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Effect of astroglial cell swelling on pH of acidic intracellular compartments

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Abstract

A variety of pathological conditions lead to swelling of astrocytes, which in turn stimulates ion release by activation of ion channels at the plasma membrane. In the present study, acridine orange and fluorescein isothiocyanate coupled to dextran (FITC-dextran) have been used to examine the effect of cell swelling on pH in acidic compartments of cultured astroglial cells. Both NH_4Cl (2 mM) and chloroquine (10 μM), known to alkalinize acidic cellular compartments, led to the expected increase in acridine orange fluorescence intensity. Similar, albeit smaller, effects were elicited by a reduction of extracellular osmolarity (−80 mOsm) and treatment of the cells with glutamate (1 mM), manoeuvres which enhanced cell volume. Determination of changes in the FITC-dextran fluorescence ratio (485/440 nm) allowed quantification of the pH changes in lysosomal compartments. Treatment with NH_4Cl, reduced extracellular osmolarity and glutamate increased lysosomal pH by 0.65 ± 0.07, 0.85 ± 0.14 and 0.25 ± 0.07, respectively. Measurement of cytosolic pH using 2',7'-bis-(2-carboxyethyl)-5- (and -6) carboxyfluorescein (BCECF) demonstrated a pronounced acidification following cell swelling, observed with both reduced extracellular osmolarity (by 0.23 ± 0.05 pH units) and 1 mM glutamate (by 0.26 ± 0.02 pH units). In conclusion, pH within lysosomes and possibly other acidic cellular compartments of astrocytes is increased by cell swelling, which may have important consequences for astrocyte function.

Keywords: Glutamate; Chloroquine; NH_4^+; Cell volume; Cytosolic pH; Lysosome

1. Introduction

Astrocytes may undergo swelling in the course of a number of pathological conditions such as cytotoxic brain edema [1], hypoglycemia [2], status epilepticus [2], ischemia [3], hypoxia [4], acidosis [5], hepatic encephalopathy [6], allergic encephalopathy [7] and head injury [8]. Cell swelling has been shown to activate ion channels in the plasma membrane of astrocytes allowing the release of ions [9]. Furthermore, cell swelling stimulates the release of cellular amino acids [10]. In liver cells, swelling has been shown to interfere with a variety of metabolic functions including proteolysis and glycogenolysis [11]. It has been demonstrated that cell swelling leads to an alkalinization of hepatic cellular lysosomes [12–15]. Since hepatic proteolysis resides largely within acidic lysosomes and is accomplished by pH-sensitive lyso-

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somal proteinases [16], the alkalinization of acidic intracellular compartments probably accounts for the antiproteolytic action of cell swelling. Further, the recycling of receptors to the membrane surface has been described as being hindered by weak bases, presumably through their alkalinization of lysosomal compartments [17]. Nothing is known about the influence of cell volume on pH in acidic intracellular compartments of brain cells. The present experiments have been performed to test whether swelling of glial cells modifies the pH in these compartments.

2. Materials and methods

Astroglial primary cultures were prepared as described previously [18,19]. Both mouse and rat astroglia-rich cultures [18] are dominated by astroglial cells and contain only a minority of oligodendroglial, microglial and ependymal cells. No neurons are present in these cultures. In addition, pure mouse astroglial cells [19] were investigated. Cells were used for experiments between days 10 and 16 after seeding. The results did not depend on culture age. Prior to experiments, cells were maintained in 90% Dulbecco's modified Eagle's medium/10% fetal calf serum under conditions of 10% CO_2/90% air at 37°C. For experimental incubations, the cells were bathed in extracellular fluid (H/S) composed of (in mM) 145 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 0.8 Na_2HPO_4, 20 Hepes adjusted to pH 7.4 and maintained at 37°C. Where indicated, osmolarity of the medium was reduced by omission of 40 mM NaCl. Reduced NaCl was substituted by 80 mM raffinose in control solution. Glutamate and chloroquine (Sigma Chemie, Deisenhofen, Germany) were used in final concentrations of 1 mM and 10 μM, respectively.

Cell volume was determined using a Coulter counter [20] with a capillary of 150 μm diameter (Casy 1 Model, Schärfe System, Reutlingen, Germany) after detaching the cells with 0.05% trypsin and resuspending them in Hepes buffer of a given osmolarity. As outlined previously [20], the method allows the determination of cell volume changes of less than 1%.

For depiction of pH changes in acidic cellular compartments acridine orange [21] and FITC-dextran [22] were used. Acridine orange accumulates in all acidic compartments of the cell including endocytic vesicles, lysosomes, certain secretory vesicles and portions of the trans-Golgi apparatus [23]. For determination of acridine orange fluorescence, the cells were incubated for 15 min with 10 μM acridine orange hydrochloride (Sigma) prior to the experiments. Light of 490 nm wavelength from a monochromator light source (Uhl, München, Germany) was directed through grey-filters (nominal transmission 1%, Oriel, Darmstadt, Germany) and was deflected by a dichroic mirror (FT 515 nm, Omega Optical, Brattleboro, VT, USA) into the objective (Plan-Neofluar 40 X, Zeiss, Oberkochen, Germany). The emitted fluorescence was directed through a 530 nm cut-off filter to a photomultiplier tube (213-1P28A, Seefelder Meßtechnik, Seefeld, Germany). Increases in the acridine orange fluorescence intensity were indicative of an alkalinization of all acidic intra-

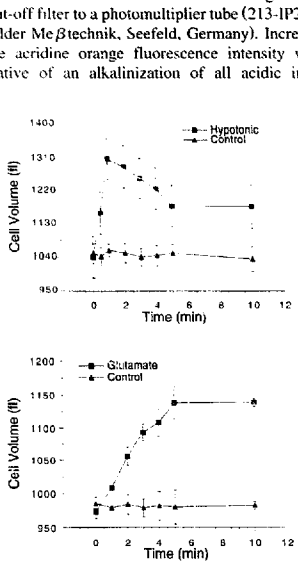

Fig. 1. Alteration in mean cell volume of rat astroglia-rich cell suspensions following treatment with either reduced extracellular osmolarity (hypotonic solution, 40 mM NaCl, upper panel) or glutamate (1 mM, lower panel). Cell volume was measured in a Coulter counter (arithmetic means ± S.E.M., $n = 4$ for each treatment).

cellular compartments. The effect of the various treatments on the pH of acidic intracellular compartments was determined by comparison of the mean acridine orange fluorescence intensity under control conditions to the mean maximal effect following initiation of treatment.

For quantitative lysosomal pH measurements, the cells were loaded with 70 μM FITC-dextran (Sigma) overnight followed by a 2-h wash in media without dye. The long wash incubation allows for the transport of dye through primary and secondary endosomal compartments following its endocytosis to the lysosomes such that the dye is mainly concentrated there. Thus, the pH measured using FITC-dextran largely reflects that of the lysosomes. Excitation light was alternated between wavelengths of 485 and 440 nm, and a dichroic mirror with 521 nm cut-off (Omega) and two emission filters of 535 nm (Ramon bandpass, Omega) and 515 nm (longpass, Schott, Mainz, Germany) were used. Specific vesicular pH was calculated following calibration of the dye [22]. Briefly, cells were superfused with a solution containing 105 mM KCl, 1 mM $MgCl_2$, 6 mM Na_2HPO_4/NaH_2PO_4 (with pH ranging from 5 to 6.5) and 10 μM nigericin. The fluorescence ratios at 485/440 were linear with vesicular pH between 5 and 6.5.

For cytosolic pH measurements, cells were incubated for 30 min with 1 μM 2',7'-bis-(2-carboxyethyl)-5- (and-6) carboxyfluorescein-methyl ster

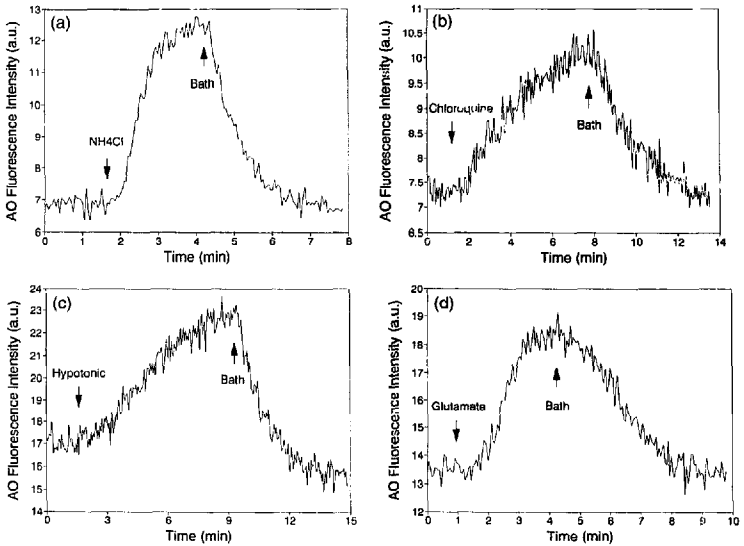

Fig. 2. Influence of 500 μM NH_4Cl (a), 10 μM chloroquine (b), reduced extracellular osmolarity (hypotonic, c) and 1 mM glutamate (d) on acridine orange (AO) fluorescence intensity in a pure population of mouse astroglial cells. Downward arrows indicate introduction of treatment solutions, while upward arrows indicate return to normal bath solution. Original tracings representative of 9, 4, 12, and 10 similar experiments, respectively (a.u. = arbitrary units).

(BCECF-AM, Molecular Probes, Eugene, OR, USA), followed by a 30 min incubation in media without dye. The same microspectrophotometry system was utilized as described for acridine orange and FITC measurements, using the same excitation light and emission filter configuration as for that of FITC. Cytosolic pH was calibrated with the high potassium/nigericin technique [22]. Briefly, cells were superfused with solutions containing 105 mM KCl, 1 mM $MgCl_2$, 30 mM Hepes (with pH ranging from 6.5 to 7.5) and 10 μM nigericin. The fluorescence ratios at 485/440 were linear with cytosolic pH between 6.5 and 7.5.

In order to reduce the region from which fluorescence was collected, a pinhole (diameter 1.5 mm) was placed in the image plane of the phototube. Data acquisition was executed using a computer program (IMG 8, Lindemann und Meiser, Homburg, Germany). Fluorescence in the absence of fluorescent dye was less than 1% of the values in the presence of the dye and was not significantly modified by the experimental manoeuvres. Fluorescence at > 630 nm did not increase following the above manoeuvres.

Data are expressed as arithmetic means ± standard error of the mean (S.E.M.). Statistical analysis was made by paired or unpaired *t*-test, where applicable. Statistically significant differences were assumed when $P < 0.05$.

3. Results

Exposure of cell suspensions derived from rat astroglia-rich cultures to reduced extracellular osmolarity (−40 mM NaCl) resulted in a rapid increase of mean cell volume from 1040 ± 50 fl ($n = 4$) to 1308 ± 55 fl ($n = 4$), followed by a partial regulatory cell volume decrease to 1178 ± 53 fl (Fig. 1). Glutamate (1 mM) treatment induced a more gradual cell volume increase from 974 ± 10 fl ($n = 4$) to 1138 ± 25 fl ($n = 4$) within 5 min, which was sustained for the 10 min period of volume measurement (Fig. 1).

An increase of acridine orange fluorescence in astroglial cells was triggered by both NH_4Cl and chloroquine. In an astroglia-rich population of mouse glial cells, acridine orange fluorescence intensity increased by 156 ± 4% ($n = 7$) within 4 min following application of NH_4Cl (? mM), and by 50 ± 3% ($n = 7$) within 5 min following addition of chloroquine (10 μM). In a pure population of mouse astroglial cells, 500 μM NH_4Cl induced an increase of acridine orange fluorescence intensity by 74 ± 1% within 4

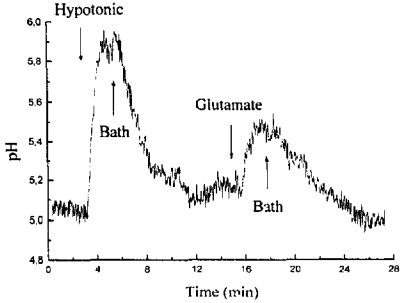

Fig. 3. Influence of reduced extracellular osmolarity and of 1 mM glutamate on lysosomal pH as indicated by measurements using FITC-dextran in a rat astroglia-rich culture. Specific pH was calibrated from ratiometric fluorescence measurement at wavelengths 485/440 nm. Original tracing representative of 5 and 4 similar experiments for reduced extracellular osmolarity and glutamate treatment, respectively.

min ($n = 9$; Fig. 2a), while 10 μM chloroquine increased it by 34 ± 2% within 5 min ($n = 4$; Fig. 2b).

Osmotic cell swelling due to reduced extracellular osmolarity increased acridine orange fluorescence by 41 ± 1% within 6 min ($n = 11$) in an astroglia-rich population of mouse glial cells and by 38 ± 1% within 6 min ($n = 12$) in a pure population of mouse astroglial cells (Fig. 2c).

Iso-osmotic swelling due to the addition of glutamate also led to an increase of acridine orange fluorescence intensity. In an astroglia-rich population of rat glial cells, acridine orange fluorescence increased by 34 ± 5% ($n = 4$) within 6 min following treatment with 1 mM glutamate. The same treatment in an astroglia-rich population of mouse glial cells elicited an increase of acridine orange fluorescence by 36 ± 2% within 6 min ($n = 4$). In a pure population of mouse astroglial cells, 1 mM glutamate increased acridine orange fluorescence intensity by 42 ± 1% within 6 min ($n = 10$; Fig. 2d).

FITC-dextran measurements were carried out in an astroglia-rich population of rat glial cells. Treatment with 2 mM NH_4Cl increased lysosomal pH from a control level of 5.45 ± 0.11 to a maximum of 6.09 ± 0.11 ($n = 7$). Osmotic cell swelling due to reduced extracellular osmolarity increased lysosomal pH from 5.03 ± 0.04 to 5.89 ± 0.10 ($n = 5$, Fig. 3). Iso-osmotic swelling due to the addition of 1 mM glutamate also led to an increase in lysosomal pH, from 5.07 ± 0.06 to 5.31 ± 0.07 ($n = 4$, Fig. 3).

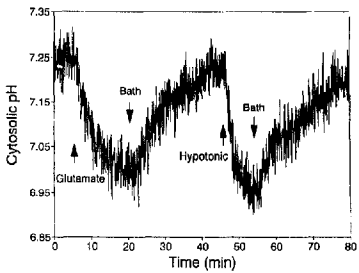

Fig. 4. Influence of reduced extracellular osmolarity and 1 mM glutamate on cytosolic pH in a rat astroglia-rich primary culture. pH was determined using the BCECF fluorescence intensity ratio at 485/440 nm. Original tracing representative of 5 similar experiments.

As determined from BCECF fluorescence, cytosolic pH in an astroglia-rich population of rat glial cells decreased from pH 7.00 ± 0.05 to 6.77 ± 0.04 ($n = 5$) following reduced extracellular osmolarity and from pH 7.17 ± 0.04 to 6.91 ± 0.05 ($n = 5$) following treatment with 1 mM glutamate (Fig. 4).

4. Discussion

Acridine orange has been demonstrated to be an effective fluorescent dye for the measurement of pH changes in acidic intracellular compartments [21]. The dye diffuses across membranes readily in the non-protonated, but not the protonated form, and is consequently trapped in all acidic compartments where it emits a weak orange fluorescence upon excitation at 490 nm. The non-protonated form of the dye which exists in alkaline fluid, however, emits an intense green fluorescence upon excitation at the same wavelength. Therefore, any alkalinization of acidic intracellular compartments results in a release of the dye and a subsequent increase in fluorescence intensity measured at 490 nm. However, the sustainment of the pH at an already alkaline pH also results in a further release of the dye and a further increase in fluorescence intensity, provided that the compartments were acidic enough to allow efficient loading. This property of the dye thereby prevents the determination of time course of pH changes. For this reason, FITC-dextran was utilized due to its properties allowing for the precise determination of absolute pH in acidic intracellular compartments, and thereby the time course of its alteration.

NH_4Cl, which alkalinizes acidic cellular compartments due to trapping of H^+ by NH_3 [24], elicited the expected increase of acridine orange fluorescence in cultured glial cells. The concentration of NH_4Cl to elicit this effect was indeed quite low and well in the range of the NH_4Cl concentrations encountered in hepatic failure [25]. The antimalarial agent chloroquine exerted a comparable effect in both astroglia-rich and pure astroglial cell cultures. Chloroquine is similarly known to interfere with lysosomal acidification [15].

Astrocytes in situ swell under a variety of conditions other than a hypotonic extracellular environment. Nevertheless, incubation of cultured cells in hypotonic medium is a manipulation believed to induce the same swelling-related processes that are encountered in vivo [26]. The glial preparations used in this study indeed swell after incubation in hypotonic medium and also show a strong regulatory volume decrease as demonstrated with cells in suspension. In a variety of glial cell preparations similar to ours, swelling and regulatory volume decrease were shown also to occur in cells still attached to the culture dish [26–28]. Therefore, it is safe to conclude that the cells used in this study behave in a comparable fashion when adhering to their substratum.

The present results demonstrate that osmotic swelling of astroglial cells leads to an alkalinization of acidic cellular compartments and lysosomes. This parallels the effect of osmotic swelling observed in other cell types such as hepatocytes [12–14,29] and kidney cells [15]. A similar alkalinization of acidic intracellular compartments and lysosomes was observed after iso-osmotic cell swelling as occurs following addition of glutamate to the perfusate. Glutamate increases cell volume by stimulation of Na^+-coupled entry of the amino acid [9,30] and by activation of kainate receptors [31], allowing the entry of Na^+ [9].

The alkalinization of intracellular compartments following cell swelling contrasts with the effect of osmotic cell swelling on cytosolic pH, which decreases upon swelling in glial cells as in other tissues [13,32]. The cytosolic acidification could either result from release of H^+ from acidic intracellular compartments or from bicarbonate exit via a HCO_3^-permeable anion channel at the plasma membrane as has been shown for MDCK cells [33]. The contrasting pH changes in intracellular compartments and the cytosol itself rule out the possibility of a reported trapping of BCECF-AM in acidic cellular organelles [34].

In addition to possible similar effects on proteolysis and glycogenolysis as described for liver cells [11], alkalinization of acidic intracellular compartments and lysosomes by swelling of glial cells may interfere with cellular degradation of internalized peptide hormones and transmitters. The latter has indeed been demonstrated for chloroquine [35] and could lead to an inhibition of recycling of receptors. In fact, altered receptor cycling could well contribute to the derangements observed following pathological glial cell swelling.

In conclusion, osmotic and glutamate-induced astroglial cell swelling leads to alkalinization of acidic cellular compartments, an effect likely modifying glial cell function.

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