Intracellular Calcium and Cell Death during Ischemia in Neonatal Rat White Matter Astrocytes In Situ

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The major pathological correlate of cerebral palsy is ischemic injury of CNS white matter. Histological studies show early injury of glial cells and axons. To investigate glial cell injury, I monitored intracellular Ca$^{2+}$ and cell viability in fura-2-loaded neonatal rat white matter glial cells during ischemia. Fura-2 fixation combined with immunohistochemistry revealed that fura-2-loaded cells were GFAP$^+$/O4$^-$ and were therefore a population of neonatal white matter astrocytes.

Significant ischemic Ca$^{2+}$ influx was found, mediated by both L- and T-type voltage-gated Ca$^{2+}$ channels. Ca$^{2+}$ influx via T-type channels was the most important factor during the initial stage of ischemia and was associated with significant cell death within 10–20 min of the onset of ischemia. The Na$^+$–Ca$^{2+}$ exchanger acted to remove cytoplasmic Ca$^{2+}$ throughout the ischemic and recovery periods. Neither the release of Ca$^{2+}$ from intracellular stores nor influx via glutamate-gated channels contributed to the rise in intracellular Ca$^{2+}$ during ischemia. Ischemic cell death was reduced significantly by removing extracellular Ca$^{2+}$ or by blocking voltage-gated Ca$^{2+}$ channels. The exclusively voltage-gated Ca$^{2+}$ channel nature of the Ca$^{2+}$ influx, the role played by T-type Ca$^{2+}$ channels, the protective effect of the Na$^+$–Ca$^{2+}$ exchanger, and the lack of significant Ca$^{2+}$ release from intracellular stores are features of ischemia that have not been reported in other CNS cell types.

Key words: axon; astrocyte; cerebral palsy; glia; ischemia; nerve fiber; white matter

Prolonged perinatal anoxia–ischemia can produce extensive CNS injury, in particular within white matter structures of the brain (Banker and Larroche, 1962; Paneth et al., 1994; Volpe, 1995). This pattern of injury (periventricular leukomalacia) is the major neurological lesion associated with cerebral palsy, a disorder affecting ~2 per 1000 live births (Kuban and Leviton, 1994). Periventricular leukomalacia is characterized by early damage of axons and glial cells (Gilles and Murphy, 1969; Leviton and Gilles, 1971; Banker and Larroche, 1974; Paneth et al., 1994; Volpe, 1995). There is subsequent microglial activation and astrocyte proliferation, followed by cavitary (Banker and Larroche, 1974; Paneth et al., 1994). Considering the role that glia play in normal CNS development (Racik, 1981), the early glial cell injury may be seminal in the development of the lesion. An ultimate reduction in oligodendrocyte numbers has been taken to indicate that the initial glial cell injury is restricted mainly to oligodendrocytes (Oka et al., 1993; Volpe, 1995). However, no information exists regarding the sensitivity of neonatal white matter astrocytes to ischemia, although astrocyte injury has been reported in periventricular leukomalacia (see Paneth et al., 1994).

In neurons, ischemic injury is mediated by Ca$^{2+}$ influx through voltage- and receptor-gated ion channels (Choi, 1988; Stiejo and Bengtsson, 1989). Astrocytes also express a number of voltage- and receptor-gated ion channels that could act as pathways for Ca$^{2+}$ influx during ischemia (Barres et al., 1990; Corvalan et al., 1990; Krieger and Chiu, 1993; Sontheimer, 1994; Gallo and Russell, 1995; Verkhratsev et al., 1996; Anoxic injury of cultured astrocytes is partly dependent on Ca$^{2+}$ influx via L-type voltage-gated Ca$^{2+}$ channels (Yu et al., 1989; Haun et al., 1992; Pappas and Ransom, 1995), whereas the importance of other routes of Ca$^{2+}$ entry remains unclear even in this reduced preparation. Ca$^{2+}$ influx during ischemia has been reported in immature gray matter astrocytes in situ (Duffy and MacVicar, 1996). Ca$^{2+}$ influx is mediated at least partly through voltage-gated channels, but its significance for cell viability is not known.

To investigate the mechanisms of Ca$^{2+}$ influx and cell death that may operate in astrocytes during periventricular leukomalacia, requires a methodology that is based on ischemia of in situ neonatal white matter astrocytes. Astrocytes in the neonatal rat optic nerve (nRON; a CNS white matter tract) were loaded with the Ca$^{2+}$-sensitive dye fura-2, and various potential routes of ischemic Ca$^{2+}$ entry were investigated. Simultaneously, astrocyte cell death was quantitated by assessing the ability of the cells to retain dye. Ischemic Ca$^{2+}$ rises were found in all cells and were associated with cell death. The Ca$^{2+}$ rises were a product of Ca$^{2+}$ influx rather than the release of Ca$^{2+}$ from intracellular stores; Ca$^{2+}$ influx was mediated via L- and T-type voltage-gated Ca$^{2+}$ channels and not by glutamate receptors. The Na$^+$–Ca$^{2+}$ exchanger acted to export Ca$^{2+}$ from the cytoplasm in the ischemic and postischemic periods. Many of these features of ischemia are unique to neonatal white matter astrocytes among the cells that have been studied.

MATERIALS AND METHODS

Dye loading. Optic nerves were dissected from postnatal (P) P0–P2 Long–Evans rats and placed in artificial CSF (aCSF) composed of (in mm) 153 Na$^+$, 3 K$^+$, 2 Mg$^{2+}$, 2 Ca$^{2+}$, 131 Cl$^-$, 26 HCO$_3^-$, 2 H$_2$PO$_4^-$, and 10 glucose. A stock solution containing 1 mm fura-2 AM (Molecular Probes, Eugene, OR) was made in dry DMSO and 10% pluronic acid. Seven microliters of the stock were added to 1 ml of aCSF to give a final fura-2 AM concentration of 7 μM, which was used for all incubations. A variety of different incubation protocols were assessed for fura-2 AM loading of the cells in the optic nerve. It was found that a 20 min period...
of exposure to collagenase (200 U/ml collagenase type I; Sigma, St. Louis, MO) was required to achieve adequate dye accumulation in cells. Presumably, the enzyme disrupted the connective tissue, allowing dye to penetrate into the tissue. nRONs were incubated for 20 min at 37°C in aCSF containing 7 μM fura-2 AM, collagenase, and a trace amount of EGTA. Then nerves were transferred to fresh aCSF and 7 μM fura-2 AM for 60 min at room temperature. The nerves were maintained in hydrated 95% O₂/5% CO₂ atmosphere for both incubation periods. The nerves were washed in aCSF before being mounted in the perfusion chamber. Imaging set-up. The ends of the optic nerves were fixed to a 22 × 44 mm glass coverslip with small amounts of cyanoacrylate glue, leaving the majority of the nerve completely free of glue. The coverslip was sealed onto a Plexiglas perfusion chamber (atmosphere chamber, Warner Instruments, Hamden, CT) with silicone grease. aCSF was run through the chamber at a rate of 2–3 ml/min, with a fluid level of ~1 mm completely covering the coverslip. The top of the chamber was sealed with a second coverslip, and 95% O₂/5% CO₂ was blown over the aCSF at a rate of 1.5 l/min. The chamber had a two-compartment design. The nerve was located in the center of the larger chamber, which contained a fluid volume of ~0.5 ml and had a lozenge shape to minimize fluid turbulence. This chamber was connected to a second, smaller chamber from which the aCSF was sucked under vacuum. aCSF was bubbled with 95% O₂/5% CO₂, then a bath at ~37°C, and then through Tygon tubing (Norton, OH) that was copper-clad to minimize gas exchange. A star valve with a purge system was used to achieve a bath washout of ~1 min.

The chamber was mounted on the stage of a Nikon Diaphot-TMD inverted epifluorescence microscope (Tokyo, Japan) equipped with a 40×/0.75 NA oil objective (Dako 40 UV; Olympus, Tokyo, Japan). Chamber temperature was maintained closely at 37°C with a flow through feedback tubing heater (Warner Instruments, Hamden, CT) positioned immediately before the aCSF entered the chamber; a feedback stage heater (Warner Instruments), which heated the metal surrounding the chamber; and a feedback objective heater (Bioptechs, Butler, PA), which warmed the objective to 37°C. This combination of heating elements regulated the temperature of the ROIs to 37°C, as established periodically with a temperature probe.

Cells within the optic nerve were visualized with a Hamamatsu C2400 intensified charge-coupled device (ICCD) video camera and image intensifier system (Hamamatsu, Bridgewater, NJ). Data were collected and stored with an image acquisition program from Photon Technology (East Brunswick, NJ) running on a Dell 486-Omniplex personal computer (Austin, TX). It was found that the preparation shifted slightly during the relatively long recording period of the experiments. For this reason the data were converted to tagged image file format (TIFF) format after the experiment and transferred to a Macintosh Power PC for off-line analysis. The data were analyzed with National Institutes of Health IMAGE software (National Institutes of Health, Bethesda, MD), which allowed the region of interest (ROI) drawn around each cell to be moved between frames. The size and shape of the ROIs were not changed at any point for any given cell.

Experimental protocol. Once mounted in the microscope, the optic nerves were left to equilibrate for 20 min. Then a 5 min period of baseline was taken before switching to ischemic conditions. Ischemia was induced by changing from aCSF to perfusion with zero-glucose aCSF that had been bubbled with 95% N₂/5% CO₂ for at least 1 hr. The atmosphere in the recording chamber was shifted simultaneously to 95% N₂/5% CO₂. Ischemia was maintained for 80 min, at which point normal conditions were reestablished for a further 60 min of recovery. Cells initially were brought into focus during illumination at 360 nm. A typical set of confocal images from a GFAP-stained nerve are shown in Figure 1. Imaging set-up. The nerve was located in the center of the larger chamber, which contained a fluid volume of ~0.5 ml and had a lozenge shape to minimize fluid turbulence. This chamber was connected to a second, smaller chamber from which the aCSF was sucked under vacuum. aCSF was bubbled with 95% O₂/5% CO₂, then a bath at ~37°C, and then through Tygon tubing (Norton, OH) that was copper-clad to minimize gas exchange. A star valve with a purge system was used to achieve a bath washout of ~1 min.

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RESULTS

Cell identification

The lineage of fura-2-loaded cells in the nRON was investigated immunohistochemically. Fura-2 was fixed in normal dye-loading protocol, fura-2 was fixed in the tissue with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). EDC (40 mg/ml) in aCSF for 90 min was found to provide excellent dye fixation (Tymianski et al., 1997). Then the nerves were washed in PBS containing 3% fetal bovine serum (FBS) and 0.1 M glycine for 10 min before being permeabilized with 1% Triton X-100 in PBS for 40 min. The nerves were washed in 3% FBS in PBS for 10 min before overnight incubation in 3% FBS in PBS with a primary antibody. Monoclonal antibodies raised against glial fibrillary acidic protein (GFAP, 1:3; Boehringer Mannheim, Indianapolis, IN) and O4 (1:5; Boehringer Mannheim) were used. The nerves were washed in 3% FBS in PBS for 2 hr before incubation with secondary antibody (Cy3, 1:30; Sigma) for 3.5 hr. The nerves were mounted in a Bio-Rad MRC-1024 UV confocal microscope (Hercules, CA), and fura-2 and Cy3 images were collected simultaneously. Statistics. Results are reported as mean ± SEM. Means represent the values of all cells studied under a particular condition (cells from all of the nerves were pooled). SEM represents the standard error of the mean values between nerves (cells pooled within nerves; error calculated between nerves). Data from P0 and P2 nerves were found to be not significantly different. Statistical significance was determined by ANOVA, with Tukey’s post-test.

Significant GFAP staining was found throughout the optic nerve. GFAP⁺ somata were noted near the outer edge of the tissue, and GFAP⁺ elements ramified throughout the nerve interior (Fig. 1, top left). Fura-2 was present within the GFAP⁺ somata (Fig. 1, top right), and combined images showed that all GFAP⁺ cells in this section of the nerve contained fura-2 (Fig. 1, bottom). In fact, all of the large numbers of cells in the two optic nerves examined that contained fura-2 were GFAP⁺. It appeared from Figure 1 that GFAP-reactive processes within the nerve also might contain fura-2, but the resolution was not adequate to prove this point. It is clear, however, that fura-2 loading into cell bodies far exceeded dye loading into astrocyte cell processes or axons. No O4⁺ cells were found in P0–P2 nerves, although O4⁺ cells were observed in P8 nerves used as a positive control (data not shown).
Ischemia

The onset of ischemia was followed by an increase in the 340:380 ratio in all cells (397 cells, 10 nerves). The majority of cells exhibited a precipitous loss of dye at some point either during ischemia or during recovery, correlating with cell death. A series of 360 images of an optic nerve at different times during an ischemia experiment is shown in Figure 2 (left). Initially, there were six cells within this region of the nerve (marked 1–6 in the line drawing to the right, Fig. 2). At various points three of the six cells disappeared from the images. For example, cell 1 was present at 0 min and 20 min, but not at 25 min. The 340:380 ratio of the three cells that died in this manner is shown in Figure 2A, and the 360 intensity is shown in Figure 2B. The values have been shifted along the y-axis to differentiate the three plots more clearly. \([\text{Ca}^{2+}]_{i}\) (340:380 ratio) started to increase within 5–10 min of the onset of ischemia in all three cells. In cell 1, \([\text{Ca}^{2+}]_{i}\) reached a peak after 20 min, and the 360 intensity was fairly stable up to that point. At 25 min the 360 intensity collapsed to the background level and stayed at that level for the rest of the experiment. The 340:380 ratio became meaningless at that point, because it reflected the ratio of the background fluorescence. The two other cells showed similar behavior, with \([\text{Ca}^{2+}]_{i}\) rising before eventual cell death.

The incidence of cell death during ischemia is shown in Figure 3A. The percentage of the initial total number of cells that died in any given 5 min epoch was plotted against time. The onset of ischemia was followed by a wave of cell death that peaked between 20 and 35 min, with significant cell death continuing through the period of ischemia. Cell death also occurred during the recovery period. In all, 46.6% of the cells died during the period of ischemia (179 of 397 cells, 10 nerves), and 59.5 ± 5.2% of the cells died over the entire 145 min period of the experiment (236 of 397 cells) (Figs. 3A, 12). There was some cell death in control experiments in which nerves were perfused continually with normal oxygenated aCSF (Figs. 3B, 12). Under these conditions, 13.3 ± 4.5% of the cells died in 145 min of recording (21 of 161 cells, 4 nerves).

\(\text{Ca}^{2+}\) influx during ischemia

Cell death and changes in \([\text{Ca}^{2+}]_{i}\) during ischemia were dependent on the presence of extracellular \(\text{Ca}^{2+}\). Astrocytes in nerves perfused with aCSF that contained 50 \(\mu\text{M}\) EGTA and no \(\text{Ca}^{2+}\) had stable \([\text{Ca}^{2+}]_{i}\) during 80 min ischemia experiments (Fig. 4A). In all, 25.5 ± 2.7% of the cells died under these conditions, significantly less than ischemia experiments performed in the presence of \(\text{Ca}^{2+}\) (55 of 216 cells, 3 nerves; \(p < 0.001\)) (Figs. 4D, 12). This degree of cell death was, however, higher than that found under control conditions in the absence of ischemia (25.4 ± 2.7% as compared with 13.3 ± 4.5%, respectively; \(p < 0.05\)). Cell death was not preceded by an increase in \([\text{Ca}^{2+}]_{i}\), within the 5 min
time resolution used for recording (Fig. 4B). This contrasts with the cell death found when nerves were perfused with normal aCSF (no ischemia), in which distinct rises in \([\text{Ca}^{2+}]_i\) were always observed before the loss of membrane integrity (Fig. 4C).

\([\text{Ca}^{2+}]_i\) changes during ischemia fell into four patterns. These were observed most clearly in astrocytes that survived ischemia (Fig. 5). In some cells, \([\text{Ca}^{2+}]_i\) increased during the early stages of ischemia before reaching a maximal value and declining toward baseline (Fig. 5A). Note from the 360 intensity plot that this cell did not die at the point of maximal \([\text{Ca}^{2+}]_i\). Small changes in the 360 signal during ischemia may reflect changes in cell volume, with accompanying changes in the concentration of fura-2. Other cells showed a slow increase in \([\text{Ca}^{2+}]_i\) that peaked toward the end of the period of ischemia (Fig. 5B). Occasionally, both patterns were apparent in a single cell (Fig. 5C). A plateau in \([\text{Ca}^{2+}]_i\), during ischemia also was observed sometimes (Fig. 5D).

The existence of both early and late components to the \([\text{Ca}^{2+}]_i\) response during ischemia correlated with the incidence of ischemic cell death (see Fig. 3A).

**Ionotropic glutamate receptors**

Both early and late ischemic \([\text{Ca}^{2+}]_i\) responses were observed in the presence of the broad-spectrum ionotropic glutamate receptor antagonist kynurenic acid (1 mM) (Fig. 6A). In all, 60 ± 4.1% of the cells died in the presence of kynurenic acid during ischemia experiments, which was not significantly different from the incidence of cell death in the absence of the antagonist (102 of 170 cells, 3 nerves; \(p > 0.5\)) (see Fig. 12). The pattern of cell death was also similar to that found with no kynurenic acid present, with an
No late increases in [Ca$^{2+}$]$_i$ were observed during ischemia in the presence of 400 µM Ni$^{2+}$ (Fig. 8B). The incidence of cell death reflected this pattern of Ca$^{2+}$ influx, with no early phase of cell death in the presence of 400 µM Ni$^{2+}$ (Fig. 8B). The incidence of cell death in the presence of Ni$^{2+}$ was 35 ± 3.3%, significantly lower than in the absence of Ni$^{2+}$ (62 of 177 cells, 3 nerves; $p < 0.001$) (see Fig. 12).

Little change in [Ca$^{2+}$]$_i$ was observed during ischemia in cells perfused with combined 400 µM Ni$^{2+}$ and 50 µM diltiazem (Fig. 9A). Occasional [Ca$^{2+}$]$_i$ changes occurred in the postischemic period and were associated with some cell death (Fig. 9B,C). The overall incidence of cell death was 26.9 ± 9.0%, which was somewhat higher than the incidence of cell death in the absence of ischemia (77 of 286 cells, 5 nerves; $p < 0.01$) (see Fig. 12), but was not significantly different from that found in ischemia experiments performed in the absence of extracellular Ca$^{2+}$ ($p > 0.1$) (see Fig. 12).

**Na$^+$$-$Ca$^{2+}$ exchange**

The Na$^+$$-$Ca$^{2+}$ exchanger plays an important role in anoxic injury in the mature RON, where changes in intracellular Na$^+$, Ca$^{2+}$, and membrane potential establish the conditions that are necessary for significant Ca$^{2+}$ influx via this protein (Stys et al., 1992). Na$^+$$-$Ca$^{2+}$ exchange was blocked with the inhibitor bepridil (50 µM). Perfusion with bepridil in the absence of ischemia had no effect on baseline [Ca$^{2+}$]$_i$ (data not shown) nor on the incidence of cell death when compared with perfusion with aCSF (14% of the cells died; 9 of 65 cells, 1 nerve). The onset of ischemia in the presence of bepridil resulted in large increases in [Ca$^{2+}$]$_i$ (Fig. 10A). [Ca$^{2+}$]$_i$ continued to rise in all cells and fell only slightly or not at all during the recovery period (Fig. 10A). The incidence of cell death in ischemia experiments performed in the presence of bepridil was 62.5 ± 12.7%, which was not significantly different from in the absence of bepridil (100 of 160 cells, 3 nerves; $p > 0.5$) (see Fig. 12). The pattern of ischemic cell death in the presence of 50 µM bepridil is shown in Figure 10B.

**Nonselective inorganic Ca$^{2+}$ channel blockers**

Ca$^{2+}$ influx across the cell membrane can be inhibited in a nonselective manner by metal ions such as La$^{3+}$, Cd$^{2+}$, and high concentrations of Ni$^{2+}$ (Hille, 1992). A complication of these metals is that they interact with dyes such as fura-2, resulting in false positive responses if the metal penetrates into the cell (Haugland, 1996). Perfusion with 100 µM La$^{3+}$ blocked all changes in [Ca$^{2+}$]$_i$ during ischemia in the majority of cells studied (Fig. 11A, open squares), but a significant increase in the 340:380 ratio was observed occasionally in some cells in the recovery period (Fig. 11A, filled circles). This late signal was not associated with cell death (Fig. 11B). The incidence of cell death was similar to that found in ischemia experiments performed in the absence of Ca$^{2+}$ (25.5 ± 5.7%; 50 cells of 196, 3 nerves; $p < 0.001$) (Fig. 12). Similar results were obtained with 2 mM Ni$^{2+}$ (data not shown). Perfusion with 100 µM Cd$^{2+}$ resulted in a rapid increase in the 340:380 ratio, which was not associated with cell death (data not shown).

The incidence of cell death during the 145 min time course of experiments under different conditions is summarized in Figure 12. A significant reduction in cell death was produced by perfusion with blockers of voltage-gated Ca$^{2+}$ channels or by the removal of extracellular Ca$^{2+}$, but not by the block of ionotropic glutamate receptors or Na$^+$$-$Ca$^{2+}$ exchange.
Delayed cell death

Cell death that occurred in the 60 min recovery period after the 80 min of ischemia was associated with two patterns of \([\text{Ca}^{2+}]_i\) change. Either cells showed an increase in \([\text{Ca}^{2+}]_i\) during ischemia that fell back to baseline and was followed by a second increase in \([\text{Ca}^{2+}]_i\) that was associated with cell death (Fig. 13A), or the cells maintained an increase in \([\text{Ca}^{2+}]_i\) that eventually was associated with cell death (Fig. 13B). Delayed cell death, defined as the percentage of the cells that were alive after the 80 min period of ischemia (or 80 min of perfusion with normal aCSF for control cells) but that subsequently died, is shown for a number of conditions in Figure 13C. Delayed cell death was significantly more common after 80 min of ischemia than after 80 min of perfusion with aCSF (27.9 ± 7.0% as compared with 3.5 ± 2.4%, respectively; \(p < 0.05\)). Delayed cell death after ischemia was reduced to control levels when \(\text{Ca}^{2+}\) was absent from the aCSF (1.1 ± 0.3%; \(p < 0.001\) as compared with ischemia in normal aCSF) and was not significantly different from that found after...
ischemia in the presence of 400 μM Ni²⁺ and 50 μM diltiazem combined (22.2 ± 8.6%; p < 0.05) or 100 μM La³⁺ (11.9 ± 2.5%; p > 0.05).

**DISCUSSION**

In situ nRON astrocytes were highly sensitive to ischemia. Rises in [Ca²⁺]i occurred within 5–10 min of ischemia, and significant cell death was apparent after 10–20 min. The high ischemic sensitivity resulted from Ca²⁺ influx through T-type channels. This is the first time such a phenomenon has been reported. No ischemic [Ca²⁺]i rises occurred in Ca²⁺-free solution, indicating that there was no significant Ca²⁺ release from intracellular stores. It is possible that transient noninflux-mediated changes in [Ca²⁺]i occurred that were not recorded with the image collection rate that was used. However, the near-complete protection from injury produced by the block of Ca²⁺ influx demonstrated that any such intracellular Ca²⁺ release was not important for cell viability. Significant prolonged ischemic Ca²⁺ release from intracellular stores occurs in gray matter astrocytes in situ (Duffy and MacVicar, 1996) and is apparently a ubiquitous feature of ischemia in neurons (Duchen et al., 1990; Dubinsky and Rothman, 1991; Friedman and Haddad, 1993; Hasham et al., 1994).

Cells responded to ischemia with either an early [Ca²⁺]i influx, a late [Ca²⁺]i influx, or a combination of the two. Both the early and late components of Ca²⁺ influx were associated with cell death. Early Ca²⁺ influx/cell death was blocked selectively by Ni²⁺, and late Ca²⁺ influx/cell death was prevented by L-type Ca²⁺ channel blockers. Both were blocked by La³⁺ and were unaffected by kynurenic acid. The results are summarized in Figure 14.

**Colocalization of fura-2 and GFAP**

Previous studies have colocalized fura-2-loaded cells indirectly with immunological markers (Porter and McCarthy, 1995). The current technique was adapted from that used to fix other BAPTA-type dyes by Tymianski et al. (1997). This allowed for the identification of fura-2-loaded cells in the optic nerve as a population of GFAP⁺ astrocytes located around the periphery of the nerve. Astrocytes present in the RON at birth constitute three morphological groups: (1) “undifferentiated” cells, (2) “trans-
verse" cells that resemble radial glia, and (3) "randomly oriented" cells found in the subpial region (Butt and Ransom, 1993). Preferential dye loading of subpial cells in the current study is presumably a consequence of the incubation method that was used. In a previous imaging study of nRON glia, fluo-3 AM was injected into the center of the nerve (Kriegler and Chiu, 1993). The particular cell type that loaded fluo-3 was not identified. In the current study only a small amount of fura-2 was found within the nerve core. It was unclear if this represented dye in out-of-focus astrocytes or whether dye had loaded to some extent into axons or astrocyte processes. Intense GFAP staining occurred in these deeper parts of the nerve, as previously reported (Ochi et al., 1993).

Ischemic Ca^{2+} influx

Astrocytes in culture can express both L- and T-type Ca^{2+} channels, depending on the culture conditions (Barres et al., 1989, 1990; Corvalan et al., 1990; Verkhratsky et al., 1998). Ca^{2+} channels at least partly mediate anoxic injury in cultured astrocytes (Yu et al., 1989; Haun et al., 1992). At P2, nRON astrocytes removed via "tissue print" exhibit both T-type and L-type channels, with T-type channels in greater density than L-type (Barres et al., 1990). The late ischemic Ca^{2+} influx and cell death in the current experiments were mediated by pharmacologically identified L-type channels. The early Ca^{2+} influx was not blocked by L-type channel blockers, but it was blocked by Ni^{2+} concentrations too low to affect L-type channels in the same cells. Greater sensitivity to Ni^{2+} than is exhibited by L-type channels is a characteristic of T-type channels (Hille, 1992). The documented presence of T-type Ca^{2+} channels in nRON astrocytes, the absence of other Ca^{2+} channels in these cells apart from L-type (Barres et al., 1990), the resistance of early Ca^{2+} influx to L-type channel blockers and its sensitivity to Ni^{2+}—all indicate that the early component of Ca^{2+} influx during ischemia is T-type channel-mediated.

Astrocytes in situ respond to ischemia with a gradual membrane depolarization from a resting potential of approximately −80 mV (Duffy and MacVicar, 1996). Depolarization from −80 mV will activate T-type channels, although they inactivate rapidly after step voltage changes (Hille, 1992). T-type channels inacti-
vate more slowly after smaller voltage changes (Fox et al., 1987; Ryu and Randic, 1990), and it may be that this voltage dependence of inactivation results in prolonged currents during gradual ischemic depolarization. Alternatively, a brief T-type Ca$^{2+}$ current during the initial ischemic depolarization may elevate [Ca$^{2+}$]$_i$ for several tens of minutes before the Ca$^{2+}$ is removed from the cytoplasm.

**Ca$^{2+}$ extrusion**

The initial phase of Ca$^{2+}$ influx during ischemia was often transitory, with [Ca$^{2+}$], declining toward baseline during the ongoing insult. Two membrane proteins are responsible for [Ca$^{2+}$], homeostasis: the Na$^+$–Ca$^{2+}$ exchanger and Ca$^{2+}$–ATPase. Bepridil, a blocker of Na$^+$–Ca$^{2+}$ exchange, removed the ability of cells to regulate [Ca$^{2+}$], during ischemia. The exchanger is therefore at least partly responsible for ischemic [Ca$^{2+}$], regulation in these cells. However, the inhibition of Ca$^{2+}$–ATPase by bepridil cannot be ruled out (Kaczorowski et al., 1989), precluding any firm conclusion regarding an exclusive role for the exchanger. Large and long-lasting increases in [Ca$^{2+}$], were found consistently during ischemia in the presence of bepridil (see Fig. 10A). However, the amount of cell death was not significantly greater than in the absence of the drug. This paradox may indicate a ceiling level of [Ca$^{2+}$], beyond which any further increase is not translated into a greater probability of cell death.

High [Na$^+$], and membrane depolarization favor the Ca$^{2+}$ import mode of the Na$^+$–Ca$^{2+}$ exchanger (Goldman et al., 1994; Stys et al., 1992). Cultured spinal cord astrocytes respond to chemical ischemia with large and rapid increases in [Na$^+$], (Rose et al., 1998). If similar changes in [Na$^+$], occur in nRON astrocytes, the exchanger would import rather than export Ca$^{2+}$. Astrocytes removed from P2 nRON by tissue print exhibit no detectable Na$^+$ conductance (Barres et al., 1990). This contrasts with astrocytes cultured in the presence of certain chemical factors (Barres et al., 1989), mature RON astrocytes (Barres et
Control aCSF 0 mM Ca2+ 50 μM D-18801 100 μM D-18801 1 mM Kynurenic acid

Figure 12. Histogram showing the incidence of cell death in ischemia experiments performed in various solutions. n represents sample size (cells); *** represents statistical significance as compared with cell death in normal aCSF (p < 0.001); †† represents statistical significance as compared with cell death in ischemia experiments performed in zero Ca2+ (p < 0.05; ††† < 0.01).

al., 1990), and mature RON axons (Stys et al., 1993). Na+ currents have been recorded from a small proportion of neonatal gray matter astrocytes in vitro (Sontheimer and Waxman, 1993). The low Na+ conductance of nRON astrocytes may limit Na+ influx during ischemia, permitting the export of Ca2+ via the exchanger. This may be a unique feature of neonatal white matter astrocytes, because in all other CNS cells that have been studied the Na++Ca2+ exchanger does not remove cytoplasmic Ca2+ effectively during ischemia, and in many cells the exchanger is a significant source of ischemic Ca2+ influx (Stys et al., 1992; Lobner and Lipton, 1993).

**Cell death**

A correlation was found between cell death and high [Ca2+]i. During ischemia and in the postischemic period cell death was always preceded by increased [Ca2+]i, as was cell death under control conditions. Manipulations that reduced ischemic [Ca2+]i rises also reduced the extent of cell death. These results are consistent with the Ca2+-mediated astrocyte death results in the absence of extracellular Ca2+ a degree of cell death occurred that was not Ca2+-mediated, as has been reported in neonatal neurons (Friedman and Haddad, 1993).

**Relevance to periventricular leukomalacia**

All evidence indicates that glial cell death occurs in the early stages of periventricular leukomalacia (see introductory remarks). There may be a causal relationship between glial injury and the subsequent development of the lesion, because both astrocytes and oligodendrocytes are involved in the normal maturation of white matter. Previous studies have focused on oligodendrocyte injury because of the role these cells play in myelination (Volpe, 1995). The current study has concentrated on neonatal white matter astrocytes. The results demonstrate unexpected novelty in the mechanisms of ischemic injury of these cells, in particular the role of T-type Ca2+ channels. Barres et al. (1990) have shown that T-type channels are expressed between P0 and P10 in optic nerve astrocytes, a period in development when this tissue closely resembles the neonatal human white matter regions that are subject to periventricular leukomalacia (DeReuck et al., 1972; Romijn et al., 1991). In addition to a potential direct contribution to the development of periventricular leukomalacia, astrocyte death during ischemia also may be a contributing factor in oligodendrocyte injury. For example, Ca2+-mediated astrocyte death results in the release of a toxic factor, probably tumor necrosis factor, that kills neighboring oligodendrocytes (Robbins et al., 1987). The current experiments show that neonatal white matter astrocytes are far more sensitive to ischemic injury than are axons at the same age in the same tissue (Fern et al., 1998). In this white matter tract astrocyte injury therefore represents the first step in the development of functional loss.

Figure 13. Two patterns of delayed cell death after ischemia. A, B, Plots of 340:380 ratio (top) and 360 intensity (bottom) from ischemia experiments. A, Ischemia is associated with an early Ca2+ influx that is not associated with cell death and a late influx that is (cell death indicated by arrows). B, Ischemia is associated with Ca2+ influx that does not return to baseline and is associated with cell death. C, Delayed cell death, defined as the percentage of cells alive at the end of ischemia that subsequently die in the recovery period, under various conditions. Delayed cell death is abolished by removing Ca2+ from the perfusing solution and is reduced to nonsignificant levels by a block of Ca2+ influx. ** represents statistical significance as compared with delayed cell death in control conditions (p < 0.05; ***p < 0.01).
REFERENCES


Figure 14. Ca$^{2+}$ influx and cell death during ischemia in neonatal optic nerve astrocytes. Ischemia is followed by a drop in ATP and breakdown in the operation of ATP-dependent membrane transport proteins (1). The resulting membrane depolarization activates Ni$^{2+}$- and La$^{3+}$-sensitive voltage-gated Ca$^{2+}$ channels (2; apparently T-type channels). T-type channel-mediated Ca$^{2+}$ influx is transitory, and [Ca$^{2+}$]i may recover because of the action of the Na$^{+}$–Ca$^{2+}$ exchanger (4). There is a subsequent Ca$^{2+}$ influx mediated by L-type Ca$^{2+}$ channels (3). Increased [Ca$^{2+}$]i, resulting from Ca$^{2+}$ influx through voltage-gated channels is associated with cell death.


