Adenosinergic Modulation of Glutamate Release in the Rat Hippocampus

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Contents

Abstract

Presynaptic adenosine A_1 receptors at synapses between Schaffer collateral commissural (SCC) fibres and hippocampal CA1 neurones, were studied using the whole cell blind patch clamp technique and determining the effects of an adenosine agonist and antagonist. The selective adenosine A_1 receptor agonist 2-chloro-N^6-cyclopenyladenosine (CCPA) and the antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) had no effect on the apparent input resistance (R_m) and membrane potential (E_m) of the postsynaptic cell and thus unlikely to alter the magnitude of the synaptic response postsynaptically. Two approaches were employed to examine the role of the adenosine A_1 receptor in the regulation of glutamate release. Firstly spontaneous miniature EPSPs (mEPSPs) were recorded and the actions of either CCPA or DPCPX were studied. Neither drug altered the mean amplitude of the mEPSPs compared to control. CCPA decreased the frequency of the mEPSPs from control, an inhibition which was completely reversed by co-application of DPCPX. DPCPX alone increased the mean frequency of the mEPSPs from control. This suggested that the CA1 neurones are subject to a tonic inhibition by endogenous adenosine. Secondly, single and pairs of evoked responses were measured during the activation of the SCC fibres using low intensity stimulation before and after exposure to either CCPA or DPCPX. After exposure to DPCPX the amplitude distributions of the singly evoked EPSPs and EPSCs were significantly shifted to the right compared to control. This was accompanied by an increase in the mean amplitude. In paired pulse experiments, carried out under current and voltage clamp, exposure to CCPA produced a leftwards shift in the amplitude distributions of both the EPSPs and EPSCs and significantly decreased the mean amplitude of both the first and second responses. After exposure to DPCPX the mean amplitude of the EPSPs and EPSCs increased, but not significantly, and their distribution was shifted to the right. A further examination of the actions of these compounds was carried out by measuring their effect on the quantal parameters m_f, m_{cv} (quantal content determined by the method of failures and the coefficient of variation respectively) and q_{cv} (quantal amplitude). When the paired pulse experiments were further examined; it was found

that paired-pulse facilitation (PPF) and paired-pulse depression (PPD) were both observed in individual neurones. No correlation between first response amplitude and whether the second response would show PPF or PPD was found. Exposure to either CCPA or DPCPX did not result in a change in the proportion of trials that showed PPD. An increase in the paired-pulse ratio was observed after exposure to CCPA and a decrease after exposure to DPCPX.

From these experiments we can conclude that these adenosinergic compounds presynaptically alter the synaptic strength of SCC synapses in the hippocampus by, presumably, changing the probability of release of individual quanta. The lack of correlation between first response amplitude and the paired-pulse ratio is in keeping with recent studies which showed considerable heterogeneity in the release parameters at glutamate mediated release sites at this location in the brain using more sophisticated statistical techniques.

Declaration

In accordance with the regulations of the University of Edinburgh I declare that this dissertation in its entirety is not substantially the same as any that the author has submitted for a degree or diploma or other qualification at any other university. The work is solely that of the author, except the few experiments indicated. These results were most generously allowed to be included by my supervisor Dr J.P. Hodgkiss.

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Publications

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Chapter 1

Introduction

Adenosine plays a crucial role in cellular energy metabolism and at the same time is recognized as an intercellular messenger that produces specific receptor mediated biochemical effects, which result in physiological responses. Adenosine acts on cells that are near the site of its formation as it is rapidly taken up and phosphorylated by adenosine kinase or deaminated by adenosine deaminase.

Adenosine as a Neuromodulator

In 1929, Drury and Szent-Györgyi reported that adenosine had cardiovascular effects. However the importance of adenosine in cellular communication in the nervous system has only been appreciated since the early 1970s from investigations of the effects of various neurohumoural substances on cyclic adenosine monophosphate (cAMP) metabolism in the brain. The concept of adenosine as a neuromodulator evolved from the initial observations of Sattin & Rall (1970), who demonstrated that adenosine and adenosine triphosphate (ATP) increased cAMP concentrations in brain slices, and that this effect was competitively antagonised by methylxanthines such as caffeine and theophylline. Based on these results they proposed that adenosine might interact with specific receptors to produce these effects. Although this work suggested that adenosine might be neuroactive, it did not indicate what type of physiological events might be initiated by the activation of adenosine receptors. Subsequent studies by Ginsbourg & Hirst (1972) established a functional role for adenosine by demonstrating that it could inhibit acetylcholine release at the neuromuscular junction. In addition a number of groups (cf; review e.g. Phillis & Wu, 1981) demonstrated that adenosine had a profound inhibitory effect on the spontaneous activity of neurones in different brain regions, an action that could be attributed, in part, to the inhibition of spontaneous excitatory synaptic inputs. It is now known that adenosine has widespread effects in several organ systems including negative chronotropic and inotropic effects, vasodilatation in almost all vascular beds, suggesting that adenosine plays a role in the local regulation of blood flow, regulation of renal renin release, inhibition of platelet aggregation and modulation of lymphocyte function. In the nervous system adenosine inhibits neurotransmitter release (cf; review e.g. Stone, 1981), suppresses spontaneous neuronal firing,

produces sedation and displays anticonvulsant activity (Harms et al. 1979; Williams, 1987; Fredholm & Dunwiddie, 1988).

Endogenous adenosine

Resting levels of extracellular adenosine in brain tissue have been estimated to be in the range 0.03 - 2μM (Zetterstrom et al., 1982; Fredholm et al., 1984; Fredholm, 1995). This level of adenosine can rise 100 fold following stimuli that cause an imbalance between adenosine triphosphate (ATP) synthesis and ATP breakdown such as ischaemia. Although adenosine does not have a large effect at these basal levels, the addition of adenosine receptor antagonists (Mitchell et al., 1993) or adenosine deaminase (Zhu & Krnjevic, 1994) elicits responses in the absence of exogenous adenosine. These results suggest that low micromolar resting levels of adenosine are adequate to activate adenosine receptors.

The intracellular concentration of adenosine is thought to be much lower than the extracellular concentration. Su et al. (1971) demonstrated that tritiated adenosine was taken up by preparations of stomach and intestine, and most of the label was found to be stored as [^{3}H]ATP in nerves. Adenosine is taken up into cells by a high affinity system with a K_m of about 3μM, and by a lower affinity system with a K_m of about 250μM (Dowdall, 1978; Hertz, 1978). Once inside cells adenosine is rapidly phosphorylated by adenosine kinase and converted into nucleotides, mainly adenosine 5'-monophosphate (AMP), adenosine 5'-triphosphate (ATP) (McIlwain, 1972) and cyclic AMP (cAMP).

A variety of factors can elicit up to a 250-fold increase in extracellular adenosine levels. Electrical stimulation of brain slice preparations has been shown to increase extracellular adenosine concentrations (McIlwain, 1972; Pull & McIlwain, 1973). Adenosine release in these experiments was determined either through overflow of radiolabeled purines, or by measuring increases in cAMP content attributable to activation of adenylate cyclase by adenosine. Release of adenosine has also been demonstrated by electrical stimulation of prelabeled synaptosomal preparations (Daval & Barberis, 1981).

Further information regarding the nature and site of adenosine has been obtained using focal stimulation techniques. [^{3}H] adenosine was injected into the entorhinal cortex (Schubert et al., 1976; Lee et al., 1981). The release of purines was enhanced both *in vivo* as well as *in vitro* by stimulation of the perforant path fibres from the entorhinal cortex. The release was not enhanced by antidromic activation of the postsynaptic neurones, indicating that the activation of the afferent nerve terminals, rather than the target neurones was essential.

However, whilst it is clear that synaptic activation is an essential element in the release process, it is not clear whether release is from the presynaptic elements, or from postsynaptic neurones activated by excitatory amino-acids; either alternative would be compatible with the evidence that is currently available (Dunwiddie, 1985). In the autonomic nervous system ATP is used as a transmitter or co-transmitter by sympathetic and parasympathetic nerves, and also by non-adrenergic, non-cholinergic neurones; e.g. the myenteric plexus of the gut. There is evidence that ATP acts as a transmitter within the central nervous system. It has been demonstrated that ATP is released from synaptosomal preparations of discrete areas of the rat brain (Barberis & McIlwain, 1976). ATP has been shown to cause excitation of neurones in the cuneate nucleus, when applied focally by iontophoresis from a microelectrode (Galindo et al., 1967). ATP also causes excitation of rat cerebellar Purkinje cells (Hoffer et al., 1971) and of cells in the sensory vestibular and trigeminal nuclei (Krishtal et al., 1983; Salt & Hill, 1983). The response to ATP in the trigeminal nucleus is not always excitatory, but is often biphasic with an initial transient excitation being followed by a period of depression (Salt & Hill, 1983). In CA1 neurones, ATP appears to have an inhibitory action, as does AMP (Di Cori & Henry, 1984). This is likely to be due to activation of adenosine receptors following its rapid degradation to adenosine by ectonucleotidases (Phillis et al., 1979).

Adenosine Formation

Adenosine can be formed by degradation of AMP and ATP by 5'-Nucleotidase enzymes (Schulz & Lowenstein, 1978). These enzymes exist in two different cytosolic forms and as a membrane bound ecto-enzyme. Adenosine

production may also occur by hydrolysis of 5-adenosylhomocysteine (SAH). SAH is formed from 5-adenosylmethionine (SAM) in the course of the transmethylation pathway by the action of SAH-hydrolase. This enzyme has been immunocytochemically localized in the neocortex, hippocampal formation, cerebellum and olfactory tubercle of rat brain (Patel & Tudball, 1986). However the involvement of SAH-hydrolase in adenosine formation has not been tested in brain.

Braas et al. (1986, 1987) used specific antisera against adenosine and the immunoreactivity was localized in neuronal cells of the brain and retina of various species. This suggested that adenosine may be a neurotransmitter, rather than a neuromodulator. It was not clear, however, if the adenosine was stored in vesicles or which stimuli were able to release it. Hypoxia increased the intensity of staining although it did not alter the pattern of staining.

Adenosine Inactivation

Adenosine must be taken up by neurones or neighbouring cells before it is inactivated either by phosphorylation by adenosine kinase or deaminated by adenosine deaminase (ADA), as localization of both these enzymes is usually cytosolic. The importance of adenosine uptake in its inactivation is emphasized by observations that potent nucleoside transport inhibitors produce vasodilatation, potentiate the ability of adenosine to decrease locomotor activity (Crawley et al., 1983; Sanderson & Schofield, 1986), depress neuronal activity (Motley & Collins, 1983) and exerts anticonvulsant effects (Dragunow & Goddard, 1984).

Adenosine is thought to be taken up into cells by a facilitated diffusion, transport process and thus largely regulated by the concentration gradient for adenosine (cf; review e.g. Thorn & Jarvis, 1996). Once taken up, adenosine may be phosphorylated by adenosine kinase or deaminated by ADA. In most preparations the values of K_m for adenosine kinase for adenosine are between one and two orders of magnitude lower than those for the deaminase (Arch & Newsholme, 1978).

Adenosine Receptors

Different classification systems for the adenosine receptor family emerged from the various groups involved in this field of research. The broadest classification is that proposed by Burnstock (1978) which differentiates between the actions of adenine nucleotides and adenosine. The purinergic receptors are divided into those preferring adenosine, referred to as P_1, and those preferring ATP, known as P_2. The basis of the further subclassification of the adenosine receptors is provided by the twofold action of adenosine and its derivatives on adenylate cyclase. Londos & Wolff (1977) demonstrated both stimulatory and inhibitory effects of adenosine on adenylate cyclase. Screening of various adenosine derivatives for their relative potencies as stimulators or inhibitors showed that an intact ribose ring was required for stimulation of adenylate cyclase and it was tolerant to modifications of the purine moiety. In contrast, inhibition of adenylate cyclase required an unmodified purine ring. This led to the designation of two sites known as the R site (the stimulatory site) and the P site (which mediates the inhibitory effect). In contrast to the R site, which represents a G protein-related receptor class, the P site appears to be located on the catalytic subunit of adenylate cyclase at an intracellular site. Londos et al. (1980) described the two receptor subtypes in fat cells and referred to the two receptors as R_i (inhibitory) and R_a (stimulatory) on the basis of their R site nature. Van Calker et al. (1979) described the two receptor subtypes in cultured astrocytes from mouse brain, and proposed the designation A_1 and A_2 for the inhibitory and stimulatory receptors respectively. The A_1/A_2 classification has now become the more common convention since it is free of any direct implication of an involvement of cAMP as the second messenger.

At least four subtypes of adenosine receptors are recognized by structural and pharmacological criteria (Fredholm et al., 1994; Fredholm, 1995). All subtypes of adenosine receptors belong to the family of rhodopsin-like G protein-coupled receptors. The A_1 receptor couples to members of the pertussis toxin-sensitive G protein family (G_{i1-3}, G_o). These G proteins can cause the inhibition of adenylate cyclase (via both the α- and the $\beta\gamma$-subunits) and can cause the activation of several types of K^+ channels and the inactivation of some types of voltage-dependent Ca^{2+}

channels (Greene & Haas, 1991). They also cause the activation of phospholipase C (via the βγ-subunits of the G protein) with subsequent activation of protein kinase C and increases in intracellular Ca^{2+} (Fredholm et al., 1994). There are also effects on phospholipase D, Cl^- channels, and gene expression (Fredholm, 1995), which may be secondary to the above mentioned actions. The A_1 receptors are widely distributed in the central nervous system.

The two adenosine A_2 receptor subtypes are both coupled via G_s proteins to increases in cAMP. The possibility exists that other G proteins, such as G_{olf}, may also be activated by A_2 subtypes and that other effector systems may sometimes be involved. It is not known if the two forms of A_2 receptors differ in pharmacology and distribution. The A_{2b} receptor is ubiquitous, whereas the A_{2a} receptor is mainly localized to the dopamine-rich regions of the brain. Lower concentrations of adenosine are required to activate A_{2a} than A_{2b} receptors. This suggests that basal levels can act on A_{2a} receptors, whereas A_{2b} receptors are mainly activated when adenosine reaches supraphysiological levels.

More recently adenosine A_3 receptors were discovered by a cloning strategy (Zhou et al., 1992), and less is known about their pharmacology, distribution, G protein coupling, and functional relevance.

The ability of adenosine and its analogues to inhibit neurotransmitter release was first discovered in the peripheral nervous system. It is now known that the release of many central neurotransmitters is also regulated by adenosine receptors. There is considerable variation in the extent of this modulation. The release of excitatory transmitters appears to be more strongly affected than that of inhibitory transmitters. This fits with the concept that adenosine acts to limit its own formation and that adenosine formation is increased by neuronal activity.

It is thought that adenosine plays a role as a major inhibitory modulator of the electrophysiological activity of the brain, one that combines both pre- and postsynaptic actions in a concerted fashion to reduce the level of excitability in many brain regions (Greene & Haas, 1991).

Presynaptic Receptors

The reflex inhibition of muscle contraction was perhaps the first example of presynaptic inhibition. Liddell & Sherrington (1925) and Cooper & Creed (1927) showed that contraction of a muscle triggered inhibition of tonus in the antagonistic muscle. This was initially attributed to postsynaptic inhibitory mechanisms acting on the motor neurones in the spinal cord (Brock et al., 1952). Barron & Matthews (1938) found that dorsal root volleys gave rise to a depolarisation that spread electrotonically along the same or adjacent dorsal roots. They further speculated that this had an inhibitory action in the spinal cord as it gave rise to electric currents that caused blockage of conduction in the collateral branches of interneurones. Subsequently there was additional evidence that inhibitory action in the spinal cord is due to block or depression of presynaptic excitatory impulses. Particularly convincing evidence of presynaptic inhibition was reported by Frank & Fuortes (1957) and Frank (1959) who showed that muscle afferent volleys produce inhibition by diminishing the size of the monosynaptic EPSP of motoneurones without having any other demonstratable action on those motoneurones - postsynaptic inhibition could not account for all of the reflex inhibition. They distinguished between two types of inhibition contributing to reflex inhibition at Ia afferents to spinal motoneurones: presynaptic inhibition and postsynaptic inhibition.

Eccles (1961) recognized that the earlier experiments of Gasser & Graham (1933) and Hughes & Gasser (1934a, b) had important implications for the mechanism by which presynaptic inhibition occurred. They demonstrated that stimulation of the afferent nerves of muscle spindles led to a depolarising potential in electrical recordings obtained from the cord dorsum. They noted a temporal correlation between the duration of this phenomenon and the duration of inhibition of reflex muscle contraction. Numerous experiments conducted since have established that there is a strong correlation between the primary afferent depolarisation (PAD) and the inhibition of synaptic transmission from muscle spindal afferents to motor neurones.

It is believed that the inhibition of transmission from afferent terminals onto motor neurones was mediated by interneurones. The interneurones that are activated

by sensory inputs release an inhibitory transmitter that produces presynaptic inhibition of transmitter release from specific afferent terminals (Eccles et al., 1962). Eccles, Schmidt and Willis (1963) showed, using picrotoxin, that presynaptic inhibition had a pharmacology that was consistent with that of a $GABA_A$ receptor, and that picrotoxin was ineffective against postsynaptic inhibtion.

In 1961, Dudel and Kuffler described presynaptic inhibition at the crayfish claw opener neuromuscular junction, where an inhibitory nerve impulse acts on the excitatory nerve terminals and decreases the probability of release of a quanta of excitatory transmitter. They used failure counting and quantal analysis, originally described by Del Castillo & Katz (1954c) at the frog neuromuscular junction, to demonstrate that the inhibition produced by the inhibitory motor axon consisted of two components, presynaptic inhibition and postsynaptic inhibition, similar to that described in the spinal cord. It was clear that presynaptic inhibition was the largest contributor to the inhibition seen, and the quantum content was reduced to 25% of control whilst the size of the quanta remained the same. It was postulated that the terminal of the inhibitor motor axon releases GABA (Dudel & Kuffler, 1961; Takeuchi & Takeuchi, 1966). GABA acts on the postsynaptic muscle by binding to a $GABA_A$ receptor and promoting the opening of a Cl^--permeable ionophore that is an intrinsic component of the $GABA_A$ receptor. It also seemed likely that the same or a very similar receptor was activated on the presynaptic terminal of the excitor axon terminal. The activation of the $GABA_A$ receptor resulted in an increase in Cl^- conductance which serves to shunt local currents that are responsible for propagation of the action potential (Baxter & Bittner, 1991). Consequently the amplitude of the action potential in the terminal is attenuated, leading to a reduction in transmitter release. If activation of the $GABA_A$ ionophore were solely responsible for presynaptic inhibition, then it would be expected that an ionophore blocker, such as picrotoxin, would block presynaptic inhibition. However it was increasingly clear that picrotoxin does not block presynaptic inhibition completely (Eccles et al., 1963). An explanation for this arose from the work of Hill & Bowery (1981). They proposed that there were two types of GABA receptors, $GABA_A$ and $GABA_B$. The $GABA_B$ receptor was shown to be a G protein-coupled neurotransmitter receptor (Bowery,

1989). These results gave rise to the suggestion of two forms of presynaptic receptor mediating presynaptic inhibition; one mediated by the direct gating of a membrane channel and another that is mediated by a G protein-coupled mechanism. However there does not appear to be any anatomical evidence to show that $GABA_B$ receptors are present on the axons and terminals of the large diameter primary afferents.

Types of Presynaptic Receptor

Presynaptic receptors can be subdivided into two categories: homoreceptors (or autoreceptors) and heteroreceptors. The term presynaptic receptor appears to have first been used by Riker et al. (1957) to describe the site at which quaternary ammonium compounds acted at motor nerve terminals, although evidence for their existence had been shown earlier (Masland & Wigton, 1940, who cited the work of Langley & Kato, 1915 in support of their findings). Carlsson (1975) coined the term autoreceptor to designate a receptor on a neurone that is acted on by the transmitter released from that neurone. The hypothesis concerning autoregulation of release states that a neurotransmitter can act on the same terminal from which it was released to impose a modulation of the subsequent release of transmitter from that terminal, a form of self-regulation. This may permit a match between the neuronal response to excitatory input and the output of the effector, so that elevated levels of input do not result in saturation of the effector output. The control of transmitter release at a terminal by heteroreceptors allows the regulation of release of a transmitter by other transmitters, whose cell bodies of origin need not be nearby. This permits a degree of flexibility in the control of transmitter release, as the modulation of a given circuit need not depend on the activity of the circuit.

Mechanisms of Presynaptic Inhibition

There are three primary mechanisms that have received the greatest attention as potential mechanisms for G protein-coupled presynaptic inhibition in the mammalian central nervous system. These mechanisms are: activation of presynaptic K^+ channels, inhibition of presynaptic Ca^{2+} channels, and inhibition of some component of the release apparatus itself.

Identified neurones in the abdominal ganglion of the marine mollusc *Aplysia* have been shown to participate in presynaptic inhibition (Waziri et al., 1969). The neuronal circuit involved in this particular example was described by Byrne (1980). The neurone identified as L10 synapses onto a number of target cells and uses acetylcholine as a neurotransmitter. Stimulation of a small cluster of histaminergic neurones, called L32 cells, produces presynaptic inhibition of transmission from L10. Kretz et al. (1986a, b) reported that application of histamine to L10 mimics all of the responses induced by stimulation of the L32 cells, including activation of a K^+ current and inhibition of Ca^{2+} current. The available evidence suggests that modulation of both K^+ and Ca^{2+} channels contributes to presynaptic inhibition in this system.

The third mechanism contributing to presynaptic inhibition was proposed by Man-Son Hing et al. (1989), and was an extension of results obtained in a variety of nonneuronal secretory cells (Ullrich & Wollheim, 1988) and at the frog neuromuscular junction (Silinsky, 1981, 1984). The peptide FMRFamide was known to produce presynaptic inhibition between a pair of synaptically coupled neurones in *Helisoma*. In addition to activating K^+ channels and inhibiting Ca^{2+} channels, these workers found that FMRFamide could inhibit release in a manner that did not involve the modulation of any membrane channels. Presynaptic inhibition by FMRFamide occurred even when the presynaptic terminal was loaded with a "caged" calcium compound and flash photolysed. The calcium released stimulated transmitter release in a manner that did not depend on Ca^{2+} influx through Ca^{2+} channels. It is thought that this occurs through some biochemical change in the secretory mechanism to Ca^{2+}, as has been proposed to occur in some secretory cells (Ullrich & Wollheim, 1988). This form of presynaptic inhibition may involve receptors on the membrane surface that couple to small G-protein like proteins that directly act upon second-messenger systems, such as cAMP formation. The second-messenger then interacts with a component of the release apparatus to inhibit release.

Evidence of these mechanisms in the CNS

Potassium Channels

Many transmitter substances have been shown to activate a K^+ current in one or more CNS neurones (North, 1989; Nicoll et al., 1990). The neurotransmitter receptor is believed to be linked to the channel through a G protein It has been proposed that activation of such a K^+ channel near the terminal could serve to reduce the propagation amplitude or duration of the action potential as it enters the terminal region. This would reduce the activation of voltage-dependent Ca^{2+} channels that participate in transmitter release.

Studies with synaptosomal preparations have indicated that many transmitters can increase ion efflux through K^+ channels in isolated synaptosomes. However there have been no reports of physiological studies that show the direct linkage of the activation of a K^+ channel in the presynaptic terminal to the inhibition of transmitter release at veterbrate central synapses.

Calcium Channels

Dunlap & Fischbach (1978) reported that noradrenaline and GABA reduced the calcium current in the cell body of sympathetic neurones. It was proposed that a similar mechanism, if active at the presynaptic terminal, could account for the inhibition of transmitter release. The inhibition of Ca^{2+} channels would reduce the Ca^{2+} influx at the transmitter release face during an action potential, thereby reducing the amount of free Ca^{2+} available to release transmitter.

In the chick ciliary ganglion, Yawo & Chuhma (1993) reported that adenosine inhibited the nerve evoked Ca^{2+} influx in the terminals by activating A1 receptors. Reduced Ca^{2+} influx was due largely to inhibition of ω-conotoxin GVIA-sensitive Ca^{2+} channels in the presynaptic terminals. It is known that there is a large family of calcium channels with different forms of the calcium channel with different voltage-sensitivities and activation characterisitcs. The different calcium channels can also be defined by differences in the blockers that they are sensitive to e.g. L-type calcium channels are dihydropyridine sensitive; N-type calcium channels are

sensitive to ω-conotoxin GVIA and P/Q-type calcium channels are sensitive to all the ω-Aga-IVA.

This hypothesis has received much more attention in peripheral neurones than in central neurones, although Anwyl (review, 1991) reported that whole cell and single channel studies have demonstrated that the N-type calcium channel is the most common type of calcium channel to be blocked by transmitters, including L-glutamate, GABA, 5-HT, Dopamine and Noradrenaline. This was demonstrated in a variety of neurones including rat hippocampal CA1 neurones, rat neocortex, pituitary cells and rat locus coeruleus. Takahashi et al. (1996) showed that the P/Q-type Ca^{2+} channels of the giant synapse - the calyx of Held - in the medial nucleus of the tapezoid body (MNTB), were responsible for the ionic basis of the metabotropic receptor mediated presynaptic inhibition. Application of 1-2-amino-4-phosphonobutyrate (L-AP4) reduced the presynaptic Ca^{2+} current, shown to be the P/Q-type Ca^{2+} current by the inhibition of the Ca^{2+} current by ω-agatoxin IVA, with a concommitant decrease in the amplitude of the EPSC.

It is thought that the neurotransmitter receptor activates a G protein that acts to inhibit the N-type Ca^{2+} channel, similar to the mechanism of activation of the K^{+} channel. There is evidence (Brown & Birnbaumer, 1990) that the inhibition of Ca^{2+} currents may involve a 'membrane delimited' action. This was first observed by Soejima & Noma (1984) and used to describe signalling mechanisms that appear not to require a cytoplasmic second messenger. However, Kalman et al. (1988) have reported examples where the activity of Ca^{2+} channels was modulated by cytoplasmic second messengers in a variety of cell types.

Modulation of the Release Apparatus

A third mechanism that has been proposed to be involved in presynaptic inhibition is an inhibition of the transmitter release apparatus, that might occur independently of modulation of membrane channels (Silinsky, 1981,1984). This mechanism has received the most attention at invertebrate synapses (Man-Son Hing et al., 1989; Dale & Kandel, 1990).There are indications that this type of mechanism may be in place at both peripheral (Silinsky, 1984) and central (Scholz and Miller,

1992) synapses of vertebrates. The basic finding is that the reduction in Ca^{2+} influx cannot account fully for the inhibition of release. A related finding is that the probability of transmitter release is reduced under conditions that negate any contribution of Ca^{2+} influx to release (i.e. when spontaneous release is measured during block of Ca^{2+} channels or when release is stimulated by release of Ca^{2+} from within the cell). The precise molecular mechanisms involved are not clear.

A role for the phosphorylation state of synapsin I has been proposed at the squid giant synapse, with dephosphorylated synapsin I reversibly inhibiting transmitter release (Llinas et al., 1991). When synapsin I is phosphorylated, by Ca^{2+}-Calmodulin-dependent Kinase II (CaM kinase II), it is activated and its binding to vesicles and/or cytoskeletal structures is reduced. Hence more vesicles are available for release during presynaptic depolarisation. The amplitude of the postsynaptic response will therefore be indirectly regulated by depolarisation induced Ca^{2+} influx, as well as more directly modulated.

It is not inconceivable that presynaptic inhibition might involve all of these mechanisms in concert. The attractiveness of such an idea is the possibility that different inhibitory neurotransmitters could utilise different mechanisms to produce inhibition of release. Under certain conditions it might be important to inhibit release without reducing Ca^{2+} influx, as the elevation of terminal Ca^{2+} concentration may be important for other functions. Thus, a transmitter could reduce release by inhibiting the release apparatus but leave Ca^{2+} influx intact.

G Proteins and Presynaptic Receptors

G proteins couple receptors to intracellular effectors or second messenger systems. The role of G proteins is strongly suspected or has been demonstrated unequivocally in most cases of presynaptic inhibition. The presynaptic receptors that have been cloned to date (adenosine A1, α-adrenergic, muscarinic acetylcholine and metabotropic glutamate) are members of the G protein-coupled receptor superfamily. More recently the $GABA_B$ receptor has been cloned (Kaupmann et al., 1997) and was also shown to belong to this superfamily of receptors. Demonstrating the involvement of G proteins in presynaptic inhibition in central neurones such as those

of the hippocampus is complicated by the inaccessibility of the presynaptic terminal itself. Unlike in invertebrates and lower vertebrate preparations (Alford & Grillner, 1991), in the hippocampal preparation only experimental 'tools' that can gain access to the terminal from the extracellular space may be used. Using a slice preparation of the auditory brainstem the medial nucleus of the trapezoid body (MNTB), whose principal neurones each receive a single giant somatic synapse called the calyx of Held, Barnes-Davies & Forsythe (1995) demonstrated that modulation of transmitter release, by metabotropic glutamate receptors (mGluRs) was achieved by a mechanism that was independent of ion channels. The implication of this work was that the G protein- coupled mGluRs can act via modulation of exocytosis itself, as well as by suppressing the high voltage activated P/Q-type calcium channel in the presynaptic terminal (Takahashi et al., 1996).

Presynaptic Adenosine Receptors

Depressant effects of adenosine have been reported at the neuromuscular junction, at putative glutamatergic synapses in the brain, such as the cerebellum (Kocsis et al., 1984), the striatum (Malenka & Kocsis, 1988), the olfactory cortex (Schofield, 1978; Okada & Kuroda, 1980) and the hippocampus (Schubert & Mitzdorf, 1979; Dunwiddie & Hoffer, 1980; Harris & Cotman, 1985; Lupica et al., 1992).

Ginsborg & Hirst (1972) reported that adenosine reversibly reduced the quantum content of end-plate potentials and the frequency of miniature end-plate potentials to about half of control in the rat phrenic nerve-diaphragm preparation. This effect of adenosine was abolished by theophylline. These actions of adenosine suggest an action at the presynaptic adenosine A1 receptor. Furthermore at the motor nerve endings to frog skeletal muscle adenosine and it analogue 2-chloroadenosine (CADO) reduced the number of quanta of acetylcholine (ACh) released in response to a nerve stimulus (Silinsky, 1984). These experiments suggested that the effect of adenosine receptor agonists is ultimately exerted at an intracellular site associated with the secretory process. When ACh release was evoked, using mechanisms that do not require calcium entry through calcium channels, the ACh release was inhibited

by adenosine. Further experiments, using perineural or patch clamp methods, have shown that adenosine receptor agonists did not affect Ca^{2+} currents at concentrations that produced maximal inhibition of ACh release (Silinsky & Solsona, 1992).

Intracellular recordings from guinea-pig submucosal plexus neurones show that inhibition of synaptic potentials is likely to be presynaptic as responses to exogenously applied ACh and noradrenaline were unaltered by CADO (Barajas-Lopez et al., 1991).

In the central nervous system, there is much evidence for the role of the presynaptic adenosine A1 receptor. A number of groups have reported that adenosine has a profound effect on the spontaneous and evoked activity of neurones in many brain regions, an action that has been attributed, in part, to the inhibition of spontaneous excitatory synaptic input. It appears that adenosine plays a role as a major inhibitory modulator of the electrophysiological activity of the brain, and that it combines both pre- and postsynaptic actions in a concerted fashion to reduce the level of excitability in many brain regions.

In the hippocampus adenosine inhibits transmission at every glutamatergic synapse that has been tested; the Schaffer collateral and commissural inputs to the CA1 region, the mossy fibre inputs to the CA3 region, and afferents to the dentate gyrus via the perforant path (Schubert & Mitzdorf, 1979; Dunwiddie & Hoffer, 1980; Harris & Cotman, 1985; Lupica et al., 1992). These depressant effects of adenosine have also been observed at other putative glutamatergic synapses in the brain, such as the cerebellum (Kocsis et al., 1984), the striatum (Malenka & Kocsis, 1988) and the olfactory cortex (Scholfield, 1978).

Mechanisms of Adenosine Mediated Presynaptic Inhibition

Attempts to define the presynatic effects of adenosine are complicated by the well defined effects of adenosine postsynaptically. Adenosine activates a G-protein coupled potassium channel in hippocampal neurones (Thompson et al., 1992; Gerber et al., 1989). This conductance could shunt synaptic currents generated in the dendrites, reducing the amount of current that reaches the cell bodies, and thereby decreasing the effectiveness of glutamatergic excitatory postsynaptic potentials

(EPSPs). Several techniques have been used in order to address this complication to experiments designed to look at the presynaptic action of adenosine.

Direct approaches:

1. Burke & Nadler (1988) looked at glutamate release from hippocampal slices and the effects of adenosine on the release, using a superperfusion method. They found that exposure to adenosine significantly decreased the amount of glutamate released into the superperfusate. This inhibition of glutamate release is most likely to be due to a presynaptic action of adenosine.

Indirect approaches have been used in other studies

2. Paired-pulse facilitation of EPSPs appears to be a presynaptic process in the hippocampus, thus agents that inhibit release might be expected to modify the extent of the facilitation, whereas postsynaptic receptor antagonists should inhibit both the conditioning and test responses equally. Adenosine has been shown to markedly potentiate paired-pulse facilitation in both the CA1 region and the dentate gyrus (Dunwiddie & Haas, 1985; Harris & Cotman, 1985).

3. Fluctuations in unitary synaptic responses, evoked by low-intensity stimulation of single afferent fibres, have been statistically characterised. The patch clamp technique has been utilised as the signal to noise ratio is higher than that when using 'sharp' electrodes, and thus the resolution of the smallest events is increased. Unitary responses are thought to reflect the amount of neurotransmitter released after the activation of a single excitatory axon, and the variability that is observed in these responses may reflect variations in the quantal release of transmitter, which is obviously of presynaptic origin (Malinow & Tsien, 1990; Bekkers & Stevens, 1990; Foster & McNaughton, 1991). However the changes may reflect a disruption, due to the whole-cell technique, in the way postsynaptic receptors couple to ion channels.

The Adenosine Receptor Involved in Presynaptic Inhibition

In order to identify the effects mediated by adenosine at the hippocampal presynaptic terminal it was necessary to characterise the adenosine receptor involved in the inhibitory effects of adenosine in the hippocampus. It has been shown that there is a significant correlation between the A_1 receptor-mediated inhibition of

adenylate cyclase and the inhibition of EPSPs when various adenosine analogues are used (Smellie et al., 1979; Dunwiddie & Fredholm, 1985). Furthermore Schild analysis of the effects of cyclopentyltheophylline (CPT) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), both potent adenosine A_1 receptor antagonists, has shown that the inhibition of field EPSPs is not significantly different from that for the A_1 receptor mediated inhibition of cAMP formation or the suppression of the postsynaptic response to adenosine (Dunwiddie & Fredholm, 1989; Alzheimer et al., 1991). Scholz & Miller (1991) reported that the presynaptic effects of adenosine on the EPSC, and its postsynaptic reduction of Ca^{2+} currents is mediated by an adenosine A_1 present on cultured hippocampal pyramidal neurones which is not readily pharmacologically distinguishable from the adenosine A_1 present on adult hippocampal neurones.

Potassium Channels

The ability of adenosine to activate a postsynaptic K^+ conductance, via pertussis toxin sensitive G proteins, in hippocampal pyramidal neurones is well documented (Trussell & Jackson, 1987; Fredholm et al., 1989; Thompson et al., 1992). This postsynaptic hippocampal K^+ conductance is reported to be blocked by extracellular barium (2mM), but not tetraethylammonium (TEA; 10mM), or concentrations of 4-aminopyridine (4-AP; 500μM), known to block transient outward K^+ currents (I_A) in the hippocampus (Gerber et al., 1989; Thompson et al., 1992). Proctor & Dunwiddie (1987) and Greene & Haas (1991) suggested that the reduction of the EPSP by adenosine is due to a postsynaptic shunt of the excitatory synaptic current, rather than a presynaptic action at the nerve terminal. This mechanism is unlikely to be the way in which adenosine acts to inhibit the EPSP as adenosine reduces the amplitude of the EPSP but does not alter its rise time (Lupica et al., 1992), which would be expected after an alteration in membrane length constant that was sufficient to shunt the excitatory currents. This study also demonstrated that the quantal content (a presynaptic parameter) was reduced by adenosine and that quantal amplitude (a pre/postsynaptic parameter) was unaffected. The observation that the actions of adenosine on the K^+ conductance are blocked by

barium, whilst leaving the inhibitory action on the EPSP intact (Dunwiddie & Proctor, 1988; Thompson et al., 1992) adds to the evidence that it is unlikely that the inhibitory effect of adenosine on glutamate mediated EPSPs in the hippocampus is due to a postsynaptic mechanism.

Some evidence has been provided for a 4-AP sensitive K^+ channel that might mediate the synaptic modulatory effects of adenosine in the hippocampal CA1 region (Klapstein & Colmers, 1992). As the postsynaptic G-protein coupled potassium channel discussed above is not sensitive to 4-AP (Greene & Haas, 1985; Gerber et al., 1989; Thompson et al., 1992), this implies that adenosine affects a distinct potassium channel on the nerve terminal. Klapstein & Colmers (1992) demonstrated that 30 μM 4-AP blocked the inhibitory effects of low, but not high, concentrations of 2-chloroadenosine. This result suggests that adenosine may inhibit neurotransmitter release presynaptically via the activation of a 4-AP sensitive K^+ channel.

Calcium Channels

The most direct mechanism by which adenosine may diminish transmitter release is the inhibition of Ca^{2+}-mediated neurotransmitter release by decreasing the activity of voltage-dependent Ca^{2+} channels. There is only indirect evidence to support or oppose this hypothesis due to the difficulties in measuring the ion channel function in central nerve terminals.

In the peripheral nervous system it has been reported that adenosine can reduce the voltage activated calcium current (Dolphin et al., 1986; MacDonald et al., 1986) and that it appears to be a N-type calcium channel that is involved (Gross et al., 1989).

Studies that have been carried out, using adenosine, in the CNS have measured the calcium current, both electrophysiologically and using synaptosomal preparations, and the effects of adenosine on it. These recordings were made from the cell body and the assumption has been made that the observations at the neuronal somata can be extended to the nerve terminals. In the hippocampus there is conflicting evidence as to the effect of adenosine on calcium currents. It has been

suggested that any adenosine effects that are observed on the calcium current are due to the shunting of the Ca^{2+} conductance by the activation of a K^+ conductance (Gerber et al., 1989; Greene & Haas., 1991). Gerber et al. (1989) reported that when barium was used as the charge carrier through Ca^{2+} channels (thus the adenosine activated K^+ conductance was completely blocked), adenosine had no effect on the voltage dependent Ca^{2+} currents. However in 1991 Scholz & Miller reported that in cultured hippocampal pyramidal neurones the voltage sensitive Ca^{2+} currents were reduced by adenosine analogues with a rank order of potency consistent with an A_1 receptor. When barium was used as the charge carrier the adenosine inhibition was still observed, and these effects were eliminated by pertussis toxin treatment, providing further evidence that a G protein is involved in the adenosine effect. A further study looking at barium currents in outside-out patches from CA3 pyramidal neurones demonstrated that 2-chloroadenosine could reduce the currents (Swartz & Bean, 1992).

Wu & Saggau (1994a) simultaneously recorded the presynaptic calcium transient, optically, and the field excitatory postsynaptic potential (fEPSP) at CA3 - CA1 synapses in the hippocampus and found that activation of presynatpic adenosine A_1 receptors inhibited the fEPSP primarily by reducing the calcium transient. They have provided evidence that presynaptic calcium channels (primarily of the N-type and possibly the Q-type) are involved in adenosine A_1 receptor mediated presynaptic inhibition.

Second Messenger Systems

The adenosine A_1 receptor has been identified as the adenosine receptor which mediates decreases in neurotransmitter release (Dunwiddie & Fredholm, 1989). However the inhibitory effects of adenosine are not affected by cAMP analogues (Dunwiddie & Fredholm, 1985).

Arachidonic acid metabolites have been proposed to inhibit synaptic transmission at sensory neurone synapses in *Aplysia* (Shapiro et al., 1988). Dunwiddie et al. (1992) looked at various agents which interact with this system in order to determine whether the adenosine receptor mediated inhibition of transmitter

release was affected. They found no evidence for modulation of synaptic transmission in the rat hippocampus by arachidonic acid or its metabolites. However Vazquez et al., (1995) observed in cerebrocortical nerve terminals that arachidonic acid suppressed the inhibitory actions of adenosine, allowing an effective facilitation of glutamate release. It is thought that arachidonic acid activates protein kinase C (PKC; Coffey et al., 1994) and that PKC would suppress the adenosine inhibitory mechanisms.

Phorbol esters activate PKC and increase inhibitory and excitatory synaptic transmission in the hippocampus (Malenka et al., 1987). It has been shown that phorbol esters block the inhibitory effects of adenosine on population spikes (Worley et al., 1987) and on field potentials (Thompson et al., 1992) in the hippocampus. Sebastião & Riberio (1990) showed that phorbol esters can also block the inhibitory effect of adenosine on ACh release at the frog sartorius neuromuscular junction, via a presynaptic mechanism. Studies using cultured hippocampal slices have shown that pre-treatment with phorbol esters do not block the activation of a postsynaptic K^+ current by adenosine (Thompson & Gähwiler, 1992).

The mechanism(s) by which phorbol esters block the adenosine A_1 receptor mediated inhibition of neurotransmitter release is unknown. The activation of PKC could result in the direct phosphorylation of a component of the adenosine A_1 receptor transduction system such as the receptor, the G protein that mediates the A_1 response, or the target of the G protein action. A further possibility is that phorbol esters change the process of transmitter release in such a way as to reduce adenosine sensitivity, for example by altering Ca^{2+} channel activity or the regulation of Ca^{2+} levels in the nerve terminal, such that the release mechanism may become 'saturated'.

The Ca^{2+}/calmodulin dependent protein kinase (CamKII) and protein phosphatase 2B (calcineurin) can control the gating of K^+-channels (Roeper et al., 1997), as well as modulating intracellular calcium levels. CamKII and calcineurin are key enzymes in the regulation of synpatic strength, they control the phosphorylation status of pre- and postsynaptic proteins. It is possible that adenosine can modulate the activity of these enzymes via an alteration in the intracellular Ca^{2+} levels, either by

Ca^{2+} entry through N- or P/Q-type Ca^{2+} channels, or via release from intracellular Ca^{2+} stores.

Tyrosine kinase inhibitors, such as genistein, have been shown to attenuate the metabotropic receptor mediated inhibition of the transient Ca^{2+}-activated K^+ current (I_{AHP}) in dentate granule neurones of the rat hippocampus (Abdul-Ghani et al., 1996). These experiments also showed that there was a tonic inhibitory influence on I_{AHP} that was reduced by tyrosine kinase inhibitors. Abdul-Ghani et al. (1996) proposed that metabotropic glutamate receptors initiated inositol 1,4,5-triphosphate production mobilized intracellular Ca^{2+} and leads to increased protein tyrosine phosphorylation, by tyrosine kinases, which in turn resulted in inhibition of I_{AHP}. The adenosine A_1 receptor may inhibit release using a similar mechanism.

As described earlier more is known about the actions of adenosine in systems such as the neuromuscular junction, although there is no evidence that adenosine activates identical mechanisms in all systems where it inhibits transmitter release, it is at least an initial hypothesis worth considering. The observation that basal rates of spontaneous release are decreased by adenosine A_1 agonists has provided support for the modulation of the secretory apparatus by second messenger systems activated by the adenosine A_1 receptor. However it has been demonstrated that adenosine analogues reduce the frequency of spontaneous release, in the presence of TTX and divalent cation calcium channel blockers, by ~45 % whilst the same concentration of agonist will inhibit evoked release by ~95 % (Scholz & Miller, 1992). This suggests that although influx of calcium through voltage-dependent calcium channels is important in regulation of neurotransmitter release, a further mechanism via a voltage-independent process also modulates transmitter release.

G Protein Involvement in Adenosine A_1 Receptor Mediated Inhibition of Transmitter Release

The bacterial exotoxin, pertussis toxin, has been used to evaluate the involvement of G proteins on the neuronal effects of adenosine. The G proteins G_i and G_o are both substrates for this toxin. Trussel & Jackson (1987) demonstrated that the G proteins which link adenosine receptors, in the hippocampus, to postsynaptic

potassium channels are sensitive to pertussis toxin. The inhibition, by adenosine, of cAMP accumulation in hippocampal slices and the reduction of the voltage sensitive Ca^{2+} currents in hippocampal cell cultures are also abolished by this toxin (Fredholm et al., 1989; Scholz & Miller, 1991). Thus most, if not all, of the postsynaptic effects of adenosine mediated by the adenosine A_1 receptor are modulated by either G_i of G_o. The inhibition, by adenosine, of electrically stimulated release of [^{3}H]-ACh and [^{3}H]-noradrenaline from hippocampal slices is not affected by pertussis toxin *in vivo* (Fredholm et al., 1989), but when pertussis toxin is injected into the hippocampus and population spikes and EPSPs are recorded from CA1 neurones it has been shown that the adenosine mediated inhibition of glutamate release is blocked (Stratton et al., 1989). Further Scholz & Miller (1991) have shown that adenosine no longer inhibits monosynaptic EPSPs recorded from pyramidal cells in culture after they have been exposed to pertussis toxin. However no effects, after pertussis toxin pretreatment, have been observed on the inhibitory effects of adenosine (Fredholm et al., 1988; Thompson et al., 1992) in the CA1 region of the rat hippocampus. It is possible that these differences are due to differences in experimental technique. These results suggest that: Firstly the G protein mediating the presynaptic effects of adenosine could be less sensitive to pertussis toxin than the postsynaptic G protein, therefore a disruption of the inhibition would only be seen if much larger injections were used or longer time points examined. Secondly, that pertussis toxin does not easily gain access to the nerve terminal, and that effects would only be seen after G proteins in the cell body have been ADP ribosylated and transported to the nerve terminal, again at much longer time points.

At present, the most likely mechanisms of adenosine action in the hippocampus are a direct action on the ion channels located in the nerve terminal either directly or via a second messenger system. In the hippocampus the synaptic modulation does not appear to involve the same potassium channel that is involved in the postsynaptic actions of adenosine, but could possibly be another potassium channel (cf. review, Lupica & Dunwiddie, 1993). Adenosine could modulate the activity of the calcium channel that mediates transmitter release either directly or via a second messenger system (Scholz & Miller, 1992) that does not involve

arachidonic acid metabolites, as in *Aplysia*, or cAMP. Alternatively adenosine could act via an unknown second messenger pathway to modulate the efficacy of the release process, perhaps by altering the calcium sensitivity, as suggested by Silinsky (1984, 1986). Trudeau et al. (1996) have shown that adenosine may exert at least part of its actions via a modulation of the secretory apparatus, the second messenger pathway being as yet unknown.

Quantal Theory

Quantal analysis has been used to determine whether changes in the synaptic response is pre- or postsynaptic, and to answer some questions about the underlying mechanism. Quantal analysis makes use of variations in an evoked synaptic potential (or current) to dissect out the quantal amplitude and its variability, the probability of transmitter release, the average number of quanta, and the maximum number of quanta released per impulse. Presynaptic factors determine release probability and the number of quanta released, whereas quantal size is usually determined by postsynaptic factors (cf. review, Redman, 1990, Walmsley, 1993). However quantal size could be modified due to the location of the release site and/or by direct changes to the release mechanism.

Quantal analysis techniques were developed from the observation that the end-plate potential at the frog neuromuscular junction appeared to be built up of small all-or-none units which were identical in size with the spontaneous 'miniature e.p.p.'s' (Del Castillo & Katz, 1954b). These could then possibly be regarded as the basic building block or 'quantum' of the end-plate response. Fatt & Katz (1952) showed that the quantal content of the e.p.p. was reduced to a small number when the external calcium concentration was lowered, and that the response approached the size of the spontaneous end-plate potential and at the same time exhibited large random fluctuations, apparently involving steps of unit size.

Electron microscopic observations of membrane bound vesicles within the presynaptic nerve terminal lead to the proposal that a spontaneous miniature endplate potential represented the postsynaptic response to the presynaptic release of the neurotransmitter release of the contents of a single vesicle. Release is thought to

occur by fusion of the vesicle and presynaptic terminal membranes at specialised regions called active zones or release sites.

The amplitude histogram of the evoked endplate potential can show multi quantal peaks, thus indicating that release of transmitter is likely to be intermittent at each release site following the arrival of the action potential, i.e. the release of the contents of a vesicle at a release site occurs in a probabilistic manner. The evoked postsynaptic potential represents the sum of such probabilistic release at all release sites in the connection. It was proposed that the probability of release, *p*, may be the same at all release sites, and that release occurs from a total pool of available vesicles, *N*. This formed the basis of the first statistical models of quantal transmission in which release was described by either a Poisson or uniform binomial process. Del Castillo & Katz (1954b) proposed a statistical treatment of the data " suppose we have, at each nerve-muscle junction, a population of *n* units capable of responding to the nerve impulse. Suppose, further, that the average probability of responding is *p*,..., then the mean number of units responding to one impulse is $m = np$. Under normal conditions *p* may be assumed to be relatively large...". However when they depressed transmission by using a low Ca^{2+}/Mg^{2+} containing bath solution " the chances of responding are diminished, and we observe mostly failures with an occasional response of one or two units". This low probability of release suggested to them that Poisson's law might describe the numbers of quanta released by successive impulses, and this was found to be the case.

The binomial model assumes that all vesicles are released with the same probability, *p*, and that the number of vesicles, *N*, available for release to a single presynaptic stimulus is small. The binomial model predicts

$$P(x) = \binom{N}{x} p^x (1-p)^{N-x}$$

If the probability that release occurs from the population of *n* units is very small, so that the mean number of releases, *m*, is a very small proportion of *N*, the binomial distribution reduces to

$$P(x) = e^{-m} m^x / x!$$

This is the Poisson distribution in which p and n have no meaning, $P(x)$ is the release probability that x vesicles will be released after a presynaptic stimulus, m is the quantal content.

Central Synapses

The methodology that has been developed for the neuromuscular junction cannot readily be applied to central synapses. The quantal synaptic potential and its variability cannot be measured easily in neurones that are contacted by many hundreds or thousands of axons, from diverse projections, over the entire dendritic and somatic membrane. Even when just one axon is stimulated, the number of synaptic contacts made by the axon and the electrotonic locations of these contacts is not known.

Quantal analysis at central synapses has been advanced by experiments that have combined quantal analysis with morphological reconstruction of the connection at which the synaptic potential was evoked (Korn et al., 1982). This has allowed a correlation of the quantal parameters with the number of synaptic contacts and their electrotonic locations, thus removing some of the uncertainties. A morphological definition of n was reached by studies of synaptic transmission in central neurones rather than at the neuromuscular junction, by recording simultaneously from pre- and postsynaptic neurones, followed by staining and reconstruction of the identified neurones. At the inhibitory synapse of the goldfish Mauthner cell it was found that the simple binomial model provided a significant description of the quantal release of glycine, and that the number of active zones established on this neurone by the afferent cell equalled binomial n calculated from statistical analysis of IPSP amplitudes (Korn et al., 1982; Triller & Korn, 1982). It was also found that at these terminals, which have the structure typical of active zones in the CNS, each release site operates in an all or none manner. The 'one vesicle hypothesis' was coined which stated that one vesicle, rather than many was released in a binary manner after an impulse, with a probability p. This view is now the relatively accepted one for most synaptic connections, despite only receiving indirect support from other studies, which confirm that when there are several active zones in a single bouton they

function as independent release sites (Korn, 1984). This provides a unifying framework for quantal release, where the synaptic connection between two cells comprises the active zone and its postsynaptic complement.

Many of the difficulties associated with quantal analysis at central synapses are due to uncertainties about the size, variance and release probability of a single quanta, as they are often small and involve the opening of just 10 - 250 channels (Edwards et al., 1990, Stricker, Field & Redman, 1996), hence single quanta cannot easily be distinguished within a synaptic bombardment generated by diverse inputs distributed on the cell. Stricker et al., (1996) demonstrated that, in rat hippocampal CA1 pyramidal neurones, quantal variance was negligible in the majority of neurones recorded from, however when quantal variability was present, it could be large, and possibly obscured an underlying quantization of transmission. They also found that the release probability was lower, on average, than that found at Ia synapses on motoneurones (Jack, Redman & Wong, 1981). Release probabilities of 1 were often found for Ia synapses, possibly reflecting the more reflexive character of these connections, whilst release probabilities at hippocampal connections are thought to be more plastic and modifiable. Turner et al. (1997) showed that the release probability, for synapses onto CA1 neurones, exhibited a wide range of values (0.03 - 0.99), and that there was a considerable intrinsic (intrasite) variability in response amplitude.

Liu & Tsien (1995a, b) have used a focal stimulation method to study neurotransmission at synapses onto hippocampal pyramidal neurones in slice cultures. Single functional boutons were visualised and then focally stimulated by local application of an elevated K^+/Ca^{2+} solution via a puffer pipette, whilst the postsynaptic currents were recorded under whole cell voltage clamp. They, also, found that although the unitary EPSCs at individual synapses varied greatly in amplitude, there was a bouton to bouton consistency in the amplitude distribution of the EPSC amplitudes.

There are two main objectives associated with quantal analysis at central connections. Firstly to determine accurately the parameters n, p and q (quantal size) and to provide a description of the release mechanisms. Secondly to ask whether a

synaptic modification is expressed, in terms of n, p and q, for example after a drug application or a stimulus protocol such as that used to induce long term potentiation (LTP), and if whether this type of analysis allows the modification to be described as pre- or postsynaptic.

Evoked Transmitter Release and Quantal Theory

The general assumption is that synaptic excitation in the central nervous system occurs via a quantal mechanism in an analogous way to that first described by Del Castillo & Katz (1954b) at the frog neuromuscular junction. At the neuromuscular junction it was observed that an individual response was made up from an integral number of units, which would vary from trial to trial. The most convincing evidence for the quantal nature of the evoked response would be an amplitude histogram that showed distinct, equally spaced peaks. Jack et al. (1990) and Redman (1990) both looked at the amplitude histograms of synaptic events obtained from *in vivo* spinal cord preparations, neither found much evidence to suggest that the distributions were 'peaky'. It was thought that the signal to noise ratio, occurring with the conventional intracellular recording, was too small. Using the patch clamp technique, with its superior signal to noise ratio, Edwards et al. (1990) demonstrated quantal behaviour for synaptic inhibition in the hippocampus but again this technique did not demonstrate equivalent peaks in the amplitude histograms for excitatory events (Malinow & Tsien, 1990; Bekkers & Stevens, 1989). Furthermore Bekkers & Stevens (1989, 1990) had concluded from their experiments, where they recorded single quantal events from a restricted region of the hippocampus (in cultured slices and dissociated neurones), that there was a substantial inherent variability in the size of quanta evoked successively from a single release site and therefore that amplitude histograms of evoked events would not show peaks. This result is contradictaory to that found by Liu & Tsein (1995a, b), and could reflect differences between acute preparations and cultured preparations. Redman (cf; review 1990) suggested that EPSP quantal size was fairly consistent as the postsynaptic receptors were saturated (i.e. all active) even with the smallest quanta. In culture there may be variations in the postsynaptic receptor distribution.

Therefore perhaps not all the receptors would be activated with the smallest quanta and variations in the quantal size may be seen.

Larkman et al. (1991), using the conventional sharp electrode recording technique, reported that they found 'peaky' histograms for synaptic activation of CA1 pyramidal cells and claimed that this demonstrated the quantal nature of excitatory transmission in this region of the hippocampus. However, as stated by Jack et al. (1994) there were several reasons for being suspicious about this data. 1 reliability: were the observed peaks spurious, arising from finite sampling of a smooth distribution? ; 2 the recording noise is expected to be higher than that found using the patch clamp technique; and 3 the best fits of the data were obtained using very low levels of quantal variance, which seemed at odds with the reported higher levels of quantal variance measured from spontaneous events in cultured slices (Bekkers et al. 1990). However Stricker et al. (1996) have also reported that quantal variation was 'negligible' for the majority of the EPSCs that they recorded from rat hippocampal CA1 neurones.

The first question as to the accuracy of this data was overcome by the development of an extension to Magleby & Miller's (1981) autocorrelation method which checked whether the peaks seen were spurious and due to the finite sampling process. This gave a numerical estimate of the likelihood that the peaks occurred due to finite sampling and the probability was found to be very low and the observation of regularly spaced peaks was reliable. The second point was about the recording noise level using the intracellular as opposed to the patch clamp recording technique. The noise level is higher using sharp electrodes, but not of the same order as the difference in the electrode resistance's (10 fold). Larkman et al. (1991) used the current clamp technique, the advantage being that the peak value of the recorded voltages do not suffer as much attenuation as the peak value of the recorded currents do for the same electrotonic distance.

As with spontaneous release the most important source of presynaptic variability in the size of unitary EPSPs/EPSCs is the number of quanta released per trial. There is no reason to assume that the probability of release is identical at different release sites. Also there is no reason to assume that the probability of

release at one site is identical from one trial to the next. Postsynaptically, the most important source of variability is probably the density of neurotransmitter receptors. There are differences in the postsynaptic densities (PSDs) facing different active zones. If all PSDs had the same number of receptors, variability in the amplitude of the event could be expected due to variations in channel gating (Faber et al., 1992). The magnitude of this variability would be largely determined by the maximum open probability of the channel(s) and the number of channels present. Some channels, such as the channels associated with the glutamate receptor family, including the AMPA/Kainate receptor, have different conductance states (Cull-Candy & Usowicz, 1987). The amount of current passed by the channel in the period that it is active may not be constant from trial to trial even if the amount of transmitter acting at that release site is constant.

When studying the amplitude fluctuations of evoked synaptic potentials or currents, it is important that the presynaptic axon(s) are reliably stimulated. Ideally just one axon is stimulated and the presynaptic action potential is measured near the site of stimulation. Intermittent conduction failures in the presynaptic fibre(s) could be interpreted, incorrectly, as release failures in the analysis and will therefore distort the measured release statistics.

When quantal analysis is used to determine whether an experimental modulation acts at a pre- or a postsynaptic site, it is necessary to assume that the hypothetical modulation affects all synapses by the same amount. E.g., a competitive antagonist acting on a uniform receptor population would be expected to modulate all synapses by the same relative amount. The converse of this is a modulation that switches on or 'unmasks' postsynaptic receptors at a previously silent synaptic bouton while leaving other boutons unaffected. This situation would be almost impossible to distinguish from presynaptic modulation using any form of quantal analysis, as it mimics an increase in the number of release sites (N in the binomial model). It has been suggested that long-term potentiation (LTP) works by unmasking silent synapses (Liao, Hessler & Malinow, 1995). The hypothesis that silent receptors are unmasked would also predict an increase in the response to bath applied agonists.

The simplest form of quantal analysis would be to estimate the mean quantal content, *m*, of an evoked postsynaptic potential or current (PSP). Once *m* is ascertained it can be used to derive other quantal model parameters. Also, any drug that acts via a presynaptic receptor to modulate synaptic transmission will produce a change in *m*. The equation

$$m = M/Q$$

where M is the mean evoked PSP amplitude and Q is the mean quantal amplitude, is the simplest method of estimating *m*. Ideally M and Q should be measured in the same cell, however the value of Q is sometimes difficult to measure, either because of problems with the recording noise or due to the slow rate of spontaneous events. The assumption that the mean miniature synaptic response amplitude accurately represents the evoked PSP quantal amplitude has been made in this method. Also this estimate of *m* cannot distinguish between a mixed pre- and postsynaptic modulation or a purely presynaptic one, and whether the presynaptic modulation is due to a change in the number of release sites or a change in the release probability.

Failure counting is another simple form of quantal analysis that can be applied to evoked data. A 'failure' is counted when no postsynaptic response, to a stimulus, is observed. Failure probability can be used to estimate *m* in the Poisson model

$$m = \ln (N/n_0)$$

where N is the total number of stimuli and n_0 is the number of failures observed. This method only provides the one value, and so cannot be used on its own to estimate parameter values for the binomial model or other models of quantal release, but can be used in conjunction with a parameter estimate obtained using another approach, e.g. combine with *q* values obtained using spontaneous release. A statistically significant change in the failure probability would indicate a presynaptic site of action for the modulation (Kuno, 1964; Malinow & Tsien, 1990).

This method requires the reliable detection of the postsynaptic response to a single quantum of transmitter, therefore experimental modulations that alter the background noise or produce a postsynaptic reduction in mean quantal amplitude may alter the number of undetected PSP and thus lead to an artifactual change in the measured failure rate. This method cannot distinguish between a mixed pre- and postsynaptic modulation or a pure presynaptic site of modulation. If *q* is unchanged, when *m* is altered the site of modulation is assumed to be presynaptic. However if the changes in *m* and *q* are correlated the modulation is assumed to be postsynaptic.This method is reliable when applied to low noise whole-cell recordings.

A versatile form of quantal analysis is based on measuring the coefficient of variation (CV) of PSP amplitude fluctuations. The measured CV is corrected for background noise.

$$CV = sqrt(\sigma_r^2 - \sigma_n^2)/M$$

where σ_r^2 is the variance of the PSP amplitude, σ_n^2 is the variance of the background noise and M is the mean PSP amplitude.

The value of CV, corrected for quantal variability, can then be used to estimate the mean quantal content in both the Poisson and the binomial models. Thus m_{cv} can be calculated from

$$m_{cv} = 1/CV^2$$

When the release probability is small (<1.0) the quantal amplitude, q, can be calculated and approximated to q_{cv}.

$$q_{cv} = (\sigma_r^2 - \sigma_n^2)/M$$

There are two general categories of analysis techniques based on PSP amplitude CV, and these can be used to investigate whether a synaptic modulation is pre- or postsynaptic in origin. There are those approaches that require the assumption

of a quantal model of transmitter release, and those that do not. The model independent approach also has the advantage that it does not require a correction for quantal variance. The ratio of CV under test and control conditions can be measured and if the modulation is postsynaptic the ratio will equal 1, if the modulation is presynaptic the ratio will not equal 1. As with the other techniques described a mixed pre- and postsynaptic modulation cannot be distinguished from a pure presynaptic modulation.

Paired-Pulse Modulation

Paired pulse facilitation can be used as an alternative approach to the study of presynaptic receptor function, as this technique does not rely upon statistical models of transmitter release. The synapses under study are stimulated twice in rapid succession (<100 ms between pulses) and there is facilitation of the second (test) response compared to the first (conditioning) response. However both false positive and false negative results can be yielded: the response to the conditioning pulse might cause receptor desensitisation and thus a diminished response to the test impulse, hence a decrease in the amplitude ratio, or alternatively a presynaptic modulation may cause no change in the amplitude ratio. Results from paired pulse experiments should ideally be cross checked using an approach based on the quantal theory.

The mechanism underlying the paired pulse facilitation is generally thought to be a transient increase in the residual calcium level (Katz & Miledi, 1968). That is, a small fraction of the calcium that enters the terminal in response to the first action potential remains for several hundred milliseconds. This amount of residual calcium is too small to trigger release itself, but adds significantly to the calcium entering the nerve terminal during the second action potential. The increase in the probability of vesicle fusion produced by the second action potential is therefore considerably enhanced compared to that of the first. Superimposed upon this facilitation under conditions of high transmitter release is a depression of the test response, which usually results from either a depletion of the pool of releasable transmitter or a decrease in the probability of its release (Del Castillo & Katz, 1954c, Katz & Miledi,

1968; Betz, 1970). There are a large number of vesicles of neurotransmitter available for release in response to a presynaptic action potential at the neuromuscular junction, but each responds with a relatively low probability (Del Castillo & Katz, 1954c), that is independent of the release of any other vesicle (Fatt & Katz, 1952). However when two stimuli are applied close together, the amplitude of the test response is reduced when compared to the conditioning response (Betz, 1970), a phenomenon known as paired pulse depression. If the same experimental protocol is repeated under conditions where the release probability has been reduced, such as increasing the $[Mg^{2+}]/[Ca^{2+}]$ ratio, the depression can be changed into a facilitation of the test response compared to the conditioning response (Del Castillo & Katz, 1954c, Katz & Miledi, 1968). This paired-pulse depression at the neuromuscular junction is not accompanied by a decrease in the sensitivity of the endplate to acetylcholine, and therefore, possibly results from a decrease in transmitter release (Thies, 1965). At central synapses it has been demonstrated that paired-pulse depression results from a decrease in release probability, rather than a decreased postsynaptic receptor sensitivity to the neurotransmitter (Debanne et al., 1996; Turner et al., 1997).

Spontaneous Transmitter Release and the Quantal Theory

As described above the spontaneous miniature endplate potential (mepp) represents the postsynaptic response to the presynaptic release of a single quantum of neurotransmitter. These appear to occur spontaneously and randomly and are seen after action potential production has been blocked, for example with tetrodotoxin (TTX). Miniature synaptic potentials (mEPSPs) are difficult to study in central neurones as they arise at random times and at various dendritic locations. mEPSPs that are produced at synapses distant from the somatic recording site are attenuated by cable properties of the dendritic tree, so that any inherent miniature synaptic response size variations are difficult to calculate as there will also be size fluctuations due to site of origin. As spontaneous synaptic releases occur at locations throughout the dendritic tree, with a somatic electrode miniature synaptic responses of different sizes would always be recorded, and without knowledge of the underlying time course it would be impossible to decide if a particular miniature synaptic response

was small as it originated at a relatively distant site or because the conductance change responsible was small.

Bekkers & Stevens (1994) looked at miniature amplitude distributions using cells in culture and locally applying hypertonic solutions, by microperfusion, to a defined position in the dendritic tree, this then increases miniature synaptic responses release locally so that the effects of cable filtering were not a factor in determining the variability in miniature synaptic response size and shape. This effect was described by Fatt & Katz (1952) at the frog neuromuscular junction when they noted that the rate of miniature synaptic response production increased when hypertonic solutions were applied to the preparation. Bekkers & Stevens (1994) found that the miniature amplitude was quite variable even when the source was at a constant electrotonic distance from the recording site, and that a 10 fold variation in amplitude was likely even in a sample of just 100 miniature synaptic responsess. This gives rise to the question of whether miniature synaptic responses are always monoquantal, or can they sometimes represent multivesicular release? This has implications if quantal analysis is carried out with the arbitrary assumption that miniature synaptic responses have a constant size, and that all of the size variations in evoked synaptic currents result from probabilistic release mechanisms.

The experiments of Bekkers & Stevens (1994) also demonstrated that miniature release occurred only if the microperfusion area included at least one synaptic bouton; and the method used had the spatial resolution, in culture, of 10 - 15μm. The effects of cable filtering on the size and shape of the recorded miniature currents by evoking release at different distances from the recording site was examined and it was concluded that this essentially did not affect these measurements.

Possible explanations for the variability of the spontaneous miniature amplitudes include: 1) each synapse may have its own distinct miniature synaptic response amplitude that varies little, but the amplitude does vary between synapses; 2) miniature synaptic response amplitudes fluctuate from one release to the next at individual synapses; 3) a mixture of both these possibilities, or 4) that release is not always monoquantal.

Bekkers & Stevens (1994) studied the source of the miniature synaptic response variability by applying hypertonic solutions to specific locations, recording the miniature synaptic responses and then staining the boutons with antibodies to synapsin in order to count them. No correlation between the variance of the miniature distribution and the number of boutons from which the miniature synaptic responses were recorded was found. It can be concluded that the major source of miniature synaptic reponse variability is present at the individual synapse although some synapse to synapse variation must occur.

The above experiments were carried out using cultured neurones. The conclusions reached have also been tested in brain slice preparations as the miniature synaptic response size distributions from a population of synapses in a slice should have about the same size and shape as the distributions found in culture. Bekkers et al. (1990) used local applications of hypertonic solution to specific sites on the surface of the brain slice and found that the resultant miniature synaptic responses had a similar time course and amplitude distribution as those observed in culture were produced. A similar conclusion was reached by Raastad et al. (1992) based on analysis of synaptic currents evoked by 'minimal' stimulation. However the question as to whether release can be multivesicular in the case of the larger miniature synaptic responses was not addressed.

As reviewed by Jack et al. (1994) there is evidence that some spontaneous synaptic events may be multiquantal. The basic evidence is that sometimes the amplitude histograms of the spontaneous events is made up of a variable number of equally spaced peaks (Edwards et al., 1990; Silver et al., 1992). In these experiments the spontaneous events arose from a relatively restricted electrotonic region. Ulrich & Luscher (1993) recorded spontaneous synaptic currents from organotypic cultures of spinal cord motoneurones and used their time course as a guide to their electrotonic locations. They were then able to correct the amplitudes for relative electrotonic attenuation and reported that the data was best fitted by the sum of two Gaussian curves of single and double the mean amplitudes. This was interpreted as reflecting a combination of either one or two quanta released spontaneously.

Two different interpretations for this data have been put forward. The more common one is that the different peaks each represent the release of an integral number of vesicles and that the mechanism by which spontaneous release occurs can lead either to a single or several vesicles to be triggered to release their contents simultaneously. This is supported by the observations of Melamed et al. (1993) at the lizard neuromuscular junction that there can be spontaneous fluctuations in intracellular calcium concentration within a synaptic bouton. The second theory is that there is just one vesicle released, but that the postsynaptic receptors are 'quantized' at different release sites. The only piece of evidence for this possibility is that the distribution of the areas of postsynaptic densities at excitatory synapses in CA1 hippocampal pyramidal cells is roughly bimodal (Harris & Stevens, 1989). However the simplest interpretation is that of multivesicular release and some direct evidence for this has been shown by Korn et al., (1993) for inhibitory synaptic events.

As discussed above spontaneous EPSPs are observed in CNS and NMJ preparations after the presynaptic action potential has been blocked by TTX. The amplitude distribution of the miniature synaptic responses provides a direct measure of mean quantal amplitude and quantal variability. An experimental condition that acts at a presynaptic site may alter the rate of occurrence of the miniature synaptic responses (Malgaroli & Tsien, 1992). However a postsynaptic modulation will not affect the spontaneous release rate (Manabe et al., 1992).

There are limitations with the technique: to reliably measure the mean amplitude and frequency of occurrence a sample size of several tens or hundreds of events is needed, this can be difficult to acquire if the rate of spontaneous events is low. The reliable detection of spontaneous events is a further consideration. If the mean quantal amplitude of the distribution is close to the background recording noise many of the smaller spontaneous events will be lost in the noise biasing the distribution towards larger events. The quantal amplitude will be overestimated and the frequency of the events will be underestimated. If an experimental modulation produced a change in the background noise or a postsynaptic change in mean quantal

amplitude this may alter the number of miniature synaptic responses that go undetected and lead to an artifactual change in their measured rate.

Spontaneous release and evoked release may occur via different mechanisms and an increase in the spontaneous release rate may not be a reliable indicator of an increase in probability of evoked release (Silinsky, 1984). In cultures of hippocampal neurones Malgaroli & Tsien (1992) found that changes in the miniature synaptic response frequency generally paralleled presynaptic modulation of evoked synaptic transmission, supporting the assumption that spontaneous and evoked release occurred via related mechanisms. This means that the quantal parameters derived from spontaneous EPSP distributions can be used as a cross check on quantal parameters found indirectly from the evoked EPSP amplitude distribution.

Spontaneous release can occur from any of the synaptic terminals on a neurone. Evoked release can only occur from the smaller number of terminals arising from the stimulated presynaptic fibre. Boutons arising from an individual fibre may be regulated by their common presynaptic neurone, and as a result their release properties may differ from the general population. A similar problem may occur when synaptic connections arise from several different pathways, e.g. in a brain slice preparation. Release properties may differ from pathway to pathway. A further potential problem is attenuation of miniature synaptic responses occurring at synapses far from the soma. If the attenuation is large and if the presynaptic fibre preferentially forms connections close to the soma, then the general population of miniature synaptic responses will have different properties than the evoked miniature synaptic responses (Clements, 1993). A correlation has been observed between mean amplitude of miniatures synaptic responses and the quantal amplitude estimated from evoked PSP amplitude distributions in several different preparations (Korn et al., 1987). Also the work of Bekkers et al. (1990) where mini synaptic responses were evoked in cultured neurones by hypertonic solutions which were applied to a small number of boutons on a short section of dendrite have a similar amplitude distribution to the general population of miniature synaptic responses from the entire dendritic tree in the same neurone. Thus it can be generally assumed that the

miniature synaptic response amplitude distribution provides a useful estimate of the quantal amplitude distribution underlying evoked PSP amplitude fluctuations.

However it cannot always be assumed that if transmitter acting at a presynaptic receptor inhibits evoked release then spontaneous release will also be inhibited. It has been demonstrated that Neuropeptide Y (NPY) inhibits evoked EPSPs in hippocampal CA1 and CA3 neurones (Klapstein & Colmers, 1993) by acting presynaptically at the Y_2 receptor, with no effect on the postsynaptic membrane properties. However when TTX is applied to the preparation and miniature synaptic responses are studied, NPY has no effect on the frequency of occurrence of the spontaneous miniature synaptic responses (McQuiston & Colmers, 1992). One explanation for this observation is that the Y_2 receptor alters the properties of a presynaptic ion channel: either by activating a K^+ channel or inhibiting a Ca^{2+} channel at the terminal, with no direct effect on the release apparatus itself.

Aims of the Study

1. To study the presynaptic Adenosine A_1 receptor, using the whole cell patch clamp technique.
2. To record from rat hippocampal CA1 neurones in the acute slice preparation.
3. To use the selective Adenosine A_1 receptor agonist 2-chloro-N^6-cyclopentyladenosine (CCPA) and antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), in order to determine the role of the adenosine A_1 receptor in modulating transmitter release.
4. The effects of activation or blockade of the Adenosine A_1 receptor was studied. Changes in quantal parameters, using evoked release was determined.
5. Paired-pulse modulation was studied using both current clamp and voltage clamp to utilise the advantages of each technique. The effects of the selective Adenosine A_1 compounds on the paired-pulse response was studied.
6. The effects of CCPA and DPCPX on spontaneous neurotransmitter release was determined.

Summary

1. Single or pairs of excitatory responses were evoked in CA1 pyramidal neurones of young rats *in vitro* by extracellular stimulation of axons in the stratum radiatum using a minimal stimulation protocol, and were recorded using the whole-cell patch-clamp technique.

2. The amplitude of successive responses to stimulation fluctuated, and was altered by the adenosine A_1 receptor agonist, 2-chloro-N^6-cyclopentyladenosine (CCPA) and antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX). It is concluded that a single, low intensity stimuli triggers the release of multiple quanta of glutamate.

3. No significant change in the resting membrane potential was observed after exposure to CCPA or DPCPX. CCPA did not significantly alter the apparent input resistance of the CA1 neurones.

4. The EC_{50} for CCPA was determined by studying the degree of inhibition of the evoked EPSP. It was found to be 114 nM.

5. DPCPX increased the amplitude of the control synaptic response (single pulse experiments) suggesting that, in the acute slice preparation, there was an appreciable level of endogenous adenosine exerting a tonic activation of adenosine A_1 receptors.

6. Quantal analysis, using the method of failures, confirmed that quantal content was decreased by CCPA, and increased by DPCPX.

7. When pairs of responses were elicited, the amplitude of the second response did not show any correlation with the amplitude of the first. Paired-pulse facilitation (PPF) and paired-pulse depression (PPD) were observed in the same neurone.

PPD was observed if the first response was > than 50 % or 70 % of the maximum control response for current- and voltage-clamp recordings respectively.

8. In paired-pulse experiments DPCPX caused a decrease in response amplitude in 8 out of 17 neurones studied. This may be due to transmitter depletion, as in 6 of these neurones a decrease in quantal content was also observed.

9. The percentage of trials that exhibited PPD was not changed by altering the release probability using CCPA and DPCPX.

10. Spontaneous minEPSPs (mEPSPs) were recorded, in the presence of TTX, from CA1 pyramidal neurones, using the whole-cell patch-clamp technique.

11. CCPA significantly decreased the frequency of mEPSPs, whilst DPCPX significantly increased the frequency of mEPSPs, possibly indicating a change in the number of vesicles of transmitter available for release. Neither CCPA or DPCPX changed the mEPSP amplitude.

12. When stimulated the presynaptic adenosine A_1 receptor inhibits transmitter release by decreasing quantal content. This is reflected in the decreased frequency of spontaneous release, and the decreased amplitude of evoked responses. When the endogenous adenosine activation of A_1 receptors was relieved by addition of DPCPX, the converse was found.

Chapter 2

Methods

Introduction

Recording from brain slices was first developed by Yamamoto & McIlwain (1966) who showed that isolated slices of prepyriform cortex could be maintained *in vitro* and still show electrical activity comparable to that obtained from the intact preparation. Skrede & Westgaard (1971) were instrumental in establishing the hippocampus as a candidate for brain slice studies. The *in vitro* hippocampal brain slice preparation with its laminated structure has now been used extensively to study, with patch and intracellular electrodes, the properties of pyramidal cells and the electrophysiological characteristics of putative neurotransmitters and drugs applied both to the whole slice and to localised areas of the slice such as the dendrites or the soma of the cell. Using the hippocampal slice preparation has enabled recordings of high quality to be made due to the slices' greater mechanical stability. The cell type recorded from can be readily identifed due to the laminated structure of the hippocampus.

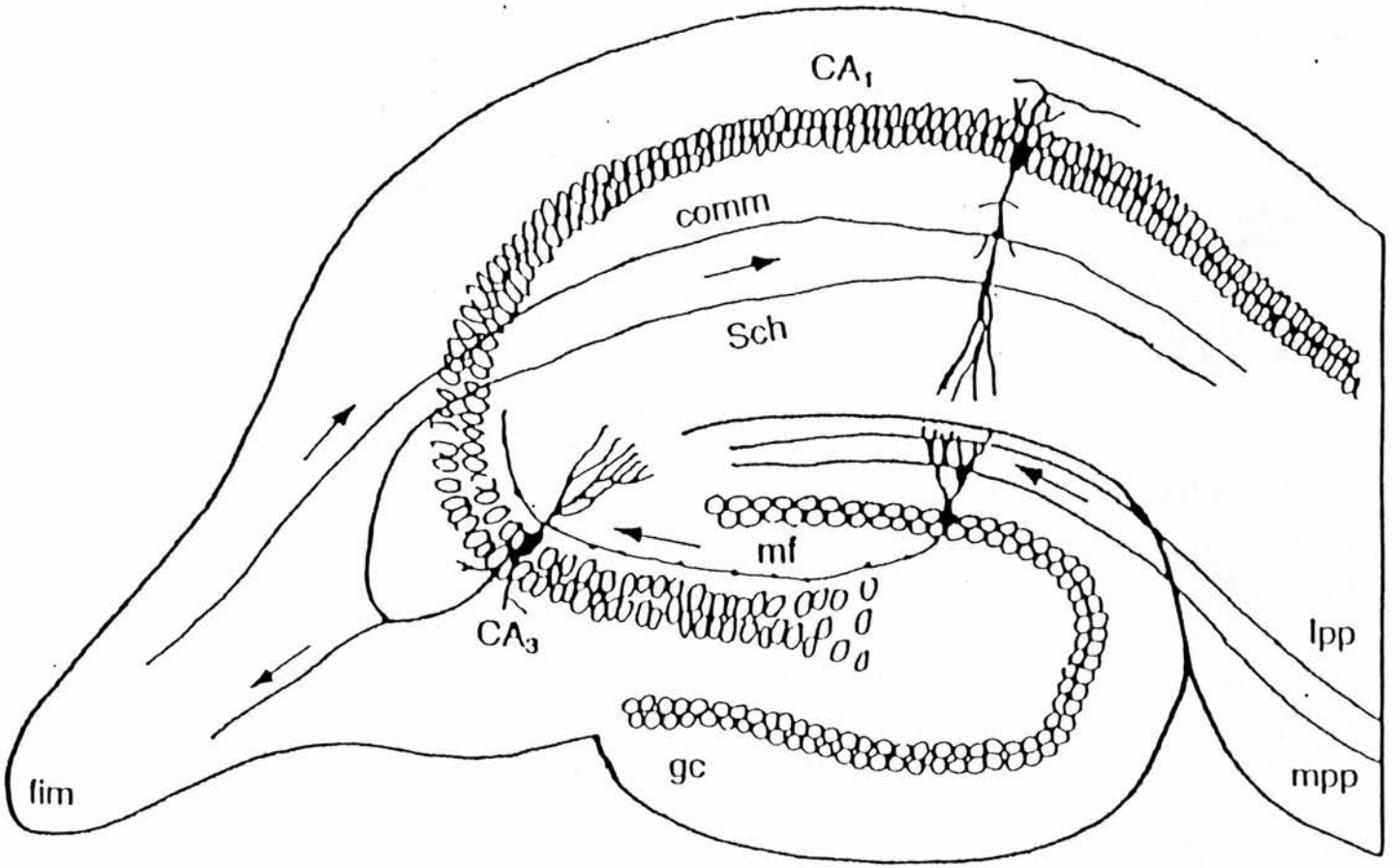
The hippocampus is a part of the limbic system and is involved in aspects of memory storage and the emotional aspects of behaviour. The hippocampus receives its major input from the entorhinal cortex by way of the perforant path (see Fig 2.1). Fibres from the entorhinal cortex that reach the hippocampus via the perforant path pass through the subiculum, an area of cortex that receives major output from the hippocampus. There are three major excitatory pathways running from the subiculum to the CA1 region of the hippocampus. The perforant pathway runs from the subiculum to the granule cells in the hilus of the dentate gyrus. The axons of the granule cells form a bundle, the mossy fibre pathway, that runs to the pyramidal cells lying in the CA3 region of the hippocampus. Finally, the pyramidal cells in the CA3 region send excitatory collaterals, the Schaeffer collaterals and commissural fibres, to the pyramidal cells in CA1. The hippocampal commissure consists of transverse fibres linking the posterior columns of the fornix on either side. These run in the stratum radiatum as do the Schaffer collateral fibres.

In this study the whole cell blind patch clamp technique was used to record from hippocampal CA1 neurones (Edwards et al., 1989). The patch clamp technique for making electrophysiological recordings from cells was first described in 1976 by

Figure 2.1 The Hippocampal Slice

The hippocampus and dentate gyrus together form an s-shaped structure as shown here in cross section. The major inputs to the dentate gyrus are the medial and lateral perforant path (mpp and lpp) fibres arriving from the entorhinal cortex and fibres arriving through the fornix (f). These fibres synapse onto the granule cells (gc) the main neurones of the dentate gyrus. These neurones give rise to the mossy fibres (mf) which synapse onto the pyramidal neurones of regions CA3 and CA2 of the hippocampus. The CA1 pyramidal neurones receive inputs from the CA3 pyramidal neurones - the Schaeffer collaterals (Sch) - and directly from the perforant (CA3) and alvear (CA1) pathways and from fibres arriving through the fornix. The commissural neurones connect the two hemispheres of the hippocampus and run in the stratum radiatum as do the Schaeffer collaterals. The basket cells are the intrinsic neurones of the hippocampus and dentate gyrus. The output from the hippocampus is carried in the axons of the pyramidal neurones through the precommissural fornix to the lateral septal nucleus and the postcommissural fornix to the mammilary body and anterior thalamus (Risold & Swanson, 1996).

See Crunelli, V., Forda, S. & Kelly, J.S. (1985). Excitatory amino acids in the hippocampus: synaptic physiology and pharmacology. *Trends in Neuroscience* **8**, 26-30. for the source of this figure.


CA1
comm
Sch
mf
CA3
lpp
mpp
gc
fim

Neher and Sakmann (cf., Neher & Sakmann, 1992). It is an extremely powerful and versatile method for studying electrophysiological properties of biological membranes. Neher & Sakmann (1976) discovered that by applying a slight suction through the pipette, when it was pressed up against a cell, could increase the resistance of the seal to gigaohm levels. Blunt electrodes are used for patch clamp recording rather than the conventional sharp intracellular electrodes. These two recording techniques are analogous, however patch clamp recording offers high resolution, low noise, excellent stability and control over intracellular constituents. These improvements are due to low electrode resistance and the tight membrane-glass seal. The whole cell patch clamp technique can be applied to smaller cells than the classic microelectrode impalement as it is better tolerated by these cells.

Since the first demonstration of single channels in a biological membrane using this methodology, several key improvements have refined its use and applicability to virtually all biological preparations (including animal and plant cells, bacteria, yeast and cell organelles). The development of the 'gigaseal' (Sigworth & Neher, 1980) and the establishment of the various recording configurations (cell-attached, inside-out, outside-out, whole cell) allowed patch recording from the cell surface or cell free membrane patches as well as intracellular recordings (Hamill et al., 1981).

Originally the patch clamp technique referred to voltage-clamp of a small membrane patch, but it now generally refers to both voltage-clamp and current-clamp measurements using 'patch-clamp'-type micropipettes. The technique allows experimental control and manipulation of the voltage of membrane patches or the whole cell (voltage-clamp), thus allowing the study of the voltage dependence of ion channels. Alternatively changes in membrane potential which constitute the physiological response of a cell in response to current flowing through ion channels (current-clamp) can be studied.

The basic approach is to measure small ionic currents in the picoampere range through single channels which requires a low-noise recording technique. This is achieved by tightly sealing a glass microelectrode onto the plasma membrane of an intact cell, thereby isolating a small patch. The currents flowing through ion channels

enclosed by the pipette tip within that patch are measured by means of a connected patch-clamp amplifier. This 'cell-attached' configuration is the precursor to all other variants of the patch-clamp technique. The resistance between pipette and plasma membrane is critical for determining the electrical background noise from which the channel currents need to be separated. The seal resistance should typically be in excess of $10^9\Omega$ ('gigaseal').

Once a gigaseal has been formed the patch can be broken by applying a pulse of negative pressure to the patch pipette, thereby creating a hole in the plasma membrane and gaining access to the cell interior. This does not compromise the gigaseal between pipette and plasma membrane. The tightness of the seal prevents leak currents flowing between pipette and the reference electrode and flooding of the cell with the constituents of the bath solution. This whole cell configuration is characterised by a low resistance access to the cell interior through the pipette tip, allowing both voltage-clamp and current-clamp of the whole cell. In this configuration the ion currents and potential changes of the whole cell are recorded - an average response of all channels in the cell membrane is obtained. The main disadvantages of this recording configuation are the possible loss of cytosolic factors and the inability to change the cytosolic solution easily. The most notable differences between recording with sharp and blunt electrodes is that patch clamp recording shows up to an order of magnitude increase in input resistance and in the membrane time constant (Edwards et al., 1989). Most patch clamp recordings are done at room temperature which will also tend to increase these parameters. Intracellular penetration of cells produces a traumatic leakage pathway which is practically absent in patch clamp recording. Therefore the measured input resistance and time constant reflect that of the intact cell.

Preparation of Hippocampal Slices

Whole cell patch clamp recordings were made from hippocampal CA1 neurones contained in acutely prepared slices. Male cobb or Wistar (Charles River) rats (50 - 100g) were decapitated and the brain was rapidly removed and dissected. Two coronal cuts were made: one at the caudal end of the frontal cortex, the other at the rostral end of the cerebellum. Next two sagittal cuts were made one to the right

hand edge of the brain and the other just left of the central line. The block of tissue containing the hippocampus was then glued (midline of the brain down) to the stage of the vibrating tissue slicer using a cyanoacrylate glue, and placed in ice-cold artificial cerebro-spinal fluid (ACSF) of the following composition (mM): 126 NaCl, 26 $NaHCO_3$, 2.75 KCl, 1.25 NaH_2PO_4, 1 $MgSO_4$, 2.5 $CaCl_2$, 10 D-glucose. This cooling of the tissue minimizes damage due to anoxia and firms the tissue for slicing. A vibratome was used to cut slices (Vibraslice, Campden Instruments). The brain was sliced down to roughly the correct level, and then 450 μm thick transverse slices were cut. Immediately after cutting, the CA3 region of the slices was removed using a blade and the slices were transferred to a holding chamber containing ACSF at room temperature, and continuously bubbled with 95% O_2/ 5% CO_2 until required. The slices remained viable for more than 10 hours. The whole operation, from decapitation of the rat to placement of the slices in the incubation chamber took approximately five minutes.

Ten minutes before the beginning of each experiment one slice was transferred to the recording chamber, with a fresh slice being transferred after approximately 1½ hours or after a successful recording had been made and the slice had been exposed to the drugs used. The recording chamber used was based upon one described by Madison (1990). The slices are maintained submerged and continuously perfused with ACSF, at 2-3 ml min^{-1}, which also contained 100 μM picrotoxin, at room temperature and bubbled with 95% O_2/ 5% CO_2. Picrotoxin was added to the ACSF to block $GABA_A$ mediated events and delineate the excitatory glutamate responses under study. The design of the chamber (Fig 2.2a) ensured that the slice was bathed in a unidirectional flow of ACSF, thus reducing dead space due to eddy currents at the edges of the chamber. The slices are held in place using U-shaped weights (Fig 2.2b) made from 0.5 mm platinum wire that has been flattened in a vice until it was approximately 400-450μm thick. Fine nylon fibres are then stretched across the frame at small intervals and superglued into place.

Micropipettes were pulled using either an horizontal puller (P80, Flaming Brown) or a vertical puller (PP83, Narishige, Japan) using a two stage pull. A filamented borosilicate capillary glass of 1.5 mm external diameter (GC150TF-15,

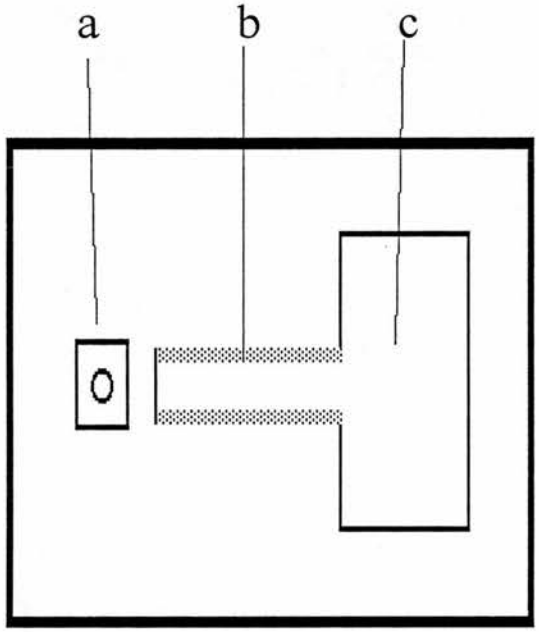
Clarke Electromedical, U.K.) was used. The pipette tips were judged to have been polished during the second pull, and no further treatment of them was required before use. They were back filled using a solution containing (mM): 130 K-gluconate, 10 KCl, 5 NaCl, 10 EGTA, 10 HEPES, 1 $CaCl_2$, 2 $MgCl_2$, 2 Na_2ATP, pH 7.3. The electrode tips were about 2 μm in diameter and typically had resistances of 4-8 MΩ. The pipettes were placed into the holder with the tip of a chlorided silver wire in contact with the pipette solution and lowered towards the surface of the slice under visual control. Postive pressure was applied to the pipette as it went through the air-water interface. Potentials were recorded, with respect to a silver/silver chloride pellet which was positioned to one end of the bath. To monitor the junction potential (a modified form of which occurs across the microelectrode tip, termed a tip potential) of the pipette with the bath solution pulses of hyperpolarising current (0.45 nA, 100 -120 ms long) were injected through the recording electrode. The tip resistance was offset with the bridge amplifier, and any potential seen between the pipette and the bath solution was zeroed with the DC offset, this is the liquid junction potential and was typically less than 5 mV. Under microscopic visualisation the pipette was lowered into the CA1 cell layer. As the cells could not be individually visualised (the blind patch clamp technique) resistance changes were monitored, a persistent increase in resistance, which was reversed when postive pressure was applied through the pipette, was judged likely to be due to a cell, in contact with the tip of the electrode. Suction was then applied to the micropipette and a seal formed. The gigaseal was then ruptured and recordings made in the whole cell configuration. Seals were always formed in current clamp mode, a 0.45 nA hyperpolarising pulse generated by an Axoclamp 2A current and voltage clamp amplifier (Axon Instruments, see fig. 2.3) was used to track resistance changes, the pulse was reduced to an intensity of 0.01 nA as the seal resistance increased. The pulse length and frequency were set using a Digitimer D4030 (Digitimer). The seal was then ruptured and whole cell access gained. The recording conditions were then optimised:

(i) in current clamp the series resistance (consisting of the electrode resistance and access resistance) was offset by balancing the 'bridge' and any stray capacitance from the microelectrode and amplifier input to ground were compensated (capacity

Figure 2.2 Bath design

The chamber used in these experiments was made from a perspex block and is shown in A. The chamber consists of a bubble trap (a) where the artificial cerebrospinal fluid (ACSF) entered the bath through small-diameter tubing using a gravity fed system. The flow rate of the ACSF was controlled using a flow regulator, and was maintained at 2 - 3 $mlmin^{-1}$. Two strips of sylgard ran along the sides of the slice bath (b), and a piece of netting was 'superglued' across these to form a base upon which the slices were placed, and the ACSF flowed across both faces of the slice thus submerging it. The ACSF was removed from the bath using a suction tube placed in the large rear well (c) and which was connected to a vacuum pump. The $Ag/AgCl_2$ electrode was also placed in the chamber at this position. The slice was held in position using horseshoe shaped platinum weights (shown in B) with a thickness of 400 μm. Fine parallel nylon threads from nylon stockings were 'superglued' to the two sides of the weight to provide a 'mesh' to place over the slice and to immobilise it. The slice was illuminated using a fibre optic tube.

A



B

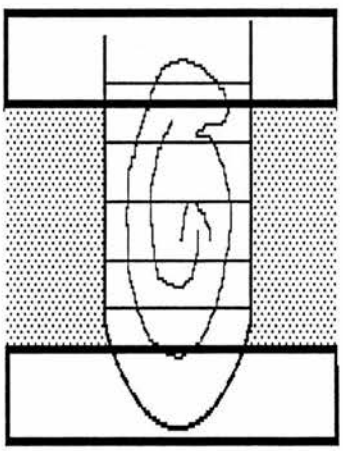
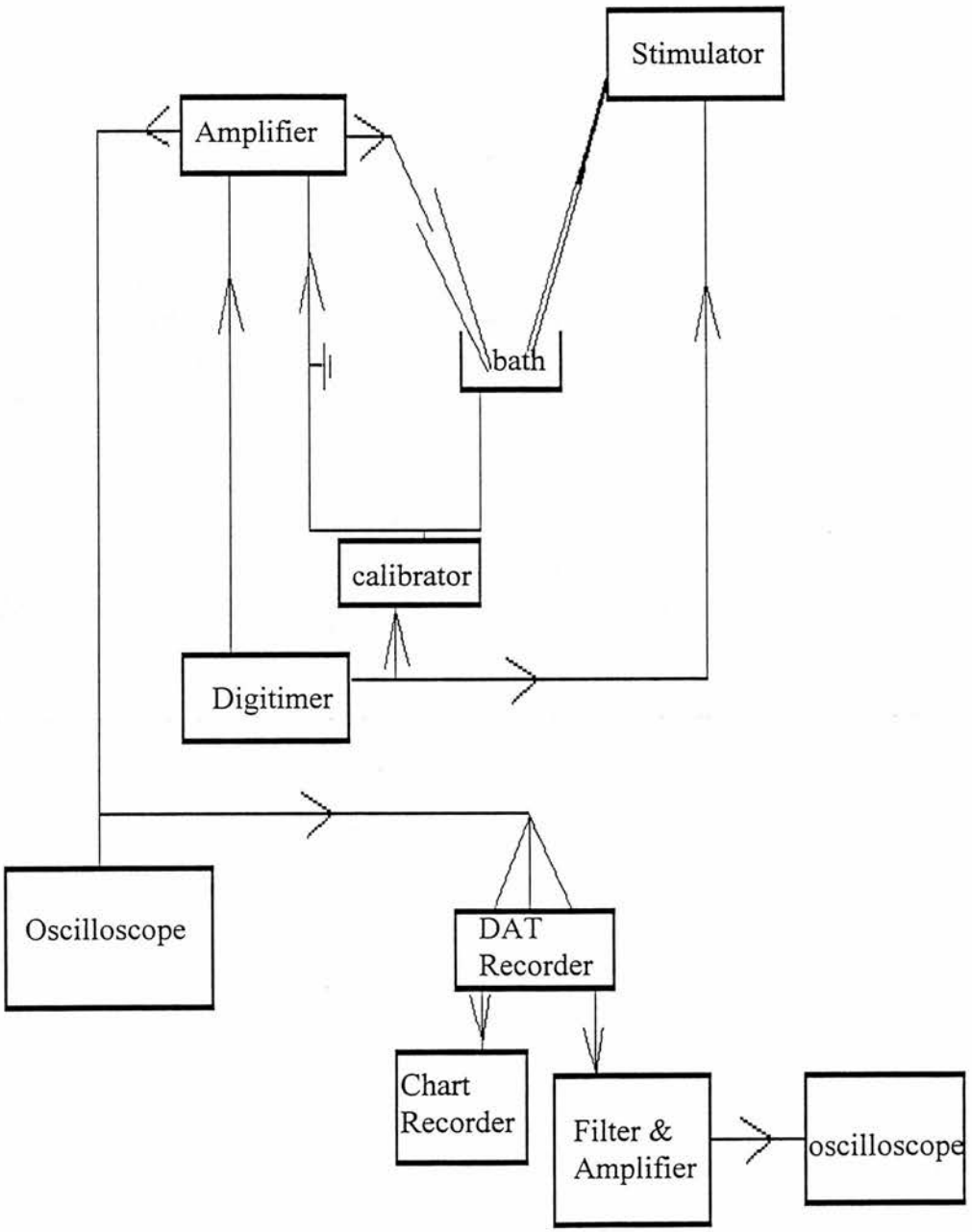


Figure 2.3 Electrical recording apparatus

Excitatory postsynaptic potentials (EPSPs) and currents(EPSCs) were recorded from hippocampal neurones using patch electrodes, the reference electrode was a silver / silverchloride pellet that was placed in the outflow chamber of the recording bath and connected to the ground input of the preamplifier. An axoclamp 2A amplifier (or axopatch 1C, used for some of the voltage clamp experiments, Axon Instruments, CA) was used to amplify the signals and to inject depolarising and hyperpolarising current pulses, or voltage commands in voltage clamp, through the recording electrode. A calibration pulse of 10 mV/1 nA was recorded either at the beginning of the experiment and was used to determine the amplitudes of the responses. A stimulator (S88, Grass) was used to electrically stimulate, using low voltages (1 - 5 V), the presynaptic axons to the cell being recorded from. The timing of all of these events was controlled using a digitimer connected to the stimulator, amplifier and the calibrator. The voltage responses were inspected on an oscilloscope and the time independent components of the voltage response evoked by a hyperpolarising current pulse offset. The signals were recorded onto either video tape (Sony) or DAT (Biologic Instruments) and displayed on another oscilloscope (Gould). The current and voltage records were further amplified before they were viewed on the oscilloscope. The responses were filtered at 1 - 2 Hz using a bessel filter and digitised at 2.5 - 7.5 kHz before data capture onto computer (486DX2, Dell) via the 1401 plus interface (C.E.D.,UK) using commercially available software (CED Cambridge).


Stimulator
Amplifier
bath
calibrator
Digitimer
Oscilloscope
DAT
Recorder
Chart
Recorder
Filter &
Amplifier
oscilloscope

compensation).

(ii) in voltage clamp recordings were made in the continuous single electrode voltage clamp mode (cSEVC), the series resistance was determined prior to switching to the whole-cell voltage clamp configuration, and the fast capacitance transient was minimised.

Recording then commenced in either current or voltage clamp. In all experiments the input resistance of the cell (R_m) was measured by plotting voltage current relationships from measurements of steady state voltage (or current) responses to families of hyperpolarising and depolarising currents (300-400 ms in duration, or voltage 1000 ms in duration) injected through the recording electrode.

From Ohm's law $V = IR$

Thus, $R_m = V/I$

The resting membrane potential of the cell was measured throughout the experiment, taken as the meter value on the axoclamp 2A amplifier, and then adjusted, after withdrawal of the electrode at the end of the experiment, for any offset from zero on the meter. In the voltage clamp experiments the cell was typically voltage-clamped at -70 mV unless otherwise stated. The holding current required to maintain the cell at this potential was measured throughout the experiment, and was taken as the meter value on the axoclamp 2A amplifier.

Two general types of experiments were undertaken: EPSPs or EPSCs were either evoked using a bipolar stainless steel stimulating electrode placed in the stratum radiatum close to the recording electrode, or spontaneous minEPSPs were recorded in the presence of 100 nM tetrodotoxin which blocked the initiation of sodium dependent action potentials. The minEPSPs observed are therefore the result of spontaneously discharged individual quanta released from synapses onto the cell being recorded from.

The data was stored on either video tape (Betamax, Sony, Japan) using a pulse code modulator (PCM, Sony, Japan) in which analogue signals were converted to pulse-code modulated signals (Lamb, 1985), or a digital audio tape (DAT, Biologic, France). The signals were also displayed on an oscilloscope (Gould Instruments) during the course of the experiment. All data was filtered (1 - 2 kHz)

and digitised (2.5 - 7.5 kHz) using an analogue-digital converter (1401 plus, C.E.D., Cambridge, UK) and stored on the hard disc of an IBM compatible computer (Dell, USA) for analysis offline. The patch and voltage clamp software and Spike 2 series of programmes (C.E.D.) were used to analyse the results.

Two adenosine A_1 receptor selective compounds were used to study the presynaptic adenosine A_1 receptor: 2-chloro-N^6-cyclopentyladenosine (CCPA) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX). CCPA is a highly selective receptor agonist with a K_i of 0.4 nM in rat brain membranes and a selectivity ratio of 1:975 for the adenosine A_1 receptor over the adenosine A_2 receptor. DPCPX is a very selective receptor antagonist with an IC_{50} of 0.69 nM and a selectivity ratio of 1:725 for the adenosine A_1 receptor over the A_2 receptor. CCPA was made up in diltue hydrochloric acid (HCl) to a concentration of 1mM, stored at -20 ^{0}C, and serially diluted in ACSF to its final concentration when required. DPCPX was made up in ethanol (100%) to 3 mM on the day of use, and then serially diluted in ACSF to the final concentration used. Other drugs used in the course of these experiments were 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) the AMPA/Kainate receptor antagonist and CGS15943 a mixed adenosine A_1 and A_{2a} receptor antagonist (K_i at the adenosine A_2 receptor = 1.08 nM). CNQX and CGS15943 were both made up in DMSO to 10 mM then serially diluted in ACSF to the required concentration. All compounds were added to the bath using a gravity perfusion system by switching to a separate reservoir containing bath solution plus the drug. Solutions were bubbled for 2-3 minutes before being added to the bath by means of a three way tap into the main perfusion tubing.

Experimental Protocols

In current clamp the series resistance of the cell was monitored, using a 0.04 nA hyperpolarising pulse, and offset using this pulse. In the voltage clamp studies the series resistance was monitored with a 10 mV hyperpolarising pulse and 60 - 70% compensation was used. Cells were voltage clamped at -70 mV unless otherwise stated.

Bipolar stainless steel stimulating electrodes were placed in the stratum radiatum close to the recording electrode. EPSPs and EPSCs were evoked using stimulation frequencies in the range 0.167 - 0.33 Hz . Events were evoked and then the stimulation intensity was decreased until occasional response failures were seen, and left unchanged at that intensity for the duration of the experiment. The effects of CCPA, CCPA co-applied with DPCPX and DPCPX were studied. In control solutions synaptic responses were collected for 10 - 15 minutes and the input resistance of the cell was determined by injecting a series of postive and negative current pulses of different intensities. The drug was washed in for 10 minutes and the synaptic responses and input resistance were measured again. The same protocol in terms of stimulation intensity was followed when a paired pulse protocol was applied; pairs of pulses were delivered at repetition rates of 0.167Hz. Stimulus intensities used were in the range 1 - 5 V. All measurements of EPSPs, EPSCs and the voltage or current responses to the steps used for plotting I-V releationships were measured from baseline to a 5 ms window around the peak. Noise amplitude measurements were made immediately before the event using the same cursor separation as used to measure response amplitude.

In the spontaneous transmitter release experiments the same drug application procedure was followed. For analysis of spontaneous minEPSPs occurring during control and in the presence of drug a 4 minute segment of data was analysed for each condition using the Spike 2 software (CED, Cambridge, UK). This was chosen at random from the stretch of data available. Noise measurements were made for an equivalent period of time, as baseline to peak response, immediately before the minEPSP. The criteria used for selecting the miniature synaptic repsonses were as follows: a fast rise time which distinguished the responses from the flutuations in the baseline noise. The baseline noise was measured for an equivalent time as baseline to peak response immediately before the miniature synaptic potential. The noise measurements were then used to determine whether the distribution of the spontaneous miniature EPSPs measured overlapped the peak of the noise distribution. Using these criteria it is possible that some of the smallest miniature

EPSPs were 'lost in the noise', however this should be consistent across all conditions used.

Quantal Analysis

The site of action of the adenosinergic compounds was determined by quantal analysis using the variance method and by the method of failures. From the Poisson distribution of the fluctuations in size of the EPSPs and EPSCs, it is possible to derive, from the variance of the EPSP amplitude and the mean EPSP amplitude, the mean quantal content of the EPSPs.

A series of EPSPs were recorded using a stimulus intensity in the control period that was adjusted to give EPSPs not larger than 10% of the membrane potential (e.g. <6 mV) to minimize problems associated with non-linear summation (McLachlan & Martin, 1981). Occassional response failures were observed using this stimulus intensity protocol. Only responses where a clear stimulus artifact and/or a clear response of the same delay as others measured in the same cell where a stimulus artifact was seen were included in the analysis. No 'failures' due to a failure of conduction rather than response were included. In each cell 120 - 150 EPSPs were collected under control conditions and in the presence of the drug.

In the spontaneous transmitter release experiments a four minute stretch of data was taken for analysis in each condition: control and in the presence of drug. The data was analysed for minEPSPs, the amplitudes and time of occurrence were recorded using a subroutine within Spike 2 (C.E.D., Cambridge). Two cursors were placed upon the trace, one at the base of the EPSP and a second one at its peak. Once these cursor postions were accepted a third cursor was placed before the first one at a distance equal to the cursor 1 - 2 separation. The temporal position of the first cursor was logged, as were the amplitudes of cursors 1, 2 and 3. From this data amplitude and interval distributions could be drawn up and quantal analysis performed using the minEPSP amplitudes. The interval distributions were compared using the Kolmogorov-Smirnov statistical test.

A series of EPSP amplitudes may be depicted as v_1, v_2, v_3, v_4. If all the quanta were the same size q then the number of quanta, m, in each EPSP would be

v_1/q, v_2/q, v_3/q, v_4/q,....

since, by definition, this series has a Poisson distribution the expected value of the variance is equal to that of the mean. Quanta are not in fact uniform in size and this contributes to the total variance of the EPSPs. Background noise also contributes to the estimated variance and noise measurements were made to allow for its contribution to the EPSP variance to be taken into consideration.

The number of failures was found to be over estimated when a visual count was performed. The noise amplitude distribution was measured and the mean and standard deviation of the noise was used to fit a normal curve to the EPSP amplitude distribution. This was fitted to the peak of the histogram class that the peak fell on and the number of failures under that curve was calculated. The mean quantal content (m) of each cell could then be compared using the two analysis methods.

Statisitics

The paired student's t-test was used for most of the statistics, $P<0.05$ was taken as the significance level and values shown are means ± standard errors. The amplitude and interval distributions were compared using the Kolmogorov-Smirnov two sample test, $P<0.05$ was taken as the significance level.

Chapter 3

Postsynaptic Responses

Introduction

Adenosine A_1 receptors are located on the terminals of the Schaffer collateral commissural fibres and on the postsynaptic CA1 pyramidal neurones. This complicates the study of the presynaptic actions of adenosine, as the postsynaptic effects of the drugs may obscure the presynaptic action. Adenosine activates a G protein-coupled potassium channel in hippocampal CA1 neurones via the adenosine A_1 receptor (Thompson et al., 1992; Greene & Haas, 1985). This conductance could shunt synaptic currents generated in the dendrites, reducing the amount of current that reaches the cell bodies, thereby decreasing the glutamatergic EPSPs. Therefore it is important to know if the membrane resistance changes, due to increased or decreased channel activation, in the presence of the adenosinergic compounds.

In these experiments the whole-cell patch-clamp technique was used to determine postsynaptic responses to evoked excitatory potentials in order to determine a concentration of the adenosine A_1 agonist, 2-chloro-N^6-cyclopentyladenosine (CCPA) that was sufficient to half maximally inhibit evoked potentials. This concentration of CCPA was used in further experiments. It should have minimal effect on postsynaptic parameters such as input resistance and membrane potential in order to determine whether changes seen are due to pre- or postsynaptic actions of the agonist.

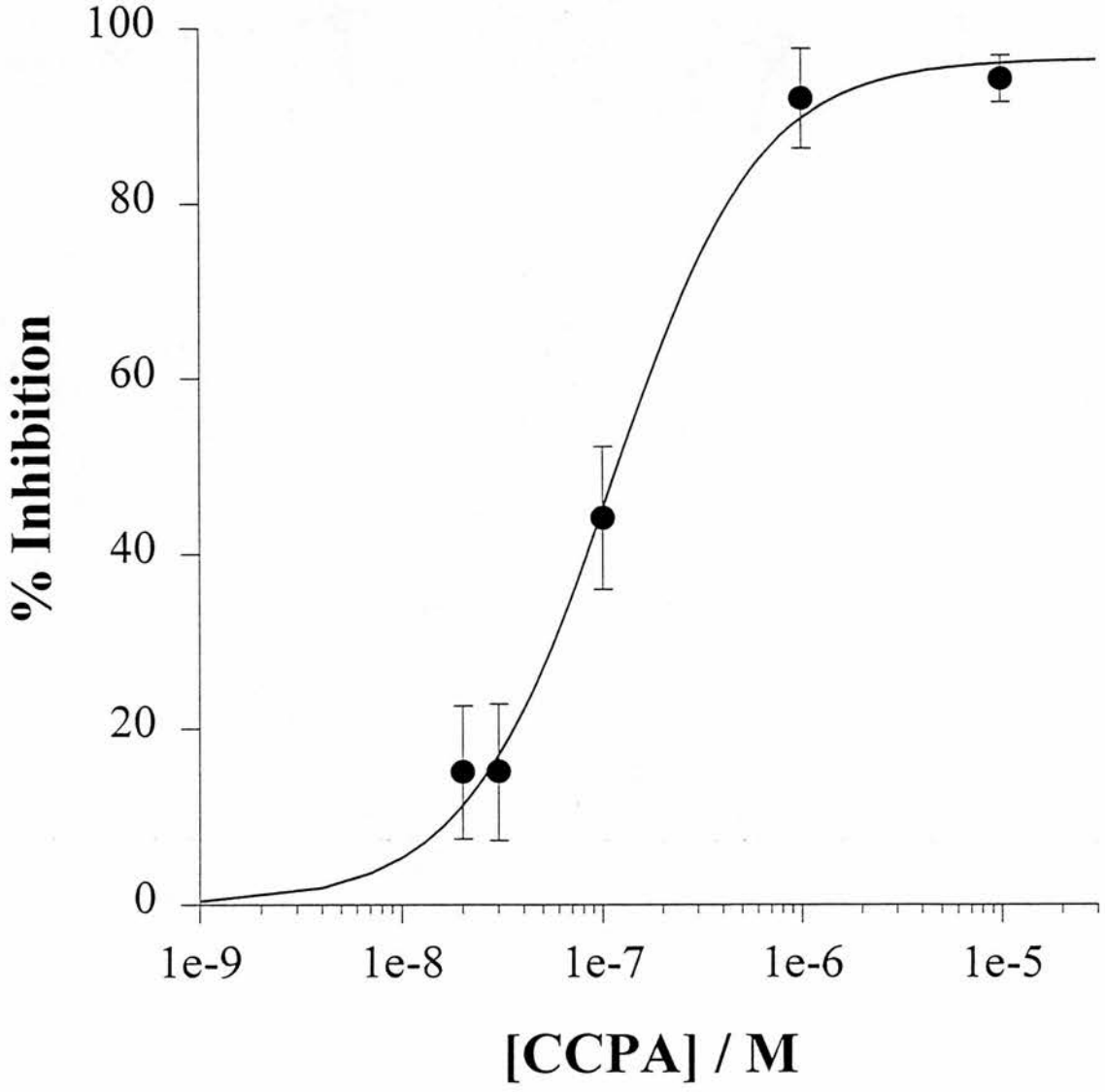
Results

The data presented here is based upon whole cell blind patch clamp recordings made from rat hippocampal CA1 neurones that had initial resting membrane potentials greater than -50 mV and had action potentials that overshot 0 mV.

The concentration of 2-chloro-N^6-cyclopentyladenosine (CCPA) that produced half maximal inhibition of evoked EPSPs (Fig. 3.1) was determined by exposing CA1 neurones to a range of concentrations of the agonist. It was found that the EPSPs could be almost completely (96 %) inhibited when exposed to 10 μM CCPA for 10 - 15 minutes, and this was accompanied by a large hyperpolarisation of the postsynaptic neurone. All other concentrations of CCPA (20 nM - 1 μM) did not

Figure 3.1 Dose Response curve for CCPA

The percentage inhibition of glutamatergic excitatory postsynaptic potentials (EPSPs), recorded from hippocampal pyramidal CA1 neurones, was determined for a range of concentrations of the adenosine A_1 agonist CCPA. The slices were bathed in artificial cerebrospinal fluid (ACSF) at room temperature, the recording pipette contained a K-gluconate/KCl based solution. CCPA was serially diluted, from stock, in ACSF to the final concentration exposed to the slice. The mean amplitude of 20 - 40 EPSPs in control and after exposure to CCPA was measured and the percentage inhibition of the control EPSP calculated. Each point is the mean and SEM of 2 - 4 individual experiments. Maximum inhibition was seen at 10 μM CCPA where the EPSP was reduced to just 4 % of control. The EC_{50} was determined by fitting a logistic curve to the data and was calculated to be 114 nM and the logistic exponent, $P = 1.179 \pm 0.173$. The membrane potential (E_m) was also recorded (data not shown) in each cell. CCPA did not significantly change E_m at concentrations between 10 nM and 1 μM. However at 10 μM CCPA the membrane potential became significantly hyperpolarised, increasing to -69.3 ± 0.88 mV from -54.0 ± 3.06 mV under control conditions ($P < 0.05$, $n = 3$).


100
80
60
40
20
0
% Inhibition
1e-9
1e-8
1e-7
1e-6
1e-5
[CCPA] / M

have a significant effect on the membrane potential although reduction in the amplitude of the EPSP from control was observed. The concentration of CCPA that produced half maximal inhibition of the EPSP was determined by fitting a logistic curve to the dose response curve using a least squares fitting routine. The EC_{50} was found to be 114 nM. Therefore in all subsequent experiments 100 nM CCPA was used.

In three experiments the evoked EPSP amplitude was measured under i) control conditions, ii) in the presence of 100 nM CCPA and iii) in the presence of CCPA and DPCPX (30 nM, Fig. 3.2). It was found that CCPA decreased the amplitude of the evoked EPSP by 42.6 %. DPCPX completely reversed this inhibition in one neurone and in three neurones the inhibition was reversed to a mean of 72.3 % of the control value. The concentration of DPCPX used was sufficient to partially reverse the CCPA inhibition.

The effect of CCPA (100 nM) on the input resistance (R_m) of the hippocampal CA1 neurones was determined (Fig. 3.3B). The cell was subjected to a series of hyperpolarising current steps (Fig. 3.3A, lower traces) and the hyperpolarising voltage responses (Fig. 3.3A upper traces) of the neurone recorded. The responses show a voltage- and time-dependent depolarising 'sag'. The data was plotted and the R_m calculated by the fitting of a first order regression line. When the data was pooled from 12 similar experiments, it was found that CCPA (100 nM) did not significantly alter the resting membrane potential (E_m) of the CA1 neurone (Fig. 3.5A) or the R_m (Fig. 3.5B) from control values. The E_m in control was -59.2 ± 1.65 mV and a small hyperpolarisation to -59.3 ± 1.80 mV ($P > 0.9$, paired student's t-test) was observed after exposure to CCPA. The R_m decreased from a control value of 131.4 ± 3.06 MΩ to 123.1 ± 6.94 MΩ in the presence of CCPA. This decrease was not significant ($P < 0.25$, paired student's t-test). The reversal potential for the change in R_m was calculated from the intersect of the I-V relationships plotted under control conditions and in the presence of CCPA. The mean reversal potential was -63.7 ± 6.02 mV (mean ± SEM, n = 12). From the Nernst equation ($E_k = (RT/F)\ln([K]_o/[K]_i)$, where room temperature is assumed to be 20 ^{0}C) the reversal potential for potassium under our experimental conditions was -99.3 mV assuming

Figure 3.2 Reversal of CCPA Inhibition of the EPSP by DPCPX

A shows an EPSP recorded from a hippocampal CA1 pyramidal neurone under control conditions, B after 15 minutes exposure to 100 nM CCPA and C after 10 - 15 minutes exposure to CCPA (100 nM) and DPCPX (30 nM). D shows the three EPSPs A, B and C superimposed. As can be seen from this figure DPCPX, at a concentration of 30 nM, reversed the CCPA inhibition. For this neurone the mean amplitude was 2.11 ± 0.03 mV under control conditions, 1.41 ± 0.02 mV in the presence of CCPA and 2.14 ± 0.07 mV after exposure to CCPA and DPCPX (n = 25 for each condition).

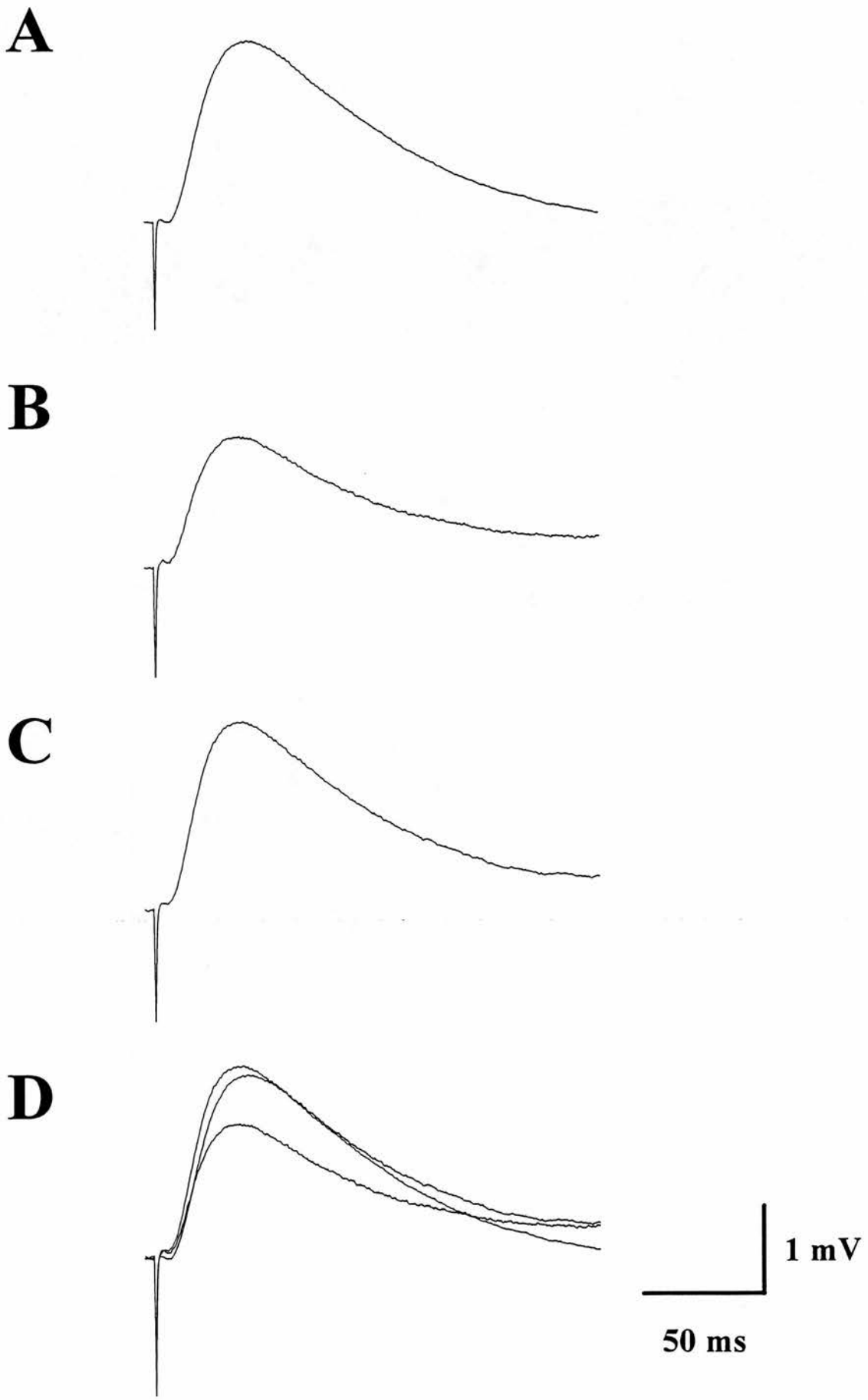
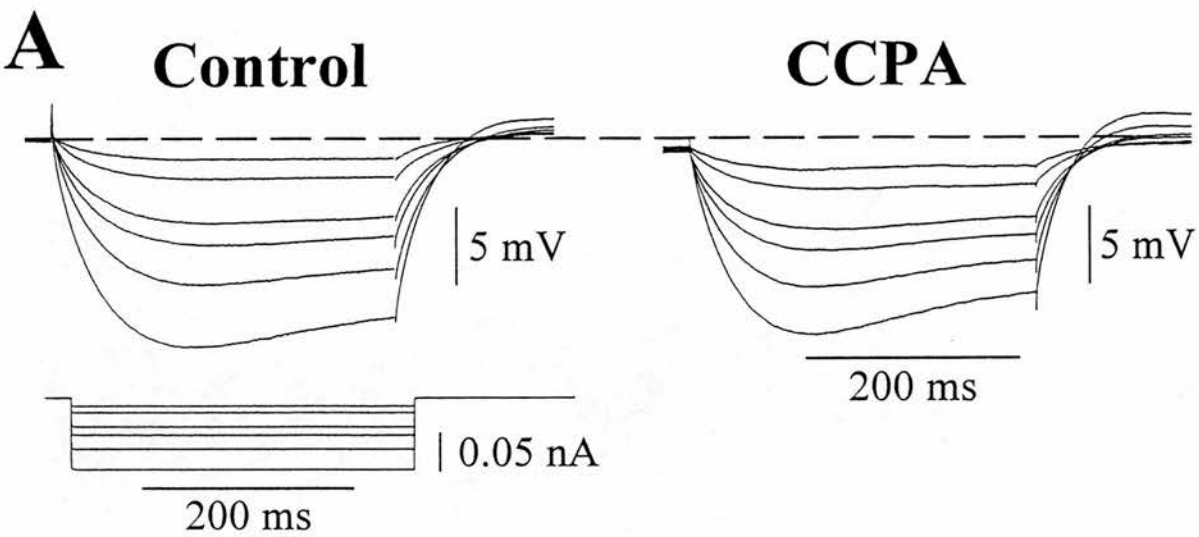
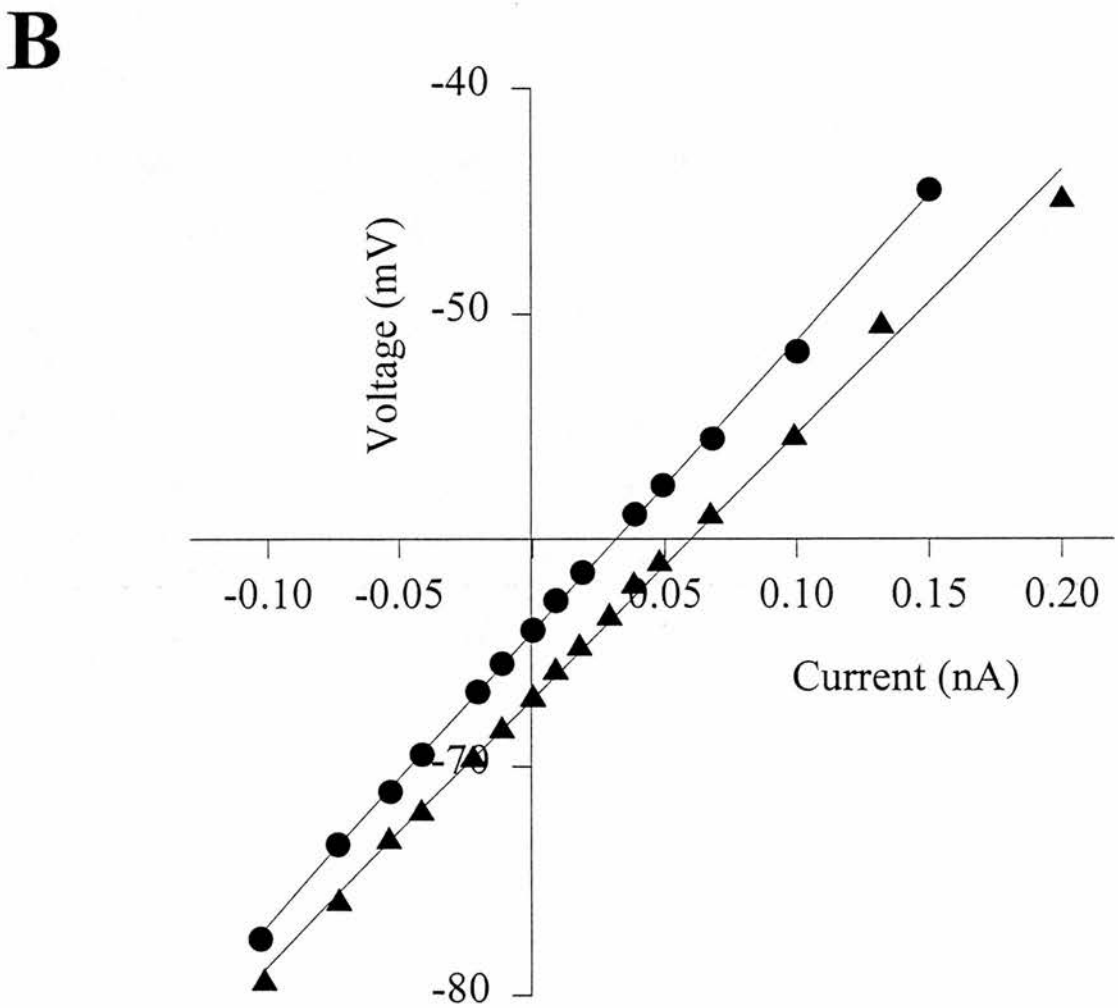

A
B
C
D
1 mV
50 ms

Figure 3.3 Effect of CCPA on the Input Resistance of a CA1 Neurone

A shows a series of hyperpolarising current steps (lower traces) and the voltage response of the hippocampal CA1 neurone to those currents (upper traces). Each trace is the average of six sweeps. From these traces the size of the response to the current steps was measured from base line to peak response and plotted as shown in B. The data could be fitted by a first order regression, the slope of which gave the input resistance (R_m) of the neurone. In control (circles) the R_m in this neurone shown was 128.9 MΩ and in the presence of 100 nM CCPA (triangles) it decreased to 117.0 MΩ. CCPA also caused a small hyperpolarisation of the CA1 neurone, the resting membrane potential (E_m) increasing to -67 mV from -64 mV in control. The reversal potential was calculated and found to be -95.9 mV for this neurone.


A
Control
CCPA
5 mV
5 mV
200 ms
0.05 nA
200 ms


B
-40
-50
-70
-80
Voltage (mV)
-0.10
-0.05
0.05
0.10
0.15
0.20
Current (nA)

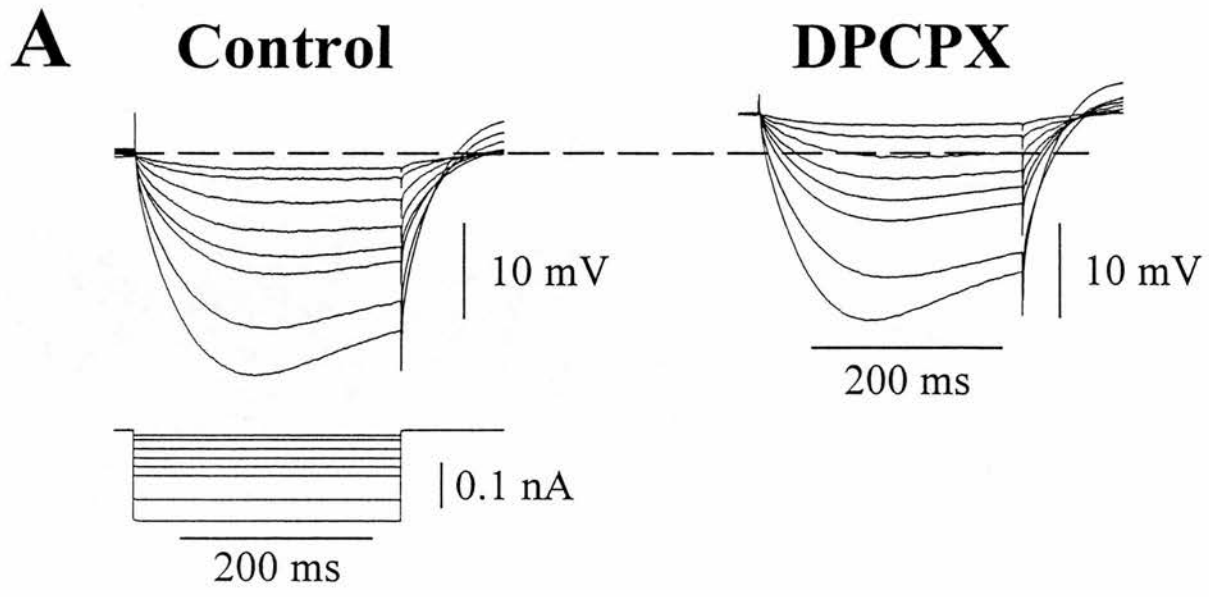
that the potassium channel does not show rectification. This result indicates that CCPA is acting via the adenosine A_1 receptor to activate potassium channels in the postsynaptic membrane. The reversal potentials for sodium and calcium ions in this system were +71.6 mV and +23.1 mV respectively.

The selective adenosine A_1 antagonist DPCPX was applied to the hippocampal slice. It was hypothesised that it would depolarise the CA1 neurones, as the endogenous adenosine, acting at the adenosine A_1 receptor, would be antagonised, and that an increase in the input resistance would be seen as the potassium conductance activated by adenosine would be shut down. Figure 3.4 shows the results of current - voltage measurements recorded in current clamp from a hippocampal CA1 neurone. A series of hyperpolarising current pulses (Fig. 3.4A, lower traces) were applied to the neurone and the hyperpolarising voltage response of the neurone recorded (Fig. 3.4A upper traces). The data were plotted and fitted with a first order regression (Fig. 3.4B). It was found, in this and 9 similar experiments, that DPCPX (30 nM) depolarised the CA1 neurones as hypothesised (Fig. 3.5A), in control E_m was -60.1 ± 1.89 mV and after 10 minutes exposure to DPCPX the E_m had decreased to -57.9 ± 2.1 mV ($P > 0.05$, paired student's t-test; $n = 10$). In addition it was found that the R_m decreased significantly (Fig. 3.5B), from 136.0 ± 8.53 MΩ under control conditions to 113.6 ± 9.72 MΩ in the presence of 30 nM DPCPX ($P < 0.005$, paired student's t-test; $n = 10$), the opposite to the result predicted.

Control experiments were carried out in which any effects due to the vehicle used were examined. DPCPX was dissolved in 100 % ethanol to make a 3 mM solution. This was then serially diluted in ACSF (1:100,000) to the final concentration of use, 30 nM. Ethanol at 0.001 % and 0.01 % were applied to the hippocampal slice, and recordings from CA1 neurones undertaken. No significant effect on E_m, R_m or on the evoked EPSP amplitude was observed (data not shown). CCPA was dissolved in 100 mM sulphuric acid to a concentration of 1 mM. This was serially diluted (1:10,000) to the final concentration of 100 nM. At this concentration no significant effects on the pH of the ACSF were observed. CNQX and CGS 15943 were both made up in DMSO, and a final concentration of 0.1 % DMSO was exposed to the slice.

Figure 3.4 Effect of DPCPX on the Input Resistance of a CA1 Neurone

A shows a family of voltage responses (upper traces) to a series of hyperpolarising current pulses (lower traces). Each trace is the average of six sweeps. The amplitude of the voltage response to the series of current steps was measured from baseline to peak response and plotted. The amplitude was measured to the near instantaneous peak as this is prior to activation of any subsequent currents e.g. the hyperpolarisation activated current (I_h). The data was plotted (B) and fitted with a first order regression, from this the R_m was calculated. In the cell illustrated the R_m in control (circles) was 131.7 MΩ and after exposure to 30 nM DPCPX (inverted triangles) for 10 - 15 minutes the R_m had decreased to 108.4 MΩ. In 10 CA1 neurones the control R_m was 136.0 ± 8.53 MΩ and after exposure to DPCPX R_m decreased significantly to 113.6 ± 9.72 MΩ ($P<0.005$, paired student's t-test). In this neurone DPCPX produced a slight depolarisation the membrane potential (E_m) falling to -53 mV from a control value of -57 mV. As can be seen from B the two plots (control and DPCPX) intersect at a depolarised potential, in this neurone the reversal potential was -34.4 mV.


A
Control
DPCPX
10 mV
10 mV
200 ms
0.1 nA
200 ms

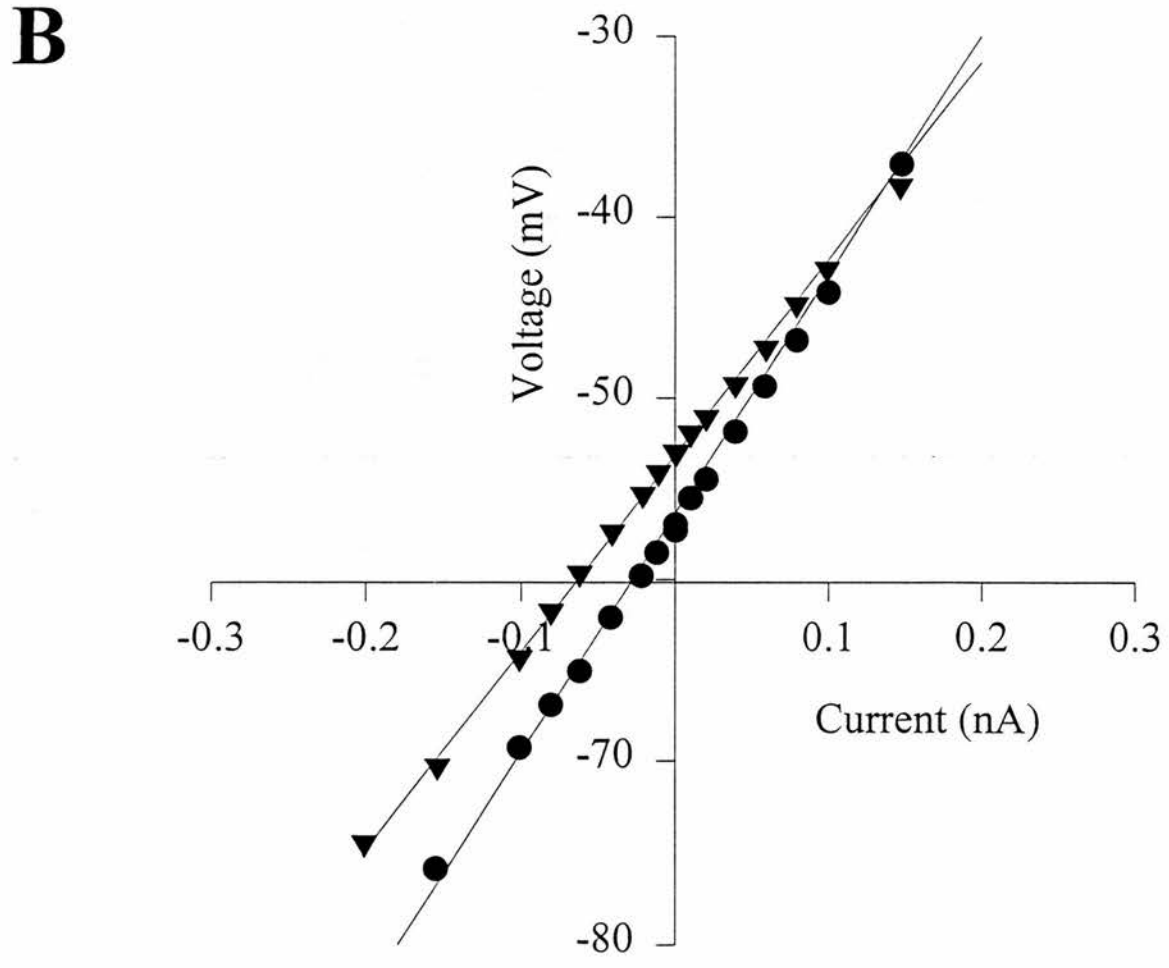
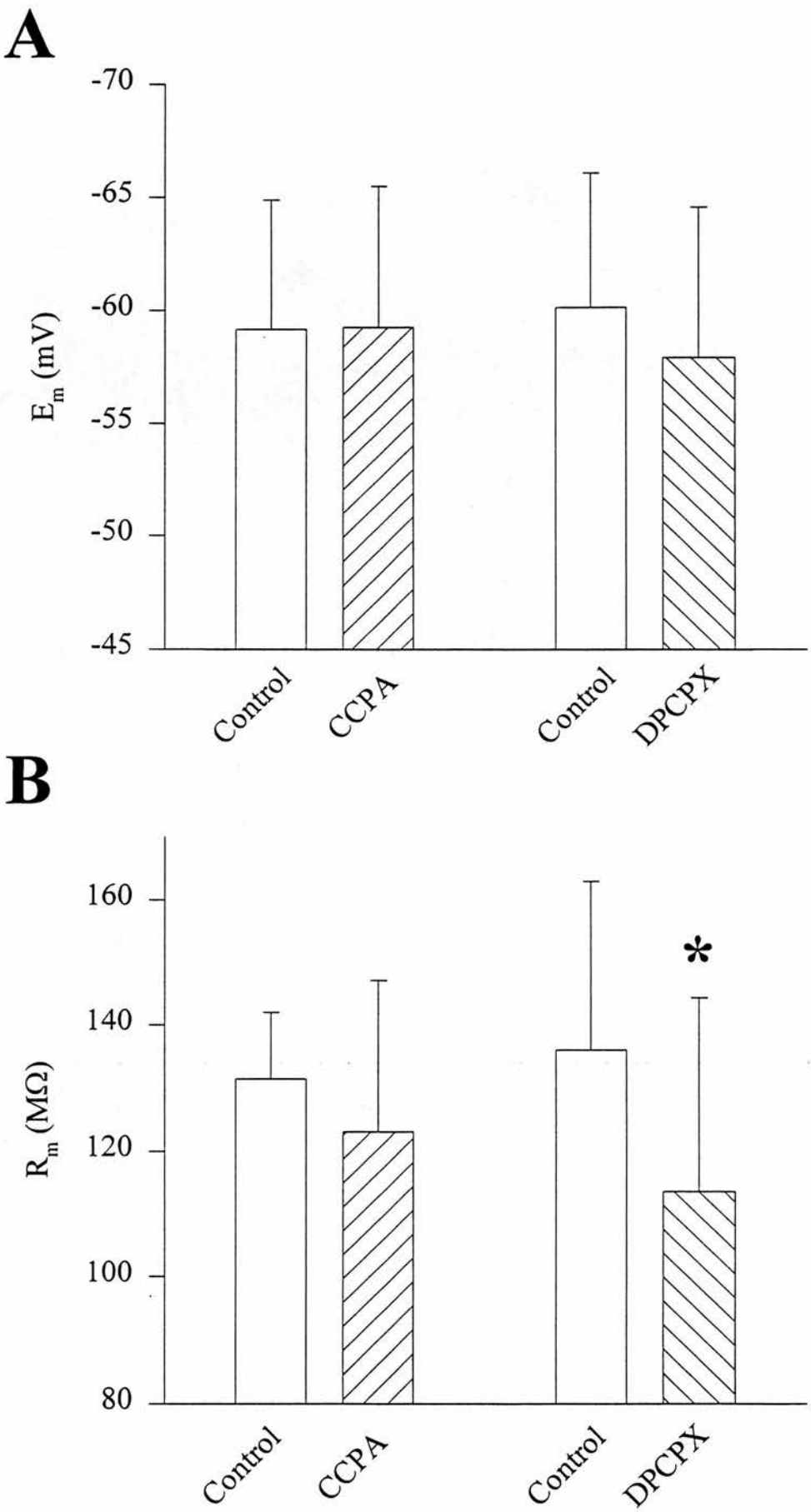

B
-30
-40
-50
-70
-80
Voltage (mV)
-0.3
-0.2
-0.1
0.1
0.2
0.3
Current (nA)

Figure 3.5 Effects of the Adenosinergic Drugs on E_m and R_m

A shows a histogram of the membrane potential of hippocampal pyramidal CA1 neurones in the presence of either 100 nM CCPA or 30 nM DPCPX (washed in for 10 minutes exposure) and their respective controls. In 12 neurones CCPA did not significantly alter the resting membrane potential; control -59.2 ± 1.65 mV, CCPA (100 nM) -59.3 ± 1.8 mV ($P > 0.9$, paired student's t-test). In 10 neurones DPCPX did not significantly change E_m; control -60.1 ± 1.9 mV, DPCPX (30 nM) -57.9 ± 2.1 mV ($P > 0.05$, paired student's t-test). B shows the mean R_m for these neurones. CCPA does not significantly decrease the R_m; control 131.4 ± 3.06 MΩ, CCPA (100 nM) 123.1 ± 6.94 MΩ ($P > 0.2$, paired student's t-test). However in the presence of DPCPX (30 nM) R_m decreases significantly (*) from 136.0 ± 8.53 MΩ to 113.6 ± 9.72 MΩ ($P < 0.005$, paired student's t-test).


A
-70
-65
-60
-55
-50
-45
E_m (mV)
Control
CCPA
Control
DPCPX
B
160
140
120
100
80
R_m (MΩ)
*
Control
CCPA
Control
DPCPX

Discussion

These experiments determined the magnitude of any postsynaptic action of the adenosinergic drugs used using the whole cell patch clamp technique.

It was found that the adenosine A_1 agonist, CCPA, at high concentrations (10 μM) significantly hyperpolarised the CA1 neurone, but at lower concentrations although a hyperpolarisation was observed it was not significantly different from control. Segal (1982), using sharp electrodes, reported that adenosine applied iontophoreically to hippocampal CA1 neurones caused a large hyperpolarisation at the neuronal membrane which was accompanied by a reduction in input resistance. At the concentration used throughout this series of experiments (100 nM) CCPA caused a small, non-significant, decrease in R_m.

The selective adenosine A_1 receptor agonist R-PIA (1 μM) was reported (Thompson et al., 1992) to cause a large hyperpolarisation in CA3 neurones, an effect comparable to 50 μM adenosine. In CA1 neurones contained in the acutely prepared hippocampal slice, 10 μM CCPA caused a hyperpolarisation which was of a similar magnitude to that observed by Thompson et al., (1992), using sharp electrode intracellular recording, in CA3 neurones contained in the organotypic hippocampal slice culture when exposed to adenosine (50 μM) or R-PIA (1 μM). At these concentrations of the adenosine A_1 agonists the evoked EPSP was inhibited to the same degree (96 %).

As CCPA did not have a significant effect on the input resistance of the CA1 neurones and no effect on their membrane potential, any effects of CCPA on the amplitude of spontaneous or evoked EPSPs can be thought to be due to a presynaptic action at the presynaptic A_1 receptor. Any postsynaptic shunting of the synaptic current generated in the dendrites occured at levels which did not cause a statistically significant effect.

The adenosine A_1 antagonist, DPCPX, had an unexpected effect on the input resistance (or input conductance in voltage clamp experiments) of the hippocampal CA1 neurone, whilst depolarising the cell membrane (not significantly) as predicted. As DPCPX is an adenosine A_1 antagonist, and the adenosine A_1 agonist, CCPA, decreases the input resistance of the CA1 neurone, blocking the effects of any

endogenous adenosine would be predicted to increase the input resistance. In all the initial experiments DPCPX decreased the input resistance of the CA1 neurones, and increased the input conductance, the opposite effect to that hypothesised. Pape (1992) reported that DPCPX had no effect on the membrane potential or the input resistance of relay neurones of the dorsal lateral geniculate nucleus (LGND) when recording with sharp electrodes. In the organotypic slice culture (Thompson et al., 1992) reported that DPCPX (200 nM) had no effect on membrane potential or on the amplitude of synaptic responses, indicating that there was no tonic activation of adenosine receptors in this preparation. In the acute slice a tonic activation of the adenosine A_1 receptor was observed: a depolarisation of the CA1 neurone was seen and in one of the reversal experiments DPCPX reversed the CCPA inhibited evoked EPSP amplitude to a greater value than that seen in control, suggesting a tonic inhibition by endogenous adenosine.

The decrease in input resistance observed in the presence of DPCPX could potentially obscure any presynaptic actions of DPCPX in this preparation.

$$V = I*R \qquad \text{(Ohm's Law) (1)}$$

Then, across a cell membrane $V_{AB} = E + IR$

(where A is the outside of the cell, B the inside, and E the membrane potential of the cell)

Rearranging gives $I = G*(V_{AB} - E)$ (where $G = I/R$) (2)

In the postsynaptic cell, blockade of the tonic inhibition due to adenosine acting at adenosine A_1 receptors may activate a conductance(s), in addition to those already active (see Figure 3.6).

$$I^1 = G(V^1 - E) \quad (3)$$

$$i = g(V^1 - e) \quad (4)$$

and $$I = I^1 + i \quad (5)$$

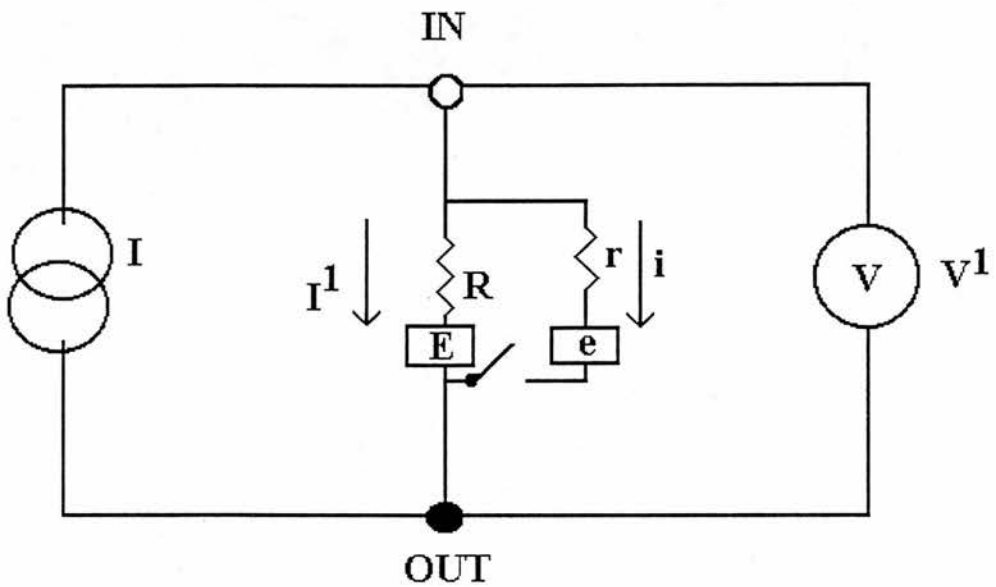
$$\Delta V = \text{synaptic potential} = V^1 - V \quad (6)$$

Substituting equations 3 and 4 into equation 5, and simplifying using 6, the result is

$$\Delta V = (g/(g + G))(e - V)$$

i.e. Synaptic potential is inversely proportional to membrane conductance or proportional to membrane resistance (see Ginsbourg, 1973).

Figure 3.6



This suggests that when a decrease in input resistance, to 83.5 % of control was observed, after application of DPCPX, a similar decrease in the amplitude of the synaptic potential would be expected, if there was no presynaptic change in quantal content.

If the response to DPCPX was an amplitude decrease it could be due to either a pre- or postsynaptic action of DPCPX. However if an increase in amplitude was observed in conjunction with a decrease in input resistance then it most probably due to a presynaptic action of DPCPX.

Chapter 4

Evoked Release

Introduction

In the central nervous system the hypothesis that adenosine acts to decrease synaptic transmission via a presynaptic action relies upon indirect evidence as excitatory neurotransmitter release is typically evoked by the simultaneous activation of many presynaptic terminals, and as adenosine also acts to hyperpolarise CNS neurones (Greene & Haas, 1985; Proctor & Dunwiddie, 1987). The coexistence of both pre- and postsynaptic actions of adenosine has made it difficult to determine the relative contribution of each of these to the overall regulation of neuronal excitability. Possible explanations that would account for the inhibition of excitatory postsynaptic responses activated through stimulation of excitatory afferents of CA1 pyramidal neurones in the hippocampus include:

Presynaptically - i) adenosine may decrease neurotransmitter release by reducing the number of vesicles released upon stimulation, ii) by limiting the amount of excitatory transmitter in each vesicle, or iii) by diminishing the number of release sites. Postsynaptically - i) adenosine could reduce neuronal sensitivity to the excitatory transmitter, e.g. at the CA1 neurone sensitivity to glutamate or ii) activate other ion channels that would shunt the excitatory response recorded at the pyramidal neuronal soma. A combination of these factors may account for the inhibition produced by adenosine.

At the neuromuscular junction there are a very large number of quanta (vesicles) available to respond to a presynaptic action potential, but the probability of release is relatively low (del Castillo & Katz, 1954a,b). The probability that one vesicle is released has been shown to be normally independent of the release of any other vesicle (Fatt & Katz, 1952; Barrett & Stevens, 1972). However when two stimuli are applied in close succession to a mammalian neuromuscular junction the amplitude of the second endplate potential is reduced compared to the first (Betz, 1970), a phenomenon known as paired pulse depression (PPD). When the two stimuli are applied under conditions where the release probability has been reduced, such as increasing the external $[Mg^{2+}]/[Ca^{2+}]$ ratio, the second response is larger than the first, this is known as paired pulse facilitation (PPF; del Castillo & Katz, 1954a,c; Katz & Miledi, 1968). In the hippocampus PPF of excitatory synaptic potentials in

areas CA1 and CA3 is observed when large numbers of axons are stimulated (Creager et al., 1980; Manabe et al., 1993).

At the neuromuscular junction it has been conclusively established, by using quantal analysis of the end-plate potentials at single nerve-muscle synapses, that adenosine reduces the average number of quanta of ACh released from the nerve terminal (quantal content, *m*), without affecting the size of individual quanta (*q*) (Ginsborg & Hirst, 1972; Silinsky, 1984). This statistical analysis of the synaptic variability is a technique that has been used in the hippocampus to determine whether alterations in synaptic efficacy are derived from pre- or postsynaptic sources (Bekkers & Stevens, 1990; Malinow & Tsien, 1990). These statistical techniques, together with the use of the low-noise whole-cell recording configuration to record the synaptic responses to applied stimuli (single or paired), from hippocampal CA1 neurones allows a detailed evaluation of the presynaptic versus the postsynaptic actions of the adenosinergic compounds. Recordings were made in both current clamp and voltage clamp and the quantal parameters *m* and *q* were determined.

In order to determine the quantal content, *m*, two different analysis methods were used: i) the method of failures (m_f) and for confirmation of the results provided by the method of failures the less accurate method ii) the coefficient of variation (m_{cv}). The coefficient of variation (CV) of a simple binomial distribution is a parameter that is dependent only on the number of release sites (*n*) and the probability of release (*p*); $CV = \sqrt{[(1-p)/np]}$. Hence CV is determined by variables that are measurements of presynaptic function and is independent of quantal amplitude (*q*). CV can be compared before and after alterations in synaptic efficacy to determine whether the change is a presynaptic phenomenon, and reflected in a change in CV. Alternatively postsynaptic changes would not be accompanied by changes in CV (Martin, 1966). A closely related statistical parameter used in quantal analysis is mean^2/variance (M^2/σ^2) (Malinow & Tsien, 1990), where M is the mean amplitude of the synaptic response and σ^2 is the variance of the mean; M^2/σ^2 is equal to $(CV)^{-2}$. In the CNS there is some controversy about the exact nature of the statistics that can be used to describe synaptic transmission. The index M^2/σ^2 has been reported to change in the expected manner after procedures known to result in

modifications of either presynaptic or postsynaptic function (Lupica et al. 1992; Malinow & Tsien, 1990). In situations where there is a non-uniform postsynaptic modulation it has been reported that M^2/σ^2 could change (Faber & Korn, 1991).

Recordings were made in current clamp and in voltage clamp from hippocampal CA1 neurones and from granule cells of the dentate gyrus. The effects of adenosinergic compounds on release were studied using a minimal stimulation protocol and either single pulses or pairs of pulses. For a more detailed analysis of the effects of the adenosinergic compounds the quantal parameters *m* and *q* were calculated and compared under control and test conditions.

Results

Single evoked responses

Single evoked potentials were evoked using stimulating electrodes placed in the stratum radiatum and were recorded from hippocampal pyramidal CA1 neurones. The Schaffer collateral commissural fibres were stimulated using a minimal stimulation protocol - EPSPs were evoked then the stimulus intensity was reduced until 'failures' were observed in response to the stimulus (Fig 4.1A). A failure was defined as an event which failed to rise above the background noise.

Current Clamp

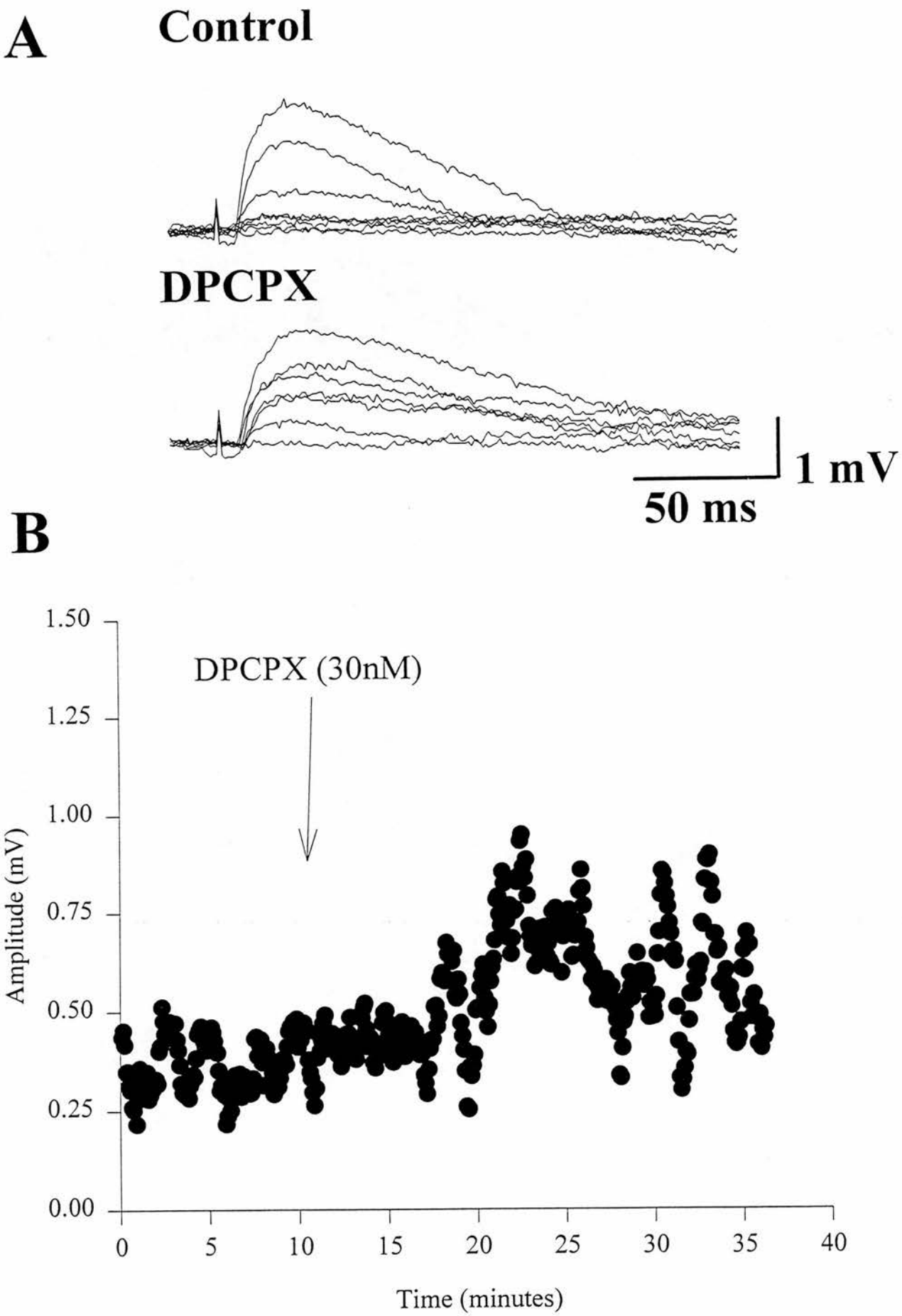
In a series of current clamp experiments the EPSPs were measured in control and in the presence of the adenosine A_1 receptor antagonist DPCPX (30 nM). This concentration of DPCPX was used as it was shown to completely reverse the inhibitory effect of 100 nM CCPA on spontaneous miniature EPSPs, also a low concentration of DPCPX would 'drive the system just a little harder' by producing a small increase in the excitatory state of the presynaptic terminals. This concentration of DPCPX should be selective for the adenosine A_1 receptors.

The amplitude of the evoked EPSPs was increased after 15 minutes exposure to DPCPX (Fig. 4.1B). The amplitude distribution was shifted to the right in the presence of DPCPX when compared to the control distribution, this change in the

Figure 4.1 Mean EPSP amplitude plotted versus Time

Excitatory postsynaptic potentials (EPSPs) were evoked by stimulation of the stratum radiatum and recorded from a CA1 neurone (E_m = -68 mV under control conditions). A: Eight consecutive representative traces showing EPSPs evoked in control (upper) and in the presence of DPCPX (30nM, Em = -64 mV, lower). The stimulation intensity was adjusted such that a low number of response failures were observed (< 15 % of total trials). Failures of transmission were judged subjectively for each trace. The integral of the mean 'failure' response did not differ from an integral calculated for the same period prior to the stimulus artefact, i.e. these responses could not be distinguished from the background noise.

B: The EPSP amplitudes were plotted as a rolling mean versus time. The rolling mean was calculated from the amplitude of 6 successive EPSPs, the first point represents the mean of the first 6 EPSPs, the second point represents the mean of EPSPs 2-7 etc... DPCPX (30 nM) was applied after a 10 minute control period, and EPSPs collected continuously for the next 25 minutes. The mean and variance of the EPSP amplitudes increased in the presence of DPCPX for this neurone. The mean amplitude increased 1.55 fold from 0.37 mV in control to 0.57 mV.


A
Control
DPCPX
1 mV
50 ms
B
DPCPX (30nM)
Amplitude (mV)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
5
10
15
20
25
30
35
40
Time (minutes)

amplitude distribution was observed in all 4 neurones and was significant (Kolmogorov-Smirnov Test, $P<0.001$).

The same neurone is illustrated in Figure 4.2, where the same number of EPSPs were compared in both conditions. The amplitude distribution is shown in control (A) and after 15 minutes exposure to DPCPX (B). The noise distribution is shown as a downwards deflection, the number of failures fitted by a normal distribution taken from the noise distribution is illustrated as a curve fitted to the histogram class where the mean falls. When the data from 4 similar experiments was combined it was found that under control conditions the mean amplitude was 0.62 ± 0.20 mV (Mean ± SEM, $n = 4$), after exposure to DPCPX the mean amplitude had increased to 0.87 ± 0.26 mV. This increase was not significant ($P = 0.055$, paired t-test). In each neurone the individual amplitudes were less than 5 % of the membrane potential, therefore they have not been corrected for non-linear summation (McLachlan & Martin, 1981). The mean membrane potential was -62 mV in control, falling to -60.5 mV in the presence of DPCPX.

Voltage Clamp

This series of experiments was repeated under voltage clamp conditions in 5 neurones (Fig. 4.3). The cell was continuously clamped at -70 mV and EPSCs evoked using the same protocol as for the EPSPs. When the data from 5 similar experiments were pooled it was found that under control conditions the mean amplitude was 21.7 ± 7.73 pA, this increased to 32.4 ± 10.9 pA after 15 minutes exposure to DPCPX. This increase was significant ($P < 0.05$, paired t-test). In all 5 neurones an increase in mean amplitude was observed.

A further series of voltage clamp recordings were made from granule cells contained in the dentate gyrus. The stimulating electrodes were placed on the perforant path and the intensity adjusted such that EPSCs were evoked and failures were observed. Under control conditions the mean amplitude was 8.02 ± 1.28 pA, after 15 minutes exposure to DPCPX the mean amplitude was 7.96 ± 1.98 pA. There was no significant difference ($n = 6$). In 3 out of 6 sites a decrease in the amplitude was seen, with an increase being observed in just 2 neurones.

Figure 4.2 Distribution of single EPSP amplitudes.

A: Amplitude distribution of 120 EPSPs in control. The noise, determined by measuring over similar intervals as used to measure EPSP amplitude, is shown as a normal distribution with a peak matched to the histogram class corresponding to the mean noise amplitude. The actual noise distribution (hatched bars) is also shown to illustrate the data needed to generate the normal distribution. The area under the curve corresponds to the number of failures and was 7 in this neurone, the mean amplitude of the EPSP was 0.37 mV. The quantal content, determined by the method failures (m_f) and the coefficient of variation (m_{cv}), was 2.84 and 6.65 respectively. B: Histogram of 120 EPSP amplitudes after exposure to DPCPX (30 nM) for 15 minutes; the EPSP amplitude increased to 0.57 mV and there were 3 response failures. The quantal content was increased to 3.69 for m_f. A 1.55 fold increase was observed in amplitude after exposure to DPCPX whilst m_f increased 1.30 fold. DPCPX caused a significant change in the EPSP amplitude distribution (Kolmogorov-Smirnov test, $P < 0.001$).

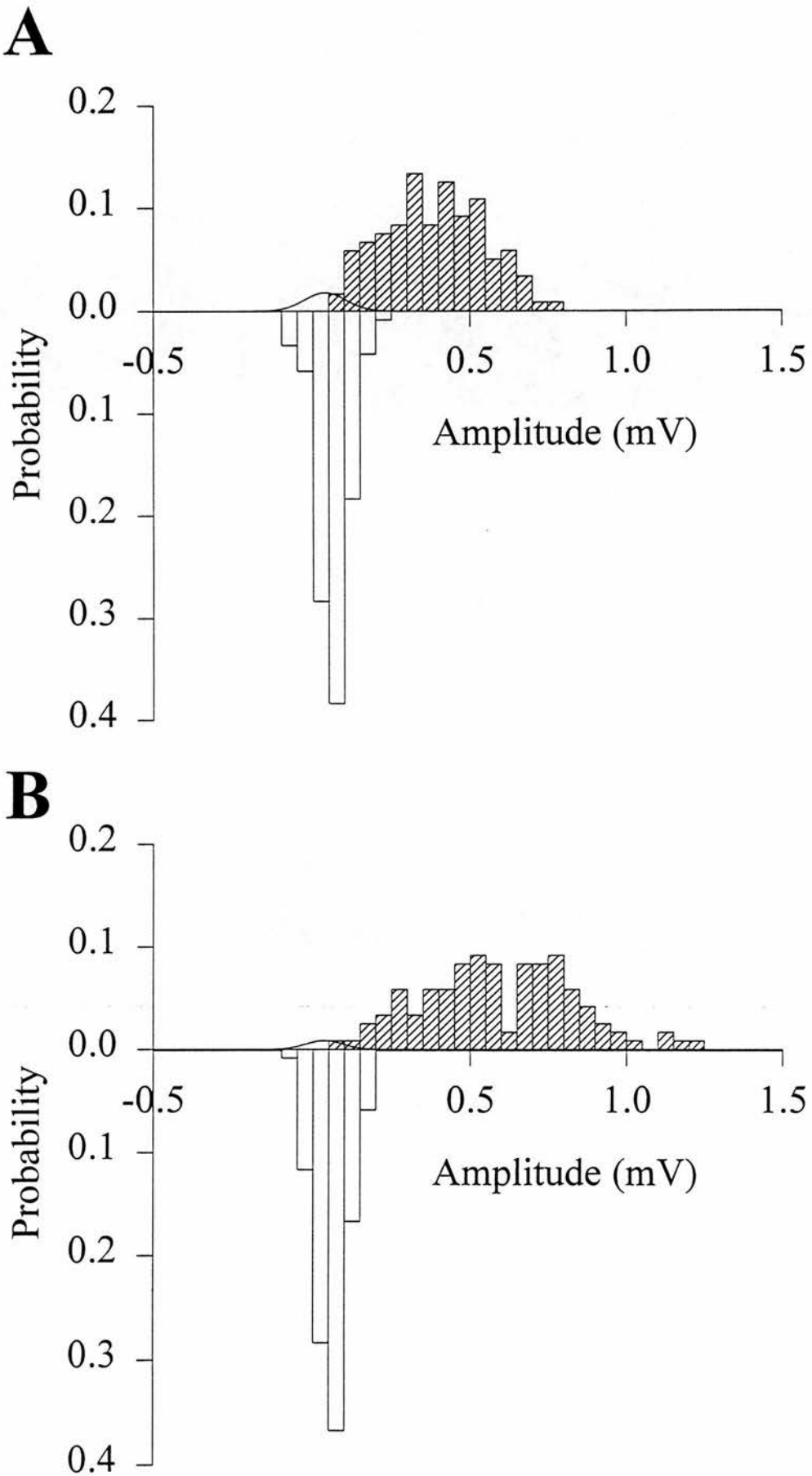
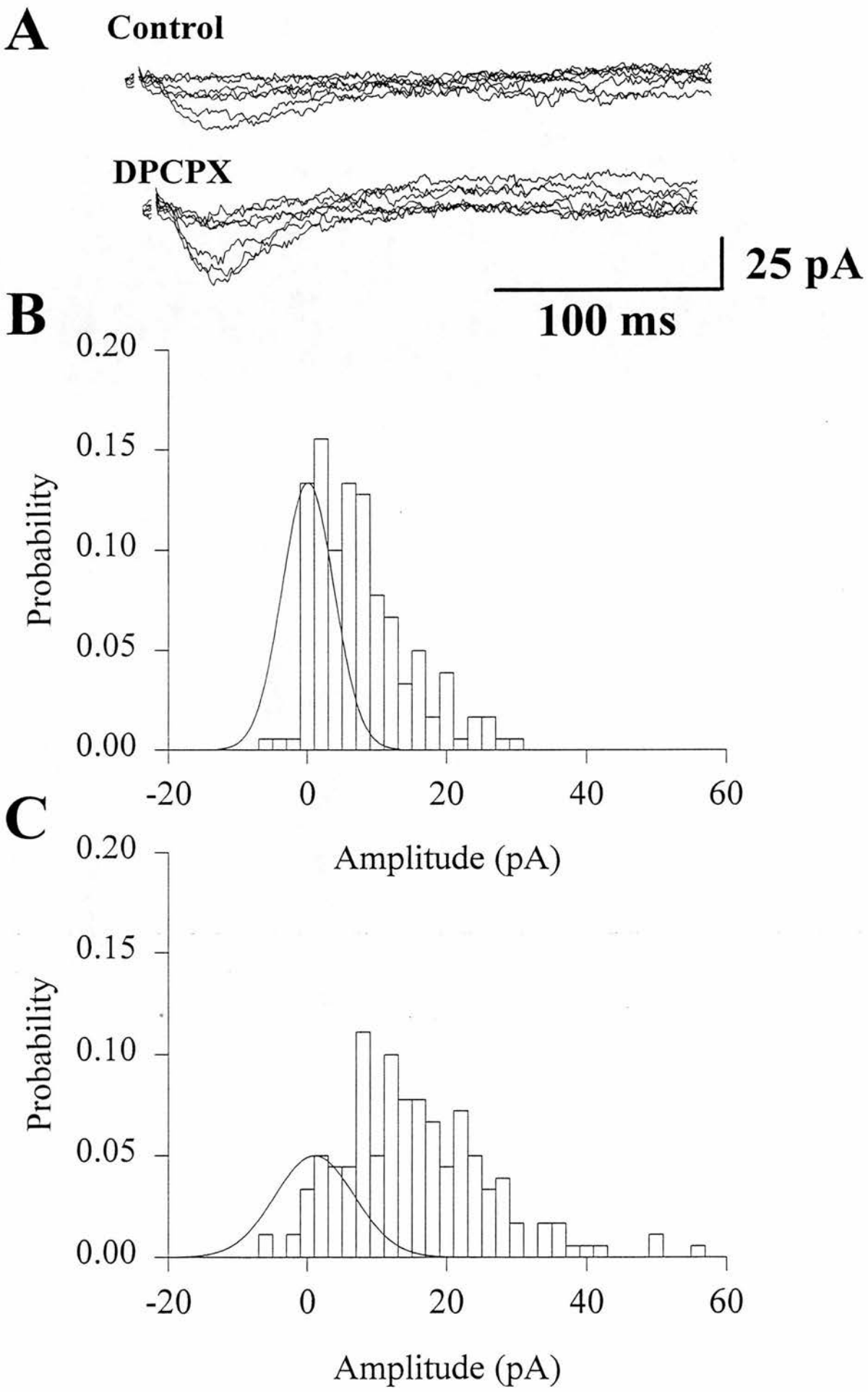

A
0.2
0.1
0.0
0.1
0.2
0.3
0.4
-0.5
0.5
1.0
1.5
Probability
Amplitude (mV)
B
0.2
0.1
0.0
0.1
0.2
0.3
0.4
-0.5
0.5
1.0
1.5
Probability
Amplitude (mV)

Figure 4.3 Distribution of single EPSC amplitudes

A: Representative traces of EPSCs measured in control (upper traces) and in the presence of DPCPX (30 nM, lower traces). The stimulating electrodes were placed in the stratum radiatum close to the CA1 pyramidal cell layer. EPSCs were recorded from a CA1 neurone that was continuously voltage clamped at -70 mV. The stimulation intensity was set such that occasional failures of response were observed. The amplitudes of 180 EPSCs, in control and DPCPX, were measured and plotted (B, C). The noise, determined by measuring over similar intervals as used to measure the ESPC amplitude, is shown as a normal distribution with the peak matched to the histogram class corresponding to the mean noise amplitude. The area under the curve corresponds to the number of failures and was 113 in this neurone under control conditions (B). The mean control EPSC amplitude was 7.07 pA. After 15 minutes exposure to DPCPX (30 nM) the mean EPSC amplitude increased to 14.86 pA, and the number of response failures decreased to 64. DPCPX caused a significant change in the amplitude distribution (Kolmogorov-Smirnov test, $P < 0.001$).


A
Control
DPCPX
25 pA
100 ms
B
0.20
0.15
0.10
0.05
0.00
Probability
-20
0
20
40
60
Amplitude (pA)
C
0.20
0.15
0.10
0.05
0.00
Probability
-20
0
20
40
60
Amplitude (pA)

In the neurone illustrated (Fig. 4.4) an early and late EPSC were observed (A, B). The separation of the two components was sufficient to be able to measure the amplitudes of both components. As can be seen (Fig. 4.4A,B) the first response was predominant under control conditions. After exposure to DPCPX it was the found that the first component was absent more frequently than under control conditions (Fig. 4.4C,D). This suggests that two sites (synaptic connections) were recorded from and that they behaved differently when exposed to DPCPX. The first fails to respond to the stimulus three times more frequently after exposure to DPCPX than under control conditions. The second responds more often to the stimulus after exposure to DPCPX. This is reflected in the mean amplitudes of the two components. The first component was 8.04 ± 0.46 pA in control and fell to 3.44 ± 0.28 pA after exposure to DPCPX. The second component was 1.83 ± 0.45 pA in control increasing to 3.66 ± 0.45 pA in the presence of DPCPX.

Quantal Parameters

The single pulse current and voltage clamp results were used to calculate the quantal parameters *m* and *q*. The quantal content was calculated using the method of failures (m_f). For confirmation of the results obtained using the method of failures the quantal content was also derived from the coefficient of variation (m_{cv}). Since the mean amplitude of the response was known the quantal amplitude (q_{cv}) could be calculated from the quantal content (m_{cv}). These values are shown in table 4.1 (m_f), Appendix 4.1 (m_{cv} and q_{cv}) and illustrated in Figure 4.5.

In the CA1 neurones, combining the results from current and voltage clamp the mean quantal content (m_f) increased from 1.67 ± 0.41 to 2.39 ± 0.45 ($P < 0.02$, Wilcoxon matched pairs signed ranks test, n = 9, Fig. 4.5A). When derived from the coefficient of variation the quantal content (m_{cv}) was 4.06 ± 1.11 in control and an increase to 7.45 ± 1.37 was seen after exposure to DPCPX ($P < 0.01$, signed ranks test, Appendix 4.1). The percentage increase in *m* in the presence of DPCPX is similar using both methods to calculate the value.

In the granule cells m_f increased to 2.03 ± 0.84 after exposure to DPCPX from a control value of 1.55 ± 0.55, m_{cv} was 2.28 ± 0.70 in control increasing to 2.91

Figure 4.4 EPSCs recorded from a dentate gyrus neurone

EPSCs were recorded from a granule cell in control (upper trace) and after exposure to DPCPX (30 nM, lower traces) in response to stimulation of the perforant path. Each record consists of 10 consecutive sweeps. In this neurone the response had two components; an early component that occurred after a latency of 7 ms which was clearly distinguishable from a second component that occurred after a latency of 14 ms. A, B; amplitude distributions of the first and second EPSCs (n = 200 for each) recorded in control conditions. The area under the curves represent 35 and 168 response failures respectively. C, D; after exposure to DPCPX the second component showed an increase in mean amplitude and a reduction in the number of failures to 134. In contrast the first component showed a fall in mean amplitude and an increase in the number of response failures to 99. DPCPX significantly changed the amplitude distributions of the first ($P < 0.01$, Kolmogorov-Smirnov test) and second ($P < 0.01$) synaptic components.

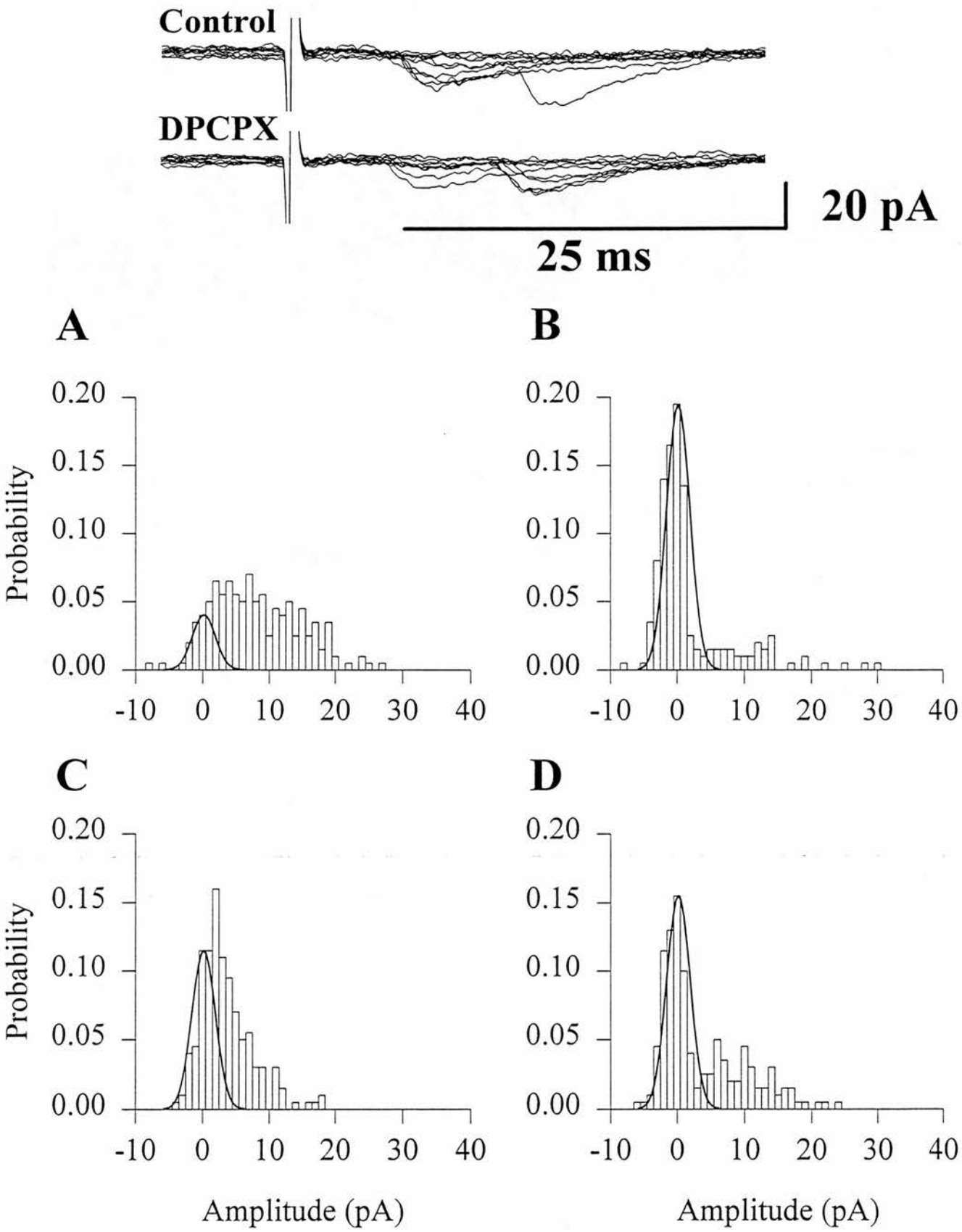

Control
DPCPX
20 pA
25 ms
A
B
C
D
Probability
0.20
0.15
0.10
0.05
0.00
-10
0
10
20
30
40
Amplitude (pA)

Table 4.1 Quantal Parameters for 'failures' experiments

This table shows the quantal content (m_f) for 4 evoked EPSP experiments, 5 evoked EPSC experiments (both recordings from the CA1) and a further 5 evoked EPSC experiments recording from dentate gyrus neurones in control and DPCPX (30 nM). The quantal content measured using the method of failures (m_f) increased in the presence of DPCPX from the value seen in control for all the EPSP and EPSC experiments.

m_f

EPSP (CA1)	Control	DPCPX
1	2.84	3.69
2	1.61	2.59
3	>4.79	>4.79
4	3.00	3.22

EPSC (CA1)	Control	DPCPX
1	0.46	1.03
2	0.82	1.12
3 *	2.36	3.69
4 *	0.59	1.40
5 *	3.35	>5.30

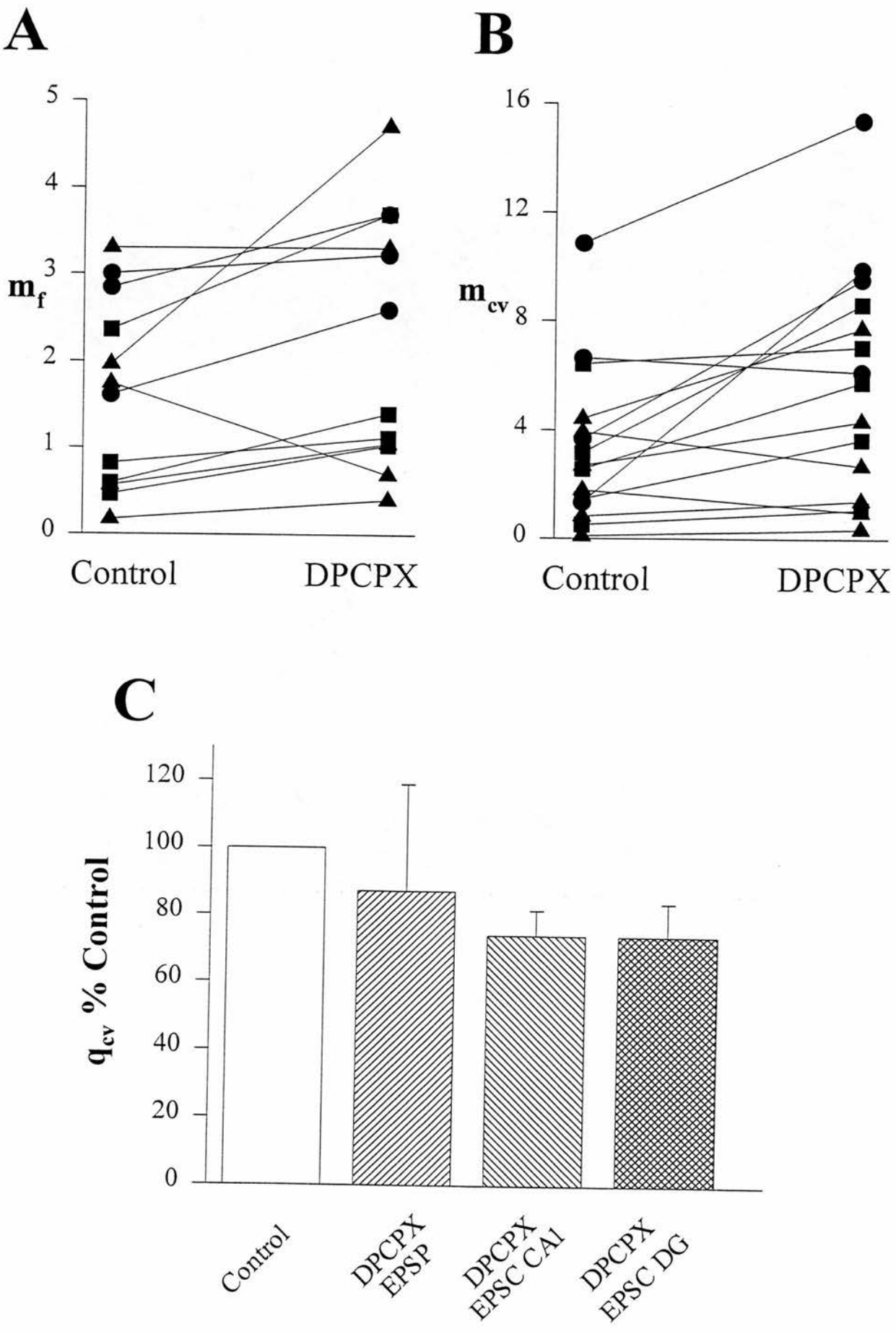
EPSC (D G)	Control	DPCPX
1 *‡	1.74	0.69
2 *‡	0.17	0.40
3 *	3.30	3.30
4 *	1.96	4.70
5 *	>5.25	1.62
6 *	0.56	1.05

* These results were kindly provided by Dr J.P. Hodgkiss

‡ These two results are for one neurone that showed two separate components to the EPSC (see fig 4.4)

Figure 4.5 Overview of m_f, m_{cv} and q_{cv} in single pulse failures experiments

A, B: Scattergrams of m_f and m_{cv} measured in control and in the presence of DPCPX (30 nM), recorded from either CA1 neurones (circles EPSP, squares EPSC) or granule cells (triangles EPSC). A: m_f was measured in 12 cells, after 15 minutes exposure to DPCPX m_f increased in 10 cells, decreased in 1 and was unchanged in another. This represents a significant change ($P < 0.02$, Wilcoxon matched-pairs signed-ranks test) for the CA1 neurones, but no significant change was seen in granule cells. B: m_{cv} was measured in 15 neurones, in 12 cells m_{cv} increased after exposure to DPCPX, but in 3 cells it decreased. These changes were significant in CA1 neurones ($P < 0.01$, $n = 9$, Wilcoxon signed-ranks test), but not in granule cells ($n = 6$). C: The quantal amplitude (q_{cv}) was measured in control and in the presence of DPCPX using both current and voltage clamp techniques. In the current clamp experiments the mean q_{cv} for the CA1 neurones was decreased to 87.2 ± 31.7 % of control (Mean ± SEM, n=4) after DPCPX application. Under voltage clamp conditions q_{cv} decreased to 74.1 ± 7.60 % of control (n=5) and 74.0 ± 9.87 % of control ($n = 6$) after 10 - 15 minutes exposure to DPCPX in CA1 and granule neurones respectively. These changes in q_{cv} were not significant (Mann-Whitney U Test).


A
B
C
5
4
3
2
1
0
m_f
Control
DPCPX
16
12
8
4
0
m_{cv}
Control
DPCPX
120
100
80
60
40
20
0
q_{cv} % Control
Control
DPCPX EPSP
DPCPX EPSC CA1
DPCPX EPSC DG

± 1.12 in the presence of DPCPX (n = 6). These increases in m_f and m_{cv}, seen after exposure to DPCPX, are of the same magnitude - 130% of the value seen in control.

The quantal amplitude (q_{cv}) was calculated from the quantal content, m_{cv}. This measure of quantal content was used as a value could be determined for each neurone under control conditions and in the presence of DPCPX, if m_f had been used some values would not be able to be determined. Although the quantal amplitude fell in the presence of DPCPX in both the CA1 neurones (87.2 ± 31.7 % and 74.1 ± 7.60 % for current and voltage clamp respectively) and the granule cells (74 ± 9.87 %), these decreases were not significant (see Appendix 4.1).

Paired Pulse Experiments

The presynaptic actions of the adenosinergic compounds, the agonist CCPA and antagonist DPCPX, were further studied in a series of paired pulse stimulation experiments, utilising both current and voltage clamp recording. Pairs of pulses, separated by 90 ms, were applied to the Schaffer collateral commissural fibres, and the responses measured in hippocampal CA1 neurones. Figure 4.6B,C show the effect of altering the separation between the stimuli, the facilitation is larger when the pulses are separated by shorter intervals, however as can be seen from Figure 4.6A a short interval combined with a large EPSP means that the second EPSP starts before the first EPSP has returned to baseline, possibly due to activation of $GABA_B$ receptors. This can also be seen for the 50 ms interval in voltage clamp recording (Fig. 4.6B). For these reasons a 90 ms separation was used in all recordings. This separation showed a facilitation of 145 % in the neurone illustrated (Fig. 4.6C).

Effect of CCPA, the adenosine A_1 agonist - Current Clamp

Pairs of pulses were applied to the Schaffer collateral commissural fibres under control conditions, after exposure to CCPA (100 nM) and CCPA co-applied with DPCPX (30 nM). The excitatory responses were measured under current clamp conditions. As can be seen from Figure 4.7A under control conditions the second EPSP was facilitated compared to the first EPSP. After exposure to CCPA the amplitude of both EPSPs had fallen and predominantly failures were observed for the

Figure 4.6 EPSCs evoked at different interstimulus intervals

A: Example of paired pulse EPSPs recorded under current clamp conditions. The EPSPs were evoked 90 ms apart, and as can be seen from this trace the first EPSP had not returned to the baseline before the second EPSP was evoked. This occurred only with large EPSPs. B: Each trace is the mean of 10 sweeps. In voltage clamp EPSCs were evoked by stimulating electrodes placed in the stratum radiatum and recorded from a CA1 neurone. The second pulse (P2) was evoked at intervals from 50 ms to 190 ms after the first pulse (P1). C: The EPSC amplitudes were measured and the mean was found for P1 and P2 from 10 consecutive trials. The P2/P1 was calculated and plotted against the EPSC separation. As can be seen from the histogram the facilitation of the second EPSC was greatest at a 50 ms separation of the pulses, but a 145% facilitation was observed at a 90 ms separation This separation was used in all further paired pulse EPSC and EPSP experiments.

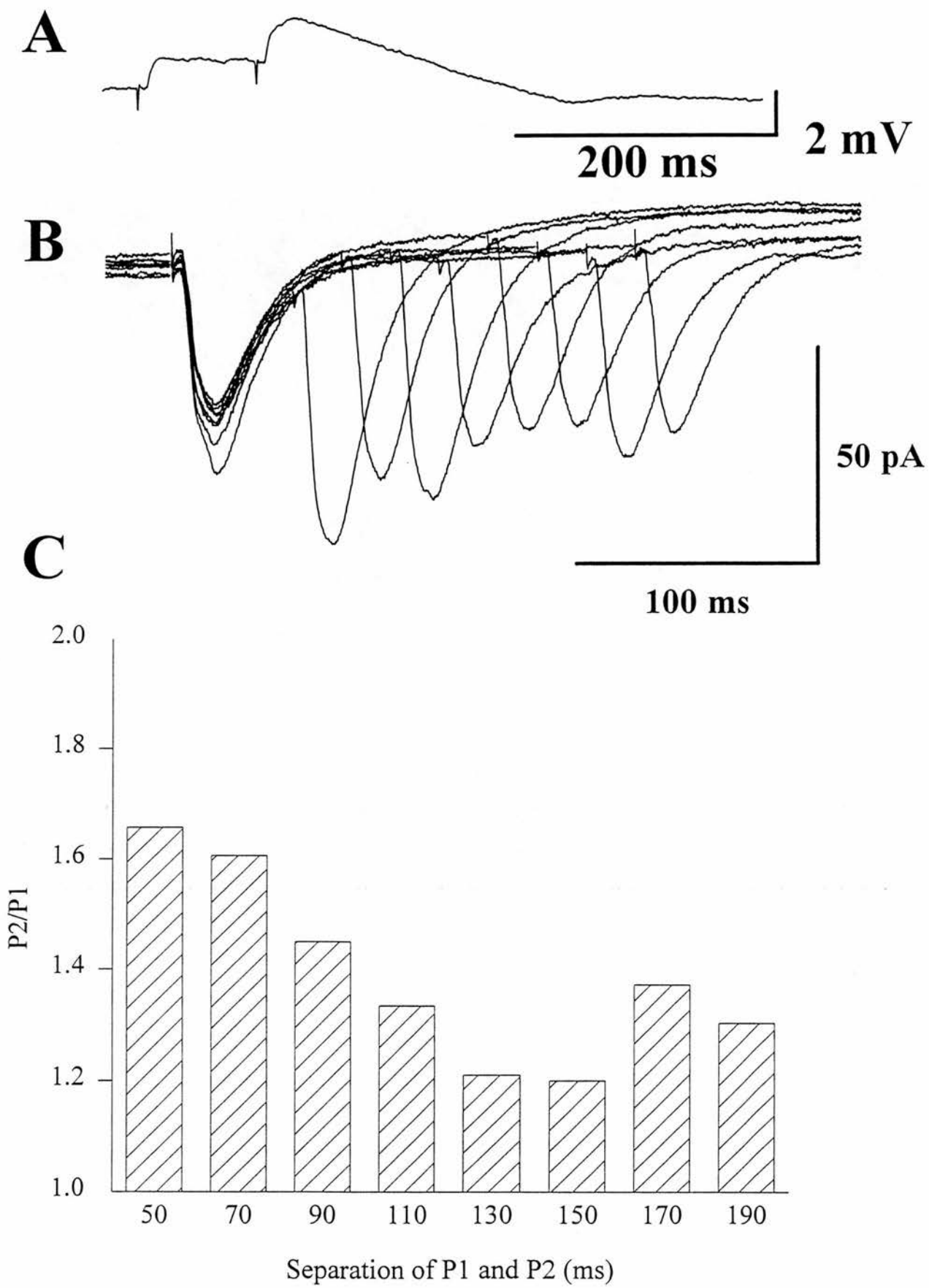
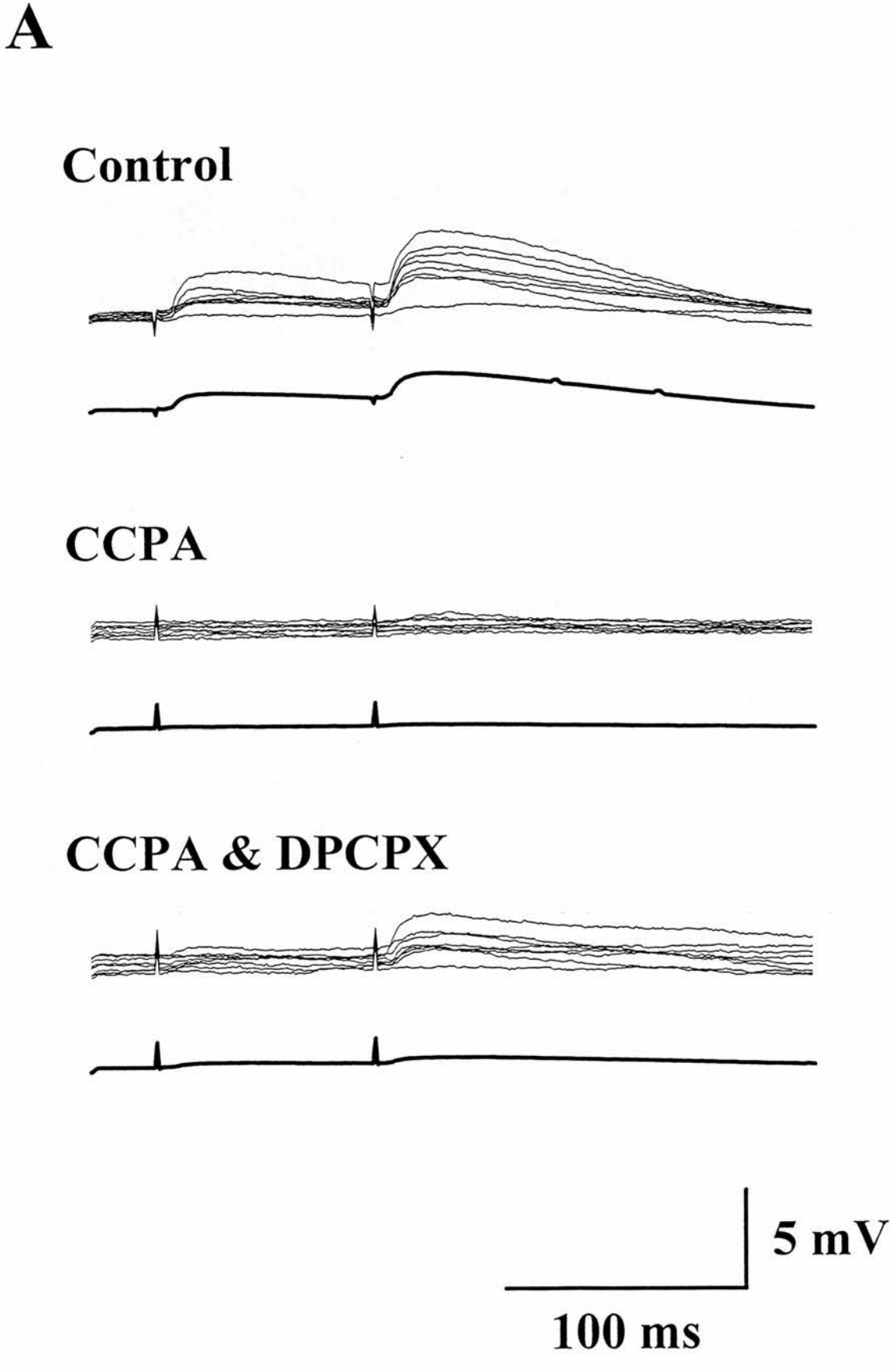
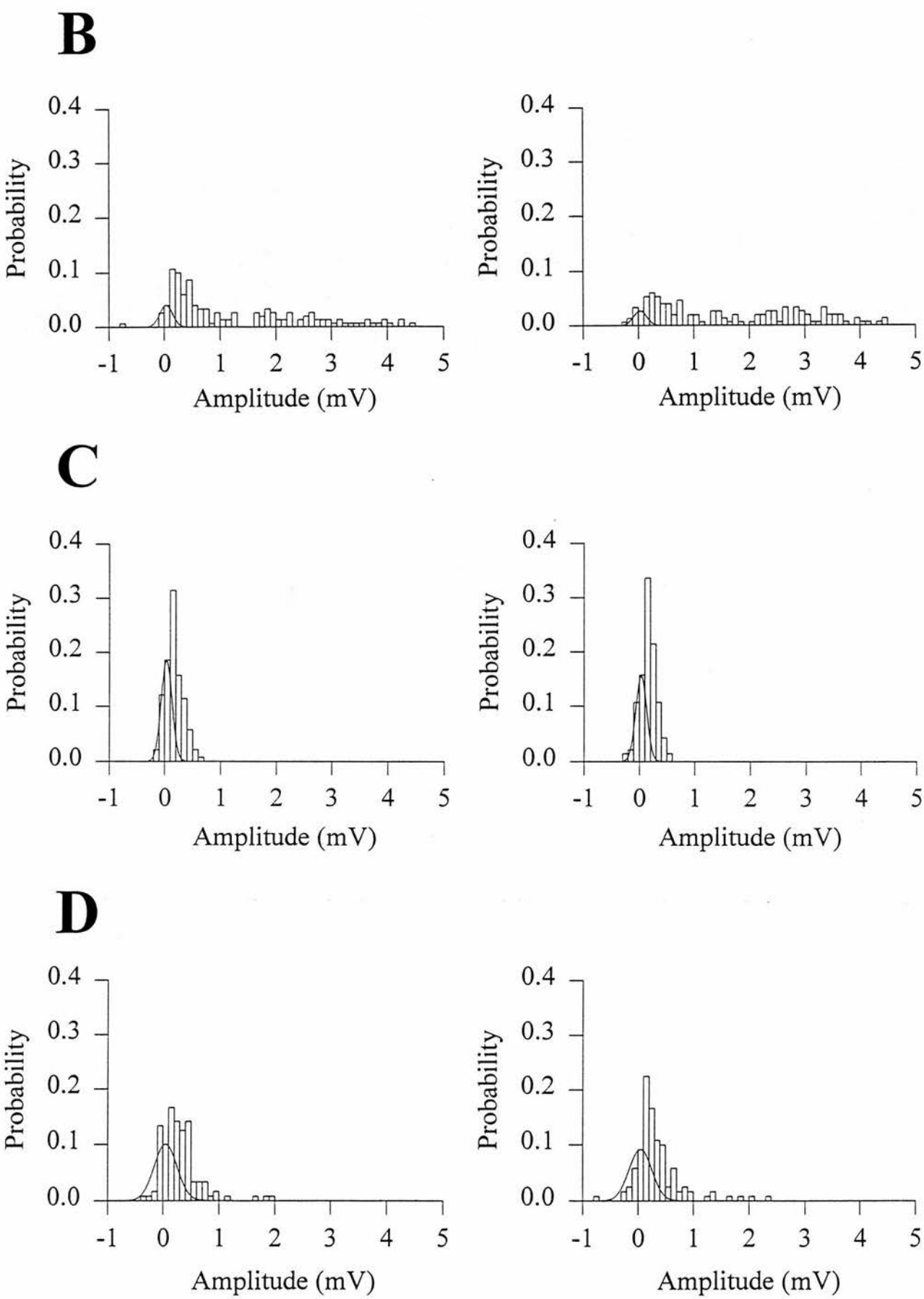

A
2 mV
200 ms
B
50 pA
100 ms
C
2.0
1.8
1.6
1.4
1.2
1.0
P2/P1
50
70
90
110
130
150
170
190
Separation of P1 and P2 (ms)

Figure 4.7 Effect of CCPA on paired pulse facilitation of EPSPs

A: shows representative traces of pairs of pulses recorded under current-clamp conditions. The EPSPs were recorded under control conditions (E_m = -56 mV, top), in the presence of the adenosine A_1 agonist, CCPA (100 nM, E_m = -56 mV, middle), and in the continuous presence of CCPA and the adenosine A_1 receptor antagonist DPCPX (30 nM, E_m = -55 mV, bottom). The trace outlined by the heavy line represents the mean paired pulse response under each condition. The stimulus intensity was set such that occasional response failures were observed. The mean paired pulse ratio was determined by taking the mean of the individual paired pulse ratios for each trial.

B: the amplitude distribution of the EPSPs evoked in control conditions. The left hand histogram represents the amplitude distribution of the first EPSP amplitudes (P1, n = 140), the right hand histogram shows the amplitude distribution of the second EPSP amplitudes (P2, n = 140). The noise is shown as a normal distribution with its peak matched to the histogram class corresponding to the mean noise amplitude. The area under the curve corresponds to the number of response failures, and was 33 for P1 and 22 for P2. The slice was then exposed to CCPA for 15 minutes and the amplitude distributions of P1 and P2 were determined (C; n = 140). The number of failures increased to 126 (P1) and 106 (P2). After a 15 minute exposure to CCPA and DPCPX the amplitude distribution was determined (D; n = 120). The number of failures was 72 for P1 and 66 for P2. In this neurone the amplitude of P1 and P2 in control was 1.30 ± 0.11 mV and 1.98 ± 0.14 mV respectively. The P2/P1 ratio was 2.95 indicating that the second EPSP was facilitated on average by 195 % with respect to the first EPSP. After exposure to CCPA, P1 and P2 amplitudes decreased to 0.061 ± 0.01 mV (Mean ± SEM) and 0.074 ± 0.01 mV respectively, the P2/P1 ratio unexpectedly decreasing to 1.64. After exposure to DPCPX and CCPA the inhibition by CCPA was partially relieved and P1 increased to 0.25 ± 0.03 mV and P2 to 0.30 ± 0.04 mV, the P2/P1 ratio decreased further to 1.19.


A
Control
CCPA
CCPA & DPCPX
5 mV
100 ms


B
C
D
Probability
Amplitude (mV)
0.0
0.1
0.2
0.3
0.4
-1
0
1
2
3
4
5

first EPSP. A partial reversal of the CCPA inhibition was observed in the presence of DPCPX.

The individual EPSP amplitudes were measured (see Fig. 4.9A) and plotted, for all conditions. The noise was measured as described earlier and fitted to the corresponding histogram class as a normal distribution. The number of failures was calculated from the area of this distribution (Fig. 4.7B - D). As can be seen under control conditions (Fig. 4.7B) the EPSP distribution was widely spread and the number of failures low. After exposure to CCPA (Fig. 4.7C) the width of the distribution was reduced and the number of failures dramatically increased. After exposure to DPCPX and CCPA (Fig. 4.7D) the number of failures was reduced towards the number seen under control conditions and the range of the distribution was increased towards control levels.

In all four neurones the EPSP amplitude distribution changed significantly after exposure to CCPA ($P < 0.001$, Kolmogorov-Smirnov test). In 3 of the 4 neurones the paired pulse protocol significantly altered the amplitude distribution such that the amplitudes observed for EPSP 2 were significantly differently distributed compared to those for EPSP 1 ($P < 0.01$, Kolmogorov Smirnov Test). In the neurones where DPCPX was co-applied with CCPA the distribution was altered significantly from that observed in the presence of CCPA alone ($P < 0.001$, Kolmogorov Smirnov test).

Under control conditions the mean EPSP 1 amplitude was 1.07 ± 0.12 mV and EPSP 2 was 1.62 ± 0.28 mV ($n = 4$) a mean facilitation ratio of 2.64 was seen (Fig. 4.9A). After 15 minutes exposure to CCPA the mean amplitudes fell significantly to 0.05 ± 0.09 mV and 0.19 ± 0.05 mV respectively ($P < 0.02$, paired student's t-test, $n = 4$), the facilitation ratio falling to 1.71. This decrease in the facilitation ratio was not expected. DPCPX partially reversed the CCPA inhibition of the EPSP and increased the amplitudes to 0.33 ± 0.04 mV and 0.37 ± 0.03 mV for first and second EPSPs respectively. The increase in amplitude was significant for the second EPSP ($P < 0.05$, paired student's t-test, $n = 3$). A further decrease in the facilitation ratio to 1.12 was observed.

Voltage Clamp

This series of experiments was repeated under voltage clamp conditions. EPSCs were recorded from hippocampal CA1 neurones that were clamped continuously at -70 mV. The effects of the adenosine A_1 receptor agonist CCPA were studied.

EPSCs were recorded under control conditions and after exposure to CCPA (Fig. 4.8A). Each EPSC amplitude was measured and the distribution plotted (Fig. 4.8B,C). As can be seen from figure 4.8B there were no response failures under control conditions, and that the range of the amplitude distribution was increased for EPSC 2 suggesting multiquantal release. After exposure to CCPA (Fig. 4.8C) there were some response failures. A greater number of 'failures' were observed for EPSC 1 than EPSC 2.

In 5 out of 6 neurones CCPA significantly altered the EPSC2 amplitude distribution, and in 4 out of 6 for EPSC 1 ($P < 0.01$, Kolmogorov Smirnov test). In 5 out of 6 neurones tested the paired pulse protocol also caused a significant change in the EPSC amplitude distribution under both control conditions and in the presence of CCPA.

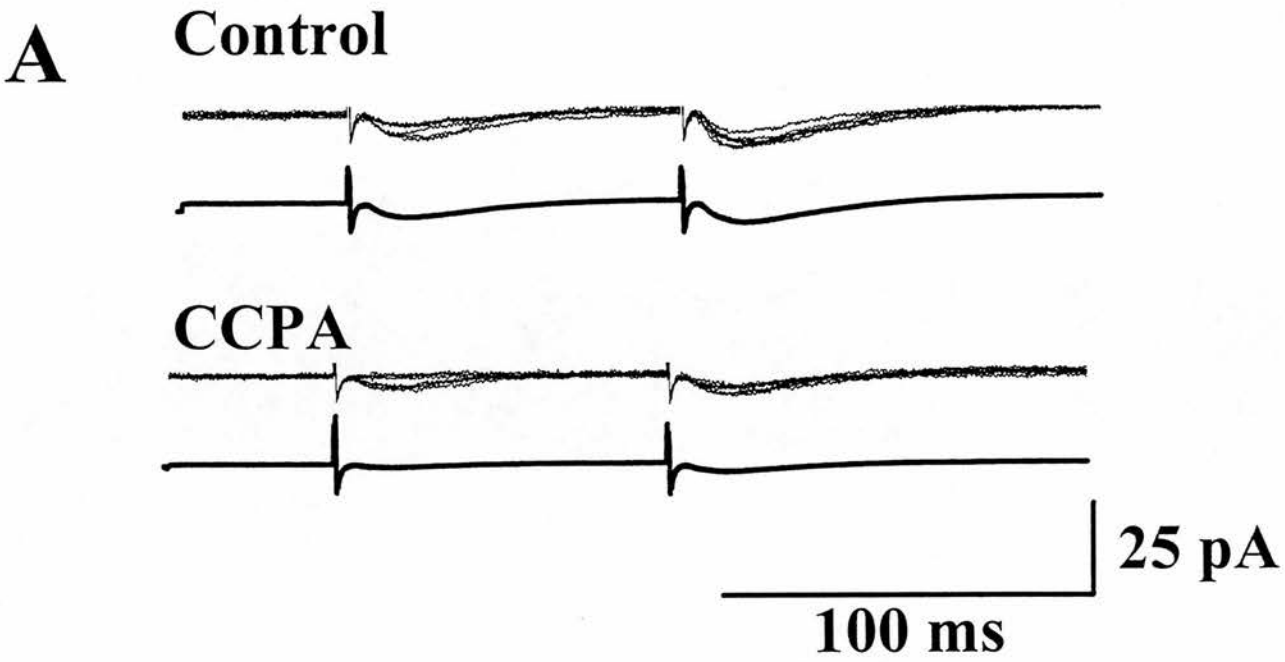
The mean amplitudes under control conditions were 27.1 ± 3.67 pA and 39.6 ± 7.13 pA for EPSCs 1 and 2 respectively. See figure 4.9B for individual amplitudes for each neurone. The paired pulse ratio, of EPSC 2 to EPSC 1, was 1.93. After 15 minutes exposure to CCPA the mean amplitude had decreased to 16.7 ± 3.22 pA and 26.0 ± 5.62 pA respectively for the two EPSCs, the facilitation ratio increased to 2.57. This inhibition of the amplitude of both EPSCs, by CCPA, was significant ($P < 0.01$, paired student's t-test).

The individual EPSC amplitudes for 1 neurone are shown in Figure 4.10A, with both EPSC 1 and EPSC 2 amplitudes indicated. This clearly shows an increase in EPSC amplitude due to the paired pulse protocol and also the inhibition of EPSC amplitude after exposure to CCPA. In Figure 4.10B the P2/P1 ratio is shown and it can be seen that after addition of CCPA the ratio increases in this neurone.

Figure 4.8 Paired pulse facilitation, effect of CCPA on evoked EPSCs

A: shows representative traces of pairs of pulses recorded under voltage-clamp conditions. The EPSPs were recorded under control conditions (holding current (hc) = -0.10 nA, left hand traces), in the presence of the adenosine A_1 agonist, CCPA (100 nM, hc = -0.10 nA, right hand traces). The trace outlined by the heavy line represents the mean paired pulse response under each condition.

B: the amplitude distribution of the EPSPs evoked in control conditions. The left hand histogram represents the distribution of the first EPSP amplitudes (P1, n = 120), the right hand graph shows the distribution of the second EPSP amplitudes (P2, n = 120). The noise, determined by measuring over similar intervals as used to measure the EPSP amplitude, is shown as a normal distribution with its peak matched to the histogram class corresponding to the mean noise amplitude. The area under the curve corresponds to the number of response failures, no failures were observed for P1 and P2. The slice was then exposed to CCPA for 15 minutes and the amplitude distributions of P1 and P2 were measured (C; n = 120). The number of failures increased to 15 (P1) and 5 (P2). In this neurone the amplitude of P1 and P2 in control was 19.4 ± 0.55 pA (Mean ± SEM) and 30.0 ± 0.93 pA respectively. The P2/P1 ratio was 1.75 indicating that the second EPSP was facilitated by 75 % with respect to the first EPSP. After exposure to CCPA, P1 and P2 amplitudes decreased to 6.5 ± 0.36 pA and 13.8 ± 0.52 pA respectively, the P2/P1 ratio increasing to 2.77.


A
Control
CCPA
25 pA
100 ms

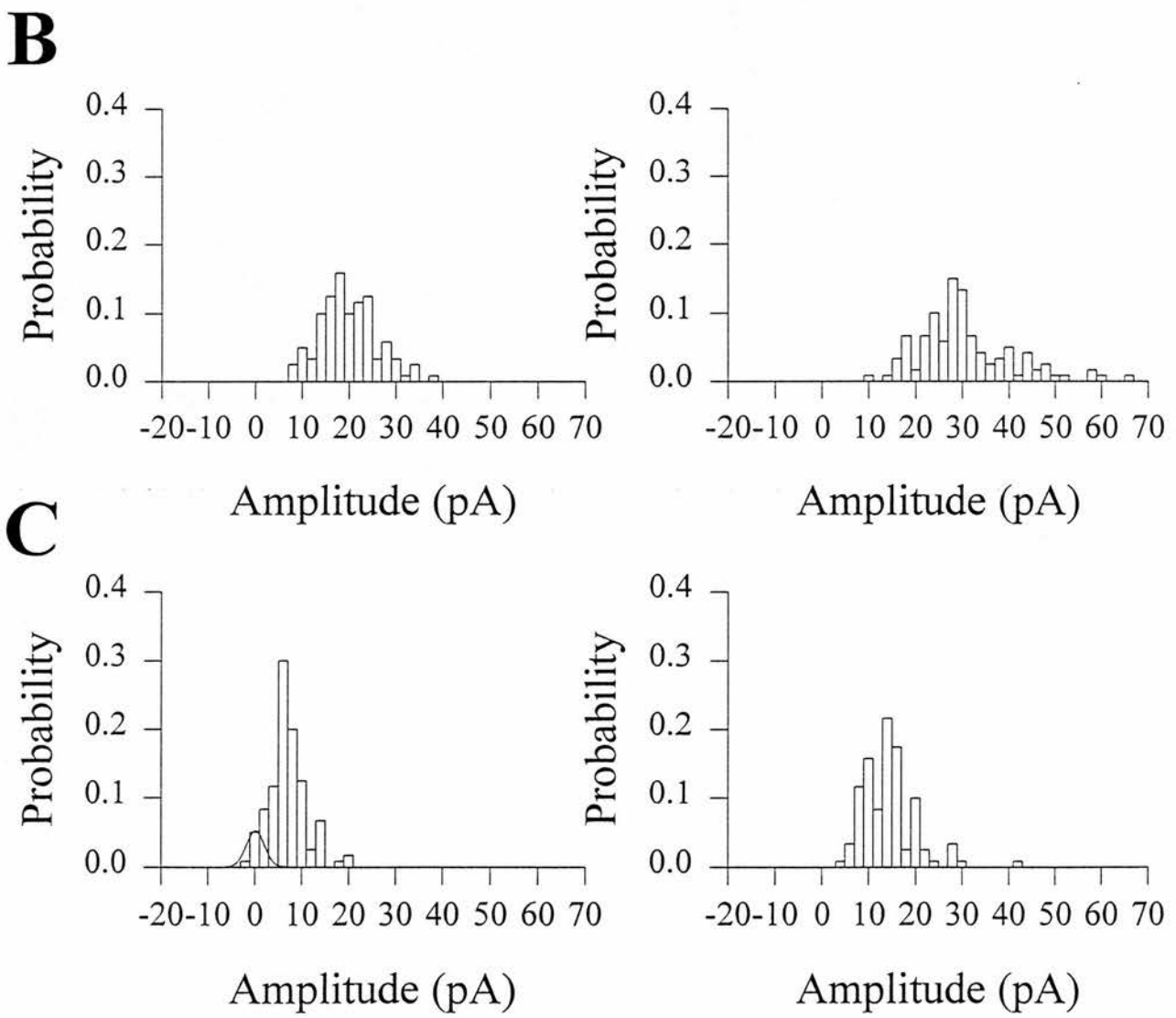

B
C
Probability
0.4
0.3
0.2
0.1
0.0
-20 -10 0 10 20 30 40 50 60 70
Amplitude (pA)

Figure 4.9 Mean amplitudes after exposure to CCPA

The graphs show the mean EPSP amplitude in control and the presence of CCPA, for EPSP 1 and EPSP 2. The same number of EPSPs were measured in control conditions and in the presence of CCPA. A: The solid line represents the control conditions, the dashed line - after 15 minutes exposure to CCPA (100 nM) and the dotted line after a further 15 minutes exposure to CCPA and DPCPX (30 nM). As can be seen the amplitude of the second EPSP was facilitated with respect to the first EPSP amplitude in all four neurones. In all neurones the EPSP amplitudes decreased in the presence of CCPA, but a facilitation of the second EPSP was still observed. A partial reversal in amplitude was observed after exposure to DPCPX. CCPA significantly decreased the amplitudes of both EPSP 1 and EPSP 2 from control ($P < 0.02$, paired t-test).

B: The left hand graph shows the mean amplitudes of EPSC 1 and EPSC 2 under control conditions, the right hand graph shows the same neurones after exposure to CCPA for 15 minutes. As can be seen the amplitude of the second EPSC (39.7 ± 7.12 pA, mean ± SEM) was facilitated with respect to the first EPSC amplitude (27.1 ± 3.69 pA, mean P2/P1 ratio of 1.93). In all neurones the EPSC amplitudes were decreased in the presence of CCPA (amplitude P1 = 16.7 ± 3.22 pA, P2 = 26.0 ± 5.62 pA), but a facilitation was still observed (P2/P1 = 2.57). CCPA significantly decreased the amplitudes of both EPSP 1 and EPSP 2 from control ($P < 0.01$, paired t-test). Also the second EPSC was significantly larger than the first ($P < 0.05$, paired t-test).

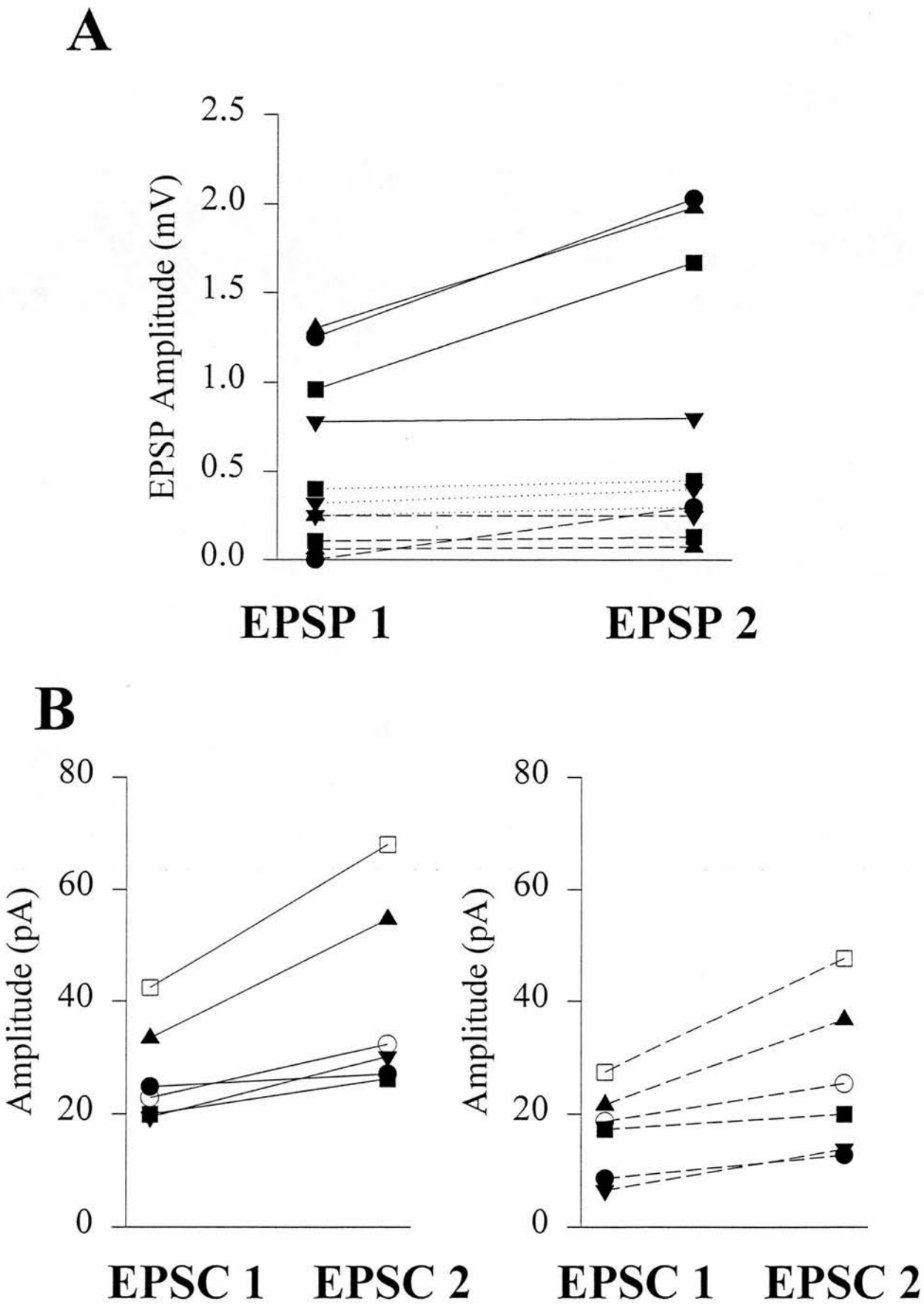
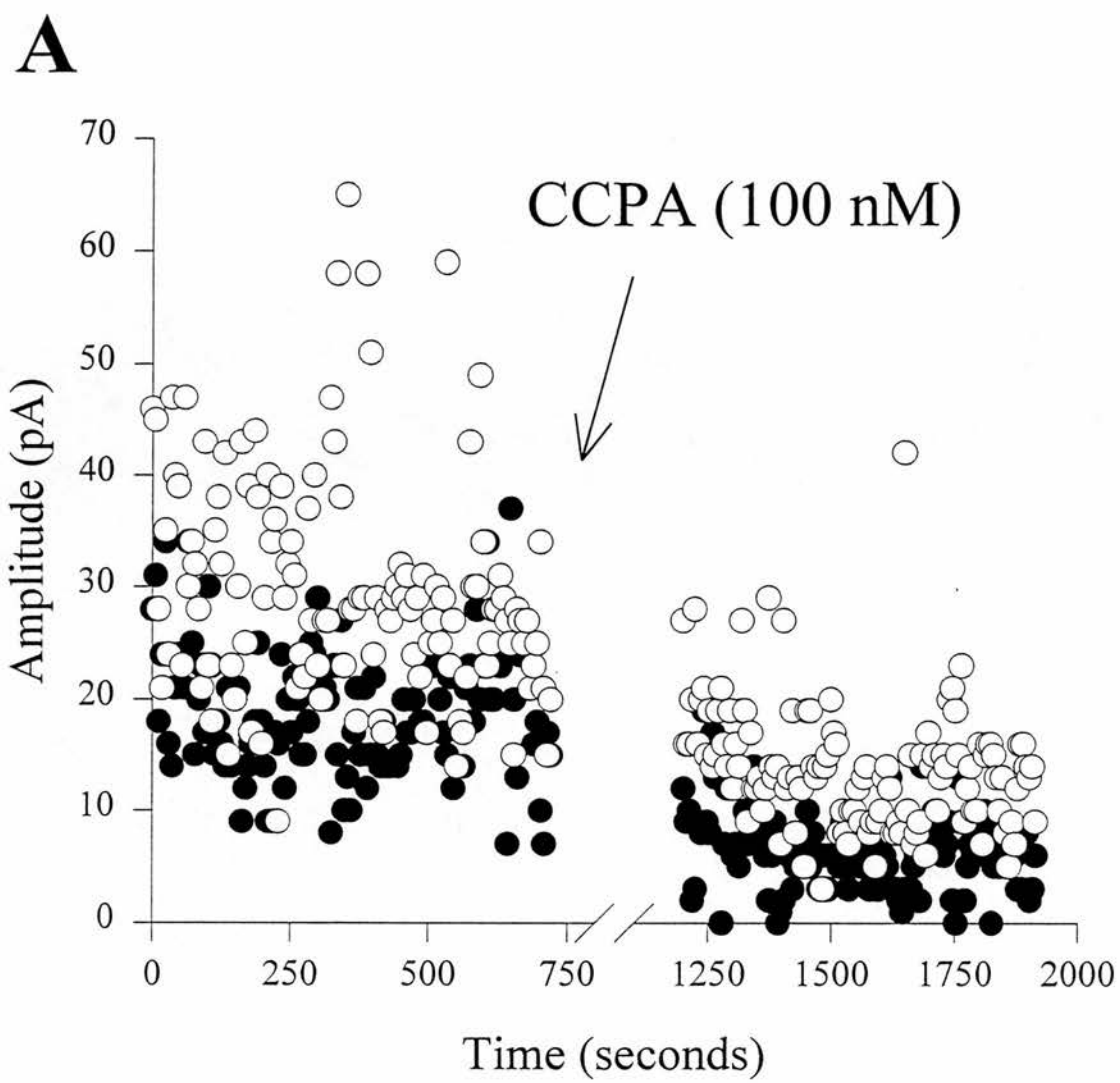
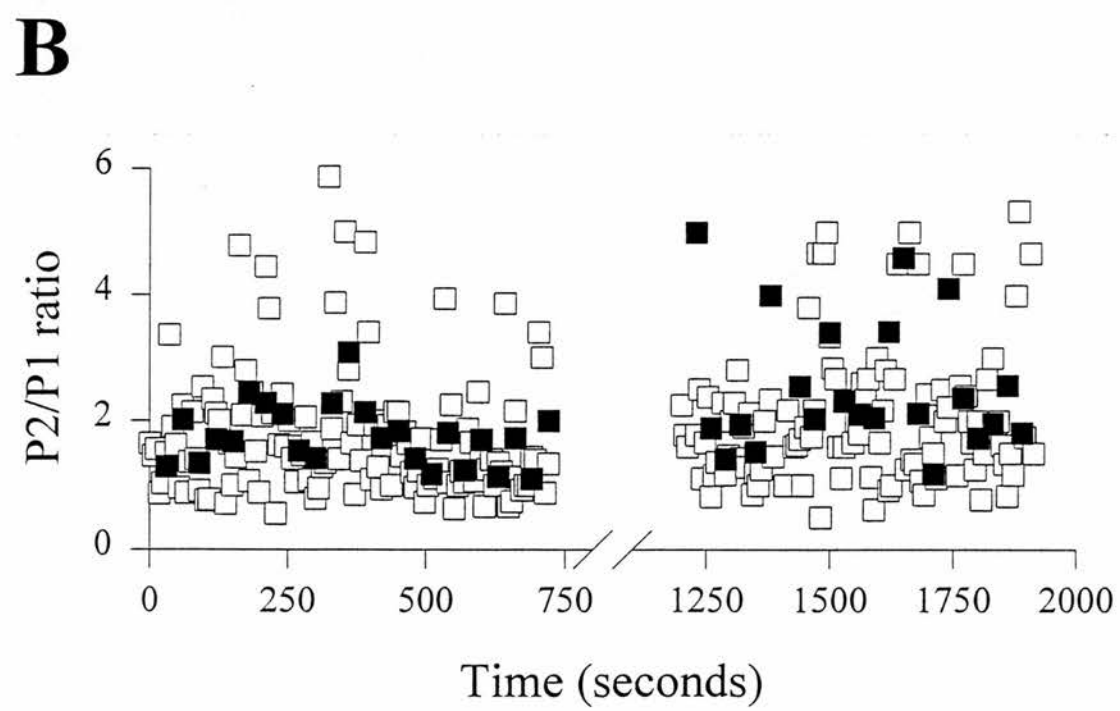

A
2.5
2.0
1.5
1.0
0.5
0.0
EPSP Amplitude (mV)
EPSP 1
EPSP 2
B
80
60
40
20
0
Amplitude (pA)
EPSC 1
EPSC 2
80
60
40
20
0
Amplitude (pA)
EPSC 1
EPSC 2

Figure 4.10 Amplitudes of P1, P2 and P2/P1 ratio against time

A: the amplitudes of individual evoked EPSCs were plotted against time; P1 (closed circles) and P2 (open circles), for a CA1 neurone voltage clamped at -70 mV. As can be seen the amplitude of the second EPSC is increased above that for the first EPSC showing facilitation. A control period of 12 minutes was recorded (until 720 seconds), CCPA was applied for 10 -15 minutes and after the break the EPSCs were recorded in the presence of CCPA (100 nM). It can be seen that the distribution of EPSC amplitudes is reduced from control in the presence of CCPA. B: the P2/P1 ratio was plotted against time (closed squares, 1 minute means; open squares individual values). The P2/P1 ratio was increased in the presence of CCPA, as can be seen from the graph.


A
CCPA (100 nM)
Amplitude (pA)
70
60
50
40
30
20
10
0
0
250
500
750
1250
1500
1750
2000
Time (seconds)


B
P2/P1 ratio
6
4
2
0
0
250
500
750
1250
1500
1750
2000
Time (seconds)

Quantal Parameters- Current Clamp

The quantal content, m_f, was calculated from the data and is shown in Table 4.2 (m_{cv} and q_{cv} are shown in Appendix 4.2). It was found that the quantal content fell after 15 minutes exposure to CCPA. The mean values of m_f and m_{cv} are shown below. Under these conditions a good correlation between m_f and m_{cv} was observed, with a correlation coefficient of 0.89.

	EPSP 1		EPSP 2	
	m_f	m_{cv}	m_f	m_{cv}
Control	2.40	2.39	3.01	3.11
CCPA	0.45	0.73	0.49	0.58
CCPA & DPCPX	0.55	1.01	0.86	1.26

CCPA significantly decreased the quantal content when measured using the method of failures (m_f, $P < 0.05$, Wilcoxon Signed ranks test). A decrease in m_{cv} was observed, this decrease was not significant. A partial reversal was seen after exposure to DPCPX and CCPA.

The quantal amplitudes were also determined (Appendix 4.2). A decrease in q_{cv} was seen for both EPSPs after exposure to CCPA. DPCPX partially reversed this decrease in q_{cv}. These changes were not significant.

	EPSP 1	EPSP 2
	q_{cv} (mV)	q_{cv} (mV)
Control	0.61	0.66
CCPA	0.15	0.39
CCPA & DPCPX	0.33	0.34

Table 4.2

This table shows the quantal parameter m_f for four hippocampal CA1 neurones, measured in control and after exposure to CCPA (100 nM). In 3 of the 4 neurones DPCPX was co-applied with CCPA.

Table 4.2

	EPSP 1 m_f			EPSP 2 m_f		
	Control	CCPA	CCPA & DPCPX	Control	CCPA	CCPA & DPCPX
1	2.53	0.11	-	3.91	0.11	-
2	3.22	0.47	0.50	3.06	0.32	0.92
3 *	1.45	0.11	0.51	1.85	0.28	0.60
4	-	1.10	0.64	3.22	1.25	1.06

* Neurone illustrated in Figure 4.7

Quantal Parameters - Voltage Clamp

The quantal content was calculated from the method of failures and using the coefficient of variation and the mean values are shown below (see Table 4.3 and Appendix 4.3).

	EPSC 1		EPSC 2	
	m_f	m_{cv}	m_f	m_{cv}
Control	2.96	6.32	4.61	9.92
CCPA	2.26	4.22	2.82	6.36

Although a decrease in m_f was observed after exposure to CCPA this was not significant for either EPSC 1 or EPSC 2. Using the coefficient of variation, m_{cv} decreased after exposure to CCPA, this decrease was significant for EPSC 2 ($P < 0.05$, Wilcoxon signed ranks test) but not for EPSC 1.

The quantal amplitude (q_{cv}, Appendix 4.3) was calculated from this data and the mean values are shown below.

	EPSC 1	EPSC 2
	q_{cv} (pA)	q_{cv} (pA)
Control	6.06	4.34
CCPA	4.56	4.32

The quantal amplitude was not significantly changed by exposure to CCPA.

When the quantal content data was pooled for current and voltage clamp experiments it was found that m_f decreased from 2.75 ± 0.29 in control to 1.53 ± 0.33 in the presence of CCPA for the first response ($n = 10$, $P < 0.05$, Mann Whitney U test) and from 3.33 ± 0.46 to 1.89 ± 0.41 for the second ($P < 0.05$, Mann Whitney U test).

Table 4.3

This table shows the quantal parameter m_f for six hippocampal CA1 neurones, measured in control and after exposure to CCPA (100 nM).

Table 4.3

	EPSC 1 m_f		EPSC 2 m_f	
	Control	CCPA	Control	CCPA
1	4.25	3.00	-	3.00
2	2.30	2.42	-	2.89
3	3.04	1.48	-	2.57
4 *	-	2.08	-	3.18
5	2.81	2.04	-	2.04
6	2.41	2.53	4.61	3.22

* Neurone illustrated in figure 4.8

Effect of DPCPX, the adenosine A_1 antagonist - Current clamp

A similar series of experiments was carried out with DPCPX, the adenosine A_1 receptor antagonist. As can be seen from Figure 4.11A DPCPX increased the amplitude of the paired pulse EPSPs, and CNQX (the AMPA and Kainate receptor antagonist) when co-applied with DPCPX completely abolished the EPSP.

The amplitude distributions for a single experiment are shown in Figure 4.11B,C,D. DPCPX (30 nM) increased the mean amplitude of both the first and second EPSPs. When the data from eight similar experiments was pooled (Fig. 4.13A) it was found that the first EPSP amplitude increased from 0.60 ± 0.08 mV to 0.67 ± 0.15 mV after 15 minutes exposure to DPCPX, and the second EPSP amplitude increased from 0.59 ± 0.11 mV to 0.73 ± 0.15 mV. These changes were not significant ($P > 0.05$, paired student's t-test, $n = 8$).

It was found when the individual experiments were studied that in 4 neurones DPCPX resulted in an increase in mean amplitude for both EPSPs whilst in 3 a decrease was observed and a further 1 neurone no effect was seen (Fig. 4.13A). When the amplitudes for just the neurones that showed an increase in amplitude after exposure to DPCPX were compared it was found that DPCPX significantly increased the mean amplitude from 0.94 ± 0.15 mV to 1.01 ± 0.19 mV for EPSP 2 ($P < 0.01$, paired student's t-test). The amplitude of EPSP 1 unaltered by DPCPX application, 0.68 ± 0.15 mV under control conditions and 0.69 ± 0.19 mV after DPCPX. The amplitudes of those neurones that showed a decrease after exposure to DPCPX (15 minutes) were not significantly changed.

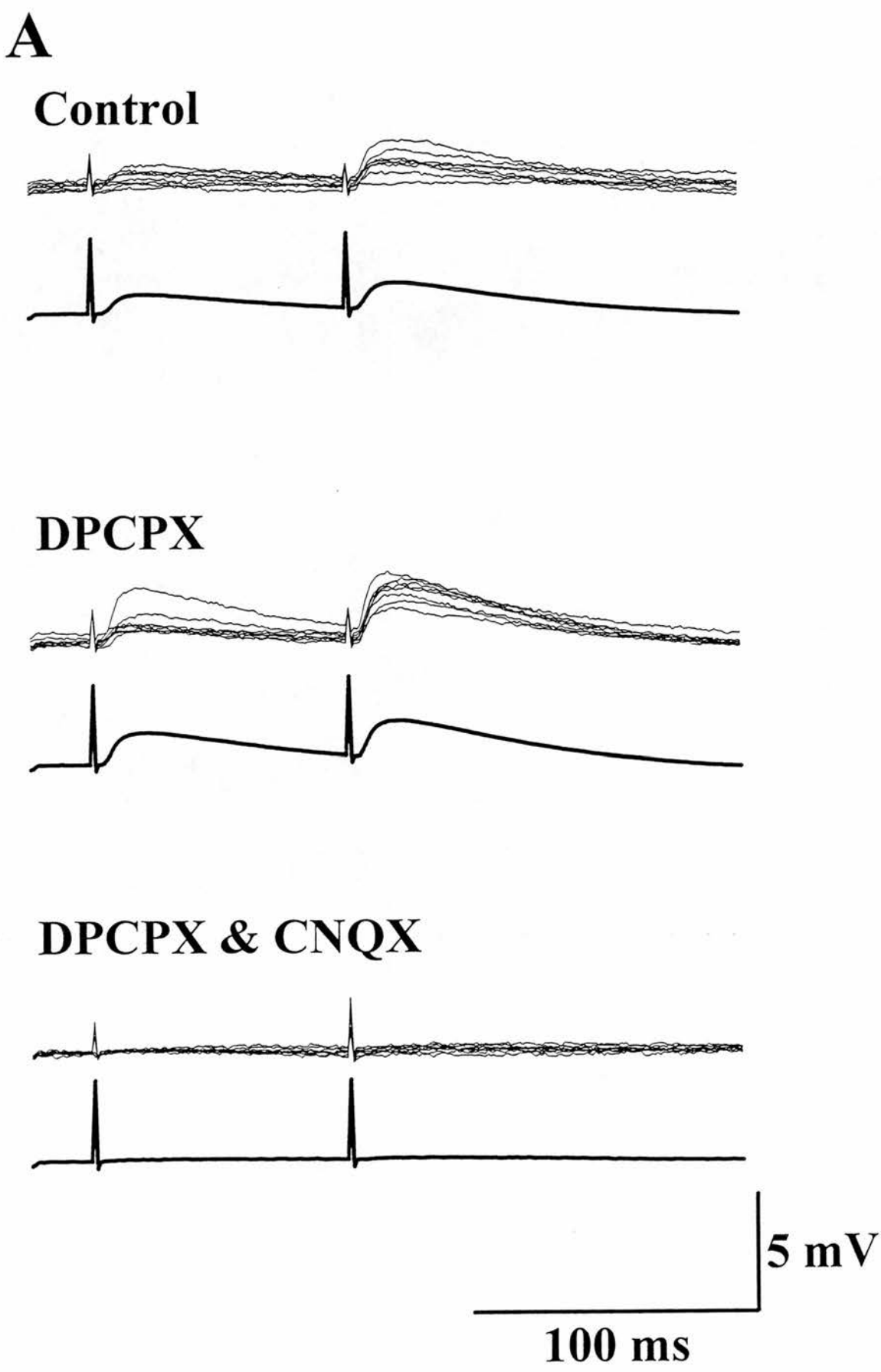
Although in 3 out of 8 neurones a decrease in mean amplitude was observed after exposure to DPCPX, there was no correlation between a decrease in mean amplitude and a decrease in quantal content. Therefore the data from the 8 experiments was pooled for these measurements.

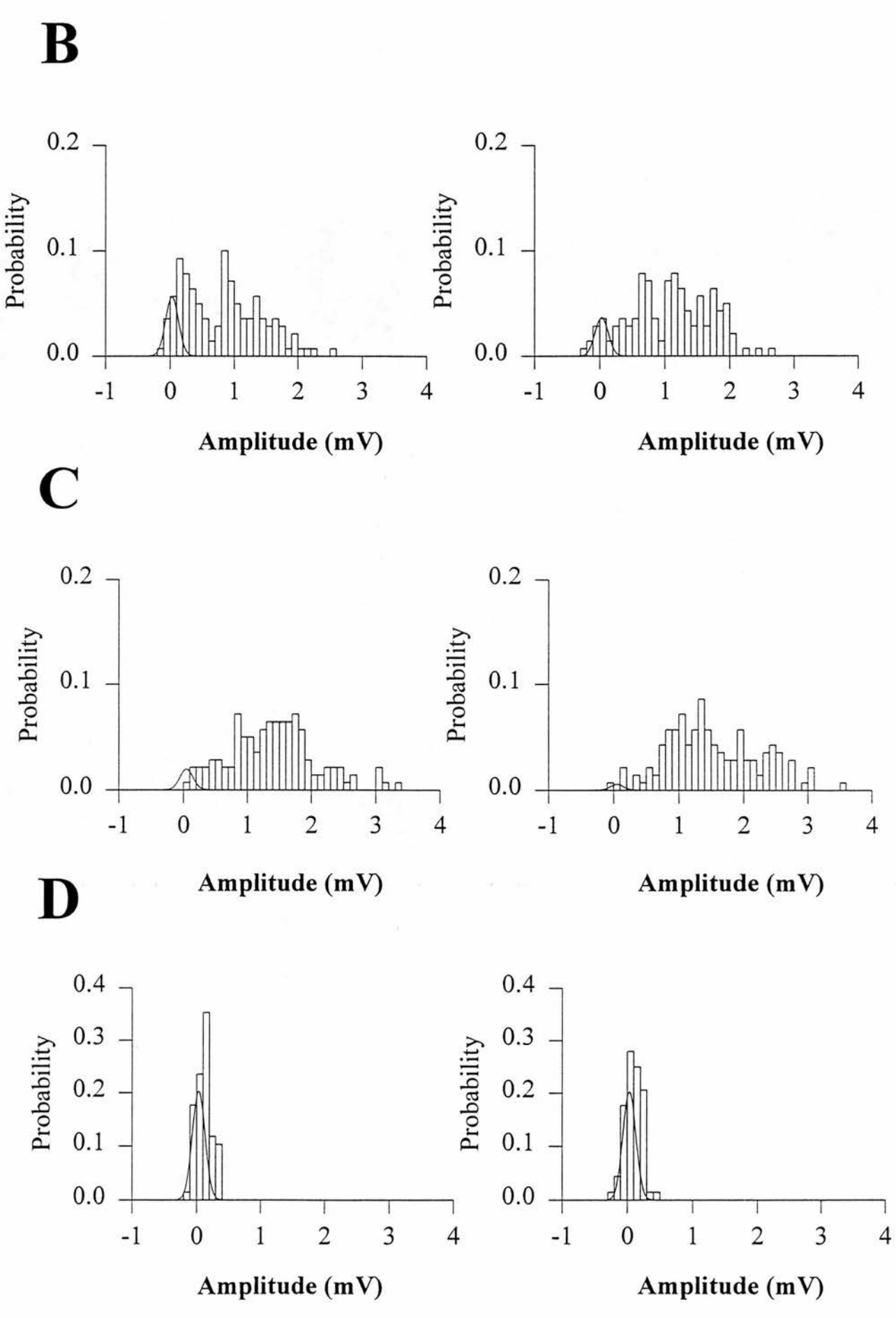
Under control conditions the paired pulse ratio was 2.21. Although the amplitude of the second EPSP was decreased when compared to the first in 4 out of 8 neurones, the mean paired pulse ratio for each neurone under control conditions showed a facilitation. After exposure to DPCPX the ratio decreased to 2.07. The depression of the second EPSP compared with the first EPSP that was observed

Figure 4.11 Effect of DPCPX on paired pulse facilitation of EPSPs

A: shows representative traces of pairs of pulses recorded under current-clamp conditions. The EPSPs were recorded under control conditions (E_m = -56 mV, left hand traces), in the presence of the adenosine A_1 antagonist, DPCPX (30 nM, E_m = -54 mV, right hand traces), and in the continuous presence of DPCPX and the AMPA/Kainate receptor antagonist CNQX (30 nM, E_m = -55 mV, traces not shown). The trace outlined by the heavy line represents the mean paired pulse response under each condition. The stimulus intensity was set such that occasional response failures were observed.

B: the amplitude distribution of the EPSPs evoked in control conditions. The left hand histogram represents the distribution of the first EPSP amplitudes (P1, n = 140), the right hand graph shows the distribution of the second EPSP amplitudes (P2, n = 140). The noise is shown as a normal distribution with its peak matched to the histogram class corresponding to the mean noise amplitude. The area under the curve corresponds to the number of response failures, and was 20 for P1 and 13 for P2. The slice was then exposed to DPCPX for 15 minutes and the amplitude distributions of P1 and P2 were measured (C; n = 140). The number of failures decreased to 7 (P1) and 2 (P2). After a 15 minute exposure to DPCPX and CNQX the amplitude distribution was determined (D; n = 70). The number of failures was 70 for P1 and 70 for P2. In this neurone the amplitude of P1 and P2 in control was 0.77 ± 0.05 mV (Mean ± SEM) and 1.01 ± 0.05 mV respectively. The mean P2/P1 ratio was 3.41 indicating that the second EPSP was facilitated by 341 % with respect to the first EPSP overall. After exposure to DPCPX, P1 and P2 amplitudes increased to 1.35 ± 0.06 mV and 1.47 ± 0.06 mV respectively, the P2/P1 ratio decreasing, as predicted, to 1.81. After exposure to DPCPX and CNQX the EPSPs were completely abolished, P1 was to 0.07 ± 0.01 mV and P2 was 0.05 ± 0.02 mV.


A
Control
DPCPX
DPCPX & CNQX
5 mV
100 ms


B
0.2
0.1
0.0
Probability
-1
0
1
2
3
4
Amplitude (mV)
C
D
0.4
0.3

when the mean amplitudes were studied under control conditions was reduced in 2 of the 4 neurones, increased in a third and became a facilitation in the fourth (Fig. 4.13A). When the mean paired pulse ratio for each neurone was studied a PPD is seen in 1 out of the 8 neurones tested.

After exposure to CNQX and DPCPX the EPSPs were abolished and could not be distinguished from the background noise. ($P < 0.005$, paired student's t-test).

As can also be seen from Fig. 4.11B and C the amplitude distribution range was increased after exposure to DPCPX (C) compared to control (B). When the distributions were examined at to determine whether any changes occurred due to the drug application it was found that in 5 out of 8 neurones the amplitude distributions changed significantly ($P < 0.05$, Kolmogorov Smirnov test) for both the first and second EPSP. Also the paired pulse protocol significantly changed the distribution in 5 out of 8 neurones under control conditions and in the presence of DPCPX.

Voltage clamp

A further set of experiments was carried out under voltage clamp conditions. The hippocampal CA1 neurone was continuously clamped at -70 mV. EPSCs were recorded under control conditions and in the presence of DPCPX (Fig. 4.12A). As can be seen from this figure the amplitude of the EPSCs increased in the presence of DPCPX. The amplitudes were measured and are plotted in Figure 4.12B, C. The amplitude distribution was shifted to the right after exposure (15 minutes) to DPCPX. A rightwards shift was seen for the distribution of the amplitudes of EPSC 2 when compared with those of EPSC 1 under control conditions and after exposure to DPCPX. Comparison of the amplitude distributions showed that in 6 out of 9 neurones there was a significant change after exposure to DPCPX for EPSC 1 and for 8 out of 9 neurones for EPSC 2 ($P < 0.01$, Kolmogorov Smirnov test). Under control conditions 8 out of 9 neurones had significantly different amplitude distributions for EPSC 2 compared with EPSC 1 and for all neurones after exposure to DPCPX ($P < 0.01$, Kolmogorov Smirnov test).

The mean amplitudes of the EPSCs 1 and 2 were 29.5 ± 6.22 pA and 43.0 ± 9.87 pA respectively. After exposure to DPCPX these amplitudes were increased to

Figure 4.12 Paired pulse facilitation, effect of DPCPX on evoked EPSCs

A: shows representative traces of pairs of pulses recorded under voltage-clamp conditions. The EPSCs were recorded under control conditions (hc = -0.38 nA), in the presence of the adenosine A_1 antagonist, DPCPX (30 nM, hc = -0.41 nA). The trace outlined by the heavy line represents the mean paired pulse response under each condition.

B: the amplitude distribution of the EPSCs evoked in control conditions. The left hand histogram represents the distribution of the first EPSP amplitudes (P1, n = 120), the right hand graph shows the distribution of the second EPSP amplitudes (P2, n = 120). The noise, was determined by measuring over similar intervals as used to measure the EPSP amplitude. In this neurone no response failures were observed either in control or in the presence of DPCPX. The slice was then exposed to DPCPX for 10 - 15 minutes and the amplitude distributions of P1 and P2 were measured (C; n = 120). In this neurone the amplitude of P1 and P2 in control was 46.1 ± 1.31 pA (mean ± SEM) and 78.8 ± 1.70 pA respectively. The P2/P1 ratio was 1.81 indicating that the second EPSP was facilitated by 81 % with respect to the first EPSP. After exposure to DPCPX, P1 and P2 amplitudes increased to 65.4 ± 1.54 pA and 102.0 ± 2.11 pA respectively, the P2/P1 ratio decreasing to 1.72.

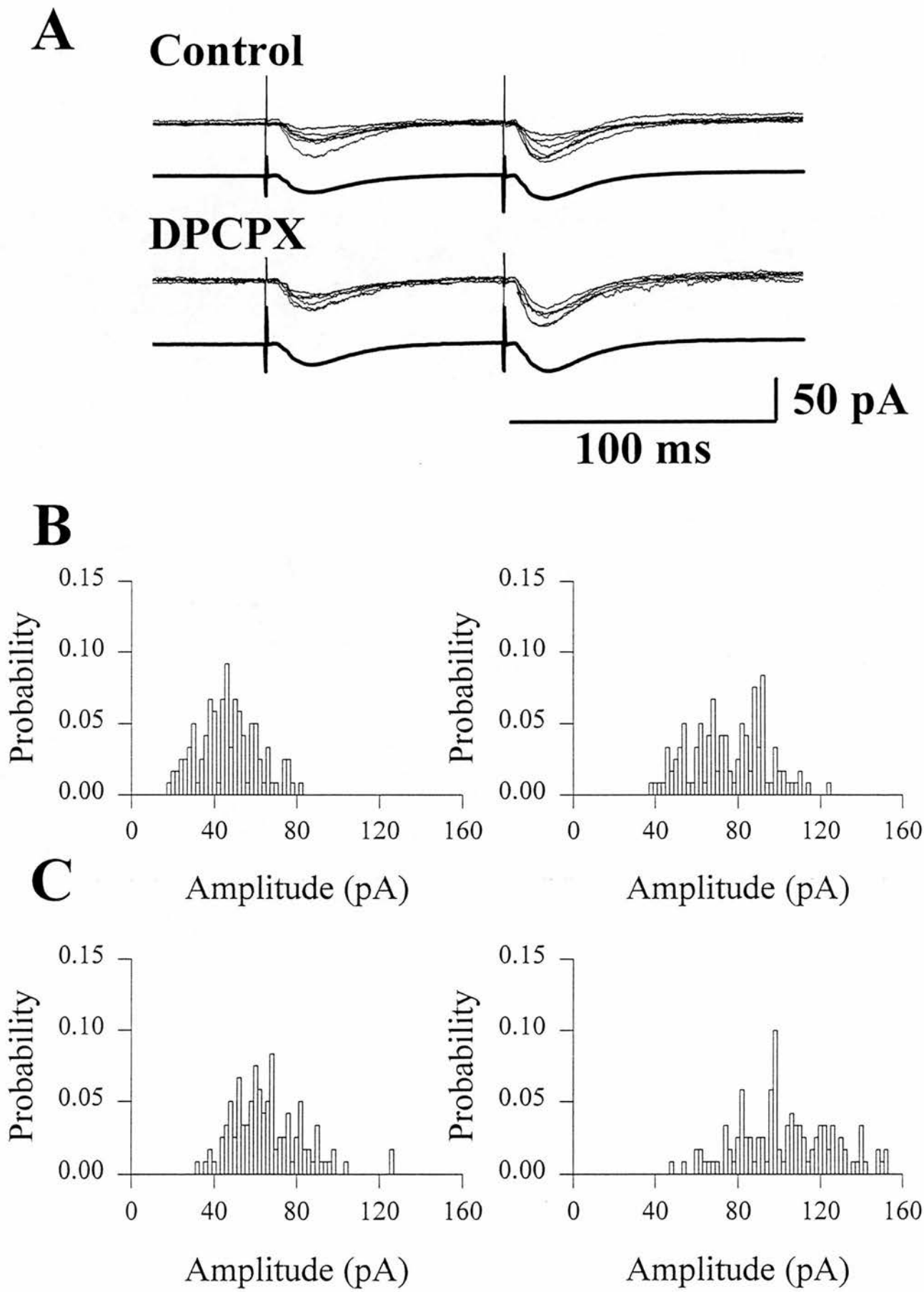
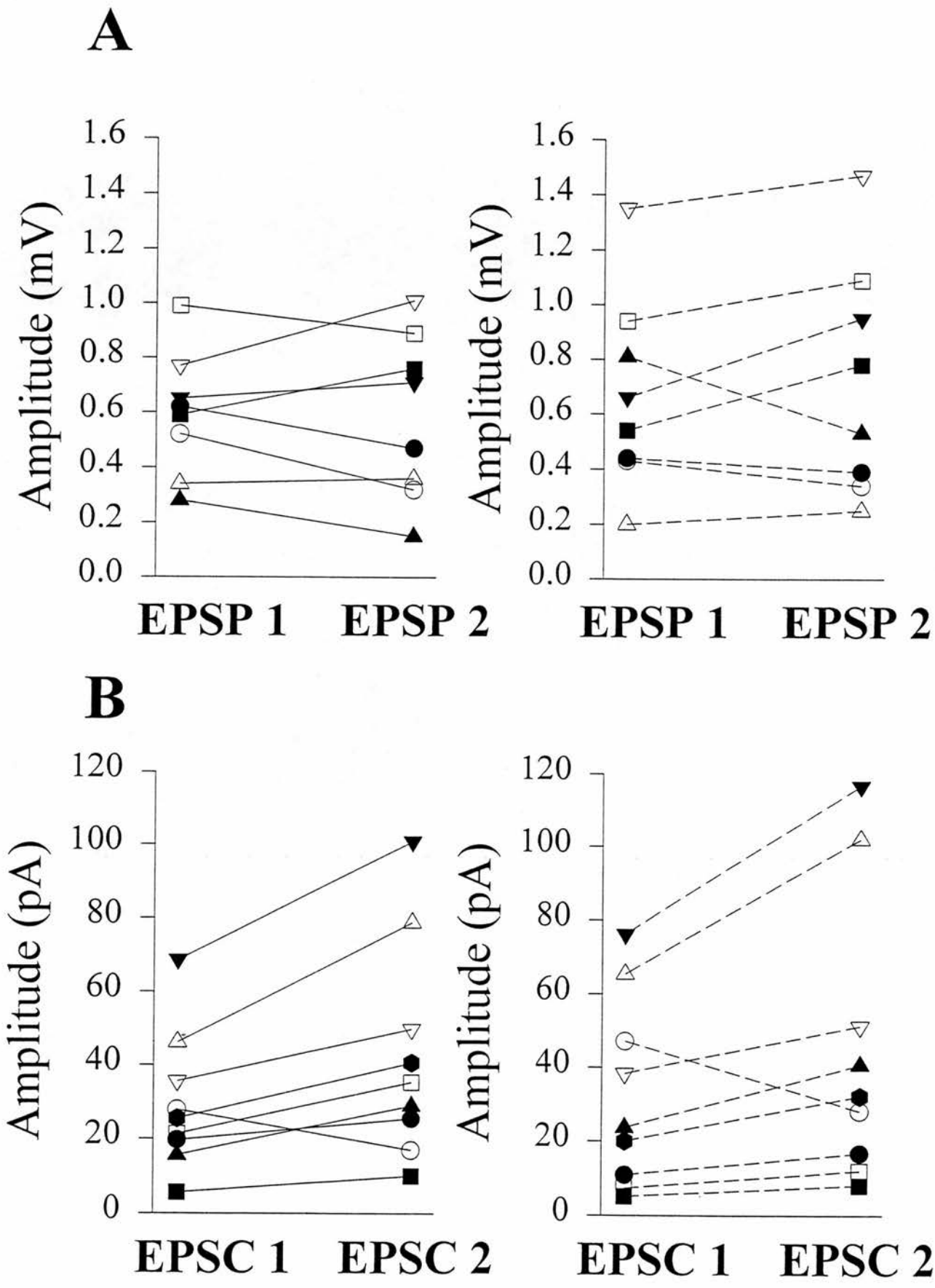

A
Control
DPCPX
50 pA
100 ms
B
C
Probability
0.15
0.10
0.05
0.00
0
40
80
120
160
Amplitude (pA)

Figure 4.13 Mean amplitudes after exposure to DPCPX

These graphs show the mean EPSC amplitude in control and the presence of the DPCPX, for EPSC 1 and EPSC 2 in the paired pulse experiments under voltage clamp conditions. The same number of EPSCs were measured in control conditions and in the presence of DPCPX. A: A second set of experiments was carried out to determine the effect of DPCPX on the mean amplitude of EPSPs 1 and 2. The left hand graph represents the control amplitudes of EPSP 1 and 2, the right hand graph shows the same neurones after 15 minutes exposure to DPCPX. When the mean amplitudes were examined there is a depression in the mean amplitude in 4 out of 8 neurones. After exposure to DPCPX 3 of the 8 neurones that showed a mean depression in amplitude of P2 compared with P1 were still depressed. In 5 out of 8 neurones the amplitude of the first EPSP decreases after exposure to DPCPX. For the second EPSP the amplitude decreases from the control value in just 2 out of 8 neurones.

B: The left hand graph represents the control amplitudes of EPSC 1 and 2, the right hand graph shows the same neurones after 15 minutes exposure to DPCPX. Under control conditions a mean facilitation was seen all 9 neurones (P2/P1 = 2.11). After exposure to DPCPX the mean facilitation ratio fell to 1.89. The paired pulse facilitation protocol significantly increased the amplitude of the second EPSC compared with the first under control conditions ($P < 0.02$, paired t-test, Wilcoxon signed ranks test). The amplitude of the EPSCs increased after exposure to DPCPX but this was not significant (Control P1 = 29.5 ± 6.22 pA, P2 = 43.0 ± 9.87 pA; DPCPX P1 = 32.6 ± 8.60 pA, P2 = 45.2 ± 13.0 pA) .


A
Amplitude (mV)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
EPSP 1
EPSP 2
Amplitude (mV)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
EPSP 1
EPSP 2
B
Amplitude (pA)
120
100
80
60
40
20
0
EPSC 1
EPSC 2
Amplitude (pA)
120
100
80
60
40
20
0
EPSC 1
EPSC 2

32.6 ± 8.60 pA and 45.2 ± 13.02 pA respectively. There were no significant differences observed. In 4 out of 9 neurones the EPSC amplitude decreased after exposure to DPCPX by 35 %, the remaining 5 neurones showed a 30 - 35 % increase in amplitude after exposure to DPCPX (Fig. 4.13B). The mean paired pulse ratio was 2.11 under control conditions and it decreased slightly to 1.89 after exposure to DPCPX.

When the amplitudes for just the neurones that showed an increase in amplitude after exposure to DPCPX were compared it was found that DPCPX significantly increased the mean amplitude from 38.3 ± 9.09 pA to 55.3 ± 14.7 pA for EPSC 1 ($P < 0.05$, paired student's t-test). The amplitude of EPSP 2 increased from 49.5 ± 9.23 pA to 69.4 ± 16.4 pA after exposure to DPCPX, this increase was significant ($P < 0.05$, paired student's t-test). The amplitudes of those neurones that showed a decrease after exposure to DPCPX (15 minutes) were not significantly changed. For those neurones which showed an increase in amplitude after exposure to DPCPX (30 nM) the paired pulse ratio decreased from a control value of 2.00 ± 0.29 to 1.65 ± 0.27 ($n = 5$). Where a decrease in amplitude was observed after exposure to DPCPX the mean paired pulse ratio was 2.22 ± 0.22 under control conditions and 2.38 ± 0.15 after exposure to DPCPX ($n = 4$). These changes were not significant.

Quantal Parameters - Current clamp

The quantal content (m_f and m_{cv}) were calculated for each neurone and the individual values are shown in Table 4.4 and Appendix 4.4 respectively. The mean values are shown below. The size of m_f for EPSP 2 was less than that for EPSP 1 under control conditions indicating a mean increase in response failures to the second stimulus in the eight neurones tested. The mean quantal content increased after exposure to DPCPX but these changes were not significant. Although the mean m_{cv} for EPSP 1 and EPSP 2 increased after exposure to DPCPX the changes were not significant (Wilcoxon signed ranks test).

When m_f and m_{cv} were studied for those neurones that showed a mean increase in amplitude after exposure to DPCPX and those that showed a mean

Table 4.4

This table shows the quantal parameter m_f for eight hippocampal CA1 neurones, measured in control and after exposure to DPCPX (30 nM).

Table 4.4

	EPSP 1 m_f		EPSP 2 m_f	
	Control	DPCPX	Control	DPCPX
1	1.88	1.08	2.01	1.12
2	1.14	1.75	1.83	1.75
3	2.30	-	1.71	-
4	1.14	3.15	1.25	3.84
5	4.32	4.32	2.45	4.32
6	3.40	-	-	3.40
7	1.03	0.95	1.03	0.80
8 *	1.95	3.00	2.38	4.25

* Neurone illustrated in figure 4.11

decrease in amplitude it was found that both m_f and m_{cv} increased regardless of the effect of DPCPX on amplitude. As described above although the mean amplitude shows both increases and decreases in individual experiments this did not correlate with any increases or decreases seen in quantal content and quantal amplitude. Thus this data was pooled for all eight experiments. EPSPs 1,2,5 and 7 in Table 4.4 are those that showed a decrease in mean amplitude after exposure to DPCPX.

	EPSP 1		EPSP 2	
	m_f	m_{cv}	m_f	m_{cv}
Control	2.14	3.77	1.81	2.58
DPCPX	2.37	7.14	2.78	4.05

The quantal amplitudes (q_{cv}) were calculated (see Appendix 4.4) and are shown below:

	EPSP 1	EPSP 2
	q_{cv} (mV)	q_{cv} (mV)
Control	0.35	0.32
DPCPX	0.24	0.32

There was no significant difference in q_{cv} under control conditions and in the presence of DPCPX.

Quantal Parameters - Voltage clamp

The quantal parameters m_f, m_{cv} and q_{cv} were determined (Table 4.5 and Appendix 4.5) from the data and the mean values are shown below.

	EPSC 1		EPSC 2	
	m_f	m_{cv}	m_f	m_{cv}
Control	2.40	6.29	2.28	8.96
DPCPX	2.17	10.39	2.77	14.79

Table 4.5

This table shows the quantal parameter m_f for nine hippocampal CA1 neurones, measured in control and after exposure to DPCPX (30 nM).

Table 4.5

	EPSC 1 m_f		EPSC 2 m_f	
	Control	DPCPX	Control	DPCPX
1	1.26	2.07	1.30	2.25
2	0.92	0.58	1.90	1.90
3	1.77	2.53	2.41	3.91
4	3.84	-	-	-
5	2.30	3.69	1.74	-
6	3.36	1.01	-	2.44
7 *	-	-	-	-
8	-	-	-	-
9	3.36	3.14	4.05	3.36

* Neurone illustrated in figure 4.12

An overall increase in m_{cv} is observed after exposure to DPCPX, this is seen for both EPSCs ($P < 0.01$, Wilcoxon signed ranks test). The value of m_f decreased after DPCPX for EPSC 1 and increased slightly for EPSC 2, these changes were not significant.

	EPSC 1 q_{cv} (pA)	EPSC 2 q_{cv} (pA)
Control	5.01	7.39
DPCPX	3.08	3.37

The quantal amplitude decreased after exposure to DPCPX, but this was not significant. There did not appear to be any differences in m_{cv} or q_{cv} for the EPSCs which showed a decrease in amplitude in response to exposure to DPCPX when compared to those that showed an increase. EPSCs 1,2,5,8 in Table 4.5 are the EPSCs that decreased in amplitude after DPCPX.

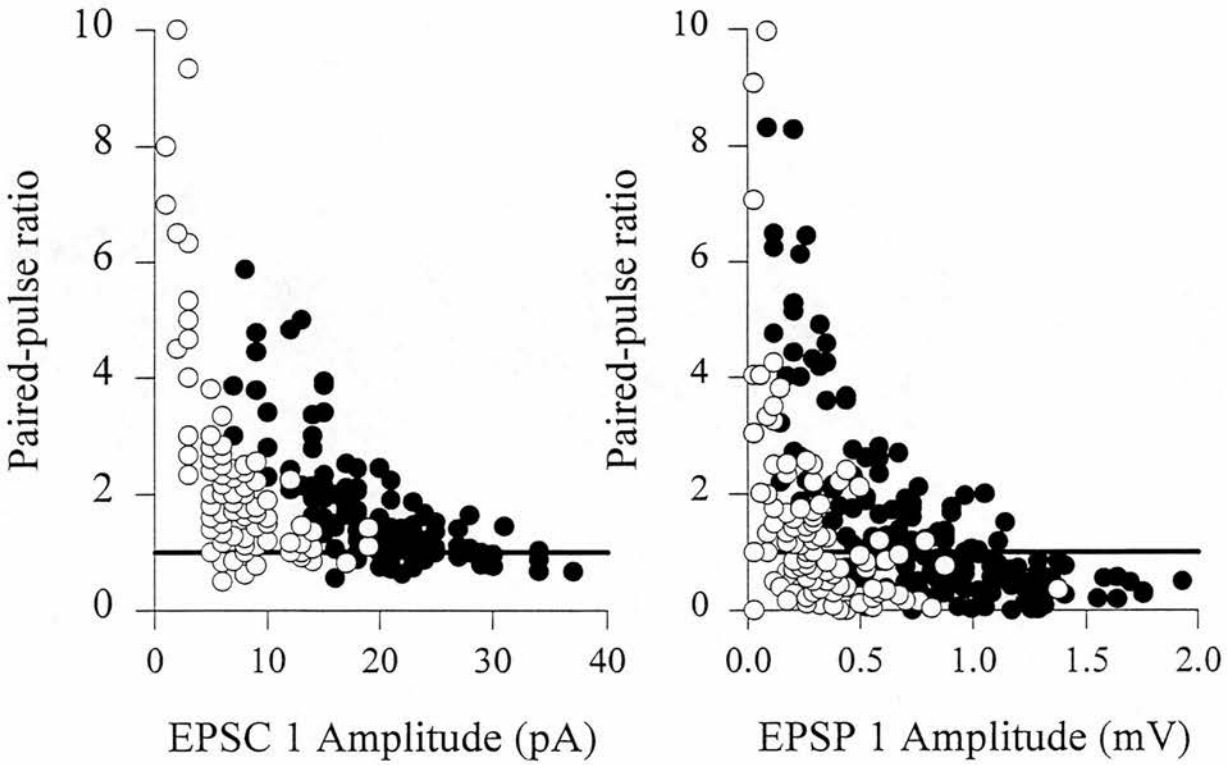
When the data were pooled for the current and voltage clamp experiments it was found that m_f was not significantly increased in the presence of DPCPX over the value found under control conditions for either response. However DPCPX significantly increased m_{cv} from 5.20 ± 0.9 in control to 8.71 ± 1.75 for response 1 and from 6.10 ± 1.59 to 8.99 ± 1.90 for response 2 ($P < 0.005$, signed ranks test).

Paired Pulse Facilitation or Depression?

The paired pulse ratio, as a function of the amplitude of the first response, was studied (Fig. 4.14). It was found that as the amplitude of the first response increased the paired pulse ratio changed from being more likely to show facilitation (PPF) to more likely to show a depression (PPD). The individual responses were recorded for all current and voltage clamp experiments under control conditions (Fig. 4.15). The effect of the adenosinergic compounds CCPA and DPCPX were studied on the pairs of pulses. It was found that when a PPF was seen the amplitude of the first response was smaller than the overall mean first response under control

Figure 4.14 Paired Pulse ratio vs Amplitude

Plot of the paired pulse ratio (EPSC2/EPSC1) as a function of the E1 amplitude under control conditions (filled circles) and after exposure to either CCPA (A; open circles) or DPCPX (B). As the E1 amplitude increased the degree of paired pulse facilitation decreased and a paired pulse depression was observed more frequently. The largest facilitation was observed with the smallest E1 amplitudes. After exposure to CCPA the maximum E1 amplitude was reduced, and the distribution shifted to the left ($P < 0.05$, Kolmogorov Smirnov test). This was found under current clamp (right hand graph) and voltage clamp conditions (left hand graph). After exposure to DPCPX the maximum E1 amplitude was increased and the distribution shifted to the right ($P < 0.05$, Kolmogorov Smirnov test). This was found under current clamp (right hand graph) and voltage clamp conditions (left hand graph). Interestingly the pattern of the distribution of the paired pulse ratios was similar under control conditions and in the presence of either CCPA or DPCPX. The magnitude of the PPF or PPD was unchanged by exposure to CCPA or DPCPX.


A CCPA
Paired-pulse ratio
EPSC 1 Amplitude (pA)
EPSP 1 Amplitude (mV)

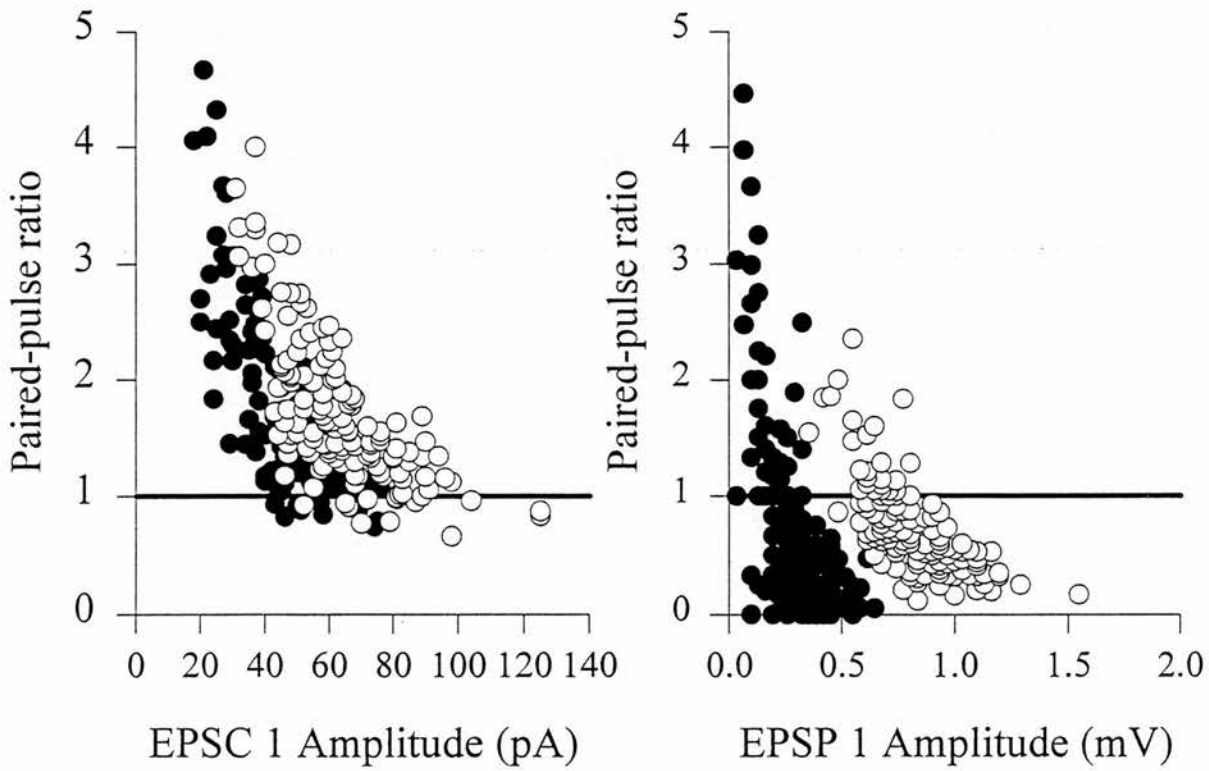
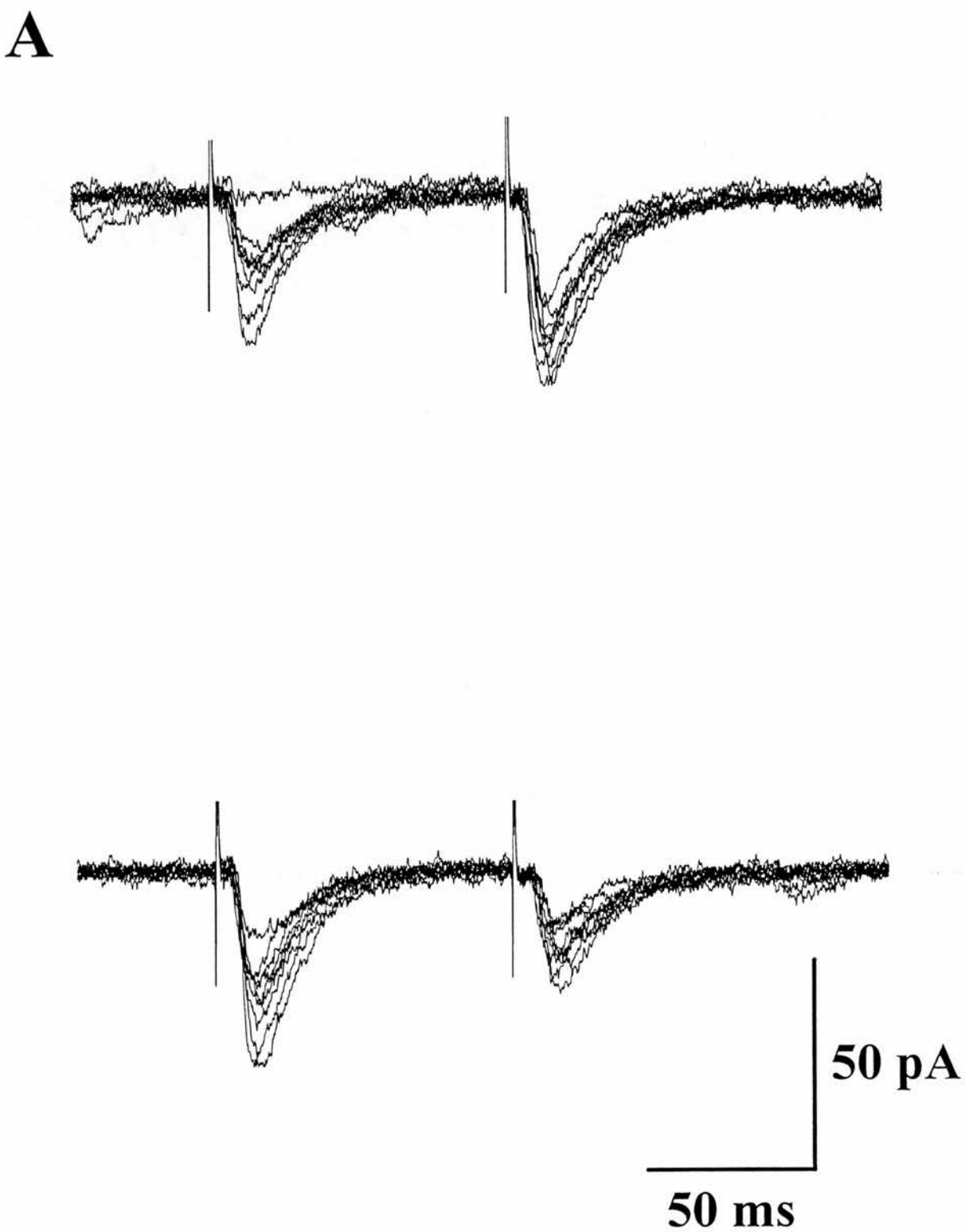
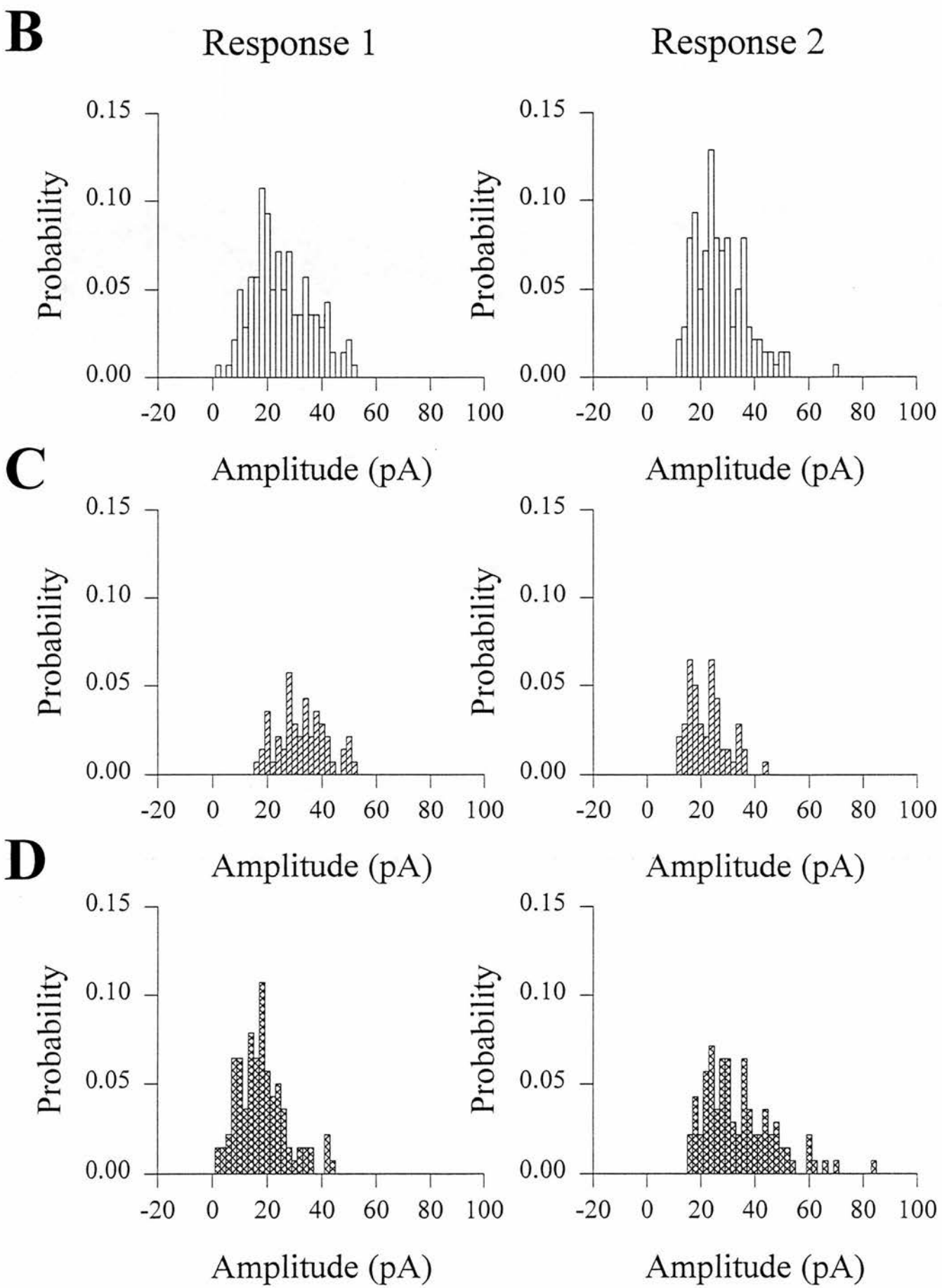

B DPCPX
Paired-pulse ratio
EPSC 1 Amplitude (pA)
EPSP 1 Amplitude (mV)

Figure 4.15 Paired Pulse Depression and Paired Pulse Facilitation

Pairs of responses were evoked and recorded from hippocampal CA1 neurones voltage clamped continuously at -70 mV. The traces shown in A are 9 that either facilitated (upper) or depressed (lower). They were taken from an arbitrary point in the data and the first 9 that showed PPD and PPF were taken. For this data amplitude histograms were drawn for all the responses (B), for those that showed PPD (C) and those that showed PPF (D).The histograms for response 1 are on the left and for response 2 on the right. The overall mean amplitude was 24.7 ± 0.91 pA for EPSC 1 and 27.0 ± 0.83 pA for EPSC 2. When paired pulse depression was observed the mean amplitudes were 32.2 ± 1.21 pA and 21.9 ± 0.94 pA for EPSCs 1 and 2 respectively. Where paired pulse facilitation was observed the mean amplitudes were 19.3 ± 0.95 pA and 30.7 ± 1.12 pA for EPSCs 1 and 2 respectively. Paired pulse depression was seen in 57 out of 140 trials.


A
50 pA
50 ms


B
Response 1
Response 2
C
D
Probability
0.15
0.10
0.05
0.00
-20
0
20
40
60
80
100
Amplitude (pA)

conditions (n = 12 current clamp, n = 15 voltage clamp) and in the presence of either the agonist (CCPA, n = 4 current clamp, n = 6 voltage clamp) or antagonist (DPCPX, n = 8 current and n = 9 voltage clamp), see Table below. The amplitude of the second response was larger than the overall mean second response amplitude when a PPF was observed under all conditions. When a PPD was seen the first response was larger than the overall mean and the second smaller than the mean, this was observed under control conditions and in the presence of the adenosinergic compounds. The values for all responses as a percentage of the overall mean under each condition is shown below and the significant differences when compared with the overall mean are indicated (Mann Whitney U Test).

		PPF		PPD	
		E1 (%)	E 2 (%)	E 1 (%)	E 2 (%)
EPSP	Control *	65.0 ± 3.7	142.6 ± 7.9	146.4 ± 5.6	57.9 ± 3.2
	CCPA §	32.4 ± 23.1	158.4 ± 5.0	183.5 ± 22.7	26.7 ± 9.8
	DPCPX#	66.4 ± 5.7	147.4 ± 8.9	137.4 ± 7.9	56.7 ± 7.9
EPSC	Control*	83.9 ± 2.4	114.9 ± 5.5	142.3 ± 5.2	70.3 ± 2.8
	CCPA ¶	83.5 ± 3.0	113.9 ± 2.7	147.8 ± 2.8	60.5 ± 2.3
	DPCPX*	82.7 ± 4.4	122.1 ± 9.4	139.7 ± 6.7	71.4 ± 2.9

* P<0.0001 # P<0.0002 § P<0.05 ¶ P<0.005

The Paired Pulse Ratio

The mean paired pulse ratio for all responses was found to be 2.35 ± 0.37 for EPSPs and 2.03 ± 0.18 for EPSCs under control conditions. Furthermore paired pulse depression was observed in 46.4 ± 4.0 % of individual trials for EPSPs (n = 12) and in 25.2 ± 3.8 % for EPSCs (n = 15), under control conditions. The results were compared before and after exposure to the agonist and antagonist. Under current clamp conditions it was found that the paired pulse ratio was decreased from 2.64 ± 0.39 to 1.71 ± 0.03 after exposure to CCPA. This result was unexpected. The percentage of trials that showed a PPD increased from 34.1 ± 4.5 % to 42.3 ± 3.1 %. DPCPX decreased the paired pulse ratio from 2.21 ± 0.36 in control to 2.07 ± 0.38.

The number of trials that showed a PPD decreased from 52.6 ± 4.2 % to 48.0 ± 5.3 %. None of these changes were significant.

Under voltage clamp conditions it was found that CCPA increased the paired pulse ratio from a control value of 1.93 ± 0.16 to 2.57 ± 0.18 ($P < 0.05$, Mann Whitney U test). The number of trials that showed a PPD decreased slightly from 25.4 ± 3.8 % to 23.8 ± 4.7 %. DPCPX decreased the paired pulse ratio from 2.11 to 1.89 and the number of trials that showed a PPD did not alter (25.0 ± 6.2 % in control and 25.1 ± 8.2 %) after exposure to DPCPX. These changes were not significant.

The current and voltage clamp data were separated into two on the basis of an increase or a decrease in mean amplitude in response to exposure to DPCPX, as described earlier. The percentage of trials that exhibited PPD were examined to determine whether the proportion that showed PPD was increased when DPCPX decreased the mean response amplitude. It was found that the percentage of trials that resulted in paired pulse depression was the same regardless of an increase or decrease in the mean amplitude after exposure to DPCPX. The effect of DPCPX on mean amplitude was not dependent on the proportion of trials that exhibited a PPD. Even when the proportion of trials that exhibited PPD was examined in neurones where a > 10 % increase in mean amplitude was observed it was found that 51.8 % and 26.5 % of trials exhibited PPD under control conditions and 49.0 % and 27.8 % of trials in the presence of DPCPX (current- and voltage-clamp respectively, $n = 4$ for each condition).

Is there a Correlation Between the Amplitudes of the First and Second Responses

The amplitudes of the first responses were looked at to determine whether there was a correlation between the amplitude of the first event and the amplitude of the second; i.e. if the first response was smaller than the mean of all first reponses would the amplitude of the second response be larger than the mean of all the second reponses (Fig. 4.14). In order to eliminate those responses close to the mean of the

first response the top 1/3 and bottom 1/3 of the first responses were looked at (the largest and the smallest only).

It was found that for the current clamp experiments that the mean EPSP2 response for the largest and smallest 1/3 of EPSP1 amplitudes was 98.8 ± 0.5 % and 100.4 ± 8.1 % of the mean EPSP2 response respectively, these were not significantly different from the mean EPSP2 response (n = 12). In the presence of the adenosine A_1 receptor agonist CCPA the largest and smallest 1/3 of EPSP1 responses were accompanied by EPSP2 amplitudes of 124.9 ± 16.3 % ($P < 0.05$, n = 4) and 78.7 ± 17.3 % of the mean EPSP2 response respectively. In the presence of the adenosine A_1 receptor antagonist DPCPX the EPSP2 responses were 98.0 ± 3.8 % and 105.3 ± 2.9 % of the mean EPSP2 response (n = 8) for the largest and smallest 1/3 of EPSP1 responses respectively. This was not significant. The only group of data in which a significant result was seen shows that a large first response also produced a larger than mean second response (124.9 ± 16.3 % of the mean EPSP2 response). This result is the opposite to that shown by Thompson et al., (1996).

Under voltage clamp conditions the mean EPSC2 response for the largest and smallest 1/3 of EPSC1 control amplitudes was 102.3 ± 3.1 % and 99.9 ± 3.2 % of the mean EPSC2 response, these were not significantly different from the mean EPSC2 response (n = 15). In the presence of the adenosine A_1 receptor agonist CCPA the largest and smallest 1/3 of EPSC1 responses were accompanied by EPSC2 amplitudes of 111.3 ± 8.7 % and 92.6 ± 9.2 % of the mean EPSC2 response respectively (n = 6). These values were not significant. In the presence of the adenosine A_1 receptor antagonist DPCPX the EPSC2 responses were 97.3 ± 2.7 % and 101.6 ± 2.7 % of the mean EPSC2 response for the largest and smallest 1/3 of EPSC1 responses respectively (n = 9), these values were not significantly different from the overall mean.

Taken to the most extreme example possible if the probability that the paired pulse stimulation will result in a PPF when the first EPSP is smaller than the mean of EPSP 1 then when the first stimulus results in a failure you would expect to see a larger than mean EPSP 2. This analysis was repeated for neurones that had greater than 10 % failures for EPSP 1. In 9 CA1 neurones when recording in current clamp

under control conditions this analysis was possible, and in 5 neurones under voltage clamp. It was found that the mean EPSP 2, when EPSP 1 was a failure, was not different from the overall mean EPSP 2. This was found for both current and voltage clamp conditions.

Although as demonstrated above it was found that when PPF was observed the first response would be smaller than the mean E1 response and the second response would be larger than the mean E2 response, and vice versa when PPD was seen, this does not translate into a correlation that would predict the E2 amplitude will be larger than the mean when E1 was smaller than the mean. PPF is observed even with the largest E1 amplitudes and PPD with the smallest.

Interestingly, when the normalised first response amplitudes were plotted against paired-pulse ratio (Fig. 4.16) it was found that when the first response amplitude was greater than 45 % and 65 % of the maximum first response amplitude observed, then a PPD was found (current- and voltage-clamp respectively). However this delineation between PPF and PPD was only observed when the data was normalised to the maximum response 1 amplitude seen. When CCPA and DPCPX were applied to the CA1 neurone it was observed that the curves did not shift, suggesting that the adenosinergic compounds did not alter the percent maximum amplitude at which a PPF became a PPD.

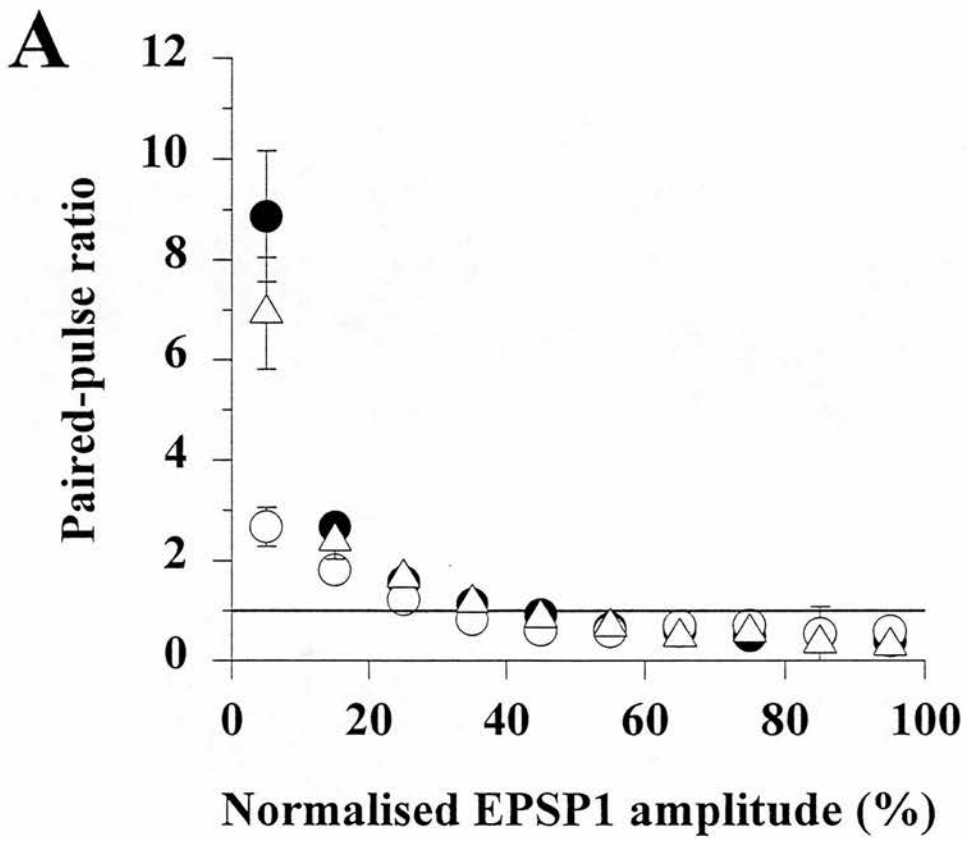
Discussion

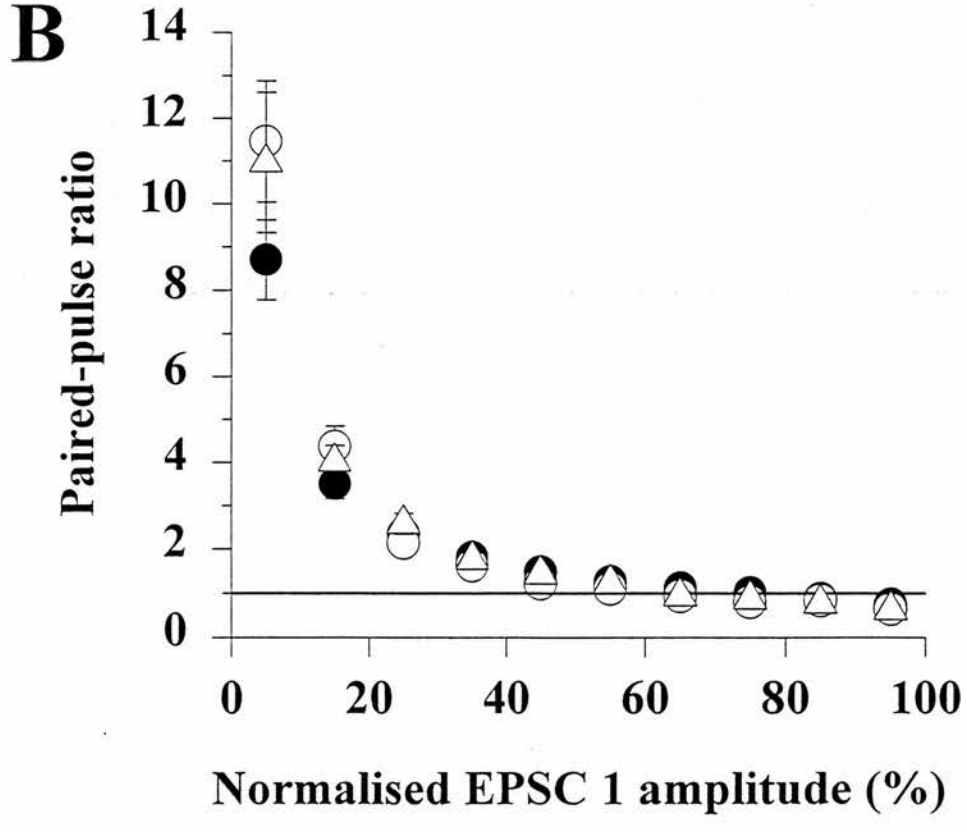
Endogenous Adenosine

An increase in the mean amplitude of EPSPs and EPSCs was observed in 20 out of 32 neurones that were exposed to DPCPX, and no change in mean amplitude was observed in a further 3 neurones. This result suggests that there is an appreciable level of endogenous adenosine present which is exerting a regulatory tonic inhibition of both hippocampal CA1 neurones and the granule cells of the dentate gyrus. In the organotypic slice culture Thompson et al., (1992) demonstrated that DPCPX (200 nM) has no effect on the amplitude of control synaptic responses although this concentration was sufficient to block the pre- and postsynaptic actions of adenosine.

Figure 4.16 Summary of normalised paired-pulse ratios

The pooled paired-pulse ratios are shown for current- (A) and voltage-clamp (B) recording. For each condition (control, filled circles; CCPA, open circles; DPCPX, triangles) the first response amplitude has been normalised to the amplitude of the largest event. Mean values of facilitation and depression were taken (± SEM) in bins of 10 %. PPF of up to 900 % was found for the smallest events and PPD of up to 60 % (current-clamp) and 40 % (voltage-clamp) were observed for the largest events under control conditions.


A
Paired-pulse ratio
12
10
8
6
4
2
0
0
20
40
60
80
100
Normalised EPSP1 amplitude (%)


B
Paired-pulse ratio
14
12
10
8
6
4
2
0
0
20
40
60
80
100
Normalised EPSC 1 amplitude (%)

In support of the findings described above are the results of Haas & Greene (1988). They demonstrated that the adenosine uptake inhibitor nitrobenzylthioinosine (NBTI) mimicked the effects of exogenously applied adenosine on evoked field EPSPs recorded from area CA1 of the rat hippocampus. Furthermore when adenosine deaminase was applied the field potentials were enhanced. It has also been shown that exposure to DPCPX facilitated long term potentiation (LTP, De Mendonça & Ribeiro, 1994), and the adenosine uptake inhibitor (NBTI) reduced LTP. From the results of the experiments showing an increase in evoked EPSP amplitudes and the evidence that endogenous adenosine modulates synaptic plasticity it appears that in the acutely prepared hippocampal slice there is sufficient adenosine present to produce a tonic inhibition.

Single Evoked Responses

Under voltage clamp conditions the increase in EPSC amplitude, recorded from CA1 neurones and from granule cells, seen in the presence of DPCPX was significant. Under current clamp conditions the amplitude increase did not reach a level of significance. This increase in amplitude could be due to either an increased postsynaptic response to a quanta of transmitter (an increase in quantal amplitude, q_{cv}), an increase in the size of the quanta released (m_f and m_{cv}) or a combination of the two. To calculate the quantal content and the quantal amplitude two different models were used: failure counting and the coefficient of variation.

When either CA1 neurones or granule cells were exposed to DPCPX the quantal content, calculated by either method, was increased over the value seen under control conditions, whilst the quantal amplitude was unchanged. In the CA1 neurones this increase in quantal content was significant. Under voltage clamp conditions it was observed in a minority of neurones recorded from that DPCPX decreased the mean EPSC amplitude, and m_{cv} was also reduced (3 out of 15 neurones). This is opposite to the effect predicted; it would be expected that these parameters would increase due to the antagonism of endogenous adenosine at the adenosine A_1 receptor, or if no adenosine was present then no change would be

observed. However in the majority of neurones an increase in quantal content was recorded.

Paired Evoked Responses

The actions of the adenosinergic agonist, CCPA, and antagonist, DPCPX, were studied on pairs of responses evoked with a 90 ms separation. It has been demonstrated that CCPA significantly decreased the mean amplitude of the synaptic potentials and that a partial reversal of the agonist actions was seen when DPCPX was coapplied. CCPA decreased quantal content, when measured using the method of failures and the coefficient of variation, whilst not changing the quantal amplitude. The antagonist, DPCPX, increased quantal content (m_{cv}), with no effect on the quantal amplitude. The evoked potential amplitude increased, but not significantly unlike the results seen with the single pulse recording.

The AMPA/Kainate receptor antagonist, CNQX, completely abolished the evoked potential demonstrating that the glutamate released at these synapses was acting through this type of postsynaptic glutamate receptor alone.

These results are consistent with previous reports using less direct measures of neurotransmitter release in the CNS (Dunwiddie & Hoffer, 1980; Fredholm, 1984; Haas & Greene, 1988; Lupica et al., 1990) and with studies of the effects of adenosine upon quantal neurotransmitter release at the neuromuscular junction (Ginsborg & Hirst, 1972; Ribeiro & Walker, 1975; Silinsky, 1984). Reduction in quantal content (m) at nerve-muscle synapses without alteration of the average size of a quantum of neurotransmitter (q) is consistent with its reduction in m_{cv} and the absence of effect on q_{cv} in this study. These results are in agreement with those of Lupica et al., (1992) who demonstrated that adenosine and the selective adenosine A_1 receptor agonist, cyclohexyladenosine (CHA) decreased m_{cv} whist not affecting q_{cv}. Further agreement is provided by Scholz & Miller (1991), who showed that the inhibitory effects of adenosine on monosynaptic EPSPs in cell culture were primarily presynaptic, and mediated by an A_1 receptor.

The Effect of the Paired Pulse Protocol on Response Amplitude

In contrast to the results reported by Debanne et al. (1996) no correlation was observed between response 1 and 2 amplitudes. They made recordings from either CA1 or CA3 neurones contained in cultured hippocampal slices, and stimulated release by eliciting an action potential in a single CA3 neurone. Under control conditions they found that PPF was observed when the first EPSC was small and that PPD was observed when the first EPSC was large. This was statistically significant. When recording from CA1 neurones contained in the acutely prepared hippocampal slices it was found that no correlation between response 1 and 2 amplitudes was observed. This was also the case when the largest 1/3 and smallest 1/3 of response 1 amplitudes and the corresponding response 2 amplitudes were looked at under both current and voltage clamp conditions. Even when taken to the extreme of looking at the amplitude of the second response when the first response was a failure, the amplitude of the second response was not significantly larger than the overall mean, or the mean of response 2 amplitudes that did not follow a failure.

When the data was looked at in terms of those responses that showed a PPD or a PPF it was found that the mean response 1 amplitude was smaller than the overall mean when PPF was observed and that the response 2 amplitude was bigger than the mean response 2 amplitude. The converse was observed in the case of those responses that showed a PPD; the first response was bigger than the mean response 1 and response 2 was smaller than the mean response 2. This was seen under current and voltage clamp conditions. This is almost, but not quite, the same as Debanne et al. (1996) observe. The major difference being that more scatter in the amplitude distribution was observed in the recordings from the acutely prepared slice. It was seen that small response 1 amplitudes can show PPD as well as PPF, and similarly for large response 1 amplitudes PPF is observed as well as PPD. The largest PPF ratios were found with the smallest response 1 amplitudes and PPD is greatest with the largest response 1 amplitudes.

Interestingly, when the first response amplitudes were normalised to the largest response seen under each condition, it was found that a PPD was observed

when the conditioning response amplitude was greater than 45 % and 65 % of the maximum amplitude seen (current- and voltage-clamp respectively). When the release probability was altered by addition of CCPA or DPCPX, this PPF versus PPD pattern of responses was not altered.

When the release probability was decreased, by the addition of CCPA, it was found that the number of trials that showed PPD decreased slightly (voltage clamp), and that the paired pulse ratio increased. In the current clamp recordings it was found that a high number of trials resulted in response failures (>60 %) after exposure to CCPA. For this reason although the paired pulse ratio was measured it is likely to be distorted by the high number of response failures seen in the presence of CCPA. The number of trials that resulted in a PPD increased after exposure to CCPA under current clamp conditions. The EPSP and EPSC amplitudes were decreased in the presence of CCPA. Reducing the release probability decreases the quantal content of the first response, indicating that action potential evoked release results in the release of more than one quantum of transmitter onto the postsynaptic CA1 neurone under control conditions. Thus as there was a reduction in the number of quanta released at the first impulse there was more transmitter available for release at the second impulse. This is confirmed by the work of Lupica et al., (1992) who concluded that adenosine presynaptically reduces synaptic strength at Schaffer collateral commissural synapses in the hippocampus by diminishing the number of quanta released, not by reducing the size of these individual quanta or postsynaptic sensitivity to excitatory neurotransmitter. This is confirmed by the m_{cv} measurements which show that CCPA decreases the quantal content of both responses compared with control conditions.

On increasing the release probability, by the addition of DPCPX, it was found that the proportion of trials that showed a PPD did not change (current and voltage clamp). The paired pulse ratio decreased after exposure to DPCPX indicating that there was less transmitter available to be released in response to the second impulse, as a larger amount had been released in response to the first stimulus. This was reflected in the quantal content values calculated for these neurones. The quantal content was increased after exposure to DPCPX for both responses, however only

m_{cv} was significantly increased after exposure to DPCPX under voltage clamp conditions.

Chapter 5

Spontaneous Release

Introduction

The observation that miniature endplate potentials occur in the absence of presynaptic activity suggested that chemical transmission was quantal at the neuromuscular junction (Fatt & Katz, 1952; Katz, 1969). The idea that miniature synaptic potentials at the neuromuscular junction, and by analogy elsewhere, reflect the presynaptic release of transmitter, was a consequence of the theory of humoral synaptic transmission. The finding that the frequency of miniature synaptic potentials depended on presynaptic polarisation, but not postsynaptic, provided strong evidence (Del Castillo & Katz, 1954d; Liley, 1956a) of the presynaptic origin of miniature synaptic potentials. Further evidence was provided by degeneration experiments. Miniature synaptic potentials would be expected to disappear with nerve terminal degeneration and appear again with re-innervation. This was found to be the case at the rat neuromuscular synapse (Liley, 1956b).

Information about the process of transmitter release may be indirectly obtained through the analysis of these spontaneous miniature synaptic potentials (Fatt & Katz, 1952; Del Castillo & Katz, 1954b, d). In the absence of action potentials, these potentials are presumed to result from the spontaneous fusion of one or more synaptic vesicles with the presynaptic membrane, resulting in the release of their neurotransmitter contents (i.e. quanta).

It is important to distinguish miniature synaptic potentials from synaptic potentials evoked by afferent nerve impulses or from potentials resulting from postsynaptic electrical activity electrotonically conducted. Methods which have been used include suppression of electrical activity by a raised concentration of Mg^{2+} or Ca^{2+}, or both (Katz & Miledi, 1963). A better method is the use of tetrodotoxin (TTX, Hubbard et al., 1967; Blakenship & Kuno, 1967) a drug which blocks action potentials based upon the electroresponsive sodium permeability system, but does not affect either spontaneous release of transmitter or its postsynaptic activity (Furukawa et al., 1959; Elmqvist & Feldman, 1965).

Spontaneous miniature synaptic potentials were initially characterised at the neuromuscular junction. In frog spinal motor neurones synaptic activity was shown to persist even after reflexes had been abolished by magnesium (Katz & Miledi,

1963) and impulse propagation blocked by tetrodotoxin (Colomo & Eruklar, 1968). These fluctuations were attributed to the spontaneous secretion of quantal packets of neurotransmitter. It is only in the last 20 years that it has been demonstrated that spontaneous miniature synaptic potentials occurred in the mammalian brain (Brown et al., 1979), where this phenomenon was observed in CA3 neurones of the guinea pig hippocampus. Spontaneous miniature synaptic potentials have been recorded in a variety of central neurones including granule cells of the dentate gyrus (Edwards et al., 1990), hippocampal CA1 and CA3 neurones (Ropert et al., 1990; Brown et al., 1979; Bekkers & Stevens, 1995) and the teleost Mauthner cell (Korn et al., 1987).

Spontaneous, miniature synaptic potentials in central neurones reflect the release properties of large numbers of presynaptic terminals. An analysis of the frequency of spontaneous events and the distribution of their amplitudes can provide useful indications of changes in the sensitivity of postsynaptic receptors and/or changes in the presynaptic release process, if they occur in all terminals (Malgaroli & Tsien, 1992; Manabe et al., 1992).

The use of spontaneous transmitter release provides several advantages to studying processes contributing to presynaptic facilitation: i) It allows modulation of release to be studied under conditions in which membrane currents essential for evoked release need play no role (Martin, 1966). ii) Facilitatory mechanisms can be studied in the absence of homosynaptic depression. This is particularly advantageous at synapses such as the sensorimotor synapses in *Aplysia*, which decrement rapidly when an action potential is fired (Castellucci et al., 1970).

Spontaneous miniature EPSPs (mEPSPs) were recorded, using the whole cell patch clamp technique, from CA1 pyramidal neurones contained in a hippocampal slice. Tetrodotoxin (100 nM) was added to the ACSF in order to block action potential evoked release. The amplitudes of and intervals between successive mEPSPs were analysed under control conditions and in the presence of the selective adenosine A_1 receptor compounds.

Results

Spontaneous miniature EPSPs were recorded under control conditions and in the presence of the adenosine A_1 agonist, CCPA, the adenosine A_1 antagonist DPCPX, or in the presence of both CCPA and DPCPX.

Figure 5.1 shows representative traces of miniature EPSPs (mEPSPs) recorded from hippocampal CA1 neurones under control conditions and in the presence of CCPA. CCPA reduces the frequency of occurrence of the mEPSPs (Fig. 5.1B) when compared with the control data (Fig. 5.1A).

The amplitude histograms for this neurone are shown in figure 5.2. The mean mEPSP amplitude was 0.43 ± 0.03 mV under control conditions, after 15 minutes exposure to CCPA (100 nM) the mean amplitude was 0.55 ± 0.08 mV. This increase was not significant ($P > 0.05$, Wilcoxon matched pairs signed ranks test, $n = 11$). Four of these neurones were then exposed to CCPA and DPCPX (30 nM) for 15 minutes. The mean amplitude of the mEPSPs recorded from these neurones was 0.35 ± 0.05 mV. This was not significantly different from the amplitude found in the presence of CCPA ($P > 0.05$, paired student's t-test).

The intervals between successive mEPSPs are shown in figure 5.3 for the data shown in Figure 5.1. The mean interval under control conditions was 1.42 ± 0.23 s, after exposure to CCPA the mean interval increased to 2.19 ± 0.33 s, this increase was significant ($P < 0.005$, paired student's t-test, $n = 11$). Co-application of DPCPX with CCPA decreased the mean interval found in the presence of CCPA to 1.29 ± 0.38 s. This decrease was not significant.

This series of experiments was repeated in the presence of DPCPX alone. Figure 5.4 shows a series of representative traces from a neurone clamped at resting membrane potential under control conditions (A) and in the presence of DPCPX (30 nM, B). The amplitude and interval data from this neurone is illustrated in Figures 5.5 and 5.6 respectively.

The mean amplitude of mEPSPs found under control conditions was 0.37 ± 0.03 mV after exposure to DPCPX the mean amplitude increased to 0.50 ± 0.12 mV ($P > 0.05$, paired student's t-test, $n = 6$).

Figure 5.1 Effect of CCPA on spontaneous miniature EPSPs

Spontaneous miniature EPSPs (mEPSPs) were recorded from hippocampal CA1 neurones bathed in a standard ACSF also containing 100 nM tetrodotoxin (TTX). Thus any synaptic potentials observed were due to spontaneous transmitter release from the nerve terminals. Min EPSPs were recorded under control conditions (A) and in the presence of 100 nM CCPA (B). Some mEPSPs are indicated by the asterisks. The frequency of occurrence of the mEPSPs was reduced to 0.79 Hz in the presence of CCPA from 1.31 Hz under control conditions for the neurone illustrated.

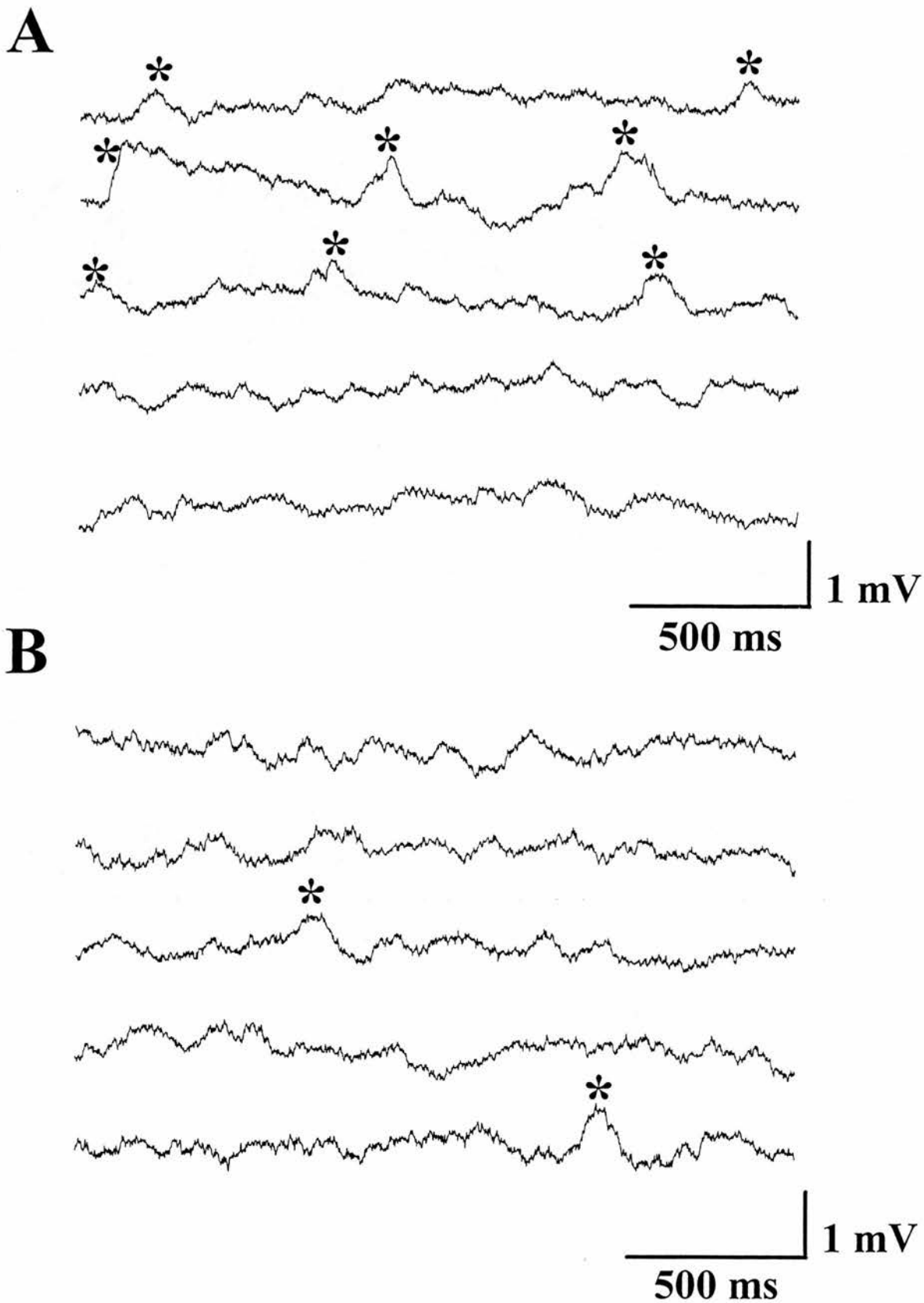

A
1 mV
500 ms
B
1 mV
500 ms

Figure 5.2 Effect of CCPA on mEPSP amplitude

Amplitude histograms for the data illustrated in Figure 5.1 are shown. A 4 minute segment of data, chosen at random from a 10 minute continuous stretch, was analysed. Amplitude histograms for the noise distribution are also represented. Noise was measured as time matched to the time for baseline-peak response from the portion of trace immediately preceding a mEPSP. As can be seen there are no spontaneous mEPSP amplitudes in the histogram class representing the peak of the noise distribution. The mean mEPSP amplitude found under control conditions (A) was 0.37 ± 0.02 mV, after 10 - 15 minutes exposure to 100 nM CCPA (B) the mean amplitude was 0.36 ± 0.04 mV. After a further 10 - 15 minutes exposure to CCPA and 30 nM DPCPX (C) the mean amplitude was 0.33 ± 0.02 mV.

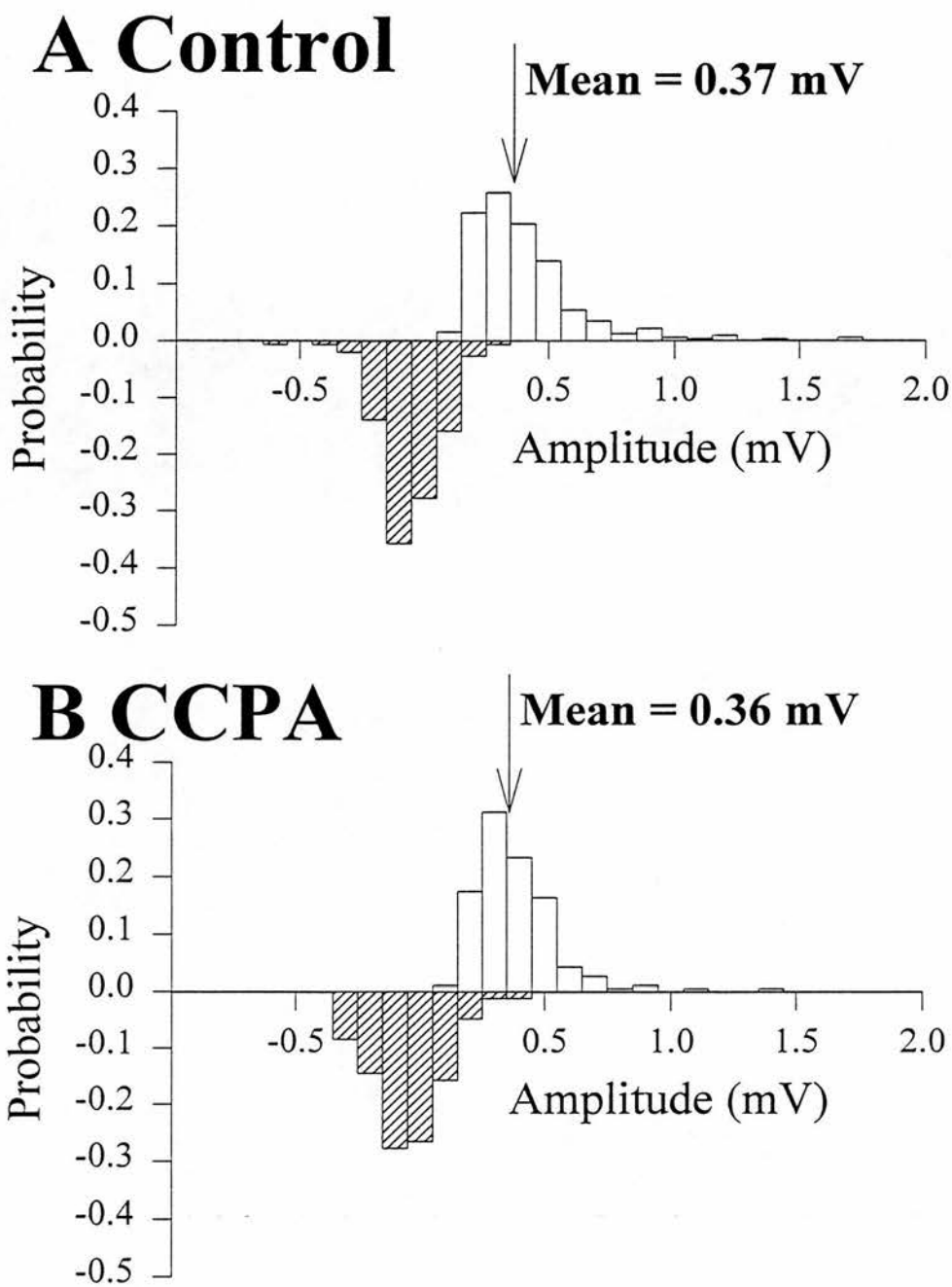
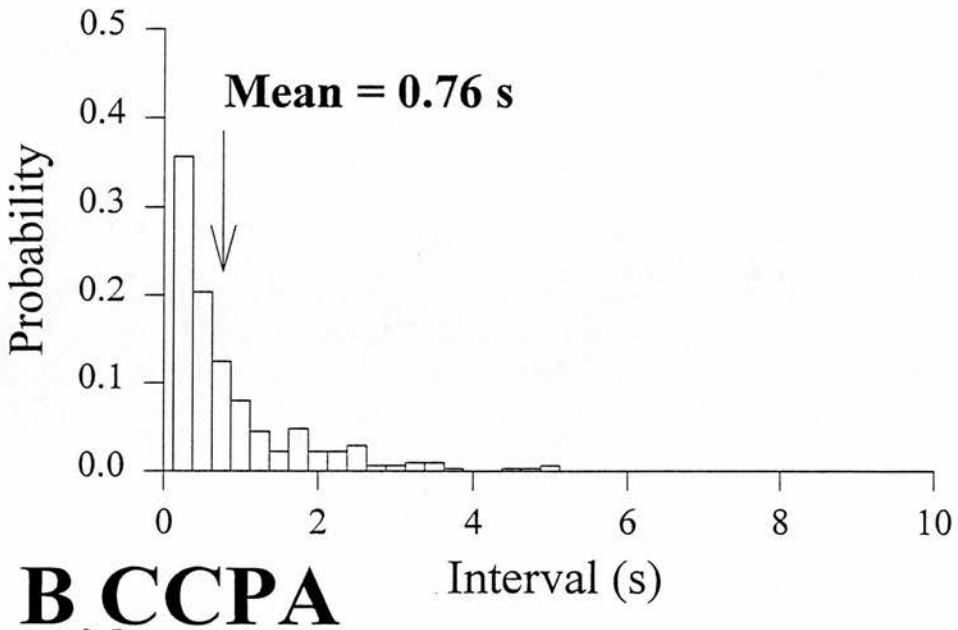
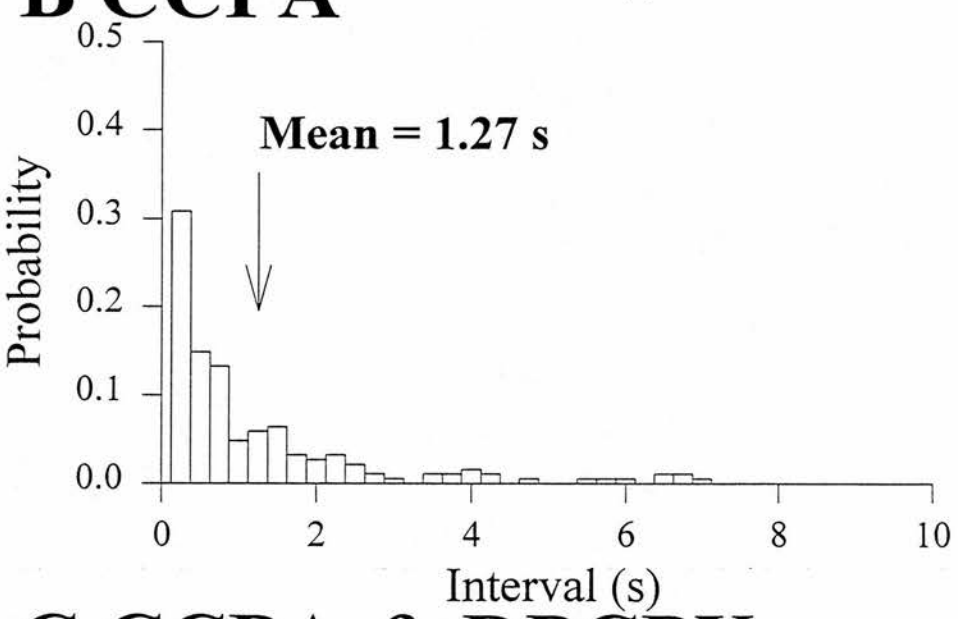

A Control
Mean = 0.37 mV
B CCPA
Mean = 0.36 mV
C CCPA & DPCPX
Mean = 0.33 mV
Probability
Amplitude (mV)
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.5
0.5
1.0
1.5
2.0

Figure 5.3 Effect of CCPA on the interval between mEPSPs

Interval histograms for the data illustrated in Figure 5.1 are shown. The mean interval under control conditions (A) was 0.76 ± 0.05 s, after 10 - 15 minutes exposure to CCPA (100 nM, B) the mean interval increased to 1.27 ± 0.14 s. After a further 10 - 15 minutes exposure to CCPA and DPCPX (30 nM, C) the mean interval returned to 0.74 ± 0.06 s. The frequency of occurrence of mEPSPs for this neurone was 1.31 Hz under control conditions, 0.79 Hz after exposure to CCPA and 1.36 Hz after exposure to CCPA and DPCPX.


A Control
Mean = 0.76 s
Probability
0.5
0.4
0.3
0.2
0.1
0.0
0
2
4
6
8
10
Interval (s)


B CCPA
Mean = 1.27 s
Probability
0.5
0.4
0.3
0.2
0.1
0.0
0
2
4
6
8
10
Interval (s)

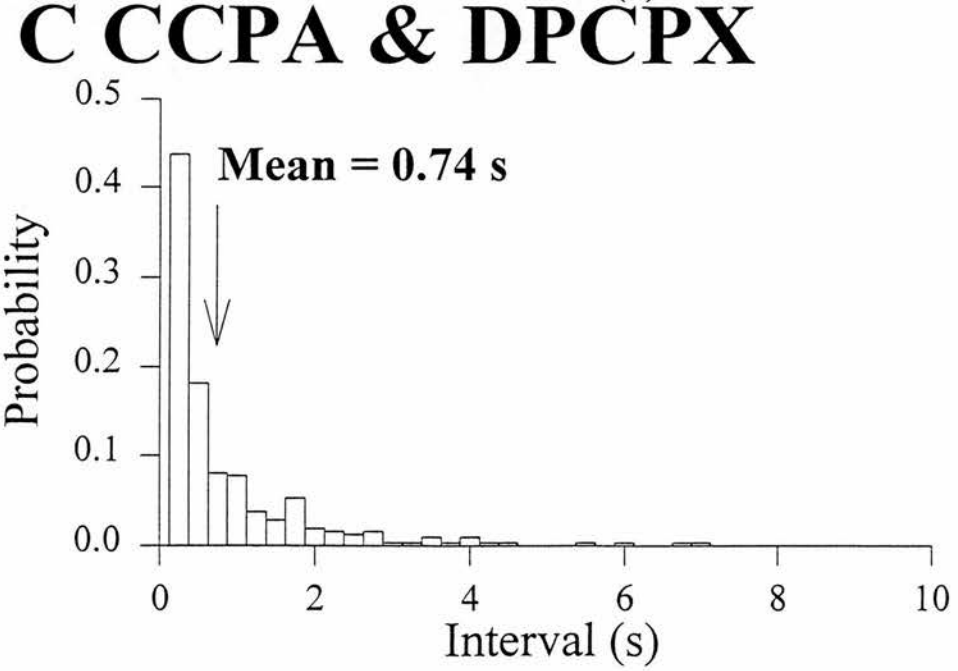
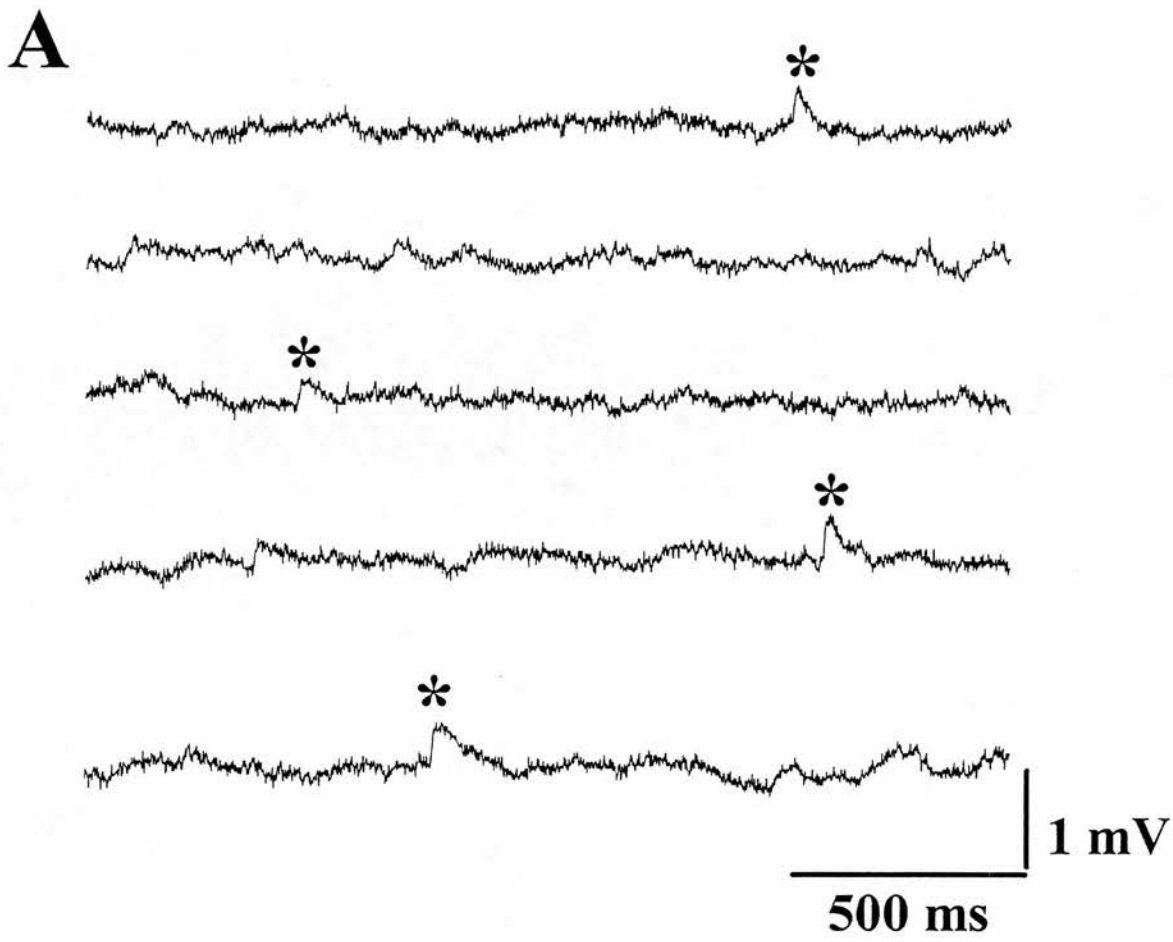

C CCPA & DPCPX
Mean = 0.74 s
Probability
0.5
0.4
0.3
0.2
0.1
0.0
0
2
4
6
8
10
Interval (s)

Figure 5.4 Effect of DPCPX on spontaneous miniature EPSPs

Spontaneous miniature EPSPs (mEPSPs) were recorded from hippocampal CA1 neurones bathed in a standard ACSF also containing 100 nM tetrodotoxin (TTX). Thus any synaptic potentials observed were due to spontaneous transmitter release from the nerve terminals. Min EPSPs were recorded under control conditions (A) and in the presence of 30 nM DPCPX (B). Some mEPSPs are indicated by the asterisks. The frequency of occurrence of the mEPSPs was reduced to 0.69 Hz in the presence of CCPA from 0.77 Hz under control conditions for the neurone illustrated.


A
1 mV
500 ms

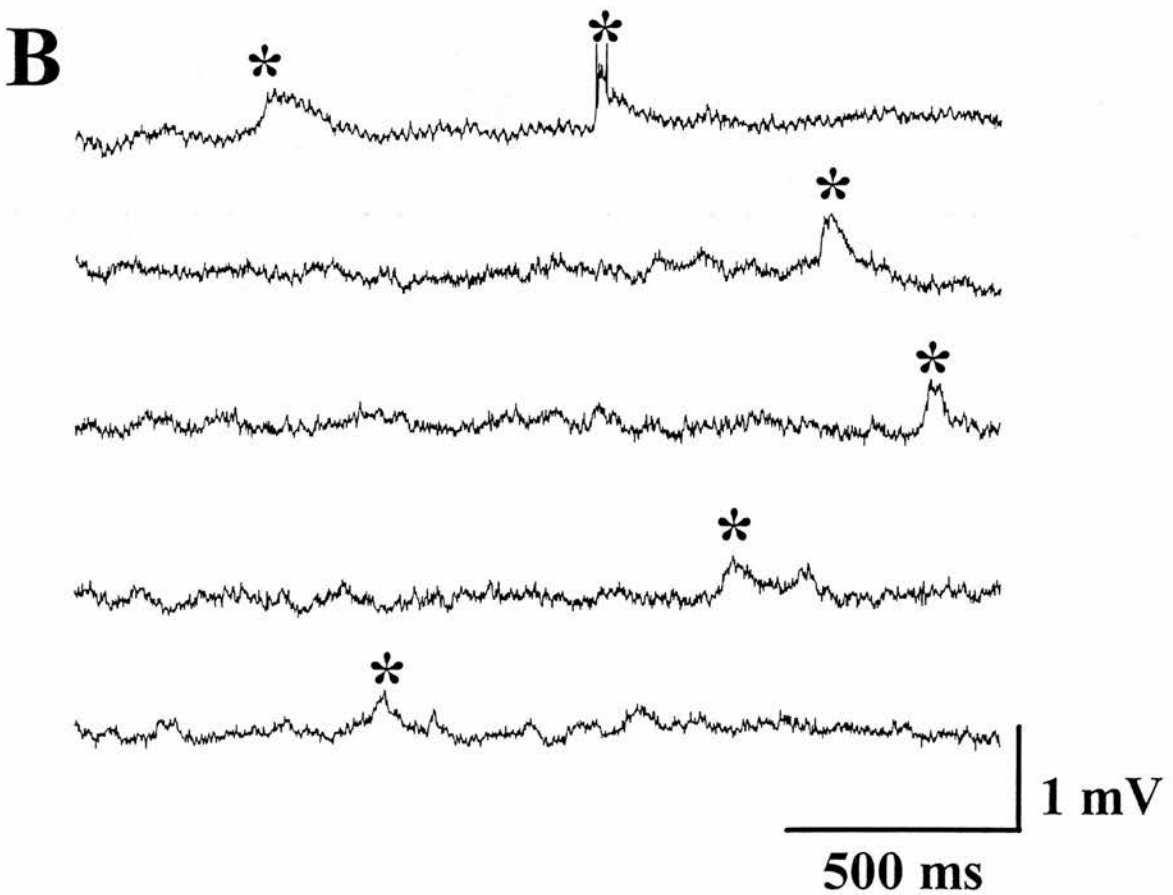
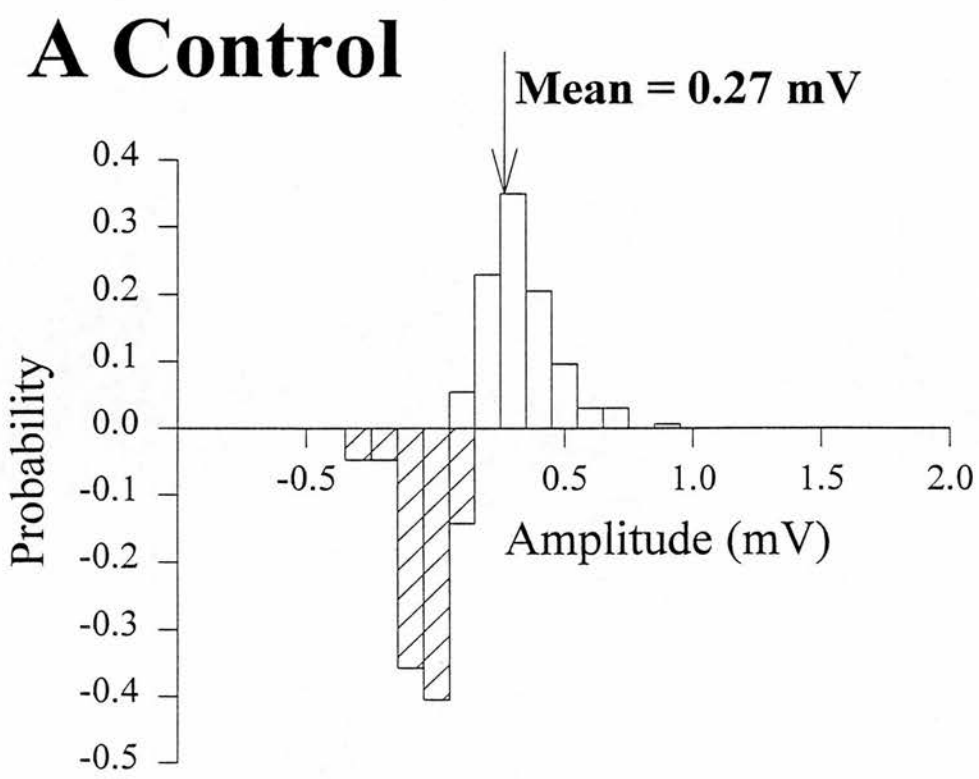

B
1 mV
500 ms

Figure 5.5 Effect of DPCPX on mEPSP amplitude

Amplitude histograms for the data illustrated in Figure 5.4 are shown. A 4 minute segment of data, chosen at random from a 10 minute continuous stretch, was analysed. Amplitude histograms for the noise distribution are also represented. Noise was measured as time matched to the time for baseline-peak response from the portion of trace immediately preceding a mEPSP. As can be seen no spontaneous mEPSP amplitudes fall in the histogram class representing the peak of the noise distribution. The mean mEPSP amplitude found under control conditions (A) was 0.27 ± 0.01 mV, after 10 - 15 minutes exposure to 30 nM DPCPX (B) the mean amplitude was 0.33 ± 0.02 mV.


A Control
Mean = 0.27 mV
Probability
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.5
0.5
1.0
1.5
2.0
Amplitude (mV)

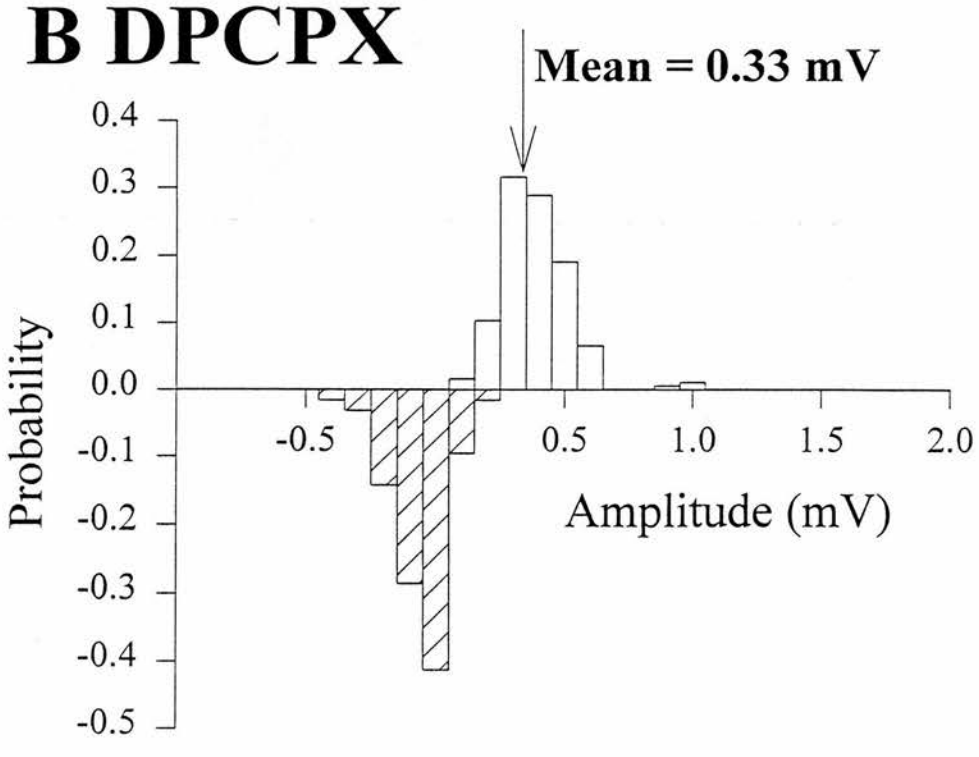
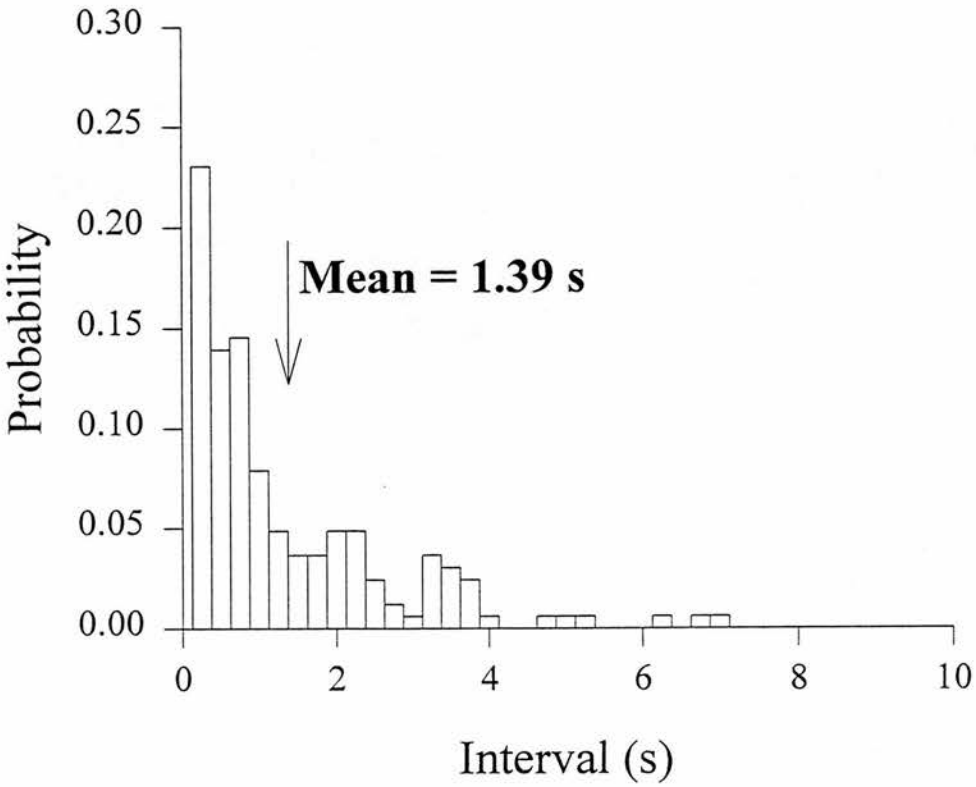

B DPCPX
Mean = 0.33 mV
Probability
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.5
0.5
1.0
1.5
2.0
Amplitude (mV)

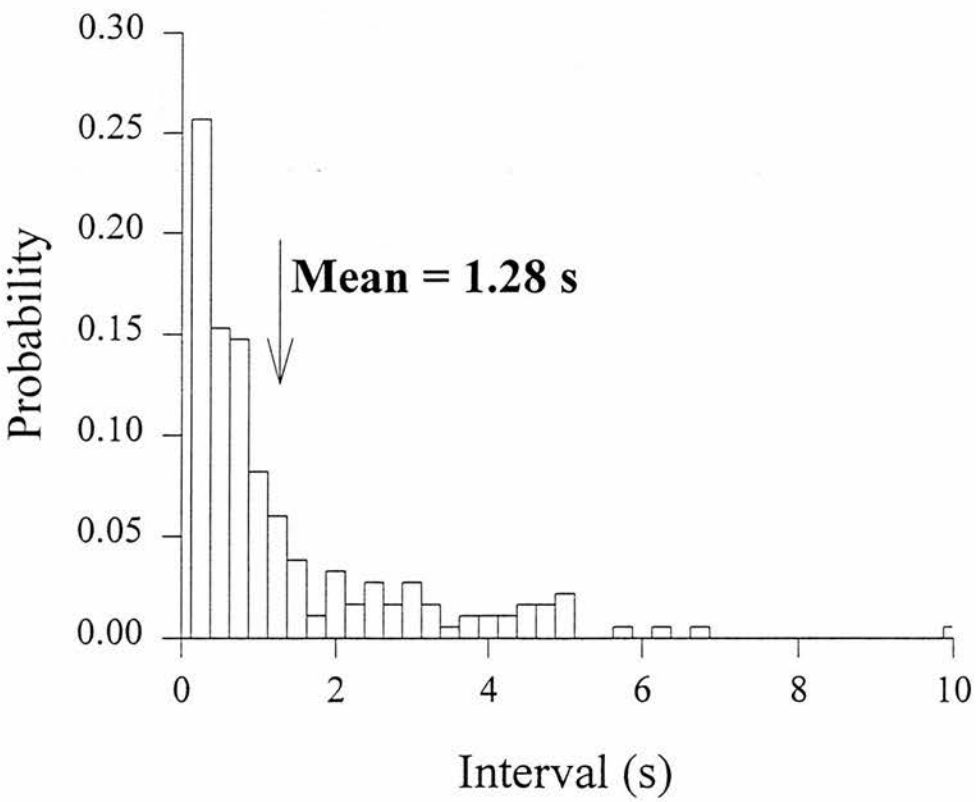
Figure 5.6 Effect of DPCPX on the interval between mEPSPs

Interval histograms for the data illustrated in Figure 5.4 are shown. The mean interval under control conditions (A) was 1.39 ± 0.14 s. After 10 - 15 minutes exposure to DPCPX (30 nM, B) the mean interval decreased to 1.28 ± 0.12 s. For this neurone the frequency of release was 0.69 Hz under control conditions and 0.77 Hz after exposure to DPCPX.

A Control



B DPCPX



The mean interval between successive mEPSPs under control conditions was 1.76 ± 0.17 s, after exposure to DPCPX the mean interval was 1.24 ± 0.19 s, this decrease in interval was significant ($P < 0.05$, paired student's t-test).

The mean data from these experiments is shown in figure 5.7. The data has been normalised to the control values found in each neurone tested. It was found that although the mean amplitude increased in the presence of both CCPA and DPCPX these increases were not significant. However when the frequency of occurrence of the mEPSPs during the 4 minute period was studied it was found that the mEPSP frequency was altered by application of the adenosinergic compounds. On average CCPA decreased the mEPSP frequency by 37.5 % from 0.92 ± 0.16 s^{-1} in control to 0.52 ± 0.07 s^{-1} (mean ± SEM, $P < 0.01$, $n = 11$). This decrease in frequency was reversed when DPCPX was coapplied with CCPA. In contrast DPCPX significantly increased mEPSP frequency to 152.4 % of control from 0.59 ± 0.05 s^{-1} to 0.88 ± 0.10 s^{-1} (mean ± SEM, $P < 0.05$, $n = 6$).

Cumulative interval and amplitude distributions were plotted (Fig. 5.8) and the Kolmogorov Smirnov test applied to determine whether the adenosinergic compounds altered the cumulative distributions. It was found that for 6 out of 11 neurones CCPA significantly altered the amplitude distribution, and for 2 out of 6 neurones DPCPX significantly altered the amplitude distribution. So although CCPA does not significantly change the mean amplitude of the mEPSPs it does significantly alter the distribution of the amplitudes in 6 out of 11 neurones. DPCPX does not alter the mean amplitude of mEPSPs but the amplitude distribution was significantly changed in just 2 out of 6 neurones.

CCPA significantly altered the interval distribution for 8 out of 11 neurones, whilst DPCPX significantly altered the interval distribution for 5 out of 6 neurones. These results indicate that a 15 minute exposure to the adenosine A_1 agonist, CCPA, or the antagonist, DPCPX, was sufficient to significantly alter the distribution of intervals between successive mEPSPs in the majority of neurones tested.

Figure 5.7 Mean data for amplitude and frequency of mEPSPs

A shows a histogram of the mean amplitude under control conditions, in the presence of CCPA (n = 11), in the presence of CCPA and DPCPX (n = 4) and in the presence of DPCPX alone (n = 6). An increase in the mean amplitude to 125.8 ± 15.3 % of control was found after exposure to CCPA, this was not significant ($P > 0.05$, signed ranks test). Co-application of CCPA and DPCPX, after exposure to CCPA, returned the mean amplitude to 100.0 ± 13.0 % of control. DPCPX increased the mean amplitude to 131.0 ± 19.1 % of control, this was not significant ($P > 0.05$, paired t-test).

B shows the frequency of occurrence of mEPSPs under control conditions, in the presence of CCPA, CCPA and DPCPX, and DPCPX alone. The star indicates changes that reached significance. CCPA decreased the mEPSP frequency to 62.4 ± 5.83 % of control ($P < 0.01$, paired t-test). DPCPX, coapplied with CCPA, reversed this decrease to 108.2 ± 15.2 % of control ($P = 0.07$, paired t-test). DPCPX increased the mean frequency to 152.4 ± 19.7 % of control when applied alone ($P < 0.05$, paired t-test).

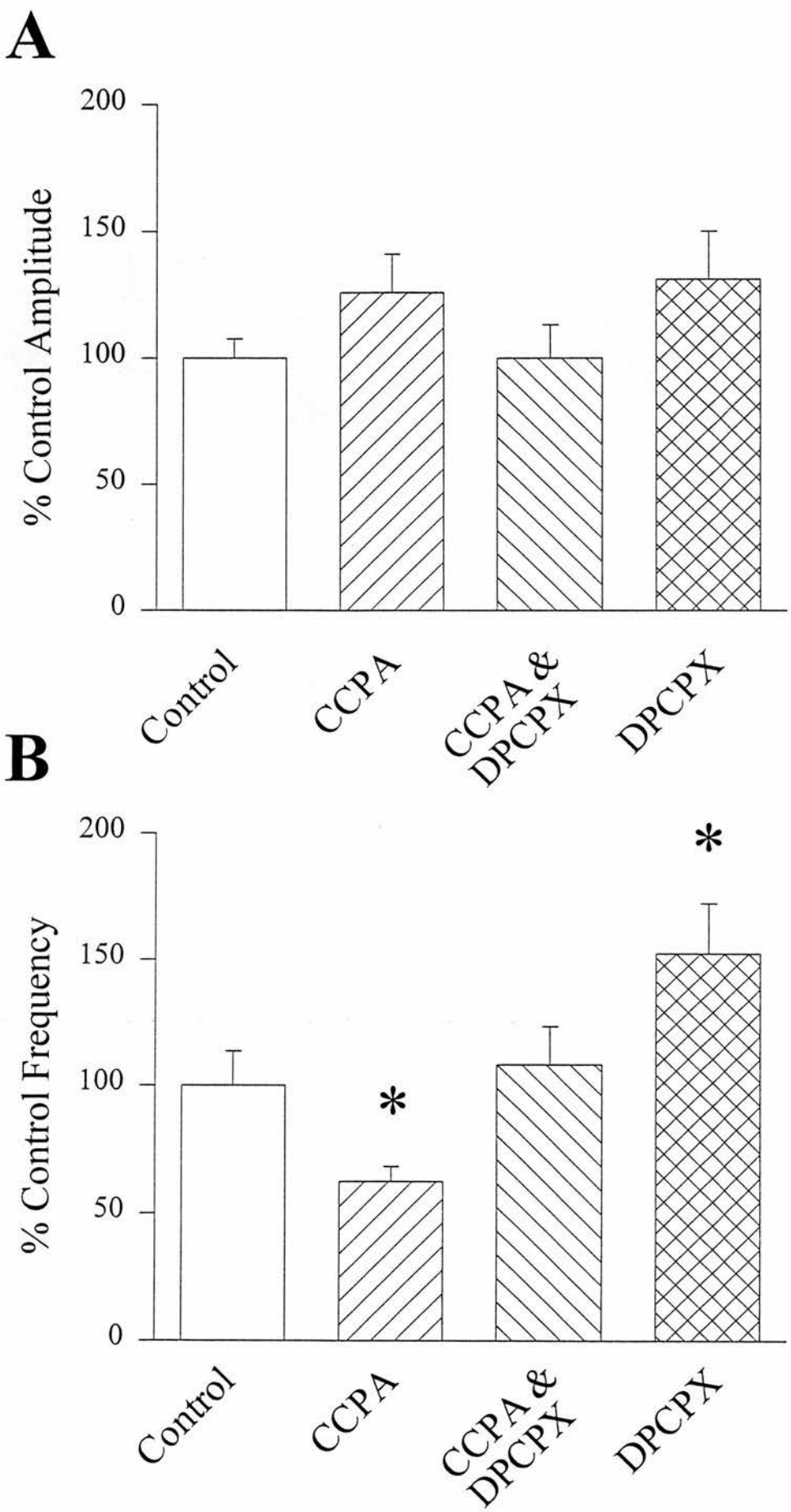
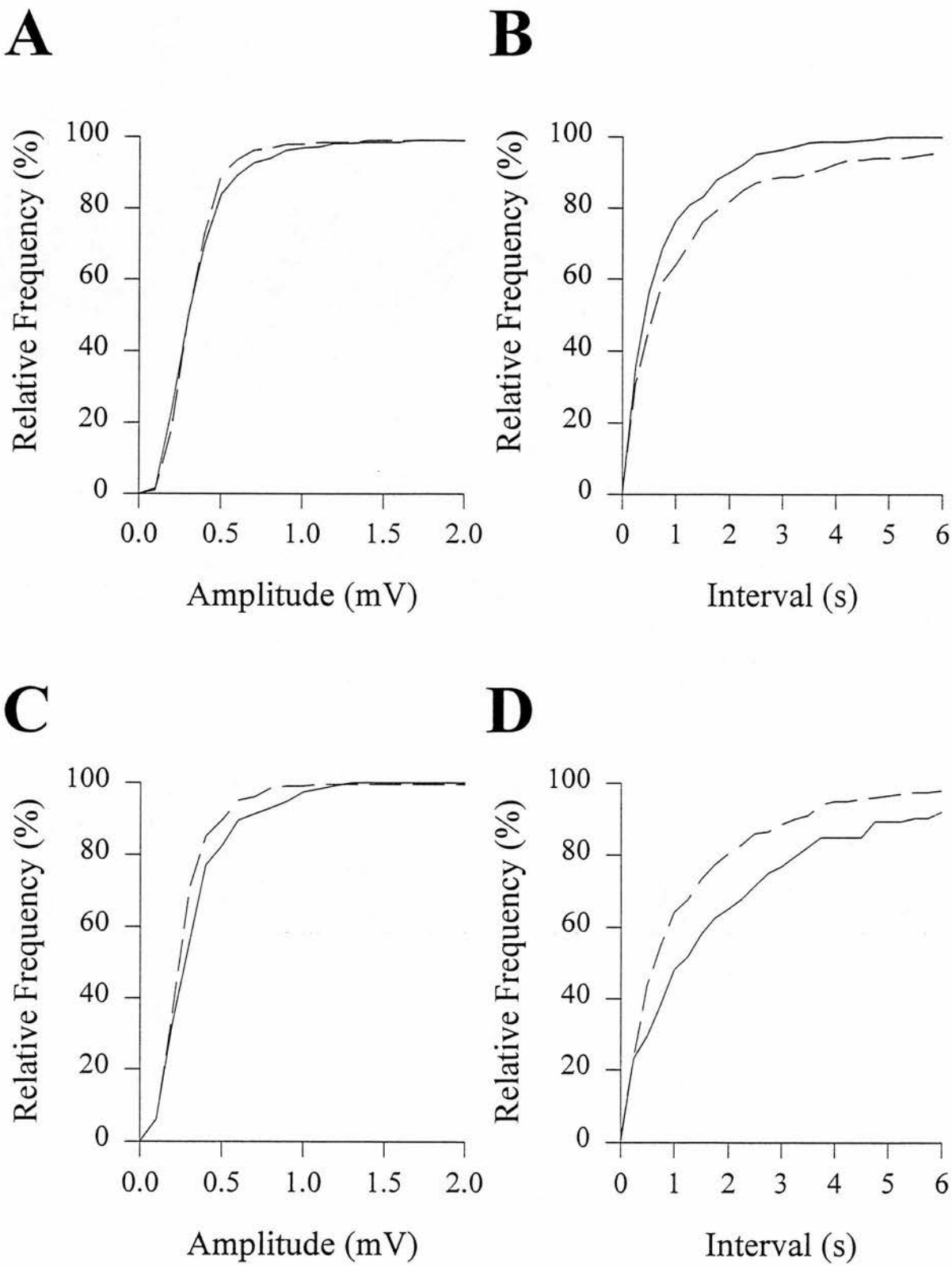

A
200
150
100
50
0
% Control Amplitude
Control
CCPA
CCPA &
DPCPX
DPCPX
B
200
150
100
50
0
% Control Frequency
*
*
Control
CCPA
CCPA &
DPCPX
DPCPX

Figure 5.8 Cumulative distributions of amplitudes and intervals

A shows the cumulative amplitude distributions of the control and CCPA amplitudes. C shows the cumulative amplitude distributions in control and in the presence of DPCPX. B shows the cumulative interval distributions under control conditions and in the presence of CCPA. D shows the cumulative interval distributions under control conditions and in the presence of DPCPX. For all graphs the solid line is the control data, and the dashed line the data in the presence of the adenosinergic compound.

In 6 out of 11 neurones the amplitude distribution changed significantly in the presence of CCPA ($P < 0.05$, Kolmogorov Smirnov test). In 2 out of 6 neurones the amplitude distribution changed significantly after 10 - 15 minutes exposure to DPCPX ($P < 0.05$, Kolmogorov Smirnov test). The interval distribution changed significantly after exposure to CCPA in 8 out of 11 neurones CCPA ($P < 0.05$, Kolmogorov Smirnov test). After exposure to DPCPX the interval distribution changed significantly in 5 out of 6 neurones CCPA ($P < 0.05$, Kolmogorov Smirnov test).


A
B
C
D
Relative Frequency (%)
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
Amplitude (mV)
0
1
2
3
4
5
6
Interval (s)

Discussion

Changes in mEPSP amplitude may reflect both pre- and postsynaptic factors. In practice, postsynaptic factors are more often responsible. Alterations in amplitude follow changes in the subsynaptic action of the transmitter or changes in the postsynaptic membrane resistance or capacitance. In these experiments the mean amplitude of the mEPSPs was not significantly altered by exposure to the adenosine A_1 agonist, CCPA or the antagonist DPCPX. This suggests that any changes seen in the presence of CCPA and DPCPX were not due to an action at the postsynaptic adenosine A_1 receptor.

These results are confirmed by the results of the frequency analysis of the rate of occurrence of mEPSPs. As miniature synaptic potentials are presynaptic in origin their frequency can only reflect presynaptic events (Katz, 1962). CCPA decreased the frequency of the mEPSPs, thus inhibiting spontaneous release. This effect was completely reversed by co-application of DPCPX (30 nM) with CCPA, suggesting that for mEPSPs this concentration of DPCPX was sufficient for reversal of the inhibition produced by the agonist. When DPCPX, alone, was applied to the hippocampal slice the frequency of the mEPSPs recorded from the CA1 neurones was increased. This indicated that the endogenous adenosine levels are sufficient to cause a tonic inhibition of the rate of occurrence of the spontaneous mEPSPs at these synapses.

These results are supported by the observations of Scanziani et al., (1992) who show that adenosine decreased the frequency of miniature excitatory postsynaptic currents (mEPSCs) recorded from hippocampal CA3 neurones in hippocampal slice cultures, without affecting the distribution of their amplitudes. The results shown above indicate that CCPA decreases mEPSP frequency by an action at the presynaptic adenosine A_1 receptor. The mean amplitude is unaffected whilst the amplitude distribution is altered in half the neurones recorded from.

These results indicate that inhibition of spontaneous transmitter release at the presynaptic adenosine A_1 receptor is via a mechanism that is not voltage dependent, i.e. not due to an activation of the adenosine A_1 receptor resulting in an decrease in cAMP production, thus leading to an decreased stimulation of either i) calcium

channels to decrease Ca^{2+} influx into the synaptic terminal or with ii) potassium channels to increase potassium permeability into the presynaptic terminal thus reducing the presynaptic action potential and thus reducing calcium influx and decreased transmitter release. Rather the adenosine A_1 receptor mediated inhibition is likely to be interfering with the release process more directly.

The mechanism by which adenosine decreases the frequency of mEPSPs is unknown. Miniature EPSPs are thought to occur by the spontaneous fusion of individual vesicles of transmitter with the active sites in the axon terminal. The rate by which this process occurs is probably dependent on several factors, especially the intracellular free Ca^{2+} concentration (Augustine et al., 1987). As it has been consistently found that blockers of voltage-dependent Ca^{2+} channels, or the presence of a calcium chelating agent such as BAPTA, have no effect on the frequency of miniature synaptic potentials and currents in many preparations (Del Castillo & Katz, 1954b; Katz & Miledi, 1963; Dale & Kandel, 1990; Hori et al., 1992; Scanziani et al., 1992), these channels are probably not tonically activated in presynaptic endings, but rather they must depend on the occurrence of action potentials for their activation. It has been shown that adenosine could alter the resting Ca^{2+} concentration in the axon terminal (Scanziani et al., 1992). Alternatively several protein kinases have been shown to phosphorylate proteins in synaptic membranes (review Augustine et al., 1987). Malenka et al., (1987) and Trimble et al., (1991) showed that stimulation of some of these kinases lead to increases in the frequency of miniature synaptic currents. These processes probably underlie the long lasting expression of excitatory transmission following tetanic stimulation (Malinow et al., 1988). Adenosine may be acting to inhibit excitatory transmitter release by decreasing the phosphorylation of these same presynaptic proteins.

Chapter 6

Anomalous DPCPX Effects

Introduction

As described in the chapter 3 the adenosine A_1 antagonist DPCPX depolarised hippocampal CA1 neurones when a concentration of 30 nM was applied for 10 minutes. However this effect was not significant. Interestingly the input resistance (R_m) of the CA1 neurones was significantly decreased by exposure to DPCPX. This was the opposite to the result that was predicted. CCPA, the adenosine A_1 agonist, decreased the R_m of hippocampal CA1 neurones whilst causing a hyperpolarisation . This is in agreement with previous results using adenosine and other adenosine A_1 receptor selective compounds (Segal et al., 1982; Greene & Haas, 1985; Thompson et al., 1992) combined with either sharp electrode or patch clamp recording. There are physiologically significant levels of endogenous adenosine present in the brain (Fredholm, 1995) and therefore in the hippocampal slice preparation (Mitchell et al., 1993). This suggests that there is a tonic inhibition of adenosine A_1 receptors. It would be expected when the adenosine A_1 receptor selective antagonist, DPCPX, is applied that it would block the adenosine effect. Therefore as the agonist decreases input resistance and produces an hyperpolarisation, an increase in input resistance accompanied by a depolarisation would be expected with application of the antagonist. DPCPX caused a depolarisation of the postsynaptic cell as expected, but a decrease in input resistance was observed. Further experiments were carried out in order to determine why DPCPX produced a decrease in input resistance, and activated a current with an apparent reversal potential of approximately -40 mV.

Glutamate is the transmitter released from the synaptic terminals of the Schaffer collateral commisural fibres. One possible explanation of the DPCPX effect on input resistance is that on blocking the adenosine A_1 receptor the glutamate released exerts a greater effect on the CA1 neurone, and a conductance is activated or enhanced which contributes to the input resistance observed.

Upon hyperpolarisation of hippocampal CA1 neurones there appears a strong time-dependent inward rectification which moves the membrane potential back towards the normal resting potential (Halliwell & Adams, 1982). This current, termed I_q or I_h, has also been observed in many types of electrically responsive cell, including neurones of the sympathetic and sensory ganglia, spinal cord, brain stem, thalamic relay neurones, cerebellum and cerebral cortex (Crunelli et al., 1987; McCormick &

Pape, 1988; McCormick & Pape, 1990). The current that underlies this phenomena is carried by both Na^+ and K^+ ions, activates upon membrane hyperpolarisation (typically beyond -60 mV), and deactivates upon depolarisation. In guinea pig hippocampal CA1 neurones I_h has been shown to half maximally activate at -86 mV when the neurones are clamped at -65 mV, and it was completely blocked by intracellular application of the voltage-activated sodium channel blocker QX-314 (Perkins & Wong, 1995). In these studies the voltage-activated sodium channels were blocked by inclusion of tetrodotoxin in the bath solution, therefore any action of QX-314 observed were solely due to its action on I_h. In the cat sensorimotor cortex this current was estimated to have a reversal potential of approximately -50 mV (Spain et al., 1987) which is 20 mV positive to E_m in these cells. Halliwell & Adams (1982) showed that the reversal potential for I_h was 'somewhat positive to -60 mV, to account for the inwardness of the small tail currents' in hippocampal CA1 neurones. Pape (review, 1996) states that the reversal potentials of I_h are between approximately -50 and -20 mV and thus in a region positive to the normal resting potential, as has been estimated from analyses of tail currents or fully activated I-V relationships in different types of cells under physiological conditions. In sino-atrial cells and Purkinje fibres of the heart, this type of current appears to be important in determining the rate of action potential generation (Noble et al., 1989). In the central nervous system the role of hyperpolarisation-activated cation currents is less clear. Potential roles in determining the resting membrane potential and modulating the response to hyperpolarising inputs have been suggested (Spain et al., 1987). I_h may also be responsible for the pacemaker depolarisation in cells that display rhythmic calcium spikes (McCormick & Pape, 1990). The actions of the adenosinergic compounds upon this conductance was investigated in order to determine whether any postsynaptic effects of the compounds were due to an alteration of this conductance.

Results

The effect of DPCPX on R_m was investigated further in a series of voltage clamp experiments. It was hypothesised that DPCPX caused an increase in glutamate release via an action at the presynaptic adenosine A_1 receptor, and hence an increased activation of the postsynaptic AMPA / Kainate receptor. In 5 experiments the

conductance of the CA1 neurone was investigated under voltage clamp conditions. The neurones were continuously clamped at -70 mV, and the cell was stepped to hyperpolarising and depolarising potentials using 10 mV voltage steps (Fig. 6.1A, lower traces), the current responses to these steps was measured (Fig. 6.1A, upper traces) at the near instantaneous peak. The data were plotted and were best fitted by a second order regression. The linear, hyperpolarising, section of the curve was used to determine the conductance of the neurone. The results from these experiments are shown in the table below:

	Control	DPCPX (30 nM)	DPCPX (30 nM) & CNQX (10 μM)
Conductance (nS)	10.8 ± 2.0	12.9 ± 2.2 *	15.1 ± 3.0 #

* P<0.05, paired student's t-test # P>0.2, paired student's t-test

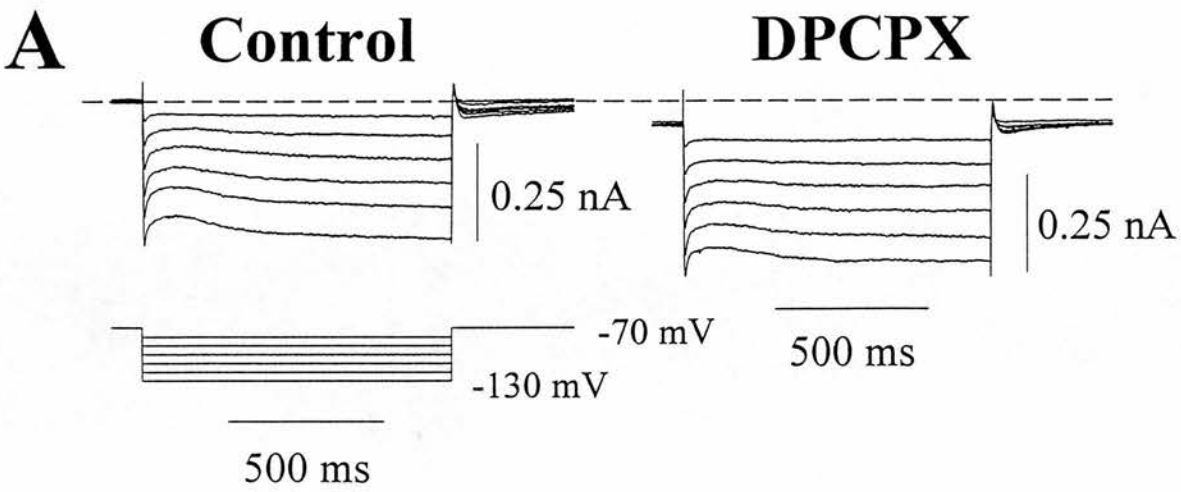
From the current clamp experiments it was found that the conductance activated by DPCPX (30 nM) had a mean reversal potential of -37.9 mV. It was hypothesised that DPCPX might be exerting an effect on I_h (the hyperpolarisation activated cation conductance). I_h has been shown to have a reversal potential that is positive to the resting membrane potential in hippocampal CA1 neurones (Halliwell & Adams, 1981).

Figure 6.2 shows the results from a series of experiments where the CA1 neurone was clamped at -70 mV and a sequence of 10 mV hyperpolarising voltage steps was applied to the neurone. I_h was measured in control, DPCPX (30 nM) and also in the presence of CNQX (10 μM) and DPCPX. As the neurone becomes more hyperpolarised the amplitude of I_h increases (Fig. 6.2A). DPCPX decreased the amplitude of I_h (Fig. 6.2B,C) and CNQX, when co-applied with DPCPX further decreased I_h. There was a significant difference in the amplitude measured at -90 mV between DPCPX (44.1 ± 10.6 nA, n = 7) and DPCPX and CNQX (27.0 ± 7.04 nA, n = 6, $P < 0.05$, unpaired student's t-test). There was no significant difference in the amplitude of I_h at any other point.

The mixed adenosine A_{2a} and A_1 antagonist CGS 15943 (100 nM and 1 μM) was co-applied with DPCPX (30 nM). It was hypothesised that by antagonising the

Figure 6.1 Effect of CNQX on the decrease in R_m seen in the presence of DPCPX

A: shows a family of voltage steps (lower traces) and the current response to those steps (upper traces) of a hippocampal CA1 neurone that was continuously clamped at -70 mV. The current response to each voltage step was measured from baseline to the near instantaneous peak. The data were plotted (B) and the conductance was determined. The conductance was determined in control, in the presence of DPCPX (30 nM), then the slice was exposed to the AMPA/Kainate receptor antagonist CNQX (10 μM) in the continuous presence of DPCPX for 10 - 15 minutes and the voltage-current relationship measured. The data were best fitted by a second order regression, the conductance was calculated from the hyperpolarising section of the data. In control (circles) the conductance of this neurone was 17.3 nS, this increased to 19.0 nS in the presence of DPCPX (30 nM, inverted triangles), a further increase to 22.8 nS was observed in the presence of CNQX (10 μM) and DPCPX (30 nM, squares).


A
Control
DPCPX
0.25 nA
0.25 nA
-70 mV
-130 mV
500 ms
500 ms

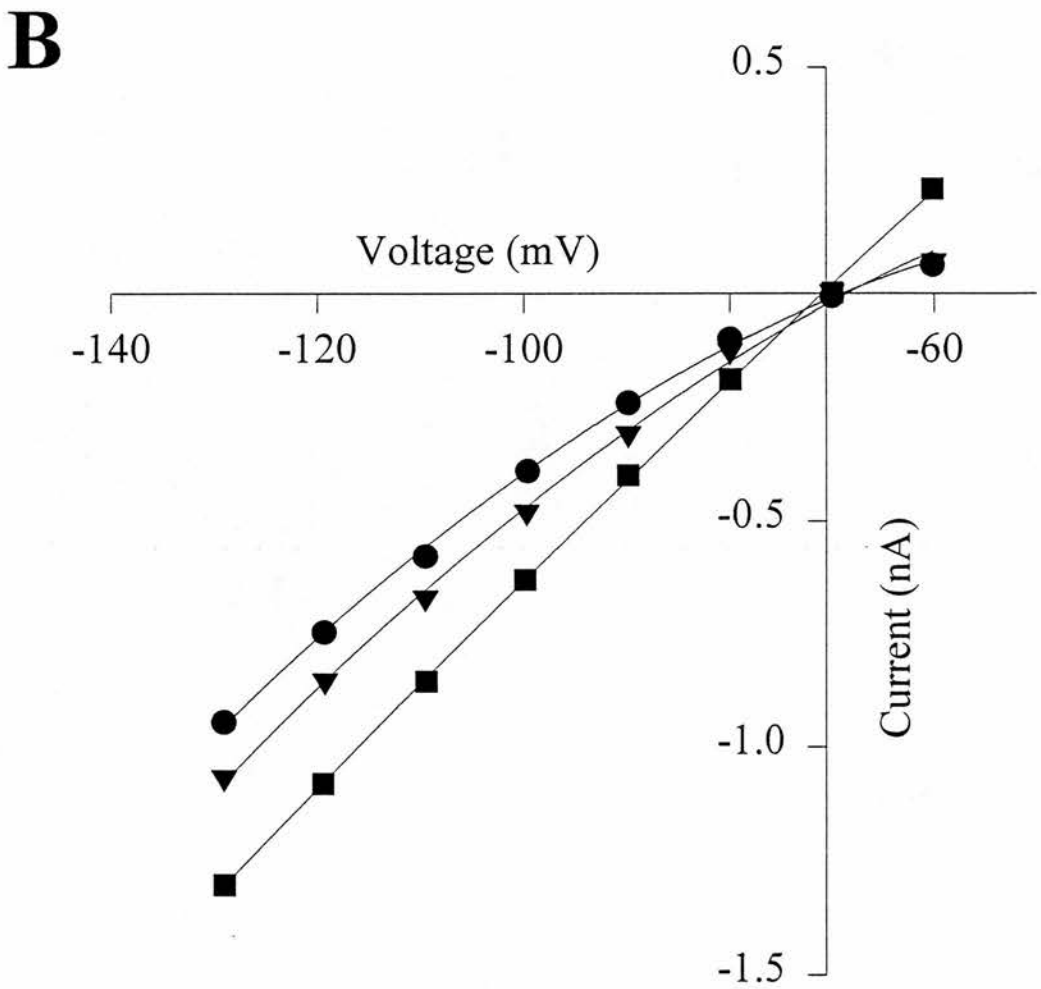
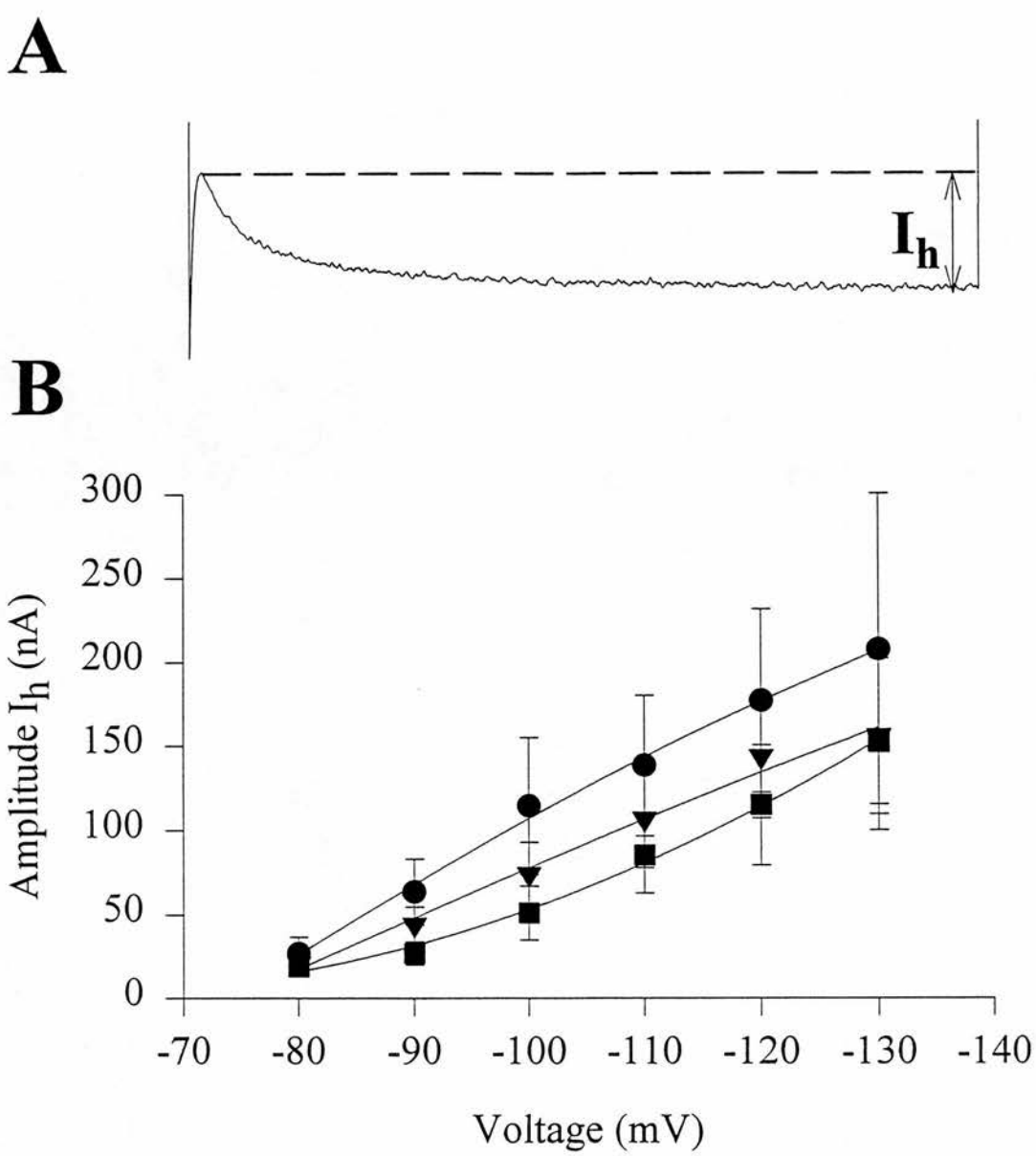

B
0.5
Voltage (mV)
-140
-120
-100
-60
-0.5
-1.0
-1.5
Current (nA)

Figure 6.2 Effect of DPCPX on a hyperpolarisation activated conductance

A: trace shows the current response to a 50 mV step from a holding potential of -70 mV. The hyperpolarisation activated current (I_h) was measured from the near instantaneous peak to just before the end of the voltage step. All voltage steps were 1 second in length.

B shows the amplitude of I_h against the holding potential of the voltage step. Each point represents the mean ± SEM of 4 - 7 recordings. I_h increases with increasing voltage steps, and was largest under control (circles) conditions. The amplitude of I_h decreased in the presence of DPCPX (inverted triangles) and further decreased when the slice was exposed to DPCPX and CNQX (squares).

C shows a histogram of the amplitude of I_h at a -50 mV step from the holding potential of -70 mV. Although the amplitude of I_h decreases from control (177.0 ± 54.58 nA, n = 7) to DPCPX (143.4 ± 36.19 nA, n = 7) and from DPCPX to DPCPX and CNQX (115.6 ± 35.52 nA, n = 6), there is no significant difference in the results.


A
I_h
B
300
250
200
150
100
50
0
Amplitude I_h (nA)
-70
-80
-90
-100
-110
-120
-130
-140
Voltage (mV)
C
250
200
150
100
Amplitude I_h (nA)
Control
DPCPX
CNQX

adenosine A_1 receptor and blocking the effects of the endogenous adenosine DPCPX was unmasking an effect at the adenosine A_2 receptor, thus promoting glutamate release and producing the opposite effect of that predicted. In these experiments (Fig. 6.3) DPCPX produced a small decrease in the R_m (in 3 experiments). This was not significantly affected by CGS 15943 at either concentration, which caused a further decrease in R_m. The mean R_m in control was 104.2 ± 8.74 MΩ, in DPCPX it fell to 98.7 ± 12.25 MΩ and in the presence of DPCPX and CGS 15942 (100 nM) a further decrease to 86.9 ± 18.19 MΩ was observed. In 2 experiments the R_m was also measured in the presence of 1μM CGS 15942, the mean R_m showed no further decrease from the value measured in the presence of 100 nM CGS 15942 and was 85.4 ± 38.6 MΩ. None of these values differed significantly from control.

The effects of DPCPX (30 nM) on the CA1 neurone were studied after 10 and 30 minutes exposure time (Fig. 6.4) in order to determine whether the decrease in R_m was a transitory fall on initial exposure, which would then increase with prolonged exposure. In one experiment DPCPX produced a small decrease in R_m after 10 minutes and a further fall was seen at the 30 minute time period. However in 2 further neurones DPCPX (10 minutes) caused a small increase in R_m, which fell back towards control at the 30 minute time point. The mean values, measured in 3 experiments, were 144.2 ± 21.5 MΩ in control, 146.5 ± 27.1 MΩ after 10 minutes exposure to DPCPX and 142.7 ± 29.2 MΩ after 30 minutes exposure. No significant difference from control was observed.

This finding was backed up in a series of sharp electrode intracellular recordings made by Dr J.P. Hodgkiss. In these experiments it was found that DPCPX (30 nM) depolarised three hippocampal CA1 neurones by 2.03 mV ± 0.18 on average, as previously shown in the patch clamp experiments. This depolarisation was accompanied by an increase in input resistance (Fig.e 6.5). The mean R_m was 36.0 ± 5.0 MΩ and it increased to 41.4 ± 5.0 MΩ after exposure to DPCPX. From the I-V relationships for these cells the reversal potential for the underlying conductance was obtained. The mean reversal potential was -75.6 ± 2.6 mV.

Figure 6.3 Effect of an Adenosine A_{2a} antagonist on the action of DPCPX on R_m

The R_m of a hippocampal CA1 neurone was determined under control (circles) conditions in the presence of DPCPX (30 nM, inverted triangles) and in the presence of DPCPX and the adenosine A_1 and A_{2a} antagonist CGS 15942 (100 nM (diamonds) and 1 μM (not shown)). The data were best fitted by a first order regression. In control the R_m was 103.8 MΩ, in the presence of DPCPX it decreased slightly to 97.3 MΩ and a further decrease to 81.8 MΩ was seen when CGS 15942 (100 nM) was present.

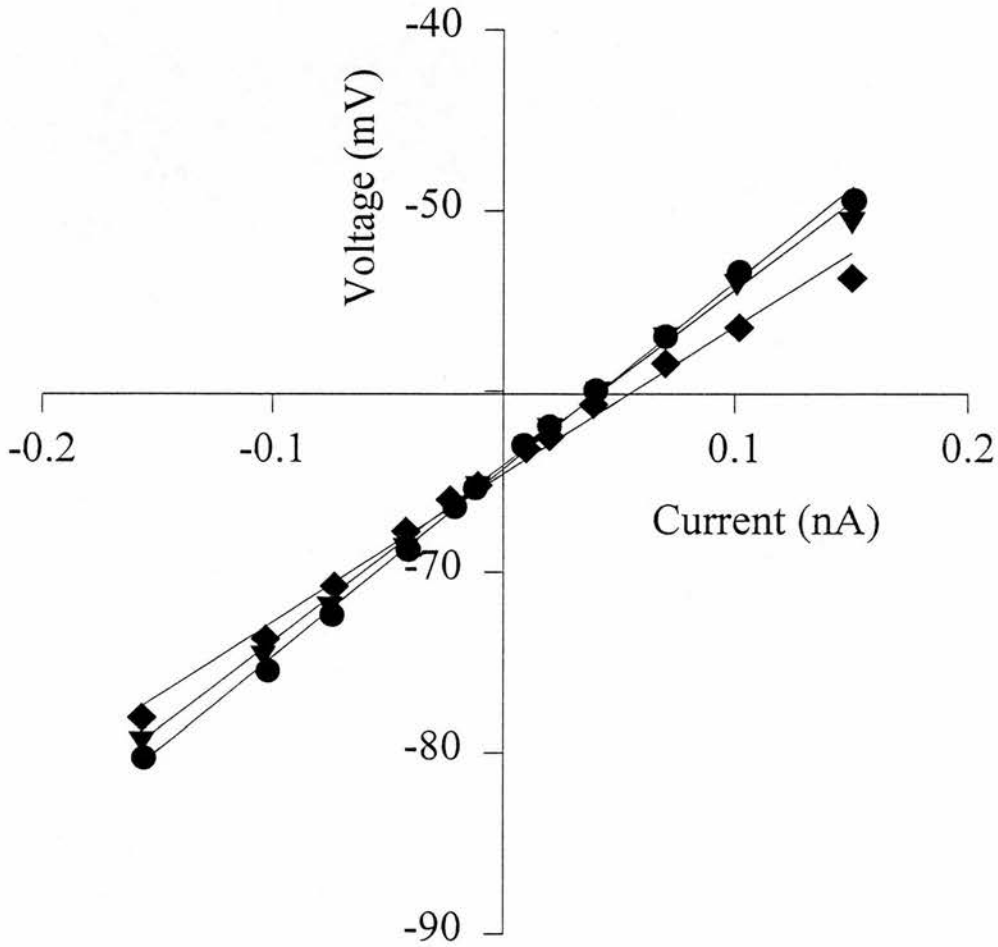

-40
-50
-70
-80
-90
Voltage (mV)
-0.2
-0.1
0.1
0.2
Current (nA)

Figure 6.4 Effects of prolonged exposure to DPCPX

The hippocampal slice was exposed to DPCPX for a prolonged period, with the input resistance being determined in control (circles) conditions, after 10 (filled inverted triangles) and 30 (unfilled inverted triangles) minutes exposure. In the neurone shown R_m in control was 137.7 MΩ, after 10 minutes exposure to DPCPX it had increased to 149.1 MΩ and after 30 minutes exposure the R_m had decreased slightly to 147.8 MΩ.

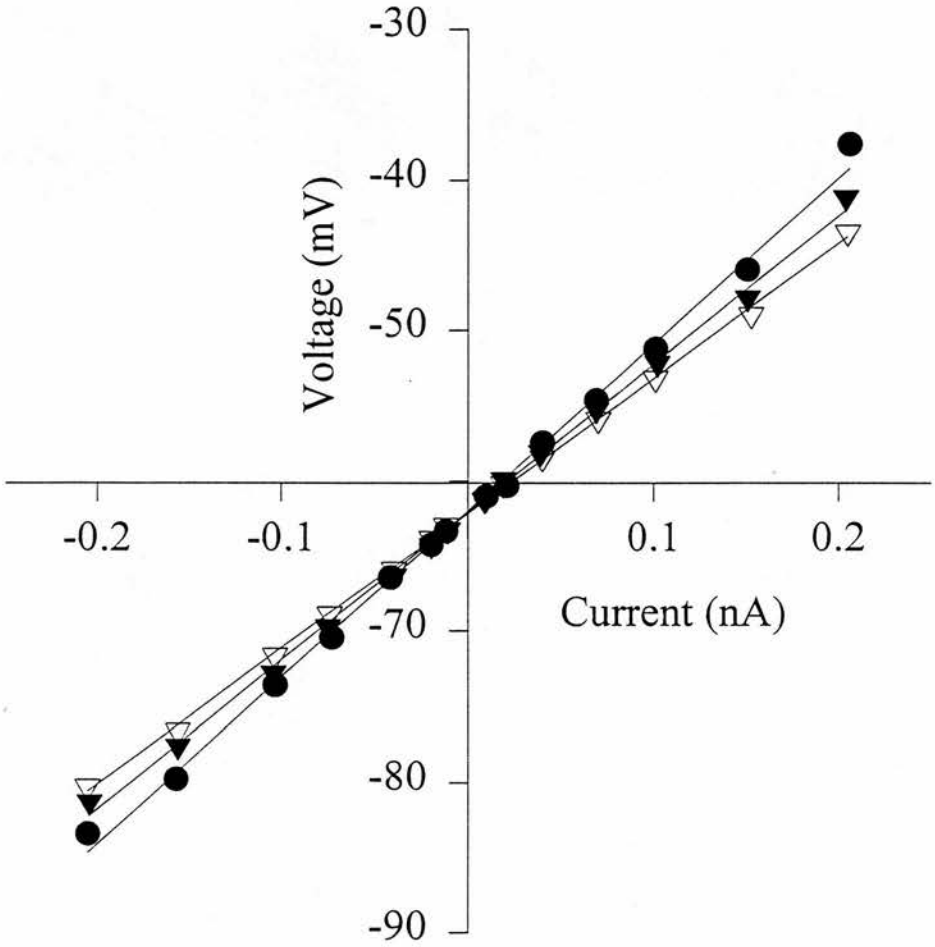
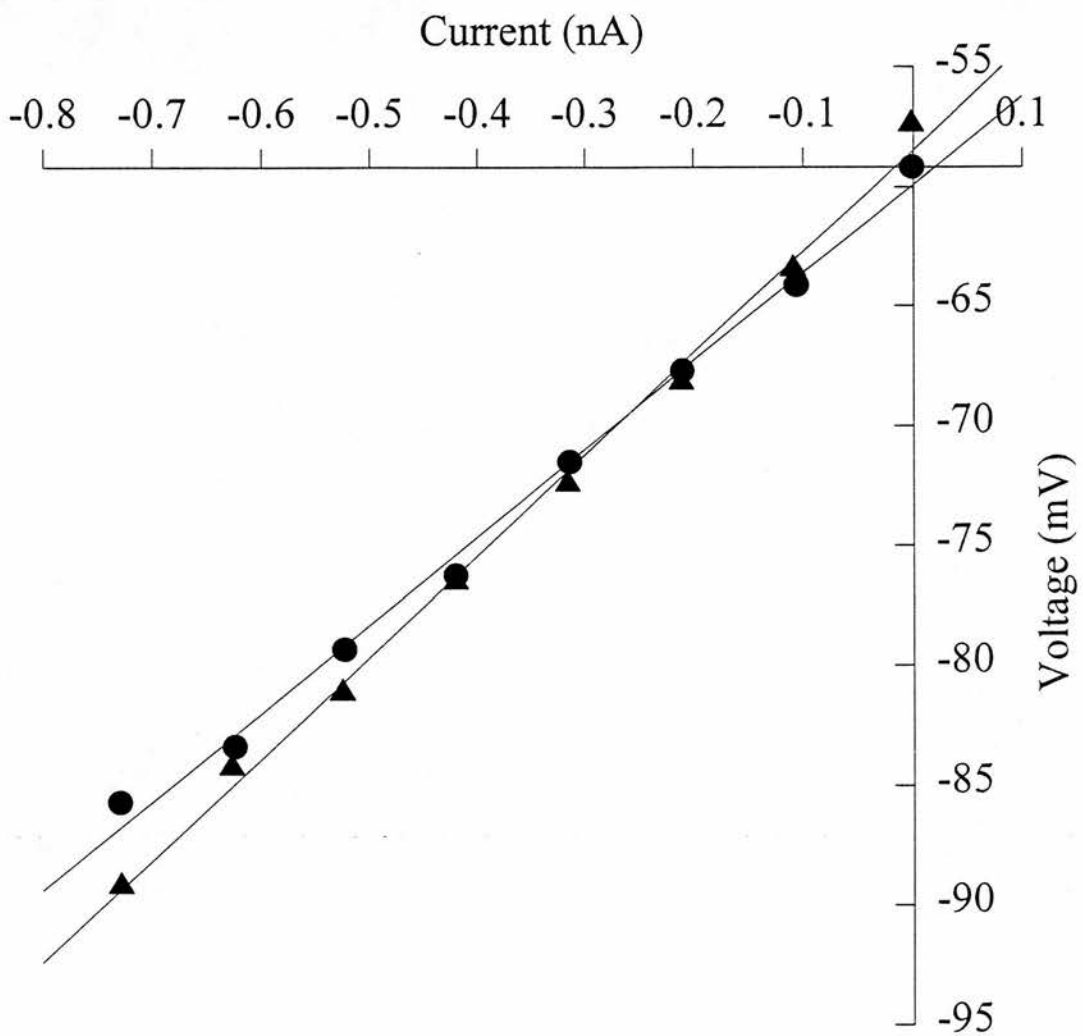

-30
-40
-50
-70
-80
-90
Voltage (mV)
-0.2
-0.1
0.1
0.2
Current (nA)

Figure 6.5 Effect of DPCPX on R_m using 'sharp' intracellular electrodes

The current-voltage relationship for a sharp electrode recording from a hippocampal CA1 neurone is shown. Under these recording conditions the input resistance was 36.9 MΩ under control conditions (circles) increasing to 42.4 MΩ after exposure to DPCPX (30 nM, triangles).


Current (nA)
-0.8
-0.7
-0.6
-0.5
-0.4
-0.3
-0.2
-0.1
0.1
-55
-65
-70
-75
-80
-85
-90
-95
Voltage (mV)

Discussion

In the LGND adenosine decreases the amplitude of I_h, as well as increasing the R_m of the relay neurone. Therefore as adenosine tonically activates CA1 neurones in the acute hippocampal slice preparation, a decrease in the amplitude of I_h (as adenosine decreases the R_m in these neurones) after exposure to DPCPX would be predicted. DPCPX did not significantly decrease the amplitude of I_h. Therefore an action of DPCPX on the hyperpolarisation activated current, I_h, does not explain its action on R_m.

When the presynaptic adenosine A_1 receptor was blocked an increase in glutamate release would be predicted. This increased release would lead to a greater postsynaptic activation of the AMPA/Kainate receptor, thus an increase in channel activation and a decrease in R_m. This mechanism did not explain the action of DPCPX on the input conductance of the hippocampal CA1 neurones. CNQX when co-applied with DPCPX did not reverse the DPCPX induced increase in input conductance, rather a further increase in the input conductance of the CA1 neurone was observed.

DPCPX did not unmask an effect due to endogenous adenosine acting at the adenosine A_{2a} receptor, which could only be observed when the adenosine A_1 receptor was blocked. The adenosine A_1 and A_{2a} antagonist CGS 15943 did not cause an increase in R_m when co-applied with DPCPX, thus either there are not adenosine A_{2a} receptors present on the cell bodies of CA1 neurones or if present they do not cause a decrease in R_m when activated by adenosine which would have contributed to the DPCPX effect. Alternatively if present on the presynaptic terminals the endogenous adenosine effect at these receptors does not contribute significantly to the DPCPX effect on R_m.

In a few later experiments (n = 2) and when the CA1 neurones were recorded from using 'sharp' intracellular electrodes R_m was found to increase after exposure to DPCPX. A likely explanation for the difference in results seen with the two recording techniques is that the intracellular contents of the CA1 neurones are fully dialysed when patch clamp electrodes are used, but little or no dialysis occurs with sharp electrodes. A further possibility is that DPCPX has wide spread effects throughout-out the slice and that its actions at the adenosine A_1 receptor, which is found on all

neurones except inhibitory GABAergic neurones, causes an increase in release of a variety of neurotransmitters which all contribute to the effect observed. This explanation is less likely as it would be expected that the same effect would be seen regardless of the type of recording electrode used.

The presynaptic actions of DPCPX may be obscured by the decrease in R_m observed. This would serve to cause amplitude measurements of spontaneous and evoked EPSPs to be under estimated, as the conductances activated would serve to shunt the synaptic currents generated in the dendrites and thus decrease the glutamatergic EPSPs directly.

Chapter 7

Discussion

These results provide evidence that, by using low concentrations of the adenosine A_1 receptor agonist, CCPA, or antagonist, DPCPX, the presynaptic adenosine A_1 receptor can be studied in isolation from any postsynaptic actions of these compounds. As the presynaptic receptor can be stimulated, or the endogenous adenosine effect blocked (Haas & Greene, 1988; Mitchell et al., 1993, these experiments), in isolation from any postsynaptic actions of these drugs, the effect of increasing or decreasing the receptor activation, hence altering the release probability at these synapses, could be studied. The effect of increasing and decreasing the probability of transmitter release from the presynaptic terminals, and thus the response to a change in the degree of excitation of the terminals at the presynaptic sites was determined by measuring both spontaneous and evoked release strategies.

The postsynaptic action of adenosinergic compounds

At the concentration used in these experiments, the adenosine A_1 receptor agonist, CCPA (100 nM), had no significant postsynaptic effects. This concentration of CCPA was close to the EC_{50} found for inhibition of evoked synaptic potentials recorded from hippocampal CA1 neurones. The membrane potential and the apparent input resistance of the postsynaptic CA1 neurones were unaltered. Interestingly DPCPX, the adenosine A_1 receptor antagonist, did not alter membrane potential but did significantly decrease the apparent input resistance. This was in the opposite direction to that predicted, as adenosine A_1 receptor agonists decrease input resistance, and if the endogenous adenosine effect was antagonised input resistance would be expected to increase. As the input resistance decreased the amplitude of evoked potentials, when recorded in current-clamp mode, would be underestimated for a constant postsynaptic current.

There are at least two possible explanations for the absence of significant postsynaptic actions of CCPA on resting membrane potential in the current study. (1) The concentration of CCPA used, which reduced the evoked potential amplitude by 50 %, was lower than the EC_{50} at postsynaptic receptors. In the current study we observed that when the CCPA concentration was increased to 10 μM the change in membrane potential observed, and percent inhibition of the evoked potential

amplitude (~95 %), was similar to that observed using 1 μM R-PIA or 50 μM adenosine (Thompson et al., 1992). The EC_{50} of adenosine at postsynaptic adenosine A_1 receptors is approximately 50 μM (Dunwiddie & Fredholm, 1989). (2) During whole-cell recording, in the absence of GTP in the patch pipette, the activation of the G-protein-linked potassium conductance that mediates the postsynaptic hyperpolarising response to adenosine is nearly eliminated within 15 - 20 minutes of rupturing the membrane patch (Trussell & Jackson, 1987). Netzer et al., (1994) also reported that use of the whole cell patch clamp technique caused a rapid relief of a tonic G protein inhibition of voltage-activated Ca^{2+} currents, this was prevented by inclusion of GTP in the pipette or by using the perforated patch clamp technique. These results suggest that phosphorylation of these ion channels is necessary in order to regulate their activity. In the experiments described herein a 15 minute control period was employed to record the synaptic responses and to construct an I-V relationship, prior to application of CCPA. GTP was not included within the pipette solution. It is seems likely that G-protein-mediated responses were not significant in these cells.

As described above GTP was not included in the patch pipette, and rundown of the postsynaptic potassium current possibly would have occurred (Trussell & Jackson, 1987), thus probably accounting for the lack of effect of CCPA and DPCPX upon the membrane potential of the CA1 neurone and of CCPA upon the apparent input-resistance. However DPCPX produced a decrease in input-resistance. A decrease in the activation of the postsynaptic potassium conductance described above, hence an increase in input resistance, would be expected in response to a relief of the endogenous adenosine effect. As it is possible that the postsynaptic potassium conductance had completely rundown by the time that DPCPX was applied to the CA1 neurones, it is likely that this conductance did not contribute to the DPCPX effect. DPCPX possibly activated another conductance that was inhibited by the endogenous adenosine.

The possibility that DPCPX activated a current in hippocampal CA1 neurones was investigated. As the reversal potential of the current activated was ~ -40 mV, and I_h, the hyperpolarisation activated current (Halliwell & Adams, 1982; McCormick &

Pape, 1990) has been shown to have a reversal potential in this region, it was speculated that DPCPX activated this current. However measurements of I_h before and after application of DPCPX (30 nM) showed that this current was unaffected by DPCPX. A second possibility studied was that glutamate release was increased, due to relief of the inhibitory endogenous adenosine tone, and by acting at AMPA / Kainate receptors to activate or enhance I_h. Application of 10 μM CNQX (the AMPA / Kainate receptor antagonist) with DPCPX did not alter the apparent input resistance from that seen in the presence of DPCPX alone, and did not significantly alter I_h. However this concentration of CNQX was sufficient to completely abolish the evoked EPSC recorded in CA1 neurones. However, the lack of effect on I_h may possibly be accounted for by the use of the whole cell patch clamp technique. I_h has been shown to be activated by a G protein mediated mechanism (Pape, 1996), and this may be impaired due to dialysis of the cellular contents by the pipette solution. Further experiments where the magnitude of I_h is examined over 30 minutes or longer are required to determine whether whole cell recording causes I_h to rundown in a similar manner to the postsynaptic G protein activated K^+ conductance (Trussell & Jackson, 1987).

The action of glutamate released spontaneously, acting at AMPA/Kainate receptors was studied in order to determine whether this conductance contributed to the increased conductance (decreased R_m) observed in the presence of DPCPX. Co-application of CNQX with DPCPX did not reverse this conductance increase, suggesting that the increase in glutamate release, due to relief of the tonic inhibition of the presynaptic adenosine A_1 receptor did not account for the decrease in R_m observed.

A further possibility studied was that blockade of the inhibitory endogenous adenosine tone at adenosine A_1 receptors unmasked an effect of the endogenous adenosine at adenosine A_2 receptors. The mixed adenosine A_1 and A_{2a} receptor antagonist CGS 15943 (at 100 nM and 1 μM) was co-applied with DPCPX, however no difference in apparent input resistance was observed from that seen in the presence of DPCPX alone. This effect of DPCPX on the decrease in input resistance was not due to activation of I_h, increased activation of AMPA / Kainate receptors or

an activation of adenosine A_{2a} receptors. It was possibly a consequence of using patch electrodes, rather than 'sharp' electrodes. When 'sharp' electrodes were used an increase in input resistance was observed in all neurones recorded from (Dr J.P. Hodgkiss). The reversal potential observed in these neurones, using this recording technique, was in the region expected of the potassium reversal potential under the conditions used (~ -99 mV), suggesting that DPCPX prevented the endogenous adenosine activation of this current, via blockade of the adenosine A_1 receptors. It is possible that Ca^{2+} or Na^+ currents are also activated, by glutamate released at presynaptic terminals, in a lesser manner, and they contribute to the reversal potential observed.

The absence of any significant postsynaptic effects of this low concentration of CCPA at the adenosine A_1 receptor meant that any presynaptic modulation of transmitter release, via the adenosine A_1 receptor, could be studied in isolation. As DPCPX did not alter membrane potential significantly, and the decrease in apparent input resistance observed which would lead to underestimated response amplitudes, the presynaptic effects of DPCPX could be studied in reasonable isolation from the postsynaptic actions.

Spontaneous transmitter release at hippocampal excitatory synapses

Spontaneous miniature EPSPs (mEPSPs) were studied following inclusion of 100 nM tetrodotoxin (TTX) in the ACSF bathing the hippocampal slices. TTX blocks the voltage activated Na^+ channels, preventing action potential evoked release and isolating the spontaneous mEPSPs (Colomo & Erulkar, 1968; Brown et al., 1979). Synaptic responses that are observed are due to the spontaneous fusion of one or more synaptic vesicles with the presynaptic terminal resulting in the release of its contents (quanta) into the synaptic cleft. The rate of release is most likely dependent entirely upon the probability of release from presynaptic elements, the amplitude of the synaptic response following vesicular release is determined 1) by the amount of neurotransmitter released; 2) the postsynaptic sensitivity to the neurotransmitter, and 3) the driving force for the ions mediating the synaptic potential.

In these experiments it was found that neither CCPA (100 nM) or DPCPX (30 nM) significantly altered the mean amplitude of the mEPSPs. However both CCPA and DPCPX altered the frequency of occurrence of the mEPSPs. CCPA decreased the mean mEPSP frequency by increasing the interval between successive mEPSPs, DPCPX increased the mean frequency by decreasing the interval between successive mEPSPs. As mEPSP frequency is determined presynaptically this result suggests that the concentrations of CCPA and DPCPX used selectively targeted presynaptic adenosine A_1 receptors.

The cumulative amplitude distributions were significantly changed in 6 out of 11 neurones after exposure to CCPA and for 2 out of 6 after exposure to DPCPX. This suggests that after exposure to CCPA, in these neurones, many small mEPSPs failed to reach the threshold for detection - they were lost in the noise. When the cumulative amplitude distributions were studied (e.g see Fig 5.8) it appears that in these neurones some of the largest mEPSPs were also absent in the presence of CCPA, again this would contribute to the apparent changes in amplitude distributions in these neurones. This would have contributed to the decrease in frequency observed after exposure to CCPA. However a significant change in the interval distribution was observed in the majority of neurones exposed to CCPA, thus suggesting that the spontaneous release probability was decreased in the presence of CCPA due to an action at the presynaptic adenosine A_1 receptor. DPCPX significantly altered the interval distribution for 5 out of 6 neurones recorded from. This suggests that DPCPX blocked the endogenous adenosine action at the presynaptic adenosine A_1 receptor, resulting in an increase in the rate of spontaneous release. This is supported by the finding that when DPCPX was co-applied with CCPA, after exposure of the slice to CCPA alone, the mean frequency of release was greater than that found under control conditions, suggesting that there was an small inhibition of the adenosine A_1 receptors under control conditions.

These results, using the highly selective adenosine A_1 receptor agonist, CCPA, showing that the mean mEPSP and therefore the mean intervals, but not the mean amplitude of the mEPSPs, was altered confirms results previously described using adenosine and the adenosine A_1 specific agonist cyclopentyladenosine (CPA;

Scanziani et al., 1992; Scholz & Miller, 1992). Similar results have been described at the rat neuromuscular junction (Ginsbourg & Hirst, 1972) and in cultured rat hippocampal neurones and slices (Scanziani et al., 1992; Scholz & Miller, 1992).

Under the conditions employed any changes in release that occur after adenosine A_1 receptor activation are probably due to the receptor coupling to a voltage-independent mechanism(s) to alter release of transmitter, as action potential evoked release was blocked by inclusion of TTX in the bathing solution. Adenosine A_1 receptor agonists have been shown to activate K^+ currents (Trussell & Jackson, 1987) and inhibit Ca^{2+} currents (Scholz & Miller, 1991) in somata of hippocampal pyramidal cells in culture. A mechanism of adenosine inhibition of transmitter release, that is independent of an inhibition of Ca^{2+} influx, has been proposed for release in many preparations including the neuromuscular junction (Katz & Miledi, 1963; Silinsky 1981, 1984), and hippocampal neurones (Scholz & Miller, 1992) as blockers of voltage-dependent Ca^{2+} channels have been shown to have no effect on the frequency of miniature synaptic currents (Scholz & Miller; 1992). These Ca^{2+} channels cannot be tonically activated in presynaptic endings, but rather they depend upon the occurrence of action potentials for their activation. It has been shown that the frequency of occurrence of spontaneous synaptic currents, recorded from hippocampal CA1 neurones, in the absence of TTX, was dependent upon the level of Ca^{2+} present in the apical dendrite area (Sastry & Bhagavatula, 1996). When Scholz & Miller (1992) compared the degree of inhibition of the frequency of spontaneous release (in the presence of TTX and Ca^{2+} channel blockers) with the inhibition of evoked release using the same concentration of CPA they observed that evoked release was inhibited by ~ 95 % compared to ~ 45 % for spontaneous release. They suggested that although inhibition of Ca^{2+} influx contributes to presynaptic inhibition, via the adenosine A_1 receptor, it is likely that other mechanisms also contribute to presynaptic inhibition. This is comparable to the results found in these spontaneous release experiments and in the evoked release experiments using the same concentration of CCPA. Under current-clamp conditions, the decrease in amplitude, in evoked release experiments, caused by CCPA was much larger (~ 50 % for non-failures experiments, ~ 95 % paired-pulse experiments) than for the change

in mEPSP frequency seen in the spontaneous release experiments (~ 38 %). Candidate mechanisms for the inhibition of transmitter release induced by activation of the adenosine A_1 receptor include a decrease in the sensitivity of the release apparatus to Ca^{2+}. Several protein kinases have been shown to phosphorylate proteins in synaptic membranes (Augustine et al., 1994; Trimble et al., 1991). Stimulation of some of these kinases has also been shown to lead to increases in the frequency of spontaneous release (Malenka et al., 1987; Finch & Jackson, 1990). A possible mechanism of action of adenosine and CCPA, is that they decrease the phosphorylation of these presynaptic proteins, and thereby inhibit excitatory transmitter release. For example an alteration in the phosphorylation state of synapsin I can change the availability of neurotransmitter for release (Llinas et al., 1991). As adenosine has been shown to inhibit adenylate cyclase in brain tissue (Fredholm et al., 1986) and synapsin I contains cAMP-dependent phosphorylation sites (Huttner & Greengard, 1979) the involvement of this system is an attractive hypothesis. A further possibility is some form of interaction of small G proteins with synaptic vesicles. The G protein dissociates from these vesicles during exocytosis (Fischer von Mollard et al., 1991).

Evoked transmitter release at hippocampal excitatory synapses

Single Pulse Experiments

When single synaptic responses (EPSPs and EPSCs) were evoked, using a low intensity stimulus, it was possible to observe response failures in a large proportion of trials. Analysis of these failures, together with an analysis of the response amplitude, was used to determine the effects of exposure of the neurone to a low concentration of the adenosine A_1 receptor antagonist, DPCPX. Hippocampal CA1 neurones and granule cells were studied in this series of experiments.

The mean amplitude and quantal content (m_f) were both increased after exposure to DPCPX for all CA1 neurones tested, under both current and voltage clamp conditions. However, it was found that the mean amplitude increased in only half of the granule cells, a decrease being observed in the remaining neurones (3 / 6). Possible explanations for this decrease in mean amplitude include: (1) depletion of

the presynaptic store of neurotransmitter, with vesicle recycling not being able to keep pace with the rate of vesicle depletion. Liu & Tsien (1995a) reported that frequent repetitive, non-physiological, stimulation produced a decline in the incidence of EPSCs as the pool of releasable vesicles was exhausted, the regeneration of the vesicular pool had a time constant of about 40 s, and the vesicular pool was capable of generating approximately 90 EPSCs without recycling. Dobrunz & Stevens (1997) showed that single synapses had a readily releasable pool of neurotransmitter that contained, on average, 5 quanta (vesicles). Once depleted that refilling rate was ~ 3 ± 2 s. In my experiments a single stimulus was applied every 3 seconds. 2) It is possible that postsynaptic receptor desensitisation occurred due to prolonged receptor exposure to glutamate, thus leaving the AMPA / Kainate receptors less responsive to the same amount of presynaptically released glutamate (Trussell & Fischbach, 1989). It has been shown that aniracetam can increase EPSC amplitudes by inhibiting AMPA receptor desenistisation, however Debanne et al., (1996) showed that aniracetam had no effect on paired-pulse depression, only upon EPSC amplitude. Receptor desensitisation is more likely to occur during paired-pulse stimulation as the released glutamate is less likely to have been cleared from the synaptic cleft by reuptake and degradation mechanisms, therefore the receptor would have had a prolonged exposure to glutamate. In the experiments described above the presynaptic axons were stimulated every 3 - 5 s, thus it is more probable that the receptor would have recovered from its exposure to the glutamate released in response to a stimulus prior to the next stimulus (Clements, 1996). There is a possibility that a longer term receptor desensitisation has occurred, e.g. receptor down regulation, and not a short term desensitisation in response to exposure to glutamate that is released after a presynaptic stimulus.

In the granule cells an increase in m_f was shown for those cells where an increase in amplitude was seen in response to DPCPX, and a decrease in m_f was found in 2 / 3 cells where a decrease in amplitude was observed. This suggests the decrease in mean amplitude is likely to be due to a decrease in the mean quantal content at these synapses.

Paired-pulse Experiments

Recordings were made from hippocampal CA1 pyramidal neurones in current and voltage clamp modes. The Schaffer collateral commissural fibres were stimulated with pairs of pulses separated by 90 ms, using a minimal stimulation protocol, and the responses of the patched CA1 neurones were measured. It was found that transmitter release fluctuated at the excitatory synapses that were stimulated, with great variation between trials. The paired pulse protocol significantly altered the amplitude distributions in response to the first compared to the second stimulus. This could be due to differences in the synapses that respond and release transmitter after the first or the second stimulus e.g. previously silent synapses were activated as has been demonstrated during LTP (Isaac et al., 1995; Liao et al., 1995). In the majority of neurones tested the amplitude distribution had shifted to the right for the second stimulus when compared with the first. This suggests that not all synapses were activated and released transmitter in response to the first stimulus. If more synapses released transmitter after the second stimulus, this could be due to (1) a priming action of the first stimulus at sites where transmitter was not released; (2) residual calcium in the presynaptic terminals which would contribute to an increased level of calcium after the second stimulus, and therefore increased transmitter release; or (3) previously silent receptors being activated. It is not known whether the synapses that released transmitter in response to the first stimulus, released transmitter in response to the second. It cannot be ruled out that some new sites also released transmitter in response to the second stimulus, but some of the sites that released transmitter in response to the initial impulse also released transmitter in response to the second stimulus.

The distribution of amplitudes was significantly altered by manipulations that changed the release probability, i.e. application of the selective adenosine A_1 receptor agonist, CCPA, or antagonist, DPCPX, to activate or block the inhibitory adenosine A_1 presynaptic receptors, respectively. These paired pulse experiments suggested that, under control conditions, stimulation of the Schaffer collateral and commissural pathways resulted in the release of more than one quantum of neurotransmitter at synapses with CA1 neurones, and that the number of quanta released fluctuated from

trial to trial. This is in agreement with other groups who have indicated that unitary excitatory synaptic responses between hippocampal pyramidal cells in acute slices are multiquantal (Foster & McNaughton, 1991; Lupica et al., 1992; Arancio et al., 1994; Debanne et al., 1996).

It is not known how many synaptic contacts are formed between the axons of the Schaffer collateral commissural fibres that were being stimulated and the CA1 neurone that was recorded from. The number of axons being stimulated was unknown, by using extracellular stimulating electrodes a large number of axons are likely to be stimulated, compared with experiments where a presynaptic action potential is elicited in a CA3 neurone that has been impaled with an intracellular electrode (Debanne et al., 1996). However a minimal number of axons were stimulated as release failures were observed in response to a large proportion of trials. It is not known if the observed multiquantal release in response to a single stimulus was due to release at a single site like that proposed for excitatory synaptic connection onto inhibitory cells in the CA3 region of the rat hippocampus (Arancio et al., 1994), or from a number of sites where a single quantum of transmitter is released in response to an action potential as at the inhibitory synapse of the goldfish Mauthner cell (Korn et al., 1982). The latter scenario is the more probable of the two, as it is likely that several axons were stimulated using the extracellular stimulation protocol.

In order to analyse the data using compound binomial statistics equal site amplitudes are required, although release probabilities and the intrinsic (intrasite) variance at each site can vary (Stricker et al., 1996). Turner et al., (1997) used a Bayesian site-directed approach to fully and distinctly analyse their data. Using this analysis technique they incorporated both inter- and intrasite variability. This type of analysis was beyond the scope of the experiments described herein, therefore for simplicity it was assumed that release was described by a compound binomial in which an axon forms n release sites with the postsynaptic CA1 pyramidal neurone, where each release site has its own release probability, P, which may or may not be uniform (review see Redman, 1990).

In these experiments only data sets which exhibited low background noise which could be fitted by a single Gaussian curve, were included. The synaptic responses were mediated entirely by AMPA / Kainate receptors, as shown by the complete inhibition of the synaptic response when 10 μM CNQX was applied to the hippocampal slice. A large proportion of failures were required in response to stimulation, in order to record from as few synapses as possible.

In these experiments we observed both PPF and PPD in the same neurone under control conditions. There was no relationship between the quantal content of the first response (under either voltage- or current-clamp conditions) and the quantal content of the second response, from trial to trial. Even when, taken to the extreme, the first response was a failure there was no difference between the quantal content of the second response and the second response quantal content when the first response was not a failure. Debanne and co-workers observed that when pairs of action potentials were elicited in a single presynaptic cell, the amplitude of the second EPSC in a postsynaptic cell depended upon the amplitude of the first EPSC (Debanne et al., 1996).

The paired-pulse ratio was a function of the quantal content of the first EPSC, and under control conditions either PPF or PPD was observed in individual trials. The amplitude of the first EPSC was determined by the number of release sites at which transmitter is released. Paired-pulse plasticity appears to occur mainly due to changes in the probability of release, as described by many groups (Redman, 1990; Manabe et al., 1993, Andreasen & Hablitz, 1994; Debanne et al., 1996). Debanne et al., (1996) suggested that, in cultured hippocampal slices, PPF would be observed if the first action potential failed to release transmitter at most of the release sites formed by the axon of the presynaptic cell, whereas PPD would be observed when the first action potential successfully triggers transmitter release at most release sites. The probability of release at a given release site depended, in part, upon its recent history. The direction of the paired-pulse plasticity depended upon the quantal content of the first action potential. However, Turner et al., (1997) showed that the degree and direction of the paired-pulse plasticity response correlated with the initial probability of release, for the critical synapse underlying most of the change,

suggesting that PPF may be present if the response starts from a lower initial probability.

Paired-pulse Facilitation

It has been suggested that the second of a pair of action potentials at the neuromuscular junction triggers release with a higher probability than the first action potential as a result of a small but long-lasting elevation of the intracellular Ca^{2+} concentration in the presynaptic axon terminal (Katz & Miledi, 1968). Using the Ca^{2+} indicator dye, Fura-2, it was shown that the presynaptic $[Ca]_{residual}$ was approximately linearly related to PPF, thus demonstrating that the residual calcium hypothesis holds for mammalian central neurones (Wu & Saggau, 1994b). The time course of PPF has been estimated, in the absence of concomitant PPD, and has been found to be complete within 500 ms of the first action potential ($Mg^{2+}/Ca^{2+} = 2.8$; Debanne et al., 1996). This, presumably, reflects the clearance of residual Ca^{2+} from the nerve terminal, and is consistent with estimates made in the hippocampus using Ca^{2+} indicator dyes (Wu & Saggau, 1994b).

Paired-pulse Depression

From these experiments it was observed that the greater the quantal content of the first response, the higher the probability that PPD occurs. Paired-pulse depression occurred when the first EPSC was > 65 % of the maximum first EPSC amplitude observed, and when the first EPSP > 45 % of the maximum first EPSP amplitude seen. Paired-pulse depression thus depends on release and is, therefore, a use-dependent form of synaptic plasticity. Debanne et al. (1996) showed that PPD was not the result of postsynaptic receptor desensitisation, as reduction of AMPA receptor desensitisation with aniracetam had no effect on PPD. Mennerick & Zorumski (1995) found an increase in the frequency of spontaneous EPSCs (sEPSC) after the conditioning pulse of a pair of pulses in microcultures of rat hippocampal neurones. These sEPSC had amplitudes which were not reduced when paired pulse depression was observed, again suggesting that receptor desensitisation did not play a part in paired pulse depression.

It might be expected that PPF would be observed in all trials, regardless of the initial response amplitude, due to the $[Ca]_{res}$ in the presynaptic terminal. However, under control conditions, in 25.2 % and 46.4 % (voltage- and current-clamp respectively) of trials a PPD was observed. Unlike the neuromuscular junction (Theis, 1965; Betz, 1970) and the cultured hippocampal slice (Debanne et al., 1996) there was no correlation of the quantal content of the second response with the quantal content of the first response. Although PPD was more likely to be observed when the first response was > 65 % and 45 % of the maximum value observed for the first response (voltage- and current-clamp respectively). It has been suggested that, after the first stimulus and subsequent transmitter release, there is a decrease in the number of quanta available to be released by the second action potential. This depletion is limited to a subset of vesicles that have a higher release probability, so that a given terminal is less likely to respond after there has been release of transmitter in response to the first action potential (review see Zucker, 1989). PPD has been estimated to have a duration of up to 5 s, occurring with a time constant of ~ 1 s. This would be the time it could take to re-prime and dock vesicles in the readily releasable pool. This is confirmed in the experiments of Dobrunz & Stevens (1997) who showed that the rate of refilling the readily releasable pool of neurotransmitter was 2.8 ± 2 s. When much larger, and less physiological, stimuli are used to trigger release the recovery from synaptic depression is much slower and has a rate constant of ~ 40 s (Liu & Tsien, 1995), indicating that restoring the number of vesicles available for refilling the readily releasable pool is much slower than re-priming.

Paired-pulse facilitation and depression have been observed under various experimental conditions in two muscles in the stomatogastic system of the lobster *Homarus americanus* that are innervated by a single motor neurone (Katz, Kirk & Govind; 1993) as well as a variety of hippocampal preparations (Harris & Cotman, 1985; Forsythe & Clements, 1990; Mennerick & Zorumski, 1995; Debanne et al., 1996; Dobrunz & Stevens; 1997). In the rat hippocampus, at the synapse formed between CA3 and CA1 neurones we, and others, have observed both forms of plasticity under control conditions (Debanne et al., 1996; Turner et al., 1997). When

the release probability was manipulated by addition of the adenosine A_1 receptor agonist, CCPA, and antagonist, DPCPX, the proportion of trials that resulted in PPD was unchanged from control conditions. The shape of the distribution of first response amplitudes (as a percentage of the maximum amplitude observed within the test period) versus the paired-pulse ratio was unaffected by altering the release probability, although the maximum amplitudes observed were changed. This was confirmed when the plots of response 1 amplitude (absolute value) vs paired-pulse ratio were examined. Although the distribution was shifted to the left (decrease in amplitude) or the right (increase) the shape of the distribution was unchanged. These plots shows that PPD was found at a wide range of response 1 amplitudes (both small and large; see Fig 4.14), indicating that a large response to the first stimulus does not, in the acutely prepared hippocampal slice, give an indication of the likely amplitude of the second response, unlike the results of Debanne et al. (1996), but in agreement with Turner et al. (1997). This result suggests that although the quantal content was altered by addition of CCPA or DPCPX, thus resulting in a decrease or increase in the amplitude of the responses, the way in which the stimulated synapses behaved in response to pairs of stimuli - i.e. showing a PPF or a PPD - did not change.

When pairs of responses that showed PPF or PPD were analysed, it was found that when PPF occurred the first response had a smaller than mean amplitude and the second response had a larger than mean response 2 amplitude. The converse was true when PPD was observed. However, as shown, PPD was observed at all initial amplitudes, it was only more likely to be observed after a larger response 1 amplitude. Unlike Debanne et al. (1996) there was no clear definition of which size of response 1 amplitude was more likely to result in the second response showing a facilitation or a depression.

It has been suggested that enhanced plasticity may be present if the first response starts from a lower initial probability (Dobrunz & Stevens, 1997; Turner et al., 1997), rather than there being a dependence upon the quantal content of the first response.. Chen et al., (1996) showed that the presence of a response (i.e. non-failure) to the first stimulus may lead to PPF compared with responses which

followed a failure to the first stimulus, which is in contrast with the study of Debanne et al. (1996).

Quantal Measurements

These experiments were designed such that under control conditions response failures were observed. The quantal content (m_f = ln (N/n_0), where n_0 = number of failures and N = total number of trials; Martin, 1966) was calculated for each neurone under all conditions. It was found that CCPA decreased both the mean amplitude and the m_f from the values found under control conditions, in both current- and voltage-clamp. This suggests that when CCPA activates the inhibitory presynaptic adenosine A_1 receptor, the resulting inhibition observed is due to a decrease in the quantal content of neurotransmitter that is released. This is confirmed by measurement of quantal content using the coefficient of variation (m_{cv}, see appendices 4.2 and 4.3). Using the coefficient of variation it was also found that the quantal amplitude (q_{cv}) was unaltered, suggesting that vesicular content was not changed by addition of CCPA or DPCPX. The coefficient of variation is a less accurate method of calculating the quantal content as it is subject to measurement errors when sample sizes are small or when signal-to-noise ratios are not favourable (Martin, 1966). This parameter also assumes that responses to individual quanta at all activated release sites are of uniform size (Korn & Faber, 1991) and that release can be described by a simple binomial model.

When DPCPX was applied to the hippocampal slice preparation it was found that the amplitude of the pairs of EPSPs and EPSCs showed increases and decreases in mean amplitude in the different cells studied. The m_f was also increased and decreased from the value found in control, but did not correlate with the changes in mean amplitude observed. In 2 cases (1 under current-clamp, and one under voltage-clamp) the value of m_f increased when the mean amplitude decreased. It is possible that the decrease in mean amplitude was due to receptor desensitisation, unlike the result shown for a single stimulation of the mossy fibre pathway and recording from the Dentate Gyrus. However measurements made using the coefficient of variation suggest that q_{cv} did not change after exposure to DPCPX (see appendices 4.4 and

4.5), whilst m_{cv} was altered, again with no relationship with the changes observed in mean amplitude. As q_{cv} was not altered by DPCPX, it is probable that receptor desensitisation did not play a large role in the decrease in mean amplitude that was observed.

A further explanation is that the increased imposition on the release sites, by increasing the transmitter release probability by blocking tonic endogenous adenosine inhibition, may have placed too great a demand on the synapses in some of the preparations, and neurotransmitter depletion occurred at these sites. The time between trials of the pairs of pulses (6 s in these experiments) may not have been long enough to allow for the transmitter pool to have been completely replenished. The time constant for replenishment has been estimated to be ~ 10 s (Stevens & Tsujimoto, 1995) in cultured slices at 37 ^{0}C, and 2.8 ± 2 s in acute slices (Dobrunz & Stevens, 1997) at room temperature.

Presynaptic Mechanisms for Inhibition of Transmitter Release

As shown above in the spontaneous release experiments, activation of the presynaptic adenosine A_1 receptor can inhibit transmitter release via a mechanism that is independent of activation of voltage-activated ion channels. Possible mechanisms include an inhibition of protein kinases (Malenka et al., 1987; Finch & Jackson, 1990), this in turn leads to a decrease in the phosphorylation status of presynaptic proteins that are involved in the release process, and a consequent decrease in the release probability. When the tonic inhibition at the adenosine A_1 receptor is relieved by addition of DPPCX the activity of the protein kinases would be enhanced and transmitter release enhanced. In the presence of CCPA the activity would be decreased, accompanied by a decrease in transmitter release. As synaptic release is mediated by a host of different proteins which play a role in vesicle filling, docking priming and release i.e. VAMP, SNAP-25, Synapsins (phosphorylated by PKA), Syntaxins, Synaptobrevin, Synaptotagmin (I - VIII), Rabphilin-3, Rab3 proteins (cf., Neuropharmacology III, Satellite to Neurosciences, Presynaptic Mechanisms of Neurotransmission Abstracts, 1995) amongst others, this provides much scope for phosphorylation and dephosphorylation of components of the release

mechanism to increase or decrease the effectiveness of the adenosine A_1 receptor in mediating synaptic release. Many of these proteins have been shown to contain PKA phosphorylation consensus sequences, thus this cAMP-dependent protein kinase may be important in regulating transmitter release at many steps in the cascade between receptor activation and exocytosis of the vesicular contents into the synaptic cleft. The adenosine A_1 receptor has been shown to decrease cAMP levels when activated (Londos & Wolff, 1977; Fredholm et al., 1986), therefore the adenosine A_1 receptor may modulate PKA dependent phosphorylation of these proteins.

Castillo et al. (1997) have recently shown that mossy fibre LTP, in mice lacking the synaptic vesicle protein, Rab3A, is abolished whilst a variety of short-term plasticities, including paired-pulse facilitation are normal. One proposed explanation for the absence of LTP is that adenylyl cyclase is not activated because the tetanus induced rise in presynaptic Ca^{2+} has been impaired. This was unlikely as paired-pulse facilitation was unaffected, and this is proposed to result due to residual calcium levels in the presynaptic terminal, after calcium influx in response to the first stimulus. A second possibility suggested is that Rab3A is involved in keeping adenylyl cyclase near to the Ca^{2+} channels. In the absence of Rab3A, Ca^{2+} would no longer activate the cyclase. However an interaction between Rab3A and adenylyl cyclase has yet to be shown. A more likely explanation is that Rab3A deletion prevents PKA-mediated modulation of some step in the release protein. As described above there are plenty of sites where this is possible. Geppert et al. (1997) observed that Rab3A may regulate the number of vesicles that are released as a function of Ca^{2+}. These results suggest that the increase in transmitter release during mossy fibre LTP occurs at a late step in the secretory process. It is possible that Rab3A is normally partially inactivated by LTP, whereas it is not affected by short term plasticities. However there are other possible components of the release mechanism that can be modulated by activation of adenosine A_1 receptors resulting in short term modulation of synaptic release.

Lüscher et al. (1997) have demonstrated that mutant mice lacking G protein-gated inwardly rectifying K^+ channels have altered postsynaptic reponses to adenosine, but presynaptic responses are unchanged. This suggests that the

presynaptic responses to adenosine are mediated by other mechanisms such as G-protein inhibition of Ca^{2+} channels, whilst postsynaptic responses are mediated by a separate mechanism. Further support for the involvement of a presynaptic G-protein coupled Ca^{2+} channels is provided by Takahashi et al. (1996). At the calyx of Held, Takahashi et al. (1996) demonstrated that mGluR agonists suppressed a high voltage-activated P/Q-type calcium conductance in the presynaptic terminal, thereby inhibiting transmitter release at this glutamatergic synapse.

These experiments have demonstrated that there is an appreciable level of endogenous adenosine, in the acutely prepared slice. The adenosine A_1 antagonist, DPCPX (30 nM), increased the amplitude of responses to single stimuli in CA1 neurones, and increased the frequency of spontaneous mEPSPs, due to relief of the endogenous adenosine inhibition of transmitter release. The results of the single-pulse recordings from granule cells and the paired-pulse experiments were more ambiguous, DPCPX decreased the mean amplitude in about half of the CA1 neurones recorded from. It is possible that relieving the endogenous adenosine inhibition of the adenosine A_1 receptor resulted in an additional load on the nerve terminal - an increase in transmitter release - resulting in a depletion of neurotransmitter with vesicle recycling being unable to keep pace with the demand placed upon the synaptic release sites.

These experiments have demonstrated that the presynaptic adenosine A_1 receptor can be studied in isolation from any postsynaptic effects. Activation of the presynaptic adenosine A_1 receptor, by CCPA (100 nM), resulted in a decrease in the amplitude of the postsynaptic response, due to a reduction its quantal content. CCPA also decreased the frequency of mEPSPs, suggesting that the presynaptic adenosine A_1 receptor can inhibit transmitter release via a mechanism that is independent of the N-type voltage-activated Ca^{2+} channel, during nerve terminal hyperpolarisation, possibly due to a direct second messenger action on the release mechanism. A larger inhibition of evoked release was observed for the concentration of CCPA used than that seen for spontaneous release, which suggests that the adenosine A_1 receptor also inhibits Ca^{2+} influx into the nerve terminal (Scholz & Miller, 1992; Takahashi et al.,

1996). The results of Scholz & Miller (1992) suggest that the adenosine A_1 receptor also inhibits transmitter release due to an inhibition of Ca^{2+} influx to the nerve terminal through the N-type calcium channel, as well as a more direct second-messenger action on the release apparatus. Takahashi et al. (1996) have demonstrated that mGluRs can inhibit transmitter release by suppressing the activity of the P/Q-type calcium conductance, at the calyx of Held. This is a further potential mechanism for adenosine A_1 receptor mediated inhibition of transmitter release at synapses onto hippocampal CA1 neurones.

Altering the release probability, by an activation of the adenosine A_1 receptor, or an inhibition of the endogenous adenosine action, did not alter the proportion of trials that exhibited PPD. PPD was observed for a wide range of P1 amplitudes, and unlike the results of Debanne et al. (1996). No correlation between P1 and P2 amplitude was found. CCPA and DPCPX can be used to decrease and increase the excitability of CA1 neurones, respectively. However they do not change the characteristics of the response of the synapses of the Schaffer collateral and commissural pathways of the hippocampus to pairs of stimuli, as evidenced by a lack of effect on the proportion of trials that exhibit PPD and the point at which PPD is seen as a percentage of maximum P1 amplitude.

The results of Turner et al (1997) showed that some sites showed a decreased release probability whilst release probability was enhanced at others, these sites could also show minor amplitude changes. The results herein suggest that synapses show heterogeneity in response to the paired-pulse stimuli, and do not all behave identically, in contrast to the results of Debanne et al (1996). This may be due to differences between cultured cells and acutely prepared slices. Global modulation of the release probability, by addition of CCPA or DPCPX, does not alter this heterogeneity, but rather causes a change in the quantal content of release in response to an action potential. This suggests that the endogenous adenosine levels may, in part influence the information filtering characteristics of the hippocampus, by modulating the amplitude of the synaptic responses. This coupled with the synaptic heterogeneity this may increase the storage capacity of the synapses and dendrites.

Future Experiments

In order to determine whether the DPCPX effect on the input resistance was due to rundown of the activity of the potassium channels in the postsynaptic cell, the experiments should be repeated with GTP (1 mM) in the pipette. The effects of CCPA could be studied under these conditions, in order to determine whether the lack of effect on input resistance was due to potassium channel rundown, or solely due to using a concentration of CCPA that was adequate to significantly reduce transmitter release with no significant postsynaptic effects. However, under the conditions used in the experiments described above, the presynaptic effects of CCPA were examined in isolation from significant postsynaptic changes, indicating that the conditions used were ideal for studying presynaptic changes.

These studies could also determine whether the lack of effect of DPCPX on the amplitude of the hyperpolarisation activated conductance (I_h) was due to rundown of this current, by measuring this conductance over 30 minutes, with and without GTP in the pipette.

A major problem with these experiments is the lack of knowledge about the number of synapses that are activated by each impulse. Even using the conditions of Debanne et al. (1996) the number of synapses are unknown. In order to obtain more detailed information about the system, experiments where action potentials are elicited in a CA3 neurone that is impaled with a sharp electrode, and responses recorded from a patched CA1 neurone, in acutely prepared slices could be carried out. The pre- and postsynaptic cells should be labelled (e.g. using biocytin) and a morphological reconstruction carried out, to determine the number of synapses made by the CA3 neurone with the CA1 neurone. By doing this, and using a detailed quantal analysis of the responses, a correlation between the number of sites determined morphologically, and physiologically (e.g. see Turner et al., 1997) may be made.

Appendix 4.1 m_{cv} and q_{cv} values for single pulse experiments

	m_{cv}		q_{cv} (mV)	
EPSP (CA1)	Control	DPCPX	Control	DPCPX
1	6.65	6.13	0.055	0.092
2	1.32	9.87	0.370	0.055
3	10.84	15.38	0.111	0.108
4	3.66	9.51	0.110	0.076

	m_{cv}		q_{cv} (pA)	
EPSC (CA1)	Control	DPCPX	Control	DPCPX
1	1.44	3.65	4.91	4.07
2	6.43	7.04	0.81	0.78
3 *	2.55	5.77	8.23	6.26
4 *	0.50	1.07	55.2	34.5
5 *	3.15	8.6	15.1	7.96

	m_{cv}		q_{cv} (pA)	
EPSC (Dentate Gyrus)	Control	DPCPX	Control	DPCPX
1 *‡	1.77	0.97	4.52	3.55
2 *‡	0.096	0.35	19.0	10.4
3 *	4.42	7.72	2.30	1.31
4 *	2.68	4.33	3.25	3.74
5 *	3.93	2.68	2.47	2.11
6 *	0.81	1.39	11.6	6.20

* These results were kindly provided by Dr J.P. Hodgkiss

‡ These two results are for one neurone that showed two separate components to the EPSC (see fig 4.4)

Appendix 4.2 m_{cv} and q_{cv} for Control and CCPA under current-clamp conditions

EPSP 1 m_{cv} EPSP 2 m_{cv}

	Control	CCPA	DPCPX	Control	CCPA	DPCPX
1	2.16	0.24	-	4.57	0.36	-
2	2.36	0.53	1.07	2.63	0.41	1.41
3 *	1.06	0.78	0.76	1.46	0.36	0.60
4	3.81	1.37	1.21	3.67	1.18	1.78

EPSP 1 q_{cv} (mV) EPSP 2 q_{cv} (mV)

	Control	CCPA	DPCPX	Control	CCPA	DPCPX
1	0.58	0.124	-	0.445	0.824	-
2	0.41	0.20	0.38	0.635	0.323	0.287
3 *	1.23	0.08	0.33	1.36	0.20	0.50
4	0.21	0.18	0.27	0.22	0.21	0.23

* neurone illustrated in Figure 4.7

Appendix 4.3 m_{cv} and q_{cv} for control and CCPA under voltage-clamp conditions

EPSC 1 m_{cv} EPSC 2 m_{cv}

	Control	CCPA	Control	CCPA
1	5.49	5.42	7.79	6.33
2	5.94	5.92	7.62	7.50
3	5.06	4.88	13.63	7.54
4 *	11.39	3.53	9.06	6.78
5	7.22	2.90	14.34	4.58
6	2.48	2.70	7.11	5.41

EPSC 1 q_{cv} (pA) EPSC 2 q_{cv} (pA)

	Control	CCPA	Control	CCPA
1	4.51	1.58	3.46	2.01
2	3.35	2.93	3.42	2.66
3	6.57	4.42	4.02	4.87
4 *	1.70	1.84	3.31	2.04
5	3.16	6.45	2.24	5.51
6	17.1	10.1	9.56	8.82

* neurone illustrated in Figure 4.8

Appendix 4.4 m_{cv} and q_{cv} values for control and DPCPX under current-clamp conditions

EPSP 1 m_{cv} EPSP 2 m_{cv}

	Control	DPCPX	Control	DPCPX
1	2.80	1.59	1.45	1.01
2	0.76	1.16	1.20	1.38
3	8.79	26.9	1.50	8.53
4	0.82	2.20	1.14	2.03
5	8.77	9.93	1.83	5.50
6	6.92	10.8	11.87	9.17
7	1.22	0.54	1.30	0.46
8 *	1.71	3.99	2.66	4.36

EPSP 1 q_{cv} (mV) EPSP 2 q_{cv} (mV)

	Control	DPCPX	Control	DPCPX
1	0.22	0.27	0.33	0.38
2	0.77	0.47	0.64	0.57
3	0.30	0.30	0.10	0.06
4	0.79	0.30	0.62	0.47
5	0.06	0.04	0.18	0.06
6	0.14	0.09	0.07	0.12
7	0.28	0.38	0.28	0.54
8 *	0.45	0.34	0.38	0.34

* neurone illustrated in Figure 4.11

Appendix 4.5 m_{cv} and q_{cv} values for control and DPCPX under voltage-clamp conditions

EPSC 1 m_{cv} EPSC 2 m_{cv}

	Control	DPCPX	Control	DPCPX
1	3.47	4.73	3.61	9.56
2	4.47	7.88	0.70	2.17
3	4.09	5.51	9.69	12.0
4	10.1	10.1	14.1	15.8
5	3.29	11.2	1.09	4.54
6	3.01	5.01	4.69	24.0
7 *	10.9	15.8	18.5	20.0
8	12.1	21.3	20.8	24.4
9	5.12	9.51	7.49	7.88

EPSC 1 q_{cv} (pA) EPSC 2 q_{cv} (pA)

	Control	DPCPX	Control	DPCPX
1	5.65	2.30	7.05	1.68
2	1.25	0.65	14.2	3.70
3	3.81	4.29	2.99	3.38
4	6.77	7.54	7.17	7.37
5	8.46	4.22	15.5	6.18
6	7.05	1.46	7.53	0.50
7 *	4.24	4.13	4.26	5.10
8	2.93	1.80	2.39	2.10
9	4.96	2.09	5.42	4.08

* neurone illustrated in figure 4.12

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