Volume regulation of C6 glial cells was studied in anoxia in vitro to improve the understanding of ischemic cell swelling in the brain. Contrary to in vivo conditions, anoxia or anoxia plus iodoacetate for additional inhibition of anaerobic energy metabolism did not induce glial swelling. However, intracellular K+ was markedly decreased while intracellular Na+ increased. Induction of energy failure by anoxia plus iodoacetate was found to prevent the regulatory volume decrease on hyposmotic exposure of the cells, which is regularly observed in normoxic control conditions. Hyposmotic exposure in anoxia plus iodoacetate led only to an initial tendency of cell volume normalization followed by secondary cell swelling. This was associated with a net increase of intracellular Na+ that may explain the failure of volume regulation under these circumstances. Maintenance of a normal glial cell size during complete energy deprivation by anoxia plus iodoacetate in isotonic medium strongly indicates that energy failure per se does not suffice to induce cell swelling. Cell swelling in cerebral ischemia in vivo thus is likely to require additional mechanisms, most likely an increase of membrane permeability to Na+, which may be caused by release and accumulation of excitotoxins such as glutamate or by an extracellular release of K+. Such a mechanism would hardly influence the extracellular homeostasis in vitro due to the large medium-to-cell volume ratio. The findings demonstrate, nonetheless, the significance of a competent energy metabolism to support cell volume regulation. This is concluded from the failure of regulatory volume decrease of hypotonically suspended glial cells in anoxia plus iodoacetate. The experimental conditions employed appear particularly useful to analyze and to differentiate on a molecular level the complex interactions resulting in ischemic cell swelling in the brain. (Stroke 1987;18:623-628)

When the cerebral circulation is interrupted or decreases below a critical threshold, drastic swelling of dendrites and glial cells ensues. A shift of fluid and Na+ from the extracellular to the intracellular space is seen after a latency of only 2–3 minutes. The resulting energy failure is thought to disturb the double Donnan equilibrium, which under physiologic conditions ensures a stable cell volume. This interpretation, however, might be too simple. The rapid onset of swelling and the complete breakdown of ion gradients suggest an involvement of additional pathogenetic mechanisms, e.g., release or activation of mediator compounds that promote the development of cytotoxic brain edema by increasing membrane permeability or by inflicting membrane damage.

Numerous in vivo studies have been performed to elucidate ischemic cell swelling in the brain. The multitude of parameters changing simultaneously, however, makes it difficult to determine the significance of individual factors. Therefore, an in vitro method has been established to study the specific processes causing cell swelling and eventually cell death under conditions providing better control of pertinent variables. The system allows one to change or keep constant any physiologic parameter of interest while monitoring cell volume, intracellular electrolytes, oxygen consumption, extracellular pH, PO2, lactate, or other metabolites.

It was the aim of the present study to examine whether the mere inhibition of cellular energy metabolism by anoxia and/or the addition of iodoacetate to block glycolysis causes swelling of glial cells, and whether and how this interferes with cell volume control mechanisms. C6 glioma cells, a well established cell line with many glia-specific properties, were used as model cells. Previous experiments have shown that C6 glioma cells spontaneously recover normal cell volume after initial swelling during exposure to a hypotonic medium. The results of this study suggest that interruption of the energy supply alone is not sufficient to induce glial swelling within a period comparable to in vivo conditions. The findings strongly support the hypothesis that cytotoxic cell swelling in ischemia involves additional factors, such as neurotoxic mediator compounds.

Materials and Methods

Preparation

Cell culture. C6 glioma cells were grown at 37°C in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% fetal calf serum (Boehringer, Mannheim, West Germany) in a humidified atmosphere of 5% CO2 and 95% room air in plastic petri
The cells were subcultivated 2–3 times per week. Confluent cultures obtained 2 days after subcultivation were used for the experiments. The cells were harvested and suspended in serum-free medium as previously described. 7

**Experimental Setup.** The cell suspension was immediately transferred to a plexiglass incubation chamber which controlled pH, PO₂, and temperature using respective electrodes. Permeable silicone rubber tubing inside the chamber was used as a membrane oxygenator to supply the medium with O₂, CO₂, and N₂ in any mixture desired. A magnetic stirrer prevented cell sedimentation. Drugs were injected and cell samples collected via cannulas inserted into the chamber.

**Induction of Anoxia.** The incubation chamber was placed in an air-tight plastic tent with rubber gloves for sample handling. The tent was filled with N₂ to prevent leakage of room air into the chamber. Fluid removed during withdrawal of samples from the chamber was automatically replaced from a connected reservoir of distilled water, frozen, sonicated, and processed for atomic absorption spectrophotometry. Remaining extracellular contamination was determined by titrated inulin previously added to the sample. Cell counts of each sample were obtained using a conventional Neubauer chamber. Exclusion of trypan blue by living cells was used to assess viability.

**Analytic Procedures**

Cell samples retrieved from the chamber were used for measurement of cell volume and intracellular and extracellular electrolytes. Lactate concentrations in the suspension medium were obtained by an enzymatic fluorometric test. 9 Cell volume was determined by flow cytometry according to the Coulter principle. The Metricell cell volume analyzer provided superior accuracy and resolution by combining the Coulter system and a hydrodynamic focusing technique. In essence, the single cell suspension was forced through a 120-μm aperture with a constant electrical current. Passage of a cell through the aperture briefly reduced the cross section of the aperture and thereby increased its electrical resistance. The height of the resulting voltage pulse was proportional to the cross section of the cell. C6 glioma cells have been shown to be nearly spherical during passage. 10

Therefore, cell volume was calculated by height analysis of the voltage pulses using a modified Maxwell equation. 10,11 The system was calibrated electrically by use of latex beads with known volume.

Intracellular and extracellular Na⁺ and K⁺ concentrations were determined by atomic absorption spectrophotometry. For studies of intracellular concentrations, the cells were briefly washed in isotonic CaCl₂ and centrifuged through phthalate oil 12 for removal of extracellular medium. The cell pellets were lysed in distilled water, frozen, sonicated, and processed for atomic absorption spectrophotometry. Remaining extracellular contamination was determined by titrated inulin previously added to the sample. Cell counts of each sample were obtained using a conventional Neubauer chamber. Exclusion of trypan blue by living cells was used to assess viability.

**Results**

**Anoxia.** Anoxia and additional inhibition of anaerobic energy production did not induce swelling of C6 glial cells (Figure 1). Cells exposed to anoxia were even slightly smaller than the normoxic controls. The combination of anoxia and iodoacetate led to a progressive decrease in intracellular K⁺ from 153.5 ± 12.6 to 38.8 ± 7.8 mM/l cell mass (Figure 2, top) together with an increase in intracellular Na⁺ from 16.5 ± 3.7 to 50.2 ± 8.5 mM/l cell mass (Figure 2, bottom). This effect is time-dependent as demonstrated in Figure 3, where the intracellular Na⁺ concentrations are plotted against the intracellular K⁺ levels. The loss of K⁺ was somewhat more pronounced than the cellular gain of Na⁺. The logarithmic regression line (p < 0.01) indicates, however, an increased net influx of sodium in the late phase of the experiment. On the other hand, intracellular K⁺ and Na⁺ concentrations studied in anoxia alone were not significantly changed during the observation period. In addition, lactate concentrations studied in the suspension medium were found to rise only in anoxia, from 1.07 ± 0.21 to 4.19 ± 0.38 mM, while addition of iodoacetate effectively blocked this response in the experimental group with exposure to anoxia (1.01 ± 0.136 mM after 2 hours of anoxia plus iodoacetate).

**Figure 1.** Cell volume (μm², mean ± SEM, n = 5–7) of C6 glioma cells incubated in normoxia (○), in anoxia (□), and in anoxia with 2.5 mM iodoacetate added to inhibit glycolysis (○). Normoxia was maintained in all groups during a control period of 60 minutes before the experiment. The onset of anoxia and the addition of iodoacetate is indicated by the arrow.
Intracellular K⁺ (top) and Na⁺ (bottom) concentrations of C6 glioma cells incubated in normoxia (○), in anoxia (○), and in anoxia with 2.5 mM iodoacetate added to inhibit glycolysis (△). A 60-minute control period of normoxia preceded the experiment. The arrow indicates the onset of anoxia and the addition of iodoacetate. Concentrations are given in mmol/l cell mass (mean ± SEM, n = 5–7). Control Na⁺ concentrations before induction of anoxia (△) were slightly higher than in the other groups. This difference, however, was not significant by analysis of variance.

**Osmotic Swelling.** Reduction of the extracellular osmolality from 300 to 160 mosmol/l resulted in immediate swelling of the C6 glioma cells (Figure 4) followed by complete volume normalization. As in previous studies, cell volume returned to the control level within 30–60 minutes, although the glial cells remained exposed to the hypotonic medium. Neither anoxia nor iodoacetate alone inhibited cell volume normalization after immediate swelling in the hypotonic suspension. However, the combination of both anoxia and iodoacetate prevented cell volume regulation under these conditions (Figure 4). At best, a tendency of volume reduction during the first 10–15 minutes was seen which, however, was followed by a secondary volume increase. Sixty minutes after hypotonic dilution of the medium, cell volume was 144% of its initial control value. Two components of the cell volume changes can be distinguished by best-fit regression analysis of the volume data obtained during the first 15 minutes (when cell volume reached a minimum) and the subsequent 45 minutes after hypotonic dilution. The cell volume decrease during the first 15 minutes obeyed kinetics similar to those observed in normoxia (Figure 4). The curve obtained for the following 45 minutes has a positive slope and a significant regression coefficient ($r = 0.45, p < 0.01$; Figure 4). Table 1 gives the intracellular electrolyte content and lactate levels in the medium of all experimental groups. Major adjustments of the intracellular electrolytes took place within 30 minutes after hypoxic exposure. In all groups, intracellular K⁺ fell to nearly half the control level. Intracellular Na⁺ increased significantly in the experiments where anoxia was combined with iodoacetate, while the increase was only marginal in the remaining groups (Table 1).

**Discussion**

Maintenance of a normal cell volume during complete shutdown of energy metabolism by oxygen deprivation and addition of iodoacetate has important implications for our understanding of cell volume control under normal and pathophysiologic conditions. According to the double-Donnan hypothesis, cell volume maintenance is believed to critically depend on the active removal of Na⁺ from the cell and a low Na⁺ permeability of the cell membranes. According to this "pump-leak" model, active transport renders the cell membrane functionally impermeable to Na⁺ and K⁺. As a consequence, failure of the ion pumps caused by metabolic deprivation should induce cell swelling due to uncontrolled cellular influx of Na⁺. The active Na⁺-K⁺ pump seems to be particularly important. Inhibition of the Mg²⁺ activated Na⁺,K⁺-ATPase by ouabain induces cell swelling in many cells. However, many studies employ epithelial cells, which are particularly sensitive to ouabain, since NaCl continuously enters these cells via the apical membrane by a coupled mediated process. Until further details become available, the active Na⁺ pump has always been considered a central element of cell volume control. Likewise, the rapid and dramatic swelling of cells in central nervous tissue in ischemia is attributed to a failing Na⁺ pump. However, the rate of intracellular Na⁺ influx is equally important. Thus, the rate of swelling is determined by the degree of membrane leakage for Na⁺.

**Figure 2.** Intracellular K⁺ (top) and Na⁺ (bottom) concentrations of C6 glioma cells incubated in normoxia (○), in anoxia (○), and in anoxia with 2.5 mM iodoacetate added to inhibit glycolysis (△). A 60-minute control period of normoxia preceded the experiment. The arrow indicates the onset of anoxia and the addition of iodoacetate. Concentrations are given in mmol/l cell mass (mean ± SEM, n = 5–7). Control Na⁺ concentrations before induction of anoxia (△) were slightly higher than in the other groups. This difference, however, was not significant by analysis of variance.

**Figure 3.** Comparison of intracellular K⁺ and Na⁺ concentrations in C6 glioma cells incubated in normoxia and in anoxia with 2.5 mM iodoacetate added. The times indicated represent the mean value of the actual sampling times for the individual electrolyte specimen. Concentrations are given in mmol/l cell mass (mean ± SEM). The regression line was obtained by best-fit analysis: $y = 130.2 - 21.8 \ln x$, $r = 0.98$, $p < 0.01$. 
The present findings illustrate perfectly that in a controlled environment energy failure can be well tolerated for > 2 hours without causing swelling or decreasing viability. The data show that loss of K\(^+\) from the cells was indeed higher than the gain of Na\(^+\).

The relevance of C6 glioma cells for the study of mechanisms involved in glial swelling in ischemic brain in vivo needs some considerations in this context. C6 glioma cells have been successfully employed in many studies of glial function. The characteristics and biochemical specifications of these cells are very similar to those found in bulk-isolated glia or glial cells obtained from primary culture. Glia-specific markers, such as the glial fibrillary acidic protein or the S-100 protein, have been demonstrated in the cell line\(^{16,17}\) as well as transmitter uptake systems\(^{18,19}\) and enzymes\(^{8,20,21}\) characteristic for glial cells. As far as the major parameters of the study, such as the cell volume or intracellular electrolytes are concerned, these are similar to those found in C6 glioma cells (unpublished data).

A major difference between the current in vitro system and ischemic brain tissue in vivo concerns changes in the suspension medium and the extracellular milieu of the cerebral parenchyma. Approximately 1-5 \times 10^7 glial cells were suspended in a 10-ml chamber. The resulting medium-to-cell volume ratio was therefore approximately 10^5-10^6:1, i.e., unlimited for all practical purposes. Thus, a potential cellular response to energy deprivation, e.g., a release of K\(^+\) or of excitatory amino acids, would be literally diluted to

Table 1. Intracellular Na\(^+\) and K\(^+\) Content of Suspended C6 Glioma Cells During Hypotonic Exposure and Inhibition of Energy Metabolism by Anoxia and/or Iodoacetate

<table>
<thead>
<tr>
<th>Treatment</th>
<th>300 mosm/l</th>
<th>160 mosm/l</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60 min preincubation</td>
<td>K(^+) Na(^+) Lactate</td>
</tr>
<tr>
<td>Normoxia</td>
<td>x</td>
<td>134.7 ± 11.0</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>x</td>
<td>134.35 ± 20.9</td>
</tr>
<tr>
<td>Anoxia</td>
<td>x</td>
<td>125.8 ± 8.3</td>
</tr>
<tr>
<td>Anoxia + iodoacetate</td>
<td>x</td>
<td>128.0 ± 13.2</td>
</tr>
</tbody>
</table>

Intracellular electrolyte content is expressed as 10^-17 mol/cell. The lactate concentrations of the medium are given as mM.

\(p < 0.05, \, p < 0.01\) compared with other experimental groups at same time of hypotonic exposure (analysis of variance and Student-Newman-Keuls test).
an ineffective level by the enormous volume of the suspension medium. Therefore, the large extracellular-to-intracellular volume ratio prevented alterations of the composition of the medium during the experiment. This is a great advantage of the current model because uncontrolled changes in the homeostasis occurring in vivo in brain tissue can be ruled out.

The significance of extracellular changes in vivo as a cause of cell swelling or cell damage in ischemia is supported by corresponding observations of Ames and Nesbett.22-24 The authors studied the influence of anoxia and metabolic deprivation on isolated retinas. Oxygen or substrate withdrawal were tolerated for an hour or more without causing tissue swelling or damage.22,23 However, when the extracellular incubation volume was restricted, O2 and substrate deprivation led to swelling of the retinas and a decrease of leucine incorporation suggestive of irreversible damage.22,24 Exposure of retinas to extracellular fluid obtained from other, anoxically incubated specimens increased damage to the former.24 These and the current findings support our conclusion that energy failure alone does not suffice to induce cell swelling, that additional mechanisms are required. One is a release into the extracellular space during ischemia or anoxia of substances that mediate cell swelling or even damage as soon as effective concentrations are reached. Such a process may explain the rapid onset of cell swelling in vivo 3-5 minutes after interruption of the circulation.

One of the earliest phenomena in cerebral ischemia is a massive release of neurotransmitters, suggested by a marked increase of cAMP within 30 seconds and an initial burst of electrical activity.25-27 A release of excitatory transmitter compounds, such as glutamate, during ischemia would increase membrane permeability for Na+ as a basis of ischemic cell swelling.4 Contrary to a normally perfused brain, persistent elevation of these powerful substances in the extracellular compartment is likely to occur in ischemia because their clearance is impeded or abolished due to failing uptake mechanisms that depend on a functional energy metabolism.28 Ischemic cell swelling may also result from Na+-H+ exchange processes in cellular acidosis as an attempt to maintain a normal intracellular pH.29 Hereby, homeostasis of the cellular pH seems to overrule cell volume control. Further, it has been shown that additional osmotic solutes are formed in ischemic brain tissue, generating an osmotic gradient and, consequently, fluid shifts resulting in swelling.30

The shortage of metabolic energy in ischemia can be considered not only to prevent the uptake and elimination of transmitters from the extracellular space but also to interfere with the active mechanisms of cell volume regulation. This conclusion is strongly supported by the current findings of maintenance of glial swelling on hypotonic exposure during complete inhibition of energy metabolism. It has been previously shown that C6 glial cells, just as other cell types, have the capacity to regulate their volume during hypoosmotic exposure.7 Under these conditions, glial cells reach a swelling maximum immediately upon hypotonic dilution of the medium due to the high hydraulic conductivity of the cell membrane. Thereafter, cell volume rapidly normalizes although hypotonicity is maintained. The mechanisms of RVD are not completely understood. Analysis of intracellular electrolytes (Table 1) confirms a marked loss of K+, which is probably accompanied by Cl- and amino acids.31,32 Since the intracellular to extracellular movement of K+ occurs downhill, it is not clear whether RVD is an active process, even though it is associated with an increased cellular O2 uptake.7 The current findings, that complete shutdown of the cellular energy metabolism by anoxia plus iodoacetate prevents RVD of hypotonically suspended glial cells, demonstrate convincingly its active nature. Under these circumstances volume normalization failed, although cell volume had a tendency to decrease during the first 10-15 minutes.

The corresponding intracellular Na+ and K+ concentrations (Table 1) demonstrate that anoxia plus iodoacetate did not prevent efflux of K+ from the hypotonically suspended cells observed in normoxia. However, contrary to normoxia, intracellular Na+ was markedly increased. The enhanced Na+ influx after hypotonic dilution thus may have contributed to the secondary swelling of the glial cells during inhibition of energy metabolism. These observations provide further support for the active nature of RVD, which cannot be explained by an activation of the Mg2+-activated Na+-K+-ATPase.5,7,11,23-35

In conclusion: Energy failure per se may not suffice to induce cell swelling in ischemia. Obviously, additional mechanisms are required. One is an abrupt change of the membrane permeability to Na+ after onset of ischemia, attributable to a release of mediator substances into the extracellular space. Accumulation of excitatory transmitters or of free fatty acids appears to be a particularly attractive mechanism. Identification of mediator substances causing ischemic cell swelling is not only of scientific interest, it is of clinical relevance as a basis for the development of better methods of treatment. The currently used in vitro system appears promising for the study of respective mechanisms and, hence, therapeutic principles on a molecular level.

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Control of glial cell volume in anoxia. In vitro studies on ischemic cell swelling.
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