

Uphill and downhill water transport in rat glioma cells

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Abstract. The cell water content determines the cell volume, which in turn controls numerous cellular functions. The mean volume of rat glioma cells was electronically measured under isotonic and anisotonic conditions. Two types of isotonic solutions were used containing either high or low concentrations of NaCl, KCl or N-methylglucamineCl. In low salt solutions, osmolarity was maintained constant by the addition of sucrose or mannitol. Anisotonicity was induced by changing the concentration of electrolytes. As expected, the cell volume increased when the concentration of electrolytes was decreased from a high (165 mM) monovalent cation concentration. In contrast, the cell volume decreased when the concentration of electrolytes was decreased from a low (85 mM) monovalent cation concentration. Reciprocally and unexpectedly, the cell volume increased during a hyperosmotic challenge when the initial cation concentration was low, whereas it decreased when the initial cation concentration was high. These opposite volume changes observed during similar anisotonic challenges but starting from different electrolyte concentrations provide the first evidence that H₂O is not only passively transported (downhill) through aquaporins but also follows ion fluxes (uphill).

Key words: Cell volume — Ion channels — Osmotic stress — H₂O flux

Introduction

Water transport through the plasma membrane of animal cells is a key feature of cell biology. Dependent on several physical parameters such as membrane tension and curvature, macromolecular crowding, extracellular osmolarity and osmolite composition, the cell volume is regulated and in turn controls numerous cellular functions including hormone and neuromediator release, cell proliferation, apoptosis and cell migration (Soroceanu et al. 1999; Lang et al. 2000; Najvirtova et al. 2003; Rouzaire-Dubois et al. 2004; Ernest et al. 2008; Okada et al. 2009). For these reasons, it is important to describe the different mechanisms that control the cell volume and that have often been underestimated.

During a hyposmotic stress, the cells swell because of a passive influx of water mainly mediated by specialised water pathways, the aquaporins. A few seconds after cell swelling, the cell volume starts slow recovery back towards its isos-

motonic value by a process of regulatory volume decrease (RVD) whereby the cell size increase activates K⁺-Cl⁻ co-transporters and Cl⁻ and K⁺ channels, inducing an efflux of K⁺, Cl⁻ and osmotically obliged H₂O (Hoffmann et al. 2009).

Unexpectedly during RVD, the cell volume can become smaller than it was before the hyposmotic challenge (Ernest et al. 2005; Rouzaire-Dubois et al. 2009). This over-regulation suggests that another unknown process is activated during a hyposmotic stress which, in addition to the classical RVD, induces cell shrinkage. In order to test this hypothesis, we electronically measured the mean cell volume of rat glioma cells under basal isotonic conditions at high and low salt concentrations compensated with sucrose or mannitol. In some cases, NaCl was substituted with KCl or N-methylglucamineCl (NMGCl). In most cases hypo- and hypertonic challenges were obtained by changes in electrolyte concentrations.

Given that in most cells, the osmotically inactive volume corresponds to only a few percent of the total cell volume (Grove et al. 1995; Rouzaire-Dubois et al. 2004; Ernest et al. 2005), any change in cell volume mainly reflects the change in cell water content due to water fluxes. Moreover, it has been suggested that H₂O could be transported against its initial osmotic gradient by Na⁺-K⁺-2Cl⁻ and Na⁺-glucose co-transporters (Hammann et al. 2005; Zeuthen et al. 2007). This

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hypothesis has been the object of an intense debate because H₂O could be either uphill transported through co-transporters (Zeuthen and Zeuthen 2007) or passively transported due to an increase in intracellular osmolarity (Charron et al. 2006). However, neither of these interpretations can explain an efflux of water in the absence of glucose or in hypotonic conditions where these transporters are not activated. For the first time, we furnish experimental arguments for a transport of H₂O against its osmotic gradient in association with passive ion fluxes likely through non selective channels.

Materials and Methods

The experiments were performed on C6 rat glioma cells at a temperature of 20–22°C. The cells were cultured as previously described (Rouzaire-Dubois et al. 2004, 2009). The cells were trypsin-detached, pelleted at 100 × g for 5 min, resuspended and mechanically stirred in isotonic solutions for 15–20 min before anisotonic challenges. Isotonic solutions ($339 \pm 2 \text{ mOsm}\cdot\text{kg}^{-1}$, $n = 15$) contained either 160 mM NaCl or 80 mM NaCl + 150 mM sucrose or mannitol. In complementary experiments, NaCl was isosmotically substituted with KCl or N-methylglucamineCl. In addition to these ions, all solutions contained (in mM): KCl 5; MgCl₂ 2; CaCl₂ 1; Hepes 10; pH adjusted to 7.40 ± 0.02 with NaOH. The osmolarity of solutions was determined using a freezing point osmometer (Knauer, Berlin, Germany). In each individual experiment, the mean cell volume of 10 000 to 70 000 cells was determined with a Z2 Coulter channelizer (Beckman-Coulter, Villepinte, France). Absolute cell volumes were obtained using beads (Coulter) as standards. The time resolution of this technique is low, in the order of 30 s to 1 min. However, we previously showed that during an instantaneous

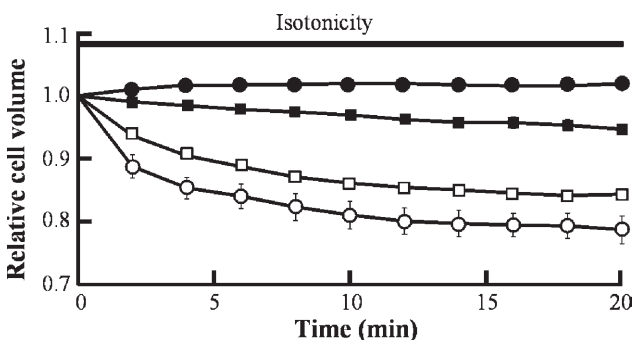


Figure 1. Evolution with time of the relative mean cell volume in isotonic solutions ($339 \text{ mOsm}\cdot\text{kg}^{-1}$). A few seconds after centrifugation, the cells were resuspended in solutions containing 160 mM NaCl (filled squares), 165 mM KCl (filled circles), 80 mM NaCl + 150 mM mannitol (open squares) or 160 mM NMGCl (open circles); $n = 3$ –5 in each condition.

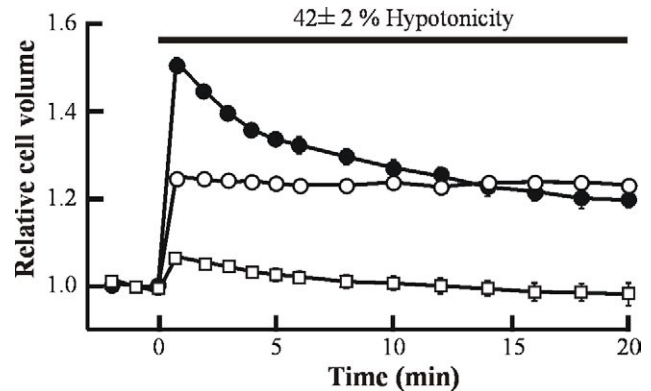


Figure 2. Time evolution of the relative mean cell volume during a $42 \pm 2\%$ hypotonic challenge from 339 to $197 \text{ mOsm}\cdot\text{kg}^{-1}$. The osmolarity was decreased in solution 1 by dilution with a NaCl-free solution (filled circles) and in solution 2 by dilution with either a sucrose-free solution (open circles) or with a NaCl- and sucrose-free solution (open squares); $n = 3$ –4 in each condition; solution 1: 160 mM NaCl, solution 2: 80 mM NaCl + 150 mM sucrose.

anisotonic challenge, the maximal volume change of calcein-loaded cells was also in the order of 1 min (Rouzaire-Dubois et al. 2009). Chemical reagents were from Sigma-Aldrich (Saint Quentin Fallavier, France). In each condition, cell volumes were expressed as the mean \pm the standard error of the mean (SEM) of n experiments. The SEM was often smaller than 2% and consequently in the figures, the SEM bars were often smaller than the symbols.

Results and Discussion

In a first series of experiments, cell pellets were resuspended in different solutions, the osmolarity of which was identical to that of the culture medium ($339 \pm 2 \text{ mOsm}\cdot\text{kg}^{-1}$, $n = 17$ and 15, respectively). When the cells were resuspended in NaCl or KCl solutions, their volume was almost constant for 20 min (decreased by 4 to 5% in NaCl solution and increased by 1 to 2% in KCl solution). In contrast, when half of the NaCl was isosmotically substituted with mannitol or NaCl was isosmotically substituted with NMGCl, the cell volume decreased by $16 \pm 0.01\%$ and $21 \pm 2\%$, respectively (Fig. 1). This was likely due to an efflux of NaCl and an associated water efflux against its osmotic gradient.

In order to test the idea that H₂O could be actively transported in association with passive ion fluxes, we measured the relative volume changes of cells submitted to a $42 \pm 2\%$ hypotonicity performed from two isotonic solutions (Rouzaire-Dubois et al. 2009) containing either 160 mM NaCl (solution 1) or 80 mM NaCl + 150 mM sucrose (solution 2). Fig. 2 clearly shows NaCl-dependent

deviation between the measured cell volume and the perfect osmometer. This confirms our recent results where, during hypotonicity, the cell volume was electronically measured or estimated from the fluorescence of calcein-loaded cells and was dependent on the net influx of Na^+ (Rouzaire-Dubois et al. 2009). Altogether, these observations provide evidence that in addition to volume changes following the prediction of the Boyle-van't Hoff law of linearity between the relative cell volume and the relative inverse osmolarity, hyposmolarity also induces Na^+ -dependent water transport

against its osmotic gradient. In other words, H_2O could be transported by two mechanisms. The first one would be passive and depend on the H_2O osmotic gradient. The second one would be associated with the net ion flux and could be opposite to the first one. To further confirm this conclusion, we measured the relative mean cell volume during 20% hypotonic challenges (from 339 to 271 $\text{mOsm}\cdot\text{kg}^{-1}$) performed from isotonic solutions 1 or 2 by dilution with NaCl-free solutions (Fig. 3a). As predicted, the cells swelled when hypotonicity was performed from isotonic solution 1.

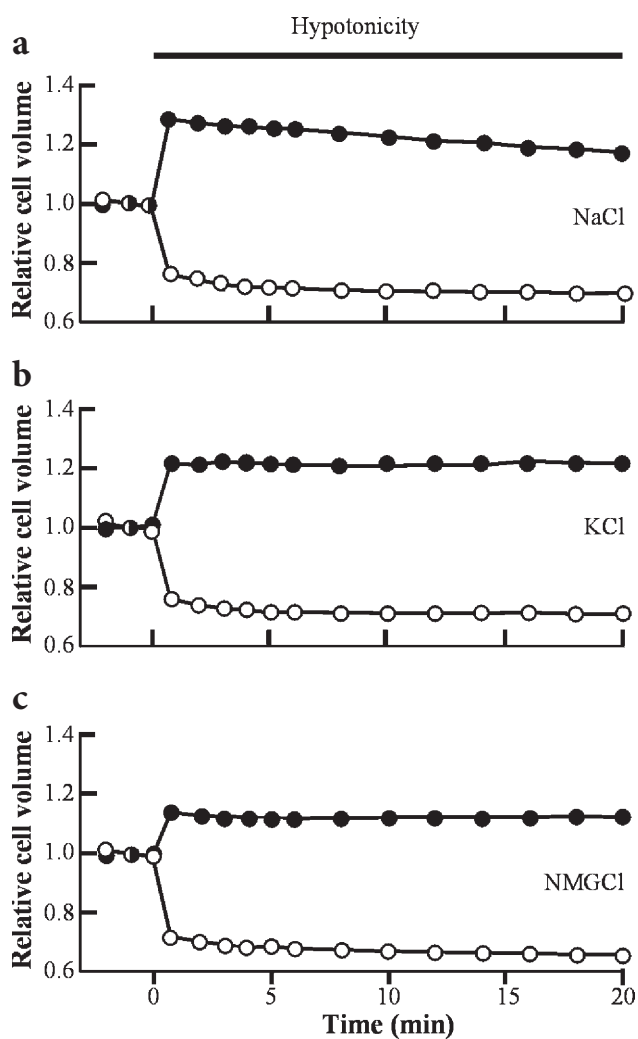


Figure 3. Hypotonicity can induce cell swelling or shrinkage. Isotonic solution 1 (filled symbols) contained 160 mM NaCl (a), 165 mM KCl (b) or 160 mM NMGCl (c). Isotonic solution 2 (open symbols) contained 80 mM NaCl + 150 mM sucrose (a), 85 mM KCl + 150 mM sucrose (b) or 80 mM NMGCl + 150 mM sucrose (c). In each condition, the osmolarity was decreased by $20 \pm 2\%$ from 339 to 271 $\text{mOsm}\cdot\text{kg}^{-1}$ with NaCl-, KCl- or NMGCl-free solutions; $n = 3-4$ in each condition.

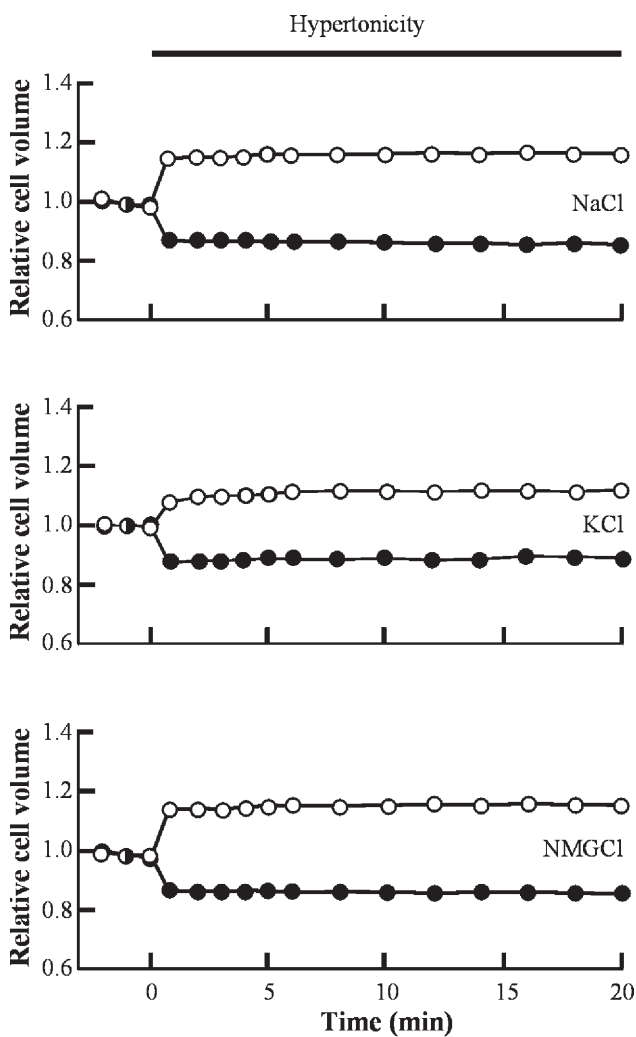


Figure 4. Hypertonicity can induce cell shrinkage or swelling. In a first series of experiments (filled circles), the isotonic solutions contained 160 mM NaCl (a), 165 mM KCl (b) or 160 mM NMGCl (c). In a second series of experiments (open circles), the isotonic solutions contained 300 mM sucrose + 40 mM NaCl (a), or 45 mM KCl (b) or 40 mM NMGCl (c). In each condition, the osmolarity was increased by 14% from 339 to 386 $\text{mOsm}\cdot\text{kg}^{-1}$ by the addition of 20 mM NaCl, KCl or NMGCl; $n = 4$ in each condition.

Unexpectedly, the cell shrank when the NaCl concentration in solution 2 was decreased from 80 to 40 mM. The same effects were observed when NaCl was substituted with KCl or NMGCl (Figs. 3b and c). These observations clearly show that when hypotonicity was induced by a decrease in electrolyte concentration from a low concentration of monovalent ions, the cell volume clearly decreased when it was expected to increase.

During hypotonicity, the cell shrinkage, observed when the external ion concentration was reduced (Fig. 3), implies an efflux of water against its osmotic gradient. This effect is not likely due to the activity of K^+Cl^- or $Na^+K^+2Cl^-$ cotransporters since their specific inhibitors dihydroindenyl-oxy-alkanoic acid (DIOA) and bumetamide had no effect (not shown).

As a corollary to the observations made during hypotonic challenges, a 14% increase in hypertonicity could also induce either cell shrinkage or cell swelling, depending on the starting ion concentration in the isosmotic solutions (Fig. 4). As expected, the cells shrank when hyperosmolarity (from 339 to 386 mOsm·kg⁻¹) was induced from a physiological concentration of monovalent ions (160 mM NaCl or 165 mM KCl or 160 mM NMGCl). In contrast, the cells swelled when the same hypertonic challenge was induced from a low concentration of monovalent ions (40 mM NaCl or 45 mM KCl or 40 mM NMGCl). Once again this shows that water can be transported against its osmotic gradient when coupled with passive ion fluxes.

Together, these observations raise the question as to how water is transported against its apparent osmotic gradient. During anisotonic challenges, H₂O could be either passively transported due to the resulting changes in intracellular osmolarity or transported uphill in association with passive ion fluxes. In ion channels, H₂O is present as hydration water of ions. According to the pore radius, one ion transports a maximum of 10 hydration H₂O molecules (Carrillo-Tripp et al. 2006). When the concentration of Na⁺ or K⁺ but also the large ion NMG⁺ was decreased or increased, the cell volume decreased or increased, respectively (Figs. 3 and 4, open circles). From these observations, we postulate that in addition to a passive transport through aquaporins (Rouzaire-Dubois et al. 2009) H₂O should be actively co-transported with ions through non selective channels. Depending on the osmotic gradient of H₂O and the electrochemical gradient of ions, these processes can be additive or opposite and can account for a deviation from the linear Boyle-vant't Hoff relationship between cell volume and inverse osmolarity. This new concept challenges the paradigm that H₂O is exclusively transported according to its osmotic gradient and provides evidence that, independently of the activity of selective ion channels and transporters, the cell volume is dependent on both the external osmolarity and ion concentration gradients.

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