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On the regulation of ischaemia-induced glutamate efflux from rat cortex by GABA; *in vitro* studies with GABA, clomethiazole and pentobarbitone

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1 Prisms of adult rat cortex were maintained *in vitro* in either aerobic conditions (control) or conditions simulating an acute ischaemic challenge (hypoxia with no added glucose).

2 Endogenous glutamate efflux increased with time in ischaemic conditions, being 2.7 fold higher than control efflux at 45 min. Returning prisms to control solution after 20 min of simulated ischaemia resulted in glutamate efflux returning to near-control values. Endogenous GABA efflux in ischaemic conditions also increased, being 4.5 fold higher than control efflux at 45 min.

3 Ischaemia-induced glutamate efflux was not accompanied by increased lactate dehydrogenase efflux and was unaltered by omitting calcium from the extra-cellular solution and adding EGTA (0.1 mM).

4 Both GABA and the GABA-mimetic clomethiazole inhibited ischaemia-induced glutamate efflux, with IC_{50} values of 26 and 24 μM respectively. The maximum inhibition by either drug was 60–70%. Bicuculline (10 μM) abolished the inhibitory effect of GABA (100 μM) but not clomethiazole (100 μM). Picrotoxin (100 μM) abolished the action of both GABA and clomethiazole.

5 Pentobarbitone inhibited glutamate efflux at 100–300 μM (maximal inhibition: 39%). Bicuculline (10 μM) abolished this effect.

6 These data suggest that ischaemia-induced glutamate efflux from rat cerebral cortex is calcium-independent and not due to cell damage up to 45 min. The inhibitory effect of GABA, clomethiazole and pentobarbitone on ischaemia-induced glutamate efflux appears to be mediated by $GABA_A$ receptors. The results suggest that clomethiazole, unlike pentobarbitone, is able to activate the $GABA_A$ receptor-linked chloride channel directly and not merely potentiate the effect of endogenous GABA.

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Keywords: Ischaemia; glutamate efflux; clomethiazole; GABA; pentobarbitone; bicuculline; picrotoxin; stroke

Abbreviations: ANOVA, analysis of variance; Bic, bicuculline; CMZ, clomethiazole; EGTA, ethylene glycol-bis (β-aminoethyl-ether)-N,N,N′, N′-tetra-acetic acid; GABA, γ-aminobutyric acid; HBS, HEPES buffered saline; LDH, lactate dehydrogenase; MCA, middle cerebral artery, NAD^+, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide, reduced form; $NADP^+$, nicotinamide adenine dinucleotide phosphate; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; Pento, pentobarbitone; Picro, picrotoxin

Introduction

Ischaemic stroke is a multi-factorial pathological process, and pharmacological intervention at numerous molecular and cellular targets has been attempted (Green & Cross, 1997a; Muir & Grossett, 1999; Lee *et al*., 1999; Dirnagl *et al*., 1999; De Keyser *et al*., 1999). Suitable *in vivo* and *in vitro* models are necessary both for examining compounds in development for the treatment of acute ischaemic stroke and also to investigate the pathological processes involved in neurodegeneration (see Hunter *et al*., 1995; Green & Cross, 1997b). *In vivo* models attempt to mimic the pathology of acute ischaemic stroke in laboratory animals (Hunter *et al*., 1995; Green & Cross 1997b) and also provide valuable data on the biochemical cascade that is initiated by ischaemia (Katsura *et al*., 1993; Saluja *et al*., 1999). However, the problems associated with *in vivo* models include the sometimes-complex surgery involved and the variations in models from one laboratory to another (Ginsberg & Busto, 1989). In addition, the surgical processes and drug administration induce physiological changes (for example hypothermia and blood pressure changes) that can markedly alter the degree of neuroprotection seen and complicate interpretation of results (see Green & Cross, 1997b). There is also a continuing difficulty of determining which models are most relevant to the clinical situation (Green & Cross, 1997b; Dirnagl *et al*., 1999; De Keyser *et al*., 1999).

In vitro models often utilize primary cultures, freshly isolated cells or cell-lines, provoking cell death by addition of excitotoxic compounds or by metabolic challenge (e.g. Goldberg *et al*., 1997; Strijbos *et al*., 1996; Nowicky & Duchen, 1998). *In vitro* techniques circumvent some of the physiological variables encountered with *in vivo* models and also remove the requirement for live animals and surgical techniques. However, the cell culture approach lacks a major feature of the intact brain, namely the cellular architecture with its specific intrinsic connections, neurotransmitter interactions and the presence of non-neuronal cellular elements that may be crucial to the action of some neuroprotective agents.

One purpose of this study was to develop an 'intermediate' approach using prisms of cerebral tissue under simulated ischaemia (hypoxia-hypoglycaemia) in which some cyto-

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architecture is retained and physiological parameters can be controlled. We have examined efflux of endogenous glutamate, the excitatory neurotransmitter believed to underlie excitoxic damage in the early phases of neuronal ischaemia (Szatkowski & Attwell, 1994; Small & Buchan, 1997; Lee *et al*., 1999). The second goal of this study was to investigate the effect of clomethiazole, which has been suggested to exert its neuroprotective effect by enhancing $GABA_A$ receptor function (Green, 1998). It has been hypothesized that enhancement of GABAergic function inhibits glutamatergic activity (Lyden, 1997; Green, 1998; Green *et al*., 2000). However it has proved difficult to explain why clomethiazole is neuroprotective while pentobarbitone, which also enhances $GABA_A$ receptor function, is not (Green *et al*., 2000). Some of these data have been published in abstract form (Nelson *et al*., 1999; 2000).

Methods

Materials

γ-Aminobutyric acid (GABA), pentobarbitone, picrotoxin, (−) bicuculline methiodide, Triton X-100, ethylene glycol-bis (β-aminoethyl-ether)-N,N,N′,N′-tetra-acetic acid (EGTA), HEPES acid, $NADP^+$, NADH and pyruvic acid were purchased from Sigma Chemical Co., Dorset, U.K. Glutamate dehydrogenase was obtained from Calbiochem-Novabiochem Corp., Nottingham, U.K and cell microsieve (100 μm) nylon mesh was purchased from BDH, Dorset, U.K. Clomethiazole edisilate was a gift from AstraZeneca R&D Södertääje, Sweden.

General procedures

Adult, female, Wistar rats (200–250 g) were killed by cervical dislocation, the brain rapidly removed and placed in ice-cold Krebs bicarbonate buffer containing (in mM) NaCl 115, KCl 4.6, $MgCl_2$ 1.2, $CaCl_2$ 2, $NaHCO_3$ 25, glucose 8.8, pH 7.4.

Prisms of cerebral cortex (cross sectional area 350 μm^2) were prepared by cross-chopping using a McIlwain tissue chopper then pre-incubated in Krebs bicarbonate buffer at 37°C for 40 min. This was followed by 30 min incubation at 37°C in aerated HEPES buffered saline (control HBS) containing (in mM) NaCl 140, KCl 2.5, $MgCl_2$ 0.5, $CaCl_2$ 2, HEPES acid 10, glucose 10, pH 7.4. Equivalent volumes of slice suspension were aliquoted into mesh baskets and incubated as described below. At the end of each experiment, baskets were transferred to 1% Triton X-100 for 5 min to lyse cell membranes and release any remaining glutamate. The supernatants were spun at 12,000 r.p.m., at 4°C, for 5 min. Glutamate was measured fluorimetrically (excitation: 366 nm, emission: 450 nm), using the conversion of $NADP^+$ to NADPH by glutamate dehydrogenase with minor modifications of published methods (Baldwin *et al*., 1994; Nicol *et al*., 1996).

Time course experiments

Cortical prisms were incubated in 2 ml of control or ischaemic HBS. Ischaemic HBS contained no added glucose and was bubbled with nitrogen for at least 2 h prior to the experiment. Using a dissolved oxygen electrode (Strathkelvin 781 with 1302 dissolved O_2 electrode) it was found that the PO_2 declined rapidly to a steady-state level within 45 min. The PO_2 values for ischaemic HBS were between 5.4–5.7 KPa (measured with an I.L.16/40 blood gas analyser). In contrast, control HBS had a PO_2 of 22.6–23.3 KPa. Baskets were transferred to fresh HBS at 5 min intervals. N_2 gas was blown over the liquid surface of the ischaemic solution throughout the experiment. The dependence of ischaemia-induced glutamate efflux on calcium was determined by incubating prisms in calcium-free ischaemic HBS containing equimolar substitution of $MgCl_2$ and 0.1 mM EGTA.

Effects of GABA-mimetics on ischaemia-induced glutamate efflux

Prisms were subjected to a 30 min uninterrupted incubation. Separate aliquots were incubated in 2 ml of either control or ischaemic HBS with the appropriate gas blown over the surface throughout the experiment (ischaemic: nitrogen, control: air). The ischaemic medium contained various concentrations of GABA (10–300 μM), clomethiazole (10–300 μM) or pentobarbitone (1–300 μM). In further sets of experiments, prisms were incubated with a single concentration of GABA, clomethiazole or pentobarbitone in the presence of bicuculline (10 μM) or picrotoxin (100 μM). Control aliquots and drug-free ischaemic aliquots were assayed in each experiment. Figures show pooled control and ischaemic data from all experiments. In some experiments, the supernatants and tissue lysates were assayed for lactate dehydrogenase (LDH) activity as an indicator of cell death. Lactate dehydrogenase (LDH) activity was measured spectrophotometrically by the rate of decrease in absorbance at 340 nm *via* the reduction of NADH to NAD^+.

Analysis of GABA

Concentrations of GABA were determined by HPLC with fluorescence detection after derivatization with o-phthaldialdehyde in mercaptoproponoic acid. The system consisted of a Hypersil ODS column (5 μm, 150 × 3 mm, with guard column; Chrompack, London, U.K.) with a CMA/280 fluorescence detector (maximum excitation, 340–360 nm; maximum emission, 495 nm: Biotech Instruments, Kimpton, U.K.). The derivatization reagent was prepared by mixing 975 μl of incomplete o-phthaldialdehyde reagent solution (Sigma, Poole, U.K.) with 25 μl of 10% (v v^{-1}) mercaptoproponoic acid in methanol. Derivatization of dialysate samples and standards, and injection on to the column were carried out with a CMA/200 refrigerated autosampler (Biotech Instruments, Kimpton, U.K.). The mobile phase gradient consisted of 50 mM sodium acetate buffer, pH 6.95, with methanol increasing linearly from 2–30% (v v^{-1}) over 27 min, and was delivered by a PM-80 twin-reciprocating pump with LC-26A vacuum degasser (BAS Technicol, Congleton, U.K.). Data was collected and analysed using EZChrom software (Aston Scientific, Stoke Mandeville, U.K.) after calibration with a range of standard aqueous amino acid solutions (0.25–5 μM).

Data analysis

All data are presented as mean ± s.e.mean unless stated otherwise. Glutamate efflux data is expressed as a percentage of the total glutamate present (i.e. $[Glut_L/(Glut_L + Glut_T)] \times 100$, where $Glut_L$ is glutamate in the supernatant and $Glut_T$ is glutamate in the tissue). IC_{50} values were calculated where appropriate by fitting Langmuir-Hill curves to glutamate efflux data after subtraction of the per cent control efflux. Curves were fitted by least squares non-linear regression (Graphpad Prism). Unpaired Student's *t*-test was used for statistical analysis (significance level $P \leqslant 0.05$) in uninterrupted incubation experiments and repeated measures, analysis of variance (ANOVA) with Bonferroni's correction was used for time course

experiments. LDH activity was expressed as a percentage of the total activity present with the equation $(100 \times LDH_L/[LDH_T + LDH_L])$, where LDH_L and LDH_T represent LDH activity from supernatant and tissue respectively.

Results

Glutamate efflux from cerebral cortical prisms in simulated ischaemic conditions

Cerebral cortical prisms maintained in control HBS (normoxic, containing 10 mM glucose) exhibited a modest glutamate efflux into the medium (Figure 1). Incubation of prisms in hypoxic HBS with no added glucose, designed to simulate ischaemic conditions, resulted in an enhanced time-dependent glutamate efflux (Figure 1). Glutamate efflux in ischaemic HBS was 2.7 ± 0.3 fold higher than in control medium at 45 min. Tissue that was returned to the normoxic medium after 20 min in ischaemic conditions exhibited a time-dependent decline in efflux, falling by 40 min, close to the level seen with tissue in the control HBS (Figure 1). Removal of calcium from the medium had no effect on the amount of glutamate efflux under ischaemic conditions (Figure 1), there being no significant difference between the efflux in the two conditions over 45 min.

LDH efflux from cerebral cortical prisms incubated in the ischaemic saline

Incubation of tissue in ischaemic conditions for 30 min did not significantly increase the fraction of LDH activity released from tissue into the supernatant (control: $26.1 \pm 2.5\%$, $n = 7$; ischaemia: $28.4 \pm 4.2\%$, $n = 7$).

Effect of GABA on ischaemia-induced glutamate efflux

GABA (100 μM) inhibited ischaemia-induced glutamate efflux (Figures 2 and 3). This inhibition was concentration-dependent, with a maximal inhibition of $66 \pm 2\%$ at 300 μM (Figure 2A). The IC_{50} for the inhibition was 26 μM (95% C.I. = 16–43 μM). Bicuculline (10 μM) and picrotoxin (100 μM) antagonized the inhibitory effect of GABA (100 μM) on ischaemia-induced glutamate efflux (Figure 3). In control or ischaemic conditions in the absence of GABA bicuculline and picrotoxin did not alter glutamate efflux (data not shown).

Effect of clomethiazole on ischaemia-induced glutamate efflux

Clomethiazole inhibited the ischaemia-induced glutamate efflux in a concentration-dependent manner (Figure 2B) with an IC_{50} of 24 μM (C.I. = 15–37.2 μM). Maximal inhibition by clomethiazole was $62 \pm 6\%$ of the enhanced efflux. Bicuculline (10 μM) did not antagonize the inhibition of glutamate efflux induced by clomethiazole (Figure 4). In contrast, picrotoxin (100 μM) abolished this inhibitory effect (Figure 4).

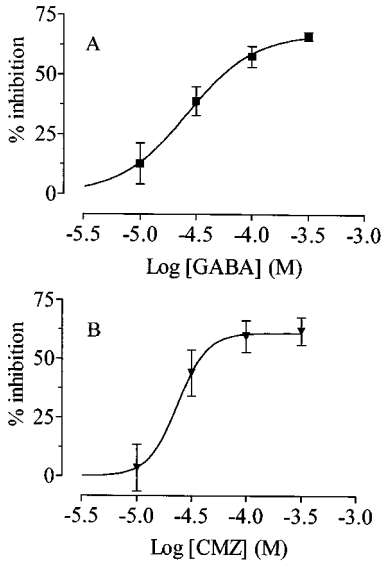

Figure 2 Effect of increasing concentrations of GABA (A) or clomethiazole (CMZ, B) on ischaemia-induced glutamate efflux from rat cortical prisms. Results reported as the per cent inhibition of the ischaemia-induced efflux are shown as mean ± s.e.mean ($n = 5$). For GABA, calculated $IC_{50} = 26$ μM and Hill coefficient = 1.4. In the case of CMZ calculated $IC_{50} = 24$ μM and Hill coefficient = 3.2.

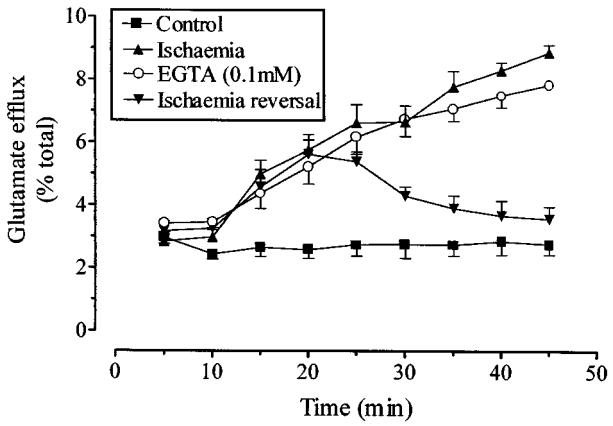

Figure 1 Effect of control conditions (Control) and simulated ischaemia (Ischaemia) on glutamate efflux from rat cortical prisms. The effects of return to normoxic medium at 20 min (Ischaemia reversal) and of a low calcium concentration in the medium (EGTA (0.1 mM); see Methods for details) are also shown. Results are shown as mean ± s.e.mean ($n = 6$). Glutamate efflux in ischaemic conditions was different from that in the control medium ($F = 139.8$, df 3,8, $P < 0.01$). There was no difference in glutamate efflux between the Control and Ischaemia reversal groups at 45 min (unpaired 't'-test). There was no effect of low calcium concentration on glutamate efflux ($P > 0.05$) over the period of observation.

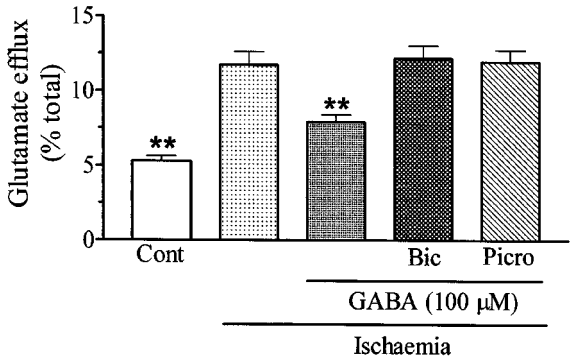

Figure 3 Effect of control conditions (Cont) or simulated ischaemia (Ischaemia) on total glutamate efflux from rat cortical prisms at 30 min and the effect of GABA (100 μM). The effect of bicuculline (Bic; 10 μM) and picrotoxin (Picro; 100 μM) on the inhibitory effect of GABA on glutamate efflux is also shown. Results shown as mean ± s.e.mean ($n = 4–9$). **Different from Ischaemia ($P < 0.01$). Addition of bicuculline or picrotoxin abolished the effect of GABA on ischaemia-induced glutamate efflux. Addition of GABA to the control medium did not affect efflux (data not shown).

Effect of pentobarbitone on ischaemia-induced glutamate release

Pentobarbitone (1–300 μM) produced a modest inhibition of ischaemia-induced glutamate release that was significant only at concentrations of 100 and 300 μM (Figure 5). The maximum inhibition by pentobarbitone (300 μM) was $39 \pm 5\%$ and this was completely antagonized by bicuculline (10 μM) (Figure 5).

GABA efflux from cerebral prisms in simulated ischaemic conditions

The concentration of endogenous GABA in the supernatant increased over time in the ischaemic medium, the major increase occurring after exposure to the ischaemic conditions for 30 min or more (Figure 6). The GABA concentration in the supernatant at 45 min was 3.8 ± 1.4 μM in ischaemic conditions and 0.8 ± 0.3 μM in control conditions (mean, s.d., $n = 3$).

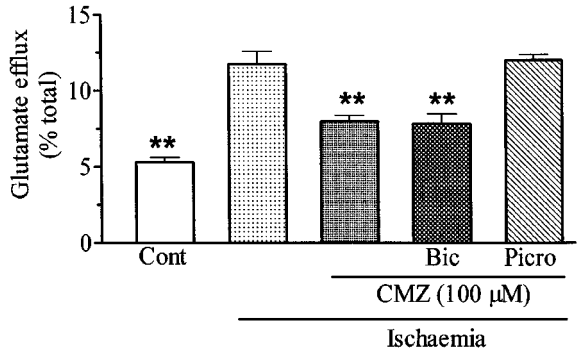

Figure 4 Effect of control conditions (Cont) or simulated ischaemia (Ischaemia) on total glutamate efflux from rat cortical prisms in 30 min and the effect of clomethiazole (CMZ; 100 μM). The effect of bicuculline (Bic; 10 μM) and picrotoxin (Picro; 100 μM) on the inhibitory effect of clomethiazole on glutamate efflux is also shown. Results shown as mean $\pm$ s.e.mean ($n = 6$). **Different from Ischaemia ($P < 0.01$). Addition of picrotoxin, but not bicuculline, abolished the effect of clomethiazole on ischaemia-induced glutamate efflux. Addition of clomethiazole to the control medium did not affect efflux (data not shown).

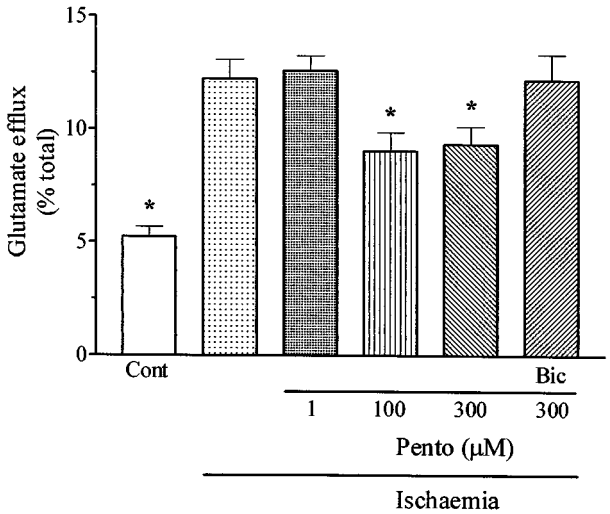

Figure 5 Effect of control conditions (Cont) or simulated ischaemia (Ischaemia) on total glutamate efflux from rat cortical prisms in 30 min and the effect of pentobarbitone (Pento). There was no effect of pentobarbitone at concentrations of 30 μM or below. The effect of bicuculline (Bic; 10 μM) on the inhibitory effect of pentobarbitone (300 μM) on glutamate efflux is also shown. Results shown as mean $\pm$ s.e.mean ($n = 5$). Addition of bicuculline abolished the effect of pentobarbitone on ischaemia-induced glutamate efflux. Addition of pentobarbitone to the control medium did not affect efflux (data not shown). *Different from ischaemia, $P < 0.05$.

Discussion

It is generally accepted that increased glutamatergic activity, resulting from the elevated extracellular glutamate which occurs in the brain during an ischaemic episode (Benveniste *et al*., 1984; Baldwin *et al*., 1994; Lee *et al*., 1999), is a crucial initiating event, leading to cell death (see Szatkowski & Attwell, 1994; Kristián & Siesjö, 1998; Green *et al*., 2000). It has previously been reported that exposing cerebral tissue to ischaemic conditions *in vitro* can induce glutamate release and this has been demonstrated using cerebral tissue from both rat (Taylor *et al*., 1995; Roettger & Lipton, 1996; Saransaari & Oja, 1997) and human (Hegstad *et al*., 1996). The results from the current study confirmed these findings in prisms of rat cortex, simulating ischaemia by use of a hypoxic medium, with no added glucose. Cortical tissue was used in the current study because this region is severely compromized by occlusion of the middle cerebral artery (MCA) *in vivo* both in animals and humans (e.g. Sydserff *et al*., 1995; Marshall *et al*., 1999). In keeping with other *in vitro* studies, the ischaemia-induced glutamate release observed here was considerably smaller in magnitude than that generally reported *in vivo* (e.g. Benveniste *et al*., 1984; Baldwin *et al* 1994). However the magnitude of release observed here was similar to that reported in human cerebral cortex tissue *in vitro* (Hegstad *et al*., 1996). Hypoxia alone (without removal of glucose) also induced glutamate release (Baldwin *et al*., 1994), albeit of a somewhat lower magnitude than that produced by simulated ischaemia (current study).

The fact that returning the tissue to the control medium reversed the ischaemia-induced glutamate efflux indicated that the increase in extracellular glutamate was not due to tissue damage. Rather, the enhanced glutamate efflux was a specific response of the tissue to simulated ischaemia. This proposal is supported by the lack of increase in LDH activity in the medium during the incubation period. It is probable that longer incubation periods in ischaemic conditions would have led to more universal damage (Goldberg *et al*., 1997).

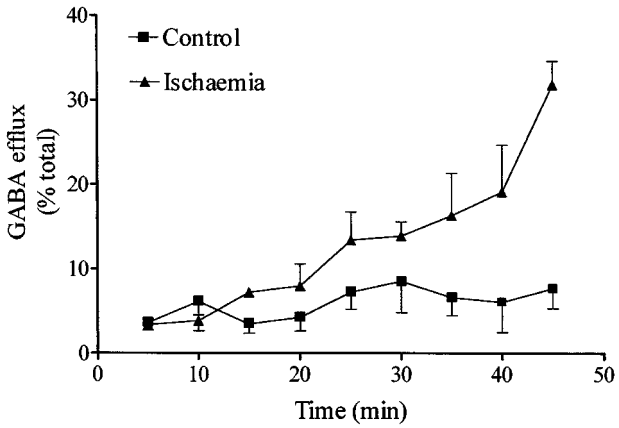

Figure 6 Effect of control conditions (Control) or simulated ischaemia (Ischaemia) on the GABA concentration in the normoxic and ischaemic medium during 45 min of incubation. Results shown as mean $\pm$ s.e.mean ($n = 3$). There was an effect of time ($F = 6.66$, df 1,8, $P < 0.001$) and also of treatment ($F = 28.06$, df 1,8, $P < 0.001$).

The ischaemia-induced efflux exhibited little sensitivity to removal of extracellular calcium. Our results cannot totally exclude a calcium-dependent component of efflux, but clearly the majority of glutamate efflux is calcium-independent, in line with that seen in other *in vitro* studies (Kauppinen *et al*., 1988; Szatkowski & Attwell, 1994; Hegstad *et al*., 1996; Polischuk *et al*., 1998; Saransaari & Oja, 1998). This calcium-independence is somewhat paradoxical, given that the excitotoxic cascade of ischaemia-induced glutamate efflux *in vivo* is usually assumed to involve some synaptic, and hence calcium-dependent, release (Strijbos *et al*., 1996; Lee *et al*., 1999; Dirnagl *et al*., 1999).

During an ischaemic episode the extracellular cerebral GABA concentration increases (e.g. Baldwin *et al*., 1994). However, GABA synthesis is decreased (Green *et al*., 1992) probably *via* an auto-inhibition mechanism (Green *et al*., 2000). It was previously proposed that enhancement of this depressed GABAergic function would be neuroprotective, by increasing inhibitory tone and thereby inhibiting glutamatergic activity (Green, 1998; Green *et al*., 2000). This proposal was initiated by the suggestion of Meldrum (1990) that the excitotoxic process depended on a balance between excitatory and inhibitory mechanisms. These speculations were supported by the observations that the GABA-mimetics muscimol and clomethiazole are neuroprotective (Cross *et al*., 1991; 1995; Shuaib *et al*., 1993; Lyden, 1997; Green, 1998) and the current data. In confirmation of earlier reports (e.g. Hegstad *et al*., 1996) ischaemia-induced glutamate release was inhibited dose-dependently by GABA, maximal concentrations inhibiting 60–70% of the ischaemia-induced glutamate efflux. The effect of GABA was fully antagonized both by bicuculline, a competitive antagonist that acts at the agonist binding site on the $GABA_A$ receptor complex and by picrotoxin, a compound which occludes the open channel of the $GABA_A$ receptor complex. There seems little reason therefore to doubt that the action of GABA on enhanced glutamate efflux is mediated by $GABA_A$ receptors.

GABA efflux also increased during exposure to ischaemic medium. The major increase occurred later than the glutamate rise and this is consistent with earlier *in vivo* observations in ischaemic tissue (Baldwin *et al*., 1994). While the concentration of endogenous GABA seen in the ischaemic supernatant at 45 min (3.8 μM) is insufficient to inhibit glutamate release (see Figure 2A, the synaptic concentration *in vivo* will be considerably higher. For example, the release of endogenous GABA *in vivo*, measured by microdialysis, gives ischaemia-induced GABA concentrations of around 10 μM (Baldwin *et al*., 1994). Given the low efficiency of microdialysis probes, the extracellular GABA concentration in ischaemic brain tissue is likely to be considerably higher, probably in the concentration range used in the current study. We also examined the effects of clomethiazole and pentobarbitone, two compounds that potentiate $GABA_A$ receptor function. Both bind to related – but not identical – sites associated with the chloride channel of the $GABA_A$ receptor ionophore complex (Cross *et al*., 1989; Moody & Skolnick, 1989; Green *et al*., 1996; Zhong & Simmonds, 1997). Neuroprotection studies have highlighted functional differences between these two drugs. Clomethiazole is an established neuroprotective drug, with demonstrated efficacy in models of both global and focal ischaemia (Green, 1998). Pentobarbitone, in contrast, has little or no efficacy as a neuroprotectant (Sternau *et al*., 1989; Cross *et al*., 1991; Ito *et al*., 1999).

The current study emphasizes differences between clomethiazole and pentobarbitone in their action on ischaemia-induced glutamate release and provides a possible explanation for the difference in their neuroprotective activity *in vivo*. Clomethiazole inhibited ischaemia-induced glutamate efflux over a very similar concentration range to GABA and the maximum degree of inhibition was also very similar. Pentobarbitone, on the other hand, inhibited glutamate efflux only at a concentration $\geqslant 100$ μM. Additionally, the inhibitory action of clomethiazole (100 μM) was abolished by picrotoxin, but unaltered by bicuculline at a concentration (10 μM) that fully antagonized the effects of GABA and pentobarbitone.

Our results with bicuculline suggest that inhibition by pentobarbitone (but not clomethiazole) in our assay results from potentiation of the action of the endogenous GABA that we have shown to be released (Figure 6). This suggests that clomethiazole can activate the chloride channel directly and that it is not merely potentiating the effect of endogenous GABA. Clomethiazole is known to potentiate the action of GABA and also to activate the receptor in the absence of GABA (Harrison & Simmonds, 1983; Hales & Lambert, 1992; Anderson *et al*., 1993). Earlier studies indicated that the direct channel opening effect occured only at high (millimolar) concentrations of clomethiazole and was bicuculline-sensitive (Hales & Lambert, 1992). The current data show that in this functional tissue prism preparation, an effect on $GABA_A$ channel function can occur at a concentration at least 10 fold lower. The experiments with bicuculline and picrotoxin further demonstrate that the effect is not due to an action of clomethiazole at the (bicuculline-sensitive) GABA recognition site.

Also noteworthy is the IC_{50} value for clomethiazole inhibiting glutamate release obtained here, 24 μM. This is in the plasma concentration range (10–30 μM) that is required for neuroprotection *in vivo* (Cross *et al*., 1995; Marshall *et al*., 1999), brain clomethiazole concentrations being approximately 40% higher than those in plasma (Green *et al*., 2000). This is the first report of an IC_{50} for clomethiazole *in vitro* that is within the *in vivo* neuroprotective range.

Pentobarbitone also potentiates the action of endogenous GABA (Study & Barker, 1981) and, at high concentrations, activates the ion channel directly (Inomata *et al*., 1988). In the present study it is likely that pentobarbitone potentiated the action of endogenous GABA with no direct effect on channel opening, since the inhibition of glutamate efflux by pentobarbitone was fully antagonized by bicuculline.

These data are consistent with previous observations that clomethiazole and pentobarbitone have distinct binding sites and support the contention that the neuroprotective action of clomethiazole results from a direct effect on channel opening. Clomethiazole is thus able to enhance GABA function in conditions of decreased GABAergic activity, such as acute ischaemia. The simple model described here is likely to be useful for investigating ischaemic brain cell responses and the mechanisms of action of neuroprotective drugs.

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