



The effects of baclofen on CaM Kinase following cerebral ischemia
by Andrea Joe Mary Everingham

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in
Applied Psychology
Montana State University
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Abstract:

One enzyme thought to be important in delayed cell death in the CA1 region of the hippocampus following cerebral ischemia is calcium calmodulin kinase II (CaM kinase), which has been shown to disappear in cells that will later die. This study was conducted to confirm the neuroprotective properties of baclofen and evaluate its effectiveness at preserving CaM kinase in the CA1 region of the hippocampus.

In Experiment I, Mongolian gerbils were divided into baclofen and saline groups and were anesthetized. Animals were given baclofen (50 mg/kg, i.p.) or saline five minutes before common carotid artery occlusion (five minutes). Animals were also injected with saline or baclofen 24 and 48 hours after ischemia. Three days after surgery sections of the hippocampus were collected and cresyl violet staining was used to assess presence of cells in the CA1 region of the hippocampus. Baclofen significantly protected the cells from damage due to ischemia.

Experiment II was conducted to determine the effect of baclofen on CaM kinase 24 hours after ischemia. Four groups of animals were used: baclofen-ischemia, baclofen-sham, saline-ischemia, and saline-sham. The animals were habituated to a T-maze for one week and then trained to perform a delayed non-matched to sample task. Animals underwent occlusion or sham operations with injections of baclofen or saline five minutes before. They were tested in the T-maze task 23 hours after ischemia and were perfused 24 hours after ischemia. Immunohistochemistry for CaM kinase was performed on the brain sections collected. There was no significant difference between the baclofen treated animals and the saline treated animals on the T-maze task, but there was a significant difference between ischemic and sham groups. We also found a significant difference between the baclofen treated animals and saline treated animals in the immunoreactivity of CaM kinase.

These studies suggest that baclofen is neuroprotective and also preserves CaM kinase in the CA1 region of the hippocampus. The behavioral test used as a marker of cell death indicates that although it is sensitive to ischemia, there may be factors influencing its effectiveness in testing with baclofen.

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FOLLOWING CEREBRAL ISCHEMIA

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**A thesis submitted in partial fulfillment
of the requirements for the degree**

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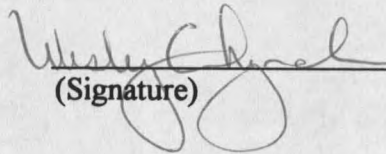
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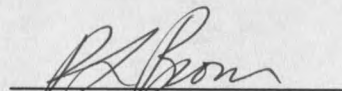
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ABSTRACT

One enzyme thought to be important in delayed cell death in the CA1 region of the hippocampus following cerebral ischemia is calcium calmodulin kinase II (CaM kinase), which has been shown to disappear in cells that will later die. This study was conducted to confirm the neuroprotective properties of baclofen and evaluate its effectiveness at preserving CaM kinase in the CA1 region of the hippocampus.

In Experiment I, Mongolian gerbils were divided into baclofen and saline groups and were anesthetized. Animals were given baclofen (50 mg/kg, i.p.) or saline five minutes before common carotid artery occlusion (five minutes). Animals were also injected with saline or baclofen 24 and 48 hours after ischemia. Three days after surgery sections of the hippocampus were collected and cresyl violet staining was used to assess presence of cells in the CA1 region of the hippocampus. Baclofen significantly protected the cells from damage due to ischemia.

Experiment II was conducted to determine the effect of baclofen on CaM kinase 24 hours after ischemia. Four groups of animals were used: baclofen-ischemia, baclofen-sham, saline-ischemia, and saline-sham. The animals were habituated to a T-maze for one week and then trained to perform a delayed non-matched to sample task. Animals underwent occlusion or sham operations with injections of baclofen or saline five minutes before. They were tested in the T-maze task 23 hours after ischemia and were perfused 24 hours after ischemia. Immunohistochemistry for CaM kinase was performed on the brain sections collected. There was no significant difference between the baclofen treated animals and the saline treated animals on the T-maze task, but there was a significant difference between ischemic and sham groups. We also found a significant difference between the baclofen treated animals and saline treated animals in the immunoreactivity of CaM kinase.

These studies suggest that baclofen is neuroprotective and also preserves CaM kinase in the CA1 region of the hippocampus. The behavioral test used as a marker of cell death indicates that although it is sensitive to ischemia, there may be factors influencing its effectiveness in testing with baclofen.

INTRODUCTION

Stroke is the third leading cause of death and disability in the United States. About thirty percent of individuals suffering stroke die within the first month following the event. Stroke is caused by a disruption in the blood flow to the brain. Focal ischemia can result following embolism, thrombosis, or hemorrhage. Cerebral embolism is a small blood clot in the general circulation of the body which lodges in one of the blood vessels of the brain and restricts blood flow to that region (Smith, 1967). Cerebral thrombosis is a blood clot that forms in the blood vessel of the brain and blocks blood flow (Smith, 1967). Cerebral hemorrhage occurs when a blood vessel ruptures in the brain (Smith, 1967).

The second type of ischemia is global or transient ischemic attacks (TIA's). These are usually caused by cardiac arrest or anoxia where blood flow to the entire brain is disrupted for a short amount of time. In all types of stroke, a brain area is deprived of blood and oxygen, which causes cell death. Neurons can also die because of events which occur during reperfusion. Depending on the site of infarct, various deficits may occur following stroke. The patient may not be able to move parts of the body, often one entire side (Smith, 1967). It is also common to see deficits in cognitive abilities such as loss of speech, confusion, difficulties in memory and judgment, and possibly dementia (Ullman,

1962). In severe cases, the patient may become abusive, resistive, and exhibit psychotic symptoms (Ullman, 1962). Even though these facts have been known for many years, the mechanism of ischemic brain damage is not fully understood.

Focal ischemia is a permanent obstruction or rupture of a blood vessel, while TIA's are temporary and do not remain following reperfusion. In focal ischemia, the area of the brain affected never receives blood or oxygen and will die as a result. Neuron death that results from TIA's occurs after blood flow to the brain has been reestablished. The cells are once again receiving blood and oxygen, but certain cells still die. The reasons for cell death is are not known.

Animal Models of Ischemia

Studying ischemia in humans is difficult because of the unpredictability and variability of the attacks. It is therefore necessary to develop appropriate animal models which approximate human ischemia. A cat model of ischemia has been developed that involves occlusion of the middle cerebral artery, which decreases blood flow to the brain (Hossmann, Mies, Paschen, Matsuoka, Schuier, and Bosma, 1983). This model is best suited to studies of pathophysiology and can be difficult to perform and interpret.

Although newer techniques limit the amount of damage caused by exposure of the artery,

it is possible to cause some lesions to parts of the brain not affected by ischemia. Primates are also used in studies of stroke. These models also involve occlusion of the middle cerebral artery (Symon, 1983). This occlusion has been shown to produce effects similar to that of stroke in humans (Symon, Dorsch, and Crockard, 1975) and the size of the primate brain makes recording from it fairly easy. Brain physiology and anatomy are also more closely related in humans and other primates than in other animals. The disadvantages of using primates include their high cost, the availability of subjects for multiple studies, and the long time required to complete experiments.

Rodents are usually chosen for studies of ischemia because they cost less to maintain and perform procedures on, they are homogenous within strains, they have small brains, which are easier to perform biochemical analysis on, and their cerebrovascular physiology and anatomy is similar to that of higher species (Ginsberg and Busto, 1989). There are models of thrombosis and embolism in the rat. In induced focal cerebral thrombosis, a photosensitizing dye is injected into the brain and a portion of the brain is irradiated with light at 560 nm which causes an aggregation of blood cells resulting in a clot (Ginsberg and Busto, 1989). This procedure is minimally invasive and the location of the thrombosis can be carefully controlled. However, the nature of the thrombosis makes it resistant to therapy and results in injury to small vessels are not representative of human thrombosis.

It is also possible to induce embolism by injecting an artificial blood clot using microspheres, into the carotid artery (Ginsberg and Busto, 1989). This procedure may be useful in specific cases, but the location and size of injury are unpredictable.

The rat model of global cerebral ischemia is similar to what occurs in humans following cardiac arrest or anoxia. In order for blood flow to be sufficiently decreased to the rat brain, the vertebral arteries are generally electrocoagulated several days before the ischemic insult. This is because there is a connection in rats and many other mammals between the vertebral arteries and the common carotid arteries. After electrocoagulation of the vertebral arteries, the animals are then subjected to common carotid artery occlusion which disrupts blood flow to the brain. This model has some drawbacks in that about 25% of the animals are lost (Ginsberg and Busto, 1989). Some of these are lost because they do not become totally unresponsive during carotid artery occlusion which suggests that they are receiving blood flow to the brain. The rest of the animals usually are lost because they have respiratory failure due to lack of blood flow to the brain stem. Another difficulty is in the electrocoagulation of the vertebral arteries. The arteries can not be seen during the procedure which can cause ineffective electrocoagulation or injury to the brain stem.

Gerbils are also used to model global ischemia. Gerbils do not possess the connection between the carotid and vertebral arteries that rats do which makes disruption of blood flow to the forebrain easier (Ginsberg and Busto, 1989). A unilateral carotid artery occlusion results in varying degrees of damage because gerbils possess a connection between the two carotid arteries. For this reason, the bilateral occlusion model is more widely used as a model of global ischemia. Clamping the carotid arteries for as little as five minutes induces delayed cell death similar to that observed in humans. The disadvantages to using gerbils as a model are that they are smaller than rats which makes monitoring (such as repeated blood sampling, which is required in autoradiographic tracer studies) more difficult. They are also susceptible to seizures which can confound findings.

The Hippocampus and Ischemia

The hippocampus is located in the temporal lobe. Although its three dimensional structure is complex, a section taken anywhere appears the same and contains the same circuitry. Incoming information from the neocortex enters the dentate gyrus which relays information to the CA3 region of the hippocampus. These CA3 neurons project to the CA1 pyramidal cells which project to the subicular complex. These neurons send

messages back to the neocortex. This circuit places the hippocampus in a position to influence the rest of the brain.

The CA1 region of the hippocampus is one of the most sensitive areas affected by TIA. Zola-Morgan, Squire, and Amaral (1986) reported the case of R.B. who had a history of heart trouble and had sustained cardiac arrest. The period of anoxia he experienced caused brain damage which manifested as anterograde amnesia. Upon his death, his brain was studied histologically and it was discovered that the CA1 region of the hippocampus had degenerated. Other parts of the hippocampus were spared from damage as well as cells outside the hippocampus (Zola-Morgan, Squire, and Amaral, 1986).

Tabuchi, Endo, Ono, Nishijo, Kuze, and Kogure (1992) reported similar damage following ischemia in monkeys. Animals subjected to 10 to 15 minute occlusions of the eight arteries which supply the brain exhibit a 40% reduction in CA1 pyramidal cells relative to controls. Five minute occlusion was not sufficient to produce damage in the CA1 region and 18 minute occlusion resulted in damage to both the CA1 region and the CA3 region. Although it is unclear why the hippocampus is particularly sensitive, significant memory impairment is frequently observed (Sarno & Sarno, 1969).

The Hippocampus and Memory

The hippocampus is important for learning and memory. Patients with hippocampal damage have difficulty consolidating certain types of new memories (Sarno & Sarno, 1969). One of the earliest clinical cases demonstrating the importance of the hippocampus in memory was that of H.M. (Milner, Corkin, and Teuber, 1968) This patient underwent an operation to reduce his frequent epileptic seizures, which involved the bilateral removal of the medial portion of the temporal lobes. H.M. experienced both mild retrograde and severe anterograde amnesia as a result of his surgery. It appeared that H.M. was unable to transfer information from short term memory to long term memory. He was able to learn new skills but could not explain when or how he learned them. H.M.'s declarative memory was impaired but his procedural memory remained intact. This example suggests that the temporal lobes are important in consolidating memories.

A study of patients with epilepsy who underwent temporal lobectomy and hippocampectomy found a correlation between severe CA3 cell loss in the hippocampus and memory deficits (Sass, Lencz, Westerveld, Novelly, Spencer, and Kim, 1991). No other subfields of the temporal lobes or the hippocampus were correlated with memory

loss. The memory test used in this study required patients to name common objects.

Therefore, it was not a test of the same type of memory deficits seen in H.M.

In a case study by Cummings, Tomiyasu, Read, and Benson (1984) a patient suffered a period of anoxia following cardiac arrest. The patient experienced severe lack of some cognitive abilities including difficulty remembering the current time and place as well as his own history. He could not remember a list of three words after a three minute time span and gave different answers to the same question when it was asked at different times. Upon his death an autopsy was performed which showed significant loss of CA1 pyramidal cells in the hippocampus while the dentate and subiculum remained intact. This study and other clinical cases suggests that the hippocampus is important in memory functions which involve acquiring new memories.

A similar finding has been demonstrated in animal models. A radial arm maze task can be used to assess memory in rats. A raised platform consisting of a center and usually eight to twelve arms with food pellets at the ends is used to test whether or not animals can remember where they have been. The animal is placed in the center of the maze and allowed to chose arms to enter. Once the food has been eaten in one arm, rats will generally not return to that arm. After hippocampal lesioning, rats enter the previously entered arms more often than normal rats (Olton and Samuelson, 1976). Ischemic subjects

performed at chance levels suggesting that they are unable to remember where they have been (Olton, 1983). Colombo, Davis, Simolke, Markley, and Volpe (1988) reported that post ischemic performance is dependent on the difficulty of the task. When only five of eight arms were baited, there was no difference observed in post ischemic rats and non-ischemic controls but a difference became apparent when the task became more difficult by baiting more arms (Colombo et al., 1988). This task is considered a measure of working memory because the animal must remember information that is important for only one trial. Reference memory is recalling information that is true each time the task is performed. If there was food placed in the same three arms for every trial, the task would be measuring reference memory.

Katoh, Ishibashi, Shiomi, Takahara, and Eigyo (1992) conducted a number of working memory tests on gerbils following 5 and 20 minute ischemia. Post ischemic gerbils (5 minute occlusion) had increased locomotor activity relative to non-ischemic controls up to five days after ischemia (Katoh et al, 1992). The authors also found that step through latency was shorter and time spent in a shock compartment longer in gerbils after both 5 minute and 20 minute ischemia when they were tested on a passive avoidance task. Finally, performance in an eight arm radial maze was also impaired in gerbils following 5 minute ischemia (Katoh et al, 1992).

Rudy and Sutherland (1989) reported that rats with hippocampal lesions were not able to perform a previously learned operant discrimination task. This paradigm required the rat to push a lever for food when a light was presented or when a tone was presented but not when both were presented. Rats with hippocampal damage could not inhibit responding when both the tone and light were presented. In a study by Ordy, Thomas, Volpe, Dunlap, and Colombo (1988), rats subjected to 30 minutes of four vessel occlusion made more errors in working memory tests such as a T-maze than sham operated animals.

Previous studies have shown that a large increase in locomotor activity following ischemia is correlated with cell death in the CA1 region of the hippocampus (Chandler, DeLeo, and Carney, 1985; Mileson and Schwartz, 1991; Wang and Corbett, 1990; Gerhardt and Boast, 1988). This increased activity has been attributed to a deficit which interferes with the animal's ability to spatially map or habituate to the environment (Wang and Corbett, 1990; Babcock, Baker, and Lovec, 1993). Spatial mapping and habituating to the environment are tasks that involve a transfer of information from short term memory to long term memory. Hippocampal damage interferes with this transfer of information and the animal is not able to effectively recall the areas where it has been.

The Role of Glutamate and Calcium in Ischemic Cell Death

There is considerable evidence suggesting that calcium influx due to glutamate stimulation is important in the chain of events that ultimately results in cell death. It has been shown that glutamate levels increase in the hippocampus during ischemia. Previous studies using microdialysis (Beneviste, Drejer, Schousboe, and Diemer, 1984; Hagberg, Lehmann, Sandberg, Nystrom, Jacobson, and Hamberger, 1985) have demonstrated a 3.5-8-fold increase in glutamate in the hippocampus during ischemia. The differences in magnitude are most likely due to experimental differences and interspecies differences. The primary input for glutamate into the CA1 cell layer is the Schaeffer collaterals. Disruption of the Schaeffer collaterals has been shown to be neuroprotective (Beneviste, Jorgensen, Sandberg, Christiensen, Hagberg, and Diemer, 1989). Studies have shown that administration of MK-801, an N-methyl D-aspartate (NMDA) receptor blocker, is neuroprotective (MacDermott, Mayer, Westbrook, Smith, and Barker, 1986). Taken together, these studies suggest that glutamate interaction with NMDA receptor types initiates the cascade of events that results in cell death. NMDA channels have a high calcium conductance and are located in the sensitive regions of the hippocampus

(Benveniste, 1991). Therefore, blocking of calcium influx is neuroprotective in some animal models (Barone, Price, Jackobsen, Sheardown, and Feurstein, 1994).

A study conducted by the Brain Resuscitation Clinical Trial II Study Group (Abramson, 1991) suggested that blocking of calcium entry by lidoflazine was ineffective in blocking cell death following anoxia due to cardiac arrest. However, lidoflazine was administered within 30 minutes of cardiac arrest, which may have been after the influx of calcium had already taken place. Extracellular levels of calcium decrease within seconds following ischemia, suggesting that the calcium has entered the cells (Hallenbeck and Dutka, 1990). This decrease in calcium is followed several hours later by an increase in extracellular calcium due to the diffusion from surrounding cells. This decrease and then increase is thought to be the cause of cell death because a similar result occurs in heart tissue which has been reperfused with calcium deficient artificial perfusate followed by reperfusion with blood (Hallenbeck and Dukta, 1990). Taken together, these studies suggest that glutamate is responsible for the observed calcium influx which leads to hippocampal cell death.

Sodium channels are also implicated in delayed neuronal cell death. Blocking sodium channels has been shown to inhibit glutamate release (Shuiab, Mahmood, Wishart, Kanthan, Murabit, Ijaz, Miyashita, and Howlett, 1995). These sodium channel blockers

have also been shown to be neuroprotective and they also enhance behavioral recovery when administered both prior to and following ischemia (Meldrum, Swan, Leach, Millan, Gwinn, Kadota, Graham, Chen, and Simon, 1992; Shuiab et al., 1995; Wiard, Dickerson, Beek, Norotn, and Cooper, 1995).

Adenosine has also been shown to be neuroprotective following cerebral ischemia (Sweeny, 1997). Adenosine is thought to inhibit glutamate through the activation of the A₁ receptor. Administration of A₁ receptor antagonists increases damage following ischemia while A₁ receptor agonists inhibit cell death (Sweeney, 1997). Adenosine does not appear as useful in focal ischemia and its effects vary greatly among brain regions (Sweeney, 1997).

Theories of Cell Death Following Calcium Influx

Reactive Oxygen Species

Reactive oxygen species (ROS), a type of free radical, have also been implicated in cell death following ischemia. Calcium influx leads to an increase in free radical production in the cell (Sussman and Bulkley, 1990) and the ROS can cause severe damage to the cell (Juurlink and Sweeney, 1997). Free radical scavengers have been shown to be neuroprotective following ischemic insult (Chan, 1994; Hall, 1993; Hall, McCall, and

Means, 1994; Siesjo, Zhao, Pahlmark, Seisjo, Katsura, and Folbergova, 1995) which suggests that ROS are important in cell death that occurs after ischemia.

GABA

GABA receptors are considered important in ischemic cell damage. GABA_B receptors are linked to calcium and potassium channels. Stimulation of these receptors has been shown to be neuroprotective (Shuaib and Beker-Klassen, 1997)

Serotonin and Norepinephrine

Serotonin receptors are abundant in the hippocampus and are inhibitory (Shuaib and Breker-Klassen, 1997). Serotonin 5HT_{1a} receptor agonists have been shown to be neuroprotective when administered both before and after ischemia (Bielenberg and Burkhardt, 1990; Zivin and Venditto, 1984). Stimulation of these receptors causes the CA1 pyramidal cells to hyperpolarize which reduces the actions of extracellular glutamate. Norepinephrine has similar effects in the hippocampus and enhancement of the noradrenergic effects appears to be neuroprotective as well (Matsumoto, Ueda, Hashimoto, and Kuriyama, 1991). This hyperpolarization of the cells decreases the amount of calcium that is allowed to enter the cell and the cascade of events leading to cell death is disrupted.

Nitric Oxide

Nitric oxide (NO) synthase is dependent on calcium and calmodulin and forms NO from L-arginine. NO increases significantly following ischemia and is implicated in the production of toxic hydroxyl radicals. Ohno, Yamamoto, and Watanabe (1994) conducted a study in which the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) was injected into the hippocampus immediately after reperfusion following global cerebral ischemia. The authors demonstrated that animals injected with L-NAME were able to perform a working memory task at the same level as non-ischemic controls. This suggests that the hippocampus is spared, however no histology was performed on the animals involved in this study.

CaM Kinase II

One approach to understanding how calcium mediates cell death is to investigate molecules that are targets for calcium. One of these molecules is the enzyme calcium calmodulin kinase II (CaM kinase) which is exquisitely sensitive to changes in calcium concentrations. CaM kinase is composed of α and β subunits which bind calmodulin and calcium. Each subunit has similar catalytic attributes but the composition varies throughout the brain (Bronstein, Farber, and Wasterlain, 1993). The ratio of α to β

subunits in the hippocampus is 3:1 respectively. CaM kinase is activated when calcium binds with calmodulin and then combines with the enzyme. After activation, CaM kinase undergoes autophosphorylation as well as phosphorylating and regulating other proteins that control neuronal functioning such as tyrosine hydroxylase, microtubule associated protein 2 (MAP 2), tau, and synapsin I (Bronstein, Farber, and Wasterlain, 1993). The two subunits of CaM kinase are cooperative but are phosphorylated independently of one another. When CaM kinase is phosphorylated, it becomes partially calcium and calmodulin independent which maximizes its activity. Synapsin I is located in the synaptic vesicle wall and inhibits the release of transmitters in its unphosphorylated form. When CaM kinase phosphorylates it in response to increased calcium, the vesicle becomes accessible for release. CaM kinase also regulates the synthesis of neurotransmitters by phosphorylating tyrosine hydroxylase which increases its action of catecholamine synthesis. In vitro, CaM kinase phosphorylates tau protein and MAP 2 inhibiting their ability to activate microtubule assembly. CaM kinase is also implicated in regulation of many other cellular processes such as potassium current, cyclic nucleotide phosphodiesterase, the inositol triphosphate receptor, voltage dependent calcium channels, and transcriptional induction by calcium (Bronstein, Farber, and Wasterlain, 1993). Unregulated changes in CaM kinase can have adverse effects on many cellular processes.

It has been shown that CaM kinase activity in the hippocampus decreases permanently shortly after an ischemic insult (Churn, Taft, Billingsley, Blair, and DeLorenzo, 1990). In a study comparing hypothermic, hyperthermic, and normal temperature gerbils in both ischemic and sham conditions, Churn and colleagues (1990) found that hypothermia protected the pyramidal cells in the CA1 region of the hippocampus from death following ischemia and that CaM kinase activity was also maintained in these animals. Hyperthermia and normal temperature both resulted in cell death in the CA1 region of the hippocampus as well as inhibition of CaM kinase activity. The mechanism responsible for the disappearance of CaM kinase and the importance of this event are unknown. It has been suggested that glutamate and subsequent stimulation of NMDA receptors may be in part responsible for CaM kinase disappearance (Churn, Limbrick, Sombati, and DeLorenzo, 1995).

Neuroprotection due to Baclofen

If glutamate is responsible for the disappearance of CaM kinase during ischemia, then inhibition of glutamate release should prevent this disappearance. Baclofen, β -(p-chlorophenyl)- γ -aminobutyric acid, was originally developed as a γ -aminobutyric acid (GABA) agonist, and has been reported to inhibit electrically evoked glutamate release in

cerebral cortex slices in guinea pigs. (Rosenbaum, Grotta, Pettigrew, Ostrow, Strong, Rhoades, Picone, and Grotta, 1990). Several studies have evaluated the effects of baclofen on cell survival with mixed results.

Using a gerbil model of ischemia, baclofen administered at a 25 mg/kg, i.p. dose was shown to significantly decrease the cell death associated with ischemia when injected five minutes before, but not five minutes after ischemia (Sternau, Lust, Ricci, and Ratcheson, 1989). The evaluation of cell survival was based on an "all-or-none" analysis in which there was either complete cell loss or no cell loss. Baclofen was 78% effective in sparing CA1 cells when injected before ischemia, while less than 10% of the animals treated with the drug after ischemia showed significant cell sparing (Sternau et al., 1989).

Lai, Shuaib, and Ijaz (1995), also using a gerbil model, reported dose related neuroprotection with baclofen when the animals were given injections at three time points (five minutes before, 24 hours after, and 48 hours after ischemia). Doses of 25, 50, and 100 mg/kg were investigated. Though there was an increase of neuroprotection as the dose increased, the difference in protection among doses was not significant (Lai, Shuaib, and Ijaz, 1995). The investigators also reported a dose related mortality rate that they attribute to an interaction with the anesthesia and the relaxant effects of baclofen on the diaphragm (Lai, Shuaib, and Ijaz, 1995).

Rosenbaum et al (1990) reported that baclofen did not protect against cell death following cerebral ischemia. In this study, rats were injected with Baclofen at 10 mg/kg, i.p. one hour before and 30-60 minutes-after ischemia. The dose was chosen because of its observed ability to reduce glutamate release without decreasing responsiveness and breathing associated with higher doses. Using a 0-4 rating system in which 0 represented no cell abnormalities and 4 represented 76-100% abnormal cells, no significant difference was found between the baclofen treated animals and the controls. The low dose chosen for this study may have been responsible for the lack of neuroprotection. Another explanation for the ineffectiveness of baclofen may be the time points at which the drug was injected (see Sternau et al., 1989).

In summary, glutamate appears to play an important role in delayed cell death following ischemia. Increased glutamate levels leads to an influx of calcium into pyramidal cells in the CA1 region of the hippocampus. This influx of calcium and disappearance of CaM kinase are implicated in the death of the pyramidal cells. Inhibition of glutamate with baclofen appears to reduce cell death in the hippocampus depending on dose and timing of administration.

EXPERIMENTS

Experiment I

Introduction

Previous studies suggest that baclofen is neuroprotective but the effect appears dose and time dependent. The first experiment was designed to confirm the neuroprotective efficacy of baclofen. This study utilized a gerbil forebrain ischemia model (Kirino, 1982; Babcock, Baker, Hallock, Lovec, Lynch, and Peccia, 1993). The dose of 50 mg/kg was evaluated because it has been shown to be neuroprotective with minimized mortality in a previous study (Lai, Shuaib, and Ijaz, 1995).

Methods

Subjects. A total of 16 male and female Mongolian gerbils weighing 60-80 g, served as subjects. They were given food and water ad lib and maintained on a 12/12 light/dark cycle. After surgery, animals were housed individually.

Procedure. Animals were anesthetized with methoxyflurane and their body temperature was maintained between 37^o and 38^oC by monitoring rectal temperature and adjusting core temperature with a heating pad. A midline incision was made in the neck of

the subject and the common carotid arteries exposed and looped with thread. Animals (n=7) were injected with 50 mg/kg, i.p. baclofen five minutes before the common carotid arteries were occluded with 85 gm pressure micro aneurysm clips. While the clips were in place, anesthesia was discontinued to assess if the arteries were correctly occluded. When the carotid arteries are correctly occluded, the animal will not awaken during surgery since blood flow to the forebrain is interrupted. Gerbils that made voluntary movement during the procedure were excluded from the study. The clips were left in place for five minutes and removed. The incision was sutured and the animal was placed in a cage and monitored until recovery from the anesthesia. The control animals (n=9) were treated the same with the exception that saline was injected and the arteries were not occluded.

Animals were given a second and third injection of baclofen (50 mg/kg) or saline at 24 hours and 48 hours after carotid occlusion (Lai, Shuaib, and Ijaz, 1995). Three days after ischemia, animals were euthanized with CO₂ and then perfused with phosphate buffered saline (PBS) and formalin. The brains were removed and allowed to postfix in formalin for one hour before being cryoprotected in 30% sucrose. Frozen sections (25 µm) were collected and stained with cresyl violet for histological assessment. Viable cells (those which were symmetrical and in which the nuclei could be seen) in the CA1 region of the hippocampus were counted using a 40X magnification and standard grid divided

into 25 squares. A random section was used from each animal and was sampled at nine different sites to determine the mean number of viable cells in the sections.

Results

Three baclofen treated animals and three saline injected animals died before assessment could take place. The baclofen treated animals (n=4) had a mean of 16.4 cells/grid square and the mean number of cells of the saline treated animals (n=6) was 5.4 cells/grid square (see Figure 1). Analysis revealed that this difference was significant [$t(8)=8.91, p=0.008$]. Representative photomicrographs of baclofen and vehicle animals are depicted in Figure 2.

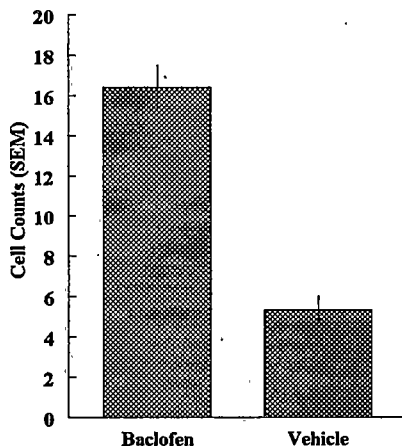


Figure 1. Number of viable cells counted in sampled region of gerbils injected with baclofen (n=4) or vehicle (n=6) 5 mins prior to, 24 hrs after, and 48 hrs after ischemic insult. Baclofen treated gerbils were found to have significantly more cells than vehicle animals.

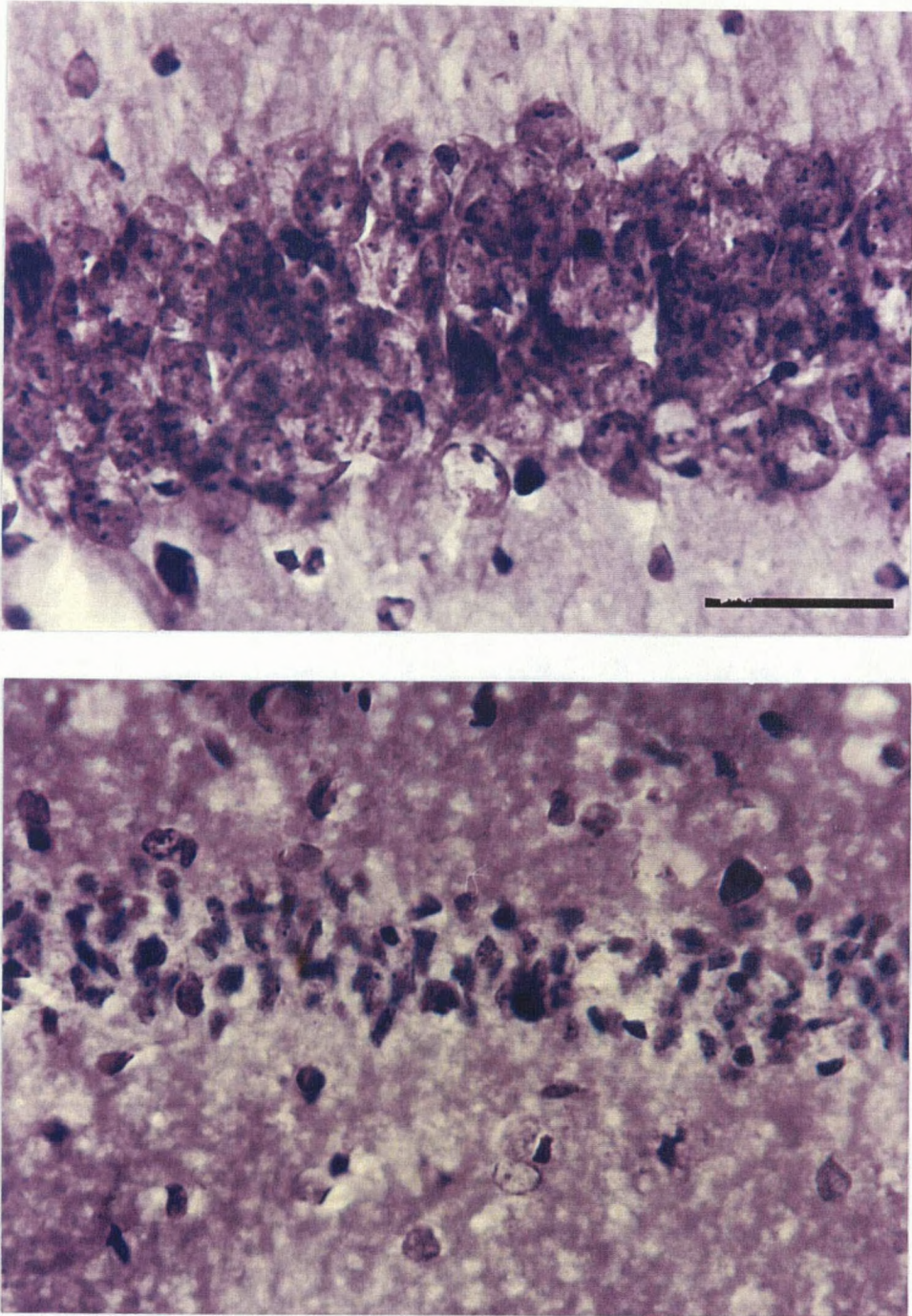


Figure 2. Representative photomicrographs of the CA1 pyramidal cell region of gerbils treated with baclofen (TOP) or vehicle (BOTTOM) prior to and after ischemic insult. The baclofen animal exhibited normal pyramidal cells while the vehicle animal exhibited significant necrosis of cells in this same region. Scale bar = 50 μ m.

Discussion

This experiment confirms that baclofen is significantly neuroprotective at a dose of 50 mg/kg, i.p. The animals receiving baclofen five minutes before, 24 hours after, and 48 hours after ischemia exhibited significantly more viable hippocampal cells than animals receiving an injection of saline at the same time points. Lai, Shuaib and Ijaz (1995) reported significant neuroprotection with injections of 50 and 100 mg/kg, i.p. while Rosenbaum et al (1990) injected only 10 mg/kg. The neuroprotection of baclofen appears to be dose dependent although very high doses tend to be fatal to the animals (Lai, Shuaib, and Ijaz, 1995).

Experiment II

Introduction

This experiment was designed to evaluate CaM kinase following neuroprotection with baclofen. We predicted that CaM kinase would be present in the CA1 region of the hippocampus of animals protected with baclofen and that it would not be present in animals given saline injections. We examined the presence of CaM kinase at 24 hours after ischemia, since cell death is not observed until three to four days following ischemia.

A non-matched to sample T-maze task was used as a behavioral marker of future cell death.

Methods

Subjects. Male and female Mongolian gerbils (n=38) 60-80 g were subjects in this study. They received food and water ad lib and were on a 12/12 light/dark cycle. All animals were housed individually and were randomly divided into four groups; baclofen-ischemia (n=12), baclofen-sham (n=6), saline-ischemia (n=11), and saline-sham (n=9).

Procedure. A delayed non-matched to sample T-maze test was used as a behavioral marker for hippocampal damage. For one week prior to surgery, gerbils were habituated to the apparatus and pretrained on the task. Animals were given unsalted sunflower seeds in their home cages in addition to their regular diet in order to habituate the animals to the seeds used in testing. The following week, animals were habituated to the T-maze. On day one of habituation, each subject was placed in the T-maze with all doors open and nine sunflower seeds scattered throughout the maze. The animals were left in the maze for five minutes or until all of the seeds were eaten. On day two seeds were placed only in the arms of the maze and on days three through five, seeds were placed in the seed cups. Again, animals were allowed to stay in the maze for five minutes or until all seeds were

eaten. Following habituation, animals were trained to complete ten trials of the delayed non-matched to sample test. One door to an arm was closed as well as the start box door. Animals were placed in the start box and the door was opened. The animal was allowed to eat the seed in the open arm and was then moved back into the start box for five seconds. At the end of the interval, the start box door was opened and the animal was allowed to choose an arm to enter. If the subject entered the arm that was opened first, it was recorded as an error and if the subject entered the arm that was opened during the interval, it was marked as correct. Each subject was given ten trials each day for five days. Subjects were divided into treatment groups by using the average performance on the T-maze test and matching animals in each group.

As in experiment I, animals were anesthetized and temperature maintained between 37° and 38° C. Gerbils received an injection of saline or baclofen five minutes before carotid occlusion or sham procedure. Common carotid artery occlusion was performed with 85 gm pressure micro aneurysm clips. While the clips were in place, anesthesia was discontinued to determine if the arteries were correctly occluded. Animals showing movement during carotid artery occlusion were excluded from the study. The sham procedure was performed similarly with the exception that the arteries were not occluded.

Twenty three hours after ischemia, the animals were tested in the delayed non-matched to sample T-maze task as in the training. Animals were each given ten testing trials.

Previous studies (Babcock et al. 1993) have shown that although CaM kinase consists of two subunits, both are altered equally following ischemia. We used a monoclonal antibody against the β subunit (gift from Dr. S.B. Churn) for the immunohistochemistry.

Animals were euthanized with methoxyflurane and perfused with chilled PBS and 4% paraformaldehyde 24 hours after the ischemic insult. Brains were removed, postfixed in the paraformaldehyde for one hour and cryoprotected in 30% sucrose. Brains were frozen and sectioned at 30 μ m. These sections were collected, rinsed with PBS, and incubated in normal horse serum containing 0.3% Triton. Sections were then incubated with a monoclonal antibody against β subunits of CaM kinase for 48 hours. The tissue was washed with PBS and biotinylated secondary antiserum (Vector kit) was added. Another wash was followed by the addition of avidin biotin peroxidase and the tissue was placed in DAB and hydrogen peroxide for five minutes to complete the reaction. Evaluation was done blind by comparing the relative amount of CaM kinase immunoreactivity in the CA1, CA3, and dentate gyrus of the two conditions using a 1-3 rating system where 3

represents immunoreactivity of the CA1 region which is equal to that of the CA3 and 1 represents a high difference in the immunoreactivity between the two regions.

Results

Three baclofen-ischemia animals, four saline-ischemia, and three saline-sham animals died before assessment could take place and were excluded from the study. An ANOVA was performed on the data collected from the non-matched to sample T-maze test. The mean percentage correct for each of the groups were baclofen-sham 75%, baclofen-ischemia 54.4%, saline-sham 65%, and saline-ischemia 44% (see Figure 3). There was a significant main effect of ischemic condition [$F(1,24)=15.48, p < 0.01$] but no main effect of baclofen treatment ($p > 0.05$) or between the baclofen sham group and the saline sham group ($p > 0.05$).

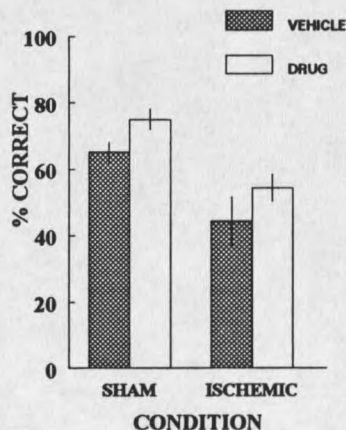


Figure 3. Mean percent correct responses (\pm SEM) for sham and ischemic gerbils pre-trained on a Win/Shift 10 sec delay task. Gerbils were given baclofen (DRUG) or saline (VEHICLE) 5 mins prior to surgery.

The results of the immunohistochemistry ratings are summarized in Figure 4. A Mann-Whitney U test was performed on the data obtained by rating the stained sections. The two sham groups were not significantly different [$U=15, p>0.05$] but there was a significant difference between the baclofen-ischemic group and the saline-ischemic group [$U=13, p=0.027$]. A representative photomicrograph illustrating the selective loss of CaM kinase immunoreactivity in the CA1 pyramidal cell layer is depicted in Figure 5. High magnification photomicrographs of the CA1 region of baclofen and vehicle gerbils are shown in Figure 6.

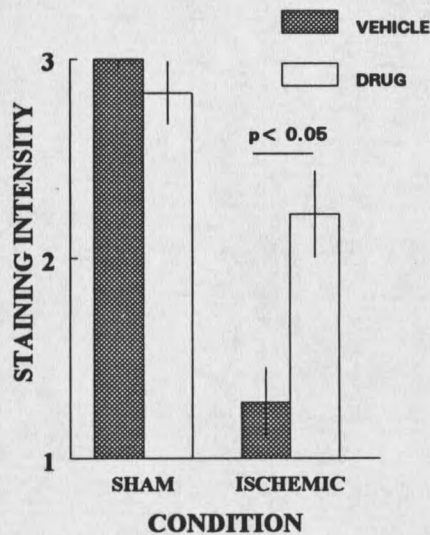


Figure 4. Staining intensity ratings for CaM kinase immunoreactivity 24 hrs following ischemic insult or sham procedure. Gerbils were pretreated with baclofen (DRUG) or saline (VEHICLE) 5 minutes prior to surgery. Baclofen treated gerbils exhibited higher staining intensities relative to vehicle animals.

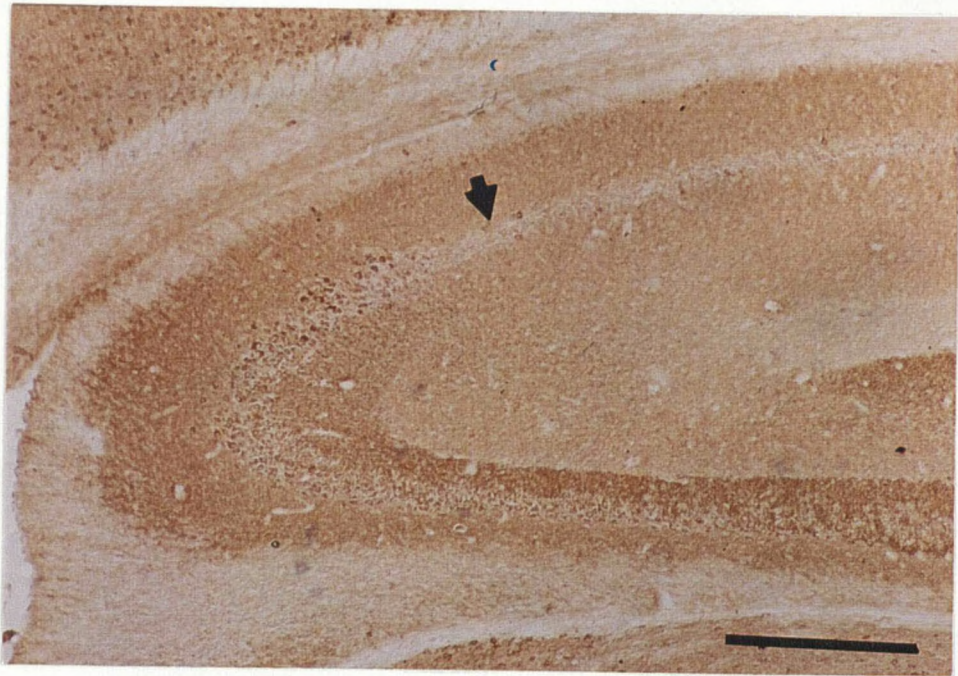


Figure 5. Photomicrograph depicting a loss in CaM kinase immunoreactivity in the CA1 pyramidal cell layer. Arrow shows CA1 region lacking CaM kinase immunoreactive cells. Gerbil was pretreated with saline 5 minutes prior to an ischemic insult and sacrificed 24 hrs later. Frozen sections of the hippocampus were processed with a monoclonal antibody against CaM kinase (β subunit). Scale bar = 500 μ m.

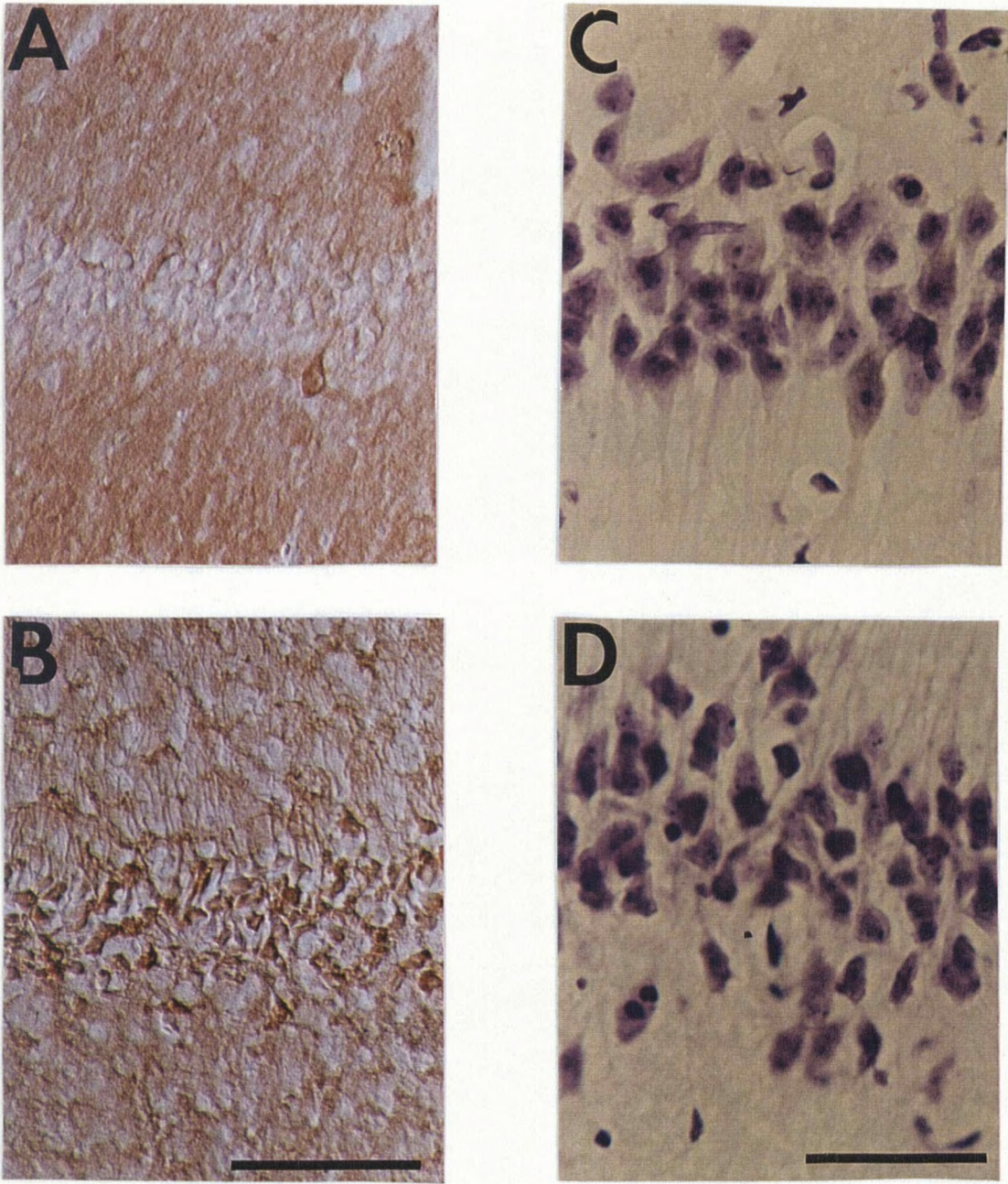


Figure 6. High magnification (40 X) photomicrographs of the CA1 pyramidal cell layer region depicting differences in CaM kinase immunoreactivity for gerbils pretreated with baclofen (A) or vehicle (B). Photomicrographs C and D are cresyl violet stained sections from the identical animals demonstrating that the loss of immunoreactivity was not associated with necrosis. Animals received an injection of baclofen or saline 5 minutes before ischemic insult and were sacrificed 24 hours later. Frozen sections were processed with monoclonal antibody against CaM kinase or stained with cresyl violet stain. Scale bar A and B = 100 μ m. Scale bar C and D = 50 μ m.

Discussion

The present study has confirmed that baclofen is neuroprotective and significantly protects against the disappearance of CaM kinase which is implicated in delayed cell death after ischemic insult. Another study conducted in our laboratory has shown that injections of glutamate into the hippocampus results in cell death and CaM kinase disappearance (unpublished observations). Churn et al (1990) has shown that hypothermia is neuroprotective and prevents the disappearance of CaM kinase. Taken together, these findings are consistent with the current speculation that CaM kinase disappearance is important in cell death following ischemia. This does not prove its role, but adds to the evidence supporting it. If we had found that CaM kinase remained while the cells disappeared, we would assume that CaM kinase is not part of the cell death cascade.

The behavioral data show a significant difference between sham and ischemic groups but there was no difference between baclofen-ischemic animals and saline-ischemic animals. This suggests that the T-maze test was sensitive to stroke but that there may be some confounding variable in the baclofen treated animals that interferes with the accuracy of the behavioral marker. A previous study (Whishaw, Rod, and Auer; 1994) has indicated that performance on a delayed non-matched to sample T-maze task was impaired by cerebral ischemia, however, this study began the behavioral testing of animals following

three weeks of recovery. It is possible that the short recovery period after surgery interferes with performance on the T-maze test. Because we were interested in the CaM kinase presence at twenty four hours, we did not have the opportunity to test animals at a later time.

Another potential confound is that animals were not performing at very high levels on the last day of training. Subjects were only performing at 70-80% on the last training day and performance at 50% or below is considered chance level performance. There is not sufficient difference between performance on the last test day and chance levels making it more difficult to show significance in score changes. Because of the staggered scheduling of testing animals, it was impossible to extend training beyond the five days.

Finally, previous studies (Stackman and Walsh, 1994; Sandyk and Gillman, 1985) suggest that baclofen may interfere with working memory. Stackman and Walsh (1994) studied the effects of intraseptal injection of baclofen in a radial arm maze and found that animals made more errors under the influence of baclofen than control animals. This deficit has also been reported in humans undergoing treatment with baclofen (Sandyk and Gillman, 1985). In three case studies, Sandyk and Gillman (1985) reported that patients had difficulty remembering names and places and experienced deficits in short term memory. It could be possible that this effect of baclofen is interfering with the

performance of the T-maze task. Although these studies suggest a deficit in working memory due to baclofen, the current study does not support that. We would expect to see a decline in performance of baclofen-sham animals if baclofen was causing a disturbance in working memory.

Although we have shown baclofen to be neuroprotective, the mechanisms mediating this effect are not clearly understood. Baclofen has been reported to be both a GABA_B agonist and a glutamate inhibitor (Rosenbaum, Grotta, Pettigrew, Ostrow, Strong, Rhoades, Picone, and Grotta, 1990). It is possible that baclofen inhibits the release of glutamate presynaptically by stimulating GABA_B receptors and it is also possible that this stimulation can cause the postsynaptic cells to be hyperpolarized which diminishes the actions of glutamate in the synapse. We can not determine this from our experiment because we did not measure glutamate concentrations. This may be an important study to conduct if we are to understand the mechanisms of baclofen better.

The current study confirms the neuroprotective ability of baclofen which appears to inhibit glutamate in the CA1 region of the hippocampus. It also supports the theory that CaM kinase is an important factor in delayed cell death following ischemia. Future studies are necessary to determine the precise mechanisms involved in this cell death that results in devastating effects on humans following anoxia.

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