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Hypoxia/Hypoglycemia-Induced Amino Acid Release Is Decreased *in Vitro* by Preconditioning

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The aim of this study was to investigate the effects of preconditioning on amino acid neurotransmitter release, induced by hypoxia/hypoglycaemia, from rat brain cortical slices. Tissue, perfused with artificial cerebrospinal fluid (aCSF) at 37°C with zero glucose and gassed with 95% nitrogen and 5% carbon dioxide, showed a fivefold increase in glutamate release with little effect on γ-aminobutyric acid (GABA) release. Preconditioning, with three 5-min periods of hypoxia/hypoglycaemia preceding continuous hypoxia/hypoglycaemia, significantly decreased glutamate release whilst significantly elevating GABA release. These results suggest that GABA may reduce the release of glutamate and consequently decrease the neurotoxic effects of glutamate.

Key Words: **hypoxia/hypoglycaemia; brain slices; amino acid release; preconditioning.**

Murry *et al.* (1) first described the phenomenon of cardiac preconditioning in that transient ischaemia afforded protection against subsequent ischaemic and reperfusion injury in dogs. This protection also occurs in the human myocardium (2).

In the brain, periods of short ischaemic insults have also been shown to have neuroprotective effects to subsequent ischaemia both *in vitro* (3) and *in vivo* (4). Neuronal toxicity following ischaemia is accepted to be as a result of excessive synaptic glutamate due to failure of adequate energy-dependent removal from the extracellular space (5). This increased level of glutamate leads to stimulation of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) receptors and subsequent excessive depolarization of the postsynaptic neurone and entry of calcium. This increased intracellular calcium concentration is considered to be the factor responsible for the initiation of a cascade of events ultimately leading to cell death (6). A recent paper (7) has reported that preconditioning of cultured cortical neurones afforded protection against subsequent ischaemia and this protection was dependent upon NMDA receptor stimulation, nitric oxide production and calcium entry through voltage-dependent channels which, in turn, induced $p21^{RAS}$ formation.

The aim of this present study was to investigate the effects of preconditioning on hypoxia/hypoglycaemia-induced release of glutamate and γ-aminobutyric acid (GABA) from rat cortical slices.

MATERIALS AND METHODS

Coronal cortical slices (250 μm) were prepared from male Wistar rats weighing 300–350 g using a McIlwain tissue chopper. The slices were immediately transferred into ice-cold artificial cerebrospinal fluid gassed with 95% oxygen and 5% carbon dioxide (normal aCSF). The slices were then placed in a sealed tissue chamber and perfused with normal aCSF at 37°C at a flow rate of 1 ml/min. The slices were allowed 1 h to equilibrate. Following equilibration the protocol for the experiment consisted of 10 min perfusion with normal aCSF followed by 30 min perfusion with aCSF containing no glucose and gassed with 95% nitrogen and 5% carbon dioxide (hypoxic/hypoglycaemic aCSF). For the preconditioning experiments the tissues were subjected to three 5-min periods of hypoxia/hypoglycaemia interspersed with 10 min of perfusion with normal aCSF. Following the final 10-min perfusion with normal aCSF the tissues were perfused for 30 min with hypoxic/hypoglycaemic aCSF.

One-minute samples of perfusate were collected and assayed by high-performance liquid chromatography following pre-column derivatization with *o*-phthaldialdehyde with subsequent fluorometric detection for glutamate and GABA. Full details of the assay methods are given elsewhere (8).

Composition of normal aCSF in mM: NaCl 124, KCl 5, NaH_2PO_4 1.25, $CaCl_2$ 2, $MgSO_4$ 2, $NaHCO_3$ 26, D-glucose 10 and the pH was 7.4. A corresponding increase in NaCl was made to compensate for the lack of glucose in the hypoxic/hypoglycaemic aCSF in order to maintain osmolarity.

Statistical analysis. Differences in glutamate and GABA release over the 40-min experimental period were analysed with 2-way ANOVA followed by Tukey's post hoc test.

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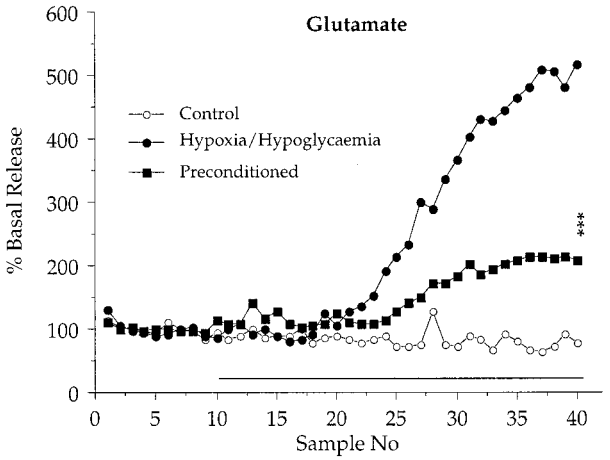

FIG. 1. The effect of preconditioning on hypoxia/hypoglycaemia-induced glutamate release from rat cortical slices. Consecutive 1-min samples of perfusate were collected. Preconditioning was carried out by perfusing hypoxic/hypoglycaemic aCSF 3 × 5 min interspersed with 10 min perfusion with normal aCSF prior to the start of the experiment. Tissues were perfused with hypoxic/hypoglycaemic aCSF for 30 min (bar in fig., *** $P < 0.001$ from both hypoxia/hypoglycaemia and control, $N = 6$).

RESULTS

Under control conditions, with perfusion of normal aCSF for 40 min, both glutamate and GABA showed no significant change from basal release (Figs. 1 and 2). Perfusion with hypoxic/hypoglycaemic aCSF induced an increase in the release of glutamate within 10 min which progressively increased over the following 20 min (Fig. 1). At the end of this perfusion the increase in glutamate release was approximately 5-fold above control release ($P < 0.001$). GABA release was not significantly affected by perfusion with hypoxic/hypoglycaemic aCSF (Fig. 2). Following preconditioning with 3 × 5 min periods of perfusion with hypoxic/hypoglycaemic aCSF, glutamate release, to the subsequent 30-min period of hypoxia/hypoglycaemia, was significantly reduced when compared to the release in the absence of preconditioning ($P < 0.001$, Fig. 1). However, this release was still significantly elevated above control release ($P < 0.001$, Fig. 1). Conversely, GABA release, to the subsequent 30-min period of hypoxia/hypoglycaemia, following preconditioning, was significantly increased to approximately 200% of control release and the release observed in the absence of preconditioning ($P < 0.001$ in both cases, Fig. 2). The periods of hypoxia/hypoglycaemia during preconditioning had no measurable effect on the release of either amino acid.

DISCUSSION

The present study demonstrates that periods of preconditioning have a significant effect on the release of both glutamate and GABA to subsequent prolonged periods of hypoxia/hypoglycaemia in cortical slices.

The mechanisms underlying the neuroprotective effect of preconditioning on subsequent periods of extended hypoxia/hypoglycaemia are not well understood. In the heart, experimental evidence suggests that adenosine-activated K_{ATP} channels are implicated in the adaptive mechanisms involved in preconditioning (9) and that these channels, by hyperpolarizing the myocyte, limit Ca^{2+} influx during reperfusion following hypoxia. A similar mechanism could account for the neuroprotective effect of preconditioning in nervous tissue. A study (10) involving anoxia-induced depolarizations in rat hippocampal slices demonstrated that three periods of 1 min of anoxia separated by 10 min was sufficient to improve the recovery of evoked potentials following a prolonged anoxic insult. This same study also showed that the potassium channel activator, pinacidil, also markedly improved the recovery of evoked potentials when perfused 30 min prior to the anoxic insult. Potassium channel (K_{ATP}) activators have been shown to decrease neurotransmitter release, in particular glutamate, from brain slices probably by maintaining the channel in its open configuration and thus inducing hyperpolarization (11). Such an action elicited by preconditioning would be compatible with the results described in this present paper. Preconditioning had a dramatic effect on the release of GABA in this study. Hypoxia/hypoglycaemia had little effect on GABA release when the tissue was not preconditioned, however, following preconditioning GABA release was increased 2-fold whilst at the same time glutamate release was significantly decreased. The decrease in hypoxia/hypoglycaemia-induced glutamate release following preconditioning

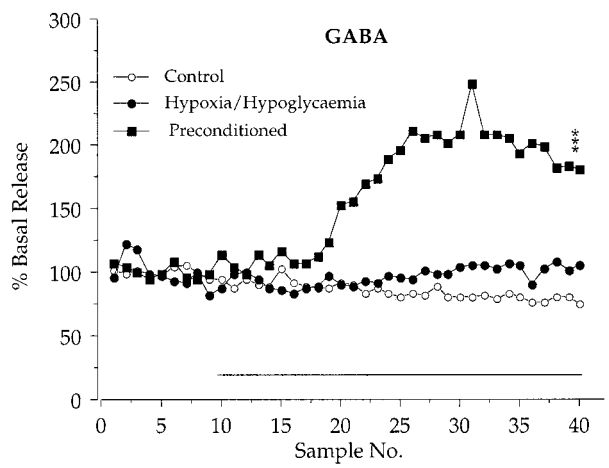

FIG. 2. The effect of preconditioning on hypoxia/hypoglycaemia-induced GABA release from rat cortical slices. Legend as for Fig. 1. (*** $P < 0.001$ from both hypoxia/hypoglycaemia and control, $N = 6$).

could be explained by an action of GABA on K_{ATP} channels. It is well documented that GABA, acting through $GABA_B$ heteroreceptors, modulates K_{ATP} channel function to decrease neurotransmitter release (12).

Brief periods of hypoxia/hypoglycaemia are probably sufficient to have a marked effect on extracellular concentrations of glutamate through decreased removal from the extracellular space. Synaptic concentrations of glutamate are of the order of 0.5–1 μM, transient increases are sufficient to excessively stimulate AMPA receptors and thus remove the voltage-dependent Mg^{2+} block on NMDA receptors and consequently initiate the cascade of events leading to increased calcium entry and nitric oxide synthesis. Another mechanistic possibility, as described in a recent paper (7), is that preconditioning is NMDA-, nitric oxide-, and calcium-dependent. Application of the NMDA channel blocker dizocilpine (MK801) or the binding site antagonist 2-amino-5-phosphonopentanoic acid (AP5), at the same time as the preconditioning event, reduced the protective effect of preconditioning. Similarly, application of the voltage-dependent calcium channel antagonists, nifedipine and nimodipine, also blocked the beneficial effects of preconditioning. In our study, the preconditioning stimuli possibly release glutamate (and/or GABA) but at a level below our limits of detection. If this is so, the released glutamate would stimulate NMDA receptors and so have a marked effect on calcium entry and consequently on nitric oxide synthesis. Gonzalez-Zulueta *et al.* (7) concluded that this sequence of events led to an increase in RAS activity downstream and that this was the critical factor underlying the effects of preconditioning.

In conclusion, we report here that preconditioning, with short periods of hypoxia/hypoglycaemia, of rat cortical slices reduces the subsequent release of glutamate elicited by prolonged hypoxia/hypoglycaemia. Whilst the exact mechanism of action of the neuroprotective effect of preconditioning stimuli is still debatable, the role of glutamate and probably GABA is central to the hypotheses that are currently being proposed.

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