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Involvement of the vacuolar proton-translocating ATPase in multiple steps of the endo-lysosomal system and in the contractile vacuole system of *Dictyostelium discoideum*

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SUMMARY

We have investigated the effects of Concanamycin A (CMA), a specific inhibitor of vacuolar type H⁺-ATPases, on acidification and function of the endo-lysosomal and contractile vacuole (CV) systems of *D. discoideum*. This drug inhibited acidification and increased the pH of endo-lysosomal vesicles both in vivo and in vitro in a dose dependent manner. Treatment also inhibited endocytosis and exocytosis of fluid phase, and phagocytosis of latex beads. This report also confirms our previous conclusions (Cardelli et al. (1989) *J. Biol. Chem.* **264**, 3454-3463) that maintenance of acidic pH in lumenal compartments is required for efficient processing and targeting of a lysosomal enzyme, α-mannosidase. CMA treatment compromised the function of the contractile vacuole complex as amoebae exposed to a hypo-osmotic environment in the presence of CMA, swelled rapidly and ruptured. Fluorescence microscopy revealed that CMA treatment induced gross morphological changes in *D. discoideum* cells, char-acterized by the formation of large intracellular vacuoles containing fluid phase. The reticular membranes of the CV system were also no longer as apparent in drug treated cells. Finally, this is the first report describing cells that can adapt in the presence of CMA; in nutrient medium, *D. dis-coideum* overcame the effects of CMA after one hour of drug treatment even in the absence of protein synthesis. Upon adaptation to CMA, normal sized endo-lysosomal vesicles reappeared, endo-lysosomal pH decreased, and the rate of endocytosis, exocytosis and phagocytosis returned to normal. This study demonstrates that the V-H⁺-ATPase plays an important role in maintaining the integrity and function of the endo-lysosomal and CV systems and that *D. discoideum* can compensate for the loss of a functional V-H⁺-ATPase.

Key words: Vacuolar ATPase, *Dictyostelium*, Concanamycin A, Endo-lysosome, Contractile vacuole

INTRODUCTION

In eukaryotic cells, many organelles including the Golgi complex, endosomes and lysosomes, are acidified (reviewed by Mellman et al., 1986). Many of these compartments rely on an electrogenic proton pumping ATPase, known as a vacuolar type ATPase (V-H⁺-ATPase), for the maintenance of their intra-luminal acidity. There is an increasing body of evidence demonstrating that a variety of cellular functions are regulated by the acidic lumens of these various organelles. For example, acidic pH is required for receptor recycling, uncoupling of receptor-ligand complexes, uptake of viruses or toxins, and for some of the functions associated with the endosomal/lysosomal (endo-lysosomal) system such as degradative processes and the biosynthesis and sorting of lysosomal hydrolases. Acidotropic agents such as chloro-quine, primaquine and ammonium chloride (reviewed by Mellman et al., 1986) have been used to study the function served by acidification in the intracellular processes; however, the concentration required to achieve intracellular alkalinization and the non-specific effects of these compounds has limited their usefulness in studies defining the role of acidic compartments.

The acidotropic amine, ammonium chloride, has been used to study the role of acidic intracellular compartments in the biosynthesis of lysosomal enzymes in the cellular slime mold *Dictyostelium discoideum,* and it was demonstrated that the maintenance of acidic pH in lumenal compartments was required for efficient processing and targeting of lysosomal enzymes (Cardelli et al., 1989). *D. discoideum* is a useful system in which to study endo-lysosomal function because it can be manipulated biochemically and genetically. Further-more, the biosynthetic and endocytic pathways to lysosomes are similar to those described in mammalian cells. For example, we and others have shown that newly synthesized precursors of lysosomal enzymes are phosphorylated and sulfated on N-linked oligosaccharide side chains in the Golgi, proteolytically cleaved in endosomes and then transported to

dense secondary lysosomes where processing is completed (reviewed by Cardelli, 1993).

During endocytosis, fluid phase markers are ingested by *D. discoideum* into clathrin-coated vesicles (O'Halloran and Anderson, 1992; Ruscetti et al., 1994) and then enter acidic lysosome-like vesicles (Padh et al., 1993; Aubry et al., 1993a). Finally, these markers enter a larger non-acidic post-lysosome prior to their egestion (Padh et al., 1993; Aubry et al., 1993a). In contrast to mammalian cells, rapid recycling of fluid phase markers to the plasma membrane, soon after their ingestion, has not been observed in *D. discoideum*. Vesicle traffic is apparently linear and ingested material must traverse the entire endocytic circuit prior to release from the cell. We and others have observed that a vacuolar ATPase (V-H$^+$-ATPase) complex is associated with endo-lysosomal membranes of *D. discoideum* (Nolta et al., 1994; Temesvari et al., 1994; Bush et al., 1994), where it likely functions in intra-lumenal acidification of this membrane system. In addition to the endosomal system, the V-H$^+$-ATPase is also associated with the membranes comprising the CV system, an organelle system involved in osmoregulation (Fok et al., 1993; Heuser et al., 1993; Bush et al., 1994).

Macrolide antibiotics that selectively block the function of proton pumps make it possible to elucidate more directly the function served by acidification in a variety of intracellular systems (van Weert et al., 1995; Reaves and Banting, 1994; Clague et al., 1994). This report describes the effects of a specific V-H$^+$-ATPase inhibitor, CMA (Kinashi et al., 1984), on acidification and the functions of the *D. discoideum* endo-lysosomal and CV systems. In vitro, CMA is an extremely potent inhibitor (IC$_{50}$ = 1 to 5 nm) of V-H$^+$-ATPases and can discriminate between mitochondrial, plasma membrane, and vacuolar ATPases (Bowman et al., 1988; Mattsson et al., 1991; Woo et al., 1992). We demonstrate that treatment of *D. discoideum* cells with CMA resulted in neutralization of the lumen of the endo-lysosomal system and inhibition of endo-lysosomal functions such as endocytosis, exocytosis and phagocytosis. Moreover, maintenance of acidic pH in lumenal compartments was required for efficient processing and targeting of a lysosomal enzyme, α-mannosidase. CMA also inhibited CV function and induced gross morphological changes in *D. discoideum* cells, characterized by a decrease in small endo-lysosomal vesicles and the formation of large intracellular vacuoles containing fluid phase and contractile vacuole markers.

MATERIALS AND METHODS

Cells and culture conditions

For all experiments, *D. discoideum*, strain Ax4, was grown axenically at 21°C, in HL5 medium (1% Oxoid proteose peptone, 1% glucose, 0.5% yeast extract (Difco Laboratories Inc., Detroit, MI), 2.4 mM Na$_2$HPO$_4$, and 8.8 mM KH$_2$PO$_4$, pH 6.5).

Measurement of endo-lysosomal pH

Endosomal pH in living *D. discoideum* amoebae (strain Ax4) was measured by a dual excitation ratio method with FITC-dextran as a pH probe (Cardelli et al., 1989). Log phase cells were harvested by centrifugation (500 ***g***, for 5 minutes), and resuspended at a concentration of 3×10^6 cells/ml in fresh HL5 medium supplemented with a fluorescein-labelled marker, FITC-dextran (70,000 M_r; Sigma

Chemical Co., St Louis, MO) (2 mg/ml) and incubated for 3 hours. Following incubation with FITC-dextran, water (no treatment), ethanol (0.01%, v/v; control) or CMA (0.1 µM to 10 µM) (Fluka) was added to the medium and the cells were incubated for various times. In some instances, the cells were exposed to CMA or ethanol in the presence of the protein synthesis inhibitor, cycloheximide (400 µg/ml). To examine the nature of adaptability to CMA (see Results), after 1 hour of CMA treatment as described above, amoebae were collected by centrifugation and resuspended in fresh HL5 supplemented with FITC-dextran (2 mg/ml) and fresh CMA (5 µM).

At the times indicated, cells were collected by centrifugation, washed and resuspended in 50 mM MES buffer (pH 6.5) at a concentration of 3×10^6 cells/ml. The cells were diluted 20× in MES buffer and fluorescence (I$_{450nm}$ and I$_{495nm}$) was measured at 520 nm using a Hitachi (Model F-4010) fluorimeter following excitation at 450 nm and 495 nm. The fluorescence excitation ratio at 495 nm and 450 nm (I$_{495nm}$/I$_{450nm}$) was calculated, and endosomal pH determined from an in vitro standard curve of FITC-dextran in a pH range of 4 to 7.

To measure pH in phosphate buffer, cells were incubated in FITC-dextran as described above, harvested and resuspended at a concentration of 3×10^6 cells/ml in 10 mM phosphate buffer (pH 6.5) supplemented with 2 mg/ml FITC-dextran in the presence or absence of CMA (5 µM). Incubation in the phosphate buffer mixture proceeded for 2 hours and endo-lysosomal pH was determined over time as described above.

Measurement of fluid phase traffic

Fluid phase endocytosis and exocytosis were measured according to the methods of Aubry et al. (1993a). Briefly, log phase cells were harvested by centrifugation, and resuspended at a concentration of 3×10^6 cells/ml in fresh HL5 medium or phosphate buffer supplemented with 2 mg/ml FITC-dextran in the presence of ethanol (0.01%, v/v; control) or CMA (5 µM). Endocytosis proceeded for 4 hours. To measure exocytosis, log phase cells were harvested by centrifugation, and resuspended at a concentration of 3×10^6 cells/ml in fresh HL5 medium supplemented with 2 mg/ml FITC-dextran. Incubation with FITC-dextran was allowed to proceed for 3 hours, after which time the cells were harvested, washed twice with HL5 medium, and finally resuspended in fresh HL5 medium or phosphate buffer in the presence of ethanol (0.01%, v/v; control) or CMA (5 µM). Exocytosis of FITC-dextran into fresh HL5 medium or phosphate buffer was allowed to proceed for 4 hours. To examine the nature of adaptability to CMA (see Results), after 1 hour of CMA treatment as described above, additional CMA was added to the medium (for endocytosis) or the amoebae were collected by centrifugation and resuspended in fresh HL5 supplemented with fresh CMA (5 µM) (for exocytosis).

At the times indicated during endocytosis or exocytosis, 1 ml samples (3×10^6 cells) were harvested by centrifugation, washed twice with HL5 medium or phosphate buffer, once with wash buffer (5 mM glycine, 100 mM sucrose, pH 8.5) and then stored on ice prior to fluorescence measurements. The cells were then lysed by the addition of 0.1 ml of 10% (v/v) Triton-X-100 to the pellets and diluted 20× in wash buffer for fluorescence measurements. Fluorescence was measured using excitation and emission wavelengths of 492 nm and 525 nm, respectively. The FITC-dextran was diluted to generate a standard curve, and the fluid phase volume taken up by 10^6 cells was calculated. All values were corrected for cellular auto-fluorescence and surface adhesion of FITC-dextran by subtracting the time zero value.

Measurement of phagocytosis

Log phase cells were harvested by centrifugation and resuspended at a concentration of 3×10^6 cells/ml in fresh HL5 medium or phosphate buffer supplemented with carboxylate-modified crimson fluorescence latex microspheres (1 µm, Molecular Probes Inc.) with or without CMA (5 µM). The particle to cell ratio was 50 to 1 and phagocytosis

was allowed to proceed for 2 hours. At the times indicated, 1 ml samples (3×10^6 cells) were harvested by centrifugation, washed and stored on ice as described above. For fluorescence measurements, the cells were lysed and diluted as described above. Fluorescence was measured using excitation and emission wavelengths of 595 nm and 625 nm, respectively. A particle standard curve was generated and the number of particles taken up per cell was calculated. As described above, all values were corrected for cellular auto-fluorescence and surface adhesion of fluorescence particles.

Morphological analyses

The morphology of the endo-lysosomal system and the subcellular distribution of fluid phase markers were examined microscopically. Log phase cells were harvested, and resuspended in HL5 medium or phosphate buffer, supplemented with FITC-dextran (2 mg/ml) in the presence or absence of CMA (5 µM) as described above. At the times indicated, 3×10^5 cells were harvested, allowed to adhere to glass slides and examined by phase contrast and fluorescence microscopy using an Olympus (Model BH2-RFL) microscope.

For sequential labelling with multiple fluorophores, amoebae were pulsed with a rhodamine-conjugated fluid phase marker, RITC-dextran (70,000 M_r; Sigma), for 15 minutes and then chased in fresh medium without marker for an additional 15 minutes. The amoebae were then incubated with a second fluid phase marker, FITC-dextran, for 15 minutes. Ethanol (0.01%, v/v; control) or CMA (5 µM) was then added to the medium, and the cells were incubated for an additional 45 minutes. Cell samples were prepared and examined by phase contrast and fluorescence microscopy as described above.

Isolation of endo-lysosomal vesicles and in vitro acidification

When 1-liter cultures reached 0.5×10^7 to 1.0×10^7 cells per ml, amoebae were harvested by centrifugation as described above and resuspended at a concentration of 5×10^7 cells/ml, in fresh HL5 medium supplemented with 1 mg/ml dextran-coated colloidal iron (Rodriguez-Paris et al., 1993) and 2 mg/ml FITC dextran. Endocytosis of these particles was allowed to proceed for 3 hours. The cells were then harvested by centrifugation and resuspended at a concentration of 2×10^8 cells/ml in 5 mM glycine (pH 8.5) supplemented with 100 mM sucrose. The cells were homogenized by passing the cell suspension through two 5 µm polycarbonate filters (Poretics). The homogenate was mixed with an equal volume of water to osmotically shock the vesicles and discharge their native acidity. Endo-lysosomal vesicles were then isolated from the homogenate by the magnetic fractionation technique of Rodriguez-Paris et al. (1993).

Measurements of in vitro acidification were performed according to the methods of Rodriguez-Paris et al. (1993), with slight modifications. Briefly, ATP-dependent quenching of the fluorescence of the isolated vesicles was used as a measure of in vitro acidification. The fluorescence intensity of the isolated vesicles was monitored spectrofluorometrically using an excitation wavelength of 492 nm and an emission wavelength of 520 nm. At time zero, isolated vesicles from 2×10^8 cell equivalents were added to a cuvette containing 2 ml of the following buffer: 30 mM KCl, 10 mM NaCl, 5 mM MgCl$_2$, 20 mM Hepes-KOH, pH 7.4, 0.1 mM EGTA. ATP was added to a final concentration of 2 mM to initiate acidification and fluorescence was recorded over time. To test the effects of CMA on isolated endo-lysosomal vesicles, ethanol (0.01%, v/v; control), CMA (1 µM), nigericin (3 µM, Sigma) or hexokinase (9 units/ml; Sigma) and glucose (10 mM) were added to the cuvette 1 minute before or 1 minute after the addition of ATP.

Measurement of ATP

Intracellular ATP was quantified using a bioluminescent somatic cell assay kit (Sigma) and a luminometer. Log phase cells were harvested, resuspended in HL5 medium in the presence or absence of CMA (5 µM) as described above. At the times indicated 1.5×10^6 cells were harvested, cell extracts were prepared, and intracellular ATP was measured according to the manufacturer's instructions.

Pulse chase analysis and immunoprecipitation

Exponentially growing cells were harvested by centrifugation, resuspended at a concentration of 10^7 cells/ml and incubated in fresh HL5 medium supplemented with 500-800 µCi of [^{35}S]methionine (NEN) for 30 minutes. Ethanol (0.01%, v/v; control) or CMA (5 µM) was then added to the medium and incubation continued for an additional 10 minutes. Cells were harvested by centrifugation, resuspended in unlabeled medium with ethanol (0.01%, v/v; control) or CMA (5 µM), and then incubated at room temperature for 4 hours (chase). A total of 10^7 cells were harvested by centrifugation at the times indicated during the chase, and resuspended in 0.5% Triton-X-100. The supernatants (medium) over the cell pellets were also preserved for analysis.

The cell and medium samples were adjusted to a final concentration of 5 mM Na$_2$EDTA, 150 mM NaCl, 50 mM Tris base, pH 7.6, 0.5% NP-40, 2 mM methionine, 1 mM NaN$_3$ (C buffer) and incubated on ice for 30 minutes with 0.1 volumes of Pansorbin (Calbiochem, San Diego, CA). Samples were centrifuged at 12,000 *g* for 3 minutes to pellet the Pansorbin and remove non-specifically bound proteins, and the resulting supernatants were incubated on ice for 1.5 to 2 hours with an excess of a monoclonal antibody against α-mannosidase (Mierendorf and Dimond, 1983). Excess Pansorbin was then added and incubation continued for an additional 60 to 90 minutes, after which time the immunoprecipitated proteins were collected by centrifugation, washed three times with C buffer, and resuspended in SDS-PAGE sample buffer (Laemmli, 1970). The proteins were separated by SDS-PAGE and visualized by fluorography.

Measurement of CV function

To assess the function of the CV system, growing amoebae were harvested by centrifugation, washed free of growth medium, and resuspended in a hypo-osmotic environment (water or 10 mM phosphate buffer) in plastic tissue culture dishes with or without CMA added to a final concentration of 5 µM. The cells were then monitored visually by phase contrast microscopy as described above.

Western blot analysis

Polyclonal antibodies recognizing the 41 kDa subunit of the *D. discoideum* V-H+-ATPase were generated by immunizing New Zealand male rabbits (Cocalico Biologicals Inc.) with bacterially expressed (recombinant) antigen. Monoclonal antibodies recognizing the 100 kDa and 70 kDa subunits of the *D. discoideum* V-H+-ATPase were a kind gift of Dr T. Steck (University of Chicago, Chicago, IL).

Amoebae were treated with CMA (5 µM) and/or cycloheximide (400 µg/ml) for 2 hours as described above. After CMA treatment, 3×10^6 cells were harvested by centrifugation, and resuspended in 45 µl of Laemmli (1970) loading buffer. One-dimensional SDS-PAGE was performed using the conventional discontinuous buffer system of Laemmli (1970). Typically, 3.3×10^5 cell equivalents of total protein were electrophoresed in 12% polyacrylamide gels. Proteins separated by SDS-PAGE were visualized by staining with 0.2% (w/v) Coomassie Brilliant Blue in 50% (v/v) methanol and 10% (v/v) acetic acid or were electroblotted onto nitrocellulose in a Towbin buffer system (Towbin et al., 1979) at 0.65 A for 3 hours at a controlled temperature of 15°C.

Nitrocellulose membranes containing immobilized protein were blocked overnight, at 4°C, in TBSTG buffer (10 mM Tris base, 150 mM NaCl, 0.05% (w/v) Tween-20, 0.1% (v/v) gelatin). After blocking, the membranes were incubated in a mixture of primary antibody (a 1/2,000 dilution, in TBSTG, of the polyclonal antibody recognizing the 41 kDa subunit and a 1/100 dilution, in TBSTG, of each of the monoclonal antibodies recognizing the 100 kDa and the 70 kDa subunits of the V-H+-ATPase). After extensive washing, the blots were incubated in TBSTG supplemented with goat anti-mouse

(Bio-Rad) (a 1/3,000 dilution in TBSTG) and goat anti-rabbit (Sigma) (a 1/30,000, dilution in TBSTG) alkaline phosphatase-conjugated secondary antibodies. The blots were developed in NBT buffer (100 mM Tris base, 100 mM NaCl, 5 mM MgCl$_2$) containing the alkaline phosphatase substrates, BCIP (1.2 mM, Amresco) and nitro blue tetrazolium (0.6 mM, Amresco).

RESULTS

Effects of CMA on intra-endosomal and lysosomal pH (pH$_{el}$)

The effects of the specific V-H$^+$-ATPase inhibitor, CMA, on the pH of endosomal and lysosomal compartments were determined by dual excitation ratio fluorimetry of cells using FITC-dextran as a pH probe. This marker is readily taken up by *D. discoideum* amoebae by pinocytosis, and the extent of fluorescence of the fluorescein moiety of this marker is dependent on the pH of the surrounding environment (Okuma and Poole, 1978). FITC-dextran has been used to measure pH$_{el}$ in a wide variety of cells, including *D. discoideum* (Yamashiro and Maxfield, 1988; Cardelli et al., 1989; Aubry et al., 1993a).

Dictyostelium amoebae were suspended in axenic growth medium in the presence of FITC-dextran for 3 hours. This time period has been shown to be sufficient for complete loading of all of the endosomal and lysosomal compartments with a fluid phase marker (Rodriguez-Paris et al., 1993). Following incubation with FITC-dextran, water (no treatment), ethanol (control) or various concentrations of CMA were added to the medium and the cells were incubated for an additional 30 minutes. The cells were then harvested and examined spectrofluorometrically. Consistent with previous reports (Cardelli et al., 1989; Bof et al., 1992; Brenot et al., 1992; Aubry et al., 1993a), the average pH$_{el}$ in untreated growing *D. discoideum* cells, was acidic, with a value of 5.4 to 5.8 (Fig. 1A). In cells treated with CMA for 30 minutes, the pH$_{el}$ increased to a value of 6.0 to 6.5 in a dose dependent fashion, with maximum effects observed at a CMA concentration of 5 µM (Fig. 1A).

Bafilomycin A1, a closely related V-H$^+$-ATPase inhibitor, had similar effects on the pH$_{el}$ of *D. discoideum* amoebae (Temesvari and Cardelli, data not shown; Aubry et al., 1993a). All subsequent experiments were performed using an extracellular CMA concentration of 5 µM. Ethanol, the CMA diluent, had no influence on the average pH$_{el}$ (Fig. 1A) and CMA by itself did not contribute to or quench fluorescence (data not shown).

To determine how rapidly the increase in pH$_{el}$ occurred after CMA treatment, FITC-dextran-loaded cells were exposed to CMA, and the pH$_{el}$ was measured over time. As seen in Fig. 1B the rise in pH was rapid, reaching a maximum value of 6.5 within 10 minutes of drug addition. However, at approximately 45 minutes of incubation with CMA, the pH$_{el}$ began to decrease to a value of 6.0. The addition of fresh CMA after 60 minutes did not result in a second rise in pH$_{el}$ (see below). The pH$_{el}$ in *D. discoideum* amoebae remained at a value of 6.0 for at least 18 hours of CMA treatment and the cells were viable over this time period, as determined by dye exclusion and recovery of clones growing as plaques on lawns of bacteria (data not shown).

CMA inhibits endosomal and phagosomal membrane traffic

Many vesicular compartments along the endo-lysosomal pathway in this organism rely on the electrogenic V-H$^+$-ATPase for maintenance of an acidic intra-organellar pH; therefore the role of a functional V-H$^+$-ATPase, in endocytosis, was examined. The fluid phase marker, FITC-dextran, was used to measure the ability of control and CMA-treated cells to take up fluid from the surrounding milieu. Cells were exposed to FITC-dextran (2 mg/ml) and CMA (5 µM) or ethanol (control) simultaneously in growth medium, and at the times indicated were harvested, lysed, and the level of intracellular fluorescence was determined spectrofluorometrically. CMA-treated cells exhibited an endocytosis rate that was approximately 80% lower (0.2 µl total uptake per 10^6 cells)
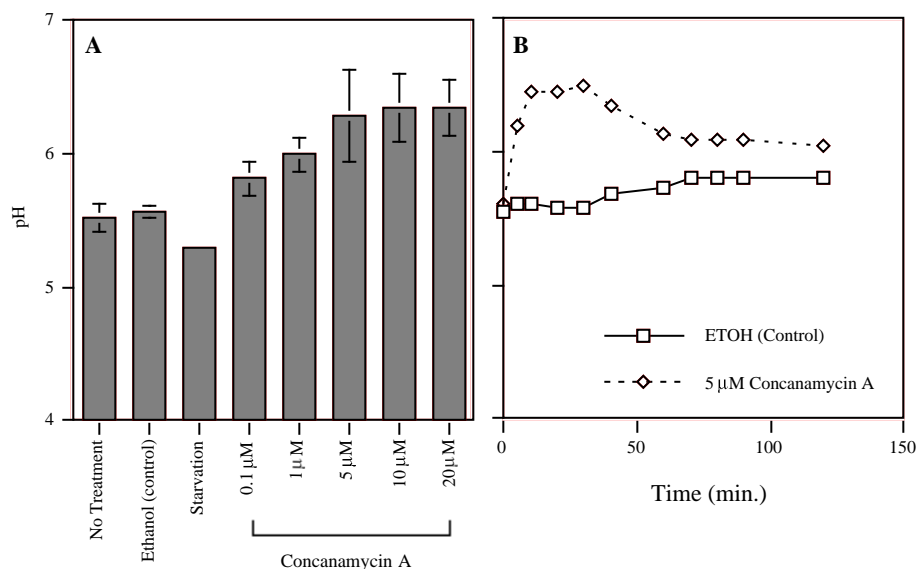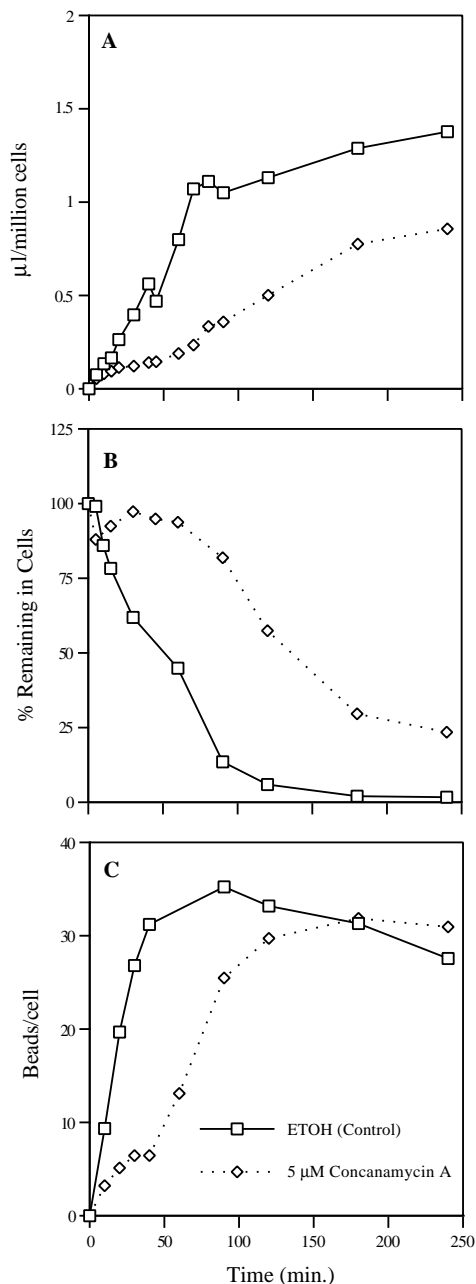

Fig. 1. The effects of CMA on pH$_{el}$. (A) Cells were incubated with FITC-dextran (2 mg/ml) in HL5 medium or phosphate buffer (starvation) for 3 hours at which time water (no treatment), ethanol (0.01%, v/v, control) or CMA (0.1 µM to 20 µM) was added and the cells were incubated for an additional 30 minutes. Cells were then collected by centrifugation, washed and resuspended in 50 mM Mes buffer (pH 6.5). Intracellular fluorescence was measured and converted to pH using a standard curve of FITC-dextran in a pH range of 4 to 7 (Cardelli et al., 1988). (B) Cells were loaded with FITC-dextran in HL5 medium and treated with ethanol (0.01%, v/v, control) or CMA (5 µM) as described above. At the times indicated during drug treatment, samples were taken and the pH was determined as described above. CMA inhibited acidification and increased the pH$_{el}$ in a statistically significant manner (*P*<0.01, *n*=3). At 60 minutes of incubation with CMA, a slight recovery of the pH$_{el}$ was observed as it decreased to a value of 6.0.
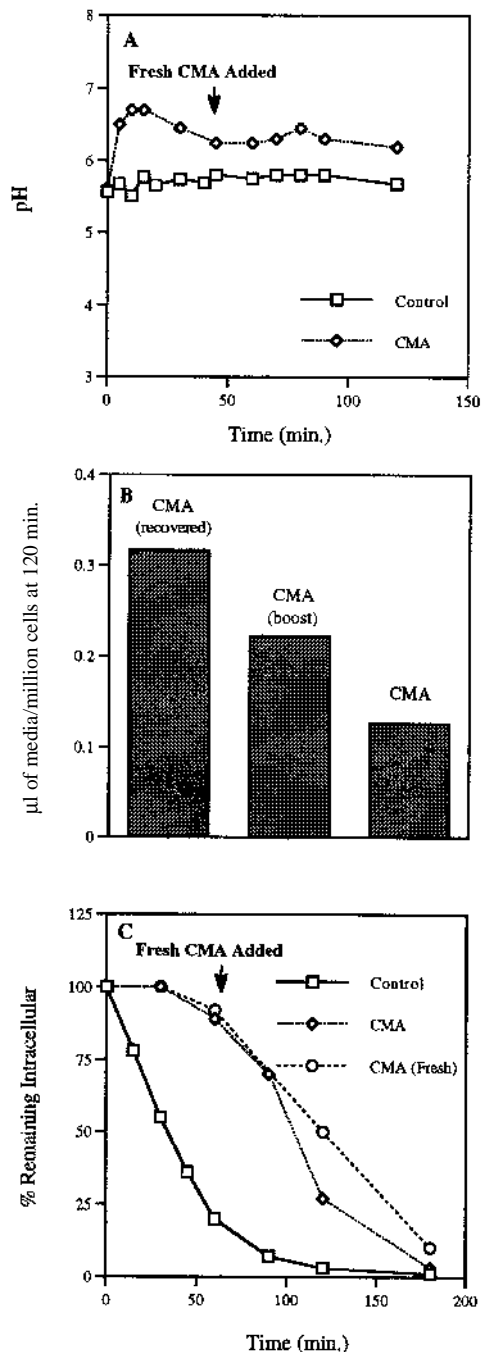
medium; this time course of feeding has been shown to be sufficient to achieve complete saturation of the endo-lysosomal compartments with a fluid phase marker (Rodriguez-Paris et al., 1993). The cells were harvested, washed free of the marker, and resuspended in fresh medium in the presence of ethanol (control) or CMA. The release of fluid over time was measured by monitoring the decrease in intracellular fluorescence. In control cells, the release of the fluid phase marker occurred immediately and was nearly complete by 2 hours. Exocytosis was severely impaired in CMA-treated cells for the first 60 minutes of treatment, after which time the cells regained their ability to release the fluid phase marker (Fig. 2B). However, after 4 hours, 25% of the fluid phase marker still remained in the treated cells. In control cells, 50% of the fluid phase marker was released by 60 minutes (Fig. 2B), while in cells treated with 5 μM CMA or 2 μM CMA, the time required to release 50% of fluid phase was 150 minutes (Fig. 2B) and 100 minutes (data not shown), respectively.

Since *D. discoideum* cells can also acquire nutrients by phagocytosis, we determined if an acidic pH$_{el}$ and a functional V-H⁺-ATPase were required for this process. Fluorescence labeled (crimson) latex microspheres were used to measure the ability of control and CMA-treated cells to take up large particles (1 μm) from the surrounding growth medium. Cells were exposed to crimson fluorescent latex microspheres (50 to 1 particle to cell ratio) and ethanol (control) or CMA simultaneously. Phagocytosis was allowed to proceed for 240 minutes and at the times indicated samples were harvested and the intracellular fluorescence was measured (Fig. 2C). Control cells phagocytosed beads at a linear rate for 30 to 45 minutes reaching a maximum of 30 to 40 beads per cell. In contrast, for the first 45 minutes of treatment, CMA-treated *D. discoideum* amoebae exhibited a phagocytosis rate that was approximately 70% lower than that of control cells. Analysis of variance demonstrated that the rate of latex bead uptake by the CMA-treated cells measured over the first 30 minutes of treatment was significantly lower than that of control cells ($P<0.01$, $n=4$). After 60 minutes the CMA-treated cells recovered their ability to take up fluorescent particles and the CMA-treated cells eventually took up the same number of

than control cells (1.05 μl total uptake per 10^6 cells) for the first 60 minutes of treatment (Fig. 2A). Analysis of variance demonstrated that the rate of fluid phase uptake by the CMA-treated cells measured over the first 40 minutes of treatment was significantly lower than that of control cells ($P<0.01$, $n=4$). Bafilomycin A1 had similar effects on the rate of endocytosis in *D. discoideum* amoebae (Temesvari and Cardelli, data not shown; Aubry et al., 1993a). CMA concentrations as low as 1 μM were effective in inhibiting endocytosis. Surprisingly, after 60 minutes, the CMA-treated cells recovered their ability to take up fluid.

The participation of V-H⁺-ATPases and the importance of acidic pH$_{el}$ in another stage of fluid phase movement, exocytosis, was also examined. *D. discoideum* amoebae were allowed to take up FITC-dextran for 3 hours in growth

Fig. 3. The nature of adaptability to CMA. (A) The effects of the addition of fresh CMA on the pH_{el} in adapted cells. After 1 hour of CMA treatment as described (see Materials and Methods and Fig. 1C), amoebae were collected by centrifugation and resuspended in fresh HL5 supplemented with FITC-dextran (2 mg/ml) and fresh CMA (5 μM). Measurement of the pH_{el} was performed as described. (B) The effects of additional CMA on the rate of endocytosis of fluid phase into adapted cells. After 1 hour of CMA treatment as described (see Materials and Methods and Fig. 2A), additional CMA was added to the medium. Measurement of the endocytosis was performed as described. (C) The effects of the addition of fresh CMA on the rate of exocytosis of fluid phase from adapted cells. After 1 hour of CMA treatment as described (see Materials and Methods and Fig. 2B), amoebae were collected by centrifugation and resuspended in fresh HL5 supplemented with fresh CMA (5 μM). Measurement of the exocytosis was performed as described. Adaptation was presumably not due to inactivation of the CMA, since replacement of the drug or addition of the drug to the surrounding medium of adapted cells did not result in significant increases in pH_{el} or re-inhibition of endocytosis or exocytosis.

pH_{el} or in re-inhibition of fluid phase exocytosis (Fig. 3A,C). During endocytosis, a similar experiment to test if adaptation was due to the inactivation of the drug was not possible, as repeated centrifugation of *D. discoideum* amoebae temporarily blocks uptake of fluid phase. Instead, in the case of endocytosis, fresh CMA was added to the existing medium of CMA-treated cells after they had adapted to the presence of the inhibitor (i.e. a 5 μM 'boost' of CMA was added to the medium). While the addition of fresh CMA (to the surrounding medium of adapted cells) had slight inhibitory activity on endocytosis (Fig. 3B), the effective final concentration of CMA could not be assessed and it is likely that cells which had adapted to 5 μM CMA were now in the presence of 10 μM CMA. Similar results were observed when the surrounding medium of cells which had adapted to the presence of 2.5 μM CMA was 'boosted' to 5 μM CMA (data not shown).

Recovery of pH_{el} in the presence of CMA does not require protein synthesis

Bafilomycin A1, a macrolide antibiotic that is structurally and functionally related to CMA, is postulated to bind to the 100 kDa subunit of the proton-translocating ATPase, and to exert its action by inhibiting proton flow through the H^{+} channel of the pump (Crider et al., 1994; Zhang et al., 1994). Adaptation to CMA may be the result of increased production of the 100 kDa and/or other subunits of the proton-translocating ATPase, such that all of the CMA is effectively absorbed leaving functional 'CMA-free' proton pumps. To investigate this, the cells were exposed to ethanol (control) or CMA (5 μM) for 15 minutes, at which time the protein synthesis inhibitor, cyclo-heximide, was added to the medium to a final concentration of 400 μg/ml. This concentration of cycloheximide has been shown to significantly inhibit protein synthesis in *D. discoideum* (Schatzle et al., 1991). The cells were incubated with cycloheximide for approximately 1.75 hours, during which time the amoebae adapted to CMA, as determined by a decrease in pH_{el} (see below). Once adapted, the cells were harvested and the relative intracellular levels of the 100 kDa, 70 kDa and 41 kDa subunits of the V-H^{+}-ATPase were determined by western blotting (see Materials and Methods). Western blot analysis (Fig. 4B) demonstrated that adaptation

beads (per cell) as control cells. Taken together, these results suggest that an acidic pH_{el} and/or a functional V-H^{+}-ATPase is required for *D. discoideum* to carry out the processes of endocytosis, exocytosis and phagocytosis. In all cases, after 60 minutes of treatment, the cells 'overcame' the action of CMA and recovered their ability to internalize and externalize membranes.

The apparent 'adaptation' to CMA was concomitant with the decrease in pH_{el} observed after 60 minutes of CMA treatment (Fig. 1B). In the case of pH_{el} and exocytosis, the adaptation was presumably not due to metabolic inactivation of CMA, since replacement of the surrounding medium with medium containing fresh CMA (5 μM) did not result in an increase in
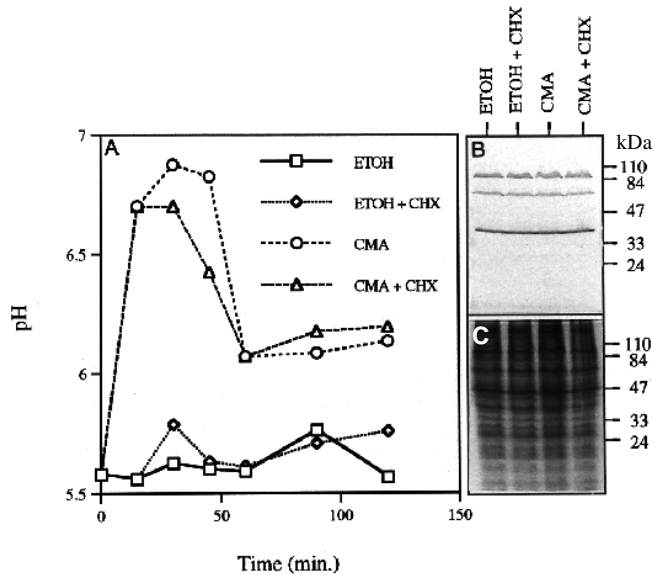
Fig. 4. The effects of the protein synthesis inhibitor, cycloheximide, on adaptation to CMA. *D. discoideum* amoebae were loaded with FITC-dextran and then treated with CMA and/or cycloheximide as described in the text. (A) At the times indicated, the cells were collected by centrifugation, intracellular fluorescence was measured, and the pH$_{el}$ was calculated. (B,C) After 2 hours of treatment in CMA and/or cycloheximide, the cells were collected and subjected to SDS-PAGE. Proteins were visualized by western blot analysis using antibodies recognizing the 100 kDa, 70 kDa and 41 kDa subunits of the V-H$^+$-ATPase (B) or Coomassie Brilliant Blue staining (C). Protein synthesis was not required for cells to adapt to CMA.

was not coupled to an increase in the relative levels of the 100 kDa, 70 kDa and 41 kDa subunits of the V-H$^+$-ATPase. These data suggest that increased synthesis and/or stability of at least one integral membrane subunit (100 kDa) and at least two outer membrane subunits (70 kDa and 41 kDa) of the V-H$^+$-ATPase is not the mechanism by which *D. discoideum* amoebae overcome the effects of CMA.

To determine if adaptation to CMA by *D. discoideum* cells required active macromolecular biosynthesis of proteins, other than the V-H$^+$-ATPase subunits described above, the following experiment was carried out. Amoebae were loaded with FITC-dextran for 3 hours as described previously. Following incubation with FITC-dextran the cells were exposed to CMA and/or cycloheximide as described. At the indicated times, cells were harvested and the pH$_{el}$ was determined as described above. As shown in Fig. 4A, cycloheximide, itself, did not significantly influence the pH$_{el}$ and this observation was consistent with that of Aubry et al. (1993b). Moreover, cycloheximide did not significantly alter the CMA-induced rise in pH$_{el}$ nor did it prevent adaptation to CMA by *D. discoideum* cells. Similar results were obtained when cycloheximide was added to the amoebae 15 to 30 minutes prior to the addition of CMA (data not shown). These results suggest that protein synthesis is not required for *D. discoideum* amoebae to overcome the effects of CMA.

CMA induces morphological changes in *D. discoideum* amoebae

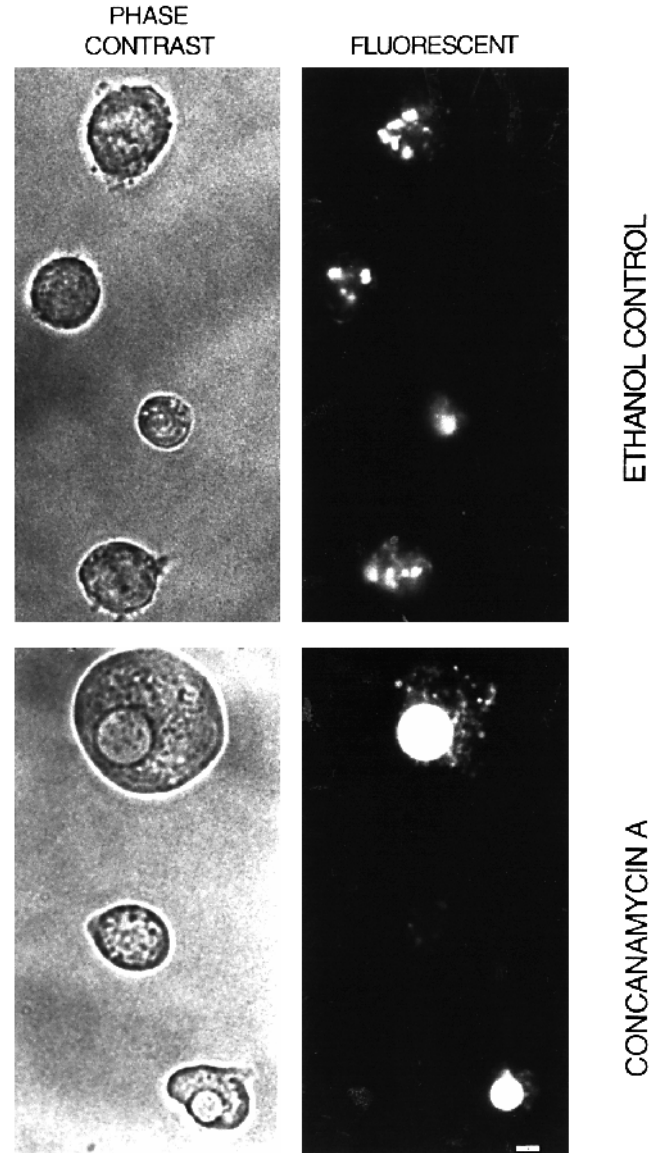CMA has been shown to induce gross morphological changes

Fig. 5. The effects of CMA on the morphology of *D. discoideum* amoebae in nutrient medium. Cells were exposed to FITC-dextran (2 mg/ml) in HL5 medium for 3 hours, at which time ethanol (0.01%, v/v, control) or CMA (5 µM) was added and the cells were incubated for an additional 15 minutes. At 15 minutes the cells were harvested, allowed to adhere to glass slides and examined by phase contrast and fluorescence microscopy. CMA induced gross morphological changes in *D. discoideum* amoebae, characterized by a marked decrease in the number of small fluorescent vesicles coupled with the appearance of 1 to 2 large vesicles per cell. Bar, 2 µm.

in the lytic granules of cytotoxic lymphocytes (Kataoka et al., 1994). We therefore examined the morphology of the endo-lysosomal system of CMA-treated *D. discoideum* amoebae. Cells were allowed to take up FITC-dextran for 3 hours in growth medium, after which time ethanol (control) or CMA was added, and the cells were incubated for an additional 15 minutes. The cells were then harvested and examined by phase contrast and fluorescence microscopy. Control cells exhibited numerous peripheral vesicles that contained FITC-dextran, indicating that these vesicles represented compartments along
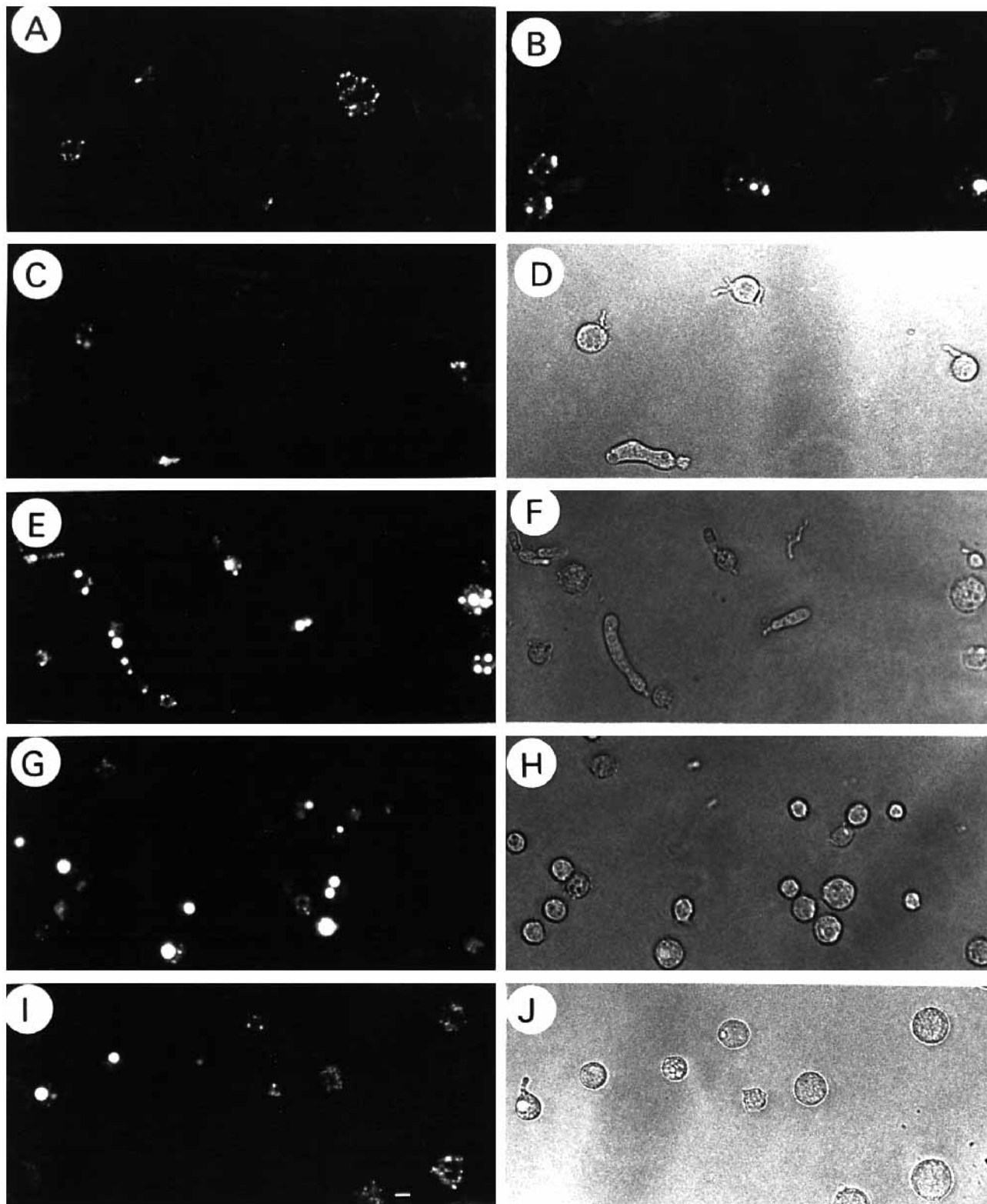
Fig. 6. The effects of CMA on the morphology of *D. discoideum* amoebae in nutrient medium over time. Cells were exposed to FITC-dextran (2 mg/ml) in HL5 medium for 3 hours, at which time ethanol (0.01%, v/v, control) or CMA (5 μM) was added and the cells were incubated for an additional 120 minutes. At the times indicated, the cells were harvested, allowed to adhere to glass slides and examined by phase contrast and fluorescence microscopy. (A) Control cells, 30 minutes. (B) CMA-treated cells, 30 minutes. (C and D) Control cells, 60 minutes. (E and F) CMA-treated cells, 60 minutes. (G and H) CMA-treated cells, 90 minutes. (I and J) CMA-treated cells, 120 minutes. CMA-treated cells exhibited 1 to 2 large vesicles for up to 60 minutes of treatment. However, after 60 minutes, these large vesicles disappeared and small fluorescent vesicles (similar to control cells) appeared. Bar, 3.5 μm.

the endo-lysosomal pathway (Fig. 5). These vesicles, generally, were small and varied in brightness, presumably due to the differences in pH between different endo-lysosomal compartments (Fig. 5). In CMA-treated cells, there was a marked decrease in the number of small fluorescent vesicles coupled with the appearance of 1 to 2 large vesicles per cell in over 80% of the cells (Fig. 5). These large vesicles were derived, at least in part, from endo-lysosomal compartments, as they harbored the fluid phase marker (Fig. 5). The large CMA-induced vesicles were also readily visible by phase contrast microscopy (Fig. 5).

We monitored the CMA-induced morphological changes over time to determine if these changes were reversible. Cells were exposed to FITC-dextran for 3 hours as described above. Ethanol (control) or CMA was then added to the medium and the incubation was allowed to continue for an additional 2 hours. At the times indicated, control and CMA-treated cells were harvested and examined by phase contrast and fluor-

escence microscopy. At 30 minutes of incubation, control cells contained numerous small endo-lysosomal vesicles (Fig. 6A) as described above, while in many of the CMA-treated cells, 1 to 5 large fluorescent vesicles were visible (Fig. 6B). The number of FITC-dextran positive vesicles in the control cells decreased by 60 minutes (Fig. 6C,D) due to efflux and little FITC-dextran remained at later time points (data not shown). Most of the CMA-treated cells continued to exhibit 1 to 2 large vesicles at 60 minutes of treatment (Fig. 6E,F). After 60 minutes, however, these large vesicles disappeared from many of the cells and small fluorescent vesicles appeared once again (Fig. 6G-J). At 120 minutes of treatment (Fig. 6I,J) most of the CMA-treated cells were indistinguishable from control cells. Interestingly, the beginning of the morphological recovery of CMA-treated *D. discoideum* amoebae was concomitant with the decrease in pH_{el}, and the apparent recovery in endocytosis, exocytosis and phagocytosis observed after 60 minutes of treatment.
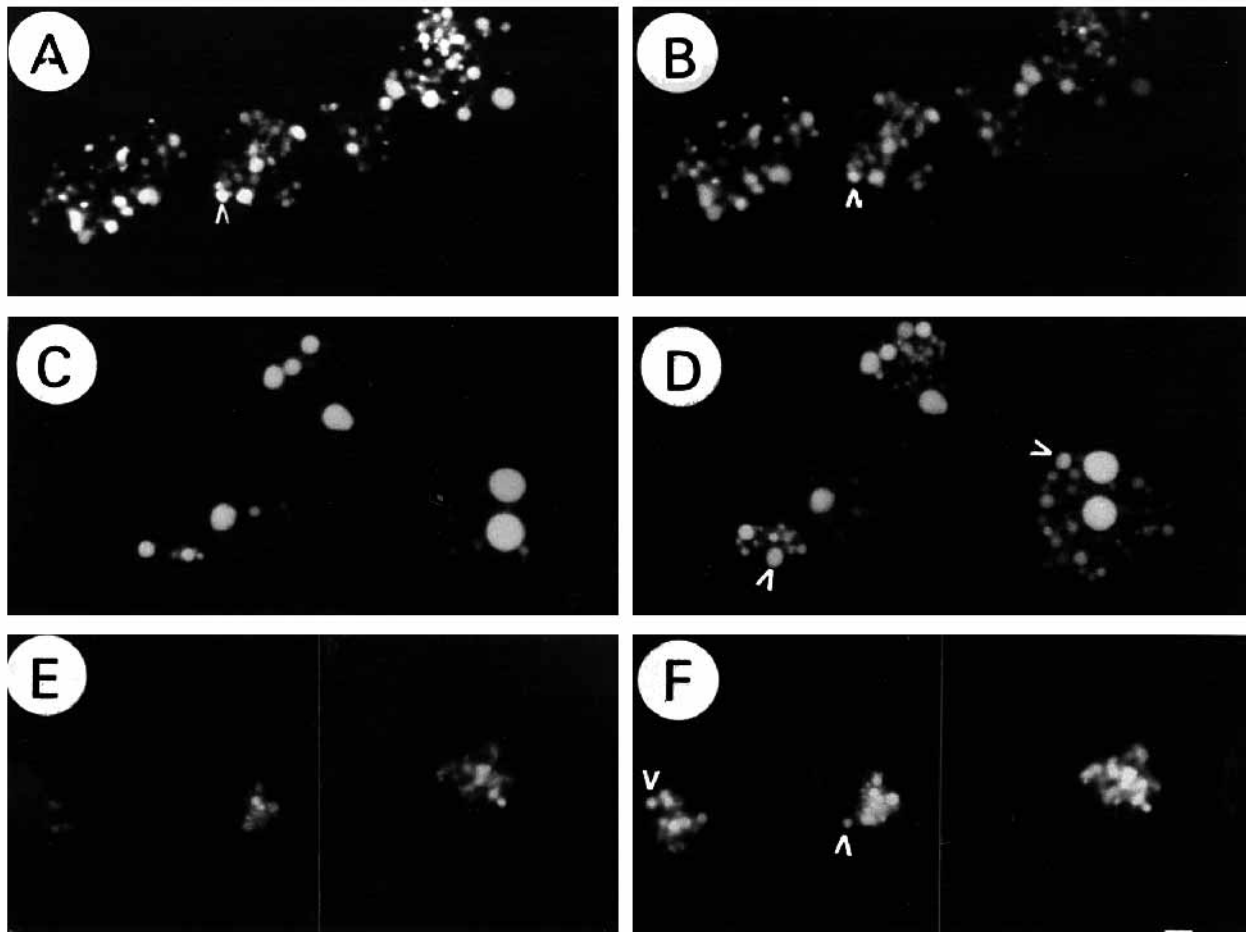

Fig. 7. The effects of CMA on early and late compartments along the endo-lysosomal pathway. Cells were pulsed with RITC-dextran, for 15 minutes, and then chased in fresh medium without marker for an additional 15 minutes. The amoebae were then pulsed with FITC-dextran for 15 minutes. This resulted in FITC-dextran labelled early endo-lysosomal compartments and RITC-dextran labelled late compartments. Ethanol (0.01%, v/v, control) or CMA (5 µM) were then added to the medium, the cells were incubated for an additional 45 minutes, and then examined by fluorescence microscopy for promiscuous marker mixing. (A) (RITC-dextran) and (B) (FITC-dextran), control cells labelled with both markers simultaneously. Arrowheads (>) represent vesicles harboring both markers. (C) (RITC-dextran) and (D) (FITC-dextran), CMA-treated cells labelled sequentially with the fluid phase markers. Arrowheads in D (>) represent FITC-dextran positive vesicles that do not contain RITC-dextran. (E) (RITC-dextran) and (F) (FITC-dextran), control cells labelled sequentially with the fluid phase markers. Arrowheads in F (>) represent FITC-dextran positive vesicles that do not contain RITC-dextran. There was limited mixing between early and late endo-lysosomal compartments during CMA treatment. Bar, 1.5 µm.

CMA does not induce promiscuous fusion between early and late compartments along the endo-lysosomal pathway

In *D. discoideum*, vesicle traffic is linear and fluid phase markers must traverse the entire endocytic circuit prior to their release from the cells. To determine if the large vesicles that formed upon CMA treatment represented retrograde fusion between late and early compartments along the endocytic circuit the following experiment was carried out. Cells were pulsed with a rhodamine-conjugated fluid phase marker, RITC-dextran, for 15 minutes and then chased in fresh medium without marker for an additional 15 minutes. The amoebae were then incubated with a fluorescein-labelled fluid phase marker, FITC-dextran, for 15 minutes. This sequential 'pulse-chase-pulse' regime resulted in FITC-dextran labelled early endo-lysosomal compartments and RITC-dextran labelled late compartments. The time elapsed prior to the addition of the second marker, ensured temporal and spatial separation between RITC-dextran and FITC-dextran. Ethanol (control) or CMA was then added to the medium, the cells were incubated for an additional 45 minutes, and then examined by fluorescence microscopy for promiscuous marker mixing.

As a control for detecting both markers within a single compartment, amoebae were exposed to both FITC- and RITC-dextran simultaneously. As can be seen in Fig. 7A and B, cells that were fed both fluid phase markers simultaneously displayed numerous small endo-lysosomal vesicles, all of which contained both FITC- and RITC-dextran. In CMA-treated cells that were sequentially labelled with the two fluid phase markers, 1 to 2 large FITC-dextran and RITC-dextran positive vesicles were visible as described above (Fig. 7C,D); however, there were also numerous FITC-dextran labelled compartments (early endosomes) that were not labelled with RITC-dextran (see arrowheads, Fig. 7D) indicating that there was limited mixing between the early and late compartments in these cells. If FITC-dextran was added with CMA to amoebae pulse-chased with RITC-dextran, none of the large RITC-dextran positive vesicles contained FITC-dextran. FITC-dextran resided solely in early small endosomal structures (data not shown). This further supports the notion that no promiscuous fusion between the early and late compartments in these cells occurred in the presence of CMA. Control cells sequentially labelled with RITC- and FITC-dextran contained numerous small endo-lysosomal vesicles (Fig. 7E,F) as described above, and also maintained separate early and late endo-lysosomal compartments (see arrowheads, Fig. 7F).

Effects of CMA on purified endo-lysosomal vesicles

To confirm the specificity of CMA action on the V-H+-ATPase, the effects of the compound on purified vesicles of the endo-lysosomal system of *D. discoideum* were examined. Intra-endosomal and lysosomal compartments were loaded with FITC-dextran and dextran-coated colloidal iron oxide for 3 hours. The cells were homogenized and the homogenate was mixed with an equal volume of water to osmotically shock the vesicles and discharge their native acidity. Endo-lysosomal vesicles were then isolated from the homogenate by the magnetic fractionation technique of Rodriguez-Paris et al. (1993). The purified vesicles were incubated in the presence of ATP. ATP-dependent quenching of fluorescence was
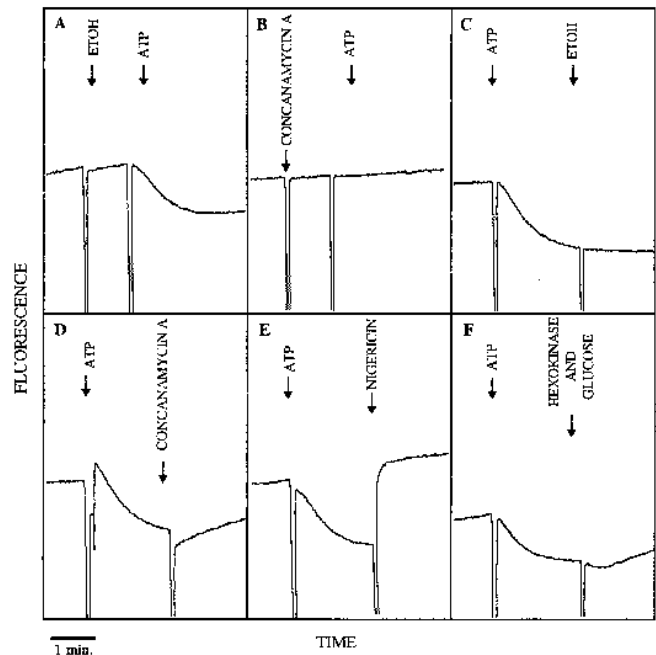

Fig. 8. The effects of CMA on acidification of endo-lysosomes in vitro. Vesicles loaded with FITC-dextran and iron/dextran were discharged of their native acidity and isolated as described in the text. Isolated vesicles from 2×10^8 cell equivalents were added to a cuvette containing 2 ml of the following buffer: 30 mM KCl, 10 mM NaCl, 5 mM MgCl$_2$, 20 mM Hepes-KOH, pH 7.4, 0.1 mM EGTA. To initiate acidification, ATP was added to a final concentration of 2 mM after the addition of ethanol (A) or CMA (B), or before the addition of ethanol (C) or CMA (D). For comparison with the effects of CMA, nigericin (3 μM) (E) or hexokinase (9 units/ml) and glucose (10 mM) (F), were added to the cuvette approximately 1 minute after the addition of ATP.

monitored on a spectrofluorometer and was used as a measure of in vitro acidification.

In control endo-lysosomal vesicles that were pre-treated with ethanol (control) and then exposed to ATP, a rapid decrease in fluorescence intensity was observed indicating that ethanol, the CMA diluent, did not inhibit in vitro acidification (Fig. 8A). Likewise, when ethanol (control) was added to the reaction mixture after ATP addition, in vitro acidification was not reversed (Fig. 8C).

In contrast, CMA (1 μM) completely inhibited in vitro acidification when added before ATP addition (Fig. 8B). Concentrations of CMA as low as 100 nM were also effective. Most importantly, the addition of CMA after acidification had been initiated resulted in only a slight increase in fluorescence intensity over time (Fig. 8D), suggesting that the drug does not act as a non-specific proton ionophore. In contrast, the addition of the ionophore, nigericin, resulted in a rapid dissipation of the pH gradient across the vesicle membrane (Fig. 8E). The rise in fluorescence intensity as a result of nigericin treatment was significantly greater than that observed for vesicles treated with CMA (Fig. 8E), supporting the notion that CMA does not exhibit ionophoric properties. To further examine the nature of the slight CMA-induced intravesicular elevation in pH, and to determine if CMA could act as a membrane permeant, the rise in pH was compared to that resulting from the natural

Table 1. Intracellular ATP levels in control and CMA-treated cells

Time (minutes)	ATP (fmoles/cell)	
	Control	Concanamycin A
0	1.1	1.1
10	1.2	1.1
20	1.25	1.45
30	1.25	1.4
40	1.2	1.75
60	1.15	1.6
80	1.15	1.25

D. discoideum amoebae were treated with ethanol or CMA and at specified times the cells were harvested and the level of intracellular ATP was determined as described in the text. The values in the table represent the mean of 2 independent experiments.

'leakiness' of these vesicles. ATP-dependent acidification of purified endo-lysosomal vesicles was initiated and then an ATP-depleting system consisting of hexokinase (9 units/ml) and glucose (10 mM) was added. The addition of the ATP-depleting components terminated acidification and a slight elevation in pH, resulting from the natural permeability of the membranes, was observed over time (Fig. 8F). Since this rise in pH was nearly identical to that observed for CMA treatment (Fig. 8D), it is unlikely that CMA permeabilized the vesicle membranes and the small effects of the drug were likely due to specific inhibitory action upon the V-H+-ATPase complex and the inherent 'leakiness' of the membranes.

CMA does not influence the levels of intracellular ATP

It has been reported that in *D. discoideum* a reduction in the intracellular levels of ATP can result in reduced endocytosis (Steinman et al., 1974). To determine if the changes observed upon CMA treatment were due simply to the drug acting as a non-specific cellular poison that could alter intracellular levels of ATP, amoebae were treated with CMA and at specified times the cells were harvested and the level of intracellular

ATP was determined. There were no significant changes in the levels of intracellular ATP during the first 30 minutes of CMA treatment (Table 1). This result indicates that CMA was not acting as an energy poison and more likely was specific for V-H+-ATPases. Interestingly, a slight but significant increase occurred in cellular ATP levels 40-60 minutes into the CMA treatment.

CMA delays processing and induces slight missorting of lysosomal α-mannosidase

Since CMA induced rapid and severe changes in the structure and function of the endosomal system of *Dictyostelium*, we examined the effects of an increase in pH_{el} on another aspect of this system: biosynthesis, processing and sorting of lysosomal enzymes. The biosynthetic pathway for the lysosomal enzyme α-mannosidase has been elucidated (Cardelli, 1993). α-Mannosidase is synthesized as a 140 kDa precursor, and a small fraction of this precursor escapes further processing and is secreted constitutively via a default secretory pathway (Cardelli et al., 1986; Mierendorf et al., 1985). The remainder of this protein is proteolytically processed to an 80 kDa intermediate form and a 58 kDa mature subunit in a late Golgi compartment or in early endosomes. The 80 kDa intermediate is further processed to a 60 kDa mature subunit in the lysosome where, together with the 58 kDa subunit, it forms a soluble multimeric holoenzyme (Richardson et al., 1988).

D. discoideum takes up radioactive label by endocytosis. Since CMA blocked endocytosis in *D. discoideum,* it was necessary to expose the amoebae to [35S]methionine for a short time prior to the addition of the inhibitor. Log phase cultures were metabolically labelled with [35S]methionine for 30 minutes. Ethanol (0.01%, v/v; control) or CMA (5 μM) was then added to the medium and incubation continued for an additional 10 minutes. The cells were then chased in the presence or absence of the drug in unlabelled growth medium for 4 hours. Samples were taken at 0, 20, 40, 60, 120 and 240 minutes of chase. The samples were separated into cellular and supernatant (extracellular) fractions by centrifugation; α-mannosidase was immunoprecipitated from both fractions and
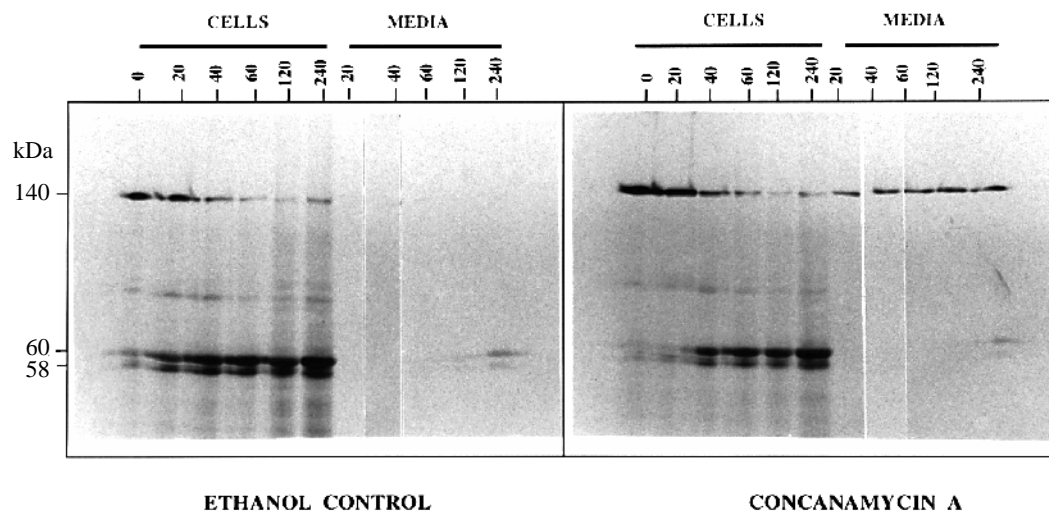

Fig. 9. The effects of CMA on the biosynthesis and processing of lysosomal α-mannosidase. Growing cells were pulsed with [35S]methionine in HL5 medium for 30 minutes, at which time ethanol (0.01%, v/v, control) or CMA (5 μM) were added and the labelling proceeded for an additional 10 minutes. Cells were then chased in unlabeled medium in the presence of ethanol or CMA. At the time points indicated (minutes), samples were taken, separated into cells and medium (extracellular) by centrifugation and incubated with monoclonal antibodies specific for α-mannosidase. SDS-PAGE and fluorography were used to separate and visualize the immunoprecipitated proteins. X-ray films were scanned with a laser densitometer to determine the relative intensities of the bands.

analyzed by SDS-PAGE and fluorography (Fig. 9). In both control and treated cells, correct processing of the α-mannosidase 140 kDa precursor, through an 80 kDa intermediate, to its mature form was evident. In control cells this processing was first observed at 20 minutes of the chase period, and displayed a half-time of 25 minutes. However, the effect of raising the pH_{el} by CMA treatment resulted in a marked delay in this processing event, such that the half-time for processing in the CMA-treated cells was 40 minutes. The delay in α-mannosidase processing was observed in the first hour of CMA treatment, a time during which the effects of the drug were maximal.

The final proteolytic processing of the 80 kDa intermediate peptide to a 60 kDa mature subunit has been shown to occur in lysosomes (Wood and Kaplan, 1985; Richardson et al., 1988; Mierendorf et al., 1985). CMA-treated cells were capable of this final processing event, suggesting that α-mannosidase reached lysosomes in these cells. Therefore, the delay in processing observed in CMA-treated amoebae may be due to a delay in the delivery of the intermediate form to the lysosomes and/or less efficient proteolytic processing of the 80 kDa intermediate once it is inside of these organelles. Negligible levels of unprocessed, missorted α-mannosidase precursor were found extracellularly for control cells; however, for CMA-treated cells approximately 20% of unprocessed precursor was found extracellularly (Fig. 9), indicating that CMA also induced some missorting of lysosomal α-mannosidase precursors.

Effects of CMA on the contractile vacuole (CV) system

In *D. discoideum* approximately 10% of the total cellular V-H$^+$-ATPase is associated with the endo-lysosomal system (Rodriguez-Paris et al., 1993). The remainder of these proton pumps are associated with tubular membranes of the CV system (Heuser et al., 1993; Fok et al., 1993; Bush et al., 1994). The proton pumps localized to this membrane network may provide energy for pumping water during osmoregulation. In addition, several lines of evidence support a functional connection between the CV and endo-lysosomal systems in *D. discoideum* (O'Halloran and Anderson, 1992; Ruscetti et al., 1994). We therefore examined the action of CMA on the function of the *D. discoideum* CV system.

To assess the function of the CV system, growing amoebae were harvested by centrifugation, washed free of growth medium, and resuspended in water or in a low ionic strength phosphate buffer (10 mM) in plastic tissue culture dishes with or without CMA. Control cells were viable in water, appeared amoeboid in shape, and adhered strongly to the surface of the plate (data not shown). In contrast, cells that were incubated in water or in a low ionic strength phosphate buffer in the presence of CMA, swelled rapidly and lost their ability to adhere to the solid surface. As well, large vesicles formed within these CMA-treated cells (see below). It appeared that CMA interfered with CV function, as treated cells were not able to overcome the stress imparted by the hypo-osmotic environment and eventually lysed.

To further explore the nature of the large vesicles that formed upon CMA treatment in water or in a low ionic strength phosphate buffer, cells were pre-loaded with FITC-dextran for 3 hours, harvested by centrifugation and resuspended in
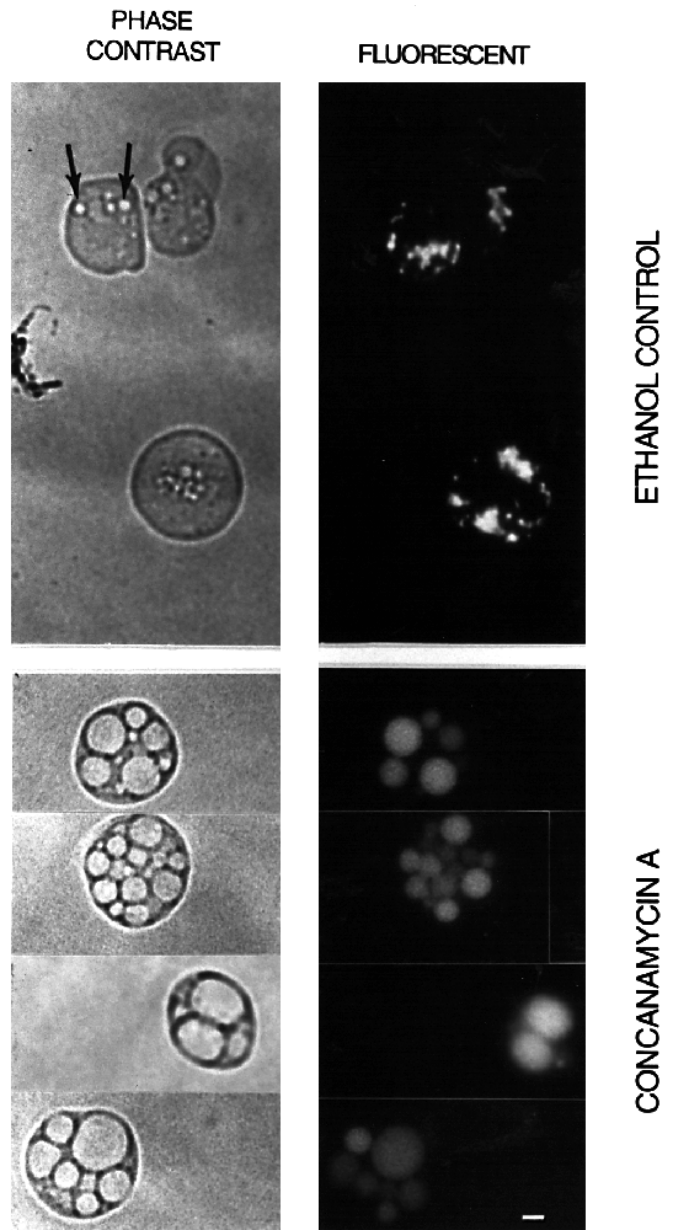

PHASE CONTRAST   **FLUORESCENT**

ETHANOL CONTROL

CONCANAMYCIN A

Fig. 10. The effects of CMA on the morphology of *D. discoideum* amoebae in phosphate buffer. Cells in HL5 were exposed to FITC-dextran (2 mg/ml) for 3 hours, harvested and resuspended in phosphate buffer supplemented with FITC-dextran (2 mg/ml). Ethanol (0.01%, v/v, control) or CMA (5 μM) was added and the cells were incubated for an additional 15 minutes. The cells were then harvested, allowed to adhere to glass slides and examined by phase contrast and fluorescence microscopy. CMA induced gross morphological changes in *D. discoideum* amoebae, characterized by a marked decrease in the number of small fluorescent vesicles coupled with the appearance of 2 to 10 large vesicles per cell. Arrows (→) represent vesicles which do not contain FITC-dextran (elements of the CV system). Bar, 2 μm.

phosphate buffer in the presence or absence of CMA. After 45 minutes the cells were examined by phase contrast and fluorescence microscopy. Control cells exhibited numerous vesicles that contained FITC-dextran, indicating that these vesicles represented compartments along the endo-lysosomal

pathway (Fig. 10). Components of the CV complex were visible in control cells as small vesicles (1 to 2 μm) that did not contain the fluid phase marker (Fig. 10, see arrows). In contrast, CMA-treated cells appeared swollen and rounded compared to control cells (Fig. 10). There was a marked decrease in the number of small fluorescent vesicles in these cells coupled with the appearance of 2 to 10 large vesicles per cell (Fig. 10). These large vesicles were derived, at least in part, from endo-lysosomal compartments as they all harbored the fluid phase marker (Fig. 10). Prior to rupturing, the CMA-treated cells excluded eosin dye, suggesting that they were still alive.

D. discoideum cells do not overcome the effects of CMA in a non-nutrient environment

It was observed that *D. discoideum* cells were not able to overcome the morphological effects of CMA in a non-nutrient environment such as phosphate buffer or water. Therefore, we examined the evolution of pH_{el} in phosphate buffer in the presence of CMA. *D. discoideum* cells were suspended in axenic growth medium in the presence of FITC-dextran for 3 hours. Following incubation with the marker, the cells were harvested by centrifugation, resuspended in phosphate buffer supplemented with FITC-dextran, in the presence of ethanol (control) or CMA, and the pH_{el} was measured over time. Starvation alone did not induce a rise in pH (Fig. 1A and Fig. 11A). Fig. 11A also demonstrates that there was a rapid rise in pH, induced by CMA, that reached a value of at least 7.0 within 10 minutes of drug addition. The pH_{el} remained at a value equal to or greater than 7.0 for at least 2.5 hours, at which time cell lysis occurred. During CMA treatment in non-nutrient phosphate buffer, *D. discoideum* cells were also unable to recover the characteristic functions of the endo-lysosomal system including exocytosis (Fig. 11B), endocytosis (data not shown) and phagocytosis (data not shown). Furthermore, cells that had adapted to CMA in growth medium, nevertheless ruptured in water in the presence of CMA (data not shown). This suggests that the function of the CV, unlike those of the endo-lysosomal system, cannot recover in the continued presence of CMA.

DISCUSSION

We have investigated the effects of a specific V-H+-ATPase inhibitor, CMA, on acidification and functions of the endo-lysosomal and contractile vacuole systems of *D. discoideum*. This drug inhibited acidification and increased the pH of endo-lysosomal vesicles both in vivo and in vitro. CMA treatment inhibited endocytosis and exocytosis of fluid phase, and phago-cytosis of large particles. CV function was also impaired in the presence of CMA. Furthermore, inhibition of acidification by CMA slowed but did not prevent proteolytic processing of the lysosomal enzyme α-mannosidase. Finally, fluorescence microscopy revealed that treatment with CMA induced gross morphological changes in *D. discoideum* cells, characterized by a decrease in small endo-lysosomal vesicles and the formation of large intracellular vacuoles containing fluid phase markers. None of the effects imparted by CMA treatment were due to non-specific actions such as altering ATP levels or per-meabilizing membranes.
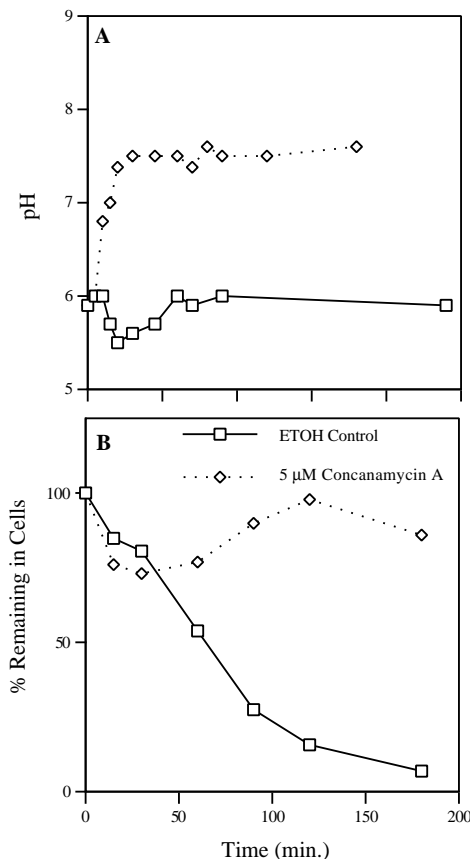

Fig. 11. The effects of CMA on the pH_{el} and on fluid phase exocytosis of *D. discoideum* cells in phosphate buffer. (A) Cells were loaded with FITC-dextran in HL5 medium, harvested, resuspended in phosphate buffer and treated with ethanol (0.01%, v/v, control) or CMA (5 μM) as described. At the times indicated during drug treatment, samples were taken and the pH was determined. (B) To measure exocytosis, cells were incubated with FITC-dextran (2 mg/ml) in HL5 medium for 3 hours, washed free of the marker with fresh medium, and then incubated in phosphate buffer in the presence of ethanol (0.01%, v/v, control) or CMA (5 μM). At the time points indicated, intracellular fluorescence was measured and the total FITC-dextran remaining in the cells was plotted. CMA significantly increased the pH_{el} and blocked exocytosis of *D. discoideum* cells in phosphate buffer.

A functional V-H+-ATPase complex was required in numerous steps along the endo-lysosomal pathway. Inhibition of acidification by CMA resulted in impaired endocytosis, exo-cytosis and phagocytosis; this was reversible over time, even in the continued presence of the drug. Reductions in cellular ATP can result in the inhibition of endocytosis (Steinman et al., 1974); however, CMA did not significantly alter ATP con-centrations in *D. discoideum*. Furthermore, we do not feel that the reduction in these processes resulted from an indirect toxic affect of the drug because this condition was reversible in cells suspended in growth medium. However, it has been shown that in mammalian cells acidification of the cytoplasm inhibits receptor mediated endocytosis from coated pits although fluid phase pinocytosis was not affected (Sandvig et al., 1987). Greater than 80% of the fluid phase that enters *Dictyostelium* is dependent on clathrin heavy chain function, suggesting entry

through coated pits. Therefore, inactivation of the vacuolar ATPase could result in acidification of the cytoplasm (see below) and inhibition of fluid phase endocytosis via clathrin coated pits.

Efflux of fluid phase was also reduced in CMA-treated cells. Consistent with this, Clague et al. (1994) have shown that in BHK cells, V-H$^+$-ATPase activity is required for the formation of endosomal carrier vesicles, an intermediate compartment between early and late endosomes. Other studies have shown that in mammalian cells an active V-H$^+$-ATPase is required for endocytic and exocytic processes that occur during viral infections (Palokangas et al., 1994) and for certain other functions of lysosomes such as protein degradation (Yoshimori et al., 1991). Finally, we have observed a significant reduction in the rate of phagocytosis in CMA-treated cells. A similar result was reported in LPS activated alveolar macrophages (Bidani and Heming, 1995).

The large fluorescent vesicles which form upon CMA treatment apparently reflect aberrant fusion of compartments along the endo-lysosomal pathway. It is important to note that the unusual fusion events that occurred upon CMA treatment in *D. discoideum* were not totally random; there was limited mixing between the early and late compartments of the endo-lysosomal pathway in the presence of CMA. van Weert et al. (1995) also demonstrated that Bafilomycin A1 inhibits transport from late endosomes to lysosomes. We postulate that the aberrant fusions that occur in *D. discoideum* in the presence of CMA are between successive compartments in the endo-lysosomal system and/or between vesicles of this pathway and the membranes of other organelle systems (see below). In another model for the appearance of large endocytic vesicles during CMA treatment, large vacuole formation could occur by swelling of the individual vesicles rather than increased fusion activity. Our data tend to support the fusion model as we observed a decrease in small endo-lysosomal vesicles simultaneous to the appearance of the large fluorescent vesicles. Similar morphological changes have been observed when cytotoxic T lymphocytes (Kataoka et al., 1994) or GH3 cells (Henomatsu et al., 1993) were treated with CMA or related V-H$^+$-ATPase inhibitors.

It is currently unclear how the V-H$^+$-ATPase functions to regulate membrane fusion. Regulation of vesicle traffic and fusion along the endo-lysosomal pathway of *D. discoideum* is not well understood; however, in vitro fusion studies, using vesicles isolated from *D. discoideum*, have shown that these processes are dependent on ATP, temperature, cytosol, and ionic strength (Lenhard et al., 1992b). In addition, the pH at or near vesicle membranes may influence vesicle fusion (Lenhard et al., 1992). For example, alkaline pH (greater than 7.5) can inhibit the fusion of *D. discoideum*-derived vesicles, while an acidic pH (less than 6.0) can stimulate the fusion of vesicles in the absence of ATP or inhibit fusion in the presence of ATP in vitro. Low cytoplasmic pH has also been shown to stimulate fusion of macrophage-derived endosomes in vitro (Lenhard et al., 1992b). In mammalian cells, plasma membrane V-H$^+$-ATPases have been implicated in the maintenance of cytoplasmic pH (Swallow et al., 1993; Nordstrom et al., 1995). While no V-H$^+$-ATPases are found at the plasma membrane of *D. discoideum*, it is conceivable that the inhibitory action of CMA on V-H$^+$-ATPases elsewhere in the cell, could alter the pH of the cytoplasmic microenvironment at endo-lysosomal

vesicles, and consequently, could influence fusions along this pathway. In support of this, it has been shown that the effects elicited simply by acidifying the cytoplasm in macrophages mimicked those observed upon treatment with bafilomycin A1, a related V-H$^+$-ATPase inhibitor (Bidani and Heming, 1995).

Cytosolic factors also play a role in regulating vesicle traffic and fusion in the endo-lysosomal pathway. For example, Ca^{2+}-regulated proteins (i.e. annexins), clathrin, adaptins, COPS and small molecular weight GTP-binding proteins such as ARFs or Rabs, may be recruited to vesicles in the course of their formation or intracellular transit (reviewed by Bauerfeind and Huttner, 1993). The V-H$^+$-ATPase could directly or indirectly regulate the cytoplasmic concentration of Ca^{2+}, an ion that has been shown to stimulate cytosolic and NSF-independent fusion between endosomes (Mayorga et al., 1994). In fact, the V-H$^+$-ATPase inhibitor, bafilomycin A1, reduced uptake of Ca^{2+} into *D. discoideum* acidosomes, an ATPase-rich membrane network (Rooney and Gross, 1992). Consistent with this pharmacological study, *D. discoideum* amoebae demonstrating a reduction (50%) in V-H$^+$-ATPase activity, brought about by antisense mutagenesis of the proteolipid subunit of the V-H$^+$-ATPase complex, also displayed reduced uptake of Ca^{2+} into ATPase-rich membranes (Xie et al., 1996). Apodaca et al. (1994) have reported that the calmodulin (Ca^{2+} binding protein) antagonist, W13, alters the morphology of the endocytic pathway in Madin-Darby canine kidney cells. It has also been demonstrated that the association of the small molecular weight GTPase, ARF, with vesicle membranes is regulated by intravesicular pH (Zeuzem et al., 1992a,b) and a role for ARFs in endosome-endosome fusion in vitro has been proposed (Lenhard et al., 1992a; Spiro et al., 1995). As well, overexpression of certain Rabs leads to the formation of abnormally large endosomes (Bucci et al., 1992). We have observed that Rab4-like and Rab7-like GTPases are associated with lysosomal (Temesvari et al., 1994; Bush et al., 1994) and phagosomal (Rodriguez-Paris and Cardelli, unpublished observations) membranes of *D. discoideum*. Finally, it has been shown that the coated vesicle V-H$^+$-ATPase associates with and is phosphorylated by AP50, a subunit of the clathrin assembly protein AP-2 (Myers and Forgac, 1993). Therefore it is conceivable that inhibition of V-H$^+$-ATPase function by CMA, leading to alkalinization of intra-endo-lysosomal compartments, could influence the recruitment of ARFs, Rabs, adaptin complexes or other cytosolic factors, to endosomes and lysosomes. In turn, this could result in aberrant fusion and transit of these vesicles and impaired endo-lysosomal function.

This report shows that treating cells with CMA delayed but did not prevent processing and correct localization of the lysosomal enzyme, α-mannosidase. This confirms our previous work using the weak base, ammonium chloride (Cardelli et al., 1989) and therefore two independent lines of evidence support the notion that acidification of the endo-lysosomal system by V-H$^+$-ATPases is only required for efficient targeting of molecules through the endo-lysosomal pathway. Our previous studies (Cardelli et al., 1989) also showed that ER to Golgi transit was not affected by alkalinization of endo-lysosomal compartments, as α-mannosidase precursors acquired endoglycosidase H resistance at the same rate in both control and treated cells. Therefore, the delay in processing observed when intracellular compartments are neutralized, must occur in a compartment in or beyond the Golgi

apparatus. The Golgi complex and distal pre-lysosomal compartments are slightly acidic (Orci et al., 1984; Griffiths and Simons, 1986; Mellman and Simons, 1992) and are thought to function as sorting depots along the biosynthetic routes of lysosomal enzymes. Therefore inadequate acidification of these compartments may result in mild missorting of uncleaved lysosomal hydrolases. CMA did, in fact, induce some missorting of the lysosomal enzyme, α-mannosidase, in *D. discoideum* cells. Similar conclusions have been reached by other investigators studying yeast (Yamashiro et al., 1990; Klionsky et al., 1992) or a mammalian cell line, HepG2 (Yilla et al., 1993).

This study also provides novel information regarding the function served by active V-H+-ATPases in the CV system. The CV is a membrane bound organelle found in most fresh water protozoa and amoebae and is thought to serve an osmoregulatory role (reviewed by Patterson, 1980). The mechanism of fluid uptake and expulsion by the CV as well as other possible functions of this organelle are not well understood. In *D. discoideum*, the CV complex is a dynamic membrane system that responds rapidly to osmotic changes in the organism's environment (Zhu and Clarke, 1992). It consists of a bladder-like pump and a tubular spongiome network; however, profound rearrangement of this membrane system can be observed throughout the cell cycle (Zhu et al., 1994). In *D. discoideum*, more than 90% of the V-H+-ATPases are associated with tubular membranes of the CV system (Rodriguez-Paris et al., 1993). The proton pumps localized to this membrane network presumably provide energy for pumping water during osmoregulation.

It is not surprising, therefore, that inhibition of proton pump action by CMA also compromised the function of the CV complex. Amoebae exposed to a hypo-osmotic environment in the presence of CMA swelled rapidly and ruptured. Immunofluorescence microscopy, using antibodies which reacted with the 100 kDa subunit of the V-H+-ATPase (CV marker), revealed that the structure of this organelle system may have also changed (data not shown). The bulk of the CV marker now appeared to localize to the large fluid phase-containing vesicles described above, suggesting that fragmentation of the CV had occurred and membranes of the CV and endo-lysosomal systems had fused. Resolution at the electron microscopic level will be necessary to confirm this rearrangement of CV markers. Communication between these two organelle systems in *D. discoideum* had been postulated previously; cells lacking the clathrin heavy chain (O'Halloran and Anderson, 1992; Ruscetti et al., 1994) or cell lines expressing a dominant negative form of a Rab4-like GTPase (Bush et al., unpublished) are defective in endocytosis and do not contain a functional CV.

This is the first report describing cells that can overcome the action of CMA. In nutrient medium, *D. discoideum* became resistant to CMA after one hour of treatment and this event was characterized by a reduction in the pH_{el} from a value of 6.5 to approximately 6.0. The nature of this adaptation is not known; however, it is probably not due to metabolic breakdown of the compound nor to adsorption of the drug by medium components, as the addition of fresh drug after the one hour time point had no effect on acidification of the endo-lysosomal system. The reduction in pH_{el} during adaptation was also not due to increased synthesis of 100 kDa subunit of the V-H+-ATPase, the proposed binding site for the CMA family of macrolide antibiotics (Zhang et al., 1994), nor to increased synthesis of

the 70 kDa and 41 kDa subunits of the V-H+-ATPase. Moreover, it was determined that protein synthesis, in general, was not required for the cells to adapt to CMA, as recovery of pH_{el} was observed even in the presence of cycloheximide. The reduction in pH_{el} during adaptation to CMA may be the result of reactivation of V-H+-ATPase function by cytosolic activators (Zhang et al., 1992). Alternatively, since ion equilibrium has been suggested to participate in pH homeostasis in lysosomes (Reijingoud and Tager, 1977), adaptation may also come about by the involvement of one or more non-vacuolar proton pump ion transporters (Rooney and Gross, 1992; Poitras et al., 1995). Finally, other non-ion pump processes (Moriyama et al., 1992) may also participate in adaptation to CMA.

During adaptation, the rate of endocytosis, exocytosis, and phagocytosis increased concomitantly with the observed decrease in pH_{el}, and the large CMA induced endo-lysosomes were replaced by smaller vesicles. It could not be determined if adaptive recovery of these features of the endo-lysosomal system also required protein synthesis because cycloheximide, itself, imparts blockage of membrane traffic in the amoebae (this study, data not shown; Gonzales and Satre, 1991). Interestingly, adaptation was not observed in cells that were treated with CMA in water or phosphate buffer, suggesting that normal growth conditions and/or normal osmolarity are required for this recovery. Surprisingly, CV function remained impaired in cells that had adapted to CMA. This may indicate that the endo-lysosomal V-H+-ATPase is regulated differently from the CV proton pump during adaptation.

Unlike for yeast, *D. discoideum* cells with disruptions in the genes coding for subunits of the V-H+-ATPases have not been obtained. In fact, only two genes encoding subunits of the *D. discoideum* V-H+-ATPase complex, the 41 kDa subunit (*DVA41*; Temesvari et al., 1994) and the proteolipid subunit (*vatP*, Xie et al., 1996), have been reported to be cloned. Antisense mutagenesis of the proteolipid subunit has been successful (Xie et al., 1996); however, the mutant cells grew slowly, died or readily reverted to wild type. Finally, *D. discoideum* cells defective in acidification have been generated by chemical mutagenesis (Bof et al., 1992), but the nature of these defects have not been elucidated. Therefore reagents such as CMA, that inhibit V-H+-ATPases and neutralize acidic compartments in a specific manner, are useful in dissecting the role of acidification and V-H+-ATPase complexes in cellular processes of *D. discoideum*.

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