Mild Acidosis Delays Hypoxic Spreading Depression and Improves Neuronal Recovery in Hippocampal Slices

Geoffrey C. Tombaugh

Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710

Severe tissue acidosis has been viewed traditionally as a damaging component of cerebral hypoxia. However, a neuroprotective action of low pH during hypoxia has been described in primary neuronal cultures. To identify and characterize this effect in mature brain tissue, adult rat hippocampal slices were made hypoxic after adjusting pH_o with HCl or NaOH. Ion-selective microelectrodes were positioned in CA1 to record evoked field potentials, extracellular DC voltage (V_o), pH_o, and $[Ca^{2+}]_o$. Orthodromic population spike amplitude was used as a measure of slice recovery 2 hr after reoxygenation. All slices became markedly acidotic during hypoxia ($\Delta pH_o \sim 0.4$ pH unit). Following restoration of O₂ and bath pH to 7.4, slice pH_o returned to its pretreatment level regardless of experimental treatment, hypoxic duration, or the degree of electrophysiological recovery. When either the period of hypoxia or the duration of HSD was held constant, acid-treated slices exhibited a significant improvement in recovery. However, in neither paradigm did the recovery of alkaline-treated slices differ from controls. Mild acidosis (bath pH = 6.9–7.3) caused a reversible depression of the orthodromic population spike, an increase in the latency of hypoxic spreading depression–like depolarization (HSD), and a decrease in the magnitude of the associated negative V_o shift. For each of these parameters, mild alkalinity (bath pH = 7.7) had the opposite effect. Acid treatment did not affect the decrease in $[Ca^{2+}]_o$ during HSD but accelerated its recovery after reoxygenation. These results suggest that mild acidosis may limit hypoxic neuronal injury *in vitro* by delaying HSD onset and by additional mechanisms unrelated to the degree of calcium influx during neuronal depolarization.

[Key words: acidosis, hypoxia, anoxic depolarization, hippocampus, calcium, microelectrodes]

A characteristic feature of hypoxic brain tissue is the sudden and gross redistribution of ions across cell membranes. This event has been labeled hypoxic spreading depression–like depolarization (HSD) (Aitken et al., 1991) due to its similarity with the spreading depression of Leao (1944), which can be

evoked in normoxic brain tissue by various stimuli. The term “HSD” is used to emphasize this similarity without necessarily implying that the two are identical. HSD is a well-described pathophysiological state, but its relationship to neuronal injury is unclear. HSD waves (or anoxic depolarizations) are commonly seen during both global and focal ischemia and have been implicated in the neurotoxic process (Hansen and Nedergaard, 1988; Iijima et al., 1992; Chen et al., 1993). During HSD, neurons undergo a large and sustained rise in intracellular calcium (Mitani et al., 1993) and lowering $[Ca^{2+}]_o$ has been shown to reduce hypoxic neuronal injury *in vitro* without preventing HSD (Balestrino and Somjen, 1986; Roberts and Sick, 1988; Amagasa et al., 1990; Young et al., 1992), suggesting that Ca^{2+} entry during HSD is critical. Such studies have depicted calcium as the underlying trigger for ischemic neuropathology, but it is clear that many factors during ischemia govern neuronal fate, not all of which necessarily play a damaging role.

Surprisingly, one important protective event may be a fall in tissue pH. Acidosis is a hallmark of cerebral hypoxia and has received considerable attention since its description nearly 40 years ago (Thorn and Hietmann, 1954; Crowell and Kaufmann, 1961; Ljunggren et al., 1974). Ischemic acidosis has been correlated with the extent of brain injury, and treatments that enhance this acidity often exacerbate tissue damage (Myers, 1979; Siesjö, 1988). Severe acidosis by itself is clearly capable of damaging brain cells: the toxicity of low-pH solutions has been well documented both *in vitro* and *in vivo* (Kraig et al., 1987; Goldman et al., 1989; Giffard et al., 1990a; Tombaugh and Sapolsky, 1990b). A causal link between severe acidosis and pannecrotic brain infarction appears well established (Plum, 1983; Kraig, 1985; Nedergaard, 1987), but such a connection between acidosis and selective neuronal injury is more tenuous (Tombaugh and Sapolsky, 1993). *In vivo*, hyperglycemia exacerbates ischemic acidosis and delays the onset of anoxic depolarization (Hansen, 1978; Ekholm et al., 1993) while it reduces both HSD frequency and neuronal injury in the penumbra of developing infarcts (Nedergaard and Diemer, 1987; Nedergaard, 1988). *In vitro*, extracellular acidity can inhibit NMDA-mediated cell currents, reduce neuronal $[Ca^{2+}]_i$ accumulation, and prevent hypoxic neuronal injury (Schurr et al., 1988; Giffard et al., 1990a; Sher, 1990; Tang et al., 1990; Tombaugh and Sapolsky, 1990a, b; Traynelis and Cull-Candy, 1990; Takadera et al., 1992), all at pH values commonly seen in the ischemic brain (Siemkowicz and Hansen, 1981).

This study examines the relationship between $[H^+]_o$, HSD, and the degree of neuronal injury induced by hypoxia in adult rat hippocampal slices. Tissue slices were exposed to brief periods of hypoxia after adjusting the ambient pH by small steps

Received Aug. 24, 1993; revised Mar. 1, 1994; accepted Mar. 15, 1994.

I am indebted to Dr. George Somjen and Dr. Peter Aitken (Department of Cell Biology, Duke University) for their expert advice and encouragement. I am also grateful to Dr. Mitchell Chesler and Dr. Robert Sapolsky for their critical readings of this manuscript. Support for this work was provided to G.C.T. by an NIH training grant and the National Stroke Association, and to G. G. Somjen by NIH Grants SP01 NS17771 and 5R01 NS18670.

Correspondence should be addressed to Dr. Geoffrey C. Tombaugh, Department of Cell Biology, Box 3709, Duke University Medical Center, Durham, NC 27710. Copyright © 1994 Society for Neuroscience 0270-6474/94/145635-09\$05.00/0

Figure 1. Summary of pH_o changes in slices treated with slightly acidic or alkaline ACSF before and during hypoxia (heavy bar). Because the peak acid shift and its recovery after 8 min of hypoxia did not differ from experiments in which hypoxia was prolonged, data from all experiments were combined ($n \geq 5$ per group). Following hypoxia, normal ACSF (pH 7.4) was restored and pH_o monitored for 2 hr. pH_o was determined at a depth where the evoked orthodromic population spike was maximal and was calculated relative to bath pH prior to any treatment (A), after exposure to experimental ACSF but before hypoxia (B), after hypoxia (C), and following a 2 hr recovery period (D). For comparison, posthypoxic pH_o was also calculated relative to bath pH (E) at the end of each experiment.

with either strong acid or base. Shifts in extracellular pH (pH_o), V_o , and $[Ca^{2+}]_o$ were monitored with ion-selective electrodes to determine whether such changes were associated with changes in neuronal recovery.

Materials and Methods

Hippocampal slice preparation. Transverse slices (400 μ m) were prepared from the middle third of hippocampi removed from male Sprague-Dawley rats (125–150 gm) immediately following decapitation under ether anesthesia (Aitken, 1985). Dissections were performed in chilled (4°C) artificial cerebral spinal fluid (ACSF) containing (in mM) NaCl, 130; KCl, 3.5; $CaCl_2$, 1.2; $MgSO_4$, 1.2; NaH_2PO_4 , 1.25; $NaHCO_3$, 24; and dextrose, 10; aerated with 95% O_2 , 5% CO_2 to maintain a nominal pH of 7.4 (294 ± 1 mOsm). Several slices were transferred immediately to each well of an "Oslo"-type dual-well interface chamber into which flowed control ACSF at ~ 1 ml/min. The same humidified O_2/CO_2 gas mixture used to oxygenate the ACSF flowed through the air space above the slices in each well. Slices were kept at 35°C and allowed to recover for 90 min prior to any experimental treatment. The pH of the experimental ACSF was adjusted by adding several drops of concentrated HCl or NaOH and measured with a Beckmann $\phi 21$ pH meter. ACSF osmolality was measured with a freezing-point depression osmometer. Acid-induced changes in ACSF $[Ca^{2+}]_o$ were monitored in some experiments with calcium microelectrodes. ACSF osmolality was unaffected by HCl addition but was markedly increased after the addition of NaOH. In these "alkaline" experiments, $[NaCl]$ was reduced by 20 mM in order to maintain osmolality at a final pH of 7.7 (298 ± 2 mOsm); control slices were exposed to ACSF in which 20 mM NaCl was replaced with 13.3 mM Na_2SO_4 (288 ± 1 mOsm).

Extracellular recordings. Double-barrel H^+ - and Ca^{2+} -selective microelectrodes (combined tip diameter of 4–7 μ m) were prepared from 1.5 mm capillary glass tubing using a vertical Narishige electrode puller. The tip of the ion-selective barrel was silanized with 5% tri-*N*-methyl-

chlorosilane (Sigma, St. Louis, MO) in xylene and filled with the appropriate ion-selective resin (Fluka, Ronkonkoma, NY) to a column height of 600–900 μ m. Ion-selective barrels were back-filled with either phosphate-buffered saline, pH 7.0 (H^+ electrode) or 1.2 mM $CaCl_2$ /150 mM NaCl (Ca^{2+} electrode). The reference barrel was filled with 150 mM NaCl and used to record both DC (V_o) changes and evoked field potentials. Microelectrodes were connected to a differential amplifier via high-impedance head stages and were calibrated before and immediately after each experiment. H^+ electrodes exhibited responses of 50–59 mV/pH unit while Ca^{2+} electrodes had slopes of 25–28 mV/decade. Electrodes were first positioned in the bath (pH = 7.4; $[Ca^{2+}]_o = 1.2$ mM) to obtain a reference pH or $[Ca^{2+}]_o$, against which subsequent ion measurements were compared after repositioning in CA1 (str. pyramidale). In some experiments $[Ca^{2+}]_o$ recordings were made in str. radiatum of CA1. Electrode depth was adjusted to maximize the amplitude of the orthodromic response evoked by electrical stimulation (10–100 μ A, 0.15 msec) of the Schaffer-collateral-commissural bundle using an insulated monopolar stimulating electrode. Slices displaying either multiple population spikes or single spikes of < 5 mV were excluded. Evoked potentials were monitored on an oscilloscope and digitized for later computer analysis (Aitken, 1985); V_o and ion signals were recorded on a polygraph and on videotape using a VHS recorder.

Experimental treatments. Evoked waveforms were recorded from all slices in both wells of the chamber. One slice in each well was then selected randomly for continuous V_o and ion recording. Control ACSF in one well was then switched to experimental acidic or alkaline ACSF. After slice pH_o had equilibrated with the new pH of the bath, slices in both wells were made hypoxic by replacing the gas phase with 95% N_2 , 5% O_2 . Two experimental paradigms were used. In one, slices were exposed to a constant 8 min period of hypoxia, a duration that had been empirically determined to result in partial recovery of the orthodromic population spike. In the second paradigm, hypoxia was extended for 5 min after the negative V_o shift occurred in order to subject CA1 neurons to a constant period of HSD. Following hypoxia, both oxygen and control ACSF (pH 7.4) were restored. Two hours later, electrophysiological recovery was measured. Evoked potentials in the remain-

ing slices were recorded from the same approximate position and depth determined at the beginning of the experiment.

Data analysis. For each slice, the ratio of the posthypoxic to pretreatment orthodromic population spike was used as a measure of functional recovery. Spike amplitude was measured from the negative peak to the midpoint of a line connecting the two shoulders of the positive wave. HSD latency was calculated from the time nitrogen was introduced to the point at which the negative V_o shift occurred and its duration measured from this negative shift to the point where oxygen was restored. Statistical comparisons were made either by Student's t test or by analysis of variance followed by a Fisher PLSD or Scheffe's F test. All data are presented as mean $\pm$ SEM.

Results

Manipulation and measurement of slice pH_o

Depending on the electrode depth, interstitial pH_o was 0.1–0.25 pH unit more acidic than the surrounding bath. A pH_o gradient existed in the upper layers of the slices, reaching a nadir of 7.15 ± 0.01 ($n = 14$) at a depth of $150 \mu\text{m}$, below which no further decrease in pH_o was seen (not shown). Maximal evoked potentials were typically found at depths of $40\text{--}80 \mu\text{m}$, corresponding to a pH_o of 7.23 ± 0.01 . During exposure to acidic or alkaline ACSF, slices reached a stable pH_o within 35–45 min; changes in pH_o closely paralleled changes in bath pH (Fig. 1). No detectable V_o changes occurred during this period.

Effects of hypoxia on slice pH_o

Regardless of the initial direction of the pH_o shift during experimental ACSF treatment, all slices became markedly more acidotic during hypoxia (Figs. 1, 2). This acidification was typically punctuated by a transient alkalinization coincident with the negative V_o shift (Fig. 2), as described previously (Kraig et al., 1983). However, slices exposed to the most acidic ACSF (pH 6.9) failed to exhibit any sudden V_o change or an abrupt alkaline shift (Fig. 2D). Interstitial pH_o always reached its nadir within 8 min and typically earlier. Following reoxygenation, slice pH_o gradually returned to its pretreatment level regardless of the experimental ACSF pH, the duration of hypoxia, or the degree of acidification during hypoxia (Figs. 1, 2). However, the time needed for pH_o recovery was generally longer in the more acidic slices.

Effects of pH_o on neuronal excitability

Under normoxic conditions, acid exposure caused a reversible dose-dependent depression of the population spike [$-10 \pm 3\%$ ($n = 10$; $\Delta\text{pH} = -0.1$) to $-58 \pm 7\%$ ($n = 12$; $\Delta\text{pH} = -0.5$)] (Fig. 3). In contrast, mild alkalinity increased the size of the population spike [$+37 \pm 13\%$, ($n = 5$; $\Delta\text{pH} = +0.3$)] and occasionally led to the appearance of a smaller secondary spike at higher stimulus intensities. These alkaline-induced changes were not due to the slightly reduced $[\text{Cl}^-]$ as they were not observed when 20 mM NaCl was substituted with 13.3 mM Na_2SO_4 (data not shown).

Effects of pH_o on neuronal recovery

In control slices, functional recovery was discontinuous with respect to the duration of HSD, declining sharply to zero as this interval exceeded 250–300 sec (Fig. 4). Following exposure to a fixed 8 min period of hypoxia (see Materials and Methods), control slices exhibited a mean recovery of $39 \pm 6\%$ while slices made slightly acidic before hypoxia showed nearly complete recovery (Fig. 5A). This protective effect was readily seen after reducing the prehypoxic pH by as little as 0.1 pH unit. In a

Figure 2. Representative pH_o and V_o recordings in slices made hypoxic during exposure to pH 7.7 (A), 7.4 (B), 7.2 (C), or 6.9 (D). Note the increase in HSD latency and the decrease in ΔV_o with declining pH_o , as well as the absence of HSD in the most acidic slice (D).

separate set of experiments, slices were exposed to a constant 5 min period of HSD (see Materials and Methods); acid-treated slices were therefore exposed to a longer total period of hypoxia than control slices, given their greater HSD latencies. These

Figure 3. Mild acidosis reversibly depresses while mild alkalinity reversibly enhances neuronal excitability ($n \geq 5$). Maximal evoked potentials were recorded before, during, and 2 hr after exposure to either pH 6.9 or pH 7.7 ACSF. The asterisks denote stimulation artifacts. The graph summarizes the maximal change in population spike as a function of extracellular pH (pH_o). Errors associated with pH_o measurements were less than 1% and have been omitted for clarity.

acid-treated slices exhibited improved recovery over controls, though this effect did not reach statistical significance until the prehypoxic pH had been reduced by 0.3 pH units (Fig. 5B). Slices treated with pH 6.9 ACSF, which did not show HSD, were exposed to a period of hypoxia nearly twice that of controls (15 min) and yet showed significantly better recovery ($73 \pm 12\%$; $p < 0.01$, $n = 7$). Surprisingly, alkaline-treated slices (ACSF pH = 7.7) and their matching Na_2SO_4 -treated controls (ACSF pH = 7.4) showed similar recovery after 8 min of hypoxia [$83 \pm 9\%$ (pH 7.4) vs $77 \pm 9\%$ (pH 7.7); $n = 12$], recoveries that were also significantly greater ($p < 0.01$) than seen in normal ACSF-treated slices ($39 \pm 6\%$). Alkaline slices also required a

longer period of HSD (> 5 min) to exhibit any measurable injury; however, after HSD duration was extended to 7 min, their recovery did not differ significantly from that of matching Na_2SO_4 -treated controls [50 ± 11 (pH = 7.4) vs 49 ± 14 (pH = 7.7); $n = 3$].

Effects of pH_o on HSD latency and ΔV_o

In control slices, HSD occurred with a latency of 212 ± 8 sec and a V_o shift of -15 ± 1 mV. Mild acidification of slices prior to hypoxia led to a dose-dependent increase in the latency of HSD during hypoxia, as well as a significant blunting of the negative DC shift (Fig. 6). Slices exposed to the most acidic ACSF (pH 6.9) never exhibited HSD even when the duration of hypoxia was extended to 15 min (Fig. 2D). In contrast, pretreatment with alkaline ACSF (pH 7.7) both shortened HSD latency and increased the size of the negative V_o shift (Fig. 6).

Effects of pH_o on $[\text{Ca}^{2+}]_o$ during hypoxia

Acidification of the ACSF to pH 7.1 led to a gradual increase in the $[\text{Ca}^{2+}]_o$ in str. pyramidale from 1.20 ± 0.01 to 1.32 ± 0.01 (Table 1), as measured with Ca^{2+} microelectrodes (Balestrino and Somjen, 1988). An identical prehypoxic rise in bath $[\text{Ca}^{2+}]$ was detected during acid treatment ($n = 12$; $p < 0.01$). During hypoxia, no consistent changes in $[\text{Ca}^{2+}]_o$ occurred in either group prior to HSD. During a fixed 5 min period of HSD, $[\text{Ca}^{2+}]_o$ fell precipitously in both groups and remained below pre-HSD levels for the duration of hypoxic exposure (Fig. 7). Both groups exhibited an identical decrease in $[\text{Ca}^{2+}]_o$ during HSD: 1.05 ± 0.02 (pH 7.4) versus 1.05 ± 0.03 (pH 7.1), $n = 13$ (Table 1). Similar results were obtained in str. radiatum ($n = 6$). Upon reoxygenation, a significant shortening of the $[\text{Ca}^{2+}]_o$ recovery time was observed in str. radiatum in the acid-treated slices, though this effect failed to reach statistical significance in str. pyramidale (Table 1).

Discussion

The cardinal findings of this study are that mild acidosis delays HSD (anoxic depolarization) and improves posthypoxic recovery of synaptic transmission in hippocampal slices. Earlier studies carried out in the presence of elevated lactic acid (Schurr et al., 1988a) revealed a similar paradoxical improvement in hypoxic slice recovery, but HSD latency was not measured. Moreover, the subsequent finding that lactate itself can be utilized as an alternative substrate in energy-deprived tissue (Schurr et al., 1988b) has served to deemphasize low pH as a protective agent. The present study resurrects the putative role of mild acidosis as an endogenous neuroprotectant during hypoxia (Giffard et al., 1990a; Tombaugh and Sapolsky, 1990a,b) and demonstrates that this protection is due in part to the ability of low pH to delay the onset of HSD. This study also strengthens earlier work that first suggested a causal relationship between HSD duration and hypoxic neuronal injury *in vitro* (Balestrino and Somjen, 1986).

In agreement with other reports, resting pH_o in control slices was found to be slightly more acidic than the surrounding bath (Balestrino and Somjen, 1988; Walz, 1989; Chen and Chesler, 1992), and ~ 0.1 pH unit more acidic than interstitial pH values commonly reported *in vivo* (cf. Chesler, 1990). Reducing pH_o further reversibly depressed the evoked population spike, while elevating pH_o had the opposite effect. Similar changes in hippocampal excitability have been seen by others, both *in vitro* and *in vivo* (Balestrino and Somjen, 1988; Walz and Harold,

Figure 4. Hypoxic neuronal injury occurs after cells undergo a critical period of HSD. Data were obtained from control slices (ACSF pH = 7.4) exposed to varying hypoxic periods.

1989; Krnjevic and Walz, 1990). Except for certain cells in the brainstem and spinal cord, acidosis appears to depress the excitability of a majority of central neurons (Meyer et al., 1961; Speckman and Caspers, 1974; Jarolimek et al., 1990).

In acid-treated slices, reduced HSD duration was associated with improved neuronal recovery, supporting a causal link between HSD and hypoxic neuronal injury (Balestrino and Somjen, 1986; Nedergaard and Diemer, 1987; Gill et al., 1992; Iijima et al., 1992; Chen et al., 1993). Moreover, slices subjected to the most severe acidosis failed to undergo HSD and exhibited improved recovery even after the hypoxic period had been nearly doubled. This indicates (1) that the duration of hypoxia in hippocampal slices does not necessarily predict neuronal recovery, and (2) that extracellular acidosis comparable in both duration and degree to that seen during transient ischemia *in vivo* (Siemkiewicz and Hansen, 1981) does not necessarily aggravate hypoxic neuronal injury *in vitro*. In control slices, a discontinuous relationship emerged between HSD duration and recovery, indicating that neuronal injury evolved once a critical period (~250–300 sec) of depolarization had been reached (Fig. 4). By delaying HSD, even very mild acid treatments could reduce HSD duration below this threshold, thereby promoting neuronal recovery.

One possible explanation for the pH effect on HSD latency may be related to changes in cell excitability. During hypoxia, hippocampal neurons undergo a transient period of spontaneous hyperexcitability prior to HSD (Sick et al., 1987; Hershkowitz et al., 1993), and a variety of agents capable of blocking synaptic transmission or cell excitability have been shown to delay HSD (Aitken et al., 1991; Jing et al., 1993). Because ATP consumption rises with increased neuronal activity (cf. Erecinska and Silver, 1989), increased miniature EPSC frequency might enhance energy demand at a time when ATP supply is severely limited, somehow accelerating the opening of membrane channels that underlie HSD. By depressing cell excitability, low pH_o may have helped maintain ATP levels and transmembrane ion gradients, thereby delaying HSD. In support of this, hypoxic CA1 neurons experience more rapid energy failure and undergo HSD sooner than the less excitable dentate granule cells (Jefferys and Haas, 1982; Balestrino et al., 1989; Kass and Lipton, 1989).

A: Fixed Duration of Hypoxia

B: Fixed Duration of HSD

Figure 5. Mild acidosis improves neuronal recovery following a fixed (8 min) period of hypoxia (A) and after a fixed (5 min) period of HSD (B). The recovery of selected slices in which V_o was monitored ($n \geq 6$; $\circ$) and the pooled recoveries of all slices ($n \geq 15$; $\bullet$) are plotted separately. The errors associated with the prehypoxic pH_o measurements were less than 1% and have been omitted for clarity. *, significantly different from control (ACSF pH = 7.4), $p < 0.05$; **, significantly different from control (ACSF pH = 7.4), $p < 0.01$; ***, significantly different from control (ACSF pH = 7.4), $p < 0.001$.

Also, glucose supplementation delays HSD (Hansen, 1978), while hypothermia, which is capable of slowing hypoxic ATP depletion (Welsh et al., 1990; Katsura et al., 1992), both delays HSD and reduces its frequency during ischemia (Nedergaard and Diemer, 1987; Katsura et al., 1992; Chen et al., 1993; Taylor and Weber, 1993). Indeed, HSD has been associated with a threshold of energy failure (Ekholm et al., 1993).

The proximal cause of HSD is not known. Several studies have implicated the excitatory amino acid glutamate (Van Har-

Figure 6. Mild acidosis increases HSD latency and reduces the size of the negative V_o shift. In both cases, mild alkalosis has the opposite effect ($n \leq 5$). *, significantly different from control (ACSF pH = 7.4), $p < 0.05$; **, significantly different from control (ACSF pH = 7.4), $p < 0.01$; ***, significantly different from control (ACSF pH = 7.4), $p < 0.001$.

reveld, 1959; Bures et al., 1974), but its role in HSD remains controversial (Hernandez-Caceres et al., 1987; Aitken et al., 1988; Silver and Erecinska, 1990; Lauritzen and Hansen, 1992). Reduced pH_o may have delayed HSD by blunting NMDA receptor activity (Tang et al., 1990; Traynelis and Cull-Candy, 1990; Jing et al., 1993); however, HSD was significantly delayed when the ACSF pH was reduced by as little as 0.1 pH unit, resulting in a pH_o during HSD (6.82 ± 0.02) capable of causing only partial NMDA receptor blockade (Tang et al., 1990). Thus, either HSD onset in acid-treated slices was very sensitive to small reductions in NMDA receptor activity, or it was delayed by additional or alternative factors. In either case, H^+ -mediated suppression of NMDA receptor-mediated Ca^{2+} influx was probably not involved, as lowering $[Ca^{2+}]_o$ actually accelerates HSD onset *in vitro* (Young et al., 1991; Mitani et al., 1993).

During HSD, neurons undergo a dramatic rise in intracellular Ca^{2+} (Silver and Erecinska, 1990; Mitani et al., 1993), and reducing extracellular Ca^{2+} enhances posthypoxic neuronal recovery in hippocampal slices (Balestrino and Somjen, 1986; Amagasa et al., 1990; Young et al., 1991). Agents that delay HSD (and thus shorten its duration) are thought to protect neurons both *in vitro* and *in vivo* by limiting the period during which intracellular calcium remains elevated (Balestrino and Somjen, 1986; Young et al., 1991; Gill et al., 1992; Iijima et al., 1992; Gido et al., 1993). Results from the present study imply that $[Ca^{2+}]_i$ must remain elevated for a certain period, given that neuronal recovery fell sharply after a critical duration of HSD had been reached. It seems unlikely that acid exposure improved recovery by curtailing Ca^{2+} influx during HSD, as reduced pH_o failed to blunt the dramatic fall in $[Ca^{2+}]_o$ during HSD. Thus,

Table 1. Extracellular calcium concentrations in control and acid-treated slices before, during, and after 5 min of HSD

ACSF pH	pH_o (HSD)	Prehypox.	$[Ca^{2+}]_o$ (mM) HSD	Posthypox.	$\Delta[Ca^{2+}]_o$	$[Ca^{2+}]_o$ recovery time (min)
Str. pyramidale						
7.4	7.08 ± 0.01	1.20 ± 0.01	0.15 ± 0.02	1.19 ± 0.01	1.05 ± 0.02	28 ± 3
7.1	$6.53 \pm 0.13^{***}$	$1.32 \pm 0.01^{**}$	$0.26 \pm 0.03^{**}$	1.21 ± 0.02	1.05 ± 0.03	20 ± 4
Str. radiatum						
7.4	—	1.24 ± 0.01	0.15 ± 0.05	1.24 ± 0.02	1.09 ± 0.05	24 ± 3
7.1	—	$1.49 \pm 0.02^{**}$	$0.34 \pm 0.12^{**}$	1.25 ± 0.02	1.16 ± 0.10	$11 \pm 3^*$

Electrodes were positioned in either str. pyramidale ($n = 13$) or str. radiatum ($n = 6$). The pH_o values were obtained from identically treated slices ($n = 10$) immediately prior to HSD onset in separate experiments.

* Significantly different from 7.4 control, $p < 0.05$.

** Significantly different from 7.4 control, $p < 0.01$.

*** Significantly different from 7.4 control, $p < 0.001$.

Str. pyramidale

Str. radiatum

Figure 7. Mild acidosis does not affect the decrease in extracellular calcium during HSD but accelerates its posthypoxic recovery: representative $[Ca^{2+}]_o$ recordings from str. pyramidale or str. radiatum (CA1) in separate pairs of slices subjected to 5 min of HSD during exposure to either pH 7.4 or 7.1. Acid-treated slices were returned to normal ACSF (pH = 7.4) immediately upon reoxygenation (asterisks). The onset of hypoxia coincides with the beginning of each recording, though a longer hypoxic exposure was used in the acid-treated slices to elicit an identical period of HSD. Note the elevated prehypoxic $[Ca^{2+}]_o$, its delayed decline, and its accelerated recovery (str. radiatum) in the acid-treated slice.

in slices exposed to a fixed period of hypoxia, mild acidity probably improved neuronal recovery by shortening the duration of Ca^{2+} overload rather than by reducing the net Ca^{2+} influx during HSD.

In slices exposed to a fixed period of HSD, the mechanism of protection afforded by low pH is less obvious but may also be related to a reduced duration of Ca^{2+} overload, as suggested by the accelerated posthypoxic recovery of $[Ca^{2+}]_o$ (see Table 1). These findings are consistent with reports that the neuroprotective action of low pH, *in vitro* is not limited to NMDA-receptor blockade (Tombaugh and Sapolsky, 1990a, 1993; Kaku et al., 1993) and clearly demonstrate that the beneficial action of low pH extends beyond its ability to delay HSD.

It should be emphasized that although a rise in $[Ca^{2+}]_i$ occurs simultaneously with a fall in $[Ca^{2+}]_o$ during HSD (Silver and Erecinska, 1990), Ca^{2+} influx sufficient to raise cytosolic calcium

significantly prior to HSD could have escaped detection, especially in the distal dendrites. Also, a rise in $[Ca^{2+}]_i$ due to its release from intracellular pools (Mitani et al., 1993) would have been undetectable. If acid treatment enhanced intracellular acidosis during hypoxia, this potentially could have increased $[Ca^{2+}]_i$, either by enhancing release from intracellular stores via H^+/Ca^{2+} exchange or by reducing intracellular Ca^{2+} buffering (Moody, 1984). Such mechanisms may underlie the recent finding that reduced pH_o enhanced $[Ca^{2+}]_i$ during hypoxia in cortical slices (O'Donnell and Bickler, 1994), further supporting the view that low pH improved neuronal recovery in ways other than simply blunting the size of the calcium signal.

The neuroprotective effects of pH_o reported here do not exclude the possibility that they resulted from actions mediated "downstream" of hypoxic calcium accumulation. As an example, the affinity of calmodulin for Ca^{2+} falls dramatically as

pH drops, potentially affecting critical second-messenger systems that mediate neuronal injury (Tkachuk and Men'shikov, 1981; Busa and Nuccitelli, 1984; Chetkovitch et al., 1991). Also, certain Ca^{2+} -sensitive enzymes may themselves be pH sensitive. In addition, ischemia-induced depression of protein synthesis has been linked to Ca^{2+} influx (Widmann et al., 1991), and such depression can be attenuated by mild acidosis *in vitro* (K. Raley-Susman, personal communication). However, whether pH_i manipulation in the present study influenced HSD latency, Ca^{2+} homeostasis, or neuronal recovery via changes in neuronal pH_i remains speculative. Several groups have reported a fall in pH_i in response to low pH_o (Whittingham et al., 1989; Ou-yang et al., 1993; O'Donnell and Bickler, 1994), but the influence of extracellular acidity on neuronal pH_i in adult hypoxic brain tissue has not been characterized.

Because acidosis clearly protected against hypoxic injury, mild alkalosis was expected to worsen cell recovery (Giffard et al., 1992). Alkaline treatment did accelerate HSD onset but failed to exacerbate injury relative to Na_2SO_4 -treated controls. Because the difference in HSD duration between these two groups became smaller as the total hypoxic duration was increased, this may have obscured any difference in neuronal recovery. In addition, damaging mechanisms triggered by hypoxia may have been maximally activated at pH 7.4, preventing a further increase at pH 7.7.

The consequence of pH shifts during hypoxic insults *in vivo* is controversial, and there are obvious limitations about extending *in vitro* findings to the intact animal. For example, certain pathological events potentially aggravated by acidosis *in situ*, such as edema and vascular damage (Paljarvi et al., 1983; Smith and Siesjo, 1986), are absent in tissue slices. Glucose, lactate, and CO_2 concentrations change dramatically *in situ* but were not adjusted in this study. Moreover, rapid functional failure in hypoxic slices is not necessarily equivalent to ischemic neuronal necrosis *in vivo*. Nevertheless, several implications for ischemic injury do arise from this study. The degree of acidification during transient global ischemia may dictate the latency of HSD, while the protective effects of lingering acidosis during reperfusion may offset some of the damaging biochemical cascades that trigger delayed neuronal necrosis. In addition, moderate but persistent acidosis in the peri-infarct zone may reduce the frequency of potentially damaging SDs. In support of this, Simon et al. (1993) have reported that hypercarbic ventilation can reduce infarction volume in focally ischemic rats.

In sum, this study characterizes a neuroprotective effect of mild acidosis in hypoxic hippocampal slices. This effect was associated with a shortening of HSD duration and involved at least one additional neuroprotective mechanism apparently unrelated to the increase in intracellular calcium during HSD. The exact nature of neuronal injury in the ischemic brain undoubtedly differs from that in tissue slices, but some overlap of the damaging cascades presumably exists, suggesting that the beneficial effects of acidosis described in this study may occur during hypoxic insults *in situ*.

References

- Aitken PG, Balestrino M, Somjen GG (1988) NMDA antagonists: lack of protective effect against hypoxic damage in CA1 region of hippocampal slices. *Neurosci Lett* 89:187–192.
- Aitken PG, Jing J, Young JN, Somjen GG (1991) Ion channel involvement in hypoxia-induced spreading depression in hippocampal slices. *Brain Res* 541:7–11.
- Amagasa M, Ogawa A, Yoshimoto T (1990) Effects of calcium and calcium antagonists against deprivation of glucose and oxygen in guinea pig hippocampal slices. *Brain Res* 526:1–7.
- Balestrino M, Somjen GG (1986) Chlorpromazine protects brain tissue in hypoxia by delaying spreading depression-mediated calcium influx. *Brain Res* 385:219–226.
- Balestrino M, Somjen GG (1988) Concentration of carbon dioxide, interstitial pH and synaptic transmission in hippocampal formation of the rat. *J Physiol (Lond)* 396:247–266.
- Balestrino M, Aitken PG, Somjen GG (1989) Spreading depression-like depolarization in CA1 and fascia dentata: relationship to selective vulnerability. *Brain Res* 497:102–107.
- Bures J, Buresova O, Krivanek J (1974) The mechanism and applications of Leao's spreading depression of electroencephalographic activity. New York: Academic.
- Busa WB, Nuccitelli R (1984) Metabolic regulation via intracellular pH. *Am J Physiol* 246:R406–R438.
- Chen JCT, Chesler M (1992) Modulation of extracellular pH by glutamate and GABA in rat hippocampal slices. *J Neurophysiol* 67:29–36.
- Chen Q, Chopp M, Bodzin G, Chen H (1993) Temperature modulation of cerebral depolarization during focal cerebral ischemia in rats: correlation to ischemic injury. *J Cereb Blood Flow Metab* 13:389–394.
- Chesler M (1990) The regulation and modulation of pH in the central nervous system. *Prog Neurobiol* 34:401–427.
- Chetkovitch DM, Gray R, Johnston D, Sweatt JD (1991) *N*-methyl-D-aspartate receptor activation increases cAMP levels and voltage-gated Ca^{2+} channel activity in area CA1 hippocampus. *Proc Natl Acad Sci USA* 88:6467–6471.
- Crowell JW, Kaufmann BN (1961) Changes in tissue pH after cardiac arrest. *Am J Physiol* 200:743–745.
- Eckholm A, Katsura K, Siesjo BK (1993) Coupling of energy failure and dissipative K^+ flux during ischemia: role of pre-ischemic plasma glucose concentration. *J Cereb Blood Flow Metab* 13:193–200.
- Gido G, Katsura K, Kristian T, Siesjo BK (1993) Influence of plasma glucose concentration on rat brain extracellular calcium transients during spreading depression. *J Cereb Blood Flow Metab* 13:179–182.
- Giffard RG, Monyer H, Christine CW, Choi DW (1990a) Acidosis reduces NMDA receptor activation, glutamate neurotoxicity, and oxygen-glucose deprivation neuronal injury in cortical cultures. *Brain Res* 506:339–342.
- Giffard RG, Monyer H, Choi DW (1990b) Selective vulnerability of cultured cortical glia to injury by extracellular acidosis. *Brain Res* 530:138–141.
- Giffard RG, Weiss JH, Choi DW (1992) Extracellular alkalinity exacerbates injury of cultured cortical neurons. *Stroke* 23:1817–1821.
- Gill R, Andiné P, Hillered L, Persson L, Hagberg H (1992) The effect of MK-801 on cortical spreading depression in the penumbral zone following focal ischaemia in the rat. *J Cereb Blood Flow Metab* 12:371–379.
- Goldman SA, Pulsinelli WA, Clarke WY, Kraig RP, Plum F (1989) The effects of extracellular acidosis on neurons and glia *in vitro*. *J Cereb Blood Flow Metab* 9:471–477.
- Hansen AJ (1978) The extracellular potassium concentration in brain cortex following ischemia in hypo- and hyperglycemic rats. *Acta Physiol Scand* 102:324–329.
- Hansen AJ, Nedergaard M (1988) Brain ion homeostasis in cerebral ischemia. *Neurochem Pathol* 9:195–209.
- Hernandez-Caceres J, Macius-Gonzalez R, Brozek G, Bures J (1987) Systemic ketamine blocks cortical spreading depression but does not delay the onset of terminal anoxic depolarization. *Brain Res* 437:360–364.
- Hershkowitz N, Katchman AN, Verege S (1993) Site of synaptic depression during hypoxia: a patch clamp analysis. *J Neurophysiol* 69:432–441.
- Iijima T, Mies G, Hossmann K-A (1992) Repeated negative DC deflections in rat cortex following middle cerebral artery occlusion are abolished by MK-801: effect on volume of ischemic injury. *J Cereb Blood Flow Metab* 12:727–733.
- Jarolimek W, Misgeld U, Lux HD (1990) Neurons sensitive to pH in slices of the rat ventral medulla oblongata. *Pflugers Arch Eur J Physiol* 416:247–253.
- Jefferys JGR, Haas HL (1982) Synchronized bursting of CA1 hippocampal pyramidal cells in the absence of synaptic transmission. *Nature* 300:448–450.

- Jing J, Aitken PG, Somjen GG (1993) Role of calcium channels in spreading depression in rat hippocampal slices. *Brain Res* 604:251–259.
- Kaku DA, Giffard RG, Choi DW (1993) Neuroprotective effects of glutamate antagonists and extracellular acidity. *Science* 260:1516–1518.
- Kass IS, Lipton P (1989) Protection of hippocampal slices from young rats against anoxic transmission damage is due to better maintenance of ATP. *J Physiol (Lond)* 413:1–11.
- Katsura A, Minamisawa H, Ekholm A, Folbergrova J, Siesjö BK (1992) Changes of labile metabolites during anoxia in moderately hypo- and hyperthermic rats: correlation to membrane fluxes of K⁺. *Brain Res* 590:6–12.
- Kraig RP, Ferreira-Filho CR, Nicholson C (1983) Alkaline and acid transients in cerebellar microenvironment. *J Neurophysiol* 49:831–850.
- Kraig RP, Petito C, Plum F, Pulsinelli WA (1987) Hydrogen ions kill brain at concentrations reached in ischemia. *J Cereb Blood Flow Metab* 7:379–386.
- Krnjević K, Walz WW (1990) Acidosis and blockade of orthodromic responses caused by anoxia in rat hippocampal slices at different temperatures. *J Physiol (Lond)* 422:127–144.
- Lao AAP (1944) Spreading depression of activity in cerebral cortex. *J Neurophysiol* 7:359–390.
- Lauritzen M, Hansen AJ (1992) The effect of glutamate receptor blockade on anoxic depolarization and cortical spreading depression. *J Cereb Blood Flow Metab* 12:223–229.
- Ljunggren B, Norberg K, Siesjö BK (1974) Influence of tissue acidosis upon restitution of brain energy metabolism following total ischemia. *Brain Res* 77:173–183.
- Meyer JS, Gotoh F, Tazaki Y (1961) Inhibitory action of carbon dioxide and acetazolamide on seizure activity. *Electroencephalogr Clin Neurophysiol* 13:762–775.
- Mitani A, Yanase H, Kimiko S, Yousuke W, Kataoka K (1993) Origin of intracellular calcium elevation induced by *in vitro* ischemic-like condition in hippocampal slices. *Brain Res* 601:103–110.
- Moody WJ (1984) Effects of intracellular H⁺ on the electrical properties of excitable cells. *Annu Rev Neurosci* 7:257–278.
- Myers RE (1979) A unitary theory of causation of anoxic and hypoxic brain pathology. *Adv Neurol* 26:195–213.
- Nedergaard M (1987) Transient focal ischemia in hyperglycemic rats is associated with increased cerebral infarction. *Brain Res* 408:97–85.
- Nedergaard M (1988) Mechanisms of brain damage in focal cerebral ischemia. *Acta Neurol Scand* 77:81–101.
- Nedergaard M, Diemer NH (1987) Focal ischemia of the rat brain, with special reference to the plasma glucose concentration. *Acta Neuropathol (Berl)* 73:131–137.
- O'Donnell BR, Bickler PE (1994) Influence of pH on calcium influx during hypoxia in rat cortical slices. *Stroke* 25:171–177.
- Ou-yang Y, Møllergaard P, Siesjö BK (1993) Regulation of intracellular pH in single rat cortical neurons *in vitro*: a microspectrofluorometric study. *J Cereb Blood Flow Metab* 13:827–840.
- Paljarvi L, Rhencrona S, Soderfeldt B, Olsson Y, Kalimo H (1983) Brain lactic acidosis and ischemic cell damage: quantitative ultrastructural changes in capillaries of rat cerebral cortex. *Acta Neuropathol (Berl)* 60:232–240.
- Plum F (1983) What causes infarction in ischemic brain?: the Robert Wartenberg lecture. *Neurology* 33:222–233.
- Roberts EL Jr, Sick TJ (1988) Calcium-sensitive recovery of extracellular potassium and synaptic transmission in rat hippocampal slices exposed to anoxia. *Brain Res* 456:113–119.
- Roberts EL Jr, Sick TJ (1992) Glucose enhances recovery of potassium ion homeostasis and synaptic excitability after anoxia in hippocampal slices. *Brain Res* 570:225–230.
- Schurr A, Dong W-Q, Reid KH, West CA, Rigo BM (1988a) Lactic acidosis and recovery of function following cerebral hypoxia *in vitro*. *Brain Res* 438:311–314.
- Schurr A, West CA, Rigo BM (1988b) Lactate supported synaptic function in the rat hippocampal slice preparation. *Science* 240:1326–1328.
- Sher PK (1990) The effects of acidosis on chronically hypoxic neurons in culture. *Exp Neurol* 107:256–262.
- Sick TJ, Solow EL, Roberts EL Jr (1987) Extracellular potassium ion activity and electrophysiology in the hippocampal slice: paradoxical recovery of synaptic transmission during anoxia. *Brain Res* 418:227–234.
- Siemkiewicz E, Hansen AJ (1981) Brain extracellular ion composition and EEG activity following 10 minutes ischemia in normo- and hyperglycemic rats. *Stroke* 12:236–240.
- Siesjö BK (1988) Acidosis and ischemic brain damage. *Neurochem Pathol* 9:31–88.
- Silver IA, Erecinska M (1990) Intracellular and extracellular changes of [Ca²⁺] in hypoxia and ischemia in rat brain *in vivo*. *J Gen Physiol* 95:837–866.
- Simon RP, Niño M, Gwinn R (1993) Brain acidosis induced by hypercarbic ventilation attenuates focal ischemic injury. *J Pharmacol Exp Ther* 267:1428–1431.
- Smith M-L, Siesjö BK (1988) Acidosis-related brain damage: immediate and delayed events. In: *Mechanisms of cerebral hypoxia and stroke* (Somjen GG, ed), pp 57–71. New York: Plenum.
- Somjen GG, Aitken PG, Czeh GL, Herreras O, Jing J, Young JN (1992) Mechanism of spreading depression: a review of recent findings and a hypothesis. *Can J Physiol Pharmacol* 70:S248–S254.
- Speckman E-J, Caspers H (1974) The effect of O₂- and CO₂-tensions in the nervous tissue on neuronal activity and DC potentials. In: *Handbook of electroencephalography and clinical neurophysiology*, Vol 2, Pt C (Remond A, ed), pp 71–89. New York: Elsevier.
- Takadera T, Shimada Y, Mohri T (1992) Extracellular pH modulates N-methyl-D-aspartate mediated neurotoxicity and calcium accumulation in rat cortical cultures. *Brain Res* 572:126–131.
- Tang C, Dichter M, Morad M (1990) Modulation of the NMDA channel by extracellular H⁺. *Proc Natl Acad Sci USA* 87:6445–6450.
- Taylor CP, Weber ML (1993) Effect of temperature on synaptic function after reduced oxygen and glucose in hippocampal slices. *Neuroscience* 52:555–562.
- Thorn WG, Heitmann R (1954) pH der Gehirnrinde vom Kaninchen *in situ* während perakuter, totaler Ischämie, reiner anoxie und in der Erholung. *Pflügers Arch Ges Physiol* 261:334–360.
- Tkachuk VA, Men'shikov MY (1981) Effect of pH on calcium binding properties of calmodulin and its interaction with the Ca-dependent form of cyclic nucleotide phosphodiesterase. *Biokhimiya* 46:779–788.
- Tombaugh GC, Sapolsky RM (1990a) Mechanistic distinctions between excitotoxic and acidotic hippocampal damage in an *in vitro* model of ischemia. *J Cereb Blood Flow Metab* 10:527–535.
- Tombaugh GC, Sapolsky RM (1990b) Mild acidosis protects hippocampal neurons from injury induced by oxygen and glucose deprivation. *Brain Res* 506:343–345.
- Tombaugh GC, Sapolsky RM (1993) Evolving concepts about the role of acidosis in ischemic neuropathology. *J Neurochem* 61:793–803.
- Traynelis S, Cull-Candy S (1990) Proton inhibition of NMDA receptors in cerebellar neurons. *Nature* 356:347–349.
- Van Harrevald A (1959) Compounds in brain extracts causing spreading depression of cerebral cortical activity and contraction of crustacean muscle. *J Neurochem* 3:300–315.
- Walz WW (1989) pH shifts evoked by neuronal stimulation in slices of rat hippocampus. *Can J Physiol Pharmacol* 67:577–581.
- Walz WW, Harold DE (1989) Brain lactic acidosis and synaptic function. *Can J Physiol Pharmacol* 68:164–169.
- Welsh FA, Sims RE, Harris VA (1990) Mild hypothermia prevents ischemic injury in gerbil hippocampus. *J Cereb Blood Flow Metab* 10:557–563.
- Whittingham TS, Warman E, Assaf H, Sick TJ, LaManna JC (1989) Manipulating the intracellular environment of hippocampal slices: pH and high-energy phosphates. *J Neurosci Methods* 28:83–91.
- Widmann R, Kuroiwa P, Bonnekoh P, Hossmann K-A (1991) [¹⁴C] leucine incorporation into brain proteins in gerbils after transient ischemia: relationship to selective vulnerability of hippocampus. *J Neurochem* 56:789–796.
- Young JN, Aitken PG, Somjen GG (1991) Calcium, magnesium, and long-term recovery from hypoxia in hippocampal tissue slices. *Brain Res* 548:343–345.