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The protective effect of 2-chloroadenosine against the development of amygdala kindling and on amygdala-kindled seizures

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Abstract

The influence of 2-chloroadenosine, a non-metabolizable adenosine A_1 receptor agonist, was tested on the development of electrically kindled amygdala and on the seizure responses of fully kindled rats. Focal intra-amygdaloid injection of 2-chloroadenosine (1–10 nmol/0.5 μl) 20 min before applying the daily kindling stimulus prevented the development of the kindling process. The behavioural seizure score and the afterdischarge duration were reduced below their initial values. The antiepileptogenic effects of 1 and 10 nmol of 2-chloroadenosine were reversible 8–10 days after withdrawal of the drug. When 2-chloroadenosine was tested on fully developed stage 5 amygdala-kindled seizures, it increased the generalised seizure threshold in a dose-dependent manner. A maximum efficiency of 125% ($P < 0.001$) was achieved with 5 nmol and the median effective dose was 0.55 nmol. Higher doses resulted in the reduced anticonvulsant effect ($P < 0.05$). With the same daily stimulation, 2-chloroadenosine 5 nmol in 0.5 μl vehicle, significantly reduced the maximum seizure score by 90%, the afterdischarge duration by 88% and completely blocked the generalised seizure duration. The antiseizure activity of the drug lasted for 3 days. In conclusion, 2-chloroadenosine not only acts as an anticonvulsant against electrically induced kindled seizures as described here, and against audiogenic seizures, electroshock and a variety of chemical convulsants as described by others, it prevents the development of the epileptic state by kindling-stimulation, i.e., it is antiepileptogenic. We theorise here that this is due to its blockade of presynaptic glutamate release.

Keywords: 2-Chloroadenosine; Electrical kindling; Seizure; Epileptogenesis; Amygdala; Adenosine A_1 receptor, pre-synaptic

1. Introduction

The key role of glutamate in basic mechanisms generating epilepsy is well established (Bradford and Dodd, 1976; Bradford and Peterson, 1987; Bradford, 1995). Thus, limbic seizures can be kindled by repeated microinjection of glutamate or aspartate (Mori and Wada, 1987; Mori et al., 1989; Croucher and Bradford, 1989) or by injection of the selective glutamate receptor agonist *N*-methyl-D-aspartate (NMDA) (Vezzani et al., 1988; Croucher et al., 1995). Antagonists of postsynaptic NMDA receptors such as 2-amino-5-phosphonopentanoic acid (AP-5), 2-amino-7-phosphonoheptanoic acid (AP-7), 3-(carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), 3-(carboxypiperazin-4-yl)-propenyl-1-phosphonic acid (CPPene), dizocilpine (MK-801), pentachlorophenol (PCP) show clear anticonvulsant and antiepileptogenic activity (Coutinho-Netto et al., 1981; Croucher et al., 1982, 1988; Peterson et al., 1983, 1984; Meldrum et al., 1983; Patel et al., 1991; Chapman et al., 1990; Kaura et al., 1993; Durmuller et al., 1994; Attwell et al., 1995).

Recently it was reported that agonists of pre-synaptic glutamate receptors are also effective in controlling glutamate release and seizure generation (Attwell et al., 1995). Cyclic glutamate ((1*S*,3*S*)-1-aminocyclopentane-1,3-dicarboxylate (1*S*,3*S*-ACPD)), which activates mainly metabotropic glutamate receptor type 2, has a depressive effect on monosynaptic excitation in neonatal rat motoneurones (Pook et al., 1992), causes depression of synaptic transmission in the hippocampus (Baskys and Malenka, 1991) and, in vivo, inhibits generalized seizure responses.

L-Amino-phosphono-butyric acid (L-AP4), which is a specific agonist of $mGlu_4$, $mGlu_6$, $mGlu_7$ and $mGlu_8$ receptors, showed strong antiepileptic effects on cobalt-induced epilepsy (Coutinho-Netto et al., 1981) and antiepileptogenic and antiseizure activity against amyg-

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dala-kindled seizures (Abdul-Ghani et al., 1997). Lamotrigine, a clinically effective anticonvulsant, works by blocking presynaptic sodium channels, thus non-specifically reducing the excessive release of glutamate which is responsible for the seizures (Leach et al., 1986; Miller et al., 1986; Bradford, 1995).

2-Chloroadenosine is a metabolically stable analogue of adenosine (Dolphin and Archer, 1983; Young and Bradford, 1991). It inhibits NMDA receptor-mediated currents in isolated rat hippocampal neurones (De-Mendonca et al., 1995) and suppresses excitatory glutamatergic inputs to rat hypoglossal motoneurones in vitro (Bellingham and Berger, 1994). It acts presynaptically to inhibit release of excitatory amino acids and is thus considered to be a neuroprotective agent. Stimulation of adenosine A_1 receptors can attenuate the basal, as well as NMDA-induced, production of nitric oxide in vivo and in vitro (Bhardwaj et al., 1995; Northington et al., 1995). 2-Chloroadenosine has been shown to inhibit dopamine release from striatal synaptosomes and slices, and from striatum in vivo using microdialysis (Ballarin et al., 1995). It inhibited evoked acetylcholine release and cyclic AMP synthesis in guinea pig superior cervical ganglia (Borasio et al., 1995). It also inhibited $GABA_B$ (γ-aminobutyric acid receptor subtype B)-mediated inhibitory postsynaptic potentials (IPSPs) by acting at adenosine A_1 receptors (Wu et al., 1995).

Analogues of adenosine are very powerful inhibitors of the presynaptic release of glutamate (Phillis and Wu, 1981; Corradetti et al., 1984). 2-Chloroadenosine suppressed epileptiform activity in hippocampal slices (Ault and Wang, 1986), and has a protective effect against pilocarpine-induced seizures (Turski et al., 1985), bicuculline-induced seizures (Franklin et al., 1989), and kindled seizures (Barraco et al., 1984; Bortolotto et al., 1985). It has shown an anticonvulsant action against electroshock seizures and audiogenic seizures (Bowker and Chapman, 1986; De-Sarro et al., 1991). The role of purinergic mechanisms in epilepsy has been fully reviewed by Dragunow (1986), as has the possibility that adenosine might be an endogenous anticonvulsant (Dragunow et al., 1985).

Previous results from this laboratory have shown the antiepileptogenic and anticonvulsant activities of two classical presynaptic agonists, (1*S*,3*S*)-ACPD (Attwell et al., 1995), and L-AP4 (Abdul-Ghani et al., 1997). The present study tested the effects of 2-chloroadenosine on the development of the kindled epileptic state, and on fully kindled seizures in amygdala.

2. Materials and methods

2.1. Drug

2-Chloroadenosine was purchased from Sigma-Aldrich (Poole, UK).

2.2. Animals and surgery

Details of the experimental procedures are described elsewhere (Croucher and Bradford, 1989; Attwell et al., 1995). Briefly, male Sprague-Dawley rats weighing 280–

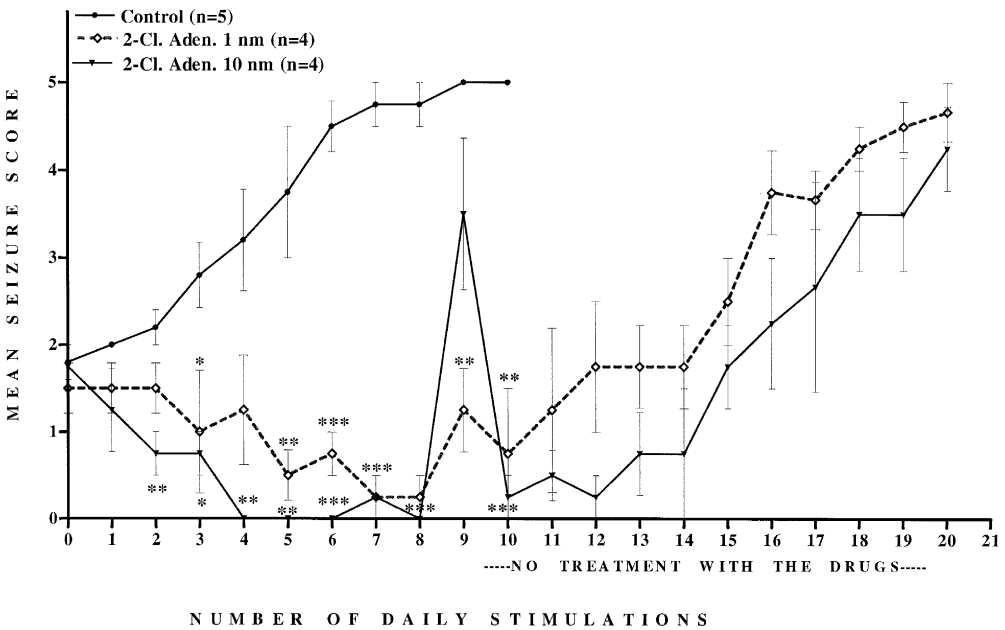

Fig. 1. Effect of 2-chloroadenosine on epileptogenesis. Values shown are mean seizure score ± S.E.M., following repeated daily stimulations. Control animals received an intra-amygdaloid injection of buffer phosphate 0.5 μl, 20 min before the stimulus. Treated animals received an intra-amygdaloid injection of 2-chloroadenosine (2-Cl. Aden.) 1 or 10 nmol in 0.5 μl vehicle. After 8 daily electrical stimulations the drug was replaced with buffer phosphate. Two-way ANOVA shows significant variation between the treatment groups ($P < 0.001$). The significance of the differences in seizure activity on individual days was calculated using Tukey's multiple comparisons test at the individual stimulation time points. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ compared to control animals.

340 g were anaesthetised with a halothane/nitrous oxide mixture and implanted with guide cannulae and stainless-steel bipolar electrodes into the right basolateral amygdala using the following coordinates for the tip of the bipolar electrodes: AP = −0.8, L = −3.8, V = −8.8 from the skull surface, and the combined unit was fixed to the skull with stainless-steel screws using cyanoacrylate cement and zinc powder. Food and water were freely available, and the animals were allowed at least 2 weeks for recovery before starting electrical kindling.

2.3. Electrical kindling procedure

The afterdischarge threshold (ADT) was estimated in each animal using the method of ascending limits as documented by Croucher and Bradford (1991) using a Grass model S88 stimulator coupled to two constant-current pulse generators (Grass model CCU1A). A stimulus of 125% of the threshold current was used for each animal for daily electrical stimulation of the amygdala and electroencephalographic responses were recorded using a Grass model 79D polygraph. Typical currents used were in the range 50–250 mA.

Motor responses were rated on a 5-point scale based on that of Racine (1972) developing from zero behavioural responses to facial muscle twitching, jaw myoclonus with head bobbing to unilateral then bilateral forelimb myoclonus, to the final stage of balance loss resulting in repeated rearing and falling. Animals were considered fully kindled after evocation of three consecutive stage 5 seizures. The afterdischarge duration (ADD) and the generalised seizure duration (GSD) were measured from the electroencephalographs. The generalised seizure threshold (GST) was defined as the minimum stimulus needed to produce a full stage 5 seizure.

2.4. Epileptogenesis

The action of 2-chloroadenosine on the development of kindling was tested by focal intra-amygdaloid microinjec-

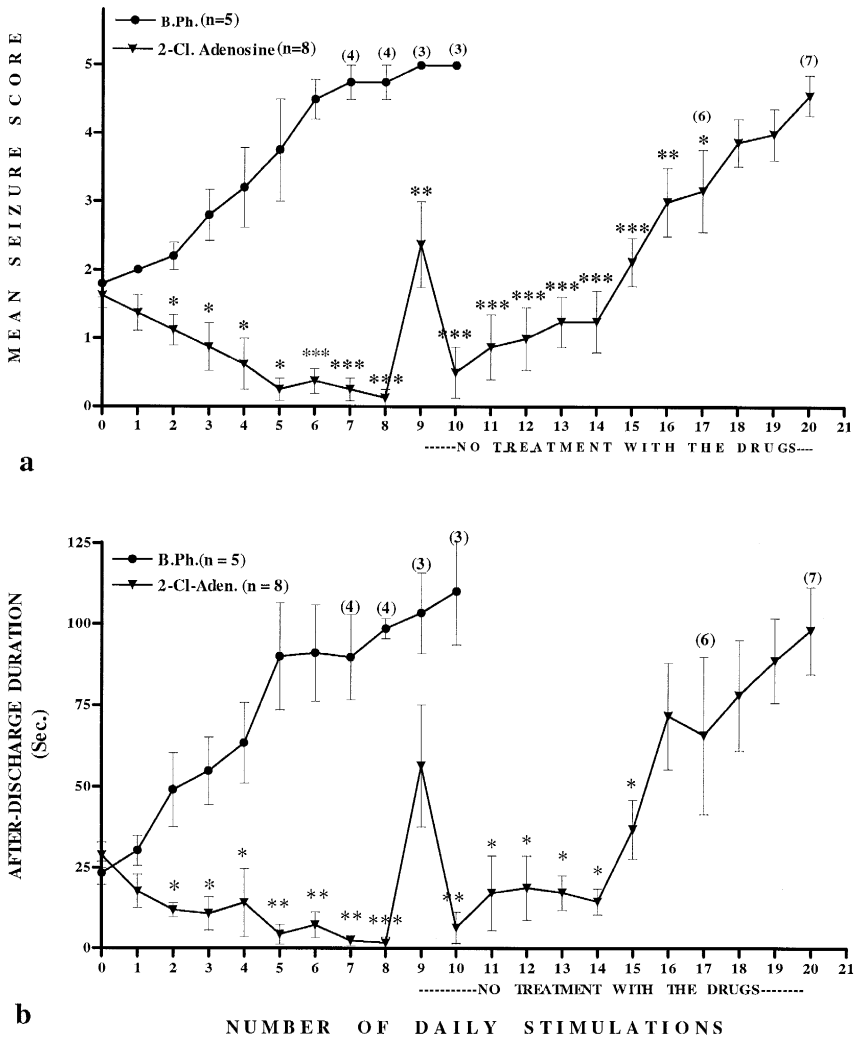

Fig. 2. Changes in mean seizure score (a) and afterdischarge duration (b) during the development of electrical kindling: Inhibition by 2-chloroadenosine. Procedures and treatments were as described in the legend to Fig. 1, except that treated animals were injected with 2-chloroadenosine (2-Cl-Aden.) 1 and 10 nmol in 0.5 μl vehicle. Values shown are mean seizure responses ± S.E.M., for the number of animals shown as *n* or in parentheses. Significant differences were calculated using Student's *t*-test. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

tion of the drug (1 or 10 nmol/0.5 μl or phosphate buffer), 20 min before applying each kindling stimulus, over the whole period. The rate of injection was 0.25 μl/min.

After 8 days the drug was withdrawn from the experimental animals, and the kindling process was continued in its absence. The composition of phosphate buffer vehicle was: NaH_2PO_4 (9.5 mM) and Na_2HPO_4 (40.5 mM) dissolved in deionised H_2O (Millipore); pH 7.4.

2.5. Anti-seizure activity

Fully kindled animals received microinjections (0.5 μl over a 2 min period) of vehicle 20 min before testing the generalised seizure threshold, and after 24 h different doses of 2-chloroadenosine (0.1–10 nmol in 0.5 μl vehicle) were injected by the same route at the same rate for re-estimation of the generalised seizure threshold. Responses to each drug dose were assessed relative to the control responses in the same animal tested 24 h previously.

In other experiments, fully kindled animals received microinjections of vehicle alone one day before treatment with 2-chloroadenosine (5 nmol) and 1, 2 and 3 days after treatment to measure the changes in mean seizure score (MSS), afterdischarge duration (ADD), and generalised seizure duration (GSD) and generalised seizure threshold (GSTs) during the recovery period.

2.6. Testing effects of intracerebrally applied 2-chloroadenosine on body temperature of rats

Before drug administration the normal body temperature of the rats was determined rectally using a small electronic thermometer. Micro-injections of 0.5 μl phosphate buffer either alone or containing 1, 5 or 10 nmol of 2-chloroadenosine were then made into the amygdala as described above (Sections 2.4 and 2.5). After 20 min the rectal temperature of the rats was redetermined to look for any drug-induced changes.

2.7. Statistical treatment of data

Treatment groups were compared using either one- or two-way analysis of variance (ANOVA) depending on their experimental design followed, where significant, by a suitable post-hoc multiple comparisons test of the individual means.

3. Results

3.1. Actions of 2-chloroadenosine on kindled epileptogenesis

In these experiments the mean afterdischarge threshold was 331 ± 76 μA (mean ± S.E.M.; $n = 13$) with an initial seizure score of 1.69 ± 0.13, and mean afterdischarge duration of 26.62 ± 2.85 s. The estimated daily stimulus current was 410 ± 93 μA ($n = 13$).

Control animals treated with the phosphate buffer vehicle developed a normal pattern of fully kindled seizures within 6–9 days. 2-Chloroadenosine (1 nmol/0.5 μl), which was focally injected into the amygdala 20 min before applying the 1 s kindling stimuli, decreased the mean seizure score by 83% from 1.500 ± 0.289 to 0.250 ± 0.250 ($n = 4$), and prevented the development of electrical kindling (Fig. 1). After withdrawal of the drug and replacement with a buffer phosphate, the effect was reversible, but at a slow rate. Thus, fully kindled seizures were achieved after 9–10 days from withdrawal of the drug. Higher doses of 2-chloroadenosine (10 nmol/0.5 μl) achieved a stronger effect, both in reducing the seizure score and the recovery period. An unexpected response was observed followed 10 nmol 2-chloroadenosine. Thus, immediately after withdrawal of the drug the seizure score was increased to 3.50 ± 0.86 (mean ± S.E.M.; $n = 4$) and subsequently reduced to very low values (Fig. 1). Similar changes occurred with afterdischarge duration (Fig. 2).

3.2. Blockade of seizures in fully electrically kindled amygdala by 2-chloroadenosine

In fully kindled animals with seizure scores of 5 on the Racine scale, and generalised seizure thresholds of 550 ± 78 μA (mean ± S.E.M.; $n = 8$) 2-chloroadenosine (0.5, 1 and 5 nmol/0.5 μl vehicle phosphate buffer) injected focally significantly increased the generalised seizure threshold by 33%, 72% and 123% respectively. Lower doses of 0.1 nmol were not effective, and with the higher

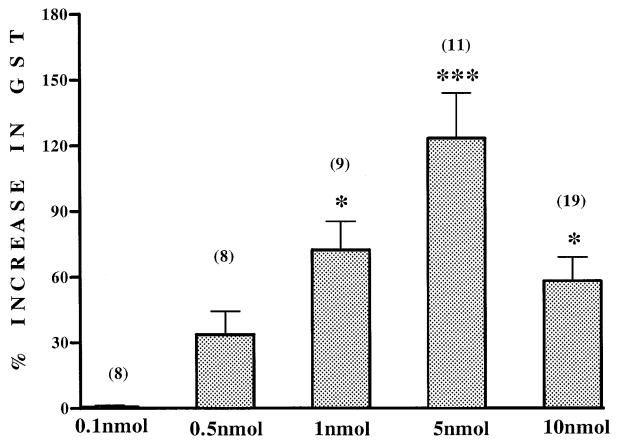

Fig. 3. Effect of different concentrations of 2-chloroadenosine on generalized seizure threshold of fully kindled animals. Fully kindled animals were intracerebrally injected with 0.1–10 nmol of the drug in 0.5 μl, 20 min before testing the generalized seizure thresholds, and 24 h after testing the effect of buffer phosphate on GSTs. Values are mean of percentage changes in generalized seizure threshold ± S.E.M. for the number of experiments indicated in parentheses. Significant differences were calculated using ANOVA with post-hoc Tukey's multiple comparisons test. * $P \leq 0.05$; *** $P \leq 0.001$.

dose of 10 nmol the increase in generalised seizure thresholds (GSTs) was reduced to 58% ($P < 0.05$) (Fig. 3).

Treatment with 2-chloroadenosine at 5 nmol/0.5 μl produced the maximum anti-seizure activity. This was paralleled by a reduction in the mean seizure score of fully kindled animals from a level of 5 on the Racine scale to 0.50 ± 0.27 on the first day, to 1.25 ± 0.62 on the second day, and 1.88 ± 0.64 on the third day. The effect was completely reversed only 72 h after injection of the drug. Similar changes were observed in the level of the afterdischarge duration, and in the generalised seizure duration (Fig. 4).

3.3. Action of 2-chloroadenosine on body temperature

Intracerebral microinjections of 2-chloroadenosine in the range at which it was shown to have anticonvulsant properties (i.e., 1–10 nmol) were tested for their actions on body temperature as such effects have been previously reported (Bowker and Chapman, 1986). Neither 1, 5 nor 10 nmol 2-chloroadenosine produced any significant change in body temperature ($+0.85 \pm 0.48$, -0.57 ± 0.35 and $+0.01 \pm 0.49$°C respectively) compared to those seen in control (buffer injected) animals ($+0.48 \pm 0.44$°C) 20 min after administration, as judged by a rectal thermometer (ANOVA; $n = 4$).

3.4. Histological assessment

In previous detailed studies from this laboratory, on completion of the electrical kindling, animals were perfused transcardially with 10% formaldehyde in 0.9% saline. The brains were then removed and immersed in fixative for at least 2 days. Serial 60 μm coronal sections were cut

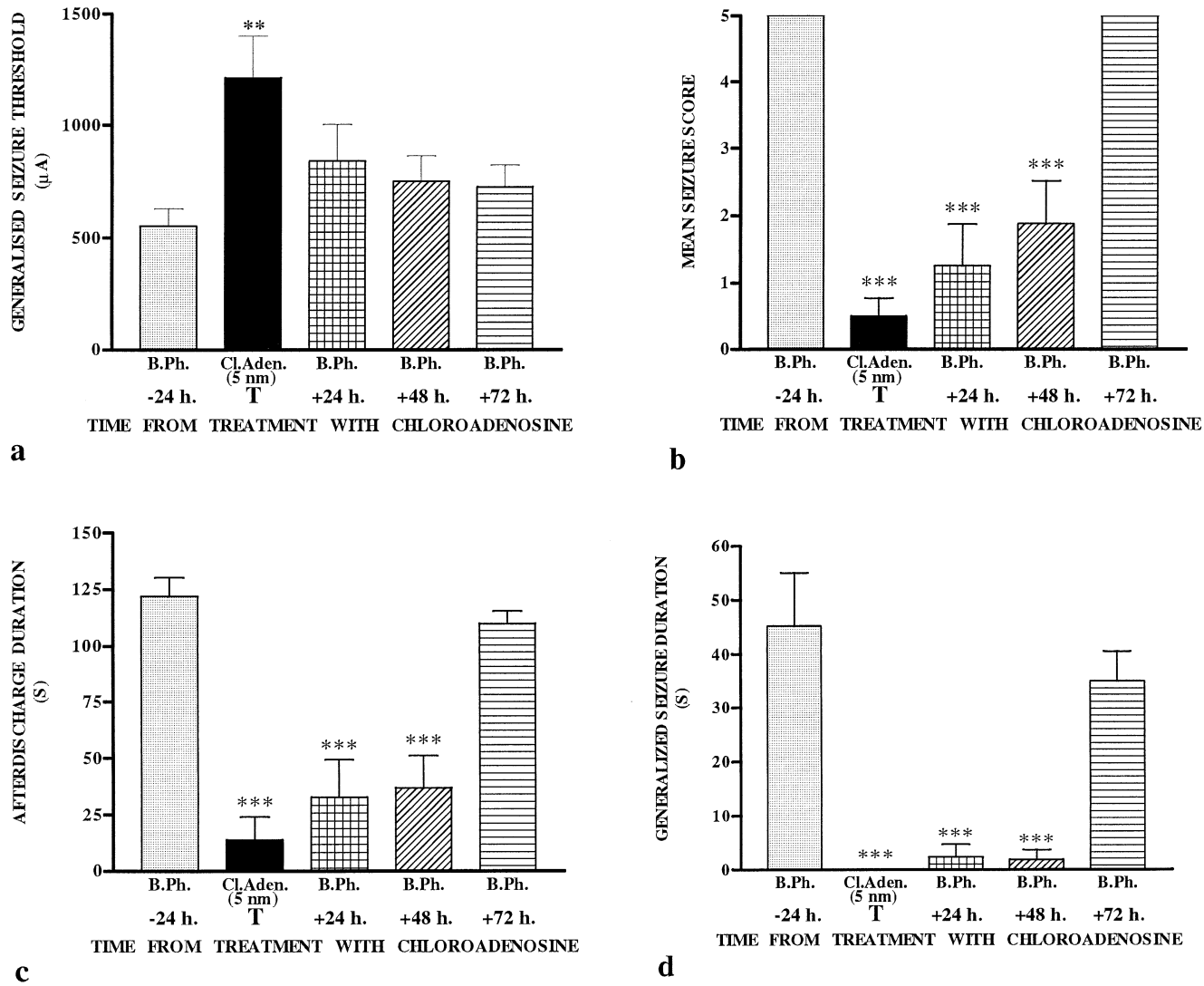

Fig. 4. The influence of intracerebral injection of 2-chloroadenosine on seizure responses of fully kindled animals. Treated animals received intra-amygdaloid injections of 2-chloroadenosine (5 nmol) in 0.5 μl buffer phosphate, 20 min before applying the electrical stimulus. The same treatment was applied with the injection of buffer phosphate 1 day before, and for 3 days after, treatment. Values are mean ± S.E.M. (a) Generalized seizure thresholds; (b) mean seizure score; (c) afterdischarge duration; and (d) generalized seizure duration. Significant differences were calculated using ANOVA with post-hoc Tukey's multiple comparisons test. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

on a freezing microtome and stained with cresyl violet. These were examined for signs of histological change, and also to confirm the correct location of the injection cannula and electrode tips in the basolateral amygdaloid nucleus. There was never any evidence of more than trivial levels of neuronal damage or glial proliferation surrounding injection cannulae or electrode tips (Croucher and Bradford, 1989, 1990, 1991; Croucher et al., 1992).

4. Discussion

The results reported here demonstrate the antiepileptogenic effect of 2-chloroadenosine, which blocks the increases in both seizure score and afterdischarge duration which normally develop during electrical kindling. The effects were significant with both 1 and 10 nmol 2-chloroadenosine. The rate of reversal after withdrawal of the drug was very slow compared to the effects of presynaptic agonists like (1*S*,3*S*)-ACPD (Attwell et al., 1995) and L-AP4 (Abdul-Ghani et al., 1997), though the adenosine A_1 receptor may be both pre- and postsynaptic in location. The unexpected increase in seizure responses which followed the first kindling stimulus after withdrawal of 2-chloroadenosine (10 nmol) could be due to the effect of the drug on other neurotransmitter systems including inhibition of the release of an inhibitory neurotransmitter. The drug was reported to inhibit dopamine release (Ballarin et al., 1995), and to inhibit $GABA_B$-mediated actions (Wu et al., 1995).

2-Chloroadenosine was also effective in reducing the generalised seizure responses of fully kindled animals. It inhibited the mean seizure score, the afterdischarge duration, and the generalised seizure duration. Generalised seizure thresholds to electrical stimuli were increased in a dose-dependent manner. Following drug doses of 5 nmol, the effects lasted for 72 h.

These results shows that 2-chloroadenosine is a very effective antiepileptogenic and anticonvulsant agent when applied to electrically kindled animals. Fig. 5 shows that it is at least as effective in blocking epileptogenesis as other presynaptically active agonists such as (1*S*,3*S*)-ACPD and L-AP4 as well as postsynaptic antagonists of glutamate receptors such as CPPene, when compared with previous results from this laboratory (Attwell et al., 1995; Abdul-Ghani et al., 1997). Also, following cessation of drug administration, this antiepileptogenic effect is longer lasting than those of these other agents (Fig. 5).

The antiseizure activity, as judged by the effect on generalised seizure thresholds (GSTs), is more potent than that of AP-5 and AP-7 (Croucher et al., 1992), and also that of L-AP4 (Abdul-Ghani et al., 1997). 2-Chloroadenosine is less potent than CGP 37849 and 39551 (Croucher et al., 1992) and CPPene (Attwell et al., 1995).

Previous studies have demonstrated the protective effect of the drug in many models of epilepsy such as electroshock-induced seizures and audiogenic seizures (Bowker and Chapman, 1986; De-Sarro et al., 1991), kindled seizures (Barraco et al., 1984), pilocarpine-induced seizures (Turski et al., 1985), and bicucculline-induced seizures (Franklin et al., 1989). The present results show that 2-chloroadenosine is also anticonvulsant against seizures

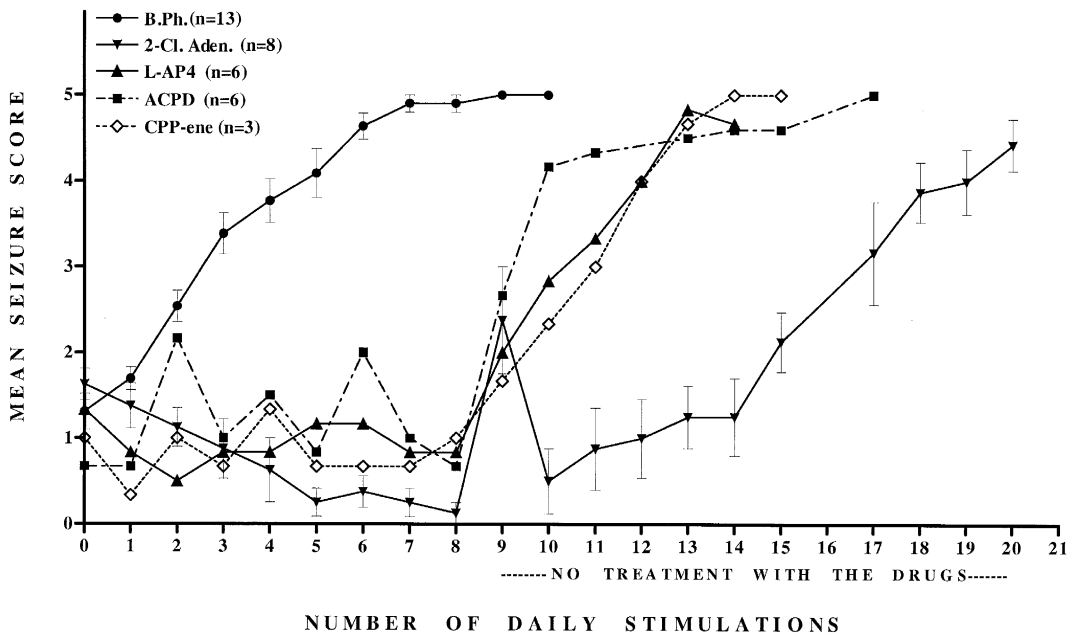

Fig. 5. Effect of 2-chloroadenosine on epileptogenesis compared to L-AP4, ACPD, and CPP-ene. The effect of 2-chloroadenosine on mean seizure score during the development of electrical kindling is compared to previous published results from this laboratory, i.e. L-AP4 (Abdul-Ghani et al., 1997), ACPD, and CPP-ene (Attwell et al., 1995). Data are mean ± S.E.M.

induced in fully kindled animals, and advances knowledge of its properties by demonstrating that it blocks development of kindled epilepsy, i.e., it is antiepileptogenic. Also, in this study the drug was delivered in small quantities by intracerebral injection in small volumes (0.5 μl) into a localised brain structure, the amygdala. Thus its actions would be local to the site of brain injection. Most other studies described above administered the drug systemically and peripherally (e.g., intraperitoneal injection), allowing the possibility that many sites of action could be involved in the subsequent responses (e.g., peripheral nervous system sites, spinal cord and medulla centres). This may explain the action of 2-chloroadenosine on temperature regulation (e.g., Bowker and Chapman, 1986). However, as shown in the present paper, intracerebral injection into the amygdala of 2-chloroadenosine at doses shown to be anticonvulsant in the present study had no action on body temperature. Such effects could not, therefore, be wholly or partially responsible for the anticonvulsant actions of 2-chloroadenosine reported here.

It is notable that various convulsive procedures cause a considerable increase in extracellular adenosine levels in the brain (Dragunow, 1991; During and Spencer, 1992). During and Spencer (1992) have argued that this rise in levels of an endogenous anticonvulsant (i.e., adenosine) may represent a feedback response of the convulsing brain to temporarily limit the extent of its own convulsions.

The mechanism of action of 2-chloroadenosine is likely to be related to its presynaptic action at adenosine receptors which results in reduced synaptic release of glutamate (Bellingham and Berger, 1994; Phillis and Wu, 1981; Corradetti et al., 1984). It inhibits NMDA receptor-mediated current in isolated hippocampal neurones (De-Mendonca et al., 1995). However, it should be noted that the effects of 2-chloroadenosine are not specific to glutamate release, since it affects the release of other neurotransmitters such as acetylcholine, dopamine and GABA (Ballarin et al., 1995; Borasio et al., 1995; Wu et al., 1995). Nevertheless, many other studies have highlighted the probable role of glutamate as the excitatory trigger in many forms of epilepsy (Attwell et al., 1995; Bradford, 1995). In this sense the antiepileptogenic and anticonvulsant actions of intracerebrally administered 2-chloroadenosine support this theory of a central involvement of the glutamate ion.

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