

## Calmodulin and the contractile vacuole complex in mitotic cells of *Dictyostelium discoideum*

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### SUMMARY

In amoebae of the eukaryotic microorganism *Dictyostelium discoideum*, calmodulin is greatly enriched on membranes of the contractile vacuole complex, an osmoregulatory organelle. Antibodies specific for *Dictyostelium* calmodulin were used in the present study to immunolocalize the contractile vacuole complex in relation to the Golgi complex (detected with wheat germ agglutinin) and the microtubule organizing center (MTOC, detected with anti-tubulin antibodies). Cells were examined throughout the cell cycle. Double-staining experiments indicated that the contractile vacuole complex extended to the MTOC in interphase cells, usually, but not always, overlapping the Golgi complex. In metaphase and anaphase cells, the Golgi staining became diffuse, suggesting dispersal of Golgi membranes. In the same mitotic cells, anti-calmodulin antibodies labeled numerous small cortical vacuoles, indi-

cating that the contractile vacuole complex had also become dispersed. When living mitotic cells were examined, the small cortical vacuoles were seen to be active, implying that all parts of the *Dictyostelium* contractile vacuole complex possess the ability to accumulate fluid and fuse with the plasma membrane. In contrast to observations reported for other types of cells, anti-calmodulin antibodies did not label the mitotic spindle in *Dictyostelium*. Despite this difference in localization, it is possible that vacuole-associated calmodulin in *Dictyostelium* cells and spindle-associated calmodulin in larger eukaryotic cells might perform a similar function, namely, regulating calcium levels.

Key words: contractile vacuole, calmodulin, *Dictyostelium*, mitosis, Golgi

### INTRODUCTION

Vegetative cells of the eukaryotic microorganism *Dictyostelium discoideum* are small, highly motile, rapidly growing amoebae (reviewed by Loomis, 1975, 1982; Spudich, 1987). Our laboratory is studying calmodulin in *Dictyostelium* cells. Calmodulin is a highly conserved, multi-functional calcium-binding protein found in all eukaryotic cells; it is implicated in the regulation of many cellular functions, including motility, secretion, ion transport and cell proliferation (reviewed by Cohen and Klee, 1988; Means et al., 1991). The calmodulin protein of *Dictyostelium* cells is very similar to mammalian calmodulin in primary structure and in ability to activate mammalian enzymes; the two proteins are 87% identical in amino acid sequence (Marshak et al., 1984; Liu et al., 1992).

We have raised polyclonal and monoclonal antibodies against *Dictyostelium* calmodulin (Bazari and Clarke, 1982; Hulen et al., 1991) and have used these antibodies to examine calmodulin localization in *Dictyostelium* cells. Initial studies using polyclonal antibodies on cells fixed in suspension showed diffuse, primarily cortical staining (Bazari and Clarke, 1982). However, when these antibodies were

affinity-purified and applied to cells that had been flattened on a substratum, the strongest staining was found on membranes of a cluster of perinuclear vacuoles and vesicles, postulated to be contractile vacuoles (Clarke, 1990). Subsequent biochemical fractionation experiments and immunolocalization studies using monoclonal anti-calmodulin antibodies confirmed that calmodulin is greatly enriched on membranes of the contractile vacuole complex (Zhu and Clarke, 1992). Contractile vacuoles are organelles found in most freshwater protozoa and amoebae, where they are thought to serve an osmoregulatory role (reviewed by Kitching, 1967; Patterson, 1980). However, neither their mechanism of fluid collection and expulsion nor their other possible functions are well understood.

Observation of contractile vacuoles in living *Dictyostelium* cells and in fixed cells immunostained with calmodulin antibodies has shown that the contractile vacuole complex is dynamic and highly responsive to changes in the cell's osmotic environment (Zhu and Clarke, 1992). The question of whether and how this vacuolar system changes in response to internal cues, as for example during the cell cycle, has not previously been explored. We show here that the contractile vacuole complex with its associ-

ated calmodulin undergoes dramatic reorganization during mitosis. This redistribution of membrane-associated calmodulin in mitotic *Dictyostelium* cells may be related to the changes in calmodulin distribution that have been observed during mitosis in other types of eukaryotic cells.

## MATERIALS AND METHODS

### Strains and growth conditions

Wild type *Dictyostelium discoideum* cells (strain NC4) were grown in association with *Klebsiella aerogenes* at 22°C on SM nutrient agar plates (Loomis, 1975) or peptone/lactose plates (Kitanishi-Yumura and Fukui, 1989). Alternatively, NC4 cells were grown on *K. aerogenes* suspended in 17 mM potassium phosphate buffer (pH 6.4); the bacterial suspension was prepared as previously described (Clarke et al., 1987). For experiments requiring exposure of wild-type cells to a medium of higher osmotic strength, the cells were placed in Bonner's salts (20 mM NaCl, 10 mM KCl, 3 mM CaCl<sub>2</sub>) containing 0.1 M sorbitol, as previously described (Zhu and Clarke, 1992). Axenic cells (strain AX2) were grown in HL5 medium (Clarke et al., 1980).

### Enrichment for mitotic cells

Two methods were used to enrich for mitotic cells. In the first, NC4 cells were grown with *K. aerogenes* on peptone/lactose plates for 24 h at 22°C; the plates were then shifted to 4°C for 40 h, and the cells were collected (Kitanishi-Yumura and Fukui, 1989). The cells were washed in cold buffer, either Na/K buffer (17 mM Na<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, pH 6.4) or MES buffer (20 mM MES, pH 6.8, 0.2 mM CaCl<sub>2</sub>, 2 mM MgSO<sub>4</sub>). Still at 4°C, the cells were placed on glass coverslips and left for 5-10 min. They were then shifted to room temperature, overlaid with agarose, and fixed as described below. Fixation was begun 8-15 min after the shift to room temperature, to capture the first round of mitoses. In some cases, after the cells had been harvested from the plate, they were incubated with a suspension of bacteria for 2.5 h at 22°C before being placed on coverslips and prepared for fixation. Thus, the cells were placed in fixative about 3 h after the shift to room temperature, corresponding to the second round of mitoses.

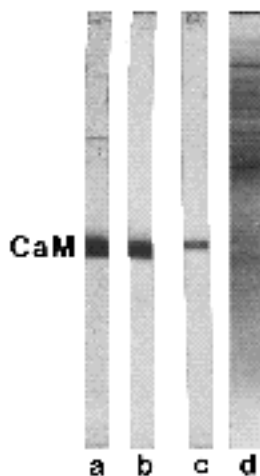
For the second method, adapted from Maeda (1986), NC4 cells at a density of  $5 \times 10^5$  cells/ml, growing on a suspension of *K. aerogenes*, were shaken at 11.5°C for 20 h, then washed in cold Na/K buffer or MES buffer. The cells were resuspended in the same buffer and placed on coverslips at 4°C. Further processing was as described above, except that the agar-overlay procedure was carried out in the cold, so that fixation was begun 2-5 min after the shift to room temperature.

### Fixation conditions

Cells were fixed using the two-step, agar-overlay procedure described by Fukui et al. (1987). The initial fixation of NC4 cells was in 2.5% formaldehyde in Na/K buffer (5 min, room temperature), and extraction was in 1% formaldehyde in methanol (-15°C, 5 min). AX2 cells were processed identically, except that the initial fixation was in 2% formaldehyde in HL5.

### Affinity purification of calmodulin-specific polyclonal antibodies

Polyclonal antibodies specific for *Dictyostelium* calmodulin were affinity-purified from a rabbit antiserum (Bazari and Clarke, 1982) using calmodulin immobilized on PVDF membrane. 2 mg of purified *Dictyostelium* calmodulin were applied to PVDF membrane (Millipore Immobilon P), which was then fixed and blocked as described by Hulen et al. (1991). The calmodulin-bearing mem-



**Fig. 1.** Immunoblot of a *Dictyostelium* cell lysate probed with polyclonal antibodies against *Dictyostelium* calmodulin. Each lane of an SDS-polyacrylamide gel was loaded with the lysate from  $2.5 \times 10^5$  *Dictyostelium* cells, strain NC4. After electrophoresis, a portion of the gel was stained with Coomassie Blue to show total protein (lane d), and proteins were transferred from the remainder of the gel to PVDF membrane. Strips of membrane were incubated with the following probes: lane a, unfractionated rabbit antiserum (1:400 dilution); lanes b and c, rabbit antibodies affinity-purified on immobilized calmodulin (1:400 dilution). The primary antibody incubations were carried out in the presence 0.1 mM CaCl<sub>2</sub> (lanes a and b) or 1 mM EGTA (lane c). CaM, calmodulin.

brane was incubated with 2.5 ml of immune serum overnight at 4°C, then washed 3 times in TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.5). Antibody was eluted by incubating the membrane in 0.5 ml of 0.1 M glycine-HCl (pH 2.5) for 5 min. The pH of the eluate was then adjusted to pH 7.4 by addition of 1 M Tris-HCl (pH 8.5). The affinity-purified antibodies recognized only calmodulin when used to stain immunoblots of *Dictyostelium* total cell lysates (Fig. 1). This figure also shows that the polyclonal antibodies recognized calcium-free as well as calcium-containing calmodulin, although reactivity was stronger with the calcium-containing form of the protein. Our electrophoresis and immunoblotting conditions for calmodulin have been previously described (Hulen et al., 1991).

### Antibodies and immunostaining conditions

Rabbit polyclonal antibodies (described above) and mouse monoclonal antibodies were used to immunostain *Dictyostelium* calmodulin. The monoclonal antibody (2D1) was previously shown to be specific for *Dictyostelium* calmodulin (Hulen et al., 1991). Tubulin was stained with a mouse monoclonal antibody (3A5) against *Drosophila* alpha-tubulin (Piperno and Fuller, 1985); this was the gift of Dr Margaret Fuller. For indirect immunofluorescence experiments, the affinity-purified, polyclonal rabbit anti-calmodulin was diluted 1:100, the 2D1 mouse ascitic fluid was diluted 1:800 or 1:400, and the 3A5 anti-tubulin was diluted 1:100. All dilutions were in TBS. Secondary antibodies were FITC-conjugated, goat anti-mouse IgG (Cappel, 1:200 or 1:400), FITC-conjugated, rabbit anti-mouse IgG (American Qualex, 1:100, or Sigma, 1:50), FITC-conjugated, sheep anti-rabbit IgG (Cappel, 1:1000), or rhodamine-conjugated, goat anti-mouse IgG (Cappel, 1:200). For double-staining with rabbit anti-calmodulin and mouse anti-tubulin, the two primary antibodies were mixed, as were the two secondary antibodies. Preparation of the antibodies and fixation and staining conditions were as described by Clarke et al. (1987).

### Other staining procedures

Cells to be stained with wheat germ agglutinin (WGA) were fixed by the two-step procedure described above, then rinsed ( $3 \times 5$  min) in PBS (10 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, pH 6.8). The fixed cells were incubated with Texas Red-labeled WGA from Molecular Probes (2 µg/ml in PBS, 30 min at room tem-

perature) and rinsed 3× in PBS. The cells were given a final rinse with TBS and then stained with anti-calmodulin or anti-tubulin as described above. DNA was labeled by incubating fixed *Dictyostelium* cells with 4',6-diamidino-2-phenylindole (DAPI, 5 mM in TBS) for 15 min at room temperature; this incubation was usually performed after all immunostaining steps had been completed.

## RESULTS

### Relationship between the contractile vacuole complex and other intracellular organelles

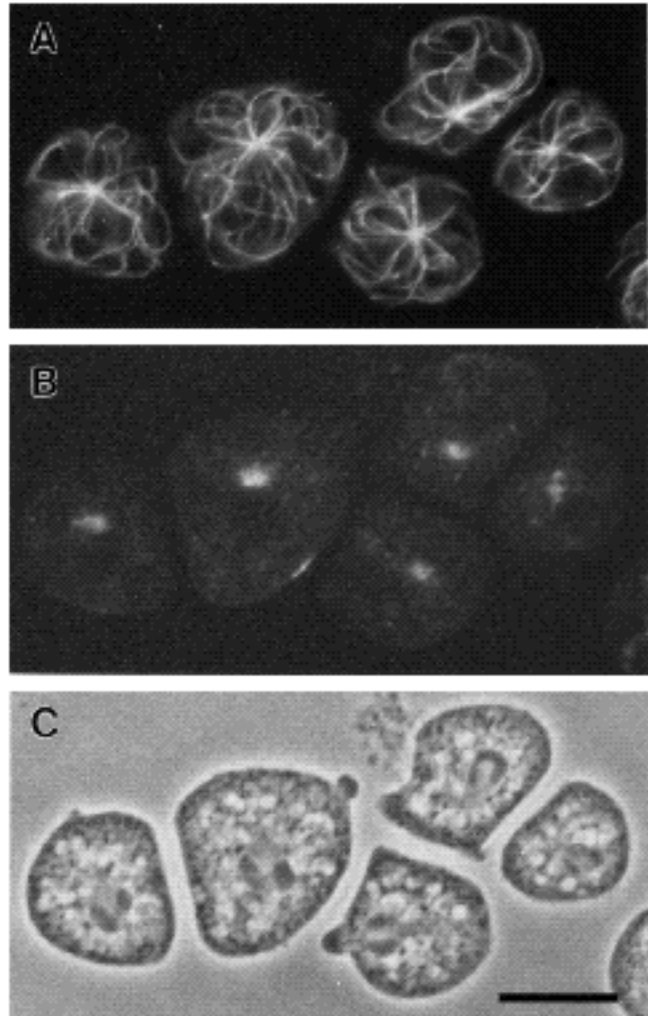
Observation of living cells, as well as inspection of immunofluorescence images of fixed cells (e.g. Zhu and Clarke, 1992), indicated that at least part of the contractile vacuole complex always lay close to the nucleus in *Dictyostelium* cells. We examined how this location related to the positions of the microtubule organizing center (MTOC) and the Golgi complex. Anti-tubulin antibodies were used to label microtubules, and Texas Red-conjugated wheat germ agglutinin (WGA) was used as a marker for Golgi membranes. (See Wuestehube et al., 1989, for a discussion of the use of WGA as a Golgi marker in *Dictyostelium*.) The staining properties of these probes are illustrated in Fig. 2, which shows vegetative *Dictyostelium* cells double-stained with anti-tubulin antibodies and with WGA. As expected, in interphase cells, the Golgi complex was always positioned at the MTOC.

The location of the contractile vacuole complex relative to that of the MTOC was examined by double-staining *Dictyostelium* cells with anti-calmodulin and anti-tubulin antibodies. Although the calmodulin-rich contractile vacuole complex sometimes extended across much of the cell, it appeared to be centered at or near the MTOC (Fig. 3).

The distribution of Golgi membranes was compared with that of contractile vacuole membranes by double-staining *Dictyostelium* cells with WGA and anti-calmodulin antibodies. In interphase cells (Fig. 4), the structures stained by the two probes were juxtannuclear and lay close together; indeed, overlapping distributions were usually observed. However, the calmodulin staining was more extensive, and in some cells the two staining patterns were wholly separated, making it clear that anti-calmodulin antibodies and WGA labeled distinct structures. Examples of both types of distribution are shown in Fig. 4. For three of the cells in this figure, the two probes stained overlapping structures, but for the fourth (lower right), the labeled structures were contiguous instead. Thus, indirect immunofluorescence did not detect calmodulin on Golgi membranes.

### Cell cycle-dependent changes in contractile vacuole organization

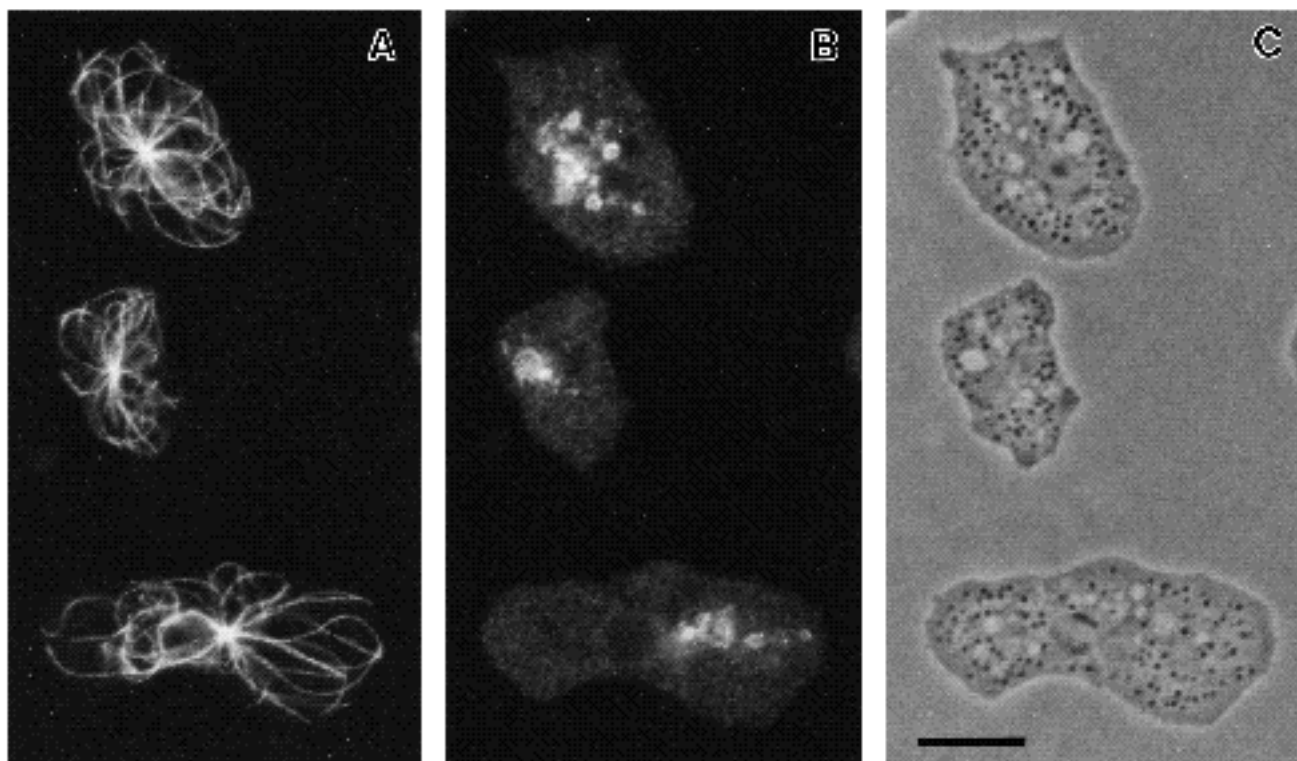
The distribution of Golgi membranes and contractile vacuole membranes was compared at different stages of the cell cycle. Typical interphase staining patterns are illustrated in Fig. 5A-C. In mitotic cells, the WGA label for Golgi membranes was diffuse (Fig. 5E), suggesting that Golgi membranes disperse during M phase in *Dictyostelium* cells, just as they do in mammalian cells (see Discussion). In telophase cells, WGA staining returned to the poles of the separated nuclei (Fig. 5H). The contractile vacuole system, detected with anti-calmodulin antibodies, also dis-



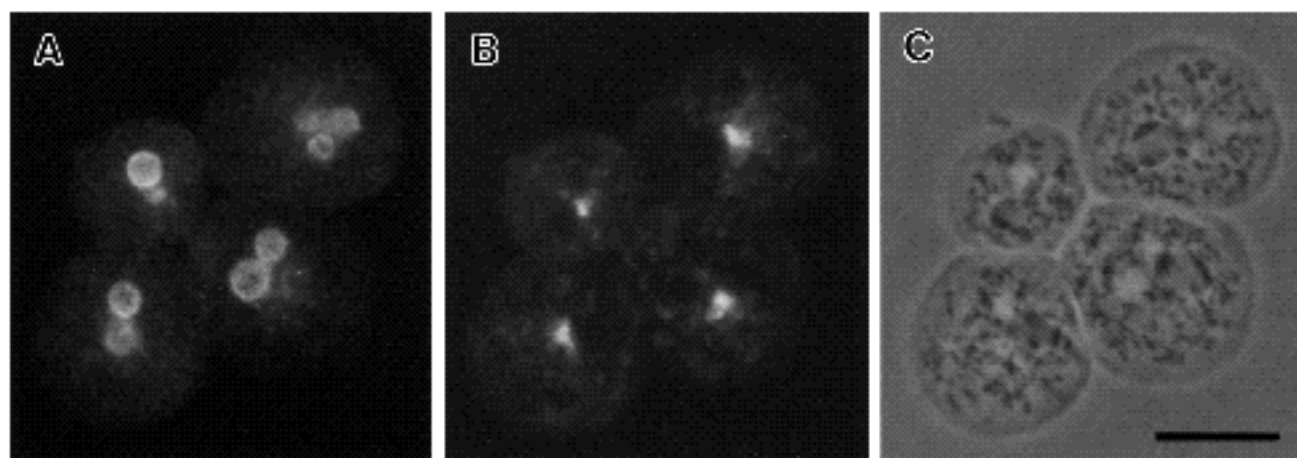
**Fig. 2.** Interphase *Dictyostelium* cells double-stained to show microtubules and the Golgi complex. NC4 cells were fixed and stained with anti-tubulin (3A5) to detect microtubules (A) and with fluorescently labeled WGA to detect Golgi membranes (B). A phase contrast image of the same cells is shown in C. The Golgi complex was positioned adjacent to the nucleus, at the MTOC. Bar, 10  $\mu$ m.

persed in mitotic cells and reaggregated during telophase near daughter cell nuclei (Fig. 5D and G). In other double-staining experiments using anti-tubulin antibodies (not shown), the dispersed state of both Golgi and contractile vacuole membranes correlated with an absence of cytoplasmic microtubules.

Cell cycle-dependent dispersal and reorganization of the contractile vacuole complex were examined in greater detail using the DNA-binding fluorochrome DAPI to visualize DNA and anti-calmodulin antibodies to label contractile vacuole membranes. Cell-cycle stages were inferred from the appearance of the cell nuclei and the DAPI-labeled chromosome masses. (Compare to Kitanishi-Yumura and Fukui (1987).) Calmodulin-immunostaining indicated that the perinuclear cluster of vacuoles characteristic of interphase cells dispersed during prophase (Fig. 6A-C), to form numerous small vacuoles in the cortical region of



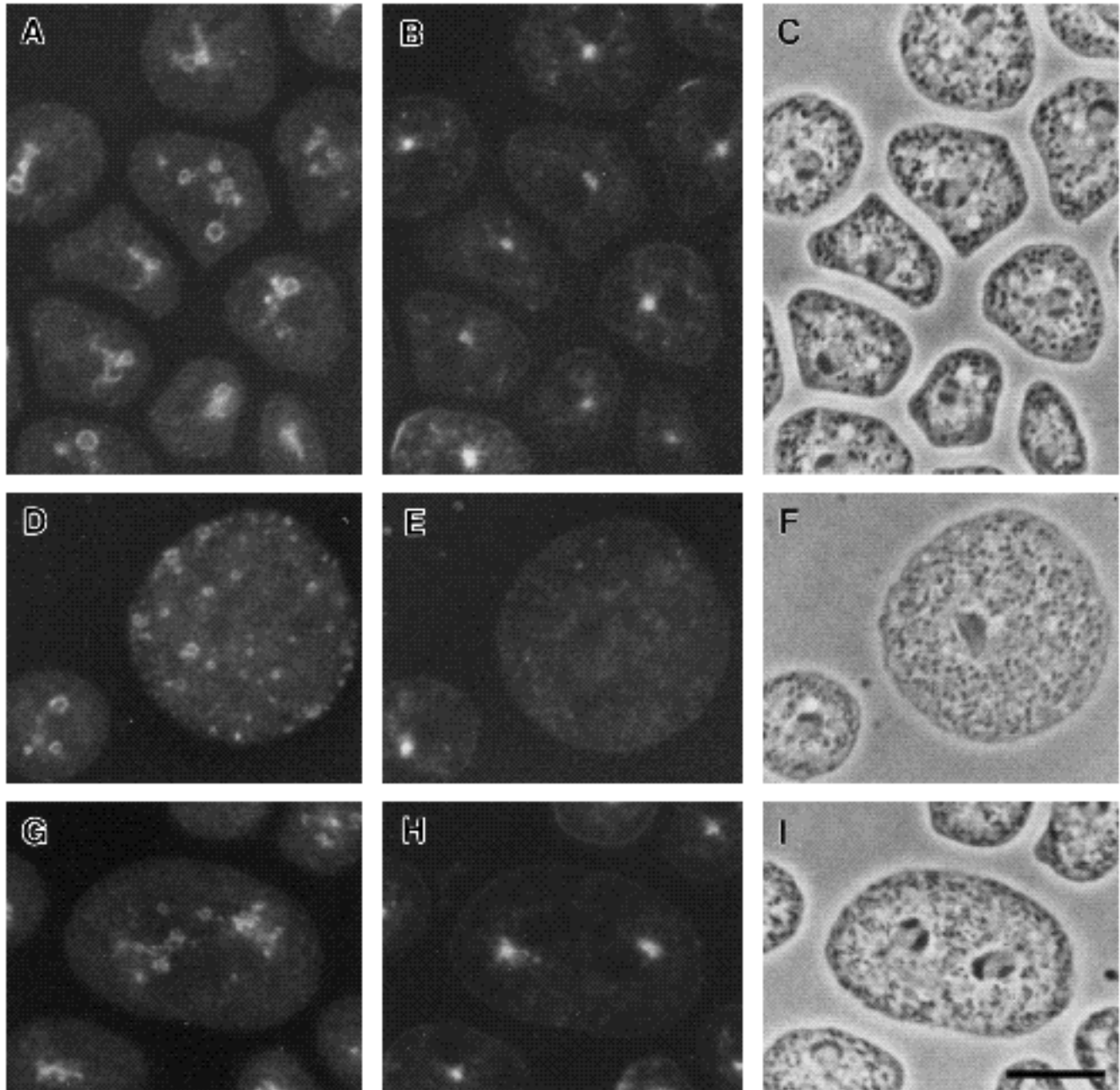
**Fig. 3.** Interphase *Dictyostelium* cells double-stained to show microtubules and the contractile vacuole complex. AX2 cells were fixed and stained with mouse anti-tubulin antibodies (3A5) to detect microtubules (A), and with affinity-purified, rabbit anti-calmodulin antibodies to label contractile vacuole membranes (B). C shows a phase contrast image of the same cells. A portion of the contractile vacuole complex extended to the nucleus, at the position of the MTOC. Bar, 10  $\mu\text{m}$ .



**Fig. 4.** Interphase *Dictyostelium* cells double-stained to show Golgi membranes and the contractile vacuole complex. NC4 cells were incubated in Bonner's salt solution containing 0.1 M sorbitol for 1 h, then shifted to a 20-fold dilution of the same medium and fixed within 10 min. The cells were double-stained with 2D1 anti-calmodulin antibodies to visualize contractile vacuole membranes (A) and with WGA to visualize Golgi membranes (B). (The shift to hypoosmotic conditions led to swelling of the contractile vacuoles, making it easier to distinguish the two membrane systems.) Three of the cells in this field displayed overlapping staining patterns for WGA and anti-calmodulin, as was commonly observed. However, in the fourth cell (lower right), the two patterns, while adjacent, were distinct. Bar, 10  $\mu\text{m}$ .

metaphase cells (Fig. 6D-F). This pattern persisted during anaphase (Fig. 6G-I). Only in telophase cells (Fig. 6J-L) did the scattered cortical vacuoles return to the poles of daughter nuclei and form the cluster of larger vacuoles characteristic of interphase cells.

An anaphase cell double-stained with anti-tubulin and anti-calmodulin antibodies is shown in Fig. 7. Using a filter set appropriate for detecting anti-calmodulin staining, successive photos were taken of the same cell, proceeding from upper (A) to central (B) to lower (C) focal planes. These

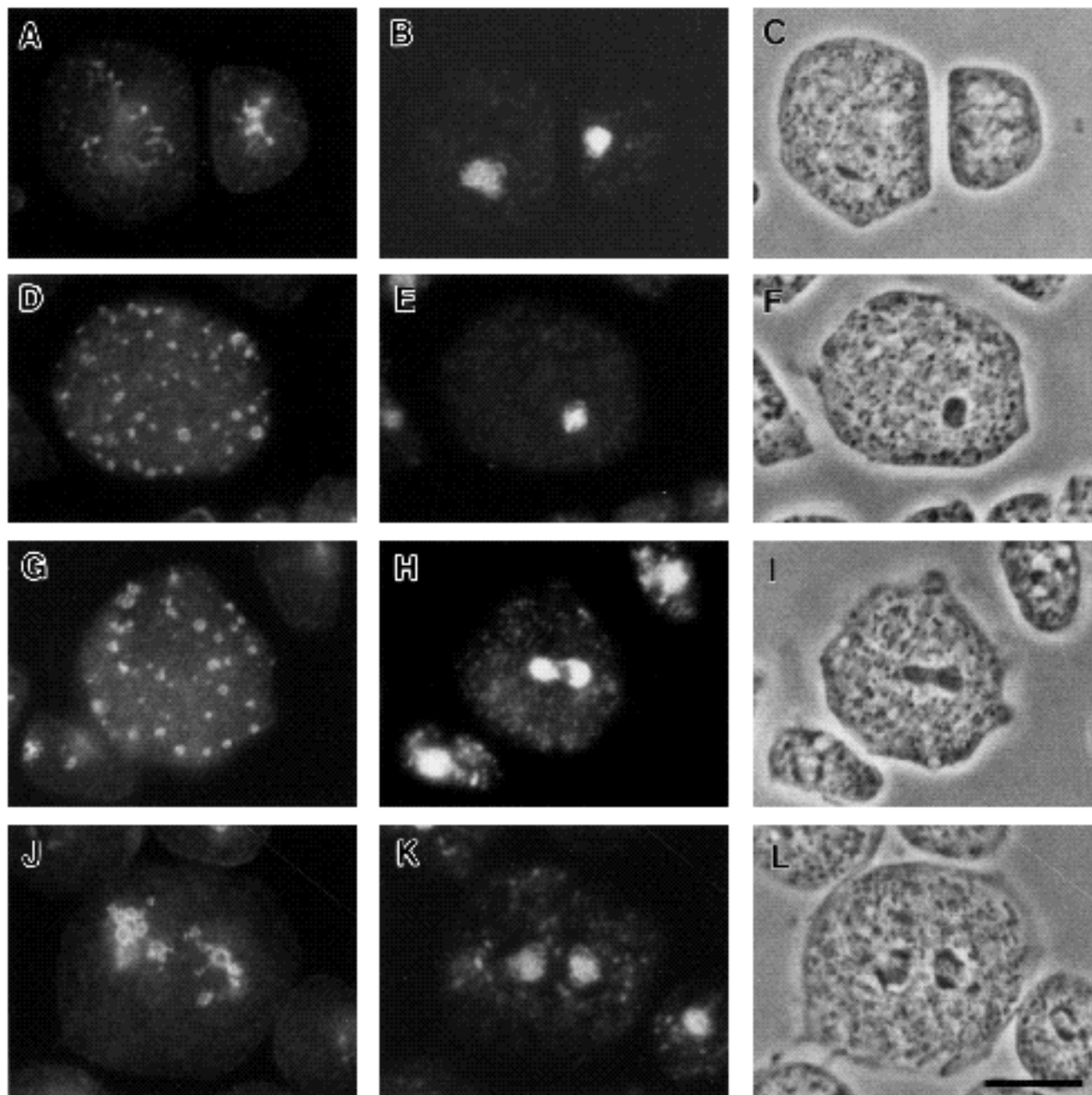


**Fig. 5.** The contractile vacuole complex and the Golgi complex in interphase and mitotic *Dictyostelium* cells. NC4 cells were double-stained with 2D1 anti-calmodulin antibodies to label contractile vacuole membranes (A,D,G) and with WGA to label Golgi membranes (B,E,H); phase contrast images are shown in C,F,I. The cells are in interphase (A-C), metaphase (D-F), and telophase (G-I). Both the Golgi complex and the contractile vacuole complex became dispersed in metaphase cells. Bar, 10  $\mu$ m.

images make clear the cortical localization of the small vacuoles labeled with calmodulin antibodies. In living mitotic cells, comparable phase-lucent cortical vacuoles could be observed filling and emptying (not shown). Anti-tubulin antibodies revealed the mitotic spindle in the central focal plane of the cell shown in Fig. 7D. Note that calmodulin was not detected in association with microtubules of the mitotic spindle.

## DISCUSSION

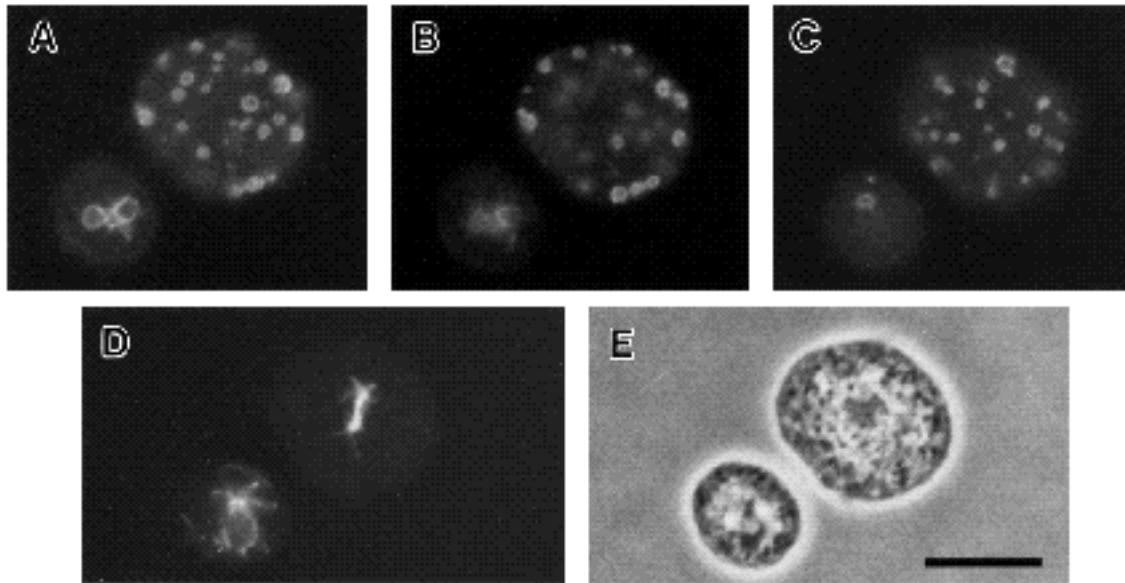
In ciliates and many protozoa, the contractile vacuole is a persistent structure located at a fixed position in the cell and associated with a specialized pore in the plasma membrane (reviewed by Kitching, 1967; Patterson, 1980). However, in *Dictyostelium* cells the contractile vacuole complex is a dynamic and highly pleomorphic structure. Its responses to changes in osmotic conditions have been previously described (Zhu and Clarke, 1992). We show here that a profound reorganization of the contractile vacuole



**Fig. 6.** Cell-cycle-dependent changes in the contractile vacuole complex of *Dictyostelium* cells. NC4 cells were stained with 2D1 anti-calmodulin antibodies (A,D,G,J) and with the DNA-binding fluorochrome DAPI (B,E,H,K); phase contrast images are also shown (C,F,I,L). The first row (A-C) shows a cell in prophase (left) next to an interphase cell. The second row (D-F) shows a metaphase cell. The third row (G-I) shows a cell in anaphase. The fourth row (J-L) shows a cell in telophase. Note that the dispersal of the contractile vacuole complex is initiated during prophase and persists through anaphase, with a return to perinuclear localization during telophase. Bar, 10  $\mu$ m.

complex occurs during a cell's progression through the cell cycle. Although commonly juxtannuclear in interphase cells, the complex disperses at prophase/metaphase to form numerous small cortical vacuoles. A corresponding redistribution of phase-lucent vacuoles can be observed in living cells, and this has been recorded in a beautiful, high resolution video of a *Dictyostelium* cell proceeding through mitosis (Fukui and Inoué, 1992). These observations suggest that all portions of the contractile vacuole complex in

*Dictyostelium* possess the ability to accumulate fluid and fuse with the plasma membrane. This interpretation is further supported by electron microscopic studies now in progress. EM images of broken, freeze-dried *Dictyostelium* cells reveal an extensive array of cisternae connected by tubular elements; the tubules appear to become incorporated into the cisternae as they swell. The membranes of the cisternae and the swollen tubules all bear vacuolar-type proton pumps, which are implicated in fluid accumulation (Heuser



**Fig. 7.** Through-focus images of contractile vacuoles in an anaphase *Dictyostelium* cell. NC4 cells were double-stained with affinity-purified rabbit anti-calmodulin antibodies (A-C) and with mouse anti-tubulin antibodies (D); a phase contrast image is also shown (E). Successive focal planes, from upper to lower, are shown in A-C, illustrating the cortical localization of the contractile vacuoles. The intranuclear mitotic spindle, found in the central focal plane, can be seen in D. Bar, 10  $\mu\text{m}$ .

et al., 1993). Thus, different parts of the contractile vacuole system appear to be functionally equivalent by this criterion as well.

In interphase *Dictyostelium* cells, double-staining experiments indicated that at least part of the contractile vacuole complex extended to the MTOC, usually overlapping the position of the Golgi complex. The fragmentation of the contractile vacuole complex in mitotic cells resembled the behavior of the Golgi complex. In spite of these similarities, however, the existence of occasional cells that displayed adjacent rather than overlapping staining patterns argued that calmodulin antibodies were not labeling Golgi membranes.

Earlier studies showed that the Golgi complex of mammalian cells becomes fragmented during mitosis, correlating with the breakdown of cytoplasmic microtubules (Lucocq and Warren, 1987; Lucocq et al., 1989; reviewed by Kreis, 1990). Golgi elements in fibroblasts also disperse when cytoplasmic microtubules are disassembled by drug or cold treatment, and, when microtubules are allowed to reassemble, the scattered Golgi elements recluster by moving along microtubules back to the region of the MTOC (Rogalski and Singer, 1984; Ho et al., 1989; Turner and Tartakoff, 1989; Corthesy-Theulaz et al., 1992). Similarly, in *Dictyostelium* cells, the dispersed state of both Golgi membranes and the contractile vacuole complex correlated with the absence of cytoplasmic microtubules. This coordinated behavior, together with the apparent enrichment of contractile vacuoles in the vicinity of the MTOC of interphase cells, suggests an interaction between contractile vacuoles and microtubules in *Dictyostelium* cells. The same implication comes from an earlier study in which an MTOC-nucleus complex was isolated from *Dictyostelium* cells; the authors reported that "peculiar vesicles... decorated with 11 nm tacks" were associated with the MTOC (Omura and Fukui, 1985). The "tacks" are indistinguish-

able from the headpieces of the vacuolar proton pumps that thickly populate the membranes of the contractile vacuole complex (Heuser et al., 1993). Finally, preliminary studies indicate that nocodazole-induced depolymerization of cytoplasmic microtubules in *Dictyostelium* cells leads to dispersal of the contractile vacuole complex (Q. Zhu and M. Clarke, unpublished observations). These indications of an apparent interaction between microtubules and contractile vacuoles can best be verified using purified components.

*Dictyostelium* amoebae, owing to their small size and their tendency to remain attached to a substratum during mitosis, have proved to be favorable subjects for observation of mitosis in living cells (Roos and Camenzind, 1981; Fukui and Inoué, 1991, 1992) and for ultrastructural analysis of mitotic spindles (Moens, 1976; Roos and Camenzind, 1981; McIntosh et al., 1985). These studies have shown mitosis in *Dictyostelium* cells to be very similar to that in higher eukaryotes, except that the spindle is smaller ( $\sim 3 \mu\text{m}$  in length), and mitosis takes place essentially within the nuclear envelope.

The lack of calmodulin-immunostaining of the mitotic spindle of *Dictyostelium* cells is an interesting difference relative to calmodulin localization in mitotic plant and animal cells. Calmodulin was found to be concentrated in the kinetocore-to-pole region of mitotic spindles in all vertebrate cells examined (Welsh et al., 1978; Anderson et al., 1978), and also in higher plant cells (Wick et al., 1985; Vantard et al., 1985). The meaning of this spindle localization is unclear, although it has led to suggestions that calmodulin may regulate microtubule disassembly (Welsh et al., 1978; Dedman et al., 1980) or stabilize kinetocore-to-pole-microtubules (Sweet et al., 1988). A direct association of spindle calmodulin with kinetocore microtubules has been postulated (Sweet et al., 1988; Welsh and Sweet, 1989). However, another possibility, perhaps more consistent with calmodulin distribution in *Dictyostelium*, is that

calmodulin is associated with endomembranes of the mitotic apparatus (Hepler, 1989).

As reviewed by Hepler (1989), in most higher eukaryotes, the nuclear membrane breaks down and becomes indistinguishable from endoplasmic reticulum as the cells enter mitosis. In cells from vertebrates, invertebrates, plants and insects, the mitotic apparatus is typically surrounded and extensively penetrated by an ER-derived membrane system. Studies of several types of plant and insect cells and a few mammalian cells have demonstrated a specific association of this membrane system with kinetocore microtubules (reviewed by Hepler and Wolniak, 1984). There are also indications that mitotic endomembranes contain bound calcium and can rapidly sequester calcium. Such observations have led to the suggestions that localized fluxes in free calcium levels may play important regulatory roles in mitosis, and that calmodulin associated with the mitotic endomembrane system could participate as an effective calcium-chelating agent, or in some other capacity (Hepler and Wolniak, 1984; Hepler, 1989, and references cited therein).

In *Dictyostelium*, as in many small eukaryotic cells, the nuclear membrane does not break down during mitosis. However, the distribution of calmodulin and the contractile vacuole system in mitotic *Dictyostelium* cells is consistent with a role in regulating calcium levels. In a small cell with a nucleus only about 3  $\mu\text{m}$  in diameter, diffusion may be sufficiently rapid to link calcium-sequestering components outside the spindle to calcium-regulated components within the spindle (Hepler and Wolniak, 1984).

Moreover, contractile vacuoles of *Dictyostelium* probably possess the necessary  $\text{Ca}^{2+}$ -transport capabilities. The vacuoles of yeast (Ohsumi and Anraku, 1983) and *Neurospora* (Miller et al., 1990) play major roles in cytosolic calcium homeostasis, calcium uptake being catalyzed by a  $\text{Ca}^{2+}/\text{H}^{+}$  antiport system, which is energized by a vacuolar  $\text{H}^{+}$ -ATPase that pumps protons into the vacuole lumen (reviewed by Klionsky et al., 1990). Our studies (Heuser et al., 1993) have shown that contractile vacuole membranes in *Dictyostelium* cells are very rich in vacuolar  $\text{H}^{+}$ -ATPase molecules. Furthermore, there is a recent report that a *Dictyostelium* membrane fraction enriched in vacuolar  $\text{H}^{+}$ -ATPase activity also possesses ATP-driven  $\text{Ca}^{2+}/\text{H}^{+}$ -antiport activity (Rooney and Gross, 1992). We think it likely that both of these activities reside in contractile vacuole membranes, and that the contractile vacuole system will prove to be a critical element in regulating intracellular calcium levels in *Dictyostelium*. If the function of calmodulin in mitosis is conserved in higher and lower eukaryotes, characterization of the contractile vacuole system in *Dictyostelium* may also provide new insights into the roles played by calcium and calmodulin in the mitotic spindle of other eukaryotic cells.

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