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European Journal of Pharmacology 265 (1994) 121–124

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Adenosine A_1 receptors in the rat brain in the kindling model of epilepsy

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Received 23 June 1994; revised MS received 26 July 1994; accepted 29 July 1994

Abstract

Adenosine and adenosine analogues have potent anticonvulsant effects on various seizure models, including kindling, an animal model of temporal lobe epilepsy. It is now reported that binding of a specific ligand (cyclohexyladenosine) to adenosine A_1 receptors is not changed in the cerebral cortex of kindled rats. However, the affinity of cyclohexyladenosine to adenosine receptors is significantly increased in the hippocampus. In addition, cyclohexyladenosine is slightly more potent to inhibit [^{3}H]D-aspartate outflow from hippocampal synaptosomes taken from kindled than from control rats. Taken together, these data suggest that an increased affinity of adenosine to A_1 receptors may play a role in the anticonvulsant effect of adenosine A_1 analogues in the kindling model.

Keywords: Adenosine; Epilepsy; Kindling; Glutamate binding; Glutamate release

1. Introduction

The term 'kindling' refers to the phenomenon in which repeated administration of an initially subconvulsant electrical stimulus results in progressive intensification of seizure activity, culminating in generalized tonic-clonic seizures. Once established, this increased excitability is essentially permanent. Kindling is believed to be an accurate model for human partial complex (temporal lobe) epilepsy (McNamara et al., 1985).

Adenosine and adenosine analogues have potent anticonvulsant effects on various models of epilepsy, including kindling (Chin, 1989). This effect appears to be mediated through the activation of adenosine A_1 receptors (Zhang et al., 1994). Drugs capable of reducing adenosine effects, such as methylxanthines, increase the duration of convulsions and facilitate the development of status epilepticus (Dragunow, 1986). Furthermore, adenosine levels in the brain are dramatically elevated following seizures (Chin, 1989). On the basis of these observations, it has been suggested that adenosine operates as an endogenous anticonvulsant substance.

In the present study, the hypothesis that biochemical changes induced by kindling contribute to the anticonvulsant properties of adenosine was tested by examining (1) binding to adenosine A_1 receptors in cortical and hippocampal membranes and (2) efficacy of a selective adenosine A_1 receptor agonist to inhibit the outflow of [^{3}H]D-aspartate from hippocampal synaptosomes. Preliminary results of this study have been reported in abstract form (Varani et al., 1994).

2. Materials and methods

2.1. Preparation of the animals

Male Sprague-Dawley rats (300 g) were divided into 3 groups: kindled, sham-stimulated and control. Under general halothane anaesthesia, a bipolar electrode was stereotaxically implanted in the right amygdala of the kindled rats (coordinates: 4.8 mm lateral and 0.8 mm posterior to bregma, 8.4 mm deep from dura). After one week's recovery, the rats were stimulated once

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Elsevier Science B.V.
SSDI 0014-2999(94)00472-2

daily with a single 1-s train of bipolar pulses (1 ms, 60 Hz, 100 μA above after-discharge threshold). Kindling criteria (3 consecutive generalized tonic-clonic convulsions, class 4 or 5 according to Racine, 1972) were reached after 18 ± 2 stimulations. The kindled rats were killed 5–7 days thereafter. Sham-stimulated rats underwent surgery and daily handling but were not stimulated. Control rats were free of any treatment.

2.2. Receptor binding assays

The rats were killed by decapitation and cortices and hippocampi were dissected on ice. The tissues were disrupted in a Polytron PTA 10 Probe (setting 5, 20 s) in 20 vols. of 50 mM Tris HCl buffer, pH 7.4. The homogenate was centrifuged ($48000 \times g$ at 4°C for 10 min) and resuspended in Tris HCl containing 2 IU/ml of adenosine deaminase (Type VI, Sigma). After a 30-min incubation at 37°C the membranes were centrifuged and the pellet was stored at −70°C. Adenosine A_1 receptor binding assay was performed essentially according to Bruns et al. (1980) using [^{3}H]N^6-cyclohexyladenosine (13.5 Ci/mmol, New England Nuclear, Boston, MA, USA). Saturation binding experiments were carried out in 1 ml of buffer containing membranes from 10–15 mg (wet weight) of tissue (cortex or hippocampus) and increasing concentrations (0.1–10 nM) of [^{3}H]cyclohexyladenosine. After a 120-min incubation at 25°C, separation of bound from free radioligand was performed by rapid filtration through Whatman GF/B filters under reduced pressure, using a cell harvester (Brandel, Gaithersburg, MD, USA). The filters were washed 3 times with ice-cold buffer, dried and treated with 5 ml Aquassure (NEN Research Products, Boston, MA, USA). Radioactivity was determined using an LS-1800 Beckman liquid scintillation counter. Non-specific binding was defined as the binding in the presence of 10 μM *R*-(−)-N^6-2-phenylisopropyladenosine (R-PIA) and was $< 10\%$ of total binding. The data were analyzed by means of the LIGAND computer program (Munson and Rodbard, 1980).

2.3. Release experiments

Hippocampi were homogenized in 0.32 M sucrose. Crude synaptosomes (P_2 pellets) were obtained by centrifuging the homogenate (10 min, $1000 \times g$, 4°C) and then centrifuging the supernatant once more (20 min, $12000 \times g$, 4°C). Synaptosomes were resuspended in oxygenated Krebs solution (mmol/l: NaCl 118.5; KCl 4.7; $CaCl_2$ 1.2; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 25; glucose 10) containing 300 nM [^{3}H]D-aspartate (New England Nuclear, Boston, MA, USA) and kept at 33°C for 30 min. The superfusion technique used was based on the one described by other authors (Raiteri et al., 1974). Aliquots of the suspension (containing 0.5–0.7 mg protein) were injected into nylon syringe filters (0.45 μm pore size, Micron Separation, Westboro, MA, USA) connected by tubing to a peristaltic pump and kept at 33°C in a thermostatic bath.

Synaptosomes were perfused with pre-oxygenated Krebs solution (Simonato et al., 1993). Stimulations were applied by pulses (1 min duration) of 50 mM potassium-Krebs solution (equimolar substitution of KCl for NaCl), 45 and 75 min after starting the superfusion. Cyclohexyladenosine was applied 5 min before the second stimulation, so that the first stimulation could be used as an internal control. The perfusate was collected at 5-min intervals, scintillation fluid was added, and was counted in a Beckman LS 1800 counter. Calculations of 50 mM KCl-evoked overflow were performed as previously described (Simonato et al., 1993).

3. Results

3.1. Binding experiments

A representative saturation curve, with the corresponding Scatchard plot, of [^{3}H]cyclohexyladenosine binding to cortical and hippocampal membranes of control and kindled rats is shown in Fig. 1A and 1B. Linearity of the Scatchard plot is indicative of the presence of a single class of high-affinity binding sites under the present experimental conditions. Computer analysis of the data failed to show a significantly better fit to a two- than to a one-site model.

Final equilibrium parameters for [^{3}H]cyclohexyladenosine binding in cortical and hippocampal membranes from the control and kindled rats are reported in Table 1. There was a statistically significant increase in affinity of cyclohexyladenosine to adenosine A_1 receptors in the hippocampus but not in the cortex of the kindled rats. Adenosine receptor density did not appear to be affected by kindling.

3.2. Release experiments

The observation that binding to adenosine A_1 receptors showed an increased affinity in the hippocampus taken from kindled rats prompted investigation into its functional relevance. In the hippocampus, adenosine A_1 receptors appear to be mainly located presynaptically, and to inhibit glutamate release (Corradetti et al., 1984; Burke and Nadler, 1988). Therefore, the decision was made to study adenosine A_1 receptor-mediated inhibition of [^{3}H]D-aspartate outflow (a marker of glutamate release) in hippocampal synaptosomes.

The selective adenosine A_1 receptor agonist, cyclohexyladenosine, reduced 50 mM KCl-evoked [^{3}H]D-

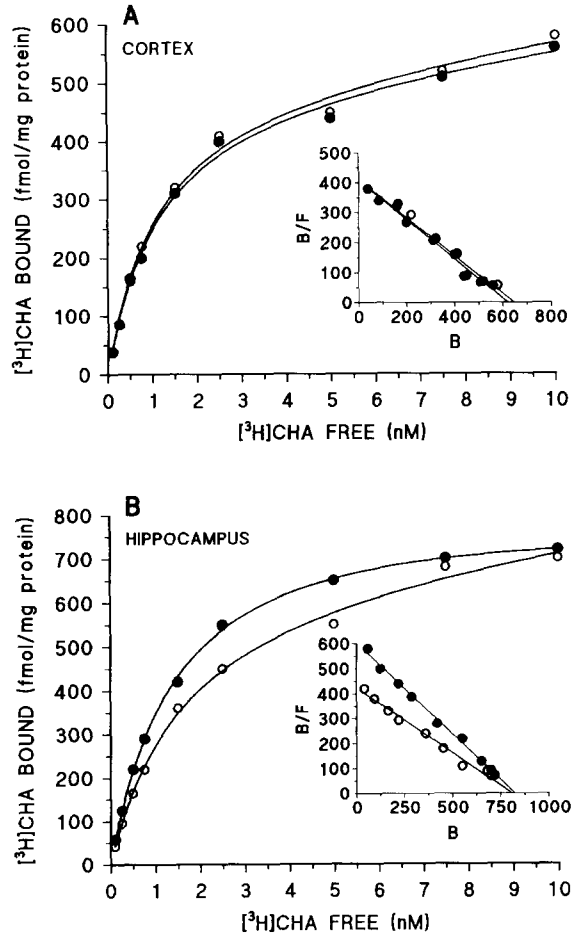

Fig. 1. (A) Saturation of [^{3}H]cyclohexyladenosine ([^{3}H]CHA) binding to cortical membranes of control (○) and kindled (●) rats; the insert is the corresponding Scatchard plot. (B) Saturation of [^{3}H]cyclohexyladenosine binding to hippocampal membranes of control (○) and kindled (●) rats; the insert is the corresponding Scatchard plot.

aspartate overflow in a concentration-dependent manner, with an EC_{50} of 66 nM. Maximal effects (−60%) were reached at 1 μM. In hippocampi taken from the kindled rats, the maximal inhibition remained unchanged, while the EC_{50} was 22 nM (Fig. 2).

4. Discussion

The main finding of this study was that the affinity of adenosine A_1 receptors to a specific ligand (cyclohexyladenosine) is increased in the hippocampus, but not in the cerebral cortex, of kindled rats. An increased binding to adenosine A_1 receptors in various brain areas following pentylentetrazole and bicuculline seizures (Daval and Sarfati, 1987; Angelatou et al., 1990; Daval and Werck, 1991) and repeated electroconvulsive shocks (Newman et al., 1984; Gleiter et al., 1989) has been reported by others. In contrast with the present results, these authors reported an increase in the B_{max} of [^{3}H]cyclohexyladenosine binding. Differences in the model employed may account for this discrepancy. Binding to adenosine A_1 receptors in another model of epilepsy, namely the tottering mouse, does not appear to be changed (Angelatou et al., 1990). Furthermore, it should be stressed that kindled rats were left unstimulated for one week before being killed: in other words, the present study dealt with the hyperexcitable (epileptic) state and not with the direct consequences of convulsions, as in the above-mentioned studies.

Table 1
K_d (nM) and B_{max} (fmol/mg protein) values of [^{3}H]cyclohexyladenosine binding to adenosine A_1 receptors

Group	Cortex		Hippocampus	
	K_d	B_{max}	K_d	B_{max}
Control	1.51 ± 0.10	655 ± 25	2.00 ± 0.04	800 ± 15
Sham-stimulated	1.50 ± 0.12	600 ± 28	1.83 ± 0.08	828 ± 14
Kindled	1.61 ± 0.16	596 ± 22	1.30 ± 0.14 [a]	822 ± 10

Means ± S.E.M. of 4 experiments, 3 animals each experiment.
[a] Significantly different from control and sham-stimulated values, $P < 0.02$, Student's t-test for non-paired data.

Binding (Patel et al., 1982), lesioning (Dragunow et al., 1988) and electrophysiology studies (Yoon and Rothman, 1991; Yamamoto et al., 1993) support the view that adenosine A_1 receptors are located mainly presynaptically in the hippocampus. Activation of presynaptic adenosine A_1 receptors reduces neurotransmitter release (Fredholm and Dunwiddie, 1988). In the hippocampus, this effect appears to be exerted on glutamatergic terminals (Corradetti et al., 1984; Burke and Nadler, 1988; Yoon and Rothman, 1991). Experiments have been made to verify whether the observed increased affinity to adenosine A_1 receptors results in a more potent inhibitory effect of cyclohexyl-

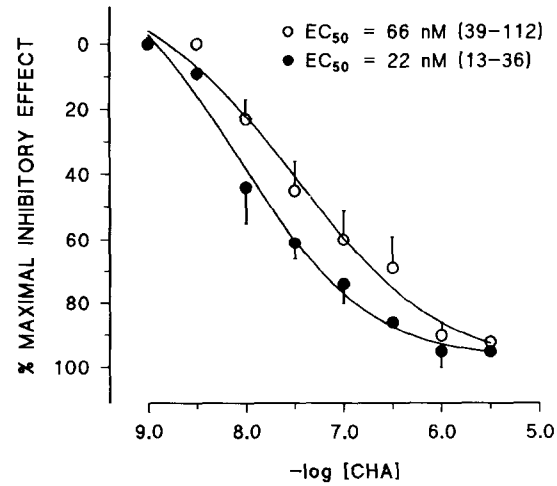

Fig. 2. Effect of cyclohexyladenosine (CHA) on 50 mM K^+-evoked [^{3}H]D-aspartate overflow in hippocampal synaptosomes taken from control (○) and kindled (●) rats (mean ± S.E.M. of 7 experiments). Confidence limits of the EC_{50} values are reported in parentheses.

adenosine on [^{3}H]D-aspartate outflow from hippocampal synaptosomes. As expected, cyclohexyladenosine is slightly more potent in kindled than in control rats. In fact, the shift in the EC_{50} for the inhibition of [^{3}H]D-aspartate outflow was slightly greater than expected on the basis of the binding experiments. Although this discrepancy may simply fall into the experimental variability, it should also be kept in mind that release experiments focus on a subset of presynaptic hippocampal adenosine A_1 receptors, i.e. those located on glutamatergic terminals, while binding experiments were run on the whole hippocampus. These results suggest that the increased affinity to adenosine A_1 receptors is functionally relevant, at least in terms of glutamate release inhibition.

An increased affinity to adenosine A_1 receptors in the hippocampus may play a role in the anticonvulsant effects of adenosine analogues in the kindling model, since this effect appears to be A_1 receptor-mediated (Zhang et al., 1994). However, the relevance of this observation in the hypothesized action of adenosine as an endogenous anticonvulsant cannot be adequately estimated because of the existence of multiple subtypes of adenosine receptors exerting opposite functional effects on hippocampal neurons (see Mogul et al., 1993). Further studies on other receptor subtypes are, therefore, required to clarify this point.

Acknowledgements

This work was supported by a MURST 60% grant. Dr. Gianni Bregola is gratefully acknowledged for technical assistance.

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