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Chronic adenosine A_1 receptor agonist and antagonist: effect on receptor density and *N*-methyl-D-aspartate induced seizures in mice

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Abstract

The effect of chronic administration of the adenosine A_1 receptor agonist N^6-cyclopentyladenosine (CPA) and the adenosine A_1 antagonist 8-cyclopentyl-1,3-dipropylxanthine (CPX) on *N*-methyl-D-aspartate (NMDA)-evoked seizures was studied in C57BL/6 mice (20/group). Animals were injected i.p. for 9 days with either 1.0 mg/kg CPA or 1.0 mg/kg CPX followed by 2 injection-free days (the washout period) and subsequent administration of a single dose of 60 mg/kg NMDA. As in our previous study, this dose of NMDA caused clonic/tonic seizures resulting in high (60%) mortality within 3 h after injection of the drug. Despite insignificant changes in seizure latency, chronic pretreatment with CPA increased the incidence of clonic/tonic episodes and end-point mortality. Conversely, chronic exposure to CPX completely eliminated clonic/tonic episodes, significantly increased average survival time, and reduced end-point mortality ($P < 0.05$). The results indicate that chronic treatment with adenosine A_1 receptor antagonist may protect against NMDA-evoked seizures to the same degree as previously observed following a single, acute exposure to CPA. Since the density of adenosine receptor binding sites was unchanged after chronic treatment with either CPX or CPA, it is likely that the mechanism behind the observed protection may rest at the level of second messenger systems coupled to adenosine A_1 receptors.

Keywords

Adenosine analog; Seizure; NMDA (*N*-methyl-D-aspartate); (Mouse)

I. Introduction

Endogenous adenosine has a protective role in the heart (Berne, 1963; Berne et al., 1974), central nervous system (CNS) (Dragunow and Faull, 1988), and in other systems (Daval et al., 1992). In the brain, adenosine inhibits calcium influx and opens presynaptic potassium channels causing a significant reduction of the release of glutamate and other neurotransmitters (for a review, see Ribeiro, 1991). Hence, adenosine is considered to provide an ‘inhibitory tone’ in the mammalian CNS (Harms et al., 1978).

Excessive release of glutamate and other excitatory amino acids is intimately involved in generation of excitotoxic damage in ischemia and epilepsy (Olney, 1978; Olney et al.,

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1986). Since adenosine inhibits release of excitatory amino acids during ischemia (Phillis et al., 1991), it is not surprising that several studies showed considerable degree of neuroprotection following both pre-and postischemic treatment with adenosine A_1 receptor agonists (reviewed by Rudolphi et al., 1992 and by Miller and Hsu, 1992). Presynaptic inhibitory effects of adenosine and its agonists are unquestionably intensified by postsynaptic modulation of both K^+ (Schubert and Lee, 1986) and Ca^{2+} (Schubert, 1988; Schubert et al., 1986, 1992) channels. The resultant hyperpolarization reduces neuronal excitability and serves to amplify the neuroprotective impact of these agents even further. Conversely, acute administration of adenosine A_1 receptor antagonists appear, on the other hand, to aggravate ischemic brain injury (Rudolphi et al., 1990).

In concert with its role as a 'retaliatory compound' (Newby, 1984), it has been also suggested that adenosine may act as an endogenous anticonvulsant (Dragunow, 1985). Extensive support of this hypothesis has been brought by studies of several authors who demonstrated amelioration of chemically and electrically elicited seizures (Dragunow et al., 1985; Barraco et al., 1986; Murray and Szot, 1986; Murray et al., 1985). We have recently described that an acute administration of a highly selective adenosine A_1 receptor agonist, N^6-cyclopentyladenosine (CPA) is highly protective against seizures and mortality evoked by NMDA, and that an equally selective antagonist, 8-cyclopentyl-1,3-dipropylxanthine (CPX) significantly worsens the effect of sublethal doses of NMDA (Von Lubitz et al., 1993a). In the present study we demonstrate that chronic administration of either CPA or CPX results in the outcome that is diametrically opposite to that seen when either of these drugs is administered acutely.

2. Materials and methods

Male C57BL/6 mice (Jackson Laboratories, Bar Harbor, MA) weighing approximately 30 g each were used in the study. All drugs were purchased from Research Biochemicals (Natick, MA). CPA and CPX were dissolved in a 20 : 80 mixture of Alkamuls EL-620 (Rhône-Poulenc, Cranbury, NJ) and saline, while NMDA was dissolved in buffered saline (pH 7.4). All drug solutions were injected i.p. at a dose of 1 mg/kg (0.15 ml/injection) using a 25 gauge needle. CPA and CPX were administered once daily for 9 days. After a 2-day injection-free wash-out period, a single injection (60 mg/kg) of NMDA was administered. The animals were immediately placed in a transparent cage for observation. The onset latency of seizures and/or other abnormal behavioral responses, and the time of death were recorded. The end-point for mortality studies was set at 24 h after injection of NMDA.

To exclude a possibility of motor disturbances caused by chronic administration of either CPA or CPX, separate groups of mice ($n = 10$/group) were also tested at the end of the wash-out period on a rotorod revolving at 5 r.p.m.

The severity of neurological impairment following drug treatment was graded on a 5 point scale (Table 1). Statistical significance of the average degree of neurological impairment, differences in the incidence and delay of seizures, and time to death was evaluated using Bonferroni's corrected Student's *t*-test. Percent incidence of seizures and other forms of neurological impairment, and the end-point survival data were determined using Fisher's exact test. $P < 0.05$ was considered significant.

The brains were removed either immediately upon death or at the survival end-point, i.e., 24 h after the injection of NMDA, and frozen on dry ice. Subsequently, they were separated according to the treatment (i.e., either NMDA, CPX + NMDA, or CPA + NMDA) and to the manner in which they were obtained (i.e., post mortem or post sacrificio). Forebrains in each category were then randomly subdivided into 3 subgroups and homogenized in 10 volumes (v/w) of 0.32% sucrose solution. The homogenate was centrifuged at 1000 × *g* for 10 min

and the supernatant was removed and recentrifuged at 32000 × *g* for 40 min. The resulting pellet was resuspended in water, recentrifuged, and the pellet resuspended in Tris · HCl buffer (pH 7.4) at a concentration of 1.5–2 mg protein/ml. All the above procedures were carried out at 4°C. Protein content was determined using the BCA protein assay reagents (Pierce Chemical Co., Rockford, IL). [^{3}H]CPX (DuPont NEN, Boston, MA) saturation studies were carried out as described (Bruns et al., 1987) at 25°C, using the radioligand in the range of 0.04–3 nM. Each incubation tube contained ca. 30–50 μg protein in a total volume of 500 μl Tris. HCl, pH 7.4, with adenosine deaminase (3 IU/ml) present. Scatchard analysis was used to determine B_{max} and K_d

3. Results

3.1. The effects of chronically administered CPA and CPX

Chronic injections of CPA or CPX alone produced no locomotor effects, and all animals stayed indefinitely on the rotorod.

3.2. Administration of NMDA

Administration of NMDA at 60 mg/kg had no effect on either B_{max} or K_d. Treatment with NMDA alone resulted in a 30 min period of locomotor hyperactivity ensuing within 5 min after the injection in 60% of animals (Tables 3 and 4). In the remaining 40% of animals, hyperactivity transformed into clonic or clonic/tonic seizures, with death following 3–10 min thereafter. Mortality at 24 h following NMDA administration was 60%.

3.3. Administration of NMDA following chronic CPX

Injection of NMDA following chronic treatment with CPX caused a significant ($P < 0.05$) reduction in the intensity of seizures and in mortality (Table 3). In all survivors a 3–4 h period of locomotor depression ensued within 2 min following administration of NMDA. However, the depressed animals were stimulus sensitive, responding to both touch and sharp noises by a rapid translocation to a different location within the cage. Neither ataxia nor any other impairment of gait were seen during such translocation.

3.4. Administration of NMDA following chronic CPA

Neither neurological impairment nor symptom latency in this group differed significantly from those seen in animals injected with NMDA alone. However, while in the NMDA group only 40% animals showed a clonic/tonic complex, in the CPA + NMDA group it was present in 70% mice. Furthermore, the mortality increased to 90% and all deaths occurred within 5 min following administration of NMDA (Tables 3 and 4).

3.5. Receptor density after acute treatment with NMDA or after chronic treatment with CPA or CPX

Chronic treatment with CPA or CPX, or acute treatment with NMDA did not affect either B_{max} or K_d. Moreover, no significant changes in receptor densities or dissociation constants were seen after chronic treatment with CPA or CPX followed by a challenge with NMDA (Table 2). The length of postictal survival did not have any influence either, and the values of B_{max} and K_d from animals whose brains were analyzed post mortem were fully comparable to those examined post sacrificio (data not shown).

4. Discussion

Pro- and anticonvulsant actions of acutely administered adenosine antagonists and agonists have been described in a variety of models by several authors (for a review see Dragunow, 1991). However, the impact of chronic administration of adenosine A_1 receptor agonists has

been hitherto unknown, and only very recently Georgiev et al. (1993) reported that the chronically administered non-selective adenosine receptor antagonist caffeine and other non-selective xanthines offer significant protection against NMDA evoked seizures. Our present observations that chronic administration of the highly selective adenosine A_1 receptor antagonist CPX results in a pronounced anticonvulsive effect is consistent with that study. Moreover, the observed behavioral responses are very similar to those seen following acute administration of the adenosine A_1 receptor agonist CPA prior to a 60 mg/kg dose of NMDA (Von Lubitz et al., 1993b).

We have previously argued that, since hypothermic effects of acutely administered CPA did not contribute to the observed protection, the chief source of amelioration of NMDA-induced seizures was related to the interaction of CPA with adenosine A_1 receptors (Von Lubitz et al., 1993b). Since during our recent experiments (Von Lubitz et al., in preparation) we have demonstrated that the acute effect of either CPA or CPX in rats is relatively short lasting and does not exceed 90 min, such explanation is less satisfactory in the present study. It is highly unlikely that effective concentration of either drug can be found in the circulation or in the interstitial space of the brain at the end of the 2-day wash-out period. Therefore, as in our previous investigation, the impact of either CPA or CPX on the body temperature can be discounted. In the absence of either drug in the circulation, it is equally unlikely that the observed effects are the result of simple and direct drug-adenosine A_1 receptor interaction. Chronic treatment with the non-selective antagonist caffeine causes significant shifts in the density of radioligand binding sites at cholinergic, serotonergic as well as adenosine A_1 (but not A_{2A}) receptors (Shi et al., 1993). Hence, it is probable that chronic treatment with ligands of much higher selectivity will also cause complex and long-lasting changes in several receptor systems, many of which may be involved either in seizure generation or spreading.

A number of prior investigations indicate that chronic treatment with adenosine A_1 receptor ligands results either in receptor up-or downregulation (Ramkumar et al., 1988; Abbracchio and Catabeni, 1992) depending whether an antagonist or agonist is used. Although neither Georgiev et al. (1993) nor we (present study) were able to demonstrate such changes, their presence cannot be definitively excluded since the nature of our methods (whole brain preparation) might have masked receptor shifts in discrete regions of the brain, e.g., hippocampus. Nonetheless, it is also possible that the source of the protection against NMDA induced seizures rests at the level of coupling of adenosine A_1 receptors to second messenger systems. Experiments using oligonucleotide probes may shed more light upon the degree to which adenosine A_1 receptors and G-proteins (e.g., G_i) are expressed under chronic treatment with highly selective adenosine A_1 receptor ligands.

Our present results emphasize that the effects of chronically vs. acutely administered agents acting at the adenosine A_1 receptor are diametrically opposed. Moreover, such differences may characterize not only the effect of these drugs on NMDA evoked seizures but possibly other processes in which NMDA receptors may be intimately involved. This notion is supported by our study in which the effects of chronically administered CPA or CPX on spatial learning and memory were investigated (Von Lubitz et al., 1993b) showing that chronically administered CPA enhanced, while CPX impaired, learning in C57/B mice. Also these results were in direct contrast to those following acute administration of adenosine receptor ligands in which an agonist impaired (Normile and Barraco, 1991), while an antagonist improved (Schingnitz et al., 1991) learning capacity. The dependence of a therapeutic result on the dosing regimen advocates caution in clinical administration of adenosinergics which has been suggested by numerous authors (see Daval et al., 1992). Nonetheless, depending upon circumstances, agents acting at adenosine A_1 receptors may

offer a very flexible approach to the treatment of several neurode-generative disorders of the brain.

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Table 1

Neurological impairment scale

No change	0
Depression	1
Scratching/biting	2
Hyperactivity	3
Clonic seizures	4
Clonig/tonic complexes	5

Table 2

Receptor densities (B_{max}) and dissociation constants of adenosine A_1 receptors in animals injected acutely with NMDA or chronically with either CPA or CPX

	B_{max} [a]	K_d [b]
NMDA	788 ± 16	0.42 ± 0.06
CPA + NMDA	844 ±40	0.42 ± 0.08
CPX + NMDA	976 ± 169	0.40 ± 0.01

Values are ± S.E.M. There are no statistically significant differences either in B_{max} or K_d values. Bonferroni's corrected Student's *t*-test. $n = 10$/group.

[a] fmol/mg tissue.

[b] nM.

Table 3

Average latency to visible behavioral changes, degree of neurological impairment, and time to death following either NMDA, chronic CPA followed by single injection of NMDA, or chronic CPX followed by single injection of NMDA

	Latency (s)	Impairment	Td (h)
NMDA	128 ± 32	2.3 ± 0.4	0.6 ± 0.2
CPA + NMDA	70 ± 17	3.0 ± 0.4	0.2 ± 0.1[a]
CPX + NMDA	165 ± 38	1.1 ± 0.3[a,b]	15.3 ± 3.6[a,b]

All values ± S.E.M. Td: time to death.

[a] $p < 0.05$ compared to controls (NMDA).

[b] $p < 0.05$ CPA compared to CPX. Bonferroni's corrected Student's *t*-test. $n = 10$/group.

Table 4

Percent incidence of seizures, locomotor depression and mortality following either a single injection of NMDA, chronic treatment with CPA followed by injection of NMDA, or chronic treatment with CPX followed by injection with NMDA

	Clon/ton	Clon/hyper	Depr	Mort
NMDA	40	60	0	60
CPA + NMDA	70 [a]	30 [a]	0	90
CPX + NMDA	0 [a,b]	30 [a]	70 [a,b]	30 [a,b]

Clon/ton: clonic/tonic complexes. Clon/hyper: either clonic seizures or hyperactivity. Depr: locomotor depression. Mort: mortality.

[a] $p < 0.05$ compared to controls (NMDA).

[b] $p < 0.05$ CPA compared to CPX. Bonferroni's corrected Student's *t*-test. $n = 10$/group.