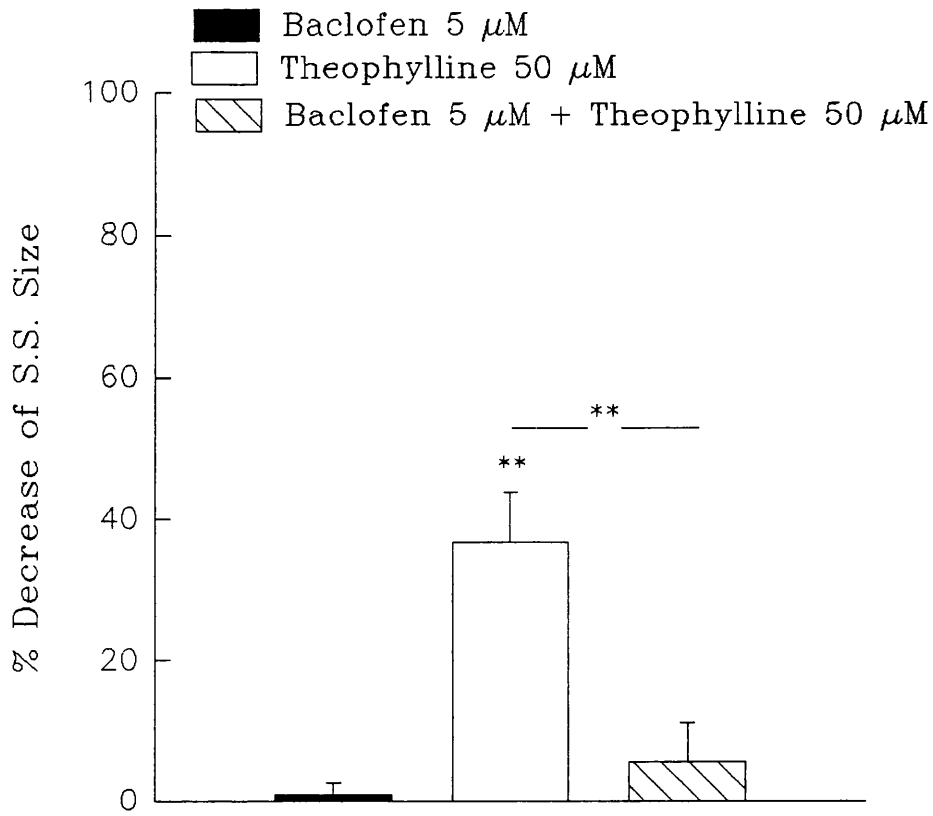

Figure 3.35. Histogram showing the effects of baclofen and theophylline on antidromically evoked CA1 secondary spike (S.S.) size in calcium-free medium. Each vertical bar represents the mean ± s.e.m. for n=6. A paired *t* test was employed to determine the significance level (**=P<0.01).

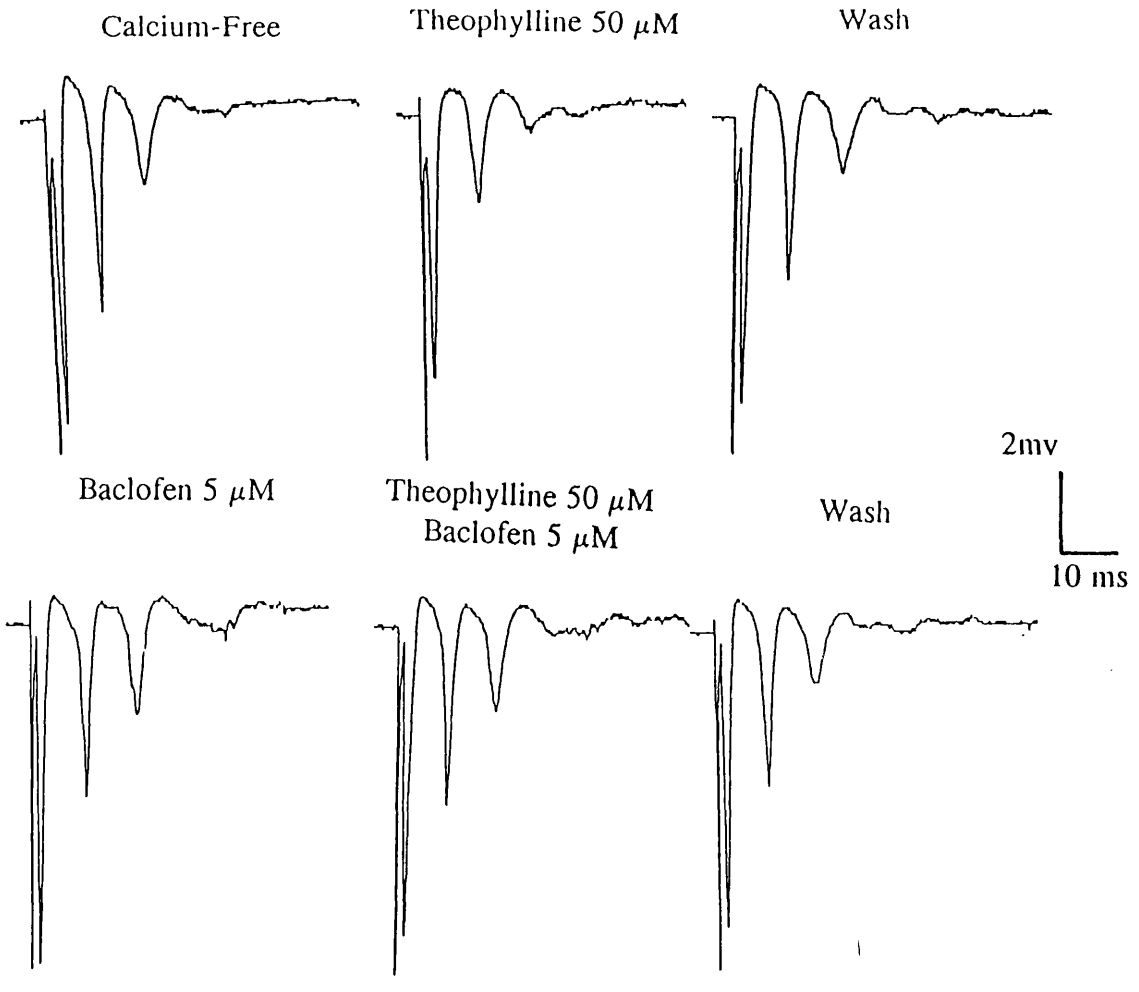

Figure 3.36. Sample records of evoked burst activity in calcium-free medium and the effect of baclofen and theophylline on secondary spike size. Baclofen preventedthe inhibitory effect of theophylline on S.S. size.

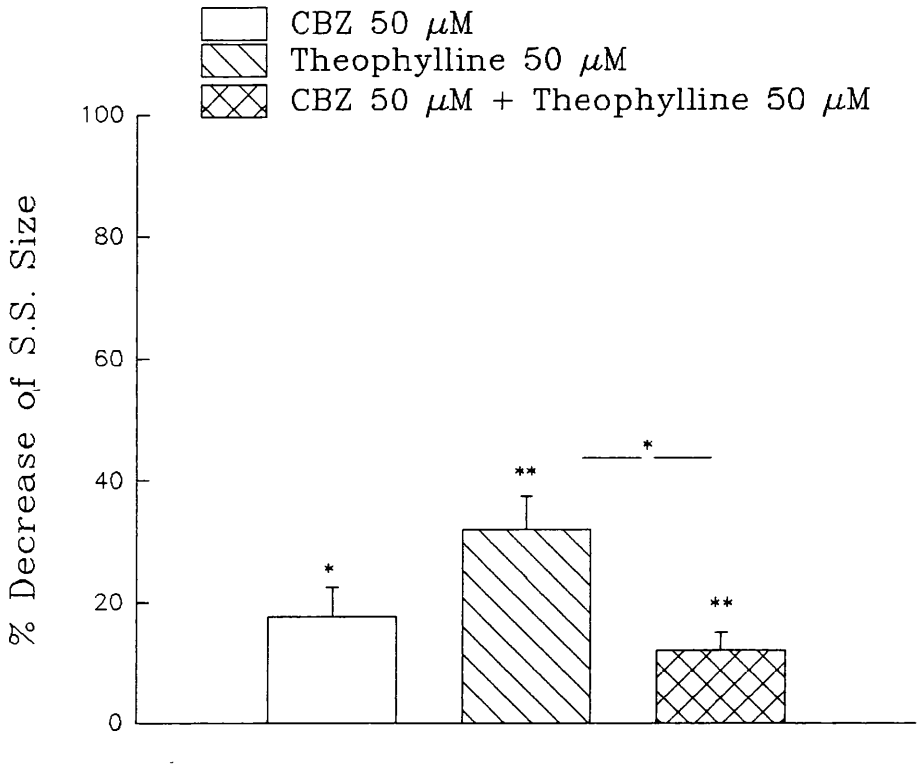

Figure 3.37. Histogram showing the effects of theophylline and carbamazepine (CBZ) on antidromically evoked CA1 secondary spikes (S.S.) in calcium-free medium. Each vertical bar represents the mean ± s.e.m. for n=6. A paired *t* test was employed to determine the significance level (*=P<0.05; **=P<0.01).

3.1.18.3.5. EFFECT OF THEOPHYLLINE ON ISOLATED CA1 ANTIDROMIC BURSTS IN CALCIUM-FREE MEDIA

Since there is a possibility that theophylline-induced pacemaker activity in the CA3 region could cause the inhibitory effect on secondary spikes in calcium-free media, CA3 and CA1 regions were disconnected and the effect of theophylline on the isolated CA1 area was studied.

Theophylline 50 μM decreased both antidromic first and secondary spikes by 19.83% ± 4.45 ($P<0.05$) and 45.11% ± 7.62 ($P<0.01$, $n=4$). These results were not significant when compared (unpaired t-test) with the results of theophylline in intact slices ($n=9$, figure 3.38.).

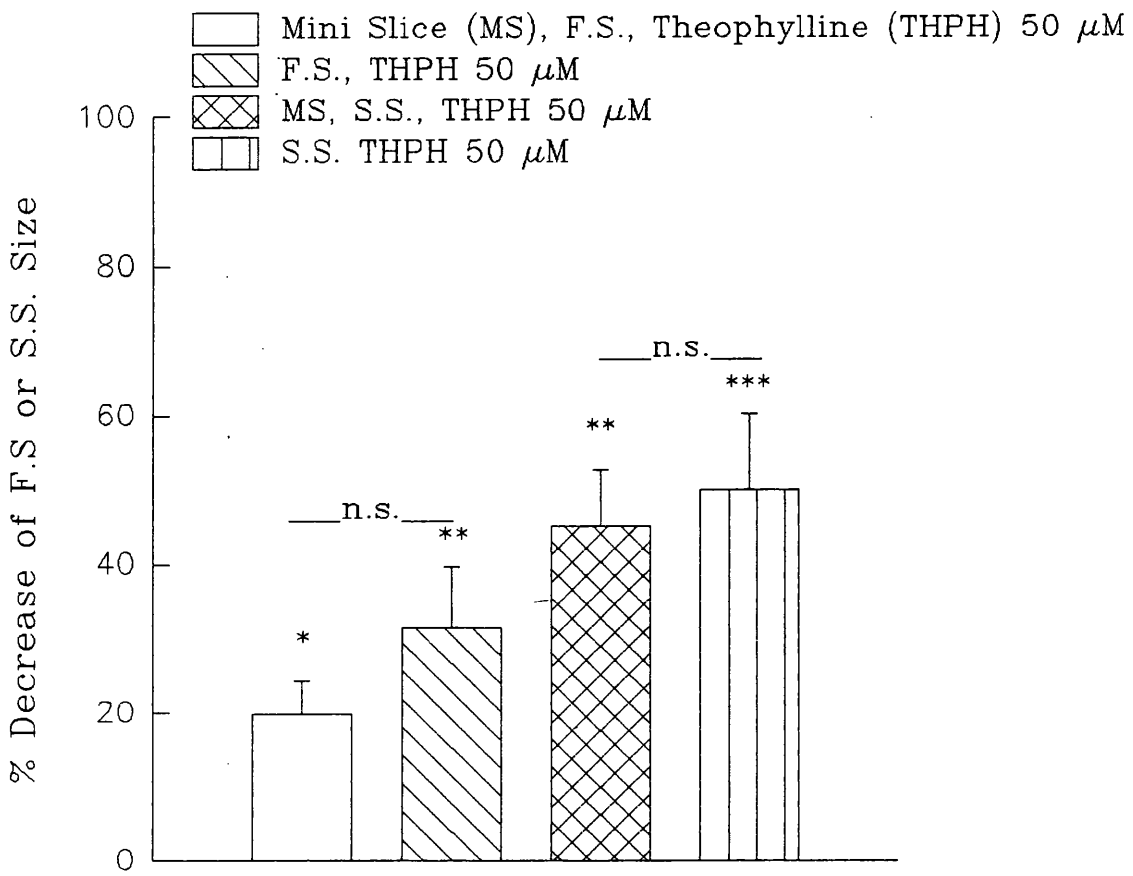

Figure 3.38. Histogram showing a comparison of the effect of theophylline 50 μM on first (F.S.) and secondary spikes (S.S.) on intact slices or isolated slices with CA3 disconnected from CA1 in calcium-free medium. Each vertical bar represents the mean ± s.e.m. for n=4 and 9 for isolated and intact slices respectively. A paired and unpaired *t* test was employed to determine the significance level (*=P<0.05; **=P<0.01; ***=P≤0.001). n.s.=non-significant.

3.1.18.3.6.1.1. EFFECT OF ADENOSINE DEAMINASE ON ORTHODROMIC EFFECT OF ADENOSINE IN NORMAL ACSF

Adenosine 50 or 100 μM completely abolished orthodromic induced P.S. size. Application of adenosine deaminase (ADA) at a concentration 0.25 IU/ml for 20 minutes had no significant effect on the orthodromic potential. Perfusion of slices with ADA at this concentration was sufficient to prevent completely the inhibitory effect of 50 μM (n=2) or 100 μM (n=1) adenosine on orthodromic potentials.

Application of adenosine 50 μM again completely abolished the P.S size after 30 minutes washout of slice with normal ACSF.

3.1.18.3.6.1.2. EFFECT OF THEOPHYLLINE ON ANTIDROMIC BURSTS INDUCED IN CALCIUM-FREE MEDIUM IN THE PRESENCE OF ADENOSINE DEAMINASE

Adenosine deaminase at a concentration of 0.25 IU/ml had only a slight depressant effect upon secondary spike size (7.94% ± 3.08, P=0.05, n=6), and did not modify the inhibitory effect of theophylline in calcium-free medium (figure 3.39).

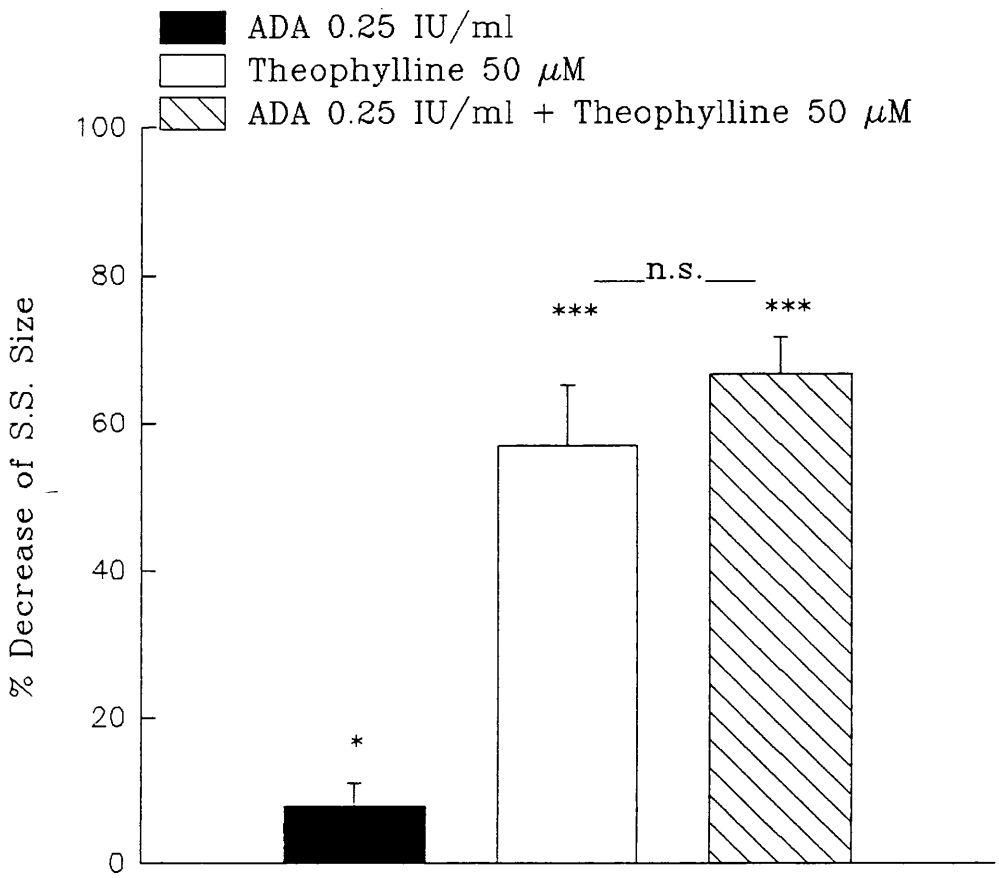

Figure 3.39. Histogram showing the effects of theophylline and adenosine deaminase (ADA) on antidromically evoked CA1 secondary spikes (S.S.) in calcium-free medium. ADA did not prevent the inhibitory effect of theophylline on S.S. size. Each vertical bar represents the mean ± s.e.m. for n=6. A paired *t* test was employed to determine the significance level (*=P=0.05; ***=P<0.001). n.s.=non-significant.

SECTION II : PHARMACOLOGICAL ANALYSIS OF ADENOSINE ACTIONS ON POTASSIUM CHANNELS

3.2. INTERACTION OF A POTASSIUM CHANNEL BLOCKER OR OPENER WITH ADENOSINE OR BACLOFEN

Since adenosine lost its inhibitory effect on secondary spikes in calcium-free media but baclofen did not, the postsynaptic sensitivity of these agonists to potassium channel blockers or openers was studieed.

3.2.1. INTERACTION OF ADENOSINE AND APAMIN ON ORTHODROMIC POPULATION SPIKES

If adenosine hyperpolarizes the soma via calcium-activated potassium channels, there is a possibility that this agonist will lose its effect in the absence of calcium. The effect of apamin, a selective blocker of small conductance calcium-activated potassium channels was therefore studied.

Bath application of 10 nM apamin had no significant effect on orthodromic CA1 field potentials (n=4). This concentration apamin reduced the relaxant effect of ATP on the carbachol contracted taenia coli of the guinea pig (Brown & Burnstock, 1981). At higher concentration, 250 nM

apamin did not have a consistent effect on orthodromic potentials. In two slices it had no significant effect and in the rest of the slices (n=5) it increased the population spikes. Overall apamin had no significant effect on the field potential (n=7, P>0.5, n.s.)

Apamin at a concentration of 250 nM (n=3, 12.37% ± 9.10, n.s.) had no significant effect on the inhibitory effect of adenosine 5 μM on orthodromic population spikes (figure 3.40.).

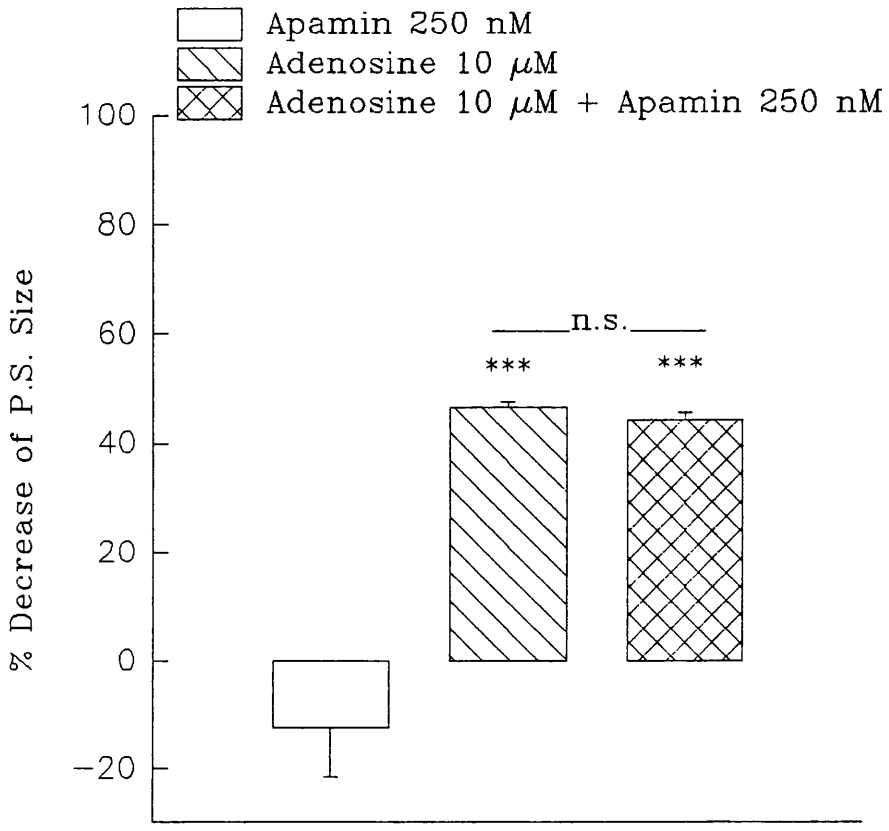

Figure 3.40. Histogram showing interaction of adenosine and apamin on orthodromically evoked CA1 population spikes in normal ACSF. Each vertical bar represents the mean ± s.e.m. for n=3. A paired *t* test was employed to determine the significance level (***=P≤0.001). n.s.=non-significant.

3.2.2. EFFECT OF ADENOSINE IN THE PRESENCE OF TOLBUTAMIDE

3.2.2.1 INTERACTION OF ADENOSINE AND TOLBUTAMIDE ON OTHODROMIC POPULATION SPIKES

Adenosine at 5 and 10 μM reduced the amplitude of orthodromic population spikes by 36.69% ± 3.56 ($P<0.01$, $n=4$) and 56.93% ± 2.2 ($n=4$, $P\leq0.001$) respectively. Tolbutamide 0.5 and 1 mM alone increased the population spikes size by 6.89% ± 4.07 (n.s., $n=3$) and 12.48% ± 3.81 ($n=4$, $P<0.05$) respectively. This effect was seen in less than 5 minutes. Tolbutamide 1 mM significantly decreased the effect of adenosine 10 μM to 32.96% ± 5.79 and markedly but not significantly decreased the response to adenosine 5 μM ($n=4$, $P<0.05$, figure 3.41).

3.2.2.2. INTERACTION OF ADENOSINE AND TOLBUTAMIDE ON ORTHODROMIC FIELD EPSPs (fEPSPs)

The inhibitory effect of adenosine was less on fEPSP size than population spike size. Adenosine at 10 and 20 μM decreased the size of the field EPSPs 13.23% ± 3.13 ($n=4$, $P<0.05$) and 39.97% ± 1.28 ($n=4$, $P\leq0.001$). Tolbutamide, 1 mM did not affect this presynaptic effect of adenosine at either concentration ($n=4$, figure 3.42.). Tolbutamide alone increased slightly the size of field EPSPs by 6.41% ± 1.99 ($n=4$, $P<0.05$).

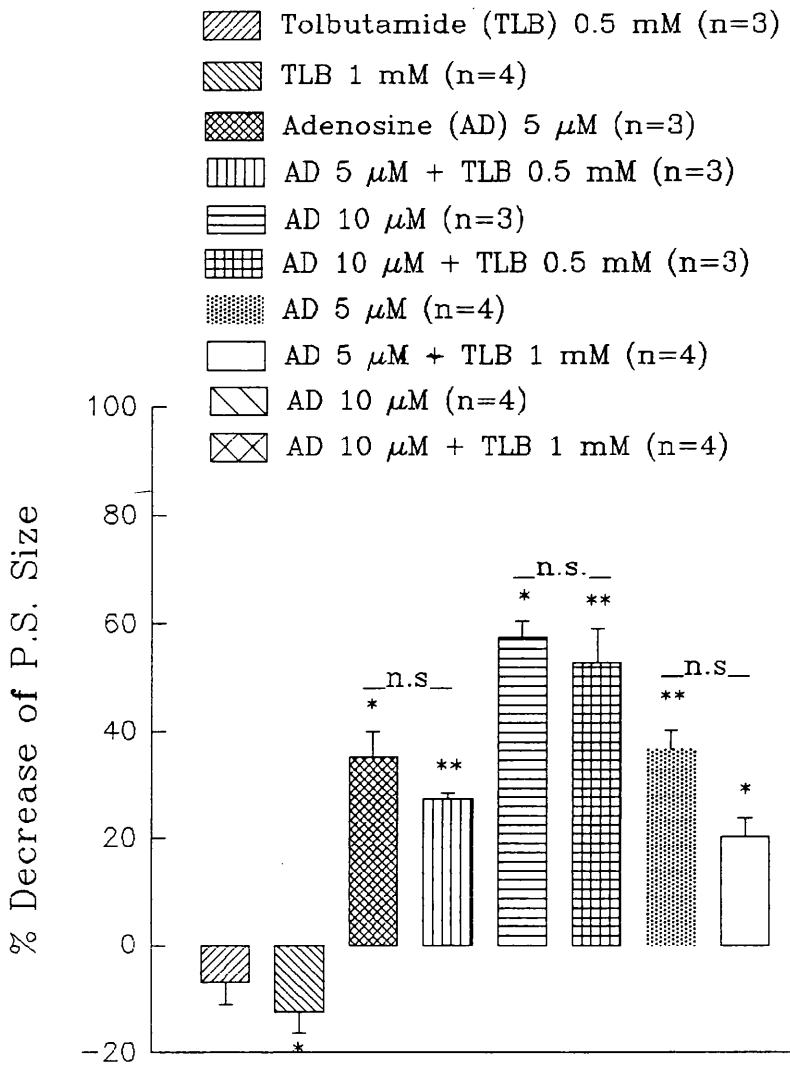

Figure 3.41. Histogram showing the effect of adenosine (AD) and tolbutamide (TLB) on orthodromically evoked CA1 population spikes (P.S.) in normal ACSF. Each vertical bar represents the mean ± s.e.m. for the number of slices indicated in parentheses. A paired *t* test was employed to determine the significance level relative to control potentials or to adenosine alone as indicated (*=P<0.05; **=P<0.01; ***=P≤0.001). n.s.=non-significant.

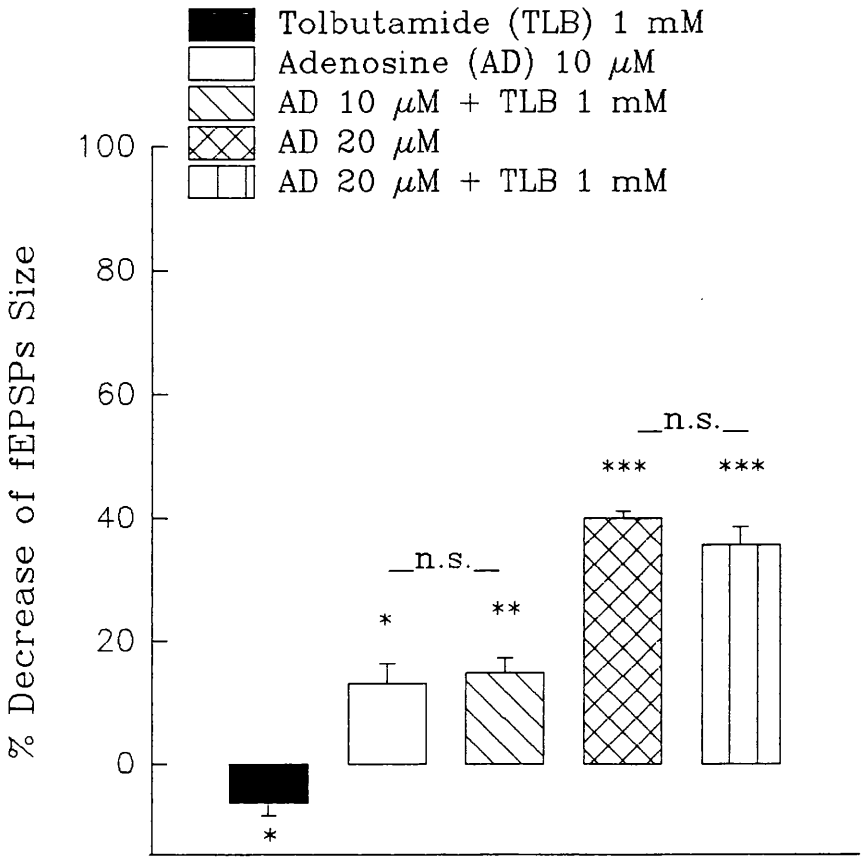

Figure 3.42. Histogram showing the effect of adenosine (AD) and tolbutamide (TLB) on orthodromically evoked CA1 field EPSPs in normal ACSF. Each vertical bar represents the mean ± s.e.m. for n=4. A paired *t* test was employed to determine the significance level (*=P<0.05; **=P<0.01; ***=P≤0.001). n.s.= non significant.

3.2.2.3. INTERACTION OF ADENOSINE AND TOLBUTAMIDE ON ANTIDROMIC BURSTS INDUCED IN CALCIUM-FREE HIGH MAGNESIUM MEDIUM

Adenosine at 5 and 10 μM decreased antidromic secondary spike size induced in calcium-free high magnesium media by 56.07% ± 12.75 (n=4, $P<0.05$) and 76.26% ± 14.67 (n=4, $P<0.05$). Tolbutamide 1 mM decreased the effect of adenosine dramatically to 9.13% ± 2.63 (n=4, $P<0.05$) and 15.36% ± 1.20 (n=4, $P<0.05$) respectively (figure 3.43 and 3.44.). Tolbutamide alone increased the size secondary spikes by 18.88% ± 7.43 (n.s., n=4).

Washout of tolbutamide 1 mM was quick and the normal inhibitory effect of adenosine was seen in about 5 minutes.

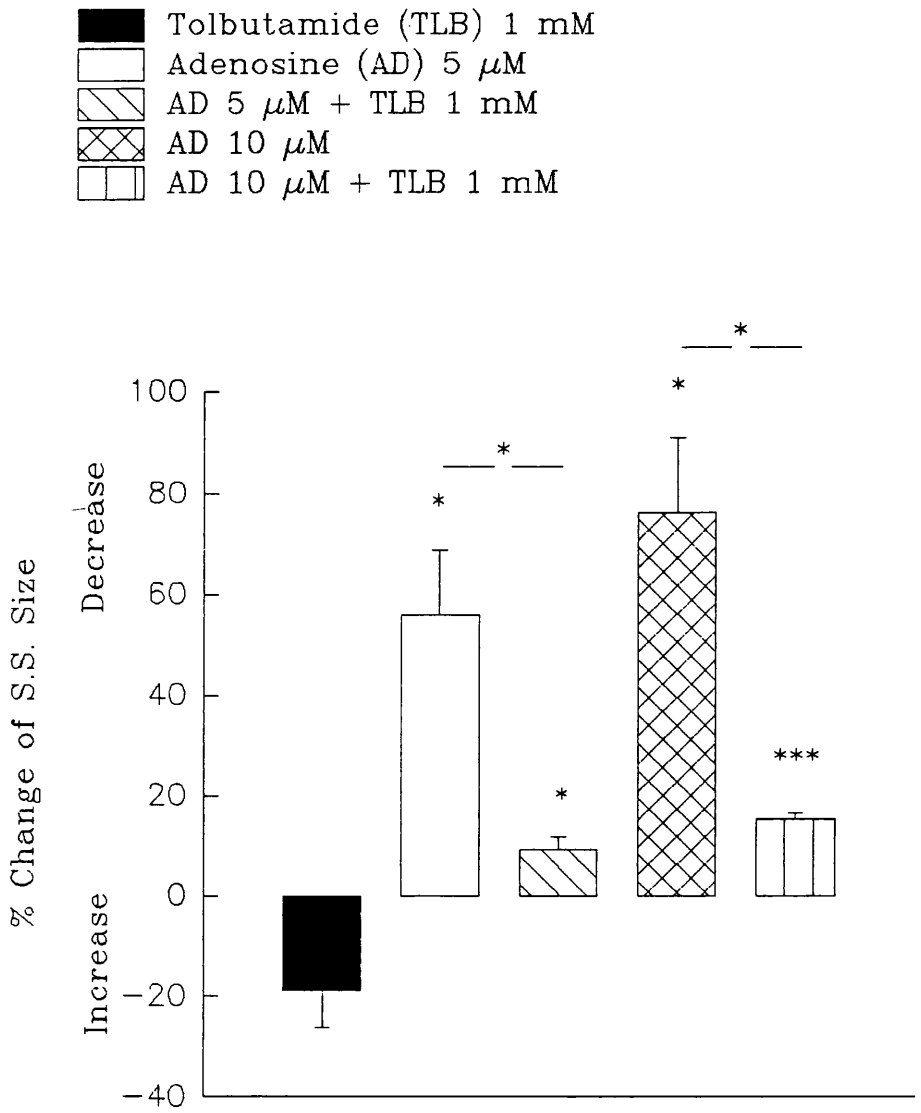

Figure 3.43. Histogram showing the effect of adenosine and tolbutamide (TLB) on antidromically evoked CA1 secondary spikes (S.S.) in calcium-free medium containing 4 mM magnesium. Each point represents the mean ± s.e.m. for n=4. A paired *t* test was employed to determine the significance level (*=P<0.05; ***=P<0.001).

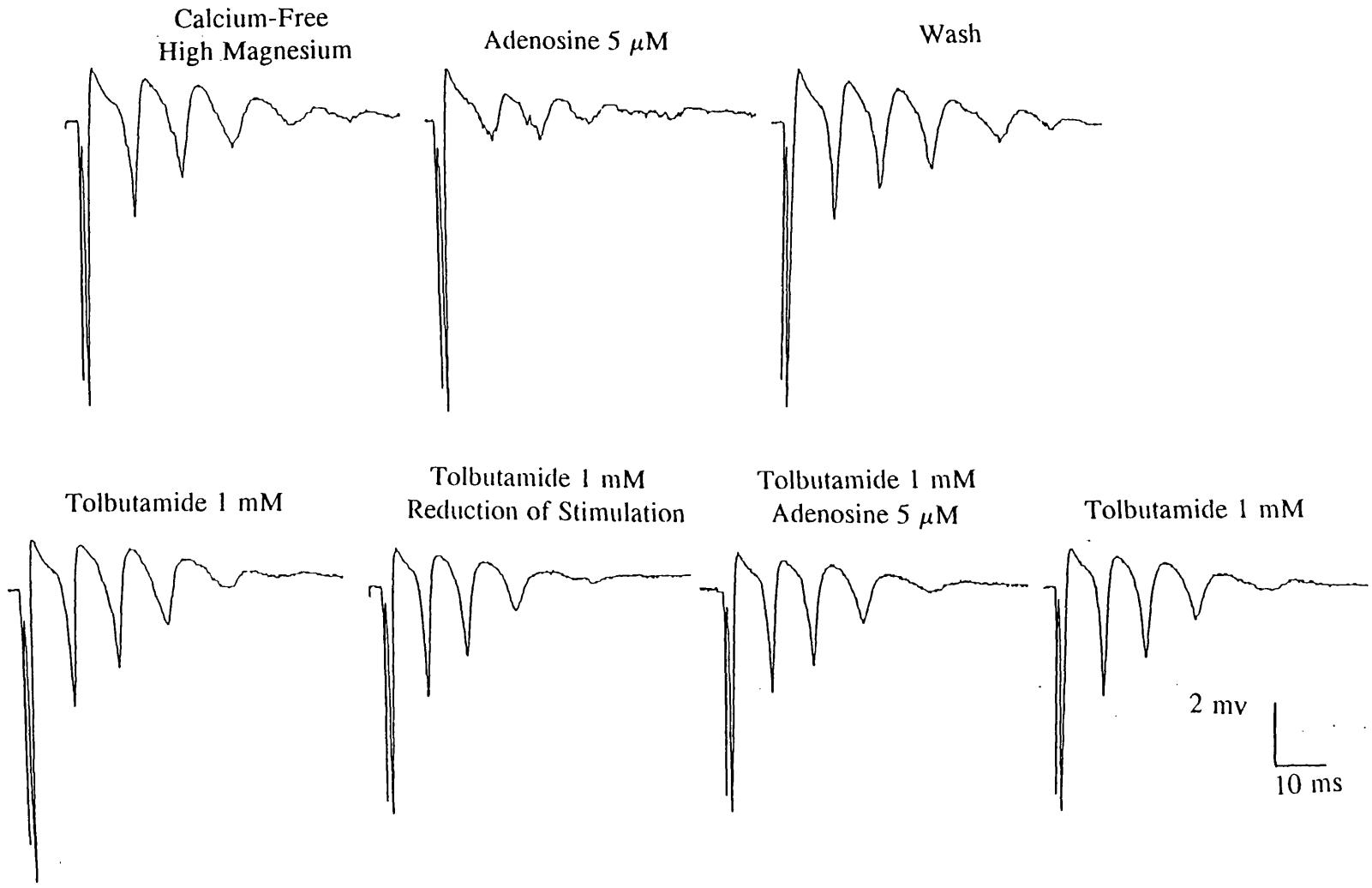

Figure 3.44. Sample records of evoked burst activity and the effect of adenosine and tobutamide on antidromically evoked CA1 secondary spike size in calcium-free medium containg 4 mM magnesium. After the first tolbutamide recorded the strength of the stimulus was reduces so as to restore the potential size to the control level seen before any drug addition.

3.2.3. EFFECT OF BACLOFEN IN THE PRESENCE OF TOLBUTAMIDE

3.2.3.1. INTERACTION OF BACLOFEN AND TOLBUTAMIDE ON ORTHODROMIC POPULATION SPIKES

Baclofen at 1 and 2 μM reduced the amplitude of population spikes by 16.90% ± 1.35 (n=4, $P<0.001$) and 46.87% ± 5.15 (n=4, $P<0.01$) respectively. Tolbutamide had no significant effect on the inhibitory effect of baclofen 1 μM and 2 μM (n=4) and itself increased the size of potential by 8.91% ± 2.73 ($P<0.05$, n=4, figure 3.45.).

3.2.3.2. INTERACTION OF BACLOFEN AND TOLBUTAMIDE ON ANTIDROMIC BURSTS INDUCED IN CALCIUM-FREE HIGH MAGNESIUM MEDIUM

Baclofen at concentrations 1, 2 and 5 μM decreased secondary spike size by 36.91% ± 7.65 ($P<0.05$, n=4), 73.06% ± 15.06 ($P<0.05$, n=4) and 96.92% ± 1.26 ($P\leq0.001$, n=4). Tolbutamide 1 mM had no significant effect on these inhibitory actions of baclofen and alone increased the amplitude of secondary spikes by 25.95% ± 7.67 ($P<0.05$, n=4, figure 3.46.) in this series of experiments.

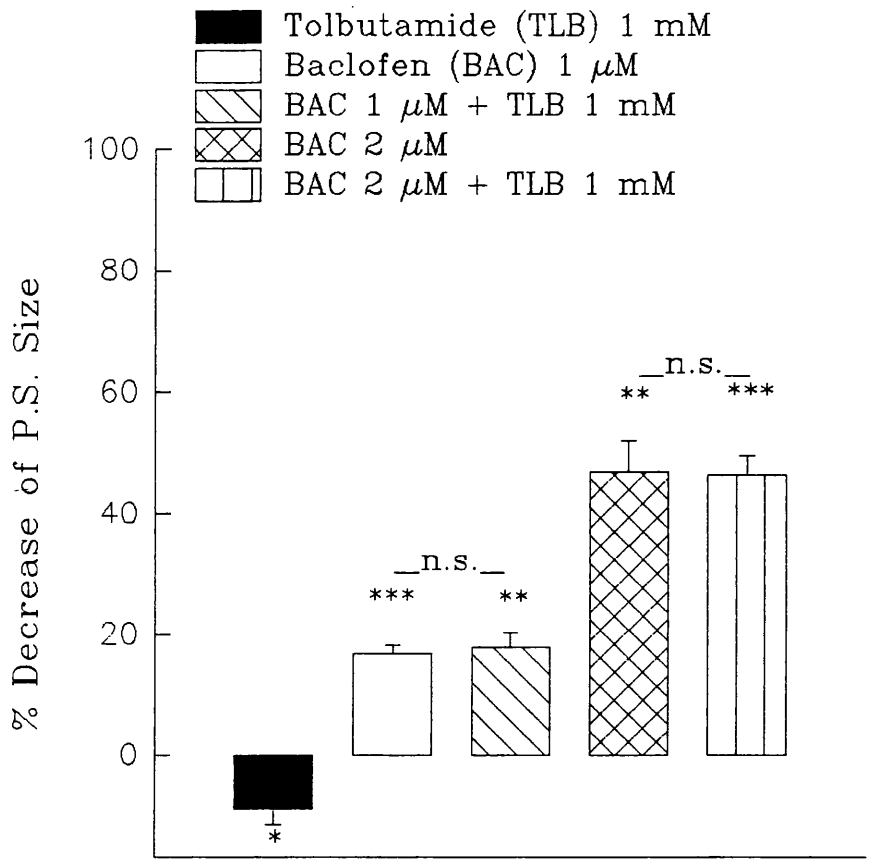

Figure 3.45. Histogram showing the effect of baclofen (BAC) and tolbutamide (TLB) on orthodromically evoked CA1 population spikes (P.S.) in normal ACSF. Each vertical bar represents the mean ± s.e.m. for n=4. A paired *t* test was employed to determine the significance level (*=P<0.05; **=P<0.01; ***=P<0.001). n.s.= non significant.

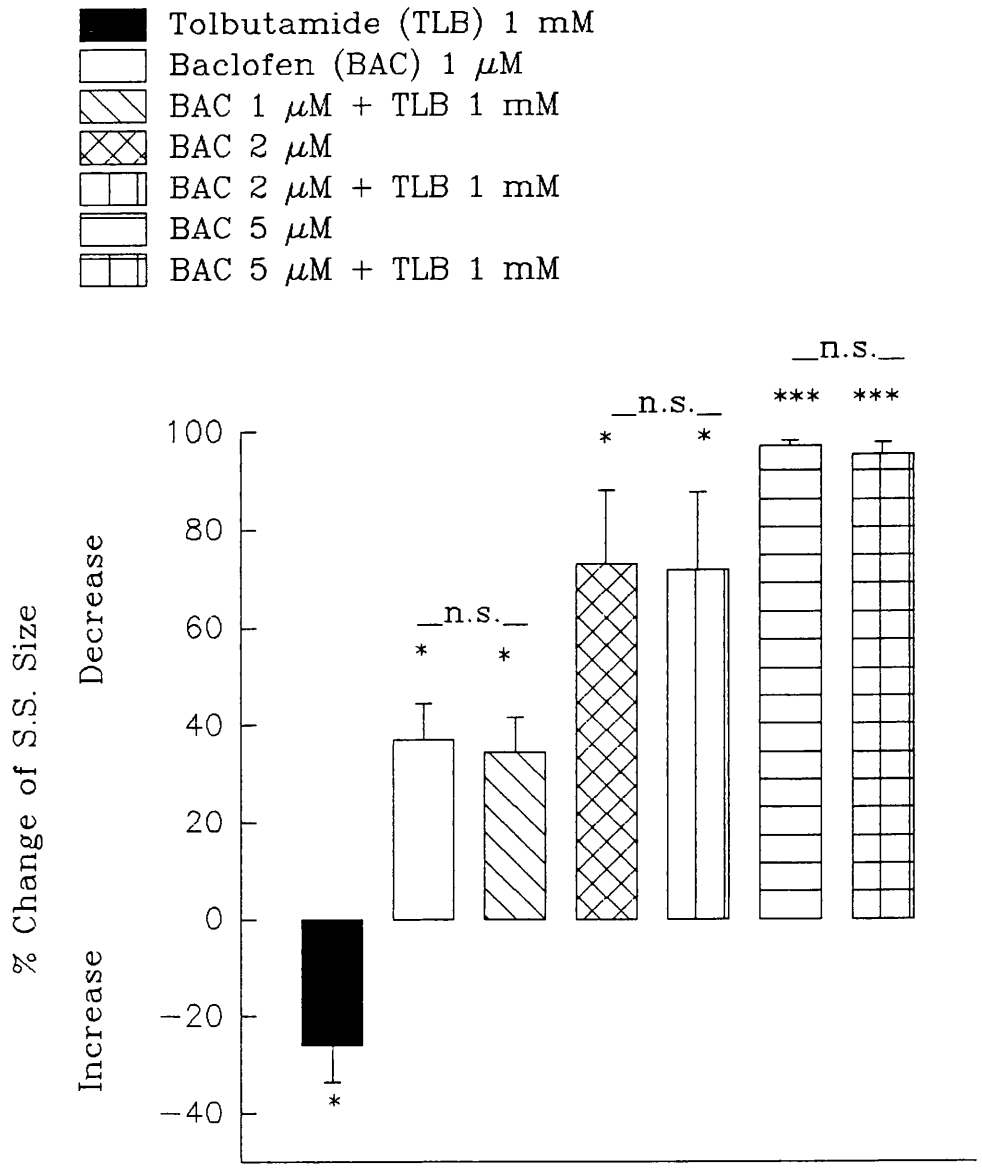

Figure 3.46. Effect of baclofen (BAC) and tolbutamide (TLB) on antidromically evoked CA1 secondary spikes (S.S.) in calcium-free medium containing 4 mM magnesium. Each vertical bar represents the mean ± s.e.m. for n=4. A paired *t* test was employed to determine the significance level (*=P<0.05; ***=P≤0.001). n.s= non-significant.

3.2.4.1. INTERACTION OF ADENOSINE AND LEVCROMAKALIM ON ORTHODROMIC POPULATION SPIKES

Levcromakalim at a concentration of 100 μM had no significant effect itself on orthodromic population spike size of CA1 pyramidal cells (9.26% ± 4.99, n=5, p=0.269, n.s.) but it reversibly potentiated the inhibitory effect of adenosine on the potential size (figure 3.47.).

3.2.4.2. INTERACTION OF BACLOFEN AND LEVCROMAKALIM ON ORTHODROMIC POPULATION SPIKES

In another series of experiments, levcromakalim 100 μM decreased population spikes slightly by 7.35% ± 1.91 ($P<0.05$, n=5) but did not change the inhibitory effect of baclofen applied at 1 or 2 μM (figure 3.48.).

3.2.4.3. INTERACTION OF LEVCROMAKALIM AND TOLBUTAMIDE ON ORTHODROMIC POPULATIUON SPIKES

Tolbutamide 1 mM increased population spike size. Levcromakalim 100 μM had no significant effect on the size of population spikes but decrease the excitatory effect of tolbutamide (figure 3.49.).

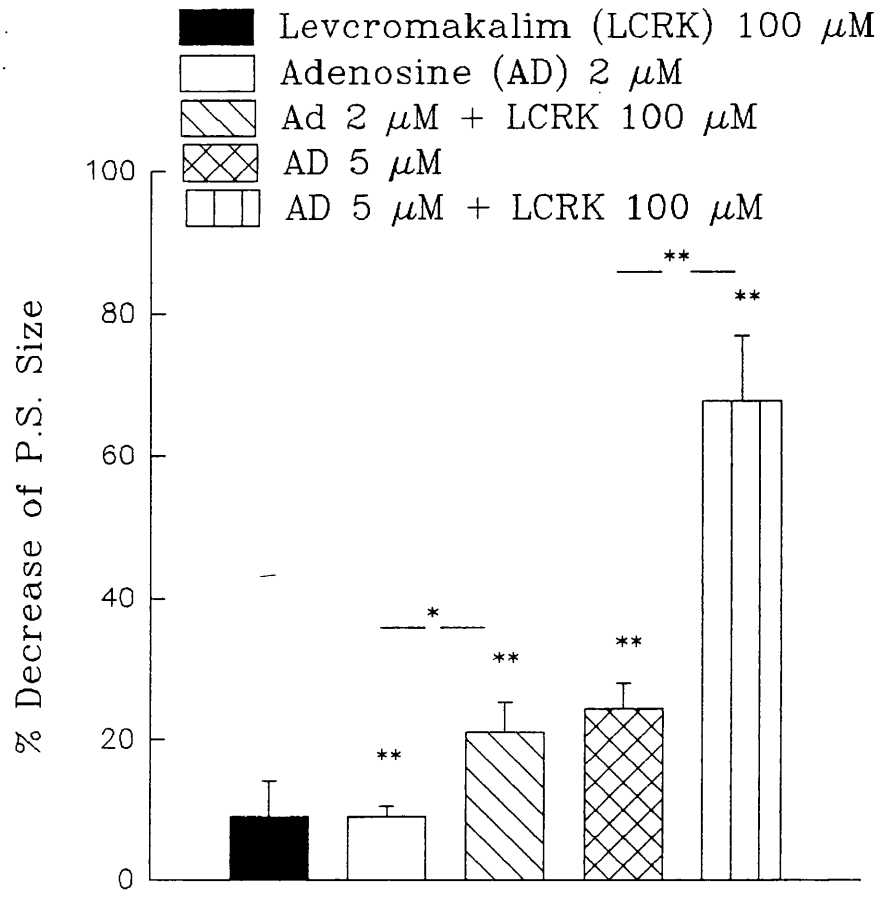

Figure 3.47 Histogram showing the effect of adenosine (AD) and levcromakalim (LCRK) on orthodromically evoked CA1 population spikes (P.S.) in normal ACSF. Each vertical bar represents the mean ± s.e.m. for n=5. A paired *t* test was employed to determine the significance level (*=P<0.05; **=P<0.01).

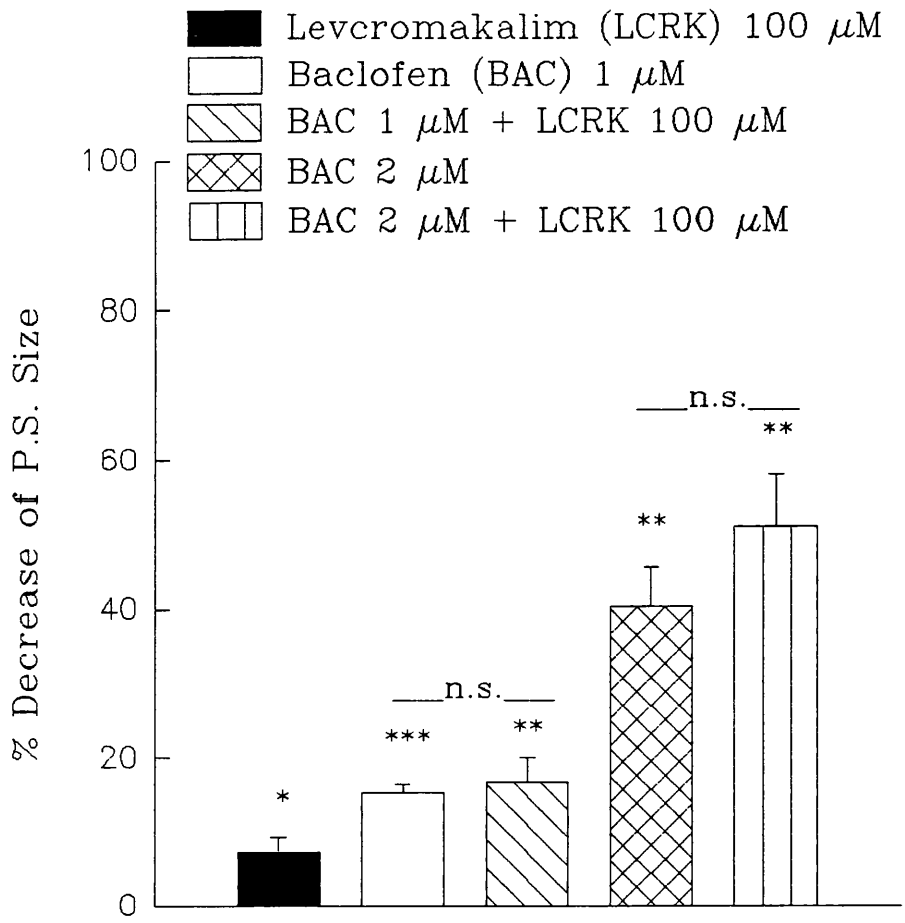

Figure 3.48. Histogram showing the effect of baclofen (BAC) and levcromakalim (LCRK) on orthodromically evoked CA1 population spikes (P.S.) in normal ACSF. Each vertical bar represents the mean ± s.e.m. for n=5. A paired *t* test was employed to determine the significance level (*=P<0.05; **=P<0.01; ***=P≤0.001). n.s.= non-significant.

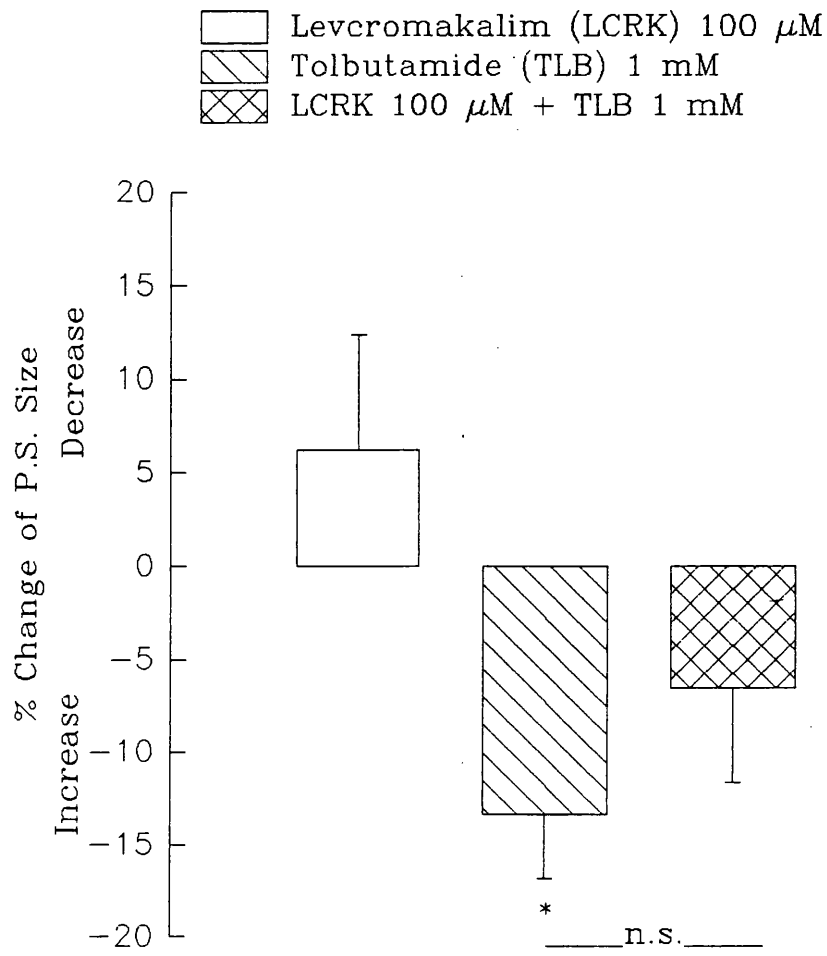

Figure 3.49. Histogram showing interaction of tolbutamide (TLB) and levcromakalim (LCRK) on orthodromically evoked CA1 population spikes (P.S.) in normal ACSF. Each vertical bar represents the mean ± s.e.m. for n=5. A paired *t* test was employed to determine the significance level (*=P<0.05). n.s.= non-significant.

DISCUSSION

4. DISCUSSION

4.1. LOSS OF ADENOSINE POSTSYNAPTIC SENSITIVITY IN CALCIUM-FREE MEDIUM

The main finding of this study was that the sensitivity of postsynaptic neurones to adenosine, as reflected in suppression of epileptiform burst discharges, was lost by removing the calcium.

In line with other literature, lowering or omitting calcium allowed CA1 pyramidal neurones to generate epileptiform bursts after antidromic stimulation (Haas & Jefferys, 1984; Taylor & Dudek, 1984). In normal ACSF, recurrent, feedforward inhibition and calcium dependent potassium mediated afterhyperpolarization oppose sustained neuronal discharges. Lowering extracellular calcium reduces these normal processes and bursts of action potentials appear (Agopyan and Avoli, 1988; Heinemann et al., 1992). Lowering calcium of the medium removes the stabilisation effect of calcium on the membrane surface charges (Begenisich, 1988; Frankenhaeuser & Hodgkin, 1957).

Measurement with ion-selective electrodes during the field bursts showed that extracellular potassium increased and extracellular sodium decreased (Yaari et al., 1983; Haas and Jefferys, 1984).

The action of calcium on the system controlling sodium and potassium permeability probably explains its effect on excitability. A nerve becomes more excitable in low calcium, because a smaller depolarization is required to increase the sodium conductance to the critical level at which the inward sodium current exceeds the outward current carried by potassium and other ions (Frankenhaeuser & Hodgkin, 1957).

Orthodromic population spikes were suppressed by changing from ACSF to nominally calcium-free or low calcium medium. In fact synaptic transmission was abolished with the removing of calcium. Thus this model of antidromic bursts induced in calcium-free medium is independent of synaptic activity and the inhibitory effects of agents refers to their postsynaptic activity.

The finding that slices become much more sensitive to reperfusion of calcium-free media after the production of full bursts with calcium-free or high potassium media and washout with normal ACSF may be related to some kind of kindling phenomenon.

4.1.1. ROLE OF THE CHANGE OF UPTAKE OR METABOLISM OF ADENOSINE ON THE LOSS OF ITS SENSITIVITY IN CALCIUM-FREE MEDIUM

The loss of adenosine sensitivity in calcium-free media is unlikely to be due to a change of uptake or metabolism of adenosine because 2-chloroadenosine and R-PIA, which are not substrates for either the nucleoside transporters or adenosine deaminase, were equally inactive in the absence of calcium. Similarly the lack of effect of HNBTI, an adenosine transport inhibitor (Hertz, 1991), showed that adenosine transport did not change or has no role in the loss of adenosine sensitivity.

4.1.2. ROLE OF HYPEREXCITABILITY ON THE LOSS OF ADENOSINE SENSITIVITY IN CALCIUM-FREE MEDIUM

The loss of efficacy of adenosine and its analogues CPA, R-PIA and 2CADO in calcium-free medium is not only due to the neuronal hyperexcitability because increasing the applied concentration up to as much as 5 mM adenosine or high concentrations of its analogues proved ineffective. Equally, reducing secondary spike size to a clearly submaximal level (less than 50% of maximum) did not restore any adenosine effect.

Of great interest are the results of combining adenosine with baclofen. When applied together with

baclofen, 20 μM which decreased secondary spikes about 50%, adenosine had no greater effect than when applied alone, supporting the earlier conclusion that adenosine's inactivity is not simply the result of neuronal hyperexcitability. The postsynaptic activity of these agonists will be explained more in section 4.3.2.

Stone et al. (1992) introduced one possibility of explaining the apparent excitatory activity of adenosine in no-magnesium low calcium (1.2 mM), on the basis that spontaneous bursts could suppress evoked potential size. If adenosine then inhibited spontaneous bursting, it could induce an increase of evoked potentials, or at least have its inhibitory activity masked. This phenomenon is not likely to explain the present loss of adenosine sensitivity since, even when the CA1 region was isolated from CA3 (the site of origin of most hippocampal bursts), adenosine still had no significant effect on secondary spikes in calcium-free medium.

4.1.3. ROLE OF EXCITATORY AMINO ACIDS IN CALCIUM-FREE MEDIUM

Another possible explanation which was considered, was that some release of transmitters might continue in the absence of calcium. Besides the universal release of neurotransmitters by calcium-dependent exocytosis, the alternative mechanism is sodium-sensitive efflux. For

example, Na^+ influx through Ca^{2+} channels can promote striatal GABA efflux in the absence of extracellular calcium in response to electrical field depolarization (Bernath et al., 1993). Excitatory amino acids participate in the initiation of seizures and their propagation (Dingledine et al ., 1990). The release of glutamate might activate the population of receptors sensitive to N-methyl-D-aspartate (NMDA), an action which has been found to diminish responses to adenosine (Bartrup & Stone, 1990; Bartrup et al., 1991). Such an explanation might also account for the ability of raised magnesium concentrations to increase adenosine responsiveness. Kynurenic acid, the excitatory amino acid antagonist (Perkins & Stone, 1984; Stone, 1993) at a concentration one tenth of that used in calcium-free media profoundly decreased orthodromic population spikes, presumably due to non-selective antagonism of excitatory amino acids in CA1 region (Stone, 1990). However, addition of kynurenic acid had no effect on burst induced by calcium-free or low calcium media.

The failure of kynurenic acid to prevent antidromic bursts does not exclude the calcium independent release of excitatory amino acids but at least shows they are not involved in this kind of burst.

Adenosine concentration-dependently decreased antidromic secondary spikes in high potassium and normal Ca^{2+}. The mechanism of these bursts may be related to: 1) decrease in

the amplitude of K^+-mediated burst afterhyperpolarization; 2) decrease in the amplitude of GABAergic IPSPs, probably secondary to a rise in intracellular [Cl^-]; 3) depolarization of pyramidal cells. These situations can enhance the activation of NMDA receptors by excitatory transmitters acting on CA1 pyramidal cells during bursts (Traynelis & Dingledine, 1988). The nature of this kind of burst is different from calcium-free bursts, since kynurenic acid abolished them, indicating the role of amino acids. Nevertheless, it is clear that, despite release of excitatory amino acids, adenosine was still active to reduce the secondary spikes. This supports the idea that the loss of adenosine sensitivity in calcium-free media is not related to the release of excitatory amino acids.

4.1.4. ROLE OF A_2 RECEPTORS IN THE LOSS OF ADENOSINE SENSITIVITY IN CALCIUM-FREE MEDIA

Bartrup and Stone (1988) showed that in the magnesium-free, reduced calcium solutions adenosine produced an increase of population spike size in half of the slices tested. This action was mimicked by NECA, which has similar potency at adenosine A_1 and A_2 receptors. The authors concluded that removal of magnesium prevents the activation of the A_1 receptor and unmasks activation of an A_2 receptor. In rat striatum slices, CPA induced a rapid functional loss of A_1 receptors (inhibitory on cAMP production time and dose dependently) which was accompanied by a concomitant

increase of A_2 receptor-mediated stimulation of cyclase activity. A_1 adenosine receptor desensitization is characterized by initial loss of receptor function in the absence of any change of receptor number or G-protein defect (Abbracchio et al., 1992). In the hippocampus the A_2 agonist, CGS21680, induced an excitatory effect in CA1 pyramidal cells of hippocampal slices (Sebastião & Ribeiro, 1992). However the very selective adenosine A_1 receptor agonist, CPA had no effect on secondary spikes in calcium-free medium. Thus it is unlikely that A_2 receptor activity is the reason for the loss of adenosine sensitivity.

4.1.5. EFFECT OF ADENOSINE ON ANTIDROMIC BURSTS INDUCED IN LOW CALCIUM OR CALCIUM-FREE PLUS MAGNESIUM

Raising or adding magnesium or calcium to calcium-free medium restored adenosine sensitivity of antidromic bursts. In calcium-free plus 4 mM magnesium, the maximum effects of adenosine, 20 μM, was the same as effect of this agonist in normal ACSF. Other studies (Lee et al., 1984) also showed that adenosine is very active in low calcium plus high magnesium medium to abolish secondary spike size induced by antidromic stimulation in the CA1 region of the hippocampal slice. Also, intracellular recordings from cell bodies showed that adenosine can hyperpolarize the cell effectively, confirming the efficacy of adenosine at postsynaptic sites (Greene & Haas, 1985; Okada & Ozawa, 1980; Segal, 1982; Thompson et al., 1992).

One of the earliest demonstrations of an interaction of cations and adenosine was the effect of magnesium on inhibitory actions of adenosine on adenylate cyclase activity in platelets (Londos & Wolff, 1977). Biochemical studies have shown that divalent cation such as calcium, magnesium and manganese could enhance A_1 receptor adenosine binding (Goodman et al., 1982, Ukena et al., 1984; Yeung et al., 1984). Recently, it was also shown that the divalent cations enhance A_2 receptor adenosine binding (Ji & Jacobson, 1993; Johansson et al., 1992; Nanoff & Stiles, 1993).

Electrophysiological experiments by Bartup and Stone (1988) also supported the concept of a divalent cation requirement for adenosine actions by revealing that a reduction of extracellular magnesium concentration in the medium perfusing hippocampal slices could reduce the potency of adenosine when inhibiting synaptically evoked potentials. However, by studying calcium fluxes, Schubert et al. (1993) have found that, in magnesium-free media, the inhibitory potency of adenosine was lost on stimulus train-evoked fluxes in the synaptic regions of the stratum radiatum, with much less change apparent in the pyramidal cell layer. This implies that the magnesium dependence of adenosine actions may be less marked in the cell body layer from which Bartrup and Stone (1988) made all their recordings, and that the requirement for magnesium may relate primarily to the inter-relationship between adenosine and calcium fluxes.

Recently Smith and Dunwiddie (1993) with an electrophysiological study of the effect of adenosine on fEPSP reported that alterations in cation concentrations do not significantly affect the sensitivity of adenosine receptors, as long as changes are made in such a way as to leave presynaptic Ca^{2+} entry unaffected. In other words, as long as there is no net effect of changes in Mg^{2+} and Ca^{2+} concentrations on transmitter release, then no changes would be expected in adenosine sensitivity. Adenosine sensitivity is dependent not on the absolute level of magnesium, but on the ratio of calcium and magnesium. They concluded that the effects of divalent cations observed in ligand studies probably reflect an effect at an intracellular site, either on the receptor itself, or perhaps on associated GTP binding proteins.

The calcium-dependency of binding at $GABA_B$ receptors has also been reported. In rat brain the binding of [^{3}H]GABA to the $GABA_B$ recognition site is dramatically stimulated by Ca^{2+} with an EC_{50} of about 10 μM. When the free Ca^{2+} concentration was below 22 nM, almost no binding of [^{3}H]GABA to $GABA_B$ sites was detected. These effects were not mimicked by other cations such as Mg^{2+}, Ba^{2+}, La^{3+}, Fe^{2+} and Zn^{2+} (Majewska & Chuang, 1984). In contrast, Hill and Bowery (1981) showed that calcium and magnesium both affect the binding of baclofen in rat brain. There are no studies to demonstrate the effect of calcium or other cations on binding of adenosine and baclofen in parallel but in

independent studies the binding of both agonists are influenced by the cations. In this electrophysiological studies, however, adenosine and baclofen showed completely different behaviour with removal of calcium. This may favour the argument that calcium or magnesium are not directly involved in restoration of adenosine sensitivity.

4.1.6. EFFECT OF THAPSIGARGIN ON ADENOSINE SENSITIVITY IN CALCIUM-FREE MEDIUM

The cation requirement of ligand binding is usually assumed to occur at the inner, cytoplasmic face of the cell membrane. The present results may indicate that a similar requirement exists at the extracellular face of the receptor. Alternatively, it may be that the removal of extracellular calcium also results in a depletion of intracellular calcium associated with membrane function. It was to test this hypothesis that thapsigargin was used. Thapsigargin inhibits the calcium-activated ATPase responsible for the uptake and storage of calcium in the endoplasmic reticulum (Inesi & Sagara, 1992); it should therefore, even in the absence of extracellular calcium, produce a rise in free intracellular calcium levels at least transiently, which might be expected to restore a temporary sensitivity to adenosine. This did not occur. It seems possible, therefore that if adenosine requires calcium it is at the external face of the membrane. This in turn would be consistent with the report of Greene and Haas

(1985) that the intracellular injection of EGTA to buffer internal calcium did not change the hyperpolarization produced by adenosine.

4.1.7. EFFECT OF PD81723 ON RESTORATION OF ADENOSINE SENSITIVITY IN CALCIUM-FREE MEDIUM

To investigate further whether the loss of adenosine sensitivity in calcium-free media is related to a decrease of adenosine receptor binding, the adenosine binding enhancer, PD81723 was used. Janusz et al. (1991) showed that PD81723 increased the binding of the A_1 agonist ligand [^{3}]CHA by up to 160% in rat hippocampus. Also this agent which itself had no effect, potentiated the inhibitory effect of adenosine on orthodromic population spikes. In another study by this group (Janusz & Berman, 1993), PD81723 alone inhibited the duration of antidromic bursts induced in low magnesium in the in vitro hippocampal slice, for which 30 μM gave a maximum effect. On the contrary, in this study, the adenosine binding enhancer alone or with adenosine had no effect on secondary spikes induced in calcium-free medium. Thus there is the possibility that removal of calcium did not decrease the binding to adenosine receptors to cause a loss of sensitivity because the adenosine binding enhancer should then restore to some extent the binding of adenosine. It should be kept in mind however that this agent increased the inhibitory effect of adenosine on orthodromic population spikes about only 20

percent. Other possibilities are that PD81723 binding it itself decreased in calcium-free medium or this agent can act only in situations in which adenosine is active.

4.1.8. DESENSITIZATION OF ADENOSINE

Desensitization is defined as a reduction of the response to a repeated or usually long-lasting stimulus (Lohse, 1993). It may be divided into two categories, i.e., "homologous" and "heterologous" desensitization. The former type refers to the situation in which there is no cross desensitization between different agonists and desensitization is specific for one kind of agonist (e.g., Anand-Srivastava et al., 1989). In heterologous desensitization, the loss of responses to one agonist may be accompanied by a decrease in the effect of other agonists (e.g., Parsons & Stiles, 1987).

Early attempts to show adenosine desensitization in brain slices was unsuccessful (Satin and Rall, 1970). The first report about desensitization of adenosine was in neuroblastoma cells in tissue culture (Green, 1977), although a clear demonstration of this phenomenon was reported by McNeal et al. (1980) in guinea-pig cerebral cortex.

Desensitization to adenosine has been reported in different tissues such as hippocampus (Lee et al., 1986; Porter et al., 1988), striatum (Abbracchio et al., 1992, 1993; Porter et al., 1988), neocortex (Abbracchio et al., 1993; McNeal, 1980; Porter et al., 1988), locus coeruleus (Regenold & Illes, 1990), neuroblastoma (Kelly et al., 1990; Kenimer & Nirenberg, 1981;) liver (Buxton, 1987), kidney (Newman & Levitzki, 1983), smooth muscle (Hayashi et al., 1985) vascular smooth muscle (Anand-Srivastava, 1989; Hussain & Mustafa, 1993), heart or myocyte (Liang & Donovan, 1990; Shryocck et al., 1989), endothelial cells (Luty et al., 1989), mast cell (Marquardt & Walker, 1987), adipocyte cells (Hoffman et al., 1989; Longabaugh et al., 1989; Parsons & Stiles, 1987; Stoneham, 1989). Adenosine desensitization was seen both in vivo (Lee et al., 1986) and in vitro (Abbracchio et al., 1992).

Abbracchio et al. (1992) have recently reported an apparent desensitization to A_1 receptor binding and Porter et al. (1988) demonstrated a decrease of A_2 receptor density and cyclase stimulation in the striatum in vivo.

Most studies of adenosine desensitization have been focused on adenylate cyclase activity (e.g., Anand-Srivastava et al., 1989; Kenimer & Nirenberg, 1981; Luty et al., 1989), although functional effects of adenosine like depressed effects in electrophysiological studies (Regenold & Illes, 1990), glycogenolysis (Buxton et al., 1987),

relaxation of smooth muscle (Hayashi et al., 1985), cardiac myocyte contractility (Shryock et al., 1989) have also been found to exhibit desensitization. In rat locus coeruleus neurones, intracellular recording showed that adenosine reduced the firing rate and caused hyperpolarization. Both effects were transient and fading occurred during contact with the drug. When adenosine was added twice at an interval of 10 minutes, the second application had no or only a slight effect (Regenold and Illes, 1990). Similarly in the present electrophysiological study adenosine lost its effect with repeated application in low calcium medium.

Lee et al. (1986) showed that a brief period of global CNS anoxia resulted in a persistent down-regulation of [^{3}H]CHA binding sites in the CA1 hippocampal pyramidal cells but not in the neocortex or striatum of gerbils. This was later shown however, to be preceded by an up-regulation of adenosine receptors (Kato et al., 1991) within 1 hour of recirculation. The relationship between this work and the present study is unclear, but ischemia may have profoundly different effects from the removal of calcium. In addition, the receptors studied here are restricted to the postsynaptic neuronal surface, whereas the changes occurring in anoxia may be far more diffusely distributed.

Despite these various studies, no desensitization to adenosine has previously been reported in electrophysiological investigations in the hippocampus.

Indeed, Thompson et al, (1992) specifically emphasised that with intracellular recordings from hippocampal pyramidal cells (in normal bathing medium) no decline of sensitivity could be detected following the repeated application of 50 μM adenosine over several hours. In this as in most earlier studies, however, the use of intrasomatic recordings would have detected primarily actions of adenosine exerted directly on the cell body. Dendritic responses would be less apparent, yet it is now recognized that dendritic receptors for adenosine do exist (Tetzlaff et al., 1987), linked to potassium and chloride channels. The use of extracellular recordings of epileptiform activity in the present study should allow any contribution of dendritic receptors to overall neuronal excitability to be observed. However, a direct examination of somatic and dendritic sensitivity revealed that adenosine lost its activity in calcium-free medium, when applied locally to either site. It is therefore concluded that adenosine receptors are similarly affected by calcium-free media along the length of the pyramidal neurone. This also strengthens the view that it is the loss of divalent cations which induces desensitization, and not the manner of recording (i.e. extracellular recording of secondary spikes rather than intracellular recording as in the work of Thompson et al. (1992). In fact in normal ACSF, repeated application of adenosine (cumulative-concentration) or prolonged (60 minutes) superfusion of 10 μM or high concentration (2 mM) did not desensitize. It should be noted, however, that in

the presence of low concentrations of calcium, locally applied adenosine responses showed some tendency to desensitize in the dendrites compared with the cell-body. This may indicate slight differences in the receptors or associated ion channels in the two regions.

The mechanism of actions and sites of desensitization and receptor(s) involved in desensitization depend on factors like design of experiment and the tissue used. Loss of agonist effects may be related to down regulation of receptors, reduction of affinity of agonist to its binding site, uncoupling of receptor from G-protein or changes in the regulatory proteins and their effectors such as adenylate cyclase and ion channels. The emergence of desensitization here is difficult to explain, but may reflect a time-dependent change of receptor state (or G-protein coupling) to an inactive form in low calcium. A change of this type has been characterized for GABA, intracellular calcium promoting a decrease of receptor affinity for GABA in bullfrog sensory neurones (Inoue et al., 1986). Previously reported desensitization to adenosine of neuronal tissue, indicated no change of G-protein or adenylate cyclase subunit function (Porter et al., 1987; Abbracchio et al., 1992). In other tissues the results are controversial. In rat adipocyte tissue, $G_{i\alpha1}$ and $G_{i\alpha2}$ but not $G_{i\alpha3}$ significantly decreased and $G_{s\alpha}$ increased following desensitization with a 6 day application of R-PIA (Longabaugh et al., 1989). However in another in vivo

experiment with 20 hours use of this A_1 receptor agonist Stoneham (1989) could not duplicate the above results in rat adipose tissue. In smooth muscle, a down-regulation of G_s protein and adenylate cyclase were seen to accompany the desensitization of the A_2 receptor system in porcine coronary artery (Hussain & Mustafa, 1993). In contrast, in rat aorta smooth muscle Anand-Srivastava et al (1989) showed that desensitization of NECA-sensitive adenylate cyclase may not be due to the decrease in activity of the catalytic subunit or of guanine nucleotide regulatory protein.

It has been reported that some occlusion occurs between adenosine and baclofen responses which might indicate the involvement of a common potassium channel or G-protein in hippocampal (Nicoll, 1988) or neocortical neurones (McCormick & Williamson, 1989). During the lack of response to adenosine in calcium-free medium, baclofen still was active to decrease or abolish the secondary spikes. This would imply that either adenosine and baclofen are not acting via common potassium channels in the hippocampal CA1 cells, or that desensitization is independent of ion channels and is confined to adenosine receptors or G-protein coupling. In fact the results showing different interactions of tolbutamide and levcromakalim with adenosine and baclofen indicate that the postsynaptic activity of these agonist are different (see section 4.3. for more explanation). Thus it may be possible

that the ionic channels related to adenosine function might be involved in desensitization.

4.1.9. ROLE OF CARBAMAZEPINE AND PROCAINE ON ADENOSINE SENSITIVITY IN CALCIUM-FREE MEDIUM

One extracellular role of calcium is its stabilising action on cell membranes. Thus two stabiliser agents, procaine, a local anaesthetic and the anticonvulsant drug carbamazepine were examined. Carbamazepine and local anaesthetics possess in common the ability to block voltage-dependent sodium channels (Catterall, 1987; Zimányi et al., 1989). Accordingly both were able to reduce or abolish the neuronal spikes.

Using autoradiographic techniques in rat brain Worley and Baraban (1987) showed that the anticonvulsants phenytoin and carbamazepine interact allosterically with scorpion venom batrachotoxin (BTX) binding to sodium channels. Electrophysiologically they also demonstrated that the synaptic block of orthodromic population spikes in the rat hippocampus produced by the sodium channel activators, aconitine and veratridine, was inhibited by phenytoin and carbamazepine but not other anticonvulsants such as diazepam and phenobarbital which did not affect the venom binding. Willow et al. (1984) showed that carbamazepine also blocked the influx of sodium into neuroblastoma cells and rat brain synaptosomes elicited by

batrachotoxin (which activates sodium channels). Local anaesthetics such as tetracaine and procaine also bind to discrete site(s) on sodium channels, which results in block of the channels and allosteric inhibition of BTX binding. Their mechanism of action involves, at least in part, high-affinity binding to inactivated states of sodium channels (Postma and Catterall, 1984).

At concentrations of either agent which produced only a small depression of these potentials, carbamazepine and procaine, yielded a markedly synergistic interaction in combination with adenosine,. This may indicate that a partial blockade of sodium conductance is sufficient to restore adenosine sensitivity and, conversely, that the loss of potency in calcium-free media may be attributable to the increased sodium current. Potentiation cannot simply be the result of combining two inhibitory agents by different mechanisms, since the effect of baclofen was not enhanced in the presence of carbamazepine and also baclofen did not restore the inhibitory effect of adenosine.

This result is reminiscent of that reached by Ribeiro and Sebastião (1984) using preparations of peripheral nerve. In a de-sheathed preparation of the frog sciatic nerve, these authors found that adenosine normally had no effect on action potential size. When voltage-dependent sodium channels were partially blocked by tetrodotoxin, however, adenosine was able to decrease action potential size.

An important influence of sodium conductance is also indicated by direct measurements of extracellular ionic concentrations with ion-selective electrodes during superfusion of hippocampal slices with low calcium media. Under these conditions, as mentioned before, there is a significant decrease of extracellular sodium concentration, consistent with a massive influx of this ion into the intracellular compartment (Heinemann et al., 1992; Yaari et al., 1983).

The mechanism linking blocking of sodium channels and restoration of adenosine sensitivity is not clear. Biochemical studies have shown that sodium chloride can decrease binding of adenosine (Johansson et al., 1992; Stiles, 1988). There is probably a regulatory site for Na^+ ion on the aspartate residue in A_1 and A_2 receptors (Jacobson et al., 1993b). This regulatory site for sodium was also observed for α-adrenergic and the dopamine D_2 receptor, located in the cytosol (Horstman et al., 1990; Motulsky & Insel, 1983; Neve et al., 1991). Thus one possibility is that during calcium-free bursts, the development of high concentrations of intracellular sodium modulate adenosine sensitivity. The calcium ions present in the extracellular medium interact with the external surface of the cell membrane. This interaction is due both to electrostatic attraction of ions by fixed anionic groups and to the binding of ions to such groups. Thus the maximal amplitude of inward sodium current induced by membrane

depolarization can be influenced by calcium. A reduction of external calcium increases the sodium influx and vice versa (Kostyuk & Krishtal, 1977; Kostyuk, 1992). In fact with removal of calcium, sodium can pass through calcium channels (Frankenhaeuser & Hodgkin, 1957). Magnesium can also block calcium channels and thus affect sodium influx (Vormann & Günther, 1993). Therefore part of the mechanism of these ionic effects on restoration of adenosine sensitivity in calcium-free media may be related to a decrease of sodium channel function.

It is not likely that the mechanism of this is activation of the sodium/ potassium exchanger by the increased sodium influx, leading to an increase of extracellular potassium and a loss of potassium gradient (although this does occur: Heinemann et al., 1992; Yaari et al., 1983), since baclofen, like adenosine is believed to act by increasing potassium conductance and yet it is still effective under the conditions of our experiments.

4.2. PARADOXICAL EFFECT OF METHYLXANTHINES IN CALCIUM-FREE MEDIA

The idea for using adenosine antagonists emerged from the restoration of adenosine sensitivity in calcium-free medium by carbamazepine. Since carbamazepine may be an adenosine antagonist (Skerritt et al., 1983a,b; Stone, 1988), xanthine adenosine antagonists, theophylline and cyclopentyltheophylline, were used.

In the present study theophylline showed two opposite effects, an expected excitatory one when examined with orthodromic stimulation in normal ACSF, but an inhibitory one when tested on responses to antidromic stimulation in calcium-free medium.

The results of antidromic stimulation in the presence of normal calcium and bicuculline to induce bursts showed that theophylline did not decrease the potential size. Thus the nature of the stimulation determines the inhibitory effect of theophylline, ie. calcium-free medium is essential for theophylline to exert an inhibitory effect.

Alkylxanthines such as theophylline and caffeine have a variety of actions in the central nervous system or peripheral tissues. These agents are antagonists at the A_1 and A_2 subtypes of adenosine receptor (Daly et al., 1981; Greene et al., 1985), and they inhibit activation of

enzymes like phosphodiesterase (Butcher & Sutherland, 1962; Fredholm et al., 1978), 5'-nucleotidase and alkaline phosphatase (Fredholm et al., 1978). In addition, the xanthines stimulate Ca^{2+} release from internal stores (Lee, 1993; Tanaka & Tashjian, 1993). Uneyama et al., (1993) have also now shown that methylxanthines can block glycine- and GABA-gated chloride currents in freshly dissociated rat hippocampal neurones.

Adenosine normally acts at A1 receptors to induce an inhibition of neuronal excitability. This is often seen as a reduced release of neurotransmitters, especially of excitatory agents such as acetylcholine (Brown et al., 1990b; Spignoli et al., 1984) and glutamate (Corradetti et al., 1984; Fastbom et al., 1985; Lambert & Teyler, 1991), but is also seen as an increase of potasssium conductance (Haas & Greene, 1984). Adenosine antagonists, therefore, including xanthines, usually exert excitatory actions on the nervous system, including an increase in the release of transmitters and an increase in the size of synaptic potentials (Bauman et al., 1992). The epileptogenic activity of xanthines is believed due to the block of adenosine receptors, since adenosine blocks the excitatory or seizure discharge induced by alkylxanthines in the hippocampus of the guinea pig in vitro (Okada & Ozawa, 1980). Theophylline has been shown to increase the frequency of epileptiform bursts of action potentials in the hippocampal slice (Dunwiddie, 1980; Dragunow et al.,

1985; Dragunow, 1988), while the A1 selective antagonist 1,3-dipropyl-8-cyclopentylxanthine causes the development of abnormal excitability in vitro which can far outlast the duration of application (Alzheimer et al., 1989a, 1993). In vivo, cyclopentyltheophylline has been found to induce status epilepticus in vivo in animals subjected to electrical stimulation (Young & Dragunow, 1994).

At sites such as the neuromuscular junction xanthine also have excitatory effects leading to an increased release of acetylcholine (Ribeiro & Sebastião, 1987).

Lowering the extracellular concentration of calcium in media perfusing brain slices blocks synaptic transmission, but leads to the development of bursts of action potentials (Taylor & Dudek, 1982; Haas & Jefferys, 1984). The mechanisms proposed to account for this include loss of the screening action of calcium on surface membrane charges, the result of which is a destabilisation of the membrane (Frankenhauser & Hodgkin, 1957; Agrigoroaei & Neacşu, 1990), and the loss of tonically active potassium conductances which are normally dependent on calcium influx (Agopyan & Avoli, 1988). Nevertheless it seems likely, from the present results, that a small amount of extracellular calcium is needed to maintain epileptiform firing, since inclusion of EGTA in the bathing medium reduces the size of the recorded population spikes.

One possible explanation of the results presented here, therefore, may be that, under conditions of low calcium-induced membrane instability the xanthines displace calcium bound to phospholipids in the neuronal membrane, thus mimicking the effect of EGTA and reducing excitability. This idea is supported by the fact that CPT and EGTA had an additive effect on spike size, and the fact that adding 100 μM calcium diminished the depressant effect of theophylline. Presumably such a displacement of calcium would occur primarily on or near the outer face of the cell membrane to induce the observed depression of excitability.

Adenosine can limit the influx of calcium stimulated by potassium or veratridine into neurones (Wu et al., 1982). Using ion-selective electrodes it has been demonstrated that adenosine can decrease the movements of calcium ions into both presynaptic and postsynaptic sites in the hippocampal slice (Schubert et al., 1986; Schubert, 1992). Conversely. the selective A1 receptor antagonist CPX enhanced stimulus train induced calcium influx in the synaptic and pyramidal cell body layers of the slices (Schubert, 1988). Inhibition of calcium movements therefore represents one possible mechanism by which adenosine could block the xanthines.

Although the precise relationship is not clear, it is possible that the depressant effect of xanthines observed here may be similar in mechanism to that reported by

Ribeiro and Sebastião (1984). These authors found that xanthines shared the ability of adenosine to block action potential production of frog sciatic nerves partially poisoned by tetrodotoxin. To date that report and the present study are among very few to show qualitatively similar neuronal depressant effects of xanthines.

The mechanism by which baclofen was able to prevent the depressant effect of theophylline on population bursts may be related to the movement of calcium postulated above. Gähwiler and Brown (1985) had failed to detect any effect of baclofen on calcium currents in CA3 neurones recorded in organotypic slice cultures of the hippocampus. However, baclofen was later reported to block calcium conductances on dorsal root ganglion neurones (Dolphin & Scott, 1987), and a similar action was observed subsequently by Scholz & Miller (1991b) using dissociated embryonic cells from the hippocampus. It is possible to speculate, therefore, that baclofen restricts the xanthine-induced movement of residual calcium across the neuronal membrane.

Since a major action of baclofen on central neurones is to open potassium channels (Thompson & Gähwiler, 1992), this might represent an alternative means by which the agonist could interact with theophylline. This idea is made attractive by recent evidence that theophylline can activate three types of potassium current, at least one of which remains active in the absence of external calcium,

(Munakata & Akaike, 1993) and which could therefore mediate the inhibitory activity of xanthines described here. However, the concentrations of theophylline used to activate these potassium channels were one to two orders of magnitude greater than the levels used here. In addition, it might be expected that the combination of theophylline and baclofen would be at least additive in producing an inhibition of neuronal excitability by a similar mechanism, whereas baclofen actually abolished the response to theophylline alone.

The anticonvulsant drug carbamazepine also proved able to prevent the inhibitory activity of theophylline on evoked action potential bursts. This may reflect carbamazepine's ability to block neuronal sodium currents (Catterall, 1987), thus acting to stabilise the membrane in a manner comparable with the addition of 100 uM calcium. Alternatively, since carbamazepine has been claimed to suppress calcium channels in ganglion cells (Schirrmacher et al., 1993) and guinea-pig hippocampal slices (Walden et al., 1993) it is possible that, like baclofen, the drug is acting by preventing xanthine induced movements of calcium.

Another different mechanism of the xanthines may be related to inhibition of phosphodiesterase and increase of cAMP (Butcher & Sutherland, 1962; Fredholm et al., 1978), although the concentrations of these agents were very low for this effect. In fact an elevation of cAMP in neurons in

response to neurotransmitters can modulate Na^+ channel function (Li et al., 1992). All agents, adenosine (via A_1 receptor, Londos et al., 1980; Van Calker et al., 1979), baclofen (Wojcik & Neff, 1984) and carbamazepine (Lewin & Bleck, 1977) can decrease levels of cAMP and could thus counteract these effect of theophylline or CPT.

The isolation of CA1 from CA3 also showed that the possible induction of spontaneous activity by the xanthines could not be the reason for their inhibitory effects.

Although CPT more potently antagonized the inhibitory effect of adenosine on orthodromic population spikes than theophylline, the xanthines were equally effective in suppressing antidromic bursts in calcium-free medium. This result may imply that this inhibitory effect is not related to the blockade of adenosine receptors. The fact that adenosine deaminase could not prevent the effect of theophylline also supports this idea.

4.3. DIFFERENT SENSITIVITY OF ADENOSINE AND BACLOFEN TO POTASSIUM CHANNEL BLOCKERS OR OPENERS

The reason that the $GABA_B$ agonist baclofen is clearly able to depress antidromic spikes, but adenosine cannot is not fully clear. One of the differences between adenosine and baclofen is in the location of their receptors. Baclofen ($GABA_B$) receptors are located on both excitatory and inhibitory presynaptic elements and can reduce the amplitude of monosynaptic IPSPs in the presence of excitatory amino acid receptor antagonists (Thompson & Gähwiler, 1992). On the contrary, adenosine receptors are located on excitatory, but not inhibitory, presynaptic elements and have no effect on the monosynaptic IPSPs (Lambert & Teyler, 1991; Thompson et al., 1992; Yoon & Rothman, 1991).

At the presynaptic sites, both agents decrease EPSPs, an effect which is blocked by activation of PKC by phorbol esters. Inhibition of EPSPs was not changed by pertussis toxin or Ba^{2+} (Thompson & Gähwiler, 1992; Thompson et al., 1992).

At the postsynaptic site, which is the main focus of the present, the inhibitory actions of these agents mainly are attributable to hyperpolarization induced by activation of potassium channels (Gähwiler & Brown, 1985; Newberry & Nicoll, 1984; Thompson & Gähwiler, 1992 for baclofen;

Greene & Haas, 1985; Okada & Ozawa, 1980; Segal, 1982; Thompson et al., 1992, for adenosine). There are some reports that adenosine can also induce a steady-state inward current involving a voltage-dependent chloride conductance (Mager et al., 1990, Schubert et al., 1991). In the CNS, the type of potassium channels activated by adenosine and baclofen, however, are controversial. Barium can block the adenosine- (Gerber et al., 1989; Thompson et al., 1992) and baclofen- (Gähwiller & Brown, 1985; Misgeld et al., 1989; Thompson & Gähwiler, 1992) activated potassium conductance in the rat hippocampus but barium has a variety of effects including blocking several different potassium currents such as I_K, I_{KATP} and I_M (Cook, 1988; Quayle et al., 1988). This does not necessarily imply that the same potassium channels are involved. The present results with tolbutamide and antidromically evoked potentials clearly showed that the postsynaptic effect of adenosine and baclofen are different since the inhibitory effect of adenosine on secondary spikes was almost abolished by tolbutamide while this compound had no effect on baclofen sensitivity. This also suggests that the effects of adenosine may involve K_{ATP} channels.

4-Aminopyridine (4-AP): 4-AP is an aminopyridine which is one of the most commonly used group of potassium channel blockers (Cook & Quast, 1990). Depending on the concentration, 4-AP blocks different potassium channels. In the hippocampus, it blocks I_D, a slowly inactivating

potassium current, at low concentration (30 μM) (Storm, 1988), and I_A, a fast transient potassium current, at high concentration (1-5 mM) (Storm, 1990). 4-AP at 5mM had no effect on I_K, the delayed rectifier potassium current in the hippocampus (Rudy, 1988). 4-AP at millimolar levels also blocked K_{ATP} in skeletal and heart muscle (Castle & Haylett, 1987; Haworth et al., 1989; Kakei et al., 1985)By blocking the potassium channel, 4-AP can affect membrane repolarization and result in prolonged action potentials and increased transmitter release and induced synchronous burst discharges (Clark & Wilson, 1992; Voskuyl & Albus, 1985).

Gerber et al. (1989) reported that adenosine had no effect on a 4-aminopyridine (4-AP) sensitive transient (I_A) and tetraethylammonium (TEA) sensitive delayed (I_K) outward currents on hippocampal neurones, but in several areas of brain including the hippocampus (Schubert & Lee, 1986), cerebral cortex (Perkins & Stone, 1980), olfactory cortex (Scholfield & Steel, 1988) and locus coeruleus (Pan et al., 1994) it has been reported that adenosine may activate I_A. Recently Li and Henry (1992) reported that adenosine-induced hyperpolarization was depressed by glibenclamide, a blocker of ATP-sensitive K^+ channels, in rat CA1 neurones.

There are also some reports that $GABA_B$ receptors may activate I_A in hippocampus (Inoue et al., 1985; Saint et al., 1990) or activate K_{ATP} in substantia nigra neurones

(Roeper et al., 1990), although Solis and Nicoll (1992) found that 4-AP at concentrations up to 1 mM did not block baclofen induced outward currents in rat hippocampus.

With intracellular recording in the rat hippocampus, in the presence of 4-AP, adenosine still produced its typical hyperpolarization (Segal, 1982; Greene & Haas, 1985). 4-AP at high concentration (5mM) blocked the inhibitory effect of adenosine on CA1 neurones, although 50 μM adenosine showed no increase of I_A in rat hippocampus slices (Gerber et al., 1989). R-PIA, a stable derivative of adenosine, with high concentration, 5 μM, had low potency against the 4-AP (100 μM) epileptiform bursting. In contrast to the negative interaction between adenosine and 4-AP, Scholfield and Steel (1988) in olfactory cortex of guinea pigs demonstrated that adenosine enhances an aminopyridine-sensitive potassium conductance in nerve terminals. Application of high Mg^{2+} or low concentrations of tetrodotoxin abolished the increased excitability but still 3,4-diaminopyridine blocked the adenosine action. These observations suggest that the action of the aminopyridine on adenosine was not solely the result of increased tissue excitability.

Because of the structural similarity of adenosine and 4-AP, the possibility of an interaction between adenosine and 4-AP at receptor sites was suggested by Perkins and Stone (1980). With a radioligand receptor binding study in

the rat striatum, Drukarch et al.,(1989) also showed that 4-AP could antagonize muscarinic receptor ligand, [^{3}H]-dexetimide, α_1-, α_2- and β-adrenoceptor radioligand, D_1-, D_2-, $5HT_{1A}$-, and $5HT_2$- receptor ligand binding at receptor sites.

250 μM 4-AP completely abolished the inhibitory effect of 50 μM adenosine on the fEPSPs in the rat hippocampus slices. This failure of adenosine to counteract 4-AP may be related to a saturation of the release process by calcium in such a way that the effects of adenosine are masked (Dunwiddie et al., 1991). Klapstein and Colmers (1992) studied the presynaptic effect of 2-chloradenosine, neuropeptide Y (NPY) and baclofen on the rat hippocampus. 4-AP (30 and 100 μM) reduced the inhibitory effect of these compounds on EPSP slope. Low calcium medium (0.75 mM) and the presence of 30 μM 4-AP, restored the effect of NPY and baclofen but not 2-chloradenosine. The authors concluded therefore that presynaptic NPY Y_2 and $GABA_B$ receptors both inhibit transmitter release by inhibition of voltage dependent calcium influx but A_1 adenosine receptors may affect on a different presynaptic mechanism. Thus these result show different sensitivity of adenosine and baclofen to 4-AP.

In general it seems that in those experiments which have been done in the presence of calcium, interaction of adenosine with 4-AP is indirect and may be related to a large release of excitatory amino acids which increase

general excitability. In the experiment of Klapstein and Colmers (1992), which used low calcium and 4-AP, so that neurotransmission continued, it is not posssible to exclude this possibility. Another possibility in relation to the interaction of adenosine with 4-AP may be related to the fact that use of high concentration of this potassium channel blocker may block K_{ATP} as well.

4.3.1. EFFECT OF APAMIN ON THE INHIBITORY EFFECT OF ADENOSINE ON ORTHODROMIC POPULATION SPIKES

One possible explanation for the loss of adenosine sensitivity is that calcium removal results in the loss of ionic conductances needed for the effect of adenosine. If adenosine enhances the activation of calcium-activated potassium conductances, this will not be operating in the absence of external calcium. Adenosine increased the slow AHP produced by increased potassium conductance (Gerber et al., 1989; Greene & Haas, 1985). Apamin is known to block the slow calcium-activated potassium current or I_{AHP} (Halliwell, 1990). Thus to study whether adenosine acts through this potassium current, the interaction of adenosine with apamin was studied.

Apamin is a bee venom which constitutes 2 % dry weight of the venom. Other toxic peptides of the venom are melittin and mast cell degranulating (MCD) peptide (Habermann, 1984). Apamin is an octadecapeptide, slightly

smaller than MCD peptide but of remarkably similar structure, with two intramolecular disulphide bridges and aminated C-terminal carboxyl group (Strong, 1990). It is the unique neurotoxic polypeptide known to pass the blood-brain barrier (Cavey et al., 1978). Apamin is a centrally acting peptide neurotoxin that produces motor hyperactivity and convulsions. This toxin blocked the slow Ca^{2+}-activated K^{+} channel in different tissues such as neocortex (Szente et al., 1988), neuroblastoma and rat muscle cells (Lazdunski et al., 1985), sympathetic neurones (Kawai & Watanabe, 1986) and ganglion cells (Goh & Pennefather, 1987; Nishimura et al., 1988).

With the voltage clamp technique, the slow AHP of rat hippocampus (Storm, 1990) and guinea pig olfactory cortex neurones (Constanti and Sim, 1987) was insensitive to apamin. Howevere, in view of the existence of apamin sensitive channels with high density in the hippocampus demonstrated by autoradiographic technique (Janicki et al., 1984; Ikeda et al., 1991; Mourre et al., 1986) it is not clear why apamin could not block this channel in these studies. In the present study also apamin did not have a consistent effect on the field potential and in two slices had no effect. These results may be related to the nature of some peptides that may be inactivated or may be adsorbed to airstone, tubes or other parts of the experimental appartus.

Apamin did not block the inhibitory effect of adenosine on orthodromic population spikes in this study. Apamin at low concentration blocked the relaxant effect of ATP and ADP (Brown & Burnstock, 1981) and NADP (Burnstock & Hoyle, 1985) but not adenosine and AMP on the carbachol contracted taenia coli of the guinea pig (Brown & Burnstock, 1981). Adenosine-activated potassium current in smooth muscle cells isolated from the pig coronary artery also were not blocked by apamin (Dart & Standen, 1993). Because of the high cost of apamin and the preliminary negative results, interaction of this peptide with adenosine was not studied antidromic bursts.

4.3.2. ROLE OF K_{ATP} IN HYPERPOLARIZATION EFFECT OF ADENOSINE AND BACLOFEN

The present results suggest 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 hyperpolarization by glibenclamide in the hippocampus (Li & Henry, 1992) and a report that in rat ventricular myocytes ATP-sensitive K^+ channels are coupled to adenosine A_1 receptors by G_i (Kirsch et al., 1990). It has been suggested that the activation of this K^+ channel may be involved in vasodilation or vasodepression by

adenosine (Dart & Standen, 1993; Furukawa et al., 1993) although in the pithed rat, no role for glibenclamide-sensitive potassium channels was seen in the action of adenosine A_1 and A_2 receptor agonists on the cardiovascular system (Fozard & Carruthers, 1993b).

The lack of K_{ATP} involvement in the activity of baclofen is consistent with behavioural evidence that, although K_{ATP} channel activation can cause antinociception (Narita et al., 1993) the antinociceptive effect of baclofen is not prevented by gliquidone, a potent K_{ATP} channel blocker (Ocaña & Baeyens, 1993).

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 of using levcromakalim, a K_{ATP} opener, also revealed a different sensitivity of adenosine and baclofen inhibitory actions. In cultured rat hippocampal neurones, glibenclamide produced a concentration-dependent depression of the cromakalim-activated current and single-channel recordings showed that similar channel openings by cromakalim were observed in patches from cells exposed to energy-depleting conditions induced by oligomycin and 2-

deoxy-D-glucose. Glibenclamide inhibited channels activated both by cromakalim and energy-depleting conditions (Politi & Rogawski, 1991). In another study in hippocampal neurones, Tromba et al. (1992) showed K_{ATP} activity which was regulated by extracellular glucose. These channels were open in resting cells and were inhibited by glibenclamide and activated by levocromakalim. In CA3 neurones of the guinea-pig hippocampus in vitro, Alzheimer et al. (1989b) showed that the cromakalim-activated potassium channel causes inward rectification and among a number of potassium channel blockers as well as tolbutamide, only caesium and barium were able to inhibit the cromakalim-induced effects. Also using patch clamp techniques on frog skeletal muscle, Quayle et al. (1988) showed that barium and caesium can block the ATP-sensitive K^+ channel. In this study levcromakalim reduced the excitatory effect of tolbutamide by about 50% although this was not statistically significant. It is possible that these agents have effects on different potassium channels besides K_{ATP}.

In isolated substantia nigra neurones, tolbutamide has been claimed to reverse membrane hyperpolarization induced by activation of dopamine D_2 receptors and $GABA_B$ receptors (Roeper et al., 1990), although in the same brain region Hicks and Henderson (1992) used applications of tolbutamide or glibenclamide and concluded that the D_2 receptor does not show the pharmacological characteristics of an ATP-sensitive potassium conductance. The $GABA_B$ receptor was not studied by this group.

When a maximal hyperpolarization response had been evoked with baclofen, Nicol (1988) found that adenosine was unable to produce any additional response. It was proposed that the receptor mechanisms activated by the two agonists might converge onto a common potassium channel. A similar proposal was made as a result of studies on cells in the cerebral cortex (McCormick & Williamson, 1989). Although this conclusion differs from that drawn from the present work, it may be related to the use of maximal concentrations of agonists by these authors. In particular, maximal concentrations may recruit a population of potassium channels which can be activated by both agents and mask the differential activation of separate channels seen in the present work.

Finally, a totally different explanation of these results should be considered. Sulphonylurea compounds which block K_{ATP} channels can also release glutamate (Kwiecien et al., 1993; Zini et al., 1993). The release of glutamate might activate the population of receptors sensitive to N-methyl-D-aspartate (NMDA), an action which has been found to diminish responses to adenosine (Bartrup & Stone, 1990; Bartrup et al., 1991). However kynurenic acid, the excitatory amino acid antagonist at high concentration (5 mM) had no effect on antidromic action potential bursts induced by calcium-free media. Thus the activation of NMDA channels or glutamate release by tolbutamide and consequent suppression of adenosine sensitivity is unlikely. In

addition, such an explanation would be expected to diminish the presynaptic effect of adenosine (effect on fEPSPs) as reported previously (Bartrup & Stone, 1990; Bartrup et al., 1991), whereas this was not observed in the present work.

The results of interaction of adenosine and the two stabilising agents suggest two conclusions. Firstly, it is clear that adenosine can still cause neuronal inhibition even in the absence of extracellular calcium, provided that sodium conductance is limited. Secondly, in the central nervous system as in the periphery, adenosine has inhibitory effects which are in part determined by the state of voltage-dependent sodium channels or the extent of sodium influx across the membrane. It is possible that, based on the results described here, interactions involving synergistic inhibitions between carbamazepine and endogenous adenosine could contribute to the anticonvulsant activity of carbamazepine and related agents.

The xanthines theophylline and CPT depressed the excitability of hippocampal pyramidal neurones in calcium-free media. This effect was prevented by adenosine, which had no effect by itself, but was not reproduced or modified by adenosine deaminase. The xanthine effects were also prevented by baclofen and carbamazepine. A common feature of adenosine, baclofen and carbamazepine which may account for their antagonism of the xanthines is the blockade of calcium channels or inhibition of cAMP. It is therefore

proposed that in the presence of low external concentrations of calcium xanthines can reduce excitability by promoting the movement of residual calcium in the medium or neuronal membranes.

With respect to the different sensitivity of adenosine and baclofen to calcium-free medium and a potassium channel blocker (tolbutamide) or opener (levcromakalim) it is concluded that the mechanisms of postsynaptic activity of adenosine and baclofen are clearly different and at postsynaptic, but not presynaptic, sites adenosine may activate ATP (tolbutamide or cromakalim)-sensitive potassium channels. The results of using procaine, carbamazepine and the adenosine binding enhancer (PD81723) also showed that the requirement of calcium for the postsynaptic effects of adenosine in calcium-free medium may be indirect and related to its stabilising action.

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5. REFERENCES

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