Proton Pumps Populate the Contractile Vacuoles of *Dictyostelium* Amoebae

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Abstract. Amoebae of the eukaryotic microorganism *Dictyostelium discoideum* were found to contain an interconnected array of tubules and cisternae whose membranes were studded with 15-nm-diameter "pegs." Comparison of the ultrastructure and freeze-fracture behavior of these pegs with similar structures found in other cells and tissues indicated that they were the head domains of vacuolar-type proton pumps. Supporting this identification, the pegs were observed to decorate and clump when broken amoebae were exposed to an antiserum against the B subunit of mammalian vacuolar H^+-ATPase. The appearance of the peg-rich cisternae in quick-frozen amoebae depended on their osmotic environment: under hyperosmotic conditions, the cisternae were flat with many narrow tubular extensions, while under hypo-osmotic conditions the cisternae ranged from bulbous to spherical. In all cases, however, their contents deep etched like pure water. These properties indicated that the interconnected tubules and cisternae comprise the contractile vacuole system of *Dictyostelium*. Earlier studies had demonstrated that contractile vacuole membranes in *Dictyostelium* are extremely rich in calmodulin (Zhu, Q., and M. Clarke. 1992. *J. Cell Biol.* 118: 347–358). Light microscopic immunofluorescence confirmed that antibodies against the vacuolar proton pump colocalized with anti-calmodulin antibodies on these organelles. Time-lapse video recording of living amoebae imaged by interference–reflection microscopy, or by fluorescence microscopy after staining contractile vacuole membranes with potential-sensitive styryl dyes, revealed the extent and dynamic interrelationship of the cisternal and tubular elements in *Dictyostelium*'s contractile vacuole system. The high density of proton pumps throughout its membranes suggests that the generation of a proton gradient is likely to be an important factor in the mechanism of fluid accumulation by contractile vacuoles.

THE contractile vacuoles in protozoa have long remained a mystery. They can be seen by light microscopy to fill and empty repetitively, and to do so more rapidly when the cells are placed in hypotonic media, suggesting that they are water-excretory organelles (Lloyd, 1928; Kitching, 1938; Patterson, 1980; Zeuthen, 1992). However, the mechanism by which they extract water from the cytoplasm has never been explained. Transfer of water from the cytoplasm to a more dilute environment inside a vacuole would seem to require a water pump, but no such entity is known. Therefore, most observers have suggested that contractile vacuoles accumulate water osmotically, via active transport of cytoplasmic ions into the contractile vacuole (for review see Zeuthen, 1992). The low ionic strength of contractile vacuole fluid is postulated to result from active reabsorption of these ions back into the cytoplasm, leaving water behind in the vacuole (Schmidt-Nielsen and Schrauger, 1963; Riddick, 1968; Mayer and Iverson, 1967). However, this would require the contractile vacuole membrane to carry out the seemingly impossible task of retaining water against an osmotic gradient, something that even the most impermeable artificial lipid bilayer cannot do (Finkelstein, 1984; Walter and Gutkneck, 1986). Contractile vacuole membranes do have special features, including a high content of alkaline phosphatase and a high calmodulin-binding activity (Bowers and Korn, 1973; Quiviger et al., 1978; Zhu and Clarke, 1992), but these properties have not suggested any solution to the enigma of how they accumulate water.

In spite of this mystery, considerable insight into contractile vacuole function has been provided by recent advances in light microscopy. Using interference–reflection microscopy (IRM),[1] Gingell and colleagues (1982) showed that *Dictyostelium* amoebae spread on glass displayed localized dark regions that typically took the form of one or more relatively large disks surrounded by a number of exceedingly thin threads. When the amoebae were photographed at ~1-min intervals, the dark disks and threads were seen to change form rapidly, making reversible connections with each other. Moreover, the dark disks abruptly disappeared every min or so, just like the phase-lucent contractile vacuoles visible by phase contrast microscopy. Gingell and co-workers inter-

1. *Abbreviations used in this paper*: IRM, interference–reflection microscopy; TEM, transmission electron microscope.

preted the IRM images to indicate that the disks were vacuoles flattened against the cell surface, creating a slab of intervening cytoplasm narrow enough to yield a black zero-order interference pattern. Consistent with this interpretation, the disks showed restricted lateral movement and tended to disappear and reappear always at the same sites, suggesting that they were adherent to the cell surface.

The present study has confirmed these important observations of Gingell and co-workers, demonstrating that the contractile vacuole system in *Dictyostelium*, like other protozoa (Patterson, 1980), is composed of vacuolar reservoirs interconnected by tubular ducts. This contrasts with an earlier view that the contractile vacuoles of *Dictyostelium* arose from the fusion of smaller "satellite vesicles" (de Chastellier et al., 1978). We also show that *Dictyostelium* contractile vacuole membranes are distinguished by an exceptionally high content of vacuolar-type proton pumps. The proton pumps may account for the remarkable ability of contractile vacuole membranes to transport and sequester excess cellular water. We suggest that contractile vacuoles cotransport bicarbonate ions along with the protons they pump, which would allow them to sequester water in their lumens in the form of an isotonic solution of bicarbonate (see Maren, 1988). Bicarbonate is perhaps the only ion that a protozoal cell living in a dilute environment could afford to mass export along with excess water.

Materials and Methods

Strains and Culture Conditions

Dictyostelium discoideum, strain NC4, was maintained in association with *K. aerogenes*, either in suspension or on SM nutrient agar plates (Loomis, 1975). To obtain newly germinated cells, sori were collected from mature fruiting bodies and suspended in bacterial conditioned buffer (Zhu and Clarke, 1992); the amoebae were examined 2 to 3 h later, shortly after emergence from their spore coats. The axenic strains AX2 and AX3 were grown on HL5 medium (Clarke et al., 1980), either in suspension or on tissue culture plates. Suspension cultures were swirled on a rotary shaker at 150 rpm. All cultures were maintained at 21–22°C.

Fixation and Staining Conditions for Indirect Immunofluorescence

The agar-overlay technique described by Fukui et al. (1987) was used for flattening the amoebae. For early experiments, fixation was in 1% formaldehyde in methanol (−15°C, 5 min) as previously described (Zhu and Clarke, 1992). For later experiments, a two-step procedure was used (2% buffered formaldehyde, room temperature, 5 min, followed by formaldehyde-methanol as above). In some instances, 0.1% DMSO was included during the first fixation step. The buffer used for the initial fixation varied. Cells to be stained for calmodulin alone were fixed in standard HL5, which is buffered with phosphate. Cells to be stained for vacuolar H^+-ATPase as well as calmodulin were fixed in HL5 that had been buffered with Bis-Tris (10 mM, pH 7.1) rather than phosphate; alternatively, they were fixed in HM buffer (20 mM Hepes/NaOH, pH 7.0; 2 mM $MgCl_2$). Antibody incubation and washing conditions were as previously described (Clarke et al., 1987). Polyclonal rabbit antiserum specific for the 57 kD (B subunit) of mammalian and yeast vacuolar H^+-ATPase (Moriyama and Nelson, 1989; Nelson and Nelson, 1990; Noumi et al., 1991) was provided by Dr. Nathan Nelson (Roche Institute of Molecular Biology; Nutley, NJ). The antiserum was preadsorbed with fixed bacteria (*K. aerogenes*) as previously described (Clarke et al., 1987). For immunofluorescence experiments, the preadsorbed antiserum was used at a dilution of 1:200. The secondary antibody was FITC-conjugated sheep anti-rabbit IgG (Cappel Laboratories, Malvern, PA), diluted 1:1000. Calmodulin was stained with the mouse mAb 2D1 as previously described (Zhu and Clarke, 1992). For double-staining experiments (calmodulin and vacuolar H^+-ATPase), the two primary antibodies were mixed, as were the two secondary antibodies. Appropriate controls were performed to ascertain that the secondary antibodies did not bind to the inappropriate primary antibody or to each other.

Immunoblotting Procedure

AX3 cells (100 ml at a density of 4×10^6 cells/ml) were harvested by centrifugation and washed once in 20 mM Tris-MOPS, pH 7.0, containing 0.25 M sucrose; the cells were suspended in 1.5 ml of the same buffer and lysed by Dounce homogenization at 4°C. The lysate was denatured and electrophoresed on an SDS polyacrylamide gel containing 10% acrylamide (Laemmli, 1970); the equivalent of 1×10^6 cells was loaded per lane. After electrophoresis, the proteins were transferred to PVDF membrane (Millipore Immobilon P) using the conditions described by Towbin et al. (1979). The membrane was blocked with 2% (wt/vol) BSA (37°C, 1 h), incubated with the vacuolar H^+-ATPase antiserum (1:1,000 dilution, 37°C, 1 h), and then with peroxidase-labeled goat anti-rabbit IgG (1:1,000 dilution, 37°C, 1 h). Washing conditions and visualization of the reaction product were as previously described (Hulen et al., 1991). Results are shown in Fig. 1.

EM of Ruptured and Freeze-dried Amoebae

Amoebae growing in suspension in HL5 were washed 2× by gentle centrifugation and resuspension in "Dicty PO_4" buffer (20 mM KH_2PO_4/Na_2HPO_4, pH 6.5, containing 1 mM $MgCl_2$) then placed on 3 × 3-mm glass coverslips that were freshly washed in chromic acid and treated with 10 mg/ml polylysine (400 kD) just before use. After 30–60 s of adherence, excess amoebae were rinsed off the glass with Dicty PO_4 buffer and the coverslips were transferred to "intracellular stabilization buffer" (70 mM KCl; 30 mM Hepes/KOH, pH 7.2; 5 mM $MgCl_2$; 2 mM EGTA; 1 mM DTT; and 0.01 mg/ml PMSF). The cells were ruptured by momentary exposure to a fine-tipped ultrasonic probe, or by rapid flow of buffer past them from a 27-gauge needle, or most successfully by inverting the coverslip and pressing it firmly against a waxed surface to squash the cells. Immediately after any of these breakage protocols, coverslips were transferred to 2% glutaraldehyde fixative in the same intracellular stabilization buffer. After ~1 h they were rinsed 5–6 times in distilled water and quick-frozen by impact against an ultra-pure copper block cooled to liquid helium temperatures, using a homemade freezing press (Heuser et al., 1979). They were then stored in liquid nitrogen until ready for mounting in a Balzer's freeze-etch machine, whereupon they were freeze-dried by maintenance in vacuo at −80°C for 15 min. They were then rotary replicated with 2 nm of platinum evaporated from an electron beam gun mounted at 24° above the horizontal and were "backed" with 4–6 nm of pure carbon evaporated from a carbon-arc source. The coverslips were then removed from the vacuum, warmed to room temperature, and floated momentarily on full-strength hydrofluoric acid to release the replicas from their surface. Each replica was washed by flotation on water, then on household bleach, then

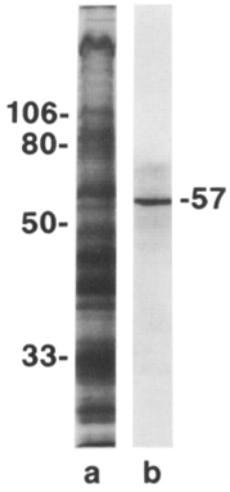


Figure 1. Specificity of the proton pump antiserum. *Dictyostelium* cells (strain AX3) were lysed, and the proteins were separated by SDS-PAGE; each lane received 1×10^6 lysed cells. Lane *a* shows a strip of the gel stained for total protein using Coomassie brilliant blue, and lane *b* shows an immunoblot of the proteins from a similar strip, transferred to a membrane and probed with an antiserum against the B subunit (57 kD) of mammalian vacuolar H^+-ATPase (Moriyama and Nelson, 1989). Migration positions of pre-stained molecular weight standards (Bio-Rad Labs., Hercules, CA) are shown to the left: phosphorylase b (106 kD), BSA (80 kD), ovalbumin (49.5 kD), and carbonic anhydrase (32.5 kD). The proton pump antiserum recognized a *Dictyostelium* polypeptide with an apparent molecular mass of 57 kD.

on water again, and finally picked up on a Formvar-coated 75 mesh grid. This was viewed in a standard transmission electron microscope (TEM) operated at 100 kV and photographed in stereo at ± 10° tilt. Final electron micrographs were printed in reverse-contrast to make platinum deposits look white.

EM of Intact Amoebae

Amoebae were gently centrifuged into soft pellets in HL5 medium and then immediately quick-frozen with the aforementioned freezing press (Heuser et al., 1979). After mounting in a Balzer's freeze-etch machine, they were warmed to −100°C and carefully freeze-fractured through the most superficial few micrometers of the pellet, where freezing would be optimal. Next they were deep-etched by simply leaving them in vacuo for 2–3 min at −100°C before replication with a 2-nm layer of platinum rotary deposited from 18° above the horizontal (Heuser, 1980). After backing with 4–6 nm of pure carbon rotary deposited from 75° above the horizontal, the pellets were thawed and floated on household bleach to release the replicas from the underlying organic material. These were washed by flotation on several changes of water, picked up on EM grids, and subjected to TEM analysis and photography as described above for the freeze-dried preparations.

Vital Staining of Dictyostelium Contractile Vacuoles with Styryl Dyes

Amoebae were gently pelleted out of HL5 axenic medium, resuspended in Dicty PO_4 buffer and immediately placed on untreated 22 × 40-mm no. 1 glass coverslips. After 30–60 min, when they had adhered to the glass and spread out well enough for viewing, the coverslips were inverted onto a glass slide made into a narrow flow-cell by two strips of vacuum grease (see Zigmond and Sullivan, 1979). They were viewed with a 63×, 1.4 NA phase-contrast objective and photographed with a Hamamatsu video camera (model 2400 SIT; Hamamatsu Photonic Sys. Corp. Bridgewater, NJ) coupled through an Argus 10 image processor to a Panasonic TQ3038F optical memory disk recorder (OMDR). For vital staining of contractile vacuoles, the styryl dye FM 4-64 (Molecular Probes, Inc., Eugene, OR) diluted to 1 μg/ml in Dicty PO_4 buffer was flowed under the coverslip, and epifluorescence imaging with a 100×, NA 1.4 bright-field objective and a rhodamine filter set was immediately initiated (Grinvald et al., 1988; Betz et al., 1992). Throughout video recording, illumination was kept to the absolute minimum needed to obtain a decent video image after 8-frame averaging and strong contrast enhancement of the output of the highly sensitive SIT camera (nothing being visible by human eye in the microscope). These precautions were mandatory to avoid photo damaging the dye-stained amoebae, this being first recognizable as a cessation of contractile vacuole activity (see Forget and Couillard, 1983). Time-lapse video records of dye redistribution from the plasma membrane to contractile vacuoles and subsequent contractile vacuole filling and discharge were obtained by recording one frame per second onto the OMDR and then playing them back at normal video rate (30 frames per second). Under optimal conditions, this permitted as many as 10 contractile vacuole cycles to be viewed before the dye began to stain the endosomes of the amoebae as well. Our development of this

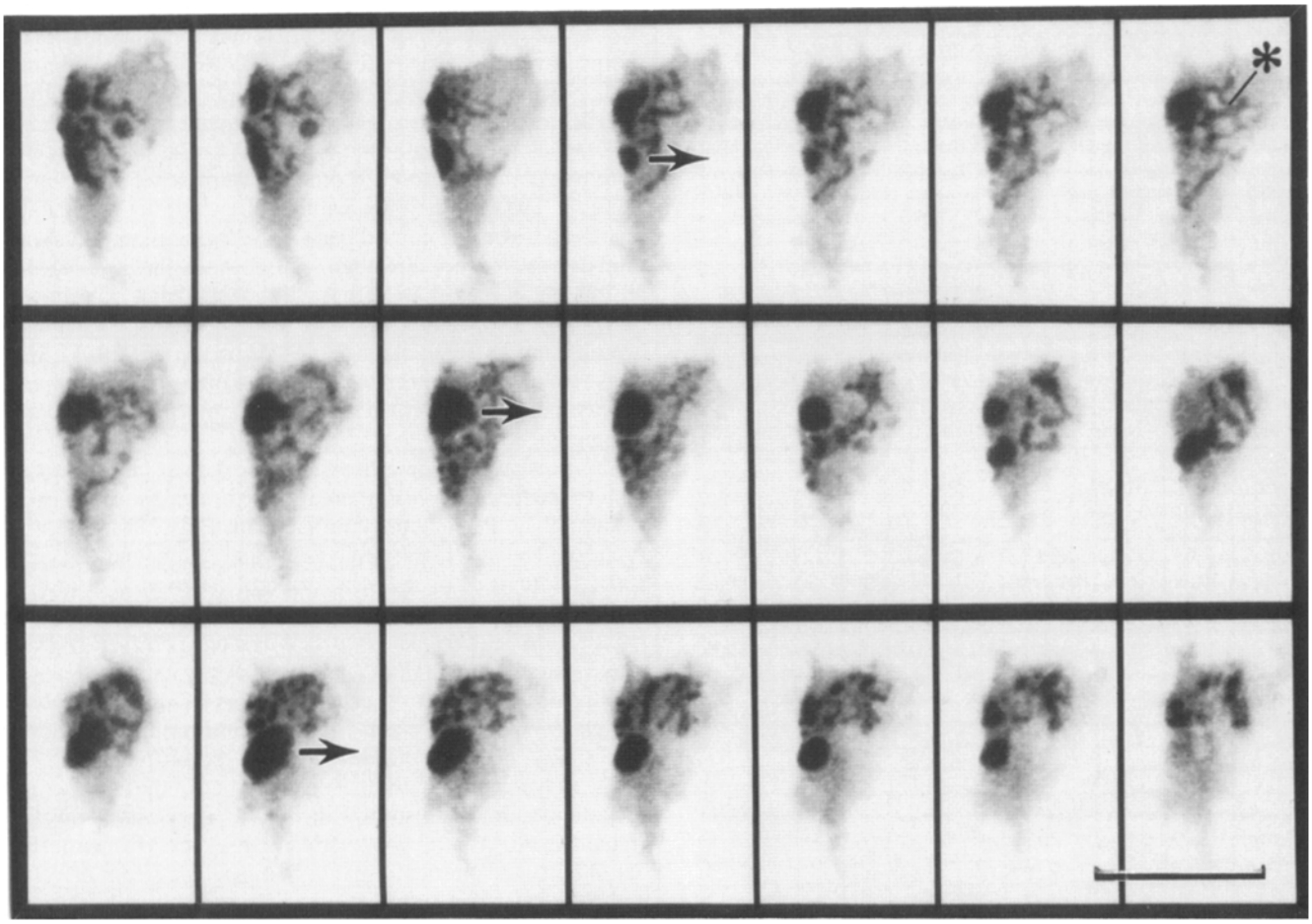


Figure 2. Time-lapse video microscopy of an AX2 *Dictyostelium* amoeba imaged by IRM. Recording starts in the upper left panel and proceeds uninterrupted at 4-s intervals to the lower right. Contractile vacuoles and their associated membrane tubules appear dark against the lighter grey of the cell bottom, due to their close approach to the plasma membrane. Arrows denote the onsets of three discharge events, which in subsequent frames can be seen to involve disappearance of the contractile vacuole and its replacement by a faintly visible and dispersed reticulum of dark dots and threads. These fine elements then gradually enlarge and appear to coalesce into a new vacuole at or near the site of the former one. A particularly clear example of a tubulo-cisternal intermediate in this process is indicated at the asterisk. Bar, 10 μm.

application for styryl dyes will be described elsewhere (Heuser, J., and J. H. Morisaki, manuscript in preparation).

IRM of Dictyostelium Contractile Vacuoles

Amoebae were prepared for epifluorescence light microscopy as described for vital staining above, but the microscope was fitted with a simple half-silvered mirror in place of the standard rhodamine fluorescence cube, thus creating the same optical conditions described in the papers that introduced IRM (Curtis, 1964; Izzard and Lochner, 1976). In this recording situation, some of the incident light is reflected by the coverglass-water boundary, due to the change in refractive index there; similarly, a portion of the light transmitted through the water is reflected on striking the ventral surface of the cell and again on striking any organelles that closely approach this ventral surface. When the intervening gaps are less than the wavelength of light, these reflected waves interact to produce visible interference patterns analogous to the production of Newton's rings in optical experiments. These patterns were recorded by time-lapse video microscopy under low-light conditions, exactly as for the vital-staining experiments described above.

Electron Microscopic Immunocytochemistry

Amoebae were ruptured as described above but fixed in 2% formaldehyde rather than 2% glutaraldehyde, the vehicle being the aforementioned "intracellular stabilization buffer" with 2 mM $CaCl_2$ rather than the usual Mg/EGTA. They were then "quenched" with 50 mM lysine and 50 mM NH_4Cl to reduce background stickiness and reacted for 60 min with the aforementioned anti-57-kD proton pump antiserum at a 1:50 dilution. Next, they were rinsed extensively over 30 min with the calcium-containing K-Hepes buffer alone and re-fixed with 2% glutaraldehyde in the same buffer, no secondary antibody being needed. Thereafter, they were prepared for EM by the quick-freeze, freeze-dry technique, exactly as described above. (We should note that in these TEM experiments, the anti-57 kD antibody did not appear to react at all with unfixed *Dictyostelium* contractile vacuoles or with ones fixed in Mg/EGTA rather than calcium, for reasons that remain obscure. This may reflect the fact that the antibody was raised to a polypeptide band cut out of a denaturing gel [Moriyama and Nelson, 1989].)

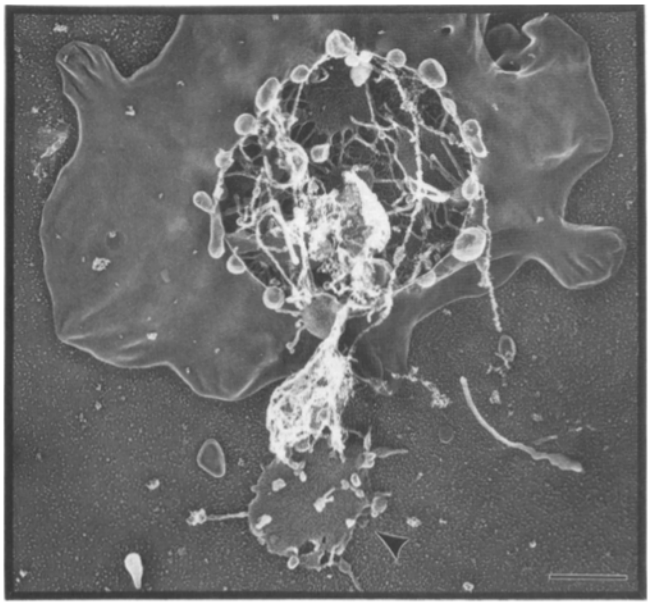


Figure 3. Ultrasonic rupture of the apex of this *Dictyostelium* amoeba revealed several flattened membranous cisternae enmeshed in a tubular reticulum, components of the contractile vacuole system seen by IRM in Fig. 2. One cisterna has been expelled onto the glass, below (*arrowhead*). Barely visible at this low magnification are the tiny white dots on the surfaces of these cisternae. These turn out to be proton pumps. Bar, 1 μm.

Results

IRM of Living Dictyostelium Amoebae

Fig. 2 shows the contractile vacuole system of *Dictyostelium* as visualized by IRM. Localized dark regions took the form of disks and threads, often interconnected into a reticulum. The disks corresponded exactly in size and location to the fluid-filled contractile vacuoles seen by phase-contrast light microscopy, and disappeared periodically like the phase-lucent vacuoles, as well. These results fully confirm the observations of Gingell et al. (1982). Furthermore, time-lapse video recording documented the dynamic interactions of the dark disks with the surrounding threads. The disks appeared to grow at the expense of the threads, and the disks regenerated local clusters of threads after their discharge and collapse, indicating that the contractile vacuole membrane is interconvertible between these two forms. Their membrane did not, in contrast, appear to coalesce with the plasma membrane during vacuole discharge.

Freeze-dried Views of Broken Dictyostelium Cells

When *Dictyostelium* amoebae were allowed to adhere briefly to polylysine-coated glass and then broken open by any of several techniques (a jet of buffer, sonication, or squashing against a waxed surface), TEM revealed on their remaining ventral surfaces the membranous organelles identified above by IRM to be constituents of the contractile vacuole system (Fig. 3). These typically took the form of flat membranous cisternae connected to each other by narrow tubules (Figs. 4 and 5). The most striking feature of the contractile vacuole membranes was the complement of ~15-nm-diam "pegs" or protrusions that covered their cytoplasmic surfaces; these are evident in Figs. 4 and 5 and are shown at high magnification in Fig. 6. Since the pegs resembled the heads of vacuolar proton pumps seen in other tissues (Brown et al., 1987), we prepared freeze-dry replicas of several other proton-pumping organelles and confirmed their close morphological correspondence (Fig. 7).

This presumptive identification of the pegs as being proton pumps was tested by using an antiserum against the 57-kD B subunit of the mammalian vacuolar H^+-ATPase (Moriyama and Nelson, 1989). (See Al-Awqati, 1986, and Nelson, 1991, for reviews of the structure and function of proton-ATPases.) This antiserum also specifically labeled a 57-kD polypeptide in *Dictyostelium* cell lysates (Fig. 1). When broken-open *Dictyostelium* amoebae were incubated with this antiserum prior to freezing, the pegs became decorated and clumped (Figs. 6 *c* and 7 *i*), thus confirming their identity as head domains of vacuolar-type proton pumps.

The range in morphology of the cisternae of the contractile vacuole system is illustrated in Fig. 8. They varied in diameter (from <1 to >3 μm), varied in thickness (from utterly flat to puffy to spherical), and varied in shape (from discoid with long narrow arms to rounded with stubby varicose arms to completely spherical without arms). We assume that these variations reflect stages of filling and discharge of contractile vacuoles, and propose that each part of the system cycles as diagrammed as in Fig. 9. Correlated with these changes in cisternal morphology were variations in proton pump distribution. Pumps were tightly packed on the flattest cisternae, in which case they were excluded from the narrow arms (Fig.

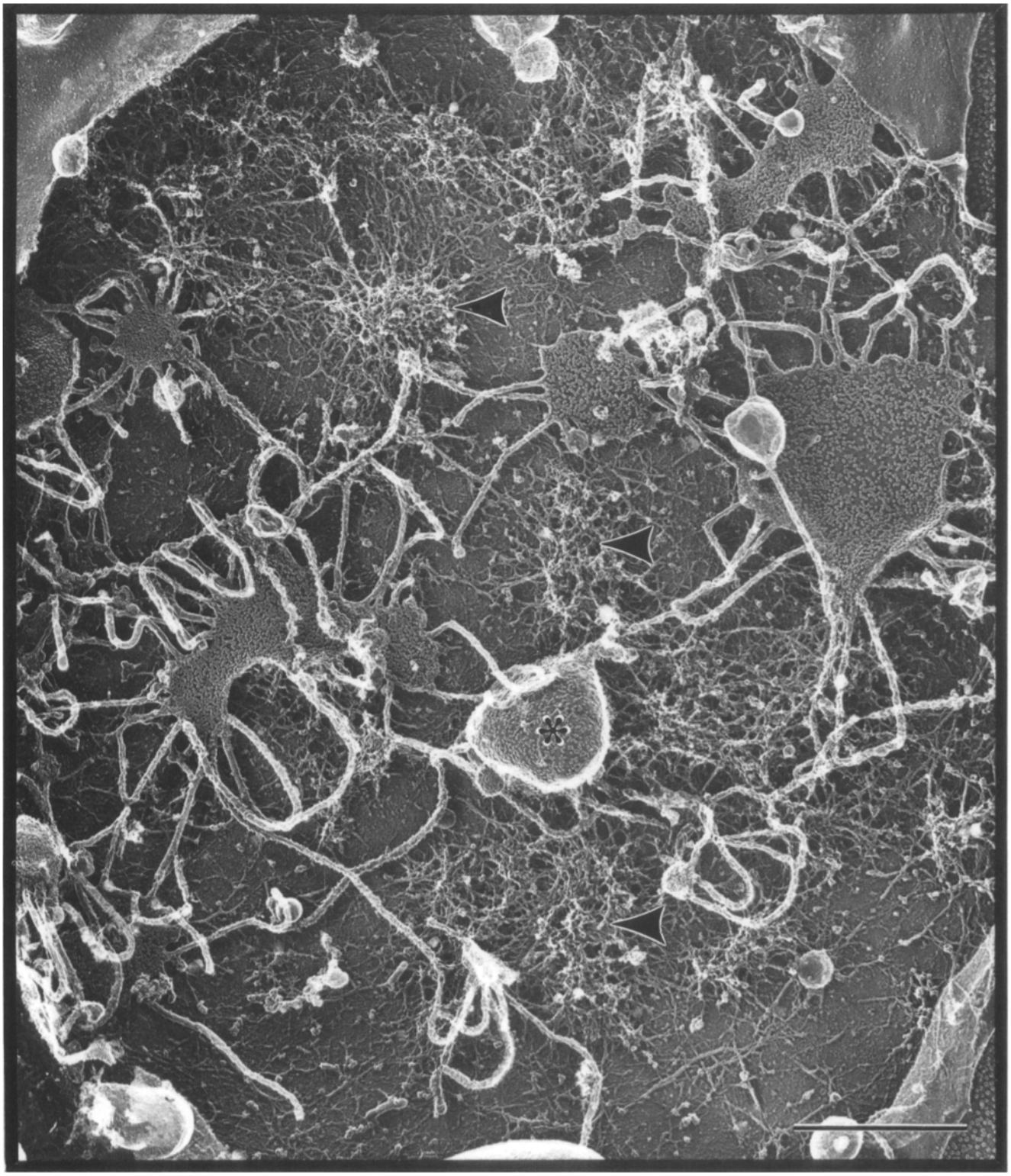


Figure 4. Expansive view of the interior of a *Dictyostelium* amoeba, achieved by adhesion to glass and rupture as in Fig. 3. Nestled between the dense plaques of cytoskeletal filaments that remain attached to the plasma membrane (*arrowheads*) are several flattened membranous cisternae with radiating tubules, some of which interconnect the adjacent cisternae. All cisternae display high concentrations of small white dots on their surfaces, including the one that looks "puffier" than the others (*asterisk*). These are all part of an extensive, interconnected contractile vacuole system, the swollen cisterna being partially filled at the moment of cell rupture and fixation. Bar, 1 μm.

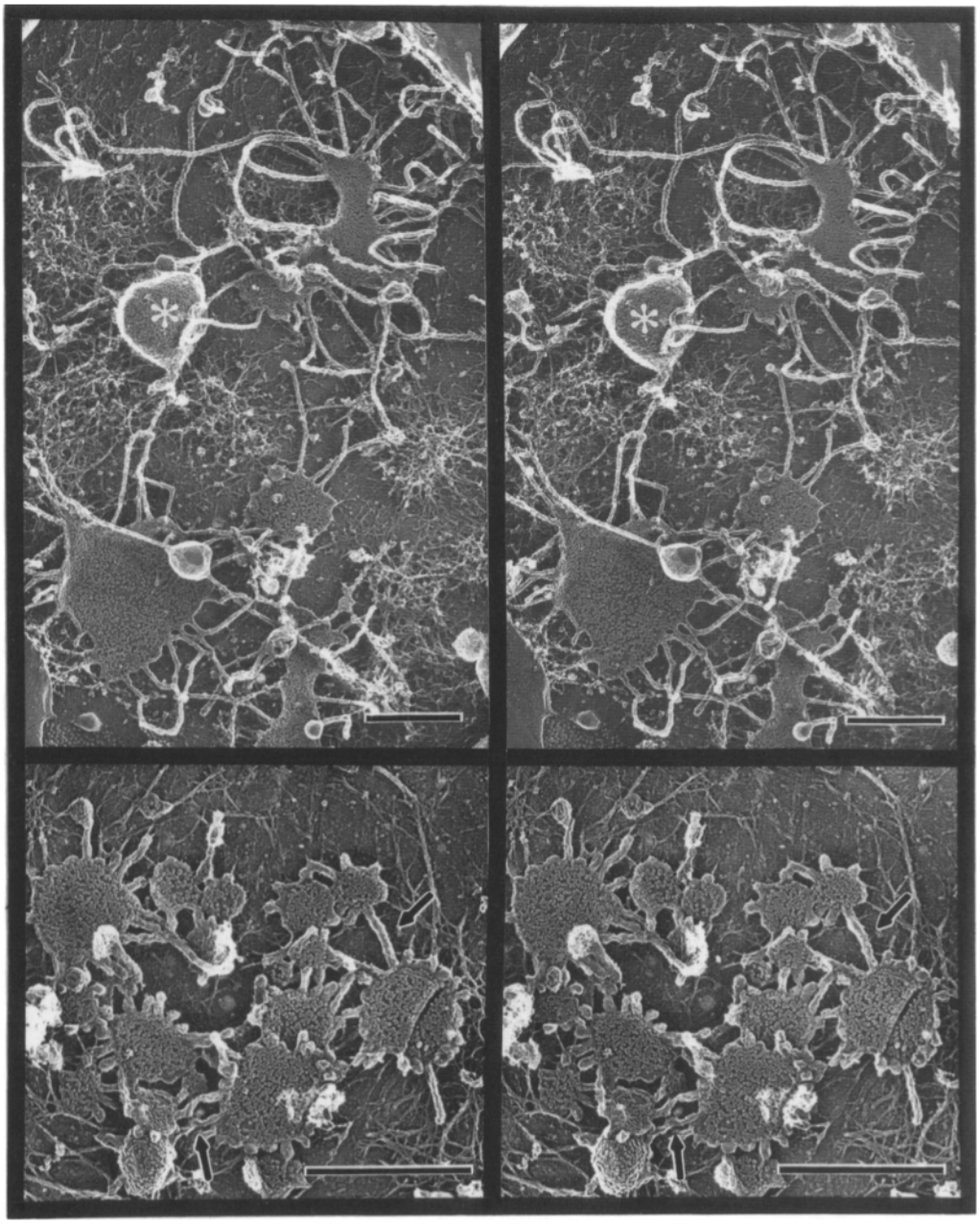


Figure 5. (*Upper panels*) Stereo view of the same field shown in Fig. 4, illustrating the raised, tuft-like configuration of F-actin in the cytoskeletal attachment plaques, the long looping arches of membrane tubules that interconnect the membrane-attached cisternae, and the one swollen cisterna at the asterisk. (*Lower panels*) Stereo-view of several closely opposed contractile vacuole elements in another *Dictyostelium* amoeba, in this case possessing only very stubby projections, an arrangement that demonstrates unambiguously their interconnection by tubules (at the *arrows*). Bar, 1 μm.

8 *a*), whereas they were more widely and uniformly dispersed on the rounder cisternae with puffy or varicose arms (Figs. 8, *b* and *c*). Thus, the freeze-dry images suggested that in *Dictyostelium*, proton pumps cannot fit into the most highly convoluted regions of contractile vacuole membrane, but can readily redistribute within it as it fills and changes shape.

Freeze Etch Views of Intact, Quick-Frozen Dictyostelium Cells

The quick-freeze, deep-etch technique revealed two important features of the contractile vacuole system (Figs. 10 and 11). First, regardless of the degree of distention of the cisternae, they appeared utterly empty inside; that is, the etching behavior of their contents was equivalent to that of pure water or a dilute electrolyte (Fig. 11 *a*). This empty appearance unequivocally distinguished elements of the contractile vacuole system from other organelles such as food vacuoles and endosomes, which were invariably filled with a dense matrix of non-etchable material (Fig. 11 *b*). It also ruled out an earlier hypothesis that contractile vacuoles might be filled with an expandable hydrocolloid that accumulated and retained water (Heywood, 1978).

The second distinctive feature of contractile vacuoles was their unique fracturing behavior. In unfixed preparations, contractile vacuole membranes displayed an unusually high concentration of large intramembrane particles on the E fracture-face (i.e., the luminal leaflet of their bilayer), corresponding in density to the 15-nm pegs on their surfaces (Figs. 10 and 11 *d*, *asterisks*). In contrast, few IMPs were present on the E fracture-faces of ER, lysosomal, or endosomal membranes (not shown). Interestingly, glutaraldehyde fixation before freezing altered the fracturing properties

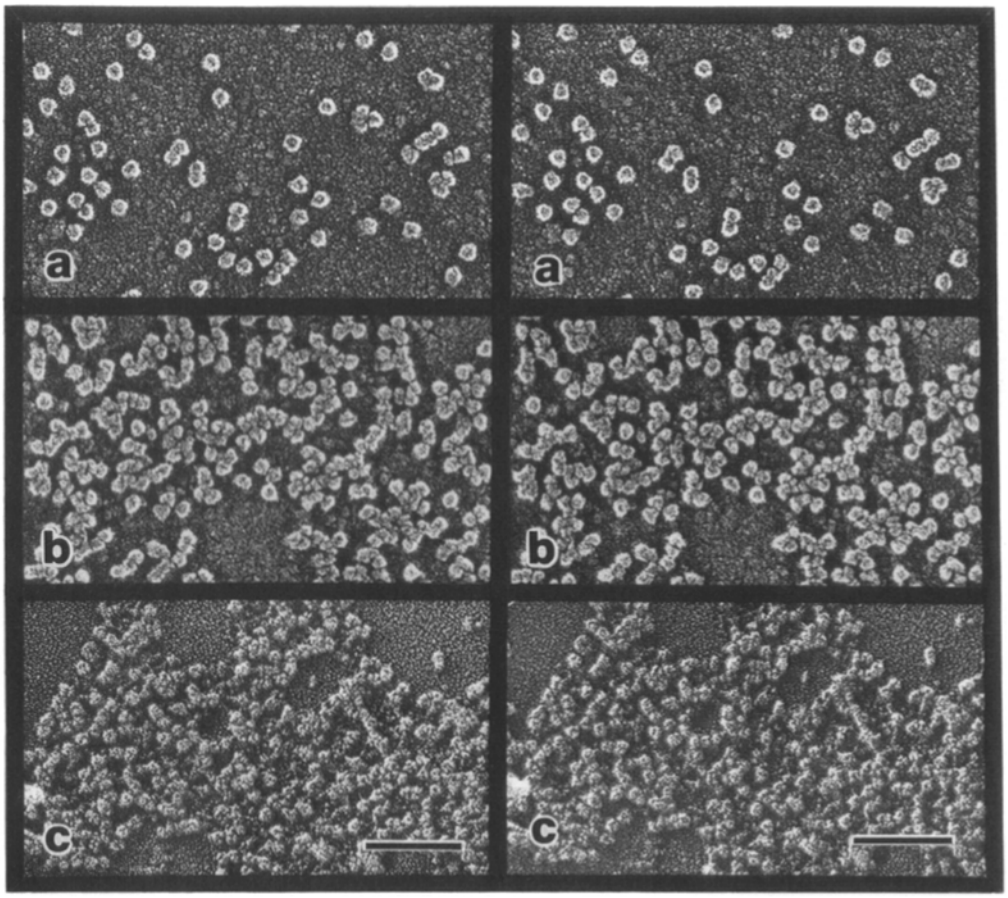


Figure 6. Stereo close-up views of the cytoplasmic surfaces of contractile vacuoles under different conditions. *a* and *b* illustrate their observed range of endowment with 15-nm membrane pegs. These tend to associate into pairs and triplets or into larger aggregates at higher concentrations, which may be an artifact of the chemical fixation used to stabilize the organelles before freeze drying. In any case, *c* illustrates that this tendency to self-associate is greatly exaggerated by exposure to the anti-57-kD anti-proton pump antibody shown in Fig. 1. Stereo viewing of *c* illustrates that this antibody piles on top of the pump heads as well as cross-links them. Bar, 0.1 μm.

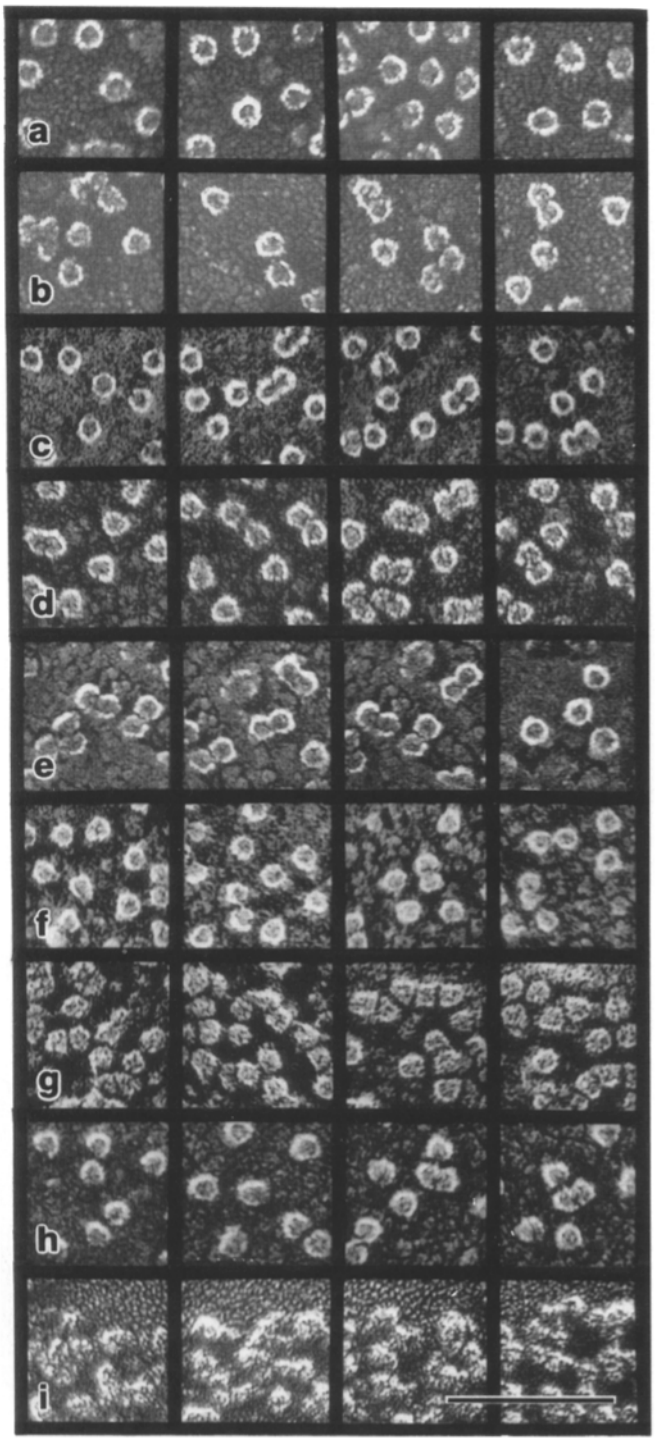
of contractile vacuole membranes, such that IMPs partitioned almost exclusively to the P fracture-face, leaving the E fracture-face so depleted of mass that it sometimes fell completely apart during deep etching (not shown). Similar fracturing properties have been reported for proton pump-bearing membranes of other aldehyde-fixed tissues (Allen and Staehelen, 1981; Orci et al., 1981; Allen, 1984; Stetson and Steinmetz, 1985; Brown, 1989).

Light Microscopic Immunocytochemistry of Contractile Vacuoles in Dictyostelium Amoebae

It was previously demonstrated that antibodies against *Dictyostelium* calmodulin strongly and selectively label contractile vacuole membranes (Zhu and Clarke, 1992). In the earlier study, cells were simultaneously fixed and extracted with cold formaldehyde–methanol, typically after exposure of the cells to hypo-osmotic buffers that caused their contractile vacuoles to distend; under those conditions, staining was restricted to the large vacuoles. With the present IRM and TEM images in mind, we tested several fixation conditions to see whether we could preserve and immunolabel the tubular elements of the system as well as the vacuoles. Best results were obtained using the two-step fixation procedure described in Methods and Materials and using cells that had been exposed to hypo-osmotic medium only briefly (<5 min) or not at all (i.e., fixation directly in HL5, the axenic growth medium). These conditions often revealed narrow extensions radiating from the vacuoles (Fig. 12, *upper panels*), and various interconnections of the system could be seen in different focal planes (Fig. 12, *lower panels*).

Double-staining experiments verified that the proton pump-bearing network of tubules and vacuoles seen by deep-etch EM is indeed the contractile vacuole system of *Dictyostelium.* The antiserum specific for the vacuolar H^+-ATPase (Moriyama and Nelson, 1989; see Figs. 1, 6, and 7), when used together with anti-calmodulin antibodies, yielded the results shown in Fig. 13. This figure shows three typical examples of interphase amoebae as well as one anaphase cell displaying the contractile vacuole dispersion that occurs in mitotic *Dictyostelium* cells (Zhu et al., 1993). In each case there was a close correspondence between the distribution of the proton pump antigen and of calmodulin, confirming that contractile vacuole membranes are the principal locus of vacuolar proton pumps in *Dictyostelium.* On the other hand, anti-proton pump staining of endosomes and phagosomes in *Dictyostelium* was generally below threshold, even though a low concentration of ~15-nm pegs was observed on these elements by TEM (data not shown).

Further confirmation of this proton pump distribution was obtained by visualizing the contractile vacuole system in newly germinated wild-type (NC4) amoebae. Newly germinated cells typically contain a single prominent, very active contractile vacuole (Cotter and Raper, 1966; Cotter et al., 1969; Zhu and Clarke, 1992). When such cells were labeled with the anti-proton pump antiserum, they displayed clear staining of this large contractile vacuole, and also diffuse



staining of material in the vicinity of the vacuole (Fig. 14 *a*). In parallel experiments, living NC4 cells were incubated with the potential-sensitive vital dye FM4-64, which partitions preferentially into their contractile vacuole systems (Heuser, J., and J. H. Morisaki, manuscript in preparation). Like the antiserum, this dye stained both the contractile vacuole proper and a diffuse cloud of associated material (Fig. 14, *b* and *c*). Time-lapse video recording of the filling and emptying cycles of contractile vacuoles in such vitally-stained NC4 amoebae illustrated that the diffusely stained material was indeed part of the contractile vacuole system and contributed to it during filling (Fig. 15). We infer that this material corresponds to the tubular elements visualized by IRM (Fig. 2) and by TEM (Figs. 4 and 5).

Proton Pump Redistribution to the Plasma Membrane

Both immunocytochemistry and TEM detected an interesting redistribution of proton pumps that occurred when axenic cells reached such high cell density that they ceased to grow and entered stationary phase. After two or three days in stationary phase, small refractile cells apparently lacking in contractile vacuoles could be detected by phase contrast microscopy. Immunostaining indicated that both proton pumps (Fig. 16) and calmodulin (not shown) had moved to the plasma membrane of such cells. Freeze etch TEM also showed that stationary cultures contained many cells with proton pumps in their plasma membranes (Fig. 17), something that is not seen in normal log-phase cells. We interpret this phenomenon to be an abortive attempt at spore formation by stationary cells, since we find a similar loss of contractile vacuoles and redistribution of calmodulin and proton pumps to the plasma membrane in the early stages of spore morphogenesis during normal development (not shown).

Discussion

Proton Pumps in the Contractile Vacuole

We have shown that membranes of the contractile vacuole system of *Dictyostelium* amoebae are endowed throughout with characteristic membrane pegs that are in fact vacuolar-type proton pumps. Early EM studies of protozoal contractile vacuoles had visualized these elements but had interpreted them to be ribosomes and thus part of the rough endoplasmic reticulum (Rudzinska, 1957). More recently, McKanna described "peg-shaped elements" on the tubular portions of the contractile vacuole systems of several pro-

Figure 7. Gallery of highly magnified membrane pegs from several different types of tissues in which vacuolar-type proton pumps have been identified, including anti-proton pump antibody decoration of them in row *i*. The top two rows (*a* and *b*) illustrate the pegs under analysis here, as they appear on the surfaces of *Dictyostelium* contractile vacuoles after fixation and freeze drying. The other rows illustrate the pegs found on the following organelles: *c*, the "spongiome" tubules of *Acanthamoeba*'s contractile vacuoles (Bowers and Korn, 1968, 1973); *d*, the contractile vacuole of the amoeba *Naegleria*; *e*, the apical membranes of the toad bladder epithelium, where the freeze etch appearance of proton pumps was first described (Brown et al., 1987); *f*, the osteoclast's cell membranes, in regions where they secrete protons to dissolve bone (Kallio et al., 1971; Blair et al., 1989); *g*, the apical membranes of vertebrate kidney distal tubule intercalated cells, the cells that pump protons into the urine (Yurko and Gluck, 1987; Brown, 1989); *h*, the "portasome" pegs found on the apical membranes of insect gut epithelial cells (Harvey et al., 1981, 1983), cells which extrude potassium by first pumping out protons and then exchanging them for potassium with an H/K antiporter (Klein et al., 1991; Wieczorek et al., 1991). These images illustrate that across all tissues and phyla, the cytoplasmic "heads" of proton pumps look remarkably similar. Row *i* further illustrates the crosslinking and decoration of *Dictyostelium* contractile vacuole pegs that is obtained with the anti-proton pump antibody shown in Figs. 1 and 6. Bar, 0.1 μm.

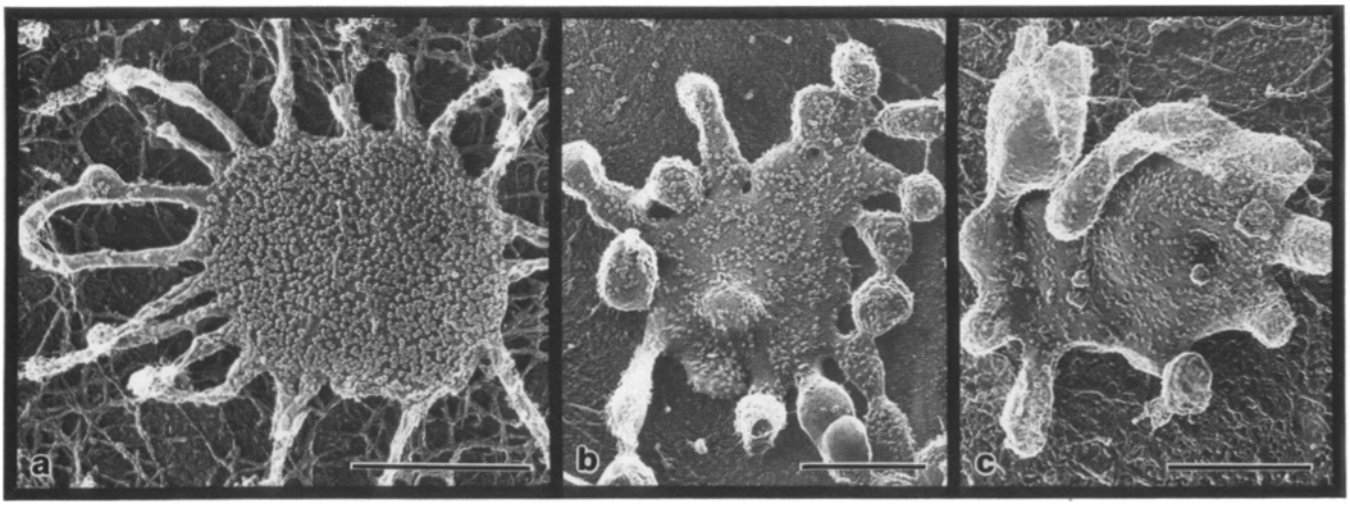


Figure 8. A gallery of three examples of subsurface cisternae that we consider to be structural intermediates in the filling and discharge cycle of *Dictyostelium*'s contractile vacuoles. The example in *a* is quite flat and crowded with pumps; this type typically displays many radiating tubules with very low densities of proton pumps. The cisternae in *b* and *c* are partially swollen. These types typically display shorter, more varicose arms with proton pump densities approaching those of the central cisternae. (Completely filled vacuoles are spherical and display no arms, but usually wash away during cell rupture and are too large to portray on the scale shown here.) We infer that the process of contractile vacuole filling involves irregular swelling of the radiating tubules with progressive incorporation of their varicosities into the growing vacuole, as diagrammed in Fig. 9. Bars, 0.5 μm.

tozoa and suggested that these were involved in water accumulation (McKanna, 1974, 1976). Here, the identification of these pegs as being vacuolar-type proton pumps was made in three ways. First, the pegs decorate with antibodies to vertebrate proton pumps, both in the TEM (Figs. 6 *c* and 7 *i*) and in the light microscope (Figs. 12–14). Second, they occur in great abundance in *Dictyostelium* membrane fractions that are enriched in proton pumps (unpublished observations with T. Steck's laboratory, Department of Biochemistry, University of Chicago; see Nolta et al., 1991). Finally, they look identical to vacuolar proton pumps seen in other tissues and phyla (Fig. 7). In particular, they look exactly like the peg-like "portasomes" seen in the plasma membrane of thin-sectioned insect gut epithelial cells (Harvey et al., 1981, 1983). These structures have recently been recognized to be vacuolar-type proton pumps (Klein et al., 1991; Wieczorek et al., 1991), and indeed, look exactly like the pegs seen in *Dictyostelium* and other protozoal contractile vacuoles (Fig. 7 *h*).

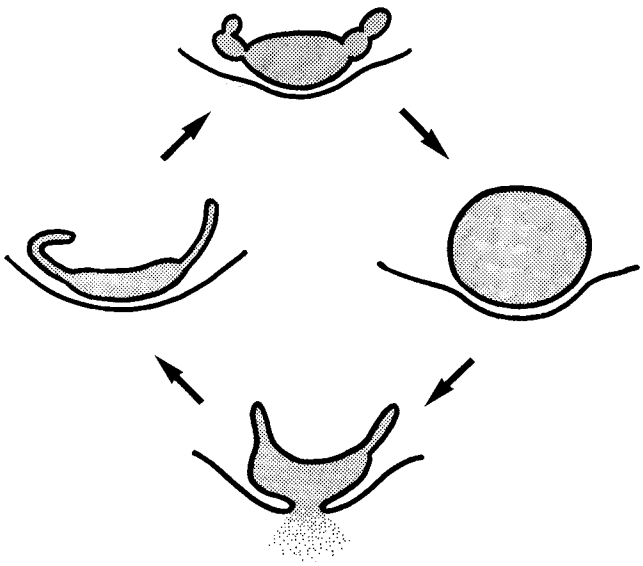


Figure 9. Proposed cycle of contractile vacuole filling and discharge in *Dictyostelium*, focusing on one portion of the broader interconnected array of membrane tubules and cisternae. Swelling of any particular cisterna during water accumulation appears to be accompanied by incorporation of membrane from the surrounding tubules, to accommodate its changing surface/volume ratio. Discharge of the resultant vacuole then seems to reverse this process, collapsing it into a flat cisterna that re-expresses its excess membrane in the form of narrow tubules. Since actin and myosin are not detected on any of these structures (see Figs. 3–6), the "contractile" nature of the discharge event may in fact be brought about by the vacuole's tendency to re-form such tubules via persistent lipid asymmetries in the two leaflets of its encompassing membrane.

Role of Proton Pumps in Water Accumulation

In defining a role for proton pumps in water accumulation, it is important first to consider the general organization of contractile vacuole systems in protozoa. In most cases, the systems consist of two phases: a central vacuole connected to a system of peripheral tubules. The tubules have been termed the "nephridial apparatus" or "spongiome" (Patterson, 1980), implying that they have a special kidney-like function in water accumulation. Indeed, the claim that membrane pegs (now seen to be proton pumps) are confined to the peripheral tubules or spongiome in most protozoa (McKanna, 1974, 1976) has helped to promote this view. However, the contractile vacuoles in most amoeboid cells (including *Dictyostelium*) have been described as being organized rather differently (reviewed in Patterson, 1980). In amoebae, the vacuoles appear by light microscopy to be transient structures that form by the coalescence of smaller vesicles (Botsford, 1926). This view seemed to be supported by early TEM studies of amoebae (Pappas and Brandt, 1958; Mercer, 1959; De Chastellier et al., 1978). However, the

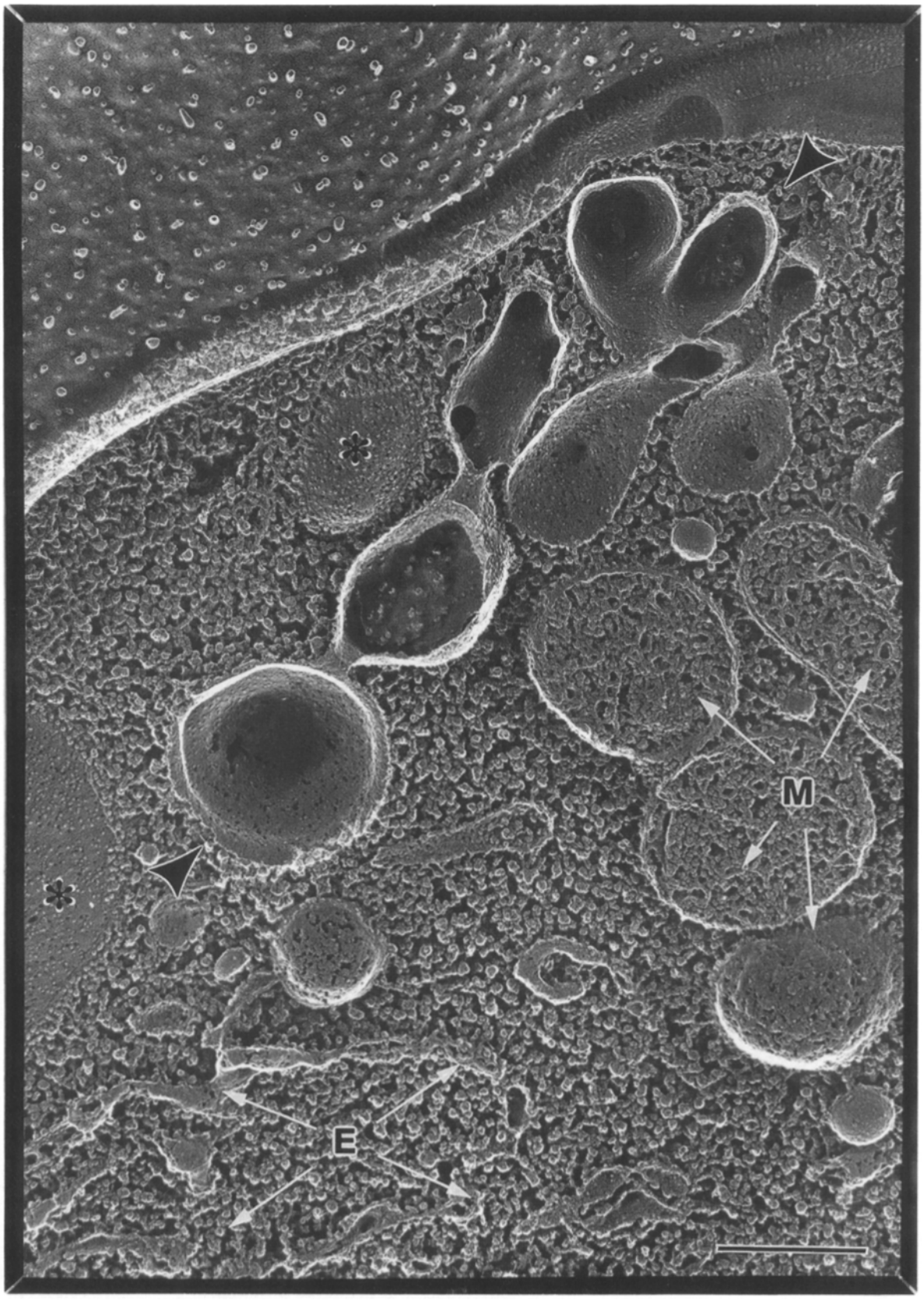


Figure 10. Freeze-fracture into the interior of a *Dictyostelium* amoeba that was quick-frozen directly from life, after brief washing in 20 mM PO_4 buffer. Among the various membrane organelles found inside it are four mitochondria (*M*), several thin tubules of endoplasmic reticulum (*E*), and most prominently, an interconnected labyrinth of varicose membrane compartments that etch deeply into their interior (bracketed by *arrowheads*). The latter we interpret to be parts of the contractile vacuole system, whose watery content has sublimed away during deep etching. Also visible are two unfractured membrane compartments (*asterisks*). Their rich endowment with large E-face intramembrane particles identifies them as additional parts of the contractile vacuole system. Bar, 0.5 μm.

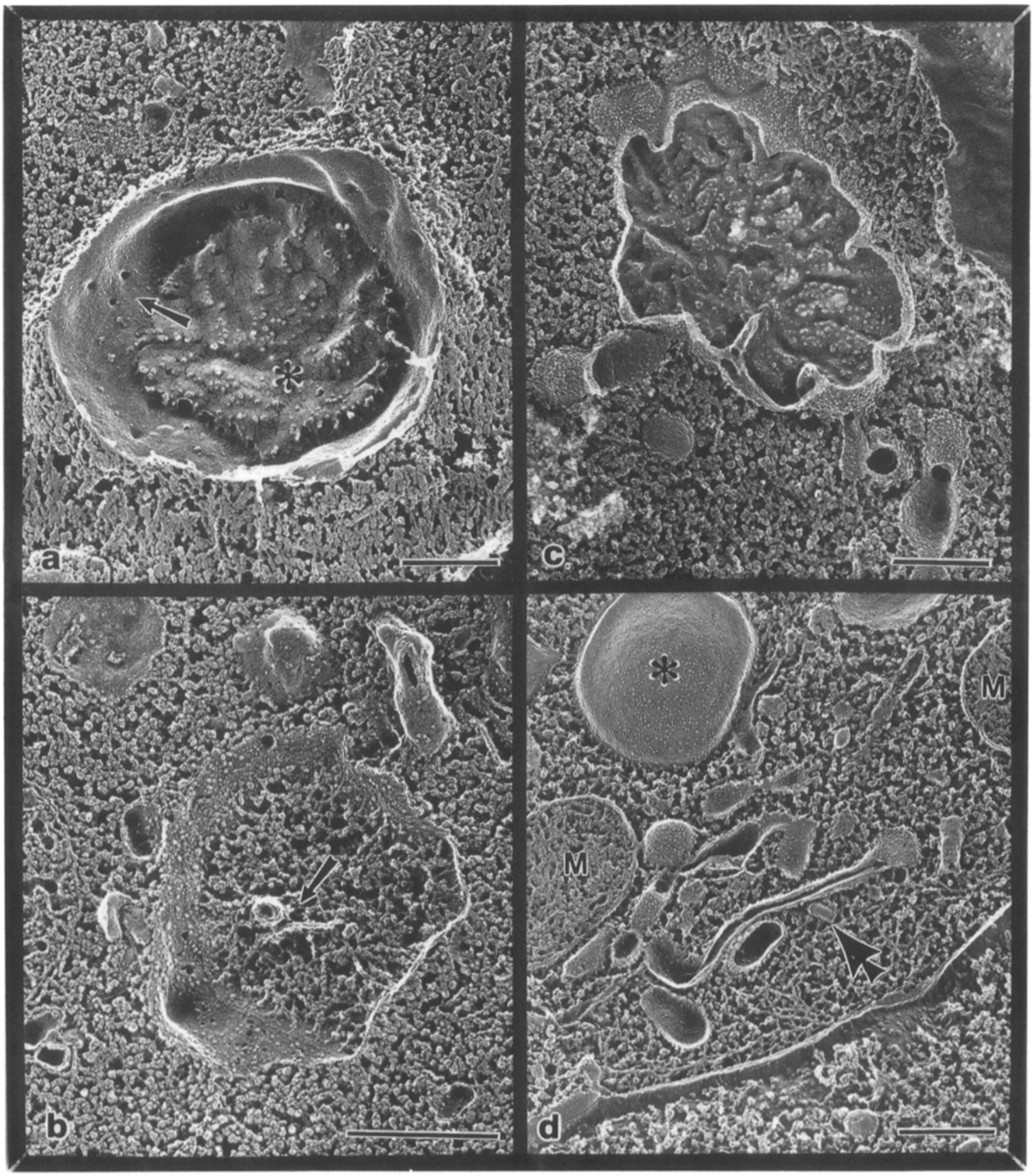


Figure 11. The etching behavior of contractile vacuoles and food vacuoles compared in *a* and *b*, and various degrees of contractile vacuole filling compared in *a*, *c*, and *d*. All four fields are from *Dictyostelium* amoebae quick-frozen under different osmotic conditions and then freeze fractured and deep etched identically. In *a* and *b* the amoebae were suspended in deionized water for 5 min to maximize their rate of contractile vacuole filling and discharge; this increased the likelihood of finding relatively swollen contractile vacuoles, as in *a*. Etching has almost entirely removed the contents of this vacuole, revealing a relatively clean internal surface with trumpet-shaped openings into the tubules that radiate from it (one of which is indicated at the arrow). The remaining puddle in the bottom of this vacuole (*asterisk*) is almost devoid of unetchable contents, as is the water surrounding such amoebae. In contrast, the fractured vacuole in *b*, from an adjacent amoeba in the same preparation as in *a*, is almost entirely filled with a meshwork of nonetchable material, including nondescript membranous debris at the arrow. This, plus subtle differences in the fracturing behavior of its surrounding membrane described in the text, identifies it as a food vacuole (e.g., a late endosome or 2° lysosome). The contractile vacuoles in *c* and *d*, again recognizable by their empty lumens and large E-face intramembrane particles, display various degrees of collapse. In *c*, partial collapse was produced artificially by sudden acidification of the amoebae (bubbling CO_2 into their PO_4 buffer 1 min before freezing; see Gittleson and Sears, 1964); while in *d*, a completely collapsed cisterna (at the *arrow*) was captured simply by freezing amoebae directly from HL5 axenic medium, in which their contractile vacuoles fill and discharge only very slowly. At the asterisk in *d* is an unfractured cisterna in the contractile vacuole system, displaying another en face view of its characteristic E-face particles, while at the *M*'s are fractured mitochondria. Bars, 0.5 μm.

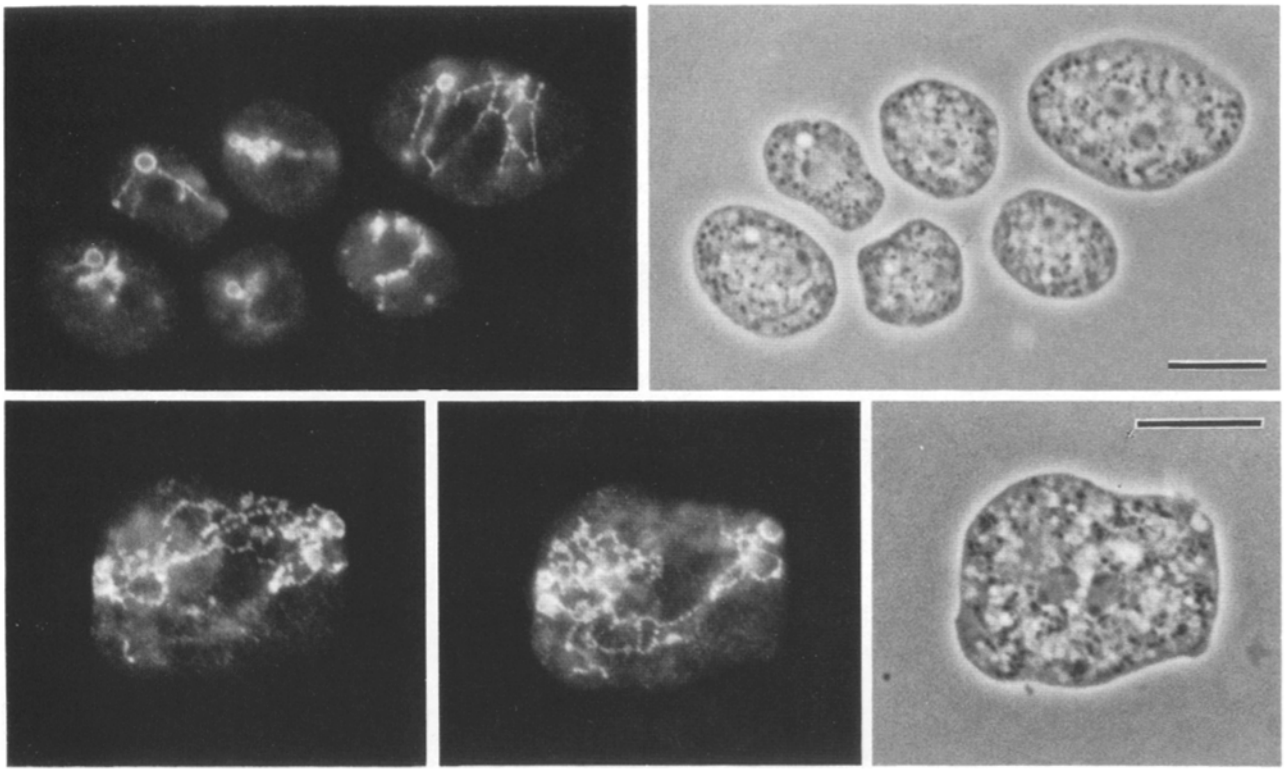


Figure 12. The contractile vacuole system of *Dictyostelium* visualized by indirect immunofluorescence using anti-calmodulin antibodies. Amoebae were fixed using the two-step procedure described in Materials and Methods, which preserves the tubular elements of the system, then stained with a mAb against *Dictyostelium* calmodulin previously shown to label contractile vacuole membranes (Zhu and Clarke, 1992). The upper panels show a group of AX2 cells fixed in HM buffer, and the lower panels show two focal planes of an AX3 cell fixed in one-third strength HL5 containing 0.1% DMSO. It is evident that even widely separated contractile vacuoles within one amoeba can be interconnected. The tendency of the tubular connections to vesiculate during fixation is suggested by the segmented or dot-like appearance along parts of their length. Bars, 10 μm.

IRM studies of Gingell et al. (1982) and the work presented here have shown that *Dictyostelium*'s contractile vacuole system actually consists of a network of relatively continuous membrane channels that are always present but simply change shape and organization during water accumulation. This is also true of several other types of amoebae that we have examined (unpublished observations.) The narrower tubular and cisternal elements of these systems would have been invisible to light microscopists working without IRM and without special stains or antibodies, and would probably have broken down into vesicles under earlier TEM fixation protocols (Doggenweiler and Heuser, 1964). Thus, the contractile vacuole system of *Dictyostelium* resembles that of other protozoa, except that its proton pumps are not confined to tubular spongiomes (and in fact are excluded from its narrowest tubular elements, as shown in Fig. 8 *a*). Instead, proton pumps populate nearly all parts of its contractile vacuole membrane, including the larger vacuoles. This would be expected if the tubules merged with the vacuoles as they grew, as suggested by our data (see Figs. 2, 8, and 15).

Implications for Contractile Vacuole Function

The clear differentiation of certain protozoal contractile vacuole systems into two physically distinct and relatively permanent phases, tubular spongiomes (collecting ducts) and central vacuoles (storage reservoirs), has long encouraged the idea that water is accumulated in a two-step reaction as well (recently reviewed by Zeuthen, 1992). The first step is thought to be ion transport into the peripheral collecting ducts, resulting in a passive or osmotic influx of water. The second step, presumably occurring in the central storage reservoir, involves pumping the same ions back into the cytoplasm, thereby preserving the cells' intracellular ions and producing a dilute contractile vacuole discharge (Schmidt-Nielsen and Schrauger, 1963; Mayer and Iverson, 1967; Riddick, 1968). For amoebae, this view was amended to state that specific "satellite" vesicles pumped ions into themselves and accumulated water osmotically, then fused to form the contractile vacuole, whereupon the ions were pumped back into the cytoplasm (Schmidt-Nielsen and Schrauger, 1963).

The basic problem with this classical view is that the final storage reservoir would have to assume an exceptionally low water permeability to resist passive water egress as its ions were being reabsorbed and its contents were becoming hypotonic relative to the cytoplasm. It is unclear how such a low permeability could be maintained, since artificial bilayer studies suggest that even pure lipid membranes with no protein insertions (pumps, channels, etc.) are relatively permeable to water (Finkelstein, 1984; Walter and Gutkneck, 1986). Moreover, isolated contractile vacuoles behave like

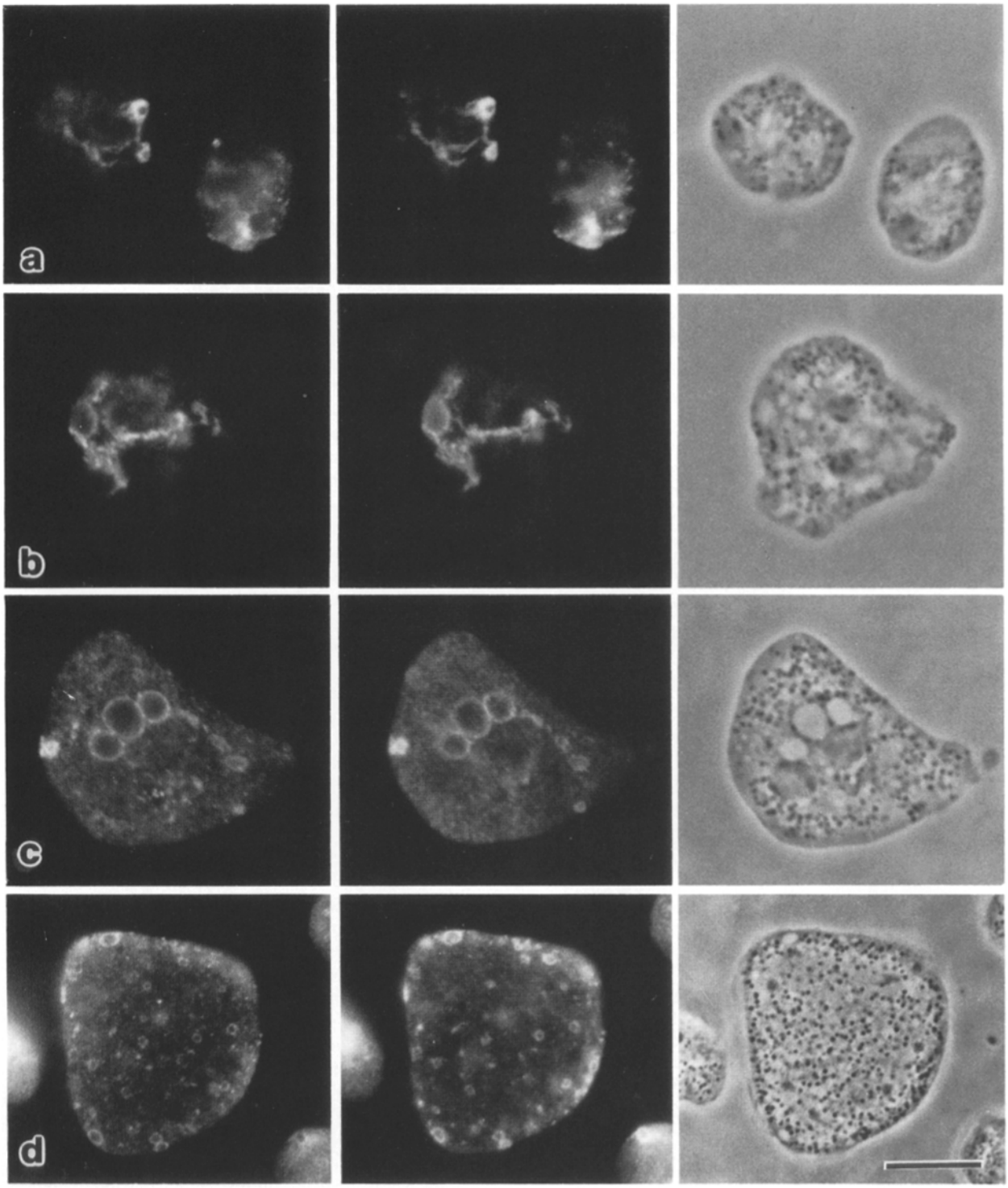


Figure 13. Dictyostelium amoebae double-stained with antibodies to calmodulin to identify their contractile vacuole systems (*center panels*), as well as with the anti-proton pump antibody used in Figs. 1, 6, and 7 (*left panels*). Note the complete correspondence of the two antibody decorations, indicating that vacuolar type proton pumps populate all parts of *Dictyostelium*'s contractile vacuole system. The two amoebae in *a* and *b* were aldehyde-fixed in HL5 medium also containing 0.1% DMSO, which better preserves their contractile vacuoles' tubular anlage (compared with the amoeba in *c* which was fixed without added DMSO). The amoeba in *d* was shown by DAPI staining to be in late anaphase and shows the characteristic dispersal of the contractile vacuole system that typifies mitosis in *Dictyostelium* (Zhu et al., 1993). Still, calmodulin and proton pump staining corresponded exactly. Bar: (*a–c*) 10 μm; (*d*) 20 μm.

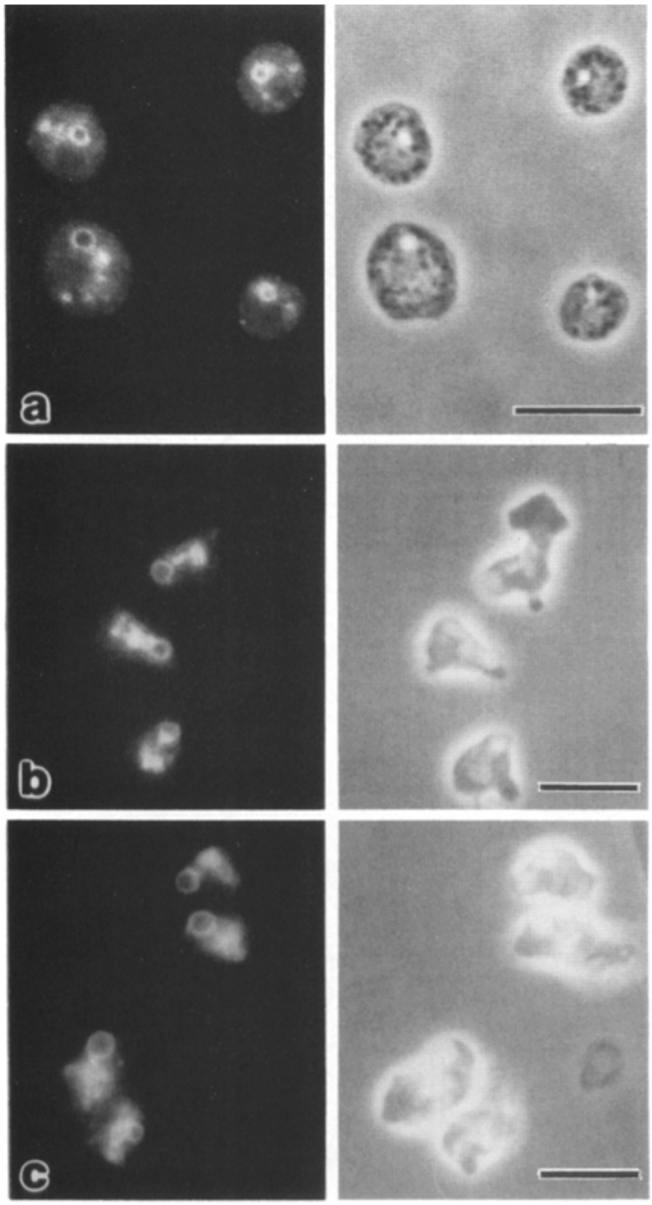


Figure 14. Contractile vacuoles in newly germinated *Dictyostelium* cells stained with anti-proton pump antibodies and with a membrane potential-sensitive styryl dye. NC4 cells that had recently emerged from their spore coats and had not yet begun to feed were fixed and immunostained with antibodies to the proton pump (*a*). A similar preparation of newly germinated cells was vitally stained with 1 μg/ml FM 4-64 (from Molecular Probes, Inc.), a potential-sensitive membrane dye (*b* and *c*). Both probes labeled the contractile vacuoles proper, as well as associated material that appears at this low resolution as diffuse "trails" of staining connected to the vacuoles. In the TEM, these diffuse deposits were seen to be central collections of the tubes and cisternae described above. Bars, 10 μm.

simple osmometers (Hopkins, 1946, and our unpublished data), further indicating that their membranes have normal water permeability.

A second problem with the classical model is presented by the actual arrangement of contractile vacuole elements in *Dictyostelium*, where all membranes of the contractile vacuole system are interconnected and relatively uniform in composition. In particular, proton pump pegs are at least as abundant in the central vacuoles as in the associated tubular and cisternal elements. This uniform pump distribution is also evident from both antibody and styryl dye staining. Such lack of intracompartmental differentiation, plus the dispersion of *Dictyostelium*'s contractile vacuole system during mitosis into dozens of small, highly active vacuoles (Zhu et al., 1993; see also Fukui and Inoué, 1992), suggests that all parts of the contractile vacuole system are capable of water transport and retention, and also of fusing with the plasma membrane.

One is thus forced to consider a water-accumulation scheme for *Dictyostelium*, and by extension for other protozoa, that does not require ions cycling in and out of two physically separate parts of the system, nor retention of water against an osmotic gradient. A simple possibility is that the cells excrete an isotonic solution of something accumulated by proton pumping into the contractile vacuole. Protons could be exchanged, for example, for Na^+ or K^+ ions via an antiporter in the contractile vacuole membrane, and these osmotically active ions could, in turn, draw in water and hold it there as an isotonic solution, obviating the need for an impermeable membrane. However, if *Dictyostelium* excreted an isotonic salt solution, it would deplete its internal supply of the salt at the same rapid rate as it discharged water. This is inconsistent with the ability of many protozoa to live in hypotonic media for hours or days, repeatedly discharging their contractile vacuoles without running out of any essential ions.

We infer that contractile vacuoles may gather and discharge other, more expendable ions. By analogy with water transport in the distal tubule of the human kidney (Boron and Boulpaep, 1983; Burckhardt et al., 1984; Maren, 1988), these ions could well be H^+ and HCO_3^-. Cotransport of these ions would create carbonic acid and its dissociation products in the lumen of the contractile vacuole. These species would be osmotically active and could draw water into the vacuole, much as bicarbonate generates water flow in the distal tubule of the vertebrate kidney (Maren, 1967, 1988). Furthermore, bicarbonate could be excreted indefinitely, since it can be readily synthesized from CO_2 and H_2O using carbonic anhydrase, an enzyme we have found to be present in the cytoplasm of *Dictyostelium* cells (Heuser, J., and W. Sly, manuscript in preparation; see also Maren, 1967). An expendable cation is also available that could facilitate this process. Ammonia is the end product of protein degradation in *Dictyostelium* and is given off by the cells (Bonner et al., 1986). Ammonia could readily diffuse into the contractile vacuole as a gas, and thereupon react with the accumulated protons to yield ammonium ions. The resultant ammonium bicarbonate would stay in solution at normal atmospheric CO_2 tension and exert the necessary osmotic effects, yet the cell could readily afford to excrete it. Consistent with this model is the observation that *Dictyostelium*'s contractile vacuoles do not accumulate weak bases like acridine orange, though its endosomes readily do (video data, not shown). Thus, contractile vacuoles are much less acid inside than are endosomes, appropriate for the presence of bicarbonate at the pK_a of ($H^+ + HCO_3^-$), which is 6.2. This model is entirely speculative, but fits the existing data and is our current working hypothesis.

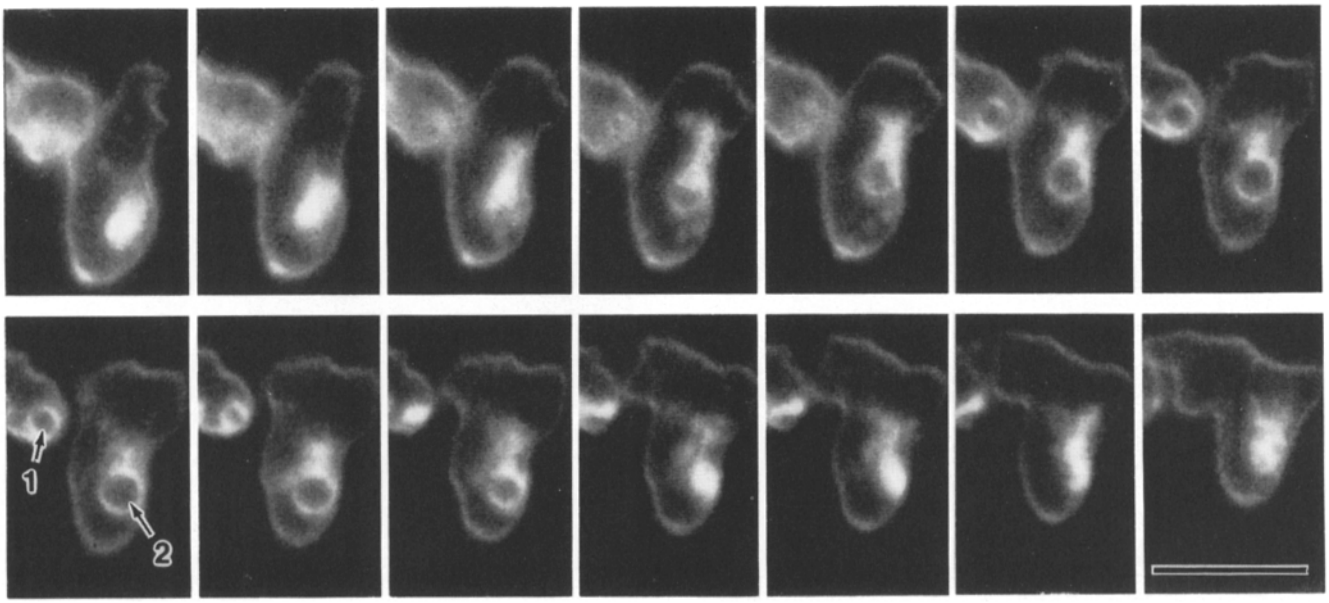


Figure 15. Time-lapse video microscopy of one minute in the life of two newly germinated NC4 *Dictyostelium* amoebae whose plasma membranes and contractile vacuole systems were vitally stained with the styryl dye FM4-64 (1 μg/ml in Dicty PO_4 buffer). Frames are separated by 4-s intervals and run sequentially from upper left to lower right. In the lower left frame, in which the individual amoebae are labeled *1* and *2*, the contractile vacuoles are nearly full. Earlier frames (*upper panels*) illustrate that filling involves the gradual incorporation into the growing vacuole of the diffusely stained associated material. Later frames illustrate that discharge of the vacuoles involves collapse and reflux of this membrane back into the diffusely stained form, but remarkably, no release of the dye. This extraordinary and unexplained retention of the styryl dyes by the contractile vacuole system permitted continuous video recording of multiple filling and discharge cycles. Bar, 10 μm.

Another area for future study is the role of contractile vacuoles in cellular activities other than osmoregulation. For instance, the peritrich protozoa have in their attachment-stalks structures called "spasmonemes" composed of dense bundles of filaments riddled with membrane tubules. A rapid, calcium-activated coiling of the filaments is thought to underlie these protozoa's escape reflex (Amos, 1972). The source of calcium, and the pool to which it is returned as coiling is relaxed, is thought to be the intervening membrane tubules (Carasso and Favard, 1966). Spasmonemal tubules would thus be functionally equivalent to the sarcoplasmic reticulum of vertebrate muscle, and so it is interesting to note that there is now evidence that these tubules are physically continuous with the tubular spongiome that typifies peritrich contractile vacuoles (Patterson, 1980). Further indication of this interrelationship came from our finding in unpublished studies that peritrich spasmonemal tubules possess surface "pegs" similar in structure to the other proton pumps studied here. Moreover, we find that the pegs on spasmonemal tubules are arranged in the same beautiful spiral patterns characteristic of the pegs on spongiome tubules in these and other ciliate protozoa (Schneider, 1960; Carasso et al., 1962; Allen, 1973; Allen and Fok, 1988). By extension, it seems possible that contractile vacuoles in these and other protozoa may also be calcium sequestering and eliminating organelles. Their rich endowment with proton pumps might, for example, allow them to accomplish this by H^+/Ca^{2+} exchange, using a strong proton gradient established across their membranes. In fact, it has been recently reported that a proton pump-rich membrane fraction isolated from *Dictyostelium* amoebae possesses ATP-driven Ca^{2+}/H^+ antiport activity (Rooney and Gross, 1992). This membrane fraction, also described by Nolta et al. (1991) as containing proton pump-rich "acidosomes," we believe to be fragmented contractile vacuole membranes. In any case, the present demonstration of the great abundance of proton pumps on bona fide contractile vacuole membranes from *Dictyostelium* will, we predict, be relevant to many aspects of protozoal cell physiology.

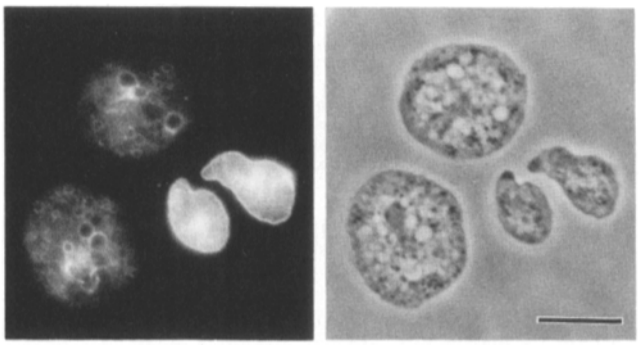


Figure 16. Stationary-phase AX3 amoebae stained with anti-proton pump antibodies, examined 2 d after a culture had reached stationary phase. Most amoebae were of normal size and still contained contractile vacuoles that became stained with the antibody. However, also present were many small refractile cells that appeared shrunken and contained no vacuoles. In these cells, the anti-proton pump antibodies strongly labeled the plasma membrane, suggesting that contractile vacuole membrane had merged with it. Bar, 10 μm.

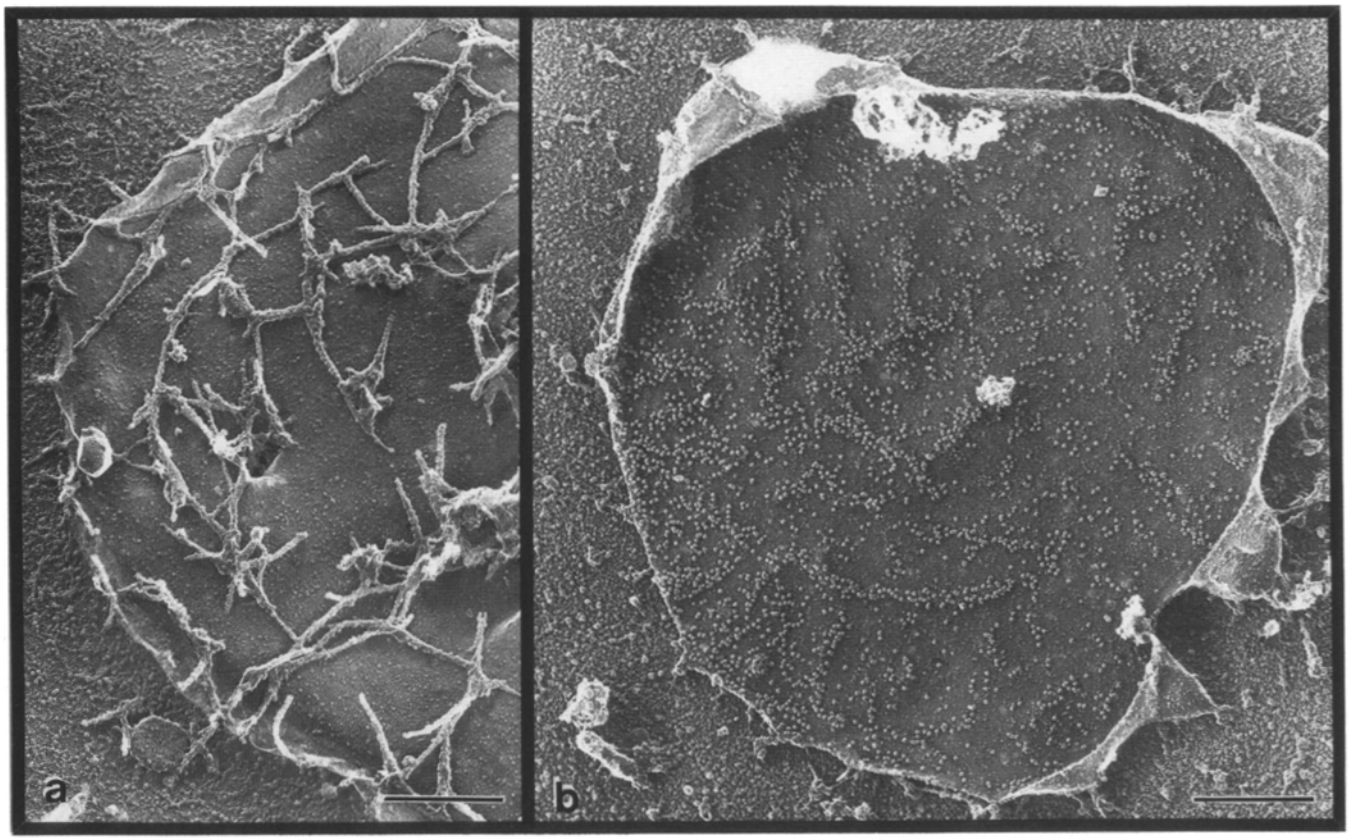


Figure 17. Freeze-dry replicas of two examples of the small refractile amoebae that appear in old stationary-phase cultures, either fixed while intact (in *a*) or broken open before fixation (in *b*), in both cases after brief attachment to polylysine-coated glass. *a* shows that such cells have begun to assemble an abortive spore coat, consisting of randomly arranged fiber bundles on the outer surface. *b* shows that the inner surface of the plasma membrane in such amoebae is utterly devoid of actin filaments and is, instead, "doped" with myriads of 15-nm pegs that look just like the proton pumps on contractile vacuoles. (Curiously, these tend to align in fixed amoebae along the extracellular fibrils, visible as embossments on the plasma membrane in *b*.) This redistribution of proton pumps correlates with the bright anti-pump staining of the plasma membrane seen in such cells in Fig. 16, and may explain the finding of electrogenic proton pumps in *Dictyostelium* plasma membranes (Van Duijn and Vogelzang, 1989). Bar, 0.5 μm.

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