NMDA antagonists increase recovery of evoked potentials from slices of rat olfactory cortex after anoxia

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Introduction

A variety of changes take place in tissues during oxygen deprivation. The central nervous system is particularly vulnerable to ischaemia partly because of glutamate release (Benveniste et al., 1984; Rothman, 1984; Drejer et al., 1985; Sanchez-Prieto & Gonzalez, 1988; Nicholls & Attwell, 1990) which causes cell death (Choi, 1988; Frandsen et al., 1989; Meldrum & Garthwaite, 1990). Under pathological conditions, glutamate acts on several receptor systems. For cell pathology, the most important of these is the N-methyl-D-aspartate receptor type (NMDA) since it is coupled to voltage-dependent channels that are Ca\(^{2+}\)permeant (Garthwaite & Garthwaite, 1986; McDermott et al., 1986; Choi, 1987; Michaels et al., 1990) producing Ca\(^{2+}\) loading of the cell. This loading in turn activates several vigorous Ca\(^{2+}\)-activated enzymes including phospholipase, proteases and nucleases leading to cell death.

There has been an assessment of several strategies that might provide some protection of neural tissue during ischaemia and one of these is based on NMDA antagonists. Several studies have found NMDA antagonists to offer some protection against ischaemia/anoxia (Park et al., 1988; Kochlar et al., 1988; Bielenberg, 1989) whilst in others, the protection has been small or absent (Perkins et al., 1988; Aitkin et al., 1988; Fleischer et al., 1989; Sterz et al., 1989; Lanter et al., 1990; McDonald et al., 1990). Most studies on the pharmacology of neuroprotection have been in vivo. Such studies are best suited to the assessment of substances that might have immediate clinical application. However, there are a large number of variables than can contribute to the tissue damage so the mechanism of action of protective agents is more difficult to analyse. For example, in cerebral ischaemia, tissue damage might result from oxygen lack directly or from accumulated metabolites. Furthermore, since most in vivo experiments have been conducted over several days, the post-ischaemic changes might be secondary to gliosis or alterations in the blood brain barrier (Giulian, 1993). Some of the problems encountered in vivo are circumvented in experiments with isolated tissues. Although most studies have concentrated on the microscopic changes after glutamate agonist insult some have looked to the effects of anoxia on evoked potentials in the hippocampus (Clark & Rothman, 1987; Rader & Lanthorn, 1989; Papas et al., 1993). In the present study, we have assessed the effects of anoxia by following the changes in electrical activity in slices of rat olfactory cortex maintained in vitro. Two goals were in mind (a) to elucidate the mechanisms underlying the anoxia-induced cell death, and (b) to find substances that might provide tissues with some protection during anoxia. We show that some NMDA antagonists produce a substantial increase in the recovery of tissues following short periods of anoxia. A preliminary account of this work has been published (Scholfield & Yassin, 1992).

Methods

Female Sprague-Dawley rats of 200–300 g weight were decapitated and their brains removed. The brain was bisected along the mid-line and pial surface slices of olfactory cortex were cut to a thickness of either 350 \(\mu\)m for studies at 35°C or 450 \(\mu\)m for 24°C. The time between decapitation and placement of the slices in oxygenated bathing solution at 24°C was 3 min, a period too short to produce permanent
changes in tissue responsiveness to electrical stimulation (see results). The two olfactory cortex slices were each subdivided along the axonal radiations into three, such that each had a length of lateral olfactory tract (LOT). The slices were placed in a 50 ml bath of Krebs solution contained within a sintered glass Buchner funnel. 95% O_2/5% CO_2 was supplied to the bottom of the funnel allowing the slices to be continually agitated in the bathing medium.

Individual slices were placed on a nylon mesh within a recording chamber (effective volume of 0.5 ml) and superfused with Krebs solution equilibrated with 95% O_2/5% CO_2 at 5 ml min^{-1} by recirculation from a 50 ml reservoir. The connections were through silicon rubber tubing the length of which was minimized to avoid gaseous de-equilibration. The gas mixture was also blown over the surface of the recording chamber to avoid gaseous exchange with air. The reservoir was water-jacketed and the flow line immediately adjacent to the recording chamber contained a heat exchanger to maintain the recording chamber at either 24° or 35°C.

A pair of stimulating electrodes was placed on the LOT and a recording pipette placed on the pial surface 2 mm away from the stimulating electrodes. The electrode was connected to a preamplifier and the waveforms were digitized, stored and analyzed by computer. The 95°C was chemically stimulated with 10 V, 0.2 ms pulses at 0.05 Hz and each response stored.

The tissue was allowed to equilibrate for 30 min in the bath before the collection of records. Antagonist was added to the reservoir and recirculated for 30 min. The recirculating solution was then changed for glucose-free solution equilibrated with 95% N_2/5% CO_2. Most experiments were performed at 24 ± 1°C (maximum range) but we also looked at some effects at a temperature nearer to that in vivo (38°C in the rat). However, at this temperature, the responses become unstable (Scholfield, 1980) presumably because of greater oxygen demands. Therefore to avoid the possibility of metabolic insufficiency and ‘sensitization’ to anoxia, experiments were conducted at 35°C rather than 38°C. Test substances were present for 30 min before and during the anoxia period. They were not present during the washout period. The concentration of NMDA antagonist used was in a series of experiments. The amplitude of the evoked potential was monitored during the application of NMDA at 5–1000 μM in normal solution and after 30 min equilibration in combination with various antagonists.

Between animals, a substantial amount of variability was observed. To reduce the effects of variability, a control experiment was performed in normal solution on each animal. The antagonist was tested on another slice from the same animal. The next pair of slices, the test experiment preceded the control. Some slices were used within 2 h of cutting whereas others were used up to 6 h after preparation. There was no time-dependent difference in control effects of anoxia. Each slice was subjected to one period of anoxia only. In some experiments, a range of anoxic periods was used. For most experiments, the same animal was used. The time period was used to produce a 5–30% control recovery, a level chosen to allow for any enhancement in the recovery of test slices. The peak amplitudes of the synaptic responses were measured and the amount of recovery expressed as a proportion of the response immediately before the anoxic period. Test and control experiments were compared by Student’s paired t test (two tailed). All values are means ± s.e. of mean and significance was taken as P < 0.05.

The Krebs solution had the following composition (mm): Na^+ 144, K^+ 5.0, Ca^2+ 2.5, Mg^2+ 1.3, Cl^- 128, HCO_3^- 25, D-glucose 2.0 and it was equilibrated with 95% O_2/5% CO_2. The rather low glucose concentration was necessary because previous work (Donaghy & Scholfield, 1990) had shown a delayed response to anoxia when slices were stored with 11 mM. During anoxia, glucose was removed since it had been shown also that responses could be sustained in glucose.

The drugs used were from the following sources: dizocilpine (MK801) Merck, Sharp and Dohme, Harlow, UK; 5,7-dinitroquinoxaline-2,3-dione (DNQX), DL-2-amino-5-phospho-pentanoic acid (AP5) and 7-chlorokynurenic acid, Tocris Neuramin, Bristol, UK; 2-[2-chlorophenyl]-2-[methylaminol]- cyclohexanone (ketamine), Sigma, Poole, UK; lignocaine, Astra Laboratories, Watford, UK.

Results

Stimulation of the LOT elicited a surface negative waveform which is the result of the release of acidic amino acid (Collins, 1979) acting on a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) type receptors (Collins & Buckley, 1989). NMDA receptors are present in olfactory cortex (Tacconi et al., 1993) and they may also be involved in synaptic transmission (Collins, 1991) but in our experiments, this component did not contribute to the evoked potential (see below). When recordings were made from an area of the slice close to the LOT, the synaptic potential was preceded by a response due to the activity of the axons underlying the recording electrode. This fibre response is not apparent in Figure 1 but can be seen with both preparations in Figure 2. The fibre potential is close to the stimulus artefact but it can be distinguished by comparing the response label ‘anoxia’ in Figure 2 where all the biologically generated responses had been lost. Without any experimental intervention, all the responses were maintained constant over a period of several hours. (Scholfield, 1980).

Effect of anoxia

When the bathing solution was changed to one equilibrated with 95% N_2/5% CO_2 there was a rapid and complete loss of both the pre- and postsynaptic responses (Figures 1 and 2). The response started to decline when the new solution arrived at the slice and this was normally complete within 4–6 min (Figure 3). When oxygenation was restored, the response recovered over a period of 30 min. In 5 slices, we followed the responses for a further 5 h after anoxia but there was no further recovery. Therefore, routinely, we studied only the initial 30 min response. In some experiments, anoxia was tested at 24° and 35°C. After 15 min of anoxia the recovery was 14.6 ± 4.1% and 3.5 ± 1.4% respectively.

With some responses, a postsynaptic population spike could be resolved and the relation between this and the excitatory postsynaptic potential (e.p.s.p.) can be used to gain insight into the changes in membrane potential (Scholfield, 1980; Richards, 1972). During the onset of anoxia, there was a large depression of the e.p.s.p. with little change in the population spike. If the e.p.s.p. was reduced by decreasing the stimulus voltage, the e.p.s.p. and population spike were reduced in sympathy. These observations indicate that anoxia causes postsynaptic depolarization.

At 35°C, slices were exposed to varying periods of anoxia between 5 and 15 min and the amount of recovery is shown in Figure 4. For short periods (5 min), recovery was complete but diminished with longer periods such that little recovery was seen after 12 min.

Effect of NMDA

Application of NMDA (5–50 μM) produced a depression in the evoked potential. The following NMDA antagonists (Kemp et al., 1987) were tested for their ability to reduce the action of NMDA: AP5 (50 and 200 μM), 7-chlorokynurenic acid (50 μM), dizocilpine (10 μM) and ketamine (200 μM). NMDA was not blocked immediately by dizocilpine or ketamine but antagonism developed over a period of 30 min.
whereas AP5 and 7-chlorokynurenate showed a full effect within 10 min of addition. The concentrations of NMDA which produced a 50% depression of the evoked response in the presence of the above antagonists are shown in Table 1. All the substances tested produced a substantial antagonism of NMDA and AP5 was dose-dependent.

**Figure 1** Single synaptically evoked potentials recorded from slices of rat olfactory cortex. In the upper traces (a), the slices was bathed in normal solution ('normal') followed 15 min in solution equilibrated with 95% N₂/5% CO₂ ('Anoxia') followed by normally oxygenated solution ('washout'). A time-course for a similar experiment is seen in Figure 3a. In the lower traces (b), another slice from the same animal was bathed in dizocilpine (10 μM) for 30 min before the period of anoxia and during the anoxia. 'S' indicates the point of stimulus application. On these traces, negative is upwards.

**Figure 2** An experiment on evoked responses from another pair of slices comparing the effect of normal solution and incubation with ketamine (200 μM). In this experiment, the presynaptic action is seen as a transient between the stimulus artifact (arrow labelled 'S') and the synaptic response. This transient has disappeared from the records labelled 'Anoxia'.
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response. The response, anoxia.
but with preincubation period of 30 min in 10 µM dizocilpine.

Effect of dizocilpine

Application of 10 µM dizocilpine in normal oxygenated solution had no effect on the synaptic response (Figures 1 and 3) confirming that any involvement of NMDA receptors in this pathway is small (Collins, 1991). The presence of dizocilpine made no difference to the onset and the decline response during anoxia. However, the speed of recovery and level of recovery attained were substantially augmented by this agent (Figure 1 and 3). Thus at 24°C, a 15 min period of anoxia produced a recovery in normal solution which was 14.6% compared to 48.3% in 10 µM dizocilpine (Table 2). The same effect was seen at 35°C for 12 min of anoxia (6.3% recovery in normal solution and 86.9% in dizocilpine).

Figure 3 Time-courses for changes in the amplitude of the evoked response. The preparation was stimulated at 0.05 Hz and for each response, the peak amplitude of the evoked potential was measured and plotted on the time-course as a single point. Each point was joined to produce the continuous trace shown. In (a) the slice was bathed successively in normal (oxygenated) solution, in solution equilibrated with N2/CO2 (12 min) and then re-oxygenated (‘washout’). (b) Shows a time-course of a slice from the same animal but with a preincubation period of 30 min in 10 µM dizocilpine.

Figure 4 Effect of varying the period of anoxia (abscissa scale) on the recovery of the evoked potential (ordinate scale) in normal solution (○) and in the presence of 10 µM dizocilpine (●). The ordinate scale is the ratio of the amplitude of the evoked potential 30 min after the period of anoxia to that before the anoxia. The lines were drawn by ‘eye’.

<table>
<thead>
<tr>
<th>Table 1 Concentrations of N-methyl-D-aspartate (NMDA) which produced a 50% depression of the evoked potential</th>
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<td><strong>Antagonist</strong></td>
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<tr>
<td>AP5 50 µM</td>
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<tr>
<td>AP5 200 µM</td>
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<tr>
<td>7-Chlorokynurenate 50 µM</td>
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<td>Dizocilpine 10 µM</td>
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<td>Ketamine 200 µM</td>
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The ratios are the values in antagonist ('Test') divided by that in normal solution ('Normal') for each slice. The n-values are the numbers of pairs of slices and the P-value calculated using the two-tailed t test.

<table>
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<th>Table 2 Effect of glutamate antagonists on recovery after a period of anoxia</th>
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<td><strong>Experiment</strong></td>
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<tr>
<td>Lignocaine</td>
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<td>AP5, 50 µM</td>
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<td>AP5, 200 µM</td>
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<td>7-Chlorokynurenate</td>
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<td>Dizocilpine</td>
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<td>Dizocilpine at 35°C</td>
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<td>Ketamine</td>
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The change was calculated by subtracting the % recovery in normal solution (control) from that in the experimental solution (test) for paired slices from the same animals and averaging these. The P values were calculated from the paired two tailed t test for the control and test groups. All experiments were done at 24°C with a period of anoxia of 15 min except those at 35°C where the period of anoxia was 12 min.
In one group of experiments, slices were exposed to varying periods of anoxia. For all periods of anoxia tested, there was an enhanced recovery in the presence of dizocilpine (Figure 4). Thus in effect, the endurance of the tissue was increased by 5–6 min. As in normal solution, there was no further change in the amplitude of the response after 30 min in the recovery solution.

Effect of ketamine

Ketamine at 200 µM had no effect on synaptic transmission (Figure 2): higher concentrations were not tested since they depress axonal responses (McGivern & Scholfield, 1990). Nor did ketamine influence the depression produced by anoxia but like dizocilpine, the rate and the amount of recovery attained were substantially increased (Table 2).

Effect of AP5

Both dizocilpine and ketamine block the channels associated with the NMDA receptor whereas AP5 was tested as a substance that competes with the NMDA binding site. It was tested at two concentrations, 50 and 200 µM. The lower concentration had no effect on responses in normoxic and anoxic conditions nor was the recovery significantly affected by its presence (Table 2). AP5 200 µM had two clear effects: (a) alone it caused a depression of the response from 1.45 ± 0.23 to 1.24 ± 0.22 mV (a 16 ± 1% depression, n = 14); (b) following anoxia, it permitted a greater recovery in the response Table 2).

Effect of 7-chlorokynurenic acid

7-Chlorokynurenic acid is an antagonist at the glycine binding site (Kemp et al., 1988). Under normoxic conditions, it produced a depressant effect on its own (Figure 5) which was concentration-dependent between 20–200 µM. To test its effect on recovery, 50 µM was used and this depressed the evoked potentials from 2.35 ± 0.20 to 1.34 ± 0.12 mV (a depression of 42.3%; n = 14). 7-Chlorokynurenic acid (50 µM) also permitted a substantial increase in the recovery after anoxia (Table 2).

Effect of DNQX

Neurotoxicity may also arise from non-NMDA receptors (Koh et al., 1990). Therefore an antagonist to the AMPA receptor (DNQX) was also tested. At a concentration of 10 µM it depressed the synthetically mediated potential confirming the role of AMPA receptors in normal transmission (Figure 6). After equilibration for 30 min with this antagonist, anoxia completely abolished the residual response. The DNQX was washed out at the same time as reoxygenation and the response recovered to a level similar to that in slices without DNQX pretreatment (Figure 6; Table 2).

Effect of other agents

In some experiments above, there was a depression of the response by the test agent (e.g., DNQX and 7-chlorokynurenic acid) before the period of anoxia. We therefore tested the effect of a relatively non-selective depressant agent, lignocaine. As shown in Table 2, there was a small but significant enhancement in the recovery provided by this agent.

![Figure 5](image)

**Figure 5** A similar time course to Figure 3 during the application of 50 µM 7-chlorokynurenic acid present during the period indicated by the solid bar. Note that there is some depression of response on adding the 7-chlorokynurenic acid.

![Figure 6](image)

**Figure 6** Another time-course using 5,7-dinitroquinoxaline-2,3-dione (DNQX, 10 µM) as the test substance. Note that this agent on its own also depressed the synaptic response. The DNQX was washed out at the end of the period of anoxia. Without the intervening period of anoxia, the response would normally recover completely from the DNQX.
Discussion
Tissue anoxia produces a complex series of events and in the present study we have looked at some of these: short-term changes in synaptic function. This is particularly vulnerable to anoxia because the presynaptic nerve terminals are very fine (<0.2 µm in olfactory cortex, Gracey & Scholfield, 1990). Cessation of ATP production will cause a run down of ionic gradients at a rate, dependent on the surface area to volume ratio and on activity. Thus ionic run-down would be rapid in cholinereceptors and rat sympathetic neurons. Thus the cell body membranes, but in the long term (in vivo studies) the effective deafferentation may induce their auto-degeneration.

In olfactory cortex, transmission is glutamate-mediated (see results) and the axonal depolarization caused glutamate to be released from the nerve terminals (see Introduction). The sustained rise in extracellular glutamate stimulates NMDA receptors and since the associated channels are depolarization-dependent, any depolarization due to metabolic insufficiency will accentuate NMDA channel opening. Several glutamate blockers were tested but none of them influenced the time course for the loss of excitability during the onset of anoxia. This would suggest that synaptic function is not lost as a consequence of any NMDA- or AMPA-mediated process. Recovery was substantially enhanced by dizocilpine, ketamine, the higher AP5 concentration and 7-chlorokynurenic acid. This would suggest that NMDA receptors are activated during anoxia and that these contribute to tissue damage.

The lower AP5 concentration provided little protection although it did produce some antagonism of NMDA. The stronger protective action of dizocilpine, ketamine and 200 µM AP5 reflect the greater effectiveness of these as NMDA channel blockers at the concentrations used. This implies that a substantial blockade of NMDA receptor/channel is required before any protective effect is obtained. This requirement for a high degree of block may explain why some studies have failed to show any effect of NMDA antagonists with anoxia and ischaemia.

On the other hand, AMPA receptor blockade by DNQX did not aid recovery. This indicates that AMPA receptors are not important in priming the depolarization for NMDA channel opening and it would suggest that depolarization results from other causes.

Although the NMDA channel antagonists were the most effective protectants, the degree of protection only extended the endurance of the tissue by around 5 min. Clearly, NMDA receptor activation is one of probably a multiplicity of degradative functions in neural tissue. However, the present results do conform the idea that NMDA receptors play an important part in the pathogenesis of cerebral anoxia.

References


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