Modulation of Epileptiform Activity by Adenosine A1 Receptor-Mediated Mechanisms in the Juvenile Rat Hippocampus

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ABSTRACT

The modulatory role played by purinergic mechanisms on the epileptiform discharges induced by 4-aminopyridine (4AP, 50 μM) in juvenile (10 to 25-day-old) rat hippocampal slices was studied with field potential recordings in the CA3 stratum radiatum. 4AP-induced activity consisted of interictal and ictal discharges along with isolated γ-aminobutyric acid-mediated potentials. The adenosine analogues 2-Cl-adenosine (10–200 μM) and N-ethylcarboxamido-adenosine (5–10 μM), the A1 receptor agonist N6-(L2-phenylisopropyl)-adenosine (2–10 μM), and the adenosine uptake inhibitor dipyridamole (1–40 μM) reduced and eventually abolished interictal and ictal discharges with IC50 values that were larger for ictal discharges as compared to interictal activity. These purinergic agents did not modify the rate of occurrence of the γ-aminobutyric acid-mediated potentials recorded during application of excitatory amino acid receptor antagonists. The changes induced by 2-Cl-adenosine, N6-(L2-phenylisopropyl)-adenosine, or dipyridamole were reversed by caffeine (500 μM) or 8-cyclopentyl-1,3-dipropylxantine (100 μM). However, these adenosine receptor antagonists did not alter the epileptiform discharges induced by 4AP. The depressant effects induced by N6-(L2-phenylisopropyl)-adenosine on the epileptiform activity were maintained in the presence of barium (2 mM), which blocks adenosine postsynaptic actions. These results demonstrate that activation of adenosine A1 receptors in the juvenile rat hippocampus leads to an anticonvulsant action that can be ascribed to a decreased release of glutamate from CA3 pyramidal cell terminals. We also propose that during the first weeks of postnatal life endogenous adenosine does not activate A1 receptors to a degree to control the ability of hippocampal neurons to generate epileptiform activity in the 4AP model.

The depressant action exerted by adenosine on cortical neuron excitability is mainly mediated through the activation of A1 receptors, and results from presynaptic (i.e., reduction of transmitter release from excitatory terminals) and postsynaptic (i.e., membrane hyperpolarization, increase in firing adaptation and enhancement of the slow afterhyperpolarization) actions (Gerber et al., 1989; Greene and Haas, 1985; Haas and Greene, 1984; Lupica et al., 1992; Scanziani et al., 1992; Thompson et al., 1992). Adenosine induces anticonvulsant effects both in vivo and in vitro models of epileptiform discharge (Ault and Wang, 1986; Barraco et al., 1984; Dunwiddie, 1980; see for review Dragunow, 1988). Furthermore, adenosine concentration rises substantially during and following seizures in animals (Dragunow, 1988) and in human beings (During and Spencer, 1992), thus suggesting that purinergic mechanisms may play a role in epileptogenesis and/or in the arrest of seizures.

The anticonvulsant action of adenosine in young animals remains as yet unestablished (but see Psarropoulou et al., 1990). Therefore, we used the in vitro hippocampal slice preparation to investigate whether modulating the efficacy of purinergic mechanisms may influence the epileptiform activity disclosed by 4AP in the juvenile rat hippocampus (Avoli et al., 1993, 1996; Fueda and Avoli, 1992). We report that adenosine and its analogues (including the adenosine uptake inhibitor dipyridamole) reduce interictal and ictal discharges recorded in the CA3 subfield of hippocampal slices from 10 to 25-day-old rats through an action on A1 receptors that are presumably located on presynaptic excitatory terminals. Our findings also indicate that 4AP-induced epileptiform discharges in juvenile hippocampus are not influenced by adenosine receptor antagonists. These results have been published in abstract form (Tancredi et al., 1994).

ABBREVIATIONS: ACSF, artificial cerebrospinal fluid; 4AP, 4-aminopyridine; CNQX, 6-cyano-7-nitro-quinoxaline-2,3 dione; CPP, 3-3-(2-carboxypiperazine-4-y1)propyl-1-phosphonate; DPCPX, 8-cyclopentyl-1,3-dipropylxantine; L-PIA, N6-(L2-phenylisopropyl)-adenosine; NECA, N-ethylcarboxamido-adenosine; NMDA, N-methyl-D-aspartate; DMSO, dimethyl sulfoxide; GABA, γ-aminobutyric acid.
Methods

Sprague-Dawley or Wistar rat pups aged 10 to 25 days postnatally were decapitated under ether or halothane anesthesia. The brain was quickly removed from the skull and placed in cold (1–3°C) ACSF. The hippocampi were dissected and cut transversely in slices (450–550 μm) using either a McIlwain tissue chopper or a vibratome. Slices were then transferred to a tissue chamber, where they were maintained at an interface between oxygenated ACSF and humidified atmosphere gassed with 95% O2/5% CO2 at a temperature of 33 ± 1°C. The ACSF composition was (in mM): NaCl 124, KCl 2, KH2PO4 1.25, MgSO4 1, CaCl2 2, NaHCO3 26 and glucose 10 (pH 7.4).

Extracellular field potential recordings were made in the stratum radiatum of the CA3 subfield with microelectrodes that were filled with either 2M NaCl (resistance 4–8 MΩ) or ACSF (resistance 1–3 MΩ). Signals were fed to a high impedance DC amplifier and were displayed on an oscilloscope and on a Gould pen chart recorder.

Drugs were added to the perfusing ACSF. Concentrations given through the paper represent the presumptive peak concentration in the bath. DPCPX was purchased from Research Biochemicals Inc.; CNQX and CPP from Tocris Cookson; 4AP, 2-Cl-adenosine, L-PIA, NECA and dipyridamole from Sigma Chemical Co. (St. Louis, MO) and the caffeine from Merck (Rahway, NJ). With the exception of DPCPX, all substances could readily be dissolved in water. DPCPX was dissolved in 0.05 ml DMSO and then into water to form a 2 mM stock solution. The maximal DMSO concentration (v/v) in the perfusing medium was 0.01%. In three experiments, DMSO alone did not influence the synchronous epileptiform activity induced by 4AP.

Slices were continuously perfused at a rate of 0.5 to 1 ml/min which allowed for complete exchange of the medium in the tissue chamber in less than 5 min. Preliminary experiments were performed to establish the minimal drug concentrations required to influence the synchronous epileptiform activity induced by 4AP in juvenile rat hippocampal slices. In the course of these studies we noticed that prolonged (>30 min) application of adenosine agonists made spreading depression-like episodes appear in most cases. Because the occurrence of spreading depression-like episodes interferes with hippocampal tissue excitability (Psarrapoulou and Avoli, 1993; Avoli et al., 1996), adenosine agonists were applied for periods of less than 25 min, unless otherwise indicated. Drug application was then followed by washout with control medium for at least 45 min. Hippocampal slices were never treated with more than three different concentrations of any given drug.

The rate of occurrence, the duration and/or the amplitude of the different types of synchronous activity induced by 4AP under control conditions were assessed for periods >5 min before adding ACSF containing any of the tested compounds. These measurements were later repeated before drug washout (i.e., at a time when the compounds had presumably reached peak concentration in the tissue).

Throughout our report measurements are expressed as mean ± S.D. and n indicates the number of slices used for any given pharmacological protocol. Statistical analysis was performed using either the paired or the unpaired t test. In addition a standard one-way analysis of variance was used to establish whether the concentrations of adenosine agonists required to abolish 4AP-induced epileptiform discharges varied as a function of the slice postnatal age. The computer software InStat was used for all statistical comparisons. Data were considered significantly different if P < .05.

Results

General features of the 4AP-induced synchronous activity. Three types of spontaneous, synchronous activity were typically recorded in the CA3 stratum radiatum of juvenile rat hippocampal slices during application of 4AP (50 μM) (cf., Avoli et al., 1993, 1996; Fueta and Avoli, 1992). These field potentials consisted of: 1) interictal-like (thereafter called interictal) positive-going discharges that lasted 0.2 to 1.2 sec and occurred at 0.2 to 1.4 Hz (arrows in fig. 1A, control); ictal-like (thereafter termed ictal) discharges that had duration = 3 to 20 sec, were made of trains of high-frequency (up to 22 Hz) positive potentials and occurred at intervals of 30 to 250 sec (continuous line in fig. 1A, control) and 2) large-amplitude (up to 8 mV), negative-going potentials that repeated every 20 to 45 sec (asterisk in fig. 1A, control). These negative-going potentials often preceded the onset of the ictal discharge.

As reported in previous studies (Avoli et al., 1993, 1996; Fueta and Avoli, 1992), none of these spontaneous, synchronous activities was influenced by the antagonist of the NMDA receptor CPP (10 μM, n = 3), although both ictal and interictal events were abolished by application of the non-NMDA receptor antagonist CNQX (10 μM, n = 3). By contrast, the negative-going potentials continued to occur during concomitant application of CPP and CNQX (10 μM for each of these compounds; n = 10), but were reduced and eventually blocked by the GABA_A receptor antagonist bicuculline methiodide (10 μM; n = 4). We have also shown that these negative-going potentials disappear during activation of mu opioid receptors (Avoli et al., 1996; Barbarosie et al., 1994). Hence these events represent synchronous, GABA-mediated potentials.

Adenosine receptor agonists. Application of 2-Cl-Adenosine (10–200 μM, n = 18) reduced and eventually abolished all epileptiform activities induced by 4AP (figs. 1 and 2). By contrast GABA-mediated field potentials continued to occur and at times (two of six slices studied with 200 μM of 2-Cl-adenosine) they increased in amplitude and rate of occurrence. However, these changes were not statistically significant. The effects induced by 2-Cl-adenosine developed over time. At first they were characterized by a progressive reduction of the rate of occurrence and of the amplitude of interictal discharges (fig. 1A, 8-min sample). Later, the intervals between ictal events increased, although interictal activity decreased further (fig. 1A, 14-min sample). Finally, both types of epileptiform discharge disappeared (fig. 1A, 19-min sample). The results obtained in the experiment shown in figure 1A are plotted in the time histograms of figure 1B.

The effects exerted by 2-Cl-adenosine on the rate of occurrence of interictal and ictal discharges were dose-dependent and had IC_50 of 21 μM (n = 17) and 59 μM (n = 9) respectively (fig. 2B). As illustrated in figure 2A, these effects were reversed by bath application of caffeine (500 μM; n = 6). Similar findings were also obtained with DPCPX (100 μM; n = 4) (not illustrated).

To establish further the action exerted by 2-Cl-adenosine on the GABA-mediated field potentials we analyzed six slices during concomitant application of CPP (10 μM), CNQX (10 μM) and 4AP (50 μM). Under these experimental conditions negative-going field potentials were recorded in isolation. As illustrated in figure 3, further application of 2-Cl-adenosine (200 μM) to these slices did not modify the rate of occurrence or the amplitude of the GABA-mediated field potentials.

Application of the specific agonist of the A1 receptor L-PIA (2–10 μM, n = 12) reduced the frequency of occurrence of both interictal (IC_50 = 5.6 μM, n = 12) and ictal discharges (IC_50 = 7.2 μM, n = 8) (fig. 4). High concentrations of L-PIA (8–10 μM, n = 8) abolished all types of epileptiform activity,
without exerting any significant change on the GABA-mediated field potentials, although a significant increase in their rate of occurrence was seen with L-PIA doses ranging 3 to 5 \(\mu\)M. However, as with 2-Cl-adenosine, L-PIA (10 \(\mu\)M, \(n = 4\)) did not induce any change in the rate of occurrence or in the amplitude of the isolated GABA-mediated potentials recorded during application of excitatory amino acid receptor antagonists (not illustrated). The effects induced by maximal concentrations of L-PIA (10 \(\mu\)M) were reversed by application of the specific adenosine A1 receptor antagonist DPCPX (100 \(\mu\)M, \(n = 4\)) (fig. 4A). Reappearance of the epileptiform activity during DPCPX
In which low concentrations of L-PIA (0.1–1 μM) were applied for periods of 40 to 60 min, although limiting the analysis to those slices where spreading depression-like episodes were not recorded. No change in the patterns of epileptiform discharge was seen with 0.1 to 0.8 μM L-PIA (n = 4), although a 20 to 30% decrease in interictal discharge rate of occurrence was induced by 1 μM L-PIA after approximately 55 min of perfusion (n = 2; not shown).

**Effects induced by the adenosine uptake inhibitor dipyridamole.** The adenosine uptake inhibitor dipyridamole (Bender et al., 1980; Stafford, 1966) was tested in 13 slices at concentrations ranging between 1 and 40 μM. Dipyridamole reduced in a dose-dependent manner the frequency of occurrence of ictal (IC₅₀ = 27 μM, n = 10 slices) and interictal (IC₅₀ = 22 μM, n = 13 slices) discharges and eventually abolished all epileptiform activity (fig. 5). Reduction and/or blockade of interictal and ictal activity were accompanied by an increase in the rate of occurrence of the GABA-mediated field potentials (Fig. 5A and B).

The effects induced by dipyridamole were reversed by DPCPX (100 μM, n = 4 slices) (fig. 5A). This antagonistic action was often accompanied by a long-lasting increase in the duration/amplitude of both interictal and ictal discharge and by a reduction in the amplitude of the GABA-mediated field potentials.

**Age-dependency of the effects induced by 2′-Cl-adenosine and dipyridamole.** The results obtained while lasting 2′-Cl-adenosine and dipyridamole on the 4AP-induced epileptiform discharges were also segregated in two age-groups (10- to 15- and 16- to 25-day-old rats) to establish whether the modulation of epileptiform activity exerted by these two purinergic agents had age-dependent features. However, we were unable to observe any significant difference between these two groups both in the IC₅₀ value of the dose-response curves and in the maximal doses required for blocking 4AP-induced epileptiform discharges.

We also used the analysis of variance test to identify differences among the concentrations of 2′-Cl-adenosine that were able to abolish the 4AP-induced epileptiform activity generated by slices of different age. However, we were unable to detect any significant difference among the concentrations of this adenosine agonist that were required to block either interictal or ictal discharges in slices obtained from 11- to 23-day-old rats (n = 11).

**Effects of adenosine receptor antagonists.** Application of caffeine (500 μM) to 4AP-containing medium prolonged interictal (from 0.9 ± 0.2 to 1.3 ± 0.3 sec) and ictal (from 7.7 ± 3.2 to 10.5 ± 3.4 sec) discharges in three of seven experiments. In the remaining four experiments caffeine did not induce any change in the duration or in the rate of occurrence of both types of epileptiform activity. However, in one experiment large amplitude (up to 11 mV), long-lasting (40–190 sec) negative-going shifts resembling spreading depression-like episodes appeared approximately 30 min after caffeine was added to the medium. Caffeine did not significantly influence the amplitude of the GABA-mediated field potentials (not shown).

Application of DPCPX (100 μM, n = 9) to medium containing 4AP did not alter the patterns of spontaneous synchronous activity including interictal and ictal discharges as well as GABA-mediated field potentials (not shown). In three experiments, further addition of L-PIA (10 μM) did not modify the synchronous activity recorded in the presence of 4AP and DPCPX; hence, DPCPX appeared to have exerted its...
an antagonist action on the A1 receptor, in spite of its inability to induce changes on the 4AP-induced activity.

Effects of barium on 4AP-induced synchronous activity. Adenosine postsynaptic actions are blocked by extracellular application of barium (Birnsgiel et al., 1992; Thompson et al., 1992). Therefore, we studied whether the depressant action of L-PIA on the epileptiform discharges elicited by 4AP was maintained in the presence of barium (2 mM, n = 6). Addition of barium to 4AP-containing medium increased the frequency of occurrence and the duration of epileptiform discharges, and often disclosed large amplitude (8–20 mV) sustained shifts of positive polarity that lasted 5 to 20 sec and were associated with repetitive, positive-going population spikes (fig. 6A). These effects were accompanied by the disappearance of the negative-going GABA-mediated potentials. Despite these dramatic effects, further application of L-PIA (10 μM) to medium containing 4AP and barium was able in all cases to abolish the epileptiform discharges (fig. 6A). Such an effect was associated with the reappearance of the GABA-mediated synchronous potentials that occurred at a higher rate than in control.

To better understand the changes induced by barium on the GABA-mediated field potentials we added CPP and CNQX to 4AP-containing ACSF before barium application (n = 9). As illustrated in figure 6B, barium increased in all experiments the rate of occurrence of the synchronous GABA-mediated events recorded during blockade of excitatory amino acid receptors.

Discussion

We have analyzed the anticonvulsant action exerted in vitro by manipulation of the adenosine receptor functions in the CA3 area of juvenile rat hippocampus. The CA3 subfield plays a key role in seizure generation (Traub and Wong, 1983; Miles and Wong, 1986) and displays a high binding density for the A1 receptor during early postnatal development (Daval et al., 1991). Our findings indicate that purinergic activation: 1) results in a powerful, A1 receptor-mediated anticonvulsant effect on both interictal and ictal discharges induced by 4AP; 2) does not influence the occurrence of glutamatergic-independent, synchronous GABA-mediated potentials and 3) is mainly exerted on presynaptic receptors that are located on (recurrent) excitatory terminals. Moreover, the lack of effects during application of adenosine receptor antagonists suggests that during the first 2 to 3 postnatal wk endogenous concentrations of adenosine may be unable to control the generation of epileptiform activity, at least in the 4AP model.

A1 receptor activation and synchronous activity induced by 4AP. In line with previous in vivo and in vitro studies (see for review Dragunow, 1988), 2-Cl-adenosine, NECA and the A1 receptor agonist L-PIA were able to reduce and abolish interictal and ictal discharges, and in this model are due to non-NMDA receptor-activated mechanisms (Avoli et al., 1993, 1996; Fueta and Avoli, 1992). We have also provided evidence indicating that the effects induced by
these adenosine agonists are blocked by both caffeine and the A1 receptor antagonist DPCPX. Hence, as documented for several models of epileptiform discharge purinergic mechanism exert anticonvulsant effects in the juvenile rat hippocampus through a mechanism that relates to the A1 receptor.

With all purinergic drugs used in this study the interictal discharges were more sensitive than ictal events as demonstrated by the IC50 of the dose-response curves obtained for 2-Cl-adenosine and L-PIA. Similar effects are also observed with baclofen (Motalli et al., 1997) that like adenosine agonists acts via a presynaptic mechanism. By contrast, we have reported that several antiepileptic drugs that interfere with Na+-dependent, repetitive action potential firing display an action that is more evident on ictal discharges as compared with the interictal activity induced by 4AP in the juvenile hippocampus (Fueta and Avoli, 1992). This difference underscores the role of presynaptic versus postsynaptic mechanisms in modulating synchronous epileptiform discharges.

We have also observed that reduction and/or blockade of epileptiform activity by 2-Cl-adenosine and L-PIA is at times associated with an increased rate in occurrence of the GABA-mediated synchronous potentials disclosed by 4AP in the hippocampus. However, these effects were not seen when the purinergic compounds were tested on the GABA-mediated synchronous potentials recorded during concomitant application of CNQX and CPP. Therefore, we interpret the facilitatory effects as due to the reduction of epileptiform activity rather than to a direct action of adenosine on the GABA-mediated potential per se. Our conclusion is in line with several studies of the cellular mechanisms of adenosine which have shown that purinergic activation controls the synaptic release of glutamic acid from excitatory terminals, although it does not affect the release of GABA from inhibitory interneuron terminals (Lupica et al., 1992; Thompson et al., 1992; Yoon and Rothman 1991; Scanziani et al., 1992).

Fig. 5. Effects induced by the adenosine uptake inhibitor dipyridamole. A, A maximal concentration of dipyridamole blocks both ictal and interictal discharges, although increasing the rate of occurrence of the negative-going field potentials. These effects are antagonized by DPCPX (100 μM). Note that during DPCPX application interictal and ictal discharge increase in amplitude and duration respectively, although the negative-going field potentials are apparently abolished. B, Dose-response curves of the changes induced by dipyridamole on the rate of occurrence of interictal and ictal discharges, as well as of the isolated negative-going potentials.

Adenosine agonist concentrations in the 4AP model. The concentrations of purinergic agents required to influence 4AP-induced epileptiform activity in the juvenile rat hippocampus are larger than those used by several investigators who have analyzed the effects of A1 adenosine agonists on synaptic transmission and epileptiform discharges in the adult hippocampus (Dunwiddie, 1980; Dunwiddie and Fredholm, 1984; Lee et al., 1984; Ault and Wang, 1986). However, such a difference is not likely to reflect the immaturity of the adenosine A1 receptor whose density is already high in the CA3 area between postnatal days 15 and 25. Moreover these receptors are already functional since they are effectively coupled to G proteins (Daval et al., 1991).

An alternative explanation for the high concentrations of adenosine receptor agonists required to control 4AP-induced epileptiform activity may reside in the experimental procedures used in our study. In the majority of the experiments we used application times <25 min to avoid the appearance of spreading depression-like episodes, that can modify neuronal excitability and thus interfere with the effects induced by the tested drugs. However, analysis of the changes induced by prolonged applications of low L-PIA concentrations in the few slices where spreading depression-like episodes did not occur, indicates that even with this procedure con-
used in our study. In line with this conclusion, we have required to influence 4AP-induced epileptiform activity in the pattern of ictal activity.

The rate of occurrence of interictal events without altering epileptiform discharges, although 1

centrations <0.8 μM were unable to modify 4AP-induced epileptiform discharges, although 1 μM L-PIA only reduced the rate of occurrence of interictal events without altering the pattern of ictal activity.

The high concentrations of adenosine receptor agonists required to influence 4AP-induced epileptiform activity in the juvenile hippocampus may indeed relate to the model used in our study. In line with this conclusion, we have reported that L-PIA reduces the interictal discharges induced by 4AP in the adult hippocampus with an IC50 = 8.75 μM (Barbarosie et al., 1994). Previous studies have shown that 4AP reverses the inhibitory effects induced by adenosine on cell firing (Perkins and Stone, 1980) and also antagonizes the anticonvulsant effects of adenosine on epileptiform activity in the hippocampal slice (Schubert and Lee, 1986). Besides possible direct postsynaptic effects of 4AP on adenosine function, the larger doses of purinergic compounds required in our study may reflect the increase in transmitter release that is exerted by 4AP at both inhibitory and excitatory synaptic terminals in the hippocampus (Perreault and Avoli, 1989, 1991). Previous in vitro studies of the effects induced by purinergic compounds on epileptiform discharges were most often performed in models where GABA_A receptor agonists were used (Dunwiddie, 1980; Dunwiddie and Fredholm, 1984; Ault and Wang, 1986).

Endogenous adenosine and anticonvulsant effects. The ability of the adenosine uptake blocker dipyridamole to reduce both interictal and ictal discharges induced by 4AP indicates that increasing the brain level of endogenous adenosine may represent an effective anticonvulsant strategy. Moreover, as with 2-Ch-adenosine and L-PIA, dipyridamole effects had IC50 values that were larger for ictal discharges as compared to interictal activity and were mediated by activation of the A1 receptor. As with the adenosine receptor agonists the concentration of dipyridamole required to abolish epileptiform discharges induced by 4AP were larger than what reported in a previous study of epileptiform discharge induced by the GABA_A receptor antagonist bicuculline (Ault and Wang, 1986).

We have also shown that caffeine and the specific A1 receptor antagonists DPCPX do not alter the pattern of synchronous activity induced by 4AP. Adenosine receptor antagonists possess proconvulsant activity in the adult rat hippocampus (Ault et al., 1987; Alzheimer et al., 1989) suggesting that endogenous adenosine may contribute to terminate the epileptic burst. Our negative findings may reflect the low concentration of adenosine in the immature brain, which between postnatal day 10 and 21 is 3- to 6-fold lower than in 2-mo-old rats (Aranda et al., 1989; Sarda et al., 1989). A previous ontogenic study of adenosine functions has shown that caffeine often fails in affecting the field EPSP recorded from slices obtained from rats younger than 10 days (Psarropoulou et al., 1990).

Pre- vs. postsynaptic sites of action. Our study provides evidence indicating that the anticonvulsant effects exerted by purinergic agents on 4AP-induced epileptiform discharges rests mainly on a presynaptic site of action. Accordingly, L-PIA maintains its ability to decrease and eventually to abolish 4AP-induced epileptiform discharges recorded in barium-containing ACSF. Barium at the concentrations used in our study blocks the potassium-mediated hyperpolarization induced by adenosine (Gerber et al., 1989; Thompson et al., 1992) have reported that barium abolishes this postsynaptic, potassium conductance without influencing the ability of adenosine to inhibit isolated EPSPs.

We are therefore inclined to conclude that the effects induced by L-PIA as well as by the other purinergic compounds tested in this study are due to the ability of A1 receptor activation to decrease the release of glutamate from CA3 pyramidal neuron terminals. A previous study performed in the CA1 subfield of the adult rat hippocampus has shown that epileptiform discharges induced by tetroethylammonium and barium are still inhibited by adenosine (Birnstiel et al., 1992).

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