AN INVESTIGATION OF PAIRED PULSE INTERACTIONS BETWEEN EVOKED FIELD POTENTIALS IN NORMAL AND BICUCULLINE-SUPERFUSED RAT HIPPOCAMPAL SLICES

by

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A Thesis Submitted in Fulfilment of the Requirements for the Degree of Doctor of Philosophy in the University of Glasgow

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August, 1996

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ABSTRACT

1. This thesis is concerned with the investigation of interactions which can be demonstrated in the CA1 area of the hippocampal slice between pairs of evoked field potentials recorded extracellularly in the stratum pyramidale.

2. Paired-pulse inhibition and paired-pulse facilitation of orthodromic population spikes by a previous conditioning stimulus were readily demonstrated using a single stimulating electrode placed in the stratum radiatum. As described by other investigators, the nature of the interaction observed depended on the interstimulus interval between test and conditioning stimuli. Inhibition predominated at short interstimulus intervals, changing to facilitation as the interstimulus interval was increased. Decreasing the strength of the conditioning stimulus increased the magnitude of the paired-pulse inhibition or changed facilitation to inhibition.

3. When the GABA-A antagonist bicuculline was added to the superfusate the single population spike normally recorded in the stratum pyramidale in response to a single stimulus in the stratum radiatum became a short burst of spikes up to 40 ms long. Under these conditions of background pyramidal cell disinhibition, using a single stimulating electrode in the stratum radiatum and a conditioning stimulus that was supramaximal for the size of the evoked potential, a consistent pattern of paired-pulse interactions was observed. Two temporally distinct phases of paired-pulse inhibition were seen, at short (< 50 ms) and at intermediate (200 to 500 ms) interstimulus intervals. At an interstimulus interval of 100

ms inhibition either showed a marked minimum or facilitation was seen. At interstimulus intervals longer than 900 ms there was a second phase of facilitation.

4. At interstimulus intervals less than 50 ms and between 300 and 900 ms, 10 μM bicuculline significantly increased inhibition or decreased facilitation compared with drug-free control in the same slices. However when the interstimulus interval was 100 ms the slices showed significantly increased facilitation in the presence of bicuculline.

5. During bicuculline superfusion, as in the bicuculline-free slice, decreasing the strength of the conditioning stimulus increased the magnitude of the paired-pulse inhibition or changed facilitation to inhibition.

6. The medium-latency (interstimulus interval of 300 ms) bicuculline-enhanced paired-pulse inhibition was decreased by the GABA-B antagonist 2-hydroxysaclofen, and by the GABA-B agonist baclofen, strongly suggesting that it was at least partly mediated by GABA released from interneurones and acting at GABA-B receptors.

7. Experiments using antidromic test stimuli to determine whether the relevant GABA-B receptors were presynaptic on the Schaffer collateral terminals or postsynaptic on the pyramidal cells were inconclusive.

8. Several lines of evidence suggested that there is an additional postsynaptic component to the medium-latency bicuculline-

enhanced paired-pulse inhibition which is not GABA- mediated and is probably related directly to the recurrent firing of the pyramidal cells. In particular, during bicuculline superfusion, the addition of baclofen or 2-hydroxysaclofen had no effect on the paired-pulse inhibition of antidromic test potentials by orthodromic conditioning potentials. Furthermore, a similar paired-pulse inhibition was demonstrated between antidromic potentials in nominally calcium-free superfusate in the absence of added agents.

9. Adenosine decreased medium-latency bicuculline-enhanced paired-pulse inhibition of orthodromic test potentials in that paired-pulse inhibition in the presence of adenosine was less than for non-adenosine superfused control potentials of the same size. In addition, adenosine inhibited the conditioned potential, which is already reduced by paired-pulse inhibition, significantly less than a matched unpaired potential. The validity of this conclusion was further supported by simple computer modelling of the effects of evoked potential size on susceptibility to inhibition.

10. A comparison was made of the concentration-response relationships for the effects of adenosine to reduce paired-pulse inhibition and to reduce the size of single evoked potentials. The experiment was carried out both during bicuculline superfusion and in the absence of bicuculline. The results ruled out the possibility that the effect of adenosine on paired-pulse inhibition was caused by the different sensitivity of two populations of adenosine receptors, those on excitatory terminals to interneurones and those on excitatory terminals to the pyramidal cells.

11.Adenosine was less effective at reducing bicuculline-enhanced paired-pulse inhibition when the conditioning stimulus was supramaximal at 1 mA.

12.These findings are all compatible with the effect of adenosine to reduce bicuculline-enhanced paired-pulse inhibition being mediated by an increase in simultaneous paired-pulse facilitation. There is some difficulty however in integrating this explanation with the residual calcium theory of paired-pulse facilitation.

13.Bicuculline-enhanced medium-latency paired-pulse inhibition of an orthodromic test potential was reduced by 1,3-dimethyl-8-cyclopentylxanthine (cyclopentyltheophylline, CPT) and adenosine deaminase. Some of this reduction was related to the occurrence of spontaneous burst potentials during superfusion of these agents and bicuculline together, but the results are consistent with the hypothesis that the release of endogenous adenosine by the conditioning stimulus contributed to the genesis of bicuculline-enhanced paired-pulse inhibition at an interstimulus interval of 300 ms. An alternative explanation, that paired-pulse inhibition was decreased by these agents as a result of a non-specific increase in neuronal excitability could not be ruled out.

14.CPT reduced short-latency (30 ms) paired-pulse inhibition of orthodromic test potentials in bicuculline- free slices. Again, this is consistent with this form of paired-pulse inhibition being partly mediated by an endogenous P1-receptor agonist although this seems intrinsically unlikely. Alternatively, a single as yet unexplained mechanism may underlie both the effect of CPT on

short latency paired-pulse inhibition in bicuculline-free slices and CPT and adenosine deaminase on bicuculline-enhanced paired-pulse inhibition.

ACKNOWLEDGMENTS

I would like to thank my supervisor Professor T.W. Stone who opened a way for me from clinical medicine into scientific research and who has provided guidance, intellectual stimulation, support and encouragement over the past few years.

I would also like to thank the many other individuals who have provided informal support and friendship during this project. In particular:

Dr Julian Bartrup, who patiently helped with many of my day to day technical problems when I was a beginner.

My fellow postgraduate students, postdoctoral colleagues, and members of the academic and technical staff of what was the Department of Pharmacology at Glasgow University and is now subsumed into the Institute of Biological and Life Sciences.

My wife Valerie.

I was supported by a Medical Graduate Fellowship from the Wellcome Trust for period of this research

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Higgins MJ, Stone TW (1993) Bicuculline-resistant inhibition between paired evoked extracellular potentials in the rat hippocampal slice. *Br J Pharmacol* **109**:29P.

Higgins MJ, Stone TW (1993) Bicuculline-resistant paired-pulse inhibition is reduced by adenosine. *Inter Congr Physiol Sci*: Abst XXXII.

Papers

Higgins MJ, Stone TW (1993) Bicuculline-resistant paired-pulse inhibition in the rat hippocampal slice. *Br J Pharmacol* **109**:1164-1168.

Higgins MJ, Stone TW (1995) Effect of adenosine on bicuculline-resistant paired-pulse inhibition in the rat hippocampal slice. *Hippocampus 5:209-216.*

Higgins MJ, Stone TW (1996) Comparative sensitivity to adenosine of paired-pulse inhibition and single field potentials in the rat hippocampus. *Neurosci Lett* **209**:69-72.

CHAPTER 1
INTRODUCTION

This thesis is concerned with the investigation of interactions, inhibition and facilitation, which can be demonstrated in the CA1 area of the hippocampal slice between pairs of evoked field potentials recorded extracellularly in the stratum pyramidale.

I Rationale

The initial observation underlying the work occurred whilst attempting to define a simple experimental paradigm to quantify inhibitory interneurone activity using paired-pulse inhibition between extracellularly-recorded field potentials, paired-pulse inhibition being the inhibition of an evoked potential by a preceding conditioning potential.

Other investigators have used paired-pulse inhibition of field potentials to quantify inhibitory neurone activity both in the intact hippocampus in vivo (for example Gilbert, 1991; Milgram et al., 1991) and in slices (for example King et al., 1985; Kostopoulos and Antoniadis, 1991; Zhao and Leung, 1991).

Paired-pulse inhibition is well described in the hippocampus, both in the Schaffer collateral / commissural-CA1 pathway and in other well characterised circuitry such as the dentate gyrus-CA3 pathway via the mossy fibres. Paired-pulse inhibition has been described both between individual cell responses using single cell recording (for example

Schwarzkroin, 1975; Lee et al., 1980) and between evoked population potentials recorded extracellularly (see below), the interstimulus interval between the test or conditioning potential being of the order of a few tens of milliseconds.

In both cases the paired-pulse inhibition is widely accepted to be caused by the action of local inhibitory interneurones releasing 4-γ-aminobutyric acid (GABA) as transmitter and activated either directly by the first (conditioning) stimulus or recurrently from pyramidal cells activated by the first stimulus. Since paired-pulse inhibition between extracellularly recorded field potentials is observed most markedly and consistently when the interstimulus interval is around 15 to 100 ms, which corresponds to the time course of the GABA-A mediated fast inhibitory potential recorded intracellularly in pyramidal cells (Davies et al, 1990), it is thought to be largely caused by the activation of GABA-A receptors. In addition, low concentrations (0.7 to 1 μM) of the GABA-A blocker bicuculline (Curtis and Felix, 1971; Curtis et al., 1971a, 1971b; Johnston et al., 1972) reduce paired-pulse inhibition in the CA1 area (Karlsson and Olpe, 1989).

Paired-pulse inhibition of population spikes recorded in the stratum pyramidale of the CA1 region and evoked from a single stimulating electrode in the stratum radiatum was readily demonstrable here, using interstimulus intervals of a few tens of milliseconds.

An initial attempt was made to confirm the proposition that this phenomenon was an example of GABA-A mediated inhibition by examining the effect of bicuculline. GABA-A antagonists, however, increase the excitability of the pyramidal cells by blocking the effects of released GABA. The single population spike normally recorded in the stratum pyramidale in response to a single stimulus in the stratum

radiatum or the alveus then becomes a short burst of spikes which may be 40 ms long. The bicuculline in the experiment described here was superfused at a concentration of 10 μM which would result in more complete GABA-A blockade than that employed by Karlsson and Olpe (1989). Under these conditions of background pyramidal cell disinhibition, paired-pulse inhibition at short latency between the evoked bursts was actually increased. In itself this is not very remarkable and probably reflects that fact that the second (test) stimulus is occurring at a time when some of the pyramidal cells are either still firing or are refractory from the first stimulus. However, in addition to this increase in short-latency paired-pulse inhibition during superfusion with bicuculline it was noticed that it was possible, in several slices, to elicit paired-pulse facilitation between the burst potentials at an interstimulus interval of about 100 ms and to demonstrate a second, marked and very consistent, paired-pulse inhibition when the interstimulus interval was about 300 ms. At longer latencies still it was possible to demonstrate a second period of facilitation. This pattern of paired pulse interactions between field potentials in the disinhibited CA1 area of the hippocampus, with its striking and consistent time course had not been previously described. Particularly interesting was longer-latency inhibition occurring after the facilitation at 100 ms. and its investigation forms the basis for most of the work documented here.

II Overview

Experiments were undertaken to systematically characterise paired-pulse interactions between orthodromic potentials in the absence of exogenous agents and to examine the action of bicuculline on these effects. A series of preliminary investigations was then undertaken into the mechanism of

the bicuculline-enhanced medium latency inhibition. The effect of several agents were examined that might be expected to influence the activity of inhibitory interneurones or to antagonise the endogenous inhibitory substance, adenosine.

In order to continue these investigations into the enhanced inhibition in more detail and, in particular, to possibly separate postsynaptic effects on pyramidal cell excitability from presynaptic effects on Schaffer-collateral terminals, a series of experiments was next undertaken to characterise the effect of bicuculline on paired-pulse inhibition of antidromic field potentials. Some experiments into the mechanism of antidromic paired-pulse inhibition were also carried out at this time.

The effect of baclofen, which inhibits GABA release from interneurones in addition to depressing excitatory transmission, the effect of the GABA-B antagonist, 2-hydroxysaclofen, and the effect of adenosine which depresses excitatory transmission but not GABA-release, were next examined on bicuculline-enhanced paired-pulse inhibition of both orthodromic and antidromic potentials. These experiments were intended to test the hypotheses that an important contribution to the enhanced inhibition was made by GABA-B receptors activated by GABA released from inhibitory interneurones. In addition it was hypothesised that the enhanced paired-pulse inhibition was, at least in part, mediated presynaptically.

An unexpected finding was that the action of adenosine in this paradigm was somewhat anomalous in that its effect on paired-pulse inhibition appeared to depend on the strength of the conditioning stimulus used to invoke the inhibition. Further investigations were thus made to clarify and attempt to explain this result.

Finally, the possibility was investigated that the action of endogenous adenosine might contribute to bicuculline-enhanced paired-pulse inhibition. As a consequence of these experiments the effect of the A1 adenosine antagonist 1,3-dimethyl-8-cyclopentylxanthine (cyclopentyltheophylline, CPT), was also examined on short-latency paired-pulse inhibition in the absence of bicuculline.

The results of these experiments are presented sequentially in Chapter 3 and discussed in detail in Chapter 4. Chapter 4 also contains a presentation of a simple computer model of pyramidal cell activation which was developed to clarify aspects of the relationship between response size, stimulus strength, and transmission at the Schaffer collateral / pyramidal cell synapses. A full listing of this program is given in the Appendix. Experimental methods are described in detail in the next chapter. The remainder of this chapter is concerned with reviewing relevant aspects of the physiology and pharmacology of paired-pulse interactions in the hippocampus, GABA, and adenosine.

III Neuronal Circuitry in the CA1 Area

Excitatory Pathways

The arrangement of the main excitatory pathway involving the CA1 pyramidal cells is shown schematically in Figure 1. The main excitatory input is from axon collaterals (Schaffer collaterals) of the CA3 pyramidal cells; the last stage of the tri-synaptic pathway from the entorhinal cortex which synapse with the pyramidal cell apical dendrites in the stratum radiatum (Andersen et al., 1966, 1971b). The main output from the CA1 pyramidal cells is to the subiculum. CA1 axons are shown bifurcating in the alveus in Figure 1 as they appear in classical histological studies (Lorente de No, 1934). However, Andersen et al.

(1973) in functional studies failed to find evidence of output in a fimbrial direction. In addition to the apical dendritic input, CA1 pyramidal cells also receive excitatory input to their basal dendrites from axons running in the stratum oriens. These includes commissural fibres from the contralateral hippocampus (Andersen et al., 1977).

Inhibitory Pathways

The basic pathways in the hippocampus were demonstrated by early electrophysiological studies. Functionally, feedback and feedforeward inhibitory pathways have been identified. In vivo electrophysiological recordings in deafferentated CA2 and CA3 pyramidal cells identified hyperpolarising inhibitory postsynaptic potentials (IPSPs) which could be evoked by antidromic stimulation of axons in the fornix with a delay of 2 to 5 ms (Kandel et al.,1961; Spencer and Kandel 1961; Andersen et al., 1964a). Current density analysis of the extracellular potential (Andersen et al., 1964b) suggested the inhibitory terminals were on or close to the pyramidal cell soma. Schwartzkroin and Mathers (1978) and Ashwood et al. (1984) described intracellular recordings from feedforward inhibitory interneurones in the CA1 area which could be activated by stimulation in the stratum radiatum. The interneurones could be activated at lower levels of stratum radiatum than the pyramidal cells, often fired before the pyramidal cells and often responded to a single stimulation with a burst of action potentials.

Subsequent physiological studies have confirmed this basic pattern of both feedforward and feedback inhibition while adding a considerable amount of detail (see for example Lacaille et al., 1987; Lacaille and Schwartkroin, 1988 for intracellular recordings from coupled pyramidal cells and interneurones in the CA1 area). Stimulation of afferents in the

stratum oriens activates feedforward inhibition similar to that evoked by stratum radiatum stimulation (Lacaille et al., 1987).

Histological studies using staining for glutamic acid decarboxylase confirmed a variety of GABAergic cell types including basket cells, with axons that ramify widely particularly in the longitudinal plain of the hippocampus i.e. perpendicular to the plane of the tri-synaptic pathway (Ribak et al., 1978, Struble et al., 1978).

IV Paired-Pulse Interactions in the CA1 Area

Paired-pulse effects between single population spikes in the hippocampus have been well characterised both in vivo and in vitro. The nature and extent of the interaction is strongly dependent on the configuration of the inputs and the temporal separation between the two stimuli (interstimulus interval).

In vivo, in the CA1 area, when test and conditioning stimuli both excite the same orthodromic pathway in the stratum radiatum, paired-pulse interactions show a biphasic pattern with inhibition at shorter interstimulus intervals up to between 80 and 200 ms and facilitation at interstimulus intervals greater than this. Facilitation may last up to several seconds (Stefensen and Henriksen, 1991). Andersen (1960) describes a similar pattern but with the change to facilitation at shorter interstimulus intervals. This pattern is different to that seen in the dentate gyrus where orthodromic paired-pulse stimulation of granule cells has a triphasic pattern with a second prolonged phase of inhibition occurring between 200 ms and 4 seconds following facilitation at interstimulus intervals between 40 and 200 ms.

In the CA1 region of the hippocampal slice preparation, with the same configuration of inputs the pattern of paired-pulse interactions is

similar to that described in vivo. Inhibition tends to occur at short interstimulus intervals with facilitation being seen when the interstimulus intervals is greater than about 40 ms (Dunwiddie et al., 1980). The latency at which inhibition changes to facilitation is shorter in the slice than in the intact animal (Dunwiddie et al., 1980). Paired-pulse facilitation can sometimes be seen up to an interstimulus interval of several seconds (Creager et al., 1980; Dunwiddie et al., 1980). Paired-pulse facilitation is strictly homosynaptic, requiring the same synapses to be stimulated twice in succession. It is not seen when different electrodes are used to stimulate different populations of afferents with the conditioning and test stimuli (Creager et al., 1980).

Inhibition of an orthodromic population spike may also be demonstrated at interstimulus intervals up to 100 ms when a conditioning stimulus sufficient to generate an antidromic population spike is given to the alveus (Creager et al., 1980; Dingledine and Langmoen, 1980; Lynch et al., 1981). Paired-pulse inhibition can demonstrated using a conditioning stimulus which stimulates a different population of afferents in the stratum radiatum to the test stimulus and it may be evoked by orthodromic conditioning stimuli which are themselves subthreshold for generating a population spike (Lynch et al.,1981; Lupica and Dunwiddie, 1991).

V GABA Responses in the Hippocampus

GABA is the main inhibitory transmitter in the mammalian brain. GABA receptors are of two types; GABA-A, a ligand-gated ion channel controlling a chloride conductance, and GABA-B, which is G protein linked.

Postsynaptic Receptors

In the CA1 area of the normal (bicuculline-free) hippocampus pyramidal cell IPSPs have been well characterised by intracellular recording. Stimulation of the Schaffer collateral pathway results in a mono- or polysynaptic hyperpolarising GABAergic IPSP which may be separated into a short-latency GABA-A-mediated component and a longer latency, longer duration GABA-B component (Alger and Nicoll, 1982a,b; Alger, 1984; Newberry and Nicoll, 1984a, 1985; Peet and Mclennan, 1986; Dutar and Nicoll, 1988; Davies et al., 1990; Lambert et al., 1991a; Bernado,1993). Davies et al. (1990) give a latency to onset of less than 3 ms, latency to peak of 17 ms and duration of 225 ms for pharmacologically isolated GABA-A evoked IPSPs in CA1 pyramidal cells. Corresponding times for the GABA-B potential are 29 ms, 139 ms and 723 ms.

Under certain conditions, a long-lasting GABA-A-mediated depolarising inhibitory potential may be seen (Alger and Nicoll, 1982a, 1982b; Newberry and Nicoll, 1985; Avoli and Perreault, 1987; Lambert et al., 1991a; Bernado, 1993). This is unlikely to be relevant to the present investigation which was mostly carried out under conditions of GABA-A blockade by bicuculline.

Both fast (GABA-A) and slow (GABA-B mediated) IPSPs can also be recorded from interneurones in the CA1 area (Segal, 1990a; Lacaille, 1991).

The GABA-B mediated postsynaptic potential in hippocampal pyramidal cells is mediated by an increase in potassium conductance (Newberry and Nicoll, 1984b, 1985; Gahwiler and Brown, 1985) which involves a pertussis toxin sensitive G protein (Andrade et al., 1986).

There is good evidence that GABA-A and GABA-B responses can be separately activated by released transmitter. Thus in CA1 pyramidal cells stimulation of feedforward interneurones in the stratum radiatum is likely to give rise to a complex IPSP with both GABA-A and GABA-B components whereas stimulation of feedback interneurones in the alveus is more likely to activate a pure GABA-A-mediated response (Dingledine and Langmoen, 1980; Alger and Nicoll 1982a; Newberry and Nicoll 1984a). In the dentate, Otis and Mody, (1992) found evoked IPSPs on the granule cells to consist of GABA-A and GABA-B components whereas spontaneous mini IPSPs were of the chloride conductance coupled GABA-A type only. Furthermore spontaneous mIPSP frequency was unaffected by baclofen activation of GABA-B autoreceptors. Segal (1990b) also in the dentate, has demonstrated isolated calcium-dependent, tetrodotoxin-sensitive isolated GABA-B responses in granule cells in response to the topical application of microdrops of 4-aminopyridine which presumably activate a subset of sensitive interneurones. Doze et al. (1991) showed that noradrenaline blocks polysynaptic but not monosynaptic GABA-A IPSPs on CA1 pyramidal cells indicating an action at excitatory terminals on the inhibitory interneurones. GABA-B IPSPs however were unaffected. In the tegmentum and amygdyla Sugita et al. (1992) demonstrated a lack of temporal correlation between spontaneous GABA-A and GABA-B potentials. The two types of IPSP were also differentially modulated by presynaptically acting muscarine and serotinin

Although these various findings may simply reflect anatomical separation of GABA-A and GABA-B receptors other more complex interpretations are possible. Otis and Moody (1992) for example consider explanations based on the higher affinity of GABA-A receptors

for the released GABA and even the possibility that the usual endogenous ligand for the GABA-B receptor is other than GABA.

Presynaptic GABA-B Receptors

In addition to the postsynaptic GABA-B receptors on pyramidal cells there are also presynaptic GABA-B receptors on both excitatory and inhibitory terminals. Activation of presynaptic GABA-B on excitatory terminals synapsing with pyramidal cell dendrites inhibits excitatory transmission (Blaxter and Carlen 1985; Forsythe and Clements 1990; Harrison et al., 1990; Solis and Nicoll, 1992; Thompson and Gahwiler, 1992; Isaacson et al., 1993).

Activation of presynaptic GABA-B autoreceptors on GABA-releasing interneurones inhibition mediates a paired-pulse inhibition of GABA-A mediated postsynaptic inhibitory potentials, both in the CA1 region of the hippocampus and elsewhere, and probably accounts, at least in part, for the observed fatigue of GABA-A mediated inhibition which occurs under conditions of repetitive stimulation (McCarren and Alger, 1985; Deisz and Prince; 1989; Davies et al., 1990; Lambert and Wilson, 1993a; Mott et al., 1993). Interestingly, though, Wilcox and Dichter, (1994) in cultured hippocampal neurones demonstrated a marked presynaptically mediated paired-pulse depression of IPSPs that was resistant to several GABA-B antagonists and was more like a negative facilitation in that it was decreased by lowering extracellular calcium and by baclofen (see p 100). Olpe et al. (1994) have also describes a component of paired-pulse depression of IPSPs in CA1 pyramidal cells which was resistant to GABA-B antagonism. Chloride accumulation in the postsynaptic cell may be a factor in frequency dependent depression of isolated fast (GABA-A) IPSPs (Bernado,

1993). Bernado (1993) has also suggested the involvement of putatively-presynaptic GABA-A receptors in frequency dependent attenuation of GABA inhibition.

Heterogeneity of GABA-B Pharmacology

A number of investigations suggest there may be differences in the pharmacology of GABA-B receptors both in regard to whether they are pre- or postsynaptic and in regard to their location on inhibitory or excitatory terminals. No clear pattern has yet emerged.

The postsynaptic potassium conductance activated by GABA-B receptors is blocked by Ba^{2+} (Gahwiler and Brown, 1985). Thompson and Gahwiler (1992) and Misgeld et al. (1989) found that GABA-B mediated inhibition of IPSPs is also blocked by Ba^{2+} while inhibition of EPSPs was not (Thompson and Gahwiler, 1992). Lambert et al. (1991b) however found baclofen (see below) induced depression of IPSPs to be resistant to Ba^{2+}. Potier and Dutar (1993) showed a quantitative difference in the susceptibility of effects on IPSPs and EPSPs to block by pertussis toxin, although both types of presynaptic response showed some pertussis toxin sensitivity. Scanzianni et al. (1992) found baclofen to reduce the frequency of miniature spontaneous EPSPs but not miniature IPSPs in CA3 pyramidal cells suggesting a difference in the mechanisms coupled to the respective populations of GABA-B receptors. Lambert and Wilson (1993b) however using low intensity stimulation to examine responses mediated by single interneurones showed that only a proportion (about half) of inhibitory nerve terminals in the CA3 area are sensitive to GABA-B inhibition raising a possible difficulty of interpretation with this study.

Olpe et al. (1994) have suggested a difference between pre and postsynaptic CA1 GABA-B receptors on the basis of a different pattern of sensitivity to a range of highly selective GABA-B antagonists (CGP55845, CGP52432, CGP46381 and CGP36742). Otis et al. (1992) have suggested that the kinetics of the presynaptic effect as demonstrated by paired-pulse depression of IPSPs are different from the kinetic of the postsynaptic potassium conductance changes. Lambert and Wilson (1993a) showed the postsynaptic potassium conductance activated by GABA-B receptors was blocked by tetrahydroaminoacridine but presynaptically mediated GABA-B effects were unaffected.

Baclofen

Baclofen (β-(p-chlorophenyl)-γ-aminobutyric acid) is a GABA-B agonist (Curtis at al., 1974; Davies and Watkins, 1974; Hill and Bowery, 1981) which has been pivotal in investigating GABA-B mediated responses.

Baclofen reduces GABA release by acting on presynaptic GABA-B receptors on inhibitory interneurone terminals (Raiteri et al.,1989; Bauman et al., 1990; Davis et al., 1990; Lambert et al., 1991b; Nathan and Lambert, 1991; Thompson and Gahwiler, 1992). It also decreases excitatory transmission in the hippocampus partly by acting at presynaptic GABA-B receptors (Bowery et al., 1980; Blaxter and Carlen 1985; Harrison et al., 1990; Hirata et al., 1992; Solis and Nicoll, 1992; Thompson and Gahwiler, 1992) and might thus be expected to reduce the activation of inhibitory neurones. Baclofen probably also depresses interneurone excitability directly since it hyperpolarises interneurones (Madison and Nicoll, 1988; Misgeld et al., 1989).

VI Adenosine

Adenosine is a neuromodulator which is also a normal product of metabolism, being a breakdown product of the energy-currency molecule adenosine triphosphate (ATP). Both adenosine and ATP are released from cells and act on neuronal receptors. In addition adenosine can be formed from adenine nucleotides in the extracellular space (see Stone et al., 1991 for review)

Adenosine Receptors

Adenosine receptors are classified into A1 and A2 on the basis of their ability to inhibit (A1) or stimulate (A2) the production of cyclic AMP (Van Calker et al., 1979). Both A1 and A2 receptors are members of the seven-transmembrane-spanning-domain G protein-coupled superfamily and can be regarded as a subclassification of the P1 purinoceptor (see Stiles, 1992 for review). A2 adenosine receptors have been subdivided into A2a (high nanomolar affinity) and A2b (micromolar affinity) on the basis of their affinity for adenosine (Daly et al., 1983). A third adenosine receptor the A3 adenosine receptor, the functional implications of which for the CNS remain to be discovered, has been described relatively recently (Zhou et al., 1992).

Autoradiographic studies show A1 receptors to be present at high density in the hippocampus. They are mostly located on pyramidal cell bodies, but a small identifiable subgroup is associated with excitatory terminals of the Schaffer collateral fibres in the CA1 area (Onudera and Kogure, 1988). By contrast, A2 receptors, at least the high affinity A2a type, are sparse in the hippocampus (Wan et al., 1990).

Transduction Mechanisms

Stimulation of postsynaptic adenosine A1 receptors hyperpolarises neurones by activating a potassium conductance. The process is mediated by a pertussis-sensitive G-protein (Trussell and Jackson, 1985, 1987; Thomson et al., 1992). The potassium conductance involved is the same conductance which is modulated by GABA-B and Seratonin receptors (Nicoll, 1988; Alzheimer and tenBruggencate, 1991; Thomson et al., 1992).

In addition, apart from cyclic AMP downregulation, A1 receptor activation has been linked to a variety of other signal transduction mechanisms including, but not exclusively, a voltage dependant calcium current (Macdonald et al., 1986), and the phosphatidylinositol / inositol triphosphate system through coupling to phospholipase C (Alexander et al., 1990; Dickenson and Hil 1993). A1 adenosine receptor activation decreases calcium entry into presynaptic terminals in the stratum radiatum of the hippocampal CA1 area (Schubert et al., 1986).

A2 receptors have also been linked to modulation of voltage dependent calcium channels. In the CA3 area of the hippocampus (Mogul et al., 1993) and the brainstem (Umemiya and Berger, 1994) A1 activation inhibits N type Calcium channels and A2 activation facilitates P type channels.

Adenosine has recently been shown to decrease action potential duration in locus coeruleus neurones by enhancing the fast voltage dependent potassium conductance I_A (Pan et al., 1994).

Endogenous Tone

Evidence for an endogenous adenosine tone in the normal hippocampus in vivo and in brain slice preparations comes from measurements of

basal adenosine concentrations and from the effects of adenosine antagonists and uptake inhibitors.

Basal Concentrations

A number of investigators have measured the concentration of adenosine in whole brain tissue with results consistently in the region of 1 μM (Rehncrona et al., 1978; Newman and McIlwain, 1977; Schrader et al., 1980; Winn et al., 1981; Fredholm et al., 1984).

Adenosine concentrations in whole brain tissue and in extracellular fluid (ECF) start to rise rapidly within seconds of the onset of ischaemia or disruption of tissue metabolism. Rehncrona (1978) estimated this rise to occur at approximately 1 μMs^{-1}over the first five minutes before levelling out and this accords well with the reports of the other investigators. Because of this lability, and the complex agonal changes that occur in tissue perfusion, the method used for quick freezing the brain tissue for analysis is important, though the impact of different techniques seems to be more important for changes in adenosine concentration rather than for determination of basal concentrations (Winn et al., 1981).

Schrader et al. (1980) measured a constant arterio-venous difference in adenosine concentration in the cerebral circulation of anaesthetised cats suggesting constant release into the brain ECF.

Newman and McIlwain (1977) found the tissue concentration of adenosine in cortical slices which had been incubated for 30 minutes and then superfused with artificial ECF for a further 17 minutes to be identical to that in quick frozen whole brain at 1 to 2 μM. Fredholm et al. (1984) measured whole tissue concentrations of 100 μM in slices of rat hippocampus immediately after cutting but this fell to 5 μM after 15

minutes (coinciding with electrical recovery) and remained relatively stable thereafter. They also measured concentrations of 0.5 to 1 μM in fluid of the slice incubation chamber after 90 minutes of incubation.

Microdialysis enables brain extracellular fluid adenosine concentrations to estimated in vivo. The technique requires estimating the true extracellular concentration of substances by a calculation based on their recovery in the dialysate from standard calibrating solutions. Errors may thus occur if there is a diffusion barrier between the relevant ECF (near the receptors) and the probe or because of mass flow effects. Further, trauma from insertion of the probe may alter normal physiology (see for example Benveniste et al., 1989).

Several investigators measure extracellular concentrations by microdialysis of around 1 μM in the anaesthetised rat hippocampus or striatum (Zetterstrom et al., 1982; Hagberg et al., 1987; Chen et al., 1992; Ogilvy et al., 1993). Hagberg et al. (1987) estimated whole tissue adenosine to be approximately twice the extracellular concentration. Ballerin (1991), and Pazzagli et al. (1995), on the other hand, have respectively estimated basal adenosine concentrations of 40 nM and about 70 nM in the striatum of recovered, awake freely moving rats. O'Regan et al. (1989) made a similar estimate using the cortical cup technique in anaesthetised rats. These differences are relevant because if the basal concentration of adenosine in the hippocampal slice is indeed around 1 μM it is difficult to integrate this fact with the excitatory effects described on superfusing hippocampal slices with low (nM) concentrations of adenosine (p. 20).

Electrically evoked adenosine release is greater from hippocampal than cortical or striatal slices (Pedata et al., 1990) and there are regional differences in the density of uptake sites (Marangos et al., 1982;

Bisserbe et al., 1985). The density of uptake sites is high in the striatum and low in the hippocampus. These findings might suggest regional differences in the turnover and concentration of adenosine in the ECF.

Effect of Antagonists and Uptake Inhibitors

Cholinergic, noradrenergic, and serotenergic nerve terminals in the hippocampus are on extrinsic projections from the septum, locus coeruleus and raphe nuclei respectively rather than on intrinsic hippocampal neurones (Lopes Da Silva et al., 1990). In the hippocampal slice these terminals are thus severed from their proximal neurones and presynaptic modulation of transmitter release can be studied free from confounding indirect postsynaptic effects on cell bodies. Adenosine antagonists (via an action on A1 adenosine receptors) and adenosine deaminase, which metabolises adenosine to inosine, increased the electrically-evoked release of all three transmitters from hippocampal slices implying background A1-adenosine receptor mediated presynaptic inhibitory tone. The adenosine uptake blocker, RE-244 decreased evoked release of the same transmitters (Jackisch et al., 1984; Feurstein et al., 1985; Jackisch et al., 1985).

Adenosine antagonists or adenosine deaminase have been shown to have excitatory effects in a variety of electrophysiological paradigms where exogenous adenosine has the opposite effect, all, therefore, indicating endogenous adenosine tone. Field potentials and intracellular EPSPs are enhanced (Haas and Green 1988; Prince and Stevens 1992). Prince and Stevens estimated an endogenous adenosine tone equivalent to 10 μM adenosine in their slices from concentration-response data. Spontaneous spiking is increased in penicillin superfused slices (Dunwiddie, 1980; Dunwiddie et al., 1981). Schubert and Heineman

(1988) used hippocampal slices superfused with a low calcium high / magnesium solution in which synaptic transmission was blocked and showed a combination of adenosine antagonists and 4-aminopyridine, which promotes calcium entry into nerve terminals, restored synaptic responses evoked by trains of stimuli. Schubert (1988) showed the selective A1-antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) increased the stimulus train-evoked uptake of calcium in the stratum radiatum and pyramidal cell layer of slices. Addition of the nonselective antagonist theophyline to the DPCPX had no further influence on uptake indicating there were no confounding A2 effects.

Effects on Synaptic Transmission.

Adenosine is known to be an effective depressant of neuronal activity, acting both postsynaptically to increase potassium conductances and induce hyperpolarisation (see above) and presynaptically at P1 receptors to suppress the release of neurotransmitters such as glutamate (Corradetti et al., 1984; Fastbom and Fredholm, 1985; Burke and Nadler, 1988) acetylcholine (Spignoli et al., 1984) dopamine (Michaelis et al., 1979; Zetterstrom and Fillenz, 1990) and 5-hydroxytryptamine (Feuerstein et al., 1985).

Adenosine has an inhibitory action in the hippocampus that in several respects is similar to baclofen. Adenosine decreases synaptic transmission and hence the evoked orthodromically evoked population spike at least in part by a presynaptic mechanism (Okada and Ozawa, 1980; Corradetti et al., 1984; Burke and Nadler, 1988; Yoon and Rothman, 1991; Thompson et al., 1992). However, although adenosine abolishes polysynaptically-mediated IPSPs in pyramidal cells, probably by decreasing transmission at excitatory synapses with interneurones, it

does not affect monosynaptic IPSPs resulting from the direct stimulation of interneurones (Lambert and Teyler, 1991; Yoon and Rothman, 1991; Thompson et al., 1992) or the potassium-evoked release of GABA from cortical slices except at extremely high (millimolar) concentrations (Hollins and Stone, 1980). This indicates that, unlike baclofen, adenosine has no presynaptic action to reduce GABA release from interneurone terminals (but does not rule out a direct action on interneurone excitability; see Lambert and Teyler, 1991).

Excitatory Actions.

In the hippocampus some investigators have reported paradoxical excitatory effects of very low concentrations of adenosine, adenine nucleotides such as adenosine triphosphate (ATP), or adenosine analogues where they find higher concentrations of these agents to be inhibitory (Wieraszko and Seyfried, 1989; Nishimura et al., 1990, 1992; Okada et al., 1990; Garaschuk et al., 1992; Wieraszko and Ehrlich, 1994). The findings are unexpected in two related respects, firstly because of the weight of evidence suggesting the inhibitory nature of the endogenous adenosine tone in the hippocampus, and secondly because several investigators have measured the basal concentration of extracellular adenosine both in vivo and in slices to be about the same as the upper limit at which these excitatory effects occur (see above).

Hydrolysis, possibly to adenosine, seems to be required for the excitatory action of adenine nucleotides (Nishimura et al., 1990; Wieraszko and Ehrlich, 1994). Intracellularly, the excitatory effect is associated with an increase in the size of the evoked EPSP with no change in resting membrane potential or conductance (Okada et al., 1992).

It is possible these effects represent a changing balance between adenosine A1-mediated and A2-mediated effects. Sebastiao and Ribeiro (1992) showed a concentration dependent increase in CA1 population spike amplitude with the A2-selective agonist CGS-21680 and Ameri and Jurna (1991) have demonstrated both A1 and A2 adenosine receptor effects on membrane properties and EPSPs in CA1 pyramidal neurones. Interpretation is not straightforward however. Nishimura et al. (1990) for instance could not demonstrate excitatory effects with the A2-selective agonist NECA

In guinea-pig superior colliculus slices adenosine mediates only excitatory effects on neurotransmission (Okada et al., 1990; Hirai and Okada, 1994; Ishikawa et al., 1994). Ishikawa et al. (1994) were unable to distinguish between an A1 and an A2 effect using selective agonists. Furthermore, the selective adenosine *antagonists* 8-cyclopentytheophyline, 3,7-dimethyl-1-propargylxanthine and CGS-15943 were also excitatory. Garashuk et al. (1992) have observed complex interactions between methlyxanthines and adenosine on excitatory transmission in CA1 area of the hippocampus. Also of possible relevance is the fact that Uneyama et al. (1993) have shown that methylxanthenes block GABA- and glycine-evoked chloride currents in dissociated hippocampal neurone cultures.

Release of Adenosine by Selective Stimulation and by Glutamate NMDA-Receptor Activation

Although adenosine is not normally regarded as being released from nerve terminals in the same manner as a classical neurotransmitter, it is known to be released into the extracellular space following neuronal

depolarisation such as that induced by raised extracellular potassium or electrical field stimulation (see Stone, 1991 for review).

Recently, it has become clear that adenosine may be released with functional consequences following localised stimulation of specific pathways. Adenosine is at least partly responsible for a prolonged (seconds to minutes) depression of CA1 pyramidal cell EPSPs caused by a long (25 Hz for 15 s or 5 Hz for 20 s) conditioning tetanus (Sekino and Koyama, 1992; Grover and Tayler, 1993). More particularly, Mitchell et al. (1993) have shown that field EPSPs recorded in the stratum radiatum of the CA1 area can be inhibited by a preceding short burst of stimuli to an independent excitatory pathway (heterosynaptic depression) and that this inhibition is mediated by adenosine. This inhibition appears with a latency of 50 ms, is maximal at about 250 ms, increases with the number of stimulations in the conditioning train and is just detectable after a single conditioning stimulus. The effect was unaffected by an inhibitor of adenine nucleotide hydrolysis suggesting it was not mediated by the release of ATP subsequently hydrolysed to adenosine.

Manzoni et al. (1994) showed that heterosynaptic depression of CA1 excitatory transmission evoked by a 100 Hz, 1 s tetanus could be abolished both by the A1 receptor antagonist 8-cyclopentyltheophylline (CPT) and by the NMDA receptor-antagonist D-2-amino-5-phosphonovalerate (APV). A similar inhibition could be evoked by direct application of NMDA to slices and this also was antagonised by CPT suggesting that the stimulation-evoked inhibition was caused by the NMDA receptor-mediated release of adenosine. Furthermore, the stimulation-evoked inhibition was antagonised by enkaphalin suggesting that the adenosine might be released from interneurones (see p. 105). This inhibition also was unaffected by inhibition of adenine nucleotide

hydrolysis suggesting it was not mediated by the release of ATP subsequently hydrolysed to adenosine.

The results of these studies are in keeping with earlier work showing that adenosine can be released from the hippocampus or cortex by the action of glutamate receptor agonists including NMDA (Perkins and Stone, 1983; Hoehn et al., 1990; Hoehn and White, 1990a, 1990b; Pedata et al., 1991), although Craig and White (1993) showed NMDA-stimulated adenosine release from cortical slices to be markedly reduced by an ecto-5′-nucleotidase inhibitor. Potassium evoked (Hoehn and White 1990a) and electrically evoked (Pedata et al., 1991) release of adenosine are also partly mediated by NMDA-receptor activation.

CHAPTER 2
MATERIALS AND METHODS

All the experiments detailed in this thesis concern the effect of agents or stimulation parameters on evoked field potentials in CA1 area of the rat hippocampal slice preparation. The general experimental set up together with the methods of slice preparation, maintenance, and superfusion with artificial cerebrospinal fluid and exogenous agents are common to all the experiments.

I Hippocampal Slice Preparation and Experimental Set-up

Slice Preparation and Storage

Male Wistar rats weighing between 150 g and 250 g were anaesthetised with intraperitoneal urethane and cooled on a bed of ice whilst breathing oxygen enriched air from a small funnel. When their rectal temperatures reached 30°C (about 15 minutes) they were killed by cervical dislocation. The brain was rapidly removed into cooled (between 1°C and 5°C) artificial cerebrospinal fluid (ACSF) of composition KH_2PO_4 2.2 mM, KCl 2 mM, $NaHCO_3$ 25 mM, NaCl 115 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.2 mM, Glucose 10 mM, which had been previously gassed with a mixture of 95 per cent oxygen and five per cent carbon dioxide for approximately 30 minutes. The hippocampi were rapidly dissected out and 450 μm-thick slices were cut on a McIlwain tissue chopper from approximately the middle half of both hippocampi. The newly cut slices were placed in a single petri dish and submerged in fresh well-gassed

ACSF. The dish was placed in an incubation chamber in an atmosphere of five per cent carbon dioxide in oxygen saturated with water vapour where the slices were maintained at room temperature until they were transferred to the recording chamber. For transfer between chambers slices were draped over a small soft camel-hair paintbrush. Great care was taken to minimise physical manipulation of slices.

In general, one set of slices was prepared each day and slices taken from the incubation chamber for experiment as required. In accordance with the larger experience in this laboratory, slices remained in good condition, in terms of their electrophysiological responses and gross morphology, for many hours when maintained in this way. Slices taken for experiments later in the day may have spent six or more hours at room temperature. All slices were allowed to rest at room temperature for at least one hour before transfer to the recording chamber. In experiments involving different treatments for two groups of slices care was taken that time in the incubation chamber was not systematically biased between groups.

For one group of experiments a cut with a scalpel blade from alveus to hippocampal fissure was made immediately after slice preparation in the CA2 area of some slices to separate the CA1 from the CA3 area. These slices were then recovered as normal in the incubation chamber.

The Recording Chamber and Superfusion Arrangements

For recording, individual slices placed on a fine wire mesh in a chamber of volume 0.5 ml and submerged in ACSF at 30°C. Slices were held gently in position by a thin bar formed from a blunted seeker needle, bent into the required shape, and mounted on a micromanipulator. The slices were superfused with gassed ACSF at between 2 and 4 $ml.min^{-1}$

(constant within each experiment) using a roller pump (gravity perfusion was used for a few of the early experiments). The ACSF was gassed in a reservoir (covered glass conical flask) with a mixture of 95 per cent oxygen and 5 per cent carbon dioxide upstream of the roller pump Two three-way taps allowed the source of ACSF to be instantly switched between any one of three such reservoirs. The ACSF was heated in a thermostatically controlled water bath immediately upstream of the recording chamber. The water in the heating bath was bubbled with the oxygen / carbon dioxide gas mixture to minimise oxygen loss though the coiled silicone tubing carrying the ACSF since this tubing is permeable to oxygen. The recording chamber was drained by a wick of tissue paper or nappy liner. The pH of the superfusate was constant at 7.4.

The recording chamber temperature was measured intermittently (several times a day) with a digital thermometer and found to be extremely constant (less than 0.5 °C variation) not only for individual experiments but, providing the apparatus was not altered, for days at a time. The apparatus was always superfused with warmed gassed ACSF for at least an hour before any slices were placed in the recording chamber to allow the temperature and oxygen tension in the heating bath and recording chamber to become stable.

A problem with the set up was the growth over days to weeks of organic matter, presumably algae, in the silicone tubing used for the ACSF superfusion. To minimise this growth the whole apparatus was flushed with distilled water at the end of each working day. Contaminated portions of tubing (usually the short sections between the reservoirs and the three-way taps or between the taps and the roller pump) were replaced as soon as there was a noticeably heavy growth. In addition, the apparatus was dismantled every few weeks, the recording

chamber cleaned using dilute hydrochloric acid and all the taps and tubing replaced with new. No attempt was made to recycle contaminated tubing.

Stimulating and Recording Arrangements

A stimulating electrode was placed in each slice either in the stratum radiatum at the CA1/CA2 junction for orthodromic activation of pyramidal cells via the Schaffer collateral pathway (S1 in Figure 1) or in the alveus on the subicular side of the recording electrode for antidromic activation (S2 in Figure 1). For some experiments an electrode was placed at both sites. For one set of experiments only, (p. 77), two stimulating electrodes were placed in the stratum radiatum, one at the CA1/CA2 junction (S1), and one in CA1 on the subicular side of the recording electrode for orthodromic activation of the pyramidal cells by antidromic stimulation of Schaffer collateral fibres (S3 in Figure 1).

Stimulation consisted of square wave constant-current pulses of 100 μs duration and up to 1 mA magnitude was delivered from a concentric bipolar stimulating electrode (Clark Electromedical Instruments Ltd.) using an A.M.P.I. Master-8 programmable stimulator and stimulus isolator. Extracellular population potentials were recorded by single glass microelectrodes (tips bumped back to about 3 μm and filled with ACSF) whose tips were located in the CA1 pyramidal cell layer. A microscope was used to aid electrode placement. With practice the tip of the recording electrode could be placed directly in the pyramidal layer amongst the pyramidal cell bodies without using the evoked potential for guidance. This was useful in standardising the stimulation regimen for different slices.

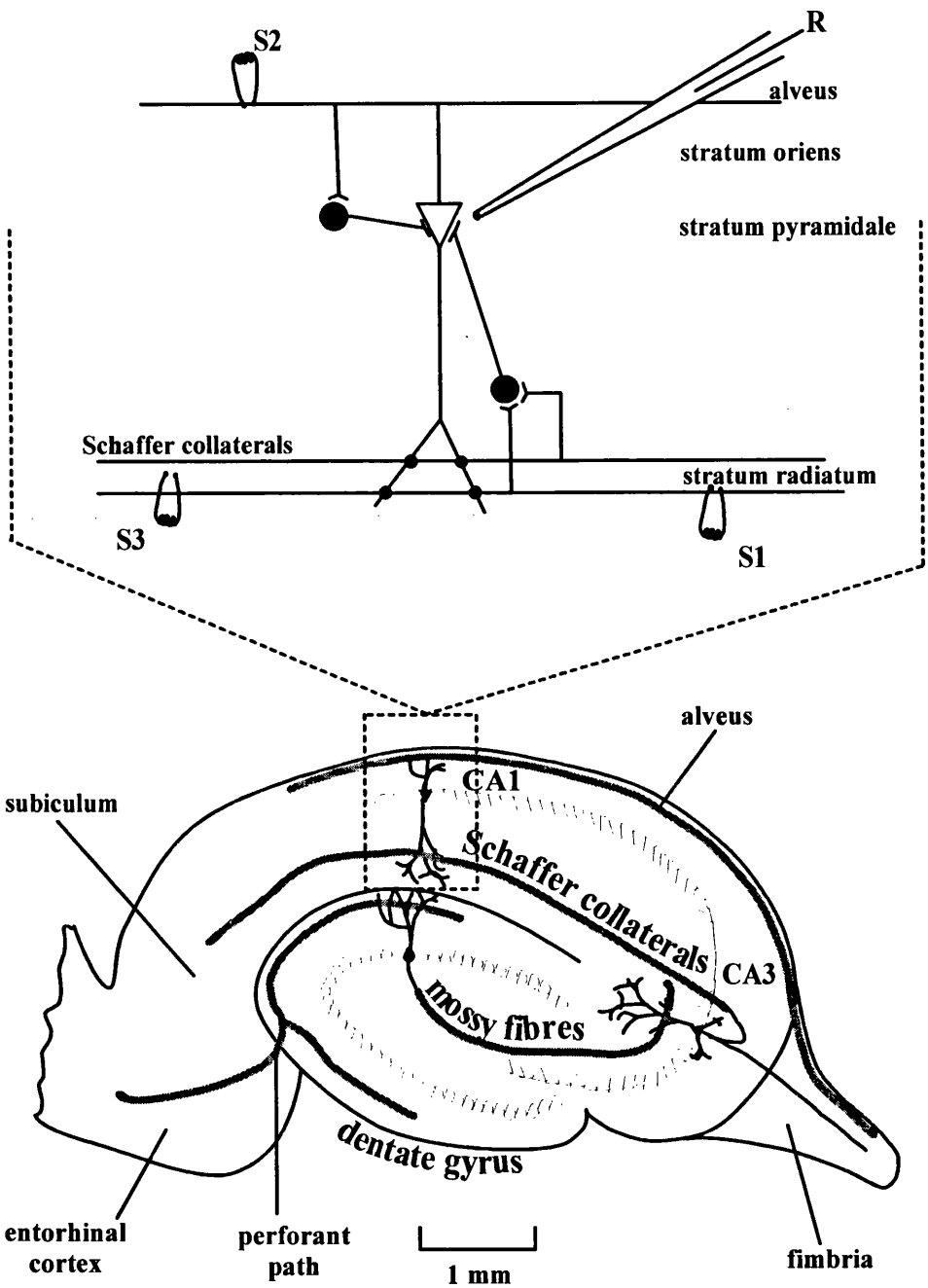

Figure 1 Diagram of a hippocampal slice. The CA1 area is enlarged in the upper drawing to show the relative placement of the recording electrode R and the stimulating electrodes S1, S2 and S3 (not to scale). The pyramidal neurone is represented as an open triangle in the upper drawing with feedforward and feedback inhibitory interneurones shown as black disks.

Recorded signals were amplified (Neurolog), and monitored on one or two digital oscilloscopes. For analysis, in early experiments 4 or 8 potentials were averaged (Neurolog averager) and plotted on a pen recorder. In later experiments potentials were captured, stored and analysed by computer (SCAN software, John Dempster, University of Strathclyde). For some experiments single responses were plotted directly on a slow-speed pen recorder.

II Slice Selection

With the exception of a few early experiments slices were only accepted for experiment if the maximum evoked orthodromic population spike in ACSF after 15 minutes in the recording chamber was at least 4 mV, stable, and free from secondary spikes. For the earliest experiments slightly less stringent criteria were used; some potentials were smaller than 4 mV and the presence of a small secondary spike was allowed.

III Test Agents and Solutions

ACSF was made up fresh each day to the composition given above using ANALAR grade reagents dissolved in distilled water.

For some experiments ACSF containing nominally zero calcium, from which the $CaCl_2$ was omitted, or raised magnesium (4.8 mM $MgSO_4$) was used. No compensation was made for the small change in total osmolality of the ACSF occasioned by these adjustments.

Test agents were made up in concentrated stock solutions and added, to give the required final concentration, to the reservoirs in which the superfusing medium was gassed with a mixture of 95 per cent oxygen and 5 per cent carbon dioxide.

Test agents other than bicuculline were generally superfused for 15 to 30 minutes (constant within each set of experiments) and washed out for 15 to 30 minutes.

Drugs and Chemicals

Drugs and chemicals were obtained from the following sources.

Baclofen, adenosine hemisulphate, 1,3-dimethyl-8-cyclopentylxanthine (cyclopentyltheophylline, CPT), adenosine deaminase and kynurenic acid were obtained from Sigma Biochemicals Ltd.

(-)-Bicuculline methobromide, 1,3-dimethyl-8-phenylxanthine (8-phenyltheophyline, 8PT) and 2-hydroxysaclofen were obtained from Research Biochemicals Inc.

Morphine sulphate was obtained from MacFarlane Smith Ltd Edinburgh.

III Stimulation Paradigms

Precise stimulation paradigms were varied for different experiments; details are given where relevant in the text and in the figure legends.

In general, after the electrodes were placed, the slice was allowed to rest for 15 minutes without stimulation. At the end of this time slice was examined for the size and shape of the evoked population spike and the slice either accepted or rejected. For experiments involving superfusion with bicuculline, this was now started and patterned stimulation was commenced, stimulation amplitude was adjusted and responses allowed to stabilise during the first 20 minutes of bicuculline superfusion. For experiments not involving bicuculline, responses were allowed to

stabilise for 10 to 20 minutes in ACSF. Patterned stimulation was continued uninterrupted for the complete duration of each experiment.

In the early experiments (characterisation of paired-pulse interactions, and pilot experiments with morphine, baclofen, adenosine and 8-phenyltheophyline) paired stimulations were delivered at 20 second intervals. In later experiments paired stimulations were delivered at 40 or 60 second intervals.

The magnitude of test and conditioning stimuli could be varied independently (where there was only a single stimulating electrode it could be connected to two different channels of the Master-8). In many experiments a conditioning stimulus was used which was supramaximal for the size of the evoked potential. This stimulus was always set at 1 mA.

In some experiments the strength of the conditioning (first of the paired stimuli) and test stimuli (second of the paired stimuli) were identical to each other throughout the experiment. In these experiments the response to the conditioning stimulus was also the control response for the calculation of facilitation or inhibition of the test potential. In other experiments the strength of the test and conditioning stimuli were different from each other, or the stimulations were delivered through different electrodes. In these experiments a separate control response was evoked by intercalating a single unpaired stimulus identical to the test stimulus in between each paired stimulus. Single and double stimuli were thus alternated.

In the experiments to examine the effect of adenosine on single evoked potentials (p. 81), single potentials were evoked at intervals of 60 seconds throughout.

IV Terminology

Designation of Stimuli and Potentials

The response to the first of a pair of stimuli, the conditioning stimulus, is designated $\mathbf{R_{cond}}$. The response to the subsequent test stimulus is labelled $\mathbf{R_{test}}$. The amplitude of R_{test} was compared with the response, $\mathbf{R_{control}}$, to an unconditioned (unpaired) stimulus of the same strength. In those experiments where conditioning and test stimuli were identical to each other throughout, R_{test} was compared with the response to the conditioning stimulus. In this case the response to the conditioning stimulus is referred to as either $R_{control}$ or R_{cond} depending on the context.

In one set of experiments (p. 75) to examine the effect of adenosine on paired-pulse inhibition stimulus strength was adjusted to evoke an unpaired potential which was the same size, before superfusion with adenosine, as the inhibited test potential R_{test}. This potential is designated $\mathbf{R_{single}}$ and is distinct from $R_{control}$.

R_{test} and $R_{control}$ were always evoked by an identical stimulus. This stimulus is referred to as the **test stimulus**.

Miscellaneous Terms

The **interstimulus interval** refers to the interval between the conditioning stimulus and its paired test stimulus.

The expression **bicuculline-enhanced paired-pulse inhibition** in the present work always refers to the inhibition measured during bicuculline superfusion which was apparent at latencies of 200 to 500 ms from the conditioning stimulus. After its initial characterisation this was always studied using an interstimulus interval of 300 ms. Unless specifically stated otherwise it refers to inhibition of an orthodromic test potential.

The **term short-latency paired-pulse inhibition** is used in the present work to describe the inhibition of orthodromic potentials which occurred in the absence of bicuculline, usually at much shorter latencies to the conditioning stimulus. Here, it was always studied using an interstimulus interval of 30 ms.

V Data Analysis

In experiments where the evoked response was a single population spike, the size of the population spike was taken to be the difference between the peak positivity and the peak negativity. In experiments where the evoked response was a short burst potential response size was taken to be the peak to peak size of the first population spike in each burst. This corresponds to the vertical distance between points a and b in Figures 6 and 14. Paired-pulse facilitation or inhibition were expressed as the percentage difference between R_{test} and $R_{control}$ compared with $R_{control}$.

One difficulty in assessing the action of substances on paired-pulse effects is that the test agents may alter the size of single evoked responses. Paired-pulse interactions during the superfusion of test agents were often calculated after the strength of the test and conditioning stimuli had been adjusted to return $R_{control}$ and R_{cond} to their control size before test agent superfusion. This is indicated where appropriate, and the problem is discussed in detail in the text.

Statistical Analysis

In general, statistical analysis was by paired t test or unpaired t test as appropriate. Data were tested for normality using a version of the Shapiro-Wilk test (Minitab Release 8). Any data where

normality could be rejected at the $P < 0.05$ level were also tested using the Wilcoxon matched pairs test, the results of which are quoted if they differ importantly from the t test. $P < 0.05$ was taken to indicate statistical significance. Results are presented as mean ± standard error of the mean (SE).

CHAPTER 3
RESULTS

The results are presented under eight main headings which correspond broadly with the chronological order in which the investigations were undertaken. First are the results of investigations into the effect of bicuculline on paired-pulse interactions between orthodromic potentials, followed by data from preliminary experiments into the mechanism of bicuculline-enhanced paired-pulse inhibition. The next two sections concern the effect of bicuculline on the paired-pulse inhibition of antidromic potentials and a short series of experiments into the mechanism of inhibition of antidromic responses during bicuculline superfusion. These are followed by the results of the experiments into the effect of baclofen, adenosine, and 2-hydroxysaclofen on the bicuculline-enhanced inhibition of orthodromic and antidromic potentials. The last three sections describes investigations carried out in an attempt to clarify the effect of exogenous adenosine on bicuculline-enhanced paired-pulse inhibition, to investigate the possibility that endogenous adenosine might contribute to the enhanced inhibition and to determine the effect of the adenosine antagonist CPT on short-latency paired-pulse inhibition in the absence of bicuculline.

I Orthodromic Responses and Interactions in the Absence and Presence of Bicuculline

Initial experiments were to characterise paired-pulse interactions

between orthodromic potentials in the absence of exogenous agents and during superfusion of bicuculline. All these experiments were carried out using stimuli evoked from a single electrode in the stratum radiatum.

Paired-Pulse Interactions between Orthodromic Stimuli in the Absence of Added Agents

Evoked responses in the stratum pyramidale which were preceded by a conditioning potential were either inhibited or facilitated compared to unpaired control potentials evoked by the same strength of stimulus (Figure 2).

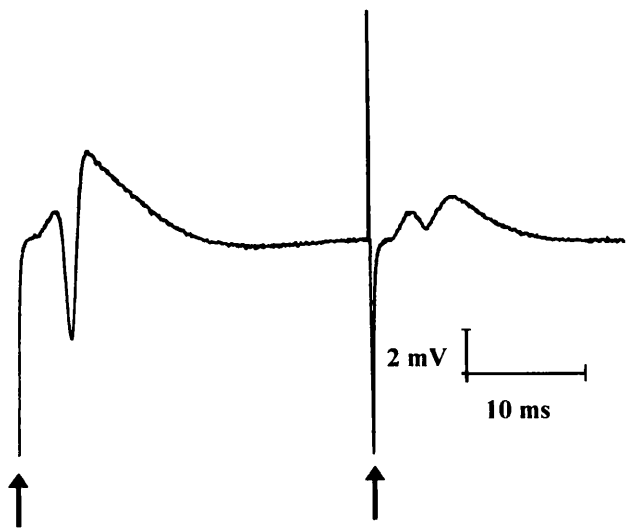

Figure 2 Typical paired-pulse inhibition in the absence of exogenous agents. All stimuli were orthodromic. Test and conditioning stimuli were identical to each other and set so that $R_{control}$ was 70% of its maximum amplitude. The interstimulus interval was 30 ms. Records were captured on computer (SCAN software, John Dempster, University of Strathclyde, UK) and plotted on a Hewlet Packard plotter. Arrows show the stimulus artefacts (the initial upstroke of the first artefact was not captured).

Whether facilitation or inhibition was seen depended on the interstimulus interval between conditioning and test potentials and on the strength of the conditioning potential.

Interstimulus Interval

The effect of varying interstimulus interval on interactions between pairs of evoked orthodromic responses was studied in the absence of added agents (no bicuculline present) in nine slices. The conditioning stimulus at 1 mA was supramaximal for the size of the evoked potential, $R_{cond.}$ $R_{control}$ was 60% maximal.

Under these conditions six of the nine slices showed an early paired-pulse inhibition at interstimulus intervals less than 50 ms although because of the admixture of slices showing facilitation this was only significant across the group at an interstimulus interval of 15 ms (Figure 3).

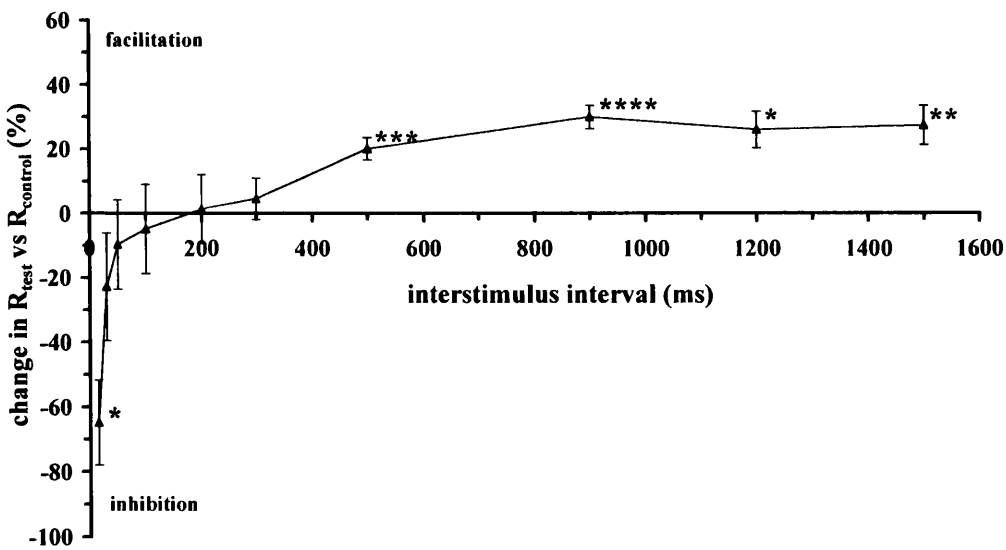

Figure 3 Effect of interstimulus interval on interactions between pairs of orthodromic evoked potentials in ACSF in the absence of added agents. The conditioning stimulus was supramaximal at 1 mA in all cases. $R_{control}$ was between 50 and 70% of maximum amplitude (constant within each experiment) in ACSF. $n = 9$ slices. Measurements at the 15 ms, 200 ms and 1200 ms points were taken in four slices only and these points are the mean of four values. Error bars show SE $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ for difference from no change in the amplitude of the evoked potential.

As interstimulus interval was increased inhibition decreased and a paired-pulse facilitation emerged in all nine slices. The interstimulus

interval at which inhibition changed to facilitation was quite variable between slices; three of the slices showed a facilitation at an interstimulus interval of 30 ms whereas three showed continuous inhibition up to an interstimulus interval of 300 ms changing to facilitation by 500 ms.

Two of the nine slices exhibited a biphasic pattern of facilitation in relation to the interstimulus interval. In these two slices facilitation showed an early maximum at an interstimulus interval about 50 ms, before decreasing to a minimum at an interstimulus interval of about 300 ms (in one case becoming an inhibition) and then returning to near its original amplitude at an interstimulus interval of 900 ms. Data from these slices are plotted individually in Figure 4.

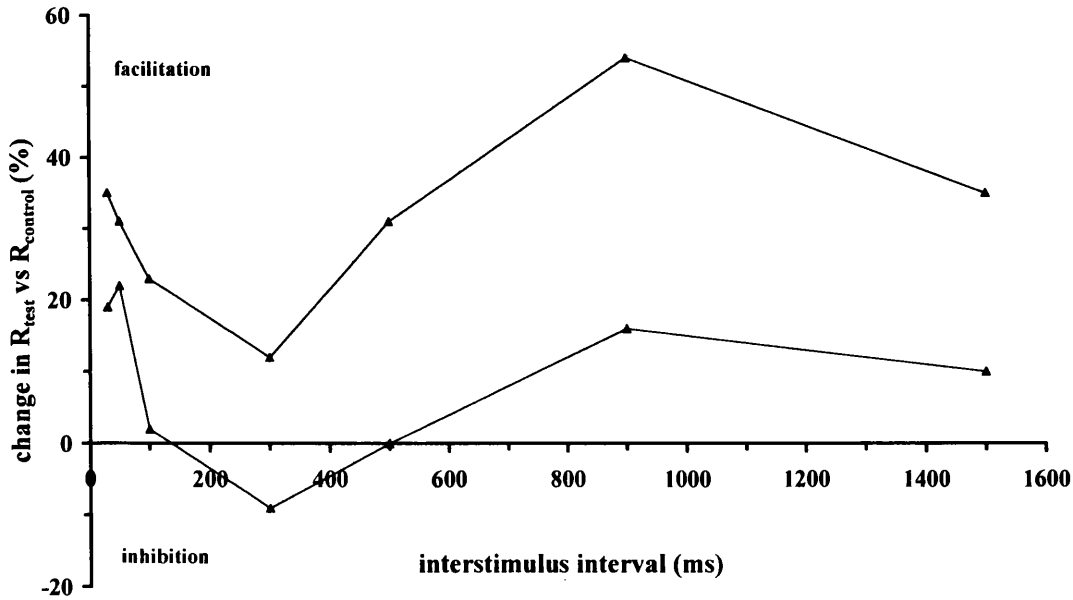

Figure 4 Interactions between paired orthodromic stimuli in ACSF in two individual slices. See Figure 3 for details.

Conditioning Stimulus Strength

In five slices the effect of using a supramaximal conditioning stimulus was compared with using a conditioning stimulus just suprathreshold for

eliciting a response. Inhibition was markedly increased at all interstimulus intervals tested (30 to 900 ms) when the smaller conditioning stimulus was used (Figure 5).

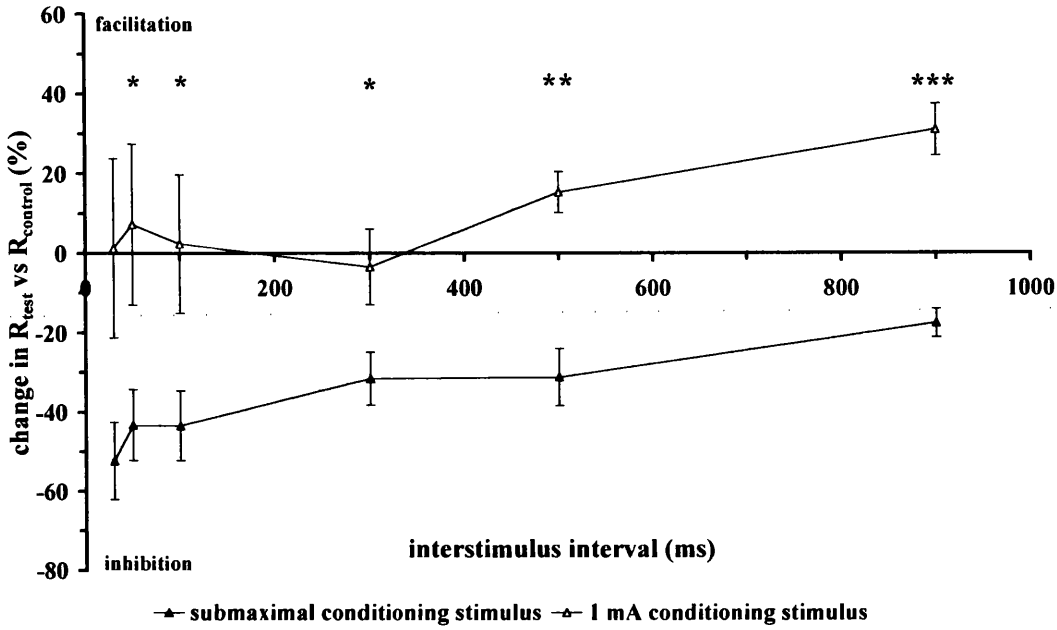

Figure 5 Influence of conditioning stimulus strength on interactions between pairs of orthodromic potentials in ACSF in the absence of added agents. Paired-pulse interactions were quantified using two different strengths of conditioning stimulus in each slice. The small conditioning potential was above the threshold for evoking a small population spike. The larger conditioning stimulus was set at 1 mA which is supramaximal for the amplitude of the evoked potential. $R_{control}$ was fixed at between 50 and 70% of maximum amplitude (constant within each slice). $n = 5$ slices. Error bars show SE. $*P < 0.05$, $**P < 0.01$. $***P < 0.001$.

Effect of Bicuculline on Orthodromic Responses and Interactions

Unpaired Orthodromic Responses

Superfusion with increasing concentrations of bicuculline increased the size of submaximal population spikes and caused the appearance of secondary population spikes such that the evoked potential resembled a short burst of population spikes 30 or 40 ms in duration. Each burst usually consisted of four or more recognisable population spikes (Figure

6). The size and shape of the evoked bursts were stable for a given set of experimental conditions, although, during the course of longer experiments (up to three hours), there was a tendency for a gradual decrease to occur in the amplitude of potentials evoked by a given sub-maximal stimulus.

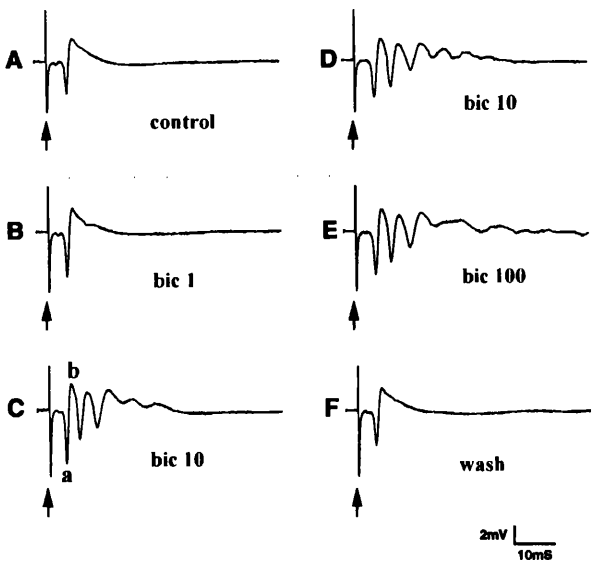

Figure 6 Effect of bicuculline on single orthodromic potentials. Bicuculline was superfused at each concentration for 20 minutes. The last record was taken after 30 minutes washout in ACSF. **A** Control potential in ACSF with the stimulus set such that the potential was 70% of maximum amplitude. **B** and **C** Evoked potentials during superfusion with bicuculline 1 μM and 10 μM respectively. Stimulus strength identical to A. **D** Superfusion with 10 μM bicuculline but with the stimulus strength reduced to return the amplitude of the potential to control size (70% maximal). **E** Superfusion with 100 μM bicuculline. Stimulus strength identical to D. **F** Wash in ACSF. Stimulus strength was the same as A. Records were captured on computer (SCAN software, John Dempster, University of Strathclyde, UK) and plotted on a Hewlet Packard plotter. Arrows mark the stimulus artefacts. The lower case letters on the spike in C are labels for discussion in the main text.

At a given concentration of bicuculline the size of the evoked potential, as measured by the size of the first population spike of the burst, varied, up to a maximum size, with stimulus strength. The strength of stimulus required to elicit a response of maximum size was reduced with bicuculline as compared with ACSF alone. Although these

observations were not formally quantified during these studies they were qualitatively very robust. Superfusion with 10 μM bicuculline always caused the evoked response to resemble the burst response shown in Figure 6.

Paired-Pulse Interactions

With a single electrode in the stratum radiatum robust and reproducible paired-pulse interactions (inhibition and facilitation) between evoked orthodromic responses were demonstrable in the presence of 10 μM bicuculline. As in the absence of exogenous agents, either facilitation or inhibition of the test response was observed depending on the interstimulus interval and on the strength of the conditioning stimulus.

Interstimulus Interval. During superfusion with 10 μM bicuculline, when a supramaximal conditioning stimulus of 1 mA was used all the nine slices examined here showed a pattern of paired-pulse interaction which changed in a characteristic fashion with the interstimulus interval. At interstimulus intervals between 15 and 1500 ms, two temporally distinct phases of inhibition were seen, at short (< 50 ms) and at intermediate (200 to 500 ms) interstimulus intervals (Figure 7). At an interstimulus interval of 100 ms inhibition either showed a marked minimum or facilitation was seen. At interstimulus intervals longer than 900 ms there was a second phase of facilitation. Eight of the nine slices showed facilitation at this latency.

At interstimulus intervals less than 50 ms and between 300 and 900 ms, 10 μM bicuculline significantly increased inhibition or decreased facilitation compared with drug-free control in the same slices. However when the interstimulus interval was 100 ms the opposite effect occurred

and the slices showed significantly increased facilitation in the presence of bicuculline.

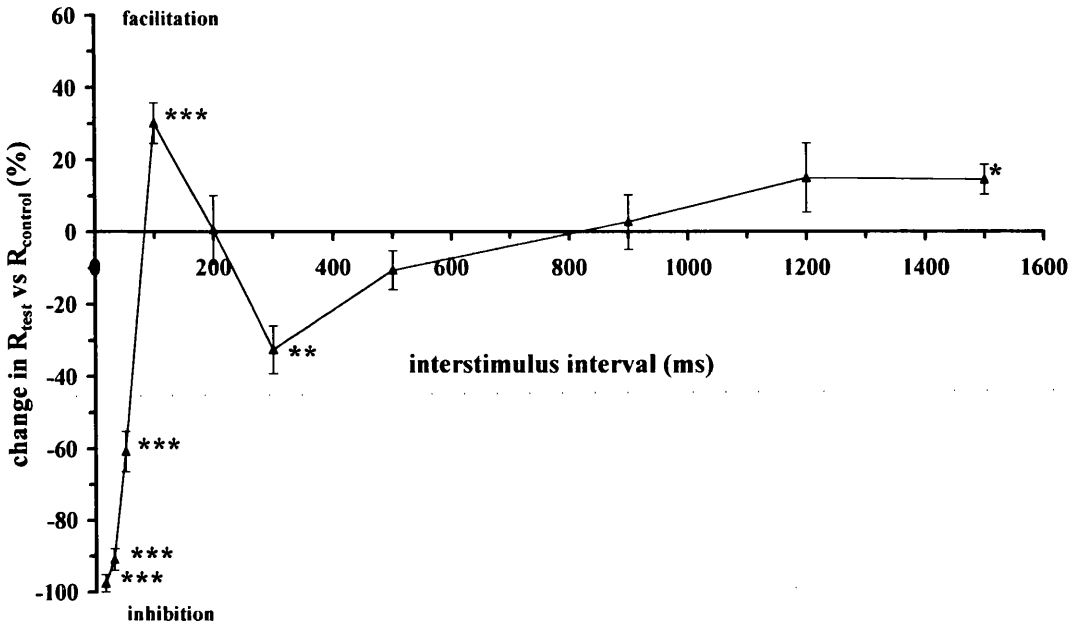

Figure 7 Effect of interstimulus interval on interactions between pairs of orthodromic evoked potentials during superfusion with 10 μM bicuculline. The conditioning stimulus was supramaximal at 1 mA in all cases. $R_{control}$ was between 50 and 70% of maximum amplitude (constant within each experiment) in bicuculline. $n = 9$ slices. Measurements at the 15, 200, 1200 ms points were taken in four slices only and these points are the mean of four values. Error bars show SE. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****$ $P < 0.0001$ for difference from no change in the amplitude of the evoked potential.

The concentration-dependence of the effect of bicuculline on the longer-latency orthodromic inhibition was examined in a further three slices at an interstimulus interval of 300 ms. Bicuculline increased inhibition in a concentration dependent manner (Figure 8).

Conditioning Stimulus Strength. When a small submaximal conditioning stimulus was used in place of a supramaximal conditioning stimulus the facilitation usually seen at 100 ms and at interstimulus intervals above 900 ms was replaced by inhibition. Data from three consecutive slices are summarised in Table 1.

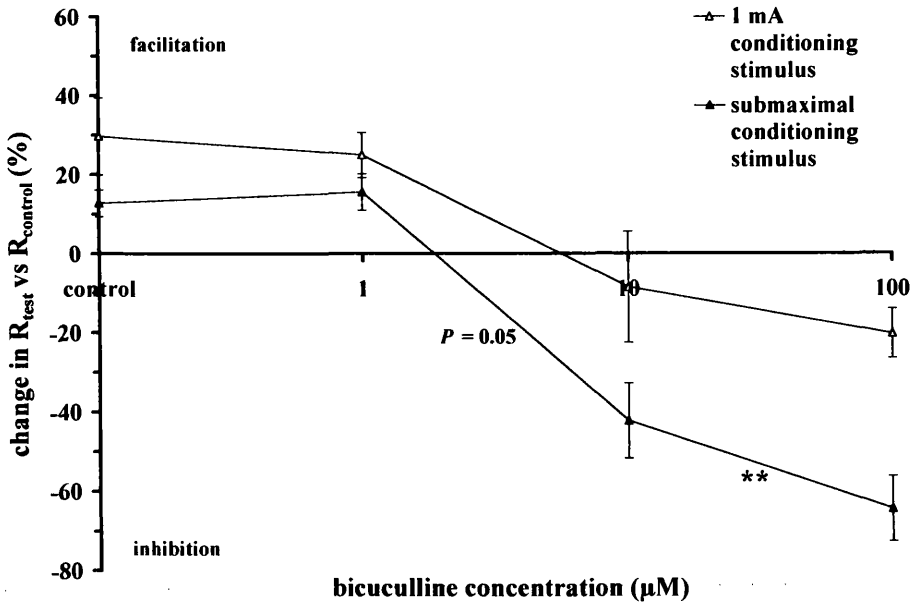

Figure 8 Concentration-response relationship for the effect of bicuculline on interactions between pairs of orthodromic potentials at an interstimulus interval of 300 ms. Bicuculline was superfused for 20 minutes at each concentration with no washout. Paired-pulse interactions were quantified using two different strengths of conditioning stimulus in each slice. The larger conditioning stimulus was set at 1 mA which is supramaximal for the amplitude of the evoked potential. The smaller conditioning stimulus was set to evoke a response of 50% of maximum amplitude under control (ACSF) conditions. $R_{control}$ was approximately 50% of maximum amplitude in ACSF (constant within each experiment). The submaximal conditioning stimulus and the unpaired test stimulus were adjusted to return $R_{control}$ and the submaximal R_{cond} to their control amplitude at each bicuculline concentration. $n = 3$ slices. $^{**}P < 0.01$ for difference between concentrations (submaximal stimulus only). Error bars show SE.

Table 1 Effect of conditioning stimulus strength on interactions between pairs of orthodromic potentials at two different interstimulus intervals in the presence of bicuculline. A negative number indicates paired-pulse inhibition, a positive number indicates paired-pulse facilitation. Paired-pulse interactions were quantified using two different strengths of conditioning stimulus in each slice; a small conditioning stimulus just above the threshold for evoking a potential and a larger 1 mA conditioning stimulus which evoked a potential of maximum size. $R_{control}$ was approximately 60% of maximum size (constant within each experiment). 10 μM bicuculline was present throughout.

	Change in Test Potential Amplitude (%)	
Amplitude of R_{cond} (% maximum)	Interstimulus Interval 100 ms	Interstimulus Interval 1500 ms
100%	29 ± 5.1	14 ± 3.8
5%	-44 ± 2.6**	-16 ± 2.9*

$^{*}P < 0.05$, $^{**}P < 0.01$ for the difference between the response when the conditioning stimulus was just suprathreshold and when it was supramaximal (n = 3).

II Preliminary Investigations into the Mechanism of Bicuculline-Enhanced Orthodromic Paired-Pulse Inhibition: Effects of Morphine, Adenosine, Baclofen and 8-Phenyltheophyline

To investigate the mechanism of the marked inhibition which consistently occurred between pairs of orthodromic potentials at an interstimulus interval of 200 to 500 ms in the presence of bicuculline (Figure 7) the effect of several different agents on this phenomenon was examined. All stimuli were delivered from a single electrode in the stratum radiatum. $R_{control}$ was set at 50% to 70% of maximum size under control conditions. Test stimuli were adjusted during superfusion of adenosine and baclofen to counteract the direct depressive action of these agents on the evoked potential and keep the size of $R_{control}$ constant. The interstimulus interval was 300 ms in all experiments and 10 μM bicuculline was present throughout.

Morphine

Morphine had no overall effect on the size of individual single evoked potentials in bicuculline ($n = 5$, $P = 0.87$) and stimulus strength was not adjusted in the presence of morphine or during washing in these experiments. When a supramaximal conditioning stimulus of 1 mA was used to evoke paired-pulse inhibition morphine 100 uM was associated with a decrease in inhibition from 61% ± 16.0 to 31% ± 14.6 (Figure 9), a change which approached statistical significance ($n = 5$, $P = 0.057$). There was no recovery of inhibition with washing in bicuculline alone for between 20 and 40 minutes but two control slices subject to an identical stimulation regimen showed no diminution of inhibition over a comparable time.

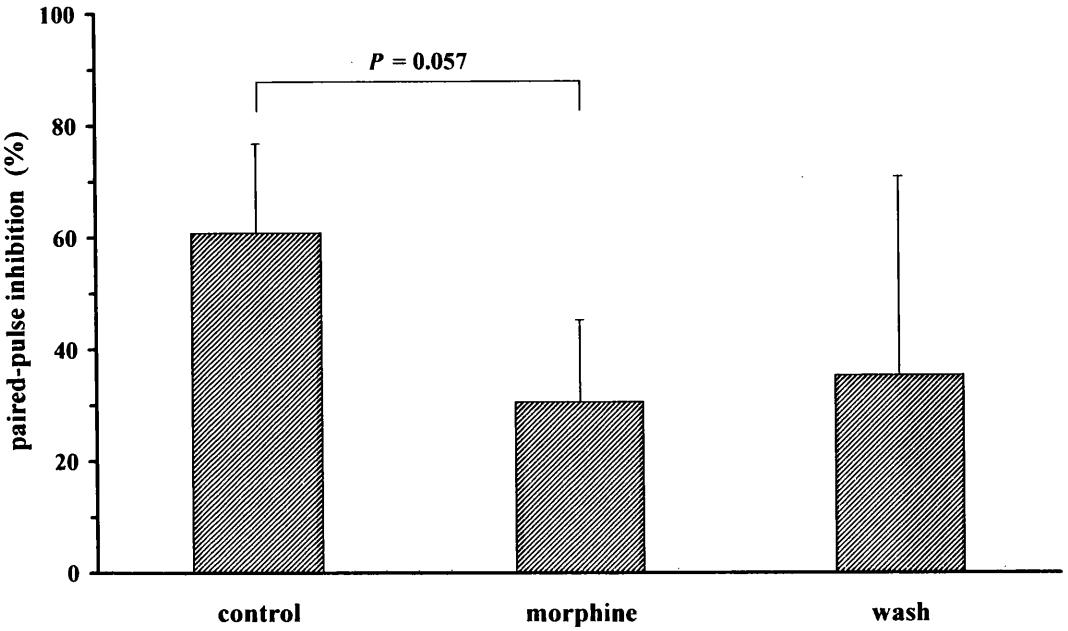

Figure 9 Effect of 100 μM morphine on paired-pulse inhibition between orthodromic potentials in the presence of bicuculline. The interstimulus interval was 300 ms. Conditioning stimuli were supramaximal (1mA). $R_{control}$ was approximately 50% of maximum amplitude. Stimuli were not adjusted in the presence of morphine. Morphine was superfused for 15 minutes. Wash was between 20 and 40 minutes. 10 μM bicuculline was present throughout. Error bars show SE. n = 5 slices.

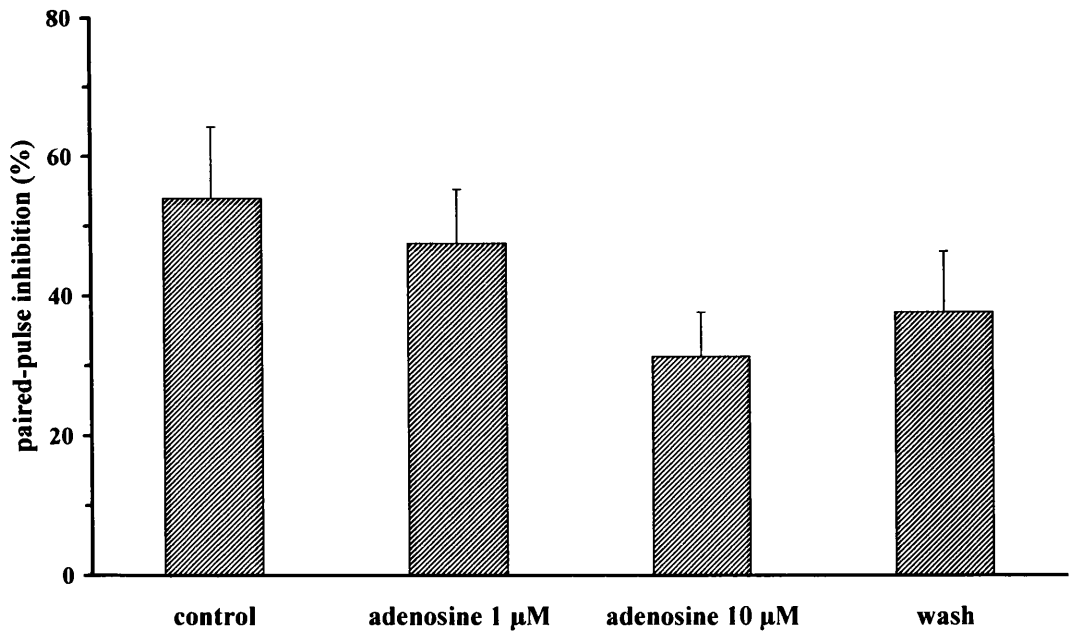

Figure 10 Effect of adenosine 1 μM and 10 μM on paired-pulse inhibition between orthodromic potentials in the presence of bicuculline. The interstimulus interval was 300 ms. Conditioning stimuli were supramaximal (1mA). $R_{control}$ was approximately 50% of maximum amplitude under control conditions. Test stimuli were adjusted to return $R_{control}$ to its control amplitude in the presence of adenosine and during subsequent washout. Adenosine was superfused for 15 minutes at 1 μM followed by superfusion with adenosine for 15 minutes at 10 μM followed by wash for 20min. 10 μM bicuculline was present throughout. Error bars show SE. n = 5 slices.

Adenosine

When the conditioning stimulus was supramaximal, adenosine 1 μM and 10 μM had no significant effect on inhibition in the presence of bicuculline ($n = 5$ slices) although there was a non-significant trend towards a decrease in inhibition with adenosine 10 μM ($P = 0.1$) which did not reverse with washing for 20 minutes (Figure 10).

Baclofen

The effect of the GABA-B agonist baclofen on bicuculline-resistant inhibition was examined in five slices at a concentration of 0.5 μM (1 slice), 0.7 μM (2 slices) and 1 μM (2 slices). In each slice paired-pulse effects were characterised using several different strengths of conditioning stimulus (Figure 11).

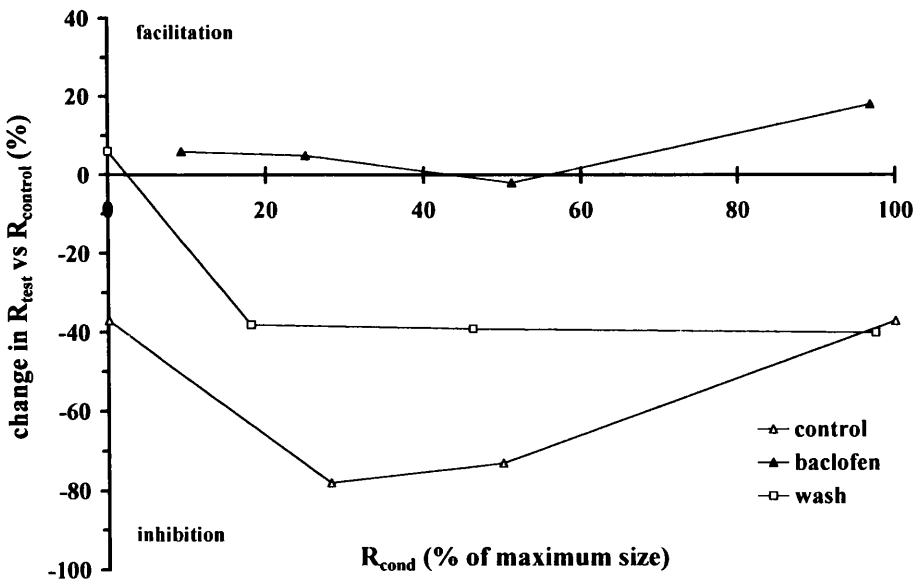

Figure 11 Data from a typical single slice showing the effect of 1 μM baclofen on paired-pulse inhibition in the presence of bicuculline. The interstimulus interval was 300 ms. Stimuli were orthodromic. Baclofen was superfused for 20 minutes and then washed out for 20 minutes. $R_{control}$ was set to 55% of maximum amplitude under control conditions. Test stimulus strength was adjusted in the presence of baclofen and during subsequent washing to keep $R_{control}$ equal to its control amplitude. Paired-pulse inhibition was measured with a range of conditioning stimulus strengths under each condition. 10 μM Bicuculline was present throughout. Similar effects were obtained in four other slices using baclofen in concentrations ranging from 0.5 μM to 1 μM (see Table 2).

In all five slices there was a decrease in paired-pulse inhibition when baclofen was superfused which recovered partially or completely with 20 minutes wash (Table 2).

Table 2 Data from five individual slices showing the effect of various concentrations of baclofen on paired-pulse inhibition in the presence of bicuculline. The interstimulus interval was 300 ms. All stimuli were orthodromic. One concentration of baclofen was superfused over each slice for approximately 20 minutes and then washed out for 20 minutes. Test stimulus strength was adjusted in the presence of baclofen and during subsequent washing to keep $R_{control}$ equal to its control size (approximately 60% of its maximal size in bicuculline alone). Paired-pulse inhibition was measured with a range of conditioning stimulus strengths under each condition. Presented here are the data obtained using a large supramaximal conditioning stimulus (1 mA) and a smaller stimulus such that R_{cond} was 50% of its maximum size. The latter data set was calculated by interpolation in plots of paired-pulse inhibition versus conditioning stimulus size (see Figure 11). Negative paired-pulse inhibition indicates facilitation of R_{test} compared to $R_{control}$. Bicuculline 10 μM was present throughout.

			Paired-Pulse Inhibition (%)		
Slice	μM Baclofen	Amplitude of R_{cond} (% maximum)	Control	Baclofen	Wash
1	0.5	100%	17	4	15
1	0.5	50%	42	14	28
2	0.7	100%	22	-1	-3
2	0.7	50%	29	5	54
3	0.7	100%	15	3	4
3	0.7	50%	60	24	38
4	1.0	100%	10	-5	*
4	1.0	50%	26	-3	7
5	1.0	100%	37	-18	40
5	1.0	50%	73	2	39

8-Phenyltheophyline (8PT)

The effect was also studied of the adenosine antagonist 8PT (in the absence of exogenous adenosine) in three slices (Figure 12). The conditioning stimuli were supramaximal. Although paired-pulse

inhibition decreased in all three slices when 1 μM 8PT was superfused the effect was not significant. In addition, there was no evidence of a concentration related effect of 8PT. No change of paired-pulse inhibition was apparent during washout of 8PT for 20 minutes.

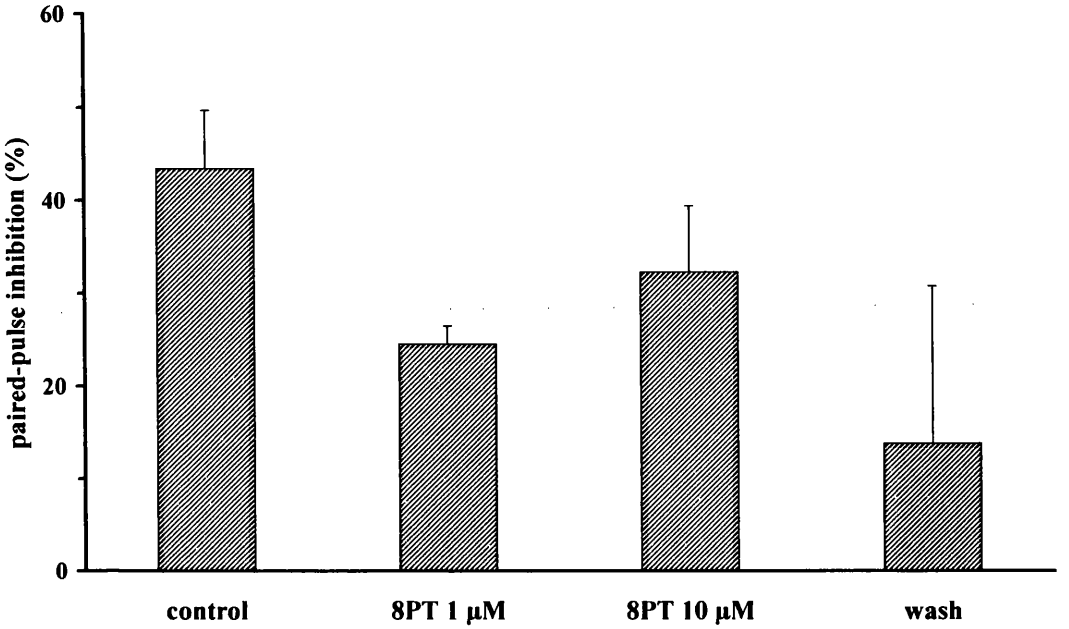

Figure 12 Effect of 8-phenyltheophyline (8PT) 1 μM and 10 μM on paired-pulse inhibition between orthodromic potentials in the presence of bicuculline. The interstimulus interval was 300 ms. Conditioning stimuli were supramaximal (1mA). $R_{control}$ was approximately 50% of maximum amplitude under control conditions. Stimuli were not adjusted in the presence of 8PT. 8PT was superfused for 15 minutes at 1 μM followed by superfusion with 8PT 10 μM for 15 minutes followed by wash for 20 minutes. 10 μM bicuculline was present throughout. Error bars show SE. $n = 3$ slices.

III Antidromic Responses and Interactions in the Absence and Presence of Bicuculline

Changes in antidromically evoked responses should reflect postsynaptic changes in the excitability of the pyramidal cells as opposed to changes in the release of transmitter from Schaffer collateral terminals. In order to discover whether postsynaptic mechanisms were involved in the effects of bicuculline on paired-pulse interactions a series of experiments was undertaken to characterise and determine the effects of

bicuculline superfusion on paired-pulse interactions between antidromic potentials.

Paired-Pulse Interactions Between Antidromic Stimuli in the Absence of Added Agents

Interactions between pairs of antidromic population spikes evoked from a single electrode in the alveus were small in comparison to interactions between orthodromic potentials. In six slices, when conditioning and test stimuli were identical to each other and adjusted so that Rcontrol was approximately 70% maximal, a small but statistically significant inhibition was seen at interstimulus intervals between 50 and 500 ms (Figure 13). Inhibition was maximal (about 4%) between 50 and 200 ms, decreased at interstimulus intervals below 50 ms and above 200 ms and was not significant between 10 and 30 ms or at interstimulus intervals above 500 ms.

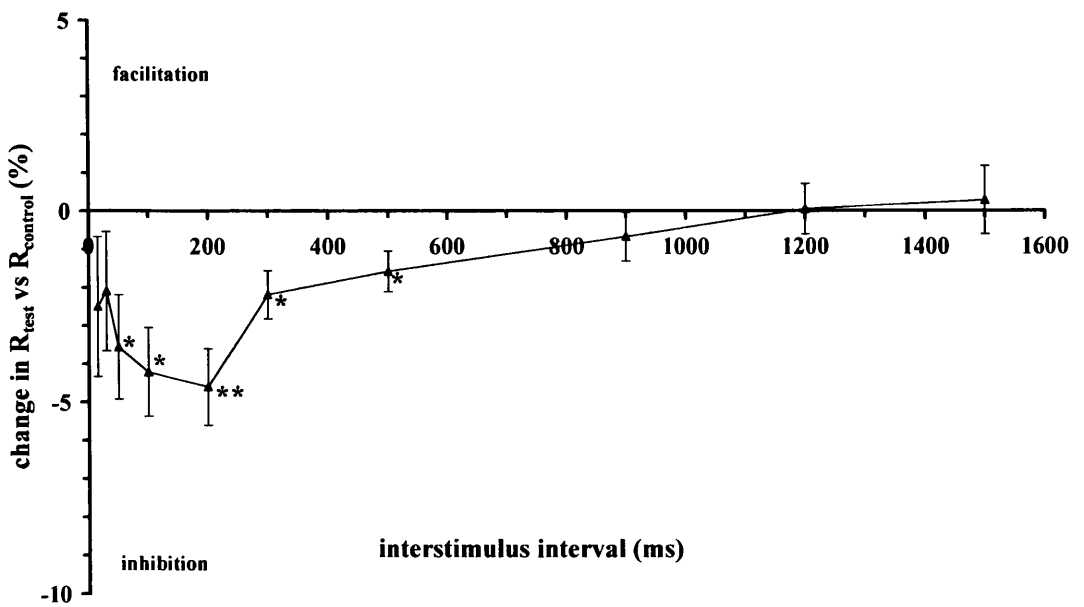

Figure 13 Effect of interstimulus interval on interactions between pairs of antidromic evoked potentials in ACSF in the absence of added agents. Conditioning and test stimuli were set to evoke responses which were 70% of maximal amplitude. Error bars show SE. *$P < 0.05$, **$P < 0.01$ for difference from no change in the amplitude of the evoked potential.

Effect of Bicuculline on Antidromic Responses and Interactions

Unpaired Antidromic Responses

In the absence of exogenous agents the typical evoked antidromic potential recorded extracellularly in the pyramidal cell layer of the CA1 area in response to a single stimulation of the alveus was a single population spike (Figure 14).

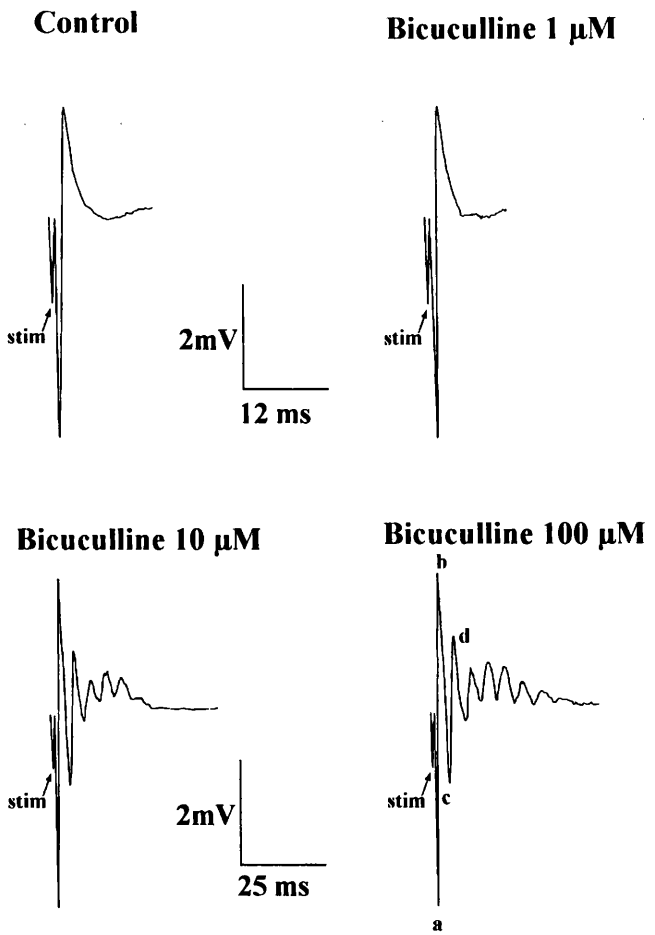

Figure 14 Effect of bicuculline on single antidromic potentials. Bicuculline was superfused at each concentration for 20 minutes. The stimulus was set such that the amplitude of the potential in ACSF was 70% of maximum and was not changed during bicuculline superfusion. The records were stored on a Neurolog averager (4 sweeps) and plotted on a chart recorder. The stimulus artefacts are marked 'stim'. The lower case letters on the spikes of the lower right potential are labels for discussion in the main text. Note the change of time scale for the lower two records.

The antidromic population spike resembled the orthodromic population spike but was of shorter latency. The size of the antidromic population spike increased, up to a maximum size, with the strength of the stimulus to the alveus. In 10 μM bicuculline this single population spike became a short burst of population spikes. This resembled the orthodromic burst evoked in bicuculline, being 30 to 40 ms in duration and usually consisting of four or more recognisable population spikes. Again, it occurred with a shorter latency than the orthodromic response (Figure 14). The size and shape of antidromic responses in bicuculline were stable for a given set of experimental conditions.

In contrast to the effect of bicuculline on orthodromic population spikes, bicuculline 10 μM had no effect on the size of antidromic potentials (as measured by the size of the first spike in the burst) evoked by submaximal stimuli ($P = 0.57$, $n = 7$).

Paired-Pulse Interactions

Interactions between pairs of antidromic population spikes evoked from a single electrode in the alveus were examined in six slices in the presence of 10 μM bicuculline. Conditioning and test stimuli which were identical to each other and adjusted so that Rcontrol was approximately 70% maximal.

Significant paired-pulse inhibition was seen at all interstimulus intervals examined between 10 and 1500 ms. Inhibition was greatest at the shortest interstimulus interval and decreased as interstimulus interval was increased (Figure15). Bicuculline 10 μm significantly increased inhibition, compared to ACSF control, between the pairs of submaximal antidromic stimuli at all interstimulus intervals apart from 50 ms (where the trend was of borderline significance ($P = 0.08$, $n = 6$).

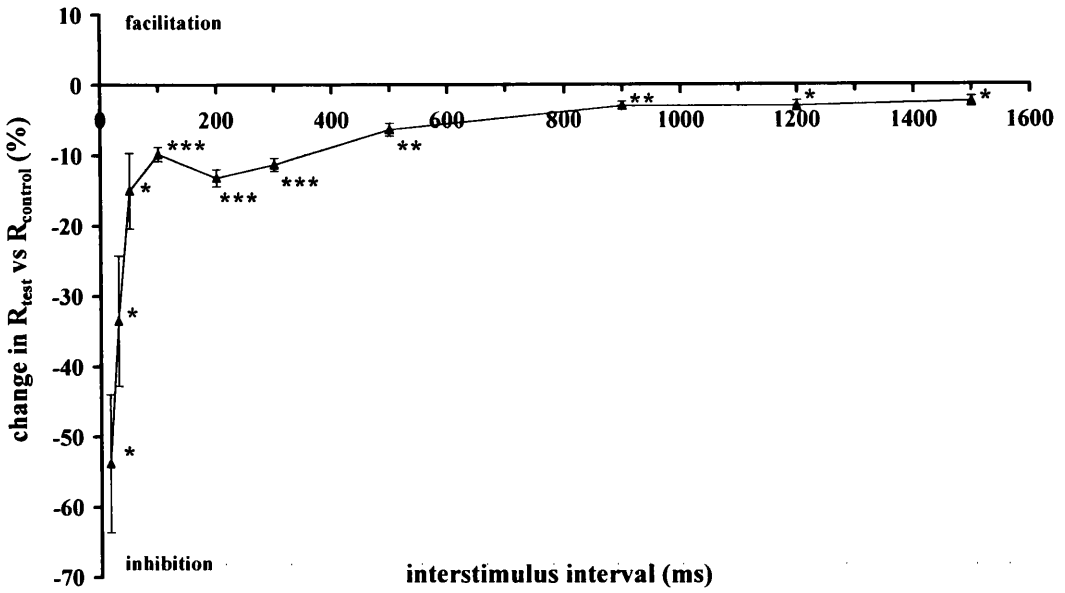

Figure 15 Effect of interstimulus interval on interactions between pairs of antidromic evoked potentials during superfusion with 10 μM bicuculline. Conditioning and test stimuli were set to evoke responses about 70% of maximal amplitude. $n = 6$ slices. Error bars show SE. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ for difference from no change in the amplitude of the evoked potential.

The concentration-dependence of the effect of bicuculline on antidromic inhibition was examined in a further three slices at interstimulus intervals of 200 and 300 ms. The magnitude of the paired-pulse inhibition tended to increase at both interstimulus intervals as the concentration of bicuculline was increased from 1 μM to 100 μM (Figure 16).

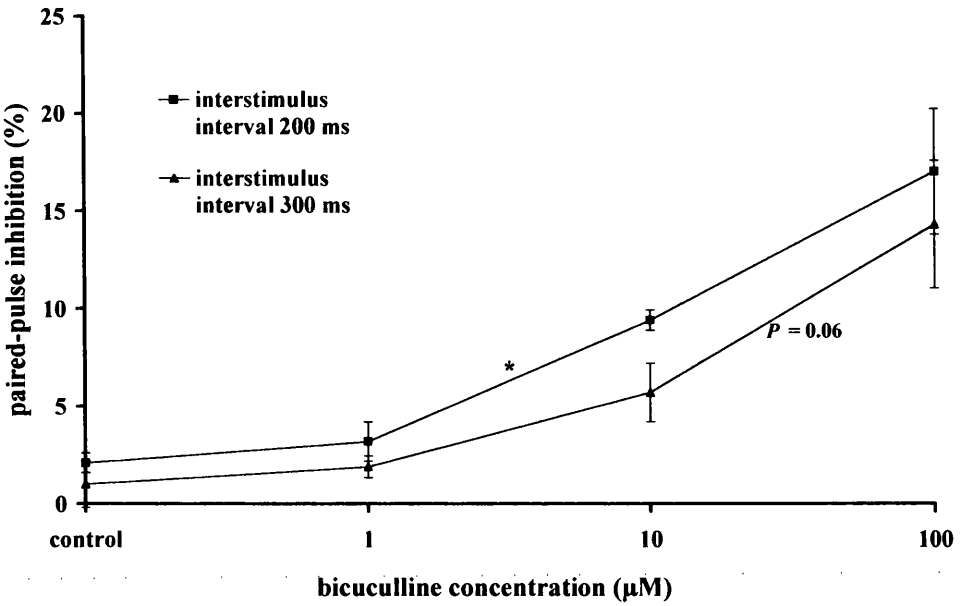

Figure 16 Concentration-response relationships for the effect of bicuculline on interactions between pairs of antidromic potentials at interstimulus intervals of 200 and 300 ms. Bicuculline was superfused for 20 minutes at each concentration with no washout. Conditioning and test stimuli were set to evoke responses about 70% of maximum amplitude and were not changed once set at the beginning of each experiment. $n = 3$ slices. $^*P < 0.05$ for difference between points. Error bars show SE.

Orthodromic and Antidromic Stimulation Paradigms in the Presence of Bicuculline

In a series of seven slices a comparison was made of the effects of the four possible combinations of submaximal antidromic and orthodromic test and conditioning responses in the presence of bicuculline methobromide 10 μM at an interstimulus interval of 300 ms (Figure 17). Antidromic and orthodromic conditioning stimuli inhibited the orthodromic test response to a similar degree ($P = 0.84$, $n = 7$). Likewise, there was no significant difference between the effects of antidromic and orthodromic conditioning stimuli on the antidromic test response ($P = 0.35$, $n = 7$). Inhibition of the antidromic test was smaller than inhibition of the orthodromic test for both antidromic and

orthodromic conditioning stimuli ($P < 0.001$, $n = 7$ for each comparison).

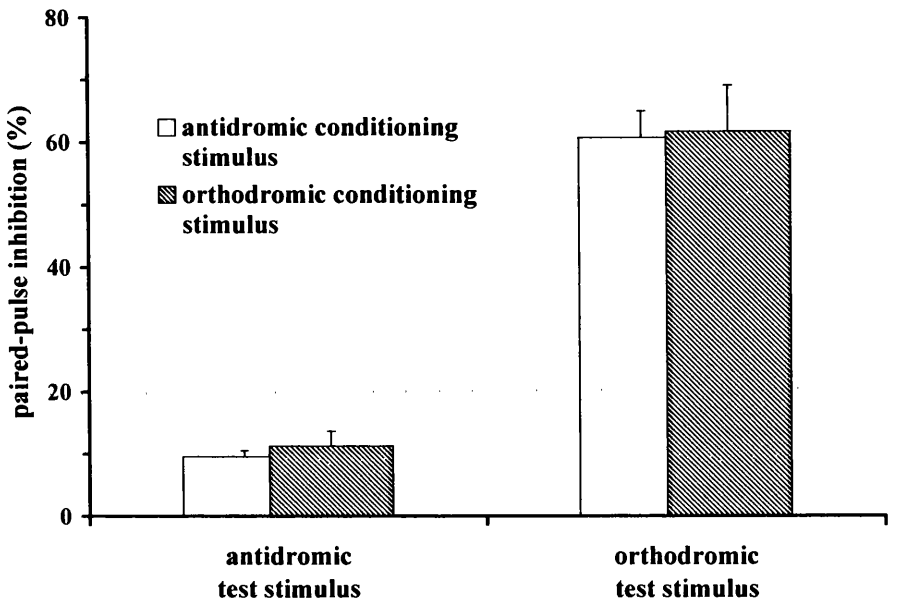

Figure 17. Comparison of paired-pulse inhibition using the four possible combinations of orthodromic and antidromic test and conditioning stimuli in 10μM bicuculline. Interstimulus interval = 300 ms. Test and conditioning stimuli were all submaximal and orthodromic responses were approximately the same amplitude as antidromic responses. Error bars show SE. $n = 7$ slices.

IV Investigations into the Mechanism of Paired-Pulse Inhibition of Evoked Antidromic Burst Potentials

A short series of experiments was undertaken to determine, firstly, whether paired-pulse inhibition of antidromic responses could be abolished by blocking excitatory synaptic components that might contaminate the antidromic response during bicuculline superfusion and secondly whether enhanced paired-pulse inhibition occurred in an alternative model of increased pyramidal cell excitability.

Effect of Kynurenic Acid on Paired-Pulse Inhibition in the Presence of Bicuculline

In two slices the effect of the non-selective glutamate antagonist kynurenic acid was examined on the paired-pulse inhibition seen between submaximal antidromic bursts in the presence of 100 μM bicuculline. 1 mM kynurenic acid reduced, but did not abolish, the burst response to an antidromic stimulus in both slices; the number and amplitude of secondary population spikes were reduced although the amplitude of the primary spike was unchanged (Figure 18). Paired pulse inhibition was still demonstrable in the presence of 1 mM kynurenic acid (Figure 18).

Paired-Pulse Inhibition between Antidromic Bursts in Calcium-Free ACSF

Evoked burst potentials resembling those seen when the slice was stimulated in the presence of bicuculline could also be demonstrated in calcium-free ACSF in the absence of exogenous agents. The occurrence of paired-pulse inhibition was studied in three slices under these conditions. Slices were superfused for 30 minutes in nominally calcium free ACSF. All stimuli were antidromic, delivered from a single electrode in the alveus, and submaximal for the size of the evoked potential. A single stimulus to the alveus evoked a short burst of population spikes similar to the antidromic potential evoked in bicuculline. In all the three slices examined marked, significant, paired-pulse inhibition of the test potential was seen when the interstimulus interval between test and conditioning stimulus was 100 ms. Smaller, but still significant, paired-pulse inhibition was seen when the interstimulus interval was 200 and 300 ms (Figures 19 and 20).

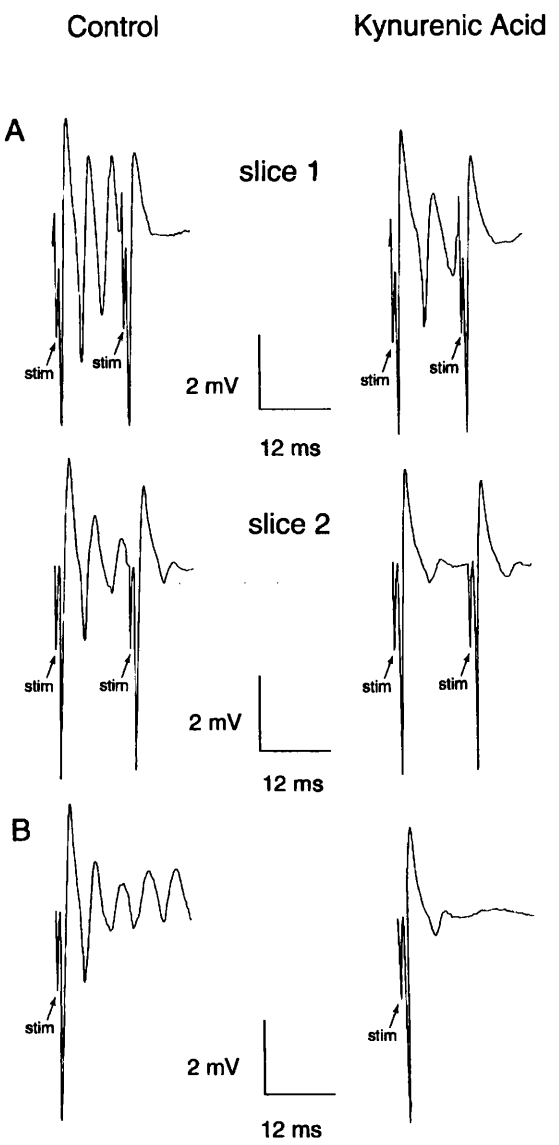

Figure 18 Records from two slices showing the effect of 1 mM kynurenic acid on paired-pulse inhibition of antidromic potentials in the presence of bicuculline. The records were stored on a Neurolog averager (4 sweeps) and plotted on a chart recorder. Conditioning and test stimuli were identical to each other and set to give $R_{control}$ 70% of maximum size. The interstimulus interval was 300 ms. 10 μM bicuculline was present throughout. **A** Pairs of potentials, $R_{control}$ and R_{test} under control conditions and during superfusion with kynurenic acid. $R_{control}$ and R_{test} were stored in succeeding halves of the same averager sweep with data acquisition suspended for most of the interstimulus interval. This results in both the potentials and the interstimulus interval appearing truncated in the figure. **B** $R_{control}$ from slice 2 shown in more detail under control conditions and during kynurenic acid superfusion. Stimulation artefacts are labelled 'stim'.

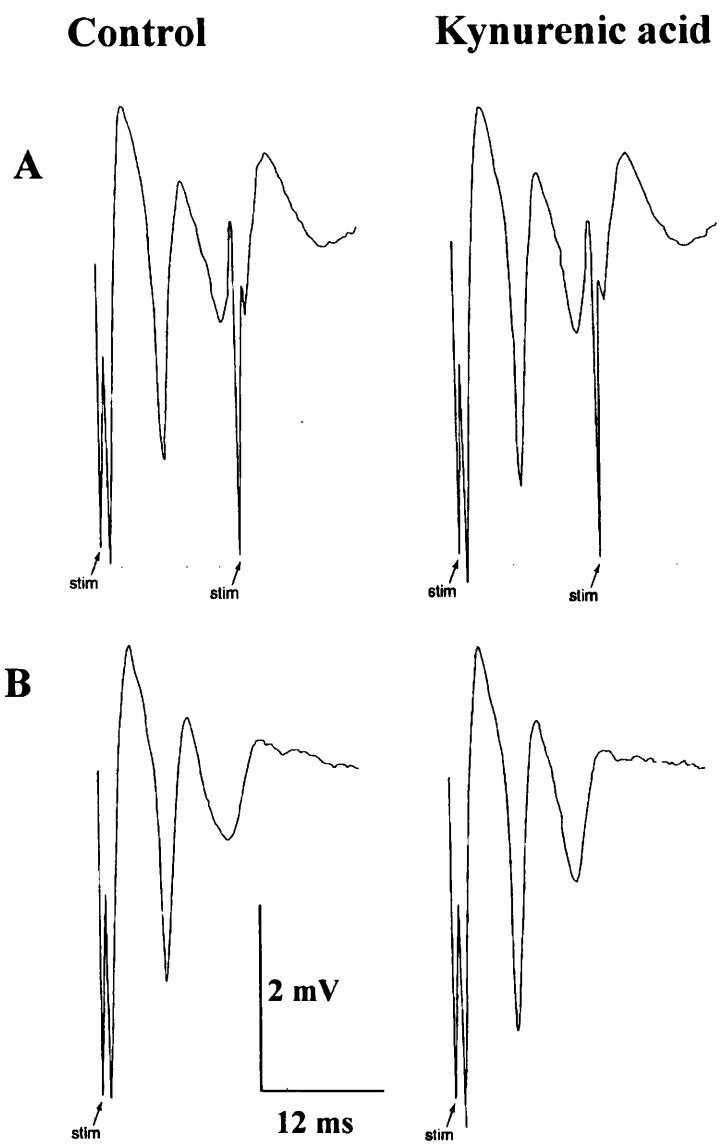

Figure 19 Records showing the effect of 1 mM kynurenic acid on paired-pulse inhibition of antidromic potentials in nominally calcium-free ACSF. The records were stored on a Neurolog averager (4 sweeps) and plotted on a chart recorder. Conditioning and test stimuli were identical to each other and set to give $R_{control}$ 70% of maximum size. The interstimulus interval was 300 ms. **A** Pairs of potentials, $R_{control}$ and R_{test} under control conditions and during superfusion with kynurenic acid. $R_{control}$ and R_{test} were stored in succeeding halves of the same averager sweep with data acquisition suspended for most of the interstimulus interval. This results in both the potentials and the interstimulus interval appearing truncated in the figure. **B** $R_{control}$ shown in more detail under control conditions and during kynurenic acid superfusion. Stimulation artefacts are labelled 'stim'.

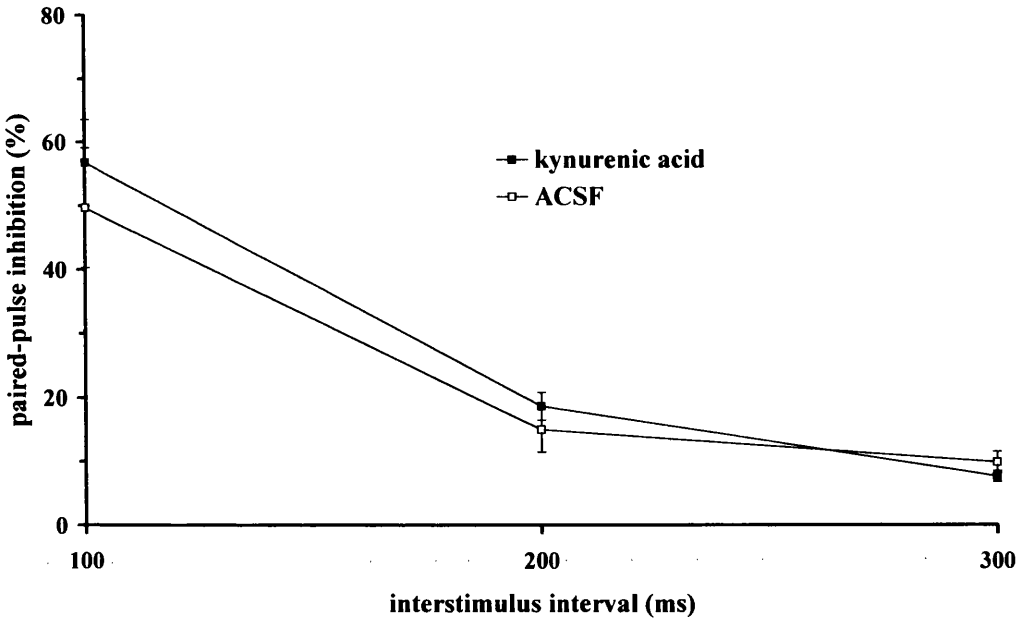

Figure 20 Effect of 1 mM kynurenic acid on paired pulse inhibition of an antidromic test stimulus by an antidromic conditioning stimulus in nominally calcium-free ACSF in the absence of other agents. Conditioning and test stimuli were both set to evoke responses about 70% of maximum amplitude. Kynurenic acid was superfused for 20 min. Stimuli were not adjusted during kynurenic acid superfusion. $n = 3$ slices. Error bars show SE.

Effect of Kynurenic Acid and Increased Magnesium Concentration

1 mM kynurenic acid had no effect either on the size and shape of the burst potentials in nominally calcium-free ACSF or on the size of the paired-pulse inhibition ($n = 3$) (Figures 19 and 20).

In two of the slices the concentration of magnesium was raised from 1.2 to 4.8 mM in calcium-free ACSF during superfusion of kynurenic acid. In both slices the amplitude of the secondary spikes on the evoked *107*response decreased, being completely extinguished in one (Figure 21). Paired-pulse inhibition at interstimulus intervals of 100 ms, 200 ms and 300 ms was abolished in both slices.

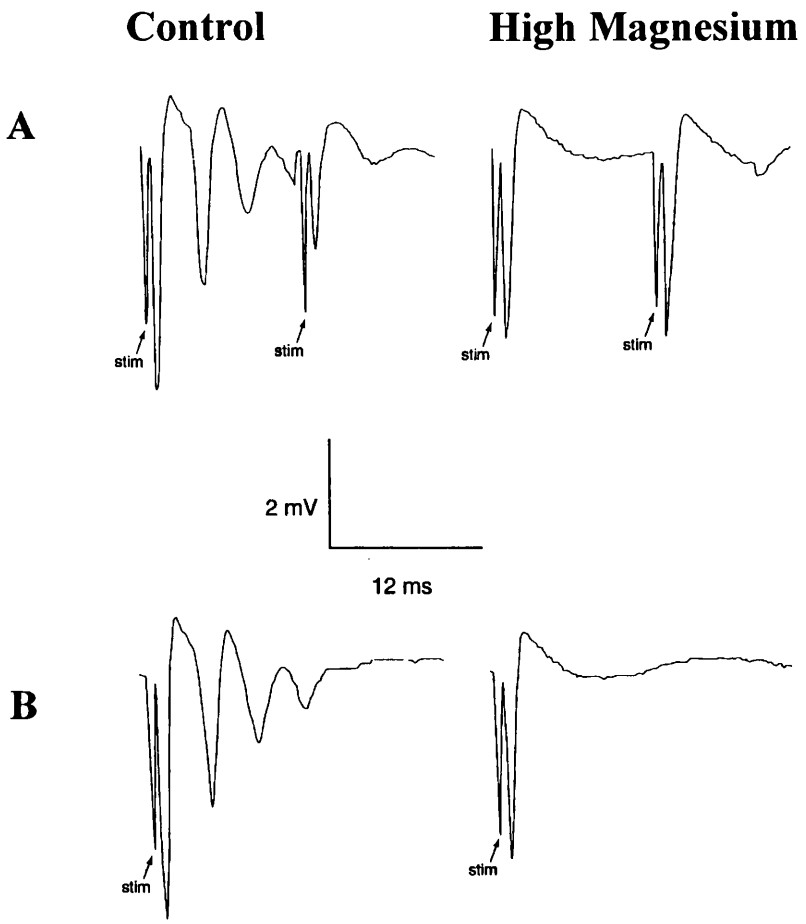

Figure 21 Records showing the effect of a high concentration of magnesium on paired-pulse inhibition of antidromic potentials in nominally calcium-free ACSF. **A** Pairs of potentials, $R_{control}$ and R_{test} during superfusion with normal (1.2 mM) and raised (4.8 mM) Mg^{2+}. The ACSF was nominally calcium-free and 1 mM kynurenic acid was present throughout. $R_{control}$ and R_{test} were stored in succeeding halves of the same averager sweep with data acquisition suspended for most of the interstimulus interval. This results in both the potentials and the interstimulus interval appearing truncated in the figure. **B** $R_{control}$ shown in more detail during superfusion with normal and high concentrations of magnesium. Stimulation artefacts are labelled 'stim'. See Figure 19 for more details.

V Effects of Baclofen, Adenosine and 2-Hydroxysaclofen on Bicuculline-Enhanced Paired-Pulse Inhibition of Orthodromic and Antidromic Potentials

A series of experiments was performed to examine the effects of baclofen, adenosine and 2-hydroxysaclofen on paired-pulse inhibition of

orthodromic potentials (evoked from the stratum radiatum) and antidromic potentials (evoked from the alveus) in the presence of bicuculline. The main experiments were conducted using orthodromic conditioning stimuli but additional experiments were performed to examine the effect of 2-hydroxysaclofen on inhibition of an orthodromic test stimulus by an antidromic conditioning stimulus. The interstimulus interval was 300 ms in all experiments.

Baclofen and adenosine were noted to reduce the size of all evoked orthodromic potentials; 20 μM adenosine often abolished R_{test} completely. Paired-pulse inhibition during superfusion with all three agents was thus measured with $R_{control}$ and R_{cond} the same size as under control conditions i.e. the test and sub-maximal conditioning stimuli were adjusted, if necessary, during superfusion of the test agents to counteract any direct action of these compounds on the evoked potential.

Paired-Pulse Inhibition before Superfusion of Test Agents

Only slices were used which showed an inhibition of an orthodromic test response by a supramaximal (1 mA) orthodromic conditioning stimulus in the presence of 10 μM bicuculline at an interstimulus interval of 300 ms. Most of the slices examined fulfilled this criterion, and all of the slices which did so also showed inhibition of an orthodromic potential by a submaximal orthodromic conditioning stimulus (R_{cond} 60% of maximum size) and of an antidromic test potential by both a submaximal and supramaximal orthodromic conditioning stimulus. Inhibition of antidromic test potentials was generally much less than inhibition of orthodromic test potentials at either conditioning stimulus strength.

When conditioning and test stimuli were both orthodromic, decreasing conditioning stimulus strength from supramaximal to submaximal caused an increase in paired-pulse inhibition in 15 of 22 slices. In the 22 slices used to examine the effect of these agents with this experimental paradigm the mean control inhibition (at 300 ms interstimulus interval in bicuculline alone) of orthodromic test potentials by orthodromic conditioning stimuli was 26.3% ± 3.45 when the conditioning stimulus was supramaximal and 39.3% ± 5.91 when the conditioning stimulus was submaximal. The difference was significant ($P < 0.05$, $n = 22$).

The mean inhibition of antidromic test potentials was 11.9% ± 1.04 by a supramaximal orthodromic conditioning stimulus and 10.3% ± 1.05 by a submaximal orthodromic conditioning stimulus. This difference was not significant ($P = 0.12$, $n = 22$).

Baclofen

Orthodromic Test Potentials

Baclofen, a GABA-B agonist, at 1 μM markedly and significantly reduced inhibition of orthodromic potentials by an orthodromic conditioning stimuli both when the conditioning stimulus was supramaximal ($P < 0.01$, $n = 7$) and when the conditioning stimulus was submaximal ($P < 0.001$, $n = 7$) (Figures 22 and 23A). The mean proportional decrease in inhibition was 92% ± 12.8 when the conditioning stimulus was supramaximal and 93% ± 10.7 when the conditioning stimulus was submaximal ($n = 7$).

The effect of baclofen on paired-pulse inhibition partially but significantly reversed when baclofen was washed from the recording chamber (Figure 23A).

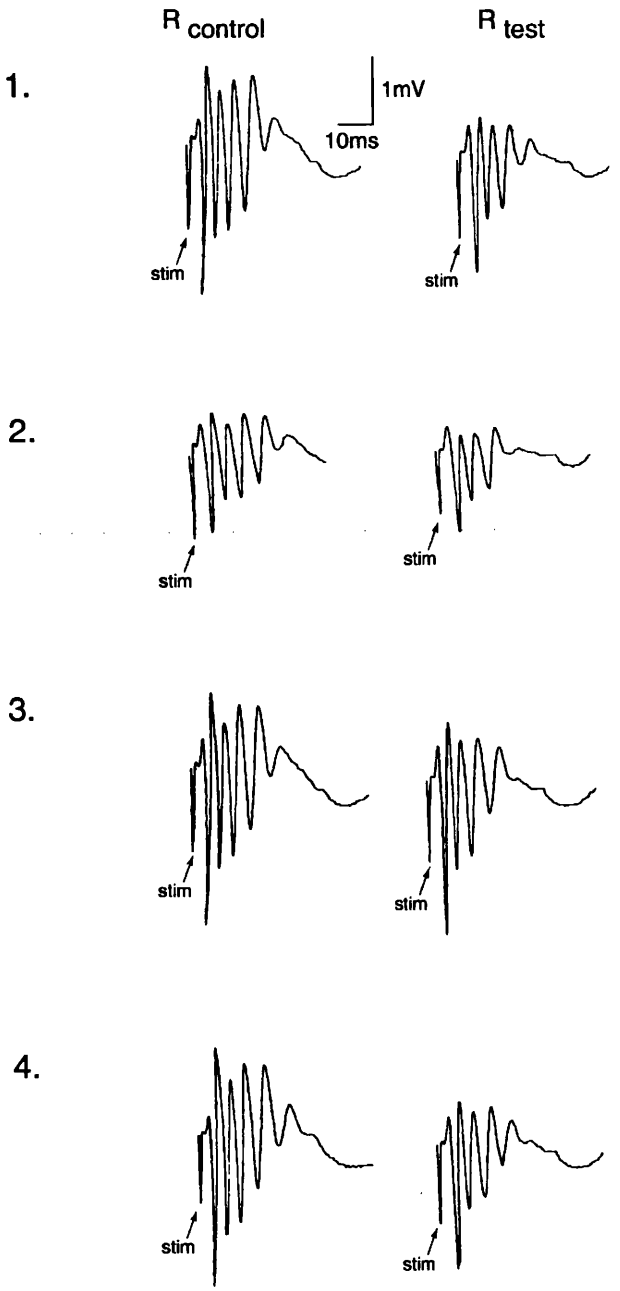

Figure 22 Records showing the effect of 1 μM baclofen on paired-pulse inhibition of orthodromic potentials in the presence of bicuculline. $R_{control}$ and R_{cond} were both set to 60% of maximum amplitude under control conditions. The interstimulus interval was 300 ms. Records were stored in a Neurolog averager (8 sweeps) and plotted on a chart recorder. The sequence shows pairs of control and test potentials; **1** Control. **2** During superfusion with baclofen, stimuli unchanged from control. **3** During superfusion with baclofen, test stimulus adjusted to restore the control amplitudes of $R_{control}$ and R_{cond}. **4** After 45 minutes washing, test stimulus adjusted to maintain the control amplitudes of $R_{control}$ and R_{cond}. See Figure 23 for further details. Stimulus artefacts are labelled 'stim'.

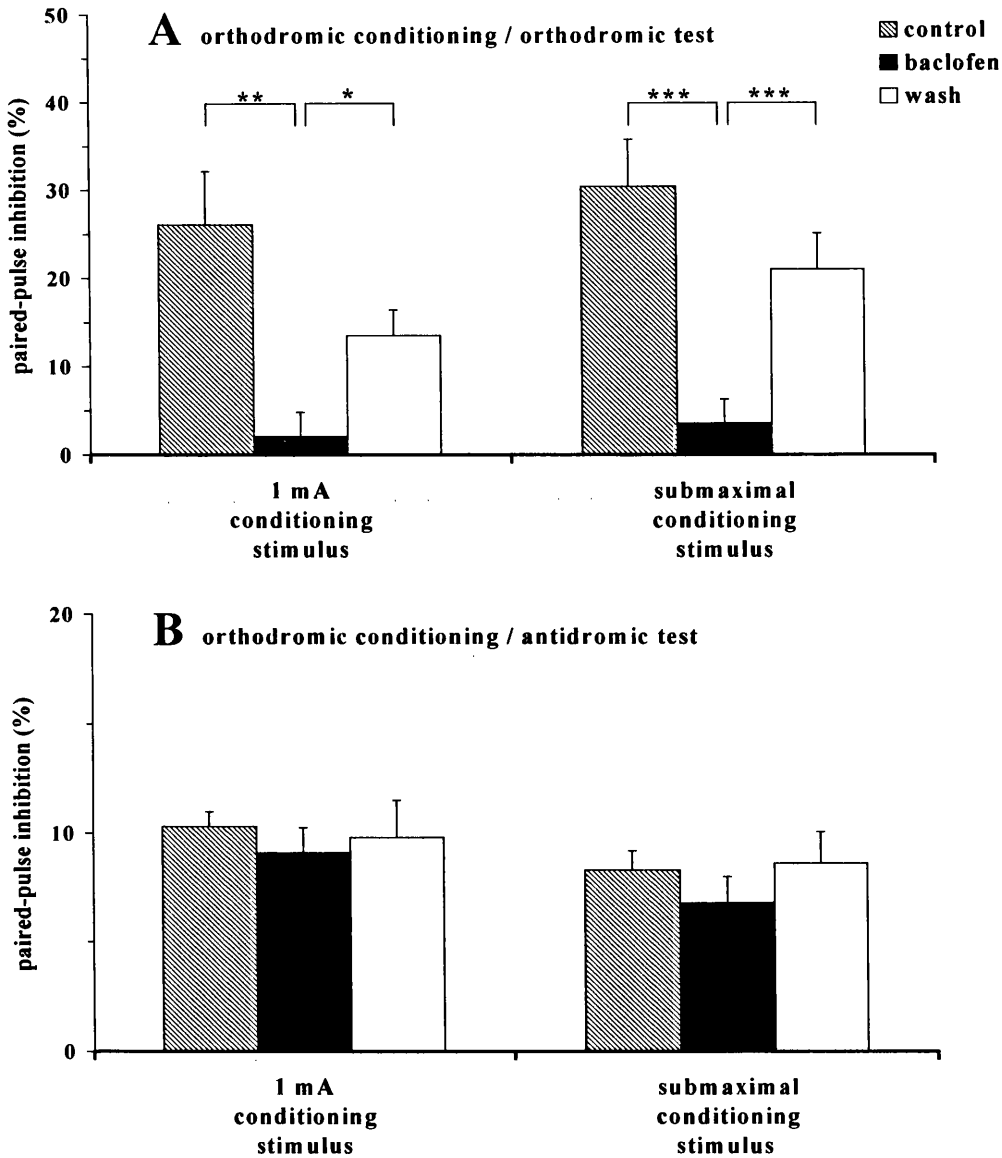

Figure 23 Effect of 1μM baclofen on paired-pulse inhibition of orthodromic and antidromic potentials by orthodromic conditioning stimuli in the presence of bicuculline. The interstimulus interval was 300 ms. Paired-pulse inhibition was measured using two strengths of conditioning stimulus, supramaximal (1 mA), and a smaller stimulus set such that R_{cond} was 60% of its maximum amplitude. $R_{control}$ was set to 60% of maximum amplitude under control conditions. The test stimulus and the smaller conditioning stimulus were adjusted if necessary during superfusion with baclofen and during subsequent wash to keep $R_{control}$ and the submaximal R_{cond} constant at their control amplitudes. Baclofen was superfused for 30 minutes with measurements taken during the second half of this period. Washout time was 45 minutes with measurements taken during the final 15 minutes. 10 μM bicuculline was present throughout. **A** Paired-pulse inhibition of orthodromic test stimuli by orthodromic conditioning stimuli. **B** Paired-pulse inhibition of antidromic test stimuli by orthodromic conditioning stimuli in the same slices. NB difference in scale between A and B. $n = 7$ slices. Error bars show SE $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Antidromic Test Potentials

Baclofen at this concentration had no significant effect on paired-pulse inhibition of an antidromic potential by an orthodromic conditioning stimulus (Figure 23B). The difference between the effect of baclofen on inhibition of orthodromic and antidromic potentials (normalised to the respective control) was significant ($P < 0.01$, $n = 7$) for both maximal and submaximal conditioning stimuli.

Adenosine

Orthodromic Test Potentials

Adenosine at 20 μM had no significant effect on inhibition of an orthodromic test potential by a supramaximal (1mA) conditioning stimulus (Figure 24A).

When a submaximal conditioning stimulus was used adenosine significantly ($P < 0.01$) and reversibly reduced inhibition of an orthodromic test potential by an orthodromic conditioning stimulus (55% ± 12.3 reduction, $n = 9$) (Figure 24A).

Antidromic Test Potentials

Adenosine at this concentration had no significant effect on paired-pulse inhibition of an antidromic potential by an orthodromic conditioning stimulus when either a supramaximal or submaximal conditioning stimulus was used (Figure 24B). The difference between the effect of adenosine on paired-pulse inhibition of orthodromic and antidromic potentials (normalised to the respective control) by a submaximal conditioning stimulus was not quite statistically significant ($P = 0.057$, $n = 9$).

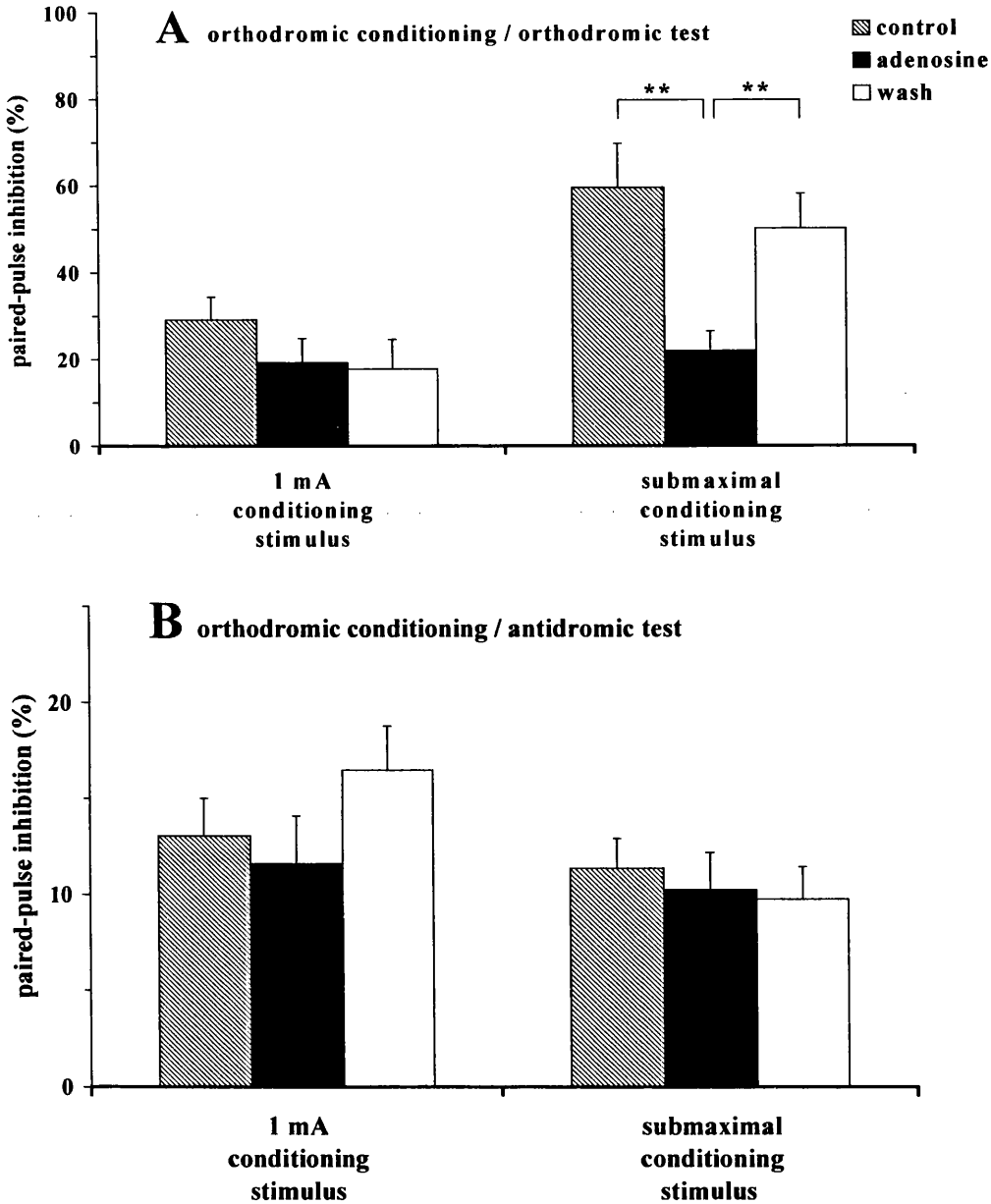

Figure 24. Effect of 20 μM adenosine on paired-pulse inhibition of orthodromic and antidromic potentials by orthodromic conditioning stimuli in the presence of bicuculline. The interstimulus interval was 300 ms. Paired-pulse inhibition was measured using two strengths of conditioning stimulus, supramaximal (1 mA), and a smaller stimulus set such that R_{cond} was 60% of its maximum amplitude. See Figure 23 for further details. **A** Paired-pulse inhibition of orthodromic test stimuli by orthodromic conditioning stimuli. **B** Paired-pulse inhibition of antidromic test stimuli by orthodromic conditioning stimuli in the same slices. NB difference in scale between A and B. n= 9 slices. Error bars show SE $^{**}P < 0.01$.

2-Hydroxysaclofen

Orthodromic test potentials

Superfusion with the GABA-B antagonist 2-hydroxysaclofen at 200 μM (in the presence of bicuculline at interstimulus interval = 300 ms) significantly reduced inhibition of an orthodromic test potential by an orthodromic conditioning stimulus both when the conditioning stimulus was supramaximal ($P < 0.05$, $n = 6$) and submaximal ($P < 0.01$, $n = 6$) (Figures 25 and 26A). Two slices showed facilitation in the presence of 2-hydroxysaclofen with both submaximal and supramaximal conditioning stimuli, a third slice showing facilitation with the larger conditioning stimulus only. When this facilitation was included in the calculation as negative inhibition the mean proportional decrease in inhibition was greater than 100% for both supramaximal (126% ± 26.7, $n = 6$) and submaximal (110% ± 18.1, $n = 6$) conditioning stimuli.

R_{test} was noted to increase in amplitude during the early minutes of 2-hydroxysaclofen superfusion while $R_{control}$ remained unchanged or decreased (see example in Figure 25). However, the experiment was designed such that stimulus adjustment to counteract changes in the amplitude of $R_{control}$ and R_{cond} was made during test agent superfusion to minimise variation in the duration of exposure to the test agents. The effect of 2-hydroxysaclofen on paired-pulse inhibition with stimulus strength kept constant throughout the experiment could not therefore be quantified through the group.

The effect of 2-hydroxysaclofen on paired-pulse inhibition partially reversed when it was washed from the recording chamber (Figure 26A).

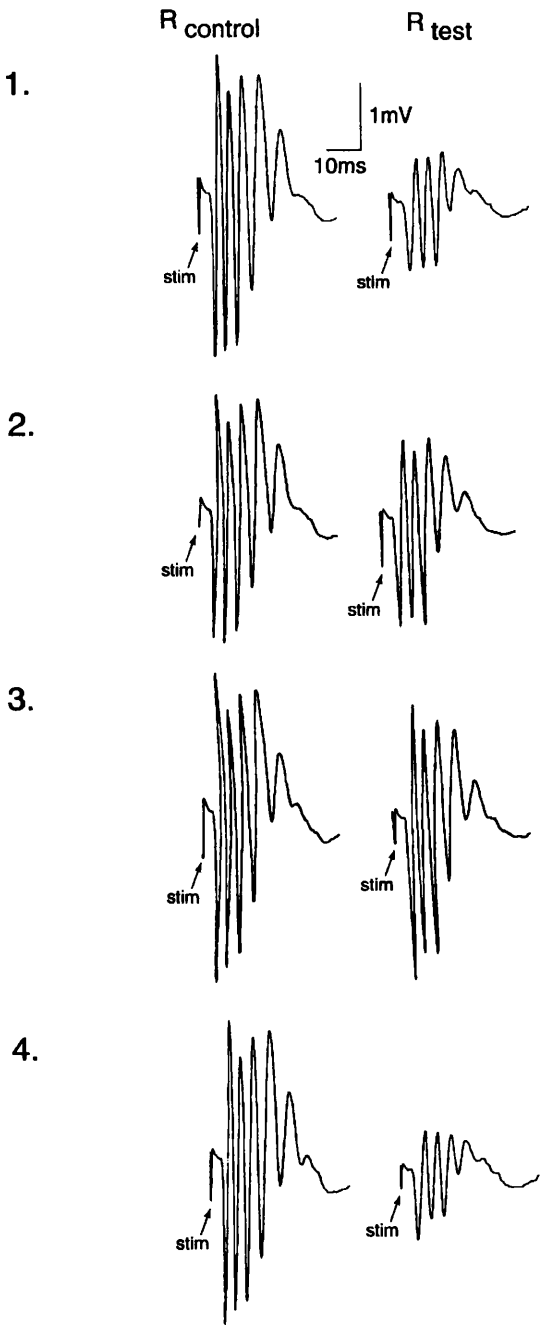

Figure 25 Records showing the effect of 200 μM 2-hydroxysaclofen on paired-pulse inhibition of orthodromic potentials in the presence of bicuculline. $R_{control}$ and R_{cond} were both set to 60% of maximum amplitude under control conditions. The interstimulus interval was 300 ms. Records were stored in a Neurolog averager (8 sweeps) and plotted on a chart recorder. The sequence shows pairs of control and test potentials; **1** Control. **2** During superfusion with 2-hydroxysaclofen, stimuli unchanged from control. **3** During superfusion with 2-hydroxysaclofen, test stimulus adjusted to restore the control amplitudes of $R_{control}$ and R_{cond}. **4** After 45 minutes washing, test stimulus adjusted to maintain the control amplitudes of $R_{control}$ and R_{cond}. See Figure 23 for further details. Stimulus artefacts are labelled 'stim'.

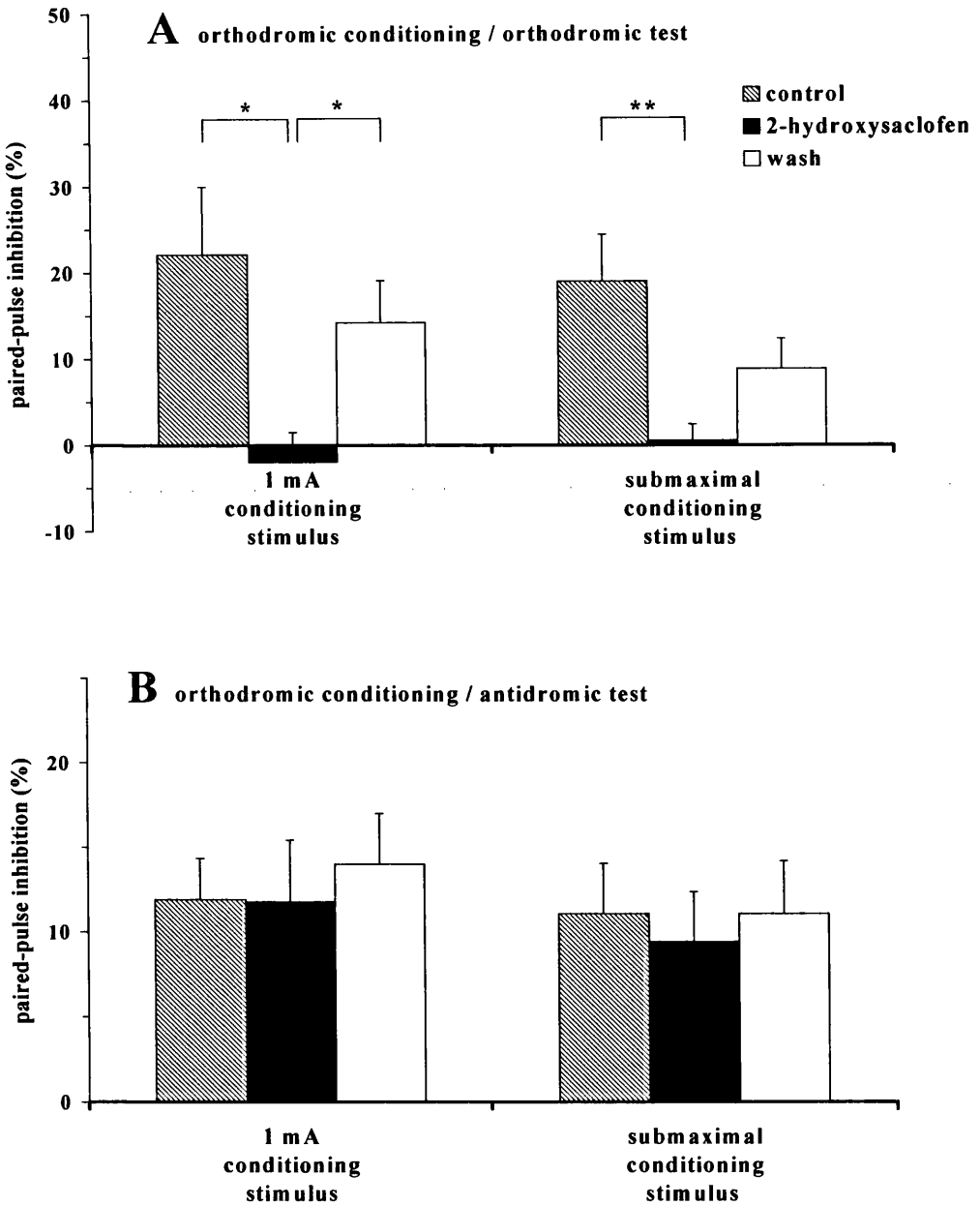

Figure 26 Effect of 200 μM 2-hydroxysaclofen on paired-pulse inhibition of orthodromic and antidromic potentials by orthodromic conditioning stimuli in the presence of bicuculline. The interstimulus interval was 300 ms. Paired-pulse inhibition was measured using two strengths of conditioning stimulus, supramaximal (1 mA), and a smaller stimulus set such that R_{cond} was 60% of its maximum amplitude. See Figure 23 for further details. **A** Paired-pulse inhibition of orthodromic test stimuli by orthodromic conditioning stimuli. **B** Paired-pulse inhibition of antidromic test stimuli by orthodromic conditioning stimuli in the same slices. NB difference in scale between A and B. Negative inhibition implies a facilitation of R_{test} compared with $R_{control}$. n= 6 slices. Error bars show SE. $*P < 0.05$, $**P < 0.01$.

Antidromic Test Potentials

In contrast to its effect on inhibition of orthodromic potentials, 2-hydroxysaclofen at this concentration had no significant effect on inhibition of antidromic test potentials by orthodromic conditioning stimuli in the same slices under the same conditions (Figure 26B). The difference between the effect of 2-hydroxysaclofen on paired-pulse inhibition of orthodromic and antidromic test potentials (normalised to the respective control) was significant ($P < 0.01$, $n = 6$) for both maximal and submaximal conditioning stimuli.

Orthodromic Test Potentials with Antidromic and Orthodromic Conditioning Stimuli

In a different set of slices the effect of 200 μM 2-hydroxysaclofen was compared on inhibition of an orthodromic test response by an antidromic and by an orthodromic conditioning stimulus (in the presence of bicuculline at interstimulus interval = 300 ms). All stimuli were submaximal. 2-Hydroxysaclofen significantly reduced paired-pulse inhibition both when the conditioning stimulus was antidromic (48% ± 9.3 reduction, $n = 5$, $P < 0.05$) and when the conditioning stimulus was orthodromic (64% ± 5.4 reduction, $n = 5$, $P < 0.05$). There was no significant difference in the effect of 2-hydroxysaclofen when the conditioning stimulus was orthodromic compared with when it was antidromic. The effect of 2-hydroxysaclofen reversed fully on washing (Figure 27).

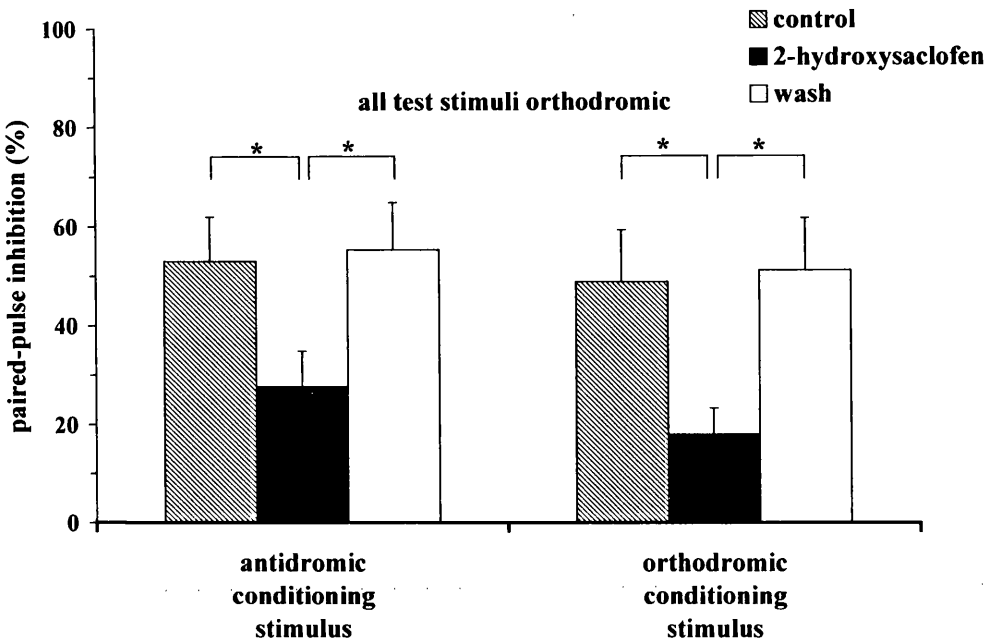

Figure 27 Effect of 200 μM 2-hydroxysaclofen on paired-pulse inhibition of orthodromic potentials by orthodromic and antidromic conditioning stimuli in the same slices in the presence of bicuculline. The interstimulus interval was 300 ms. Conditioning and test stimuli were both set to evoke responses 60% of maximum amplitude. Stimuli were adjusted in the presence of 2-hydroxysaclofen and during subsequent washing, if necessary, to return $R_{control}$ and R_{cond} to their control amplitude. Orthodromic responses were approximately the same amplitude as antidromic responses. Test stimuli were all orthodromic. 2-hydroxysaclofen was superfused for 30 minutes with 30 minutes wash. $n = 5$ slices. Error bars show SE. $^*P < 0.05$.

VI Further Experiments to Define the Effect of Adenosine on Orthodromic Bicuculline-Resistant Paired-Pulse Inhibition

A series of experiments was undertaken to further define the effect of adenosine on orthodromic bicuculline-resistant paired-pulse inhibition. The previous investigations into the effect of adenosine on paired-pulse inhibition of an orthodromic test potential were repeated using different experimental parameters and extended. The concentration-response relationships for the effects of adenosine on single evoked potential amplitude and on paired-pulse inhibition were compared and a comparison was also made of the effects of adenosine on single evoked population spikes and short latency paired-pulse inhibition in the

absence of bicuculline. The influence of conditioning stimulus strength on adenosine's effect on bicuculline-enhanced paired-pulse inhibition was examined in comparison to its influence on the effect of baclofen.

All stimuli were orthodromic. The interstimulus interval was 300 ms in the experiments with bicuculline and 30 ms in the experiments in ACSF.

Effect of Stimulus Strength on Bicuculline-Enhanced Paired-Pulse Inhibition

The effect was studied of varying stimulus strength on the amount of paired-pulse inhibition between orthodromic stimuli when conditioning and test stimuli were identical. Stimulus strength / response profiles for paired-pulse inhibition were generated in control solution (10 μM bicuculline) before superfusion with adenosine in 7 slices.

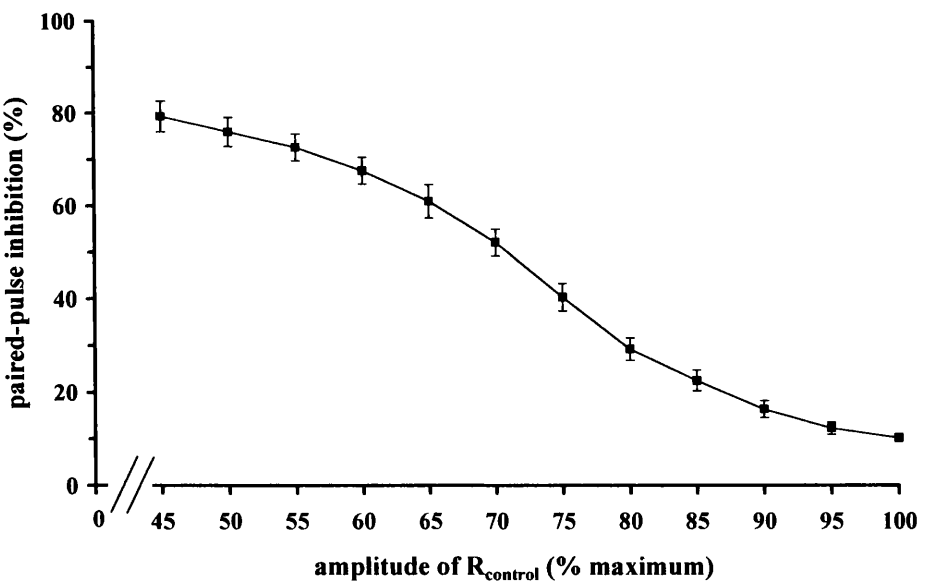

Figure 28 Effect of evoked potential amplitude on paired-pulse inhibition of a test stimulus by an identical conditioning stimulus in the presence of bicuculline. All stimuli were orthodromic. Inhibition was measured using a range of between 6 and 12 different stimulus strengths in each slice with the conditioning stimulus always identical to the test stimulus. Slices were selected to show inhibition in the range 20 to 40% when $R_{control}$ was 80% maximal. The maximum amplitude of $R_{control}$ was evoked by a supramaximal stimulus of 1 mA. 10 μM bicuculline was present throughout. Interpolated values were used for averaging between slices. Error bars show SE. n = 7 slices.

When pairs of identical stimuli were used to elicit the conditioning potential $R_{control}$ and the test response R_{test}, the size of the paired-pulse inhibition between them was found to depend on the evoked potential amplitude. When stimulus strength was such that $R_{control}$ was 50% of its maximum size the test response was inhibited by approximately 80%. As stimulus strength was increased towards that yielding a maximal potential, paired-pulse inhibition declined to less than 20% (Figure 28).

Effect of Adenosine on Bicuculline-Enhanced Paired-Pulse Inhibition

Constant Stimulus Strength

A series of experiments was undertaken to determine the effect of adenosine on bicuculline-enhanced paired-pulse inhibition when no adjustment of stimulus strength was made to compensate for the direct depressive effect of adenosine on the evoked potentials (these experiments also used to investigate the effect of adenosine on matched unpaired and paired-inhibited responses; see below). Pairs of identical stimuli were used to elicit the conditioning potential ($R_{control}$) and the test potential (R_{test}).

Adenosine at a concentration of 10 μM reversibly decreased the size of both unpaired control ($R_{control}$) and paired test evoked potentials (R_{test}) (Figure 29 and Figure 30; open and crosshatched columns). Adenosine 10 μM caused a proportionately greater decrease in R_{test} than of $R_{control}$ leading to an apparent increase in paired-pulse inhibition. Control inhibition of 26% ± 2.0 increased to 56% ± 5.4 in the presence of adenosine ($n = 10$ slices, $P < 0.0001$). $R_{control}$ was approximately 80% of maximum size before adenosine superfusion.

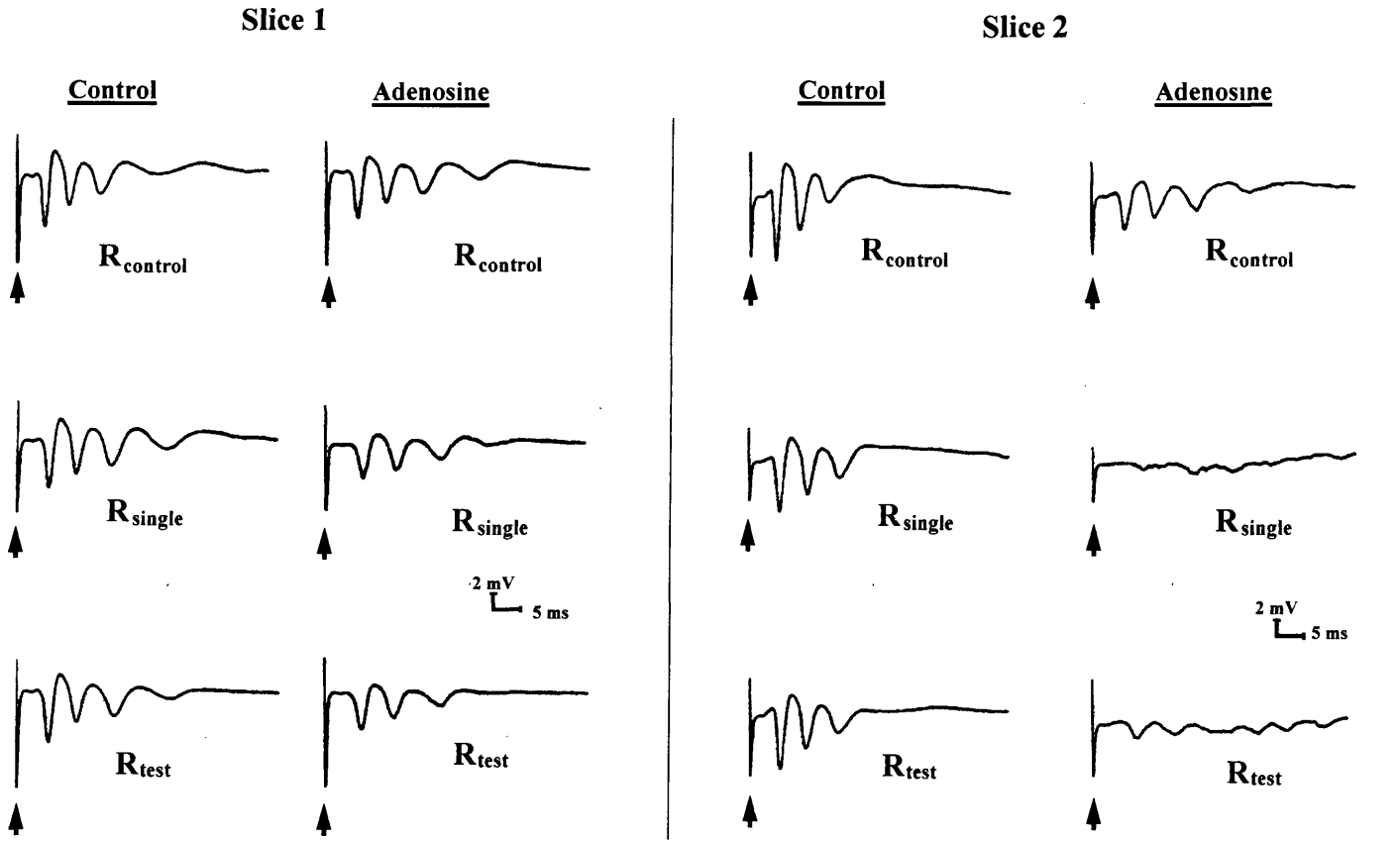

Figure 29 Typical records from two slices showing the effect of 10 μM adenosine on the amplitude of paired and unpaired orthodromic potentials in the presence of bicuculline. Pairs of equal stimuli 300 ms apart were used for the paired potentials ($R_{control}$ and R_{test}). Pairs of stimuli were alternated at 1 min intervals with single evoked potentials (R_{single}). The stimulus used to evoke R_{single} was adjusted so that R_{single} and R_{test} were the same amplitude in control solution. Stimulus strength in the presence of adenosine was unchanged from control solution. For the paired potentials $R_{control}$ was approximately 80% maximal in control solution and slices were selected to show paired-pulse inhibition in the range 20 to 40% in control solution. Adenosine was superfused for 15 minutes. Records were captured on computer (SCAN software, John Dempster, University of Strathclyde, UK) and plotted on a Hewlet Packard plotter. Bicuculline 10 μM was present throughout. Arrows show stimulus artefacts.

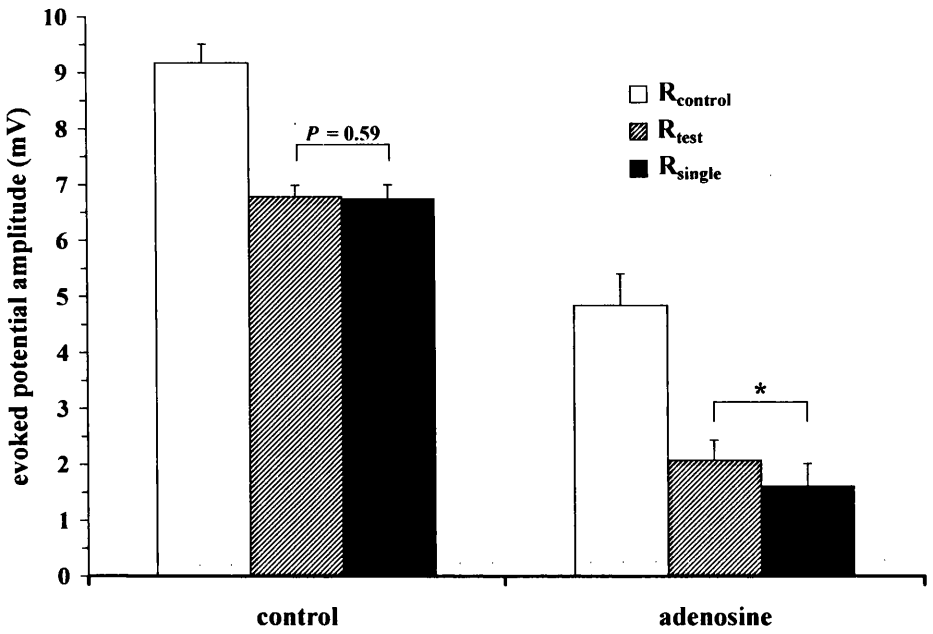

Figure 30 Effect of 10 μM adenosine on the amplitude of paired and unpaired orthodromic potentials in the presence of bicuculline. See Figure 29 for details. Effects of adenosine reversed on washing for 20 minutes. $*P < 0.05$, $n = 10$ slices. Error bars show SE.

Constant Control Potential Amplitude

Stimulus strength / response profiles for paired-pulse inhibition were generated, in seven slices, in control solution before superfusion with adenosine in the experiment described above (Figure 28). These were used to compare paired-pulse inhibition in adenosine with paired-pulse inhibition in control solution when $R_{control}$ was the same size in control and adenosine solutions. In this case adenosine significantly reduced paired-pulse inhibition; inhibition of 56 ± 8% in the presence of adenosine 10 μM corresponded to control inhibition of 79 ± 4.4% where $R_{control}$ was about 50% maximal in control solution ($P < 0.01$; $n = 5$ slices: in two of the seven slices the size of $R_{control}$ in adenosine was outside the range of the profile determined in the control solution). Furthermore, in two different sets of experiments (see below) where stimulus strength in adenosine was adjusted to return $R_{control}$ and R_{cond} to

the same size as in control solution, adenosine consistently reduced paired-pulse inhibition:

1. In an investigation of concentration-response relationships adenosine at concentrations between 4 μM and 40 μM caused a significant decrease in paired-pulse inhibition between pairs of equal stimuli (Figure 31; filled squares). $R_{control}$ was 70% of maximum size under control conditions.
2. In the experiments to examine whether the effect of adenosine on paired-pulse inhibition was modulated by the strength of the conditioning stimulus, 20 μM adenosine caused a highly significant decrease in paired-pulse inhibition at all sizes of conditioning stimulus (for instance 47 ± 5.5% decrease in paired-pulse inhibition when $R_{control}$ and R_{cond} were both 60% of maximum in control solution; $n = 9$ slices, $P < 0.0001$) (Figure 36A; filled circles).

Effect of Adenosine on Matched Unpaired and Paired-Inhibited Test Potentials in the Presence of Bicuculline

The effect of adenosine was examined on single unpaired potentials (R_{single}, adjusted, before superfusion with adenosine, to the same size as the inhibited test response (R_{test}) of a pair. During adenosine superfusion R_{test} was smaller than R_{single} ($P < 0.05$, $n = 10$ slices) and adenosine 10 μM caused a small but significantly greater decrease in R_{single} (77 ± 5.8%) than in R_{test} (69.5 ± 5.0%; $P < 0.01$, $n = 10$ slice) (Figures 29 and 30). 10 μM bicuculline was present throughout the experiment.

Concentration-Response Relationships in the Presence of Bicuculline

Adenosine depressed single evoked potentials in a concentration dependent manner (Figure 31; open circles). When $R_{control}$ was adjusted

during superfusion with adenosine to compensate for this direct depressant effect the inhibitory effect of adenosine on paired-pulse inhibition between pairs of equal submaximal stimuli was also found to be concentration dependent (Figure 31; filled squares). At low concentrations adenosine produced a similar change of paired-pulse inhibition and single potentials but at concentrations above 4 μM adenosine had a smaller effect on paired-pulse inhibition than on single evoked potentials.

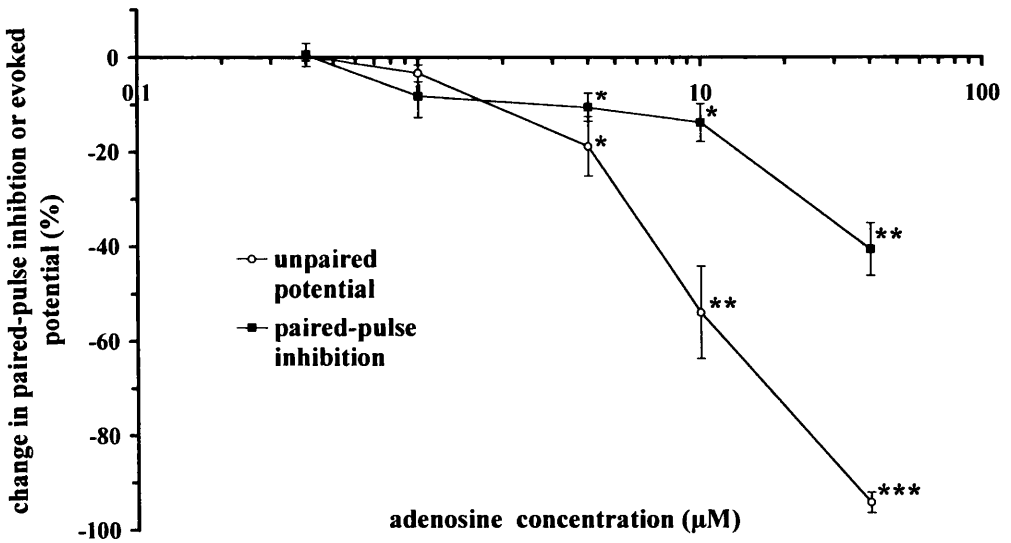

Figure 31 Concentration-response relationships for the effect of adenosine on paired-pulse inhibition and on single evoked potential amplitude in the same slices in the presence of bicuculline. All stimuli were orthodromic. Pairs of equal stimuli were used for the paired potentials. $R_{control}$ and unpaired potentials were 70% maximal in control solution. Stimulus strength was adjusted in adenosine to return $R_{control}$ to control amplitude and paired-pulse inhibition was quantified after stimulus readjustment. Adenosine was superfused for 15 minutes at each concentration followed by 15 minutes wash. The interstimulus interval was 300 ms. Bicuculline 10 μM was present throughout. $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$ for difference between adenosine and control. The percentage change in paired-pulse inhibition and in single evoked potential amplitude were not significantly different from each other at 0.4 μM, 1 μM or 4 μM adenosine. $n = 6$ slices. Error bars show SE.

Concentration-Response Relationships in the Absence of Bicuculline

The concentration-response relationships were examined for the effect of low concentrations of adenosine on short-latency paired-pulse inhibition and single population spikes in the normal hippocampal slice in the absence of other agents. The interstimulus interval was 30 ms. All potentials were orthodromic but conditioning and test stimuli were delivered from different stimulating electrodes in the stratum radiatum, one to either side of the recording electrode (see Figure 1). In order to determine whether the effect of adenosine on paired-pulse inhibition in this paradigm was influenced by the strength of the conditioning stimulus, paired-pulse inhibition was measured at each concentration of adenosine and corresponding wash/control using a range of conditioning stimuli from supramaximal (1 mA) to just below threshold for evoking a population spike. The test stimulus was adjusted as necessary to keep $R_{control}$ constant throughout each experiment. No bicuculline was present.

Evoked Responses and Paired-Pulse Inhibition before Adenosine Superfusion

Stimulus strength and the amplitude of R_{cond}. The amplitude of the evoked potential R_{cond} increased as the strength of the stimulus used to evoke it was increased; the maximum size of potential was evoked by stimuli in the range approximately 400 to 600 μA. However, in six of the nine slices examined a supramaximal stimulus of 1 mA evoked a potential that was *smaller* than the maximum size (maximum potential size range 3 to 26% larger than the potential evoked by a supramaximal stimulus of 1 mA) (Figure 32). This phenomenon was also noticed when testing slices during other experiments described in this thesis (see p. *156* for further comment).

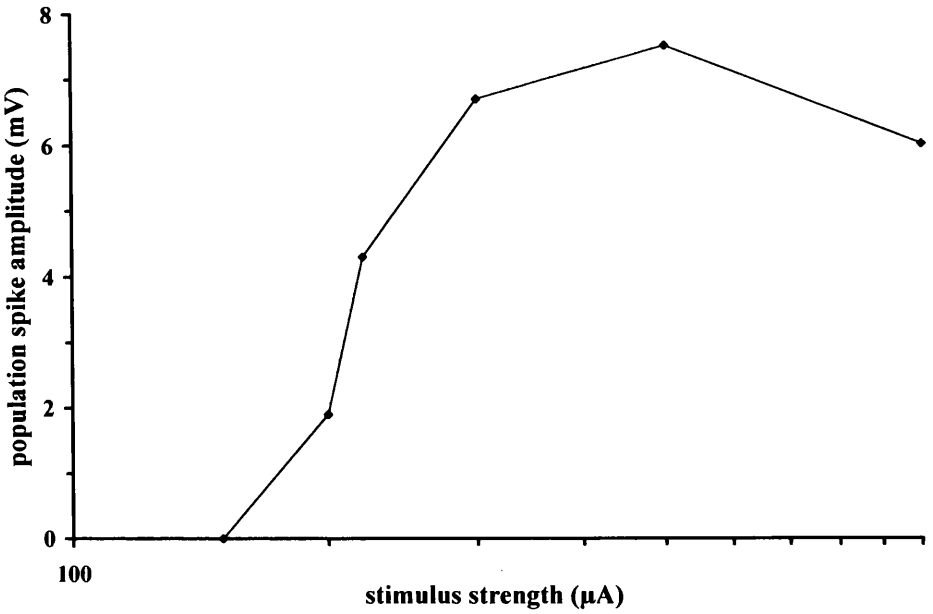

Figure 32 Relationship between the amplitude of unpaired orthodromic population spikes and stimulus strength in the absence of added agents in a single slice. Of the nine slices used in the experiment described in Figure 33 below, this slice showed the most marked decrease in population spike amplitude with a supramaximal stimulus.

Conditioning stimulus strength and paired-pulse inhibition. All the examined slices showed paired-pulse inhibition at this latency when separate electrodes were used for conditioning and test stimuli. At the lowest strength of conditioning stimulus examined, which was below the threshold for evoking a population spike but above the threshold for evoking a small field EPSP response, paired-pulse inhibition was 26% ± 9.0, $n = 9$. As the strength of the conditioning stimulus was increased, paired-pulse inhibition also increased so that when the conditioning stimulus was strong enough to evoke an R_{cond} which was 30 to 40% of maximum size, paired-pulse inhibition was about 80%. As conditioning stimulus strength was increased above this threshold the amount of paired-pulse inhibition remained constant. In particular, paired-pulse inhibition evoked using a supramaximal stimulus of 1 mA was similar to that evoked using smaller conditioning stimuli (Figure 33).

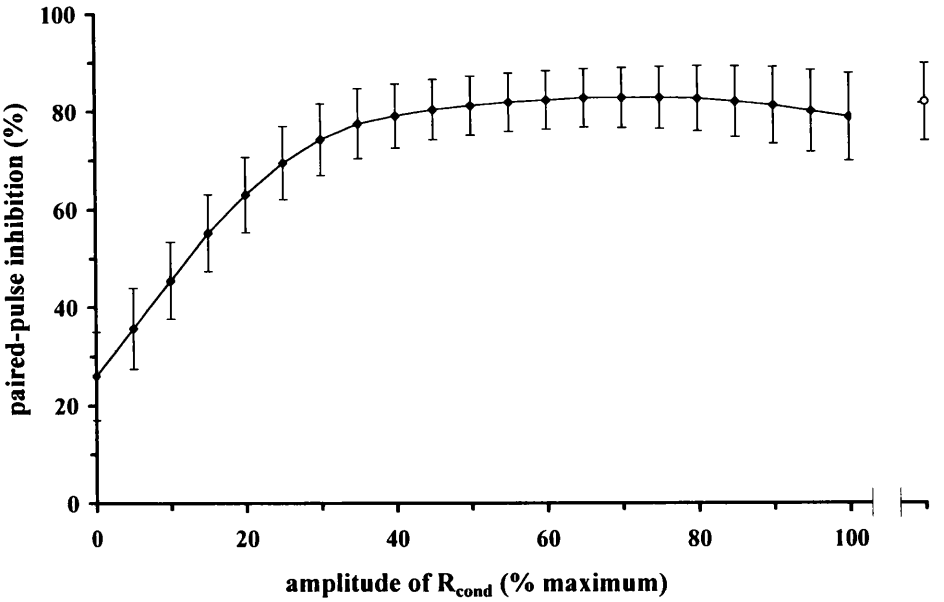

Figure 33 Effect of conditioning stimulus strength on paired-pulse inhibition between orthodromic potentials in ACSF. Two stimulating electrodes were placed in the stratum radiatum (see Figure 1). Test responses were evoked at 40 s intervals from one electrode. Conditioning stimuli were delivered 30 ms before alternate test stimuli using the other stimulating electrode. $R_{control}$ was constant at 70 % of maximum amplitude. Conditioning stimulus strength was varied in each slice from supramaximal (1 mA) to just suprathreshold for a field EPSP response but subthreshold for a population spike. Paired-pulse inhibition was measured using 6 or 7 different conditioning stimuli per slice and interpolated values were used for averaging between slices. Because the amplitude of R_{cond} evoked by a supramaximal 1 mA stimulus was sometimes *smaller* than that evoked by a slightly smaller stimulus the data obtained with conditioning stimulus = 1 mA has been plotted separately (open circle). R_{cond} = 100% was the maximum amplitude of response obtainable (usually at a stimulus strength between 400 and 700 μA). The data plotted here are the control data (before the first adenosine superfusion) from the experiment described in Figure 35. $n = 9$ slices. Error bars show SE.

Concentration-Response Relationships

Adenosine at 4 μM and 10 μM caused a concentration-dependent decrease in the size of both single evoked population spikes ($n = 9$) and the magnitude of paired-pulse inhibition ($n = 8$) (Figures 34 and 35). The effect of adenosine on paired-pulse inhibition was similar irrespective of the size of R_{cond}, for instance there was no significant difference between the effect of adenosine 10 μM on paired-pulse inhibition when R_{cond} was evoked using a supramaximal stimulus of 1

mA and when R_{cond} was 30%, 40%, 50%, or 60% of maximum size (data for the range R_{cond} 70% to 100% of maximum size were missing because of the direct effect of adenosine to decrease the evoked potential). Data calculated using R_{cond} = 60% of maximum size was used for the comparison presented in Figure35.

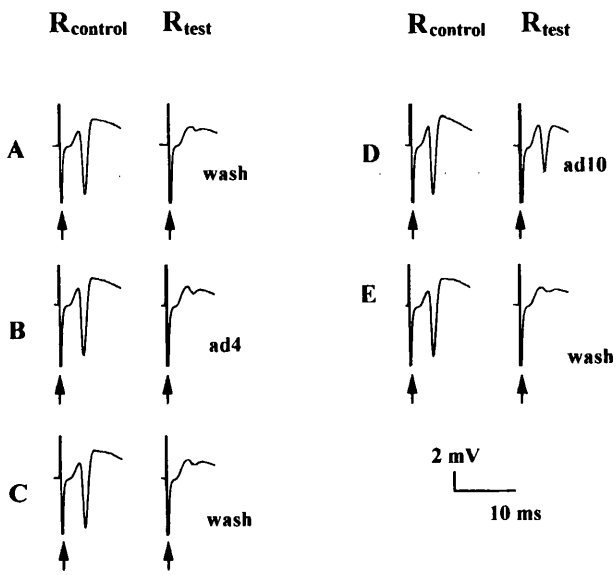

Figure 34 Records showing typical effect of 4 μM and 10 μM adenosine on short-latency paired-pulse inhibition in the absence of other agents. The figure shows unconditioned responses ($R_{control}$) and the corresponding conditioned responses (R_{test}). Responses to conditioning stimuli are not shown but were similar in amplitude to the unconditioned responses. Test and conditioning stimuli were adjusted in adenosine to counteract the direct depressant effect of adenosine on single evoked potentials. See Figure 35 for further details. Records were captured on computer (SCAN software, John Dempster, University of Strathclyde) and plotted on a Hewlet Packard plotter. Arrows mark stimulus artefacts.

Using these data adenosine had no significant effect on either paired-pulse inhibition or single evoked population spikes at concentrations of 100 nM and 1μM although a tiny (3.9 ± 1.5%, n = 8) increase in paired-pulse inhibition and a similarly small (2.7 ± 0.74%, n = 9) decrease in single evoked population spikes with adenosine 400 nM were both

significant (Figure 35). No slices showed an increase in evoked single population spike size with any concentration of adenosine.

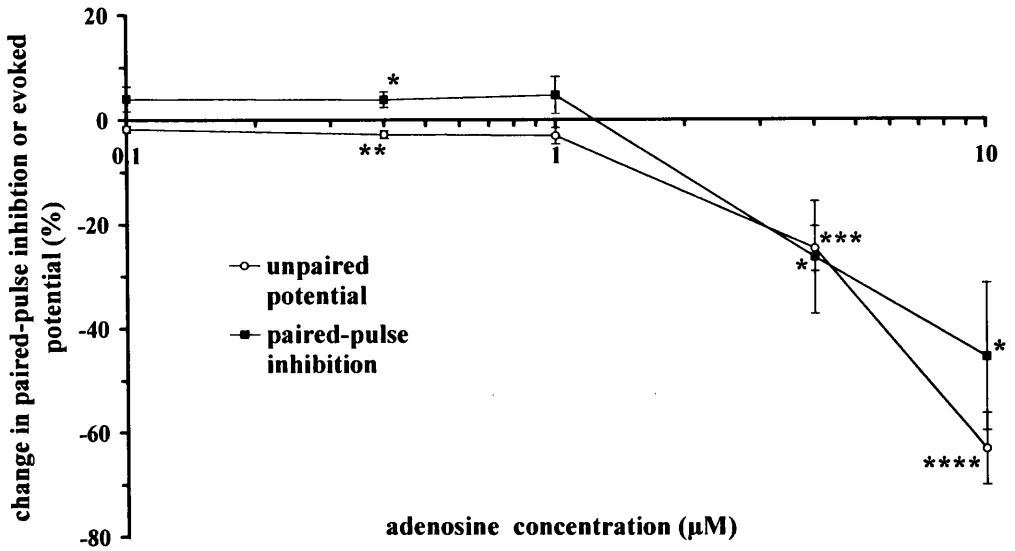

Figure 35 Concentration-response relationships for the effect of adenosine on paired-pulse inhibition and on single orthodromic population spikes in the normal hippocampal slice. Experiments were carried out in ACSF in the absence of other agents. The concentration-response relationships for the effect of adenosine on single population spikes and on paired-pulse inhibition were derived in different slices, $n = 9$ and $n = 8$ slices per group respectively. Single potentials were 70% maximal before adenosine superfusion. For the measurement of paired-pulse inhibition stimulation strength was adjusted to keep $R_{control}$ constant at 70 % of its initial maximum amplitude throughout the experiment. Paired-pulsed inhibition was measured using a range of conditioning stimulus strengths at each condition. The interstimulus interval was 30 ms. Data plotted here were obtained by interpolation for R_{cond} = 60% of maximum amplitude (see Figure 33 for further details of stimulation regimen). Adenosine was superfused for 20 minutes at each concentration followed by 15 minutes wash. Error bars show SE. $*P < 0.05$ $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ for difference between adenosine and control. Single evoked potential and paired-pulse inhibition data differed significantly from each other only at the 400 nM datapoint ($P < 0.01$).

Conditioning Stimulus Strength and the Effect of Adenosine and Baclofen on Bicuculline-Enhanced Paired-Pulse Inhibition

The effects of baclofen 1 μM and adenosine 20 μM were tested on bicuculline-resistant inhibition using a range of different conditioning stimulus strengths. (These concentrations were chosen to match those used in the work described on p. 59).Control paired-pulse inhibition was

similar in the two groups of slices used for the adenosine and baclofen experiments (Figure 36A). Changes in paired-pulse inhibition were measured in the presence of adenosine or baclofen after adjusting $R_{control}$ and R_{cond} to the size they had been before adenosine or baclofen superfusion.

Adenosine at 20 μM caused a similar decrease in bicuculline-resistant paired-pulse inhibition regardless of conditioning response size in the range of 20-80% of maximum size (Figure 36B; filled circles). When a large supramaximal conditioning stimulus (1mA) was used adenosine caused a decrease in paired-pulse inhibition of 34 ± 6.3% ($P < 0.001$, $n = 9$). (Figure 36B; open circle). This decrease in paired-pulse inhibition was significantly smaller ($P < 0.05$, $n = 9$) than the decrease caused by adenosine in the range of R_{cond} from 20% to 80% of maximum (NB. The size of the maximal R_{cond} was reduced in adenosine to 84±1.8% compared to the original control size, hence the lack of data points at the 90 and 100% levels in Figure 36B).

In contrast, baclofen tended to be more effective at decreasing paired-pulse inhibition as R_{cond} increased above 50% of maximum (Figure 36B; filled squares). When a large supramaximal conditioning stimulus (1mA) was used baclofen caused a decrease in paired-pulse inhibition of 70 ± 4.8% ($n = 9$; $P < 0.001$) (Figure 36B; open square). This was significantly greater than the decrease caused by adenosine under the same conditions ($P < 0.001$, $n = 9$ slices per group). (NB. As for adenosine the supramaximal R_{cond} was slightly smaller than in control solution and hence the 100% data point is absent).

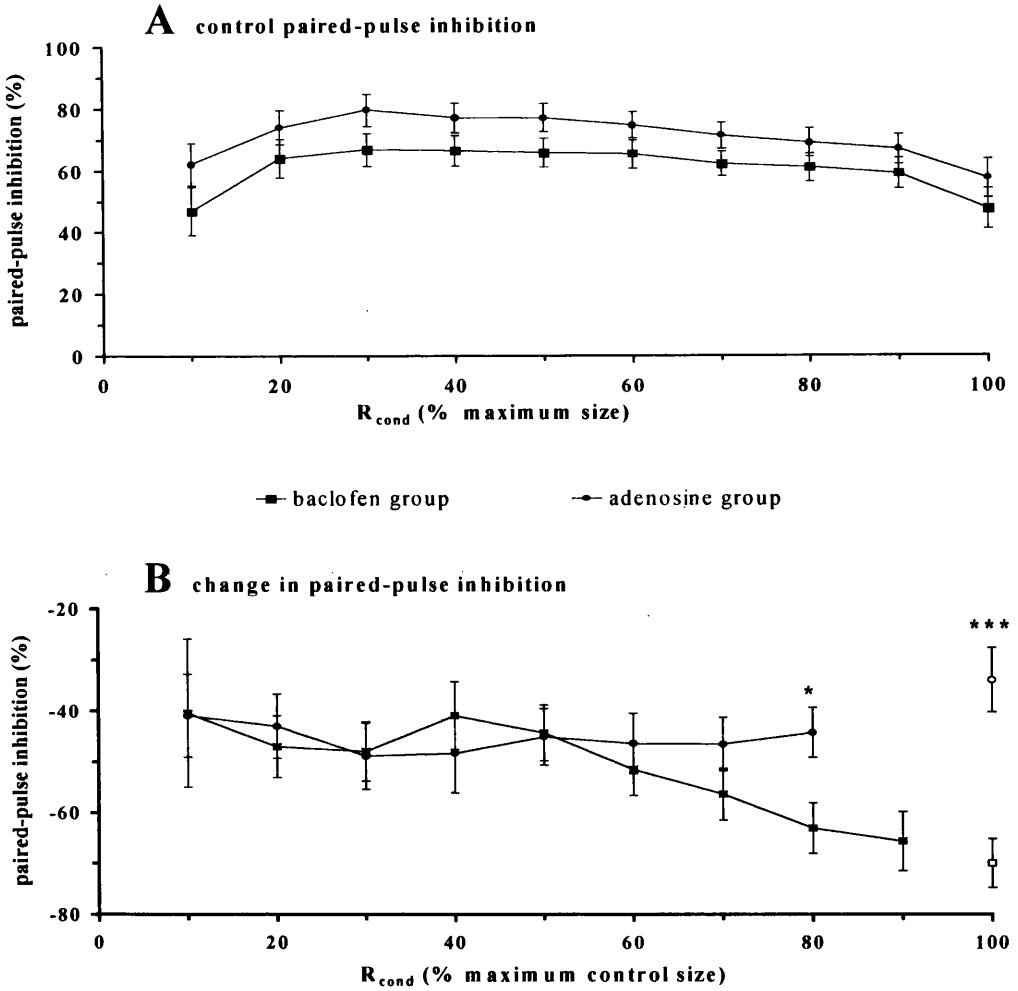

Figure 36 Influence of conditioning potential amplitude on the effect of baclofen and adenosine to reduce paired-pulse inhibition in the presence of bicuculline. All stimuli were orthodromic. $R_{control}$ was 60% maximal in control solution. Stimulus strength was adjusted to keep $R_{control}$ constant during adenosine and baclofen superfusion and subsequent wash. For each slice under control conditions, and again during baclofen or adenosine superfusion, paired-pulse inhibition was measured for a range of conditioning stimulus strengths from just suprathreshold for an evoked potential to supramaximal. Each profile comprised 9 or 10 data points. Interpolated points were used for averaging between slices. The interstimulus interval was 300 ms. Agents were superfused for approximately 30 minutes with measurements made during the second half of this period. Bicuculline 10μM was present throughout. **A** Control paired-pulse inhibition for the adenosine group and baclofen group. The groups were not significantly different at any point. The inhibition corresponding to R_{cond} = 100% maximal was evoked by a supramaximal (1mA) conditioning stimulus. **B** Change in paired-pulse inhibition during superfusion with 1 μM adenosine or 20 μM baclofen compared with paired-pulse inhibition evoked by an R_{cond} of the same amplitude under control conditions. As the maximum amplitude of R_{cond} was reduced in adenosine and to a lesser extent in baclofen there are no data points for adenosine at R_{cond} = 90% and 100% or for baclofen at R_{cond} = 100%. The change in paired-pulse inhibition with baclofen and adenosine when the supramaximal conditioning stimulus of 1 mA was used have been plotted separately on the same axes using open symbols but their position on the x-axis is arbitrary. The effect of adenosine and baclofen reversed on washing. $***P < 0.001$, $*P < 0.05$ for difference between baclofen and adenosine. n = 9 slices per group. Error bars show SE.

VII Investigations into the Role of Endogenous Adenosine in Mediating Orthodromic Bicuculline-Enhanced Paired-Pulse Inhibition

A series of experiments was carried out to investigate the effect of the adenosine A1 antagonist 1,3-dimethyl-8-cyclopentylxanthine (cyclopentyltheophylline, CPT) and the enzyme adenosine deaminase on medium-latency paired-pulse inhibition in the presence of bicuculline. All stimuli were orthodromic, delivered from a single electrode in the stratum radiatum. Test and conditioning stimuli were identical to each other, and stimulus strength was such that the response to the conditioning stimulus ($R_{control}$) was 70% maximal under control conditions in ACSF. The interstimulus interval was 300 ms. 10 μM bicuculline was present throughout all experiments.

Ability of CPT to Antagonise the Effects of Exogenous Adenosine

Two slices were superfused with 20 μM adenosine in ACSF in the absence of other agents. Single orthodromic population spikes were reduced to 44% and 39% of control. When 100 nM CPT was added to the superfusate, in addition to the adenosine, population spike size increased to 83% and 118% of control (ACSF) respectively.

Effect of CPT on Unpaired potentials

Normal slices

In the presence of 10 μM bicuculline, 100 nM CPT increased the size of single evoked potentials in nine of 12 slices (range 6 to 24% of control), whereas three slices exhibited a large decrease of up to 24% relative to

controls. Overall there was no significant change of single evoked potential size in these slices (Figure 37A).

In 11 of 12 slices tested, the amplitudes of the single evoked potentials remained elevated above control potential size after washing out CPT for 20 minutes. (In one slice the amplitude of the potential decreased in CPT and decreased further on washing. When the data from this slice were included in the analysis, the differences in potential size between control, CPT and wash were far from normally distributed). The median amplitude on washing for 20 minutes was 6% larger than control potentials ($n = 12$; $P < 0.05$; Wilcoxon matched pairs).

Cut slices

Ten additional slices were prepared in which the CA1 and CA3 regions were isolated from each other by means of a surgical cut in the CA2 region. In these experiments, all individual slices showed an increase in the size of single evoked potentials during superfusion with CPT in the presence of bicuculline. CPT increased single evoked potential amplitude by 13% ± 2.0 ($n = 10$; $P < 0.0001$) (Figure 38A). This effect reversed upon washing for 20 minutes.

Effect of CPT on Paired-Pulse Inhibition

Normal slices

Most slices examined in the presence of bicuculline demonstrated marked paired-pulse inhibition at an interpulse interval of 300 ms. In the presence of bicuculline, CPT decreased paired-pulse inhibition by 62% ± 4.9 (± = 12; $P < 0.0001$) when stimuli were not adjusted throughout the experiment, and by 55% ± 7.5 ($n = 12$; $P = 0.0001$) when stimuli

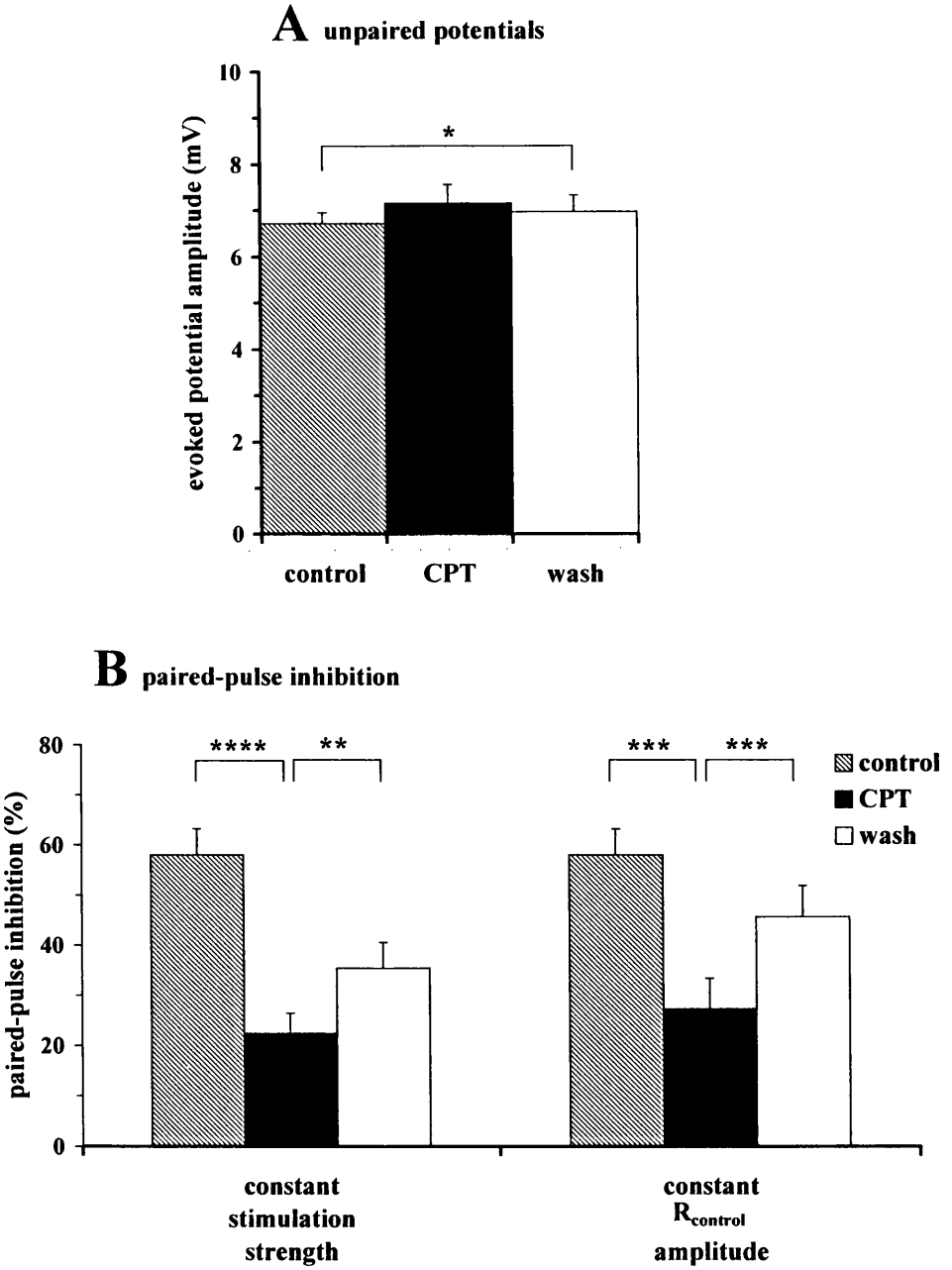

Figure 37 Effect of CPT on the amplitude of single evoked potentials and on the magnitude of paired-pulse inhibition in the presence of bicuculline in the same slices. All stimuli were orthodromic. CPT was superfused for 20 minutes, with data being collected during the last five minutes of this period, followed by 20 minutes wash in ACSF. Bicuculline 10 μM was present throughout. **A** Effect on single potentials. Potentials were 70% of maximal amplitude in control solution (bicuculline) **B** Effect on paired-pulse inhibition. Test and conditioning stimuli were identical. The interstimulus interval was 300 ms. Stimulus strength was initially set such that the response to the conditioning stimulus ($R_{control}$) was 70% maximal under control conditions in bicuculline. In the presence of CPT and after washing paired-pulse inhibition was calculated both using the same stimulus strength as under control conditions and with stimulus strength adjusted to counteract the direct effect of CPT on response amplitude and return $R_{control}$ to the same amplitude as under control conditions. Error bars show SE. $n = 12$ slices. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

were adjusted to restore the size of the first potential in CPT to the same size as the control potential (Figures 37B and 39).

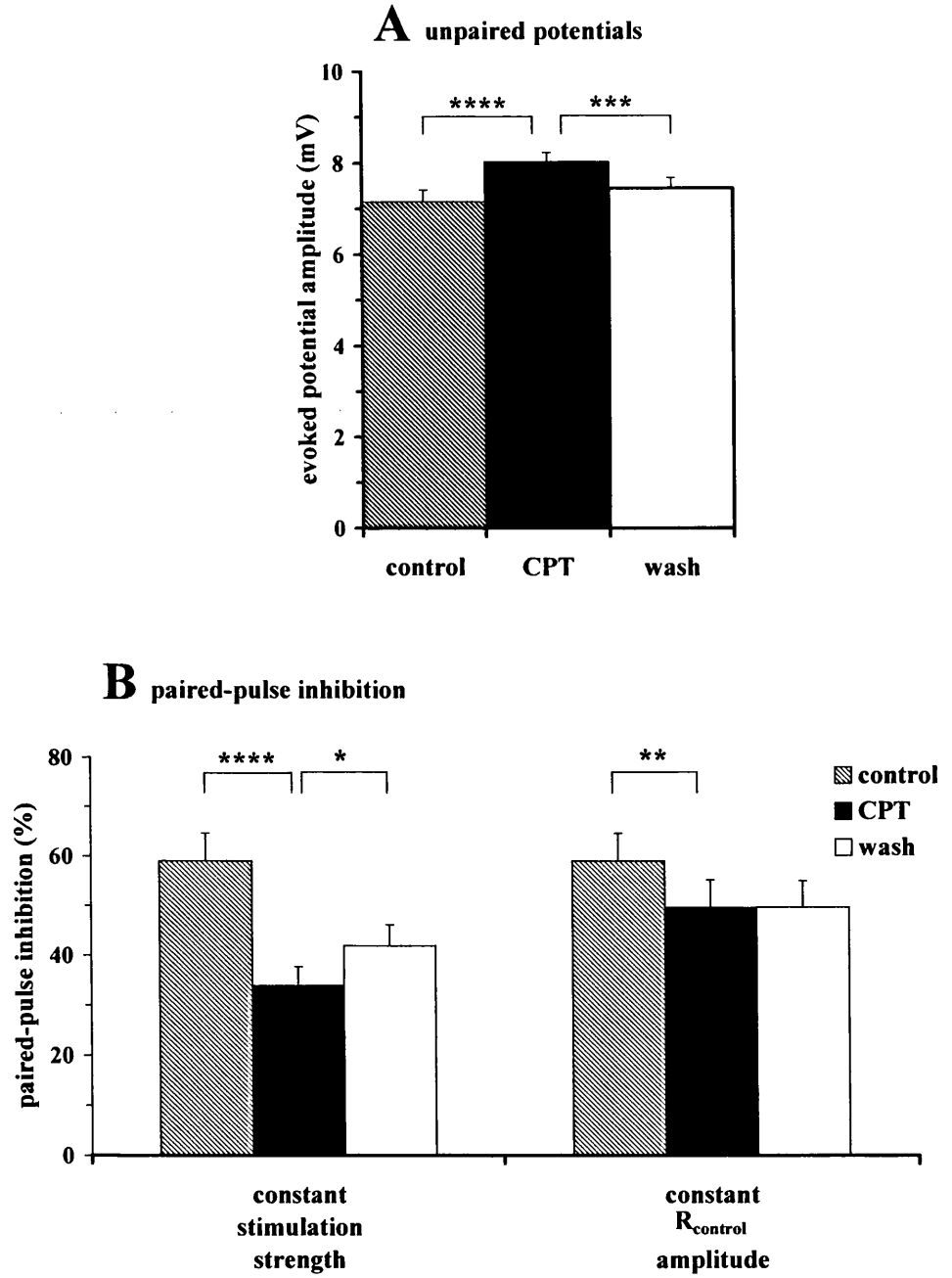

Figure 38 Effect of CPT on the amplitude of single evoked potentials and on the magnitude of paired-pulse inhibition in the presence of bicuculline in the same slices: cut slices. All slices were cut to divide the CA1 from the CA3 area. Experimental details are otherwise as for Figure 37 **A** Effect on single potentials. **B** Effect on paired-pulse inhibition. In the presence of CPT and after washing paired-pulse inhibition was calculated both using the same stimulus strength as under control conditions and with stimulus strength adjusted to counteract the direct effect of CPT on response amplitude and return $R_{control}$ to the same amplitude as under control conditions. Error bars show SE. $n = 12$ slices. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

The CPT-induced decrease in paired-pulse inhibition was significantly ($P < 0.01$) reversed upon washing for 20 minutes whether or not stimulus strength was adjusted to account for the effect of CPT on individual evoked potentials. Reversal was greater when the stimulus strength had been adjusted: when stimulus strength was adjusted the recovering slices showed paired-pulse inhibition which was only 19% ± 7.9 ($n = 12$; $P < 0.05$) less than controls after 20 min wash (Figure 37B), but when stimulus strengths were not adjusted, potentials in washed slices showed paired-pulse inhibition which was still 41% ± 6.2 ($n = 12$; $P < 0.0001$) less than controls.

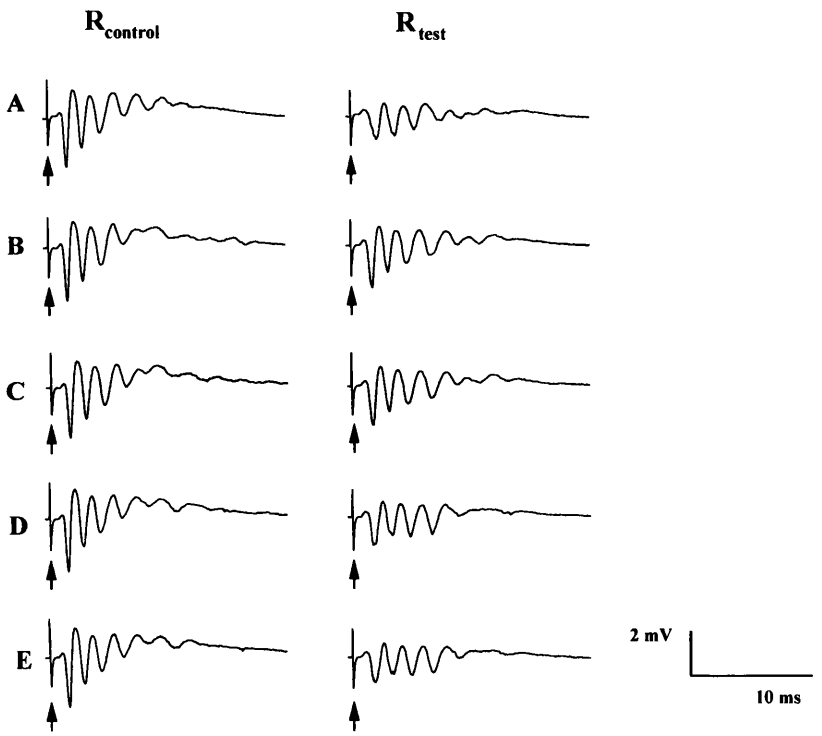

Figure 39 Typical records of pairs of evoked conditioning ($R_{control}$) and corresponding test (R_{test}) potentials showing the effect of CPT 100 nM on paired-pulse inhibition at an interstimulus interval of 300 ms in the presence of 10 μM bicuculline. See Figure 37 for experimental details. **A** Control **B** CPT 100 nM. **C** CPT 100 nM with stimulus readjusted to return $R_{control}$ to control amplitude. **D** Wash. **E** Wash with stimulus readjusted to return $R_{control}$ to its control amplitude in bicuculline alone. Stimulus strength was identical for records A, B and D. Bicuculline 10 μM was present throughout. Records were captured on computer (SCAN software, John Dempster, University of Strathclyde) and plotted on a Hewlet Packard plotter. Arrows mark stimulus artefacts.

Cut slices

In the ten slices which had been cut to separate the CA1 area from the CA3 area superfusion with CPT at 100 nM decreased paired-pulse inhibition by 42% ± 4.1 ($n = 10$; $P < 0.0001$) when stimuli were not adjusted. This effect was partly but significantly reversible ($n = 10$; $P < 0.05$) (Figure 38B); inhibition remained decreased by 28% ± 6 compared with controls after 20 minutes washing ($n = 10$; $P < 0.01$).

When stimulus strength was adjusted so as to restore the size of the first potential in CPT to the same size as the control potential, the xanthine still decreased paired-pulse inhibition significantly, though to a lesser extent (16% ± 3.7; $n = 10$; $P < 0.01$). In this case there was no significant reversal of the effect on washing for 20 minutes (Figure 38B).

Effect of Adenosine Deaminase on Unpaired Potentials

At an activity of 1 $U.ml^{-1}$ adenosine deaminase had no significant effect on the mean amplitude of single orthodromic potentials in 10 slices (Figure 40A). Individual potentials showed a range of effects, with 5 slices exhibiting decreases (range 6 to 48%) in potential size and 4 slices showing increases (range 1 to 13%).

Effect of Adenosine Deaminase on Paired-Pulse Inhibition

Adenosine deaminase decreased the extent of paired-pulse inhibition by 43% ± 6.2 ($n = 10$; $P < 0.001$) when stimulus strength was not adjusted to compensate for the effect of the enzyme on individual single evoked potential size (Figure 40B). An even greater effect was observed when stimulus strength was adjusted, paired-pulse inhibition now being reduced by 50% ± 6.2 ($n = 10$; $P < 0.001$).

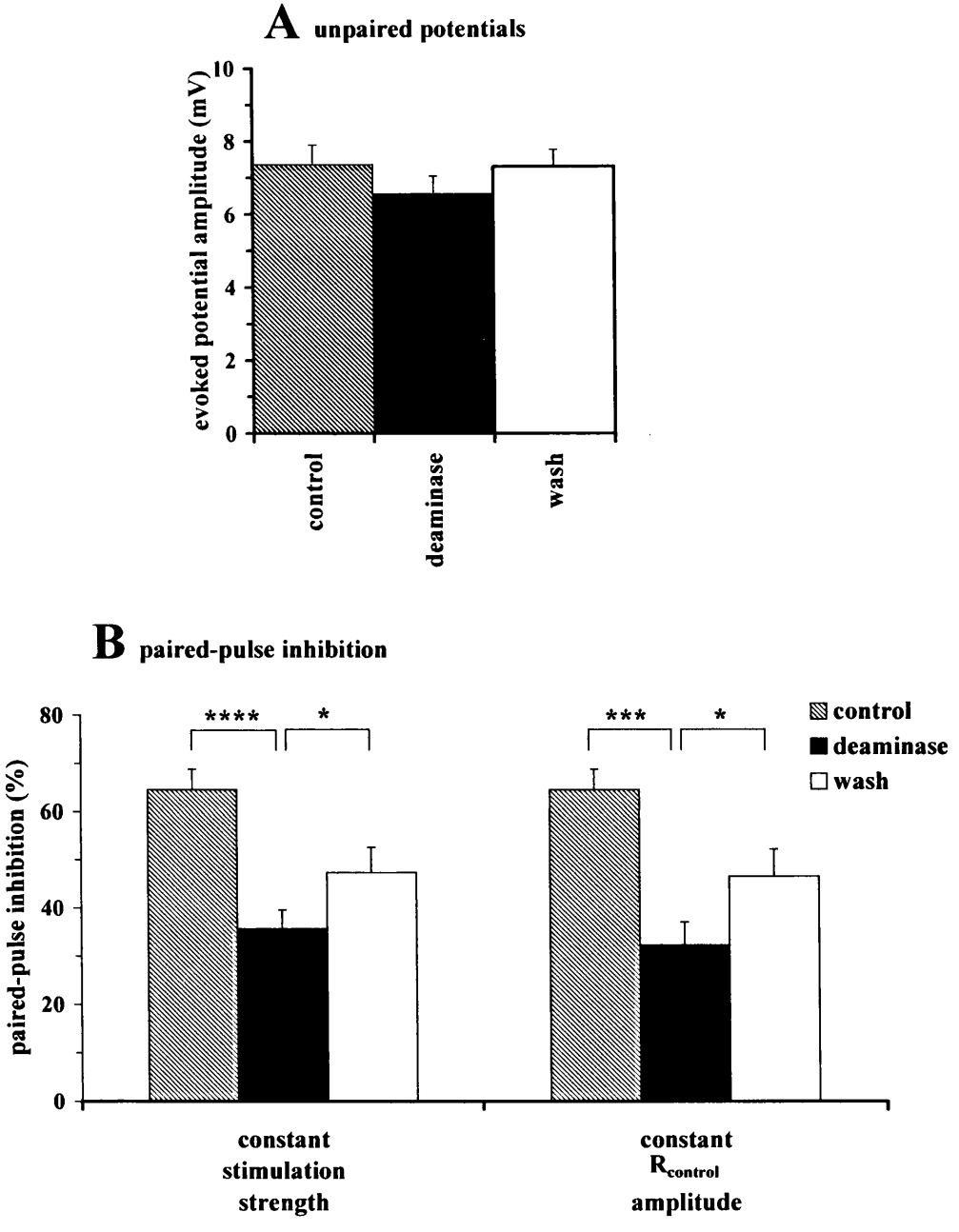

Figure 40 Effect of adenosine deaminase 1 U.ml^{-1} on the amplitude of single evoked potentials and on the magnitude of paired-pulse inhibition in the same slices in the presence of bicuculline. Experimental details are as for Figure 37 except that the slices were superfused with adenosine deaminase in the place of CPT. **A** Effect on single potentials. **B** Effect on paired-pulse inhibition. In the presence of adenosine deaminase and after washing paired-pulse inhibition was calculated both using the same stimulus strength as under control conditions and with stimulus strength adjusted to counteract the direct effect of the enzyme on response amplitude and return $R_{control}$ to the same amplitude as under control conditions. Error bars show SE. $n = 12$ slices. *P <0.05, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

The decrease in inhibition was partly but significantly reversed after 20 minutes washing in experiments with or without stimulus adjustments (Figure 40B). When stimulus strength was adjusted the recovering slices showed paired-pulse inhibition which was only 28% ± 7.6 ($n = 10$; $P < 0.01$) less than controls after 20 minutes wash (Figure 40B), and when stimulus strengths were not adjusted, potentials in washed slices showed paired-pulse inhibition which was 27% ± 5.7 ($n = 10$; $P < 0.01$) less than controls.

Spontaneous Bursts during Superfusion of CPT and Adenosine Deaminase

Many of the slices which were exposed to bicuculline developed spontaneous bursts when they were additionally superfused with CPT or adenosine deaminase (Figures 41 and 42). The spontaneous bursts had comparable amplitudes and durations to the evoked potentials although some bursts were noted with amplitudes less than 1 mV (Figure 41).

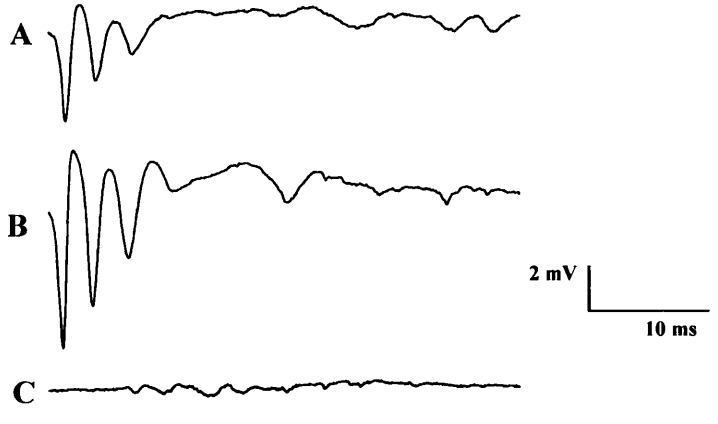

Figure 41 Examples of spontaneous bursts, in three separate slices, during superfusion with 100 nM CTP and 10 μM bicuculline. All slices were uncut. Records were captured on computer (SCAN software, John Dempster, University of Strathclyde) and plotted on a Hewlet Packard plotter.

The bursts generally occurred with a frequency of about 0.1Hz or less, were not present before the addition of adenosine deaminase or CPT and decreased in frequency or disappeared when slices were washed in bicuculline alone (Figure 42) Such spontaneous bursts did not occur in any of slices in which a surgical cut in CA2 divided the CA1 region from the CA3.

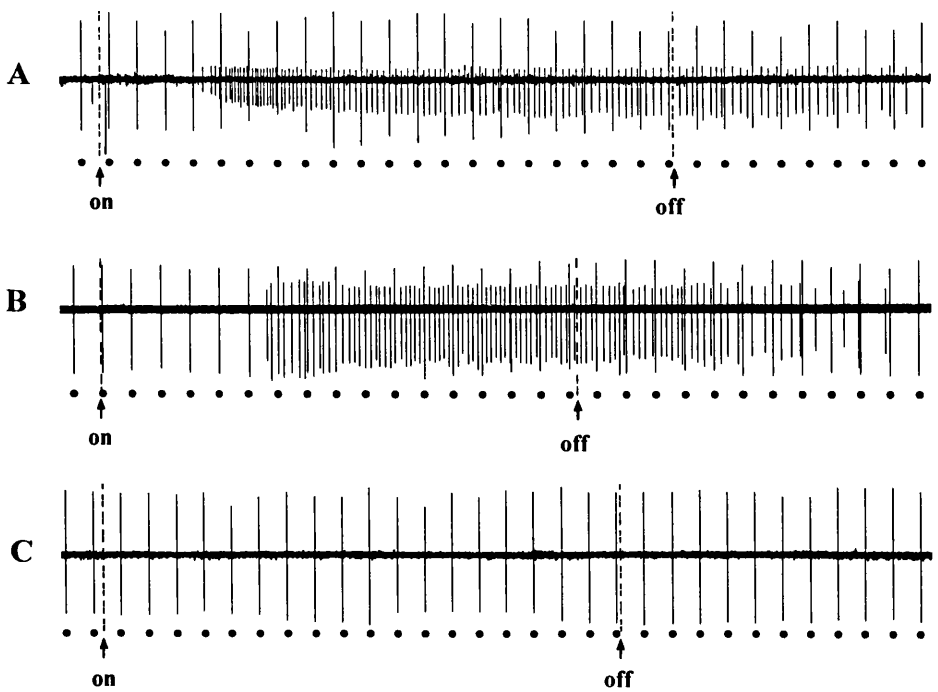

Figure 42 Typical slow speed chart recordings showing effects of CPT and adenosine deaminase on the development of spontaneous bursting in three slices in the presence of bicuculline. Arrows mark the onset and termination of superfusion with 100 nM CPT or adenosine deaminase 1 $U.ml^{-1}$. 10 μM bicuculline was present throughout. Pairs of evoked potentials,each showing as a single spike on the slow speed recording, occur at 1 minute intervals in all three records (marked with dots). Records **A and B** which are normal hippocampal slices (uncut) show the development of slightly smaller amplitude spontaneous bursts, each represented by a single spike, during superfusion with adenosine deaminase (record A) or CPT (record B). The spontaneous bursts disappeared on washing in bicuculline alone. Record **C** is from a slice in which a cut has been made to isolate the CA1 from the CA3 area and shows no spontaneous bursting in response to superfusion with CPT.

VIII Effect of CPT on Unpaired Responses and on Short-latency Paired-Pulse Inhibition in the Absence of Other Agents

The effect of the adenosine A1 antagonist 1,3-dimethyl-8-cyclopentylxanthine (cyclopentyltheophylline, CPT) was examined on short-latency paired-pulse inhibition in the normal hippocampal slice in the absence of other agents. All stimuli were orthodromic, delivered from a single electrode in the stratum radiatum. Test and conditioning stimuli were identical to each other and stimulus strength was such that the response to the conditioning stimulus ($R_{control}$) was 70% maximal under control conditions in ACSF alone. The interstimulus interval was 30 ms. Slices were initially selected to show paired-pulse inhibition of at least 10% under control conditions. At this latency paired-pulse inhibition was the most commonly occurring response.

Unpaired Population Spikes

In the absence of other agents CPT at a concentration of 100 nM increased the size of single orthodromic evoked potentials by 14% ± 2.9 ($n = 20$; $P < 0.001$). This increase was reversed on washing for 20 minutes (Figure 43A). The mean size of the potentials in the washed slices was 8% ± 2.9 smaller than control ($n = 20$; $P < 0.01$).

Paired-Pulse Inhibition

When CPT was superfused, 12 of the 20 slices tested showed a reduction in paired-pulse inhibition of at least 38% both when stimuli were adjusted to keep the amplitude of $R_{control}$ constant and when the strength of test stimulus kept constant throughout the experiment (Table 3, Figures 43B and 44).

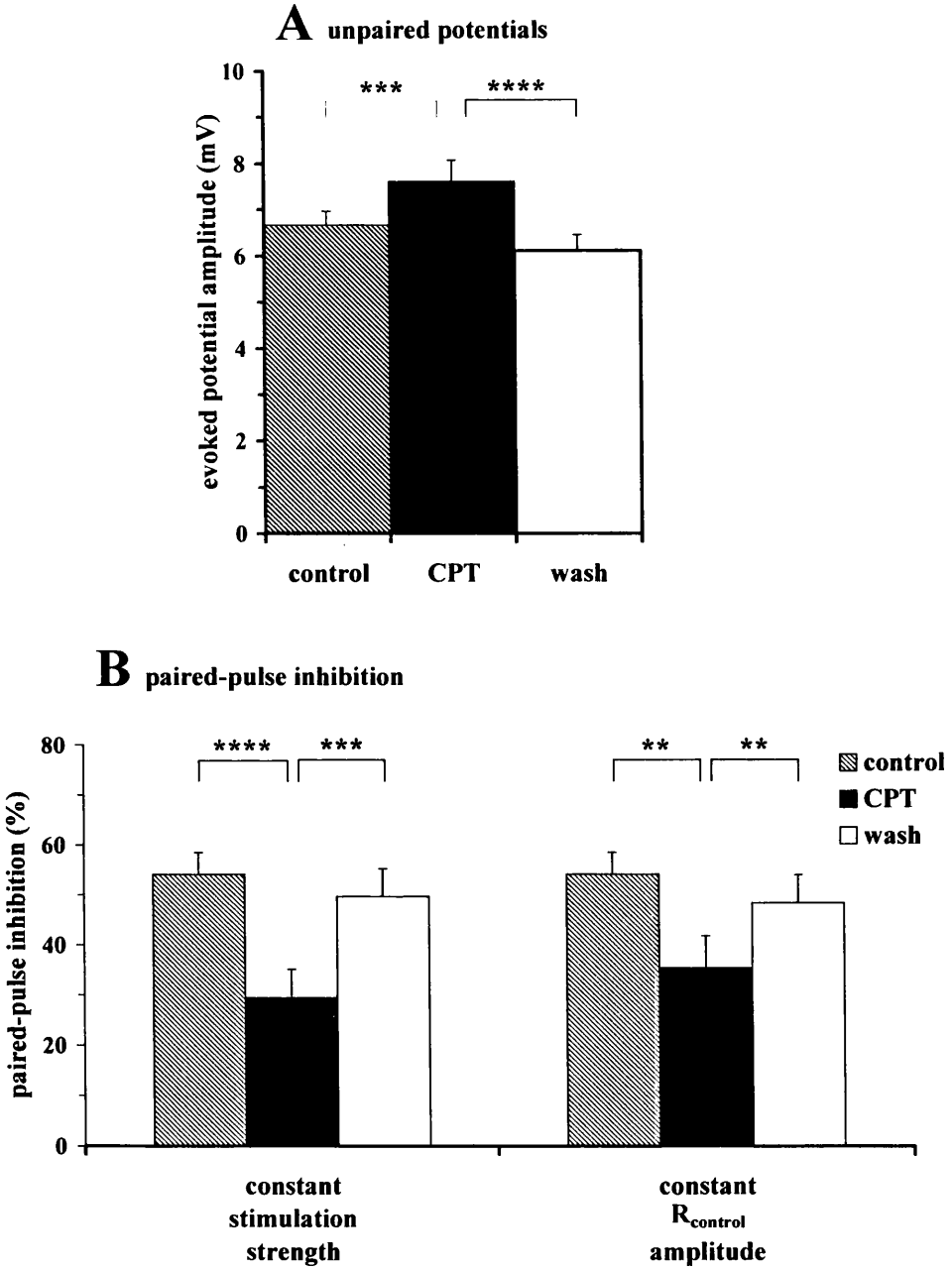

Figure 43 Effect of CPT on the amplitude of single population spikes and on the magnitude of short-latency paired-pulse inhibition in the same slices. Experiments were carried out in ACSF in the absence of other agents. All stimuli were orthodromic. CPT was superfused for 20 minutes, with data being collected during the last five minutes of this period, followed by 20 minutes wash in ACSF. **A** Effect on single potentials. Potentials were 70% of maximal amplitude in control solution (ACSF). **B** Effect on paired-pulse inhibition. Test and conditioning stimuli were identical. The interstimulus interval was 30 ms. Stimulus strength was initially set such that the response to the conditioning stimulus ($R_{control}$) was 70% maximal under control conditions in bicuculline. In the presence of CPT and after washing paired-pulse inhibition was calculated both using the same stimulus strength as under control conditions and with stimulus strength adjusted to counteract the direct effect of CPT on response amplitude and return $R_{control}$ to the same amplitude as under control conditions. Slices were initially selected to show obvious paired-pulse inhibition under control conditions. Error bars show SE. $n = 20$ slices. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

When stimuli were not adjusted throughout the experiment the amount of inhibition produced was significantly reduced by 47% ± 8.1 ($n = 20$; $P < 0.0001$) (Figure 43B and Table 3). The effect of CPT reversed virtually completely after washing.

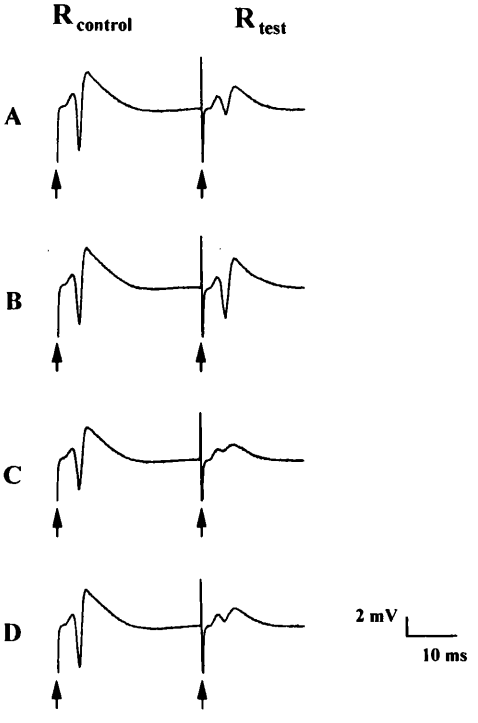

Figure 44 Records of pairs of evoked conditioning ($R_{control}$) and test (R_{test}) potentials from the first slice in Table 3 showing the effect of CPT 100 nM on paired-pulse inhibition at an interstimulus interval of 30 ms in ACSF. See Figure 43 for experimental details. **A** ACSF control. **B** CPT 100 nM. **C** wash in ACSF. **D** wash with stimulus readjusted to return $R_{control}$ to its control amplitude in ACSF. Stimulus strength is identical for records A to C. $R_{control}$ in this slice showed no change in amplitude on superfusion with CPT and a slight decrease on washing. Arrows mark stimulus artefacts. Records were captured on computer (SCAN software, John Dempster, University of Strathclyde) and plotted on a Hewlet Packard plotter.

When stimulus strength was adjusted to compensate for the direct effect of CPT on evoked potentials and to keep $R_{control}$ constant throughout the experiment CPT decreased the mean paired-pulse inhibition by 34% ± 10.0 ($n = 20$; $P < 0.01$). Again the effect was

substantially and significantly ($P < 0.01$) reversed on washing (Figure 43B and Table 3).

Table 3 Effect of superfusion with 100 nM CPT on paired-pulse inhibition at an interstimulus interval of 30 ms in the absence of other agents. Data from individual slices. The effect of CPT on the amplitude of unpaired population spikes (PS) is given for comparison. Note that the PS data is expressed as the per cent change in PS with CPT superfusion whereas the paired-pulse data is expressed as absolute values; a negative number in the leftmost column indicates a decrease in PS with CPT. A negative number in the last four columns indicates paired-pulse facilitation. Slices in which CPT caused a substantial decrease in paired-pulse inhibition are grouped towards the top of the table. See Figure 43 for further details.

Change in PS with CPT (%)	Paired-Pulse Inhibition (%)			
	Control	CPT*	CPT**	wash
2	64	19	17	84
35	50	19	26	51
14	56	33	32	42
17	88	20	31	74
22	43	8	17	30
3	57	26	26	51
11	46	-5	17	28
-2	45	28	23	43
19	47	-6	-14	16
-5	65	14	14	68
2	56	24	30	35
26	85	80	75	83
30	64	49	69	66
15	41	41	44	35
27	46	51	72	65
-3	14	11	11	17
-8	32	11	11	16
25	89	85	91	90
19	25	16	32	22
23	70	66	85	78

*Stimulus strength unchanged from control. **Stimulus strength adjusted to counteract the direct effect of CPT on the size of $R_{control}$. Stimulus strength for wash data was the same as for control.

CHAPTER 4
DISCUSION

This chapter begins with a short section considering aspects of the experimental methods. After this the various findings are discussed in roughly the same order as they were originally presented. Occasional liberties have been taken with this arrangement for the sake of logical flow. For the same reason, section headings differ from those in the previous chapter.

I Experimental Methods

The general experimental technique employed in these experiments has been used in this laboratory for some years with good results in terms of consistency and stability of electrophysiological responses from the slices.

The working temperature, at 30°C, was within, but at the at the low end of, the range encompassing the practice of other groups using mammalian brain slices (Reid et al., 1988). A low working temperature reduces metabolic rate and has advantages in terms of improving the balance between oxygen utilisation and delivery. This in turn means the relatively thick, physically robust slices, ideal for extracellular recordings in a high flow superfusion chamber, were unlikely to be chronically hypoxic in the deeper layers and therefore more likely to be stable for long experiments. On the other hand many properties of neurones are altered by unphysiological temperatures. In the CA1 area

of the hippocampal slice raising the temperature from 29 to 37°C decreases the amplitude of the population spike (Schiff and Somjen, 1985a). Scott et al. (1985) showed an increase with cooling in the amplitude and duration of action potentials recorded in CA1 neurones. Furthermore there was an increase in the size of the K_{ca}-mediated afterhyperpolarisation, a finding that might be relevant to the present work.

The main departure from the laboratory 'standard' technique was in cooling the rats to 30°C before killing. Cooling was rapid as rats were anaesthetised sufficiently deeply with urethane to prevent shivering. During cooling they breathed additional oxygen from a funnel to prevent hypoxia from respiratory depression or from the effects of deep anaesthesia on ventilation-perfusion matching in the lungs. Mild to moderate hypothermia has a marked protective effect on brain tissue against the deleterious effects of hypoxia and ischaemia (reviewed by Ginsberg et al., 1992). Pre-cooling was initiated on theoretical grounds when the investigator was in the early stages of learning the technique of slice preparation, and a strong impression was gained that it improved the consistency with which viable slices were obtained. Soon afterwards Newman et al. (1992) showed that cooling to 31°C prior to killing decreased histologically apparent damage when slices were incubated at 37°C immediately after preparation, although the effect was obtunded by the protective effect of cooling the slices to 21°C after preparation.

II. Orthodromic Responses and Interactions in the Absence and Presence of Bicuculline

The finding in these investigations that, in the absence of added agents and using a single electrode in the stratum radiatum, paired-pulse inhibition can be observed between orthodromic population spikes at short interstimulus intervals, changing to facilitation at longer interstimulus intervals is consistent with previous findings (p. 7).

Superfusion with bicuculline had marked effects both on unpaired evoked potentials and on paired pulse interactions between evoked orthodromic potentials. It increased both paired-pulse inhibition and paired-pulse facilitation in a characteristic pattern that depended on the interstimulus interval and the strength of the conditioning stimulus. The effect of bicuculline on unpaired potentials, on the general pattern of paired-pulse interactions and on paired-pulse facilitation are discussed below.

Single Population Spikes in the Absence of Bicuculline

In ACSF, in the absence of added agents, the field potential recorded in the pyramidal cell layer of the CA1 area in response to a single stimulation of the stratum radiatum has a characteristic shape. The main feature of the potential is a single negative going (downward) population spike. This is the extracellularly recorded manifestation of action potentials firing in cell bodies in the pyramidal cell layer. The population spike is superimposed on a positive going population field excitatory postsynaptic potential (fEPSP) which reflects a current sink in the stratum radiatum caused by depolarisation of pyramidal cell dendrites. The population spike / fEPSP complex may be preceded, immediately following the stimulus artefact, by a small negative

(downward) deflection, the axonal volley, caused by action potentials in the Schaffer collateral axons (Andersen, 1960; Andersen et al., 1966; Andersen et al., 1971a; Anderson et al,.1977; Leung, 1979).

Anderson et al. (1971a) simultaneously analysed smoothed population spikes and extracellular single unit responses from the same recording electrode by using two amplifiers with different time constants. They showed convincingly on experimental and theoretical grounds that population spike size amplitude directly reflects the number of pyramidal cells firing action potentials in the vicinity of the recording electrode.

Effect of Bicuculline on Unpaired Orthodromic Potentials

The ability of bicuculline and other GABA-A antagonists to increase the size of single orthodromic field potentials and to convert single evoked population spikes into bursts is well documented. The short bursts of population spikes reflect synchronised multiple firing of the pyramidal cells which, in the CA1 area, normally respond to a single orthodromic stimulus with a single action potential (Dingledine and Gjerstadt, 1980; Traub and Wong, 1982; Wong and Traub, 1983; Hablitz 1984). Heyder et al. (1982) have suggested that in addition to its property of GABA-A antagonism bicuculline may have a direct postsynaptic effect to block a potassium conductance and prolong action potentials.

Paired-Pulse Facilitation

The mechanism of paired-pulse facilitation is not clear. A commonly accepted explanation is based on the fact that the test stimulus may occur when presynaptic cytosolic calcium concentration is still raised from the conditioning stimulus. This results in a higher peak cytosolic calcium concentration and hence increased excitatory transmitter

release, compared to a situation where the calcium signal rises from the resting baseline (Hess and Kuhnt, 1992; Wu and Saggau, 1994; Reviewed by Zucker 1989, and Greengard et al., 1993). Alternatively, in the hippocampus, it has been argued that facilitation occurs because of paired-pulse depression of concomitant inhibitory activity (Nathan et al., 1990; Nathan and Lambert, 1991; Brucato et al., 1992; Kahl and Cotman, 1993), the depression of inhibitory activity being mediated by released GABA acting on presynaptic GABA-B receptors on inhibitory interneurones to inhibit further GABA release.

These explanations are not necessarily mutually exclusive. GABA-B mediated paired-pulse inhibition of GABA-A mediated postsynaptic inhibitory potentials clearly does occur, both in this part of the hippocampus and elsewhere (p. 11). The persistence of facilitation in the present experiments, however, under conditions of markedly reduced GABA-A inhibition, would favour the residual-calcium hypothesis rather than paired-pulse depression of underlying GABA-A mediated inhibition as the main mechanism of the facilitation observed here. This though, does not explain why facilitation at 100 ms should be increased by superfusion with bicuculline.

The main direct effect GABA-A blockade is probably to increase postsynaptic excitability. It may be that the increased sensitivity of the post-synaptic response during this bicuculline-mediated disinhibition magnifies the effects of small paired-pulse increases in transmitter release. In addition, disinhibition of interneurones will result in increased GABA-release which may in turn mean a greater tonic activation of presynaptic GABA-B receptors on the Schaffer collateral terminals (see p. 119 for further discussion). A number of investigators have described enhanced paired-pulse facilitation under conditions of

increased presynaptic inhibition where evoked transmitter release is decreased by agents or conditions such as adenosine or low extracellular calcium both in the hippocampus (Creager et al., 1980; Dunwiddie and Haas, 1985; Muller and Lynch, 1989; Sagratella et al., 1991, Kahl and Cotman, 1993) and elsewhere (Katz and Miledi, 1968; Mallart and Martin, 1968; Harris and Cotman, 1983).

Effect of Interstimulus Interval on Paired-Pulse Interactions Using a 1 mA Conditioning Stimulus During Bicuculline Superfusion:
If the mechanism of paired-pulse facilitation observed during bicuculline superfusion involves residual calcium in presynaptic terminals then facilitation should be maximal at short interstimulus intervals and decrease monotonically thereafter. However, when the conditioning stimulus was set at 1 mA a robust and repeatable pattern of alternate inhibition and facilitation was observed as the interstimulus interval was progressively increased.

It seems most likely that, under these conditions, the apparent minima in or absence of facilitation at very short interstimulus intervals and again at around 300 ms are caused by marked superimposed inhibitions. This implies that although inhibition is observed at these latencies the mechanism underlying facilitation is also operating simultaneously but the effects are masked. It also suggests that the mechanism of the medium latency inhibition is different to that of the short latency inhibition since a biphasic pattern of inhibition is not compatible either with a decrease in pyramidal cell excitability consequent on repetitive firing or with transiently decreased availability of transmitter for release.

The theory of superimposed inhibition was adopted as the working hypothesis at an early stage although the possibility of a true of biphasic

pattern to the paired-pulse facilitation could not be unequivocally excluded.

Effect of Conditioning Stimulus Strength on Paired-Pulse Interactions

Paired pulse facilitation at an interstimulus interval of 100 ms tended to be observed during bicuculline superfusion when a supramaximal (1 mA) conditioning stimulus was used but not when the conditioning stimulus was smaller (Table 1). In addition, in ACSF alone, when bicuculline was not present, paired pulse inhibition of a given test response was also *increased* when a *smaller* conditioning stimulus was used compared with a larger conditioning stimulus (Figure 5). A similar effect could be seen for bicuculline-enhanced paired-pulse inhibition at an interstimulus interval of 300 ms (Figure 8 and when data from later experiments were analysed; p. 61).

An increased tendency to facilitation rather than inhibition with a large conditioning stimulus would explain these observations. This effect of conditioning stimulus strength is discussed in some detail below (p. 155).

Summary

Consistent patterns of paired-pulse interaction were demonstrated both in ACSF alone and when bicuculline was added to the superfusate. Superfusion with bicuculline increased both paired-pulse inhibition and paired-pulse facilitation depending on the interstimulus interval and the strength of the conditioning stimulus. Decreasing the conditioning stimulus tended to increase paired-pulse inhibition both during bicuculline superfusion and in ACSF alone. During bicuculline superfusion, using a conditioning stimulus supramaximal for the size of

the evoked potential, paired-pulse interactions between orthodromic potentials alternated between inhibition and facilitation as the interstimulus interval was increased. This strongly suggested a process of medium-latency paired-pulse inhibition, superimposed on the paired-pulse facilitation, and of different genesis to the marked paired-pulse inhibition which was also observed between evoked bursts at short latencies.

III Preliminary Investigations into the Mechanism of Medium-Latency Bicuculline-Enhanced Paired-Pulse Inhibition

A series of pilot experiments was performed on the medium-latency paired-pulse inhibition seen at 300 ms in bicuculline (p. 44). The actions of morphine, adenosine and baclofen were examined for their known effects (or lack of effects) on inhibitory interneurones. 8PT was examined as an antagonist of the endogenous inhibitory modulator adenosine.

Experimental Design

The conditioning stimulus of 1 mA was supramaximal for the size of the evoked potential under control (agent-free) conditions. This was intended to simplify the interpretation of any changes in paired-pulse inhibition; an inhibitory stimulus that remained supramaximal would be comparable both under control conditions and during superfusion with test agent, despite any direct inhibitory or facilitatory effects of the various agents on the single evoked potential. The control responses ($R_{control}$) must necessarily be submaximal to be sensitive to inhibition or facilitation. As some of the agents used had direct inhibitory actions on single evoked potentials the test stimulus had to be adjusted in their

presence to return control potentials ($R_{control}$) to their initial size before paired-pulse inhibition could be quantified for comparison with the agent-free condition.

Morphine

Opioid-receptor agonists, particularly μ-receptor agonists, are known to suppress GABAergic inhibition in the hippocampus. They hyperpolarise (Lee et al., 1980; Madison and Nicoll, 1988) and inhibit firing (Pang and Rose, 1989) of interneurones. They decrease IPSPs recorded in pyramidal cells or block evoked inhibition of pyramidal cell firing (Zieglgansberger et al., 1979; Nicoll et al., 1980; Siggins and Zieglgansberger, 1981; Lambert et al., 1991c). Gahwiler (1980) showed their excitatory effect on pyramidal cells in organotypic culture was blocked by blocking synaptic transmission. In addition they have been shown to decrease paired-pulse inhibition of field potentials in the hippocampus (Dunwiddie et al., 1980; Lupica and Dunwiddie 1991). Some investigators, however, have failed to find an effect on inhibition of pyramidal cells (Haas and Ryall, 1980; Dingledine, 1981; Lynch et al., 1981) which Lupica and Dunwiddie (1991) have ascribed to different effects of μ and δ-receptor activation. Lynch et al. (1981) found paired-pulse inhibition unchanged by enkephalin.

In the present work morphine had no effect on the size of (submaximal) unpaired potentials. Most other investigators have found an increase in evoked field potential amplitude with opioids including morphine. The concentration used here (100 μM) was relatively high. Lynch et al. (1981), Valentino and Dingledine (1982), and Wimpey et al. (1989) all found a threshold concentration of 0.1 to 1 μM for the effect of morphine on the population spike. The lack of effect in the

present work probably reflects the presence of bicuculline in the superfusate and is consistent with the explanation that morphine normally causes an increase in population spike size by decreasing GABA-A mediated inhibition.

The decrease in the medium-latency inhibition caused by 100 μM morphine in the present experiments just failed to reach arbitrary statistical significance in the five slices tested ($P = 0.057$) and was not reversible on washing for between twenty and forty minutes (Figure 9). However, because of the lack of effect on the size of single evoked potentials interpretation was not complicated by the necessity to adjust the size of the control response in the presence of morphine. Furthermore, two control slices showed no diminution in paired-pulse inhibition over a comparable time. The results are compatible with the medium latency inhibition being mediated through the action of interneurones.

Adenosine and Baclofen

Adenosine and baclofen are both agents which act presynaptically to decrease transmitter release. Baclofen is a GABA-B agonist which is known to decrease both GABA release from interneurones and glutamate release from excitatory terminals (p.13). Adenosine acts mainly at excitatory terminals to decrease glutamate release with little or no action to inhibit GABA-release from interneurones (p. 19). As expected both baclofen and adenosine decreased the size of single evoked potentials and control stimuli had to be adjusted in the presence of these agents. Baclofen (0.5 μM to 1 μM) appeared reliably and reversibly to reduce paired-pulse inhibition under these conditions although it was not possible to pool the results of the experiments for

statistically analysis because of the different concentrations of baclofen employed. Adenosine on the other hand was less obviously effective at reducing paired-pulse inhibition.

The data again are in keeping with the 300 ms-latency paired-pulse inhibition being an interneuronally-mediated phenomenon. Interpretation of the results is, however, not completely clear cut since adenosine unequivocally and reversibly reduced paired-pulse inhibition in one slice in which a submaximal conditioning stimulus was used (data not presented). The trend to a reduction in paired-pulse inhibition (P =0.1) with 10 μM adenosine across the five slices shown in Figure 10 is also compatible with a small effect of adenosine to reduce paired-pulse inhibition when a supramaximal conditioning stimulus was used.

8-Phenyltheophyline (8PT)

Endogenous adenosine can be released by selective neuronal depolarisation (p. 21) and would be a potential candidate as an agent causing inhibition at this latency. Three speculative experiments with the non-selective adenosine antagonist 8-phenyltheophyline (Smellie et al., 1979) were therefore performed at this time. Although the results of these limited experiments gave no support to the possibility that endogenous adenosine might have a role to play in mediating the enhanced paired-pulse inhibition they were not comprehensive enough to rule it out and the question was later investigated further (see p. 164).

Summary

Preliminary data were broadly consistent with a role for inhibitory interneurones in the mechanism of medium-latency bicuculline-enhanced paired-pulse inhibition.

IV Paired-Pulse Interaction of Antidromic Potentials

The mechanism of bicuculline-enhanced paired-pulse inhibition could be presynaptic involving a decrease in transmitter release at the Schaffer collateral terminals or it could be postsynaptic involving a decrease in the excitability of the pyramidal cells. In turn, a decrease in postsynaptic excitability might occur because of events intrinsic to the pyramidal cell membrane or might be a consequence of synaptically-released inhibitory transmitter such as GABA. None of these possibilities are mutually exclusive.

Changes in the excitability of the pyramidal cells should be reflected in the size of antidromically evoked potentials. In order to determine whether enhanced paired-pulse inhibition in bicuculline involved a transient decrease in pyramidal cell excitability and as a preliminary to further experiments into the mechanism of the inhibition, the effect of bicuculline was examined on paired-pulse inhibition of antidromic test potentials. Paired-pulse interactions were also examined in calcium-free ACSF to determine whether enhanced paired-pulse inhibition could be demonstrated, in the absence of synaptic transmission, when pyramidal cell excitability was increased by a mechanism other than GABA-A disinhibition.

Paired-Pulse Interactions in the Absence of Bicuculline.

It was consistently possible to demonstrate paired-pulse inhibition of an antidromic test stimuli using antidromic conditioning stimuli in the absence of bicuculline, Interestingly, maximum paired-pulse inhibition was not seen at the shortest interpulse intervals but between 50 and 200 ms (Figure 13). This may have been an artefact caused by averaging values over the six slices; one slice showed a small facilitation at short

interstimulus intervals. On the other hand, three of the six slices showed an increase in inhibition at this latency compared with shorter interstimulus intervals. This observation would suggest that the inhibition is not a consequence of a monotonic change in excitability of the postsynaptic pyramidal cell membrane following its initial firing.

Effect of Bicuculline

Unpaired Potentials As with the orthodromic potentials, the development of secondary spikes on the antidromic potential during bicuculline superfusion probably reflects repeated pyramidal cell action potentials following a single stimulation.

Paired-Pulse Inhibition Although paired-pulse inhibition of an antidromic test stimulus was consistently and significantly increased during bicuculline superfusion using an antidromic conditioning stimulus, the magnitude of the inhibition as measured by the decrease in amplitude of the first spike in the evoked burst was smaller at a latency of 300 ms than when conditioning and test stimuli were orthodromic. An antidromic rather than an orthodromic conditioning stimulus was used for these exploratory experiments in order to be able to examine the effects on this paired-pulse inhibition of manoeuvres which blocked synaptic transmission (see below). The difference in the magnitude of paired-pulse inhibition was likely to be a consequence of the orientation of the test rather than the conditioning stimulus but in order to be certain that the antidromic conditioning stimulus was not less effective at causing paired-pulse inhibition a comparison was made between the effects of various combinations of test and conditioning stimuli. When the test stimulus was orthodromic the effect of orthodromic and antidromic conditioning stimuli were indistinguishable in terms of the

magnitude of inhibition of the test response at an interstimulus interval of 300 ms, or in terms of effects on the overall shape of the test burst response. Similarly, changing the orientation of the test stimulus had no effect on paired-pulse inhibition at 300 ms when the test stimulus was antidromic (Figure 17).

Integrity of Antidromic Responses

The residual-calcium hypothesis (p. 100) requires that paired-pulse facilitation is a synaptic phenomenon and furthermore that it is homosynaptic, requiring repeat activation of the synapses involved. Two of the six slices examined in the absence of bicuculline demonstrated slight but definite facilitation (repeatable within each slice and at different latencies in the two slices). This despite the fact that both test and conditioning stimuli involved direct antidromic stimulation of the pyramidal cell axons in the alveus. This would imply either that there was some synaptic component to the antidromic population spike, perhaps through stimulation of synapses on pyramidal cell dendrites in the stratum oriens, or that the facilitation was mediated by some non-synaptic mechanism intrinsic to the post-synaptic membrane. This second possibility should not be discounted; on one later occasion during a different series of experiments paired-pulse facilitation of an antidromic potential was observed in a calcium-free, high-magnesium solution. The facilitation took the form of secondary spike activity in the test potential which was not present in the control potential. Although, tantalisingly, this observation was reproducible within the same slice it was not possible despite attempts on different occasions to repeat it in three other slices.

Two observations suggest that any synaptically-mediated contribution to the antidromic potential in ACSF or during bicuculline superfusion is likely to be small. Firstly, a clear shift in the latency of the first population spike is apparent when an antidromic stimulus is substituted for an orthodromic stimulus both in ACSF alone and during bicuculline superfusion (compare Figures 6 and 14). Contamination with a large synaptic component would obscure this clean difference. Secondly, the size of submaximal primary antidromic spikes was unaffected by superfusion with bicuculline (p. 50) in contrast to the orthodromic population spikes which were markedly increased (p. 40). Any important synaptic contribution to the antidromic spike should be revealed by causing the antidromic spike amplitude to increase in bicuculline.

Effect of Kynurenic Acid. The possible contamination of the antidromic potential with a synaptically mediated contribution, albeit comparatively small, raises the question of whether the observed paired-pulse inhibition might be explained by a marked paired-pulse inhibition of this synaptic component. In order to address the question of whether paired-pulse inhibition could be demonstrated in the absence of synaptic activity the effect of the non-selective glutamate antagonist kynurenic acid (Perkins and Stone, 1982; Ganong et al., 1983; Jahr and Jessell, 1985) was examined on the paired-pulse interaction between antidromic potentials in the presence of bicuculline. Superfusion with kynurenic acid at this concentration would be expected to markedly diminish or abolish any glutamatergic synaptically mediated component of the evoked antidromic potential.

Superfusion with kynurenic acid reduced paired-pulse inhibition in the two slices examined but did not abolish it (Figure 18). However

kynurenic acid also reduced (though did not abolish) the burst response to a single stimulation compared with the response in bicuculline alone. Thus the reduction in paired-pulse inhibition may well have been secondary to the decrease in burst activity associated with the conditioning stimulus.

Paired-Pulse Inhibition In Calcium-Free ACSF

In very low or calcium-free solution synaptic transmission is abolished and the excitability of nerve cell membranes is increased (Frankenhaueser and Hodgkin, 1957; Brismar and Frankenhaueser, 1975) Under these conditions field potentials, consisting of a burst of population spikes, occur both spontaneously and in response to antidromic pyramidal cell activation by a single stimulus in the alveus (Taylor and Dudek, 1982; Haas and Jefferys, 1984; Konnerth et al., 1986; Agopyan and Avoli, 1988). Simultaneous intra- and extracellular recording shows the bursts to be caused by repetitive synchronised firing of pyramidal cells. Evoked bursts gradually increase in duration over tens of minutes to several hours until they are several seconds long (Agopyan and Avoli, 1988). Spontaneous bursts, which also are often several seconds in duration may take up to two hours to develop (Agopyan and Avoli, 1988). The short (30 ms) evoked bursts seen in the present work resembled the evoked burst responses seen in bicuculline. This probably reflects the short exposure time of the slices to calcium-free ACSF (30 minutes before the start of each experiment). No marked change in the length or shape of the bursts occurred during the further 30 minutes or so required to complete the experiments.

Konnerth et al. (1986) describe an absolute refractory period which may be up to 22 seconds in length after long (several seconds)

spontaneous or evoked low calcium bursts. The demonstration of marked paired-pulse inhibition at interstimulus intervals of up to at least 300 ms between the short evoked bursts in the present work suggests that paired-pulse inhibition similar to that seen during superfusion with bicuculline can be mediated by mechanisms intrinsic to the post synaptic membrane.

Increasing the concentration of magnesium in the superfusate acts to counteract the effects of low or zero calcium on membrane excitability (Frankenhaeuser and Hodgkin, 1957; Somjen and Kato, 1968; Haas and Jefferys, 1984). The disappearance (Figure 21) of the enhanced paired-pulse inhibition at high concentrations of magnesium (which also abolished or much reduced the secondary spikes of the evoked potentials) gives further support to the idea that enhanced paired-pulse inhibition is closely associated with the mechanism underlying or consequences of repetitive pyramidal cell firing.

Kynurenic acid. The lack of effect of kynurenic acid on either the size and shape of the bursts seen in calcium-free solution or on paired-pulse inhibition between the bursts makes it unlikely that the action of kynurenic acid in bicuculline was caused by a non-specific stabilising effect on the post-synaptic membrane rather than by its effect on glutamate receptors. It also suggests that glutamate receptor activation is involved in the mechanism of bicuculline-mediated evoked bursts but not in zero-calcium-mediated evoke bursts, although the small number of slices involved in these experiments must make any such conclusion very tentative.

Summary

The results suggest that bicuculline-enhanced medium-latency paired-pulse inhibition is at least partly mediated postsynaptically. A single explanation consistent with all the findings in this section is that paired-pulse inhibition of antidromic population potentials occurs as a result of mechanisms acting at the pyramidal cell membrane, the occurrence of marked paired-pulse inhibition being directly related to the occurrence of burst activity (which reflects repetitive firing of pyramidal cells) in the evoked potentials, independently of the mechanism of the bursting. On the other hand, although the results show that a synaptic component is not necessary for marked paired-pulse inhibition to occur they do not exclude a synaptically-mediated contribution to antidromic paired-pulse inhibition during bicuculline superfusion.

V Mechanism of Bicuculline-Enhanced Paired-Pulse Inhibition: Effects of Baclofen, Adenosine, and 2-Hydroxysaclofen on Inhibition of Orthodromic and Antidromic Potentials

The results of the preliminary investigations (p. 44) suggested that further investigations should be undertaken to confirm the effect of baclofen in reducing paired-pulse inhibition and to clarify any effects of adenosine because of the implications of these findings for the involvement of GABA-releasing interneurones.

In addition it was decided to examine the effect of the GABA-B antagonist 2-hydroxysaclofen since if the inhibition was in fact mediated by GABA-releasing interneurones during GABA-A blockade, GABA-B receptor activation was a possible mechanism.

In an attempt to distinguish between postsynaptic effects on pyramidal cells and presynaptic effects on Schaffer collateral terminals

paired pulse inhibition (by orthodromic conditioning stimuli) of both orthodromic and antidromic potentials were studied.

Experimental Design

The test stimulus was set to give a response $R_{control}$ which was between 50 and 70% maximal in bicuculline alone and readjusted if necessary to keep $R_{control}$ the same size during superfusion of agent. A submaximal test response is important to allow the response to reflect sensitively any inhibitory influences or to show facilitation. A discussion of the rationale for readjusting the strength of the test stimulus to counteract direct inhibitory effects of the superfused agents is presented in some detail below (p. 138).

Paired-pulse inhibition was examined using two strengths of conditioning stimulus, one supramaximal for the evoked response in ACSF at 1 mA. This stimulus was kept constant throughout the experiment. The second strength of stimulus gave R_{cond} approximately the same size as $R_{control}$ in ACSF. This smaller conditioning stimulus was adjusted to keep $R_{control}$ the same size throughout. The supramaximal conditioning stimulus remained supramaximal in most slices during superfusion with baclofen and adenosine at the concentrations employed, although in some slices small gains in potential size could be made under these conditions by large increases (e.g. to 2 mA) in stimulus strength. This was not formally investigated or quantified.

The use of two strengths of conditioning stimulus was primarily intended to increase confidence that any change in inhibition was not the result of changes in pyramidal cell activity in the conditioning burst in the presence of the test agents whilst retaining comparability with the earlier experiments. This was possible since neither 2-hydroxysaclofen,

adenosine nor baclofen had any marked effect on the shape of the evoked orthodromic potentials in bicuculline once the stimulus had been adjusted to return the size of the primary spike to control. Had these agents selectively suppressed secondary spikes in the evoked potentials, for instance, interpretation would have been more difficult.

The experiments into the inhibition of an antidromically-initiated test potential had given reasonable grounds for confidence that this arrangement could be used to examine a component of the paired pulse inhibition which was caused by a transient decreased excitability of the pyramidal cells and was independent of any effects on transmitter release at Schaffer collateral synapses. The experiments were therefore also performed using antidromically evoked test responses to examine excitability of the pyramidal cells. The same pattern of orthodromic conditioning stimuli was used as was used with the orthodromic test potentials.

Involvement of GABA-B Receptors

Evidence for the involvement of GABA-B receptors in bicuculline-enhanced medium-latency paired-pulse inhibition is provided by the effect of 2-hydroxysaclofen, by the time course of the inhibition, by comparison with other experimental paradigms and by the known effect of GABA-A antagonists on postsynaptic intracellular responses.

2-Hydroxysaclofen

2-Hydroxysaclofen caused a marked reduction in the 300 ms-latency bicuculline-enhanced inhibition of orthodromic test potentials both when supramaximal and submaximal conditioning stimuli were used (Figures 26A and 27). This would strongly support the view that this inhibition was at least partially mediated by GABA-B receptors (Kerr et

al., 1988). The inference could be made with even greater confidence if it had been possible to examine the effect of other GABA-B inhibitors. However, more recent GABA-B antagonists such as CGP-35348 (Olpe at al., 1990) were only just becoming available when these experiments were carried out. 2-Hydroxysaclofen was the most potent and selective GABA-B antagonist available at the time. Stanford et al. (1995) have recently shown that medium-latency bicuculline-enhanced inhibition in the CA1 area of the hippocampus is reduced by the GABA-B antagonist phaclofen.

Time Course

The time course of the medium latency bicuculline-enhanced paired-pulse inhibition is consistent with its being caused by GABA-B-receptor activation. Davies et al. (1990) measured a latency to onset of 29 ms, with a peak at 135 ms and duration of 723 ± 135 ms for pure isolated GABA-B IPSPs on CA1 pyramidal cells.

Other Paired-Pulse Paradigms

Morrisett et al. (1991) have described a paired-pulse inhibition between field potentials in the CA1 area of rat hippocampal slices superfused with the GABA-A blocker picrotoxin and the non-NMDA glutamate receptor antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX). This paired-pulse pulse inhibition of an isolated NMDA response is very similar to the medium-latency bicuculline-enhanced paired-pulse inhibition described in the present work and has an identical time course. It was almost completely abolished by phaclofen and 2-Hydroxysaclofen indicating GABA-B receptor involvement. Interestingly, pharmacologically isolated non-NMDA responses showed

no paired-pulse inhibition in the same preparation. Responses in the absence of glutamate antagonists were not studied.

Isaacson et al. (1993) used a complex paired-pulse paradigm consisting of a short burst of conditioning stimuli (3 to 5 pulses at 50 Hz) from one electrode and a pair of test stimuli 50 ms apart from a second electrode. This enabled them to simultaneously study 'heterosynaptic' paired-pulse inhibition and homosynaptic paired pulse facilitation between the test stimuli. In the absence of exogenous agents they found a marked inhibition of the test responses with a time course very similar to the bicuculline-enhanced paired-pulse inhibition in the present work with a peak at a latency of 300 ms. The inhibition was completely abolished by the GABA-B receptor antagonist CGP35348.

Other observers have recently described a biphasic pattern to simple paired-pulse inhibition of field potentials in hippocampal slices in the absence of exogenous agents. The late phase has a time course similar to the bicuculline-enhanced inhibition observed here and is reduced by 2-Hydroxysaclofen and Phaclofen indicating the involvement of GABA-B receptors (Caddick et al., 1995;Stanford et al., 1995). The inhibition or a loss of facilitation at a latency of 300 ms which was seen under control (bicuculline free) conditions in two slices in an early experiment (Figure 4) is in keeping with these findings.

Postsynaptic IPSPs and GABA-A Antagonists

In the presence of GABA-A antagonists the GABA-B IPSP recorded in the pyramidal cells is enhanced and merges with a prolonged K_{Ca}-mediated membrane after-hyperpolarisation (Schwartzkroin and Stafstrom, 1980; Newberry and Nicoll, 1984a). A similar phenomenon

can be demonstrated in organotypic hippocampal cell cultures (Scanziani et al., 1991).

Involvement of GABAergic Interneurones

The release of GABA by GABAergic interneurones is likely to be increased during bicuculline superfusion. As a result of the reduced GABA-A-mediated inhibition, pyramidal cells respond to a single stimulation with a repetitive burst of action potentials. This will cause increased activation of recurrent interneurones and hence increased GABA release. Activation of feedforward interneurones, either by direct stimulation by the electrode in the stratum radiatum or synaptically via Schaffer collateral fibres is also likely to increase as they too will be subject to decreased GABA-A-mediated inhibition (Lacaille, 1991), again resulting in increased GABA release. Finally Bernado (1993) has demonstrated, in the CA1 area of guinea-pig hippocampal slices that blockade of GABA-A receptors decreased the depression of GABA-B IPSPs that occurs with repetitive stimulation. The possible interpretations of this finding include the existence of presynaptic GABA-A inhibitory autoreceptors whose blockade would increase GABA release.

Such increased GABA-release could account for both the enhanced GABA-B IPSP noted by other investigators and the 2-hydroxysaclofen-sensitive inhibition in the present work.

The marked decrease in bicuculline-enhanced inhibition of orthodromic potentials during superfusion with baclofen suggests that GABAergic inhibitory interneurones are involved in the inhibition studied here.

Baclofen

Baclofen is known to reduce GABA release from inhibitory interneurones (p. 13). Furthermore, in the absence of other drugs baclofen is known to decrease paired-pulse inhibition of single population spikes in the CA1 area in vivo (Steffensen and Henriksen, 1991) and in vitro (Ault and Nadler, 1983; Karsson and Olpe, 1989).

It may seem paradoxical that the bicuculline-enhanced paired-pulse inhibition was decreased both by baclofen, a GABA-B agonist, and by 2-hydroxysaclofen, a GABA-B antagonist. In fact, it would only be difficult to reconcile these findings if the action of released GABA did not make a substantial direct contribution to the paired-pulse inhibition. The effect of baclofen superfusion is to create a marked *tonic* GABA-B influence in the slice decreasing both evoked pyramidal cell activity and evoked inhibitory transmitter release from interneurones (conditioning and test responses to given stimuli were both decreased by baclofen superfusion). Baclofen, providing it acts on the relevant terminals might still be expected to decrease paired-pulse inhibition even if the paired-pulse inhibition were solely mediated by transmitters other than GABA. Alternatively tonic activation of postsynaptic pyramidal cell GABA-B receptors could attenuate paired-pulse inhibition through some unknown mechanism.

In either of these cases superfusion with 2-hydroxysaclofen would be expected either to have the reverse effect to baclofen and increase paired-pulse inhibition or to have no effect depending on whether or not intrinsic GABA-B mechanisms in the absence of baclofen normally had an (indirect) influence on the magnitude of paired-pulse inhibition. On the other hand, if GABA-release did contribute directly to paired-pulse inhibition, any action of 2-hydroxysaclofen to decrease GABA-B effects

on transmitter release or interneurone stability would be set against the effects of antagonising the acute increase in GABA-B activation caused by the GABA released by the conditioning stimulus. In this case 2-hydroxysaclofen might be expected to cause a decrease in paired-pulse inhibition, particularly if the influence of *intrinsically mediated* GABA-B receptor activation on GABA-release by the conditioning stimulus is normally small. Alternatively it is possible, but unlikely, that the paired-pulse inhibition is not mediated by GABA but that 2-Hydroxysaclofen has a powerful effect to decrease paired-pulse inhibition through a separate mechanism unrelated to GABA antagonism.

The qualitative effects of the two agents were consonant with the GABA-B theory in that baclofen appeared to decrease both test and conditioning responses but to decrease conditioning responses to a greater extent (Figure 22), whereas 2-hydroxysaclofen appeared to increase test responses (Figure 25). In retrospect, it is a pity this aspect was not investigated more carefully; in some slices adjustment of stimulus strength was made during the early minutes of superfusion with baclofen or 2-hydroxysaclofen and the effect of these agents on paired-pulse inhibition with stimulus strength kept constant throughout the experiment was therefore not properly quantified. Had this been done, as well as increasing confidence that the effects which were observed at constant stimulus strength were typical, the result would have been useful for comparison with the effect of adenosine (see below). Furthermore it would have been interesting to determine the effects, if any, of the GABA-B agonists and antagonists on the unpaired (control) antidromic potentials since, in particular, an effect of baclofen to decrease the size of the primary spike of the antidromic potential would

have been evidence that this potential was indeed sensitive to the effects of postsynaptic GABA-B receptor activation (see p. 124).

The experiments were designed as they were, precluding the gathering of these data, to prevent experiments becoming overlong. This was partly to avoid artefacts arising from changes in the condition of the slice and partly out of consideration of the duration of superfusion with the expensive 2-hydroxysaclofen. Keeping the superfusion time shorter allowed more experiments with a given quantity of this agent. If adjustments had only been made to relevant potentials after the full periods of agent wash in and wash out, without speeding up the frequency of slice stimulation, and if a full set of duplicate measurements (before and after adjustment) each composed of several potentials for averaging had been gathered for each of the four stimulation conditions, it would have added considerably to the length of time during which agent must be superfused. Performing fresh experiments to gather the data was not a practical option at that time.

Presynaptic versus Postsynaptic GABA-B Receptors

The GABA-B receptors involved in medium-latency bicuculline-enhanced paired-pulse inhibition may be those known to be located on the pyramidal cell membrane and which are responsible for the enhanced GABA-B IPSP, discussed above, which has been described under conditions of GABA-A antagonism. This fits well with the idea that short-latency paired-pulse inhibition between single population spikes in the normal hippocampus (no bicuculline) is thought to be mediated through GABA-A mediated IPSPs at the pyramidal cell membrane. Morrisett et al. (1991) argue that their GABA-B mediated paired-pulse inhibition of pharmacologically-isolated NMDA field

potentials is largely a post synaptic phenomenon as pharmacologically-isolated non-NMDA potentials do not show paired-pulse inhibition in the same preparation and post synaptic NMDA receptors are more likely to be sensitive to GABA-B mediated hyperpolarisation than non-NMDA receptors. Furthermore the inhibition was magnesium sensitive.

On the other hand, enhanced paired-pulse inhibition of an orthodromic test response might result from activation of presynaptic GABA-B receptors located on the Schaffer collateral terminals on pyramidal cell dendrites instead of or as well as postsynaptic receptors. An alternative explanation of the apparent postsynaptic site of Morrisett and colleagues' inhibition (above) is that NMDA-receptor activation on inhibitory interneurones is implicated in increased GABA-release during bicuculline superfusion (see Scanziani et al., 1991). Isaacson et al. (1993) argued that the paired-pulse inhibition they evoked in the normal hippocampal slice using a short tetany (p 118) was mediated presynaptically since it was associated with a simultaneous paired-pulse facilitation of the paired test response.

Slices in bicuculline consistently showed paired-pulse inhibition of an antidromic response although, when quantified by the size of the first spike in the evoked burst, the degree of inhibition was much smaller than that occurring when the test stimulus was orthodromic. These antidromic test stimuli were paired with orthodromic conditioning stimuli identical to those used with the orthodromic test stimuli and thus, with the caveats discussed above (p. 110), allowed an examination of the purely postsynaptic component of orthodromically evoked inhibition. If the effects of 2-hydroxysaclofen, baclofen and adenosine on this postsynaptic component of the paired-pulse inhibition and on the paired-pulse inhibition of orthodromic potentials were quantitatively

similar it would be a strong argument that there was a substantial contribution from enhanced postsynaptic GABA-B inhibitory potentials to the enhanced orthodromic paired-pulse inhibition.

In fact antidromic test responses were not changed significantly by 2-hydroxysaclofen nor by baclofen or adenosine (but see p. 129). This would indicate the main mechanism of the postsynaptic inhibition demonstrated by this paradigm did not involve GABA-B receptors and was at least partially different from the inhibition demonstrated using an orthodromic test potential. One explanation of this finding is that the enhanced paired-pulse inhibition of orthodromic responses is largely mediated through activation of presynaptic GABA-B receptors on the Schaffer collateral terminals. An alternative explanation of the finding is that the GABAergic component of paired-pulse inhibition of orthodromic responses *is* mediated postsynaptically but that postsynaptic GABAergic inhibition is not effective at decreasing the antidromic test response.

In favour of this latter argument is the finding that, in the initial exploratory experiments with bicuculline, although the potentials were prolonged by secondary spikes, submaximal single antidromic population spikes in ACSF were not increased in amplitude by bicuculline (p. 50) whereas orthodromic ones were (p. 40). If it is assumed that there is normally a *constant* GABA-A inhibitory tone, the result of spontaneous interneurone activity, which is antagonised by bicuculline, this observation would seem to suggest that the size of the primary single antidromic spike is not changed by alterations in postsynaptic *GABA-A-mediated* inhibition. Microdialysis measurements suggest a constant concentration of GABA of 0.2 to 0.8 μM in hippocampal ECF in vivo (Lerma et al., 1986; Tossman et al., 1986).

Against an effective constant background GABA inhibitory tone is the fact that none of the GABA-B antagonists CGP35348 (Davies at al., 1991; Isaacson et al., 1993), Phaclofen (Caddick et al., 1995; Stanford et al., 1995) or 2-hydroxysaclofen (Caddick et al., 1995) increased the size of single evoked potentials. Indeed, phaclofen had no effect on the size of evoked field potentials even in the presence of low concentrations of bicuculline when any background GABA tone might be expected to be increased (Stanford et al., 1995).

The sensitivity of the amplitude of the primary orthodromic spike to GABA-A blockade does not necessarily imply a background GABAergic tone. It may well reflect the fact that the field EPSP which gives rise to it is normally contaminated with a fast (GABA-A) IPSP as a result of the activation of feedforward interneurones by the stimulus in the stratum radiatum. Intracellular recordings in the pyramidal layer show evoked fast IPSPs and EPSPs to overlap (see p. 153). The antidromic spike on the other hand, reflecting direct activation of pyramidal cells by stimulation of their axons in the alveus will occur a millisecond or two before the corresponding synaptically mediated inhibitory potentials and while this inhibition might well normally prevent repetitive pyramidal cell firing it would not be expected to affect the chances of an initial action potential occurring or, therefore, to have an effect on the size of the primary spike.

An investigation into the effects of baclofen and a GABA-A agonist such as muscimol on the amplitude of unpaired antidromic potentials would resolve these questions.

In some slices perfusion with baclofen or 2-hydroxysaclofen reversed the paired-pulse inhibition of orthodromic test potentials completely (six slices showed paired-pulse facilitation) while these slices still showed

inhibition of an antidromic test potential under the same conditions. Since identical orthodromic conditioning stimuli were used with both orthodromic and antidromic test stimuli this suggests that paired-pulse facilitation, occurring when the test stimuli were also orthodromic, was strong enough to overcome the simultaneous decrease in excitability of the postsynaptic cells revealed by using an antidromic test stimulus. This provides another argument against there being a marked postsynaptic GABAergic component which does not reveal itself as a decrease in the amplitude of the antidromic potential. If this were the case it would imply that the (non-GABAergic) postsynaptic inhibition which is detected is much stronger than the postulated GABA-B inhibition while the effect of the non-GABAergic inhibition could itself easily be overcome by the increase in transmitter release associated with paired-pulse facilitation.

The present investigation does not provide any further evidence as to the mechanism of the postsynaptic non-GABAergic inhibition. Possible explanations include:

1. Membrane effects such as the K_{Ca} mediated after-hyperpolarisation (Alger and Nicoll, 1980b; Schwartzkroin and Stafstrom, 1980; Nicoll and Alger, 1981; Newberry and Nicoll, 1984a; Lancaster and Adams, 1986).
2. A non-GABA-mediated IPSP (Williams and Lacaille, 1990). (Although this mechanism could contribute to bicuculline-enhanced paired-pulse inhibition it could not account for the paired-pulse inhibition demonstrated in calcium-free ACSF).
3. Ionic or pH changes in the extracellular fluid secondary to repetitive firing. The extracellular concentration of potassium is known to rise steeply to as high as 9 to 10 mM during low-calcium bursting (Haas

and Jefferys, 1984; Yaari et al., 1986) and it has been suggested that this could cause a depolarisation block that terminates the burst (Sypert and Ward, 1971). Both Haas and Jefferys (1984) and Yaari et al., (1986) argue that this is unlikely for a number of reasons including the fact that superfusion with high potassium ACSF (8 mM) increases excitability. In any case the burst associated rise in potassium occurs over several seconds and is unlikely to be relevant to the short (less than 40 ms) bursts demonstrated in the present work. Indeed any burst-related changes in the extracellular environment are likely to increase in magnitude with the duration of the burst. Given this and the fact that in other circumstances spontaneous and evoked burst potentials can last for several seconds (p 112) it seems unlikely that such a mechanism could play an important role in the marked inhibition apparent here after such short conditioning bursts.

Feedforward vs. Feedback Interneurones

An orthodromic conditioning stimulus might be expected to activate both feedforward inhibitory interneurones and feedback inhibitory interneurones via firing of the pyramidal cells whereas an antidromic conditioning stimulus might be expected to activate mainly feedback interneurones of the stratum oriens.

In the normal hippocampus there is evidence that stimulation of feedforward interneurones in the stratum radiatum is likely to give rise to both GABA-A and GABA-B IPSPs on the pyramidal cells whereas stimulation of feedback interneurones in the alveus is more likely to activate a pure GABA-A-mediated response (p. 6). If this were the case in the bicuculline-perfused preparation, and if postsynaptic GABA-B potentials were important in mediating the enhanced paired-pulse

inhibition, it might be expected that an orthodromic conditioning stimulus would be more effective at evoking the 300 ms latency paired-pulse inhibition (of an orthodromic conditioning stimulus) than an antidromic conditioning stimulus. Furthermore, the inhibition caused by an orthodromic conditioning stimulus should be more susceptible to attenuation by the GABA-B antagonist 2-hydroxysaclofen.

In fact there was no difference between the magnitude of inhibition of an orthodromic test potential produced by orthodromic and antidromic conditioning stimuli of similar magnitude (Figures 17 and 27). In addition, paired-pulse inhibition was decreased to a similar extent by 2-hydroxysaclofen independently of the orientation of the conditioning stimulus (Figure 27). These data can be explained in a number of ways but in common with the other results discussed above they fail to give any specific support to the theory that enhanced *post-synaptic* GABA-B activity is of marked importance in the mechanism of the enhanced paired-pulse inhibition.

It seems likely that in the disinhibited (GABA-A blocked) slice discriminatory effects resulting from the differential activation of discrete populations of interneurones may not be maintained. Firstly the stimulus may spread to involve a larger group of neurones as a result of the repetitive firing of excitatory synapses and greater excitability of interneurones. Secondly, inhibitory synapses are likely to fire repetitively both as a result of intrinsic burst firing by interneurones and of repetitive drive by excitatory synapses. The consequent increase in GABA release may cause overspill to activate receptors remote from the point of release (see Isaacson et al., 1993).

Adenosine

Adenosine had no significant effect to reduce paired-pulse inhibition when the conditioning stimulus was supramaximal. This is in agreement with the exploratory experiments (p. 44). As in the exploratory experiments the trend ($P = 0.19$, $n = 9$) was towards a decrease in paired-pulse inhibition with adenosine superfusion with little or no reversal on washing (Figure 24A). When a smaller conditioning stimulus was used, however, adenosine had a highly significant reversible effect to decrease paired-pulse inhibition. This apparent dependence of the effect of adenosine on conditioning stimulus strength is in contrast to the effect of baclofen on paired-pulse inhibition which was independent of the size of the conditioning stimulus (compare Figures 11, 23A and 24A). This suggested that baclofen and adenosine were acting on paired-pulse inhibition through different mechanisms and lead directly to a further series of experiments. The question is discussed in detail later in the chapter (p. 136).

Reanalysis of the Inhibition of Antidromic Bursts using Measurements of Second Spike Amplitude

Some investigators (for example Schubert and Lee, 1986; Fowler 1988; Lupica et al., 1990; Lupica and Dunwiddie, 1991; Hosseinzadeh and Stone, 1994) interested in the modulation of antidromically evoked potentials have used the size of the second spike of antidromic bursts as their main measure of postsynaptic excitability. These investigators have been working with low calcium or calcium-free solutions where synaptic transmission is blocked. In the present experiments the primary spike was selected for a number of reasons:

1) Although inhibition of the primary spike of the antidromic evoked potential in bicuculline was small compared both with the size of the uninhibited spike and with inhibition of orthodromic potentials, it was nevertheless extremely consistent.
2) The interest was in comparing effects on antidromic potentials with effects on orthodromic potentials and with effects in the presence and absence of disinhibition by bicuculline. Using the primary spike of the antidromic evoked potential allowed direct comparison with effects on the primary spike of the orthodromically evoked burst and with single spikes in the absence of bicuculline.
3) As for the single population spike in the absence of agents the main determinant of the amplitude of the primary population spike during bicuculline superfusion is probably the number of pyramidal cells firing action potentials in the vicinity of the recording electrode. A decrease in the amplitude of the spike implies that fewer pyramidal cells are firing and should be a fairly direct measure of pyramidal cell inhibition. In the case of the antidromically evoked potential this may be because pyramidal cell axons become less excitable or because there is an increase in the failure rate for the conduction of action potentials from axon to cell body. However, changes in the synchrony of firing of pyramidal cells will also affect the size of the population spike. This is unlikely to be a major factor in the amplitude of the primary spike where the action potentials are synchronised by the evoking stimulus but may well be an important determinant of secondary spike amplitude, secondary spikes being a manifestation of repetitive firing of the pyramidal cells. Andersen and colleagues describe reduction in antidromic population spike amplitudes due to

failure of invasion of excitation into hippocampal pyramidal cells in vivo (Andersen and Lomo, 1966; Andersen et al., 1973).

4) The antidromic primary spike is probably a purely postsynaptic phenomenon with regard to the pyramidal cells (but see p. 110). In many experiments it would be difficult to rule out a synaptically-mediated component to the longer-latency secondary spikes, as a result for instance of activation of pyramidal dendritic synapses in the stratum oriens. This is important in the arguments concerning the differentiation of pre- and postsynaptic GABA-B effects.

5) It is easy, by changing stimulus strength, to adjust the size of the primary spike to compensate for any effects of exogenous agents on spike amplitude.

6) No compelling published arguments were encountered against using a measurement of the primary spike

The original data using antidromic bursts were gathered and analysed with these arguments in mind. In reviewing the experiments for this thesis, however, it seemed interesting to re-examine the results of the antidromic burst experiments using measurements of secondary antidromic spikes.

For the original analysis the primary spike was measured as the difference between the initial negative and the following positive deflection (ab in Figure 14). The size of the first secondary spike would be best measured by the difference between the first negative deflection of the secondary spike and the following positive (cd in Figure 14]). This measurement is zero when there is no secondary spike, and increases with the size of the secondary spike. The measurement from the peak positivity of primary spike to the following peak negativity (bc in Figure 14) also reflects the size of the first secondary spike but, as is

apparent from examining Figure 14, does not fall to zero when there is no secondary spike.

On re-examining the original paper traces it was possible to make the measurement bc for almost all the potentials used for the original analyses. Unfortunately, particularly in the experiments into the effects of baclofen, adenosine and 2-hydroxysaclofen on paired-pulse inhibition of antidromic burst potentials, the majority of records were truncated such that measurement of cd was not possible. A further problem with these experiments was that adenosine and baclofen had a marked suppressive effect on the presence of the secondary potentials (i.e. both $R_{control}$ and R_{test} were markedly diminished or abolished when they were measured by the size of the secondary spike).

Time Course of Antidromic Inhibition

Figure 45 is a reanalysis of the experiment into interactions between pairs of antidromic evoked potentials in the presence of bicuculline at interpulse intervals between 10 and 1500ms (p. 51). Figure 45 shows the original data using measurements of the primary antidromic spike (ab in Figure 14) and the corresponding plots obtained by substituting measurement of bc or cd (Figure 14). Almost a full set of data was available for this analysis.

Particularly at shorter interpulse intervals, inhibition of the secondary spike was greater than inhibition of the primary spike. However, the time course of the inhibition was similar whichever burst parameter was measured including, interestingly, a small relative decrease in inhibition at an interstimulus interval of about 200 ms.

When the antidromic potential was represented by the measurements ab or bc paired-pulse inhibition was significant at all time intervals

measured. When the antidromic potential was represented by the measurement cd, which reflects only the size of the secondary potential, there was markedly greater interindividual scatter in values between slices and paired-pulse inhibition was not significant at interstimulus intervals from 900 to 1500 ms.

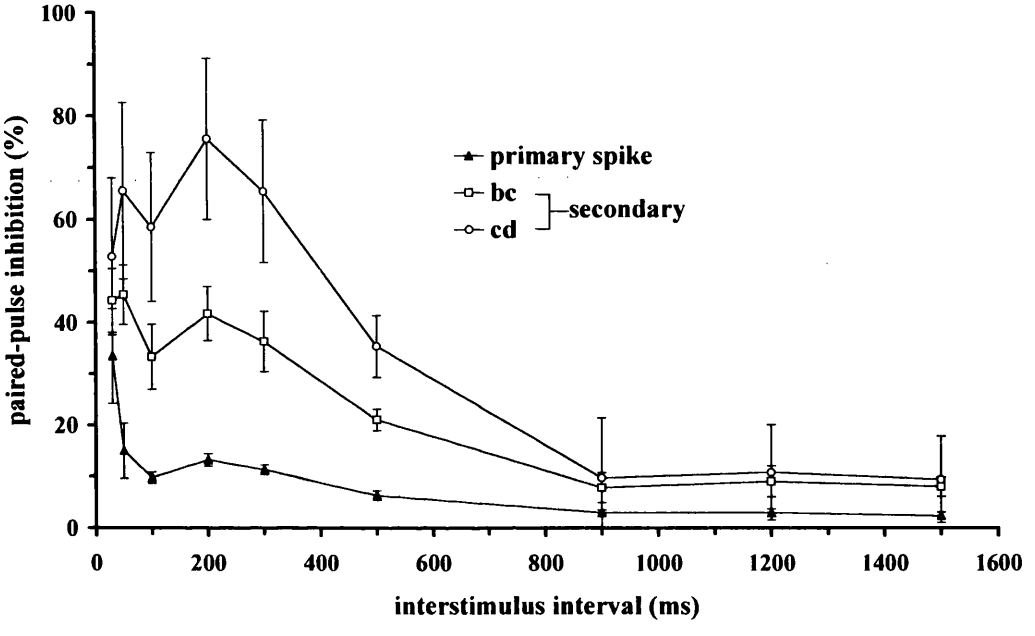

Figure 45 Paired-pulse interactions between pairs of antidromic potentials in 10 μM bicuculline analysed according to different parameters of the evoked potential. The graph labelled primary spike represents the same data as Figure 15. The graphs showing paired-pulse inhibition of the secondary spike are based on measurements of the difference between the peak positivity of primary spike and the following peak negativity (bc) or the difference between the peak of the first negative deflection of the secondary spike and the following positive peak (cd). The letters refer to the labels in Figure 14.

Effects of Baclofen, 2-Hydroxysaclofen and Adenosine on Paired-Pulse Inhibition of Antidromic Potentials

It was not possible to make the measurement cd on most of the records but bc was measurable for most potentials in the adenosine and 2-hydroxysaclofen experiments. There were not enough data to make a useful reanalysis of the experiment into the effect of baclofen.

Using bc as the measurement of the antidromic secondary spike, when the orthodromic conditioning potential was submaximal 10 μM adenosine caused a significant decrease in paired-pulse inhibition (39%, $P < 0.05$, $n = 7$). The effect of adenosine on paired-pulse inhibition was not significant when a supramaximal conditioning stimulus was used ($P = 0.1$, $n = 6$).

Any interpretation of these observations is difficult, however, since superfusion with adenosine itself caused a marked and significant decrease in the size of the secondary spikes which was reflected in an 18% decrease in the measurement bc of unpaired potentials ($R_{control}$, $n = 13$, $P < 0.0001$). 1 μM baclofen caused a similar suppression of secondary spikes (12% decrease in the measurement bc of unpaired potentials, $R_{control}$, $n = 8$, $P < 0.05$). 2-Hydroxysaclofen at 200 μM, in contrast, had no effect by itself on the size of the secondary spikes of the antidromic potentials (1.2% decrease in bc, $P = 0.7$, $n = 12$).

2-hydroxysaclofen had no significant effect on paired-pulse inhibition evoked by a supramaximal conditioning stimulus although there was a strong trend ($P = 0.1$) to a reversible decrease in paired-pulse inhibition on superfusion with 2-hydroxysaclofen (5 of six slices showed a >33% decrease in paired-pulse inhibition calculated from the measurement of bc. This was reversible in three of the slices and unchanged on washing in one slice. Data for the fifth slice were not measurable). When a submaximal orthodromic conditioning stimulus was used 200 μM 2-hydroxysaclofen significantly decreased paired-pulse inhibition measured according to the change in bc (65% decrease, $P < 0.05$, $n = 6$ slices). Reversal of the decrease was not significant over the five slices for which data were available. The trend was towards a return of paired-pulse inhibition on washing.

There is some disagreement then, between the findings on initial analysis of the primary spike data and this later analysis which used data influenced by effects on the secondary spike. This is most problematic with regard to 2-hydroxysaclofen where the reanalysed data is compatible with quite a large decrease in paired-pulse inhibition of the antidromic response, comparable in fact to the effect of 2-hydroxysaclofen on paired-pulse inhibition of the primary spike of orthodromic responses (see Figures 26A and 27).

Interpretation of effects on the secondary spike is complicated by the factors discussed above (p. 129). The action of 2-hydroxysaclofen is consistent with a post-synaptic GABA-B contribution to the paired-pulse inhibition but could also be mediated by an effect on presynaptic GABA-B receptors whose activity influences a synaptic component of secondary spike activity. It is also possible that the secondary spike is very sensitive to small changes in post-synaptic GABA-B-receptor activity. This might be the case if GABA-B activation had an effect on the rate of repetitive firing of pyramidal cells and thus on pyramidal cell synchronisation. The amplitude of the second and subsequent spike will reflect the extent to which pyramidal cells firing together at the initial stimulus retain their synchrony during subsequent action potentials. Interestingly in this context a recent paper by Cobb et al. (1995) shows that individual GABAergic neurones can cause spontaneous firing and subthreshold oscillations in CA1 pyramidal cells to become synchronised at frequencies of 4-7 Hz although this frequency is slower than the within-burst repetitive firing demonstrated in the present work and the phenomenon was related to GABA-A-receptor activation.

Summary

Taking all the findings together, antagonism of bicuculline-enhanced medium latency paired-pulse inhibition of orthodromic potentials by 2-hydroxysaclofen indicated that a substantial component is mediated by GABA-B receptor activation. This conclusion is supported by the time course of the inhibition, by its similarity to other GABA-B-mediated paired-pulse inhibitions described in different experimental paradigms, and by the known effects of bicuculline on interneurone activity and GABA-B receptor activation. The action of baclofen to decrease paired-pulse inhibition supports the hypothesis that GABAergic interneurones are the source of the GABA. The question of whether the relevant GABA-receptors are mainly presynaptic, postsynaptic or a mixture of both remains open. The evidence is compatible with the effect being substantially mediated presynaptically but was far from conclusive. Similarly, experiments with antidromic test potentials failed to find any *unequivocal* evidence for a large postsynaptic GABA effect. Whatever the loci of the GABA-B effect both the evidence of these experiments and the evidence discussed in the previous section suggest that there is an additional postsynaptic component to the bicuculline-enhanced paired-pulse inhibition which is not GABA-mediated and is probably related directly to the recurrent firing of the pyramidal cells.

VI Further Investigations into the Effect of Adenosine on Orthodromic Bicuculline-Enhanced Inhibition

In the experiments discussed above, baclofen appeared unequivocally to reduce 300 ms latency bicuculline-enhanced paired-pulse inhibition. This fits well with the known actions of baclofen and with the theory

that GABA, released from interneurones and acting on GABA-B receptors contributes substantially to the paired-pulse inhibition.

Adenosine has an inhibitory action in the hippocampus that in several respects is similar to baclofen although, unlike baclofen, adenosine has no presynaptic action to reduce GABA release from interneurone terminals (p. 19).

It would have been in keeping with the proposed mechanism of the bicuculline-enhanced paired-pulse inhibition therefore if the inhibition had been unchanged by adenosine. Instead, oddly, and in contrast to baclofen, adenosine appeared to have an effect which was dependant on the strength of the conditioning stimulus, reducing paired-pulse inhibition only it was when evoked by a conditioning stimulus which was submaximal for the size of the evoked potential and not when a larger 1 mA stimulus was used.

This apparent ability of adenosine to reduce the paired-pulse inhibition caused by a submaximal conditioning stimulus was observed after the test stimulus had been adjusted during adenosine superfusion to return $R_{control}$ to its pre-adenosine size. Baclofen, in some experiments, was observed to decrease paired-pulse inhibition despite decreasing the amplitude of both $R_{control}$ and R_{test} before the test stimulus was adjusted during baclofen superfusion. By contrast, the effect of adenosine on paired-pulse inhibition in these circumstances (i.e. before stimulus strength was adjusted to return $R_{control}$ to its pre-adenosine size) was not observed since at the stimulation parameters used, the inhibited potential R_{test} was completely or almost completely suppressed by 20 μM adenosine.

Defining the Effect of Adenosine

In view of the above, the effect of adenosine was re-examined on bicuculline-enhanced paired-pulse inhibition, but deliberately selecting slices that showed a relatively modest control inhibition (range 20 to 40%). A lower concentration of adenosine (10 μM) was used and a higher initial stimulus strength so that paired-pulse inhibition could be quantified both before and after stimulus adjustment in adenosine. The conditioning stimulus in these experiments was submaximal and equal to the test stimulus, conditioning and test stimuli being delivered through the same electrode.

Under these conditions, *when the same stimulus strengths were maintained in the control and adenosine solutions* paired-pulse inhibition was greater during adenosine superfusion. On the other hand, paired-pulse inhibition in the presence of adenosine was still less than for non-adenosine-superfused control potentials of the same size (p. 74). This presents a difficulty of interpretation since, if the stimulus is not adjusted, adenosine reduces the size of both $R_{control}$ and R_{cond} but paired-pulse inhibition normally increases when (in bicuculline in the absence of other agents) $R_{control}$ and R_{cond} are decreased by diminishing the stimulus strength (Figure 28).

The Problem of Test Stimulus Adjustment: Relationship between Test Potential Amplitude and Susceptibility to Inhibition

The increase in paired-pulse inhibition which occurs with decreasing stimulus strength, when test and conditioning stimuli are identical to each other, could be a consequence of a decrease in the conditioning stimulus or it could reflect an increased sensitivity of smaller test

potentials to a given inhibitory influence (i.e. it could be essentially an experimental artefact).

When test and conditioning stimuli are delivered from a single electrode using an interstimulus interval of 300ms and the test stimulus is held constant, paired-pulse inhibition does decrease slightly as the conditioning stimulus is increased from that required to produce a 30% maximal potential to that supramaximal for response size (see Figure 36A). Although the size of this effect may vary between different groups of slices (see control groups in Figures 23A, 24A and 26A) a comparison of the gradient of the relationship in Figure 36A (test stimulus constant) with that in Figure 28 (both test and conditioning stimuli changed) suggests that most of the increase in bicuculline-enhanced paired-pulse inhibition in Figure 28, when both stimuli are changed, is related to the decrease in test stimulus strength. Furthermore, although formal experiments were not carried out, the qualitative observation was made many times while stimuli were being adjusted during various experiments in bicuculline, that smaller test responses were more inhibited than larger test responses by a constant conditioning stimulus.

One possible explanation of this could be a non-linear relationship between the density of activated pyramidal cells in the vicinity of the recording electrode and the amplitude of the population spike, so that as successive pyramidal cells were recruited by the increasing stimulus they added proportionately less to the amplitude of the population spike. Inhibition of a given fraction of cells would then decrease larger population spikes proportionately less.

It is not necessary, however, to dispense with the linear relationship between activated-cell density and population spike size in order to

explain the finding; a simple explanation can be deduced from the arrangement of pyramidal cells and activating fibres. Increasing the strength of a stimulus in the stratum radiatum will increase the number of axons in the vicinity of the stimulating electrode which are triggered to fire action potentials. This increase in the size of the axonal volley results in turn in an increase in the synaptic input to the dendritic fields of pyramidal cells in the vicinity of the recording electrode. Not only will this result in more cells reaching firing threshold and contributing to the population spike but, in addition, because there is overlap in the populations of pyramidal cells contacted by individual fibres synaptic input to pyramidal cells already over firing threshold will also increase, giving these cells a greater margin of safety for firing and greater resistance to inhibition. In other words smaller potentials evoked by a smaller stimulus will be proportionately more affected by a given inhibitory influence. This argument is not restricted to paired pulse inhibition. It explains, for instance, the common observation that, within a given slice, superfusion with exogenous inhibitors such as adenosine has a proportionately greater effect on smaller population spikes.

The argument should not only apply to potentials which are smaller because of decreased stimulus strength, but also to potentials which are decreased in size at constant stimulus strength as a result of background inhibition by an agent such as adenosine. In this case it is easiest to follow the argument for a falling concentration of inhibitory agent; as the concentration of agent decreases more pyramidal cells achieve their firing threshold in response to a given stimulus but because the agent affects the input to all pyramidal cells, the input to cells previously over threshold also increases, rendering these cells less susceptible to inhibition from another source.

Given this equivalence between the effects of inhibitors like adenosine and the effect of decreasing stimulus strength on the susceptibility of the test response to further inhibition, it should be possible to counteract the effect of adenosine on the susceptibility of a control potential to further inhibition by increasing stimulus strength. If this is correct then the pattern of results found in these experiments, i.e. an increase in paired-pulse inhibition during adenosine superfusion when the control stimulus was not adjusted and $R_{control}$ was allowed to decrease, but a diminution in paired-pulse inhibition when the size of $R_{control}$ was maintained in adenosine, suggests that adenosine does cause a decrease in the influence of paired-pulse inhibition on the control potential.

Test Stimulus Adjustment: A Computer Simulation

Because of the importance of testing the validity of these arguments and in particular of determining whether there was any reason in principle that 'adjusted responses' should be systematically more sensitive to the effects of paired-pulse inhibition it was decided to address the questions by constructing a simple computer model.

Design. The model comprises 100 pyramidal cells and 50 stratum radiatum fibres. Each fibre contacts a random 40% of the pyramidal cells. Each fibre has only one synapse with each cell it contacts. Each fibre when activated contributes an amount towards the activation of each cell it contacts. This amount has a default value of one unit. The size of this input can be continuously varied to simulate presynaptic inhibition and facilitation but is always identical for all synapses in the model. The number of active fibres can be varied to simulate changes in stimulus strength.

The model is arranged to allow presynaptic changes to be entered by means of two separate factors (for example to simulate the interaction between adenosine inhibition and presynaptic paired-pulse inhibition). In this case the inhibitory factors multiply. For instance, if the first inhibitory factor is 20% and the second 10% then the synaptic value is reduced from 1 unit to 0.72.

Firing of a pyramidal cell requires synaptic input to reach a certain threshold. By default the threshold of individual cells is set randomly to a value between 9 and 11 units to simulate variability in biological firing threshold and synaptic input.

Postsynaptic inhibition can be simulated also, by changing the pyramidal cell firing threshold by a fixed amount (for example 2 units) which is added to all synapses in the model and is the same for all synapses.

The model will simulate different slices by re-randomising the connections and pyramidal cell thresholds. There is a 'default slice' which always has the same pattern of connectivity and thresholds.

A full listing of the program is given in the Appendix *Simulations*. Figure 46 shows the results from a set of simulations into the effect of stimulus strength on susceptibility to presynaptic inhibition. Smaller responses are more susceptible to inhibition than larger responses evoked by activating more fibres. This finding is robust in the face of changes to the size of the presynaptic inhibition and across different 'slices'.

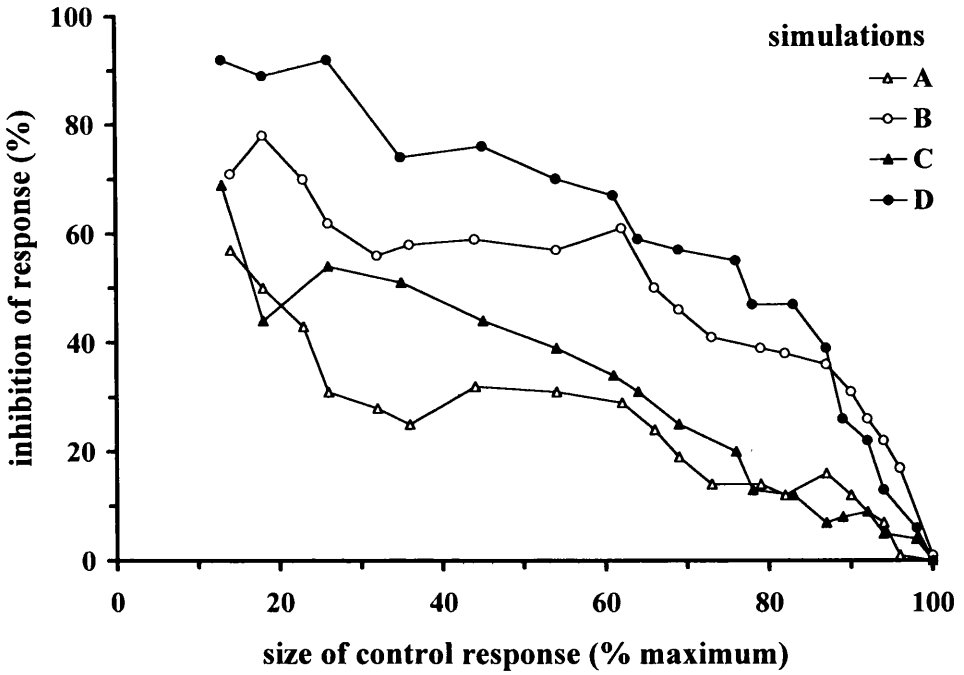

Figure 46 Computer simulation of the influence of control response size on susceptibility to presynaptic inhibition. The size of the control response was changed by systematically varying the number of stratum radiatum fibres activated. Four separate simulations (A to D) were undertaken. The amplitude of each control response is plotted against the percentage inhibition of each control response which occurred when synaptic strength was reduced by a constant factor. Response size was calculated as the number of activated pyramidal cells. In graphs **A** and **B** the inhibitory factor was set to reduce synaptic strength by 10% and 20% respectively in the model's default 'slice'. Graphs **C** and **D** show the inhibition caused by synaptic inhibitory factors of 10% and 20% respectively in an alternative 'slice'.

Figure 47 shows the results from a set of simulations into the effect of background presynaptic inhibition (for example such as that by adenosine) on susceptibility to further presynaptic inhibition at constant stimulus strength. Responses which are smaller because of pre-existing presynaptic inhibition are more susceptible to further presynaptic inhibition than responses which are larger because of less background inhibition. This finding is robust at different amounts of background inhibition, at different 'stimulus strengths' and across different 'slices'. Similar results which are not shown were obtained with combinations of presynaptic and postsynaptic inhibition.

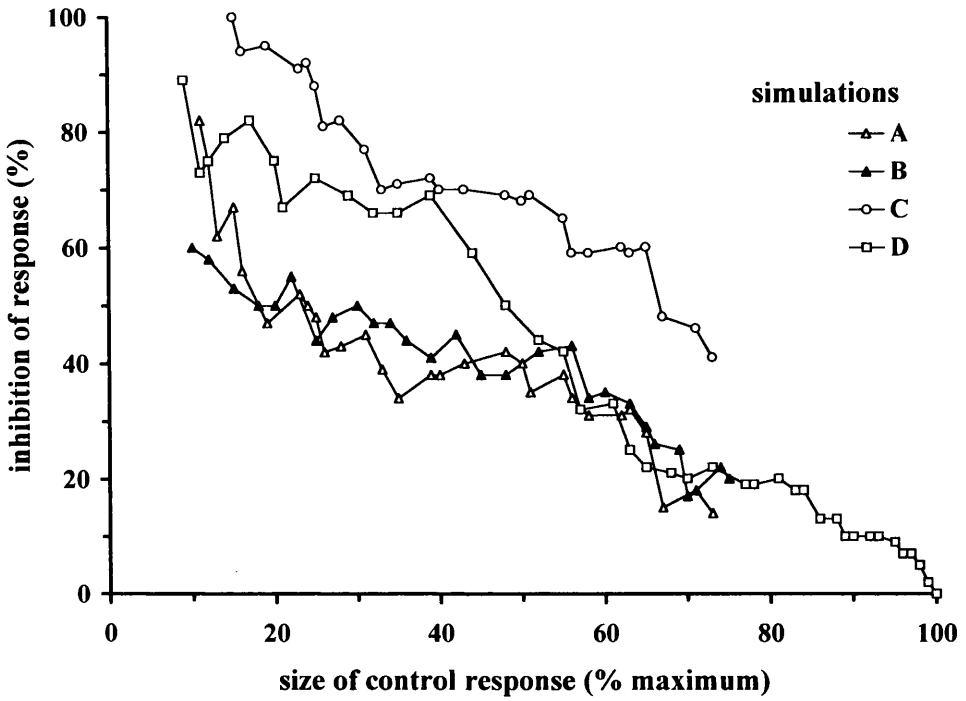

Figure 47 Computer simulation of the influence of control response size on susceptibility to presynaptic inhibition. The size of the control response was changed by systematically varying the strength of the synaptic connections between stratum radiatum fibres and pyramidal cells while the number of active stratum radiatum fibres was held constant. Four separate simulations (A to D) were undertaken. The size of each control response is plotted against the percentage inhibition of each control response which occurred when synaptic strength was further reduced by a constant factor, the second inhibitory factor. Response size was calculated as the number of activated pyramidal cells. In graph **A** the second synaptic inhibitory factor was set to 10% using the model's default 'slice'. 'Stimulation strength' was set to activate 60% of the stratum radiatum fibres which resulted in a submaximal pyramidal cell response before synaptic strength was decreased. In graph **B** the model parameters are the same as in graph A except that a different 'slice' was used. In Graph **C** the second synaptic inhibitory factor was set to 20% using in the model's default 'slice'. 'Stimulation strength' was again set to activate 60% of the pyramidal cell fibres. In graph **D** the second synaptic inhibitory factor was set to 10% using the model's default slice but 'stimulation strength' was set to activate 100% of the stratum radiatum fibres and was supramaximal for the activation of all the model's pyramidal cells before synaptic strength was decreased.

Figure 48 shows the results of several simulations in three different consecutive 'slices' in which a background presynaptic inhibition was balanced by increasing the number of active fibres. In each experiment increasing amounts of presynaptic inhibition (simulating increasing concentrations of adenosine) were balanced by increasing the number of active fibres to restore the original size of the response. The

susceptibility of these corrected responses to further presynaptic inhibition was then tested. The relationship between the inhibition of the corrected response by the second presynaptic inhibitory factor ('paired-pulse inhibition') and the amount of background presynaptic inhibition ('adenosine concentration') was virtually flat in all cases. This result was robust across the three slices, at two different sizes of initial control potential, and at two different strengths of 'paired-pulse inhibition' inhibitory factor. None of the simulation results demonstrated decreasing 'paired-pulse inhibition' with increasing background inhibition.

Conclusions. It would be relatively easy to construct models of greater complexity and fidelity at the cost of spending more time and effort on quantification and verification (see Leaning,1980). Despite the simplicity of this particular model it nevertheless simulates the general arrangement of pyramidal cell activation well enough for the results to suggest that the experimental effect of adenosine on paired-pulse inhibition are not a systematic artefact consequent on this arrangement. The ability of the model to mimic the empirical results of the experiments into the interaction between response size and susceptibility to inhibition give further confidence in the model's validity. In passing, it also interesting to note that even such a simple model can show remarkable biological-type variability in its output.

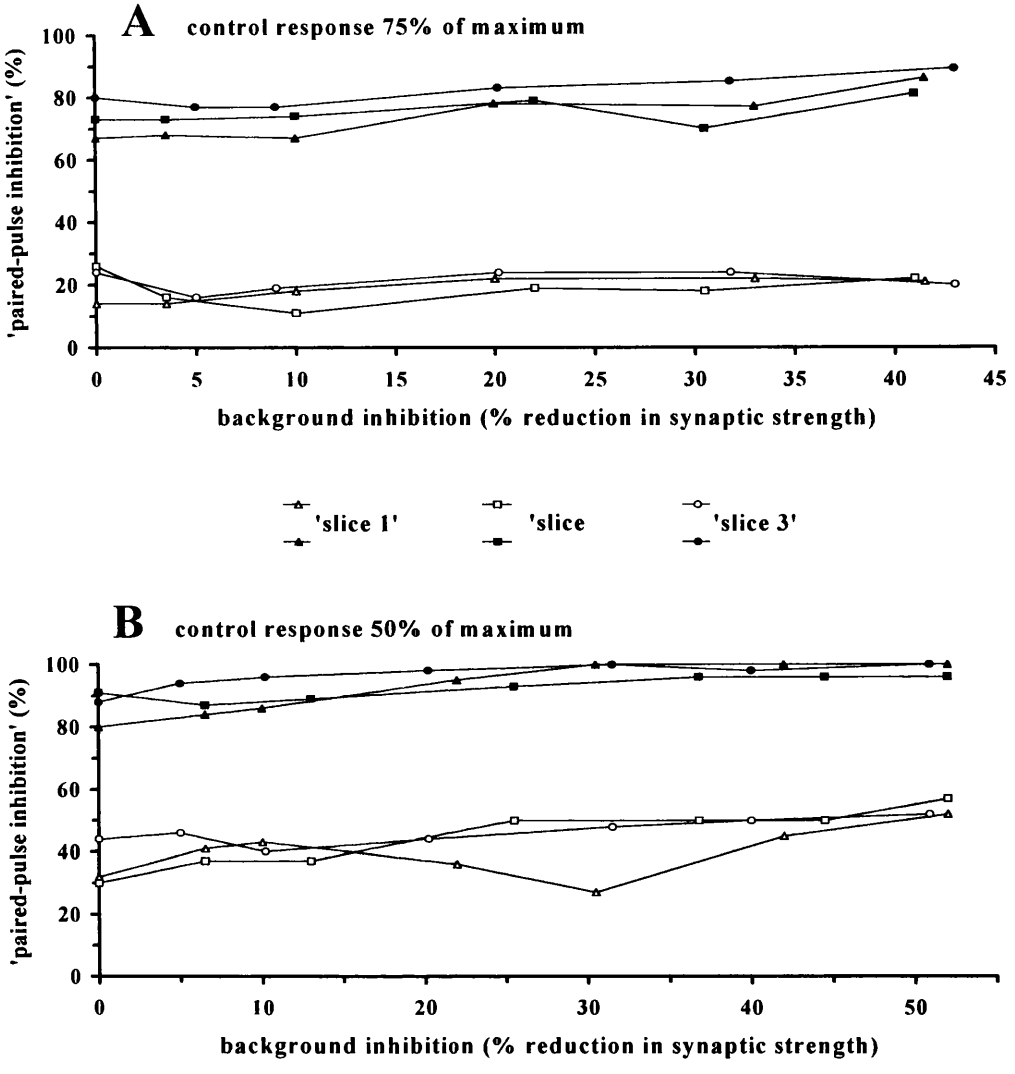

Figure 48 Computer simulations of experiments in three different consecutive 'slices' into the susceptibility to further presynaptic inhibition of control responses where a background presynaptic inhibition (first synaptic inhibitory factor) was balanced by increasing the number of active fibres. In each simulation increasing reductions in the strength of the synapses between stratum radiatum fibres and pyramidal cells were balanced by increasing the number of active stratum radiatum fibres to restore the original size of the control response. The susceptibility of these corrected control responses to further constant decreases in synaptic strength (second synaptic inhibitory factor) was then tested. The percentage inhibition of each control response ('paired-pulse inhibition') in response to the second synaptic inhibitory factor is plotted against the size of the corrected (first) synaptic inhibitory factor for each control response. Response size was calculated as the number of activated pyramidal cells. In **A** the control response size without any background inhibition was set to about 75% of maximal (kept constant within each 'slice'. The filled and open symbols show the effects of setting the second inhibitory factor to cause a 30% and 10% reduction respectively in synaptic strength in each of three 'slices'. In **B** the control response size without any background inhibition was set to just below 50% of maximal (kept constant within each 'slice'. The filled and open symbols show the effects of setting the second inhibitory factor to cause a 30% and 10% reduction respectively in synaptic strength in the same three 'slices' as plotted in A.

Effect of Adenosine on Matched Unpaired and Paired-Inhibited Test Potentials

The simulated experiments suggest there is no reason arising from the *general arrangement* of pyramidal cells and stratum radiatum fibres why the adjusted responses should be more susceptible to adenosine. Nevertheless, the excitatory input to the cells forming $R_{control}$ before adenosine superfusion will be different from the excitatory input to the cells forming $R_{control}$ during adenosine superfusion; the adenosine superfused population is activated by a larger axonal volley and therefore influenced by a larger number of excitatory synapses, each synapse on average being less effective than in the non-adenosine superfused population. If adenosine inhibition selectively affects a subgroup of pyramidal cells the two populations may then each be made up of a different set of pyramidal cells which differ systematically in their connectivity, receptor profile, and susceptibility to paired-pulse inhibition. This possibility is difficult to refute. Finally, it is also possible (although there is no obvious reason why this should be the case) that the relationship between population spike size and the density of active pyramidal cells is changed during adenosine superfusion.

In order to provide a complimentary paradigm to the adjustment of control potentials during adenosine superfusion the effect of adenosine was compared on the inhibited potential R_{test} and on single potentials which were adjusted to the same size as R_{test} in the absence of adenosine. In this case, the effect of adenosine is examined on two same-sized populations of active cells, one of which is subject to the depressant effect of paired-pulse inhibition which has been compensated for by increased excitatory input. If adenosine does decrease the influence of paired-pulse inhibition then adenosine should have a

greater depressant effect on the single potentials than on R_{test} since in the case of R_{test} its direct inhibitory effect of adenosine will be offset to some extent by the decrease in paired-pulse inhibition.

Adenosine did, in fact, inhibit R_{test} significantly less than unpaired potentials of the same size (Figure 30). The effect was small compared to the apparent effect of adenosine to decrease paired-pulse inhibition when it was examined directly but this may simply reflect the strength of the direct depressant effect of adenosine on the evoked potential compared to its indirect effect through modulation of paired-pulse inhibition. In the previous experiments the effect of adenosine on paired-pulse inhibition was only unmasked when the direct depressant effect was cancelled out by increasing the stimulus. This was in marked contrast to baclofen where the effect of baclofen on paired-pulse inhibition was great enough in some experiments to overcome the direct depressant effect of baclofen on R_{test} (Figure 22).

Summary

The experimental effect of adenosine on bicuculline-enhanced paired-pulse inhibition (i.e. that paired-pulse inhibition in the presence of adenosine was less than for non-adenosine superfused control potentials of the same size) was robust. It was seen in several different experimental groups (pp. 64 and 74). It was seen at concentrations of adenosine from 4 μM to 40 μM, and showed evidence of a concentration-response relationship (Figure 31). In addition, the phenomenon could be demonstrated both when the initial control potential ($R_{control}$ before adenosine superfusion) was 70% maximal (Figure 31) and when it was much smaller at 50% maximal (p. 74) indicating that the reduction in paired-pulse inhibition was not related to

the relatively large stimuli required to restore the 70% response in adenosine. Simple computer modelling suggests that there is no reason in principle why control potentials in adenosine should be more sensitive to inhibition than non-adenosine superfused potentials of the same size. Furthermore, adenosine inhibited the potential R_{test}, which is reduced by paired-pulse inhibition, significantly less than an unpaired amplitude-matched potential.

Although other possible interpretations may be considered, the evidence suggests that the reduction in paired-pulse inhibitory effect during adenosine superfusion is not an artefact caused by a change in the sensitivity of $R_{control}$.

Mechanism of the Effect of Adenosine on Orthodromic Bicuculline-Enhanced Inhibition

One possible mechanism whereby adenosine might decrease paired-pulse inhibition despite having no direct effect on the release of GABA from interneurones would be if adenosine preferentially inhibited transmission at excitatory terminals onto interneurones.

An alternative explanation for adenosine's reduction of bicuculline-resistant paired-pulse inhibition is that it is acting presynaptically at the Schaffer collateral synapses with the pyramidal cell dendrites to increase concomitant facilitation. It has already been argued that, at least when a supramaximal conditioning stimulus is used, that paired-pulse facilitation is occurring simultaneously with the medium-latency paired-pulse inhibition although it is masked by the magnitude of the inhibition (pp. 100 and 102). In addition, adenosine has been reported to increase paired-pulse facilitation of the field excitatory post synaptic potential (recorded extracellularly in the stratum radiatum of CA1) by a

presynaptic mechanism at the Schaffer collaterals (Dunwiddie and Haas, 1985).

Concentration-Response Relationships

If the effect of adenosine to decrease observed paired-pulse inhibition is mediated by an increase in paired-pulse facilitation consequent on the decrease in transmitter release caused by activation of these same receptors, then the concentration-response relationships for depression of paired-pulse inhibition and depression of single evoked potentials should be similar. In particular the minimally effective concentration of adenosine should be similar in both cases.

On the other hand if adenosine is decreasing paired-pulse inhibition by suppressing transmission at excitatory terminals onto interneurones (an explanation not ruled out by Dunwiddie and Haas, 1985) it might suggest either that these receptors are more sensitive to adenosine than those on the Schaffer collateral/pyramidal cell synapses or that there was a greater density of adenosine receptors on the Schaffer collateral/interneurone synapses. This is because adenosine decreases paired-pulse inhibition when stimulus strength for $R_{control}$ is increased to compensate for the effect of adenosine at the excitatory Schaffer collateral synapses onto pyramidal cell dendrites. Unless Schaffer collateral synapses onto interneurones are preferentially sensitive to adenosine the initial ratio of pyramidal cells to interneurones should be maintained in the adenosine-inhibited potential and in the new population of cells recruited by the increased stimulus.

If there are two such different populations of adenosine receptors, or if adenosine receptors are distributed preferentially on the interneuronal terminals of the collateral fibres, then adenosine would be expected to

reduce paired-pulse inhibition at a lower concentration than is required for depression of single evoked potentials. In view of this the concentration-response relationships for adenosine were investigated on single evoked potentials in the presence of bicuculline and on bicuculline-enhanced paired-pulse inhibition at 300ms.

The results indicate the potency of adenosine is similar at low concentrations when depressing paired-pulse inhibition and when depressing the control potentials. In particular, paired-pulse inhibition was not decreased at a concentration of adenosine less than that required for depression of single evoked potentials (Figure 31). This result fails to provide any evidence for two qualitatively or quantitatively different populations of adenosine receptors and is consistent with the explanation that adenosine decreases paired-pulse inhibition by increasing paired-pulse facilitation.

The result does not rule out a direct inhibitory effect of adenosine on interneurone excitability. Such an explanation, however, would require that interneurones were *particularly* sensitive to postsynaptic inhibition by adenosine in comparison to pyramidal cells, since compensation for the small postsynaptic effect of adenosine on pyramidal cell excitability is provided by the stimulus readjustment which restores $R_{control}$.

Furthermore, adenosine may well act predominantly presynaptically, at least on pyramidal cell excitation, at the relatively low concentrations employed in these experiments (see Yoon and Rothman, 1991; Prince and Stevens 1992; Scanziani et al. 1992) although this is by no means clear. Adenosine decreases evoked and spontaneous bursting of pyramidal cells in low calcium / high magnesium media at concentrations from 20 μM down to less than 1 μM (Lee et al., 1984, Haas and Green, 1988). This would seem to be a postsynaptic effect but

it would be difficult to exclude the possibility that tiny amounts of released transmitter were critically influencing cell firing even in low calcium solution. Investigators have generally used concentrations of 50 μM or more when studying postsynaptic membrane effects directly. Gerber et al. (1989) described increases in potassium conductance of pyramidal cell membranes with bath-applied concentrations down to 10 μM although only results of applying 50 μM adenosine were presented. Green and Haas (1985) found no changes in resting membrane properties at applied concentrations less than 50 μM although concentrations less than this increased the stimulus evoked afterhyperpolarisation.

An effect on the activation of *recurrent* interneurones by pyramidal cells remains a possibility; in the absence of confounding factors recurrent interneurones should be activated in proportion to the number of active pyramidal cells but during adenosine superfusion, although the same number of pyramidal cells contribute to the adjusted $R_{control}$ as before adenosine superfusion, excitatory transmission from pyramidal cells to recurrent interneurones is likely to be inhibited to some extent by the adenosine. Whether this is important will depend partly on the extent to which this group of interneurones contributes to bicuculline-enhanced paired-pulse inhibition. There is some evidence that in the CA1 area of the normal (bicuculline-free) hippocampus recurrent interneurones are less likely to contribute to postsynaptic GABA-B receptor activation than feedforward interneurones (Alger and Nicoll 1982a; Davis et al., 1990; Newberry and Nicoll 1984a). In the earlier experiments reported here, however, it was not possible to define separate feedforward and feedback components of paired-pulse

inhibition using orthodromic and antidromic conditioning stimuli (p. 127).

Aside on the Reported Excitatory Effects of Low Concentrations of Adenosine.

In the hippocampus some investigators have reported paradoxical excitatory effects of very low concentrations of adenosine (p 20). This phenomenon, like the effect of adenosine on paired-pulse inhibition, could possibly be explained by a disinhibition if the activation of interneurones were preferentially sensitive to the effects of low concentrations of adenosine. This is plausible since GABA antagonists such as bicuculline (this work) or picrotoxin (Landgren, 1991) increase the amplitude of evoked field potentials in the hippocampus. Furthermore feedforward inhibition from a single stimulation (in the absence of bicuculline) influences the shape of the resulting intracellularly recorded EPSP (Dingledine et al., 1987) or field EPSP (Dingledine et al., 1987; Nathan et al., 1990). Ashwood et al. (1984) reported that feedforward interneurons often fired before pyramidal cells in response to stratum radiatum stimulation.

A preferential sensitivity to adenosine of excitatory terminals on interneurones could also explain the comparative vulnerability to hypoxia of polysynaptically-mediated but not monosynaptically-mediated IPSPs reported by Khazipov et al. (1993) in CA1 pyramidal cells since the concentration of endogenous adenosine is increased by hypoxia (p. 16).

To test this hypothesis, in addition to the investigations of concentration-response relationships during bicuculline superfusion (as discussed above, concentration-response relationships were compared

for the effect of adenosine on single evoked population spikes and on short-latency paired-pulse inhibition at an interstimulus interval of 30 ms in the normal hippocampal slice in the absence of bicuculline.

In the absence of bicuculline, just as during bicuculline superfusion the concentration-response relationships for both effects were similar; adenosine did not decrease paired-pulse inhibition at concentrations less than those required for inhibition of single evoked potentials.

These experiments were carried out at a later date than the experiments in bicuculline and in order to minimise the potential contribution of homosynaptic facilitation to the paired pulse interactions separate electrodes, one on either side of the recording electrode, were used for conditioning and test stimuli (Figure 1). It is interesting that adenosine still had a depressive effect on paired-pulse inhibition in these circumstances. However, although overt facilitation was not observed with this arrangement the relatively coarse electrodes used are likely to stimulate fibres over a large volume of the Schaffer collateral bundle and it may well be that there was an overlap in the population of synapses stimulated by each electrode; an increase in facilitation was thus not excluded as an explanation for the action of adenosine on paired-pulse inhibition.

None of the slices in the present experiment exhibited an increase in population spike size with adenosine so differential sensitivity to adenosine of excitatory terminals onto interneurones and pyramidal cells cannot with certainty be excluded as an explanation for the reported paradoxical direct excitatory effects of adenosine. However, by demonstrating that such differential sensitivity to adenosine is not a general property of hippocampal slices this explanation is made less likely.

Influence of Conditioning Stimulus Strength on the Effects of Baclofen and Adenosine

In the pilot experiments (p. 44) and in the subsequent set of systematic experiments (p. 64), adenosine had no significant effect on bicuculline-enhanced paired-pulse inhibition (after stimulus readjustment) when the paired-pulse inhibition was evoked using a 1mA conditioning stimulus that was considerably supramaximal for the size of the evoked potential. This was in direct contrast to the effect of baclofen, which was to reduce paired-pulse inhibition whatever the strength of the conditioning stimulus, and suggested it would be interesting to make a systematic investigation into the effect of conditioning stimulus strength on the ability of adenosine and baclofen to reduce bicuculline-enhanced paired-pulse inhibition.

In these new experiments adenosine again had a significantly smaller effect on paired-pulse inhibition when a supramaximal (1mA) conditioning stimulus was used than when the conditioning stimulus was submaximal. On this occasion however, adenosine did significantly decrease paired-pulse inhibition (after stimulus readjustment) when the conditioning stimulus was supramaximal (p. 81, Figure 36B). The effect was not graded; when the conditioning stimulus was submaximal the effect of adenosine on paired-pulse inhibition was independent of conditioning potential size. This was in contrast to baclofen which tended to be more effective at reducing paired-pulse inhibition as the size of the conditioning stimulus was increased from that evoking a 50% maximal R_{cond} to supramaximal at 1 mA (Figure 36B).

When conditioning and test stimuli are delivered orthodromically from the same electrode, both in ACSF alone and during bicuculline superfusion, paired-pulse inhibition tends to be smaller when the

conditioning stimulus is supramaximal than when the conditioning stimulus is smaller. In some cases paired-pulse inhibition evoked with a small conditioning stimulus becomes facilitation when a supramaximal conditioning stimulus is used (Figure 5 for example). It has already been suggested (p. 103) that these observations could be explained by a shift towards facilitation with large conditioning stimuli. This conclusion is strengthened by the observation that short-latency paired pulse inhibition did not decrease with increasing conditioning stimulus strength when the test and conditioning stimuli were delivered from *separate* electrodes in the stratum radiatum (p. 78).

Marked paired-pulse inhibition can be obtained from conditioning stimuli small enough to be subthreshold for population spikes (Lynch et al., 1981; Peet and Mclennan, 1986) and feedforward inhibitory interneurons can be activated with a lower threshold in terms of stratum radiatum stimulation than pyramidal cells (Ashwood et al,. 1984). This in turn suggests that maximal or near maximal inhibitory influences, at least from feedforward interneurones, should be obtained from conditioning stimuli submaximal for the associated population spike. If paired-pulse facilitation increases with the strength of the conditioning stimulus, then the balance of paired-pulse effect should move towards facilitation with larger conditioning stimuli. There is no direct evidence for this conclusion, but, if paired-pulse inhibition (either normally or during bicuculline superfusion) is caused by the activation of interneurones by the conditioning stimulus, it is difficult to see how increasing the size of the conditioning stimulus could decrease the size of the direct inhibitory effect.

An observation that may possibly be relevant in this context is the finding that in some slices, particularly in ACSF, the maximum size of

single (unpaired) potentials was evoked by a stimulus well below 1 mA and the potential evoked by a supramaximal 1 mA stimulus was smaller than the maximal potential (p. 77, Figure 32). The effect was generally small, appeared to be much less marked during bicuculline superfusion (although this question was not formally addressed) and was not present in all slices. Nevertheless, it may reflect a particular inhibitory mechanism associated with very large stimuli and it is conceivable that the effect, whatever it is, extends to inhibition of inhibitory interneurones.

The last observation aside, if paired-pulse facilitation does indeed contribute proportionately more to the paired-pulse interaction when the conditioning stimulus is large, then the influence of conditioning stimulus strength on adenosine's action to reduce paired-pulse inhibition is consistent with adenosine, in contradistinction to baclofen, acting to reduce paired-pulse inhibition by causing an increase in simultaneous paired-pulse facilitation. That is adenosine decreased inhibition only under circumstances where underlying facilitation was likely to be smaller and there was greater scope for an increase in facilitation.

Correlation between Effect of Adenosine and Effect of Stimulus Adjustment. Since the balance between underlying inhibition and facilitation is variable in individual slices, if the above explanation is correct, then a negative correlation might be expected between the increase in control paired-pulse inhibition which occurred on decreasing conditioning stimulus strength in an individual slice and the size of the adenosine effect on paired-pulse inhibition which occurred in the same slice when the conditioning stimulus was supramaximal; slices which showed a larger reduction in control inhibition with stimulus adjustment presumably having more facilitation underlying the paired-pulse effect

evoked by the supramaximal stimulus than slices which showed a smaller reduction in paired-pulse inhibition.

The effect, on the actions of baclofen and adenosine, of varying the strength of the conditioning was examined in 18 slices in two different sets of experiments (pp. 59 and 81). Figure 49A shows the relationship, across all these 18 slices, between the increase in paired-pulse inhibition which occurred with adenosine superfusion when the conditioning stimulus was supramaximal and the change in control paired-pulse inhibition which occurred when a stimulus evoking a 60% maximal R_{cond} was substituted for a supramaximal 1 mA conditioning stimulus. There was a significant correlation ($r = 0.63$, $P < 0.01$) in the expected direction. By contrast there was no significant correlation for the same relationship in the case of baclofen (Figure 49B).

Furthermore, when the data are examined in terms of the two different experiments, the theory provides an explanation for the fact that adenosine (when the conditioning stimulus was 1 mA) had no significant effect in the first set of slices but did cause a significant reduction in paired-pulse inhibition in the second set. The change in paired-pulse inhibition with conditioning stimulus decrease under control conditions was particularly marked in the first group of slices ($39 \pm 13.9\%$ decrease in paired-pulse inhibition with a supramaximal conditioning stimulus compared with a 60% maximal conditioning stimulus, the comparable value in the second study being $24 \pm 5.6\%$).

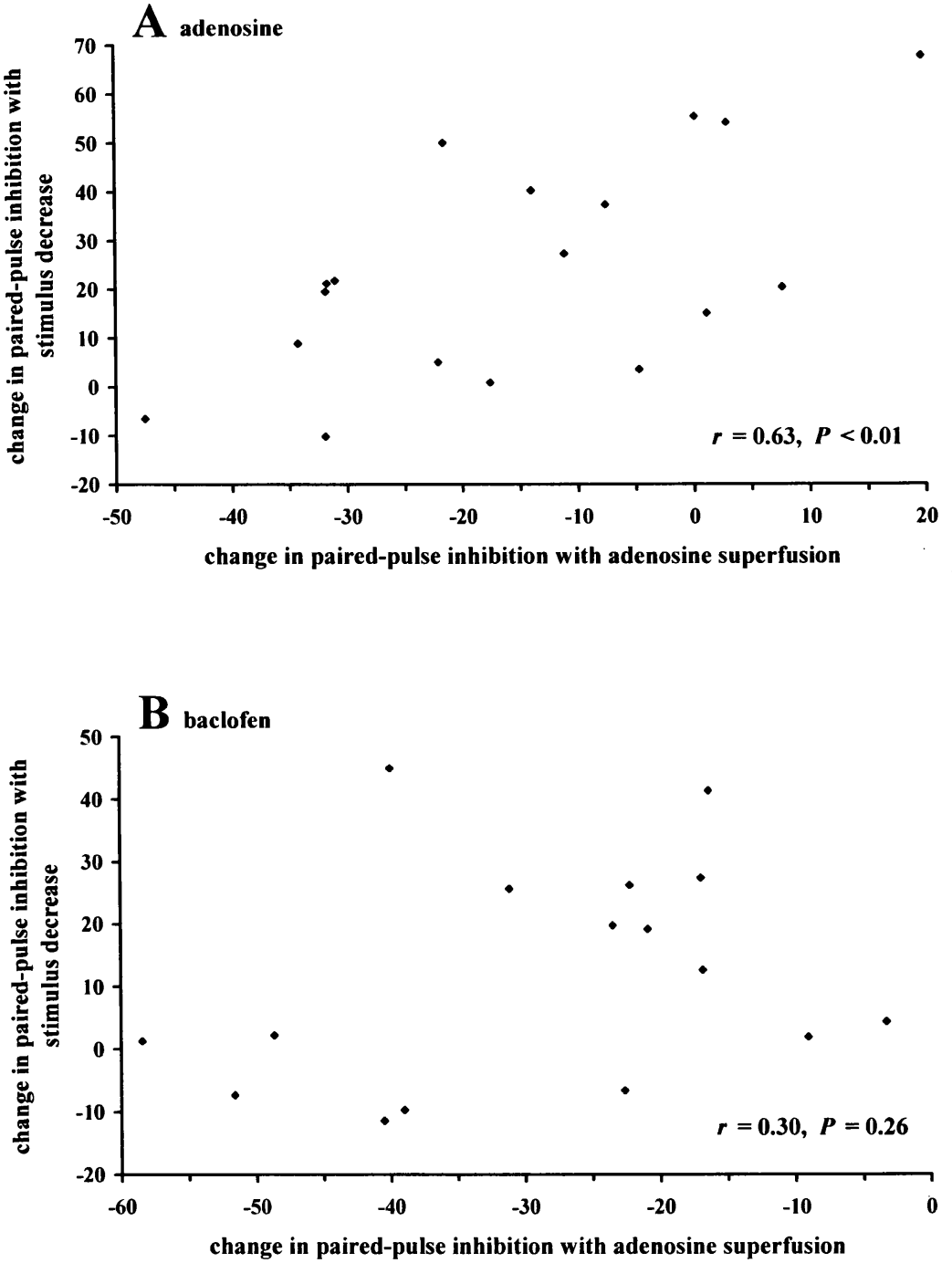

Figure 49 Relationship between change in bicuculline-enhanced paired-pulse inhibition which occurred on decreasing conditioning stimulus strength from supramaximal (1mA) and that which occurred with adenosine superfusion (**A**) or baclofen superfusion (**B**). The smaller conditioning stimulus in the stimulus change data set was such that R_{cond} was approximately 60% maximal. The conditioning stimulus in the adenosine / baclofen data set was kept at 1 mA. Each data point represents one slice. Data are taken from two groups of experiments (see pp. 59 and 81). Change in paired-pulse inhibition is given in paired-pulse inhibition units (i.e. percentage change in the amplitude of the original control potential).

Problem of the Residual Calcium Theory. Despite the attractiveness of these arguments, there is a potential difficulty with the idea that facilitation increases with the strength of the conditioning stimulus. The residual calcium theory of paired-pulse facilitation (p. 100) is widely accepted. This requires facilitation to be strictly homosynaptic i.e. to occur only when the same synapse is activated twice in close succession. On this basis, given that test and conditioning stimuli are delivered from the same electrode, facilitation of the field potential should increase as the conditioning stimulus increases from just sub-threshold until it is equal to the test stimulus. At this point all the synapses activated by the test stimulus are also activated by the conditioning stimulus. Increasing the strength of the conditioning stimulus beyond this point recruits stratum radiatum fibres and synapses that are not reactivated by the test stimulus and hence should cause no further increase in facilitation.

In fact, in many of the slices in which an effect of interchanging two different conditioning stimuli was demonstrated, the *smaller* conditioning stimulus was approximately equal to the control stimulus (see Figure 24A for example). Furthermore, in the baclofen and adenosine experiments which systematically examined the effect of varying conditioning stimulus strength, most of the decrease in paired-pulse inhibition tended to occur at higher conditioning stimulus strengths where the associated evoked potential was larger than $R_{control}$ (Figures 36A and 50). None of the 18 slices demonstrated a pattern where paired-pulse inhibition decreased with increasing conditioning stimulus strength and then levelled off at the point when R_{cond} was equal to $R_{control}$.

If the observed changes in paired-pulse inhibition are indeed caused by changes in facilitation then these observations are difficult to reconcile with the residual calcium theory.

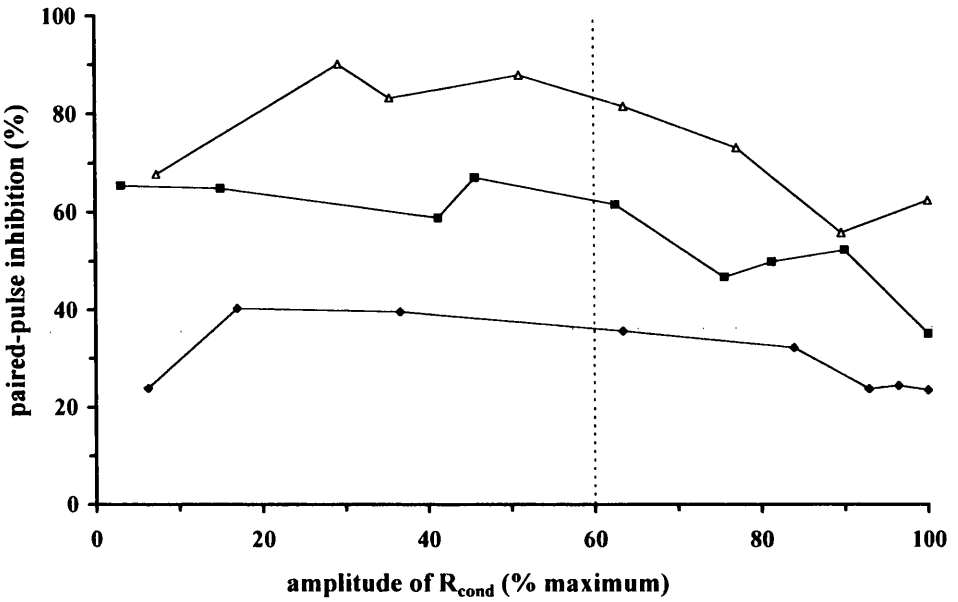

Figure 50 Effect of conditioning response amplitude on bicuculline-enhanced paired-pulse inhibition. Data from three individual slices. The inhibition corresponding to R_{cond} = 100% maximal was evoked by a 1 mA conditioning stimulus. Stimuli were orthodromic. The control response $R_{control}$ was 60% of maximum amplitude. These slices are included in the data represented in Figure 36. See Figure 36 for further details.

Possibility of Separating Conditioning and Test Stimuli. Some of the questions discussed above might be further explored using separate electrodes for the test and conditioning stimuli. In the normal slice in the absence of bicuculline adenosine did decrease short-latency paired-pulse inhibition (30 ms) when two separate electrodes, in the stratum radiatum, one on either side of the recording electrode, were used to deliver the test and conditioning stimuli (Figure 35). With this arrangement the contribution of homosynaptic facilitation is reduced. It was possible in some individual slices to demonstrate facilitation when both stimuli were delivered from the right hand ('test') electrode but

inhibition when the conditioning stimulus was delivered from the left hand electrode and the test stimulus from the right. Unfortunately, even when *overt* facilitation cannot be demonstrated there may be an unquantifiable degree of overlap between the populations of synapses stimulated by the two electrodes. This problem may be overcome by using an antidromic conditioning stimulus in the alveus although this may mean that a different set of interneurones is involved in mediating the inhibition.

It would be interesting to determine whether adenosine reduced bicuculline-enhanced paired-pulse inhibition when the conditioning stimulus was delivered antidromically from an electrode in the alveus. It would conversely also be interesting to determine whether baclofen, like other inhibitors of transmitter release increased paired-pulse facilitation (p. 100). It is even more difficult, however to design an experiment in which facilitation is uncontaminated by inhibition than vice versa. Wilcox and Dieter (1994) working with pairs of synaptically coupled hippocampal neurones in low density tissue culture have shown that baclofen, in common with lowered extracellular calcium, decreases paired pulse inhibition between pharmacologically isolated IPSPs. GABA-B antagonists had no effect on this paired-pulse inhibition indicating it was not an autoinhibition mediated by GABA released by the conditioning stimulus and suggesting the effect of baclofen was related to its background effect to reduce transmitter release. Kahl and Cotman (1993) found baclofen to decrease paired-pulse facilitation in the dentate although it was increased by adenosine and the glutamate-release inhibitor L-α-amino-4-phosphonobutyric acid, an effect they ascribed to a large component of facilitation there being mediated by paired-pulse depression of GABA-A inhibition (p. 100). Baclofen

apparently has less direct depressant effect on excitatory pathways in the dentate than in the CA1 and actually increases the amplitude of unpaired population spikes (Steffensen and Henriksen, 1991. Kahl and Cotman, 1993).

Summary

Adenosine was less effective at reducing bicuculline-enhanced paired-pulse inhibition when the conditioning stimulus was supramaximal at 1 mA. Control paired-pulse inhibition was less with a 1 mA conditioning stimulus than with a submaximal conditioning stimulus and there was a correlation between the effect of adenosine on paired-pulse inhibition in individual slices and the decrease in control paired-pulse inhibition which occurred when the conditioning stimulus was increased to 1 mA. A comparison of the concentration-response relationships for the effects of adenosine to reduce paired-pulse inhibition and to reduce the size of single evoked potentials ruled out the possibility that the effect of adenosine on paired-pulse inhibition was caused by the different sensitivity of two populations of adenosine receptors, those on excitatory terminals to interneurones and those on excitatory terminals to the pyramidal cells.

These observations are all compatible with the effect of adenosine to reduce bicuculline-enhanced paired-pulse inhibition being mediated by an increase in simultaneous paired-pulse facilitation. There is some difficulty however in integrating this explanation with the residual calcium theory of paired-pulse facilitation.

VII Investigations into the Role of Endogenous Adenosine in Mediating Orthodromic Bicuculline-Enhanced Paired-Pulse Inhibition and Short-Latency Paired-Pulse Inhibition in the Absence of Bicuculline

Although bicuculline-enhanced paired-pulse inhibition was reduced by 2-hydroxysaclofen it was not completely abolished by this agent in many slices (see Figure 27). This may have been because the 2-hydroxysaclofen was not used at a high enough concentration. At the time the experiments were carried out the cost of this agent precluded concentration-response studies with higher concentrations. The observation however leaves open the possibility other factors in addition to GABA-B activation were contributing to the paired-pulse inhibition.

The observation may be explained by effects consequent on disinhibited burst firing which are intrinsic to the postsynaptic membrane as suggested above (pp. 114 and 126). It is also possible that there is a contribution from other endogenous substances. The results of the early pilot experiments into the effect of the non-selective adenosine receptor antagonist 8PT in just three slices (Figure12) did not suggest that bicuculline-enhanced paired-pulse inhibition was substantially mediated by endogenous adenosine, but neither did they rule out a role for this substance.

This, together with the reported findings on release of endogenous adenosine by NMDA-receptor activation and by short bursts of repetitive stimuli (p. 21) suggested it was worth pursuing the question of whether adenosine contributed to paired-pulse inhibition in bicuculline-treated slices. To this end the effects were examined of the adenosine

A1-receptor antagonist CPT (Williams, 1991) and the enzyme adenosine deaminase on bicuculline-enhanced paired-pulse inhibition.

More speculatively, since Mitchell et al. (1993) were able to detect an adenosine effect in the normal (bicuculline-free) hippocampus after a single conditioning stimulus the effect of CPT was also examined on short-latency (30ms) homosynaptic paired-pulse inhibition demonstrable in the absence of added agents.

Effect of CPT and Adenosine Deaminase on Bicuculline-Enhanced Paired-Pulse Inhibition

Both CPT and adenosine deaminase proved to have a marked effect on paired-pulse inhibition at an interstimulus interval of 300 ms in the presence of bicuculline, producing a highly significant decrease (Figures 37 and 40). This effect was seen in most of the individual slices tested and occurred irrespective of whether the stimulus strength was adjusted to compensate for the effect of these agents on control potential size. Adenosine deaminase, the enzyme responsible for metabolising adenosine to inosine was used in order to exclude possible non-specific effects of CPT.

Taken at face value these results might suggest that, in the presence of bicuculline, adenosine is released as a result of the conditioning stimulus and the resulting short synchronised burst of pyramidal cell firing and that this adenosine contributes directly to the suppression of the subsequent test response. This interpretation is consistent with the results of Mitchell et al. (1993) (above). However, both adenosine deaminase and CPT induced spontaneous bursts in the bicuculline-treated slices. Wong and Traub (1983) showed spontaneous bursts in the disinhibited hippocampus to be initiated in the CA3 region and

transmitted along the stratum radiatum to the CA1. In the present work surgical isolation of the CA1 and CA3 regions eliminated spontaneous bursts of amplitude large enough to be detected by the slow-speed chart recorder. CPT had a smaller effect on paired-pulse inhibition in these cut slices suggesting that the presence of spontaneous bursts per se decreased paired-pulse inhibition and that some of the effect of CPT and adenosine deaminase to reduce paired-pulse inhibition in the uncut slices was related to the induction of spontaneous bursts.

Although it was reduced, the effect of CPT was still significant in these cut slices (Figure 38). It is possible that, although large synchronised bursts were abolished in the cut slices, non-synchronised spontaneous repetitive firing of individual neurones was still occurring during CPT superfusion and that this could mediate the remaining effect on paired-pulse inhibition. If undetected burst firing was occurring in the cut slices however, unlike the large synchronised bursts it did not interfere with the ability of CPT to increase the size of single evoked potentials.

The effect of spontaneous bursting on paired-pulse inhibition is itself an interesting observation. The mechanism is by no means clear but may be related to the fact that GABA-mediated inhibition in the normal hippocampus is known to fatigue at frequencies greater than 0.1Hz at least partly because of the activation of inhibitory GABA-B autoreceptors on inhibitory interneurones (Nathan and Lambert, 1991; Davies et al., 1990). As shown earlier baclofen decreases bicuculline-enhanced paired-pulse inhibition.

Lack of spontaneous bursting during superfusion with bicuculline alone. Spontaneous bursting in the presence of GABA-A antagonists alone has been reported by other investigators (Schwartzkroin and Prince, 1978;

Wong and Traub, 1983; Hablitz, 1984; Stanford et al., 1995). The lack of spontaneous bursting during superfusion with bicuculline alone in the present experiments may reflect the method of slice preparation with protective cooling of the anaesthetised rat prior to decapitation. Transient hypoxia is known to make slices more excitable partly, but not solely, because of a failure of GABAergic inhibition (Haas and Jefferys, 1984; Schiff and Somjen, 1985b; Khazipov et al., 1993). The relatively low working temperature of 30°C would also tend to be protective against ongoing chronic hypoxia in the slice interior. In addition the slice selection criteria may have introduced a bias to less excitable slices since only slices demonstrating large 'clean' single population spikes during initial ACSF perfusion were accepted for experiment. Slices showing any signs of the development of secondary potentials at this stage were rigorously rejected.

Finally the lack of spontaneous bursting possibly reflects a characteristic of the rats used to prepare the slices: in their study of low calcium-induced spontaneous bursting in hippocampal slices Haas and Jefferys (1984) note that "Sprague-Dawley rats from one supplier proved resistant to low Ca^{2+} field bursts".

Unpaired Potentials

CPT significantly increased size of *single* orthodromic population spiked evoked in the absence of bicuculline but had no significant effect when bicuculline was present. The ability of CPT, in the absence of other agents, to increase significantly the size of single orthodromic evoked potentials is consistent with several previous studies and probably reflects the presence of sufficient endogenous adenosine in the hippocampal slice preparation to exert a tonic restraint on transmitter

release (Dunwiddie, 1980). It is curious, however, that this effect is no longer clearly observed in the presence of bicuculline. It might be anticipated that the increased neuronal excitability engendered by the block of GABA receptors would increase the ambient level of adenosine and thus invite a greater relative increase of potential size by CPT than in drug-naive slices. For the same reason it is unexpected that adenosine deaminase had no significant overall effect on the size of unpaired potentials during bicuculline superfusion.

The lack of effect may be related to the production of spontaneous bursts in bicuculline-treated slices during superfusion with CPT and adenosine deaminase. Surgical isolation of the CA1 and CA3 regions restored the ability of CPT to increase evoked potential size.

Effect of CPT on Short Latency Paired-Pulse Inhibition in the Absence of Bicuculline

CPT also reduced paired-pulse inhibition at an interpulse interval of 30 ms in drug naive slices. The effect was not seen in all slices but 11 out of the 20 slices tested showed an obvious marked decrease in paired-pulse inhibition with perfusion of CPT (first 11 slices in Table 3) and the effect was highly significant over the group of 20 slices (Figure 32). In each of the eleven slices the effect of CPT on paired-pulse inhibition was substantially reversible on washing in ACSF for 20 minutes.

This marked effect of CPT on paired-pulse inhibition in drug-naive slices was an unexpected finding, particularly in view of the short-latency (30ms) between conditioning and test response. This interval seems unlikely, but not impossible, for the generation of a response mediated by a G protein coupled receptor. Based on data from the frog retina (Vuong et al., 1984), Otis et al. (1992) calculated a necessary

delay of 6 to 8 ms for G protein activation before the start of the GABA-B mediated increase in potassium conductance in dentate granule cells. The time constant of the increase was then 45 ms. Andreason and Lambert (1991) have described a component of the fast IPSP in CA1 pyramidal cells which is abolished by-adrenoceptor blockers in such a fashion as to suggest direct β_1-adrenergic activation of a subset of inhibitory interneurones, with a the time course similar to glutamatergic transmission. β_1-receptors are normally G protein-linked to adenylate cyclase.

Although Mitchell et al. (1993) demonstrated an apparently adenosine-mediated depression of field EPSPs in the CA1 after a single conditioning stimulus the effect of a single compared with multiple conditioning stimuli was relatively small (about 6% inhibition) and occurred with a latency of greater than 50ms, with a maximal effect at about 250ms.

In the present work it is unlikely that the effect of CPT on short-latency paired-pulse inhibition was a direct consequence of its facilitatory effect on single population spikes since, like the effect on bicuculline-enhanced paired-pulse inhibition, it could be demonstrated both before and after compensatory adjustment of stimulus strength in CPT. Furthermore, there was no significant correlation between the effect of CPT on PS size and on paired-pulse inhibition ($r = 0.297$, $n = 20$, not significant) with the non-significant trend in the wrong direction to favour this explanation. It is, further, unlikely that the effect of CPT on paired-pulse inhibition in bicuculline-free slices is mediated by a non-specific increase in neuronal excitability since although CPT caused a small increase in the size of single evoked population spikes, unlike bicuculline it did not cause repetitive firing of pyramidal cells as

evinced by the development of secondary potentials. This does not remove the possibility that the CPT-mediated reduction in background adenosine tone may have other effects, not directly reflected by changes in PS size, which may account for the decrease in paired-pulse inhibition.

Exogenous adenosine, though, itself decreases short-latency paired-pulse inhibition in the absence of other drugs (Dunwiddie and Haas, 1985) and at 300 ms in the presence of bicuculline (this work). The effect of CPT on paired-pulse inhibition was thus the reverse of that which would be expected from decreasing tonic adenosine receptor activity. In a manner directly analogous to the effects of the GABA-B agonist baclofen and the GABA-B antagonist 2-hydroxysaclofen these findings could be reconciled if P1-receptor activation did contribute directly to paired-pulse inhibition since the effects of antagonising background adenosine tone could be set against the effects of antagonising the acute increase in adenosine (or other P1 agonist) caused by the conditioning stimulus.

Since no other adenosine antagonist was tested it was not possible to exclude a non-purine-related effect of CPT, although CPT was used at a minimally effective concentration to minimise any non-specific effects. Pilot experiments showed 100 nM CPT to be sufficient to reduce by about 70% the effect of 20 mM adenosine on single evoked potentials. 20 mM adenosine itself produced an approximately 60% decrease in the size of single evoked potentials in ACSF, a comparable decrease in potential size to that observed as a result of paired-pulse inhibition.

The observation, then, is intriguing and on consideration remains consistent with the possibility that release of adenosine or another P1-agonist may have contributed directly to short-latency (30 ms) paired-

pulse inhibition in more than half of the hippocampal slices tested. Clearly further work would be required to substantiate or refute the suggestion; it would be interesting initially to examine the effect of adenosine deaminase.

Speculations on Synaptic Plasticity

The effects of CPT on single evoked potentials and paired-pulse inhibition in drug naive-slices in ACSF were readily reversible. In contrast, in the presence of bicuculline, the suppression of paired-pulse inhibition by CPT was only partly, though still significantly reversible. It is possible that this represents a problem of washout of the xanthine, but unlikely in view of the ready reversibility in normal slices and the relatively low concentration at which it was superfused. The effect of adenosine deaminase on paired-pulse inhibition was also only partly reversible. It is also possible that the observation simply reflects a time-dependant decrease in paired-pulse inhibition in some slices (compare Figures 23, 24, 26, and 27 for the reversibility of the effects of other agents on bicuculline-enhanced paired-pulse inhibition).

On the other hand given the increasingly-evident role of plasticity in hippocampal transmission, the observation may reflect a role for adenosine in the long-term control of synaptic transmission such that in the absence of GABA-mediate inhibition, a blockade of adenosine receptors yields a long-term depression of neuronal inhibition. This could be a non-specific effect of the increased transmitter release consequent on blocking adenosine receptors or reflect a specific interaction between GABA and adenosine receptors. Akhondzadeh and Stone (1994) have reported a mutual potentiation between these agonists on pyramidal neurones. Manzoni et al. (1994) noted that the effect of

CPT to block synaptic depression induced by a heterosynaptic tetany in the presence of picrotoxin was only partially reversible after an hour of washing whereas a similar effect of the NMDA antagonist APV reversed fully within 20 minutes.

The observation that CPT under some circumstances could induce a long-term decrease of paired-pulse inhibition would also help to account for the long-lasting development of spontaneous epileptiform bursting reported by Alzheimer et al. (1989, 1993) following their use of the less hydrophilic adenosine receptor antagonist 1,3,-dipropyl-8-cyclopentylxanthine. Alzheimer et al. (1993) also noted a similar apparently irreversible effect in a small number of slices exposed transiently to adenosine deaminase. In the same context, De Mendonça and Ribeiro, (1994) have shown that long term potentiation of the Schaffer collateral / CA1 pyramidal cell synapses is increased in the presence of adenosine antagonists.

These discussions are purely speculative and further investigation would require the use of careful controls giving consideration to slice selection, slice handling, stimulation parameters, and slice preparation-experiment time.

Summary

Bicuculline-enhanced paired-pulse inhibition was reduced by CPT and adenosine deaminase. Some of this reduction was related to the occurrence of spontaneous burst potentials during superfusion of these agents and bicuculline together, but the results remain consistent with the hypothesis that the release of endogenous adenosine by the conditioning stimulus contributed to the genesis of bicuculline-enhanced paired-pulse inhibition at an interstimulus interval of 300 ms. This is

consistent with published findings by other investigators. An alternative explanation, that paired-pulse inhibition is decreased by these agents as a result of a non-specific increase in neuronal excitability could not be ruled out.

In addition, surprisingly, CPT reduced short-latency (30 ms) paired-pulse inhibition in bicuculline-free slices. Again, this is consistent with this form of paired-pulse inhibition being partly mediated by a P1-receptor agonist although this seems intrinsically unlikely. Alternatively, a single as yet unexplained mechanism may underlie both the effect of CPT on short latency paired-pulse inhibition in bicuculline-free slices and CPT and adenosine deaminase on bicuculline-enhanced paired-pulse inhibition.

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APPENDIX

LISTING OF PROGRAM FOR SIMULATION OF PYRAMIDAL CELL ACTIVATION

The program is written in Turbo Basic, a compiled form of Basic. This is a word-processed version of the compiled program used for the simulations presented in the main text. Comments are italicised to improve clarity.

```
                                        'Introductory screen 1
CLS
PRINT "-This is a simple model of pyramidal cell activation by fibres in"
PRINT "  the stratum radiatum."
PRINT ""
PRINT "-There are 100 pyramidal cells and 50 stratum radiatum fibres."
PRINT ""
PRINT "-Each fibre contacts a random 40% of the pyramidal cells."
PRINT ""
PRINT "-You can stimulate as many of the fibres as you choose. "
PRINT ""
PRINT "-Each fibre when stimulated activates one synapse on each pyramidal cell"
PRINT "  it contacts. The default value of this input is set to one unit,"
PRINT "  but this value can be continuously varied to simulate presynaptic"
PRINT "  inhibition and facilitation."
PRINT ""
PRINT ""
PRINT "Press any key to continue"
WHILE NOT INSTAT : WEND
                                        'Introductory screen 2
CLS
PRINT "-You may set presynaptic inhibition as two separate factors which
PRINT "  multiply together; eg to simulate effects of two agents."
PRINT ""
PRINT "-Each pyramidal cell has a threshold for activation randomly set between"
PRINT "  9 and 11 units. A constant value can be added to this threshold to
PRINT "  simulate postsynaptic inhibition."
PRINT ""
PRINT "-If you choose 'new slice' connections and thresholds are re-randomised."
PRINT ""
PRINT ""
nul$ = INKEY$
PRINT "Press any key to continue"
WHILE NOT INSTAT : WEND
                                        'Get location for results file
CLS
nul$ = INKEY$
e$ = ""
DO UNTIL UCASE$(e$) = "Y" OR UCASE$(e$) = "N"
   LOCATE 20,1
   PRINT "Results to file Y or N?";
```

```
   e$ = INKEY$
LOOP
IF UCASE$ (e$) = "Y" THEN
 nul$ = INKEY$
 e$ = ""
 file = 1
 DO UNTIL UCASE$(e$) = "Y"
  CLS
  LOCATE 2,1
  PRINT "Give full name (including path) of file for results"
  PRINT "e.g. c:\pyramid.dat"
  PRINT ""
  INPUT, results$
  nul$ = INKEY$
  e$ = ""
  DO UNTIL UCASE$(e$) = "Y" OR UCASE$(e$) = "N"
   LOCATE 22,1
   PRINT "Happy Y or N?";
   e$ = INKEY$
  LOOP
 LOOP
 CLS
 e$ = ""
 nul$ = INKEY$
ELSE
 nul$ = INKEY$
 e$ = ""
END IF
file = 0
CLS
                                        'define and set variables
DEFDBL A-Z
synapse = 1                             'default value of activated synapse
facil = 0                               'presynaptic facilitatory factor
inhib1 = 0                              'presynaptic inhibitory factors
inhib2 = 0
threshold = 10                          'default pyramidal cell threshold
postvary = 0                            'postsynaptic inhibitory factor
stimsize = 1                            'number of fibres activated
response = 0                            'number of pyramidal cells activated
oldresponse = 0                         'indicator
count = 1                               'indicator
CLS
LOCATE 20,1
PRINT "Calculating connections and thresholds"
                                        'set up slice
                                        'set up array to store connections
DIM connect(1:50,1:100)                 '50 fibres x 100 cells
FOR f = 1 to 50
```

```
 FOR c = 1 to 100
  rany = int(rnd*10)                    'get random number in range 0 to 9
  IF rany >3 THEN indivcon = 0
  IF rany <4 THEN indivcon = 1          'each fibre has 0.4 probability of
  connect(f,c) = indivcon               'connecting with a given cell
 NEXT c
NEXT f
                                        'set up array to store pyramidal
                                        cell thresholds
DIM thresholds(1:100)
FOR c = 1 to 100
 rany = int(rnd*201)                    'set each threshold randomly between
 rany = rany/100                        '9 and 11 in steps of 0.01
 thresholds(c) = 9 + rany
NEXT c
again = 1                               'indicator
DO UNTIL again = 0
 NUL$ = INKEY$
 e$ = ""
 DO UNTIL UCASE$(e$) = "Y"
  CLS                                   'get data for individual simulation
  LOCATE 1,1
  PRINT "enter stimulation size (must be a whole number between 1 and 50)"
  INPUT, stimsize
  PRINT "enter first factor (%) for PRESYNAPTIC inhibition"
  PRINT "-must be a whole number between 1 and 100"
  PRINT "-eg entering 20 will reduce synaptic strength by 20%"
  PRINT "-zero or RTN will leave synaptic strength at default"
  INPUT, inhib1
  PRINT "enter second factor (%) for PRESYNAPTIC inhibition"
  PRINT "-will multiply with first factor"
  PRINT "-zero or RTN will first factor in operation"
  INPUT, inhib2
  PRINT "enter factor (%) for PRESYNAPTIC FACILITATION"
  PRINT "-must be a whole number between 1 and 100"
  INPUT, facil
  PRINT "enter value for POSTSYNAPTIC inhibition"
  PRINT "normal threshold is 10 synaptic units"
  PRINT "postsynaptic inhibition will add directly to theshold"
  PRINT "eg factor of 2 will change threshold to 12"
  INPUT, postvary
  e$ = ""
  DO UNTIL UCASE$(e$) = "Y" OR UCASE$(e$) = "N"
   LOCATE 24,1
   PRINT "Happy Y or N?";
   e$ = INKEY$
  LOOP
 LOOP
```

```
                                        'recalculate  value of activated synapse after
                                        'presynaptic inhibition and facilitation
 synapse = synapse - ((inhib1/100)*synapse) + ((facil/100)*synapse)
 synapse = synapse - ((inhib2/100)*synapse)
 CLS
 LOCATE 22,1
 PRINT "Calculating active cells"
 DIM cells(1:100)                       'calculate input to individual
 FOR c = 1 to 100                       'cells and store in array
  FOR f = 1 to stimsize
   cells(c) = cells(c) + (connect(f,c) * synapse)
  NEXT f
 NEXT c

                                        'calculate no. of cells
 FOR  x = 1 to 100                      'over threshold
  IF cells(x) = (thresholds(x)+postvary) OR cells(x) > (thresholds(x)+postvary)
THEN  response = response + 1
 NEXT x
                                        'results to screen
 CLS
 LOCATE 10,1
 PRINT "Stimulation size is (maximum is 50) ";stimsize
 PRINT ""
 PRINT "Synaptic factor is (normal is 1) ";synapse
 PRINT "First presynaptic inhibitory factor ";inhib1
 PRINT "Second presynaptic inhibitory factor ";inhib2
 PRINT "Postsynaptic inhibition is (normal threshold is about 10 units) ";postvary;"
units"
 PRINT ""
 PRINT "Response size is ";response;"% maximal"
 PRINT ""
 IF oldresponse > 0  THEN
  PRINT USING "This response = ###% of last
response";(response/oldresponse)*100
  PRINT USING "This response is ###% inhibited compared to last response";100 -
(response/oldresponse)*100
 END IF
 PRINT ""
                                        'results to file
 IF file = 1 THEN
  OPEN results$ FOR APPEND AS #1
  PRINT #1, "Stimulation size is (maximum is 50) ";stimsize
  PRINT #1, ""
  PRINT #1, "First presynaptic inhibitory factor ";inhib1
  PRINT #1, "Second presynaptic inhibitory factor ";inhib2
  PRINT #1, "Synaptic factor is (normal is 1) ";synapse
  PRINT #1, "Postsynaptic inhibition is (normal threshold is about 10 units)
";postvary;" units"
```

```
   PRINT #1, "Cell coverage per fibre = 40%"
   PRINT #1, ""
   PRINT #1, "Response size is ";response;"% maximal"
   PRINT #1, ""
   IF oldresponse > 0  THEN
     PRINT #1, USING "This response = ###% of last
response";(response/oldresponse)*100
     PRINT #1, USING "This response is ###% inhibited compared to last
response";100 - (response/oldresponse)*100
   END IF
   PRINT #1, ""
   PRINT #1, ""
   CLOSE #1
  END IF

  e$ = ""
  LOCATE 23,30
  PRINT "Press any key to continue";
  WHILE NOT INSTAT : WEND
  nul$ = INKEY$
  CLS
                                          'loop program for repeat simulation
  e$ = ""
  DO UNTIL UCASE$(e$) = "Y" OR UCASE$(e$) = "N"
    LOCATE 20,1
    PRINT "another stimulation Y or N?";
    e$ = INKEY$
  LOOP
  IF UCASE$(e$) = "N" THEN
    CLS
    END
  ELSEIF UCASE$(e$) = "Y" THEN
    e$ = ""
    CLS
    DO UNTIL UCASE$(e$) = "Y" OR UCASE$(e$) = "N"
      LOCATE 21,1
      PRINT "new slice Y or N?";
      e$ = INKEY$
    LOOP                                  'optional rerandomise connections
                                          'and thresholds
    IF UCASE$(e$) = "Y" THEN CALL model
    e$ = ""                               'reset variables and counters
    again = 1
    ERASE cells
    synapse = 1
    facil = 0
    inhib= 0
    threshold = 10
    postvary = 0
```

```
   stimsize = 1
   oldresponse = response
   response = 0
  END IF
LOOP

SUB model                                   'rerandomises connections and
SHARED connect(), thresholds()              'thresholds for repeat simulations
  ERASE connect
  ERASE thresholds
  RANDOMIZE TIMER
  CLS
  LOCATE 20,1
  PRINT "Calculating connections and thresholds"
  DIM connect(1:50,1:100)                   '50 fibres x 100 cells
  FOR f = 1 to 50
    FOR c = 1 to 100
      rany = int(rnd*10)
      IF rany >3 THEN indivcon = 0
      IF rany <4 THEN indivcon = 1
      connect(f,c) = indivcon
    NEXT c
  NEXT f
  DIM thresholds(1:100)
  FOR c = 1 to 100
    rany = int(rnd*201)
    rany = rany/100
    thresholds(c) = 9 + rany
  NEXT c
END SUB
```