Role of sodium and potassium ions in regulation of glucose metabolism in cultured astroglia

\(([^14C]d\text{eoxyglucose}/Na^+,K^+-\text{ATPase/glutamate/astrocyte})\)

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ABSTRACT Effects of increasing extracellular K+ or intracellular Na+ concentrations on glucose metabolism in cultures of rat astroglia and neurons were examined. Cells were incubated in bicarbonate buffer, pH 7.2, containing 2 mM glucose, tracer amounts of \(^{14}\text{C}\)deoxyglucose (\(^{14}\text{C}\)dGlc), and 5.4, 28, or 56 mM KCl for 10, 15, or 30 min, and then for 5 min in \(^{14}\text{C}\)dGlc-free buffer to allow efflux of unmetabolized \(^{14}\text{C}\)dGlc. Cells were then digested and assayed for labeled products, which were shown to consist of 96–98% \(^{14}\text{C}\)deoxyglucose 6-phosphate. Increased K+ concentrations significantly raised \(^{14}\text{C}\)deoxyglucose 6-phosphate accumulation in both neuronal and mixed neuronal-astroglial cultures at 15 and 30 min but did not raise it in astroglial cultures. Veratridine (75 \(\mu\text{M}\)), which opens voltage-dependent Na+ channels, significantly raised rates of \(^{14}\text{C}\)dGlc phosphorylation in astroglial cultures (+20%), and these elevations were blocked by either 1 mM ouabain, a specific inhibitor of Na+,K+-ATPase (7). Opening voltage-dependent Na+ channels with the carboxylic sodium ionophore, monensin (10 \(\mu\text{M}\)), more than doubled \(^{14}\text{C}\)dGlc phosphorylation in astroglial cultures (+20%), and these elevations were blocked by ouabain (7).

The function-driven increases in \(\text{ICMR}_{\text{glc}}\) in \textit{vivo} occur mainly in neuropil or synapse-rich regions (4, 6). The spatial resolution of the autoradiographic \(^{14}\text{C}\)dGlc method is limited to 100–200 \(\mu\text{m}\) (8); this is insufficient to identify the specific cellular or subcellular elements in the neuropil that share in the \(\text{ICMR}_{\text{glc}}\) increases. Whether the function-driven increases in energy metabolism are limited to axonal and/or dendritic processes or include astrocytic processes that envelop the synapses therefore remains uncertain.

Action potentials reflect Na+ influx and K+ efflux in neurons, and increases in energy metabolism are proportional to their frequency. Presumably, with higher spike-frequencies, increases in intracellular Na+ ([Na+]i) and extracellular K+ ([K+]o) concentrations are greater, and more Na+, K+-ATPase activity and energy metabolism are needed to restore ionic gradients to resting levels. Such restoration must occur in neuronal elements from which the action potentials are derived, but astrocytic processes might also be involved. Astrocytes are believed to regulate [K+]o, either by passive diffusion (9, 10) or active transport (11) after increases in [K+]o resulting from neuronal excitation (12, 13), and there have been reports based on studies with tissue sections (14–17) or cultured cells (18–22) that energy is consumed in the process. In the present study we have attempted to simulate \textit{in vitro} changes in the extracellular environment that might result from increased spike activity \textit{in vivo} and to examine their effects on glucose metabolism of neurons and astroglia in culture. Effects of elevated [K+]o, and [Na+]o and of glutamate, the most prevalent excitatory neurotransmitter in brain, were examined.

Glucose is normally the main substrate for the brain’s energy metabolism (1). Applications of the \(^{14}\text{C}\)dGlc method for determination of local rates of glucose utilization (ICMRg) in neural tissues to conditions with altered local functional activities have established that energy metabolism and functional activity are closely linked and that the increases in ICMRg are quantitatively related to the magnitude of functional activation (2–4). During retinal stimulation with random light flashes, ICMRg increases proportionately to the logarithm of the light intensity in regions receiving direct projections from the retina (3, 4). Electrical stimulation of the cervical sympathetic trunk or sciatic nerve increases ICMRg linearly with the spike frequency in the superior cervical ganglion and dorsal lumbar spinal cord, respectively (5, 6).

These function-related increases in metabolism depend on Na+ or K+-ATPase activity. In neurohypophysial preparations stimulated electrically \textit{in vitro} the increases in \(^{14}\text{C}\)dGlc uptake were blocked by ouabain, a specific inhibitor of Na+,K+-ATPase (7). Opening voltage-dependent Na+ channels with veratridine and allowing Na+ entry into cells also stimulated ICMRg; this stimulation was also blocked by ouabain (7).

**MATERIALS AND METHODS**

**Animals.** Pregnant Sprague–Dawley rats with known dates of conception were purchased from Taconic Farms. All procedures on animals were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the National Institute of Mental Health Animal Care and Use Committee.

**Materials.** 2-Deoxy-D-[\(^1\text{C}\)]glucose (\(^{14}\text{C}\)dGlc; specific activity, 53 mCi or 1.96 GBq per mmol; 1 Ci = 37 GBq) was purchased from DuPont/NEN. Other chemicals and materials were obtained as follows: high-glucose (25 mM) Dulbecco’s modified Eagle medium, penicillin, and streptomycin from Life Technologies (Gaithersburg, MD); defined fetal bovine serum from HyClone; Dulbecco’s phosphate-buffered saline, l-glutamate, choline bicarbonate, choline chloride, poly(L-lysine), cytosine arabinoside, DL-2-amino-5-phosphonovaleric acid.

**Abbreviations:** \(^{14}\text{C}\)dGlc, \(^{14}\text{C}\)deoxyglucose; \(^{14}\text{C}\)dGlc, \(^{14}\text{C}\)dGlc, \(^{14}\text{C}\)dGlc; GFAP, glial fibrillary acidic protein; CNQX, 2,3-dihydro-7-nitro-2-oxo-\(\beta\)-ketobutyric acid; [K+]o, extracellular K+ concentration; [Na+]o, intracellular Na+ concentration; NMDA, N-methyl-d-aspartate.

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acid, DL-threo-β-hydroxyaspartic acid, ouabain, tetrodotoxin, veratridine, and monensin from Sigma; 6-cyano-7-nitroquinoline-2,3-dione (CNOX) from Research Biochemicals International (Natick, MA); trypsin-EDTA from Boehringer Mannheim; and N-6,2’-O-dibutyryl cAMP (dbcAMP) from Calbiochem.

Neuronal and Mixed Neuronal–Astroglial Cultures. Neuronal and mixed neuronal–glial cultures were prepared from striatum of fetal rats on embryonic day 16. Striatal tissue was excised and mechanically disrupted by passage through a 22-gauge needle. The dissociated cells were counted and cultured in high-glucose (25 mM) Dulbecco’s modified Eagle medium containing 10% (vol/vol) fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in humidified air/7% CO₂. For neuronal cultures viable cells (1.5 x 10⁶ cells per ml) that excluded trypan blue were placed in poly(l-lysine) (5 μg/ml)-coated 6-well culture plates or the eight center wells of 24-well culture plates. Cytosine arabinoside (10 μM) was added 2–3 days later. For mixed neuronal–astroglial cultures, 1.0 x 10⁶ cells per ml were plated in poly(l-lysine)-coated plates, and no cytosine arabinoside was added. Assays were done on 6- to 8-day-old cultures.

Astroglial Cultures. Astroglial cultures were prepared from cerebral cortex of newborn rats or striatum removed on embryonic day 16. Results were the same with both; therefore, only results obtained with astroglia of cortical origin are reported here. Meninges and blood vessels were removed from brains obtained from newborn rats, and the fronto–parietal cortices were dissected out and mechanically disrupted. The dissociated cells (2.5 x 10⁶ cells per ml) were cultured in medium like that used for mixed neuronal–astroglial cultures in uncoated 75-cm² culture flasks at 37°C in humidified air/7% CO₂. Culture medium was changed every 2 days until the cultures reached confluence; the flask was then shaken overnight at room temperature to eliminate loosely adherent process-bearing cells. The adherent cells were treated with trypsin-EDTA solution diluted 1:5 with Dulbecco’s phosphate-buffered saline for 1–2 min at 37°C, suspended in 4 vol of fresh culture medium, and placed in uncoated 6-well culture plates or the eight center wells of 24-well culture plates (secondary culture). Culture medium was changed every 3 days, and the cultures were used when confluent (day 19–22) or 1 week later (day 26–29). Some cultures were treated with dbcAMP (0.5 mM) for 24 or 72 hr before the experiment to induce morphological differentiation of the astroglia.

Assay of [14C]dGlc Phosphorylation. The reaction mixture for assay of [14C]dGlc phosphorylation contained 110 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄, 0.9 mM Na₂HPO₄, 44 mM NaHCO₃, 2.0 mM D-glucose, and 50 μM [14C]dGlc (2.5 μCi or 92.5 kBq per ml) in a final volume of 0.2 ml per well in 24-well plates or 1.0 ml per well in 6-well plates. pH was adjusted with CO₂ to 7.2 just before use. When KCl content was raised, NaCl concentration was decreased equally to maintain constant osmolality. Immediately before assay the culture medium was replaced by [14C]dGlc-free reaction mixture. After 15 min of preincubation at 37°C in humidified air/7% CO₂, the preincubation medium was replaced by reaction mixture containing [14C]dGlc, and one of four concentrations of K⁺ (2.7, 5.4, 28, or 56 mM), and incubation was continued for 10, 15, or 30 min.

Effects of veratridine (75 μM), monensin (10 μM), and glutamate (500 μM) were examined with K⁺ (10 mM) at 5.4 mM and 15 min of incubation. When choline was substituted for Na+ to obtain a Na⁺-free medium, or inhibitors (e.g., 1 mM ouabain, 10 μM tetrodotoxin, 1 mM DL-threo-β-hydroxyaspartic acid, 1 mM DL-2-amino-5-phosphonovaleric acid, or 100 μM CNOX) were added, cultures were first preincubated with the appropriate medium without [14C]dGlc for 15 min before final incubation with [14C]dGlc.

At end of the incubation, the reaction mixture was replaced by fresh reaction mixture lacking [14C]dGlc, and incubation was continued for 5 min to allow efflux of residual [14C]dGlc from the cells. The cell carpets were washed quickly three times with ice-cold Dulbecco’s phosphate-buffered saline and digested for 2 hr in 0.2 ml of 1 M NaOH at room temperature. Cell digests were assayed for protein content (23), and ¹⁴C concentration was measured by liquid scintillation counting with external standardization. Results are expressed as mean dpm/μg of protein ± SEM of quadruplicate wells. Separate experiments with anion-exchange (Dowex-1-formate) column chromatography confirmed that 96–98% of the total radioactivity recovered by this procedure was contained in [14C]deoxyglucose 6-phosphate and further acidic metabolites of [14C]dGlc.

RESULTS

Effects of Increased [K+]₀ on [14C]dGlc Phosphorylation. Increasing [K⁺]₀ from 5.4 to 28 and 56 mM stimulated [14C]dGlc phosphorylation in neuronal and mixed neuronal–astroglial cultures (day 6) incubated for 15 and 30 min (P < 0.05); there was a lag in the stimulation because it was not seen after 10 min of incubation (Fig. 1). In pure secondary astroglial cultures baseline rates of [14C]dGlc phosphorylation varied considerably from preparation to preparation, but no stimulation by elevated [K⁺]₀ was found in any of them (Fig. 1, Table 1). Even when the range of [K⁺]₀ was expanded from 2.7 to 56 mM, no K⁺-induced increases in rates of [14C]dGlc phosphorylation were observed in 20-day-old astroglial cultures (Table 1).

![Fig. 1. Effects of [K⁺]₀ on rates of [14C]dGlc phosphorylation in cultures of neurons (day 6), mixed neuronal–astroglia (day 6), and astroglia (day 19, no dbcAMP). Values are means ± SEM obtained from quadruplicate wells. Numbers above bars indicate the duration of incubation. Data are representative of a minimum of three such experiments for each condition. * P < 0.05; ** P < 0.01 compared with the 5.4 mM [K⁺]₀ (Dunnett’s test for multiple comparison).](image-url)
effects of increased [K⁺]₀ on rates of phosphorylation of [¹⁴C]dGlc in cultured astroglia

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Incubation time, min</th>
<th>Rate, dpm/μg of protein</th>
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<tbody>
<tr>
<td>2.7 mM [K⁺]₀</td>
<td>5.4 mM [K⁺]₀</td>
<td>28 mM [K⁺]₀</td>
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<td>20-day culture</td>
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<td>15</td>
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<td>30</td>
<td>144 ± 3</td>
<td>142 ± 2</td>
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<tr>
<td>22-day culture</td>
<td>No dbcAMP</td>
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<td>15</td>
<td>143 ± 4</td>
<td>131 ± 1*</td>
</tr>
<tr>
<td>30</td>
<td>273 ± 8</td>
<td>251 ± 3*</td>
</tr>
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<td>29-day culture</td>
<td>No dbcAMP</td>
<td>10</td>
</tr>
<tr>
<td>15</td>
<td>147 ± 2</td>
<td>144 ± 3</td>
</tr>
<tr>
<td>30</td>
<td>328 ± 3</td>
<td>283 ± 6*</td>
</tr>
<tr>
<td>22-day culture</td>
<td>No dbcAMP</td>
<td>15</td>
</tr>
<tr>
<td>+ dbcAMP-1 day</td>
<td>96 ± 3*</td>
<td>90 ± 3*</td>
</tr>
<tr>
<td>+ dbcAMP-3 days</td>
<td>116 ± 1*</td>
<td>106 ± 1*</td>
</tr>
</tbody>
</table>

Effects of treatment of cultures with dbcAMP

| 22-day culture | No dbcAMP | 15 | 73 ± 1 | 75 ± 3 | 81 ± 3 | 81 ± 8 |
| + dbcAMP-1 day | 96 ± 3* | 90 ± 3* | 100 ± 2* | 95 ± 2 |
| + dbcAMP-3 days | 116 ± 1* | 106 ± 1* | 125 ± 3* | 116 ± 7* |

All values are means ± SEM of quadruplicate wells. The results are representative of at least three experiments for each condition; the culture ages in these experiments were 19-22 days for the first experimental series, 19-22 and 26-29 days for the effects of culture age, and 19-22 days for the last group of experiments.

**Effects of Veratridine and Monensin on [¹⁴C]dGlc Phosphorylation.** Facilitating Na⁺ entry into cells with veratridine (75 μM), which opens membrane voltage-dependent Na⁺ channels, or with the Na⁺ ionophore monensin (10 μM) stimulated [¹⁴C]dGlc phosphorylation in astroglia by 20% and 171%, respectively (Table 2). Ouabain (1 mM) suppressed the basal rate of phosphorylation by 20%, completely eliminated the stimulation by veratridine, but only partially suppressed the stimulation by monensin (Fig. 2). Tetrodotoxin (10 μM) did not alter basal rates of [¹⁴C]dGlc phosphorylation in astroglia; it did, however, eliminate the effects of veratridine but had no effect on the stimulation by monensin (Fig. 2). Replacement of Na⁺ with choline eliminated the stimulations by both veratridine and monensin (Fig. 2).

**Effects of Glutamate on [¹⁴C]dGlc Phosphorylation in Astroglia.** Glutamate stimulated [¹⁴C]dGlc phosphorylation in astroglia (Fig. 3); this stimulation increased progressively with glutamate concentrations between 0 and 500 μM (data not shown). The glutamate stimulation was eliminated by 1 mM ouabain or was absent with incubations in Na⁺-free medium but was unaffected by tetrodotoxin or N-methyl-D-aspartate (NMDA) and non-NMDA receptor antagonists (Fig. 3). Addition of DL-threeo-β-hydroxyaspartate, an inhibitor of glutamate-Na⁺ cotransport (24), markedly stimulated [¹⁴C]dGlc phosphorylation by itself, and glutamate had no additional stimulating effect in its presence (Fig. 3). The stimulation by hydroxyaspartate was blocked by ouabain or incubation in Na⁺-free medium but was unaffected by tetrodotoxin and inhibitors of NMDA or non-NMDA receptors (data not shown).

**DISCUSSION**

Resting [K⁺]₀ in brain in vivo is normally ~3 mM and increases during neuronal activation (25) to ~12 mM with normal neuronal excitation and as high as 50–80 mM in seizures or spreading cortical depression (12, 13, 25). IC₅₀ in vivo is increased in all these conditions (3). In the present study, increasing [K⁺]₀ between 5.4 and 56 mM stimulated energy metabolism in neuronal and mixed neuronal-astroglial cultures but did not stimulate this metabolism in astroglial cultures over a [K⁺]₀ range of 2.7 to 56 mM.

Neuronal excitation generates action potentials produced by depolarization-induced rapid inward Na⁺ current and K⁺ efflux. The consequence increases in [K⁺]₀ and [Na⁺]₀ can then activate neuronal Na⁺,K⁺-ATPase and stimulate energy metabolism needed to restore ionic gradients to resting levels. In the present studies depolarization by elevated [K⁺]₀ stimulated glucose utilization in neurons as expected, but in astroglia glucose utilization was unaffected. Astroglia, however, do not produce action potentials, and depolarization by K⁺ might then not lead to increased [Na⁺]₀. The possibility that stimulation of energy metabolism by depolarization depended on an associated increase in [Na⁺]₀ rather than on increased [K⁺]₀, or depolarization per se was examined by the use of veratridine and monensin to increase [Na⁺]₀ in astroglia. Veratridine opens voltage-dependent Na⁺ channels (26, 27), allowing [Na⁺]₀ to increase. Because astroglial [Na⁺]₀ is ~10–20 mM, and the Km of Na⁺,K⁺-ATPase for Na⁺ is ~10 nM (28), astroglial Na⁺,K⁺-ATPase can readily respond to elevated [Na⁺]₀. Veratridine did indeed stimulate glucose metabolism not only in neurons (data not shown) but also in astroglia, and this stimulation was completely blocked by ouabain, indicating that it depended on Na⁺,K⁺-ATPase activity. The veratridine stimulation of [¹⁴C]dGlc phosphorylation was also blocked by tetrodotoxin, which blocks voltage-dependent Na⁺ channels, indicating that the stimulation was mediated by Na⁺ entry through these Na⁺ channels. Monensin, a carboxylic Na⁺ ionophore that mediates exchange of external Na⁺ for internal H⁺, thus raising Na⁺ and lowering H⁺ concentrations in the cells (29), also stimulated [¹⁴C]dGlc phosphorylation, but this stimulation was only partially blocked by ouabain. Both increased [Na⁺]₀ and decreased intracellular H⁺ concentration, however, should stimulate glucose utilization—the former by activating Na⁺,K⁺-ATPase activity, which is ouabain-sensitive, and the latter by stimulating glycolysis through pH effects on
phosphofructokinase (30), which is not ouabain-sensitive. These results agree with those of Erecinska et al. (31) in rat synaptosomes but agree only partly with those of Yarowsky et al. (16), who found the stimulation of [14C]dGlc uptake by monensin in astroglia to be completely inhibited by ouabain.

The fact that [14C]dGlc phosphorylation in astroglia is not stimulated by increased [K+]o, but is stimulated by veratridine or monensin-induced increases in [Na+]o, indicates that energy metabolism in astroglia can be stimulated, but the stimulation requires an increase in intracellular Na+ concentration. Astroglial membrane can be depolarized by increased [K+]o, but the depolarization is not associated with action potentials (32) and may, therefore, not lead to increased Na+ entry into the cells. This result may be due to either low density or different properties of voltage-dependent Na+ channels in astroglia (33). Barres et al. (34) observed that type I astrocytes prepared from optic nerve do have voltage-dependent Na+ channels, but they open at more negative potentials and more slowly than neuronal Na+ channels in response to depolarization. Also, Hisanaga et al. (35) have reported that high [K+]o elicits c-fos expression in neurons but does not elicit it in astroglia, further indicating that neurons and astroglia may respond differently to increased [K+]o.

The failure of increased [K+]o to alter energy consumption in astroglia also indicates that K+ uptake by astrocytes does not require energy. That astroglia do take up K+ is manifested by the ability of an astroglial syncytium to move K+ from areas of neuronal stimulation to areas of lower [K+]o, e.g., spatial buffering of K+ (33). Also, astroglia in primary cultures have been reported to take up K+ avidly (32, 36), and the uptake rate is higher than that of neurons (37). Astroglial membranes, however, have a much higher K+ conductance than that of neurons, and K+ uptake may, therefore, not be energy-dependent.

cAMP and its analogue, dbcAMP, induce morphological differentiation in cultured astroglia (38), and dbcAMP has been reported to induce increased Na+,K+-ATPase activity (39). dbcAMP did increase the basal rate of [14C]dGlc phosphorylation in our astroglial cultures, but increased [K+]o still did not stimulate this rate. Age of the culture could be a factor. Central nervous system cultures become more anaerobic and consume glucose more rapidly with age of the culture (40), and Na+,K+-ATPase has been reported to require 28 days in culture to reach mature differentiation (39). In our studies the basal rate of [14C]dGlc phosphorylation was indeed higher in 26- to 29- than in 19- to 22-day-old cultures but was not stimulated by increased [K+]o, in either.

Contrary to our results, there have been reports that increased [K+]o stimulates [14C]dGlc uptake in cultured astroglia (18–22). The reasons for this discrepancy are not obvious. Differences in the cell cultures could possibly be involved. The astroglia derived from the cortex of newborns that we used were secondary cultures and were very pure; they contained no neurons and virtually no cells without glial fibrillary acidic protein (GFAP). Some of our primary astroglial cultures, particularly those from embryonic tissue, appeared to be pure when tested with anti-GFAP alone, but staining with anti-GFAP plus antivimentin revealed the presence of vimentin+/GFAP− cells. Such cells were never found in the secondary cultures. Inasmuch as mixed neuronal–astroglial cultures do
exhibit increased [14C]dGlc phosphorylation in response to elevated [K+]o, contamination with other cell types must be considered in the reported appearance of metabolic responses in primary astroglial cultures to increased [K+]o. Furthermore, neurons influence astrocytic differentiation in vitro (41). For example, [3H]ouabain binding in cultured astroglia, which reflects the level of the functional alpha2/alpha3 catalytic subunit of Na+,K+-ATPase, has been reported to be low in pure astrocytic cultures and much higher in neuron-conditioned mixed cultures (42); this suggests an effect of neurons on the level or properties of Na+,K+-ATPase in astroglia. Glutamate uptake in astroglia is also linked to increased function-related spike activity in neuropil. The present results indicate that neuronal elements can respond with increased glucose utilization to changes in the extracellular ionic environment associated with spike activity (i.e., increased [K+]o), astroglial energy metabolism is, however stimulated by increased Na+ entry and not increased [K+]o. Spike activity in axonal terminals has, however, additional consequences to those on [K+]o; it also leads to release of neurotransmitters that could activate Na+ entry into astrocytes and thus stimulate astroglial energy metabolism. Glutamate is the most common and widely distributed excitatory neurotransmitter in the central nervous system. Astroglia actively take up glutamate, and at least three different Na+-dependent glutamate transporters have been cloned (44–46); one is expressed mainly in astrocytes (47). Glutamate uptake in astroglia is associated with cotransport of Na+, leading to increased [Na+]o, and depolarization (48), and, according to the present studies, stimulation of glucose utilization. This stimulation of metabolism is eliminated when Na+ in the external medium is replaced by choline. These results are in accord with a recent report by Pellerin and Magistretti (49). It would appear then that astroglia can participate in the increased energy metabolism associated with functional activity; in contrast to neurons, however, the stimulation is due not to increased [K+]o but rather to another mechanism, such as the release of glutamate and/or possibly other neurotransmitters that promote Na+ entry into the cells.