Protective Effects of Felbamate Against Hypoxia in the Rat Hippocampal Slice

R.A. Wallis, MD; K.L. Panizzon, BS, BA; M.D. Fairchild, PhD; and C.G. Wasterlain, MD

Background and Purpose: Felbamate is a new dicarbamate anticonvulsant with low toxicity currently being investigated in human clinical epilepsy trials. In this study, we examined the protective effects of felbamate against hypoxia.

Methods: We exposed paired rat hippocampal slices to hypoxia with and without felbamate treatment while monitoring the CA1-evoked population spike.

Results: Felbamate provided dose-dependent neuroprotection against hypoxia at concentrations of 45 mg/l and greater (p<0.05). At a felbamate concentration of 300 mg/l, recovery of CA1 evoked population spike amplitude after hypoxic exposure was 99% compared with 0.5% for unmedicated paired slices. The appearance and disappearance of the hypoxic injury potential was delayed in slices treated with 300 and 400 mg/l (p<0.05).

Conclusions: In this model of hypoxia, felbamate provided neuroprotection against hypoxia at concentrations similar to serum felbamate levels currently being used in human clinical epilepsy trials. (Stroke 1992;23:547-551)

Many anticonvulsants exhibit neuroprotective properties against hypoxia and ischemia.1-3 Although many of these agents display potent anti-ischemic properties,4 they also manifest considerable toxicity.5,6 Felbamate is a new dicarbamate anticonvulsant currently being tested in human clinical epilepsy trials. It has proved to be remarkably safe in animal trials, with a toxic dose 50 (TD 50) of over 3,000 mg/kg in the dog (personal communication from Dr. Duane Sofia). Additionally, it has shown little neurotoxicity in human clinical trials in patients treated with up to 3,600 mg felbamate per day.7,8

We examined the antihypoxic properties of felbamate using the hippocampal slice. This in vitro model simulates in vivo hypoxic neuronal insults that occur in stroke and other cerebral ischemic events. Additionally, it allows relative preservation of neuronal-glial relations yet provides ease of environmental manipulation.

Materials and Methods
Twenty-four male Sprague-Dawley rats weighing 197-410 g were briefly anesthetized with halothane and decapitated. The brains were removed within 60 seconds and placed for 1 minute in cold artificial cerebrospinal fluid composed of (mM) NaCl 126, KCl 4.0, KH2PO4 1.4, MgSO4 1.3, CaCl2 2.4, NaHCO3 26, and glucose 4.0, pH 7.4, and saturated with 95% O2-5% CO2. Hippocampi from each rat were rapidly dissected and 475-μm transverse slices were cut using a McIlwain tissue chopper. Hippocampal slices were placed in two recording wells with temperature maintained at 34.0±0.5°C. Each recording well had a capacity of 2.0 ml and was perfused at a rate of 2.5 ml/min, with upper slice surfaces submerged under 2 mm artificial cerebrospinal fluid. A humidified 95% O2-5% CO2 gas mixture was directed above the fluid surface.

Sixty minutes after slice placement, the orthodromic CA1 population spike amplitude of each slice was tested by stimulating the Schaffer collaterals and recording the response in the CA1 pyramidal cell layer. A bipolar electrode consisting of a pair of twisted insulated nichrome wires was placed on the stratum lacunosum and stimulated with square wave pulses of 40 μsec duration. To record population spike responses, a tungsten electrode was placed in the CA1 pyramidal cell layer with stimulating currents and recording depths adjusted until a maximal population spike amplitude was obtained, generally at a depth of about 200 μm. Only slices with a population spike of 3.0 mV or greater were used.

In each recording well, one slice was stimulated every 30 seconds to monitor evoked responses throughout the experiment. These slices were designated as "stimulated slices." Remaining slices in each well received stimulation to assess evoked responses before and after hypoxic exposure and were designated as "nonstimulated slices."

Twenty-four paired trials were performed. Each trial monitored two stimulated slices during hypoxia, with one slice receiving felbamate and the other receiving no medication. Additionally, 214 nonstimulated slices were assessed, of which 111 received felbamate and 103 were unmedicated during hypoxia.

Before hypoxic exposure, one well received oxygenated artificial cerebrospinal fluid with added felbamate...
for 30 minutes. Felbamate was added directly to the artificial cerebrospinal fluid and dissolved with gentle warming to 45°C. The pH of the artificial cerebrospinal fluid with added felbamate was then measured, and no change was detected. The antihypoxic properties of felbamate were tested at concentrations of 1, 45, 90, 200, 300, and 400 mg/l (0.004, 0.190, 0.840, 1.300 and 1.700 mM, respectively). Hypoxic conditions were introduced simultaneously in both recording wells by changing the perfusion fluid to artificial cerebrospinal fluid saturated with 95% N₂-5% CO₂. Slices pretreated with felbamate continued to receive felbamate during hypoxia.

Hypoxic exposure was continued until 5 minutes beyond the disappearance of the hypoxic injury potential in the unmedicated, stimulated slice. This point for hypoxia termination was chosen because we have found that the disappearance of this potential is a reliable, temporal indicator of irreversible hypoxic neuronal injury. Oxygenated conditions were then restored for all slices. Felbamate-treated slices continued to receive felbamate for the first 15 minutes of this recovery period. Population spike amplitude was measured 60 minutes after reoxygenation. Preliminary experiments indicated that by that time, population spike recovery had reached a plateau that was maintained for several hours.

Population spike amplitude, excitatory postsynaptic potential slope, and fiber volley amplitude in all slices were examined before and after hypoxic exposure. Population spike amplitude was measured as the distance between the negative peak of the population spike and the second excitatory postsynaptic potential peak that followed. Slope of the excitatory postsynaptic potential was measured by assessing the slope between the first excitatory postsynaptic potential peak and the second excitatory postsynaptic potential peak. To determine baseline evoked responses, we followed the population spike over 20 minutes. If the amplitude of this response did not vary more than 10% during that time, we then proceeded with the experiment. The last evoked response of that time period was used as the baseline for further analysis. Percent recovery of population spike amplitude, excitatory postsynaptic potential slope, and fiber volley amplitude was calculated as the final value, divided by the initial value, multiplied by 100. For stimulated slices, evoked responses of felbamate-treated and unmedicated slices were compared using Student's correlated t tests. For nonstimulated slices, evoked responses were compared by Wilcoxon rank sum test. To take into consideration the population spike recovery seen in unmedicated, nonstimulated slices, a measure of hypoxic protection was calculated. For this purpose, mean percent damage was defined as 100 minus mean recovery. Hypoxic protection was calculated as the mean percent damage seen in unmedicated, nonstimulated slices minus the mean percent damage in felbamate-treated slices divided by the mean percent damage seen in unmedicated slices. Percent recoveries greater than 100 were taken as zero damage. To assess effects of felbamate during hypoxia, evoked responses for hypoxic trials using felbamate 300 mg/l were analyzed and compared using Student's correlated t test. This concentration of felbamate was chosen because it provided virtually full protection against hypoxic damage. Additionally, mean hypoxic exposure times for each felbamate concentration were compared with one-way analysis of variance.

Results

Felbamate exposure significantly protected against hypoxic exposure in stimulated hippocampal slices, beginning with a felbamate concentration of 45 mg/l (p < 0.05). Following hypoxia and reoxygenation, slices treated with 300 mg/l felbamate showed a mean±SE population spike amplitude recovery of 98.8±2.4%. In contrast, paired, unmedicated slices showed a mean recovery of 50.0±0.5% (p < 0.05), as illustrated in Figure 1.

Felbamate treatment also provided hypoxic protection in nonstimulated slices. This was first observed at a concentration of 90 mg/l (p < 0.05) (Table 1). This protection of nonstimulated slices was nearly complete with felbamate treatment of 300 and 400 mg/l.

Hypoxic protection of population spike amplitude with felbamate proved to be concentration-dependent in both stimulated and nonstimulated slices. At felbamate concentrations of 1 mg/l, essentially no hypoxic protection was observed, whereas nearly full protection was seen with felbamate concentrations of 300 mg/l and above. The EC₅₀ for hypoxic protection by felbamate was 198.7 mg/l (Table 1).

Treatment with 300 mg/l felbamate during hypoxia produced significant recovery of mean excitatory postsynaptic potential slope (66±4%) after hypoxia.

<table>
<thead>
<tr>
<th>Table 1. Felbamate Hypoxic Protection in Nonstimulated Felbamate-treated and Paired, Unmedicated Rat Hippocampal Slices</th>
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</thead>
<tbody>
<tr>
<td>Felbamate concentration</td>
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<tr>
<td>-------------------------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>45</td>
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<tr>
<td>90</td>
</tr>
<tr>
<td>200</td>
</tr>
<tr>
<td>300</td>
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<tr>
<td>400</td>
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Values are mean±SE % recovery. *p < 0.05 different from unmedicated slices by Wilcoxon rank sum test.
yielded fiber volley responses that averaged 101 ±6% of when compared with paired unmedicated slices (0%) (Table 2). Mean fiber volley responses were somewhat resistant to hypoxia and did not differ significantly in felbamate-treated and paired, unmedicated slices, although a trend toward felbamate protection was seen. Treatment with 300 mg/l felbamate during hypoxia yielded fiber volley responses that averaged 101 ± 6% of initial potential, whereas paired, unmedicated slices showed 83 ± 18% mean recovery.

Evoked potential evolution during hypoxia in unmedicated, stimulated slices was also examined. Initially, population spike amplitude showed a progressive decline and then disappeared. As hypoxia continued, the hypoxic injury potential appeared and, after further hypoxic exposure, disappeared.9 This progression of population spike amplitude changes during hypoxia is illustrated in Figure 2.

Stimulated slices treated with felbamate showed a similar sequence of changes in population spike amplitude during hypoxia but with a significantly delayed time course of the hypoxic injury potential. No significant difference in time of initial population spike disappearance was seen between slices with or without felbamate. In slices treated with 300 mg/l felbamate, the population spike disappeared at a mean of 6.3 ± 1.0 minutes compared with 6.0 ± 0.6 minutes in paired, unmedicated slices. The appearance of the hypoxic injury potential was, however, significantly delayed in slices treated with felbamate concentrations of 300 and 400 mg/l (p < 0.05). In slices treated with 300 and 400 mg/l felbamate, the hypoxic injury potential appeared at means of 15.7 ± 0.7 and 15.7 ± 3.0 minutes, respectively, whereas in paired, unmedicated slices, this potential appeared at means of 9.3 ± 1.5 and 11.0 ± 2.1 minutes, respectively. Additionally, in several instances with felbamate concentrations of 300 and 400 mg/l, the hypoxic injury potential did not appear during the entire hypoxic exposure. Data from these particular cases were not included in the above comparison but may indicate an even further delay in the progression of the hypoxic injury potential. In slices treated with 400 mg/l felbamate, disappearance of the hypoxic injury potential occurred at a mean of 22.7 ± 3.4 compared with 18.3 ± 2.7 minutes in paired, unmedicated slices. This analysis, as well, did not include one case in which the hypoxic injury potential never disappeared during hypoxic exposure. Again, this may be further evidence of delayed hypoxic injury potential evolution in the presence of felbamate.

Although slices treated with felbamate showed protection of excitatory postsynaptic potential slope against hypoxia, no significant difference in mean excitatory postsynaptic potential slope during hypoxia was seen in felbamate-treated and paired, unmedicated slices. Peak amplitude of the hypoxic injury potential in slices treated with 300 mg/l felbamate occurred at a mean hypoxic exposure of 18 ± 1 minutes (Table 2). At this time, the mean excitatory postsynaptic potential slope had declined to 4 ± 7% in felbamate-treated slices and to 8 ± 8% in paired, unmedicated slices. Subsequently, at the time of hypoxic termination, recovery of excitatory postsynaptic potential slope was sometimes seen in felbamate-treated but not in paired, unmedicated slices (Table 2).

To assess the effect of felbamate without hypoxia on evoked potentials without hypoxia, responses of stimulated slices were examined after treatment for 30 minutes with 300 mg/l felbamate and compared with slices receiving only artificial cerebral spinal fluid for 30 minutes. No significant changes in fiber volley amplitude, excitatory postsynaptic potential slope, or population spike amplitude were seen (Table 2).

In slices not treated with felbamate, significant differences in population spike recovery were seen in stimulated and nonstimulated slices exposed to hypoxia. Nonstimulated slices showed a mean population spike recovery of 24.3 ± 3.3% after hypoxia, whereas stimulated slices exposed to hypoxia showed a mean recovery of 0.7 ± 0.4% (p < 0.05). Thus, stimulation given during hypoxia had a clearly detrimental effect on slice recovery, even with a low stimulation rate of once every 30 seconds.

No indication of toxicity by felbamate was found at concentrations up to 400 mg/l, which was the limit of felbamate solubility in artificial cerebrospinal fluid.
Excitatory postsynaptic potential slope

<table>
<thead>
<tr>
<th>Response</th>
<th>Initial potential</th>
<th>% of initial potential after 30 minutes of preincubation</th>
<th>% of initial potential at 18 minutes of hypoxia*</th>
<th>% of initial potential at hypoxia termination†</th>
<th>% of initial potential after 60 minutes of recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fiber volley</td>
<td>2.0±0.4 mV</td>
<td>81±7</td>
<td>69±11</td>
<td>72±12</td>
<td>101±6</td>
</tr>
<tr>
<td>Felbamate-treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unmedicated</td>
<td>1.9±0.4</td>
<td>92±5</td>
<td>66±20</td>
<td>63±36</td>
<td>83±18</td>
</tr>
<tr>
<td>Excitatory postsynaptic</td>
<td>0.6±0.2 mV/mS</td>
<td>76±27</td>
<td>4±6</td>
<td>45±24</td>
<td>66±37‡</td>
</tr>
<tr>
<td>potential slope</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Felbamate-treated</td>
<td>0.6±0.1</td>
<td>100±7</td>
<td>8±8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Unmedicated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Population spike</td>
<td>5.6±1.0 mV</td>
<td>95±5</td>
<td>29±9</td>
<td>9±3</td>
<td>99±3‡</td>
</tr>
<tr>
<td>Felbamate-treated</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Unmedicated</td>
<td>6.2±0.8</td>
<td>102±2</td>
<td>5±5</td>
<td>0</td>
<td>0.5±0.5</td>
</tr>
</tbody>
</table>

Values are mean±SE.
*Mean time of maximal amplitude of hypoxic injury potential in felbamate treated slices.
†Total hypoxic exposure averaged 24±2 minutes.
‡p<0.05 by Student’s correlated t test.

Initial mean population spike amplitude of slices given felbamate treatment and paired, unmedicated slices were similar. Slices later receiving felbamate treatment showed a mean initial population spike amplitude of 5.5±0.2 mV, and unmedicated slices showed an initial mean population spike amplitude of 5.4±0.2 mV.

Hypoxic exposure time for each trial was determined by the disappearance of the hypoxic injury potential in the stimulated slice not receiving felbamate. Overall mean hypoxic exposure time was 28.4±2.0 minutes. Mean hypoxic exposure times for each tested concentration group did not differ significantly by one-way analysis of variance.

Discussion

These experiments indicate that the anticonvulsant felbamate provided concentration-dependent protection of population spike amplitude and excitatory postsynaptic potential slope against hypoxia in the hippocampal slice model. The effective concentration50 (EC50) for felbamate hypoxic protection of population spike amplitude in rat hippocampal slices was 198.7 mg/L. This value compares with the oral anticonvulsant effective dose50 (ED50) of felbamate for maximal electroshock seizures in the rat (48 mg/kg) and the ED50 for pentylenetetrazol (Metracor)-induced seizures in the rat (238 mg/kg).11

Human subjects in antiepileptic trials of felbamate have been recorded as having peak felbamate serum levels averaging up to 96.1 mg/L.12 These felbamate serum concentrations, even with the presence of valproate, carbamazepine, phenytoin, and phenobarbital, did not produce any evidence of toxicity. Other trials have recorded felbamate serum trough levels of 32–54 mg/L.13 Therefore, felbamate serum concentrations reached in human anticonvulsant trials are similar to the lower range of concentrations found to be neuroprotective for hypoxic rat CA1 pyramidal cells in the present study.

The mechanism of felbamate hypoxic protection appears to involve a delay in the evolution of hypoxic injury. At higher concentrations, felbamate-treated slices show a significant delay in the appearance and disappearance of the hypoxic injury potential. Although disappearance of the initial evoked potential during hypoxia appears to reflect neuronal hyperpolarization,13–15 our preliminary results suggest that hypoxic injury potential onset correlates intracellularly with mild neuronal depolarization. Additionally, the disappearance of the hypoxic injury potential disappearance appears to correlate with severe neuronal depolarization. In keeping with these findings, our previous studies have indicated that the hypoxic injury potential is a reliable temporal marker of irreversible neuronal injury during hypoxia in the hippocampal slice. Termination of hypoxia before the beginning of hypoxic injury potential decline produces nearly full recovery, whereas termination of hypoxia beyond the disappearance of the hypoxic injury potential results in limited recovery of the population spike amplitude. Therefore the delay of the appearance and disappearance of the hypoxic injury potential by felbamate may delay the onset of irreversible detrimental processes during hypoxia.

Several mechanisms of damage may be operative during hypoxia. Excitatory amino acid neurotransmitters are released during hypoxic exposure and bind to N-methyl-D-aspartate (NMDA) and non-NMDA receptors.16 Binding of these receptors opens ion channels that are permeable to calcium and sodium and facilitates postsynaptic neuronal depolarization.17 Accumulation of cytosolic calcium appears to induce permanent neuronal injury.18 Additionally, energy failure during hypoxia may exacerbate neuronal damage by preventing excitotoxic transmitter reuptake, which allows greater NMDA receptor activation.19 Energy failure may also interfere with intracellular calcium sequestration and active calcium extrusion, thus contributing to further hypoxic damage.20

The mechanism of felbamate protection against seizures and hypoxia is not understood. Felbamate has been found to weakly inhibit adenosine uptake.21,22 It is also a weak agonist of y-aminobutyric acid and benzodiazepine receptors. Felbamate actions at these receptor complexes, however, occur at concentrations too high to account for its neuroprotective effects.23,24 Alternatively, felbamate may interact directly with NMDA
receptors, possibly through redox modulation. The NMDA receptor is selectively modulated by redox state with reduction-potentiating NMDA responses.\textsuperscript{25,26} Additionally, many anticonvulsants reduce cerebral metabolic rate. With such an effect, felbamate could also delay energy failure during hypoxia.\textsuperscript{3} By sustaining energy reserves for longer periods of time, extracellular excitatory amino acid concentrations might be reduced and excitotoxic injury minimized.

This study also revealed increased damage during hypoxia to those slices that were stimulated. One likely mechanism of damage in stimulated slices may be release of excitatory amino acids. Metabolic depletion may also occur earlier in stimulated than in nonstimulated slices. This metabolic depletion could prevent excitotoxic transmitter reuptake, resulting in greater accumulation of excitotoxic neurotransmitters. This adverse effect of stimulation on hypoxic neurons may be important because neurons are commonly found at the periphery of ischemic infarcts, where neurons are alive but unable to generate evoked potentials.\textsuperscript{27} The current findings might suggest that stimulation of such hypoxic neurons can adversely affect their survival.

In conclusion, felbamate provided potent, dose-dependent protection against hypoxia in the hippocampal slice. This protection extended to stimulated as well as nonstimulated slices. During hypoxia, felbamate significantly delayed the appearance and disappearance of the hypoxic injury potential. The EC\textsubscript{50} for felbamate protection was close to the ED\textsubscript{50} for felbamate anticonvulsant activity. Additionally, this EC\textsubscript{50} for felbamate hypoxic protection was in the range of therapeutic serum levels achieved in human clinical trials for epilepsy. In view of its low toxicity and good protection against hypoxia, felbamate may warrant further evaluation in the treatment of acute cerebral hypoxia-ischemia.

References

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