Vacuolar H⁺-ATPase of *Dictyostelium discoideum*

A monoclonal antibody study

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SUMMARY

A *Dictyostelium* membrane fraction rich in vacuolar proton pumps, previously described by Nolta et al. (*J. Biol. Chem.* 266, 18,318-18,323, 1991), was used as the immunogen for production of monoclonal antibodies. We obtained antibodies that recognized polypeptides of 100 kDa and 68 kDa, corresponding to the two largest subunits of the vacuolar proton pump. In indirect immunofluorescence experiments, these two subunits were located on an interconnected collection of tubules and vacuoles. On frozen thin sections they were found principally on membranes of vacuoles and collections of small vesicles typically located just internal to the plasma membrane. These vesicles and vacuoles had electron-translucent lumens. No other structures in axenically grown *Dictyostelium* cells were labeled to a significant extent by these two antibodies. Using an affinity-purified antibody to calmodulin and a monoclonal antibody to the B subunit of the chromaffin granule vacuolar ATPase, markers known to label the membranes of the contractile vacuole complex in *Dictyostelium* (Zhu and Clarke, *J. Cell Biol.* 118, 347-358, 1992; Heuser et al., *J. Cell Biol.* 121, 1311-1327, 1993), we showed that the 100 kDa and 68 kDa subunits had the same distribution as these two markers. Co-localization was seen in both interphase and mitotic cells. Thus, our results support the conclusion that vacuolar proton pumps are located principally on the membranes of the contractile vacuole complex in *Dictyostelium*. In addition, in indirect immunofluorescence experiments, these monoclonal antibodies provided improved images of the organization of the contractile vacuole system.

Key words: V-ATPase, proton pump, contractile vacuole complex, calmodulin, *Dictyostelium*, monoclonal antibody, osmoregulation, *Paramecium*

INTRODUCTION

Our two laboratories are studying the contractile vacuole systems of the protozoan *Paramecium multimicronucleatum* (Allen and Fok, 1988; Allen et al., 1990) and the amoeba *Dictyostelium discoideum* (Zhu and Clarke, 1992; Zhu et al., 1993; Heuser et al., 1993). The contractile vacuole system is an osmoregulatory organelle found in freshwater amoebae and other protozoa. Although this organelle has attracted the interest of several generations of biologists, remarkably little is understood about the mechanisms by which it functions. In protozoa such as *Paramecium*, the contractile vacuole is a permanent structure located at a fixed position in the cell, adjacent to a specialized pore in the plasma membrane through which the vacuole discharges its contents. Associated with the vacuole is a membranous system of tubules and vesicles called the spongione or nephridial apparatus, which is thought to play a role in water accumulation. In amoebae, neither the number of vacuoles nor their location is fixed, and no defined pore structures have been detected. The mechanisms that allow the contractile vacuole system to collect, sequester and expel excess fluid remain to be elucidated. (See Kitching (1967), Patterson (1980), and Zeuthen (1992) for reviews.)

Early electron microscopic studies revealed the presence of distinctive pegs on certain membranes of the contractile vacuole system in protozoa, leading to the suggestion that these structures might play a role in water sequestration (McKanna, 1974, 1976). It has now been demonstrated that the peg-decorated membranes are the locus of fluid segregation in *Paramecium* (Ishida et al., 1993). A recent freeze-dry electron microscopy survey of several organisms showed that these pegs, visualized on the contractile vacuole or spongione membranes of *Dictyostelium, Acanthamoeba* and *Naegleria*, were identical in morphology to the head domains of vacuolar proton pumps on the apical membranes of toad bladder epithelium and the membranes of osteoclasts (Heuser et al., 1993). The high concentration of proton pumps on these membranes suggests that they are likely to play an important role in contractile vacuole function.

Vacuolar proton pumps (also called vacuolar H⁺-
ATPases or V-ATPases) are multimeric enzymes that transport protons across the membranes of eukaryotic cells. The structure of these enzymes is highly conserved in organisms as diverse as yeast and mammals. The enzyme consists of two domains, a cytoplasmic (V1) domain that hydrolyses ATP, and an integral membrane (V0) domain that carries the protons produced by ATP hydrolysis across the membrane. Each domain is composed of multiple subunits. Vacular proton pumps serve many functions. In the endomembranes of eukaryotic cells, they acidify organelles such as clathrin-coated vesicles and lysosomes. In plasma membranes, they allow renal intercalated cells to acidify urine and osteoclasts to resorb bone. The proton gradient that they establish across organelar membranes is also used to energize transport, such as the uptake of neurotransmitter amines into synaptic vesicles. The properties and roles of vacular proton pumps have been recently reviewed (Nelson, 1992).

An examination was undertaken of the distribution of proton pumps in the contractile vacuole system of Dictyostelium amoebae (Heuser et al., 1993). Electron microscopy of freeze-dried broken cells revealed that the contractile vacuole system consisted of an interconnected array of cisternae and tubules, with all parts of the system except the narrowest tubules being richly endowed with proton pumps. The pumps became clumped and decorated when exposed to polyclonal antibodies specific for the B subunit of the V-ATPase of chromaffin granules (Moriyama and Nelson, 1989). The same antibodies labeled contractile vacuole membranes in indirect immunofluorescence experiments. However, these antibodies did not react strongly with Dictyostelium and their availability was limited, so they were not the ideal reagents for additional immunocytochemical studies. We therefore undertook to raise monoclonal antibodies against the vacular proton pumps of Dictyostelium for use as probes in further analysis of the structure and composition of the contractile vacuole system.

A membrane fraction rich in V-ATPase activity had previously been isolated from Dictyostelium amoebae (Nolta et al., 1991). The major polypeptides present in this membrane fraction, which was given the name ‘acidosomes’, had apparent molecular masses of 90, 68, 53, 42, 37, 25, 17 and 15 kDa, with the 90 and 17 kDa polypeptides behaving as integral membrane proteins. This composition resembles the subunit composition of mammalian and fungal V-ATPases (Nelson, 1992). Thus, this membrane fraction appeared to be a suitable immunogen for the production of monoclonal antibodies against vacular proton pumps of Dictyostelium. A sample of this proton pump-rich membrane fraction, generously provided by Drs K. Nolta and T. Steck, was used for this purpose.

We describe here the production and characterization of two monoclonal antibodies that recognize subunits of the vacular proton pump of Dictyostelium. One monoclonal antibody recognizes a subunit of the catalytic head domain, and the other a subunit of the membrane domain. The distribution of these antigens has been examined by indirect immunofluorescence and in frozen thin sections. The results confirm that membranes of the contractile vacuole complex are the primary locus of vacular proton pumps in Dictyostelium. Furthermore, indirect immunofluorescence studies with these antibodies have provided clearer visualization of the extent and complexity of the contractile vacuole system.

**MATERIALS AND METHODS**

**Materials**

Dictyostelium discoideum strains AX2 and AX3 were grown on HL5 medium at 21-22°C (Clarke et al., 1980), either in suspension or on tissue culture plates. Suspension cultures were swirled on a rotary shaker at 180 rpm. Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA), American Qualex (La Mirada, CA), Cappel (Organon Teknika Corp., Durham, NC), and Sigma (St. Louis, MO). Paraformaldehyde and glutaraldehyde were from Ladd (Burlington, VT).

**Production of monoclonal antibodies to the V-ATPase subunits**

A membrane fraction rich in V-ATPase isolated from Dictyostelium was a gift from Drs K. Nolta and T. Steck (Department of Biochemistry and Molecular Biology, The University of Chicago). Briefly, Balb/c mice were immunized with this fraction in Freund’s complete adjuvant, and monoclonal antibodies were produced as described previously (Fok et al., 1988). The hybridomas were screened using an enzyme-linked immunosorbent assay (ELISA). A total of four hybridoma cell lines were produced, and two of these lines, B8-3-2-2 (N2) and B30-2-3 (N4), were selected for this study. The first line was subcloned three times, while the second was subcloned twice.

**Immunoblotting procedure**

Samples of the proton pump-rich membrane fraction used as the immunogen and of a total lysate of Dictyostelium cells were immunoblotted. For preparation of the lysate, exponentially growing AX2 cells were harvested, washed and lysed as described by Heuser et al. (1993). Proteins were reduced and processed for SDS-polyacrylamide (10%) gel electrophoresis according to Laemmli (1970) and subsequently transferred to PVDF (Millipore Immobilon P) membrane according to Towbin et al. (1979). The membranes were blocked with 2% bovine serum albumin. The blots were incubated sequentially with one of the monoclonal antibodies (N2 or N4 culture supernatants at 1:50 and 1:20, respectively) and then with peroxidase-conjugated goat anti-mouse IgG (American Qualex, 1:2000). Enzyme reaction products were visualized using 3-amino-9-ethylcarbazole and hydrogen peroxide according to Hulen et al. (1991).

**Fluorescence and electron microscopy**

In preparation for indirect immunofluorescence experiments, Dictyostelium cells were subjected to the two-step agar-overlay fixation procedure described by Fukui et al. (1987). The buffer used for the first step of fixation was one-third strength HL5 containing 2% formamide and 0.1% dimethyl sulfoxide (DMSO) or, in the case of experiments involving the polyclonal anti-B subunit antiserum, 20 mM HEPES-NaOH (pH 7.0), 1 mM MgCl2 also containing formaldehyde and DMSO. After 5 minutes at room temperature, the cells were transferred to cold (~15°C) 1% formaldehyde in methanol for 5 minutes. Immunostaining procedures were as described by Clarke et al. (1987) and Heuser et al. (1993). Rabbit anti-Dictyostelium calmodulin antibodies were affinity-purified using calmodulin blotted onto PVDF membrane (Zhu et al., 1993). Monospecific polyclonal rabbit antiserum against the B subunit (57 kDa) of the chromaffin granule mem-
brane V-ATPase (Moriyama and Nelson, 1989) was a generous gift from Dr Nathan Nelson (Roche Institute of Molecular Biology, Nutley, NJ). In double-labeling studies for both immunofluorescence and immunoelectron microscopy, the two primary antibodies were mixed before incubation, and the two secondary antibodies were also pooled before use.

For immunoelectron microscopy, cells were fixed in 0.25% glutaraldehyde and 4% paraformaldehyde in half-strength HL5 for 45 minutes at room temperature. In some cases the cells were exposed to 0.2% DMSO in HL5 for 10-30 minutes prior to being fixed; this treatment had no significant effect on the quality of fixation. After fixation, the cells were washed 3× in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl (TBS), quenched with two treatments of sodium borohydride (0.5 mg/ml in TBS), washed again in TBS, and then processed for cryosectioning and immunogold staining as published earlier (Allen et al., 1990). To reduce nonspecific staining, all sections were preincubated with 5% normal goat serum prior to each incubation with primary and secondary antibodies. Controls consisted of using irrelevant primary antibodies or omitting the primary antibody.

RESULTS

Specificity of the monoclonal antibodies N2 and N4

The proton pump-rich membrane fraction isolated from Dictyostelium cells contained multiple polypeptides, the major polypeptides being presumptive subunits of the vacuolar proton pump (Nolta et al., 1991). When we used this membrane fraction as an immunogen for monoclonal antibody production, we obtained hybridoma cell lines producing antibodies that recognized each of the two largest major polypeptides (Fig. 1A). Antibodies from two of those cells lines, N2 and N4, are characterized here. The relevant polypeptides had been assigned molecular masses of 90 kDa and 68 kDa by Nolta et al. (1991), but our size determination, based on the SDS-gel migration rate of these polypeptides relative to Bio-Rad markers, gave a somewhat different value (100 kDa) for the larger polypeptide (Fig. 1A). When the N2 and N4 monoclonal antibodies were used to probe an immunoblot of total Dictyostelium cell proteins, N2 antibodies recognized only a 100 kDa polypeptide, and N4 recognized only a 68 kDa polypeptide (Fig. 1B). Thus, the epitopes recognized by the N2 and N4 antibodies appeared to be restricted to single presumptive subunits of the vacuolar proton pump.

Immunolocalization studies at the light microscopic level using N2 and N4

The monoclonal antibodies N2 and N4 were used in indirect immunofluorescence experiments to examine the distribution of the 100 kDa and 68 kDa polypeptides in Dictyostelium cells. Earlier studies (Heuser et al., 1993) had indicated that a major locus of vacuolar proton pumps in Dictyostelium is the membranes of the contractile vacuole system. We therefore began by comparing the distribution of the polypeptides recognized by N2 and N4 to the distribution of calmodulin, a marker for contractile vacuole membranes. (Under the fixation and permeabilization conditions that we use for indirect immunofluorescence, soluble calmodulin is retained only poorly, but membrane-associated calmodulin remains, acting as a selective label for the contractile vacuole system (Zhu and Clarke, 1992).) We double-stained exponentially growing Dictyostelium cells with either N2 or N4 monoclonal antibodies, together with affinity-purified anti-calmodulin antibodies, as previously described (Zhu et al., 1993).

In vegetative Dictyostelium cells, N2 antibodies co-localized with anti-calmodulin antibodies (Fig. 2A-I), and N4 antibodies also co-localized with anti-calmodulin (Fig. 3A-F). Usually the staining appeared as a bright area surrounding and extending from one or a few phase-lucent vacuoles. This type of image is commonly observed for the
Fig. 2. Indirect immunofluorescence localization of the 100 kDa polypeptide recognized by N2. Exponentially growing *Dictyostelium* cells were fixed and double-stained with N2 antibodies (A,D,G,J) and with anti-calmodulin antibodies (B,E,H) or with anti-V-ATPase B subunit antibodies (K). For each cell, a phase-contrast image is also shown (C,F,I,L). The primary antibodies were N2 hybridoma culture supernatant (mouse) at 1:25 or 1:50, affinity-purified anti-calmodulin (rabbit) at 1:50 or 1:100, and anti-V-ATPase B subunit (rabbit) at 1:50. The secondary antibodies were FITC-conjugated goat anti-mouse IgG (Sigma) at 1:50 and rhodamine-conjugated goat anti-rabbit IgG (Cappel) at 1:400. The 100 kDa polypeptide co-localized with calmodulin and the V-ATPase subunit, indicating that it is found in membranes of the contractile vacuole system. Bar, 10 µm.
contractile vacuole system under conditions in which its tubular elements are not individually resolved (see Discussion). Sometimes, as in the upper cell of Fig. 2A-C, the staining was confined to the periphery of a large, easily identified contractile vacuole.

We also compared the distribution of the 100 kDa and 68 kDa polypeptides to that of the 57 kDa polypeptide recognized by an antiserum against the B-subunit of the chromaffin granule V-ATPase (Moriyama and Nelson, 1989). Earlier studies had demonstrated that this antiserum labels vacuolar proton pump heads in Dictyostelium cells (Heuser et al., 1993). Both N2 (Fig. 2I-L) and N4 antibodies (Fig. 3G-L) co-localized with the anti-B subunit antibodies.

The distribution of the 100 kDa and 68 kDa polypeptides was also examined in mitotic cells (Fig. 4). It has been shown that the contractile vacuole system disperses in mitotic Dictyostelium cells, behavior resembling that of Golgi membranes (Zhu et al., 1993). Fig. 4A-C shows an

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**Fig. 3.** Immunolocalization of the 68 kDa polypeptide recognized by N4. Exponentially growing Dictyostelium cells were double-stained with N4 antibodies (A,D,G,J) and with anti-calmodulin (B,E) or with anti-V-ATPase B subunit antibodies (H,K). Phase-contrast images are shown to the right (C,F,I,L). The primary antibodies were N4 culture supernatant at 1:20, anti-calmodulin at 1:50, and anti-V-ATPase B subunit at 1:100. The secondary antibodies were as described for Fig. 2. The 68 kDa polypeptide co-localized with calmodulin and the V-ATPase subunit on membranes of the contractile vacuole system. Bar, 10 µm.
anaphase cell stained with N4 monoclonal antibodies. At a focal plane near the upper surface of the cell (A), many tiny vacuoles are seen. At a slightly deeper focal plane (B), the vacuoles at the cell periphery are more prominent. These images are indicative of the cortical localization of the tiny contractile vacuoles, a characteristic of mitotic cells. Fig. 4D-F shows a single focal plane of another anaphase cell, double-stained with N2 (D) and anti-calmodulin (E). In this cell, the two types of antibodies labeled the tiny scattered vacuoles in a similar pattern.

There are two important implications from these double-staining experiments. First, the antigens recognized by N2 and N4 co-localized with the B subunit of the vacuolar proton pump, supporting the identification of the 100 kDa and 68 kDa polypeptides as proton pump subunits. Second, their distribution closely resembled that of calmodulin, a known marker for the contractile vacuole system, in both vegetative and mitotic cells. These results support an earlier finding that contractile vacuole membranes are the principal locus of vacuolar proton pumps in *Dictyostelium* cells (see Discussion).

The immunofluorescent signal from double-stained cells was always fainter than that from cells stained with a single type of antibody, possibly because of steric interference among antibody molecules bound to antigens lying close together. Thus, details in such images were limited. However, in some preparations stained with either N2 or N4 antibodies alone, we were able to obtain better resolution of the small elements of the contractile vacuole system (Fig. 5). Fig. 5A-D shows two examples of cells stained with N2 antibodies. The tubules interconnecting distant parts of the contractile vacuole system are visible in these images. Fig. 5E-H shows three focal planes of a single cell stained with N4 monoclonal antibodies. Two large vacuoles extend from near the upper surface (E) through the center of the cell (F). The third image (G), in the region of the cell close to the substratum, shows tubular elements interconnecting these vacuoles. Note that regions of diffuse fluorescence in one image are often revealed to be out-of-focus views of structures clearly defined in another focal plane. The phase-contrast image (H) corresponds approximately to the upper immunofluorescence image (E). Although some tubules are visible in all three immunofluorescence images, the tubules appear to be most abundant close to the membrane that is associated with the substratum. This is consistent with data obtained using other techniques (see Discussion). These images suggest that the monoclonal antibodies N2 and N4 will be powerful probes for resolving the structure of the contractile vacuole system.

**Immunolocalization studies at the electron microscopic level**

The distribution of the 100 kDa and 68 kDa polypeptides was also examined by immunogold labeling of cryosections of *Dictyostelium* cells. The labeling intensity of the two monoclonal antibodies was found to depend strongly on the
fixation conditions. Initial experiments showed that the antigenicity of the epitopes recognized by N2 and N4 was completely suppressed when the cells were fixed with 0.5% glutaraldehyde. To combine adequate antigenicity with reasonable preservation of the fine structure of these cells, we adopted a fixative of 4% paraformaldehyde and 0.25% glutaraldehyde.

In frozen thin sections of axenically grown Dictyostelium cells (strains AX2 and AX3), N2 antibodies typically labeled a few vacuoles and a collection of small vesicles, the latter often lying adjacent to the vacuoles (Fig. 6A). Some of these vacuoles appeared to be collapsed, in that they were non-spherical in profile. Fig. 6B shows an especially large collection of the small vesicles. The labeled vacuoles and vesicles were frequently found immediately adjacent to the plasma membrane, which was never labeled. Aside from this group of vacuoles and small vesicles, no other organelles were found to be significantly labeled. Mitochondria, the nucleus and the cytosol were essentially devoid of gold particles. Similar results were obtained using N4 antibodies. Fig. 6C shows a non-spherical vacuole that was labeled with N4. Some vesicles nearby also bore a few gold particles. N4 gave weaker labeling than N2. Control sections, incubated only in secondary antibody, were devoid of label.

As axenically grown cells contained both labeled and unlabeled vacuoles, it was necessary to ascertain the identity of the labeled vacuoles and the adjacent small vesicles. Since calmodulin has been shown to be highly enriched on contractile vacuole membranes and not on the membranes of food vacuoles in Dictyostelium (Zhu and Clarke, 1992), we double-labeled the cryosections using both affinity-purified rabbit anti-calmodulin and N2 monoclonal antibodies. Our results showed that calmodulin and the 100 kDa polypeptide co-localized on the membranes of the same vacuoles (Fig. 7A) and small vesicles (Fig. 7B). With anticalmodulin labeled cryosections, a low level of gold particles was also found in the background cytosol, suggesting that soluble calmodulin was at least partially retained in frozen thin sections of cells that had not been permeabilized.

The distribution of the 100 kDa polypeptide was also compared with that of the V-ATPase B subunit in cryosections. Double-labeling experiments showed that these two antigens were present on the same large vacuoles and assortment of small vesicles and tubules (Fig. 8A,B). The
results from immunogold labeling thus were consistent with the immunofluorescence experiments and suggested that subunits of the vacuolar proton pump were principally localized on contractile vacuole membranes.

**DISCUSSION**

We have produced and characterized two monoclonal antibodies raised against a vacuolar proton pump-rich membrane fraction from *Dictyostelium* cells. The N2 monoclonal antibodies specifically label a 100 kDa polypeptide in whole cell lysates. This polypeptide corresponds to band 1 of the V-ATPase preparation described by Nolta et al. (1991). In preliminary studies, we have obtained independent verification that this polypeptide is a subunit of the vacuolar proton pump. N2 monoclonal antibodies were used to screen a λgt11 expression library of *Dictyostelium* cDNA.
A 1.1 kb cDNA insert identified by this screen encodes an open reading frame whose amino acid sequence is 39% identical (77% similar) to the amino terminus of the 116 kDa subunit of the rat synaptic vesicle/clathrin-coated vesicle V-ATPase (Perin et al., 1991). The Dictyostelium cDNA fragment is currently being used as a probe to isolate the remainder of the gene (Liu and Clarke, unpublished studies). This V-ATPase subunit is a highly conserved integral membrane protein that is postulated to play a role in targeting proton pumps to the correct endomembrane (Gluck, 1992). We intend to explore its function in Dictyostelium cells through the use of molecular genetic techniques.

The second monoclonal antibody, N4, specifically recognizes a 68 kDa polypeptide in Dictyostelium total cell lysates. This polypeptide corresponds to band 2 of the Nolta et al. (1991) V-ATPase preparation. Based on its size and its release from membranes by dilute chaotropic agents, this polypeptide was postulated to be the A subunit of the vacuolar proton pump, part of the catalytic head domain (Nolta et al., 1991). In preliminary studies, we have confirmed this identification. N4 monoclonal antibodies, used to screen a Dictyostelium cDNA expression library, led to the isolation of a 2.0 kb cDNA encoding an open reading frame whose predicted amino acid sequence is 61% identical to that of the Neurospora V-ATPase A subunit (Bowman et al., 1988). We are currently characterizing this Dictyostelium cDNA (Burdine and Clarke, unpublished studies). Thus, the N2 and N4 monoclonal antibodies recognize subunits in both the V₀ and V₁ domains of the V-ATPase molecule.

In immunolocalization studies at both the light and electron microscopic levels, N2 and N4 co-localized with antibodies against the V-ATPase B subunit, as would be expected for different subunits of the same enzyme. The membranes labeled by these antibodies were identified as contractile vacuole membranes on the basis of several criteria. First, the N2 and N4 antibodies co-localized with anti-calmodulin antibodies, which were previously shown to be markers for contractile vacuole membranes (Zhu and Clarke, 1992; Zhu et al., 1993). Second, their co-localization with the anti-B subunit antibodies also supported this identification, since the latter antibodies had previously

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**Fig. 7.** Double-labeling with N2 (1:20, 18 nm gold, large arrowheads) and anti-calmodulin (1:100, 12 nm gold, small arrowheads). (A) N2 and affinity-purified rabbit anti-calmodulin antibodies co-localized on contractile vacuole compartments next to the plasma membrane. (B) Co-localization of the same antibodies as in A on a mass of tubules. Bars, 0.5 μm.
been shown to cause the proton pump heads visible on contractile vacuole membranes in freeze-dried Dictyostelium cells to become decorated and clumped (Heuser et al., 1993). Finally, the structure of the contractile vacuole system revealed by the N2 and N4 antibodies is very similar to that visualized in living cells by interference reflection microscopy (Heuser et al., 1993). In particular, these antibodies labeled an array of tubules closely associated with the cytoplasmic face of the plasma membrane attached to the substratum, the tubules being connected to each other and to phase-lucent vacuoles. Thus, our immunolocalization studies of the 100 kDa and 68 kDa proton pump subunits support the conclusion of Heuser et al. (1993) that contractile vacuole membranes are the principal locus of vacuolar proton pumps in Dictyostelium.

Our results must be compared with those of Nolta et al. (1993). These workers raised a polyclonal antiserum against the same proton pump-rich membrane fraction used here to prepare monoclonal antibodies, except that they first enriched the V-ATPase complex by detergent extraction of the membranes. Their polyclonal antiserum labeled many organelles in Dictyostelium cells, predominantly what we would identify as the contractile vacuole system, but also lysosomes, phagosomes and multivesicular bodies. The more extensive labeling was probably due both to the polyclonal nature of the antiserum and to the very high antibody concentration used for immunostaining (a 1:1000 dilution of an antiserum whose specificity had been tested by immunoblot at a 1:50,000 dilution). This high antibody concentration may have led to the labeling of organelles containing low levels of proton pumps and possibly even to the detection of antigens other than proton pump subunits. Our preliminary studies suggest that proton pumps may be present, although not abundant, on endosomal membranes in Dictyostelium.

The major discrepancy between our results and those of Nolta et al. (1991, 1993) is their identification of the predominant immunoreactive organelles, composed of complexes of vacuoles and tubules, as ‘acidosomes’ rather than elements of the contractile vacuole system. In their view,
acidosomes are distinct pump-rich organelles. However, our current study, as well as an earlier examination of living and freeze-dried cells (Heuser et al., 1993), failed to detect any organelle in *Dictyostelium* aside from the contractile vacuole system that bears a high concentration of proton pumps. Moreover, the pump-rich ‘acidosome’ fraction used as our immunogen elicited the monoclonal antibodies shown here to label contractile vacuole membranes. We infer that acidosomes isolated biochemically are probably fragments of the contractile vacuole system. Thus, the biochemical characterization of this membrane fraction described by Nolta et al. (1991) and related papers may help to shed light on the properties of contractile vacuole membranes.

The N2 and N4 monoclonal antibodies described here represent powerful probes for examining the organization and dynamics of the contractile vacuole system in *Dictyostelium*. Through molecular genetics, they also provide the means of exploring the function of these two highly conserved subunits of the V-ATPase enzyme complex in *Dictyostelium*. Exciting preliminary data suggest that they may be equally useful in characterizing the V-ATPase of mammalian cells (Sturgill-Koszycki et al., unpublished data). Thus, biochemical, immunological and molecular biological approaches are now available for analysis of this ubiquitous and highly conserved enzyme complex. We hope to use these tools to elucidate the role of proton pumps in contractile vacuole function.

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