Effects of the Na\(^+\)/H\(^+\) exchanger monensin on intracellular pH in astroglia

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Abstract

Stimulation of astroglial glucose utilization by the Na\(^+\)/H\(^+\) exchanger monensin is only partially blocked by ouabain. The present studies show that monensin also raises intracellular pH in astroglia. Because increased pH stimulates phosphofructokinase activity, the ouabain-insensitive portion of the stimulation of cerebral glucose utilization (CMR\(_{\text{glc}}\)) appears to be due to stimulation of glycolysis by intracellular alkalinization.

Theme: Other systems of the CNS

Topic: Brain metabolism and blood flow

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Changes in the rate of local cerebral glucose utilization (CMR\(_{\text{glc}}\)) are linearly related to changes in spike frequency in the terminal zones of an activated neural pathway [6]. CMR\(_{\text{glc}}\) has also been reported to be stoichiometrically coupled to the rate of glutamate–glutamine cycling [4]. Because both neuronal firing and/or glutamate release are generally associated with functional activation, measurement of local CMR\(_{\text{glc}}\) is often used to map local changes in neuronal functional activity [5]. Because the effects of stimulation of a pathway, e.g. the hypothalamic–neurohypophyseal pathway, on local CMR\(_{\text{glc}}\) are inhibited by ouabain, a specific inhibitor of Na\(^+\),K\(^+\)-ATPase, the increased energy metabolism is attributed to activation of Na\(^+\),K\(^+\)-ATPase activity, in the neurons to restore the ionic gradients partially degraded by the spike activity [2] and in the astroglia by the Na\(^+\)-dependent uptake of glutamate released by the nerve terminals [3,4,7]. There are, however, processes other than Na\(^+\),K\(^+\)-ATPase activity that can require energy. For example, the conversion of the glutamate taken up by the astroglia to glutamine by glutamine synthetase consumes ATP. Also, the Na\(^+\) ionophore monensin stimulates astroglial CMR\(_{\text{glc}}\) [7,10], but, unlike the stimulation produced by opening voltage-dependent Na\(^+\) channels with veratridine, this stimulation is only partially sensitive to Na\(^+\),K\(^+\)-ATPase inhibition by ouabain [7]. Because monensin is a Na\(^+\)/H\(^+\) exchanger, the ouabain-sensitive component of the stimulation of CMR\(_{\text{glc}}\) can be explained by Na\(^+\) entry into the cells. The ouabain-insensitive part was postulated to be due to intracellular alkalinization [7] because phosphofructokinase activity, the rate-limiting step in glycolysis, is known to be stimulated by increased pH [9]. The present study was undertaken to determine if monensin does indeed raise intracellular pH (pH\(_i\)) in astroglia under the conditions in which it stimulates CMR\(_{\text{glc}}\). The results demonstrate that it does.

All procedures on animals were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the National Institute of Mental Health Animal Care and Use Committee. Pregnant Sprague–Dawley rats were purchased from Taconic Farms (Germantown, NY, USA). Astroglial cultures were prepared from the cerebral cortex of the newborn pups. After removal of the meninges and blood vessels from brains, the fronto-parietal cortices were dissected out and mechanically disrupted by passage of the tissue through a 22-gauge hypodermic needle. The dissociated cells (2.5 × 10\(^5\) cells/ml) were cultured at 37°C in
humidified air/7% CO₂ in uncoated 75 cm² culture flasks (Costar Corp., Cambridge, MA, USA) containing high-glucose Dulbecco’s Modified Eagle Medium (DMEM) (Life Technologies, Grand Island, NY, USA), 10% (v/v) fetal bovine serum (HyClone Laboratories, Inc., Logan, UT, USA), penicillin (100 units/ml), and streptomycin (100 µg/ml) (Life Technologies). The culture medium was changed every 2 days until the cultures reached confluence, generally about 1 week. The flask was then shaken overnight at room temperature to eliminate loosely adherent process-bearing cells. The adherent cells were washed with Ca²⁺- and Mg²⁺-free Dulbecco’s phosphate-buffered saline (DPBS) (Life Technologies), treated for 1–2 min at 37°C with 0.5% (w/v) trypsin (ICN Biochemicals, Aurora, OH, USA) in DPBS, suspended in four volumes of fresh culture medium, and placed in 35 mm glass bottom microwell dishes (MatTek Corp., Ashland, MA, USA) for pH measurement. The culture medium was changed every 3 days, and the cultures were allowed to reach confluence and used after 18–22 days in secondary culture. Some of the secondary culture cells were also plated in 6-well culture plates and examined immunohistochemically with antibodies against glial fibrillary acidic protein (GFAP) (DAKO, Carpinteria, CA, USA) and α-vimentin (Roche Molecular Biochemicals, Indianapolis, IN, USA).

The cells in the glass bottom culture dish were incubated with the fluorescent dye 2’,7’-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF)-acetoxymethylester (10 nM) (Molecular Probes, Eugene, OR, USA) at 37°C for 15 min. After washout of extracellular dye, the culture dish was mounted in an inverted fluorescent microscope (Leitz, Wetzlar, Germany) fitted with a Nikon objective lens (Fluor 40 Ph3DL). Excitation light at 495 and 440 nm was alternately projected from a mercury arc lamp (#688.50, Oriel Instruments, Stanford, CT, USA) by means of a Lambda 10 optical filter changer (Sutter Instrument Co., Novato, CA, USA), and fluorescent light at 535 nm was captured by a signal intensifier (VS2525, Video Scope International, Ltd, Sterling, VA, USA) and CCD camera (CCDC72, Dage-MTI, Inc., Michigan City, IN, USA). Images were recorded and stored by means of image-analyzing software (Synapse-IP, Synergy Research Inc., Monrovia, MD, USA) and a Power Macintosh computer. The region of interest (300×500 µm) for measuring average fluorescent intensities was fixed, and the ratio of fluorescence intensity obtained with excitation light at 495 nm to that obtained at 440 nm was calculated. Astroglial cultures were first incubated with Hanks’ balanced salt solution (HBSS) containing 2 mM glucose for 15 min of pH measurement. The medium was then replaced by HBSS containing 2 mM glucose and 10 µM monensin, and the pH of the pH was measured for the next 15 min. The pH of both media was adjusted to pH 7.20 before use.

The relationship between pH and the fluorescent intensity ratio was calibrated by the high-K⁺ nigericin method [8], as adapted for use in cultured rat astroglia [1]. Calibration curves were determined by incubation of the cultures for 10 min in the same media as those normally used in the pH assays, except for the addition of 106 mM K⁺ and 10 µM nigericin at several standard pH values, i.e. 7.0, 7.6, and 8.4. Two complete sets of calibrations were

![Fig. 1](image-url)  
Fig. 1. Representative calibration curve for pH in the range of 7.0–8.4 with respect to the ratio of fluorescence intensities obtained with excitation light at 495 nM to that obtained at 440 nm. The curve was calculated by least squares best-fit of the quadratic equation.

\[ \text{pH} = 2.29R^2 - 2.39R + 7.09 \]
carried out and averaged. A standard calibration curve was obtained from a computed least squares best-fit of a quadratic equation to the pH values and the corresponding measured intensity ratios (Fig. 1). In the pH assays pH\textsubscript{i} was calculated by this equation from the ratio of the signal intensities at 495 and 440 nm (Fig. 1).

The time course of a representative assay is shown in Fig. 2. With the cells in the culture medium pH\textsubscript{i} remained relatively stable for at least 15 min. When the control incubation medium was replaced with similar culture medium containing 10 μM monensin, pH\textsubscript{i} rose steeply from 7.23 to 7.32 within 2 min. Replacement with culture medium alone had negligible effects on pH\textsubscript{i}. For each well the individual pH\textsubscript{i} values obtained during the 10–15 min period following addition of medium were averaged to obtain a mean pH\textsubscript{i} for that well under the control and monensin-treated conditions. A total of four wells were assayed, and the pH\textsubscript{i} values in each of them before and after monensin addition are graphically presented in Fig. 3. In the control medium the astroglial pH\textsubscript{i} was 7.25±0.03 (mean±S.E.M., n=4), and after addition of 10 μM monensin, the mean pH\textsubscript{i} rose statistically significantly to 7.33±0.03 (P<0.05, paired t-test).

Phosphofructokinase activity is extremely sensitive to small changes in pH, an increase in pH leading to greater affinity of the enzyme for its substrate fructose-6-phosphate (F-6-P) [9]. The magnitude of the effect depends on many other factors, such as the concentrations of F-6-P, AMP, etc. [9]. Without knowledge of these potentially influential intracellular factors it is difficult to relate

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**Fig. 2.** Representative time course of pH\textsubscript{i} in astrocytes during incubation in control medium and following addition of monensin. pH\textsubscript{i} increased steeply from 7.23 to 7.32 within 2 min after replacement of control medium with one containing 10 μM monensin. Replacement with control medium alone had no significant effect.

**Fig. 3.** Effects of monensin on pH\textsubscript{i} in astrocytes in four wells. Each line represents pH\textsubscript{i} from one well averaged over the 10–15 min period following medium change. The mean pH\textsubscript{i} in all four wells increased from 7.26±0.03 (mean±S.E.M.) to 7.33±0.03 (P<0.05, paired t-test).
accurately the changes we observed in $\text{pH}_i$ to the ouabain-insensitive increases in $\text{CMR}_{\text{glc}}$ caused by monensin [7]. Under most of the conditions examined [9], however, a rise in $\text{pH}_i$ of as much as 0.08 units is sufficient to enhance phosphofructokinase activity several fold, more than enough to explain the less than 100% monensin-induced ouabain-insensitive increase in astroglial glycolysis. These findings also reinforce the caveat that not all increases in local cerebral glucose utilization or associated changes in blood flow should be interpreted as evidence of increased functional neuronal activity. Other intracellular processes that influence glucose utilization may also be operating.

References


