ELSEVIER

Pharmacology Biochemistry and Behavior, Vol. 53, No. 2, pp. 249–255, 1996

0091-3057/96 $15.00 + .00

0091-3057(95)00208-1

# Chronic Ethanol Intoxication Enhances [$^3$H]CCPA Binding and Does Not Reduce $A_1$ Adenosine Receptor Function in Rat Cerebellum

ALESSANDRA CONCAS,[1] MARIA P. MASCIA, TONINO CUCCHEDDU, STEFANIA FLORIS, MARIA C. MOSTALLINO, CHIARA PERRA, STEFANIA SATTA AND GIOVANNI BIGGIO

*Department of Experimental Biology, Chair of Pharmacology, University of Cagliari, Italy*

Received 17 February 1995; Accepted 10 May 1995

CONCAS, A., M. P. MASCIA, T. CUCCHEDDU, S. FLORIS, M. C. MOSTALLINO, C. PERRA, S. SATTA AND G. BIGGIO. *Chronic ethanol intoxication enhances [$^3$H]CCPA binding and does not reduce $A_1$ adenosine receptor function in rat cerebellum.* PHARMACOL BIOCHEM BEHAV **53**(2) 249–255, 1996.—The effects of acute and chronic treatment with ethanol on the function of $A_1$ adenosine receptor in the rat cerebellar cortex were investigated. Acute administration of ethanol (0.5–5 g/kg) had no effect on the binding of the $A_1$-receptor agonist [$^3$H]2-chloro-$N^6$-cyclopentyladenosine ([$^3$H]CCPA) or that of the antagonist [$^3$H]8-cyclopentyl-1-3-dipropylxanthine ([$^3$H]DPCPX) in rat cerebellar cortical membranes. Rats were rendered ethanol dependent by repeated forced oral administration of ethanol (12–18 g/kg per day) for 6 days. [$^3$H]CCPA binding was increased by 23% in cerebellar cortical membranes prepared from rats killed 3 h after ethanol withdrawal compared with saline-treated animals. The increase in [$^3$H]CCPA binding was still apparent 12–24 h after the last ethanol administration, but was no longer detectable 3–6 days after ethanol withdrawal. In contrast, the binding of [$^3$H]DPCPX was not modified in the cerebellar cortex of rats killed at various times after ethanol withdrawal. The acute administration of CCPA [0.25–1 mg/kg, intraperitoneally (IP)] suppressed the tremors and audiogenic seizures apparent 24 h after ethanol withdrawal. Moreover, repeated coadministration of CCPA (0.5 mg/kg, IP, four times daily) and ethanol did not prevent the generation of audiogenic seizures during withdrawal but completely prevented mortality. Finally, CCPA antagonized with similar potencies and efficacies the isoniazid-induced convulsions observed in control and ethanol-withdrawn rats. These results indicate that long-term treatment with intoxicating doses of ethanol enhances [$^3$H]CCPA binding but does not reduce the anticonvulsant efficacy of CCPA or the function of $A_1$ adenosine receptors.

Chronic ethanol  $A_1$ receptors  CCPA  Withdrawal  Rat

ADENOSINE has been characterized as a homeostatic neuromodulator. This nucleoside is released as a consequence of neuronal activity and participates in the regulation of multiple physiologic functions in various mammalian tissues (11). The major behavioral properties of adenosine include sedation, anticonvulsant activity, and neuroprotective effects. These actions are mediated through at least two types of membrane-bound extracellular receptors, termed $A_1$ and $A_2$ (17,32), that have been distinguished on the basis of both pharmacologic and biochemical criteria. Activation of $A_1$ receptors both decreases neurotransmitter release, probably via presynaptic activation of a $K^+$ conductance or inhibition of $Ca^{2+}$ currents (12,14,18,31), and reduces the neurotransmitter sensitivity of the postsynaptic membrane, via inhibition of adenylate cyclase (16,32).

The availability of high-affinity ligands for $A_1$ adenosine receptors has contributed to the pharmacologic characterization of these binding sites. Among these ligands, the 2-chloro-$N^6$-cyclopentyladenosine (CCPA) shows agonist properties (23) and binds with subnanomolar affinity and a high selectivity (10,000-fold) to $A_1$ receptors (21). Moreover, CCPA exhibits its potent anticonvulsant activity against seizures induced by

[1] Requests for reprints should be addressed to A. Concas, Department of Experimental Biology, University of Cagliari, Via Palabanda, 12, 09123, Cagliari, Italy.

various convulsant drugs (7), an effect specifically antagonized by 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), the most selective $A_1$ receptor antagonist available (22).

Recent evidence suggests that $A_1$ adenosine receptors may be important in the effects of ethanol on the CNS. Thus, Clark and Dar (4) showed that acute ethanol administration increases the density of $A_1$ receptors in the rat cerebellum. On the other hand, studies on the effects of chronic ethanol administration on $A_1$ receptors have yielded conflicting results, showing a decrease in the number of $A_1$ receptors or no effect (9,10).

We investigated the effects of chronic ethanol intoxication on [$^3$H]CCPA binding and [$^3$H]DPCPX binding to $A_1$ adenosine receptors in the rat brain. Moreover, to understand further the functional role of $A_1$ receptors in the central effects of chronic ethanol exposure, we examined changes in the binding of these ligands during ethanol withdrawal syndrome and the ability of CCPA to reduce or abolish withdrawal symptoms (tremors and convulsions) in rats physically dependent on ethanol. A preliminary account of a part of this work has appeared as a rapid communication (6).

## METHODS

### *Rats and Intoxication Procedure*

We studied male Sprague–Dawley rats (Charles River, Como, Italy), with body masses of 250–300 g at the beginning of the experiments. After delivery to our animal facilities, rats were allowed to acclimatize to the new housing conditions for at least 1 week. Animals were housed four per cage under an artificial 12 L : 12 D cycle (light on from 0800–2000 h) at a constant temperature of 22 ± 2°C and a relative humidity of 60%. Animals were rendered ethanol dependent by the method of Majchrowicz (26), which consisted of the repeated administration of a 20% (w/v) ethanol solution (per os, by gavage) four times daily (0000, 0600, 1200, and 1800 h) for 6 days, to maintain a constant blood ethanol concentration. Each rat received a daily dose of 12–18 g ethanol/kg body mass in four divided doses (maximum 5 g/kg). Experiments were initiated by administering a priming dose of ethanol (5 g/kg), and subsequent doses were determined for each animal at every administration on the basis of the observed degree of intoxication. According to Majchrowicz (26), the doses given were as follows: no signs of intoxication, 5 g/kg; sedated 4 g/kg; ataxia 1, 3 g/kg; ataxia 2, 2 g/kg; and ataxia 3, 1 g/kg. No dose was given to animals with loss of writhing reflex or coma. Observation of the behavioral states of intoxication and ethanol administration was performed by the same operator to avoid variation in evaluation criteria. During the experimental treatment, the animals spent most of the time in a severe state of intoxication, unable to eat the food pellets provided. Consequently, we observed a loss in body mass of 10–15%; the lack of source food intake was partially compensated for by the daily oral administration (at 0900 h) of a liquid diet (20 g/kg Similac; M & R Laboratoria B.V., Zwolle, Netherlands).

For the first 8–9 h after the last ethanol administration, the extent of ethanol intoxication gradually decreased as blood ethanol concentrations declined (26). After this period, ethanol dependence was demonstrated by the development of the typical symptoms of withdrawal syndrome: tremors of the tail and head, rigidity of the tail and body (with the consequent characteristic body position and posture), bizarre behavior such as "wet dog" shakes and teeth chattering, vocalization, running episodes, and spontaneous convulsions. All these signs were routinely recorded; they were most severe 12–24 h after withdrawal, and gradually disappeared over the next 2–6 days.

Rats of a second group acted as pair-fed controls. They were maintained under the same housing conditions and were administered isocaloric sucrose by oral gavage four times daily at the same time schedule as for ethanol-treated rats. Moreover, control rats received the same amount of liquid diet (20 g/kg) (Similac) once a day. By this method, control and ethanol group were given equal numbers of oral injections, equal calories, and the some volume of fluid daily.

Immediately after the last administration of ethanol, rats were separated one per cage until they were tested in the biochemical and behavioral experiments.

For studies of the effects of acute ethanol administration, rats were deprived of food and water for 12–15 h before experiments, and were then killed 3 h after intragastric injection of ethanol (0.5, 1, 3, or 5 g/kg; $n = 4$ for each group).

### *Killing of Animals*

For biochemical studies, after the last ethanol administration, we separated (four animals for each group) and decapitated rats at various times as follows: at 3 h, when they still had high blood ethanol concentrations (470 ± 87 mg/dl) and showed no symptoms of withdrawal; at 12–24 h, when blood ethanol concentrations had decreased (12 h: 136 ± 33 mg/dl; 24 h: not detectable) and rats presented severe signs of withdrawal; and at 3–6 days, when signs of withdrawal were no longer apparent.

### *[$^3$H]CCPA Binding and [$^3$H]DPCPX Binding Assays*

Binding assays were performed according to Marangos et al. (27) with minor modifications. The brain was removed immediately after death, and cerebellar cortex, cerebral cortex, hippocampus, and striatum were separated and homogenized with a Polytron PT 10 (setting of 5 for 20 s) in 25 vol. (w/v) of ice-cold 50 mM Tris-HCl (pH 7.4 at 25°C). The homogenates were centrifuged at 48,000 × *g* for 10 min, and the resulting pellets were washed once by resuspension and centrifugation in the same ice-cold buffer. The tissues were then resuspended in 25 vol. of Tris-HCl buffer and incubated for 30 min at 37°C in the presence of adenosine deaminase (2 U/ml). After centrifugation at 48,000 × *g* for 10 min, the final pellets were stored at −20°C until use (1–15 days later).

On the day of the assay, the pellets were thawed and reconstituted in 50 vol. of Tris-HCl buffer. [$^3$H]CCPA and [$^3$H]DPCPX binding assays were performed in a final volume of 500 μl, consisting of 100 μl of membrane suspension (0.1–0.15 mg of protein), 50 μl of [$^3$H]CCPA or [$^3$H]DPCPX at final assay concentrations of 0.5 or 0.2 nM, 50 μl of 1 M $MgCl_2$, and 300 μl of 50 mM Tris-HCl (pH 7.4 at 25°C). Incubations (25°C) were initiated by addition of membranes and were terminated after 90 min by rapid filtration through glass fiber filter strips (Whatman GF/B; Whatman Int'l, Maidstone, Kent, UK). The filters were rinsed twice with 4 ml of ice-cold Tris-HCl buffer in a Cell-Harvester filtration manifold (model M-24; Brandel, Gaithersburg, MD). Filter-bound radioactivity was quantitated by liquid scintillation spectroscopy. Nonspecific binding was defined as binding in the presence of 10 μM $N^6$-cyclohexyladenosine (CHA). Saturation analysis was performed with seven different concentrations of ligand (0.1–6.4 nM). Scatchard analysis of the binding data was performed with an iterative computer program (LIGAND).

Protein was assayed by the method of Lowry et al. (25), with bovine serum albumin as standard.

*Tremors and Seizure Studies*

Because they are the most reliable signs of ethanol withdrawal syndrome in animals and humans, only tremors were evaluated for the scoring of treatment effectiveness. Tremors were rated on the basis of their intensity according to the method of Hunt and Majchrowicz (20). Tail, caudal, head, and general tremors were rated as mild (rating 1), moderate (rating 2), or severe (rating 3), and the sum of the ratings was used as the withdrawal score. The total maximum possible score any animal could attain for tremors was 12. Withdrawal tremors of 12–20 animals per group were recorded after the last ethanol administration by an observer who was unaware of the treatment.

At 24 h after the last ethanol administration, saline- and ethanol-treated rats (15–20 animals/group) were tested for their sensitivities to seizures induced by sound stimulation or isoniazid [300–500 mg/kg, subcutaneously (SC)]. Susceptibility to audiogenic seizures was evaluated by placing rats in a cage in which they were exposed one by one to the sound generated by an electric bell (100 dB) for 1 min. This sound induced a running episode that preceded the loss of the righting reflex, the development of tonic-clonic seizures, and death. At the moment of drug administration, handling-induced withdrawal seizures were observed in some animals. Rats were observed for 3 h after isoniazid administration, during which the latency of convulsions, mortality, and the pattern of tonic-clonic seizures were recorded.

CCPA (0.25–1 mg/kg) was dissolved in saline and administered intraperitoneally (IP) simultaneously with isoniazid, 30 min before scoring of tremors, or 60 min before audiogenic seizure assessment. DPCPX was dissolved in distilled water with one drop of Tween 80/5 ml and was administered IP 5 min before CCPA.

In some experiments, animals were chronically treated with ethanol (12–18 g/kg daily) in combination with CCPA (0.5 mg/kg, IP, four times daily) for 6 days.

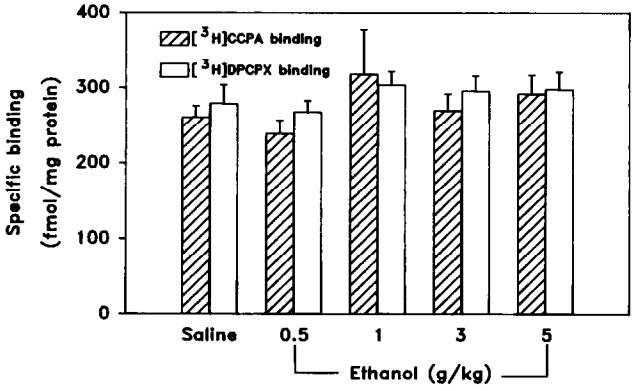


FIG. 1. Effect of acute intragastric administration of ethanol on [$^3$H]CCPA and [$^3$H]DPCPX binding to rat cerebellar cortical membrane preparations. Ethanol (0.5–5 g/kg) was administered by intragastric injection and the animals were killed 3 h later. Membranes were incubated in the presence of 0.5 nM [$^3$H]CCPA or 0.2 nM [$^3$H]DPCPX for 90 min at 25°C. Data are expressed as specific binding and are the means ± SEM of three to five separate experiments, each performed using four animals for each group.

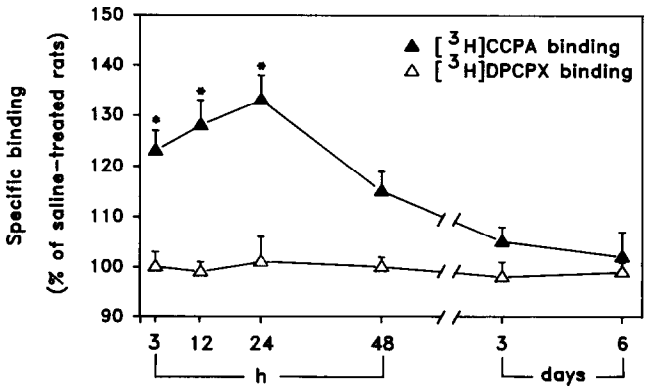


FIG. 2. Effect of chronic ethanol intoxication on [$^3$H]CCPA and [$^3$H]DPCPX binding to rat cerebellar cortical membranes. Rats chronically treated for 6 days with saline or ethanol (12–18 g/kg per day) were killed at the indicated times after the last administration. Membranes were incubated in the presence of 0.5 nM [$^3$H]CCPA or 0.2 nM [$^3$H]DPCPX for 90 min at 25°C. Data are expressed as a percentage of control (saline-treated rats) and represent the means ± SEM of four separate experiments, each performed using four animals for each group. Specific binding of [$^3$H]CCPA and [$^3$H]DPCPX in the saline-treated group was 256 ± 16 fmol/mg protein and 317 ± 25 fmol/mg protein, respectively. $*p < 0.05$ vs. saline-treated rats (ANOVA followed by Scheffé's test).

*Statistical Analysis*

Biochemical data were evaluated by analysis of variance (ANOVA) followed by Scheffé's test. Behavioral data were analyzed by Fisher's exact probability test and the Kruskal–Wallis test followed by the Tukey-type test.

*Chemicals*

CCPA, CHA, and DPCPX were obtained from RBI, Amersham (Milan, Italy). Adenosine deaminase was from Boehringer Mannhein (Milan, Italy). Isoniazid (isonicotinic acid hydrazine) was from Sigma (St. Louis, MO). [$^3$H]CCPA (specific activity, 35 Ci/mmol) and [$^3$H]DPCPX (specific activity, 109 Ci/mmol) were from Dupont NEN Products (Milan, Italy). Ethanol and other chemicals were obtained from commercial sources.

## RESULTS

*Biochemical Studies*

Acute intragastric administration of ethanol (0.5–5 g/kg) had no significant effect on [$^3$H]CCPA or [$^3$H]DPCPX binding measured with cerebellar cortical membranes prepared from rats that were killed 3 h after treatment (Fig. 1).

In contrast, [$^3$H]CCPA binding was significantly increased (+23 ± 4%) in cerebellar cortical membranes prepared from rats chronically exposed to ethanol (12–18 g/kg per day) for 6 days and killed 3 h after the last ethanol administration, when they showed a severe state of intoxication (Fig. 2).

To evaluate whether the increase in [$^3$H]CCPA binding in the cerebellar cortex was apparent also during ethanol withdrawal syndrome, we killed the animals at various times after the last ethanol administration. The increase in [$^3$H]CCPA

TABLE 1

CHRONIC ETHANOL TREATMENT ENHANCED THE NUMBER OF [$^3$H]CCPA BINDING SITES IN RAT CEREBELLAR CORTICAL MEMBRANES

| | | Specific [$^3$H]CCPA binding | | |
|---|---|---|---|---|
| Treatment | Withdrawal | $B_{max}$ (fmol/mg protein) | % Changes | $K_d$ (nM) |
| Control | | 855 ± 48 | 100 | 1.1 ± 0.09 |
| Ethanol | 3 h | 1051 ± 52* | 123 | 0.9 ± 0.07 |
| Ethanol | 24 h | 1101 ± 61* | 128 | 1.1 ± 0.06 |
| Ethanol | 6 days | 901 ± 53 | 105 | 1.3 ± 0.12 |

Pair-fed controls and ethanol-treated (12–18 g/kg per day) rats were killed at the indicated times after the last administration. Membranes were incubated in the presence of seven concentrations (0.1–6.4 nM) of [$^3$H]CCPA. Date represent the means ± SEM of four separate experiments, each performed using four animals for each group.

*$p < 0.05$ vs. saline-treated rats (ANOVA followed by Scheffé's test).

binding was observed 12 and 24 h after the last ethanol administration (maximal enhancement, +33 ± 5% at 24 h), when severe signs of withdrawal syndrome were evident (Fig. 2). [$^3$H]CCPA binding was not increased 3–6 days after ethanol discontinuation, when signs of the abstinence syndrome were no longer apparent. [$^3$H]DPCPX binding showed no difference between ethanol- and saline-treated rats at any time after withdrawal.

Scatchard analysis of saturation isotherms indicated that the increase in [$^3$H]CCPA binding observed in cerebellar cortical membranes of rats killed 3 and 24 h after withdrawal was attributable to an increase in the total number of recognition sites ($B_{max}$) with no change in apparent affinity ($K_d$) (Table 1).

Chronic ethanol intoxication did not modify [$^3$H]CCPA binding (Table 2) and [$^3$H]DPCPX binding (data not shown) in membrane preparations from cerebral cortex, hippocampus, or striatum.

*Behavioral Studies*

To investigate the functional consequences of the increase in [$^3$H]CCPA binding observed in ethanol-dependent rats, we tested the ability of CCPA to antagonize tremors and audiogenic seizures that occur during the withdrawal syndrome in rats rendered physically dependent by forced ethanol administration.

In a dose-dependent manner, CCPA (0.25–1 mg/kg, IP) reduced the intensity of withdrawal tremors apparent 24 h after the last ethanol administration (Fig. 3). The highest dose tested (1 mg/kg) completely suppressed tremors, an effect mimicked by the acute administration of ethanol (3 g/kg per os).

Similarly, CCPA treatment abolished in a dose-dependent manner the generation of convulsions induced by an intense audiogenic stimulus (Fig. 4). The lowest dose (0.25 mg/kg, IP) of CCPA reduced the number of animals showing seizures by 50%, whereas the highest dose (1 mg/kg, IP) completely antagonized audiogenic seizures. Prior administration of DPCPX (1 mg/kg, IP) completely abolished the antagonistic effects of CCPA on both tremors (Fig. 3) and audiogenic seizures (Fig. 4).

The role of $A_1$ receptors in the effects elicited by ethanol intoxication was further investigated in animals chronically treated with ethanol in combination with CCPA (0.5 mg/kg, IP, four times daily) for 6 days. This combined treatment did not significantly reduce the number of animals presenting audiogenic seizures (10 of 15 vs. 13 of 15 for rats treated with ethanol alone), but attenuated the severity of convulsions and completely prevented mortality (0 of 15 vs. 13 of 15; $p < 0.005$, Fisher's test).

We also studied the ability of CCPA to prevent convulsions induced by isoniazid, an inhibitor of γ-aminobutyric acid (GABA) synthesis (19), in both control and ethanol-intoxicated rats (Table 3). Because ethanol-withdrawn rats

TABLE 2

EFFECT OF CHRONIC ETHANOL TREATMENT ON [$^3$H]CCPA BINDING IN MEMBRANE PREPARATIONS FROM DIFFERENT RAT BRAIN AREAS

| | Specific [$^3$H]CCPA Binding (% of Control) | |
|---|---|---|
| Brain Areas | 3 h Withdrawal | 24 h Withdrawal |
| Cerebellar cortex | 123 ± 4* | 133 ± 5* |
| Cerebral cortex | 108 ± 5 | 110 ± 6 |
| Hippocampus | 113 ± 8 | 111 ± 9 |
| Striatum | 107 ± 6 | 117 ± 8 |

Rats chronically treated for 6 days with saline or ethanol (12–18 g/kg per day) were killed 3 or 24 h after the last administration. Membranes were incubated in the presence of 0.5 nM [$^3$H]CCPA, for 90 min at 25°C. Data are expressed as a percentage of control (saline-treated rats) and represent the means ± SEM of three separate experiment, each performed using four animals for each group.

*$p < 0.05$ vs. saline-treated rats (ANOVA followed by Scheffé's test).

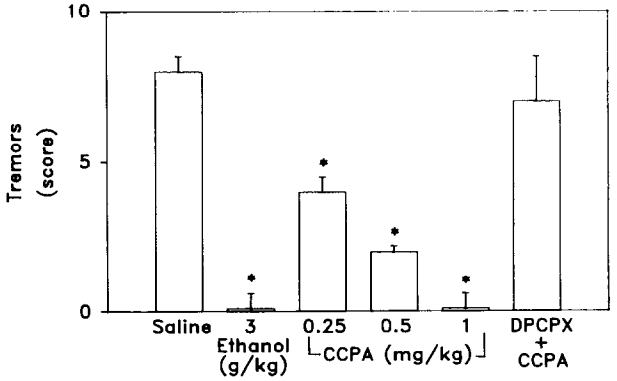


FIG. 3. Effect of CCPA on withdrawal tremors in ethanol-dependent rats. Rats chronically treated with ethanol (12–18 g/kg per day) were tested 24 h after the last administration. Withdrawal tremors were scored between 30 and 60 min. after saline ($n = 20$), acute ethanol ($n = 12$), or CCPA administration (0.25–1 mg/kg; $n = 20$ for each group). DPCPX (1 mg/kg, IP) was administered 5 min before CCPA (1 mg/kg) ($n = 20$). *$p < 0.01$ vs. saline-treated rats (Tukey-type multiple comparison test for nonparametric analysis).

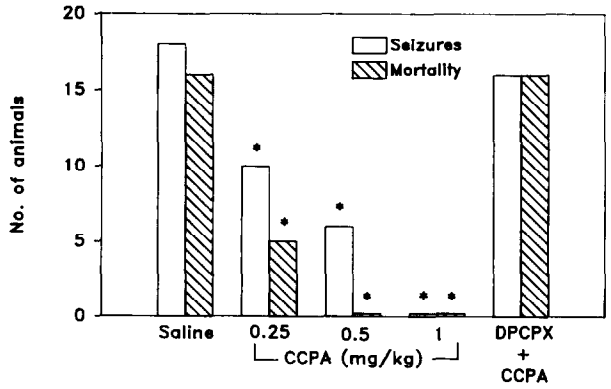


FIG. 4. Effect of CCPA on audiogenic seizures in ethanol-dependent rats. Rats chronically treated with ethanol (12–18 g/kg per day) were tested 24 h after the last administration. Susceptibility to audiogenic seizures was assessed 60 min after saline ($n$ = 20) or CCPA administration (0.25–1 mg/kg; $n$ = 20 for each group). DPCPX (1 mg/kg, IP) was administered 5 min before CCPA (1 mg/kg, IP) ($n$ = 20). *$p < 0.01$ vs. saline-treated rats (Fisher's exact probability test).

tested at 24 h show an increased sensitivity to the convulsant action of isoniazid (29), ethanol-intoxicated rats received a lower dose (300 mg/kg) of isoniazid than saline-treated animals (500 mg/kg). This approach yielded similar patterns of convulsions with regard to latency, severity, number of animals showing seizures, and mortality between the two experimental groups. CCPA (0.5 mg/kg, IP) induced a significant delay (~30 min; $p < 0.01$) in the onset of convulsions and a marked reduction (65%; $p < 0.05$) in mortality in both control and withdrawn rats. Moreover, at a higher dose (1 mg/kg, IP), CCPA also markedly reduced the number of animals presenting convulsions ($p < 0.05$). Thus, CCPA showed similar efficacies and potencies in antagonizing isoniazid-induced convulsions in the two experimental groups.

## DISCUSSION

A possible role for adenosine in the pharmacology of ethanol has recently been suggested. Thus, brain adenosine modulates the motor impairment effect of ethanol (8), whereas ethanol increases the extracellular concentration of adenosine by increasing the release and inhibiting the uptake of this neuromodulator (5,28). However, the role of the purinergic system in the development of ethanol tolerance and dependence, as well as in the onset of the withdrawal syndrome, is not well established.

Our results further suggest the participation of $A_1$ adenosine receptors in the molecular events that underlie intoxication and physical dependence after chronic administration of ethanol. This conclusion is supported by the increase in the number of [$^3$H]CCPA binding sites observed in the cerebellar cortex of rats chronically exposed to ethanol for 6 days. The effect of ethanol on [$^3$H]CCPA recognition sites was apparent 3 h after the last ethanol administration, when the animals were still intoxicated and showed no signs of withdrawal, as well as during the withdrawal syndrome (12–24 h after the last ethanol administration). Although cerebellum might play a key role in the motor impairment effects of ethanol, the lack of changes of [$^3$H]CCPA binding in cerebral cortex, hippocampus, and striatum is difficult to explain. Further studies using more direct approaches (quantitative authoradiography and adenylate cyclase assay) have been undertaken to better elucidate the effect of chronic ethanol intoxication on the function of $A_1$ adenosine receptors in these brain areas.

In contrast, the acute administration of ethanol failed to modify [$^3$H]CCPA binding sites, indicating that $A_1$ receptors are altered only after repeated exposure to this drug.

These observations are not consistent with previous data of Dar et al. (10), who showed a marked decrease in the dissociation constant ($K_d$) of $A_1$ adenosine receptors in mice chronically fed with ethanol. Indeed, subsequest studies from the same group (9) showed no difference in the $B_{max}$ and $K_d$ values of $A_1$ receptors between ethanol-withdrawn and control mice. In addition to the species difference, our experiments differ from those of Dar et al. (9,10) in the intoxication procedure,

TABLE 3

EFFECT OF CCPA ON ISONIAZID-INDUCED SEIZURES IN CONTROL AND ETHANOL-WITHDRAWN RATS

| Experimental Group (mg/kg) | Latency of Convulsions (min) | Rats Presenting Convulsions | Pattern of Convulsions | | Mortality |
|---|---|---|---|---|---|
| | | | Tonus | Clonus | |
| Isoniazid (500) | 45 ± 3.0 | 15/15 | 15/15 | 15/15 | 9/15 |
| Isoniazid + CCPA (0.25) | 56 ± 4.1 | 15/15 | 15/15 | 15/15 | 8/15 |
| Isoniazid + CCPA (0.5) | 78 ± 6.2* | 13/15 | 13/15 | 13/15 | 3/15† |
| Isoniazid + CCPA (1.0) | 68 ± 4.0* | 6/15† | 6/15 | 6/15 | 2/15† |
| Ethanol + isoniazid (300) | 31 ± 1.5 | 15/15 | 14/15 | 15/15 | 11/15 |
| Ethanol + isoniazid + CCPA (0.25) | 41 ± 3.5 | 15/15 | 15/15 | 15/15 | 12/15 |
| Ethanol + isoniazid + CCPA (0.5) | 57 ± 4.5‡ | 14/15 | 14/15 | 12/15 | 4/15§ |
| Ethanol + isoniazid + CCPA (1.0) | 66 ± 9.2‡ | 9/15§ | 8/15 | 9/15 | 3/15§ |

Rats chronically treated with saline or ethanol (12–18 g/kg per day) were injected with CCPA (IP) and isoniazid (SC) 24 h after the last administration.

*$p < 0.01$ vs. isoniazid-treated rats (ANOVA followed by Scheffé's test).

†$p < 0.05$ vs. isoniazid-treated rats (Fisher's test).

‡$p < 0.01$ vs. ethanol + isoniazid (ANOVA followed by Scheffé's test).

§$p < 0.05$ vs. ethanol + isoniazid (Fisher's test).

dosing regimen, duration of treatment, and radioactive ligand used for the biochemical analysis.

The lack of changes in [$^3$H]DPCPX binding in both intoxicated and withdrawn animals is difficult to interpret, because DPCPX has been shown to bind with high affinity to $A_1$ receptors (22). The lower selectivity of DPCPX for $A_1$ receptors (700-fold) compared with that of CCPA (10,000-fold) might contribute to the difference in binding between the two ligands. Moreover, evidence suggests that the binding properties of agonists and antagonists of $A_1$ adenosine receptors differ. Thus, differences in terms of metal modulation, thermal, and proteolytic denaturation, coupling to G proteins, and thermodynamic characteristics between agonist and antagonist binding to membrane $A_1$ adenosine receptors have been demonstrated (3,24,27).

Evidence suggests that endogenous adenosine may function as an inhibitory modulator in epileptogenenesis (13). Accordingly, an increase in the number of $A_1$ receptors in various brain areas of animals treated with chemoconvulsants as well as in the brains of epileptic patients, has been demonstrated (1,2). Furthemore, the brain content of adenosine increases within seconds after the onset of experimental seizures (15,30). Thus, the upregulation of [$^3$H]CCPA binding sites observed 12–48 h after termination of chronic ethanol administration might be related to the behavioral changes (tremors and audiogenic convulsions) apparent during withdrawal. However, the fact that the number of [$^3$H]CCPA binding sites is also increased in the cerebellum of rats killed 3 h after the last ethanol administration, when no signs of withdrawal are yet apparent, suggests that the modification of $A_1$ receptors is attributable to the long-term exposure of animals to ethanol rather than to the development of withdrawal syndrome.

To clarify whether the changes in [$^3$H]CCPA binding were related to changes in the function of $A_1$ receptors, we evaluated the efficacy of CCPA in antagonizing the withdrawal symptoms apparent 24 h after the last ethanol administration. CCPA inhibited in a dose-dependent manner both tremors and audiogenic seizures. The antagonism of the withdrawal syndrome occurred at doses of CCPA (0.25 mg/kg) that do not alter exploratory motility (data not shown), indicating that a general sedative effect was not responsible for the decrement in seizure activity. Moreover, the effects of CCPA were mediated by the activation of $A_1$ adenosine receptors, because they were completely abolished by prior administration of DPCPX.

Thus, long-term treatment with intoxicating doses of ethanol does not reduce the anticonvulsant efficacy of CCPA or the function of $A_1$ adenosine receptors. This conclusion is further supported by the observation that CCPA antagonized with similar potencies and efficacies the occurrence and severity of seizures and the mortality induced by the GABA synthesis inhibitor isoniazid in both control and ethanol-withdrawn rats. Indeed, the administration of CCPA at a dose (0.25 mg/kg) unable to antagonize seizures induced by chemical convulsants (Table 2) (7) was efficacious in reducing (by 50%) the expression of withdrawal signs.

These pharmacologic data contrast with previous evidence showing a cross-tolerance between chronic ethanol administration and adenosine receptor agonists. Dar and Clark (9) showed a reduction in the efficacy of the adenosine receptor agonist CHA in accentuating the motor incoordination induced by acute ethanol administration in animals chronically fed with ethanol. This discrepancy may be attributable to the different treatments used to render animals ethanol dependent; however, the possibility that cross-tolerance develops to the effect of adenosine receptor agonists on motor impairment but not to the anticonvulsant effects cannot be ruled out.

The high efficacy of CCPA together with the lack of cross-tolerance with ethanol suggests that this $A_1$ adenosine receptor agonist may be a potential tool with which to prevent or reduce the withdrawal syndrome in ethanol-dependent rats. Although the coadministration of CCPA and ethanol failed to reduce significantly the number of animals showing audiogenic seizures, this treatment attenuated the severity of convulsions and completely abolished mortality.

In conclusion, our data further suggest that $A_1$ adenosine receptors are important in the neurochemistry and pharmacology of ethanol intoxication and dependence. Given that chronic ethanol does not reduce the sensitivity of these receptors, our results suggest that highly selective agonists of $A_1$ receptors may show therapeutic efficacy in the treatment of alcoholism.

## REFERENCES


1. Angelatou, F.; Pagonopoulou, O.; Kostopoulos, G. Alterations of $A_1$ adenosine receptors in different mouse brain areas after pentylenetetrazol-induced seizures, but not in the epileptic mutant mouse "tottering." Brain Res. 534:251–256; 1990.
2. Angelatou, F.; Pagonopoulou, O.; Maraziotis, T.; Olivier, A.; Villemeure, J. G.; Avoli, M.; Kostopoulos, G. Upregulation of $A_1$ adenosine receptors in human temporal lob epilepsy: A quantitative autoradiographic study. Neurosci. Lett. 163:11–14; 1993.
3. Casadò, V.; Allende, G.; Mallol, J.; Franco, R.; Lluis, C.; Canela, E. I. Thermodynamic analysis of agonist and antagonist binding to membrane-bound and solubilized $A_1$ adenosine receptors. J. Pharmacol. Exp. Ther. 266:1463–1474; 1993.
4. Clark, M.; Dar, M. S. Mediation of acute ethanol-induced motor disturbances by cerebellar adenosine in rats. Pharmacol. Biochem. Behav. 30:155–161; 1988.
5. Clark, M.; Dar, M. S. Effect of acute ethanol on release of endogenous adenosine from rat cerebellar synaptosomes. J. Neurochem. 52:1859–1865; 1989.
6. Concas, A.; Cuccheddu, T.; Floris, S.; Mascia, M. P.; Biggio, G. 2-Chloro-N$^6$-cyclopentyladenosine (CCPA), an adenosine $A_1$ receptor agonist, supresses ethanol withdrawal syndrome in rats. Alcohol Alcohol. 29:261–264; 1994.
7. Concas, A.; Santoro, G.; Mascia, M. P.; Maciocco, E.; Dazzi, L.; Ongini, E.; Biggio, G. Anticonvulsant doses of 2-chloro-N$^6$-cyclopentyladenosine, an adenosine $A_1$ receptor agonist, reduce GABAergic transmission in different areas of the mouse brain. J. Pharmacol. Exp. Ther. 267:844–851; 1993.
8. Dar, S. M. Central adenosinergic system involvement in ethanol-induced motor incoordination in mice. J. Pharmacol. Exp. Ther. 255:1202–1209; 1990.
9. Dar, S. M.; Clark, M. Tolerance to adenosine's accentuation of ethanol-induced motor incoordination in ethanol-tolerant mice. Alcohol. Clin. Exp. Res. 16:1138–1146; 1992.
10. Dar, S. M.; Mustafa, S. J.; Wooles, W. R. Possible role of adenosine in the CNS effects of ethanol. Life Sci. 33:1363–1374; 1983.
11. Daval, J. L.; Nehlig, A; Nicolas, F. Physiological and pharmacological properties of adenosine: Therapeutic implications. Life Sci. 49:1435–1453; 1991.
12. Dolphin, A. C.; Forda, S. R.; Scott, R. H. Calcium-dependent currents in cultured rat dorsal root ganglion neurones are inhibited by an adenosine analogue. J. Physiol. (London) 373:47–61; 1986.
13. Dragunow, M. Adenosine: The brain's natural anticonvulsant? Trends Pharmacol. Sci. 7:128–130; 1986.
14. Dunwiddie, T. V. Interactions between the effects of adenosine

and calcium on synaptic responses in rat hippocampus in vitro. J. Physiol. (London) 350:545–559; 1984.
15. During, M. J.; Spencer, D. D. Adenosine: A potential mediator of seizure arrest and postictal refractoriness. Ann. Neurol. 32: 618–624; 1992.
16. Ebersolt, C.; Premont, J.; Prochiantz, A.; Perez, M.; Bockaert, J. Inhibition of brain adenylate cyclase by $A_1$ adenosine receptors: Pharmacological characteristics and locations. Brain Res. 267:123–129; 1983.
17. Fredholm, B. B.; Abbracchio, M. P.; Burnstock, G.; Daly, J. W.; Harden, K. T.; Jacobson, K. A.; Leff, P.; Williams, M. VI. Nomenclature and classification of purinoceptors. Pharmacol. Rev. 46:143–156; 1994.
18. Fredholm, B. B.; Dunwiddie, T. V. How does adenosine inhibit transmitter release? Trends Pharmacol. Sci. 9:130–134; 1988.
19. Horton, W. R. GABA and seizures induced by inhibitors of glutamic acid decarboxylase. Brain Res. Bull. 5:605–608; 1980.
20. Hunt, W. A.; Majchrowicz, E. Suppression of the ethanol withdrawal syndrome by aliphatic diols. J. Pharmacol. Exp. Ther. 213:9–12; 1980.
21. Klotz, K. N.; Lohse, M. J.; Schwabe, U.; Cristalli, G.; Vittori, S.; Grifantini, M. 2-Chloro-$N^6$-cyclopentyladenosine ([$^3$H]CCPA), a high affinity agonist radioligand for $A_1$ adenosine receptors. Naunyn-Schmied. Arch. Pharmacol. 340:679–683; 1989.
22. Lohse, M. J.; Klotz, K. N.; Lindenborn-Fotinos, J.; Reddington, M.; Schwabe, U.; Olsson, R. A. 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), a selective high affinity antagonist radioligand for $A_1$ adenosine receptors. Naunyn-Schmied. Arch. Pharmacol. 336:204–210; 1987.
23. Lohse, M. J.; Klotz, K. N.; Schwabe, U.; Cristalli, G.; Vittori, S.; Grifantini, M. 2-Chloro-$N^6$-cyclopentyladenosine: A highly selective agonist at $A_1$ adenosine receptors. Naunyn-Schmied. Arch. Pharmacol. 337:687–689; 1988.
24. Lohse, M. J.; Lenschow, V.; Schwabe, U. Two affinity states of $R_i$ adenosine receptors in brain membranes. Analysis of guanine nucleotides and temperature effects on radioligand binding. Mol. Pharmacol. 26:1–9; 1984.
25. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275; 1951.
26. Majchrowicz, E. Induction of physical dependence upon ethanol and the associated behavioral changes in rats. Psychopharmacologia 43:245–254; 1975.
27. Marangos, P. J.; Patel, J.; Martino, A. M.; Dilli, M.; Boulenger, J. P. Differential binding properties of adenosine receptor agonists and antagonists in brain. J. Neurochem. 41:367–374; 1983.
28. Nagy, L. E.; Diamond, I.; Casso, D. J.; Franklin, C.; Gordon, A. S. Ethanol increases extracellular adenosine by inhibiting adenosine uptake via the nucleoside transporter. J. Biol. Chem. 265:1946–1951; 1990.
29. Sanna, E.; Serra, M.; Cossu, A.; Colombo, G.; Follesa, P.; Cuccheddu, T.; Concas, A.; Biggio, G. Chronic ethanol intoxication induces differential effects on $GABA_A$ and NMDA receptor function in the rat brain. Alcohol. Clin. Exp. Res. 17:115–123; 1993.
30. Schrader, J.; Wahl, M.; Kuschinsky, W.; Kreutzberg, G. W. Increase of adenosine content in cerebral cortex of the cat during bicuculline-induced seizures. Pflugers Arch. 387:245–251; 1980.
31. Trussel, L. O.; Jackson, M. B. Adenosine-activated potassium conductance in cultured striatal neurons. Proc. Natl. Acad. Sci. USA 82:4857–4861; 1985.
32. Van Calker, D.; Müller, M.; Hamprecht, B. Adenosine regulates, via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. J. Neurochem. 33:999–1005; 1979.