# The modulation of epileptiform activity in rat hippocampal slices by adenine nucleotides.

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## Abstract

In this study the most consistent method of inducing epileptiform activity in hippocampal slices involved recording from the pyramidal cell layer of the CA3 region whilst bathing the slice in a medium containing no added magnesium and 4-aminopyridine (4AP) (50μM). The bursts produced were interictal in nature and inhibited by kynurenate (1mM) or 2-amino-5-phosphonopentanoic acid (20 and 40μM) suggesting that both NMDA and non-NMDA receptors were involved.

The effect of ATP and other adenine nucleotides on the rate of spontaneous epileptiform activity was investigated. ATP and adenosine were equipotent at decreasing discharge rate at concentrations above 10μM. The depression produced by ATP was characterised by being inhibited by the $A_1$ receptor antagonist 8-cyclopentyl, 1,3-dimethylxanthine (CPT) but resistant to adenosine deaminase (at a concentration which annulled the effect of adenosine). $P_2$ receptor antagonists (pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) and suramin) were also unable to block ATP depression of bursting.

AMP depressed epileptiform activity similarly to ATP. Its effect was inhibited by CPT and 5'-adenylic acid deaminase (AMPase). AMPase also blocked the depression in discharge rate elicited by ATP. 5'-nucleotidase is involved in the metabolism of AMP to adenosine. The combination of 5'-nucleotidase and adenosine deaminase with AMP or ATP resulted in no significant change in the frequency of activity. These later results, therefore, suggest that ATP acts through

metabolism to AMP. The antagonism by CPT supports the involvement of $A_1$ receptors.

The ATP analogues α, β-methyleneATP and 2-methylthioATP were also tested as well as uridine triphosphate. Only α, β-methyleneATP (10μM) altered the discharge rate producing an increase in the frequency of spontaneous activity which was inhibited by suramin and PPADS. These results are indicative of an excitatory P2X receptor in the hippocampal CA3 region. Several have been cloned and found expressed in the hippocampus but none with the characteristics displayed in this study.

BzATP, an agonist at the $P_{2Z}$ receptor, decreased the discharge rate during a 10 minute perfusion. This depression was not reversed upon washing but continued to progress. The combination of BzATP and CPT produced an increase in discharge rate which was smaller than that caused by CPT alone. This suggests that $A_1$ receptors may not be involved in producing the effect of BzATP.

The alpha, omega-adenine dinucleotides $Ap_4A$ and $Ap_5A$ produced a concentration-dependent depression in discharge rate. Adenosine deaminase inhibited the effect of 1μM but not the early stages of the depression produced by 10μM $Ap_4A$ and $Ap_5A$. CPT but not AMPase antagonised the depression in rate produced by $Ap_4A$. This suggests that in the CA3 region of the hippocampus $Ap_4A$ and $Ap_5A$ act partly by stimulating xanthine sensitive receptors directly and partly through the formation of the metabolite, adenosine.

AMPase initially elevated the discharge rate to a small extent but a subsequent fall in rate occurred during the wash period which continued until a plateau was reached at which the rate remained for up to 90 minutes. IMP was ineffective. Dialysis of AMPase neither affected the enzymatic activity of AMPase nor the following depression. Boiling the enzyme rendered it inactive in that it could no longer prevent the effect of AMP. The sustained depression in activity produced by AMPase was also inhibited by denaturisation. It was proposed, therefore, that AMPase induces a form of long term depression (LTD). The induction of this LTD, as measured by an effect on discharge rate, was not altered by antagonists of $A_1$, opioid or $GABA_A$ receptors nor inhibitors of cyclo-oxygenase, nitric oxide synthase or protein kinase.

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## Abbreviations

| | |
|---|---|
| A3 | *N*-(2-aminoethyl)-5-chloro-1-naphthalene sulphonamide |
| aCSF | artificial cerebrospinal fluid |
| AMPA | α-amino-3-hydroxy-5-methyl-isoxazole propionic acid |
| AMPase | 5'-adenylic acid deaminase |
| $ANAPP_3$ | arylazidoaminopropionyl-ATP |
| 4AP | 4-aminopyridine |
| AP5 | 2-amino-5-phosphonopentanoic acid |
| APNEA | $N^6$-(4-aminophenyl)-ethyladenosine |
| BzATP | benzoylbenzoicATP |
| CGS21680 | 2-(*p*-(-carboxyethyl)-phenylethlyamino)-5'-*N*-ethylcarboxamidoadenosine |
| CHA | $N^6$-cyclohexyladenosine |
| Cl-IB MECA | 2-chloro-N6-(3-iodobenzyl)-MECA |
| CNQX | 6-cyano-7-nitroquinoxaline-2,3-dione |
| CPA | $N^6$-cyclopentyladenosine |
| CPP | 3-(±2-carboxypiperazine-4-yl)propyl-1-phosphonic acid |
| CPT | 8-cyclopentyl-1,3-dimethylxanthine |
| DMSO | dimethylsulphoxide |
| DNQX | 6,7-dinitroquinoxaline-2,3-dione |
| DPCPX | 8-cyclopentyl-1,3-dipropylxanthine |
| EPSP | Excitatory postsynaptic potential |
| GABA | gamma aminobutyric acid |
| IPSP | Inhibitory postsynaptic potential |

| | |
|---|---|
| *L*-NAME | *N* ω-nitro-*L*-arginine methyl ester |
| (+)MCPG | α-methyl-4-carboxyphenylglycine |
| mGLU | metabotropic glutamate |
| α, β-meATP | α, β-methyleneATP |
| MECA | methylcarboxamidoadenosine |
| 2meSATP | 2-methylthioATP |
| NECA | 5'-*N*-ethylcarboxamidoadenosine |
| NMDA | *N*-methyl-*D*-aspartate |
| *R*-PIA | *R*-phenylisopropyladenosine |
| PPADS | pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid |
| 8pSPT | 8-(*p*-sulphophenyl)theophylline |
| ZM241385 | (4-(2-[7-amino-2-(2-furyl)[1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-yl amino]ethyl) phenol) |

## Publications

Ross, F.M., Brodie, M.J. & Stone, T.W. (1996) Modulation of epileptiform activity by purines. *Drug Dev. Res.*, **37**, 141.

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Ross, F.M., Brodie, M.J. & Stone, T.W. (1997) Dinucleotide modulation of epileptiform activity in rat hippocampal slices. *Brain Res. Assoc. Abstr.*, **15**

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## Declaration

I declare that all the work in this thesis was carried out by myself except where referenced and that it has not been submitted for any previous higher degree.

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My heartfelt thanks also go to my grandma for just being who she was. I hope that in life I can follow the great example she set. It is in her memory that this thesis is dedicated.

**Two-minute tonic**

Sometimes, thankfully, we have days when everything goes right. And you don't need me to remind you that there are also days when every blessed thing goes wrong.

Sometimes we have a gorgeous summer, sometimes not.

There are days when you'll spot a real bargain while you are out shopping-and there are, of course, times when you could look forever and see nothing you like, at any price.

No matter how happy, or important, or well-off you happen to be, now and again you're going to have a run of bad luck that tests your patience and makes you wonder if it will ever end.

But now and then, we all tend to forget that after rain comes sunshine, after a bad spell there comes a lucky beak.

The pattern of life for all of us-rich and poor alike, young and old-is a complex weave of both bright and sombre colours.

So, the next time you feel near despair hang on a bit longer, smile if you can and keep cheery even if its a struggle.

A change for the better may only be a moment away, while each day brings new opportunities and challenges.

Abstract from the Weekly News.

# 1.0 Introduction

## 1.1 Epilepsy

Epilepsy is a neurological disorder which affects approximately 0.5-1% of the population (Rogawski & Porter, 1990) and has been defined as "an episodic disorder of the nervous system arising from the excessively synchronous and sustained discharge of a group of neurones" (Jackson, 1890). The term epilepsy or epileptic refers to numerous and unpredictable episodes of seizures. Seizures are characterised by excessive firing of CNS neurones and are not necessarily epileptic. Within the broad title of epilepsy lies a widespread variety of symptoms which range from vacant stares in absence seizures, to loss of consciousness accompanied by muscle spasm and thence jerking of the limbs which is characteristic of tonic-clonic seizures. Seizures are initially characterised into two groups depending on whether the abnormal discharge remains localised in a brain region, partial seizures, or whether it spreads rapidly, generalised seizures.

Clinically, the common feature of a brain with increased susceptibility to epileptic episodes is the presence of an abnormal waveform of electrical activity in an electroencephalogram (EEG) out with a seizure. Such activity is referred to as an interictal spike. The intracellular correlate is a paroxysmal depolarising shift (PDS) which consists of depolarisation of the cellular membrane along with bursts of action potentials (Ayala *et al.*, 1970). Initially it was proposed that the PDS was a summated or 'giant' excitatory postsynaptic potential (EPSP) (Dichter & Spencer, 1969). However, evidence also points to it being an intrinsically generated event (Prince, 1978; Schwartzkroin & Prince, 1978; Mesher &

Schwatrzkroin, 1980). Ictal discharges refer to the patterns of electrical activity that occur during a seizure. The extent of depolarisation and spread of activity is prolonged and more diverse when compared to interictal events thus producing a full blown seizure.

## 1.2 Neurotransmitters and epilepsy

The normal functioning of the brain rests on maintaining a fine balance between excitation and inhibition. Disruption in this balance may lead to a predisposition for epileptic tendencies.

### *1.2.1 Gamma-aminobutyric acid (GABA)*

Reduction of inhibition by GABA antagonists generates epileptiform activity both in *in vivo* and *in vitro* (Ault *et al.*, 1986; Knowles *et al.*, 1987; Kohr & Heinemann, 1990; Colom & Saggau, 1994; Ameri *et al.*, 1997) models raising the possibility that epilepsy arises from a loss of inhibition. Decreased concentrations of GABA were found in tissue resected from epileptic patients when compared to 'normal' concentrations determined from non-epileptic patients (van Gelder *et al.*, 1972). When the concentrations of GABA in tissue resected from epileptic foci and surrounding non-epileptic areas were compared no significant differences were found (van Gelder *et al.*, 1972; Peeling & Sutherland, 1993). Human studies pose problems in that normal values are hard to establish. Tissue for the determination of so-called normality is either postmortem tissue with which time delays may be complicating, or from areas adjacent to epileptic tissue in fresh specimens which also may not reflect a true normal value. The use of *in vivo*

micro-dialysis found lower GABA levels in the epileptic compared with the non-epileptic hippocampus (During & Spencer, 1990; During & Spencer, 1993). Immunohistochemistry revealed a decreased number of GABA-immunoreactive neurones in epileptic tissue. The induction of status epilepticus in animals results in a loss of GABA binding sites in the rat forebrain (Kapur *et al.*, 1994). However *in situ* hybridisation histochemistry of hippocampal slice cultures treated with convulsants found no change in the mRNA levels for $GABA_A$ receptor subunits (Gerfin-Moser *et al.*, 1995). The administration of maximal electroshock, to induce generalised tonic-clonic seizures, in the absence of calcium results in no change in hippocampal GABA concentration, suggesting that seizures directly reduce the neuronal release of GABA (Rowley *et al.*, 1995). Glutamate induced, calcium-dependent release of GABA was markedly reduced in human epileptic hippocampi (During *et al.*, 1995) an action which is secondary to a 48% loss in the number of GABA transporters. Reduced inhibition may also result from an impaired excitatory input to interneurones in the hippocampus in a theory known as the 'dormant basket cell' hypothesis (Sloviter, 1991) which is discussed in section 1.3.5. Analysis of paired-pulse depression (PPD) in granule cells of brain slices from patients with temporal lobe epilepsy found that although in some cases impaired inhibition was apparent due to a weak PPD, a strong level of inhibition was still evident in others (Uruno *et al.*, 1995). This is in agreement with studies where intracellular recordings have demonstrated preserved GABAergic inhibitory postsynaptic potentials in sclerotic, epileptic hippocampi (see Schwartzkroin, 1994 for review). A clear cut reduction in either GABA concentration or in the activity of GABAergic neurones is therefore not the significant underlying cause

that it was initially thought to be. Although some alteration in inhibitory processes may cause or result from seizures, the exact nature and extent of this involvement is not clear.

### *1.2.2 Glutamate*

The alternative explanation of increased excitability is enhanced excitation which probably involves glutamate. Glutamate receptor agonists, *N*-methyl-*D*-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-isoxazole propionic acid (AMPA) & kainate, are convulsants producing seizures both *in vivo* (Koek *et al.*, 1990; Chiamulera *et al.*, 1992) and *in vitro* (Fisher & Alger, 1984). The administration of kainate to rats produces hippocampal cell loss which resembles hippocampal sclerosis found in temporal lobe epileptic brains (Nadler *et al.*, 1980; Nadler, 1981; Ben-Ari, 1985). Kindling, the repeated stimulation of a pathway at intervals until seizures are initiated, is highly dependent on NMDA-receptor activation in some models (Cain *et al.*, 1988; Gilbert, 1988; Robinson, 1991). Glutamate receptor antagonists are good at inhibiting seizures in models where the involvement of either NMDA or non-NMDA receptors is clear, for example zero magnesium or kainate, but they are not such potent anticonvulsants in other models such as those produced by GABA antagonists (Scharfman, 1994), $K^+$ (7mM) or kainate (Neuman *et al.*, 1988). A major contention in the theory that enhanced glutamate levels are responsible for seizures are the conflicting reports regarding the concentration of glutamate before or during seizures. In epileptic patients undergoing surgery, an increase in glutamate levels was detected in samples of extracellular fluid extracted by microdialysis (Hamberger *et al.*, 1991;

Ronne-Engström *et al.*, 1992; During & Spencer, 1993) although no change was apparent in seizures induced in animals by kainate, bicuculline, quinolinic acid, picrotoxin or electrical stimulation (Lehmann *et al.*, 1985; Vezzani *et al.*, 1985; Obrenovitch *et al.*, 1996). Additionally, glutamate *per se* is not a very potent convulsant, with 1300-fold higher concentrations than kainate required, in some instances, to produce only transient seizures (Mizoule *et al.*, 1985). The artificial enhancement of glutamate concentrations by more than 20-fold using the uptake inhibitor *L*-trans-pyrrolidine-2,4-dicarboxylate did not result in electrophysiological changes indicative of seizure activity (Obrenovitch *et al.*, 1996). Thus it is proposed that seizures and raised glutamate levels are not necessarily related.

Enhanced excitability can be attributed to other mechanisms apart from elevated glutamate levels. An increased density of NMDA and kainate receptors has been described in the entorhinal cortex and hippocampal CA1 region (Geddes *et al.*, 1990; McDonald *et al.*, 1991) and an increase in AMPA receptor density in the dentate gyrus has also been shown (Hosford *et al.*, 1991) in tissue removed from patients suffering from intractable epilepsy. Decreases in receptor subunit expression have also been reported for NMDA and non-NMDA receptors in the hippocampus (Gerfin-Moser *et al.*, 1995) but this may correlate with a loss of neurones (Lynd-Balta *et al.*, 1996). Altered patterns of glutamate receptor subunit immunoreactivity were found in the temporal lobe of epileptic patients, including staining in areas which are not stained in normal tissue, for example the molecular layer of the dentate gyrus. This suggests that changes in hippocampal circuitry

could affect the degree of excitability (Lynd-Balta *et al.*, 1996). Over-expression of glutamate receptors can be directly convulsant in that injection of a virus vector containing a glutamate subunit results in seizure generation (During *et al.*, 1993). Enhanced efficiency of glutamatergic transmission may be responsible for elevated excitability in seizures. Potentiated responses to the application of glutamate agonists occur in slices from kindled rats (Mody *et al.* 1988; Martin *et al.*, 1992). NMDA mediated responses are recorded in the hippocampus after seizures when, under normal circumstances non-NMDA currents are involved. Receptor characteristics such as blockage by $Mg^{2+}$ for NMDA receptors may also be subject to change. Therefore there exist numerous mechanisms whereby the basal level of excitability can be elevated.

## 1.3 The hippocampus

### *1.3.1 Hippocampal anatomy*

The hippocampus is a bilateral curved structure found within the forebrain located beneath the posterior and temporal neocortex (O'Keefe & Nadel, 1978). The term hippocampus is used to designate the hippocampus proper and the *fascia dentata* or dentate gyrus. This has not to be confused with the hippocampal formation which includes not only the hippocampus proper and dentate gyrus but also the surrounding cortical areas. Division of the hippocampus proper into *the regio superior* and *regio inferior* was based on differences in cell morphology and fibre projections (Cajal, 1911; Blackstad, 1956). Lorente de Nó (1934), using the Golgi method of staining, divided the hippocampus proper into four subfields described as *cornu ammonis* (CA) 1-4, thus the hippocampal formation is also known as

Ammon's horn. CA1 corresponds to the *regio superior* and CA2 and CA3 to the *regio inferior*. The CA4 represents the cells at the transition of the hippocampus and dentate gyrus, also known as the hilar region, which in some respects resemble those in the CA3.

CA1 cells are of medium size with a principle apical dendrite which has only small side branches. In contrast the CA3 cells have apical dendrites that divide close to the soma and which contain thorny spines near their origin. According to Lorente de Nó (1934) CA2 cells differed from CA3 cells in that they were not innervated by the mossy fibres of the dentate granule cells since their dendrites lacked thorny spines. However Blackstad (1956) found no distinction between the two cell types with regard to their histology or connectivity. A more recent study using horseradish peroxidase along with a computer aided digitalising system supports the existence of an anatomically distinct CA2 region (Ishizuka *et al.*, 1990). Dentate granule cells differ from the cells of the hippocampus proper in that they have mainly apical with very few basal dendrites.

Although the hippocampus is considered primitive in comparison to the cortex Cajal (1968) divided the hippocampus proper and the dentate gyrus into several layers. The three dentate layers are the granule layer which contains the granule cell bodies, the molecular layer containing the apical dendrites of the granule cells and thirdly the polymorph layer containing the granule cell axons. The hippocampus proper also contains a molecular layer or *stratum moleculare* which is considered to be the outermost region of the hippocampus. This area contains

distal apical dendrites. Next is the *stratum lacunosum* in which bundles of fibres are found of which some are from the CA3 region and also a number of cells of random organisation. The *stratum radiatum* consists mainly of proximal dendrites and their arborizations from the pyramidal cells in the adjacent layer, *stratum pyramidale*. Below this is the *stratum oriens* which contains the basal dendrites of the pyramidal cells. The white coat of the hippocampus is produced by the axons of the pyramidal cells which are grouped to form the *alveus*. An additional layer is described for the CA3 region, *stratum lucidum*, between the pyramidal cell layer and the *stratum radiatum* which receives the mossy fibre input from the dentate granule cells.

### *1.3.2 Hippocampal connectivity*

The major input into the hippocampus is from the entorhinal cortex, a transitional area between the cortex and hippocampus in which pathways from all over the neocortex converge. Three pathways have been proposed: perforant pathway, alvear pathway and the crossed temporo-ammonic tract (Cajal, 1911). Of these the perforant pathway (PP) is considered to be the most prominent. This pathway was initially observed to originate in the lateral entorhinal cortex with fibres terminating in either the hippocampus proper or dentate gyrus (Lorente de Nó, 1934; Raisman *et al.*, 1965). More recent work has shown that the pathway can originate in the medial and lateral entorhinal cortex terminating in the medial third and distal third of the dentate gyrus dendrites respectively. The perforant pathway has been reported to make contact with apical arborizations of pyramidal cell dendrites from all regions of the hippocampus (Blackstad, 1958) however others

have located terminals primarily to CA3 and dentate regions with relatively few in the CA1 (Nafstad, 1967) or to only the dentate gyrus and *regio* inferior with none in the superior region (Hjorth-Simonsen & Jeune, 1972). The perforant path is an excitatory pathway both to cells in the dentate gyrus (Lomo, 1971) and CA3 (Gloor *et al.*, 1963). Input also arises from the medial septum which is generally more diffuse, ending in all fields of the hippocampus.

The axons of the dentate granule cells form the mossy fibres which make contact with mossy cells in the hilus and ipsilateral CA3 cells. In the hilus the mossy fibres branch, with the long branches grouping into two bundles going to the CA3 region. The infrapyramidal bundle travels in the stratum oriens and terminates on the proximal basal dendrites whereas the suprapyramidal bundle forms the lucidum layer in the CA3 and reaches to the extremities of the CA3 area. The mossy fibres have varacisities along their length which can be impaled by the thorny spines of the pyramidal and mossy cells (Blackstad & Kjaerhelm, 1961).

Axons of the CA3 cells divide with one division heading out of the hippocampus towards the fimbria and septum and the other remaining within the hippocampus. This latter branch further divides into three. The Schaffer collaterals run from the CA3 to the CA1 stratum radiatum forming excitatory synapses *en passage* (Lorente de Nó, 1934; Hjorth-simonsen, 1973). In combination the perforant pathway, mossy fibres and Schaffer collaterals make up the basic trisynaptic circuit of the hippocampus.

Out-with this basic system other forms of connectivity exist. Excitatory monosynaptic afferents between cells in the same field were initially thought only to occur in the CA3 region (Lebovitz *et al.*, 1971). These connections are highly organised such that cells in the CA3 subregions are only connected to cells in the same subfield. Indirect inhibition of pyramidal cells occurs via a feedback loop involving interneurones known as basket cells. Pyramidal cells can excite basket cells which in turn inhibit pyramidal cells by forming basket like plexuses around their cell bodies. Connections can also occur between different lamellae of the hippocampus along the longitudinal axis in what is known as the longitudinal association pathway of Lorente de Nó. Fibres also project to the contralateral hippocampus. Evidence exists for homotopic projections from the CA3 to CA3 and non-homotopic inputs to CA1 and dentate gyrus such that the CA1 and DG can receive input from the CA3 on both sides. This takes place in a highly organised fashion.

Axons of the CA1 pyramids go to the subiculum and in turn to the entorhinal cortex and also through the fornix system to lower brain areas.

### *1.3.3 Neurotransmission in the hippocampus*

Glutamate is considered to be the major excitatory transmitter within the CNS. Afferent pathways into the hippocampus as well as intrinsic connections and outputs are all glutamatergic. Both ionotropic and metabotropic subclasses of glutamate receptor exist. Three types of ionotropic receptor: NMDA, AMPA and kainic acid receptors along with numerous subtypes of G-protein coupled

receptors are known. AMPA and kainate receptors are collectively referred to as non-NMDA receptors. Under normal conditions transmission within the hippocampus is carried out by non-NMDA receptors (Collingridge *et al.*, 1982; Collingridge *et al.*, 1983). NMDA receptors may be activated but current flow is inhibited by a voltage-dependent block generated by physiological concentrations of magnesium (Nowak *et al.*, 1984; Coan & Collingridge, 1987).

### *1.3.4 The hippocampus and epilepsy*

The majority of models used in the investigation of the mechanisms responsible for epilepsy are centred around the hippocampus. The focus of attention on one region of the temporal lobe is not purely because the hippocampus, as a structure, is viable as a slice preparation or responds well *in vivo*, but also because the hippocampus plays an important role in the generation and spread of seizure activity within the temporal lobe. Support for this comes from patient studies. Surgery to remove areas of the temporal lobe usually include regions of the hippocampus (Nayel *et al.*, 1991). Electrophysiological recordings from the hippocampus of epileptic patients either *in situ* or after resectioning reflect epileptiform activity (Babb *et al.*, 1987). Many features that are found in common between human epileptic tissue and in experimental models of epilepsy are hippocampal in origin, for example hippocampal sclerosis, mossy fibre sprouting, dendritic changes. One question which arises is what makes the hippocampus so prone to epileptic tendencies?

The cells of the CA3 field can generate paroxysms which propagate to the CA1 region in a number of models independent of the dentate gyrus (Schwartzkroin & Prince, 1978; Lothman *et al.*, 1981). The CA3 region has thus been named the 'epileptic pacemaker' for the hippocampus (Lothman *et al.*, 1981). Intrinsic properties of these cells may underlie this behaviour. The basal level of cell excitability is determined by ionic movement through numerous ligand and voltage gated channels. One such channel is a high threshold calcium channel located in the soma and dendrites (Wong *et al.*, 1979; Fisher *et al.*, 1990). Even under so-called normal conditions CA3 cells can discharge spontaneously producing activity which resembles paroxysmal depolarisation shifts (PDS) (Wong & Prince, 1978). Under these circumstances, this bursting activity lacks synchronisation. Connections between CA3 cells are numerous and excitatory such that excitation in one cell can be communicated to the next and so forth. This excitatory network is under control from inhibitory influences. Other features of the hippocampus which could enhance synchronisation, which is essential for epileptogenesis, are electronic synapses, ephatic interactions mediated through the extracellular space (Dudek *et al.*, 1986) and feedback loops (Buckmaster *et al.*, 1992). Due to this organisation with so much potential for aberrant excitation the hippocampus could be described as an accident waiting to happen.

### *1.3.5 Dormant basket cell hypothesis*

Basket cells are important inhibitory interneurones which are more resistant than hilar mossy cells or pyramidal cells to degeneration resulting from adverse activity. Basket cells receive excitatory input from mossy cells and dentate granule cells and in turn inhibit granule cells. Loss of mossy cells can occur in hippocampal sclerosis resulting in reduced activation of the basket cells and hence a partial loss of inhibition producing a reciprocal increased excitation (Sloviter, 1991).

### *1.3.6 Mossy fibre sprouting*

Synaptic contacts lost due to cell degeneration are proposed to initiate the sprouting of axon collaterals. Initial experiments to substantiate the proposal that mossy fibres innervated granule cells studied paired-pulse stimulation in slices from rats treated with kainate. In this paradigm paired pulse depression should be converted to paired-pulse facilitation if sprouting is present. Trauk & Nadler (1985) found a small but consistent potentiation of responses. An *in vivo* study comparing responses to perforant path stimulation a few days and then months after kainate treatment showed that initially multiple population spikes were evoked, but after 2 months, when sprouting was proposed to have been established, responses were normal (Sloviter, 1992). The basis for this involves the death of mossy cells and inhibitory interneurones (excluding basket cells) and reduced dentate inhibition. Mossy fibre axons sprout collaterals to compensate for this loss, making synapses with basket cells in the inner molecular layer and hence providing a compensatory mechanism against developing hyperexcitability.

Similar results were found by Cronin *et al.* (1992) again using slices from kainate treated rats. However, when a medium containing bicuculline was used, both stimulation-induced and spontaneous bursts of action potentials were produced which suggested that recurrent excitatory collaterals had innervated granule cells during sprouting which were previously masked.

The use of human tissue exhibiting mossy fibre sprouting supports the occurrence of a new excitatory circuit (Houser *et al.*, 1990; Pokorny *et al.*, 1991; Masukawa *et al.*, 1992; Isokawa & Fried, 1996). More recent studies using similar experimental techniques have shown that during reduced inhibition, spontaneous seizure-like bursts with tonic-clonic components can result (Wuarin & Dudek, 1996).

Mossy fibre sprouting in response to kindling is dependent on NMDA receptor activation, in that MK801 can prevent it (Sutula *et al.*, 1996). Histological approaches, using mainly Timms staining, have been used to corroborate the existence of mossy fibre sprouting although no direct morphological evidence of functional collateral synapses is yet available. Sprouting of fibres after kainate treatment to form recurrent excitatory circuits in the CA1 has also recently been reported (Meier & Dudek, 1996; Perez *et al.*, 1996).

### *1.3.7 Hippocampal sclerosis*

Hippocampal or Ammon's horn sclerosis is a prominent feature occurring in brains of patients with temporal lobe epilepsy and is characterised by neuronal cell death and gliosis particularly within the hilus and CA1 and CA3 subfields (Babb & Brown, 1987; Gloor, 1991). A great debate was started, which still continues to date, when Sommer (1880) proposed that hippocampal sclerosis was a cause of epilepsy. Temporal lobectomy, in which sclerotic regions of the hippocampus are removed, results in a dramatic improvement in epileptic patients thus supporting the view that the sclerotic hippocampus is causing the seizures. Slices of sclerotic hippocampi when stimulated *in vitro* contain reduced GABAergic potentials (Knowles *et al.*, 1992) and sprouting of excitatory axons (Bekenstein & Lothman, 1993) both of which would cause enhanced excitation. Cell loss in hippocampal slice cultures in response to convulsants has been attributed to the activity *per se* and not the convulsants due to the inclusion of TTX to inhibit action potential generation (Thompson, 1993).

However, evidence for hippocampal sclerosis being a consequence of seizure activity also exists. For example correlation exists between seizure intensity and the extent of neuronal damage (Mouritzen-Dam, 1980; DeGiorgio *et al.*, 1992) with status-epilepticus producing extensive damage to the hippocampus and surrounding cortical areas (Hauser, 1983). Hippocampal lesioning is also not found in newly diagnosed epileptic patients (Saukkonen, 1994). Hence the question of whether hippocampal sclerosis is a cause or consequence remains unanswered. The possibility exists that some insult in childhood, for example

febrile seizures, could affect vulnerable areas which in later life give rise to temporal lobe epilepsy.

### *1.3.8 Hippocampal Slices*

Since it was shown that slices of brain tissue could be maintained *in vitro* (McIlwain *et al.*, 1951) and also respond in an expected manner to electrical stimulation, slices have been extensively used as *in vitro* models of CNS activity (Yamamoto & McIlwain, 1966). The hippocampus consists of parallel lamellae organised perpendicular to the longitudinal axis (Andersen *et al.*, 1971) with each section containing all the functional areas described previously. This apparent two-dimensional structure favours the use of the hippocampus as a slice preparation since each individual slice has the ability to act as a single unit, with slices not being substantially different from each other. *In vitro/in vivo* studies comparing the effects of electrical stimulation have shown that the fundamental properties of the hippocampus such as inhibitory mechanisms, postactivation facilitation, frequency potentiation and post-tetanic potentiation are preserved *in vitro* (Skrede & Westgaard, 1971).

The slice preparation has a number of advantages:

1. the distinct pathways of the hippocampus are found intact within the slice and can be selectively activated (Skrede & Westgaard, 1971).
2. the different subfields can be visualised under low magnification allowing accurate positioning of electrodes.
3. the ionic environment can be tightly controlled allowing ease of manipulation.

4. drugs can be applied without the restriction of the blood-brain barrier to contend with and also are not subject to systemic metabolism.
5. Stable potentials can be recorded over several hours.

However, as with most preparations, disadvantages are also apparent:

1. the trauma of dissection. In general the slice preparation contains a region of intact tissue surrounded by two layers of damaged tissue (Bak *et al.*, 1980) and hence the metabolism of the slice may be altered from *in situ* (Reid *et al.*, 1988).
2. the ionic environment does not mimic exactly normal extracellular conditions *in vivo* and could influence responses.
3. although the hippocampus can be considered as a two-dimensional structure longitudinal and contralateral pathways do exist which are severed during slicing thus altering both excitatory and inhibitory inputs.
4. noradrenergic, cholinergic and serotonergic afferents are lost in the slicing procedure.
5. variability between different areas in the one subfield can occur (Grinvald & Segal, 1984) although this could also apply *in vivo*.

Despite these points hippocampal slices are widely regarded as an important *in vitro* preparation.

## 1.4 *In vitro* epileptiform models

*In vitro* models of epileptiform activity have been used extensively over the last few decades to investigate the genesis of epilepsy. These models generally involve slices of brain tissue, for example hippocampus (Voskuyl & Albus, 1985; Watts & Jefferies, 1993; Morris *et al.*, 1996), neocortex (Mattia *et al.*, 1993) and amygdala (Arvanov *et al.*, 1995). These preparations can be manipulated by either changing the medium used for perfusion or by the direct application of compounds to the slice. Epileptiform activity is generated by raising the basal level of excitation by the use of excitatory compounds or procedures, compounds which reduce inhibition or by altering the ion content of the perfusing medium. Epileptiform activity can be illustrated as spontaneous bursts of population spikes or as multiple wave forms in response to stimulation. Epileptiform activity *in vitro* consisting of short periods of defined activity are referred to as interictal due to the resemblance to this activity *in vivo*. Similarly periods of intense activity lasting seconds is denoted ictal activity. Although caution must be exercised when making direct comparisons between situations *in vitro* and *in vivo*, *in vitro* models have been beneficial in understanding the fundamental mechanisms which may underlie the generation of human epilepsy.

### *1.4.1 4-aminopyridine induced epileptiform activity*

4-aminopyridine (4AP) can induce epileptiform activity *in vitro* (Chesnut & Swann, 1988; Watts & Jefferies, 1993; Arvanov *et al.*, 1995; Traub *et al.*, 1995; Avoli *et al.*, 1996a) and *in vivo* (Szenet & Pongracz, 1979; Morales-Villagrán *et al.*, 1996) as well as producing clinical seizures in man (Spyker *et al.*, 1980; Thesleff, 1980). The principal action of 4AP is the blockade of neuronal potassium currents including the transient A and slowly-inactivating 'delay' (D) currents. The A current has been recorded in hippocampal neurones *in situ* (Gustefsson *et al.*, 1982) and in culture (Segal & Barker, 1984; Segal *et al.*, 1984) and this rapidly activating and deactivating current is involved in controlling excitability via a role in spike repolarisation. Concentrations of 4AP greater than 100μM will block this current (Storm, 1988). The D current has a rapid activation but a slow inactivation, which introduces a long delay in firing induced by just threshold depolarisations (Storm, 1988). The D current is more sensitive to 4AP with complete blockage occurring between 30-40μM (Storm, 1988). Other potassium currents are active in hippocampal neurones but are not affected by 4AP. An enhanced release of neurotransmitters can also be stimulated by 4AP (Thesleff, 1980) which is thought to occur secondary to a prolongation of action potential duration allowing a greater influx of calcium into the presynaptic membrane (Molgo *et al.*, 1977; Flores-Herández *et al.*, 1994). A direct effect on voltage-gated calcium channels could also be an effect of 4AP (Lundh & Thesleff, 1977; Rogawski & Barker, 1983; Segal & Barker, 1986).

Three types of burst characteristics have been documented to result from the use of 4AP. The most prevalent is the generation of frequently occurring bursts of short duration, namely interictal activity (Watts & Jefferies 1993; Mattia *et al.*, 1994; Avoli *et al.*, 1996a; Morris *et al.*, 1996). Less frequent negative potentials which have been proposed to be GABAergic have been found in some instances (Michelson & Wong, 1991; Perreault & Avoli, 1991; Perreault & Avoli, 1992; Mattia *et al.*, 1994). Prolonged seizure-like bursts, ictal activity, lasting up to tens of seconds can also occur (Gean *et al.*, 1990; Mattia *et al.*, 1993; Watts & Jefferies, 1993; Arvanov *et al.*, 1995; Bianchi & Wong, 1995; Avoli *et al.*, 1996a; Morris *et al.*, 1996).

The involvement of glutamatergic transmission in 4AP-induced epileptiform activity has been investigated using selective NMDA and non-NMDA receptor antagonists. In experiments using slices from rats, epileptiform activity of interictal and ictal characteristics induced by 4AP is suppressed by non-NMDA antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6,7-dinitroquinoxaline-2,3-dione (DNQX) (Gean *et al.*,1990; Perreault & Avoli, 1991; Avoli *et al.*, 1996a). NMDA receptor antagonists generally have no or very little effect on 4AP induced bursts (Gean *et al.*, 1990; Perraeult & Avoli, 1992; Avoli *et al.*, 1993). However, exceptions have been reported. Ictal events in the entorhinal cortex were abolished by 3-(±-2-carboxypiperazine-4-yl)propyl-1-phosphonic acid (CPP), an NMDA receptor antagonist (Avoli *et al.*, 1996a). Inter-species differences in the susceptibility to NMDA receptor antagonists may prevail as ictal bursts in guinea-pig neocortex slices were inhibited by CPP and not affected by

not affected by CNQX, a non-NMDA receptor antagonist, whereas the opposite was found with slices of rat origin (Mattia *et al.*, 1993). The disappearance of 4AP bursts in low or zero calcium media suggests a dependence on synaptic activity for generation (Voskuyl & Albus, 1985; Gean *et al.*, 1990). α-methyl-4-carboxyphenylglycine ((+)MCPG), a metabotropic glutamate receptor (mGluR) antagonist, blocked the induction of ictal but not interictal activity in slices of rat amydgala induced by 4AP, but was ineffective on established ictal activity thus suggesting that mGlu receptors may be involved in the transition between normal and epileptic activity (Arvanov *et al.*, 1995).

To a large extent models of epileptiform activity, especially early ones, were based on a reduction in inhibition. One of the unusual features of 4AP induced epileptiform activity is that both excitatory and inhibitory synaptic transmission is enhanced (Thesleff, 1980; Kita *et al.*, 1985; Rutecki *et al.*, 1987; Perreault & Avoli, 1991). This can be shown by enhanced IPSP's recorded in response to Schaffer collateral stimulation.

### *1.4.2 Zero magnesium induced epileptiform activity*

Reduced levels of magnesium have been associated with symptoms of clinical epilepsy (Duralach, 1967). Conversely, magnesium sulphate is used to treat seizures associated with eclampsia (Pritchard *et al.*, 1984). Slice preparations of various central nervous system tissues generate epileptiform activity when perfused with a medium devoid of added magnesium: hippocampus (Mody *et al.*, 1987; Scheiderman & MacDonlad, 1987; Whittington *et al.*, 1995), entorhinal cortex (Stanton *et al.*, 1987; Jones, 1989; Behr & Heinemann, 1996), neocortex (Horne *et al.*, 1986; El-Beheiry & Puil, 1990) and amydgala (Gean & Shinnick-Gallagher, 1988). Three mechanisms, which are not necessarily involved to an equal extent, have been proposed to underlie burst generation. Magnesium and calcium, as divalent cations, are involved in membrane charge screening which acts to stabilise membrane currents. The removal of magnesium, therefore, reduces membrane screening, facilitating the production of inward ionic currents and action potentials (Frankenhaeuser & Hodgkin, 1957; McLaughlin *et al.*, 1971). Mody *et al.* (1987) showed that the contribution of reduced charge screening to $OMg^{2+}$ activity was quite small and hence not the sole mechanism involved.

Magnesium and calcium are mutual antagonists with magnesium blocking calcium entry into pre- and postsynaptic membranes (Katz & Miledi, 1969; Czèh & Somjen, 1989). Relief of this action of magnesium facilitates neurotransmitter release and enhanced excitability of the postsynaptic neurone, allowing recruitment of a sufficient number of cells for a population burst to occur (Mody

*et al.*, 1987). A leftward shift of input-output curves, lower EPSP threshold and larger stimulus-induced calcium signals in zero magnesium medium reflect the involvement of this mechanism in zero magnesium bursts (Mody *et al.*, 1987).

The participation of NMDA receptors in neurotransmission is regulated by a number of modulators which act both positively e.g. glycine or negatively e.g. magnesium. At normal physiological concentrations magnesium exerts a voltage-dependent block on NMDA receptors by binding to a site which is thought to be located within the ion channel (Mayer *et al.*, 1984; Nowak *et al.*, 1984; Ascher & Nowak, 1988). Relief of this block can ensue during periods of depolarisation, allowing the movement of $Na^+$, $K^+$ and $Ca^{2+}$ through the receptor channel. The activation of NMDA receptors following removal of magnesium from the bathing medium is considered to be the principal mechanism involved in the generation of epileptiform activity in the zero magnesium model. Evidence comes from the total or partial reduction of epileptiform activity in the presence of selective NMDA receptor antagonists (Horne *et al.* 1986; Mody *et al.*, 1987; Schneiderman & MacDonald, 1987; Neuman *et al.*, 1988; Tancredi *et al.*, 1990; Psarropoulou & Kostopoulos, 1991). The fact that, in some instances, epileptiform activity is still evident during perfusion with NMDA receptor antagonists, suggests that the other two mechanisms also play an important role.

In general the epileptiform activity instigated by zero magnesium medium is interictal, characterised by burst of milliseconds as opposed to seconds in duration (Mody *et al.*, 1987; Tancredi *et al.*, 1990; Drier & Heinemann, 1991; Whittington

*et al.*, 1995). Anderson *et al.* (1986) recorded ictal events with corresponding tonic and clonic periods of activity in hippocampal slices using a zero magnesium medium. These events were preceded by periods of interictal activity. These periods of ictal activity did not continue throughout the total period of zero magnesium perfusion. The addition of baclofen, a $GABA_B$ receptor agonist, prolonged the appearance of ictal activity (Swartzwelder *et al.*, 1987; Swartzwelder *et al.*, 1988; Lewis *et al.*, 1989; Andersen *et al.*, 1990). Ictal events are promoted under zero magnesium conditions in slices incorporating the entorhinal cortex and hippocampal regions (Jones, 1989; Drier & Heinemann, 1991; Velíšek *et al.*, 1994; Behr & Heinemann, 1996).

GABAergic inhibition is still evident during zero magnesium epileptiform activity due to the maintained ability to evoke inhibitory postsynaptic potentials (IPSP's) (Tancredi *et al.*, 1990) and thus may act to limit the extent of firing. In other studies inhibition has been found to be reduced in comparison to normal circumstances (El-Beheiry & Puil, 1990; Jefferies *et al.*, 1994). Phosphorylation of a site on the $GABA_A$ receptor is required for normal functioning. Use of a medium devoid of magnesium can result in decreased MgATP levels which could reduce the extent of phosphorylation. Thus erosion of inhibition is a proposed mechanism for the progression of epileptic activity (Whittington *et al.*, 1995).

### *1.4.3 Elevated potassium induced epileptiform activity*

The extracellular concentration of potassium rises during seizures induced *in vivo* in cats (Moody *et al.*, 1974; Fisher *et al.*, 1976) and *in vitro* hippocampal slices (Yaari *et al.*, 1986). Basal potassium levels are approximately 3mM and can rise to around 12mM. This change in potassium concentration was simulated by raising the concentration of potassium in the medium bathing slices to between 5 and 10mM. This resulted in the generation of epileptiform activity *in vitro* (Stringer & Lothman, 1988; Traynelis & Dingledine, 1988; Leschinger *et al.*, 1993; McBain, 1994).

Interictal activity in the CA3 region of the hippocampus occurs when slices are bathed in a high potassium medium (Rutecki *et al.*, 1985; Korn *et al.*, 1987; Traynelis & Dingeldine, 1988). This activity propagates to the CA1 where intense seizure-like activity results (Traynelis & Dingeldine, 1988; Leschinger *et al.*, 1993). This ictal activity is focal in nature in that it does not spread to incorporate the CA3. The disconnection of the CA1 from the CA3 region prevents the induction of seizure activity in the CA1 illustrating that CA3 interictal activity is required. Activity can be restored in the CA1 by stimulation mimicking CA3 interictal activity (Traynelis & Dingeldine, 1988).

Several contributing factors have been postulated to produce a positive feedback loop resulting in sustained reverberating activity. This model suggests that the initial consequences of elevated potassium results in an increased number and synchrony of CA1 pyramidal cells. Each ictal burst further increases the

potassium released into the extracellular space causing a regenerative cycle. Increased potassium levels, paralleled with a decreased potassium driving force, cause

1. a decrease in the amplitude of a potassium mediated after hyperpolarising potential (AHP) which normally acts to limit repetitive firing.
2. a decrease in the amplitude of GABAergic inhibitory postsynaptic potentials (IPSP) (Korn *et al.*, 1987; McBain, 1993).
3. depolarisation of the pyramidal cells (Alger *et al.*, 1983). CA1 cells and glia but not CA3 cells are depolarised immediately prior to an ictal discharge (Dichter *et al.*, 1972; Traynelis & Dingeldine, 1988). Depolarisation of glia has also been reported to occur during interictal activity (Dichter *et al.*, 1972).
4. reduction of spike threshold.
5. cell swelling. Increased extracellular potassium reduces potassium efflux as a result of a modified concentration gradient (Dietzel *et al.*, 1980) and increases glial uptake (Kimelberg *et al.*, 1982) thus reducing the size of the extracellular space and increasing ephaptic interactions between pyramidal cells (Taylor & Dudek, 1984).

It is postulated that seizures develop when the CA1 region can no longer clear the excess potassium from the interstitial space before the next interictal burst (Dichter *et al.*, 1972; Traynelis & Dingeldine, 1988).

The depolarisation that precedes seizure discharges could potentially remove the voltage-dependent block of NMDA receptors by $Mg^{2+}$ (Dingledine *et al.*, 1990). Selective NMDA-receptor antagonists totally (Traynelis & Dingledine, 1988) or

partially blocked CA1 electrographic seizures (Leschinger *et al.*, 1993) but not CA3 interictal bursts (Traynelis & Dingledine, 1988). The combination of NMDA and non-NMDA antagonists abolished all epileptiform activity (Leschinger *et al.*, 1993); synaptic interactions seem to play a role seizure activity. Metabotropic glutamate receptors may also be involved in the maintenance of CA3 interictal activity (McBain, 1994).

During normal circumstances the spread of excitation is controlled by a high level of spontaneous IPSP's . When potassium concentration is elevated synchronised GABAergic inhibitory activity is reduced in a time dependent manner as mentioned above. The mechanism under-lying activity dependent changes in IPSP are not clear but may be due to an increase in the intracellular chloride concentration and subsequent reduction in driving force for IPSP formation (Hugenard & Alger, 1986; Thompson & Gähwiler, 1989a; Thompson & Gähwiler, 1989b).

The transition from interictal to ictal activity has been postulated to revolve around an increase in potassium concentration although other contributing factors are required (Dichter *et al.*, 1972).

### *1.4.4 Low calcium induced epileptiform activity*

Epileptiform activity is accompanied by a decrease in the concentration of extracellular calcium (Heinemann *et al.*, 1986). Synaptic transmission is thought to be involved in developing the degree of synchrony required for the spread of epileptiform activity. However in circumstances of reduced or zero calcium, epileptiform activity of an ictal nature can be generated in hippocampal slices (Jefferys & Haas, 1982; Yaari *et al.*, 1983; Konnerth *et al.*, 1986; Heinemann *et al.*, 1992; Watson & Andrew, 1995). In some studies increased magnesium or EDTA accompanied calcium changes in the medium. Activity of this nature was generated in the CA1 region with no involvement of the CA3 or other hippocampal regions (Jefferys & Haas, 1982; Yaari *et al.*, 1983; Konnerth *et al.*, 1986). As with the zero magnesium model a reduction in the concentration of divalent cations reduces membrane charge screening, thus facilitating membrane depolarisation (Frankenhaeuser & Hodgkin, 1957; McLaughlin *et al.*, 1971). No apparent change in action potential threshold during low calcium perfusion occurred, suggesting that reduced charge screening is not the primary mechanism involved (Jefferies & Haas, 1982). A reduction in calcium dependent potassium currents which are responsible for after-hypolarisation (Jefferies & Haas, 1982) and a reduced synaptic GABAergic inhibition by spontaneously active interneurones (Jones & Heinemann, 1987) also contribute to zero/low calcium epileptogenesis.

The above factors contribute to increased hippocampal excitability but the generation of seizure like events requires sustained regenerating activity. Rises in

extracellular concentration of potassium accompany low/zero calcium epileptiform discharges (Jefferies & Haas, 1982; Yaari *et al.*, 1983; Yaari *et al.*, 1986; Heinemann *et al.*, 1992). Thus spread occurs by spatial dispersion of potassium via diffusion and spatial glial buffering (Gardner-Medwin, 1983; Somjen, 1984). The potassium induced depolarisation and sustained extracellular currents excite adjacent neuronal aggregates. This process is repeated laterally causing spread of the paroxysm (Yaari *et al.*, 1986). Ephaptic interactions are another possible means of seizure spread (Yaari & Konnerth, 1986; Heinemann *et al.*, 1992). CA1 cells are closely packed and this, when combined with a reduced extracellular space during seizures, can enhance field effects between neurones. Extracellular electric fields produced by active CA1 cells cause currents to flow passively across adjacent inactive cell membranes, depolarising their somata. This synchronises the firing in the two cells and so on (Yaari & Konnerth, 1986). Electronic coupling between gap junctions may also contribute to synchronising interneuronal activity (Valliante *et al.*, 1995). Thus non-synaptic mechanisms, in addition to synaptic ones, may be of importance in the synchronisation of epileptiform activity.

### *1.4.5 Epileptiform activity induced by GABA receptor antagonists*

Several *in vitro* models have produced epileptiform activity by using various GABA receptor antagonists, for example picrotoxin (Lee & Halbitz, 1989; Knowles *et al.*, 1987; Kohr & Heinemann, 1990) and bicuculline (Herron *et al.*, 1985; Burke & Hablitz, 1994; Colom & Saggau, 1994; Ameri *et al.*, 1997). In respect to the action of these compounds epileptiform activity results primarily from reduced inhibition.

### *1.4.6 Other models*

Numerous other compounds, in addition to the ones already mentioned, have been used both *in vivo* and *in vitro* to generate epileptiform activity:

- kainic acid (Westbrook & Lothman, 1983; Stringer & Sowell, 1994)
- tetanus toxin (Whittington & Jefferies, 1994)
- penicillin (Wong & Prince, 1979; Gjerstad *et al.*, 1981; Traub & Wong, 1981; Lathers *et al.*, 1993).
- pentylenetetrazol (Mirski *et al.*, 1994; Stringer, 1994; Stringer & Sowell, 1994)
- pilocarpine (Nagao *et al.*, 1996)

### *1.4.7 Stimulus trains*

Trains of electrical stimuli represent a non-pharmacological, non-ionic manipulation which results in epileptogenesis (Stasheff *et al.*, 1985; Anderson *et al.*, 1987).

## 1.5 Adenosine triphosphate

Intracellular adenosine triphosphate (ATP) is fundamentally involved in energy metabolism, enzyme regulation and nucleic acid synthesis, but, ATP also participates in numerous extracellular activities. Drury & Szent-Györgyi (1929) reported that adenine compounds were active on preparations of mammalian heart causing sinus bradycardia, complete atrioventricular block, a negative ionotropic effect on the atrium and cessation of atrial fibrillation. Further reports followed regarding the actions of ATP and adenosine on the cardiovascular system (Gillespie, 1933; Green & Stoner, 1950).

The presence of ATP in the perfusate of rabbit ear artery after nerve stimulation (Holton, 1959) first suggested that ATP may act as a neurotransmitter. Autonomic nerves innervating the gastro-intestinal tract were found to be neither adrenergic nor cholinergic (Burnstock *et al.*, 1963; Martinson & Muren, 1963). Similar components of the autonomic nervous system also supplied other viscera including the urinary bladder and vas deferens. Burnstock *et al.* (1970) proposed that the substance released in response to autonomic nerve stimulation was ATP and termed the nerves involved purinergic (Burnstock, 1972). Since this discovery the role of ATP as an extracellular mediator and neurotransmitter has flourished with substantial evidence to support the original concept.

### *1.5.1 Source of ATP*

ATP is a ubiquitous substance and as a result any cell can potentially act as a source of extracellular ATP (Gordon, 1986). The concentration of ATP within the cytosol may be up to 10mM (Dubyak & El-Motassim, 1993; Sperlagh & Vizzi, 1996) although a large proportion of cytosolic ATP is bound and thus is not available for neural function. It is uncertain whether ATP can be stored and released from secretory vesicles in its own right or directly from the cytosol. ATP can be found co-packaged in vesicles along with acetylcholine (Dowdall *et al.*, 1974; Volknandt & Zimmerman, 1986) and noradrenaline (Winkler, 1977). ATP is located in numerous brain regions including hippocampal synaptosomes (Terrian *et al.*, 1989) and presynaptic terminals (Wieraszko *et al.*, 1989), neostriatal neurones (Zhang *et al.*, 1988), sensory motor cortex (Wu & Phillis, 1978) and caudate nucleus cholinergic nerve terminals (Richardson & Brown, 1987).

### *1.5.2 Release of ATP*

Substantial evidence exists regarding the release of ATP in response to depolarising stimuli in various peripheral preparations. Included in this are preparations innervated by sympathetic nerves e.g. rat vas deferens (Vizi & Burnstock, 1988; Kurz *et al.*, 1994), rat tail artery (Westfall *et al.*, 1987; Bao & Stjärne, 1993), cultured sympathetic neurones (von Kügelgen *et al.*, 1994) and cholinergic nerves e.g. ileum longitudinal muscle (Nitahara *et al.*, 1995), taenia coli (Su *et al.*, 1971). ATP can be released not only from neuronal cells but also

non-neuronal cells such as endothelial or smooth muscle cells (Vizi *et al.*, 1992; Sperlágh & Vizi, 1996).

ATP release has been demonstrated in response to chemical stimulation in brain synaptosomal preparations. White (1978) detected an increase in luminescence, which correlated to ATP release using the firefly luciferin/luciferase assay, after adding a high $K^+$ solution to hypothalamic synaptosomes. High frequency electrical stimulation of hippocampal slices resulted in ATP release (Wieraszko *et al.*, 1989; Wieraszko & Seyfried, 1989; Cunha *et al.*, 1996). This release was not apparent in low calcium medium, showing calcium dependence, and was not modulated by kynurenic acid, a glutamate receptor antagonist, or glutamate application suggestive of a presynaptic origin of release (Wieraszko *et al.*, 1989). Tetrodotoxin (TTX) virtually abolishes the electrically evoked release of ATP which is also suggestive of nerve terminal origin (White & MacDonald, 1990). Low frequency stimulation produced no (Wieraszko *et al.*, 1989) or only a small release of ATP (Pedata *et al.*, 1993). Similar release has been reported in the habenula (Barajas-López *et al.*, 1995). The release of ATP through classical exocytosis has been disputed by Hamann & Attwell (1996) who propose that ATP release in response to electrical stimulation of slices of hippocampus, cerebellum and habenula is through electroporation, the transient production of holes in the cell membrane in response to electrical activity. The release of ATP with acetylcholine has also been demonstrated in nerve terminals derived from the caudate nucleus (Richarson & Brown, 1987).

### *1.5.3 ATP receptors*

One of the criteria that are used for defining a compound as a neurotransmitter (Eccles, 1964) is the requirement for receptor mediated events. Subsequent to the proposal of purinergic transmission, Burnstock (1978) clarified the mass of often conflicting data regarding the action of nucleotides and nucleosides by subdividing purinergic receptors into two subclasses according to four main criteria:

1. rank order of potency of nucleotides and nucleosides
2. action of methylxanthines
3. transduction mechanism, with particular regard to adenylate cyclase
4. effect on prostaglandins

Thus $P_1$ and $P_2$ purinoceptors were defined as having the following characteristics:

*$P_1$ purinoceptors* have an agonist potency order of adenosine > AMP > ADP > ATP, are antagonised by methylxanthines and receptor activation results in changes in intracellular cAMP levels through modulation of adenylate cyclase activity.

*$P_2$ purinoceptors* have an agonist potency order of ATP > ADP > AMP > adenosine, methylxanthines are not antagonists and receptor activation does not change intracellular cAMP level but on occasions prostaglandin synthesis is stimulated. This classification has been verified extensively and in general still holds weight.

The arrival of agonists and antagonists proposed to be selective for $P_2$ receptors for example 2-methylthio-adenosine triphosphate (2meSATP) and

α, β-methylene-adenosine triphosphate (α, β-meATP), receptor agonists, and arylazidoaminopropionyl-ATP ($ANAPP_3$), a receptor antagonist, increased the understanding of $P_2$ receptors while at the same time revealing differing resultant effects depending on which agonists were used. Thus Burnstock & Kennedy (1985) suggested that the $P_2$ receptor subclass was not homogeneous and proposed a further division according to agonist potency relative to ATP, the effect of $ANAPP_3$ and receptor desensitisation on prolonged exposure to α, β-meATP. The resulting subtypes of $P_2$ receptors were:

*$P_{2x}$ receptors*: have an agonist potency order of α, β-meATP, β, γ-meATP > ATP = 2meSATP, are antagonised by $ANAPP_3$, are selectively desensitised by α, β-meATP and in general mediate excitation.

*$P_{2y}$ receptors*: have an agonist potency order of 2meSATP >> ATP > α, β-meATP, β, γ-meATP, are weakly antagonised by $ANAPP_3$ and mediate relaxation.

Two further subtypes were proposed by Gordon (1986), the $P_{2T}$ and the $P_{2z}$ based on the actions of ATP on platelets, lymphocytes and mast cells.

*$P_{2T}$ receptors* are found mainly on platelets, are activated by ADP causing platelet aggregation and antagonised by ATP.

*$P_{2Z}$* receptors are found within the immune system on lymphocytes and mast cells, are activated by the tetrabasic form of ATP, $ATP^{4-}$ and antagonised by oxidised ATP.

The receptor classification has been forced to expand further due to the use of the pyrimidine uridine triphosphate (UTP) whose interaction did not fit into any of the afore-mentioned categories.

$P_{2U}$ receptor has a rank order of potency of UTP >=ATP >> 2meSATP.

α, ω-adenine dinucleotides or diadenosine polyphosphates ($Ap_nA$) are a group of naturally occurring compounds which have been implicated in extracellular communication (Baxi & Vishwanatha, 1995). Much debate exists as to whether dinucleotides activate just $P_{2x}$ and/or $P_{2y}$ receptors or whether a distinct dinucleotide receptor exists. Displacement studies using [$^3$H]$Ap_4A$ and competition studies using [$^{35}$S]ADP-β-S in rat brain synaptic terminals led to the proposal of the existence of a receptor distinct from previously defined $P_2$ receptors and designated $P_{2d}$ (Pintor *et al.*, 1993a).

### *1.5.4 Ionotropic and metabotropic ATP receptors*

Nucleotide receptors, based on structure and mechanism, can be divided into two classes, ligand-gated intrinsic ion channel receptors and G-protein coupled receptors.

Ion channels directly activated by extracellular ATP were first described in rat and cat sensory neurones (Krishtal *et al.*, 1983) and dorsal horn neurones (Jahr & Jessel, 1983). Such receptors have now been found extensively both centrally and peripherally. The channel properties can differ slightly between cell types with regard to ion permeability, desensitisation and size. The ion channel, in general, is

a non-selective cationic channel permeable to sodium, potassium and calcium (Benham & Tsien, 1987; Bean, 1992). In a few instances ATP-activated ion channels were found to be permeable to anions in addition to cations (Thomas & Hume, 1990; Balachadran & Bennett, 1996). The responses produced by P2X receptor activation are characterised by being rapid: currents can turn on within milliseconds (Bean, 1992). They are dependent on the ion composition of the surrounding medium (Thomas & Hume, 1990) and are unaffected by G-protein inhibitors (Bolego *et al.*, 1995), all which support the hypothesis of the involvement of an integral ion channel receptor.

Upon cloning of the P2X receptor, it was found to have a sequence which predicts an unusual subunit structure (Brake *et al.*, 1994). The subunit contains two transmembrane domains which are joined by a long extracellular loop with both the C- and N- termini located intracellularly. Thus P2X receptors define a new family of ligand-gated ion channels (Valera *et al.*, 1994).

P2Y receptors are coupled to G-proteins and therefore show slower responses to ATP when compared to P2X receptors (Boyer *et al.*, 1989). A number of transduction pathways are subsequently activated following ATP occupation of P2Y receptors: phospholipase C activation results in the production of inositol triphosphate ($IP_3$) and diacylgylcerol (DAG) (Webb *et al.*, 1993; Parr *et al.*, 1994; Communi *et al.*, 1995; Chang *et al.*, 1995), adenylate cyclase can be modulated (Boyer *et al.*, 1993; Boyer *et al.*, 1995; Berti-Mattera *et al.*, 1996) and phospholipase $A_2$ can also be activated (Firestein *et al.*, 1996; Stella *et al.*, 1997).

Cloned P2Y receptors have a predicted structure of seven transmembrane domains, sites for N-glycosylation near the N-terminal and conserved cysteine residues in the first two extracellular loops which are characteristic features of G-protein-coupled receptors.

The classification system mentioned previously has been regarded as too complicated with not enough scope for further expansion. A different system based initially on transduction mechanism followed by agonist and antagonist potency order has been proposed (Abbracchio & Burnstock, 1994). In this method P2X receptors are ligand gated and P2Y receptors are G-protein coupled with numbers used to designate further subtypes in each class. To date seven P2X receptors ($P2X_1$ - $P2X_7$) and eight P2Y receptors have been discovered. This system has recently been reviewed and been essentially accepted as the new nomenclature for P2 receptors, no longer purinoceptors due to the activity of pyrimidines (Fredholm *et al.*, 1997).

### *1.5.5 Cloning and distribution of $P_2$ receptors*

#### *1.5.5.1 P2Y purinoceptors*

The first $P_2$ purinoceptor cloned was the $P2Y_1$ receptor from chick brain (Webb *et al.*, 1993). When expressed in Xenopus oocytes the receptor displayed an agonist potency order similar to the initial order proposed for the P2Y receptor. $P2Y_1$ receptor homologues have also been cloned from turkey (Filtz *et al.*, 1994), bovine (Henderson *et al.*, 1995), rat (Tokuyama *et al.*, 1995), mouse (Tokuyama *et*

*al.*, 1995) and human (Schachter *et al.*, 1996; Ayyanathan *et al.*, 1996; Janssens *et al.*, 1996).

The mouse (Lustig *et al.*, 1993) and human (Parr *et al.*, 1994) $P2Y_2$ or $P_{2U}$ receptors have been cloned and are activated equally by ATP and UTP but not by their dinucleotide counterparts.

A third P2Y receptor, namely $P2Y_3$, was cloned from chick brain and expressed in Xenopus oocytes and human Jurkat cells (Webb *et al.*, 1996a). This receptor, which has a high affinity for both ADP and UDP.

The cloned human $P2Y_4$ receptor (Communi *et al.*, 1995; Nguyen *et al.*, 1995) could be considered a pyrimidine receptor due to its potency order of UTP>UDP=deoxyUTP> 5-bromo-UTP (Communi *et al.*, 1996a). The coupling of the receptor to phospholipase C may involve two classes of G-protein. ATP has been proposed to act as an antagonist (Nguyen *et al.*, 1995), partial agonist (Communi *et al.*, 1995) and full agonist (Nicholas *et al.*, 1996).

Webb *et al.* (1996b) identified an orphan G-protein receptor cDNA, 6H1, as a member of the P2Y receptor family with characteristics differing from those mentioned above with regard to potency order. This receptor is not activated by UTP and has a 10-fold lower affinity for 2meSATP. Due to the low sequence homology of this receptor to others in the P2Y family this receptor has been classed as $p2y_5$ (Fredholm *et al.*, 1997)

Cloned rat $P2Y_6$ receptor (Chang *et al.*, 1995) displayed a novel agonist sequence. Expression of the receptor in 1321N1 cells found it to activate phospholipase C, with UDP and 5-bromo-UDP being most potent. ATP was inactive (Nicholas *et al.*, 1996). Similar characteristics were described for the human homologue which was detected in spleen, placenta, thymus, intestine and blood leukocytes (Communi *et al.*, 1996b).

The seventh P2Y receptor has been cloned form human erythroleukaemic cells and has a potency order of ATP>2meSATP>β, γ-meATP>ADP=UTP (Akbar *et al.*, 1996). In agreement with the other P2Y receptors, $P2Y_7$ is coupled via G-proteins to PLC and thus acts through $IP_3$ and calcium.

*1.5.5.2 P2X purinoceptors*

Radioligand binding (Michel & Humphrey, 1993) and autoradiography studies (Balcar *et al.*, 1995) using [$^3$H] α, β-meATP have been used to detect areas of binding. These methods localised high areas of binding to several brain regions. The existence of P2X receptors was further supported by the cloning of P2X receptors from both the vas deferens (Valera *et al.*, 1994) and PC12 cells (Brake *et al.*, 1994). To date seven subtypes of P2X receptor have now been cloned, all with distinctive characteristics regarding desensitisation and agonist/antagonist potency orders.

$P2X_1$ receptors were cloned using cDNA libraries from rat vas deferens (Valera *et al.*, 1994). Northern blotting detected expression of this receptor in the vas deferens, urinary bladder, lung, spleen, spinal cord and coeliac ganglia. *In situ*

deferens, urinary bladder, lung, spleen, spinal cord and coeliac ganglia. *In situ* hybridisation using 5 day old rat brain sections showed labelling in the cerebellum, striatum, CA1-3 regions of the hippocampus, dentate gyrus and cortex (Kidd *et al.*, 1995). However no RNA was detected in adult rat brain (Collo *et al.*, 1996). This receptor is activated by α, β-meATP, antagonised by suramin and pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS), and shows desensitisation.

The $P2X_2$ receptor was cloned from phaeochromocytoma (PC12) cells (Brake *et al.*, 1994). Northern blotting and *in situ* hybridisation have found labelling in the striatum, CA1-3 regions of the hippocampus, dentate gyrus, medial amydgala and cortex (Kidd *et al.*, 1995). Other studies found no signals in the hippocampus or cerebellum and weak detection in the perirhinal and pyriform cortex (Collo *et al.*, 1996). This receptor is characterised by being unresponsive to α, β-meATP, not subject to desensitisation but antagonised by suramin and PPADS.

The $P2X_3$ receptor, which is located exclusively in sensory neurones, was initially cloned from rat dorsal root ganglion cells (Chen *et al.*, 1995a; Lewis *et al.*, 1995). This receptor is sensitive to α, β-meATP and rapidly desensitises when expressed in Xenopus oocytes or HEK293 cells. In nodose and dorsal root ganglion neurones the current produced by ATP or α, β-meATP was not subject to desensitisation. This prompted the suggestion that P2X subunits could be co-expressed. Lewis *et al.* (1995) found that co-expression of $P2X_2$ and $P2X_3$ receptors conferred the characteristics seen in sensory neurones. The $P2X_3$

receptor has recently been cloned from human heart and spinal cord (Garcia-Guzman *et al.*, 1997a).

Several groups have cloned the $P2X_4$ receptor which has the highest expression of all the P2X receptors in the CNS (Bo *et al.*, 1995; Buell *et al.*, 1996b; Séguéla *et al.*, 1996; Soto *et al.*, 1996; Wang *et al.*, 1996; Garcia-Guzman *et al.*, 1997b). Slightly different characteristics have been reported for this receptor subtype but in general ATP is the preferred ligand with α, β-meATP displaying very weak or no activity. The $P2X_4$ receptor differs from the other P2X receptors in that suramin and PPADS are not antagonists although the human isoform shows higher sensitivity to PPADS than the rat form (Garcia-Guzman *et al.*, 1997b). In all cases the ion channel was permeable to sodium and potassium but calcium can also be transported through the channel (Buell *et al.*, 1996b; Soto *et al.*, 1996). *In situ* hybridisation reveals mRNA expression predominantly in neurones with a moderate to high level of staining in the pyramidal cell layers of the CA1 and CA3 regions of the hippocampus and also the mossy cells of the hilar region and the dentate granule cells (Buell *et al.*, 1996b; Soto *et al.*, 1996; Garcia-Guzman *et al.*, 1997b). Other central regions contain $P2X_4$ mRNA.

The $P2X_5$ receptor, isolated from rat coeliac ganglia, has an activity order of ATP>2meSATP>ADP, displays minimal desensitisation and is inhibited by suramin and PPADS (Collo *et al.*, 1996). In situ hybridisation found no labelling in the brain.

An insensitivity to α, β-meATP, little desensitisation and resistance to suramin and PPADS are the characteristics of the $P2X_6$ receptor (Collo *et al.*, 1996). mRNA was expressed heavily in the CNS with strong hybridisation in all hippocampal subfields and the dentate gyrus.

A P2X receptor cloned from rat brain and macrophages and human monocytes displays the characteristics of the $P_{2Z}$ receptor in that receptor activation leads to channel activation then pore formation, has a low potency for ATP and high affinity for benzoylbenzoicATP (BzATP) and thus has been termed $P2X_7$ (Surprenant *et al.*, 1996; Rassendren *et al.*, 1997). Differences regarding agonist sensitivity, current duration and uptake of a propidium dye exist between the human and rat receptors.

### *1.5.6 Functions of extracellular ATP*

The initial actions of extracellular ATP documented involved the cardiovascular system, but functions of ATP have now been established within the autonomic nervous system, supplying both viscera and the vascular system, and in various regions of the central nervous system.

#### *1.5.6.1 ATP as a co-transmitter with noradrenaline*

Stimulation of the sympathetic nerves supplying the vas deferens produces a biphasic contraction of the smooth muscle consisting of a fast twitch followed by a slower maintained contraction. Only the sustained contraction was blocked by α-adrenoceptor antagonists (Fedan *et al.*, 1981) although the rapid twitch was

inhibited by the $P_2$ receptor antagonists $ANAPP_3$ (Fedan *et al.*, 1981), suramin (Dunn & Blakeley, 1988) and PPADS (McLaren *et al.*, 1994) and was also selectively desensitised by α, β-meATP (Meldrum & Burnstock, 1983). Responses to applied ATP were inhibited in a similar manner, supporting the view that ATP was responsible for the rapid twitch component resulting from sympathetic nerve stimulation.

In the vascular system ATP, when released, activates P2X receptors producing excitatory junction potentials, as a result of depolarisation, which summate to form action potentials leading to vasoconstriction. In the vascular system the contribution of ATP to the control of tone varies depending on the vessel in question (von Kügelgen & Starke, 1991). Vasodilatation due to P2Y receptors can also occur in response to ATP (Corr & Burnstock, 1991).

*1.5.6.2 ATP as a co-transmitter with acetylcholine*

A large amount of the work regarding the co-transmission of ATP and acetylcholine (ACh) in response to parasympathetic nerve stimulation has been carried out on the urinary bladder. Stimulation of these nerves produces contraction of the bladder which in humans and primates is more or less totally inhibited by atropine, a muscarinic cholinergic receptor antagonist. In other mammals e.g. rats, guinea-pigs and cats the inhibition by atropine is only partial. This is suggestive of the existence of an additional non-cholinergic transmission (Brindley & Craggs, 1975; Craggs & Stephenson, 1982; Hoyle, 1995). ATP has been proposed to be responsible for the residual contraction during atropine

application. When ATP is applied to preparations of bladder it mimics the effect of nerve stimulation (Burnstock *et al.*, 1978). Both the effect of applied ATP and the contraction in the presence of atropine are inhibited by $ANAPP_3$ (Theobald, 1986), suramin (Hoyle *et al.*, 1990; Bailey & Hourani, 1994) and PPADS (Ziganshin *et al.*, 1993) and are desensitised by α, β-meATP (Kasakov & Burnstock, 1982; Hoyle & Burnstock, 1985; Moss & Burnstock, 1985; Sneddon & McLees, 1992).

The receptors involved in the production of excitatory junction potentials in the bladder display properties which are characteristic of the P2X subtype, fast activation of a rapidly desensitising inward current (Inoue & Brading, 1991).

*1.5.6.3 ATP effects in the central nervous system*

Exogenous ATP applied to dorsal horn neurones produced an inward current providing the first evidence that ATP could act in an extracellular manner within the CNS (Jahr & Jessel, 1983). ATP has since been reported to exert similar effects in other central neurones. In cultured rat hypothalamic neurones ATP induced a rapid dose-dependent increase in the concentration of intracellular calcium in a suramin-sensitive manner suggesting the involvement of P2 receptors (Chen *et al.*, 1994). ATP produces an inward cationic current in rat nucleus solitarii neurones which has a potency order resembling that of P2Y receptors but has a high permeability to calcium (Ueno *et al.*, 1992). Outward potassium currents are generated in response to the application of ATP in rat striatal neurones (Ikeuchi & Nishizaki, 1995) and rat cerebellar neurones (Ikeuchi &

Nishizaki, 1996) and in response to ADP in rat medullary neurones (Ikeuchi *et al.*, 1995). In all three instances the involvement of P2Y receptors was implicated due to agonist rank order of potency and the inhibition by GDPβS, a G-protein inhibitor.

In pontine slices containing the locus coeruleus, ATP analogues, α, β-meATP and 2meSATP but not UTP, enhanced the spontaneous firing rate and produced depolarisation of the cells (Harms *et al.*, 1992; Tschopl *et al.*, 1992; Fröhlich *et al.*, 1996). These effects were displayed by ATP in the presence of 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX), an adenosine receptor antagonist (Harms *et al.*, 1992; Tschopl *et al.*, 1992). Intracellular recordings suggest that P2 receptor activation causes a reduction in a potassium current and the opening of non-selective cationic currents or a calcium-sensitive sodium current (Harms *et al.*, 1992).

Locus coeruleus neurones are central nor-adrenergic neurones which respond to stimulation by producing a postsynaptic depolarisation, due to glutamate and GABA, followed by an inhibitory postsynaptic potential, due mainly to noradrenaline (Cherubini *et al.*, 1988). The use of glutamate and GABA antagonists showed that a residual depolarisation is inhibited by suramin and PPADS thus implicating ATP as a transmitter. It has been proposed that ATP and noradrenaline, similarly to the peripheral nervous system, are co-released (Illes *et al.*, 1996). Noradrenergic neurones of the caudal medulla directly excite supraoptic vasopressin cells. This response is not antagonised by adrenoceptor

antagonists but is mimicked by ATP and α, β-meATP and inhibited by suramin, suggesting that central noradrenergic neurones can use ATP as a transmitter (Day *et al.*, 1993).

Evoked and miniature excitatory postsynaptic currents in the habenula are still apparent under conditions of glutamate and GABA receptor blockade. The residual current is depressed exclusively by suramin and desensitised by α, β-meATP. In addition exogenous application of α, β-meATP produced currents. The fast rise time of the currents was suggestive of the involvement of an ion channel coupled receptor. ATP was therefore suggested to be a chemical mediator of fast excitatory transmission in the CNS (Edwards *et al.*, 1992). In support of this ATP release from the habenula has been demonstrated (Sperlágh *et al.*, 1995).

*1.5.6.4 ATP in the hippocampus*

A number of studies regarding ATP and P2 receptors have concentrated on the hippocampus both in slice form and as neuronal cultures, resulting in somewhat conflicting results. Stimulation of slices in a manner that brought about ATP release also produced long term potentiation (LTP) of evoked responses recorded as population spikes (Wieraszko *et al.*, 1989). The application of exogenous ATP at nanomolar concentrations also enhanced the CA1 population spike in a persistent manner suggestive of LTP (Wieraszko & Seyfried, 1989). However the same group also reported that high micromolar concentrations of ATP permanently depressed CA1 population spikes, suggesting that ATP exerts a dose-dependent biphasic effect on hippocampal CA1 population spikes. ATP and AMP

also caused a persistent depression of neuronal excitation produced by glutamate application in the CA1 region of hippocampal slices, suggesting that ATP acted postsynaptically to modulate hippocampal activity (Di Cori & Henry, 1984). P2 receptor antagonists, suramin, reactive blue 2 and PPADS, all decreased excitatory postsynaptic currents recorded in the CA1 region on stimulation of the Schaffer collaterals. However, the response to iontophoretically applied glutamate was resistant to inhibition by PPADS. Therefore these results suggest that both pre- and post-synaptic P2 receptors can modulate the release and action of endogenous glutamate (Motin & Bennett, 1995).

Inward currents mediated by channels carrying calcium have been shown to be activated in hippocampal neurones (Inoue *et al.*, 1992; Inoue *et al.*, 1995; Dave & Mogul, 1996). The ATP-induced calcium current has two components, an indirect transient current due to glutamate release and a direct sustained current (Inoue *et al.*, 1992). Ion substitution experiments revealed that ATP can activate anionic in addition to cationic conductances in cultured hippocampal neurones (Balachandran & Bennett, 1996). ATP can also evoke an outward potassium current which may result from the opening of calcium sensitive potassium channels (Ikeuchi *et al.*, 1996).

Although the above evidence points to a functional role for ATP in the hippocampus, other reports have not shared the same view. Stone & Cusack (1989) reported that stable ATP analogues produced no consistent effect on either CA1 population spike amplitude or single cell firing in response to glutamate

application. ATP depressed both parameters in a manner similar to that of adenosine such that the effect was inhibited by 8-phenyltheophylline, a P1 receptor antagonist.

### *1.5.7 Metabolism*

One of the criteria that must be met when establishing a compound as a transmitter is the existence of a means of removing the transmitter and thence terminating its action. ATP is metabolised to form adenosine by the action of ecto-ATPases (Maire *et al.*, 1984; Richardson & Brown, 1987). These enzymes are located on the membrane and are found within the hippocampus. The products of ATP metabolism are ADP, AMP and adenosine in order of production.

## 1.6 Adenosine

Adenosine, an extracellular neuormodulator, can be synthesised intracellularly *de novo* and transported across the membrane or can result from the metabolism of ATP.

### *1.6.1 Adenosine receptors*

Adenosine acts extracellularly to modulate neuroexcitability through the activation of P1 purinoceptors. Originally P1 receptors were classified according to their effect on adenylate cyclase activity and the ability of methylxanthines to antagonise them (Sattin & Rall, 1970). Nanomolar concentrations of adenosine agonists inhibited adenylyl cyclase activity expressed as decreased cAMP

production in mouse brain cells whereas micromolar concentrations increased cAMP production. $A_1$ receptors were designated as sites that inhibited adenylate cyclase and $A_2$ receptors as those that stimulated adenylate cyclase (Van Calker *et al.*, 1978). Similar results were obtained in liver cells (Londos *et al.*, 1980). These effects on adenylate cyclase are mediated through the activation of G-proteins namely $G_i$ for $A_1$ receptors (Munshi & Linden, 1989) and $G_s$ for $A_2$ receptors (Morgan, 1991).

The receptors are also characterised according to agonist potency. In general $N^6$ substitution on the adenine moiety confers higher potency for the $A_1$ receptor. The most potent and selective $A_1$ receptor agonists are $N^6$-cyclopentyladenosine (CPA) (700 fold $A_1$ over $A_2$), $N^6$-cyclohexyladenosine (CHA) (400 fold selective) and $N^6$-phenylisopropyladenosine (*R*-PIA) (100 fold selective) (Bruns *et al.*, 1986; Jarvis *et al.*, 1989). A split of the $A_1$ receptor into 1a and 1b was proposed by Gustafsson *et al.* (1990) on the basis that the receptor in rat brain, $A_{1a}$, is a high affinity receptor and that a lower affinity receptor found in guinea-pig ileum and rat vas deferens is $A_{1b}$.

$A_2$ receptors can be distinguished from $A_1$ receptors with respect to agonist potency order. 5'-*N*-ethylcarboxamidoadenosine (NECA) is equipotent at both receptor subtypes but displays greater potency at the $A_2$ receptor compared with CPA, CHA or *R*-PIA. Division into $A_{2A}$ and $A_{2B}$ was based on the presence of a high affinity site in striatal membranes ($A_{2A}$) and a lower affinity site expressed throughout the brain ($A_{2B}$). Both, when activated, enhanced cAMP levels (Daly *et*

*al.*, 1973). A similar low affinity site occurs in human fibroblasts (Bruns *et al.*, 1986). 2-(p-(-carboxyethyl)-phenylethylamino)-5'-N-ethylcarboxamidoadenosine (CGS21680) is considered to be selective for the $A_{2A}$ receptor (Jarvis *et al.*, 1989; Lupica *et al.*, 1990).

A third adenosine receptor, $A_3$, has been proposed which can bind both NECA and $N^6$-(4-amiophenyl)-ethyladenosine (APNEA), an $A_1$ receptor agonist, but not CGS21680 or $A_1$ antagonists and can inhibit adenylate cyclase (Zhou *et al.*, 1992). Several compounds have been synthesised which display selectivity towards the $A_3$ receptor, many of which are derivatives of methylcarboxamidoadenosine (MECA). 2-chloro-N6-(3-iodobenzyl)-MECA (2-Cl-IB-MECA) is 2500- and 1400-fold selective for $A_3$ receptors over $A_1$ and $A_{2A}$ receptors respectively (Kim *et al.*, 1994), whereas the N6-(3-isothiocyanobenzyl) derivative is 14-fold selective for $A_3$ over $A_1$ receptors (Ji *et al.*, 1994). A recent study has demonstrated an interaction between $A_3$ and $A_1$ receptors (Dunwiddie *et al.*, 1997). 2-Cl-IB-MECA alone had no effect on normal responses in the CA1 region nor on LTP, but antagonised the $A_1$ receptor mediated inhibitory responses on neurotransmission by reducing receptor affinity for adenosine (Dunwiddie *et al.*, 1997). $A_3$ receptor activation has also been proposed to potentiate calcium currents in dissociated hippocampal CA3 neurones through a mechanism involving protein kinase A (Fleming & Mogul, 1997).

### *1.6.2 Actions of adenosine*

The majority of work surrounding adenosine has labelled this purine as an inhibitory neuromodulator (Stone, 1981). Early work demonstrated the ability of adenosine to act presynaptically to depress transmission at the neuromuscular junction (Ginsborg & Hirst, 1971; Ribeiro & Walker, 1973). Within the CNS adenosine depressed activity in a number of brain regions including the cerebral cortex (Phillis *et al.*, 1974; Stone & Taylor, 1977) striatum, hippocampus (Dunwiddie & Hoffer, 1980) and thalamus (Kostopoulos & Phillis, 1977). This action has been attributed to both pre-and postsynaptic mechanisms involving inhibition of neurotransmitter release (Ribeiro, 1995 for review). The actual receptor mediated transduction mechanisms involved include not only modulation of cAMP levels but also activation of potassium channels (Gerber *et al.*, 1989; Thompson *et al.*, 1992), inactivation of calcium channels (Greene & Haas, 1991) and activation of protein kinase C (Gerwins & Fredholm, 1995; Henry *et al.*, 1996; Marala & Mustafa, 1995). In contrast to these effects adenosine can, in some instances, also be excitatory (Nishimura *et al.*, 1990; Okada *et al.*, 1992).

## 1.7 Aim

There is a considerable amount of literature regarding the effect of adenosine on epileptiform activity both *in vitro* and *in vivo*, but there is very little concerning ATP *per se* and not as a source of adenosine. In view of the evidence for receptor-mediated actions of ATP on single neurones, it was of interest to investigate the effect of ATP and related analogues on the behaviour of a neuronal population. Although CA1 population spikes had proved resistant to ATP analogues, there exists both pharmacological and biochemical evidence surrounding ATP in the hippocampus to justify the use of this CNS region, especially since the hippocampus has been implicated in seizure generation. The present study, therefore, was designed to examine the modulation by nucleotides of epileptiform bursting which can be triggered from the hippocampal CA3 region.

## 2.0 Methods

Male Wistar rats (170-250g) were anaesthetised with urethane (1.3g/kg i.p.) and cooled on a bed of ice whilst breathing oxygen enriched air until the rectal temperature had reached 31°C. Animals were subsequently killed by cervical dislocation. After decapitation, by means of a guillotine, the brain was carefully removed and placed in oxygenated ice-cold artificial cerebrospinal fluid (aCSF). In a petri dish lined with filter paper and filled to overflowing with aCSF the cerebellum was removed and the two cerebral hemispheres separated using a scalpel blade. The hippocampi were dissected from each hemisphere using micro-spatulas. Transverse hippocampal slices (450μm) were prepared using a McIlwain Tissue Chopper and separated using sealed fine glass capillaries. The slices were kept in a petri dish with a small volume of aCSF, adequate to cover the slices, within an interface chamber containing artificial cerebrospinal fluid (aCSF) gassed with 95%$O_2$-5%$CO_2$ . Slices were incubated for at least one hour prior to use.

The composition of the aCSF was as follows (mM): NaCl 115; $NaHCO_3$ 25; KCl 2; $KH_2PO_4$ 2.2; $CaCl_2$ 2.5; $MgSO_4$ 1.2; glucose 10; saturated with 95%$O_2$-5%$CO_2$.

After incubation individual slices were transferred using a fine brush to a 1ml submersion chamber which was continually perfused with aCSF or modified aCSF at a rate of 3.5-4 ml/min via a peristaltic pump. The temperature of the chamber was maintained at approximately 34°C. Drugs were added to the perfusion medium and applied for a minimum of 10 min.

Recording electrodes were made from borosilicate glass capillaries with an outside diameter of 1.5mm and internal diameter of 0.86mm (GC150F). A Kopf vertical electrode puller was used to form two electrodes of a fine tip diameter. The tip

diameter was broken back to 2-4μm under low magnification using a glass probe. Electrodes were filled with sodium chloride (2M) using a fine 36 gauge needle.

### *2.1 Generation of epileptiform activity*

A concentric bipolar stimulation electrode was placed either in the stratum radiatum of the hippocampal CA3 or CA1 region to allow orthodromic stimulation of the mossy fibers or Schaffer collaterals respectively. The response was recorded via a glass capillary electrode in the pyramidal cell layer of the CA3 or CA1 region (Fig. 1). Stimulation (0.2Hz.) was applied briefly to check the viability of the slice and the correct positioning of the recording electrode, after which stimulation was halted and the perfusing medium changed from normal aCSF to a modified medium.

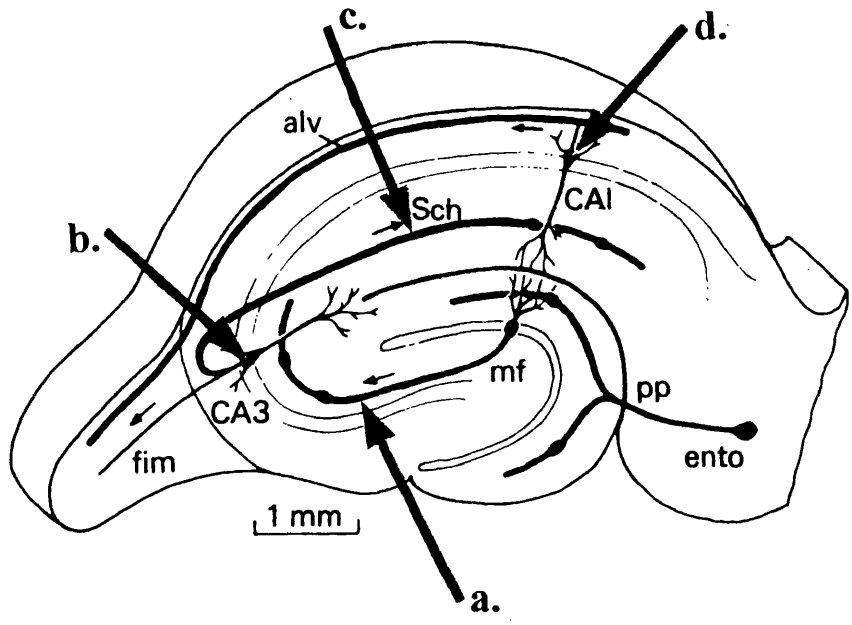



**Figure 1** A diagram showing the organisation of a hippocampal slice in which alv: alveus, ento: entorhial cortex, fim: fimbria, pp: perforforant path, Sch: Schaffer collateral and mf: mossy fibres. Electrodes a and b were used for stimulating and recording in the CA3 region respectively, whereas electrodes c and d were used for investigation in the CA1 region. (Adapted from Andersen et al, 1971).

In our initial attempts to generate epileptiform activity several media of various ionic compositions with and without drug additions were used. Details of these are given in appendix 1.

The most frequently employed medium was magnesium-free aCSF containing 4-aminopyridine (4AP) at 50μM. After approximately 5-20 minutes spontaneous bursts of population spikes occurred which were continuously recorded on a Gould storage oscilloscope and a Grass pen recorder and subsequently plotted as frequency against time.

### *2.2 Data analysis and statistics*

The control frequency (bursts per minute) was calculated as the mean of the 3 observations immediately preceding start of drug perfusion. The effect of added agents was taken as the mean of the final 3 observations made during the 10 minute period of perfusion and as the maximum amount of inhibition produced during the period of application. Results are expressed as a percentage of the control rate ± standard error of the mean (s.e.m.) for n slices. A paired Student's t-test using raw data obtained before and after drug perfusion was used to determine the degree of significance and is denoted on figures by the # symbol. Comparison between two data groups was made using a paired Student's t-test if both treatments were performed sequentially on the same slice, if different slices were used then a non-paired Student's t-test was used. Multiple comparisons involving more than two different sets of data were made using analysis of variance (ANOVA) followed by a Student-Newman-Keul's test in the case of histograms and Dunnett's post-test in time plots. In the figures comparisons between different treatment groups are denoted with a *. $P<0.05$ was taken to indicate significance.

*2.3 Generation of ictal activity*

In one set of experiments male Wistar rats at a lower body weight (60-100g) were used to increase the probability of generating ictal activity. In addition, the slice thickness was increased to 650μm. Several combinations of zero magnesium solutions, stimulus trains, addition of 4-aminopyridine and baclofen were used as described in appendix 2.

*2.4 Recording of epileptiform activity and population potentials simultaneously*

In some instances both spontaneous and evoked activity were recorded simultaneously. For this a stimulation electrode was placed as before in the stratum radiatum of the CA3 region. Two recording electrodes were placed in the CA3 pyramidal cell layer. One electrode was connected as for the recording of epileptiform activity alone and thus gave a continuous account of the activity displayed on the oscilloscope and Grass recorder. The second electrode was used to record evoked potentials and was connected via an averager to a Gould oscilloscope and also a Par 1000 thermal recorder.

Stimulation was applied at 0.2Hz until two potentials could be recorded after which stimulus frequency was changed to 0.1Hz at an intensity between 200 and 300μA. After 15 minutes the perfusion medium was changed to one containing no added magnesium and 4AP (50μM). In these experiments the stimulation was continued during the perfusion of the changed medium. At given time points eight sequential population potentials were averaged and recorded. Once the potentials had become stabilised a further set were averaged and used as the control response. The population spike amplitude was determined as the height from midway between the population spike and pre-synaptic volley to the lowest part of the population potential (fig. 2). The effect of added agents was determined after 5 and 10 minutes perfusion and expressed as a percentage of the

control. The frequency of spontaneous activity was calculated as described for bursts alone.

Similar experiments were also performed in the CA1 region of the hippocampus. For these the stimulation electrode was placed in the stratum radiatum of the CA1 region to allow stimulation of the Schaffer collaterals. Population spikes were recorded in the CA1 pyramidal cell layer.

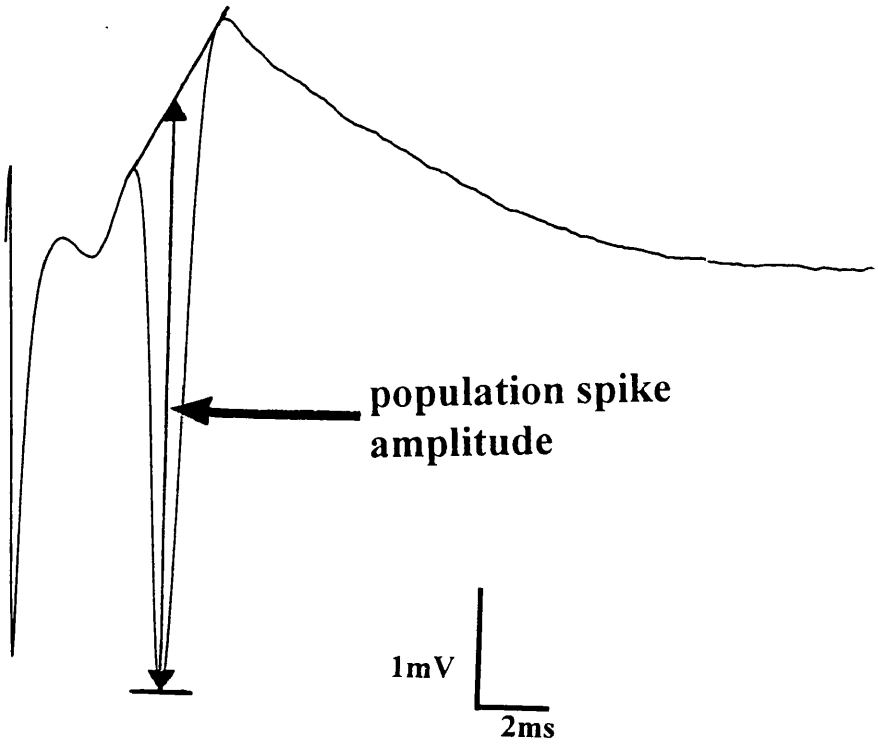



**Figure 2** An example of a population potential recorded from the CA1 region. The downward deflections represent in sequence the stimulus artifact, pre-synaptic volley and population spike. A line was drawn between the pre-synaptic volley and population spike. The vertical distance between the lowest point of the population spike and this joining line was taken as the amplitude of the population potential.

### *2.5 Recording of evoked population spikes*

For some experiments drug effects were examined on evoked population spikes only. For this the electrodes were placed as mentioned above with only one recording electrode being used. In these cases all experiments were performed using normal aCSF.

### *2.6 Drugs*

The majority of drugs were dissolved in distilled water to form a stock solution before being diluted further in normal or modified aCSF. AMPase was dissolved directly in modified aCSF, then filtered to remove a resulting residue. This process did not alter the activity of the enzyme. Adenosine deaminase was also dissolved directly in the perfusion medium. 8-cyclopentyl-1,3-dimethylxanthine (CPT) was dissolved in a 1:1 combination of NaOH (0.5M) and distilled water. Kynurenate was dissolved in NaOH (0.5M). AP5 was dissolved using 1:4 combination of NaOH (0.5M):distilled water. 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) was dissolved in dimethylsulphoxide (DMSO) (100%), but the final concentration of DMSO never exceeded 0.1%. (4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-yl amino]ethyl) phenol) (ZM241385) and aspirin were dissolved in DMSO (50%). Methanol was used to dissolve indomethacin.

Adenosine, 5'-Adenylic acid deaminase (AMPase), ADP, adenosine deaminase (type II), 4-aminopyridine, aspirin, ATP, 2 & 3'-O-(4-benzoylbenzoyl)ATP, diadenosine tetraphosphate, diadenosine pentaphosphate, IMP, indomethacin, kynurenate, 2-methylthioATP, α, β-methyleneATP, α, β-methyleneADP, *N* ω-nitro-*L*-arginine methyl ester (L-NAME), 5'-nucleotidase, UMP and UTP were purchased from the Sigma Chemical Company. AMP and zinc acetate were from British Drug Houses (BDH). PPADS and *N*-(2-aminoethyl)-5-chloro-1-naphthalene sulphonamide (A3) were bought from Tocris Cookson. AP5,

bicuculline methbromide, CPT, DPCPX and 8-(*p*-sulphophenyl)theophylline were from Research Biochemicals Incorporated (RBI). Naloxone was from Endo laboratories Inc. Suramin was a gift from Bayer and ZM241385 a gift from Zeneca.

# 3.0 Results

## 3.1 Generation of epileptiform activity

Whilst recording in the CA1 region, perfusion with media with the following alterations resulted in spontaneous activity: zero magnesium, kainic acid (2μM), 4-aminopyridine (100μM), zero calcium + EDTA (1mM) + 4-aminopyridine (50μM) and 4-aminopyridine (150μM) + $K^+$ (6mM). However, in general, the frequency of bursts was low and irregular with a low incidence of occurrence. Bursts could be more regularly recorded from the CA3 hippocampal subfield.

In the majority of hippocampal slices perfusion with a medium containing no added magnesium and 4-aminopyridine (50μM) (4AP) resulted in the generation of epileptiform activity of an inter ictal nature in the CA3 region. Individual bursts had a duration of between 150 and 500ms with either single or multiple peaks per burst (fig. 3). The frequency of bursts was in the range of 0.08-0.6 Hz. Burst duration, frequency and amplitude varied among slices but remained stable during rest and washout periods in individual slices.

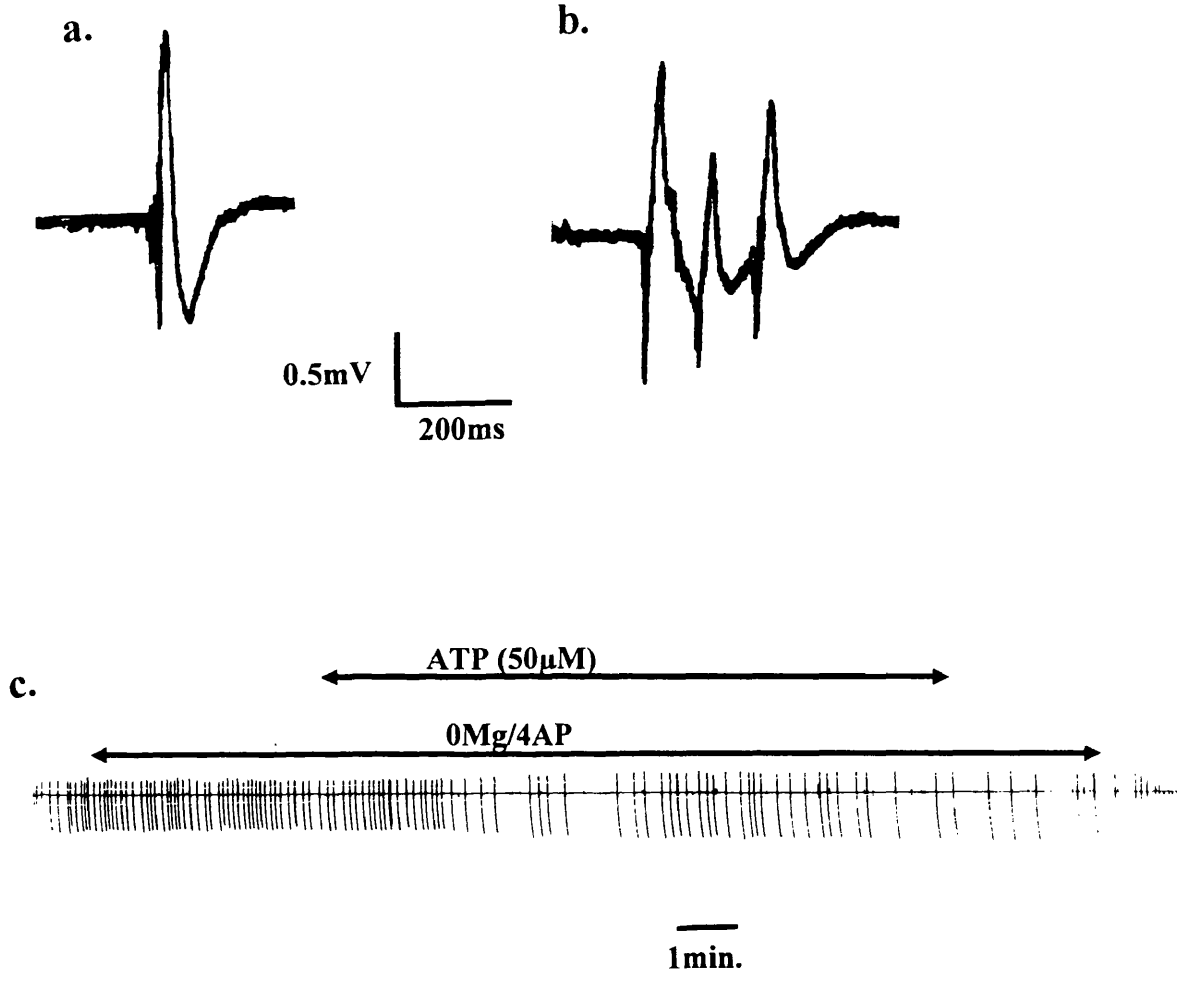



**Figure 3** (a) and (b) represent individual bursts of spontaneous activity recorded on a fast time scale which display either single or multiple peaks per burst respectively. An example of a slow time scale trace is shown in (c). Each vertical line represents an individual burst of activity. These deflections were counted to give the frequency in bursts per minute.

The involvement of NMDA and non-NMDA receptors in the generation of epileptiform activity in this model (0Mg/4AP) was investigated using kynurenate and 2-amino-5-phosphonopentanoic acid (AP5). Kynurenate at 200μM and 1mM decreased the rate of activity to 45.52%±9.92 (n=4, P>0.05) and 9.39%±5.19 (n=4, P<0.01) control respectively at the end of a 10 minute perfusion (fig.4). AP5 (20 and 40μM) reduced the discharge rate to a similar extent 64.43%±1.22 (n=3, P<0.01) and 60.53%±4.14 (n=3, P<0.5) respectively (fig. 5). The depression produced by both kynurenate and AP5 was fully reversible with rate often exceeding control levels during the subsequent wash period.

All the epileptiform activity described above had interictal characteristics. The use of young rats, thicker hippocampal slices and a variety of techniques described in appendix 2 resulted in either no spontaneous activity or interictal bursts but never ictal activity.

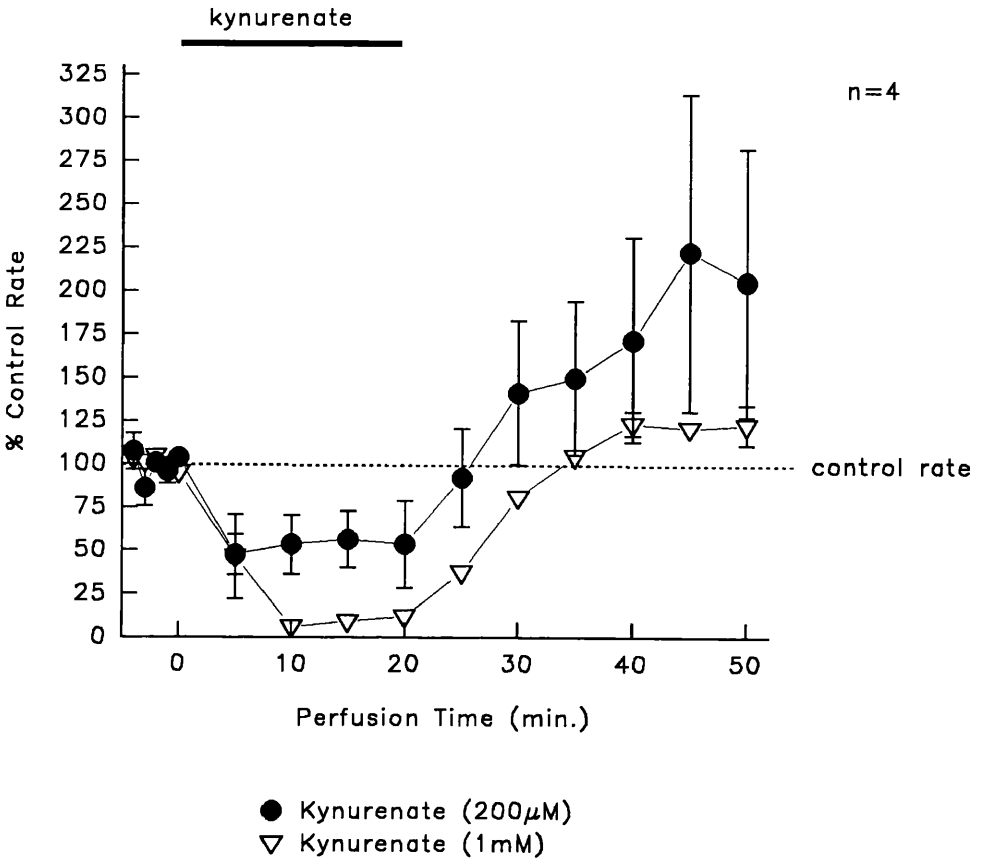



**Figure 4** This shows the time course of the effect of kynurenate applied for 20 minutes on the rate of epileptiform activity. Although kynurenate (200μM) decreased the rate of activity, this only became significant at the higher concentration of 1mM. At both concentrations the depression was fully reversible.

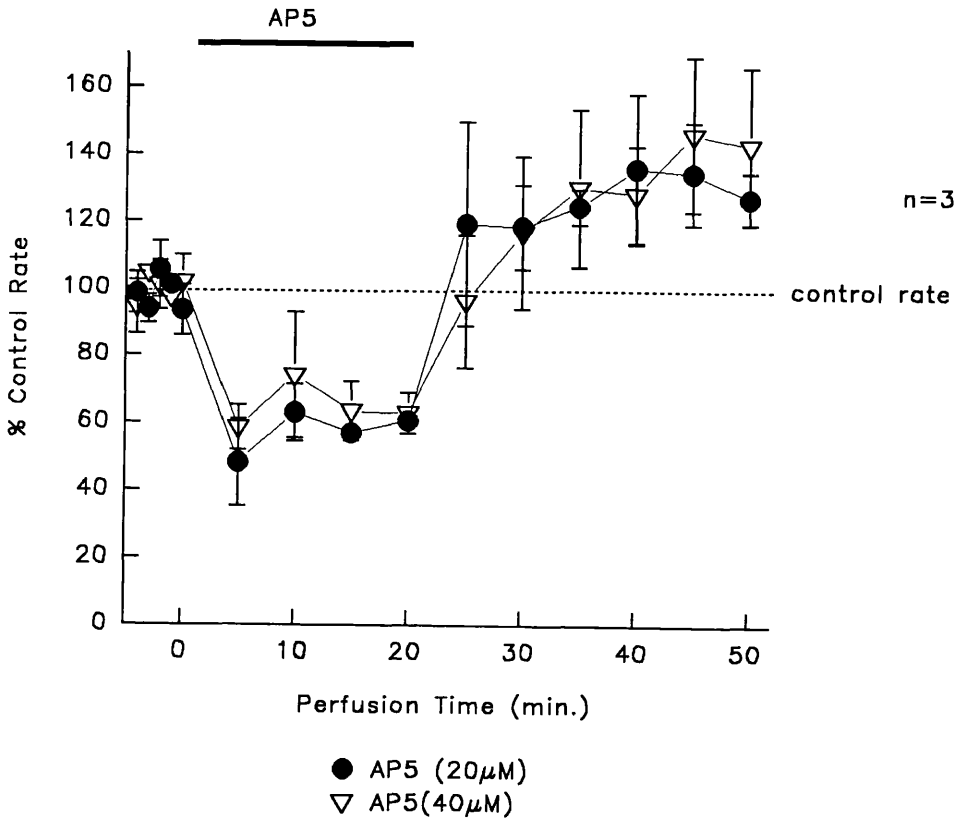



**Figure 5** 2-amino-5-phosphonopentanoic acid (AP5) was perfused for 20 minutes. During this time the discharge rate was significantly decreased to a similar extent by 20 and 40μM AP5. During the wash period the rate returned to slightly above control values but this did not reach significance.

## 3.2 Modulation of epileptiform activity by ATP and adenosine

Adenosine produced a concentration-dependent reduction in the rate of epileptiform activity (fig. 6). ATP at concentrations greater than 10μM depressed spontaneous activity to a degree comparable with adenosine (fig. 6). However, at a concentration of 2μM the concentration-response graphs of adenosine and ATP diverged, with ATP tending to increase the burst frequency, though this did not reach significance.

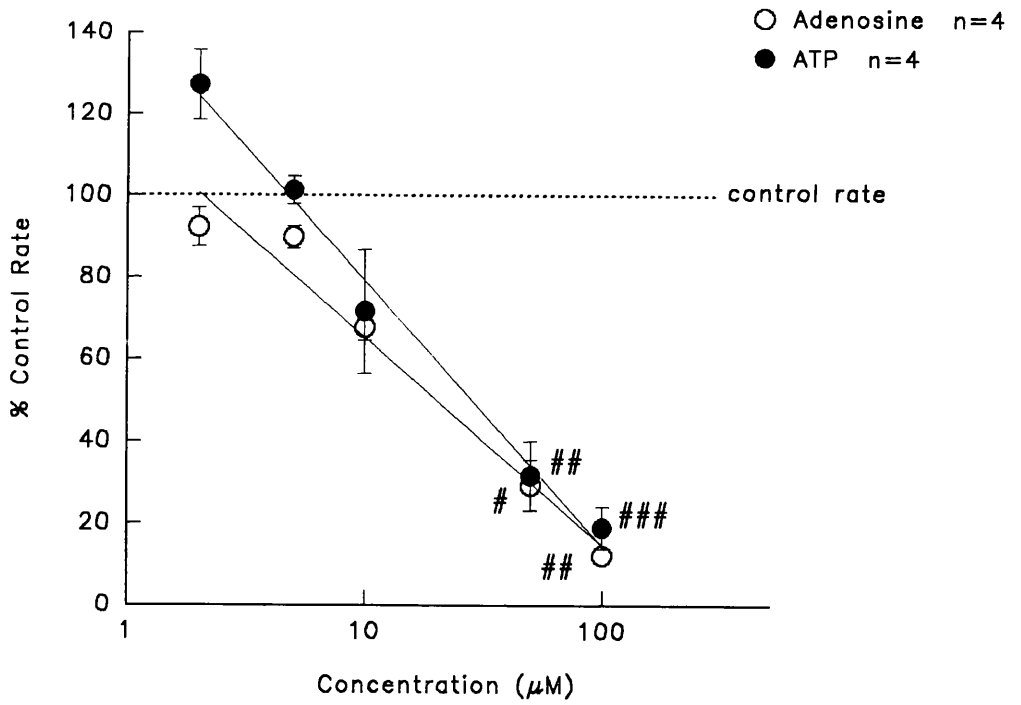



**Figure 6** Concentration-response curves for the effect of ATP and adenosine on the frequency of epileptiform activity. # P<0.05, ## P<0.01, ### P<0.001.

### *3.2.1 Adenosine Deaminase*

ATP is metabolised to adenosine by ecto-nucleotidases. In order to inactivate any adenosine formed during the perfusion of ATP, adenosine deaminase was co-perfused with ATP. The time course of the depression of activity induced by ATP at 50μM is plotted in fig. 7a. The onset of the ATP effect is almost instantaneous when the perfusion system lag time of 1.5-2 min. is taken into consideration, with some subsequent evidence of recovery towards an equilibrium effect after 10 minutes. This may reflect a degree of desensitisation.

Adenosine deaminase itself (0.2U/ml) tended to increase the rate of activity to a small extent but this did not reach significance (figs. 7b and 8b). Adenosine deaminase (0.2U/ml) did not significantly reduce the extent of the ATP (50μM) induced depression at equilibrium (10 min.) (fig. 7b). The depression of burst rate by adenosine (50μM) followed a similar time course to that of ATP, but when perfused in identical experimental conditions the same concentration of adenosine deaminase (0.2U/ml) totally abolished any effect of adenosine as shown in fig. 8.

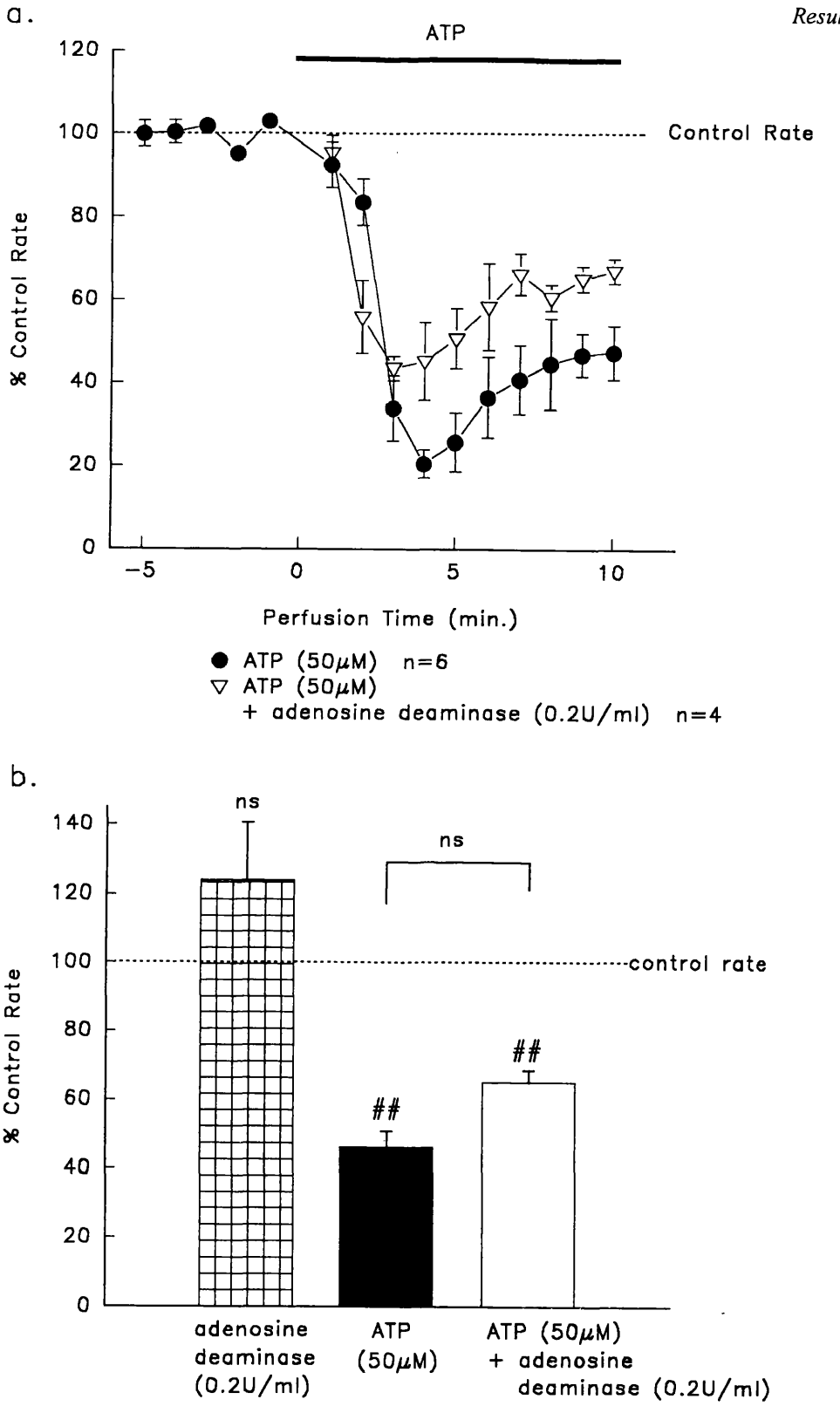



**Figure 7** The effect of adenosine deaminase on the depression of epileptiform activity produced by ATP. (a) shows the time course of ATP effect when perfused alone and in combination with adenosine deaminase. (b) is a summary of the effects at the end of the perfusion period. Adenosine deaminase did not significantly alter the discharge rate whereas ATP and ATP + adenosine deaminase did, ## $P<0.01$.

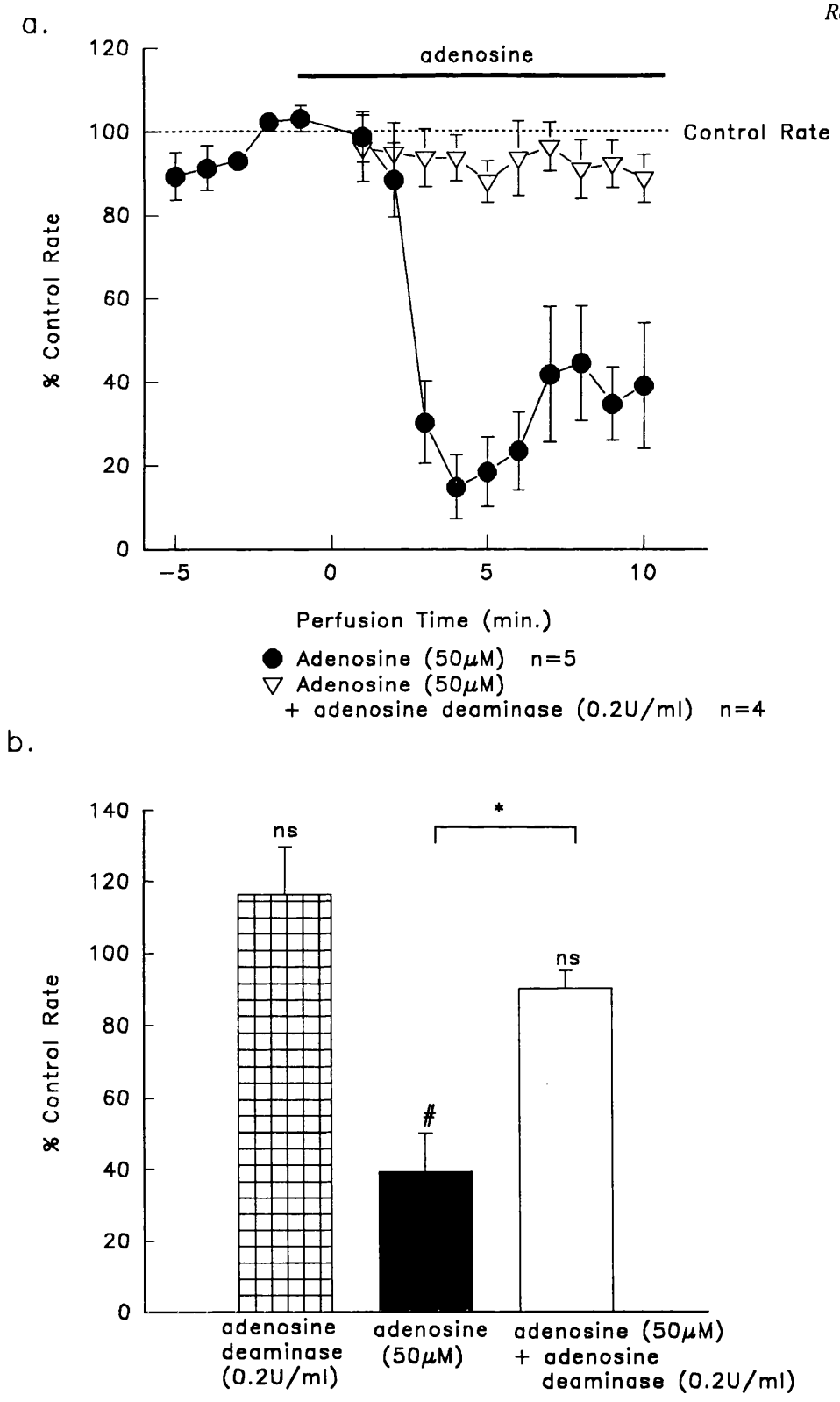



**Figure 8** The time course of the decrease in discharge rate by adenosine and the inhibition of this by adenosine deaminase is shown in (a). (b) represents the mean effect at the end of 10 minute perfusion. Only adenosine significantly alters the rate from control, # $P<0.05$. The addition of adenosine deaminase reduces the effect of adenosine to a significant degree, * $P<0.05$.

### *3.2.2 Cyclopentyltheophylline*

8-Cyclopentyl-1, 3-dimethylxanthine (CPT), an $A_1$ receptor antagonist, at 100nM elevated the rate of burst activity by approximately 40% (fig. 9). When CPT was perfused simultaneously with ATP or adenosine (50μM) it completely prevented the effect of the purine such that the CPT plus purine curves were almost superimposable upon the effect of CPT alone. The time course of the effects of adenosine and ATP are illustrated in fig. 10a and b, and a summary plot of the changes in burst frequency and their statistics are given in fig. 10c.

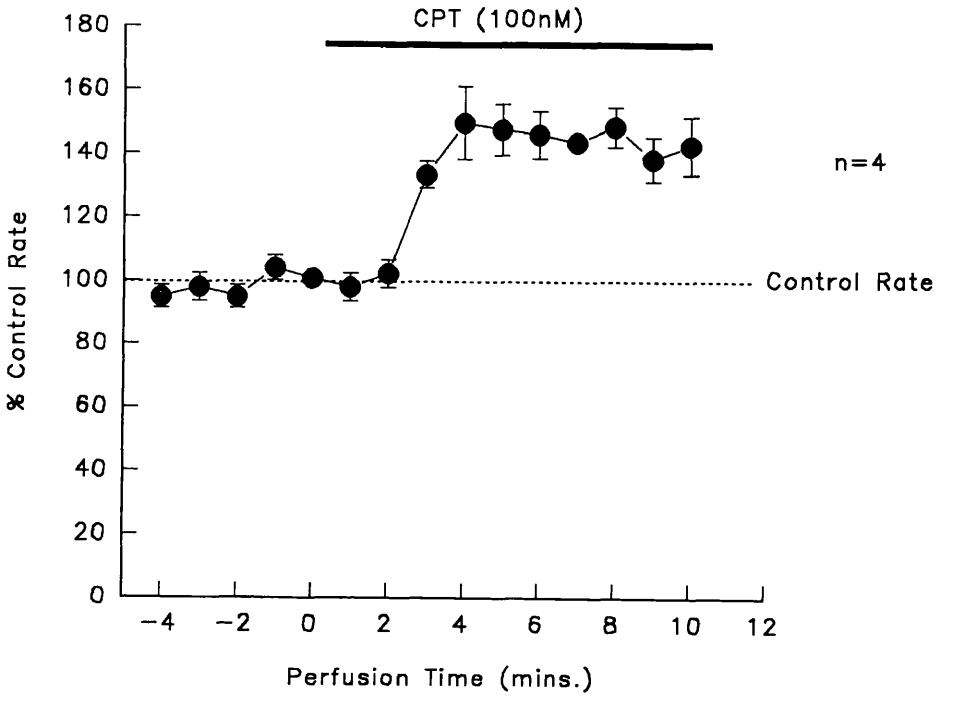



**Figure 9** This shows the time course of the significant increase in discharge rate resulting from perfusion with CPT, an $A_1$ receptor antagonist.

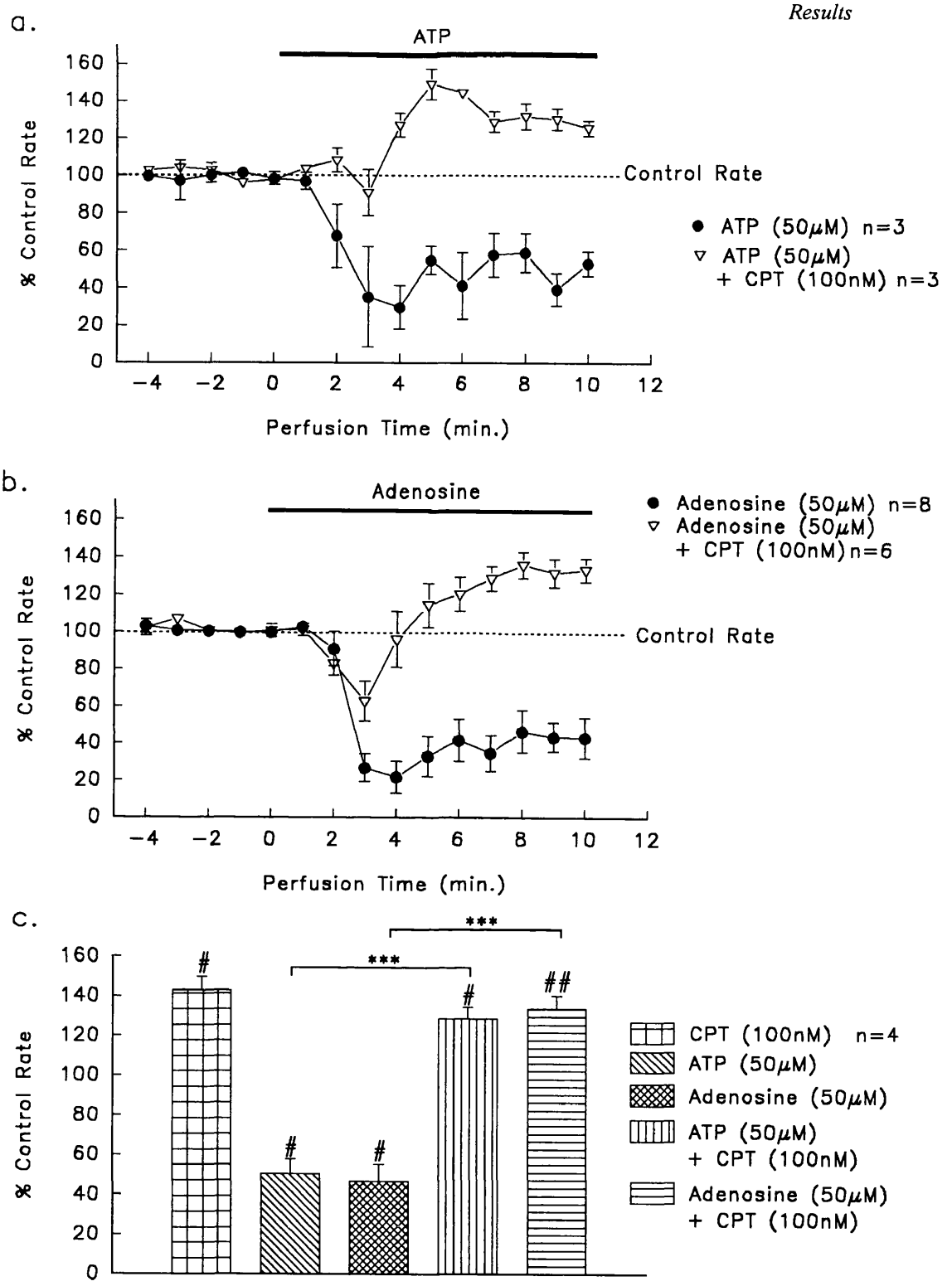



**Figure 10** The effect of CPT on the depression of spontaneous activity by ATP and adenosine. The time course of the effect of ATP and adenosine are represented in (a) and (b) respectively. The net effect after 10 minutes perfusion of CPT, ATP and adenosine, singly or in combination is shown in (c). All combinations altered the rate significantly from control, # $P<0.05$, ## $P<0.01$. CPT significantly inhibited the effect of both ATP and adenosine with *** $P<0.001$.

### *3.2.3 Sulphophenyltheophylline*

8-(*p*-sulphophenyl)theophylline (8pSPT), a P1 receptor antagonist, increased the rate of epileptiform activity to approximately 130 and 180% of control at concentrations of 1 and 5μM respectively but was ineffective at 0.1μM (figs. 11 and 12). 8pSPT (1μM), when perfused with ATP (50μM), reduced the extent of inhibition to a significant extent. However ATP in the presence of 8pSPT still significantly reduces discharge rate (fig.13). Since both ATP and SPT were perfused simultaneously with no prior exposure to 8pSPT the excitatory effect of the antagonist must be considered as possibly being superimposed on the ATP induced depression.

Adenosine (50μM) significantly decreased activity rate in the presence of 8pSPT at 1μM but not at 5μM (fig. 14b). The time course of events is shown in fig. 14a. In all instances there is an initial depression with a subsequent varying degree of recovery. Unlike the situation with CPT the graphs of 8pSPT alone and in combination with ATP and adenosine are clearly distinguishable (figs. 12, 13 and 14).

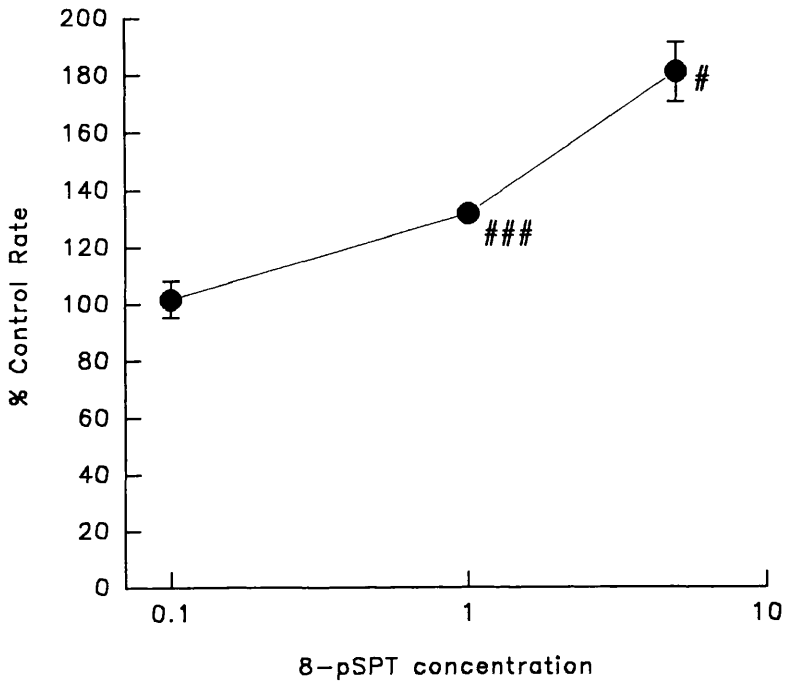



**Figure 11** Concentration response curve for the $P_1$ receptor antagonist 8pSPT. The rate was significantly increased from control values at concentrations of 1 and 10μM. # $P<0.05$ and ### $P<0.001$.

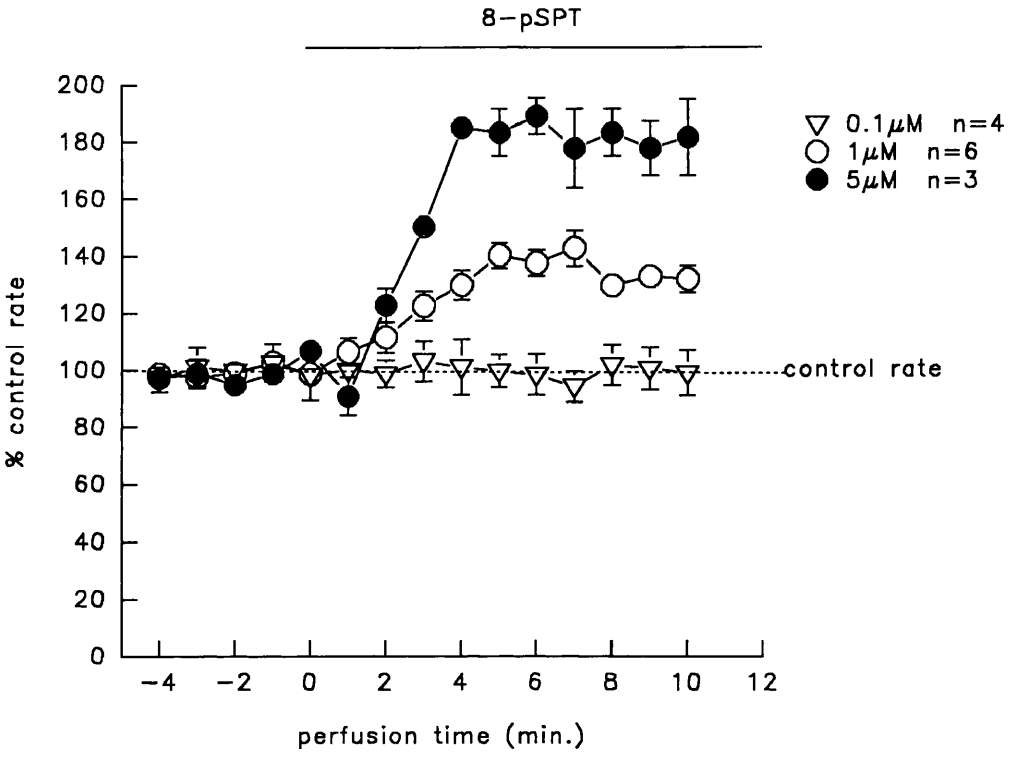



**Figure 12** This figure illustrates the time course of the increase in discharge rate produced by 8pSPT.

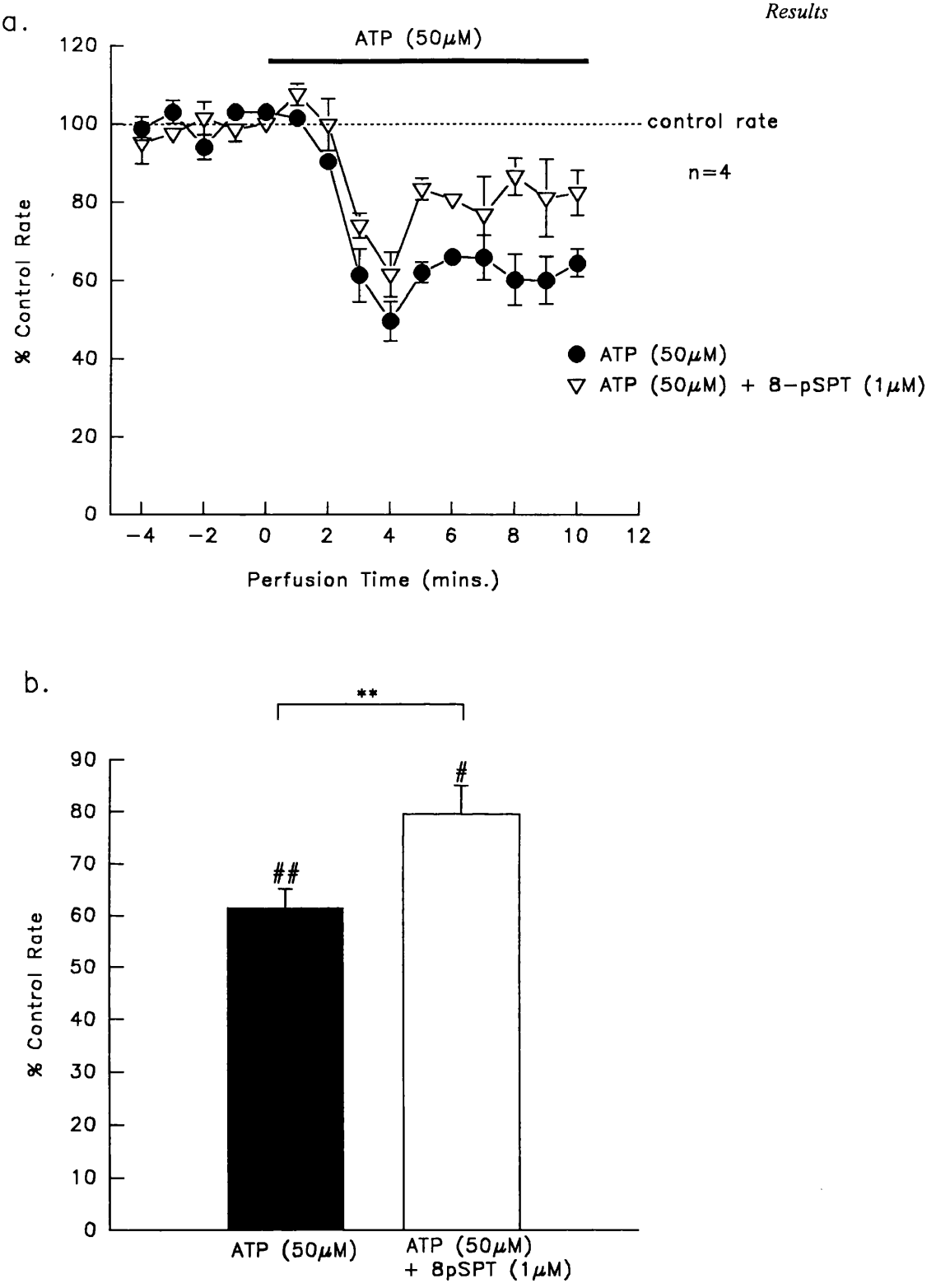



**Figure 13** (a) shows the time course of the effect of ATP when perfused alone and in combination with 8pSPT. The mean effect at the end of a 10 minute perfusion is shown in (b). ATP and ATP + 8pSPT both significantly decreased the activity rate from control (# $P<0.05$, ## $P<0.01$). There was a significant difference between the extent of inhibition displayed, ** $P<0.01$.

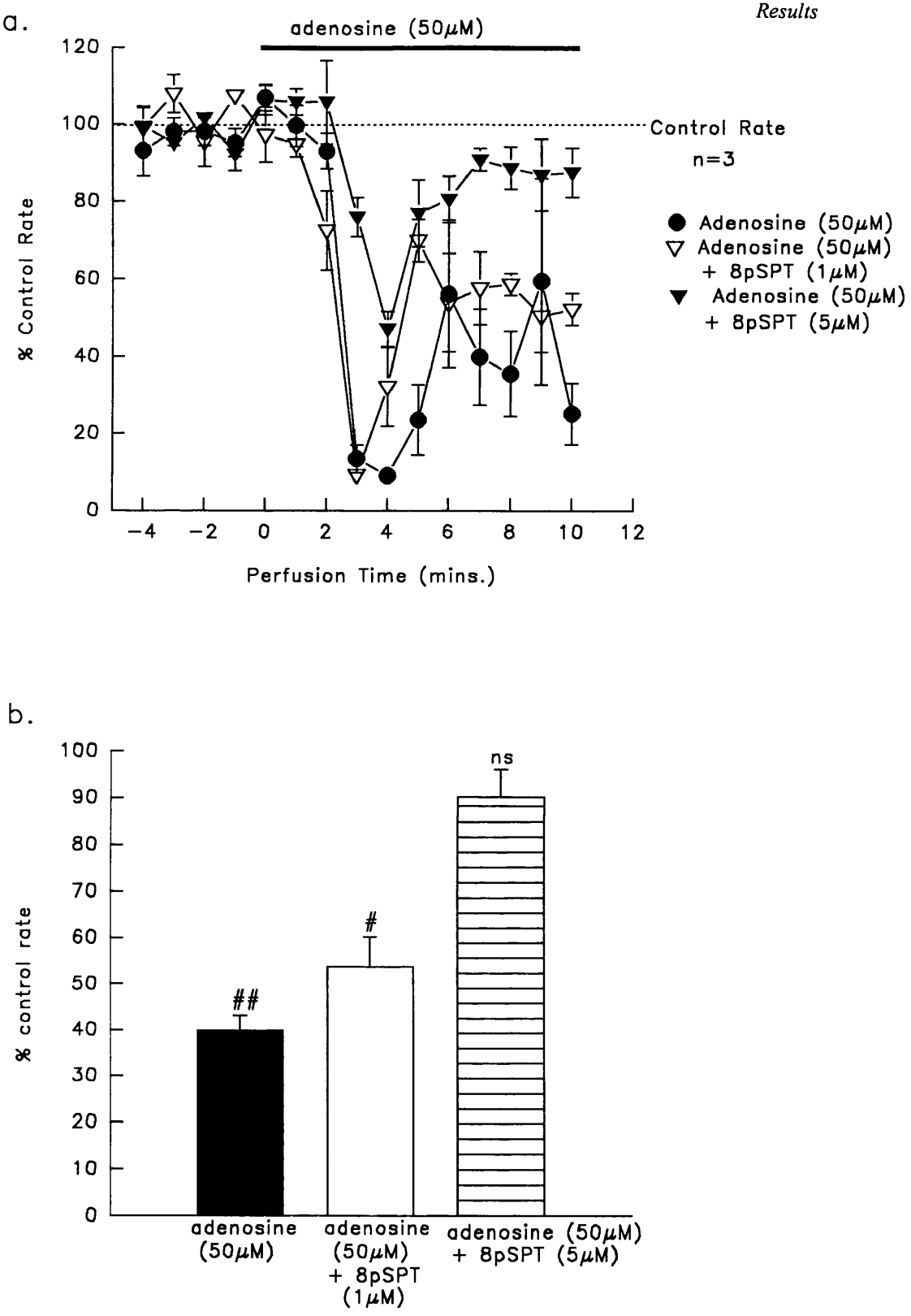



**Figure 14** The time course of the effect of 8pSPT on the depression of activity produced by adenosine is illustrated in (a). The mean effect and analysis is shown in (b). Adenosine alone and in the presence of 8pSPT (1μM) significantly decreases the discharge rate, # $P<0.05$, ## $P<0.01$, but this effect is lost in the presence of 8pSPT (5μM).

### *3.2.4 Nucleotide Analogues*

The ATP analogue α, β-meATP is relatively resistant to degradation by ecto-nucleotidases and is considered to activate several sub-types of P2X receptors. At a concentration of 5μM α, β-meATP had no effect on burst frequency, whereas at 10μM the frequency was increased significantly (fig. 15). This excitatory effect was not altered when α, β-meATP was co-perfused with adenosine deaminase (data not shown).

2-MethylthioATP (2-meSATP) is susceptible to a similar degree of metabolic breakdown as ATP and can activate P2Y receptors. 2-meSATP failed to alter significantly the frequency of spontaneous activity at concentrations of 10 and 50μM (fig. 12). However, 2-meSATP is susceptible to metabolism by nucleotidases and the experiments were therefore also performed in the presence of α, β-meADP (50μM), an inhibitor of 5'-nucleotidase. 2-meSATP was still ineffective under these conditions (fig. 16). The pyrimidine nucleotide UTP was also not significantly active at 50μM in this model of epileptiform activity.

ADP modified spontaneous epileptiform activity to a significant degree at 50 but not 10μM (fig. 17). Adenosine deaminase (0.2U/ml) reduced the effect of ADP (50μM), although ADP still depressed burst rate to a significant degree in the presence of the enzyme.

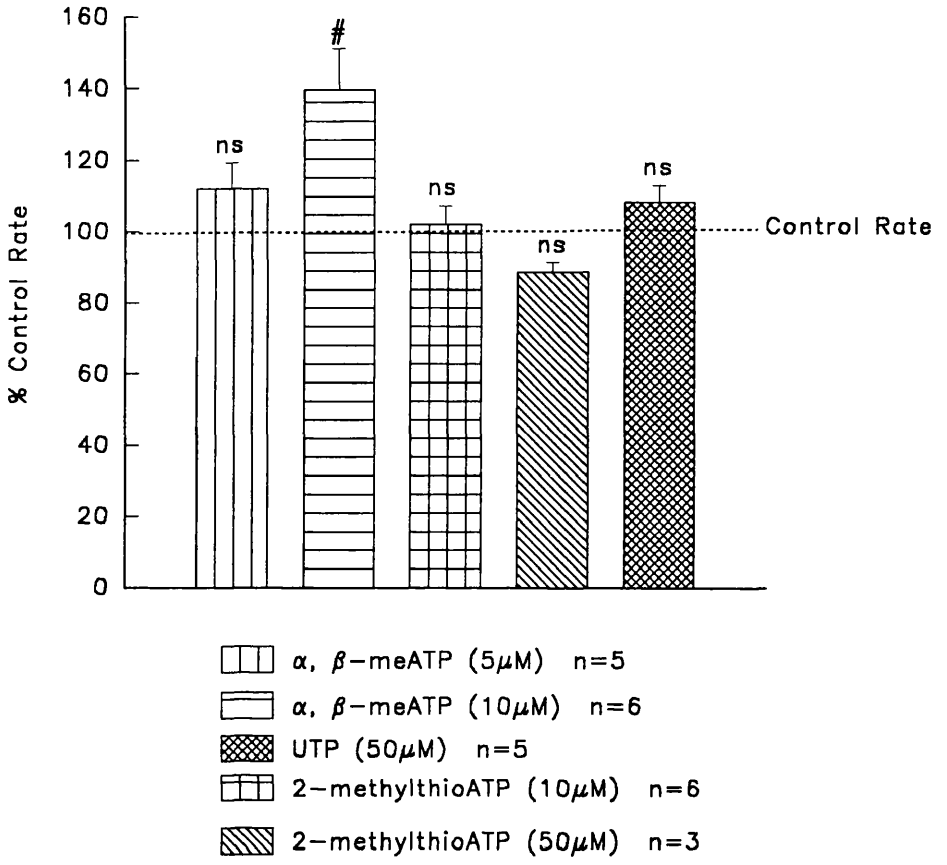



**Figure 15** Effect of ATP analogues on epileptiform activity. Results are taken at the end of a 10 minute perfusion. Only α, β-meATP (10μM) significantly changed the rate from control, # $P<0.05$.

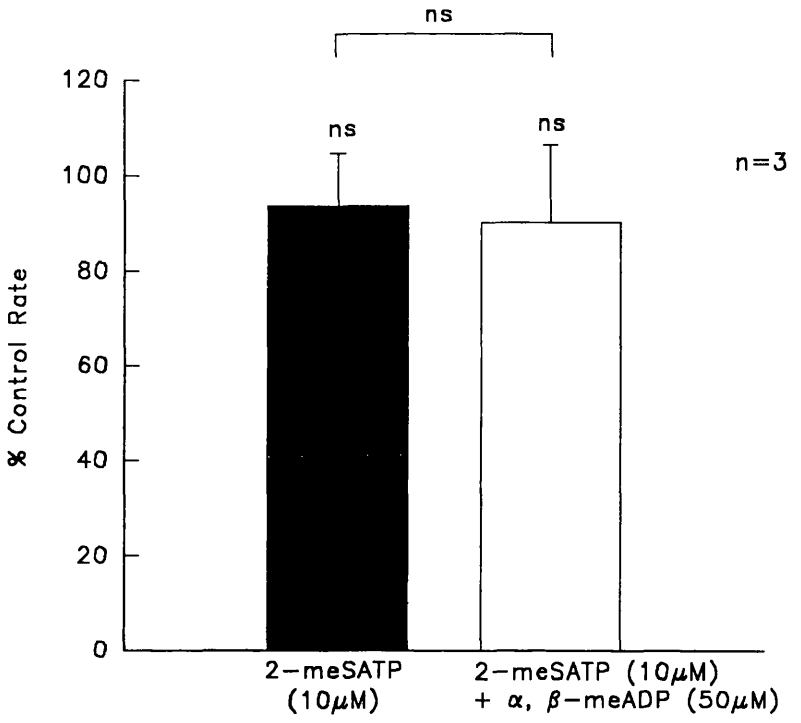



**Figure 16** The mean effect of perfusion with α, β-meADP, a 5'nucleotidase inhibitor, and 2meSATP. 2meSATP, neither alone nor alone or in combination with α, β-meADP, changed the rate of epileptiform activity.

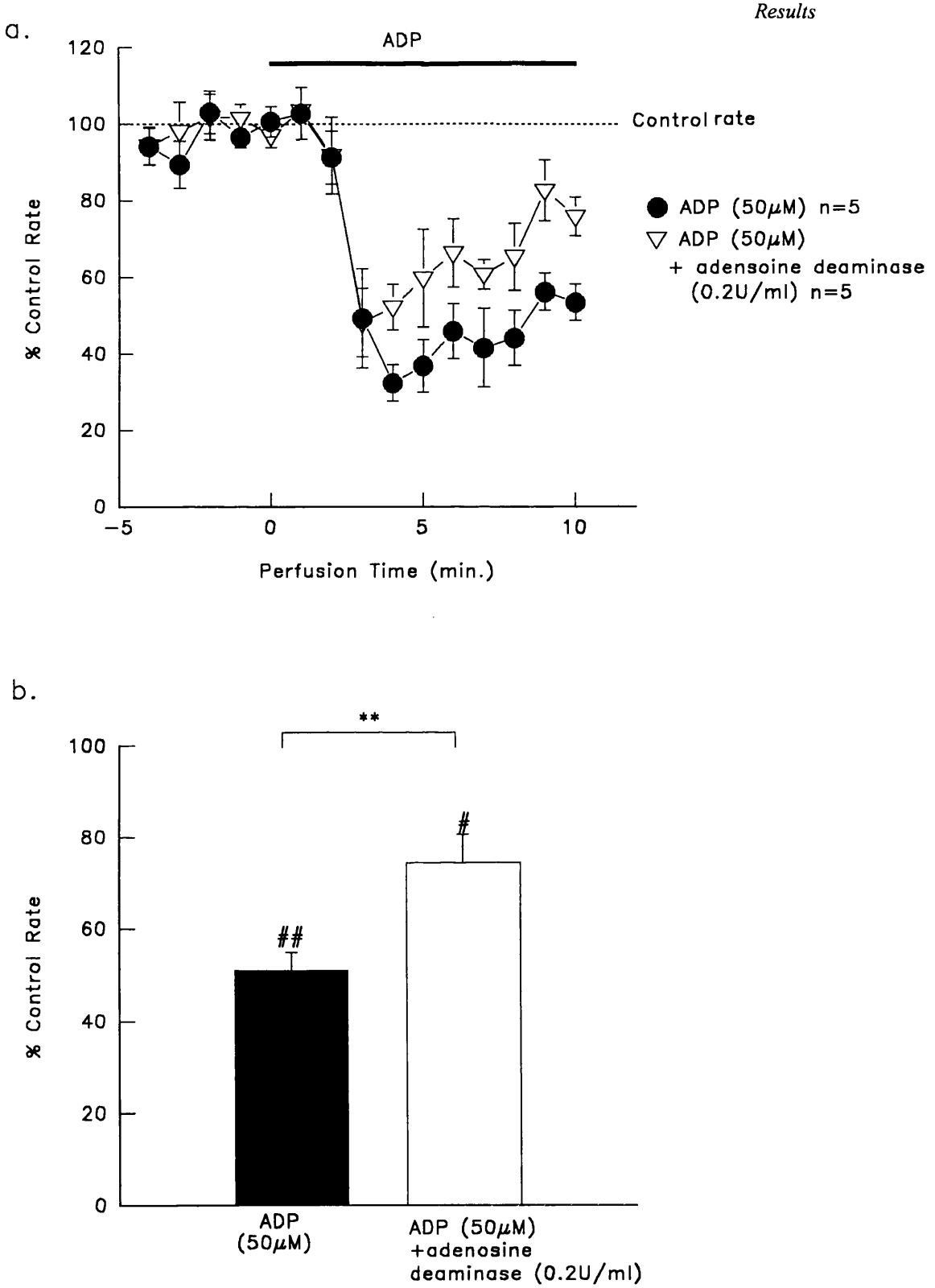



**Figure 17** The time course of the effect of adenosine deaminase on the depression of discharge rate by ADP is shown in (a). (b) is a summary of the effect at the end of a 10 minute perfusion. ADP significantly decreases the rate when perfused alone or with adenosine deaminase, # $P<0.05$, ## $P<0.01$. Adenosine deaminase reduced the effect of ADP, ** $P<0.01$.

### 3.2.5 *Nucleotide Antagonists*

Suramin and pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS), P2 receptor antagonists, were perfused independently to determine any effect on discharge frequency.

Preliminary experiments indicated that suramin alone had no effect on burst frequency at concentrations of 10 and 50μM whereas at 200μM there was a significant depression of activity (fig. 18a). A concentration of 50μM was therefore selected for use in experiments with ATP. Similarly PPADS at a concentration of 5μM did not alter the basal rate of activity (fig. 18b). Slices were exposed to suramin (50μM) and PPADS (5μM) for periods of 10 and 15 min. respectively prior to the addition of ATP or related analogues.

In experiments examining antagonism of the ATP depression, adenosine deaminase was always perfused concurrently with ATP to ensure the elimination of any effect of adenosine. Neither suramin (fig. 19) nor PPADS (fig. 20) inhibited the depressant action of ATP (50μM). In fact with both compounds there was a marked tendency for the effect of ATP to be enhanced and this enhancement was statistically significant in the case of PPADS (fig. 20c).

Similar conditions were used to investigate the effect of P2 antagonists on the increase of burst frequency induced by α, β-meATP. As in the earlier experiments with α, β meATP alone, this nucleotide again increased bursting rate, an effect which was prevented by suramin (50μM) (fig.21). Similarly in the presence of PPADS (5μM) α, β-meATP (10μM) no longer significantly altered the rate from control (fig. 22).

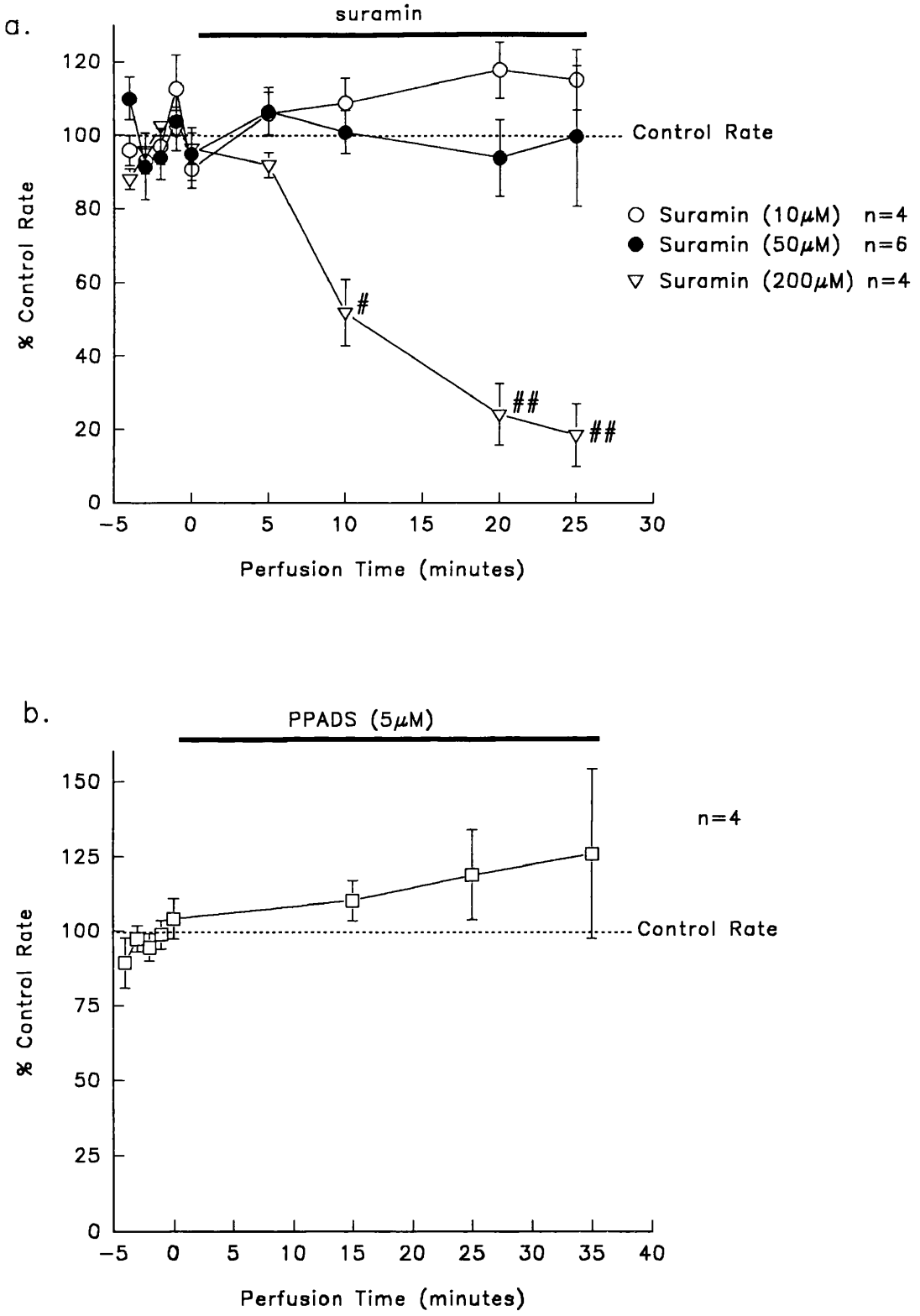



**Figure 18** (a) shows the time course of perfusion with suramin. Only at 200μM did suramin significantly depress the basal discharge rate. This was evident after 10 minutes perfusion until the end, # $P<0.05$, ## $P<0.01$. Perfusion with PPADS for 35 minutes did not significantly alter the frequency of spontaneous activity (b).

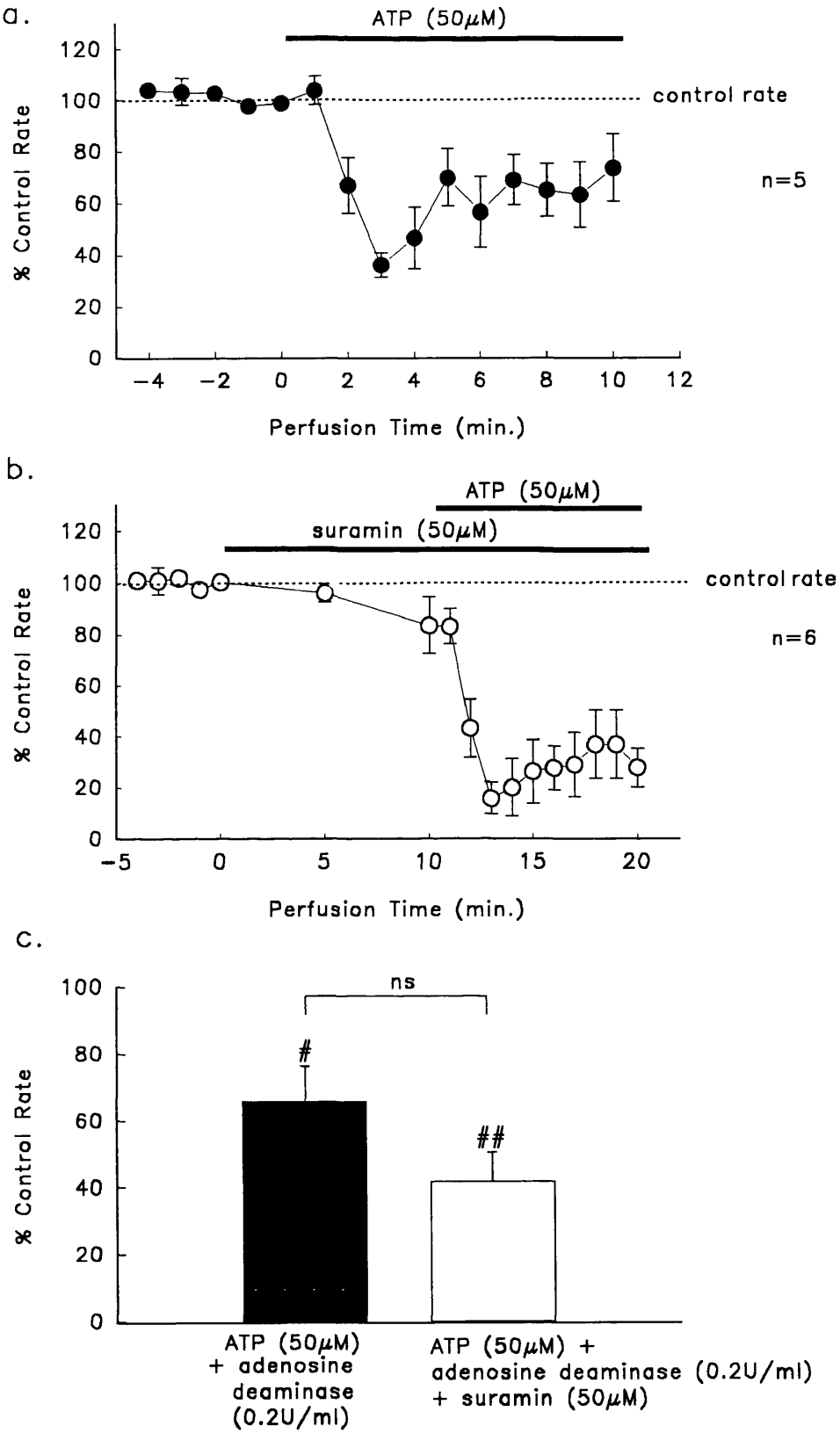



**Figure 19** (a) shows control responses to ATP in the presence of adenosine deaminase. Slices were washed with 0Mg/4AP medium for a minimum of 15 minutes between subsequent ATP additions. Suramin was perfused for 10 minutes prior to ATP and adenosine deaminase addition (b). Statistical analysis of the resultant effects are shown in (c) with # $P<0.05$, ## $P<0.01$.

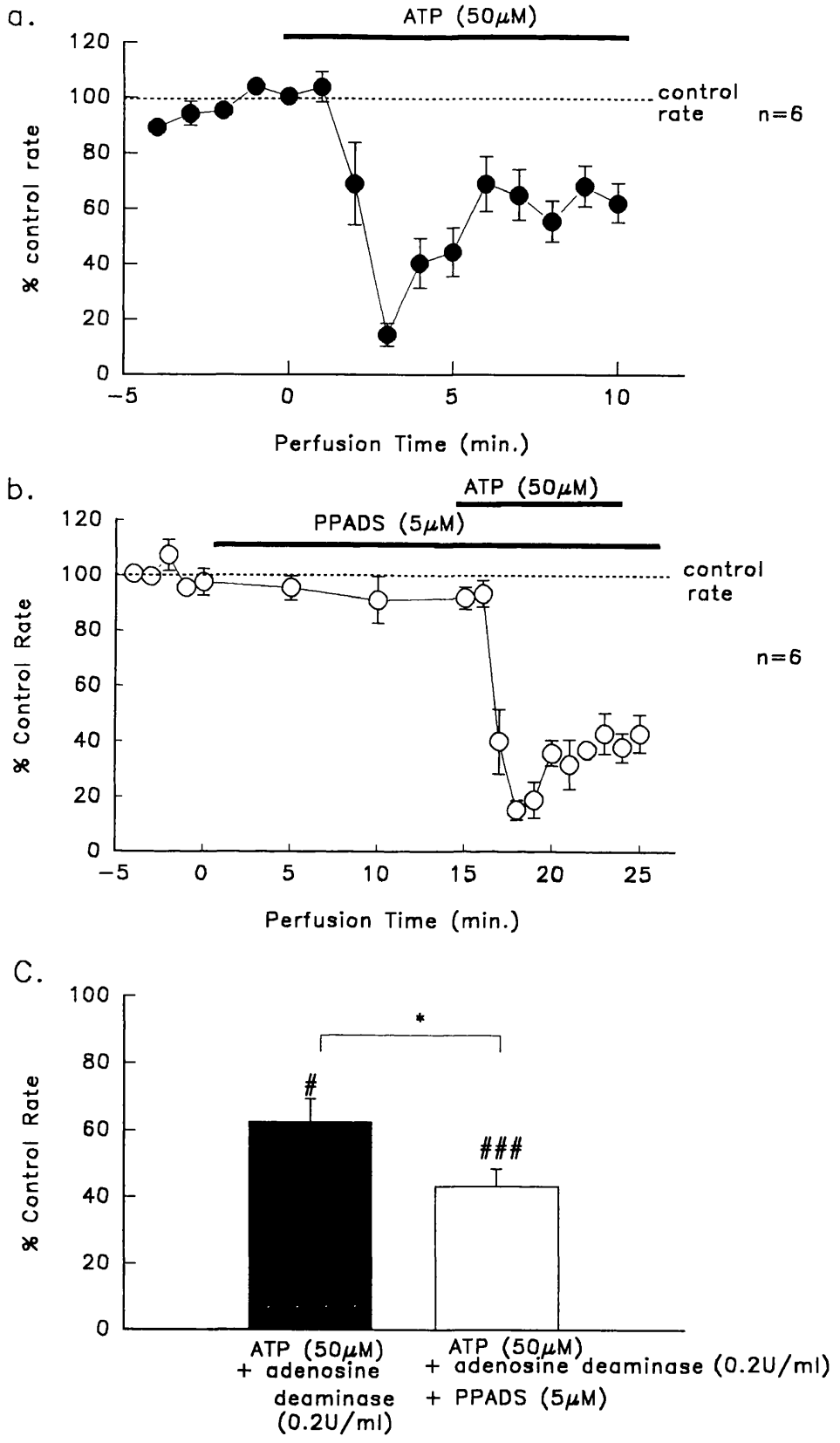



**Figure 20** (a) shows control responses to ATP and adenosine deaminase. PPADS was perfused for 15 minutes before the subsequent addition of ATP and adenosine deaminase (b). The resultant effects are summarised in (c). # $P<0.05$, ### $P<0.001$, * $P<0.05$.

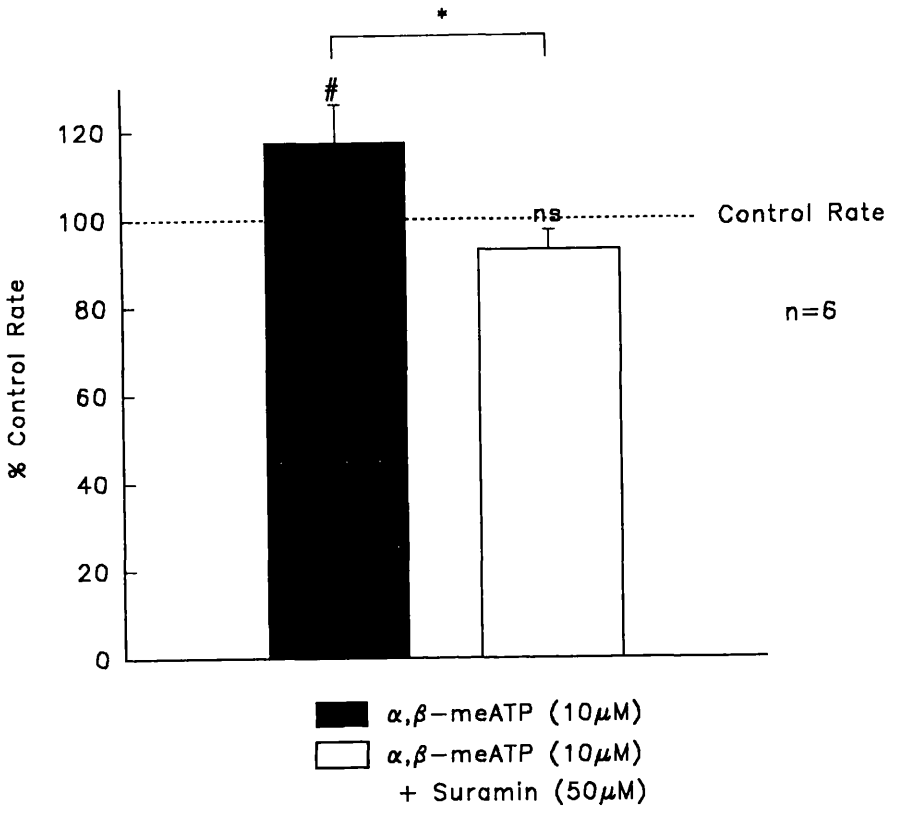



**Figure 21** The effect of the P2 antagonist suramin on the excitatory response of α, β-meATP. α, β-meATP increases burst rate to a significant level, # $P<0.05$. Suramin was perfused for 10 minutes before α, β-meATP was added. Suramin inhibited this excitation, * $P<0.05$.

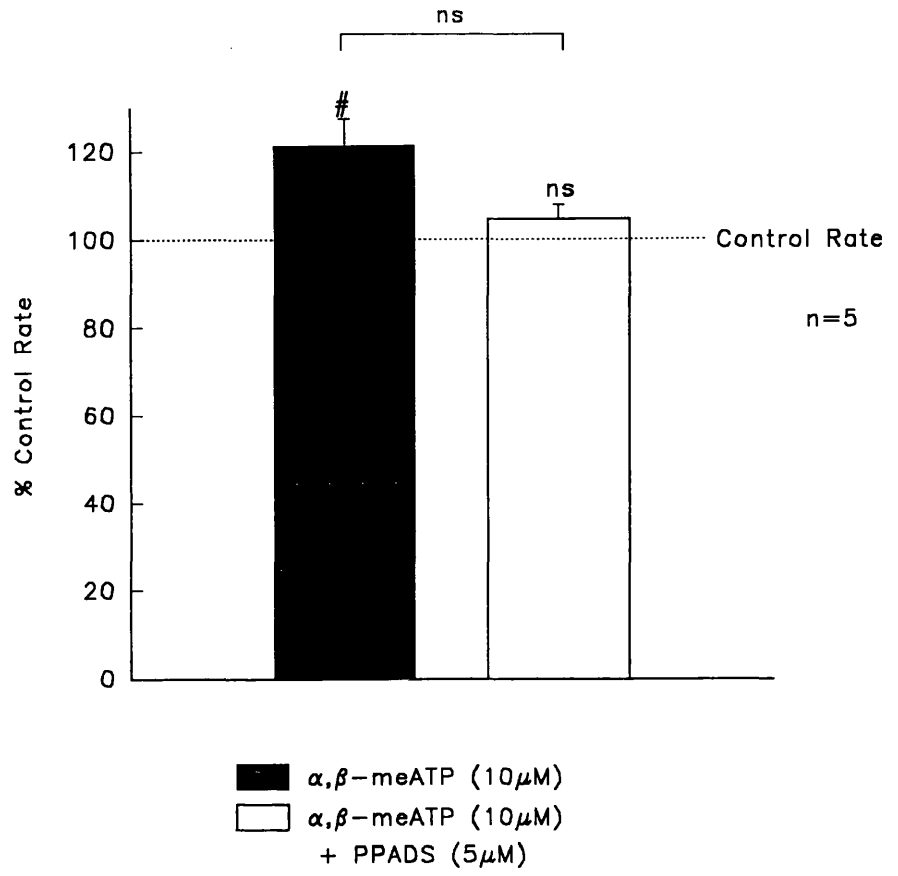



**Figure 22** α, β-meATP enhances the frequency of epileptiform activity, # $P<0.05$. The P2 antagonist PPADS was perfused for 15 minutes prior to the addition of α, β-meATP. PPADS inhibited the increase in rate from control.

### *3.2.6 Dependence of effect on the perfusion medium composition*

One of the major problems when using a modified medium is that the effect of the compound in question may vary according to the make-up of the medium. For these experiments epileptiform activity was generated using 0Mg/4AP medium. Once bursts were established the medium was changed to either 4AP (50μM) or $0Mg^{2+}/K^{+}$(6mM) and left for a minimum of 30 minutes to form a new burst pattern. In the initial experiments investigating the use of different media each slice was only used for one medium and hence only one addition of ATP or adenosine.

ATP (50μM), in the presence of adenosine deaminase (0.2U/ml), significantly reduced the rate of spontaneous activity (fig. 23). This depression produced by ATP (50μM) + adenosine deaminase (0.2U/ml) was significantly greater if the perfusion medium was changed to either OMg/K (6mM) or 4AP (50μM) (fig. 23). Figure 24 shows the extent of depression elicited by adenosine at the end of a 10 minute perfusion period. In all three media, OMg/4AP, 0Mg/K (6mM) and 4AP (50μM), adenosine significantly decreased the discharge rate. In the OMg/K (6mM) medium the effect of adenosine was significantly potentiated when compared to a 0Mg/4AP medium.

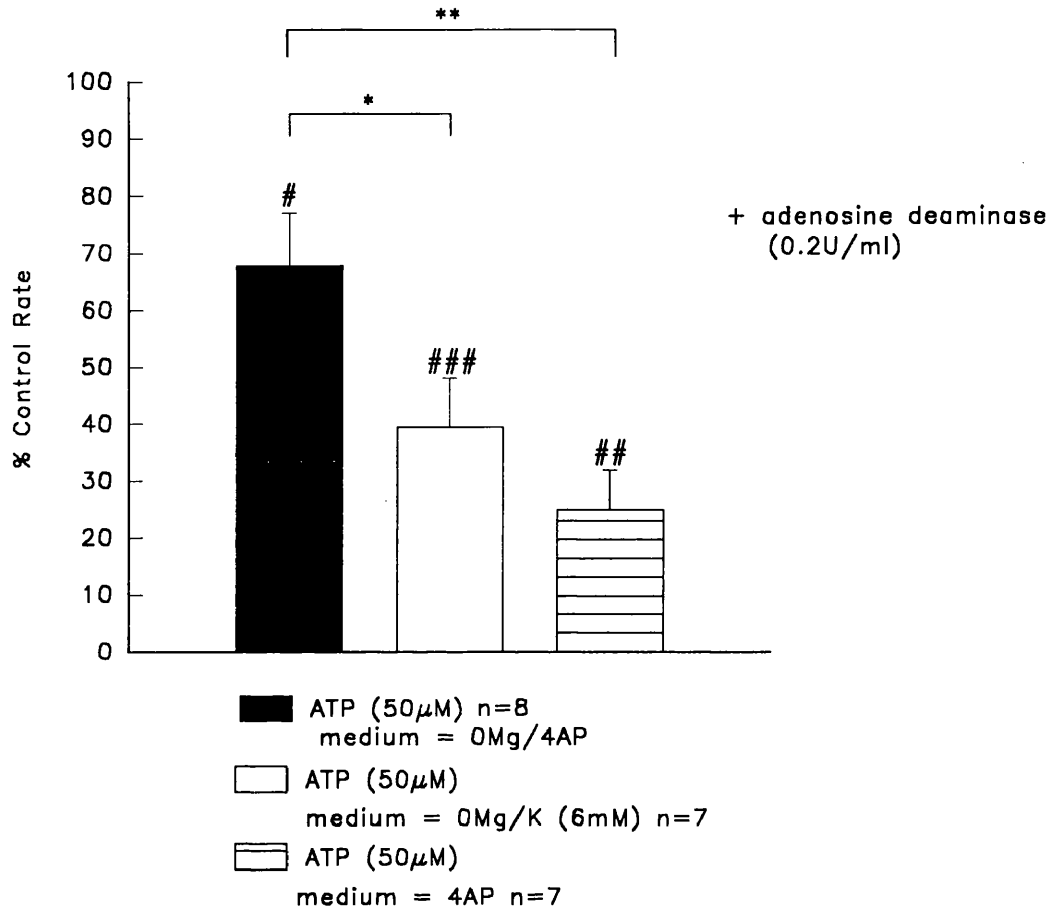



**Figure 23** The use of different media and the effect on the extent of ATP induced depression of activity. ATP, in the presence of adenosine deaminase, significantly decreased the discharge rate from control regardless of which medium was used, # $P<0.05$, ## $P<0.01$. The extent of inhibition exerted by ATP perfusion was potentiated significantly in a medium containing 0Mg/K (6mM) (* $P<0.05$) and in one with 4AP added (** $P<0.01$). For these experiments each slice did not have its own control.

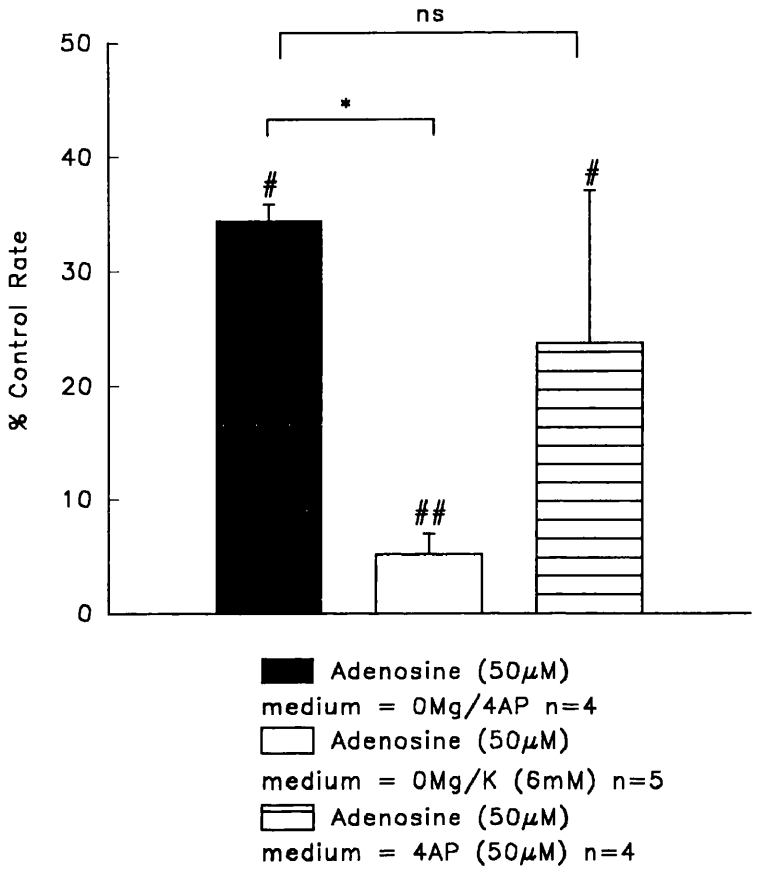



**Figure 24** Adenosine significantly depressed the frequency of activity in all three different media with # $P<0.05$ and ## $P<0.01$. The extent of inhibition produced by adenosine was potentiated in a medium without added 4AP (OMg/K (6mM)), * $P<0.05$. In these experiments different slices were used for each addition of adenosine.

### *3.2.7 Combination of ATP and adenosine*

The combined effect of ATP and adenosine was investigated at 10 and 50μM. ATP (10μM) had no significant effect on the frequency of spontaneous activity whereas adenosine decreased discharge rate by approximately 35%. The combined effect of ATP and adenosine (10μM) depressed activity by about 45% which is significantly different from that of ATP but not from adenosine perfused alone (fig. 25). At the higher concentration of 50μM the effect of neither ATP, adenosine nor ATP + adenosine are significantly different from each other (fig. 26).

In the majority of experiments a control response to either ATP, adenosine or any other compound that was being investigated was undertaken on each slice with a minimum of 15 minutes between subsequent additions. To verify that this is a sufficient period two sequential responses to ATP (both in the absence and presence of adenosine deaminase) and adenosine were measured. Figure 27 shows that both the first and second additions of ATP or adenosine result in effects which are not significantly different from each other.

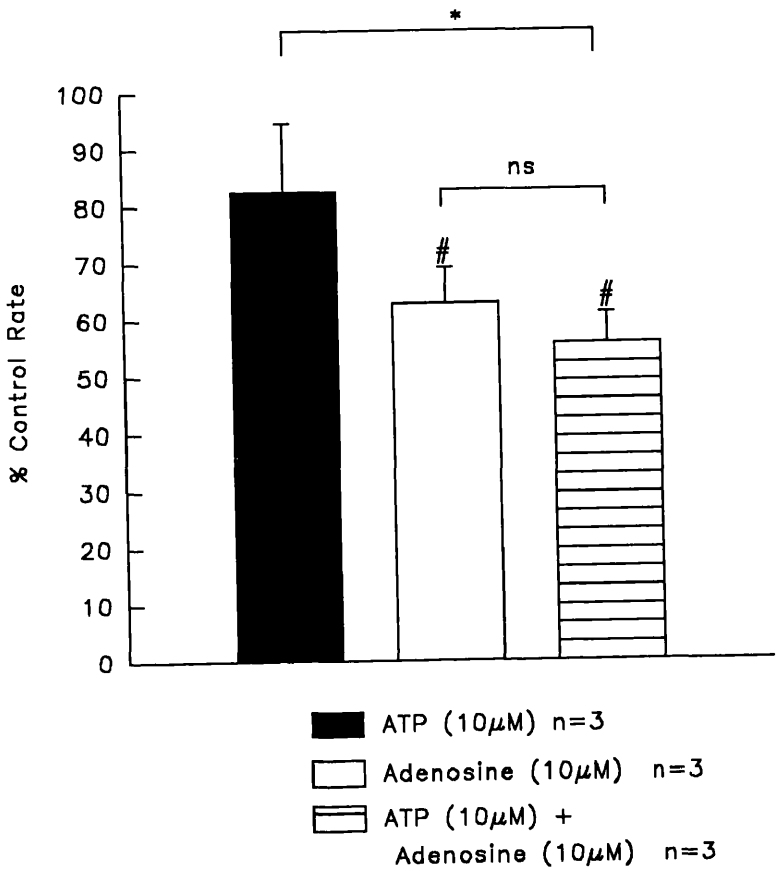



**Figure 25** The combined effect of ATP and adenosine. Each slice was exposed to ATP, adenosine and ATP + adenosine with at least 15 minutes between each addition. Adenosine and ATP + adenosine significantly depressed the rate of activity, # $P<0.05$. The effect of ATP and adenosine combined was not different from that of adenosine alone but was significantly lower than that of ATP alone, * $P<0.05$.

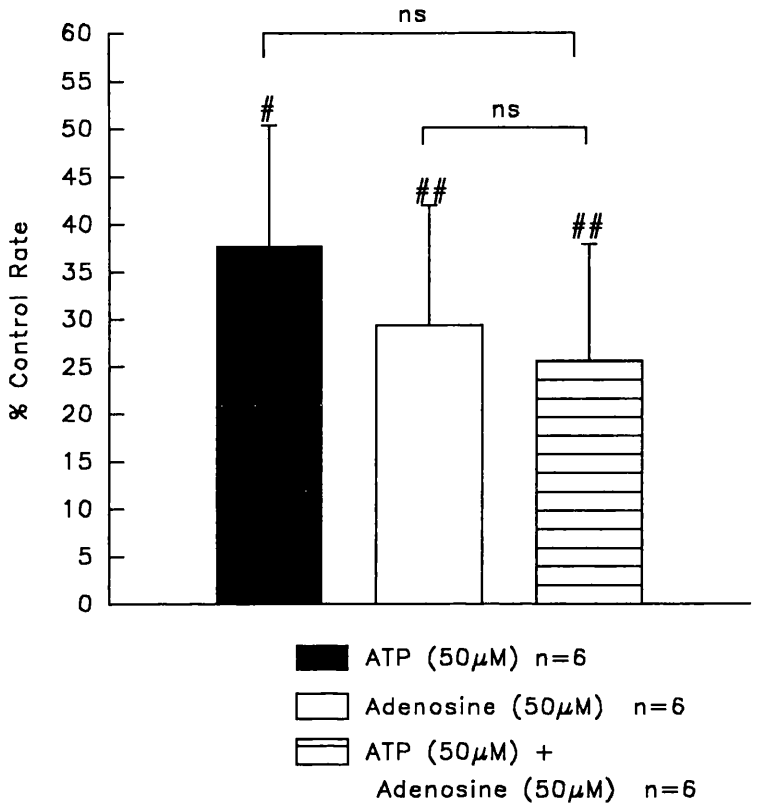



**Figure 26** The combined effect of ATP and adenosine at a higher concentration. Each slice was exposed to ATP, adenosine and ATP + adenosine with at least 15 minutes between each addition. ATP and adenosine alone and in combination depressed the discharge rate with no significant difference between each other. # $P<0.05$, ## $P<0.01$.

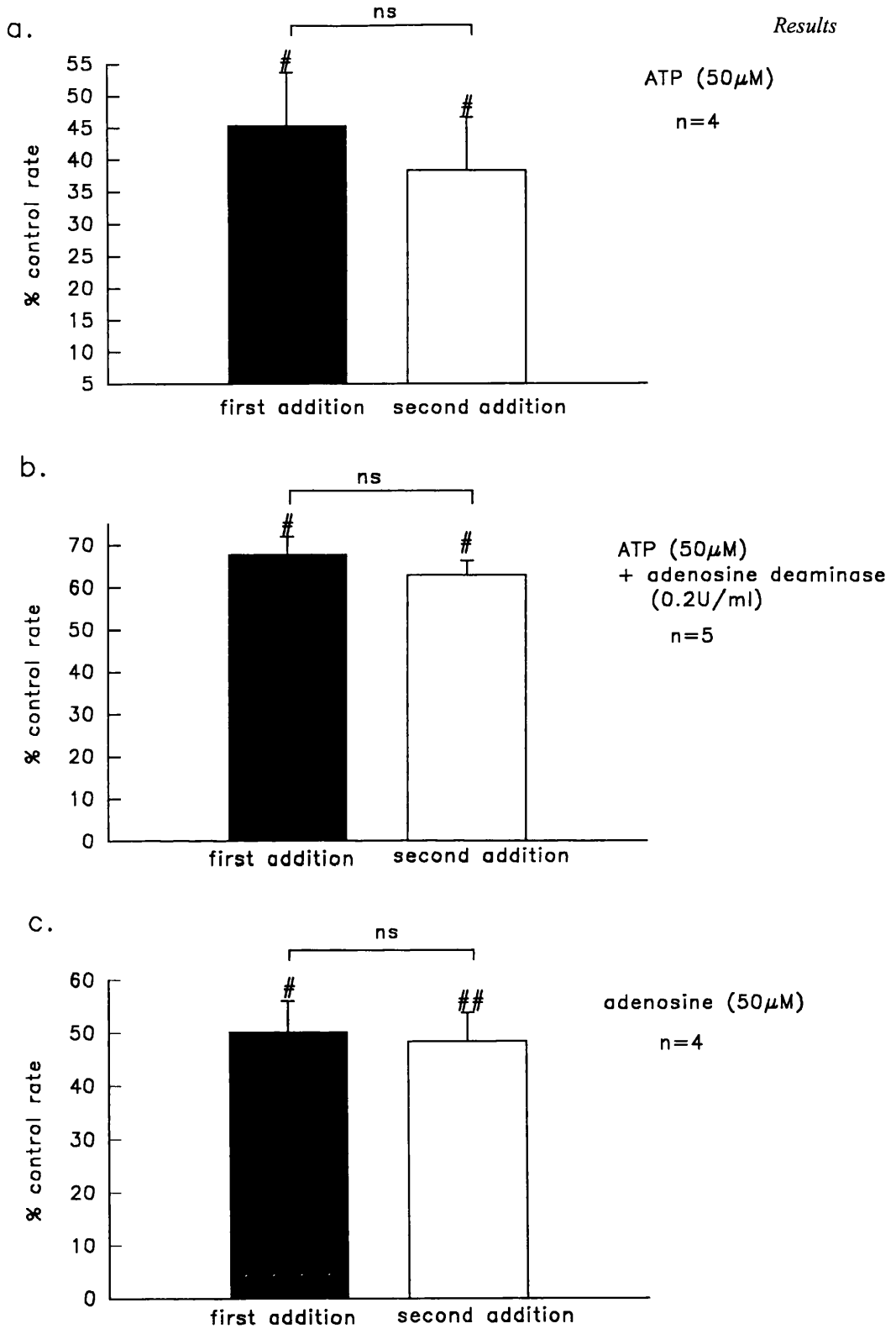



**Figure 27** Slices were exposed to ATP or adenosine for 10 minutes, washed for 15 minutes before ATP or adenosine was reperfused for a further 10 minutes. ATP, with or without adenosine deaminase, and adenoisne significantly depressed the discharge rate with # $P<0.05$ and ## $P<0.01$. (a), (b) and (c) illustrate that the there is no significant difference in the extent of inhibition exerted between the first and subsequent additions of ATP or adenosine.

### *3.2.8 Zinc*

In control experiments zinc acetate (10 or 100μM), the source of zinc used, did not alter the rate of epileptiform activity to any significant degree over the 40 minute perfusion period (fig. 28a). Neither concentration of zinc potentiated or decreased the depression of discharge rate produced by ATP (50μM) (fig. 28b, c).

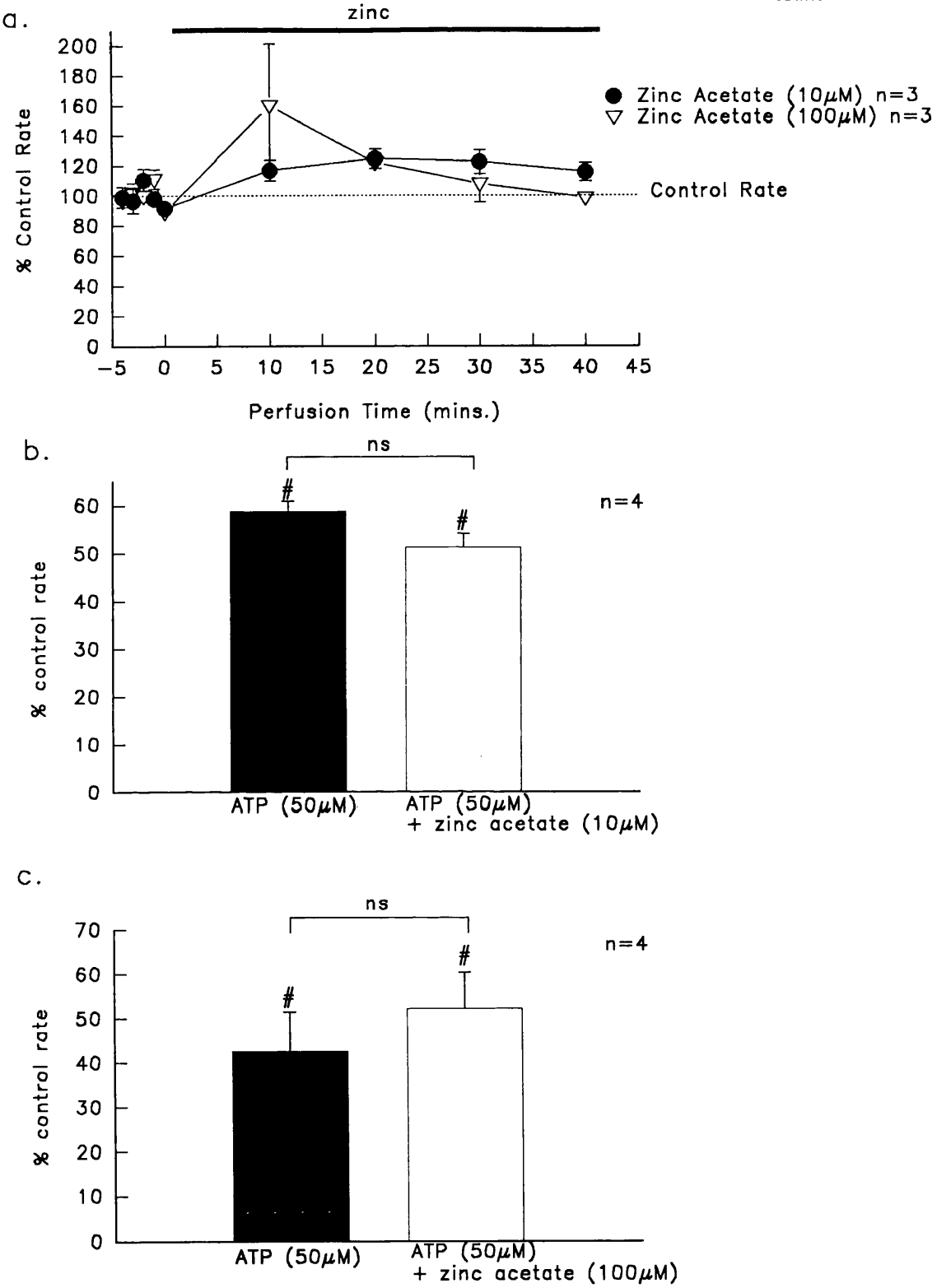



**Figure 28** (a) show the time course of perfusion with zinc acetate for 40 minutes. ATP significantly decreased the discharge rate at the end of a 10 minute perfusion period with # $P<0.05$. Zinc was perfused for 10 minutes before ATP was added to the perfusing medium. (b) and (c) show that the presence of zinc at 10 and 100μM respectively does not alter the effect of ATP on the rate of epileptiform activity.

### *3.2.9 AMP*

AMP at 50μM depressed the rate of epileptiform activity by approximately 60% (fig. 29). This effect was rapid in onset and, as with ATP and adenosine, had a slight tendency to drift back towards control during the perfusion time (fig. 29a).

5'-adenylic acid deaminase (AMPase) deaminates AMP to IMP. AMPase (0.2U/ml) decreased the rate of activity to a small extent, but over a 10 minute perfusion period this did not reach significance (fig. 30). The depression of activity caused by AMP (50μM) was totally inhibited when AMPase (0.2U/ml) was co-perfused with AMP (fig. 29).

Adenosine deaminase (0.2U/ml), a concentration which totally inhibited the effect of adenosine (fig. 8), decreased both the maximum and the mean inhibition at 10 min. produced by AMP (50μM) (fig. 31b, c). However AMP in the presence of adenosine deaminase still depressed the rate of epileptiform activity to 36.39%±7.94 of control maximally and 60.93 %±2.92 of control at the end of a 10 minute perfusion.

Fig. 32a and b illustrate the time course of the depression caused by AMP (50μM) and the inhibition of this effect by CPT (100nM) respectively. In these experiments CPT was perfused for 10 minutes prior to the addition of AMP in the continued presence of CPT. In the histogram (fig. 32c) the effect of AMP + CPT is expressed as a percentage of the rate after CPT had been perfused for 10

minutes. From this it can be seen that CPT significantly inhibited any depression caused by AMP. This is similar to the findings with ATP and adenosine.

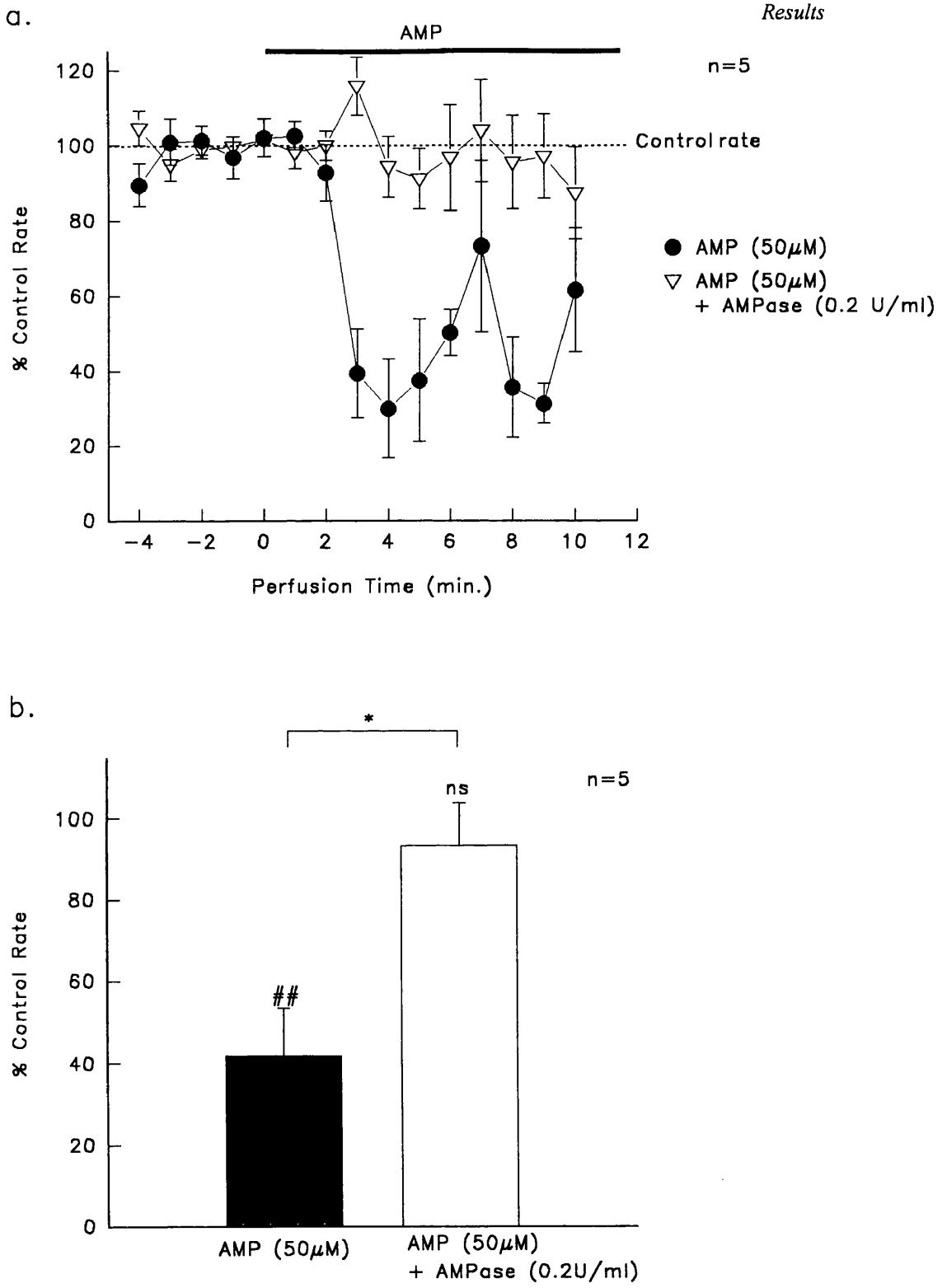



**Figure 29** The time course of the decrease in discharge rate by AMP and the inhibition by AMPase is shown in (a). The mean effect at the end of a 10 minute perfusion is shown in (b). AMP significantly alters the rate from control, ## $P<0.05$. AMPase significantly reduces the effect of AMP, * $P<0.05$.

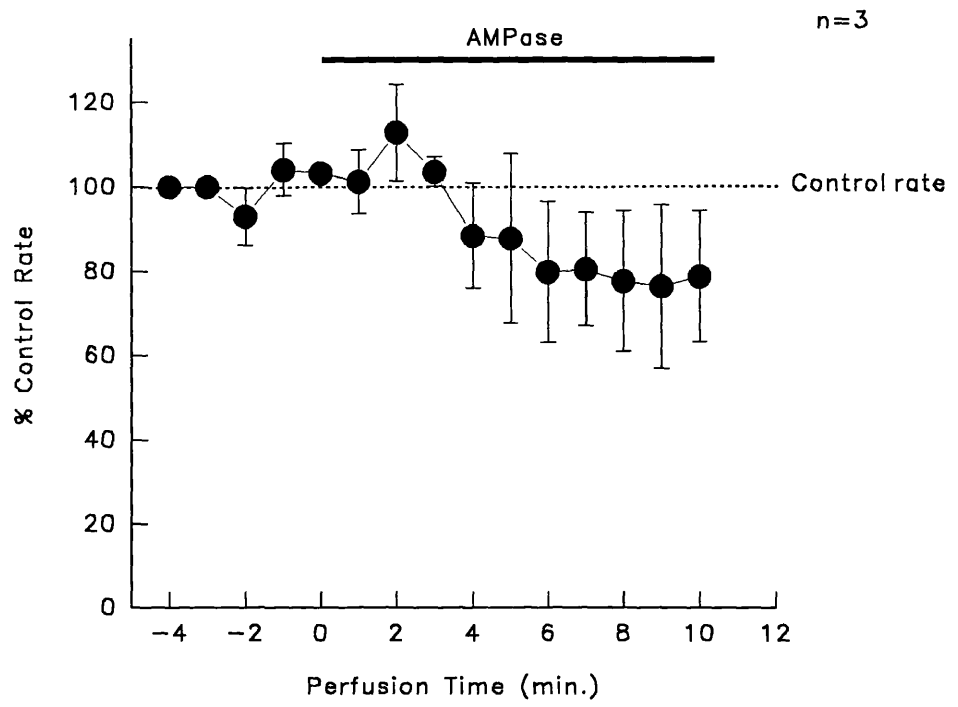



**Figure 30** The effect of AMPase on the rate of epileptiform activity. During the 10 minute perfusion the rate did not differ significantly from control.

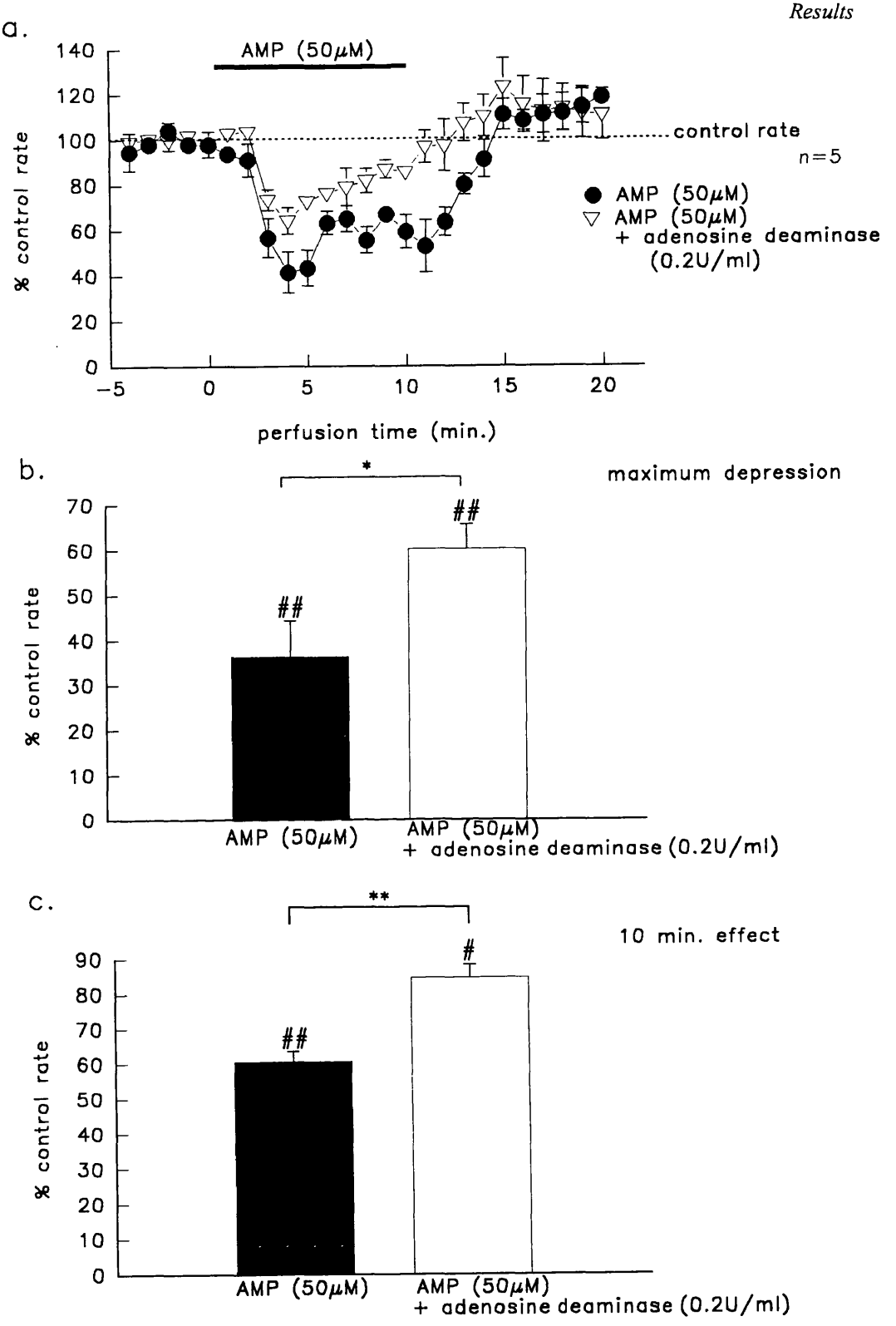



**Figure 31** (a) shows the time course of the effect of AMP alone and in the presence of adenosine deaminase. The maximum degree of inhibition and the mean effect at the end of a 10 minute perfusion of AMP and AMP + adenosine deaminase are represented in (b) and (c) respectively. # $P<0.05$, ## $P<0.01$. Both the maximum and 10 minute effect of AMP alone and in the presence of adenosine deaminase are significantly different with * $P<0.05$ and ** $P<0.01$.

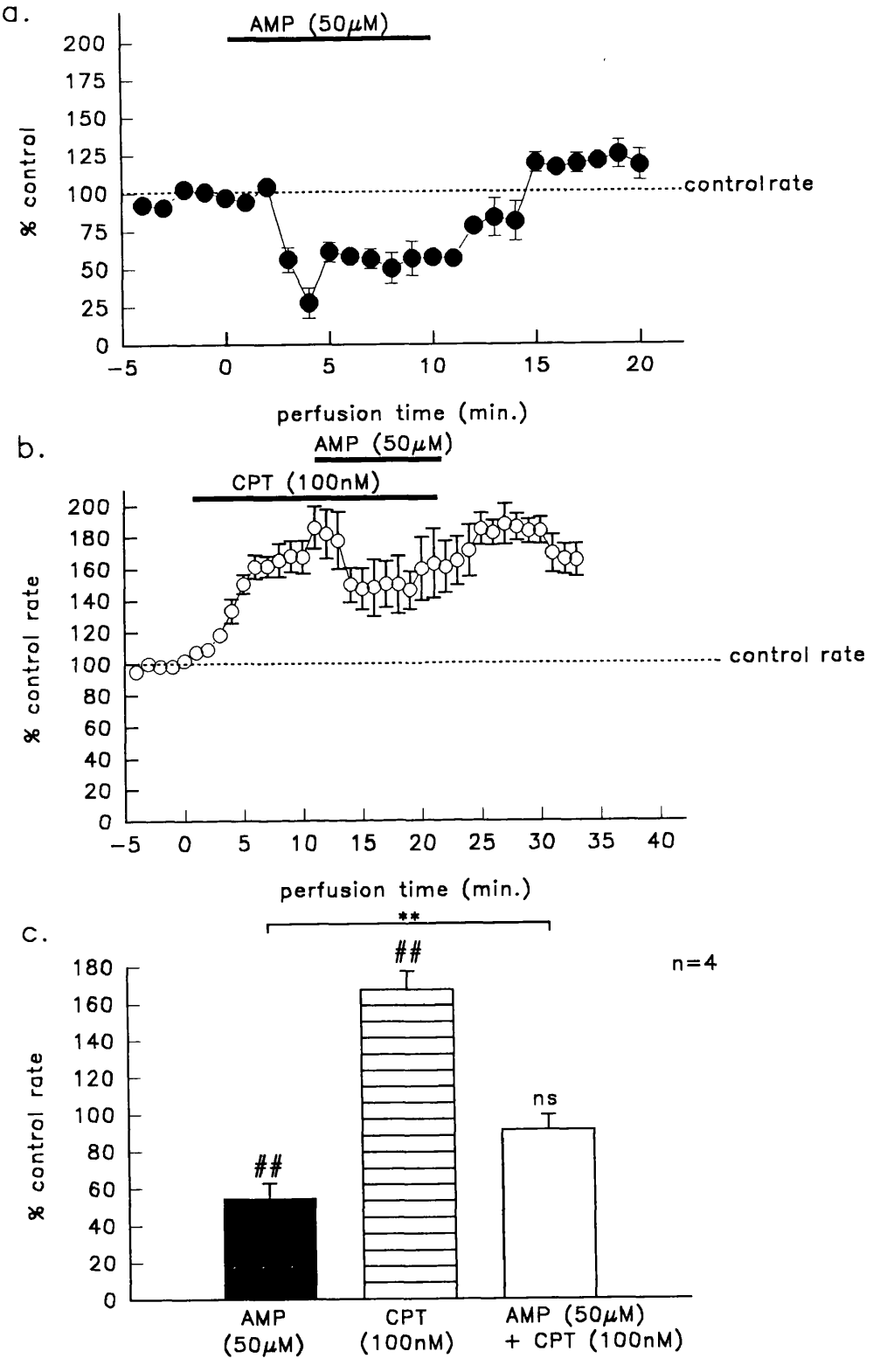



**Figure 32** The time course of a control response to AMP and the affect of CPT on this effect is shown in (a) and (b) respectively. CPT was perfused for 10 minutes prior to the perfusion of both CPT and AMP. (c) shows that AMP significantly decreased the burst rate whereas CPT increased it, ## $P<0.01$. The effect of CPT + AMP is expressed as a percentage of the rate after 10 min. perfusion with CPT. CPT inhibits the depression of activity caused by AMP, ** $P<0.01$.

*3.2.10 5'-adenylic acid deaminase*

ATP is metabolised through a number of stages by ecto-ATPases. One such metabolite is AMP. In order to neutralise any AMP produced during the perfusion of ATP, AMPase was used to the concentration which inhibited the effect of AMP in the experiments just described. Early in the perfusion of ATP the inhibition of burst frequency was fully prevented by AMPase, although the rate of bursting declined progressively throughout the application. Nevertheless, by the end of the ATP application, AMPase still reduced the effect sufficiently that ATP no longer depressed burst frequency below control levels (fig. 33c). Indeed, the degree of inhibition produced by ATP plus AMPase was similar to that produced by the enzyme alone (fig. 30) and may therefore be attributed to an action of the enzyme itself. The maximum amount of inhibition produced by ATP was reduced from 75%±5.71 to 39%±10.28 (fig. 33c).

ATP at the higher concentration of 200μM depressed the activity rate to approximately 30% of control (fig. 34). AMPase (0.2U/ml) inhibited the depression of activity caused by ATP (200μM), with a significant difference between the effect of ATP alone and in the presence of AMPase. The maximum amount of inhibition produced by ATP was reduced from 96%±3.45 to 20%±11.98 (fig. 34b).

The metabolism of AMP to adenosine involves the action of 5'-nucleotidase. AMP (50μM), in the presence of 5'-nucleotidase (0.2U/ml) and adenosine deaminase (0.2U/ml) to inactivate any adenosine formed, no longer reduced the

rate of epileptiform activity with both the maximum and 10 min. effects not differing significantly from control values (fig. 35). In the presence of 5'-nucleotidase and adenosine deaminase, ATP (50μM) showed an initial depression which did not occur with AMP, although the inhibitory effect of ATP was reduced to a level which was not significantly different from control values (fig. 36b). By the end of the 10 minute perfusion period the discharge rate had returned to control rate (fig. 36). α, β-meADP (50μM), an inhibitor of 5'-nucleotidase, failed to alter the depression produced by ATP (25μM) in 2 experiments (fig. 37).

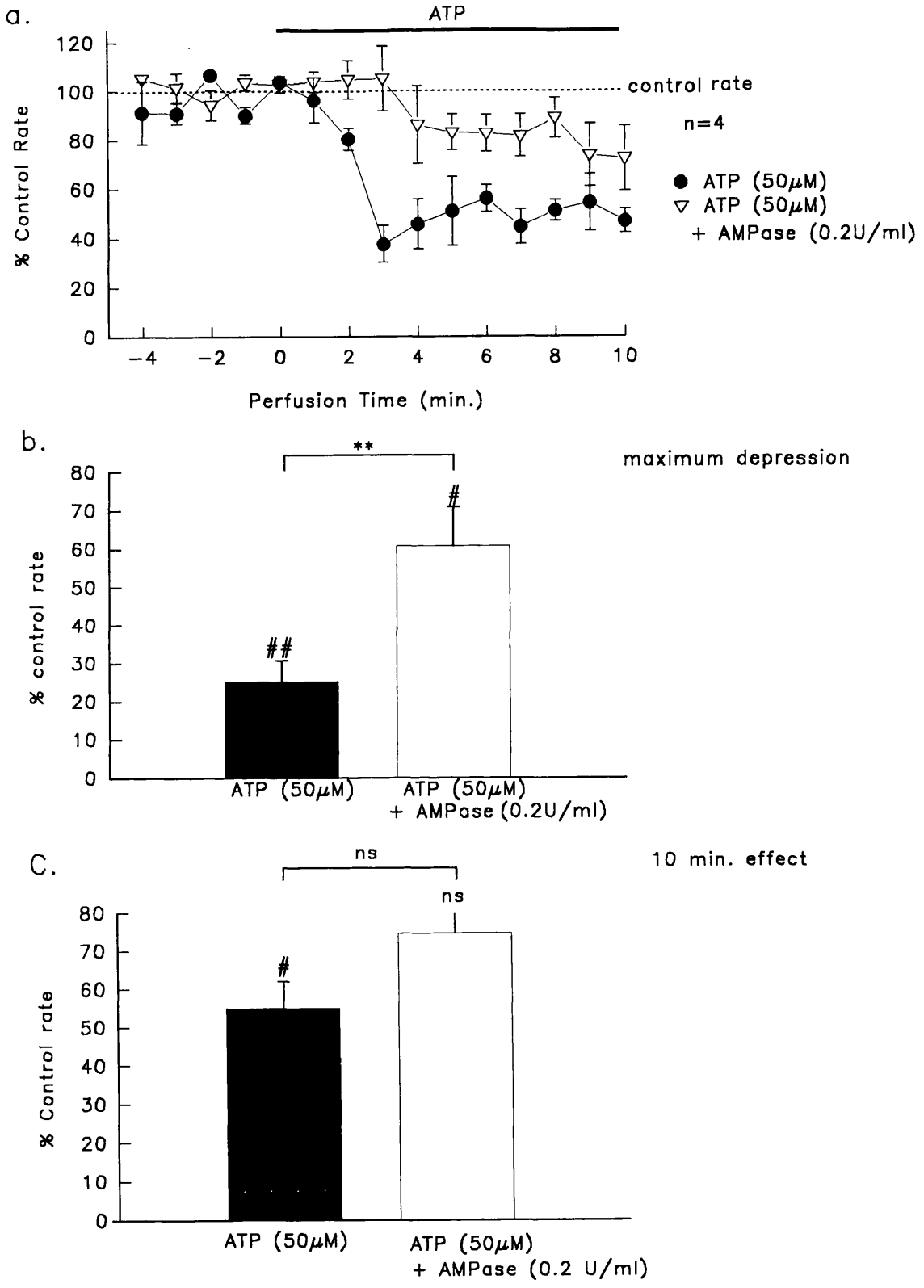



**Figure 33** The effect of AMPase on the depression of activity produced by ATP. (a) shows the time course of both ATP alone and when perfused with AMPase. The resulting effect at the end of 10 minute perfusion are shown in (c). ATP significantly alters the rate from control. (b) shows the mean ± s.e.m. of the maximum extent of inhibition in individual slices. # $P<0.05$, ## $P<0.01$, ** $P<0.01$.

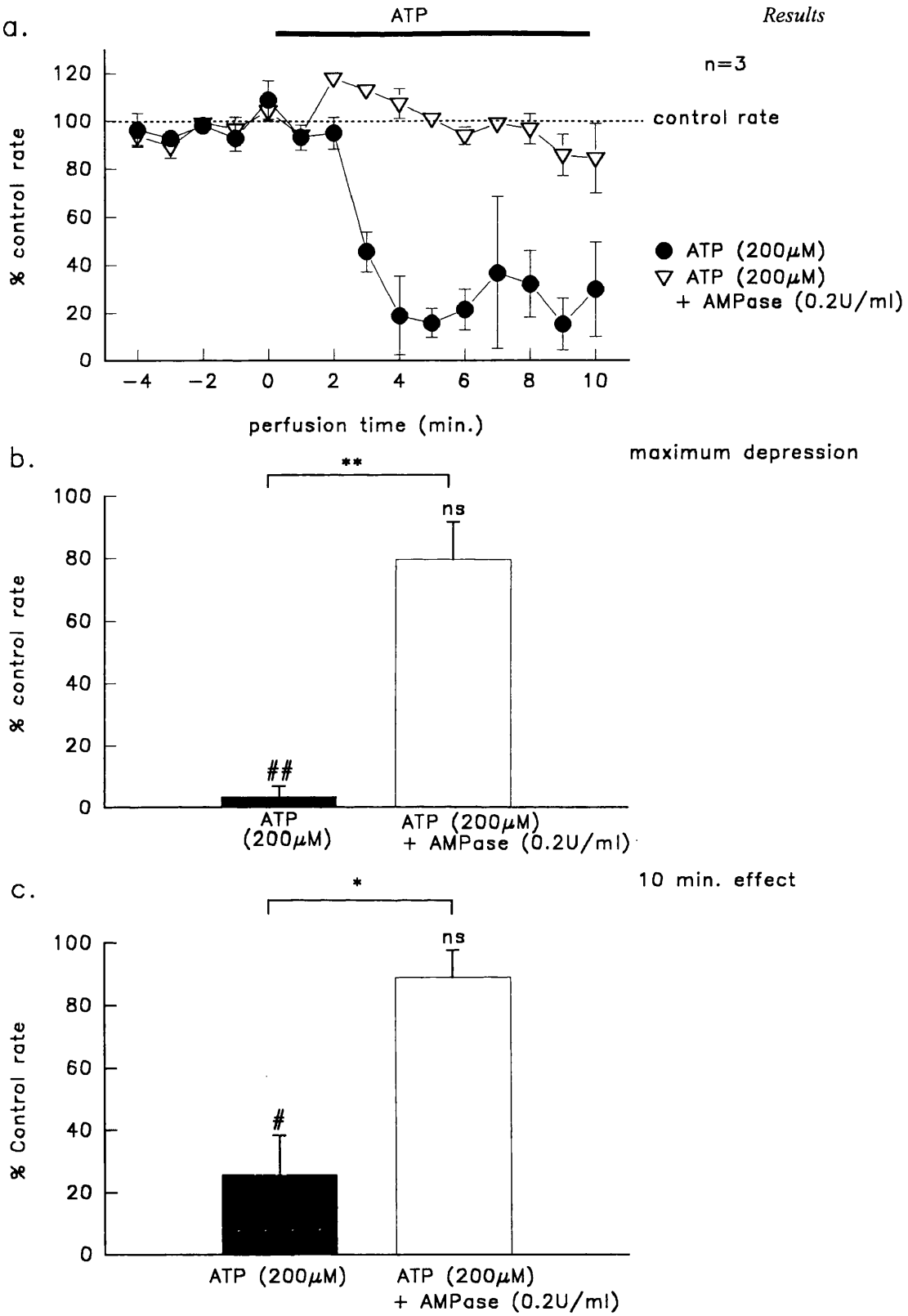



**Figure 34** (a) represents the time course of the effect of ATP (200μM) ± AMPase on the rate of activity. (b) and (c) show the maximum inhibition and net effect at the end of 10 minutes perfusion produced by ATP resepectively. The results are calculated as the mean ± s.e.m. of the maximum effect in individual slices, not necessarily at the same time point. # $P<0.05$, ## $P<0.01$, * $P<0.05$, ** $P<0.01$.

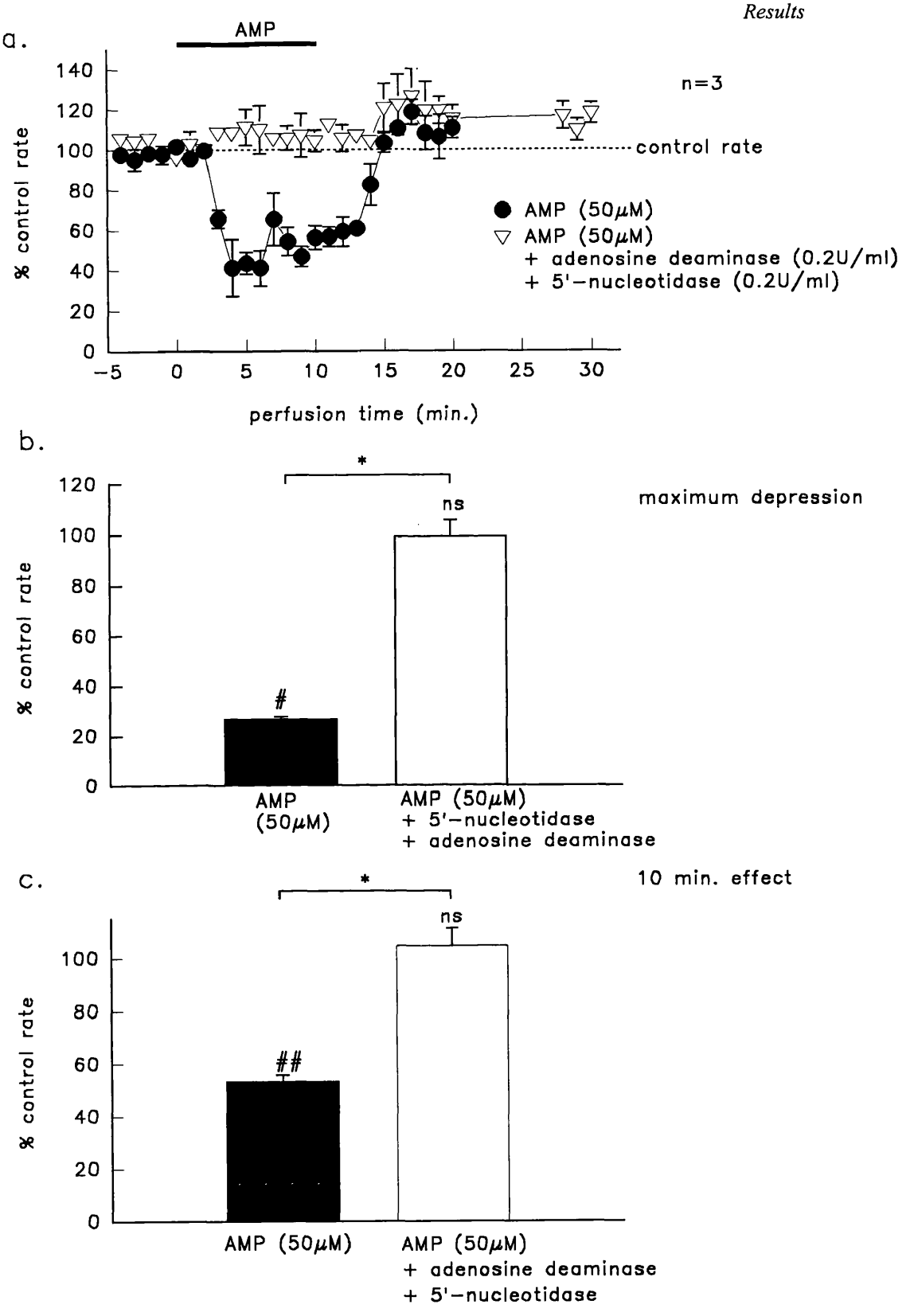



**Figure 35** The time course of the effect of AMP alone and with 5'-nucleotidase and adenosine deaminase is shown in (a). The maximum extent of inhibition and the 10 min. effect are analysed in (b) and (c) respectively. # $P<0.05$, * $P<0.05$, ** $P<0.01$.

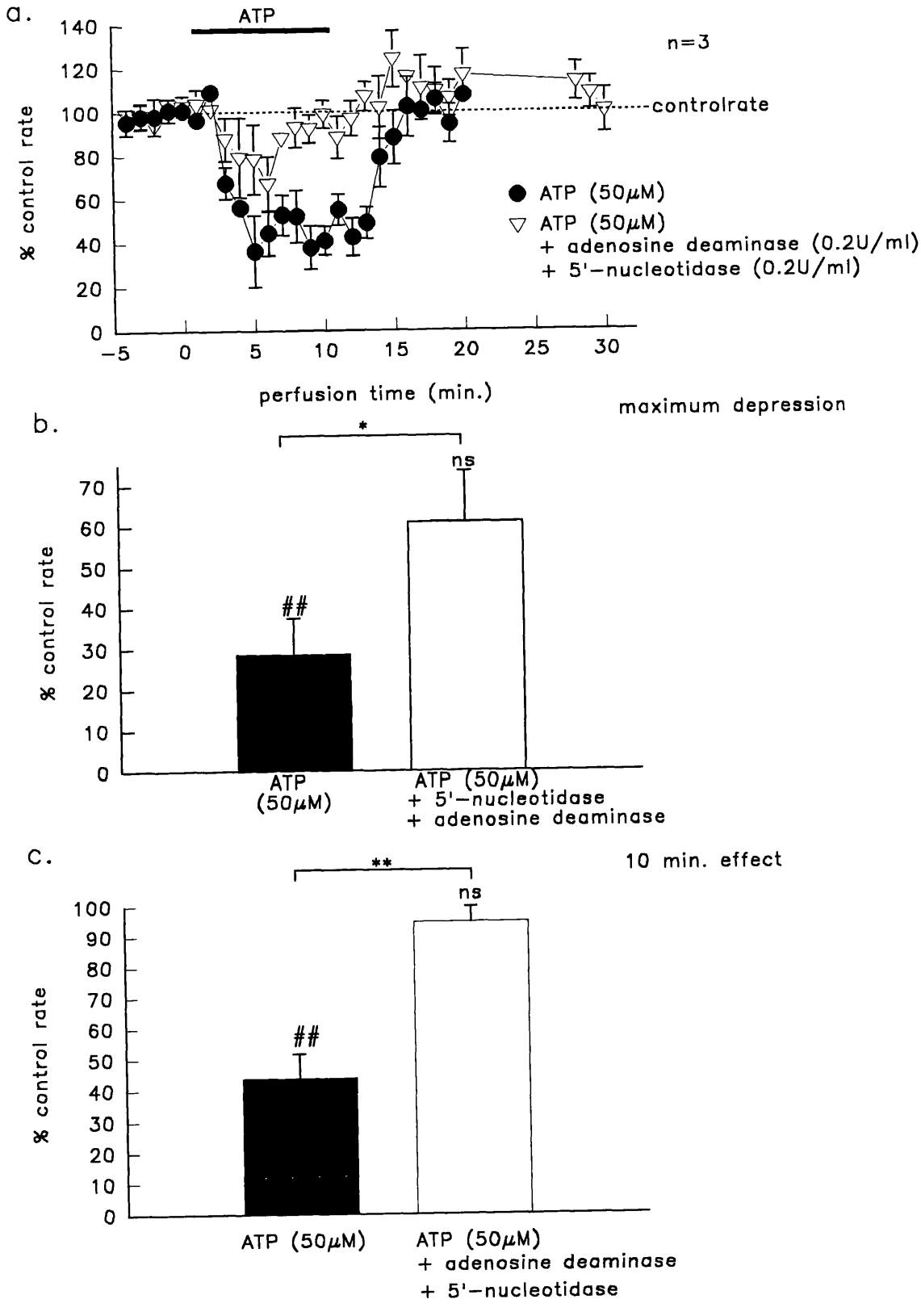



**Figure 36** (a) illustrates the inhibition by 5'-nucleotidase and adenosine deaminase on the depression of activity produced by ATP (50μM). (b) and (c) respectively represent the maximum and 10 min. effect of ATP both alone and in the presence of 5'-nucleotidase and adenosine deaminase. ## $P<0.01$, * $P<0.05$, ** $P<0.01$.

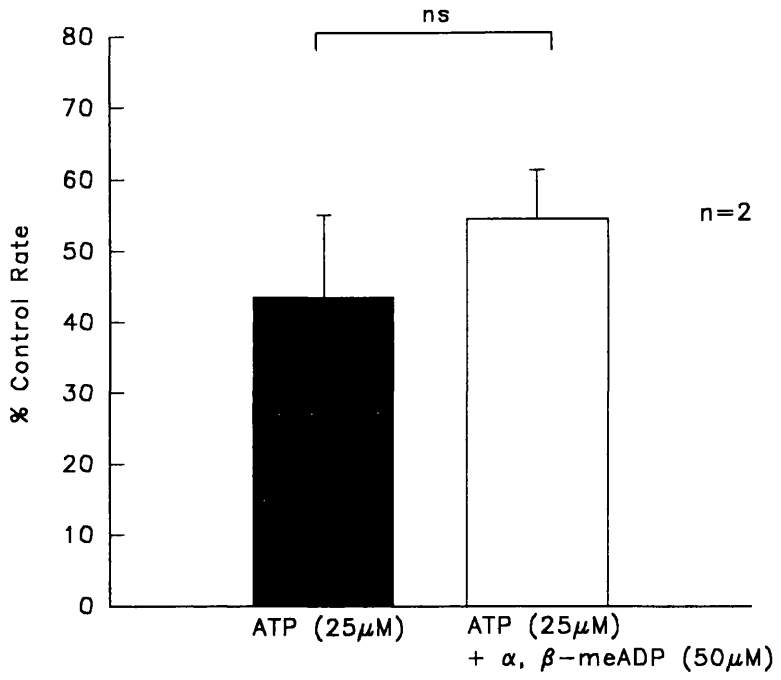



**Figure 37** The effect of inhibition of 5'nucleotidase. In the two experiments in which ATP was perfused with α, β-meADP, the effect of ATP at the end of a 10 minute perfusion period did not appear to be altered to any great extent. Due to the low n number no statistical analysis was performed on this set of results.

### 3.3 BenzoylbenzoicATP

BenzoylbenzoylATP (BzATP) is the most potent agonist at the $P2X_7$ or $P_{2Z}$ receptor which is characterised by forming pores in the membrane allowing the passage of small molecules. BzATP (10μM) had no effect on the rate of activity. However at 50μM, BzATP caused a decrease in rate of approximately 25% (fig. 38). In contrast to the other nucleotides used, the effect of BzATP (50μM) did not reverse during the wash period, but instead the rate continued to fall over the subsequent 10 minutes (fig. 38a). Unfortunately the wash period was not extended further and hence whether a recovery or a further drop in rate would follow is not apparent.

CPT (100nM), as reported previously, increased the frequency of spontaneous activity in this instance to approximately 170% control (fig. 39b). The perfusion of BzATP (50μM) with CPT (100nM) resulted in a significant elevation in the discharge rate compared with control (fig. 39) which was significantly lower than that of CPT alone thus implying an addition of excitatory and inhibitory responses. However, the situation is complicated by the fact that in this instance the control CPT response is larger than when performed earlier in the project (fig. 9). It is possible that the effect of CPT, and indeed any other $A_1$ antagonist, may be dependent on the level of endogenous adenosine. Correlation graphs between initial rate and effect of CPT were therefore constructed both from this experiment and from the ATP/adenosine one (fig. 40). First order (linear) regression analysis was performed. In fig. 40a the initial rate is plotted against the % control rate after

perfusion with CPT ($r = 0.6668$; $P<0.05$). In fig. 40b initial rate is plotted against the actual rate achieved after perfusion with CPT ($r = 0.9657$; $P<0.0001$).

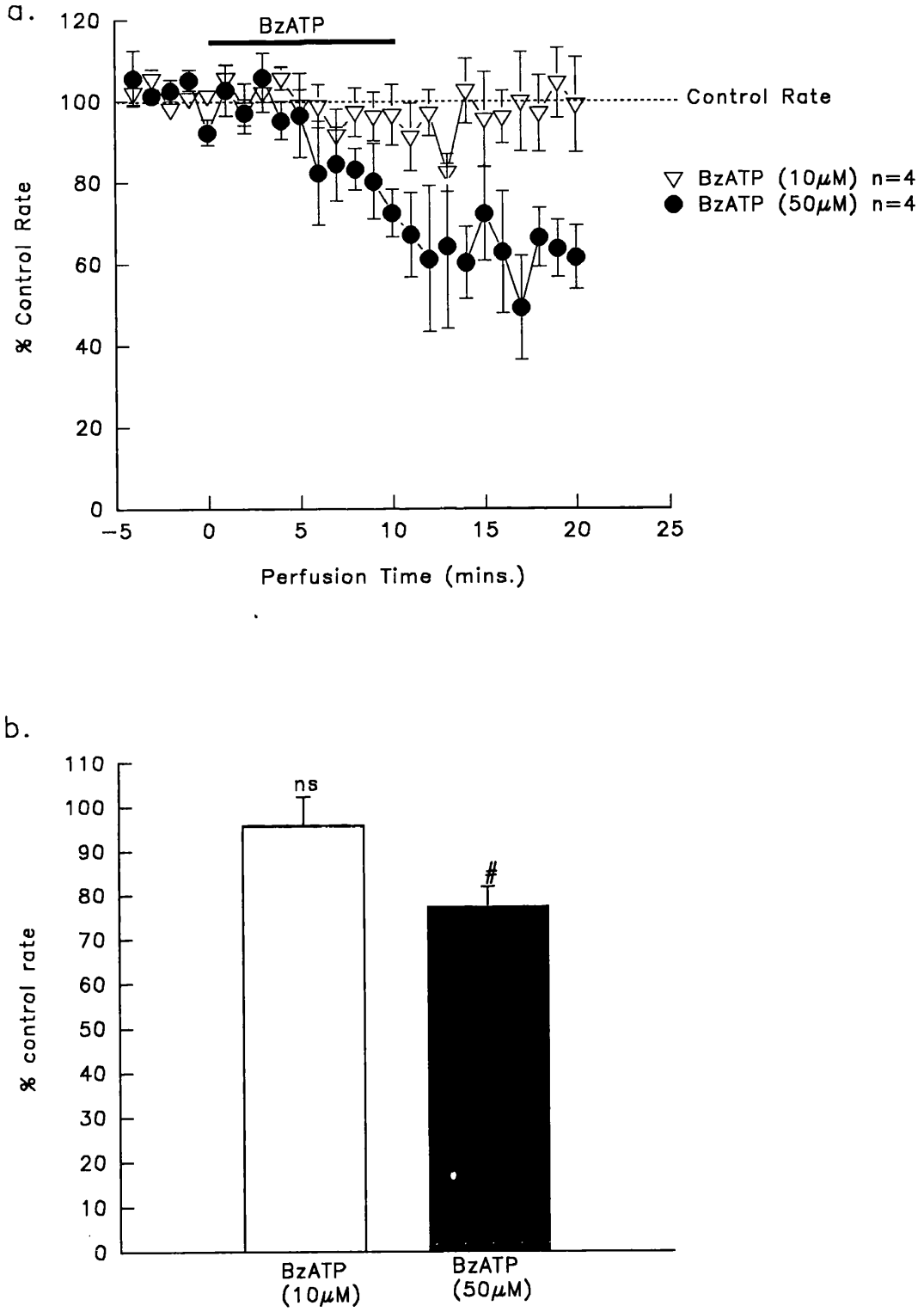



**Figure 38** The time course of the effect of BzATP on the discharge rate is illustrated in (a) with the effect at the end of the 10 minute perfusion and analysis shown in (b). BzATP at 50μM significantly decreased the rate (# $P<0.05$), an effect which was not reversed during the subsequent 10 minute wash period.

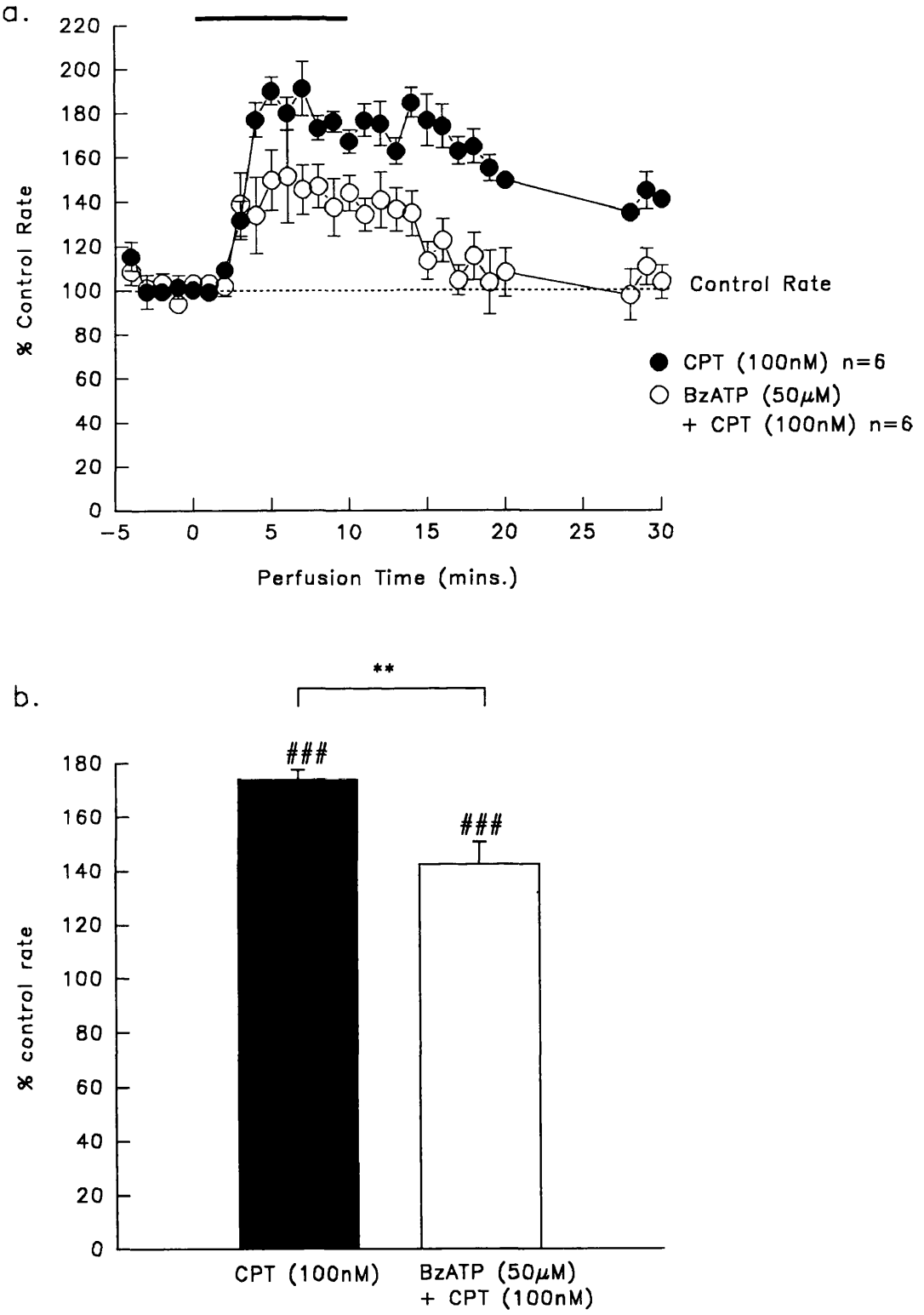



**Figure 39** CPT and BzATP were perfused together for 10 minutes (a). In these experiments no prior exposure to CPT was used. CPT and CPT + BzATP increased the discharge rate, ### $P<0.001$. The extent of the CPT induced increase in activity rate was significantly reduced in the presence of BzATP, ** $P<0.01$ (b).

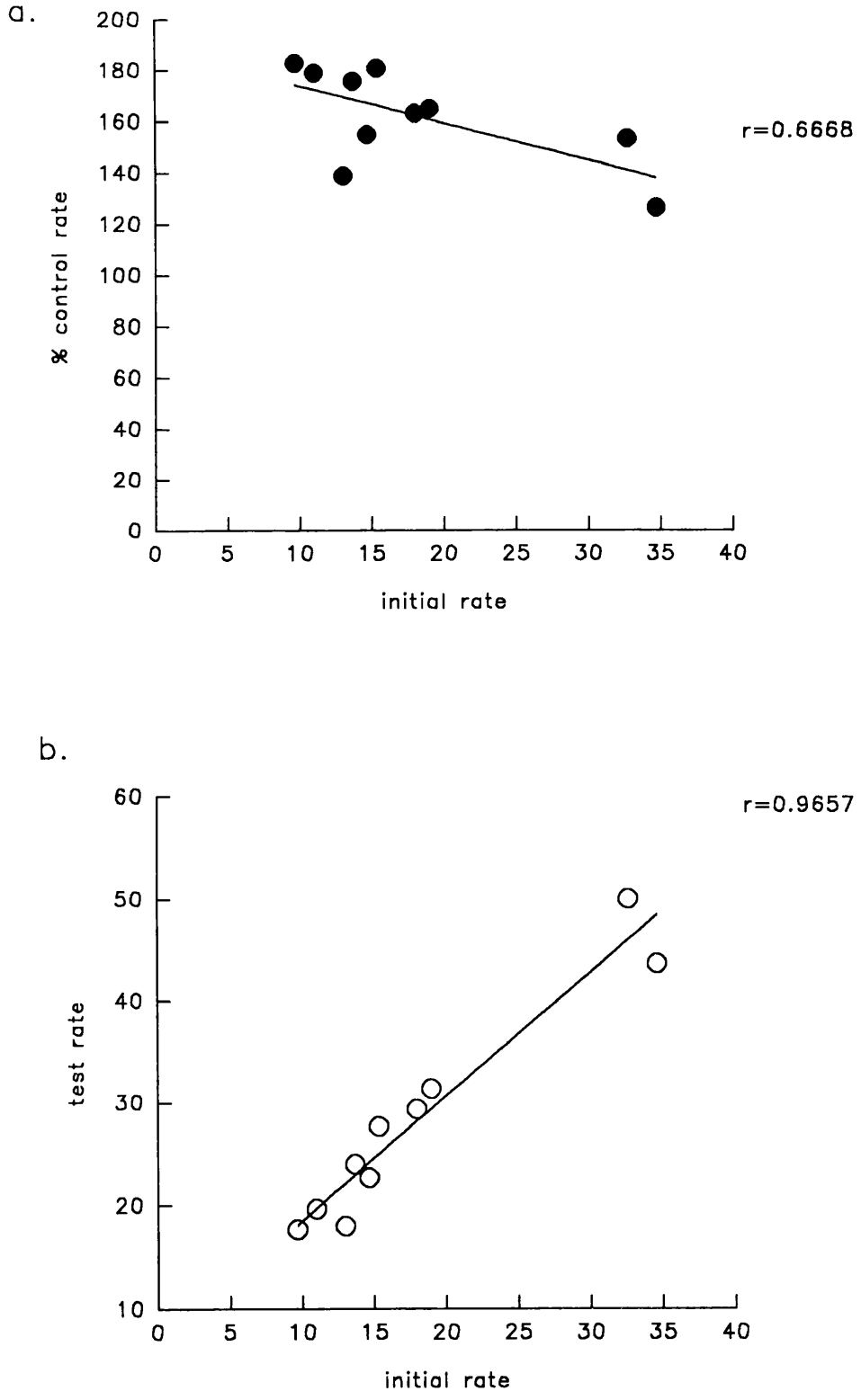



**Figure 40** Regression curves of initial rate against the effect produced by CPT on discharge rate expressed as % control rate in (a) and as the actual raw data in (b). The graph contains data pulled from control curves for CPT in the BzATP and ATP/adenosine set of experiments. The figures presented are used to determine the degree of correlation to first order regression.

### 3.4 The effect of adenosine on simultaneously generated evoked and spontaneous activity in the CA1 region.

Population potentials evoked during the perfusion of 0Mg/4AP medium often contain secondary and tertiary peaks in addition to the main primary peak (fig. 41). In these experiments evoked potentials and spontaneous activity occurring simultaneously were investigated. Adenosine (50μM), as expected, significantly depressed the rate of spontaneous activity. However, the amplitudes of the population potentials, in contrast, were not reduced, in fact the opposite occurred. The primary peak was not changed whereas the amplitude of both the secondary and tertiary peaks were significantly increased to 131.42%±8.61 and 162.08%±17.68 of control respectively (fig. 42). During the wash period a significant rebound effect was produced with regard to the discharge rate, with a recovery beyond control levels being produced. The three peaks of the population potential returned to below control levels, although only the primary peak was significantly lower.

Similar experiments were conducted using adenosine in combination with both $A_1$ and $A_2$ receptor antagonists, namely DPCPX and ZM241385 respectively. For the DPCPX experiments only primary and secondary peaks were examined. As before, adenosine decreased activity rate but in these experiments the size of the primary and secondary peaks was significantly increased (fig. 43a). DPCPX (50nM) inhibited both the depression of rate and the increase in peak amplitude (fig. 43b). Control responses to adenosine (50μM) for the ZM241385 experiments are found in fig. 44a. ZM241385 (250nM), a concentration which

should inhibit both $A_{2A}$ and $A_{2B}$ receptors, had no effect on either the depression of spontaneous activity or the rise in the amplitude of evoked potentials produced by adenosine (50μM) (fig. 44b).

The effect of adenosine on evoked activity during ongoing spontaneous activity differed if the bursts were generated in a 4AP (50μM) medium containing normal levels of magnesium. Adenosine decrease the discharge rate and also significantly decreased, instead of increasing, the tertiary peak (fig. 45). The primary and secondary peaks were not significantly altered.

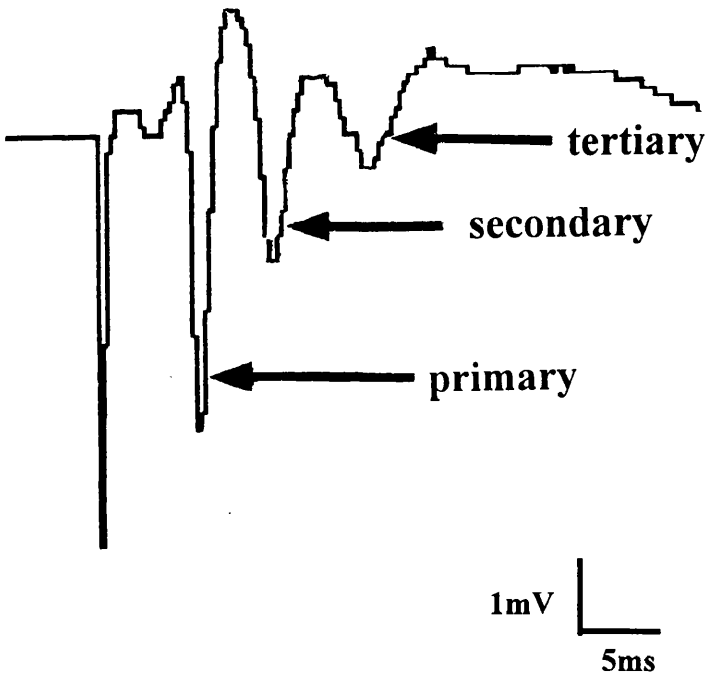



**Figure 41** A population spike recorded in the CA1 region during perfusion with 0Mg/4AP medium displaying secondary and tertiary peaks in addition to the main primary one.

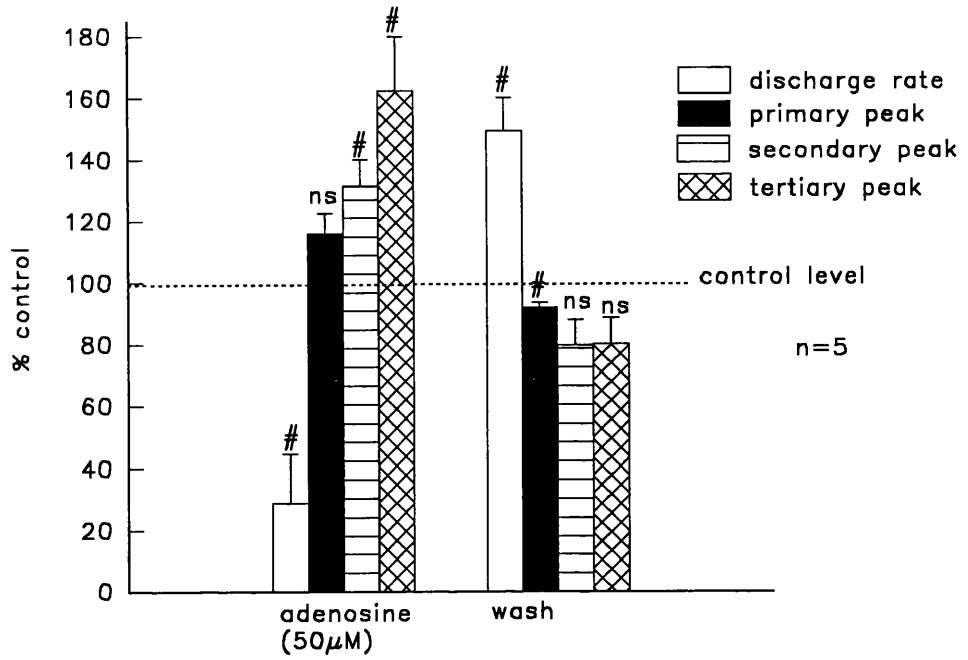



**Figure 42** The effect of adenosine on simultaneously generated evoked population spikes and spontaneous activity. The first set of bars on the histogram represent the effect of adenosine on discharge rate, primary, secondary and tertiary peaks at the end of a ten minute perfusion. The second set show the results after a 10 minute wash period. # $P<0.05$.

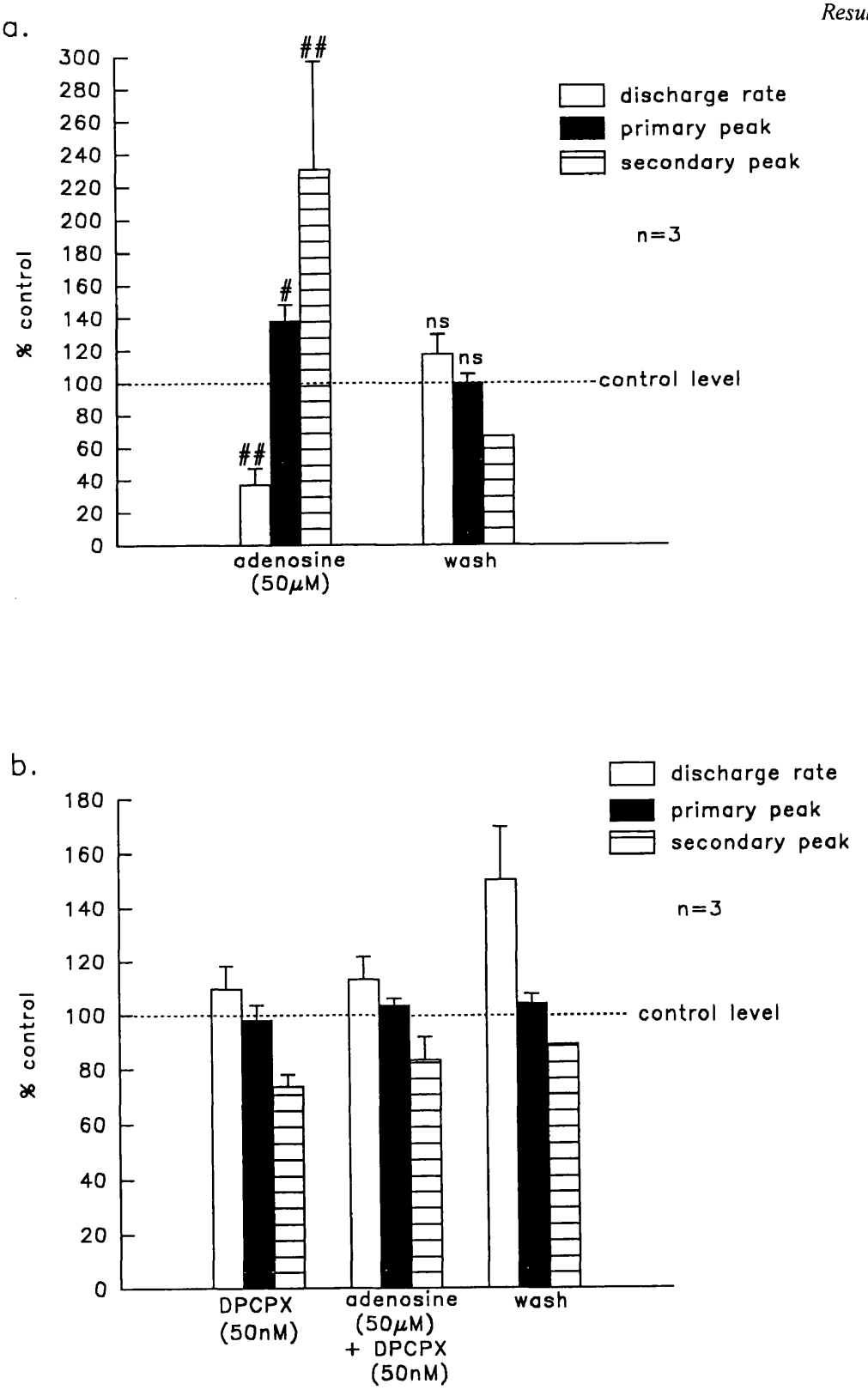



**Figure 43** Control responses to adenosine after perfusion for 10 minutes and the subsequent 10 minute wash period are shown in (a). DPCPX was perfused for 10 minutes before adenosine was added. (b) shows the results after perfusion of DPCPX alone, then after perfusion of adenosine + DPCPX and finally after 10 minute wash period. Discharge rate and the amplitude of primary and secondary population spikes were analysed in each treatment. # $P<0.05$, ## $P<0.01$.

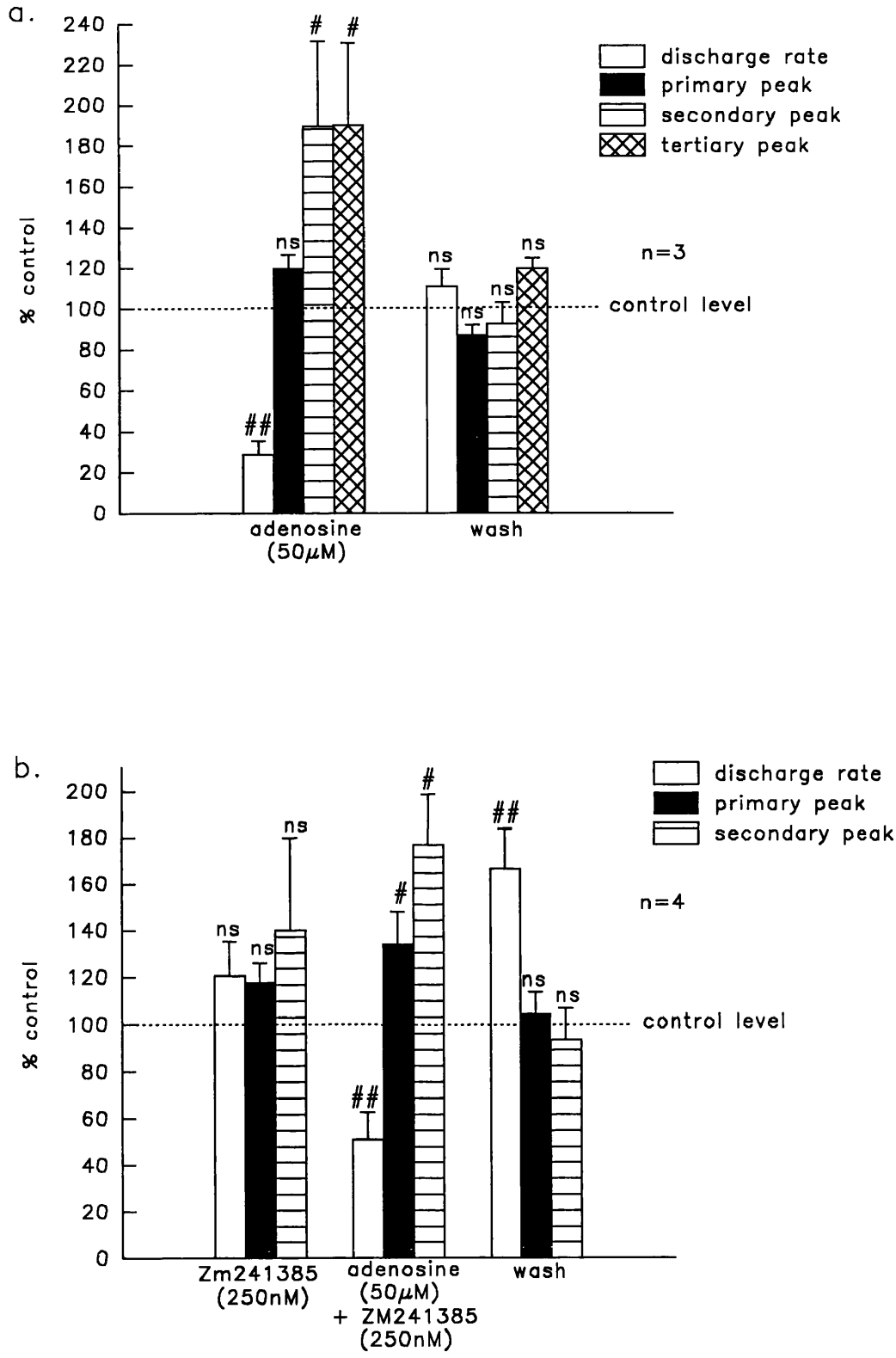



**Figure 44** (a) illustrates control responses to adenosine after 10 minutes perfusion on discharge rate and the amplitude of the evoked primary population spike and subsequent afterdischarges. The effect at the end of the wash period is also shown. The effect of a 10 minute perfusion of ZM241385 alone, then together with adenosine and the subsequent wash period on discharge rate and the population spike amplitude is given in (b). # $P<0.05$ and ## $P<0.01$.

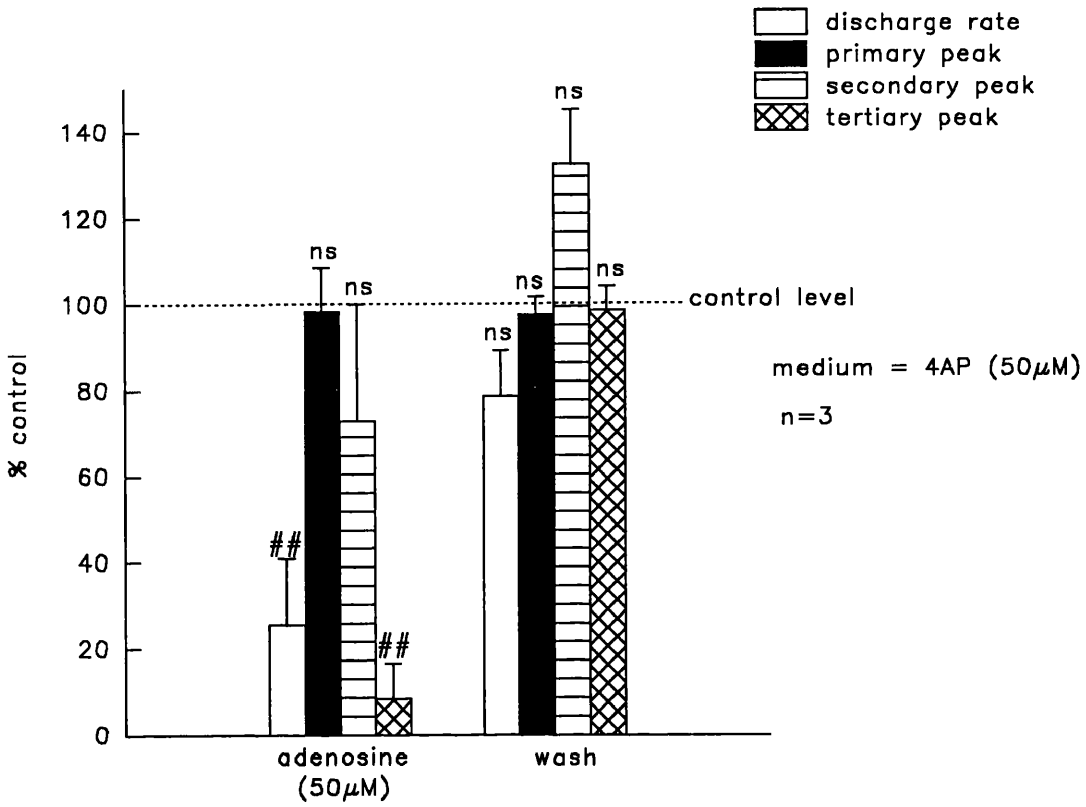



**Figure 45** Spontaneous bursts and evoked population spikes were analysed simultaneously in a medium containing normal magnesium and 4AP (50μM). Adenosine was perfused for 10 minutes. The effect on discharge rate and the amplitude of the primary, secondary and tertiary peaks of the population potential at the end of this perfusion and after a 10 minute wash period are shown. ## $P<0.01$.

### 3.5 P1 receptor antagonists

The $A_1$ receptor antagonist CPT can induce epileptiform activity as shown in fig. 46. CPT (10μM) was perfused for 15 minutes in an otherwise normal aCSF. Bursts were generated within 5 minutes of the start of perfusion. Control rate was defined as the mean of the rates in the last three minutes of CPT perfusion. The introduction of normal aCSF produced a gradual decline in burst frequency to approximately zero percent of control (fig. 46).

In addition to $A_1$ receptor inhibition, it has been proposed that $A_1$ antagonists increase basal activity by freeing endogenous adenosine to act at $A_2$ receptors. In order to investigate this in our 0Mg/4AP model of epileptiform activity ZM241385, an $A_2$ receptor antagonist, was perfused in combination with CPT. CPT (100nM) alone increased the rate to 169.31%±13.41 (fig. 47). ZM241385 (100nM) alone had no effect on epileptiform activity rate nor did it alter the response to CPT (fig. 48). At a higher concentration, ZM241385 (2μM) increased the discharge rate to 170.53%±30.84 control. The subsequent addition of CPT (100nM) further increased the rate to a small degree although there was no significant difference between ZM241385 alone and in combination with CPT (fig. 49).

The perfusion of CPT (100nM) increased the rate of activity generated in a 4AP (50μM) medium to 286.38%±37.15 of control after 10 minutes perfusion (fig. 50). This increase is significantly larger compared with the increase in rate produced by CPT in previous experiments (controls for ATP/adenosine, BzATP and ZM241385) in which a 0Mg/4AP medium was used (figs. 9, 39 and 48).

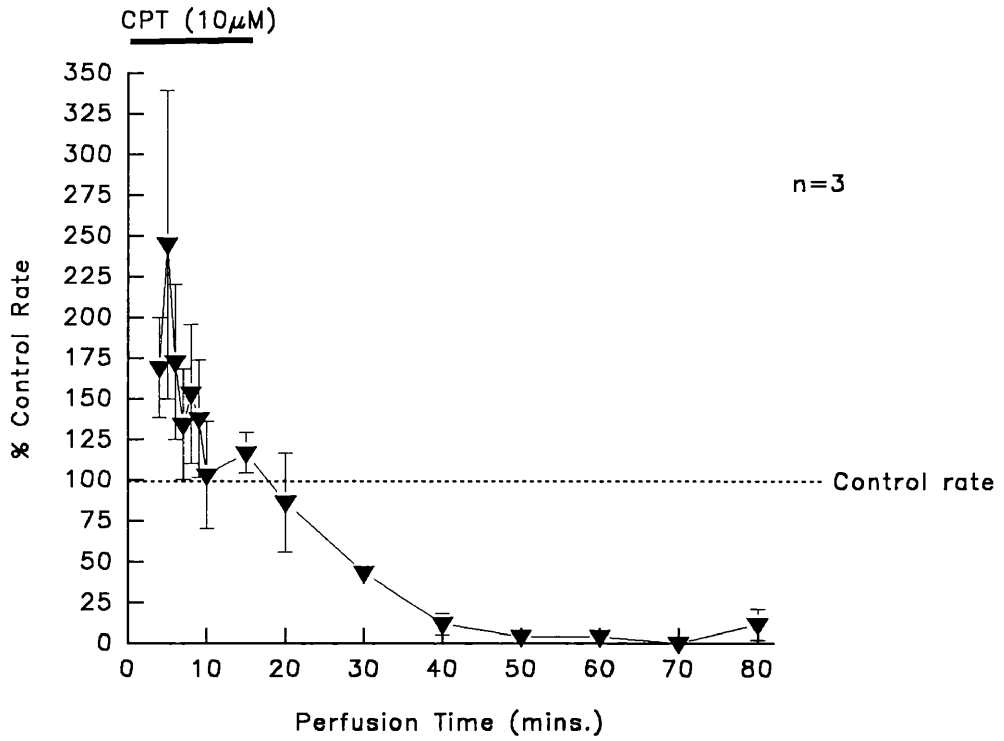



**Figure 46** CPT was perused for 15 minutes in an otherwise normal aCSF. Spontaneous activity was initiated after 3-5 minutes. Control rates were determined as the mean of the last three minutes of CPT perfusion.

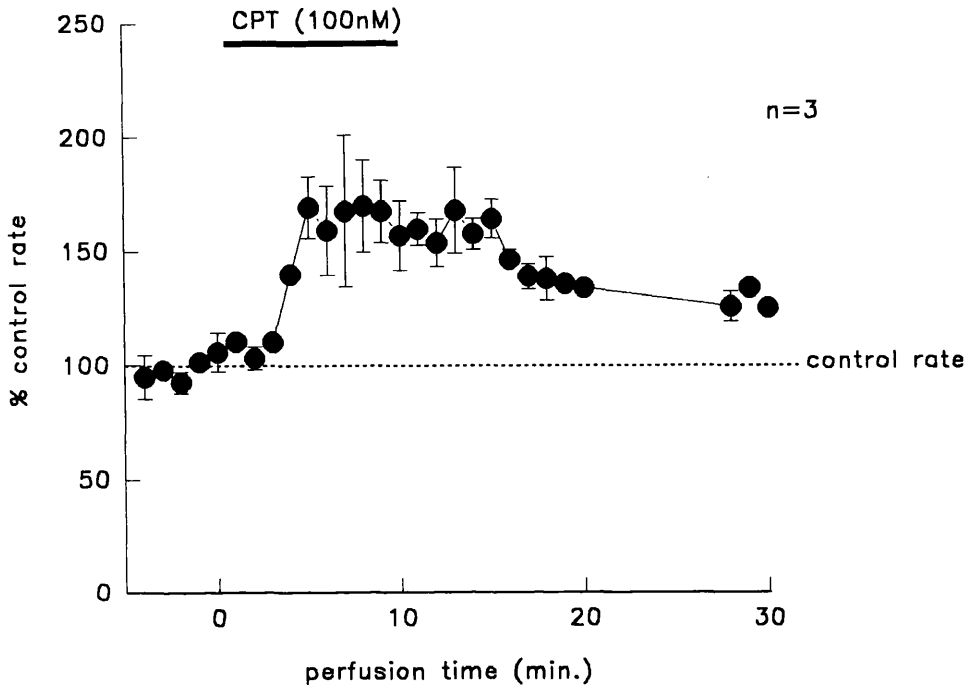



**Figure 47** The effect of CPT on discharge rate generated using a zero magnesium/4-aminopyridine medium.

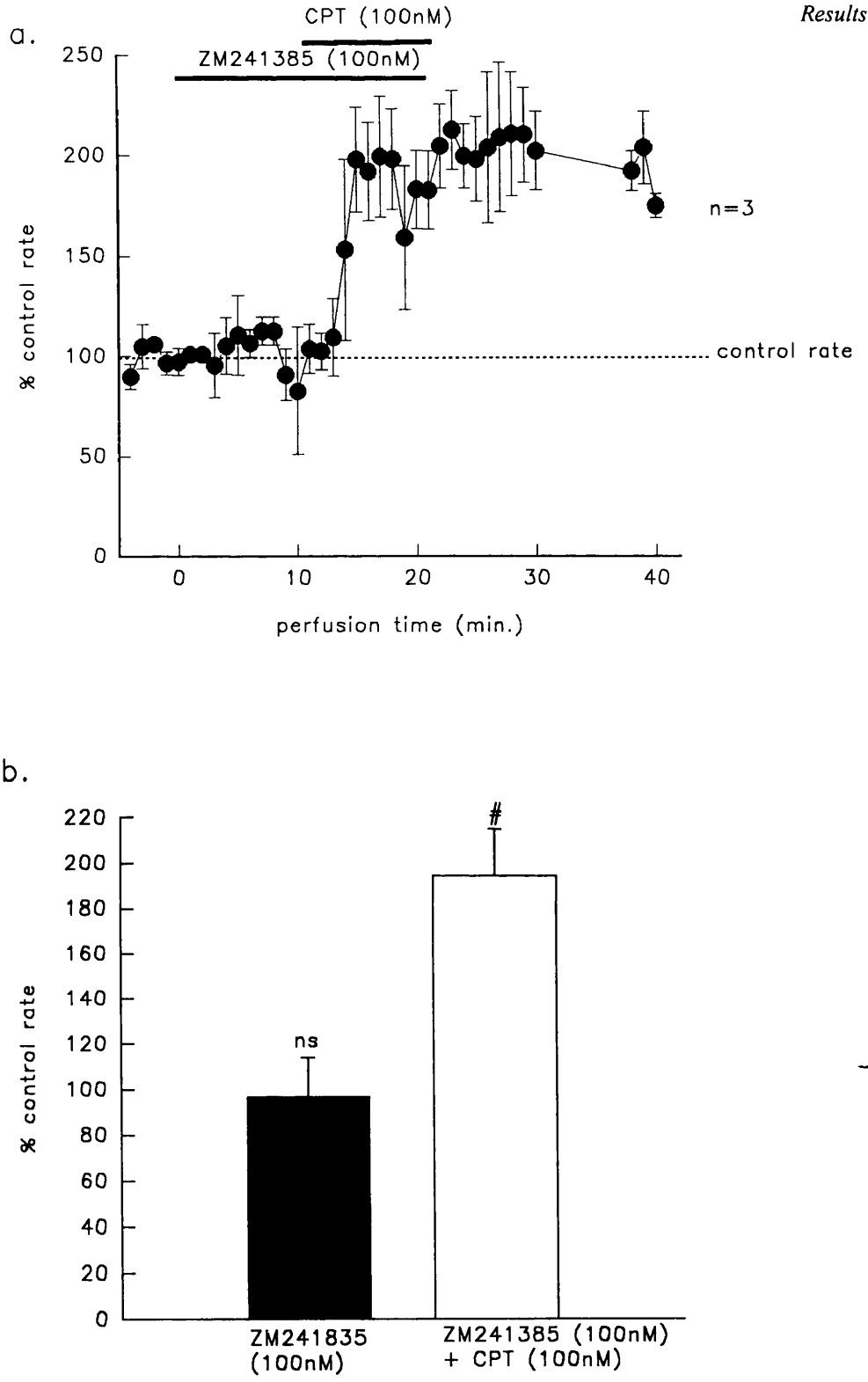



**Figure 48** ZM241385 was perfused for 10 minutes prior to the addition of CPT (a). The mean effect of ZM241385 perfused alone and in combination with CPT is shown in (b). The effect of CPT is expressed as a percentage of the rate at the end of perfusion with ZM241385.

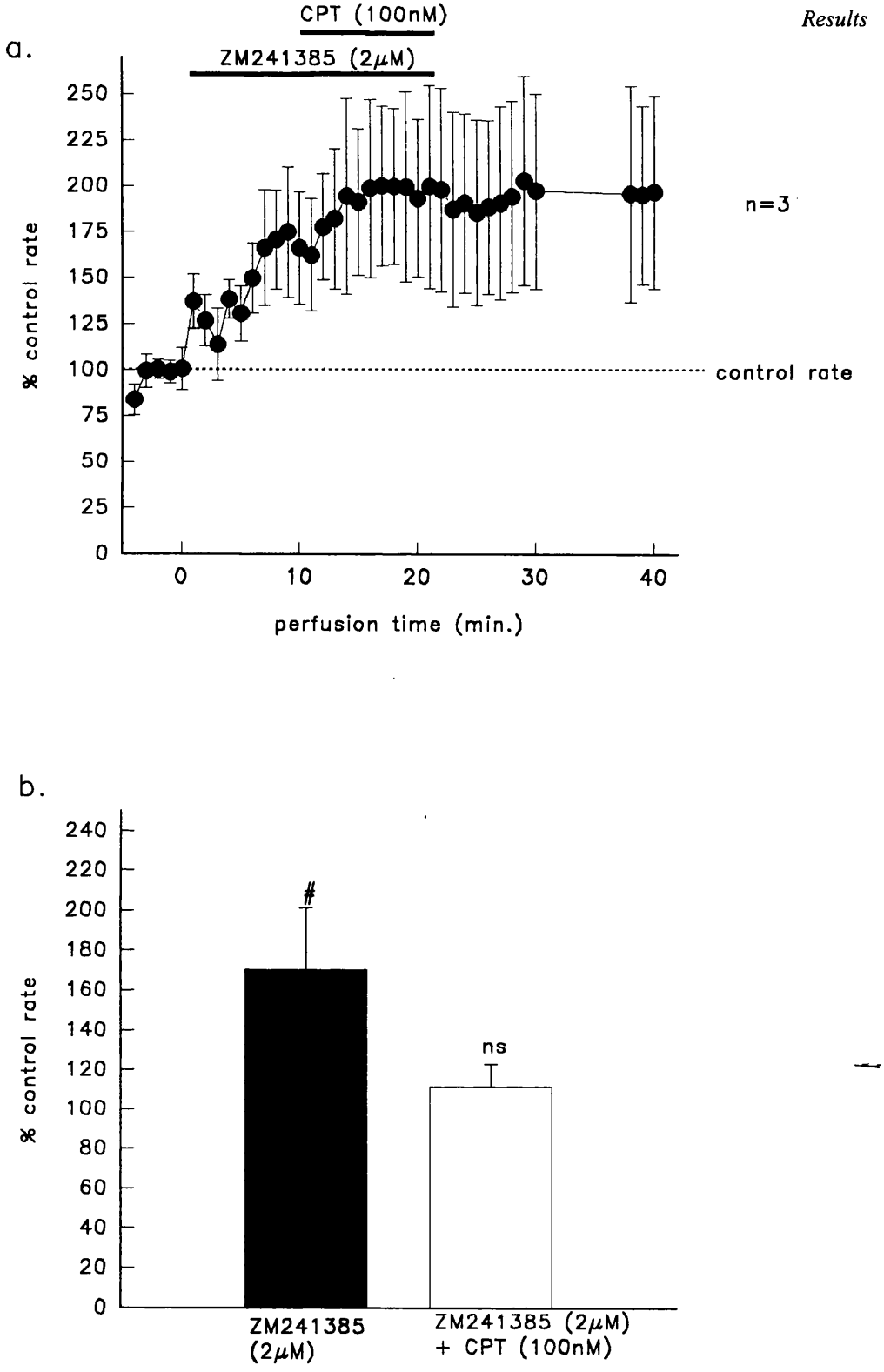



**Figure 49** ZM241385, at the higher concentration of 2μM, had a pre-exposure period of 10 minutes (a). The mean effect of ZM241385 perfused alone and in combination with CPT is shown in (b). Results with CPT are expressed as a percentage of the rate after perfusion with ZM241385.

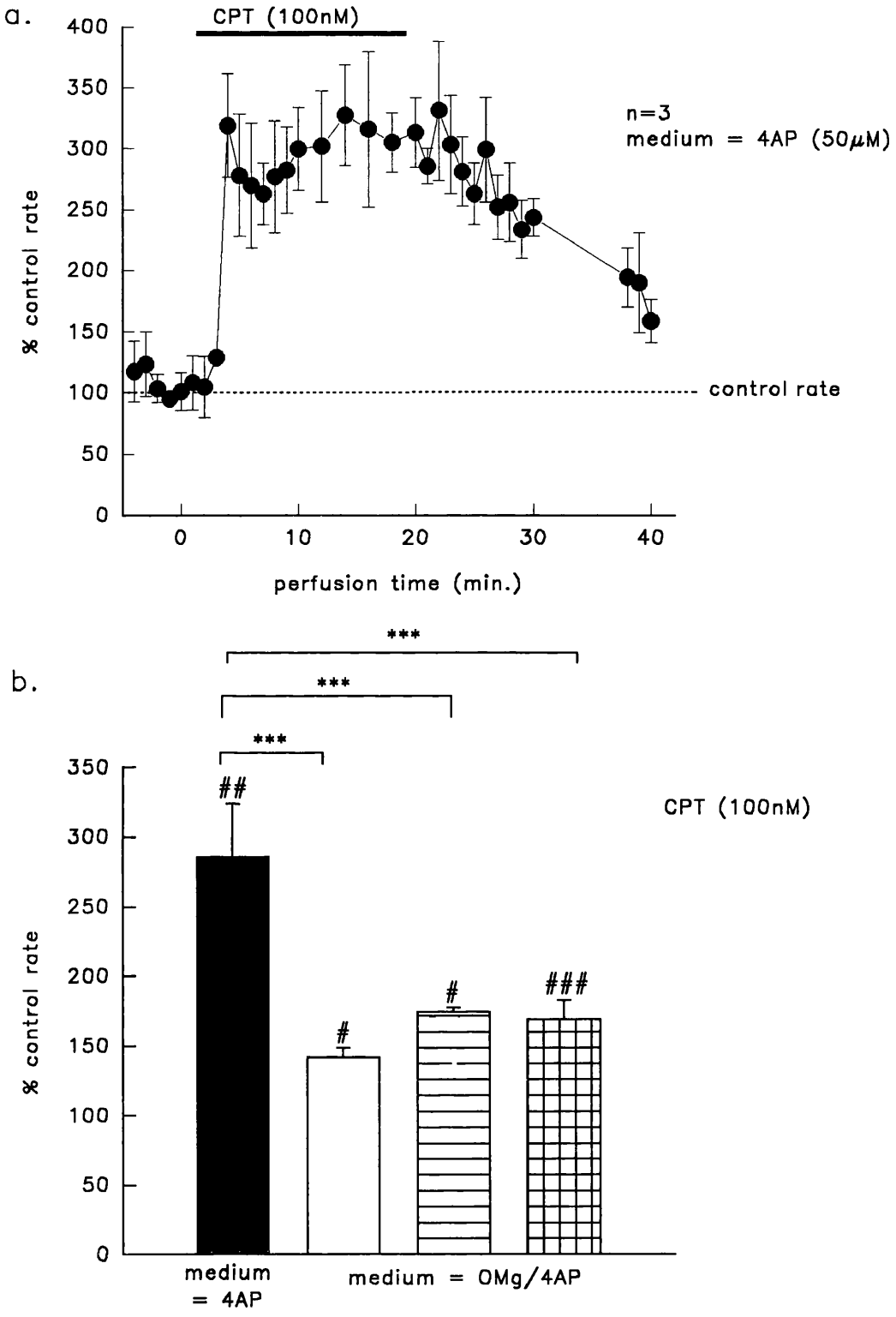



**Figure 50** CPT was perfused for 20 minutes in a medium containing 4AP (50μM) (a). The effect after 10 minutes is shown in (b) together with previcus CPT results used as controls in experiments with ATP/adenosine, BzATP and ZM241385 in which a 0Mg/4AP medium was used. In all instances CPT increased the discharge rate with # $P<0.05$ and ## $P<0.01$. The use of a 4AP medium significantly potentiated the effect of CPT (*** $P<0.001$).

## 3.6 Diadenosine polyphosphates

Alpha, omega-adenine dinucleotides ($Ap_nA$) or diadenosine polyphosphates consist of two adenosine molecules linked at their 5' region by a number of phosphate groups, with n denoting the number of phosphates. In view of the evidence that adenosine dinucleotides can exert an effect within the brain including the hippocampus, it was a natural progression to investigate whether $Ap_4A$ and $Ap_5A$ could influence the frequency of epileptiform activity.

Adenosine at a concentration of 1μM failed to alter the rate of epileptiform activity (fig. 51). Diadenosine tetraphosphate ($Ap_4A$) and diadenosine pentaphosphate ($Ap_5A$) at 1μM depressed the discharge rate to 83.88%±2.82 and 88.03%±1.44 respectively, values which are significantly different from control in both instances (fig. 52a, b and 53a, b respectively). The depression caused by $Ap_4A$ was apparent earlier in the period of perfusion but both effects were fully reversible (fig. 52a and 53a).

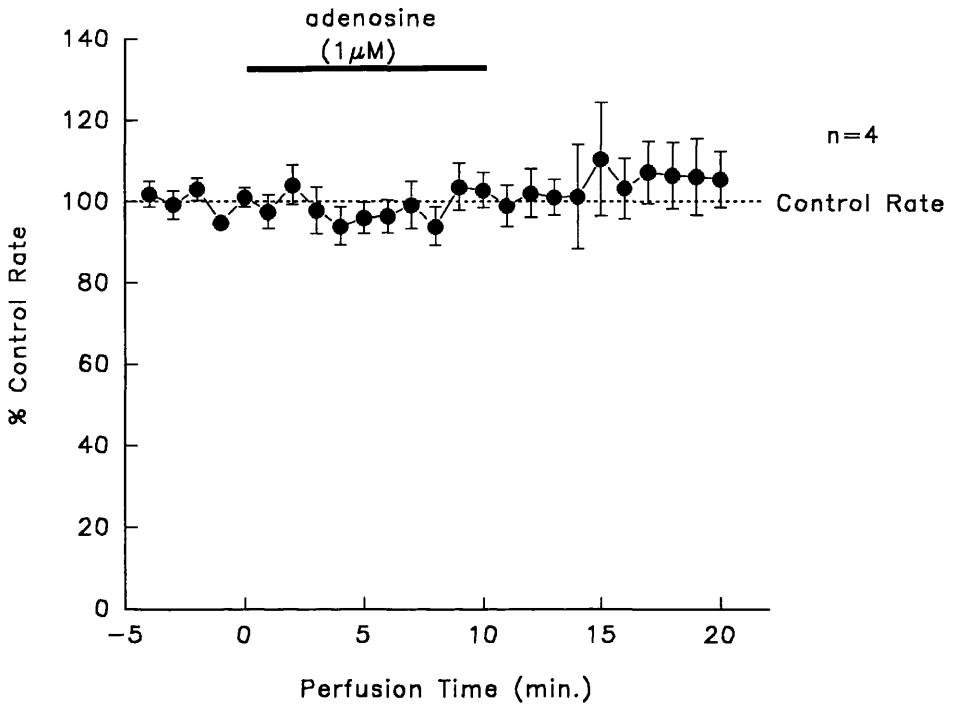
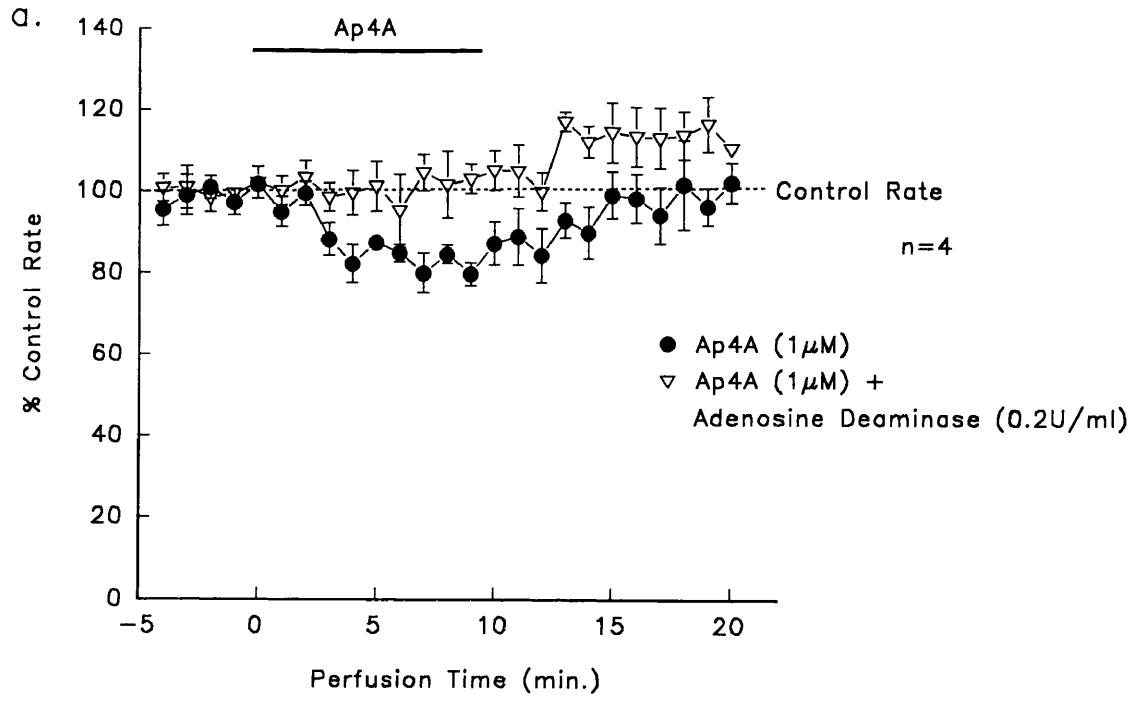



**Figure 51** The effect of adenosine (1μM) on the rate of epileptiform activity induced by 0Mg/4AP medium.



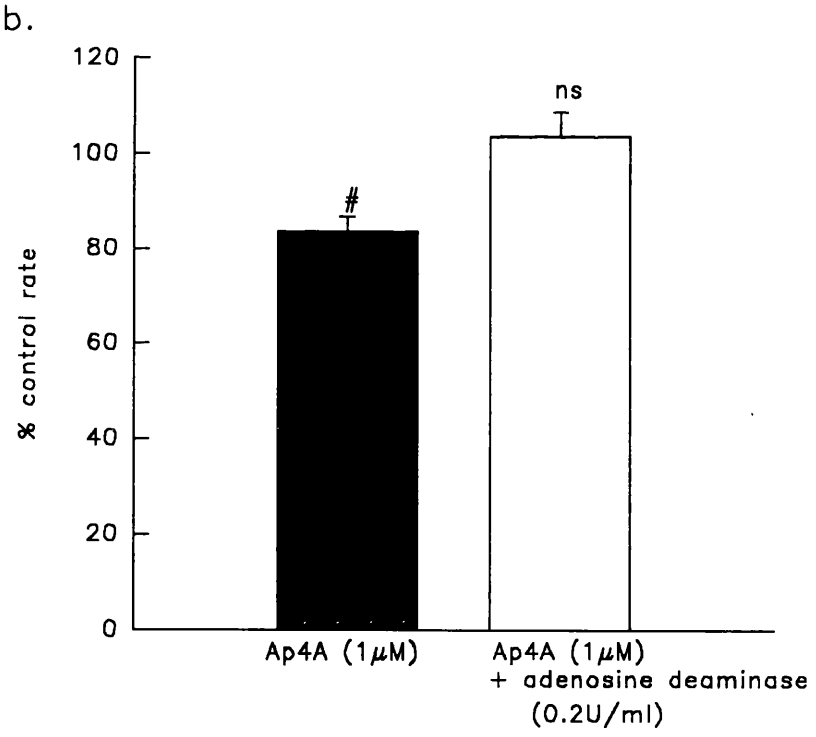
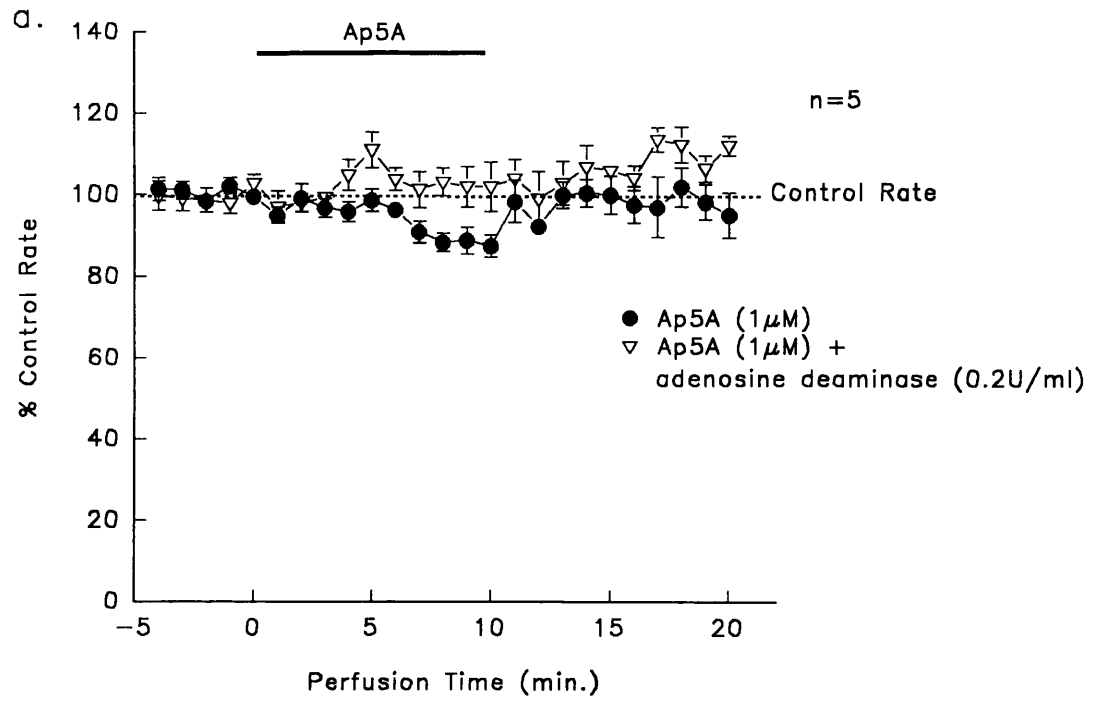




**Figure 52** The effect of diadenosine tetraphosphate on the frequency of epileptiform activity. (a) shows the time course of the depression caused by $Ap_4A$ (1μM) alone and with adenosine deaminase (0.2U/ml). The mean effect at the end of a 10 min. perfusion is shown in (b). $Ap_4A$ (1μM) significantly decreases the rate from control, # $P<0.05$.



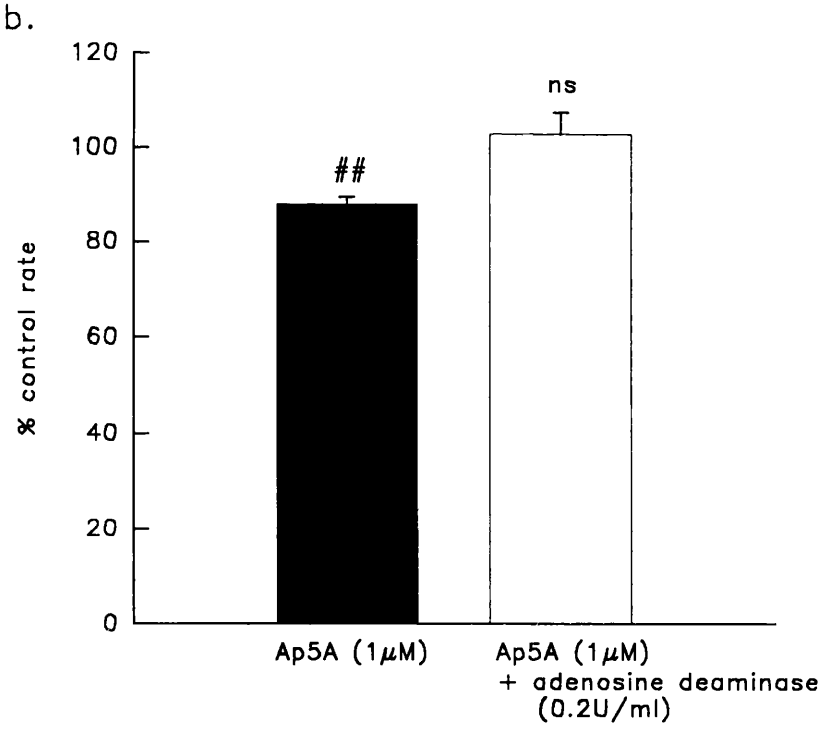




**Figure 53** The time course of the effect of $Ap_5A$ on discharge rate is shown in (a). (b) shows the mean effect at the end of a 10 min. perfusion in which $Ap_5A$ significantly alters the rate from control, ## $P<0.01$.

At a higher concentration of 10μM, adenosine, $Ap_4A$ and $Ap_5A$ all significantly decreased the discharge rate (fig. 54, 55 and 56). With regards to adenosine this is different from what was obtained in the concentration response curve of fig. 6. Although the degree of inhibition was similar 74.81 %±5.78 against 67.79%±3.2, the effect was previously found to be not significant when compared to control (P=0.0539, n=4). This effect was rapid, producing an initial maximal depression within 2-3 minutes of the start of perfusion. A subsequent recovery towards control rate was noted during the 10 minute perfusion. However, the maximum extent of inhibition (fig. 54b, 55b and 56b) and the rate of epileptiform activity at the end of the drug perfusion (fig. 54c, 55c, 56c) were both significantly lower when compared with control. Perfusion of $Ap_4A$ (10μM) for 30 minutes showed a continuation of the trend for recovery during the $Ap_4A$ perfusion time with a rebound excitation revealed on washing (fig. 57). The effect of $Ap_4A$ (10μM) was enhanced if the perfusing medium was changed from 0Mg/4AP to 4AP (50μM) with the 10 min. effect increased from a 29%±6.45 decrease to 60.04%±8.88 (fig. 58)

### *3.6.1 Adenosine deaminase*

Adenosine deaminase (0.2U/ml) totally annulled the inhibition of activity produced by adenosine (10μM) which was expected since previous results showed that this concentration of adenosine deaminase inhibited the effect of 50μM adenosine (fig. 8). Indeed, in this instance, an increase in activity was now revealed (fig. 54c). $Ap_4A$ (1μM), when perfused with adenosine deaminase (0.2U/ml), no longer significantly altered the discharge rate from control (fig.

52). The same applied to $Ap_5A$ (1μM) when co-perfused with the enzyme (fig. 53b). However, at the same concentration which inhibited the effect of adenosine at 10μM, adenosine deaminase did not change significantly the maximum depression of epileptiform activity produced by $Ap_4A$ at 10μM (fig. 55b). When the mean effect at the end of a 10 min. perfusion was analysed, $Ap_4A$ (10μM) no longer significantly altered the rate from control (fig. 55c). The same situation was apparent when $Ap_5A$ (10μM) was perfused in combination with adenosine deaminase (0.2U/ml) with the maximum effect not changed (fig. 56b) but the 10 min. depression being lost (fig. 56c).

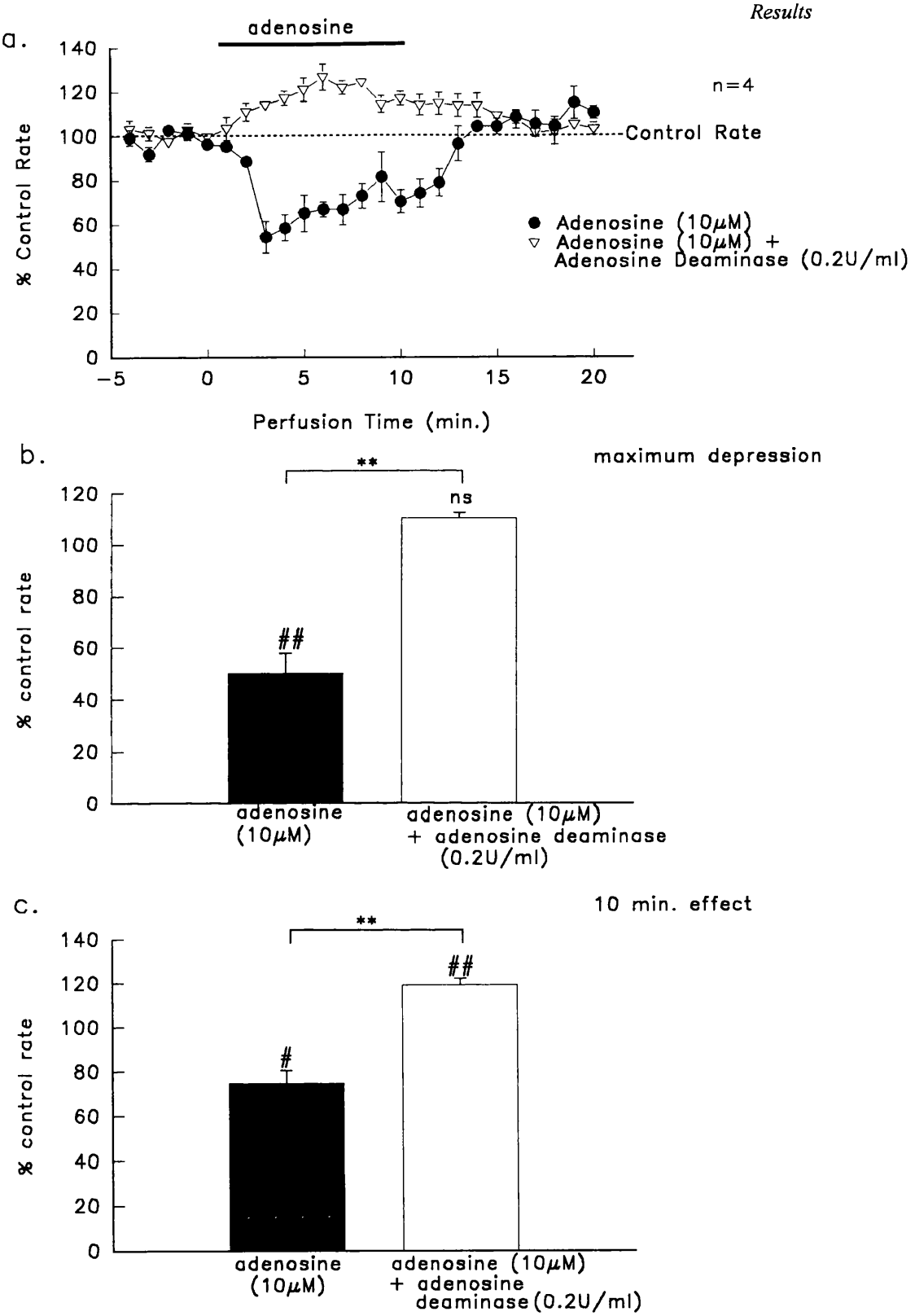



**Figure 54** Adenosine can change the rate of epileptiform activity at a concentration of 10μM. (a) displays the time course of this effect and the subsequent inhibition by adenosine deaminase. Statistical analysis on the maximum depression is shown in (b) and mean effect after 10 min. perfusion is shown in (c.). # $P<0.05$, ## $P<0.01$, ** $P<0.01$.

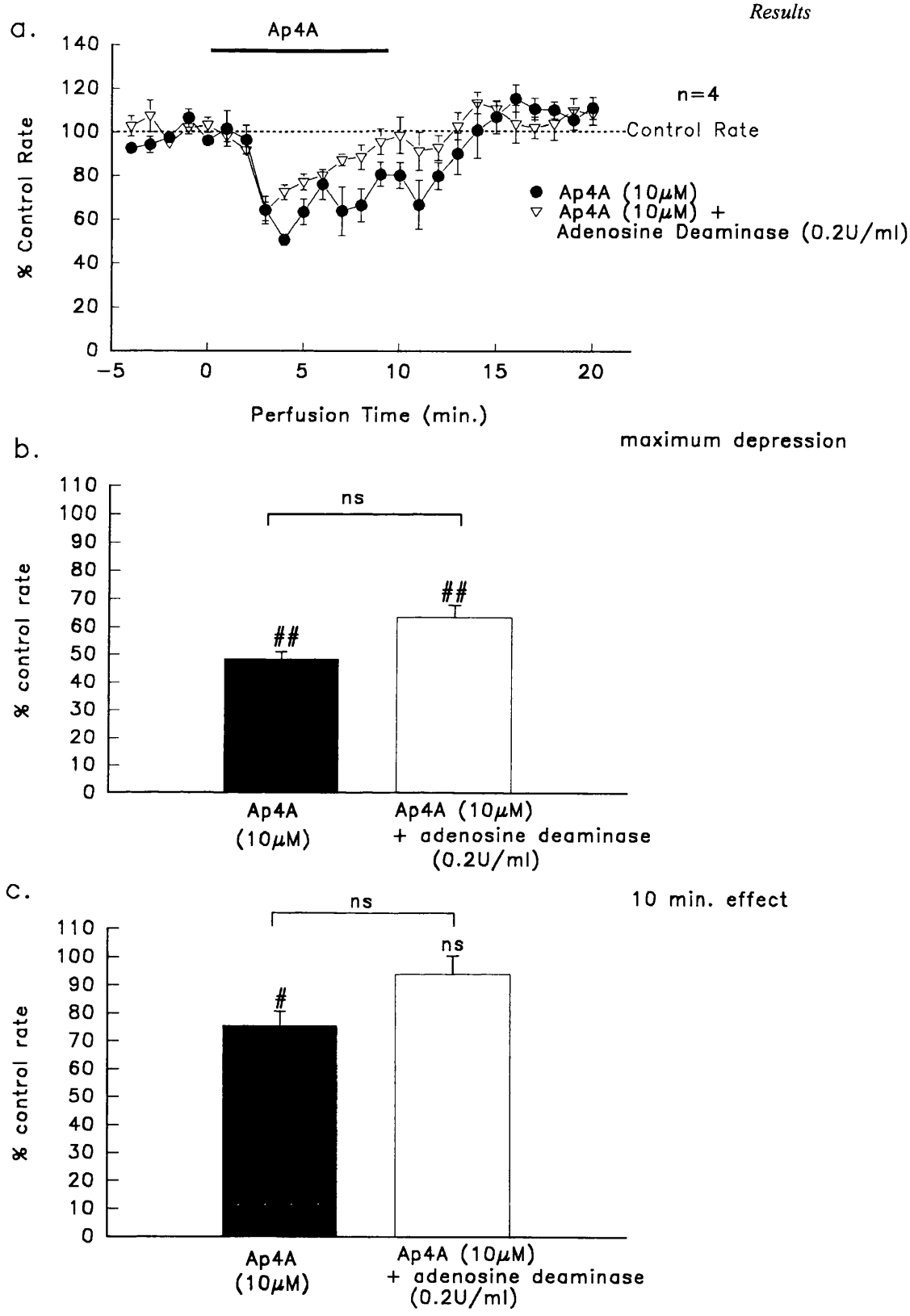



**Figure 55** The effect of $Ap_4A$ (10μM) on the rate of activity and how this is modulated by adenosine deaminase is shown in (a) as time against % control rate. (b) represents the mean of the maximal depression elicited in each slice. $Ap_4A$ alone and with adenosine deaminase significantly inhibits spontaneous activity from control. The effect after 10 min. perfusion is shown in (c). # $P<0.05$, ## $P<0.01$.

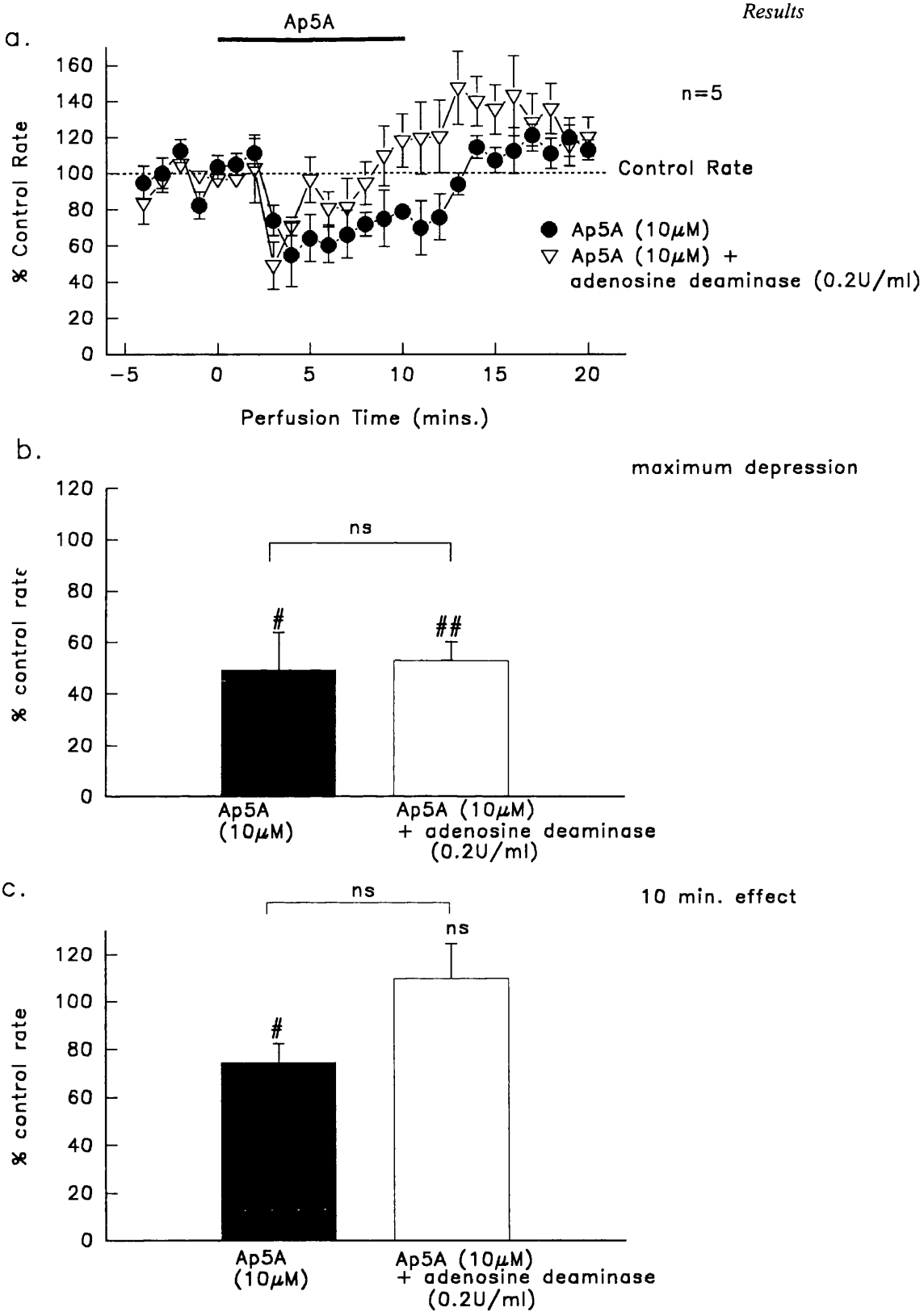



**Figure 56** The modulation of the effect of $Ap_5A$ by adenosine deaminase. (a) represents the time course of events with the maximum and 10 min. effects shown and analysed in (b) and (c) respectively. # $P<0.05$, ## $P<0.01$.

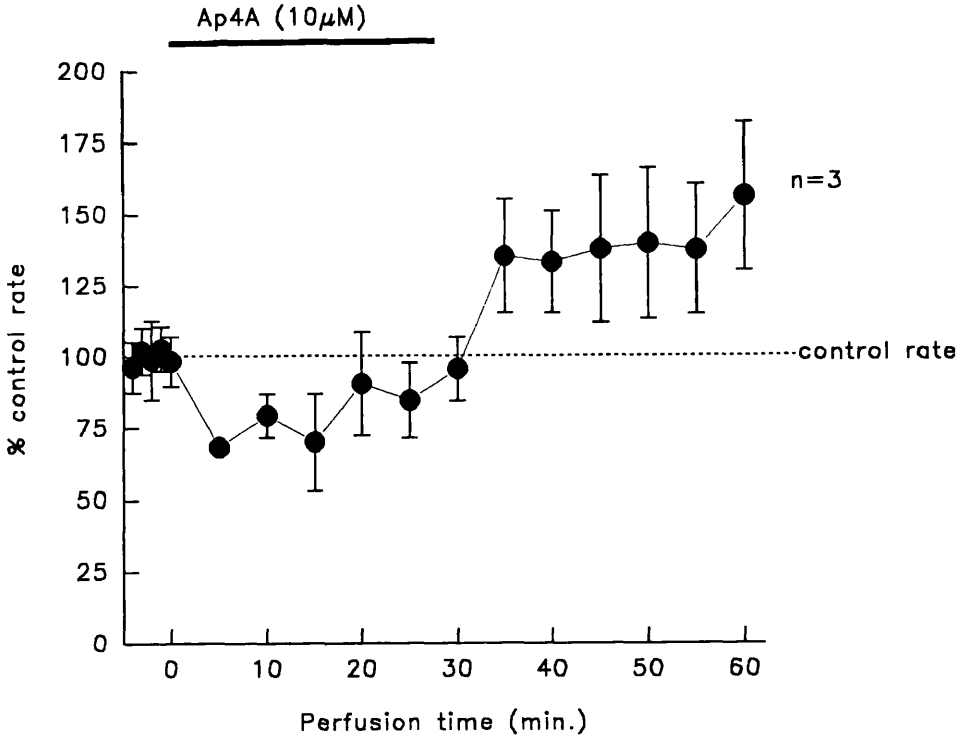



**Figure 57** The time course of a 30 minute perfusion with $Ap_4A$.

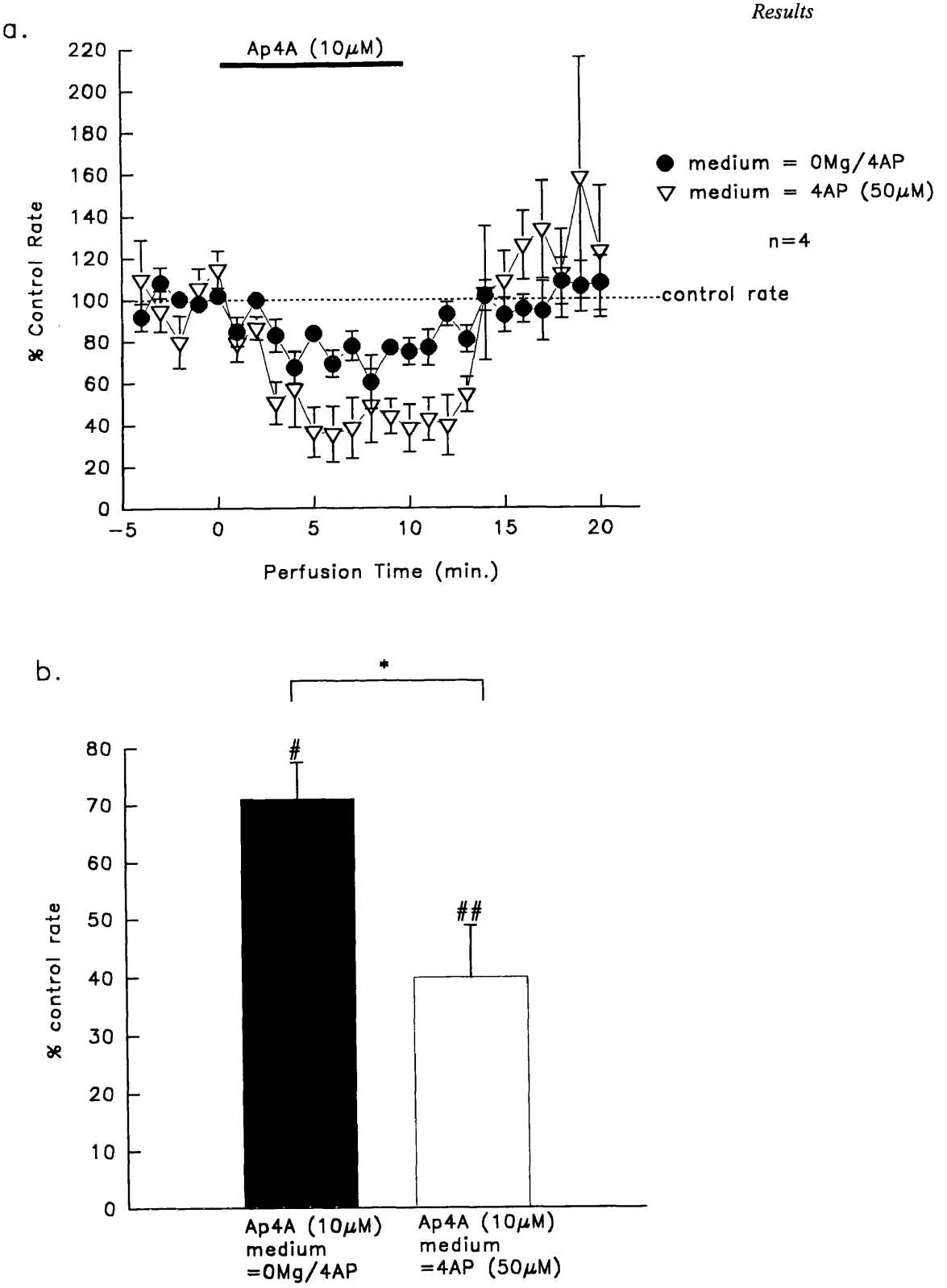



**Figure 58** The time course of the effect of $Ap_4A$ in both a 0Mg/4AP and a 4AP (50μM) medium is shown in (a). Analysis after 10 minutes perfusion is displayed in (b). In both media $Ap_4A$ significantly depresses discharge rate from control (# $P<0.05$, ## $P<0.05$). This effect is potentiated significantly in a 4AP medium, * $P<0.05$.

### *3.6.2 Cyclopentyltheophylline*

8-cyclopentyl-1, 3-dimethylxanthine (CPT) was perfused for 10 min. prior and during perfusion with $Ap_4A$. $Ap_4A$ (10μM) alone decreased activity rate to a significant extent as reported above (fig. 59a). CPT (100nM) by itself, as before, increased the rate of spontaneous activity to approximately 150% control (fig. 59b), but in the presence of CPT $Ap_4A$ (10μM) no longer depressed the rate of epileptiform activity (fig. 59b). If the rate of epileptiform activity recorded at the end of perfusion with CPT alone was taken as the new control rate, then the effect of $Ap_4A$ was reduced to a significant extent from 66.77%±8.26 to 97.74%±3.63 (fig. 59c).

### *3.6.3 AMP deaminase*

AMPase was used at the concentration which inhibited the effect of AMP (fig. 29). When perfused with AMPase neither the maximum effect (fig. 60b) nor the mean 10 min. effect (fig. 60c) of $Ap_4A$ (10μM) was significantly changed from control.

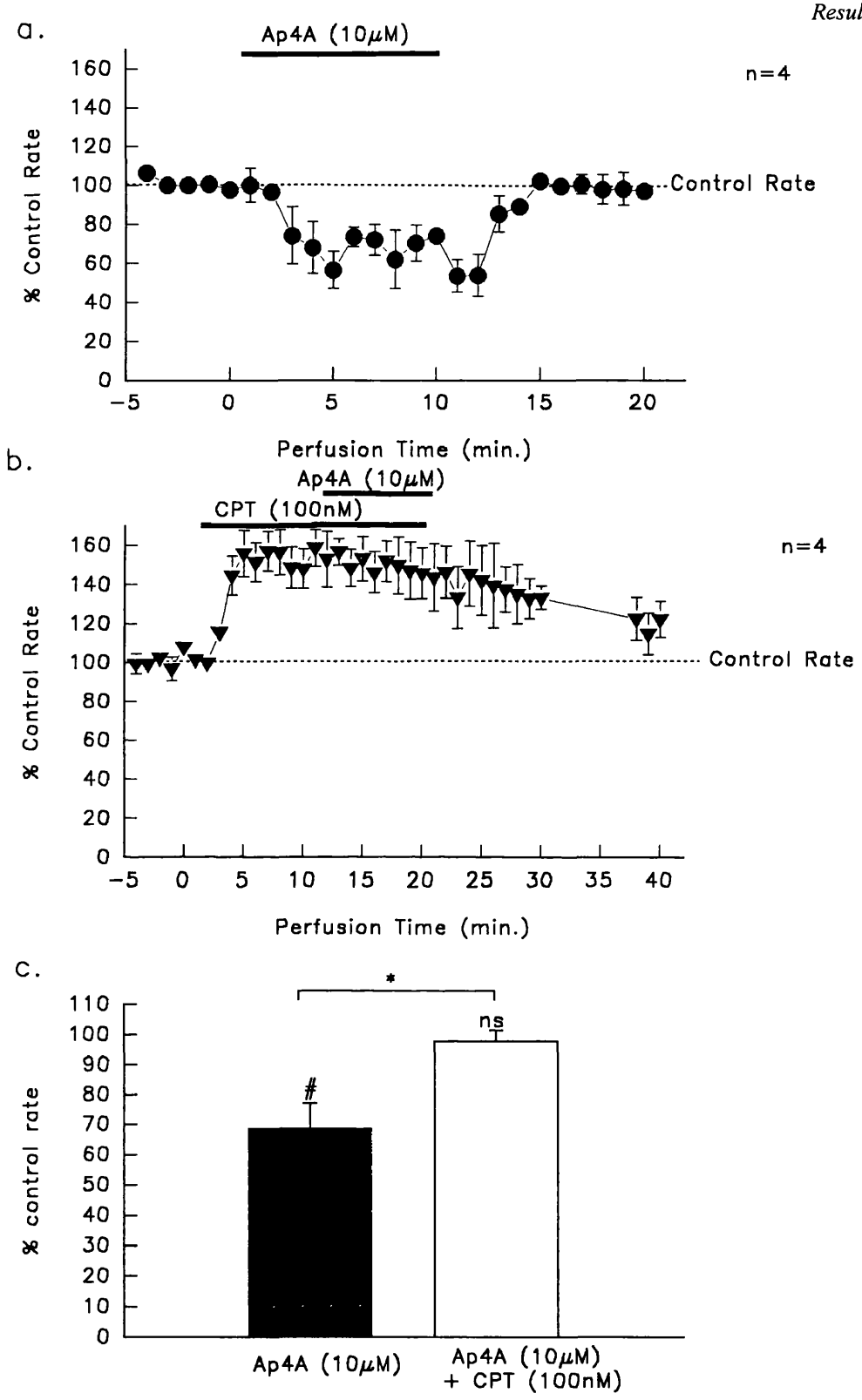



**Figure 59** The effect of CPT on the depression of activity produced by $Ap_4A$. A control response to $Ap_4A$ is seen in (a). CPT was perfused for 10 min. prior to the addition of $Ap_4A$ (b). Statistical analysis is shown in (c). For the comparison of $Ap_4A$ + CPT and control, the mean of the last three values during CPT perfusion alone were taken to be control. # $P<0.05$, * $P<0.05$.

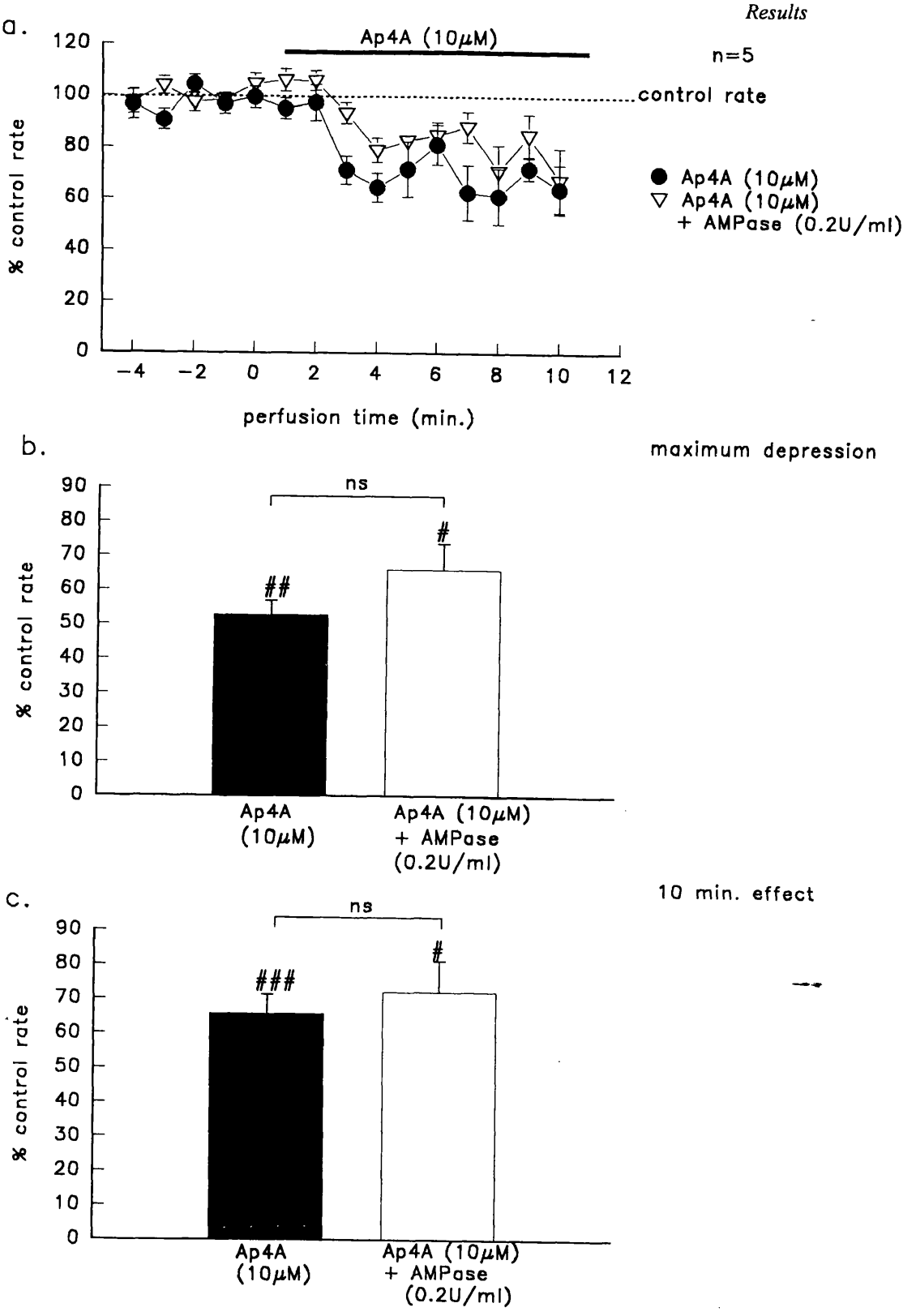



**Figure 60** AMPase was perfused together with $Ap_4A$ (10μM). The time course of a control $Ap_4A$ response and AMPase plus $Ap_4A$ is shown in (a). AMPase does not significantly change either the maximum (b) nor the mean 10 min. effect (c) of $Ap_4A$. # $P<0.05$, ## $P<0.01$, ### $P<0.001$.

### 3.7 5'-adenylic acid deaminase

During the use of AMPase in the characterisation of the response produced by ATP it became apparent that after perfusion with AMPase a gradual decline in activity rate took place during the subsequent wash period which either continued decreasing or reached a plateau below the control level with no sign of recovery. This unexpected effect of AMPase was therefore investigated further.

AMPase (0.2U/ml) during a 10 minute perfusion significantly potentiated the discharge rate to 116.16%±3.72 of control (fig. 61) after which, during perfusion with the control medium 0Mg/4AP, the rate decreased to approximately 50% control with no apparent recovery for up to 90 minutes. This effect of AMPase on epileptiform activity was concentration dependent in that perfusion of AMPase (0.1U/ml) resulted in a depression of about 30% with a slight recovery during the following hour to a reduced, but non significantly affected level (fig. 62). At 0.02U/ml an initial depression was followed by a return to control rates although some fluctuation followed (fig. 63).

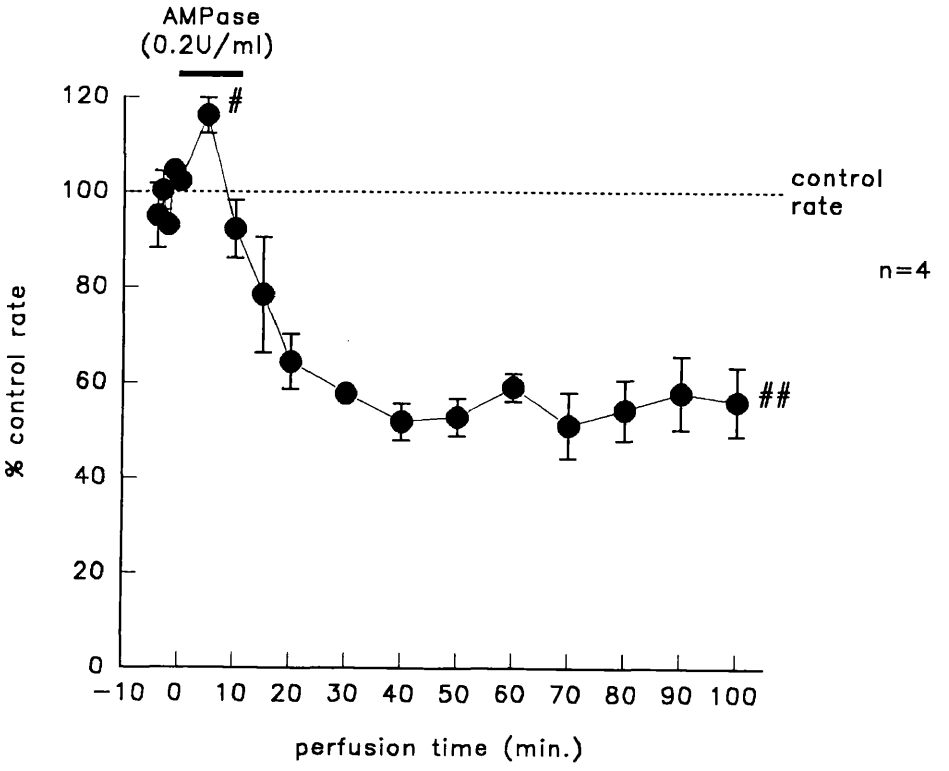



**Figure 61** AMPase was perfused for 10 minutes with a subsequent 90 minute wash period. An initial increase in rate (# $P<0.05$) followed by a significant depression (## $P<0.01$) was produced.

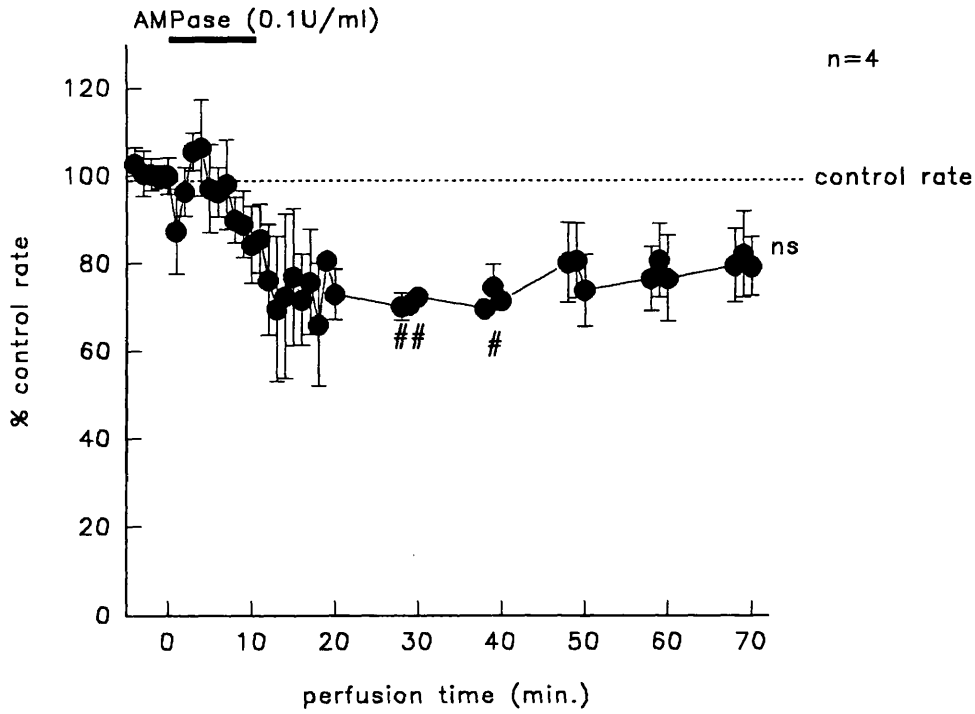



**Figure 62** The effect of AMPase (0.1U/ml) on discharge rate. # $P<0.05$ and ## $P<0.01$.

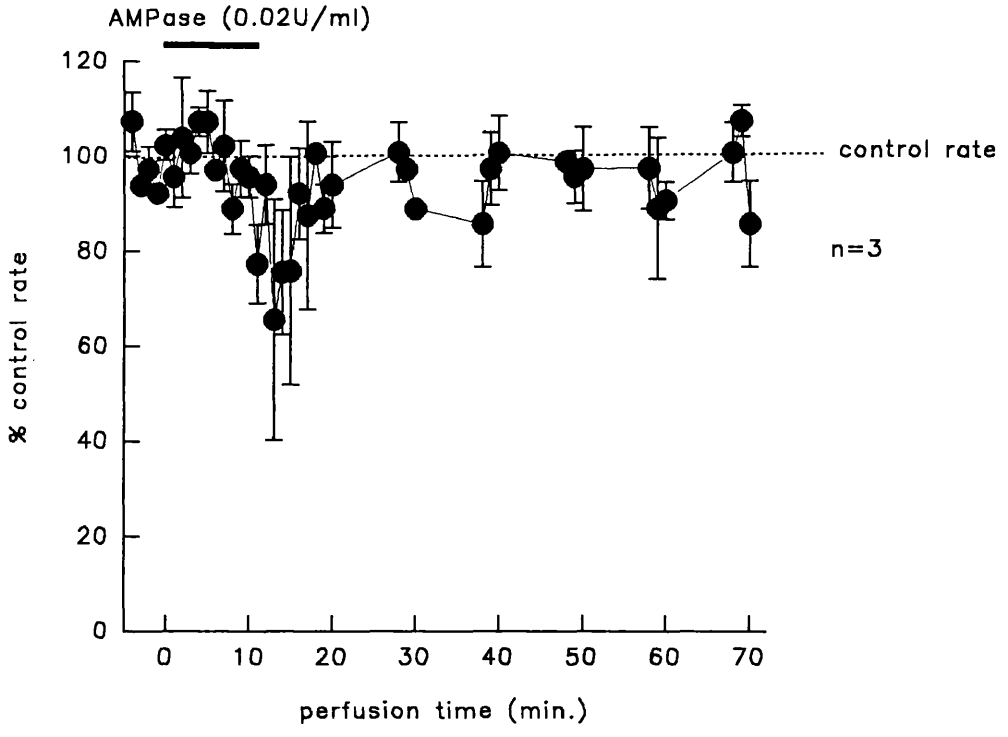



**Figure 63** The effect of AMPase (0.02U/ml) on discharge rate.

In order to verify to some extent that the inhibition produced by AMPase on the effect of AMP and ATP is specific and not just a general inhibition of slice responsiveness baclofen, a $GABA_B$ agonist, was used as an independent arbiter. Baclofen (1μM) depresses the rate of epileptiform activity to 41.78%±6.12 control when perfused alone and 32.66%±13.16 control when perfused with AMPase (0.2U/ml) (fig. 64). AMPase, therefore does not impair the action of baclofen. The maintained depression of activity due to the enzyme is still apparent in fig. 64a.

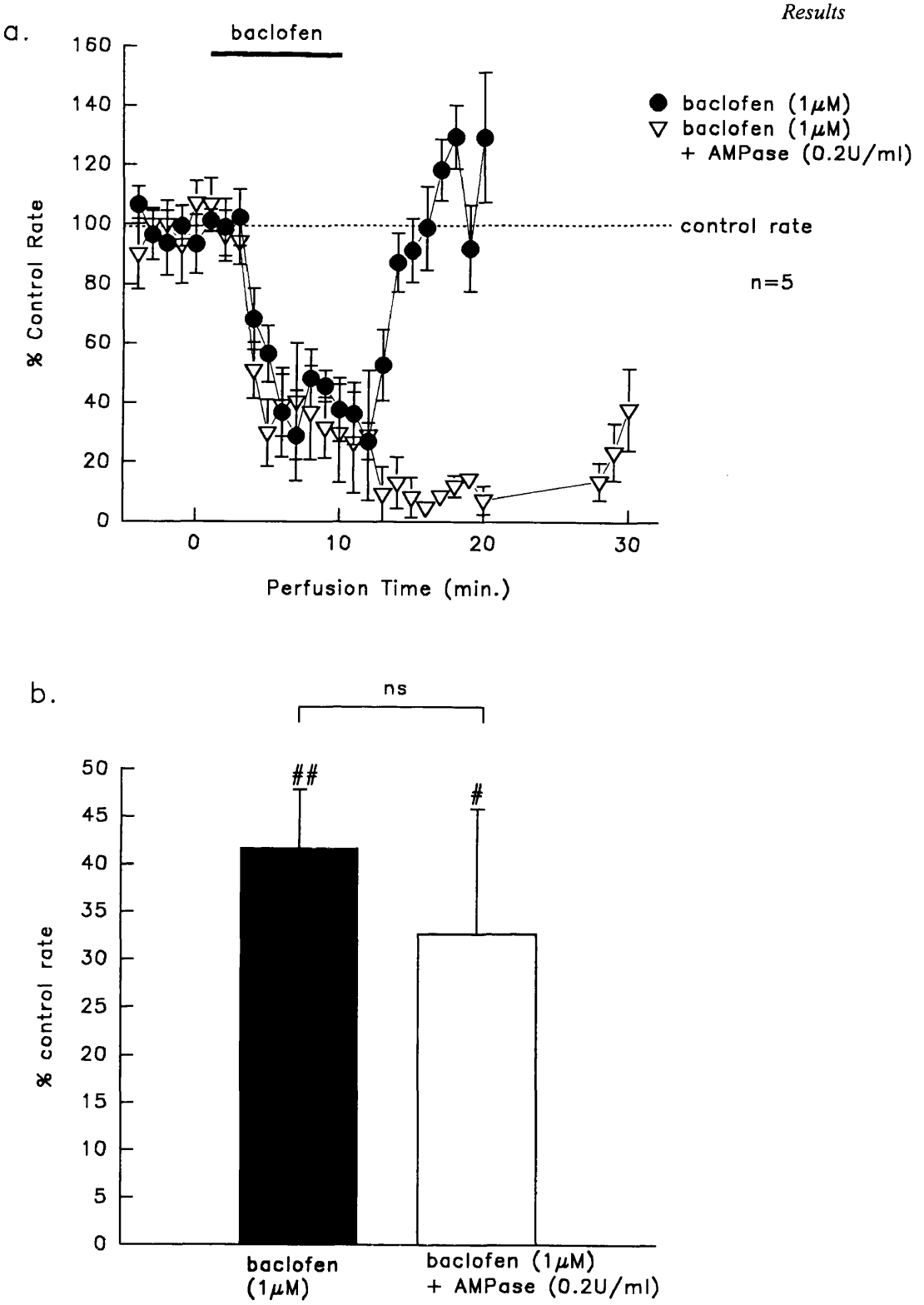



**Figure 64** (a) illustrates the time course of the effect of baclofen alone and in the presence of AMPase (0.2U/ml). In both instances discharge rate is significantly depressed with # $P<0.05$, ## $P<0.01$ (b). AMPase did not alter this effect of baclofen.

### *3.7.1 IMP*

AMPase deaminates AMP to form IMP, and it was possible that the metabolite was responsible for the reduction in burst frequency by AMP deaminase. IMP (5, 10, 50, 200 and 500μM) failed to alter to any significant extent the rate of activity (fig. 65a). Even during the following 15 min. wash period the rate was not changed. Fig. 65b shows the time course of the effect or non-effect of IMP (200μM).

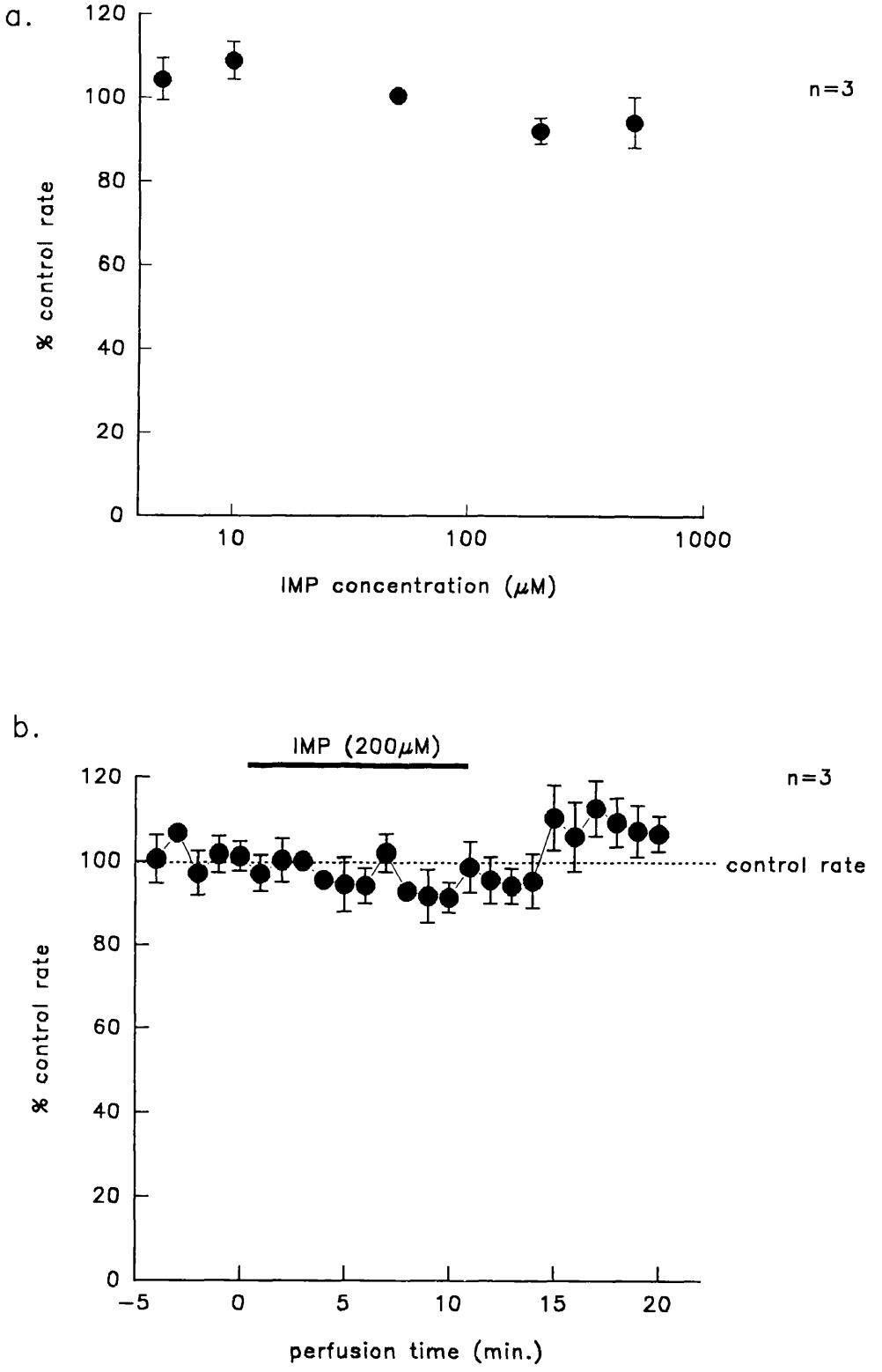



**Figure 65** (a) a concentration response curve to IMP. (b) shows the time course of perfusion with IMP (200μM).

### *3.7.2 Dialysis*

Commercial enzymes can, in some instances, contain high levels of ions which can interfere and complicate results. AMPase was dissolved in water and dialysed in a continuous flow of water for 4 days, after which the normal composition of 0Mg/4AP was made up. Dialysed AMPase (0.2U/ml) depressed activity subsequent to its perfusion (fig. 66) in a manner similar to fresh AMPase. The process of dialysis did not affect the activity of AMPase as dialysed AMPase (0.2U/ml) reduced the extent of inhibition produced by AMP from 50.4%±9.01 to 9.28%±4.88 which was not significantly different from control rates (fig. 67).

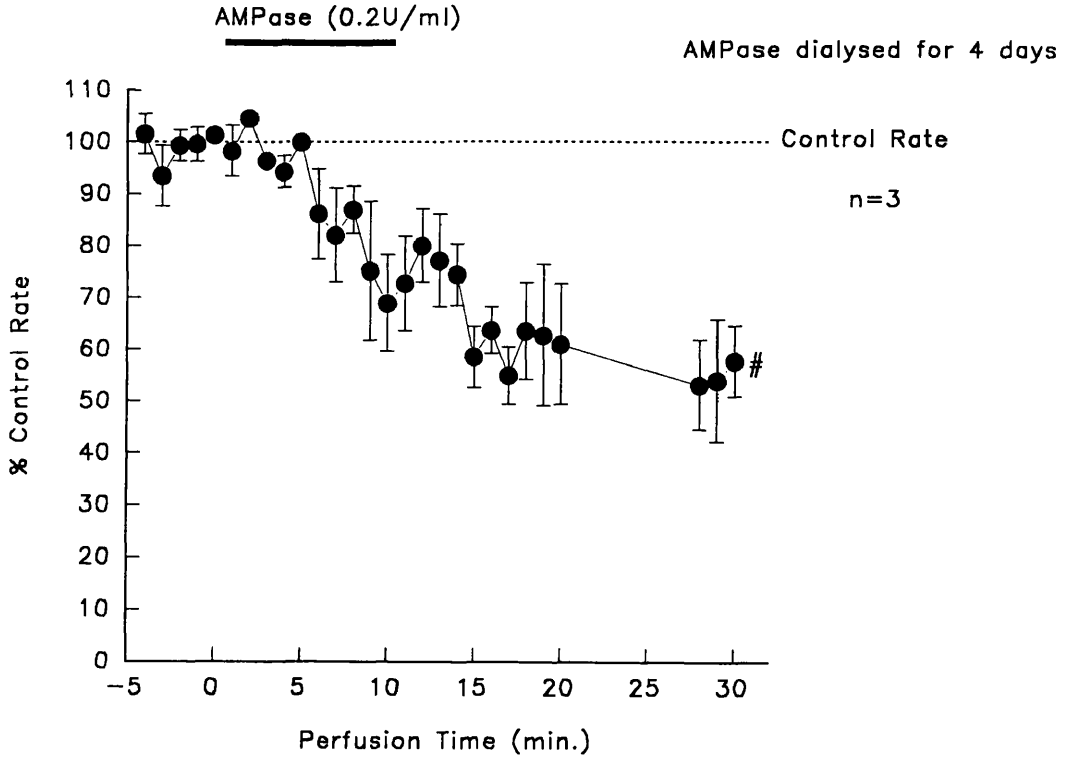
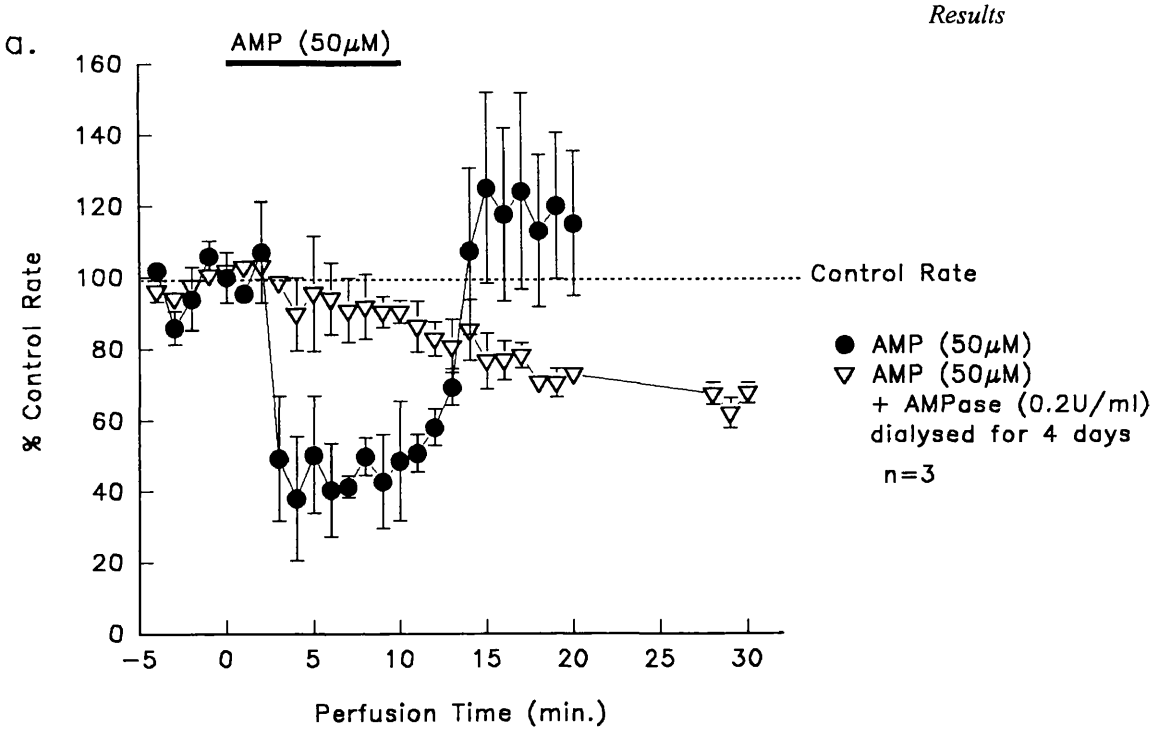



**Figure 66** AMPase was dialysed for 4 days in a continuous flow of water before being perfused for 10 minutes. # $P<0.05$.



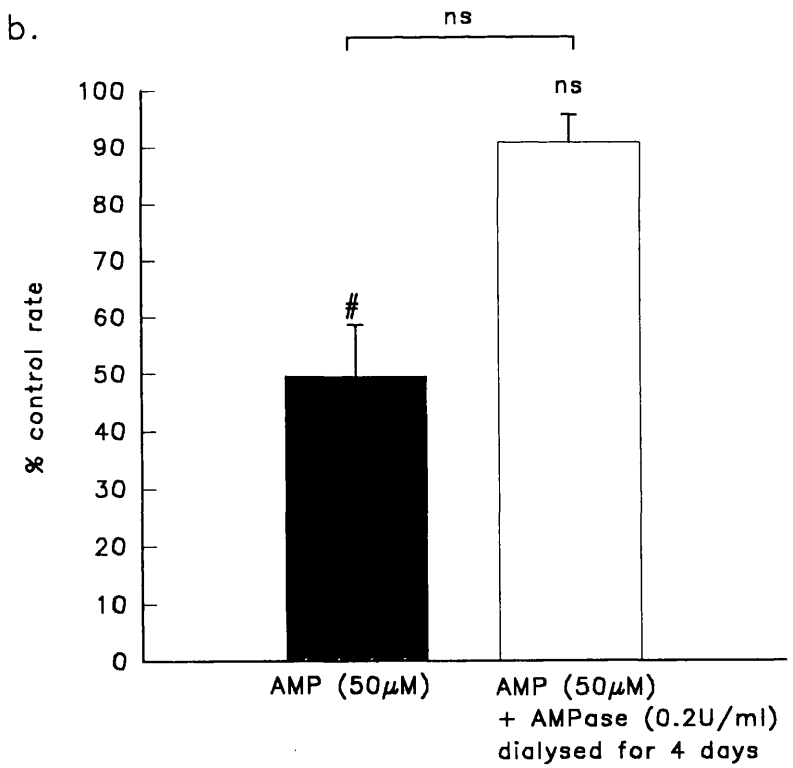




**Figure 67** The activity of dialysed AMPase was tested by perfusing dialysed AMPase in combination with AMP. A time course is shown in (a) with the mean effect analysed in (b). # P<0.05 and ## P<0.01.

### *3.7.3 Enzyme denaturisation*

The question of whether the enzyme itself was producing this effect or whether a contaminant was responsible remained unanswered. AMPase was dissolved in water and boiled for 10 minutes in an attempt to denature the enzyme. Although the perfusion of boiled AMPase (0.2U/ml) produced a fluctuation in rate both above and below control, no distinct depression of epileptiform activity ensued (fig. 68). AMP (50μM) in the presence of boiled AMPase (0.2U/ml) still inhibited the discharge rate to 62.33%±6.93 of control which was not significantly different from the control response (fig. 69). Furthermore, a recovery towards control values occurred after the perfusion of AMP + boiled AMPase (fig. 69a).

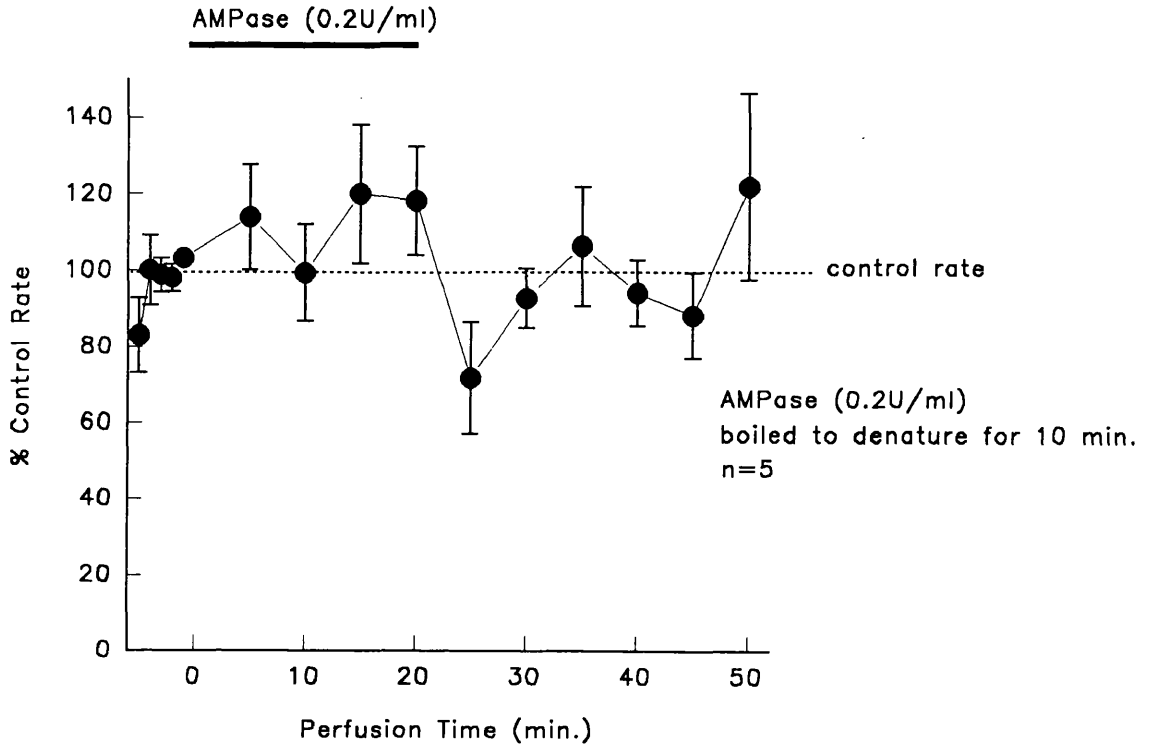



**Figure 68** AMPase was dissolved in water and boiled for 10 minutes. The normal composition of 0Mg/4AP was made up and the boiled AMPase perfused for a period of 10 minutes.

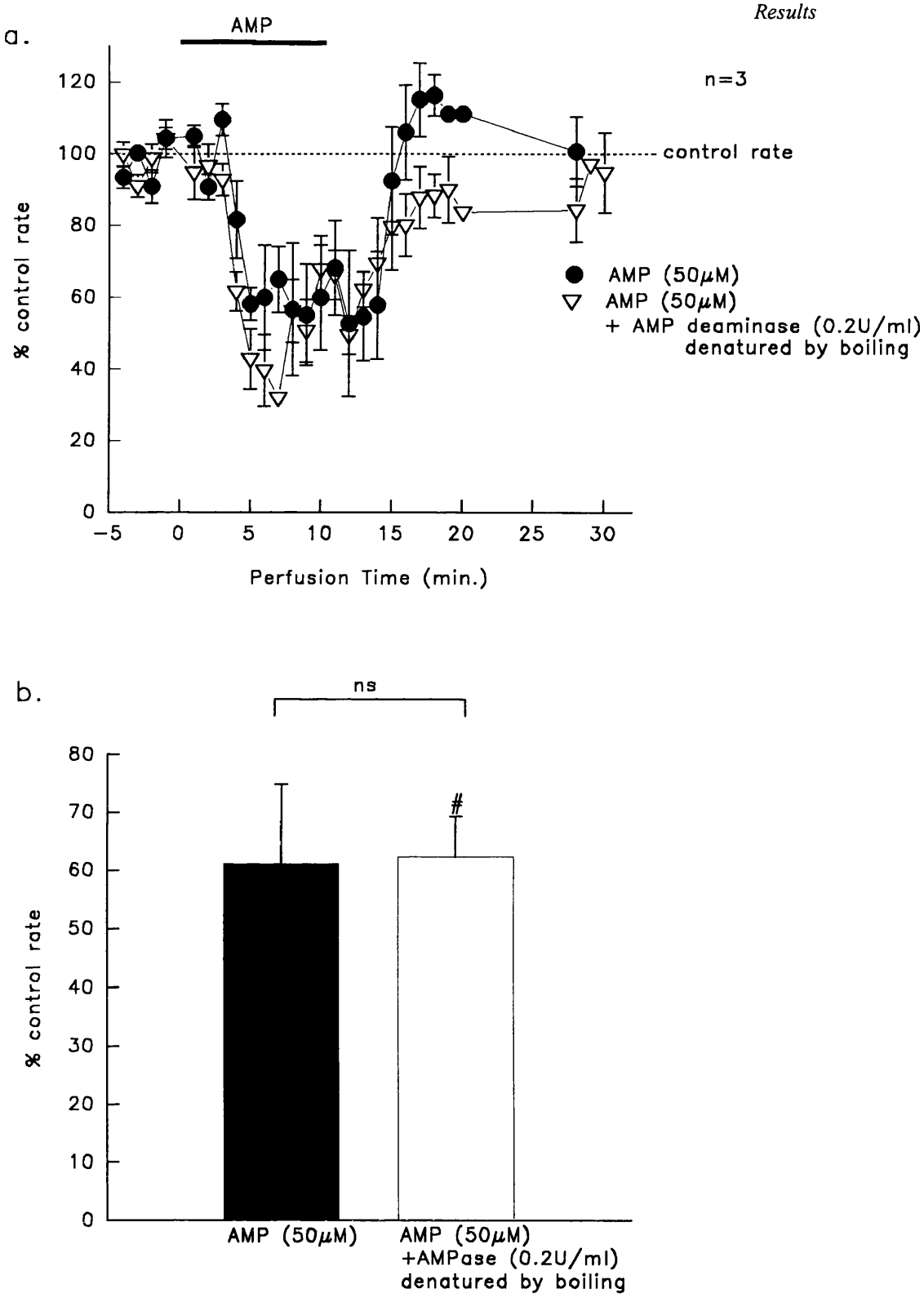



**Figure 69** The affect of boiled AMPase on the depression of activity caused by AMP. A time course of the effect is shown in (a) with the mean effect analysed in (b). AMP alone and in the presence of boiled AMPase decreased the discharge rate to a significant extent with # $P<0.05$.

### *3.7.4 Different media*

Using a $0Mg^{2+}/K^{+}$ (8mM) medium, AMPase (0.2U/ml) decreased the rate to greater extent than in 0Mg/4AP with a recovery to around 50% control (fig. 70). In a medium with normal magnesium and 4AP (50μM) an increase to 281.39%±10.15 control occurred during AMPase (0.2U/ml) perfusion (fig. 70b). During the wash the rate decreased to very low levels.

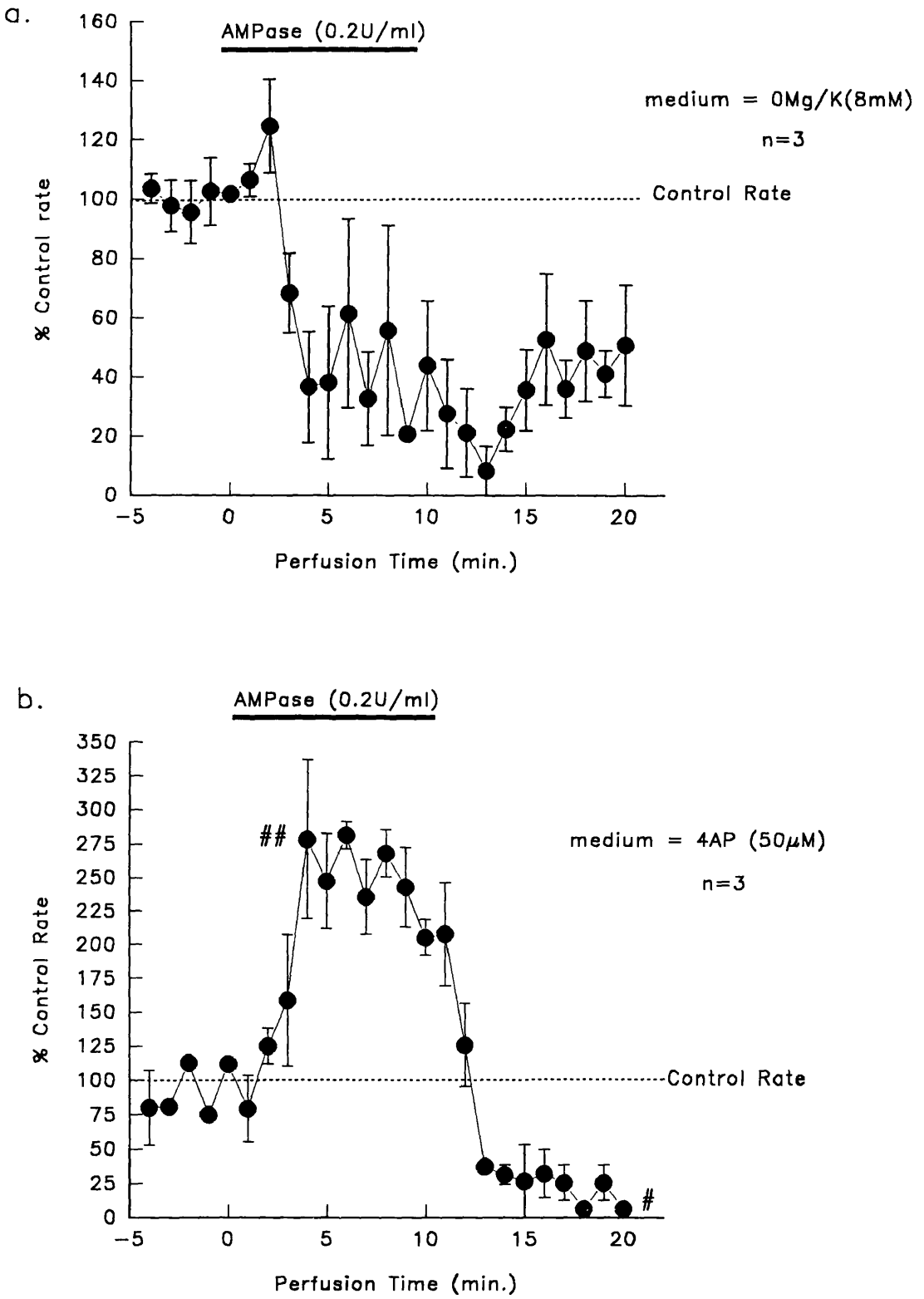



**Figure 70** The time course of the effect of AMPase in a OMg/K (6mM) and a 4AP (50μM) medium is shown in (a) and (b) respectively. # $P<0.05$, ## $P<0.01$.

### *3.7.5 Simultaneous evoked potentials and spontaneous activity*

Simultaneously produced evoked population potentials and spontaneous epileptiform bursts were recorded in both the CA3 and CA1 regions of the hippocampus. In the CA3 region neither discharge rate nor primary population spike amplitude changed during AMPase perfusion (fig. 71a). During the following 20 minutes discharge rate decreased, significant at 10 minutes, whereas population spike amplitude increased, significant at 20 minutes. During AMPase perfusion and in the subsequent 20 minute wash period discharge rate recorded from the CA1 was significantly reduced whereas in contrast the population spike amplitude increased (fig. 71b).

### *3.7.6 Population spikes*

The effect of AMPase (0.2U/ml) on the amplitude of evoked population spikes alone was investigated in both the CA1 and CA3 hippocampal subfields. In the CA3 region AMPase increased the spike size to 177.6%±8.98 of control. This enhancement was sustained for approximately 10 minutes before the potential began to decrease in size until only a very small population response was evoked after stimulation (fig. 72a). In the CA1 area AMPase also had a tendency to increase spike amplitude although this did not reach significance and was not maintained but instead decreased to around 40% control (fig. 72b). This level of inhibition was conserved for up to 90 minutes after the perfusion of AMPase.

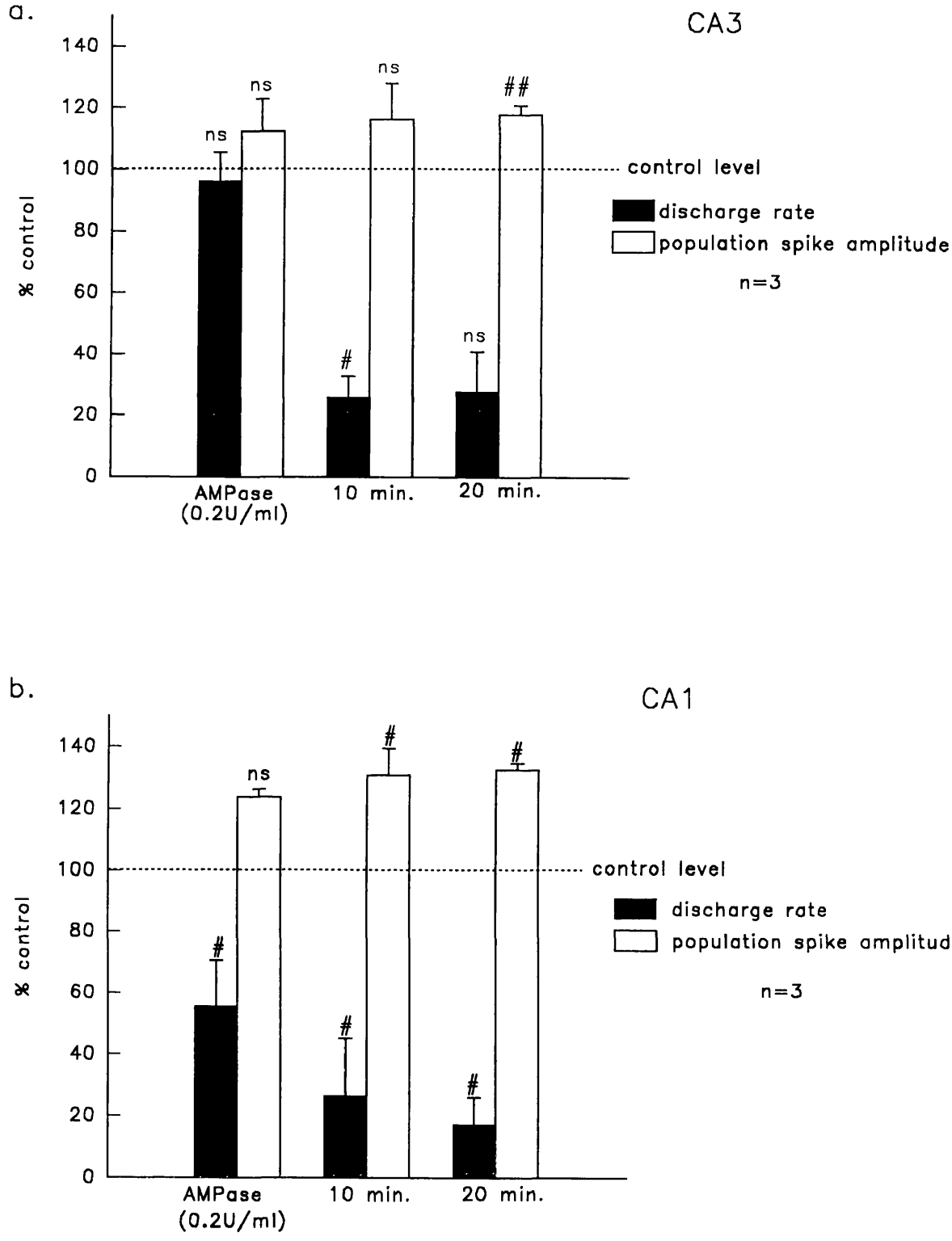



**Figure 71** The effect of AMPase on simultaneously recorded spontaneous activity and evoked population potentials in the CA3 (a) and CA1 (b) regions of the hippocampus. The three groups of bars represent the effect after a 10 minute perfusion with AMPase and during the wash period at the 10 and 20 minute time points respectively. # $P<0.05$.

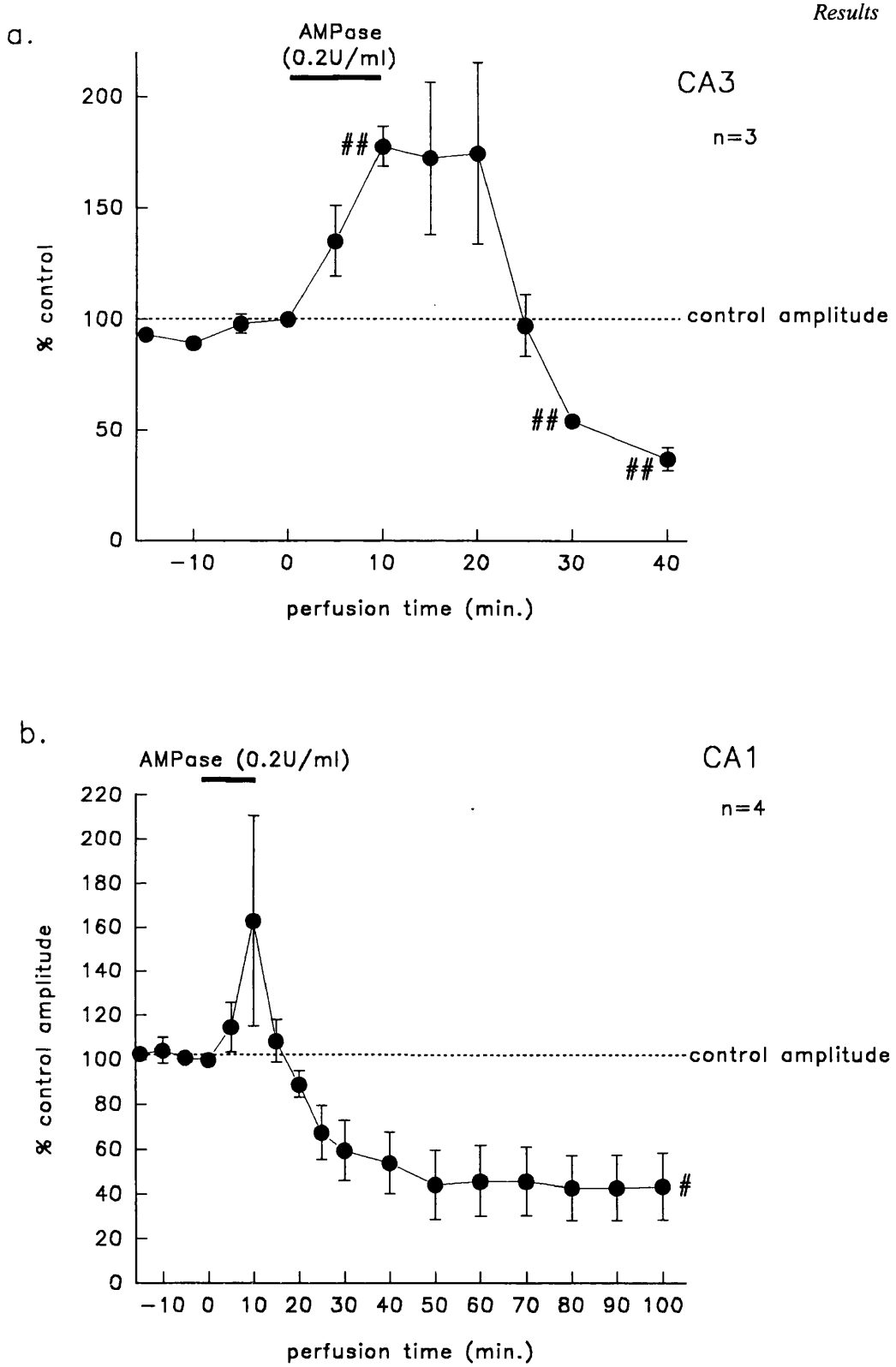



**Figure 72** The effect of AMPase on population spike amplitude in the CA3 (a) and CA1 (b) subfields of the hippocampus with # $P<0.05$ and ## $P<0.01$.

### *3.7.7 Antagonists and inhibitors*

In an attempt to inhibit the depression of epileptiform activity resulting from AMPase perfusion numerous receptor antagonists and pathway inhibitors were perfused both with and following AMPase (0.2U/ml). These experiments allow insight into the initiation of depression but not its long term maintenance as perfusion was only continued for 20-30 minutes after AMPase perfusion.

The modulation of the effect of AMPase (0.2U/ml) by DPCPX (50nM) was investigated on simultaneously generated spontaneous and evoked activity (fig. 73). DPCPX (50nM) significantly increased the activity rate whilst not changing the population spike amplitude. AMPase addition further increased the burst frequency before a subsequent decline resulted which persisted at least 70 minutes after the washout period for AMPase. The population spike amplitude remained at approximately control size for the period of recording.

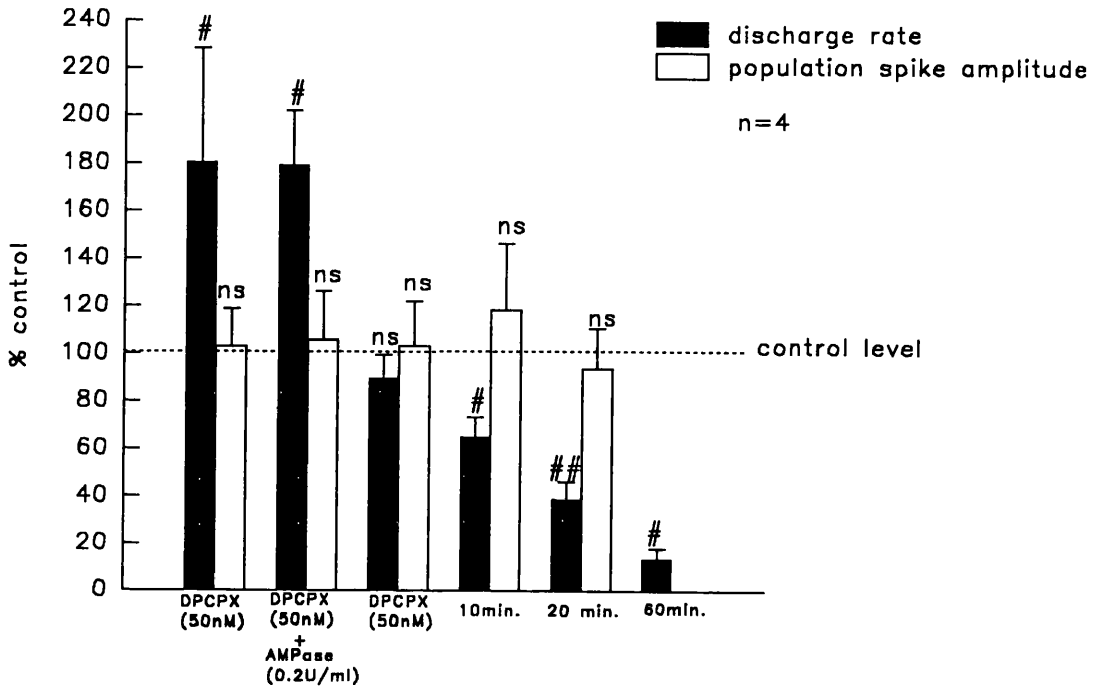



**Figure 73** Both spontaneous activity and evoked potentials were investigated in this set of experiments. The bars represent in the following order the effect at the end of a 10 minute perfusion with DPCPX alone, DPCPX + AMPase, reperfusion with DPCPX alone and at the 10, 20 and 60 minute time points of the wash period. # $P<0.05$ and ## $P<0.01$.

Naloxone, an opioid antagonist, at concentrations of 1, 10 and 50μM had no effect on the rate of CA3 activity when perfused alone (fig. 74a). Naloxone (10μM) did not alter the depressant effect of AMPase (fig. 74b). Similarly bicuculline, a $GABA_A$ receptor antagonist, at 10μM failed to affect the decrease in activity rate caused by AMPase (fig. 75).

Indomethacin and aspirin inhibit cyclo-oxygenase and hence the metabolism of arachidonic acid. AMPase, in the presence of either indomethacin (50μM) or aspirin (500μM), continued to cause a resultant depression of activity to 45.3%±6.79 and 53.57%±3.57 of control respectively after 20 minutes wash (fig. 76a and b).

*N*-(2-aminoethyl)-5-chloro-1-naphthalene sulphonamide (A3) is a non-selective protein kinase inhibitor. AMPase + A3 (100nM) potentiated the rate to a significant extent 141.58%±14.6 of control before a reduction occurred which reached a plateau during the perfusion of A3 alone and then the wash period (fig. 77).

Nitric oxide has been postulated to be both neurotoxic and neuroprotective and also to act in a convulsive and anti-convulsive manner. L-NAME, an inhibitor of nitric oxide synthase (NOS), alone caused no significant changes in the frequency of epileptiform activity. The presence of L-NAME (30μM) did not impede the depression of activity resulting from AMPase perfusion (fig. 78).

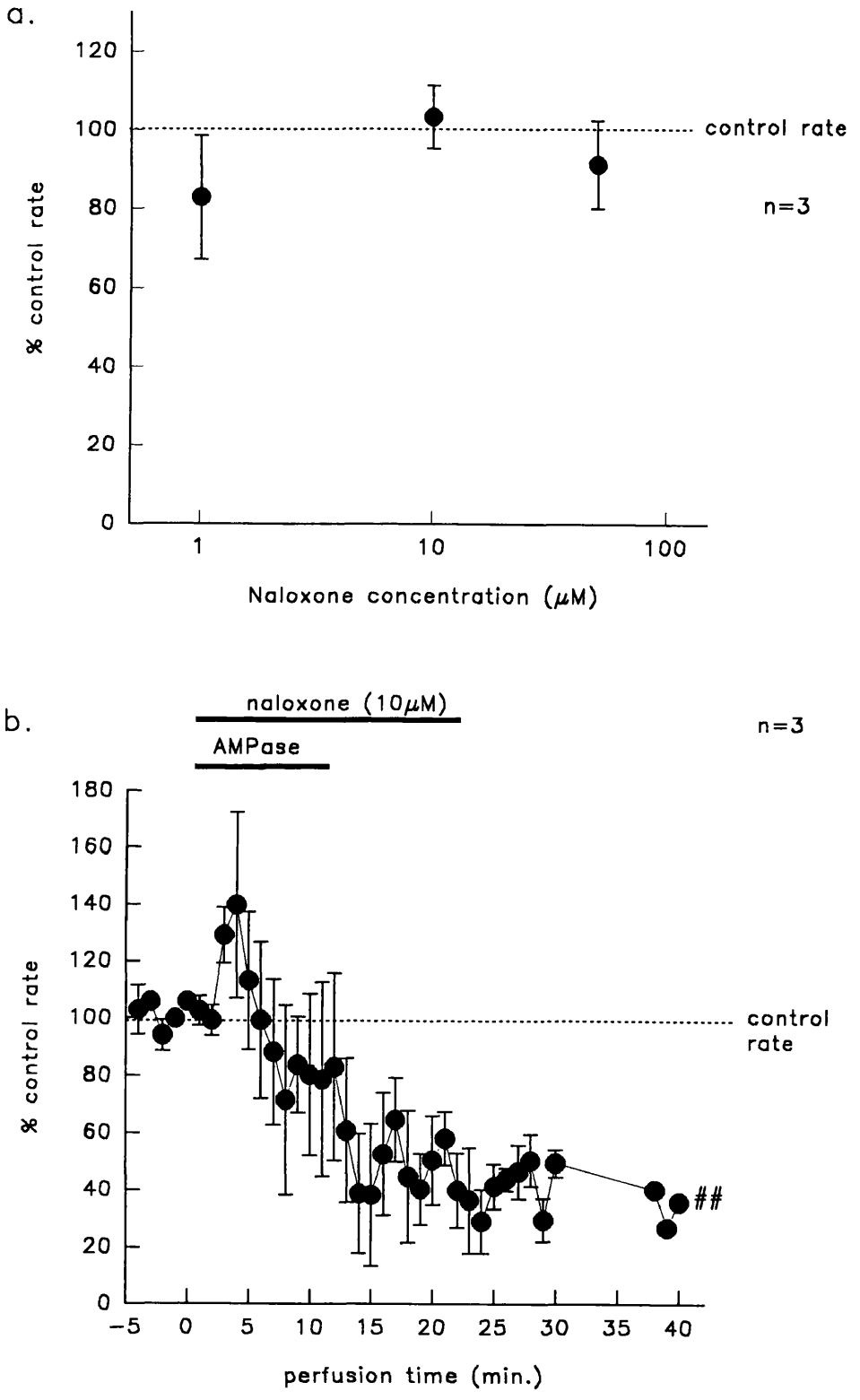



**Figure 74** (a) shows a concentration response curve to naloxone. Each concentration was perfused for 10 minutes with a minimum of 15 minutes between subsequent additions. Naloxone was perfused together with AMPase for 10 minutes followed by a period of 10 minutes in which naloxone was perused alone (b). ## $P<0.01$.

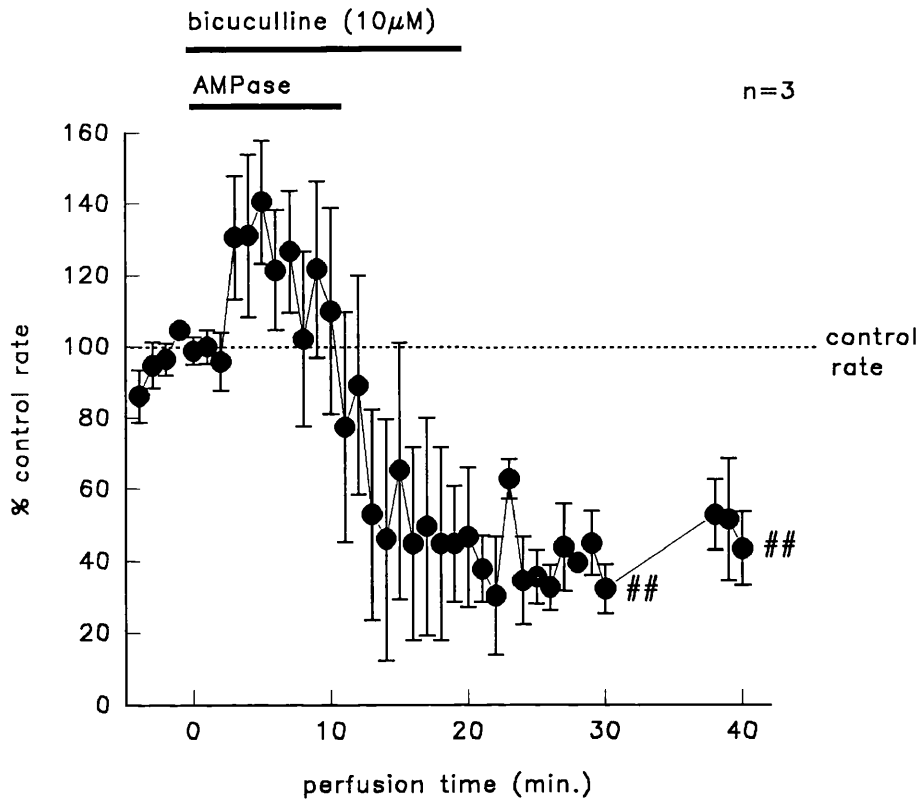



**Figure 75** The effect of bicuculline on the effect produced by AMPase. Bicuculline was perfused with AMPase and then alone both for periods of 10 minutes.

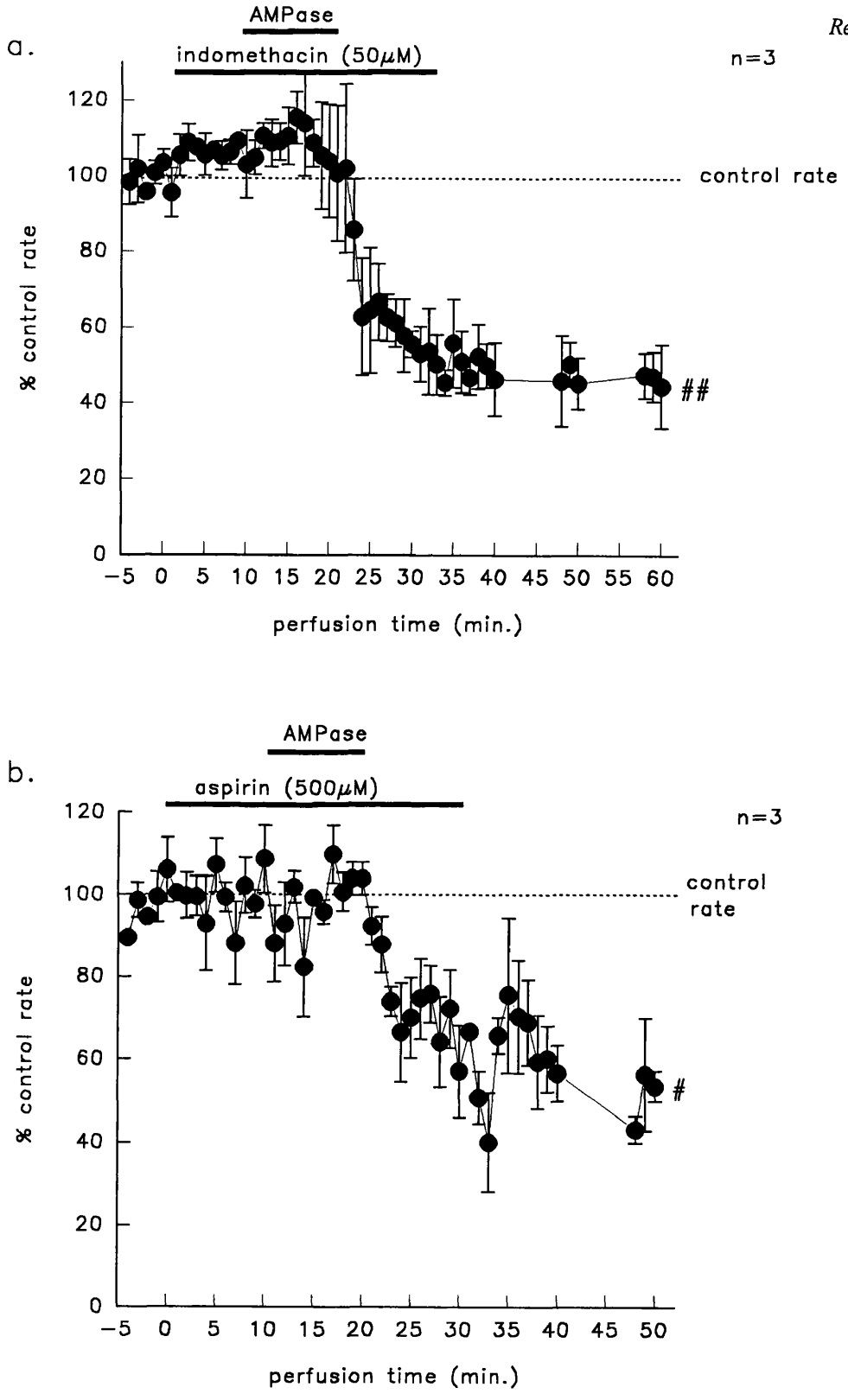



**Figure 76** The effect of indomethacin (a) and aspirin (b) on the long term depression produced by AMPase. Indomethacin and aspirin were perused for 10 minutes before and after perfusion with AMPase. # $P<0.05$, ## $P<0.01$.

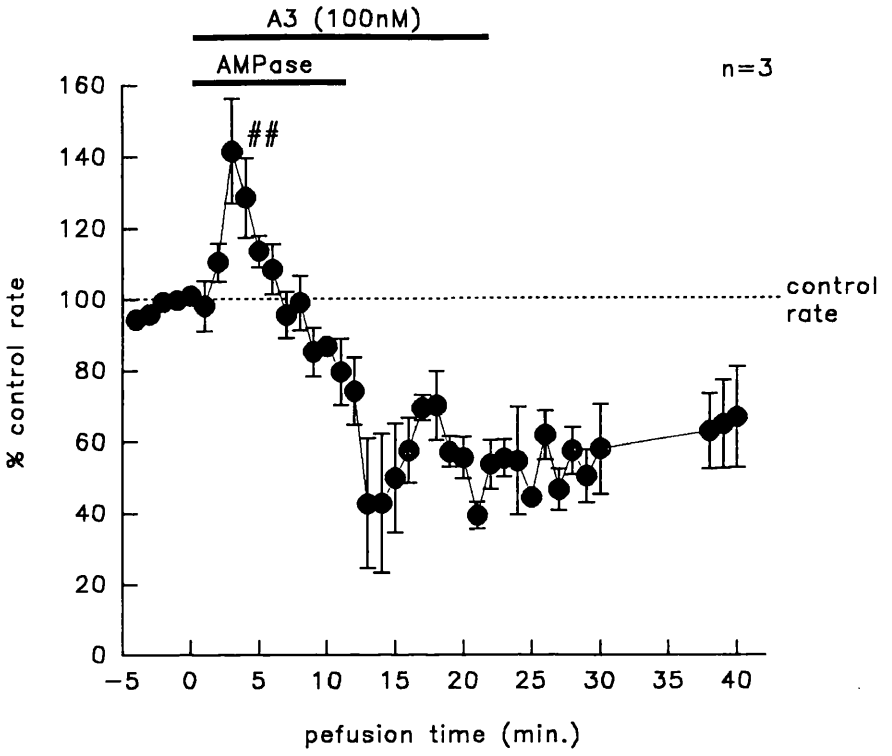



**Figure 77** A3, a protein kinase inhibitor, was co-perfused with AMPase and for the following 10 minutes perfused alone. # $P<0.05$.

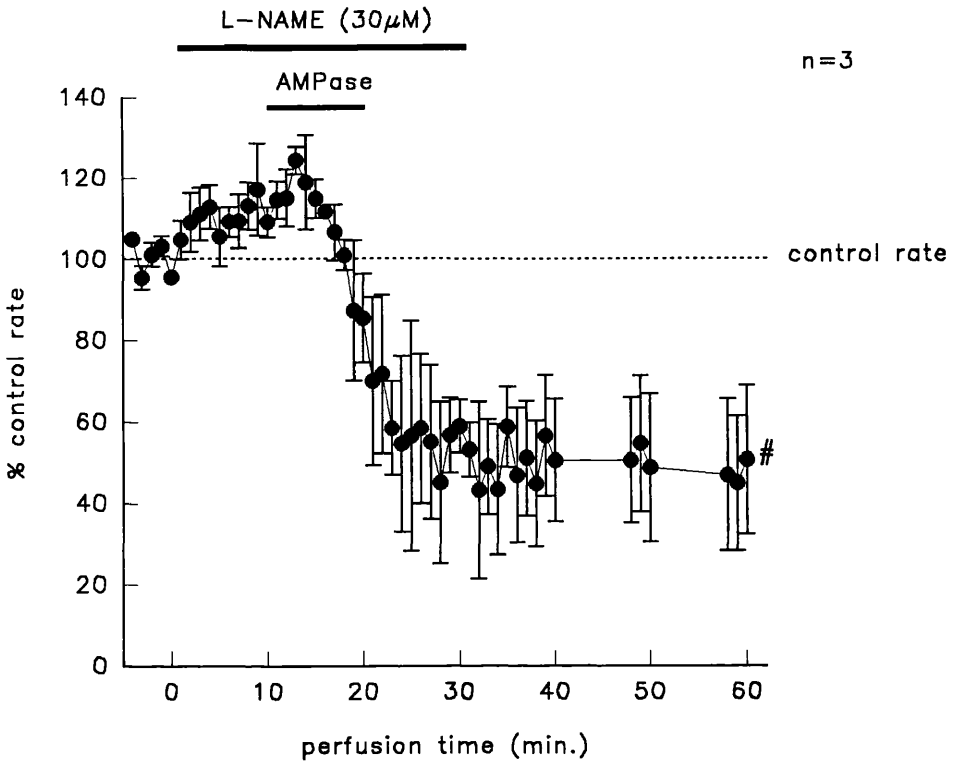



**Figure 78** The effect of L-NAME on the effect of AMPase. L-NAME was perfused alone for 10 minutes, with AMPase for 10 minutes and then alone for a further 10 minutes. # $P<0.05$.

# 4.0 Discussion

## 4.1 Use of hippocampal slices

Hippocampal slices have been used extensively over the last few decades as an indispensable research tool with far too many references to quote individually. The basis for using the hippocampus as a slice preparation stemmed from its ability to respond in a similar manner *in vitro* to that *in vivo* (Skrede & Westgaard, 1971) and also due to the lamellar organisation which meant that each slice could respond similarly to the next in a fashion typical of the hippocampus (Andersen *et al.*, 1971). Recent anatomical studies have argued against the lamellar hypothesis (Amaral & Witter, 1989) showing that the projections arising from injections of horseradish peroxidase into the CA3 region can extend to three quarters of the septotemporal CA1 region (Ishizuka *et al.*, 1990). This suggests that longitudinal projections are as important to the normal functioning of the hippocampus as those at the level of origin and that the hippocampus should be considered three dimensional instead of two. If this view is to be taken literally then the use of *in vivo* studies should be favoured in preference to slice preparations and also cell cultures.

The basic method for the preparation of slices appears to be consistent between different laboratories with the main variables being:

1. McIlwain tissue chopper or Vibratome cutter. The McIlwain chopper allows the production of reliable slices with little handling of the tissue required and is the system used in this study and by many other groups (Lipton *et al.*, 1995).

2. the type of chamber used: interface, submerged or static. In a submerged chamber the slice sits on a wire mesh held in place by a wire foot which extends around the edges of the slice and is completely submerged in oxygenated aCSF. Both advantages and disadvantages exist.
3. the most prominent difference between experimenters is the composition of the aCSF used. For the aCSF used in this study the proportion of ions used are within the range used by other groups.
4. temperature has a critical effect on the functioning of the hippocampus. Increasing temperature reduces the field excitatory postsynaptic potential (fEPSP) latency and increases the fEPSP slope in the CA1 region of hippocampal slices (Schiff & Somjen, 1985). In general temperatures used range between 29 and 35 $^{o}$C (Teyler, 1980). A temperature of between 33 and 35 $^{o}$C was used in this study which is within the range used by several other groups (Yaari *et al.*, 1983; Sagratella *et al.*, 1987; Perreault & Avoli, 1991; Watts & Jefferies, 1993; Whittington *et al.*, 1995; Behr & Heinemann, 1996). Electrographic seizures generated by raising extracellular potassium but not interictal activity were abolished if the temperature was reduced to 27-29 $^{o}$C (Traynelis & Dingeldine, 1988).
5. animal age or weight. The animals used for the majority of our experiments were between 170-250g which is regarded as being representative of an adult animal. Many groups studying epileptiform activity use young rats because of reports that it is easier to generate ictal activity in these animals when compared to adults (Anderson *et al.*, 1986). At this weight there was no

difficulty in getting consistent generation of epileptiform activity of an interictal nature although ictal activity was never obtained.

6. treatment of the animal prior to slice preparation. Hypothermia can protect against ischaemic brain injury in animal models. A study in which the rat was placed on a bed of ice whilst under anaesthesia until the rectal temperature reached 31 °C found that the subsequent survival of hippocampal slices was remarkably improved, indicating that hypothermia should be used in brain slice preparation (Newman *et al.*, 1992). The majority of rats in the present work were cooled under anaesthesia until rectal temperature reached 31 °C whilst breathing $O_2$ enriched air.
7. the anaesthetic used. Many conflicting reports are available regarding the use of urethane (ethyl carbamate) as an anaesthetic. Urethane washes quickly out of *in vitro* preparations, taking approximately 10 min. (Bagust & Kerkut, 1981). Urethane has been reported to depress both glutamate release (Moroni *et al.*, 1981) and depolarisation evoked by glutamate addition (Evans *et al.*, 1981) but to have no effect on GABAergic transmission (Moroni *et al.*, 1981). Depression of hippocampal unit activity by urethane showed differential rates of recovery (Mecer *et al.*, 1978). Paired-pulse inhibition was also reduced when urethane was used (Shirasaka & Wasterlain, 1995). Although in some instances urethane does not interfere with seizure activity (Sloviter, 1983) in other cases anticonvulsant effects of urethane have been reported (Cain *et al.*, 1989, 1992; Heltovics *et al.*, 1995). When using *in vitro* slice preparations this anticonvulsant property of urethane should not be a problem due to its rapid washout. In contrast pentobarbitone has a long-lasting effect (Versteeg, 1984).

8. Another area is the analysis of results. In a number of occasions in this study the results have been expressed as time v's % control rate graphs. This method of presenting results has been questioned for the use of *in vitro* slice preparations (Reid *et al.*, 1988). The major draw-back in using this type of analysis is that between different experiments, no matter how regulated it is, the perfusion rate is going to vary. Even if this is only to a small extent any change could still affect the time the drug takes to reach the bath and hence the pattern of effect produced. In this study variations in the time for maximum responses to be elicited could vary with time but whether this was an actual effect or simply due to perfusion changes could not be determined. Therefore to combat this time graphs were used to illustrate the general characteristics of an effect but in a number of experiments either the mean effect of a few minutes was used which should help minimise changes in perfusion rate or the maximum effect from each individual slice was measured and analysed.
9. Although in most instances consistent effects were elicited, on some occasions large discrepancies were apparent. Two variations in the analysis of results may have removed these problems and given additional insight into drug effects. The frequency of epileptiform activity varied between slices, a property which is common as previously mentioned. However this does pose a problem in that the relative change in spike number does not always correlate well with percentage change such that with slow rates a decrease by even 1 burst/minute produces a substantial percentage decrease. Therefore a minimum rate should be obtained or the slice abandoned. Although slices producing very low rates were not normally used, in conditions of different media frequencies

lower than would normally have been expected may have been used. Regression analysis, however, found no linear correlation between the initial discharge rate and the effect of adenosine or ATP.

10. The second point is that burst duration also varied between slices. The reason for this was not investigated, but a possibility is that within individual slices the contribution of removing magnesium and adding 4AP to burst generation may not have been consistent. Although it was not clearly evident the overall effect produced by some drugs may have been dependent on the burst type i.e. single or multiple spikes. Drugs may also have acted to limit burst duration instead of or in addition to burst frequency. Again maybe only bursts exhibiting certain characteristics should have been used. Bursts were only used if their amplitude exceeded 0.5 mV.

## 4.2 Generation of epileptiform activity

Several *in vitro* models of epileptiform activity have been used in order to gain greater understanding of the epileptic brain, including the perfusion of hippocampal slices in such a way as to either increase excitation or reduce inhibition.

Intrinsic properties of the CA3 subfield have led to its being dubbed the pacemaker for epileptic activity within the hippocampus, with activity thence being propagated to the CA1 region. Epileptiform activity recorded in the CA3 and CA1 regions simultaneously is synchronised with bursts in the CA3 area preceding those in the CA1 by 5-15 milliseconds (Lothman *et al.*, 1981). The

onset of epileptiform activity, being recorded first in the CA3 and thereafter in the CA1, is a feature noted by several groups (Schwartzkroin & Prince, 1978; Tancredi *et al.*, 1990) The severing of connections between the CA3 and CA1 regions does not alter activity in the CA3 but abolishes discharge in the CA1 (Swartzkroin & Prince, 1978) which suggests that abnormal input from the CA3 in to the CA1 is responsible for burst generation in the CA1. However a study by Mesher & Schwartzkroin (1980) in which the excitatory substance penicillin was restricted to the CA3 region showed that abnormal input from CA3 to CA1 alone was not adequate to evoke epileptiform activity in the CA1 and that changes intrinsic to the CA1 are also required. More recently Perez *et al.* (1996) showed the existence of sprouted CA1 axon collaterals in slices obtained from rats which had received intracerebroventricular injections of kainate. Therefore, recurrent excitation may contribute to epileptic activity in this area. In support of this proposal, slices from kainate treated rats, in which the CA1 region had been isolated, generated burst afterdischarges upon stimulation when bathed in a medium containing bicuculline (Meier & Dudek, 1996). In the present study the generation of epileptiform activity in the CA1 region was inconsistent. However if the recording electrode was re-situated in the CA3 pyramidal cell layer spontaneous activity was apparent. It is possible that the placement of the stimulating electrode in the CA1 stratum radiatum in some way damaged the connections between the two regions and hence the propagation of epileptiform activity. In the latter stages of this study epileptiform activity was generated in combination with stimulation in area CA1 using a medium containing no added magnesium and 4-aminopyridine (50μM).

The discharges produced were interictal in nature and consisted of defined bursts of millisecond duration containing one or more spikes (fig. 3). Between slices the discharge rate, burst amplitude and burst duration varied to some extent but remained similar in individual slices. This variability is commonly noted (Jefferys & Haas, 1982; Ault & Wang, 1986; Konnerth *et al.*, 1986; Stringer & Lothman, 1988; Kostopoulos *et al.*, 1989; Tancredi *et al.*, 1990). The omission of magnesium or the addition of 4-aminopyridine have been used extensively as separate ways to induce epileptiform activity (Mody *et al.*, 1987; Schneiderman & MacDonald, 1987; Chestnut & Swann, 1988; Watts & Jefferies, 1993; Arvanov *et al.*, 1995; Traub *et al.*, 1995; Whittington *et al.*, 1995; Avoli *et al.*, 1996a) but their combined use is not well established. A recent report using rat coronal slices and intracellular recording techniques found a combination of zero magnesium and 4-aminopyridine (100μM) to generate spontaneous paroxysmal depolarising shifts and postsynaptic depolarising potentials (Sisiscalchi *et al.*, 1997). The concentration of 4AP used was similar to that used by other groups (Voskuyl & Albus, 1985; Avoli *et al.*, 1996a; Psarropoulou & Avoli, 1996). In this study the individual components, zero magnesium and 4AP (50μM), when perfused alone did not consistently generate epileptiform activity, in contrast to their combined effect, but would sustain bursts already generated.

In general epileptiform activity generated under zero magnesium conditions is highly dependent upon NMDA receptor activation (Horne *et al.*, 1986; Mody *et al.*, 1987; Schniederman & MacDonald, 1987; Neuman *et al.*, 1988; Tancredi *et al.*, 1990; Psarropoulou & Kostopoulos, 1991) whereas activity induced by 4AP in

rats involves to a large extent the activation of non-NMDA receptors (Gean *et al.*, 1990; Perreault & Avoli, 1991; Avoli *et al.*, 1996a). 2-amino-5-phosphonopentanoic acid (AP5), a selective NMDA receptor antagonist (Perkins *et al.*, 1981; Davies *et al.*, 1981), decreased but did not totally abolish epileptiform activity suggesting the involvement of NMDA receptors in the maintenance of bursting in this model of epileptiform activity. Kynurenate was initially described as a selective amino acid antagonist in the cerebral cortex (Perkins & Stone, 1982) causing inhibition of both NMDA and non-NMDA receptors. However, in certain regions of the CNS kynurenate shows greater inhibition of NMDA responses (Ganong *et al.*, 1983; Elmslie & Yoshikami, 1985).

Kynurenate selectively inhibited the response to iontophoretically applied NMDA in the hippocampal CA3 region of normal slices, being ineffective against kainate and quisqualate up to concentrations of 1mM (Stone, 1990). In this study kynurenate produced a dose dependent reduction in discharge rate with 200μM decreasing the rate by approximately half and 1mM exerting close to a total inhibition of epileptiform activity.

The complexity of the antagonism exerted upon ionotropic glutamate receptors by kynurenate makes a clear interpretation hard. Two explanations are possible. The epileptiform activity generated by a 0Mg/4AP medium is dependent solely on NMDA receptors if kynurenate is selective for NMDA receptors in the CA3 region although the concentration for total block is higher than that previously reported (Stone, 1990). However, the lack of total inhibition by AP5 suggests that

this is not the case. The fact that both concentrations of AP5 used had similar effects rules out the possibility that a high enough concentration of antagonist had not been used. Concentrations are within the range used by other groups (Mody *et al.*, 1987; Neuman *et al.*, 1988). Therefore in this instance it appears that kynurenate is acting in a non-selective manner to inhibit both NMDA and non-NMDA receptors. The use of a selective non-NMDA receptor antagonist such as CNQX would allow a clearer insight in to receptor involvement. In slices from rats treated with colchicine to destroy the mossy fibre projection to the CA3 cells kynurenate equally inhibited NMDA and kainate responses (Stone, 1990) revealing a discrimination between pre- and post-synaptic kainate receptors with regard to antagonism by kynurenate. In the zero magnesium/4-aminopyridine model of epileptiform activity activation of both NMDA and non-NMDA receptors is involved. This is in agreement with the study of Sisiscalchi *et al.* (1997) in which the application of either AP5 or CNQX totally abolished PDS's induced by OMg/4AP.

Slices (650μm) prepared from 3-4 week old rats were used in an attempt to generate bursts with ictal characteristics using a number of strategies which have been reported to result in such behaviour (see appendix 2 for details). However, these attempts were in vain, with only interictal activity being generated, if any at all. Although protocols that had been reported to generate ictal activity were followed, a number of variables exist that could be responsible for our unsuccessful attempts.

Age is a major factor. A greater incidence of ictal activity being generated in slices from young rats, although the occurrence of ictal activity has been reported in adult tissue (Swatzwelder *et al.*, 1987). This was addressed by using animals in the weight range of 60-100g which is within the range used by several groups (Anderson *et al.*, 1986; Anderson *et al.*, 1990). In a number of studies the stage of animal development is given in days old as opposed to weight. In this study animals were between 25-30 days old which corresponds to postnatal week 4. Psarropoulou & Avoli, (1996) found that the incidence of ictal activity decreased profoundly in the $4^{th}$ postnatal week. In agreement with this Gloveli *et al.* (1995) found that slices from rats of postnatal day 15-25 were the most epileptogenic. In contrast slices prepared from rats of 22-35 and 30-45 days old still produce seizure-like activity in response to excitatory media (Swartzwelder *et al.*, 1988; Lewis *et al.*, 1989).

Temperature can also affect the response of hippocampal slices. For all the experiments in this study the temperature in the bath was approximately 34°C which should allow the induction of ictal epileptiform activity (Lewis *et al.*, 1989; Anderson *et al.*, 1990; Velísek *et al.*, 1994; Avoli *et al.*, 1996a). The pH of the perfusing medium was 7.4. Again this should not affect the ability to generate ictal activity.

Other variables exist in the preparation of the slices for example the method used to kill the rat or whether anaesthetic was used. In this study rats were killed by cervical dislocation under urethane anaesthesia before being decapitated.

Decapitation under anaesthesia induced by halothane (Avoli *et al.*, 1996a), ether (Velisek *et al.*, 1994; Gloveli *et al.* 1995) and chloroform (Lewis *et al.*, 1989) have subsequently not interfered in the induction of ictal activity. Although urethane has been reported to act as an anticonvulsant (Heltovics *et al.*, 1985; Cain *et al.*, 1989) its rapid washout from slices should not have interfered with slice excitability.

The rat strain and sex may have contributed to the differences between this and other studies. In the majority of cases Sprague-Dawley rats were the strain used (Anderson *et al.*, 1986; Swartzwelder *et al.*, 1988; Lewis *et al.*, 1989; Anderson *et al.*, 1990; Velísek *et al.*, 1994; Avoli *et al.*, 1996a; Psarropoulou & Avoli, 1996). Wistar rats were used by Jones (1989) but in this instance slices of entorhinal cortex and not hippocampus were used. As with this study male rats were most frequently used.

In addition to interictal and ictal activity, perfusion with 4AP produced negative potentials which are blocked by the $GABA_A$ receptor antagonist bicuculline (Mattia *et al.*, 1994; Avoli *et al.*, 1996b). These GABA mediated potentials, which are associated with an increase in extracellular potassium concentration, precede the incidence of ictal activity. It has been proposed, therefore, that these GABA mediated events are involved in synchronising neurones resulting in the initiation of ictal events (Avoli *et al.*, 1996b). Potentials with these characteristics were not observed in this study. However such events have not been reported yet for a zero magnesium medium. Either one or a combination of these slight

differences could, therefore, possibly be the reason for our inability to generate ictal activity.

### 4.3 Modulation of epileptiform activity by ATP

Over the last few years investigations into the role of extracellular ATP and P2 purinoceptors have established that ATP is an important extracellular compound acting through a variety of P2X and P2Y receptors (Burnstock, 1990; Chen *et al.*, 1995). However, there is very little information regarding the effect of ATP on epileptiform activity. The study of ATP is hampered by its degradation to metabolites which are active in their own right, especially adenosine.

ATP and adenosine depressed epileptiform activity in our model to a similar extent which raised the question of whether ATP was acting directly or after metabolism to adenosine. Adenosine or adenosine analogues reduce spontaneous or evoked epileptiform activity induced *in vitro* by media containing no added magnesium (O'Shaughnessy *et al.*, 1988; Kostopoulos *et al.*, 1989), penicillin (Lee *et al.*, 1984) or bicuculline (Ault & Wang, 1986). *In vivo* adenosine is a potent anticonvulsant against pentylenetetrazole induced (Malhotra & Gupta, 1997), kindled (Dragunow & Goddard, 1984) and audiogenic seizures (Maitre *et al.*, 1974). Inhibitors of adenosine uptake enhance the depressive effect of adenosine, whereas P1 receptor antagonists inhibit this effect on epileptiform activity (Ault & Wang, 1986; O'Shaughnessy *et al.*, 1988; Barbarosie *et al.*, 1994).

In addition to portraying anticonvulsive tendencies when applied exogenously, adenosine has been postulated to represent an endogenous anticonvulsant. Several lines of evidence support such a theory. Dipyridamole, an inhibitor of adenosine

uptake (Bender *et al.*, 1980), decreased bicuculline induced epileptiform activity in hippocampal slices in a dose-dependent manner (Ault & Wang, 1986). Similarly nitrobenzylthioinosine, also an adenosine uptake blocker (Hammond & Clanachan, 1984), decreased the rate of occurrence, duration and amplitude of spontaneous epileptiform events generated in human neocortical slices by a magnesium free-medium (Kostopoulos *et al.*, 1989) in the absence of exogenous adenosine. P1 receptor antagonists caffeine and aminophylline induced seizures *in vivo* after intraperitoneal administration (Chu, 1981). Likewise, in slices of hippocampus, theophylline induced bursting of an interictal nature in the CA3 region (Ault & Wang, 1986). In this study the perfusion of CPT in an otherwise normal medium resulted in spontaneous activity within 5 minutes of the start of perfusion. Such activity was gradually reversed upon return to perfusion with a normal medium. This is in contrast to the persistence of epileptiform activity that occurs under normal conditions following DPCPX addition in guinea-pig hippocampal slices (Alzheimer *et al.*, 1989).

During experimental seizures induced by bicuculline in rats (Winn *et al.*, 1979) and cats (Schrader *et al.*, 1980) the concentration of adenosine rapidly rises above basal levels. The concentration of adenosine in the extracellular fluid of patients suffering from complex partial seizures both prior to, during and after a seizure was investigated using a non-invasive *in vivo* microdialysis technique (During & Spencer, 1992). Basal levels were calculated to be approximately 2μM and rose up to 60-fold during a seizure and remained elevated for at least 18 minutes. Furthermore pentylenetetrazol-induced seizures in mice induce an upregulation of $A_1$ receptor density which is not uniform throughout the brain but is restricted to distinct anatomical areas including the hippocampus where the biggest change was in the stratum oriens and stratum radiatum of the CA1 region (Angelatou *et al.*, 1990; Pagonopoulou *et al.*, 1993). Significant elevation of adenosine receptors also occurred in the same layers of the CA3 subfield. Whether a similar scenario

is apparent in humans in response to seizures is not clear as conflicting reports have been published (Angelatou *et al.*, 1993; Glass *et al.*, 1996).

Whether endogenous adenosine acts to limit seizure spread or initiation is also a source of debate. The depression of epileptiform activity by adenosine that occurs in *in vitro* models, including the present one, is often accompanied by an increase in burst amplitude and duration, which is indicative of an effect on burst initiation and not spread (Ault & Wang, 1986). However in a kindling model of seizures in the amygdala adenosine reduced the severity of motor seizure duration and spread but not the afterdischarge threshold (Dragunow *et al.*, 1985).

The anticonvulsant action of adenosine and related analogues is mediated through the activation of $A_1$ receptors (O'Shaughnessy *et al.*, 1988; Dunwiddie & Fredholm, 1989). Both pre- and post-synaptic mechanisms have been postulated to be involved (Dunwiddie, 1980; Fredholm & Hedquist, 1980; Lee *et al.*, 1984; Alzheimer & ten Bruggencate, 1991; Scholz & Miller, 1991). Adenosine acting on receptors located on pre-synaptic terminals can alter the release of glutamate (Corradetti *et al.*,1984), acetylcholine (Jackisch *et al.*, 1984) and noradrenaline (Jonzon & Fredholm, 1984) in the hippocampus. The exact mechanism by which adenosine receptor activation results in decreased synaptic transmission has not been fully established although reduced calcium entry and an effect on the release mechanisms have been proposed. The activation of postsynaptic $A_1$ receptors results in membrane hyperpolarisation in response to the generation of potassium currents (Gerber *et al.*, 1989; Thompson *et al.*, 1992).

### *4.3.1 Adenosine deaminase*

The action of adenosine *in vivo* is regulated both enzymatically and by uptake. In the present work adenosine deaminase was used at a concentration of 0.2U/ml which was sufficient to abolish fully the effect of added adenosine. At the same concentration adenosine deaminase did not block the inhibitory activity of ATP to a significant extent suggesting that the depression was not mediated by the formation of adenosine. The use of adenosine deaminase has been criticised due to the large size of the enzyme which may restrict entry to small compartments where the production of adenosine from ATP is occurring. Hence in ctivation of locally formed adenosine would not occur (Rubio *et al.*, 1987). However adenosine deaminase has been used in many tissues with great success to inhibit the production of adenosine from ATP (Côte *et al.*, 1993; Barajas-Lopez *et al.*, 1995; King *et al.*, 1996).

### *4.3.2 $A_1$ receptor antagonists*

Cyclopentyltheophylline (CPT), an $A_1$ receptor antagonist with a 140-fold selectivity for $A_1$ over $A_2$ receptor subtypes (Bruns *et al.*, 1986), increased the rate of burst discharge substantially, a result which is consistent with the view that $A_1$ receptor antagonists are proconvulsants due to inhibiting the basal activation of $A_1$ receptors. Although a slight decrease towards control levels occurred during the wash period, a reduction back to control was never fully achieved. CPT has been shown previously to be difficult to wash out of slices (Mitchell *et al.*, 1993). 8-(*p*-sulphophenyl)theophylline (8pSPT) which is a non-selective P1 receptor antagonist also increased the basal rate of activity in a concentration-dependent manner. As with CPT this is thought to result primarily from reduction of a tonic purinergic tone.

The extent of excitation produced by CPT may be dependant on the basal concentration of endogenous adenosine. If high levels of adenosine are present

then theoretically the control rate of epileptiform should be lower than in circumstances of low concentrations of endogenous adenosine. If the initial rate of activity is low (i.e. high concentrations of basal adenosine) CPT should produce a more substantial increase in rate. Such a theory was investigated by regression analysis of initial rate against both the percentage of control rate and the actual rate of activity after CPT perfusion. In both instances a significant correlation was obtained suggesting a linear relationship could exist between initial rate and the extent of the effect.

The discovery of binding sites for [$^3$H]CGS21680, a selective agonist for $A_{2A}$ receptors, detection of mRNA for $A_{2A}$ receptors by *in situ* hybridisation studies (Cunha *et al.*, 1994a) and the enhanced excitability produced in a few occasions by adenosine (Nishimura *et al.*, 1992; Okada *et al.*, 1992) all support the theory that adenosine, acting via $A_{2A}$ receptors, can act as an excitatory neuromodulator in addition to an inhibitory one. Hence it was possible that $A_1$ receptor antagonists unmasked an excitatory effect involving the activation of $A_{2A}$ receptors by endogenous adenosine. Such a theory has been postulated to explain, in part, the persistent spiking activity of DPCPX in hippocampal slices (Chesi & Stone, 1997). ZM241385 is 30-80 fold selective for $A_{2A}$ over $A_{2B}$ receptors and 400-1000 fold selective over $A_1$ receptors in guinea-pig isolated tissues (Poucher *et al.*, 1995). At a concentration which should only inhibit $A_2$ receptors, ZM241385 did not alter the rate of epileptiform activity which suggests a lack of basal activity of $A_2$ receptors. A complicating factor when considering the activation of both $A_1$ and $A_2$ receptors is not just the opposite modulation of excitability that they induce but the ability of $A_2$ receptors to negatively modulate $A_1$ receptors which has been reported *in vitro* (Cunha *et al.*, 1994a; O'Kane E.M., unpublished observations) and *in vivo* (Jones P.J., unpublished observations). The involvement of $A_2$ receptors in the excitatory effect of CPT is not substantiated due to the ineffectiveness of ZM241385. In contrast the discharge rate was

substantially elevated from control values when a higher concentration was used. However at this concentration of 2μM the selectivity of ZM241385 for $A_2$ over $A_1$ receptors may be compromised, such that the excitation produced results from $A_1$ receptor inhibition.

As expected, CPT completely prevented the inhibitory effect of adenosine, supporting previous conclusions that adenosine inhibits epileptiform activity through $A_1$ receptors (Lee *et al.*, 1984; Thompson *et al.*, 1992; Barbarosie *et al.*, 1994). The combination of adenosine and 8pSPT, however, produced an additive effect comprising the depression of adenosine and the excitation of 8pSPT. From this it is proposed that adenosine can overcome the antagonism of 8pSPT due to its reduced selectivity for the $A_1$ receptor compared with CPT. Matsuoka *et al.* (1995) found that concentrations of 10-100μM 8pSPT were required to inhibit the effect of adenosine.

Unexpectedly, however, in view of the results with adenosine deaminase, CPT but not 8pSPT, reversed the effect of ATP. This combination of results with CPT and adenosine deaminase strongly suggest that the inhibitory effect of ATP on burst frequency may be mediated by the nucleotide acting directly at a P1 receptor, or at a xanthine-sensitive P2 receptor. In slices of cortex ATP and related analogues decrease the evoked overflow of [$^3$H] noradrenaline in a manner which is not sensitive to adenosine deaminase or suramin but which is inhibited by 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX) (von Kügelgen *et al.*, 1992). This led the authors to propose that nucleotides could act directly on P1 receptors located presynaptically. A similar suggestion has been made more recently that adenosine dinucleotides may act directly on P1 receptors in the heart (Hoyle *et al.*, 1996). A third subtype of purine receptor, the $P_3$ purinoceptor, has been proposed. Shinozuka *et al.* (1988) found that both nucleotides and nucleosides could modulate the electrically evoked release of noradrenaline from the rat caudal

artery suggesting a potency order which was distinct from previously documented receptors. This proposed $P_3$ receptor, which is inhibited by P1 receptor antagonists and α, β-meATP (Shinozuka *et al.*, 1990; Todorov *et al.*, 1994), has also been described in the rabbit vas deferens (Todorov *et al.*, 1994) and guinea-pig submucosal neurones (Barajaz-López, 1995). A binding protein which could be classified as a putative $P_3$ receptor has been purified from rat brain membranes (Saitoh & Nakata, 1996). In general the $P_3$ purinoceptors are located on the pre-synaptic membrane.

### *4.3.3 Nucleotide analogues*

The initial subclassification of P2 purinoceptors into P2X and P2Y was based on the differential effects of two ATP analogues, α, β-meATP and 2-meSATP (Burnstock & Kennedy, 1985). α, β-meATP is relatively resistant to degradation by ecto-ATPases thus reducing the complicating factor of metabolism to adenosine (Welford *et al.*, 1986). Earlier studies have failed to demonstrate a consistent effect of α, β-meATP in hippocampal slices (Stone & Cusack, 1989). The LTP reported to be induced by ATP was not imitated, but rather inhibited by α, β-meATP (Wieraszko & Ehrlich, 1994). In the present study α, β-meATP was found to elevate the level of epileptiform activity. α, β-meATP is considered to activate preferentially P2X receptors (Burnstock & Kennedy, 1985). These receptors involve an intrinsic ion channel which is generally considered to be non-specific for cations (Benham & Tsien, 1987; Bean, 1992) and activation of P2X receptors is usually coupled to situations which involve an increase in cell excitability, for example muscle contraction (Meldrum & Burnstock, 1983; Burnstock & Kennedy, 1985; Khakh *et al.*, 1995b) and neuronal depolarisation (Illes *et al.*, 1994).

2-meSATP is subject to the same metabolism as ATP but can induce inward currents in cultured hippocampal neurones with a greater potency than ATP and α,

β-meATP (Balachadran & Bennett, 1996). In this study 2-meSATP failed to alter the rate of epileptiform activity. Due to the potential metabolism of this compound, α, β-meADP, a 5'-nucleotidase inhibitor, was applied together with 2-meSATP but the latter remained ineffective. The lack of effect of 2-meSATP would suggest an absence of P2Y receptors in the hippocampal CA3 region.

Much evidence is available surrounding the existence of a receptor which is activated by uridine triphosphate (UTP). In many instances it is suggested that ATP and UTP are acting at different receptors since, for example, PPADS antagonises responses to ATP but not UTP (Connolly, 1994) and there are dissimilarities in the effects produced by the two nucleotides (Häussinger *et al.*, 1984; von Kügelgen *et al.*, 1987). However, cross-desensitisation between ATP and UTP (Brown *et al.*, 1991) and their equipotency in a number of cell types has been demonstrated (Lustig *et al.*, 1993; Parr *et al.*, 1994) suggesting that both compounds may activate the same receptor in some systems or some circumstances. Several receptors have been cloned which have varying affinities for purine and pyrimidine tri- and di-phosphates which may be able to shed light on the often confusing data surrounding the action of UTP. A receptor activated by ATP and UTP which depressed potassium currents has been demonstrated in hippocampal neurones (Nakazawa *et al.*, 1994). In the present study the inactivity of UTP, at a concentration at which ATP produced a clear effect, suggests that a $P_{2U}$ or similar receptor subtype was not involved in the modulation of CA3 neuronal bursting.

### *4.3.4 Nucleotide antagonists*

In addition to agonist potency orders, the classification of P2 receptors has been based on antagonist activity. Three main antagonists are used: suramin - a non-selective P2 antagonist (Dunn & Blakeley, 1988), PPADS - an agent with limited selectivity for P2X receptors but which can also inhibit P2Y but not $P_{2U}$ receptors at higher concentrations (Lambrecht *et al.*, 1992; Windscheif *et al.*, 1994; Brown

*et al.*, 1995) and reactive blue 2 (RB2) - which has a small concentration window in which it is considered to be selective for P2Y receptors (Burnstock & Warland, 1987).

Suramin does not affect responses to carbachol and noradrenaline on mouse vas deferens (Dunn & Blakeley, 1988) nor acetylcholine and 5-hydroxytryptamine in cultured coeliac ganglion neurones (Evans *et al.*, 1992; Silinsky & Gerzanich, 1993) but does inhibit the actions of ATP in these tissues and in many others which support its use as a selective P2 receptor antagonist. At concentrations within the range for P2 receptor antagonism, suramin increases both the EPSP slope and population spike amplitude recorded from the CA1 region of the mouse hippocampus in response to stimulation of the Schaffer collaterals in a manner which resembles long term potentiation (Wieraszko, 1995). In contrast to this suramin has been reported to block currents generated by the application of GABA, kainate and NMDA to cultured hippocampal neurones (Nakazawa *et al.*, 1995). Suramin, at a concentration within the range that inhibited glutamate currents, decreased the rate of epileptiform activity in our model. Since the generation of bursts involves NMDA and non-NMDA receptor activation it seems plausible to assume that this inhibition of spontaneous activity results from miscellaneous actions of suramin and not from antagonism of a basal excitatory tonus portrayed by P2 receptors. The lack of a similar effect of PPADS is supportive of this conclusion.

Suramin and PPADS did not antagonise the inhibitory action of ATP on epileptiform activity. PPADS, and to a small extent suramin, potentiated the effect of ATP, which may be explained if ATP were capable of causing both excitation and inhibition. This possibility is supported by the excitatory effect of $\alpha$, $\beta$-meATP and its inhibition by suramin and PPADS. Overall the results are consistent with the idea that P2X receptors exist in the CA3 region, which are

capable of increasing neuronal excitability. Seven subtypes of P2X purinoceptor have so far been cloned, all with distinct characteristics with regard to agonist potency, antagonism and desensitisation. $P2X_2$ (Kidd *et al.*, 1995), $P2X_4$ (Bo *et al.*, 1995; Buell *et al.*, 1996; Soto *et al.*, 1996) and $P2X_6$ (Collo *et al.*, 1996) receptors are expressed in regions of the hippocampus and it is possible, therefore, that one of these subtypes mediates the excitatory effect of α, β-meATP in the CA3 region. However, the characteristics of the cloned receptors with expression in the brain do not correlate with those found in this study. It is possible that other subtypes of P2X receptors exist which have yet to be cloned or that heterologous expression of receptor subtypes, as seen with $P2X_2$ and $P2X_3$ receptors, produces characteristics dissimilar from that of the cloned receptor.

### *4.3.5 Zinc*

Zinc is an endogenous trace element found within regions of the brain including the hippocampus where it is highly associated with the mossy fibres (Crawford & Conner, 1972) and can be released in response to excitatory stimulation (Assaf & Chung, 1984; Howell *et al.*, 1984). The ability of low micromolar concentrations of zinc to potentiate currents through ATP activated P2X receptors has been reported in rat pheochromocytoma PC12 cells (Koizumi *et al.*, 1995a), superior cervical ganglion neurones (Cloues *et al.*, 1993) and nodose ganglion neurones (Li *et al.*, 1993; Wright & Li, 1995). Zinc has been proposed to increase the affinity of the P2X receptor for ATP (Cloues, 1995; Koizumi *et al.*, 1995a; Wright & Li, 1995). Previous reports of inhibition of NMDA receptor channels (Peters *et al.*, 1987; Westbrook & Mayer, 1987) $GABA_A$ receptor channels (Smart, 1992) and potentiation of non-NMDA receptors (Peters *et al.*, 1987) by zinc together with a reduction in synaptic activity in the CA3 hippocampal region (Gutierrez *et al.*, 1995) would predict that zinc should modulate epileptiform activity. However, zinc had no effect on the rate of epileptiform activity in this model. Since a multitude of mechanisms are involved in burst production, it is possible that zinc

does affect epileptiform activity but that opposing effects cancel each other out. If ATP was acting at an excitatory P2X receptor in addition to an inhibitory one, it was postulated that the addition of zinc should augment the excitation resulting not necessarily in a positive response to ATP but in a reduced inhibition. Zinc neither potentiated or reduced the depression of epileptiform activity by ATP. This could mean either that ATP only acts in an inhibitory manner or that the excitatory receptor involved is not sensitive to modulation by zinc. Zinc differentially affects P2X and $P_{2U}$ receptors in PC12 cells (Koizumi *et al.*, 1995b).

### *4.3.6 Combination of ATP and adenosine*

The simultaneous addition of receptor agonists can be used to give an indication of whether the same or different receptors or transduction mechanism are being activated. At a low concentration the combined effect of ATP and adenosine was greater than that of ATP. This could be accounted for by the action of adenosine alone. At a higher concentration no additive effect (resulting in a significantly greater inhibition of epileptiform activity) resulted from the perfusion of ATP and adenosine. Therefore it is difficult to take anything from these experiments except that the combination of ATP and adenosine does not result in a response greater than the addition of either when perfused alone.

### *4.3.7 AMP*

In the classification of purinoceptors as P1 and P2, AMP was proposed to activate preferentially P1 receptors (Burnstock, 1978). AMP exerted a depressant effect in our model of epileptiform activity which is in agreement with the inhibitory effects of AMP on hippocampal population spikes (Dicori & Henry, 1984; Salter & Henry, 1985). This depression was reduced by adenosine deaminase at the concentration that annulled the effect of adenosine but which did not effect ATP. Adenosine deaminase will to some extent also deaminate AMP which could account for this effect. Although adenosine deaminase when perfused alone did

not significantly increase the rate of epileptiform activity a small rise was frequently seen. Therefore it is also possible that adenosine deaminase can reduce inhibitory responses in an additive manner. As for ATP and adenosine, CPT totally inhibited the action of AMP which suggests that AMP acts through $A_1$ receptors or possibly even through the same mechanism as ATP.

### *4.3.8 ATP metabolism*

One of the major problems with investigating the effect of ATP is that metabolism occurs. The metabolism of ATP is carried out primarily by a group of enzymes known as ectoATPases or ectonucleotidases, which were first described by Engelhardt (1957). These enzymes, which have their active site facing the extracellular medium (Nagy *et al.*, 1983; Grondal & Zimmerman, 1986), differ from their intracellular counterparts, mitochondrial ATPase, $Na^+/K^+$-ATPase and $Ca^{2+}$-transport ATPases (Nagy *et al.*, 1986). The metabolism of ATP by ectoenzymes has been reported in numerous brain regions including vestibular neurones (Cummins & Hyden, 1962), hippocampal neurones (Cunha *et al.*, 1992; Cunha *et al.*, 1994b) and synaptosomes from a number of brain regions including the cortex (Lin & Way, 1982) and the hippocampus (Nagy *et al.*, 1986). Thus, these enzymes represent a method for terminating the action of ATP released endogenously (Richardson & Brown, 1987; Terrain *et al.*, 1989; Kennedy *et al.*, 1996). EctoATPases are calcium and magnesium dependent, although only one cation has to be present for the enzymes to be active (Grondal & Zimmerman, 1986; Nagy *et al.*, 1986). Therefore, the enzymes should continue to be active in the present work despite the omission of magnesium from the medium. Several ectoATPases have been cloned (Lin & Guidotti, 1989; Bermudes *et al.*, 1994; Asai *et al.*, 1995). A discrepancy was discovered between the breakdown of neuronally released ATP, which was rapidly metabolised, and that of exogenous ATP, which persisted in the perfusate of isolated vas deferens for longer periods of time (Todorov *et al.*, 1997). However when $1N^6$-etheno-ATP was added during

stimulation of the sympathetic input into the vas deferens breakdown was enhanced. This led to the proposal that in addition to membrane bound ectoATPases a soluble ATPase exists which can be released upon nerve stimulation (Todorov *et al.*, 1997).

The formation of AMP from ADP results from the action of either ectoATPase, ectoADPase or adenylate kinase which is also thought to be an ecto-enzyme (Nagy *et al.*, 1989). The final enzyme involved is 5'-nucleotidase which is also located on the external aspect of membranes (Lee *et al.*, 1986). This dephosphorylates AMP to form adenosine.

In some instances it has been reported that the majority of ATP is metabolised in a biological system within 30 seconds (Green *et al.*, 1995), although an equally convincing body of work has failed to find substantial metabolism (Welford & Anderson, 1988; Matsuoka *et al.*, 1995). The major metabolites are, in order of their formation, ADP, AMP and adenosine. All three compounds can activate receptors: P2 receptors for ADP and P1 receptors for AMP and adenosine, and all these ATP metabolites can thus be agonists in their own right. Adenosine, as discussed above, was not the causative agent of the inhibitory effect of ATP. However, when attributing an effect to ATP the possible involvement of all the metabolites should be considered.

A number of mechanisms are available to rule out the involvement of metabolites. Enzymes which degrade the resulting metabolites to inactive compounds are commonly used. AMPase deaminates AMP to IMP which is not active. In the present model of epileptiform activity AMPase tended to decrease the discharge rate, but this was not significant over the 10 minute perfusion period used for drug application.

AMPase at a concentration of 0.2U/ml, which annulled the effect of AMP, totally inhibited the action of ATP. The time course of inhibition was different for ATP and ATP plus AMPase and the extent of inhibition produced by ATP was markedly reduced. At equilibrium, this concentration of AMPase reduced the effect of ATP in that it no longer produced a depression which was significantly different from the control rate. The percentage inhibition of activity rate noted in the later stages of perfusion with AMPase and ATP was similar to that seen with AMPase perfused alone and thus could be due to an action of the enzyme. When a higher concentration of ATP was used (200μM) AMPase totally inhibited the depression of discharge rate by ATP.

The attribution of an effect to the direct action of ATP is usually substantiated by the use of non-hydrolysable ATP analogues which are P2 receptor agonists, for example α, β-methyleneATP or ATPγS, or the inhibition by known selective P2 antagonists. However, a number of recent studies have reported effects of ATP which are inhibited by P1 receptor antagonists (von Kügelgen *et al.*, 1992; Cunha *et al.*, 1994b; Barajas-Lopez *et al.*, 1995; King *et al.*, 1996) but not P2 antagonists, leading to the concept that nucleotides can activate P1 receptors. Our earlier results were entirely consistent with this since ATP depressed epileptiform activity in a manner insensitive to adenosine deaminase but inhibited by the $A_1$ antagonist cyclopentyltheophylline. The role of metabolites in these studies and our own was ruled out mainly by using adenosine deaminase to degrade any adenosine formed, and α, β-methyleneADP, a 5'-nucleotidase inhibitor, to halt the production of adenosine from AMP. However, the involvement of AMP was not considered by us or previous groups (von Kügelgen *et al.*, 1992; Cunha *et al.*,1994b; Barajas-Lopez *et al.*, 1995; King *et al.*, 1996). The fact that adenosine antagonists blocked the effect of ATPγS was further evidence for the direct activation of P1 receptors by ATP and related analogues (von Kügelgen *et al.*, 1994; von Kügelgen *et al.*, 1992; Cunha *et al.*, 1994b). Although ATPγS is resistant to degradation in

endothelial cells and smooth muscle (Cusack *et al.*, 1983; Welford *et al.*, 1986) metabolism in skeletal muscle and potentiation of responses in a calcium/magnesium free medium suggest that in some instances ATPγS may be subject to metabolism (Cascalheira & Sebastião, 1992; Trezise *et al.*, 1994). Some groups, however, have failed to block ATP responses with AMPase, thus substantiating their suggestion that ATP activated P1 receptors (Griese *et al.*, 1991; Bo *et al.*, 1993 Côte *et al.*, 1993)

### *4.3.9 Interference by magnesium, calcium and 4-aminopyridine*

In a study investigating the effect of differing magnesium concentrations on the inhibition of synaptic transmission by adenosine in the hippocampal CA1 region, Bartrup & Stone (1988) found that reducing the concentration of magnesium in the perfusing medium produced a parallel decline in the ability of adenosine and related analogues to decrease population spike amplitude. The removal of magnesium ions increases excitability due to removal of the voltage-dependent block of NMDA receptor channels and relief of calcium antagonism. A simple elevation in excitability from normal which would require higher levels of inhibitory compounds to depress was ruled out as an explanation for reduced adenosine responses. In agreement with such an interaction the concentration of adenosine required to depress epileptiform activity to a similar extent is greater in conditions of zero magnesium compared with models in which bicuculline or penicillin are used to induce excitatory activity (Lee *et al.*, 1984; Ault & Wang, 1986; Kostopoulos *et al.*, 1989; Janusz & Berman, 1993). The potency of ATP but not adenosine was potentiated when a medium containing a normal complement of magnesium and 4AP was used to sustain epileptiform activity. A dependence on magnesium ions for receptor inhibition of adenylate cyclase (Yeung *et al.*, 1985) and an enhancement of cyclohexyladenosine binding by magnesium (Goodmann *et al.*, 1982) support the proposal that magnesium ions are required for $A_1$ receptor activation (Batrup & Stone, 1988). However a direct

interaction between magnesium and adenosine has been disputed (Smith & Dunwiddie, 1993) with suggestions that only manipulations which alter pre-synaptic calcium entry interfere with the ability of adenosine to modulate synaptic transmission.

Ecto-ATPases, enzymes responsible for ATP metabolism, are dependent on magnesium and calcium. The bathing of a preparation of vagus nerve (Trezise *et al.*, 1994) in media that was devoid of magnesium and calcium altered the potency order for activation of P2X receptors such that ATP and hydrolysable analogues, for example 2meSATP, were more potent than α, β-meATP. The measurement of inorganic phosphate as an indicator of metabolism revealed that under conditions of zero calcium/magnesium negligible metabolism of ATP and 2meSATP took place (Khakh *et al.*, 1995). The synthesis of the selective ecto-ATPase inhibitor ARL 67156 (formally know as FPL 67156) demonstrated that under normal circumstances, for example when ATP acts postjunctionally in the vas deferens or urinary bladder, the actions of ATP are limited by enzymatic breakdown (Westfall *et al.*, 1996; Westfall *et al.*, 1997). The function of ARL 67156 as an inhibitor of ectoATPase was investigated by using a human blood cell assay in which the dephosphorylation of [$\gamma^{32}$P]-ATP was analysed (Crack *et al.*, 1995). ARL 67156 inhibited [$\gamma^{32}$P]-ATP metabolism. This action of ARL 67156 was further substantiated by potentiating the contraction elicited by ATP and UTP but not α, β-meATP of rabbit ear artery (Crack *et al.*, 1995). This revelation regarding ATP, metabolism and receptor potency has a profound effect on receptor classification.

Attempts to investigate the effect of ATP on epileptiform activity under conditions of reduced or zero magnesium and calcium were to no avail. Numerous combinations of differing concentrations of magnesium, calcium and potassium were used as dictated in appendix 3 but in no instance could bursts be induced or maintained under such conditions. In a number of instances after a change from

0Mg/4AP the activity rate increased until the bursts disappeared. On reintroduction of 0Mg/4AP activity returned. Hence it is possible that these conditions over-depolarised the cells so that they could no longer respond. However, as mentioned previously the presence of either calcium or magnesium is enough for enzyme activation, so in this situation potentiation of ATP is not due to reduced metabolism. In light of the inhibition of ATP by AMPase, any potentiation of ATP by magnesium is likely to involve $A_1$ receptors. However, the lack of a similar interaction with adenosine brings this into doubt. Greater differences in adenosine potency between the two media may be more apparent lower down the concentration scale.

Another aspect of the interaction between adenosine and magnesium arises when considering the mechanism of zero magnesium epileptiform activity. If magnesium at normal physiological concentrations positively modulates $A_1$ receptor activation removal of this ion would enhance excitability through a reduction in basal purinergic inhibitory tone. The combination of low magnesium and 8-phenyltheophylline, a P1 receptor antagonist, augments population spike amplitude to no greater extent than each component individually which suggests that both processes involve removal of the action of endogenous adenosine (Stone *et al.*, 1990). Here both CPT and 8pSPT enhanced the rate of epileptiform activity induced by 0Mg/4AP. However CPT potentiated the rate of activity more substantially in a medium with the normal concentration of magnesium, suggesting that the removal of endogenous adenosine may play a small part in the generation of epileptiform activity.

4-aminopyridine inhibited almost completely the depression in antidromic spike amplitude produced by adenosine and *L*-PIA in hippocampal slices (Schubert & Lee, 1986) and the decrease in response to glutamate application produced by ATP in cortical neurones (Perkins & Stone, 1980). This indicates that purines

require the potassium channels inhibited by 4AP to be active for postsynaptic inhibition. Since $A_1$ receptors were known to interact with potassium channels it was proposed that these 4AP sensitive channels were involved. The inhibitory actions of both ATP and adenosine were potentiated by the removal of 4AP from the perfusing medium. Despite this interaction $A_1$ receptor ligands can reduce epileptiform activity induced by 4AP (Barbarosie *et al.*, 1994; Longo *et al.*, 1995) with a pre-synaptic mechanism of action being suggested.

### 4.4 Modulation of simultaneously evoked and spontaneous activity

The perfusion of hippocampal slices with a medium modified to increase excitability can result not only in spontaneous but also evoked epileptiform activity. This consists of a single population spike followed by a number of afterdischarges or secondary peaks. In some studies it is these evoked potentials that are investigated (Lee *et al.*, 1984). By using two electrodes it was possible to investigate the modulation of evoked and spontaneous activity simultaneously. For this part of the study recording took place in the CA1 region of the hippocampus. In general it was easier to generate more consistently population spikes in this hippocampal subfield. In the majority of slices perfusion with OMg/4AP medium resulted in spontaneous activity and population potentials with at least one secondary potential if not more.

Adenosine reduced the discharge rate similarly to that produced previously on bursts in the CA3. However, in contrast to this the primary peak (population spike) was either not significantly altered or increased in size. Accordingly the amplitude of the afterdischarges (secondary and tertiary peaks) was potentiated by adenosine. Both the decrease in rate and increase in amplitude were reversible. Adenosine, in a concentration dependent manner, reduced the population spike and/or eliminated the afterdischarges produced by perfusion with penicillin G (Lee *et al.*, 1984). In a low calcium model of epileptiform activity in which potentials

were generated by antidromic stimulation, adenosine had no effect on the primary spike but reduced the afterdischages. Adenosine also depressed the spontaneous activity that was produced. However whether these events occurred simultaneously was not apparent (Lee *et al.*, 1984).

ZM241385, an antagonist at $A_2$ receptors, did not inhibit the increase in spike amplitude caused by adenosine, ruling out an involvement of $A_2$ receptors in this effect. DPCPX, in contrast to CPT and 8pSPT, did not elevate the basal rate of epileptiform activity. The involvement of DPCPX in the generation or potentiation of epileptiform activity is complex. In some studies DPCPX induced the generation of epileptiform activity in the form of spontaneous bursts and evoked afterdischarges (Alzheimer *et al.*, 1989; Alzheimer *et al.*, 1993) whereas in others a form of priming was required before epileptiform activity was instituted (Chesi & Stone, 1997). DPCPX is also reported to either increase (Thompson *et al.*, 1992) or have no effect (Barbarosie *et al.*, 1994) on established epileptiform activity. In the presence of DPCPX any effect of adenosine was totally quashed.

Cell firing and evoked potential amplitude were reduced by the appearance of spontaneous activity. Therefore, the inhibition of spontaneous activity could indirectly cause a reciprocal increase in the size of evoked potentials (Stone *et al.*, 1992). Such an effect could explain the situation in this study. The switch from normal to modified aCSF produces an initial increase in population spike size and the appearance of afterdischarges. Once spontaneous activity starts and increases in frequency to a steady rate, evoked activity decreases from this original elevated level to a steady size which is still usually of larger amplitude than the potentials generated in normal aCSF. It would be of interest to see if, under circumstances of increased bursting frequency, the amplitude of evoked potentials is reduced.

This theory of opposite modulation of simultaneously evoked and spontaneous activity is challenged somewhat by the depression of both discharge rate and evoked afterdischarges by adenosine in a medium in which activity was sustained by 4AP addition. It is possible that adenosine, in the presence of magnesium, can overcome the potentiation of evoked activity that follows a reduction in spontaneous activity ultimately producing a reduction in the population spike amplitude. Such a situation would support the theory that magnesium is required for optimal action of adenosine. Previously, in this study, magnesium availability increased the effect produced by ATP but not that of adenosine.

Under conditions of elevated calcium/magnesium ratios, such as would the case in a zero magnesium medium, CPT has been shown to permanently alter the relative contribution of NMDA and non-NMDA receptors to evoked population spikes (Klishin *et al.*, 1994a; Klishin *et al.*, 1995a; Klishin *et al.*, 1995b). An increased level of calcium is also a requirement. Another proposal is that adenosine is not equally effective on NMDA and non-NMDA mediated events and that under certain conditions this becomes apparent. The generation of evoked population spikes and spontaneous bursts will not necessarily involve the activation of NMDA and non-NMDA receptors to the same extent. Therefore in different media, with varying calcium/magnesium ratios, adenosine may produce differential effects on evoked and spontaneous activity. Whether there is an actual discriminatory effect by adenosine of abnormal activity and so called normal activity represented by spontaneous bursts and population spikes respectively requires more detailed investigation.

## 4.5 BenzoylbenzoicATP

In a small range of cell types: mast cells (Dahlquist, 1974; Cockcroft & Gomperts, 1979), chronic lymphocytic leukaemia lymphocytes (Wiley & Dubyak, 1989), macrophages (Sung *et al.*, 1985;Greenberg *et al.*, 1988), acinar cells (Soltoff *et al.*, 1992) and transformed fibroblasts (Rozengurt & Heppel, 1975) the activation of $P_{2Z}$ receptors alters plasma membrane permeability allowing the influx of cations. Variability exists in pore size among cell types allowing ethidium bromide passage into macrophages, mast cells and lymphocytes but not acinar cells. In addition to a non-selective increase in membrane permeability, the nvolvement of $P_{2Z}$ receptors is characterised by restricted nucleotide selectivity. BenzoylbenzoylATP (BzATP) is the preferred agonist with a far higher potency than ATP (Gonzalez *et al.*, 1990). Thirdly the effect is dependant on the concentration of the tetrabasic form of ATP ($ATP^{4-}$). This is reflected in the increased potency of ATP in the absence of magnesium and the positive correlation between the magnitude of the effect and the concentration of $ATP^{4-}$ and not total ATP.

BzATP produced a smaller degree of depression of epileptiform activity after a 10 minute perfusion than ATP at the same concentration, a relative potency which is not characteristic of the $P_{2Z}$ receptor. However, in contrast to the effect of ATP and other nucleotides the depression of discharge rate not only persisted after perfusion with BzATP had stopped but was increased in size. Such an effect would fit in with the formation of pores in the membrane which produce a prolonged effect which can become irreversible (Nuttle *et al.*, 1993). $P_{2Z}$ receptors have been implicated in the spontaneous death of J774 macrophage cells due to the protective effect of the selective $P_{2Z}$ receptor antagonist oxidised ATP (Murgia *et al.*, 1993) and the low incidence of cell death in cell clones that have reduced expression of the $P_{2Z}$ receptor (Chiozzi *et al.*, 1996). Necrosis or

apoptosis can be induced by ATP in some tumour cell lines, a process which is believed to involve the activation of $P_{2Z}$ receptors (Zheng *et al.*, 1991).

In order to substantiate a proposed difference in the effect of ATP and BzATP on epileptiform activity, the effect of CPT on the depression of discharge rate caused by BzATP was investigated. The potentiation of activity produced by the combination of BzATP and CPT was less than that of CPT alone by approximately the degree of inhibition produced by BzATP when perfused alone. This would suggest that CPT was not able to prevent the effect of BzATP leading to the proposal that BzATP acts independently of ATP or its metabolites would seem justified.

However a problem exists in that the potentiation produced by CPT alone is greater than that noted in previous experiments. As mentioned previously the extent of the effect of CPT may be dependent on the basal concentration of adenosine. However, this does not solve the problem of whether BzATP is acting in a different manner than ATP and if so whether the $P_{2Z}$ receptor is involved. Further investigation is required to answer this question starting with the use of oxidised ATP.

## 4.6 Diadenosine polyphosphates

Alpha, omega-adenine dinucleotides ($Ap_nA$) or dinucleoside polyphosphates consist of two adenosine molecules linked at their 5' region by a number of phosphate groups, with n denoting the number of phosphates. Diadenosine diphosphate ($Ap_2A$) through to diadenosine hexaphosphate ($Ap_6A$) are known to exist naturally. The majority of work carried out on the diadenosine polyphosphates has concentrated on the higher phosphate groups, $Ap_4A$, $Ap_5A$ and $Ap_6A$, especially $Ap_4A$, with a smaller volume of work available regarding $Ap_2A$ and $Ap_3A$. The earliest role to be proposed for dinucleotides was in DNA replication (see Green *et al.*, 1995) but since then a role in extracellular communication has also been established (for rev. see Baxi & Vishwanatha, 1995) with earlier work showing potent effects on smooth muscle systems (Stone, 1981).

Diadenosine polyphosphates have been investigated in numerous cell and tissue systems. These compounds play a dual role in platelet aggregation, causing both inhibition (Harrison *et al.*, 1975) and initiation (Lüthje & Ogilvie, 1985) depending on the size of the phosphate chain. Dinucleotides are also vasoactive pointing to a possible role in blood pressure regulation (Tepel *et al.*, 1995; Zidek *et al.*, 1995) and are believed to modulate release of catecholamines from adrenal chromaffin cells (Castro *et al.*, 1992).

More recently, diadenosine polyphosphates have been found stored in brain synaptosomes (Pintor *et al.*, 1992). The release of the dinucleotides diadenosine tetraphosphate ($Ap_4A$) and diadenosine pentaphosphate ($Ap_5A$) from rat cerebral cortex synaptosomes by the action of depolarising agents, such as veratridine and 4-aminopyridine, has been demonstrated (Pintor *et al.*, 1992). Phosphodiesterases were used to degrade the released dinucleotides with the resulting hplc analysis showing the products expected from $Ap_4A$ metabolism. This release occurred in a calcium-dependent manner. Amphetamine sulphate, through the involvement of

dopamine receptors, can stimulate the release of dinucleotides from basal ganglia (Pintor *et al.*, 1993b) and neostriatum (Pintor *et al.*, 1995) *in vivo*. Autoradiography using [$^3$H]-diadenosine tetraphosphate has been used to investigate the areas of dinucleotide binding within rat brain. Binding was found to be widespread with an intermediate density of binding in the pyramidal layers of the hippocampal formation (Rodríguez-Pascual *et al.*, 1997).

$Ap_4A$ and $Ap_5A$ both depressed the rate of epileptiform activity in the present study in a concentration dependent manner. At both concentrations used of 1 and 10μM, $Ap_4A$ and $Ap_5A$ were approximately equipotent. This is in agreement with previous studies in the hippocampus (Klishin *et al.*, 1994b), vas deferens (Hoyle *et al.*, 1995) and left atrium (Hoyle *et al.*, 1996). However this comparability of potency is not always apparent, with $Ap_4A$ being more active in some instances (Castro *et al.*, 1990; Kleta *et al.*, 1995) and $Ap_5A$ being the more potent in others (Frohlich *et al.*, 1996).

The depressant nature of the dinucleotides in this study is in agreement with previous work carried out using hippocampal slices (Klishin *et al.*,1994b) in which $Ap_5A$ and $Ap_4A$ at 5μM inhibited extracellular postsynaptic field potentials and intracellular postsynaptic currents recorded from the CA1 region in a reversible manner, with excitability recovering to control level or beyond. Similarly in this study control values were resumed within 5 min. of washing with control medium. $Ap_5A$ and $Ap_3A$ also depressed the firing of spontaneously active cortical neurones (Stone & Perkins, 1981).

In isolated hippocampal CA3 neurones the application of $Ap_5A$ at 5μM enhanced the amount of inward current through calcium channels in a rapid and reversible manner (Panchenko *et al.*, 1996). The use of the antagonist ω-conotoxin GVIA suggested the involvement of N-type calcium channels. In rat midbrain

synaptosomes $Ap_4A$ and $Ap_5A$ induced calcium entry in a voltage-independent process, although in this case known calcium antagonists were unable to block the effect (Pintor *et al.*, 1993b). A similar rise in intracellular calcium levels in response to extracellular $Ap_4A$ was noted in deermouse brain synaptosomes (Pivorun & Nordone, 1996), and enhanced calcium activity following dinucleotide application occurred in human neutrophils (Gasmi *et al.*, 1997), human fibroblasts (Tepel *et al.*, 1995), rat hepatocytes (Green *et al.*, 1995) and adrenal chromaffin cells (Castro *et al.*, 1992). The elevation of intracellular calcium concentration is usually associated with an increase in excitability and hence an increase in calcium following dinucleotide application may account for the rebound effect reported by Panchenko *et al.* (1996). A similar tendency was apparent in some of the present experiments.

In a similar manner to ATP, adenine dinucleotides are subject to metabolism by ecto-enzymes. Ecto-hydrolases cleave the dinucleotide to yield AMP and the corresponding residual moiety (Ogilvie, 1992). For example $Ap_4A$ is reduced to AMP and ATP. These enzymes differ from their intracellular counterparts in that they are not specific for a given phosphate chain length (Ogilvie & Lüthje, 1988; Ogilvie *et al.*, 1989). The nucleotides formed following the action of hydrolases on $Ap_nA$ can in turn be metabolised by ecto-nucleotidases and 5'-nucleotidase producing adenosine as the end metabolite. Adenine dinucleotides have a longer half-life in the extracellular fluid in comparison to ATP (Lüthje & Ogilvie, 1988; Miras-Portugal *et al.*, 1990; Green *et al.*, 1995) and thus metabolic breakdown is not considered as much of a problem. Hplc analysis of liver cell membranes showed no significant breakdown of $Ap_4A$ occurring during a 20 min. time period (Edgecombe *et al.*, 1996). In other studies, constant perfusion systems (Green *et al.*, 1995; Kleta *et al.*, 1995) or low temperatures (Pintor *et al.*, 1993a) have been used in order to combat the interference by metabolites.

As before, adenosine reduced the frequency of discharge rate, an effect that was inhibited by adenosine deaminase (0.2U/ml). $Ap_4A$ and $Ap_5A$ depressed activity at low concentrations (1μM) at which adenosine itself was inactive. However this action of $Ap_nA$ was inhibited by adenosine deaminase at the concentration which eliminated responses to adenosine. One explanation of this paradox may be that $Ap_5A$ can increase the spontaneous efflux of [$^3$H] adenosine from neurones (Stone & Perkins, 1981). Thus, at low concentrations $Ap_nA$ may be able to increase the extracellular concentration of adenosine to a level which can depress epileptiform activity.

At the higher concentration of 10μM $Ap_4A$ and $Ap_5A$ depressed the rate of epileptiform activity to a similar maximum level in the presence and absence of adenosine deaminase, suggesting that adenosine is not the mediator of this depression. However, if the extent of the inhibition was analysed as the mean change in rate at the end of a 10 min. perfusion then adenosine deaminase did reduce the response to $Ap_4A$ and $Ap_5A$. The results may, therefore, be indicating that $Ap_4A$ and $Ap_5A$ have direct inhibitory effects on epileptiform activity which are not mediated by adenosine, but that these effects decline with time, perhaps due to desensitisation. This would be in agreement with previous studies carried out in which a return towards basal activity levels occurred in the maintained presence of dinucleotides (Klishin *et al.*, 1994b). A return almost to control values occurred after perfusion of $Ap_4A$ for 30 minutes. This early direct action of the dinucleotides is then continued by the formation or release of adenosine.

Adenosine monophosphate (AMP) is part of the extracellular hydrolysis pathway for $Ap_nA$. Although AMP is not often considered as a major effector it is able to activate P1 receptors in a manner similar to adenosine (Burnstock, 1978). AMPase did not significantly alter either the maximum or the end of perfusion depression of activity exerted by $Ap_4A$ suggesting that AMP is not the mediator of

this effect. A similar conclusion was reached by Gu and Geiger (1994) in rat cerebellum.

Much debate exists surrounding the receptors activated by adenine dinucleotides. Inhibition by the P2X receptor antagonist pyridoxal-6-azophenyl-2,4-disulphonic acid (PPADS), and desensitisation by α, β-methyleneATP suggest the involvement of receptors of the P2X subclass (Hoyle *et al.*, 1989; Tepel *et al.*, 1996). Alterations in intracellular calcium concentrations in chromaffin cells (Castro *et al.*, 1992) and liver cells (Edgecombe *et al.*, 1996) by dinucleotides have been attributed to P2Y receptors due to inhibition by cibachrome blue and the displacement of dinucleotide binding by 2-methylthioATP. In contrast, displacement studies using [$^3$H]$Ap_4A$ and competition studies looking at the displacement of [$^{35}$S]ADP-β-S by diadenosine polyphosphates in rat brain synaptic terminals found a binding profile which was distinct from previous P2 receptors and had a high affinity for diadenosine polyphosphates (Pintor *et al.*, 1993a). This receptor was designated as $P_{2d}$. Functional studies in rat brain synaptosomes have also shown that ATP and $Ap_nA$ display different response profiles with regard to antagonism and cross desensitisation, thereby supporting the existence of a $P_{2d}$ receptor (Pintor & Miras-Portugal, 1995; Pivorum & Nordone, 1996). The $P_{2d}$ receptor has been classed as part of the P2Y family and is referred to as $P2Y_{Ap4A}$ (Fredholm *et al.*, 1997). The depression of hippocampal field potentials and excitatory postsynaptic currents by $Ap_5A$ was inhibited by the $A_1$ receptor antagonist 8-cyclopentyl-1,3-dimethylxanthine (CPT) (Klishin *et al.*, 1994b) suggesting that adenine dinucleotides can directly activate P1 receptors. Similarly theophylline, a P1 receptor antagonist, reduced the inhibition of cortical neurones by dinucleotides (Stone & Perkins, 1981). CPT totally prevented any depression of epileptiform activity by $Ap_4A$. $A_1$ receptor antagonists have inhibited responses to $Ap_nA$ in Xenopus oocytes (Pintor *et al.*, 1996), heart (Hoyle *et al.*, 1996; Rubino & Burnstock, 1996; Vahlensieck *et al.*, 1996) and vas

deferens (Hoyle *et al.*, 1995). Taken together, these results strongly suggest that dinucleotides are able to activate P1 receptors.

### 4.7 5'adenylic acid deaminase

5'adenylic acid deaminase (AMPase) catalyses the conversion of AMP to yield equimolar amounts of IMP and ammonia (Zielke & Swelter, 1971; Ogasawara *et al.*, 1975). This enzyme has been isolated and characterised from rabbit heart (Thakker *et al.*, 1993b), human uterine smooth muscle (Nagel-Starczynowska *et al.*, 1993) and skeletal muscle. The addition of AMPase to inhibit the action of exogenous AMP or to rule out the involvement of AMP as a metabolite is not common practise with only a few cases documented (Griese *et al.*, 1991; Bo *et al.*, 1993; Côte *et al.*, 1993). The majority of investigations involve isolation and characterisation of AMPase from a variety of tissues with special interest in the enzyme from skeletal muscle where 2-3% of muscle biopsies contain deficient AMPase levels (Thakker *et al.*, 1993a). A role for AMPase in regulating adenylate metabolism in conditions of myocardial ischaemia has also been proposed (Thakker *et al.*, 1993a; Thakker *et al.*, 1993b; Thakker *et al.*, 1994).

AMPase initially elevated the discharge rate to a small extent. Although there is much documentation regarding the existence of endogenous adenosine, the involvement of endogenous AMP in producing basal inhibitory tone is not usually considered. Such an increase in rate could result from the removal of endogenous AMP. During the perfusion of AMPase, as noted previously, the activity did have a tendency to drop below control values during the 10 minute perfusion and to continue to fall during the wash period until a plateau was reached at which the rate remained at for up to 90 minutes. The prolonged depression produced by AMPase in the absence of a similar scenario with adenosine deaminase prompted the question of what was responsible for this marked effect.

The possibility existed that IMP produced by the action of AMPase was not inactive as previously thought but could in fact cause substantial neuro-depression. This proposal was ruled out by the apparent ineffectiveness of IMP at concentrations up to 500μM. AMPase is not specific for AMP and can also deaminate adenosine (Margolin *et al.*, 1994). Thus, it was also possible that inosine could be the mediator but the lack of similar effects with adenosine deaminase argues against this theory.

Although so far this type of effect was only seen with AMPase it did not necessarily mean that the enzyme was directly responsible. It was possible that some contaminant in the enzyme product could be having a detrimental effect on the slices resulting in loss of activity. This was investigated in several ways. Baclofen is a $GABA_B$ receptor agonist which has been shown to inhibit interictal activity allowing the generation of seizure like activity (Swartzwelder *et al.*, 1987; Lewis *et al.*, 1989). The activation of postsynaptic $GABA_B$ or adenosine receptors results in membrane hyperpolarisation due to the activation of the same potassium current (Dunwiddie & Fredholm, 1989). Baclofen reversibly depressed the rate of epileptiform activity. AMPase did not alter the potency of baclofen, the only difference in the response between baclofen alone and baclofen + AMPase being the prolonged depression that again was apparent after AMPase perfusion. The inhibitory effect of AMPase on AMP and ATP can therefore be considered to result from specific deamination and not a general blanket depression of cell responsiveness.

To rule out contaminating ions as the cause of this AMPase induced depression, AMPase was dialysed for a period of 4 days. This treatment affected neither the enzymatic activity of AMPase nor the now characteristic long term decrease in bursting activity. For these experiments the AMPase was dissolved in water and then the normal constituents of a 0Mg/4AP medium added after the period of

dialysis. To rule out any difference between the two methods of drug preparation: directly dissolved in aCSF against dissolving in water then adding in the required salts, a few experiments were conducted in which AMPase was prepared by the later method. This resulted in a situation of reduced activity similar to previous instances.

A further means of discriminating between an actual enzyme mediated effect and that of a contaminant involved denaturing the enzyme by heat. This treatment reduced the activity of AMPase in that it could no longer inhibit the effect of AMP. Perfusion with denatured AMPase produces only small fluctuations around the control discharge rate which did not develop into a prolonged depression. Hence enzyme inactivation correlated with removal of the long term depression in activity rate.

The effect of AMPase on simultaneously induced discharges and population spikes was investigated in both CA3 and CA1 subfields. In both areas reciprocal effects were evident on bursts and spikes with AMPase, as usual, reducing spontaneous activity but increasing population spike amplitude. As explained in section 4.4 this opposition of effects may be due to the AMPase reducing spontaneous firing and thus removing the inhibitory effect of such activity on population spike amplitude. However, if AMPase was being toxic to the slices a parallel decline in population spike amplitude would be expected to occur. This represents another piece of evidence against a contaminant being the active agent causing depression of bursts.

Consistent with the effects on population spikes recorded in the presence of spontaneous activity, AMPase potentiated the size of evoked activity in the CA1 and CA3 regions although to a far greater extent in CA3. As with the increase in discharge rate this could result from inhibiting endogenous AMP. However in

contrast this potentiation was reversed during the wash period to below control values from which there was no recovery even after 90 minutes in the case of the CA1 area. Therefore, the perfusion of hippocampal slices with AMPase not only results in sustained depression of spontaneous activity but also of evoked population potentials.

The apparent lack of a toxic effect of AMPase, as taken from the sustained population spike in the presence of spontaneous activity, is substantiated by the ability of short bursts of high frequency stimulation or increased stimulus intensity to reverse the depression (undocumented observations). This finding, together with the relationship that appears to exist between enzyme activity and resulting depression supports the notion that the enzyme itself is the mediator of a form of hippocampal long term depression.

### *4.7.1 Long term depression*

Long term depression (LTD) is a persistent depression of synaptic efficacy which is not secondary to deterioration of the slice (Christie *et al.*, 1994). Many different stimulation patterns have been used to induce LTD with the most commonly used in the hippocampus being prolonged periods of low frequency stimulation, for example 1Hz. (900 pulses) (Dudek & Bear, 1992; Manahan-Vaughan, 1997) or 3Hz. for 5 min. (Hrabetova & Sacktor, 1997). Three different models of LTD exist:

1. heterosynaptic- this is used to describe LTD induced in an inactive pathway either by conditioning stimuli such that would induce long term potentiation in a converging pathway or by antidromic stimulation causing activation of the postsynaptic neurone.
2. homosynaptic- in this case activation of a pathway results in LTD of that same pathway.

3. associative- this describes the situation where two converging pathways are active but not synchronised with each other (Christie *et al.*, 1994).

The ability of the different paradigms to induce LTD in several regions of the hippocampus have been demonstrated. The induction of a long term depression after a period of long term potentiation is known as depotentiation.

A few prerequisites are required for the induction and maintenance of LTD. Depolarisation of the postsynaptic membrane is required for LTD. Stimuli that produce only a transient depression cause LTD when combined w: h postsynaptic depolarising pulses (Gean & Lin, 1993). Also, hyperpolarisation of the postsynaptic neurone prevents the induction of LTD (Mulkey & Malenka, 1992). The concentration of intracellular calcium is also important in that influx of calcium into the postsynaptic cell is required for LTD (Mulkey & Malenka, 1992; Bolshakov & Siegelbaum, 1994; Cummings *et al.*, 1996). Buffering of intracellular calcium with a calcium chelator, for example BAPTA, prevents LTD in the CA1 region (Mulkey & Malenka, 1992; Debanne *et al.*, 1994). Both membrane depolarisation and calcium influx can result from the activation of NMDA receptors. NMDA receptor antagonists block hippocampal LTD (Dudek & Bear, 1992; Mulkey & Malenka, 1992; Debanne *et al.*, 1994; Abraham, 1996). A recent study found differential block of LTD and LTP in hippocampal slices by the NMDA receptor antagonists AP5 and CPP suggesting that these antagonists can block subtypes of NMDA receptors which are not equally involved in the induction of LTD and LTP (Hrabetova & Sacktor, 1997). In a few instances AP5 failed to inhibit the induction of LTD whereas the metabotropic glutamate (mGlu) receptor antagonist MCPG prevented LTD induction (Bolshakov & Siegalbaum, 1994; O'Mara *et al.*, 1995). Other reports confirm a dependence on mGlu receptors activation as a characteristic of LTD (Bashir *et al.*, 1993; Manahan-Vaughan, 1997) although this has been disputed (Selig *et al.*, 1995). The activation of protein phophatases is required for this kind of plasticity due to the

attenuation of LTD by protein phosphatase inhibitors, okadaic acid and calyculin A (Mulkey *et al.*, 1993). FK506, a calmodulin inhibitor, also prevented LTD being induced by low frequency stimulation of rat hippocampal slices (Mulkey *et al.*, 1994). The following scheme was proposed for the mechanism involved in LTD: calcium enters the cell through NMDA receptor channels and binds to calmodulin which results in calcineurin (protein phosphatase 2B) activation and the subsequent dephosphorylation and hence activation of protein phosphatase 1 (PP1) (Mulkey *et al.*, 1994). Whether a common mechanism is responsible for the induction of LTD regardless of the situation i.e. homosynaptic, heterosynaptic or associative remains to be determined.

AMPase produced a depression which persisted for 90 minutes, which was as long as the experiment was carried out. Although a number of *in vivo* studies have shown LTD to persist from hours up to 7 days (Krug *et al.*, 1985; Abraham, 1996; Manahan-Vaughan, 1997) persistence for 30 minutes seems acceptable for the term LTD when using *in vitro* studies (Christie *et al.*, 1994; Hrabetova & Sacktor, 1997; Kemp & Bashir, 1997).

The addition of the $A_1$ receptor antagonist DPCPX or adenosine deaminase to slices of adult rat hippocampus during low frequency stimulation produced LTD which was not generated by stimulation alone in the CA1 region (Kemp & Bashir, 1997). DPCPX enhances LTD and depotentiation in the CA1 subfield of hippocampal slices from young rats (de Mendonça *et al.*, 1997) suggesting that endogenous adenosine acting through $A_1$ receptors attenuates long term depression and depotentiation in the hippocampus. This would seem in contrast to the usual inhibitory nature of the responses elicited by $A_1$ receptor activation. However LTD involves NMDA receptors which adenosine is known to inhibit. Although the inhibition of endogenous AMP and to some extent even endogenous adenosine by AMPase would support the generation of LTD, no protocol such as

low frequency stimulation that generates LTD was followed in this study. The possibility that spontaneous activity or continuous stimulation at 0.1 Hz. in conditions of reduced endogenous adenosine produce LTD is ruled out by the lack of a similar effect with adenosine deaminase or the $A_1$ receptor antagonists CPT and DPCPX.

DPCPX potentiated the rate of activity to a far greater extent in these set of experiments compared to when it was used to investigate the effect of adenosine on simultaneously generated spontaneous evoked activity. In a similar manner to CPT (section 4.3.2), the degree of potentiation produced by DPCPX, could be dependent on the basal concentration of adenosine. Prior exposure of the perfusion system to DPCPX could influence the effect elicited, for example first exposure to DPCPX could cause a large increase in rate whereas a subsequent addition resulted in much smaller change in rate (undocumented observations). Persistence in the silicone tubing of the perfusion system was proposed to be responsible for this effect such that inhibition of $A_1$ receptors was instrumental in determining the basal discharge rate. A study by Starke *et al.* (1978) showed that silicone tubing could absorb lipophilic drugs. In a few instances the addition of CPT after DPCPX resulted in an uncharacteristic depression of activity (undocumented observations). Such a situation has been described in hippocampal slices between DPCPX and theophylline in which theophylline could reduce the frequency of spontaneous activity induced by DPCPX (Chesi & Stone, 1997). Therefore the use of DPCPX can involve complications which make interpretation of the findings more complex.

The depression of discharge rate produced by AMPase was in general larger after perfusion with DPCPX than that under control conditions although no exact comparison was made. This could be interpreted, in view of the above statements, that LTD induced by AMPase is potentiated by DPCPX. However the perfusion

of AMPase during constant stimulation produced a similar degree of inhibition in discharge rate as to that which is seen after perfusion with DPCPX.

Since AMPase appears to be inducing a form of LTD by a method distinct from deamination of AMP or adenosine and thus inhibition of a basal inhibitory purinergic tone, inhibitors of other receptor and pathways were investigated.

Endogenous and exogenous opioid peptides act on distinct receptor subtypes within the hippocampus to increase excitatory synaptic transmission indirectly through inhibition of GABA release from interneurones (Zieglangansberger *et al.*, 1979; Madison & Nicoll, 1988). The activation of mu and delta opiate receptors enhance the amplitude of CA1 population spikes and intracellular EPSP's in a naloxone sensitive manner (Lupica *et al.*, 1992; Sagratella & DeScotti, 1993; Miller & Lupica, 1994). An underlying involvement of endogenous opioids in regulating the discharge rate was not apparent due to the lack of effect of naloxone, a non-selective opioid receptor antagonist. LTD induced by high intensity tetanic stimulation of the Schaffer collaterals was blocked by naloxone (1μM) (Francesconi *et al.*, 1997). However in this study naloxone at 10μM was unable to prevent the long term depression induced by AMPase.

The $GABA_A$ receptor antagonist bicuculline had no effect on LTD induced in hippocampal slices obtained from young animals whereas the $GABA_B$ receptor antagonist CGP35348 inhibited LTD (Wagner & Alger, 1995). In slices from adult animals bicuculline enhanced the expression of LTD by low frequency stimulation suggesting that $GABA_A$ receptors limit the activation of NMDA receptors during low frequency stimulation decreasing the probability of inducing LTD (Wagner & Alger, 1995). Bicuculline failed to inhibit LTD induction in slices of striatum (Calabresi *et al.*, 1992). Similarly to this bicuculline did not alter the induction nor the persistence of AMPase induced depression of

spontaneous activity suggesting a lack of involvement of $GABA_A$ receptor activation.

Activation of phospholipase $A_2$ ($PLA_2$) results in the formation of arachidonic acid (AA) which is subsequently metabolised through the action of cyclo-oxygenase and lipoxygenase to form a group of compounds collectively known as the eicosanoids. The $PLA_2$ inhibitor bromophenacylbromide reduced or inhibited LTD in the CA1 region of hippocampal slices (Fitzpatrick & Baudry, 1994; Normandin *et al.*, 1996). Aspirin and indomethacin, irreversible inhibitors of cyclo-oxygenase, did not substantially modify the basal rate of activity nor the AMPase induced depression which is in agreement with a previous study in which indomethacin failed to reduce hippocampal LTD (Normandin *et al.*, 1996). However in that study a lipoxygenase inhibitor nordihydroguaiaretic acid partially blocked hippocampal LTD. Therefore although the $PLA_2$/AA pathway involving cyclo-oxygenase is not involved in AMPase induced LTD, the possibility still exists that other components of this pathway may be implicated.

Nitric oxide has been postulated to act as a mediator in numerous situations including the induction and maintenance of LTP and LTD although conflicting reports are abundant. In hippocampal slices the induction of LTD was not affected by nitric oxide synthase inhibitors, L-N(G)-nitroarginine (L-NOArg) or L-NAME (Cummings *et al.*, 1994; Malen & Chapman, 1997) nor the nitric oxide donor hydroxylamine (Malen & Chapman, 1997) which fits in with the observed lack of effect of L-NAME in this study. However in contrast L-N(G)-monomethylarginine, L-NOArg and haemoglobin, which all interfere with the action of nitric oxide, blocked LTD induced by low frequency stimulation of the Schaffer collateral pathway suggesting that nitric oxide was the mediator of this form of LTD (Izumi & Zorumski, 1993).

Dephosphorylation and phosphorylation have been proposed to be important for the induction and maintenance of LTD and LTP respectively. The non-selective protein kinase inhibitor H7 and the selective protein kinase C inhibitor chelerythrine mimic the maintenance phase of LTD. Further to this biochemical analysis illustrates a decrease in protein kinases during the maintenance phase of LTD (Hrabetova & Sacktor, 1996). In general stimulation at high frequencies induces LTP whereas if low frequencies are used LTD is produced. However in between these two extremes there exists frequencies which neither produced LTP or LTD. Stimulation at such frequencies in the presence of kinase inhibitors generated LTD (Coussens & Teyler, 1996). In contrast to this, $Ca^{2+}$/calmodulin dependent kinase II (CaMKII) activity is necessary for LTD induction (Stanton & Gage, 1996). AMPase initially produces a small potentiation of 10-20% which does not always reach significance, but in the presence of the non-specific protein kinase inhibitor N-(2-aminoethyl)-5-chloro-1-naphthalene-sulphonamide (A3) (Lu *et al.*, 1996) this increase was 40%. This increase is due to inhibition of endogenous AMP which may vary from slice to slice. It is possible, therefore, that the increase in rate produced by AMPase would also be prone to variation. The presence of A3 did not alter the induction or maintenance phases of AMPase induced LTD.

In one slice an error resulted in 100 times the usual concentration of A3 being used. AMPase and A3 resulted in almost a four fold increase in discharge rate which declined over the following 20 minutes to just below (10-15%) control levels. The lack of more substantial depression could be accounted for by the large decrease encountered from the elevated to wash rates. However similar degrees of potentiation were reached in the presence of CPX and when a normal medium containing 4AP was used to sustain spontaneous activity. In both instances a marked depression ensued. This does only represent one slice and

therefore further investigation is required before anything can be concluded from it.

# 5.0 Conclusions

Both the CA3 and CA1 subfields of rat hippocampal slices can generate spontaneous activity when bathed in a medium containing no added magnesium and 4-aminopyridine (50μM). The activity produced consists of defined individual bursts lasting up to 500 ms, with each one containing one or more peaks. Although there are no intracellular recordings to corroborate this, it is proposed, on their characteristics, that these bursts define a form of interictal activity. The relationship between interictal and ictal activity is still poorly understood although proposals that interictal activity inhibits the generation of seizures and that a rise in potassium concentration either directly or indirectly is required for the induction of seizures have been put forward. However, there is clearly a need for a greater understanding of this transition from interictal to ictal activity.

A tremendous volume of work in the last two decades has been dedicated to developing the early theory that adenosine triphosphate has an equally important role to play extracellularly as intracellularly. To date 15 receptor subtypes have been cloned with a widespread distribution throughout the central nervous system and periphery, with agonist studies showing that more may yet be discovered. Hence what started out as a theory has blossomed in to the discovery of a major natural neuromodulator.

Our initial findings of an ATP induced depression of spontaneous activity which was inhibited by the $A_1$ receptor antagonist CPT but not by the P2 receptor

antagonists suramin and PPADS were not consistent with the characteristics of known P1 or P2 receptors. Metabolism to adenosine was counteracted by the addition of adenosine deaminase, which did not affect the depression resulting from ATP application. Hence the involvement of a xanthine-sensitive nucleotide receptor was suggested. However the metabolism of ATP also involves the production of AMP which is postulated to activate $A_1$ receptors. The depression of epileptiform activity by ATP was found to be annulled by the enzyme AMPase suggesting that AMP is the mediator of this ATP effect. AMP induced depression was inhibited by CPT and AMPase and also to a small extent by adenosine deaminase. Although not to a significant degree, adenosine deaminase also decreased the effect of ATP which would fit in with an effect of adenosine deaminase on AMP. The only discrepancy in this theory is that both ATP and AMP were approximately equipotent when it would be expected that the metabolite would be less active. The inhibition of the ATP response by cyclopentyltheophylline suggests that $A_1$ receptors are indeed involved. This study, therefore, suggests that caution should be made when using ATP in the absence of ecto-ATPase inhibitors and that all possible metabolites, including AMP, need to be considered and not just adenosine. The lack of effect of 2-methylthioATP and uridine triphosphate suggests the absence P2Y or $P_{2U}$ receptors respectively in the hippocampal CA3 region. The excitatory nature of $\alpha$, $\beta$-methyleneATP is indicative of the existence of a P2X receptor able to increase the excitability of CA3 neurones.

The alpha, omega-adenine dinucleotides $Ap_4A$ and $Ap_5A$ depressed the rate of epileptiform activity in a manner which was insensitive to AMPase, partially reduced in the later stages of the response by adenosine deaminase and totally antagonised by cyclopentyltheophylline. This profile suggests that in this model of hippocampal epileptiform activity $Ap_5A$ and $Ap_4A$ act partly by stimulating xanthine sensitive receptors directly, an effect which declines during perfusion for several minutes, and partly through the metabolite adenosine.

The most unexpected finding in this study was the sustained depression of spontaneous discharge rate and evoked population spike amplitude produced by 5'adenylic acid deaminase in both area CA3 and CA1 of hippocampal slices. This depression has tentatively been described as a form of long term depression. That the enzyme itself is the mediator of this depression is supported by the relationship that exists between enzyme activity and the induction of LTD: inactivated enzyme does not generate LTD. However application of adenosine deaminase or CPT, which both act to inhibit basal purinergic tone as does AMPase, does not induce LTD. In fact with CPT a small persistent elevation in activity results. So what is the difference that allows AMPase to induce LTD? $A_1$ receptors, nitric oxide, $GABA_A$ receptors, the cyclo-oxygenase pathway and opioids are not mediators of AMPase induced LTD.

AMPase, in contrast to adenosine deaminase, is not very specific with a broad substrate specificity. Due to this it was possible that AMPase inhibited the response to ATP due to direct deamination of ATP. However the use of

5'nucleotidase verified the conversion to AMP. AMPase can deaminate cAMP to form cIMP albeit at a much reduced rate (Margolin *et. al.*, 1994) compared with the natural substrate AMP. Factors which either increase dephosphorylation or decrease phosphorylation are proposed to enhance the induction of LTD. A number of protein kinases are cAMP dependent and a reduction in basal cAMP levels due to deamination by AMPase could reduce the extent of protein phosphorylation leading to the induction of a form of LTD. If this is the case protein kinase inhibitors would be expected to also induce LTD when given alone or to potentiate that generated by AMPase. However the application of A3 did not alter the effect of AMPase. It is possible that if both A3 and AMPase were acting through the same mechanism, then AMPase could occlude any effect produced by A3 and vice versa. The added inhibition of endogenous AMP and maybe even adenosine which inhibits LTD may be the extra stimulus that is required in addition to inhibition of protein kinases to induce LTD. One way to investigate this theory is the use of cAMP inhibitors with and without inhibition of $A_1$ receptor or endogenous agonists.

ATP has been implicated in inducing a form of long term potentiation through acting as an orthophosphate donor for ecto-protein kinase (Fujii *et al.*, 1995; Fujii *et al.*, 1995; Chen *et al.*, 1996). The possible deamination of ATP by AMPase would result in reduced availability of phosphate for use by protein kinases. However this theory relies on a basal concentration of ATP. ATP is released in the hippocampus but only in response to high but not low frequency stimulation.

Due to the broad substrate specificity of AMPase it is possible that other non purinergic related compounds could be either inhibited or activated by an action of AMPase. For example the ester *p*-nitrophenylacetate can serve as a substrate for AMPase (Quenelle & Melander, 1975).

BenzoylbenzoylATP caused a dose dependent reduction in discharge rate which continued to decrease during the wash period in a similar manner to the effect of AMPase. This was proposed to possibly involve the formation of pores due to activation of $P_{2Z}$ ($P2X_7$) receptors but this is merely a speculation. Whether this depression was resistant to reversal after prolonged wash periods was also not fully investigated during this study. In view of the induction of LTD by AMPase, further study into the effect of BzATP would be warranted although whether a relationship exists between AMPase, LTD and the effect of BzATP is no more than supposition at this stage.

In summary, the epileptiform activity generated by zero magnesium/4-aminopyridine perfusion of hippocampal slices can be regulated in numerous manners by different members of the purinergic family. Further work is required to fully decipher by which means some of this modulation takes place.

## 5.1 Future work

The use of ecto-ATPase inhibitors to prevent the metabolism of ATP would help verify that ATP is acting through its metabolites. Hplc analysis of perfusate might also confirm the presence of AMP. Increasing the number of experiments using α, β-methyleneADP in combination with a higher concentration of ATP would support the theory that ATP needs to be metabolised to AMP to be active but not to adenosine. The use of oxidised ATP, an inhibitor of the $P_{2Z}$ ($P2X_7$) receptor, would clarify whether BzATP reduces discharge rate by stimulating this receptor subtype to increase membrane permeability. It would also be of interest to combine oxidised ATP and AMPase. The mechanism behind the induction of a form of long term potentiation by AMPase remains undetermined. Coformycin, at higher concentrations than is required to inhibit adenosine deaminase, inhibits the action of AMPase. This would confirm that the enzyme itself is the active compound producing LTD. If cAMP is implicated in causing this form of LTD then inhibitors of cAMP action might give us some insight into the mechanism of this form of LTD. In order to confirm that this is not merely slice deterioration experiments showing reversal of LTD on high frequency stimulation need to be carried out properly with analysis. On this line it would also be interesting to see if AMPase further reduces slice activity after LTD has been induced by low frequency stimulation and vice versa. There exists a number of directions that could be followed in the quest to determine what underlies this AMPase induced long term depressive action.

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## Appendix 1-media used to induce epileptiform activity.

The table contains details of the media that were used in the initial attempts to generate spontaneous epileptiform activity. The right hand column contains the alterations that were made to the normal aCSF to get the desired medium.

| method | aCSF composition |
|---|---|
| $0Mg^{2+}$ | normal minus $MgS0_4$ |
| $0Mg^{2+}/0Ca^{2+}$/EDTA | normal minus $MgSO_4$ and $CaCl_2$ with EDTA (1mM) added. |
| $0Mg^{2+}/0Ca^{2+}/K^{+}$(5mM)/ EDTA | normal minus $MgSO_4$ and $CaCl_2$, KCl (2.8mM), NaCl (114.2mM) with EDTA (1mM) added. |
| $0Mg^{2+}/0Ca^{2+}/K^{+}$(7mM)/ EDTA | normal minus $MgSO_4$ and $CaCl_2$, KCl (4.8mM), NaCl (112.2mM) with EDTA (1mM) added. |
| $0Mg^{2+}/0Ca^{2+}/K^{+}$(10mM)/ EDTA | normal minus $MgSO_4$ and $CaCl_2$, KCl (5.8mM), NaCl (110.2mM) with EDTA (1mM) added. |
| $0Mg^{2+}/K^{+}$ (5mM) | normal minus $MgSO_4$, KCl (2.8mM) and NaCl (114.2mM). |
| $0Mg^{2+}/K^{+}$ (7mM) | normal minus $MgSO_4$, KCl (4.8mM) and NaCl (112.2mM). |

| | |
|---|---|
| $0Mg^{2+}/K^{+}$ (7mM) | normal minus $MgSO_4$, KCl (5.8mM) and NaCl (110.2mM). |
| $K^{+}$ (10mM) | normal with KCl (5.8mM) and NaCl (110.2mM). |
| $0Ca^{2+}/K^{+}$ (7mM)/EDTA | normal minus CaCl, KCl (4.8mM), NaCl (112.2mM) and EDTA (1mM) added. |
| Kainate | normal with kainate (2μM) added. |
| bicuculline | normal with bicuculline (1/5μM) added |
| $OMg^{2+}$/ bicuculline | normal minus $MgSO_4$ with bicuculline (1μM) added |

## Appendix 2- Methods used to generate ictal activity

The table contains the methods that were used in our attempts to generate ictal epileptiform activity. The left hand column details the aCSF composition, drug additions and whether any stimulation was used. The slice thickness is displayed in the right hand column.

| aCSF composition/ slice treatment | slice thickness |
|---|---|
| $0Mg^{2+}$ (normal aCSF minus $MgSO_4$). | 450μm |
| $0Mg^{2+}$ (normal aCSF minus $MgSO_4$). | 625μm |
| $0Mg^{2+}$ (normal aCSF minus $MgSO_4$)/ stimulate for 2 sec. at 50/sec. at 10 minute intervals. | 450μm |
| $0Mg^{2+}$ (normal aCSF minus $MgSO_4$)/ stimulate for 2 sec. at 50/sec. at 10 minute intervals. | 625μm |
| $0Mg^{2+}$ (normal aCSF minus $MgSO_4$)/ stimulate for 2 sec. at 50/sec. at 5 minute intervals. | 625μm |
| $0Mg^{2+}$ (normal aCSF minus $MgSO_4$) plus baclofen (2μM). | 450μm |
| $0Mg^{2+}$ (normal aCSF minus $MgSO_4$) plus baclofen (2μM). | 625μm |
| 4-aminopyridine (normal aCSF with 4AP (50μM) added. | 450μm |
| 4-aminopyridine (normal aCSF with 4AP (50μM) added). | 625μm |

| **aCSF composition/ slice treatment** | **slice thickness** |
|---|---|
| 4-aminopyridine (normal aCSF with 4AP (50μM) added)/ stimulate 1/100 sec. | 625μm |
| 4-aminopyridine (normal aCSF with 4AP (50μM) added)/ stimulate for 2sec. at 50/sec. every 5 min. | 625μm |
| 0Mg/4AP (normal aCSF minus $MgSO_4$ with 4AP (50μM) added). | 450μm |
| 0Mg/4AP (normal aCSF minus $MgSO_4$ with 4AP (50μM) added). | 625μm |

## Appendix 3-Low/zero calcium and magnesium media.

The alterations to the normal aCSF composition used in an attempt to generate/maintain bursts in zero or low calcium and magnesium conditions included:

$0Mg^{2+}/0Ca^{2+}$

$0Mg^{2+}/Ca^{2+}$ (0.1mM)

$0Mg^{2+}/Ca^{2+}$ (0.5mM)

$Mg^{2+}$ (0.1mM) / $0Ca^{2+}$

$Mg^{2+}$ (0.5mM) / $0Ca^{2+}$

$Mg^{2+}$ (0.8mM)/ $K^{+}$ (10mM)/ $Ca^{2+}$ (1.67mM)

$Mg^{2+}$ (1.0mM)/ $K^{+}$ (10mM)/ $Ca^{2+}$ (0.5mM)