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Volume changes induced by osmotic stress in freshly isolated rat hippocampal neurons

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Abstract The degree to which osmotic stress changes the volume of mammalian central neurons has not previously been determined. We isolated CA1 pyramidal cells and measured cell volume in four different ways. Extracellular osmolarity (π_o) was lowered by omitting varying amounts of NaCl and raised by adding mannitol; the extremes of π_o tested ranged from 134 to 396 mosm/kg. When π_o was reduced, cell swelling varied widely. We distinguished three types of cells according to their response: "yielding cells" whose volume began to increase immediately; "delayed response cells" which swelled after a latent period of 2 min or more; and "resistant cells" whose volume did not change during exposure to hypo-osmotic solution. When π_o was raised, most cells shrank slowly, reaching minimal volume in 15–20 min. We observed neither a regulatory volume decrease nor an increase. We conclude that the water permeability of the membrane of hippocampal CA1 pyramidal neurons is low compared to that of other cell types. The mechanical support of the plasma membrane given by the cytoskeleton may contribute to the resistance to swelling and protect neurons against swelling-induced damage.

Key words Cell swelling · Cell volume · Cerebral oedema · Dissociated neurons · Hypotonia · Osmotic effects

Introduction

The swelling of neurons is an important feature of many neuropathological conditions, including cerebral hypoxia, ischaemia and excitotoxicity [29, 34]. Haemodilution and hyponatremia cause cerebral oedema and a rise of intracranial pressure [37] because the blood-brain barrier is more permeable to water than to most of the solutes in blood plasma, but the water loading of the brain is less than expected for "passive" osmotic flow [22, 30]. Excess water in cerebral tissue accumulates both in the interstitial space and in cells [24], but from whole-brain measurements it is not clear whether this occurs equally in glial and neuronal elements. In experimental hypernatremia the volume of brain cells appears to be maintained whereas the interstitial space shrinks [8]. Osmotically induced volume changes have been described in some detail for isolated glial cells [9, 19, 20] and molluscan neurons [10, 36], but data on mammalian neurons are incomplete.

Exposure in vitro of mammalian neurons and brain slices to osmotic stress has yielded seemingly disparate results. Isolated hippocampal CA1 neurons exposed briefly and suddenly to strongly hypo-osmotic solution retained their size and shape [31], while during hyper-osmotic treatment many but not all isolated hippocampal neurons shrank [35]. The main focus of these earlier studies was electrophysiology, and cell size and shape were examined only by low-resolution microscopy. Seemingly in contrast to isolated cells, when brain tissue slices were bathed in a solution of low osmolarity, cell swelling was indicated by a reduction of interstitial space [6], increased tissue resistance [3, 6] and increased tissue translucence [2, 3, 21]. Neither interstitial space nor optical signals distinguish, however, glial cells from neurons. Andrew and MacVicar [2] concluded that neuronal dendrites and glial elements swell more than neuron somata. In recent experiments we found a reversible increase of the apparent capacitance, as measured by whole-cell recording from the cell soma of CA1 neurons in hippocampal tissue slices, during a moderate decrease of interstitial osmolarity (π_o), suggesting expansion of the cell surface and therefore of the cell volume [17].

As a result of these seemingly disparate observations, we have re-examined the responses of isolated neurons to graded changes of π_o of varying lengths of time, using improved methods for measuring cell size. Some of the cells did respond with a concentration-dependent revers-

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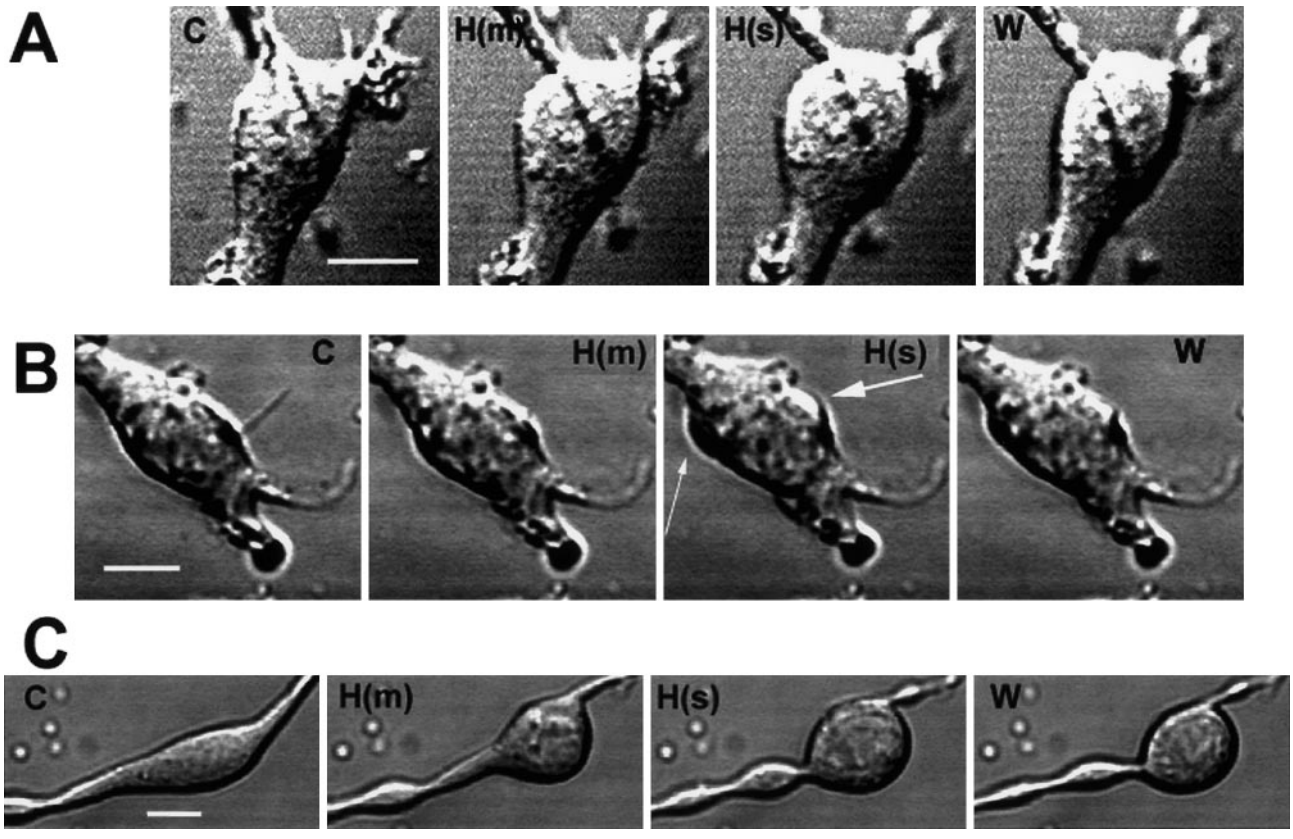

Fig. 1A–C Images of three neurons exposed to two different hypo-osmotic solutions. [*C* Control, after 5–10 min of observation and about 1 min before changing the bath, *H(m)* after 5 min of moderate hypotonia (40 mM NaCl removed from the medium), *H(s)* after 5 min of severe hypotonia (60 mM NaCl removed), *W* after 5–6 min of washing in normal solution.] Note reversible swelling of cell in **A**; bleb (membrane blister), marked by *white arrows*, of cell in **B**; and irreversible distortion of shape in **C**. *Calibration bars*: 10 μm

ible volume change but others seemed to resist the hypotonic challenge. In all cases the rate of volume change was slow, and its magnitude varied widely among individual cells.

Some of the results have appeared in an abstract [32].

Materials and methods

We isolated rat hippocampal CA1 pyramidal cells according to the method of Kay and Wong [18]. Briefly: Rats of 80–150 g body mass were decapitated under ether anaesthesia. Brains were removed and 400- to 500-μm-thick slices were cut from the hippocampus. The CA1 region was cut into smaller pieces of about 0.4 mm×0.4 mm. These tissue fragments were transferred to a glass vessel for trypsin digestion for 45–60 min. The digestion medium contained (in mmol/l): NaCl 125, KCl 5, $CaCl_2$ 1, $MgCl_2$ 2, D-glucose 25, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) 10, pH 7.0, with 1 mg trypsin/ml, at 35°C. The digestion medium was oxygenated and stirred gently. After digestion the tissue pieces were washed 3 times and then incubated in trypsin-free oxygenated medium at room temperature. Tissue fragments were dispersed by trituration with a graded series of Pasteur pipettes. Cells were maintained in flowing HEPES-buffered medium of the following composition: (in mmol/l) NaCl 130, KCl 3.5, $CaCl_2$ 1.2, $MgCl_2$ 1.0, glucose 25, HEPES 10, pH 7.3, either at 22–26°C (Duke University) or 32°C (Amsterdam).

Extracellular osmolarity (π_o) was lowered by omitting NaCl from the bathing fluid and raised by adding mannitol. The osmolality of the bathing solutions, measured by freezing point or vapor pressure osmometry, in mosm/kg, was – normal: 297±2.4; hypotonic: omitting 25 mM NaCl, 247±1.08, omitting 40 mM NaCl, 217; omitting 50 mM NaCl, 211±2.2, omitting 60 mM NaCl, 189; omitting 90 mM NaCl, 134; hypertonic: 50 mM mannitol added, 341±2.4; +100 mM mannitol: 396±2.3.

The experiments were conducted at two different sites (Duke University and the University of Amsterdam). Cell volume changes were determined in four ways:

1. The cell suspension was placed into a 70-μl miniature flow cell [4] and the cells were allowed to settle and to adhere to the bottom. They were, viewed using an inverted microscope with a 100× oil immersion objective of 1.2 n.a. At a flow rate of 0.6 ml/min, the exchange of solutions in this miniature chamber took less than 0.5 s from the moment of turning the taps. A selected cell (sometimes two cells) was imaged either in white light or, after loading with the dye BCECF (3–10 μM), in fluorescent light (excitation 450–490 nm, emission 515–565 nm). Images were collected using MetaMorph software (Universal Imaging, West Chester, Pa., USA). The diameter of the cell was defined as its greatest width measured along a line normal to the cell's long axis, and volume changes were computed as the third power of changes in this measurement. The integrity of the plasma membrane of 4 neurons out of a total of 17 was tested after the completion of recording sessions by adding propidium iodide (0.5 μM) to the bathing solution. If the membrane is abnormally permeable, this dye attaches to the nuclear membrane and causes it to fluoresce [5, 23]. None of the tested neurons showed this reaction, even after repeated exposure to anisosmotic solutions. When, however, the cell was exposed to the detergent Triton (0.1%) or to distilled water, the nucleus did become intensely fluorescent.

2. Cells were loaded with an indicator dye, PBFI or SBFI (Molecular Probes), by incubation in 5–10 μM of the acetoxymethyl (AM) ester of the dye. In a minority of trials loading was aided by adding 0.001% pluronic acid. The cell suspension was then transferred in a chamber of approximately 1.3 ml volume, flow rate 1.0–1.4 ml/min on the stage of a Nikon Diaphot inverted microscope. In this chamber the near-complete exchange of solutions, including the 10- to 20-s delay due to the tubing dead space, took approximately 90 s. Cells were imaged with a Nikon Fluor 40× objective of 1.3 n.a. The image of selected cells was captured by a cooled CCD camera and stored on computer. The fluorescence values at both 340 and 380 nm excitation, (emission at 515 nm) were recorded and stored in memory. The wavelength 380 nm is approximately isosbestic for changes in ion activity and it was used for volume estimation. Cell volume was estimated by fitting the experimental data to a theoretical cell constructed as an ellipsoidal solid of revolution filled with uniform fluorescence. The distribution of pixel intensities of near-isosbestic 380 nm excitation, within the boundary of the cell from which recordings were made, was fitted (least-squares criterion) to the distribution of this model cell adapting its size, whereafter cell volume was calculated. This method provides the correct volume for spherical or ellipsoid cell shapes. The fluorescence ratio of excitation at 340/380 nm should be a measure of K^+ or Na^+ activity, but calibration both inside cells and in vitro of the fluorescence ratio against K^+ concentration indicated insufficient sensitivity and therefore we deleted ratiometric data from this report.
3. The fluorescence intensity of the ion-sensitive dyes PBFI and SBFI was measured in a small area near the centre of the image of the cell. A volume increase caused dilution, whereas a volume decrease caused concentration of the fluorescent dye measured at the isosbestic 380 nm excitation. Methods 2 and 3 were applied concurrently to the same cells.
4. Cell suspensions were placed in a chamber of about 0.7 ml capacity on the stage of a Zeiss Axioskop. Images of cells were recorded in the "transmitted light" mode of a confocal microscope system (BIO-RAD MRC 600), using 40× water immersion objective (Fig. 1). Using COMOS software, an outline of the cell was drawn and the image area determined. Volume was computed as the 3/2 power of the image area and changes were expressed as a percentage of the control volume.

Results

Hypotonic swelling

The response of individual neurons to lowering of π_o varied greatly. Figure 1 illustrates various size and shape changes. The cell shown in Fig. 1A swelled in hypotonic medium and shrank when washed in solution of normal π_o. However, this cell did not completely regain its original pyramidal shape. The cell soma of Fig. 1B retained its normal size and shape during treatment with hypo-osmotic medium, but the membrane developed two small blisters or "blebs" [11, 23]. These blebs were reduced in size but they did not entirely disappear during 6 min of washing with normal solution. The cell of Fig. 1C swelled irregularly; its soma became more spherical in shape and its dendrites developed varicosities but no membrane blebs. During washing in normal solution its volume decreased but the shape remained irregular, indicating irreversible structural changes.

Not only the degree but also the time course of size change varied among neurons. Various temporal patterns of volume changes are presented in graphical form in Fig. 2. The data for the three cells shown in Fig. 2 were recorded on different occasions, but all three were maintained in the standard (larger) chamber and all three were exposed to the same degree of hypotonia (omitting 50 mM of NaCl, bath osmolality approximately 211 mosmol/kg). In these experiments relative volume changes were computed from the area occupied by the fluorescent image as well as from the dilution of the fluorescence at the isosbestic wavelength of the dye, as described under points 2 and 3 in Materials and methods. The cell whose data are illustrated in Fig. 2A began to swell soon after the arrival of the hypo-osmotic solution and then continued to gradually increase its size during the 5 min of treatment with hypo-osmotic solution. The cell represented in Fig. 2B retained its size for more than 2 min of exposure to hypo-osmotic solution and then it swelled rather suddenly. The third cell (Fig.

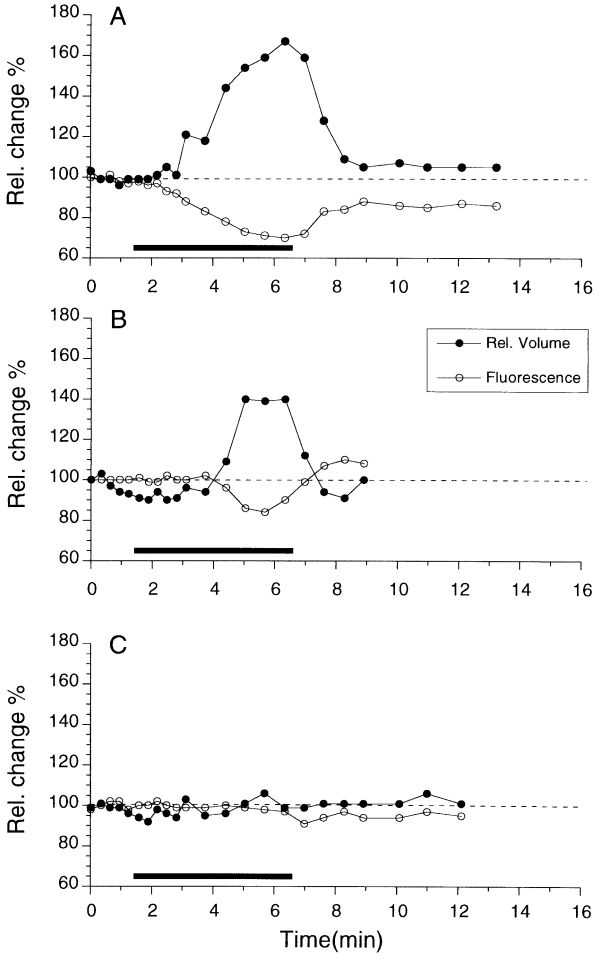

Fig. 2A–C Changes in the size of three cells before, during and after exposure to hypo-osmotic solution [50 mM NaCl deleted from bathing fluid; extracellular osmolality (π_o) approximately 211 mosmol/kg]. *Filled circles* indicate volume as a percentage of initial control, computed from the fluorescent image ("Method 2"). *Open circles*: fluorescence intensity at the near-isosbestic (380 nm) excitation wavelength ("Method 3"). **A** Example of a cell "yielding" to hypotonic challenge; **B** example of delayed swelling; **C** example of resistance to hypo-osmotic challenge

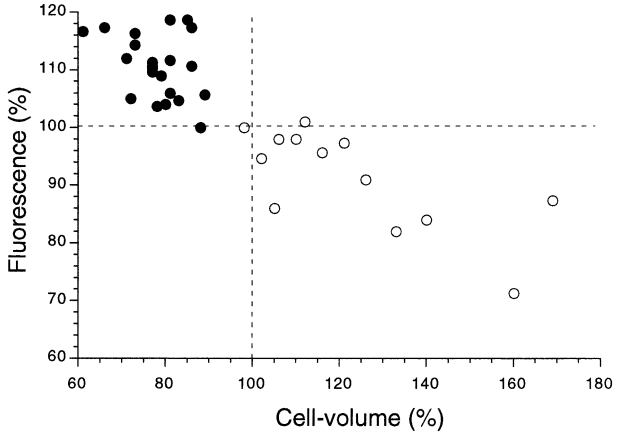

Fig. 3 Normalized maximal and minimal computed volumes and isosbestic fluorescence intensities reached after 5 min of treatment of individual neurons with anisosmotic solution. *Filled circles*: elevated π_o (396 mosm/kg); *open circles*: reduced π_o (211 mosm/kg). Correlation of fluorescence and volume, R=–0.865 (P<0.001); regression coefficient –40±0.04

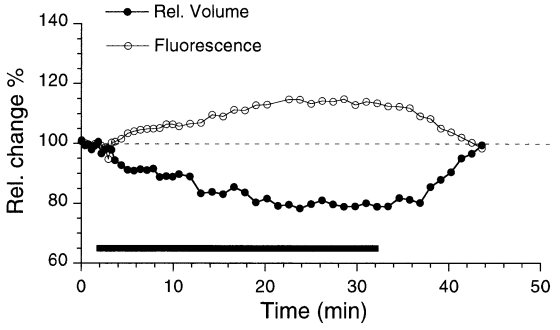

Fig. 4 The course of the average relative computed volume and relative isosbestic fluorescence of 7 cells during 30 min of exposure to mildly hypertonic (341 mosm/kg) solution

2C) did not change its volume for the entire period of treatment with hypo-osmotic solution. We propose to characterize the behaviour of the cell shown in Fig. 2A as "yielding", that of the cell of Fig. 2B as "delayed response" (at least 2 min between the start of exposure and the start of detectable swelling) and that of the cell in Fig. 2C as "resistant" (volume change no greater than 5% of control volume in 5 min of exposure). The volumes of individual cells reached after 5 min of exposure to a π_o of about 211 mosmol/kg (–50 mM NaCl) in the standard chamber are shown for each cell so treated as the open circles in Fig. 3. These volumes varied from 100% (i.e. no change) to swelling up to as much as 170% (i.e. an increase by +70%).

Eight cells were exposed in the standard chamber to a milder degree of hypotonia (–25 mM of NaCl, 247 mosmol/kg) for 30 min. Two of these cells retained their size and shape for the entire treatment period and the subsequent 10 min of washing with normal solution. Four cells swelled gradually, reaching an average maximum of 126% in 25–30 min. A regulatory volume decrease (RVD) was not observed. During 10 min of washing with normal solution after such a prolonged hypotonia-induced swelling these four cells recovered on average only about one-third of their excess volume (final recovered volume 118%). Two more cells swelled and formed membrane blisters or "blebs" which did not entirely disappear.

Sixteen neurons were studied in the miniature chamber. Each of these cells was exposed to several π_o levels. Images of additional 22 neurons were recorded by confocal microscopy, of which five were exposed to moderate hypotonia (40 mM NaCl deleted), 9 to severe hypotonia (60 mM NaCl deleted) and the remaining 8 to both levels in succession. In this series 4 out of 22 cells developed membrane blebs.

Hypertonic shrinkage

Most, but again not all, cells shrank when bathed in hypertonic solution. After 5 min of exposure to a solution of 396 mosmol/kg (100 mM mannitol) in the standard chamber, cell size varied from 95 to 62% (Fig. 3). Mean and standard error of sizes reached after 5 min of varying degrees of hypertonia in both the miniature and the standard cell chamber are shown in Fig. 5A.

Seven cells were treated for 30 min in mildly hypertonic solution (50 mM mannitol, 341 mosmol/kg). All seven shrank during treatment with hypertonic solution and then recovered their normal volume after 10 min of washing with normal solution. The minimum size of 79% of control volume was reached in about 20 min, as illustrated in Fig. 4. A regulatory volume increase was not observed.

Computed volume versus fluorescence changes

In the cells that were filled with the dye PBFI or SBFI, the intensity of fluorescence at the near-isosbestic excitation wavelength of 380 nm was recorded in a small central area of the image of the cell soma. In all cases, when the computed volume of a cell changed, so did the isosbestic fluorescence intensity, in the opposite direction but with similar time course (Figs. 2–4). The normalized computed volume change and the normalized fluorescence change recorded after 5 min of treatment with anisosmotic solution were negatively correlated (Fig. 3, R= –0.86, n=34). The change of the normalized fluorescence was, however, consistently less than that of the computed volume, yielding a regression coefficient of –0.40± 0.04. For reasons explained in the Discussion, the fluorescence intensity, as measured, underestimates the volume change.

Comparing volume changes estimated by methods 1, 2 and 4

Figure 5A shows the dependence of average cell volume changes on π_o, as determined by three different methods. Figure 5B shows the same data plotted against the

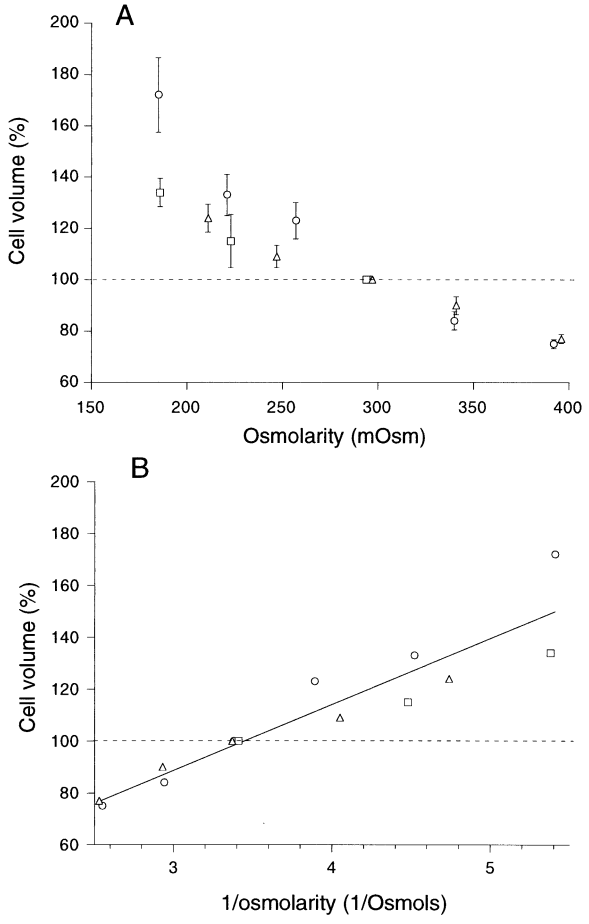

Fig. 5. A The mean (±SEM) average volumes of isolated neurons after 4.5–5.5 min of exposure to anisosmotic solutions of varying concentrations. Note that some of the cells were treated for 30 min, but the data used for this figure were measured at 5 min. *Circles* indicate data from cells maintained in the miniature chamber;, normalized volume change was calculated from the 3rd power of the diameter ("method 1")., *Triangles* indicate data from cells maintained in the standard chamber; cell volume was computed from the fluorescent image, ("method 2"). *Squares* indicate data from cells viewed by confocal microscopy in a 0.7-ml chamber;, cell volume was calculated as 3/2 power of image area ("method 4"). **B** The dependence of cell volume on the inverse of osmolarity. From the same data as **A**. (R=0.93; coefficient of regression: 25.4)

inverse of osmolality. Size changes are linearly related to the inverse of osmolality; the coefficient of correlation for each of the three methods being $R \geq 0.99$. The coefficients of regression of volume increases on the inverse of osmolality were as follows – for "method 1" (volume change estimated from the 3rd power of the diameter): 33.7±1.9; for "method 2" (matching fluorescent image to theoretical model): 17.2±2.0; for "method 4" (the 3/2 power of the confocal image area): 20.1±1.6. For the three data sets combined, R=0.93; the combined regression coefficient was 25.4, the Y-intercept, 12.5. Methods 2 and 4 agree well (the difference between the coefficients is less than the sum of the standard errors), but method 1 appears to overestimate changes (see Discussion).

Differences in onset time between cell swelling and shrinkage

Cells started to shrink in high π_o sooner than they started to swell in low π_o. For example the cell represented in Fig. 2B, which swelled after a latent period of more than 2 min in low π_o, was, after recovery in normal solution, exposed to hypertonic solution and started to shrink within 10 s of the arrival of the solution in the chamber. Since the cell did not move, it is clear that its position in the bath or similar incidental conditions were not the determining factor in the delay.

In the standard chamber the mean delay between switching solutions to the onset of cell volume change was 71.9 s (n=40, range 20–164 s; SEM ±7.9 s) for hypertonia-induced shrinkage, and 154.4 s (n=14; range 58–344 s, SEM ±24.9 s) for hypotonia-induced swelling. The cells that did not swell at all but retained their size within 5% of control volume are not included in these average times. These latency times include the time during which the anisosmotic solution arrived at the cell.

Possible sources of variability

The degree to which individual cells swelled or shrank was independent of the initial size of the cell. Computed cell volumes varied from 0.7 to 4.4 pl. The degree of osmotically induced volume change was random across this range.

We considered the possibility that cell preparations could deteriorate in the course of storage. There was, however, no correlation between relative cell volume change and the time of day of the measurement (from 13:00 hours to 18:00 hours), indicating that the length of time spent in oxygenated solution at room temperature did not influence the outcome.

In most cases, isosbestic fluorescence intensities recovered to pre-exposure levels to the same degree as did computed volumes (see e.g. Figs. 2B, 4; but also Fig. 2A for an exception), indicating that bleaching or dye loss from cells was usually negligible during the time of observation.

Discussion

Osmotically induced volume changes and the permeability of the plasma membrane to water

The plots of Fig. 5 suggest a precise dependence of cell volume on bath osmolality, and the linear regression of the average cell volume on the inverse of osmolality conforms to the Boyle-van't Hoff formulation and is in line with findings in studies of other cell types [12] (Fig. 5B). Yet interpretation of these figures must be tempered, because after 5 min of treatment most cells did not reach their final volume. Besides, the average values conceal the widely variable behaviour of individual neurons.

Each of the methods we used to estimate cell volume changes has its own technical limitation. From Fig. 5 it would seem that the cells in the miniature chamber swelled more in strongly hypo-osmotic solution than did the cells in the larger baths (Fig. 5A and Results). In part this divergence may be due to the error inherent in using the 3rd power of the diameter as the index of relative volume. Such use of diameter (or radius) is valid as long as the cell expands in equal proportion in all directions, but cell somata may have expanded more in girth than in length (Fig. 1A). Computations based on the area of the image are less prone to error due to shape changes, but suffer loss of precision if the cell rises or collapses so that the image no longer presents the cell's greatest cross-sectional area. In spite of these limitations, and even though the three sets of volume estimates were obtained using different cell chambers in different laboratories, they yielded similar results and reinforce one another.

Volume measurement based on the dilution of an intracellular marker substance avoids the errors of geometrical estimates. Unfortunately, however, the fluorescence intensity as measured in these trials was not a true measure of dye concentration, because the volume within which the detector captured photons was not constant. Fluorescence was detected within a cylinder of defined radius but undefined height. As cells swelled, the depth within which photons were detected increased. As a result, the fluorescence intensity changes were consistently smaller than the volume changes computed from the image area of the same neurons. Even so, the timing and magnitude of fluorescence and volume changes measured concurrently in a population of neurons were closely correlated (Figs. 2–4) and confirm the validity of the data.

The variability of the response, especially in hypo-osmotic solutions, is one of the remarkable findings. This variability is not explained by inadequate mixing of test solutions in the chamber. Cells that responded with a delay to hypo-osmotic solution responded promptly to hyper-osmotic medium, even though the position of the cell in the chamber and the direction and the rate of flow was the same in the two conditions. Furthermore, in the miniature chamber exchange of solution was very rapid (<0.5 s) and complete. It is well to remember that methods frequently used to measure aggregate volumes of large cell populations conceal the variability of the effect.

The slowness of the change even of those cells whose volume did yield to osmotic stress in both hypo- and hyperosmotic solutions indicates that the permeability to water across the plasma membrane in these cells is low. Many other cell types, including cultured cerebellar granule cells and glia, change volume much faster [7, 27, 28, 33]. The rate of osmotically induced swelling of cultured neurons decreases with age [14, 15]. Swift volume change is due to a brisk transmembrane flow of water, believed to be mediated through water channels formed by proteins known as aquaporins [1]. In mammalian brain, aquaporins have, so far, been found only in glial cell membranes and not in neurons [26].

In the absence of specialized channels, water may seep through either the lipid bilayer or through ion channels that are believed to have a water-filled pore [13]. In the latter case it is thinkable that tight closure of ion channels could cut off the flow of water. This would be in line both with the absence of detectable cell swelling and with the generalized "ion channel shutdown" phenomenon shown by neurons exposed briefly to strongly hypo-osmotic solutions, described in our earlier report [31]. In those trials test solutions were blown onto neurons from a nearby micropipette for periods of 20–75 s, which is shorter than the delay period that preceded the swelling of most of the cells observed in this study.

Resistance to osmotically induced deformation could also be explained by mechanical resilience, due in part or whole to the support lent to the plasma membrane by the cytoskeleton [15, 25]. The irregularly distributed varicosities of the cell shown in Fig. 1C, as well as the delayed but sudden swelling illustrated in Fig. 2B, could be due to the yielding of cytoskeletal support. The relatively rare blistering (blebbing) represents detachment of the membrane from the cytoskeleton. In the example of Fig. 1B the blister accommodated the excess fluid entering the cell so that the soma retained its normal size and shape. Also, the greater resistance against hypotonic swelling compared to hypertonic shrinkage agrees with the idea of a force restraining outward expansion more effectively than inward crinkling of the membrane. The two explanations, restricted water flow and mechanical resistance, are not mutually exclusive. In either case, resistance to cell swelling could be protective of the integrity of the membrane and of its supporting structure. As we have seen, the recovery of cell volume from swelling, once it did occur, was often less complete than the recovery from shrinkage.

Isolated cells differ from cells in situ

Dispersed neurons differ in several respects from neurons in cerebral tissue. In the course of the dissociation procedure the cells lose most of their dendritic tree. They are not surrounded by other neuronal elements, but are suspended in a large volume of solution. In addition, they are maintained at room temperature in a HEPES-buffered solution without bicarbonate and without amino acids but containing an excess of glucose. It may well be that fine dendritic branches behave differently from cell somata and the main proximal dendritic trunk. Furthermore, close apposition of cellular elements could limit the degree of hypotonic swelling of neurons in situ.

From our measurements of interstitial space changes in the CA1 region of hippocampal tissue slices we calculated the average cell expansion in response to severe hypotonia (134 mosmol/kg) lasting 30 min to be about 11% over the "resting" volume, assuming even distribu-

tion of excess water among all cells [6]. Even allowing for uneven swelling of different types of cellular elements and for possible underestimation of swelling (see Discussion in [6]), it seems that, on average, brain cells swell less in their natural environment than in the dissociated state. In addition, the absence of mechanical support provided by adjacent cells and by the interstitial matrix, the trypsin treatment, the low temperature or the lack of appropriate osmolytes in the bathing fluid may have prevented isolated neurons from regulating their cell volume.

Those cells that yielded to 30 min of hypotonic treatment, as well as some of the cells that were exposed to 5–6 min of hypotonia, did not completely return to their control volume during subsequent washing with normal solution. A few were injured, in the form of irreversible, irregular localized varicosities or membrane "blebbing". By contrast, neurons in hippocampal tissue slices recovered full function as well as input capacitance following 30 min of even more severe hypo-osmotic treatment [16, 17]. An important difference between the two conditions is the slow equilibration of the interstitial spaces of tissue slices after bath solution change, which makes the onset of osmotic challenge more gradual for the cells in the slice compared with that experienced by dispersed cells.

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