Hyperosmotic stress leads to reversible dissociation of the proton pump-bearing tubules from the contractile vacuole complex in *Paramecium*

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SUMMARY

To study the effect of hyperosmotic stress on the structure and function of the contractile vacuole complex of *Paramecium multimicronucleatum*, we employed two different monoclonal antibody markers: one to a decorated spongiome antigen (A4) and a second to an antigen found on all other membranes of the contractile vacuole complex (G4). A hyperosmotic condition was produced by adding sorbitol to the axenic culture medium which induced both dose- and time-dependent decreases in the vacuole’s expulsion rate. The addition of 150 mM sorbitol to the medium (making a final osmolarity of 230 mOsmol) was sufficient to completely stop the expulsion of the contractile vacuole. Immunofluorescence demonstrated that the blocking of fluid output was accompanied by the disappearance of most fluorescence labeling from the decorated spongiome (the A4 antigen). Electron microscopy revealed that the disappearance of the labeling was accompanied by the disappearance of the decorated tubules from around the collecting canals. These tubules vesiculate. The other membranes of the contractile vacuole complex remained unaffected which was demonstrated by both electron microscopy and indirect immunolabeling using the mAb against the G4 antigen. These results show that the decorated spongiome is formed from a distinct membrane pool separate from that of the smooth spongiome, collecting canals and the contractile vacuole. Recovery of the decorated spongiome rapidly followed the return of the cell to an isotonic environment and was completed within 3 hours. Decorated tubule recovery paralleled the recovery of the function of the contractile vacuole. Recovery was also observed during continuous hyperosmotic treatment with the reappearance of the contractile vacuole activity starting at 3 hours and stabilizing at around 10 hours of incubation. Functional recovery under these conditions was accompanied by a reappearance of the decorated tubules but the total fluid output was always lower than for cells in an isotonic environment. Thus, cells were shown to be capable of adapting to high hyperosmotic conditions. We conclude that the dissociation and reassociation of the decorated spongiome is an important regulatory feature controlling the activity of the contractile vacuole complex and of intracellular osmoregulation in *Paramecium*.

Key words: Contractile vacuole, Decorated spongiome, *Dictyostelium discoideum*, Osmoregulation, *Paramecium multimicronucleatum*, Vacuolar-type proton ATPase

INTRODUCTION

Contractile vacuole complexes (CVCs) are widely distributed in protozoa and are also found in freshwater sponges and zoospores of some fungi. The CVCs are thought to be osmoregulatory organelles as their contraction frequency and the diameter of their contractile vacuoles change in response to changes in the extracellular osmolarity (reviewed by Kitching, 1956, 1967; Patterson, 1980; and Zeuthen, 1992). However, the molecular mechanisms by which they extract and expel water from the cytoplasm remain unknown.

Early comparative ultrastructural studies (McKanna, 1973a,b, 1974, 1976) showed the presence of 12 nm pegs on the cytosolic side of the membranes of the contractile vacuole system. These pegs found in both protozoa and fresh water sponges, were considered by this author to be important in water sequestration. Thus, McKanna referred to these peg-bearing compartments as fluid segregating organelles. By microinjecting a monoclonal antibody into *Paramecium*, we have recently demonstrated that such peg-decorated membranes are indeed the locus of fluid segregation (Ishida et al., 1993). Several recent studies have shown that calmodulin (Zhu and Clarke, 1992), α-actinin (Furukawa and Fechheimer, 1994), unconventional myosin (Baines and Korn, 1990; Zhu and Clarke, 1992), alkaline phosphatase (Quiviger et al., 1978; Nolta and Steck, 1994), Rab 4-like GTPase (Bush et al., 1994) and a vacuolar-type proton ATPase (V-ATPase)(Nolta et al., 1993; Heuser et al., 1994) are all associated with the membranes of the CVC of the amoebas, *Dictyostelium discoideum* and *Acanthamoeba castellanii*. Exactly what role any of these proteins play in osmoregulation remains to be determined. The CVC in *Dictyostelium* fragments into small vacuoles during cell division (Zhu et al., 1993) and its contractile vacuoles are no longer visible when these cells are exposed to a sufficiently high hyperosmotic culture medium (Zhu and Clarke, 1992). Freeze drying of quick-frozen fragmented *Dictyostelium* showed the
CVC in this cell to be composed of vacuoles interconnected by an extensive labyrinth of membrane channels and flattened cisternae located just beneath the plasma membrane (Heusser et al., 1993). All of these membranes were studded with 15 nm ‘ pegs ’ . When exposed to the antiserum against the 57 kDa B subunit of the V ATPase of chromaffin granules, Heusser et al. (1993) showed that the 15 nm pegs were both decorated and aggregated by the B subunit antibody.

In Paramecium interphase cells, only the smooth spongione of the CVC next to the collecting canals is surrounded by and in continuity with the peg-bearing membrane, called the decorated spongione (McKanna, 1976). For P. multimicronucleatum, we recently showed that these pegs on the cytosolic surface of the membrane of the decorated spongione are a part of the V ATPase (Fok et al., 1995). In collaboration with other laboratories, we obtained antibodies specific for the V ATPase and applied these to Paramecium. These included polyclonal antibodies against the V ATPase of Dicyostelium discoideum (Nolta et al., 1993), and of the V ATPase of chromaffin granules (Nelson, 1992). By using indirect immunofluorescence as well as immunogold electron microscopy, we showed that each of these antibodies labeled the decorated spongione giving the same pattern as that revealed by our own monoclonal antibody DS 1, which labels an uncharacterized antigen termed A4 (Allen et al., 1990; Fok et al., 1995). Also, in these studies, we showed: (1) that a V ATPase specific inhibitor, concanamycin B, inhibits the CVC’s fluid output but does not affect the integrity of the decorated tubules; and (2) that cold treatment, known to cause the dissociation of the isolated VI V 0 complexes, causes the in vivo dissociation of the decorated tubules so that the antigens become dispersed throughout the cytosol. These results confirmed our notion that the pegs on the decorated spongione are, in fact, components of a V ATPase in Paramecium (Fok et al., 1995). The antibody DS 1, therefore, must also recognize this same V ATPase, however, it has so far failed to react with any polypeptide on immunoblots.

In the current study, we employed an additional monoclonal antibody, SS 1 to an antigen termed G 4 , which was previously shown to react with the membranes of the smooth spongione, collecting canal, ampullae and contractile vacuole of the CVC as well as to the cell’s plasma membrane (Fok et al., 1995). By using DS 1 and SS 1 as morphological markers for the CVC membranes, we examined the effect of a range of hyperosmotic conditions on the CVC’s fluid output. The fluid output of the cell was obtained by multiplying the number of expulsions of the two CVCs by the average volume of the CVCs at each timepoint (Ishida et al., 1993). The specificities of mAbs DS 1 and SS 1 have been published (Allen et al., 1990; Fok et al., 1995). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). All other chemicals were obtained from Sigma (St Louis, MO).

For making hyperosmotic solutions, an amount of sorbitol needed to produce a final osmolality of approximately 300 mOsmol was added to the axenic medium, thereby maintaining a nearly constant concentration of ions within the hypertonic solutions. The hyperosmotic condition was induced by the dilution of the axenic culture medium with deionized distilled water (DDW).

RESULTS

The effect of hyperosmotic stress on CV function

Hyperosmotic conditions were induced by the addition of a series of concentrations of sorbitol to the axenic culture medium. The osmolality of each solution was determined with a freezing point depression osmometer (Advanced Instruments, Norwood, MA). The osmolality of the sorbitol-free axenic culture medium was approximately 80 mOsmol. As shown in Fig. 1, a stepwise increase in hyperosmotic conditions induced a decrease in the expulsion frequency of the contractile vacuole (CV). Thus, CV activity was determined to be time- and dose-dependent. Addition of 80 mM sorbitol to the growth medium essentially stopped pulsation of the CV after 45 minutes. Maximum inhibition of expulsion was obtained within 10 minutes following the addition of 160 mM sorbitol, at which time the CVs were completely collapsed. By measuring CV diameters and the expulsion frequencies while under the influence of the 160 mM sorbitol, the total fluid output of the cells was calculated (Fig. 2). The total fluid output was drastically diminished over time and was essentially eliminated by 10 minutes. Thus, the CVC becomes inactive under such high osmotic conditions.

MATERIALS AND METHODS

Materials

Paramecium multimicronucleatum, syngen 2, cultured in an axenic medium as previously described (Fok and Allen, 1979), was harvested at mid-log phase of growth. Expulsion frequencies and contractile vacuole (CV) sizes were measured in cells whose movements were restricted by pressure from the cover glass. The total fluid output of the cell was obtained by multiplying the number of expulsions of the two CVCs by the average volume of the CVCs at late diastole as described previously (Ishida et al., 1993). The specificities of mAbs DS 1 and SS 1 have been published (Allen et al., 1990; Fok et al., 1995). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). All other chemicals were obtained from Sigma (St Louis, MO).

Fluorescence- and electron-microscopy

In preparation for indirect immunofluorescence experiments, Paramecium cells were fixed in 3% formaldehyde in 50 mM phosphate buffer (pH 7.2), acetone permeabilized at -20°C for 20 minutes, and incubated sequentially with a monoclonal antibody and then a secondary antibody as previously described (Allen et al., 1990). Cells were examined in a Zeiss microscope (Thornwood, NY) equipped with epifluorescence illumination.

For conventional electron microscopy cells were fixed in 1% glutaraldehyde buffered with 0.05 M cacodylate (pH 7.2) for 30 minutes at room temperature (Allen, 1974). For immuno electron microscopy, cells were fixed in 0.25% glutaraldehyde in 50 mM phosphate buffer (pH 7.2) at room temperature for 15 minutes. These cells were embedded in 4% gelatin and infiltrated with 2.3 M sucrose before being processed for cryosectioning and immunogold staining as previously described (Allen et al., 1990). To reduce nonspecific staining, all sections were preincubated with 5% normal goat serum before the primary antibody as well as 1% bovine serum albumin prior to incubation with primary and with secondary antibodies.

Materials

Paramecium multimicronucleatum, syngen 2, cultured in an axenic
To investigate the effect of hyperosmotic conditions on the structure of the CVC, we employed the mAb DS-1 as a tag to visualize the decorated spongiome which has been demonstrated to label the A4 antigen on the decorated spongiome membrane (Allen et al., 1990). Increased hyperosmotic conditions were accompanied by decreases in the fluorescent labeling of the decorated spongiome. During the first few minutes, the decorated spongiome varied little from the control (Fig. 3A,B). By 10 minutes in 80 mM sorbitol solution the labeling of the decorated spongiome began to disappear from the distal ends of the collecting canals (Fig. 3C) while after 10 minutes in 160 mM sorbitol solution a much greater effect was observed (Fig. 3D). Disappearance of label was most pronounced after 45 minutes (Fig. 3E,F), particularly in those cells treated with 160 mM sorbitol solution (Fig. 3F). Along with the reduction in decorated spongiome labeling, we observed an increase in background labeling with the DS-1 mAb (Fig. 3C-F) as well as a tendency for the label to extend to and surround the contractile vacuole itself (Fig. 3F). On the other hand, a hypoosmotic condition produced by a 128 times dilution of the external axenic culture medium with DDW induced an increase in total fluid output of the cell (data not shown). Immunofluorescence staining revealed that such a strong hypoosmotic change induced no observable structural change of the decorated tubules (Fig. 4). Thus, the disappearance of the decorated tubules is observed only under hyperosmotic con-
conditions in which the expulsion of the contractile vacuole essentially stops.

To investigate the basis for these sorbitol-induced morphological changes, cells treated with sorbitol were fixed, serial sectioned and observed by electron microscopy. Fig. 5A and B show oblique sections of the radial arms of the CVC obtained from a control and a 10 minutes sorbitol-treated cell, respectively. Instead of the decorated tubules being of uniform width, like those found next to the smooth spongiome of untreated cells (Fig. 5A), the decorated tubules in sorbitol treated cells have swollen ends (arrows and inset, Fig. 5B), which apparently represents an initial stage of expanding tubules (asterisks, Fig. 5B). Fig. 5C shows an oblique section of the decorated spongiome obtained from a cell treated with 150 mM sorbitol for 45 minutes. The area next to the smooth spongiome is packed with vesicles (asterisks), which are believed to be derived from expanded decorated spongiomes. On the other hand, the smooth spongiome and the other components of the contractile vacuole complexes were normal after 10 minutes.

Fig. 4. Indirect immunofluorescence labeling of a hypoosmotically treated cell showing the existence of the decorated spongiome in the CVC. No change was seen even after 1 hour of treatment in 128 times DDW-diluted axenic culture medium. Bar, 50 μm.

Fig. 5. Transmission electron micrographs showing part of the decorated spongiome (ds) in an untreated control cell (A) and in a cell treated with 150 mM sorbitol for 10 minutes (B) and for 45 minutes (C). Inset in B shows the expanding decorated spongiomes in another cell. cc, collecting canal; ss, smooth spongiome; arrows, expanded ends of decorated tubules; asterisks, vesicles in area of decorated spongiome. Bars, 0.2 μm.
(ss, Fig. 5B) and are still present after 45 minutes (Fig. 5C, also see Fig. 9B).

Further immunocytochemical investigations designed to determine the cause for the disappearance of the decorated spongiome in the cytoplasm were carried out. Cryosections of sorbitol treated and untreated cells were incubated with DS-1 followed by secondary antibody complexed with gold. Gold particles were prevalent only on the decorated tubules in the untreated cell (arrows, Fig. 6A), and seemed to be specific for the pegs (inset, Fig. 6A, also see Fig. 6 in Fok et al., 1995). No gold particles were observed on the cross sections of the radial arm of a 150 mM sorbitol treated cell in which the decorated spongiome was no longer visible (Fig. 6B). In this micrograph, the smooth spongiome is surrounded by some vesicles (asterisks) which are believed to be remnants of the decorated tubules. We then attempted to determine the fate of the vesiculated decorated tubules in the cytoplasm. However, as these vesicles no longer react with DS-1 (Fig. 6B), we were unable to identify such vesicles in the cytosol. To account for the higher background fluorescence (Fig. 3F) observed in sorbitol-treated cells, we assume that the A4 antigens are released from the decorated tubules into solution and become randomly scattered throughout the cytoplasm.

These results suggest that the disappearance of the fluorescent labeling from the CVC is due both to: (1) the vesiculation and release of the decorated spongiome from around the smooth spongiome; and (2) the loss of either the antigenicity of A4 or, more likely, the release of the antigens from the vesiculated decorated tubules.

Recovery from the effect of hyperosmotic stress
To investigate the process of recovery from hyperosmotic stress, cells treated 45 minutes with 150 mM sorbitol were transferred to fresh axenic culture medium. The expulsion frequency and the structure of the decorated spongiome were monitored sequentially over the next several hours. The recovery of the expulsion frequency was very rapid, even after treatments which induced almost total disappearance of the decorated spongiome. Recovery of function started soon after transfer to the fresh axenic medium, and was essentially completed within 3 or 4 hours (Fig. 7). Indirect immunofluorescence labeling with mAb DS-1 showed that recovery of the expulsion frequency was accompanied by reappearance of the decorated spongiome (Fig. 8). After 1 hour, the expulsion frequency had recovered to more than half of its normal frequency (Fig. 7), and the structure of the decorated spongiome revealed by mAb-DS-1 labeling had also begun to recover (Fig. 8B), more along some radial arms than along others. Recovery of the decorated spongiome began at the more proximal ends of the radial arms (Fig. 8A, B) and
extended to the distal portion over the succeeding two to three hours (Fig. 8C-E). Such a rapid recovery of the structure and function of the decorated spongiome suggests that the disappearance of the fluorescence labeling may have resulted from the dissociation of the decorated spongiome from the CVC as intact vesicles still bearing intact or partially intact proton pumps rather than to a complete proteolysis and resynthesis of the decorated spongiome and its elaborate pump complexes.

Cellular adaptation to hyperosmotic stress
Reassociation of the decorated spongiome with the collecting canals was also observed during prolonged high hyperosmotic treatment (medium was made 230 mOsmol by adding 150 mM sorbitol). We found that long-term treatment had no effect on the osmolarity of the external solution, it remained 230 mOsmol. To follow the structures of the CVC through such long-term treatments, we carried out double labeling experiments with the two different mAbs, DS-1 for A4 and the SS-1 for G4, detected by FITC and Texas Red, respectively. As we demonstrated in a previous study, indirect double immunofluorescence staining using these two mAbs, DS-1 and SS-1, shows two distinctive patterns of labeling (Fok et al., 1995). Label with mAb SS-1 for G4 was observed on the contractile vacuole, ampullae, and the full extent of the collecting canals, but not on the decorated spongiome. Thus, we employed the antibody to G4 to monitor the structures of the CVC membranes other than those revealed by mAb DS-1 which labels only the decorated spongiome’s A4 antigen. Indirect immunostaining for A4 shows a recovery of the decorated spongiome in cells continuously exposed to hyperosmotic stress that begins around 3 hours (Fig. 9E) and is completed by 9-10 hours of incubation (Fig. 9I). The other membrane structures of the CVC, labeled with the mAb SS-1 to G4 antigen, did not change during this prolonged hyperosmotic treatment (Fig. 9B,D,F,H,J,L). This restoration of the decorated spongiome along the collecting canals was accompanied by a recovery of CVC function (Fig. 10). Partial recovery to control levels of total fluid output from complete inhibition in the 150 mM sorbitol solution (230 mOsmol) seemed to begin after 3 hours of incubation. Recovery stabilized at approximately 1/3 of the fluid output of control cells by 8 hours of incubation. Even after 17 hours of incubation, total fluid output remained at this same level, 4.84±2.38 pl/cell/minute. Although the fluid output did not reach control cell levels, the structure of the CVC as judged by immunofluorescence had returned to normal during this time period (Fig. 9I). As shown in Fig. 9K and L, the structure of the CVC at 24 hours was identical to that of 9 hours from the start of the hyperosmotic treatment. Moreover, cells survived throughout these prolonged treatments (more than 18 hours), even though fluid output was reduced to zero for 3 hours. Cellular adaptation to the prolonged hyperosmotic stress appears to involve the reconnection of the decorated spongiome with the rest of the CVC.

Fig. 7. Recovery of contractile vacuole (CV) activity following inhibition produced by sorbitol treatment. Cells acclimated to axenic culture medium containing 160 mM sorbitol (final osmolarity of 240 mOsmol) for 45 minutes were transferred to fresh culture medium (80 mOsmol) at time 0. Each point represents the mean ± s.d. from 10 CVs. Broken line indicates the average in control cells (n=30).

Fig. 8. Recovery of the decorated spongiome following sorbitol treatment. Cells acclimated to axenic culture medium containing 160 mM sorbitol (240 mOsmol) for 45 minutes were transferred back into fresh culture medium. In all micrographs, the cell anterior is directed toward the left. Micrographs were selected to reveal a typical immuno-stained image of the cell at a given time. (A) soon after the transfer. (B) 1 hour after transfer. (C) 2 hours after transfer. (D) 3 hours after transfer. (E) 4 hours after transfer. Bar, 50 μm.
DISCUSSION

To study the effect of osmotic stress on Paramecium, we subjected the cells to increasing hypertonic concentrations by adding sorbitol to the external medium while keeping the external ionic concentration constant. As Paramecium mult micronucleatum is a fresh water ciliate and has an internal osmolarity which we estimate to be about 140 mOsmol when growing in axenic medium, we increased the osmolarity of the culture medium stepwise from 80 mOsmol to a final osmolarity of 240 mOsmol. The most obvious effects of this increase in hypertonicity on the cell when viewed by fluorescence and light microscopy were a marked reduction in the CVC expulsion rate and the apparent disappearance of the decorated spongiome (Figs 3-6) from the CVC. In contrast, under hypotonic conditions, the expulsion rate and total fluid output of the cell was increased without any observable change in structure of the decorated spongiome (Fig. 4). Thus, the disappearance of the decorated spongiome was observed only under hypertonic conditions. This response was reminiscent in some respects to changes in the CVC following the microinjection of mAb DS-1 into the cell (Ishida et al., 1993). This mAb has recently been shown to be specific for V-ATPase complexes (Fok et al., 1995). However, unlike the recovery from mAb microinjection, which required 48 hours during which the vesiculated or expanded decorated spongiomes were sequestered into autophagic vacuoles together with the bound DS-1 antibody (Ishida et al., 1993), immunostaining of fixed sorbitol-treated cells indicated that the antigen labeled by DS-1, became dispersed throughout the cytosol, and was not sequestered into autophagic vacuoles. As a consequence, the time required for recovery was far shorter following hypertonic stress than was the case following the microinjection of antibodies (Fig. 7). Thus, the fate of the A4 antigens following sorbitol treatment is entirely different from the fate of the antigen-antibody complexes formed after microinjection of mAb DS-1.

At the electron-microscopic level, the tubules of the
decorated spongiome following sorbitol treatment rounded up and became disconnected from the smooth spongiome (Fig. 5). Vesiculation was indicated in regular thin sections as well as in cryosections where vesicles, presumably formed from the decorated spongiome, were observed occupying the space next to the smooth spongiome. However, such vesicles were rarely labeled with gold in cryosections following the use of DS-1 as the primary antibody (Fig. 6). Thus we were unable to pinpoint the exact localization of the V-type proton pumps of the decorated spongiome once the spongiome was dispersed into the cytoplasm even though indirect immunofluorescence staining with mAb DS-1 had indicated that the fluorescence of the decorated spongiome was broadly distributed throughout the cytoplasm (Fig. 3F). We conclude that the A4 antigen (part of the V-ATPase) has been released from the membrane into the soluble cytoplasm or, alternatively, has lost its antigenicity during the preparation for cryo-immunogold localization. The latter seems less likely as the antigen is still reactive in cryosections when it is bound to the decorated tubules.

Thus, the cells appear to respond to changes in hyperosmotic conditions by first disconnecting and then reconnecting their V-ATPase-bearing decorated spongiome with the smooth spongiome of the CVC. These results suggest that the dissociation and reassociation of the decorated spongiome is a key response brought about by sudden changes in extracellular osmolarity and has both structural and physiological consequences. A similar dissociation of the decorated spongiome from the rest of the CVC was also observed when cells were exposed to cold temperature (Fok et al., 1995). The time required for the recovery from this cold shock was about the same as that for sorbitol-treated cells, suggesting that similar reversible changes occur during cold shock as during hyperosmotic stress. However, since the disconnected decorated spongiome was not followed by thin section electron microscopy, we did not determine its fate in the cold study (Fok et al., 1995). Therefore, it is not yet possible to tell if the disappearance of the decorated spongiome from the CVC in cold treated cells is the same as in sorbitol treated cells.

Restoration of the decorated spongiome was also observed in cells maintained under continuous high hyperosmotic conditions (Fig. 9) thus the cell is capable of both functional and structural adaptation to these hyperosmotic conditions. The time required for the reassociation of the decorated spongiome to the radial arms was much longer, 6 to 9 hours, than that needed for recovery when the cells are returned to an isotonic solution, 3 hours. Although the recovery of the structure was complete, total fluid output was stabilized at approximately 1/3 of the control rate at around 8 hour. As reviewed by Patterson (1980), when protozoa are exposed to osmotic stress, their cell volumes and vacuolar outputs are initially changed but tend to drift back towards their original values after some time. Ciliates, such as Tetrahymena pyriformis, Miamensis avidus and Paramecium calkinsi, are capable of adjusting both their inorganic (Dunham, 1964; Kaneshiro et al., 1969a) and organic osmolyte concentrations (Kaneshiro et al., 1969b; Cronkite and Pierce, 1989) during osmotic adaptation. A similar adaptation process may be functional in P. multimicronucleatum and may lead to a new osmotic balance and a new level of CVC activity after 8 hours. This may explain the reduced rate of CVC activity following hyperosmotic adaptation.

In this study, we employed the two mAbs DS-1 and SS-1 as morphological markers to distinguish the decorated spongiome from other membranes of the CVC, and to allow us to follow the disappearance of the V-ATPase bearing decorated spongiome when the cell was under the influence of high hyperosmotic stress. This had never been attempted in Paramecium, although a somewhat similar study had been reported by Zhu and Clarke (1992) for Dictyostelium discoideum. By using an anti-calmodulin antibody for indirect immunostaining, Zhu and Clarke reported the dispersal of the contractile vacuole membrane as a result of the exposure of Dictyostelium to hyperosmotic solutions produced by the addition of sorbitol. Immunostaining indicated that the calmodulin-stained membranes condensed next to the nucleus and then reappeared as contractile vacuole membrane very rapidly after the sorbitol was washed out. In contrast to Paramecium, this amoeba’s CVC is less highly organized and has a variable number of contractile vacuoles which move about in the cell. It also lacks permanent pore structures (Patterson, 1980). The cytosolic surfaces of all the membranes of the CVC in Dictyostelium have 15 nm pegs which have recently been demonstrated to be the V1 complexes of V-ATPases (Heuser et al., 1993; Fok et al., 1993). A similar characteristic of these peg-bearing membranes, in both Dictyostelium and Paramecium, is that they undergo vesiculation in response to hyperosmotic stress. Thus vesiculation of the membranes that bear the V-ATPase proton pumps and the separation of these vesicles from other components of the CVC may be a general regulatory response in fresh-water protozoa to abrupt rises in external hypertonicity. Such vesiculation may also be induced by other environmental changes such as temperature, but this must be confirmed by further study.

In summary, Paramecium responds to hyperosmotic stress by disconnecting and vesiculating its proton pump-bearing membrane from the bulk of its CVC membrane. This is a completely reversible phenomenon requiring less time to recover in cells returned to isotonic conditions than in cells kept under
continuous hypertonic conditions. Such obvious ultrastructural changes triggered by environmental changes may occur in other cell organelles but none readily come to mind. The changes in the grana of chloroplasts when deprived of light comes close, but here a continuous membrane system reshapes from stacks of grana to a prolamellar body without actually vesiculating. A search for other clear examples of the reversible dissociation of pump-bearing membranes or membranes bearing other enzymes from an organelle in response to changes in the environment may establish this mechanism of adaptation to have a wider application in nature then is currently known.

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