Characterization and Immunocytochemical Distribution of Calmodulin in Higher Plant Endosperm Cells: Localization in the Mitotic Apparatus

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ABSTRACT In this study we have examined the immunocytochemical distribution of calmodulin during mitosis of higher plant endosperm cells. Spindle development in these cells occurs without centrioles. Instead, asterlike microtubule converging centers appear to be involved in establishing spindle polarity. By indirect immunofluorescence and immunogold staining methods with anti-calmodulin antibodies, we found endosperm calmodulin to be associated with the mitotic apparatus, particularly with asterlike and/or polar microtubule converging centers and kinetochore microtubules, in an immunocytochemical pattern distinct from that of tubulin. In addition, endosperm calmodulin and calcium showed analogous distribution profiles during mitosis. Previous reports have demonstrated that calmodulin is associated with the mitotic apparatus in animal cells. The present observation that calmodulin is also associated with the mitotic apparatus in acentriolar, higher plant endosperm cells suggests that some of the regulatory mechanisms involved in spindle formation, microtubule disassembly, and chromosome movement in plant cells may be similar to those in animal cells. However, unlike animal cell calmodulin, endosperm calmodulin appears to associate with kinetochore microtubules throughout mitosis, which suggests a specialized role for higher plant calmodulin in the regulation of kinetochore microtubule dynamics.

Calcium has been implicated as a mediator of a variety of cellular functions (for review, see reference 1). Numerous observations have suggested that calcium acts as a modulator in the process of mitosis and chromosome movement in animal cells. Part of this regulation may be through modulation of the assembly and disassembly of microtubules. Cytoplasmic and mitotic spindle microtubules are sensitive to calcium ions, both in living cells after microinjection of calcium (2, 3) and in detergent-treated or ionophore-permeabilized cells (4–6). In addition, several studies (7–10) have shown that partially purified preparations of microtubules can be depolymerized in vitro by calcium. The molecular mechanisms by which calcium exerts its regulatory role on microtubule depolymerization are not known. However, several lines of evidence suggest that the effects of calcium on microtubules are mediated at least in part by calmodulin, a ubiquitous, highly conserved, calcium binding protein that regulates multiple calcium-dependent processes (for recent reviews, see references 11 and 12). Calmodulin enhances the calcium-sensitivity of microtubules in vitro (4, 13–16), and microinjection of calcium-calmodulin into fibroblasts results in a localized depolymerization of microtubules around the site of microinjection (17). Furthermore, ultrastructural localization studies of calmodulin by immunocytochemical procedures (18–22) or after microinjection (23) have demonstrated that calmodulin is associated with the mitotic apparatus in several types of animal cells. The observation that calmodulin is closely associated with the spindle poles, the pericentriolar region, and the kinetochore-to-pole microtubules supports the idea that calmodulin may be involved in

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the ordered disassembly and rearrangement of microtubules that occur during anaphase chromosome movement.

However, the regulation of microtubule depolymerization by calcium and calmodulin appears to be a complex process. The sensitivity of different microtubule preparations to calcium can be affected by ionic strength, temperature, tubulin concentration, presence of microtubule-associated proteins, and calmodulin concentration (4, 8, 16); and some microtubule preparations are insensitive to calmodulin (24, 25). In addition, an in vitro effect of calmodulin on microtubules does not necessarily mean that calmodulin is the endogenous regulator. Thus, the regulatory mechanisms that operate on microtubules from different species and tissues may vary.

In this regard, little is known about the potential regulation of mitosis by calcium and calmodulin in plant cells. Endosperm cells of the blood lily, *Haemanthus*, have been used for many years in studies of chromosome dynamics during mitosis. Endosperm cells lack a cellulose wall and thus allow easier penetration of antibodies. This feature makes endosperm cells particularly useful for immunocytochemical studies. We previously reported (26, 27) the development and application of immunocytochemical procedures for visualization of microtubules in *Haemanthus* endosperm cells. Those studies yielded new information about how interphase microtubules rearrange during mitosis into the bipolar architecture that characterizes the mitotic spindle. In most higher plant cells, this process occurs without centrioles, organelles that appear to play an essential role as microtubule nucleation or orienting centers during spindle development in animal cells. We have identified (26, 27) asterlike centers of microtubular structures that are involved in the reorganization of microtubule arrays both at the onset of mitosis and during telophase–interphase transition in endosperm cells. It is possible, but has not been demonstrated yet, that these asterlike microtubule converging centers may function as microtubule organizing centers in endosperm cells. Thus, it appears that microtubule rearrangement and dynamics during mitosis of these cells involve at least three processes: microtubule assembly/disassembly, reorientation of microtubules, and change in microtubule properties reflected in their lateral interaction during spindle development.

Whether calmodulin is involved in the regulation of these processes has not been examined. Analysis of the distribution of calcium at various stages of mitosis in *Haemanthus* suggests that calcium is associated with the mitotic apparatus and that redistribution of calcium within the spindle accompanies anaphase chromosome movement (28, 29). However, no studies have been reported on the localization of calmodulin during mitotic chromosome movement in higher plant cells. We have examined the immunocytochemical localization of calmodulin in interphase and mitotic endosperm cells. We report here that endosperm calmodulin is associated with the mitotic apparatus, particularly with the asterlike centers and kinetochore microtubules, in a pattern distinct from that of tubulin, and that calcium and calmodulin are co-localized during mitosis.

MATERIALS AND METHODS

Preparation of Cells: Living mitotic endosperm cells of *Haemanthus katherinae* Bak were prepared as previously described (30, 31). Endosperm preparations were fixed and permeabilized as described (26). Some cell preparations were perfused with colchicine (Merck Chemical Div., Merck & Co., Inc., Darmstadt, West Germany) solution at a concentration of 8×10^{-5} M for 3–5 min and processed for immunocytochemical observations (see below). For cold treatments, cells were cooled rapidly to 3°C using either a cooling perfusion chamber or directly immersing the preparations in a cooling bath as previously described (31). During colchicine or cold treatment, chromosome movements of individual cells were constantly monitored by phase-contrast microscopy. Cells were fixed and perfused for immunocytochemistry within 1–3 min after colchicine or cold treatment.

Preparation of Antibodies: Affinity-purified rabbit antibodies against dog brain tubulin (32) or dog brain calmodulin (22) were prepared as described. Rabbit antibody against spinach calmodulin was produced as described for vertebrate calmodulin (33), and an IgG fraction was prepared as described (34). Radioimmunoassays were done as described (33). The antibody prepared against spinach calmodulin shows good reactivity with higher plant calmodulins but little, if any, reactivity with vertebrate calmodulins (12). The affinity-purified antibody against vertebrate brain calmodulin (22) reacts with both vertebrate and higher plant calmodulins.

Immunocytochemical Procedures: For light- and electron-microscopic localization of calmodulin, indirect immunofluorescence (27) and immunogold staining (26) procedures were done. After fixation and permeabilization as previously described (26, 27), cells were incubated with 5% (vol/vol) normal goat serum in Tris-buffered saline (TBS)[1] for 20 min, and then incubated with appropriate antibodies either for 4 h at 37°C or overnight at room temperature. For tubulin localization, rabbit antibody to highly purified dog brain tubulin was used at a concentration of 5 μg/ml in 1% (vol/vol) normal goat serum in TBS. For calmodulin localization, rabbit antibodies against spinach calmodulin or dog brain calmodulin were used at concentrations of 10 and 1–2 μg/ml, respectively. After incubation with antibody, cells were washed in TBS containing 0.1% (vol/vol) bovine serum albumin before being processed further for indirect immunofluorescence or immunogold staining. Control experiments were done with preimmune sera.

For indirect immunofluorescence, cells were incubated for 1 h at 37°C with fluorescein-labeled goat anti-rabbit serum added with Evans blue, and then mounted in glycerol as described (27). Observations were made with a Leitz Orthoplan microscope, in epifluorescence, using an HBO 100/W2 lamp as a light source, fluorescein isothiocyanate Ploemopack filters, and a 63X oil fluorescence objective numerical aperture 1.30 (Leitz, Wetzlar, West Germany; Wild, Heerbrugg, Switzerland). Micrographs were taken with a Wild-Leitz Vario-Orthomat camera using Kodak Ektachrome ASA 125, or Tri-X Pan ASA 400 film.

For immunogold staining, cells were incubated for 1 h at room temperature with a preparation of GAR G5 (colloidal gold of 5 nm diam [Janssen Life Sciences Products, Beerse, Belgium] diluted 1/10 in TBS containing 1% (vol/vol) bovine serum albumin and coated with goat anti-rabbit IgG) (32). Cell preparations were washed in TBS containing 0.1% (vol/vol) bovine serum albumin and then fixed, dehydrated, and flat-embedded as previously described (31). Serial sections were made with a Reichert UMU_2 ultramicrotome (Reichert Scientific Instruments, Buffalo, NY) and observed with a Philips 410 electron microscope at 100 kV.

Chlorotetracycline: Chlorotetracycline (CTC) (Fluka, Buchs, Switzerland) was used as a membrane-Ca^{2+} probe (35, 36). It was applied to living mitotic endosperm cells at a concentration of 10 μM for 10–20 min, in the dark, before observation. Observations were made with a low-light video camera (Heinmann RKH85; Wiesbaden, West Germany) mounted on the Leitz MPV_2 Orthoplan microscope equipped with epifluorescence as described above. Leitz filter (513 413), excitation 335–425 nm/emission above 460 nm was used in the Ploemopack system. Pictures were taken directly from the television screen; this permits shorter exposure time and therefore reduces potential light damage to the living cell as well as fading. Tri-X films were used and processed with Diafine.

Purification of Endosperm Calmodulin: White corn (*Zea mays*) was obtained from a local supplier. Endosperm calmodulin was isolated essentially as described for spinach calmodulin (37, 38). In brief, endosperm tissue was removed, ground using a mortar and pestle, and homogenized in 50 mM Tris-HCl, 5 mM EGTA, 1 mM β-mercaptoethanol, 0.5% (wt/vol) polyvinylpolypyrrolidone, pH 7.5. After centrifugation at 10,000 *g* for 30 min, the supernatant was subjected to ammonium sulfate fractionation, and precipitation at pH 4.1 as described (38). Ion-exchange chromatography was done on DEAE cellulose DE-52 resin (Whatman Chemical Separations Inc., Clifton, NJ) equilibrated in column buffer (20 mM Tris-HCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.1 M NaCl, pH 7.5). The sample was applied to the column, and endosperm calmodulin was step-eluted with column buffer containing 0.4

[1] *Abbreviations used in this paper*: CTC, chlorotetracycline; GAR G5, 5-nm-diam colloidal gold coated with goat antibodies to rabbit IgG; STOPs, stable tubule-only polypeptides; TBS, Tris-buffered saline.

M NaCl. After calcium-dependent, affinity-based chromatography over phenothiazine-Sepharose, the final step in the purification of endosperm calmodulin was reverse-phase chromatography as described (37).

RESULTS

Characterization of Endosperm Calmodulin

Calmodulins from dicotyledons (spinach) and monocotyledons (barley) have been shown to be indistinguishable by a number of criteria, including cyclic nucleotide phosphodiesterase activator activity, immunoreactivity, electrophoretic mobility, amino acid composition, peptide maps, and limited amino acid sequence analysis (38, 39). However, calmodulin from endosperm tissue had not previously been characterized. Because of the reported (40–45) alterations in characteristics of calmodulins from certain organisms of divergent phylogenetic groups, including higher plants, and the presence of multiple forms of calmodulin or calmodulin-like proteins in some tissues (46–48), it was important to determine the characteristics of endosperm calmodulin in order to evaluate our immunocytochemical results. Therefore, we purified endosperm calmodulin from a monocotyledon (corn) and characterized the protein in terms of its biochemical and immunochemical properties. Endosperm calmodulin is very similar to other higher plant calmodulins in terms of electrophoretic mobility, phosphodiesterase activator activity, and amino acid composition (data not shown). In addition, as shown in Fig. 1, endosperm calmodulin reacts quantitatively in competition radioimmunoassay with anti-spinach calmodulin antibody that distinguishes higher plant calmodulin from other calmodulins. These data demonstrate that calmodulin is present in endosperm tissue and indicate that the validity of the immunochemical procedures used in this study to detect calmodulin in endosperm cells.

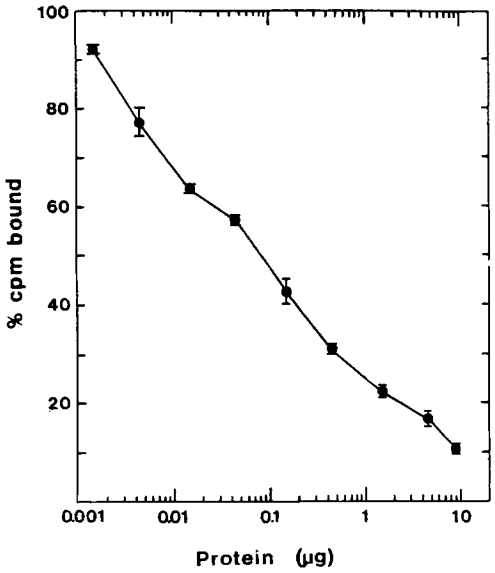

FIGURE 1 Competition radioimmunoassay of endosperm calmodulin. The ability of increasing amounts of purified endosperm calmodulin (●) to compete with a limiting dilution (1:300 final dilution) of anti-spinach calmodulin serum for binding to ^{125}I-labeled spinach calmodulin was examined. The ordinate expresses the percent counts per minute bound, where 100% is the amount bound in the absence of competing protein. Points represent the mean ± range of duplicate determinations.

Localization of Endosperm Calmodulin

We examined the immunocytochemical distribution of calmodulin in endosperm cells during interphase and mitosis by using either affinity-purified antibody against vertebrate calmodulin or an IgG fraction of antibody against spinach calmodulin. Both antibodies gave indistinguishable results. In control experiments, preimmune sera were used and these controls gave no specific immunoreactive product. We also examined the microtubule and calcium distributions by using anti-tubulin immunofluorescence and CTC fluorescence, respectively, and compared these with the calmodulin distribution.

Interphase

As described before (26, 27), interphase cells contain elaborate, randomly distributed microtubule arrays radiating from the surface of the nucleus toward the cell membrane. In interphase cells, calmodulin shows a diffuse, homogeneous localization throughout the cytoplasm.

Interphase–Prophase Transition

At the onset of prophase, the microtubule distribution begins to rearrange from the uniform distribution seen in interphase cells. As the chromosomes begin to condense, microtubule density increases around the nucleus. The microtubules begin to form two or three transitory converging centers, two of which will correspond to the future polar regions of the spindle. Close to the nuclear surface, microtubules tend to arrange parallel to this spindle axis (Fig. 2*A*).

As prophase develops, the microtubule converging centers become more defined and pointed due to the increasingly closer association of microtubules within the converging arrays. If there are more than two converging centers, they fuse into two dominant centers on opposite sides of the nucleus (Fig. 2*B*). On the cytoplasmic periphery only few microtubules remain. When the nuclear envelope begins to break down, some microtubules form thin distinct bundles that may contribute to the formation of kinetochore bundles, while the nucleus zone is still surrounded by the mantle of oriented microtubules. During these microtubule rearrangements, the calmodulin distribution also becomes more regular and is concentrated at these regions where microtubule asterlike converging centers develop (Fig. 2*C*).

Prophase-Metaphase

During the progression of prometaphase, when chromosomes move individually to the equator plane, the spindle shape gradually changes. The polar region is no longer pointed but becomes diffuse and not well defined. Multiple subpoles are distinguished and each of them results from the convergence of two or three kinetochore bundles intermingling with nonkinetochore microtubules that extend from one polar region to the other (Fig. 3*A*).

The distribution of calmodulin during the establishment of metaphase coincides with the polar converging centers and, later, with the kinetochore microtubule bundles (Fig. 3*B*). The calmodulin associated with the asterlike converging centers reorganizes into a bipolar spindlelike arrangement. The staining is most intense at the diffuse spindle poles and gives the appearance of thin, inverted conelike fibers toward the equatorial plane. This calmodulin distribution is similar to

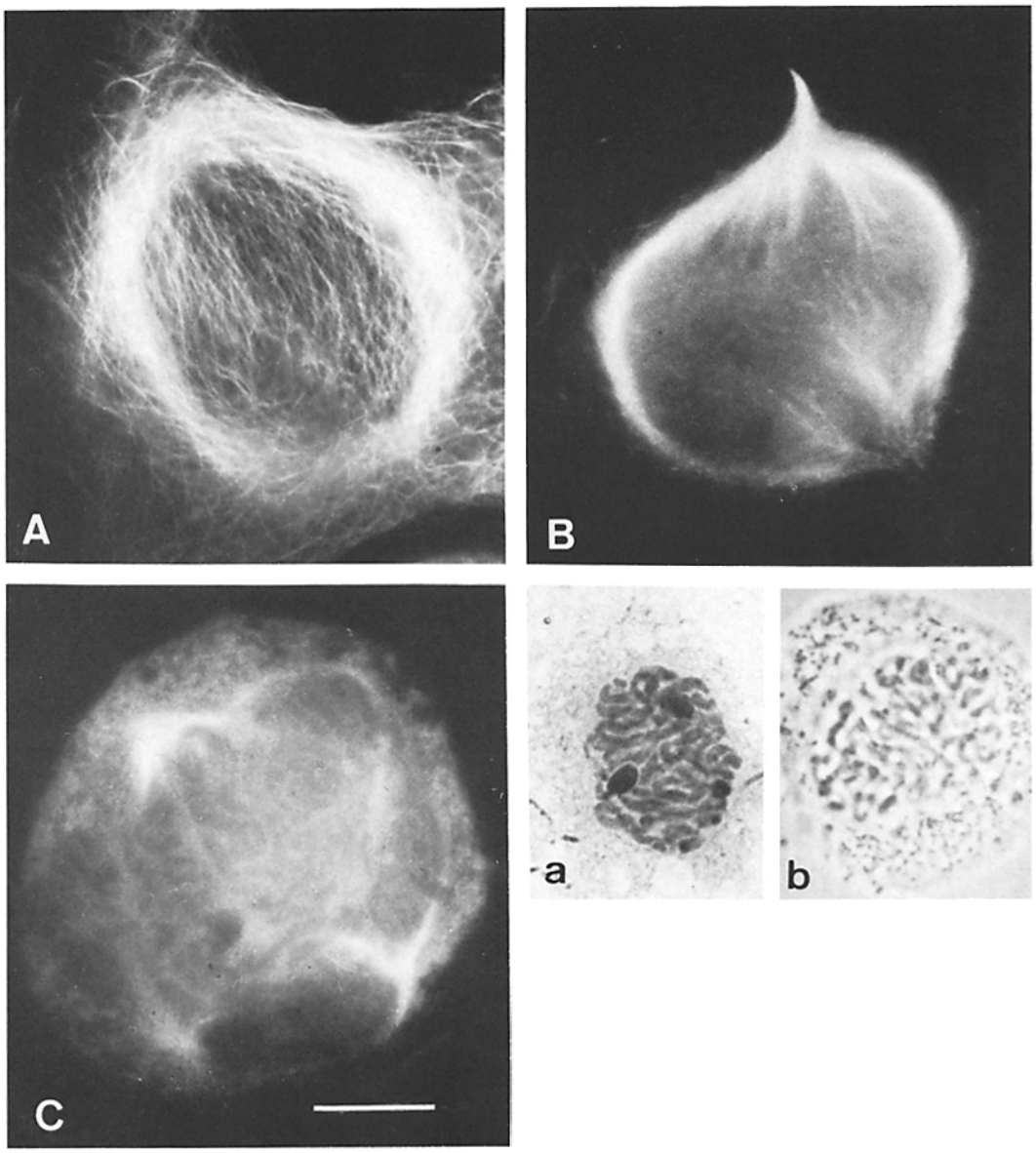

FIGURE 2 Tubulin (*A* and *B*) and calmodulin (*C*) immunofluorescence during prophase progression in acentriolar mitotic cells of *Haemanthus katherinae*. (*A*) Early prophase. The cytoplasmic interphase microtubule meshwork is transformed. Microtubule density is increased around the nucleus and transitory microtubule converging centers appear on opposite sides of the nucleus and form the future spindle poles. On the nuclear surface, microtubules tend to align parallel to this predominant axis. (*a*) The cell in phase contrast. (*B*) Late prophase when the nuclear envelope starts to break. The prophase spindle is formed around the nucleus. In the periphery, the cytoplasm is devoid of microtubules. The poles are well defined and pointed. Thin microtubule bundles form and will contribute to kinetochore fibers formation. (*b*) The cell in phase contrast. (*C*) Calmodulin distribution in late prophase. The calmodulin distribution is similar to the localization of the microtubule converging centers, i.e., the future spindle poles. (*A*, *B*, and *C*) Bar, 10 μm. × 1,600.

the distribution of kinetochore microtubule fibers, with almost no calmodulin detected between neighboring kinetochore bundles.

Anaphase

At anaphase, the microtubule distribution undergoes a drastic change as described (26). The kinetochore fibers begin to shorten and the number of microtubules at the equatorial region of the interzone decreases. Nonkinetochore microtubules form distinct bundles in the interzone. The microtubules converge to form more well-defined polar regions. In later anaphase when kinetochores are close to the poles, new microtubules assemble at the polar regions and radiate into the interzonal region (Fig. 4*A*). This polar microtubule population will participate in phragmoplast formation.

During all stages of anaphase, calmodulin is localized in the half-spindles on the polar sides of the kinetochores (Fig. 4*B*). Little if any calmodulin is seen in the interzone region. As the kinetochore fibers begin to shorten and converge to the poles, calmodulin shows a similar rearrangement with a localization that is concentrated at the polar regions, perhaps in association with the polar ends of kinetochore microtubules.

Telophase-Interphase

The phragmoplast that develops during this stage is com-

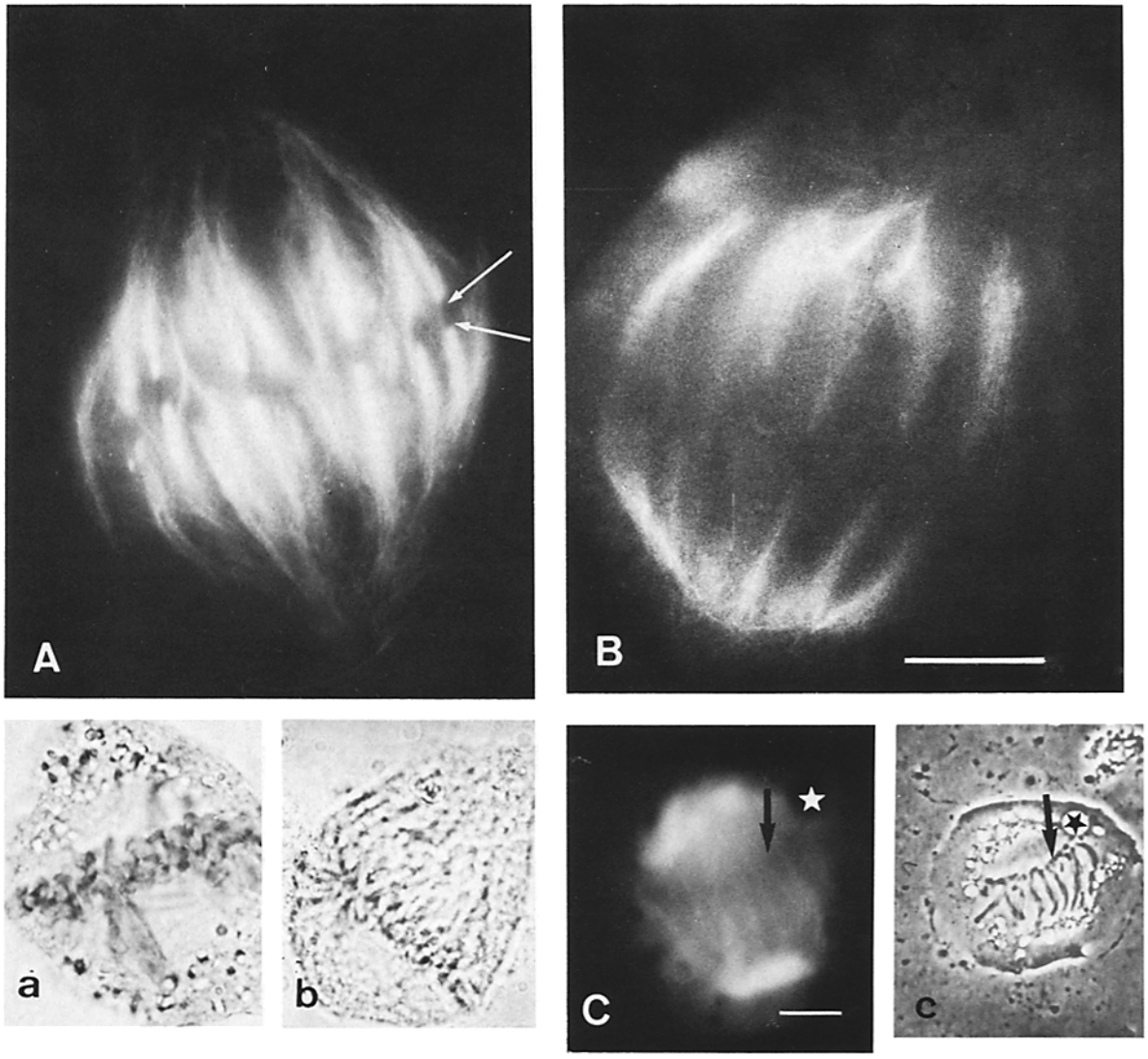

FIGURE 3 Tubulin (*A*) and calmodulin (*B*) immunofluorescence and CTC fluorescence (C) during metaphase. (*A*) Metaphasic spindle with kinetochore bundles and nonkinetochore arrays which cross the equatorial plane. The polar regions are diffuse. Neighboring kinetochore fibers tend to converge and form subpoles. White arrows indicate two sister kinetochores. (*a*) The cell in phase contrast. (*B*) Calmodulin distribution is intense at the polar regions and forms an inverted conelike pattern similar to the distribution of kinetochore bundles. (*b*) The cell in phase contrast. (*A* and *B*) Bar, 10 μm. × 1,600. (*C*) CTC fluorescence and phase contrast (*c*) of the same metaphase cell. Black arrows indicate the location of kinetochores at the equatorial plane. Star: one vacuole. CTC fluorescence is dense at the polar regions where membrane systems (mitochondria, endoplasmic reticulum) are accumulated. Diffuse fluorescence is observed in the spindle region. The upper half-spindle of the cell (*c*) is devoid of chromosome arms and therefore permits visualization of thin inverted conelike distribution of CTC fluorescence that corresponds to the localization of kinetochore bundles. The tips of these cones appear to end at kinetochores (compare black arrow in *C* with that in *c*). A comparison between *B* and *C* shows that calmodulin distribution and CTC fluorescence are similar. (*C*) Bar, 10 μm. × 900.

posed of nonkinetochore, polar microtubules that have assembled in late anaphase and radiated in an asterlike fashion toward the interzone, remnants of nonkinetochore microtubules, and microtubules that possibly arise at the cell plate (26, 27). The interzone region where the phragmoplast forms becomes filled with bundles of microtubules, and the interphase, cytoplasmic microtubule network of the daughter cells forms around the telophasic chromosomes and later the daughter nuclei (Fig. 5*A*).

As in the other stages of mitosis, the calmodulin localization during telophase is similar to the localization of kinetochore microtubules. Only the polar regions that contain remnants of kinetochore microtubules are intensely stained, whereas no calmodulin staining is observed in association with the interzonal microtubules (Fig. 5*B*).

Association of Calmodulin with Kinetochore Microtubules

To determine whether the calmodulin localization was restricted to the kinetochore microtubules, we compared the distribution of calmodulin in untreated cells with the tubulin pattern in cells subjected to experimental conditions that induce selective disassembly of nonkinetochore microtubules. These conditions were treatment with colchicine (8×10^{-5} M) for 3 to 5 min or with cold (3°C) for 3 min as described

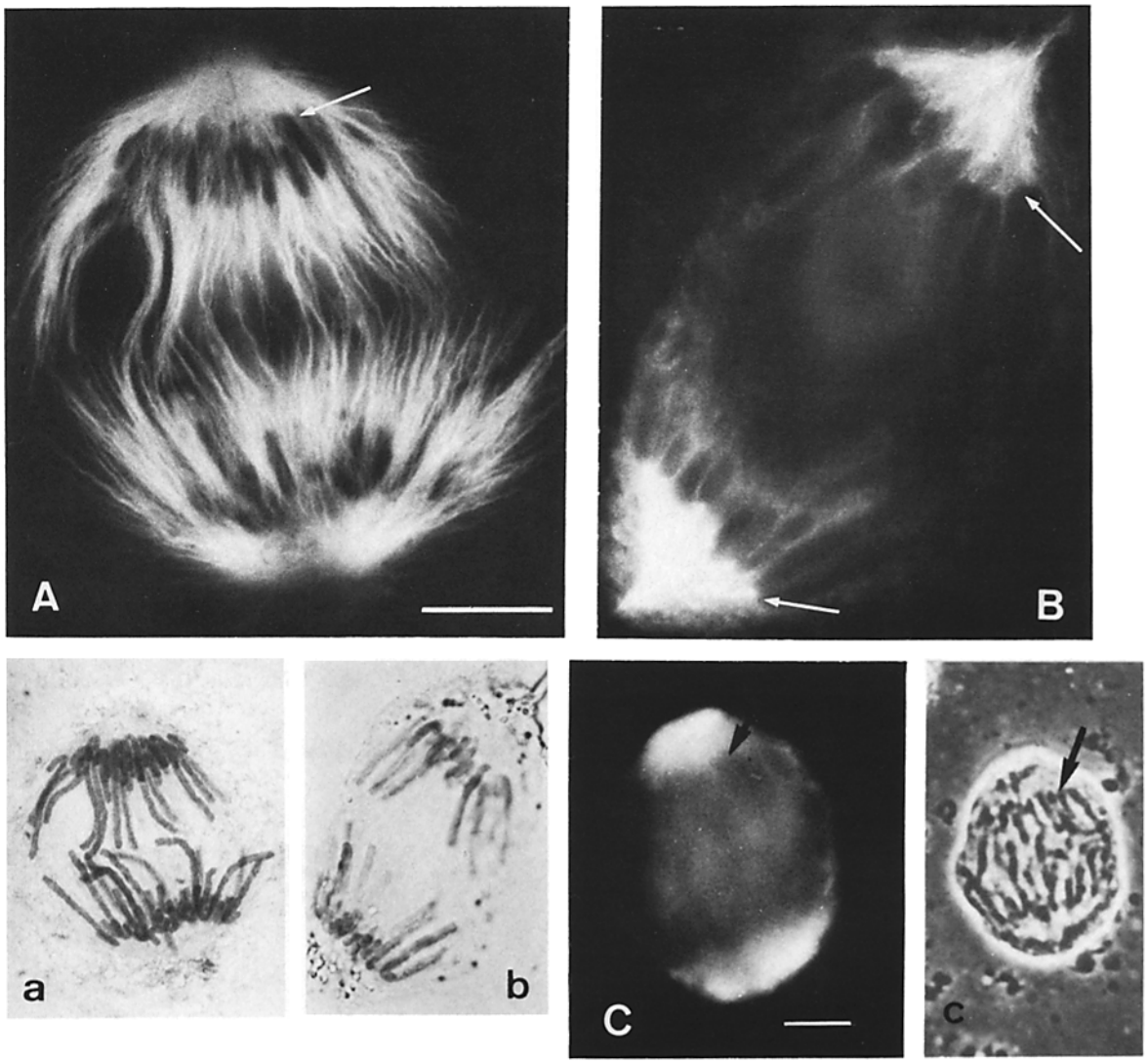

FIGURE 4 Tubulin (*A*) and calmodulin (*B*) immunofluorescence and CTC fluorescence (*C*) during late anaphase. White arrows in *A* and *B* indicate location of kinetochores. (*A*) Short kinetochore fibers remain while chromosomes are near the polar regions. The interzone is invaded with new polar microtubules that assemble in late anaphase and elongate toward the equator. Microtubule density behind the kinetochores increases enormously and remnants of nonkinetochore bundles are mixed with polar microtubules. Chromosomes stained with Evans blue are visible in phase contrast (*a* and *b*). (*B*) Calmodulin pattern exhibits a conelike distribution and is restricted to the half-spindle in front of kinetochores. No calmodulin fluorescence is detected in the interzone. (*A* and *B*) Bar, 10 μm. × 1,600. (*C*) CTC fluorescence and phase-contrast (*c*) micrographs of the same living cell at a similar stage of anaphase. CTC fluorescence is intense in the half-spindles between kinetochores (black arrows) and the poles, and the intensity is highest at the polar regions. In the interzone, diffuse fluorescence is present both between chromosome arms and at the equatorial region. A comparison between *B* and *C* suggests strongly that calmodulin distribution is directly related to CTC fluorescent regions. (*C*) Bar, 10 μm. × 900.

in Materials and Methods. Under these conditions, the kinetochore microtubules are relatively stable, whereas the nonkinetochore and polar microtubules rapidly disassemble. Fig. 6, *A* and *B*, illustrates the calmodulin pattern in normal cells just at the moment of kinetochore splitting, i.e., the onset of anaphase. Calmodulin fluorescence is restricted to areas analogous to areas of kinetochore bundle that remain after cold treatment (Fig. 7). However, calmodulin distribution is always denser at the polar area. Similar observations are made in late anaphase (Fig. 8) where normal calmodulin distribution (Fig. 8 *B*) is comparable to the remaining kinetochore bundles after colchicine treatment (Fig. 8 *A*).

We also studied the calmodulin distribution in cells pretreated with colchicine or cold. We found that after treatment with colchicine (Fig. 9) or cold (data not shown), the pattern of calmodulin localization remained essentially the same as before treatment (compare Fig. 8 *B*), being very similar to the kinetochore microtubule localization. Although these results do not rule out the possibility that calmodulin is resistant to release from nonmicrotubule binding sites during these treatments, they are also consistent with the possibility that calmodulin is associated specifically with the kinetochore microtubule fibers.

To try to determine more precisely the localization of calmodulin, we examined cells by electron microscopy and immunogold staining procedures as described in Materials

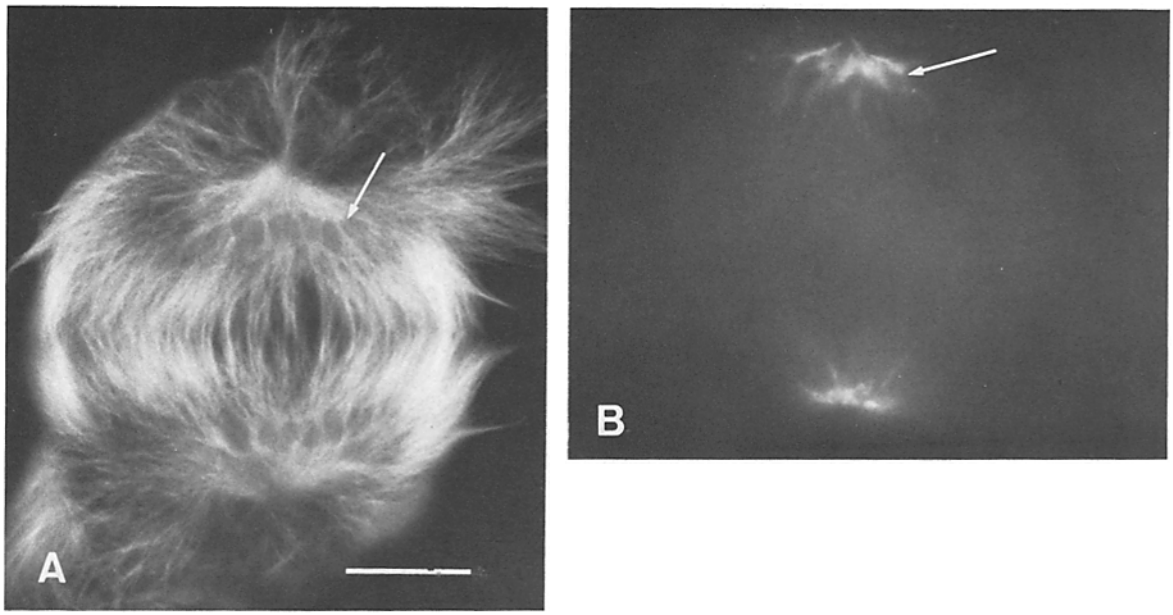

FIGURE 5 Tubulin (*A*) and calmodulin (*B*) immunofluorescence during early telophase. White arrows indicate location of kinetochores. (*A*) Polar microtubules assembled in late anaphase radiate in an asterlike fashion at the polar regions and will contribute to the formation of the future interphase meshwork. The interzone is entirely invaded with microtubules, mostly of polar origin, which form the phragmoplast. Short kinetochore microtubules are still present. (*B*) Anti-calmodulin staining at the same stage. The fluorescence is restricted to the localization of the remnants of kinetochore microtubules at the polar regions. Bar, 10 μm. × 1,600.

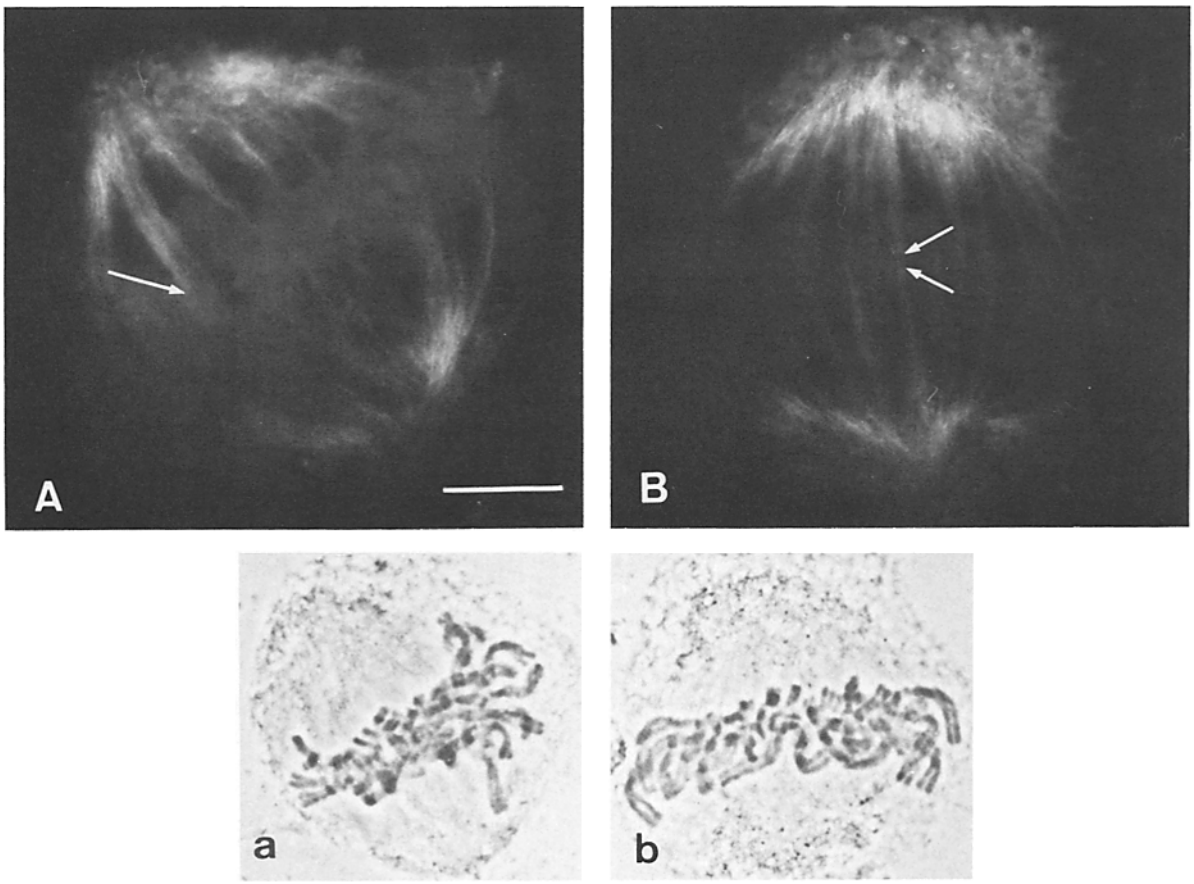

FIGURE 6 Calmodulin distribution at the start of anaphase, during kinetochore splitting (the cells in phase contrast are in *a* and *b*). White arrows indicate location of kinetochores. Calmodulin fluorescence is superimposed on kinetochore bundles and can be followed up to the kinetochore. Fluorescence density is higher at the poles. The fluorescence pattern is indistinguishable using either plant calmodulin (spinach) antibodies (*A*) or vertebrate (dog brain) calmodulin antibodies (*B*). (*A* and *B*) Bar, 10 μm. × 1,600.

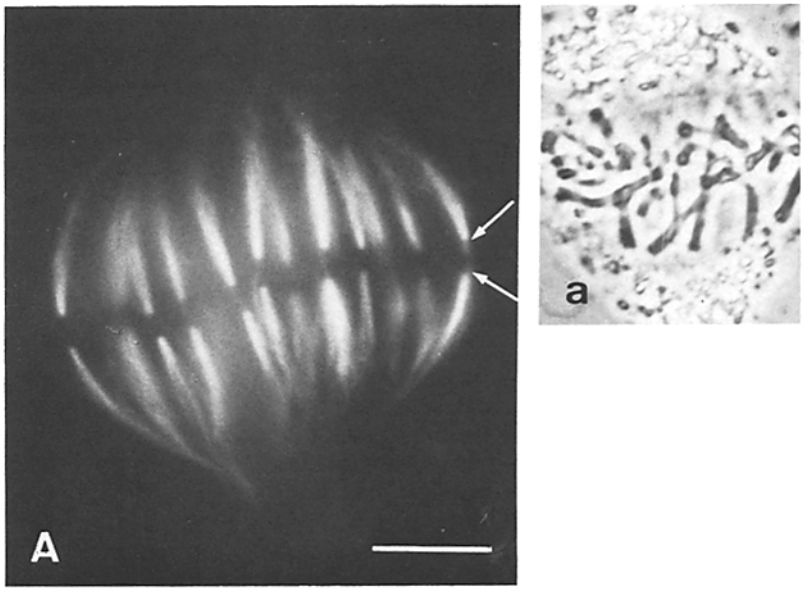

FIGURE 7 Anti-tubulin staining of a cold-treated metaphase cell. White arrows indicate location of kinetochores. Nonkinetochore microtubules rapidly disassemble while kinetochore microtubules remain as cold-stable bundles. Comparison with Fig. 6 indicates that calmodulin localization in untreated cells is essentially the same as the distribution of kinetochore fibers. Bar, 10 μm. × 1,600.

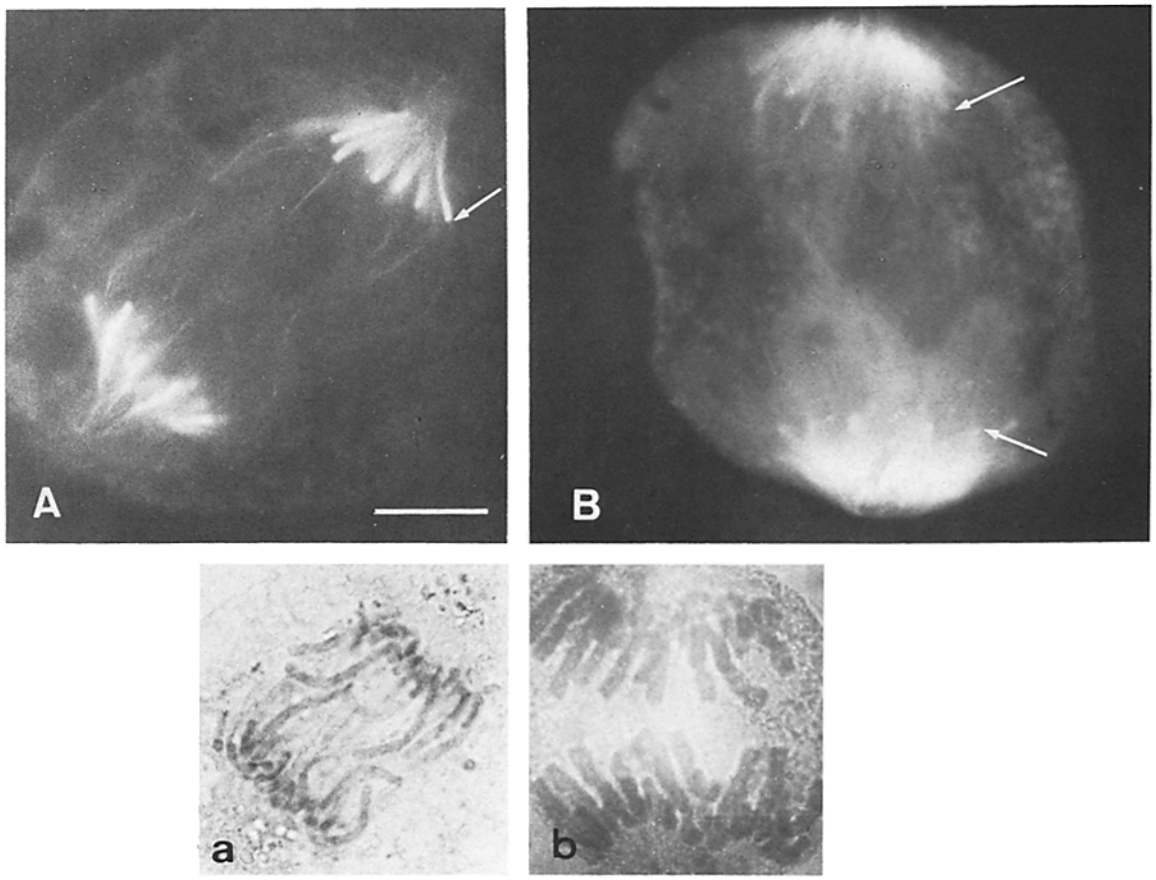

FIGURE 8 Comparison between tubulin immunofluorescence of a colchicine-treated cell (*A*) and calmodulin localization at a similar stage of chromosome movement in an untreated cell (*B*). White arrows indicate location of kinetochores. (*A*) After 5 min of colchicine perfusion, kinetochore microtubules remain selectively and nonkinetochore microtubules are almost gone in the interzone. Chromosome movement is arrested as controlled in vivo before fixation. (*a*) The cell in light microscopy. (*B*) Untreated cell stained with anti-calmodulin antibodies at similar mitotic stage. (*b*) The cell in light microscopy. Comparison with *A* shows that calmodulin localization is similar to the distribution of kinetochore bundles. Higher density of calmodulin fluorescence is observed at the polar regions. Bar, 10 μm. × 1,600.

and Methods. Serial sections of anaphase cell preparations were analyzed for calmodulin localization. As shown in Fig. 10, we detected gold particles near microtubules, although we could not distinguish precisely where the calmodulin was binding. Some microtubule fibers were not labeled, which suggests that calmodulin shows a specificity for certain microtubule fibers. Fig. 11 illustrates the distribution of gold particles (5 nm) in the half-anaphase spindle and shows the increase of density from the kinetochore towards the polar region. Gold particles were counted in consecutive areas of 3.75 μm^2. These data are consistent with the results obtained with immunofluorescence localization of calmodulin.

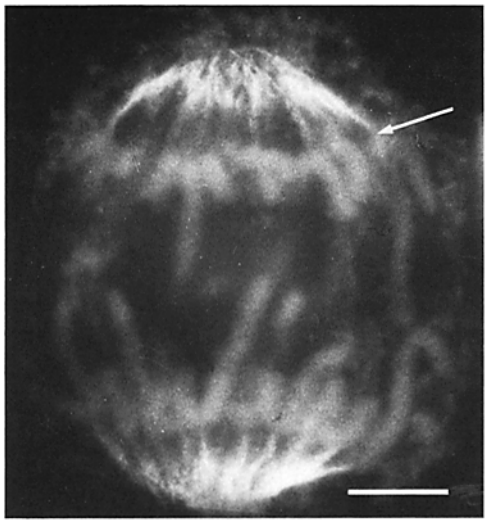

FIGURE 9 Calmodulin fluorescence pattern in an anaphase cell pretreated with colchicine. White arrow indicates location of kinetochores. Cells were fixed 5 min after colchicine perfusion, when chromosome movements were blocked. In such a cell, only kinetochore microtubules remain. The calmodulin pattern is comparable to that of a normal untreated cell (compare Fig. 8 *B*), which suggests therefore that it is directly related to the distribution of kinetochore microtubules. Bar, 10 μm. × 1,600.

Co-localization of Calcium and Calmodulin

We examined the distribution of calcium in endosperm cells by using the fluorescent probe CTC. CTC was used in earlier studies (28, 29) to localize membrane-associated calcium in mitotic endosperm cells, and we obtained similar results. The previous reports demonstrated a calcium localization in the chromosome-to-pole region of the mitotic apparatus that appears similar to the pattern of calmodulin localization we observed above for mitotic endosperm cells. It was of interest, therefore, to directly determine whether the distributions of calcium and calmodulin are coincident during mitosis. We found that the pattern of CTC fluorescence clearly coincides with the immunocytochemical localization of calmodulin (Figs. 3*C* and 4*C*).

DISCUSSION

We have shown here that (*a*) calmodulin is present in endosperm tissue and is biochemically similar to other higher plant calmodulins; (*b*) endosperm calmodulin is associated with the mitotic apparatus, particularly with the asterlike microtubule converging centers and with kinetochore microtubules, in an immunocytochemical pattern distinct from that of tubulin; and (*c*) endosperm calmodulin and calcium show analogous distribution profiles during mitosis. Fig. 12 is a schematic drawing that summarizes the association of calmodulin with kinetochore microtubules and the dynamic changes in calmodulin distribution in the acentriolar mitotic spindle of endosperm cells.

The demonstration that calmodulin isolated from higher plant endosperm tissue is similar in its biochemical properties and immunochemical characteristics to other higher plant calmodulins indicated the validity of using endosperm cells

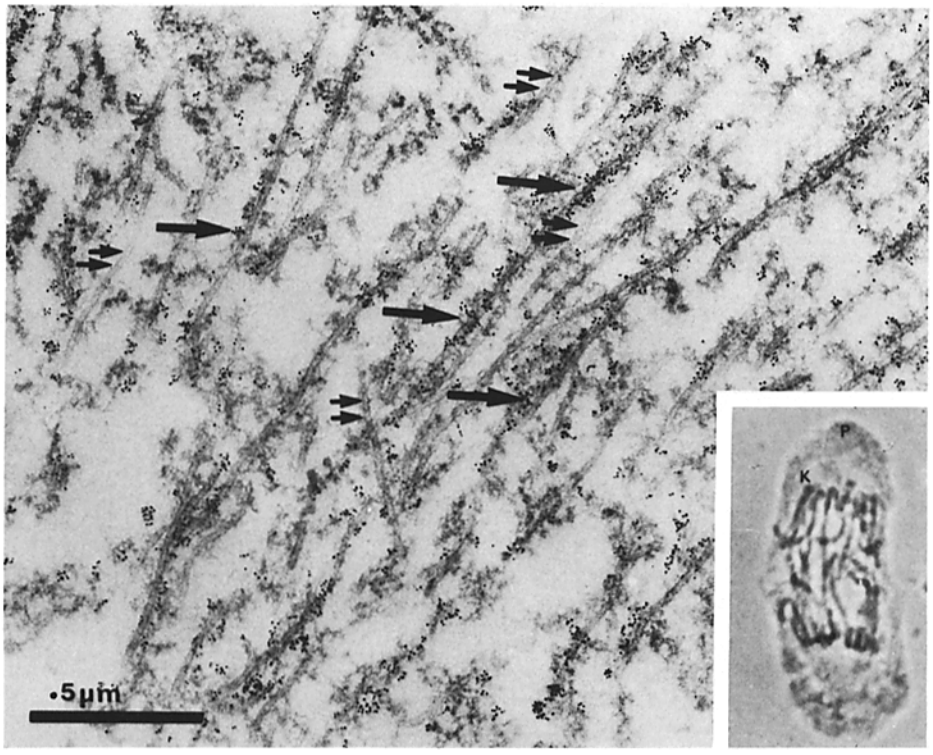

FIGURE 10 Thin section of an anaphase cell. *Inset*: the cell in plastic. Detail of the half spindle in front of kinetochore region. The cell was fixed as described in Materials and Methods, treated with anti-calmodulin antibodies, and immunogold stained using colloidal gold 5 nm, GAR G5. Gold particles are detected in the vicinity of microtubules (thick arrows) and often exhibit a periodic pattern. Some microtubules (double small arrows) are not labeled, which suggests that calmodulin shows a specificity for certain microtubules (kinetochore bundles). × 48,000.

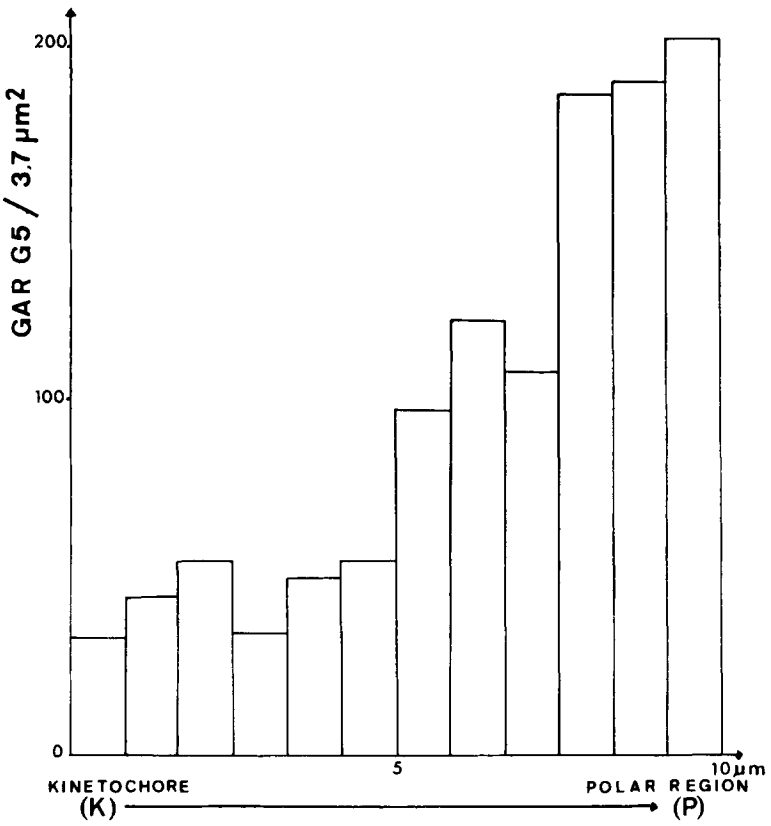

FIGURE 11 Distribution of gold particles in the half spindle of the anaphase cell shown in Fig. 10. 5-nm gold particles were counted per 3.75 μm^2, from the kinetochore region toward the poles. A few micrometers after the kinetochores the density of gold particles increases rapidly and may be correlated with the convergence of kinetochore fibers nearer the polar regions. At the poles, the density is the highest as seen qualitatively with immunofluorescence staining.

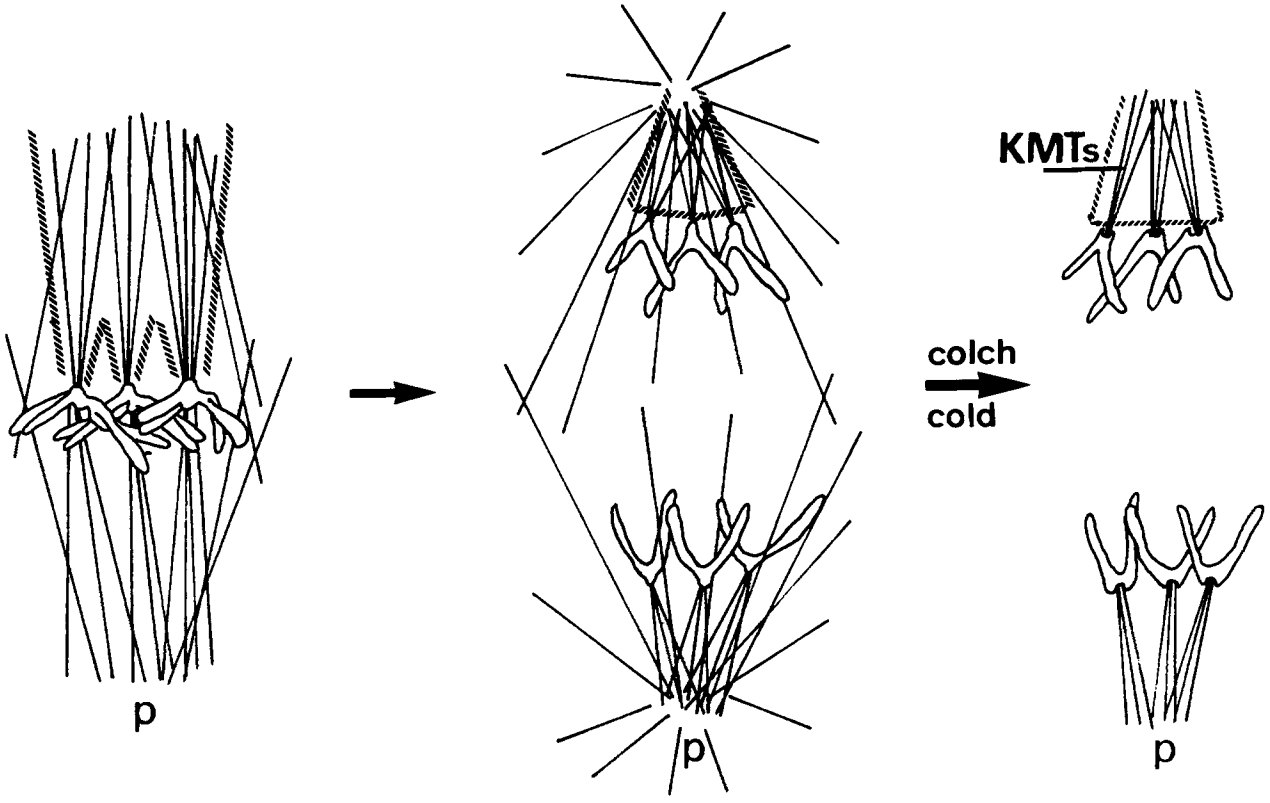

FIGURE 12 Schematic interpretation of the dynamic changes in calmodulin distribution in the acentriolar mitotic spindle of higher plant cells during metaphase-anaphase chromosome movements. Comparison of untreated and cold- or colchicine-treated cells shows that the calmodulin distribution is coincident with the distribution of kinetochore microtubule bundles, as the pattern is comparable after nonkinetochore microtubules are selectively disassembled. Changes in calmodulin distribution are directly related to the shortening of kinetochore microtubules and not to the dynamics of nonkinetochore or polar microtubules. Our data suggest that in higher plant cells calmodulin is involved in the regulation of kinetochore microtubule dynamics. *p*, poles; KMTs, kinetochore microtubules; colch, colchicine.

to examine the immunocytochemical distribution of higher plant calmodulin. Endosperm cells are particularly useful for immunocytochemical procedures. These cells lack a cell wall and thus allow antibody penetration without enzyme pretreatment that may induce changes in antigen distribution. In addition, their largeness permits analysis of the entire microtubule population at the light-microscopy level. As with any immunocytochemical procedure, there is the possibility of artifactual localizations due to nonspecific staining, antigenic masking, or redistribution of antigen upon processing. However, we found no specific staining for calmodulin with preimmune sera; we obtained indistinguishable calmodulin localization with two different anti-calmodulin antibodies prepared against calmodulins from vertebrate and plant origin; and, as noted above, we tried to avoid treatments that might induce redistribution.

Within the limitations of immunocytochemistry, the distribution of endosperm calmodulin we obtained is in some aspects strikingly similar to the calmodulin distribution seen by others (18–23) using a variety of animal cells. For example, endosperm calmodulin shows a diffuse, apparently random distribution throughout the cytoplasm of interphase cells. In addition, during mitosis, endosperm calmodulin is associated with the mitotic apparatus and is concentrated in the regions of kinetochore microtubule fiber activity.

However, there are certain differences in the localization of calmodulin in higher plant and animal cells. First, animal cell calmodulin is concentrated at the centriolar and pericentriolar regions and on radiating spindle microtubules during mitosis, and speculations have therefore arisen that calmodulin may play a role in regulation of spindle assembly and disassembly. In most higher plant cells, spindle development occur without centrioles. We previously identified (26, 27) asterlike microtubule converging centers that appear to be directly involved in establishing spindle polarity in endosperm cells. We have shown here that calmodulin is associated with these microtubule converging centers during prophase and with the diffuse spindle poles during metaphase. Thus, even though endosperm calmodulin is concentrated at microtubule converging centers instead of at centriolar and pericentriolar regions as in animal cells, these data are consistent with the hypothesis that calmodulin may regulate spindle assembly/disassembly in acentriolar endosperm cells.

A second difference in the localization of calmodulin in higher plant and animal cells is seen at later stages of mitosis. During late anaphase-early telophase in animal cells, calmodulin distribution shifts from the pericentriolar region to the interzonal region, localizing in distinct areas on the distal ends of the midbody (18–21). In contrast, endosperm calmodulin is not seen in the interzonal region in late mitosis but instead remains associated with the kinetochore microtubule fibers at the polar regions. There was no apparent localization of calmodulin coincident with the interzonal microtubule population that is involved in phragmoplast formation. These data suggest a specialized role for endosperm calmodulin in the dynamic equilibrium of kinetochore microtubules and anaphase chromosome movement.

The observation that the distribution of endosperm calmodulin is similar to that of kinetochore microtubules during mitosis suggests that calmodulin may regulate assembly/disassembly of kinetochore microtubules, as has been postulated for calmodulin in animal cells (16, 19). However, the molecular targets and mechanism of this regulation are not well defined. The binding of calmodulin directly to the tubulin dimer has not been clearly demonstrated. It is possible that calmodulin may regulate microtubule disassembly through its interaction with microtubule-associated proteins. For example, calmodulin has been reported to bind to high molecular weight microtubule-associated proteins (49), and the presence of microtubule-associated proteins can affect the depolymerization activity of calmodulin in vitro (4, 8, 50). It has also been shown (16) that calmodulin will enhance the calcium sensitivity of a subpopulation of vertebrate brain microtubules, the cold-stable microtubules, possibly through interaction with microtubule-associated polypeptides referred to as stable tubule-only polypeptides (STOPs). STOPs are tubulin- and calmodulin-binding proteins (51) that have been reported (52) to act on microtubules in concentrations substoichiometric to that of tubulin to render the microtubules stable to cold or high calcium concentrations. The stable state can be rapidly reversed by the presence of calmodulin or by calmodulin-dependent or -independent phosphorylation reactions (16, 51, 52). Because kinetochore microtubules are of the cold-stable type and because of our observations that calmodulin is localized near the kinetochore microtubule fibers, especially at the polar region, during mitosis in endosperm cells, it is attractive to speculate that calmodulin may regulate microtubule dynamics and anaphase chromosome movement in higher plant cells through interaction with STOPs at specific disassembly sites. However, the presence of calmodulin-binding STOPs in plant cells or calmodulin-dependent kinases that can phosphorylate STOPs has not been demonstrated yet.

In addition to the lack of definition of calmodulin-binding target proteins in the mitotic apparatus of plant cells, the molecular signals that induce calmodulin action are unknown. Our observations of the co-localization of calcium fluorescence and calmodulin during mitosis of endosperm cells suggest that fluxes in intracellular calcium concentration may be such a molecular signal. It has been observed (29) that there is a reduction in membrane-associated calcium at the onset of anaphase in endosperm cells, which suggests that an efflux of calcium from sequestered stores may trigger anaphase chromosome movement. A localized increase in calcium concentration in the particular microenvironment of kinetochore microtubules and subsequent calcium–calmodulin effects of kinetochore microtubule disassembly would allow spatially limited control of microtubule equilibrium. The overall low calcium concentration around nonkinetochore microtubules would tend to stabilize and permit assembly of these microtubules. Such a model is consistent with our observations that calmodulin is associated with kinetochore microtubules in endosperm cells, and that disassembly of kinetochore microtubules during anaphase is accompanied by concomitant assembly of nonkinetochore, polar microtubules. A recent interesting suggestion (53) is that calmodulin associates with mammalian microtubules in a calcium-independent manner but affects microtubule disassembly only in the presence of micromolar calcium. If such a situation exists in plant cells, calmodulin may interact with microtubules without necessarily effecting depolymerization until triggered by localized calcium fluxes.

In summary, we have determined the immunocytochemical localization of calmodulin in acentriolar higher plant cells and have shown that endosperm calmodulin, like animal cell calmodulin, is associated with the mitotic apparatus. These

data suggest that some of the regulatory mechanisms operating on microtubules from animal and plant cells may be similar. However, the observation that calmodulin associates with microtubules that form the kinetochore fibers in endosperm cells throughout mitosis also suggests a specialized role for higher plant calmodulin in the regulation of kinetochore microtubule dynamics. Although the molecular basis of the calcium-dependent regulation of microtubule disassembly and chromosome movement is not understood, our results are consistent with the hypothesis that calmodulin may be involved in this regulation.

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