Detection of the Membrane-Calcium Distribution during Mitosis in *Haemanthus* Endosperm with Chlorotetracycline

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ABSTRACT The distribution of membrane-associated calcium has been determined at various stages of mitosis in *Haemanthus* endosperm cells with the fluorescent chelate probe chlorotetracycline (CTC). CTC fluorescence in *Haemanthus* has two components: punctate, because of mitochondrial and plastid membrane-Ca^{++}; and diffuse, primarily because of Ca^{++} associated with endoplasmic reticulum membranes. Punctate fluorescence assumes a polar distribution throughout mitosis. Cones of diffuse fluorescence in the chromosome-to-pole regions of the metaphase spindle appear to coincide with the kinetochore fibers; during anaphase, the cones of fluorescence coalesce and this region of the spindle exhibits uniform diffuse fluorescence. Perturbation of the cellular Ca^{++} distribution by treatment with lanthanum, procaine, or EGTA results in a loss of diffuse fluorescence with no accompanying change in the intensity of punctate fluorescence. Detergent extraction of cellular membranes causes a total elimination of CTC fluorescence. CTC fluorescence of freshly teased crayfish claw muscle sarcoplasmic reticulum coincides with the A bands and is reduced by perfusion with lanthanum, procaine, and EGTA in a manner similar to that for diffuse fluorescence in the endosperm cells. These results are consistent with the hypothesis that a membrane system in the chromosome-to-pole region of the mitotic apparatus functions in the localized release of sequestered Ca^{++}, thereby regulating the mechanochemical events of mitosis.

The formation and operation of the mitotic apparatus implies the presence of a system to regulate the assembly and function of motile elements responsible for chromosome movement. Although great effort has been directed toward determining the distribution and chemical nature of motile elements of the mitotic apparatus, such as tubulin (20, 31, 39) and actin (21, 22), the distribution and functions of membranes in the spindle have received little attention until recently (25–28).

One of the plausible roles for a membrane system in the mitotic apparatus is the sequestration and localized release of calcium (26, 27). Calcium exerts profound effects on the stability of spindle microtubules both in vivo (32, 33) and in vitro (40), as well as on chromosome displacement in vitro (40), an effect that may be important in the regulation of mitotic events. Recent studies have demonstrated the redistribution of cytoplasmic membranes (the nuclear envelope-endoplasmic reticulum [NE-ER] complex) during mitosis in fixed cells of barley and the association of membranes with kinetochore fibers (28). Ca^{++}-sequestering capabilities of spindle membranes, from isolated sea urchin mitotic apparatus, are increased by ATP (41–43), and it is interesting that the distribution of these vesicles parallels that observed in fixed cells (28).

In this paper we provide evidence for changes in the distribution of membrane-associated calcium in the mitotic apparatus of living endosperm cells of *Haemanthus katherinae* Baker using the fluorescent chelate probe chlorotetracycline (CTC). CTC has been used to localize membrane-associated Ca^{++} in a variety of cells and isolated organelles (3, 10–16, 19, 23, 36). It is nontoxic when applied in concentrations <100 μM (11, 15), and freely passes through membranes, thereby entering all cytoplasmic compartments (11, 16, 23). Complexed with a diamagnetic ion (Mg^{++} or Ca^{++}) at physiological pH, the probe emits maximal fluorescence in an apolar environment (i.e., membrane) when stimulated by blue excitation light (12–14).

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Both the emission and excitation spectra for fluorescence are characteristic for the cation bound, and Ca^{++} binding can be distinguished from Mg^{++} binding to CTC by fluorometric techniques (11). The intensity of fluorescence by Ca-CTC in an aqueous environment is increased approximately fivefold in an apolar environment (23), whereas, Mg-CTC exhibits only slight augmentation of fluorescence emission intensity (13, 14, 23). Spectral elimination of a portion of Mg^{++}-based emission through longer wavelength cutoff barrier filter combinations (1) coupled with irradiation at longer wavelengths minimizes Mg-CTC excitation (15, 16) and results in fluorescence patterns caused largely by Ca^{++} binding to the probe in apolar environments (11, 15, 16). Perturbations of the Ca^{++} distribution in the cells caused by agents such as lanthanum, procaine, and EGTA provide further evidence for Ca^{++} binding by the probe (11); changes in fluorescence resulting from these treatments permit comparisons of membrane-associated Ca^{++} in the dividing endosperm cell and a known Ca^{++}-sequestering system, the sarcoplasmic reticulum. Preliminary reports of this study have appeared in abstract form (46, 47).

MATERIALS AND METHODS

Chemicals

CTC, oxytetracycline (OTC), procaine, dinitrophenol (DNP), carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), EGTA, poly D-L-lysine, and Ficoll 400 were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Cells

Endosperm cells were expressed from immature seeds of *Haemanthus* as described by Jackson (30) onto glass microscope slides precoated with 0.1-1% Ficoll 400 according to the methods described by Hard and Allen (24). The seeds were obtained from plants growing in the Murdough Experimental Greenhouse at Dartmouth College, Hanover, N. H. (30).

To the cell suspension from one to two seeds (having a volume of 25–50 μl), 1–5 μl of freshly prepared unbuffered 3.5% glucose with 200 μM CTC was added, yielding a final concentration of 10–15 μM CTC. The cover glass, precoated with either 0.1-1% Ficoll 400, or 0.25 mg/ml poly D-L-lysine buffered with phosphate to pH 7.0 (48), was gently placed on the cell suspension. Small dabs of Vaseline, placed at the corners of the cover glass, served as spacers and alleviated excess pressure on the cells. The cover glasses were not sealed to the slides for these experiments. CTC fluorescence was a function of cell thickness; very thin cells generally had lower levels of fluorescence than did thicker cells. We believe that this is because of the greater superposition of cytoplasmic membranes (source of CTC fluorescence) in thicker cells. Cells were incubated in CTC at least 20 min before observation to assure that the probe had entered all cytoplasmic compartments (15, 16).

Fresh claw muscle from the crayfish (*Cambarus* spp.) was dissected into phosphate-buffered saline (PBS; 0.145 M NaCl, 0.01 M NaH_2PO_4/Na_2HPO_4, pH 7.1) and placed on ice. The muscle tissue was teased apart and incubated for at least 20 min with 20–50 μM CTC before microscope observation (15, 16).

Microscopy

Fluorescence was monitored with either Zeiss or Leitz epifluorescence optics using a × 40 objective lens (NA 0.65). CTC fluorescence was induced by broadband blue excitation light (BG-12 filters; peak approximately at 410 nm; a 400-nm dichroic beam splitter and a red-suppressor filter, BG-38, were also included) from a mercury arc lamp and emission was limited by a 520-nm barrier filter on the Leitz microscope. The cells were photographed on Kodak Tri-X film processed in Diafine (Acufine, Inc. , Chicago, Ill.) with an effective ASA of 1,600. Cells were photographed within 10 s of initial illumination with blue excitation light. The duration of photographic exposure was <15 s.

Fluorometric Measurements

Microfluorometric measurements of CTC fluorescence were made with a Ferrand spectral analyzer (Ferrand Optical, Valhalla, N. Y.) coupled to the Leitz microscope. Values of relative fluorescence at 520 and 530 nm (emission peaks for Mg^{++}-CTC and Ca^{++}-CTC, respectively [11]) were obtained from various regions of cells, with a 15-μm aperature and a 1.5-mm slit width. Excitation for the mercury lamp on the Leitz microscope was held constant at 405 nm by a monochromator.

Fluorometry on a bulk endosperm cell suspension was performed using an Aminco SPF-500 fluorometer (American Instrument Co., Silver Spring, Md.). Cells from 40 seeds were gently suspended in 20% (wt/vol) Ficoll 400. With the fluorescence emission wavelength held constant at 520 or 530 nm, excitation wavelengths were scanned from 300 to 450 nm.

Perfusion Studies

After a 20-min incubation with CTC, endosperm cells were post-treated with a test solution by perfusion beneath the cover glass. A solution of 3.5–5.0% glucose with 20 μM CTC was used as a perfusion medium in all experiments. Results were not significantly different with CTC absent from the perfusion medium. Polylysine-anchored endosperm cells exhibited no apparent anomalous morphological or developmental changes as a result of the substitution of endosperm fluid by the glucose solution. The perfusion medium for crayfish claw muscle was PBS with 20-50 μM CTC.

RESULTS

When endosperm cells are observed by phase-contrast microscopy with incident green light, most cells proceed through mitosis. Neither the rate nor extent of anaphase chromosome movement appears to be affected by concentrations of CTC <50 μM. Cells are killed by long exposure to the high energy blue light used to excite fluorescence. In addition, exposure of the cells to the excitation beam for periods of time exceeding 45 s resulted in a change in the color of fluorescence, with the cells becoming increasingly yellow. Caswell (11) attributes this color change to photo-oxidation of the CTC. For these reasons, fluorometric measurements were made or photographs obtained within 10 s of exposure to the blue excitation light. Because it was possible to obtain only one fluorescent image of a cell, different cells (in various stages of mitosis) were photographed. With greatly reduced intensity of the excitation light, we found it possible to observe CTC fluorescence in the same cells over an extended period of time using an image intensifier to enhance the fluorescence signal (46). In that study, all six of the cells observed completed anaphase and produced a cell plate, demonstrating nontoxic effects of the probe on the cells.

Normal cells immersed in endosperm liquid or in 3.5% glucose do not exhibit significant autofluorescence under the conditions of the experiments (Fig. 1). Dead cells do exhibit autofluorescence but are easily distinguished from living cells. Living cells treated with 20–50 μM OTC, a Ca^{++}-insensitive analogue of CTC (15, 16), exhibit very low levels of fluorescence, only slightly more intense than untreated cells (Fig. 1, additional data not shown).

Ca-CTC can be distinguished from Mg-CTC by fluorometric analysis because the emission peak of the former is at 530 nm, whereas that for the latter is at 520 nm (11, 15, 19). Experimental measurements of single endosperm cells reveal a twofold greater signal at 530 nm than at 520 nm with excitation at 405 nm. Further delineation between Ca- and Mg-CTC can be achieved through analysis of the excitation spectra. Maximal excitation occurs at 390 nm for Ca-CTC and at 375 nm for Mg-CTC (11). Fluorometric analysis of a *Haemanthus* cell suspension treated with 10 μm CTC yields excitation peaks at 377 nm for Mg-CTC (520 nm emission) and at 385 nm for Ca-CTC (530 nm emission).

The fluorescence pattern generated by CTC in living cells has two components: punctate and diffuse (Fig. 2). The punctate fluorescence in the interphase cell lies in a broad perinuclear band (Fig. 2) that forms two polar aggregates before nuclear envelope breakdown (Fig. 3). By observing individual

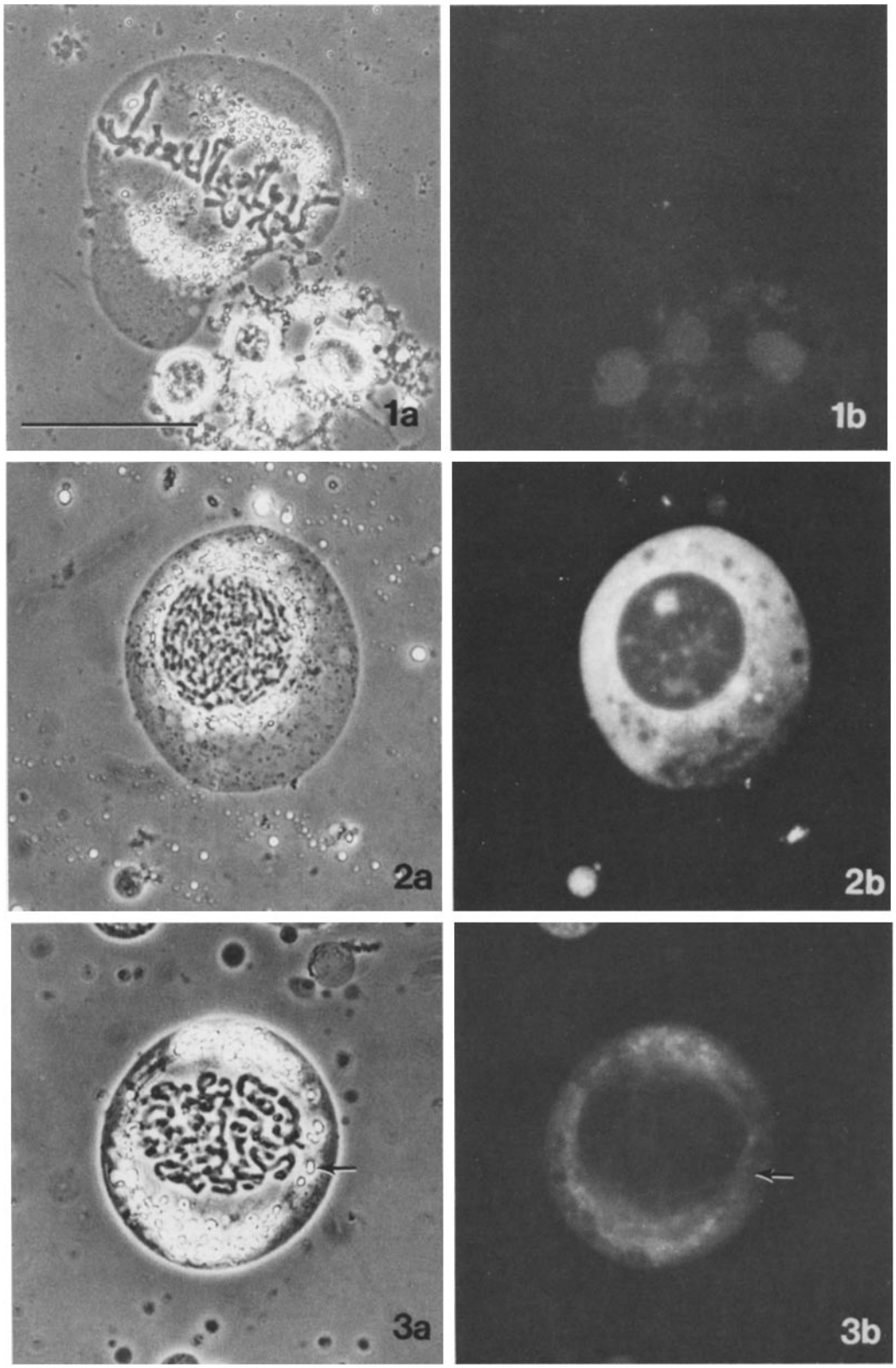


FIGURE 1 Autofluorescence control. Figs. 1–11 show paired phase contrast (*a*) and fluorescence (*b*) micrographs of *Haemanthus* endosperm cells in various stages of mitosis. Bars, 50 μm. × 540.

FIGURE 2 Early prophase cell treated with 20 μM CTC.

FIGURE 3 Prometaphase cell treated with 20 μM CTC. Plastids and mitochondria aggregate at sites of incipient spindle poles. Arrows show lack of CTC fluorescence from the starch grains. The nuclear envelope appears to be intact at this stage and emits fluorescence with CTC.

cells proceeding through mitosis with phase-contrast microscopy, we were able to conclude that these were the incipient spindle poles. Punctate fluorescence remains localized at the spindle poles through the onset of telophase (Figs. 4-7). Subsequently, there is a gradual disaggregation and a return to the perinuclear pattern observed during interphase (Fig. 7).

Because we were not able to focus on the diffuse fluorescence at the cell surface, we conclude that this fluorescence is not primarily associated with the plasmalemma. During mitosis, the pattern of diffuse fluorescence remains bright in regions outside the mitotic apparatus (Figs. 4–6). Within the spindles, the region occupied by the chromosomes is dark and the chromosome-to-pole region of the spindle exhibits bright "cones" of fluorescence in positions that are assumed to be occupied by kinetochore fibers (Fig. 4). In Fig. 4*a* (phase contrast) the kinetochores are seen as white dots on the chromosomes. The spindle region in the lower portion of the cell is largely devoid of chromosome arms (Fig. 4*a*); the alternations in fluorescence intensity (Fig. 4*b*, arrows) are clearly not caused by the presence of chromosomes in this part of the spindle. The bright fluorescent cones each point to a kinetochore in the metaphase plate (arrows, Figs. 4*a* and *b*) and the bright cones of CTC fluorescence coincide with the location of each kinetochore fiber. These discrete cones disperse during anaphase; the chromosome-to-pole region of the anaphase spindle exhibits uniformly diffuse CTC fluorescence (Figs. 5 and 6). The timing of this change is not known at present.

The cell plate formed during telophase emits bright CTC fluorescence (Fig. 7). Plate initiation during late anaphase is accompanied by an accumulation of fluorescence in the equatorial region of the spindle, interrupted by the trailing chromosome arms (Fig. 6). As chromosome migration nears completion (Figs. 6 and 7), the equatorial fluorescence becomes increasingly apparent in the region of the cell plate. At this stage, the coalescence of cell plate vesicles is visible with phase-contrast optics (data not shown). Adjacent to the cell plate, on either side, is a zone of reduced fluorescence (Fig. 7).

Perfusion Studies

Endosperm cells, incubated for 20 min in 20–50 μM CTC and anchored to polylysine-coated cover glasses, show no alternation of fluorescence patterns when they are perfused with 20 μM CTC in either endosperm liquid or in 3.5% glucose. The fluorescence pattern, however, is altered by several agents known to modify calcium distribution in living cells. The most obvious effect of lanthanum, EGTA, and procaine is the reduction in diffuse fluorescence with no significant loss of punctate fluorescence (Table I, and compare Figs. 8 and 9 with Figs. 3 and 4).

In contrast to the effects of La^{+++}, procaine and EGTA, the metabolic uncouplers DNP and CCCP cause an increase in diffuse fluorescence that appears to accompany a slight reduction in punctate fluorescence (compare Fig. 3 with Fig. 10). Much of the increase in diffuse fluorescence occurs in the plasma membrane.

Intact cells, pretreated with CTC, undergo an increase in diffuse fluorescence in response to perfusion with 1 mM $CaCl_2$. Much of the increase occurs at the cell surface; the pattern of fluorescence is similar to perfusion with metabolic uncouplers (Fig. 10). No change in punctate fluorescence was observed with perfusion of $CaCl_2$ (Table I). There was a slight decrease in both punctate and diffuse fluorescence after perfusion of the metabolic poison, NaN_3 (Table I).

Detergent extraction with 1% Nonidet P-40, which causes extensive dissolution of membranes (S. M. Wolniak and W. T. Jackson, unpublished observations; [40]), completely eliminates CTC fluorescence (Table 1, Fig. 11).

Fluorescence in the forming cell plate is reduced by perfusion of Nonidet and EGTA (Table II). The fluorescence emission from the cell plate changed from green to yellow with EGTA perfusion. The introduction of calcium to EGTA-perfused cells did not reverse this effect. Perfusion of DNP or CCCP had no effect on cell plate fluorescence (Table II).

Freshly teased crayfish claw muscle incubated with CTC also emits yellow-green fluorescence in response to broad-band blue excitation light (Fig. 12, and references 3, 14, 19). CTC fluorescence is at least ten times brighter than autofluorescence levels (Fig. 13) and is brightest in regions where the sarcoplasmic reticulum (SR) coincides with the A bands of the myofibrils (Fig. 12). CTC fluorescence of claw muscle is abolished (to levels similar to autofluorescence) by perfusion with La^{+++}, EGTA, procaine, and Nonidet P-40 (data not shown).

DISCUSSION

CTC, at concentrations <100 μM, permits detection of membrane-associated cations by fluorescence microscopy in living cells. Blue excitation light evokes maximal yellow-green fluorescence from CTC complexed to Ca^{++} or Mg^{++} in apolar (i.e., membrane) environments (11). The conformational shift in the Ca-CTC complex in apolar surroundings (13) appears to be responsible for a large (fivefold) augmentation in the fluorescence emission intensity (23), while little change in emission intensity is observed with the Mg-CTC complex in either polar or apolar environments (23). In apolar environments, CTC is three times more likely to bind Ca^{++} than Mg^{++} (11) and, additionally, the excitation and emission spectra for the Mg-CTC and the Ca-CTC complexes differ sufficiently to be resolved with fluorometric techniques; in an apolar environment in the physiological pH range, the Ca-CTC complex excites maximally at 390 nm and emits maximally at 530 nm (11, 13, 15, 19, 23), while excitation and emission optima for the Mg^{++} complex are at 375 and 520 nm, respectively (12–14, 19, 23). Exclusion of much of the Mg-CTC emission spectrum through cutoff barrier filter combinations, and reduction of the Mg-CTC excitation spectrum through illumination with longer wavelength blue light (>390 nm; 15, 16) optimize conditions for detection of Ca-CTC fluorescence, making the probe relatively specific for studies on Ca^{++}-membrane distribution in living systems (15, 16). Our microscopes were outfitted to meet these requirements.

The intracellular distribution of Mg^{++} is assumed to be uniform throughout the cytoplasm, in the concentration range of 0.5–1.0 mM (8). In marked contrast, the cytoplasmic-free Ca^{++} concentration is several orders of magnitude lower than $[Mg^{++}]$, except in localized, membrane-bound domains (e.g., mitochondria) where millimolar levels of the cation may exist (8). The intracellular CTC distribution in mitotic endosperm cells of *Haemanthus* more closely parallels the nonrandom, membrane-enclosed, distribution of Ca^{++} than the uniform pattern expected with Mg^{++}-binding by the probe. Our microfluorometric measurements on regions of the cytoplasm in individual cells indicate that much of the fluorescence is caused by Ca^{++} binding. Because of rapid photo-oxidation of the probe with high intensity excitation light (11), the degree of overlap in emission spectra for Mg-CTC and Ca-CTC could

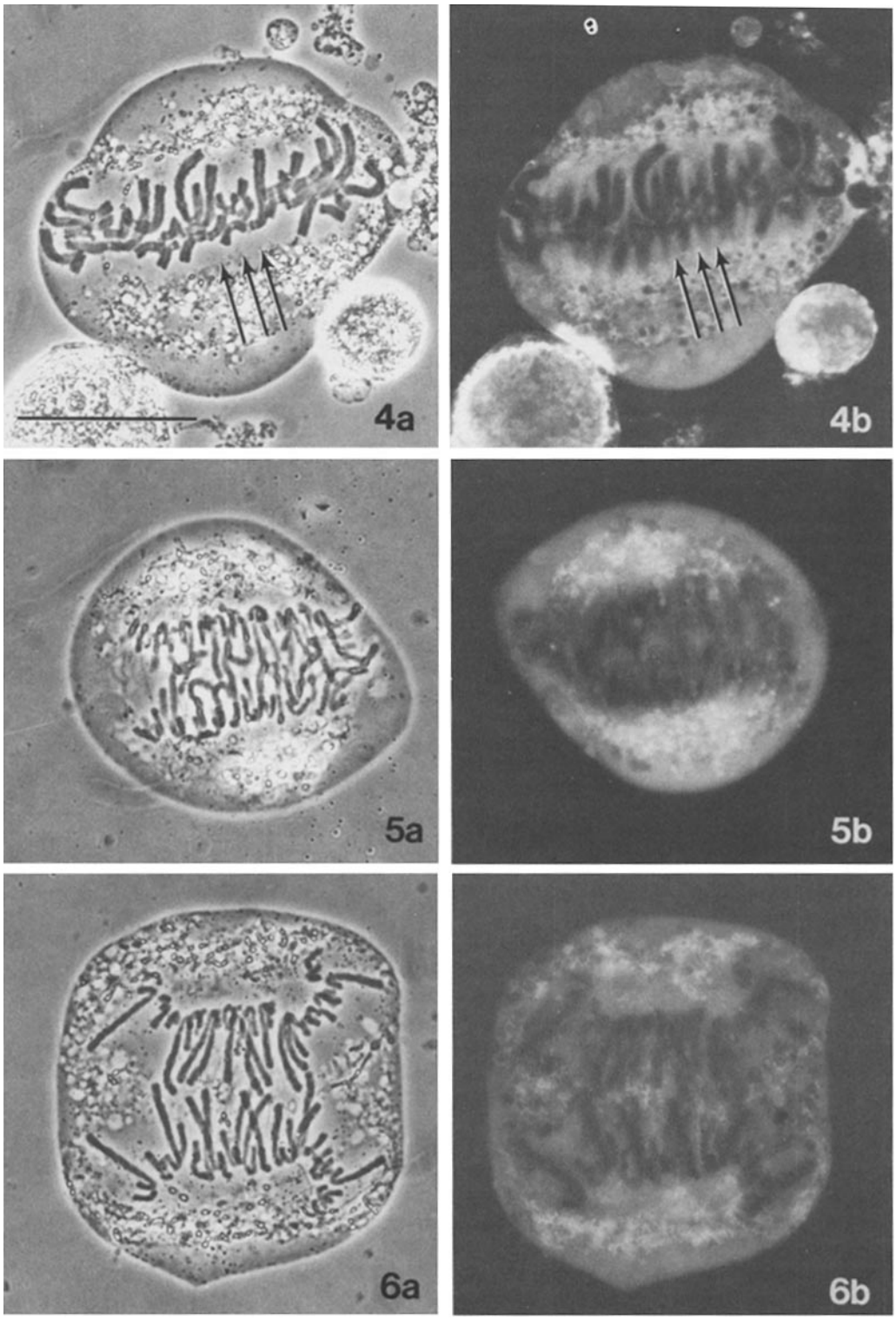


FIGURE 4 Metaphase endosperm cell treated with 20 μM CTC. "Cones" of diffuse CTC fluorescence (arrows in *b*) coincide with location of kinetochore fibers (arrows in *a*). Plastids and mitochondria are restricted to spindle pole regions and contribute to "punctate" component of CTC fluorescence.

FIGURE 5 Mid-anaphase endosperm cell treated with 20 μM CTC. The spindle midzone region is a zone of reduced fluorescence; punctate fluorescence is restricted to the poles and diffuse fluorescence to the chromosome-to-pole region of the spindle. The chromosomes emit no fluorescence with CTC.

FIGURE 6 Late anaphase; 20 μM CTC. Diffuse fluorescence in the chromosome-to-pole region of the spindle is uniform. In the zone of cell plate formation, phase-dense particle aggregation is accompanied by an increase in CTC fluorescence.

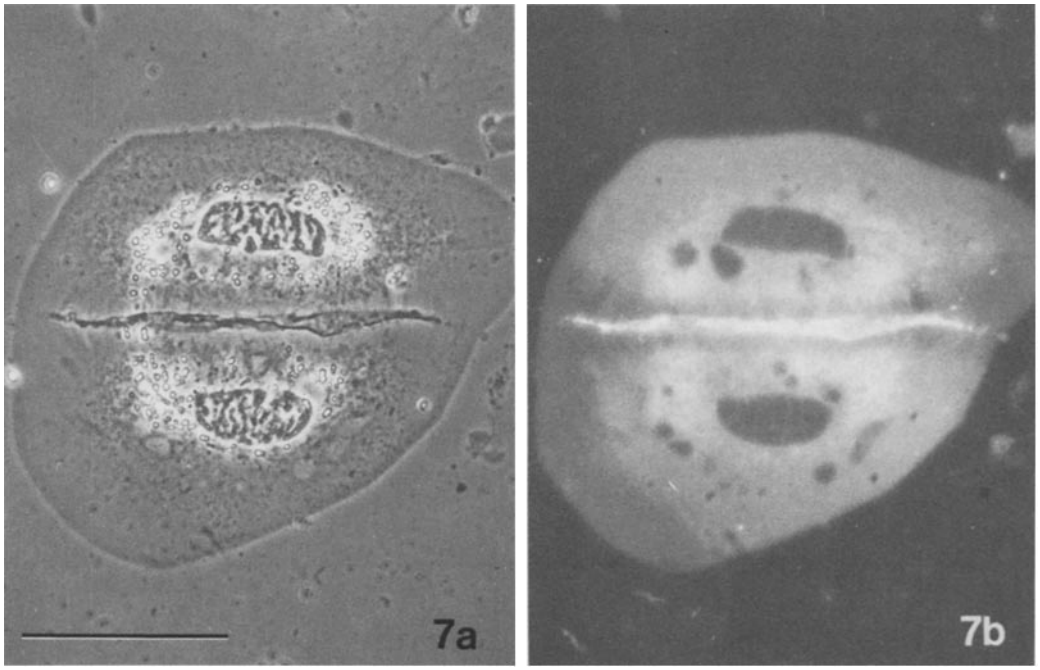


FIGURE 7 Late telophase; 20 μM CTC. With cell plate formation essentially complete, the plastids and mitochondria assume a perinuclear distribution. The phragmoplast region, extending ~5 μm on either side of the cell plate is a zone of reduced CTC fluorescence. The plate itself is brightly fluorescent with the probe.

TABLE I

Perturbation of the Cellular Ca^{++} Distribution by Perfusion: Effects on Punctate and Diffuse CTC Fluorescence in Dividing Endosperm Cells

Treatment	Punctate	Diffuse
2 mM $LaCl_3$, 20 min	No effect	Reduced
1 mM procaine, 20 min	No effect	Reduced
1 mM EGTA, 20 min	No effect	Reduced
1 μM DNP, 20 min	Slightly reduced	Increased
5 μM CCCP, 20 min	Slightly reduced	Increased; bright surface fluorescence
1 mM NaN_3, 30 min	Slightly reduced	Slightly reduced
3.5% Glucose, 20 min	No effect	Slightly reduced, then returned
1% Nonidet, P-40, 2 min	Abolished	Abolished
1 mM $CaCl_2$, 20 min	No effect	Increased; bright surface fluorescence

Endosperm cells were incubated with 10-50 μM CTC for 20 min before perfusion of these test solutions. After exchange of the endosperm fluid by the test solution, the cells were incubated as indicated above before illumination with broad-band blue excitation light for fluorescence photomicrography (see Materials and Methods).

TABLE II

Effects of Uncouplers, Chelators, and Detergent on CTC Fluorescence in the Endosperm Cell Plate

Treatment	Effect on cell plate-CTC fluorescence
1 mM EGTA, 20 min	Reduced, became progressively yellow
1 μM DNP, 20 min, 20 min	No effect
5 μM CCCP, 20 min	No effect
1% Nonidet, P-40, 2 min	Abolished

Endosperm cells were incubated in 10-50 μM CTC for 20 min before perfusion with these agents. After exchange of the endosperm fluid with the test solution, the cells were incubated as indicated above before illumination with broad-band blue excitation light for fluorescence photomicrography (see Materials and Methods).

not be assessed. Fluorometric analysis of excitation peaks of CTC in a bulk endosperm cell suspension indicates that CTC binds both Mg^{++} and Ca^{++} in these cells. Technical problems in the preparation of a bulk cell suspension prevent quantitation in these experiments, however. Extraction of all membranes with the nonionic detergent Nonidet P-40 (40; S. M. Wolniak and W. T. Jackson, unpublished observations) results in a total loss of CTC fluorescence, indicating that fluorescence is associated with membranes in the cell. Fluorescence with the antibiotic OTC was very weak in endosperm cells though its pattern of distribution was similar to CTC. OTC fluorescence was not perturbed by perfusion of 1 mM procaine (data not shown), indicating that CTC fluorescence patterns are membrane- and cation-associated (15, 16).

The distribution of punctate CTC fluorescence in endosperm cells of *Haemanthus* coincides with the distribution of mitochondria and plastids in the cytoplasm (4, 5, 29; P. K. Hepler and W. T. Jackson, unpublished observations). The starch present within the plastids does not fluoresce with the probe (Fig. 3, arrows) and detergent extraction of membranes abolishes CTC fluorescence without removal of starch (Fig. 11). CTC fluorescence associated with the plastid is most likely because of cation binding to the probe in or near the plastid membrane.

A second component of CTC fluorescence in *Haemanthus* endosperm is the "diffuse" fluorescence, and it is probably caused, in part, by cation-chelate localizations in the plasma membrane. However, the plasma membrane does not appear to be the exclusive source of diffuse CTC fluorescence. Perfusion with DNP, CCCP, or $CaCl_2$ results in a general increase

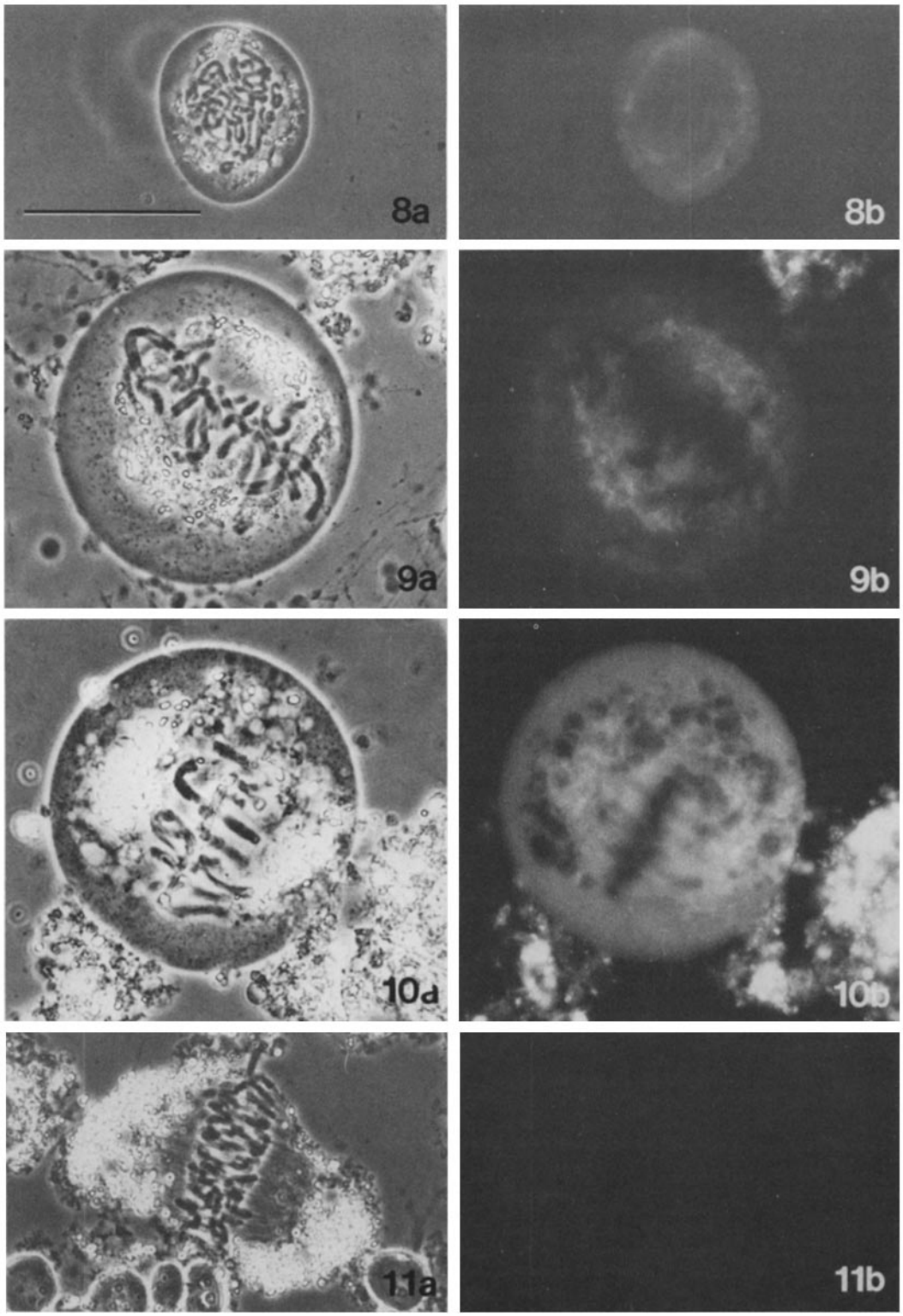
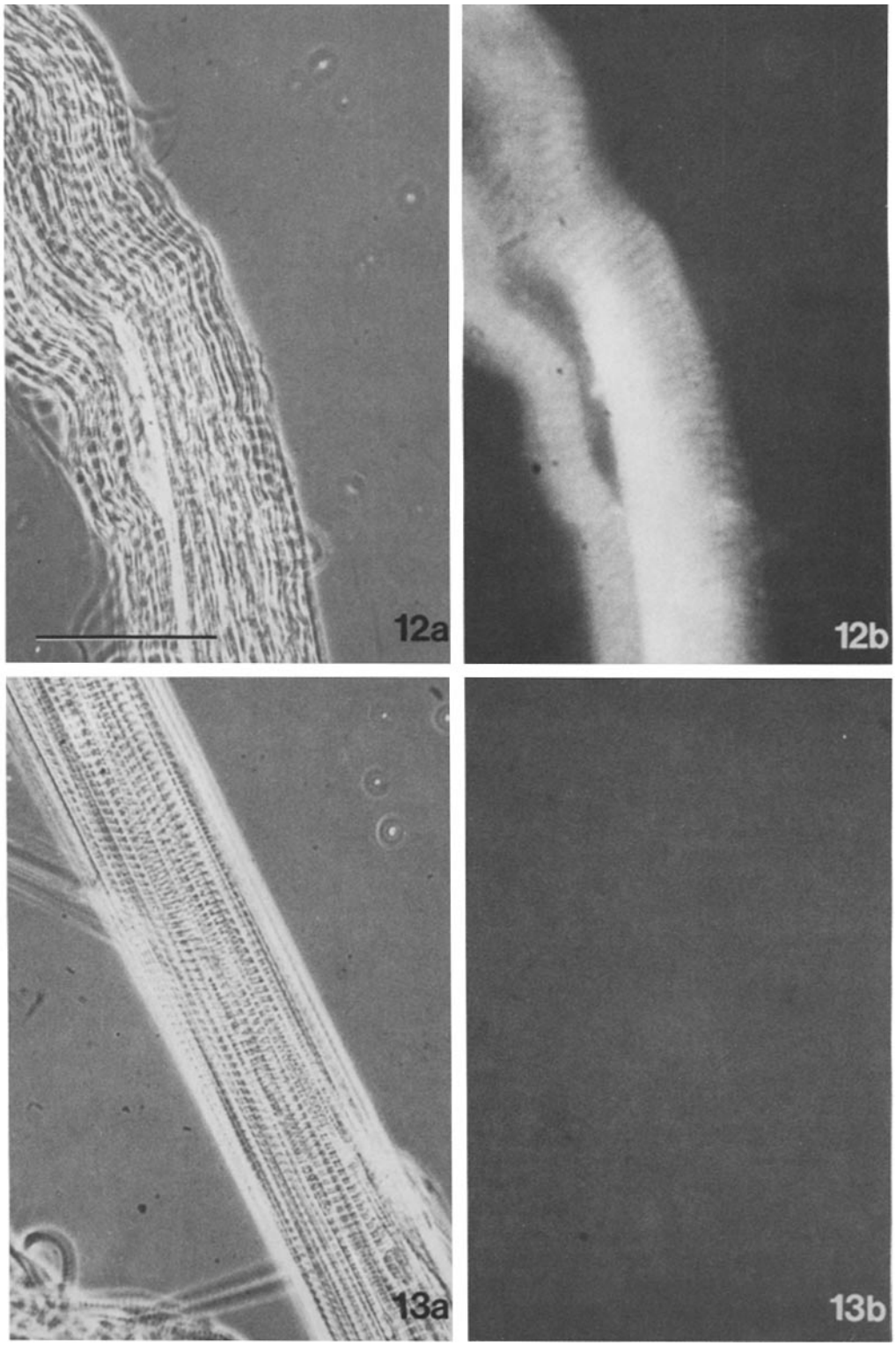


FIGURE 8 Prometaphase endosperm cell pretreated with 20 μM CTC, then perfused with 2 mM $LaCl_3$. Diffuse fluorescence is reduced, whereas punctate fluorescence is unaffected.

FIGURE 9 Metaphase endosperm cell pretreated with 20 μM CTC, then perfused with 1 mM procaine. As with the treatment with $LaCl_3$, diffuse fluorescence is reduced, whereas punctate fluorescence is unaffected.

FIGURE 10 Metaphase endosperm cell pretreated with 20 μM CTC, then perfused with 1 μM DNP. Punctate fluorescence is slightly reduced and diffuse fluorescence increases at the cell surface.

FIGURE 11 Anaphase endosperm cell pretreated with 20 μM CTC, then perfused with 1% (wt/vol) Nonidet P-40 in 20 mM PIPES, 4 mM $MgSO_4$, 1 mM EGTA (pH 6.95). Punctate and diffuse fluorescence are completely eliminated with this treatment.



FIGURES 12 and 13 Freshly teased crayfish claw muscle in PBS. Bar, 50 μm. × 540.

FIGURE 12 Crayfish claw muscle treated with 50 μM CTC in PBS. Fluorescence coincides with A bands.

FIGURE 13 Crayfish claw muscle; autofluorescence control.

in diffuse fluorescence, a major portion of which can be focused at the plasma membrane (Fig. 10), and differs in appearance from the normal pattern of CTC fluorescence. In non-perfused cells, much of the diffuse fluorescence is not at the cell surface. These results suggest that diffuse fluorescence originates largely from membranes within the cytoplasm. Because of the limits of resolution with the light microscope, CTC fluorescence originating from endomembranes will assume a diffuse appearance.

Osmium-ferricyanide staining techniques have been adapted

recently for mapping the distribution of the NE-ER complex in dividing plant cells (28). In barley cells during mitosis (28), the NE-ER complex possesses a pattern similar to that for the diffuse CTC fluorescence depicted here, and suggests that the endomembranes contribute to this component of the fluorescent emission. Especially noteworthy are the cones of fluorescence that correlate in position with the kinetochore bundles in the chromosome-to-pole region of the mitotic apparatus. We suggest that the fluorescence indicates membrane associations with the kinetochore microtubules. The disappearance of discrete cones of diffuse CTC fluorescence during anaphase (Figs. 5 and 6) coincides with the pattern of kinetochore-bundle dissociation observed at this stage by Hard and Allen (24). The spindle pole region also contains an extensive network of ER (5, 29; P. K. Hepler and W. T. Jackson, unpublished observations; B. Doyle and W. T. Jackson, personal communication). We conclude that diffuse fluorescence may be attributed mainly to Ca^{++} bound to or associated with the ER.

Punctate and diffuse fluorescence are differentially sensitive to agents affecting the Ca^{++} distribution in the cells. Perfusion of $LaCl_3$, an inhibitor of Ca^{++} transport across the plasma membrane (34, 44), reduced diffuse fluorescence, but had little effect on punctate fluorescence. The effect of La^{+++} on fluorescence may be the result of a net efflux of Ca^{++} from the ER into nonmembrane cytoplasm, into the mitochondria and plastids, or into the external medium. Perfusion with EGTA, a strong Ca^{++} chelator, or with procaine, an anesthetic that modifies the Ca^{++} distribution in sarcoplasmic reticulum (37), causes a marked reduction in diffuse fluorescence, without modifying punctate CTC fluorescence in endosperm cells. These treatments suggest that the Ca^{++} giving rise to the diffuse fluorescence is more easily exchanged than that in mitochondria and plastids, although this result may merely reflect significant differences in total Ca^{++} content in various cytoplasmic membrane compartments. It appears that a DNP- or CCCP-induced Ca^{++} efflux from the mitochondria (7, 9) causes an increase in diffuse fluorescence. The slight decrease in both punctate and diffuse fluorescence induced by NaN_3 treatment suggests that the effects of DNP and CCCP are more than a nonspecific result of cell death. Perfusion of $CaCl_2$, either in the presence or absence of CTC in the perfusion medium results in increased diffuse fluorescence primarily from cation binding to CTC in the plasma membrane (data not provided).

The similarity of the reduction of claw muscle SR and endosperm diffuse fluorescence by perfusion of La^{+++}, procaine, and EGTA evokes the exciting notion that the endosperm ER may function in Ca^{++} sequestration in a manner analogous to muscle SR (18). Although CTC fluorescence shifts alone are inadequate evidence to document functions of membrane systems, it is conceivable that modulation of localized Ca^{++} uptake or release by vesicles or cisternae could control motile events during anaphase (25, 27). The existence of a membrane fraction located in the chromosome-to-pole regions of the spindle, exhibiting an ATP-dependent Ca^{++}-uptake function, has recently been demonstrated by Silver et al. (42).

Recent studies have demonstrated that calmodulin confers Ca^{++} sensitivity to many cellular processes. Immunological localizations of calmodulin in the mitotic apparatus of mammalian cells (2, 6, 38, 45) are strikingly similar to the pattern of diffuse CTC fluorescence in dividing endosperm cells. Because of the hydrophobic requirement for maximal fluorescence with CTC (11), it is unlikely that calmodulin, with its hydrophilic properties, is responsible for the Ca-CTC fluorescence. However, we think it is possible that both Ca^{++} and calmodulin could be associated with the membrane system of the mitotic apparatus. We further suggest that calmodulin in the mitotic apparatus, in addition to its postulated role in tubulin depolymerization (6, 38, 45) and actomyosin activation (1, 17), could interact with the membrane transport system and control Ca^{++} sequestration and release, consonant with its role in controlling Ca^{++} transport across membranes in other systems (35).

We would like to thank Stuart Gelder and Randall Alberte for their help in the use of their fluorometric instruments, Robert Barlow for the use of his darkroom laboratory space at the Marine Biological Laboratory (MBL), and Victor Laronga of Atlantex & Zieler Instrument Corp. for the loan of the Zeiss epifluorescence system used at the MBL. We would also like to express appreciation for the helpful comments and criticisms from our colleagues at the MBL, Dartmouth College, and the University of Massachusetts.

This research was supported by a grant from the National Institutes of Health (NIH) (GM-25120) to P. K. Hepler and a grant from the National Science Foundation (PCM-7805172) to W. T. Jackson. S. M. Wolniak is supported by a postdoctoral fellowship from the NIH.

Received for publication 15 January 1980, and in revised form 9 May 1980.

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