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# Regulation of Adenosine Receptors in Rat Brain following Chronic Carbamazepine Treatment[δ]

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Carbamazepine (CBZ), an anticonvulsant, has beeen reported to displace ligands at adenosine receptors. Several studies have demonstrated that as far as $A_2$ adenosine receptors is concerned, CBZ acts as an antagonist. However, the situation with regard to $A_1$ receptors is less straightforward. In this study, we describe the effects of one-week CBZ treatment (25 mg/kg/day) on cerebrocortical $A_1$ adenosine receptors. $A_1$ adenosine receptor bindings as determined by using [$^3$H]DPCPX was not significantly altered in membranes prepared from CBZ-treated rats. However, there was a significant decrease in the $A_1$ adenosine receptor-mediated stimulation of [$^{35}$S]GTP$_\gamma$S binding to cerebrocortical membranes prepared from CBZ-treated rats (20.0% decrease in basal activity; 17.8% decrease in maximal activity). The basal and $10^{-4}$ M forskolin-stimulated adenylyl cyclase activities were relatively unaffected by CBZ treatment, but 10 mM NaF-stimulated adenylyl cyclase activity was significantly reduced in CBZ-treated rats. It appears that one-week CBZ treatment caused an uncoupling of adenosine receptors from G proteins without alteration of $A_1$ adenosine receptor molecules, suggesting that CBZ acts as an agonist at $A_1$ adenosine receptors in rat brain.

Key Words: Carbamazepine, $A_1$ Adenosine receptor, Cerebral cortex, Adenylyl cyclase activity, [$^{35}$S]GTP$_\gamma$S binding, [$^3$H]DPCPX binding

Abbreviations: CBZ, carbamazepine; PIA, R-phenylisopropyladenosine; DPCPX, 8-Cyclopentyl-1,3-dipropylxanthine; BSA, Bovine serum albumin; [$^{35}$S]GTP$_\gamma$S, [$^{35}$S]Guanosine-5'-($_\gamma$- thio)triphosphate; CHA, $N^6$-Cyclohexyladenosine

## INTRODUCTION

The tricyclic imino-derivative carbamazepine (CBZ) is well known for its anticonvulsive properties and its efficacy in the treatment of paroxysmal pain syndroms (Rogawski and Porter, 1990). The idea that the anticonvulsive properties of CBZ may predominantly be due to allosteric blockade of voltage-sensitive $Na^+$ channels, while $GABA_B$ mechanisms may account for its antinociceptive properties. The concept that the anticonvulsant action of CBZ may relate to an interaction with adenosine receptors was developed by Lewin and Bleck (1977), who demonstrated that CBZ but not phenytoin or phenobarbital could inhibit adenosine stimulation of cAMP production in rat cortical slices. Subsequent studies have shown that CBZ specifically inhibit adenosine agonist ([$^3$H]CHA) and antagonist ([$^3$H]DPCPX) bindings to central $A_1$ adenosine receptors in the range of $10^{-6}$ to $10^{-5}$ M (Marangos et al, 1983; Weir et al, 1984) . These levels of CBZ are in the range of those reported to exist in brain at therapeutic doses of this drug (Morselli et al, 1976).

An important question concerning the interaction of CBZ with adenosine receptors relates to whether it acts as an agonist or antagonist. The CBZ-induced reduction of adenosine receptor-mediated increase in cAMP levels suggests that CBZ acts as an $A_2$ adenosine antagonist. However, the sedative and anticon-

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vulsant effects of CBZ is more consistent with $A_1$ agonist effects. In an effort to clarify the effect of CBZ at $A_1$ adenosine receptors, we carried out this study, which involved chronic treatment of rat with CBZ using mini-osmotic pumps.

## MATERIALS AND METHODS

### *Chemicals*

[$^3$H]DPCPX (120 Ci/mmol) and [$^{35}$S]guanosine 5'-($\gamma$-thio)triphosphate (1000-1500 Ci/mmol) were obtained from DuPont NEN® (Boston, MA, U. S. A.); [$^{32}$P]ATP and [$^3$H]cAMP from Amersham International plc; mini-osmotic pumps from Alza corp (Palo Alto, CA, U. S. A.); DPCPX and PIA from Research Biochemicals Inc. (Natick, MA, U. S. A.); BSA, adenosine deaminase from calf intestine, GDP, Tris-HCl and EDTA from Sigma Chemicals (St. Louis, MO, U. S. A.); GF/B glass microfiber filters from Whatman International Ltd. (Maidstone, U. K.). All other chemicals were of analytical grade.

### *Animals*

Male Sprague-Dawley rats weighing 170 to 230 g, which have been acclimatized in the animal care facilities of the university for more than a week, were used in all experiments. Rats were allowed free access to food and tap water, under a light-dark cycle with the light on from 6 a.m. to 6 p.m.

### *Treatment of rats with carbamazepine*

Carbamazepine was administered to rats at a rate of 25 mg/kg/day subcutaneously for 1 week using the Alzet® mini-osmotic pumps.

### *Preparation of crude brain membranes*

Crude brain membranes were prepared as described by Lorenzen et al, (1993). Briefly, rats were sacrificed by decapitation around 10 a.m. The cerebrocortical layers were dissected out and homogenized in 20 volumes of ice-cold 0.32 M sucrose buffer using a Potter-Elvejehm homogenizer. The resulting homogenates were then centrifuged at 3,500 rpm (1,000 × g) for 10 min in a Sorvall RC-5B centrifuge. The supernatant was centrifuged at 18,000 rpm (20,000 × g) for 30 min. The supernatant was discarded, and the pellet was washed twice in 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA. The final pellet was resuspended in the same buffer, treated with adenosine deaminase (0.5 units/ml) at 37°C for 15 min, and frozen in −70°C.

### *Determination of adenylyl cyclase activity*

Adenylyl cyclase activity was determined by the method of Salomon *et al.* (1974). The total volume of the incubation medium was 100 $\mu$l which contained 0.1 mM [$^{32}$P]ATP, 0.1 mM cAMP, 4 mM $MgCl_2$, 1 mM GTP, 1 mg/ml BSA, 2 mM creatine phosphate, 25 units/ml creatine phosphokinase, 0.5 units/ml adenosine deaminase and 30 mM Tris-HCl (pH 7.5) (Londos et al, 1978). [$^3$H]cAMP was included in the reaction mixture to check the recovery. Reaction was started by adding membrane fractions into the tubes and carried out at 30°C for 15 min. [$^{32}$P]cAMP formed was separated from [$^{32}$P]ATP using alumina and Dowex 50 columns, and the radioactivity from [$^{32}$P]cAMP was counted in Beckman liquid scintillation counter.

### *Determination of [$^{35}$S]GTP$_\gamma$S bindings*

[$^{35}$S]GTP$_\gamma$S bindings were determined by the method of Lorenzen et al (1993). The incubation mixture contained in a total volume of 100 $\mu$l, 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM $MgCl_2$, 10 $\mu$M GDP, 1 mM DTT, 100 mM NaCl, 0.2 units/ml adenosine deaminase, 0.3 ~ 0.5 nM [$^{35}$S]GTP$_\gamma$S (about 50,000 cpm) and 0.5% BSA. The incubation was carried out for 30 min at 25°C. Incubations were terminated by rapid filtration of the samples through glass fiber filters (Whatman GF/B), followed by two 5 ml washes of the same buffer. After tranferring the filters into a vial containing 13 ml of scintillation cocktail, the radioactivity was determined in a Beckman scintillation counter.

### *Determination of [$^3$H]DPCPX bindings*

Determination of $A_1$ adenosine receptor binding to rat cerebrocortical membranes was carried out using [$^3$H]DPCPX by a modification of the method of Bruns et al (1987). Briefly, Aliquots of crude cerebrocortical membranes (approximately 40 $\mu$g protein/tube) were incubated with 0.1 to 3 nM [$^3$H]DPCPX, 5 mM $MgCl_2$, 0.5 units/ml adenosine deaminase, 50 mM Tris-HCl (pH 7.4) at 25°C for 90 min. The total volume of the reaction mixture was 1 ml. Bound and free ligands were separated by rapid filtration of the reaction mixture through Whatman GF/B glass filters.

The filters were immediately washed with two 5 ml-portions of ice-cold 50 mM Tris-HCl buffer (pH 7.4). The radioactivity bound to the filters was determined in a liquid scintillation counter. Specific binding was defined as the amount of the radioligand bound in the absence of competing ligand minus the amount of that bound in the presence of 10 $\mu$M PIA.

*Determination of protein concentrations*

Protein concentrations were determined by the method of Bradford using BSA as standard (Bradford, 1976).

*Data analysis*

Analyses of saturation binding assays and concentration-response curves were carried out using the GraphPad Prism® (GraphPad Software Inc., San Diego, CA, U. S. A.). Comparisons between groups were carried out using the unpaired Student *t*-test.

## RESULTS

*Changes in [$^3$H]DPCPX bindings*

Table 1 shows the response of the adenosine receptors to 1 week CBZ treatment using mini-osmotic pumps. In cerebral cortex, both the $K_d$ (dissociation constant) and $B_{max}$ (receptor density) remained relatively unaffected by CBZ treatment.

*Changes in [$^{35}$S]GTP$_\gamma$S bindings*

In an effort to determine if $A_1$ adenosine receptors-G proteins coupling has been modified following CBZ treatment, PIA-stimulated [$^{35}$S]GTP$_\gamma$S and PIA inhibition of adenylyl cyclase were studied. As shown in Fig. 1 and Table 2, PIA caused a increase in [$^{35}$S]GTP $_\gamma$S bindings in a concentration-dependent manner. Thus, in CBZ-treated animals, the basal and PIA-stimulated [$^{35}$S]GTP$_\gamma$S bindings were reduced by 20.0% and 18.7%, respectively. However, the $EC_{50}$ for the concentration-response curve was not changed significantly, with values of 104.4±14.1 nM in control rats and 91.3±16.8 nM in CBZ-treated rats. There were no significant changes in GDP and Na+ sensitivity to [$^{35}$S]GTP$_\gamma$S bindings in CBZ-treated groups (Lorenzen et al, 1993) (data not shown).

*Changes in adenylyl cyclase activity*

The effect of CBZ treatment on the adenylyl cyclase activity was assessed in rat cerebrocortical membranes.

**Table 1.** Effect of carbamazepine treatment on [$^3$H]DPCPX bindings in rat cerebrocortical membranes

| | Control | CBZ-treated |
|---|---|---|
| $K_d$(nM) | 0.87±0.05(5) | 0.83±0.05(5) |
| $B_{max}$(fmol/mgprotein) | 1091.0±31.3(5) | 999.3±26.0(5) |

Data are presented as means±standard errors of quintuplicate samples. No significant differences between CBZ-treated and control groups were observed.

**Table 2.** Effect of carbamazepine treatment on PIA-stimuated [$^{35}$S]GTP$_\gamma$S binding in rat cerebrocortical membranes

| | Control | CBZ-treated |
|---|---|---|
| Basal activity (fmol/mg protein) | 65.2± 3.2(5) | 52.2±1.4*(5) |
| Maximal activity (fmol/mg protein) | 181.4± 6.6(5) | 147.4±5.0*(5) |
| $EC_{50}$(nM) | 104.4±14.1(5) | 91.3±16.8(5) |

Data are presented as means±standard errors of quintuplicate samples. *$p<0.05$

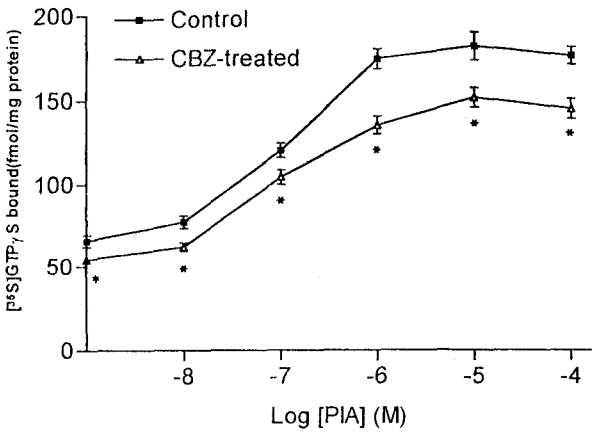


**Fig. 1.** Concentration-response curves for PIA-stimulated [$^{35}$S]GTP$_\gamma$S bindings in cerebrocortical membranes prepared from CBZ-treated and control rats. Crude membranes were incubated with indicated concentrations of PIA under the "[$^{35}$S]GTP$_\gamma$S binding assay" conditions for 45 min. Data are presented as means±standard errors of quintuplicate samples. *<0.05 by Student's t-test.

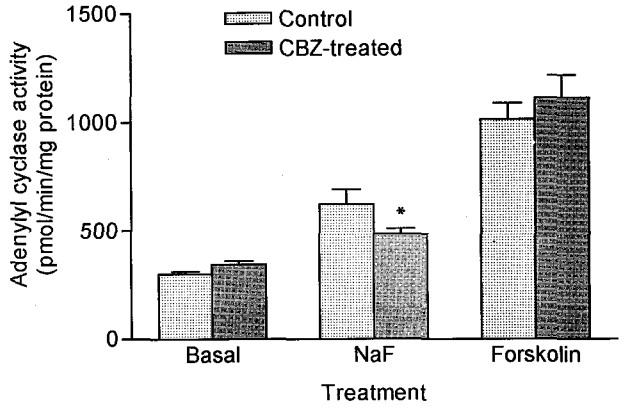


**Fig. 2.** Stimulation of adenylyl cyclase activity by NaF and forskolin in cerebrocortical membranes prepared from CBZ-treated and control rats. Crude membranes were incubated with 10 mM NaF or $10^{-4}$ M forskolin under the "adenylyl cyclase assay" conditions for 15 min. Data are presented as means±standard errors of quintuplicate samples. *<0.05 by Student's t-test.

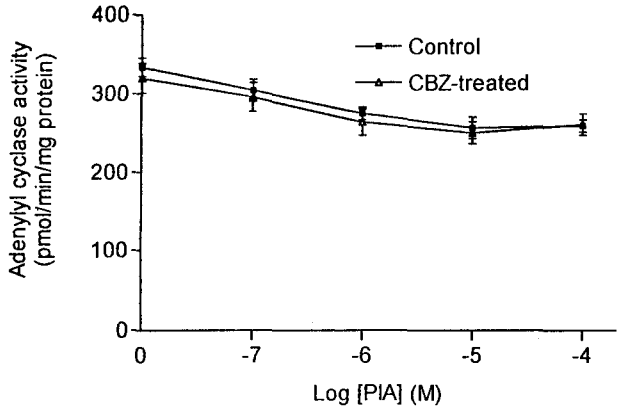


**Fig. 4.** Concentration-response curves for inhibition of adenylyl cyclase activity by PIA in cerebrocortical membranes prepared from CBZ-treated and control rats. Crude membranes were incubated with indicated concentrations of PIA under the "adenylyl cyclase assay" conditions for 15 min. Data are presented as means±standard errors of quintuplicate samples.

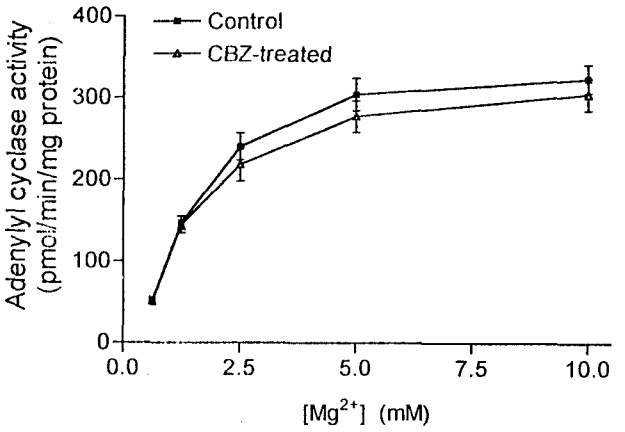


**Fig. 3.** Effect of magnesium concentration on basal adenylyl cyclase activity in cerebrocortical membranes prepared from CBZ-treated and control rats. Crude membranes were incubated with indicated concentrations of magnesium chloride under the "adenylyl cyclase assay" conditions for 15 min. Data are presented as means± standard errors of quintuplicate samples.

CBZ treatment had little effect on basal and $10^{-4}$M forskolin-stimulated adenylyl cyclase activity. However, it was found that 10 mM NaF-stimulated adenylyl cyclase activity was decreased by 22% in CBZ-treated groups compared to control rats. $Mg^{2+}$ caused increases in basal adenylyl cyclase activity in a concentration-dependent manner, and there were no significant changes in the $Mg^{2+}$ effect on the basal adenylyl cyclase activity (Fig. 3). Fig. 4 shows that PIA decreased the basal adenylyl cyclase activity, but the inhibition of basal adenylyl cyclase activity by PIA was relatively unaffected by CBZ treatment. The PIA inhibition of $10^{-4}$ M forskolin-stimulated adenylyl cyclase activity was unchanged in a similar manner (data not shown).

## DISCUSSION

An important question relating to the mechanism of CBZ action is whether it acts as an agonist or antagonist at the adenosine receptors. Previous results have shown that CBZ blocks cAMP production, suggesting that it is an $A_2$ adenosine receptor antagonist (Lewin and Bleck, 1977; Weir et al, 1984). In order to further investigate this problem, we assessed the effects of chronic CBZ treatment on the $A_1$ adenosine receptors based on the generalization that chronic agonist administration would lead to a downregulation and chronic antagonist administration causes an upregulation of the receptors. Our results show that one-week treatment of rats with CBZ causes an uncoupling of adenosine receptors from G proteins without alteration of $A_1$ adenosine receptor molecules, suggesting that CBZ acts as an agonist at $A_1$ adenosine receptors in rat brain. It is possible that a change in the number of adenosine receptors may have been

observed, if we administered CBZ for a longer period of time. Although the effects of CBZ on adenosine receptors are considered to be direct since CBZ inhibits the binding of [$^3$H]CHA to the cerebral cortical membranes from the rat brain (Weir et al, 1990), we cannot rule out the indirect actions of CBZ.

Our findings are consistent with the expected profile of an anticonvulsant drug and the stimulant and proconvulsant effects of adenosine antagonists such as caffeine. Adenosine and metabolically stable adenosine receptor agonists have anticonvulsant activity in animals (Dunwiddie and Worth, 1982; Albertson et al, 1983; Barraco et al, 1984) and inhibit epileptiform activity in the hippocampal slice preparation (Dunwiddie, 1980).

Marangos et al (1985) showed that chronically administered CBZ by oral route produces a selective increase in adenosine receptors labeled with [$^3$H]CHA or the adenosine antagonist [$^3$H]DPCPX in virtually all brain areas, supporting the concept that the drug acts as an antagonist. In accordance with this conclusion is the observation of Weiss et al (1985) that adenosine antagonists fail to block the anticonvulsant effect of CBZ as they should if the anticonvulsant activity of CBZ were due to its action as an adenosine agonist. Further study of the mechanism of CBZ action and whether or not actions at the adenosine receptor mediate its therapeutic or side effects may be needed to provide insights into the role of the adenosine systems.

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