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CONTRIBUTION OF ASTROCYTE GLYCOGEN STORES TO PROGRESSION OF SPREADING DEPRESSION AND RELATED EVENTS IN HIPPOCAMPAL SLICES

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Abstract—Spreading depression (SD) is a wave of coordinated cellular depolarization that propagates slowly throughout brain tissue. SD has been associated with migraine aura, and related events have been implicated in the enlargement of some brain injuries. Selective disruption of astrocyte oxidative metabolism has previously been shown to increase the propagation rate of SD *in vivo*, but it is currently unknown whether astrocyte glycogen stores make significant contributions to the onset or propagation of SD. We examined SD in acutely-prepared murine hippocampal slices, using either localized microinjections of KCl or oxygen and glucose deprivation (OGD) as stimuli. A combination of glycogenolysis inhibitors 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) and 1-deoxynojirimycin (DNJ) increased the propagation rates of both high K^+-SD and OGD-SD. Consistent with these observations, exposure to L-methionine-DL-sulfoximine (MSO) increased slice glycogen levels and decreased OGD-SD propagation rates. Effects of glycogen depletion were matched by selective inhibition of astrocyte tricarboxylic acid (TCA) cycle activity by fluoroacetate (FA). Prolonged exposure to reduced extracellular glucose (2 mM) has been suggested to deplete slice glycogen stores, but significant modification SD of propagation rate was not observed with this treatment. Furthermore, decreases in OGD-SD latency with this preexposure paradigm appeared to be due to depletion of glucose, rather than glycogen availability. These results suggest that astrocyte glycogen stores contribute to delaying the advancing wavefront of SD, including during the severe metabolic challenge of OGD. Approaches to enhance astrocyte glycogen reserves could be beneficial for delaying or preventing SD in some pathologic conditions.

Key words: spreading depression, glycogen, astrocyte, hippocampal slice, ischemia.

Spreading depression (SD) is characterized by a wave of severe depolarization that can propagate throughout CNS tissue and result in suppression of electrical activity and disruption of ion homeostasis. These events can provide repetitive large metabolic burdens to both neurons and astrocytes (Hansen, 1985; Somjen, 2001). If SD is generated in otherwise healthy tissue, neuronal ionic gradients and brain function can be fully restored after SD (Nedergaard and Hansen, 1988), and there is evidence that such recoverable events are related to migraine aura (Okada et al., 1988; Hadjikhani et al., 2001). However, SD triggered in the context of ischemia can result in irrecoverable injury if metabolic substrates are not resupplied immediately following SD onset (Rader and Lanthorn, 1989; Tanaka et al., 1997), in both animal models (Nedergaard and Astrup, 1986; Hossmann, 1996; Hartings et al., 2003) and in human trauma and stroke patients (Fabricius et al., 2006; Dohmen et al., 2008). Therefore, inhibition of SD-like events following ischemia is being considered a potential therapeutic approach to limit injury progression (Lauritzen et al., 2011). Much work to date has concentrated on targeting neurons to limit these spreading depolarizations, but astrocytes may be a useful additional target.

Although astrocytes may not be the primary direct contributors to generation of SD events (Zhou et al., 2010), pharmacological evidence supports a significant role in regulating SD progression. Selective disruption of astrocyte oxidative metabolism with fluorocitrate (FC) has been shown to increase the rate of SD propagation in rat cortex *in vivo* (Largo et al., 1997). In rat brain slices, the related gliotoxin fluoroacetate (FA) accelerated onset of SD generated by hypoxia (Muller and Somjen, 1999), disrupted prodromal oscillations prior to SD (Larrosa et al., 2006), and with extended exposures, was sufficient to initiate SD under some experimental conditions (Canals et al., 2008).

The aim of the present study was to determine the role of astrocytic glycogen stores in the regulation of SD-like events studied in brain slices. Astrocytes are the primary source of glycogen in the brain (Cataldo and Broadwell, 1986; Wender et al., 2000; Brown, 2004). Glycogen stores are known to be depleted following SD events (Selman et al., 2004) and inhibition of glycolysis or glycogenolysis results in decreased glutamate uptake by cultured astrocytes (Swanson, 1992; Sickmann et al., 2009), but the possible contributions of astrocyte glycogen stores in the initiation and/or propagation of SD are currently unclear. A previous study suggested that the latency to single cell anoxic depolarizations in rat hippocampal slices was determined by depletion of astrocytic glycogen (Allen et al., 2005). However, it is not yet known (1) whether the glucose depletion approach used previously did in fact deplete glycogen stores, (2) whether the delay in the onset of a single neuron anoxic depolarization also applies to SD initiation, and (3) whether initiation and/or propagation of

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Abbreviations: ACSF, artificial cerebrospinal fluid; ATP, adenosine triphosphate; DAB, 1,4-deoxy-1,4-imino-D-arabinitol; DNJ, 1-deoxynojirimycin; FA, fluoroacetate; FC, fluorocitrate; fEPSP, excitatory postsynaptic potential; MSO, L-methionine-DL-sulfoximine; NADH, nicotinamide adenine dinucleotide (reduced); OGD, oxygen and glucose deprivation; SD, spreading depression; TCA, tricarboxylic acid cycle.

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coordinated waves of SD are significantly influenced by astrocyte glycogen stores.

In the present study, we have examined these questions by studying SD-like events in murine hippocampal slices. SD-like events were generated either by oxygen glucose deprivation (OGD) or by localized high K^+ stimuli, and astrocyte metabolism was disrupted by using FA, putative inhibitors of glycogen metabolism, or the glucose depletion approach previously suggested to deplete glucose/glycogen stores prior to SD onset. We conclude that availability of astrocyte glycogen stores can modify the latency to SD onset generated in ischemia-like conditions, but that lack of availability of glucose (rather than glycogen) likely explains the effects of low glucose preexposure strategies in our preparations. SD propagation rates appear to be significantly regulated by glycogen availability, likely by reducing the rate of extracellular K^+ and/or glutamate accumulation within astrocytes at the advancing wave front of SD generated in both normoxic and ischemic-like conditions.

EXPERIMENTAL PROCEDURES

Slice preparation

Male mice (FVB\N) were obtained from Harlan Laboratories (Indianapolis, IN, USA) at 4–6 weeks of age and were housed in standard conditions (12 h light/dark cycle) for up to 2 weeks prior to euthanasia. Mice were deeply anesthetized with a mixture of ketamine and xylazine (85 and 15 mg/ml, respectively, s.c.) and decapitated. Brains were rapidly removed and placed in ice-cold cutting solution (see below for composition). Coronal sections (250 μm) were cut on a Vibratome (Technical Products International, St. Louis, MO, USA) and slices were subsequently transferred to oxygenated room temperature artificial cerebrospinal fluid (ACSF) (see below). Cutting and recording solutions were both 300–305 mOsm/L. After warming to 34 °C for 1 h, the ACSF was exchanged again and slices were then held at room-temperature. Individual slices were then transferred to a recording chamber and superfused with oxygenated ACSF at 2 ml/min at 35 °C.

Electrical recording

Extracellular measurements of slow DC shifts characteristic of SD were made using borosilicate glass microelectrodes, filled with ACSF (~5 MΩ) and placed in stratum radiatum ~45 μm below the surface of the slice and approximately 150 μm from the pyramidal cell body layer. In some experiments, Schaffer collateral inputs to the CA1 region were stimulated using a bipolar electrode (25 μm tip) placed on the surface of stratum radiatum. Single shocks (80 μs, 0.1–1.5 mA) were applied using a constant-current stimulus isolation unit (Isoflex, AMPI, Israel). Stimulus intensity was chosen based on an input/output curve generated in each slice, to produce responses ~60% of maximal amplitude (0.4–0.55 mA). Signals were amplified (Neurodata IR-283), digitized (Digidata 1322A, Axon Instruments, Union City, CA, USA) and then acquired using Axoscope software (v 8.1, Axon Instruments).

Autofluorescence measurements

NAD(P)H autofluorescence was used to assess the inhibition of slice mitochondrial function during OGD exposures, and also to track the progression of high K^+-SD and OGD-SD. This was performed as previously described (Shuttleworth et al., 2003) with minor modifications. In most experiments, 360 nm excitation was delivered via a fiber optic/monochromator system (Polychrome IV; Till Photonics, Grafelfing, Germany) and reflected onto the slice surface using a dichroic mirror (DMLP 400 nm, Chroma Technology, Brattleboro, VT, USA). Fluorescence emission (>410 nm) was collected with a cooled interline transfer CCD camera (IMAGO, Till Photonics). Image data were background-subtracted to account for camera noise, and presented as the changes in fluorescence intensity/prestimulus fluorescence intensity ($\Delta F/F_o$) from stratum radiatum. All imaging was performed after focusing onto the surface of the slice with a 10× water immersion objective (NA 0.3, Olympus) and fluorescence collected after 2×2 binning of the 640×480 line image. Single images or image pairs were acquired every 2.5 s.

Tissue glycogen levels

Glycogen measurements were performed following previously-described methods (Cruz and Dienel, 2002). Isolated hippocampal slices were allowed to recover in normal artificial cerebral spinal fluid (nACSF) for at least 4 h following slicing, to allow glycogen levels recover (Lipton, 1989) prior to different exposure paradigms. Tissues were flash-frozen and added to ice-cold 0.3 ml 65% ethanol/phosphate-buffered saline solution and then homogenized and centrifuged at 9000×g for 10 min. The supernatant was discarded, and the extraction procedure repeated four to five more times, before pellets were freeze dried. Samples were acidified (0.4 ml 0.03 N HCl), boiled (45 min), and stored at 4 °C. Glycogen levels were determined indirectly, after hydrolysis to glucose. Samples were acidified (final concentration of 0.1 M acetate), divided and incubated (2 h, 37 °C) in either the presence or absence of amylo-α-1,4-α-1,6-glucosidase (30 μg/ml). Samples were centrifuged to remove particulate matter and glucose levels were analyzed by fluorometry (hexokinase/glucose-6-phosphate dehydrogenase procedure), and responses compared with a glucose standard curve. Tissue levels were within the linear detection range of the assay and an approach expected to deplete tissue glycogen (20 min OGD, see (Lipton, 1989)) abolished glycogen signals in slices (-1.18 ± 0.13 nmol/mg protein, $n=2$, $P<0.0001$) when compared with controls (11.59 ± 0.53 nmol/mg protein, $n=7$). We used the rough estimate of 150 mg protein per 1 g wet tissue wt to compare our measurement to published work, and found that while our values were lower (~2 μmol/g wet wt vs. 12.5–5.4 μmol/g wet wt (Cruz and Dienel, 2002)), they were within an order of magnitude of previous work utilizing larger volumes of tissue.

Reagents and solutions

Except where noted, all drugs and salts were obtained from Sigma Chemical Co (St Louis, MO, USA). ACSF contained, in mM: 126 NaCl, 2 KCl, 1.25 NaH_2PO_4, 1 $MgSO_4$, 26 $NaHCO_3$, 2 $CaCl_2$, and 10 glucose, equilibrated with 95% O_2/5% CO_2. Cutting solution contained, in mM: 2 KCl, 1.25 NaH_2PO_4, 6 $MgSO_4$, 26 $NaHCO_3$, 0.2 $CaCl_2$, 10 glucose, 220 sucrose, and 0.43 ketamine. For OGD studies, ACSF was modified by replacement of glucose with equimolar sucrose, and equilibration with 95% N_2/5% CO_2, rather than 95% O_2/5% CO_2. Studies of hypoxia alone, or hypoglycemia alone were performed using ACSF equilibrated with 95% N_2/5% CO_2 or replacement of 10 mM glucose by 10 mM sucrose, respectively.

Statistical analysis

Significant differences between group data were evaluated using unpaired Student's *t*-tests or one-way ANOVA. Bonferroni's multiple-comparison test or contrast comparisons were used for post hoc analysis in which the effects of multiple treatments were compared against each other. A value of $P<0.05$ was considered significant in all cases. Numbers in the study refer to the number of slices, with a maximum of three slices from an individual animal used for each experimental protocol.