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The pegs on the decorated tubules of the contractile vacuole complex of *Paramecium* are proton pumps

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SUMMARY

Our previous study has shown that the decorated tubules (collectively known as the decorated spongiome) of the contractile vacuole complex (CVC) in *Paramecium* are the site of fluid segregation, as the binding of microinjected monoclonal antibody (mAb) DS-1 to the tubules reduced the CVC's fluid output. In this study, we showed by immunogold labeling on cryosections that the antigenic sites for mAb DS-1 were located on the 15 nm 'pegs' protruding from the cytosolic surface of the decorated tubules. In immunofluorescence studies, both polyclonal antibodies against the subunits of the V-ATPase of *Dictyostelium discoideum* and against the 57 kDa B-subunit of the V-ATPase of chromaffin granules gave identical labeling patterns to that produced by mAb DS-1. On cryosections, all three antigens were located most consistently near or on the pegs of the decorated tubules. These data support the notion that the pegs on the membrane of the decorated tubules represent the V_1 complex of a proton pump. Concanamycin B, a potent inhibitor of V-ATPase activity and of acidification of lysosomes and endosomes, strongly and reversibly inhibited fluid output from the CVC but had minimal effect on the integrity of the decorated spongiome as observed by immunofluorescence. Such inhibition suggests that a V-ATPase is intimately involved in fluid segregation. Exposing *Paramecium* to 12°C or 1°C for 30 minutes resulted in the dissociation of the decorated tubules from the smooth spongiome that borders the collecting canals; thus the DS-1-reactive A4 antigen, the 75 kDa and 66 kDa antigens were all found dispersed in the cytosol. Fluid output in these cells also ceased. In summary, the results obtained from proton pump antibodies, a V-ATPase inhibitor and cold treatment showed that the pegs on the decorated spongiome of *Paramecium* are V_1 subunits of the V-ATPase and that these V_1V_0 complexes must remain active and in place for normal fluid segregation to occur.

Key words: concanamycin B, contractile vacuole complex, decorated spongiome, *Dictyostelium*, fluid segregation, *Paramecium*, V-ATPase, proton pump

INTRODUCTION

Though one of the first organelles to be visualized within *Paramecium* (see Patterson, 1980) or any cell following the development of the light microscope, the precise function of contractile vacuole complexes (CVCs) has yet to be resolved. It is generally assumed that this organelle system is involved in osmoregulation and that it accumulates and expels a hypotonic fluid. However, the ionic composition of this fluid and how fluid segregation is achieved are not known.

Recent studies have shown that the membranes of the CVC of *Dictyostelium discoideum* are enriched in vacuolar-type proton pumps (V-ATPase) (Heuser et al., 1993; Fok et al., 1993; Nolta et al., 1993). The V-ATPase is one of three major ATP-hydrolyzing proton pump classes known in nature (see Harvey, 1992; Nelson, 1992), possibly the primordial class (Nelson, 1994). These pumps have been visualized in replicas of quick-freeze freeze-dried preparations of *Dictyostelium* as 15 nm 'pegs' scattered liberally over the cytosolic surface of the membranes of the CVC. This has led to the suggestion that these V-type proton pumps may produce a proton gradient to drive other ions such as sodium, bicarbonate or ammonium ions into the CVC (Heuser et al., 1993). The presence of such pegs on the CVC membranes of *Paramecium* (McKanna, 1976) and *Vorticella* (McKanna, 1974) has been known for many years, based on thin-section electron-microscopic images. Pegs were also reported in the CVC of the fresh water sponge *Spongilla lacustris* (Brauer and McKanna, 1978). We have frequently observed indications of these pegs in our own studies of *Paramecium* both in thin-section and freeze-fracture electron microscopy (Hausmann and Allen, 1977; Allen et al., 1990). They decorate the bundles of membrane tubules that are collectively known as the decorated spongiome. These bundles are connected to a network of smooth tubules (collectively called the smooth spongiome) along the collecting canals. All of these structures are components of the 5 to 10 radial arms of each CVC.

Recently, we showed that in this ciliate these bundles of

decorated membrane tubules form the locus of fluid segregation (Ishida et al., 1993). In the present study and by using two antibodies known to be specific for the V_1 complexes in other cells, we show that these pegs on the decorated tubules of *Paramecium* are, in fact, the V_1 complexes of the V-ATPases. We also show: (1) that the ATPase 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 V_1V_0 complexes, causes the in vivo dissociation of the decorated tubules so that the antigens become dispersed throughout the cytosol.

MATERIALS AND METHODS

Materials

Cells of *Paramecium multimicronucleatum*, syngen 2, cultured in an axenic medium (Fok and Allen, 1979), were harvested at mid-log phase of growth. Concanamycin B, a potent and specific inhibitor of ATP-dependent acidification of macrophage lysosomes and endosomes (Woo et al., 1992), was a gift from Dr K. Miwa (Central Research Laboratories, Ajinomoto Co., Japan). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). All other chemicals were obtained from Sigma Chemical Co. (St Louis, MO).

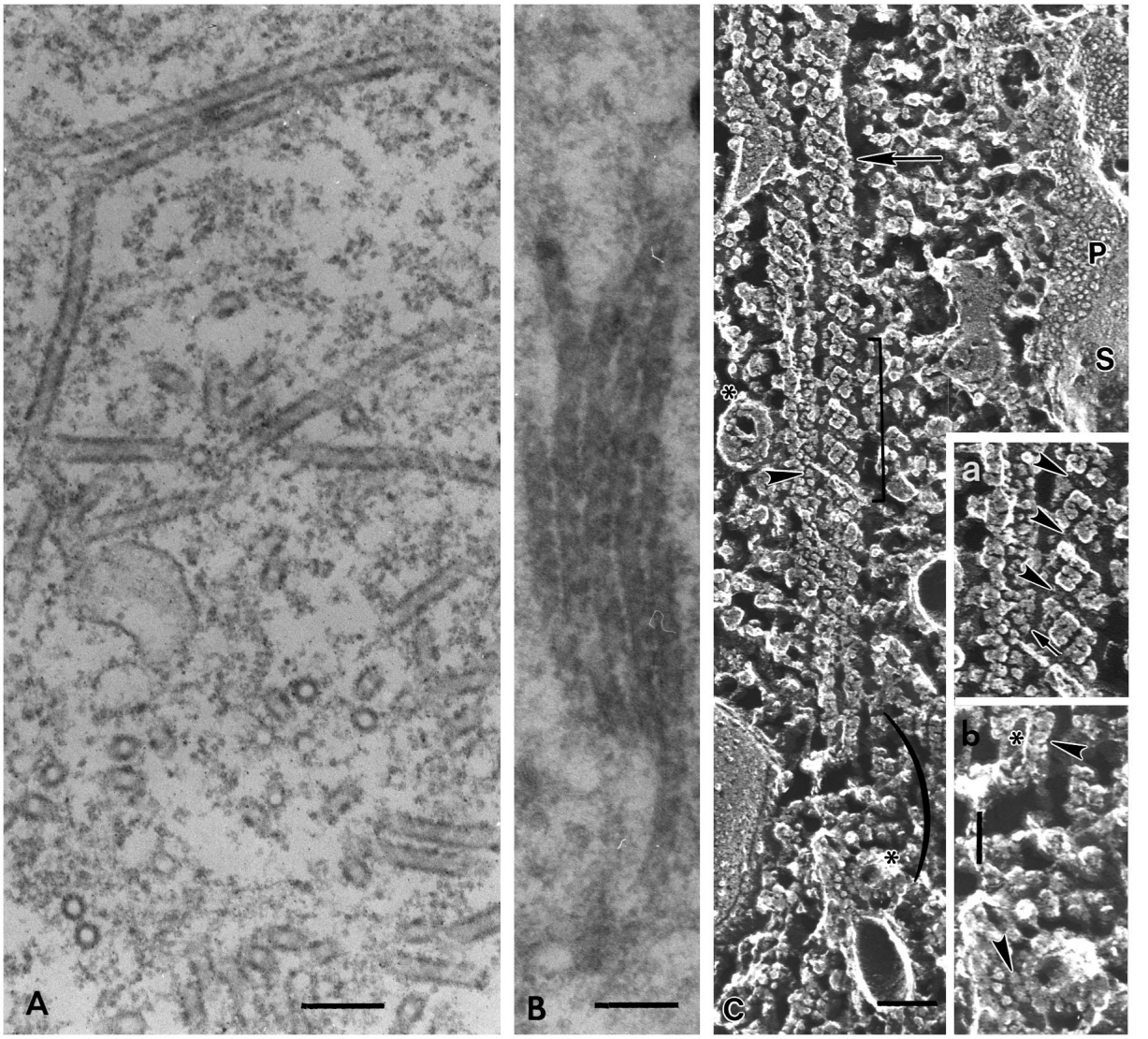

Fig. 1. The decorated tubules of the CVC of *Paramecium multimicronucleatum.* (A) Sections processed to show horseradish peroxidase and enhanced with merthiolate show the electron-opaque two-start helical pattern of pegs that wrap around the 50-nm tubules. Bar, 0.2μm. (B) These tubules are also easily recognized in lightly fixed cryo-sections. Bar, 0.2μm. (C) The pegs on the tubules are best seen in QF-DE images. Each helical ridge is composed of two rows of pegs (arrow). Each 15 nm peg appears to be composed of several subunits. The lumen of the tubules supports a number of smaller 11 nm globules (arrowhead) which may be the transmembrane extensions of the pegs or of the complexes to which the pegs attach. Asterisks, cross fractured tubules. P, P-fracture face of the collecting canal membrane. S, true luminal face of collecting canal membrane. Bar, 0.1 μm. Inset a, enlargement of area by square bracket. The ridges, each composed of two rows of pegs slanting around the tubule, are separated by grooves (arrowheads) which display small 'cogs' at the bottom of the grooves. Globular protrusions of 11 nm (arrow) also line the luminal aspect of the fractured tubules. Inset b, enlargement of area by curved bracket. A cross-factured and a tangential fractured (asterisk) decorated tubule bear 15 nm globular protrusions (arrowheads). The lumen of the tubules is 30 nm across. Bar, 0.05μm for insets.

Antibodies

Monoclonal antibody (mAb) DS-1, whose specificity has been described (Allen et al., 1990), was used to identify the decorated tubules of the decorated spongiome. For simplicity, the antigen to which DS-1 binds will be called A4. Monoclonal antibody SS-1 (Clone C38.2.2) was obtained according to standard procedures for hybridoma production (Kohler and Milstein, 1975). This mAb labeled the smooth spongiome at the light microscopic level and will be called the G4 antigen. In contrast to the DS-1-specific A4 antigen, the SS-1-specific G4 antigen was not reactive after glutaraldehyde fixation. Also, neither the A4 nor the G4 antigens under either reduced or non-reduced conditions were detectable on immunoblots.

A polyclonal antibody that was raised against the V-ATPase subunits of *Dictyostelium discoideum*, and affinity purified using purified V-ATPase (*Dictyostelium* proton pump antibody; Nolta et al., 1993) was used to identify the general location of the V-ATPase in *Paramecium*. A monospecific polyclonal antibody against the 57 kDa B-subunit of the V-ATPase of chromaffin granules (Nelson, 1992), referred to in this paper as anti-57 kDa antibody, was a gift from Dr N. Nelson (Roche Institute of Molecular Biology, Nutley, NJ). Antigens for these two polyclonal antibodies were determined using SDS-PAGE and immunoblotting as described (Fok et al., 1988).

Quick freeze-deep etch electron microscopy

Living cells were concentrated, placed on copper mounts and slam-frozen onto polished copper blocks at –195°C. Fractures, etching and platinum-carbon replicas were made as described (Allen et al., 1989). Replicas were viewed in a Zeiss 10A transmission electron microscope (TEM).

Fluorescence and electron microscopy

For indirect immunofluorescence experiments, *Paramecium* was fixed in 3% formaldehyde in 50 mM phosphate buffer (pH 7.2), and acetone permeabilized at –20°C for 20 minutes. These cells were then incubated sequentially with a primary antibody followed by a secondary antibody, as previously reported (Allen et al., 1990). For double labeling, cells were incubated first with a mixture of two primary antibodies, followed by their respective secondary antibodies. Cells were examined in a Zeiss light microscope (Thornwood, NY) equipped with epifluorescence illumination.

For immunoelectron microscopy, cells were fixed in 0.25% gultaraldehyde in 50 mM phosphate buffer (pH 7.2) at room temperature for 15 minutes. These cells were pelleted and embedded in 4% gelatin and processed for cryosectioning and immunogold staining as described (Allen et al., 1990). To reduce the nonspecific staining, all sections were preincubated with 5% normal goat serum and/or bovine serum albumin prior to incubation with a primary antibody.

Determination of the CVC's fluid output

The expulsion frequency and the diameter of the contractile vacuole were measured in cells whose motility was restricted. Fluid output of each contractile vacuole was obtained by multiplying its expulsion frequency by its maximum volume at late diastole (Ishida et al., 1993).

Concanamycin B treatment

A stock solution of concanamycin B (10 mM) in dimethylsulfoxide was diluted immediately before use in axenic culture medium which lacked phosphatidylethanolamine. Preliminary experiments had shown that the potency of this inhibitor was much lower when phosphatidylethanolamine was present. This could be caused by the binding of concanamycin B to the phospholipid vesicles thereby lowering its effective concentration. To determine the dose effect of concanamycin B on the total contractile-vacuole fluid output, cells were incubated in varying concentrations of concanamycin B while keeping the dimethylsulfoxide concentration at 0.01% or less.

Cold treatment

For cold treatment, cells in axenic culture medium were quickly cooled to 12°C or 1°C for 30 minutes. Cells were then fixed for immunofluorescence and cryoimmunogold labeling as above. For recovery experiments, cells were returned to room temperature and the fluid output of their contractile vacuoles was measured.

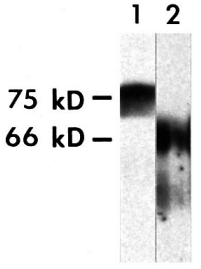

Fig. 2. A microsomal fraction was obtained by differential centrifugation and was processed for SDS-PAGE and immunoblotting. Nitrocellulose blots were incubated with either the *Dictyostelium* proton pump antibody (lane 1) or anti-57 kDa antibody (lane 2) and processed by enhanced chemiluminescence.

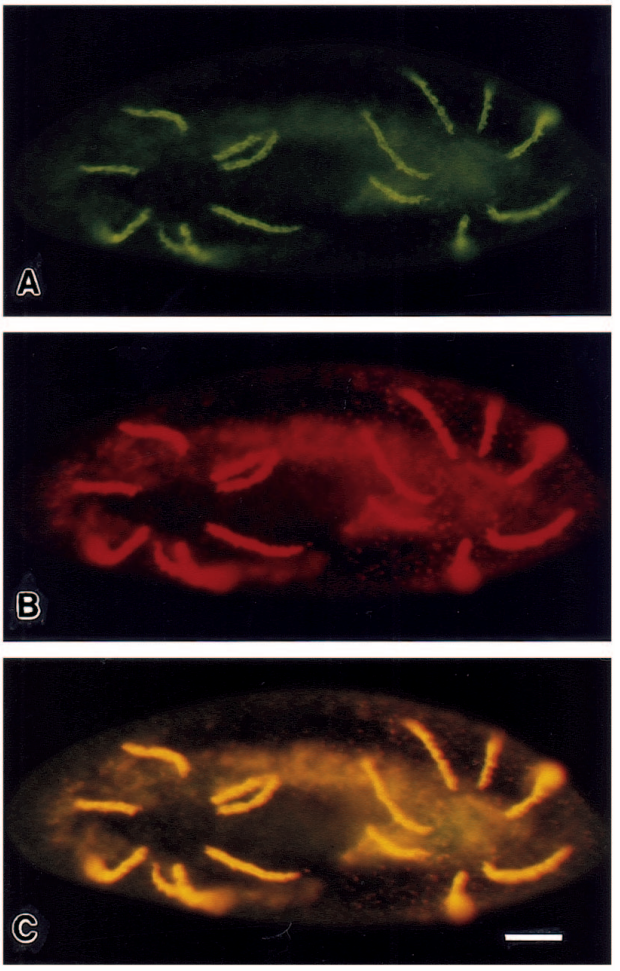

Fig. 3. Evidence of colocalization of the A4 antigen and the antigen reactive with the *Dictyostelium* proton pump antibody. (A) FITC labeling by *Dictyostelium* proton pump antibody. (B) Texas Red labeling by mAb DS-1. (C) Double exposure of FITC and Texas Red shows the complete overlap of these two antigens confirming their colocalization. Bar, 20 μm.

RESULTS

Quick freeze-deep etch and TEM studies of the decorated tubules and their pegs

Decorated tubules of the decorated spongiome of the CVCs of *Paramecium* are often arranged as bundles of 50 nm diameter, membrane-bound cylinders. These cylinders are 'decorated' by rows of ~15 nm globular pegs helically wound around their cytosolic surfaces when viewed in both conventional thin sections (Fig. 1A) as well as in cryo-thin sections (Fig. 1B). However, they can be visualized in greatest detail using the quick-freeze deep-etch (QF-DE) technique. By QF-DE, it can be seen that ridges formed from a two-start helical pattern of 30 nm wide compact protrusions are separated by grooves of 10 nm in width (arrow, Fig. 1C). This is reminiscent of the F_1F_0 subunits on the mitochondrial cristae of the same cell, although a one-start helix with a longer pitch is found on the cristae (Allen et al., 1989). Each ridge is composed of a double row of pegs spaced at 15 nm (center-to-center). Thus, using a formula for the length of a helix (Allen et al., 1989), the number of pegs along a decorated tubule can be calculated to be ~467/μm. For each micrometer of a decorated tubule, the membrane surface area and the enclosed volume (inset b, Fig. 1C) are about 0.16 μm^2 and 7×10^{-4} μm^3, respectively. Thus, the ratios of membrane surface to luminal volume and of pegs to luminal volume are very high along the length of these tubules.

At high magnification in our best QF-DE replicas, each peg appears to be composed of several subunits, and many resemble tetrads (arrow, Fig. 1C) or three rows of diads (insert a, Fig, 1C). In favorable fractures, apparent luminal extensions of the protein complexes can be seen in a region where the tubule has been fractured obliquely (arrowhead, Fig. 1C, and small arrow in inset a). These luminally exposed globular units are 11 nm in diameter.

To estimate the cell's total load of pegs on its two CVCs, we obtained a rough approximation of the total length of decorated tubules in 16 randomly selected cross sections of CVC radial arms. The average total length of such tubules was determined to be roughly 16 μm per 70 nm thick cryosection. Earlier measurements had shown the average length of the decorated spongiome obtained from measuring 505 radial arms to be 37.4 μm in interphase cells (Allen et al., 1990). From these numbers we could roughly estimate that there were 8,600 μm of decorated tubules per radial arm. At 467 pegs per μm of decorated tubules, an average radial arm would contain around 4 million pegs. Since there are 5 to 10 radial arms per CVC, and two CVCs per cell, a rough estimate of the total load of pegs per cell would be between 40 and 80 million.

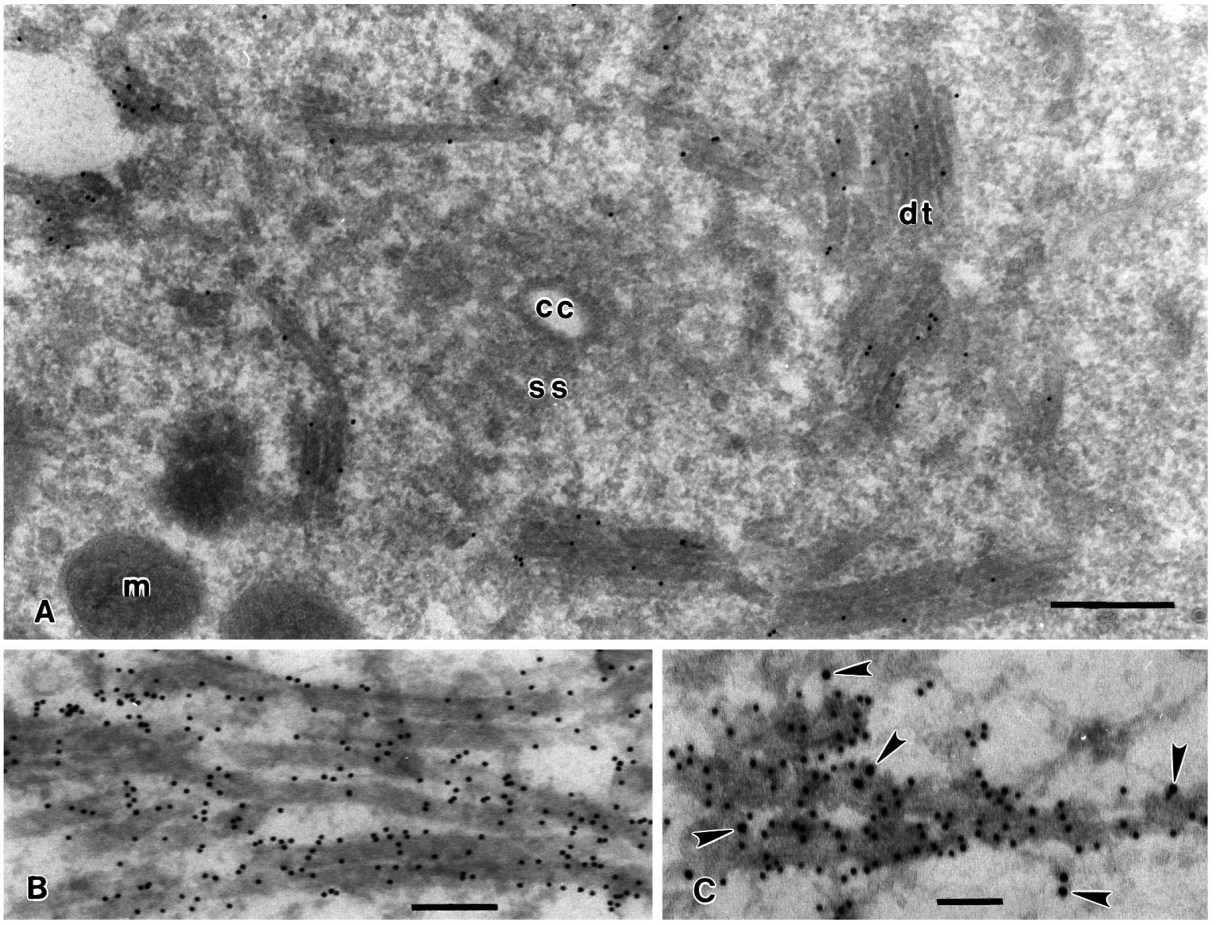

Fig. 4. Immunogold-labeled cryosections of the decorated tubules. (A) A cross section of a radial arm of a CVC labeled with mAb DS-1. Only the decorated tubules (dt) are labeled with gold. cc, collecting canal; ss, smooth spongiome; m, mitochondrion. Bar, 0.5 μm. (B) Tubules labeled with *Dictyostelium* proton pump antibody. Bar, 0.2 μm. (C) Decorated tubules double-labeled with DS-1, 18 nm gold (arrowheads), and *Dictyostelium* proton pump antibody, 12 nm gold, show that both antibodies label the same structure. Bar, 0.1 μm.

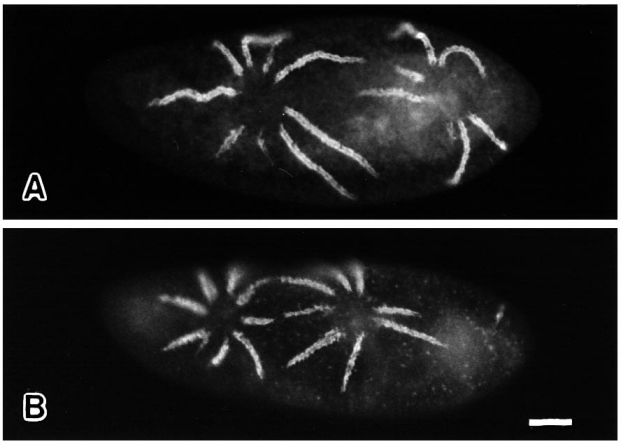

Fig. 5. Cells labeled with FITC showing (A) the location of the 66 kDa polypeptide, the antigen to the anti-57 kDa antibody and (B) the unaltered appearance of the decorated spongiomes following concanamycin B treatment. Bar, 20 μm.

Localization of the proton pump antigens on the decorated tubules

Previous work in our laboratory has shown that mAb DS-1 to the A4 antigen specifically labels the decorated tubules of the CVC (Allen et al., 1990). Unfortunately, A4 was shown to be unreactive on blots of either whole cell homogenates or isolated membrane fractions known to be enriched in the decorated tubules. Thus the identity of this antigen could not be determined. To determine if the pegs were part of the V-ATPase, we used antibodies specific for the V-type proton pumps of other organisms to see if they would cross-react with the decorated tubules of *Paramecium*. The *Dictyostelium* proton pump antibody reacted with a 75 kDa polypeptide in blots of *Paramecium* (Fig. 2, lane 1) and strongly labeled the decorated tubules (Figs 3 and 4). Double labeling showed that both the A4 (Fig. 3B) and the 75 kDa antigens (Fig. 3A) were located on this decorated spongiome, while double exposure showed the colocalization most dramatically (Fig. 3C). Similar results were obtained using cryo-thin sections where antigens were located on the decorated tubules singly (Fig. 4A,B) or in double labeling experiments (Fig. 4C).

To confirm that the decorated tubules have authentic V-type proton pump polypeptides, we obtained a monospecific polyclonal antibody to the 57 kDa B-subunit of the V_1 complex of chromaffin granules. Immunoblotting showed that this antibody cross-reacted with a somewhat larger 66 kDa polypeptide in *Paramecium* (Fig. 2, lane 2). This anti-57 kDa antibody gave essentially the same labeling pattern as mAb DS-1 (compared Fig. 5A with Fig. 3B) or as the *Dictyostelium* proton pump antibody by both immunofluorescence (compare Fig. 5A with Fig. 3A) and immunogold labeling (Fig. 6D).

With all three antibodies, the label was mostly on the cytosolic sides of the tubules of the decorated spongiome where the 15 nm pegs are located (Fig. 6A-D). None of the membranes of the smooth spongiome, the collecting canals, or the contractile vacuole itself was labeled, and nonspecific background labeling was minimal. These results showed that the pegs on the decorated tubules of *Paramecium* are, beyond doubt, components of a V-ATPase.

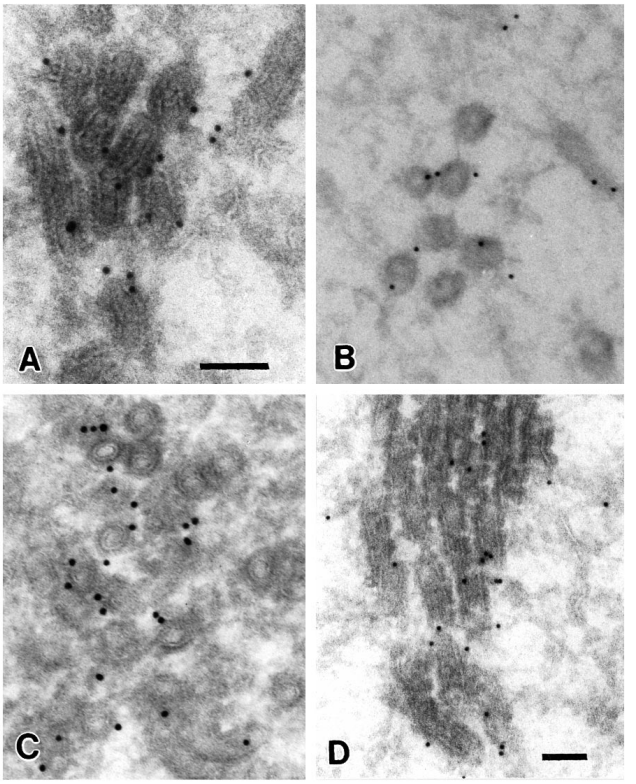

Fig. 6. Immunogold is located almost exclusively at the outer surface of cryosectioned decorated tubules. (A,B) Transverse sections labeled by the *Dictyostelium* proton pump antibody. (C) DS-1 mAb and (D) the anti-57 kDa antibody. Bars, 0.1 μm.

Effects of concanamycin B on the structure and fluid output of the CVC

Concanamycin B is a macro-cyclic lactone structurally related to the V-ATPase inhibitor bafilomycin A. The latter drug was the first specific V-ATPase inhibitor to be discovered and was found to be effective in fungi, plants, and in animal cells (Bowman et al., 1988), but it had no noticeable effect on the function of the CVC in *Paramecium* (unpublished observation). Concanamycin B, which was found to potently and specifically inhibit the V-ATPase activity of endosomes and lysosomes in J774 macrophages (Woo et al., 1992), was shown to reversibly block the CVC function in *Dictyostelium discoideum* (Heuser et al., 1993). In the present study, we found that it also inhibited the fluid output of the contractile vacuoles of *P. multimicronucleatum* in a dose-dependent manner after cells were exposed to this drug for 30 minutes (Fig. 7A). Half maximal inhibition was achieved at approximately 15 nM of the drug. In one experiment where cells were exposed to 30 nM concanamycin B for 30 minutes and then transferred to a hypotonic solution containing this same concentration of drug, the cells became swollen and lysed (data not shown). Thus, these cells were not able to protect themselves from a hypo-osmotic shock when the V-ATPase was inhibited.

The inhibitory effect of concanamycin B on fluid output in *Paramecium* was reversible. After a 30 minute incubation in 30 nM concanamycin B, cells transferred to a fresh axenic culture medium began to recover their CVC's activity soon

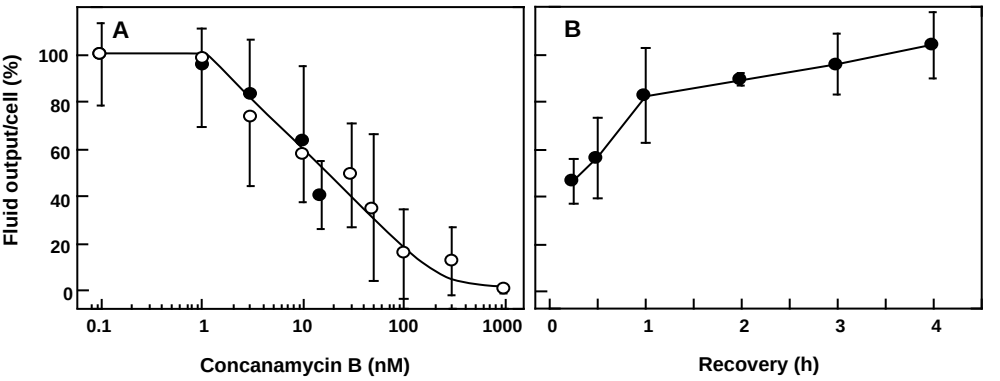

Fig. 7. Fluid output was obtained in cells whose movement was restricted by multiplying the maximal diameter of the contractile vacuole at late diastole by the expulsion frequency. Each point represents the mean ± s.d. of fluid output from 10 CVCs in 5 cells. The control fluid output was 12.5 pl/cell per minute. (A) Cells were exposed to 0.1 to 1,000 nM concanamycin B for 30 minutes before measurements were taken. The open and filled circles represent data from two separate experiments. (B) After 30 minutes exposure to 30 nM concanamycin B, cells were washed and fluid output was determined.

after washout. Recovery was essentially complete within 3 hours. This dramatic and reversible inhibitory effect produced by concanamycin B indicated that an ATP-dependent acidification mechanism, the putative V-ATPase, is intimately involved in the water segregation of the CVC of *Paramecium*.

To test for the effects of concanamycin B on the structure of the CVCs, cells were treated with this drug (30-1,000 nM) for 30 minutes, and then fixed and processed for immunolabeling with mAb DS-1. Interestingly, concanamycin B had almost no effect on the structure of the decorated spongiome (Fig. 5B).

Effects of cold treatment on CVCs

It is known that cold causes the dissociation of some isolated proton pump complexes into their subunit polypeptides. This was first shown for the F_1F_0-ATPase of mitochondria (Pullman et al., 1960) and eubacteria. More recently, it has also been shown that cold together with ATP and $MgCl_2$ cause the dissociation of the V_1 from the V_0 subunit of the V-ATPase of chromaffin granule membrane vesicles (Moriyama and Nelson, 1989). To see what effect cold treatment would have on the decorated tubules in vivo, *Paramecium* was incubated at 12°C or 1°C for 30 minutes, fixed and then processed for immunofluorescence using mAbs DS-1 and SS-1. Functionally, when the cells were at 1 °C for 30 minutes, all fluid output stopped. However, upon warming, fluid output rapidly resumed, returning to normal in a few hours.

The morphology of the decorated spongiome in these cells could be divided into three categories. The first category consisted of cells with normal morphology (Fig. 8A). The second category consisted of cells with remnants of the

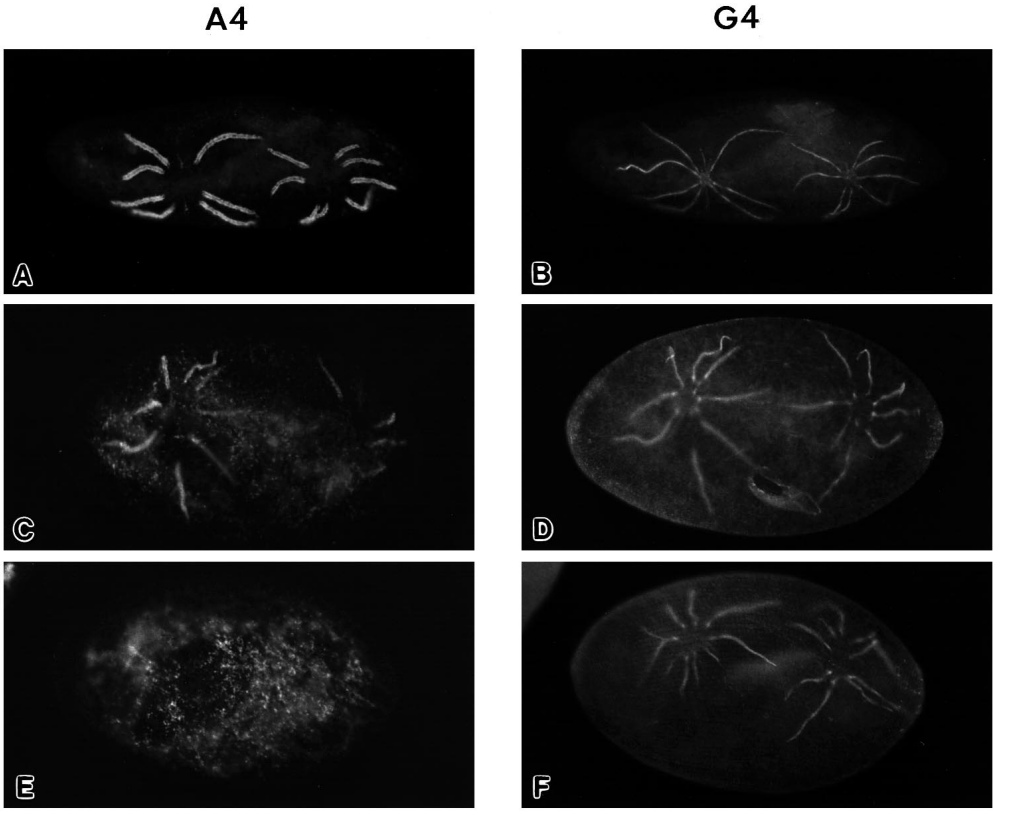

Fig. 8. Effect of cold treatment (1°C for 30 minutes) on the CVC of *Paramecium*. Cells were double labled with mAb DS-1 to the A4 antigen (FITC; A,C and E) and mAb SS-1 to the G4 antigen (Texas Red; B,D and F). (A and B) Control cell. (C and D) A cell showing partial dissociation of the decorated spongiome (C) and an unchanged smooth spongiome (D). (E and F) An example of complete dissociation of the decorated spongiome (E) but an intact smooth spongiome (F). Bar, 20 μm.

decorated spongiome along the radial arms but the decorated spongiome appeared to be in the process of dissociating or disintegrating (Fig. 8C). The last group consisted of cells that had no discernible decorated spongiome and the A4 antigen was dispersed throughout the cytosol (Fig. 8E). That the smooth spongiome was still intact in all cases is shown in Fig. 8B,D and F. The distribution of cells in the three structural categories is shown in Fig. 9. At ambient temperature, less than 10% of the cells were in category 2. Some of these cells might have been in the process of cell division, as it is known that the CVCs undergo partial dissociation (Allen et al., 1990) during this stage. After 30 minutes at 12°C, almost half of the cells had a decorated spongiome that belonged to category 2. When cells were exposed to 1°C, almost 90% of the cells had a decorated spongiome belonging to either category 2 or 3. Fig. 10 shows that the decorated spongiome is entirely absent in a cell exposed to 1°C for 30 minutes that was in category 3. These data verified that cold temperature had little effect on structures of the CVC other than the decorated spongiome which, for the most part, was dissociated from the smooth spongiome (Fig. 10). However, whether the V_1 subunits themselves were dissociated from their basepieces in the dispersed decorated spongiome could not be determined in this study.

Identical results were obtained when these cells were labeled with either *Dictyostelium* proton pump antibody or the anti-57 kDa antibody (data not shown).

DISCUSSION

It has been known for some time that the decorated tubules of the CVCs in *Paramecium* are decorated with pegs (McKanna, 1976). Using QF-DE we confirm here that the decorated tubules support two helically wound arrays of pegs (Fig. 1C). These pegs are similar to those in the CVC of *Dictyostelium* in both appearance and in size. The pegs in *Dictyostelium* were identified as the V-ATPase by showing their cross-reactivity with the antibody to the B subunit of the V-ATPase of chromaffin granules (Heuser et al., 1993). However, while it might be reasonable to speculate that the pegs on the decorated tubules of the CVCs in *Paramecium* are also proton pumps, definitive evidence for this conclusion was lacking.

In this report, we provide four lines of evidence to show that the pegs on the decorated tubules of *Paramecium* are parts of these proton pumps. Firstly, these pegs have a topography and structural complexity that closely resembles the F_1F_0 proton pumps on the cristae of the mitochondria of *Paramecium* (Allen et al., 1989). Both the pegs on the CVC and the proton pumps in mitochondria are complex globular units of ~15 nm and 10 nm, respectively, which are located on the cytosolic or matrix sides of the membrane. At high magnification in QF-DE replicas, the pegs appear similar to the F_1 complex in the mitochondria and each peg appears to be composed of several subunits. Pegs in both the CVC and in the mitochondria exist as two closely spaced rows that wind around the supporting membrane tubules in a helical pattern, although, in the CVC the pegs are arrayed in a two-start helical pattern. Secondly, an affinity-purified *Dictyostelium* proton pump antibody and an antibody to the B subunit of the chromaffin granule V-ATPase both cross-react with these pegs. Immunological labeling of cryosections shows that the antigens are located predominantly at the surface of the membrane tubules where the pegs of the decorated spongiome are located (Fig. 6A-D). These results show that the pegs are probably part of the V_1V_0 proton pumps. Thirdly, concanamycin B, a specific V-ATPase inhibitor, completely and reversibly inhibited the fluid output of the CVCs. Lastly, subjecting cells to a cold temperature results in the dissociation of the decorated spongiome from the smooth

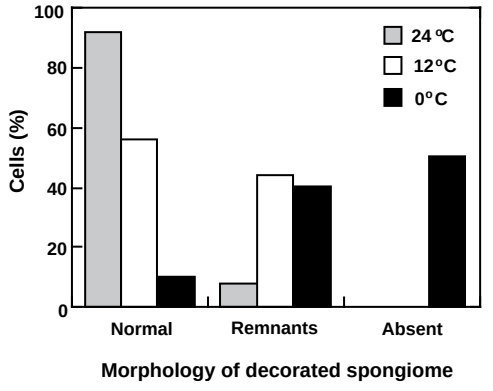

Fig. 9. Cells were first exposed to 24°C, 12°C or 0°C for 30 minutes, fixed with 3% formaldehyde in phosphate buffer (0.05 M, pH 7.6) and processed for indirect immunofluorescence observation. The morphology of the decorated spongiome in the treated cells was divided into 3 categories: 1, normal morphology; 2, with remnants of decorated spongiome remaining; and 3, no discernible decorated spongiome. For each temperature 100 CVCs in 50 cells were scored.

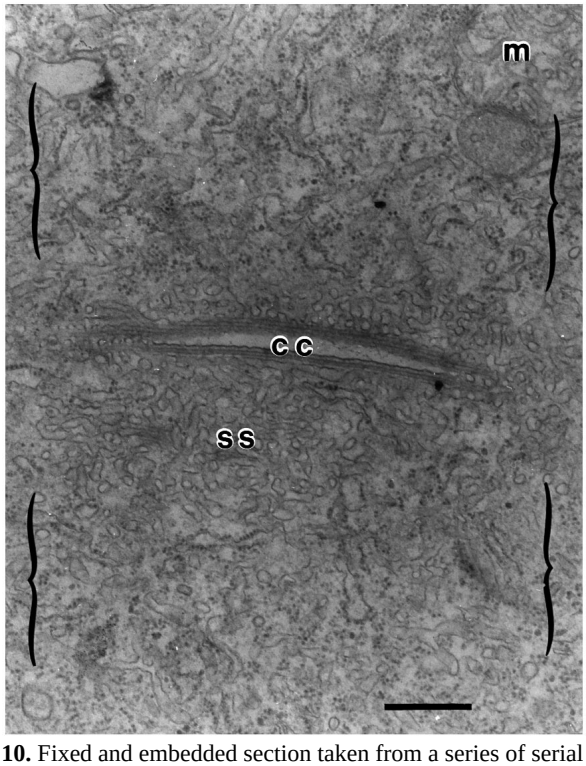

Fig. 10. Fixed and embedded section taken from a series of serial sections of a radial arm of the CVC in a cell that was exposed to 1°C for 30 minutes. The collecting canal (cc) and smooth spongiome (ss) appear to be unchanged but there are no decorated tubules (between brackets) to be seen (this was true of the full length of the radial arm). m, mitochondrion. Bar, 0.4 μm.

spongiome so that the A4 antigen, as well as the 75 and 66 kDa polypeptides of the proton pump are found dispersed in the cytosol instead of being specifically associated with the intact smooth spongiome along the collecting canals.

What is the significance of the proton pumps on the decorated tubules?

In this study, we determined that *Paramecium*, like *Dictyostelium*, has a large number of pegs/proton pumps associated with its CVC membranes. However, unlike *Dictyostelium* where most of the membrane compartments of the CVC bear the pumps, the pumps in *Paramecium* are found only on the decorated spongiome, the locus of water segregation in this cell. The smooth spongiome, collecting canals, ampullae and the contractile vacuole, presumably have other functions such as ion exchange, fluid transport, fluid storage, or membrane storage, as they are normally devoid of proton pumps. Thus, it appears there may be even more 'division of labor' in the CVC of *Paramecium* than has been described in the CVC of *Dictyostelium* (Nolta and Steck, 1994).

The decorated tubules in *Paramecium* have an extremely high proton pump-to-luminal volume ratio. We estimate there are 6.6×10^5 pumps per μm^3 of lumen or 467 pumps per μm of tubule length. This comes out to 1 proton pump for every 1,500 nm^3, which is roughly the volume of one V_1 complex. One *Paramecium* cell of average size has roughly 1.3×10^5 μm of total decorated tubule length or a total of 40 to 80 million proton pumps in its two CVC. Such a large number of pumps around a total volume of 85.7 μm^3 has the potential of moving a great number of protons into a very small volume within a very short time. Yet the pH of the culture medium in which the *Paramecium* cells are multiplying exponentially and which contains the fluid expelled by the CVs not only does not become acid but actually becomes slightly alkaline with time (A. K. Fok, unpublished observation). Thus, any protons generated by these pumps must be quickly removed from solution or neutralized by anions present in the CVC. It seems reasonable to begin to look for ion exchangers in the CVC which could be driven by the potential energy developed by this very large number of proton pumps.

V-ATPase activity and integrity of the decorated spongiome are necessary for fluid segregation

Our results from the experiments using concanamycin B, a specific V-ATPase inhibitor, show that this ATPase activity is necessary for fluid segregation to continue. This drug strongly inhibits fluid segregation, but has minimal effect, if any, on the integrity of the decorated tubules. On the other hand, cold treatment results in the loss of the decorated spongiome from around the smooth spongiome without affecting the smooth spongiome itself. In preliminary TEM experiments we have seen evidence of vesiculated decorated tubules which apparently have lost their V_1 complexes as they are no longer labeled with mAb DS-1. These experiments as well as the effects of hyperosmotic treatment on the CVC are currently under study.

In summary, while our earlier paper showed that the decorated tubules are the sites of fluid segregation, the results of the present report demonstrate that the pegs on the decorated tubules are proton pumps whose integrity and whose ATPase activity are essential for ongoing fluid segregation.

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