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Extracellular adenosine concentrations during *in vitro* ischaemia in rat hippocampal slices

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

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

1 The application of an ischaemic insult in hippocampal slices results in the depression of synaptic transmission, mainly attributed to the activation of A_1 adenosine receptors by adenosine released in the extracellular space.

2 To estimate the concentration of endogenous adenosine acting at the receptor level during an ischaemic episode, we recorded field e.p.s.ps (fe.p.s.ps) from hippocampal slices, and evaluated the ability of the selective A_1 receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), to reverse the fe.p.s.p. depression induced by *in vitro* ischaemia. A relationship between the IC_{50} of an antagonist and the endogenous concentration of a neurotransmitter has been used for pharmacological analysis.

3 The complete and reversible depression of fe.p.s.p. in the CA1 region induced by 5 min ischaemia was decreased in the presence of DPCPX (50–500 nM). 8-Phenyltheophylline (10 μM) abolished the depression of fe.p.s.ps during the ischaemic period, while a small (peak effect $12 \pm 4\%$) decrease in fe.p.s.ps was observed during the initial phase of reperfusion.

4 In the time-interval of maximal depression of fe.p.s.ps., IC_{50} and adenosine concentration changed as function of time with a good degree of correlation. The maximal value of adenosine concentration was 30 μM.

5 Our data provide an estimation of the adenosine concentration reached at the receptor level during an ischaemic episode, with a higher time discrimination (15 s) than that achieved with any biochemical approach. This estimation may be useful in order to establish appropriate concentrations of purinergic compounds to be tested for their pharmacological effects during an ischaemic episode.

Keywords: Adenosine; DPCPX; 8-PT; adenosine deaminase; synaptic responses; A_1 receptors; hippocampal slices; *in vitro* ischaemia; anoxia

Abbreviations: aCSF, artificial cerebral spinal fluid; Ado, adenosine; ADA, adenosine deaminase; CNS, Central Nervous System; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; DPY, dipyridamole; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine hydrochloride; fe.p.s.p., field excitatory post synaptic potential; NBTI, S-(4-nitrobenzyl)-6-thioinosine; 8-PT, 8-phenyltheophylline

Introduction

In the Central Nervous System (CNS) adenosine is an important neuromodulator which exerts an inhibitory tonus on synaptic transmission, principally mediated by an inhibition of neurotransmitter release and by a reduction of postsynaptic excitability (Corradetti *et al*., 1984a; Dunwiddie, 1985; Proctor & Dunwiddie, 1987). This well established action of adenosine on neurotransmission is classically associated with the activation of the first (A_1) of four adenosine receptor types which have been cloned and identified in the CNS: A_1, A_{2A}, A_{2B}, A_3 (Fredholm *et al*., 1994). In the brain region such as the hippocampus, under physiological conditions, adenosine A_1 receptors are tonically exposed to extracellular endogenous adenosine, as demonstrated by excitatory effects of adenosine receptor antagonists on electrophysiological responsiveness (Corradetti *et al*., 1984b; Dunwiddie & Hoffer, 1980; Schubert, 1988; Wu & Saggau, 1994). On the other hand, an excitatory action of A_{2A} adenosine receptors on synaptic responses has been demonstrated in the hippocampus by use of the selective agonist CGS 21680 (Cunha *et al*., 1994; Sebastião & Ribeiro, 1992), while A_3 adenosine receptors may be responsible for a desensitization of A_1 receptor-mediated responses (Dunwiddie *et al*., 1997). No information has been provided until now on the role of A_{2B} adenosine receptors on synaptic transmission.

Brain extracellular concentration of adenosine can be increased by several stimuli, including electrical stimulation, hypoxia and ischaemia (Pedata *et al*., 1991; 1993; Phillis *et al*., 1987). It appears that in the hippocampus the application of ischaemic or hypoxic insults is associated with depressed synaptic transmission, mainly evoked by the activation of A_1 adenosine receptors by endogenous adenosine released in the extracellular space. Non-selective antagonists of adenosine receptors, like 8-phenyltheophilline (8-PT), have been found to delay or to prevent the hypoxia- or ischaemia-induced depression of synaptic transmission in the hippocampus (Fowler, 1989; 1990; Pedata *et al*., 1993), and the selective A_1 receptor antagonist, DPCPX, has been found to prevent the inhibition of synaptic responses evoked by hypoxia (Canhao *et al*., 1994; Lucchi *et al*., 1996). While an important neuroprotective role of A_1 adenosine receptor activation against ischaemic damage has been clearly demonstrated (Domenici *et al*., 1996; Heron *et al*., 1994), very few reports describe the role of A_2 and A_3 adenosine receptor during cerebral ischaemia. Since A_1, A_2 and A_3 adenosine receptors may be activated by different ranges of concentrations of endogenous adenosine (Fredholm *et al*., 1994), knowledge of adenosine concentrations reached in the extracellular space

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

during an ischaemic event, is critical to evaluate the involvement of different adenosine receptors.

In a recent work, Barlow (1995) explicitly set out the relationship between the IC_{50} of an antagonist and the endogenous concentration of a neurotransmitter and suggested that by using an antagonist of known K_B it is possible to estimate the concentration of an agonist of known K_A and $[A_{50}]$ acting at receptor level for producing the recorded pharmacological effect. By using this relationship, in the present study we estimated the concentrations of adenosine acting at the receptor level at various times during an ischaemia-like episode *in vitro*. To monitor the effect of endogenously released adenosine during ischaemia we recorded fe.p.s.ps and we evaluated the ability of the selective A_1 receptor antagonist, DPCPX, to reverse fe.p.s.p. depression induced by an ischaemic episode on hippocampal slices.

Methods

Preparation of hippocampal slices

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 and their hippocampi rapidly removed and placed on ice-cold oxygenated (95% O_2-5% CO_2) artificial cerebral spinal 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 (0.5 ml) and superfused through a peristaltic pump with oxygenated aCSF (30–32°C) at a constant flow rate of 2 ml min^{-1}. The treated solutions reached the preparation in 90 s and this delay was taken into account in our calculations. *In vitro* ischaemia-like conditions were applied by superfusing the slice for 5 min with aCSF without glucose and gassed with nitrogen (95% N_2-5% CO_2) (Pedata *et al*., 1993). 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 50 min.

Extracellular recording

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 excitatory post synaptic potential (fe.p.s.p.) whose slope was 40–50% of the maximum and was kept constant throughout the experiment. The amplitude of fe.p.s.p. was routinely measured and expressed as the percentage of the average amplitude of the potentials measured during 10 min that preceded exposure of the hippocampal slices to ischaemic conditions. In some experiments both the amplitude and the initial slope of fe.p.s.p. were quantified, but since no appreciable differences were observed in the effect of drugs and of *in vitro* ischaemia, usually only the measure of the amplitude was expressed in figures.

Pharmacological approaches

The first step of our study was to estimate the concentration of endogenous adenosine in our preparations using an approach similar to that described by Dunwiddie & Diao (1994). This allowed us to compare our conditions with those of previously published studies, and permitted us to implement our calculations with the pharmacological parameters obtained in these works. In parallel we tested whether DPCPX was able to antagonize a high concentration of adenosine (20 μM) under conditions of substantial block of adenosine uptake and deamination. This permitted the choice of antagonist concentrations to be used to block the effects of adenosine released by the ischaemic episode. Finally, to estimate the concentrations of endogenous adenosine, data were analysed as suggested by Barlow (1995).

Collection of data and pharmacological analysis

To generate data for DPCPX concentration-response curves, the amplitude of fe.p.s.ps evoked by test stimuli was measured while slices were superfused with increasing concentrations of the antagonist using a cumulative protocol (unless otherwise stated). The per cent changes in amplitude of recorded potential were fitted to a hyperbolic function (equation 1):

$$E = E_{max}/[1 + ([B_{50}]/[B])^n] \quad (1)$$

where E is the per cent change in fe.p.s.p. amplitude produced by the antagonist at the concentration [B], E_{max} is the maximum change in response, $[B_{50}]$ is the concentration of the antagonist producing a half-maximum effect and n is the slope index. Non-linear regression fitting was carried out with Prism 2.0 (GraphPad) software facilities.

The maximum response achievable in the preparation was used to calculate the maximum fractional increase in the response. To estimate the concentration of endogenous adenosine acting on A_1 receptors under control conditions, this value was introduced in equation 2 (Dunwiddie & Diao, 1994):

$$[A_{end}] = (FI)^{1/H} * [A_{50}] \quad (2)$$

where $[A_{end}]$ is the concentration of endogenous adenosine, FI is the fractional increase in the response produced by an antagonist in the presence of endogenous adenosine (e.g.: if DPCPX increases the control response by 16% FI = 0.16), and H is the Hill slope of the concentration-response curve of the agonist. In our calculations we introduced a Hill slope of 1.52 as obtained in the work by Dunwiddie & Diao (1994).

The estimation of adenosine concentration during the ischaemic episode was based on the relationship among the degree of agonist stimulation, the concentration of an antagonist producing 50% inhibition of agonist stimulation, and the dose ratio as defined by the Gaddum-Schild equation. These relationships have been explicitly set out by Barlow (1995). In brief, the relationship between response, E, and the concentration of the agonist [A] is described by equation 3:

$$E = E_{max}[[A]^n/([A]^n + [A_{50}]^n)] \quad (3)$$

where E_{max} is the maximum response, $[A_{50}]$ is the agonist concentration producing a half-maximum response, and n is the slope index (Hill coefficient). In the presence of an antagonist producing a dose ratio of r, the response is described by equation 4:

$$E' = E_{max}\{([A]/r)^n/[([A]/r)^n + [A_{50}]^n]\} \quad (4)$$

which, resolved for the percentage of reduction (Z) defined by $[(E-E')/E]*100$, can be reduced to equation 5:

$$Z = 100(r^n - 1)/[r^n + ([A]/[A_{50}])^n] \quad (5)$$

If the antagonist is competitive, the Gaddum-Schild equation defines the dose ratio as (equation 6):

$$r = 1 + ([B]/K_B) \quad (6)$$

and allows the relation between percentage of inhibition, Z, and the antagonist concentration expressed as a fraction of the dissociation constant (K_B), that is $[B]/K_B$ to be expressed by using equation 7:

$$Z = 100([B]/K_B)^n/[([B]/K_B)^n + ([A]/[A_{50}])^n] \quad (7)$$

Therefore when K_B of the antagonist and $[A_{50}]$ are known, the equation can be resolved and the concentration of the agonist [A] calculated.

The assumptions and limitations in application of this equation are fully developed in Barlow (1995). In the context of our approach the use of equation 7 requires that the value of $[B]/K_B$ is >1 which justifies the use of the logistic expression. This can be assumed when the IC_{50} is $>>K_B$. In our experiments the IC_{50} of the competitive antagonist DPCPX can be calculated and the assumption verified.

We calculated the IC_{50} of DPCPX using the logistic equation 1 adapted for inhibition curves.

Drugs

Adenosine, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 8-phenyltheophylline (8-PT), dipyridamole (DPY), *erythro*-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA) and S-(4-nitrobenzyl)-6-thioinosine (NBTI) were purchased from Research Biochemicals International (Natick, MA, U.S.A.). Adenosine deaminase (ADA; 220 U mg^{-1}) was from Boehringer Mannheim (Monza, Italy). DPCPX and NBTI were dissolved in DMSO and stock solutions were made to obtain concentrations of DMSO of 0.05 and 0.01% in aCSF,

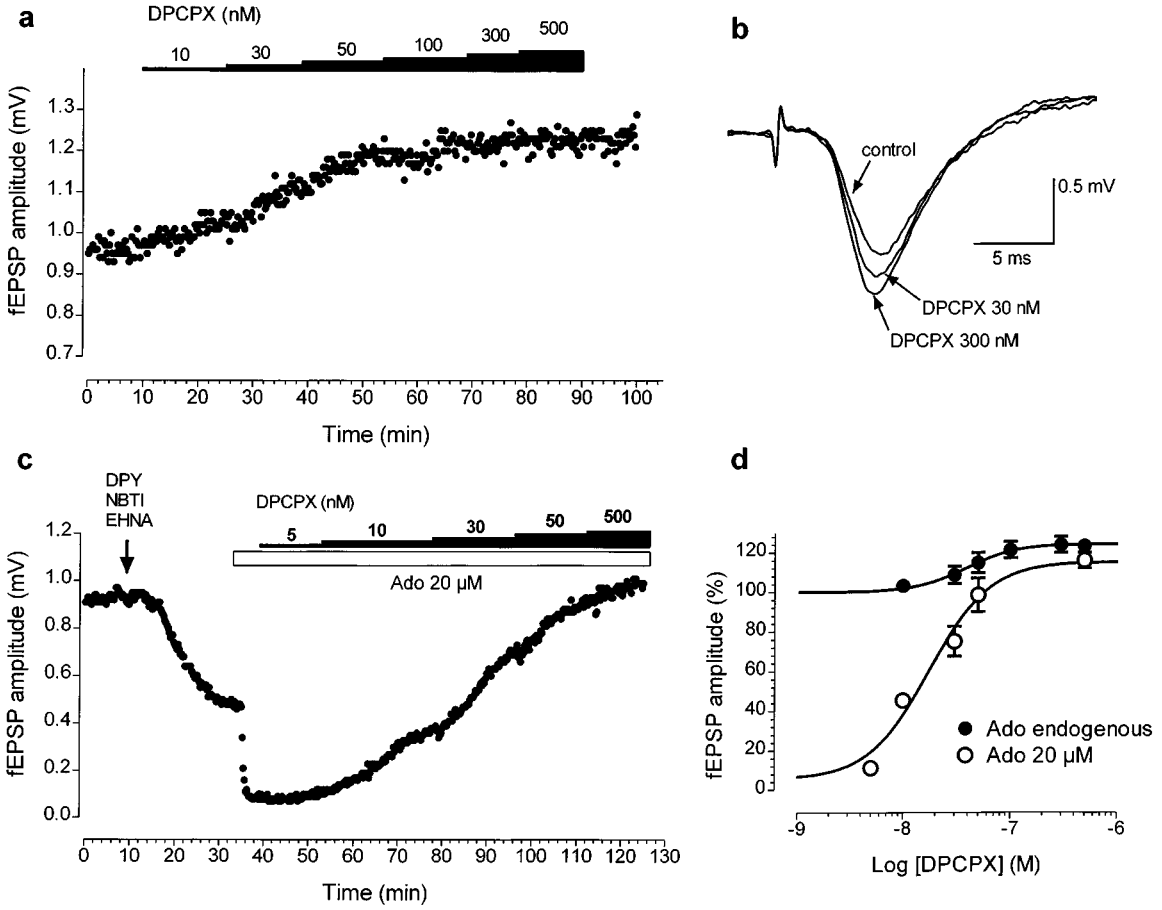

Figure 1 Effects of DPCPX on fe.p.s.ps in normoxic slices and antagonism of adenosine-induced depression of fe.p.s.ps. (a) Time course of the changes in fe.p.s.p. amplitude elicited by application of DPCPX (10–500 nM) in one typical of five experiments. Increasing concentrations of the antagonist were applied with a cumulative protocol at times indicated by the staircase bar. (b) fe.p.s.ps recorded from the same experiment using the protocol shown in (a). Traces illustrate potentials evoked by stimulation of the stratum radiatum and recorded from the dendrite region of CA1 pyramidal cells. (c) DPCPX antagonized the effect of adenosine (Ado, 20 μM; open bar) applied in the presence (throughout the experiment starting from arrow) of uptake and deaminase inhibitors (DPY: 5 μM; NBTI: 100 nM; EHNA: 5 μM). Stepwise application of increasing concentrations of DPCPX (5–500 nM) is indicated by the staircase bar. Data shown are from one typical of four experiments. (d) Concentration-response curves for DPCPX were fitted to experimental points obtained from the protocols illustrated in (a) and (c), using nonlinear regression (see Methods). Symbols are means$\pm$s.e.mean of fe.p.s.p. amplitude values (expressed as per cent of predrug baseline) and show the effects of DPCPX on fe.p.s.ps recorded from normoxic slices in control conditions ($n=5$) or in the presence of adenosine and uptake/deaminase inhibitors ($n=4$).

respectively. Experiments carried out in parallel for an unrelated project showed that this concentration of DMSO did not affect the depression induced by the second ischaemic episode. Dipyridamole was dissolved in ethanol and stock solution were made to obtain a final concentration of ethanol of 0.05% in aCSF. Stock solutions of 8-PT (10 mM) were made up in alkalyne ethanol.

Statistics

All numerical data are expressed as the mean ± s.e.mean. Statistical significance was determined by paired Student's *t*-test or by analysis of variance followed by the Fisher *post hoc* test.

Results

The major task of the present work was to produce experimental data whose analysis could allow estimation of endogenous adenosine concentrations acting at the receptor level during an ischaemia-like episode *in vitro*. However, since we needed to introduce into our calculations some pharmacological parameters already available in the literature, we considered it necessary to evaluate whether our experimental conditions were similar enough to include previously published values in our analysis. In addition, we investigated whether the adenosine A_1 receptor antagonist was able to substantially antagonize high concentrations of adenosine within a range of concentrations which insured maintenance of A_1 receptor selectivity.

Effects of DPCPX on fe.p.s.ps in normoxic conditions

In a first series of experiments we tested the effects of DPCPX (10 – 500 nM) on the fe.p.s.p. evoked in normoxic conditions. DPCPX was allowed to equilibrate for at least 15 min, and a steady-state effect was considered to be reached when eight consecutive fe.p.s.ps (2 min) showed a reproducible, constant amplitude. As shown in Figure 1a and b, in one typical of five experiments, superfusion of slices with the antagonist elicited a concentration-dependent increase in fe.p.s.p. amplitude.

The maximum increase in fe.p.s.p. amplitude was 24.4 ± 0.9% ($P<0.05$, ANOVA) (Figure 1d), a value similar to those found by Dunwiddie & Diao (1994) (19%) and by Brundege & Dunwiddie (1996) (22%) using theophylline or 8-cyclopentyltheophylline as antagonists.

DPCPX also antagonized the effects of 20 μM adenosine applied in the presence of DPY (5 μM), NBTI (100 nM), and EHNA (5 μM), which substantially blocked the uptake and deamination of adenosine (Dunwiddie & Diao 1994). Figure 1c shows the time course of the changes in fe.p.s.p. amplitude in one typical experiment. The application of uptake and deaminase inhibitors significantly decreased fe.p.s.p. amplitude (−47.7 ± 9.2% vs control, $P<0.04$, Student *t*-test, $n=4$), and subsequent application of adenosine 20 μM produced a further, almost complete, inhibition of fe.p.s.ps

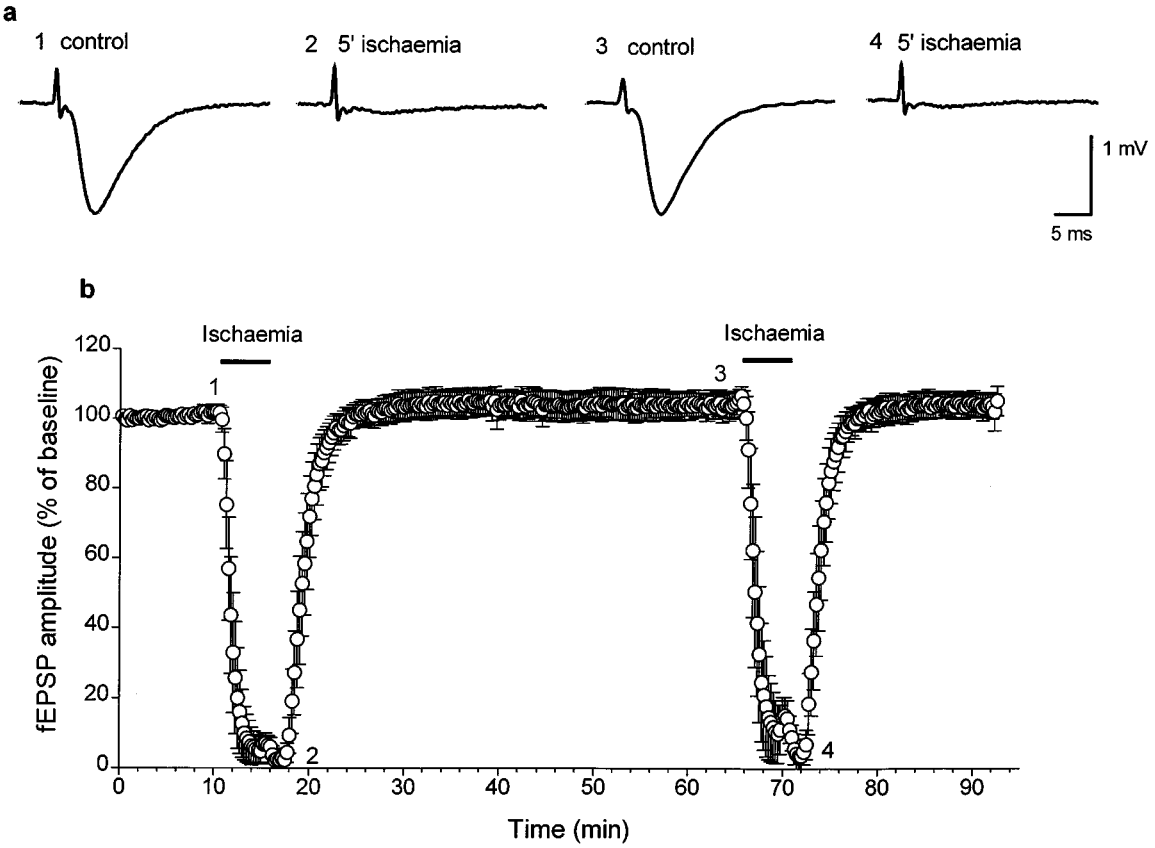

Figure 2 Decrease in the amplitude of synaptic potentials induced by 5 min *in vitro* ischaemia. (a) Traces are fe.p.s.ps recorded from the CA1 stratum radiatum in a typical experiment at the times indicated by corresponding numbers in (b). The second ischaemic period administered 50 min after the end of the first period elicited a comparable depression of synaptic potentials. (b) Time-course of fe.p.s.p. amplitude before, during and after the application of two consecutive ischaemic insults of 5 min duration (filled bars). Data are expressed as per cent of baseline values. Amplitudes of fe.p.s.p. in normoxic conditions (100%) were: 1.3 ± 0.1 mV before the first period of ischaemia and 1.4 ± 0.2 mV before the second (means ± s.e.mean; $n=5$).

($-77.2\pm3.1\%$ vs uptake and deaminase inhibitors effect, $P<0.04$; $-90\pm1.9\%$ vs control $P<0.005$, Student t-test, $n=4$). In the absence of uptake and deaminase inhibitors, 20 μM adenosine induced a $55.1\pm6.5\%$ depression of fe.p.s.p. amplitude (data not shown, $P<0.006$, Student t-test, $n=3$). Superfusion of DPCPX (5–500 nM), still in the presence of 20 μM adenosine, elicited a concentration-dependent reappearance of fe.p.s.ps, which in the presence of 500 nM were increased over control (pre-treatment) values by $15.5\pm0.1\%$ (Figure 1d; $P<0.05$; Student t-test $n=4$). Therefore, we showed that DPCPX was able to antagonize the effect of a high concentration of adenosine.

Effects of in vitro *ischaemia on fe.p.s.ps*

The application of ischaemia-like conditions for 5 min on hippocampal slices resulted in a progressive depression of synaptic potentials recorded in the CA1 region, with a characteristic time-course. As illustrated in Figure 2b, the depression of synaptic potentials started about 45 s after the beginning of the ischaemic period and reached complete inhibition of fe.p.s.ps after about 3 min. Immediately after starting reperfusion with the oxygenated aCSF solution, there was a transient reappearance of synaptic potentials, followed by complete inhibition of fe.p.s.p. amplitude for about 3 min. After this period, fe.p.s.p. progressively reappeared and reached a complete recovery of amplitude within 10 min from the beginning of reperfusion. The application of a second period of 5 min *in vitro* ischaemia, 50 min after the end of the first period, resulted in a similar depression of fe.p.s.p. amplitude, followed by a faster recovery of fe.p.s.ps in comparison with the first period, i.e. after 7–8 min of reperfusion. This difference was consistently reproducible and, though small, was statistically significant from the fifth min of reperfusion with normoxic aCSF ($P<0.05$, Student t-test) until complete recovery.

In each experiment a first ischaemic insult was applied to evaluate the responsiveness of slice preparation to *in vitro* ischaemia, and all the pharmacological evaluations were carried out on the second ischaemic episode.

Effects of DPCPX on fe.p.s.p. depression elicited by an ischaemic episode

The application of the selective A_1 receptor antagonist DPCPX (50–500 nM) resulted in a concentration-dependent increase in fe.p.s.p. amplitude and slope, and in a significant reduction of the depression of fe.p.s.ps produced by ischaemia. Figure 3 summarizes the effects of 100 nM DPCPX on baseline fe.p.s.p. after recovery from the first ischaemic period and on the depression induced by the second ischaemic treatment. As shown in Figure 3a, 5 min of ischaemia completely and reversibly (see Figure 3b) depressed the fe.p.s.p. Subsequent application of DPCPX increased the field synaptic response and partially prevented the effects of ischaemia. The time-course of fe.p.s.p. modifications produced by ischaemia-like

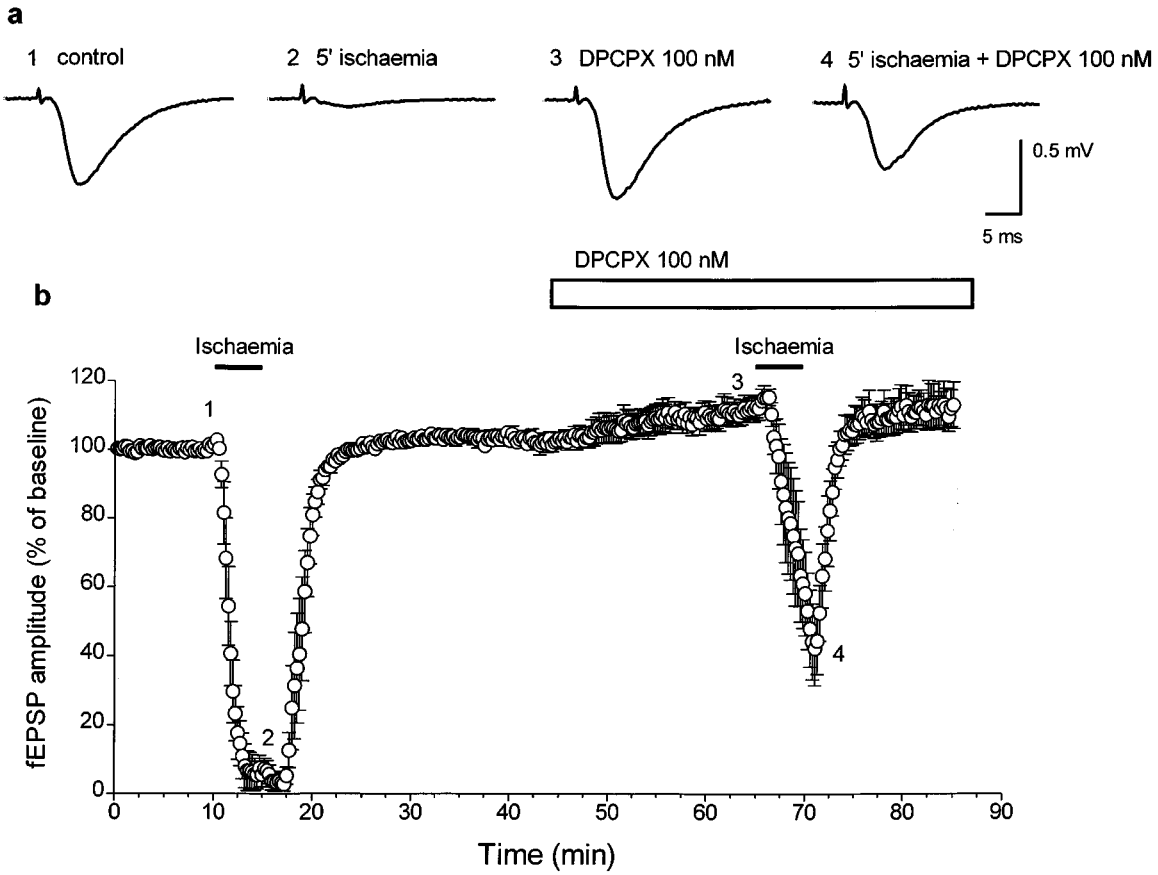

Figure 3 Effects of the selective A_1 receptor antagonist, DPCPX, on fe.p.s.p. depression induced by 5 min *in vitro* ischaemia. (a) Traces are fe.p.s.ps recorded from the CA1 stratum radiatum in a typical experiment at the times indicated by corresponding numbers in (b). DPCPX (100 nM) was applied 20 min before the second ischaemic period and maintained thereafter. (b) Time-course of fe.p.s.p. amplitude changes elicited by 5 min ischaemia (filled bars) in the absence and presence of DPCPX (100 nM; open bar). Data are expressed as per cent of baseline values. Amplitudes of fe.p.s.p. in normoxic conditions (100%) were: 1.1 ± 0.1 mV before the first period of ischaemia and 1.3 ± 0.1 mV before the second (means$\pm$s.e.mean; $n=5$).

treatment in the absence and presence of 100 nM DPCPX is depicted in Figure 3b. The concentration-dependent effects of DPCPX on ischaemia-induced depression of fe.p.s.ps is shown at an expanded time scale in Figure 4a. Statistical significance of the antagonism of the ischaemia-induced depression produced by DPCPX at various times of the ischaemic treatment is shown in Figure 4b. DPCPX antagonized in a concentration-dependent manner the ischaemia-induced depression of fe.p.s.p. In the interval between the second min of ischaemia and the fourth min of recovery the changes were statistically significant for all concentrations tested.

Evaluation of the contribution of adenosine receptors to the fe.p.s.p. depression induced by in vitro *ischaemia*

A separate set of experiments was addressed to assess if, in our experimental conditions, the depression of excitatory neurotransmission observed during the ischaemic episode was due to the action of adenosine. Due to the poor solubility of DPCPX, the concentration could not be raised above 500 nM. We therefore tested whether the presence of adenosine deaminase (ADA) during the second ischaemic episode, by decreasing the concentration of adenosine at the receptor level, allowed for a full block of A_1 receptors by 500 nM DPCPX. As shown in Figure 5a, the superfusion of ADA (4 U ml^{-1} + DPCPX 500 nM) elicited a reproducible increase in the amplitude of the fe.p.s.ps ($15 \pm 4\%$; $n=7$). When the second ischaemic episode was induced in the presence of DPCPX + ADA, the depression was completely antagonized for the first 3–4 min of ischaemia (Figure 5a). In the following 2–3 min (i.e. starting from the fifth min of ischaemia through the third min of reperfusion), the fe.p.s.ps were contaminated by dendritic and/or somatic spikes. This hampered precise measuring of fe.p.s.ps, but confirmed that the depressant effects of ischaemia on excitatory neurotransmission are mediated by adenosine. To further confirm this conclusion, we tested the effects of 8-phenyltheophylline (8-PT, 10 μM; $n=5$), a non-selective, potent adenosine antagonist on the fe.p.s.p. depression induced by ischaemia. As illustrated by Figure 5b, 8-PT increased the amplitude of the fe.p.s.ps ($22 \pm 3\%$) and delayed any decrease in fe.p.s.ps for at least 4 min. In three out of five preparations, a decrease was found only after the fifth min of ischaemia (i.e. during the first 2 min of reperfusion: Figure 5b and c). The peak of the depression was observed within the first min of reperfusion ($12 \pm 4\%$, $n=5$), and the values of residual depression observed in the interval between the fourth min of ischaemia and the second min of reperfusion were used for correcting our calculations of adenosine concentration (see below).

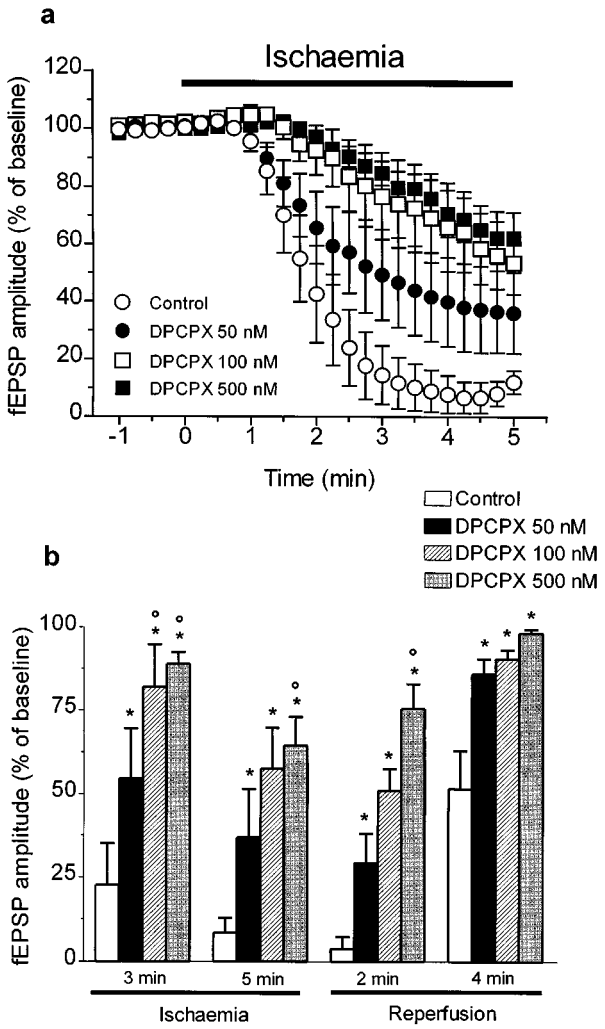

Figure 4 Effect of DPCPX on fe.p.s.p. amplitude during *in vitro* ischaemia. (a) The graph shows the effects of DPCPX (50, 100 and 500 nM) during the first 2.5 min of *in vitro* ischaemia. In each curve the fe.p.s.p. amplitude is expressed as the percentage of the average amplitude of the potentials measured during 2 min that preceded the second ischaemic period. Each point represents the mean ± s.e.mean of five experiments for control, 100 and 500 nM DPCPX, and six experiments for 50 nM DPCPX. (b) Statistical analysis of results illustrated in (a). Each bar represents the average amplitude of four consecutive fe.p.s.ps recorded during the third and the fifth min of ischaemia and the second and the fourth min of reperfusion, in control conditions or in the presence of several concentrations of DPCPX: 50, 100 and 500 nM. Differences between data were analysed by two-way analysis of variance ($P<0.001$) followed by *post hoc* Fisher's test: $^*P<0.05$ vs control, $^\circ P<0.05$ vs DPCPX 50 nM.

Pharmacological analysis and estimation of adenosine concentrations in normoxic conditions

To estimate the concentration of adenosine under control normoxic conditions we calculated the maximum fractional increase in fe.p.s.p. amplitude which was estimated by the effects of the A_1 receptor antagonist DPCPX. As shown in Figure 1d the changes in fe.p.s.p. amplitude observed in the presence of DPCPX in normoxic aCSF were fitted using the logistic equation (equation 1) and led to estimation of a maximum fractional increase of about 25%. By applying equation 2, the calculated concentration of endogenous adenosine in control conditions was 240 nM; 95% C.L.: 225–255 nM ($n=5$). This value was in agreement with those (120–200 nM) found by Dunwiddie & Diao (1994) and that calculated from the experiments devoted to studying the effects of DPCPX during ischaemia (180 nM; 95% C.L.: 175–185 nM; see e.g. Figure 3b).

Pharmacological analysis and estimation of adenosine concentrations in ischaemic conditions

The depression of fe.p.s.ps which developed during *in vitro* ischaemia and reperfusion is temporally coincident with the increase in adenosine flowing out of the cells (Latini *et al.*, 1998a). During the first minutes of ischaemia the changes in fe.p.s.p. amplitude produced by the ischaemic episode, and their modification by DPCPX, were too small to allow calculations of adenosine extracellular concentrations at any experimental times sampled by fe.p.s.p. recording. Therefore we chose the time interval between the last minutes of ischaemia and the beginning of reperfusion (from 3.25 min of ischaemia

to 2.50 min of reperfusion), when maximal depression of fe.p.s.p. is reached and when the effects of DPCPX on the ischaemia-induced depression of fe.p.s.ps were measurable and permitted accurate calculations of adenosine concentration.

Since it is known that the IC_{50} of an antagonist depends on the concentration of the agonist, it was conceivable that the IC_{50} of DPCPX was changing as a function of time, according to the concentration of adenosine present at each time. We therefore tested whether our data could generate estimations of IC_{50} which were sensitive to the presumed changes in adenosine concentration which developed over time.

The depression of fe.p.s.ps (expressed as per cent of baseline values) in the absence and presence of DPCPX was fitted to a logistic equation and led to an IC_{50} estimation for DPCPX (e.g. Figure 6a) which changed over time with a good degree of correlation (Figure 7). The IC_{50} of DPCPX varied according to the initial degree of synaptic depression and the values were greater at times when the fe.p.s.ps were abolished in control ischaemia. For clarity of illustration only the curves obtained every 30 s in the 1–4 min interval of reperfusion are shown in Figure 6a. The slopes of the curves are constrained to the average value (0.68 ± 0.05, $n=7$) obtained from fitting the same data with a variable slope. The curves obtained in the time interval 1.5–5 min of ischaemia are almost symmetrical and gave similar IC_{50} values (range: 53–353 nM; see also Figure 7). These results indicated that our protocol and measurements were sensitive enough to accurately follow the changes in the IC_{50} of DPCPX. In addition, at any time considered the IC_{50} of DPCPX was much higher than its K_B (0.45–3.6 nM, see below). This allowed the application of the equation explicitly set out by Barlow (1995).

We therefore fitted, by using equation 7, the values of fe.p.s.p. depression induced by ischaemia in the control and in the presence of DPCPX. This equation permits estimation of the concentration of an agonist (whose A_{50} is known) from the IC_{50} of an antagonist of known K_B. For our calculations we introduced the following parameters: A_{50} of adenosine 610 nM, K_B of DPCPX 1.5 nM (-8.82). These values were taken from Dunwiddie & Diao (1994) and from Zocchi *et al.* (1996), respectively (see also Methods). Figure 6b illustrates examples of the curves obtained with this procedure for the same times shown in Figure 6a. The adenosine concentrations estimated by these calculations in the interval between the fifth min of ischaemia (peak effect) and the third min of recovery ranged between 30 and 7.5 μM and changed as a function of time (Figure 7). Similar results were obtained by introducing into the equations the values of K_B for DPCPX measured in functional experiments in hippocampal slices (3.3 nM: -8.48 from Alzheimer *et al.*, 1991; 0.45 nM: -9.35 from Sebastião *et al.*, 1990). Using these alternative parameters, the ranges of adenosine concentrations in the time interval shown in Figure 7 were 20–5 and 45–10 μM respectively.

A residual depression of the fe.p.s.ps was still seen in the presence of 8-PT and although the concentration (10 μM) of the antagonist did not insure full occupancy of the A_1 receptors, the participation of other neurotransmitters or mechanisms in the transient depression observed could not be discarded (see Discussion). We corrected the calculations

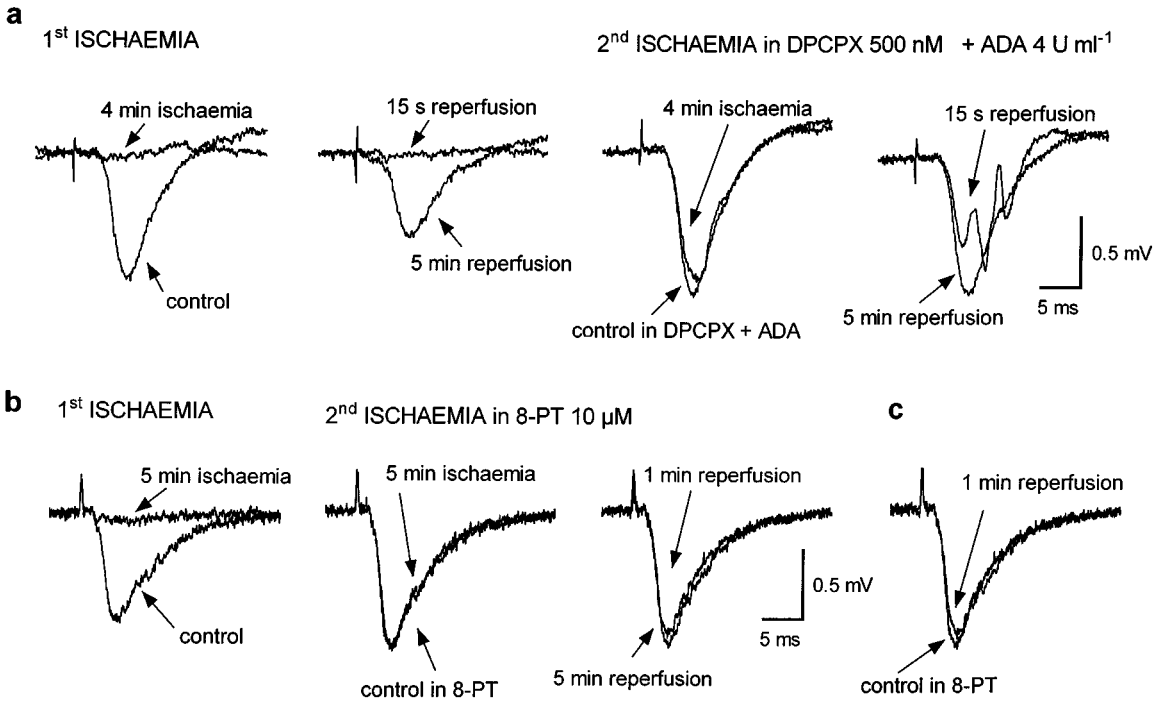

Figure 5 Effects of DPCPX combined with adenosine deaminase (ADA) and the effects of 8-PT alone on fe.p.s.p. depression induced by 5 min *in vitro* ischaemia. (a) fe.p.s.ps recorded from a typical experiment ($n=7$) during and after the first period of *in vitro* ischaemia, in the absence of drugs and during and after the second period of ischaemia in the presence of 500 nM DPCPX + ADA (4 U ml^{-1}). DPCPX and DPCPX + ADA were applied 25 and 15 min before the second ischaemic period respectively, and maintained thereafter. Note the contamination of the fe.p.s.p. by dendritic and/or somatic spikes during reperfusion, and the complete recovery after 5 min. (b) fe.p.s.ps recorded from a typical experiment ($n=5$) during the first period of *in vitro* ischaemia and during and after the second period of ischaemia in the presence of 10 μM 8-PT. 8-PT was applied 20 min before the second ischaemic period and maintained thereafter. (c) fe.p.s.p. recorded at the point of maximal depression in the presence of 8-PT (after 1 min of reperfusion) and fe.p.s.p. recorded under control conditions in the presence of 8-PT are superimposed to show the maximal effect of ischaemia in this experiment. Note the increase in the amplitude of the fe.p.s.p. during 8-PT application, and the absence of contamination by dendritic and/or somatic spikes.

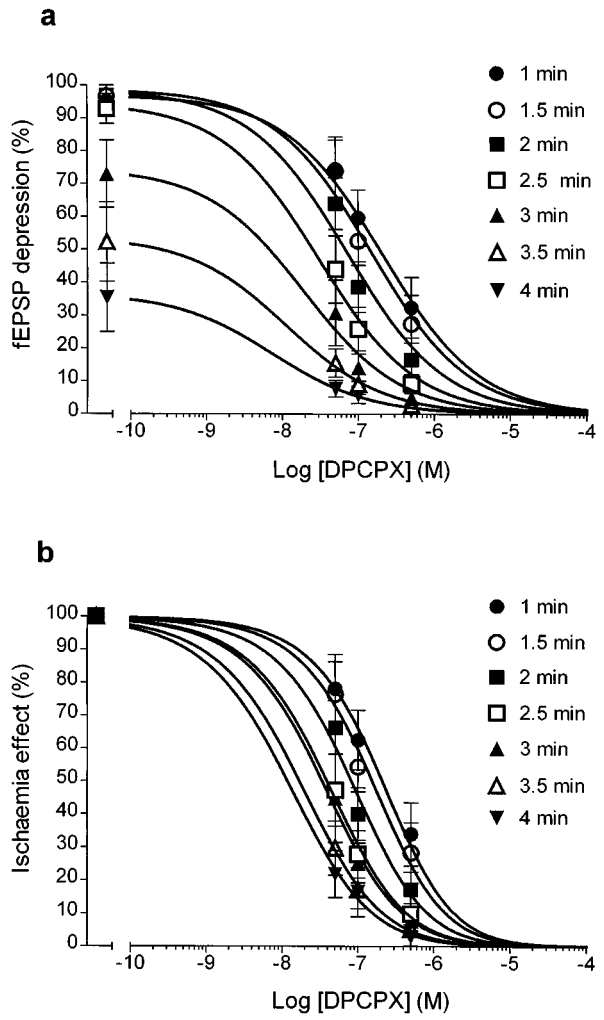

Figure 6 Estimation of the IC_{50} of DPCPX at 30 s intervals during recovery (min 1–4) from an ischaemic episode of 5 min. (a) Each curve describes the concentration-dependent antagonism of fe.p.s.p. depression by DPCPX for a given time of recovery. The upper curve shows the effects of DPCPX at the first min of recovery and the lowest is that obtained at the fourth. Note that with increasing recovery time the initial degree of depression is progressively reduced. Each concentration of the antagonist was tested in a different group of preparations. DPCPX was applied at 50 nM ($n=6$), 100 nM ($n=5$), or 500 nM ($n=5$). In each preparation fe.p.s.ps were evoked every 15 s and the per cent depressions elicited by the ischaemic episode were averaged at corresponding times for each experimental condition (i.e. absence or presence of DPCPX). For clarity plots are shown at 30 s intervals (values calculated every 15 s are shown in Figure 7). For each interval the fe.p.s.p. depression (expressed as per cent decrease compared to baseline fe.p.s.p. amplitude) is plotted against the concentration of DPCPX present during the ischaemic episode. Curves were obtained by nonlinear fitting (for parameters see text) of data in the logistic equation. (b) The ischaemia effect in the absence or presence of the antagonist (at the same recovery times shown in (a)) is plotted against the concentration of DPCPX. Curves were obtained by nonlinear fitting of data to the equation (7), keeping the slope index fixed to the average value of 14.5 ± 0.7 obtained by fitting the same data with unconstrained, variable slope.

by subtracting the residual depression observed in 8-PT (during the interval between the fourth min of ischaemia and the second min of reperfusion) from the values implemented in Barlow's equation. In the interval considered, the resulting concentrations of adenosine were accordingly lower than those in Figure 7 and peaked at 30 s reperfusion with 23 μM.

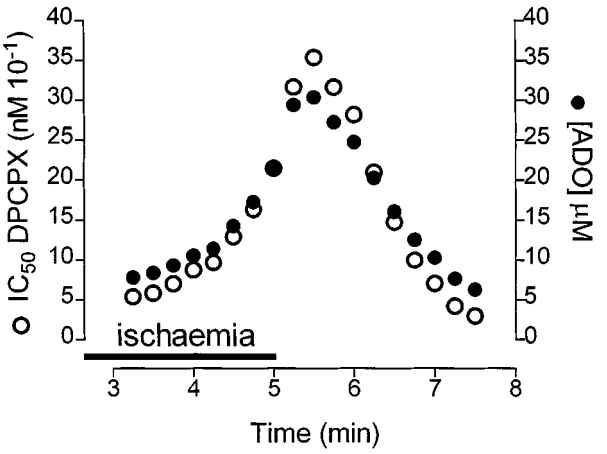

Figure 7 Time-course of the changes in IC_{50} of DPCPX and adenosine concentrations during ischaemia and recovery. The values obtained from the analyses shown in Figure 6 are plotted against time to show the time-related changes of the IC_{50} of the antagonist, and the estimated concentrations of adenosine during the ischaemic-like episode and reperfusion with control aCSF. Note that 3 min after the beginning of the ischaemic episode, adenosine concentration is already 24 times higher than basal levels.

Discussion

Our results provide the first estimation of the concentration of adenosine acting at the receptor level during an ischaemia-like episode in the CNS.

We monitored the effects of an ischaemia-like episode on the amplitude of extracellularly recorded fe.p.s.ps, which mainly reflect the currents generated by the inflow of cations into CA1 pyramidal cell dendrites due to activation of glutamate receptors (Hubbard *et al*., 1969). The reduction of the fe.p.s.p. amplitude can be produced through several mechanisms whose principles are: (i) competitive or non competitive antagonism of glutamate receptors; (ii) depolarization of the postsynaptic cell elicited by excitatory receptor stimulation or by potassium ion accumulation in the extracellular space and subsequent spreading depression; (iii) decrease in glutamate release by actions at presynaptic terminals. Among these possibilities the most likely mechanism of fe.p.s.p. reduction during ischaemia is a decrease in glutamate release caused by activation of presynaptic A_1 receptors (Corradetti *et al*., 1984a). In addition, it has been shown that the postsynaptic action of adenosine does not affect the size of miniature postsynaptic excitatory currents recorded from CA1 pyramidal cells (Prince & Stevens, 1992; Scholz & Miller, 1992), which suggests that the main effect of A_1 receptor stimulation on fe.p.s.p. is a decrease in glutamate release.

Other mechanisms not involving adenosine receptors might participate in the decrease in fe.p.s.ps in our experiments. For instance, ATP-dependent potassium channels are known to open during a hypoxic and/or hypoglycaemic episode (Ben-Ari, 1989; Mourre *et al*., 1989; Tromba *et al*., 1992). However it is generally accepted that synaptic depression during ischaemia is due to A_1 adenosine receptor stimulation (Fowler, 1990), and since the presence of the adenosine receptor antagonist 8-phenyltheophylline (8-PT) fully prevents the disappearance of fe.p.s.p. during an ischaemic episode (Pedata *et al*., 1993), it is conceivable that additional non adenosine-mediated mechanisms represent a negligible source of error in our conditions.

This assumption was confirmed by our experiments performed in the presence of DPCPX + ADA or 8-PT alone

where the effect of the ischaemia was blocked for as long as 5 min (i.e. the whole duration of the ischaemic episode). However, at the end of the 5 min ischaemic period and during the first 2–3 min of reperfusion, a depression was still observed in the presence of any of the treatments. In addition, the treatment with DPCPX + ADA produced, mainly during reperfusion, a contamination of the fe.p.s.p. by dendritic and/or somatic spikes. It is known that the ischaemic insult increases spontaneous release of glutamate and that A_1 receptor stimulation can greatly limit the outflow of glutamate (Heron *et al*., 1994). On the other hand, once the inhibitory effect of adenosine is completely blocked, glutamate can freely flow out of cells and, by stimulating ionotropic and metabotropic receptors, depolarize pyramidal neurones and increase their excitability. This produces a decrease in the amplitude of the fe.p.s.ps and the appearance of multiple action potentials. Furthermore, ADA might enhance neurone excitability through unspecific effects, as suggested by the modest, if any, hyperexcitation observed during ischaemia when the adenosine receptors are blocked by 8-PT. On the basis of this interpretation, we considered the experiments with 8-PT more reliable for correcting our calculations for the participation of unidentified mechanism(s) of fe.p.s.p. depression during reperfusion. However, an alternative explanation should be taken into account based on the fact that 8-PT is a competitive antagonist. From our calculations, the concentrations of adenosine at the end of 5 min ischaemia approached 20 μM and, considering the affinity of 8-PT for A_1 receptors ($K_i = 86$ nM) relative to that of adenosine ($EC_{50} = 610$ nM) the fraction of A_1 receptors still occupied by the agonist (calculated with Gaddum's equation; see Kenakin (1993)) is not negligible (about 20%). Under our conditions of competitive antagonism, it is likely that the residual depression (10–15%) observed during reperfusion was still due to A_1 receptor stimulation.

Since A_{2A} adenosine antagonists, (e.g. ZM 241385), do not modify the depression of fe.p.s.p. induced by the ischaemic episode (Latini *et al*., 1998b; Latini *et al*., manuscript in preparation) and since 8-PT does not antagonize A_3 receptors (Fredholm *et al*., 1994), A_{2A} and A_3 receptors do not appear directly involved in the depression of fe.p.s.p. induced by ischaemia. The role of A_{2B} receptors, which are uniformly distributed in the CNS (Dixon *et al*., 1996), remains to be elucidated.

In our experiments, we found that the recovery of the fe.p.s.ps after the second ischaemic episode was slightly faster than after the first episode. This suggests a small but statistically significant 'preconditioning effect' by the first ischaemic episode, possibly due to desensitization of the A_1 receptors. It has been shown that prolonged (>20 min) stimulation of A_3 receptors by mM concentrations of adenosine blocks A_1 receptor-mediated responses in the CA1 region of the hippocampus (Dunwiddie *et al*., 1997). Such a mechanism is unlikely to occur in our conditions because the first ischaemic episode lasted only 5 min and it is difficult to envisage that adenosine reached mM concentrations. The stimulation of A_{2A} adenosine receptors also might desensitize A_1 receptors in the CA1 (Cunha *et al*., 1994; O'Kane & Stone, 1998). However, O'Kane & Stone (1998) showed that stimulation of A_{2A} receptors does not modify A_1 receptor-mediated inhibitory action on fe.p.s.ps, and convincingly demonstrated that the possible interaction between A_{2A} and A_1 receptors is selectively postsynaptic in the CA1 region. We favour an alternative interpretation based on measurements of endogenous adenosine flowing out of hippocampal slices during two ischaemic episodes in similar (though not identical) experimental conditions. We found a $36 \pm 8\%$ ($P < 0.05$; $n = 7$) reduction in the total amount of adenosine released over basal levels during the second ischaemic insult when compared to the first. Consistent with the present findings, the time-course of the outflow of adenosine after the second ischaemic insult was faster than after the first (unpublished observations). These results suggest a reduction in adenosine availability after the first ischaemic episode.

Our approach is based on the assumption that to decrease fe.p.s.ps, adenosine acted on a single class of receptors with identical properties, an assumption which has been supported by previous work (Alzheimer *et al*., 1991; Dunwiddie *et al*., 1986; Dunwiddie & Diao, 1994; Dunwiddie & Fredholm, 1989; Scholz & Miller, 1992; Wu & Saggau, 1994). For our calculations we introduced several parameters which were obtained from previous work (Alzheimer *et al*., 1991; Dunwiddie & Diao, 1994) conducted in hippocampal slices by using methods similar to those applied in the present research. Our estimation of the concentrations of adenosine is based on the analysis proposed by Barlow (1995); (see Methods). On the basis of this analysis the calculated concentrations of adenosine represent the degree of receptor stimulation. From our data, the most conservative estimation of adenosine concentrations at the peak, after the ischaemic episode, at the receptor level is between 20 and 30 μM, depending on the parameters used for estimation and/or on the correction of raw data for the residual depressant effects observed in the presence of 8-PT. The rise in adenosine concentration was time-locked to the ischaemic episode, reaching its maximum within 45 s after reintroduction of oxygenated control aCSF and rapidly declining thereafter. These results confirm the observation by Latini *et al*. (1998a) that an increase in adenosine efflux from hippocampal slices closely parallels in time the depression of fe.p.s.ps produced by an ischaemic episode similar to that used in this study.

Under normoxic conditions the adenosine extracellular concentration estimated in the brain by microdialysis 24 h after fibre implantation (40–120 nM, values corrected for recovery of the fibre) (Ballarin *et al*., 1991; Pazzagli *et al*., 1993; 1994; 1995) or by the cortical cup technique (30–50 nM) (Phillis, 1989) is in about the same concentration range as that calculated in the present work (180–240 nM) and by Dunwiddie & Diao (1994) (120–200 nM) who used the same preparation. This supports the interpretation that in normoxic conditions the released adenosine equilibrates in the extracellular fluid and therefore the degree of tonic stimulation at its receptors can be inferred from the measure of adenosine concentration in the CSF using the microdialysis technique.

After the *in vitro* ischaemic episode, adenosine increases about 100–150 fold, reaching an estimated value of 30 μM. This increase is much higher than that calculated in the fluid collected from hippocampal slices (4–6 fold) (Latini *et al*., 1998a; Pedata *et al*., 1993) or from the hippocampus *in vivo* (19–23 fold) (Andinè *et al*., 1990; Dux *et al*., 1990) after inducing ischaemia. However, it should be noted that the time discrimination of adenosine concentrations (15 s) allowed by the present approach is greater than that achieved with any biochemical approach, where the sampling periods are more likely to represent the equilibrium reached in the CSF after partial reuptake and degradation of released adenosine. Taking into consideration these observations, it is likely that the adenosine concentration of 30 μM estimated in our study represents an adenosine concentration acting on receptors before diffusion and equilibration in the CSF. Therefore the degrees of receptor stimulation is greater than that extrapolated by the microdialysis adenosine measurement after

ischaemia. It deserves mention that adenosine concentrations as high as 24–40 μM have been measured by microdialysis after induction of global ischaemia in the rat and gerbil (Dux *et al*., 1990; Hagberg *et al*., 1987). However these values were reached on the first day after microdialysis tube implantation when the estimated basal adenosine extracellular concentration is 20 times higher than those found in experiments conducted 24 h after surgery (Pazzagli *et al*., 1993). The effect of ischaemia immediately after tube implantation of the probe may be therefore compounded by that of trauma due to the microdialysis probe insertion.

In agreement with the view that the presence of adenosine on receptors, close to release sites, is functionally important, Brundege & Dunwiddie (1996) have demonstrated, by applying adenosine by the recording pipette, that the degree of synaptic depression in the CA1 hippocampus is related to the adenosine concentration in the electrode. Their data strongly suggest that the principal source of adenosine, acting at the presynaptic level to decrease the excitatory input on the recorded CA1 pyramidal neurone, is the outflow of adenosine from the cell from which the postsynaptic current was recorded. This implies that during the ischaemic episode the outflow of adenosine from CA1 pyramidal neurones is likely to greatly affect excitatory neurotransmission.

In the CNS adenosine may act through four receptor subtypes to which the endogenous ligand shows varying affinity: a higher affinity for A_1 and A_{2A} (1–30 nM) than for A_{2B} and A_3 (>1 μM) receptors (Fredholm *et al*., 1994). Under normoxic conditions the adenosine present at the receptor level is therefore likely to preferentially activate A_1 and A_{2A} receptors. On the other hand, if we assume that our estimation of adenosine concentration reached during an ischaemia-like episode reflects the order of magnitude of adenosine present at the receptor level during an *in vivo* ischaemic episode, it is conceivable that under these conditions all adenosine receptors subtypes are activated during *in vivo* ischaemia. Stimulation of at least A_1, A_{2A} and A_3 adenosine receptors may modify the outcome of an ischaemic episode. It appears that during *in vivo* ischaemia A_1 receptor stimulation protects cerebral tissue from damage (Rudolphi *et al*., 1992) whereas the role of A_{2A} (O'Regan *et al*., 1992; Phillis, 1989) and/or A_3 adenosine receptors remains to be clarified (Von Lubitz *et al*., 1994).

In conclusion our data provide a 'dynamic' estimation of the concentration of adenosine reached at the receptor level during a transient ischaemic episode. This information may be useful in designing appropriate concentrations of purinergic compounds to be tested for their pharmacological effects during *in vitro* and/or *in vivo* ischaemic episodes.

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