Magnetic resonance spectroscopy studies on Ca$^{2+}$, Zn$^{2+}$ and energy metabolism in superfused brain slices

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Introduction

Current views on the biochemical basis for the cell death which occurs in ischaemia and after prolonged convulsions are based on the toxicity caused by excessive release of excitant amino acids (glutamate and possibly also aspartate). The events known to be involved include depolarization, disturbance of the energy state and release of glutamate and other neurotransmitters, and are thought to be mediated by an increase in the intracellular calcium concentration [Ca$^{2+}$], which triggers off a cascade of damaging biochemical reactions. However, the temporal and causal relationships between these various events have not been clearly established. A major route for the toxic increase in [Ca$^{2+}$], is thought to be the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor, which operates a Mg$^{2+}$-gated direct Ca$^{2+}$ channel, modulated by glycine, polyamines and also possibly by Zn$^{2+}$.

Some years ago, we developed a superfusion system for magnetic resonance spectroscopy (m.r.s.) studies on cerebral metabolism, where continuous observations could be made directly and non-invasively on actively metabolizing slices of the cerebral cortex [4], and applied the $^{19}$F-m.r.s. indicator for Ca$^{2+}$, 1,2-bis(2-amino-5-fluorophenoxy)-ethane-$N,N',N'$-tetraacetic acid (5FBApTA developed by Smith et al. [5]) to the measurement of [Ca$^{2+}$], in our system [6].

We have now compared the effects of depolarization, metabolic deprivation (hypoxia/hypoglycaemia) and excitotoxic amino acids (glutamate and NMDA) on [Ca$^{2+}$], and the energy state. During the course of these investigations, we observed in the $^{19}$F-magnetic resonance spectra a release of free intracellular zinc ([Zn$^{2+}$]) after exposure to the excitotoxins, but not after depolarization or metabolic deprivation.

Methods

Tissues

Tissue slices (0.35 mm thick) from the guinea pig cerebral cortex were prepared as previously described [4]. After a pre-incubation period of 30 min in control media to restore normal levels of metabolites such as lactate, ATP and phosphocreatine (PCr), the slices were transferred to the superfusion apparatus and placed within the m.r. magnet. The control superfusion medium contained (mM): NaCl, 124; KCl, 5; KH$_2$PO$_4$, 1.2; MgSO$_4$, 1.2; CaCl$_2$, 2.4; NaHCO$_3$, 26 and glucose, 10, gassed with O$_2$/CO$_2$ (95:5, v/v) at 37°C. After control spectra had been acquired, the superfusion medium was replaced by the media indicated in the text. Further n.m.r. spectra were then acquired. In studies on the effects of glutamate or NMDA, these were present in the superfusing media throughout the experiment after control spectra had been acquired. When MK801 was used, the tissues were exposed to it at a concentration of 10 μM for 1 h before treatment with glutamate or NMDA.

Measurement of [Ca$^{2+}$], in the superfused metabolizing tissues was performed using the $^{19}$F-n.m.r. indicator (5FBApTA) as previously described [6]. The energy state was monitored in the superfused tissues by interleaved $^{31}$P-spectra.

M.R. spectroscopy

M.r.s. data were obtained using an AMX-500 Bruker spectrometer operating at 202.46 MHz for $^{31}$P and 470.51 MHz for $^{19}$F. The magnetic field was shimmed using the $^1$H resonance of the water of the superfusing medium to achieve a line-width usually of <5 Hz, which increased to <12 Hz in the presence of the metabolizing tissues.

$^{31}$P-spectra were accumulated as 360 transients using 60° radiofrequency pulses repeated every 2 s. $^{19}$F-Data were accumulated in blocks of 5000 transients using 45° radiofrequency pulses, a sweep width of 10 KHz and an interpulse interval of 0.2 s. $^{19}$F-Spectra were obtained by Fourier transformation using a line-broadening (exponential weighting) of 70 Hz. Values for [Ca$^{2+}$], were calculated from the ratios of the areas of bound and free resonances in the $^{19}$F-spectra, multiplied by the dissociation constant of the Ca$^{2+}$-5FBApTA complex (600 mM [7]).

Abbreviations used: [Ca$^{2+}$], intracellular [Ca$^{2+}$]; 5FBApTA, 1,2-bis(2-amino-5-fluorophenoxy)ethane-$N,N',N'$-tetraacetic acid; m.r., magnetic resonance; m.r.s., m.r. spectroscopy; NMDA, N-methyl-D-aspartate; PCr, phosphocreatine; [Zn$^{2+}$], intracellular [Zn$^{2+}$].

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Results and discussion

Figure 1 shows control $^{19}$F-m.r. spectra (Figure 1a) and those observed in tissues depolarized with extracellular K$^+$ (40 mM) (Figure 1b), exposed to combined hypoxia/hypoglycaemia (Figure 1c), to 0.5 mM glutamate (Figure 1d) and to 0.02 mM NMDA (Figure 1e). The increase in [Ca$^{2+}$], was much higher (~450% of control) after metabolic deprivation (Figure 1c) than after exposure to depolarization or excitotoxins (150–200%, Figures 1b, 1d and 1e). Zn$^{2+}$ was only observed after treatment with the excitotoxic amino acids (Figures 1d and 1e); the increase in [Ca$^{2+}$], and the appearance of Zn$^{2+}$ were both considerably slower after exposure to NMDA than to glutamate [7].

No increase in [Ca$^{2+}$], or appearance of Zn$^{2+}$ was observed with 0.2 mM glutamate, and the results were the same for 0.5 and 1 mM glutamate. Increasing the NMDA concentration from 0.02 to 0.2 mM had no significant further effect on [Ca$^{2+}$], or Zn$^{2+}$, and the effects did not seem to be stereospecific because similar results were obtained with NMDA, N-methyl-D-aspartate and N-methyl-L-aspartate. We have reason to believe that the resonances observed in the $^{19}$F-m.r. spectra are attributable to intracellular Zn$^{2+}$: from its chemical shift, and because it disappeared in the presence of N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine, a divalent cation chelator with high affinity for zinc [7].

Use of the NMDA receptor blocker MK 801 produced interesting differences in the effects of glutamate and NMDA (Figure 2). Pretreatment for 1 h with 10 μM MK801 had no effect on the increase in [Ca$^{2+}$], or the decrease in PCr produced by glutamate, but it suppressed the appearance of Zn$^{2+}$. In contrast, identical pretreatment with MK 801 blocked all responses to NMDA: the appearance of Zn$^{2+}$ was suppressed and the levels of [Ca$^{2+}$], and of PCr remained at control values.

These results (summarized in Table 1) suggest that the consequences of metabolic deprivation as may occur in ischaemia, sufficient to produce severe energy failure and a large increase in [Ca$^{2+}$], cannot be readily and simply attributed to release of excitotoxic amino acids, where the increase in [Ca$^{2+}$], was far less with similar energy failure. Also, the release of intracellular Zn$^{2+}$ (which has been suggested to be potentially neuroprotective [8]) was
Effect of pretreatment with MK801 on changes in [PCr], [Ca\(^{2+}\)], and [Zn\(^{2+}\)], caused by glutamate (a,b) and NMDA (c,d).

The increase in [Ca\(^{2+}\)] and decrease in [PCr] caused by glutamate (Figure 1) are not affected by MK 801 but the appearance of Zn\(^{2+}\) is suppressed. The changes caused by NMDA (Figure 1) are all prevented by MK801 where the spectra (c,d) are the same as control spectra (N. Thatcher, P. G. Morris and H. S. Bachelard, unpublished work).

![Figure 2](image)

**Table 1**

Changes in [Ca\(^{2+}\)], [Zn\(^{2+}\)], and PCr in superfused cerebral tissues under the conditions described in the text.

Values of [Ca\(^{2+}\)] and [PCr]/[P] (% of control) are means ± S.D. (n = 2–12). Control values for [PCr]/[P] were ~1.5 and for [Ca\(^{2+}\)], 181 ± 29 nM in current studies. In all studies, parallel experiments were conducted in the absence of SFAPTA to ensure that there was no effect of the indicator on control values. NMDA was tested in the absence of Mg\(^{2+}\).

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>[Ca(^{2+})] (%)</th>
<th>Zn(^{2+})</th>
<th>[PCr]/[P] (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>100</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Low O(_2) + low glucose</td>
<td>2</td>
<td>459*</td>
<td>-</td>
<td>23</td>
</tr>
<tr>
<td>Depolarization</td>
<td>2</td>
<td>163*</td>
<td>-</td>
<td>35*</td>
</tr>
<tr>
<td>NMDA (20–100 μM)</td>
<td>4</td>
<td>168*</td>
<td>+</td>
<td>39*</td>
</tr>
<tr>
<td>(with MK 801)</td>
<td>2</td>
<td>100</td>
<td>-</td>
<td>95</td>
</tr>
<tr>
<td>Glutamate (50 μM)</td>
<td>3</td>
<td>171</td>
<td>+</td>
<td>43*</td>
</tr>
<tr>
<td>(with MK 801)</td>
<td>2</td>
<td>177*</td>
<td>-</td>
<td>27*</td>
</tr>
</tbody>
</table>

*P < 0.01 compared with control values.
observed neither on metabolic deprivation nor on depolarization, but only after treatment with excitotoxins [7,9]. In related experiments (results not shown) using 13C-m.r. to study flux through metabolic pathways, we observed quite different patterns between these various insults. NMDA caused glycaemic coma, is probably always caused by rapidly decreased N-acetylaspartate and increased lactate, with slow decreases in pool sizes of amino acids [10], in marked contrast to the changes caused by severe hypoxia [11] or depolarization [12].

Our preliminary unpublished results on the effects of MK801 (Figure 2, Table 1) tend to confirm suggestions [8] that Zn+2 may be associated particularly with the NMDA receptor, and provide further evidence for the importance of non-NMDA receptors in the damaging consequences of metabolic deprivation.

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Energy metabolism, ion homeostasis, and cell damage in the brain
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Acute brain damage, as it occurs after ischaemia, intracranial haemorrhage, gross trauma, and hypoglycaemic coma, is probably always caused by cellular energy failure, secondary to reduced delivery of oxygen or glucose [1–3]. Under such circumstances, oxidative phosphorylation and ATP production are insufficient to sustain normal metabolic functions. A decline in ATP production per se is potentially harmful because spontaneous degradation of macromolecules and macromolecular assemblies is no longer matched by a correspond-

ing resynthesis. However, the secondary effects of energy failure represent an additional threat to cell viability. These effects encompass membrane depolarization, with presynaptic release of transmitters, including excitatory amino acids (EAAs) such as glutamate, and loss of ion homeostasis, as well as lactic acidosis (see [4,5]). Over-bombardment of glutamate receptors and Ca2+ influx, in turn, activate enzymes which degrade cell structure (e.g. phospholipases and proteases), as well as those altering the activity of proteins which regulate receptor function, gene expression and protein synthesis (mainly kinases and phosphatases). In this way, primary energy failure triggers a cascade of metabolic events which have potentially adverse effects.

Under certain circumstances, cell death occurs in settings in which energy failure is less obvious. For example, head trauma without lacer-