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Cell volume control in *Paramecium*: factors that activate the control mechanisms

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Summary

A fresh water protozoan *Paramecium multimicronucleatum* adapted to a given solution was found to swell until the osmotic pressure difference between the cytosol and the solution balanced the cytosolic pressure. The cytosolic pressure was generated as the cell swelled osmotically. When either one or both of these pressures was somehow modified, cell volume would change until a new balance between these pressures was established. A hypothetical osmolyte transport mechanism(s) was presumably activated when the cytosolic pressure exceeded the threshold value of ~1.5 × 10^5 Pa as the cell swelled after its subjection to a decreased osmolarity. The cytosolic osmolarity thereby decreased and the volume of the swollen cell resumed its initial value. This corresponds to regulatory volume decrease (RVD). By contrast, another hypothetical osmolyte transport mechanism(s) was activated when the cell shrank after its subjection to an increased osmolarity. The cytosolic osmolarity thereby increased and volume of the shrunken cell resumed its initial value. This corresponds to regulatory volume increase (RVI). The osmolyte transport mechanism responsible for RVD might be activated again when the external osmolarity decreases further, and the cytosolic osmolarity thereby decreases to the next lower level. Similarly, another osmolyte transport mechanism responsible for RVI might be activated again when the external osmolarity increases further, and the cytosolic osmolarity thereby increases to the next higher level. Stepwise changes in the cytosolic osmolarity caused by a gradual change in the adaptation osmolarity found in *P. multimicronucleatum* is attributable to these osmolyte transport mechanisms. An abrupt change in the amount of fluid discharged from the contractile vacuole seen immediately after changing the external osmolarity reduces an abrupt change in cell volume and thereby protects the cell from the disruption of the plasma membrane by excessive stretch or dehydration during shrinkage.

Key words: cell volume control, osmoreception, osmoregulation, RVI, RVD, cytosolic pressure, osmotic pressure, bulk modulus, contractile vacuole, *Paramecium.*

Introduction

We previously found that the cytosol of the fresh water ciliate *Paramecium multimicronucleatum*, when adapted to a given solution, always ended up hypertonic to the adaptation solution and that the cytosolic osmolarity changed in a stepwise fashion as the adaptation osmolarity changed (Stock et al., 2001), i.e. the approximate cytosolic osmolarity was (in mosmol l^{-1}) 75, 160 and 250 when the adaptation osmolarity was (1) less than (in mosmol l^{-1}) 75, (2) more than 75 but less than 160 and (3) more than 160, respectively. These findings imply that a hypothetical osmolyte-transport mechanism(s) is activated to increase the cytosolic osmolarity when the external osmolarity is increased beyond either 75 or 160 mosmol l^{-1} and that another hypothetical osmolyte-transport mechanism(s) is activated to decrease the cytosolic osmolarity when the external osmolarity is decreased beyond either 75 or 160 mosmol l^{-1}.

Conversely, the contractile vacuole complex (CVC) has been regarded to be the organelle responsible for osmoregulation in fresh water protozoans and fresh water sponges (Jepps, 1947; Kitching, 1967; Dunham and Kropp, 1973; Patterson, 1980; Allen and Naitoh, 2002). As a matter of fact, the amount of fluid discharged from the contractile vacuole (CV) to the cell's exterior markedly increases when the cell is exposed to a hypotonic solution, while the fluid discharge ceases when the cell is exposed to an isotonic or hypertonic solution. Dunham and Kropp (1973) suggested the presence of cell volume control mechanisms in *Tetrahymena*, a fresh water ciliate, in addition to the CVC. It is, therefore, interesting and important to know how much the CVC contributes to the osmoregulation or volume control in the *Paramecium* cell.

In the present study, we examined the effects of a change in

the external osmolarity on both cell volume and the amount of fluid discharged from the CV in *P. multimicronucleatum*. We give evidence for the presence of two hypothetical osmolyte transport mechanisms in *Paramecium* that are responsible for regulatory volume increase (RVI) and regulatory volume decrease (RVD), respectively (Strange, 1994; Hoffmann and Dunham, 1995; Lang et al., 1998; Baumgarten and Feher, 2001). These two transport mechanisms fill the major role in regulating cell volume and the cytosolic osmolarity. The CVC seems to fill a lesser role in cell volume regulation.

Materials and methods

Cells

Paramecium multimicronucleatum (syngen 2) (Allen and Fok, 1988) cells were grown in an axenic culture medium (Fok and Allen, 1979), which has an osmolarity of ~84 msomol l^{-1} (Ishida et al., 1993) at 25°C. The cells harvested at the mid-logarithmic growth phase were centrifuged (~120 ***g***) for 25 s to form a loose pellet. The cells were then suspended in an experimental solution and centrifuged again. This procedure was repeated twice to wash away the culture medium. The cells were kept suspended in the experimental solution for 12–18 h to ensure their adaptation to the solution. The adapted cells were washed twice with the same adaptation solution and kept immersed in this final wash for more than 30 min prior to experimentation. All the experiments were performed at a regulated room temperature of ~25°C.

Experimental solutions

All the experimental solutions, each with a different osmolarity (4–204 mosmol l^{-1}), were made by adding different amounts of sorbitol to a standard saline solution that contained (mmol l^{-1} in final concentration) 2.0 KCl, 0.25 $CaCl_2$ and 1.0-MOPS-KOH (pH 7.0). Osmolarity of the standard saline solution without sorbitol was 4 mosmol l^{-1} (Naitoh et al., 1997a). In some experiments, a 30 mmol l^{-1} KCl or 10 mmol l^{-1} tetraethylammonium (TEA) chloride-containing solution was used. The osmolarity of each solution was determined by using a freezing-point osmometer (Advanced Instruments, Inc., Norwood, MA, USA).

Experimental procedures

An experimental trough (18 mm in length) with a rectangular cross section (0.2 mm in depth, 6 mm in width) was made from cover glass. The trough was first filled with 0.02% (v/v) poly-L-lysine (Sigma, St Louis, MO, USA) solution, then cells suspended in an adaptation solution were introduced into the trough from its one end, while excess solution in the trough was removed from the other end by using filter paper. Cells that adhered to the bottom surface of the trough were subjected to a change in the external osmolarity. This was done by introducing an experimental solution with an osmolarity different from the adaptation solution into the trough as described above. Images of the cells were magnified through a phase contrast objective (×40) of an inverted microscope (Olympus America Inc., Lake Success, NY) and video-recorded by using a CCD camera (Dage MIT Inc., Michigan City, IN) and a video cassette recorder (Sony Corp., Park Ridge, NY) at 30 frames s^{-1}.

Estimation of cell volume and its relative change

Video-recorded images of a cell were fed into a computer (Apple Computer Inc. Cupertino, CA) and the area (A) and length (l) for each image were determined by using NIH Image (downloaded from http://rsb.info.nih.gov/nih-image/). If we assume that a *Paramecium* cell approximates a solid of revolution, the volume of the cell (v) can be approximated by an equation as,

$$v = \frac{1}{6}\pi w^2 l\,, \tag{1}$$

where w is the width of the cell. An area and a length measured at t min after changing the external osmolarity, A_t and l_t can be formulated as,

$$A_t = a_t A_0 \tag{2}$$

and

$$l_t = b_t l_0 \tag{3}$$

respectively, where A_0 and l_0 are A and l at the start of the osmolarity change (at time 0), respectively. The width measured at t min after changing the external osmolarity, w_t, can be formulated as,

$$w_t = \frac{a_t}{b_t} w_0\,, \tag{4}$$

where w_0 is w at the start of the osmolarity change. Cell volume measured at t min after changing the external osmolarity, v_t, can be formulated as,

$$v_t = \frac{1}{6}\pi w_t^2 l_t = \frac{1}{6}\pi\left(\frac{a_t}{b_t} w_0\right)^2 b_t l_0 = \frac{1}{6}\pi w_0^2 l_0 \frac{a_t^2}{b_t} = v_0 \frac{a_t^2}{b_t}\,, \tag{5}$$

where v_0 is v at the start of the osmolarity change. Relative change in cell volume measured at t min after changing the external osmolarity can be formulated as,

$$\frac{v_t}{v_0} = \frac{a_t^2}{b_t}\,, \tag{6}$$

where a_t is A_t/A_0 (the relative change in cell area; see Equation 2) and b_t is l_t/l_0 (the relative change in cell length; see Equation 3). Actually, the values for A and l could be estimated more accurately than that for w. We, therefore, employed equation 6 for estimating cell volume.

Determining the rate of fluid discharge from the CV

The rate of fluid discharge from the CV ($\dot{R}_{CVC}$) was determined on the replayed images of the CV. $\dot{R}_{CVC}$ was obtained by dividing the maximum volume of the spherical CV immediately before the start of discharge, that is calculated

from the CV diameter, by the period of time from the start of fluid filling immediately after the previous fluid discharge to the start of the present fluid discharge, i.e. the time between two successive fluid discharges (Naitoh et al., 1997a; Stock et al., 2001).

Results

Volume of cells adapted to varied osmolarities

Six groups of *Paramecium* cells obtained from the same culture were adapted to different osmolarities, i.e. 4, 64, 84, 144, 164 or 204 mosmol l^{-1}, for 18 h, then cell volume for each group was determined. Cell volume was presented as a value relative to that for cells adapted to 84 mosmol l^{-1}, the osmolarity of the axenic culture medium (Ishida et al., 1996), and plotted against the adaptation osmolarity in Fig. 1. Cell volume was larger in cell groups adapted to a lower osmolarity.

Time course of change in cell volume after changing the external osmolarity

Decreasing the osmolarity

Five groups of *Paramecium* cells adapted to different osmolarities, i.e. 164, 144, 124, 84 or 64 mosmol l^{-1}, for 18 h were subjected to a 60 mosmol l^{-1} decrease in the external osmolarity and the time course of change in cell volume for each group was determined, and shown in Fig. 2. Each cell volume is presented as a value relative to the cell volume before decreasing the external osmolarity (at time 0).

The volume of 164 mosmol l^{-1}-adapted cells increased to its peak value of ~1.07 times the initial volume (at time 0) in ~10 min after decreasing the external osmolarity to 104 mosmol l^{-1} (Fig. 2A). The volume then decreased to its initial value in ~30 min after decreasing the external osmolarity and continued to decrease to the lowest value of ~0.95 by ~50 min. The volume then tended to increase slowly.

The volume of 144 mosmol l^{-1}-adapted cells increased to a plateau value of ~1.20 times the initial volume in ~20 min after decreasing the external osmolarity to 84 mosmol l^{-1} (Fig. 2B). In contrast to the previous case of 164 mosmol l^{-1}-adapted cells, the cells remained swollen at this plateau level after that. A series of three pictures of a representative 144 mosmol l^{-1}-adapted cell taken at 0, 15 or 30 min after decreasing the external osmolarity is shown in Fig. 4A. The cell at 15 min was thicker and longer than the cell at 0 min, and the cell at 30 min was thicker and longer than that at 15 min.

The volume of 124 mosmol l^{-1}-adapted cells increased to its peak value of ~1.23 times the initial volume in 30 min after decreasing the external osmolarity to 64 mosmol l^{-1}. The volume then decreased to resume its initial value (Fig. 2C).

As is shown in Fig. 2D, the volume of 84 mosmol l^{-1}-adapted cells increased to its peak value of ~1.14 in ~10 min after decreasing the external osmolarity to 24 mosmol l^{-1}. The volume then decreased to its initial value at time 0 in ~30 min and continued to decrease to ~0.95 in 40 min. The volume then tended to increase slowly. A series of three pictures of a representative 84 mosmol l^{-1}-adapted cell taken at 0, 15 or 30 min after decreasing the external osmolarity, is shown in Fig. 4B. The cell at 15 min was thicker and longer than the cell at 0 min, while the cell at 30 min was thinner than the cell at 15 min.

As is shown in Fig. 2E, the volume of 64 mosmol l^{-1}-adapted cells increased to a plateau value of ~1.17 in 20 min after decreasing the external osmolarity to 4 mosmol l^{-1}. The cells remained swollen at this level after that.

Increasing the osmolarity

Five groups of *Paramecium* cells adapted to different osmolarities, i.e. 104, 84, 64, 24 or 4 mosmol l^{-1}, for 18 h, were subjected to a 60 mosmol l^{-1} increase in the external osmolarity and the time course of change in cell volume for each group was determined and shown in Fig. 3.

The volume of 104 mosmol l^{-1}-adapted cells decreased to its lowest value of ~0.84 times the initial volume in ~10 min after increasing the external osmolarity to 164 mosmol l^{-1} (Fig. 3A). The volume then increased to ~0.95 in 60 min after increasing the external osmolarity.

The volume of 84 mosmol l^{-1}-adapted cells decreased to its lowest value of ~0.74 times the initial volume in ~20 min after increasing the external osmolarity (Fig. 3B). In contrast to the previous case of 104 mosmol l^{-1}-adapted cells, the cells remained shrunken at this lowest level after that.

The volume of 64 mosmol l^{-1}-adapted cells decreased to its lowest value of ~0.65 times the initial volume in ~10 min after increasing the external osmolarity to 124 mosmol l^{-1} (Fig. 3C). The volume then increased to ~0.85 in ~60 min after increasing the external osmolarity. A series of three pictures of a representative 64 mosmol l^{-1}-adapted cell taken at 0, 15 or 30 min after increasing the external osmolarity to

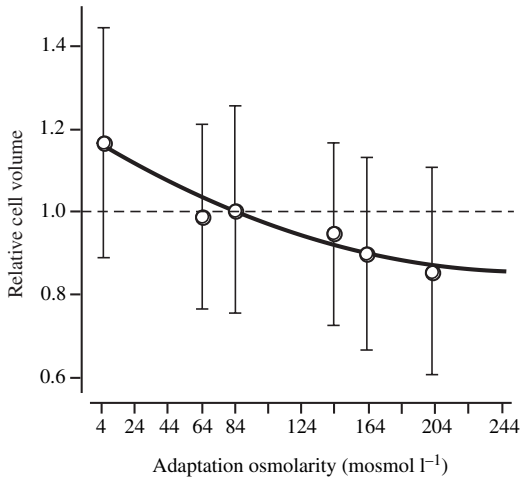

Fig. 1. The relationship between cell volume and the adaptation osmolarity in six different groups of *Paramecium multimicronucleatum* cells adapted to different osmolarities. Each plot for cell volume is the mean±S.D. (N=74–94) and is presented as a value relative to the mean volume of 84 mosmol l^{-1}-adapted cells. A solid line is a binomial approximation of the plots.

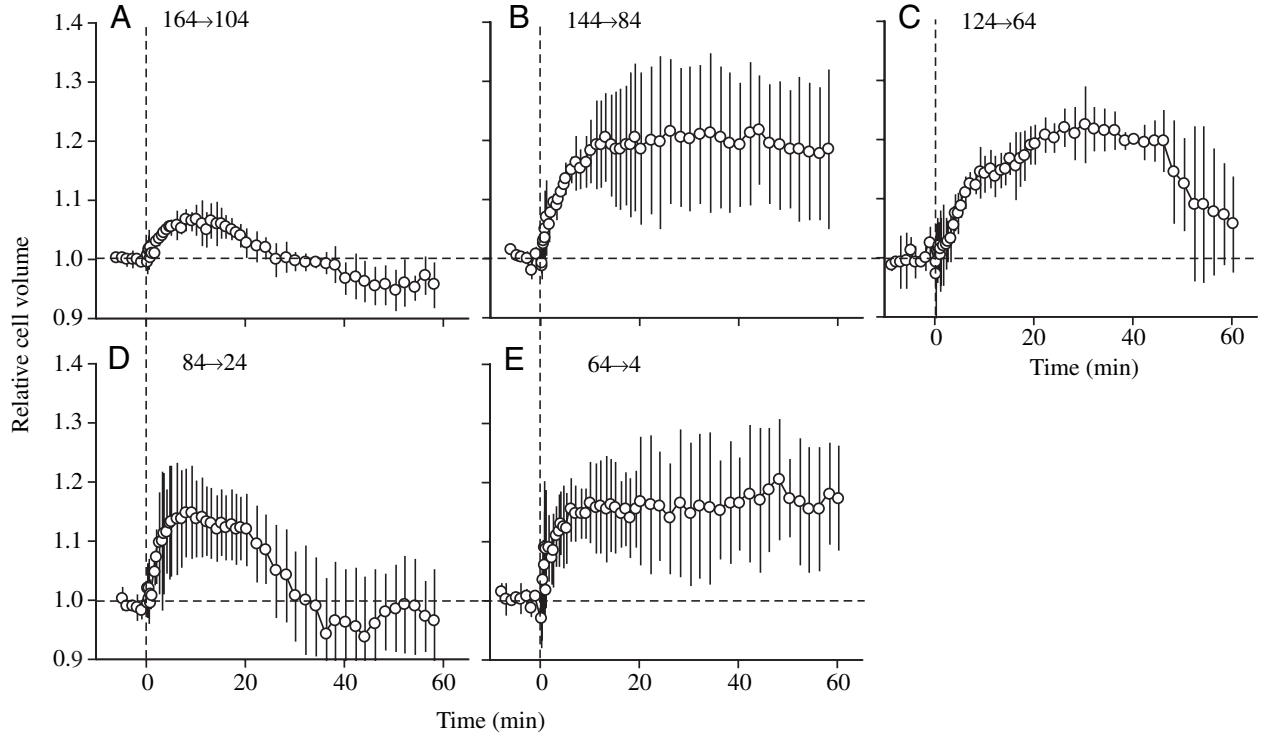

Fig. 2. Time courses of changes in cell volume after decreasing the external osmolarity by 60 mosmol l^{-1} in five different groups of *P. multimicronucleatum* adapted to different osmolarities. (A) 164 mosmol l^{-1}-adapted cells were subjected to 104 mosmol l^{-1}. (B) 144 mosmol l^{-1}-adapted cells were subjected to 84 mosmol l^{-1}. (C) 124 mosmol l^{-1}-adapted cells were subjected to 64 mosmol l^{-1}. (D) 84 mosmol l^{-1}-adapted cells were subjected to 24 mosmol l^{-1}. (E) 64 mosmol l^{-1}-adapted cells were subjected to 4 mosmol l^{-1}. Each plot for cell volume is the mean±S.D. (*N*=3) and presented as a value relative to the cell volume before decreasing the external osmolarity (at time 0).

124 mosmol l^{-1} is shown in Fig. 4D. A marked indentation was seen in the posterior one third of the cell at 15 min. This indentation was less pronounced in the cell at 30 min.

The volume of 24 mosmol l^{-1}-adapted cells decreased to its lowest value of ~0.80 times the initial volume in ~10 min after increasing the external osmolarity to 84 mosmol l^{-1} (Fig. 3D). The volume then increased to ~0.99 in 40 min after increasing the osmolarity and tended to decrease slowly after that.

The volume of 4 mosmol l^{-1}-adapted cells decreased to its lowest value of ~0.75 times the initial volume in ~40 min after increasing the external osmolarity to 64 mosmol l^{-1} and remained unchanged after that (Fig. 3E). A series of three pictures of a representative 4 mosmol l^{-1}-adapted cell taken at 0, 15 or 30 min after increasing the external osmolarity to 64 mosmol l^{-1} is shown in Fig. 4C. The cell at 15 min was thinner and shorter than the cell at 0 min, and the cell at 30 min was shorter than the cell at 15 min.

Effects of the degree of change in the external osmolarity on the time course of change in cell volume

To know the effects of the degree of change in the external osmolarity on the time course of change in cell volume after changing the external osmolarity, the time courses were compared among different groups of cells adapted to the same osmolarity and then subjected to different changes in osmolarity.

Decreasing the osmolarity

When 144 mosmol l^{-1}-adapted cells were subjected to 104 mosmol l^{-1}, where osmolarity decrease was 40 mosmol l^{-1}, cell volume increased to a plateau value of ~1.05 in ~10 min and remained unchanged at this level after that (Fig. 5A, black open circles). This time course was essentially the same as that seen after their subjection to 84 mosmol l^{-1}, where osmolarity decrease was 60 mosmol l^{-1} (Fig. 2B; the same data is shown in Fig. 5A by gray open circles for the sake of comparison), but the plateau value was far smaller than that for the 60 mosmol l^{-1} decrease (~1.20).

When 84 mosmol l^{-1}-adapted cells were subjected to 44 mosmol l^{-1}, where the osmolarity decrease was 40 mosmol l^{-1} (black open circles) or to 64 mosmol l^{-1}, where osmolarity decrease was 20 mosmol l^{-1} (red open circles), cell volume increased to a peak value of ~1.14, then decreased to resume its initial value (Fig. 5B). These time courses are essentially the same with that seen after their subjection to 24 mosmol l^{-1}, where osmolarity decrease was 60 mosmol l^{-1} (Fig. 2D; also shown in Fig. 5B by gray open circles for the sake of comparison). The peak values were almost the same among these three cases (~1.14) independently of the total decrease in the external osmolarity. However, the time to reach the peak was shorter when the decrease in the external osmolarity was larger, i.e. the time was ~10, 20 and 30 min for a 60, 40 and 20 mosmol l^{-1} decrease, respectively.

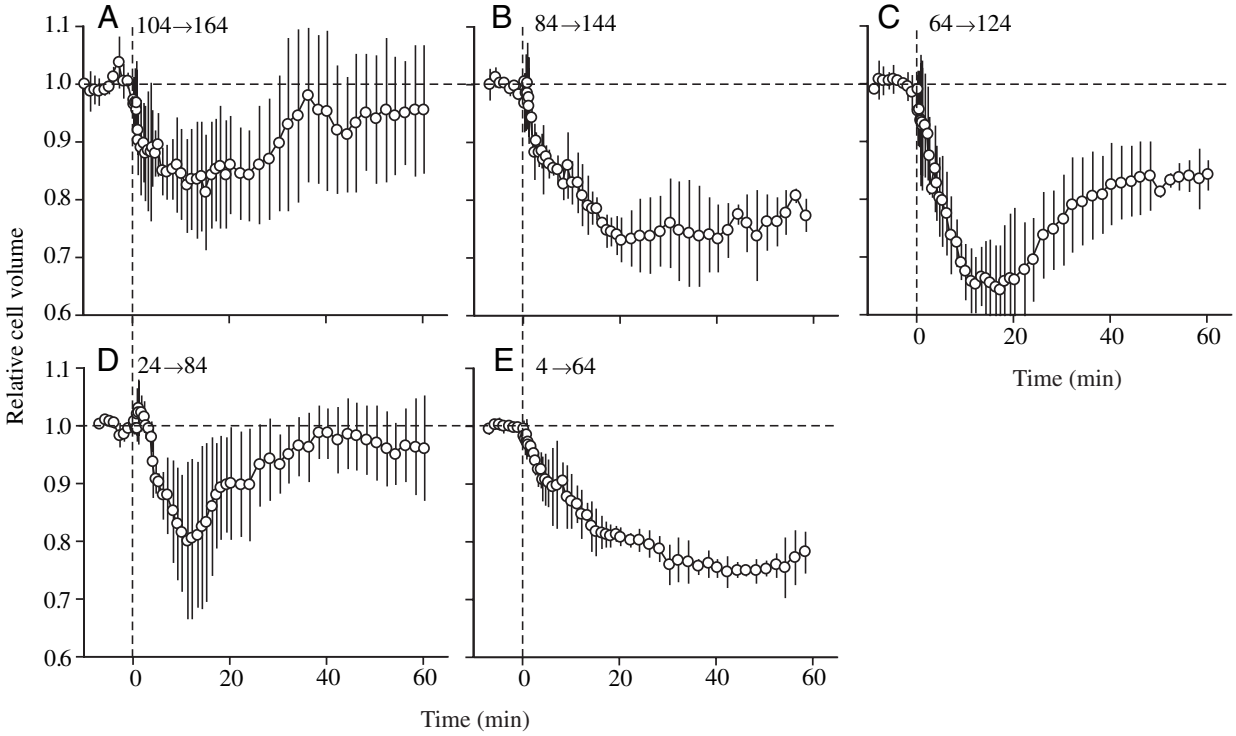

Fig. 3. Time courses of changes in cell volume after increasing the external osmolarity by 60 mosmol l^{-1} in five different groups of *P. multimicronucleatum* adapted to different osmolarities. (A) 104 mosmol l^{-1}-adapted cells were subjected to 164 mosmol l^{-1}. (B) 84 mosmol l^{-1}-adapted cells were subjected to 144 mosmol l^{-1}. (C) 64 mosmol l^{-1}-adapted cells were subjected to 124 mosmol l^{-1}. (D) 24 mosmol l^{-1}-adapted cells were subjected to 84 mosmol l^{-1}. (E) 4 mosmol l^{-1}-adapted cells were subjected to 64 mosmol l^{-1}. Each plot for cell volume is the mean±S.D. (*N*=3) and presented as a value relative to the cell volume before increasing the external osmolarity (at time 0).

Increasing the osmolarity

When 4 mosmol l^{-1}-adapted cells were subjected to 44 mosmol l^{-1}, where osmolarity increase was 40 mosmol l^{-1}, cell volume decreased to its lowest value of ~0.79 in ~40 min and then remained almost unchanged at this level (Fig. 6A, black open circles). This time course is essentially the same with that seen after their subjection to 64 mosmol l^{-1}, where osmolarity increase was 60 mosmol l^{-1} (Fig. 3E; also shown in Fig. 6A by gray open circles for the sake of comparison). However, decrease in the volume was smaller than that for a 60 mosmol l^{-1} decrease (~0.75).

When 64 mosmol l^{-1}-adapted cells were subjected to 84 mosmol l^{-1} (Fig. 6B), where osmolarity increase was 20 mosmol l^{-1} (black open circles), or to 104 mosmol l^{-1}, where osmolarity increase was 40 mosmol l^{-1} (red open circles), cell volume first decreased to its lowest value, then increased to resume its initial value. These time courses are essentially the same with that seen after their subjection to 124 mosmol l^{-1}, where osmolarity increase was 60 mosmol l^{-1} (Fig. 3C; also shown in Fig. 6B by gray open circles for the sake of comparison). However, the degree of volume decrease was larger when osmolarity increase was larger, i.e. the lowest volume was ~0.91, 0.80 and 0.64 for an osmolarity increase of 20, 40 and 60 mosmol l^{-1}, respectively.

Effects of TEA^+ and K^+ in the external solution on cell volume change

The major osmolytes in the cytosol of *Paramecium* are K^+ and Cl^- (Akita, 1941; Stock et al., 2002). It is, therefore, highly probable that a K^+ transport system(s) in the plasma membrane is involved in restoring cell volume in *Paramecium* (see Fig. 2A,C,D; Fig. 3A,C,D; Fig. 5B; Fig. 6B). We, therefore, examined the effect of the presence of 10 mmol l^{-1} TEA^+, a potent K^+ channel inhibitor, or excess (30 mmol l^{-1} instead of a normal 2 mmol l^{-1}) K^+ in the external solution on the time course of the change in cell volume after changing the external osmolarity (Fig. 7).

Decreasing the osmolarity

164 mosmol l^{-1}-adapted cells continued to swell after decreasing the external osmolarity to 104 mosmol l^{-1} in the presence of 10 mmol l^{-1} TEA^+ (Fig. 7A; black open circles) or 30 mmol l^{-1} K^+ (Fig. 7B; black open circles) in contrast to the control cells that returned to their initial volume after a temporary swelling (Fig. 7A,B; gray circle data from Fig. 2A).

Increasing the osmolarity

In the presence of 10 mmol l^{-1} TEA^+, 104 mosmol l^{-1}-adapted cells shrank to a more-or-less stationary volume of ~0.75 after increasing the external osmolarity to 164 mosmol l^{-1}

and did not return to their initial volume (Fig. 7C; black open circles); the cells returned to their initial volume after a temporary shrinking in the absence of TEA^+ (Fig. 7C; gray circles data from Fig. 3A).

Conversely, the cells returned to their initial volume after they showed a temporary shrinking upon increasing the external osmolarity to 164 mosmol l^{-1}. They responded more quickly in the presence of 30 mmol l^{-1} K^+ (Fig. 7D; black open circles) than in the presence of a normal 2.0 mmol l^{-1} K^+ (Fig. 7D; gray circles data from Fig. 3A). The time needed to return to the initial volume was ~20 min as opposed to 60 min or more in the control. The degree of shrinking was smaller (~0.90) than for the control (~0.84).

No change in the osmolarity

To determine whether the presence of TEA^+ or excess K^+ in the external solution itself affects cell volume, the time course of change in volume of the cells adapted to 164 mosmol l^{-1} after they were transferred into a 10 mmol l^{-1} TEA^+ or a 30 mmol l^{-1} K^+-containing 164 mosmol l^{-1} solution was determined. The cells were not subjected to a change in the external osmolarity. Cell volume increased very slowly to ~1.05 in 60 min after they were subjected to 10 mmol l^{-1} TEA^+. Cell volume scarcely changed after the cells were subjected to 30 mmol l^{-1} K^+-containing solution (data not shown).

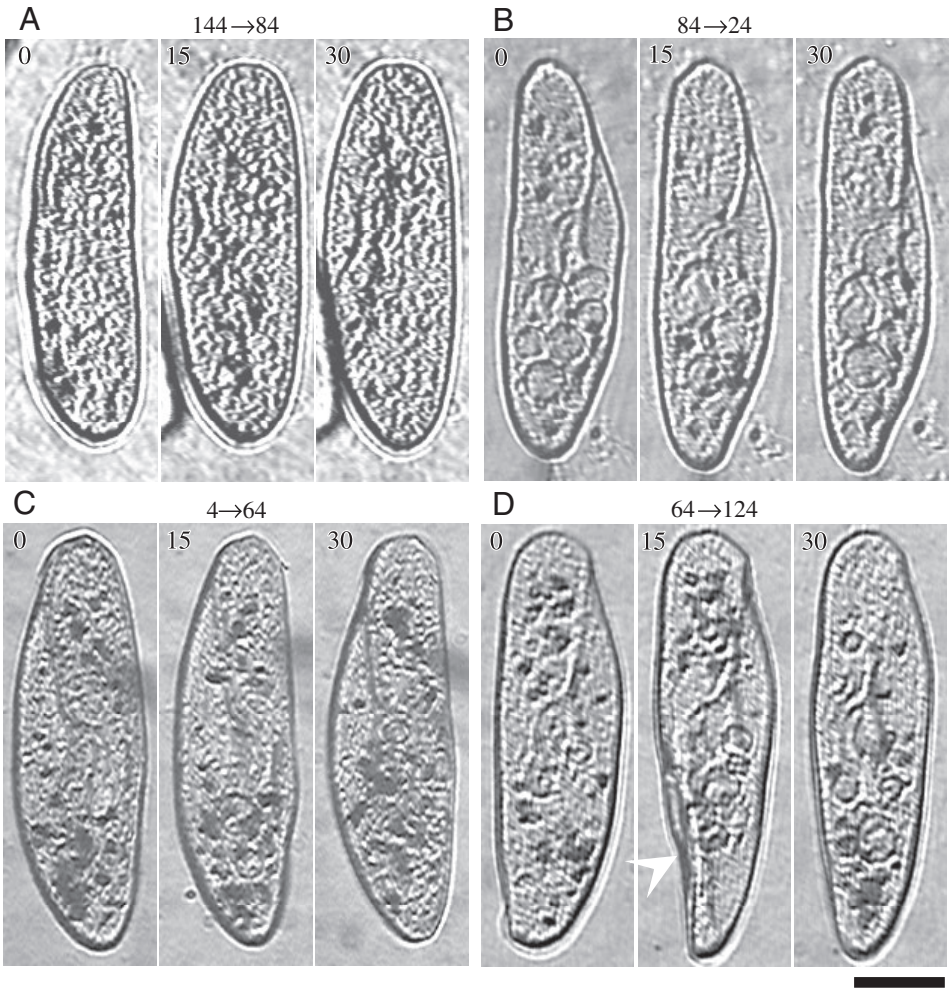

Fig. 4. Four sets of three consecutive pictures of a representative cell obtained from four different groups of *P. multimicronucleatum* cells adapted to different osmolarities taken at 0, 15 and 30 min, respectively, after changing the external osmolarity by 60 mosmol l^{-1}. (A) A 144 mosmol l^{-1}-adapted cell was subjected to 84 mosmol l^{-1}. (B) A 84 mosmol l^{-1}-adapted cell was subjected to 24 mosmol l^{-1}. (C) A 4 mosmol l^{-1}-adapted cell was subjected to 64 mosmol l^{-1}. (D) A 64 mosmol l^{-1}-adapted cell was subjected to 124 mosmol l^{-1}. A white arrowhead points to an indentation of the cell. The top of each cell image corresponds to the anterior portion of the cell. A number in the upper left corner of each picture is the time in min after changing the external osmolarity when the picture was taken. Scale bar, 50 μm.

Change in the rate of fluid discharge from the CV, $\dot{R}_{CVC}$, after changing the external osmolarity

To examine how much the CV affects cell volume, the time course of change in the rate of fluid discharge, $\dot{R}_{CVC}$ (Naitoh et al., 1997a), after changing the external osmolarity was determined. Four representative results are shown in Fig. 8, where $\dot{R}_{CVC}$ is presented as a value relative to its initial value before changing the external osmolarity.

Decreasing the osmolarity

When a 164 mosmol l^{-1}-adapted cell was transferred into a 104 mosmol l^{-1} solution, $\dot{R}_{CVC}$ increased to its peak value of ~6.5 in ~13 min after the transfer, then decreased to a more-or-less stationary value of ~2.5 (Fig. 8A). The initial value for $\dot{R}_{CVC}$ in this cell was ~11.9 fl s^{-1}.

When a 144 mosmol l^{-1}-adapted cell was transferred into a 84 mosmol l^{-1} solution, $\dot{R}_{CVC}$ increased to a plateau value of ~6.5 in ~13 min after the transfer. $\dot{R}_{CVC}$ then decreased gradually with time (Fig. 8B). The initial value for $\dot{R}_{CVC}$ in this cell was ~9.2 fl s^{-1}.

Increasing the osmolarity

When a 104 mosmol l^{-1}-adapted cell was transferred into a 164 mosmol l^{-1} solution, the cell suddenly ceased its CVC activity so that $\dot{R}_{CVC}$ became 0 in less than 10 min after the transfer. $\dot{R}_{CVC}$ activity started to recover gradually ~30 min after the transfer (Fig. 8C). The initial value for $\dot{R}_{CVC}$ in this cell was ~22.7 fl s^{-1}. The similar time course was observed when a 84 mosmol l^{-1}-adapted cell was transferred into a 144 mosmol l^{-1} solution

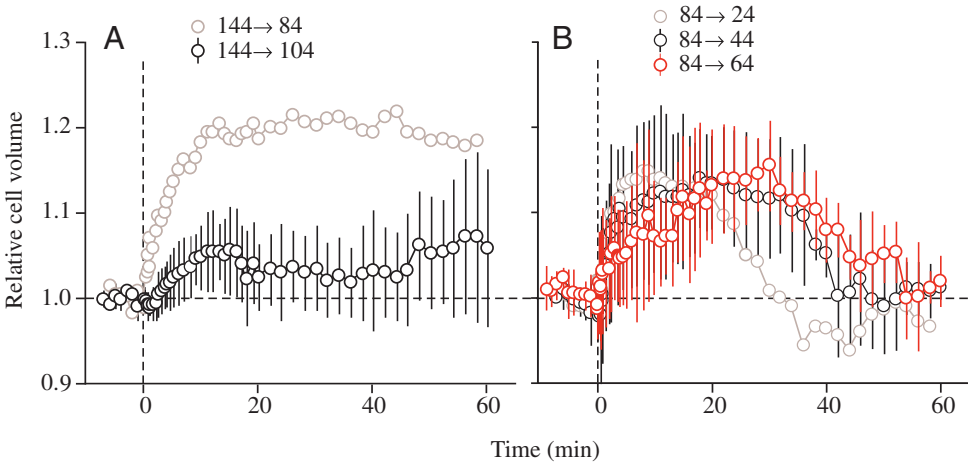

Fig. 5. Effects of the degree of decrease in the external osmolarity on the time course of change in cell volume after decreasing the external osmolarity in *P. multimicronucleatum*. (A) Two groups of cells adapted to 144 mosmol l^{-1} were subjected to 84 mosmol l^{-1} (the degree of decrease; 60 mosmol l^{-1}, gray open circles) or to 104 mosmol l^{-1} (the degree of decrease; 40 mosmol l^{-1}, black open circles). (B) Three groups of cells adapted to 84 mosmol l^{-1} were subjected to 24 mosmol l^{-1} (the degree of decrease; 60 mosmol l^{-1}, gray open circles), 44 mosmol l^{-1} (the degree of decrease; 40 mosmol l^{-1}, black open circles) or 64 mosmol l^{-1} (the degree of decrease; 20 mosmol l^{-1}, red open circles). Each plot for cell volume is the mean±S.D. (N=3) and presented as a value relative to the cell volume before decreasing the external osmolarity.

(Fig. 8D). The initial value for $\dot{R}_{CVC}$ in this cell was ~12.7 fl s^{-1}.

Discussion

Fig. 9B uses arrows to summarize and help to visualize the adaptation osmolarity and the degree of change in the external osmolarity for the series of 16 experiments that are presented in this paper. Each arrow lies in parallel with the abscissa of Fig. 9A that corresponds to the external osmolarity. The position of the tail end of each arrow, i.e. an intersection with a vertical broken line drawn from the tail end to the abscissa, corresponds to the adaptation osmolarity, while that of the head end to the osmolarity to which the cells are subjected. These two osmolarity values (mosmol l^{-1}) are shown by bold numbers beside the ends of each arrow. The length of each arrow, therefore, corresponds to the degree of change in the external osmolarity to which the cells are subjected, and is shown by an italic number in each arrowhead. A negative number corresponds to a decrease, while a positive number to an increase in the external osmolarity. The leftward arrow corresponds to a decrease, while the rightward arrow to an increase in the external osmolarity. Figure number(s) in parentheses beside the tail end of each arrow refers to the figure in this paper corresponding to the time course of change in cell volume.

An osmotic pressure difference between the cytosol and the adaptation solution balances a hydraulic pressure within the cytosol in a cell adapted to a particular osmolarity

We previously found that the cytosolic osmolarity of a *Paramecium* cell following adaptation to an osmolarity in a range from 4 to 204 mosmol l^{-1} always became higher than the osmolarity of the adaptation solution (Stock et al., 2001). This implies that an adapted *Paramecium* cell is always swollen to an extent determined by the osmotic pressure difference between the cytosol and the adaptation solution (π_{cyt}). This osmotic pressure difference balances a hydraulic pressure in

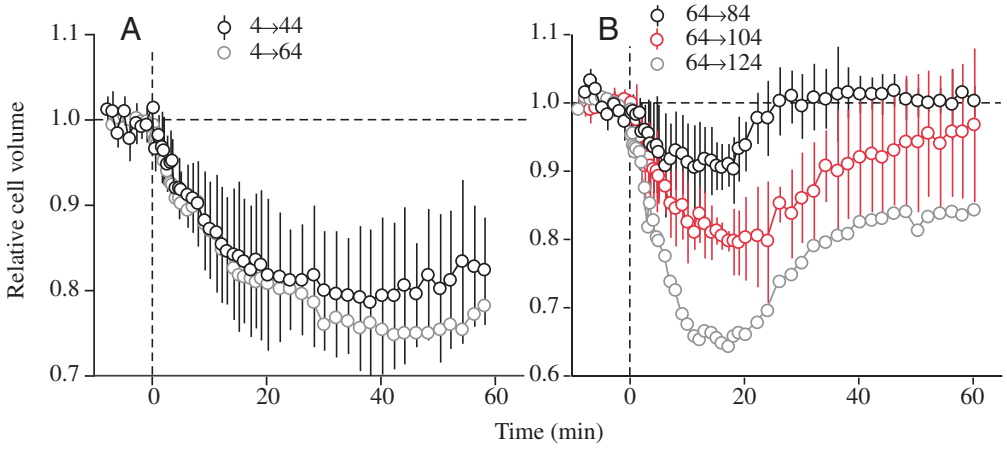

Fig. 6. Effects of the degree of increase in the external osmolarity on the time course of change in cell volume after increasing the external osmolarity in *P. multimicronucleatum*. (A) Two groups of cells adapted to 4 mosmol l^{-1} were subjected to 64 mosmol l^{-1} (the degree of increase; 60 mosmol l^{-1}, gray open circles) or to 44 mosmol l^{-1} (the degree of increase; 40 mosmol l^{-1}; black open circles). (B) Three groups of cells adapted to 64 mosmol l^{-1} were subjected to 124 mosmol l^{-1} (the degree of increase; 60 mosmol l^{-1}, gray open circles), 104 mosmol l^{-1} (the degree of increase; 40 mosmol l^{-1}, red open circles) or 84 mosmol l^{-1} (the degree of increase; 20 mosmol l^{-1}, black open circles). Each plot for cell volume is the mean±S.D. (N=3) and presented as a value relative to the cell volume before increasing the external osmolarity.

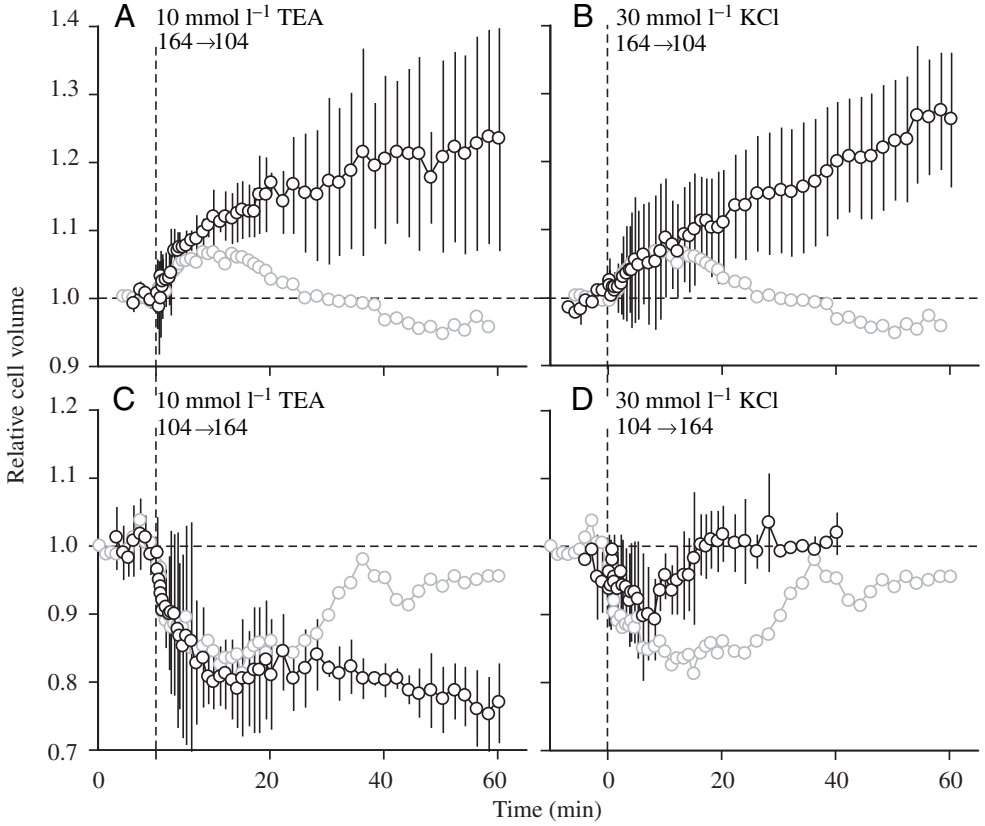

Fig. 7. Effects of TEA^+ and K^+ in the external solution on the time course of change in cell volume after changing the external osmolarity in *P. multimicronucleatum*. (A) 164 mosmol l^{-1}-adapted cells were transferred into a 10 mmol l^{-1} TEA^+-containing 104 mosmol l^{-1} solution, (B) 164 mosmol l^{-1}-adapted cells were transferred into a 30 mmol l^{-1} KCl-containing 104 mosmol l^{-1} solution. (C) 104 mosmol l^{-1}-adapted cells were transferred into a 10 mmol l^{-1} TEA^+-containing 164 mosmol l^{-1} solution. (D) 104 mosmol l^{-1}-adapted cells were transferred into a 30 mmol l^{-1} KCl-containing 164 mosmol l^{-1} solution. Each plot for cell volume is the mean±S.D. (N=3) and presented as a value relative to the cell volume before changing the external osmolarity.

the cytosol, the 'cytosolic pressure' (P_{cyt}), which is generated by an elastic membrane surrounding the cell as the cell becomes osmotically swollen. If either one or both of these pressures are somehow modified, the cell volume changes until a new balance between these pressures is established.

The presence of a cytosolic pressure in adapted cells was clearly demonstrated when 4 mosmol l^{-1}-adapted cells were subjected to 64 mosmol l^{-1} (Fig. 3E) or 44 mosmol l^{-1} (Fig. 6A). In these cases, the cells shrank even though the cytosolic osmolarity of 4 mosmol l^{-1}-adapted cells, i.e. ~75 mosmol l^{-1} (Stock et al., 2001), was higher than the osmolarity to which the cells were subjected, i.e. 44 or 64 mosmol l^{-1}. The cytosolic pressure of 4 mosmol l^{-1}-adapted cells exceeded the osmotic pressure difference that was lowered by raising the external osmolarity, and thereby compressed the cells. Similarly, 84 mosmol l^{-1}-adapted cells shrank when they were subjected to 144 mosmol l^{-1} (Fig. 3B), even though the cytosolic osmolarity of 84 mosmol l^{-1}-adapted cells, i.e. ~160 mosmol l^{-1} (Stock et al., 2001), was higher than 144 mosmol l^{-1}. We previously demonstrated the presence of a cytosolic pressure in *Paramecium* cells was the basis for the discharge of the contractile vacuole fluid (Naitoh et al., 1997a,b).

Cell volume control mechanisms are activated when the external osmolarity is changed beyond a critical osmolarity

We previously found that the cytosolic osmolarity of the *Paramecium* cell changed stepwise at around 75 or 160 mosmol l^{-1} when the adaptation osmolarity was changed gradually (Stock et al., 2001). This implies that an active change in the cytosolic osmolarity takes place when the external osmolarity is changed beyond these osmolarities. Hereafter, these two osmolarities will each be termed a 'critical osmolarity' (C_N) for causing an active change in the cytosolic osmolarity.

It can, therefore, be predicted that when the external osmolarity is decreased beyond a critical osmolarity, an osmolyte-transport mechanism(s) in the plasma membrane is activated to decrease the cytosolic osmolarity, so that a cell that has been swollen by its subjection to a decreased osmolarity may resume its initial volume. When the external osmolarity is increased beyond a critical osmolarity, another osmolyte-transport mechanism(s) is activated to increase the cytosolic osmolarity, so that the cell that has been shrunken by its subjection to an increased osmolarity may resume its initial volume. By contrast, when the external osmolarity is changed within an osmolarity range where no critical osmolarity is included, the cell will remain swollen or shrunken, since the osmolyte transport mechanisms are not activated.

These predictions proved correct. That is, cell volume recovery from its temporary swelling caused by a decrease in the external osmolarity was observed when the decrease took place beyond a critical osmolarity, i.e. a decrease from (in mosmol l^{-1}) 164 to 104 (Fig. 2A; the critical osmolarity is 160), that from 124 to 64 (Fig. 2C; the critical osmolarity is 75), that from 84 to 24 (Fig. 2D; the critical osmolarity is 75), that from 84 to 44 (Fig. 5B; the critical osmolarity is 75) and that from 84 to 64 (Fig. 5B; the critical osmolarity is 75). Leftward arrows with a red asterisk in Fig. 9B correspond to

these experiments. Each arrow crosses with either one or the other of two red vertical lines that corresponds to a critical osmolarity of 75 or 160 mosmol l^{-1}, respectively. By contrast, cells remained swollen when a decrease in the external osmolarity took place within an osmolarity range where no critical osmolarity was included, i.e. a decrease from (in mosmol l^{-1}) 144 to 84 (Fig. 2B), that from 144 to 104 (Fig. 5A) and that from 64 to 4 (Fig. 2E). Leftward arrows without an asterisk in Fig. 9B correspond to these experiments. These arrows cross neither one or the other of the two red vertical lines.

Paramecium showed recovery from its temporary shrinkage after its subjection to an increase in the external osmolarity when the increase took place beyond a critical osmolarity, i.e. an increase from (in mosmol l^{-1}) 104 to 164 (Fig. 3A; the critical osmolarity is 160), that from 64 to 124 (Fig. 3C; the critical osmolarity is 75), that from 64 to 104 (Fig. 6B; the critical osmolarity is 75), that from 64 to 84 (Fig. 6B; the critical osmolarity is 75) and that from 24 to 84 (Fig. 3D; the critical osmolarity is 75). Rightward arrows with a blue asterisk in Fig. 9B correspond to these experiments. These arrows cross either one or the other of the two red vertical lines. By contrast, the cell remained shrunken when an increase in the external osmolarity took place within an osmolarity range where no critical osmolarity was included, i.e. an increase from (in mosmol l^{-1}) 84 to 144 (Fig. 3B) and that from 4 to 64 (Fig. 3E). Rightward arrows without an asterisk in Fig. 9B correspond to these experiments. These arrows cross neither of the two red vertical lines.

Thus two hypothetical osmolyte transport mechanisms are proposed for *Paramecium*, i.e. (1) responsible for cell volume recovery from osmotic swelling and (2) responsible for cell volume recovery from osmotic shrinkage. These hypothetical transport mechanisms may correspond to osmolyte transport mechanisms found in the cells of multicellular animals that are responsible for (1) regulatory volume decrease, RVD, and for (2) regulatory volume increase, RVI, respectively (Strange, 1994; Hoffmann and Dunham, 1995; Lang et al., 1998; Baumgarten and Feher, 2001).

Bulk modulus of the cell changes as the cytosolic osmolarity changes

It was predicted that a cell adapted to an osmolarity very close to a critical osmolarity for activation of RVI, i.e. 75 mosmol l^{-1} in an osmolarity range of less than 75 mosmol l^{-1} or 160 mosmol l^{-1} in an osmolarity range from 75 to 160 mosmol l^{-1}, shows neither osmotic swelling nor shrinkage, since the cytosolic osmolarity is nearly the same as the critical osmolarity (see Fig. 9A). Hereafter, the volume of a cell under this condition will be termed the 'natural cell volume' (v_N). The natural cell volume is assumed to be determined by the structural and mechanical properties of the cytoskeleton and the associated pellicular membranes.

Conversely, the volume of a cell adapted to an osmolarity lower than the critical osmolarity is larger than the natural cell volume, since the external osmolarity is lower than the cytosolic osmolarity, so that the cell will be osmotically swollen. The cell volume becomes maximum when the adaptation osmolarity is the lowest in an osmolarity range where no critical osmolarity is included, since the difference in the osmolarity between the cytosol and the external solution becomes maximum. It was, therefore, predicted that the cell volume might change stepwise at each critical osmolarity as the adaptation osmolarity changes.

This prediction, however, proved incorrect. As is shown in

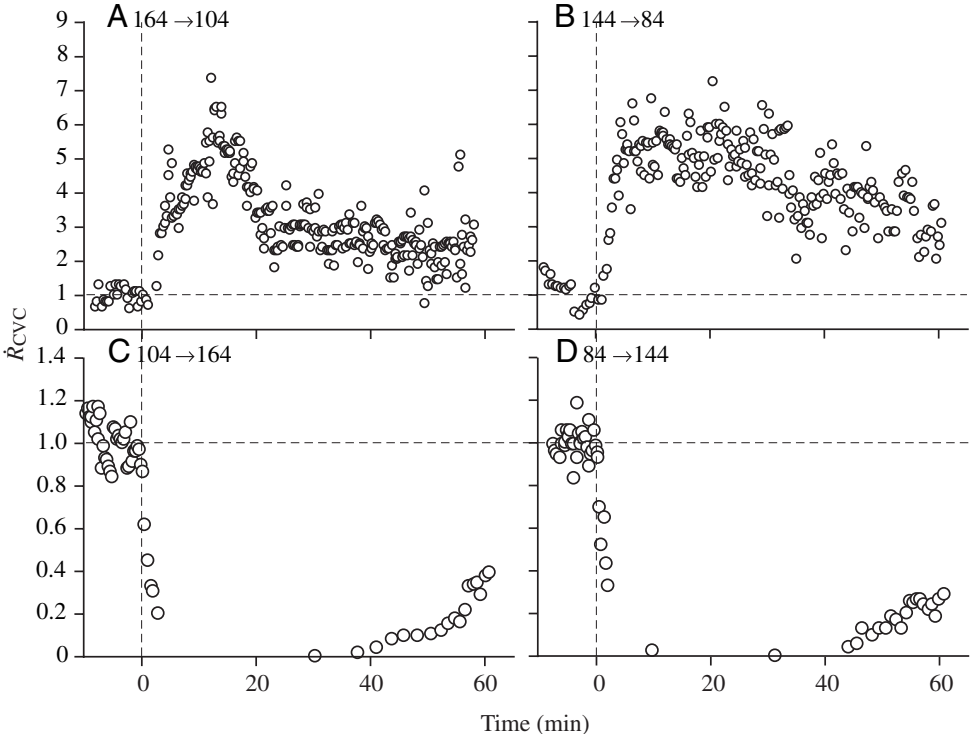

Fig. 8. Four representative time courses of change in the rate of fluid discharge from the contractile vacuole after changing the external osmolarity in *P. multimicronucleatum*. (A) A 164 mosmol l^{-1}-adapted cell was subjected to 104 mosmol l^{-1}. (B) A 144 mosmol l^{-1}-adapted cell was subjected to 84 mosmol l^{-1}. (C) A 104 mosmol l^{-1}-adapted cell was subjected to 164 mosmol l^{-1}. (D) A 84 mosmol l^{-1}-adapted cell was subjected to 144 mosmol l^{-1}.

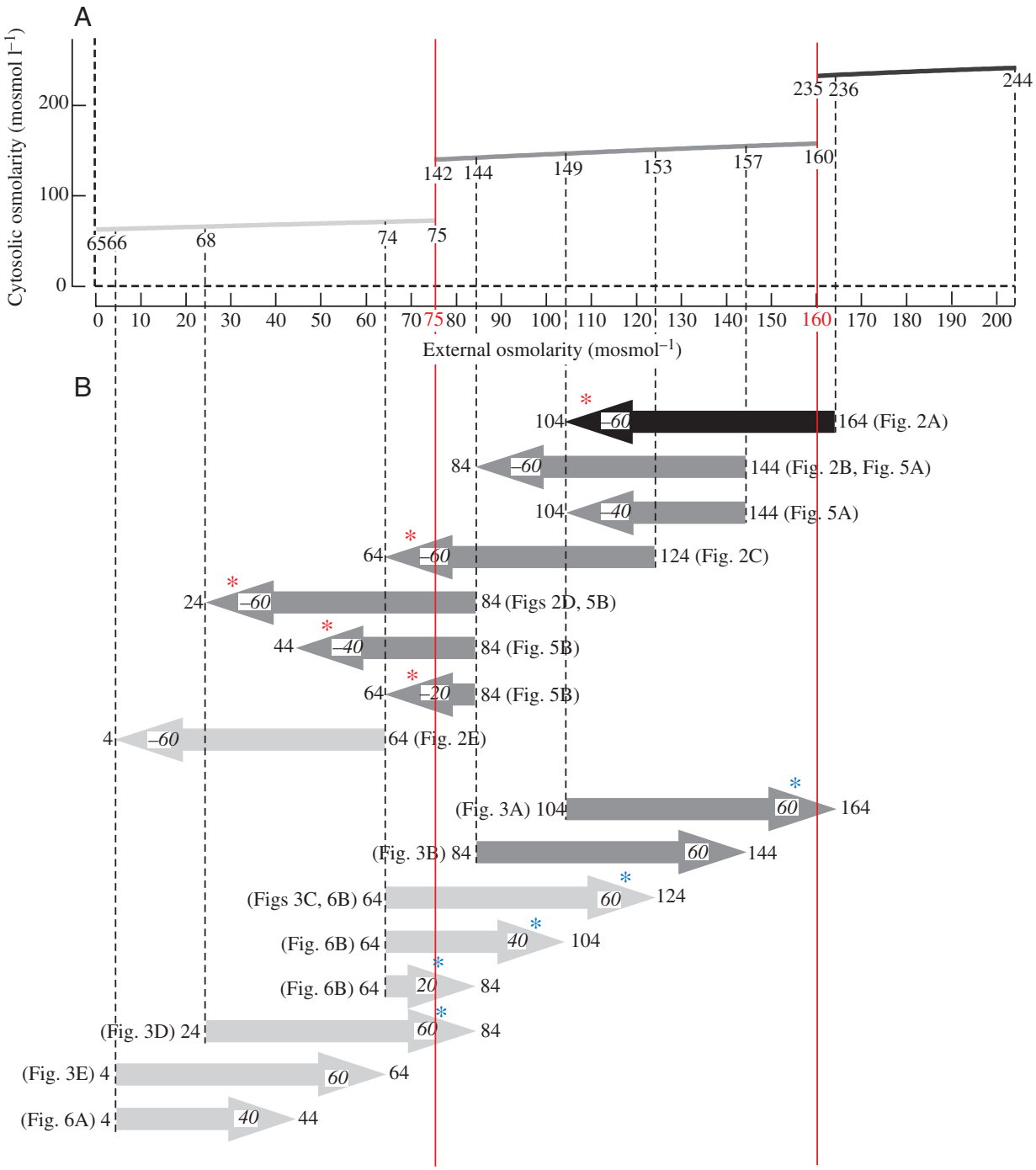

Fig. 9. (A) Estimated cytosolic osmolarity of *P. multimicronucleatum* as a function of the adaptation osmolarity. Light, medium and dark gray lines correspond to the cytosolic osmolarities for three different adaptation osmolarity ranges, i.e. less than (in mosmol l^{-1}) 75, from 75 to 160 and more than 160, respectively. (B) A profile of changes in both adaptation osmolarity and osmolarity to which the adapted cells are subjected for 16 experiments done in the present study. 16 arrows correspond to these 16 different experiments, respectively. Light gray arrows, medium gray arrows and dark gray arrows correspond to the experiments with cells adapted to an osmolarity in an osmolarity range of less than 75 mosmol l^{-1}, those with cells adapted to an osmolarity in an osmolarity range from 75 to 160 and those with cells adapted to an osmolarity in an osmolarity range of more than 160 mosmol l^{-1}, respectively. Estimated cytosolic osmolarity of each cell is shown by a number under a line for the cytosolic osmolarity in A. Leftward arrows with a red asterisk beside each arrow are the cases where RVD takes place after cell's subjection to a decreased osmolarity. Rightward arrows with a blue asterisk beside each arrowhead show the cases where RVI takes place after cell's subjection to an increased osmolarity. Abscissa (external osmolarity) corresponds to the adaptation osmolarity for A and to both adaptation osmolarity and osmolarities to which the adapted cells are subjected for B. See the first paragraph of the Discussion section for more detail.

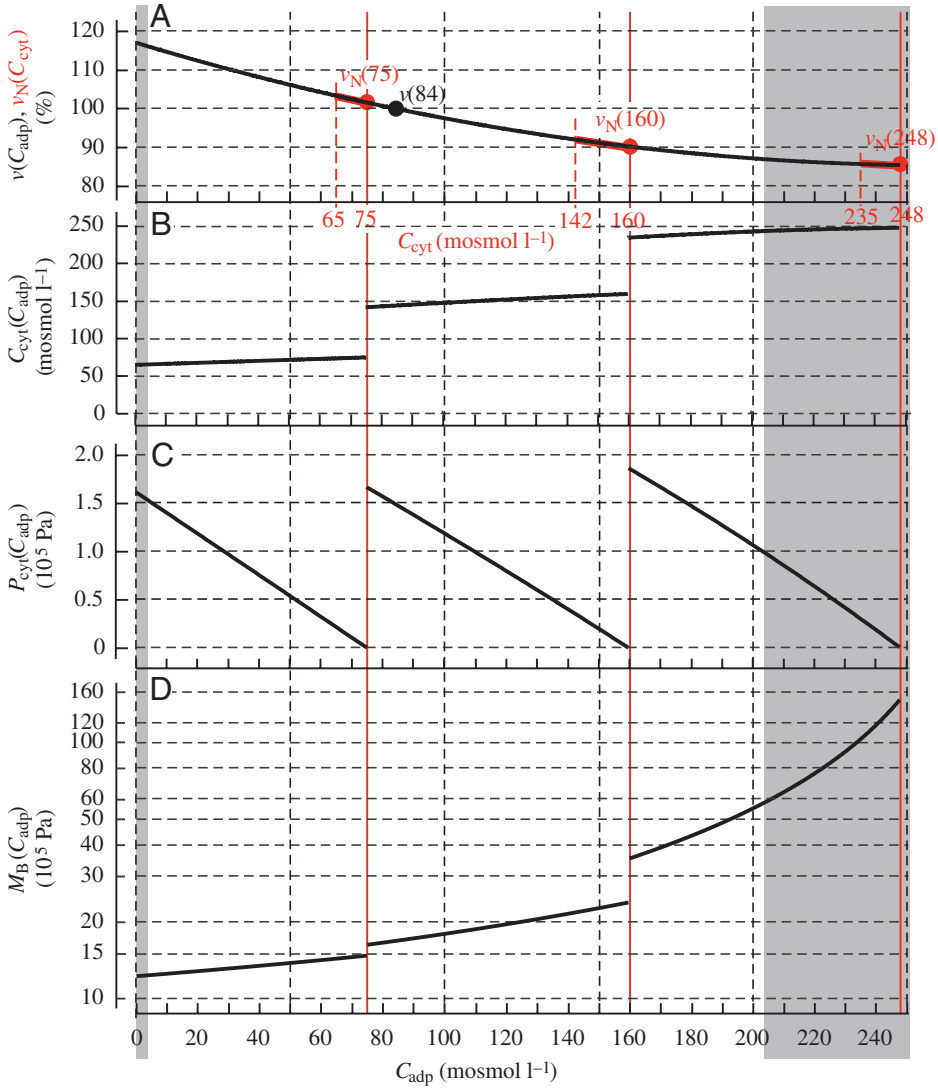

Fig. 10. Values for cell volume, $v(C_{adp})$, (A), cytosolic osmolarity, $C_{cyt}(C_{adp})$, (B), cytosolic pressure, $P_{cyt}(C_{adp})$, (C) and the bulk modulus, $M_B(C_{adp})$, (D), in *P. multimicronucleatum* cells plotted against the adaptation osmolarity, C_{adp}. Values for the natural cell volume, $v_N(C_{cyt})$ (thick red lines), are plotted against the cytosolic osmolarity, C_{cyt} (red letters), instead of C_{adp}. A black filled circle labelled $v(84)$ corresponds to volume of the cell adapted to 84 mosmol l^{-1} and all the cell volumes are presented as a percentage of $v(84)$. Data on gray columns are estimated by extrapolation of the actual data for C_{adp} ranging from 4 to 204 mosmol l^{-1}. Left gray column; for C_{adp} ranging from 0 to 4 mosmol l^{-1}, right gray column; for C_{adp} ranging from 204 to 250 mosmol l^{-1}. See the text for detail.

Fig. 1, the cell volume of 4 mosmol l^{-1}-adapted cells was significantly (P<0.01) larger than those cells adapted to an osmolarity higher than 4 mosmol l^{-1}, i.e. (in mosmol l^{-1}) 64, 84, 144, 164 or 204, whereas there is no significant difference in cell volume between 64 mosmol l^{-1}- and 84 mosmol l^{-1}-adapted cells (P=0.691, N=56–64), or between 144 mosmol l^{-1}- and 164 mosmol l^{-1}-adapted cells (P=0.158, N=95–96). These facts imply that cell volume changes continuously without showing a stepwise change at each critical osmolarity as the adaptation osmolarity changes.

Cell volume (v) plotted against adaptation osmolarity (C_{adp}) as shown in Fig. 1 can be approximated by a binomial equation as,

$$v(C_{adp}) = 0.000454C^2_{adp} - 0.240C_{adp} + 117, \quad (7)$$

where $v(C_{adp})$ is the volume of cells adapted to a C_{adp} (in mosmol l^{-1}), and expressed by the percentage of volume of cells adapted to 84 mosmol l^{-1}. Equation 7 is depicted as a thick line in Fig. 1 (and also in Fig. 10A).

Our finding that cell volume decreases continuously as the adaptation osmolarity increases, while the cytosolic osmolarity increases stepwise (compare Fig. 10A with Fig. 10B) implies that stiffness of the cell against its osmotic swelling increases stepwise at each critical osmolarity as the adaptation osmolarity increases. To quantify the stiffness, the idea of the bulk modulus was introduced for the *Paramecium* cells adapted to different osmolarities. The bulk modulus of the *Paramecium* cell adapted to a C_{adp}, $M_B(C_{adp})$, is defined as,

$$M_B(C_{adp}) = \frac{P_{cyt}(C_{adp})}{\frac{v(C_{adp}) - v_N(C_N)}{v_N(C_N)}}, \quad (8)$$

where $P_{cyt}(C_{adp})$ and $v_N(C_N)$ are a cytosolic pressure and a natural cell volume for a cell adapted to C_{adp}, respectively. $P_{cyt}(C_{adp})$ balances an osmotic pressure difference between the cytosol and an adaptation solution, $\pi_{cyt}(C_{adp})$. This balance can be written as,

$$P_{cyt}(C_{adp}) = \pi_{cyt}(C_{adp}). \quad (9)$$

The number of osmolytes in the cytosol of a single cell, N,

is assumed to be the same among cells adapted to a C_{adp} varied within an osmolarity range where no critical osmolarity is included, since no osmolyte transport mechanism(s) is activated by varying C_{adp} in this range. $C_{cyt}(C_{adp})$, therefore, can be written as,

$$C_{cyt}(C_{adp}) = \frac{N}{v(C_{adp})} \ . \quad (10)$$

$C_{cyt}(C_{adp})$ in each of three different osmolarity ranges was calculated according to equation 10 and shown as three gray lines with different gray scales in Fig. 9A (also in Fig. 10B), i.e. light gray for the 0–75 mosmol l^{-1} range, medium gray for the 75–160 mosmol l^{-1} range and dark gray for the 160–204 mosmol l^{-1} range. N value for a 0–75 mosmol l^{-1} range and that for a 75–160 mosmol l^{-1} range were estimated as a product of $v(75) \times 75$ mosmol l^{-1} and that of $v(160) \times 160$ mosmol l^{-1}, respectively. N for a 160–204 mosmol l^{-1} range was estimated as a product of $v(160) \times 235$ mosmol l^{-1}. It is clear from the figure that C_{cyt} gradually increases as C_{adp} increases within each osmolarity range, since v decreases as C_{adp} increases (compare Fig. 10B with Fig. 10A). The values for $\pi_{cyt}(C_{adp})$ can be estimated according to van't Hoff's formula for a diluted solution as,

$$\pi_{cyt}\,(C_{adp}) = (C_{cyt}(C_{adp}) - C_{adp})\,RT\,, \quad (11)$$

where R is the gas constant and T is the absolute temperature. Estimated values for $\pi_{cyt}(C_{adp})$ are shown in Fig. 10C.

Each red solid circle on the thick black line in Fig. 10A shows the natural cell volume at each corresponding critical osmolarity, $v_N(C_N)$. According to the definition of the natural cell volume, the cytosolic osmolarity of a cell adapted to a C_N, $C_{cyt}(C_N)$, equals C_N. The thick line in Fig. 10A corresponding to $v(C_{adp})$ (equation 7), therefore, can be regarded as a line that corresponds to the natural cell volume as a function of the cytosolic osmolarity, $v_N(C_{cyt})$, and can be written as,

$$v_N\,(C_{cyt}) = 0.000454C^2{}_{cyt} - 0.240C_{cyt} + 117\,. \quad (12)$$

As is shown in Fig. 10B, C_{cyt} changes (in mosmol l^{-1}) from ~65 to 75 when C_{adp} changes from 0 to 75 and from ~142 to 160 when C_{adp} changes from 75-160. Changes in v_N corresponding to these changes in C_{cyt} are depicted in Fig. 10A as two thick red lines superimposed behind the black line for $v(C_{adp})$.

The values for the bulk modulus of cells adapted to different osmolarities were then calculated according to Equations 8 in consideration of a change in v_N due to a change in C_{cyt} (Equation 12; Fig. 10A, red lines), and plotted against C_{adp} in Fig. 10D. It is clear from the figure that the bulk modulus increases stepwise at each critical osmolarity as the adaptation osmolarity increases. It is also seen that the bulk modulus gradually increases as the cytosolic osmolarity increases within each osmolarity range. These facts imply that the bulk modulus of *Paramecium* is primarily dependent on the cytosolic osmolarity.

A major cytosolic osmolyte in *Paramecium* is KCl (Akita, 1941; Stock et al., 2002). It is, therefore, highly probable that the bulk modulus of the *Paramecium* cell increases as cytosolic KCl concentration increases. Cytosolic KCl concentration might modify the elastic property of the cytoskeleton and its associated pellicular membrane. The concentration effect of cytosolic KCl on the mechanical properties and electron microscopical structures of the cytoskeleton of *Paramecium* could be examined to try to understand the mechanism by which the cell's bulk modulus is modified when changing the adaptation osmolarity, although such changes might be too subtle to be recognized by conventional fine structure analysis.

Factors that activate the osmolyte transport mechanisms

Decreasing the external osmolarity

The volume of 84 mosmol l^{-1}-adapted cells increased to a maximum value of ~1.15 times that of its initial value after the cells were subjected to 24, 44 and 64 mosmol l^{-1}, respectively, then began to decrease (Fig. 5B). The time needed to reach the maximum volume was shorter when the degree of decrease in the external osmolarity was larger. These results would seem to imply that RVD is activated when the cell is swollen to a certain extent (1.15) independently of the rate of swelling of the cell.

Conversely, the volume of 144 mosmol l^{-1}-adapted cells increased to a plateau value of ~1.20 times that of its initial value and remained unchanged after reducing the external osmolarity to 84 mosmol l^{-1} (Figs 2B, 5A). RVD was not activated even though the cells were swollen to an extent larger than 1.15. Furthermore, the 164 mosmol l^{-1}-adapted cells showed RVD when they were swollen to an extent of ~1.07. i.e. smaller than 1.15, by decreasing the external osmolarity to 104 msomol l^{-1} (Fig. 2A). These results imply that an osmotic swelling of a cell to a critical size is not the primary factor for activation of RVD. These results also exclude the possibility that a decrease in the concentration of a certain chemical(s) in the cytosol by osmotic swelling is the primary factor for activation of RVD.

A plausible factor for activation of RVD is an increase in the cytosolic pressure to a critical extent. As is previously mentioned, the cytosolic pressure is generated as the cell is osmotically swollen. To examine this possibility, the cytosolic pressure of cells showing their maximum swelling, after their subjection to a decreased osmolarity, was estimated by using the data from eight experiments as shown in Figs 2 and 5. Estimated values for the cytosolic pressure are shown in Table 1 together with values for the parameters needed for the estimation.

As is shown in Table 1, the cytosolic pressure of cells showing maximum volume after their subjection to a decreased osmolarity (P_{cyt2}) was larger than $\sim 1.5 \times 10^5$ Pa, when they showed RVD (+ in the RVD column). Conversely, P_{cyt2} was smaller than $\sim 1.5 \times 10^5$ Pa, when the cells did not show RVD (– in the RVD column). It can, therefore, be said that an increase in the cytosolic pressure over a threshold value of $\sim 1.5 \times 10^5$ Pa is a plausible factor for activation of RVD.

Table 1. *Cytosolic pressure of* P. multimicronucleatum *cells after their subjection to a decreased external osmolarity*

C_{adp} (mosmol l^{-1})	C_{stm} (mosmol l^{-1})	v_2/v_1	C_{cyt1} (mosmol l^{-1})	C_{cyt2} (mosmol l^{-1})	P_{cyt1} (10^5 Pa)	P_{cyt2} (10^5 Pa)	RVD	Data
164	104	1.07	236	221	1.78	2.9	+	Fig. 2A
144	84	1.20	157	131	0.32	1.2	–	Fig. 2B
144	104	1.06	157	148	0.32	1.1	–	Fig. 5A
124	64	1.21	153	126	0.72	1.6	+	Fig. 2C
84	24	1.14	144	126	1.49	2.5	+	Fig. 2D
84	44	1.14	144	126	1.49	2.1	+	Fig. 5B
84	64	1.14	144	126	1.49	>1.5	+	Fig. 5B
64	4	1.17	74	63	0.25	<1.5	–	Fig. 2E

C_{adp}, osmolarity to which cells were adapted; C_{stm}, osmolarity to which adapted cells were subjected; v_1, volume of cells adapted to C_{adp}; v_2, maximum volume of cells after their subjection to C_{stm}; C_{cyt1}, cytosolic osmolarity of cells adapted to C_{adp}; C_{cyt2}, cytosolic osmolarity of cells showing maximum cell volume after their subjection to C_{stm}; P_{cyt1}, cytosolic pressure of cells adapted to C_{adp}; P_{cyt2}, maximum cytosolic pressure of cells showing maximum cell volume after their subjection to C_{stm}; RVD, the regulatory volume decrease. + and – correspond to the presence and absence of RVD after decreasing the external osmolarity, respectively; Data, figures corresponding to respective estimations of P_{cyt1} and P_{cyt2}. The values for C_{cyt1} were obtained from Fig. 10B. C_{cyt2} was estimated based on the assumption that the total osmolyte(s) number in the cytosol remains unchanged before RVD takes place according to the equation as,

$$\frac{100 \cdot v_{CVC}}{\Delta v - v_{CVC}} ;$$

P_{cyt1} was calculated according to the equation, $P_{cyt1}=(C_{cyt1}-C_{adp})RT$, where R is gas constant and T is absolute temperature (298 K). Similarly, P_{cyt2} was calculated according to the equation, $P_{cyt2}=(C_{cyt2}-C_{stm})RT$.

Table 2. *Participation of the contractile vacuole complex activity in the control of cell volume in* P. multimicronucleatum *cells*

C_{adp} (mosmol l^{-1})	C_{stm} (mosmol l^{-1})	R_{CVC0} (fl s^{-1})	v_0 (fl)	t (min)	v_{CVC} (fl)	Δv (fl)	Participation of CVC (%)	Data
164	104	11.9	220 000	7.9	–31 000	15 500	–67	Fig. 2A, Fig. 8A
144	84	9.2	234 000	13.2	–36 400	48 200	–43	Fig. 2B, Fig. 8B
104	164	22.7	244 000	10.6	14 400	–38 600	–27	Fig. 3A, Fig. 8C
84	144	12.7	249 000	19.0	14 500	–65 900	–18	Fig. 3B, Fig. 8D

C_{adp}, osmolarity to which the cells were adapted; C_{stm}, osmolarity to which the adapted cells were subjected; $\dot{R}_{CVC0}$, the rate of fluid discharge from the CV, $\dot{R}_{CVC}$, at the start of osmolarity change; v_0, cell volume at the start of osmolarity change; t, the period from the start of osmolarity change to the time when cell volume reached its peak or plateau value; v_{CVC}, volume of fluid expelled from the cytosol (–) through the CVC (fluid outflow) or supplied to the cytosol (+) from the cell exterior through a hypothetical transporter responsible for fluid inflow during t; Δv, an increase (+) or a decrease (–) in cell volume during t; participation of CVC, v_{CVC} presented in percentage of the total change in cell volume during t, and formulated as,

$$C_{cyt2} = \frac{v_1}{v_2} C_{cyt1} .$$

Data, figures from which the parameters for estimation of the the degree of participation of CVC were obtained.

This threshold pressure is in a range of so called 'micro-pressure' (Macdonald and Fraser, 1999), that is far lower than a hydrostatic pressure that causes disintegration of cytoskeletal filamentous structures (Macdonald, 2001). Finding cellular structures or molecules responsible for sensing this small change in the cytosolic pressure and activating the osmolyte transport mechanism would be interesting and important.

Increasing the external osmolarity

Fig. 6B shows that the volume of 64 mosmol l^{-1}-adapted cells decreased to ~0.90, 0.80 and 0.65 times that of their initial volume after their subjection to 84, 104 and 124 mosmol l^{-1}, respectively. Cell volume, then began to increase, i.e. RVI was activated. The rate and the degree of decrease in cell volume were larger when the degree of increase in the external osmolarity was larger.

Conversely, as is shown in Fig. 6A, the volume of 4 mosmol l^{-1}-adapted cells decreased to ~0.80 and 0.75 times that of their initial volume after their subjection to 44 and 64 mosmol l^{-1}, respectively. Even though the rate and the degree of decrease in cell volume were comparable with or even larger than those for 64 msomol l^{-1}-adapted cells after their subjection to an increased osmolariy (Fig. 6B), RVI was not activated. These results imply that neither the rate nor the degree of decrease in cell volume is the primary factor for activation of RVI. These results also exclude the possibility

that RVI is activated when cytosolic concentration of a certain chemical(s) reaches a threshold value as cell volume decreases.

As was previously mentioned, RVI is activated when the external osmolarity is increased beyond a critical osmolarity. This increase in the external osmolarity causes a decrease in cell volume to an extent that is smaller than the natural cell volume, i.e. an osmotic shrinkage of the cell, since the external osmolarity exceeds the cytosolic osmolarity.

Conversely, when an increase in the external osmolarity takes place within an osmolarity range where no critical osmolarity is included, no osmotic shrinkage occurs, although a decrease in cell volume takes place, since the external osmolarity never exceeds the cytosolic osmolarity. It can, therefore, be said that a slackening of the cytoskeleton-associated membrane surrounding the cell by an osmotic shrinkage of the cell, or a cytosolic pressure that is generated as the cell shrinks osmotically and antagonizes the osmotic pressure difference between the cytosol and the external solution is a plausible factor for activation of RVI. As a matter of fact, an indentation on the cell surface, i.e. osmotic shrinkage of the cell, was always observed when the external osmolarity was increased beyond a critical osmolarity and RVI took place (Fig. 4D, a white arrowhead).

Factors that deactivate the osmolyte transport mechanisms

The fact that the cytosolic osmolarity of *Paramecium* changes stepwise as the adaptation osmolarity changes (Stock et al., 2001) implies that the hypothetical osmolyte transport mechanism responsible for RVD remains active until the cytosolic osmolarity decreases to the next lower step level. Several hours are needed for this osmotic adaptation of the cell (Ishida et al., 1996). Conversely, only 20–80 min were needed for restoration of cell volume (Fig. 2A,C,D) This implies that the cytosolic pressure decreases below the threshold for activation of the transport mechanism in this period of time, while the mechanism remains active. It can, therefore, be said that deactivation (or inactivation) of the transport mechanism is most likely to be cytosolic osmolarity dependent.

Similarly, the hypothetical osmolyte transport mechanism responsible for RVI remains active until the cytosolic osmolarity increases to the next higher level. Several hours are needed for this osmotic adaptation of the cell (Ishida et al., 1996). Conversely, only 40–100 min were needed for restoration of cell volume (Fig. 3A,C,D). This implies that the cell recovers from its shrinkage in this period of time, while the transport mechanism remains active. It can, therefore, be said that deactivation (or inactivation) of the transport mechanism is most likely to be cytosolic osmolarity dependent.

K^+ channels are involved in the regulatory volume control mechanisms

Neither RVD nor RVI took place in the presence of 10 mmol l^{-1} TEA^+ (Fig. 7A,C). Furthermore, an increase in KCl concentration in the external solution to 30 mmol l^{-1} inhibited RVD, so that cell volume continued to increase after a cell's subjection to a decreased osmolarity (Fig. 7B). By contrast, the presence of 30 mmol l^{-1} KCl in the external solution enhanced RVI, so that recovery of cell volume from its temporary shrinkage caused by the cell's subjection to an increased osmolarity took place faster than that in the presence of normal 2 mmol l^{-1} KCl (Fig. 7D). These results strongly support the idea that K^+ channels in the plasma membrane are involved in cell volume control mechanisms in *Paramecium* cells. Involvement of several kinds of K^+ channels in regulatory cell volume control has been demonstrated in several kinds of cells (Montrose-Rafizadeh and Guggino, 1990; Tang et al., 2004).

How much does the contractile vacuole complex participate in the control of cell volume?

It is well known that the rate of fluid discharge from the CV, $\dot{R}_{CVC}$ (Naitoh et al., 1997a), increases when the external osmolarity decreases, while it decreases when the external osmolarity increases (Kitching, 1967). Conversely, the volume of an adapted cell remains unchanged even though the CV discharges its fluid content to the cell exterior. This implies that an amount of fluid discharged from the CV (fluid outflow) is somehow supplied to the cell (fluid inflow). If this fluid outflow–inflow balance has been kept during changes in the external osmolarity, a change in $\dot{R}_{CVC}$ will have no affect on an osmotic change in cell volume. However, if a change in the external osmolarity affects only the outflow, a change in $\dot{R}_{CVC}$ will have an affect on the osmotic change in cell volume. That is, an osmotic swelling of the cell upon decreasing the external osmolarity is buffered by an increase in $\dot{R}_{CVC}$, while an osmotic shrinkage of the cell upon increasing the external osmolarity is buffered by a decrease in $\dot{R}_{CVC}$, since the inflow does not change.

We estimated how much an osmotic change in cell volume is buffered by a change in $\dot{R}_{CVC}$ by using data shown in Figs 2, 3 and 8. The basic assumption for this estimation is that the fluid inflow remains unchanged until cell volume reaches its peak or plateau value after changing the external osmolarity. As is shown in Table 2, when the cells were subjected to a 60 mosmol l^{-1} decrease in the external osmolarity (from 164 to 104 mosmol l^{-1} or from 144 to 84 mosmol l^{-1}), ~50% (–67% and –43%) of the water that entered the cell osmotically was discharged through the CV until the cell volume reached its highest value. Conversely, when the cells were subjected to a 60 mosmol l^{-1} increase in the external osmolarity (from 104 to 164 mosmol l^{-1} or from 84 to 144 mosmol l^{-1}), ~20% (–27% and –18%) of the osmotically expelled water was compensated for by the CVC until the cell volume reached its lowest value. It is, therefore, conceivable that an immediate change in $\dot{R}_{CVC}$ after changing the external osmolarity buffers a large initial osmotic change in cell volume, so that the cell will not be subjected to mechanical disruption. In their physiological studies on the contractile vacuole function of *Tetrahymena pyriformis*, Dunham and his colleagues had earlier suggested a role for the CV in buffering the osmotic changes in cell volume (Stoner and Dunham, 1970; Dunham and Kropp, 1973, see also Patterson, 1980).

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References

Akita, Y. K. (1941). Electrolytes in *Paramecium*. *Memoir. Fac. Sci. Agric. Taihoku Imp. Univ*. **13**, 99-120.

Allen, R. D. and Fok, A. K. (1988). Membrane dynamics of the contractile vacuole complex of *Paramecium*. *J. Protozool.* **35**, 63-71.

Allen, R. D. and Naitoh, Y. (2002). Osmoregulation and contractile vacuoles of protozoa. *Int. Rev. Cytol.* **215**, 352-394.

Baumgarten, C. M. and Feher, J. J. (2001). Osmosis and regulation of cell volume. In *Cell Physiology Source Book, A Molecular Approach*, 3rd edn (ed. N. Sperelakis), pp. 319-355. San Diego, San Francisco, New York, Boston, Sydney, Tokyo: Academic.

Dunham, P. B. and Kropp, D. L. (1973). Regulation of solute and water in *Tetrahymena*. In *Biology of* Tetrahymena (ed. A. M. Elliot), pp. 165-198. Dowden, Stroudsburg, PA, USA: Dowden, Hutchinson & Ross.

Fok, A. K. and Allen, R. D. (1979). Axenic *Paramecium caudatum*. I. Mass culture and structure. *J. Protozool*. **26**, 463-470.

Hoffmann, E. K. and Dunham, P. B. (1995). Membrane mechanisms and intracellular signalling in cell volume regulation. *Int. Rev. Cytol.* **161**, 173-262.

Ishida, M., Aihara, M. S., Allen, R. D. and Fok, A. K. (1993). Osmoregulation in *Paramecium*: the locus of fluid segregation in the contractile vacuole complex. *J. Cell Sci*. **106**, 693-702.

Ishida, M., Fok, A. K., Aihara, M. S. and Allen, R. D. (1996). Hyperosmotic stress leads to reversible dissociation of the proton pump-bearing tubules from the contractile vacuole complex in *Paramecium*. *J. Cell Sci*. **109**, 229-237.

Jepps, M. W. (1947). Contribution to the study of the sponges. *Proc. R. Soc. Lond. B Biol. Sci.* **134**, 408-417.

Kitching, J. A. (1967). Contractile vacuoles, ionic regulation and excretion. In *Research in Protozoology*, vol. 1 (ed. T. T. Chen), pp. 307-336. London, UK: Pergamon.

Lang, F., Bush, G. L., Ritter, M., Volkl, H., Waldegger, S., Gulbins, E. and Haussinger, D. (1998). Functional significance of cell volume regulatory mechanisms. *Physiol. Rev.* **78**, 247-306.

Macdonald, A. G. (2001). Effects of high pressure on cellular processes. In *Cell Physiology Source Book, A Molecular Approach* 3rd edn (ed. N. Sperelakis), pp. 1003-1023. San Diego, San Francisco, New York, Boston, London, Sydney, Tokyo: Academic.

Macdonald, A. G. and Fraser, P. J. (1999). The transduction of very small hydrostatic pressures. *Comp. Biochem. Physiol. A*. **122**, 13-36.

Montrose-Rafizadeh, C. and Guggino, W. B. (1990). Cell volume regulation in the nephron. *Annu. Rev. Physiol.* **52**, 761-772.

Naitoh, Y., Tominaga, T., Ishida, M., Fok, A. K., Aihara, M. S. and Allen, R. D. (1997a). How does the contractile vacuole of *Paramecium multimicronucleatum* expel fluid? Modelling the expulsion mechanism. *J. Exp. Biol.* **200**, 713-721.

Naitoh, Y., Tominaga, T. and Allen, R. D. (1997b). The contractile vacuole fluid discharge rate is determined by the vacuole size immediately before the start of discharge in *Paramecium multimicronucleatum*. *J. Exp. Biol.* **200**, 1737-1744.

Patterson, D. J. (1980). Contractile vacuole and associated structures: their organization and function. *Biol. Rev*. **55**, 1-46.

Stock, C., Allen, R. D. and Naitoh, Y. (2001). How external osmolarity affects the activity of the contractile vacuole complex, the cytosolic osmolarity and the water permeability of the plasma membrane in *Paramecium multimicronucleatum*. *J. Exp. Biol*. **204**, 291-304.

Stock, C., Grønlien, H. K., Allen, R. D. and Naitoh, Y. (2002). Osmoregulation in *Paramecium*: *in situ* ion gradients permit water to cascade through the cytosol to the contractile vacuole. *J. Cell Sci*. **115**, 2339-2348.

Stoner, L. C. and Dunham, P. B. (1970). Regulation of cellular osmolarity and volume in *Tetrahymena*. *J. Exp. Biol.* **53**, 391-399.

Strange, K. (1994). *Cellular and Molecular Physiology of Cell Volume Regulation*. Boca Raton, FL, USA: CRC.

Tang, X. D., Santarelli, L. C., Heinemann, S. H. and Hoshi, T. (2004). Metabolic regulation of potassium channels. *Annu. Rev. Physiol.* **66**, 131-159.