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Acute Volume Regulation of Brain Cells in Response to Hypertonic Challenge

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Background: **Hypertonic dehydration of the brain through administration of osmotic agents, either alone or in combination with "loop" diuretics, has been a mainstay in the treatment of increased intracranial pressure for decades. Controversy exists, however, as to the mechanism and long-term value of such therapy. Although many cell types possess volume regulatory mechanisms capable of opposing hypertonic dehydration, such behavior in the brain is poorly understood.**

Methods: **As a model for the mammalian central nervous system, the real-time volume behavior of rat C6 glioma cells was observed by laser light scattering during hypertonic challenge. Cells were allowed to equilibrate in isotonic balanced salt solutions at physiologic pH and temperature, and then rapidly exposed to hypertonic solutions. Experiments were conducted in the presence and absence of sodium, chloride, and the loop diuretic bumetanide to assess their roles in volume regulation.**

Results: **In response to acute, large (70 mOsm) hypertonic exposures, cells immediately shrank and then rapidly regulated their volume completely back to control within minutes. In the presence of the loop diuretic bumetanide, the volume regulatory process was significantly inhibited with only 54% recovery observed at concentrations of 10^{-4} M. Volume regulation was also significantly inhibited by removal of extracellular sodium and chloride.**

Conclusions: **Brain cells possess powerful, electrolyte-dependent and bumetanide-sensitive volume-regulatory mechanisms that directly oppose attempted osmotic shrinkage. These observations suggest a possible new mechanism for the clinically observed synergistic effects of loop and osmotic diuretics in reduction of brain volume. (Key words: Brain: cell volume; diuretics; hypertonic; intracranial pressure; osmolarity.)**

CURRENT management of cerebral edema and increased intracranial pressure is predicated on a three-compartment model of central nervous system (CNS) volume relationships: Vtotal = Vblood + Vtissue + Vcsf. Since Weed and McKibben's demonstration of reduced cerebrospinal fluid pressure and brain mass in response to intravenous injection of hypertonic solutions,[1,2] it has been presumed that the brain tissue volume compartment behaves as an osmometer, thus clinically permitting osmotic dehydration with agents such as mannitol, glycerol, urea, and hypertonic saline.

Although much data has accumulated demonstrating the rapid and transient reduction of intracranial pressure following intravenous infusion of agents such as mannitol, there is conflicting evidence as to whether this arises from hemodynamic, rheologic, or osmotic mechanisms.[3–7] Additionally, there is evidence from whole animal models to suggest that, while extracellular water content may be influenced, brain intracellular volume is preserved during hypertonic challenge.[8] Therefore, to investigate the phenomenon of osmotic dehydration at the cellular level, we have developed a cell culture model for continuous observation of brain cell volume. With this model, we are now able to demonstrate the rapid and complete regulation of volume in cells of central nervous system origin after hypertonic shrinkage. In other cell types, this behavior has been classically referred to as regulatory volume increase (RVI).[9,10]

Because rapid volume regulation suggests the presence of associated ion transport pathways,[9,10] this process was studied in the presence of bumetanide (analogous to the clinical practice of simultaneous administration of loop and osmotic diuretics) and in the absence of sodium and chloride.

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Materials and Methods

Cell Culture

Rat C6 glioma cells, purchased from the American Type Culture Collection (Rockville, MD), were cultured in Eagle's minimal essential medium (MEM) with 10% fetal bovine calf serum and penicillin/streptomycin. Cultures were maintained in a humidified 5% CO_2/95% air atmosphere at 37° C and growth media changed every 48 h. Cells were seeded at low concentrations and all experiments conducted after 3–5 days' growth when cultures had reached approximately 80–90% confluence. Before each experiment, microscopic examination of each slide confirmed the presence of healthy cells with normal morphology. Further evidence of viability is provided by the observed volume regulatory behavior described below.

Cell Volume Observation Technique

An apparatus (fig. 1) similar to that of Fishbarg *et al.*[11] was constructed for continuous observation of cell volume changes. Cells were cultured on 11 × 22-mm rectangular glass cover slips to near confluency. Cover slips were mounted in a custom-designed cuvette of 2 ml total volume that was then continuously perfused with experimental solutions. All solutions were maintained at 36–38° C in equilibrium with 5% CO_2/95% O_2. During each experiment, the cuvette was perfused with experimental solutions at a rate of 3–5 ml/min. During solution exchanges, this rate was briefly (10–15 s) increased to 10–15 ml/min after it was determined that such increases in rate allow nearly complete solution replacement within approximately 10 s.

An 8-mm-diameter spot on the cover slip was illuminated with a 5-mW red helium-neon laser (Model 05-LHP-151; Melles Griot, Irvine, CA) while the coverslip was oriented at an angle of 30° to the incident. Scattered light was detected by a photomultiplier tube (Model 9826B; Thorn EMI, Fairfied, NJ) oriented 90° to the incident light. The apparatus was enclosed within a light-tight housing and photomultiplier tube (PMT) voltage output conducted sequentially through: 1) a signal preamplifier and voltage converter (Model A1; Thorn EMI), 2) a low-pass filter (Model 902; Frequency Devices, Haverhill, MA), and 3) an A/D conversion board (Model DT2821; Data Translation, Marlborough, MA) interfaced to an 80286 computer and sampled at 1 Hz.

Though a complex phenomenon, light scattering is proportional to the number and density of illuminated scatterers.[12,13] Although large particles are believed to scatter *via* a combination of interference and diffraction (best observed in the near-forward direction), small particles will scatter similarly in all directions and may

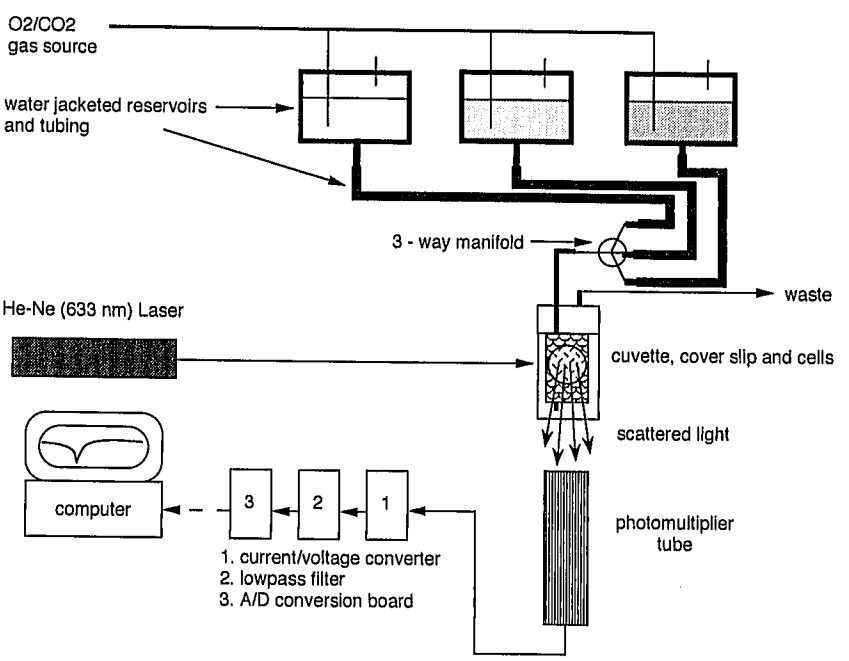

Fig. 1. Laser light scattering system for study of cell volume changes (details in text).

be observed at greater angles.[2,12,13] A biological cell, in addition to transmitting light, has a gross structure that allows it to scatter as a large particle and an internal structure that allows it to scatter as a collection of small particles. Therefore, both forward and large-angle light scattering have been used in the past to study cell volume, morphology, and physiology. In our system, the intensity of scattered light is related to the concentration of intracellular scatterers and closely tracks cell volume.[11–15] Thus, osmotic shrinkage may be expected to concentrate cellular contents and increase light scattering, while swelling will dilute cellular materials and decrease scattering. We have verified this relationship with the C6 cell line and other cell types through numerous calibration studies, including microscopic video-image analysis and optical sectioning.[15,16]

Because, for any given culture preparation, specific light scattering will be unique and dependent on such variables as cell type and confluency, light scattering provides *relative* volume information and each experimental substrate must serve as its own control. Therefore, cells are initially equilibrated for approximately 30 min under baseline conditions and the photomultiplier tube output set to zero volts. Experimental perturbations are then conducted and changes in scattered light intensity measured as voltage deflections from zero. The maximal voltage change for a given perturbation is then defined as 100% and steady-state recovery voltage as a fraction thereof.

Experimental Solutions

Isotonic (290 mOsm) control solution was bicarbonate-buffered (pH 7.4) and composed of (in mM): KCl 5.4, NaCl 116.4, NaHCO3 26.2, NaHPO4 1.0, MgSO4 0.8, CaCl2 1.8, glucose 5.5, glutamine 2, and inositol 0.02 with 30 nM selenium, 10 mg/l biotin, 5 mg/l insulin, 10 nM hydrocortisone, 5 pM tri-iodothyronine, and 25 μg/l PGE_1. Hypertonic (360 mOsm) solutions were produced by addition of NaCl and osmolarities were measured by freezing point depression (Osmette A; Precision Systems, Sudbury, MA). For inhibitor studies, both control and experimental solutions were prepared containing either 10^{-4} or 10^{-5} M bumetanide. For ion substitution experiments, isotonic and hypertonic sodium-free solutions were prepared using N-methyl-d-glucamine in place of sodium, and sodium isethionate (2-hydroxyethanesulfonate) in place of chloride.

Measurement of Cell Volume Changes

Cover slips were mounted in the study cuvette and perfused with control solution for 15–30 min to allow equilibration and stabilization of the PMT signal. Isotonic solution perfusate was then abruptly replaced with experimental solutions (as described above) and volume behavior observed.

Experimental Protocol

All experiments involved initial equilibration of cells in isotonic solution until a steady light scattering signal was observed for 15–30 min. For experiments involving bumetanide, the isotonic equilibration solution contained bumetanide; for ion-substitution experiments, the equilibration solution was sodium or chloride-free. It was previously shown that exposure of C6 cells to bumetanide alone does not cause volume pertubation (unpublished observations and Chassande *et al.*[17]).

Statistical Analysis

Data are expressed as means ± SE. Statistical analyses of the effects of bumetanide and ion substitution on response to hypertonic challenge were performed using Student's two-way *t* test for independent means with response to hypertonic saline alone taken as control. Initial rates of recovery were obtained by linear regression analysis of the first 60 data points during recovery. In all cases, $r > 0.96$.

Results

Stable light scattering signals with only minor drift were consistently demonstrable during equilibration periods of up to 1 h in isotonic solution. Exposure of cell monolayers to sudden hypertonic saline challenge resulted in immediate shrinkage, as evidenced by increased light scattering. Seconds later, regulatory volume increase became apparent as a steady decrease of light scattering back to control values. The RVI process was complete and new steady state achieved within 2–4 min (fig. 2). Mean recovery ($n = 6$) was 105 ± 5.4% and mean initial rate of recovery was 1.1 ± 0.1%–s^{-1}.

In the presence of bumetanide, RVI was substantially inhibited (fig. 3), in terms of both rate and extent of recovery. At concentrations of 10^{-4} M ($n = 4$), mean recovery was 54 ± 1.6% with rates of 0.5 ± 0.1%–s^{-1} (fig. 3). At concentrations of 10^{-5} M, recovery was 63

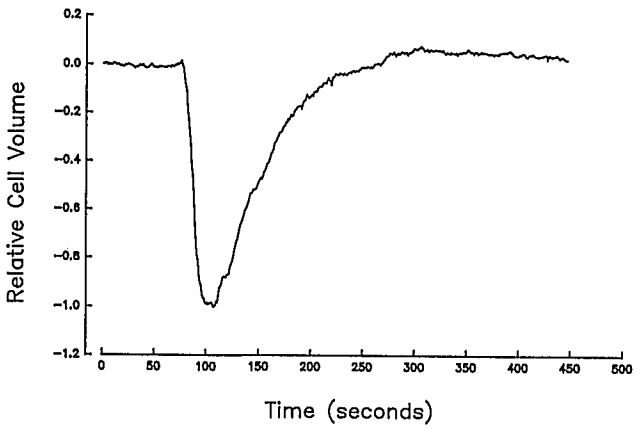

Fig. 2. Volume behavior of C6 cells exposed to 70 mOsm hypertonic saline challenge. Cells are equilibrated in isotonic solution until the voltage signal has remained stable for 15–30 min and are then abruptly exposed to the anisosmotic experimental solution. Light scattering voltages are normalized to the maximal voltage change and presented as relative changes in cell volume. Note rapid and complete recovery of cell volume after hypertonic shrinkage.

± 3.9% with rates of 0.7 ± 0.1%–s^{-1}. Further recovery was not observed throughout subsequent 30–45-min observation periods.

In the absence of sodium (n = 4), the extent (60 ± 3.8%) and rate (0.5 ± 0.08%–s^{-1}) of recovery was similar to that observed in the presence of bumetanide. Chloride removal (n = 5) produced the most profound inhibition of RVI extent 39 ± 2.5%, and inhibition of rate identical to that of sodium removal (table 1).

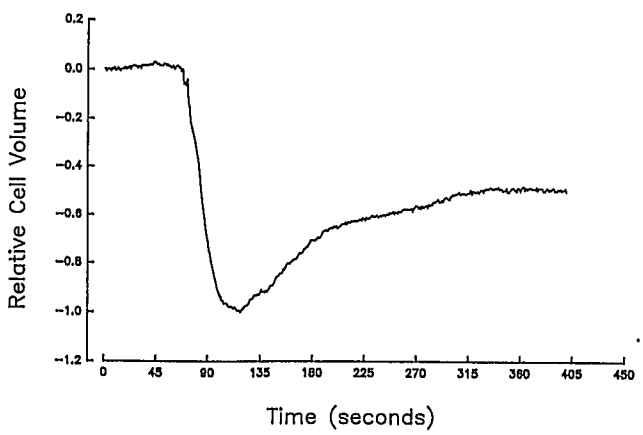

Fig. 3. Volume behavior of C6 cells exposed to 70 mOsm hypertonic saline challenge in the presence of 10^{-4} M bumetanide. Experimental protocol as in figure 2.

Table 1. Extent and Rate of C6 Cell Volume Regulation in Response to 70 mOsm Hypertonic Challenges

Experimental Solution	% Recovery	Rate of Recovery (% per second)
Hypertonic saline (control) (n = 6)	105 ± 5.4	1.1 ± 0.1
Bumetanide 10^{-4} M (n = 4)	54 ± 1.6 (<0.0005)	0.5 ± 0.1 (<0.0001)
Bumetanide 10^{-5} M (n = 6)	63 ± 3.9 (<0.001)	0.7 ± 0.1 (<0.02)
0 Sodium (n = 4)	60 ± 3.8 (<0.001)	0.5 ± 0.08 (<0.001)
0 Chloride (n = 5)	39 ± 2.5 (<0.0001)	0.5 ± 0.08 (<0.0001)

Values are mean ± SEM. Values in parentheses are *P* values *versus* control.

Discussion

For decades, the administration of hypertonic solutions of osmotic diuretics has been considered one of the few available therapies for cerebral edema and increased intracranial pressure. Although mannitol, first introduced clinically by Wise and Chater in 1962,[18] is probably the agent most widely used for this purpose, there is ample evidence that administration of simple hypertonic saline solutions may produce similar effects.[19–22] This practice is predicated in part on the basic assumption that brain tissue behaves as an osmometer and that associated brain water may be removed by exposure to an osmotic gradient. Because mannitol is believed to slowly equilibrate across the blood–brain barrier, the initial response to intravenous injection will include osmotic movement of water from the brain's extracellular space to the intravascular space. Because sodium and chloride are the primary extracellular solutes, any osmotic water movement from that space will principally result in cellular exposure to a hypertonic saline gradient. Our observations here demonstrate that, under physiologic conditions, cells of central nervous system origin possess potent mechanisms for the rapid regulation of their volume that directly oppose such attempted hypertonic shrinkage. The phenomenon of cell volume regulation, although poorly understood in the brain, is widely appreciated in other cell types and has been identified as the basis of other physiologic responses relevant to anesthesiology.[23,24]

Demonstration of RVI in the intact mammalian brain has been provided recently by the work of Cserr *et al.*[8]

Using microelectrode techniques and tissue water measurements in a rat model of hypernatremia, it was shown that, after raising serum osmolarity through intraperitoneal injection of hypertonic saline, a significant decrease in extracellular, but not intracellular, water can be observed.

In such a model, as in clinical practice, several minutes are required before equilibration has occurred across the blood–brain barrier and brain cells are fully exposed to hypertonicity. Therefore, it is possible that the more gradual stresses encountered *in vivo* are met by regulatory mechanisms capable of maintaining nearly constant cell volume. Data presented here support this hypothesis, demonstrating that RVI mechanisms in glial cells are extremely vigorous and capable of complete volume regulation even during large, rapid hypertonic exposures. Recently, using microscopic video-imaging techniques, we observed similar volume regulatory behavior in primary cultures of rat cortical neurons, and, by light scattering, in primary cocultures of neurons and astrocytes (Churchwell, McManus, and Strange, unpublished observations).

Acute volume regulation is primarily accomplished through the action of volume-activated electrolyte transporters. Thus, in the face of acute hypertonic challenge and water efflux, the cell engages mechanisms capable of rapidly transporting solute and water inward. Examples (reviewed in Siebens[9]) include Na^+/Cl^- and $Na^+/K^+/2Cl^-$ cotransporters[25] and Na^+/H^+ or K^+/H^+ transport coupled with $Cl^-/HCO3^-$ exchange.[26] Because the $Na^+/K^+/2Cl^-$ cotransporter is inhibited by loop diuretics and because both clinical experience and numerous animal and human studies[27–31] have documented the synergy of osmotic and loop diuretics in reduction of brain volume, we investigated the effects of the diuretic bumetanide in this model. Bumetanide was selected in preference to the more clinically common furosemide because of its enhanced specificity for the Na-K-2Cl cotransporter.

Bumetanide was found to substantially inhibit volume regulatory processes in this model and caused cultured brain cells to remain shrunken for a prolonged period in response to hypertonicity. Because bumetanide and similar agents produce their effects through inhibition of Na^+ and Cl^- transport, it is not surprising that removal of these ions from the extracellular fluid also led to marked inhibition of volume control. It is important to note, however, that inhibition was incomplete and, thus, the bumetanide-sensitive pathway probably represents only one of several such transport mechanisms involved in the fundamental process of cell volume regulation.

Several possible mechanisms for the synergistic effects of loop and osmotic diuretics have been suggested. These include enhancement of diuresis through combined renal effects, inhibition of CSF production, and differential effects of different agents on normal and abnormal brain tissue.[27–32] In 1972, Buhrley and Reed[33] observed that sodium-22 uptake into the CSF and cerebral cortex of nephrectomized rats was markedly reduced by intraventricular injection of furosemide. However, because of the free communication between cells and CSF, these authors were unable to characterize the cellular component of this reduction. Work described here suggests that cellular transport of sodium in the brain is, indeed, inhibited by loop diuretics. More specifically, our experiments provide evidence that inhibition of RVI, mediated substantially by sodium and chloride uptake through bumetanide-sensitive pathways, may represent an important mechanism by which osmotic and loop diuretics exert their synergistic effects in reduction of brain volume. Although research involving a transformed cell line must be interpreted with caution, these data are consistent with available *in vivo* observations, clinical experience, and the growing body of knowledge concerning cell volume regulation.

The observations presented here imply that physical dehydration of the brain must be accomplished through removal of water from the extracellular compartment while the intracellular compartment regulates its volume. This volume regulation, however, may be overcome by inhibitors of electrolyte transport, such as those diuretics commonly employed in clinical practice. Although much research has been directed toward the understanding of cerebral edema and its mechanisms, little is known regarding homeostasis of the brain intracellular volume compartment. Currently, a three-compartment model of the central nervous system permits therapy aimed at reduction of blood, cerebrospinal fluid, or brain tissue volumes, with the last accomplished through osmotic dehydration. Investigations such as those presented here indicate that further understanding of cerebral edema requires expansion of our traditional three-compartment model of brain volume relationships. A four-compartment model, as proposed by Cserr and Patlak,[34] provides the next logical step: $V_{total} = V_{blood} + (V_{extra\text{-}cellular} + V_{intra\text{-}cellular}) + V_{csf}$.

Further investigation into the regulation of intracellular compartments is necessary to define the extent to which acute volume regulatory mechanisms in the brain oppose current clinical strategies and may be inhibited or stimulated by the anesthesiologist to improve patient outcome.

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