Sodium influx blockade and hypoxic damage to CA1 pyramidal neurons in rat hippocampal slices


Department of Biology, Vassar College, Poughkeepsie 12604; and Department of Anesthesiology and Department of Physiology and Pharmacology, State University of New York Health Science Center, Brooklyn, New York 11203

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INTRODUCTION

Ischemia and hypoxia of the brain lead to irreversible neuronal damage and cause death and disability. In this paper we investigate the effect of interrupting sodium influx, one component of the ischemic and hypoxic cascade.

It is known that during hypoxia and ischemia there is a depolarization of the neurons that is associated with a change in their intracellular ionic composition; altering the ionic environment of these neurons attenuates this change (Hansen 1985; Tanaka et al. 1997). Sodium influx increases early during hypoxia and appears to be an important component of the rapid depolarization (Hansen 1985; Tanaka et al. 1997). Sodium is an ion that is central to cellular homeostasis; its gradient across the cell membrane is used as an energy source to transport calcium, glutamate, and hydrogen (Choi 1992; Siesjo and Siesjo 1996). These components are thought to be important determinants or triggers of neuronal damage. Glutamate-induced excitotoxicity has been implicated as an important trigger of hypoxic damage (Choi 1992). High cytosolic sodium concentrations can reverse the glutamate transporter, cause release of glutamate, and enhance excitotoxicity (Roettger and Lipton 1996). Much evidence exists to establish the importance of calcium as a trigger of hypoxic damage (Kass and Lipton 1982, 1986; Lipton 1999; Siesjo 1991); there is little evidence of sodium’s role during hypoxia independent from its effect on calcium. This paper examines whether reducing the intracellular sodium accumulation and the depolarization during hypoxia without significantly altering cytosolic calcium can improve recovery after hypoxia.

It has been established that blockade of sodium channels can reduce hypoxic or ischemic damage (Astrup et al. 1981; Boening et al. 1989; Lucas et al. 1989; Stys et al. 1992; Weber and Taylor 1994). Lidocaine and tetrodotoxin block sodium channels; lidocaine at high concentrations can also block potassium efflux and other cellular processes (Astrup et al. 1981; Strichartz and Ritchie 1987). Tetrodotoxin is highly specific for the sodium channel. The low concentration of lidocaine used in our study is similar to an antiarrhythmic dose used clinically and does not disrupt neuronal activity (Fried et al. 1995; Lucas et al. 1989; Stys et al. 1992; Weber and Taylor 1994). There is evidence that lidocaine can improve recovery both in animals in vivo and clinically (Lei et al. 2001; Liu et al. 1997; Mitchell et al. 1999; Shokunbi et al. 1990). We have used intracellular recording techniques, histological analysis, measurement of protein synthesis, and cytosolic calcium concentration measurements to improve our understanding of how the blockade of sodium influx during hypoxia improves recovery.

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METHODS

Slice preparation

The experiments were approved by the Institutional Animal Care and Use Committee and conform to National Institutes of Health guidelines. Many of the techniques used in this paper have been reported by us before and will be described briefly (Fried et al. 1995; Kass et al. 1992; Raley-Susman and Lipton 1990; Raley-Susman et al. 1997; Wang et al. 1999, 2000). For each experiment an adult Sprague-Dawley rat (110–120 days old) was anesthetized with 2% isoflurane until it lost its righting reflex and was unresponsive to touch (Zhu et al. 1997). The rat was then decapitated, its brain removed and placed in ice-cold (2–4°C) artificial cerebrospinal fluid (ACSF). The hippocampus was dissected from the brain and sliced transversely. ACSF contained (in mM) 126 NaCl, 3 KCl, 1.4 KH2PO4, 26 NaHCO3, 4 glucose, 1.3 MgSO4, and 1.4 CaCl2, with pH 7.4, and was equilibrated with 95% O2-5% CO2. Hypoxia was generated by switching to ACSF preequilibrated with 95% N2-5% CO2. During hypoxia the slice is continuously superfused with ACSF containing 4 mM glucose. This is a less severe insult than that used in many other slice studies of in vitro ischemia in which the ACSF is switched to 0 glucose during the hypoxia. Both models wash potentially toxic metabolites from the slice during the hypoxic/ ischemic period. Unless indicated in the text, the duration of hypoxia is 10 min. In the drug-treated groups, slices were exposed to drug 10 min before and during 10 min of hypoxia. Two concentrations of lidocaine (10 and 100 μM) and tetrodotoxin (6 and 63 nM) were examined. The slices were maintained at 37°C during the experiments.

Electrophysiology

For the electrophysiology experiments, 400-μm slices were prepared with a vibratome, incubated at room temperature, and then placed in a tissue chamber (Fine Science Tools) at 37°C. Multiple slices from the same animal were transferred to the tissue chamber serially to minimize the number of animals used. The slices were submerged 1 mm below the ACSF surface in the tissue chamber and superfused at a rate of 3 ml/min. Intracellular recordings of CA1 pyramidal neurons were made with glass micropipettes filled with 4 M KAc. The resistance of the electrodes ranged from 70 to 120 MΩ. A bipolar stimulating electrode was placed in the Schaffer collateral pathway before impaling the CA1 neuron. Only neurons with stable resting potentials of at least −55 mV for 15 min with high-amplitude, short-duration action potentials that showed spike frequency accommodation and were activated by short-latency Schaffer collateral stimulation were examined. These parameters are characteristic of CA1 pyramidal cells, and our recordings were typically stable for over 1 h. Analog signals were recorded with an AxoClamp 2B amplifier and an IBM-compatible computer.

Protein synthesis and morphology

In the experiments that examined protein synthesis and morphology, slices were exposed to either lidocaine or TTX 15 min before, during, and 15 min after 10 min of hypoxia. Forty-five minutes after the end of the 10-min hypoxia, 4.5 μCi/ml [3H]leucine was added to each beaker, and the slices were allowed to incorporate the label into protein for an additional 75 min after the insult. Slices were washed in ice-cold buffer for 3 min to remove extracellular unincorporated label and were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer at 4°C. [3H]Leucine incorporated into protein, but not free unincorporated intracellular leucine, is retained by the tissue (Raley-Susman and Lipton 1990). Based on our previous measurements in response to anoxia/aglycemia, changes in incorporation reflect changes in protein synthesis, not changes in the specific activity of the precursor pool or in the pool of free unlabeled leucine (Raley-Susman and Barnes 1998; Raley-Susman and Lipton 1990). Slices were dehydrated and embedded in methyl acrylate. Five-micrometer sections were mounted on coated slides and dipped in NTB2 emulsion (Kodak) for autoradiographic assessment of protein synthesis. Autoradiographs were digitized and analyzed as described previously (Wang et al. 1999), where silver grain density is proportional to the amount of radioactivity incorporated into protein. Densities from experimental groups were compared with densities of normoxic control sections treated identically on the same slides.

Morphological assessment was performed on adjacent 20-μm tissue sections using light microscopy as described by us previously (Wang et al. 1999). In brief, we scored the histologic class of all neurons in the CA1 pyramidal layer of a tissue section from each slice. Healthy neurons (class A) had intact cell boundaries, a clear uniform nucleus and clear cytoplasm. Unhealthy neurons (class C) were either indistinct, lacking a clear cell boundary, with a small darkened nucleus, or darkened, shrunken cells. There were a number of neurons in each slice that exhibited an intermediate damaged morphology, class B, suggestive of a progression of damage. For simplicity, we focused our analysis on the class A and class C neurons. The percentage of the intermediate neurons (class B) did not change between normoxic and hypoxic conditions (data not shown). We examined the morphology of neurons 2 h after the hypoxic insult to correspond temporally with the protein synthesis measurement. This time point is after many reversible changes have recovered toward normoxic levels, thus at a time preceding overt cell death.

ATP, Na, and K measurements

Slices (500 μm) were prepared using a manual tissue slicer and mounted on nylon mesh attached to a Plexiglas grid. The use of a manual tissue slicer allowed a greater number of slices to be obtained from each animal and substantially reduced the number of animals required for these experiments. The grids were then placed in beakers containing ACSF. Slices from the same animal were distributed to beakers subjected to either control or experimental treatments. The slices were incubated at room temperature for 45 min, and then the temperature was increased to 37°C and maintained at that temperature for the rest of the experiment. The slices were subjected to hypoxia 75 min after shifting to 37°C. The ACSF in the beaker was aerated with 95% O2-5% CO2; to generate hypoxia the ACSF in the beaker was aerated with 95% N2-5% CO2. Sodium, potassium, and ATP levels in tissue from the CA1 region were measured before and during hypoxia. ATP concentrations were measured in the CA1 regions from slices frozen in liquid nitrogen and lyophilized to complete dryness (4.5 h). The CA1 regions were microdissected and weighed, and the ATP was extracted in perchloric acid frozen on dry ice (Kass 1986). The neutralized extract was assayed in a photometer using the luciferin-luciferase assay (Lust et al. 1981).

Sodium and potassium were measured using a flame photometer. At each time point, slices were placed in agitated ice-cold (4°C) isotonic sucrose for 10 min to wash ions from the extracellular space (Kass et al. 1992). The CA1 regions of the slices were rapidly microdissected, placed on preweighed aluminum foil boats, dried at 85°C for 48 h, and weighed. Each boat, which contained the pooled CA1 regions of all the slices from a single beaker, was placed in a 0.5-ml microcentrifuge tube. Dilute nitric acid (0.1 M) was added to the tubes that were then shaken for 16 h to extract ions from the tissue. The tubes were centrifuged at 13 K for 1 min, and the supernatant was assayed in a flame photometer (Fried et al. 1995).

To measure cellular sodium accurately, the sodium in the extracellular space of the slice needs to be washed out; however, there is a small loss of ions from the intracellular space while washing out the extracellular space of the slice. We have optimized the wash out conditions for sodium (Kass et al. 1992) such that there is minimal loss of cellular sodium. There is a sharp difference in slope of the sodium wash out indicating two major pools: an initial fast component indicative of the extracellular pool and a much slower component is
indictive of wash out from the intracellular space. At 10 min of wash out in ice-cold isotonic sucrose, the slope of the sodium in the fast component extrapolated to 0 while there was only a minimal effect on sodium from the slow component (the intracellular pool) (Kass et al. 1992). Potassium wash out did not separate neatly into two very different slopes, and 10 min in isotonic sucrose caused a relatively greater loss of potassium during wash out. One interpretation of this is that there is enhanced cellular permeability to potassium replenishing the extracellular space leading to a reduced fast component (Kass et al. 1992). Therefore the potassium concentrations measured are lower than their true level. We are more interested in the sodium levels and that is why we optimized the technique to measure sodium. All the tissue in our study was treated identically, and relative changes in the amount of sodium and potassium are compared.

Calcium imaging

Slices for Ca imaging were sectioned to a thickness of 300 μm on a vibratome. These thinner slices improved the visualization of tissue in the microscope and dye loading. Slices were incubated in oxygenated ACSF for at least 2 h before dye loading. Two slices were then placed in a small beaker with 6 ml ACSF containing 9 μM Fura 2 AM (Molecular Probes; Eugene, OR), 0.01% pluronic acid and 50 μM stratum pyramidale and the proximal third of the stratum oriens and light source and the CCD camera for image acquisition. The calcium attached to a filter changer (340- and 380-nm filters), a 300-W Xenon violet objective (n.a. 0.5) and a Nikon TMS inverted microscope were cinnati, OH). A long working distance Nikon Plan Fluor dual wave length imaging system with a low light level charge-coupled device (CCD) camera was used (Intracellular Imaging, Cincinnati, OH). An InCyt Im2 from the initial incubation until they were placed in a tissue chamber on the microscope stage: maintenance at this temperature improved dye loading. The slices were maintained at 37°C in the tissue chamber on the microscope stage throughout the experiment. An InCyt Im2 dual wave length imaging system with a low light level charge-coupled device (CCD) camera was used (Intracellular Imaging, Cincinnati, OH). A long working distance Nikon Plan Fluor ×20 ultraviolet objective (n.a. 0.5) and a Nikon TMS inverted microscope were attached to a filter changer (340- and 380-nm filters), a 300-W Xenon light source and the CCD camera for image acquisition. The calcium concentrations were measured from a region that included the CA1 stratum pyramidale and the proximal third of the stratum oriens and the stratum radiatum.

We used Ca^2+ buffers in solution and Fura-2 salt (Molecular Probes) to calibrate the imaging system for Ca concentration. All values represent Ca concentrations corrected for background fluorescence in time-matched, unlabeled (no Fura2) slices subjected to hypoxia (Wang et al. 2000). This was done to correct for the increase in background fluorescence due to the increase in NADH levels during hypoxia (Brooke et al. 1996).

Statistics

A χ^2 test was used to analyze recovery in the electrophysiological experiments. To statistically analyze the morphologic changes, data were transformed using an arc sine transformation of the square root of each value (Motulsky 1999). These transformed data then fit a normal distribution and were analyzed using an ANOVA followed by the Newman-Keuls test for multiple comparisons. The protein synthesis, ATP, sodium, potassium, and calcium data were analyzed using an ANOVA followed by the Newman-Keuls multiple comparison test (Prism, GraphPad Software, San Diego, CA). P < 0.05 was considered significant for all analyses.

RESULTS

Membrane potential changes

During hypoxia there is an initial hyperpolarization followed by a slow then a rapid depolarization (Fig. 1A). In untreated hippocampal slices, 10% of the CA1 pyramidal cells recovered their normal resting potential and their ability to generate action potentials after 10 min of hypoxia. A low concentration of lidocaine (10 μM), which did not alter the excitability of the neurons before hypoxia, significantly improved recovery of membrane potentials and excitability after hypoxia; 67% of the CA1 pyramidal neurons recovered (Fig. 1B). A higher concentration of lidocaine (100 μM) also significantly improved recovery when compared with untreated slices (80% of the neurons recovered); there was no significant difference in recovery between the 10- and 100-μM lidocaine groups after 10 min of hypoxia. If the hypoxic period was extended to 15 min, then 100 μM, but not 10 μM lidocaine, significantly improved recovery (Fig. 2B). Thus the higher concentration is more potent against the effects of prolonged hypoxia.

The slow depolarization during hypoxia is prolonged and attenuated by 10 μM lidocaine such that the neuron does not become completely depolarized after 10 min of hypoxia (Fig. 1A). One hundred micromolar lidocaine further reduced this depolarization. At 15 min of hypoxia, the 10-μM lidocaine group was completely depolarized and did not recover while the 100-μM lidocaine group was depolarized to the same level as the 10 μM after 10 min of hypoxia (Fig. 2A). Thus it seems that complete depolarization during hypoxia leads to the loss of membrane potential recovery after hypoxia.

A high concentration of lidocaine (100 μM) markedly decreases excitability to both orthodromic activation and intracellular current injection before hypoxia; 10 μM lidocaine has no effect on excitability (Fig. 1C). After 10 min of hypoxia, CA1 cells from both the 10- and 100-μM lidocaine groups recover their resting and action potentials.

The mean time after hypoxia for recovery to 50% of the resting potential with 10 μM lidocaine was 2.95 ± 0.70 (SE) min, while 100 μM lidocaine shortened this time to 1.17 ± 0.25 min; these values were not significantly different.

The threshold for action potential generation with current injection was elevated in both the 10- and the 100-μM lidocaine groups 60 min after hypoxia (15 and 0% of the neurons generated action potentials, respectively, at 100 pA; 54 and 67% at 200 pA; 100 and 100% at 300 pA) compared with the threshold before hypoxia and drug addition (82% at 100 pA). This indicates a reduction of excitability even though action potential generation is preserved. There was no significant difference between the two lidocaine concentrations.

Lidocaine has actions other than blocking sodium channels on the cell membrane; we therefore examined the effect of tetrodotoxin (TTX), a drug that selectively blocks certain sodium channels. Any effects on other ions or cellular processes would therefore be secondary to the blockade of sodium influx.

The effect of TTX on the membrane potential changes during hypoxia was similar to that of lidocaine. A low concentration of TTX (6 nM) prolonged and attenuated the slow depolarization and delayed the onset of the rapid depolarization (Fig. 3A). A higher concentration (63 nM) further prolonged the slow depolarization and blocked the rapid depolarization (Fig. 3A).

The mean time after hypoxia for recovery to 50% of the resting potential with 6 nM TTX was 3.88 ± 1.44 min, 60 nM TTX shortened this time to 1.10 ± 0.20 min, these values were significantly different.

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FIG. 1. Effect of lidocaine on recovery of membrane and action potentials after 10 min hypoxia. A: the membrane potentials (mean ± SE) of CA1 pyramidal cells during hypoxia with 0, 10, and 100 μM lidocaine. Lidocaine significantly delays the onset of the rapid depolarization and the level of depolarization at 10 min hypoxia. B: percent of CA1 pyramidal neurons that recover their resting and action potentials after 10 min hypoxia. In slices treated with lidocaine 10 μM (n = 21) and 100 μM (n = 10), there was significantly better recovery compared with untreated slices (n = 21) after 10 min hypoxia (*P < 0.01, χ² test). C: an example of intracellular electrophysiologic recordings of an individual experiment from each treatment group. The 1st trace in each set is the response recorded from a CA1 pyramidal cell after stimulation of the Schaffer collateral pathway; the 2nd and 3rd trace in each set is during 100- and 200-pA current injection through the recording electrode. Sets of traces are shown in each treatment group: before drug, 10 min after drug application before hypoxia, during 10 min hypoxia with drug, and 60 min after reoxygenation and drug wash out. The excitability of neurons before hypoxia was not altered by 10 μM lidocaine. The neurons treated with 10 and 100 μM lidocaine recovered their ability to be excited; however, the threshold to evoke action potentials was increased, and their response to stimulation was not as robust after hypoxia.
Both concentrations of TTX examined (6 and 63 nM) improved the recovery of the membrane potential and the ability of neurons to generate action potentials after hypoxia (Fig. 3B). In untreated slices, no cells recovered after 10 min of hypoxia; 31% of the CA1 pyramidal cells recover if the slices were treated with 6 nM TTX, while 73% recovered in slices treated with 63 nM TTX.

**Morphology**

Two hours after 10 min hypoxia, there is distinct and significant morphological damage (class C cells; see Methods for a description). We assessed the percentage of CA1 neurons in each slice that exhibited this damaged morphology and the corresponding percentage of healthy cells (class A cells).
treated slices showed a substantial loss in the percentage of class A cells in the CA1 region, with a corresponding increase in class C cells (Fig. 4A). Ten micromolar lidocaine significantly reduced the percentage of damaged CA1 pyramidal cells 2 h after 10 min of hypoxia (47 vs. 78% in untreated hypoxic slices). One hundred micromolar lidocaine had a similar effect on the morphology (51% damaged cells); there was no significant difference between the morphology with the two concentrations of lidocaine.

TTX (63 μM) significantly improved the histology of hypoxic neurons following 10 min hypoxia (Fig. 4B). In untreated slices 70% of the CA1 pyramidal neurons appeared damaged (class C cells); this compares with 40% damaged cells from slices treated with 63 nM TTX. Six nanomolar TTX did not significantly reduce the percentage of damaged neurons after 10 min hypoxia (63%).

Protein synthesis

There was dramatic and persistent inhibition of new protein synthesis in the CA1 neurons 2 h after 10 min hypoxia, to 17.3% of normoxic levels (Fig. 5A). Ten micromolar lidocaine significantly attenuated the inhibition of protein synthesis (to 42% of normoxic levels), and 100 μM lidocaine preserved protein synthesis to an even greater degree (67% of normoxic levels). One hundred micromolar lidocaine-treated hypoxic slices had significantly more new protein synthesis than 10 μM lidocaine-treated hypoxic slices. Thus while both concentrations of lidocaine improved the recovery of protein synthesis, the 100-μM concentration was more effective.

In the TTX study, hypoxia caused an inhibition of new protein synthesis to 21.9% of normoxic levels when measured 2 h after the insult (Fig. 5B). Six nanomolar TTX did not significantly attenuate this inhibition (levels were 44% of normoxic protein synthesis rates). However, 63 nM TTX significantly improved protein synthesis rates after hypoxia to 73% of normoxic levels.

ATP concentrations

ATP fell to 10% of its normoxic concentration during 10 min of hypoxia; this fall was attenuated to only 22% by 100
µM lidocaine (Fig. 6A). Ten micromolar lidocaine did not significantly alter the ATP depletion during 10 min of hypoxia; it fell to 15% of its normoxic concentration.

The fall in ATP during hypoxia was significantly attenuated by 63 nM but not 6 nM TTX (Fig. 6B). ATP fell to 11 and 24% of its normoxic concentration after 10 min hypoxia with 6 and 63 nM TTX, respectively.

**Sodium and potassium concentrations**

During hypoxia, ATP concentrations were reduced; this should inhibit the Na/K pump and cause sodium to increase dramatically. Sodium increased to 218% of its normal concentration after 10 min of hypoxia (Fig. 7A). A low concentration of lidocaine (10 µM) significantly attenuated the increase in sodium during hypoxia (to 181% of its concentration with normal oxygen); a higher concentration of lidocaine (100 µM) further attenuated this increase (155%). Lidocaine significantly reduced the hypoxic sodium influx during hypoxia; 100 µM was significantly more effective than 10 µM.

The sodium concentration increased to only 179% of its normoxic concentration in slices treated with 63 nM TTX (Fig. 7B). This was significantly attenuated compared with untreated hypoxic tissue (226%). There was a small, but not significant, attenuation in the rise in sodium with 6 nM TTX; it rose to 207% of its normoxic concentration.

There was a decrease in intracellular potassium to 43% of its normal concentration at 10 min of hypoxia; 10 µM lidocaine did not significantly attenuate the fall in potassium during hypoxia (51% of normoxic concentration) (Fig. 8A). The high concentration of lidocaine (100 µM) significantly attenuated the fall in intracellular potassium; potassium fell to only 71%.

Thus there is an important difference between 10 µM lidocaine’s effect on potassium and sodium; the low concentration appears to be selective for sodium.

**Calcium concentrations**

Increased intracellular calcium concentrations have been implicated as a trigger of hypoxic neuronal damage. During 10 min of hypoxia, calcium increased to 241% of its concentration during normal oxygenation (Fig. 9A). Ten micromolar lido-
lidocaine, but not TTX at 10 nM, significantly attenuated the rise in cytosolic calcium, concentrations and reduced the depolarization during hypoxia, though it improved the recovery of physiologic properties and morphology after 10 min of hypoxia (Table 1). This indicated that neurons could be protected against hypoxic damage even if calcium levels rise during hypoxia.

During hypoxia the neurons slowly depolarize, reach a threshold, and then undergo a rapid depolarization to 0 mV (Hansen 1985). The reduced cellular ATP concentration during hypoxia is thought to inhibit the sodium-potassium pump and allow potassium to build up outside the neuron. This would slowly depolarize the neurons, open more sodium channels, and accelerate the depolarization; lidocaine and TTX would reduce this action. Aside from the effect of depolarization on sodium channel activation, it has been demonstrated that hypoxia can directly open sodium channels (Hammarstrom and Gage 2000). This may be an important component of the slow depolarization. The hypoxic activation of these sodium channels was blocked by lidocaine and TTX (Hammarstrom and Gage 2000); these results are consistent with our finding that lidocaine and TTX attenuate the slow depolarization, thereby delaying the onset of the rapid depolarization. It is thought that the hypoxic depolarization is an important aspect of the pathophysiologic events (Balestrino 1995; Fung and Haddad 1997; Perez-Pinzon et al. 1998; Tanaka et al. 1997). There are large fluxes of sodium and calcium into the cell and potassium out of the cell when the neurons completely depolarize (Hansen 1985). There is also a massive release of glutamate, which opens ligand-gated ion channels and accelerates these ion fluxes (Choi 1992). The changes in cytosolic ion concentrations caused by the increased membrane conductance are exacerbated by an inhibition of membrane ion pumps due to decreased ATP concentrations. It is thought that the reduced ATP and the altered cellular ionic environment during hypoxia triggers processes leading to delayed damage. The current paper examines how sodium channel blockade alters the primary effects of hypoxia; it does not look at delayed damage or secondary biochemical processes triggered by these initial changes.

High cellular sodium levels and depolarization can have direct deleterious effects on cells. The increase in sodium can lead to cell swelling and direct histologic damage; it will also alter the sodium electrochemical gradient and inhibit or reverse important membrane transporters for H⁺, Ca²⁺, and glutamate (Lipton 1999; Roettger and Lipton 1996). Since depolarization also affects the sodium electrochemical gradient in a similar manner, it will also affect these transporters. The depolarization and high cellular sodium would lead to cellular acidosis and calcium loading as well as the release of glutamate into the extracellular space. The first two are directly damaging to cells; the release of glutamate can lead to excitotoxic damage, which is an important component of ischemic damage (Choi 1992; Lipton 1999; Siesjo and Siesjo 1996).

There is substantial evidence that increased cytosolic calcium concentrations are an important trigger for hypoxic and ischemic damage (Kass and Lipton 1982, 1986; Roberts and Sick 1988; Siesjo 1991; Tymianski and Tator 1996). Calcium is known to trigger a number of biochemical cascades that may lead to neuronal damage (Lipton 1999; Siesjo and Siesjo 1996; Tymianski and Tator 1996). Others have also found evidence that the blockage of sodium influx can improve recovery after hypoxia and ischemia (Astrup et al. 1981; Boening et al. 1989; Lucas et al. 1989; Stys et al. 1992; Weber and Taylor 1994). It

DISCUSSION

We have examined the mechanisms by which blocking sodium channels attenuates hypoxic damage. Both TTX and lidocaine were examined since lidocaine has other actions aside from its ability to block sodium channels (Astrup et al. 1981; Das and Misra 1992). We found that concentrations of lidocaine and TTX that attenuated the increase in sodium concentrations and reduced the depolarization during hypoxia, but did not significantly attenuate the rise in cytosolic calcium, improved the electrophysiology, protein synthesis, and morphology after 10 min of hypoxia (Table 1). This indicated that neurons could be protected against hypoxic damage even if calcium levels rise during hypoxia.

FIG. 8. Effect of lidocaine and tetrodotoxin on the potassium concentration in the CA1 pyramidal cell layer at 10 min of hypoxia. Values are the means ± SE (*P < 0.01, vs. untreated hypoxic tissue; Newman-Keuls test). A: 100 μM lidocaine, but not 10 μM lidocaine, significantly attenuated the fall in potassium (nM/mg dry weight) compared with untreated tissue during hypoxia (n = 6 for all groups). B: 63 nM TTX, but not 6 nM TTX, significantly attenuated the fall in potassium (nM/mg dry weight) compared with untreated tissue during hypoxia (n = 6 for all groups).

The increase in calcium during hypoxia with 63 nM and 6.3 nM TTX was to 186 and 225% of its normoxic concentration, respectively; the calcium concentration at the end of 10 min of hypoxia after the application of these TTX concentrations was not significantly different from untreated tissue (Fig. 9B). Thus 63 nM TTX did not block the change in cytosolic calcium even though it improved the recovery of physiologic properties and protein synthesis after 10 min of hypoxia.
has been unclear whether this blockage of sodium influx was indirectly attenuating the rise in cytosolic calcium by improving the pumping of calcium out of the cytosol and reducing the calcium influx through ion channels (Zhang and Lipton 1999). In the present study, a low concentration of lidocaine reduced the hypoxic changes in sodium and attenuated the hypoxic depolarization, but did not significantly alter the changes in ATP, calcium, or

TABLE 1. Effect of lidocaine and tetrodotoxin on damage after and cellular conditions at 10 min of hypoxia

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<tr>
<th>Condition</th>
<th>Resting Potential</th>
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<th>Histology</th>
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A plus sign indicates significantly different from untreated tissue subjected to the same hypoxic period; 0 indicates no significant difference. The recovery of the Resting Potential, Protein Synthesis, and Histology are measured 1–2 h after 10 min of hypoxia. The other parameters are measured at 10 min of hypoxia.
potassium at 10 min of hypoxia. These results indicate that depolarization and the increase in sodium during hypoxia are important triggers for the loss of viability after hypoxia, independent of their effect on calcium.

We are only measuring short-term indicators of recovery; it is possible that the calcium increase during hypoxia has a greater influence on delayed damage, which we cannot measure in acute brain slices. However, a clinical study indicated that low concentrations of lidocaine (serum levels between 16.6 and 7.8 \( \mu \text{M} \)) protect against cognitive deficits, which are common after open heart surgery (Mitchell et al. 1999). These deficits are thought to be due to ischemia from microemboli. We found that a low concentration of lidocaine, equivalent to 10 \( \mu \text{M} \) concentration used in the current study, reduces infarct size 7 days after middle cerebral artery occlusion (Lei et al. 2001). In an in vivo forebrain ischemia model, Liu et al. (1997) found that lidocaine improved histologic outcome and increased the latency and reduced the amplitude of the extracellularly measured depolarization during ischemia. This is analogous to the changes we found in vitro with intracellular recording techniques. The studies described above give us confidence that our current study is examining mechanisms that are applicable to ischemia in vivo and long-term damage even though we can only examine the acute effects of these agents.

The length of the hypoxic depolarization correlates with damage both in vivo and in vitro (Liu et al. 1997; Perez-Pinzon et al. 1998). Both 10 and 100 \( \mu \text{M} \) lidocaine and 63 nM tetrodotoxin delayed the hypoxic depolarization. In addition to delaying the hypoxic depolarization, all the concentrations of lidocaine and TTX tested reduced the final level of the depolarization at 10 min of hypoxia. Thus the recovery of the resting and action potentials seem to be determined, at least in part, by the extent and magnitude of the hypoxic depolarization.

In support of this was the finding that 10 \( \mu \text{M} \) lidocaine did not improve recovery of the resting and action potentials after 15 min of hypoxia; this period of hypoxia allowed the membrane potential to reach 0 mV even in the presence of 10 \( \mu \text{M} \) lidocaine. The membrane potential did not completely depolarize after 15 min of hypoxia with 100 \( \mu \text{M} \) lidocaine, and both membrane and action potentials recovered after this period of hypoxia. In our studies when the membrane potential was prevented from completely depolarizing, the membrane and action potentials recover after the hypoxia. In two other studies from our laboratory, thiopental and hypothermia attenuated the final level of depolarization during hypoxia and improved recovery (Wang et al. 1999, 2000).

TTX (63 nM) and lidocaine (10 \( \mu \text{M} \)) did not significantly affect calcium but did significantly attenuate the depolarization and reduce cellular sodium levels during hypoxia. There was improved cell morphology, protein synthesis, and electrophysiologic recovery under these conditions indicating that blocking the increase in sodium and the hypoxic depolarization can improve recovery even if calcium levels still rise. This is a major finding of this paper since most previous work emphasized the importance of calcium in leading to damage and ignored sodium’s role.

Blocking sodium channels during hypoxia has two primary effects that are difficult to separate; it reduces cytosolic sodium levels and attenuates the hypoxic depolarization. Both of these effects are significantly attenuated by concentrations of blockers that improve recovery. We separately plotted the membrane potential and the sodium concentration at 10 min of hypoxia versus the normalized percentage of protein synthesis recovery, resting potential recovery, and normal morphologic appearing neurons (type A) 1–2 h after 10 min hypoxia. The percentage recovery for each category was calculated as the value 1–2 h after hypoxia divided by its prehypoxic value times 100. The numbers in parentheses indicate the linear correlation coefficient \( (r^2) \), the number sign indicates that the slope of the regression line was significantly different from zero. A: the less the CA1 neurons depolarize at 10 min of hypoxia the greater was the recovery of protein synthesis, histology, and resting potential after hypoxia. B: protein synthesis but not histology or resting potential correlated with sodium levels at 10 min of hypoxia. C: none of the indicators of recovery correlated with cytosolic calcium levels at 10 min of hypoxia.
A) 1–2 h after 10 min hypoxia (Fig. 10). The percentage recovery for each category was calculated as the value 1–2 h after hypoxia divided by its prehypoxic value times 100. We found an inverse correlation between the level of depolarization during hypoxia and the recovery of protein synthesis, histology, and resting potential after hypoxia ($R^2 = 0.93, 0.68, 0.93$; all slopes significantly non-zero; Fig. 10A). Protein synthesis but neither histology nor resting potential correlated with sodium levels during hypoxia ($R^2 = 0.75, 0.64, 0.44$; only the slope of the protein synthesis was significantly different from zero; Fig. 10B). Thus an attenuation of the membrane potential during hypoxia correlates better with recovery of the resting potential and improved morphology; interestingly both membrane potential and sodium concentrations correlated with improved protein synthesis.

Using the same analysis techniques, we demonstrated that the level of cytosolic calcium during hypoxia did not correlate well with protein synthesis, histology, or the resting potential ($R^2 = 0.35, 0.13, 0.64$; none of the slopes were significantly different from zero). This is further evidence of protection from damage with sodium channel blockers that is not dependent on cytosolic calcium levels.

A previous study (Fried et al. 1995) found that 10 $\mu$M lidocaine significantly attenuated the increase in sodium and the fall in ATP concentrations at 5 min of hypoxia, indicating that 10 $\mu$M lidocaine delays the fall in ATP. Thus the changes in depolarization, sodium, and ATP may play a role in the protection by 10 $\mu$M lidocaine even though no improvement in ATP is measured at 10 min of hypoxia.

ATP levels fell during hypoxia; this fall was attenuated by both lidocaine and TTX. However, even untreated hypoxic tissue recovered 60% of their prehypoxic ATP concentration by 30 min after hypoxia (Kass and Lipton 1982; Wang et al. 1999); the energy charge recovered almost completely. ATP and energy charge are low only during and shortly after the hypoxic period. The sodium and potassium levels also recover substantially within 30 min of reoxygenation; sodium recovers to 118% of prehypoxic levels, and potassium recovers completely (Wang et al. 1999). Even though cells will not recover function as assayed by protein synthesis, histology, and recovery of the resting potential, their ATP, sodium, potassium, and calcium levels recover after hypoxia. Thus these changes must be triggering other events during hypoxia that lead to the damage. Our results are different from Taylor et al. (1999), who did not find recovery of the ions after in vitro ischemia. Their ischemic lesion was more severe since they not only subjected their slices to hypoxia but removed glucose from the ACSF, we maintain glucose at 4 mM during hypoxia and generate a milder lesion.

There was no further improvement in cellular morphology when the lidocaine concentration was increased from 10 to 100 $\mu$M lidocaine. This suggests that the increase in the hypoxic depolarization and sodium were primarily responsible for the histologic damage and that calcium played only a minor role in altering cell structure shortly after hypoxia. The blockade of sodium channels would reduce cell swelling and improve the histologic state.

The alteration of protein synthesis after hypoxia may be due to the hypoxic depolarization, the increase in cytosolic sodium, and the decrease in ATP; our results indicate that calcium may not be responsible for it. TTX (63 nM) demonstrated the same improvement of protein synthesis as 100 $\mu$M lidocaine, yet only 100 $\mu$M lidocaine blocked the increase in cytosolic calcium. TTX and lidocaine had similar effects on sodium and ATP concentrations.

Some possible mechanisms by which damage could be prevented independent of calcium are by 1) preserving ATP during hypoxia, which would improve protein synthesis and other anabolic processes; 2) attenuating the hypoxic depolarization, which might block direct damage to ion channels; depolarized or inactivated channels might be more sensitive to damage due to proteolysis during hypoxia; and/or 3) reducing glutamate efflux by a reversed transporter and thereby attenuate excitotoxic damage.

There was improved recovery with 100 $\mu$M compared with 10 $\mu$M lidocaine after 15 min of hypoxia; the former powerfully attenuated the increase in cytosolic calcium. This indicates that calcium may be an important trigger for neuronal damage. While calcium appears not to be responsible for the inhibition of protein synthesis, there is evidence that it enhances protein degradation by activating caspases and proteases, this will exacerbate the effects of protein synthesis inhibition (Lipton 1999). One hundred micromolar lidocaine further reduced sodium and improved ATP at 10 min of hypoxia; these actions may also help explain the improved recovery with 100 $\mu$M lidocaine.

Our data indicate that selective blockade of the hypoxic depolarization and the increase in intracellular sodium with a low concentration of lidocaine or with TTX can improve recovery after hypoxia. This blockade improved the resting and action potentials, histologic state, and protein synthesis of CA1 pyramidal neurons in rat hippocampal slices after 10 min of hypoxia. A higher concentration of lidocaine, which also improved ATP, potassium, and calcium concentrations during hypoxia, protected against more prolonged hypoxia. Thus while the depolarization and increased sodium concentrations during hypoxia can alone account for some of the neuronal damage after hypoxia, changes in other components, such as calcium, ATP, and potassium also contribute to hypoxic neuronal damage.

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