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Effect of A_{2A} adenosine receptor stimulation and antagonism on synaptic depression induced by *in vitro* ischaemia in rat hippocampal slices

[1]Serena Latini, [1]Francesca Bordoni, [1]Renato Corradetti, [1]Giancarlo Pepeu & *,[1]Felicita Pedata

[1]Department of Preclinical and Clinical Pharmacology, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy

1 In the present study we investigated the role of A_{2A} adenosine receptors in hippocampal synaptic transmission under *in vitro* ischaemia-like conditions.

2 The effects of adenosine, of the selective A_{2A} receptor agonist, CGS 21680 (2-[p-(2-carboxyethyl)-phenethylamino]-5′-N-ethylcarboxamidoadenosine), and of selective A_{2A} receptor antagonists, ZM 241385 (4-(2-[7-amino-2-(2-furyl)-{1,2,4}-triazolo{2,3-a}{1,3,5}triazin-5-ylamino]ethyl)phenol) and SCH 58261 (7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine), have been evaluated on the depression of field e.p.s.ps induced by an *in vitro* ischaemic episode.

3 The application of 2 min of *in vitro* ischaemia brought about a rapid and reversible depression of field e.p.s.ps, which was completely prevented in the presence of the A_1 receptor antagonist DPCPX (1,3-dipropyl-8-cyclopentylxanthine) (100 nM). On the other hand both A_{2A} receptor antagonists, ZM 241385 and SCH 58261, by themselves did not modify the field e.p.s.ps depression induced by *in vitro* ischaemia.

4 A prolonged application of either adenosine (100 μM) or CGS 21680 (30, 100 nM) before the *in vitro* ischaemic episode, significantly reduced the synaptic depression. These effects were antagonized in the presence of ZM 241385 (100 nM).

5 SCH 58261 (1 and 50 nM) did not antagonize the effect of 30 nM CGS 21680 on the ischaemia-induced depression.

6 These results indicate that in the CA1 area of the hippocampus the stimulation of A_{2A} adenosine receptors attenuates the A_1-mediated depression of synaptic transmission induced by *in vitro* ischaemia.

Keywords: Adenosine; A_{2A} and A_1 adenosine receptors; CGS 21680; SCH 58261; ZM 241385; synaptic responses; hippocampal slices; *in vitro* ischaemia

Abbreviations: aCSF, artificial cerebral spinal fluid; ADO, adenosine; CGS 21680, (2-[p-(2-carboxyethyl)-phenethylamino]-5′-N-ethylcarboxamidoadenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; field e.p.s.p., field excitatory post synaptic potential; SCH 58261, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine; ZM 241385, 4-(2-[7-amino-2-(2-furyl)-{1,2,4}-triazolo{2,3-a}{1,3,5}triazin-5-ylamino]ethyl)phenol

Introduction

In the central nervous system (CNS), adenosine is an important neuromodulator which acts on four different receptors: A_1, A_{2A}, A_{2B} and A_3 (Fredholm *et al*., 1994). The well established inhibitory tonus exerted by endogenous adenosine on synaptic transmission is mainly attributed to activation of the A_1 adenosine receptor type (Wu & Saggau, 1994), which is expressed with high density and widespread distribution in the brain (Fastbom *et al*., 1987).

After introduction of the A_{2A} adenosine receptor agonist CGS 21680 (Hutchinson *et al*., 1989), several excitatory actions of A_{2A} adenosine receptor stimulation have been described in the brain (see: Latini *et al*., 1996; Sebastiao & Ribeiro, 1996). In the rat brain CGS 21680 is 180 and 40 fold more selective for A_{2A} than for A_1 and A_3 receptors, respectively, and virtually inactive on A_{2B} receptors (Jarvis *et al*., 1989; Williams *et al*., 1989).

Early autoradiographic (Jarvis & Williams, 1989; Martinez-Mir *et al*., 1991) and molecular biology studies (Schiffmann *et al*., 1990; 1991; Fink *et al*., 1992) showed that A_{2A} adenosine receptors were mainly confined to the striatal region. However, lower levels of expression of A_{2A} receptor mRNA (Cunha *et al*., 1994; Dixon *et al*., 1996) and of [^{3}H]-CGS 21680 binding sites (Wan *et al*., 1990; Cunha *et al*., 1994; Johansson & Fredholm, 1995) have also been detected in the hippocampus and cortex. Finally, a immunohistochemical analysis of A_{2A} receptor distribution in the rat brain has recently confirmed their presence in the hippocampus and cortex (Rosin *et al*., 1998).

Electrophysiological investigations of the role of A_{2A} adenosine receptors in synaptic functions have shown both an increase (Sebastiao & Ribeiro, 1992; Cunha *et al*., 1994; 1997; Li & Henry, 1998) and no significant effect (Dunwiddie *et al*., 1997; O'Kane & Stone, 1998) of A_{2A} receptor stimulation on hippocampal neurotransmission. Although the mechanisms by which A_{2A} receptors increase excitatory neurotransmission are not fully understood, it has been shown that CGS 21680 may decrease the ability of A_1 receptor agonists to inhibit hippocampal excitatory neurotransmission (Cunha *et al*., 1994; O'Kane & Stone, 1998).

In the hippocampus it appears established that, under hypoxic and ischaemic conditions, a consistent increase in the extracellular concentration of endogenous adenosine (Pedata *et al*., 1993; Latini *et al*., 1998b; 1999) is associated with activation of A_1 adenosine-receptors, which results in a significant depression of synaptic transmission (Fowler, 1989;

*Author for correspondence; E-mail: pedata@server1.pharm.unifi.it

1990; Canhao *et al*., 1994; Lucchi *et al*., 1996; Latini *et al*., 1999). This activation of A_1 receptors and the subsequent reduction in glutamate release is believed to be one of the principal neuroprotective mechanisms of adenosine against ischaemic brain damage (Rudolphi *et al*., 1992). On the other hand, the role of A_{2A} adenosine receptors under brain ischaemia is still not clear (see Ongini & Schubert, 1998) and no information on their involvement in synaptic transmission during an ischaemic episode is available.

The aim of this investigation was to study whether the stimulation of A_{2A} receptors might counteract the synaptic depression induced by an *in vitro* ischaemia model. The effects of adenosine and of selective compounds such as the agonist CGS 21680 and the antagonists ZM 241385 and SCH 58261, which are active on A_{2A} adenosine receptors, were studied on the depression of field e.p.s.ps produced by *in vitro* ischaemia in rat hippocampal slices.

A preliminary account of these results has been previously communicated (Latini *et al*., 1998a).

Methods

Preparation of hippocampal slices and induction of in vitro *ischaemia*

Experiments were carried out on rat hippocampal slices, prepared as previously described (Corradetti *et al*., 1983). Charles River male Wistar rats, 150–200 g body weight, were killed by decapitation, their hippocampi rapidly removed and placed on ice-cold oxygenated (95% O_2/5% CO_2) artificial cerebrospinal fluid (aCSF) of the following composition (mM): NaCl 124, KCL 3.33, KH_2PO_4 1.25, $MgSO_4$ 2, $CaCl_2$ 2, $NaHCO_3$ 25 and D-glucose 10. Slices (400 μm thick) were cut by a McIlwain tissue chopper and kept in oxygenated aCSF for at least 1 h at room temperature. A single slice was then placed on a nylon mesh, completely submerged in a small chamber and superfused with oxygenated aCSF (30–32°C) using a peristaltic pump at a constant flow rate of 2 ml min^{-1}. *In vitro* ischaemia-like conditions were induced by superfusing the slice for 2 min with aCSF without glucose and gassing with nitrogen (95% N_2/5% CO_2). At the end of the ischaemic period, the slice was again superfused with normal oxygenated aCSF. Each slice was exposed to two periods of ischaemia-like conditions, with a time interval of 45 min. Drugs were applied before the second ischaemic episode.

Extracellular recording of synaptic transmission in hippocampal slices

Test pulses (80 μs, 0.06 Hz) were delivered through a bipolar nichrome electrode positioned in the stratum radiatum. Evoked extracellular potentials were recorded with glass microelectrodes (2–10 MΩ) filled with 3 M NaCl, placed in the CA1 region of the stratum radiatum. Responses were amplified (Neurolog NL 104, Digitimer Ltd), digitized (sample rate, 33.33 kHz), and stored on floppy disks for later analysis using pCLAMP 6 software facilities (Axon Instruments Inc.). Stimulus-response curves were obtained by gradual increases in stimulus strength. The test stimulus pulse was then adjusted to produce a field e.p.s.p. whose slope was 40–50% of the maximum and was kept constant throughout the experiment. The field e.p.s.p. amplitude was routinely measured and expressed as the percentage of the average amplitude of the potentials measured during the 10 min preceding exposure of the hippocampal slice to *in vitro* ischaemia. In some experiments both the amplitude and initial slope of field e.p.s.ps were quantified, but since no appreciable differences between the effect of *in vitro* ischaemia on both parameters were observed, usually only amplitude measurement is shown in the figures.

Statistical analysis

All numerical data are expressed as the mean ± s.e.mean. Data were analysed for their statistical significance using the paired Student's *t*-test and analysis of variance (ANOVA) followed by Fisher *post hoc* test.

Drugs

Adenosine was purchased from Calbiochem (La Jolla, CA, U.S.A.); CGS 21680 (2-[p-(2-carboxyethyl)-phenethylamino]-5′-N-ethylcarboxamido adenosine) and DPCPX (1,3-dipropyl-8-cyclopentylxanthine) from Research Biochemicals International (Natik, MA, U.S.A.); ZM 241385 (4-(2-[7-amino-2-(2-furyl)-{1,2,4}-triazolo{2,3-a}{1,3,5}triazin-5-ylamino]ethyl)-phenol) from Tocris Cookson (Bristol, U.K.). SCH 58261 (7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine) was a generous gift of Schering-Plough Research Institute (Milan, Italy). Drugs were dissolved in 100% DMSO and then diluted in distilled water to obtain a concentration of DMSO $<0.05\%$ in the final aCSF solution. The solvent concentration was kept constant throughout the experiment by adding the same per cent of DMSO to the aCSF solution without drugs.

Results

Effect of in vitro *ischaemia on field e.p.s.ps in the CA1 region of hippocampal slices*

We had previously observed that the application of an *in vitro* ischaemic insult of 5 min duration to hippocampal slices resulted in a complete and reversible depression of field e.p.s.ps (Latini *et al*., 1998b; 1999). In this work, we have utilized a shorter period of *in vitro* ischaemia (2 min) to obtain a submaximal depression of field e.p.s.ps and to allow detection of drug effects in either the reduction or increase in depression.

The application of an *in vitro* ischaemic episode of 2 min resulted in a partial and reversible depression of synaptic potentials recorded in the CA1 region. The depression of synaptic potentials started about 45 s after the beginning of the *in vitro* ischaemic insult, reaching a maximal inhibition of 70%, 15–30 s after reperfusion with the oxygenated aCSF solution (Figure 1). The application of a second period of 2 min of *in vitro* ischaemia, 45 min after the end of the first period, resulted in an identical depression of field e.p.s.p. amplitude, with the same time course. No significant differences were found by comparing synaptic potentials at any time during the first and second *in vitro* ischaemic periods ($n=6$, $P>0.05$). For this reason the effects of all pharmacological treatments on synaptic depression were evaluated during the second period of *in vitro* ischaemia in comparison with the depression induced by the first ischaemic period.

Effect of A_1 adenosine receptor antagonist on synaptic depression induced by in vitro *ischaemia*

During the ischaemic episode the depression of field e.p.s.ps is mostly ascribed to A_1 receptor-mediated presynaptic

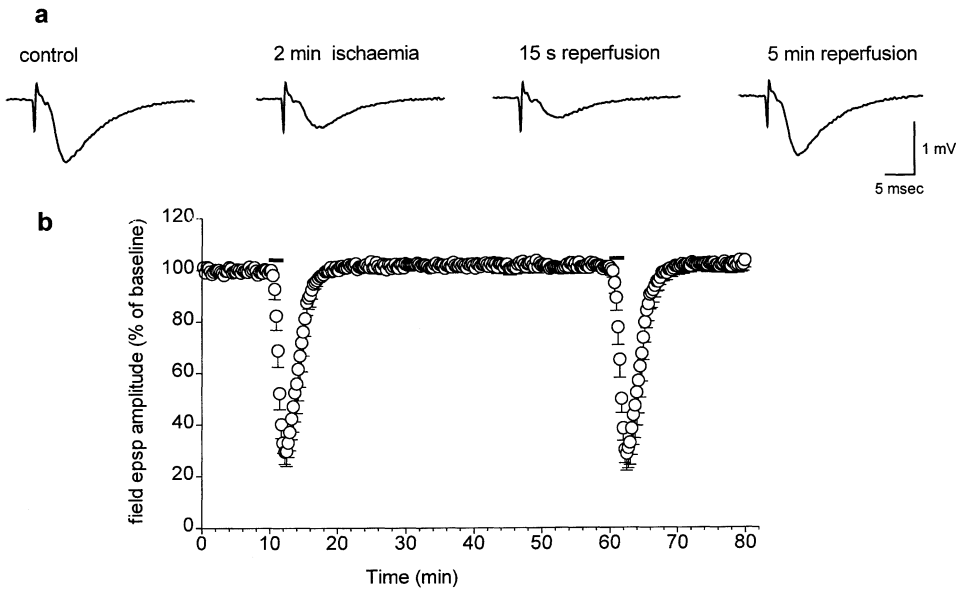

Figure 1 Modifications in the amplitude of synaptic potentials induced by 2 min of *in vitro* ischaemia. (a) Traces of field e.p.s.ps recorded during a typical experiment, under control conditions, at the end of 2 min of *in vitro* ischaemia and after 15 s and 5 min of reperfusion. (b) Time-course of field e.p.s.p. amplitude, expressed as per cent of baseline, before, during and after the application of two consecutive *in vitro* ischaemic insults of 2 min (indicated by bars on graph). Each value represents the mean ± s.e.mean of six experiments. Absolute values (means ± s.e.mean) of field e.p.s.p. amplitude in normoxic conditions (100%) were 1.06 ± 0.04 mV before the first period of *in vitro* ischaemia and 1.07 ± 0.05 mV before the second.

inhibition of glutamate release exerted by the large amounts of adenosine flowing out of cells. Therefore the ability of the A_1 receptor antagonist DPCPX to block the ischaemic depression of field e.p.s.ps was assessed in our experimental conditions.

Perfusion of slices for 20 min with 100 nM DPCPX, before application of the second ischaemic insult, brought about a 10 and 12% ($n=3$, $P<0.05$, Student t-test) increase in field e.p.s.p. amplitude and slope, respectively. This confirmed an effective antagonism of A_1 receptor-mediated inhibition of field e.p.s.ps caused by endogenous adenosine under normoxic conditions (Dunwiddie & Diao, 1994).

As shown in Figure 2, 100 nM DPCPX almost completely prevented the synaptic depression induced by 2 min of *in vitro* ischaemia. The effect of DPCPX was statistically significant at all recording times ($P<0.05$, paired Student's t-test) between 1 min after the beginning of the ischaemic insult up to 3.5 min of reperfusion.

A_{2A} adenosine receptor antagonists do not affect field e.p.s.ps under normoxic conditions and during in vitro *ischaemia*

We have previously observed that at the end of 2 min of *in vitro* ischaemia, the adenosine concentration at the receptor level increases about 25 fold, starting from a basal value of 180–240 nM under normoxic conditions, and reaching an estimated value of 5 μM after the ischaemic insult (Latini *et al*., 1999). Considering the affinity of each adenosine receptor subtype for the endogenous ligand (1–30 nM for A_1 and A_{2A} receptors and >1 μM for A_{2B} and A_3 receptors) (Fredholm *et al*., 1994), it is conceivable that under these conditions all adenosine receptor subtypes could be stimulated by endogenous adenosine flowing out of cells during the ischaemic episode.

The question therefore arises as to whether, during the ischaemic period, A_{2A} adenosine receptors are also stimulated and partially counteract the depressant effects exerted by A_1 receptor stimulation. To test this possibility we evaluated the effects of two selective A_{2A} receptor antagonists, ZM 241385 and SCH 58261, under normoxic and ischaemic conditions. It was expected that, if A_{2A} receptors were stimulated during the ischaemic insult, their antagonism would have unmasked a greater depression during ischaemia.

As shown in Table 1, ZM 241385 (100 nM) and SCH 58261 (50 nM) did not significantly modify either the excitatory neurotransmission under normoxic conditions or the maximal ischaemic depression of field e.p.s.p. amplitude observed during ischaemia.

Effects of prolonged application of adenosine on ischaemia-induced depression of field e.p.s.ps

In spite of the negative results obtained using A_{2A} selective antagonists, the possibility that A_{2A} receptor stimulation could modulate the depression caused by ischaemia could not be discarded. A slow-time course of excitatory responses induced by CGS 21680 and exogenous adenosine is observed in the hippocampus (Li & Henry, 1998) and our previous findings demonstrated that, using longer (5 min) ischaemic episodes, the second episode recovered more rapidly from depression than the first (Latini *et al*., 1999). These findings suggest that periods of *in vitro* ischaemia longer than 2 min may be necessary to activate A_{2A} receptors. Unfortunately a 5-min *in vitro* ischaemia produces a complete disappearance of field e.p.s.ps (Latini *et al*., 1999), likely due to supra-maximal stimulation of A_1 receptors. This makes it unlikely to detect any possible effect of A_{2A} receptor antagonists. We therefore devised a different approach to stimulate A_{2A} receptors. Thus hippocampal slices were exposed for 20 min to a concentration of adenosine (100 μM) which should be high enough to stimulate all adenosine receptor subtypes, even in the presence of adenosine re-uptake and degradation.

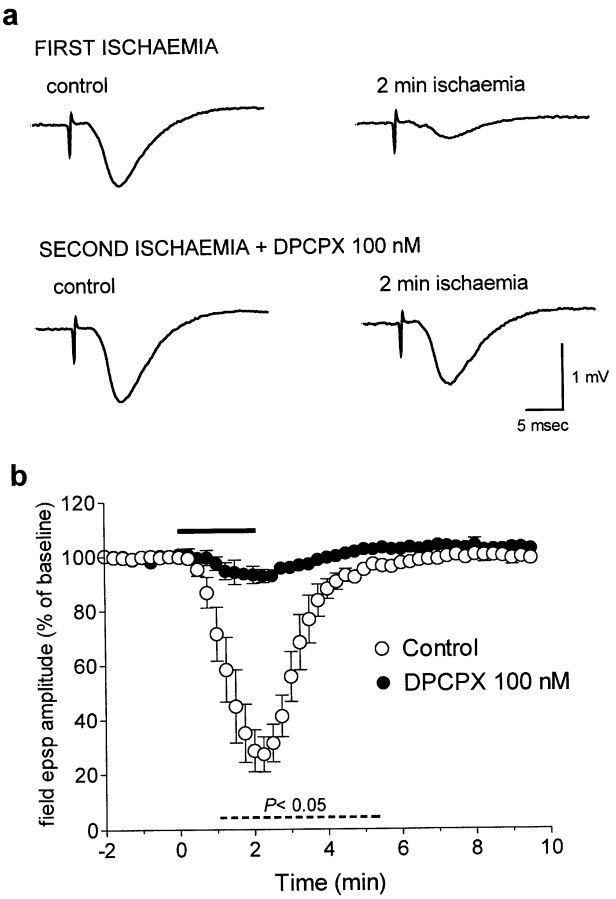

Figure 2 Effect of the A_1 receptor antagonist DPCPX, on the field e.p.s.p. depression induced by 2 min of *in vitro* ischaemia. (a) Traces of field e.p.s.ps recorded during a typical experiment, under control conditions and at the end of 2 min of *in vitro* ischaemia both in the absence and in the presence of DPCPX. (b) Time-course of field e.p.s.p. amplitude modifications during the first and second ischaemic episodes. Field e.p.s.p. amplitude is expressed as the percentage of averaged potentials recorded before the respective ischaemic periods. Each value represents the mean$\pm$s.e.mean of three experiments. Absolute values (means$\pm$s.e.mean) of field e.p.s.p. amplitude in normoxic conditions (100%) were 0.92 ± 0.06 mV before the first period of *in vitro* ischaemia and 1.01 ± 0.07 mV ($+10\%$, $P<0.05$) before the second, in the presence of DPCPX. The dotted line under the graph indicates the statistical significance of the effect of DPCPX, evaluated with the paired Students *t*-test at each time.

As shown in one typical experiment out of four (Figure 3a), the application of 100 μM adenosine, after recovery from the first ischaemic period, resulted in a complete depression of field e.p.s.ps, followed by a rapid recovery after adenosine washout. The application of the second ischaemic insult, immediately after the complete recovery of field e.p.s.p. amplitude, resulted in a significant reduction in field e.p.s.p. depression in comparison with the first ischaemic episode. The peak depression (i.e. during the first min of reperfusion) of the field e.p.s.p. for the first ischaemic period was $84.6\pm0.6\%$, and for the second ischaemic period $39.7\pm4.7\%$, with a significant reduction of $53\pm5.6\%$ ($P<0.0001$, $n=4$, ANOVA).

As shown in Figure 3b, the attenuation of field e.p.s.p. ischaemic depression induced by the prolonged application of adenosine is significantly smaller when adenosine is applied in the presence of the A_{2A} selective antagonist ZM 241385 (100 nM). Under these conditions the peak depression (i.e. during the first min of reperfusion) of the field e.p.s.ps during the second ischaemic period ($62.8\pm10.6\%$) was not statistically different from that of the first ischaemic period ($81.4\pm6.3\%$). On the other hand, the reduction in ischaemic depression ($24\pm7.2\%$) was significantly different ($P<0.005$, Students *t*-test) from that caused by adenosine in the absence of ZM 241385. These results strongly support the notion that a prolonged activation (>2 min) of A_{2A} receptors is needed to counteract the A_1 mediated effects of field e.p.s.ps.

Table 1 Effect of A_{2A} receptor antagonists on field e.p.s.p. amplitude under normoxic and *in vitro* ischaemic conditions

	n	*Normoxic conditions* (mV)	*Ischaemic conditions* (% depression)
Control	5	1.06 ± 0.07	63.3 ± 10.6
ZM 241385 (100 nM)		1.10 ± 0.09	55.3 ± 7.5
Control	5	1.06 ± 0.04	54.4 ± 7.6
SCH 58261 (50 nM)		1.11 ± 0.05	53.3 ± 3.9

Drugs were applied 20 min before the second ischaemic insult. Values are shown as means$\pm$s.e.mean. For normoxic conditions, reported values represent the field e.p.s.p. amplitude of averaged potentials during 2 min of registration. For the ischaemic conditions, reported values correspond to the maximal depression of field e.p.s.ps during the first 2 min of reperfusion, SCH 58261 checked at 100 nM concentration in two experiments did not affect the ischaemic field e.p.s.ps depression.

Effect of the A_{2A} adenosine receptor agonist CGS 21680 on synaptic depression induced by in vitro *ischaemia*

In order to confirm the involvement of A_{2A} adenosine receptors in the attenuation of field e.p.s.p. ischaemic depression observed after the prolonged application of adenosine, we have evaluated the effect of the selective A_{2A} receptor agonist CGS 21680. Consistent with the findings obtained with adenosine, the application of CGS 21680, 20 min before the second *in vitro* ischaemic insult, resulted in a significant reduction in field e.p.s.p. ischaemic depression. As shown in Figure 4a, 100 nM CGS 21680 did not affect field e.p.s.p. amplitude under normoxic conditions (absolute values of field e.p.s.p. amplitude were 1.28 ± 0.08 mV before and 1.27 ± 0.1 mV after the application of 100 nM CGS 21680, $n=5$) but partially reversed the depression of field e.p.s.p. recorded at the end of the ischaemic period. The effects of two concentrations of CGS 21680 (30 and 100 nM) and the time-course of ischaemic depression are shown in Figure 4b. Statistically significant differences between the ischaemic depression in the absence and in the presence of CGS 21680 were found during the second minute of *in vitro* ischaemia and during the first and second minute of reperfusion (Figure 4c). The maximal depression of field e.p.s.p. amplitude (i.e. during the first minute of reperfusion) was $29\pm5.4\%$ and $43\pm4.5\%$ smaller in the presence of 30 and 100 nM CGS 21680, respectively, than without drug application.

The effect of 30 nM CGS 21680 on the *in vitro* ischaemic-depression was antagonized by the selective A_{2A} receptor antagonist, ZM 241385, at the concentration of 100 nM (Figure 5a). As shown in Figure 5b, no significant effect of CGS 21680 was observed in the presence of ZM 241385, both during and after the *in vitro* ischaemic insult.

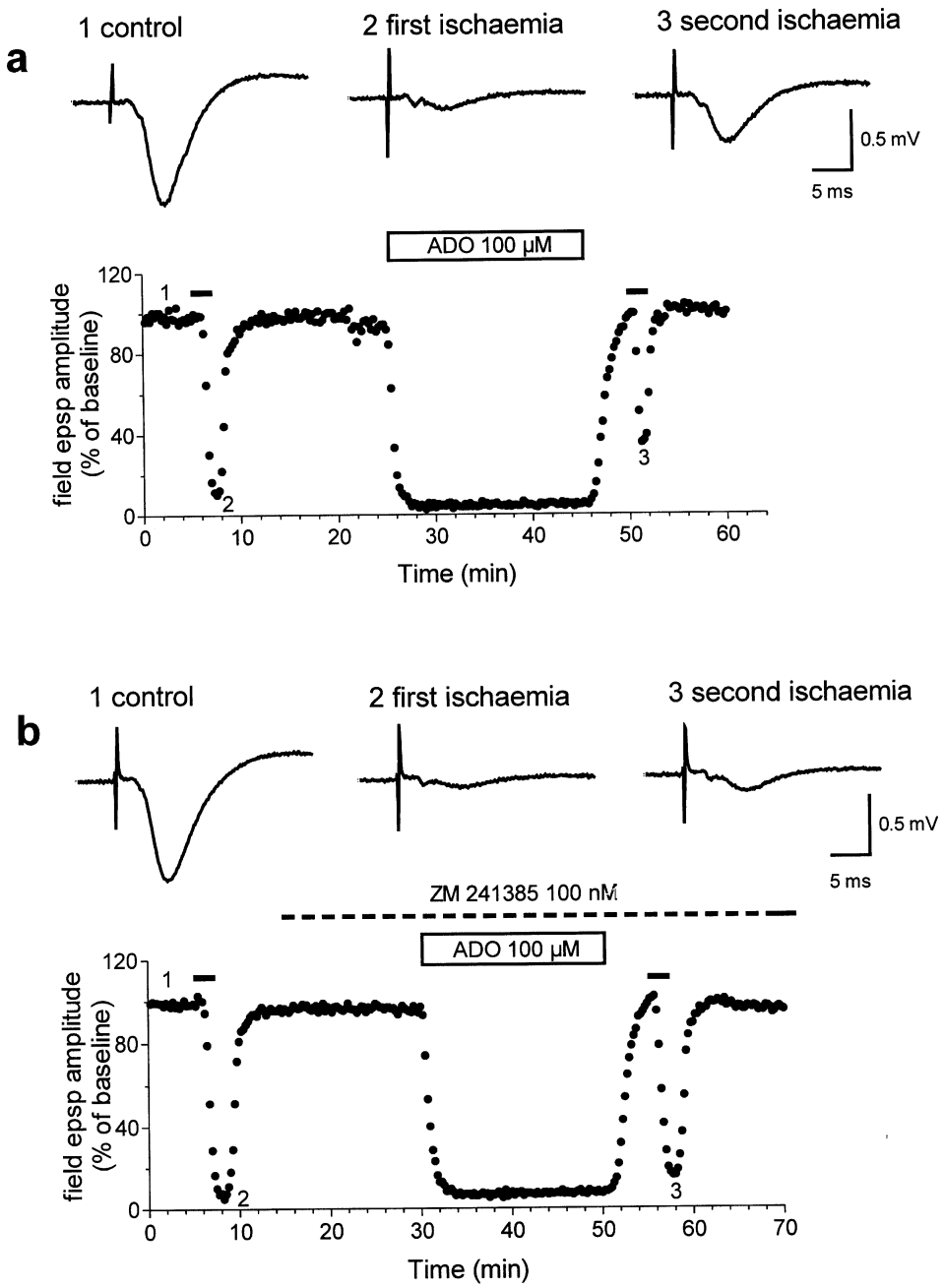

Figure 3 Effect of a prolonged application of adenosine on field e.p.s.p. depression induced by *in vitro* ischaemia. (a) Time-course of changes in field e.p.s.p. amplitude elicited by application of adenosine (100 μM) in one typical of four experiments. Traces show field e.p.s.ps recorded at the time indicated by numbers in the graph. Adenosine was applied 15 min after the recovery of field e.p.s.p. amplitude from the first ischaemic insult, and was maintained for 20 min. The second ischaemic episode was applied immediately after recovery from adenosine effect. (b) Time-course of changes in field e.p.s.p. amplitude elicited by application of adenosine (100 μM) in the presence of the A_{2A} adenosine receptor antagonist, ZM 241385 in one typical of four experiments. Traces show field e.p.s.ps recorded at the time indicated by numbers in the graph. ZM 241385 (100 nM) was applied 15 min before adenosine and was maintained during the second ischaemic episode and until the end of the experiment. (a) and (b) are from different slices.

On the other hand, the application of 1 nM ($n=4$, data not shown) and 50 nM ($n=5$, Figure 6) SCH 58261, did not antagonize the effect of 30 nM CGS 21680 during and after the *in vitro* ischaemic insult. As shown in Figure 6b, at peak depression of field e.p.s.p. amplitude (i.e. during the first min of reperfusion), there was a $34\pm7.6\%$ reduction ($P<0.05$, ANOVA) in synaptic depression in the presence of both SCH 58261 and CGS 21680, in comparison to control.

Discussion

In this study we show that the synaptic depression observed in the CA1 area under *in vitro* ischaemic conditions is reduced by prolonged stimulation of A_{2A} adenosine receptors.

In our experiments the application of a short *in vitro* ischaemic insult (2 min) caused a submaximal and repeatable depression of field e.p.s.ps amplitude. As previously shown in the hippocampus, the induction of *in vitro* ischaemic

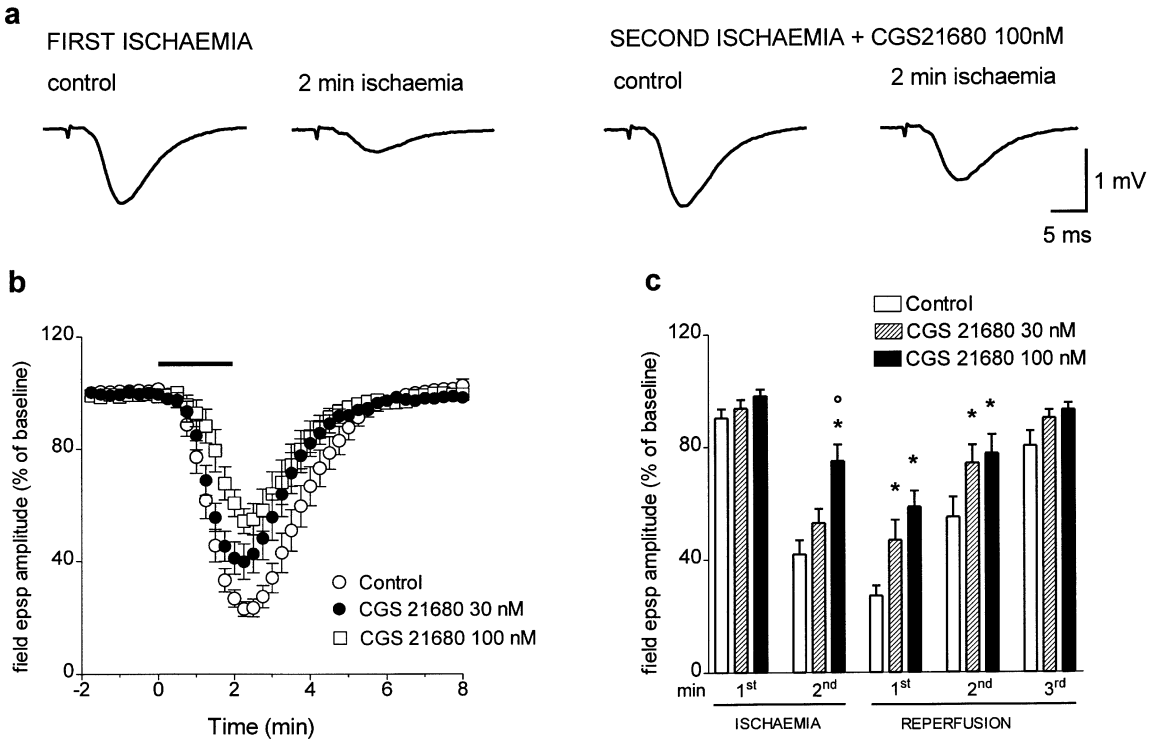

Figure 4 Effect of the selective A_{2A} receptor agonist, CGS 21680, on the field e.p.s.p. depression induced by 2 min of *in vitro* ischaemia. CGS 21680 was applied 20 min before the second ischaemic period and maintained until the end of the experiment. (a) Traces of field e.p.s.ps recorded during a typical experiment, under normoxic and ischaemic conditions, either in the absence or presence of 100 nM CGS 21680. (b) Time course of field e.p.s.p. amplitude modifications and effect of two concentrations of CGS 21680 (30 and 100 nM). Field e.p.s.p. amplitude is expressed as the percentage of averaged potentials recorded before the respective ischaemic periods. Since no significant differences were found between the first ischaemic depression in the group of experiments with 30 nM and in the group with 100 nM CGS 21680, the values from these groups are shown in the figure averaged and compared with those obtained during the second ischaemic depression in the presence of CGS 21680. Each value represents the mean$\pm$s.e.mean of 12 experiments for control, seven experiments for 30 nM CGS 21680 and five experiments for 100 nM CGS 21680. (c) Each bar represents the average amplitude of four consecutive field e.p.s.ps (1 min), recorded during the first and second minute of *in vitro* ischaemia and during the first, second and third minute of reperfusion (expressed as per cent of controls). Differences among data were analysed by ANOVA ($P<0.001$) followed by *post hoc* Fisher's test: $^{*}P<0.05$ vs control, $^{\circ}P<0.05$ vs 30 nM CGS 21680.

conditions is associated with an increase in the extracellular concentration of adenosine (Lloyd *et al*., 1993; Pedata *et al*., 1993; Latini *et al*., 1995; 1999) and with depression of synaptic responses (Fredholm *et al*., 1984; Fowler 1989; 1990; Gribkoff *et al*., 1990; Pedata *et al*., 1993). In the CA1 area the two phenomena are temporally correlated (Latini *et al*., 1998b). The adenosine-mediated depression of field e.p.s.ps is mainly attributed to a decrease in glutamate release by activation of presynaptic adenosine A_1 receptors (Corradetti *et al*., 1984; Latini *et al*., 1999). Our findings that the selective A_1 receptor antagonist DPCPX prevents the synaptic depression induced by *in vitro* ischaemia, confirms the preponderant role of A_1 receptors in inhibiting synaptic responses in the present experimental conditions.

In a previous work (Latini *et al*., 1999) we estimated that the concentration of adenosine reaching the receptor level at the second min of ischaemia was 5 μM, a concentration well above that needed to stimulate A_{2A} receptors (see Fredholm *et al*., 1994). This raised the possibility that, during the ischaemic episode A_{2A} receptors were also stimulated and acted by limiting the depression of field e.p.s.ps produced by A_1 receptor activation. In our experimental conditions the effects of the two brief (2 min) ischaemic episodes on field e.p.s.ps were submaximal and superimposable in amplitude and time-course. This indicated that the first episode did not produce lasting modifications in excitatory neurotransmission sensitivity to the action of adenosine released by the second episode. The application of the A_{2A} selective antagonist ZM 243985 between the two ischaemic episodes neither affected by itself the baseline neurotransmission nor increased the ischaemic synaptic depression. This demonstrates the absence of a detectable stimulation of A_{2A} receptors by endogenous adenosine in normoxic conditions and by that released during the 2 min ischaemic period.

On the other hand, during ischaemic insults *in vivo* the levels of adenosine might rise for longer than 2 min, and the more persistent resulting activation of A_{2A} receptors could lead to detectable effects on the outcome of the ischaemia as revealed by the hippocampal neuroprotection exerted by A_{2A} antagonists in cerebral damage induced by global ischaemia (see: Von Lubitz 1999).

In our conditions, a 5 min episode of ischaemia speeded up the recovery time-course from a subsequent similar ischaemic episode (Latini *et al*., 1999). Unfortunately, this 'pre-conditioning' effect was too small to be investigated pharmacologically, and periods of ischaemia longer than 5 min will result in the death of the preparation (Pedata *et al*., 1993). However, when the action of adenosine released during a longer ischaemic episode was mimicked by the application of exogenous adenosine, a significant reduction (53%) in field e.p.s.p. depression by a 2 min ischaemic episode became apparent. The fact that ZM 243985

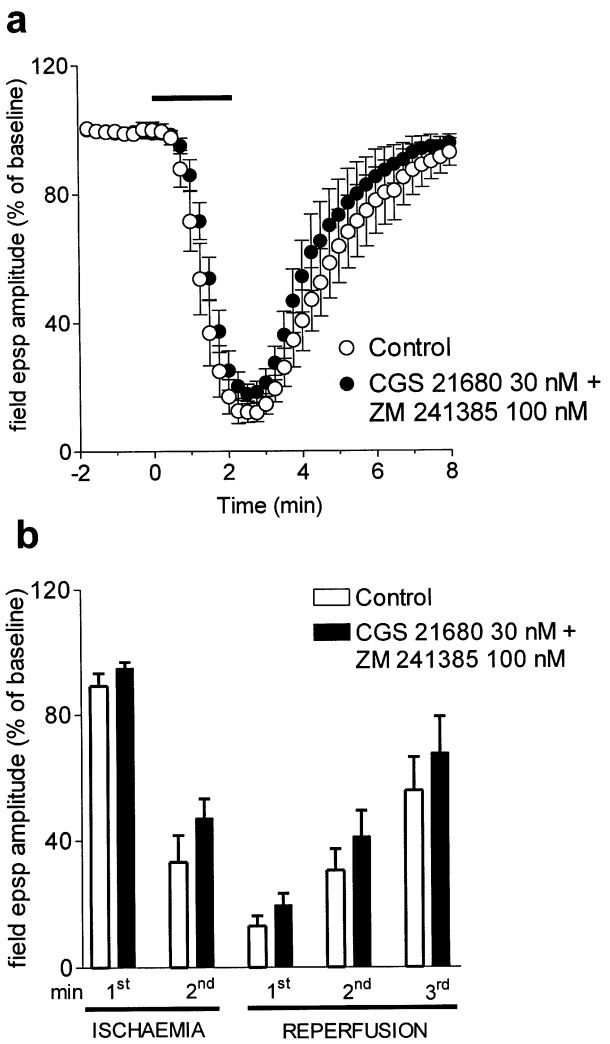

Figure 5 Effect of the A_{2A} adenosine receptor antagonist ZM 241385 on the CGS 21680-induced reduction of *in vitro* ischaemic depression. (a) 100 nM ZM 241385 was applied 20 min before 30 nM CGS 21680 and maintained during the second period of *in vitro* ischaemia and until the end of the experiment. Field e.p.s.p. amplitude is expressed as the percentage of averaged potentials recorded before the respective ischaemic periods. Each value represents the mean ± s.e.mean of five experiments. Averaged (mean-s ± s.e.mean) field e.p.s.p. amplitudes in normoxic conditions (100%) were: 1.13 ± 0.06 before the first period of ischaemia and 1.18 ± 0.06 before the second, in the presence of drugs. (b) Each bar represents the average amplitude of four consecutive field e.p.s.ps recorded during the first and second minute of *in vitro* ischaemia and during the first, second and third minute of reperfusion. No statistically significant differences among data were observed by the application of ANOVA followed by *post hoc* Fisher's test.

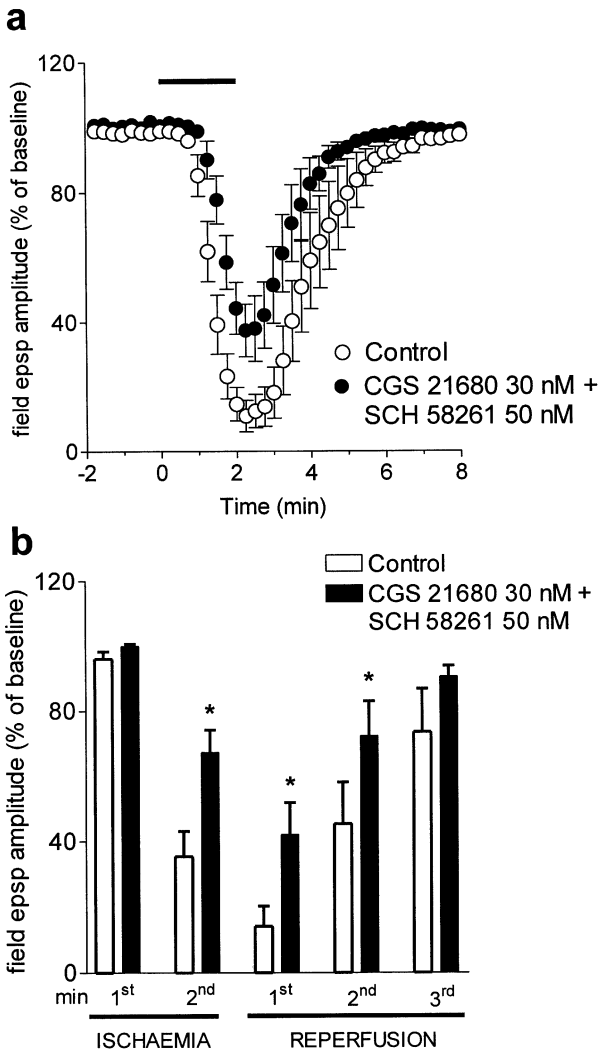

Figure 6 Effect of the A_{2A} adenosine receptor antagonist SCH 58261 on the CGS 21680-induced reduction in *in vitro* ischaemic depression. (a) 50 nM SCH 58261 was applied 20 min before 30 nM CGS 21680 and maintained during the second period of *in vitro* ischaemia and until the end of the experiment. Field e.p.s.p. amplitude is expressed as the percentage of averaged potentials recorded before the respective ischaemic periods. Each value represents the mean ± s.e.mean of five experiments. Averaged (mean-s ± s.e.mean) field e.p.s.p. amplitudes in normoxic conditions (100%) were: 0.98 ± 0.02 mV before the first period of *in vitro* ischaemia and 1.02 ± 0.03 before the second, in the presence of drugs. (b) Each bar represents the average amplitude of four consecutive field e.p.s.ps recorded during the first and second minute of *in vitro* ischaemia and during the first, second and third minute of reperfusion. Differences among data were analysed by ANOVA ($P < 0.001$) followed by *post hoc* Fisher's test: $^*P < 0.05$ vs control.

substantially prevented the effect of adenosine on the response to ischaemia, strongly suggests that the action of adenosine was through A_{2A} receptor stimulation and not to be ascribed to changes in adenosine re-uptake kinetics or in cell metabolism. This notion is confirmed by the similar action of the A_{2A} selective agonist CGS 21680, which is unlikely to modify adenosine re-uptake and cell metabolism and whose effect is fully prevented by the A_{2A} receptor antagonist ZM 243985. The residual attenuation of peak depression by adenosine (24%), observed when A_{2A} receptors are blocked, may be attributed to other mechanisms, including a desensitization of A_1 receptors elicited by activation of A_3 receptors (Dunwiddie *et al*., 1997).

The mechanism by which A_{2A} adenosine receptors counteract the A_1 receptor-mediated effects of adenosine are at present elusive.

CGS 21680 has been shown to increase the release of excitatory neurotransmitters like acetylcholine (Cunha *et al*., 1995) and excitatory amino acids (Popoli *et al*., 1995). A facilitation of glutamate release could thus counteract the A_1-mediated synaptic depression during *in vitro* ischaemia. This possibility is strengthened by the demonstration that CGS 21680 increases the cortical release of glutamate evoked by *in vivo* ischaemia (O'Regan *et al*., 1992).

The finding that CGS 21680 is able to decrease the synaptic depression produced by *in vitro* ischaemia is in agreement with

previous observations showing that, under normoxic conditions, inhibition of population spike amplitude induced by the selective A_1 receptor agonist, N^6-cyclopentyladenosine (CPA), is attenuated in the presence of CGS 21680 (Cunha *et al*., 1994; O'Kane & Stone, 1998). A reduced sensitivity of A_1 receptors to adenosine has therefore been proposed as a mechanism explaining the decrease in A_1 receptor-mediated responses also in the CA1 region (O'Kane & Stone, 1998).

The fact that a prolonged (>2 min) stimulation of A_{2A} receptors is required to produce detectable effects on excitatory neurotransmission suggests the need for a persistent intracellular signalling which eventually leads to a decrease in sensitivity of A_1 receptors to adenosine. In rat striatal synaptosomes, A_{2A} receptor activation by CGS 21680 reduces the affinity of the A_1 receptor for its agonists, *via* a cytoplasmic pathway involving the action of a protein kinase C (Dixon *et al*., 1997). The reasons why activation of A_{2A} receptors for less than 2 min is unable to develop the suggested A_{2A}/A_1 receptor interactions remains unclear and requires further investigation. Nevertheless, at least two possibilities can be envisaged to explain the need for long activation of adenosine receptors to develop the effect: (1) a persistent stimulation is required to trigger the intracellular response. This might be due to the relatively low density of hippocampal A_{2A} receptors (Dixon *et al*., 1996) and/or to slow production kinetics of ternary complexes with G-proteins (Kenakin, 1993; 1997). The result will be a slow accumulation of intracellular messenger(s) which might require reaching a 'threshold' level to start the machinery responsible for A_1 receptor desensitisation. (2) Once triggered, if the target of the intracellular signal is the activated conformation of A_1 receptors, the machinery might require sustained stimulation of A_1 receptors to be fully effective. It deserves mention also that A_3 receptor stimulation needs to be maximal and prolonged to produce a delayed desensitization of A_1 receptors (Dunwiddie *et al*., 1997).

In our experiments the effect of CGS 21680 in reducing ischaemic depression is completely prevented by ZM 241385, which is selective for A_{2A} vs A_1 (500–1000 fold) and A_{2A} vs A_{2B} (80 fold), but does not bind to A_3 receptors (Poucher *et al*., 1995). ZM 241385 has been shown to displace with similar potency both striatal (K_i = 0.35 nM) and hippocampal (K_i = 0.52 nM) [^{3}H]-CGS 21680 binding sites in the rat (Cunha *et al*., 1997) and to antagonize the effects of CGS 21680 on the depression of synaptic transmission in the hippocampus induced by N^6-cyclopentyladenosine (O'Kane & Stone, 1998). In contrast the effect of CGS 21680 was not antagonized by the selective A_{2A} receptor antagonist, SCH 58261 (K_i = 2.3 nM, in the rat striatum), which is 50 fold A_{2A}/A_1 selective and does not bind to A_{2B} and A_3 receptors (Zocchi *et al*., 1996). SCH 58261 has been shown to discriminate between the two different binding sites observed in the rat brain for [^{3}H]-CGS 21680 (James *et al*., 1992; Johansson *et al*., 1993; Cunha *et al*., 1996). Thus, SCH 58261 is able to displace the binding of [^{3}H]-CGS 21680 from striatal membranes, but is virtually ineffective in hippocampal and cortical membranes with an estimated K_i greater than 1 μM (Lindström *et al*., 1996). Our finding that ZM 241385, but not SCH 58261, is able to antagonize the CGS 21680 effect is in agreement with these previous studies and suggests that the CGS 21680 effect in the hippocampus is mainly mediated by receptors different from the A_{2A} receptor population predominant in the striatum.

One conclusion of our work is that stimulation of A_{2A} receptors for longer than 2 min is required to detectably modify the responsiveness of excitatory neurotransmission to ischaemia. Our results therefore suggest that, after prolonged stimulation by adenosine, the effect of A_{2A} receptor stimulation becomes detectable and net excitatory synaptic activity may be the result of decreased adenosine A_1-mediated inhibitory action and of A_{2A}-mediated stimulatory effects.

This mechanism may be of relevance in pathological conditions since, during *in vivo* ischaemic episodes, adenosine levels rise considerably and A_{2A} receptors attenuate the inhibitory and neuroprotective effect elicited by A_1 receptor stimulation. In accordance it has been shown that A_{2A} receptor antagonists administered systemically (Gao & Phillis, 1994; Phillis, 1995; Von Lubitz *et al*., 1995; Bona *et al*., 1997; Jones *et al*., 1998b; Monopoli *et al*., 1998) or injected locally in the hippocampus (Jones *et al*., 1998a) show neuroprotective effects in *in vivo* models of brain ischaemia or kainate-induced excitotoxicity. Although interpretation of our *in vitro* experiments should be conservatively limited to the demonstration of an increase in excitatory neurotransmission by A_{2A} receptors, it is conceivable that blockade of this effect concurs with the neuroprotective action of A_{2A} antagonists. It is worth mentioning that the protective effects of A_{2A} receptor blockade observed *in vivo* might comprise additional mechanisms unrelated to glutamate release and/or depression of synaptic activity (see Ongini & Schubert, 1998).

In conclusion, our results demonstrate that, in the CA1 area of the hippocampus, stimulation of A_{2A} receptors attenuates the depression of excitatory neurotransmission induced by adenosine through A_1 receptor activation during *in vitro* ischaemia. This finding supports the notion that blockade of the negative interaction between A_{2A} and A_1 adenosine receptors may represent a possible mechanism of protection against ischaemic neuronal damage.

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