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Neuroprotective activity of chlormethiazole following transient forebrain ischaemia in the gerbil

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1 The effect of chlormethiazole, and other drugs which potentiate γ-aminobutyric acid (GABA) function on delayed neuronal death in the hippocampus has been examined in the gerbil.

2 Chlormethiazole (100 mg kg^{-1}, i.p.) and two other drugs previously reported to be neuroprotective (dizocilpine, 3 mg kg^{-1}, i.p. and ifenprodil, 4 mg kg^{-1}, i.p.) were all found to prevent neurodegeneration of CA1/CA2 neurones in the hippocampus when given 30 min before a 5 min episode of bilateral carotid artery occlusion.

3 Chlormethiazole (100 mg kg^{-1}) was neuroprotective when given up to 3 h, after the ischaemic episode.

4 Given 1 h after the cartoid artery occlusion, chlormethiazole produced significant protection against hippocampal neurodegeneration at a dose of 50 mg kg^{-1}, but not at 25 mg kg^{-1}.

5 Phenobarbitone (100 mg kg^{-1}, i.p.) and Saffan (alphaxalone, 45 mg kg^{-1} plus alphadalone, 15 mg kg^{-1}, i.p.) were not protective when given 1 h after the ischaemic episode while pentobarbitone (30 mg kg^{-1}, i.p.) had a modest protective effect.

6 Evidence is presented to show that neither the operating procedure nor the chlormethiazole administration lowered rectal or cerebral temperature.

7 The data suggest that chlormethiazole may be a useful treatment in the prevention of neurodegeneration following stroke or cardiac arrest.

Keywords: Ischaemia; neuroprotection; chlormethiazole; hippocampal neurodegeneration; barbiturates; dizocilpine; ifenprodil

Introduction

Transient forebrain ischaemia can be induced in the Mongolian gerbil by bilateral occlusion of the common cartoid arteries (Crockard *et al.*, 1980). Following a brief episode of ischaemia a characteristic and highly specific pattern of neuronal degeneration is observed, with the large neurones of the hippocampal CA1/CA2 subfields being particularly sensitive (Brown *et al.*, 1979). The degeneration of these neurones develops following a period of 24 h during which no obvious morphological changes are apparent, a process which has been termed 'delayed neuronal death' (Kirino, 1982).

A large number of studies have examined the efficacy of various pharmacological agents in preventing the delayed degeneration of hippocampal CA1 neurones. Most prominent amongst these agents are antagonists of excitatory amino acid receptors, particularly the N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) preferring subtypes (see Meldrum, 1990 for review). It is clear that NMDA receptor antagonists exert a powerful neuroprotective action, consistent with the concept that release of glutamic acid (or another excitatory amino acid) mediates the delayed neuronal death. It is thought likely that glutamic acid initiates an 'excitotoxic' process (Olney, 1988) involving excessive membrane depolarization and pathological rises in internal calcium ion concentrations (Simon *et al.*, 1984).

Many other drugs have been examined in the gerbil model of transient forebrain ischaemia. Several compounds which enhance inhibitory processes in the hippocampus have been shown to have some neuroprotective effect, particularly when treatment is initiated around the time of the ischaemic episode (Kirino *et al.*, 1986; De Leo *et al.*, 1988; Alps *et al.*, 1988; Taft *et al.*, 1989). Chlormethiazole is an anticonvulsant, sedative and hypnotic drug (Ogren, 1986; Green & Murray, 1989), which acts at the γ-aminobutyric acid (GABA) receptor both to potentiate the effects of GABA and to modulate channel opening directly (Harrison & Simmonds, 1983; Moody & Skolnick, 1989; Cross *et al.*, 1989). In the present study we have examined the effects of chlormethiazole in the gerbil model of transient forebrain ischaemia, and compared these effects with those of other compounds which modulate $GABA_A$ receptor function.

Some of these studies have been described in a communication to the British Pharmacological Society (Cross *et al.*, 1991).

Methods

Animals and operative procedures

Male Mongolian gerbils weighing 60–80 g were used. They were maintained on a 12 h light/12 h dark cycle (lights on: 07 h 00 min–19 h 00 min) and given food and water *ad libitum*.

Animals were anaesthetized with Saffan (alphaxalone 45 mg kg^{-1} plus alphadalone 15 mg kg^{-1}, i.p.) placed on an electrically heated mat and operated on while under heating lamps to maintain body temperature at 37°C (continuously monitored by rectal probe). The carotid arteries were exposed by cervical midline incision, separated from the vagus nerve and occluded for 5 min with aneurism clips, the effectiveness of the occlusion being confirmed visually.

Following the period of ischaemia the clips were removed and the incision sutured. Sham operated animals had their arteries exposed but not occluded.

In some experiments halothane anaesthesia was employed. In this case anaesthesia was induced with 5% halothane in 70% N_2O/30% O_2, and maintained with 1.5% halothane in 70% N_2O/30% O_2.

Measurement of hippocampal damage

Four days following carotid artery occlusion the animals were anaesthetized with pentobarbitone (300 mg kg^{-1}, i.p.) and perfused transcardially with 10% formaldehyde in 5% sucrose. Brains were removed and placed in 5% formaldehyde in 30%

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sucrose at 4°C for 2 days. Coronal 20 μm sections were cut through the hippocampus with a cryostat and stained with cresyl violet. Sections from each brain taken at the level of the hippocampus (analogous to level A 3290 μm of the rat according to the atlas of Konig & Klippel, 1963) were used to assess the extent of damage to hippocampal neurones. All assessments of histological sections were made by an observer who was unaware of the drug treatment of the animals.

The extent of neurodegeneration in the hippocampal CA1/CA2 region was routinely determined by a method similar to that described by Gill *et al.* (1987). One section from each brain was photographed and measurements taken from both hippocampi. The total length of the CA1/CA2 region containing degenerated neurones was measured. This was possible as the degeneration occurred in discrete patches (see Gill *et al.*, 1987). The length of the degenerated CA1/CA2 region was expressed as a percentage of the entire CA1/CA2 region (i.e. % degeneration), in this way correcting for any minor differences in the level of sectioning through the hippocampus.

Brain temperature measurement

Brain temperature was measured with a thermocouple probe (29 gauge, Model 3AT-12, Physitemp). Gerbils were anaesthetized and a 7.5 mm stainless steel cannula (23 gauge) implanted above the hippocampus (stereotaxic coordinates from bregma; 0 mm anterior, 2 mm lateral, 1 mm ventral). The following day the gerbils were anaesthetized with Saffan and the temperature probe inserted into the cannula. Bilateral carotid artery occlusion was performed as above, and 1 h post-occlusion the animals received chlormethiazole (100 mg kg^{-1}, i.p.). Throughout the experiment (i.e. 2 h) rectal temperature was maintained at 37°C, thus mimicking exactly the neuroprotection study.

Drugs

Drugs were obtained from the following sources (in parentheses): Saffan, (Veterinary Drug Co, U.K.), sodium pentobarbitone (Sagatal, RMD Animal Health Ltd, U.K.), phenobarbitone (Sigma London Ltd, U.K.), dichlormethiazole ethane disulphonate (Astra Research Centre, Sodertalje, Sweden). Ifenprodil and dizocilpine (MK-801) were generous gifts from Synthelabo Recherche, France and Merck, Sharp & Dohme, U.K. respectively. Drugs were administered intraperitoneally in isotonic saline, and all doses refer to the free base. Control animals received saline injections.

Statistics

Each set of data was evaluated by Student's t test or one way analysis of variance as appropriate. In the latter case, individual group differences were determined by use of adjusted t-tests with significance levels corrected by the Bonferroni method.

Results

Pretreatment with chlormethiazole and other drugs

Transient occlusion of the carotid arteries of the gerbil for 5 min resulted in marked degeneration of hippocampal CA1/CA2 neurones 4 days post-ischaemia (Figure 1). This damage did not extend into the CA3 region or the dentate gyrus. Pretreatment with dizocilpine (3 mg kg^{-1}, i.p.) or ifenprodil (4 mg kg^{-1}, i.p.) resulted in a significant reduction in the extent of damage to the CA1/CA2 pyramidal neurones (Figure 2). In most cases the CA1 region of animals pretreated with

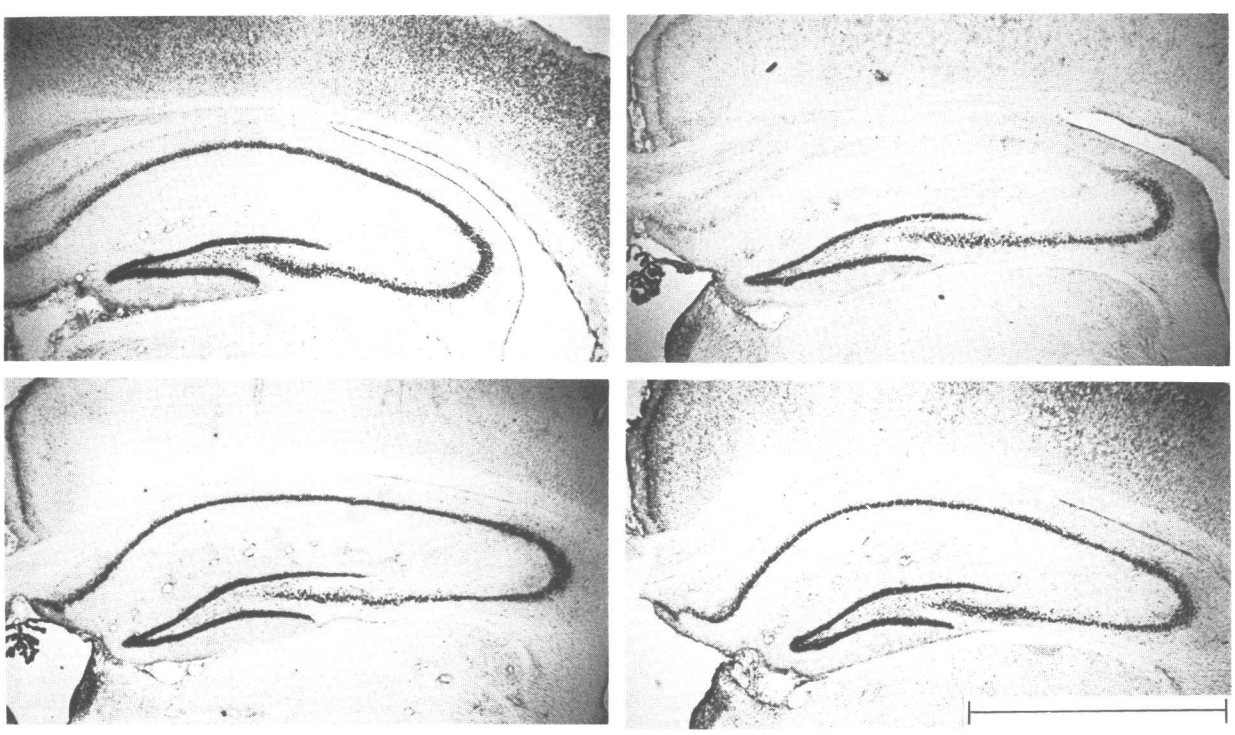

Figure 1 Neuroprotective effect of dizocilpine (3 mg kg^{-1}) and chlormethiazole (100 mg kg^{-1}) when given 30 min before 5 min bilateral carotid artery occlusion. Micrographs of the hippocampus (20 μm coronal sections) stained with Cresyl Violet, 4 days post-ischaemia. Upper left: control; upper right: ischaemia; lower right: chlormethiazole/ischaemia; lower left: dizocilpine/ischaemia. Note almost complete destruction of CA1/CA2 region in ischaemic hippocampus, and protection of this region by both chlormethiazole and dizocilpine. Scale bar = 2 mm.

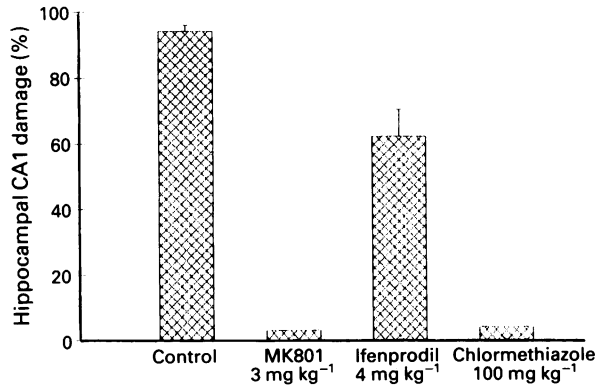

Figure 2 The effect of chlormethiazole, dizocilpine (MK-801) and ifenprodil on hippocampal CA1/CA2 degeneration following 5 min transient ischaemia. All drugs were administered as i.p. injections 30 min before bilateral carotid artery occlusion; controls received saline. Columns represent mean with s.e.mean shown by vertical bars; $n = 6$–10. ANOVA $F = 143$, d.f. = 3, 26, $P < 0.01$. All treatment groups are significantly different from control ($P < 0.01$).

dizocilpine (30 min) showed no obvious damage (Figure 1). Pretreatment with chlormethiazole (100 mg kg^{-1}, i.p.) also resulted in marked protection of hippocampal CA1/CA2 neurones (Figure 2) and again no obvious histological damage

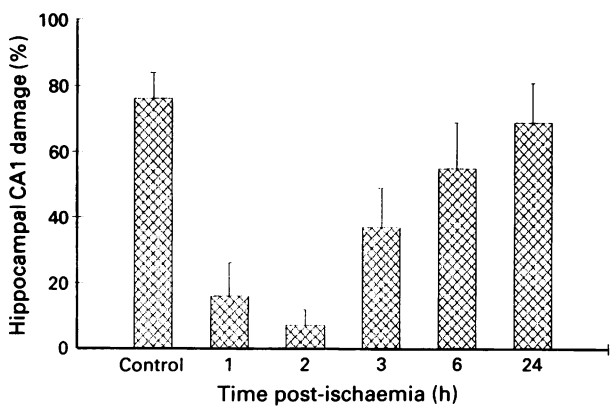

Figure 3 The effect of chlormethiazole on hippocampal CA1/CA2 pyramidal cell degeneration when administered post-ischaemia. Chlormethiazole (100 mg kg^{-1}) was administered i.p. in saline at the indicated times after 5 min bilateral carotid artery occlusion, controls received saline. Columns represent mean with s.e.mean shown by vertical bars, $n = 8$–24. ANOVA $F = 7.08$, d.f. = 5, 62, $P < 0.01$. Treatment groups at 1 h and 2 h ($P < 0.01$) and 3 h ($P < 0.05$) were significantly different from control.

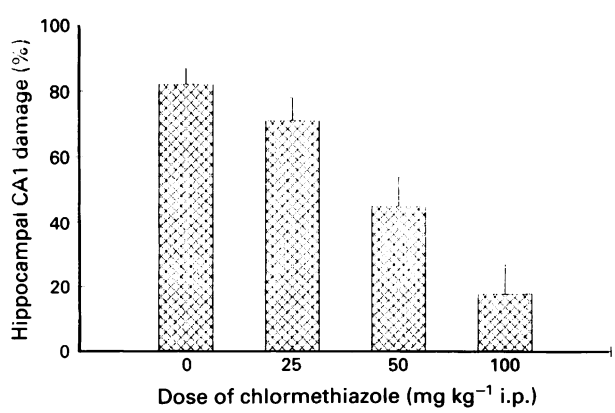

Figure 4 The effect of various doses of chlormethiazole on hippocampal CA1/CA2 degeneration following 5 min ischaemia. Chlormethiazole was administered by i.p. injection in saline 1 h post-ischaemia, controls received saline. Columns represent mean with s.e.mean shown by vertical bars; $n = 11$–12. ANOVA $F = 16.3$, d.f. = 3, 43, $P < 0.01$. Treatment with 50 and 100 mg kg^{-1} chlormethiazole produced significantly less damage ($P < 0.01$).

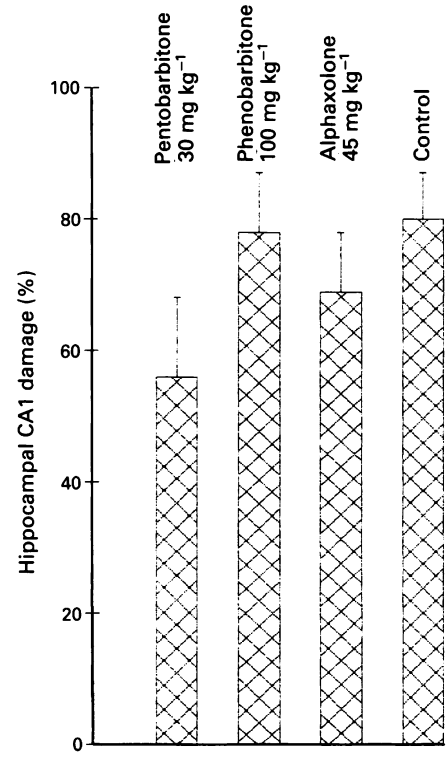

Figure 5 The effect of γ-aminobutyric acid (GABA)-potentiating drugs on hippocampal CA1/CA2 degeneration following 5 min ischaemia. Drugs were given i.p. in saline 1 h post-ischaemia, controls received saline. Columns represent mean with s.e.mean shown by vertical bars; $n = 6$–24. ANOVA $F = 3.06$, d.f. = 3, 56, $P < 0.05$. Treatment with phenobarbitone (100 mg kg^{-1}) or alphaxolone (45 mg kg^{-1} + 15 mg kg^{-1} alphadalone) did not significantly reduce hippocampal damage. Treatment with pentobarbitone (30 mg kg^{-1}) resulted in significantly less damage ($P < 0.05$).

was present in the hippocampus of most chlormethizole treated animals (Figure 1). This dose of chlormethiazole induced sedation but not loss of the righting reflex.

Treatment with chlormethiazole post-ischaemia

Chlormethiazole (100 mg kg^{-1}) produced a marked neuroprotective effect when given up to 3 h after the carotid artery

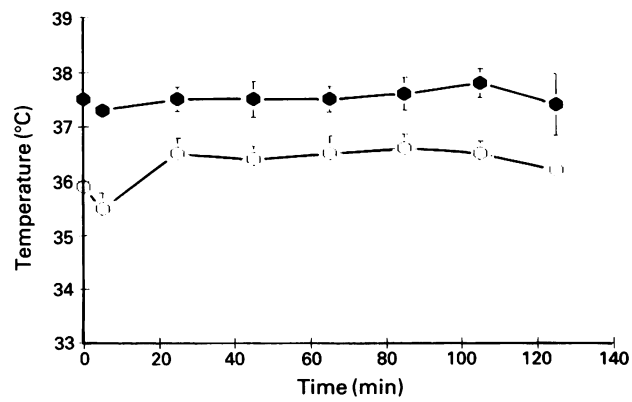

Figure 6 Brain and rectal temperature following transient forebrain ischaemia and subsequent chlormethiazole treatment. Rectal temperature (●) and hippocampal temperature (○) were measured as described in the text. Bilateral carotid artery occlusion was performed immediately after the first temperature measurement (t = 0) for 5 min; 60 min later (t = 65) animals received chlormethiazole (100 mg kg^{-1} i.p.). Rectal temperature was maintained between 37–38°C with heated mats and lamps. Symbols represent mean with s.e.mean shown by vertical bars; $n = 4$.

occlusion (Figure 3). The degree of protection seen when the drug was given 6 h after the ischaemic episode failed to reach statistical significance and no protection was observed when the drug was given 24 h after the insult (Figure 3).

Various doses of chlormethiazole were examined for their protective effect when given 1 h after the ischaemic episode (Figure 4). Whilst 50 mg kg^{-1} chlormethiazole produced significant protection, this was not apparent following a dose of 25 mg kg^{-1}. A log dose versus % protection plot was linear (not shown), suggesting that a protection of 50% neurone loss would be achieved with a dose of chlormethiazole of 55 mg kg^{-1}.

A series of experiments were performed with halothane anaesthesia. In these experiments the duration of occlusion was limited to 4 min, to produce the same degree of neuronal damage as under Saffan anaesthesia. When chlormethiazole (100 mg kg^{-1}) was given 1 h after the ischaemic episode, a marked neuroprotective effect was still observed (saline: 72 ± 10, $n = 12$, chlormethiazole: 37 ± 7, $n = 12$, $P < 0.01$).

The effects of GABA potentiating drugs

The effect of administering 3 other GABA potentiating drugs after the ischaemic episode was also investigated. Saffan had no significant effect on CA1 neurone degeneration and phenobarbitone (100 mg kg^{-1}, i.p.) was also without effect (Figure 5). A sedative dose of pentobarbitone (30 mg kg^{-1} i.p.) did produce a small but significant reduction of the damage to CA1/CA2 neurones (Figure 5).

The effects of chlormethiazole on brain temperature

Brain and rectal temperatures were recorded in 4 gerbils during carotid artery occlusion and subsequent chlormethiazole treatment. Following the protocol described, rectal temperature was successfully maintained between 37–38°C (Figure 6). While the temperature of the hippocampus did not decrease during the occlusion there was a small but statistically significant rise afterwards (Figure 6). Administration of chlormethiazole did not alter cerebral temperature (Figure 6).

Discussion

The present study clearly demonstrates that chlormethiazole prevents the degeneration of hippocampal CA1/CA2 pyramidal neurones following transient forebrain ischaemia in the gerbil. There are, however, a couple of factors which need to be considered when comparing the results of the present study with other published studies.

The current study generally used the injectable anaesthetic Saffan rather than an inhalant anaesthetic such as the commonly used halothane. It has previously been shown that the severity of hippocampal damage following ischaemia varies with the anaesthetic agent (Lightfoote *et al.*, 1977; Kuroiwa *et al.*, 1989). However, chlormethiazole was protective when either Saffan or halothane had been used. Furthermore, dizocilpine was protective in Saffan-anaesthetized animals (this study) to a similar degree to that seen in halothane anaesthetized animals (Gill *et al.*, 1987). Additionally, ifenprodil, which is neuroprotective in the rat and cat (Gotti *et al.*, 1988) was shown to be neuroprotective in our experiments in the gerbil. We do not feel therefore that the choice of anaesthetic influenced the results obtained. This view is supported by the failure of a second anaesthetic dose of Saffan to reduce hippocampal damage following ischaemia. We would also suggest that Saffan is easier to use than an inhalant anaesthetic in the operative procedure.

A second point is that chlormethiazole administration produces hypothermia in gerbils (unpublished observations) and it is well established that hypothermia can be neuroprotective (Busto *et al.*, 1987). Care was therefore taken to maintain body temperature during the ischaemic episode and subsequent drug treatment. The success was evidenced by the observation that brain as well as body temperature was unaltered by drug treatment, rendering unlikely any proposal that chlormethiazole was neuroprotective because it produced hypothermia.

In common with several other anticonvulsant drugs with diverse mechanisms of action, including phenytoin, barbiturates, diazepam and etomidate (Taft *et al.*, 1989; Sternau *et al.*, 1989; Hermans *et al.*, 1983), chlormethiazole was an effective neuroprotective drug when given just prior to the ischaemic episode. What distinguishes chlormethiazole from these other drugs is its high degree of efficacy as a neuroprotectant when given up to several hours after the ischaemic insult. It has previously been shown that phenytoin, diazepam and etomidate are ineffective when given after ischaemia (Sternau *et al.*, 1989) and the current study demonstrated a lack of effect of phenobarbitone. Only pentobarbitone had any effect when given 60 min after the ischaemic episode, and this was marginal.

While it is possible therefore that the neuroprotective effect of chlormethiazole is related to its anticonvulsant activity when given before the ischaemic episode, the failure of other anticonvulsants to exert neuroprotection when given post-ischaemia suggests a different mechanism of action is required to explain its efficacy when given 2–3 h after the ischaemic insult.

Electrophysiological and biochemical experiments have demonstrated that whilst chlormethiazole can potentiate the action of GABA acting at the $GABA_A$ receptor, chlormethiazole can also directly modulate $GABA_A$ receptor function independent of agonist (Harrison & Simmonds, 1983; Moody & Skolnick, 1989). In contrast to benzodiazepines and barbiturates, this action may occur at lower concentrations of chlormethiazole than those required to potentiate GABA agonists (Simmonds & Turner, 1987). It may well be that this distinct profile of action accounts for the neuroprotective effect of chlormethiazole during the post-ischaemic period. It is clear that during ischaemia and immediately following reperfusion there is a massive efflux of neuroactive amino acids, including GABA and glutamate (Benveniste *et al.*, 1984; Hagberg *et al.*, 1985; Globus *et al.*, 1988). However, extracellular concentrations of GABA and glutamate return to normal shortly after reperfusion. Whilst GABA-containing interneurones in the hippocampus have generally been found to be remarkably resistant to ischaemic damage (Francis & Pulsinelli, 1982; Johansen *et al.*, 1983), there is some evidence to suggest that a reversible functional deficit may occur shortly after ischaemia (Johansen *et al.*, 1987; Grimaldi *et al.*, 1990). It may well be that the ability of chlormethiazole to activate postsynaptic $GABA_A$ receptors in the absence of sufficient endogenous GABA, explains its efficacy.

Following an intraperitoneal dose of 100 mg kg^{-1}, the peak plasma concentration of chlormethiazole in the gerbil is around 200 nmol ml^{-1} (unpublished observations). In man, similar plasma levels have been observed during clinical use of the drug (Christensen *et al.*, 1983). This concentration of chlormethiazole corresponds well with that required for inhibition of [^{35}S]-t-butylbicyclophosphorothionate ([^{35}S]-TBPS) binding to the GABA receptor (IC_{50} 140 μM, Cross *et al.*, 1989).

As has been discussed by Meldrum (1990), the excitotoxic process probably depends on a balance between excitatory and inhibitory mechanisms. Within the hippocampus of the rat the majority of inhibitory interneurones use GABA as transmitter, and $GABA_A$ receptors are present on hippocampal pyramidal neurones (Bowery *et al.*, 1987). Thus any imbalance leading to a net overactivity of excitatory amino acid systems may be counteracted by increasing the activity of the major inhibitory system mediated by GABA. In this respect it is interesting to note that chlormethiazole is an effective inhibitor of the tonic convulsions induced by peripherally administered NMDLA (unpublished data).

Chlormethiazole was effective in reducing the extent of hip-

pocampal damage administered at least 3 h post-ischaemia. Although many compounds have been shown to reduce ischaemic damage in the hippocampus when administered during the ischaemic period, few are effective when administered post-ischaemia. Prominent amongst these are excitatory amino acid antagonists, acting at NMDA (Boast *et al.*, 1987; Gill *et al.*, 1988) or AMPA (Sheardown *et al.*, 1990) subtypes of receptor. However, there are problems with the use of the non-competitive NMDA antagonists since in rodents they have been found to induce a complex motor syndrome and ataxia (See Tricklebank *et al.*, 1989) and can also produce transient pathological changes in the morphology of specific populations of brain neurones (Olney *et al.*, 1989). Both these observations raise questions as to the safety of such drugs in clinical use. No evidence has been obtained for chlormethiazole acting at the glutamate, glycine or polyamine sites of the NMDA receptor complex or at the AMPA receptor (Addae & Stone, 1988; Cross *et al.*, unpublished observations). It therefore seems likely that the neuroprotective action of chlormethiazole results from the known interaction of the drug with the GABA receptor complex (Cross *et al.*, 1989; Moody & Skolnick, 1989; Vincens *et al.*, 1989).

Chlormethiazole has been used clinically as a sedative, hypnotic and anticonvulsant drug for over 25 years (Evans *et al.*, 1986). A safe approach to the treatment of ischaemic stroke involving GABAergic mechanisms can therefore be proposed.

In conclusion, the present results demonstrate that following transient forebrain ischaemia a single dose of chlormethiazole prevents the degeneration of hippocampal pyramidal neurones. Chlormethiazole is effective when administered at least 3 h after ischaemia, suggesting that it may be useful clinically in attenuating the neurodegeneration following stroke or cardiac arrest.

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