

Association of Calmodulin and an Unconventional Myosin with the Contractile Vacuole Complex of *Dictyostelium discoideum*

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Abstract. mAbs specific for calmodulin were used to examine the distribution of calmodulin in vegetative *Dictyostelium* cells. Indirect immunofluorescence indicated that calmodulin was greatly enriched at the periphery of phase lucent vacuoles. The presence of these vacuoles in newly germinated (non-feeding) as well as growing cells, and the response of the vacuoles to changes in the osmotic environment, identified them as contractile vacuoles, osmoregulatory organelles. No evidence was found for an association of calmodulin with endosomes or lysosomes, nor was calmodulin enriched along cytoskeletal filaments. When membranes from *Dictyostelium* cells were fractionated on equilibrium sucrose density gradients, calmodulin cofraction-

ated with alkaline phosphatase, a cytochemical marker for contractile vacuole membranes, at a density of 1.156 g/ml. Several high molecular weight calmodulin-binding proteins were enriched in the same region of the gradient. One of the calmodulin-binding polypeptides (molecular mass \sim 150 kD) cross-reacted with an antiserum specific for *Acanthamoeba* myosin IC. By indirect immunofluorescence, this protein was also enriched on contractile vacuole membranes. These results suggest that a calmodulin-binding unconventional myosin is associated with contractile vacuoles in *Dictyostelium*; similar proteins in yeast and mammalian cells have been implicated in vesicle movement.

WE are exploring the roles played by calmodulin in the eukaryotic microorganism *Dictyostelium discoideum*. Calmodulin is a small, highly conserved, calcium-binding protein found in all eukaryotic cells; it has been implicated in the regulation of many aspects of cellular metabolism and cell motility (reviewed in Manalan and Klee, 1984; Cohen and Klee, 1988). *Dictyostelium* calmodulin closely resembles mammalian calmodulin in its physical properties and in vitro activities, although there are some amino acid differences between *Dictyostelium* and mammalian calmodulins (Marshak et al., 1984; Clarke, 1990).

One means of examining calmodulin function is to use antibodies as probes to determine how calmodulin is localized within the cell. Several laboratories have used this approach in mammalian cells. A variety of staining patterns have been reported, ranging from diffuse cytoplasmic fluorescence (Anderson et al., 1978) to specific labeling of microfilaments (Dedman et al., 1978), lysosomes (Nielsen et al., 1987), and, in mitotic cells, spindle poles and chromosome-to-pole microtubules (Anderson et al., 1978; Welsh et al., 1978; 1979). The diversity of staining patterns could reflect the multi-functional nature of calmodulin, although differences in cell types, fixation techniques, and antibody preparations may also contribute.

We have raised mAbs against *Dictyostelium* calmodulin and have demonstrated by immunoblot that these antibodies recognize only calmodulin in total cell lysates (Hulen et al., 1991). These antibodies have been used to examine calmodu-

lin distribution in vegetative *Dictyostelium* cells by indirect immunofluorescence, EM, and subcellular fractionation. Although nearly two-thirds of the cell's calmodulin is soluble, both immunolocalization and subcellular fractionation revealed that some *Dictyostelium* calmodulin is membrane-associated, and this calmodulin is greatly enriched on membranes of the contractile vacuole complex, an osmoregulatory organelle.

Several calmodulin-binding proteins were also detected in membrane fractions rich in calmodulin. Preliminary data suggest that one of these, a 150-kD polypeptide, is an unconventional myosin not previously identified in *Dictyostelium*.

Materials and Methods

Cell Growth Conditions

Dictyostelium discoideum strains NC4 (wild type) and AX3 (axenic) were maintained on SM nutrient agar plates (Loomis, 1975) in association with *K. aerogenes*. For examination of exponentially growing NC4 cells, spores were collected from a single sorocarp with a sterile loop, inoculated into a 5-ml suspension of *K. aerogenes* prepared as described in Clarke et al. (1987), and incubated with shaking at 22°C. Amebas were harvested the next morning in log phase growth (density \sim 1 \times 10⁶ cells/ml), and washed free of bacteria by differential centrifugation (200 g, 3 min, three cycles) in 17 mM Na₂HPO₄/KH₂PO₄ buffer, pH 6.4 (Na/K buffer). AX3 cells were grown on HL5 medium (Clarke et al., 1980) and harvested in log phase growth (density 1–4 \times 10⁶ cells/ml). The cells were pelleted by centrifugation and washed once in Na/K buffer or as indicated.

Spore Germination

For examination of newly germinated amebas, NC4 spores were inoculated into Na/K buffer that had been "conditioned" by the presence of *K. aerogenes*. This technique was based on the observations of Hashimoto et al. (1976) and Dahlberg and Cotter (1979) that bacterial cells produce a substance that triggers spore germination. The bacterial suspension was prepared as previously described (Clarke et al., 1987) and shaken for 2 or 3 d at 22°C; the resulting conditioned buffer was clarified by passage through a 2- μ m filter. Spores from four or five sori were washed once in Na/K buffer, then suspended in 2 ml of the bacterial conditioned buffer. Approximately 2 h later, the amebas emerged from their spore coats. They were harvested by centrifugation and fixed at this time.

Cell Fractionation

AX3 cells growing on HL5 were harvested at a density of 4×10^6 cells/ml. (Alternatively, NC4 cells growing on a suspension of *K. aerogenes* were harvested at a density of 2×10^6 /ml and washed free of bacteria as described above.) The last wash was in 50 mM Tris-HCl (pH 7.5), 25 mM KCl, 5 mM MgCl₂, 0.1 mM CaCl₂ (TKMC) containing 0.25 M sucrose. The cell pellet (1×10^9 cells) was resuspended in 3 ml of the same buffer containing protease inhibitors (50 ng/ml TLCK, 10 ng/ml leupeptin, and 10 ng/ml chymostatin). Using procedures described by Cardelli et al. (1987), the cells were disrupted by Dounce homogenization and then subjected to low-speed centrifugation (1,500 g, 5 min) to remove nuclei and unbroken cells. The supernatant (2 ml) was layered on top of a 9.5-ml continuous sucrose gradient (25–61% sucrose [wt/vol], prepared in TKMC), and centrifuged for 14 h at 100,000 g (Beckman SW41 rotor). 0.9-ml fractions were collected from the top. The density of each fraction was determined by measurement of refractive index, and the fractions were assayed for protein content and enzyme activity. A portion of each fraction was diluted with an equal volume of TKMC, and membranes were sedimented at 100,000 g (45 min, 4°C). Membrane proteins in the pellets were subjected to SDS gel electrophoresis, then analyzed by immunoblot for content of calmodulin or myosin I.

Electrophoresis and Immunoblotting Methods

Electrophoresis was carried out in SDS polyacrylamide gels using the discontinuous buffer system described by Laemmli (1970). Western blots of calmodulin were carried out as previously described (Hulen et al., 1991). The primary antibody was 2D1, a mouse mAb described in the same report; 2D1 ascitic fluid was diluted 1:500 in TBS (150 mM NaCl, 20 mM Tris-HCl, pH 7.5). For other proteins, transfer was conducted as described by Towbin et al. (1979), except that PVDF membrane (Millipore Immobilon P) was used. Myosin I was detected using an antiserum against *Acanthamoeba* myosin IC (Baines and Korn, 1990) diluted 1:500 in TBS, followed by peroxidase-conjugated goat anti-rabbit IgG (1:2,000; American Qualex). *Dictyostelium* cell surface proteins were labeled with biotin as described by Goodloe-Holland and Luna (1987). After sucrose gradient fractionation, membrane proteins were electrophoresed and blotted to PVDF membrane. The biotinylated cell surface polypeptides were stained with peroxidase-conjugated streptavidin (0.04 μ g/ml; Pierce Chemical Co.) under conditions described by Goodloe-Holland and Luna (1987); quantitation was by scanning densitometry.

Calmodulin-binding proteins were detected by a modified immunoblot procedure. Samples were denatured at 65°C (rather than 100°C) before SDS-PAGE. Transfer was conducted by the procedure of Towbin et al. (1979) onto PVDF membrane; the transfer buffer contained 0.1 mM CaCl₂. The membrane was washed with TBS, blocked with 2% BSA, washed with TBS containing 0.1 mM CaCl₂, then incubated in a solution containing *Dictyostelium* calmodulin (100 ng/ml) and BSA (1 mg/ml) in TBS (37°C, 45 min). After being washed in TBS, the membrane was stained with 2D1 anticalmodulin and secondary antibody as usual.

Protein and Enzyme Assays

Protein was determined using the Coomassie protein assay (Pierce Chemical Co., Rockford, IL) with BSA as a standard. Alkaline phosphatase was determined essentially as described by Quiviger et al. (1980); 20 μ l of each sucrose gradient fraction was assayed. The reaction conditions described by Padh et al. (1989a) were also tested and found to yield identical results. Acid phosphatase was assayed as described by Padh et al. (1989a). NADH cytochrome c reductase was assayed as described by Beaufay et al. (1974).

Fixation Methods, Antibodies, and Staining Procedures

Our usual method of fixation has been described previously (Clarke et al., 1987). In brief, cells were placed on a glass cover slip, covered with a thin layer of agarose, blotted to flatten the cells, and fixed in cold formaldehyde-methanol. (To verify that this procedure did not cause a rearrangement of the contractile vacuole system, we also examined cells that had not been flattened under agarose; the appearance of the contractile vacuole complex was similar under both conditions.) An alternative fixation method, adapted from Baines and Korn (1990), was also employed. For this procedure, cells under a layer of agarose were covered with 5–10 μ l of a fixative solution containing 0.25% glutaraldehyde and 4% paraformaldehyde in half-strength HL5 (40 min, room temperature.) The cells were washed once in HL5 and once in PBS (150 mM NaCl in 20 mM sodium phosphate buffer, pH 7.5), then immersed in 0.5% saponin in PBS (30–45 min, room temperature). After three washes in TBS, the cells were treated with freshly prepared sodium borohydride (0.5 mg/ml in TBS) for 10 min at room temperature; this step was repeated once. The cells were washed three times in TBS and then immunostained.

All immunostaining procedures, including preabsorption of the antibodies, were conducted as previously described (Clarke et al., 1987). Calmodulin was stained with 2D1 ascitic fluid (1:800) followed by FITC-conjugated goat anti-mouse IgG (1:400; Cappel). Myosin I was stained with anti-*Acanthamoeba* myosin IC (1:50) followed by FITC-conjugated goat anti-rabbit IgG (1:1,500), or (for double-staining experiments) rhodamine-labeled goat anti-rabbit IgA + IgG + IgM (1:400; Cappel). In double-staining experiments, the two primary antibodies were mixed, as were the two secondary antibodies.

Electron Microscopy

NC4 cells were washed free of bacteria and suspended in Na/K buffer at a density of 2×10^6 /ml, and a drop of cell suspension (~ 100 μ l) was placed on a coverslip. After 5–10 min, the coverslip was dipped briefly into 1% formaldehyde in methanol (-15°C , 1 s) and then immersed in extraction buffer containing 10 mM sodium phosphate (pH 7.4), 150 mM NaCl, and 0.25% Triton X-100 (room temperature, 15–120 s). The cover slip was transferred to fixation medium (4% paraformaldehyde and 0.25% glutaraldehyde in half-strength HL5, room temperature, 45 min), washed in TBS (three changes, 5 min each), and treated with sodium borohydride as described above. Antibody (2D1 ascitic fluid at 1:200 dilution) was applied, and the cover slip was incubated 2 h at room temperature. After 3×5 min washes in TBS, the cover slip was incubated with 5-nm colloidal gold-conjugated goat anti-mouse IgG + IgM (Janssen Life Science) diluted 1:10 in TBS, for 2 h at room temperature. After 3×5 min washes in TBS and a final 5-min wash in 0.1 M sodium phosphate buffer (pH 7.2), the cover slip was fixed with 2.5% glutaraldehyde in the same phosphate buffer (45 min, room temperature), then washed 3×10 min in the same buffer. Postfixation (0.5% OsO₄, 10 min, on ice) and the final washes (3×10 min, room temp) were also carried out in this phosphate buffer. The cells were dehydrated and embedded in a mixture of Epon-Araldite (Condeelis et al., 1987). Thin sections were cut in a plane parallel to that of the cell monolayer; sections were counter-stained with uranyl-lead. Micrographs were taken using a Jeol JEM-1200 CX electron microscope.

Membranes from the sucrose density gradient fraction most enriched in alkaline phosphatase and calmodulin (e.g., fraction 7 in Fig. 5) were stained cytochemically for alkaline phosphatase. The fraction was diluted with 1 vol of TKMC, and the membranes were pelleted by centrifugation (100,000 g, 45 min). The membrane pellet was covered with 2.5% glutaraldehyde prepared in 50 mM cacodylate buffer (pH 6.8), and fixation was carried out for 1 h at room temperature. The fixed pellet was cut into small fragments and stained as described by Quiviger et al. (1978). The control sample was incubated without substrate.

Immunoprecipitation Methods

Immunoprecipitation was carried out as described by Yurko and Gluck (1987), with some modifications. Affi-Gel-Protein A (Bio-Rad Laboratories, Cambridge, MA) was washed three times with buffer I (20 mM Tris-HCl [pH 7.5], 500 mM NaCl, 0.1% BSA, and 0.005% Tween-20); 40 μ l of the washed resin was incubated with 300 μ l of rabbit anti-mouse IgG (American Qualex, diluted 1:20 in buffer I) for 1.5 h at room temperature with gentle mixing. The resin was washed three times in buffer I by centrifugation (200 g, 2 min). The resin was then mixed with 300 μ l of either anti-

calmodulin (2D1 ascitic fluid diluted 1:10 in buffer I) or a similar dilution of a control antibody (P3.26; described in Reines and Clarke, 1985) and incubated 1.5 h at room temperature. The resin was washed three times in buffer I, then mixed with 300–700 μ l of sucrose gradient fraction 7 (see Fig. 5). Additions were made to obtain the following final concentrations in the incubation mixture: 0.1% BSA, 0.005% Tween-20, 10 ng/ml leupeptin, 10 ng/ml chymostatin, and 50 ng/ml TLCK. Incubation was carried out for 1.5 h at room temperature. The resin was washed with TKMC containing 0.25 M sucrose, 0.1% BSA, and 0.002% Tween-20, and was then assayed for alkaline phosphatase activity. (For some experiments, formalin-fixed *Staphylococcus aureus* cells [Bethesda Research Laboratories, Bethesda, MD] were used instead of Affi-Gel-Protein A, and minor modifications of buffer conditions were employed. These variations did not yield significant differences in results.)

For some experiments, membranes were immunoprecipitated from a cruder membrane fraction. This fraction was prepared essentially as described in Cell Fractionation, with the following differences. The homogenization buffer was buffer II (20 mM Tris-HCl [pH 7.5], 0.25 M sucrose, 1 mM DTT, 10 ng/ml leupeptin, 10 ng/ml chymostatin, 50 ng/ml TLCK, and 0.1 mM PMSF). The supernatant from the initial low-speed spin of the homogenate was centrifuged again (7,600 g, 4 min). From that supernatant (S2), membranes were collected by centrifugation at 100,000 g (45 min, 4°C). The membrane pellet was resuspended in buffer II, and the centrifugation step was repeated. The membrane pellet was resuspended in buffer II (to one-half the original volume of S2) plus an equal volume of buffer I. The membrane suspension was centrifuged briefly (at the same speed used to collect Affi-Gel-Protein A or *S. aureus* cells) to remove any aggregates, then mixed with the antibody-coated resin or *S. aureus*, prepared as described above.

Labeling of Endosomes and Lysosomes/Acidic Compartments

Acidic compartments in living cells were labeled essentially as described by Padh et al. (1989b), except that cells (AX3 or NC4) were allowed to adhere and spread on glass cover slips before labeling. The cells were incubated with 3 μ M Acridine Orange (Sigma Chemical Co., St. Louis, MO) for 10–20 min, then rinsed and examined. For observation of the cells, a cover slip was inverted onto supports consisting of narrow strips of another cover slip.

To visualize endosomes in living cells, Texas-red-dextran (\sim 70 kD; Molecular Probes, Inc., Eugene, OR) was added to a final concentration of 2 mg/ml to AX3 cells growing in HL5. After 1 h, the cells were washed in 17 mM potassium phosphate buffer (pH 6.4) and placed on cover slips. Observation was with a Zeiss Axiophot microscope equipped with phase-contrast and epifluorescence optics and a heat reflecting filter.

For visualization of the endosomal/lysosomal system in fixed cells, *Dictyostelium* cells were fed labeled bacteria. *K. aerogenes* were suspended at a density of \sim 3 \times 10¹⁰/ml ("1 \times " concentration) in 17 mM potassium phosphate buffer, pH 8.0, containing 0.2 mg/ml sulfo-NHS-biotin (Pierce Chemical Co.). The suspension was swirled for 25 min at room temperature, then washed four times in potassium phosphate buffer, pH 6.5, and resuspended in this buffer. NC4 cells were mixed with a 1 \times concentration of biotinylated bacteria and swirled at 22°C for 1 h. The cells were processed by our standard procedures for indirect immunofluorescence of calmodulin, except that rhodamine-labeled ExtrAvidin (Sigma Chemical Co.), diluted 1/500 in TBS, was added along with the FITC-labeled secondary antibodies.

Results

Indirect Immunofluorescence Localization of Calmodulin in Newly Germinated and Growing NC4 Cells

In indirect immunofluorescence experiments, antibodies against calmodulin were enriched at the periphery of a vacuolar compartment in *Dictyostelium* cells. To obtain an unambiguous identification of this compartment, newly germinated wild type (NC4) cells were examined. Since such cells have not yet begun to feed, they do not contain the extensive system of endosomal and digestive vacuoles found in

growing cells. Instead, the only phase lucent vacuoles that they contain are one or two contractile vacuoles, which are quite prominent and active during spore germination (Cotter and Raper, 1966; Cotter et al., 1969). Calmodulin immunostaining was concentrated at the periphery of these vacuoles (Fig. 1), suggesting an association of calmodulin with contractile vacuole membranes. This staining pattern persisted during cell growth and early development.

The relationship of the contractile vacuole complex to the cytoskeletal system and to other intracellular organelles was examined in double-labeling experiments. There was no enrichment of filamentous actin, detected with rhodamine-phalloidin, or of conventional myosin, detected with a mAb against that protein (Reines and Clarke, 1985), near the calmodulin-labeled vacuoles (not shown). In interphase cells, the contractile vacuole complex always lay near the nucleus, in the vicinity of the microtubule organizing center; in mitotic cells, the calmodulin-labeled vacuole complex became fragmented and dispersed (Zhu, Q., and M. Clarke, manuscript in preparation). The possibility of an interaction between the contractile vacuole complex and microtubules is under investigation. The calmodulin-stained membranes were not part of the endosomal/lysosomal system, as shown by visualization of both that compartment and calmodulin in exponentially growing cells (Fig. 2).

Observation of Contractile Vacuoles and Other Vesicular Compartments in Living Cells

In living cells placed in hypotonic medium, contractile vacuoles appeared as large, phase lucent organelles whose filling and emptying action could be readily observed by phase-contrast or differential interference contrast microscopy. Typically, an interphase wild type cell contained one or two contractile vacuoles, located near the cell nucleus; three or more were sometimes observed. If a cell contained two (or more) vacuoles, they filled and emptied in an alternating fashion. Sometimes fluid appeared to move from one vacuole to another, suggesting that they were part of a single interconnected system.

Endosomal and acidic compartments in living cells were identified by the use of fluorescent markers. Pinocytosis of Texas red-dextran was observed in AX3, a mutant derivative of NC4 that is capable of a rapid fluid uptake. Acidic compartments in both AX3 and NC4 were labeled with Acridine Orange. After the cells had been rinsed with fresh medium, neither type of marker was found in contractile vacuoles. However, these markers persisted in numerous small vesicles that underwent rapid saltatory motion. In AX3 cells, the endosomal marker also labeled a few large vacuoles that were comparable in size to contractile vacuoles; some of these were phase lucent. However, the persistence of the endosomal marker during the half-hour observation period indicated that these vacuoles did not empty, identifying them as compartments on the endosomal pathway rather than contractile vacuoles.

Osmotic Effects on Contractile Vacuole Organization in Wild Type and Axenic Cells

Contractile vacuoles are thought to play an osmoregulatory role in *Dictyostelium* cells, as in other fresh-water amoebae. By observing living cells, and by using calmodulin immuno-

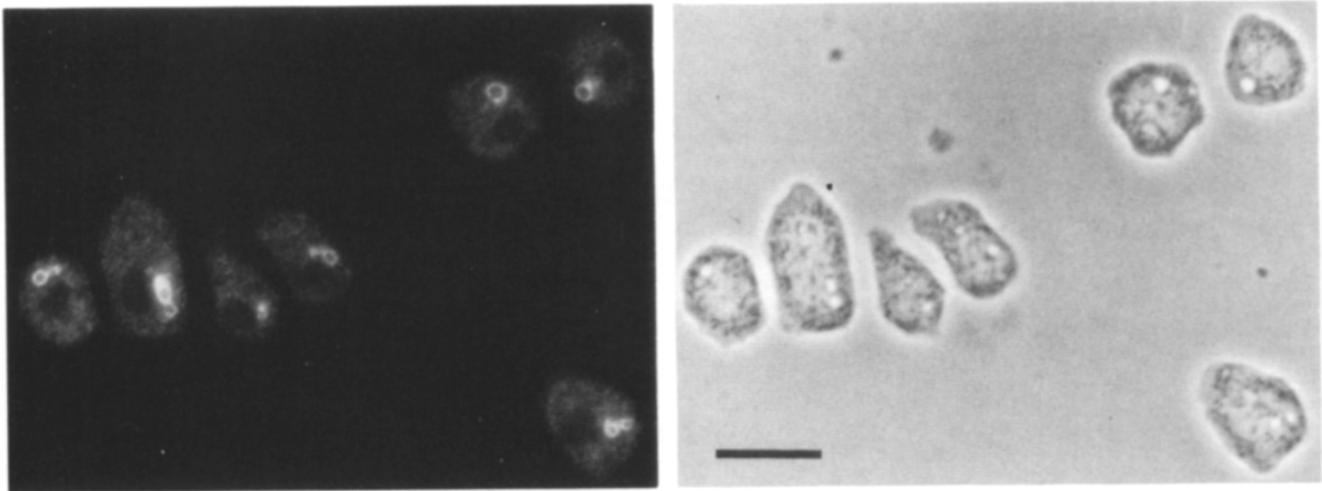


Figure 1. Immunolocalization of calmodulin in newly germinated *Dictyostelium* cells. Spores of *D. discoideum* strain NC4 were induced to germinate by exposure to buffer conditioned by *K. aerogenes*. 2 h later, shortly after the amoebas had emerged from their spore coats, the cells were fixed in formaldehyde-methanol and immunostained to detect calmodulin. Immunostaining indicated that calmodulin was enriched on the membranes of contractile vacuoles. The left panel shows an indirect immunofluorescence image and the right panel a phase-contrast image of the same field of cells. Bar, 10 μm .

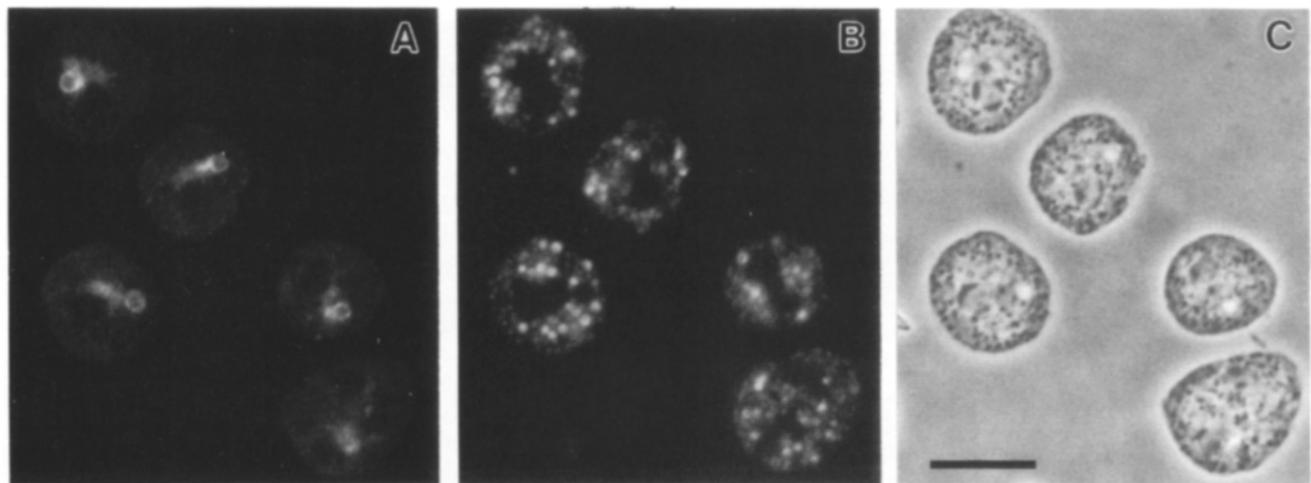
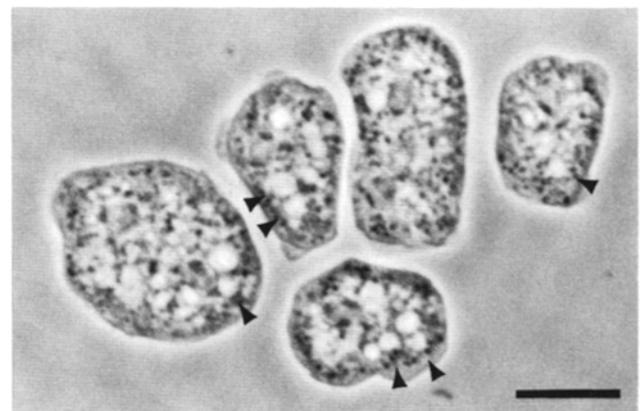
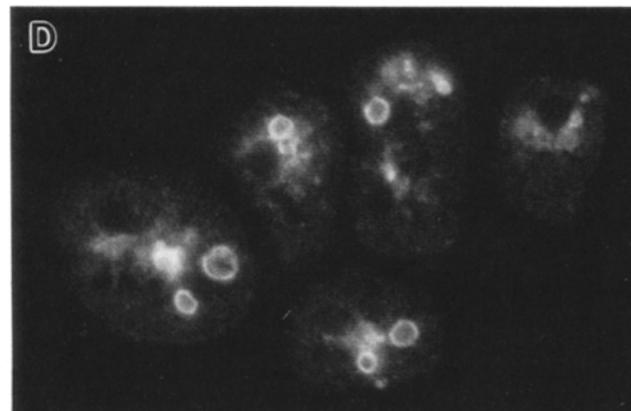
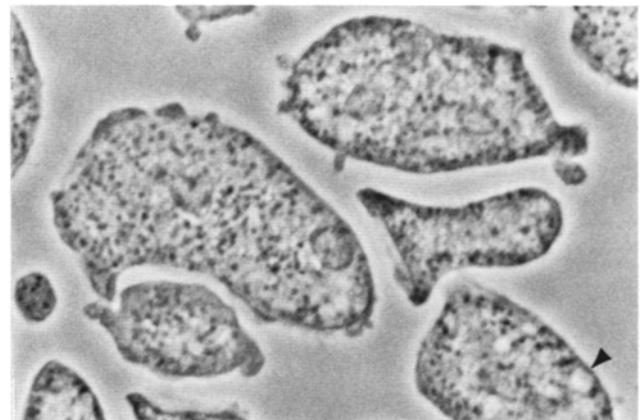
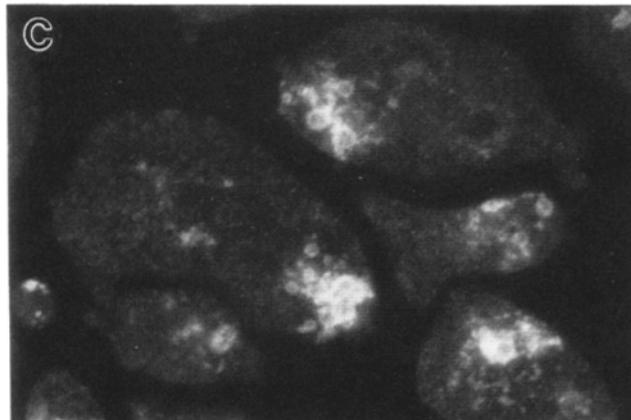
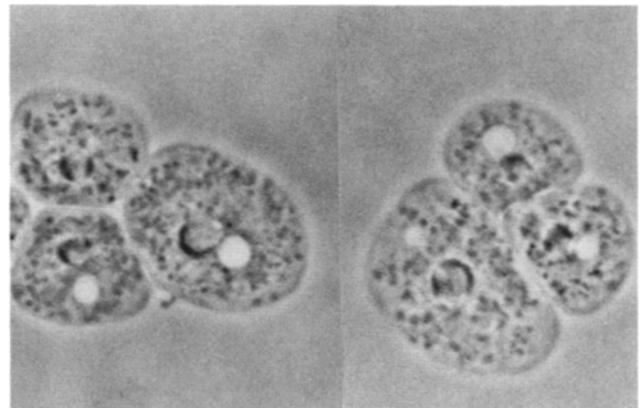
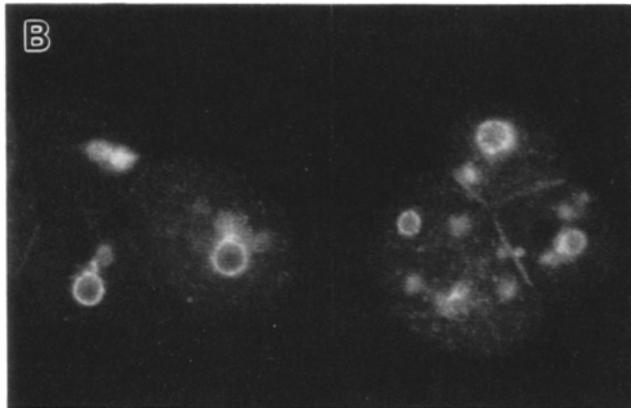
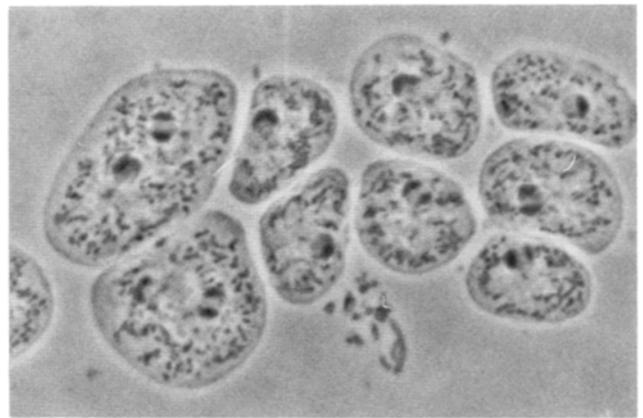
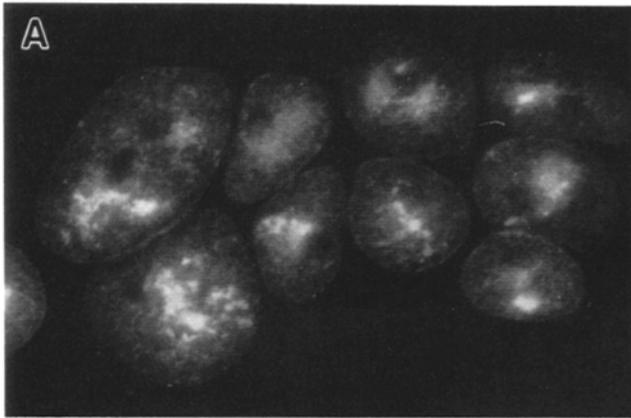


Figure 2. *Dictyostelium* cells double-stained to detect calmodulin and ingested bacteria. NC4 cells were fed biotinylated bacteria for 1 h, then washed, fixed, and stained with rhodamine-conjugated avidin to visualize the endosomal/lysosomal compartment (B). The same cells were immunostained with FITC-conjugated antibodies to detect calmodulin (A). The vacuoles labeled with calmodulin antibodies were distinct from those containing biotinylated bacteria. A phase-contrast image of the cells is shown in C. Bar, 10 μm .

staining as a marker for contractile vacuole membranes in fixed cells, we examined the effect of changes in the osmotic environment on the organization of the contractile vacuole complex. For these experiments, NC4 cells were grown on bacteria suspended in Bonner's salt solution (20 mM NaCl, 10 mM KCl, 3 mM CaCl₂). A shift to higher osmotic

strength was provided by the addition of 0.1 M sorbitol to the cell suspension. The sorbitol had no toxic effect on the cells; they were able to grow and develop normally in this medium. Soon after sorbitol addition, no vacuoles were visible in either living or fixed cells, and immunostaining indicated that the calmodulin-stained membranes were condensed next to

Figure 3. Osmotic effects on calmodulin staining and contractile vacuole organization in *Dictyostelium* cells. Wild type cells (NC4) are shown in A and B. Sorbitol (0.1 M) was added to NC4 cells growing on *K. aerogenes* suspended in Bonner's salt solution. After 1 h, some cells were fixed in formaldehyde-methanol (A); other cells were diluted to low sorbitol concentration and fixed 10 min later (B). The cells were immunostained to detect calmodulin. Indirect immunofluorescence (left) and phase-contrast (right) images are shown. Axenic cells (AX3) are shown in C and D. AX3 cells growing in HL5 medium were fixed in formaldehyde-methanol either without first washing the cells (C), or after washing the cells in 17 mM potassium phosphate buffer (D). The arrows mark phase lucent vacuoles that did not become labeled with calmodulin antibodies; these are probably endocytic vacuoles. In B and D, there are some immunostained vacuoles slightly out of the plane of focus; these appear as fluorescent patches. Bar, 10 μm .



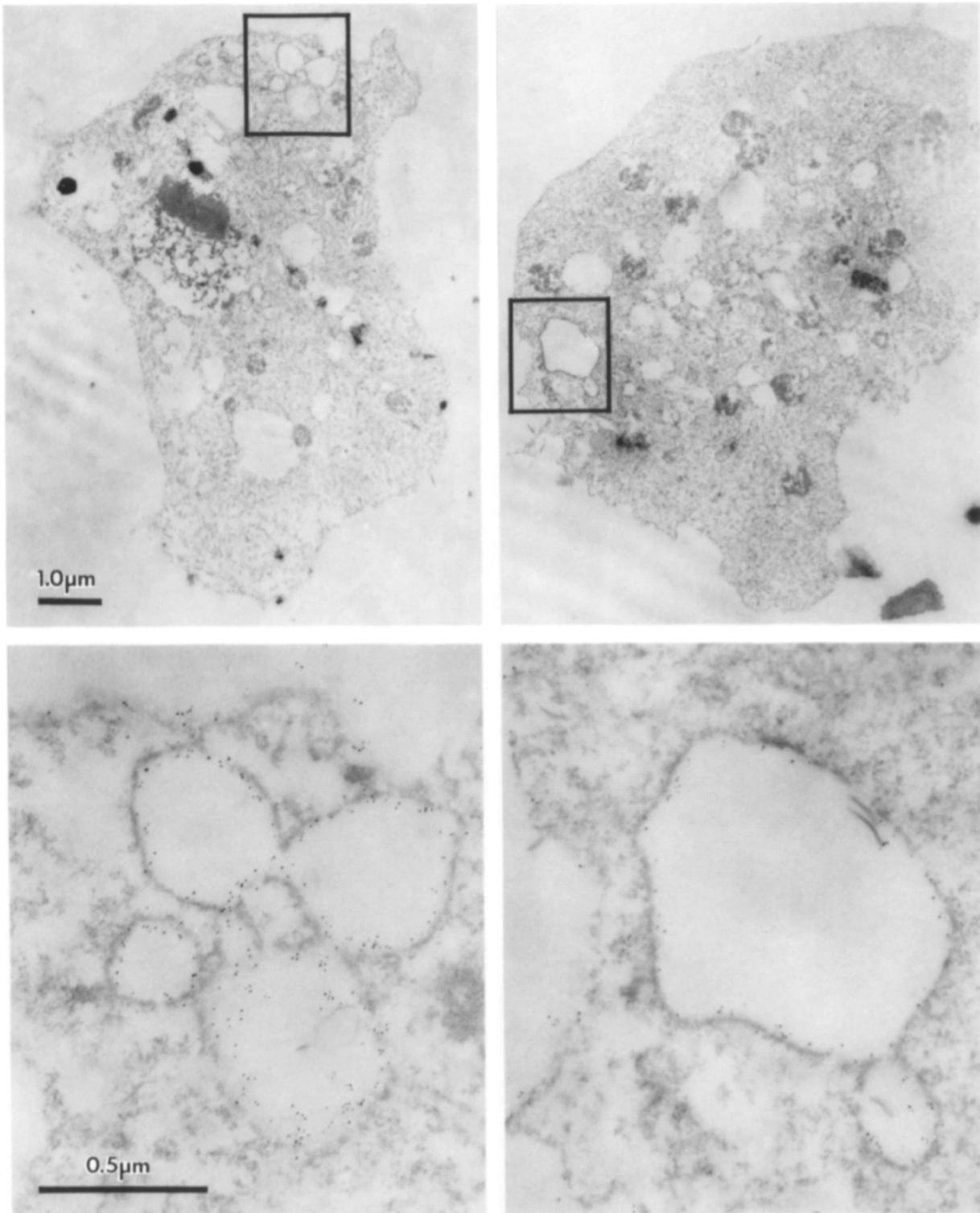


Figure 4. Immunogold labeling of calmodulin in *Dictyostelium* cells. NC4 cells were permeabilized by brief exposure to 0.25% Triton X-100, then fixed in a mixture of glutaraldehyde and paraformaldehyde. The cells were incubated with anticalmodulin primary antibodies and gold-conjugated secondary antibodies, fixed again in glutaraldehyde, postfixed in osmium, embedded in Epon-Araldite, and thin-sectioned. (Details are provided in Materials and Methods.) In each cell, gold particles were restricted to a vacuole or cluster of vacuoles; two examples are shown.

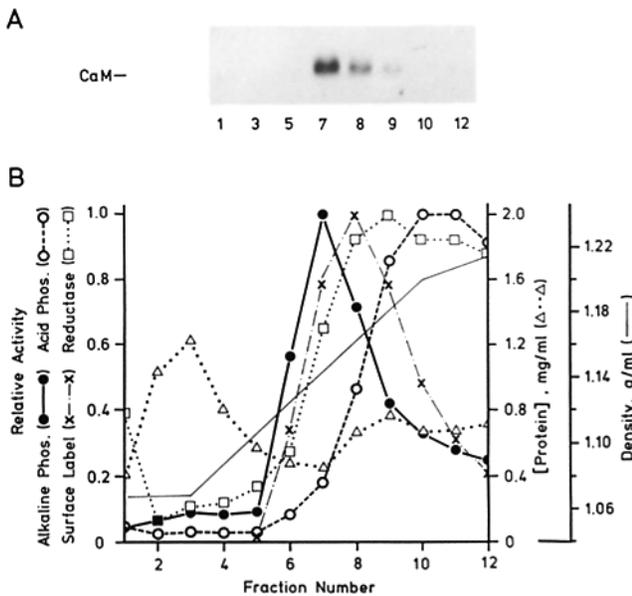


Figure 5. Cofractionation of calmodulin and alkaline phosphatase on a sucrose density gradient of *Dictyostelium* cell membranes. A postnuclear supernatant of AX3 cells was fractionated on a continuous sucrose density gradient. Membrane-bound calmodulin, detected by immunoblot, is shown in *A*. *B* shows the densities of the fractions and the distribution of protein and enzyme markers across the gradient. The enzymes assayed were alkaline phosphatase (contractile vacuole marker), acid phosphatase (lysosome marker), and NADH cytochrome *c* reductase (ER marker). The distribution of biotinylated cell surface proteins (plasma membrane marker) is also shown. (For each marker, values are plotted as fractions of the peak value for that marker.) In five experiments, the highest levels of alkaline phosphatase activity and calmodulin protein were found in the same fraction, with a density of $1.156 (\pm 0.010)$ g/ml.

the nucleus (Fig. 3 *A*). After the cells had been incubated 60–90 min to allow them to adapt to this osmotic environment (Wetterauer and MacWilliams, 1990), they were shifted to Bonner's salt solution containing 5 mM sorbitol. The cells were fixed within 10 min after the shift to lower osmotic strength. At this time, the cells contained very large and prominent contractile vacuoles, the periphery of which became labeled with antibodies to calmodulin (Fig. 3 *B*).

In cells subjected to this hypo-osmotic shift, a segment of plasma membrane labeled with calmodulin antibodies was occasionally observed. These images were presumed to represent recently emptied vacuoles, collapsed against the plasma membrane. Aside from this, calmodulin antibodies did not immunostain plasma membranes.

AX3 cells were also examined. When grown on bacteria, AX3 cells resembled wild type cells. When grown axenically (i.e., on liquid nutrient medium), AX3 cells contained an extensive contractile vacuole complex as well as some phase lucent vacuoles that did not become labeled with antibodies to calmodulin (Fig. 3 *C*); the latter presumably corresponded to the endocytic vacuoles observed in living cells. If the cells were washed in a hypo-osmotic buffer before being fixed, both types of vacuoles became swollen, indicating that both compartments had received an osmotically induced influx of water (Fig. 3 *D*). Thus, in axenically-grown cells subjected

to common washing conditions, the phase-contrast appearance of contractile vacuoles and endocytic vacuoles was similar, at least in static images. Only in living (or appropriately labeled) cells could the two compartments be easily distinguished.

Immunogold Labeling of Calmodulin in Vegetative *Dictyostelium* Cells

The compartment labeled by calmodulin antibodies in *Dictyostelium* cells was also examined by EM, using secondary antibodies conjugated to gold particles. Both preembedding and postembedding staining techniques were tested. Only preembedding methods, which involved extensive extraction of cellular membranes, yielded sufficient labeling to be meaningful. The best labeling was obtained for cells extracted briefly with Triton X-100. In such cells, the gold particles were concentrated at the periphery of a cluster of cytoplasmic vacuoles (Fig. 4). These vacuoles were surrounded by a membrane residuum that appeared thicker and more electron-dense than that of other cytoplasmic vacuoles. An unexpected finding was that most of the gold particles lay on the luminal side of the membrane residuum (see Discussion).

Cofractionation of Calmodulin and Alkaline Phosphatase

Cytochemical methods have demonstrated that alkaline phosphatase is a marker for contractile vacuole membranes in *Dictyostelium* cells (Quiviger et al., 1978). We examined whether calmodulin was enriched in the membrane fractions that possessed alkaline phosphatase activity. A postnuclear supernate from a *Dictyostelium* cell homogenate was fractionated on a continuous sucrose density gradient. The fractions were assayed for protein and enzyme content, and membranes were collected from each gradient fraction and analyzed for calmodulin by immunoblot (Fig. 5). In five experiments, the peak of alkaline phosphatase activity and the highest level of calmodulin protein coincided in a membrane fraction recovered at a density of 1.156 g/ml ($SD \pm 0.010$). Although markers for other types of cellular membranes were also present in this fraction, the peak levels of markers for cell surface proteins, ER, and lysosomes were always found in denser regions of the gradient. An estimate of the purity of the membranes in gradient fraction 7 was obtained by cytochemically staining the membranes in this fraction for alkaline phosphatase. Approximately 30% of the membrane vesicles in fraction 7 became labeled with the lead phosphate reaction product, suggesting that $\sim 30\%$ of these membranes were derived from contractile vacuoles (Fig. 6).

To verify that calmodulin was associated with the same population of membrane vesicles that was rich in alkaline phosphatase, vesicles were immunoprecipitated from gradient fraction 7, as well as from a cruder, pregradient membrane fraction, using anticalmodulin antibodies. In each of three experiments, the immunoprecipitate obtained from fraction 7 with anticalmodulin antibodies was two-to-three-fold enriched in alkaline phosphatase activity relative to that obtained with control antibodies, and the immunoprecipitate obtained from the crude membrane fraction was six-to-nine-fold enriched. These results argue for a preferential association between calmodulin and contractile vacuole mem-

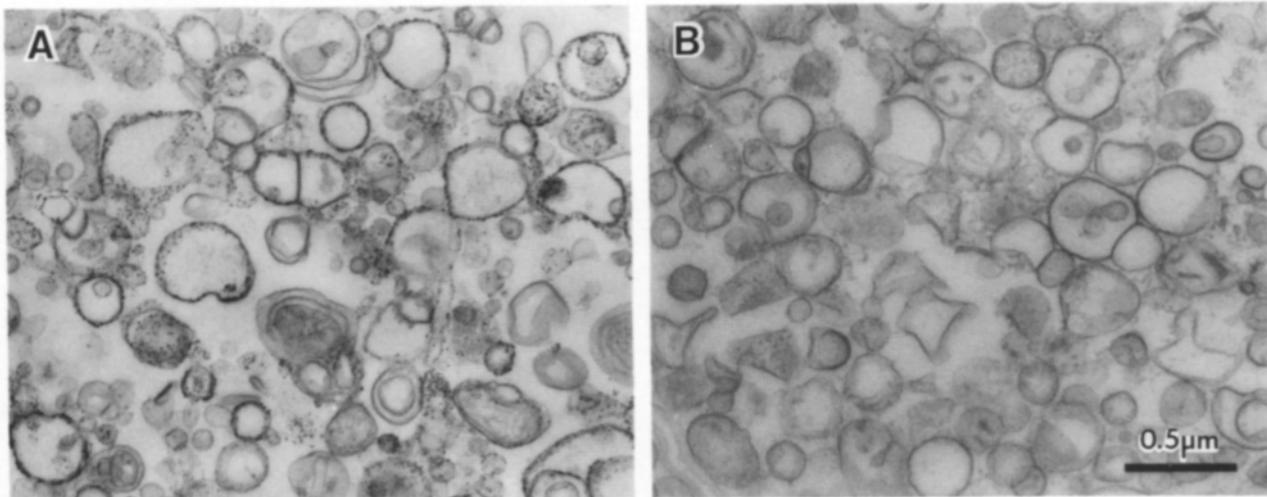


Figure 6. Alkaline phosphatase detected by cytochemical staining of membrane vesicles from a sucrose gradient. Membranes were pelleted from the sucrose gradient fraction richest in alkaline phosphatase and calmodulin. After glutaraldehyde fixation, the membranes were cytochemically stained for alkaline phosphatase, postfixed in osmium, embedded, and thin-sectioned (*A*). The lead phosphate reaction product appeared as a granular black precipitate along membranes of approximately one-third of the vesicles. Membrane vesicles from the same fraction, identically processed except without added substrate, are also shown (*B*). The membrane vesicles averaged $0.3 \mu\text{m}$ in diameter.

branes. However, that association was not strong enough to survive the additional washes needed for immunogold-labeling of the vesicles, so a direct visualization of this linkage could not be obtained.

Detection of Membrane-associated Calmodulin-binding Proteins

Another means of characterizing the association between calmodulin and contractile vacuoles is to identify calmod-

ulin-binding proteins enriched on contractile vacuole membranes. To that end, we developed a modified immunoblot procedure for detecting calmodulin-binding proteins (see Materials and Methods). Total soluble and particulate proteins as well as membrane-associated proteins from the sucrose density gradient were analyzed. The immunoblot procedure detected distinct populations of calmodulin-binding proteins in soluble and particulate fractions of *Dictyostelium* cells (Fig. 7, *A* and *B*, lanes *S* and *P*). For some of these

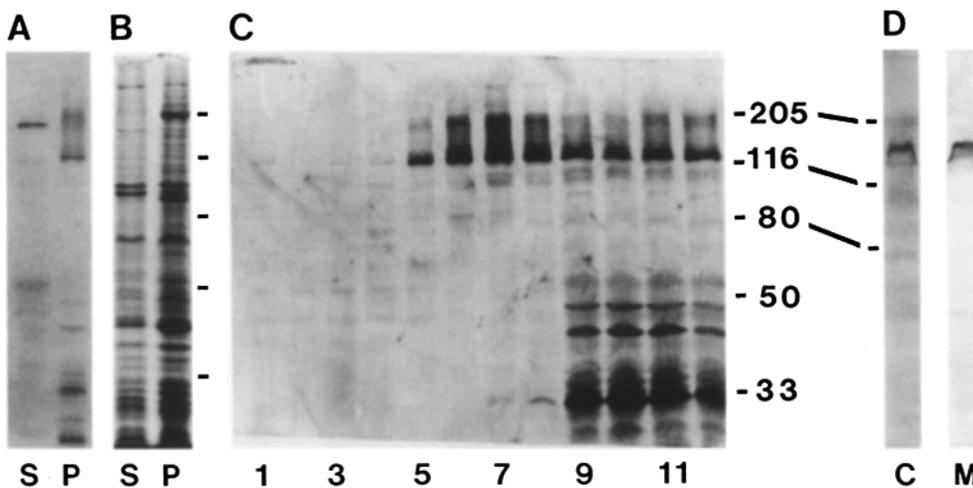


Figure 7. Calmodulin-binding proteins, including an unconventional myosin, detected by immunoblot. Soluble (*S*) and particulate (*P*) proteins from AX3 cells homogenized in TKMC buffer plus 0.25 M sucrose and protease inhibitors were separated by centrifugation at $100,000 \text{ g}$ for 45 min. Material derived from 1×10^6 cells was loaded in each lane of an SDS polyacrylamide gel. One pair of lanes was stained to show total protein (*B*), and another pair was blotted and stained with calmodulin plus anticalmodulin to detect calmodulin-binding

proteins (*A*). In *C*, membrane proteins from the sucrose density gradient fractions shown in Fig. 5 *B* were separated on an SDS polyacrylamide gel (an equal portion of the total sample being loaded in each lane), then blotted and stained to detect calmodulin-binding proteins. *D* shows the identification of one of the high molecular weight calmodulin-binding proteins as an unconventional myosin. AX3 cells were extracted and DE-52 chromatography was performed as described by Cote et al. (1985) for the initial steps in the isolation of myosin I from *Dictyostelium* cells. DE-52 fractions with K^+/EDTA ATPase activity were pooled, and a sample of this pool was immunoblotted. One strip from this blot was probed with calmodulin plus anticalmodulin (*C*), and the other was probed with an antiserum against *Acanthamoeba* myosin IC (*M*). A polypeptide of the same mobility was recognized by both probes. The migration positions of prestained molecular weight markers (Bio-Rad Laboratories) are indicated for each blot; the markers and their apparent molecular weights are myosin (205,000), beta-galactosidase (116,500), BSA (80,000), ovalbumin (49,500), and carbonic anhydrase (32,500).

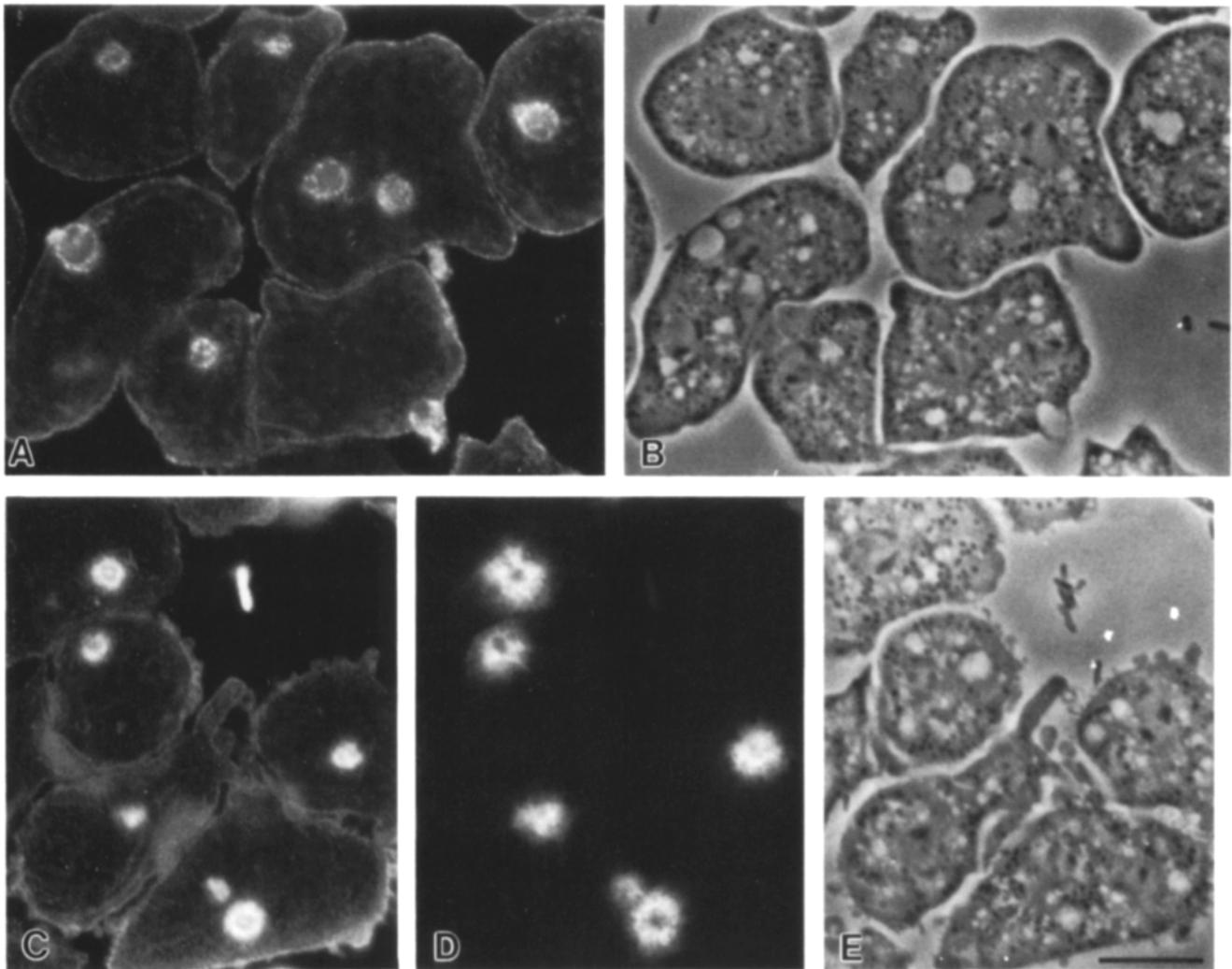


Figure 8. Colocalization of an unconventional myosin with calmodulin on *Dictyostelium* contractile vacuole membranes. AX3 cells fixed in paraformaldehyde-glutaraldehyde and permeabilized with saponin were immunostained with an antiserum against *Acanthamoeba* myosin IC (A) or were double-stained with this antiserum (C) and anticalmodulin (D). Bar, 10 μ m.

calmodulin-binding proteins, polypeptides with the same mobilities had previously been labeled using 125 I-calmodulin (Winckler et al., 1991); however, additional polypeptides were detected using the present technique. Several high molecular weight calmodulin-binding polypeptides found in the particulate fraction were enriched in membranes from the same region of the sucrose density gradient as the alkaline phosphatase peak, while most of the smaller calmodulin-binding polypeptides were enriched in denser fractions (Fig. 7 C). Although the identity of most of these proteins is not yet known, one of the high molecular weight calmodulin-binding proteins appears to be an unconventional myosin, as described below.

Identification of an Unconventional Myosin Associated with Contractile Vacuoles

An antiserum against the C isoform of *Acanthamoeba* myosin I, which labels contractile vacuole membranes and plasma membranes in *Acanthamoeba* (Baines and Korn,

1990), was used to probe immunoblots of *Dictyostelium* cells and cell fractions. This antiserum cross-reacted with a *Dictyostelium* protein of \sim 150 kD that was present in membranes across most of the sucrose gradient and was enriched in the region of the alkaline phosphatase peak (not shown). Purification of this presumptive unconventional myosin is in progress. Fig. 7 (D) shows two strips from a blot of the partially purified protein; one strip was incubated with calmodulin plus anticalmodulin and the other with the antiserum against *Acanthamoeba* myosin IC. Both probes appeared to recognize the same polypeptide.

The same antiserum was used for indirect immunofluorescence staining of *Dictyostelium* cells; it labeled contractile vacuole membranes and, to a lesser extent, plasma membranes (Fig. 8, top). The cells shown were fixed in glutaraldehyde-formaldehyde and permeabilized with saponin, as recommended by Baines and Korn (1990) for best reactivity of the epitope recognized by this antiserum. This procedure yielded poorer preservation of membranes and more diffuse calmodulin immunostaining than our standard fixation in

formaldehyde-methanol. However, double-labeling experiments (Fig. 8, *bottom*) made it clear that calmodulin and the protein recognized by the antimyosin IC antiserum were associated with the same vacuole population.

Discussion

Contractile vacuoles are found in most freshwater protozoa and amebas, where they are thought to serve an osmoregulatory function (reviewed by Kitching, 1967; Patterson, 1980). A typical contractile vacuole complex consists of a system of membranous tubules and/or vesicles (the presumptive site of fluid accumulation) feeding into a terminal reservoir, the contractile vacuole; the vacuole discharges its contents by means of transient fusion with the plasma membrane. In ciliates, the contractile vacuole is a fixed structure that empties through a permanent pore or diaphragm in the plasma membrane; the pore is surrounded and stabilized by helically wound microtubules. In amebas, the contractile vacuole complex moves about the cell, and no defined pore structures have been detected (Patterson, 1980). It is evident that a soil ameba such as *Dictyostelium*, exposed to rapid osmotic changes (e.g., rainfall), would require an efficient method of expelling excess fluid. However, the mechanism and regulation of contractile vacuole function are not well understood in *Dictyostelium* or any other organism.

For ciliates, there were earlier suggestions that calmodulin might be involved in contractile vacuole function. Peroxidase-tagged calmodulin antibodies were found to label the surfaces of several types of vacuoles, including a presumptive contractile vacuole, in *Paramecium tetraurelia* (Momayezi et al., 1986). Indirect immunofluorescence detected calmodulin at the pore of the contractile vacuole complex in *Tetrahymena pyriformis* (Suzuki et al., 1982), and drug studies, although inconclusive, were consistent with a role for calmodulin in excretion of vacuole contents (Suzuki et al., 1982; Bergquist, 1989). However, for both *Paramecium* and *Tetrahymena*, calmodulin was found to be associated with a great many organelles, and its possible link to contractile vacuoles (or pores) was never verified or further explored.

In *Dictyostelium*, we have found that membranes of the contractile vacuole complex are so highly enriched in calmodulin that this protein is effectively a marker for contractile vacuole membranes. This association persists whether the vacuoles are in a condensed or distended state, permitting visualization of this dynamic system throughout the vacuole cycle by indirect immunofluorescence. The evidence that the structures labeled by calmodulin antibodies are in fact contractile vacuoles is indirect but strong. In newly germinated cells, contractile vacuoles are the only large, phase lucent organelles present in the cell, and these organelles became labeled. In growing cells, the effect of osmotic shifts on the calmodulin-stained vacuoles mimicked the behavior of contractile vacuoles in living cells. Finally, calmodulin and alkaline phosphatase cofractionated on sucrose density gradients of *Dictyostelium* membranes.

Alkaline phosphatase has been shown to be a cytochemical marker for contractile vacuoles in *Dictyostelium* (Quiviger et al., 1978) and *Acanthamoeba* (Bowers and Korn, 1973). Quiviger and co-workers (1978) detected alkaline phosphatase activity on the membrane of the large vacuole of the *Dictyostelium* contractile vacuole complex, but not in

satellite vacuoles, along the plasma membrane, or in digestive vacuoles. Earlier biochemical studies had found that intact, living *Dictyostelium* cells manifested some alkaline phosphatase activity, suggesting that at least part of the enzyme was located on the exterior surface of the cell (Parish and Pelli, 1974; Lee et al., 1975). We too have found that ~5% of the total alkaline phosphatase activity in log phase AX3 cells and 15% of the activity in stationary phase cells is accessible in living cells (our unpublished data). However, a possible alternative to plasma membrane localization for this enzyme is that it lies on the luminal surface of the contractile vacuole membrane and is accessible to exogenous substrate during only that fraction of the pumping cycle when the vacuole fuses with the plasma membrane. This interpretation is consistent with the observation that horseradish peroxidase can enter contractile vacuoles during the interval between the expulsion of vacuole contents and the sealing of the pore (De Chastellier et al., 1978).

Immunogold EM of permeabilized cells showed that calmodulin was enriched at the periphery of a cluster of intracellular vacuoles. Most of the gold particles lay close to the luminal side of the membrane residuum. However, this apparent luminal localization may be misleading, since the detergent permeabilization conditions that enabled gold-conjugated antibodies to penetrate into the cell also caused substantial dissolution of membrane structure, possibly including rearrangement of membrane components. There are several examples in other cell types of an antigen with a known orientation appearing to lie on the "wrong" side of a membrane, in an open space, when labeled with immunogold (Van Hooff et al., 1989; Nakata et al., 1990); *Acanthamoeba* myosin IC is a particularly relevant example (Baines and Korn, 1990). Thus our EM data, while confirming the association of calmodulin with vacuole membranes, are inconclusive regarding the orientation of calmodulin.

In contrast to results reported for mammalian cells (see Introduction), we found no indication from immunofluorescence or EM that calmodulin was enriched along cytoskeletal filaments or was associated with subcellular organelles other than contractile vacuoles. The lack of staining of microfilaments is not surprising. *Dictyostelium* cells are highly motile and thus do not contain stress fibers (closely packed arrays of actin filaments and associated proteins), which are the structures reported to be labeled by calmodulin antibodies in fibroblasts (Dedman et al., 1978). *Dictyostelium* cells also appear to lack a calmodulin-regulated myosin light chain kinase (Tan and Spudich, 1991).

A more significant result is the absence of any evidence for an association of calmodulin with lysosomes. Nielsen and co-workers (1987) reported that in several types of cultured cells, similar vesicle populations (postulated to be lysosomes) were labeled with Acridine Orange in living cells and with calmodulin antibodies in fixed cells. However, in living *Dictyostelium* cells, acidic compartments visualized with Acridine Orange were clearly distinct from contractile vacuoles. In fixed cells, lysosomes detected by immunostaining with antibodies against *Dictyostelium* alpha-mannosidase or acid phosphatase have a scattered, punctate distribution (Bush and Cardelli, 1989; our unpublished observations). A similar but more extensive vesicle/vacuole population, probably including both endosomes and lysosomes, was labeled

in the present study by feeding cells biotinylated bacteria. That compartment did not overlap with the compartment stained by calmodulin antibodies. Furthermore, membrane-bound calmodulin segregated from lysosomal markers on a sucrose density gradient. Thus, several methods failed to detect an association between calmodulin and lysosomes. While *Dictyostelium* cells might differ from mammalian cells in this regard, it seems worthwhile to reexamine mammalian cells using the highly specific anticalmodulin mAbs now available.

Although our study has focused on the contractile vacuole, this focus is not intended to imply that calmodulin plays no other roles in *Dictyostelium*. In spite of the high concentration of calmodulin on contractile vacuole membranes, this is only a small part of the total calmodulin in the cell. Fractionation experiments indicate that nearly two-thirds of the cell's calmodulin is soluble under the lysis conditions used in the present study (our unpublished observations); in an EGTA-containing lysis buffer, this fraction is even higher (Clarke et al., 1980). Soluble calmodulin probably contributes to the general cytoplasmic fluorescence seen in immunostained cells. That calmodulin presumably carries out several of the myriad functions assigned to calmodulin by studies of other cell types. Calmodulin may also be associated with other intracellular membranes, but at levels not detected by methods used here.

The implications of the association of calmodulin with contractile vacuole membranes are being explored. The availability of high affinity, specific anticalmodulin antibodies has enabled us to develop a modified immunoblot procedure for detecting calmodulin-binding proteins. Among the membrane-associated calmodulin-binding proteins detected in contractile vacuole-rich fractions is a polypeptide of ~150 kD, which appears to be a new unconventional myosin in *Dictyostelium*. It is larger than the myosin I protein(s) previously purified from *Dictyostelium* (Cote et al., 1985) and larger than the predicted products of the myosin I genes that have been cloned from *Dictyostelium* (Jung and Hammer, 1990; Titus et al., 1989). Antibodies recognizing the small myosin I isoforms of *Dictyostelium* have been shown to label the leading edge of migrating cells (Fukui et al., 1989). Thus, both the size of the 150-kD protein and its association with contractile vacuoles are novel, consistent with earlier predictions from molecular biology data that several additional unconventional myosins remained to be identified in *Dictyostelium* (Titus et al., 1989).

The 150-kD protein may be the *Dictyostelium* homologue of a class of unconventional myosins recently identified in mammals (Larson et al., 1990; Espreafico et al., 1991; Mercer et al., 1991) and yeast (Johnston et al., 1991). These unconventional myosins (*Dilute*/p190/*MYO2*) are high molecular weight proteins whose sequence includes a cluster of putative calmodulin-binding domains (reviewed in Cheney and Mooseker, 1992). A temperature-sensitive mutation in the yeast gene, *MYO2*, suggests that this gene product may be involved in targeting or transporting vesicles to the site of bud formation (Johnston et al., 1991). In *Dictyostelium*, the enrichment of the 150-kD protein along with calmodulin on the membrane of contractile vacuoles is consistent with a role for this protein in movement of the vacuole to the plasma membrane. Purification of the *Dictyostelium* 150-kD protein is in progress.

The high level of calmodulin and the presence of multiple calmodulin-binding polypeptides in contractile vacuole-rich fractions suggest that calmodulin may serve more than one function. Another plausible role is the regulation of ion transport across contractile vacuole membranes, a necessary aspect of osmoregulation. Calmodulin is known to regulate Ca^{++} -gated K^+ and Na^+ channels in *Paramecium tetraurelia* (reviewed by Preston et al., 1991). In fungi, a vacuolar H^+ -ATPase provides the electrochemical potential used to drive the transport of calcium and other ions across the vacuole membrane (reviewed by Klionsky et al., 1990; Anraku et al., 1991). Preliminary studies indicate that contractile vacuole membranes of *Dictyostelium* cells do contain a vacuolar H^+ -ATPase (our unpublished observations). Thus, it is plausible that this enzyme may potentiate, and calmodulin may help to regulate, transport of ions between the contractile vacuole complex and the cytosol. This possibility is also under investigation.

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