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CELL VOLUME CHANGES UPON SODIUM PUMP INHIBITION IN *HELIX ASPERSA* NEURONES

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SUMMARY

1. Identified neurones of the suboesophageal ganglia of *Helix aspersa* were loaded with tetramethylammonium (TMA^+). Experimentally induced changes in cell water volume and membrane potential were measured continuously by monitoring changes in intracellular [TMA^+] using ion-sensitive double-barrelled microelectrodes. The technique allowed measurements of cell water volume changes of less than 5%.

2. Exposure to hyperosmotic (up to +24%) or hyposmotic (up to about −10%) solutions caused reversible decreases and increases in cell water volume respectively, which agreed with near-ideal osmometric behaviour. Upon exposure to hyposmotic solutions whose osmolality was decreased by 30–40%, the cell water volume increased to maximum values below those expected for ideal osmometric behaviour and exhibited partial regulatory volume decrease.

3. The sodium pump was inhibited in twenty identified neurones by sustained exposure to 1 mM ouabain. In every case ouabain caused cell membrane depolarization, as expected for inhibition of an electrogenic sodium pump.

4. Upon pump inhibition most cells ($n = 14$) shrank by up to 13% of their initial water volume. In five of these cells, shrinkage was preceded by one or more short-lived swelling phases. In two other neurones short-lived swelling was followed by cell volume recovery without appreciable shrinkage. In four out of the twenty cells, there were no measurable volume changes.

5. The lack of an initial swelling phase in the cells that shrank, as well as the absence of detectable volume changes in some of the neurones, was not due to loss of ion-selective electrode sensitivity since predictable changes in cell volume elicited by osmotic challenges were monitored in the same cells.

6. It is concluded that neurones can be endowed with ouabain-insensitive mechanisms of volume control, whose activation following Na^+ pump inhibition prevents them from short-term swelling and lysis.

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INTRODUCTION

Animal cells contain impermeant solutes, such as negatively charged macromolecules, and this results in a high intracellular colloid osmotic pressure relative to that of the extracellular fluid. Since the plasma membrane of most cells is permeable to water, the colloid osmotic pressure gradient acts as a driving force for net water influx. If cells were not endowed with mechanisms for maintaining their volume constant under isosmotic conditions, colloid osmotic swelling and eventual lysis would ensue. According to the pump–leak hypothesis, colloid osmotic swelling is prevented by the operation of pumps that extrude Na^+ at a rate equal to that at which they leak into the cell (Tosteson & Hoffman, 1960). Hence, inhibition of the Na^+ pump should result in cell swelling as has been shown in many tissues (Tosteson & Hoffman, 1960; Macknight & Leaf, 1977; Strange, 1989). However, some cells apparently do not swell after Na^+ pump inhibition with ouabain (Macknight & Leaf, 1977; Kempski, Staub, Rosen, Zimmer, Neu & Baethmann, 1988; Strange, 1989). This seems to contradict the pump–leak hypothesis and suggests that in some cases only ouabain-insensitive mechanisms control cell volume (Kleinzeller, 1985; Van Rossum, Russo & Schiselbauer, 1987; Proverbio, Marín & Proverbio, 1989). In addition, some cells first swell and then decrease their volume in the presence of cardiac glycosides (Jensen, Fisher & Spring, 1984; Van Rossum, Russo & Schiselbauer, 1987; Strange, 1990). This indicates that a compensatory decrease in cell volume can occur under conditions in which the Na^+ pump is inhibited. These discrepancies could reflect differences in the rates and time course of ouabain-induced swelling and subsequent volume decrease. A short-lived or small swelling could pass undetected due to inadequate time resolution or sensitivity of the methods commonly used to assess cell volume. On the other hand, the discrepancies could reflect real differences resulting from the magnitude and direction of net transmembrane flow of osmotically active solutes. For instance, if the efflux of osmotically active particles is larger than their influx, cell shrinkage will ensue.

Little is known about the mechanisms underlying cell volume regulation and maintenance in neurones (Ballanyi & Grafe, 1988). Cell volume studies in neurones are important for the following reasons. First, because of their small volume/surface ratio, and the net ion movements underlying synaptic and all-or-none electrical activity, neurones are vulnerable to undergo volume changes (cf. Serve, Endres & Grafe, 1988). Secondly, because Na^+ pump inhibition secondary to ATP depletion is currently accepted as a major factor underlying cytotoxic brain oedema (Klatzo, 1985; Rosenberg, 1990), and yet no direct measurements of cell volume of neurones exposed to ouabain are available.

Here we report changes in cell water volume in response to Na^+ pump inhibition or to experimentally induced alterations of extracellular osmolality in cell bodies of identified *Helix aspersa* neurones. Cell water volume changes and membrane potential were measured with electrophysiological techniques (Reuss, 1985). We found that upon pump inhibition most neurones shrink. Only in a few cases is the shrinkage preceded by a short-lived swelling phase. There were few cells in which ouabain did not elicit measurable volume changes. The results suggest that while the sodium pump may be necessary for long-term cell volume maintenance, neurones are

endowed with ouabain-insensitive mechanisms which prevent them from swelling and lysis after Na^+ pump inhibition. Brief accounts of some of these findings have been published (Alvarez-Leefmans, Gamiño & Reuss, 1990; Gamiño, Alvarez-Leefmans & Reuss, 1992).

METHODS

General

Most of the experiments were done with neurones 1F, 2F, 76F and 78F (Kerkut, Lambert, Gayton, Loker & Walker, 1975) of the right parietal suboesophageal ganglion of *Helix aspersa*. Experiments were carried out at room temperature (20–25 °C). Animals were kept in a terrarium at room temperature and fed lettuce twice a week. The ganglia were removed and pinned to the bottom of a Sylgard-lined Petri dish filled with 5 ml snail Ringer solution. The thick external layer of connective tissue surrounding the ganglia was removed with fine forceps and microscissors under a dissecting microscope. Then the capsule enveloping the ganglia was softened by treating the preparation with snail Ringer solution containing 1 mg ml^{-1} protease Type XIV (Sigma, St Louis, MO, USA) for 15 min at room temperature. After the enzyme treatment the capsule was carefully removed and the ganglia were transferred with a pipette to the experimental chamber, which also had a Sylgard-lined bottom to which the ganglia were pinned down. The ganglia were positioned in the centre of the chamber, separated from fluid inlet and outlet by two vertical parallel partitions. This allowed the perfusion fluid to be directed fairly uniformly towards the ganglia, as evidenced with dye tests. The chamber, which had a volume of 0·5 ml, was continuously perfused with bathing solutions at a flow rate of 5 ml min^{-1}. The preparation was transilluminated by means of a dry dark-field condenser (Leitz, D 0·80–0·95, Wetzlar) and viewed through a dissecting microscope (Wild M8, Heerbrugg), as described previously (Alvarez-Leefmans, Gamiño & Rink, 1984).

Solutions

The standard snail Ringer solution contained (mM): NaCl, 82·5; KCl, 4; $CaCl_2$, 7; $MgCl_2$, 5; HEPES, 5; dextrose, 5 and was equilibrated with air. The pH was adjusted to 7·5 with NaOH and the osmolality was 206–211 mosmol $(kg\ water)^{-1}$. The TMA^+-loading solution was made by isosmolar replacement of NaCl with tetramethylammonium chloride (TMACl). In a few cases the TMA^+-loading solution was made by replacing NaCl with TMAOH (40 mM) and sodium gluconate (40 mM), adjusting the pH to 7·5 with D-gluconic acid. The osmolality of the loading solution was the same as that of the standard snail Ringer solution. To measure steady-state changes in cell water volume in response to anisosmotic solutions, once loaded with TMA^+, the ganglion was bathed with standard snail Ringer solution for a few minutes and then with control isosmotic solution in which 40 mM NaCl was omitted and sucrose added to match the osmolality of the standard snail Ringer solution. Anisosmotic solutions were prepared by sucrose addition or removal keeping the ionic concentrations of the solution constant and at the same value as the isosmotic control. The changes in osmolality were nominally ± 10, ± 20, ± 30 and -40%. In some experiments the hyperosmotic solution ($+20\%$) was prepared by adding sucrose to the control snail Ringer solution. Similarly, some hyposmotic (-5 to -12%) solutions were prepared by NaCl removal from the control snail Ringer solution. In these cases the control readings were taken when the preparation was bathed with standard snail Ringer solution. Osmolalities of all solutions were measured with a vapour pressure osmometer (model 5100 B: Wescor Inc., Logan, UT, USA). Ouabain (Sigma, St Louis, MO, USA) was added to the standard snail Ringer solution at a final concentration of 1 mM.

Electrodes

The bath reference electrode was a low resistance (< 1 MΩ) 3 M KCl microelectrode. Double-barrelled microelectrodes were used to measure intracellular TMA^+ activity and transmembrane potentials. To prepare them (Cotton, Weinstein & Reuss, 1989) segments of 9·5 cm length of double-barrelled laterally fused borosilicate glass with an inner filament (each barrel 1·0 mm o.d., 0·43 mm i.d.; Hilgenberg, Malsfeld, FRG) were cleaned with nitric acid. The glass was rinsed and boiled in five changes of deionized water. After drying in an oven, each fused doublet was held on a horizontal puller (PD-5, Narishige, Tokyo), heated, twisted 360 deg, allowed to cool down for

20–30 s, and then pulled. The back of one of the barrels was broken by inserting a sharp blade between both barrels. The reference barrel was partially filled with deionized water to prevent its silanization. This was done by touching the back end of the short barrel with a droplet of deionized water, which partially filled the shank by capillarity. Similarly, the shank of the other barrel was

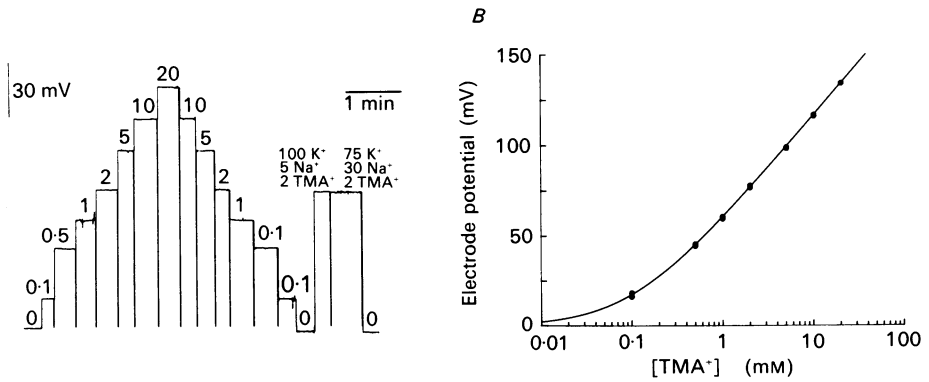

Fig. 1. Calibration and selectivity test of a double-barrelled TMA^+-sensitive microelectrode, tip external diameter approximately 1 μm. *A*, record of the differential signal $E_{TMA}-E_{ref}$ obtained in various calibration solutions. The numbers indicate the TMA^+ concentration (mM) of the different solutions, all of which contained a constant background [KCl] of 100 mM. The differential signal recorded in the TMA^+-free solution containing 100 mM KCl is denoted 0. Just after the calibration test, the selectivity of the microelectrode for K^+ and Na^+ was assessed within the levels at which the latter cations were expected to change in the cytosol during ouabain treatment. The composition of each solution for the selectivity test is indicated (in mM). *B*, calibration plot of electrode potential against [TMA^+]. The continuous line is a fit of the Nikolsky–Eisenman equation by non-linear least-squares regression: $E = E_0 + S\log([TMA^+] + k_{TMA,K}[K^+])$; where E is electrode potential; E_0, constant reference potential; S, electrode potential change for a tenfold increase in [TMA^+] in the absence of interfering ions and $k_{TMA,K}$ the selectivity coefficient. Since ionic strengths were kept within 0·1–0·12 M, concentrations rather than activities were used. The linear part of the plot had a slope of 56·4 mV/log [TMA^+].

partially filled with hexamethyldisilazane (Sigma). The microelectrodes were then placed, tip upwards, on a preheated (275 °C) aluminium plate sitting on a hot plate (Corning, 351, NY, USA) and a stream of hot air from a hair dryer was directed until the silane, and the water, evaporated completely (usually 2 h). The micropipettes were allowed to cool, then the silanized barrel of each micropipette was injected with a drop of a liquid cation exchanger (5 mg potassium tetrakis *p*-chlorophenylborate in 0·1 ml 3-nitro-*O*-xylene) and backfilled with 0·1 M KCl. This barrel was used to record intracellular TMA^+ activity. The reference barrel was filled with a 1 M sodium formate–0·01 M KCl solution, and in a few cases with 3 M KCl. This barrel was used to record the membrane potential. When necessary, bubbles were removed by gentle local heating with a microforge. Chloridized silver wires were wax sealed in each barrel for electrical connections. The microelectrodes responded to TMA^+ only after their tips were broken back to about 1–1·5 μm. Broken-tip microelectrode resistances measured in 0·1 M KCl solution were 80–150 MΩ for the reference barrel (filled with the sodium formate solution) and 7–10 GΩ for the TMA^+-sensitive barrel. Neglecting the capacitative transients the DC coupling between barrels, assessed by passing a 1 nA square pulse through the reference barrel was less than 1%.

Calibration and recording procedures

The potential from the ion-sensitive barrel of the double-barrelled microelectrode (E_{TMA}) was monitored with a WPI FD-223 electrometer (New Haven, CT, USA). The potential of the reference barrel (E_{ref}) was recorded using a WPI M-707 electrometer (New Haven, CT) and subtracted

electronically from E_{TMA} to give the differential signal ($E_{TMA}-E_{ref}$), which is proportional to [TMA^+]. The outputs of the electrometers were low-pass filtered (0–5·3 Hz), displayed on a chart recorder, digitized with an analog–digital converter, displayed and stored on a Zenith 158 PC for subsequent analysis. Each channel was sampled at 10 Hz. Data acquisition was carried out with a commercial system (Asyst, Macmillan Software Co., New York, NY, USA). Recorded signals were analysed with customized programs to calculate changes in cell water volume and other parameters.

The TMA^+-sensitive microelectrodes were calibrated before and after each impalement with solutions containing 100 mM KCl plus 0·1–20 mM TMACl. Slopes ranged between 53 and 65 mV/log [TMA^+], for [TMA^+] between 0·5 and 20 mM. Between 0·1 and 0·5 mM [TMA^+], slopes ranged from 25 to 35 mV/log [TMA^+]. The mean slope for the precalibration curves in the relevant range of intracellular TMA^+ concentrations (0·5–20 mM) was $59·6 \pm 0·6$ mV/log [TMA^+] ($n = 21$) and that for the postcalibrations was $69·4 \pm 1·8$ mV/log [TMA^+] ($n = 19$). For the measurements reported here we used only the precalibration curves. Figure 1 shows the calibration of one such electrode. The selectivity coefficients for TMA^+/K^+ were 10^2–10^3 and for TMA^+/Na^+, 10^3–10^4, respectively. In identified *Helix* neurones, basal $[Na^+]_i$ measured with neutral carrier-based microelectrodes (ETH-227, Fluka, Buchs, Switzerland) was $6·2 \pm 0·9$ mM ($n = 14$) for cells 1F and 2F, and $5·8 \pm 0·7$ mM ($n = 13$) for cells 76F and 77F (F. J. Alvarez-Leefmans, S. Márquez & A. Nani, unpublished observations) and $[K^+]_i = 91 \pm 2·2$ mM (Alvarez-Leefmans, Gamiño & Rink, 1984). Hence for $[TMA^+]_i \geqslant 0·5$ mM, the ion-selective barrel virtually responds only to TMA^+. During ouabain treatment cells were expected to gain Na^+ and Ca^{2+} and lose K^+ (cf. Deitmer & Schlue, 1983; Schlue, 1991). As expected from selectivity coefficient measurements, reducing [K^+] from 100 to 75 mM and increasing Na^+ from 5 to 30 mM against a background [TMA^+] of 2 mM gave no measurable response of the ion-sensitive microelectrode (Fig. 1*A*). We also tested for possible interference from Ca^{2+} on TMA^+- sensitive microelectrode response. Varying free [Ca^{2+}] between 10^{-7} and 10^{-4} M in EGTA-buffered solutions having a background of 100 mM KCl plus 2 mM TMACl gave no measurable response of the TMA^+-sensitive microelectrode. The Nikolsky–Eisenman equation was fitted to the calibration data points for each electrode by non-linear least-squares regression using the program ELCAL (Barolet, Andrews & Morris, 1989). Figure 1*B* shows the calibration curve so obtained built with the raw data points illustrated in Fig. 1*A*.

TMA^+ loading and recording procedure. Once calibrated, the double-barrelled ion-sensitive microelectrode was positioned in the control bathing solution and the potentials with respect to the bath reference electrode were taken as zero. Then the cell was impaled. The transmembrane potential difference (E_m), measured with the reference barrel, was subtracted from the voltage recorded with the TMA^+-sensitive barrel (E_{TMA}) giving the differential signal ($E_{TMA}-E_m$) which indicates the $[TMA^+]_i$. Once the records were stable, cells were loaded with TMA^+ simply by exposing the ganglion to the TMA^+-loading solution (see above). Neurones were loaded with TMA^+ to a final concentration of $3·5 \pm 0·6$ mM (range 0·6–8·9 mM, $n = 20$). As required for a good intracellular water marker, TMA^+ was virtually trapped inside the neurones. Once the cells were loaded to the desired $[TMA^+]_i$ the bathing fluid was changed to a control solution to obtain readings of stable baseline. TMA^+ leaked out from the cells at a rate that resulted in a concentration change well below 0·5 mM h^{-1}. This gave a baseline on which volume changes, assessed from changes in $[TMA^+]_i$ could be continuously measured over periods of several minutes. The noise levels of the reference and the ion-sensitive barrels allowed for measurements with a sensitivity of 1 mV or less in the differential voltage trace, even in spiking neurones. In the latter case measurements were made within the interspike intervals. Cell volume changes of 5% or less could be detected as illustrated in Fig. 3*A*.

An example of the TMA^+ loading of a neurone exposed to a TMA-gluconate solution is shown in Fig. 2. Note that upon exposure to TMA^+ the cell depolarized and fired action potentials, which in the case illustrated appear truncated due to low-pass filtering. The mechanism by which this depolarization is produced is not clear. However, since these and other *Helix* neurones are depolarized by quaternary ammonium compounds, including nicotinic agonists, it is likely that the depolarization is due to activation of acetylcholine receptors (cf. Kazachenko, 1990).

One of the assumptions underlying the present technique to measure cell water volume is that TMA^+ is uniformly distributed in the cytoplasm, and not taken up or released by organelles. This implies that TMA^+ should not be compartmentalized within the cell. To test the latter hypothesis neurones were permeabilized by exposing them to the cation channel-forming ionophore nystatin

(100 μg ml^{-1}), and then loaded with TMA^+. The kinetics of TMA^+ exit from the cell in the continued presence of nystatin was studied. If the intracellular water volume in which TMA^+ is diluted behaves as a single compartment we would expect that the efflux of TMA^+ should follow an exponential time course with a single rate constant. We found that this is the case. The efflux of TMA^+, in the range from 10 mM down to 0·1 mM, followed a single-exponential time course with a mean rate constant of $1{\cdot}11 \pm 0{\cdot}01$ min^{-1} ($n = 3$ cells).

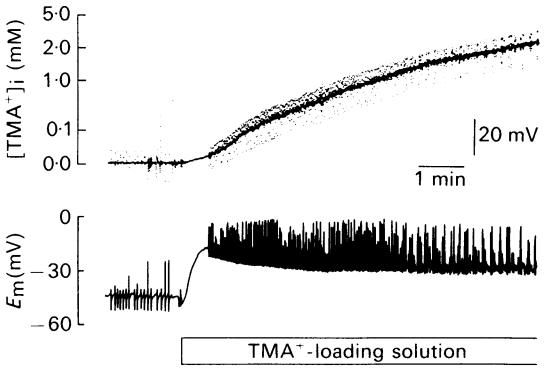

Fig. 2. TMA^+-loading procedure. The cell was impaled with a double-barrelled microelectrode. The transmembrane potential (E_m) recorded through the reference barrel (lower trace) was subtracted from the potential recorded through the TMA^+-sensitive barrel (E_{TMA}) giving the differential signal ($E_{TMA} - E_{ref}$). When inside the cell $E_{ref} = E_m$ and the differential signal denotes $[TMA^+]_i$, the intracellular concentration of TMA^+ (upper trace). The $[TMA^+]_i$ scale was obtained from the differential voltage ($E_{TMA} - E_{ref}$) recorded by the electrode in the calibration solutions containing known TMA^+ concentrations as explained in the Methods. On the $[TMA^+]_i$ scale 0·0 indicates the differential voltage for a TMA^+-free calibration solution containing 100 mM K^+, which in this cell corresponded to $[K^+]_i$. The cell (1F) was exposed to the loading solution as indicated by the box. In the example shown, initial, net TMA^+ influx was estimated to be about 27×10^{-12} mol cm^{-2} s^{-1}, assuming constant cell volume. The near spherical cell body of this neurone had a radius of 75 μm.

Another assumption of the technique is that TMA^+ diffuses to equilibrium very rapidly within the cell. For a spherical cell having a radius of 75 μm and considering that the free diffusion coefficient of TMACl is $1{\cdot}39 \times 10^{-5}$ cm^2 s^{-1} it would take 2·5 s for TMA^+ to diffuse to equilibrium (99·5% of final concentration) from the periphery to the centre of the cell and 5 s if its diffusion coefficient in the cytosol is assumed to be half of that in free solution (see Crank, 1975, p. 91, equation 6.19).

Calculation of cell water volume

The TMA technique permits measurement of changes in the fraction of the total cell volume corresponding to solvent or osmotically active water. We refer to this fraction as the cell water volume, which of course does not include the so-called non-solvent or structured water (Dick, 1979). For practical purposes the latter is considered to be part of the non-aqueous volume of the cell. In other words, the measured parameter in the present work is the difference between total cell volume and non-aqueous volume, the latter being the part of the cellular volume which does not participate in the osmotic swelling or shrinkage.

Normalized cell water volume changes (V_t/V_0) were computed from changes in $[TMA^+]_i$ elicited by exposure to anisosmotic solutions or upon Na^+ pump inhibition with ouabain, according to the equation

$$V_t/V_0 = [TMA^+]_0/[TMA^+]_t, \quad (1)$$

where V_t is the cell water volume at time t, V_0 is the initial cell water volume, $[TMA^+]_0$ is the intracellular $[TMA^+]$ at time = 0, and $[TMA^+]_t$ is the intracellular $[TMA^+]$ at time t. Unless otherwise noted, experimental values are expressed as means ± standard error of the mean (S.E.M.).

RESULTS

The relation between neuronal cell water volume and external osmotic pressure

The osmotic behaviour of *Helix* neurones was studied by exposure to anisosmotic solutions having osmolalities which ranged between 60 and 124% of that of the control solution (i.e. the changes in osmolalities ranged between −40 and +24% relative to that of the control solution). Apparent steady-state changes in cell water volume in response to anisosmotic challenges were measured in twenty-one cells. Figure 3*A–C* shows typical records in three of these identified neurones during exposure to either hyposmotic or hyperosmotic solutions. In the cases illustrated the cells swelled by 5% in the 5% hyposmotic solution (Fig. 3*A*) and by 8% in the 12% hyposmotic solution (Fig. 3*B*). For a solution 20% hyperosmotic (Fig. 3*C*) the cell shrank by 16% of its initial volume. Upon returning to the isosmotic solution, the cells recovered their initial volume. The relatively slow rates of change in cell water volume upon exposure to anisosmotic solutions can be explained by (1) the time it took for complete exchange of fluid in the chamber (approximately 18 s); (2) the unavoidable presence of unstirred fluid layers; and (3) an unknown water permeability of the cell membrane. Inasmuch as the time courses of the changes in osmolality at the cell surface were not measured, a quantitative analysis of the apparent rates of change in cell water volume is not possible.

The apparent steady-state changes in water volume measured in the twenty-one cells exposed to anisosmotic solutions are illustrated in Fig. 4. The data points corresponding to hyperosmotic solutions and to small degrees of hyposmolality fall close to the line describing ideal osmometric behaviour. This theoretical line was calculated from the following equation:

$$V_t/V_0 = \pi_0/\pi, \tag{2}$$

where V_0 is the volume of the cell in an isotonic Ringer solution or in a solution isosmotic with the Ringer solution, having an osmotic pressure π_0 and V_t is the corresponding volume of the cell in equilibrium with a solution of osmotic pressure, π. The theoretical line is the plot of the relative cell volume (V_t/V_0) as a function of the reciprocal of the relative osmotic pressure, i.e. π_0/π. This relationship is valid if (1) there is no net loss or gain of intracellular solute during the interval of exposure to the test anisosmotic solutions and (2) the activity coefficient of intracellular TMA^+ does not change significantly during the experiment. When the osmolality was reduced by 30% or more, the maximal change in cell water volume was less than the theoretical expectation. This indicates that at least one of the above assumptions does not hold under these conditions.

One possibility is that the dilution of intracellular electrolytes during osmotic swelling reduces the ionic strength so that the intracellular [TMA^+] is overestimated because of a change in activity coefficient. However, decreasing [KCl] from 100 to 60 mM at 6 mM [TMA^+] changed the TMA^+ electrode signal by 1·5 mV, which yields an error of 0·3 mM in the estimate of [TMA^+]. This explains about one-third of the deviation depicted in Fig. 4 for the data with $\pi_0/\pi > 1{\cdot}5$. In addition, the apparent steady-state volume changes could include a volume-regulatory response. These two factors may explain the apparently lower than osmometric response of the cells when

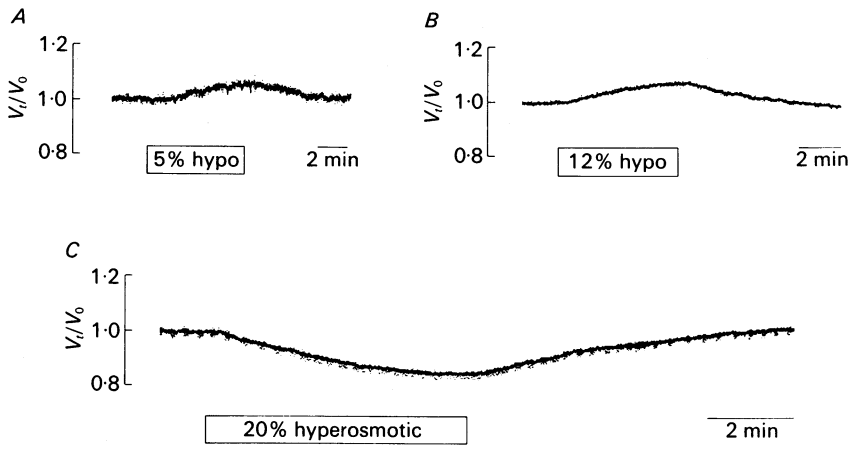

Fig. 3. Changes in cell water volume measured in three neurones exposed to anisosmotic solutions of various osmolalities as indicated in each box. *A*, neurone 76F; *B*, neurone 76F; and *C*, neurone 1F. In all cases the normalized cell water volume (V_t/V_0) was computed from the changes in $[TMA^+]_i$ according to eqn (1).

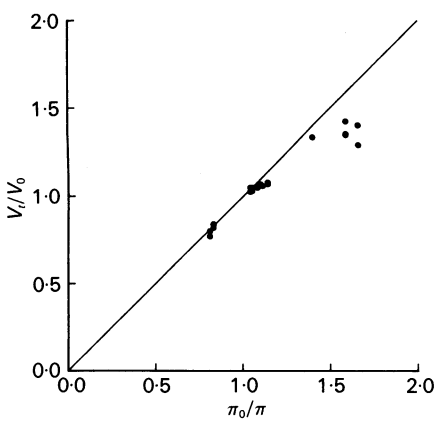

Fig. 4. The relationship between the apparent steady-state relative cell water volume (V_t/V_0) and the reciprocal of the relative osmotic pressure of the medium (π_0/π). The neurones were assumed to be at osmotic equilibrium prior to exposure to each anisosmotic solution ($n = 21$ cells in 20 animals). The line denotes the predicted behaviour of a perfect osmometer according to eqn (2).

exposed to hyposmotic solutions whose osmolality was reduced more than 30% relative to that of the control solution. Hence, we tested whether osmotically swollen *Helix* neurones down-regulate their volume in the sustained presence of hyposmotic medium.

Five neurones were initially superfused with an isosmotic solution containing 40 mM NaCl and appropriate amounts of sucrose to achieve an osmolality equal to that of snail Ringer solution. Then the isosmotic solution was replaced with a solution about 40% (38·1 ± 0·7%, range 37–39·8%, $n = 5$) hyposmotic, made by

sucrose removal keeping the ionic strength constant. The cells swelled to a maximum of $36{\cdot}5 \pm 2{\cdot}3\%$ over their initial water volume, at an initial rate of $4{\cdot}7 \pm 0{\cdot}7\%$ min^{-1}, reaching their maximal swelling in $14{\cdot}2 \pm 1{\cdot}2$ min. Then the cells started to down-regulate their volume at an initial rate of $-0{\cdot}8 \pm 0{\cdot}2\%$ min^{-1}. When cell water volume had recovered to $19{\cdot}7 \pm 3{\cdot}2\%$ above control, the hyposmotic solution was replaced with isosmotic solution and the cells further decreased their water volume at an initial rate of $-5{\cdot}2 \pm 0{\cdot}5\%$ min^{-1}. A transient shrinkage peaking at $15 \pm 4\%$ below control was observed.

The regulatory volume decrease was observed only when cells were exposed to solutions about 40% hyposmotic. Solutions ranging between 10 and 28% hyposmotic were ineffective in producing regulatory volume decrease. Similar volume regulatory responses have been described for vertebrate neurones (cf. Falke & Misler, 1989) as well as for other cell types (Hoffmann & Simonsen, 1989). An illustrative experiment done in a neurone 78F is shown in Fig. 5.

Effects of Na^+ pump inhibition on neuronal cell volume and membrane voltage in isosmotic medium

To elucidate the role played by the Na^+–K^+ pump in the maintenance of cell volume we measured the cell water volume changes elicited in twenty neurones by exposure to standard Ringer solution containing ouabain at a concentration (1 mM) expected to inhibit completely the electrogenic Na^+ pump (Thomas, 1969). The cells were initially superfused with standard snail Ringer solution and once stable traces were obtained ($E_m = -53{\cdot}4 \pm 1{\cdot}8$ mV), the bathing solution was changed to one containing the cardiac glycoside. All the cells ($n = 20$) were depolarized by ouabain as expected for inhibition of an electrogenic Na^+ pump (Thomas, 1972). The initial rate of depolarization was $21{\cdot}8 \pm 6{\cdot}7$ mV min^{-1}. Some cells ($n = 3$) depolarized in a continuous fashion during exposure to ouabain and repolarized upon readmission of the control Ringer solution. In the other cells ($n = 17$) the ouabain-induced depolarization peaked at $14{\cdot}8 \pm 2{\cdot}6$ mV above the resting potential, with a mean time to peak of $4{\cdot}6 \pm 0{\cdot}6$ min. In the latter group of cells the depolarization was followed by repolarization and even hyperpolarization. In a few cases the cells depolarized to a plateau. Three major types of cell water volume response were observed upon sustained exposure to ouabain: (1) shrinkage, (2) shrinkage preceded by one or more short-lived swelling phases, and (3) no measurable volume changes. None of the responses appeared to be correlated with any specific cell type. The first type of response was observed in nine out of the twenty cells. These neurones shrank to $6{\cdot}1 \pm 0{\cdot}6\%$ (range 3–9%) below their initial water volume at an initial rate of $-2{\cdot}7 \pm 1\%$ min^{-1}. Shrinkage began 114 ± 24 s after the onset of the ouabain-induced depolarization and peaked at 386 ± 60 s. One of these experiments is illustrated in Fig. 6*B*. It is important to emphasize that in these cells the shrinkage was not preceded by detectable swelling (Fig. 6*B*). This response was unexpected on the basis of the simplest predictions of the pump–leak hypothesis. The lack of ouabain-induced swelling in these cells was not due to inadequate electrode response since exposure of the same cells to nominally 5% hyposmotic solutions prior to ouabain exposure did produce the expected osmotic swelling which was reversible upon readmission of standard Ringer solution (Fig. 6*A*).

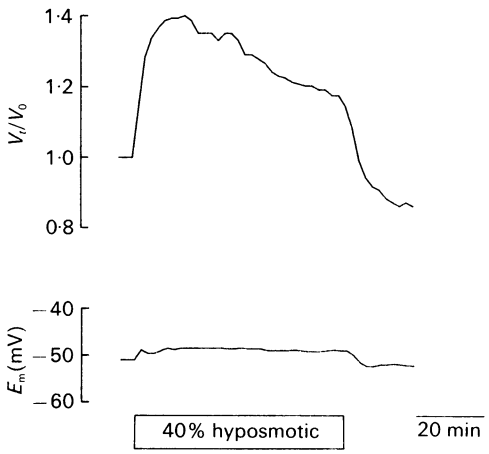

Fig. 5. Effect of a long-lasting exposure to a 40% hyposmotic solution on cell water volume (neurone 78F). Upper trace: normalized cell water volume (V_t/V_0). Lower trace: transmembrane potential (E_m). Data plotted by sampling at a rate of one point every 2 min. The neurone was initially superfused with a solution isosmotic to snail Ringer solution containing 45 mM Na^+, 40 mM Cl^- and *ca.* 65 mM sucrose (osmolality = 209 mosmol kg^{-1}), the other solutes being the same as those contained in the snail Ringer solution, pH = 7·5. During the time indicated by the box, the isosmotic solution was replaced by a solution 40% hyposmotic (osmolality = 126 mosmol kg^{-1}), by sucrose removal keeping the ionic strength constant. The cell swelled by 40% of its initial water volume, at an initial rate of about 7·5% min^{-1}, reaching maximum volume at 13 min; thereafter, regulatory volume decrease was observed at an initial rate of −0·6% min^{-1}. When cell water volume had recovered to 18% above control, the hyposmotic solution was replaced with isosmotic solution and the cell further decreased its water volume at an initial rate of −4·6% min^{-1}, shrinking to 14·4% below control. Thereafter, the impalement was lost. Similar experiments in other cells revealed that shrinkage was transient.

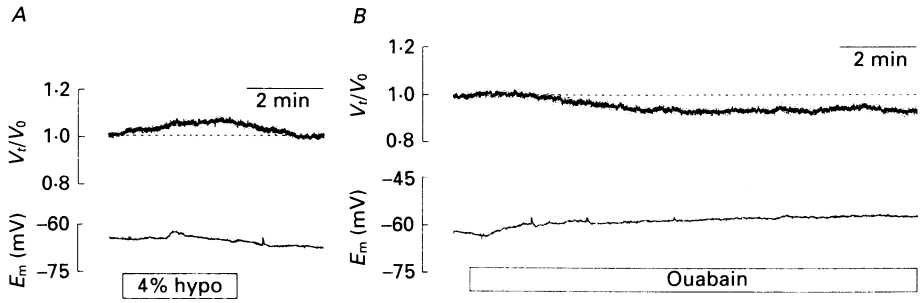

Fig. 6. Shrinkage of a nerve cell upon exposure to 1 mM ouabain. In this and subsequent figures, on each panel, the upper trace corresponds to the normalized cell water volume (V_t/V_0), the lower trace to the transmembrane potential (E_m), and the box indicates the length of exposure to 1 mM ouabain or to anisosmotic solutions. *A*, exposure of the neurone (cell 77F) to a hyposmotic solution to test that the electrode was able to respond to increases in cell volume as little as 4%. *B*, ouabain-induced decrease in cell water volume. Shrinkage ensued with a latency of 80 s, at an initial rate of −1·7% min^{-1}. The latency was measured from the onset of the ouabain-induced membrane depolarization to the onset of the cell volume changes. The cell shrank to 7% below its initial water volume.

The second major type of response observed upon the sustained presence of ouabain consisted of one or more short-lived swelling phases followed by recovery of cell volume and, in most cases, eventual shrinkage. This type of response was found in seven out of the twenty cells. Two examples are shown in Fig. 7. The swelling

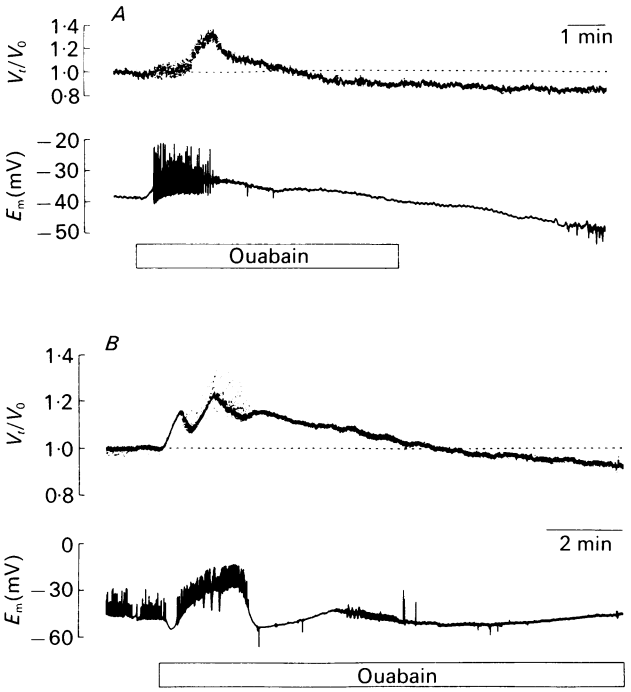

Fig. 7. Ouabain-induced swelling followed by shrinkage in two 1F neurones. *A*, a slow and a rapid swelling phase preceded cell shrinkage. *B*, multiple swelling phases preceded cell shrinkage. Symbols as in Fig. 6.

response was quite variable, sometimes monophasic (Fig. 7*A*), but in most cases was composed of two or more short-lived swelling phases (Fig. 7*B*). Sometimes ($n = 3$) the first swelling phase presented two rates of rise in cell volume, one slow ($7{\cdot}5 \pm 2{\cdot}1$ % min^{-1}) followed by a faster one (see below). In other cells ($n = 4$) the first swelling phase ensued with a single fast rate of rise. The fast initial rate of rise in cell volume for the whole population ($n = 7$), whether preceded or not by the slow rate, was 50 ± 17 % min^{-1}. The cells swelled by $18{\cdot}7 \pm 5{\cdot}1$ % above their initial volume and down-regulated their volume very rapidly, at a rate of $-24{\cdot}4 \pm 8{\cdot}1$ % min^{-1}. The second swelling phase, when present ($n = 4$), had an initial rate of $19{\cdot}8 \pm 7{\cdot}9$ % min^{-1}, the mean swelling was $20{\cdot}1 \pm 9{\cdot}6$ % above the control volume, and cell volume decrease occurred at an initial rate of $-12{\cdot}3 \pm 4{\cdot}4$ % min^{-1}. The third swelling phase, ($n = 3$) occurred with a mean initial rate of $10{\cdot}9 \pm 2{\cdot}8$ % min^{-1}, a mean increase in cell volume of $19{\cdot}7 \pm 8{\cdot}6$ % and a rapid down-regulatory phase at a rate of $-7{\cdot}2 \pm 1{\cdot}1$ % min^{-1}. Thereafter, cell volume recovered relatively slowly at an initial

rate of $-4{\cdot}8 \pm 1{\cdot}6\%$ min^{-1}. Most of these ouabain-treated cells not only fully recovered their original volume in the presence of the glycoside but shrank by up to 13% of their initial volume (Δ volume $= -7{\cdot}1 \pm 1{\cdot}7\%$, range -3 to $-13{\cdot}3\%$, $n = 5$) and remained shrunken for at least 10 min.

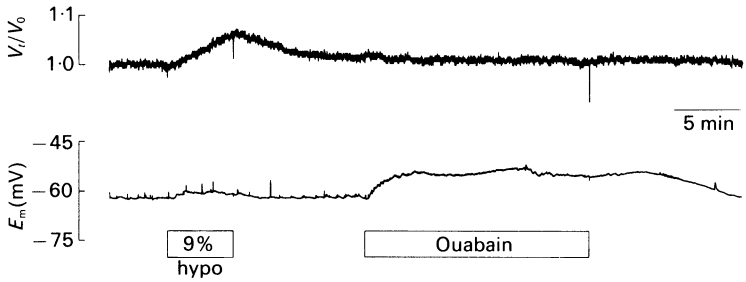

Fig. 8. Lack of ouabain-induced changes in cell water volume in neurone 76F. To test the adequacy of the response of the intracellular TMA^+-sensitive double-barrelled microelectrode, the cell was exposed to a 9% hyposmotic solution prior to exposure to ouabain. Symbols as in Fig. 6.

Finally, in four out of the twenty cells there were no measurable volume changes during 10–15 min exposure to ouabain. This observation was validated by assessment, in the same cells, of the changes in cell volume elicited by osmotic challenges. It was found that prior to ouabain application the cells exhibited the predicted osmotic response to 4–12% hyposmotic solutions. One of these experiments is shown in Fig. 8.

DISCUSSION

The role of the Na^+–K^+ pump in cell volume maintenance in the animal kingdom has reached the status of textbook knowledge (e.g. Alberts, Bray, Lewis, Raff, Roberts & Watson, 1989; Rowland, Fink & Rubin, 1991). The tenet is that cells contain impermeant anionic macromolecules which create an inwardly directed colloid osmotic pressure gradient resulting in net entry of permeable solutes and water. This influx is balanced by extrusion of solutes and water at a rate equal to that of the dissipative leak entry. According to this view, known as the pump–leak hypothesis (Tosteson & Hoffman, 1960; Tosteson, 1964), the Na^+–K^+ pump maintains ionic gradients and osmotic balance across the plasma membrane thereby preventing colloid osmotic cell swelling. A simple prediction of this hypothesis is that inhibition of the Na^+–K^+ pump should result in cell swelling and eventual lysis. The present results show that upon Na^+–K^+ pump inhibition nerve cells respond in the short term (15–20 min) in a manner which deviates from the behaviour expected from the simplest predictions of the pump–leak hypothesis. The two major findings of the present study are that (1) most cells shrink upon sodium pump inhibition, albeit sometimes the shrinkage is preceded by one or more short-lived swelling phases; (2) some cells do not change their water volume after blocking the sodium pump. These findings suggest that the predictions and assumptions of the pump–leak

hypothesis as originally formulated and as commonly taken for granted are neither complete nor universal. Inadequate time resolution and sensitivity of the existing techniques for measuring cell volume has made it difficult to validate and demonstrate experimentally these assumptions and predictions. Indeed, the fast cell volume changes following sodium pump inhibition described in this paper would have been difficult to demonstrate with commonly used techniques for measuring cell volume changes. Our results suggest that cell volume is maintained under isosmotic conditions not only by adjustment of pump rates balancing inward Na^+ leaks but also by changes in other membrane transport pathways activated as a consequence of sodium pump inhibition. Based on our results and those of others (see below), we hypothesize that *Helix* neurones have a relatively low passive Na^+ permeability, i.e. the inward leak is relatively small. When the pump is inhibited the inward leak is insufficient to cause measurable cell swelling. However, the rise in intracellular Na^+ might promote an increase in $[Ca^{2+}]_i$ which activates a K^+ conductance, causing membrane hyperpolarization and K^+ efflux. The end result could be that upon pump inhibition net solute (i.e. K^+ and an accompanying anion) and water efflux outweigh net solute and water influx resulting in cell shrinkage.

Clearly the validity of our conclusions rests upon the reliability of the technique we have used. For the present study we adapted to neurones a technique for measuring changes in cell water volume using TMA^+ as an intracellular volume marker and measuring its activity with an ion-sensitive microelectrode (Reuss, 1985). Besides adequate sensitivity and time resolution, the technique allowed simultaneous recording of membrane potentials. The responses of the ion-sensitive microelectrodes *in vitro* indicate that they can measure intracellular free TMA^+ concentrations down to 0·1 mM in the presence of the expected intracellular levels of native cations. We have experimental evidence suggesting that TMA^+ distributes intracellularly in a single compartment. We estimated that TMA^+ diffuses intracellularly to 99·5% equilibrium in less than 3 s. The technique was further validated by determining the apparent steady-state and time-dependent changes in cell water volume measured by exposing the cells to solutions of various osmolalities. We found that exposure of the neurones to anisosmotic solutions, having osmolalities in the range between -12 and $+24$% of that of the control solution, resulted in apparent steady-state changes in cell water volume which conform to a near-ideal osmometric behaviour. We were able to show that with this technique it is possible to detect changes in cell water volume of less than 5%. In addition it was found that upon prolonged exposure to 40% hyposmotic solutions the cells showed regulatory volume decrease following a time course similar to that described for other cells (Hoffmann & Simonsen, 1989), including neurones (Falke & Misler, 1989).

Most neurones shrank upon inhibition of the sodium pump

Unexpectedly, most cells (14 out of 20) responded to Na^+ pump inhibition by shrinking. Only in a few cases (5 out of the 14 cells) was the shrinkage preceded by one or more transient swelling phases. This indicates that the mechanisms underlying the shrinkage are not necessarily triggered by preceding increases in cell volume which could in turn activate transport mechanisms by membrane stretch, changes in cell shape or dilution of an intracellular molecule.

The onset of the first swelling phase occurred concomitantly with the onset of the membrane depolarization. Measurements with intracellular Na^+-sensitive microelectrodes (Cruzblanca, Nani, Altamirano, Márquez, Merediz & Alvarez-Leefmans, 1992) revealed that $[Na^+]_i$ increases monotonically, at a rate of less than 1 mM min^{-1} (cf. Thomas, 1972). Assuming that this reflects net salt uptake, the initial rate of change of intracellular osmolality would be about 2 mosmol (kg water)$^{-1}$ min^{-1}. Assuming that intracellular and extracellular osmolalities are equal, i.e. about 200 mosmol (kg water)$^{-1}$, cell swelling would occur at a maximum possible rate of 1% min^{-1}. However, in our experiments, cell volume increased at rates ranging between 7·5 and 50% min^{-1} for some of the swelling phases reported here. This would require inward Na^+ leaks larger than the ones expected from the recorded rates of change in intracellular $[Na^+]$. This clearly shows that Na^+ entry through leak pathways alone cannot explain the initial swelling phase and some other unknown factors are involved, e.g. Ca^{2+}-triggered release of some intracellular organic solute (Chamberlin & Strange, 1989). Further studies on the changes in $[Na^+]_i$ and other intracellular osmotically active particles will be necessary to elucidate the mechanisms by which ouabain induced the swelling phases. We can rule out the possibility that recorded swelling phases resulted from artifactual movement of the microelectrode since anisosmotic challenges up to 40% hyposmotic, which are expected to produce considerable cell expansion, never reproduced the transient swelling phases elicited by ouabain. In addition, we have unpublished evidence showing that ouabain induces oscillatory increases in $[Ca^{2+}]_i$ which could in turn produce oscillations in Ca^{2+}-activated K^+ efflux which could underlie the multiphasic short-lived swellings.

The ouabain-induced short-lived swelling phases were followed by a decrease in cell water volume proceeding at a rate of about -5% min^{-1} and leading to a mean final cell shrinkage of about 7% below initial water volume. This regulatory phase started within 2–6 min of the onset of the effect of ouabain and was concomitant with membrane hyperpolarization which is due to Ca^{2+}-activated K^+ efflux (Cruzblanca *et al.* 1992). We hypothesize that the ouabain-induced cell shrinkage may be due to opening of Ca^{2+}-activated K^+ channels. Recent studies in leech neurones support this contention of activation of K^+ conductance upon exposure to ouabain (Schlue, 1991). The loss of solute (K^+ and an accompanying anion) and osmotically committed water outweighs the solute and water entry through Na^+ leak pathways or the increase in putative internal organic solutes released from internal sources, leading to cell shrinkage. The same explanation would hold for the cases in which inhibition of the Na^+–K^+ pump resulted in cell shrinkage which was not preceded by measurable swelling. In these cases shrinkage ensued within 2–4 min of ouabain perfusion. The predicted ouabain-induced increase in intracellular $[Ca^{2+}]$ has been observed in invertebrate neurones (Deitmer & Schlue, 1983; Schlue, 1991) and other excitable cells (Török, 1989). This rise in $[Ca^{2+}]_i$ has been attributed to reversal of Na^+–Ca^{2+} exchange and calcium-induced calcium release from internal stores (reviewed by Török, 1989). In neurones, Ca^{2+} entry can also occur through stretch-activated channels (Morris, 1990).

Some neurones showed no volume changes upon sodium pump inhibition

About 20% of the neurones studied showed no measurable water volume changes within 10–15 min of exposure to ouabain. It was demonstrated that the absence of measurable volume changes was not due to inadequate electrode response. This observation suggests that under certain circumstances, upon Na^+ pump inhibition, net solute and water fluxes could remain balanced in such a way that cell volume is kept constant. It cannot be argued that the cells were insensitive to ouabain since all of them depolarized upon exposure to the glycoside. A similar lack of ouabain-induced cell volume response has been reported for astrocytes and it has been attributed to limited membrane permeability to Na^+ and Cl^- (Kimelberg & Ransom, 1986). In addition, a closely related observation has been reported for proximal tubule cells of rabbit kidney when external osmolality is gradually changed at a relative slow rate (1·5 mosmol kg^{-1} min^{-1}). The latter phenomenon has been named isovolumetric regulation (Lohr & Grantham, 1986).

Implications for cell survival at the onset of ischaemia and pathophysiology of cytotoxic neuronal oedema

It is generally accepted that Na^+ pump inhibition secondary to ATP depletion underlies nerve cell swelling (cytotoxic oedema), resulting from ischaemia or trauma (Klatzo, 1985; Rosenberg, 1990). Although in the experiments described in this paper Na^+ pump inhibition was achieved by pharmacological means and was not a consequence of ATP depletion, the present results may be relevant for understanding the cell volume changes expected to occur in hypoxia of neural tissues. Our results suggest that contrary to what is commonly expected and accepted, at least within 10–15 min after complete inhibition of the Na^+ pump, the dominant response of nerve cells may be shrinkage rather than swelling. This shrinkage probably results from activation of net solute and water efflux, and prevents cell lysis resulting from Na^+ pump inhibition. Assuming that cells really swell during hypoxia, the explanation underlying the increase in cell volume probably does not involve inhibition of the Na^+ pump but other metabolic factors such as an increase in organic osmotically active solutes (Chamberlin & Strange, 1989). On the other hand, it has recently been shown that during hypoxia neurones become depleted of K^+ through activation of ATP-sensitive K^+ channels and Ca^{2+}-activated K^+ channels (Leblond & Krnjević, 1989; Jiang & Haddad, 1991).

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