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Effects of repeated administration of selective adenosine A_1 and A_{2A} receptor agonists on pentylenetetrazole-induced convulsions in the rat

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Abstract

The protective effects of the selective adenosine A_1 receptor agonist, 2-chloro-N^6-cyclopentyladenosine (CCPA), the selective adenosine A_{2A} receptor agonist, 2-hexynyl-5'-*N*-ethylcarboxamidoadenosine (2HE-NECA), and the non-selective agonist, 5'-*N*-ethylcarboxamidoadenosine (NECA) were studied against lethal seizures induced by intraperitoneal (i.p.) injection of pentylenetetrazole (80 mg/kg). In acute studies there was a dose-dependent reduction of lethal seizures, as shown by the low dose's protecting 50% of animals (PD_{50}): 0.11, 0.05 and 0.05 mg/kg i.p. for CCPA, 2HE-NECA and NECA, respectively. In the repeated administration studies the animals received either vehicle or drug i.p. twice daily for 12 days. The drug doses were twice the PD_{50} value: 0.3 mg/kg for CCPA or 0.1 mg/kg for both 2HE-NECA and NECA. 2HE-NECA and NECA maintained their protective activity against pentylenetetrazole-induced seizures (63% or 60% vs. 60% or 58% in acute studies, respectively). Conversely, repeated treatment with CCPA resulted in a marked decrease of its effects (67% vs. 30% in acute studies; $P < 0.05$). The data indicate that in addition to adenosine A_1 the A_{2A} receptors also appear to be involved in the protection from seizures. The anticonvulsant effects induced by repeated stimulation of adenosine A_1 receptors are subject to tolerance, whereas effects depending on adenosine A_{2A} receptor activation are maintained.

Keywords: Adenosine receptor; Adenosine A_1 receptor agonist; Adenosine A_{2A} receptor agonist; Seizure; Pentylenetetrazole; (Rat)

1. Introduction

In the mammalian brain, endogenous adenosine acts as a neuromodulator which exerts sedative, anxiolytic and anticonvulsant activities (Dunwiddie, 1985). The actions of adenosine on the central nervous system (CNS) are mediated through interactions mainly with adenosine A_1 and A_2 receptor subtypes, the latter being subdivided into A_{2A} and A_{2B} (Fredholm et al., 1994). All these receptors have recently been cloned (Linden et al., 1993), even though they had already been classified on the basis of physiological and pharmacological criteria (Fredholm et al., 1994). A new member of the adenosine receptor family, the A_3 receptor, also has been cloned (Zhou et al., 1992). Selective agonists for the adenosine A_1 receptor (Williams et al., 1986; Monopoli et al., 1994a) and the adenosine A_{2A} receptor subtypes (Hutchison et al., 1989; Monopoli et al., 1994b) are useful pharmacological tools for exploring the function of each specific receptor.

Consistent with its role as 'retaliatory metabolite' (Newby, 1984), there is evidence that adenosine, whose brain levels increase dramatically within seconds of seizure onset (Winn et al., 1979), may act as an endogenous anticonvulsant (Dragunow et al., 1985; Berman et al., 1990). In fact, adenosine has been reported to have an inhibitory tone on synaptic transmission in that it reduces Ca^{2+} influx and opens presynaptic potassium channels (Ribeiro, 1991). It also limits excitation of the effector cells by hyperpolarizing postsynaptic membranes (Dunwiddie, 1985). Furthermore, there is evidence that activation of the adenosine A_1 receptor inhibits the release of excitatory amino acids (Corradetti et al., 1984), which are mainly in-

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volved in the generation of excitotoxic damage in ischemia and epilepsy (Dragunow, 1991; Rudolphi et al., 1992). All these actions may be responsible for the anticonvulsant effects produced by adenosine A_1 receptor activation in a variety of experimental models of epilepsy. The anticonvulsant effects of adenosine A_1 receptor agonists have been well documented against either electrically (Dragunow et al., 1985; Berman et al., 1990) or chemically induced seizures, using the chloride channel blocker, pentylenetetrazole (Dunwiddie and Worth, 1982; Murray et al., 1985; Concas et al., 1993), or the specific γ-aminobutyric acid$_A$ ($GABA_A$) receptor antagonist, bicuculline (Concas et al., 1993). Conversely, the involvement of adenosine A_{2A} receptors in the protective mechanisms of adenosine is still to be explored.

Although the protective effects of acute administration of adenosine A_1 receptor agonists in experimental models of seizures have been well characterized, only a few studies have assessed the neuropharmacological effects of adenosine analogs following chronic administration, as in the treatment of epileptic diseases. This is particularly important in view of recent findings showing that prolonged exposure of adenosine receptors to A_1 agonists leads to rapid receptor desensitization (Abbracchio et al., 1993) and tolerance to anticonvulsant (Von Lubitz et al., 1994) or hypotensive actions (Casati et al., 1994). The aim of the present study was to evaluate the central effects of repeated administration of selective adenosine agonists which we have previously characterized in in vivo and in vitro models: 2-chloro-N^6-cyclopentyladenosine (CCPA), the adenosine A_1 receptor agonist with high potency and selectivity for A_1 receptors (Lohse et al., 1988; Monopoli et al., 1994a) and 2-hexynyl-5′-*N*-ethylcarboxamidoadenosine (2HE-NECA), the potent adenosine A_{2A} receptor agonist with good A_{2A} vs. A_1 receptor selectivity (Cristalli et al., 1992; Monopoli et al., 1994b). 5′-*N*-Ethylcarboxamidoadenosine (NECA), the non-selective adenosine agonist (Bruns et al., 1986), was also used for comparison. Firstly, we studied the dose-dependent protection of a single administration of these adenosine agonists against lethal seizures induced by pentylenetetrazole in the rat. We then investigated the effects induced by repeated treatment with these agonists. The effects on adenosine A_1 and A_{2A} receptor binding in brain tissues were also evaluated.

2. Materials and methods

2.1. Animals

Male Sprague Dawley rats (175–200 g), supplied by Charles River (Calco, Italy), were used. The animals were allowed to settle under standard conditions with free access to food and water for one week before the start of the experiment.

2.2. Dose-response studies

The convulsant agent, pentylenetetrazole, was given intraperitoneally (i.p.) at the dose of 80 mg/kg 1 h after either drug or vehicle administration. After pentylenetetrazole injection, the rats were observed for 2 h in order to assess the incidence of clonic/tonic seizures and lethality. The efficacy of adenosine derivatives was determined as their PD_{50} value, the dose which protected 50% of the animals against pentylenetetrazole-induced lethal seizures. CCPA (0.03, 0.1, 0.3 and 1 mg/kg i.p.), 2HE-NECA (0.003, 0.01, 0.03 and 0.1 mg/kg i.p.) or NECA (0.01, 0.03, 0.1, 0.3 and 1 mg/kg i.p.) were administered to 9–16 rats for each dose group.

2.3. Repeated drug treatment

Two treatment schedules were used for each drug with a dose about 2-fold the PD_{50} value, calculated from the dose-response studies. In the first treatment regimen, 20–30 rats for each group received CCPA (0.3 mg/kg), 2HE-NECA (0.1 mg/kg) or NECA (0.1 mg/kg) twice daily for 12 days. For comparison, the effects of single drug administration (acute) were assessed in groups of animals that had received repeated treatment with vehicle for the entire length of the experiment (twice daily for 12 days). The last treatment was a single dose of CCPA (0.3 mg/kg), 2HE-NECA (0.1 mg/kg) or NECA (0.1 mg/kg). One hour later, the animals were challenged with pentylenetetrazole (80 mg/kg) and observed for 2 h. Latency of the onset of seizures and incidence of lethality were recorded.

2.4. Receptor binding assay

Adenosine A_1 and A_{2A} receptor binding assays were performed on cerebral cortex and striatum respectively, obtained from animals that had received repeated drug administration. After a 36-h washout period, the rats were decapitated and tissues were pooled and disrupted in a Polytron PTA 10 probe (setting 5.30 s) in 25 vols. of 50 mM Tris-HCl buffer, pH 7.4. The homogenates were centrifuged twice at 40 000 and once at 48 000 × *g*, respectively, for 10 min at 4°C and resuspended in Tris-HCl containing 2 units/ml adenosine deaminase. After 30-min incubation at 37°C, the membranes were centrifuged, and the pellet was stored at −70°C.

Adenosine A_1 and A_{2A} receptor binding assays were performed according to Bruns et al. (1980) and Jarvis et al. (1989) using [^{3}H]N^6-cyclohexyladenosine and [^{3}H]2-[4-(2-carboxyethyl)phenethylamino]-5′-*N*-ethyl-

Table 1
Protective effects of the adenosine agonists, CCPA, 2HE-NECA and NECA, against pentylenetetrazole-induced lethal seizures in the rat

Drug	Adenosine receptor subtype	Dose range (mg/kg i.p.)	PD_{50} (mg/kg i.p.) (95% confidence limits)
CCPA	A_1	0.03 –1	0.11 (0.03–0.25)
2HE-NECA	A_{2A}	0.003–0.1	0.05 (0.02–0.26)
NECA	A_1/A_{2A}	0.01 –1	0.05 (0.02–0.10)

Pentylenetetrazole (80 mg/kg i.p.) was injected 1 h after drug administration. The PD_{50} values with 95% confidence limits were determined by PROBIT analysis. Number of animals for each dose group was 9–16.

carboxamidoadenosine (CGS 21680) as radioligands, respectively. Non-specific binding at adenosine A_1 and A_{2A} receptors was defined in the presence of cyclohexyladenosine (10 μM) and NECA (100 μM), respectively, and was $\leq 10\%$ of total binding. Radioactivity was determined using a LS-6000 Beckman liquid scintillation counter (Beckman Instruments, Fullerton, CA, USA) at an efficiency of 50–60%. Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard. Saturation studies at A_1 cortical receptors were carried out by incubating membranes with 12 different concentrations of [^{3}H]cyclohexyladenosine ranging from 0.125 to 128 nM. At adenosine A_{2A} striatal receptors, 'mixed curves' (Rovati et al., 1991) with [^{3}H]CGS 21680 were performed by using five radioligand concentrations ranging between 1 and 16 nM for the 'saturation' part of the curve, and four concentrations of cold ligand (32–128 nM) against 16 nM [^{3}H]CGS 21680 for the 'displacement' part of the curve. The total number of receptor sites (B_{max}) and the dissociation constant (K_d) values were determined from Scatchard analysis of saturation binding experiments.

2.5. Statistical analysis

In dose-response studies PD_{50} values with 95% confidence limits were determined by PROBIT analysis (Finney, 1971). In chronic studies, statistical differences in seizure latencies were tested using Dunnett's t-test. Comparisons between the incidence of lethality in single-treated or chronic-treated groups and control groups were performed using Fisher's exact test. In binding studies, differences in K_d and B_{max} between treated and control groups were evaluated according to Dunnett's t-test. All computations were performed by means of the SAS PROC GLM program (SAS Institute, 1987).

2.6. Drugs

CCPA and NECA were purchased from Research Biochemicals (Natick, MA, USA). 2HE-NECA was synthesized at the Department of Chemical Science of the University of Camerino, Italy. The drugs were administered i.p. (3 ml/kg) as aqueous suspensions obtained by adding a few drops of Tween 80 (ICN Biochemical, Milan, Italy). Pentylenetetrazole was purchased from Aldrich Chimica (Milan, Italy).

3. Results

3.1. Dose-response studies

Pentylenetetrazole-induced seizures were characterized by tremors and Straub tail, followed by myoclonic jerks of the limbs, leading to full generalized clonic/tonic seizures. Generally, death occurred within a few minutes of the onset of clonic/tonic seizures or, in any case, within 1 h. CCPA, 2HE-NECA and NECA showed protection against pentylenetetrazole-induced

Table 2
Effects of acute or chronic administration of CCPA (0.3 mg/kg), 2HE-NECA (0.1 mg/kg) and NECA (0.1 mg/kg) on pentylenetetrazole-induced lethal seizures in rats

Treatment	Seizure latency (s)	Clonic/tonic seizures	Lethality	Protection from lethality
Vehicle	69 ± 7	27/30	25/27	7%
CCPA acute	104 ± 10 [a]	18/20	6/18 [c]	67%
CCPA chronic	85 ± 9	20/22	14/20 [d]	30%
2HE-NECA acute	122 ± 12 [b]	30/30	11/30 [c]	63%
2HE-NECA chronic	103 ± 12	30/30	12/30 [c]	60%
NECA acute	113 ± 24	20/20	8/20 [c]	60%
NECA chronic	170 ± 26 [b]	21/22	10/21 [c]	58%

The animals received either vehicle or drug i.p. twice daily for 12 days. Pentylenetetrazole (80 mg/kg i.p.) was administered 1 h after the last drug treatment. To study the effects of single administration (acute) drugs were injected on day 12, 1 h before pentylenetetrazole. Lethality was estimated as the ratio between number of dead animals and number of animals showing convulsions. Statistical comparison of data was performed with Dunnett's t-test: [a] $P < 0.05$, [b] $P < 0.01$ vs. vehicle. Fisher's exact test: [c] $P < 0.01$ vs. vehicle, [d] $P < 0.05$ vs. acute treatment.

lethality, being effective over a low dose range (Table 1). At a dose 2-fold the PD_{50} value of each drug, both CCPA and 2HE-NECA significantly delayed the seizure latency (104 ± 10 or 122 ± 12 vs. 69 ± 7 s of vehicle, $P < 0.05$ and $P < 0.01$, respectively), whereas the effect of NECA was not significant (Table 2). However, none of the drugs reduced frequency or severity of seizures.

3.2. Repeated drug treatment

A dose twice the PD_{50} value, calculated for each drug, was used for the repeated-dose regimen studies. The results obtained from the two administration schedules, i.e. acute and chronic drug administration, are summarized in Table 2. Independently of the drug treatment regimen, at least 90% of animals in each group showed clonic/tonic seizures following pentylenetetrazole injection. The acute treatment with each compound produced significant protection against pentylenetetrazole-induced lethal seizures ($P < 0.01$). This protective activity was also observed following repeated administration of either 2HE-NECA or NECA. Seizure latency remained elevated after repeated treatment with 2HE-NECA even though the effect was not significant, whereas NECA significantly delayed the onset of seizures in the repeated-dose regimen ($P < 0.01$). Conversely, the adenosine A_1-selective agonist, CCPA, lost its protective effects against seizures ($P < 0.05$ vs. acute treatment). CCPA, which delayed seizure onset after single administration, was unable to increase seizure latency following the 12-day dose regimen.

3.3. Receptor binding studies

Repeated treatment with CCPA, 2HE-NECA and NECA did not affect either adenosine A_1 or A_{2A} receptor binding parameters in rat cerebral cortices and striata, respectively (Table 3). Scatchard plot analysis of [^{3}H]cyclohexyladenosine and [^{3}H]CGS 21680 showed that none of these adenosine compounds changed either B_{max} or K_d of radioligands.

4. Discussion

Adenosine is thought to play an important role in neuronal functioning by interacting with specific adenosine receptors (Dunwiddie, 1985). Activation of these receptors has an important adaptive function in states of neuronal hyperactivity such as epileptic seizures (Dragunow, 1991). In addition, adenosine modulates the activity of ion fluxes and neurotransmitters at both presynaptic and postsynaptic sites (Ribeiro, 1991). Together with an increase in local vascular supply, these effects tend to minimize any cellular damage potentially resulting from metabolic stress (Williams, 1987).

In agreement with these findings, our results indicate that the selective adenosine A_1 receptor agonist, CCPA, the selective A_{2A} receptor agonist, 2HE-NECA, and the non-selective A_1/A_{2A} receptor agonist, NECA, all produce dose-dependent protection against pentylenetetrazole-induced lethality. These data suggest that adenosine A_1 and A_{2A} receptors are both involved in the protective mechanisms of adenosine.

Our findings with CCPA are also consistent with data showing the anticonvulsant action of adenosine A_1 receptor stimulation (Dunwiddie and Worth, 1982; Dragunow et al., 1985; Von Lubitz et al., 1993). Specifically, it was demonstrated that the A_1 agonist, L-phenylisopropyl adenosine (L-PIA), gives only a relatively low percentage of protection (35%) against pentylenetetrazole-induced lethality in mice, when compared with the effect of diazepam (100%) (Dunwiddie and Worth, 1982). More recently, Concas et al. (1993) reported that, in spite of its negative modulation of $GABA_A$ receptor function, CCPA (1 mg/kg i.p.) markedly antagonizes convulsions induced by a sublethal dose of pentylenetetrazole (55 mg/kg) in mice. In our studies, CCPA exerted a protective action against pentylenetetrazole-induced lethal seizures at a low dose range, the PD_{50} value being 0.11 mg/kg i.p. However, given at 0.3 mg/kg, CCPA protected only 67% of the animals. Using different convulsant agents, Klitgaard et al. (1993) as well as Dunwiddie and Worth (1982) found that adenosine analogs have low efficacy

Table 3
Effects of chronic treatment with CCPA, 2HE-NECA and NECA on receptor density (B_{max}) and dissociation constant (K_d) of A_1 and A_{2A} adenosine receptors

Treatment	A_1		A_{2A}	
	B_{max} (fmol/mg protein)	K_d (nM)	B_{max} (fmol/mg protein)	K_d (nM)
Vehicle	468 ± 18	1.15 ± 0.05	1281 ± 183	26 ± 4.8
CCPA	467 ± 18	1.23 ± 0.06	1228 ± 184	24 ± 2.6
2HE-NECA	510 ± 15	1.31 ± 0.16	1328 ± 114	24 ± 3.5
NECA	489 ± 13	1.20 ± 0.11	1239 ± 120	26 ± 3.8

Values are means ± S.E. of 4 experiments. There is no statistically significant difference either in B_{max} or K_d values. Dunnett's t-test.

against seizures induced by pentylenetetrazole, even though this animal model is widely used for testing new anticonvulsant agents (Swinyard et al., 1986). This may well depend upon the mechanism by which stimulation of adenosine A_1 receptors ultimately changes the neurotransmission mediated by GABA or excitatory amino acids, whose net effects appear to be influenced by adenosine (Williams, 1987). The mechanism underlying the anticonvulsant activity of adenosine A_1 receptor agonists still needs to be clarified.

An important result of the present study is that 2HE-NECA, a compound interacting selectively with the adenosine A_{2A} receptor, is protective against seizures induced by pentylenetetrazole. In addition, 2HE-NECA and the non-selective agonist, NECA, seem to be more potent than the adenosine A_1-selective agonist, CCPA, as shown by their lower PD_{50} values. To our knowledge, this is the first evidence for the clear action of adenosine A_{2A} receptor activation in protecting animals from seizures. There are indeed few data on the involvement of the adenosine A_{2A} receptor in the anticonvulsant activity of adenosine. Janusz and Berman (1993) observed that cerebral infusion of the adenosine A_{2A} receptor agonist, CGS 21680, does not affect behavioral seizure stage or seizure threshold in kindled rats but they also observed that simultaneous activation of adenosine A_1 and A_{2A} receptors leads to the potentiation of the anticonvulsant effects of adenosine itself. The concomitant activation of both adenosine receptors may also account for the higher potency of NECA, the non-selective A_1/A_{2A} receptor agonist, to protect against pentylenetetrazole-induced lethal seizures as compared to the selective A_1 receptor agonist CCPA. Probably, the additional effects induced by adenosine A_{2A} receptor activation during the critical postictal period may contribute significantly to minimizing cell damage by increasing blood flow and reducing free radical formation (Fredholm et al., 1993). However, the exact role of adenosine in the regulation of cerebral blood flow (CBF) is still to be clarified, since there is evidence for either positive (Van Wylen et al., 1989) or negative (McBean et al., 1989) modulation of CBF by adenosine. Moreover, Rosen and Berman (1985) have demonstrated that the effects of adenosine analogs on postictal events in amygdala-kindled rats are not related to their hypotensive activity, since hydralazine, a drug which decreases blood pressure through a direct dilator action on vascular smooth muscle, does not affect ictal or postictal events. In view of these findings it seems that the protection exerted by the adenosine A_{2A} receptor agonist, 2HE-NECA, against postictal lethality may well be independent from its hemodynamic effects, even though further investigation is needed.

Recent work has demonstrated that 2HE-NECA also interacts with A_3 receptors, displaying a K_i value of 26 nM (Siddiqi et al., 1995) versus 2.2 nM at A_{2A} receptors (Monopoli et al., 1994b). This result is particularly interesting considering the recent observation by Von Lubitz et al. (1995) that the adenosine A_3-selective agonist, N^6-(3-iodobenzyl) adenosine-5′-*N*-methylcarboxamide (IB-MECA), provides significant protection under both acute and chronic conditions in the model of pentylenetetrazole-induced seizures. Although full characterization of the physiological functions of the recently discovered adenosine A_3 receptors is still to be made, it appears that A_3 receptor activation may be partially responsible for the protective effects of 2HE-NECA observed under our conditions.

The controversy concerning the actual site of action of adenosine agonists has not yet been resolved. Although the systemic administration of stable adenosine analogs has been widely proved to induce behavioral changes (Dunwiddie and Worth, 1982; Durcan and Morgan, 1989) and anticonvulsant effects (Dragunow et al., 1985; Berman et al., 1990; Concas et al., 1993), it is unclear to what extent they pass through the blood-brain barrier and act directly on central neurons: evidence for (Rosen and Berman, 1985; Durcan and Morgan, 1989) and against (Brodie et al., 1987) has been offered. Unfortunately, our study, though confirming the anticonvulsant effects of peripherally administered adenosine receptor agonists, does not help to resolve this long-standing controversy.

Prolonged exposure of rat brain tissues to adenosine analogs results in selective and dose-dependent desensitization of adenosine A_1 receptors (Abbracchio et al., 1993). This observation is supported by in vivo data showing the loss of A_1-mediated anticonvulsant effects after repeated treatment with the adenosine A_1 receptor agonist, N^6-cyclopentyl adenosine (CPA), in *N*-methyl-D-aspartate-evoked seizures in mice (Von Lubitz et al., 1994). Conversely, cerebral adenosine A_{2A} receptors seem to be resistant to agonist-induced desensitization (Abbracchio et al., 1993). In accordance with these observations, our study showed that repeated stimulation of the adenosine A_1 receptor by CCPA leads to tolerance to anticonvulsant activity, whereas both the A_{2A} agonist, 2HE-NECA, and the non-selective agonist, NECA, maintain their protective effects against pentylenetetrazole-induced seizures. In previous studies we found that, also in the cardiovascular system, repeated administration of CCPA leads to tolerance to both hypotensive and bradycardiac effects, whereas 2HE-NECA and NECA maintain their antihypertensive properties throughout the 21-day experimental period (Casati et al., 1994).

In agreement with results of previous studies (Von Lubitz et al., 1994; Casati et al., 1994), our data also showed that receptor binding parameters are not af-

fected by prolonged stimulation of adenosine receptors. Other mechanisms such as uncoupling of the second messenger system (Abbracchio et al., 1993) may well underlie the reduction of activity upon repeated stimulation of central or peripheral adenosine A_1 receptors.

In conclusion, our results provide evidence for the involvement of the adenosine A_{2A} receptor, as well as the A_1 receptor, in the anticonvulsant activity of adenosine. Moreover, these A_{2A}-mediated effects do not undergo adaptive changes after repeated treatment with either selective or non-selective agonists. The data available do not, however, allow us to identify the mechanisms of action by which stable adenosine analogs, interacting with the adenosine A_{2A} receptor, exert anticonvulsant activity. In addition, this study further confirms the well known anticonvulsant activity of acute administration of adenosine A_1-selective agonists, an action to which tolerance develops when the agonist is given repeatedly. Adenosine agonists do not appear to offer a potential as anticonvulsant agents because of the variety of peripheral, mainly cardiovascular, effects associated with the central action (Dunwiddie and Worth, 1982; Monopoli et al., 1994a,b). These studies help, however, to clarify the mechanisms governing the different actions of the adenosine A_1 compared with A_{2A} receptors in the CNS.

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