Swelling of glial cells in lactacidosis and by glutamate: significance of Cl−-transport

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Swelling of glial and nerve cells is characteristic of brain damage in cerebral ischemia or trauma. The therapeutical efficiency of inhibition of Cl−-transport by a novel antagonist, the diuretic torasemide, on cytotoxic swelling of glial cells from lactacidosis, or glutamate was analyzed. Lactacidosis and the interstitial accumulation of glutamate are hallmarks of the pathophysiological alterations in ischemic or traumatic brain tissue. C6 glioma cells harvested from culture and suspended in a physiological medium were either exposed to pH 6.2, or 5.0 by lactic acid, or exposed to 1 mM glutamate at normal pH. Cell swelling and viability were quantified by flow cytometry. Lactacidosis of pH 6.2 led to an increase in cell volume to $117.9 \pm 0.7\%$ within 60 min. Torasemide (1 mM) inhibited the swelling response by 50% ($P < 0.01$). Cell swelling at pH 5.0, although more severe, was again attenuated by torasemide ($P < 0.01$). No effect was seen on the decrease in cell viability at this level of acidosis. Addition of glutamate led to a steady increase in cell volume which, contrary to cell swelling from lactacidosis, was not inhibited by torasemide. Inhibition of cell swelling from acidosis by this diuretic may be attributed to blocking of Cl^-/HCO_3^- exchange mechanisms activated by acidosis. The lack of effect by torasemide in glial cell swelling from glutamate indicates operation of a different mechanism inducing cell swelling, for example cellular accumulation of the amino acid together with Na^+ and water.

INTRODUCTION

Swelling of glial and nerve cells, i.e. cytotoxic brain edema, is a common finding in cerebral ischemia, trauma, metabolic disorders, and also occurs under conditions leading to vasogenic brain edema[2,23]. In order to explore causal, cell biological factors leading to cytotoxic brain edema under these conditions, our laboratory has established an in vitro model[19,21]. Mechanisms of cell swelling and damage can therefore be analyzed in isolation without interference from the many, complex phenomena taking place in damaged brain tissue in cerebral ischemia or trauma in vivo. Such an approach, although limited to observations of a well-defined cell type in isolation, may yield information on the mechanisms underlying cytotoxic cell swelling at a quantitative level and with a dynamic resolution, hardly available under the respective in vivo conditions. In previous studies, the significance of acidosis for cell swelling and irreversible cell damage was examined by the use of C6 glioma cells or astrocytes from primary culture[21,35]. Cell swelling from acidosis occurs once the pH is decreased to 6.8 or below, whereas cell viability starts to decline at pH 5.6.

Another objective of the present study was to address the cell swelling inducing properties of glutamate. This excitatory neurotransmitter is released in large amounts in ischemic or injured brain tissue[3,5]. Since both acidosis, and accumulation of glutamate in the extracellular space must be considered as key factors in cytotoxic cell swelling[8,21,23,37] and cell damage from cerebral ischemia and trauma, specific inhibition of the resulting mechanisms may be therapeutically beneficial. The purpose of the present study was to investigate the therapeutic potential of torasemide, an efficient ion transport and channel inhibitor, on cell

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swelling and cell injury from lactacidosis or glutamate, respectively. Torasemide is structurally related to furosemide, and so is a blocker of $Na^+/K^+/Cl^-$ co-transport systems and of Cl^--channels in cell membranes[11,38]. The inhibitory potential of torasemide on the acidosis-induced swelling of C6 glioma was currently being investigated since Cl^-/HCO_3^- exchange mechanisms are likely to play a role in the swelling process[21,24,35]. Respective effects against cell swelling by glutamate were analyzed for comparison.

MATERIALS AND METHODS

Cell culture

C6 glioma cells were cultured using Dulbecco's modification of minimum essential medium Eagle (DMEM; Boehringer Mannheim, Mannheim, FRG) supplemented with 100 IU/ml penicillin G, 50 μg/ml streptomycin, and 10% fetal calf serum (FCS). The cells were grown at 37°C in humidified room air containing 5% CO_2. For the experiments, confluent cultures were harvested with 0.05% trypsin/0.02% EDTA, washed twice in serum-free medium and resuspended in medium[19,21]. For experiments on cell swelling by glutamate, an amino acid- and FCS-free medium was used for cell washing and resuspension. The amino acid-free medium was made by addition of 5 ml MEM-vitamins (100×, Boehringer Mannheim, Mannheim, FRG), 5 ml Na^+-pyruvate (100 mM; Gibco, Eggenstein, FRG), 7.5 mg phenolred, 550 mg glucose, 1850 mg $NaHCO_3$, 3100 mg NaCl, 200 mg KCl, 100 mg $CaCl_2$, 100 mg $MgSo_4\cdot7H_2O$, and 62.5 mg $NaH_2PO_4\cdot H_2O$ to 500 ml destilled water. Osmolality of the medium was adjusted with NaCl to 300 mosmol/l. The cell suspension was transferred to an incubation chamber to allow for continuous control of pH, temperature, and pO_2. A gaspermeable silicone rubber tube served as a membrane oxygenator supplying the chamber with CO_2, O_2, and N_2. Details of the incubation chamber have been published[19,21].

Experimental groups

The experiments were preceded by a 60-min control period for measurement of cell volume, viability, and medium osmolality under normal conditions. The arithmetic mean of three cell volume determinations during the last 15 min of the control period was taken as the reference. In a control group ($n = 3$), effects of torasemide ((1-isopropyl-3-((4-(3-methylphenylamino)pyrid-3-yl)-sulfonyl)urea), 1 mM, Boehringer Mannheim, Mannheim, FRG) were tested on volume and viability of the glial cells under normal conditions: pH 7.4 and pO_2 of 80–100 mmHg. Acidosis was induced by addition of isotonic lactic acid (300 mosmol/l) without changing osmolality of the suspension medium (i.e. 300 mosmol/l). The osmolality in the medium prior to and after addition of lactic acid was frequently examined. The pH of the medium was lowered from 7.4 (control) to 6.2 or 5.0, respectively ($n = 4$). To compensate for a loss of CO_2 from the medium buffered by bicarbonate (25 mM) and, therefore, a secondary recovery of pH, CO_2 was added to maintain the pCO_2 at 80–100 mmHg. This level is comparable with in vivo findings in ischemic brain tissue[33]. Cell volume and viability were monitored for 60 min during lactacidosis. In parallel experiments torasemide (1 mM) was administered 15 min prior to induction of acidosis ($n = 4$ or 5). In further studies at normal pH, the cell suspension was added with 1 mM glutamate (final concentration), and the cell volume and viability were measured over 120 min ($n = 4$). Glutamate concentrations in the medium were assessed by high performance liquid chromatography (HPLC). Inhibition of cell swelling from glutamate was studied by addition of torasemide (1 mM) 15 min prior to administration of glutamate to the suspension ($n = 4$).

Measurement of cell volume and viability

Cell volume was determined by flow cytometry based on an advanced Coulter system and hydrodynamic focusing[18]. Accuracy of the method allows detection of cell size alterations of ~1%. The system is calibrated electrically and by use of latex beads of known size. Cell viability was also assessed by flow cytometry[18] (Fluvo Metricell, HEKA-Elektronic, Lambrecht/Pfalz, FRG) using the exclusion of propidium iodide[29]. Aliquots of 100 μl of cell suspension were added to 2 μl of 2 mg/ml propidium iodide in dimethylformamide and incubated at 37°C for 1 min. The fluorescence of propidium iodide was excited by a high pressure mercury arc lamp through a 500 nm short-pass filter. Maximum emission of propidium iodide (630 nm) was measured by using a 580 nm long-pass filter. A window integration system was employed for discrimination of propidium-positive (dead) from propidium-negative (viable) cells[17].

Further analytical procedures

Measurement of glutamate in the medium by HPLC (Pharmacia LKB, Freiburg, FRG) was made after precolumn fluorogenic derivatization with ortho-phthaldialdehyde (OPA fluoraldehyde reagent solution, Pierce, Rockford, Illinois, USA). A mixture of acetonitril and phosphate buffer served as mobile phase. The system was calibrated daily using amino acids standards. Osmolality of the suspension medium was measured by freezing point depression (Osmomat 030, Gonotec, Berlin, FRG). Results are expressed as mean ± S.E.M.. The data were analysed for statistical significance using the Kruskal–Wallis test for non-parametric one-way analysis of variance and multiple comparisons on ranks for unpaired samples[36].

RESULTS

The average volume of the C6 glioma cells during a control period at pH 7.4 was 774.9 ± 11.4 μm^3 (mean ± S.E.M.), 87.4 ± 0.8% of the cells were viable. Incubation of the cells under control conditions for up to 2 h did not affect cell volume or viability[21,35]. Likewise, torasemide (1 mM) added to the cell suspension did not influence cell volume or viability during a 60-min observation period. Lactacidosis of pH 6.2, led to an increase in cell volume to 108.5 ± 0.3% of control within 1 min followed by further swelling to 117.9 ±
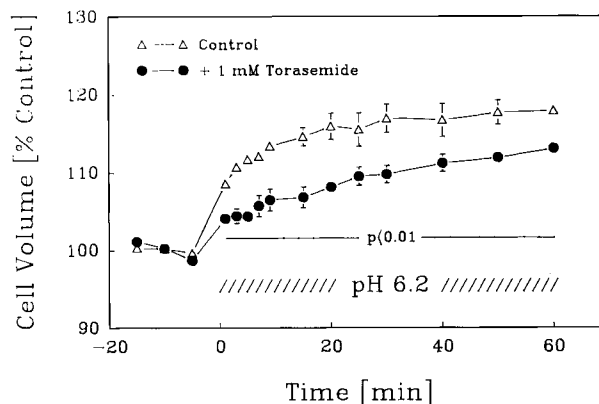

Fig. 1. Inhibition of swelling of C6 glioma cells from lactacidosis at pH 6.2 by torasemide (1 mM). Cell volume is given in percent of control obtained during the last 15 min prior to acidification of the medium. Four experiments were performed in each group. The medium was titrated to pH 6.2 by addition of isotonic lactic acid. Inhibition of cell swelling from lactacidosis by torasemide was statistically significant.

0.7% after 60 min (Fig. 1). The swelling of C6 glioma cells at pH 6.2 was significantly attenuated by torasemide during the whole observation period (Fig. 1, $P < 0.01$). The increase in cell size was 50% of that found without addition of torasemide. Cell viability remained normal when the pH was lowered to 6.2[35].

As seen in Fig. 2, lactacidosis of pH 5.0 led to a more intensive cell swelling. Cell size during the first minute of exposure reached $115.7 \pm 0.8\%$ of control and amounted to $146.9 \pm 2.3\%$ after 60 min. The swelling response was again significantly inhibited by torasemide (Fig. 2, $P < 0.01$). Yet, in contrast to pH 6.2, the number of viable cells was continuously decreasing (Fig. 2). After 60 min at pH 5.0 only $46.7 \pm 4.4\%$ of the glial cells were viable. It is noteworthy that torasemide, although markedly attenuating cell swelling, had no effect on the cell viability deterioration from acidosis (Fig. 2).

Addition of glutamate at a final concentration of 1 mM led to a linear increase in cell size over time, irrespective of whether torasemide was present (Fig. 3). After 120 min, cell swelling was $111.3 \pm 0.5\%$ in the
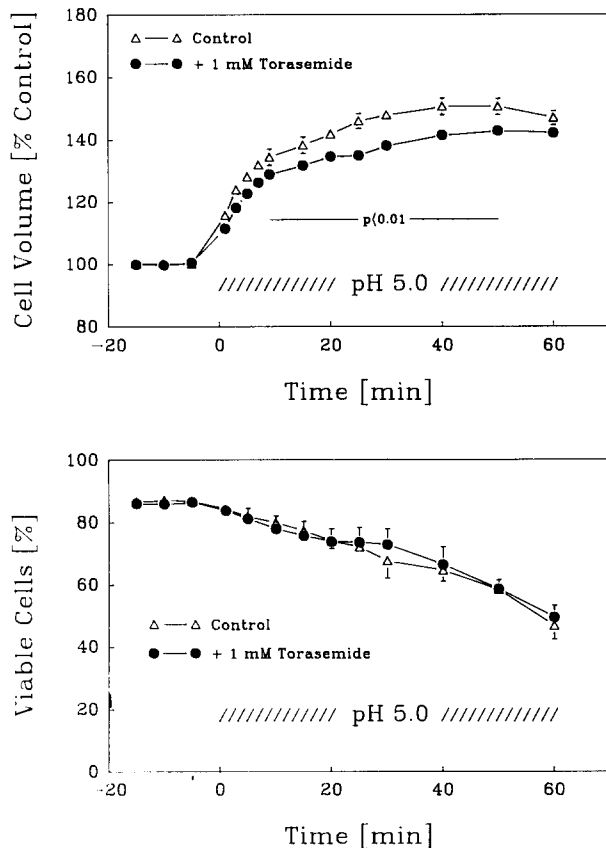

Fig. 2. Cell volume (top) and viability (bottom) of C6 glioma cells in percent of control during lactacidosis of pH 5.0 with and without addition of torasemide. The diuretic led to a significant attenuation of cell swelling without impairment of the cell viability from acidosis. Symbols represent means ± S.E.M. of four or five experiments per group.
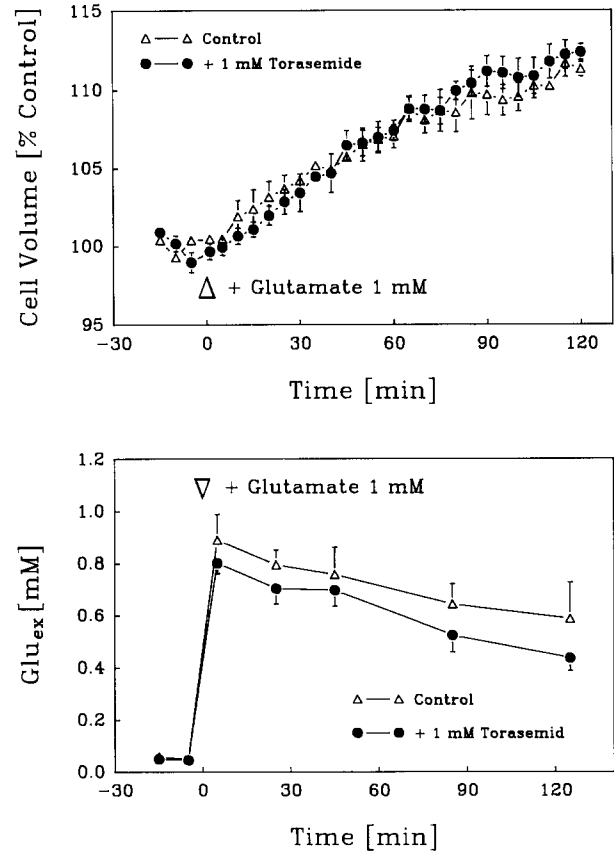

Fig. 3. Steady increase in cell volume of C6 glioma cells by glutamate in the presence or absence of torasemide (top). Four experiments were made per group. Torasemide was administered 15 min prior to addition of glutamate to the cell suspension. Torasemide had no effect on the volume increase of the glial cells by glutamate. The concentration profile of glutamate in the medium with and without administration of torasemide is shown at the bottom. It can be seen that glutamate was continuously decreasing in the suspension medium indicating clearance by the glial cells. This process was not affected by torasemide

absence of torasemide as compared to $112.4 \pm 0.5\%$ in the presence of torasemide. Viability of the C6 glioma cells was not affected by glutamate (data not shown). In the control, the spontaneous glutamate concentration in the suspension was approximately 0.05 mM (Fig. 3). Addition of glutamate resulted initially in a medium concentration of ca. 1 mM. Shortly thereafter, however, the concentration decreased continuously, indicative of clearance of glutamate by the glial cells. Five minutes after glutamate was administered, the concentration had fallen to 0.89 ± 0.10 mM, and in the presence of torasemide to 0.81 ± 0.04 mM. At 125 min, glutamate was decreased to 0.58 ± 0.14 mM, or 0.44 ± 0.05 mM, respectively (Fig. 3).

DISCUSSION

Loop diuretics such as furosemide and ethacrynic acid have been employed in the past for the treatment

of brain edema[12]. In human red blood cells, these diuretic compounds inhibit ion transport systems, such as the $Na^+/K^+/Cl^-$-cotransporter[7], and a SITS- or DIDS-sensitive anion carrier[6]. Other components inhibiting anion carriers, for example alcanoic acids and their analogues, have a limited saluretic activity but have also properties which therapeutically inhibit brain edema and cell swelling in vitro and in vivo[9,25]. Torasemide is structurally related with furosemide, interfering with the $Na^+/K^+/Cl^-$-cotransporter and blocking Cl^--channels in isolated tubules of mouse or rat kidney[11,38]. According to clinical findings torasemide is a more potent diuretic than furosemide with less kaliuretic side effects and a longer half life in plasma[11]. Analogues of torasemide were found to inhibit the swelling of brain slices and of astrocytes from potassium[28]. For the current in vitro studies, torasemide was administered at a final medium concentration of 1 mM in order to obtain a therapeutically effective level. For example, plasma concentrations of 0.3 mM were found in rats after oral or intravenous administration of 10 mg torasemide/kg b.wt.[13]. In studies on inhibition of anion exchange mechanisms by SITS in vitro, a similar dose level has been employed[24].

C6 glioma having several properties of astrocytes[30] are frequently used as model cells in studies on glial swelling and damage[15,19,21,35]. Specific markers, such as glial fibrillary acidic protein[1], or S-100 protein[39] are found in this cell line, as well as glial specific enzymes[10], uptake systems for neurotransmitters[30], and ion carriers[16]. An active respiratory metabolism in C6 glioma cells has been shown in previous studies of this laboratory[19]. Moreover, the extent of cell swelling of C6 glioma from acidosis and high potassium is comparable to that of astrocytes obtained from primary culture[21,22,35].

The baseline cell volume is similar to the results of former studies in this laboratory. Although cell viability might have been affected somewhat by the harvesting procedures for the experiment, the resulting level of approximately 90% viable cells under control conditions is in accordance with other studies using C6 glioma[14].

Lactacidosis

A significant role of lactacidosis in brain damage from ischemia or trauma leading to cell swelling and cell necrosis is widely accepted[27,32]. In experiments of this laboratory with lactic or sulfuric acid, glial swelling commenced as soon as the pH was lowered to 6.8 or below. Inhibition of the Na^+/H^+-antiporter by amiloride or replacement of Na^+ by choline in the medium were effective in reducing, or preventing swelling from acidosis[21,35]. The following concept was developed on the underlying mechanisms[21,24]: addition of acid to a bicarbonate-buffered medium leads to formation of carbonic acid, which is immediately dissociating into CO_2 and water. CO_2 accumulating in the intracellular compartment forms H_2CO_3 when catalyzed by carbonic anhydrase. H^+- and bicarbonate-ions generated from the dissociation of carbonic acid are exchanged against extracellular Na^+- and Cl^--ions. Consequently, net amounts of Na^+ and Cl^- are shuttled into the cell, whereas CO_2, H^+, and HCO_3^- ions are recycled into and out of the cell. The intracellular accumulation of Na^+ and Cl^- is the final step of the acidosis-induced glial swelling.

Lactic acid has in addition specific swelling-inducing properties. The compound may enter the intracellular compartment as a non-polar, undissociated molecule, followed by its dissociation. Due to their hydrophilic properties, the lactate anions formed in the cell, have a low membrane permeability, resulting in trapping of the ion species inside the cell, thereby raising the intracellular osmotic concentration[35].

To evaluate the contribution of the cellular uptake of lactate ions per se to the swelling process, experiments were made by exposure of glial cells under isotonic conditions in medium containing Na^+-lactate (20 mM) at normal pH. The procedure raised the glial cell volume to ca. 104% of normal during an observation period of 60 min[35]. Specificity of cell swelling from lactic acid was further compared to experiments using sulfuric acid at the same degree of acidosis[21]. Accordingly, acidosis of pH 6.2 using sulfuric acid resulted in swelling of 109.8 ± 1.4% of control within 10 min whereas lactic acid raised cell size to 113.4 ± 0.8% (cf. results). At 60 min, cell volume was increased to 110.7 ± 1.4% by sulfuric acid as compared to 117.9 ± 0.7% by lactacidosis. At pH 5.6, sulfuric acid led to cell swelling of 115.5 ± 3.0%, and lactic acid to 122.9 ± 1.7% after 10 min and to 118.8 ± 1.6% as compared to 139.9 ± 2.0% after 60 min[35]. The findings suggest that an initial, rapid increase in cell volume is brought about by activation of the Na^+/H^+- and Cl^-/HCO_3^--antiporters, since this phase is similar when sulfuric or lactic acid were used. The subsequent slower volume increase might be caused by uptake of lactic acid in its protonated form followed by accumulation of lactate anions in the cell after dissociation.

As shown, swelling of the C6 glioma cells was significantly inhibited by torasemide at both, pH 6.2 or 5.0 (Figs. 1 and 2). Thus, apart from a function of the Na^+/H^+-antiporter in the acidosis-induced cell swelling, inhibition of glial swelling by torasemide might

be attributable to antagonism of anion exchange mechanisms, such as the Cl^-/HCO_3^--antiporter.

The present findings on attenuation of the lactacidosis-induced cell swelling by torasemide, that might be attributable to inhibition of the Cl^-/HCO_3^--antiporter, confirm respective investigations of this laboratory with the classical chloride exchange inhibitor SITS (4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid). SITS led to significant inhibition of cell swelling at pH 6.2 from sulfuric acid[34]. Fifteen minutes after addition of sulfuric acid, cell volume increased to 110.1 $\pm$ 1.2% of control, but to only 106.1 $\pm$ 1.2% with 1 mM SITS, given 15 min prior to acidification ($P < 0.01$, $n = 6$ in each group). After 75 min, cell volume amounted to 110.2 $\pm$ 1.3% without and to 104.4 $\pm$ 1.0% with SITS ($P < 0.01$). Moreover, another inhibitor of anion exchange, the alkanoic acid 5B[9], was found to reduce glial swelling from sulfuric acid at pH 6.2 in the initial phase[20]. These findings when taken together show obviously that activation of anion exchange mechanisms, of the Cl^-/HCO_3^--antiporter in particular, plays a role in glial swelling from acidosis. As to the mechanism exerted by torasemide on the acidosis-induced swelling of glial cells, the following explanation might be offered. Inhibition of the exchange of intracellular bicarbonate against extracellular Cl^--ions conceivably results in an increase in the intracellular buffer capacity, attenuating the intracellular acidosis from acidification of the cell suspension. Consequently, a major factor in the acidosis-induced glial swelling, the Na^+/H^+-antiporter, is hardly activated.

Some comments are appropriate concerning the cell viability resulting from acidosis. In previous studies, a pH threshold of 5.6 was identified, below which cell viability deteriorated in a dose-dependent fashion[35]. Cell viability was found to decrease to 50% of normal within 60 min at pH 5.0, irrespective of whether torasemide was present (Fig. 2). It is therefore concluded that in acidosis, cell volume and cell viability were affected by mechanisms which are different from each other. This conclusion is supported by studies on inhibition of the lactacidosis-induced glial swelling with replacement of Na^+-ions in the suspension medium by choline chloride. Under these conditions, at pH 5.6, although the acidosis-induced glial swelling was more or less completely abolished, loss of cell viability was significant as compared to acidosis experiments at the same pH in the presence of a normal Na^+-ion concentration in the medium[35]. The findings suggested that absence of Na^+-ions led to a failure of the Na^+/H^+-antiporter, inhibiting regulation of the intracellular pH by the glial cells, thus facilitating development of an intracellular acidosis as the ultimate mechanism of cell

death[32]. Despite inhibition of cell swelling, torasemide did not protect – but also did not impair – viability of the C6 glioma cells at this advanced level of acidosis. This may indicate that the mechanisms of torasemide in prevention of the acidosis-induced cell swelling were independent of the mechanisms controlling the intracellular pH.

Glutamate

Glutamate is an important excitatory neurotransmitter in the brain and has neurotoxic properties at higher than normal extracellular concentrations[31]. In cerebral ischemia or trauma, release and extracellular accumulation of glutamate can be excessive and is likely to cause additional damage to neurons and glial cells[3,5]. Glutamate may also have a pathophysiological function in brain edema[8,37]. Addition of glutamate to the cell suspension (final concentration: 1 mM) led to a steady cell volume increase to 110% of control within 120 min, associated with a decrease in the glutamate level in the medium. Torasemide neither reduced cell swelling from glutamate nor influenced its clearance from the medium by the cells (Fig. 3). Evidence is available that swelling of glial cells by glutamate is based on an active intracellular accumulation of the amino acid[4,26]. Clearance of glutamate from the extracellular space by the glial cells is a requirement for normal neuronal function. The glutamate uptake occurs against a steep intra- to extracellular concentration difference, and is fueled by a concurrent influx of Na^+-ions along their electrochemical gradient[4,26]. The intracellular accumulation of glutamate together with Na^+-ions raises cell osmolality as an ultimate mechanism of swelling. The present findings indicate that activation of the $Na^+/K^+/Cl^-$-cotransporter, or opening of Cl^--channels was not involved in the glial swelling from glutamate or its clearance by the cells.

Taken together, the present results demonstrate a therapeutical potential for inhibition of the Cl^--transport by torasemide in the swelling of glial cells from lactacidosis, which makes in vivo studies promising to inhibit acute brain edema from cerebral ischemia or trauma.

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