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Adenosine is considered to be a neuromodulator or

cotransmitter in the periphery and CNS. This neuromodulatory

action of adenosine may be observed as an anticonvulsant effect.

Dose-response curves for R-phenylisopropyladenosine (PIA),

cyclohexyladenosine (CHA), 2-chloroadenosine (2-ClAdo), N-

ethylcarboxamidoadenosine (NECA) and S-PIA were generated against

PTZ seizure thresholds in the rat. The rank order of potency for

adenosine agonists to elevate PTZ seizure threshold was R-PIA >

2-ClAdo > NECA > CHA > S-PIA. R-PIA was approximately 80-fold

more potent than S-PIA. This 80-fold difference in potency

between the the diasteriomers of PIA was consistent with an A_1

adenosine receptor-mediated response. The anticonvulsant action

of 2-ClAdo was reversed by pretreatment with theophylline.

Theophylline alone produced a proconvulsant effect on PTZ seizure

thresholds. This anticonvulsant action of 2-ClAdo and the

proconvulsant effect of theophylline were observed with other

chemoconvulsants such as bicuculline (BIC) and picrotoxinin (PTX). The respective ED_{50} values for 2-ClAdo and theophylline were similar regardless of the chemoconvulsant used. The inability of theophylline to antagonize the anticonvulsant action of diazepam and pentobarbital demonstrates the selectivity of the anticonvulsant effect of 2-ClAdo. 2-ClAdo-induced elevation of BIC seizure threshold was not antagonized by the benzodiazepine antagonists RO 15-1788 and CGS 8216, establishing the specificity of 2-ClAdo as an adenosine receptor agonist.

Chronic administration of theophylline significantly increased the specific binding of ^{3}H-cyclohexyladenosine in membranes of the cerebral cortex and cerebellum of the rat. Chronic exposure to theophylline produced a significant increase in the densities of both the high- and low-affinity forms of A_1 adenosine receptors in the cerebral cortex. The high- and low-affinity dissociation constants obtained from theophylline-exposed membranes of the cerebral cortex did not differ significantly from the corresponding values in saline-treated animals. Qualitatively and quantitatively similar results were observed in cerebellar membranes. Chronic exposure to theophylline resulted in significant increases in the doses of PTZ, BIC and PTX required to elicit a clonic convulsion. Thus, these results suggest that the reduced susceptibility to seizures induced by a chronic theophylline regimen represents a functional correlate of the upregulation of the A_1 adenosine receptor.

ADENOSINE RECEPTOR MODULATION OF SEIZURE SUSCEPTIBILITY IN RATS

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Introduction: Biochemical and Physiological
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A. Overview of Adenosine Metabolism

Adenosine, a purine nucleoside, is an intermediate in the
pathway of purine nucleotide degradation. Adenosine is
considered to be one of the most ubiquitous metabolic
intermediates in the body. The level of adenosine in the brain
varies with the physiological state of the animal. Hence,
accurate measurement of adenosine concentrations, especially in
the central nervous system (CNS) has proven to be a difficult
task. During hypoxia, adenosine levels can increase one hundred
to one thousandfold with a concomitant reduction in adenosine
triphosphate (ATP) levels (1,2). Therefore, to obtain reliable
measures of adenosine concentrations in the CNS, it is essential
that the tissue be fixed without interruption of the circulation
or traumatization of the cerebral tissues. Depending on the
technique used and the lability of ATP, adenosine levels have
been reported to range from 0.52 $\pm$ 0.11 umol/kg (3) to 19 umol/kg
(4). There is evidence that adenosine is localized selectively
within the neurons in some brain areas. Immunohistochemical
studies with antibodies to adenosine showed discrete localization
of adenosine to the cell groups such as pyramidal cells of the

hippocampus, deep layers of the medial cerebral cortex, the caudate, the basolateral amygdala with fibers most apparent in the spinal tract of the trigeminal (8).

The synthesis of adenosine can potentially be by a variety of different metabolic pathways which include: (a) the dephosphorylation of adenosine 5'-monophosphate (5'-AMP); (b) hydrolysis of S-adenosylhomocysteine that was formed by methylation; (c) the dephosphorylation of adenosine 3'-monophosphate (3'-AMP); (d) the adenosine end groups of ribonucleic acid (RNA); and (e) the reaction of adenine and ribose-1-phosphate (8,53). The most likely biosynthetic source of adenosine is the dephosphorylation of 5'AMP by the enzyme 5'-nucleotidase. The hydrolysis of S-adenosylhomocysteine by S-adenosylhomocysteine hydrolase has been shown to be an important pathway of adenosine production (Fig. 1). The condensation of adenine and ribose-1-phosphate is not a likely source for most adenosine production because the substrate is not readily available and the enzyme activity is low (8,53). The direct generation of adenosine from RNA is unlikely, due to the fact that the degradation of RNA is to adenosine-3'-monophosphate and not to adenosine (8,53). Sources of 5'-AMP for the synthesis of adenosine by 5'-nucleotidase include the turnover of adenine nucleotides from ATP and cyclic adenosine-3',5'-monophosphate (cAMP), or from the turnover of RNA that takes place during normal cellular function (53). The enzyme, 5'-nucleotidase, is localized predominantly on the outer surface of cell membranes
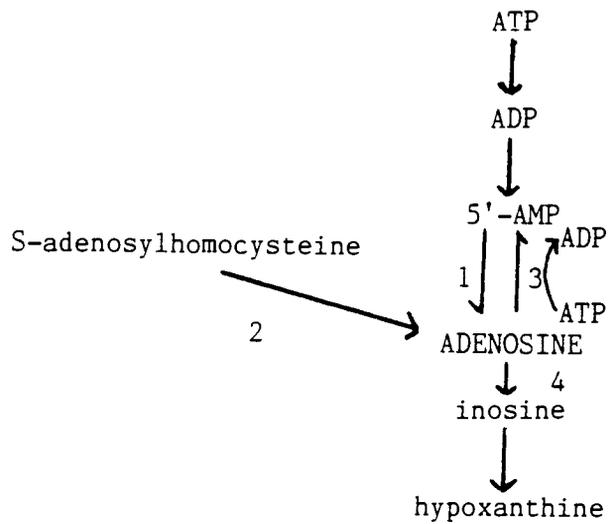
This diagram illustrates the pathways by which adenosine is formed (1 = 5'-nucleotidase and 2 = S-adenosylhomocysteine hydrolase) and degraded (3 = adenosine kinase and 4 = adenosine deaminase) within the neuron.

Figure 1. Schematic of adenosine synthesis and metabolism

(6,7). Some cells are known to have a soluble, intracellular 5'-nucleotidase in addition to, or even instead of the membrane form (8). The distribution of 5'-nucleotidase is rather discrete as shown by histochemical studies (9). The highest density of 5'-nucleotidase is in the molecular layer of the cerebellum (9,10), and in the molecular and polymorphic layers of the dentate gyrus and hippocampus (10). It has been suggested that this enzyme is responsible for the formation of extracellular adenosine because the hippocampus (9) and cerebellum (11) also contain a high density of adenosine receptors. However, 5'-nucleotidase probably has numerous other functions besides possibly serving as a biosynthetic enzyme for adenosine, because the enzyme is also located on glial cells and myelin (144).

Once adenosine is formed it may be removed from cells by being released extracellularly or degraded to purine end products. Release of adenosine directly into the extracellular environment occurs mainly in the myocardium and CNS in response to the lack of oxygen (4,20-22). Adenosine in the cytosol may be removed by its conversion to adenine nucleotides by the enzyme adenosine kinase, or alternatively metabolized to inosine by adenosine deaminase (6) (Fig. 1). The activity of these two cytoplasmic enzymes results in the maintenance of low intracellular concentrations of adenosine.

B. <u>History and Introduction to the Characterization of the</u>
    <u>Adenosine Receptor</u>

An abundant amount of evidence supporting a
physiological role for adenosine in both the periphery and the
CNS has stimulated attempts to characterize the adenosine
receptors mediating these responses. Holton and Holton in 1954
were able to demonstrate the release of ATP from central and
peripheral nerve fiber terminals (55). The released ATP may then
be rapidly hydrolyzed by the enzyme 5'-nucleotidase to form
adenosine. The free adenosine could then act at specific
receptors. At the present time, the only neural tissue so far
studied which has not been shown to corelease ATP with
acetylcholine during activity is the sympathetic ganglion (56).

Burnstock first classified purine receptors with respect to
the relative potencies of adenosine and ATP in a wide variety of
tissues (57). Receptors at which adenosine was more potent than
ATP were called P_1, while at P_2 receptors ATP was more potent
than adenosine. The P_1 receptor, but not the P_2 receptor, is
antagonized by methylxanthines such as theophylline and caffeine
(57).

Sattin and Rall in 1970 were the first to biochemically
characterize the response to adenosine receptor activation (58).
They showed that adenosine enhanced cAMP accumulation in brain
slices, and that this response was competitively blocked by the
methylxanthine theophylline (58). Adenosine actions upon cAMP

levels in the CNS could be potentiated by including

dipyridamole, an adenosine uptake inhibitor, in the brain slice

preparation (59). This indicated that adenosine was mediating

this alteration in cAMP production by a cell surface receptor.

Further support of this hypothesis came when Olsson and coworkers

demonstrated adenosine covalently bound to oligosaccharide

stacchyose beads produced identical responses to free adenosine

in the periphery (60).

Londos and collaborators observed that adenosine produced

either an increase or a decrease in cAMP levels in a variety of

tissue cell cultures in the periphery (61,62). These

investigators demonstrated that the ribose ring on adenosine

analogs must be intact to produce these alterations in cAMP. Due

to this structural requirement they classified Burnstock's P_1

receptor as R_a or R_i adenosine receptors. R_a receptors were

associated with adenylate cyclase in a stimulatory manner

resulting in elevated cAMP levels, while the R_i receptors were

associated with adenylate cyclase in an inhibitory fashion

producing a decrease in intracellular cAMP levels (61,62). Van

Calker, Muller and Hamprecht at the same time as Londos and

coworkers, demonstrated that adenosine and adenosine analogs

have stimulatory and inhibitory action on adenylate cyclase in

the brain (63). Van Calker et al. classified the receptors

associated with adenylate cyclase in a stimulatory manner as A_2

and the receptors associated with adenylate cyclase in an

inhibitory fashion as A_1. This group also showed that A_1

receptors required nanomolar concentrations of adenosine to elicit a response, whereas A_2 receptors required micromolar concentrations. The A_1 receptor exhibited marked stereoselectivity toward the R-isomer of phenylisopropyladenosine (PIA) relative to the S-isomer, while the A_2 receptor displayed much less stereoselectivity for these diastereomers. Both the A_1 and A_2 receptors were competitively antagonized by methylxanthines such as caffeine and theophylline. Xanthine antagonists theophylline and caffeine are not selective for blockade at either the A_1 or A_2 receptor.

At the present time, detailed structure activity requirements for adenosine analogs at the A_1 and A_2 receptors have been characterized. The most potent adenosine analogs at the A_1 receptor are N^6-substituted compounds. N^6-cyclohexyladenosine (CHA), N^6-phenyladenosine, and N^6-phenylisopropyladenosine (R-PIA) are very potent, while N^6-methyladenosine, N^6-benzyladenosine, and the S-diastereomer of N^6-PIA are considerably less potent. 2-Chloroadenosine (2-ClAdo) and adenosine are potent at the A_1 receptor, while 5'-carboxamide derivatives of adenosine are somewhat less potent (64). The characteristic rank order potency of adenosine analogs at A_1 receptors coupled to adenylate cyclase is R-PIA > 2-ClAdo > NECA > S-PIA (65). At the A_1 receptor R-PIA is 20-100 fold more potent than S-PIA, while at A_2 receptors R-PIA is 3-5 fold more potent than S-PIA (130).

The most potent adenosine analogs at the A_2 receptor are the
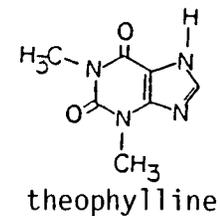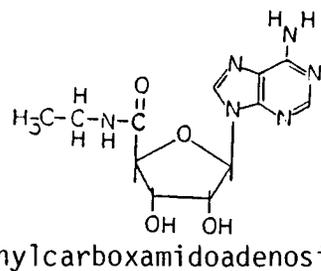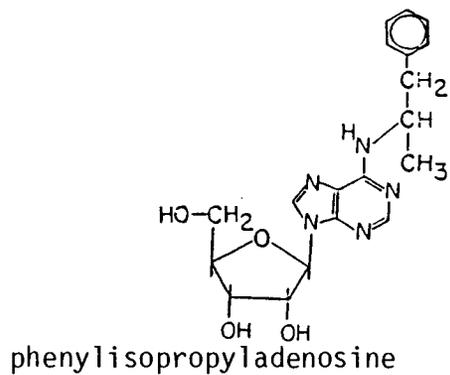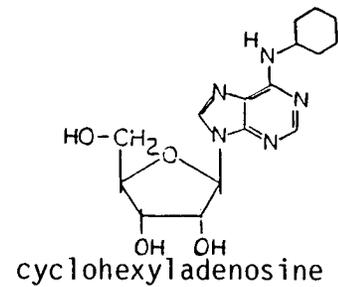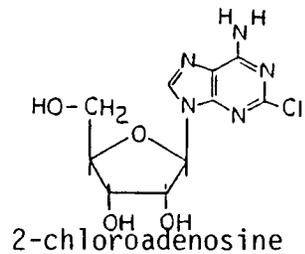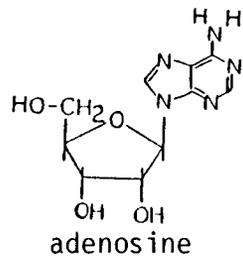
Figure 2. Structures of adenosine, adenosine analogs, theophylline and caffeine

5'carboxamides adenosine derivatives such as 5'-ethyl and
5'cyclopropylcarboxamides. Adenosine and 2-ClAdo are about 10-
fold less potent. The N^6-substituted analogs at the A_2 receptor
are less potent than 2-ClAdo, which is opposite to that observed
at the A_1 receptor. The typical rank order potency of adenosine
analogs at A_2 receptors coupled to adenylate cyclase is NECA > 2-
ClAdo > R-PIA $\geq$ S-PIA (65).

C. Historical Overview of Adenosine as a Physiological
    Regulator

    The first research group to acknowledge that adenosine
produced a physiological response was Drury and Szent-Gyorgi in
1929 (12). They found that extracts of heart muscle, brain,
kidney, and spleen when injected intravenously into a dog had
profound effects upon the cardiovascular system that were
identical to adenosine. The cardiovascular responses observed
were a slowing of heart rate, lowering of arterial pressure and
dilation of coronary vessels. A few years later Gaddam and Holtz
(13), and Green and Stoner (14) further studied these effects of
adenosine upon the myocardium and the vasculature in the
periphery. Their data further corroborated the role of adenosine
as a physiological regulator in the cardiovasculature.

    Since this discovery of adenosine's cardiovascular effects, a
great deal of information has accumulated concerning the role
adenosine plays as a physiological regulator in the periphery and

CNS. I will briefly describe the action of adenosine on specific biological processes in the periphery. The main focus of my thesis is the central action of adenosine as an anticonvulsant agent and will be discussed in greater detail in another section (Page 13).

1. Myocardium.

The physiological response to adenosine in the myocardium is a negative chronotropic effect on the sinoatrial node (12,29), a negative inotropic effect on atrial muscle (8), and a negative dromotropic effect on the atrioventricular node (16). Adenosine has also been shown to attenuate the catecholamine-induced increase in ventricular force (18). Adenosine mediates these effects upon myocardial tissue by inhibiting adenylate cyclase activity (145,146) and interfering with calcium movement and/or availability (19). The antiadrenergic activity of adenosine is mediated by the inhibition of the catecholamine-induced elevation of cAMP (18).

2. Vasculature.

As already stated adenosine is known to be a potent vasodilator of the coronary vasculature (12,15), and also produces vasodilation in skeletal muscle and brain (13, 23-27). The vasodilation produced by adenosine in animals results in

severe hypotension (28). The dose of adenosine required to produce coronary vasodilation is 100 times higher then the dose required to produce negative inotropic and chronotropic effects in the myocardium (29). The vasodilatory response is associated with cell surface receptors resulting in an activation of adenylate cyclase with a concomitant change in intracellular levels of cAMP (30-32). Verhaeghe (33) showed that adenosine produced a response by inhibiting the release of norepinephrine from adrenergic vasomotor nerves. The vasculature is not the only peripheral smooth muscle that adenosine acts on. Adenosine mediates relaxation of the alimentary canal, the urinary bladder and the reproductive organs by acting on adenosine receptors (54).

3. Platelet.

Adenosine has been shown to have an effect on platelet aggregation that is independent of adenosine diphosphate's (ADP) action upon platelets (34). Adenosine inhibits platelet aggregation induced by various agonists like ADP, epinephrine, serotonin and vasopressin (34-37). The ability of papaverine, an adenosine uptake inhibitor, to enhance adenosine's action indicates a cell surface mediated response. Adenosine is able to inhibit platelet aggregation by other agonists because it stimulates adenylate cyclase on platelet membranes (39) resulting in an increase in intracellular cAMP levels (40). There is a close correlation between elevated cAMP and the degree of

inhibition of aggregation observed by adenosine (41).

4. Adipose.

The physiological role of adenosine in regulating lipolysis has been demonstrated in a variety of adipose tissue preparations (42-46). Adenosine released from the fat cells (47) regulates norepinephrine induced lipolysis by inhibiting norepinephrine-stimulated cAMP accumulation in the fat cells (45). This response of adenosine upon cAMP is mediated by a cell surface A_1 adenosine receptor (46) which results in inhibition of hormone stimulated adenylate cyclase activity (45).

5. Pulmonary.

A role for adenosine in mediating bronchial asthma has been speculated for some time due to the wide use of theophylline (an adenosine receptor antagonist) in the treatment of pulmonary obstructive disorders. Adenosine has been shown to release histamine from mast cells and basophil cells in the rat (48) and guinea pig (49) by interacting with a cell surface receptor which stimulates adenylate cyclase and results in an elevation of intracellular cAMP levels (50,51). The exact role adenosine plays in mediating bronchoconstriction following an immunological response in humans is still unresolved (50,52).

6. Central Nervous System.

The administration of adenosine and adenosine analogs is associated with sedation (66-71), analgesia (67,68), hypnosis (69-71), hypothermia (67) and anticonvulsant activity (72) in a wide variety of animals. These pharmacological actions of adenosine have been attributed to the pronounced effect that adenosine has on the spontaneous firing rate of central neurons. It is now well established that the iontophoretic application of adenosine and various adenine nucleotides result in a decreased firing rate of rat cerebral cortex (73), cerebellum Purkinje neurons (74), hippocampus, caudate nucleus, thalamus, superior colliculus and olfactory bulb neurons (75). The hippocampus, cerebral cortex, caudate nucleus and thalamus appear to be the most sensitive to the application of ionophoretically applied adenosine (75). This decrease in neuronal activity by adenosine was blocked by pretreatment with the methylxanthine theophylline (76,78,80). The effects of adenosine on cell firing can be enhanced by adenosine uptake inhibitors (76,78) or by inhibitors of the enzyme adenosine deaminase (76,78). These results supported the notion that adenosine was producing its effect via an activation of an extracellular receptor. When adenosine uptake inhibitors and adenosine deaminase inhibitors were administered alone intracerebroventricularly, a decrease in cortical neuronal activity was observed (76,78). In contrast, the methylxanthine theophylline produced an increase in

spontaneous cell activity (76,78). These results indicate that under normal in vivo conditions, endogenous adenosine exerts a tonic inhibition of neuronal excitability.

Similar results have been obtained in experiments using an in vitro slice preparation. A slice preparation allows one to measure the intracellular and extracellular activity of pre- and postsynaptic potentials of a monosynaptic response. Utilizing this preparation it was demonstrated that the presynaptic fiber potential was not altered by adenosine, while the postsynaptic spike potential was decreased in amplitude (81,83,84,86,87). The decreases in spontaneous and evoked postsynaptic potentials by adenosine was not accompanied by a change in resting input resistance (81,83). The most plausible explanation for this depression of postsynaptic potential without any alteration in presynaptic volley or postsynaptic membrane conductance would be a reduction in the efficacy of presynaptic transmission. These actions by adenosine upon neuronal activty in an in vitro preparation were antagonized by theophylline (82-86) and enhanced by adenosine uptake inhibitors (84). Adminstration of adenosine deaminase alone (85) or theophylline (82) resulted in an elevation of the population spike amplitude in a hippocampal preparation, while hexobendine, an adenosine uptake inhibitor, produced a depressant effect on the population spike amplitude (85). This suggests that the endogenous release of adenosine is sufficient to exert a tonic inhibitory influence upon neuronal activity in the hippocampal slice preparation. A study of the

inhibitory actions of a number of adenosine analogs in the
olfactory cortical slice indicated that the 6-aminopurine ribose
moiety was essential for activity, as was the presence of at
least one hydrogen atom in the amino group at the
6-position of the purine, and the OH group at the 2-position of
the ribose (86). These structural requirements demonstrated that
the observed response was mediated by A_1 adenosine receptor.
Smellie and coworkers provided further support for an A_1 receptor
mediated inhibition of hippocampal activity by demonstrating that
R-PIA was approximately 100 times more potent than S-PIA in
producing neuronal depression (88). However, the difference in
potency of these diastereoisomers to elicit an accumulation of
cAMP was only 4-5 fold (88). This suggested that an elevation in
cAMP levels did not mediate the neuronal depression of adenosine
and adenosine analogs. Reddington and Schubert showed that the
concentration range of adenosine and 2-ClAdo required to
stimulate cAMP synthesis was the same, while a 50 fold difference
in concentration was required for electrophysiological response
(89). This difference in ED_{50} values of adenosine and 2-ClAdo
further indicates a lack of a relationship between cAMP and
neuronal depression of evoked potential. It was noted by Okada
and coworkers that adenosine produced an elevation in cAMP levels
in the superior colliculus but it did not depress the
postsynaptic potential in this region (87). Finally,
administration of dibutyryl cAMP, a synthetic analog of cAMP that
can diffuse across neuronal membranes, produced an increase in

neuronal activity (83). It was therefore concluded that the decreased neuronal response produced by adenosine in the CNS was not mediated by an A_2 receptor and elevated cAMP levels.

A presynaptic site of action of adenosine first became evident when it was shown that adenosine depressed the spontaneous and evoked release of acetylcholine from the rat phrenic nerve (90). Presently, there is evidence that adenosine is capable of depressing the release of several putative neurotransmitters in the CNS. This action of adenosine was first demonstrated in cerebral cortical tissue where it inhibited the spontaneous release of acetylcholine, and this effect was selectively blocked by theophylline (91). In addition, adenosine has been shown to depress the release of norepinephrine, dopamine, serotonin and gamma-aminobutyric acid from brain slices or synaptosomes (92-95). This inhibitory effect of adenosine on transmitter release is antagonized by the methylxanthine theophylline (92-95). The methylxanthines when adminstered alone enhanced resting acetylcholine release from rat neocortical slices (96) and cerebral cortex in situ (97) suggesting that the extracellular levels of endogenously released adenosine were sufficient to depress the release of acetylcholine. Adenosine may produce this depressant action on neurotransmitter release by blocking calcium (Ca^{++}) uptake transport. The effects of adenosine on transmitter release are most apparent when extracellular Ca^{++} is reduced, and can be abolished by elevating extracellular Ca^{++} levels (98). Adenosine has been shown to

reduce the uptake of $^{45}Ca^{++}$ in potassium (K^+)-depolarized synaptosomal preparations (99). The effect of adenosine on K^+-induced Ca^{++} in rat cerebral cortical synaptosomes is antagonized by theophylline and enhanced by dipyridamole, an adenosine uptake inhibitor (100). Though conflicting evidence does exist concerning the action of adenosine on presynaptic Ca^{++} uptake. Barr and coworkers have demonstrated an inability of adenosine and 2-ClAdo to alter fast-and slow-phase voltage-dependent Ca^{++} entry into synaptosomes of rat whole brain (164). There have also been suggestions that adenosine may reduce the level of intracellular free Ca^{++} (90,101).

A postsynaptic action of adenosine has been documented in hippocampal CA1 pyramidal neurons (102). The postsynaptic hyperpolarizing action produced by adenosine was hypothesized to be mediated by an activation of an outward K^+ current. Segal determined that the hyperpolarization produced by adenosine in the CA1 region of the hippocampus had a reversal potential of 90mV, suggesting an increase in K^+ conductance (103). Therefore in the hippocampus, an area very sensitive to the suppressant actions of adenosine, both a pre- and post-synaptic action of adenosine have been demonstrated. Another postsynaptic action mediated by adenosine is the inhibition of an inward Ca^{++} current (165,166). A reduction in Ca^{++} influx by adenosine and adenosine analogs has been demonstrated in rat cervical sympathetic ganglion (165) and in mouse dorsal root ganglion (166) resulting in hyperpolarizing afterpotential (165). The action of adenosine

on Ca^{++} influx appears to be mediated through a cell surface

adenosine receptor. The action of dipyridamole, an adenosine

uptake inhibitor, to potentiate the effects of adenosine on Ca^{++}

influx provides futher evidence for the existance of an external

receptor. Support for the involvement of an adenosine receptor

was shown by the antagonism of adenosine's action on the inward

Ca^{++} current by methylxanthines such as theophylline (165,166),

p-sulpho-8-phenyltheophylline and 1,3-dipropyl-8-(2-amino-4-

chlorophenyl)xanthine (PACPX) (166).

D.   The Anticonvulsant Properties of Adenosine and

     Adenosine Analogs

In 1974, Maitre and coworkers provided the first evidence

for an anticonvulsant action of exogenously administered

adenosine (72). The anticonvulsant activity of adenosine

activity was examined utilizing a strain of mice susceptible to

seizures induced by auditory stimuli. Adenosine administered

intraperitoneally to audiogenic seizure sensitive mice prevented

convulsions induced by a 100 dB (7500 Hz) sound stimulus. The

time course of adenosine's anticonvulsant action differed from

the time course of the adenosine-induced sedation and

hypotension. These data indicate that adenosine's anticonvulsant

action in the audiogenic seizure sensitive mouse was not due to

generalized sedative effect or decreased blood pressure.

More recently, Dunwiddie has examined the anticonvulsant activity

of adenosine in the hippocampal slice preparation (105).The

hippocampus is an appropriate tissue to study the anticonvulsant

action of adenosine because of its propensity for seizure

activity and its sensitivity to neuronal depression produced by

adenosine. When sodium penicillin-G and elevated K^+ levels are

included in the hippocampal perfusion medium, the hippocampal

slice _in vitro_ generates repetitive interictal spikes that are

recorded intracellularly in the CA1 region of the hippocampus.

These interictal spikes are responsive to convulsant and

anticonvulsant drugs. The amplitude of the spikes correspond

roughly to the number of neurons being activated synchronously.

When adenosine was applied to these hyperexcitable hippocampal

slices the interictal spiking was slowed or eliminated depending

on the dose of adenosine. This demonstrated adenosine's ability

to suppress seizure-induced activity by depressing neuronal

activity in the hippocampus. Adenosine's decrease in interictal

spiking was antagonized by concurrently perfusing the medium with

theophylline. When theophylline or adenosine deaminase were

added to the perfusion medium, an increase in the interictal

spiking rate was recorded in the CA1 pyramidal neurons. The

resultant increase in interictal spiking elicited by theophylline

or adenosine deaminase established a role for endogenous

adenosine as a modulator of neuronal excitability in the

hippocampus.

Since Dunwiddies's work, the anticonvulsant activity of

adenosine in hippocampal CA1 pyramidal neurons has been

attributed in part to a postsynaptic affect on Ca^{++}-dependent K^+

current (106,107,122). Activation of the Ca^{++}-dependent K^+

current results in a long-lasting afterhyperpolarization and

slower accommodation of firing. Modulation of this Ca^{++}-

dependent K^+ current by transmitters results in an altered

afterhyperpolarization in the CA1 hippocampal pyramidal neurons.

Application of adenosine to the perfusion medium resulted in an

enhancement of the slow afterhyperpolarization and accommodation

of the CA1 neurons (107). Adenosine decreased the amplitude of

the slow spikes and the duration of the depolarizing plateau

(107). A decrease in input resistance was also observed (107).

These results demonstrate adenosine's ability to stabilize

neurons to the effects of depolarizing action potentials.

Further support for a postsynaptic action of adenosine on

Ca^{++}-stimulated K^+ current was demonstrated by Lee and coworkers

(106,122). They measured the afterpotential spiking in the

pyramidal CA1 neurons that followed an antidromic stimulation of

the same neuron. To eliminate the presynaptic effects of

adenosine, experiments were performed in a low calcium medium.

Adenosine decreased the afterdischarge spiking in CA1 pyramidal

neurons with no effect on the initial antidromic stimulation

(106,122). This effect of adenosine on afterpotential spiking

was antagonized by theophylline (106). The adenosine agonist

R-PIA was shown to be 33 fold more potent than the diasteriomer

S-PIA in reducing the afterdischarge spiking, indicating an

adenosine A_1 receptor mediated process (106). To establish the

mechanism of adenosine's action on afterdischarge spiking, 4-aminopyridine was included in the incubation medium. 4-Aminopyridine selectively blocks the Ca^{++}-stimulated K^+ current (170). When adenosine and R-PIA were examined in the presence of 4-aminopyridine, the effects of adenosine and R-PIA in reducing afterpotential spiking were attenuated. Since 4-aminopyridine antagonized the effect of adenosine and R-PIA on afterpotential spiking, a Ca^{++}-sensitive K^+ channel was implicated in the anticonvulsant action of adenosine. These data are in agreement with those previously discussed regarding an action of adenosine on postsynaptic Ca^{++}-dependent K^+ channels in the hippocampus as the underlying antiepileptic mechanism (107).

The CA1 region of the hippocampus is not the only area sensitive to the anticonvulsant action of adenosine. Ault and Wang studied the effects of adenosine and adenosine analogs in the pyramidal CA3 region of the hippocampus (109). The CA2/CA3 pyramidal cells are the primary generators of epileptiform activity which may then invade the CA1 region (110). When bicuculline and slightly elevated K^+ levels were included in the perfusion medium of the in vitro hippocampal slice preparation, a synchronous discharge of neurons in the CA3 region was recorded as bursts of population spikes. Adenosine and adenosine analogs reduced the rate of bursting of the CA3 pyramidal neurons. The reduced rate of firing occurred as a result of an increase in the interval between each burst potential. The rank order potency of the adenosine agonists

employed to decrease bursting was CHA > R-PIA > NECA > 2-ClAdo >

adenosine (109). The effect of adenosine on CA3 bursting was

antagonized by theophylline. Conversely, theophylline by itself

enhanced the bicuculline-induced bursting of CA3 neurons. In

hippocampal slice preparations untreated with bicuculline,

application of theophylline or adenosine deaminase to the

perfusion medium induced bursting activity in the CA3 pyramidal

neurons. These data confirm Dunwiddie's (105) hypothesis that

endogenous adenosine may be a modulator of neuronal excitability

in the hippocampus. The anticonvulsant effect of adenosine in

the _in vitro_ hippocampal slice can be attributed to the

suppression of seizure initiation in area CA3 rather than the

spread of neuronal activity.

Dunwiddie and Worth examined the effects of adenosine

agonists on seizures induced by a variety of chemoconvulsant

agents _in vivo_ in the mouse and rat (111). In mice, R-PIA

produced sedation, hypothermia and anticonvulsant activity

against pentylenetetrazol (PTZ) seizures. The sedation and

hypothermia induced by R-PIA was antagonized in mice by

theophylline pretreatment. Theophylline pretreatment, however,

did not antagonize R-PIA's increase the latency to PTZ seizures.

In contrast, in rats, this anticonvulsant action of R-PIA was

reversed by theophylline pretreatment. R-PIA was 20-fold more

potent than S-PIA as an anticonvulsant against PTZ seizures in

mice. This difference in potency between the PIA

diastereoisomers is characteristic of an adenosine A_1

receptor mediated response. However, R-PIA, CHA and 2-
ClAdo displayed varying degrees of potency as anticonvulsants
when treated against a variety of chemoconvulsants including
strychnine, kainic acid, picrotoxinin, PTZ and 3-
mercaptoproprionic acid (111). When EC_{50} values of the
anticonvulsant activity of R-PIA, CHA and 2-ClAdo were compared,
R-PIA displayed a profile which differed from that of 2-ClAdo and
CHA (111). These differences in relative potencies of R-PIA, CHA
and 2-ClAdo against various chemoconvulsants was interpreted as
evidence for the involvement of multiple adenosine receptors.

In mice treated with a subconvulsant dose of PTZ,
theophylline produced a proconvulsant effect (111). In
additional experiments in mice seizures were induced by
theophylline treatment alone; however, these theophylline-induced
seizures were not antagonized by pretreatment with R-PIA, CHA or
2-ClAdo (111). Because adenosine analogs did not protect the
mice from theophylline-induced seizures, it was suggested that
this neurotoxic effect of theophylline was not mediated via an
antagonism of the effects of endogenous adenosine (111).
Although Dunwiddie and Worth demonstrated anticonvulsant
properties of adenosine analogs against a wide spectrum of
convulsant agents, this anticonvulsant action did not appear to
be mediated through a homogenous receptor population (111).

More recently, Turski and coworkers have examined the
effect of 2-ClAdo on pilocarpine-induced seizures and correlated
the convulsant activity with EEG seizures in the hippocampus

(108). The effects of 2-ClAdo and theophylline on pilocarpine-induced epileptiform activity in the hippocampus were recorded using EEG tracings. 2-ClAdo prevented the development of pilocarpine-induced seizures, while theophylline decreased the threshold for seizure activity. The EEG activity in the hippocampus closely correlated with the convulsant activity of the animal.

The anticonvulsant properties of adenosine are not limited solely to chemoconvulsant agents. Burley and Ferrendelli demonstrated an anticonvulsant action of adenosine in maximum electroshock induced seizures in mice (112). The refactory time was the most sensitive parameter to the anticonvulsant action of adenosine (112). Refactory time was the minimal time interval required before the animal could receive another stimulus to elicit a seizure (112). Adenosine and 2-ClAdo was shown to increase the refractory period (112). This indicated an involvement of adenosine in the initiation and maintenance of a seizure (112).

Chemically-induced seizures and maximum electroshock are not the only available models of epilepsy to study the anticonvulsant properties of drugs. In 1967, Goddard and colleagues introduced an animal model of epilepsy called kindling (117). Kindling phenomenon occurs when repeated adminstration of an initially subconvulsive electrical stimulus results in progressive intensification of seizure activity, culminating in a generalized seizure. Kindling can be induced by electrical stimulation of

most, but not all, sites in the brain (162). The amygdala is the

most sensitive area to the action of kindling , though kindling

has occurred with stimulation to the cortex, olfactory bulb,

septal area, preoptic area, caudate-putamen and hippocampus

(162). Electrical stimulation is not the only means to induce

kindling. Animals have been kindlied with repeated

administration of subconvulsive doses of carbachol,

chlorodimeform, cocaine, fluorothyl, lidocaine, PTZ (161) and

theophylline (114). The importance of kindling as a model of

epilepsy is the permanently-induced alteration in neuronal

excitability. The possibility that kindling may provide insight

into the mechanism of neuronal plasticity and human epilepsy has

led to an extensive investigation of this model.

Kindling as an experimental model of epilepsy provides a

variety of indicies which can be used to assess the

anticonvulsant or convulsant property of a drug. On the fully

kindled seizure the following indicies can be measured:

afterdischarge duration (s), motor seizure duration (s),

afterdischarge threshold (uA), generalized seizure threshold (uA)

and behavioral rank. On kindling development the number of

stimulation required to elicit a stage 5 seizure, seizure

duration, and afterdischarge threshold (uA) can be measured. The

effects of adenosine analogs and adenosine antagonists on pre-

and post-kindled seizures have been fully characterized.

Adenosine, adenosine analogs and papaverine reduced the severity

and the afterdischarge duration (s) of amygdala-kindled

seizures (113-115,120). Additionally in amygdala-kindled animals an increase in latency to bilateral forelimb clonus was observed following 2-ClAdo (115). Barraco and coworkers showed NECA to be 4 times more potent then R-PIA in reducing seizure severity and duration in amygdala-kindled rats (113). 2-ClAdo did not alter the afterdischarge threshold (uA) in the amygdala-kindled seizure (115) suggesting that adenosine had little effect on seizure initiation in this model and that the action of adenosine may be to modulate the development or expression of amygdala-kindled seizure. Aminophylline has been shown to increase the afterdischarge duration in rats not fully kindled using both cerebral cortical and amygdala stimulation (114,115,120). A decrease in the number of stimulations required to reach a stage 5 rank seizure was also observed in amygdaloid kindling following aminophylline pretreatment (114). The effect of methylxanthine's on fully kindled seizures has been examined in the amygdala (114,119-121), cortex (120) and hippocampus (118) model of kindling. In all of the kindled models studied, theophylline increased the afterdischarge duration (114,118-120) and the seizure severity (118,119,121). These data suggest that the methylxanthines interfere with the processes which terminate electrical seizure activity.

It is apparent from the existing literature that adenosine and adenosine agonists are potent anticonvulsants against a wide variety of convulsant agents. Adenosine analogs may produce their anticonvulsant action _in vitro_ and _in vivo_ by modulating

the initiation of a seizure. In the kindling model, adenosine

analogs produced an anticonvulsant action by modulating the

spread and the termination of seizure activity. Adenosine also

modulated the kindling process. Therefore the actions of

adenosine as an anticonvulsant will vary according to the model

of epilepsy used. The receptor subtype responsible for

these actions of adenosine has not been clearly established. The

in vitro hippocampal slice preparation indicates the A_1 adenosine

receptors are involved, while the amygdaloid-kindled model

indicates an A_2 receptor. Moreover, Dunwiddie and Worth (111)

have suggested the involvement of more than one receptor subtype

in the anticonvulsant action of adenosine analogs versus

seizures induced in vivo by chemoconvulsants. In addition the

effects of R-PIA on PTZ seizure latency in mice reported by

Dunwiddie and Worth were not antagonized by theophylline (111).

Thus, the mechanism by which adenosine analogs produce an

anticonvulsant response remains to be elucidated.

E. Adenosine Receptor Modulation of Seizure Susceptibility

   in rats: A Statement of the Problem

From the literature it is apparent that adenosine possesses

anticonvulsant properties. Based on in vitro studies, this

anticonvulsant activity of adenosine has been attributed to its

interaction with CNS adenosine receptors. Dunwiddies and Worth's

in vivo data has questioned the role of the adenosine receptor in

the anticonvulsant action of adenosine analogs. The increase in PTZ seizure latency induced by R-PIA in mice was not antagonized by theophylline pretreatment (111). Moreover, R-PIA, CHA and 2-ClAdo displayed varying degrees of potency as anticonvulsants when tested against a variety of chemoconvulsants (111). When EC_{50} values of the anticonvulsant activity of R-PIA, CHA and 2-ClAdo were compared, R-PIA displayed a profile which differed from that of 2-ClAdo and CHA (111). These differences in relative potencies of R-PIA, CHA and 2-ClAdo against various chemoconvulsants were interpreted as evidence for the involvement of more than one adenosine receptor or the involvement of a non-purinergic receptor (111). Barraco and coworkers data suggest the anticonvulsant response of NECA and R-PIA on kindled amygdaloid seizures to be mediated through an adenosine A_2 receptor (113). A non-purinergic action of adenosine has been proposed to exist at the benzodiazepine receptor based on benzodiazepine binding studies (147). Further support for adenosine analogs action at sites other then the adenosine receptor was the inability of theophylline to block the anticonvulsant action of R-PIA on pentylenetetrazol seizures in mice (111).

Characterizing the role and adenosine receptor subtype involved in the regulation of seizure susceptibility may help to understand the pathophysiology of epilepsy. If endogenous adenosine subserves a physiological relevant role in modulating seizure activity. Seizures have been shown to be induced in rats

(135,137,139,138) and in humans (136) by the administration of an

adenosine antagonist, theophylline. Status epilepticus was

observed in patients with no prior history of seizure activity

when they were treated with theophylline medication and received

electroshock therapy (140). Antagonism of the adenosine receptor

by methylxanthines such as caffeine or theophylline has been

shown to increase the CNS neuronal activity . If an underlying

predisposition to epilepsy exists in a person, then consumption

of a methylxanthine may precipitate a seizure. The exact

mechanism of theophylline-induced seizures is still unclear. In

the rat, seizures induced by theophylline were not attenuated

by the administration of the adenosine agonist R-PIA (111).

Also, aminophylline has been shown to antagonize the

anticonvulsant properties of diazepam, diphenylhydantoin,

phenobarbital and valproic acid on electroconvulsions in mice

(132,134) Therefore, it is important to establish the exact role

the adenosine receptor plays in modulating seizure activity

because of the therapeutic implications regarding the consumption

of caffeinated beverages and the therapeutic use of theophylline

in pulmonary obstructive diseases.

Therefore, this thesis research project was undertaken

to characterize CNS adenosine receptor subtype involved in

the anticonvulsant action of adenosine analogs and to establish a

modulatory role for endogenous adenosine on seizure

susceptibility. A time-infusion technique was employed as the

method for determining the seizure threshold to the

chemoconvulsant pentylenetetrazol (PTZ). The time-infusion technique is considered to be a reliable and sensitive method for measuring the seizure threshold value because the first indication of convulsant activity correlated closely with the first EEG seizure activity (124,127). Therefore a chemically-induced change in neuronal activity would be observed as an increase or decrease in the PTZ seizure threshold value from control. The specific aims of these investigations were as follows:

1. To classify the receptor subtype involved in mediating the anticonvulsant effect of adenosine analogs;

2. To determine the effect of the adenosine receptor antagonist theophylline on seizure threshold of PTZ;

3. To determine the generality of 2-ClAdo's anticonvulsant action and theophylline's proconvulsant action against a variety of chemoconvulsant agents;

4. To determine the effect of benzodiazepine receptor antagonists on the anticonvulsant effect of 2-ClAdo;

5. To examine the effect of PTZ kindling on the adenosine A_1 receptors in the CNS with the use of radioligand binding;

6. To examine the relationship between A_1 adenosine receptor upregulation and seizure susceptibility.

These results will further our understanding of adenosine's modulation of seizure susceptibility.

Chapter 2

Effects of Acute Administration of Adenosine Analogs Upon
Pentylenetetrazol Seizure Threshold in Rats

Introduction

There is considerable evidence to suggest a
neuromodulatory role for adenosine in the CNS. This
neuromodulatory action of adenosine may be manifested
behaviorally as an anticonvulsant effect. The ability of
adenosine to suppress seizure activity has been
demonstrated in a variety of anticonvulsant studies.
Adenosine depresses chemically induced epileptiform
activity in the in vitro hippocampal slice preparation
(105-107,122,110). In vivo studies have shown adenosine
and its analogs to posses anticonvulsant properties to
audiogenic seizures (72), maximum electroshock (112),
kindled seizures (113-115) and chemoconvulsant agents
(111,108). Dunwiddie and Worth (111) showed adenosine
analogs to prolong the latency for a clonic
convulsion induced by the following chemoconvulsants:
pentylenetetrazol (PTZ), picrotoxinin (PTX), strychnine,
kainic acid, and 3-mercaptoproprionic acid. Measuring the
latency to a seizure is one of many different in vivo
methods that can be used to observe an anticonvulsant
action of a compound. The in vivo experimental method
chosen to use to demonstrate the anticonvulsant action of

adenosine receptor agonists was a time infusion technique. The

time infusion technique was recently demonstrated to be the most

consistent method for determining the seizure

threshold to the chemoconvulsant PTZ (123,124). The

threshold method is considered to be a reliable method

because a change in the threshold is a reflection of the

compound being tested and is not due to changes in the

pharmacokinetic parameters of PTZ (126). Therefore, the

PTZ seizure threshold technique was used to asses the

anticonvulsant effects of adenosine agonists and adenosine

uptake inhibitor, dipyridamole in rats.

Materials and Methods

Male Sprague-Dawley rats (Simonsen Labs, Gilroy, Ca.)

weighing 140-180 g were used. The animals were housed upon

delivery, in groups of 4-6 at 22°C with food and water provided

ad libitum. A standard light-dark cycle was maintained with a

timer-regulator light period from 0800 to 2000 hr. Each rat was

used only once for any determination.

The seizure thresholds for PTZ in rats were determined by

the method of Nutt et. al. (123) and described in an outline in

Figure 3. Rats were lightly restrained and the PTZ was infused

via a 25 guage butterfly needle inserted into the lateral tail

vein. The butterfly needle was connected with PE tubing to a

syringe mounted in a Harvard infusion pump. The solution of PTZ

Figure 3. Method for the Determination of Pentylenetetrazol
Seizure Thresholds

1. PTZ (5 mg/ml) is continously infused into a lateral tail
    vein of the rat via a 25 guage butterfly

2. The time (sec) to the first myoclonic twitch was
    recorded

3. The PTZ seizure threshold is calculated from the recorded
    time, the infusion rate of PTZ (1.6 ml/min), the
    concentration of PTZ (5 mg/ml) and the weight (kg) of the
    animal

$$\text{Threshold Dose (mg/kg)} = \frac{\text{time (min) x infusion rate (ml/min) x PTZ conc. (mg/ml)}}{\text{weight (kg)}}$$

(5 mg/ml) was infused at a constant rate of 1.6 ml/min. The end point of the infusion was taken as the first myoclonic jerk of the head and neck. The first myoclonic jerk of the head and neck has been reported to occur synchronously with the first EEG discharge (124,127) and most closely correlates with the levels of PTZ in the brain (125,126). The seizure threshold for PTZ are expressed in units of mg/kg of PTZ infused intravenously. The time to the first myoclonic twitch was recorded to the nearest 0.1 sec,and the dose of PTZ required to elicit the seizure was calculated from this time, the concentration of the solution of PTZ, and the weight of the animal. Due to day to day variability in seizure thresholds, the appropriate control animals injected with saline were always tested at the same time as the experimental animals (123,124).

Time-response experiments were performed with 2-ClAdo (1 mg/kg), NECA (1 mg/kg) and dipyridamole (30 mg/kg). Following intravenous administration of the analogs, PTZ seizure thresholds were determined at 1, 5, 10, 20 and 30 min for 2-ClAdo and 1, 5, 15, 30 and 60 min for NECA and dipyridamole. Due to the marked hypothermia induced by intravenous NECA, the animals body temperature was monitered with a rectal temperature probe and maintained at 37°C with a heat lamp. The hypothermia was most evident at the 60 min post-injection of NECA where a significant decrease in body temperature was observed.

Dose-response curves were generated for R-PIA (0.01-1.0 mg/kg), 2-ClAdo (0.1-10 mg/kg), CHA (0.1-10 mg/kg), S-PIA (0.3-10

mg/kg) and NECA (0.03-10 mg/kg) to PTZ seizure threshold. The seizure thresholds were determined 1 min post-intravenous injection of the adenosine analogs. Modified Hill plots were used to compare quantitatively the dose-response curves for adenosine analogs. For each agonist, the log $[E/(100-E)]$ was plotted against the log dose (in umol/kg) where E represents the effect of the drug as a percentage of the maximum response. The Hill plots permitted the estimation of the ED_{50} values (x-intercepts) for the various agonists employed. The 95% confidence limits for the ED_{50} values were estimated by fitting a least squares regression line to the points and determining the confidence limits for the x-intercept (128). A dose-response curve was also generated for the adenosine uptake inhibitor, dipyridamole. The PTZ seizure threshold was determined 1 min post-intravenous injection.

The mean arterial blood pressure was recorded in pentobarbital anesthetized rats before and after the intravenous administration of R-PIA and CHA. The mean arterial blood pressure was recorded in the femoral artery, while CHA or R-PIA was administered via the femoral vein. A Grass Model 7, 6 channel recorder, previously calibrated with the transducer by a sphygmomanometer, was used for recordings. The body temperature was monitered and maintained at 37°C with an electric heating pad controlled by a rectal probe.

Statistical significance was determined in treatment groups to control by performing a Student's two-tailed t test.

Results

When 2-ClAdo was administered intravenously at a dose of 1 mg/kg, there was a significant increase in the dose of PTZ required to elicit a seizure. As shown in Figure 4, the peak effect for this dose of 2-ClAdo on the seizure threshold for PTZ occurred between 1 and 5 min after injection. The 1 mg/kg dose of 2-ClAdo increased the seizure threshold for PTZ from a control value (time 0) of 22.9 $\pm$ 0.8 to 31.4 $\pm$ 1.0 mg/kg at 1 min after an intravenous bolus (Fig. 4). The 10 and 20 min time post-injection of 2-ClAdo also resulted in a significant elevation of PTZ seizure threshold from time 0. The PTZ seizure threshold of 2-ClAdo returned to control by 30 min, illustrating a short anticonvulsant action of 2-ClAdo. The intravenous administration of NECA at a dose of 1 mg/kg produced an elevation in PTZ seizure threshold only at the 1 min post-injection (Fig.5), while the remainder of the time-response curve was not significantly different from control. It was very important to maintain the animals body temperature for the 60 min post-injection of NECA. If the body temperature was not maintained a 5.4 $\pm$ 0.3°C drop in body temperature was observed, with a nonsignificant increase in PTZ seizure threshold from 18.6 $\pm$ 0.6 to 19.6 $\pm$ 0.45 mg/kg. These preliminary experiments established the time of 1 min following injection of adenosine analogs to produce a maximum anticonvulsant effect. Therefore, using a time of 1 min, dose-response curves for each of the analogs were generated (Fig.6).
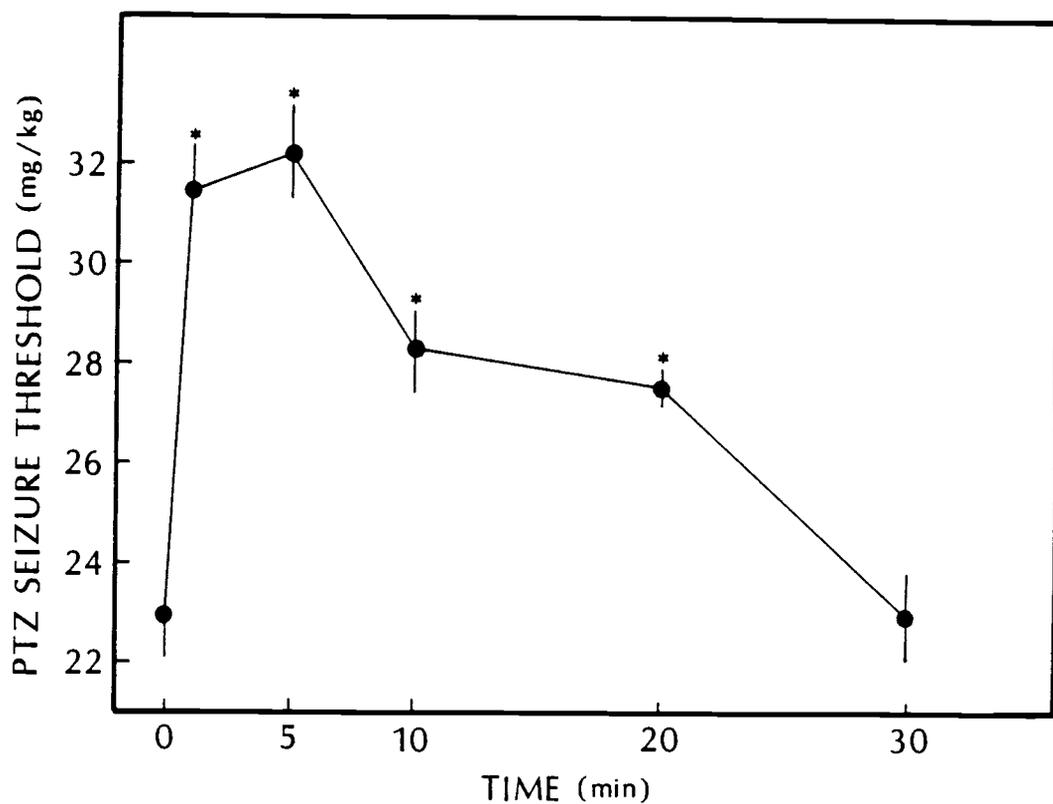
Figure 4. Time-response relationship for the effect of
2-ClAdo (1 mg/kg, i.v.) on the seizure threshold to
pentylenetetrazol in rats. Values represent the
mean ± SEM for 8 to 12 animals for each time.
Asterisks indicate significantly different ($P <$
0.05) values from time 0 controls.

Figure 5. Time-response relationship for the effect of NECA
(1 mg/kg, i.v.) on pentylenetetrazol seizure
threshold in rats. Values represent the mean ± SEM
for 8 to 12 animals for each time. Asterisks
indicate significantly different (P < 0.05) values
from time 0 controls.
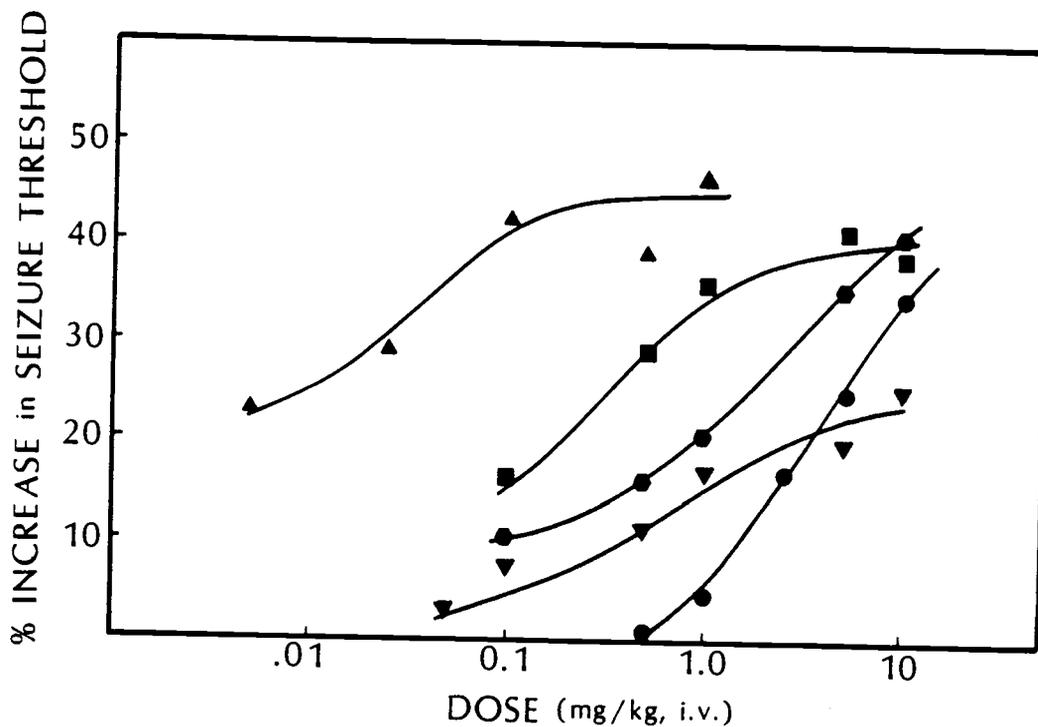
Figure 6.   Dose-response curves for the effects of R-PIA (▲),
2-ClAdo (■), CHA (◕), S-PIA (●) and NECA (▼)
on the seizure threshold for pentylenetetrazol. All
doses of analogs of adenosine were administered
intravenously in a volume of 2 ml/kg and all tests
were performed 1 min after injection. Values are
expressed as the mean percentage increase in seizure
threshold as compared to saline injected controls.
Each value represents the mean of 8 to 16 rats.

The results of these experiments indicated that R-PIA, 2-ClAdo, CHA, NECA and S-PIA all displayed conventional log dose-response curves. Moreover, examination of the dose-response curves depicted in Figure 6 indicates that all adenosine analogs except NECA produced a maximal elevation of PTZ seizure threshold of approximately 35-45%. The maximum elevation of PTZ seizure threshold produced by NECA was 25%. Thus, NECA was the least efficacious of the adenosine analogs tested. R-PIA was the most potent analog tested with a dose as low as 5 ug/kg (i.v.) eliciting a 23.2 $\pm$ 4.1% (P < 0.05) increase in the threshold dose of PTZ required for inducing a seizure. The ED_{50} values and respective 95% confidence limits for each of the analogs of adenosine tested are shown in Table 1. The rank order of potency for adenosine receptor agonists in elevating PTZ seizure threshold was R-PIA > 2-ClAdo > NECA > CHA > S-PIA, with R-PIA being approximately 80 times more potent then S-PIA.

The intravenous administration of 5 ug/kg dose of R-PIA elicited a 20.3 $\pm$ 2.9 mmHg reduction in femoral arterial blood pressure when administered to rats anesthetized with pentobarbital (Table 2). This decrease in the mean arterial pressure (MAP) of 100 mmHg in anesthetized rats was dose-dependent for R-PIA and CHA as shown in Table 2. Phillis and Wu (129) have previously shown that reductions in neuronal firing rates were observed only when the fall in blood pressure exceeded 40-50 mmHg. Thus, the effect of 5 ug/kg dose of R-PIA on the seizure threshold would not appear to be a consequence of its

Table 1. Relative Potencies of Adenosine Analogs on PTZ Seizure
          Threshold in Rats

Compound	ED_{50} (umoles/kg)	95% Confidence Limits
R-PIA	0.086	0.054 - 0.118
2-ClAdo	0.418	0.302 - 0.534
NECA	1.75	0.94 - 2.55
CHA	4.12	3.95 - 4.29
S-PIA	6.80	5.79 - 7.18

The ED_{50} values and confidence limits were derived from modified
Hill plots of dose-response data shown in Figure 6.

Table 2. Alteration in Mean Arterial Blood Pressure Induced
          by R-PIA and CHA

Compound	Dose (ug/kg)	Decrease in MAP (mmHg)	% Control
R-PIA	1	0	100
	5	20.3 ± 2.9	79.7 ± 1.8
	50	68.3 ± 8.8	27.3 ± 4.1
	500	77.3 ± 5.0	24.0 ± 1.2
CHA	1	0	100
	5	7.4 ± 1.7	93.4 ± 1.6
	25	78.2 ± 2.4	30.3 ± 1.4
	50	83.2 ± 7.2	24.2 ± 0.9
	500	82.0 ± 4.7	27.1 ± 1.6
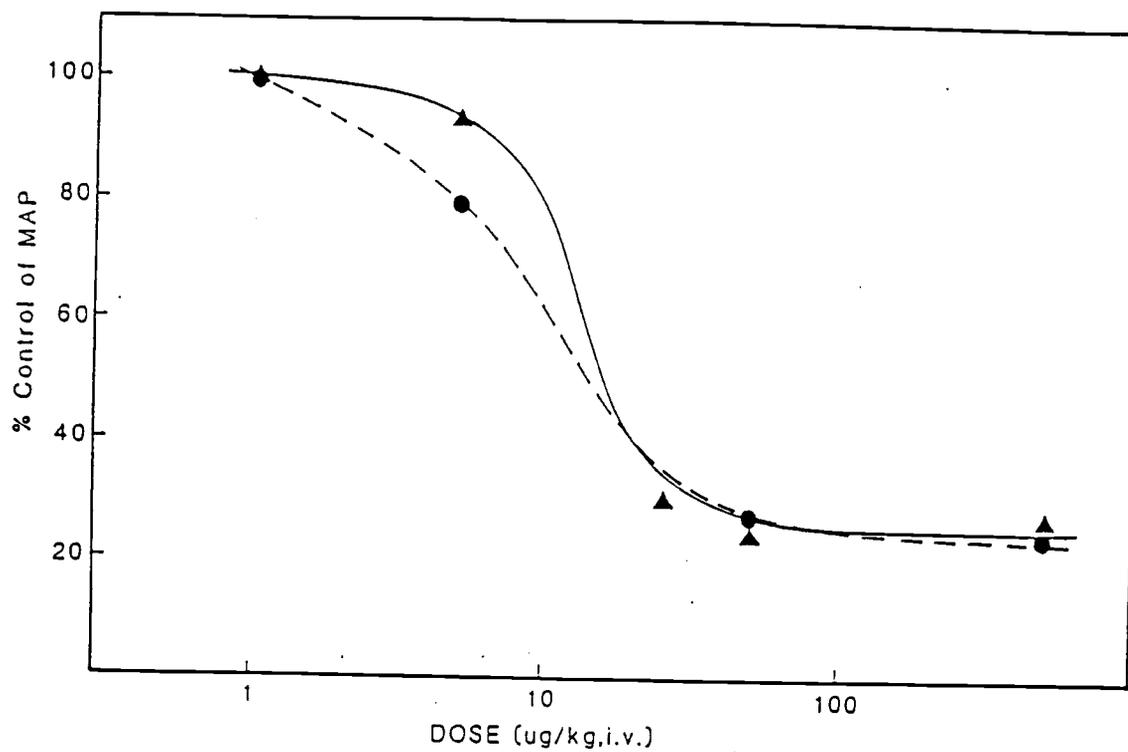
Figure 7. Dose-response curves for the effects of R-PIA (●)
and CHA (▲) on the mean femoral arterial pressure
in rats. All doses of R-PIA and CHA were
administered intravenously in a volume of 2 ml/kg.
Values are expressed as the percentage of control
of the mean arterial pressure before R-PIA or CHA
were given. Each value represents the mean of 3 to
4 animals.
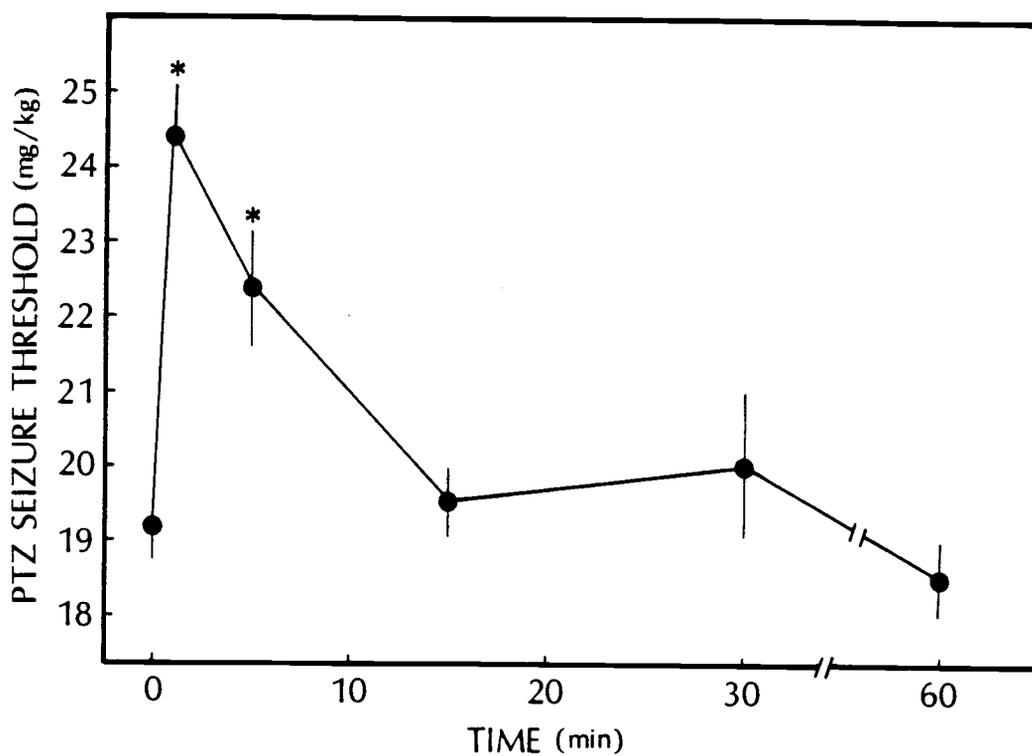
Figure 8. Time-response relationship for the effect of
dipyridamole (30 mg/kg, i.v.) on pentylenetetrazol
seizure threshold in rats. Values represent the
mean ± SEM for 8 to 10 animals for each time.
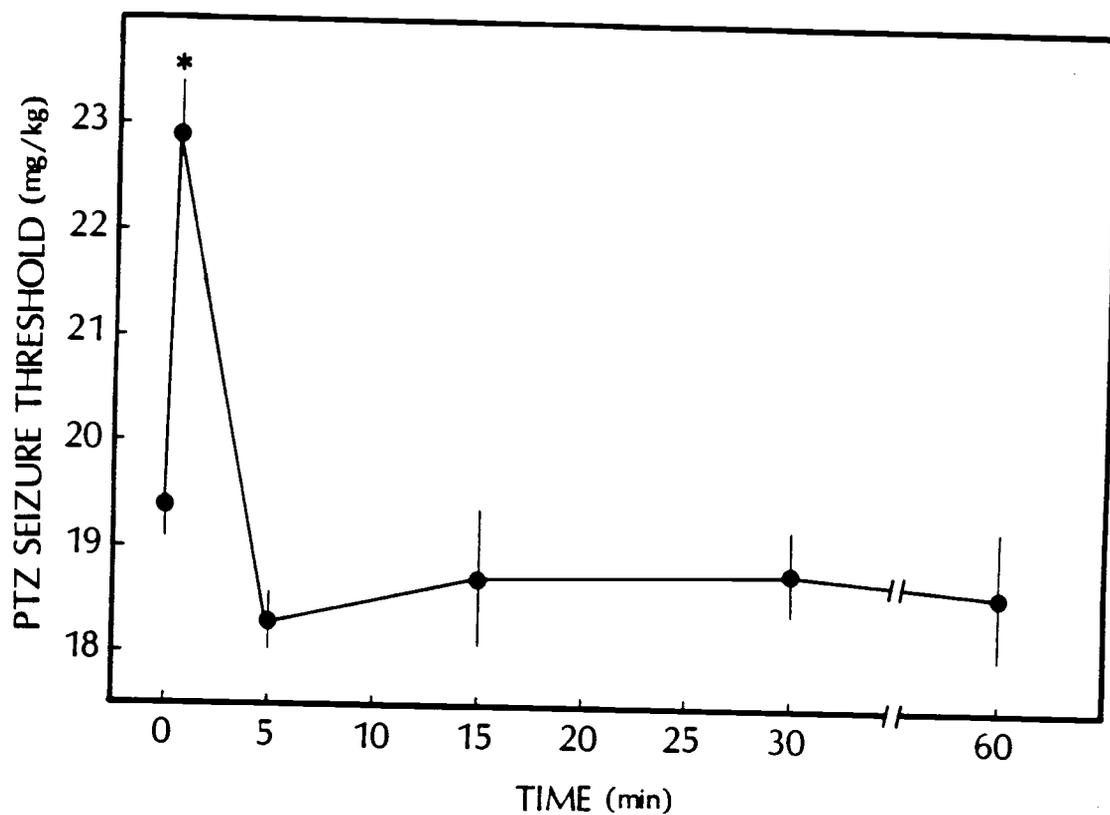Asterisks indicate significantly different (P < 0.05)
value from time 0 controls.
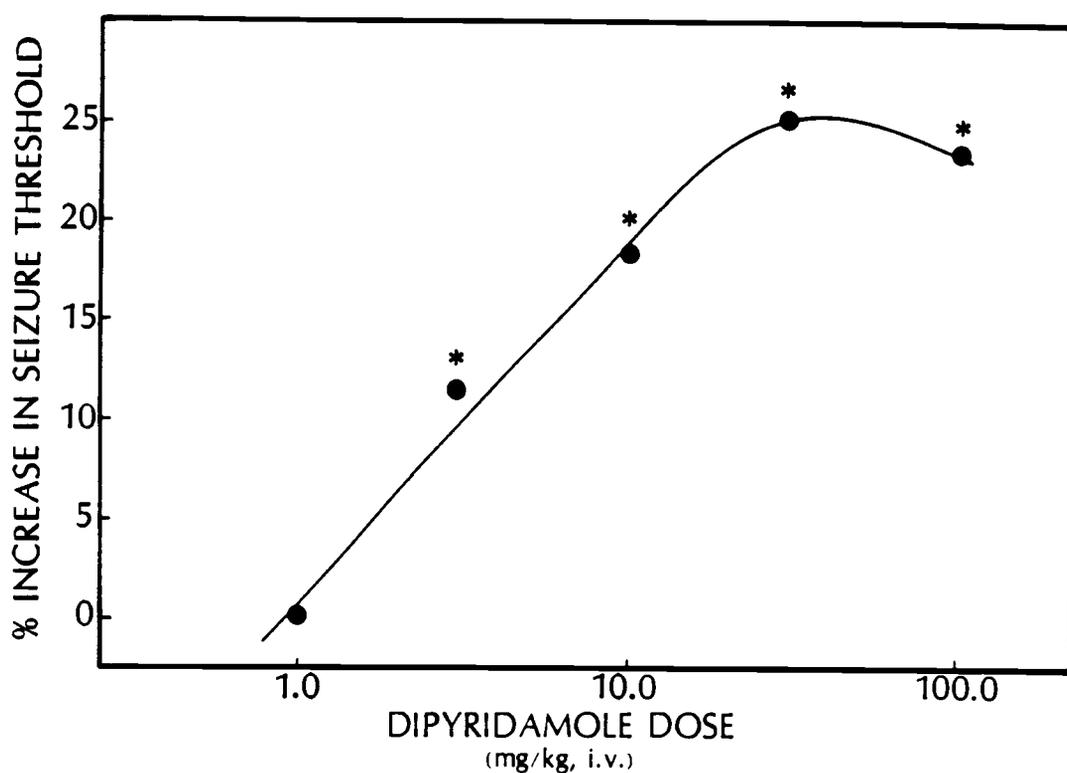
Figure 9.   Dose-response curve for the effect of dipyridamole
on pentylenetetrazol seizure threshold in rats.
All doses of dipyridamole were administered
intravenously in a volume of 2 ml/kg and seizure
thresholds were determined 1 min after injection.
Values represent the mean percentage increase in
seizure threshold of 8 to 10 rats at each dose.
Asterisks indicates significant difference (P < 0.05)
from saline-injected group calculated before
normalizing data.

hypotensive action. R-PIA and CHA appear to be equipotent in
producing this hypotensive effect as seen in Figure 7. The ED_{50}
values for R-PIA and CHA in reducing MAP are 32 and 38 ug/kg,
respectively. The approximate equipotency of R-PIA and CHA as
hypotensive agents shown here and by Phillis and Wu (129) versus
the fact that R-PIA was 48 times more potent then CHA as an
anticonvulsant agent, is further evidence against changes in
blood pressure as the underlying mechanism for the anticonvulsant
effect of adenosine analogs as shown in the present study.

The adenosine uptake inhibitor dipyridamole at a dose of 30
mg/kg, also resulted in an elevation in PTZ seizure threshold 1
min following its intravenous administration (Fig.8). An
elevation in PTZ seizure threshold was still observed at the 5
min time, but returned to control levels by 15 min. This is
similar to the time-response curves for 2-ClAdo and NECA on PTZ
seizure threshold. Dipyridamole was also found to produce a
dose-dependent elevation of PTZ seizure threshold when
administered intravenously (Fig.9). The maximum elevation in
seizure threshold (25% increase) was observed after the 30 mg/kg
dose of dipyridamole.

Discussion

The results of the present investigation have demonstrated
that adenosine receptor agonists produce dose-dependent reduction
in the susceptibility of rats to the convulsant effects of PTZ.

Of the five analogs of adenosine tested in the seizure threshold
model for PTZ, R-PIA was the most potent with doses as small as 5
ug/kg producing a significant increase in seizure threshold
without any apparent sedative or hypothermic effects. These
results are in agreement with the findings of Dunwiddie and Worth
(111) who reported that R-PIA induced a dose-dependent delay in
the onset of seizures induced by PTZ in mice. Moreover, Barraco
et. al. (113) have recently shown that both R-PIA and NECA
produced a dose-dependent suppression of amygdaloid-kindled
seizures in rats when administered by intracerebralventricular
injection.

In the present investigation, the rank order of potency of
adenosine analogs in producing an increase in the threshold dose
for PTZ seizures was: R-PIA > 2-ClAdo > NECA > CHA > S-PIA. The
80 fold potency difference between the R- and S-diastereoisomers
of PIA is consistent with an A_1 adenosine receptor mediation of
the seizure-attenuating action of these adenosine analogs, since
this degree of stereoselectivity is characteristic of A_1 but not
A_2 adenosine receptors (130). In accordance with these results
R-PIA has been reported to be 20 times more potent then S-PIA as
an anticonvusant in mice (105). Moreover, Daly (131) has
suggested that a rank order of potency of R-PIA > 2-ClAdo > NECA
> S-PIA may be considered as evidence supporting the involvement
of the A_1 subtype of adenosine receptors. Thus, the rank order
of potency for these 4 adenosine analogs observed in the study
are consistent with an A_1 adenosine receptor mediation of the

seizure suppressant effects of these compounds. The finding that CHA was less potent then R-PIA and 2-ClAdo in elevating PTZ seizure threshold is, however, not characteristic of the A_1 receptor subtype. It is noteworthy that Dunwiddie and Worth (111) reported that CHA and 2-ClAdo were relatively ineffective in affording protection against PTZ and kainic acid seizures in mice when compared to R-PIA. Further investigations employing intracerebroventricular injections may be required to determine whether this apparent anomaly is the result of either dispositional mechanisms or multiple receptor interactions.

Dipyridamole, the adenosine uptake inhibitor, produced an anticonvulsant effect by elevating PTZ seizure threshold in a manner similar to the adenosine analogs. Adenosine uptake inhibitors were also shown to posses agonist like anticonvulsant action in the hippocampal CA3 preparation (109) and in amygdaloid-kindled animal. This indicates a possible role for endogenous adenosine to modulate chemically and electrically induced seizures.

Chapter 3

Effects of Acute Theophylline on Pentylenetetrazol
Seizure Threshold

<u>Introduction</u>

The ability of adenosine analogs to elevate PTZ seizure

threshold indicates an involvement of the adenosine receptor in

mediating this anticonvulsant effect. If the adenosine receptor

is involved, then adenosine receptor antagonists should enhance

seizure activity. Administration of theophylline to a hippocampal

preparation resulted in epileptiform activity of the neuron

(105,109). This hyperexcitable state of the neuronal preparation

was measured as an increase in interictal spiking (105) and an

increase in bursting activity (109). In the rat, pretreatment

with theophylline or aminophylline magnified the convulsant

action of pilocarpine (108), pentylenetetrazol (133,138) and

maximum electroshock (138). In amygdaloid kindled rats, the

methylxanthines prolonged the after discharge duration and

severity of the seizure (114,115,118-121). Theophylline has also

been shown to induce convulsions in rats (135,137,139) and in

humans (136). Therefore, depending on the dose and route of

administration of theophylline, a proconvulsant or convulsant

effect can be observed. To further establish a role for the

adenosine receptor as the site of anticonvulsant action of the

adenosine analogs, pretreatment with the methylxanthines should

attenuate or block the action of adenosine. Dunwiddie (105), and

Ault and Wang (109) demonstrated the ability of theophylline to antagonize the anticonvulsant activity of adenosine in the hippocampal preparation. Barraco et al. (113) showed that caffeine antagonized the action of NECA on kindled seizures. Therefore it was important to study the effect of theophylline on seizure threshold for PTZ, and to observe the effect of theophylline pretreatment on the anticonvulsant action of adenosine analogs.

Materials and Methods

Male Sprague-Dawley rats (Simonsen Labs, Gilroy, Ca.) weighing 140-180 g were used. The animals were housed upon delivery, in groups of 4-6 at 22°C with food and water provided ad libitum. A standard light-dark cycle was maintained with a time-regulator light period from 0800 to 2000 hr. Each rat was used only once for any determination.

The seizure threshold for PTZ were determined by the method described in Chapter 2.

Time-response experiments were performed with theophylline (30 mg/kg) on PTZ seizure threshold in rats. PTZ seizure thresholds were determined 15, 30, 60 120 and 240 min following the intraperitoneal injection of theophylline. Dose-response curve for theophylline to PTZ seizure threshold was determined 15 min post-intraperitoneal injection.

To evaluate whether the increase in the seizure threshold

induced by analogs of adenosine was mediated by stimulation of adenosine receptors, the effect of pretreatment with theophylline on the response to 2-ClAdo was examined. Groups of rats received an intravenous injection of either saline or theophylline (5 mg/kg) 15 min prior to the intravenous administration of 2-ClAdo (1, 5 and 10 mg/kg), and seizure thresholds to PTZ were determined 1 min later.

To observe the selectivity of theophylline for the adenosine receptor, the anticonvulsant activity of diazepam and pentobarbital were determined in animals pretreated with theophylline. Groups of rats received intraperitoneal injections of diazepam (1.25 mg/kg) or pentobarbital (5 mg/kg) 15 min prior to the intravenous administration of either saline or theophylline (5 mg/kg), and the seizure threshold for PTZ was determined 15 min later.

To evaluate the role of endogenous adenosine in the anticonvulsant effect of dipyridamole, the effect of theophylline pretreatment was determined on dipyridamoles response. Groups of rats received either saline or theophylline (5 mg/kg) intravenously 15 min prior to the intavenous administration of dipyridamole (30 mg/kg), and the seizure threshold for PTZ was determined 1 min later.

Statistical analyses of data were computed using a Student's two-tailed t test.

Results

In contrast to the anticonvulsant action of the adenosine agonists, acute theophylline administration produced a reduction in PTZ seizure threshold. As shown in Figure 10, the minimum threshold occurred 15 min after the administration of theophylline (30 mg/kg, i.p.) and a significant reduction was still observed after 120 min. The maximum reduction of threshold elicited by theophylline was approximately 27% after the 30 mg/kg dose (Fig. 11). While the maximum response to theophylline was associated with the 30 mg/kg dose, as shown in Figure 11, doses of 15 and 60 mg/kg also produced significant reductions in seizure threshold. The biphasic nature of the dose-response curve for theophylline is most likely a function of the mutiple actions of this methylxanthine. While methylxanthines certainly possess multiple actions, they antagonize the effects of adenosine at doses 3-300 times smaller than those causing inhibition of phosphodiesterase or mobilization of calcium (142).

The ability of theophylline pretreatment to antagonize the anticonvulsant action of 2-ClAdo is shown in Figure 12. Theophylline (5 mg/kg, i.v.) was administered 15 min prior to the intravenous injection of various doses of 2-ClAdo. As shown in Figure 12, this dose of theophylline administered alone had no significant effect on the seizure threshold for PTZ. However, pretreatment with theophylline did result in a significant antagonism of the increase in the seizure threshold induced by 2-
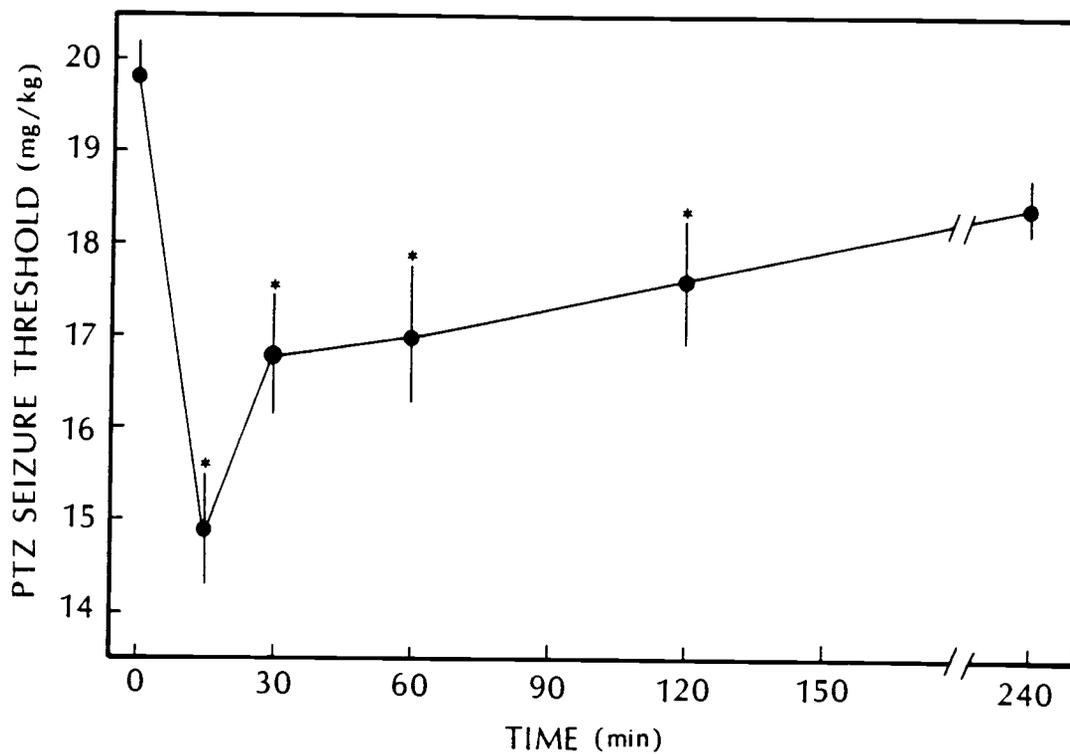
Figure 10. Time-response relationship for the effect of
theophylline (30 mg/kg, i.p.) on the seizure
threshold to pentylenetetrazol (PTZ) in rats.
Values represent the mean ± SEM for 8 to 14 animals
for each time point. Asterisks indicate
significantly different (P < 0.05) values from time
0 controls.
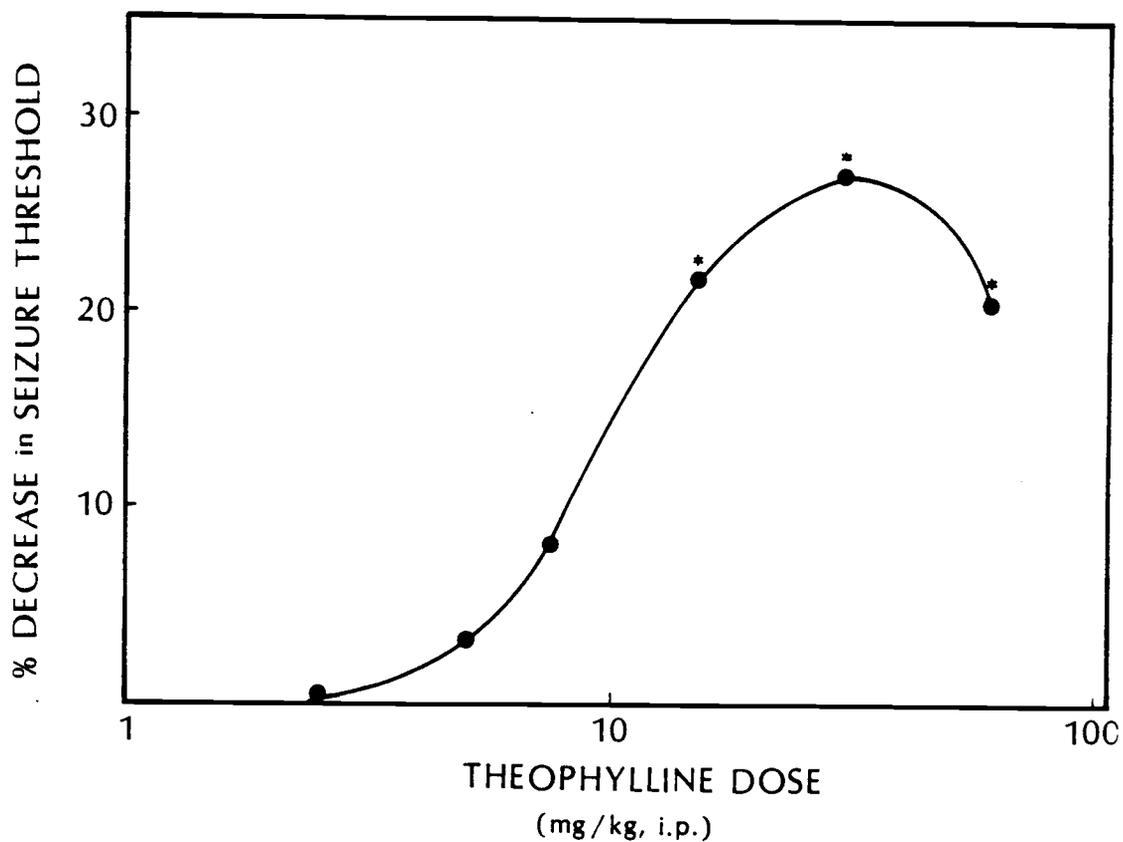
Figure 11. Dose-response curve for the effect of theophylline on the seizure threshold to pentylenetetrazol in rats. All doses of theophylline were administered intraperitoneally in a volume of 2.5 ml/kg and the seizure threshold was determined 15 min after injection. Values represent the mean percentage decrease in seizure threshold of 8 to 16 rats at each dose. Asterisks indicate significant difference (P < 0.05) from saline-injected group calculated before normalizing the data.
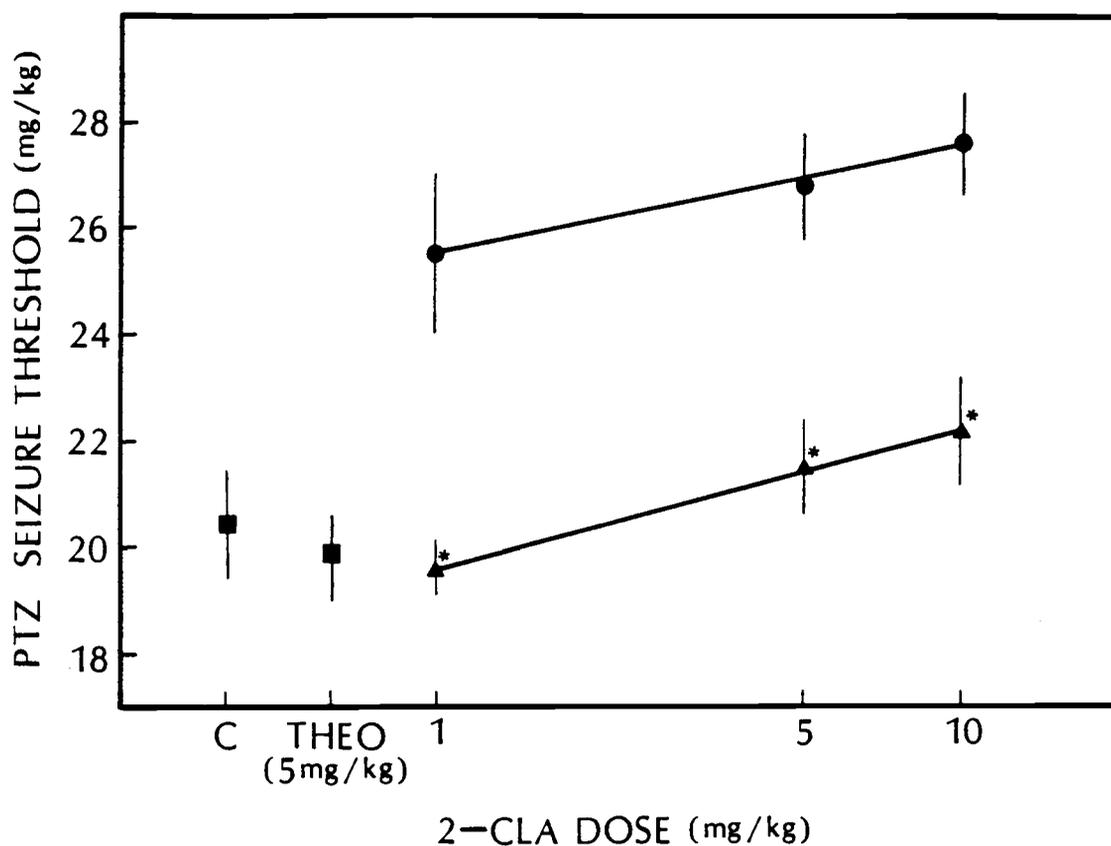
Figure 12. Antagonism of the anticonvulsant effects of 2-ClAdo
by theophylline (THEO). Groups of rats (n = 8 for
each value) received an intravenous injection of
either saline (●) or 5 mg/kg theophylline (▲) 15
min prior to the intravenous administration of
various doses of 2-ClAdo (1,5 and 10 mg/kg), and
seizure threshold was determined 1 min later. As
indicated this dose of theophylline (■) did not
alter the seizure threshold for pentylenetetrazol
(PTZ) when administered alone. Asterisks indicate
that a statistically significant-reduction (P < 0.05)
of the effect of all doses of 2-ClAdo was observed
in rats pretreated with theophylline.

ClAdo at each of the doses tested (Fig. 12). The selectivity of the theophylline-induced antagonism of the anticonvulsant effect of 2-ClAdo was assessed by examining the influence of theophylline administration on the response to both diazepam and pentobarbital. As shown in Figure 13, diazepam (1.25 mg/kg) and pentobarbital (5 mg/kg) both produced increases in the dose of PTZ required to elicit a seizure when administered alone. Treatment with theophylline (5 mg/kg, i.v.) failed to antagonize the increase in the seizure threshold for PTZ induced by diazepam and actually produced a significant enhancement of the anticonvulsant effect of pentobarbital (Fig. 13). These results suggest that the anticonvulsant effects of 2-ClAdo is mediated through an interaction with adenosine receptors.

Interestingly, the anticonvulsant action of dipyridamole was not antagonized by the theophylline pretreatment (Fig. 14). In fact, theophylline (5 mg/kg, i.v.) pretreatment actually enhanced the effect of dipyridamole on seizure threshold. Theophylline also enhanced the anticonvulsant action of pentobarbital, and this interaction may involve dispositional mechanisms. The lack of antagonism by theophylline of dipyridamole's effect on seizure threshold suggests that the observed anticonvulsant action of dipyridamole was not mediated by a potentiation of the effects of endogenous adenosine. Hammond and Clanachan (143) have shown dipyridamole to have a low affinity for the adenosine uptake site as labeled by [^{3}H]-nitrobenzylthioinosine in rat brain tissue.
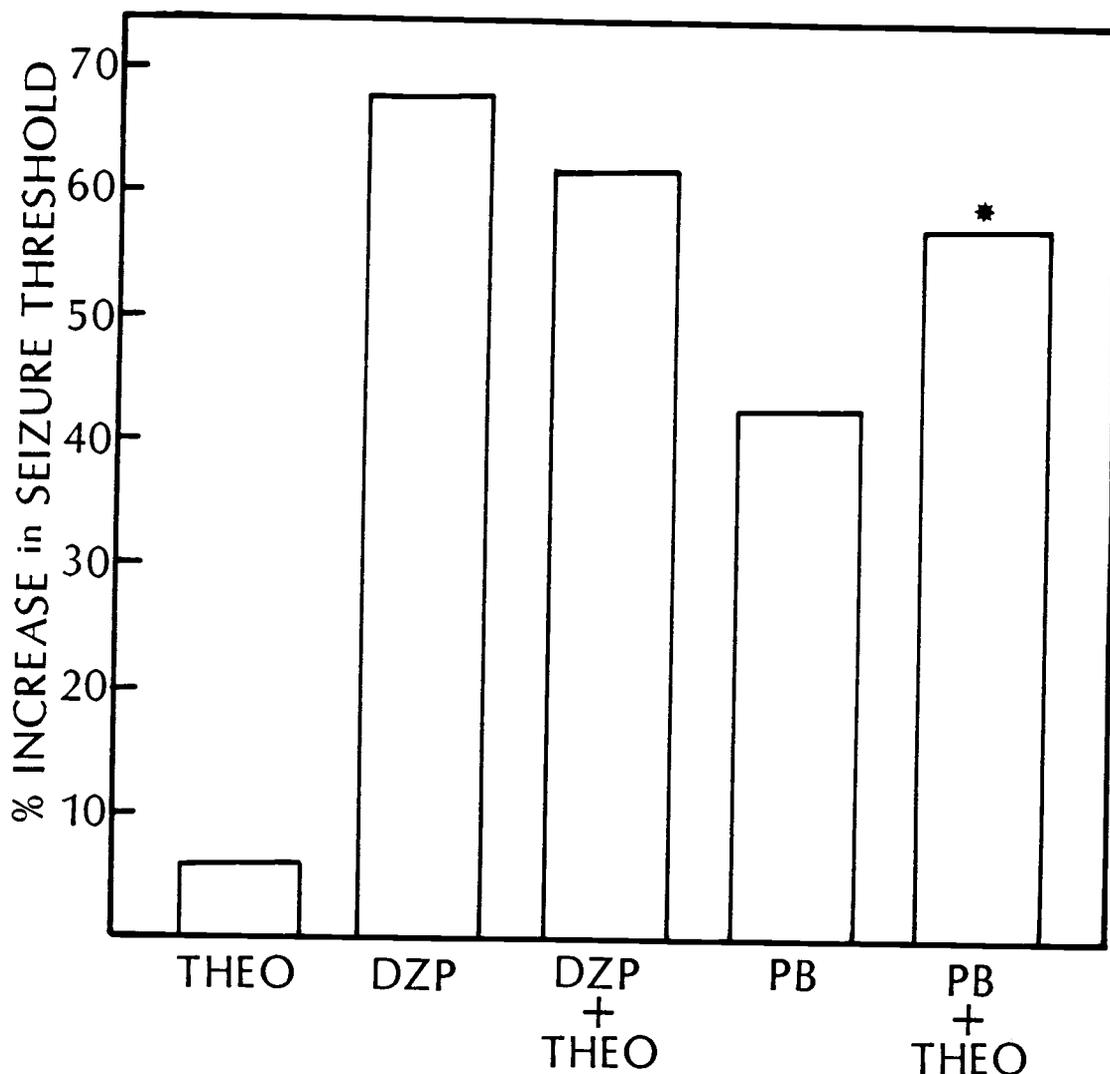
Figure 13. Lack of effect of pretreatment with theophylline on the anticonvulsant activity of diazepam (DZP) and pentobarbital (PB). Groups of rats (n = 8 for each bar) received intraperitoneal injections of either diazepam (1.25 mg/kg) or pentobarbital (5 mg/kg) 15 min prior to the intravenous administration of either 5 mg/kg theophylline (THEO) or saline, and the seizure threshold for pentylenetetrazol was determined 15 min later. Values represent the mean percentage increase in seizure threshold relative to saline-injected controls. As indicated this dose of theophylline (5 mg/kg,i.v.) did not alter the seizure threshold for pentylenetetrazol when administered alone. The asterisk indicates a statistically significant (P < 0.05) increase in the seizure threshold for pentylenetetrazol when compared to the group treated with pentobarbital (PB) alone.
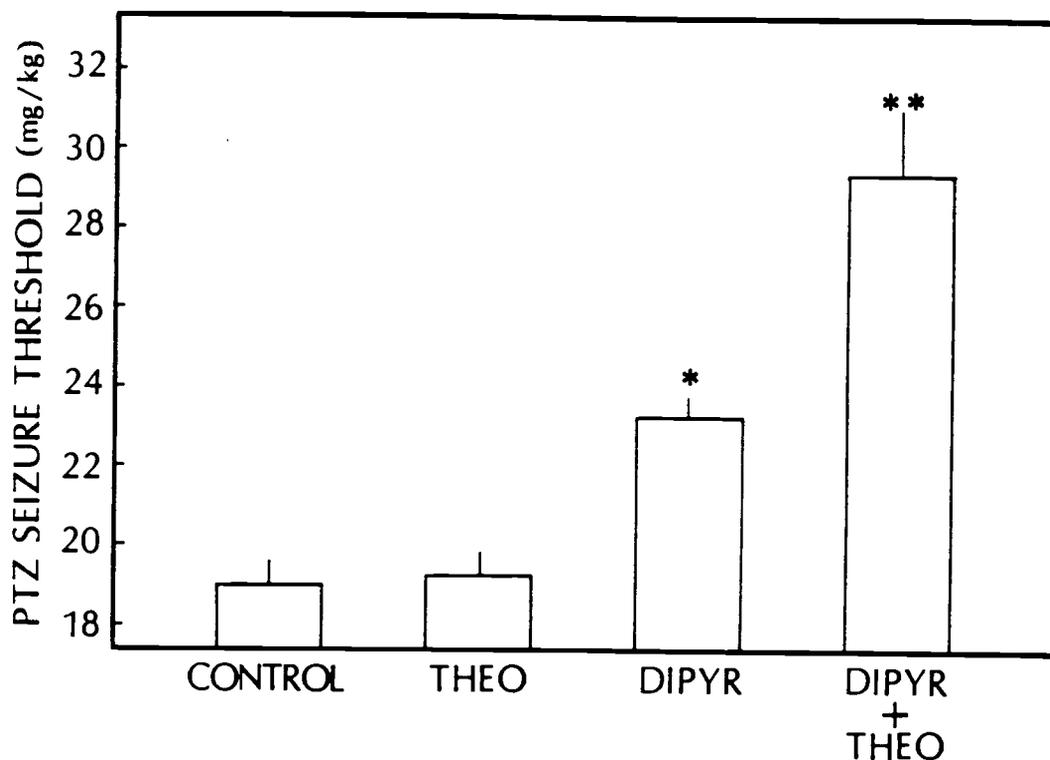
Figure 14. Lack of effect of pretreatment with theophylline
(THEO; 5 mg/kg,i.v.) on the anticonvulsant action
of dipyridamole (DIPYR; 30 mg/kg,i.v.). Groups
of rats (n = 8 for each bar) received intravenous
injections of saline or theophylline 15 min prior
to the administration of dipyridamole, and seizure
threshold was determined 1 min later. Single
asterisk indicates dipyridamole (DIPYR) alone
produced a statistically significant (P < 0.05)
increase in pentylenetetrazol (PTZ) seizure
threshold when compared to the saline control group.
Double asterisks indicate a statistical difference
(P < 0.01) from the group treated with dipyridamole
alone.

Discussion

Evidence in support of a tonic activation of adenosine receptors was provided by the demonstration of dose-related proconvulsant action of theophylline in the present study. These results confirm a previous report of the ability of aminophylline to reduce the seizure threshold for PTZ in rats (138). The proconvulsant activity of theophylline and aminophylline implies that endogenous adenosine functions as a modulator of excitability in the CNS. Moreover, the 2-ClAdo-induced elevation of the seizure threshold for PTZ was antagonized by pretreatment with theophylline, suggesting that an activation of adenosine receptors was operative in the observed anticonvulsant action of 2-ClAdo. In accordance with these results, Barraco et al (113) have recently demonstrated that pretreatment with caffeine antagonized the anticonvulsant effects of NECA in the amygdaloid kindling model in the rat. Moreover, Dunwiddie and Worth (111) have previously reported that theophylline reversed the increase in the seizure latency for PTZ induced by R-PIA in rats. The inability of theophylline to antagonize the anticonvulsant action of diazepam or pentobarbital is further support for the involvement of the adenosine receptor in the anticonvulsant action of 2-ClAdo.

Chapter 4

Modulation of Seizure Susceptibility by
2-Chloroadenosine and Theophylline on the Chemoconvulsants
Bicuculline and Picrotoxinin

Introduction

Adenosine analogs and theophylline appear to modulate

pentylenetetrazol seizure activity through an interaction with

CNS adenosine receptors. If this alteration in convulsant

activity is due to the modulation of neuronal activity, then

adenosine analogs should possess anticonvulsant activity to a

wide variety of chemoconvulsants with different mechanisms of

action. To evaluate the general regulatory role of the adenosine

receptor on CNS excitability induced by chemoconvulsants,

bicuculline and picrotoxinin seizure thresholds were determined

in the presence of 2-ClAdo or theophylline.

Materials and Methods

Male Sprague-Dawley rats (Simonsen Labs, Gilroy, Ca.)

weighing 140-180 g were used. The animals were housed upon

delivery, in groups of 4-6 at 22°C with food and water provided

ad libitum. A standard light-dark cycle was maintained with a

timer-regulator light period from 0800 to 2000 hr. Each rat was

used only once for any determination.

The timed-infusion technique described in detail in Chapter

2 was used to determine seizure thresholds for the
chemoconvulsants, bicuculline (BIC) and picrotoxinin (PTX). BIC
and PTX concentrations and infusion rates were altered to
compensate for the difference in potency as convulsant agents
compared to PTZ. The mg/kg seizure threshold value for BIC was
calculated from a concentration of 0.05 mg/ml infused at a rate
of 1.2 ml/min, while PTX seizure threshold value was determined
from a concentration of 0.5 mg/ml at an infusion rate of 1.6
ml/min.

Dose-response curves were generated for 2-ClAdo to BIC and
PTX seizure threshold. The seizure thresholds were determined 1
min post-intravenous injection of 2-ClAdo. Modified Hill plots
were used to compare quantitatively the dose-response curves of
2-ClAdo to PTZ, BIC and PTX seizure threshold. The modified Hill
plots were determined as described in detail in Chapter 2.

Dose-response curves were generated for theophylline to BIC
and PTX seizure threshold. The seizure thresholds were
determined 15 min post-intraperitoneal injection of theophylline.
Modified Hill plots were used to compare quantitatively the dose-
response curves of theophylline to PTZ, BIC and PTX seizure
threshold. The modified Hill plots were determined as described
in detail in Chapter 2.

The ability of theophylline pretreatment to antagonize 2-
ClAdo's elevation of BIC seizure threshold was studied to
establish the role of the adenosine receptor in this response.
Groups of rats received intravenous injection of either saline or

theophylline (5 mg/kg) 15 min prior to the intravenous

administration of 2-ClAdo (3 mg/kg), and seizure threshold to BIC

was determined 1 min later.

Results

The dose-response curves of 2-ClAdo to bicuculline (Fig. 15)

and picrotoxinin (Fig. 16) seizure threshold resembles the dose-

response curve to pentylenetetrazol seizure threshold (Fig. 6).

The maximum elevation in seizure threshold to BIC and PTX

elicited by 2-ClAdo was 42% and 20%, respectively. Of the three

chemoconvulsants studied it appears that the BIC seizure

threshold is the most sensitive to the modulatory action of 2-

ClAdo, while PTX seizure threshold was the least sensitive. The

ED_{50} values and their corresponding 95% confidence limits for 2-

ClAdo to elevate the seizure threshold to PTZ, BIC and PTX are

shown in Table 3. The similarity in ED_{50} values with overlapping

of the 95% confidence limits suggest that 2-ClAdo is producing

its anticonvulsant action to these chemoconvulsants via a similar

mechanism of action.

To substantiate the role of the adenosine receptor as a

modulator of seizure activity, theophylline dose-response curves

were generated to BIC (Fig. 17) and PTX (Fig. 18) seizure

threshold in rats. Theophylline resulted in an increased

sensitivity to the convulsant action of BIC and PTX very similar

to the effect theophylline had on PTZ seizure threshold (Fig.
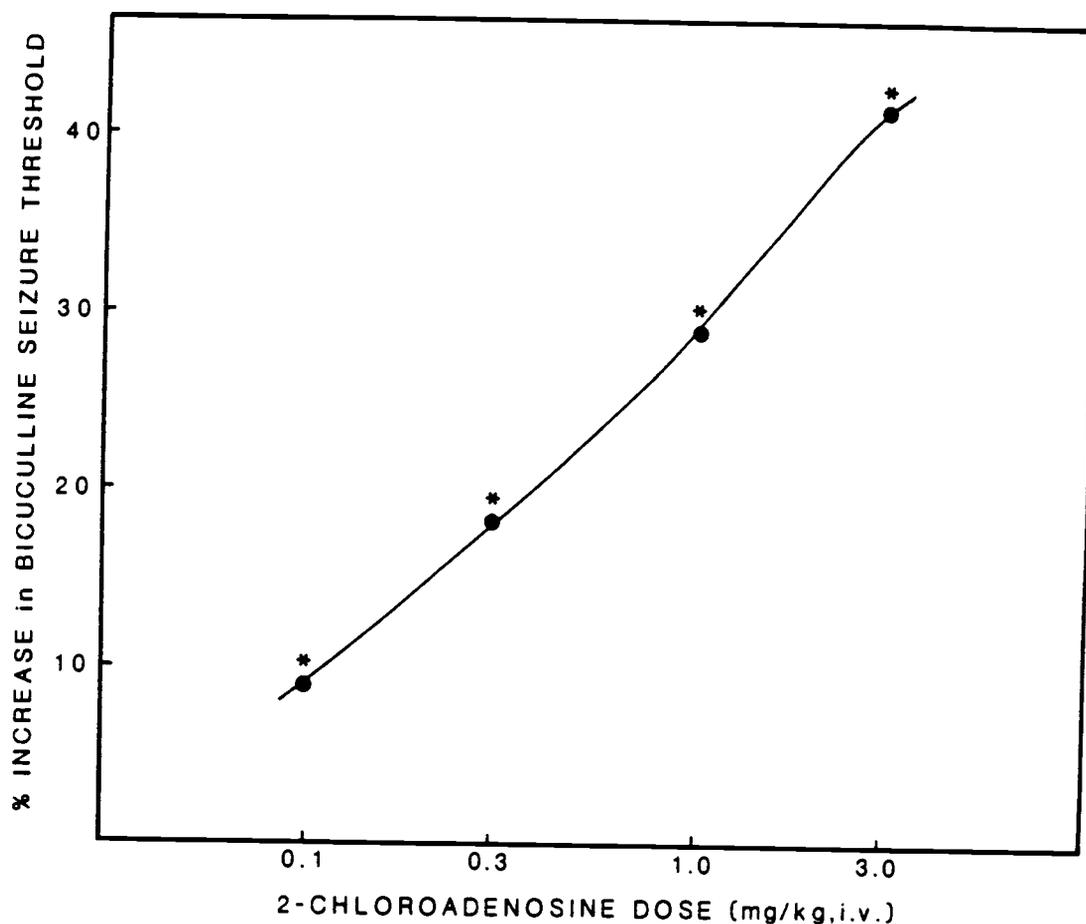
Figure 15. Dose-response curve for the effect of 2-chloro-
adenosine (2-ClAdo) on the seizure threshold for
bicuculline. All doses of 2-ClAdo were administered
intravenously in a volume of 2 ml/kg and all tests
were performed 1 min after injection. Values are
expressed as the mean percentage increase in seizure
threshold as compared to saline injected controls.
Each value represents the mean of 8 to 12 rats.
Asterisk indicates significant difference (P < 0.05)
from saline-injected group calculated before
normalizing the data.
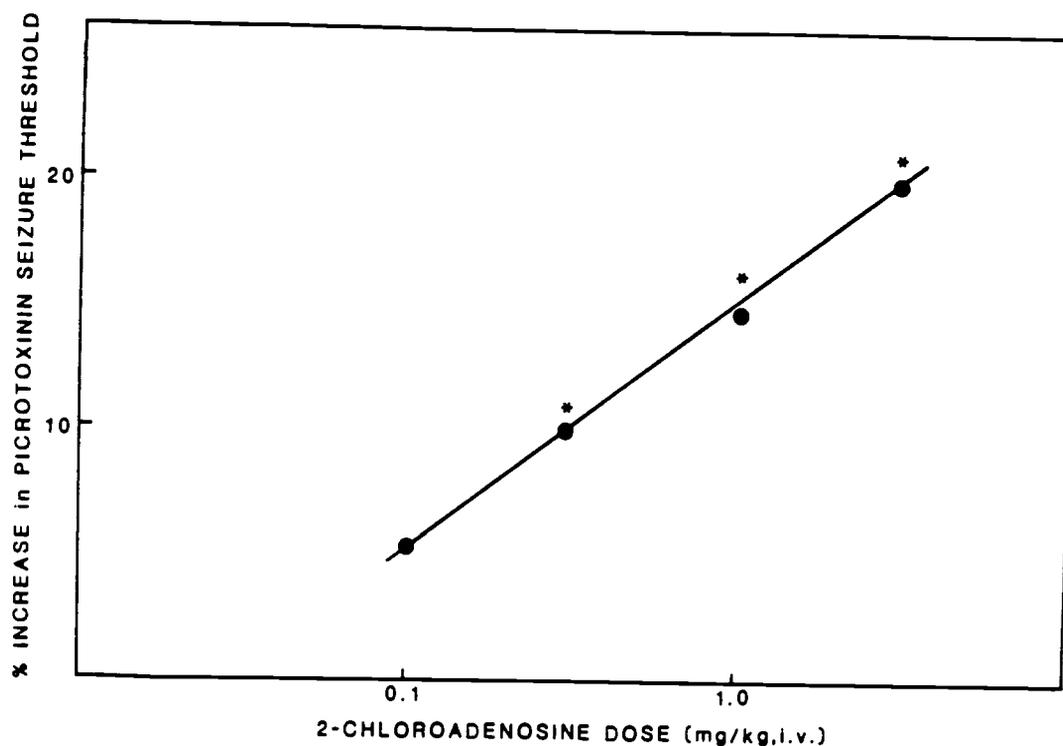
Figure 16. Dose-response curve for the effect of 2-chloro-
adenosine (2-ClAdo) on the seizure threshold for
picrotoxinin. All doses of 2-ClAdo were
administered intravenously in a volume of 2 ml/kg
and all tests were performed 1 min after injection.
Values are expressed as the mean percentage increase
in seizure threshold as compared to saline-
injected controls. Each value represents the mean
of 8 to 12 rats. Asterisk indicates significant
difference (P < 0.05) from saline-injected group
calculated before normalizing the data.

Table 3. ED_{50} Values for 2-Chloroadenosine-induced Elevation of Seizure Threshold to Various Chemoconvulsants

Chemoconvulsant	ED_{50} (mg/kg, i.v.)	95% Confidence Limits
Pentylenetetrazol	0.126	0.091 – 0.161
Bicuculline	0.353	0.096 – 1.30
Picrotoxinin	0.311	0.226 – 0.427

The ED_{50} values and confidence limits were derived from modified Hill plots of the dose-response data shown in Figure 6, 15 and 16

11). A biphasic dose-response curve was observed for theophylline to BIC (Fig. 17) and PTX (Fig. 18) seizure threshold, with a maximum reduction in seizure threshold occurring at the 30 mg/kg dose of theophylline. These results are identical to the effect theophylline produced on PTZ seizure threshold (Fig. 11). ED_{50} values and their corresponding 95% confidence limits for theophylline to PTZ, BIC and PTX seizure threshold are shown in Table 4. ED_{50} values for the proconvulsant effect of theophylline on the three chemoconvulsants are comparable, with overlapping in the 95% confidence limits, indicating a similar mechanism of action. The ability of theophylline to produce a proconvulsant effect demonstrates a role for endogenous adenosine as a modulator of CNS excitability.

The ability of theophylline pretreatment to antagonize the anticonvulsant action of 2-ClAdo on BIC seizure threshold is shown in Figure 19. The intravenous administration of theophylline (5 mg/kg) had no significant effect on the seizure threshold for BIC (Fig. 19). However, pretreatment with theophylline did result in a significant antagonism of the increase in BIC seizure threshold induced by 2-ClAdo (3mg/kg) (Fig. 19). Therefore, 2-ClAdo appears to be mediating its anticonvulsant action through the adenosine receptor.
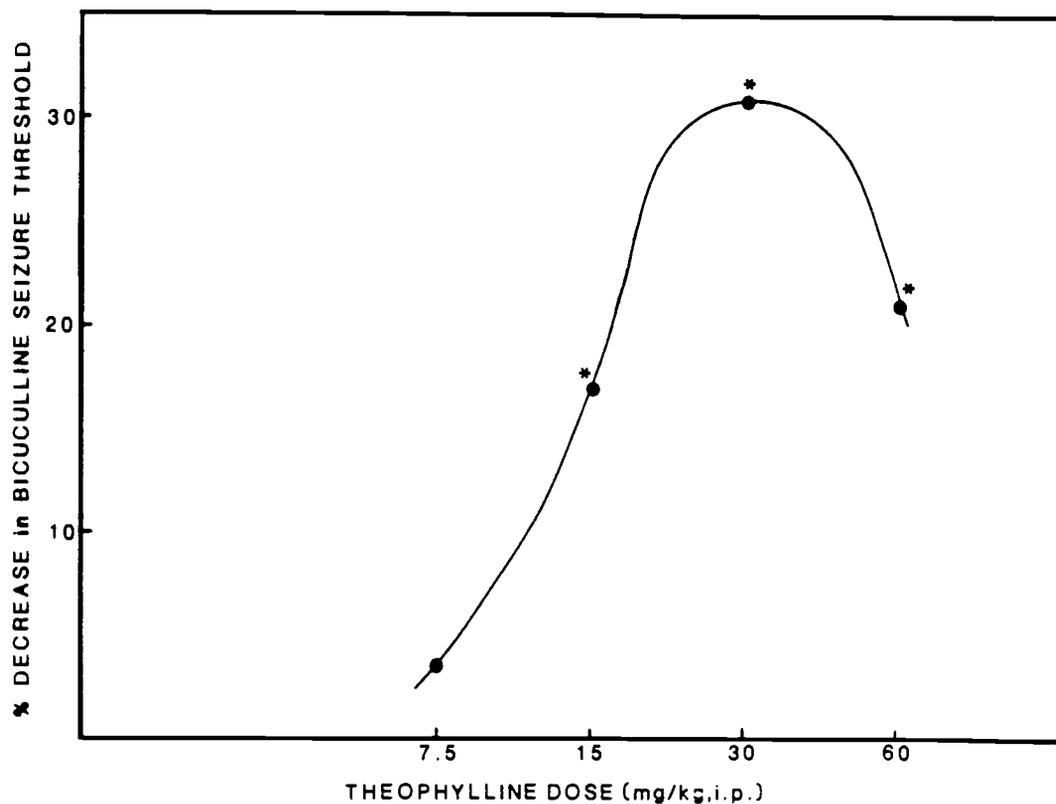
Figure 17. Dose-response curve for the effect of theophylline
on the seizure threshold of bicuculline in rats.
All doses of theophylline were administered
intraperitoneally in a volume of 2.5 ml/kg and the
seizure threshold determined 15 min after injection.
Values represent the mean percentage decrease in
seizure threshold of 8 to 12 rats at each dose.
Asterisk indicates significant difference ($P < 0.05$)
from the saline-injected group calculated before
normalizing the data.
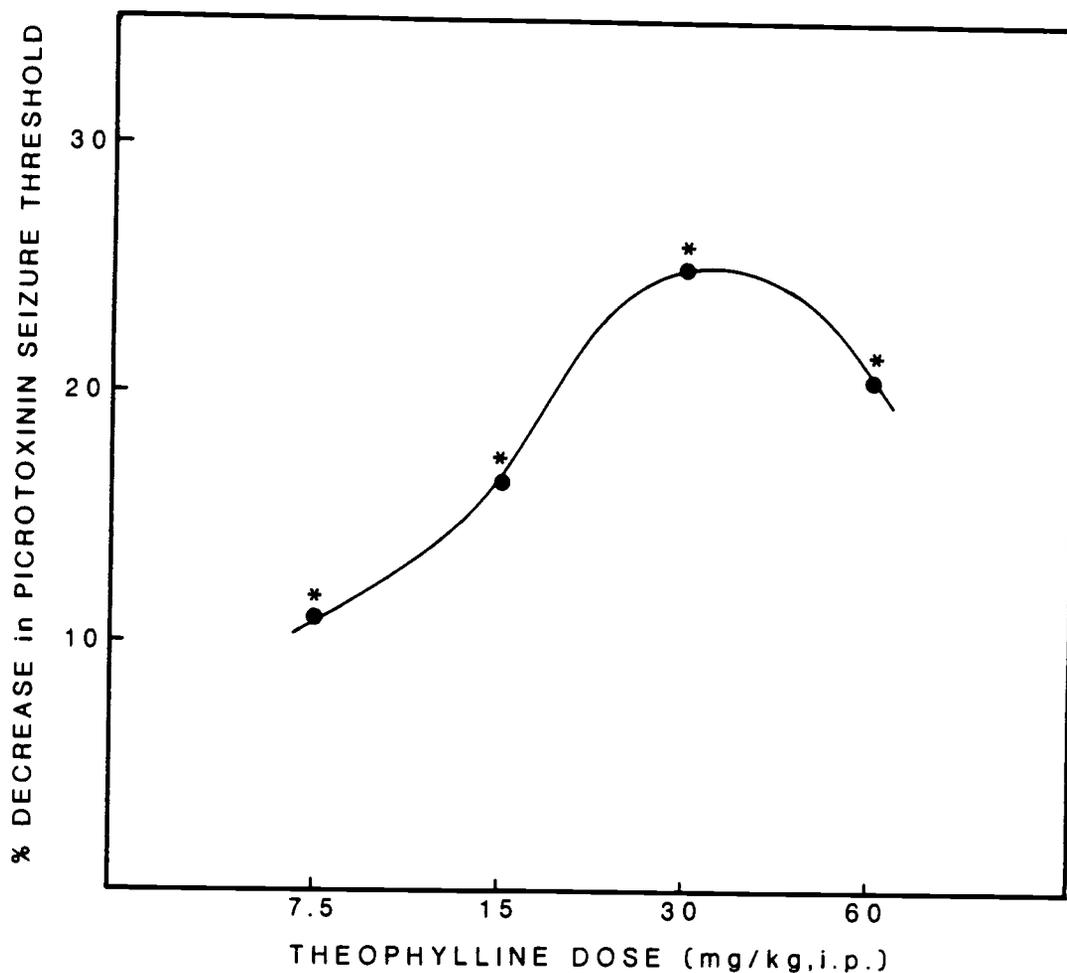
Figure 18. Dose-response curve for the effect of theophylline
on the seizure threshold of picrtoxinin in rats.
All doses of theophylline were administered
intraperitoneally in a volume of 2.5 ml/kg and the
seizure threshold determined 15 min after injection.
Values represent the mean percentage decrease in
seizure threshold of 8 to 12 rats at each dose.
Asterisk indicates significant difference ($P < 0.05$)
from saline-injected group calculated before
normalizing the data.

Table 4. ED_{50} Values for Theophylline-induced Decrease
of Seizure Threshold to Various Chemoconvulsants

Chemoconvulsant	ED_{50} (mg/kg, i.p.)	95% Confidence Limits
Pentylenetetrazol	9.81	3.9 - 25.0
Bicuculline	12.50	3.0 - 52.0
Picrotoxinin	9.40	0.3 - 268

The ED_{50} values and confidence limits were derived from modified
Hill plots of the dose-response data shown in Figure 11,17 and
18
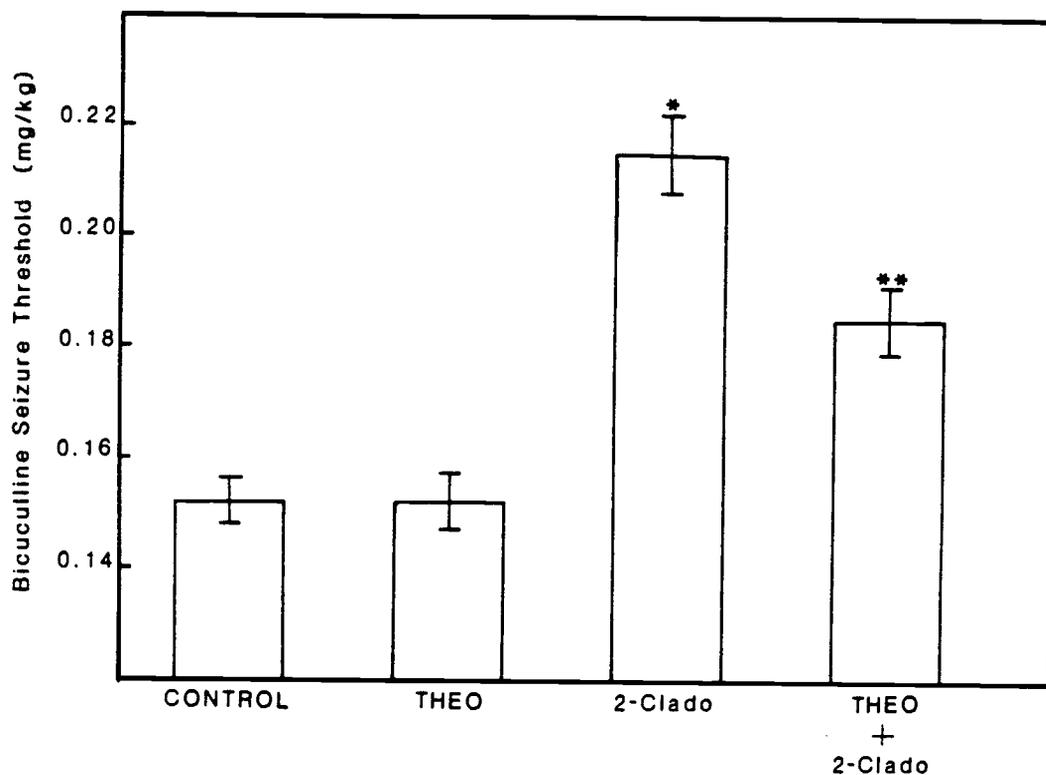
Figure 19. Antagonism of the anticonvulsant effect of 2-ClAdo
by theophylline (THEO). Groups of rats (n = 8 for
each bar) received an intravenous injection of
either saline or 5 mg/kg theophylline 15 min prior
to the intravenous administration of 2-ClAdo
(3 mg/kg) and seizure threshold to bicuculline was
determined 1 min later. As indicated this dose of
theophylline (THEO) did not alter the seizure
threshold for bicuculline when administered alone.
Single asterisk indicates a statistically
significant (P < 0.05)increase in the seizure
threshold for bicuculline when compared to the
saline control group. Double asterisk indicates
a statistical difference (P < 0.05) from the group
treated with 2-ClAdo alone.

Discussion

The ability of 2-ClAdo and theophylline to alter the seizure

activity of three different chemoconvulsant agents is indicative

of a general regulatory action of the adenosine receptor on CNS

excitability. Dunwiddie and Worth showed adenosine analogs to

prolong the latency time to seizure activity induced by

pentylenetetrazol, strychnine, kainic acid, 3-mercaptoproprionic

acid and picrotoxinin (111). The similarity in ED_{50} values for

2-ClAdo to elevate the seizure threshold for PTZ, BIC and PTX

indicates that 2-ClAdo's anticonvulsant action was due to a

similar mechanism of action. However, this data was not in

agreement with Dunwiddie and Worth. Dunwiddie and Worth

demonstrated a varying degree of potency of 2-ClAdo, R-PIA and

CHA as anticonvulsants (111). These differences in relative

potencies of R-PIA, CHA and 2-ClAdo against various

chemoconvulsants was interpreted as evidence for the involvement

of multiple adenosine receptors (111). The discrepancy in

results may be due to the different techniques used to measure

convulsant activity. Theophylline produced a proconvulsant

effect against PTZ, BIC and PTX. The similarity in ED_{50} values

for theophylline to decrease the seizure threshold for PTZ, BIC

and PTX indicates a similar mechanism of action. Theophylline

also produced a proconvulsant effect when a subconvulsant dose of

PTZ was administered to mice (111). Anatagonism of the

anticonvulsant effect of 2-ClAdo by theophylline establishes the

involvement of the adenosine receptor in the action of these compounds.

These results imply that agonists at the adenosine receptor suppress the excitatory effect of chemoconvulsants on neuronal activity. This supports the theory that adenosine and adenosine analogs can delay the initiation of a seizure by stabilizing the excitability of the neuronal activity.

Chapter 5

Effect of Benzodiazepine Antagonists on 2-Chloroadenosine's
Elevation of Bicuculline Seizure Threshold in rats.

Introduction

A great deal of interest in the interaction of adenosine at

benzodiazepine receptors has been generated since purines were

shown to inhibit the specific binding of ^{3}H-diazepam (193,194)

and ^{3}H-flunitrazepam (196) in rat brain. Of the endogenous

purines examined, adenine was the most potent purine tested in

displacing ^{3}H-diazepam and ^{3}H-flunitrazepam (193,194,196), while

adenosine was the least potent naturally occurring purine (199).

The methylxanthines such as caffeine and theophylline were the

most potent purine derivatives to inhibit the specific binding of

[3]H-diazepam and ^{3}H-flunitrazepam in rat brain membranes

(193,194,196). The methylxanthines have also been shown to

inhibit other pharmacological actions of the benzodiazepines.

The anticonvulsant action of diazepam against maximum

electroshock (188,189) and PTZ-induced (195) seizures in mice

were blocked by pretreatment with aminophylline. Flurazepam-

induced depression of spontaneous firing rate of rat cerebral

cortical neurons was attenuated by the prior administration of

theophylline to the perfusion medium (197). Caffeine antagonized

the effects of diazepam on dorsal root potential and ventral

root-evoked Renshaw cell discharge in cats (195). Moreover, the

benzodiazepine agonists such as diazepam, clonazepam and

flunitrazepam were shown to inhibit seizures induced by caffeine in mice (191). The benzodiazepine antagonist CGS 8216 was five-fold more active than theophylline in displacing the specific binding of ^{3}H-cyclohexyladenosine in rat brain (184) and blocked the activation of adenylate cyclase by adenosine in guinea pig brain (192). These results suggest that the methylxanthines and the benzodiazepine agonists may be acting at similar sites. Based on these results it was important to evaluate the involvement of benzodiazepine receptors in the anticonvulsant action of 2-ClAdo. Therefore, the anticonvulsant activity of 2-ClAdo was examined in the presence of two selective benzodiazepine receptor antagonists, RO 15-1788 and CGS 8216.

Materials and Methods

Male Sprague-Dawley rats (Simonsen Labs, Gilroy, Ca.) weighing 180-250 g were used. The animals were housed upon delivery, in groups of 4-6 at 22°C with food and water provided ad libitum. A standard light-dark cycle was maintained with a timer-regulator light period from 0800 to 2000 hr. Each rat was used only once for any experimental determination.

BIC seizure threshold was determined by the method described in Chapter 4.

Animals were pretreated with benzodiazepine receptor antagonists to evaluate the involvement of the benzodiazepine receptor in the anticonvulsant action of 2-ClAdo. The

benzodiazepine receptor antagonists that were used were RO 15-1788 and CGS 8216. Groups of rats received intraperitoneal injections of saline or RO 15-1788 (1 mg/kg) 15 min prior to intravenous administration of 2-ClAdo (3.0 mg/kg), and seizure thresholds for BIC were determined 1 min later. A second group of rats received intravenous injections of saline or CGS 8216 (0.5 mg/Kg or 2.0 mg/Kg) 10 min prior to intravenous 2-ClAdo administration (3.0 mg/Kg). BIC seizure threshold determinations were made 1 min later.

The benzodiazepine antagonist properties of RO 15-1788 and CGS 8216 were evaluated by examining the increase in BIC seizure threshold induced by diazepam (DZ) in their respective presence. Groups of rats received intraperitoneal injections of diazepam (1.25 mg/kg) 15 min prior to a second intraperitoneal injection of saline or RO 15-1788 (1.0 mg/kg), and seizure thresholds were performed 15 min after the last injection. Intraperitoneal injections of diazepam (1.25 mg/kg) were administered to a second group of rats 20 min prior to intravenous saline or CGS 8216 (0.5 mg/kg), and BIC seizure thresholds were then determined 10 min later.

Results

The benzodiazepine antagonist RO 15-1788 by itself produced a dose-dependent elevation of BIC seizure threshold in rats as shown in Figure 20. The 1 mg/kg dose of RO 15-1788 resulted in a
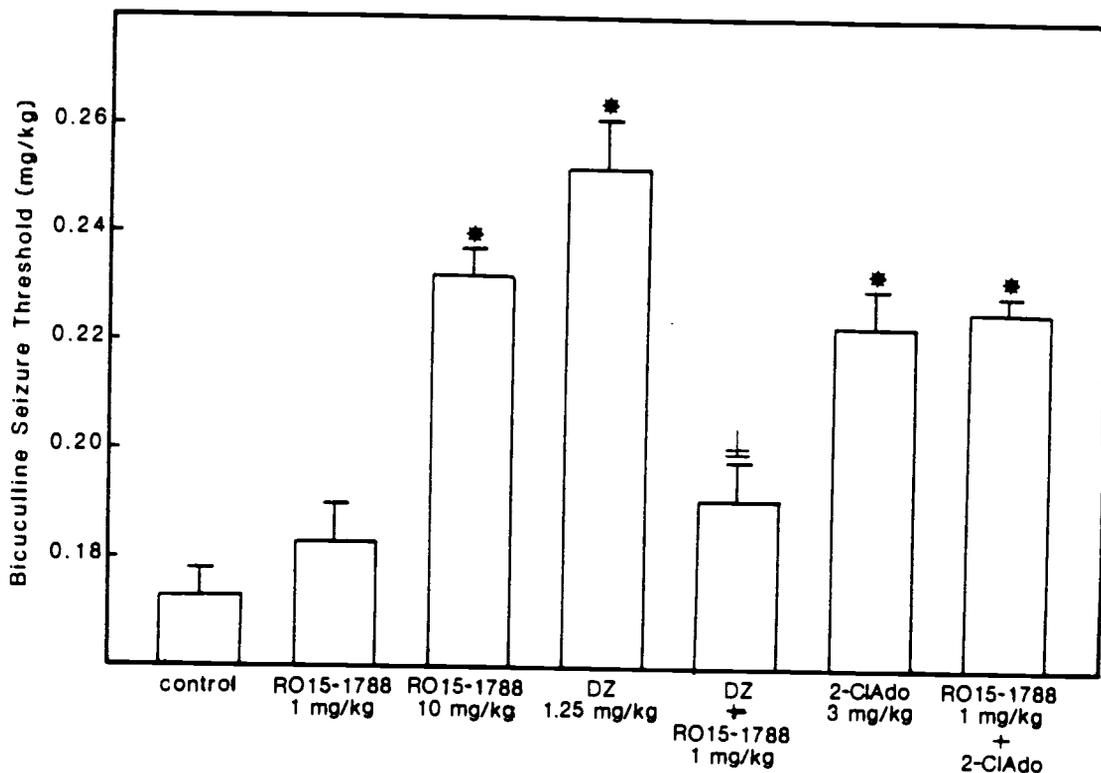
Figure 20. The ability of RO 15-1788 to selectively antagonize
the anticonvulsant action of diazepam on
bicuculline seizure thresholds in rats. Groups of
rats (n = 8-12 for each bar) received an intra-
peritoneal injection of diazepam (DZ) 15 min prior
to the intraperitoneal injection of saline or
RO 15-1788, and the seizure threshold for
bicuculline was determined 15 min later. Another
group of rats (n = 8-12 for each bar) received
an intraperitoneal injection of saline or RO 15-1788
15 min prior to the intravenous administration of
2-ClAdo, and seizure threshold for bicuculline was
determined 1 min later. Asterisk indicates a
statistically significant increase (P < 0.05) in
bicuculline seizure threshold compared to control
group.   indicates a statistical difference (P <
0.05) from the group treated with DZ alone.

nonsignificant increase in seizure threshold of BIC, while the 10

mg/kg dose of RO 15-1788 produced a significant elevation in BIC

seizure threshold of approximately 34 %. RO 15-1788's ability to

depress seizure induced activity was an indication that RO 15-

1788 possessed some intrinsic action at the benzodiazepine

receptor. It has been suggested that RO 15-1788 at low

concentrations is a potent benzodiazepine antagonist, while at

higher doses RO 15-1788 possess benzodiazepine like properties

(186,187,190). The data shown here was support for a partial

benzodiazepine like property of RO 15-1788. DZ (1.25 mg/kg,

i.p.) resulted in a significant elevation of BIC seizure

threshold of approximately 46%. Administration of RO 15-1788 (1

mg/kg) to rats treated with DZ resulted in a significant

antagonism of DZ induced elevation in BIC seizure threshold (Fig.

20). However, pretreatment with RO 15-1788 did not antagonize

the 29% increase in BIC seizure threshold elicited by 2-ClAdo

(Fig. 20). Therefore, RO 15-1788 (1 mg/kg) selectively

antagonized the anticonvulsant action of DZ. The inability of RO

15-1788 to antagonize the anticonvulsant action of 2-ClAdo was an

indication that 2-ClAdo did not produce its anticonvulsant action

through the benzodiazepine receptor.

CGS 8216 administered alone produced a dose-dependent

reduction in BIC seizure threshold in rats (FIG. 21). CGS 8216

at a dose of 0.5 mg/kg resulted in a nonsignificant decrease in

seizure threshold, while the 2 mg/kg dose of CGS 8216 resulted in

a 28% decrease in BIC seizure threshold that was statistically
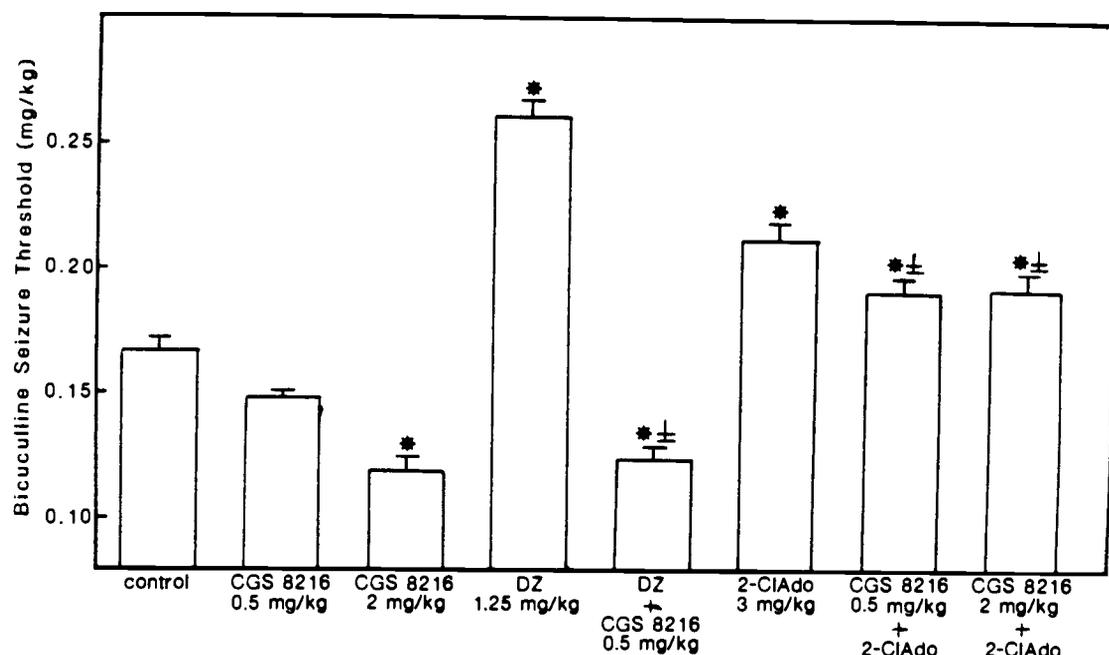
Figure 21. The ability of CGS 8216 to antagonize the anti-
convulsant action of diazepam and 2-chloroadenosine
on bicuculline seizure thresholds in rats. Groups
of rats (n = 8-12 for each bar) received an intra-
peritoneal injection of diazepam (DZ) 20 min prior
to the intravenous administration of saline or
CGS 8216, and the seizure threshold for bicuculline
was determined 10 min later. Another group of rats
(n = 8-12 for each bar) received an intravenous
injection of saline or CGS 8216 (0.5 and 2.0 mg/kg)
10 min prior to the intravenous injection of
2-chloroadenosine (2-ClAdo), and the seizure
threshold for bicuculline was determined 1 min later.
Asterisk indicates a statistically significant
(P < 0.05) change in bicuculline seizure threshold
compared to the control group.   indicates a
statistical (P < 0.05) decrease in the combination
treatment group from the respective group treated
with DZ or 2-ClAdo alone.

significant. This proconvulsant action of CGS 8216 has been shown by other researchers and was attributed to an inverse agonist property of CGS 8216 at the benzodiazepine receptor (187,205,206). The anticonvulsant action of DZ was completely antagonized by the administration of CGS 8216 (0.5 mg/kg) as shown in Figure 21. The administration of CGS 8216 (0.5 mg/kg) to DZ treated rats resulted in a signficant decrease in BIC seizure threshold of approximately 25.3% (Fig. 21). This data indicates CGS 8216 to be a potent benzodiazepine antagonist. Pretreatment with CGS 8216 at a dose of 0.5 or 2 mg/kg resulted in a modest, though significant, reduction in the anticonvulsant activity of 2-ClAdo (Fig. 21). The change in seizure activity induced by 2-ClAdo was still significantly elevated in the presence of either dose of CGS 8216 (Fig. 21). The antagonism of 2-ClAdo's anticonvulsant effect by CGS 8216 was not dose-dependent, the 0.5 and 2 mg/kg dose of CGS 8216 decreased the BIC seizure threshold of 2-ClAdo by approximately 12%.

Discussion

These results indicate an interaction of the benzodiazepine antagonist CGS 8216 with 2-ClAdo at a common receptor. CGS 8216 may antagonize 2-ClAdo's anticonvulsant action by interacting at the A_1 adenosine receptor. CGS 8216 has been shown to displace ^{3}H-cyclohexyladenosine (^{3}H-CHA) binding in rat brain with a potency 5 fold greater then theophylline (184). CGS 8216 blocked

the activation by adenosine on cGMP formation in vesicles prepared from guinea pig brain (192). The CGS 8216 IC_{50} value was equivalent to the IC $_{50}$ value for theophylline (192). These data indicate CGS 8216 to be as potent as theophylline as an adenosine antagonist, however, the antagonism of 2-ClAdo's anticonvulsant action by CGS 8216 was modest (only 12%) and not dose-dependent. The inability of CGS 8216 to completely reverse the 2-ClAdo induced elevation in BIC seizure threshold indicates that CGS 8216 was not acting at the A_1 adenosine receptor. The reduction in 2-ClAdo's anticonvulsant effect may be due to the proconvulsant action of CGS 8216 at the benzodiazepine receptor.

RO 15-1788 appears to have little or no activity at the A_1 adenosine receptor. This was demonstrated by RO 15-1788's inability to antagonize the anticonvulsant action of 2-ClAdo against BIC seizure threshold, to displace ^{3}H-CHA binding in rat cortical membranes (184) and to inhibit the activation of adenylate cyclase by adenosine in guinea pig CNS (192).

The anticonvulsant property of RO 15-1788 against BIC seizure threshold was not a novel finding. Since the introduction of RO 15-1788 as a selective benzodiazepine antagonist (185), a great deal of research has shown RO 15-1788 to possess benzodiazepine-like activty at high doses (186,187,190,200-202). Nutt and coworkers have shown RO 15-1788 to elevate the seizure threshold of PTZ and BIC in rats (186). Jensen, Peterson and Braestrup showed that audiogenic seizure sensitive mice given RO 15-1788 were protected from sound-induced

seizures (187). Moreover, RO 15-1788 reduced the rank but not the afterdischarge duration in the kindled amygdaloid model (201) and prolonged the development of kindled amygdaloid seizures in rats (202). Vellucci and Webster showed that RO 15-1788 protected rats against convulsions induced by PTZ, BIC and the convulsant benzodiazepine RO 5-3663, only when just maximal doses of the convulsant were given, but not at supramaximal doses (200). Grecksch and coworkers demonstrated similar results in that RO 15-1788 elicited an anticonvulsant action against PTZ seizures only when a submaximal dose of PTZ was used (190). These data indicated that the method employed to measure the anticonvulsant action of RO 15-1788 must be sensitive to detect minor changes in neuronal activity. Administration of a supramaximal convulsant dose may mask the anticonvulsant property of RO 15-1788. This may explain why Hunkeler and coworkers failed to observe an anticonvulsant action of RO 15-1788 against PTZ seizures (185).

There have also been reports that RO 15-1788 may have proconvulsant activity. Corda, Costa and Guidotti have shown that RO 15-1788 was proconvulsant when it was combined with the chemoconvulsant isoniazid (203). An increase in duration and severity of isoniazid seizures were observed when rats were pretreated with RO 15-1788 (203). However, when Pieri and Biry repeated this experimental protocol, RO 15-1788 failed to produce any relevant shift of the isoniazid dose-response curve (204).

The ability of CGS 8216 to elicit a proconvulsant action has

been demonstrated by other researchers (187,205,206). Jensen and coworkers showed that pretreatment of audiogenic seizure sensitive mice with CGS 8216 facilitated in a dose-dependent manner the sound induced clonic seizures of a subthreshold sound exposure (187). Rats pretreated with CGS 8216 showed an increased sensitivity to the convulsant effects of PTZ (205). The CD_{50} for PTZ in the presence of CGS 8216 was almost one-half the CD_{50} for control rats (205). File also showed a proconvulsant action of CGS 8216 in mice given subconvulsant doses of PTZ or PTX (206). This proconvulsant property of CGS 8216 was attributed to an inverse agonist property of CGS 8216 at the benzodiazepine receptor (187,205,206).

Chapter 6

Effect of Acute and Chronic (Kindling) Pentylenetetrazol
Administration on ^{3}H-Cyclohexyladenosine Binding
in the CNS

Introduction

Elevation of chemoconvulsant seizure thresholds by adenosine

analogs indicates an involvement of the adenosine receptor in the

modulation of seizure susceptibility in rats. The proconvulsant

effect of theophylline on seizure threshold demonstrates a role

for endogenous adenosine in the modulation of seizure

excitability. Seizures induced by bicuculline have been shown to

produce an increase in the CNS level of adenosine (4). Wybenga

and coworkers demonstrated a reduction in the density of A_1

adenosine receptors in the rat cerebellum following a PTZ seizure

(148). This indicated that an acute epileptic episode could

alter the density of the adenosine A_1 receptor in specific

regions of the brain. The kindling model of epilepsy has been

shown to alter the binding parameters of a variety of

neurotransmitter receptors. Kindling is an animal model of

epilepsy that is used to represent the conditions of human

epilepsy because of the permanent change in electrical activity

induced by this phenomenon (159). Kindling is the progressive

development of seizures that occurs with repeated subconvulsive

stimuli of the forebrain (117). Kindling can be induced by

electrical stimulation of most, but not all, sites in the brain

(162). The amygdala is the most sensitive area to the action of kindling, though kindling has occurred with stimulation to the cortex, olfactory bulb, septal area, preoptic area, caudate-putamen and hippocampus (162). Electrical stimulation is not the only means to induce kindling, animals have been kindled with repeated administration of subconvulsive doses of carbachol, chlordimeform, cocaine, fluorothyl, lidocaine, pentylenetrazol (162), bicuculline, picrotoxinin (161) and theophylline (114). Researchers have felt that the identification of the brain structures and neurotransmitters responsible for kindling may lead to an understanding of the cellular and molecular mechanisms involved in epilepsy (161). When examining alterations induced by the kindling phenomenon two important components become apparent: 1. the temporary neuronal changes produced by the kindled seizure and 2. the permanent neuronal alterations that represent the kindled state. A great deal of research has been devoted to identifying neurotransmitter systems that are altered as a result of kindling. Kindling model of epilepsy has resulted in alterations in the following neurotransmitter systems: (a.) a decrease in muscarinic cholinergic receptors in the rat hippocampus and amygdala (153,154); (b.) an increase in benzodiazepine receptor density in the rat hippocampus (155,156); (c.) a decrease in B-adrenergic receptor in the amygdala measured by ^{3}H-dihydroalprenolol binding (157); (d.) a decrease in ^{3}H-spiroperidol binding in the amygdala with a parallel decrease in dopamine-sensitive adenylate cyclase activity (150,158), and e.

an increase in ^{3}H-glutamate binding to quisqualate-sensitive site in the hippocampus (160). These changes in neurotransmitter systems induced by kindling were not permanent. This suggests that the alterations were due to the kindled seizure and not due to the kindling process (162).

Materials and Methods

Male Sprague-Dawley rats (Simonsen Labs, Gilroy, Ca.) weighing 180-250 g were used. The animals were housed upon delivery, in groups of 4-6 at 22°C with food and water provided ad libitum. A standard light-dark cycle was maintained with a timer-regulator light period from 0800 to 2000 hr. Each rat was used only once for any determination.

To determine the effects of acute pentylenetetrazol (PTZ) on CNS adenosine A_1 receptors, rats were injected intraperitoneally with either saline or PTZ (65 mg/kg) in a volume of 2 ml/kg. Only rats demonstrating tonic-clonic convulsions within 4 min post-injection of PTZ were used for the study. All animals were sacrificed by decapitation 1 hr following the injection of saline or PTZ. The brains were rapidly removed, dissected into various brain regions and stored at -70°C until the binding experiments were performed.

Pentylenetetrazol kindling in rats was performed according to the method described by Mason and Cooper (151). The experimental protocol consisted of 3 treatment groups. One group

of rats received PTZ intraperitoneally at a dose of 30 mg/kg (2

ml/kg) every 48 hr between the hours of 1600 to 1800. This dose

of PTZ is considered to be a subconvulsive dose with a single

administration. After each drug injection, the animals was

observed for 20 min. During this time, behavioral changes were

recorded and classified according to a rank system described

by Gee et al. (150). A brief discription of the ranking system

is as follows:

        0 = control behavior

        1 = nodding or head clonus

        2 = salivation and forelimb clonus

        3 = rearing on hindlimbs

        4 = falling backward and hindlimb clonus

An animal was considered to be kindled when it was given a

ranking of 4 two times in a row. Most animals were kindled after

the 15th injection. Those animals that did not demonstrate two

stage 4 ranking in a row were not used. A second group of

animals received saline intraperitoneally every 48 hr between the

hours of 1600 to 1800. On the 16th injection, a convulsant dose

of PTZ (50 mg/kg) was administered instead of saline. The third

group of animals did not receive any injections and were

sacrificed by decapitation 30 days later. All other animals were

sacrificed by decapitation 24 hr after the last injection. The

brains for each treatment group were rapidly removed, dissected

and stored at -70°C until the binding experiment could be

performed.

The brain regions were weighed and homogenized in 100 volume of ice-cold 50 mM Tris-HCl buffer at pH 7.6 which contained 5 mM MgCl. The tissue was homogenized using a Polytron (Brinkman Instruments, Westbury, N.Y.) at a setting of 8 for 15 sec. The homogenate was centrifuged at 48,000 x g for 10 min at 4°C. The pellet was suspended in Tris-MgCl buffer, centrifuged, and resuspended in Tris-MgCl containing 2 IU of adenosine deaminase (type III) per ml. After a 30 min incubation at 25°C, the membranes were recentrifuged and the pellet resuspended in ice-cold Tris-MgCl buffer. This final homogenate was then used in the ^{3}H-cyclohexyladenosine binding experiment.

The binding of the A_1 selective ligand ^{3}H-cyclohexyladenosine (^{3}H-CHA) to brain membranes was measured using an incubation volume of 1 ml consisting of 400 ul of the homogenate described above, 50 ul of ^{3}H-CHA (25 Ci/mmol; Dupont NEN, Boston, Ma.) and 50 ul of buffer or R-PIA dissolved in buffer. Incubation was carried out at 25°C for 2 hr. Separation of free ^{3}H-CHA from membrane bound was performed using a vacuum filtration on Whatman GF/B glass fiber filters with four 4 ml washes of ice-cold Tris-MgCl buffer. Each filter was placed in a vial with 8 ml of ACS II (Amersham, Arlington Heights, Il.) and counted by a liquid scintillation counter. Specific binding was defined as the excess over blanks determined in the presence of 2 uM R-PIA.

Protein concentration of each sample was determined by a modified Lowery (163), using 0.5 N NaOH to solubilize the

protein. Crystalline bovine serum albumin was used as a standard.

Each value is expressed as fmol/mg protein of ^{3}H-CHA bound determined from triplicate counts.

Results

The effect of an acute PTZ-induced seizure on ^{3}H-CHA binding in the cerebral cortex, cerebellum and hippocampus are shown in Table 5. Seizure activity induced by an acute PTZ injection did not alter the ^{3}H-CHA specific binding in any of the brain regions studied.

The time course for the development of kindling to occur in rats is shown in Figure 22. The majority of the rats appeared to be kindled by the 11th injection of PTZ (Fig. 20). The effect of PTZ kindling on ^{3}H-CHA binding in specific brain regions is shown in Table 6. An acute PTZ seizure resulted in a nonsignificant change in ^{3}H-CHA specific binding in cerebellar and hippocampal membranes. This is in agreement with the results reported in Table 5. PTZ kindling resulted in a nonsignificant 16.2% increase in cerebellar membranes and no change in the specific binding of ^{3}H-CHA in hippocampal membranes. Apparently then, PTZ-induced seizure activity either by acute or chronic treatment, does not alter the specific binding of the adenosine A_1 selective radioligand ^{3}H-CHA in the CNS of rats.

Table 5. Lack of Effect of an Acute Pentylenetetrazol-induced
Seizure on ^{3}H-Cyclohexyladenosine Binding in the
CNS of Rats

Brain Area	^{3}H-CHA Bound (fmol/mg prot. ± SEM)		% Increase
	Saline	PTZ	
Cortex	432 ± 31	440 ± 37	1.7
Cerebellum	546 ± 18	582 ± 43	6.6
Hippocampus	527 ± 45	527 ± 39	0

Values given are the mean ± SEM of triplicate determination
in 6 animals for each brain region. ^{3}H-CHA binding was
determined at a final concentration of 5 nM. Specific binding
was defined as the total binding minus the nonspecific binding
determined in the presence of 2 uM R-PIA.
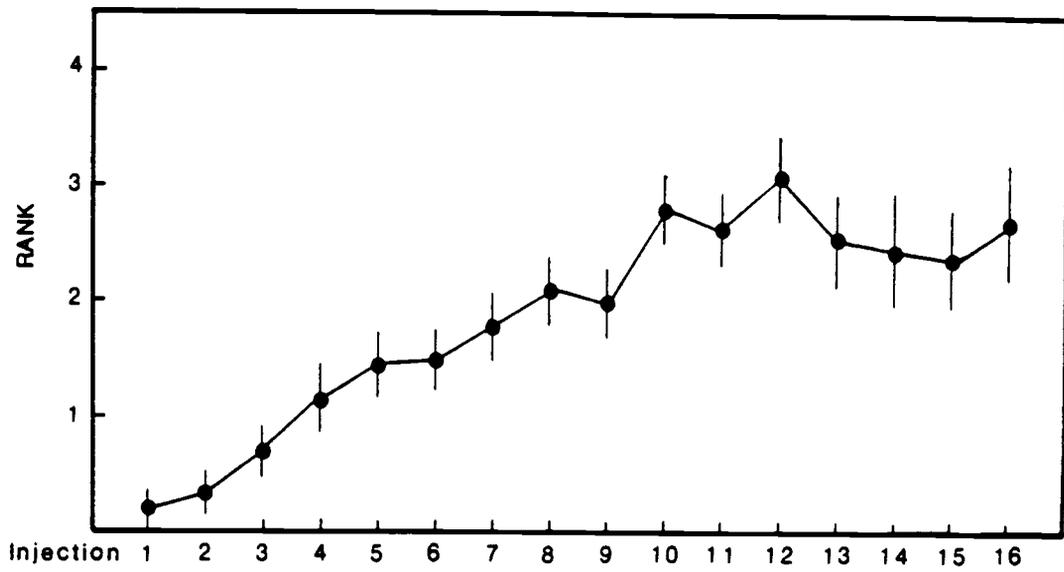
Figure 22. Time-response of pentylenetetrazol-kindled seizure
development. Each point represents the mean ± SEM
of 12 animals.

Table 6. Lack of Effect of Pentylenetetrazol Kindling on
^{3}H-Cyclohexyladenosine Binding in the Cerebellum
and Hippocampus of Rats

| Treatment | ^{3}H-CHA Bound (fmol/mg protein ± SEM) | |
	Cerebellum	Hippocampus
Control	297 ± 31	283 ± 8
Saline – PTZ	278 ± 29	272 ± 9
PTZ Kindling	345 ± 49	280 ± 14

Values given are the mean ± SEM of triplicate determinations in
4 animals for each brain region. ^{3}H-CHA binding was determined
at a final concentration of 1 nM. Specific binding was defined
as the total binding minus the nonspecific binding determined
in the presence of 2 uM R-PIA.

Discussion

The results obtained from the present investigation indicate that seizure activity, either by acute or chronic PTZ, does not alter the binding of the selective A_1 ligand ^{3}H-cyclohexyladenosine. Alterations in neuronal activity induced by the kindling phenomenon can be either temporary or permanent in nature. Temporary changes in neuronal activity are the result of seizures induced by the process of kindling, while the kindled state results in a permanent alteration in neuronal activity. If the adenosine A_1 receptor is involved in the production of kindling, then the density of this receptor should be altered permanently (161,162). Because kindling did not alter the binding of ^{3}H-CHA in the hippocampus, an area very sensitive to the effects of kindling (161,162), it is concluded that the A_1 adenosine receptor as labeled by ^{3}H-CHA is not involved in the kindling process. This lack of effect of seizure activity on ^{3}H-CHA binding was not unexpected. Previous experiments examining the effects of adenosine analogs on amygdaloid-kindling indicate that adenosine has little effect on seizure initiation. The actions of adenosine and adenosine analogs on amygdaloid-kindled seizures were to reduce the severity of the seizure and to reduce the afterdischarge duration (113-115,120). An increase in latency to bilateral forelimb clonus was also observed after the administration of 2-ClAdo to amygdaloid-kindled rats (115). 2-ClAdo did not alter the afterdischarge threshold in the

amygdala-kindled seizure (115). Alterations in the afterdischarge threshold and the generalized seizure threshold are the two most important indicies for measuring the effectiveness of a treatment to modify either the development of kindling or the expression of a fully kindled seizure (162). Because adenosine did not alter the afterdischarge threshold, it was concluded that the adenosine A_1 receptor was not involved in the process of kindling. The methylxanthines increased the afterdischarge duration (114,118-120) and seizure severity (118,119,121), while the afterdischarge threshold of amygdaloid-kindling remained unaffected. These actions of the methylxanthines on kindled seizures were further support for the lack of involvement of the A_1 receptor in the kindling phenomenon. It is evident that adenosine possesses anticonvulsant activity against amygdaloid-kindled seizures by virtue of its ability to alter the severity and duration of the seizure activity. It has been suggested that this anticonvulsant action of adenosine was due to the modulation of the spread of the seizure (114).

Barraco and coworkers have shown the intracerebroventricular adminstration of NECA to be 4 times more potent then R-PIA in decreasing seizure severity and afterdischarge duration of amygdaloid-kindled seizures (113). These data indicate an involvement of the A_2 adenosine receptor in the anticonvulsant action of adenosine on amygdaloid-kindling. The rank order potency of adenosine analogs as anticonvulsants on amygdaloid-

kindling does not agree with the rank order potency of adenosine agonists to elevate PTZ seizure threshold. Data presented earlier in this document in Figure 6 demonstrated an adenosine A_1 receptor response mediated the alteration of PTZ seizure threshold in rats. R-PIA was the most potent adenosine analog tested, while its diastereoisomer S-PIA was the least potent. R-PIA was approximately 20 fold more potent then NECA in elevating the seizure threshold of PTZ. In addition, NECA was less efficacious then R-PIA as an anticonvulsant. These differences in the relative potency of NECA and R-PIA to alter amygdala-kindled seizures versus PTZ seizures suggest a different receptor subtypes are involved in mediating the anticonvulsant effects in these two models.

Chapter 7

Effect of Chronic Theophylline on Seizure Threshold of
Chemoconvulsants and ^{3}H-Cyclohexyladenosine
Binding in the CNS

Introduction

Methylxanthines, such as caffeine and theophylline, are
adenosine receptor antagonists and it is now generally accepted
that their central stimulant effects are mediated through a
competitive antagonism of A_1 adenosine receptors in the brain
(167). The chronic adminstration of theophylline (168) or
caffeine (169) have been shown to produce an upregulation of
adenosine A_1 receptors in rat cerebellar membranes. In both
reports chronic exposure to the methylxanthines resulted in a
significant increase in the maximum number of binding sites with
no change in the affinities of the radioligands used. These
results suggest that the A_1 adenosine receptors of the cerebral
cortex of the rat are responsive to fluctuations in their access
to endogenous adenosine. However, the functional consequences of
these alterations in receptor density remain to be elucidated.
The present study was undertaken to further characterize the
upregulation induced by theophylline, by investigating both the
neuroanatomical pattern of this compensatory response as well as
the effect of chronic exposure to theophylline on agonist
affinity states of A_1 adenosine receptors. In addition, in an
attempt to establish a functional correlate of upregulation of A_1

adenosine receptors, the time-infusion technique was employed to determine seizure thresholds to convulsants in control and chronic-theophylline treated rats.

Materials and Methods

Male Sprague-Dawley rats (Simonsen Labs, Gilroy, Ca.) weighing 180-250 g were used. The animals were housed upon delivery, in groups of 4-6 at 22°C with food and water provided ad libitum. A standard light-dark cycle was maintained with a timer-regulator light period from 0800 to 2000 hr. Each rat was used only once for any determination.

The chronic treatment regimen consisted of twice-daily injections with either theophylline (50 mg/kg, i.p.) or saline (3 ml/kg) for 14 consecutive days. All determinations of seizure threshold with convulsants were performed 48 hr after the last injection of theophylline or saline. Additional animals, receiving an identical chronic treatment regimen, were sacrificed by decapitation 48 hr after last injection. The brains were rapidly removed, dissected and stored at -70°C until the ^{3}H-CHA binding could be performed.

Seizure threshold for pentylenetetrazol, bicuculline and picrotoxinin were determined using a timed-infusion method described in Chapter 2 and 4.

Preparation of tissue membranes and the binding of the specific A_1 ligand ^{3}H-cyclohexyladenosine (^{3}H-CHA) to the brain

membranes are described in detail in Chapter 5.

In equilibrium saturation experiments ^{3}H-CHA was used in
concentrations ranging from 0.1 to 52 nM. Analysis of agonist
interactions with A_1 adenosine receptors has demonstrated that
the A_1 receptor can exist in two affinity states for agonists
(171,172). The equilibrium binding isotherms of the agonist
radioligand ^{3}H-CHA were therefore analyzed using the iterative
public procedure FITFUN on the PROPHET system, assuming both a
one-site and a two-site model. Individual saturation isotherms
were analyzed by nonlinear regression analysis, assuming either
ligand binding to a single receptor site or binding to two
independent species of receptor. These analyses assume that the
ligand interactions with one or two species of receptor conforms
to mass action principles as follows: One-site:

$$B = \frac{(R)\ (L)}{(L)\ +\ K_D}$$

Two-site:

$$B = \frac{(R_H)\ (L)}{(L)\ +\ K_H} + \frac{(R_L)\ (L)}{(L)\ +\ K_L}$$

Where B is the amount of specifically bound ^{3}H-CHA; R, R_H and R_L
are the maximum number of binding sites in either the one- or
two-site model; L is the concentration of free radioligand; and
K_D, K_H and K_L are the respective equilibrium dissociation
constants for individual receptor species in the two models. It
is assumed that in the two-site model that K_H is the equilibrium
dissociation constant for the formation of the high-affinity
complex and K_L is the equilibrium constant for the formation of

the low-affinity complex. Binding to a single site was assumed
unless the more complex two-site model accounted better for the
experimental data. Data are described as better fit by one model
of ligand binding than another when a partial F-test, comparing
the two models indicated significant improvement in residual sum
of squares (P < 0.01), as described by Hoyer, Reynolds and
Molinoff (173). The F-value for comparing the two models was
calculated from an analysis of residuals, according to the
following equation:

$$F = \frac{SS_1 - SS_2/df_1 - df_2}{SS_2/df_2}$$

where SS_1 and SS_2 are residual sums of squares with corresponding
degrees of freedom df_1 and df_2, associated with the simpler and
more complex model, respectively. The F-values were calculated
using df_1 - df_2 degrees of freedom in the numerator and df_2
degrees of freedom in the denominator (173). Although a ternary
complex model provides a mechanistically more correct quantitive
description of agonist-receptor-N protein interactions, the two-
independent-site model described above can be used to provide
estimates of the affinity of ligands for different states of the
receptor (174,175,176). It has also been found that the
parameter estimates for one- and two-site models, derived from
the use of the Lundon-1 iterative curve-fitting program (177),
provided virtually identical results to those obtained using the
FITFUN on the PROPHET system.

To determine the functional activity of the upregulated
adenosine A_1 receptors, the anticonvulsant property of 2-ClAdo

was examined in animals chronically treated with theophylline or saline. The effect of 2-ClAdo on animals receiving the chronic treatment was determined 48 hr after the last injection of theophylline or saline. 2-ClAdo (3.0 mg/kg) or saline was administered intravenously to rats in a volume of 2 ml/kg, and bicuculline seizure thresholds were determined 1 min later.

To examine the effect of other CNS stimulants on seizure threshold, amphetamine was chronically administered to rats. Animals received twice daily injections of amphetamine (5 mg/kg, i.p.) or saline (2 ml/kg) for 14 days. Bicuculline seizure threshold was determined 48 hr after the last injection of amphetamine or saline.

Results

In agreement with a previous report (168), chronic theophylline treatment produced a significant increase in the specific binding of ^{3}H-CHA to membranes of the cerebral cortex of the rat as shown in Table 7. The specific binding of ^{3}H-CHA in three additional regions of the brain revealed that exposure to theophylline also significantly increased the binding of ^{3}H-CHA in membranes of the cerebellum, but not in membranes from the hippocampus or diencephalon (Table 7). These results indicate that the adenosine antagonist-induced upregulation of binding sites for ^{3}H-CHA displayed a heterogenous neuroanatomical pattern.

Table 7. Effect of Chronic Exposure to Theophylline on the Specific Binding of ^{3}H-CHA to Various Regions of the Rat Brain

| Brain Area | ^{3}H-CHA Bound (fmol/mg protein ± SEM) | | % Increase |
	Control	Theophylline	
Cortex	176.7 ± 12.3	227.4 ± 7.9[*]	28.7
Hippocampus	335.1 ± 19.6	382.5 ± 38.9	14.1
Cerebellum	335.5 ± 16.5	422.7 ± 24.0[*]	26.0
Diencephalon	168.5 ± 12.8	181.9 ± 9.8	8.0

Values are the means (fmol/mg protein) ± SEM of triplicate determinations in 4 animals for each region of the brain. ^{3}H-CHA binding was determined at a final concentration of 1 nM. Asterisk indicates significant difference (P <0.05) in treated group from control.

In order to assess the effects of chronic administration of theophylline on the densities and agonist affinity states of A_1 adenosine receptors, saturation analysis was performed in membranes from the cerebral cortex and cerebellum in both saline- and theophylline-treated animals. Using a concentration range of 0.1 to 52 nM, the specific binding of ^{3}H-CHA saturated, while the nonspecific binding increased linearly as a function of concentration of ^{3}H-CHA. Representative binding isotherm in membranes from cerebral cortex shown in Figure 23 reveal that the saturation data were better fit by a two-site model in both saline- and theophylline-treated animals. Computer-assisted analysis of these data using equations based on mass action principles, indicated that in both the cortex from saline- and theophylline-treated animals, the binding isotherms could be resolved into two receptor affinity states having, respectively, high (K_H) and low (K_L) affinity for ^{3}H-CHA. The resulting parameter estimates for both the one- and two-site model are summarized in Table 8. In both saline- and theophylline-treated cortical membranes, in 5 of 5 experiments the two-site model provided a significant improvement in the fit, compared to that obtained from the one-site fit ($P < 0.01$). These data indicate that ^{3}H-CHA distinguished between two agonist affinity states in saline-treated membranes from cerebral cortex with a K_H of 0.96 $\pm$ 0.18 nM and a K_L of 16.8 $\pm$ 2.74 nM. The corresponding high and low affinity dissociation constants obtained from theophylline-exposed membranes from cerebral cortex were 1.14 $\pm$ 0.05 nM and

Figure 23. Equilibrium saturation analysis of binding of ^{3}H-CHA
to membranes of the cerebral cortex of saline- (●)
and theophylline- (▲) treated rats. Membranes
from both treatment groups were incubated for 2 hr
at 25°C with 12 concentrations of ^{3}H-CHA, ranging
from 0.1 to 52 nM. Specific binding (filled
symbols) is defined as the total binding minus the
nonspecific binding (open symbols), determined in
presence of 30 uM R-PIA. Values shown are from a
single representative experiment which was repeated
four times. The saturation isotherms for both the
one-site (dotted lines) and two-site (solid lines)
models were generated by computer-assisted analysis
as described under "Methods". In this experiment
the two-site fit described the data significantly
better than the one-site fit for both the saline-
(F = 33.0, P < 0.001) and theophylline-treated (F =
136.3, P < 0.001) membranes from cerebral cortex.

Table 8. Effect of Chronic Treatment with Theophylline on Binding Parameters Derived from Computer-assisted Analyses of ^{3}H–CHA Saturation Isotherms in Cerebral Cortical Membranes of the RAT.

Treatment (n)	1-Site		2-Site				$\%R_L$
	K_d	R_t	K_H	R_H	K_L	R_L	
Saline (5)	4.13±0.23	298.7±11.6	0.96±0.18	108.5±17.5	16.8±2.74	251.9±13.9	70.2±4.5
Theophylline (5)	4.81±0.42	375.0±2.76*	1.14±0.05	186.4±19.2**	25.1±4.97	331.3±18.2**	64.1±2.1

The membranes were incubated with 12 concentrations of ^{3}H–CHA, ranging from 0.1 to 52 nM in 5 separate experiments for each treatment group. Values given are the mean ± SEM for both the 1-site and 2-site analyses. K_d is the dissociation constant for the one-site model, while the high-affinity and low-affinity dissociation constants in the two-site model are designated K_H and K_L, respectively. R_t is the maximum number of binding sites for the one-site model, and R_H and R_L are the corresponding densities for the high- and low-affinity forms in the two-site model. The percentage of the total receptor population present in the low-affinity state in the two-site model is $\%R_L$. *Indicates significantly different from corresponding value of saline-treated group, $P < 0.05$; **$P < 0.01$.

25.2 ± 4.97 nM and did not differ significantly from the saline-treated group. Exposure to theophylline did, however, produce significant increases in the densities of both the high and low affinity forms of A_1 adenosine receptors in the cerebral cortex. The mean percentage increase in the densities of the high affinity state of the A_1 receptor was 71.8% while the low affinity state was increased by 31.5% in theophylline-treated membranes from cerebral cortex (Table 8). The low affinity state receptors comprised similar proportions of the total receptor population in the two treatment groups. These results suggest that chronic exposure to theophylline increases the density of A_1 receptor in the cerebral cortex with no concomitant changes in the ability of ^{3}H-CHA to distinguish separate agonist affinity states of the receptor.

Representative saturation isotherms for ^{3}H-CHA in cerebellar membranes, shown in Figure 24, demonstrated that these data were also better described by a two-site rather than a one-site model. In 6 of 6 experiments, using membranes from cerebellum, prepared from animals treated chronically with either saline or theophylline, the two-site model provided a significant improvement in the fit compared to that obtained with the one-site fit (P < 0.01). The parameter estimates for binding isotherms for ^{3}H-CHA in membranes from cerebellum, summarized in Table 9, demonstrated that these results were qualitatively and quantitatively similar to those obtained in the cerebral cortex. Thus, exposure to theophylline produced significant increases in
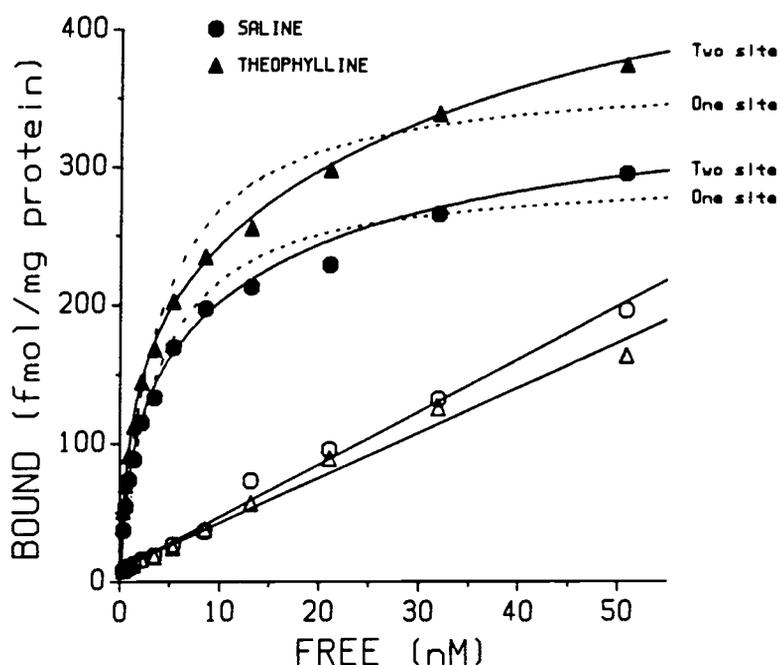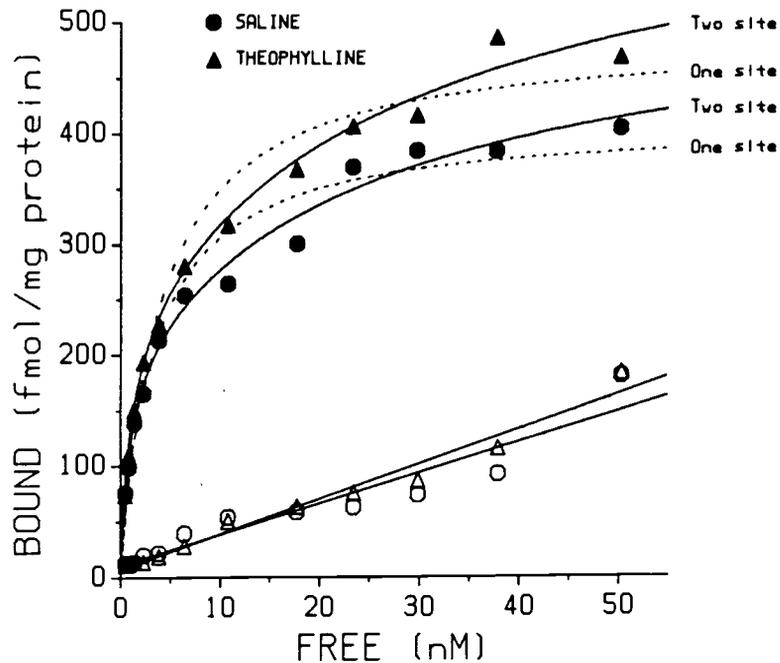
Figure 24. Equilibrium saturation analysis of binding of ^{3}H-CHA
to membranes from the cerebellum of saline- (●) and
theophylline- (▲) treated rats. Membranes from
both treatment groups were incubated for 2 hr at
25°C with 12 concentrations of ^{3}H-CHA, ranging from
0.1 to 52 nM. Specific binding (filled symbols) is
defined as the total binding minus the nonspecific
binding (open symbols), determined in the presence
of 30 uM R-PIA. Values shown are from a single
representative experiment which was repeated five
times. The saturation isotherms for both the one-
site (dotted lines) and two-site (solid lines)
models were generated by computer-assisted analysis
as described under "Methods". In this experiment
the two-site fit described the data significantly
better than the one-site fit for both the saline-
(F = 10.0, P < 0.01) and theophylline- (F = 16.6,
P < 0.005) membranes from cerebellum.

Table 9. Effect of Chronic Treatment with Theophylline on Binding Parameters Derived from Computer-assisted Analyses of ^{3}H–CHA Saturation Isotherms in Cerebellar Membranes of the Rat.

Treatment (n)	1-Site		2-Site				$\%R_L$
	K_d	R_t	K_H	R_H	K_L	R_L	
Saline (6)	3.30±0.29	379.6±39.2	0.72±0.33	126.6±28.3	22.8±11.9	276.8±23.0	68.6±9.3
Theophylline (6)	3.78±0.17	488.4±36.7*	0.92±0.18	193.8±38.5*	24.1±8.49	382.9±65.3*	66.4±5.1

The membranes were incubated with 12 concentrations of ^{3}H–CHA, ranging from 0.1 to 52 nM in 6 separate experiments for each treatment group. Values given are the mean ± SEM for both the 1-site and 2-site analyses. K_d is the dissociation constant for the one-site model, while the high-affinity and low-affinity dissociation constants in the two-site model are designated K_H and K_L, respectively. R_t is the maximum number of binding sites for the one-site model, and R_H and R_L are the corresponding densities for the high- and low-affinity forms in the two-site model. The percentage of the total receptor population present in the low-affinity state in the two-site model is $\%R_L$. * Indicates significantly different (P < 0.05) from corresponding value of saline-treated group.

the density of A_1 adenosine receptors in both the high-affinity

(53.1% increase) and low-affinity (38.3% increase) forms of the

receptor, with no concomitant effects on the affinities per se

(Table 9). In addition, exposure to theophylline was not

accompanied by any alterations in the percentage of A_1 receptors

comprising the low-affinity state. Thus, in both the cerebral

cortex and cerebellum of the rat, treatment with theophylline

elicited a coordinated upregulation of high- and low-affinity

forms of A_1 adenosine receptors with no apparent effect on the

ability of ^{3}H-CHA to distinguish separate affinity states of the

receptor.

In an attempt to assess the physiological significance of

the theophylline-induced upregulation of A_1 adenosine receptors

seizure thresholds to selected convulsants were determined in

rats treated chronically with either saline or theophylline. In

animals that has been treated chronically with theophylline, the

threshold dose of PTZ for inducing seizures was significantly

elevated. In three separate experiments, in which saline- and

theophylline exposed animals were tested 48 hr after the end of

the chronic treatment regimen, the seizure threshold for PTZ

was 20-21% higher in the chronic theophylline-treated animals.

As shown in Table 10, the magnitude of the increase in the dose

of PTZ required to elicit a seziure was highly significant and

remarkably consistent in all three experiments. The

generalization of the alteration in the seizure threshold for PTZ

associated with chronic exposure to theophylline was assessed by

Table 10. Effect of Chronic Exposure to Theophylline on
Seizure Thresholds for Convulsants in Rats.

Experiment	Control(n)	Theophylline(n)	%Increase	P
	Seizure Threshold to PTZ (mg/kg, i.v.)			
1.	18.4 ± 0.23(8)	22.4 ± 0.81(7)	21.3	0.001
2.	15.2 ± 0.49(15)	18.4 ± 0.43(15)	20.1	0.001
3.	17.9 ± 0.76(9)	21.5 ± 1.07(8)	20.3	0.025
	Seizure Threshold to BIC (mg/kg, i.v.)			
4.	0.24 ± .008(11)	0.31 ± .010(12)	28.2	0.001
	Seizure Threshold to PTX (mg/kg, i.v.)			
5.	4.7 ± 0.10(10)	5.4 ± 0.09(11)	15.3	0.001

Values given are the mean ± SEM (mg/kg, i.v.) of convulsant
agents required to elicit a seizure. Seizure thresholds were
determined 48 hr after the last injection of saline or
theophylline

Table 11. Effect of 2-Chloroadenosine (2-ClAdo) on Bicuculline
Seizure Threshold of Chronic Saline- and Theophylline-
treated Rats

Treatment	BIC Seizure Threshold (mg/kg, i.v.)	%Increase
Saline/Saline	0.151 ± 0.006 (6)	
Saline/2-ClAdo	0.176 ± 0.005 (6)	16.2
THEO/2-ClAdo	0.204 ± 0.008 (6)	35.1

Values given are the mean ± SEM (mg/kg, i.v.) of bicuculline
required to elicit a seizure. Seizure thresholds were
determined 48 hr after the last injection of saline or
theophylline

determining seizure thresholds for bicuculline (BIC) and

picrotoxinin (PTX). The results of these experiments, presented

in Table 10, indicate that animals that had been treated

chronically with theophylline displayed significant elevations in

the dose of BIC (P < 0.001) and PTX (P < 0.01) required to elicit

a clonic convulsion. Thus, chronic exposure to an adenosine

receptor antagonist resulted in a reduced susceptibility to

seizures induced by three convulsant agents. To further

establish a functional significance of the upregulated A_1

adenosine receptor, 2-ClAdo was administered to rats chronically

exposed to saline and theophylline. These results are shown in

Table 11 and demonstrate an increased sensitivity of the

theophylline-treated rats to the anticonvulsant effect of 2-

ClAdo. The administration of 2-ClAdo resulted in a significant

(P < 0.05) elevation of 16.7% and 35.1% in BIC seizure threshold

for the chronic saline- and theophylline-treated rats. When

animals that were exposed to chronic theophylline were given

given 2-ClAdo the increase in seizure threshold of BIC was

significantly (P < 0.05) greater then the 2-ClAdo induced

increased in BIC seizure threshold of the chronic saline group.

This enhanced anticonvulsant effect of 2-ClAdo in the chronic

theophylline group represents a physiological action of the

upregulated A_1 adenosine receptor. As it has previously been

shown that the effect of acute treatment with theophylline is to

lower seizure thresholds for PTZ (Chapter3), the presence of any

residual quantities of theophylline in the CNS was presumably not

Table 12. Effect of Chronic Amphetamine Exposure on Bicuculline
Seizure Threshold in Rats

Treatment	BIC Seizure Threshold (mg/kg, i.v.)
Saline	0.22 ± 0.007 (10)
Amphetamine	0.19 ± 0.007 (8)[*]

Values given are the mean ± SEM (mg/kg, i.v.) of bicuculline
required to elicit a seizure. Seizure thresholds were
determined 48 hr after the last injection of saline or
amphetamine. Asterisk indicates a significant change ($P < 0.05$)
in bicuculline seizure threshold of chronic amphetamine-treated
group compared to the chronic saline-treated group

a significant factor. The specificity of this response to

chronic threatment with theophylline was ascertained by

determining seizure thresholds for BIC in rats treated

chronically with either saline or amphetamine (Table 12). The

mean dose of BIC required to elicit a seizure in saline-treated

animals was 0.22 ± 0.007 mg/kg, while the corresponding value for

the threshold for BIC seizure in animals exposed to amphetamine

was 0.19 ± 0.007 mg/kg. This 13.1% reduction in the seizure

threshold for BIC in rats treated chronically with amphetamine

was statistically significant ($P < 0.01$). These results indicate

that the reduced sensitivity to chemoconvulsant seizures induced

by convulsants in rats treated chronically with theophylline as

not merely due to a compensatory response to a CNS stimulant.

Considered together, these data suggest that the reduced

susceptibility to seizures observed in animals chronically

exposed to theophylline, represents a functional correlate of

upregulation of the A_1 adenosine receptor.

Discussion

The regulation of the number of neurotransmitter receptors

can occur in response to treatment with a variety of agonist and

antagonist drugs and represents an important adaptive mechanism

for the regulation of neuronal sensitivity (178). A_1 adenosine

receptors resemble many other neurotransmitter receptors in that

chronic exposure to antagonists, such as caffeine (169,179) and

theophylline (168), produces an increase in the number of A_1

recognition sites in the brain of the mouse and rat. The present

study demonstrates that the theophylline-induced upregulation of

the A_1 receptors exhibits a heterogenous neuroanatomical pattern

with the cerebral cortex and cerebellum showing the greatest

increases in binding of ^{3}H-CHA. In contrast, no significant

increases were observed in either the hippocampus or

diencephalon. Such regional differences in plasticity of A_1

receptors may reflect different levels of adenosine-related

neuromodulation or "inhibitory tone"; however, further studies

are required to elucidate the underlying mechanism responsible

for these differences.

The present study further characterized the theophylline-

induced upregulation of A_1 receptors by examining the influence

of exposure to theophylline on agonist affinity states of the

receptor in cerebral cortex and cerebellum of the rat. The

saturation isotherms for ^{3}H-CHA were best fit by a two-site model

having distinct receptor affinity states in both structures of

the brain. The mean values for the high (K_H) and low (K_L)

affinity states for ^{3}H-CHA obtained in the present study compare

favorably with those previously reported for ^{3}H-PIA binding to A_1

receptors of fat cells of the rat (172) and membranes from the

cerebral cortex of the rat (180). In these previous reports,

using ^{3}H-PIA, the results were interpreted as representing high-

and low-affinity agonist states of the A_1 adenosine receptor. By

analogy with other neurotransmitter receptors, which are coupled

to adenylate cyclase, adenosine agonists are thought to be capable of inducing a high-affinity form of the receptor (171,172,181). These high-affinity states for agonist binding have been demonstrated to represent a ternary complex, consisting of the hormone or neurotransmitter receptor and a guanine nucleotide binding protein (182). In this ternary complex model guanine nucleotides are thought to bind to the guanine nucleotide binding protein and destablize the complex, yielding a low-affinity agonist-receptor complex. Chronic exposure to theophylline did not have any effects on the affinities of A_1 receptors per se, but rather produced a coordinated upregulation of the densities of both the high- and low-affinity states of the receptor in the cerebral cortex and cerebellum. In addition, treatment with theophylline did not alter the proportion of receptors in the two agonist affinity states in either area of the brain. These results are consistent with ^{3}H-CHA labeling one population of receptors, which can exist in two interconvertable agonist affinity states. These findings suggest that the principle effect of chronic exposure to theophylline is to increase the density of A_1 adenosine receptors in areas of brain examined. Green and Stiles have recently reported that chronic ingestion of caffeine shifts all of the A_1 receptors as labeled by ^{3}H-PIA to the high-affinity state in rat cerebral cortex (180). Possible explanations for this apparent discrepancy include different membrane preparations and ionic constituents of the assay buffers in the two investigations.

Analogs of adenosine have been shown to be remarkably potent as anticonvulsants in seizure threshold model using PTZ as shown in Chapter 2. Chapter 3 has shown the suppressant effects of 2-ClAdo on seizures are antagonized by theophylline and, when administered alone, theophylline produces a significant proconvulsant effect on seizures induced by PTZ. These results provided evidence in support of the idea that endogenous adenosine functions as a regulator of susceptibility to seizures (105). Although the precise mechanism by which analogs of adenosine produce these suppressant effects on seizures remains to be established, evidence for the involvement of A_1 receptors has been provided by Dunwiddie and Worth (111) and this document in a previous section. These findings led to the proposal that the densities of A_1 adenosine receptors in discrete loci of the brain may be involved in the regulation of susceptibility to seizures. In accordance with this proposal, the results of the present study demonstrate that upregulation of A_1 receptors induced by adenosine antagonist is accompanied by an elevation of seizure thresholds for convulsants. The reduced sensitivity to seizures produced by selected convulsants in animals treated chronically with theophylline may therefore represent a functional correlate of upregulation of A_1 receptors. Thus, the increased thresholds for seizures induced by convulsants in theophylline-exposed animals may represent a functional supersensitivity to the neuromodulatory actions of endogenous adenosine. The enhanced anticonvulsant action of 2-ClAdo in rats

chronically treated with theophylline further established a functional upregulation of the A_1 adenosine receptor. These data are in accordance with the recent demonstration that the dose-response curve for adenosine to depress evoked potentials was shifted significantly to the left in slices of hippocampus prepared from rats treated chronically with caffeine (183). These results suggested that the increase in the number of binding sites for ^{3}H-PIA in animals exposed to caffeine was translated into an increase in the neuromodulatory action of adenosine (183). Considered together, these results suggest that physiologically-relevant changes in the density of A_1 adenosine receptors occur in the CNS. Further investigations will be required to understand more fully the functional anatomy involved in the modulation of seizure susceptibility by A_1 receptors, given the known importance of both cortical and subcortical events in the expression of seizures induced by PTZ.

Chapter 8

Summary and Conclusions

Adenosine is considered to be a neuromodulator or
cotransmitter in the periphery and CNS. This neuromodulatory
action of adenosine may be observed as an anticonvulsant action.
The precise mechanism of adenosine and structural analogs of
adenosine to produce this anticonvulsant effect remains to be
elucidated. The studies presented in this thesis were performed
to characterize the adenosine receptor subtype involved in
mediating the anticonvulsant action of adenosine analogs in rats.

A timed-infusion technique was employed to measure the
anticonvulsant properties of the adenosine analogs. The
threshold method was considered to be reliable because
alterations in seizure threshold were a direct reflection of the
compound of interest and not due to alterations in the
pharmacokinetic parameters of the chemoconvulsant agent. The end
point of the infusion was taken as the first myoclonic jerk of
the head and neck, a phenomenon which has been reported to occur
synchronously with the first EEG (124,127) and most closely
correlates with the levels of PTZ in the brain (125,126).
Utilizing this protocol rendered the experimental method
sensitive to chemically-induced changes in neuronal activity.

Administration of 2-ClAdo (1 mg/kg, i.v.) and NECA (1 mg/kg,
i.v.) resulted in a significant increase in PTZ seizure threshold
at the 1 min post-injection time. Therefore, dose-response

curves for R-PIA, CHA, 2-ClAdo, NECA and S-PIA were generated against PTZ seizure thresholds using a time of 1 min. The adenosine analogs displayed conventional log dose-response curves. All adenosine analogs except NECA produced a maximal elevation of PTZ seizure threshold of approximately 35-45%. Of the adenosine analogs tested, NECA was the least efficacious. The rank order of potency for adenosine receptor agonists in elevating PTZ seizure threshold was R-PIA > 2-ClAdo > NECA > CHA > S-PIA, with R-PIA being 80 fold more potent then S-PIA. This 80 fold difference in potency between the R- and S-diastereoisomers of PIA was consistent with an A_1 adenosine receptor-mediated response.

The decrease in seizure susceptibility by the adenosine analogs was not due to hypotension. This was established by measuring the reduction in mean arterial blood pressure of pentobarbital anesthetized rats following the intravenous administration of R-PIA and CHA. R-PIA and CHA were approximately equipotent as hypotensive agents, while R-PIA was 48 fold more potent then CHA as an anticonvulsant agent. This difference in potency profile of R-PIA and CHA as an anticonvulsant agent versus a hypotensive agent indicated the involvement of multiple adenosine receptors.

Because the central stimulant effects of theophylline have been attributed to its ability to antagonize adenosine receptors in the CNS, the influence on seizure threshold for PTZ were evaluated. In contrast to the anticonvulsant effect of the

adenosine receptor agonists, the acute administration of theophylline produced a reduction of the seizure threshold for PTZ. The minimum threshold occurred 15 min after the administration of theophylline (30 mg/kg, i.p.). The maximum reduction elicited by theophylline was approximately 27% after the 30 mg/kg dose. The biphasic nature of the dose-response curve for theophylline was most likely a function of the multiple actions of this methylxanthine. While methylxanthines possess multiple actions, they antagonize the effects of adenosine at doses 3-300 times smaller than those causing inhibition of phosphodiesterase or mobilization of calcium (142). The proconvulsant activity of theophylline implies that endogenous adenosine functions as a modulator of excitability in the CNS.

To evaluate whether the increase in the seizure threshold induced by the adenosine analogs was mediated by stimulation of adenosine receptors, the effect of pretreatment with theophylline (5 mg/kg, i.v.) on the response to 2-ClAdo was examined. This dose of theophylline alone did not alter the seizure threshold for PTZ. Pretreatment with theophylline (5 mg/kg, i.v.) resulted in a significant antagonism of the increase in the seizure threshold induced by 2-ClAdo. The selectivity of theophylline for the adenosine receptor was established by its inability to antagonize the anticonvulsant action of diazepam and pentobarbital.

The anticonvulsant properties of the adenosine agonists were not limited to the chemoconvulsant PTZ. 2-ClAdo elevated the

seizure threshold of bicuculline (BIC) and picrotoxinin (PTX) in a dose-dependent manner. The ED_{50} values and their corresponding 95% confidence limits for 2-ClAdo to elevate PTZ, BIC and PTX seizure threshold were similar with overlapping of the 95% confidence limits. These data suggest that 2-ClAdo was producing its anticonvulsant action via a similar mechanism of action. Theophylline administration decreased the BIC and PTX seizure thresholds in a biphasic dose-response curve. The ED_{50} values for the proconvulsant action of theophylline were comparable, indicating a single mechanism of action. The increase in BIC seizure threshold by 2-ClAdo was antagonized by pretreatment with theophylline (5 mg/kg, i.v.). These results further establish the role of the adenosine receptor as a modulator of seizure activity.

Benzodiazepine receptor antagonists were used to evaluate the involvement of the benzodiazepine receptor in 2-ClAdo's anticonvulsant action. The selective benzodiazepine antagonists RO 15-1788 and CGS 8216 were unable to reverse the elevation in BIC seizure threshold induced by 2-ClAdo. This established the adenosine receptor as the receptor subtype responsible for the anticonvulsant action of 2-ClAdo.

Chronic administration of theophylline (50 mg/kg twice daily for 14 consecutive days) significantly increased the specific binding of ^{3}H-CHA in membranes of the cerebral cortex and cerebellum of the rat, but not in membranes derived from the hippocampus or diencephalon. To characterize further the

upregulation of A_1 adenosine receptors induced by theophylline,
saturation analysis with ^{3}H-CHA was performed in membranes of the
cerebral cortex and cerebellum. In cortical membranes of both
saline- and theophylline-treated rats the binding isotherms for
^{3}H-CHA could be resolved into receptor affinity states having
respectively high- (K_L) and low- (H_L) affinity for ^{3}H-CHA. The
high- and low-affinity dissociation constants obtained from
theophylline-exposed membranes of the cerebral cortex were 1.14
nM and 25.2 nM and did not differ significantly from the
corresponding values in saline-treated animals. Chronic exposure
to theophylline did, however, produce a significant increase in
the densities of both the high- (71.8% increase) and low-affinity
(31.5% increase) forms of A_1 adenosine receptors in the cerebral
cortex. Qualitatively and quantitatively similar results were
observed in cerebellar membranes. The high- and low-affinity
dissociation constants obtained from theophylline-exposed
membranes of the cerebellum wer 0.92 nM and 24.1 nM and did not
differ significantly from the corresponding values in saline-
treated animals. Chronic theophylline exposure produced a
significant increase in the densities of both the high- (53.1%
increase) and low-affinity (38.3% increase) forms of the
receptor. These results suggest that chronic exposure to
theophylline increases the density of A_1 receptors in the
cerebral cortex and cerebellum with no concomitant changes in the
ability of ^{3}H-CHA to distingiush separate agonist affinity states
of the receptor. The physiological significance of theophylline-

induced upregulation was assessed by determining seizure thresholds for convulsants in rats treated chronically with saline or theophylline. Chronic exposure to theophylline resulted in significant increases in the dose of PTZ (20% increase), BIC (28% increase) and PTX (15% increase) required to elicit a clonic convulsion. These data suggest that the reduced susceptibility to seizures observed in animals chronically exposed to theophylline, represents a functional correlate of upregulation of the A_1 adenosine receptor.

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