

# 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*

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Accepted 28 October 2000; published on WWW 3 January 2001

## Summary

The rate of fluid expulsion,  $R_{CVC}$ , from the contractile vacuole complex (CVC) of *Paramecium multimicronucleatum* was estimated from the volume of the contractile vacuoles (CVs) immediately before the start of fluid discharge and from the time elapsing between discharges. The  $R_{CVC}$  increased when the cell was exposed to a strongly hypotonic solution and decreased in a weakly hypotonic solution. When the cell was exposed to an isotonic or a hypertonic solution,  $R_{CVC}$  fell to zero. The time constant,  $\tau$ , used to describe the change in  $R_{CVC}$  in response to a change in external osmolarity shortened after a short-term exposure to a strongly hypotonic solution and lengthened after a short-term exposure to a less hypotonic solution. A remarkable lengthening of  $\tau$  occurred after a short-term exposure to isotonic or hypertonic solution. Under natural conditions, mechanisms for controlling  $R_{CVC}$  are effective in maintaining the cytosolic osmolarity hypertonic within a narrow concentration range despite changes in the external osmolarity, which is normally hypotonic to the cytosol. Cells exposed to an isotonic or hypertonic solution resumed CV activity when left in the

solution for 12 h. The cytosolic osmolarity was found to increase and to remain hypertonic to the external solution. This will permit cells to continue to acquire water. The increase in the cytosolic osmolarity occurred in a stepwise fashion, rather than linearly, as the external osmolarity increased. That is, the cytosolic osmolarity first remained more-or-less constant at an increased level until the external osmolarity exceeded this level. Thereupon, the cytosolic osmolarity increased to a new higher level in 12 h, so that the cytosol again became hypertonic to the external solution and the cells resumed CV activity. These results imply that the cell needs to maintain water segregation activity even after it has been exposed to an isotonic or hypertonic environment. This supports the idea that the CVC might be involved not only in the elimination of excess cytosolic water but also in the excretion of some metabolic waste substances.

Key words: contractile vacuole complex, fluid segregation activity, cytosolic osmolarity, water permeability of plasma membrane, exocytotic cycle, *Paramecium multimicronucleatum*.

## Introduction

In its normal habitat, a freshwater protozoan is always faced with hypotonic conditions. The external water, therefore, passes osmotically into the cell through the semipermeable plasma membrane. To keep the cytosol hypertonic to the environment and to prevent the cell from bursting, water is continuously expelled from the cell through the contractile vacuole complex (CVC) (Kitching, 1956; Patterson, 1980; Paulin, 1996; Zeuthen, 1992). In *Paramecium multimicronucleatum*, the contractile vacuole (CV), the central exocytotic vesicle of the CVC, swells as cytosolic water, segregated by the radial arms of the CVC (Fok et al., 1995; Ishida et al., 1993; Ishida et al., 1996), flows into the CV (fluid-filling phase; diastole). The CV then rapidly collapses as the fluid is expelled through the CV pore (fluid-discharge phase;

systole) (Hausmann and Allen, 1977; Patterson, 1977). When the cell is exposed to a strongly hypotonic solution, more water enters the cell, and this excess water must be expelled. In contrast, when the cell is exposed to a weakly hypotonic solution, less water enters the cell osmotically, so less will be expelled. When the cell is exposed to an isotonic solution, net osmotic water flow stops and water discharge from the CV also stops temporarily (Kitching, 1956; Patterson, 1980; Paulin, 1996; Zeuthen, 1992).

Previous investigators (Dunham and Kropp, 1973; Ishida et al., 1996) have reported that the cells of *Paramecium multimicronucleatum* and *Tetrahymena pyriformis* resumed their CV activity after they had remained in an isotonic or hypertonic solution for more than 10 h. This indicates that

water once again enters the cell following this period of adaptation. In fact, it was found (Stoner and Dunham, 1970) that in *Tetrahymena pyriformis* the cytosolic osmolarity increased until it became hypertonic to the external osmolarity as this was increased by the addition of either sucrose or sorbitol.

To understand the mechanism by which the activity of the CVC is modified by a change in the external osmolarity, it is first necessary to determine the quantitative relationship between the water expulsion activity of the CVC and changes in external osmolarity. The present study will deal with (i) the time courses of change in the rate of fluid expulsion of the CVC in response to change in the external osmolarity, i.e. the short-term effects of a change in external osmolarity on CVC activity, (ii) the relationship between the rate of fluid expulsion and the osmolarity of the solution to which cells have been adapted, i.e. the long-term effects of changes in the external osmolarity on the CVC activity, (iii) the long-term effects of the external osmolarity on the cytosolic osmolarity and (iv) the long-term effects of the external osmolarity on the water permeability of the plasma membrane.

## Materials and methods

### Cells

Cells of *Paramecium multimicronucleatum* (syngen 2) (Allen and Fok, 1988) were grown in an axenic culture medium at 24 °C (Fok and Allen, 1979) and harvested at the mid-logarithmic growth phase. The cell density was between  $3 \times 10^6$  and  $4 \times 10^6$  cells  $l^{-1}$ . The cells in their culture medium (12 ml) were centrifuged (1000 revs  $min^{-1}$ ; Centra-CL2 centrifuge, Needham Heights, MA, USA) for 25 s to form a loose pellet. The cells were then suspended in a standard saline solution containing (in  $mmol\ l^{-1}$ ) 2.0 KCl, 0.25  $CaCl_2$ , 20 sorbitol and 1.0 Mops-KOH buffer (pH 7.0) and again pelleted by centrifugation. This washing procedure was repeated twice, and the cells were finally suspended in 5 ml of the standard saline solution. The cells were kept in this solution for more than 12 h prior to experimentation. All experiments were performed at room temperature (25–27 °C).

### Experimental solutions

Eleven different experimental solutions were prepared, each with a different sorbitol concentration. The sorbitol was varied in steps of  $20\ mmol\ l^{-1}$  from 0 to  $200\ mmol\ l^{-1}$ . Each solution contained the same inorganic components as those present in the standard saline solution used for the washing and adaptation of the cells (in  $mmol\ l^{-1}$ : 2.0 KCl, 0.25  $CaCl_2$  and  $1.0\ mol\ l^{-1}$  Mops-KOH buffer; pH 7.0). The osmolarities of these solutions, measured by using a freezing-point osmometer (Micro-Osmometer, model 3MO plus, Advanced Instruments, Inc., Norwood MA, USA), ranged from 4 to  $204\ mosmol\ l^{-1}$  in steps of  $20\ mosmol\ l^{-1}$ . The  $24\ mosmol\ l^{-1}$  solution corresponded to the standard saline solution.

## Experimental procedure

### Experimental chamber

An experimental chamber with a volume of approximately  $20\ \mu l$  was constructed on a coverslip (18 mm  $\times$  18 mm) by placing two strips of coverslip (18 mm  $\times$  4 mm) on opposite sides of the larger coverslip to form a trough. The trough was covered by another coverslip (18 mm  $\times$  10 mm) to form a chamber with openings at both ends. To capture cells inside the chamber, it was first filled with a 0.02 % (v/v) poly-L-lysine (Sigma, St Louis, MO, USA) solution. The cell suspension was then introduced into the chamber at one end, while the poly-L-lysine solution was removed from the chamber at the other end by absorption with filter paper. During this process, some cells adhered to the surface of the coverslip within the chamber. These cells were then subjected to experimentation.

### Video-recording of contractile vacuoles

The CVs of cells adhering to the chamber were recorded under Nomarski microscope optics (Leitz  $\times 63$  objective, Leica Mikroskop. u. Sys. GmbH, Wetzlar, Germany) using a video recorder (AG-6300, Panasonic Indust. Co., Secaucus, NJ, USA). A CCD camera (CCD-72, Dae MIT Inc., Michigan City, IN, USA) and a video timer (FOR. A. Japan) completed the apparatus.

### Adaptation and solution exchange

Cells originally adapted to the standard saline solution were next adapted to an experimental solution (solution 1) for more than 12 h. The cells were then washed twice with the same solution by centrifugation and resuspension as described above. These washes were used to eliminate the possibility of contamination produced by the cells during adaptation. The cells were then introduced into the experimental chamber, washed twice with solution 1 and kept in the chamber for 15 min before video-recording the CV for 13–15 min to determine the CV activity of the cell adapted to solution 1. A test solution (solution 2) was then introduced into the chamber, while the CV activity was video-recorded for another 30 min. The solution 2 in the chamber was then changed back to solution 1, and recording of CV activity was continued for a final 15 min. To examine the mechanical effects of solution exchange in the chamber on CV activity, cells adapted to the standard saline solution were subjected to a change to fresh standard saline solution. The mechanical effect of solution exchange on the CV activity was negligible.

### Determination of CVC activity

On the replayed images of the CV, we measured the time between two successive fluid discharges ( $T$ , the period for the fluid discharge cycles) and the maximum diameter of the spherical CV immediately before the start of fluid discharge ( $D_{max}$ ). Only one CV in each cell was measured and evaluated. The number of measurements,  $N$ , therefore, represents both the number of cells and the number of CVs investigated.

The rate of fluid expulsion by the CVC,  $R_{CVC}$ , was calculated by dividing the maximum volume of the contractile

vacuole immediately before the start of fluid discharge by  $T$  ( $\pi D_{\max}^3/6T$ ). The value thus calculated corresponds to the mean rate of fluid expulsion during the fluid-filling phase before fluid discharge. The time constant,  $\tau$ , for the change in  $R_{CVC}$  upon solution exchange was defined as the time from the beginning of solution exchange to the time when  $R_{CVC}$  reached 63% of its new plateau value.

#### Determination of the cytosolic osmolarity

The cytosolic osmolarities of 11 groups of cells, each having been adapted to one of the 11 different experimental solutions, prepared as described above, were determined as described previously (Stoner and Dunham, 1970). However, we used Congo Red and a spectrophotometer instead of [ $^{14}\text{C}$ ]inulin and a scintillation counter.

The cells at late logarithmic or early stationary growth phase, which had been cultured in a 11 Erlenmeyer flask at 24°C in 300 ml of axenic culture medium, were pelleted in a 35 ml conical tube by centrifugation at 1000 revs  $\text{min}^{-1}$  for 1 min (Centra-CL2 centrifuge, Needham Heights, MA, USA). The supernatant was decanted, and 30 ml of one of the 11 experimental solutions was added to the tube to resuspend the cells. The cells were then allowed to adapt to this solution for more than 12 h before washing and resuspension in the same adaptation solution.

A 450  $\mu\text{l}$  sample of the cell suspension and a 50  $\mu\text{l}$  sample of 1  $\text{mmol l}^{-1}$  Congo Red (Sigma, St Louis, MO, USA) dissolved in the same experimental solution were introduced into an Eppendorf tube. The cells were then centrifuged at approximately 11 000 revs  $\text{min}^{-1}$  for 10 s (Microfuge E, Beckman Instruments, Inc., Fullerton, CA, USA) to obtain a cell pellet. We measured the overall osmolarity of the cell pellet ( $Osm_p$ ) using a freezing-point osmometer. A 40  $\mu\text{l}$  sample of the same cell pellet was resuspended in the corresponding experimental solution to give a final volume of 4 ml. This suspension was spun down gently, to ensure that cells would not be broken, and the supernatant was filtered under sterile conditions using a microfilter (pore size 1.2  $\mu\text{m}$ ; Gelman Sciences Inc., Ann Arbor, MI, USA) to obtain a cell-free suspension solution. The concentration of the Congo Red in this filtered suspension solution ( $C_{CR}$ ) was measured using a spectrophotometer (model DU-65, Beckman Instruments, Inc., Fullerton CA, USA).

The volume of a sample of the cell pellet,  $V_p$  can be expressed as:

$$V_p = V_c + V_s, \quad (1)$$

where  $V_c$  is the space in the sample occupied by the cells and  $V_s$  is the rest of the space in the sample, i.e. the space occupied by Congo-Red-containing experimental solution (extracellular space). The ratio ( $R$ ) of the extracellular space ( $V_s$ ) to the volume of the pellet ( $V_p$ ) can be written as:

$$R = \frac{V_s}{V_p} = \frac{V_p - V_c}{V_p}. \quad (2)$$

Equation 2 implies that  $R$  is 1 if the sample contained no cells

( $V_c=0$ ). In this case,  $C_{CR}$  is 1  $\mu\text{mol l}^{-1}$ .  $R$  is 0 if the sample contained only cells ( $V_c=V_p$ ), in which case  $C_{CR}$  would be 0  $\mu\text{mol l}^{-1}$ . We can, therefore, estimate the value of  $R$  from the value of  $C_{CR}$ .

The relationship between  $R$  and the cytosolic osmolarity,  $Osm_c$ , can be formulated as:

$$Osm_c = \frac{Osm_p - Osm_s R}{1 - R}, \quad (3)$$

where  $Osm_p$  is the osmolarity of the pellet and  $Osm_s$  is the osmolarity of the experimental solution, i.e. the extracellular space.

The significance of all data was tested using the Mann-Whitney  $U$ -test ( $P < 0.05$ ). Values are presented as means  $\pm$  S.E.M.

## Results

### Short-term effects of external osmolarity change on the CVC activity as the cells adapt to three different osmolarities

Three groups of *Paramecium* were adapted to 4, 24 or 84  $\text{mosmol l}^{-1}$ , respectively, for 12 h. The cells of each group were then exposed to a test solution with an osmolarity different from that of the adaptation solution. The maximum diameter ( $D_{\max}$ ) and  $T$  were measured before, during and after exposure of the cell to the test solution, and  $R_{CVC}$  was then calculated. The value of  $\tau$  was estimated from the time course of the change in  $R_{CVC}$  upon changing the osmolarity.

### Cells adapted to 4 $\text{mosmol l}^{-1}$

Fig. 1 shows representative results obtained with two different cells (cells 1 and 2) initially adapted to a 4  $\text{mosmol l}^{-1}$  solution. As shown to the left of the vertical broken line for cell 1,  $D_{\max}$  remained almost constant at approximately 13  $\mu\text{m}$  (Fig. 1Bi), while  $T$  was also constant at 10 s (Fig. 1Ci), so that  $R_{CVC}$  was almost constant at approximately 100  $\text{fl s}^{-1}$  (Fig. 1Ai) in the 4  $\text{mosmol l}^{-1}$  adaptation solution. As shown to the right of the vertical broken line for cell 1,  $D_{\max}$  decreased to a lower steady value of approximately 9  $\mu\text{m}$  (Fig. 1Bi), while  $T$  increased slightly to approximately 12 s (Fig. 1Ci) after changing the external solution to a 24  $\text{mosmol l}^{-1}$  solution. The decrease in  $D_{\max}$  together with the slight increase in  $T$  corresponded to a decrease in  $R_{CVC}$  to a lower steady level of approximately 30  $\text{fl s}^{-1}$  (Fig. 1Ai), completed in approximately 300 s.

As shown to the right of the vertical broken line for cell 2, when the cell was re-exposed to a 4  $\text{mosmol l}^{-1}$  solution after its exposure to a 24  $\text{mosmol l}^{-1}$  solution for approximately 30 min,  $D_{\max}$  increased to a higher steady value of approximately 12  $\mu\text{m}$  (Fig. 1Bii), whereas  $T$  decreased to a lower steady value of approximately 7 s (Fig. 1Cii). These changes in  $D_{\max}$  and  $T$  corresponded to an increase in  $R_{CVC}$  to a higher steady value of approximately 114  $\text{fl s}^{-1}$  (Fig. 1Aii), completed in approximately 300 s.

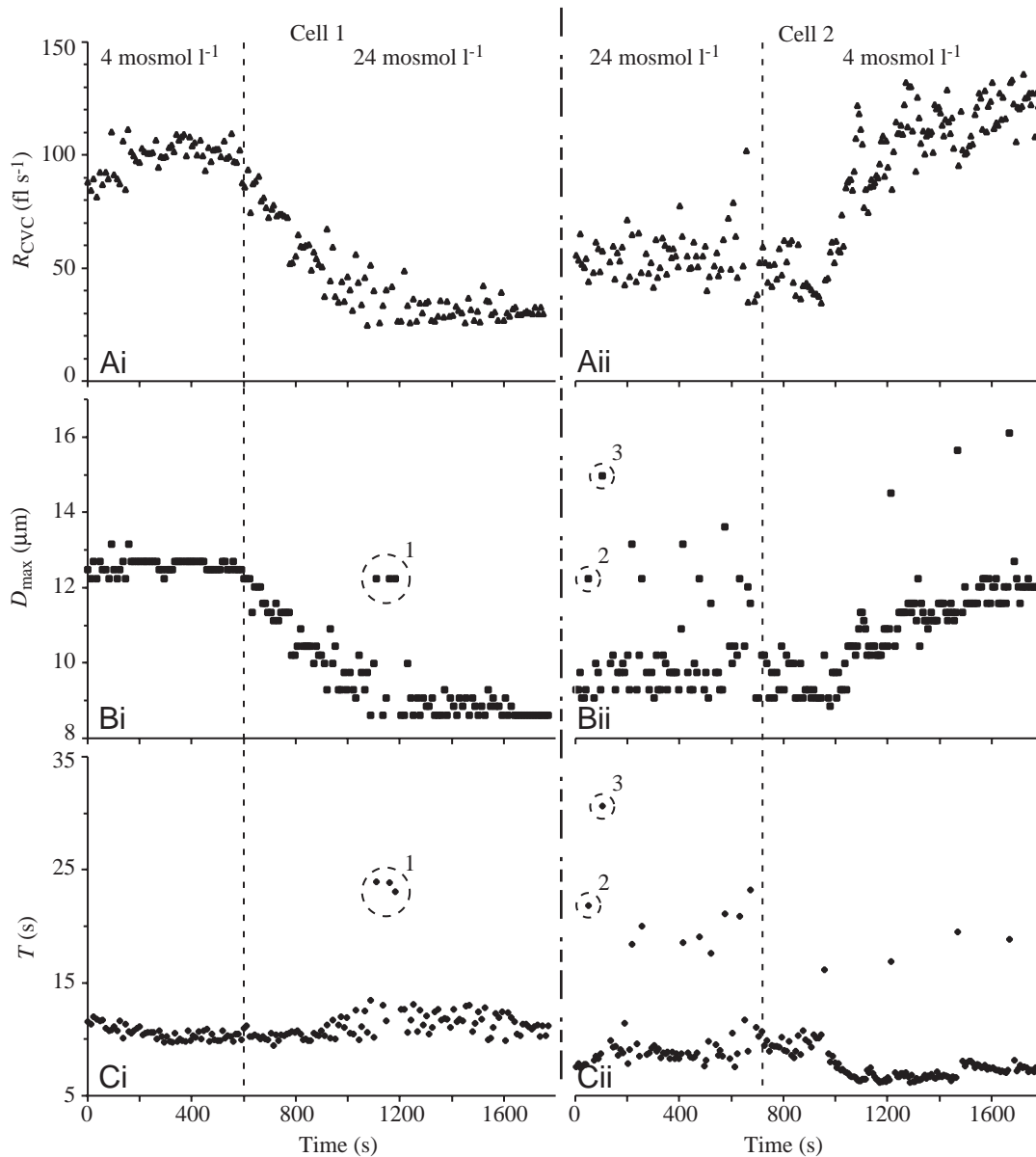


Fig. 1. Activity of the contractile vacuole complex (CVC) in two representative cells (cells 1 and 2) of *Paramecium multimicronucleatum* adapted to 4 mosmol l<sup>-1</sup>. (A) Changes in the rate of fluid expulsion from the CVC,  $R_{CVC}$ ; (B) the maximum diameter of the contractile vacuole immediately before the start of fluid discharge,  $D_{max}$ ; and (C) the time between two successive fluid discharges (the period of the fluid discharge cycle),  $T$ . Cell 1, exposed to a 24 mosmol l<sup>-1</sup> solution at the time indicated by a vertical dotted line. Cell 2, re-exposed to the 4 mosmol l<sup>-1</sup> adaptation solution after its 30 min exposure to a 24 mosmol l<sup>-1</sup> solution at the time indicated by a vertical dotted line. For encircled points, see text.

#### Cells adapted to 24 mosmol l<sup>-1</sup>

Fig. 2 shows representative results obtained with four different cells (1, 2, 3 and 4) initially adapted to a 24 mosmol l<sup>-1</sup> solution. As shown to the left of the vertical broken line for cell 1,  $D_{max}$  was almost constant at approximately 9 μm (Fig. 2Bi), while  $T$  was also constant at 7 s (Fig. 2Ci) in the adaptation solution (see also Fig. 2, cell 3), so that  $R_{CVC}$  was constant at approximately 67 fl s<sup>-1</sup> (Fig. 2Ai). When the cell was exposed to a 4 mosmol l<sup>-1</sup> solution (to the right of the vertical broken line),  $D_{max}$

increased to a steady value of approximately 11 μm (Fig. 2Bii), while  $T$  remained unchanged at approximately 7 s (Fig. 2Cii). This increase in  $D_{max}$  corresponded to an increase in  $R_{CVC}$  to approximately 107 fl s<sup>-1</sup> (Fig. 2Aii), completed in approximately 300 s.

As shown for cell 2,  $D_{max}$  decreased to a lower steady value of approximately 9 μm (Fig. 2Bii), whereas  $T$  increased to a slightly higher steady value of approximately 8 s (Fig. 2Cii), when the cell was re-exposed to the 24 mosmol l<sup>-1</sup> solution after its prior exposure for approximately 30 min to a

4 mosmol l<sup>-1</sup> solution. These changes in  $D_{\max}$  and  $T$  corresponded to a decrease in  $R_{\text{CVC}}$  to a lower steady value of approximately 50 fl s<sup>-1</sup> (Fig. 2Aii), completed in approximately 100 s.

The values plotted to the left of the vertical broken line for cell 3 show that  $D_{\max}$  was almost constant at approximately 10  $\mu\text{m}$  (Fig. 2Biii) and  $T$  was also constant at 7 s (Fig. 2Ciii), so that  $R_{\text{CVC}}$  was approximately 70 fl s<sup>-1</sup> (Fig. 2Aiii) in the

adaptation solution. When the cell was exposed to an 84 mosmol l<sup>-1</sup> test solution,  $D_{\max}$  decreased to approximately 6  $\mu\text{m}$  (Fig. 2Biii), while  $T$  increased to more than 7 min (Fig. 2Ciii). These changes in  $D_{\max}$  and  $T$  corresponded to a decrease in  $R_{\text{CVC}}$  to less than 1 fl s<sup>-1</sup> (Fig. 2Aiii), completed in approximately 300 s.

As shown to the right of the vertical broken line for cell 4, when a cell first adapted to a 24 mosmol l<sup>-1</sup> solution and then

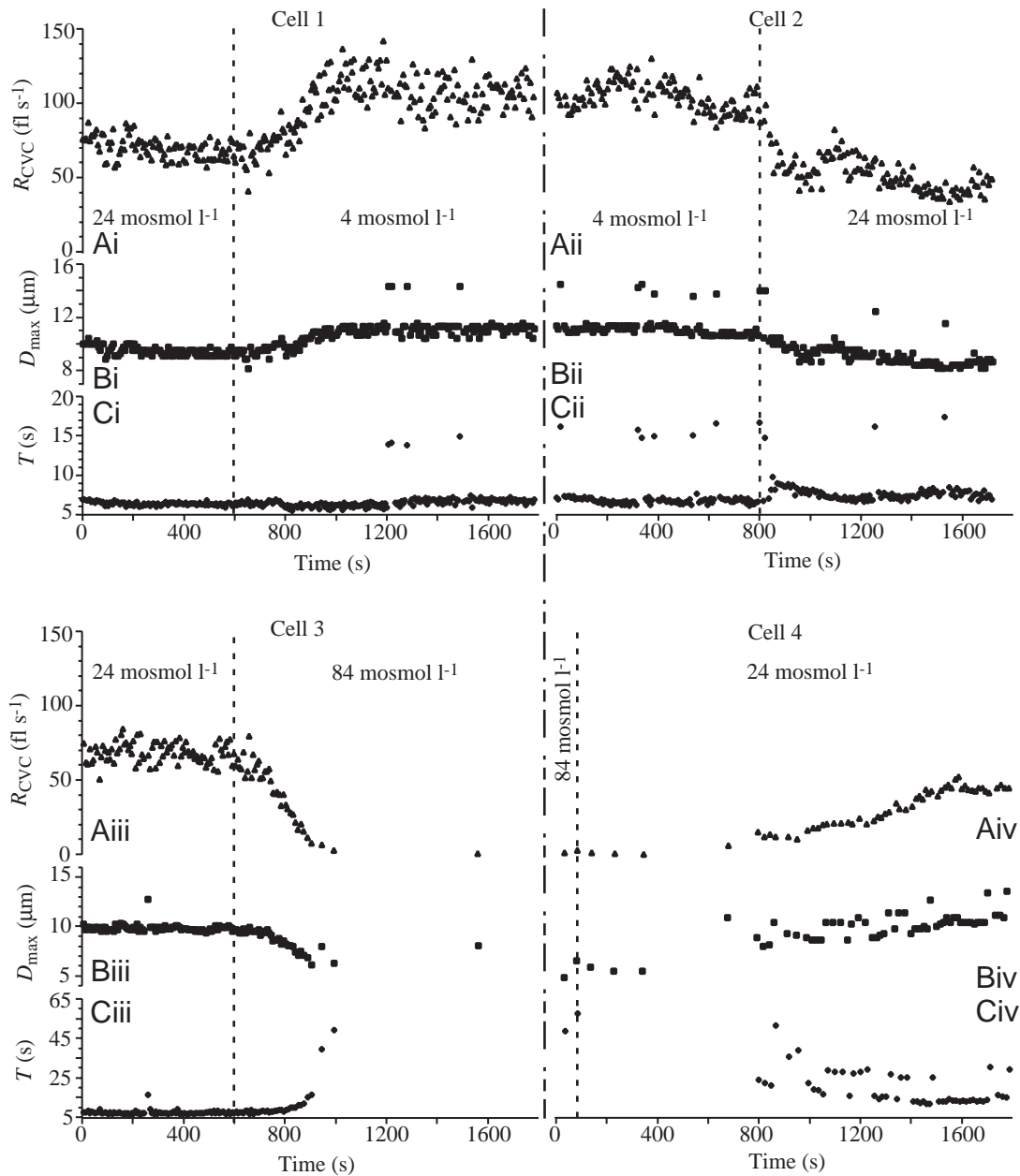


Fig. 2. Activity of the contractile vacuole complex (CVC) in four representative cells (cells 1–4) of *Paramecium multimicronucleatum* adapted to 24 mosmol l<sup>-1</sup>. (A) Changes in the rate of fluid expulsion from the CVC,  $R_{\text{CVC}}$ ; (B) the maximum diameter of the contractile vacuole immediately before the start of fluid discharge,  $D_{\max}$ ; and (C) the time between two successive fluid discharges (the period of the fluid discharge cycle),  $T$ . Cell 1, exposed to a 4 mosmol l<sup>-1</sup> solution. Cell 2, re-exposed to the 24 mosmol l<sup>-1</sup> adaptation solution after a prior 30 min exposure to a 4 mosmol l<sup>-1</sup> solution. Cell 3, exposed to an 84 mosmol l<sup>-1</sup> solution. Cell 4, re-exposed to the 24 mosmol l<sup>-1</sup> adaptation solution after a prior 30 min exposure to an 84 mosmol l<sup>-1</sup> solution. The times at which the cells were exposed to different solutions are indicated by vertical dotted lines.



exposed to an 84 mosmol<sup>-1</sup> solution for approximately 30 min was subsequently re-exposed to a 24 mosmol<sup>-1</sup> solution,  $D_{\max}$  slowly increased to a steady value of approximately 11  $\mu\text{m}$  (Fig. 2Biv), while  $T$  decreased to a steady value of approximately 15 s (Fig. 2Civ). These changes in  $D_{\max}$  and  $T$  corresponded to an increase in  $R_{\text{CVC}}$  from its very low value of approximately 1 fl s<sup>-1</sup> in an 84 mosmol<sup>-1</sup> solution to a higher value of approximately 50 fl s<sup>-1</sup> (Fig. 2Aiv), completed in approximately 1200 s.

#### Cells adapted to 84 mosmol<sup>-1</sup>

Fig. 3 shows representative results obtained with three different cells (1, 2 and 3) initially adapted to an 84 mosmol<sup>-1</sup> solution. As shown to the left of the vertical broken line for cell 1, both  $D_{\max}$  and  $T$  were almost constant with approximate values of 10  $\mu\text{m}$  for  $D_{\max}$  (Fig. 3Bi) and 10 s for  $T$  (Fig. 3Ci), so that  $R_{\text{CVC}}$  was also constant at approximately 50 fl s<sup>-1</sup> (Fig. 3Ai) in the adaptation solution. As shown to the right of the vertical broken line, when this cell was exposed to a 144 mosmol<sup>-1</sup> solution,  $D_{\max}$  decreased to a value of

approximately 7  $\mu\text{m}$ , while  $T$  increased to be more than 400 s (not shown on graph). These changes in  $D_{\max}$  and  $T$  corresponded to a decrease in  $R_{\text{CVC}}$  to a value of approximately 0.6 fl s<sup>-1</sup>, completed in approximately 180 s.

As shown to the left of the vertical broken line for cell 2, when a cell adapted to an 84 mosmol<sup>-1</sup> solution and exposed to a 144 mosmol<sup>-1</sup> solution for approximately 30 min was re-exposed to an 84 mosmol<sup>-1</sup> solution,  $D_{\max}$  slowly increased to a steady value of approximately 9  $\mu\text{m}$  (Fig. 3Bii), while  $T$  decreased to a steady value of approximately 18 s (Fig. 3Cii). These changes in  $D_{\max}$  and  $T$  corresponded to an increase in  $R_{\text{CVC}}$  from its very low value of approximately 0.6 fl s<sup>-1</sup> in the 144 mosmol<sup>-1</sup> solution to a higher value of approximately 15 fl s<sup>-1</sup> (Fig. 3Aii), which was achieved in approximately 15 min.

As shown to the right of the vertical broken line for cell 3, when a cell adapted to an 84 mosmol<sup>-1</sup> solution was exposed to a 24 mosmol<sup>-1</sup> solution,  $D_{\max}$  increased from its steady value of approximately 11  $\mu\text{m}$  to a higher steady value of approximately 12  $\mu\text{m}$  (Fig. 3Biii), while  $T$  remained almost

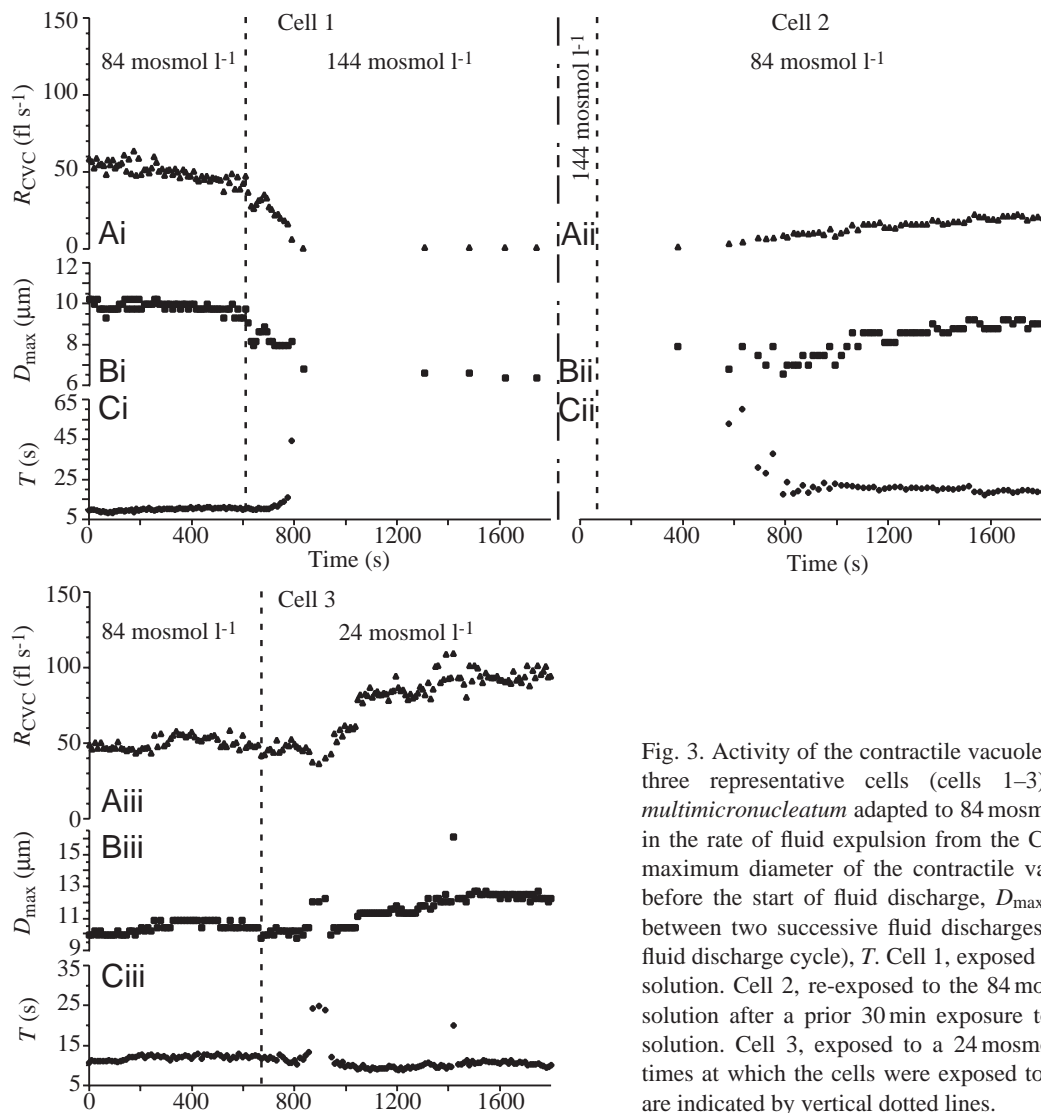


Fig. 3. Activity of the contractile vacuole complex (CVC) in three representative cells (cells 1–3) of *Paramecium multimicronucleatum* adapted to 84 mosmol<sup>-1</sup>. (A) Changes in the rate of fluid expulsion from the CVC,  $R_{\text{CVC}}$ ; (B) the maximum diameter of the contractile vacuole immediately before the start of fluid discharge,  $D_{\max}$ ; and (C) the time between two successive fluid discharges (the period of the fluid discharge cycle),  $T$ . Cell 1, exposed to a 144 mosmol<sup>-1</sup> solution. Cell 2, re-exposed to the 84 mosmol<sup>-1</sup> adaptation solution after a prior 30 min exposure to a 144 mosmol<sup>-1</sup> solution. Cell 3, exposed to a 24 mosmol<sup>-1</sup> solution. The times at which the cells were exposed to different solutions are indicated by vertical dotted lines.

constant at approximately 12 s (Fig. 3Ciii). This change in  $D_{\max}$  corresponded to an increase in  $R_{\text{CVC}}$  from its steady value of approximately  $50 \text{ fl s}^{-1}$  in the adaptation solution to a higher steady value of approximately  $92 \text{ fl s}^{-1}$  (Fig. 3Aiii), completed in approximately 480 s.

The mean values for  $D_{\max}$ ,  $T$ ,  $R_{\text{CVC}}$  and  $\tau$  obtained from the above set of experiments are summarized in Table 1.

*Sporadic discrete increases in  $D_{\max}$  and  $T$*

It was sometimes observed that  $D_{\max}$  increased abnormally in association with an abnormal increase in  $T$ . These sporadic extra increases in  $D_{\max}$  and  $T$  were observed most frequently after the external solution had been changed, as can be seen in Figs 1–3. Three representative pairs of  $D_{\max}$  and  $T$  are shown in Fig. 1 as pairs of plots surrounded by broken circles.

In the pairs of  $D_{\max}$  and  $T$  values labelled 1 and 2,  $T$  increased by a value twice as large as that for the preceding normal discharge cycles, while  $D_{\max}$  took a value that was approximately 1.26 times as large as that for normal discharge cycles. In the pair labelled 3,  $T$  increased by a value three times as large as that for normal discharge cycles, while  $D_{\max}$  took a value that was approximately 1.44 times as large as that for the normal discharge cycles. The rate of fluid expulsion,  $R_{\text{CVC}}$ ,

therefore, remained unchanged at the previous value for the normal discharge cycles in spite of these dramatic changes in  $D_{\max}$  and  $T$ .

*Long-term effects of a change in the external osmolarity on the CVC activity*

When a cell adapted to  $4 \text{ mosmol l}^{-1}$  was exposed to an  $84 \text{ mosmol l}^{-1}$  solution, it ceased its CV activity, so that the CV became invisible ( $D_{\max}$  and  $R_{\text{CVC}}$  were regarded as zero). Similarly, in a cell adapted to  $24 \text{ mosmol l}^{-1}$  exposed to an  $84 \text{ mosmol l}^{-1}$  solution or a cell adapted to  $84 \text{ mosmol l}^{-1}$  exposed to a  $144 \text{ mosmol l}^{-1}$  solution, the CV became invisible as the CV activity nearly stopped (Fig. 2, cell 3; Fig. 3, cell 1). However, the cells resumed their CV activity after adaptation for 12 h to solutions with osmolarities higher than  $84 \text{ mosmol l}^{-1}$ .

To examine the long-term effects of external osmolarity on CV activity, 11 groups of cells were adapted to 11 different solutions of different osmolarities varying from 4 to  $204 \text{ mosmol l}^{-1}$ , in steps of  $20 \text{ mosmol l}^{-1}$ , for more than 12 h. No differences in morphology or locomotory activity were observed between the cells adapted to different solutions. Values for  $D_{\max}$  and  $T$  were then determined, and  $R_{\text{CVC}}$  was

Table 1. Activity of the contractile vacuole complex of *Paramecium multimicronucleatum* cells adapted to different osmotic solutions

| A                                       | Osmolarity ( $\text{mosmol l}^{-1}$ ) |           | 4                    | ⇒          | 24                    | ⇒          | 4               |             |               |
|---|---------------------------------------|-----------|----------------------|------------|-----------------------|------------|-----------------|-------------|---------------|
| $D_{\max}$ ( $\mu\text{m}$ )            |                                       |           | <b>11.5±2.3 (5)</b>  |            | 10.7±2.1 (10)         |            | 12.5±0.6 (5)    |             |               |
| $T$ (s)                                 |                                       |           | <b>11.0±1.6 (5)</b>  |            | 16.9±6.7 (10)         |            | 10.7±3.4 (5)    |             |               |
| $R_{\text{CVC}}$ ( $\text{fl s}^{-1}$ ) |                                       |           | <b>98.2±50.3 (5)</b> |            | 46.9±22.6 (10)        |            | 104.5±23.0 (5)  |             |               |
| $\tau$ (s)                              |                                       |           |                      |            | 302±99 (5)            |            | 335±73 (5)      |             |               |
| B                                       | 24                                    | ⇐         | 4                    | ⇐          | <b>24</b>             | ⇒          | 84              | ⇒           | 24            |
| $D_{\max}$ ( $\mu\text{m}$ )            | 11.8±1.6 (4)                          |           | 12.6±1.4 (10)        |            | <b>11.1±1.9 (10)</b>  |            | 7.3±1.6 (6)     |             | 10.8±1.6 (3)  |
| $T$ (s)                                 | 8.9±1.8 (4)                           |           | 10.2±3.6 (10)        |            | <b>10.9±4.5 (10)</b>  |            | 144.4±50.5 (6)  |             | 14.0±3.3 (3)  |
| $R_{\text{CVC}}$ ( $\text{fl s}^{-1}$ ) | 89.3±26.8 (4)                         |           | 117.4±42.4 (10)      |            | <b>69.9±14.7 (10)</b> |            | 1.9±0.9 (6)     |             | 48.8±14.5 (3) |
| $\tau$ (s)                              |                                       | 94±62 (4) |                      | 348±56 (5) |                       | 228±31 (5) |                 | 867±210 (3) |               |
| C                                       | Osmolarity ( $\text{mosmol l}^{-1}$ ) |           | 24                   | ⇐          | <b>84</b>             | ⇒          | 144             | ⇒           | 84            |
| $D_{\max}$ ( $\mu\text{m}$ )            |                                       |           | 12.6±0.8 (4)         |            | <b>10.8±1.0 (8)</b>   |            | 8.4±1.5 (8)     |             | 8.5±0.4 (3)   |
| $T$ (s)                                 |                                       |           | 13.8±4.3 (4)         |            | <b>15.1±4.0 (8)</b>   |            | 597.1±292.7 (8) |             | 32.3±10.6 (3) |
| $R_{\text{CVC}}$ ( $\text{fl s}^{-1}$ ) |                                       |           | 82.4±19.3 (4)        |            | <b>47.1±14.6 (8)</b>  |            | 0.7±0.2 (8)     |             | 12±6.3 (3)    |
| $\tau$ (s)                              |                                       |           |                      |            | 390±94 (4)            |            | 142±43 (5)      |             | 785±105 (3)   |

Cells were first adapted to different osmolarities, 4 (A), 24 (B) and 84 (C)  $\text{mosmol l}^{-1}$ , respectively for 12 h (bold type), exposed to a second solution, different from each adaptation solution, for approximately 30 min and then re-exposed to the original adaptation solution.

The activity variables measured are  $D_{\max}$ , the maximum diameter of the contractile vacuole immediately before its fluid discharge;  $T$ , the time needed for fluid filling of the contractile vacuole before the corresponding fluid discharge;  $R_{\text{CVC}}$ , the rate of fluid expulsion from the contractile vacuole complex calculated from  $D_{\max}$  and  $T$ , and;  $\tau$ , the time constant for the change in  $R_{\text{CVC}}$  following the change in the external osmolarity.

Arrows indicate the sequence of exposure of the cells to different solutions. The data presented in bold type were obtained from the 12-h-adapted cells.

Each value is a mean ± S.E.M.

Numbers in parentheses are numbers of measurements of different contractile vacuoles, one from each cell.

calculated for the cells of each group. The results are shown in Fig. 4 (Fig. 4A,  $R_{CVC}$ ; Fig. 4B,  $D_{max}$ ; Fig. 4C,  $T$ ).

As shown in Fig. 4A,  $R_{CVC}$  decreased linearly to a value of approximately  $20 \text{ fl s}^{-1}$  as the osmolarity of the adaptation solution increased from 4 to  $64 \text{ mosmol l}^{-1}$ . When the external osmolarity was increased further to  $84 \text{ mosmol l}^{-1}$ ,  $R_{CVC}$  increased significantly ( $P < 0.05$ ) to a value of approximately  $45 \text{ fl s}^{-1}$ . Values for  $R_{CVC}$  then decreased

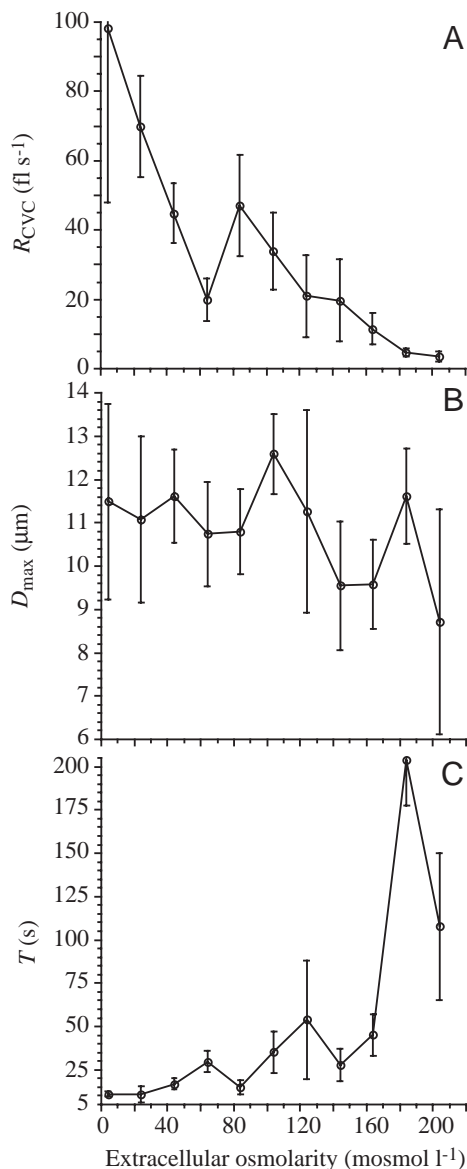


Fig. 4. Effects of 12 h adaptations to a series of external osmolarities, from 4 to  $204 \text{ mosmol l}^{-1}$  in steps of  $20 \text{ mosmol l}^{-1}$ , on the contractile vacuole complex (CVC) activity of *Paramecium multimicronucleatum*. (A) Changes in the rate of fluid expulsion from the CVC,  $R_{CVC}$ ; (B) the maximum diameter of the contractile vacuole (CV) immediately before the start of its fluid discharge,  $D_{max}$ ; and (C) the time between two successive fluid discharges (the period of the fluid discharge cycle),  $T$ . The error bars are the S.E.M. for 5–10 measurements on different contractile vacuoles in different cells.

linearly again to a value of approximately  $20 \text{ fl s}^{-1}$  when the osmolarity was increased to  $124 \text{ mosmol l}^{-1}$ . The  $R_{CVC}$  value then remained unchanged when the osmolarity was increased to  $144 \text{ mosmol l}^{-1}$ . Further increases in the osmolarity to  $204 \text{ mosmol l}^{-1}$  led to a decrease in  $R_{CVC}$ .

As shown in Fig. 4B,  $D_{max}$  was more-or-less constant at approximately  $11 \mu\text{m}$  regardless of the change in the osmolarity of the adaptation solution. No significant differences ( $P > 0.05$ ) in the values for  $D_{max}$  were found from 4 to  $184 \text{ mosmol l}^{-1}$  solutions. In contrast, the value for  $T$  was significantly ( $P < 0.05$ ) higher in cells adapted to  $64 \text{ mosmol l}^{-1}$  than in those adapted to  $44 \text{ mosmol l}^{-1}$  (Fig. 4C), and it was significantly ( $P < 0.05$ ) lower in cells adapted to  $84 \text{ mosmol l}^{-1}$  than in those adapted to  $64 \text{ mosmol l}^{-1}$ . Changes in  $R_{CVC}$  as a function of the external osmolarity (Fig. 4A) were, therefore, mostly caused by changes in  $T$  as the external osmolarity increased (Fig. 4C).

#### Determination of the cytosolic osmolarity

We determined the cytosolic osmolarity of 11 groups of cells adapted to 11 different solutions, each with a different osmolarity, using the same series of osmolarities as that employed in the previous experiment. As shown in Fig. 5, the cytosolic osmolarity was almost the same, approximately  $70 \text{ mosmol l}^{-1}$  ( $70.4 \pm 3.6 \text{ mosmol l}^{-1}$ ;  $N=5$ ), among the four groups of cells that had been adapted to 4, 24, 44 and  $64 \text{ mosmol l}^{-1}$ , respectively. The cytosolic osmolarity increased significantly ( $P < 0.05$ ) to a value of approximately  $170 \text{ mosmol l}^{-1}$  ( $173.4 \pm 51.5 \text{ mosmol l}^{-1}$ ;  $N=10$ ) when the osmolarity of the adaptation solution was increased to  $84 \text{ mosmol l}^{-1}$  and remained at this value in the four groups of cells adapted to solutions with osmolarities ranging between 84 and  $144 \text{ mosmol l}^{-1}$ . When the osmolarity of the adaptation solution increased to more than  $144 \text{ mosmol l}^{-1}$  (164, 184,  $204 \text{ mosmol l}^{-1}$ ), the cytosolic osmolarity increased significantly ( $P < 0.05$ ) again to approximately  $250 \text{ mosmol l}^{-1}$  ( $237.4 \pm 15.9 \text{ mosmol l}^{-1}$ ;  $N=5$ ).

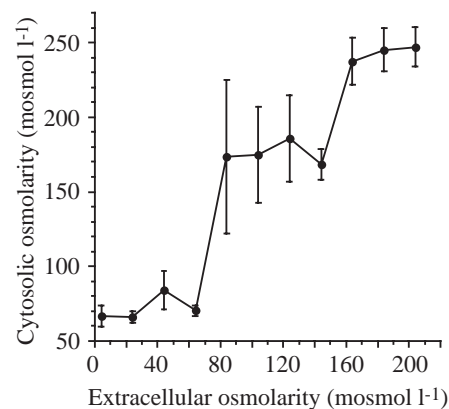


Fig. 5. Change in the cytosolic osmolarity of cells of *Paramecium multimicronucleatum* as a function of the osmolarity of their adaptation solution. Values are means  $\pm$  S.E.M. for measurements using 6–10 different cell cultures.



## Discussion

### Paramecium multimicronucleatum osmoregulates by changing the rate of fluid expulsion

The cytosol of the freshwater protozoan *P. multimicronucleatum* is hypertonic to the normal environment to which it is exposed, such as pond water or culture medium. External water can, therefore, enter the cell continuously across the plasma membrane down the concentration gradient of water as long as the plasma membrane remains permeable to water. To regulate its cytosolic osmolarity at a hypertonic level, *P. multimicronucleatum* expels the excess cytosolic water through its CVC.

When a *P. multimicronucleatum* adapted to 4 mosmol<sup>-1</sup> was exposed to the higher osmolarity of 24 mosmol<sup>-1</sup>, which was still lower than the cytosolic osmolarity (approximately 70 mosmol<sup>-1</sup>; see Fig. 5), the rate of water expulsion from the CVC,  $R_{CVC}$ , decreased markedly (Fig. 1Ai). In contrast, when the cell was re-exposed to 4 mosmol<sup>-1</sup> after its exposure to 24 mosmol<sup>-1</sup> for a short period (approximately 30 min),  $R_{CVC}$  increased to the value previously measured in the 4 mosmol<sup>-1</sup> adaptation solution (Fig. 1Aii).

When a *P. multimicronucleatum* adapted to 24 mosmol<sup>-1</sup> was exposed to a lower osmolarity, such as 4 mosmol<sup>-1</sup>,  $R_{CVC}$  markedly increased (Fig. 2Ai). In contrast, when the cell was re-exposed to 24 mosmol<sup>-1</sup> after short-term exposure to 4 mosmol<sup>-1</sup>,  $R_{CVC}$  decreased to its previous lower value in the 24 mosmol<sup>-1</sup> solution (Fig. 2Aii). Moreover, when a *P. multimicronucleatum* adapted to 24 mosmol<sup>-1</sup> was exposed to 84 mosmol<sup>-1</sup>, which was higher than the cytosolic osmolarity,  $R_{CVC}$  decreased to almost zero, i.e. water expulsion from the CVC essentially stopped (Fig. 2Aiii).

These results clearly show that the CVC changes its fluid expulsion rate according to the osmotic entry of water into the cell, i.e. the rate was higher when the potential for water entry was higher. The use of the CVC to control the fluid expulsion rate is effective in keeping the cytosolic osmolarity constant at a level higher than the external osmolarity in a range from less than 4 mosmol<sup>-1</sup> to that of the cytosol (approximately 70 mosmol<sup>-1</sup>).

### The relationships between $R_{CVC}$ , $D_{max}$ and $T$

As described above,  $R_{CVC}$  was modified by exposing the cell for short periods (approximately 30 min) to an osmolarity different from that of its adaptation solution. In this process, changes in  $R_{CVC}$  led to the changes in  $D_{max}$  and  $T$ . Our observations are consistent with classical observations (Kitching, 1956; Patterson and Sleight, 1976; see Kitching, 1967; Dunham and Kropp, 1973; Patterson, 1980). We found that, when a cell adapted to 4 mosmol<sup>-1</sup> was exposed to 24 mosmol<sup>-1</sup>,  $D_{max}$  decreased while  $T$  stayed almost constant. The result was a decrease in  $R_{CVC}$  (Fig. 1, cell 1). In contrast, when a cell adapted to 4 mosmol<sup>-1</sup> was re-exposed to 4 mosmol<sup>-1</sup> after a short-term exposure to 24 mosmol<sup>-1</sup>,  $T$  decreased while  $D_{max}$  increased only slightly and  $R_{CVC}$  consequently increased (Fig. 1, cell 2). Which variable is

predominantly affected by the change in  $R_{CVC}$  remains unpredictable.

In cells adapted to different osmolarities for 12 h,  $D_{max}$  remained almost the same at approximately 11.5  $\mu\text{m}$  irrespective of the osmolarity (Fig. 4B), while  $R_{CVC}$  differed according to the osmolarity (Fig. 4A). It can, therefore, be said that  $T$  is affected by the change in  $R_{CVC}$  caused by a change in the osmolarity of the adaptation solution. In fact, the plots of  $T$  against the osmolarity of the adaptation solution are roughly a mirror image of the plots of  $R_{CVC}$  against the osmolarity (compare Fig. 4C with Fig. 4A).

In some cases, a doubling or tripling of  $T$  was observed after the external osmolarity had been changed (see the data surrounded by broken circles in Fig. 1 as well as uncircled examples in Figs 1–3). The maximum volume of the CV calculated from  $D_{max}$  in cases where  $T$  was two or three times its normal length, was two or three times as large as it was in normal expulsion cycles, indicating that  $R_{CVC}$  remained unchanged when  $T$  doubled or tripled. This suggests that the water expulsion activity in the radial arms is independent of the timing of the fluid discharge of the CV. This is consistent with a previous finding (Naitoh et al., 1997), in which it was reported that  $R_{CVC}$  remained unchanged even after the pore formation of the CV was inhibited by an application of cationized ferritin to the external medium, i.e. the volume of the CV continued to increase when fluid discharge was blocked.

Stretch-sensitivity of the CV membrane may be involved in the control of  $D_{max}$  and  $T$ . It has been proposed (Tominaga et al., 1998) that periodic changes in the CV membrane tension govern fluid discharge cycles of the CV. An increase in the tension will cause severing of the CV from its radial arms as well as fusion of the CV membrane with the plasma membrane at the pore. A decrease in the tension promotes closure of the pore and reconnection of the CV to the radial arms, through which the fluid that has continued to be segregated by the radial arms can now enter the CV. It has also been demonstrated (Tominaga et al., 1998; Tani et al., 2000) that the CV continues cyclic tension development after the CV has been isolated from the cell, which implies that the periodic tension-developing mechanism resides in the CV membrane and that this mechanism is independent of the decorated spongiome and water segregation. Moreover, tension development was found to be triggered by applying tension to any localized area of the isolated CV *in vitro* (Tani et al., 2000). The mechanosensitive tension-developing mechanism in the CV membrane might be involved in the control of  $T$ . How cytosolic change(s) induced by osmotic stimulation affects the mechanism should be examined in future work.

### Discontinuous changes in $R_{CVC}$ and stepwise change in the cytosolic osmolarity as functions of the osmolarity of the adaptation solution

As shown in Fig. 4A,  $R_{CVC}$  showed one abrupt increase as the osmolarity of the adaptation solution rose from 4 to 204 mosmol<sup>-1</sup>. This large increase in  $R_{CVC}$  occurred between

external osmolarities of 64 and 84 mosmol $l^{-1}$  and was followed by a linear decrease before the  $R_{CVC}$  levelled out at 144 mosmol $l^{-1}$ . It showed that the cytosolic osmolarity underwent an abrupt increase at this external osmolarity. In fact, Fig. 5 shows that the cytosolic osmolarity increased stepwise at 84 and 164 mosmol $l^{-1}$ . That is, the cytosolic osmolarity was approximately 70 mosmol $l^{-1}$  for cells adapted to 4–64 mosmol $l^{-1}$ , approximately 175 mosmol $l^{-1}$  for cells adapted to 84–144 mosmol $l^{-1}$  and approximately 240 mosmol $l^{-1}$  for cells adapted to 164–204 mosmol $l^{-1}$ . These stepwise increases in the cytosolic osmolarity are consistent with the increase in  $R_{CVC}$  at 84 mosmol $l^{-1}$  and the levelling of the  $R_{CVC}$  at approximately 144 mosmol $l^{-1}$ .

These results strongly support the idea that *P. multimicronucleatum* cells are capable of maintaining a more-or-less constant cytosolic osmolarity irrespective of the changes that occur in external osmolarity as long as this does not exceed the cytosolic osmolarity of the cells. As soon as the external osmolarity approaches the cytosolic osmolarity, *P. multimicronucleatum* increases its cytosolic osmolarity to a higher value. This then remains essentially unchanged irrespective of an increase in the external osmolarity until the external osmolarity again approaches the cytosolic osmolarity.

Linear, rather than stepwise, increases in the cytosolic osmolarity were reported to be induced by hypertonic stimulation of *Tetrahymena pyriformis* (Dunham and Kropp, 1973; Stoner and Dunham, 1970). In this species, external osmolarities higher than 40 mosmol $l^{-1}$  caused intracellular osmolarity to increase linearly so that the cytosol constantly remained hypertonic to the surrounding solution. It is interesting to note that neither species seems to be able to tolerate an isotonic or hypertonic condition, i.e. a prolonged stoppage of its CV activity.

$Na^+$  and  $K^+$  were shown to be the osmoregulatory ion species in *Tetrahymena pyriformis* (Dunham and Child, 1961; Dunham and Kropp, 1973) and in the ciliate *Miamiensis avidus* (Kaneshiro et al., 1969a) that keep the cytosol hypertonic; if  $Cl^-$  and  $Ca^{2+}$  have any role, it is only minor. In the present study, the external osmolarity was adjusted by changing the sorbitol concentration while  $K^+$ ,  $Ca^{2+}$  and  $Cl^-$  concentrations remained unchanged. Since our experimental solutions did not contain  $Na^+$ , an involvement of  $Na^+$  in the control of cytosolic osmolarity can be excluded.  $K^+$  and  $Cl^-$  might play a major role, as they do in many other cells such as human erythrocytes (Garay et al., 1988), human lymphocytes (Sarkadi et al., 1984) and Ehrlich ascites tumour cells (Hoffmann and Mills, 1999; Hoffmann et al., 1984). We recently found a stepwise increase in the cytosolic  $K^+$  concentration as the external osmolarity increased in *P. multimicronucleatum* (Stock et al., 2000).

In addition to inorganic ions, free amino acids, such as glycine, alanine and proline, have been found to act as osmolytes in *Miamiensis avidus* (Kaneshiro et al., 1969b), *Tetrahymena pyriformis* (Stoner and Dunham, 1970) and *Paramecium calkinsi* (Cronkite et al., 1993; Cronkite and Pierce, 1989). In the process of adaptation to hyperosmotic stress, the amino acids are not taken from the surrounding

medium, but are probably released by protein hydrolysis (Deaton et al., 1984; Kaneshiro et al., 1969b). The osmolytes that are used to keep the cytosol hypertonic to the surrounding solution in *P. multimicronucleatum* have yet to be determined.

#### Water permeability of the plasma membrane estimated from $R_{CVC}$

We estimated the water permeability coefficient of the plasma membrane of *P. multimicronucleatum* adapted to different external osmolarities from their  $R_{CVC}$  on the assumptions (i) that water uptake by the cell through food vacuole activity is negligible (Dunham and Kropp, 1973; Pal, 1972; Patterson, 1980), (ii) that cell volume does not differ among cells adapted to different osmolarities and (iii) that the rate of osmotic uptake of water by the cell through the plasma membrane is equal to the rate of fluid expulsion from the CVC. The relationship between  $R_{CVC}$  and the water permeability coefficient,  $\alpha$ , can be formulated as:

$$2R_{CVC} = \alpha A(P_i - P_o), \quad (4)$$

where  $A$  is the area of the plasma membrane (the surface area of a cell),  $P_i$  is the osmotic pressure inside and  $P_o$  is the osmotic pressure outside the cell.  $2R_{CVC}$  corresponds to the amount of water that enters the cell osmotically, since there are usually two CVs in a single cell of *P. multimicronucleatum*. Equation 4 can be rewritten as:

$$2R_{CVC} = \alpha \Delta C R T, \quad (5)$$

where  $\Delta C$  is the difference in the osmolarity inside and outside of the cell,  $R$  is the gas constant (8.31 N m K $^{-1}$  mol $^{-1}$ ) and  $T$  is the absolute temperature (298 K for a room temperature of 25 °C).

$A$  was estimated to be  $3.99 \times 10^4 \pm 0.90 \times 10^4 \mu m^2$  (mean  $\pm$  S.E.M.,  $N=10$ ) for cells in culture medium on the assumption that the overall cell shape is represented by the geometry of two facing frusta and that the total surface area of cilia is equal to that of the deciliated cell body (Dunlap, 1977). The cytosolic osmolarities used for the estimation of the water permeability were 71.8 mosmol $l^{-1}$  for cells adapted to 4, 24, 44 and 64 mosmol $l^{-1}$ , 176 mosmol $l^{-1}$  for cells adapted to 84, 104, 124 and 144 mosmol $l^{-1}$ , and 243 mosmol $l^{-1}$  for cells adapted to 164, 184 and 204 mosmol $l^{-1}$  (Fig. 5). The values for  $\alpha$  were calculated by introducing these values into equation 5 and are given in Table 2.

The water permeability of the plasma membrane of *P. multimicronucleatum* remains virtually unchanged irrespective of the change in external osmolarity in a range where the cytosolic osmolarity remains unchanged. It decreases stepwise as the cytosolic osmolarity increases stepwise when the external osmolarity approaches the cytosolic osmolarity ( $\alpha$  is approximately  $0.22 \times 10^{-5} \mu m \text{ min}^{-1} \text{ Pa}^{-1}$  for the 4–64 mosmol $l^{-1}$  range,  $0.063 \times 10^{-5} \mu m \text{ min}^{-1} \text{ Pa}^{-1}$  for the 84–144 mosmol $l^{-1}$  range and  $0.013 \times 10^{-5} \mu m \text{ min}^{-1} \text{ Pa}^{-1}$  for the 164–204 mosmol $l^{-1}$  range). Although, at present, we do not know the biological significance of this decrease in water permeability of the plasma membrane that accompanies

Table 2. Water permeability coefficients of the plasma membrane in *Paramecium multimicronucleatum* cells adapted to different solutions with different osmolarities ranging from 4 to 204 mosmol l<sup>-1</sup>

| Osmolarity of adaptation solution (mosmol l <sup>-1</sup> ) | Water permeability coefficient (μm min <sup>-1</sup> Pa <sup>-1</sup> ) |
|---|---|
| 4   | 0.18×10 <sup>-5</sup>   |
| 24  | 0.18×10 <sup>-5</sup>   |
| 44  | 0.20×10 <sup>-5</sup>   |
| 64  | 0.31×10 <sup>-5</sup>   |
| 84  | 0.06×10 <sup>-5</sup>   |
| 104   | 0.06×10 <sup>-5</sup>   |
| 124   | 0.05×10 <sup>-5</sup>   |
| 144   | 0.08×10 <sup>-5</sup>   |
| 164   | 0.02×10 <sup>-5</sup>   |
| 184   | 0.009×10 <sup>-5</sup>  |
| 204   | 0.01×10 <sup>-5</sup>   |

increasing cytosolic osmolarity, it is certain that it is effective in reducing the probability of cell disruption when a cell with higher cytoplasmic osmolarity encounters a very dilute environment, since osmotic entry of water into the cell is slowed down by the lowered water permeability, thus giving the water segregation mechanism of the CVC time to adjust to this hypotonic challenge by increasing its activity.

The water permeability coefficients estimated for *P. multimicronucleatum* are consistent with that for a freshwater amoeba, *Amoeba proteus* (0.03×10<sup>-5</sup> μm min<sup>-1</sup> Pa<sup>-1</sup>) (Mast and Fowler, 1935) and for the eggs of a frog, *Xenopus* sp. (0.07×10<sup>-5</sup> μm min<sup>-1</sup> Pa<sup>-1</sup>) (Dick, 1959; Prescott and Zeuthen, 1953). These values for  $\alpha$  are much lower than the water permeability coefficients for human erythrocyte membrane (3.06×10<sup>-5</sup> to 5.81×10<sup>-5</sup> μm min<sup>-1</sup> Pa<sup>-1</sup>) (Jacobs, 1932; Sidel and Solomon, 1957).

*The time constant for the change in R<sub>CVC</sub> upon osmotic stimulation can be used as an index for the water expulsion activity of the CVC*

It is now known that, in *P. multimicronucleatum*, segregation of excess cytosolic water is mediated by proton pumps residing in the membrane of the decorated spongiome of the CVC, where they form densely packed helices of protein granules (Fok et al., 1995; Ishida et al., 1996). These proton pumps are a prominent feature of the CVC of *Dictyostelium discoideum* (Fok et al., 1993; Heuser et al., 1993; Nolte et al., 1993) and are also found in the CVC of the zoospores of *Phytophthora nicotianae* (Mitchell and Hardham, 1999).

The time needed for  $R_{CVC}$  to reach its new value after a change in the external osmolarity is assumed to be dependent in large part on proton-pump-mediated water transport activity in the radial arms. We, therefore, arbitrarily chose the time constant,  $\tau$ , to represent a change in  $R_{CVC}$  as an index of the water expulsion activity of the CVC.

As shown in Table 1A, in cells adapted to 4 mosmol l<sup>-1</sup>,  $\tau$

for cells exposed to 24 mosmol l<sup>-1</sup> was nearly the same as that for the cells re-exposed to 4 mosmol l<sup>-1</sup> after a short-term (approximately 30 min) exposure to 24 mosmol l<sup>-1</sup>. This implies that the water transport activity of the radial arms was little affected by the short-term exposure of the cells to an osmolarity slightly higher than that of the adaptation solution. In cells adapted to 24 mosmol l<sup>-1</sup>,  $\tau$  for the cells re-exposed to 24 mosmol l<sup>-1</sup> after their short-term exposure to a lower 4 mosmol l<sup>-1</sup> was significantly ( $P < 0.05$ ) shorter than that for cells exposed to 4 mosmol l<sup>-1</sup> (Table 1B). This result implies that the water transport activity of the radial arms is enhanced by short-term exposure of the cell to an osmolarity lower than that of the original adaptation solution. In contrast,  $\tau$  for the cells adapted to 24 mosmol l<sup>-1</sup> re-exposed to 24 mosmol l<sup>-1</sup> after a short-term exposure to a higher 84 mosmol l<sup>-1</sup>, which was slightly higher than the cytosolic osmolarity (see Fig. 5), was far longer than that for cells exposed to 84 mosmol l<sup>-1</sup> (Table 1B). Similarly in cells adapted to 84 mosmol l<sup>-1</sup> (Table 1C),  $\tau$  for cells re-exposed to 84 mosmol l<sup>-1</sup> after a short-term exposure to 144 mosmol l<sup>-1</sup>, which was nearly isotonic to the cytosol (see Fig. 5), was far longer than that for cells exposed to 144 mosmol l<sup>-1</sup>. These results imply that the water transport activity in the membrane of the radial arms is depressed by short-term exposure of the cell to an osmolarity equal to or larger than the cytosolic osmolarity.

It has been demonstrated (Ishida et al., 1996), by using both immunogold and immunofluorescent labelling using a decorated spongiome reactive antibody, that exposure of *P. multimicronucleatum* to a sorbitol-containing hypertonic solution caused disruption of the decorated spongiome leading to a retardation of the water expulsion rate (see also Naitoh et al., 1997). A change in CV membrane morphology as a result of cells being exposed to a hypertonic solution was also reported for *Dictyostelium discoideum* (Zhu and Clarke, 1992). We therefore conclude that the prolongation of  $\tau$  during the recovery of  $R_{CVC}$  after the cells have been returned to the lower-osmolarity adaptation solution from a hypertonic solution of 84 mosmol l<sup>-1</sup> or above is caused by a partial disruption of the decorated spongiome. Reassembly of active decorated spongiomes may require time.

In cells adapted to 84 mosmol l<sup>-1</sup>,  $\tau$  for cells exposed to 144 mosmol l<sup>-1</sup> was significantly ( $P < 0.05$ ) shorter than that for cells exposed to 24 mosmol l<sup>-1</sup> (Table 1C). Similarly, in cells adapted to 24 mosmol l<sup>-1</sup>,  $\tau$  for cells exposed to 84 mosmol l<sup>-1</sup> was significantly ( $P < 0.05$ ) shorter than that for cells exposed to 4 mosmol l<sup>-1</sup> (Table 1B). We assume that this shortening of  $\tau$  for cells exposed to an isotonic or hypertonic solution is attributable to a relatively rapid disruption of the decorated spongiome.

It should be noted that the values for  $\tau$  for the cells adapted to 84 mosmol l<sup>-1</sup> exposed to 24 mosmol l<sup>-1</sup> (Table 1C), for the cells adapted to 24 mosmol l<sup>-1</sup> exposed to 4 mosmol l<sup>-1</sup> (Table 1B) and for the cells adapted to 4 mosmol l<sup>-1</sup> exposed to 24 mosmol l<sup>-1</sup> (Table 1A) were very similar. This implies that the water expulsion activities of the radial arms of *P.*



*multimicronucleatum* are the same in cells in different solutions that have different osmolarities after the cells have adapted to a solution for a sufficient period (12 h), at least in the osmolarity range employed in the present experiments (4–84 mosmol l<sup>-1</sup>). It has been clearly demonstrated (Ishida et al., 1996) that it took more than 10 h for the decorated spongione to recover morphologically as well as physiologically, as far as the fluid expulsion activity was concerned, after the cells had been transferred to a hypertonic solution.

#### *Functional significance of the CVC activity other than osmoregulation*

It is well known that the primary response of the cells of multicellular organisms exposed to a hypertonic stimulation is to increase their cytosolic osmolarity to keep the volume of the cells or tissues constant (Baumgarten and Feher, 1995). If a *P. multimicronucleatum* cell needs to maintain its volume when faced with an increase in the external osmolarity, an increase in the osmolarity of the cytosol should be enough to fulfil this goal. However, we have clearly demonstrated that the cytosolic osmolarity of *P. multimicronucleatum* exceeds the external osmolarity during its adaptation (12 h) to an initially isotonic or hypertonic solution, and this increase in the cytosolic osmolarity is always accompanied by reactivation of the fluid expulsion activity of the CVC that had ceased immediately after the exposure of the cell to either of these solutions (Figs 4, 5).

This finding, together with our previous findings (i) that in *P. multimicronucleatum* the decorated spongione of the radial arms, which plays a central role in water segregation in the CVC, disintegrated upon exposure of the cell to an isotonic or hypertonic solution, and (ii) that the decorated spongione recovered both morphologically and functionally during the adaptation of the cell to the hypertonic solution (Ishida et al., 1996; Naitoh et al., 1997), strongly supports the idea that the fluid segregation activity of the CVC has biological significance not only for the elimination of excess cytosolic water but also for a second function. This second function may be the elimination of a metabolic waste substance(s) from the cytosol. It seems to be essential for *P. multimicronucleatum* cell survival to keep fluid expulsion via the CVC active.

In this connection, recovery of the CVC activity in a brackish-water ciliate *Paramecium calkinsi* during adaptation to a highly concentrated solution with an osmolarity over 1000 mosmol l<sup>-1</sup> has been reported (Cronkite et al., 1991). Moreover, several marine ciliates, such as *Uronema filificum*, *U. elegans* (Thompson and Kaneshiro, 1968), *Euplotes raikovi* (Dallai et al., 1985) and *Miamiensis avidus* (Kaneshiro et al., 1969a), continue to show CVC activity in sea water, in which most marine invertebrates, including these ciliates, are assumed to encounter osmolarities high enough to be isotonic with cytosolic osmolarities and would not need to become hypertonic. These facts also support the idea that the CVC has a function other than pure osmoregulation, although this

function is not identified at present. The CVC activity of a marine ciliate is, of course, effective in keeping the cytosolic osmolarity constant when it encounters a hypotonic environment.

#### *Further considerations*

Hypotonic stimulation of a cell causes an increase in the net water influx across the plasma membrane and a decrease in the cytosolic osmolarity due to this potential water influx. This may be accompanied by osmotic swelling of the cell. In *P. multimicronucleatum*, hypotonic stimulation causes an increase in  $R_{CVC}$  and a shortening of  $\tau$  when the cell is returned to the adaptation solution (Fig. 2; Table 1B). However, hypertonic stimulation of a cell should lead to a reversal in the direction of net water flux across the plasma membrane and an increase in the cytosolic osmolarity. Such an efflux may be accompanied by cell shrinkage. In *P. multimicronucleatum*, hypertonic stimulation causes cessation of the fluid expulsion from the CVC not only because of a lack of water flowing into the cytosol but also because the hydrogen pumps on the radial arms disintegrate. Hypertonic stimulation of *P. multimicronucleatum* leads to the eventual increase in the cytosolic osmolarity that renders it hypertonic again when the stimulation is long-lived (Fig. 5). It is not known how changing the external osmolarity can trigger changes in CVC activity and how hypertonic stimulation can lead to an increase in the cytosolic osmolarity.

To our knowledge, the existence of a sensor for the direction of the net water flux in the plasma membrane has never been reported. Nevertheless, we found that the water permeability of the plasma membrane of *P. multimicronucleatum* decreased as the cytosolic osmolarity increased (Table 2). This suggests the presence of mechanisms in the membrane for sensing the cytosolic osmolarity of the cell and for changing its water permeability.

The osmotic sensitivity of the membrane has been reported to depend on the degree of acyl chain saturation (Lehtonen and Kinnunen, 1995). It has been shown (Hantz et al., 1986) that stretching the plasma membrane by osmotic swelling of the cell reduces the lateral packing of lipids. This lateral packing is known to modulate the catalytic activities of phospholipases A<sub>2</sub> and C (Boguslavsky et al., 1994; Burack et al., 1993; Grainger et al., 1990; Lichtenberg et al., 1986) and protein kinase C (Souvignet et al., 1991). To understand the mechanisms that govern the osmotic-stimulation-activated cellular events in *P. multimicronucleatum*, it may be worthwhile considering the possible involvement of changes in the lateral packing of plasma membrane lipids during osmotic swelling or shrinkage in allowing the cell to react to osmotic changes.

This work was supported by NSF grant MCB 98 09929. We also acknowledge the German Academic Exchange Service, DAAD, for financial support for C.S. (Stipendium im Rahmen des Gemeinsamen Hochschulsonderprogramms III von Bund und Ländern).

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