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Research Report

A pH-dependent increase in neuronal glutamate efflux in vitro: Possible involvement of ASCT1

Michael D. Weiss [a,*], Candace Rossignol [a], Colin Sumners [c], Kevin J. Anderson [a,b,d]

[a] Department of Pediatrics, PO Box 100296, University of Florida, Gainesville, FL 32610-0296, USA
[b] Department of Physiological Sciences, University of Florida, Gainesville, FL 32610-0296, USA
[c] Department of Physiology and Functional Genomics, University of Florida, Gainesville, FL 32610-0296, USA
[d] Mcknight Brain Institute, University of Florida, Gainesville, FL 32610-0296, USA

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Abstract

Efflux of glutamate from intracellular pools during hypoxia–ischemia has been postulated to be mediated by amino acid transporters and can lead to excitotoxicity. In addition, a decrease in pH seen during global hypoxia–ischemia may influence which transporter is responsible for this glutamate efflux. For example, the neutral amino acid transporter ASCT1 is an effective transporter of glutamate at low pH. We have examined the effects of pH, pH and temperature, and hypoxia on glutamate efflux in a rat primary neuronal cell culture model. We observed a marked increase of glutamate efflux as pH was decreased from 7.4 to 5.5. This pH-dependent efflux is likely due to a transporter-mediated process because it was seen in the presence of tetrodotoxin and was blunted by decreasing the temperature to either 35 °C or 33 °C. In addition, no increase in LDH was seen at pH 5.5 suggesting that increased glutamate levels were not due to cellular death. No change in glutamate levels was seen when the oxygen tension of the medium was lowered from 150 mm Hg to either 30 or 15 mm Hg. Given that EAAT transporters are inhibited by low pH, other transporters, such as ASCT1, may be responsible for this pH-dependent efflux of glutamate.

Theme: Neurotransmitters, Modulators, Transporters, and Receptors
Topic: Uptake and transporters

Keywords: Amino acid transport; Glutamate; Efflux; pH; Neurotoxicity

1. Introduction

Glutamate is the primary excitatory neurotransmitter in the brain and the concentration in the neurosynaptic junction is tightly regulated. If the glutamate level rises above 100 μM for more than a few minutes, neuronal death is triggered [1]; a process termed "excitotoxicity" (Reviewed by [8]). Microdialysis measurements have indicated that extracellular levels of glutamate are increased during global hypoxia–ischemia [3,12] and the tissue pH drops to as low as 6.2 [22,33].

Glutamate over-stimulation of the post-synaptic neuron leads to cell damage and death during global hypoxia–ischemia. What is the cellular mechanism by which abnormally high levels of glutamate are released into the neurosynaptic junction during hypoxic–ischemic states? Several possibilities exist: (1) Ca^{+}-dependent release of vesicular glutamate [10,19,20]. (2) A calcium-independent release of cytoplasmic glutamate that may be mediated by a transporter [1,24], and/or (3) Lysis of dead cells, resulting in escape of cytoplasmic glutamate [26]. Of these mechanisms, transporter-dependent efflux may be the more important during global hypoxia–ischemia for two reasons. First, neuronal vesicular release of glutamate is blunted during anoxia due to the release of adenosine that

* Corresponding author. Fax: +1 352 392 4533.
E-mail address: weissmd@peds.ufl.edu (M.D. Weiss).

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reduces presynaptic calcium influx [1,13]. Second, after a few minutes of anoxia/ischemia, $[K^+]_0$ rises to about 60 mM, depolarizing cells to approximately −20 mV, while $[Na^+]_0$ falls by about the same amount as $[K^+]_0$ rises [1]. These ionic changes could promote the operation of a Na^+-dependent transporter to favor efflux. Under these conditions, it has been estimated to take approximately 3 s to raise the neurosynaptic levels of glutamate to a toxic level of 100 μM [1].

Recently, we have described the density and localization of ASCT1, a neutral amino acid transporter, in the developing brain [40]. ASCT1 was localized to both neurons and glia in the developing brain and to areas of the brain that are vulnerable to neuronal injury during hypoxic–ischemic encephalopathy (HIE). ASCT1 demonstrates a unique property by switching substrates as the pH is lowered from 7.5 to 5.5 [37]. At pH 7.5 ASCT1 primarily exhibits affinity for neutral amino acids and is a relative poor mediator of anionic amino acid transport. As the pH drops, ASCT1 becomes much more efficient transporter of anionic amino acids such as glutamate. Given that the primary glutamate transporters, EAATs (EAAT1-3), do not function well at a lower pH [5], we hypothesized that ASCT1 is a key amino acid transporter in mediating the glutamate efflux at the lower pH values seen during HIE. As an initial step to study the possible physiologic role of ASCT1 in effluxing glutamate during hypoxia–ischemia, we have examined the effects of pH, temperature, and oxygen concentration on glutamate efflux from neurons in vitro.

2. Materials and methods

2.1. Cell culture

Primary neuronal cortical cultures were obtained from 1-day-old Sprague–Dawley rats and grown for 14 days in a 37 °C humidified incubator with 90% air–10% $C0_2$. The cultures yield a mixture of 90% neurons and 10% glia (primarily astrocytes). Full details of the neuronal isolation and culture have been described [7].

2.2. pH experiments

Cells were washed twice with artificial cerebrospinal fluid (ACSF) (122 mM NaCl, 3.3 mM KCl, 1.2 mM $CaCl_2$, 0.4 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 5 mM glucose) for 1 min each while the plates were floated in a water bath warmed to 37 °C. The final wash was performed for 5 min at 37 °C using ACSF with tetrodotoxin (TTX, 0.1 μM). The cells were then exposed to 1 mL of ACSF with TTX (0.1 μM) at pH 7.4, 7.0, 6.5, 6.0, and 5.5 at 37 °C. One pH condition was chosen per plate ($n = 4$ pH 7.4, 7.0, 6.5, and 6.0 at all time points, $n = 5$ for pH 5.5 at all time points). ACSF samples (120 μL) were taken at 0, 30, 60, 90, and 120 s and underwent analysis for glutamate content using a Amplex Red glutamic acid/glutamate oxidase assay kit (Molecular Probes—see below). Following the final sample at 120 s, 250 μL of ACSF was removed for LDH analysis to validate that potential differences in glutamate levels between pH values was not due to cell lysis. The cells were then harvested from the plates for protein analysis.

2.3. Temperature experiments

To study the effects of temperature on the efflux of glutamate, cells were washed twice with ACSF (122 mM NaCl, 3.3 mM KCl, 1.2 mM $CaCl_2$, 0.4 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 5 mM glucose) for 1 min each while the plates were floated in water bath warmed to 37 °C. The final wash was performed for 5 min using ACSF with TTX (0.1 μM) at 37 °C. The cells were then exposed to 1 mL of ACSF with TTX (0.1 μM) at pH 7.4 or 5.5 at 37, 35, and 33 °C. One temperature and pH condition was chosen per plate ($n = 4$ for all pH, temperatures and time points with the exception of pH 5.5 at 37 °C, $n = 5$). The temperature of the ACSF was manipulated to see if temperature had an effect on glutamate efflux between the two pH values. 120 μL samples of the ACSF were taken at 0, 30, 60, 90, and 120 s and underwent analysis for glutamate content using an Amplex Red glutamic acid/glutamate oxidase assay kit (Molecular Probes—see below). Following the final sample at 120 s, 240 μL samples of ACSF was removed for LDH analysis to validate that potential differences in glutamate levels between pH values was not due to cell lysis. The cells were then harvested for protein analysis.

2.4. Hypoxia

To study the effects of hypoxia on the efflux of glutamate, cells were washed twice with ACSF (122 mM NaCl, 3.3 mM KCl, 1.2 mM $CaCl_2$, 0.4 mM $MgSO_4$, 1.2 KH_2PO_4, 25 mM $NaHCO_3$, 5 mM glucose) for 1 min each while the plates were floated in a water bath warmed to 37 °C. The final wash was performed for 5 min using ACSF with TTX (0.1 μM) at 37 °C. The cells were then exposed to 1 mL of ACSF with TTX (0.1 μM) at 37 °C at a pH 7.4 and placed in a Billups–Rothemberg chamber (Billups–Rothemberg, Del Mar, California). The chamber was modified to allow the insertion of probes for monitoring and sampling. Monitoring was performed using a Paratrend monitoring system (Diametrics Medical, Roseville, Minnesota). This apparatus allows for real time continuous monitoring of pH, pO_2, pCO_2, and base deficit. The chamber was flushed with a gas mixture of 1% O_2, 5% CO_2, 94% N_2 continuously. The gas was warmed to 37 °C before entry into the chamber. When the pO_2 reached the desired oxygen concentration (either 30 mm Hg or 15 mm Hg), 120 μL samples were drawn from