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Major Differences in Ca_i^{2+} Response to Anoxia between Neonatal and Adult Rat CA1 Neurons: Role of Ca_o^{2+} and Na_o^+

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Although we and others have previously shown that newborn central mammalian neurons are more tolerant to anoxia than their adult counterparts, we do not know whether neonatal nerve cells accumulate free cytosolic calcium (Ca_i^{2+}) less than adults in response to O_2 deprivation. In order to determine whether anoxia increases Ca_i^{2+} in adult and neonatal neurons, we monitored calcium in CA1 hippocampal neurons using the calcium-sensitive probe fluo-3 and confocal microscopy. These neurons were studied in the dissociated state in order to study their inherent response to anoxia without the influence of modulatory factors such as synaptic input and neurotransmitters.

Severe anoxia caused a rapid increase in Ca_i^{2+} in adult CA1 hippocampal neurons, followed by swelling and bleb formation. In neonatal neurons, the latency of this calcium rise was about five times longer than in the adult. Removal of extracellular calcium and addition of calcium channel blockers (Co^{2+}) greatly attenuated the increase in Ca_i^{2+} in response to anoxia but did not prevent cell swelling and injury. The addition of glutamate antagonists MK-801 and 6-cyano-7-nitroquinoxaline-2,3-dione did not affect the increase in Ca_i^{2+} induced by anoxia. Replacing extracellular sodium with impermeant cations (*N*-methyl-D-glucamine) prevented anoxia-induced nerve injury. In addition, Ca_i^{2+} levels dropped, rather than increased, during the anoxic period in the absence of sodium; Ca_i^{2+} returned toward baseline levels upon reoxygenation.

Glutamate was also studied, in the presence of O_2, to compare with the effects of anoxia. Adult neurons responded with similar latencies to both anoxia and glutamate (10–500 μM). Neonatal neurons, however, exhibited a more rapid increase in Ca_i^{2+} in response to glutamate than they did when exposed to anoxia. We conclude (1) that neonatal neurons increase Ca_i^{2+} more slowly than adults in response to anoxia and that this response is due to an influx of Ca_o^{2+} into the cytosol, (2) that an increase in Ca_i^{2+} is not necessary to induce nerve injury when nerve cells are deprived of O_2, (3) that the removal of Na_o^+ prevents cell swelling and blebbing and is associated with a decrease in Ca_i^{2+} during anoxia, and finally (4) that glutamate exposure may not be a completely valid model for the direct action of anoxia on neurons.

[Key words: hippocampus, ischemia, neurotoxicity, calcium, sodium, glutamate]

It has been known for a long time that oxygen deprivation, even for relatively short periods of time, can cause irreversible damage to the mammalian brain. In the past decade, a wealth of information has been gathered on the response of neurons to O_2 deficiency (cf. *Stroke*, Vol. 21, Suppl. III, 1990; Siesjö, 1981; Siesjö and Bengtsson, 1989; Silver and Ereciñska, 1990; Stys et al., 1990). What has not yet been determined, however, is the exact sequence of events that lead to anoxia-induced nerve injury. The leading hypothesis in neurons is the so-called Ca^{2+} hypothesis, which involves an increase in intracellular calcium (Ca_i^{2+}), an observation made in both *in vivo* and cultured cells during anoxia (Siesjö and Bengtsson, 1989; Silver and Ereciñska, 1990). The importance of this hypothesis stems from the fact that calcium is known to modulate the activity of a number of enzymes including phosphatases and kinases, and to play a primary role in influencing the state and integrity of the cytoskeleton (Orrenius et al., 1989; Harris and Morrow, 1990).

Newborn mammals are known to be more resistant to anoxia than adults (Duffy et al., 1975; Ferriero et al., 1988; Cherubini et al., 1989; Haddad and Donnelly, 1990). We have recently shown that neonatal neurons depolarize much less with hypoxia or anoxia, increase their intracellular chloride after a longer latency, and decrease much less their intracellular potassium than do adult neurons (Haddad and Donnelly, 1990; Jiang et al., 1992a,b). Whether this apparent tolerance to the effects of anoxia is associated with or due to a lack of rise in Ca_i^{2+} is not known.

We decided to investigate the direct effect of anoxia on CA1 hippocampal neurons since these cells are among the most sensitive neurons to anoxia in the mammalian CNS (Kawasaki et al., 1990; Silver and Ereciñska, 1990; Imon et al., 1991). Since neonatal CA1 neurons are more resistant to anoxia than adult ones (Ferriero et al., 1988; Cherubini et al., 1989; Choi and Rothman, 1990; Haddad and Donnelly, 1990), our goal was to determine whether the increase in Ca_i^{2+} is smaller or follows a different pattern in the neonate than in the adult. We simultaneously monitored Ca_i^{2+} and morphology of neurons before, during, and after an anoxic period. We chose to use freshly dissociated cells in order to examine the intrinsic neuronal responses to anoxia without the potentially confounding effect of synaptic connections, nerve–glia interactions, or release of endogenous neurotransmitters, including glutamate.

Since anoxia is generally difficult to induce *in vitro* in culture, or in the absence of tissue that consumes enough O_2, and since glutamate has been shown to be released during anoxia (Ben-

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veniste et al., 1984; Siesjö and Bengtsson, 1989), anoxic stress has frequently been modeled by exposing slices or cells in primary cultures to glutamate (Choi and Rothman, 1990; Michaels and Rothman, 1990). Therefore, we have also endeavored to determine whether glutamate mimics the effect of anoxia in newborn and adult neurons with respect to changes in intracellular calcium.

Materials and Methods

Preparation of slices for dissociation. Dissociated neurons were prepared according to a modified procedure of Kay and Wong (1986; Cummins et al., 1991). In brief, Sprague–Dawley rats of the desired age were anesthetized by inhalation of methoxyflurane (Pitmann-Moore) and decapitated with a guillotine, and the brain was rapidly removed and placed into ice-cold oxygenated dissociation buffer [DB; 120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM glucose, and 20 mM HEPES (Sigma), pH 7.4). The hippocampi were removed and cut into 400–500 μm slices. The slices were placed into a dissociation chamber containing 10 ml of DB plus 5–15 mg trypsin (Sigma) for 40 min followed by the addition of 2.5–5 mg pronase (Sigma) for another 20–40 min. The buffer was replaced with fresh DB, without enzymes, for another 20 min. Slices could be maintained in oxygenated DB for up to 6 hr. Based on our electrophysiological data (Haddad and Donnelly, 1990; Cummins et al., 1991), we defined neonates as being 1–8 d old, and adults as greater than 21 d. We did not dissociate slices from animals over 40 d of age, as the cell yield becomes poor after that age: Cell yield, after loading the cells with fluo-3 and washing, was 10–20 apparently healthy neurons per coverslip for neonates, half that for adults aged about 22 d, and 2–3 neurons for rats approximately 30 d old.

Dissociation of CA1 neurons and loading with fluo-3. The CA1 layer was cut out from several (two or three) hippocampal slices using a dissecting microscope, and these were dissociated by delicate trituration with fire-polished Pasteur pipettes in DB plus 3 μM fluo-3 acetoxymethyl ester (fluo-3/AM; Molecular Probes) plus 3 μl/ml pluronic F-127 (25%; Molecular Probes) (Kao et al., 1989). The dissociated cells were pipetted onto ethanol-cleaned coverslips, coated with 2 μl of Cell-Tak (Collaborative Research Inc.), and allowed to settle and load the dye at room temperature for 30–40 min. The cells were then washed with DB and allowed to equilibrate for a further 20 min.

Recording of intracellular calcium. A coverslip containing the fluo-3–loaded, freshly dissociated neurons was placed on a heated (35 ± 2°C) coverslip holder on a Zeiss Axiovert microscope attached to a Bio-Rad MRC 500 Laser Confocal Scanning unit. The unit's argon laser emits at 488 nm, the excitation wavelength of fluo-3. Emission was monitored using a 515 nm barrier filter. The exciting beam was attenuated to 0.1 mW transmission using the built-in filters. Transmitted (nonconfocal differential interference contrast) and reflected (fluorescence) images were acquired simultaneously every 30 sec (Figs. 1, 2). We estimate the optical sections to be 1–2 μm thick, focusing on the central, nucleus-containing region of the cell. The images were recorded using a Panasonic high-resolution monochrome TQ-2028F optical memory disk recorder. The sample was perfused at a rate of 2–3 ml/min with oxygenated O_2 (95%), CO_2 (5%) Ringer's buffer [RB; 125 mM NaCl, 26 mM $NaHCO_3$, 3 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose, pH 7.4]. To induce anoxia, the perfusing buffer was changed, using a solenoid switch (General Valve), to NRB [RB equilibrated with N_2 (95%), CO_2 (5%) to which 2 mM $Na_2S_2O_4$, an oxygen scavenger, was added]. O_2 concentration (PO_2) in the buffer was measured by the use of an oxygen microelectrode (built by Dr. S. Agulian, Department of Physiology, Yale University). PO_2 dropped rapidly in the bath within 30 sec and reached zero torr within approximately 45 sec. In order to ascertain that the effect obtained by $Na_2S_2O_4$ is only related to O_2 deprivation, we have also used argon (Ar_2), which is heavier than air, to saturate the RB as well as superfuse the flow chamber containing the neurons (Aw et al., 1987).

In addition, we verified that our observations were not an artifact of the addition of $Na_2S_2O_4$. A calcium clamp was applied across a neuron by incubating it for 5 min in RB containing only 100 nM Ca^{2+} (using a Ca^{2+}-EGTA buffer) plus 10 μM 4-bromo-A23187. Subsequent perfusion for 10 min with N_2-saturated low-calcium RB plus 10 μM 4-bromo-A23187 and 2 mM $Na_2S_2O_4$ yielded no change in fluorescence.

Na^+-free media. NaCl was replaced with *N*-methyl-D-glucamine (NMDG)-HEPES titrated with 124 mM HCl in most studies (80%) or with choline chloride (choline-HEPES buffer). A control Na-HEPES buffer was also used. The buffers were comprised of (in mM) NaCl, choline Cl, or NMDG (titrated with HCl), 135; K_2CO_3, 1; $CaCl_2$, 2.4; KH_2PO_4, 1; $MgSO_4$, 1.3; glucose, 10; and HEPES, 25; pH 7.4.

Cell viability. The ability of a neuron to cleave the fluo-3/AM into its cell-impermeant fluorescent form and contain the dye is taken as a sign of viability. In addition, propidium iodide (3 μM) was added to the perfusion buffer and both transmission and fluorescence images were monitored. When the plasma membrane became damaged, propidium iodide entered neurons and fluoresced upon binding to nucleic acids (Sasaki et al., 1987; Macklis and Madison, 1990). Control studies show that freshly isolated neurons from both neonates and adults have stable morphological properties and fluo-3 fluorescence levels for up to 90 min of recording at intervals of 15 sec.

Cell volume. Swelling was measured using the transmitted light images from the confocal microscope. The major (d_1) and minor (d_2) diameters of the soma were measured from the recorded OMDR image at 2 min time intervals. Volume (V) for an ellipsoid was calculated relative to the volume (V_0) at the start of the anoxic period:

$$V/V_0 = \{(d_1 \times d_2)^{3/2}\}/\{(d_{1_0} \times d_{2_0})^{3/2}\}$$

(Fischbarg et al., (1990).

Experimental protocol. Freshly dissociated CA1 neurons were found to be highly fluorescent following loading and incubation periods, although the extent varied widely within the population. We selected moderately fluorescent cells, with two or three primary processes each and no observable dye sequestration. These cells exhibited typical morphology of pyramidal CA1 neurons with a short basal process and a longer apical dendrite (Figs. 1, 2). The cells studied and analyzed did not show any visible sign of injury: flat or swollen cells or those with grainy appearance were considered to be injured (Kay and Wong, 1986; Cummins et al., 1991).

A minimum of a 10 min baseline was recorded to determine stability of intracellular Ca^{2+}, that is, minimal variation in fluorescence intensity. Only cells whose fluorescence was stable (less than 5% variation) during the baseline recording were used. Following baseline recordings, the perfusing buffer was changed to NRB, for 10 min, and then back to RB. Recordings continued for up to 90 min.

The internalized fluo-3 was found to be responsive to changes in extracellular calcium ranging from 0.02 μM to 1 μM in the presence of the calcium ionophore 4-bromo-A23187 (10 μM; Molecular Probes). Fluorescence could also be quenched by the addition of 10 mM $MnCl_2$. Since bleaching of fluo-3 has been reported to be a possible problem with laser confocal scanning microscopy (Pawley, 1991), we checked for bleaching and cell viability at a higher sampling rate (measurements every 15 sec as compared to our usual 30 sec protocol) and with a higher intensity (we used a 1% transmission filter instead of the 0.1% filter we normally employ). No change in morphology and minimal (<0.1%/min) bleaching were observed during 90 min of recording.

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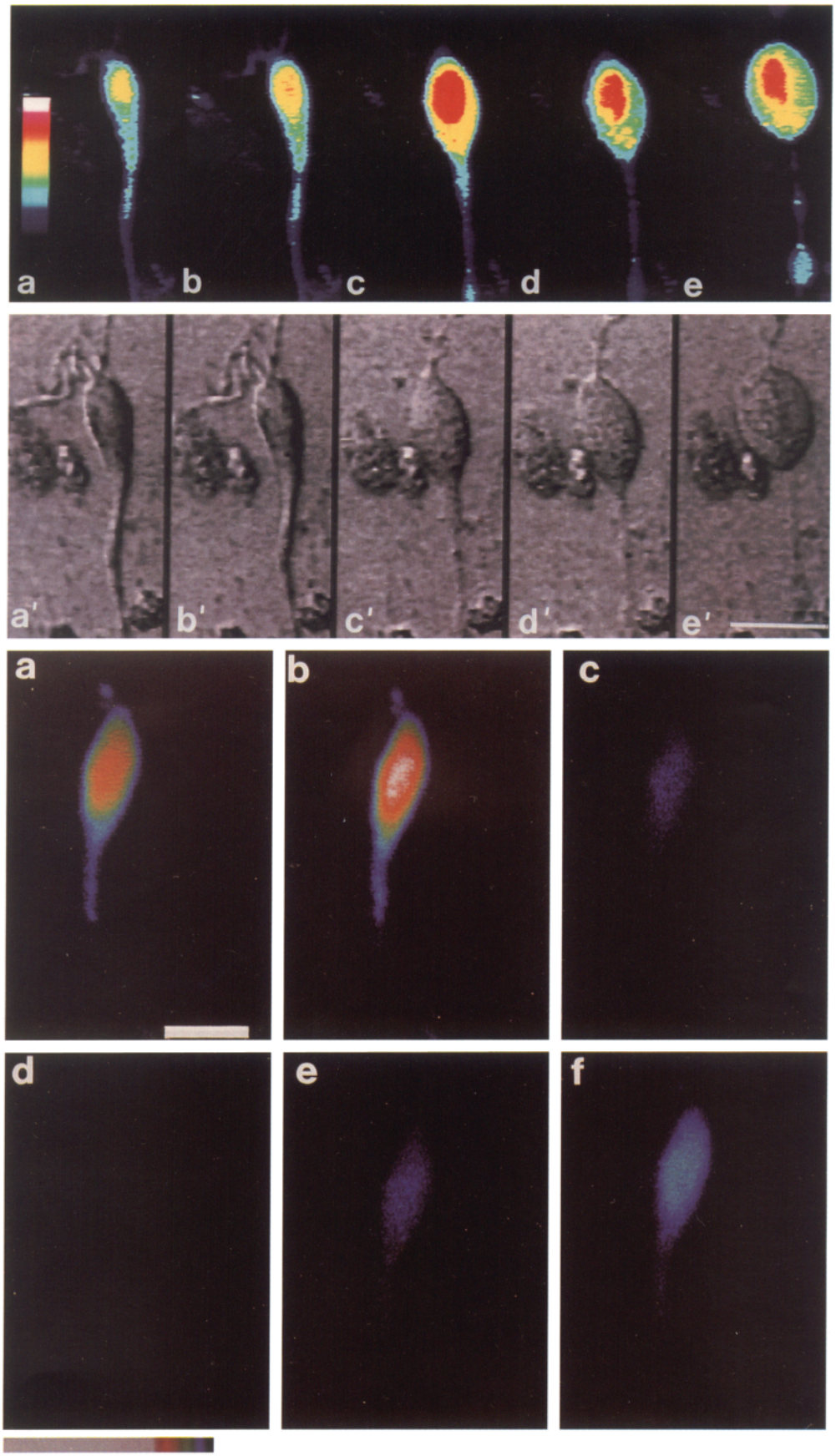
Figure 1 (opposite, top two rows). Anoxia-induced change in intracellular calcium in an adult CA1 neuron: pseudocolor images of the fluo-3 fluorescence from a freshly dissociated, 28-d-old rat CA1 neuron before, during, and after exposure to anoxia for 10 min using confocal microscopy, 25×/M.A. 0.8 Zeiss oil objective. The pseudocolor scale ranges from *red* (high Ca_i^{2+}) to *violet* (low Ca_i^{2+}). *a*, Taken during baseline recording, 1 min before application of anoxia. *b–e*, Taken 1, 6, 8, and 15 min after start of the anoxic period. *a′–e′*, Transmitted light images taken concurrently with the fluo-3 fluorescence images. Note the changes in morphology at the same time points as changes in Ca_i^{2+}. Scale bar, 25 μm.

Figure 2 (opposite, lower two rows). Intracellular calcium decreases during anoxia in absence of extracellular sodium: pseudocolor pictures of a CA1 neuron from a 30-d-old rat during baseline recording, 5 min before addition of NMDG (*a*), 13 min after Na^+ replacement with NMDG (*b*), anoxia (5 min after induction) in the presence of NMDG (*c*), anoxia 10 min after induction (*d*), reoxygenation 5 min after start of oxygenation (*e*), and reoxygenation 20 min after oxygenation (*f*). Note that Ca_i^{2+} increases during NMDG (before anoxia) but decreases during anoxia in the absence of Na_o^+. Scale bar, 10 μm.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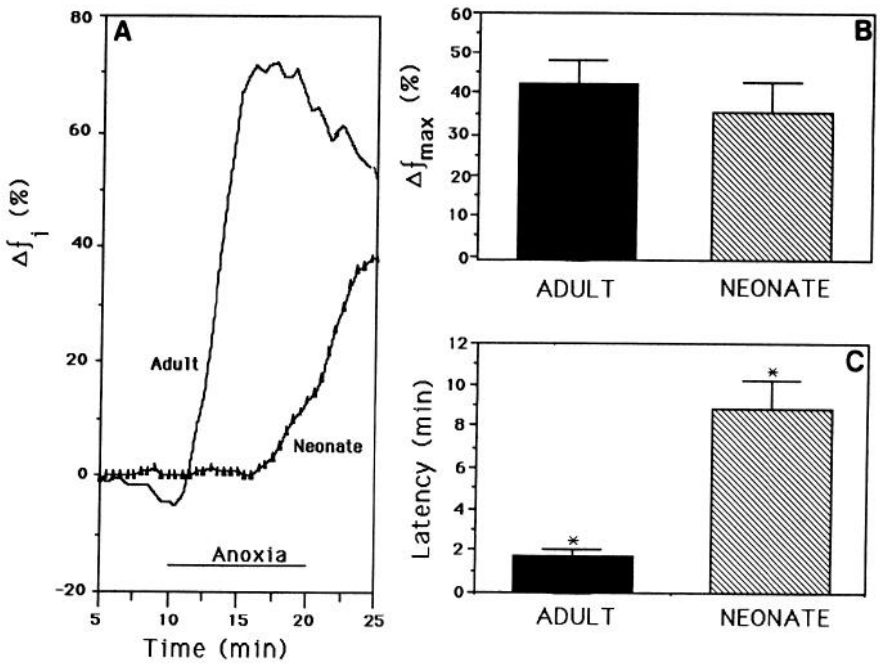

Figure 3. Intracellular calcium increases in newborn CA1 neurons during anoxia but after a longer latency than in adults. *A*, Two curves showing $\%\Delta f_i = [(f_i - f_0)/f_0] \times 100$ for an adult and a neonatal CA1 neuron. The *bar* indicates the 10 min anoxic period. The protocol used is defined in Materials and Methods. Note the longer latency of increase in the neonate's $\%\Delta f_i$. *B*, The maximum fluorescence change, Δf_{max} (%), for both neonates and adults, following a 10 min anoxic exposure. *C*, Latency of increase in fluorescence following anoxia. Note that the average latency of increase is about five times longer in the neonate. *, $p < 0.0001$.

Analysis. The recorded images were played back, frame by frame, and a best-fitted rectangle was placed within the soma. For each frame, i, the average fluorescence intensity within the rectangle, f_i, was calculated and recorded. $\Delta f_i = (f_i - f_0)/f_0$ was determined, where Δf_i is the normalized change in fluorescence (with respect to the baseline) for any given frame i, and f_0, the average of the fluorescence values acquired during the 10 min baseline recording (Yuste and Katz, 1991). Statistical significance was determined by the Student's t test as well as the Wilcoxon rank sum test. Values are presented as mean ± SEM.

Materials. NaCN (Aldrich) was prepared fresh as a 1 M stock solution. 4-Bromo-A23187 (Molecular Probes) was prepared as a 1 mM solution in ethanol and stored at −20°C. Fluo-3/AM (1 mM; Molecular Probes) was prepared weekly in dimethyl sulfoxide (DMSO; Aldrich) and stored at −20°C. Carbonyl cyanide *p*-fluoromethoxyphenylhydrazone (FCCP; 10 mM; Sigma) was dissolved in DMSO. Ultrapure argon (Ar_2) was obtained from Linde. 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) and MK-801 were obtained from Research Biochemicals, Inc. TTX was purchased from Sigma. Nimodipine, a gift from Miles Pharmaceuticals (West Haven, CT), was dissolved in ethanol and kept at −20°C.

Results

Response of adult and neonatal CA1 neurons to anoxia. Freshly dissociated adult CA1 hippocampal neurons were found to re-

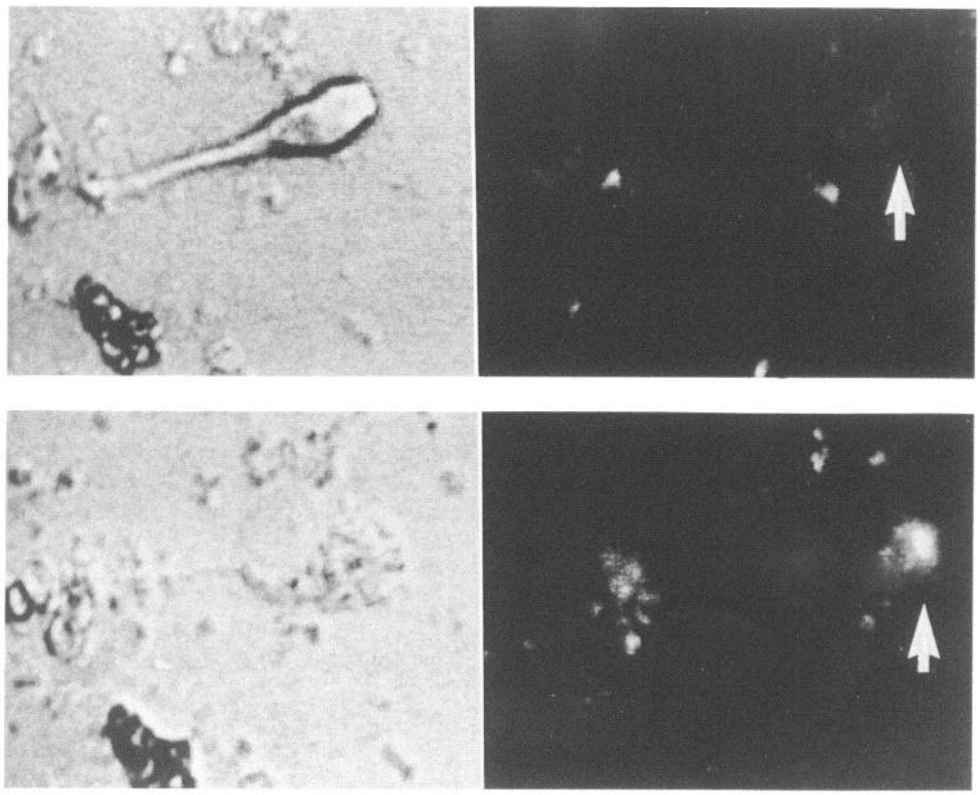

Figure 4. Propidium iodide stains nuclear region following anoxia. (*Left*, transmitted light images; *right*, fluorescence images.) *Top*, CA1 neuron from 32-d-old rat does not take up propidium iodide (*arrow*). *Bottom*, After 10 min anoxia period, the cell has swollen, permitting entry of propidium iodide and staining of the nucleus (*arrow*).

spond rapidly to anoxia. Within 2 min (1.7 ± 0.3; $n = 19$) of anoxic exposure, these neurons showed an increase in free intracellular Ca^{2+}, as demonstrated by increased fluo-3 fluorescence (Figs. 1, 3). The average maximum percentage increase in fluorescence, $\%\Delta f_{max} = [(f_{max} - f_0)/f_0] \times 100$ was 42.8 ± 6% ($n = 11$) (Fig. 3*B*). Following this increase, cells exhibited a decrease in fluorescence (Fig. 3*A*) even though these neurons were still exposed to anoxia. Upon reoxygenation, fluorescence continued to decline steadily, eventually leveling off. Concurrent with the increase in calcium fluorescence with anoxia, we also observed changes in morphology: retraction of processes, appearance of blebs, and swelling of soma (see Figs. 1, 6). Blebs started to appear within 5 min (4.9 ± 2.8 min), initially as small blebs on the dendrites, increasing in diameter as each process was retracting (see Figs. 1*e,e'*; 6). The apparent volume of the soma increased (within 9.8 ± 3.5 min) from 50% to 200% of the original value (Fig. 1). Every neuron exposed to 10 min of anoxia exhibited an increase in fluorescence and swelling. Within 5–15 min after reoxygenation, 80% of the neurons had a sudden, near-complete loss of dye from the cell, indicating cell death. This percentage increased to 97% within 30 min. This estimate of cell death was corroborated in independent experiments by the application of propidium iodide, which stained the nucleus at a similar time after anoxia (Fig. 4). In order to verify that our anoxia-induced observations in the presence of $Na_2S_2O_4$ were related to the fact that $PO_2 = 0$ torr rather than to a nonspecific effect of $Na_2S_2O_4$, we saturated RB with Ar_2, instead of N_2 and $Na_2S_2O_4$. PO_2 was measured using Ar_2, and within 2.5 min of perfusion, PO_2 was reduced to 0 torr. Perfusion with Ar_2-saturated RB resulted in a rapid increase in intracellular calcium, swelling, and surface blebbing, a pattern that was similar to that obtained with $Na_2S_2O_4$.

Neonatal rat CA1 neurons had a markedly different response to the same anoxic conditions. The average maximum increase in $\%\Delta f_{max}$ was smaller (36.2 ± 7; $n = 11$) than in adult neurons, but it was not significantly different (Fig. 3*B*). The pattern of increase was, however, very different. The increase started toward the end of the anoxic period and continued during reoxygenation, in contrast to the adult. The latency of the onset of rise in fluorescence was 8.9 ± 1.3 min ($n = 11$), which is significantly longer ($p < 0.0001$) than the latency found in adults (Fig. 3*C*). Neonatal neurons also exhibited morphological changes concurrent with the changes in Ca_i^{2+}, as described for adult neurons, although with a delay.

Calcium blockade. In adult neurons, baseline fluorescence decreased within 5 min of the addition of $CoCl_2$ (1 mM) to the perfusate. When $CoCl_2$ was added in the presence of Ca^{2+}, baseline fluorescence decreased 25%, while in the absence of extracellular Ca^{2+} baseline dropped 45% (Fig. 5*A*). In the presence of cobalt, anoxia induced an increase in $\%\Delta f_{max}$ of 19.1 ± 3.6 ($n = 4$) (Fig. 5*B*), which is significantly lower ($p < 0.05$) than the response to anoxia in normal buffer. Removal of extracellular Ca^{2+} in solutions containing cobalt caused not only a decrease in baseline fluorescence but also a decreased response to anoxia (Fig. 5*B*). The increase in $\%\Delta f_{max}$ in response to anoxia from this new baseline was only 7% ($n = 4$) (Fig. 5*B*). Latency was not affected. Replacement of extracellular calcium by 1–5 mM EGTA inhibited any increase in fluorescence in response to anoxia. In the presence of cobalt, neonates exhibited a small (2.3% ± 1.3; $n = 4$) fluorescence increase during or after the anoxic episode (Fig. 4*B*). Without calcium, or in the presence of EGTA, no fluorescence increase was seen at all ($n = 3$). In all cases ($n = 15$), even in the absence of any increase in Ca_i^{2+},

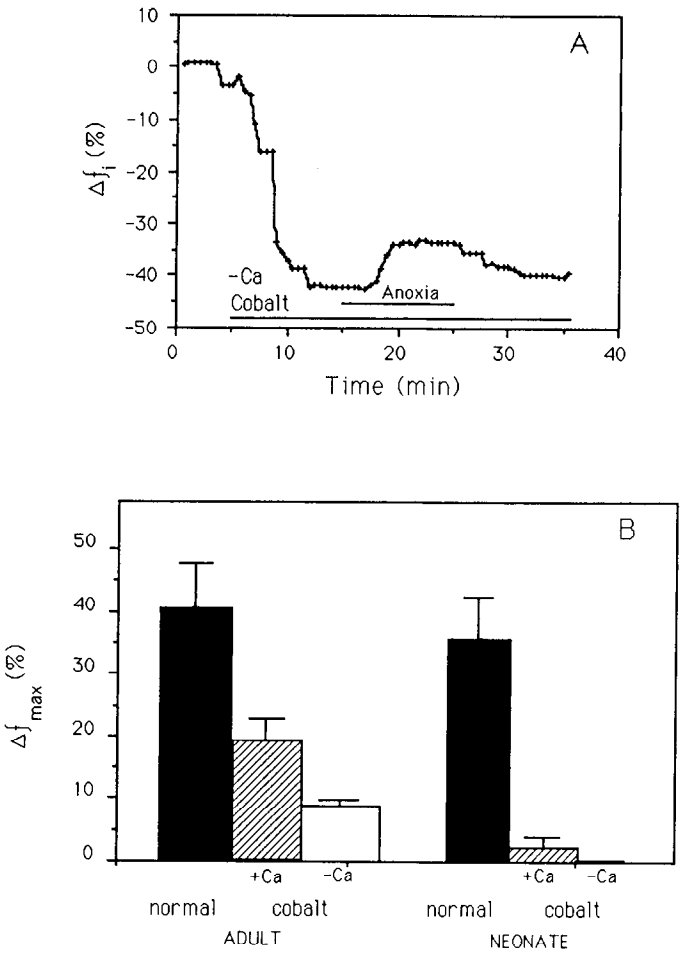

Figure 5. Cobalt abolishes most of the anoxia-induced rise in intracellular calcium. *A*, Freshly dissociated adult CA1 neurons perfused with normal buffer and then switched to buffer containing 1 mM Co^{2+} and nominally zero Ca^{2+} (indicated by the *bar*). Induction of anoxia was as per Materials and Methods. Note that Δf_i (%) increases much less with anoxia in the presence of Co^{2+}. *B*, Δf_{max} (%) for both neonates and adults, following a 10 min anoxic exposure, in the absence (*normal*) or presence of cobalt, with or without extracellular calcium. All results are significantly different from one another ($p < 0.05$) with the exception of the normal adults versus normal neonates.

swelling of the neurons still occurred during and following anoxia, although the rate of swelling was reduced, with a 50% increase in diameter occurring in 15.5 min as compared to 9.8 min in the presence of Ca^{2+} (see above). Initiation of blebbing, however, had a similar time course as in the presence of Ca^{2+}. Sudden loss of dye and apparent loss of three-dimensional morphology occurred within 45 min. Control experiments have shown that the removal of extracellular calcium per se did not cause swelling over the time course of our experiments.

Since the decrease in baseline fluorescence in the presence of Co^{2+} possibly reflects depletion of intracellular stores, we applied cyanide (CN) to these neurons after Ca_i^{2+} had reached a minimum (Nicotera et al., 1989). In the absence of extracellular Ca^{2+} and in the presence of both $CoCl_2$ (1 mM) and nimodipine (10 μM), CN resulted in a fluorescence increase of approximately 30% ($n = 3$). This demonstrated that releasable intracellular Ca^{2+} stores were not depleted during perfusion with calcium-free buffer.

Glutamate antagonists. If endogenous glutamate were released by the dissociated neurons during anoxia resulting in

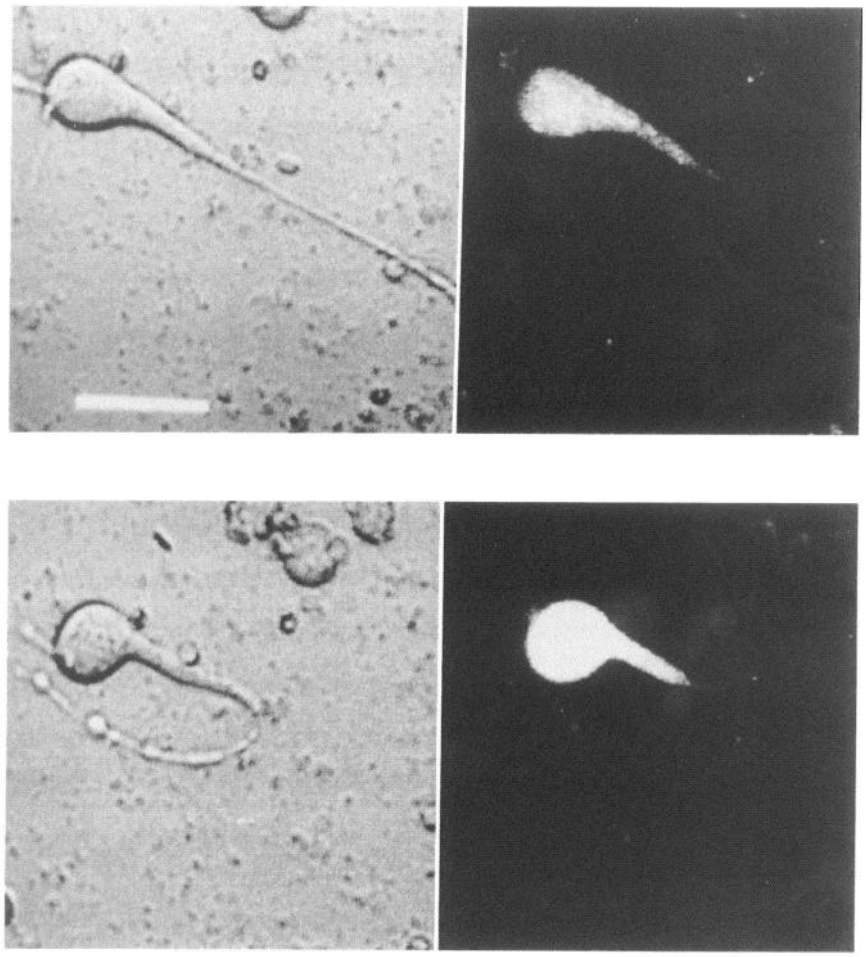

Figure 6. Anoxia causes increase in Ca_i^{2+} in the presence of MK-801: same conditions as for Figure 1, except that the CA1 cell from a 22-d-old rat was preincubated with 15 μM MK-801 for 10 min before, during, and after anoxia. *Top,* Before anoxia. *Bottom,* Two minutes after start of anoxia period. (*Left,* transmitted light images; *right,* fluorescence images.) Scale bar, 25 μm.

locally elevated levels in the cellular microenvironment, then it would be conceivable that NMDA or non-NMDA mechanisms could play a role in the anoxia-induced increase in Ca_i^{2+}. This was tested by exposing neurons to CNQX (20 μM) or MK-801 (15 μM) for 10 min before anoxia and during and after the period of anoxia. Using MK-801, the anoxia-induced Ca_i^{2+} increase had a latency of 1.75 ± 0.18 min (n = 3) with a fluorescence increase of 110 ± 8%. CNQX also did not significantly affect the rise in Ca_i^{2+}, having a latency of 2.5 ± 0.7 min (n = 3) and a fluorescence increase of 70.2 ± 10%. Both of these sets of results fall within the range of the anoxia-induced latency of Ca_i^{2+} increase for adult rats and on the high side of the increase in fluo-3 fluorescence. Figure 6 demonstrates the change in both morphology and fluorescence of an adult CA1 neuron preincubated with MK-801 and subsequently exposed to anoxia.

Replacement of sodium. In an attempt to study how CA1 neurons swell during anoxia, we replaced Na^+ with the impermeant cation choline or NMDG. A baseline was first established using the Na-HEPES buffer. Upon replacing the perfusate with the equivalent Na-free NMDG or choline-HEPES buffer, baseline fluo-3 fluorescence increased (Figs. 2, 7). Cells decreased in size by about 30% of the original surface area. Upon exposing adult neurons to anoxic conditions, no swelling was observed (Fig. 2). In addition, we made the surprising observation that fluorescence decreased to almost background level (i.e., zero), with a latency of 3 min or less (n = 5) (Figs. 2, 7). Upon reoxygenation, fluorescence reappeared to 50–75% of the original baseline level. No swelling was observed during or following the anoxic period, as long as the perfusate does not contain Na^+. Subsequent reperfusion with Na^+-containing RB resulted in an immediate (within 2 min) surface blebbing and swelling of the neuron.

In order to determine whether the decrease in fluorescence during anoxia in the absence of Na_o^+ was due to sequestration of Ca^{2+} into intracellular organelles, we applied 2 mM NaCN following a 10 min perfusion with oxygenated NMDG-HEPES buffer and after 7.5 min of N_2+$Na_2S_2O_4$–induced anoxia (at which time the fluorescence level had apparently dropped to a minimum). The application of 2 mM NaCN (n = 2) had no effect on fluorescence during the 5 min of application, nor did the addition of 10 μM FCCP (n = 2), which causes calcium release from the mitochondria by dissipating the proton gradient. Control experiments in the presence of extracellular Na^+ using CN or FCCP (without anoxia) on these neurons produced a rapid (within 2 min) increase in Ca_i^{2+} and similar morphological changes as observed during anoxia.

Since our results suggested that Na^+ entry was responsible for swelling, we examined the possibility that Na^+ penetrated through TTX-sensitive Na^+ channels. Following a 5 min preincubation with 3 μM TTX, adult neurons were exposed to our 10 min anoxic protocol. TTX did not protect the cells (data not shown), as evidenced by an increase in fluorescence, swelling, and surface blebbing.

When neonates were exposed to the Na^+-substituted NMDG-

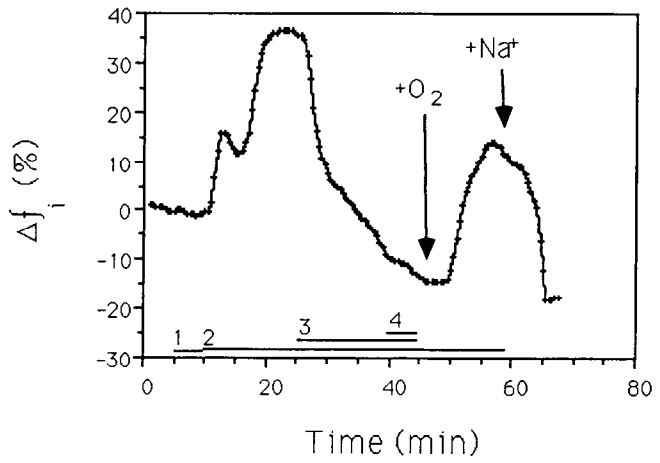

Figure 7. Removal of extracellular sodium results in decreases in intracellular calcium during anoxia: calcium is not accessible by FCCP. This graph shows fluorescence response of an adult CA1 neuron to anoxia in the absence of Na^+. The neuron was first perfused with RB, and then switched to Na-HEPES buffer at *1* followed by NMDG-HEPES buffer at *2*. At *3*, anoxia was applied, followed by 10 μM FCCP at *4*. Anoxia and FCCP application were terminated simultaneously while the neuron continued to be bathed with oxygenated NMDG-HEPES buffer. After a total of 45 min with NMDG-HEPES buffer, the neuron was reperfused with RB. Note that there was no increase in the fluorescence with FCCP. Also, note that the addition of Na_o^+ at the end of the experiment induced swelling, blebbing, and loss of dye.

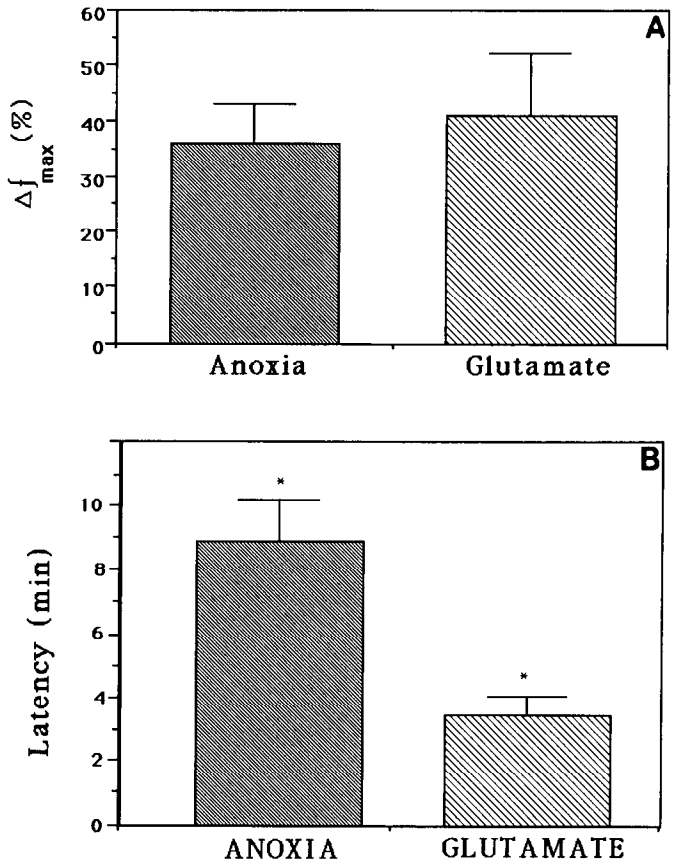

Figure 8. Latency of glutamate-induced rise in intracellular calcium in neonatal neurons is shorter than that induced by anoxia: fluorescence response of neonatal CA1 neurons (in the absence of Mg^{2+} and presence of 5 μM glycine) to 10 μM glutamate. *A*, Comparison of anoxia induced $\%\Delta f_{max}$ with that induced by 10 μM glutamate. *B*, Comparison of latency between the neonatal neuronal response to anoxia versus glutamate ($p < 0.0001$). Note that latency is shorter with glutamate than with anoxia.

HEPES buffer, fluorescence increased, as with adult neurons. Upon exposure to anoxia, fluorescence decreased, but to a lesser extent than adult neurons. Comparing the change in fluorescence from the NMDG-induced maximum to the anoxia-induced minimum, the $\Delta f_{adults} = 74.9 \pm 14\%$ ($n = 5$) while for neonates $\Delta f_{neo} = 31.3 \pm 7\%$ ($n = 6$) ($p < 0.01$).

Comparison of anoxia and glutamate effects. We made a direct comparison between the effect of glutamate (at different doses) and that of anoxia on neonatal and adult CA1 neurons. In the absence of Mg^{2+} and presence of 5 μM glycine, the dynamics of adult neuronal response to 10–500 μM glutamate was similar to the response to anoxia. The magnitude of the increase in fluorescence was dose dependent. Upon adding 10–500 μM glutamate to neonatal CA1 neurons under the same conditions as for adults, an increase in fluorescence with a latency of 3.4 ± 0.7 min ($n = 6$) was observed (Fig. 8*B*). This was significantly shorter than the anoxia-induced latency of 8.9 min ($p < 0.001$) (Fig. 3*C*), although still significantly longer ($p < 0.005$) than that of adults. The increase in $\%\Delta f_{max}$ (41.1 ± 11), evoked by 10 μM glutamate, was similar to that induced by anoxia (Fig. 8*A*). Increasing the concentration of glutamate from 10 to 500 μM caused an increase in the magnitude of the response but did not apparently affect the latency.

Discussion

The experiments presented in this article lead us to make four main observations. (1) Freshly dissociated CA1 neurons from both neonatal and adult rats show an increase in Ca_i^{2+} during anoxia, with the neonate having a much longer latency of increase. The increase in Ca_i^{2+} is followed by cellular swelling and somatic and dendritic bleb formations and retractions. (2) The increase in Ca_i^{2+} with anoxia depends to a large extent on the presence of extracellular Ca^{2+} in both age groups. The lack of increase in Ca_i^{2+} that occurred with Ca^{2+} channel blockers and absence of Ca_o^{2+} did not prevent swelling and neuronal death. (3) In the absence of Na_o^+, Ca_i^{2+} decreased rather than increased with anoxia and neurons did not swell, lose dye, or show any evidence of membrane damage with anoxia. (4) Anoxia-induced changes in Ca_i^{2+} are not necessarily similar to those of glutamate and hence should not be modeled by glutamate excitotoxicity.

A major hypothesis that has been put forth to explain anoxic neuronal injury and death in mammalian central neurons is that, during anoxia, intracellular calcium levels increase, resulting in a myriad of events including the activation of proteases, kinases, and other enzymes (Choi, 1987; Orrenius et al., 1989). This formed the basis of our rationale for performing these studies, in particular comparing neonates with adults. We have presented evidence in this work that neonatal rat CA1 neurons differ from adults in their sensitivity to anoxia in terms of their handling of cytosolic free calcium. The primary difference is that the latency of onset of rise in intracellular calcium is longer in neonates than in adult rat CA1 neurons. This is in line with previous observations from our laboratory showing that depolarization and changes in Ca_o^{2+}, Na_o^+, Cl_o^-, and Cl_i^- take much longer to develop during anoxia in the neonate than in the adult (Haddad and Donnelly, 1990; Jiang and Haddad, 1991; Jiang et al., 1992a,b).

The longer latency (about five times) in the neonate as com-

pared to the adult is most likely a reflection of different regulatory mechanisms of cytosolic free Ca^{2+} between the two age groups. Possible differences include better buffering capacity (Blaustein, 1988; Mattson et al., 1991), lower rate of inward Ca^{2+} leakage, fewer active calcium-conducting channels, increased extrusion of Ca_i^{2+}, or increased ability of sequestration into intracellular stores in the newborn. Although evidence for such differences is lacking, neonatal handling of Ca_i^{2+} in muscle fibers does not indicate that the newborn has an enhanced ability to sequester Ca_i^{2+} (Maxwell et al., 1983). A potentially important explanation for the increased latency in the neonate may be, then, the ability of the neonatal tissue to preserve ATP levels or produce ATP from alternative pathways such as glycolysis (Xia et al., 1992), and therefore energy-requiring processes for Ca^{2+} homeostasis may be sustained for longer periods.

One important consideration in this work is whether the differences we observed between the neonate and adult nerve cells are due to the methods used and to potential nerve injury in the adult. We believe that the differences are not related to the methods used for several reasons. Upon comparing the electrophysiologic properties of freshly isolated CA1 neurons with those of neurons in the CA1 region in brain slices (i.e., cells that were not subjected to enzyme treatment or trituration), we found that the cellular and membrane properties that exist in the slice are also present in isolated cells for both neonates and adults (Cummins et al., 1991). This was true not only of CA1 neurons but also for brainstem nerve cells (Jiang and Haddad, 1991; Jiang et al., 1991). In addition, adult and neonatal CA1 neurons in the slice exhibit a similar intracellular pH level as do freshly dissociated neurons, indicating that the sensitive regulation of pH_i has not been affected by the isolation procedures (T. R. Cummins and G. G. Haddad, unpublished observations). Furthermore, as presented in the Results, our experiments with propidium iodide dye exclusion show that adult cells do not take up the dye. That the neurons, both adult and neonatal, are stable and exhibit minimal changes in fluorescence (<5%) during 90 min of baseline recording is another indication that damage has not occurred during the preparation, although absolute levels of Ca_i^{2+} were not determined in each of the preparations. Although it is unlikely, the possibility exists that a higher initial resting level of Ca_i^{2+} in adults might result in a different sensitivity to anoxia, resulting in the calcium changes that we have observed.

The substantial attenuation of the anoxia-induced Ca_i^{2+} increase in the presence of extracellular Co^{2+} and/or EGTA suggests that during anoxia the increase in Ca_i^{2+} is dependent, to a great extent, on Ca^{2+} entry from the extracellular fluid. These experiments indicate that the primary mode of entry is through Co^{2+}-blockable ion channels in both neonate and adult. The fact that the increase in Ca_i^{2+} with anoxia is attenuated in the presence of Co^{2+} does not necessarily indicate that intracellular Ca^{2+} stores are depleted. Indeed, we do not believe so since our data and those of others demonstrate that NaCN or FCCP (Aw et al., 1987; Fulceri et al., 1991) causes a substantial increase in Ca_i^{2+} in the absence of extracellular Ca^{2+}.

Although our data show an anoxia-induced increase in Ca_i^{2+} that could have led to nerve injury, they also demonstrate that the lack of increase in Ca_i^{2+} during severe anoxia does not, by itself, eliminate or change the course of the somatic and dendritic swelling, cell injury, and death in CA1 hippocampal neurons. This latter finding is in disagreement with the Ca^{2+} hypothesis, which maintains that anoxia neurotoxicity is mediated by the influx of extracellular Ca^{2+} (Berdichevsky et al., 1983; Choi, 1987; Mattson et al., 1989; Siesjö and Bengtsson, 1989; Goldberg and Choi, 1990; Silver and Ereciñska, 1990; Somjen et al., 1990). Our data therefore support the idea that nerve damage during anoxia is not necessarily or solely related to an increase in intracellular calcium (Rothman, 1985; Choi, 1987; Michaels and Rothman, 1990; Dubinsky and Rothman, 1991). Another important conclusion that one may draw from our data is that the increase in Ca_i^{2+} that we observe in our CA1 neurons when Na^+ was replaced with NMDG is not sufficient to induce neuronal injury. Indeed, this question has raised much controversy in the literature. For example, Rothman (1985) and Dubinsky and Rothman (1991) found that elevated levels of Ca_i^{2+} per se, induced by A23187 or NaCN, did not cause nerve damage in cultured neonatal rat hippocampal cells, whereas Mattson et al. (1989) found, in a similar culture system, that elevated Ca_i^{2+} levels induced by A23187 did cause degeneration, albeit after a longer period of time.

That neurons swell and produce blebs during and following a 10 min period of complete anoxia is not surprising. These major morphological changes in the soma and processes could be related to several factors, as has been previously suggested by a number of investigators, including ourselves, for different cell types (Lemasters et al., 1987; Nicotera et al., 1989). Na^+ and Cl^- have both been incriminated in cell damage and death during anoxia (Rothman, 1985; Jiang et al., 1992b), and our present data lead us to conclude that Na_o^+ is crucial in the initiation of swelling and nerve damage (Fig. 2): Na^+ substituted with NMDG or choline did prevent cell swelling during and following 10 min of anoxia. Boening et al. (1989) found that when CA1 hippocampal slices were exposed to anoxia for 5 min, the preapplication of TTX permitted recovery of 70% of the evoked population spike but had no effect when anoxia was applied for 10 min, indicating that Na^+ entry occurred through other pathways. Based on our present observations, it would seem that a Na^+-dependent process triggers a cascade of events that leads to swelling and cell injury. It is interesting to note that swelling and cell injury occurred when Na^+ was added subsequent to the anoxic exposure (during reoxygenation), indicating that CA1 neurons were "primed" during anoxia and could still be influenced by addition of Na^+ during reoxygenation. Replacement of Na^+ with impermeant cations has also prevented or postponed glutamate toxicity (Choi, 1987; Dubinsky and Rothman, 1991), although this is not a universally accepted idea (Mattson et al., 1989).

The results of Na^+ replacement with impermeant cations may not lend themselves to easy interpretation since Na^+ replacement can lead to a complex chain of events. In addition to the (Na^+/K^+)ATPase, it can affect intracellular calcium (as we have shown) and possibly pH by modulating Na^+/H^+ and Na^+/Ca^{2+} exchange. In the slice, removal of Na^+ hyperpolarizes neurons modestly but does not inhibit totally the anoxia-induced depolarization. Hence, we cannot postulate that there is less influx of Ca^{2+} on the basis of inhibition of voltage-dependent Ca^{2+} channels.

We were therefore intrigued by our observation that, in the absence of Na_o^+, anoxia induces a decrease, rather than an increase, in Ca_i^{2+} as would have happened in normal RB. It is clear that this decrease in Ca_i^{2+} during anoxia is not an artifact since (1) this fluorescence attenuation does not occur in the absence of cells (data not shown) and (2) there is a subsequent increase in fluorescence with reoxygenation. Although we do

not have an explanation for this finding at present, we believe that Ca_i^{2+} does not get sequestered in intracellular organelles that are known to be sensitive to FCCP and CN (Fig. 7).

Our experiments on dissociated CA1 neurons are important not only because they provide new data regarding the effect of anoxia on Ca_i^{2+} in CA1 neurons, but also because they prove that Ca_i^{2+} can increase during anoxia in the absence of any accumulation of glutamate in the bathing fluid. Therefore, the increase in Ca_i^{2+} in neonatal versus adult neurons in our studies depends on the inherent properties of these neurons and not on synaptic input, hormonal, or neurotransmitter changes in the microenvironment.

Glutamate excitotoxicity is based on the findings that glutamate, which is released during anoxia, causes Ca^{2+} and Na^+ entry into neurons (Choi, 1987). However, the presence of glutamate can obfuscate studying the direct effect of anoxia on cells since the combined action of glutamate and anoxia may be different than that of each stimulus alone. Our studies that are done on isolated neurons with no synaptic input strongly suggest that glutamate release is not at the basis of the anoxia-induced increase in Ca_i^{2+}. This argument is strengthened by our data demonstrating that glutamate blockers do not attenuate the increase in Ca_i^{2+} during anoxia.

The latency response observed in neonatal neurons upon the addition of glutamate under our conditions was longer than expected. However, we would like to point out that most of the data presented in the literature demonstrating the effect of glutamate (or NMDA) on hippocampal neurons use cultured heterogeneous populations of neurons that are not necessarily of CA1 origin (Choi, 1987; McNamara and Dingledine, 1990; Michaels and Rothman, 1990; Greengard et al., 1991), rendering comparisons difficult. Our electrophysiological data (Cummins and Haddad, unpublished observations) indicate that whereas glutamate currents are present in dissociated cells, the response of neonates is much smaller in magnitude than that of adults. In addition, data from our laboratory show that in the neonate, levels of the ion-carrying NMDA and AMPA channels are low, increasing with age. Since we have shown that the response of neonatal CA1 neurons to anoxia is significantly different from that to glutamate, we suggest that the use of glutamate as a model for the direct, primary effect of anoxia on neurons is not valid.

We can therefore conclude the following. (1) Anoxia, in and of itself (i.e., without any microenvironmental neurotransmitter changes or synaptic input) causes an increase in intracellular Ca^{2+} in CA1 neurons, as well as swelling and bleb formation. (2) Neonatal CA1 neurons are much more slowly responsive to anoxia than adult neurons vis-à-vis calcium and swelling. (3) Neither Ca_o^{2+}o nor an increase in Ca_i^{2+} is necessary to induce nerve cell injury during anoxia. (4) Replacement of Na_o^+ with NMDG prevents anoxia-induced nerve injury. (5) Glutamate neurotoxicity may not be a valid model for the direct effect of anoxia on neurons.

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