

THE USE OF *IN VITRO* MODELS FOR NEURAL INJURY WITH SUPERIMPOSED HYPOXIA IN THE DEVELOPMENT OF NEW HEAD INJURY TOLERANCE CRITERIA

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ABSTRACT

Acceleration of the head produces a deformation of the neural and vascular components of the brain. Previous work indicates that the vasculature responds to dynamic deformation by vasoconstriction. This work and other work showing that there is an increase in intracranial pressure after traumatic head injury suggest that a venous constriction can produce regional cerebral hypoxic ischemia. Dynamic mechanical deformation of isolated neural cells results in increases in intracellular calcium concentration. The magnitude of the transient rise in concentration and therefore the rate of return of the concentration to normal depend on the magnitude and time course of the deformation. In a hypoxic ischemic environment, increases in intracellular calcium concentration that are slightly injurious are hypothesized to become more injurious.

An *in vitro* model of isolated neural cells is employed to determine the effect of hypoxic ischemia on the recovery of neural cells from mechanical insult. The model is composed of a neuroblastoma x glioma cell hybrid (NG108-15), which is grown in a chamber designed to deliver a known strain and strain rate to the cell while the chemical environment (of the cell) is independently controlled. The actual cell deformation is determined in the form of the average strain in the cell membrane using fluorescent microspheres. To produce hypoxic ischemia, the normal bathing media is replaced with media containing 2-deoxyglucose (25 mM) and salicylate (3 mM). The intracellular concentration of calcium is used as an index of cell injury and is measured fluorimetrically in real time using a calcium indicator dye.

The responses of cells to mechanical stimulus with and without hypoxic ischemia are compared. Experiments were performed in which the cells were exposed to mechanical deformation only, hypoxic ischemia only, and the combination. These data will be used to improve injury tolerance criteria by relating the response of single cells to mechanical insults and the effects of systemic responses such as hypoxic ischemia.

INTRODUCTION

Traumatic injury to the head is responsible for 60,000 fatalities per year, and another 40,000 severely injured people per year in the United States. It is estimated that 400,000 people have mild head injuries that produce some degree of functional disability for a period of months after the initial injury. In the United States alone, nearly two million people per year suffer some type of injury [1].

The current head injury tolerance criteria are not based upon epidemiological studies, or on results from animal and physical models. These more recently developed animal and physical models have been useful in elucidating the deformations and microscopic injuries induced in the brain during trauma.

Non-impact injuries to the head have been studied in our lab using a subhuman primate model for neural injury [2]. The crania of these primates were subjected to rotational inertial

accelerations that produced a range of injuries including diffuse brain injuries and subdural hematomas. These results indicate that both the neural and vascular components of the brain undergo deformations during a head injury. In order to quantify these deformations, physical models of the primate skulls were developed using a surrogate brain material [3, 4]. These models were subjected to the same kinematic loading conditions as the primates and the resultant displacements in the brain material were measured.

The response of blood vessels to axial deformations applied at the same rate and to the same deformation as seen in the physical models was studied *in vitro* [5, 6]. Femoral arteries and veins were isolated from a rat preparation and stretched uniaxially using a small scale materials testing device. The study showed that veins respond to mechanical stimuli of the form seen in traumatic injury with an immediate and prolonged vasoconstriction, while arteries do not. These studies and others done with the piglet fluid percussion model [7] indicate that there is a decrease in intracranial blood flow during a traumatic head injury (ischemia). This decrease in blood flow and the continued energy requirements of the cells results in a decrease in metabolites available to the cells, a condition termed hypoxic ischemia.

The decrease in metabolites available to the brain has been seen indirectly in the subhuman primate experiments [8, 9]. In certain inertial experiments, the brain was fixed and sectioned. For brains subjected to rotational accelerations, the adenosine triphosphate (ATP) content of various cortical regions in the sections was found to be between 25 and 80% of corresponding regions in non-accelerated brains. ATP is the major energy carrying component in all cells. These results therefore indicate that the cells did not have enough energy available in the form of metabolites to maintain their normal ATP levels.

The intracellular calcium concentration of neural-like cells *in vitro* has been shown to increase in response to mechanical deformation [10]. *In vivo*, the calcium concentration is four orders of magnitude higher outside of the cell than inside and is maintained by the cell at an intracellular value of order 10^{-7} M. Calcium is a secondary messenger in the cell and is used in the preservation of the cell's cytoskeleton. The maintenance of the intracellular calcium concentration is thus of paramount importance to the cell and is used in this study as an index of cell injury.

To develop more comprehensive injury tolerance criteria that include the effects of hypoxic ischemia on the recovery of brain tissue from traumatic injury, the response of the underlying brain components to these stimuli must be determined. In this study, a model for isolated neurons has been developed in which a single cell can be deformed while its chemical environment is controlled. The model relates the effects of mechanical stimuli and hypoxic ischemia on intracellular calcium concentration.

METHODS

A system capable of applying a uniform biaxial deformation to cultured cells has been developed [11]. In this system, the cells are grown on the exposed surface of an elastic substrate that is attached to the bottom of a circular well, which is attached to a base (see Figure 1). The elastic substrate is a transparent silicone elastomer (Sylgard 184, Dow Corning) that has been coated with polylysine and laminin to promote cell attachment and neurite outgrowth. The cells are deformed by decreasing the pressure below the elastic substrate to which they are attached. Due to the cell attachments to the substrate, the substrate deformation is transferred to the cell. The chemical environment of the cell can be controlled independently of the mechanical deformation by altering the composition of the cell bathing media.

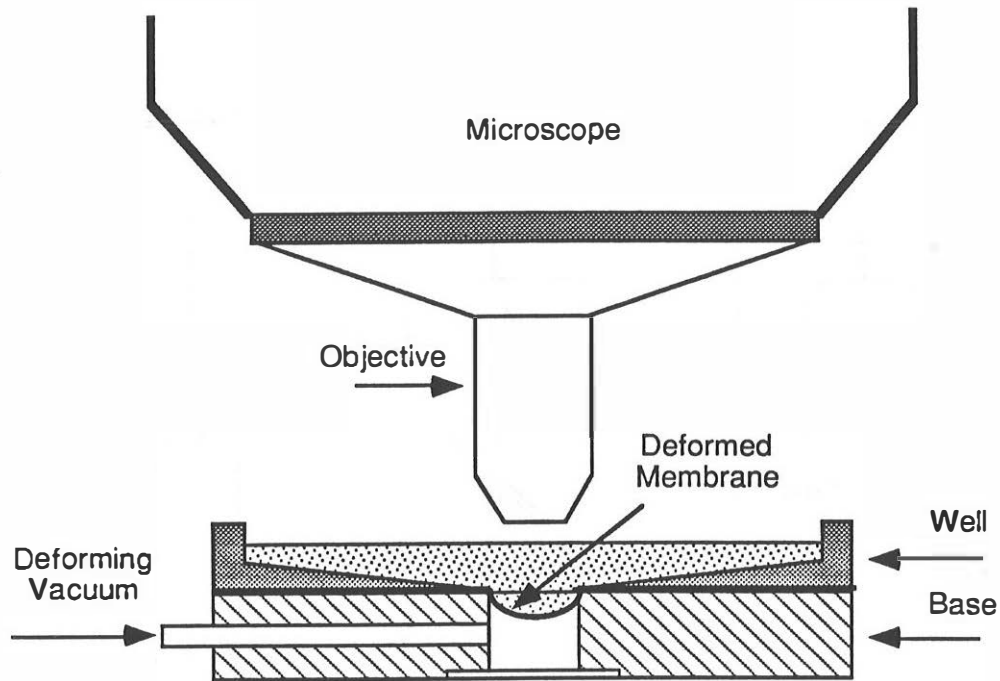


FIGURE 1

A neuroblastoma cross glioma cell line (NG108-15) is used as the neural cell model. These cells become neural-like when they are differentiated with dibutyryl cyclic adenosine monophosphate (dBcAMP). The neural-like properties of these cells have been well documented and include electrical excitability and receptors for morphine [12]. The cells also respond to laminin [13], secrete acetylcholine and form synapses with muscle cells in the manner of primary neural cells. This cell line has been used as a model for neural cells by many other researchers in the past [13-17].

The cells are cultured in Dulbecco's Modified Eagle Medium (DMEM) fortified with 10% Fetal Bovine Serum. In preparation for an experiment, the cells are plated onto the exposed elastic substrate in the bottom of the well and left to differentiate for 5 days in DMEM containing 1 mM dBcAMP. The cells are then loaded with 2 μ M Fura-2AM in phosphate buffered saline (PBS). The loading is performed at room temperature to avoid compartmentalization. The PBS used for these preparations and the normoxic (normal oxygen level) experiments is a phosphate buffer fortified with 25 mM glucose and equilibrated with room air. After loading with dye, the well is attached to its base and placed on the microscope stage.

The intracellular calcium concentration is measured using the calcium indicator dye Fura-2 (Molecular Probes, Eugene, OR, USA) and a custom designed quantitative fluorimeter. The ratio of fluorescent emissions of the calcium indicator Fura-2 for two excitation wavelengths provides a quantitative measure of the calcium concentration in the cell. The fluorimeter is designed around a Nikon Optiphot microscope configured for epifluorescence measurements and includes a photomultiplier tube (PMT) to measure the intensity of the fluorescent emissions. The source for the two excitation wavelengths is a xenon lamp directed through a chopper wheel with two filters. The emission intensity data from the PMT is demodulated with position data from the filter wheel into two signals representing the emission at each of the two excitation wavelengths (see Figure 2). This data is transferred into a computer through an analog to digital converter and stored.

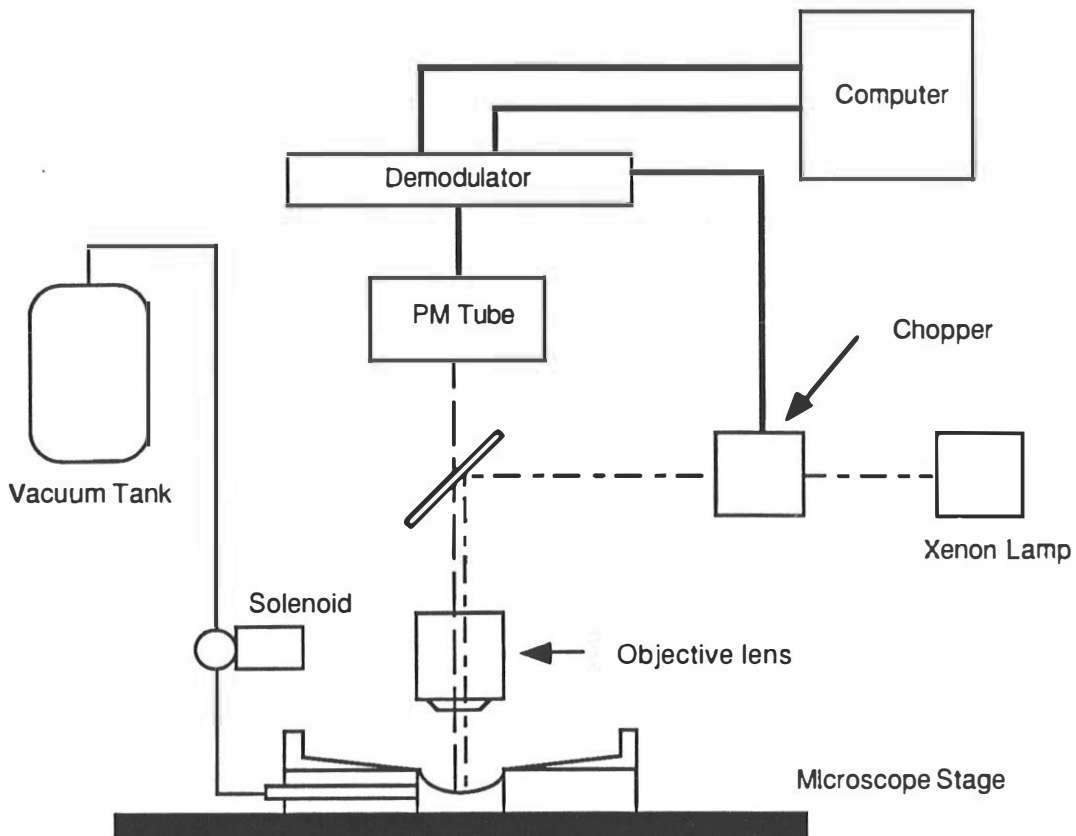


FIGURE 2

An experiment is begun by obtaining the baseline fluorescence of the cell of interest and a background reading of the substrate with no cell. Impulse deformations of the substrate are then applied by evacuating a tank to the desired vacuum level and applying a narrow width square wave to the solenoid valve between the tank and base. The result is a rapid deformation and relaxation of the elastic substrate and a corresponding cellular deformation. Fluorescence data is acquired via computer before and after the deformation for 2 minutes. The background fluorescence of the substrate is subtracted from each of the emission data sets and the ratio is taken, producing a quantitative value for the change in calcium concentration.

Chemical poisoning with 25 mM 2-deoxyglucose and 3 mM salicylate is used to mimic hypoxic ischemia. The 2-deoxyglucose inhibits anaerobic metabolism by blocking the glycolytic pathway and is used in the place of glucose in the cell media. Salicylate uncouples oxidative metabolism in the mitochondria and is used in the form of the sodium salt in the cell media. This combination results in the cell acting as if the glucose and oxygen had been removed from the surrounding media. This is the equivalent of hypoxic ischemia in the brain. To perform an experiment with chemical hypoxic ischemia, the same preparation as described above is followed. After the well with loaded cells is attached to the base, the normal PBS is replaced with hypoxic ischemic PBS. The experiment is then performed as before.

The actual strain produced in the cell for a given substrate deformation is determined using fluorescently labeled microspheres (Polysciences, Warrington, PA, USA) [18]. These microspheres are 0.7 microns in diameter and fluoresce at the same wavelengths as fluorescein. A solution of 40 μ l of microspheres in 10 ml of PBS is placed in the well for 15 minutes.

Approximately 4 microspheres attach to the apical surface of each cell and some attach directly to the elastic substrate. The excess beads are rinsed off with PBS. The well is then placed on the base and the microsphere positions are recorded using a camera attached to the microscope. The elastic substrate is then inflated quasistatically to produce a known substrate strain, the microscope is refocused and the microsphere positions are recorded. The microsphere positions for each level of substrate strain are digitized and fed into a computer program that calculates the strains between microspheres with respect to the unstrained positions.

RESULTS

The results of the cell membrane strain measurements are shown in Figure 3. These strains are calculated using the microsphere technique described in the methods section. The strains are referenced to the distances between the microspheres prior to substrate deformation. The solid line is the strain corresponding to the substrate deflection as calculated from the analysis of Winston, *et al.* [11] for a circularly clamped elastic membrane. The open diamonds represent the strains calculated from microspheres attached to the substrate and agrees with the theory within the error of the measurement technique. The open squares represent the strain calculated using microspheres attached to the cell membrane of a single cell. The cell membrane strain is less than the substrate strain; however, it increases in proportion to that of the substrate. This proportionality constant was found to be approximately 0.8 and will be used when converting between substrate strain and cell strain.

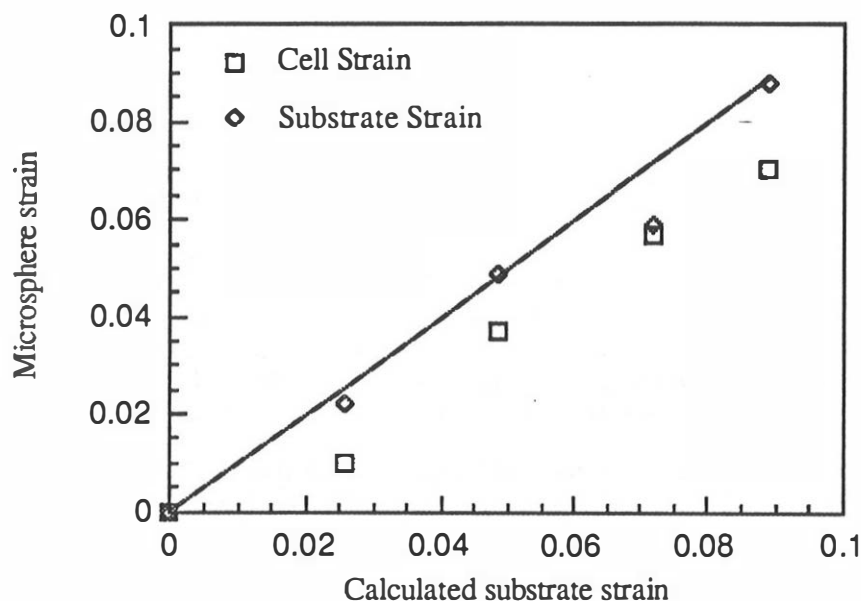


FIGURE 3

Control experiments were performed in which the intracellular calcium concentration was measured while the cells were exposed to hypoxic ischemic media with no mechanical stimulus. The results in Figure 4 show that the cells do not begin to show an increase in calcium until one half hour after the addition of the hypoxic ischemic media. The small break in the data results from a limitation of the data acquisition software, which can only acquire data for a maximum of 30

minutes. The break represents the delay between the end of one data set and the beginning of the next.

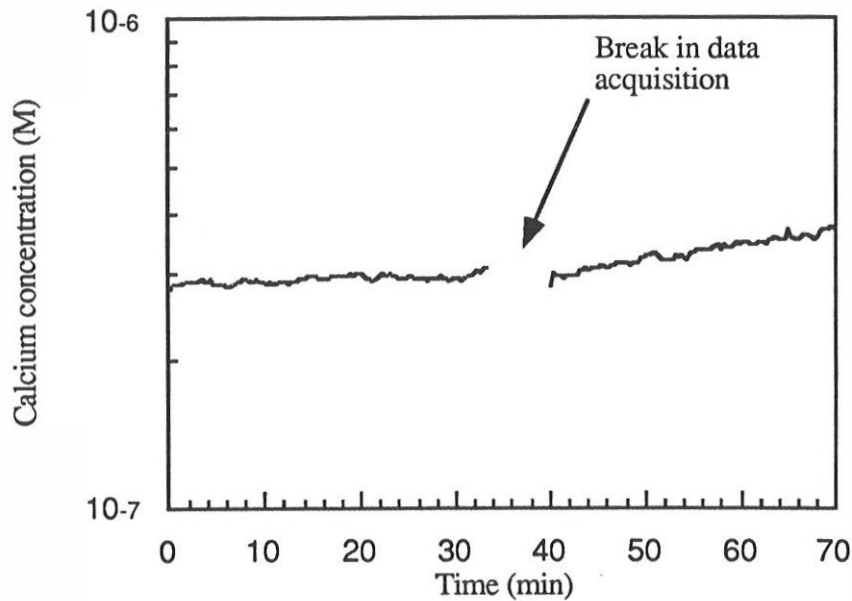


FIGURE 4

The effect of a rapid biaxial stretch under normoxic conditions on the cells for four experiments is shown in Figure 5. For each experiment, the stretch was applied between 5 and 10 seconds after the initiation of data acquisition. The form of the pressure change was an impulse with a rise and fall time of 10 msec and a dwell time of approximately 0.1 sec. The four lines from top to bottom represent substrate strains of 0.23, 0.19, 0.35, and 0.45, respectively. The two uppermost traces show an increase in intracellular calcium concentration of approximately 250 nM occurring in 12 seconds, yielding an average rate of 20 nM/sec. The next lowest trace requires 30 seconds for the intracellular calcium concentration to increase by 100 nM.

The combined effects of rapid biaxial stretch and hypoxic ischemia are shown in Figure 6. The stretch was applied 5 seconds on the time axis. The load was an impulse with a rise and fall time of 10 msec and a dwell time of approximately 0.1 sec. The calcium concentration increased by 5 μ M in 8 seconds, yielding an average rate of 600 nM/sec. The peak substrate strain was 0.50.

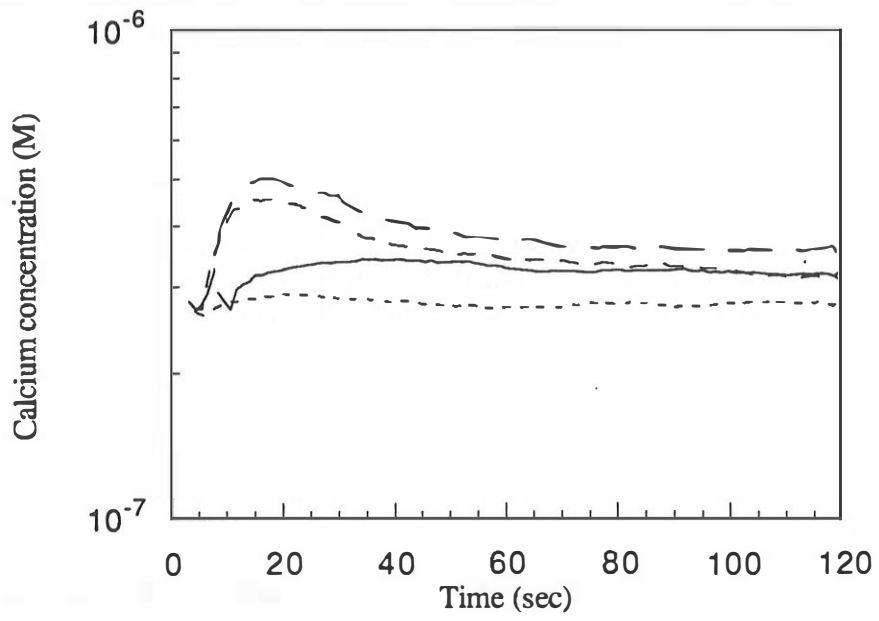


FIGURE 5

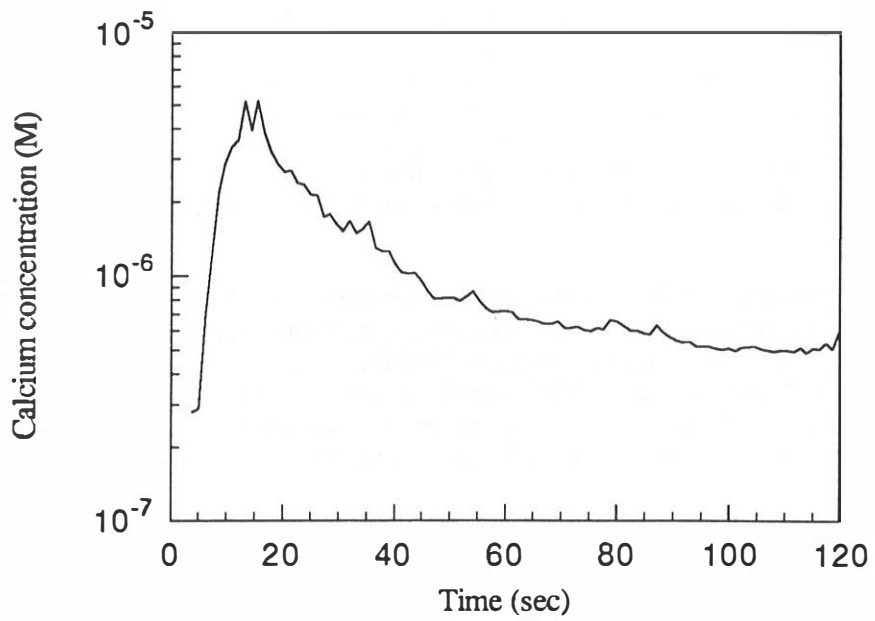


FIGURE 6

DISCUSSION

Neuroblastoma cross glioma cell hybrids were subjected to mechanical deformation with superimposed hypoxic ischemia or normoxia. The mechanical deformation applied to the cells is representative of that seen in traumatic head injury as determined by physical model studies. In the physical model experiments, the strains induced in the cortical regions of the brain range up to 0.50 at a strain rate of order 100 s^{-1} [19]. The cellular responses seen in this study are therefore indicative of the *in vivo* responses of neural cells in traumatic head injury.

The results of this study indicate that there is both a qualitative and a quantitative difference between the response of neural cells to normoxic mechanical stimulus and hypoxic ischemic mechanical stimulus. The hypoxic ischemic cells subjected to mechanical deformation have a more rapid increase in calcium concentration (600 nM/sec) that ultimately leads to an intracellular calcium concentration that is one order of magnitude higher than in the normoxic experiments (20 nM/sec). The higher final value of intracellular calcium seen in the hypoxic ischemic cells indicates that the cells have been injured to a larger degree than the normoxic cells.

Currently, alterations in intracellular calcium concentration are thought to be the underlying mechanism in cell death [20]. The immune complement system [21], excitotoxicity [22], and platelet activating factor [23] all produce an increase in intracellular calcium concentration that ultimately leads to cellular destruction. One example of a calcium dependent injury mechanism is the calcium activated neutral proteases (CANPs) that have been found to exist in neural cells [24]. These proteases become active when the intracellular concentration of free calcium exceeds approximately one micromolar. The active proteases then begin to disassemble the actin filaments that make up the cytoskeleton of the cell, causing cell destruction. This dependence of the viability of the cell on calcium concentration indicates the importance of the effect of hypoxic ischemia on the increase in intracellular calcium concentration of neural cells resulting from mechanical insults.

Calcium is continuously removed from the cell to maintain the concentration gradient. This continuous active transport requires metabolic resources from the cell. In a hypoxic ischemic cell, these resources are no longer available. If, in addition to hypoxic ischemia, there is an increase in calcium influx due to mechanical deformation, the cell's metabolic resources will be further burdened. The result of this synergy between the mechanical deformation and hypoxic ischemia is the inability of the cell to remove calcium from its intracellular space as fast as it is entering. This analysis is supported by the rate data obtained from these studies. The rate of increase in calcium in the hypoxic ischemic cell (600 nM/sec) is 30 times higher than the rate for the normoxic cells (20 nM/sec).

These data present the case that the outcome from mechanically induced neural injury will depend upon neuronal deformation and compromise of the metabolic machinery in a synergistic fashion. The latter is the result of a localized reduction in cerebral blood flow also induced by the mechanical deformation of the cerebral blood vessels. In other words, a reversible and recoverable form of trauma to the neural elements can become an irreversible event when one considers the simultaneous events occurring in concert with the mechanical deformations.

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