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Magnesium and Cobalt, not Nimodipine, Protect Neurons Against Anoxic Damage in the Rat Hippocampal Slice

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Brain tissue, maintained *in vitro*, was used to determine whether agents that block calcium entry into neurons can improve the recovery of evoked responses after anoxia. The hippocampus was dissected from a rat brain and sliced perpendicular to its long axis such that its main neuronal circuits remain functional. A pathway in the slice was stimulated electrically, and an extracellular potential, the evoked population spike, recorded from the neurons postsynaptic to that pathway. A bipolar stimulating electrode was placed in either the perforant path or the Schaeffer collaterals and a monopolar metal microelectrode placed, respectively, in either the dentate granule cell layer or the CA1 pyramidal cell layer. The slices were maintained *in vitro* by superfusing them with oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (aCSF). In order to generate anoxia, the tissue was superfused with aCSF bubbled with 95% N_2, 5% CO_2 for either 5 or 10 min. All drugs examined were present in the aCSF before, during, and immediately after the anoxic period. Percentage recovery was expressed as the amplitude of the evoked population spike 60 min after anoxia divided by its preanoxic amplitude. Protection in this model is defined as a significant ($P < 0.05$) improvement in percentage recovery compared with the recovery of untreated slices. There was no recovery of the response recorded from untreated dentate granule cells after 10 min of anoxia (0 ± 0%, n = 5; mean ± SE), whereas 5 min of anoxia was sufficient to cause damage to the untreated CA1 pyramidal cells (4 ± 3%, n = 6). When nimodipine (10^{-7} M) was present, there was no significant improvement in the recovery of the evoked population spike from either the dentate granule cells (11 ± 11%, n = 5) or the CA1 pyramidal cells (5 ± 5%, n = 5). Cobalt (2 mM), which had improved the recovery of dentate granule cells,[1] protected the CA1 pyramidal cells from anoxic damage (64 ± 12%, n = 5). Magnesium (10 mM) significantly improved recovery of both the dentate granule cells (76 ± 5%, n = 5) and the CA1 pyramidal cells (35 ± 10%, n = 8) after anoxia in this *in vitro* model. ATP levels during anoxia were measured in order to determine how magnesium might protect against the anoxic damage. ATP was maintained at a significantly higher level during anoxia when 10 mM magnesium was present in the bathing medium (1.7 ± 0.2 *vs.* 1.1 ± 0.15 nM/mg dry weight). Nimodipine did not maintain ATP levels during anoxia. The authors conclude that magnesium and cobalt, but not nimodipine, protect against anoxic damage to the hippocampus in this *in vitro* model. Any potential clinical benefit of magnesium (cobalt is highly toxic) would have to be tested in an *in vivo* model, and serious problems such as the limited permeability of the blood–brain barrier to magnesium would have to be overcome. Their results support the importance of calcium influx as one trigger for anoxic damage. (Key words: Brain, hippocampal slice: ATP; ischemia. Ions: cobalt; magnesium. Pharmacology, calcium channel blocking drugs: nimodipine.)

A BRIEF INTERRUPTION of oxygen supply to the brain may lead to irreversible loss of brain function. Recent studies have focused on the role that calcium plays in triggering neuronal damage.[1–6] During ischemia and anoxia the calcium concentration in the extracellular space is reduced and it is postulated that this results from calcium influx into neurons.[6] Recently, net calcium influx has been shown to increase during anoxia *in vitro*.[1]

Nimodipine, a dihydropyridine calcium entry blocker, has been shown to provide protection from ischemia *in vivo*.[7,8] Although this drug decreases the postischemic hypoperfusion seen after ischemia,[8] it is unclear to what extent other effects contribute to its protection. It is possible that nimodipine blocks calcium entry into neurons and protects by this mechanism as well. Magnesium, an ion that interferes with calcium entry into neurons, protects against spinal cord ischemia *in vivo*.[9] It, like nimodipine, could protect the neurons either directly or by better maintaining blood flow after ischemia. Cobalt, which is toxic *in vivo*, is a potent and highly specific calcium channel blocker. It would be less likely than magnesium to have beneficial effects other than blocking calcium entry into neurons. In addition, previous studies[1] have demonstrated that cobalt does indeed reduce net calcium entry during anoxia in the hippocampal slice preparation.

In the current study we use an *in vitro* model[10,11] to assess whether agents that block calcium entry protect against anoxic neuronal damage. In this model neurons are superfused directly, independent of the vasculature. Thus we can determine the direct effect of various agents on the neurons. Any protection found would be independent of an agent's effect on ischemic or postischemic perfusion. A further advantage of this model is that it allows examination of agents that are toxic *in vivo* because of complicating effects on systems other than the nervous system. Although these agents would be of no use clinically, they can provide a powerful tool for examining the mechanisms of anoxic and ischemic brain damage.

In the current study the effect of nimodipine and magnesium on anoxic damage to CA1 pyramidal and dentate granule cells and the effect of cobalt on anoxic damage to the CA1 pyramidal cells are examined.

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Methods

The experiments reported in this article have been approved by the Animal Care and Use Committee of the State University of New York Health Science Center at Brooklyn. The methods have been described in detail elsewhere,[1,10,12] therefore, only a brief description will be provided here. White, male Sprague-Dawley rats (100–120 days old) were decapitated, and their hippocampi were dissected from the brain, sliced transverse to their long axis, and placed on nylon mesh held to a Plexiglas® grid.[13] The slices were deeply submerged (1 cm below the air–fluid interface) and superfused with artificial cerebrospinal fluid (aCSF) at 60 ml/min.[12,14] The composition of the aCSF was (mM) as follows: NaCl, 126; KCl, 4.0; KH_2PO_4, 1.4; $MgSO_4$, 1.3; $CaCl_2$, 2.4; $NaHCO_3$, 26; and glucose, 4. The aCSF was equilibrated with 95% oxygen, 5% carbon dioxide and the temperature maintained at 37° C. The *p*H was 7.4.

One hour after the dissection, a bipolar stimulating electrode and a monopolar metal recording electrode were placed in the slice. In order to record the population spike evoked from the dentate granule cells, the stimulating electrode was placed in the perforant path, which monosynaptically activates these cells. The recording electrode was placed in the dentate granule cell layer, which recorded the summed action potentials of many granule cells that are activated simultaneously. The size of the population spike is proportional to the number of neurons generating action potentials. The CA1 pyramidal cells can likewise be examined if a stimulating electrode is placed in the Schaeffer collaterals, a pathway that monosynaptically excites these pyramidal cells, and a recording electrode placed in the CA1 pyramidal cell layer. A detailed description and diagrams of these pathways were provided by Andersen *et al.*[15] The stimulating voltages were biphasic (100 μs each phase), and the voltage was adjusted to provide a maximal response at the beginning of the experiments; parameters were not adjusted afterwards. The slice was stimulated once every 10 s throughout the experiment. The population spikes were observed with the use of a 100 × gain preamplifier (filters set to pass potentials with a frequency between 10 Hz and 3 KHz) connected to a digital oscilloscope. Responses were monitored for 1 h to assure stability of the preparation. Only slices that had a stable response were used for the experiment. If slices are not subjected to anoxia, they demonstrate stable responses for 6 h.

Anoxia was generated by superfusing the slice with aCSF preequilibrated with 95% N_2, 5% carbon dioxide. Neuronal transmission was examined before, during, and after short periods of anoxia. The dentate granule cells were exposed to 10 min of anoxia; there is little recovery of the evoked response after this amount of anoxia in untreated slices. The CA1 pyramidal cells are more sensitive to anoxia than the dentate granule cells[1] and were examined after 5 min of anoxia. The untreated pyramidal cells show little recovery after this period of anoxia. The population spike is measured as the mean amplitude, in microvolts, of the negative and positive arms of the population spike.[10] The recovery of neuronal transmission was expressed as the amplitude of the evoked population spike after 60 min of normoxic reperfusion after anoxia divided by its preanoxic amplitude.

Nimodipine was first dissolved in dimethyl sulfoxide (DMSO) and then added to the aCSF. The concentration of DMSO never exceeded 0.5%. We have found no effect of DMSO on either the size of the population spike or the recovery of the response from anoxia.§ Because light accelerates the breakdown of nimodipine, experiments using it were performed in the dark. Nimodipine was added to the aCSF superfusing the slice 15 min before anoxia, was present during anoxia, and was washed from the system 15 min after reperfusion with normoxic aCSF. We superfused the slices with nimodipine for 15 min before the anoxic period to ensure that the drug reached its site of action before the anoxia began.

Magnesium chloride (9 mM) was added to normal aCSF for the high magnesium studies (final concentration 10 mM). For the cobalt studies, 2 mM cobalt chloride was added to aCSF from which KH_2PO_4 was removed to prevent precipitation of cobalt phosphate. Magnesium and cobalt were added to the aCSF superfusing the slice 5 min before anoxia, were present during anoxia, and were washed from the system 5 min after reperfusion with normoxic aCSF. Because both magnesium and cobalt had a clear effect on the preanoxic population spike, we could be certain that these drugs reached effective concentrations within 5 min, and therefore we superfused the slices with these drugs for only 5 min before anoxia.

The results are expressed as the mean ± the standard error (SE). For each group in the electrophysiology experiments, n includes between five and eight animals; the actual numbers are given in table 1. The percentage recovery of the dentate granule cells for each drug treatment was compared with the percentage recovery of the untreated dentate granule cells. Significance was determined with the use of the unpaired Student's *t* test with the Bonferroni correction. The same comparison was performed on data from the CA1 region. No comparisons were made between the dentate and CA1 regions.

Adenosine triphosphate (ATP) levels were measured

§ Abramowicz AE, Kass IS, Cottrell JE: The effect of midazolam and gabaergic inhibition on anoxic damage in the rat hippocampal slice. Presented at the American Society of Anesthesiology October, 1988, meeting.

TABLE 1. Percentage Recovery of Evoked Population Spike

	Mean	Standard Error	n
Dentate granule cells			
Untreated	0	0	5
Nimodipine (10^{-7})	11	11	5
Nimodipine (10^{-5})	12	12	5
Magnesium (10 mM)	76†	5	5
CA1 Pyramidal Cells			
Untreated	4	3	6
Nimodipine (10^{-7})	5	5	5
Magnesium (10 mM)	35*	10	8
Cobalt (2 mM)	64†	12	5

* $P < 0.05$, or † $P < 0.005$, compared with untreated group from the same region.

in slices treated similarly to those in the electrophysiologic experiments. The tissue was prepared and placed on Plexiglas® grids as described above. Each grid was then placed in a small beaker with aCSF and bubbled with 95% oxygen–5% carbon dioxide. To generate anoxia, the aCSF was bubbled with 95% nitrogen–5% carbon dioxide for 10 min. At this point the grids were removed from the beakers and rapidly frozen in liquid nitrogen. The tissue was then lyophilized and the dentate and CA1 regions dissected and weighed.[6] ATP was measured for each region with the use of the luciferin–luciferase photometric assay.[16]

The biochemical experiments used multiple slices from single animals. In each case slices from a single animal were distributed to the different treatment groups. The number of slices in each group was between 11 and 16; the actual numbers are provided in table 2. ATP levels at the end of anoxia with nimodipine and magnesium treatments were compared with ATP levels at the end of anoxia in untreated tissue. No comparison was made either between drug treatments or between normoxic and anoxic treatments; previous studies have demonstrated significant reduction in ATP levels during anoxia.[1,10] Significance was determined with the unpaired Student's *t* test with the Bonferroni correction.

Results

Dentate Granule Cell Region

There is damage in the dentate gyrus of the rat hippocampal slice after 10 min of anoxia. The population spike evoked by stimulation of the perforant path does not recover in untreated anoxic slices (0 ± 0%; mean ± SE) (table 1).

Nimodipine (10^{-7} M), a concentration commonly used *in vivo*, was not followed by increased recovery of dentate granule cells after 10 min of anoxia. This is shown for a single experiment in figure 1. The response amplitude recovered to 11 ± 11% of its preanoxic level (table 1). This was not significantly different from untreated slices. The responses with 10^{-5} M nimodipine treatment recovered to 12 ± 12% of their preanoxic amplitude after 10 min of anoxia. These results also were not significantly different from anoxia with no drug treatment.

Nimodipine had little effect on the evoked response before anoxia (fig. 1). In the preanoxic period, nimodipine (10^{-7} M) caused only a slight decrease in the population spike to 82 ± 11% (n = 5) of its predrug amplitude; 10^{-5} M nimodipine diminished the preanoxic spike to 90 ± 6% (n = 5).

Magnesium (10 mM) blocked the evoked population spike in the dentate region during the preanoxic period. The response that recovered from 10 min of anoxia (fig. 2) was measured after the magnesium was washed from the system. The mean amplitude of the postanoxic population spike was 76 ± 5% of its preanoxic value (table 1). There was significant protection of the dentate granule cells in slices treated with magnesium compared with control slices ($P < 0.005$).

CA1 Pyramidal Cell Region

The evoked population spike measured in the CA1 pyramidal cell region does not recover after 5 min of anoxia (4 ± 3%) (table 1). When nimodipine (10^{-7} M) was present, the population spike recorded from the CA1 pyramidal cells did not recover after 5 min of anoxia (table 1). The mean postanoxic amplitude of the evoked population spike in the CA1 region of slices treated with nimodipine was 5 ± 5% of its preanoxic amplitude.

Nimodipine had a very small effect on the size of the evoked population spike recorded from the CA1 region in the preanoxic period. The spike was reduced to 94 ± 4% (n = 5) of its predrug amplitude. This was unlike magnesium and cobalt; both of which completely blocked the population spike in the preanoxic period (n = 8, magnesium; n = 5, cobalt).

When cobalt (2 mM) is present in the aCSF bathing the slices during anoxia, the response of the CA1 pyramidal cells demonstrates significantly better recovery after 5 min of anoxia ($P < 0.005$) (table 1). The mean postanoxic amplitude of the response in the CA1 pyramidal cells treated with cobalt was 64 ± 12% of its preanoxic amplitude.

Slices were superfused with aCSF containing 10 mM magnesium during 5 min of anoxia; the mean recovery from anoxia with this treatment was 35 ± 10%. There was significantly better recovery ($P < 0.05$) of the population spike from anoxia in slices treated with magnesium than in untreated slices.

ATP Levels

Maintaining ATP levels during anoxia has been shown to protect against anoxic damage,[10] therefore magnesium

TABLE 2. ATP nM/mg Dry Weight

	Normoxic (mean ± SE)	n	Anoxic (mean ± SE)	n
Denate granule cells				
Untreated	4.7 ± 0.33	11	1.1 ± 0.15	15
Nimodipine (10^{-7} M)			1.0 ± 0.12	15
Magnesium (10 mM)			1.7 ± 0.2*	15
CA1 Pyramidal Cells				
Untreated	4.4 ± 0.2	12	0.72 ± 0.08	15
Nimodipine (10^{-7} M)			0.60 ± 0.05	16
Magnesium (10 mM)			1.4 ± 0.1†	15

*$P < 0.02$, or †$P < 0.005$, compared with ATP in untreated cells from the same region.

and nimodipine were tested for their effect on ATP. Nimodipine did not maintain ATP levels in either the dentate or the CA1 region during 10 min of anoxia (table 2). ATP in the dentate region decreased to 23% of normoxic levels after 10 min of anoxia in untreated slices; in slices treated with 10 mM magnesium, ATP decreased to only 36% of its normoxic level. After 10 min of anoxia, the CA1 region ATP levels were 16% of their normoxic values in untreated slices and 32% in slices treated with magnesium. Thus, 10 mM magnesium, which protected the electrophysiologic response from anoxic damage, significantly reduced the decrease in ATP during anoxia in both the dentate ($P < 0.02$) and the CA1 ($P < 0.005$) regions. Nimodipine, which did not protect, did not reduce the decrease in ATP in either region.

Discussion

Our experiments use the hippocampal slice model of anoxic damage to examine the degree of protection provided by agents that block calcium entry into neurons. This model subjects the neurons to a severe interruption of energy metabolism but allows for the continued supply of glucose and the washout of lactic acid and other metabolites. Results with this model have been confirmed by *in vivo* studies. For example, the CA1 pyramidal cells demonstrate greater susceptibility to anoxic damage than dentate granule cells in this *in vitro* and other long-term *in vivo* models.[1,17] Similarly, young animals show greater resistance to anoxic damage in this model and *in vivo*.[18,¶] Aminophosphonovaleric acid, a blocker of the n-methyl-d-aspartate (NMDA) type of glutamate receptor, protected against ischemic and anoxic damage in an *in vivo* and a hippocampal slice model.[19,20] Barbiturates have also demonstrated protection *in vivo* and *in vitro*.[11,21]

In this model we define protection against anoxic damage as a significant increase in the recovery of the evoked

¶ Kass IS, Lipton P: Differential sensitivity of pyramidal and granule cell neurons to anoxic damage in hippocampi from young rats (abstract). Society of Neuroscience 9:473, 1983

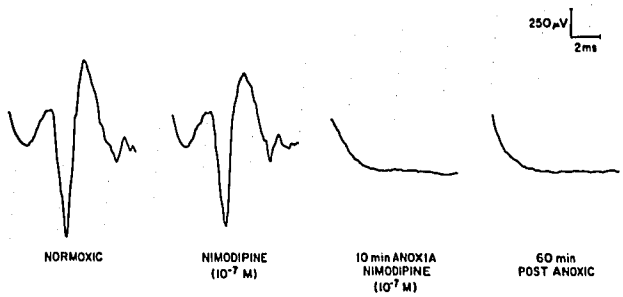

FIG. 1. The effect of nimodipine (10^{-7} M) on the recovery of responses evoked from the dentate granule cell layer after 10 min of anoxia. Responses are recorded in the dentate granule cell layer after stimulation of the perforant path. Representative traces are shown before anoxia, before anoxia with nimodipine, during anoxia with nimodipine, and 60 min after anoxia. Nimodipine is added to the aCSF 15 min before the beginning of anoxia and washed out 15 min after the end of the anoxic period.

population spike 60 min after anoxia when drug-treated slices are compared with untreated slices. The amplitude of the postanoxic spike is maximal after 30 min of reoxygenation and will remain at this level for at least 3 h (the longest time we examined). Because this brain slice preparation maintains a stable population spike amplitude (± 10%) for 6 h in slices not subjected to anoxia, our ability to confirm long-term protection is limited.

Calcium influx has been postulated as an important factor in anoxic and ischemic damage.[1-6] Previous studies[1,10] have demonstrated that reducing net calcium influx during anoxia with either 10 mM magnesium–0 calcium or 2 mM cobalt protects against anoxic damage; 10 mM magnesium–0 calcium was found to protect both the dentate granule cells and the CA1 pyramidal cells in the hippocampal slice preparation.[1] Cobalt (2 mM) had only been examined for its effect on the dentate granule cells.[1] In the current study, these experiments have been extended and 2 mM cobalt was shown to protect the CA1 pyramidal cells, which are more sensitive to anoxic damage.

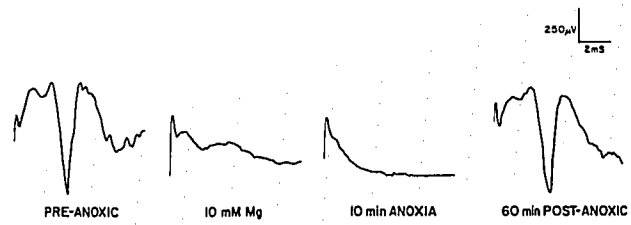

FIG. 2. The effect of magnesium (10 mM) on the recovery of responses evoked from the dentate granule cell layer. Responses are recorded in the dentate granule cell layer after stimulation of the perforant path. Representative traces are shown before anoxia, before anoxia with magnesium, during anoxia with magnesium, and 60 min after anoxia. Magnesium is added 5 min before a 10-min anoxic period and washed out 5 min after the end of the anoxic period.

Cobalt and magnesium block both voltage-sensitive and NMDA-activated channels that allow calcium into neurons. In contrast, nimodipine, a dihydropyridine-type calcium channel blocker, does not block NMDA-coupled channels and is effective against the L-, but not the T- and N-type voltage-sensitive calcium channels.[22] The dihydropyridine blockers do not block synaptic transmission and therefore are probably not effective against the voltage-sensitive calcium channels responsible for transmitter release. It is not known which calcium channels are responsible for neurotransmitter release. Our data suggest that the L channel is not important for transmitter release. Cobalt and magnesium did block synaptic transmission. We were unable to demonstrate protection against anoxic damage with nimodipine. Possible explanations are that 1) calcium entry into neurons during anoxia is by a nimodipine-insensitive calcium channel; 2) transmitter release has a major influence on the damage; and/or 3) NMDA receptor activation influences anoxic damage.

Clinical[23] and *in vivo*[7,8] studies with nimodipine have demonstrated protection against damage from ischemia. The *in vivo* studies showed an increased postischemic perfusion after treatment with nimodipine. Although this is a possible vascular mechanism for nimodipine's protective effect, an additional direct effect by blocking calcium entry into neurons has also been suggested.[8,23] Our data do not support a direct protective effect of nimodipine on the neurons. In the hippocampal slice preparation, the neurons are superfused directly with oxygenated artificial cerebrospinal fluid and any effect of nimodipine on the vasculature would not be observed.

Magnesium is protective against anoxic damage *in vitro*. In addition, and perhaps related to this, magnesium blocks synaptic transmission presumably by preventing the release of neurotransmitters. Low doses of magnesium sulfate are used safely in humans to treat preeclampsia, although the serum concentration achieved is lower than the 10 mM used in our study.[24] Magnesium sulfate has been shown to decrease seizures during epilepsy when administered intravenously,[25] but it may not normally cross the blood–brain barrier.[26] It is possible that the blood–brain barrier is altered during ischemia, as it is during epilepsy, however, there is no convincing evidence supporting this possibility. Thus it may be required that magnesium be administered directly into the cerebrospinal fluid or the brain extracellular fluid in order to test its effectiveness *in vivo*.

In other studies we have been able to correlate the decrease in ATP during anoxia with recovery of electrophysiologic activity after anoxia.[10] Therefore, nimodipine and magnesium were examined to determine if they attenuated this decrease in ATP. Magnesium, but not nimodipine, reduced the decrease in ATP during anoxia. This correlates with electrophysiologic recovery of the evoked response. The difference in ATP levels at the end of anoxia between the magnesium-treated slices, which recover physiologic activity, and the untreated slices, which do not recover, is small but statistically significant. In order for those differences to be physiologically significant, one needs to argue that there is an ATP threshold (between 1.1 and 1.7 nM/mg dry weight for the dentate tissue) below which anoxic damage proceeds. Possible mechanisms by which magnesium could help maintain ATP levels during anoxia are by blocking calcium channels, neurotransmitter release, and/or the NMDA–glutamate receptor channel. Both cobalt and magnesium block the NMDA-coupled channel.[27] The consequent reduced activation of the postsynaptic neurons should reduce the sodium and calcium influx during anoxia. This in turn would reduce the activity of both the Na–K ATPase pump and the Ca ATPase pump, thereby reducing ATP use.

We do not mean to suggest that the maintenance of ATP levels during anoxia is the only or even the principal method by which magnesium protects against anoxic damage. Other possibilities include the blockade of calcium entry into neurons. Calcium entry has been implicated as a major trigger of anoxic damage.[1,28] We believe the evidence points to a multifaceted cause of anoxic damage including, but not limited to, reduced ATP levels and increased calcium influx.

In summary, we have examined the efficacy of agents that block calcium entry for their ability to protect against anoxic damage in an *in vitro* model. Cobalt (2 mM) and magnesium (10 mM) increase the recovery of neuronal transmission in the hippocampus, but nimodipine is ineffective. The decrease in ATP during anoxia is attenuated when magnesium is present in the aCSF. Nimodipine does not affect the decrease in ATP during anoxia.

Care should be taken in using this information to predict *in vivo* or clinical results because this model superfuses the neurons independently of the vasculature and any effect of these agents on the vasculature or any tissue other than brain tissue is eliminated in this system. Another difference is that agents used in this *in vitro* model need not pass the blood–brain barrier to reach the neurons. Thus, this model does not necessarily predict clinical efficacy but is useful in examining mechanisms of protection and revealing potentially protective agents for more exhaustive study *in vivo*.

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