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Hypo-osmotic or Ca^{2+}-rich external conditions trigger extra contractile vacuole complex generation in *Paramecium multimicronucleatum*

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Summary

The freshwater ciliated protozoan, *Paramecium multimicronucleatum*, usually possesses two contractile vacuole complexes (CVCs). The number of CVCs in a single cell, however, may vary from 1 to 7. We found that the number of cells that have more than two CVCs increased after the cells were exposed to a hypo-osmotic or a high Ca^{2+} condition. It is assumed that the biological significance of this increase in the number of CVCs is to enhance the cell's ability to eliminate excess water or Ca^{2+} from the cytosol. An extra CVC was either generated *de novo* in the posterior region of the cell or, when in the anterior region, by binary fission of the anterior CVC. Generation of these extra CVCs was not inhibited by aphidicolin, a potent inhibitor of DNA synthesis in the micronuclei of *Paramecium*, even though normal duplication of the CVC that accompanies normal cell division was completely inhibited by this inhibitor. These results suggest that generation of extra CVCs is controlled by a hypothetical regulatory mechanism that is activated either by a hypo-osmotic or by a Ca^{2+}-rich condition and that differs from the regulatory mechanism that governs normal CVC duplication during cell division.

Key words: contractile vacuole complex, osmoregulation, Ca^{2+} regulation, organelle biogenesis, *Paramecium multimicronucleatum*.

Introduction

In the freshwater protozoan, *Paramecium multimicronucleatum*, the osmolarity of the cytosol is always higher than that of the external environment (Kitching, 1956; Stock et al., 2001). Water, therefore, constantly enters the cell through the plasma membrane by diffusion. The cell possesses osmoregulatory organelles, the contractile vacuole complexes (CVCs), which segregate excess cytosolic water and discharges it to the cell's exterior (Ishida et al., 1993; Naitoh et al., 1997; Stock et al., 2001). Segregation and discharge of the cytosolic water by the CVC temporarily ceases immediately after the cell is exposed to an iso- or hyperosmotic condition, and therefore the water influx through the plasma membrane ceases. However, the cells resume their CVC activities if kept in the iso- or hyperosmotic solution (Dunham and Kropp, 1973; Ishida et al., 1996). This resumption of the CVC activity is accompanied by an increase in the osmolarity of the cytosol over that of the external solution, and therefore the external solution is no longer iso- or hyperosmotic (Stock et al., 2001). It was, therefore, reasonable to assume that the CVC may also have a role in expelling some waste substances from the cytosol along with excess water. Recent direct measurements of the ionic composition of the fluid of the *in vivo* CV, using ion-selective microelectrodes, have revealed that excess cytosolic Ca^{2+} can be expelled through the CVC along with excess cytosolic water (Stock et al., 2002a,b).

Many species of *Paramecium* possess two CVCs in a single cell. Individual cells in a population, however, have been reported to possess three or more CVCs, i.e. *P. multimicronucleatum* (Powers and Mitchell, 1910; King, 1935; Allen et al., 1990), *P. caudatum* (Bhatia, 1923; Wichterman, 1946), *P. aurelia* complex (King, 1954) *P. ugandae* (Wichterman, 1986). What factor(s) causes generation of extra CVCs has not been determined.

In the present study, we demonstrate that in *P. multimicronucleatum* generation of extra CVCs is enhanced by exposing the cells to either lowered osmolarity or tincreased Ca^{2+} concentration. Furthermore, we found that generation of extra CVCs under these conditions was controlled by a mechanism that differs from the mechanism that controls normal duplication of CVCs during cell division.

Materials and methods

Cells

Paramecium multimicronucleatum (syngen 2) (Allen and Fok, 1988) were cultured in an axenic medium as previously described (Fok and Allen, 1979). Osmolarity and free Ca^{2+} concentration of the culture medium was approximately 84 mosmol l^{-1} (Ishida et al., 1993) and less than 0.05 mmol l^{-1} (M. Iwamoto, R. D. Allen and Y. Naitoh, unpublished

preliminary data), respectively. Cells in the mid-logarithmic growth phase were centrifuged (at approximately 120 ***g***) for 25 s to form a loose pellet. The cells were then suspended in the experimental solution, which was changed twice to wash the cells. The cells were then incubated in the same solution for an extended adaptation period. Cell culture, adaptation and experimentation were performed at a room temperature of 25±1°C, regulated by a window-type air conditioner.

Experimental solutions

The standard saline solution for adaptation was a mixture containing (mmol l^{-1} final concentration) 2.0 KCl, 0.25 $CaCl_2$ and 1.0 Mops-KOH (pH 7.0), which had an osmolarity of 4 mosmol l^{-1} (Naitoh et al., 1997). Osmolarities of some experimental solutions were varied by adding sorbitol to the standard saline solution. In some experiments, concentrations of KCl and/or $CaCl_2$ were varied. The osmolarity of each solution was determined by using a freezing-point osmometer. A stock solution of aphidicolin (Sigma, St Louis, MO, USA) dissolved in dimethyl sulphoxide (DMSO) at 25 mmol l^{-1} was added to the cell suspension to inhibit cell cycle progression.

Immunofluorescence microscopy of the CVC

For counting the number of CVCs in *Paramecium*, cells were fixed for 30 min in formaldehyde (3% in 50 mmol l^{-1} phosphate buffer, pH 7.4) and permeabilized for 20 min in cold (–20°C) acetone before treatment with a monoclonal antibody (mAb) raised against the smooth spongiome (SS-1 mAb, IgM) (Ishida et al., 1996). This was followed by treatment with fluorescein-isothiocyanate (FITC)-conjugated rabbit anti-mouse IgM. The cells were observed by using a Zeiss microscope equipped with epifluorescence illumination and a filter appropriate for FITC (B-2E, Nikon, Tokyo, Japan). Images of the cells were taken on Kodak Tri-X film. The number of CVCs per cell (N_{CVC}), in more than 300 cells, was determined for each experiment.

Results

Frequency distribution of the number of CVCs in a single cell (N_{CVC}) in cells growing in axenic culture medium compared to cells adapted to a 4 mosmol l^{-1} standard saline solution

The frequency distribution of *P. multimicronucleatum* cells with different N_{CVC} growing in an axenic culture medium (84 mosmol l^{-1}) was compared to the distribution of cells in a population of *Paramecium* cells adapted for 24 h to a 4 mosmol l^{-1} standard saline solution. As is clearly shown in Fig. 1A (black columns), approximately 79% of the cells growing in the culture medium possessed the normal number of two CVCs. Less than 10% of the cells possessed three CVCs. In contrast, the number of cells with two CVCs (Fig. 1B1) decreased to approximately 51%, while that with three CVCs (Fig. 1B2) increased to approximately 43% after the cells were adapted to the standard saline solution (Fig. 1A, white columns). Some cells in both cell populations possessed CVCs of more than three while a few cells possessed only one CVC. The maximum N_{CVC} so far observed was seven (Fig. 1B3).

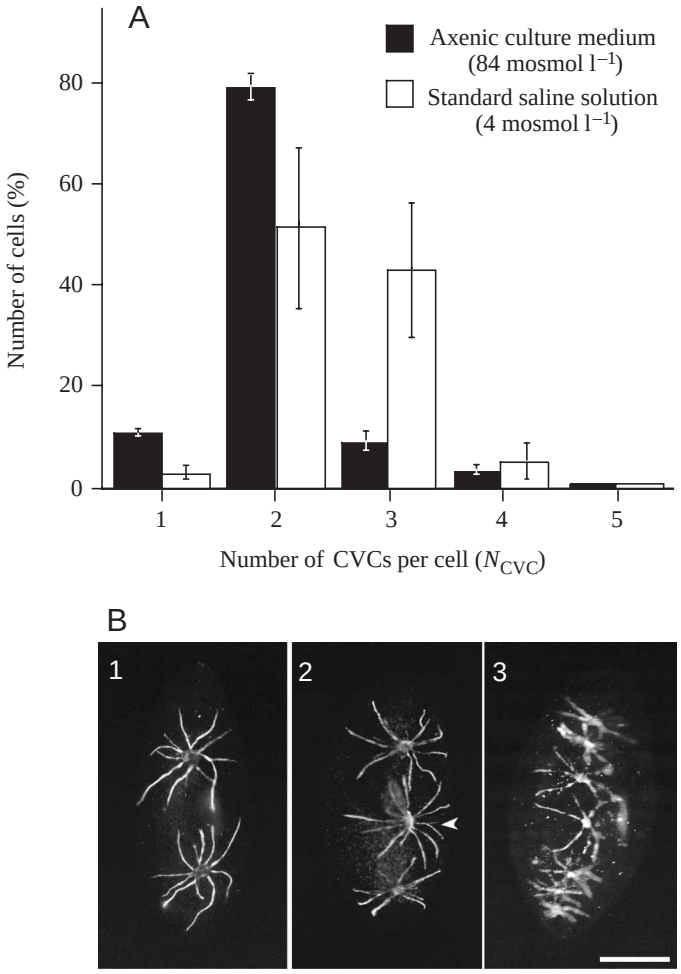

Fig. 1. (A) A frequency histogram for the number of cells containing different numbers (1–5) of CVCs obtained for two different populations of *Paramecium multimicronucleatum* cells. Black columns: cells cultured in a 84 mosmol l^{-1} axenic culture medium. White columns: cells adapted to a 4 mosmol l^{-1} standard saline solution. Values are means ± S.D. (N=3). (B) SS-1 labeling of the smooth spongiome, visualized by its immunological fluorescence image in cells adapted to a 4 mosmol l^{-1} standard saline solution for 24 h. (1) A cell with two CVCs. (2) A cell with three CVCs; an arrowhead indicates branching of a radial arm. (3) A cell with seven (maximum) CVCs. Scale bar, 50 μm.

Frequency distribution of the number of radial arms in a single CVC (N_{RA}) in cells growing in axenic culture medium compared to cells adapted to a 4 mosmol l^{-1} standard saline solution

CVCs of cells adapted to a 4 mosmol l^{-1} standard saline solution for 24 h differed from those growing in axenic culture medium not only in N_{CVC} but also in the number of radial arms in a single CVC (N_{RA}). N_{RA} was larger in cells adapted to the saline solution than those in the axenic culture medium. Fig. 2 shows the frequency distributions of N_{RA} for the two groups

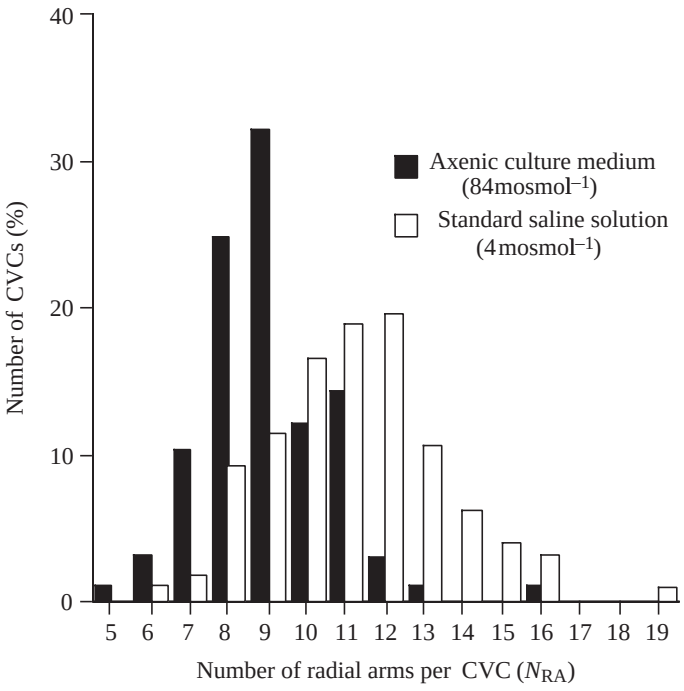

Fig. 2. A representative frequency histogram for the number of CVCs with different numbers of radial arms obtained for two different populations of *P. multimicronucleatum* cells. Black columns: cells cultured in a 84 mosmol l^{-1} axenic culture medium. White columns: cells adapted to a 4 mosmol l^{-1} standard saline solution.

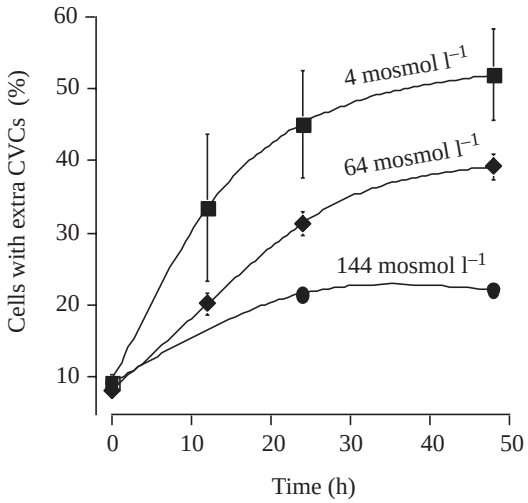

Fig. 3. The number of *P. multimicronucleatum* cells with extra CVCs as a function of the time after the cells were transferred from their 84 mosmol l^{-1} culture medium into three different saline solutions with different osmolarities (4, 64 and 144 mosmol l^{-1}). The number is percentage of cells in each population of cells examined. Values are means ± S.D. (*N*=3).

of cells, growing in the culture medium (black columns) or adapted to the standard saline solution (white columns). The mean N_{RA} for cells adapted to the standard saline solution was 11.2±2.2 (*N*=135) and was significantly (*t*-test, $P<10^{-18}$) larger than that for the cells in the axenic culture medium (9.0±1.5; *N*=141). Moreover, N_{RA} exhibited a wider distribution (variance s=4.8) in cells adapted to the saline solution than in cells in the axenic culture medium (s=2.3). In addition, the radial arms of the cells adapted to the standard saline solution frequently exhibited branching (Fig. 1B2; white arrowhead).

Change in the number of cells with extra CVCs after transferring the cells into saline solutions that have different osmolarities from that of the axenic culture medium

The time course of change in the number of cells that possess three or more CVCs (the cells with extra CVCs) was determined after transferring the cells from their culture medium to one of three saline solutions with different osmolarities 4, 64 or 144 mosmol l^{-1}, respectively. Fig. 3 shows the number of cells with extra CVCs as a function of time after the transfer. The number increased with time in all cases, even when the osmolarity was higher than the axenic culture medium (84 mosmol l^{-1}). The increase was the highest in the solution with the lowest osmolarity, i.e. 4 mosmol l^{-1}.

Change in the number of cells with extra CVCs after transferring the cells into saline solutions with different K^+ or Ca^{2+} concentrations

In order to investigate external factors other than the osmotic factor that affect the extra CVC generation, the time course of change in the number of cells with extra CVCs was determined after transferring the cells from their culture medium to several different saline solutions with (1) a varied K^+ concentration (0.1, 2.0 or 5.0 mmol l^{-1}) at a constant Ca^{2+} concentration (0.25 mmol l^{-1}) or (2) a varied Ca^{2+} concentrations (0.001, 0.1, 0.25 or 1.0 mmol l^{-1}) at a constant K^+ concentration (2.0 mmol l^{-1}). The osmolarity of each saline solution was kept constant at 84 mosmol l^{-1}, which equalled the osmolarity of the axenic culture medium. These cells were, therefore, not subjected to a change in the osmolarity upon their transfer into any of the saline solutions.

As is shown in Fig. 4A, the time course for the cells in each of three different solutions having different K^+ concentrations was essentially identical with each other. The number increased with time from its initial value of approximately 12% to approximately 40% at 48 h.

Fig. 4B shows the time course for the cells in each of four different solutions with different Ca^{2+} concentrations. In each case the number increased with time and reached its plateau value at around 48 h. The plateau value was larger as the Ca^{2+} concentration increased.

Change in the number of cells with extra CVCs after returning cells to axenic culture medium

In order to determine how the number of cells with extra CVCs changes after the cells are returned to the culture medium from a saline adaptation solution, a sample of cells obtained from the axenic culture was first transferred into a 4 mosmol l^{-1} saline solution and kept immersed in this solution

for 18 h. The cells were then returned to fresh axenic culture medium and kept immersed in this solution for 60 h. The number of cells that possess extra CVCs was determined for cells obtained from the sample at intervals of time during this 60 h. The cell density was also determined at the same time and presented as a percentage of the density of cells at –18 h, corresponding to the time when the cells were transferred into the 4 mosmol l^{-1} saline solution.

As shown in Fig. 5, the number of cells with extra CVCs (solid circles) that had increased to approximately 45% during the adaptation of the cells to the saline solution (from –18 to 0 h) did not change during the first 12 h after returning the cells to the axenic culture medium. The cell density (Fig. 5, open circles) was also unchanged during this same period. The number began to decrease between 12 and 24 h and continued to decrease. By 60 h the number had returned to its initial value of approximately 12%. The cell density had begun to increase by 24 h and continued to increase to the end of the 60 h experiment.

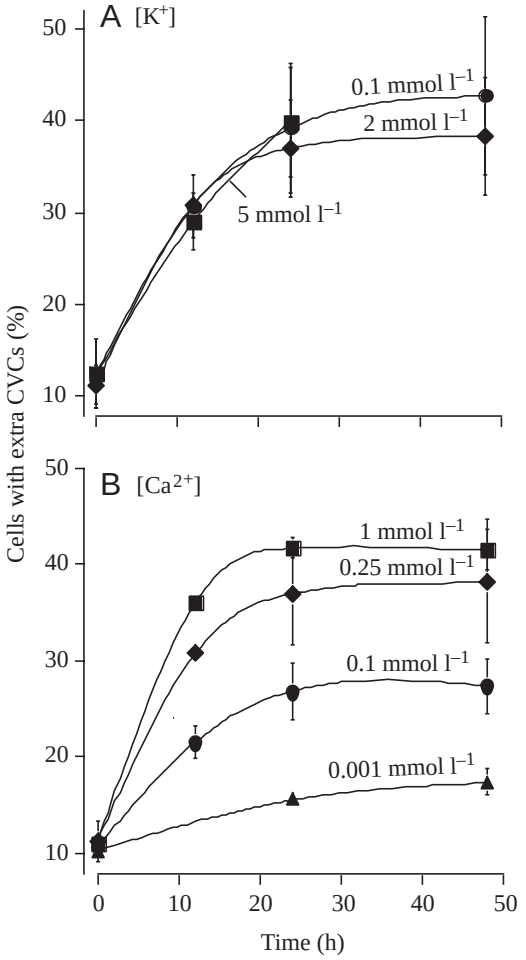

Fig. 4. The number of *P. multimicronucleatum* cells with extra CVCs as a function of the time after the cells were transferred from their 84 mosmol l^{-1} culture medium into different saline solutions with different ionic compositions but with the same overall osmolarity of 84 mosmol l^{-1}. (A) The K^+ concentrations were 5.0, 2.0 and 0.1 mmol l^{-1}, respectively. (B) The Ca^{2+} concentrations were 1.0, 0.25, 0.1 and 0.001 mmol l^{-1}, respectively. The number is percentage of cells in each population examined. Values are means ± S.D. (*N*=3).

Locations in cells where extra CVCs are generated

In a normal cell division cycle, each 'daughter' CVC will be formed anterior to a pre-existing 'mother' CVC immediately before the start of cell division (King, 1935; Allen et al., 1990). *Paramecium* cells, therefore, typically have four CVCs just before cytokinesis occurs (Fig. 6A).

On the other hand, as described above, under a condition of either lowered osmolarity or higher Ca^{2+} concentration or both, some non-dividing cells formed one extra CVC at either one of two different locations. Firstly, a new CVC could be generated in the posterior region of the cell posterior to the posterior CVC. This newly generated CVC frequently seemed to be connected to a long radial arm of the mature posterior CVC (Fig. 6B). Secondly, a new CVC could also be generated by the apparent binary division of the anterior CVC (Fig. 6C). The extra CVC formed by binary division was similar to its mother CVC in both size and shape.

Effects of aphidicolin on the extra CVC generation

To determine whether extra CVC generation is related to germinal DNA synthesis in the micronuclei, the effect of aphidicolin, a potent inhibitor of DNA synthesis in the micronuclei of *Paramecium* (Sabaneyeva et al., 1999), as well as in the nuclei of other eukaryotic cells, on extra CVC generation was examined. Two groups of cells obtained from

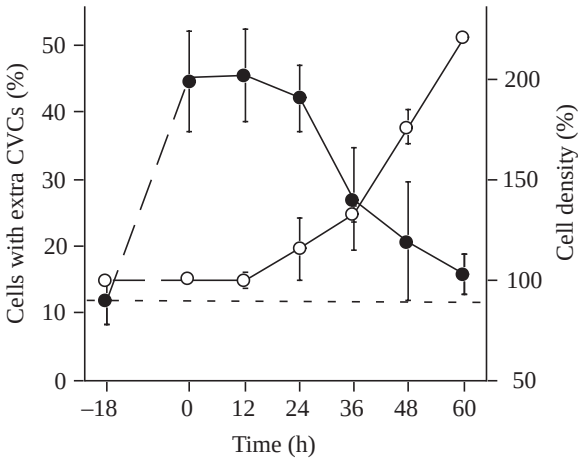

Fig. 5. The number of *P. multimicronucleatum* cells with extra CVCs (filled circles) and the cell density (open circles) as a function of the time after the cells were returned to their 84 mosmol l^{-1} axenic culture medium (at 0 time) from a 4 mosmol l^{-1} standard saline adaptation solution. The percentages of cells with extra CVCs were determined from 300 cells at each time selected at random. The cell density is the percentage compared to the density at –18 h, the time when the cells were transferred for adaptation into the standard saline solution from the axenic culture medium. Each symbol is the mean of two calculations obtained from two experimental series. Bars indicate ± S.D.

the axenic culture were transferred, one into a 4 mosmol l^{-1} standard saline solution and the other into the same saline solution containing 25 μmol l^{-1} aphidicolin. The numbers of cells that had 1, 2, 3, 4 or 5 or more CVCs, respectively, were determined for these two cell groups after they had been adapted to the respective saline solutions for 18 h. As is clearly shown in Fig. 7, the frequency distribution of cells with different N_{CVC} was identical between these two cell groups.

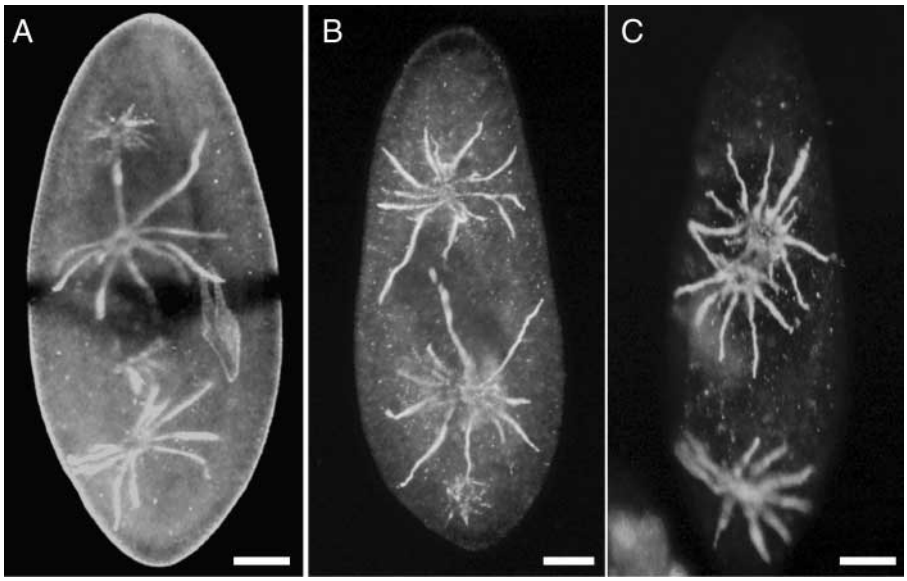

Fig. 6. Generation of new CVCs in *P. multimicronucleatum* cells. Top of each photograph corresponds to the anterior end of the cell. (A) Normal generation in an axenic culture medium that precedes cell division. Each new CVC is generated anterior to each old CVC. (B) An extra CVC generated posterior to the posterior CVC. (C) An extra CVC generated by division of the anterior CVC. Scale bars, 20 μm.

Discussion

Factors that cause an increase in N_{CVC} and their physiological significances

Most *P. multimicronucleatum* cells in an axenic culture medium have two CVCs, although some do have more than two CVCs (extra CVCs) (Fig. 1A, black columns). We found that the number of cells with extra CVCs increased while that of the cells with two CVCs decreased, after the cells were transferred into a 4 mosmol l^{-1} standard saline solution (Fig. 1A, white columns). Transferred cells encounter both (1) a large decrease in the external osmolarity and (2) changes in the external chemical factors, since the osmolarity of the saline solution (4 mosmol l^{-1}) is far lower than that of the axenic culture medium (84 mosmol l^{-1}; Ishida et al., 1993) and the chemical composition in the saline solution is much simpler than that in the axenic culture medium. The major cation species in the saline solution are K^+ (2.0 mmol l^{-1}) and Ca^{2+} (0.25 mmol l^{-1}).

The number of cells with extra CVCs increased when the cells were transferred into a series of saline solutions with decreasing osmolarities but in which the ionic compositions remained the same (Fig. 3). This implies that lowering the external osmolarity is one factor that influences the extra CVC generation.

However, it was also observed that the number of cells with extra CVCs also increased slightly in a 144 mosmol l^{-1} saline solution (Fig. 3). This osmolarity is higher than the osmolarity of the culture medium (84 mosmol l^{-1}), and the result implies that external factors other than lowering the osmolarity can also influence the mechanism for generation of extra CVCs.

The time course required for a population of cells to acquire extra CVCs after transfer from an 84 mosmol l^{-1} axenic culture medium into saline solutions also at 84 mosmol l^{-1}, but with different concentrations of K^+ (0.1, 2.0 or 5.0 mmol l^{-1}) or Ca^{2+} (0.001, 0.1, 0.25 or 1.0 mmol l^{-1}), clearly demonstrated that generation of extra CVCs was essentially unaffected by varying the external K^+ concentration (Fig. 4A), while it was enhanced by increasing the external Ca^{2+} concentration (Fig. 4B). This implies that an increase in the external Ca^{2+} concentration is a second factor that promotes the extra CVC generation. This hypothesis may account for the increase in the number of cells with extra CVCs found in 144 mosmol l^{-1} saline solutions (Fig. 3; filled circles) which, in fact, did have

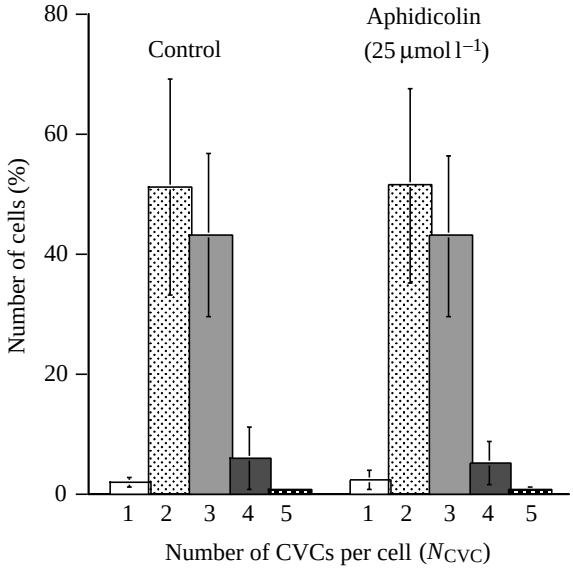

Fig. 7. Two sets of frequency histograms for the number of cells with different numbers (1–5) of CVCs (N_{CVC}) obtained for two different populations of *P. multimicronucleatum* cells. (Left) Cells adapted to a 4 mosmol l^{-1} standard saline solution for 18 h (control). (Right) Cells adapted to a 4 mosmol l^{-1} standard saline solution containing 25 μmol l^{-1} aphidicolin for 18 h. Values are means ± S.D. (N=3).

higher Ca^{2+} concentrations than the axenic culture medium, since the free Ca^{2+} concentration in the axenic culture medium we used was less than 0.05 mmol l^{-1} (M. Iwamoto, R. D. Allen and Y. Naitoh, unpublished preliminary data) while that in the standard saline solution was 0.25 mmol l^{-1}.

It was also expected that more Ca^{2+} ions would enter the cell through Ca^{2+} channels in the cell surface membrane when the cells were in the standard saline solution than in the axenic culture medium. Voltage-dependent Ca^{2+} channels (Naitoh and Eckert, 1968; Naitoh, 1979; Ehrlich et al., 1984; Machemer, 1988) have been reported to be present in the ciliary membrane of *Paramecium*, while mechano-sensitive Ca^{2+} channels (Naitoh and Eckert, 1969) are found in the somatic membrane (Dunlap, 1977; Ogura and Takahashi, 1976; Ogura and Machemer, 1980). Elimination of excess Ca^{2+} from the cytosol is extremely important for the cell to be able to maintain its Ca^{2+}-mediated intracellular signaling systems. The cytosolic free Ca^{2+} concentration in the *Paramecium* cell was assumed to be around 10^{-8} mol l^{-1} by Naitoh and Kaneko (1972). More recently Plattner's group determined the cytosolic Ca^{2+} concentration of *Paramecium* to be $5–8\times10^{-8}$ mol l^{-1}, using a fluorochrome analysis method (Klauke and Plattner, 1998; Plattner and Klauke, 2001). Many cells have Ca^{2+}-pumps (Ca^{2+}-ATPases) in their plasma membranes to eliminate excess cytosolic free Ca^{2+} ions (Edes and Kranias, 2001). In *P. multimicronucleatum* a Ca^{2+}-pump has not yet been identified in the plasma membrane; however, we recently found that the CVC of *P. multimicronucleatum* can accumulate as much as 20 mmol l^{-1} Ca^{2+} (Stock et al., 2002b). Such a high concentration of Ca^{2+} in the CVC suggests that the CVC plays an important functional role in segregating excess cytosolic Ca^{2+} and subsequently eliminating it from the cell. To achieve Ca^{2+} regulation we now find that the *Paramecium* cell can also generate extra CVCs under conditions of high external Ca^{2+} concentration.

Ca^{2+}-pumps have been reported in the CV membrane of *Dictyostelium*, and have a high homology to other P-type pumps (Moniakis et al., 1995), so that Ca^{2+} transport into the CV by this pump may require a proton gradient between the cytosol and the inside of the CV (Moniakis et al., 1999). We have demonstrated that numerous proton pumps are indeed present in CV membranes of *Paramecium* (Fok et al., 1995). Heuser et al. (1993) demonstrated V-ATPases are also found in the CV membrane of *Dictyostelium*. Thus CVCs in *Dictyostelium* may also play a role not only in osmoregulation but also in Ca^{2+} discharge from the cytoplasm. In fact V-ATPases seem to be a constant component of contractile vacuole systems as they have also been shown in the tiny CVs of *Phytophthora* zoospores (Mitchell and Hardham, 1999). We now need to see if there is a functional link between proton pumps and Ca^{2+} uptake in the CVCs of *Paramecium*.

Another finding is that the number of radial arms in a single CVC (N_{RA}) is larger in cells adapted to a 4 mosmol l^{-1} standard saline solution than those in cells in the axenic culture medium (Fig. 2). It can be assumed that an increase in N_{RA} will enhance the rates of segregation of both excess cytosolic water and Ca^{2+} in a cell in addition to an increased N_{CVC}.

Cell division normalizes N_{CVC}

As is clearly shown in Fig. 5, the number of cells with extra CVCs that increased during adaptation of the cells to a 4 mosmol l^{-1} saline solution started to decrease to its normal value (approximately 12%) as the cells began to multiply, approximately 24 h after the cells were returned to a normal axenic culture medium. This implicates cell division in the reduction of N_{CVC}. It can, therefore, be expected that N_{CVC} might increase, if cell division is inhibited. In fact, a slight increase in the number of cells with extra CVCs was observed in an 84 mosmol l^{-1} saline solution containing a very low Ca^{2+} concentration (0.001 mmol l^{-1}; Fig. 4B, filled triangles). Upon transferring into this solution, the cells encountered neither a lowering of the external osmolarity nor an increase in the external Ca^{2+} concentration, but they did encounter a deletion of the external nutrients that are necessary for cell division (Fig. 5; open circles).

Extra CVC generation is governed by a mechanism(s) that differs from duplication of CVCs during normal cell division

We clearly demonstrated that extra CVC generation was enhanced by transferring the cells into hypo-osmotic or Ca^{2+}-rich saline solution (Figs 1, 3, 4) but in which normal cell division stopped (Fig. 5). It is known that during normal cell division, the appearance of new daughter CVCs is followed immediately by the first signs of the formation of a division zone across the middle of the cell (Fig. 6A) (Allen et al., 1990; Fok et al., 2002). It is therefore suggested that extra CVC generation is independent of cell cycle progression. In fact, addition of aphidicolin, which inhibits micronuclear DNA synthesis in *Paramecium* (Sabaneyeva et al., 1999), had no effect on the generation of extra CVCs (Fig. 7). This supports the hypothesis that extra CVC generation is independent of micronuclear DNA synthesis on which normal CVC duplication cycles depend.

In addition, the manner in which extra CVC generation occurs differs morphologically from that of daughter CVC generation during normal cell division. The two daughter CVCs always arise anterior to their mother CVCs (King, 1935; Kaneda and Hanson, 1974; Allen et al., 1990) at nearly the same time in the cell cycle, so that cells showing signs of a division furrow almost always have four CVCs (Fig. 6A). On the other hand, in many cells one extra CVC is generated posterior to the posterior CVC (Fig. 6B) or an extra CVC can be generated by the division of the anterior CVC (Fig. 6C). These morphological differences also strongly support the idea that the mechanism(s) that governs the generation of the extra CVCs differs from that which governs generation of new CVCs during cell division.

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References

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

Allen, R. D., Ueno, M. S., Pollard, L. W. and Fok, A. K. (1990). Monoclonal antibody study of the decorated spongiome of contractile vacuole complexes of *Paramecium*. *J. Cell Sci.* **96**, 469-475.

Bhatia, B. L. (1923). On the significance of extra contractile vacuoles in *Paramecium caudatum*. *J. R. Microsc. Soc.* **262**, 69-72.

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: Hutchinson & Ross.

Dunlap, K. (1977). Localization of calcium channels in *Paramecium caudatum*. *J. Physiol.* **271**, 119-133.

Edes, I. and Kranias, E. G. (2001). Ca^{2+}-ATPases. In *Cell Physiology Source Book* (third edition) (ed. N. Sperelakis), pp 271-282. San Diego, San Francisco, New York, Boston, London, Sydney, Tokyo: Academic Press.

Ehrlich, B. E., Finkelstein, A., Forte, M. and Kung, C. (1984). Voltage-dependent calcium channels from *Paramecium* cilia incorporated into planar lipid bilayers. *Science* **225**, 427-428.

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

Fok, A. K., Aihara, M. S., Ishida, M., Nolta, K. V., Steck, T. L. and Allen, R. D. (1995). The pegs on the decorated tubules of the contractile vacuole complex of *Paramecium* are proton pumps. *J. Cell Sci.* **108**, 3163-3170.

Fok, A. K., Yamauchi, K., Ishihara, A., Aihara, M. S., Ishida, M. and Allen, R. D. (2002). The vacuolar-ATPase of *Paramecium multimicronucleatum*: gene structure of the B subunit and the dynamics of the V-ATPase-rich osmoregulatory membranes. *J. Eukar. Microbiol.* **49**, 185-196.

Heuser, J., Zhu, Q. and Clarke, M. (1993). Proton pumps populate the contractile vacuoles of *Dictyostelium* amoebae. *J. Cell Biol.* **121**, 1311-1327.

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.

Kaneda, M. and Hanson, E. D. (1974). Growth patterns and morphogenetic events in the cell cycle of *Paramecium aurelia*. In *Paramecium, A Current Survey* (ed. W. J. van Wagtendonk), pp. 219-262. Amsterdam, London, New York: Elsevier.

King, R. L. (1935). The contractile vacuole of *Paramecium multimicronucleata*. *J. Morphol.* **58**, 555-571.

King, R. L. (1954). Origin and morphogenetic movement of the pores of the contractile vacuoles in *Paramecium aurelia*. *J. Protozool.* **1**, 121-130.

Kitching, J. A. (1956). Contractile vacuole of protozoa. In *Protoplasmatologica*, vol. III (ed. L. V. Heilbrunn and F. Weber), pp. 1-45. Wien: Springer-Verlag.

Klauke, N. and Plattner, H. (1998). Caffeine-induced Ca^{2+} transients and exocytosis in *Paramecium* cells. A correlated Ca^{2+} imaging and quenched-flow/freeze-fracture analysis. *J. Membr. Biol.* **161**, 65-81.

Machemer, H. (1988). Electrophysiology. In *Paramecium* (ed. H.-D. Görtz), pp. 185-215. Berlin, Heidelberg, New York, London, Paris, Tokyo: Springer-Verlag.

Mitchell, H. J. and Hardham, A. R. (1999). Characterisation of the water expulsion vacuole in *Phytophthora nicotianae* zoospores. *Protoplasma* **206**, 118-130.

Moniakis, J., Coukell, M. B. and Forer, A. (1995). Molecular cloning of an intracellular P-type ATPase from *Dictyostelium* that is up-regulated in calcium-adapted cells. *J. Biol. Chem.* **270**, 28276-28281.

Moniakis, J., Coukell, M. B. and Janiec, A. (1999). Involvement of the Ca^{2+}-ATPase PAT1 and the contractile vacuole in calcium regulation in *Dictyostelium discoideum*. *J. Cell Sci.* **112**, 405-414.

Naitoh, Y. (1979). Membrane currents in voltage-clamped *Paramecium* and their relations to ciliary motion. *Acta Protozool.* **18**, 1-6.

Naitoh, Y. and Eckert, R. (1968). Electrical properties of *Paramecium caudatum*: Modification by bound and free cations. *Z. Vergl. Physiol.* **61**, 427-452.

Naitoh, Y. and Eckert, R. (1969). Ionic mechanisms controlling behavioral responses in *Paramecium* to mechanical stimulation. *Science* **164**, 963-965.

Naitoh, Y. and Kaneko, H. (1972). Reactivated triton-extracted models of *Paramecium*: Modification of ciliary movement by calcium ions. *Science* **176**, 523-524.

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

Ogura, A. and Machemer, H. (1980). Distribution of mechanoreceptor channels in the *Paramecium* surface membrane. *J. Comp. Physiol.* **135**, 233-242.

Ogura, A. and Takahashi, K. (1976). Artificial deciliation causes loss of calcium-dependent responses in *Paramecium*. *Nature* **264**, 170-172.

Plattner, H. and Klauke, N. (2001). Calcium in ciliated protozoa: sources, regulation, and calcium-regulated cell functions. *Int. Rev. Cytol.* **201**, 115-208.

Powers, J. H. and Mitchell, C. (1910). A new species of *Paramecium* (*Paramecium multimicronucleata*) experimentally determined. *Biol. Bull.* **19**, 324-332.

Sabaneyeva, E., Tao, W. and Verbelen, J. P. (1999). Aphidicolin inhibits DNA replication in the micronucleus and blocks cytokinesis in *Paramecium caudatum*. *Cell Biol. Int.* **23**, 859-862.

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. (2002a). Osmoregulation in *Paramecium*: in situ ion gradients permit water to cascade through the cytosol to the contractile vacuole. *J. Cell Sci.* **115**, 2339-2348.

Stock, C., Grønlien, H. K. and Allen, R. D. (2002b). The ionic composition of the contractile vacuole fluid of *Paramecium* mirrors ion transport across the plasma membrane. *Eur. J. Cell Biol.* **81**, 505-515.

Wichterman, R. (1946). Unstable micronuclear behavior in an unusual race of *Paramecium*. *Anat. Rec.* **94**, 94.

Wichterman, R. (1986). Classification and Species of *Paramecium*. In *The Biology of Paramecium* (second edition), pp. 1-59. New York, London: Plenum Press.