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Attenuation by chlormethiazole administration of the rise in extracellular amino acids following focal ischaemia in the cerebral cortex of the rat

H.A. Baldwin, J.L. Williams, M. Snares, T. Ferreira, [1]A.J. Cross & A.R. Green

Astra Neuroscience Research Unit, 1 Wakefield Street, London WC1N 1PJ

1 *In vivo* microdialysis has been used to investigate the concentration of various amino acids and lactate in the extracellular fluid of the rat cortex following focal ischaemia, the probe being placed in the core of the infarct area.

2 An ischaemic infarct was produced in the cortex by use of a photochemical dye (Rose Bengal) and light irradiation. There was a marked increase in lactate concentration (300%) over the next 4 h. Substantial increases were also seen in the concentration of the excitatory (glutamate and aspartate), inhibitory (GABA and taurine) and other amino acids (serine, alanine, asparagine).

3 Administration of chlormethiazole (200 mg kg^{-1}, i.p.) 5 min after the onset of ischaemia reduced the ischaemia-induced neurodegeneration by approximately 30%, measured histologically 24 h later.

4 Chlormethiazole (200 mg kg^{-1}, i.p.) administration also reduced the rise in the concentration of lactate and all the amino acids by between 30–60% during the first 4 h after the onset of ischaemia.

5 Analysis of the time course of the amino acid changes suggested that chlormethiazole is not neuroprotective because of the inhibition of excitatory amino acid release but rather that the attenuated rise in the concentration of all the amino acids is reflective of neuroprotection and therefore decreased cell death.

6 This conclusion was supported by the observation that the enhanced efflux of glutamate from slices of cerebral cortex which had been induced by incubation of the slices in an hypoxic medium was unaltered by the presence of a high concentration of chlormethiazole (1 mM) in the medium.

7 Overall the data strengthen the evidence for the neuroprotective effect of chlormethiazole in this model of focal ischaemia.

Keywords: Focal ischaemia; chlormethiazole; excitatory amino acids; amino acids; neuroprotection; glutamate; GABA; lactate; *in vivo* microdialysis

Introduction

Watson and colleagues (1985) have developed a relatively non-invasive method of producing an ischaemic infarct in the cortex by means of a photochemically induced thrombosis of cerebral arteries. An intravenous injection of the photosensitive dye, Rose Bengal, is given and a green light used to penetrate the skull of anaesthetized rats and illuminate subdural blood vessels. A photochemical reaction occurs with subsequent damage to the endothelial lining of blood vessels, platelet aggregation and thrombosis (Dietrich *et al.*, 1987a,b; 1988; Grome *et al.*, 1988; De Ryck *et al.*, 1989; Laursen *et al.*, 1991). In the irradiated area of the cortex there is vascular stasis and ischaemic cell death (Watson *et al.*, 1985; Snape *et al.*, 1993; Baldwin *et al.*, 1993b,c).

Recently we reported that chlormethiazole, a drug already shown to be an effective neuroprotective agent in the gerbil model of transient global ischaemia (Cross *et al.*, 1991; Baldwin *et al.*, 1993a) reduced the size of the infarction in the photochemical model of focal ischaemia (Snape *et al.*, 1993). Dizocilpine, the N-methyl-D-aspartate antagonist, despite being neuroprotective in the gerbil model (Gill *et al.*, 1987; 1988; Cross *et al.*, 1991) was without protective effect in the photochemical model as was the AMPA antagonist NBQX [2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo (F) quinoxaline] (Baldwin *et al.*, 1993c). As both dizocilpine and NBQX are protective in other models of focal ischaemia (Gill *et al.*, 1988; 1992; Park *et al.*, 1988; Sheardown *et al.*, 1990) this raises questions as to the involvement of glutamate in the mechanisms involved in cell death in the photochemical model.

The current study was undertaken therefore to investigate by use of *in vivo* microdialysis, the extracellular concentrations (and therefore presumably efflux) of various transmitter and non-transmitter amino acids and also lactate following a photochemically induced ischaemic episode and the effect of chlormethiazole on these concentrations. In this way it was hoped that knowledge would be gained both of the changes that follow photochemically induced ischaemia and also the mechanisms involved in the neuroprotective action of chlormethiazole.

Since a previous study (Baldwin *et al.*, 1993c) found that the extracellular concentrations of amino acids and lactate did not change in non-ischaemic animals in the course of the experiment, it was considered reasonable to perform the study comparing only drug and non-drug treated ischaemic rats.

Methods

Animals

Male Lister hooded rats (Olac, U.K.) weighing 280–360 g at the time of surgery were used. Rats were housed in groups of 5 in a room with a 12 h light:12 h dark cycle (lights on at 07 h 00 min) and given food and water *ad libitum*.

Implantation of microdialysis probes

Rats were anaesthetized with halothane (1.5–5.0%) in a O_2/N_2O mixture (1:2) and secured in a Kopf stereotaxic frame with the tooth bar at −3.3 mm below interaural zero. A 3 mm concentric dialysis probe (240 μm diameter; CMA

[1] Author for correspondence.

Microdialysis AB, Sweden) was implanted horizontally via a guide cannule (CMA11, CMA Microdialysis AB) into the right cortex: −2.3 mm posterior and −2.0 mm ventral to the skull surface at bregma and the tip of the probe was located at ±0.0 mm from the midline. A 6 mm diameter plastic ring was placed directly over the right cortex, behind bregma and tangential to the bregmoidal and midline skull sutures. The probe and plastic ring were secured to the skull with 2 screws and dental acrylic, leaving the skull inside the ring, free of acrylic. Rats were given 2 ml of 4% glucose in saline (s.c.) as a nutrient and placed in individual perspex cages (30 × 30 × 25 cm) to recover for 20 h.

Induction of ischaemia

Rats were anaesthetized with halothane (1.5–5.0%) in an oxygen/nitrous oxide mixture (1:2). Rose bengal (7.5 mg ml^{-1}) in saline was then injected slowly into the right jugular vein at a dose of 20 mg kg^{-1} (Ca. 0.5–0.8 ml injected over 20 s). Immediately after the injection a fibre optic was positioned directly over the centre of the plastic ring above the skull (distance from fibre to skull <2.0 mm). This area of skull was irradiated for 7 min with light from a 150 W halogen source that had been passed through a green filter (Olympus Highlight 3000, Olympus, U.K.).

Five minutes after induction of ischaemia rats received an injection of either chlormethiazole (200 mg kg^{-1}, i.p.) or NaCl, 0.9% w/v (saline). Since the chlormethiazole-treated animals were sedated, they were placed on a heating mat under a blanket for the experiment. Saline-treated animals were replaced in the perspex boxes. The rectal temperature of both groups of animals was monitored and maintained at 37°C throughout the remainder of the experiment.

Collection of dialysate samples

Dialysis probes were perfused with artificial cerebrospinal fluid (composition, mM: NaCl 125, KCl 2.5, $MgCl_2$ 1.18 and $CaCl_2$ 1.26) at a rate of 1 μl min^{-1}. The first 60 min sample was discarded and the next three 30 min baseline samples collected. Rats were then anaesthetized for the induction of ischaemia, replaced in the perspex boxes and samples collected every 30 min for the next 4 h.

After collection, animals were replaced in their cages overnight before perfusion and histological assessment.

Each dialysis sample was divided into 2 aliquots and frozen at −30°C until assay; 5 μl was taken for lactate determination and 25 μl for amino acid determination.

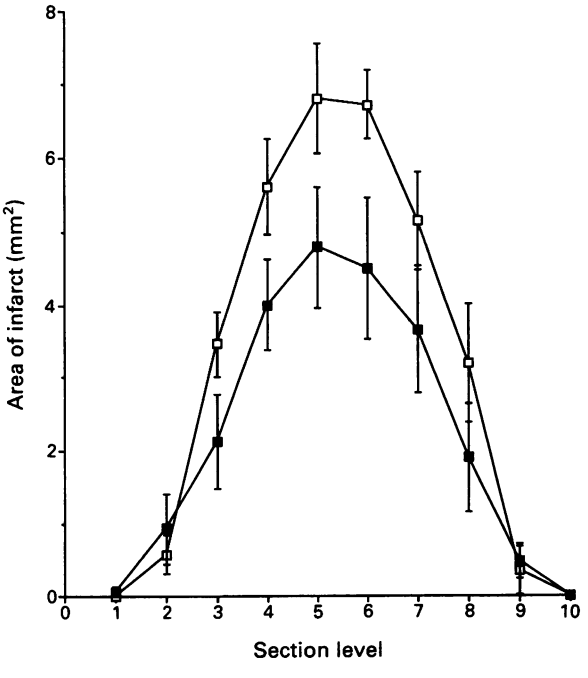

Figure 1 The effect of chlormethiazole (200 mg kg^{-1}, i.p., $n = 9$; ■) or saline (i.p., $n = 10$; □) given 5 min post-ischaemia, on the size of the cortical infarct shown by tetrazolium chloride staining measured 24 h post-ischaemia. Values are mean (± s.e.mean) area of infarct (mm^2) at each section level (i.e. 1 mm sections taken from anterior to posterior of brain). There was a significant effect of 'level' (ANOVA $F(8,136) = 40.59$, $P<0.001$) and a significant 'drug' × 'level' interaction (ANOVA $F(8,136) = 2.02$, $P<0.05$).

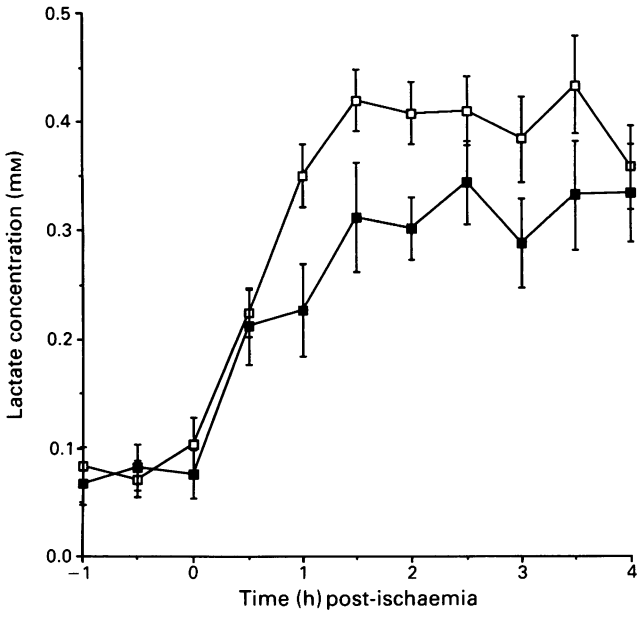

Figure 2 Measurement of lactate concentration in dialysate from ischaemic rats. Mean (± s.e.mean) lactate concentration (mM) from rats receiving either chlormethiazole (200 mg kg^{-1}, i.p., $n = 10$; ■) or saline (i.p., $n = 10$; □) 5 min after induction of ischaemia. There was a significant effect of 'time' (ANOVA $F(10,180) = 55.45$, $P<0.001$) and a significant 'drug' × 'time' interaction (ANOVA $F(10,180) = 1.97$, $P<0.05$).

Measurement of lactate and amino acids concentrations in dialysate

Lactate concentrations were determined by the enzymatic assay method of Lowry & Passonneau (1972) as described by Baldwin *et al.* (1993c). Amino acid concentrations were measured by high performance liquid chromatography (h.p.l.c.) with fluorometric detection following precolumn derivatisation with *o*-ptthalaldehyde as described by Lindroth *et al.* (1985) and briefly detailed by Baldwin *et al.* (1993c).

Histological assessment of damage

Twenty four hours after induction of ischaemia rats were perfused with 2,3,5-triphenyltetrazolium chloride (4% w/v in saline) and the brain subsequently prepared for fixing and slicing into 1 mm thick sections. The stained slices were photographed under a dissecting microscope and measures of the infarct size made with a digitizing tablet and computer by an observer unaware of treatment condition. Full experimental details are given in the paper of Snape *et al.* (1993) which also shows photographs of typical infarct damage.

Measurement of glutamate release from cortical slices in vitro

Rats were killed by cervical dislocation, the brains removed and cortical slices prepared with a McIlwain tissue chopper

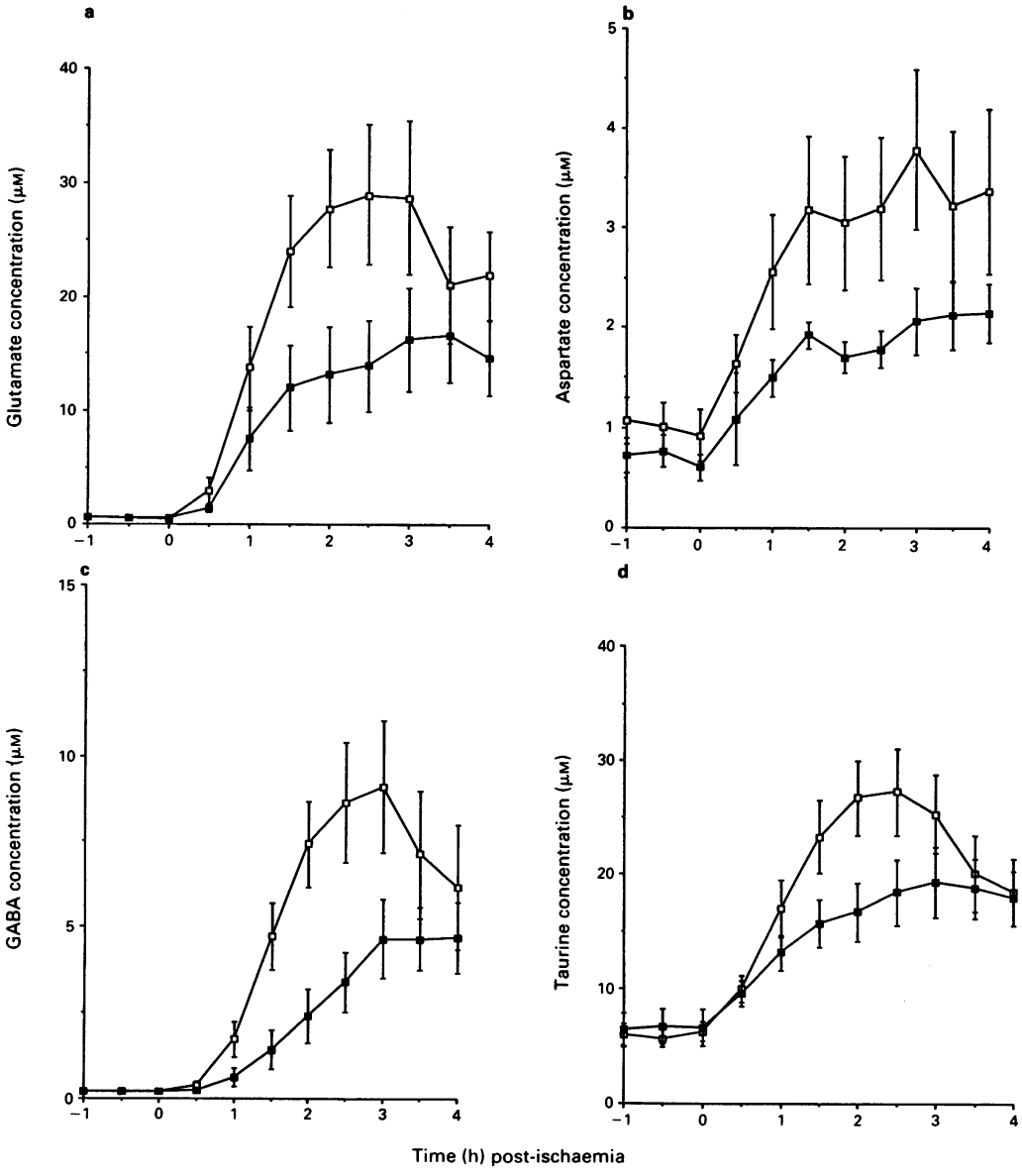

Figure 3 Measurement of glutamate, aspartate, GABA and taurine concentration in dialysate from ischaemic rats. Rats received either chlormethiazole (200 mg kg^{-1}, i.p., $n = 10$; ■) or saline (i.p., $n = 10$; □) 5 min after induction of ischaemia. (a) Mean (± s.e.mean) glutamate concentration (μM) in dialysate. There was a significant effect of 'time' (ANOVA $F(10,180) = 24.04$, $P < 0.001$) and a significant 'drug' × 'time' interaction (ANOVA $F(10,180) = 2.50$, $P < 0.05$). (b) Mean (± s.e.mean) aspartate concentration (μM) in dialysate. There was a significant effect of 'time' (ANOVA $F(10,180) = 16.34$, $P < 0.001$) but no significant 'drug' × 'time' interaction. (c) Mean (± s.e.mean) GABA concentration (μM) in dialysate. There was a significant effect of 'drug' (ANOVA $F(1,18) = 4.56$, $P < 0.05$) and 'time' (ANOVA $F(10,180) = 26.96$, $P < 0.001$) and a significant 'drug' and 'time' interaction (ANOVA $F(10,180) = 3.90$, $P < 0.001$). (d) Mean (± s.e.mean) taurine concentration (μM) in dialysate. There was a significant effect of 'time' (ANOVA $F(10,180) = 34.24$, $P < 0.001$) and a significant 'drug' × 'time' interaction (ANOVA $F(10,180) = 3.00$, $P < 0.005$).

(500 μm, cut in two perpendicular directions). Slices were incubated in oxygenated Krebs buffer (pH 7.4, 37°C) for 15 min. The buffer was removed and the procedure repeated two further times. The final wash buffer was removed and 100 μl of packed slices was transferred to mesh baskets. The baskets were placed in vials containing 2 ml of oxygenated Krebs buffer and incubated for 3 min. Baskets were then transferred to vials containing 2 ml of fresh solution at 3 min intervals. In some cases the buffer had been bubbled with N_2 instead of O_2 and chlormethiazole (1 mM) was added to some of the buffer solutions. After 18 min, baskets were placed in HCl (1 M) for 60 min to release all remaining amino acids. Aliquots of the release medium and tissue extract were frozen until assay for amino acids. Amino acid release was expressed as a percentage of the total content of the tissue slices plus medium.

Drugs and reagents

The following drugs and reagents were used (source in parentheses): dichlormethiazole edisylate (Astra Arcus, Södertälje, Sweden); halothane (ICI, U.K.); sodium pentobarbitone ('Sagatal', RMD Animal Health Ltd., U.K.); rose bengal [acid red 94; tetraiodotetrachlorofluorescein sodium salt], 2,3,5-triphenyltetrazolium chloride, *o*-phthalaldehyde, lactic acid glutamic acid, γ-aminobutyric acid (GABA), aspartic acid, taurine, serine, alanine and asparagine (Sigma Chemical Co., Poole, U.K.). All other reagents were obtained from Merck Ltd., Poole, U.K.

Statistics

Separate analyses were performed for each amino acid and

for lactate. Dialysate amino acid and lactate concentrations were analysed by two-way Analysis of Variance (ANOVA) with 'drug' (i.e. saline or chlormethiazole) as the between groups factor and 'time' as the repeated measure. The histological data from the dialysis study were analysed by 2-way ANOVA with 'drug' as the between groups factor and 'section level' as the repeated measure. Data from the *in vitro* release experiments were analysed by 2-way ANOVA with 'condition' (i.e. O_2, hypoxia or hypoxia with chlormethiazole) as the between groups factor and 'time' as the repeated measure.

Results

Effect of chlormethiazole on the infarct size following the ischaemic episode

The size of the cortical damage 24 h after the induction of ischaemia was measured in a series of sections taken through the irradiated area of cortex. The area of damage was reduced by approximately 30% in animals given chlormethiazole (200 mg kg^{-1}, i.p.) 5 min after the ischaemic episode (Figure 1).

Effect of ischaemia on dialysate lactate concentrations

The concentration of lactate in the dialysate rose rapidly following the light exposure and onset of the ischaemic episode with a final concentration 4 h after the onset of ischaemia being around 300% higher than baseline (Figure 2). Administration of chlormethiazole (200 mg kg^{-1}, i.p.) 5 min after the onset of ischaemia attenuated this change although there was no difference in the first 30 min sample following the start of the ischaemic episode (Figure 2).

Effect of chlormethiazole on glutamate, aspartate, GABA and taurine concentrations in the dialysate

The glutamate concentration in the dialysate rose nearly 30 fold within 2 h of the start of ischaemia and this increase was decreased by approximately 40% in the chlormethiazole-treated rats (Figure 3a). The ischaemia-induced rise in aspartate concentration was much smaller, but was also decreased by nearly 40% in chlormethiazole-injected rats (Figure 3b).

The concentration of GABA and taurine in the dialysate also rose significantly following the start of the ischaemic episode and these increases were attenuated by administration of chlormethiazole (Figures 3c and d). The peak concentration of all 4 compounds was observed to occur approximately 2 h after the onset of ischaemia (Figure 3).

Effect of chlormethiazole on serine, alanine and asparagine concentrations in the dialysate

The concentrations of serine, alanine and asparagine amino acids increased in the dialysate following the onset of ischaemia with the concentration appearing to increase throughout the 4 h collection period (Figure 4). Chlormethiazole-treated animals had a significantly smaller increase in the concentration of these amino acids in the dialysate in each case (Figure 4).

Effect of hypoxia and chlormethiazole on glutamate release from cortical slices

Incubation of cortical slices in an oxygenated buffer resulted in a steady and modest release of glutamate into the medium (Figure 5). When the slices were transferred to an hypoxic medium there was a marked increase in glutamate release, which was not affected by the presence of a high concentration (1 mM) of chlormethiazole in the buffer (Figure 5).

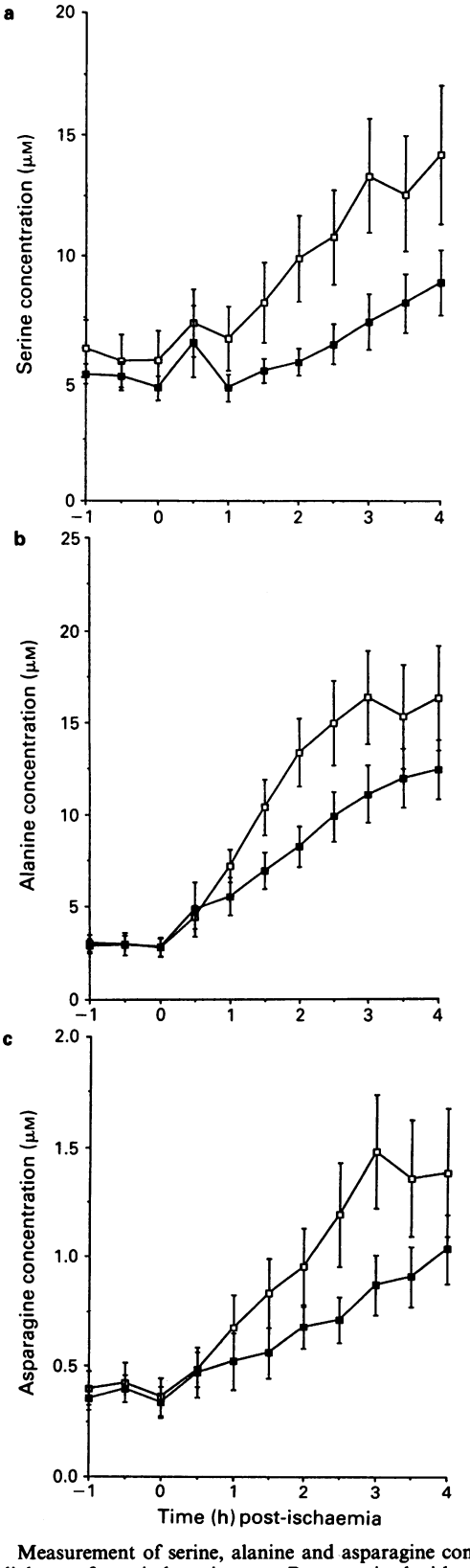

Figure 4 Measurement of serine, alanine and asparagine concentration in dialysate from ischaemic rats. Rats received either chlormethiazole (200 mg kg^{-1}, i.p., $n = 10$; ■) or saline (i.p., $n = 10$; □) 5 min after induction of ischaemia. (a) Mean (± s.e.mean) serine concentration (μM) in dialysate. There was a significant effect of 'time' (ANOVA $F(10,180) = 15.64$, $P < 0.001$) and a significant 'drug' × 'time' interaction (ANOVA $F(10,180) = 2.93$, $P < 0.005$). (b) Mean (± s.e.mean) alanine concentration (μM) in dialysate. There was a significant effect of 'time' (ANOVA $F(10,180) = 41.80$, $P < 0.001$) and a significant 'drug' × 'time' interaction (ANOVA $F(10,180) = 2.44$, $P < 0.01$). (c) Mean (± s.e.mean) asparagine concentration (μM) in dialysate. There was a significant effect of 'time' (ANOVA $F(10,180) = 23.17$, $P < 0.001$) and a significant 'drug' × 'time' interaction (ANOVA $F(10,180) = 2.49$, $P < 0.01$).

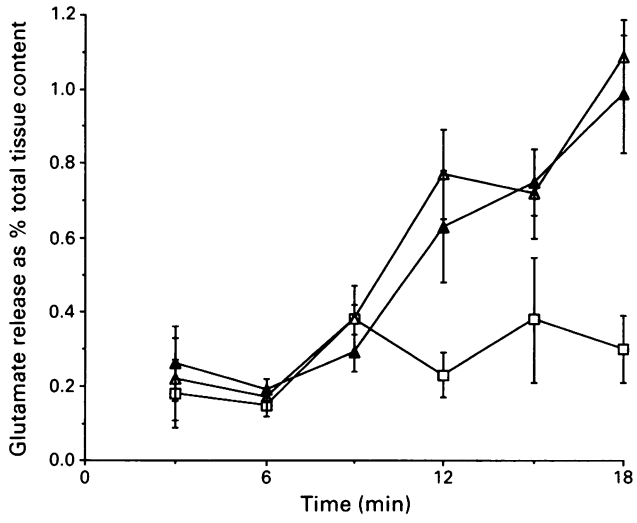

Figure 5 The effect of hypoxia on glutamate release from cortical slices. Slices of cortex were incubated in oxygenated buffer. After 6 min one group was continued in oxygenated buffer (□) while two other groups were incubated with either buffer bubbled with N_2 (△) or N_2 buffer containing 1 mM chlormethiazole (▲). Results shown as glutamate release as % of total glutamate content ± s.e.mean.

Discussion

The finding of Snape *et al.* (1993) that chlormethiazole is neuroprotective in the photochemical model of ischaemic cell death was confirmed in the current investigation (Figure 1), thereby allowing confidence in the data obtained when the drug was used to investigate changes in the extracellular neurotransmitter amino acid concentrations which occur following ischaemia.

Previous data from this laboratory (Snape *et al.*,1993) have indicated that neuronal damage occurs very rapidly after the onset of ischaemia in the photochemical model of stroke, the damage observed at 4 h being approximately 75% of the total damage present at 24 h, when damage is near maximal. This degree of damage is almost identical to that reported by Grome *et al.* (1988). It therefore seems reasonable to suppose that the major biochemical changes which lead to the observed pathology are also occurring in the first 4 h.

We previously demonstrated by use of *in vivo* microdialysis that there was a marked increase in the extracellular concentration of lactate following the induction of focal ischaemia using the photochemical model (Baldwin *et al.*, 1993c). This increase presumably indicated an increase in anaerobic metabolism and was consistent with findings in other ischaemia models (Kuhr & Korf, 1988). We also found a clear increase in the efflux of the excitatory amino acid, glutamate (Baldwin *et al.*, 1993c), in agreement with the findings of others using different models of focal and global ischaemia (Benveniste *et al.*, 1984; Globus *et al.*, 1988; Butcher *et al.*, 1990). The current study has confirmed these observations and also found a marked increase in the excitatory amino acid, aspartate. All these data could therefore be used to suggest a key role for the excitatory amino acids in the development of the neurodegenerative changes which follow an ischaemic episode in the model, as has been proposed in others (Rothman & Olney, 1986; Choi *et al.*, 1988; Meldrum, 1990).

The efflux of the inhibitory amino acid, GABA, was also found to increase at the same time (Baldwin *et al.*, 1993c; this paper) as did the concentration of taurine, in agreement with studies in other ischaemic models (Benveniste *et al.*, 1984; Butcher *et al.*, 1990; Lekieffre *et al.*, 1992). It remains uncertain as to whether taurine is an inhibitory neurotransmitter. However, there is certainly evidence for an inhibitory neuromodulatory role in the brain, possibly through an action at GABA receptors (see Oja & Kontro, 1990) and taurine has also been shown to assist in the recovery of neuronal function following hypoxia (Schurr *et al.*, 1987). There is also evidence that taurine is an important factor in cerebral osmoregulation (Kimelberg *et al.*, 1990; Lehmann, 1990; Puka *et al.*, 1991). It could be postulated therefore that the release of taurine may be indirectly linked to mechanisms involved in limiting the development of the infarct, particularly as the infarct in this model is associated with a significant degree of oedema (Dietrich *et al.*,1987a; Laursen *et al.*, 1991; Snape *et al.*, 1993; Green & Cross, unpublished observations).

Chlormethiazole administration decreased the efflux of the excitatory amino acids in the infarct region, which would provide an attractive explanation for its neuroprotective action. However, it was also observed to decrease the efflux of the inhibitory amino acids, GABA and taurine. Furthermore the efflux of the amino acids not thought to have a neurotransmitter role in the brain (serine, alanine and asparagine) was decreased by a similar amount.

Since the dialysis probe was placed in the centre of the infarct region, directly under the illumination source, the changes in dialysate concentration reflect biochemical changes in the core of the infarct. This is important as chlormethiazole decreases the spread of damage (Snape *et al.*, 1993; this paper). We therefore have confidence that the neurochemical changes discussed above reflect neurochemical differences in the infarct region, not the fact that less tissue has been damaged in the region of the probe.

Following occlusion of the middle cerebral artery in rats, the rise in extracellular glutamate and aspartate peaked within the first hour post-ischaemia (Butcher *et al.*,1990). After transient forebrain ischaemia, the extracellular concentrations of these amino acids rose even more rapidly (Benveniste *et al.*, 1984). In both models, extracellular levels of 'metabolic' amino acids (such as alanine and serine) increased relatively slowly and reached peak concentrations which were considerably lower than those of the neuroactive amino acids. In the present study the extracellular concentrations of neuroactive amino acids also rose to a greater extent than the metabolic amino acids; however, peak concentrations were not reached until at least 2 h after the onset of ischaemia.

What seems probable therefore is that the rise in the extracellular concentrations of all amino acids reflects the breakdown of damaged cells in the ischaemic area. The attenuation of this rise in the chlormethiazole-treated rats therefore relates to the neuroprotective effect of the drug, and is a reflection of fewer cells being damaged, rather than an inhibition of the ischaemia-induced release of glutamate and aspartate. Consistent with this contention was the observation that chlormethiazole, even at a high (1 mM) concentration, did not inhibit the hypoxia-induced release of glutamate *in vitro*.

Further support for these proposals comes from the fact that chlormethiazole administration afforded around 30% protection measured histologically and also decreased the efflux of amino acids into the extracellular space in the infarct area by approximately the same amount.

It should also be noted that the increase in extracellular lactate concentrations was diminished in the chlormethiazole-treated rats. However, there was no difference between control and chlormethiazole-treated rats in the lactate concentration in the dialysate collected over the first 30 min following the ischaemic episode. This suggests that chlormethiazole did not affect this immediate effect of ischaemia. In this model, damage occurs rapidly (Grome *et al.*, 1988) with Snape *et al.* (1993) observing that approximately 50% of the area of oedema and extravasation measured at 24 h was apparent 30 min after the infarct, a time when chlormethiazole had no effect on lactate concentrations.

Again it is likely that the difference in extracellular lactate after this time relates to neuroprotection. However, what cannot be ruled out at present is that a difference was not observed in lactate concentration between control and experimental groups in the first 30 min because chlormethiazole was either altering blood flow or routes of energy metabolism.

As stated earlier glutamate has often been claimed to play a key role in the pathological processes associated with neurodegeneration following an ischaemic episode. We have previously questioned its involvement in the photochemical model, based on our finding that dizocilpine and NBQX were not neuroprotective (Baldwin *et al.*, 1993a). The current study does not suggest that this opinion is unreasonable. Chlormethiazole did decrease the rise in extracellular glutamate which followed the ischaemic insult. However, it failed to have any effect on the release of this neurotransmitter from brain slices exposed to an hypoxic insult. We are therefore forced to conclude that the chlormethiazole-induced effect on glutamate *in vivo* is a reflection of its neuroprotective action which prevents cell death and concomitant glutamate release rather than an inhibition of glutamate release thereby affording neuroprotection. In this model of ischaemia therefore increased extracellular glutamate concentrations appear to be a consequence of neurodegeneration rather than the cause.

In conclusion, the *in vivo* microdialysis data described in this study have provided neurochemical data which support the histological evidence for the neuroprotective action of chlormethiazole in the photochemical model (Snape *et al.*, 1993; this paper). Although the results do not give any plausible explanation for the mechanism(s) by which chlormethiazole produces its protective effect, they do indicate that a specific action on excitatory amino acid release is unlikely.

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