Adenosine inhibits epileptiform activity arising in hippocampal area CA3

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1   The ability of adenosine and structurally-related compounds to inhibit epileptiform activity induced by bicuculline in the CA3 region of the hippocampal slice of the rat was examined.

2   Bath application of all purinoceptor agonists tested reduced the frequency of generation of burst potentials. Analysis of dose-response curves yielded the following IC_{50} values: adenosine, $1.5 \mu M$; 2-chloroadenosine, $0.144 \mu M$; 5'-(N-ethyl)carboxamidoadenosine, $30.2 nM$; L-phenylisopropyladenosine, $12.1 nM$; cyclohexyladenosine, $7.9 nM$.

3   Theophylline ($30 \mu M$) increased the rate of bursting and antagonized the effect of exogenous adenosine.

4   Dipyridamole ($0.03–1 \mu M$) reduced the occurrence of burst firing.

5   In slices untreated with bicuculline, theophylline ($30 \mu M$) and adenosine deaminase ($10 \mu g\, ml^{-1}$) induced bursting activity.

6   These results demonstrate that purinoceptor agonists can suppress epileptiform activity in the hippocampus and suggest that adenosine may act as an endogenous anticonvulsant.

Introduction

Studies in the peripheral nervous system suggest that adenine nucleotides and nucleosides are either transmitters or modulators of neuronal activity, hence the term 'purinergic transmission' (Burnstock, 1975). In the CNS, adenosine is the principal neuroactive purine released into extracellular fluid (Pull & McIlwain, 1972; Jonzon & Fredholm, 1985). Numerous reports have demonstrated that adenosine can act at specific cell surface receptors (Daly *et al.*, 1981; Reddington *et al.*, 1983; Marangos, 1984; Daly, 1985) to depress neuronal firing (Phillis *et al.*, 1979; Stone, 1981; Patel, *et al.*, 1984; Snyder, 1985). Antagonism of adenosine receptors in the CNS is thought to underlie the stimulatory actions of the alkylxanthines theophylline and caffeine (Sattin & Rall, 1970; Daly *et al.*, 1981; Snyder *et al.*, 1981; Katims *et al.*, 1983).

Particular attention has been paid to the hippocampal formation since this brain area possesses one of the highest concentrations of cyclohexyladenosine binding sites (Lewis *et al.*, 1981; Murray & Cheney, 1982). The release of adenosine subsequent to field stimulation of hippocampal slices also suggests a transmitter role (Jonzon & Fredholm, 1985). Electrophysiologically, adenosine can inhibit neurotransmission between Schaffer collateral-commisural fibres

and CA1 pyramidal cells in the hippocampal slice by depressing the release of transmitter (Schubert & Mitzdorf, 1979; Dunwiddie & Hoffer, 1980; Dunwiddie, 1984) and by reducing the excitability of the postsynaptic cells (Segal, 1982).

The effects of adenosine upon CA1 pyramidal cells are remarkably similar to those of baclofen, a γ-aminobutyric acid$_B$ ($GABA_B$) receptor agonist (Bowery *et al.*, 1980; Hill & Bowery, 1981). Baclofen, like adenosine, suppresses the firing of CA1 pyramids by reducing transmitter release from terminals of Schaffer collateral-commissural fibres (Lanthorn & Cotman, 1981; Ault & Nadler, 1982; Olpe *et al.*, 1982) and depressing cell excitability (Ault & Nadler, 1983b; Newberry & Nicoll, 1984; 1985; Inoue *et al.*, 1985).

An anticonvulsant action of both adenosine and baclofen is indicated by their ability to block afterdischarges of CA1 pyramidal cells evoked by electrical stimulation in the presence of convulsant agents (Ault & Nadler, 1983a; Lee *et al.*, 1984). In the hippocampus, CA2/CA3 pyramidal cells are the primary generators of epileptiform activity which then invades area CA1 (Wong & Traub, 1983). It has been demonstrated that baclofen blocks intrinsically-arising burst discharges in area CA3 at concentrations up to an

order of magnitude lower than those necessary to inhibit electrically-evoked firing (Gruenthal et al., 1984).

In this study we have investigated the ability of adenosine to depress burst firing in the CA3 region, and the potential anticonvulsant role of endogenous adenosine. A brief account of this study has been published previously (Ault & Wang, 1985).

Methods

Preparation of slices

Hippocampi were dissected from the brains of adult female Sprague-Dawley rats (supplier, Charles River) which had been killed by cervical dislocation. Transverse slices of 425 μM nominal thickness were quickly prepared and placed on nylon nets in a tissue bath (Fine Science Tools). Slices were initially placed in a static chamber, then, after 1–1.5 h incubation in order to restore synaptic responsiveness, transferred to a smaller (approximately 0.5 ml) bath for recording. Slices in the recording chamber were superfused at 1.2–1.5 ml min^{-1} with Elliott's (1969) medium (composition (mM): NaCl 122, NaHCO$_3$ 25, KCl 3.1 or 4.6, CaCl$_2$ 1.3, MgCl$_2$ 1.2, KH$_2$PO$_4$ 0.4 and dextrose, 10, gassed with 95% O$_2$:5% CO$_2$) which was warmed to between 30 and 31°C.

Recording of field potentials

Extracellular potentials were recorded from the CA3 cell body layer using glass microelectrodes of 2–5 MΩ impedance filled with 3M NaCl. In order to optimize the recording position the Schaffer-commissural fibres were stimulated at 0.5 Hz and the electrode was lowered into the tissue until a maximal evoked potential was obtained. A diagrammatic representation of the stimulating and recording sites has been published (Ault & Nadler, 1983a). Stimulation was then stopped and spontaneously arising potentials were amplified, filtered below 1 Hz and above 3 kHz, and displayed on an oscilloscope. The incidence of bursting was continuously monitored on a chart recorder. Some potentials were photographed from the oscilloscope screen with a Hewlett-Packard type 197A camera using Polaroid film.

Application of drugs

During the period of recording, the slices were submerged to facilitate exchange of drugs between the superfusion medium and the extracellular fluid in the slice. This process did not adversely affect the physiological responsiveness of the tissue. Normal or drug-containing media were introduced into the tissue

chamber from pressurized flasks by the use of a rotary valve. In the majority of experiments bursting was induced by addition of bicuculline (Bic) to medium which contained a slightly elevated K concentration (total of 5 mM). Bic was therefore usually superfused continuously. Only in the two series of experiments when theophylline (30 μM) and adenosine deaminase (10 μg ml^{-1}) were added to standard medium (3.5 mM K) was Bic not used. Purinoceptor agonists were dissolved in 20 ml of Bic-containing medium and superfused for 10 min periods, which was found to be sufficient to allow equilibrium of the agonists before the measurement of the rate of bursting. When dose-response curves were studied, increasing concentrations of agonist were applied cumulatively from separate flasks. To determine the antagonistic action of theophylline upon exogenous adenosine or baclofen, a dose-response curve was initially generated in Bic-containing medium. The slice was then washed for 10–20 min to reverse the effect of the agonist and theophylline was then added to the medium. After a further 15–25 min the depressant action of adenosine or baclofen was again determined in the presence of theophylline. The adenosine uptake inhibitor dipyridamole was found to have a longer equilibrium time than purinoceptor agonists and was thus applied for 1.5 min periods. Adenosine deaminase was applied for 10–20 min.

Analysis of drug effects

The frequency of bursting was assessed for the 4 min period prior to application of test compounds, and during the last 4 min of the drug superfusion periods. For purinoceptor agonists, the degree of change in bursting rate was expressed as the percentage inhibition of the initial rate determined in agonist-free medium. The effects of theophylline and dipyridamole were expressed as a fraction of the initial bursting rate.

IC$_{50}$ values were calculated from dose-response curves by a least squares fit of logarithmic transformations of dose and response data. The dose-ratio is defined as the IC$_{50}$ determined in the presence of theophylline divided by the IC$_{50}$ measured in theophylline-free medium. Averaged data are expressed as mean $\pm$ s.e.mean for n = number of experiments on separate slices.

Materials

Bicuculline methiodide was obtained from Pierce Chemicals (Rockford, IL, U.S.A.) and ($\pm$)-baclofen was provided by Ciba-Geigy Corp. (Ardsley, NY, U.S.A.). Other chemicals were purchased from Sigma Chem. Co. (St Louis, MO, U.S.A.). Adenosine deaminase (type VII) had an activity of 37 or 39 units mg^{-1} of powder.

Results

Bursts of population spikes, which reflect the synchronous discharge of neurones in the vicinity of the recording electrode (Anderson *et al.*, 1981), were induced in area CA3 of the hippocampal slice by superfusing medium containing Bic (50 µM) and a slightly elevated level of K (5 mM). Bursting in the presence of Bic was also seen at a normal level of K (3.5 mM): however, the rate of burst firing was found to be more consistent over several hours at the higher K concentration. A typical potential consisted of a burst of multiple population spikes superimposed on a slow positive-going potential of 60–80 ms duration (Figure 1b) which is the extracellular correlate of the paroxysmal depolarizing shift and neuronal firing recorded intracellularly (Schwartzkroin & Prince, 1978; Prince, 1978). The negative-going undershoot

which followed the positivity was mainly due to the a.c. coupling of the amplification system. Between experiments the rate of burst discharges varied from 4 to 22 min^{-1} (mean ± s.e. of 10.4 ± 0.6 bursts min^{-1}, $n = 49$) but was relatively constant in individual slices for up to 4 h.

Effects of purinoceptor agonists upon burst firing

Superfusion of adenosine produced a dose-dependent reduction in the rate of burst firing (Figure 1a and b). The concentration necessary for a threshold effect was about 0.25 µM and a complete cessation of activity was observed at 4–8 µM. Figure 1a illustrates the rapid rate of onset and offset of a single application of adenosine. Routinely, however, cumulative dose-response curves
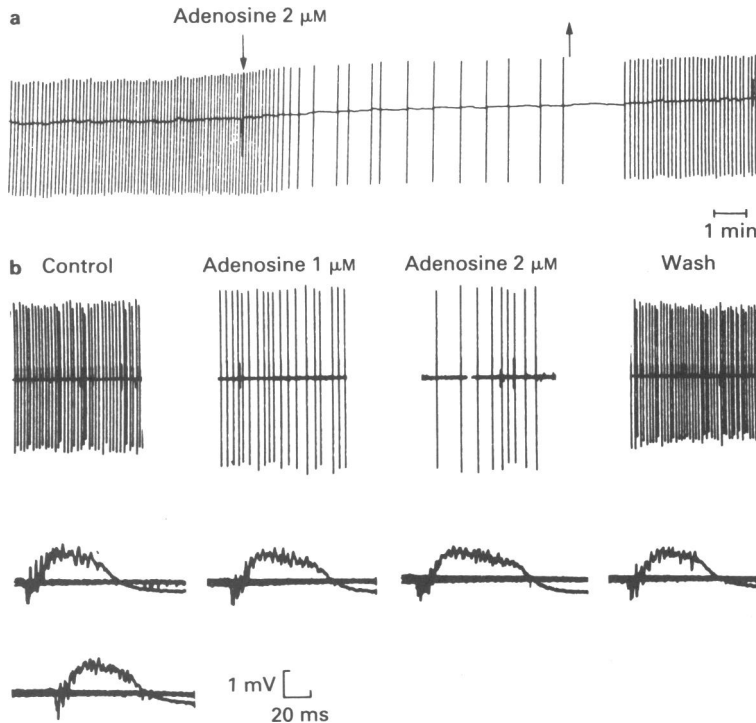
Figure 1 Depressant effect of adenosine upon the rate of burst firing in hippocampal slice of rat. The incidence of bursting was recorded on a chart recorder, with positive-going potentials producing an upward deflection. Chart records do not accurately represent the amplitude of the bursts because of the low frequency response of the recorder and are therefore not calibrated. However, the illustrated records would be equivalent to a d.c. voltage step between 0.5 and 1 mV. The rapid onset and offset of a single dose of adenosine is shown in (a). Amplifier offset drift caused the slow change in baseline. Cumulative dose-response curves were normally generated and (b) illustrates the inhibition of bursting by increasing doses of adenosine. Typical potentials captured on the oscilloscope during this experiment are shown under the appropriate chart record and indicate the prolongation of burst firing observed upon reduction of burst frequency.

were utilized since this procedure was more convenient and appeared to be comparable to single dose applications. Examination of oscillographs indicated that adenosine acted primarily to depress the rate of burst discharge. Athough a systematic analysis was not performed due to the incomplete photographic record, it appeared that when the rate of burst firing was inhibited more than about 50% that there was usually a prolongation of the duration of each burst potential (Figure 1b lower records). Evidence of this can also be seen in the increase of the amplitude of the chart recordings, which reflect the area bounded by the waveform due to the low frequency response of the chart recorder (Figure 1a and b).

All of the purinoceptor agonists tested were capable of completely suppressing the generation of burst discharges and the following IC_{50} values were determined from dose-response curves: adenosine, $1.5 \pm 0.17 \mu M$, $n = 12$; 2-chloroadenosine (2-Cl-AD), $144 \pm 28 \, nM$, $n = 9$; 5'-(N-ethyl)carboxamidoadenosine (NECA), $30.2 \pm 1.5 \, nM$, $n = 4$; N^6-(L-phenylisopropyl)adenosine (L-PIA), $12.1 \pm 1.8 \, nM$, $n = 6$; N^6-cyclohexyladenosine (CHA), $7.9 \pm 1.7 \, nM$, $n = 4$.

Action of theophylline

In 6 slices, addition of theophylline $(3-100 \, \mu M)$ to the superfusion medium increased the rate of burst firing in a dose-related manner (Figure 2). The increase in frequency of bursting was accompanied by a decrease in burst duration which is reflected in the reduction of the amplitude of the chart record (Figure 2).

The antagonistic action of theophylline was investigated by comparing cumulative dose-response curves for adenosine in the absence and then the presence of theophylline. Theophylline $(30 \, \mu M)$ increased the rate of bursting and shifted the adenosine dose-response curve to the right (Figure 3). A comparison of IC_{50} values gave dose-ratios between 3 and 9 (mean $\pm$ s.e. of 6.4 ± 1.7, $n = 4$). To determine whether the increase in the rate of bursting reduced the apparent potency of adenosine and overestimated the true pharmacological antagonism, a similar series of experiments was performed with baclofen. Dose-response curves for baclofen were shifted to the right in the presence of theophylline with an average dose-ratio of 1.7 ± 0.32, $n = 3$. Analysis by Student's t test indicated a significantly greater antagonism of adenosine ($P < 0.025$), the difference reflecting the specific receptor blockade.

Effect of uptake inhibition

Slices or synaptosomes prepared from cerebral cortical tissue have been shown to accumulate adenosine actively, by a sodium-dependent mechanism (Banay-Schwartz et al., 1980; Bender et al., 1980). The effect of dipyridamole, an inhibitor of adenosine uptake (Bender et al., 1980), was thus examined. In four experiments cumulative doses of dipyridamole produced
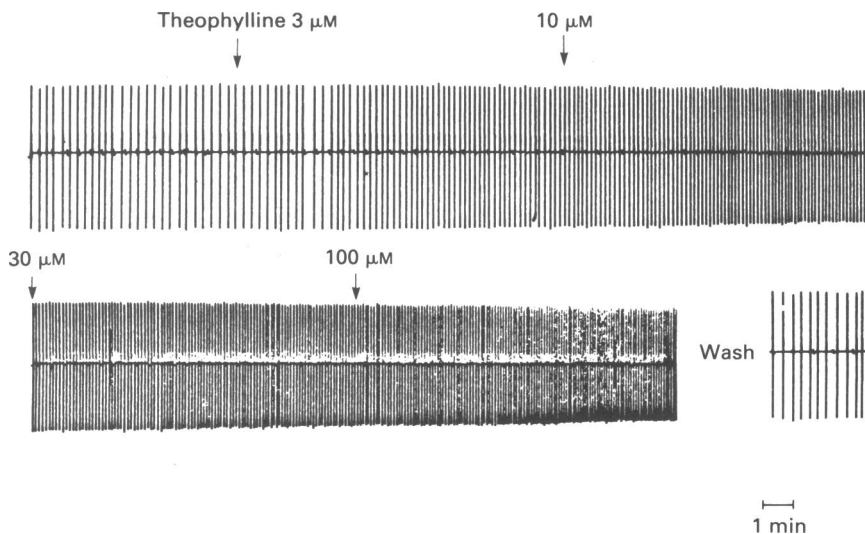
Figure 2 Effect of theophylline on burst frequency in hippocampal slice of rat. Increasing concentrations of theophylline superfused for 10 min periods enhanced the rate of bursting. A coincident decrease of burst duration is reflected in the amplitude of pen deflection. Superfusion of theophylline-free medium for 15 min reversed the action of theophylline.
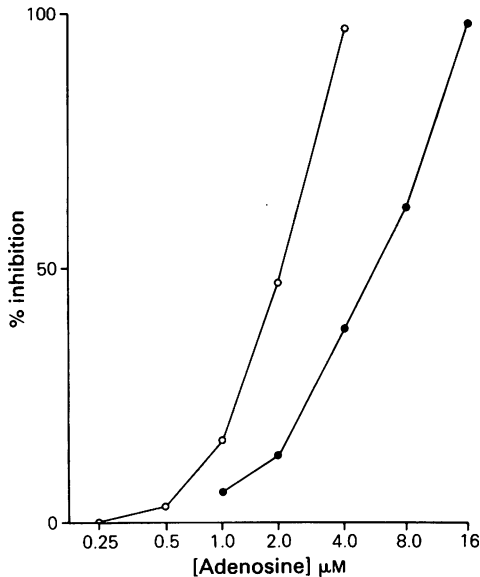
Figure 3 Antagonism of adenosine. Cumulative dose-response curves were generated in the absence (O) and presence (●) of theophylline (30 μM). A single example is shown because doses of adenosine varied between experiments.
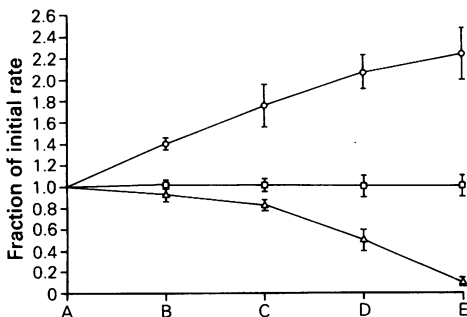
Figure 4 Comparison of the action of theophylline and dipyridamole. Cumulative doses of theophylline and dipyridamole were superfused in the presence of Bic. Values of A-E for each curve: 0, 15, 30, 45 and 60 min (control, □); 0, 3, 10, 30 and 100 μM (theophylline, O); 0, 0.03, 0.1, 0.3 and 1.0 μg ml⁻¹ (dipyridamole, Δ). The superfusion period for theophylline was 10 min, therefore the experimental duration was shorter than for the other curves. Data points indicate the mean rate of bursting compared to the initial rate for 6 slices (control and theophylline) and 4 slices (dipyridamole); vertical lines show s.e.mean.

a progressive decrease in the rate of bursting (Figure 4). The threshold concentration was approximately 30 nM and 1 μM nearly abolished epileptiform activity.

Physiological role of endogenous adenosine

Whilst the data obtained from superfusion of theophylline and dipyridamole indicated a tonic effect of endogenous adenosine, it was possible that such modulation might only occur subsequent to the neuronal activity induced by Bic. When incubated in normal medium (K 3.5 mM) which did not contain Bic, hippocampal slices did not usually exhibit extracellular bursting activity. Ten such slices from four rats were studied using theophylline and adenosine deaminase, which metabolizes adenosine to inosine. Superfusion of theophylline (30 μM, $n = 4$) or adenosine deaminase (10 μg ml⁻¹, $n = 6$) induced bursting in all slices tested (Figures 5 and 6). Theophylline took 2–4 min to have an observable effect, whilst adenosine deaminase had a delay of up to 10 min. In four cases, two in each treatment group, the burst duration was 25–50 ms, but in the other slices the bursts were comparable to those recorded in the presence of Bic.

Discussion

Our findings demonstrate that adenosine and structurally-related compounds are potent inhibitors of bursting activity induced in the hippocampal slice by the GABA antagonist Bic. These observations suggest an anticonvulsant action of purinoceptor agonists since burst firing in the hippocampus *in vitro* is representative of interictal spiking *in vivo* (Prince, 1978). Studies of burst potentials (Dunwiddie, 1980) or electrically-evoked afterdischarges (Lee *et al.*, 1984) induced by penicillin in hippocampal area CA1 also are in agreement with this conclusion.
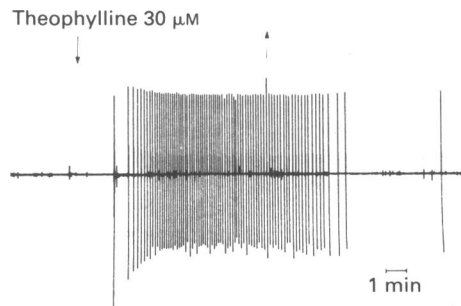
Figure 5 Induction of bursting by theophylline. Superfusion of theophylline led to generation of burst discharges in the absence of bicuculline. Washing the slice readily reversed this action.

Adenosine deaminase 10 μg ml^{-1}

1 min
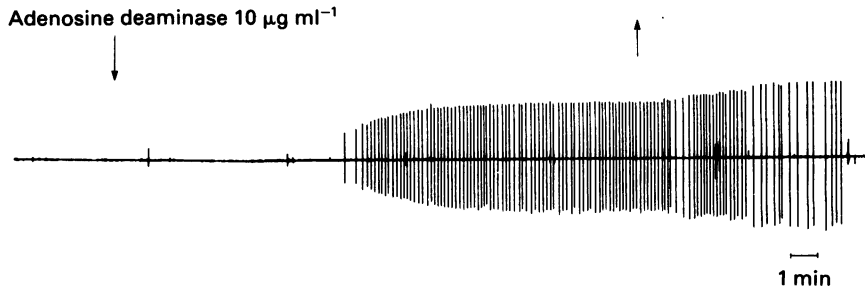
Figure 6 Induction of bursting by adenosine deaminase. Adenosine deaminase had a similar effect to theophylline but had a longer onset time and was only slowly reversed upon washing (at upward arrow) with normal medium.

The predominant effect of the purinoceptor agonists was to reduce the rate of bursting, which was often associated with an increase in the duration of each burst potential. Adenosine therefore acted to suppress the initiation rather than the spread of seizure activity. A number of factors could influence the likelihood of bursting in a population of CA3 pyramidal cells. These are the spontaneous firing of neurones, intrinsic bursting behaviour, excitatory interconnections between cells, and inhibitory processes. Adenosine is capable of altering these parameters by increasing K^+ conductance (Segal, 1982; Haas & Greene, 1985), and inhibiting transmitter release (Schubert & Mitzdorf, 1979; Dunwiddie & Hoffer, 1980; Siggins & Schubert, 1981; Segal, 1982; Dunwiddie, 1984). An increase in K^+ conductance of pyramidal cells results in hyperpolarization (Segal, 1982; Haas & Greene, 1985), a decrease in calcium conductances (Halliwell & Scholfield, 1984), and an enhancement of burst afterhyperpolarization (Haas & Greene, 1985). These factors would account for the observed responses by reducing spontaneous firing and increasing the interburst interval which is mainly determined by the afterhyperpolarization (Suppes et al., 1985). The observation that dopamine can also decrease the frequency of bursting with little effect on individual bursts (Suppes et al., 1985), strengthens this conclusion. However, it is possible that a small decrease in synaptic transmission might also be of importance since the population burst is thought to be dependent on reverberating activity through many synapses in the CA3 neuronal circuit (Traub & Wong, 1983). The apparently paradoxical increase in burst duration seen at higher concentrations of adenosine may be explained by disinhibition (Lee & Schubert, 1982), an action shared by baclofen (Ault & Nadler, 1983a). It should be noted, though, that most studies have examined the effects of much higher concentrations of adenosine than used in our experiments upon physiological processes in area CA1, therefore the relative contribution of these changes should be determined by intracellular recording of CA3 neurones.

The effects of adenosine in area CA1 are essentially identical to those of baclofen, which decreases postsynaptic excitability (Ault & Nadler, 1983b; Newberry & Nicoll, 1984; 1985; Inoue et al., 1985) and blocks transmitter release (Ault & Nadler, 1982; Olpe et al., 1982). This correspondence may be due to the linkage of receptors for baclofen and adenosine to a common adenylate cyclase catalytic unit (Wojcik et al., 1985). Although at this time there is no direct evidence that inhibition of adenylate cyclase activity results in the observed electrophysiological effects of baclofen or adenosine, it may be relevant that pertussis toxin, which prevents coupling of inhibitory receptors to adenylate cyclase, reverses purinergic inhibition of glutamate release (Dolphin & Prestwich, 1985).

Adenosine receptors in the CNS have usually been categorized into A_1 and A_2 subtypes based on their inhibition or activation of adenylate cyclase respectively (Van Calker et al., 1979; Londos et al., 1980; Daly, 1985) and the relative potencies of adenosine analogues, in particular the stereoisomers of PIA (Daly et al., 1981; Reddington et al., 1983). Both the A_1 and A_2 receptors are blocked by alkylxanthines (Daly et al., 1981). As discussed above, it is likely that if adenosine influences the activity of adenylate cyclase it would be inhibitory, suggesting mediation by the A_1 receptor. The relative potencies of the agonists tested and the antagonism of adenosine by theophylline would be consistent with this proposal. However, our data are also compatible with the existence of other receptors which have been recently characterized (Chin et al., 1985). A quantatitive analysis of the receptors mediating the electrophysiological action of adenosine, especially by the use of antagonists (Kenakin & Leighton, 1985), therefore appears necessary.

In these studies we have demonstrated a modulatory effect of endogenous adenosine by a number of pharmacological manipulations. Theophylline, at a concentration that antagonized adenosine, induced

epileptiform activity and elevated the rate of bursting in the presence of Bic. Similarly, removal of adenosine by adenosine deaminase induced bursting activity. Conversely, inhibiting adenosine uptake with dipyridamole produced a dose-dependent reduction of burst discharges. These data support other groups who have previously demonstrated that endogenous adenosine can affect the excitability of CA1 pyramidal cells (Dunwiddie, 1980; Dunwiddie et al., 1981; Greene et al., 1985) and reduce the release of radiolabelled noradrenaline (Jonzon & Fredholm, 1984), acetylcholine (Jackisch et al., 1984) and 5-hydroxytryptamine (Feuerstein et al., 1985). An important additional observation in our study was that in unstimulated slices, adenosine was obligatory for the prevention of generation of burst discharges. Correspondingly, adenosine has been shown to be released continuously from hippocampal slices (Dunwiddie et al., 1981). Thus, in the maintenance of physiological equilibrium, adenosine has a similar importance to GABA in area CA3. The sensitivity of burst firing to concentrations of adenosine that have little effect on electrically-evoked potentials in area

CA1 (Dunwiddie & Hoffer, 1980; Reddington et al., 1983) also suggests that the predominant action of endogenous adenosine will be upon CA3 neurones, assuming a similar extracellular concentration.

We conclude from these experiments that purinoceptor agonists can preferentially inhibit epileptiform potentials generated by CA3 pyramidal cells and that endogenous adenosine tonically suppresses such activity. Two hypotheses can be drawn from this conclusion. Firstly, drugs that mimic or enhance purinergic inhibition might be useful anticonvulsants in the treatment of temporal lobe epilepsy. Interestingly, carbamazepine, a drug used in the treatment of complex partial seizures (Mattson et al., 1985), may interact with adenosine receptors (Marangos et al., 1983) although effects in the hippocampus are not blocked by caffeine (Olpe et al., 1985). Secondly, a deficit in the action of endogenous adenosine could contribute to the generation of limbic epilepsy. In the latter regard it is surprising that little attention has been paid to the possible pro-convulsant effects of dietary alkylxanthines (Aird et al., 1984).

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