British Journal of Pharmacology (1996) 118, 1551-1557

Block by N^6-L-phenylisopropyl-adenosine of the electrophysiological and morphological correlates of hippocampal ischaemic injury in the gerbil

M.R. Domenici, A. Scotti de Carolis & [1]S. Sagratella

Laboratorio di Farmacologia, Istituto Superiore di Sanita', Viale Regina Elena 299, 00161, Roma, Italy

1 The effects of the mixed A_1 and A_2 adenosine receptor agonist N^6-L-phenyl-isopropyladenosine (L-PIA) were tested on ischaemia-induced hippocampal neuronal injury in gerbils subjected to 5-min bilateral carotid occlusion. For comparison, the effects of the selective A_2 adenosine receptor agonist, CGS 21680 were tested.

2 Five-min bilateral carotid occlusion produced within 1 week an irreversible suppression of the CA1, but not of the dentate extracellular electrical somatic responses, in 30% of gerbil hippocampal slices with respect to controls. In addition, a significant reduction occurred in the density of CA1 hippocampal pyramidal neurones but not of dentate granule cells with respect to controls.

3 Injection 1 h before or after bilateral carotid occlusion of L-PIA (0.8-1.5 mg kg^{-1}, i.p.) but not of CGS 21680 (5 mg kg^{-1}, i.p.), significantly prevented the irreversible disappearance of the CA1 extracellular electrical somatic responses with respect to controls. In addition, the CA1 pyramidal neuronal loss was also prevented.

4 The results show that activation of A_1 adenosine receptors is able to prevent or block the electrophysiological and morphological correlates of hippocampal neuronal injury after global ischaemia in the gerbil, suggesting that adenosine receptor agonists might have a useful role in the treatment of neuronal functional and anatomical injury due to ischaemia.

Keywords: Gerbil; ischaemia; electrophysiology; L-PIA; hippocampal slices

Introduction

Adenosine is an endogenous nucleoside that has been shown to play an important role in the regulation of neuronal function in a variety of mammalian tissues and has been recognized as a homeostatic neuromodulator (Greene & Haas, 1991). It exerts its action via membrane-bound receptors. Membrane-bound adenosine receptors have been demonstrated in the central nervous system as well as in peripheral tissues. Two receptor subtypes named A_1 and A_2 have been distinguished on the basis of their affinity for adenosine and their coupling to adenylate cyclase (Van Calker *et al.*, 1979). Stimulation of neuronal adenosine A_1 receptor leads to depression of neuronal excitability and firing rate (Phillis *et al.*, 1974; Dunwiddie, 1980; Lee *et al.*, 1984; Frank *et al.*, 1988) and reduction of release of neurotransmitters including the excitatory amino acids (Dolphin & Archer, 1983; Corradetti *et al.*, 1984; Fastbom & Fredholm, 1986; Burke *et al.*, 1988), that have been implicated in the pathophysiology of epilepsy and brain injury (Meldrum, 1985).

Adenosine accumulates *in vivo* in the extracellular space of brain tissue after several pathological conditions such as seizure, hypoxia-ischaemia and hypoglycaemia (McIlwain & Poll, 1986; Butcher *et al.*, 1987; Hagberg *et al.*, 1987; Barraco *et al.*, 1991). It may be released as an endogenous mediator and act to limit the development of excitability and brain injury by interference with specific adenosine receptors, at either a pre- or post-synaptic level. In fact, adenosine and adenosine derivatives have been reported to produce anticonvulsant and neuroprotective effects (Dragunow & Faull, 1988). In agreement with the neuroprotective role of adenosine in the brain, some groups have demonstrated that acute treatment with selective adenosine receptor agonists prior to hypoxia or hypoglycemia or ischaemia, leads to a significant morphological neuroprotection (Evans *et al.*, 1987; Goldberg *et al.*, 1988; Rudolphi *et al.*, 1992). On the other hand, acute treatment with adenosine receptor antagonists has an opposite effect and aggravates the ischaemia-induced damage (Sutherland *et al.*, 1991; Von Lubitz *et al.*, 1994). Electrophysiological studies have also added further information on the neuroprotective effects of adenosine, or adenosine derivatives in neuronal injury. The adenosine reuptake inhibitor, soluflazine, delayed the hypoxic depolarization in rat hippocampal slices (Boissard & Gribkoff, 1993). During short-lasting but not during long-lasting hypoxia, A_1 adenosine antagonists delayed the hypoxia-induced reversible suppression of CA1 synaptic transmission in rat hippocampal slices, giving physiological evidence for the hypoxia-induced increase of CNS levels of adenosine (Fowler, 1989; Zeng *et al.*, 1992). In addition, A_1 adenosine agonists prevented morphological signs of brain injury induced by systemic injection of the excitatory amino acid, kainic acid (McGregor *et al.*, 1993), but did not display significant protection against neuronal injury by glutamate or NMDA (Golberg *et al.*, 1988; Frank *et al.*, 1994).

The Mongolian gerbil is a useful model for studying cerebral ischaemia (Kahn, 1972). In this species, forebrain ischaemia can be easily produced by clamping both common carotid arteries, since the circle of Willis connecting the carotid and vertebrobasilar circulations is incomplete. In this study, using electrophysiological *in vitro* techniques, we have explored the possibility that cells, which appear to be protected histologically by treatment with adenosine agonists, are actually functional. In particular, we have tested the effects of two adenosine receptor agonists having different affinities for A_1 and A_2 adenosine receptors: N^6-L-phenyl-isopropyladenosine (L-PIA) and 2-[*p*-(carboxyethyl)phenylethylamino]-5-N-carboxamidoadenosine (CGS 21680) (Lupica *et al.*, 1990).

[1] Author for correspondence.

Methods

Animals

Male Mongolian gerbils (Charles River, Italy) weighing 60–80 g and fed *ad libitum* were used. The animals were divided in 7 groups. One hour before operation, 3 of these groups, which were later subjected to 5 min of forebrain ischaemia, were treated with a low (0.2 mg kg^{-1}, i.p., $n=6$), intermediate (0.8 mg kg^{-1}, i.p., $n=6$) or high dose (1.5 mg kg^{-1}, i.p., $n=9$) of L-PIA in aqueous solution at 10 ml kg^{-1} body weight. One group received L-PIA (1.5 mg kg^{-1}, i.p., $n=18$) 1 h after the forebrain ischaemia; another group was pretreated (1 h before the operation) with CGS 21680 (5 mg kg^{-1}, i.p., $n=6$). A further group served as ischaemic control and did not undergo any treatment ($n=18$); the last was the sham-operated group and underwent the same experimental procedures except for the artery occlusion ($n=5$).

Experimental procedures

Gerbils were subjected to a 5-min period of bilateral carotid artery occlusion under anaesthesia using a mixture of 3% isofluorane, 5% CO_2 and 95% O_2. They were fixed in the supine position on a temperature-controlled operating table, a 2 cm anterior midline cervical incision was made and both common carotid arteries were carefully dissected free of accompanying tissues. Bilateral carotid arterial occlusion was produced by clamping the vessels with microaneurysm clips. During operation, anaesthesia was maintained with 1% isofluorane in the same gas mixture.

The animals were allowed to survive for 7 days following the ischaemia. Then they were killed by decapitation under light ether anaesthesia. The skull was opened and the hippocampus rapidly removed. The left hippocampus of some ischaemic control animals ($n=6$), of some animals treated 1 h after forebrain ischaemia with L-PIA 1.5 mg kg^{-1}, i.p. ($n=6$), and all of the sham-animals and the animals pretreated with L-PIA or with CGS 21680, was processed for slice preparation and electrophysiological studies. The right hemisphere of these animals was used for the histological analysis of neuronal damage in the dorsal region of the hippocampus. In 6 ischaemic control animals and in 6 animals treated 1 h after forebrain ischemia with L-PIA 1.5 mg kg^{-1}, i.p. both hippocampi were processed for slice preparation and electrophysiological studies. In another 6 ischaemic control animals and another 6 animals treated 1 h after forebrain ischaemia with L-PIA 1.5 mg kg^{-1}, i.p., all the brain was used for the histological analysis of neuronal damage in the dorsal region of the hippocampus.

Slice preparation

Slices of hippocampus (450 μm thick) were cut with a tissue chopper (McIlwain) and immediately placed in the recording chamber, where they were constantly perfused (at a rate of 2–3 ml min^{-1}) with an artificial cerebral spinal fluid (CSF) saturated with 95% O_2:5% CO_2. The composition of the artificial CSF was the following (mM): NaCl 122, KH_2PO_4 0.4, KCl 3, $MgSO_4$ 1.2, $NaHCO_3$ 25, $CaCl_2$ 1.3, glucose 10 (pH 7.3). The temperature of the perfusion chamber was maintained at 33 ± 1°C. An interval of 60–90 min was allowed between the time the slices were cut and the start of the recording session.

Recording session

Field potentials (FPs) were recorded through 3 M NaCl-filled glass microelectrodes (1–5 megaohms) in the CA1 and dentate area after electrical stimulation (0.1 Hz, 70 μs, 100–200 μA) of the *stratum radiatum* or *stratum moleculare*. Electrical potentials, in particular population spike (PS) magnitude or excitatory post-synaptic potential (e.p.s.p.) slope (measured from the beginning to the maximum of the negative or positive deflexion, respectively), were amplified, recorded on tape (Racal 4DS), digitized at 10 kHz, averaged (five consecutive recordings) and analyzed on line by an *ad hoc* software package on a PS2 IBM computer. Three slices from each gerbil were used for the electrophysiological studies.

When a stable FP was obtained, a stimulus-response curve was performed. In slices obtained from each animal, we measured the amplitude of the PS at intermediate stimulation levels. FPs in amplitude less than 0.5 mV were not considered. For each group, the average of the PS amplitude recorded in CA1 and dentate areas of hippocampal slices was recorded. Differences among groups were evaluated by the Newman-Keul's test. Moreover, we considered in each group, the percentage of failures to find FPs or the rate of occurrence of FPs in CA1 and dentate area.

Histology

The right hemisphere was immersed in a solution of formaldehyde buffered with 1% PBS, where it was left until histological processing. Paraffin sections (7 μm) of the hippocampus were then cut on a microtome and stained with Cresyl violet. These sections were assessed for qualitative and quantitative analysis of the neuronal degeneration. Qualitative analysis was performed at the light microscope level and the severity of neuronal damage in the hippocampus was graded on the score of 0–3, according to Pulsinelli *et al.* (1982), with 0 = normal tissue; 1 = few neurones damaged; 2 = many neurones damaged; 3 = majority of the neurones damaged. For quantitative analysis, sections were viewed under a light microscope and the number of CA1 pyramidal cells and the area occupied by the cells was counted in 3–4 consecutive fields of 10,000 μm^2 of the CA1 *stratum pyramidale* using an automatic image analysis system (Leica, Cambridge, U.K.) connected via a TV camera to the microscope. In each group the qualitative scores or the quantitative values were measured. Differences among groups were evaluated by the Newman-Keul's test or the Mann-Whitney test.

Drugs

L-PIA was obtained from the Sigma Chemical Company (St Louis, MO, U.S.A.) and dissolved in distilled water containing a few drops of HCl 0.1N. CGS 21680 was synthesized and generously donated by Ciba-Geigy (Summit, NJ, U.S.A.); it was dissolved in distilled water.

Results

Behaviour

All the gerbils undergoing 5-min bilateral carotid occlusion survived up to 1 week after the surgical procedures. The treatment with adenosine receptor agonists did not modify the survival of the animals.

Electrophysiology

In the sham-operated group, CA1 and dentate somatic FPs consisted of an e.p.s.p. (2–3 mV, 3–4 ms) and a single superimposed PS (3–5 mV, 2–3 ms). The magnitude of the FPs was higher in the CA1 than in the dentate area (data not shown).

In 30% of the left hippocampal slices and in 33% of the right hippocampal slices obtained from ischaemic control animals, no FPs could be recorded in the CA1 area (Figure 1, Table 1). Moreover in this group, a significant decrease ($P<0.05$) of the CA1 PS amplitude was found with respect to the sham-group (Figure 2). No significant differences occurred in the amplitude of the dentate PS between the sham and the ischaemic control group (Figure 1).

The treatment with low doses of L-PIA (0.2 mg kg^{-1}, i.p.)

did not affect the CA1 PS amplitude or the percentage of success in finding a CA1 FP with respect to the ischaemic control group (Table 1, Figure 2).

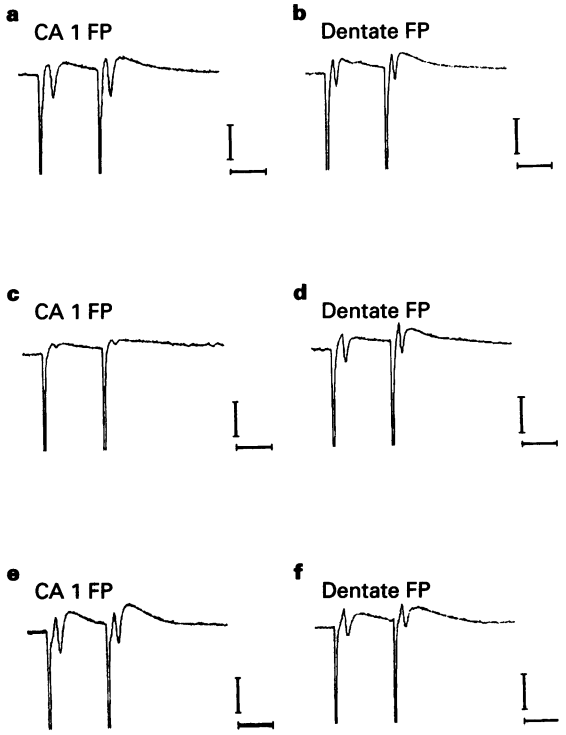

Figure 1 N^6-L-phenyl-isopropyl-adenosine (L-PIA) blocks hippocampal CA1 electrical failure 1 week after global ischaemia in the gerbil. (a) Control CA1 extracellular somatic field potential (FP) in a slice obtained from a sham animal; (b) control dentate extracellular somatic FP in a slice obtained from a sham animal. (c) Suppression of the CA1 FP in a slice obtained from an ischaemic animal; (d) persistence of the dentate FP in a slice obtained from an ischaemic animal. (e) Persistence of the CA1 FP in a slice obtained from an ischaemic animal treated with 1.5 mg kg^{-1}, i.p. of L-PIA 1 h after carotid occlusion; (f) persistence of the dentate FP in a slice obtained from an ischaemic animal treated 1 h after carotid occlusion with 1.5 mg kg^{-1} i.p. L-PIA. Calibrations: 5 mV; 5 ms.

Between slices obtained from the ischaemic control animals and those obtained from animals pretreated with higher doses of L-PIA (0.8–1.5 mg kg^{-1}), significant differences ($P<0.05$) in the CA1 PS amplitude and in the percentage of finding a CA1 FP, were obtained. The CA1 PS amplitude and the rate of occurrence of CA1 FPs was dose-dependently and significantly higher in the slices obtained from animals pretreated with 0.8–1.5 mg kg^{-1} of L-PIA compared to slices obtained from the ischaemic control group (Table 1, Figure 2).

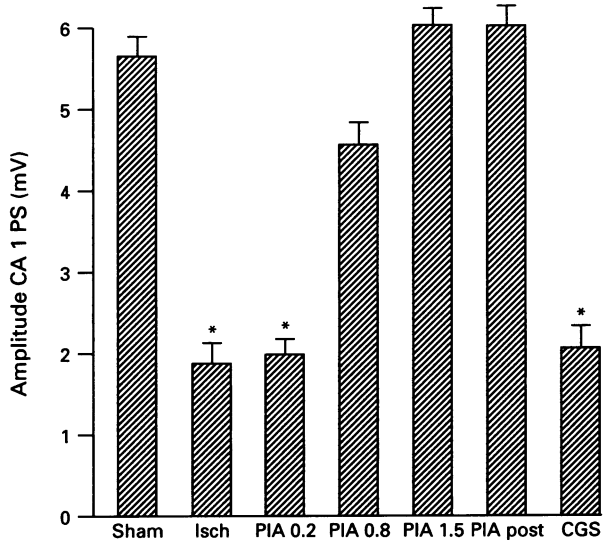

Figure 2 Effects of adenosine receptor agonists on the ischaemia-induced decrease of the amplitude of CA1 extracellular somatic responses in left gerbil hippocampal slices. The histograms show the protective effects of high doses of N^6-L-phenyl-isopropyl-adenosine (L-PIA) injected 1 h before or after carotid occlusion on the ischaemia-induced decrease of the amplitude of the CA1 population spike (PS). Abbreviations: Sham, sham-operated gerbil; Isch, ischaemic gerbil; PIA, ischaemic gerbil treated 1 h before carotid occlusion with L-PIA; PIA post, ischaemic gerbil treated 1 h after carotid occlusion with 1.5 mg kg^{-1}, i.p. of L-PIA; CGS, ischaemic gerbil treated 1 h before carotid occlusion with 5 mg kg^{-1}, i.p. of CGS 21680; *significantly different from Sham ($P<0.05$ according to Newman-Kuel's test).

Table 1 Influence of adenosine receptor agonists on the electrophysiological correlates of the neuronal injury in CA1 hippocampal area after global ischaemia

Drugs	*Dose* (mg kg^{-1})	*n/N*	*Suppression CA1 FP* %	A	%A
Sham	–	0/15*	0.0§	5	0±0&
Isch	–	12/36	30.3	12	33±4
R-Isch	–	6/18	33.3	6	33±8
PIA	0.2	7/18	38.8	6	38±5
PIA	0.8	1/18*	5.5§	6	5±5&
PIA	1.5	0/27*	0.0§	9	0±0&
PIA post	1.5	0/36*	0.0§	12	0±0&
R-PIA post	1.5	0/18$	0.0#	6	0±0@
CGS	5	4/18	18.2	6	22±6

The table shows the effects of adenosine receptor agonists on CA1 field potentials recorded in slices obtained from gerbils subjected to 5-min bilateral carotid occlusion.
Abbreviations: *n/N* = number of slices in which no field potentials could be recorded/total number of slices used; % = percentage of slices showing electrical failure; A = number of animals; % A = percentage of slices showing electrical failure per animal; Isch = ischaemic control animals in which left hippocampus was analyzed; R-Isch = ischaemic control animals in which right hippocampus was analyzed; PIA = animals pretreated 1 h before carotid occlusion with L-PIA in which left hippocampus was analyzed; PIA post = animals treated 1 h after carotid occlusion with 1.5 mg kg^{-1}, i.p. of L-PIA in which left hippocampus was analyzed; R-PIA post = animals treated 1 h after carotid occlusion with 1.5 mg kg^{-1}, i.p. of L-PIA in which right hippocampus was analyzed; CGS = animals pretreated 1 h before carotid occlusion with 5 mg kg^{-1} CGS 21680 in which left hippocampus was analyzed; *significantly different from Isch ($P<0.05$ according to the Fisher exact test); §significantly different from Isch ($P<0.05$ according to the chi square test). $significantly different from R-Isch ($P<0.05$ according to the Fisher exact test); #significantly different from R-Isch ($P<0.05$ according to the chi square test); & significantly different from Isch ($P<0.05$ according to the Newman-Keul's test); @ significantly different from R-Isch ($P<0.05$ according to the Newman-Keul's test).

In the right and left hippocampal slices obtained from animals treated with L-PIA (1.5 mg kg^{-1}) 1 h after a 5-min carotid occlusion period, a significant increase ($P<0.05$) in the CA1 PS amplitude and in the rate of occurrence of FPs were found compared with slices taken from ischaemic control animals (Table 1, Figures 1 and 2).

The treatment with CGS 21680 (5 mg kg^{-1}, i.p.) did not modify, with respect to ischaemic controls, the electrical activity of CA1 pyramidal neurones (Table 1, Figure 2).

In the dentate area no differences were found among the different groups, either in the PS amplitude, or in the rate of occurrence of FPs (data not shown).

Histology

The morphology of the CA1 and dentate area in the non-ischaemic sham group did not differ from that described by other authors (Von Lubitz *et al.*, 1994). Qualitative analysis showed a widespread destruction of CA1 pyramidal neurones in both hippocampi of all the gerbils of the ischaemic control group. The pyramidal neurones were either absent or badly shrunken. Numerous microglia were seen around the dendrites in the lower *stratum radiatum*. Astrocytes were swollen within the entire CA1 region. Cells of the CA2–CA3 region were also affected. The morphology of the dentate area was significantly less affected than the pyramidal area (Figures 3 and 4).

Qualitative analysis revealed that damage to CA1 pyramidal neurones or gliosis was absent or reduced in both hippocampi of the L-PIA-treated groups (Table 2). Quantitative studies showed a significant ($P<0.01$) increase in the number of neurones per field in all L-PIA-treated groups (Figure 5) and a dose-dependent increase of the area occupied by the neurones per field in the L-PIA-treated groups with respect to the ischaemic control group (Figure 6). In contrast, no significant neuroprotection occurred in the group treated with CGS 21680 (Table 2, Figure 5 and 6).

Discussion

The Mongolian gerbil is a useful model for studying cerebral ischaemia because of the lack of a complete circle of Willis. For this reason, cerebral infarction is easily induced, and testing the effect of drugs on ischaemia-induced neurodegeneration is readily achieved.

It has been shown that discrete areas in the brain, and certain types of neurones such as the CA1 pyramidal cells in the hippocampus or the Purkinje cells in the cerebellum, are selectively vulnerable to ischaemic insults. Following a brief episode of ischaemia, the pyramidal cells of the CA1 subfield of the hippocampus undergo a so-called 'delayed neuronal

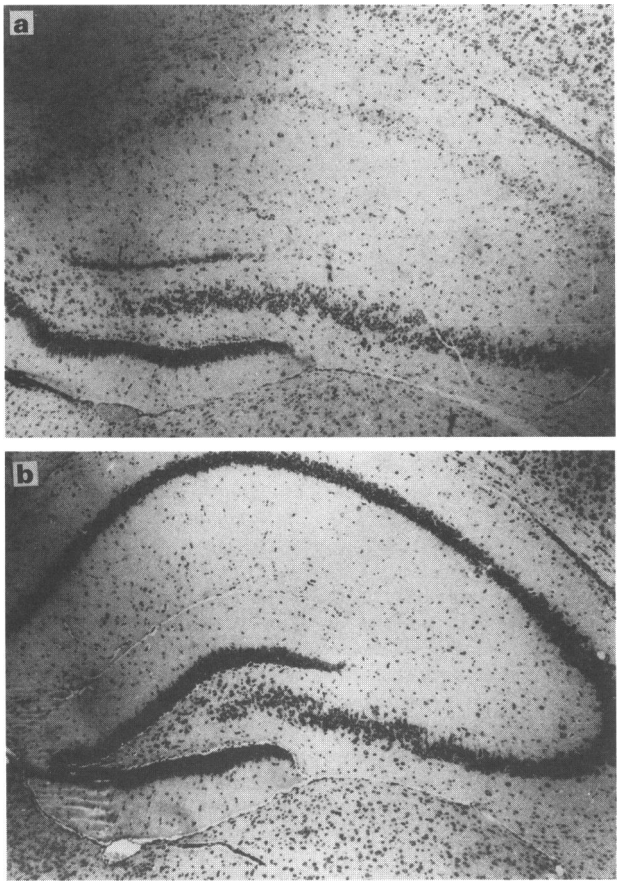

Figure 3 N^6-L-phenyl-isopropyl-adenosine (L-PIA) blocks hippocampal morphological injury due to global ischaemia in the gerbil: (a) hippocampal ischaemic injury 1 week after bilateral carotid occlusion in the gerbil; (b) protective effects of 1.5 mg kg^{-1}, i.p. L-PIA injected 1 h after bilateral carotid occlusion in the gerbil.

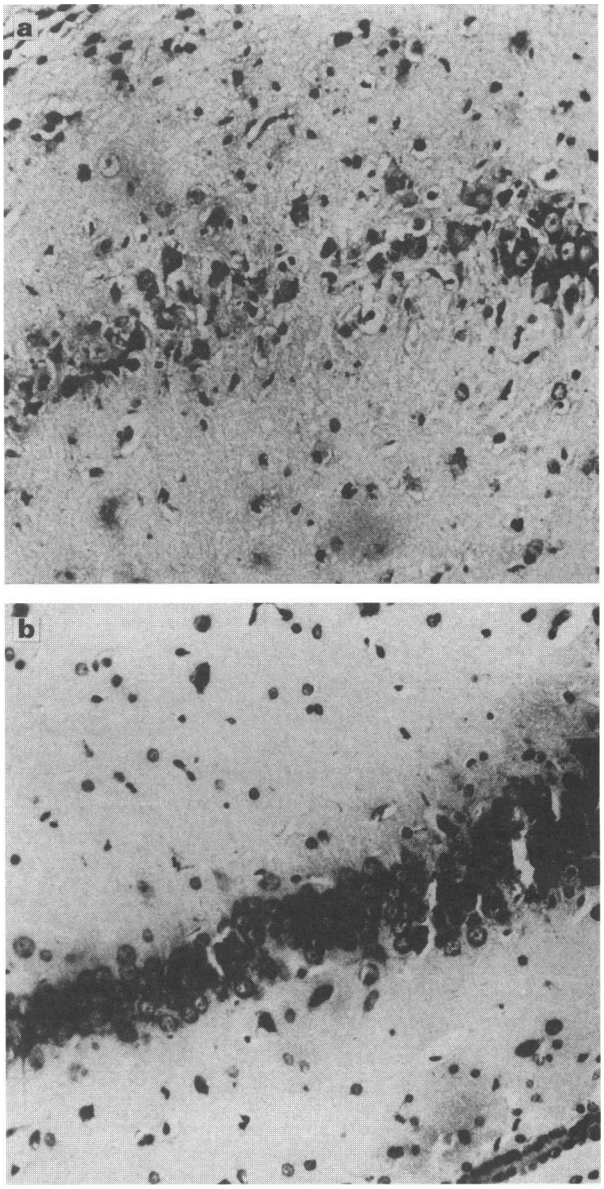

Figure 4 N^6-L-phenyl-isopropyl-adenosine (L-PIA) blocks hippocampal CA1 morphological injury due to global ischaemia in the gerbil. (a) Hippocampal CA1 ischaemic injury 1 week after bilateral carotid occlusion in the gerbil; (b) protective effects of 1.5 mg kg^{-1}, i.p. of L-PIA injected 1 h after bilateral carotid occlusion in the gerbil.

Table 2 Influence of adenosine receptor agonists on the morphological correlates of the neuronal injury in CA1 hippocampal area after global ischaemia

Drugs	*Dose* (mg kg^{-1})	*N*	*Score*
Sham	–	5	0±0*
Isch	–	12	2.3±0.3
L-Isch	–	6	2.6±0.5
PIA	0.2	6	1.8±0.4
PIA	0.8	6	0.7±0.3*
PIA	1.5	9	0.5±0.2*
PIA post	1.5	12	1.1±1.0*
L-PIA post	1.5	6	0.9±0.5§
CGS	5	6	1.5±0.4

Values are ±s.e.mean.
The table shows the effects of adenosine receptor agonists on the morphological correlates of hippocampal neuronal injury in gerbils subjected to 5 min bilateral carotid occlusion.
Abbreviations: *N* = number of animals; Isch = ischaemic control animals in which right hippocampus was analyzed; L-Isch = ischaemic control animals in which left hippocampus was analyzed; PIA = animals pretreated 1 h before carotid occlusion with L-PIA in which right hippocampus was analyzed; PIA post = animals treated 1 h after carotid occlusion with 1.5 mg kg^{-1}, i.p. of L-PIA in which right hippocampus was analyzed; L-PIA post = animals treated 1 h after carotid occlusion with 1.5 mg kg^{-1}, i.p. L-PIA in which left hippocampus was analyzed; CGS = animals pretreated 1 h before carotid occlusion with 5 mg kg^{-1}, i.p. CGS 21680 in which right hippocampus was analyzed; *significantly different from Isch ($P<0.05$ according to the Mann-Whitney test); §significantly different from L-Isch ($P<0.05$ according to Mann-Whitney test).

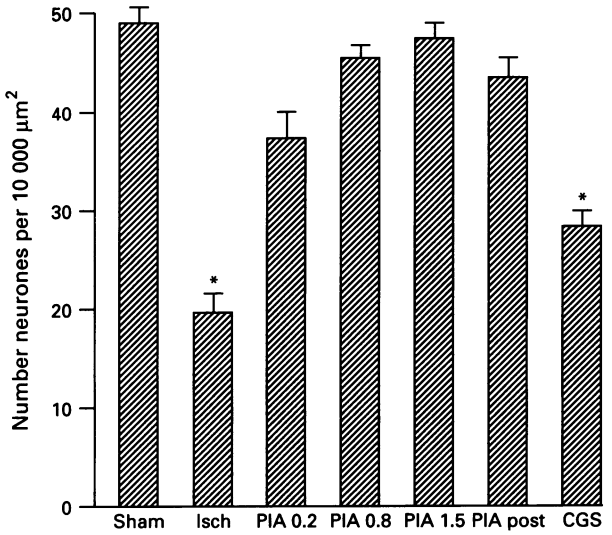

Figure 5 Effects of adenosine receptor agonists on the ischaemia-induced decrease of the number of CA1 pyramidal neurones in the right hippocampus of gerbil. The histograms show the protective effects of N^6-L-phenyl-isopropyl-adenosine (L-PIA) injected 1 h before or after carotid occlusion on the ischaemia-induced decrease of number of CA1 neurones. Abbreviations: Sham, sham-operated gerbil; Isch, ischaemic gerbil; PIA, ischaemic gerbil treated 1 h before carotid occlusion with L-PIA; PIA post, ischaemic gerbil treated 1 h after carotid occlusion with 1.5 mg kg^{-1}, i.p. L-PIA; CGS, ischaemic gerbil treated 1 h before carotid occlusion with 5 mg kg^{-1}, i.p. CGS 21680; *significantly different from Sham ($P<0.05$ according to Newman-Kuel's test).

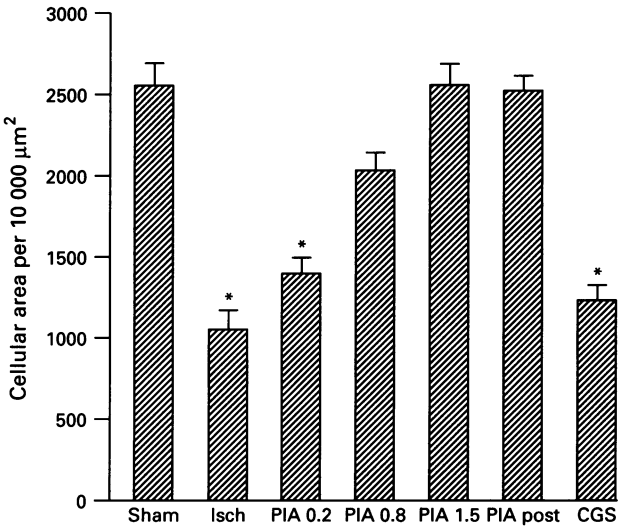

Figure 6 Effects of adenosine receptor agonists on the ischaemia-induced decrease of the area occupied by CA1 pyramidal neurones in the right hippocampus of gerbil. The histograms show the protective effects of N^6-L-phenyl-isopropyl-adenosine (L-PIA) injected 1 h before or after carotid occlusion on the ischaemia-induced decrease of the area occupied by CA1 neurones. Abbreviations: Sham, = sham-operated gerbil; Isch, ischaemic gerbil; PIA, ischaemic gerbil treated 1 h before carotid occlusion with L-PIA; PIA post, ischaemic gerbil treated 1 h after carotid occlusion with 1.5 mg kg^{-1}, i.p. L-PIA; CGS, ischaemic gerbil treated 1 h before carotid occlusion with 5 mg kg^{-1}, i.p. CGS 21680; *significantly different from Sham ($P<0.05$ according to Newman-Kuel's test).

death' (Kirino, 1982) and several lines of evidence suggest that post-ischaemic synaptic release of excitatory amino acids, mainly glutamate, is responsible for transient cell hyperexcitability (Chang *et al.*, 1989; Urban *et al.*, 1989), and is a causative factor in the development of neuronal death (Jorgensen & Diemer, 1982). This would lead to glutamate receptor activation and to calcium influx, production of free radicals, lipid peroxidation and cell death (Choi, 1987; Hartley & Choi, 1989; Domenici *et al.*, 1993).

In the present study, we compared the electrophysiological and morphological counterparts of the hippocampal ischaemic neuronal injury after global ischaemia in the gerbil. It has been shown that medially more than the 50% of pyramidal neurones disappeared within one week after 5-min of global ischaemia. From an electrophysiological point of view, these morphological changes were accompanied by a complete suppression of the CA1 PS in 30% of the slice-experiments and by a significant reduction of the magnitude of the PS in almost 40% of the slice-experiments. The incomplete correlation between slice viability and morphological damage (i.e. 50% of neuronal decrease against 70% of slice viability) might be due to the possibility of the recording electrode detecting electrical activity from areas around the CA1 where the neuronal decrease may not be maximal, as in the CA1 area. The lack of correlation is unlikely to depend on a difference in the incidence of ischaemic damage after global ischaemia between the right (where the histological studies were carried out routinely) and the left hemisphere (which was used for the electrophysiological studies), because in separate experiments we found similar degrees of slice viability and neuronal damage in either hemisphere.

In the present study, we found that acute treatment with the higher doses of the A_1/A_2 adenosine agonist, L-PIA, is able to prevent the ischaemia-induced reduction in CA1 hippocampal neuronal density (expressed as number of neurones and as area occupied by the neurones) and the electrical failure observed in the CA1 area in a proportion of animals after ischaemia. Treatment with 0.8–1.5 mg kg^{-1} of L-PIA is also able to prevent the decrease of the PS amplitude noted in the ischaemic control group and to restore the normal magnitude of the PS in the CA1 subfield of the hippocampus. Low doses of L-PIA (0.2 mg kg^{-1}) do not have the same effect, in accordance

with the findings of others (Von Lubitz *et al.*, 1994). Under our experimental conditions, treatment with 0.2 mg kg^{-1} of L-PIA, even though it significantly decreased the neuronal loss with respect to ischaemic controls, did not prevent the CA1 electrical failure. This suggests that the surviving neurones have lost function though they may be histologically 'normal'. In fact, in agreement with the electrophysiological findings we also found that the low dose of L-PIA failed to prevent the ischaemia-induced changes in neuronal size (expressed as area occupied by the residual neurones), confirming the altered functionality of the surviving neurones. On the whole, the data indicate the importance of the association of functional and morphological studies in the experimental model of neurotoxicity.

L-PIA exerts its effect if administered either 1 h before the ischaemia, or 1 h after a 5-min carotid occlusion. The neuroprotective action of the adenosine receptor agonist is similar to that produced by the AMPA antagonist, NBQX. This drug was also able to counteract ischaemia-induced morphological changes if its administration was delayed some hours after bilateral carotid occlusion (Sheardown *et al.*, 1993). By contrast, the NMDA antagonist, MK-801, failed to affect ischaemia-induced neuronal injury if its administration was delayed (Sheardown *et al.*, 1993; Valentino *et al.*, 1993). The differential neuroprotective influence of adenosine receptor agonists is further evinced by the findings with models of hypoxic injury in rat hippocampal slices. Whereas adenosine receptor agonists and non-NMDA receptor antagonists, in contrast to NMDA antagonists, fail to prevent the irreversible disappearance of CA1 electrical synaptic responses after a long-lasting hypoxic period (Zeng *et al.*, 1992; Yassin & Scholfield, 1994). The differential spectrum of neuroprotection by adenosine receptor agonists and glutamate receptor antagonists suggests a differential involvement of subtypes of glutamate receptors and of adenosine receptors in the different models of brain injury.

The neuroprotection observed with treatment with L-PIA is probably due to its interaction with A_1 receptors because the administration of CGS 21680, a selective A_2 agonist, does not exert any protection, either from an electrophysiological point of view, or histologically. This finding is in agreement with biochemical studies where the density of the adenosine A_1 receptors correlates with the distribution of the selectively vulnerable neurones. A_1-receptors are highly concentrated in the dendritic zones of the CA1 subfield of the hippocampus (Murray & Cheney, 1982).

Stimulation of A_1 receptors can inhibit glutamate release and the related calcium influx into the neurone via glutamate-operated calcium channels. Adenosine also directly limits membrane depolarization and reduces the opening of voltage-sensitive calcium channels (Dolphin, 1983). Adenosine exerts its stabilizing effect through a membrane hyperpolarization due to an increased K^+ permeability, which prevents sustained discharge (Greene & Haas, 1991). Furthermore, adenosine activates a well defined voltage-dependent Cl^- conductance, that facilitates an outward flux of accumulated intracellular Cl^- and adds to the stabilizing effect of adenosine (Mager *et al.*, 1990). All these pre- and postsynaptic effects of adenosine receptor agonists might contribute to their neuroprotective effect.

In previous studies of excitotoxicity, we found L-PIA unable to affect NMDA-induced hippocampal neuronal injury. In particular, L-PIA did not ameliorate (or aggravate) the NMDA-induced irreversible disappearance or reduction in amplitude of the hippocampal CA1 PS. In contrast to this finding, the NMDA receptor antagonist, MK 801, preserved the presence of the hippocampal CA1 PS after the application of a high concentration of NMDA (Frank *et al.*, 1994). These data are in agreement with other reports showing the inability of adenosine to alter the neurotoxic morphological changes of exogenously applied glutamate in cortical cell cultures (Golberg *et al.*, 1988). In addition, in unpublished experiments, we have found L-PIA (5 μM) unable to affect the irreversible electrophysiological changes due to high concentrations of the non-NMDA excitatory amino acid, AMPA (25 μM) in rat hippocampal slices. The lack of effect of L-PIA in postsynaptic models of excitotoxicity presumably points to the anti-ischaemic neuroprotective effects of the drug being dependent mainly on a pre-synaptic mechanism.

Thus adenosine, by acting at specific presynaptic A_1 receptors, may inhibit the release of several neurotransmitters including glutamate and aspartate, in control conditions and during ischaemic episodes, leading to an attenuation of electrophysiological and morphological changes in neuronal injury.

The authors thank Drs S. Pestalozza and Y.C. Zeng for their helpful suggestions during the course of these studies.

References

BARRACO, R.A., WALTER, G.A., POLASEK, P.M. & PHILLIS, J.W. (1991). Purine concentrations in the cerebrospinal fluid of unaesthetized rats during and after hypoxia. *Neurochem. Int.*, **18**, 243–248.

BOISSARD, C.G. & GRIBKOFF, V.K. (1993). The effects of the adenosine reuptake inhibitor soluflazine on synaptic potentials and population hypoxic depolarizations in area CA1 of rat hippocampus *in vitro*. *Neuropharmacology*, **32**, 149–155.

BURKE, L.P. & NADLER, J.V. (1988). Regulation of glutamate and aspartate release from slices of the hippocampal CA1 area: effect of adenosine and baclofen. *J. Neurochem.*, **51**, 1541–1551.

BUTCHER, S., HAGBERG, H., SANDBERG, M. & HAMBERGER, M. (1987). Extracellular purine catabolite and amino acid levels in the rat striatum during severe hypoglycemia: effects of 2-amino-5-phosphonovalerate. *Neurochem. Int.*, **11**, 95–99.

CHANG, H., SASAKI, T. & KASSELL, N. (1989). Hippocampal unit activity after transient cerebral ischemia in rats. *Stroke*, **20**, 1051–1055.

CHOI, D.W. (1987). Ionic dependence of glutamate excitotoxicity. *J. Neurosci.*, **7**, 369–379.

CORRADETTI, R., LO CONTE, G., MORONI, F., PASSANI, M.B. & PEPEU, G. (1984). Adenosine decreases aspartate and glutamate release from rat hippocampal slices. *Eur. J. Pharmacol.*, **104**, 19–25.

DOLPHIN, A.C. (1983). The adenosine agonist 2-chloroadenosine inhibits the induction of long-term potentiation in the perforant path. *Neurosci. Lett.*, **39**, 83–87.

DOLPHIN, A.C. & ARCHER, E.R. (1983). An adenosine agonist inhibits and a cyclic AMP analogue enhances the release of glutamate but not GABA from slices of rat dentate gyrus. *Neurosci. Lett.*, **43**, 49–54.

DOMENICI, M.R., LONGO, R., SCOTTI DE CAROLIS, A., FRANK, C. & SAGRATELLA, S. (1993). Protective actions of 21-aminosteroids and MK-801 on hypoxia-induced electrophysiological changes in rat hippocampal slices. *Eur. J. Pharmacol.*, **233**, 291–293.

DRAGUNOW, M. & FAULL, R.L.M. (1988). Neuroprotective effects of adenosine. *Trends Pharmacol. Sci.*, **9**, 193–195.

DUNWIDDIE, T.V. (1980). Endogenously released adenosine regulates excitability in the *in vitro* hippocampus. *Epilepsia*, **21**, 541–548.

EVANS, M.C., SWAN, J.H. & MELDRUM, B.S. (1987). An adenosine analogue, 2-chloroadenosine, protects against long term development of ischemic cell loss in the rat hippocampus. *Neurosci. Lett.*, **83**, 287–292.

FASTBOM, J. & FREDHOLM, B.B. (1986). Inhibition of [H3]glutamate release from rat hippocampal slices by 1-phenylisopropyladenosine. *Acta Physiol. Scand.*, **125**, 121–123.

FOWLER, J.C. (1989). Adenosine antagonists delay hypoxia-induced depression of neuronal activity in hippocampal brain slice. *Brain Res.*, **490**, 378–384.

FRANK, C., SAGRATELLA, S., BENEDETTI, M. & SCOTTI DE CAROLIS, A. (1988). Comparative influence of calcium blocker and purinergic drugs on epileptiform bursting in rat hippocampal slices. *Brain Res.*, **441**, 393–397.

FRANK, C., SCOTTI DE CAROLIS, A. & SAGRATELLA, S. (1994). Influence of adenosinergic drugs on the epileptiform and neurotoxic effects of N-methyl-d-aspartate: comparison with the effects of MK801. *Arch. Int. Pharmacodyn.*, **327**, 3–12.

GOLDBERG, M.P., MONYER, H., WEISS, J.H. & CHOI, D.W. (1988). Adenosine reduces cortical neuronal injury induced by oxygen or glucose deprivation *in vitro*. *Neurosci. Lett.*, **89**, 323–327.

GREENE, R.W. & HAAS, H.L. (1991). The electrophysiology of adenosine in the mammalian central nervous system. *Prog. Neurobiol.*, **36**, 329–341.

HAGBERG, H., ANDERSSON, P., LACAREWICZ, J., JACBSON, I., BUTCHER, S. & SANDBERG, M. (1987). Extracellular adenosine, inosine, hypoxanthine, and xanthine in relation to tissue nucleotides and purines in rat striatum during transient ischemia. *J. Neurochem.*, **49**, 227–231.

HARTLEY, D.M. & CHOI, D.W. (1989). Delayed rescue of N-methyl-D-aspartate receptor-mediated neuronal injury in cortical culture. *J. Pharmacol. Exp. Ther.*, **250**, 752–758.

JORGENSEN, M.B. & DIEMER, N.H. (1982). Selective neuron loss after cerebral ischemia in the rat: possible role of transmitter glutamate. *Acta Neurol. Scand.*, **66**, 536–546.

KAHN, K. (1972). The natural course of experimental cerebral infarction in the gerbil. *Neurology*, **22**, 510–515.

KIRINO, T. (1982). Delayed neuronal death in the gerbil hippocampus following ischemia. *Brain Res.*, **239**, 57–69.

LEE, K.S., SCHUBERT, P. & HEINEMANN, U. (1984). The anticonvulsive action of adenosine: a postsynaptic dendritic action by a possible endogenous anticonvulsant. *Brain Res.*, **321**, 160–164.

LUPICA, C.R., CASS, W.A., ZAHNISER, N.R. & DUNWIDDIE, T.W. (1990). Effects of the selective adenosine A2 receptor agonist CGS 21680 on in vitro electrophysiology, cAMP formation and dopamine release in rat hippocampus and striatum. *J. Pharmacol. Exp. Ther.*, **252**, 1134–1141.

MAGER, R., FERRONI, S. & SCHUBERT, P. (1990). Adenosine modulates a voltage-dependent chloride conductance in cultured hippocampal neurones. *Brain Res.*, **532**, 58–62.

MCGREGOR, D.G., MILLER, W.J. & STONE, T.W. (1993). Mediation of the neuroprotective action of R-phenylisopropyl-adenosine through a centrally located adenosine A_1 receptor. *Br. J. Pharmacol.*, **110**, 470–476.

MCILWAIN, H. & POLL, J.D. (1986). Adenosine in cerebral homeostatic role: appraisal through actions of homocysteine, colchicine and dipyridamole. *J. Neurobiol.*, **17**, 39–49.

MELDRUM, B.S. (1985). Possible therapeutic applications of antagonists of excitatory amino acid neurotransmitters. *Clin. Sci.*, **68**, 113–122.

MURRAY, T.F. & CHENEY, D.L. (1982). Neuronal localization of N-Cyclohexyl-[^{3}H]-adenosine binding sites in rat and guinea pig brain. *Neuropharmacology*, **21**, 575–580.

PHILLIS, J.W., KOSTOPOULUS, G.K. & LIMACHER, J.J. (1974). Depression of corticospinal cells by various purines and pyrimidines. *Can. J. Physiol. Pharmacol.*, **52**, 1226–1234.

PULSINELLI, W.A., BRIERLEY, J.B. & PLUM, F. (1982). Temporal profile of neuronal damage of transient forebrain ischemia. *Ann. Neurol.*, **11**, 491–498.

RUDOLPHI, K.A., SCHUBERT, P., PARKINSON, F.E. & FREDHOLM, B.B. (1992). Adenosine and brain ischemia. *Cerebrovasc. Brain Metab. Rev.*, **4**, 346–358.

SHEARDOWN, M.J., SUZDAK, P.D. & NORDHOLM, L. (1993). AMPA, but not NMDA receptor antagonism is neuroprotective in gerbil global ischaemia, even when delayed 24 h. *Eur. J. Pharmacol.*, **236**, 347–353.

SUTHERLAND, G.R., PEELING, J., LESIUK, H.J., BROWNSTONE, R.M., RYDZY, M., SAUNDERS, J.K. & GEIGER, J.D. (1991). The effects of caffeine on ischemic neuronal injury as determined by magnetic resonance imaging and histopathology. *Neuroscience*, **42**, 171–182.

URBAN, L., NEILL, K., CRAIN, B., NADLER, J.V. & SOMJEN, G. (1989). Postischemic synaptic physiology in area CA1 of the gerbil hippocampus studied *in vitro*. *J. Neurosci.*, **9**, 3966–3975.

VALENTINO, K., NEWCOMB, R., GADBOIS, T., SINGH, T., BOWERSOX, S., BITNER, S., HOFFMAN, B.B., CIARANELLO, R., MIJANICH, G. & RAMACHANDRAN, J. (1993). A selective N-type calcium channel antagonist protects against neuronal loss after global cerebral ischemia. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 7894–7897.

VAN CALKER, D., MULLER, M. & HAMPRECT, B. (1979). Adenosine regulates via two different types of receptors the accumulation of cyclic AMP in cultured brain cells. *J. Neurochem.*, **33**, 999–1005.

VON LUBITZ, K.J.E., LIN, R.C.S., MELMAN, N., XIAO-DUO, J., CARTER, M.F. & JACOBSON, K.A. (1994). Chronic administration of selective adenosine A_1 receptor agonist or antagonist in cerebral ischemia, *Eur. J. Pharmacol.*, **256**, 161–165.

YASSIN, M. & SCHOLFIELD, C.N. (1994). NMDA antagonists increase recovery of evoked potentials from slices of rat olfactory cortex after anoxia. *Br. J. Pharmacol.*, **111**, 1221–1227.

ZENG, Y.C., DOMENICI, M.R., FRANK, C., SAGRATELLA, S. & SCOTTI DE CAROLIS, A. (1992). Effects of adenosinergic drugs on hypoxia-induced electrophysiological changes in rat hippocampal slices. *Life Sci.*, **51**, 1073–1082.

(Received July 31, 1995
Revised October 17, 1995
Accepted April 4, 1996)