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Astrocytic Glycogen Influences Axon Function and Survival during Glucose Deprivation in Central White Matter

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We tested the hypothesis that astrocytic glycogen sustains axon function during and enhances axon survival after 60 min of glucose deprivation. Axon function in the rat optic nerve (RON), a CNS white matter tract, was monitored by measuring the area of the stimulus-evoked compound action potential (CAP). Switching to glucose-free artificial CSF (aCSF) had no effect on the CAP area for ~30 min, after which the CAP rapidly failed. Exposure to glucose-free aCSF for 60 min caused irreversible injury, which was measured as incomplete recovery of the CAP. Glycogen content of the RON fell to a low stable level 30 min after glucose withdrawal, compatible with rapid use in the absence of glucose. An increase of glycogen content induced by high-glucose pretreatment increased the latency to CAP failure and improved CAP recovery. Conversely, a decrease of glycogen content induced by norepinephrine pretreatment decreased the latency to CAP failure and reduced CAP recovery. To determine whether lactate represented the fuel derived from glycogen and shuttled to axons, we used the lactate transport blockers quercetin, α-cyano-4-hydroxycinnamic acid (4-CIN), and *p*-chloromercuribenzene sulfonic acid (*p*CMBS). All transport blockers, when applied during glucose withdrawal, decreased latency to CAP failure and decreased CAP recovery. The inhibitors 4-CIN and *p*CMBS, but not quercetin, blocked lactate uptake by axons. These results indicated that, in the absence of glucose, astrocytic glycogen was broken down to lactate, which was transferred to axons for fuel.

Key words: astrocytes; α-cyano-4-hydroxycinnamate; glucose; hypoglycemia; lactate; p*-chloromercuribenzene sulfonic acid; quercetin; rat optic nerve*

The function of brain glycogen is not well understood. Glycogen turns over rapidly in the brain, however, and turnover is enhanced when adjacent neural activity is increased (Orkand et al., 1973; Pentreath and Kai-Kai, 1982; Swanson et al., 1992). It is appealing to imagine that glycogen might serve to provide fuel to the brain when glucose is in short supply. Indeed, astrocytic glycogen *in vitro* is degraded rapidly when glucose is withdrawn (Dringen et al., 1993), and glycogen falls rapidly *in vivo* during ischemia, with a time course that is closely related to the depletion of ATP and the accumulation of lactate (Swanson et al., 1989a). These observations are consistent with the action of glycogen as a fuel source during glucose shortage, but they do not prove this hypothesis. Glycogen content varies by a factor of two or more between brain regions [it is highest in the brainstem and cerebellum and lowest in the striatum and white matter (Swanson et al., 1989a)]. Energy metabolism also varies significantly between different brain regions (Sokoloff et al., 1977). Therefore, glycogen could be more protective against glucose depletion in some areas than in others.

Given all of the above, it is natural to wonder whether glycogen can enhance the survival and function of brain tissue in the absence of glucose. Surprisingly, only a single study, done on cultured cells, has tested this question. Neurons grown in astrocyte-rich cultures are injured less severely by glucose withdrawal than are neurons in astrocyte-poor cultures (Swanson and Choi, 1993). This benefit appears to derive from the presence of greater amounts of glycogen in the astrocyte-rich cultures. Depleting the astrocyte-rich cultures of glycogen negates the benefit (Swanson and Choi, 1993). Two possible mechanisms for this benefit, not mutually exclusive, were suggested but not tested: (1) astrocytes themselves use the energy from glycogen breakdown to prevent the accumulation of toxic levels of glutamate (removing it by a sodium gradient-dependent transporter), or (2) glycogen provides fuel to neurons to sustain their energy metabolism.

We have studied the role of astrocytic glycogen in an *in vitro* preparation of CNS white matter, the isolated rat optic nerve (RON). An advantage of this preparation is that function can be monitored continuously. Optic nerve function persists for ~30 min in the absence of glucose (Ransom and Fern, 1997; Fern et al., 1998), suggesting the presence of an intrinsic energy reserve such as astrocytic glycogen. It also is known that the optic nerve, like other neural tissues, can survive on substrates other than glucose, making it feasible that a breakdown product of glycogen other than glucose could mediate the energy transfer between astrocytes and axons (Schurr et al., 1988; Larrabee, 1995; Ransom and Fern, 1997). We tested the hypothesis that axon function and survival depend on astrocytic glycogen when glucose is withdrawn. Our results indicate that glycogen content strongly affected the duration of function and survival of axons after glucose removal and that lactate was probably the molecule that shuttled from astrocytes to axons to mediate energy transfer.

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MATERIALS AND METHODS

Preparation. Long–Evans rats were anesthetized deeply with CO_2 and then decapitated. The optic nerves were exposed, gently freed from their dural sheaths, and placed in an interface perfusion chamber (Medical Systems, Greenvale, NY) (see also Stys et al., 1990). Nerves were maintained at 37°C and perfused with artificial CSF (aCSF) that contained (in mM): 153 Na^+, 3 K^+, 2 Mg^{2+}, 2 Ca^{2+}, 143 Cl^-, 26 HCO_3^-, 1.25 HPO_4^{2-}, and 10 glucose. The aCSF was bubbled with a 5% CO_2-containing gas mixture (95% N_2/5% CO_2) to maintain the pH at 7.45. The tissue was oxygenated by a humidified gas mixture (95% O_2/5% CO_2) that flowed over its surface; anoxia was achieved by switching to an O_2-free mixture (95% N_2/5% CO_2).

Suction electrodes filled with aCSF (of the same composition as the test perfusate for each experiment) were attached to the nerve for stimulation and recording of the compound action potential (CAP) after the nerves were given a 60 min equilibration period in control aCSF. Stimulus strength was adjusted to evoke the maximum amplitude CAP and then was increased another 25% to ensure that stimulus strength was always supramaximal.

Data were acquired online (Digidata 1200A, Axon Instruments, Foster City, CA) with proprietary software (Axotape, Axon Instruments). CAP area was calculated with Clampfit (Axon Instruments).

Curve fitting. To standardize data interpretation, we used a mathematical approach to analyze CAP area. This approach is based on the Boltzmann equation because, as illustrated in Figure 1*C*, plotting CAP area against time during glucose removal (our standard insult) resulted in a trace that can be resolved into two sigmoidal curves, each of which can be fit by a Boltzmann function, one with a negative (falling) slope and the other with a positive (rising) slope. Our goal was to use the Boltzmann equation to define precisely the point of CAP decline and the maximum amount of CAP recovery (see Fig. 1*C*). The Boltzmann relationship is described by:

$$y = \frac{max}{1 - \exp\left(\frac{V - t}{k}\right)},$$

where y is the area under the CAP curve, max is the maximum value of the described sigmoidal (designated max_1 for the first sigmoidal, of negative slope, and max_2 for the second sigmoidal, of positive slope), V is the time at which the CAP area is 50% of max, t is the time, and k is the slope at point V. It is important to note that max_1 and max_2 are not necessarily the highest CAP area values from the baseline and recovery periods, respectively, but rather are the maximum values of the described sigmoidals. The minimum value for any data set is defined by the function as zero. The solid line superimposed on the data set shown in Figure 1*C* was generated by fitting the Boltzmann equation to the data. The break in the curve, indicated by the downward-pointing vertical arrow, identifies the point at which the equation has identified the zero point. The equation is applied separately to the second curve (of positive slope). In this case the maximum point as determined by curve fitting is represented by max_2, which describes CAP recovery. When this equation was applied to every data set, it was possible to calculate the latency of onset of CAP decline, defined as $t = 0.95{\cdot}max_1$, and CAP recovery, defined as $(max_2/max_1) \times 100\%$.

Transmission electron microscopy. Adult male Long–Evans rats were anesthetized deeply with ketamine/xylazine (40/2.5 mg/kg of body weight, i.p.) and perfused transcardially, first with a PBS solution and then with a fixative solution containing 2% paraformaldehyde and 2% glutaraldehyde in 0.14 M phosphate buffer, pH 7.4. Optic nerves were freed carefully and placed in fresh fixative overnight at 4°C. Then the tissue was rinsed several times in 0.14 M phosphate buffer, post-fixed in 1% OsO_4 and 1.5% potassium ferrocyanide in 0.14 M phosphate buffer for 3 hr at 4°C, and rinsed several times in phosphate buffer. The nerves were dehydrated in a graded ethanol series and embedded in Epon. Silver-gray sections were cut with a Reichardt Ultracut E and contrasted with uranyl acetate and lead citrate.

Glycogen and protein assays. Nerves were placed immediately in 3 ml of ice-cold 85% ethanol/15% 30 mM HCl. This instantly stops glycogen metabolism. The tissue (in solution) was stored at −20°C until assays were performed. Assays were performed as previously described (Swanson and Choi, 1993). Briefly, the nerves in the ethanol/HCl solution were warmed to room temperature, and the tissue was agitated gently for several hours to permit egress of all glucose (glucose is soluble in this solution, but glycogen is not). Each determination required four optic nerves (~8 mg of tissue total). The nerves were transferred to 0.3 ml of 30 mM HCl and sonicated to suspension. Then 50 μl of the suspension was removed and added to 200 μl of 0.1N NaOH for protein assay by using the Lowry method, in triplicate (Lowry et al., 1951). The remainder was divided into two 100 μl fractions. Glycogen was determined by the amyloglucosidase method of Passonneau and Lauderdale (1974). Amyloglucosidase completely hydrolyzes glycogen to glucose. One of the two 100 μl fractions (fraction A) was treated with amyloglucosidase, and the other (fraction B) was not. Then the glucose in both fractions was quantified by the glucose-6-phosphate dehydrogenase/NADP fluorescence method. Glucose in fraction B, which reflects endogenous true glucose in the nerves, was subtracted from glucose in fraction A, which reflects the sum of endogenous glucose and glucose derived from glycogen hydrolysis, to yield glycogen expressed as glucosyl equivalents. In practice, the soaking of the nerves in the ethanol/acid solution removes all detectable glucose such that glucose measured in fraction B was negligible, and all of the glucose measured in fraction A reflects hydrolyzed glycogen. Standards were prepared either from glucose or from rabbit liver glycogen after desiccation at 120°C. These are found to be equivalent, i.e., the desiccated glycogen digested with amyloglucosidase yields almost exactly the predicted amount of glucose.

Data analysis. Data are presented as means and SEM. Significance was determined by ANOVA with Tukey's post-test, where $p < 0.05$ was taken to indicate statistical significance.

RESULTS

The effects of glucose deprivation on CAP area in adult RONs are shown in Figure 1. CAPs were evoked every 30 sec. During a 60 min period of glucose withdrawal the CAP was maintained, on average, for 28.8 ± 2.1 min ($n = 15$) before it began to fail (Fig. 1*A*). It fell rapidly from that point to zero. The CAP recovered to an average of 45.3 ± 3.7% ($n = 15$) of the control CAP after a 60 min recovery period in normal aCSF (i.e., containing 10 mM glucose), indicating that irreversible injury had occurred. This agreed with previously published results (Ransom and Fern, 1997). Figure 1*B* shows representative CAPs from one of the nerves represented in Figure 1*A* before the removal of glucose (*a*), at the conclusion of 60 min of glucose deprivation (*b*), and after maximum recovery (*c*). The pattern of CAP recovery shown here was typical; the first peak of the CAP was best preserved. This suggested relative preservation of the larger-diameter axons, but further morphological analysis would be necessary to confirm this. To quantify the effects of glucose withdrawal on the CAP, we adopted a curve-fitting protocol to standardize the analysis of latency to CAP decline and CAP recovery magnitude (Fig. 1*C*; see Materials and Methods for details).

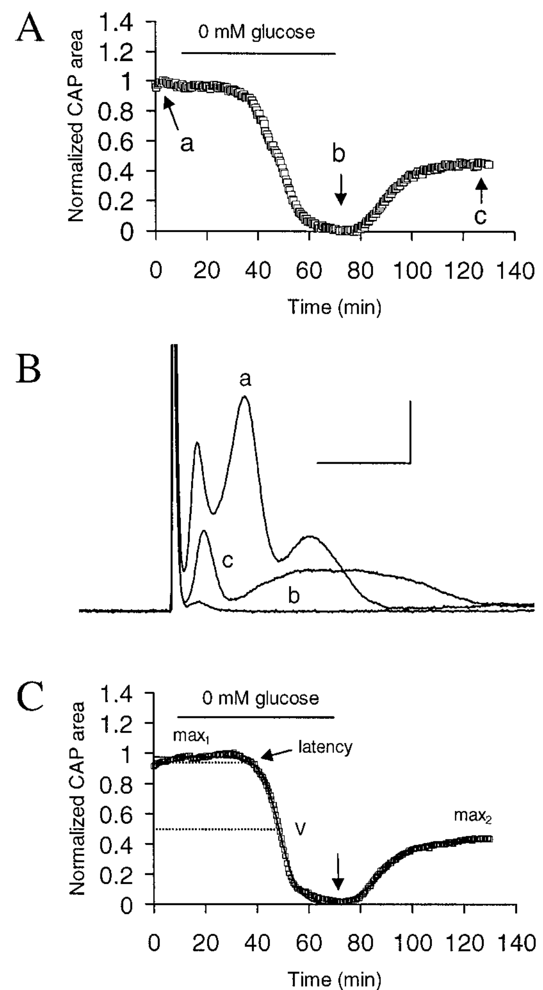

Figure 1. Effects of glucose withdrawal for 60 min on the rat optic nerve CAP area. *A*, A 60 min period of glucose withdrawal caused failure of the CAP after ~30 min and resulted in incomplete CAP recovery. Each *symbol* represents average CAP area (evoked every 30 sec; $n = 15$). *B*, Representative CAPs recorded from one of the nerves averaged in *A*. The recordings were taken at the time points indicated (*a–c*). Calibration: 0.5 mV, 1 msec. *C*, Representative trace from an individual nerve included in *A* to illustrate the curve-fitting protocol that was used to quantify latency and recovery.

Ultrastructural identification of astrocytic glycogen in the RON

Electron microscopy studies performed on perfusion-fixed RONs (Fig. 2) showed granules of glycogen located within most astrocytes. No glycogen was seen in axons or oligodendrocytes. No

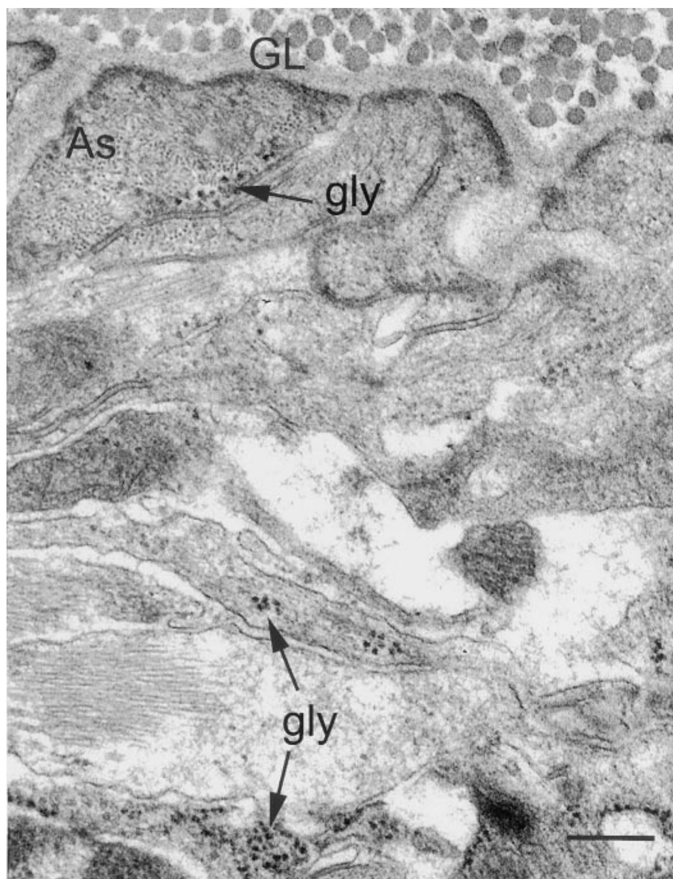

Figure 2. Ultrastructural evidence of glycogen deposition in astrocytes in adult rat optic nerve. Electron micrograph shows accumulations of glycogen granules (*gly*), which are ~20–25 nm in diameter, within processes of astrocytes (*As*) at the glia limitans (*GL*). Glycogen granules were not seen in axons nor in oligodendrocytes. Scale bar, 0.25 μm.

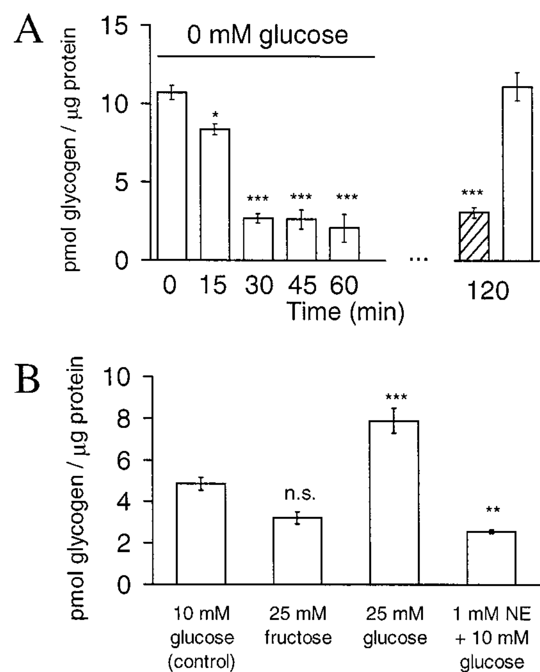

Figure 3. Glycogen content of rat optic nerves after glucose deprivation and pharmacological manipulation. *A*, In the absence of glucose, glycogen content declined over time and reached a low stable level at 30 min. The *cross-hatched bar* at 120 min shows the glycogen content of nerves that were allowed to recover for 60 min in control aCSF after the 60 min period of glucose withdrawal. The *clear bar* at 120 min represents the glycogen content of nerves that were perfused with control aCSF for the entire 120 min test period, with no exposure to glucose-free aCSF. *Time (min)* refers to the time elapsed from the beginning of glucose-free perfusion. All of the nerves in this experiment were incubated first in control aCSF for 60 min; $n = 6$ for all conditions except 60 min ($n = 4$). Error bars indicate SEM. $^{*}p < 0.05$ and $^{***}p < 0.001$ as compared with *0 min*. *B*, Incubation of nerves with 25 mM glucose increased glycogen content, and incubation with NE caused glycogen to decline. Glycogen content in nerves pretreated with 25 mM fructose was not significantly different from control. All of the nerves were incubated for 60 min in the indicated substrate beginning immediately after their removal from the animal; $n = 6$ for each group except NE ($n = 7$). Error bars indicate SEM. *n.s.*, Not significant; $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared with control.

attempts were made to quantify the glycogen seen in this manner. These results agreed with previous studies on other neural areas (Cataldo and Broadwell, 1986; Magistretti et al., 1993).

Glycogen content of RON

The levels of glycogen in RONs under different conditions were determined by biochemical assay (Fig. 3). Glycogen content declined *in vitro* after the removal of nerves from the animal. In one set of experiments, for example, glycogen content fell from 10.10 ± 0.72 pmol of glycogen/μg of protein ($n = 6$) immediately after dissection (i.e., the nerves were never placed in the tissue chamber) to 4.85 ± 0.31 pmol of glycogen/μg of protein ($n = 6$; $p < 0.001$) in companion nerves that were perfused with control aCSF for 60 min (see Fig. 3*B, first bar*). Nonetheless, glycogen content of RONs was quite stable after 60 min of incubation in control aCSF containing 10 mM glucose (Fig. 3*A*, compare the *first* and *last bars*). It should be noted that the absolute values of glycogen in nerves under control conditions (60 min incubation in aCSF containing 10 mM glucose) were variable between assay sets (e.g., Fig. 3, compare the *first bar* in *A* with the *first bar* in *B*), but results within each set of assays were internally consistent.

In one set of experiments we investigated the effects of glucose removal on glycogen content. All nerves were given an initial 60 min incubation period in control aCSF containing 10 mM glucose. The glycogen content fell rapidly with glucose removal (Fig. 3*A*). From an initial value of 10.70 ± 0.45 pmol of glycogen/μg of protein ($t = 0$ min; $n = 6$), glycogen fell to 8.37 ± 0.35 pmol of glycogen/μg of protein after 15 min of glucose deprivation ($n = 6$; $p < 0.05$ vs 0 min group) and then to a low stable level after 30 min (2.69 ± 0.30 pmol of glycogen/μg of protein; $n = 6$). One group of nerves was allowed to recover for 60 min in control aCSF after the 60 min period of glucose withdrawal (*cross-hatched bar* at *120 min* in Fig. 3*A*; 3.06 ± 0.33 pmol of glycogen/μg of protein; $n = 6$). Glycogen content in this group was not significantly different from glycogen content in nerves that were not given a recovery period ($p > 0.05$ compared with nerves collected at 30, 45, and 60 min). The glycogen content of nerves that were simply perfused with control aCSF for the entire 120 min test period, with no period of glucose deprivation, was not significantly different from the control nerve glycogen content (11.1 ± 0.89 pmol of glycogen/μg of protein; $n = 6$; $p > 0.05$ compared with control and $p < 0.001$ compared with *cross-hatched bar*).

As a crucial step to testing the effects of glycogen on RON function, we determined whether RON glycogen content could be modulated. These results are shown in Figure 3*B*. For this set of nerves the control population incubated for 60 min in normal aCSF contained 4.85 ± 0.31 pmol of glycogen/μg of protein ($n = 6$). In other preparations exposure to high glucose concentration increases glycogen content (Prasannan and Subrahmanyam, 1966; Swanson et al., 1989b; Dringen and Hamprecht, 1992), whereas exposure to norepinephrine causes glycogen content to fall (Quach et al., 1978; Magistretti, 1988; Magistretti et al., 1993). Incubation of nerves in 25 mM glucose for 60 min induced an increase in glycogen stores to 7.90 ± 0.59 pmol of glycogen/μg of protein ($n = 6$; $p < 0.001$ vs control); conversely, pretreatment for 60 min with 1 mM norepinephrine led to a decline in RON glycogen (2.56 ± 0.08 pmol of glycogen/μg of protein; $n = 7$; $p < 0.01$ vs control). Fructose can sustain the CAP in the absence of glucose (R. Wender,

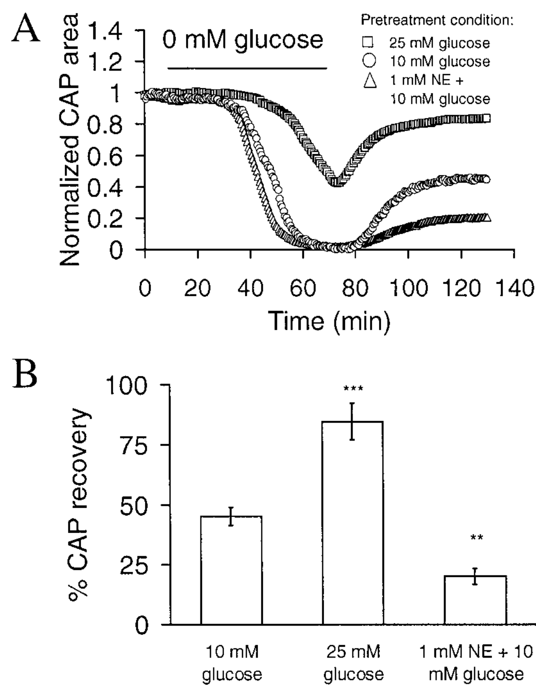

Figure 4. Effect of nerve glycogen content on axon function during, and recovery from, a 60 min period of glucose deprivation. *A*, Increase of glycogen by pretreatment of optic nerves with 25 mM glucose ($n = 6$) delayed the onset of CAP area decline during 60 min of glucose deprivation as compared with control ($n = 15$). Under these conditions the magnitude of CAP recovery was greater. Decrease of glycogen by pretreatment with 1 mM norepinephrine decreased the extent of CAP recovery ($n = 9$). *B*, Percentage of CAP recovery 60 min after exposure to glucose-free aCSF for the nerves represented in *A*. Control nerves showed recovery of CAP area to 45.3 ± 3.7%. Pretreatment of nerves with 25 mM glucose increased CAP recovery, and incubation with 1 mM norepinephrine had the opposite effect. $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared with *10 mM glucose* group.

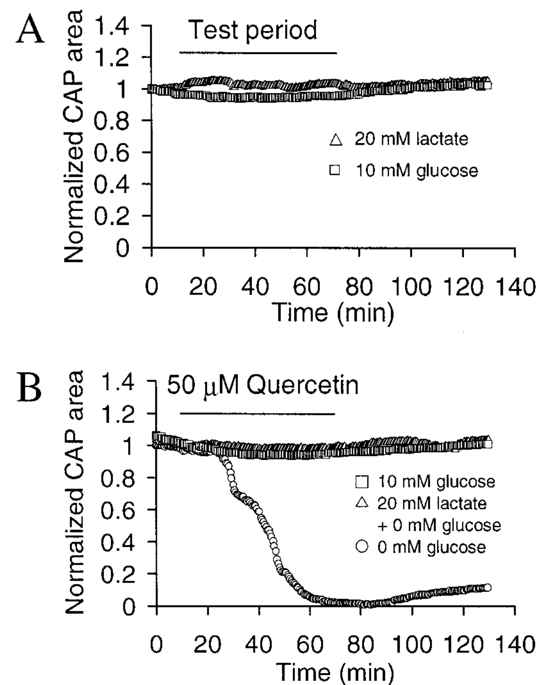

Figure 5. Lactate supported RON function in the absence of glucose and in the presence of the lactate transport blocker quercetin. *A*, Nerves continually perfused with control aCSF showed no decline in average CAP area over 120 min ($n = 6$). Lactate could substitute for glucose in maintaining the CAP for the 60 min period shown by the *solid horizontal line* ($n = 6$). *B*, Effect of quercetin (50 μM) on RONs subjected to 60 min of glucose withdrawal. Quercetin had no effect on the average CAP of nerves perfused with 10 mM glucose ($n = 6$). Quercetin also had no effect on the CAP of nerves exposed to 20 mM lactate during glucose withdrawal ($n = 6$). When quercetin was applied during 0 mM glucose exposure, however, there was a decline in CAP area and reduced recovery ($n = 7$).

A. Brown, and B. Ransom, unpublished observations) but does not lead to glycogen formation *in vitro* (Wiesinger et al., 1997). As expected, nerves equilibrated for 60 min in 25 mM fructose had no change in glycogen content as compared with control (3.23 ± 0.29 pmol of glycogen/μg of protein; $n = 6$; $p > 0.05$ vs control); the significance of this observation is discussed later.

Glycogen content and axon function

To determine whether RON glycogen content affected axon function during glucose withdrawal, we assessed the CAP during glucose withdrawal in nerves for which the glycogen was increased or decreased (Fig. 4). Nerves with increased glycogen (i.e., preincubated with 25 mM glucose), as compared with control nerves, showed increased latency to CAP area decline during glucose deprivation [41.5 ± 4.9 min ($n = 6$) vs control at 28.9 ± 2.1 min ($n = 15$); $p < 0.05$]. The CAP never fell to zero during aglycemia in the high-glycogen nerves (Fig. 4*A*). Nerves with decreased glycogen (i.e., preincubated with 1 mM norepinephrine) all had latencies to CAP decline of <28 min, the average latency to CAP decline in control nerves, but this trend did not reach statistical significance (23.2 ± 1.1 min; $n = 9$; $p > 0.05$ vs control). The magnitude of post-aglycemia CAP recovery for nerves with variable glycogen content is illustrated in Figure 4*B*. CAP recovery after glucose withdrawal was significantly greater in high-glycogen nerves (85.8 ± 7.2%; $n = 6$; $p < 0.001$) and significantly less in low-glycogen nerves (20.2 ± 3.3%; $n = 9$; $p < 0.01$) as compared with control tissue (45.3 ± 3.7%; $n = 15$).

To confirm that the results observed with 25 mM glucose were attributable to the presence of increased glycogen stores and were not merely a consequence of "loading" of the extracellular space with elevated glucose, we performed a series of experiments with 25 mM fructose. Fructose, like glucose, sustains the CAP, but it does not induce glycogen synthesis (Wiesinger et al., 1997) (see Fig. 3*B*). There was no statistically significant difference in either latency to CAP decline or CAP recovery between nerves pretreated with 25 mM fructose versus control nerves pretreated with 10 mM glucose (latency, 27.6 ± 2.2 min; recovery, 59.0 ± 7.4%; $n = 6$; $p > 0.05$). This result with fructose suggested that the effects of 25 mM glucose were attributable to glycogen and not to lingering amounts of substrate in the extracellular space.

We determined whether glycogen content would affect the latency of CAP failure or the degree of CAP recovery after an anoxic insult as opposed to an aglycemic insult. Nerves with increased or decreased glycogen content were subjected to 60 min periods of anoxia. RON glycogen content had no apparent effect on the time course of CAP failure or on the degree of CAP recovery from anoxia (data not shown).

Blockade of lactate transport and axon function during glucose withdrawal

Nerves in control aCSF containing 10 mM glucose maintained robust CAPs for several hours (Fig. 5*A*), in agreement with Stys et al. (1991). A metabolically equivalent concentration of lactate (i.e., 20 mM) could be substituted for glucose for a 60 min test period with no loss of CAP area (106 ± 8.9% of baseline CAP area; $n = 6$; at $t = 130$ min; Fig. 5*A*). These data strongly supported the hypothesis that lactate can support axon function as effectively as can glucose, at least for the period that was tested.

Because lactate served as an effective energy source for axon function and appears in the extracellular space with glycogen breakdown (Dringen et al., 1993; Wiesinger et al., 1997), we attempted to interfere with lactate transport to test the theory that lactate was transferred from astrocytes to axons during glucose deprivation. We first tested the bioflavonoid quercetin, which preferentially blocks extrusion of lactate (Belt et al., 1979; Volk et al.,

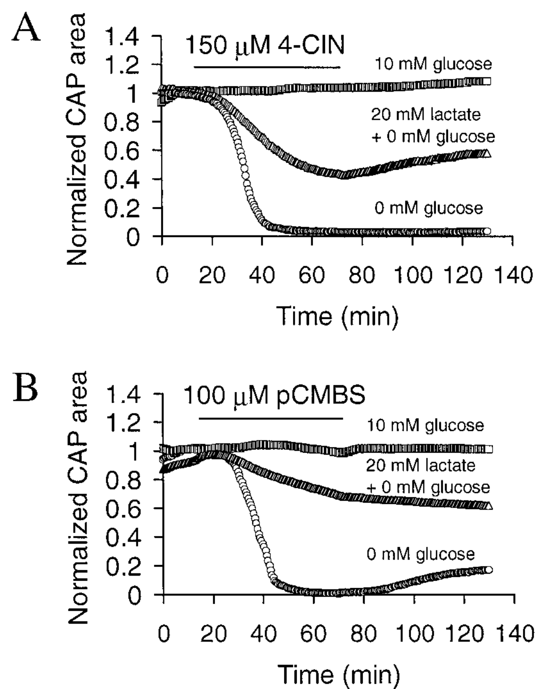

Figure 6. Effect of the lactate transport inhibitors 4-CIN and *p*CMBS on RONs exposed to 0 or 10 mM glucose or 20 mM lactate for 60 min. *A*, α-Cyano-4-hydroxycinnamic acid (*4-CIN*;150 μM) had no effect on the average CAP in the presence of glucose. 4-CIN led to a loss of function in nerves substituted with 20 mM lactate. 4-CIN caused rapid CAP failure in nerves exposed to 0 mM glucose and a lower recovery of baseline CAP area. *B*, *p*-Chloromercuribenzene sulfonic acid (*pCMBS*; 100 μM) had no effect on the CAP in the presence of glucose. *p*CMBS led to a loss of function in nerves perfused with aCSF in which glucose had been substituted with 20 mM lactate. When *p*CMBS was applied to nerves exposed to unsupplemented 0 mM glucose, CAP recovery declined as compared with control conditions. All traces in *A* and *B* represent an average of six experiments.

1997). Nerves were perfused with 50 μM quercetin for 20 min before and during the 60 min period of glucose withdrawal. In the presence of quercetin the latency to CAP failure was 23.3 ± 2.8 min ($n = 7$) as compared with 28.9 ± 2.1 min in control experiments ($p > 0.05$; Fig. 5*B*). These nerves sustained a greater degree of irreversible injury than did control nerves (12.3 ± 3.4% vs 45.3 ± 3.7% control; $p < 0.001$). It appeared that quercetin blocked lactate *efflux* in the RON, because quercetin did not prevent lactate, exogenously applied, from supporting the CAP in the absence of glucose (CAP area = 105 ± 7.1% at $t = 130$ min; $n = 6$; Fig. 5*B*). As a control, quercetin was applied during continuous perfusion with glucose-containing aCSF. It was without effect under these conditions (Fig. 5*B*).

Two other lactate transport inhibitors, α-cyano-4-hydroxycinnamic acid (4-CIN) and *p*-chloromercuribenzene sulfonic acid (*p*CMBS), were tested for their effects on axon function during glucose deprivation (Fig. 6). Both compounds had no effect on the CAP in the continuous presence of glucose. 4-CIN (150 μM), applied 20 min before, and during, 60 min of glucose deprivation, decreased latency to CAP decline to 17.5 ± 3.2 min ($n = 6$; $p < 0.05$ vs control; Fig. 6*A*). 4-CIN-treated nerves recovered only minimally (3.5 ± 1.0%; $n = 6$; $p < 0.001$ vs control). It appeared that 4-CIN blocked lactate *uptake* by RON axons because, in the presence of 4-CIN, 20 mM lactate in glucose-free aCSF was not able to support the CAP fully (Fig. 6*A*). In the presence of 4-CIN and lactate, CAP area declined and showed irreversible injury (58.2 ± 5.4%; $n = 6$; $p < 0.001$ compared with nerves perfused with lactate alone).

When *p*CMBS, a specific blocker of the monocarboxylate transporter isoform MCT1 (Halestrap and Price, 1999; Juel and Halestrap, 1999), was applied during glucose withdrawal, latency to CAP decline was 19.5 ± 2.6 min ($n = 6$; $p > 0.05$ vs 0 mM glucose control), and CAP recovery was reduced to 17.1 ± 1.9% of baseline CAP area ($n = 6$; $p < 0.01$ compared with recovery under control conditions; Fig. 6*B*). When applied in the presence of 20 mM lactate for the test period, *p*CMBS caused a fall in CAP area beginning at 29.5 ± 2.9 min and an inevitable loss of CAP area (62.0 ± 4.4% of baseline CAP area; $n = 6$; $p < 0.001$). These results suggested that MCT1 must be present on axons in adult RON. The effects of the lactate transport blockers on the percentage of CAP recovery from 60 min of aglycemia or on exposure to 20 mM lactate are summarized in Figure 7.

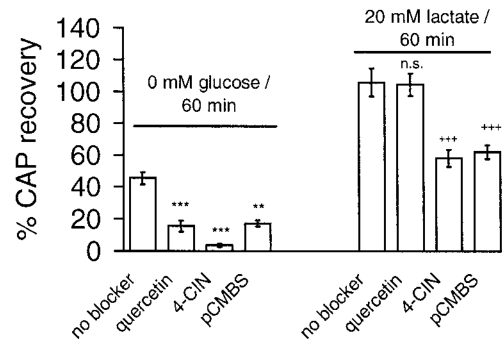

Figure 7. Summary of CAP recoveries after aglycemia with inhibition of lactate transport, with and without lactate supplementation. The *four bars* on the *left* represent nerves that were subjected to a 60 min period of glucose withdrawal, i.e., no exogenous substrate was provided. A second set of nerves, represented by the *four bars* on the *right*, was exposed to the indicated lactate transport blockers during aglycemia but in the presence of 20 mM lactate. Each *bar* represents a minimum of six experiments. Error bars indicate SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared with *no blocker* in *0 mM glucose* group; $^{+++}p < 0.001$. *n.s.*, Not significant as compared with *no blocker* in *20 mM lactate* group.

DISCUSSION

Our results support the hypothesis that, during glucose deprivation in white matter, astrocytes supply energy substrate to axons in the form of lactate derived from glycogen. This is, to our knowledge, the first demonstration of the importance of astrocytic glycogen for axon function and survival during glucose withdrawal. Our conclusions are based on the following observations: (1) the RON contained glycogen localized exclusively in astrocytes; (2) in the absence of glucose, RON axons remained functional for 30 min and then failed with a time course that mirrored glycogen loss; (3) RON function during and after 60 min of glucose removal was enhanced by increasing glycogen content and decreased by decreasing glycogen content; (4) lactate supported RON function in the absence of glucose; and (5) blockade of transmembrane lactate transport decreased RON function in the absence of glucose.

The persistence of the CAP for 30 min without glucose suggested the presence of an intrinsic energy reserve. Brain glycogen, localized almost exclusively in astrocytes (see Fig. 2), is a prime candidate to fill this role (Cataldo and Broadwell, 1986; Magistretti et al., 1993). The preservation of the CAP in glucose-free aCSF was not attributable to persistent glucose within the tissue. Glucose concentration in the extracellular space ($[\text{glucose}]_o$) would be less than in the bulk perfusate because diffusion of glucose is likely to be slow compared with glucose use. Even *in vivo*, in which the diffusion distances would be much less than in the isolated RON, the $[\text{glucose}]_o$ is only approximately one-third of that in blood (Silver and Erecinska, 1994). Direct measurements of $[\text{glucose}]_o$ in cortex indicate that it falls within minutes to unmeasurable levels when the exogenous supply is interrupted (Siesjö, 1978; Silver and Erecinska, 1994), consistent with the high rate of glucose consumption by brain tissue at 37°C (Siesjö, 1978).

We feel confident that the electron-dense particles located in glial end-feet (see Fig. 2) are glycogen, on the basis of two observations. First, the only other structure with which these might be confused is free ribosomes, which at 10–15 nm are too small to have accounted for the structures in Figure 2 (which are 20–40

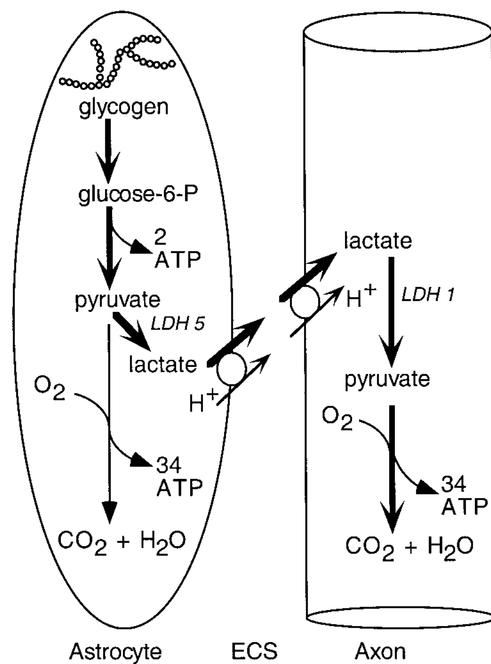

Figure 8. Schematic illustration of how astrocytic glycogen appears to fuel axons in the absence of glucose. In the absence of glucose the astrocytic glycogen is broken down to lactate, which is transported to the extracellular space (*ECS*) via a MCT. Then it is taken up by a MCT in axons and is metabolized oxidatively to produce the energy needed to sustain excitability. *LDH5* preferentially reduces pyruvate to lactate, and *LDH1* preferentially oxidizes lactate to pyruvate. This scheme recognizes that astrocytes can subsist, at least transiently, on glycolytic energy metabolism, whereas axons require oxidative metabolism.

nm). Second, the pattern of glycogen distribution illustrated in Figure 2 is consistent with what has been shown in previous ultrastructural studies (Peters and Palay, 1976; Cataldo and Broadwell, 1986; Clarke and Sokoloff, 1999). This ultrastructural picture is consistent with *in vitro* experiments indicating that only astrocytes generate measurable quantities of glycogen (Dringen et al., 1993; Wiesinger et al., 1997).

In the absence of glucose the glycogen content fell to a low, stable level by 30 min (see Fig. 3*A*), closely corresponding to the time at which axonal conduction failed. Glucose controls glycogen content by binding and inactivating the glycogen breakdown enzyme phosphorylase A (Stryer, 1995). It is not surprising that nerve glycogen content remained at a low plateau level after 30 min of glucose withdrawal rather than falling to zero. Cultured astrocytes are not able to mobilize their glycogen stores completely in the absence of glucose (Lomako et al., 1993, 1995). Glycogen is composed of a protein core, glycogenin, with many attached glucose residues. Astrocytes do not degrade glycogen all the way to the free glycogenin (Wiesinger et al., 1997).

Changes in RON glycogen content had significant functional consequences. Nerves with amplified glycogen stores maintained normal conduction for 12 min longer than did control nerves during glucose deprivation, and they showed much higher levels of CAP recovery after the insult. Moreover, CAP area never fell to zero in the high glycogen-containing nerves during aglycemia. In NE-treated nerves, with diminished glycogen, CAP recovery was less than in control nerves.

Our results supported the model shown in Figure 8. The model asserts that astrocytes contain glycogen, which is converted into lactate for transport to axons during glucose deprivation. Lactate is the most likely fuel to be transferred from astrocytes to axons for the following reasons: (1) astrocytes, but not neurons, are known to extrude large amounts of lactate (Walz and Mukerji, 1990); (2) astrocytes, but not neurons, can survive for a limited time on anaerobic metabolism alone (Goldberg and Choi, 1993; Pappas and Ransom, 1995; Ransom and Fern, 1996) and could "afford" to export large amounts of lactate; (3) lactate, but not glucose, is released from astrocytes when glucose is removed (Dringen et al., 1993); (4) lactate has been shown to be an effective fuel in numerous types of CNS tissue (Larrabee, 1983; Schurr et al., 1988; Izumi et al., 1994; Izumi et al., 1997), including RON axons (Wender et al., 1999); (5) lactate from Müller cells fuels photoreceptors in guinea pig retina (Poitry-Yamate et al., 1995). According to the model, axons import lactate for subsequent oxidative metabolism to generate ATP. This model reflects the constraint that lactate could be metabolized by axons *only* in the presence of oxygen, and our results confirm this. Clinically, our model would operate during hypoglycemia. Astrocytes also may supply axons with lactate from glycogen breakdown during high energy demand, when extracellular glucose levels would be expected to fall.

For astrocytes to convert glycogen to lactate for transfer to axons as a fuel in the absence of glucose (i.e., Fig. 8), several conditions must be met. There must be appropriate enzymes for the creation of lactate in astrocytes and for its use in axons, and there also must be appropriate transport mechanisms for lactate movement. Indeed, the expression patterns in the CNS of lactate dehydrogenase (LDH) and the monocarboxylate transporter (MCT) seem well suited to accommodate these needs. LDH, the enzyme catalyzing interconversion of pyruvate and lactate (Stryer, 1995), is composed of various combinations of two subunits, H (or LDH1) and M (or LDH5). Tetramers composed primarily of the former subunit preferentially oxidize lactate to pyruvate, and tetramers composed principally of the latter subunit predominantly reduce pyruvate to lactate (Stryer, 1995). Neurons, highly dependent on oxidative metabolism (Siesjö, 1978), stain exclusively with anti-H (anti-LDH1) antibodies (Bittar et al., 1996). Astrocytes, which have less active oxidative enzymes (Friede, 1962), are stained by both M (LDH5) and H antibodies. Thus astrocytes, expressing at least some LDH5, can convert pyruvate to lactate readily, and neurons, expressing LDH1, are specialized to oxidize lactate to pyruvate.

MCTs, of which there are several isoforms, shuttle lactate and pyruvate across cell membranes by using proton symport (Poole and Halestrap, 1993). There are several isoforms of MCTs. MCT1 appears to be expressed in tissue that preferentially releases lactate, and MCT2 is expressed in tissues that mainly consume lactate (Jackson and Halestrap, 1996; Broer et al., 1997). MCT1 is the only MCT expressed by astrocytes, whereas neurons express MCT2 (Broer et al., 1997; Koehler-Stec et al., 1998). MCT2 has a 10-fold higher affinity for substrates than does MCT1 and is, therefore, ideally suited for uptake at low substrate concentrations (Halestrap and Price, 1999). Additionally, because pH gradients drive transmembrane lactate movement (Poole and Halestrap, 1993; Juel, 1997), glycolytically generated lactic acid could initiate its own export. The expression patterns of LDH and MCT isoforms would tend to make astrocytes a lactate source and make axons a lactate sink.

The model is supported by the results of inhibiting lactate transport, which would be predicted to block the movement of lactate between astrocytes and axons. All three MCT blockers that were tested reduced CAP recovery after a 60 min period of glucose withdrawal. Quercetin preferentially inhibits lactate efflux from cells (Belt et al., 1979) (see also McKenna et al., 1998). When 20 mM lactate was added to the glucose-free perfusate, quercetin had no effect on the CAP (see Fig. 5*B*), consistent with the idea that the drug did not affect lactate influx into axons. 4-CIN preferentially blocks MCT2 (Halestrap and Price, 1999). In contrast to quercetin, 4-CIN partially blocked the ability of exogenous lactate to support axonal function during glucose removal (see Fig. 6*A*). Because 4-CIN is a competitive inhibitor, it is not surprising that axon function was maintained to some degree during perfusion with 20 mM lactate plus 4-CIN (Edlund and Halestrap, 1988).

The inhibitor 4-CIN can have effects on mitochondrial pyruvate transport (Juel and Halestrap, 1999) that can lead to a misinterpretation of results. For this reason we used a third lactate transport blocker. *p*CMBS, which is highly specific for MCT1 (Broer et al., 1998; Broer et al., 1999; Halestrap and Price, 1999; Juel and

Halestrap, 1999), gave similar results to those observed with 4-CIN (see Figs. 6*B*, 7). Considering that *p*CMBS partially blocked the ability of exogenous lactate to sustain the CAP, our data suggest that axon membranes express some MCT1.

Our results support the idea that astrocytic glycogen acted as a readily available source of energy (i.e., lactate) for axons when glucose was withdrawn. This glial–neuronal interaction, although long a theoretical possibility and suggested by earlier tissue culture experiments (Swanson and Choi, 1993), had not been demonstrated previously. It was surprising that glycogen was able to sustain nerve function for up to 30 min. It has been assumed that glycogen content in the brain could sustain neural function for <5 min (Clarke and Sokoloff, 1999). It may be that white matter, with a lower metabolic rate than gray matter, is unique in this regard.

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