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Title: Adenosine Analogs Suppress Seizures Induced by Bicuculline Methiodide in the Rat Prepiriform Cortex

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Although considerable experimental evidence suggests that endogenous adenosine may function as a modulator of neuronal activity in the etiology or control of seizures, the neuroanatomical and neurochemical basis for the anticonvulsant actions of adenosine have not been well established. A discrete brain region of possible fundamental importance to epileptogenisis within the rat prepiriform cortex (PC) has been described (Piredda and Gale, 1985). The objectives of this study were to determine whether adenosine analogs exert anticonvulsant effects by an action at the seizure focus - a site within the PC, - elicited by a chemoconvulsant, bicuculline; and to pharmacologically characterize the adenosine receptor mediating the seizure suppressant effects in the rat prepiriform cortex.

Unilateral focal microinjection of 118 pmol bicuculline methiodide (BMI) into the PC elicited bilateral motor seizures and these seizures were potently inhibited by prior injection of the adenosine receptor agonist, 2-chloroadenosine (2-ClA). In addition,

this anticonvulsant action of 2-ClA at a dose of 41.4 pmol was completely prevented when co-administered with the specific adenosine receptor antagonist 8-(p-sulfophenyl)theophylline (8-pSPT). These results not only indicate that the PC is one potential site of adenosine modulation of seizure susceptibility, but also demonstrate that an activation of adenosine receptor is operative in the observed suppression of the behavioral seizures. Furthermore various adenosine analogs , N-ethylcarboxamidoadenosine (NECA), (-)N^6-(R-phenylisopropyl)adenosine (R-PIA), (+)N^6-(S-phenylisopropyl)adenosine (S-PIA), N^6-cyclopentyladenosine (CPA) and N^6-cyclohexyladenosine (CHA) were applied to the PC and produced a dose-dependent reduction in the severity of seizures evoked by BMI. The most potent analog tested was NECA with an ED_{50} value as low as 4.0 ± 3.0 picomoles. Other adenosine analogs were also potent antiepileptic agents and had ED_{50} values which ranged from 7.76 to 207.8 picomoles. The rank order potency was NECA $\geq$ CHA > CPA > R-PIA > 2-ClA >> S-PIA. R-PIA was found to be 10.2 times more potent than its S-diastereoisomer as an antiepileptic in the PC. Finally, the selective A_2 adenosine receptor agonist, 2-phenylaminoadenosine (CV-1808), was devoid of seizure suppressant activity when focally injected in the PC in a dose as high as 334.8 picomole. This rank order potency, degree of stereoselectivity for R-PIA vs S-PIA and lack of anticonvulsant effect of CV-1808 suggest that activation of A_1 adenosine receptors is the basis for protection afforded by focal injections of these analogs against seizures induced by bicuculline from a site within the rat prepiriform cortex.

Adenosine Analogs Suppress Seizures Induced by Bicuculline Methiodide in the Rat Prepiriform Cortex

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INTRODUCTION

The epilepsies are a family of neurological disorders that have in common a transient, recurrent, self-sustained interruption of normal brain function and a simultaneous hypersynchronous activation of a large population of neurons in one focal area or generally throughout the brain (Dichter et al., 1987). Over many years, advances in understanding central nervous system (CNS) function, especially in the context of contemporary concepts of cellular and synaptic processes, have provided progressively more sophisticated hypotheses to explain epilepsy. Epilepsy researchers have attempted to understand the altered physiology of individual neurons or synaptic function that underlies regional epileptiform activity, with the hope of developing pharmacological tools and therapeutic agents that could be used to suppress such abnormal activity. The transition to a seizure appears to be due to simultaneous increments in excitatory influences and decrements in inhibitory processes (Dichter et al., 1987). However, the specific anatomical and neurochemical substrates involved in epilepsies are not fully understood. A growing

body of evidence suggests that endogenous adenosine as an inhibitory neuromodulator plays important roles in the regulation of seizure activity and cell homeostasis via activation of specific membrane receptors (Dragunow, 1988). Although systemic injections of chemoconvulsant agents had been used to create experimental models of generalized epilepsy, the neuroanatomic site of epiletogenisis was unknown. Recently, one specific anatomic locus in the deep prepiriform cortex which appears to be involved in the development of generalized clonic seizures induced by local injection of bicuculline has been described (Piredda and Gale, 1985). Considered together, the prepiriform cortex, which is rich in adenosine receptors and appears to be involved in epileptogenisis, may play some role in adenosine's effects. My hypothesis underlying this study is that focal activation of adenosine receptors suppresses behavioral seizure activity evoked by a chemoconvulsant, bicuculline , from the rat prepiriform cortex.

I. Adenosine and Adenosine Receptors

Adenosine is an endogenous purine substance that subserves many modulatory functions in both the periphery and brain (Arch and Newsholme, 1978). In the periphery, adenosine exerts a wide range of effects, including (1) negative inotropic, chronotropic and dromotropic effects in the heart (Belardinelli et al., 1983; Belhassen et al., 1986); (2) vasodilation: its vasodilatory effects on numerous vascular beds may mediate postischemic hyperemia of the coronary blood vessels since adenosine levels dramatically increase

during hypoxemia with an associated increase of coronary blood flow (Berne et al., 1980); (3) inhibition of platelet aggregation: it is possible that the build up of adenosine following cardiac ischemia could thus influence blood clotting (Born et al., 1965) ; (4) inhibition of lipolysis (Snyder, 1985); (5) modulation of respiration (Eldridge et al., 1985; Watt et al., 1985); (6) modulation of the autonomic nervous system: adenosine inhibits norepinephrine release from sympathetic neurons as well as acetylcholine release at the neuromuscular junction and in ganglia (Fredholm and Hedqvist, 1980).

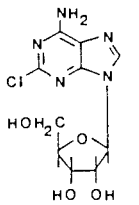
Adenosine as a neuromodulator exerts characteristic inhibitory actions on neuronal firing rates, synaptic transmission and neurotransmitter release in the central nervous system (CNS). Adenosine inhibits the spontaneous firing of most central neurons (Phillis et al., 1981). Adenosine and adenosine analogs also depress evoked field potential amplitudes in the rat olfactory cortex slice via A_1 receptors (Collins et al., 1985). Direct postsynaptic inhibition is represented by the adenosine agonist-induced decrease in the amplitude of the excitatory postsynaptic potential (EPSP) response in the CA_1 region of rat hippocampal (Dunwiddie et al., 1985). The predominant presynaptic effect is blockade of excitatory transmitter release. Acetylcholine release by guinea pig neocortex synaptosomes is inhibited by adenosine derivatives (Corrieri et al., 1981). Adenosine decreases aspartate and glutamate but not GABA release from the rat brain (Dolphin, et al., 1983; Corradetti et al., 1984), and adenosine antagonists enhance glutamate release from the rat cerebellar granule cells (Prestwich et al., 1987).

Autoradiographic evidence for adenosine receptor location on axon terminals of excitatory neurons has been reported (Goodman et al., 1983). Behaviorally, adenosine has marked depressant, sedative and hypnotic effects and decreases locomotor activity (Dunwiddie et al., 1982; Snyder et al., 1981; Radulovacke et al., 1984; Phillis, et al., 1986). Seizures are associated with large increases in adenosine levels, suggesting that adenosine may be involved in the modulation of seizure susceptibility after seizure onset (Dragunow, 1988). Thus considerable evidence suggests that endogenous adenosine is an important modulator involved in various physiological functions and pathological processes.

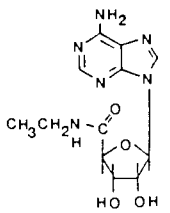
Adenosine depresses neuronal activity by an action at specific extracellular receptors that may be coupled to adenylate cyclase, and are antagonized by methylxanthines such as theophylline and caffeine (Phillis and Wu, 1981; Stone, 1981; Williams, 1984; Snyder, 1985). It is now well established that many tissues contain at least two classes of adenosine membrane receptors referred as A_1 and A_2 on the basis of effects on adenylate cyclase and structure-activity profiles of agonists (Hamprecht and VanCalker, 1985; Stone, 1985). At A_1 adenosine receptors the rank order of potency of analogs of adenosine <u>in vitro</u> is CPA $\geq$ R-PIA $\geq$ CHA > NECA > 2-ClA >> S-PIA >> CV-1808; the A_1 adenosine receptors are negatively coupled to adenylate cyclase. The structures of these adenosine analogs are shown in Fig.1. In contrast, at A_2 adenosine receptors the potency series for these agonists <u>in vitro</u> is NECA > CV-1808 $\geq$ 2-ClA > R-PIA > CHA $\geq$ CPA > S-PIA; the A_2 receptors are positively coupled to adenylate cyclase

Figure 1. Structures of adenosine and xanthine derivatives. These 8 compounds were used in my studies. 2-Chloroadenosine and 5'-N-ethylcarboxamidoadenosine are nonselective adenosine analogs in terms of A_1 and A_2 adenosine receptor subtypes. The diastereoisomers of N^6-(2-phenylisopropyl)adenosine, R-PIA and S-PIA have greater affinity for A_1 than A_2, and also A_1 receptor shows greater stereoselectivity for R-PIA than S-PIA. Two other N^6-substitutes, CPA and CHA ,are selective A_1 adenosine receptor agonists. 2-Phenylaminoadenosine (CV-1808) possesses selective adenosine receptor agonist properties. 8-(p-Sulfophenyl)theophylline, a xanthine derivative, is a specific adenosine receptor antagonist.

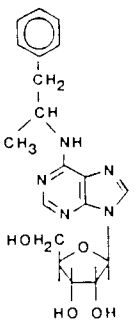
Figure 1.



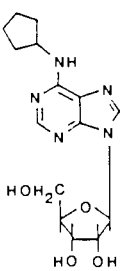
2-Chloroadenosine
(2-ClA)



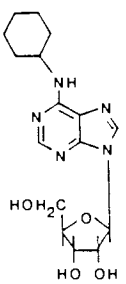
5'-N-Ethylcarboxamidoadenosine
(NECA)



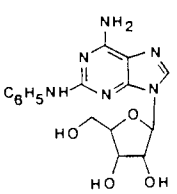
N^6-(2-Phenylisopropyl)adenosine, R(-) and S(+)-isomer
(R-PIA and S-PIA)



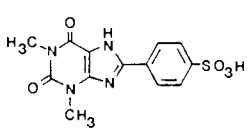
N^6-Cyclopentyladenosine
(CPA)



N^6-Cyclohexyladenosine
(CHA)



2-Phenylaminoadenosine
(CV-1808)



8-(p-Sulfophenyl)theophylline
(8-pSPT)

(Bruns et al., 1987; Daly et al.,1987). In addition, A_1 receptors are affected by nanomolar concentrations of adenosine, whereas A_2 receptors require micromolar levels. A_1 sites demonstrate marked stereoselectivity toward PIA, with the R-isomer being over ten times more potent, whereas much less stereoselectivity is apparent for A_2 receptors (Snyder, 1985; Daly et al., 1987).

II. Anticonvulsant Effects of Adenosine and Its Analogs

Adenosine and its analogs have anticonvulsant effects on a range of seizure models. An anticonvulsant action of adenosine was first reported by Maitre et al. (1974) who found that systemic injection of adenosine alone protected mice from audiogenic seizures. The adenosine agonist R-PIA, injected intraperitoneally (i.p.) protected mice from strychnine- and metrazol-induced seizures (Snyder et al., 1981) and systemic injection of mice and rats with the adenosine agonists R-PIA, CHA, and 2-ClA delayed the onset of seizures induced by a range of chemoconvulsants such as pentylenetetrazol (PTZ), strychnine, picrotoxin, kainic acid and 3-mercaptopropionic acid (Dunwiddie et al., 1982). It has also been observed that intravenously (i.v.) injected R-PIA, S-PIA, NECA, CHA and 2-ClA raised seizure threshold for PTZ, picrotoxin and bicuculline in a theophylline-reversible fashion in rats (Murray et al., 1985; Murray et al., 1986; Szot et al., 1987). Intracerebroventricular (i.c.v.) injections of adenosine analogs, R-PIA and NECA decreased both afterdischarges and behavioral convulsions in amygdala-kindled rats

and the anticonvulsant effect of NECA was antagonized by caffeine (Barraco et al., 1984). These results on kindled, audiogenic, and chemically-induced seizures indicate that adenosine and its analogs have powerful anticonvulsant effects after both systemic and intracerebroventricular adminstration.

These in vivo results are supported by the in vitro studies on epileptiform activity. Studies using penicillin and low-calcium induced spiking in hippocampal slices also suggested that adenosine inhibits seizure-like activity (Dunwiddie and Fredholm, 1984; Haas et al., 1984). In addition, it was demonstrated that adenosine, R-PIA and S-PIA also blocked epileptiform afterdischarges elicited by a low calcium, high magnesium medium in hippocampal slices (Lee et al., 1984). An increase in the levels of adenosine in the brain of rats after seizures induced by bicuculline has been demonstrated (Winn et al., 1980), suggesting that adenosine's anticonvulsant effects may be maximal after seizure onset.

The above evidence indicates that endogenous adenosine may function as a neuromodulator involved in the seizure mechanisms. However, peripheral effects of adenosine analogs complicate the interpretation of the anticonvulsant effects after systemic injections (e.g. respiratory depression, hypotension, hypothermia, sedation, muscle weakness). Adenosine analogs produced sedation and muscle weakness even after intracerebroventricular adminstration (Barraco et al., 1983), indicating that this route of injection does not overcome this problem. Therefore, the neuroanatomical and neurochemical basis for the anticonvulsant actions of adenosine are

not well understood. Recently, it was reported that injection of R-PIA and NECA at kindled foci blocked amygdala, hippocampal and caudate kindled seizures (Rosen et al., 1987), suggesting that activation of adenosine receptors at a seizure focus can inhibit seizure generation. Thus more definitive studies are clearly warranted to elucidate the mechanisms by which adenosine modulates seizure-initiating mechanisms in select brain regions.

III. An Epileptogenic Site in the Rat Prepiriform Cortex

A recently identified site sensitive to chemoconvulsants in the prepiriform cortex (Fig.2) has been described as a " crucial epileptogenic site " (Piredda and Gale, 1985). This is the first identification of a site in the forebrain from which generalized convulsions can be elicited by a focal injection of bicuculline in low doses. It was found that picomole amounts of bicuculline, kainic acid, or carbachol injected unilaterally into this area resulted in contralateral (followed by bilateral) clonus of forelimbs, rearing and falling, accompanied by epileptiform activity in EEG recordings from cortical and subcortical sites.

Previously, although certain brain regions such as hippocampus and amygdala are especially sensitive to the seizure-inducing actions of some epileptogenic agents as evidenced electrographically or metabolically (Ben-Ari et al., 1981; Evans et al., 1984; Lothman et al., 1981), a single injection of a low dose of a chemoconvulsant is placed directly into these and other brain regions have generally

Figure 2. The anatomical view of piriform cortex in the rat brain. The entire expanse of olfactory cortex bound rostrally by the anterior olfactory nucleus, caudally by the lateral entorhinal area, medially by the olfactory tubercle and cortical areas associated with the amygdala, and laterally by neocortex was termed the piriform cortex (Haberly et al., 1978).

A. Coronal section of the rat brain. Because this section is 2.7 mm anterior to bregma, piriform cortex shown here is prepiriform cortex.

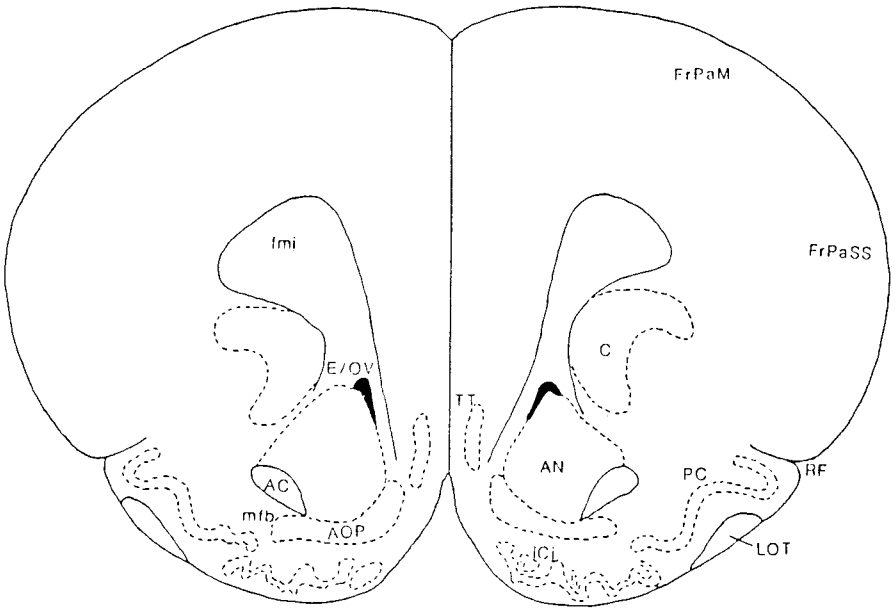
B. Reconstruction of the olfactory areas on a ventral veiw of the rat brain. The boundary between anterior piriform cortex (heavy stippling) and posterior piriform cortex (light stippling) is indicated by the dashed line.

Abbreviations:

AC	anterior commissure
AN	accumbens nucleus
AON	anterior olfactory nucleus
AOP	anterior olfactory nucleus, post
C	claustrum
COa	anterior cortical nucleus of the amygdala
COp	posterior cortical nucleus of the amygdala
fmi	forceps minor corpus callosum
FrPaM	frontoparietal cortex, motor area
FrPaSS	frontoparietal cortex, somatosensory area
ICj	islands of Calleja
LEA	lateral entorhinal area
LOT	lateral olfactory tract
M	medial nucleus of amygdala
MEA	medial entorhinal area
mfb	median forebrain bundle
NLOT	nucleus of the lateral olfactory tract
OB	olfactory bulb
OT	olfactory tubercle
OV	olfactory ventricle
PAC	periamygdaloid cortex
PC	piriform cortex
PCa	anterior piriform cortex
PCp	posterior piriform cortex
RF	rhinal fissure
S	subiculum
TT	taenia tecta
TTv	ventral taenia tecta

Figure 2.

A.


FrPaM
FrPaSS
fmi
E/OV
C
TT
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PC
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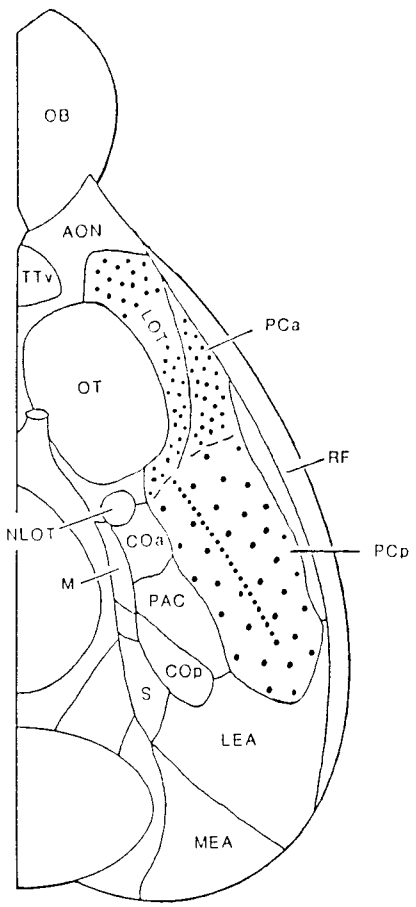
B.


OB
AON
TTv
LOT
PCa
OT
RF
NLOT
COa
PCp
M
PAC
COp
S
LEA
MEA

failed to elicit bilateral motor seizures. According to Gale's studies (1985), the most consistently effective site in the rat prepiriform cortex (PC) for obtaining motor seizures, corresponds to the following stereotaxic coordinates when incisor bar is 5.0 mm above the interaural line: 4.0 mm anterior to bregma, 3.0 mm lateral to midline and 6.5 mm below dura. Intracerebral injection of relatively high doses (20-40 times higher than the doses Gale used in the PC) of bicuculline or carbachol to hippocampus and amygdala do not produce clinical seizure activity. Intracerebral administration of nanomole amounts of kainic acid (30-40 times higher than the doses administered in the PC) can elicit strong and long-lasting bilateral motor seizures, but this effect is not anatomically selective, as it can be evoked from many injection sites in the forebrain. In contrast, it was found that after infusion of bicuculline, carbachol or kainic acid 1.0-2.0 mm away from the " specific " site described above, in most cases, no behavioral seizure activity was observed (Piredda et al., 1985). The authors concluded that this site in the forebrain was distinct from other sites in three important ways: (1) it was considerably more sensitive; (2) it did not require repeated stimulation in order to elicit bilateral motor seizures; and (3) it was responsive to chemoconvulsants with a variety of mechanisms of action: including manipulation of GABAergic, cholinergic and excitatory amino acid receptors.

Subsequent studies by Gale and coworkers provided the hypothetical neurochemical circuity underlying the development of seizures from the prepiriform cortex, an output neuron of the

circuity which is driven negatively by GABA and positively by excitatory amino acids (Gale et al., 1986). It is apparent that the prepiriform cortex is a crucial site in chemically-induced epileptogenisis. The site described by Gale et al. may represent the neuroanatomic substrate for the anticonvulsant effect of adenosine analogs.

The goals of this study were (1) to determine whether the rat prepiriform cortex is a neuroanatomical substrate for the suppressant effects of adenosine receptor agonists on bicuculline induced seizures; and (2) to pharmacologically characterize the adenosine receptors mediating the seizure suppressant effects in this site.

MATERIALS AND METHODS

I. Animals

Male Sprague-Dawley rats, weighing 250-400g at the time of surgery, were used in these experiments. Animals were housed in groups of 4-5 upon delivery and maintained with food and water for at least one month prior to use. Animals were kept at 22 ± 1°C on a standard 12 hour light/dark schedule.

II. Stereotaxic Surgery

Under Equithesin anaesthesia (Equithesin 2.7 ml/kg, i.p.) rats were stereotaxically implanted with a single stainless-steel 22 gauge guide cannula (16.8 mm length) and 28 gauge injection cannula which extended at least 1 mm below the tip of the guide cannula (Plastic Products) into a location within the right prepiriform cortex. The stereotaxic coordinates for the site in the prepiriform cortex with the incisor bar 5 mm above the interaural line were: 3.5-4.0 mm anterior to bregma, 3.5 mm lateral to midline and 6.5 mm below dura. The guide cannulae were held in place with Fastcure dental repair material (Kerr) which was anchored to one small screw inserted through the skull. Finally, the injection cannula was taken out and a fine wire stilette was inserted into the guide cannula to prevent clogging.

III. Drugs and Drug Administration

The drugs used in this study were : (-)-bicuculline methiodide (BMI), 8-(p-sulfophenyl)theophylline (8-pSPT), N^6-cyclopentyladenosine (CPA) and 2-phenylaminoadenosine (CV-1808) purchased from Research Biochemicals Inc.(Wayland, MA); N-ethylcarboxamidoadenosine (NECA), (-)N^6-(R-phenyl-isopropyl)-adenosine (R-PIA) and (+)-N^6-(S-phenyl-isopropyl)-adenosine (S-PIA) were purchased from Boehringer Mannheim (Mannheim, West Germany); N^6-cyclohexyladenosine (CHA) was obtained from CALBIOCHEM-BEHRING; 2-chloroadenosine (2-ClA) was purchased from Sigma Chemicals (St.Louis, MO).

All drugs were dissolved in normal saline at the desired concentrations except R-PIA and CV-1808. R-PIA was prepared by dissolving 2 mg in 200 μl of 0.1 N HCL and 400 μl of normal saline to achieve complete solubilization, then adding 600 μl of 40 mM Na_2HPO_4 buffer, final dilutions were made with normal saline to reach desired concentrations. CV-1808 was prepared by dissolving 1 mg into 60 μl of of 0.1 N HCL and 400 μl of normal saline, then adding 80 μl of 40 mM $NaHCO_3$ buffer, final dilution was made with 460 μl normal saline to 1 mg/ml concentration. In addition, NECA, 8pSPT, R-PIA and CV-1808 required gentle heating and sonication to achieve complete solubilization.

Intracerebral microinjections were performed using a modification of the method described by Piredda and Gale (1985). The

28 gauge injection cannula was connected with a PE 20 tubing to a Hamilton microsyringe (1 μl) mounted in a Harvard infusion pump. The solutions of all drugs were injected at a constant rate of 0.9 nl/sec in a volume of 120 nl over a 2 min and 7.2 sec period via injection cannulae which extended at least 1 mm below the tip of the guide cannulae into the defined site in the right prepiriform cortex of each rat. The injection cannula was left in place for 1 min after the termination of injections. Drugs were not administered more than once a day to each rat. Rats were tested a maximum of 2-6 times.

IV. Behavioral Assay

After drug administration animals were placed in a 40 x 40 x 30 cm plexiglass testing chamber for observation of seizure activity. The observation period for seizure activity induced by bicuculline methiodide (BMI) was 30 min from the beginning of the infusion of BMI. During the observation period, every sign and the latency of seizure activity were recorded in each animal and the highest seizure score was used in data analysis.

According to preliminary studies, 118 pmol BMI unilaterally injected into the site within the prepiriform cortex produced stereotyped bilateral motor seizures (Fig.3). The latency of the seizures was from 3 min to 15 min in most animals. In most cases, the seizures began with mouth and jaw clonus and myoclonic jerks which rapidly progressed to bilateral clonic movements of the forelimb, finally with rearing and falling occurring (stage 4 and 5)

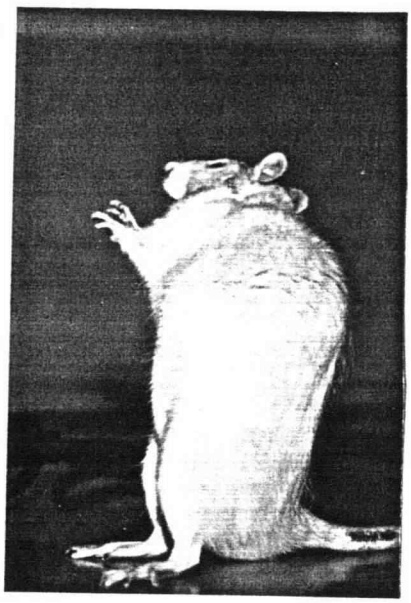
Figure 3. Representative example of bilateral motor seizures in the rat resulting from unilateral microinjection of bicuculline (118 pmol) in the prepiriform cortex. A. forelimb clonus with jaw clonus; B. start to rearing with severe forelimb clonus; C. rearing; D. falling.

A.

B.

C.

D.



within 8 - 30 seconds. In some animals, the seizures started with myoclonic jerks which progressed to clonic movement of the forelimb contralateral to the injected side and repeated several times, then progressed to rearing and falling in 10 - 15 minutes. In most rats, more than one seizure was observed during the 30 min following the infusion of bicuculline and seizures recurred continually for less than 15 min, but occasionally lasted for more than 30 min.

Severity of seizure activity induced by bicuculline methiodide was quantified by assignment of a score ranging from 0-5 on a scale of increasing seizure activity: 0, no seizure; 1, myoclonic jerks of the contralateral forelimb; 2, mild forelimb clonus (with/without mouth and facial movements - clonus of jaw and vibrissae and head nodding) lasting at least 5 sec; 3, severe forelimb clonus lasting more than 15 sec; 4, rearing in addition to forelimb clonus; 5, loss of balance and/or falling in addition to rearing and forelimb clonus.

V. Experimental Protocol

Animals were allowed a minimum of 24 hour recovery from surgery before any drug testing. All experiments were carried out during 12 hour light cycle.

A. Control

All rats were first challenged by a dose of 118 pmol bicuculline methiodide (BMI) that was microinjected into the prepiriform cortex (PC). Only those rats with seizure scores of 4 and 5 were used for

subsequent studies and each rat served as its own control.

B. Anticonvulsant Evaluation

Some animals were pretreated with various doses of adenosine analogs that were injected into the PC for 15 min prior to the administration of a challenging dose of BMI. If any sign of seizure behavior was not observed or seizure score was less than control response in animals following BMI challenge by any adenosine analog treatment, anticonvulsant effects were confirmed by elicitation of a control level response to BMI 24 hour later. Otherwise if animals post-tested with BMI did not remain the same magnitude of sensitivity to BMI, those animals were excluded from data analysis.

C. Coadministration

In some animals the adenosine analog 2-ClA at a dose of 41.4 pmol was coadministered with the adenosine receptor antagonist 8-(p-sulfophenyl)theophylline (8pSPT) at a dose of 1.61 nmol in a volume of 120 nl into the PC 15 min prior to injection of a challenging dose of BMI.

VI. Statistical Analysis

Ranking of behavioral seizures was analyzed using a Rank Sum test (Devore et al., 1986) which is a nonparametric test for the difference of 2 population means.

ED_{50} values for anticonvulsant effect of adenosine analogs were

estimated by a four-parameter logistic equation using the iterative public procedure FITFUN on the PROPHET computer system.

RESULTS

I. Anticonvulsant Potential of Adenosine Agonist in the Rat Prepiriform Cortex (PC)

First to determine whether the rat prepiriform cortex is an neuroanatomical substrate for the seizure suppressant effects of a adenosine agonist , 2-chloroadenosine (2-ClA), an agonist which acts at both A_1 and A_2 adenosine receptors (Daly et al., 1987) was applied to prevent seizures induced by bicuculline methiodide in the rat prepiriform cortex. Doses of 2-ClA at 20.7 pmol, 41.4 pmol, 82.9 pmol, 166 pmol and 1 nmol/rat were focally injected into the PC 15 min prior to BMI administration. The effect of 2-ClA on behavioral seizure stage is summarized in Table 1. 2-CLA significantly reduced the seizure stages at 41.4 pmol and 82.9 pmol ($P < 0.05$) compared to control responses. It was also shown that 2-ClA decreased mean seizure scores in dose-dependent manner and completely protected at doses greater than 82.9 pmol against seizures induced by BMI in the rat PC. This protection by 2-ClA was also quantified as the percent reduction in the mean seizure score as compared to the mean control response (n=2-6). The range of 2-ClA doses provided 10.6 % to 100 % protective effect on behavioral seizures induced by BMI in the PC. Saline did not have any protective effect on seizure activity because its mean seizure score (n=4) was the same as control response (data not shown).

Table 1. Protection by 2-Chloroadenosine (2-ClA) Against Bicuculline Methiodide-Induced Seizures in the Rat Prepiriform Cortex (PC)

Treatment	Distribution of Seizure Scores[a]						Mean Seizure Score (n)[b]	% Protection[c]
	0	1	2	3	4	5		
Control					1	1	4.5	
1 nmol 2-ClA	2						0.0 (2)	100
Control					1	1	4.5	
166 pmol 2-ClA	2						0.0 (2)	100
Control					2	1	4.3	
82.9 pmol 2-ClA	3						0.0 *(3)	100
Control					3	2	4.4	
41.4 pmol 2-ClA	4					1	1.0 *(5)	77.3
Control					2	4	4.7	
20.7 pmol 2-ClA	1					5	4.2 (6)	10.6

a Below each seizure score, the number of rats reaching that score are shown.

b n indicates number of animals in each group.

c Percent protection is given as the percent reduction of mean seizure score from mean control response for groups of animals receiving doses of 2-ClA.

* Statistically significant difference from control response ($P < 0.05$, one tail Rank Sum test).

II. Antagonism of Protection by an Adenosine Agonist Against Seizures Elicited by BMI in the PC

In order to demonstrate that 2-ClA was exerting its anticonvulsant behavioral effect by activation of adenosine receptors, the specific adenosine receptor antagonist 8-(p-sulfophenyl)theophylline (8-pSPT) was used in this experiment. As shown in Tab.1, 2-ClA at a dose of 41.4 pmol significantly reduced the mean seizure score from 4.25 to 1.13 ($P < 0.05$) and provided 77.4 % reduction of seizure severity against BMI-induced seizures in the prepiriform cortex. When 8pSPT was coadministratered with 2-ClA, the mean seizure score was not significantly different from that of control. Also 8pSPT pretreated alone did not have any effect on seizure activity induced by BMI (Table 2). However, 8pSPT pretreatment decreased the latency and increased the recurrence of seizures induced by BMI (data not shown). It was clear that the anticonvulsant effect of 2-ClA at a dose of 41.4 pmol was completely prevented when coinjected with the specific adenosine receptor antagonist 8pSPT at a dose of 1.61 nmol (Fig.4).

III. Pharmacological Characterization of Adenosine Receptor Mediating Suppressant Effect in the PC

The dose response relationships for a series of adenosine analogs including NECA, R-PIA, S-PIA, CPA and CHA which possess high

Table 2. Antagonism by 8-(p-Sulfophenyl)theophylline (8pSPT) of 2-Chloroadenosine (2-ClA) Protection Against Bicuculline Methiodide-Induced Seizures in the Rat Prepiriform Cortex

Treatment	Distribution of Seizure Scores						Mean Seizure Score (n)[a]
	0	1	2	3	4	5	
Control					6	2	4.25
41.4 pmol 2-ClA	6				1	1	1.13** (8)
Control					3		4.0
41.4 pmol 2-ClA + 1.6 nmol 8pSPT						3	5.0 (3)
Control					1	3	4.75
1.6 nmol 8pSPT						4	5.0 (4)

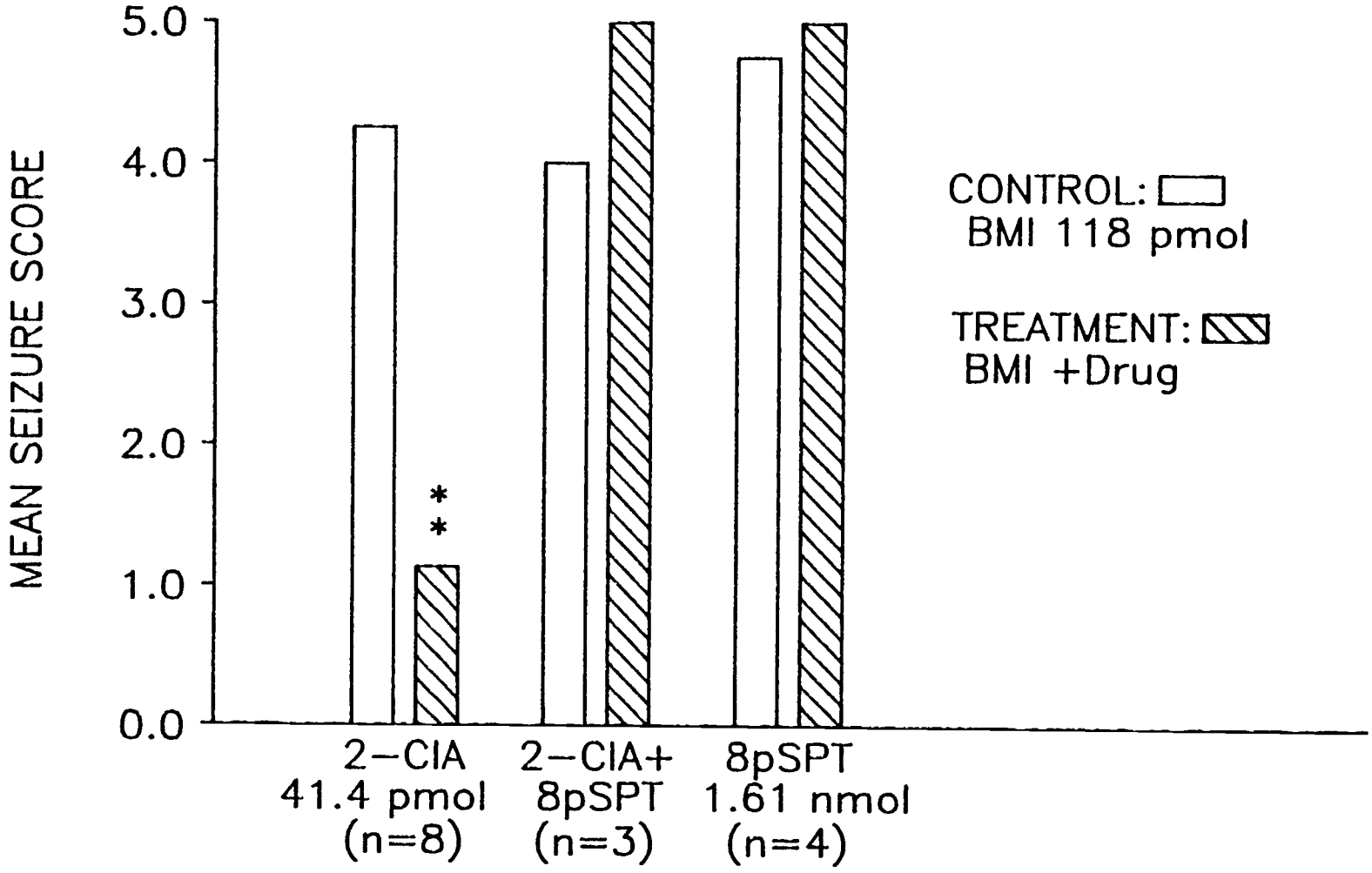
a n indicates number of animals in each group.

b Values of mean seizure scores are the means for each group of animals of the highest seizure observed in individuals during a 1 hour epoch following microinjection of bicuculline methiodide (118 pmol) into prepiriform cortex alone or 15 min after pretreatment with either 2-ClA (41.4 pmol), 8pSPT (1.6 nmol) or 2-ClA (41.pmol) + 8pSPT (1.6 nmol). In each instance experimental treatments followed control injections by no less than 24 h.

** Significantly different from the control ($P < 0.01$, one tailed Rank Sum test).

Figure 4. Antagonism by 8-(p-sulfophenyl)theophylline (8pSPT) of protection by 2-chloroadenosine (2-ClA) against seizures induced by bicuculline methiodide (BMI) in the rat prepiriform cortex. * At a dose of 41.4 pmol 2-ClA, which protects 77.4 % against BMI seizures in the prepiriform cortex ($P < 0.01$, one tailed Runk sum test), the anticonvulsant effect was completely antagonized by co-adminstration of 8pSPT which alone was not different from control. Other details are as described in the legend of Tab.1.

Figure 4.


MEAN SEIZURE SCORE
5.0
4.0
3.0
2.0
1.0
0.0
**
CONTROL:
BMI 118 pmol
TREATMENT:
BMI +Drug
2-CIA
41.4 pmol
(n=8)
2-CIA+
8pSPT
(n=3)
8pSPT
1.61 nmol
(n=4)

affinity for A_1 adenosine receptors were determined. NECA, like 2-ClA, when administered in the rat prepiriform cortex, provided a potent dose-dependent protection against the BMI-induced seizures. At doses of 6.5 pmol and 16 pmol NECA significantly reduced the mean seizure scores ($P < 0.01$, $P < 0.05$, respectively). This range of doses of NECA (1.6 pmol - 6.5 pmol/rat) afforded 10.5 % to 100 % reduction in seizure severity. Doses greater than 1.6 pmol of NECA completely prevented seizures elicited by BMI in the PC (Table 3).

R-PIA also has very potent anticonvulsant effect on seizure activity induced by BMI in the PC of rat. As shown in Table 4, R-PIA (14, 28, 56 and 112 pmol/rat) intracerebrally administered into the PC produced significant inhibition of BMI-induced seizures in dose-dependent manner (28, 56 and 112 pmol). The range of R-PIA doses used afforded 0 % - 84.5 % suppressant effect on behavioral seizures induced by BMI. S-PIA, a diastereoisomer of R-PIA, was less potent than R-PIA. Doses of S-PIA greater than 104 pmol were required to depress seizure activity evoked by BMI in the PC (Table 5). Doses of 104 pmol, 208 pmol and 311 pmol S-PIA provided 0 %, 42.1 % and 100 % protection, respectively.

The adenosine analog, N^6-cyclopentyladenosine (CPA) which has very high affinity and specificity for A_1 adenosine receptors was employed to inhibit behavioral seizures in the PC. The doses of CPA used produced a very potent dose-related decreases in mean seizure scores and provided 0 % - 100 % protection against BMI-induced seizure activity (Table 6). N^6-cyclohexyladenosine (CHA) like CPA is a very selective A_1 agonist, also was a very potent anticonvulsant

Table 3. Anticonvulsant Effect of N-Ethylcarboxamidoadenosine (NECA) Against Bicuculline Methiodide-Induced Seizures in the Rat Prepiriform Cortex

Treatment	Distribution of Seizure Scores						Mean Seizure Score (n)	% Protection
	0	1	2	3	4	5		
Control						1	5.0	
162 pmol NECA	1						0.0 (1)	100
Control					1	1	4.5	
81 pmol NECA	2						0.0 (2)	100
Control					1	2	4.67	
16 pmol NECA	3						0.0 *(3)	100
Control					2	2	4.5	
6.5 pmol NECA	4						0.0**(4)	100
Control					1	3	4.75	
3.2 pmol NECA				1	1	2	4.25 (4)	10.5
Control					1	3	4.75	
1.6 pmol NECA	1					3	3.75 (4)	21.1

Statistically significant differences between control and treated responses were assessed by one tailed Rank Sum test : * $P < 0.05$ ** $P < 0.01$.

Other details are as described in legend of Table 1.

Table 4. Anticonvulsant Effect of R(-)-Phenylisopropyladenosine (R-PIA) Against Bicuculline Methiodide-Induced Seizures in the Rat Prepiriform Cortex

Treatment	Distribution of Seizure Scores						Mean Seizure Score (n)	% Protection
	0	1	2	3	4	5		
Control						5	5.0	
112 pmol R-PIA	4				1		0.8** (5)	84.5
Control					4	1	4.2	
56 pmol R-PIA	4				1		0.8 * (5)	80.9
Control					2	2	4.5	
28 pmol R-PIA	2	1			1		1.25* (4)	72.4
Control					3	1	4.25	
14 pmol R-PIA	1				2	1	3.25 (4)	23.5
Control					2	2	4.5	
2.8 pmol R-PIA					2	2	4.5 (4)	0.0

Statistically differences between control and treated responses were assessed by a one tailed Rank Sum test: * $P < 0.05$ ** $P < 0.01$.

Other details are as described in the legend of Table 1.

Table 5. Anticonvulsant Effect of S(+)-Phenylisopropyladenosine (S-PIA) Against Bicuculline Methiodide-Induced Seizures in the Rat Prepiriform Cortex

Treatment	Distribution of Seizure Scores						Mean Seizure Score (n)	% Protection
	0	1	2	3	4	5		
Control					2	2	4.5	
311 pmol S-PIA	4						0.0** (4)	100
Control					1	3	4.75	
208 Pmol S-PIA	1	1				2	2.75 (4)	42.1
Control					3	1	4.25	
104 pmol S-PIA					3	1	4.25 (4)	0.0
Control					1	1	4.5	
26 pmol S-PIA					1	1	4.5 (2)	0.0

** Statistically significant difference from control response ($P < 0.01$, one tailed Rank Sum test).

Other details are as described in the legend of Table 1.

Table 6. Anticonvulsant Effect of N^6-Cyclopentyladenosine (CPA) Against Bicuculline Methiodide-Induced Seizures in the Rat Prepiriform Cortex

Treatment	Distribution of Seizure Scores						Mean Seizure Score (n)	% Protection
	0	1	2	3	4	5		
Control					1	4	4.8	
29.8 pmol CPA	5						0.0** (5)	100
Control						4	5.0	
22.4 pmol CPA	3				1		1.0** (4)	80.0
Control					1	6	4.86	
14.9 pmol CPA	1	1			1	4	3.75 (7)	26.5
Control					2	2	4.5	
2.98 pmol CPA					2	2	4.5 (4)	0.0

** CPA treatment differs from control by $P < 0.01$ (one tailed Rank Sum test).

Other details are described in the legend of Table 1.

agent (Table 7). The doses of 2.9 pmol, 14.3 pmol,57.2 pmol and 229 pmol CHA applied to the PC produced 4.1 % to 100 % protection and dose greater than 57.2 pmol/rat fully prevented seizures elicited by BMI.

In contrast to these results obtained with adenosine analogs which possess a high affinity for A_1 adenosine receptors, the selective A_2 adenosine receptor agonist, CV-1808(2-phenylaminoadenosine), was devoid of seizure suppressant effect when focally injected in the PC in a dose as high as 334.8 picomoles (Table 8).

The dose-response curves for a series of adenosine analogs effect on seizure activity (as percent reduction of BMI control) are shown in Fig.5 and Fig.6, and the calculated picomole doses required to suppress seizure severity by fifty percent (ED_{50}) are given in Table 9. These results indicated that all adenosine analogs used except CV-1808 produced highly significant dose-dependent decreases in behavioral seizure activity. NECA was the most potent analog tested, and as little as 4.0 pmol/rat of NECA was sufficient to reduce seizure activity by fifty percent. Many of the N^6-substituted analogs were also comparatively potent in suppression of seizure activity and had ED_{50} values which ranged from 7.76 to 207.8 picomoles. The ED_{50} values derived from analysis of the dose-response data indicated that the rank order potency was NECA (4.0 ± 3.0 pmol) $\geq$ CHA (7.76 ± 0.07 pmol) > CPA (17.0 ± 0.8 pmol) > R-PIA (20.3 ± 7.0 pmol) > 2-ClA (32.2 ± 0.3 pmol) >> S-PIA (207.8 ± 0.1 pmol) >> CV-1808 (>334.8 pmol) (Table 9). R-PIA was approximately 10 times more

Table 7. Anticonvulsant Effect of N^6-Cyclohexyladenosine (CHA) Against Bicuculline Methiodide-Induced Seizures in the Rat Prepiriform Cortex

Treatment	Distribution of Seizure Scores						Mean Seizure Score (n)	% Protection
	0	1	2	3	4	5		
Control					1	3	4.75	
229 pmol CHA	4						0.0** (4)	100
Control					1	3	4.75	
57.2 pmol CHA	4						0.0** (4)	100
Control						5	5.0	
14.3 pmol CHA	3	2					0.4** (5)	92
Control					1	4	4.8	
2.9 pmol CHA					2	3	4.6 (5)	4.1

** $P < 0.01$, statistically significant difference between control and treated responses as assessed by a one tailed Rank Sum test.

Other details are as described in the legend of Table 1.

Table 8. Effect of 2-Phenylaminoadenosine (CV-1808) on Seizure Activity Induced by Bicuculline Methiodide in the Prepiriform Cortex of Rat

Treatment	Distribution of Seizure Scores						Mean Seizure Score	(n)
	0	1	2	3	4	5		
Control						4	5.0	
334.8 pmol CV-1808					1	3	4.75	(4)

Figure 5. The suppressant effect of four adenosine analogs on seizure activity induced by bicuculline methiodide (BMI) in the rat prepiriform cortex. (●——●) N-ethylcarboxamidoadenosine (NECA); (□——□) N^6-cyclohexyladenosine (CHA); (▲——▲) N^6-cyclopentyladenosine (CPA); (○——○) 2-chloroadenosine (2-ClA). Adenosine analogs were focally microinjected 15 min prior to challenge of bicuculline. Values are expressed as percent reduction of mean seizure score of control response receiving BMI in the rat prepiriform cortex.

Figure 5.

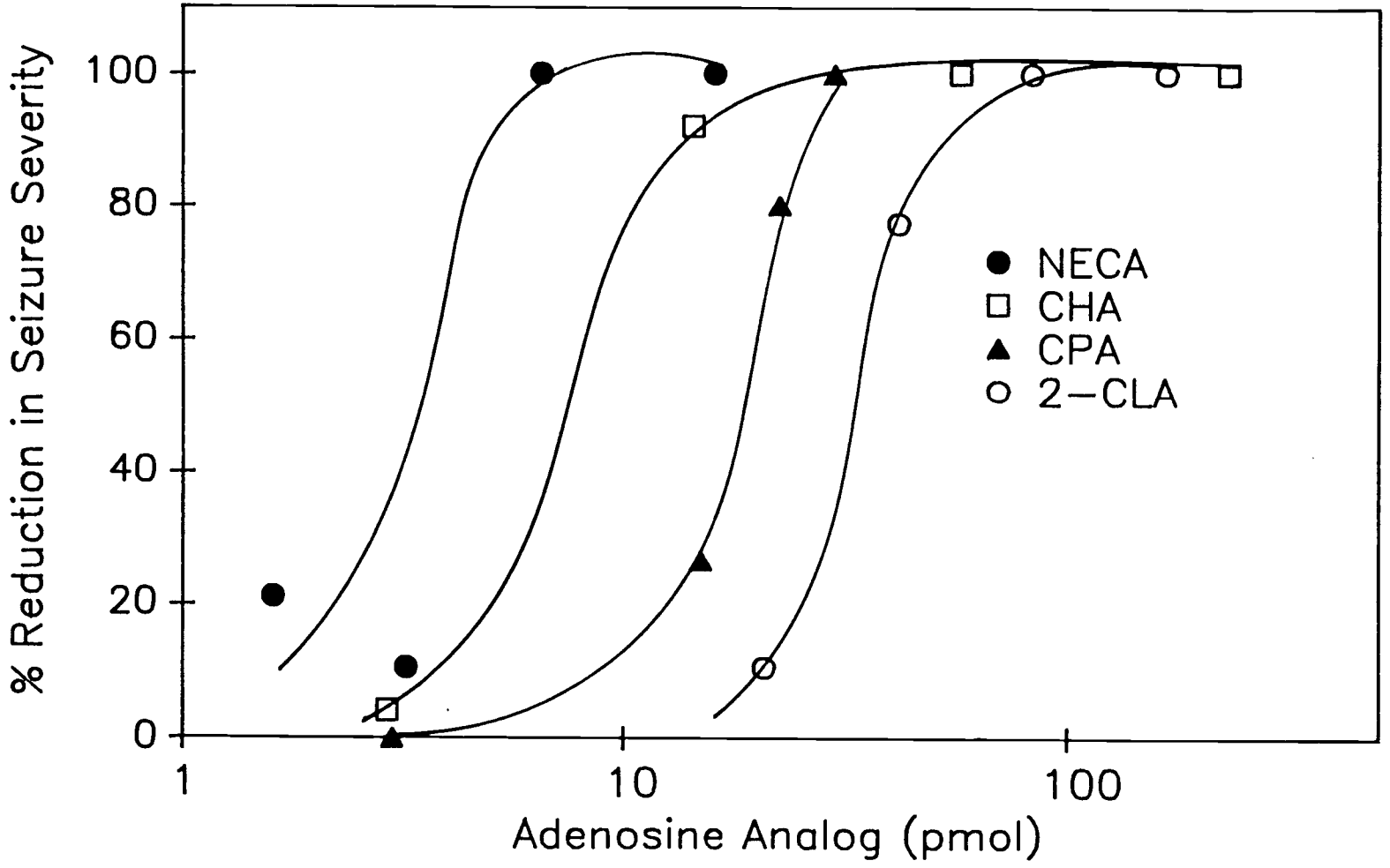

% Reduction in Seizure Severity
100
80
60
40
20
0
1
10
100
Adenosine Analog (pmol)
NECA
CHA
CPA
2—CLA

Figure 6. Anticonvulsant effect of R(-) and S(+)-phenylisopropyladenosine (R-PIA and S-PIA) against bicuculline methiodide (BMI) evoked seizures in the rat prepiriform cortex. Values are expressed as percent reduction in seizure severity of control (mean seizure score) receiving BMI.

Figure 6.

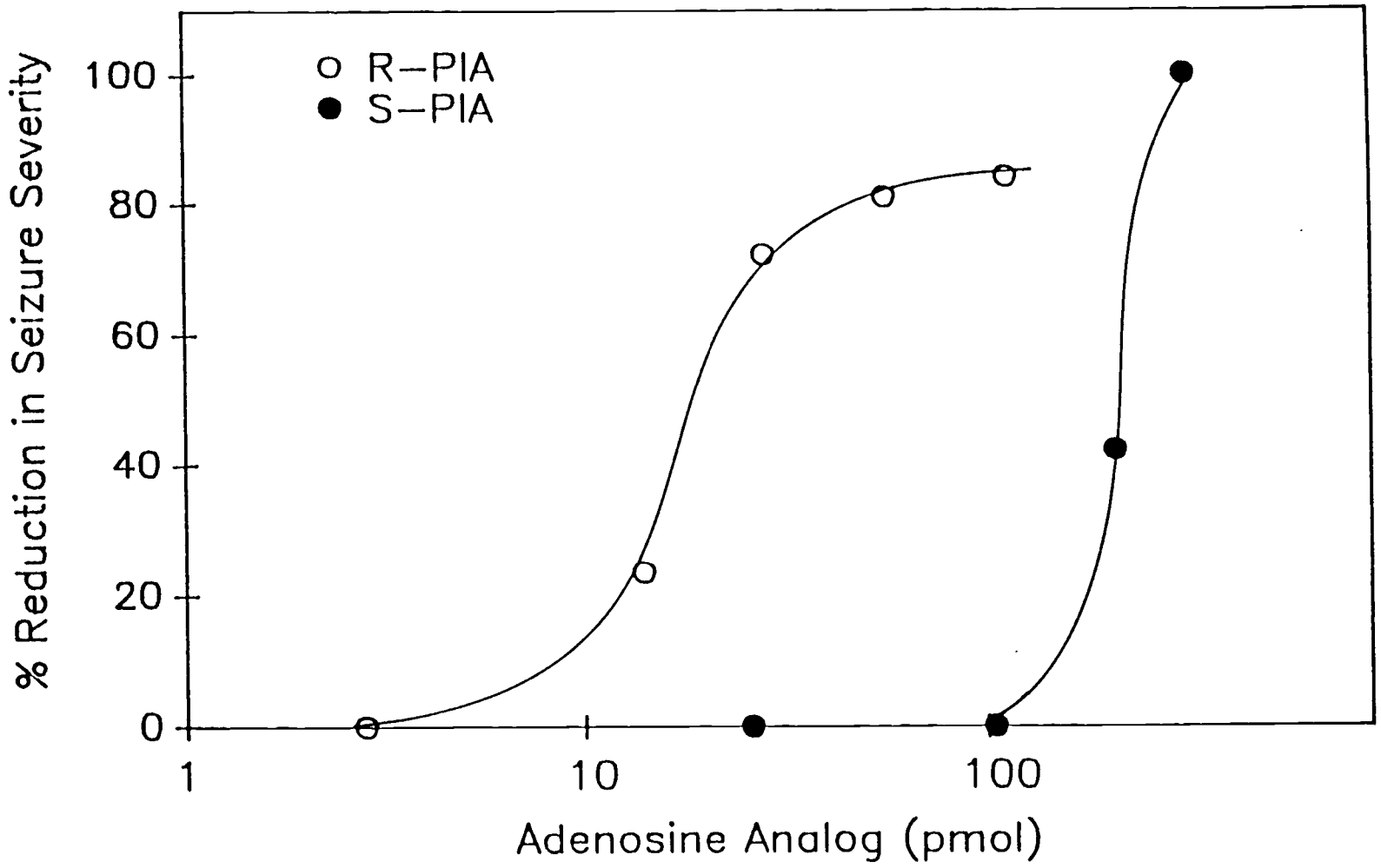

% Reduction in Seizure Severity
100
80
60
40
20
0
R-PIA
S-PIA
1
10
100
Adenosine Analog (pmol)

Table 9. Potency of Adenosine Analogs in Protecting Against Bicuculline Methiodide-Induced Seizures in the Rat Prepiriform Cortex

Compound	ED_{50} ± S.D. (pmol)
NECA	4.0 ± 3.0
CHA	7.76 ± 0.07
CPA	17.9 ± 0.8
R-PIA	20.3 ± 7.0
2-ClA	32.2 ± 0.3
S-PIA	207.8 ± 0.1
CV-1808	> 335.0

This table presents the doses of intracerebrally administered adenosine analogs required to elicit a 50 % protection against BMI-induced behavioral seizure activity in the prepiriform cortex. S.D. indicates standard deviation. Values in this table were calculated from data in Tab.1, and Tab.3 - 8.

potent than its S-diastereoisomer as an anticonvulsant agent in the PC. This rank order potency, stereoselectivity for R-PIA and S-PIA and lack of effect of CV-1808 argues against the involvement of the A_2 adenosine receptor subtype in the adenosine analog-produced protection against seizures induced by bicuculline in the PC.

DISCUSSION

The results of this study provide the first demonstration that the adenosine analogs have extremely potent anticonvulsant properties when preinjected focally to block seizures induced by administration of bicuculline methiodide into the prepiriform cortex. All adenosine analogs tested except CV-1808 provided a powerful consistent dose-dependent protection against BMI-induced seizures (Fig.5 and Fig.6).

The first adenosine analog tested, 2-chloroadenosine (2-ClA), provided very potent anticonvulsant action by focal injection against seizures initiated by bicuculline with ED_{50} value as low as 32.2 picomole. For comparison, 2-ClA was over 10,000 times less potent by i.p. administration in mice (Dunwiddie et al., 1982) and more than 2000 times less potent by i.v. injection (Murray et al., 1985) in rats against PTZ induced seizures. In addition, the potent suppressant effect of 2-ClA in the prepiriform cortex was fully prevented by coadministration of a xanthine derivative, 8-(p-sulfophenyl)theophilline (1.61 nmole/rat) which is a selective adenosine receptor antagonist (Bruns et al., 1986; Daly et al., 1987). This result was also consistent with previous investigations that the anticonvulsant effect on PTZ seizures by i.v. injection of 2-ClA was reversed by i.p. pretreatment of theophylline (Murray et al., 1985) and the anticonvulsant effect of NECA against amygdala-kindled seizures was antagonized by caffeine (Barraco et al., 1984). The present data not only indicate that the prepiriform cortex is one

potential site of adenosine modulation of seizure susceptibility, but also demonstrate that this anticonvulsant response is an adenosine receptor-mediated phenomena.

NECA was the most potent adenosine analogs with ED_{50} value 4.0 pmol/rat as an antiepileptic in the PC tested in these studies. Previous observations revealed that i.v. injection of NECA was over 10,000 times less potent in the PTZ seizure model (Murray et al., 1985) and even i.c.v. administration of this compound was approximately 80 times less potent in a kindling model (Barraco et al., 1984) compared to focal microinjection in the PC. The difference in potency might be partially due to elimination of potential problems associated with systemic injection (i.e. side effects and limitation of diffusion of drugs into CNS). Preliminary quantitative autoradiographic data from our lab using [^{3}H]NECA focally injected into the PC demonstrated that NECA was localized around the injected site about 1.5 mm rostrally or caudally, and ventrally or dorsally (Franklin et al., in preparation), suggesting further that the protective effects of this adenosine analog observed in this study were due to a direct action of this compound in a site in the PC. However, because 2-ClA and NECA both are relatively nonselective adenosine receptor agonists (Ki ratio A_2/A_1 for 2-ClA and NECA are 6.77 and 1.64, respectively, Bruns et al., 1986), the identification of the subtype of adenosine receptor mediating this anticonvulsant response required further pharmacological characterization.

To further characterize the suppressant effects of adenosine analogs on BMI-induced seizures in the PC, five other adenosine

analogs were applied focally. Dose-dependent inhibition of seizures evoked by BMI were afforded by R-PIA and S-PIA in the PC (Fig.6). R-PIA was 10.2 times more potent than its S-diastereoisomer as an anticonvulsant agent in the PC. This result suggests that the anticonvulsant effect may be mediated by A_1 receptors, since this degree of stereoselectivity is consistent with that of A_1 receptors. Observations from our lab indicated that the S-PIA/R-PIA affinity ratio for A_1 sites *in vivo* functional assays (approximately 10 - 21) was similar to those of *in vitro* radioligand binding assays (about 15-36) in various tissues.

In an attempt to further confirm the A_1 receptor involvement in this specific response , two adenosine analogs, CPA and CHA, that possess high affinity and high selectivity for A_1 adenosine receptors (Ki for A_2/A_1 was 784 and 392, respectively, Bruns et al., 1986) were used. CPA and CHA both produced extremely potent protective effects against BMI-induced seizures in the PC. In contrast, a very selective A_2 adenosine receptor agonist CV-1808, lacked anticonvulsant action when administered focally in a dose as high as 334.8 pmol/rat. If the anticonvulsant effect of adenosine analogs was via A_2 adenosine receptors, CV-1808 should have produced an effect at this dose since either *in vivo* or *in vitro* assays indicate that CV-1808 is more potent or at least equal potent at A_2 sites compared to 2-ClA (Daly et al., 1987).

As shown in Table 9, this rank order of potency of protection by adenosine agonists against BMI-evoked seizures in the PC, determined in the present study was NECA $\geq$ CHA > CPA > R-PIA > 2-ClA

>> S-PIA >> CV-1808. Moreover, this rank order of potency in vivo also suggests that the suppressant effects of adenosine analogs on seizures induced by BMI in the PC is mediated through an interaction with adenosine A_1 receptors. The finding that NECA was more potent than CPA, CHA, and R-PIA in suppression of seizures in the PC is likely to be a function of differences in physicochemical properties of these compounds which may influence their disposition following intracerebral injection.

In accordance with these results, Murray et al. (1985) have demonstrated that the anticonvulsant effects of adenosine analogs by i.v. injection to elevate PTZ seizure threshold was through activation of A_1 adenosine receptors. Moreover, Szot et al. (1987) have recently reported that chronic exposure to the adenosine antagonist theophylline produced an upregulation of A_1 adenosine receptors in the rat cerebral cortex which was associated with a reduction of sensitivity to chemoconvulsants. Studies employing quantitative autoradiographic analysis of the specific binding of [^{3}H]CHA have revealed that A_1 adenosine receptors are highly enriched in the rat prepiriform cortex (Goodman et al.,1982). Taken together, these results suggest that adenosine may play an important role in the modulation of seizure susceptibility via an activation of A_1 adenosine receptors. The precise mechanisms of the anticonvulsant action of adenosine in the CNS are not clear. Adenosine exerts direct anticonvulsant action probably due to (1) postsynaptic inhibition of spontaneous and evoked neuronal firings (Phillis et al., 1981), and/or (2) presynaptic inhibition of excitatory neurotransmitter

release (Corrieri et al., 1981; Corradetti et al., 1984). In the rat prepiriform cortex, adenosine receptors may reduce the excitatory drive of an important output pathway from this brain area, either by exerting a postsynaptic hyperpolarization or by inhibiting excitatory neurotransmitter release at a presynaptic site in the domain of the output neuron. Future studies should, therefore, attempt to investigate more fully the mechanisms by which adenosine modulates the initiation of convulsions in this epileptogenic site in the rat forebrain.

In summary, the prepiriform cortex appears to be one of neuroanatomical sites for adenosine analogs exerting anticonvulsant effect on bicuculline-induced seizures. It is likely that activation of A_1 adenosine receptors is the basis for protection by adenosine analogs against seizures elicited by bicuculline in the prepiriform cortex of rat.

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