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

by

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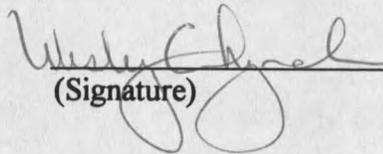
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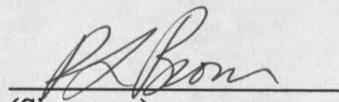
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VITA

Andrea Joe Mary (Daniel) Everingham was born August 27, 1973 in Havre, Montana to Michael J. and Susan J. Daniel. She attended Havre High School and received a Bachelor of Science degree in Psychology from Montana State University in May, 1995. She entered the Graduate Program in Applied Psychology in June 1995.

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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.

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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 A_1 receptor. Administration of A_1 receptor antagonists increases damage following ischemia while A_1 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 5HT1a 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° and 38°C 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 CO_2 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
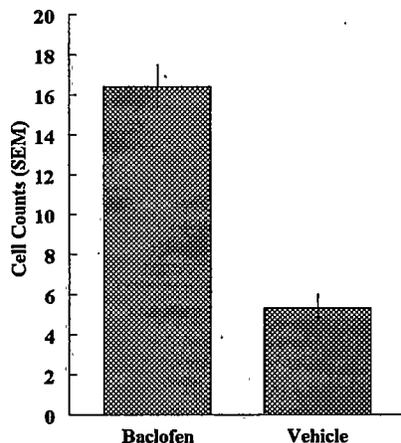
are depicted in Figure 2.

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.