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In Vitro Ischemia Promotes Calcium Influx and Intracellular Calcium Release in Hippocampal Astrocytes

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The intracellular calcium concentration ($[Ca^{2+}]_i$) of astrocytes within rat hippocampal slices was measured during simultaneous hypoxia and hypoglycemia to examine the early intracellular signaling events induced by this *in vitro* model of ischemia. Hypoxia–hypoglycemia for 3.3–7.5 min evoked $[Ca^{2+}]_i$ increases in astrocytes iontophoretically loaded with calcium orange (11/14 slices; 2.5 min to peak $[Ca^{2+}]_i$, 5 min to >60 min duration). Calcium elevations also were observed in the absence of extracellular calcium ($[Ca^{2+}]_o$) (4/4 slices), indicative of Ca^{2+} release from internal stores. Hypoxia–hypoglycemia depolarized astrocytes (51 ± 16 mV), suggesting additional contribution from voltage-gated Ca^{2+} influx. Depolarization of a similar magnitude (51 ± 4 mV) by 50 mM extracellular potassium ($[K^+]_o$) triggered $[Ca^{2+}]_i$ increases (20/24 slices), which were blocked by removal of $[Ca^{2+}]_o$ (8/8 slices) indicating that depolarization promoted Ca^{2+} influx. Voltage-gated Ca^{2+} influx and internal release were measured in acutely isolated astrocytes during *in vitro* ischemia to examine these processes in the absence of surrounding neurons. Hypoxia–hypoglycemia (7.5–34.0 min) induced only modest, slow increases in the basal $[Ca^{2+}]_i$ of Fura-2-loaded isolated astrocytes (average 12% increase in Fura-2 ratio $R_{340/380}$ after 10 min) that were blocked by $[Ca^{2+}]_o$ removal. Voltage-gated Ca^{2+} influx was still functional under ischemia, however, as 50 mM $[K^+]_o$ evoked $[Ca^{2+}]_i$ increases (14/14 cells, $\Delta R_{340/380}$ of 48%) approximately equal to preischemic responses. Isolated neurons displayed large irreversible increases in basal $[Ca^{2+}]_i$ after 1.5–6.5 min *in vitro* ischemia (10/12 cells; average $\Delta R_{340/380}$ of 152%). The absence of significant basal $[Ca^{2+}]_i$ increases in isolated astrocytes indicates that ischemia-induced Ca^{2+} influx and internal release in astrocytes within slices depend on signals released from neurons (K^+, neurotransmitters). Ischemic $[Ca^{2+}]_i$ elevations may constitute a signaling mechanism for postischemic reactive responses.

Key words: astrocyte; ischemia; intracellular calcium; calcium orange; hippocampus; Ca channels; pyramidal neurons

Astrocytes express complex $[Ca^{2+}]_i$ signaling properties (Finkbeiner, 1993), and the induction of $[Ca^{2+}]_i$ signals by neurotransmitters and other messengers in the extracellular microenvironment could mediate a functional coupling of astrocyte physiology to neuronal activity. Direct evidence for $[Ca^{2+}]_i$-dependent neuron-to-glial communication, however, requires examination of astrocytic $[Ca^{2+}]_i$ responses during well defined physiological or pathological processes in intact tissue. Astroglial responses in several models of ischemia have been studied extensively and are characterized by a progressive series of morphological and biochemical alterations collectively referred to as reactive transformation (for reviews, see Lindsay, 1986; Reier, 1986; Kraig and Jaeger, 1990; Landis, 1994). Conversely, the intracellular signaling mechanisms that induce reactive responses remain obscure. Given the ubiquity of $[Ca^{2+}]_i$ elevations as a physiological signaling mechanism, we tested for the presence of $[Ca^{2+}]_i$ signals in the early response of hippocampal astrocytes to simultaneous hypoxia and hypoglycemia, a widely used *in vitro* model of ischemia. Astrocytes express high-threshold Ca^{2+} currents (MacVicar, 1984; Barres et al., 1989, 1990; Corvalan et al., 1990), and elevating $[K^+]_o$ to levels observed during ischemia *in vivo* (Somjen, 1979; Hansen, 1985) evoked significant $[Ca^{2+}]_i$ elevations in isolated hippocampal astrocytes by promoting voltage-dependent influx (Duffy and MacVicar, 1994b). Therefore, we directly tested for the activation of voltage-gated Ca^{2+} influx by anoxic depolarization. Because ischemic tissue also releases many neurotransmitters and second messengers, which could mobilize Ca^{2+} from internal stores, we also tested for such Ca^{2+} release during brief ischemic episodes.

In this study, we used two complementary approaches to study ischemic $[Ca^{2+}]_i$ signals in astrocytes. Fluorometric $[Ca^{2+}]_i$ measurements from astrocytes iontophoretically loaded with the Ca^{2+}-indicator calcium orange within hippocampal slices were performed to study ischemic responses in intact tissue. Additionally, $[Ca^{2+}]_i$ of acutely isolated astrocytes and neurons was measured during *in vitro* ischemia to assess the intrinsic ischemic responses in these cell types. These results show that brief episodes of simultaneous hypoxia and hypoglycemia induce $[Ca^{2+}]_i$ elevations in astrocytes that are mediated by voltage-gated influx and internal release. The $[Ca^{2+}]_i$ of acutely isolated astrocytes is much less sensitive to ischemia than astrocytes in intact tissue or isolated neurons, indicating that astroglial $[Ca^{2+}]_i$ responses in hippocampal slices are mediated primarily by extracellular messengers released during ischemia (e.g., $[K^+]_o$ accumulation, neurotransmitters).

Some results have appeared in abstract form (Duffy and MacVicar, 1993, 1994a).

MATERIALS AND METHODS

Preparation and maintenance of hippocampal slices. Transverse hippocampal slices (450 μm) from P21–P42 Sprague–Dawley rats were prepared using a manual tissue chopper (Stoelting) in artificial cerebrospinal fluid (aCSF) containing (in mM): 124 NaCl, 5 KCl, 26

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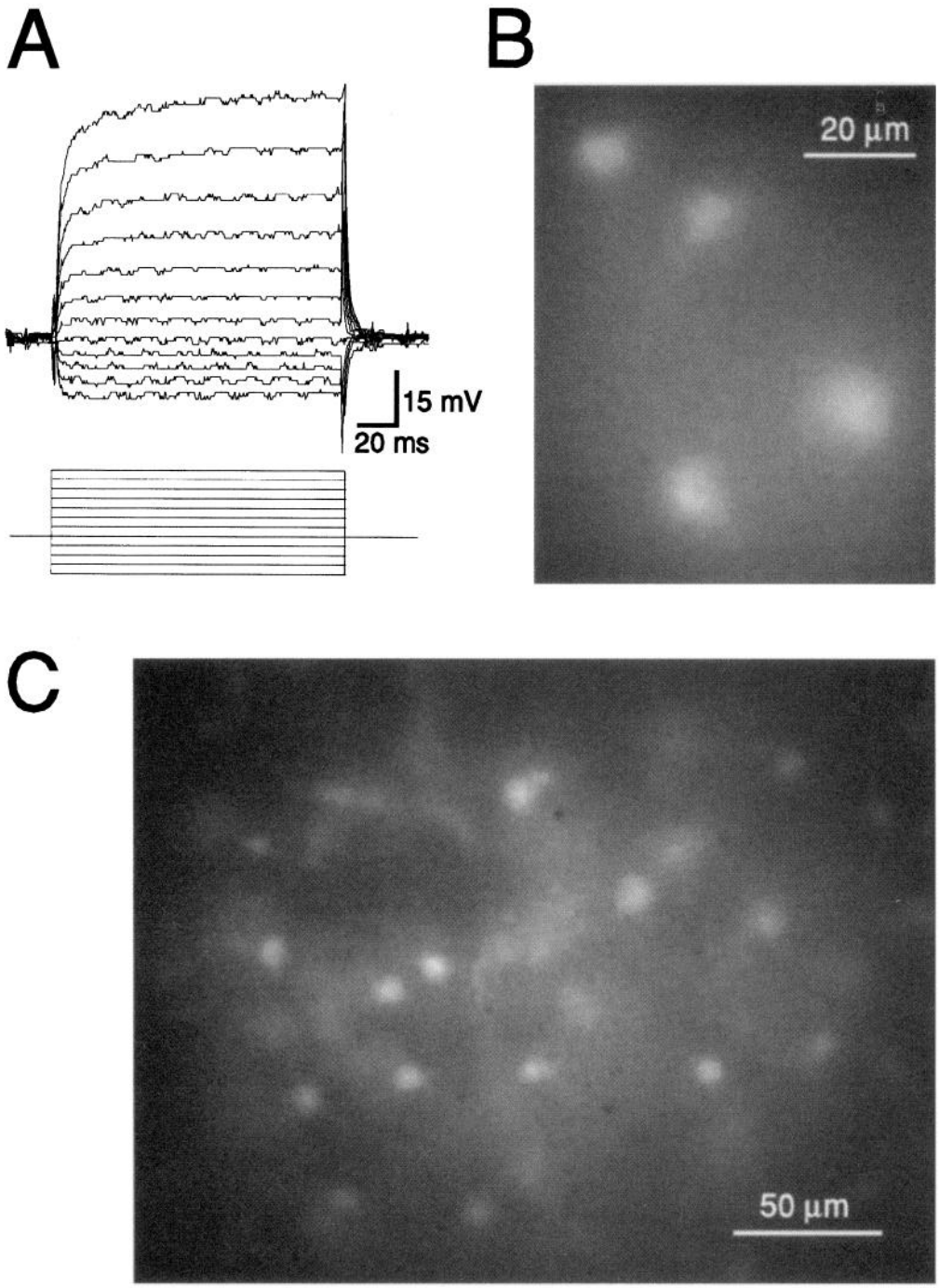

Figure 1. Iontophoretic injection of calcium orange into a single astrocyte resulted in the dye loading of many cells. *A,* The current–voltage relationship for an astrocyte impaled with a dye-filled electrode. No stimulus-evoked action potentials were observed in response to 140 msec current pulses between −0.8 and 1.4 nA. *B,* Fluorescence emission image at 580 nm showing the intracellular staining pattern after injection of the cell in *A*. Only a subfield of the injected cells is shown. *C,* A lower magnification image from another slice, demonstrating the full extent of dye coupling that resulted from ~20 min injection of calcium orange into a single astrocyte.

$NaHCO_3$, 1.3 $MgCl_2$, 2 $CaCl_2$, 10 glucose; pH = 7.35–7.40. Slices were stored on filter paper saturated with aCSF in an aerated (95% O_2/5% CO_2) interface chamber. For $[Ca^{2+}]_i$ imaging experiments, individual slices were transferred to a submerged perfusion bath on the stage of an inverted epifluorescence microscope (Axiovert 10, Zeiss). Slices were superfused with aCSF at a rate of 0.5–0.6 ml/min using a peristaltic pump (Gilson). Calcium-free aCSF was prepared by omitting $CaCl_2$ and adding 40–100 μM of the Ca^{2+} chelator ethylene glycol bis(β-aminoethyl)ether-*N,N,N′,N′*-tetra-acetic acid (EGTA). For high (50 mM) $[K^+]_o$ aCSF, 45 mM KCl was substituted for NaCl. Hypoxia and hypoglycemia were initiated by superfusing slices with glucose-free aCSF deoxygenated with 95% N_2/5% CO_2 at 35 ± 1°C. All solutions contained 10 or 20 μM of the heavy metal chelator tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) (Arslan et al., 1985).

Iontophoretic loading of calcium orange and imaging of hippocampal astrocytes. The techniques for iontophoretic loading of astrocytes with calcium orange were described in detail previously (Duffy and MacVicar, 1995). Briefly, a microelectrode was filled with 100 mM K acetate plus 5 mM calcium orange, tetrapotassium salt (final electrode resistance: 75–150 MΩ) and mounted to the head stage of a physiological amplifier (Neurodata). Astrocytes 30–100 μm below the top cut surface were impaled, and hyperpolarizing current (−0.2 to −0.5 nA) with superimposed square current pulses (−0.4 to −1.0 nA, 200–500 msec duration, 1 Hz) was passed through the electrode. Dye injection was continued as long as electrode resistance and membrane potential remained stable (10–30 min). For recording changes in astroglial membrane potential in response to 50 mM $[K^+]_o$ aCSF or during hypoxia–hypoglycemia, cells were impaled with lower-resistance electrodes (30–50 MΩ) containing 2 M K acetate or 1 M KCl.

After injection, the slice was turned over so that loaded cells were within working distance of the objective (50× Achroplan or 25× Plan-Neofluar, Zeiss), and the slice was secured with small pieces of platinum wire. Excitation light from a 75 W Xenon arc lamp (Zeiss) was gated by an electronic shutter (Uniblitz T-132) controlled by Axon Imaging Work-

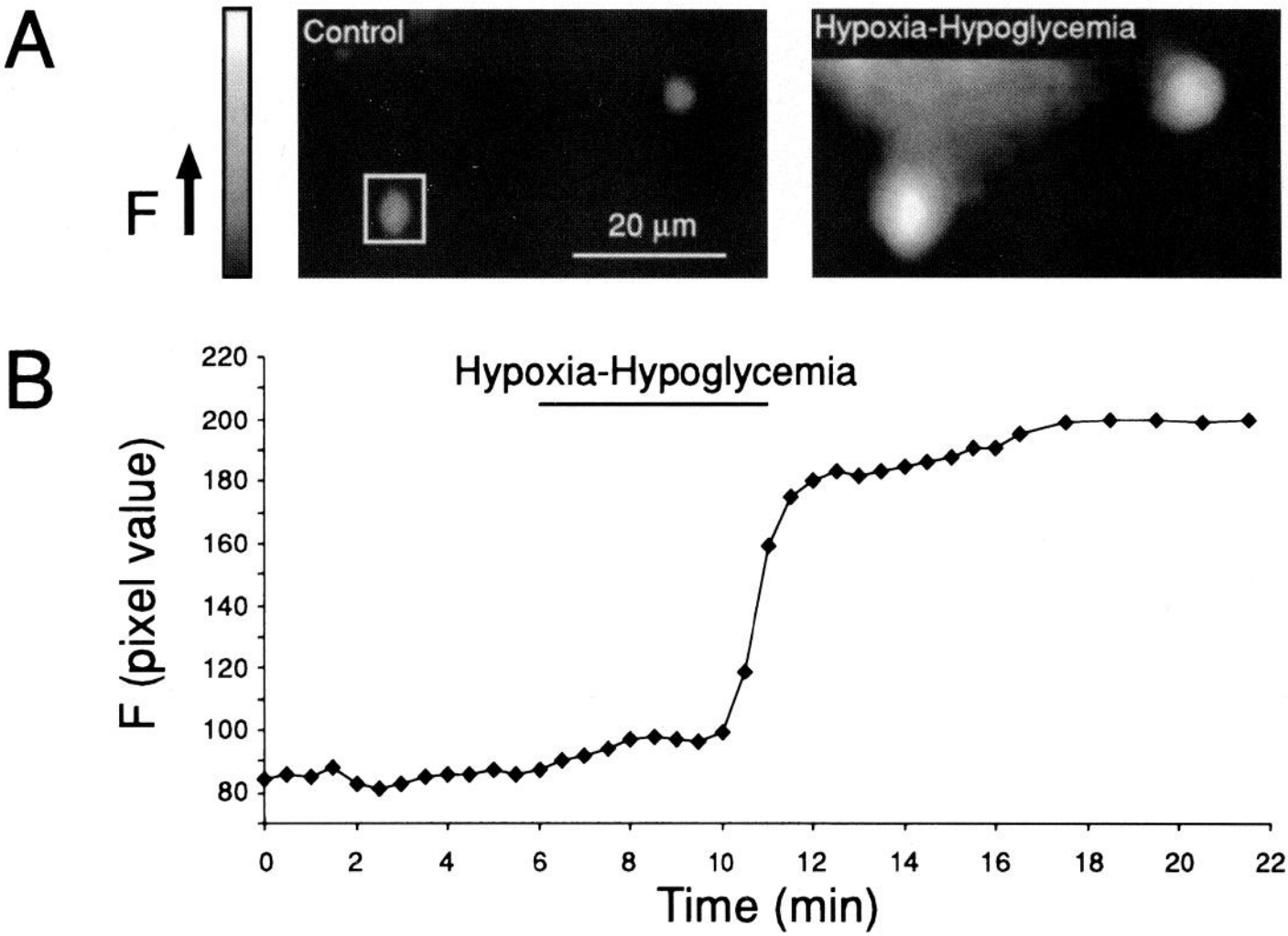

Figure 2. Simultaneous hypoxia–hypoglycemia induced an increase in astroglial $[Ca^{2+}]_i$. *A,* In response to 5 min superfusion of glucose-free aCSF aerated with 95% N_2/5% CO_2, an increase in calcium orange fluorescence emission (F) was observed, as indicated by increased brightness of the gray scale. Two astrocytes at the periphery of a cluster of dye-loaded cells are shown. *B,* The time course of the initial rise in ΔF for the cell demarcated by the box in *A*.

bench software (Axon Instruments). Rhodamine filters (Omega Optical) with excitation and emission maxima at ~550 and 580 nm were used to visualize fluorescence of the rhodamine-based dye calcium orange. Video images of calcium orange fluorescence emission (F_{580} or ΔF_{580}) were recorded using a Cohu 6500 CCD camera coupled to an image intensifier (KS-1381, Video Scope). Video frames were digitized and averaged (32 video frames/image) using an eight-bit A/D board (DT 2867, Data Translation) controlled by Axon Imaging Workbench software on a 486 microcomputer (Atman). Acquisition rates ranged from one image every 10–30 sec. Changes in F_{580} were quantified by averaging the eight-bit pixel values (between 0 and 255) within a box overlapping an image of the astrocyte cell body. Because calcium orange is a nonratiometric dye with a narrow dynamic range (approximately four- to fivefold increase in F_{580} from 0 to 39.8 μM $[Ca^{2+}]$, Molecular Probes Handbook of Fluorescent Probes and Research Chemicals 1994), fluorescence measurements were not calibrated for absolute or relative changes in $[Ca^{2+}]_i$. A slow decrease in baseline fluorescence was observed in many experiments presumably because of continuous diffusion of dye through gap junctions into surrounding astrocytes (Duffy and MacVicar, 1995). For accurate determination of response duration, this decrease was compensated by a linear extrapolation:

$$\Delta F = \{[- (F_n - F_1)/(t_n - t_1)]t_i + F_i\} - F_1,$$

where F_i is the uncorrected fluorescence at time t_i, and F_1 and F_n are the first and last F values at times t_1 and t_n.

Intracellular calcium measurements from acutely isolated astrocytes and neurons. Astrocytes and neurons were acutely isolated as described previously (Duffy and MacVicar, 1994b), with a few modifications. Hippocampal slices (500 μm) were prepared and incubated for 1–2 hr in control aCSF. Slices were placed on nylon mesh at the liquid–gas interface in an aerated (95% O_2/5% CO_2) interface chamber. The chamber well contained 20 ml of aCSF plus 2 mg/ml of the protease papain, 0.24 mg/ml of the papain activator L-cysteine, and 1 mM of the glutamate receptor antagonist kynurenic acid. The enzyme solution was stirred using a magnetic stirrer (Bellco) for 1 hr at 32°C. After digestion, slices were washed several times in aCSF. The CA1–CA3 regions from two or three enzyme-treated slices were dissected free using a scalpel blade, and these sections were added to Dulbecco's Modified Eagle's Medium containing 16.6 μM Fura-2 AM, predissolved in dimethyl sulfoxide (DMSO) with 10% pluronic acid, plus 1 mM kynurenic acid. Hippocampal cells were acutely isolated by repeated passage of tissue through fire-polished glass pipettes. The resultant cell suspension was allowed to settle for ~20 min on poly-L-lysine-coated coverslips. Cells were superfused with aCSF containing 40 mM sucrose at 35 ± 1°C. Hypoxic–hypoglycemic conditions were initiated as described for hippocampal slice experiments. Calcium imaging methodology was similar to that used for calcium orange experiments, except Fura-2 excitation filters (340, 360, 380 nm; Omega Optical) were mounted on a Lambda-10 rotating filter changer (Sutter Instruments) interposed between the shutter and the microscope.

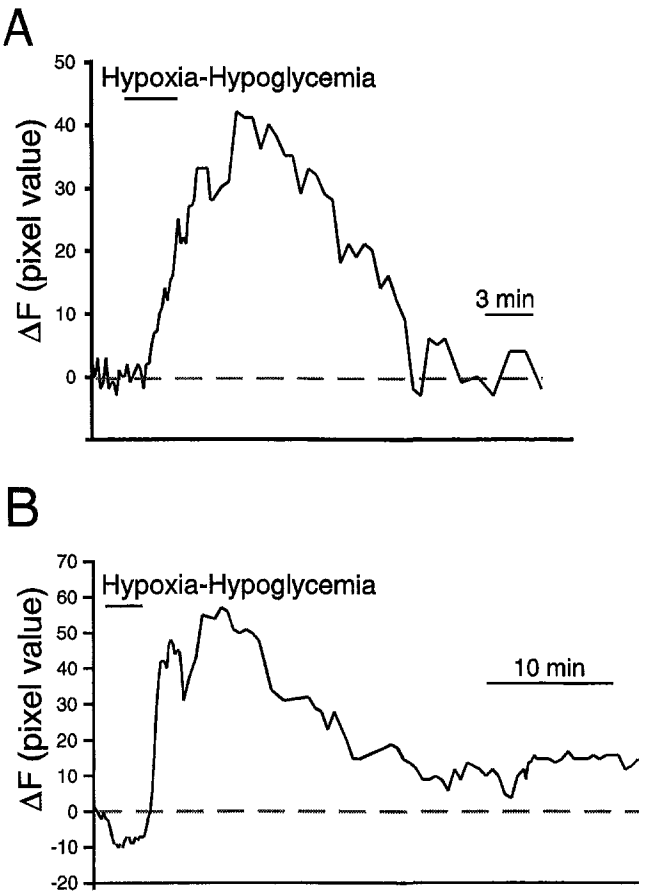

Figure 3. Variability in the duration of $[Ca^{2+}]_i$ elevations produced by hypoxia–hypoglycemia. *A*, A 3.3 min episode evoked a reversible increase of ~17 min duration, whereas in another slice (*B*) an episode of the same duration produced a maintained increase (>40 min).

RESULTS

Effect of simultaneous hypoxia and hypoglycemia on astrocyte intracellular calcium

Astrocytes in CA1–CA3 stratum radiatum were identified by their characteristic membrane potential (75 ± 3 mV, n = 36), absence of impalement- or stimulus-evoked action potentials (Fig. 1*A*), and extensive dye-coupling after 10–30 min ionotophoretic injection of calcium orange (Fig. 1*B,C*). To examine the effects of ischemia on astrocyte $[Ca^{2+}]_i$, microfluorometric $[Ca^{2+}]_i$ measurements were performed during superfusion of glucose-free aCSF deoxygenated with 95% N_2/5% CO_2. Under these hypoxic–hypoglycemic conditions, an increase in calcium orange fluorescence, indicating an increase in $[Ca^{2+}]_i$, was observed in most slices within 7.5 min (n = 11/14). A typical experiment is shown in Figure 2. After 5 min of hypoxia–hypoglycemia, a sudden rise in fluorescence emission was observed. The duration of hypoxia–hypoglycemia required to initiate these $[Ca^{2+}]_i$ increases ranged from 3.3 to 7.5 min (5.5 ± 1.4 min), after which $[Ca^{2+}]_i$ rose to a peak within 1.5 to 4 min (average 2.5 min).

After reoxygenation, $[Ca^{2+}]_i$ remained elevated for a highly variable period of time. The shorter $[Ca^{2+}]_i$ elevations ranged from 5 to 17 min in duration (5/11 slices, Fig. 3*A*). The remaining responses were much longer, lasting at least 30–60 min (Fig. 3*B*). We could not accurately determine the exact duration of these longer $[Ca^{2+}]_i$ responses because of uncertainty in extrapolating the baseline shift over long intervals (see Materials and Methods). Response duration was inversely related to the ischemic episode required to evoke a $[Ca^{2+}]_i$ signal. The minimum ischemic period required was 4.6 ± 0.7 min (range, 3.3–5.5 min), for the longest responses (17 min to >1 hr), compared with 7.0 ± 0.6 min for the shortest (4.0–7.5 min) responses. A minority of the slices tested (3/14) were unresponsive to 10 min of hypoxia–hypoglycemia (the longest duration tested).

The role of calcium release from intracellular stores

To examine the source of this $[Ca^{2+}]_i$ elevation, we first examined the dependence on $[Ca^{2+}]_o$. A $[Ca^{2+}]_i$ increase was still observed in response to hypoxia–hypoglycemia after washout of extracellular calcium (Fig. 4, six responses in four slices). Unlike $[Ca^{2+}]_i$ increases in the presence of $[Ca^{2+}]_o$, these responses were always brief (mean duration, 16.8 min) and of relatively constant duration (range, 16.5–17.5 min). A second ischemic episode in 0 $[Ca^{2+}]_o$ also induced a $[Ca^{2+}]_i$ increase (n = 2/2; 13.5 and 12.25 min).

The role of voltage-dependent calcium influx

Intracellular recordings in hippocampal slices from astrocytes, which were not dye-loaded, revealed a depolarization in response to hypoxia and hypoglycemia (Fig. 5; peak ΔE_m = 51 ± 16 mV, range, 30–70 mV, n = 9). This measurement of peak depolarization underestimated the true value, however, because electrode penetration usually was lost during the most rapid phase of depolarization probably because of tissue swelling (Andrew and MacVicar, 1994).

The observation that hypoxia–hypoglycemia both depolarized astrocytes and increased $[Ca^{2+}]_i$ suggested that another mechanism mediating these responses was influx through voltage-gated Ca channels (Duffy and MacVicar, 1994b). This putative ischemia-induced Ca^{2+} influx could not be isolated from the total $[Ca^{2+}]_i$ response because this would require either specific blockade of astroglial internal Ca^{2+} stores or quantitative comparison of responses in the presence and absence of $[Ca^{2+}]_o$. This later option was not possible using the nonratiometric calcium orange, and iontophoretic injection of Fura-2 did not yield satisfactory resolution of individual astrocytes over background autofluorescence. Thus, ischemic Ca^{2+} influx was studied indirectly, first by establishing the presence of voltage-dependent Ca^{2+} influx in astrocytes under normoxic conditions and subsequently by demonstrating that such influx is operational under ischemia.

To investigate the role of voltage-gated Ca^{2+} influx independently from other putative ischemia-specific $[Ca^{2+}]_i$ mobilizing pathways, we first measured E_m and $[Ca^{2+}]_i$ during elevation of $[K^+]_o$ under normoxic conditions. Increasing the $[K^+]_o$ from 5 to 50 mM for 3 or 4 min depolarized astrocytes by 51 ± 4 mV (average of eight cells at 22–24°C). In dye-loaded slices, superfusion of 50 mM $[K^+]_o$ aCSF also caused a $[Ca^{2+}]_i$ increase in astrocytes (20 of 24 slices, Fig. 6). In those slices that showed a $[Ca^{2+}]_i$ increase (80% of slices tested), such $[Ca^{2+}]_i$ signals were observed in all visible astrocytes (66/66 cells from 20 slices). These increases followed a nearly synchronous time course in neighboring cells (19/20 slices, 61/66 cells). Responses were never observed after removal of $[Ca^{2+}]_o$ (Fig. 6*A*; 0/5 slices at 22–24°C, 0/3 slices at 35 ± 1°C). The Ca channel blocker verapamil (70 μM), which blocks voltage-dependent Ca^{2+} influx in acutely isolated astrocytes (Duffy and MacVicar, 1994b), also inhibited $[Ca^{2+}]_i$ elevations induced by 50 mM $[K^+]_o$ in astrocytes within slices (n = 5/5 slices, data not shown). In contrast, elevation of $[K^+]_o$ to 15 mM (4–7 min), which corresponds to the ceiling $[K^+]_o$ increases ob-

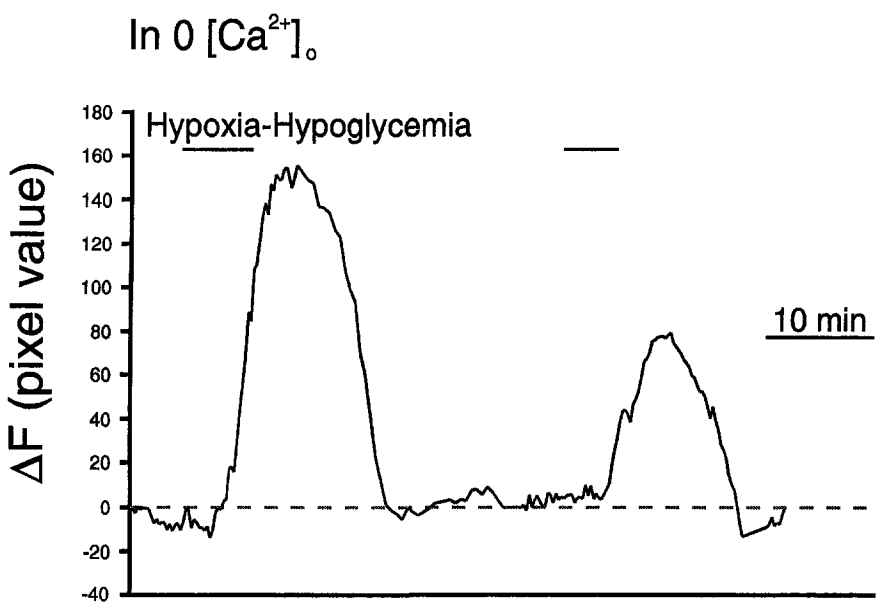

Figure 4. Two episodes of hypoxia–hypoglycemia (6.5 and 5.0 min) induced $[Ca^{2+}]_i$ increases in the absence of $[Ca^{2+}]_o$.

served *in situ* during intense neuronal activity, did not increase $[Ca^{2+}]_i$ (Fig. 6*B*). Rather, a transient decrease in calcium orange fluorescence was observed, likely reflecting dilution of dye concentration concomitant with $[K^+]_o$-dependent astroglial swelling (Walz, 1989). In all cases, subsequent application of 50 mM $[K^+]_o$ did induce a response (n = 6/6 slices, n = 4/4 at 22–24°C, n = 2/2 at 35 ± 1°C). Response duration was markedly temperature-sensitive; at 35 ± 1°C, 2.5–3.0 min of 50 mM $[K^+]_o$ evoked $[Ca^{2+}]_i$ increases >35 min in duration (Fig. 6*A*, n = 3/3), whereas 4 min of 50 mM $[K^+]_o$ at 22–24°C triggered biphasic $[Ca^{2+}]_i$ increases lasting 13.65 ± 4.05 min (Fig. 6*B*, n = 5).

Anoxic depolarization and normoxic spreading depression cause astrocyte volume increases (Walz, 1989; Landis, 1994), and this response can promote Ca^{2+} influx into cultured glia by activating stretch-sensitive Ca channels (Puro, 1991). To determine whether cell swelling in the absence of depolarization can influence $[Ca^{2+}]_i$ in hippocampal astrocytes that have not been cultured, hypo-osmotic aCSF (−60 to −120 mOsm) was superfused onto dye-loaded slices. In response to 3–5 min of hypo-osmolar aCSF, $[Ca^{2+}]_i$ did not increase; rather, a decrease in fluorescence emission was observed (Fig. 7; n = 5/5) possibly because of dilution of the intracellular dye concomitant with the cell volume increase.

Ischemic responses in acutely isolated astrocytes and neurons

Because *in vitro* ischemia and $[K^+]_o$ elevation in intact tissue depolarize both astrocytes and neurons, it is difficult to differentiate the direct effect of depolarization on astroglial $[Ca^{2+}]_i$ from effects mediated by signals (i.e., neurotransmitters) released from adjacent neurons. We therefore measured ischemic $[Ca^{2+}]_i$ responses in acutely isolated astrocytes and neurons. In contrast to the rapid $[Ca^{2+}]_i$ elevations evoked in astrocytes within brain slices, hypoxia–hypoglycemia triggered slowly developing increases in the basal $[Ca^{2+}]_i$ of acutely isolated astrocytes (Fig. 8). The average increase in Fura-2 ratio $R_{340/380}$ was 6% after 5 min, 12% after 10 min, and 15% after 12–15 min continuous *in vitro*

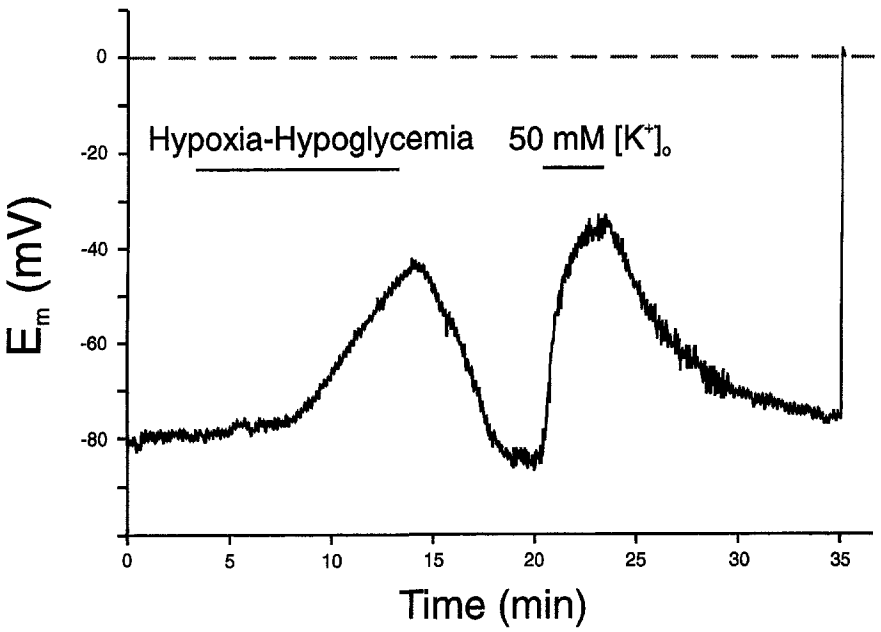

Figure 5. Hypoxia–hypoglycemia depolarized astroglia within hippocampal slices. Ten minutes of hypoxia–hypoglycemia induced a large depolarization, which was approximately equivalent in magnitude to a depolarization evoked by subsequent addition of 50 mM $[K^+]_o$.

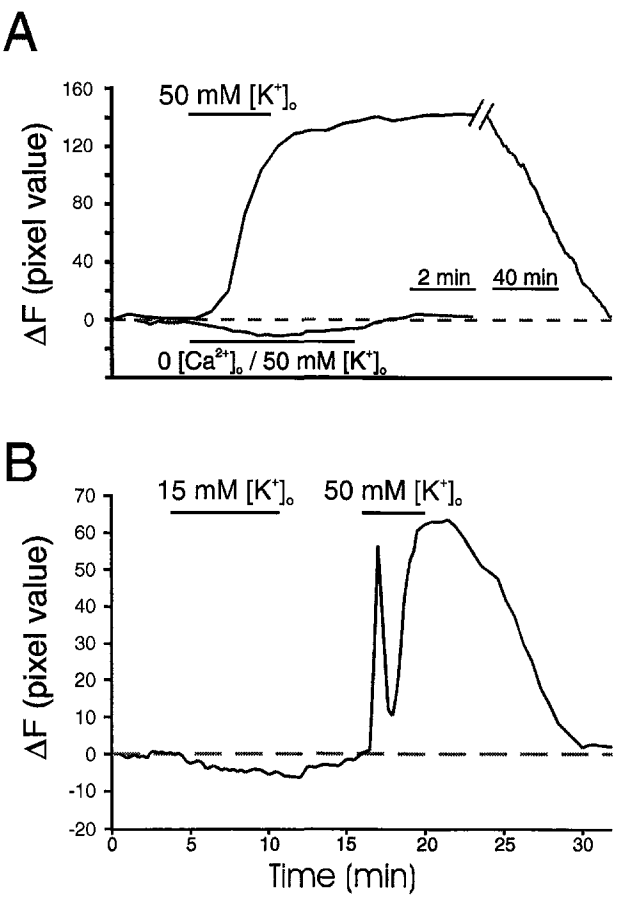

Figure 6. Elevating $[K^+]_o$ from 5 mM to 50 mM increased astrocyte $[Ca^{2+}]_i$. *A*, In response to 3 min superfusion of 50 mM $[K^+]_o$, a $[Ca^{2+}]_i$ increase was observed in 2 mM $[Ca^{2+}]_o$ (*top*), but not in $[Ca^{2+}]_o$-free saline (*bottom*). T = 35°C. *B*, Superfusion of 15 mM $[K^+]_o$ for 7 min did not increase $[Ca^{2+}]_i$; rather, a modest fluorescence decrease was observed. Subsequent addition of 50 mM $[K^+]_o$ for 4 min caused a $[Ca^{2+}]_i$ increase (T = 22°C).

ischemia. This increase was completely reversible by removal of $[Ca^{2+}]_o$ (Fig. 8*A*; n = 8/8 cells), indicating that ischemia caused a small net increase in resting Ca^{2+} influx. Depolarization by 45–90 sec superfusion of 50 mM $[K^+]_o$ during prolonged hypoxia–hypoglycemia (7.5–34 min) evoked more rapidly developing and larger $[Ca^{2+}]_i$ increases (14/14 cells; average ΔR of 48%), which were also blocked by $[Ca^{2+}]_o$ removal (n = 3/3 cells; Fig. 8*B,C*). Average response magnitude was not significantly different from preischemic $[K^+]_o$-evoked $[Ca^{2+}]_i$ responses (average $\Delta R_{ischemia}$ 95% of control, n = 9 cells). Astroglial Ca channels, therefore, are functional under ischemia, but significant Ca^{2+} influx requires additional depolarization.

In contrast to acutely isolated astrocytes, acutely isolated pyramidal neurons showed large, irreversible $[Ca^{2+}]_i$ responses after brief (1.5–6.5 min) episodes of hypoxia–hypoglycemia (n = 10/12 cells, ΔR of 152%), which were not reversed by removal of $[Ca^{2+}]_o$ (Fig. 9; n = 5/5 cells). These differences in the ischemic $[Ca^{2+}]_i$ responses of acutely isolated neurons, isolated astrocytes, and astrocytes within intact tissue are summarized in Figure 10. In some experiments, coisolated astrocytes and neurons within the same field of view (100–300 μm apart) were imaged simultaneously during hypoxia–hypoglycemia. These experiments demonstrate the marked differences in intrinsic ischemic-sensitivity under identical conditions (Fig. 10*A*, n = 3). Finally, in Figure 10*B*, the ischemic response of an astrocyte within a slice is compared with an acutely isolated cell to demonstrate the dramatically different kinetics of the basal $[Ca^{2+}]_i$ increase.

DISCUSSION

Brief episodes of simultaneous hypoxia and hypoglycemia, a common *in vitro* model of ischemia, increased $[Ca^{2+}]_i$ in rat hippocampal astrocytes by promoting voltage-dependent Ca^{2+} influx and Ca^{2+} release from internal stores. Intracellular calcium increases have been measured previously in cultured astrocytes during anoxia (Haun et al., 1992), but our results represent the first direct measurements of this response in intact tissue that has not been cultured. In cultured astrocytes, moreover, significant $[Ca^{2+}]_i$ elevations and cellular dysfunction (possibly mediated by $[Ca^{2+}]_i$) require many hours of continuous hypoxia and hypoglycemia (Yu et al., 1989; Haun et al., 1992), although we report that $[Ca^{2+}]_i$ signaling is a predominant component of the very early ischemic response. Similar to results in culture, acutely isolated astrocytes showed no significant increase in basal $[Ca^{2+}]_i$ during brief hypoxia–hypoglycemia, indicating that extracellular messengers released from ischemic tissue stimulate these responses in intact brain.

The mechanisms of astroglial $[Ca^{2+}]_i$ increases during hypoxia–hypoglycemia

Increases in $[Ca^{2+}]_i$ were induced by *in vitro* ischemia in the absence of $[Ca^{2+}]_o$, indicative of Ca^{2+} release from internal stores. Whether this release was mediated by activation of receptors linked to inositol triphosphate production or by Ca^{2+} store disruption because of ATP depletion is uncertain. Ischemia could induce the release of a large number of transmitters or messengers, which mobilize Ca^{2+} stores in hippocampal astrocytes. Our observations that the transient $[Ca^{2+}]_o$-independent responses observed in slices were not seen in isolated astrocytes suggest the influence of neurotransmitters or intercellular messengers, although the identity of these messengers is unknown. Glutamate is one obvious candidate, as ischemia is associated with large elevations in extracellular glutamate (Szatkowski and Attwell, 1994), but glutamate receptor agonists did not increase $[Ca^{2+}]_i$ of astrocytes within hippocampal slices (Duffy and MacVicar, 1995). Alternatively, reductions in intracellular ATP also could result in rapid efflux of stored Ca^{2+} because these stores are maintained by ATPase activity and the stored Ca^{2+} is in rapid equilibrium with the cytosol (Pozzan et al., 1994).

The observation that $[K^+]_o$ depolarization induced Ca^{2+} influx suggests that the ischemic depolarization also promotes influx. However, Ca^{2+} currents can be suppressed by anoxia (Krnjevic and Leblond, 1989; Young and Somjen, 1992), so we examined $[K^+]_o$-induced Ca^{2+} influx in isolated astrocytes in which pre- and postischemic $[Ca^{2+}]_i$ could be quantified and compared. Under these conditions, significant $[Ca^{2+}]_i$ elevations were observed, but only upon $[K^+]_o$ depolarization. This strongly suggests that Ca^{2+} influx in intact tissue is triggered by depolarization concomitant with deregulation of $[K^+]_o$ rather than by an intrinsic depolarizing response. Other routes of Ca^{2+} influx also are possible. For example, reoxygenation could lead to the formation of free radicals, which promote Ca^{2+} release or inhibit $[Ca^{2+}]_i$ buffering. We also have shown that GABA promotes Ca^{2+} influx via $GABA_A$ receptor-mediated depolarization (Fraser et al., 1995), suggesting that nonglutamatergic transmitters may contribute to the observed $[Ca^{2+}]_i$ response. Alternatively, internal Na^+ accumulation

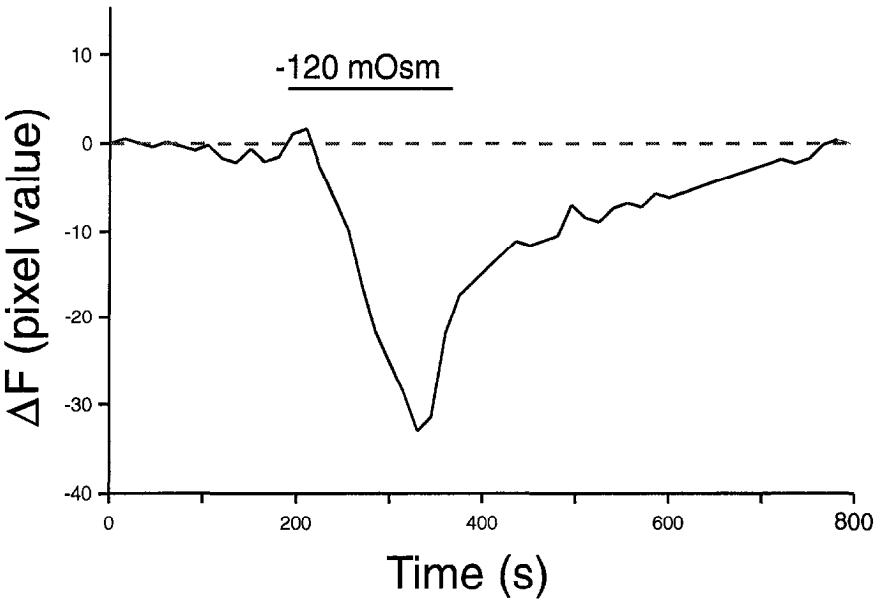

Figure 7. Hypo-osmotic aCSF did not increase $[Ca^{2+}]_i$. Reducing the osmolarity from 290 to 170 mOsm caused a decrease in fluorescence emission (ΔF).

could lead to Ca^{2+} influx through reversal of the Na^+–Ca^{2+} exchange (Waxman et al., 1991; Kim-Lee et al., 1992).

Ischemic $[Ca^{2+}]_i$ increases in astrocytes versus neurons

The electrophysiological and biochemical responses of neurons to ischemia have been documented extensively, and it is widely held that a sustained increase in $[Ca^{2+}]_i$ (so-called $[Ca^{2+}]_i$ deregulation) is one important factor mediating neuronal damage (for reviews, see Orrenius et al., 1989; Siesjo and Bengtsson 1989; Choi, 1990; Nicotera et al., 1992; Martin et al., 1994). However, many studies of neuronal $[Ca^{2+}]_i$ during ischemia have measured $[Ca^{2+}]_i$ in tissue bulk-loaded with Ca^{2+} indicators. The results presented here indicate that these measurements probably include both a neuronal and an astroglial component. Moreover, these components are not easily separable because they share similar kinetics. For instance, the duration of *in vitro* ischemia required to induce astroglial $[Ca^{2+}]_i$ responses falls within the range that induces anoxic depolarizations (Clark and Rothman, 1987; Grigg and Anderson, 1990; Rader and Lanthorn, 1989) and $[Ca^{2+}]_i$ increases in neurons (Silver and Erecinska, 1990; Lobner and Lipton, 1993; Mitani et al., 1993). Moreover, ischemic $[Ca^{2+}]_i$ responses in both astrocytes (reported here) and neurons (Katchman and Hershkowitz, 1993; Lobner and Lipton, 1993; Mitani et al., 1993) involve Ca^{2+} influx and release from internal stores. The quantitative contribution made by astrocytes to the total tissue $[Ca^{2+}]_i$ response would depend on the relative volume of the astroglial compartment. On average, astroglia comprise one-third of the cellular volume of gray matter (Pope, 1978). In areas such as the stratum radiatum, where neuronal somata are sparse, the contribution of astrocyte $[Ca^{2+}]_i$ signals could be substantial.

Although both neurons and astrocytes display ischemic $[Ca^{2+}]_i$ elevations, there are dramatic differences in postischemic viability. A few minutes of focal ischemia can trigger neuronal degeneration, whereas significant astroglial death requires many hours (Yu et al., 1989; Haun et al., 1992). The viability of isolated neurons was not directly assessed in this study, although we noted a significant reduction in Fura-2 fluorescence 10–15 min after the initial $[Ca^{2+}]_i$ increase, suggesting loss of membrane integrity (our unpublished observations). Neither dye leakage nor irreversible

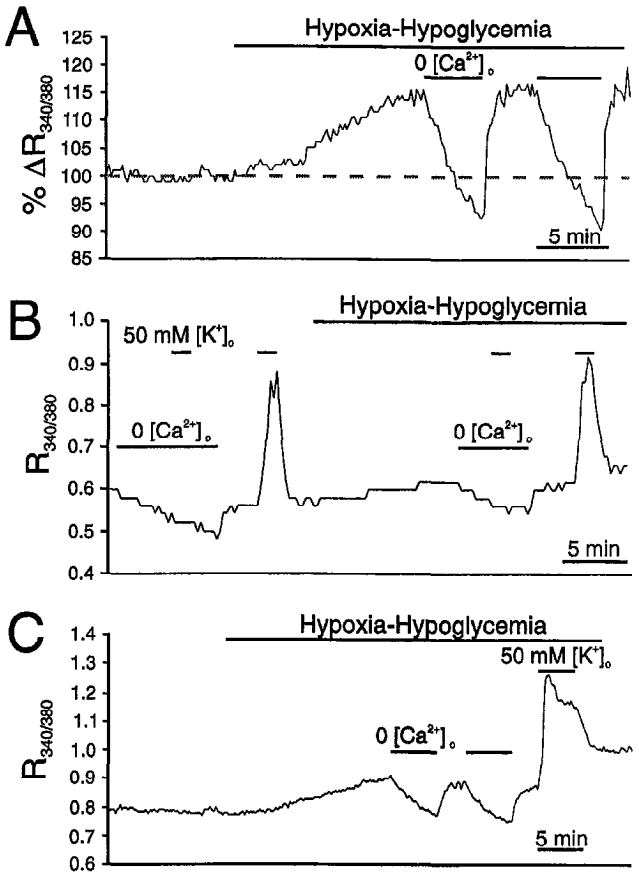

Figure 8. Effect of hypoxia–hypoglycemia on basal and $[K^+]_o$-evoked $[Ca^{2+}]_i$ increases in acutely isolated astrocytes loaded with Fura-2. *A*, Hypoxia–hypoglycemia caused a modest increase in basal $[Ca^{2+}]_i$, as revealed by the change in Fura-2 ratio $R_{340/380}$ of 14% over 13 min. This increase was reversed by $[Ca^{2+}]_o$ removal. Subsequent readdition of $[Ca^{2+}]_o$ resulted in re-elevation of $[Ca^{2+}]_i$. *B*, Superfusion of 50 mM $[K^+]_o$ increased $[Ca^{2+}]_i$ under normoxic conditions and after 20 min of hypoxia–hypoglycemia. No response was observed under either condition in the absence of $[Ca^{2+}]_o$. *C*, 50 mM $[K^+]_o$ still elicited a $[Ca^{2+}]_i$ increase after prolonged (34 min) hypoxia–hypoglycemia.

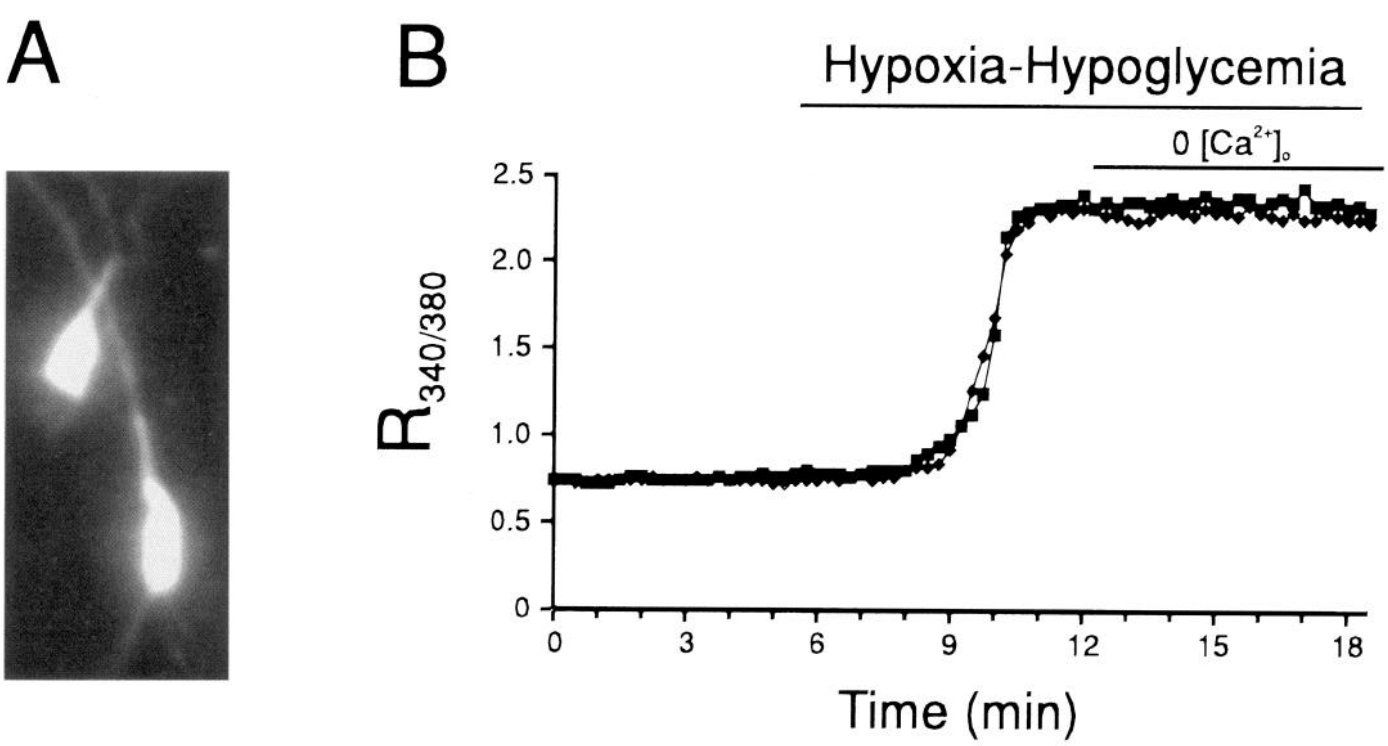

Figure 9. Hypoxia–hypoglycemia induced rapid $[Ca^{2+}]_i$ increases in Fura-2-loaded acutely isolated neurons, which were not reversed by removal of external $[Ca^{2+}]_o$. *A*, Fluorometric image of two closely spaced isolated pyramidal neurons. *B*, After 2.8 min of hypoxia–hypoglycemia, a rapid increase in basal $[Ca^{2+}]_i$ was initiated (ΔR of 221 and 214% in these two cells), which was not reversed by removal of $[Ca^{2+}]_o$.

$[Ca^{2+}]_i$ increases were observed in astrocytes even after very prolonged *in vitro* ischemia. It is reasonable, therefore, to speculate that the differences in viability of postischemic astrocytes and neurons relate to ischemic $[Ca^{2+}]_i$ metabolism. For example, $[Ca^{2+}]_i$ elevations in isolated ischemic astrocytes, but not in isolated neurons, were reversed by $[Ca^{2+}]_o$ removal. This suggests that neuronal Ca^{2+} efflux is markedly suppressed under hypoxic conditions, whereas astroglial Ca^{2+} extrusion is maintained at rates sufficient to buffer hypoxia-induced influx or release. Reduced neuronal Ca^{2+} efflux is likely given that the high density of voltage-gated Na channels and ionotropic transmitter receptors would result in intracellular Na^+ loading with subsequent suppression or reversal of Ca^{2+} extrusion via Na^+–Ca^{2+} exchange. Differences in the nature of internal Ca^{2+} stores also may contribute. In addition to the inositol triphosphate-sensitive store common to both astrocytes and neurons, hippocampal neurons express a $[Ca^{2+}]_i$-dependent Ca^{2+} release store (Simpson et al., 1995), which is either absent or of considerably lower capacity in astrocytes (Duffy and MacVicar, 1994b, 1995; Salter and Hicks, 1994). Thus, in neurons, Ca^{2+} influx may further augment Ca^{2+} release from internal stores leading to saturation of $[Ca^{2+}]_i$ buffering mechanisms and subsequent activation of catabolic processes (i.e., activation of Ca^{2+}-dependent proteases, lipases, and nucleases, and free-radical formation; for review, see Coyle and Puttfarcken, 1993).

Astroglial $[Ca^{2+}]_i$ during pathological and nonpathological $[K^+]_o$ elevations

During spreading depression (SD) *in vivo*, $[K^+]_o$ can be elevated to levels (50–80 mM; Somjen, 1979) sufficient to increase astroglial $[Ca^{2+}]_i$, as shown in this paper. SD also results in reactive transformation (Kraig et al., 1991), suggesting a link between these reactive responses and $[Ca^{2+}]_i$ signaling. It has been proposed that SD is propagated by regenerative intercellular $[Ca^{2+}]_i$ waves through astrocyte networks (Nedergaard, 1994; Parpura et al., 1994). If this is the case, then the present results indicate that one source of $[Ca^{2+}]_i$ is voltage-dependent influx. In contrast, elevating $[K^+]_o$ to 15 mM did not increase $[Ca^{2+}]_i$. This result implies that $[K^+]_o$ elevations resulting from intense neuronal activity, which normally do not exceed a ceiling level of 8–12 mM (Somjen 1979), do not directly trigger voltage-dependent Ca^{2+} influx into astrocytes. Voltage-gated Ca^{2+} influx, therefore, is unique to pathological conditions associated with deregulation of $[K^+]_o$.

Functional implications of astroglial $[Ca^{2+}]_i$ signaling during ischemia: possible roles in neuroprotection and neurotoxicity

The rapid changes in astroglial physiology after reoxygenation and SD, most notably a dramatic increase in protein synthesis (Gunn et al., 1990; Raley-Susman and Lipton, 1990; Petito et al., 1990; Bonthius and Steward, 1993; Hori et al., 1994), suggest the influence of early intracellular signals generated by these insults. Moreover, morphological transformation and proliferation, the hallmark reactive responses, can be initiated by stimuli that increase $[Ca^{2+}]_i$ or stimulate $[Ca^{2+}]_i$-sensitive enzymes (Harrison and Mobley, 1990; Puro and Mano, 1991; Yong, 1992), further supporting the involvement of $[Ca^{2+}]_i$ signaling in the initiation of reactive transformation.

Many postischemic responses could confer neuroprotection. Among the earliest ischemic responses is astrocytic swelling from K^+, Cl^-, and Na^+ uptake with obligatory water movement (Walz, 1989; Andrew and MacVicar, 1994; Landis, 1994). The ensuing regulatory volume decreases (RVDs) are dependent on increased $[Ca^{2+}]_i$ (Olson et al., 1990; Pierce and Politis, 1990; Bender and Norenberg, 1994) and subsequent activation of Ca^{2+}-dependent

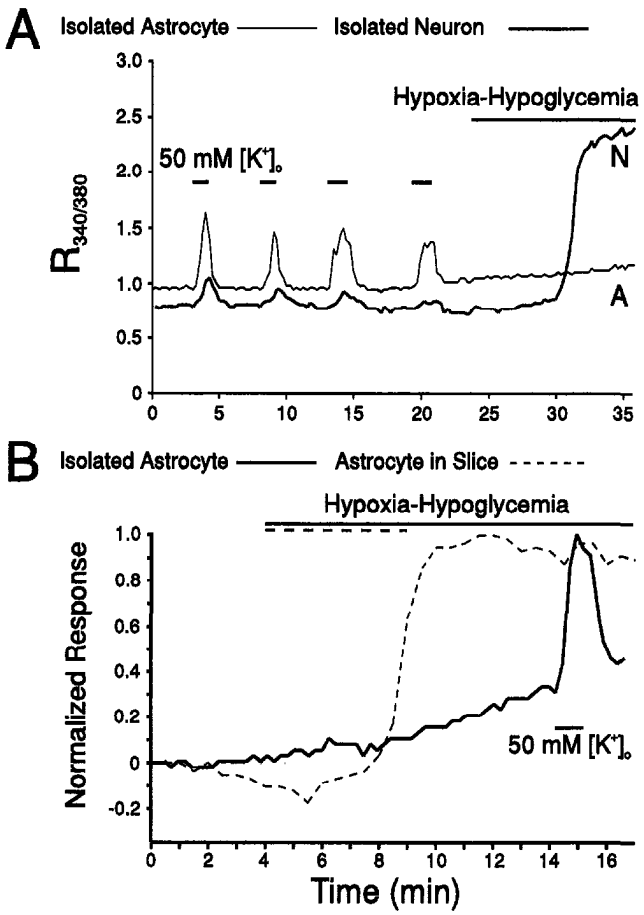

Figure 10. Comparison of ischemic $[Ca^{2+}]_i$ responses in coisolated hippocampal astrocytes and neurons (*A*) and between astrocytes in slice and in isolation (*B*). *A*, An acutely isolated astrocyte (*trace* marked *A*) and neuron (*trace* marked *N*) in the same field of view showed reversible $[Ca^{2+}]_i$ increases in response to multiple applications of 50 mM $[K^+]_o$. Subsequent hypoxia–hypoglycemia caused a rapid elevation in basal $[Ca^{2+}]_i$ in the neuron after 6.5 min (ΔR of 239%), whereas astrocytic basal $[Ca^{2+}]_i$ was not significantly affected (ΔR of 10% after 10 min hypoxia–hypoglycemia). *B*, A response, normalized to peak, from an astrocyte in slice (- - - -) is superimposed onto a response from an isolated astrocyte (——). After 5.5 min hypoxia–hypoglycemia, $[Ca^{2+}]_i$ of an astrocyte within a hippocampal slice rose rapidly to peak within 2 min (*dashed bar*). In contrast, $[Ca^{2+}]_i$ of the isolated astrocyte did not increase markedly until the cell was depolarized by application of 50 mM $[K^+]_o$.

K channels (Quandt and MacVicar, 1986; Butt et al., 1990; Olson et al., 1990). Thus, one purpose for these $[Ca^{2+}]_i$ transients might be to regulate volume recovery. This, in turn, could ameliorate neuronal pathology because astroglial swelling both directly causes the release of neurotoxic glutamate and aspartate (Kimelberg et al., 1990) and exacerbates the extracellular accumulation of excitotoxins by reducing the extracellular volume fraction (McBain et al., 1990). An interesting possibility is that swelling-induced activation of mechanosensitive Ca channels (Puro, 1991) or internal release (Charles et al., 1991) provides the RVD $[Ca^{2+}]_i$ signal. However, cell swelling alone did not increase $[Ca^{2+}]_i$ in acutely isolated cells (Duffy and MacVicar, 1994b) or in intact slices. In addition, $[Ca^{2+}]_i$ increases may reduce external glutamate through upregulation of glial glutamate transport (Flott and Seifert, 1991). Astroglial $[Ca^{2+}]_i$ signals also might trigger the release of auxiliary metabolites from astroglial glycogen stores (Phelps, 1972). Elevations in external $[K^+]_o$ can trigger glycogenolysis in both brain slices (Ververken et al., 1982; Hof et al., 1988) and cultured astrocytes (Cambray-Deakin et al., 1988). Loading of these stores can reduce neuronal death from hypoglycemia in neuron–astrocyte cocultures (Swanson and Choi, 1993) and possibly ischemic neuronal death *in vivo* (Swanson et al., 1990), whereas inhibition of glial glycogen synthesis or glucose uptake can potentiate hypoxic neuronal death *in vitro* (Virgin et al., 1991; Tombaugh et al., 1992). Finally, astroglial Ca^{2+} influx may reduce neurotoxicity simply by lowering $[Ca^{2+}]_o$.

Although there is much circumstantial evidence for a neuroprotective function of astroglial $[Ca^{2+}]_i$ increases, it is possible that, at least under some conditions, these signals are deleterious to ischemic astrocytes and neurons. Prolonged ischemia (hours) *in vitro* does result in astroglial death, and this can be partially reversed by Ca channel antagonists (Yu et al., 1989; Haun et al., 1992). Also, Ca^{2+} deposits within mitochondria, a possible sign of cytotoxic $[Ca^{2+}]_i$ increases, have been observed in astrocytes after ischemia *in vivo* (Dux et al., 1987). Finally, the increase in $[K^+]_o$ and the decrease in $[Na^+]_o$ associated with hypoxia can induce reverse glutamate transport (efflux) from glial cells (Szatkowski et al., 1990), which could exacerbate extracellular glutamate increases and contribute to neuronal death (Szatkowski and Attwell, 1994). The recent demonstration of glutamate receptor-dependent $[Ca^{2+}]_i$ signals in cultured neurons triggered by $[Ca^{2+}]_i$ waves through adjacent astrocytes (Parpura et al., 1994) suggests that astroglial glutamate efflux may be stimulated by $[Ca^{2+}]_i$.

Our results indicate that in response to ischemia, astrocytes exhibit a rise in $[Ca^{2+}]_i$ mediated by voltage-dependent influx and release from internal stores. The role of these signals is a matter of conjecture. Elucidation of these postischemic signaling pathways has gained added significance with recent evidence, indicating that astrocytes can exert neuroprotective effects on neurons in some *in vitro* models of ischemia or glutamate toxicity (Vibulsreth et al., 1987; Nieto-Sampedro et al., 1988; Mattson and Rychlik, 1990; Petito et al., 1992; Vaca and Wendt, 1992; Hori et al., 1994). The possibility that these signals activate endogenous neuroprotective mechanisms is an important area for future research.

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