TUBULIN AND CALMODULIN

Effects of Microtubule and Microfilament Inhibitors on Localization in the Mitotic Apparatus

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ABSTRACT

Indirect immunofluorescence was used to determine the distribution of calmodulin in the mitotic apparatus of rat kangaroo PtK_2 and Chinese hamster ovary (CHO) cells. The distribution of calmodulin in PtK_2 cells was compared to the distribution of tubulin, also as revealed by indirect immunofluorescence. During mitosis, calmodulin was found to be a dynamic component of the mitotic apparatus. Calmodulin first appeared in association with the forming mitotic apparatus during midprophase. In metaphase and anaphase, calmodulin was found between the spindle poles and the chromosomes. While tubulin was found in the interzonal region throughout anaphase, calmodulin appeared in the interzone region only at late anaphase. The interzonal calmodulin of late anaphase condensed during telophase into two small regions, one on each side of the midbody. Calmodulin was not detected in the cleavage furrow. In view of the differences in the localization of calmodulin, tubulin, and actin in the mitotic apparatus, experiments were designed to determine the effects of various antimitotic drugs on calmodulin localization. Cytochalasin B, an inhibitor of actin microfilaments, had no apparent effect on calmodulin or tubulin localization in the mitotic apparatus of CHO cells. Microtubule inhibitors, such as colcemid and N_2O, altered the appearance of tubulin- and calmodulin-specific fluorescence in mitotic CHO cells. Cold temperature (0°C) altered tubulin-specific fluorescence of metaphase PtK_2 cells but did not alter calmodulin-specific fluorescence. From these studies, it is concluded that calmodulin is more closely associated with the kinetichore-to-pole microtubules than other components of the mitotic apparatus.

KEY WORDS mitosis · microtubules microfilaments · calcium · immunofluorescence · calcium-dependent regulator (CDR)

In an effort to understand the role of calcium in the regulation of certain cell functions, we have been investigating the properties of a low molecular weight, calcium-binding protein which is widely distributed in eukaryotic cells. The protein is similar to the calcium-binding protein of muscle, troponin-C, sharing ~50% amino acid sequence homology (1). Further, this calcium-binding protein has been shown to regulate several cellular

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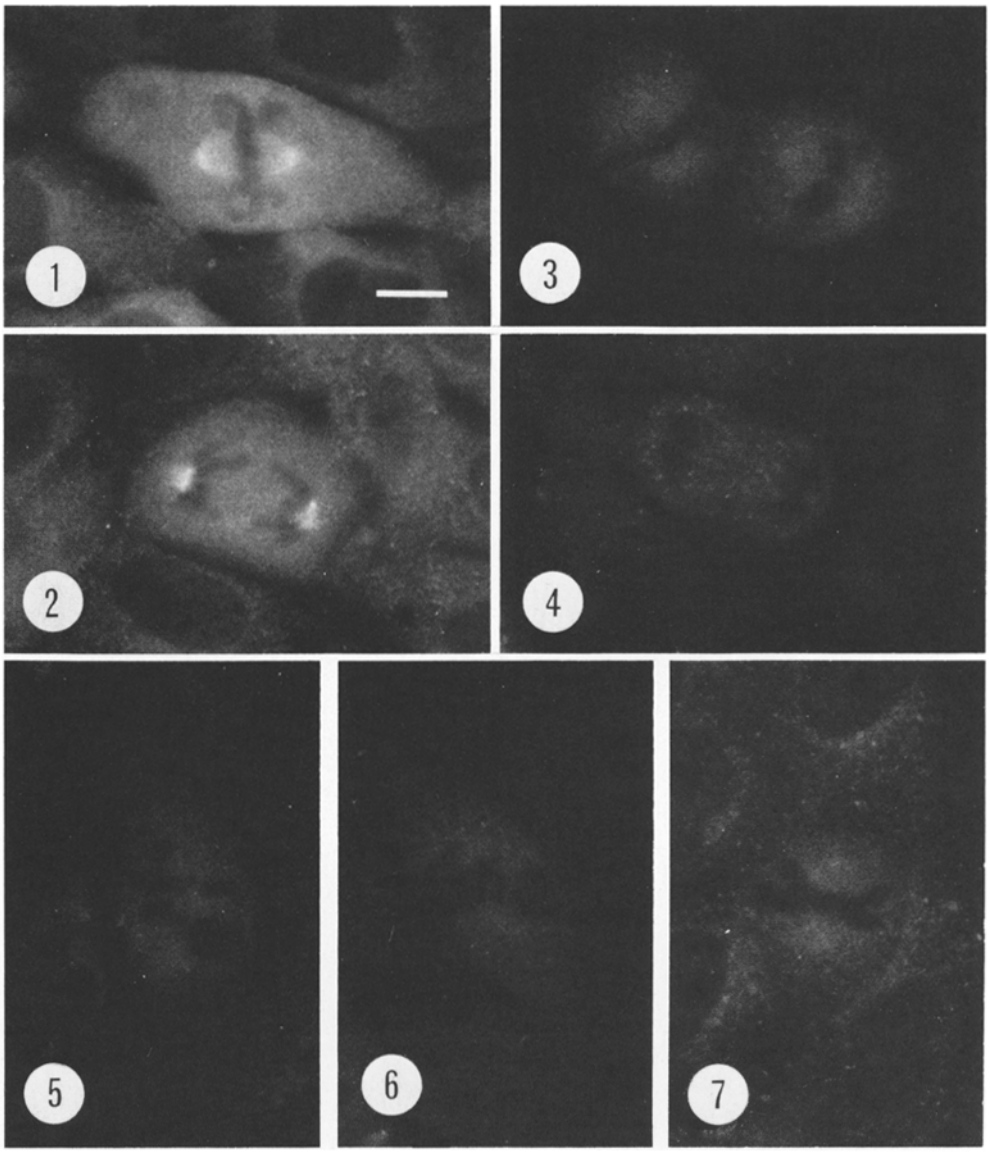


FIGURES 1–7 Calmodulin immunofluorescence in mitotic rat kangaroo PtK_2 cells. All figures × 900. Bar in Fig. 1, 10 μm.

FIGURE 1 Calmodulin in spindle of metaphase cell.

FIGURE 2 Calmodulin in half-spindles of anaphase cell.

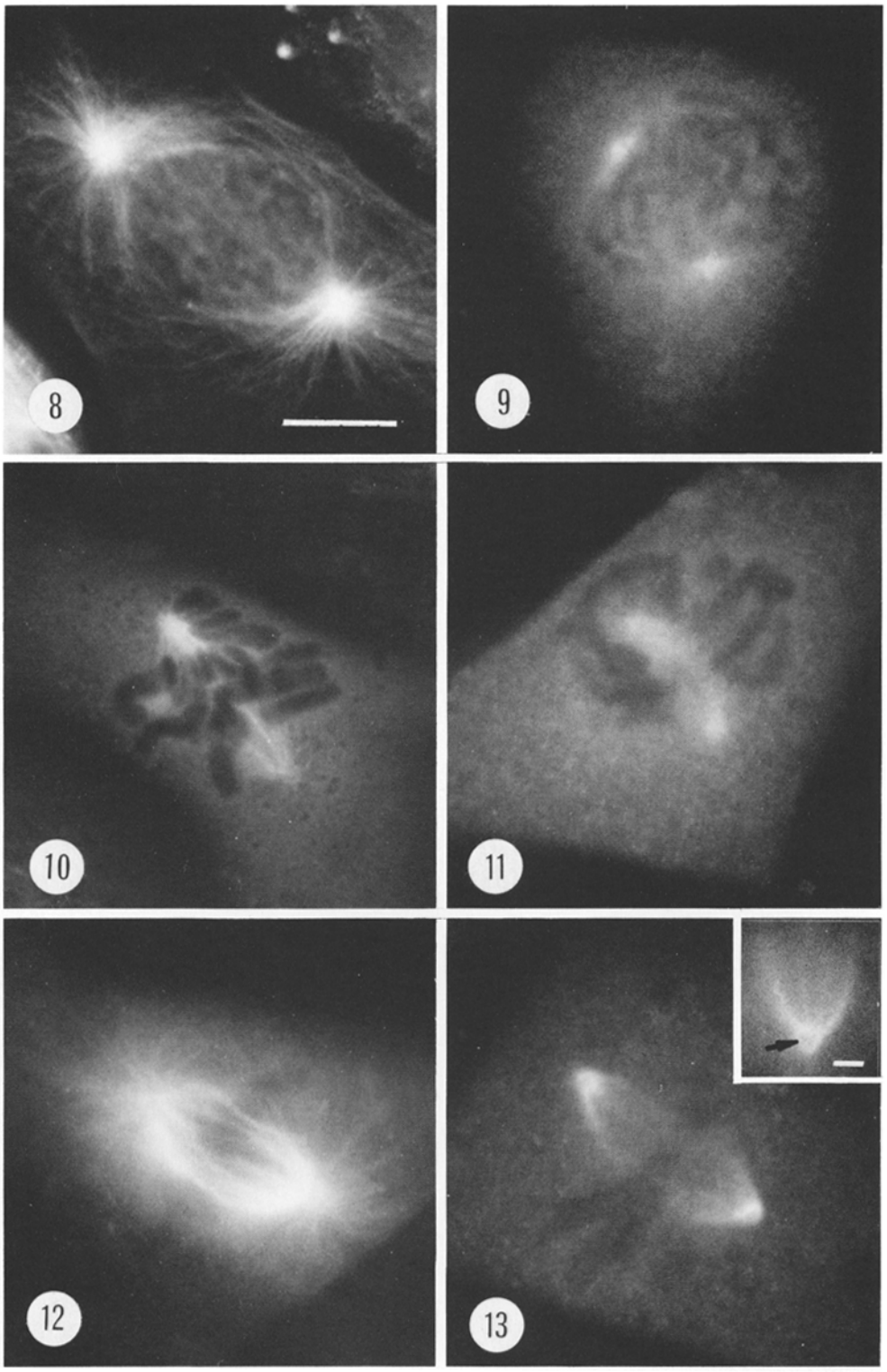
FIGURE 3 Two metaphase cells show no specific fluorescence when calmodulin antibody is preabsorbed for 1 h at 23°C with fivefold excess antigen.

FIGURE 4 Anaphase cell also exhibits no localization of calmodulin in half-spindles when calmodulin antibody is preabsorbed with fivefold excess antigen.

FIGURE 5 Metaphase cell incubated with pre-immune IgG instead of calmodulin antibody exhibits no specific localization of calmodulin in the spindle.

FIGURE 6 Metaphase cell incubated with fluorescent conjugated second antibody only exhibits no fluorescence in the spindle.

FIGURE 7 Metaphase cell incubated with immunized goat IgG which was not retained on the calmodulin affinity column also exhibits no fluorescence in the mitotic apparatus.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activities in a calcium-dependent manner (2–11). The protein has been referred to by many names, including activator protein, modulator protein, and calcium-dependent regulatory protein (CDR). Recently, the term calmodulin has been accepted as a compromise and will be used in the present communication.

We have developed and purified monospecific antibodies to calmodulin isolated from rat testes (12). By use of indirect immunofluorescence, we have shown calmodulin to be localized in the mitotic apparatus of cells in culture (13) and associated with stress fibers of fibroblasts in interphase (12, 13). The demonstrated functions of calmodulin in various tissues and the distribution of calmodulin throughout mitosis suggested several possible functions for the protein in the mitotic apparatus. The localization of calmodulin to a structure composed largely of microtubules suggested that calmodulin might mediate calcium-dependent effects on the microtubules of the mitotic apparatus (13). Indeed, Marcum et al. have shown that calmodulin does enhance calcium sensitivity of the assembly-disassembly of bovine brain tubulin in vitro (14). Such in vitro results, however, may not necessarily reflect conditions within living cells. Alternately, the similarity of calmodulin to troponin-C and the localization of calmodulin to stress fibers of many interphase cell types (12) suggests that calmodulin might be a component of a microfilamentlike contractile system in the mitotic apparatus. Moreover, recent results have shown calmodulin to regulate myosin-linked ATPase of muscle and nonmuscle cells by promoting the phosphorylation of a myosin light chain (10, 15). In a further effort to examine the role and distribution of calmodulin in mitosis, we have compared the localization of calmodulin and that of tubulin in mitotic cells. We describe also the effects on calmodulin and tubulin localization of agents which selectively disrupt microfilaments or microtubules. Agents or treatments used included cytochalasin B to disrupt microfilaments, and colcemid, exposure to 0°C, or nitrous oxide to disrupt microtubules or spindle structure.

MATERIALS AND METHODS

Cells

Rat kangaroo cells (PtK_2) and Chinese hamster ovary (CHO) cells were obtained from the American Type Culture Collection, Rockville, Md. Cells were grown at 37°C in 95% air-5% CO_2 in Eagle's minimal essential medium supplemented with 10% fetal calf serum. The cells were grown in Corning plastic culture dishes (Corning Glass Works, Science Products Div., Corning, N. Y.) (35 mm) containing two 11 × 22-mm glass coverslips per dish.

Antibodies

The production and isolation of rabbit antibodies to bovine brain tubulin were performed as described by Fuller et al. (16). Antibodies to rat testis calmodulin were produced in a goat, purified, and characterized as described by Dedman et al. (12).

Indirect Immunofluorescence

Cells grown on coverslips were prepared essentially as described by Fuller et al. (16). Cells were fixed for 30

FIGURES 8–19 Indirect immunofluorescence localization of tubulin or calmodulin in PtK_2 cells at similar stages of mitosis. All figures × 1,660. Bar in Fig. 8, 10 μm.

FIGURE 8 Tubulin-specific fluorescence in prophase. The asters are at opposite sides of the nucleus and a spindle structure is beginning to form.

FIGURE 9 Prophase cell showing calmodulin in two regions on opposite sides of the nucleus.

FIGURE 10 A spindle structure has formed in a prometaphase cell as the condensed chromosomes proceed to align on the metaphase plate.

FIGURE 11 Localization of calmodulin in prometaphase cell is similar to that of tubulin.

FIGURE 12 At metaphase, tubulin-specific fluorescence shows a complete spindle with microtubule bundles passing from the spindle poles to the chromosomes and also microtubules traversing the metaphase plate.

FIGURE 13 Calmodulin at metaphase. Calmodulin is concentrated near the poles with fiberlike strands of fluorescence projecting toward the chromosomes. The *inset* shows one half-spindle of another PtK_2 cell (at × 2,200) illustrating that calmodulin in the pericentriolar region is reduced at the centrioles (arrow) and in their immediate vicinity. Bar in *inset*, 2 μm.

min in 3% formalin, post-fixed for 7 min in acetone, −20°C, then incubated inverting the coverslip on a drop (20 μl) of primary antibody (rabbit anti-tubulin or goat anti-calmodulin, 100 μg/ml) for 45 min. Cells were washed twice for 10 min in Dulbecco's phosphate-buffered saline (PBS) and subsequently incubated with the appropriate fluorescein-conjugated second antibody (goat anti-rabbit IgG or rabbit anti-goat IgG). After two 10-min washes in PBS, the coverslips were mounted in buffered glycerol (pH 9.0). Cells were viewed and photographed with a Leitz Orthoplan microscope (E. Leitz, Inc., Rockleigh, N. J.) equipped with a Polemak 2.1 illumination system with a 100-W mercury lamp. Images were viewed through a 63× Fl Oel objective (NA 1.3). Images were recorded on Kodak Tri-X or Plus-X film.

Controls for calmodulin immunofluorescence consisted of (*a*) cells incubated with second antibody only, (*b*) Ig G not retained on the respective antigen affinity column (500 μg/ml), (*c*) Ig G from the pre-immune animals (1 mg/ml), or (*d*) antibodies preabsorbed with antigen (100 μg/ml IgG with fivefold molar excess [60 μg/ml] of antigen).

Treatment of cells

Immediately before formalin fixation and processing for immunofluorescence, some cells (on coverslips) were subjected to various treatments as follows: (*a*) incubation of cells with 10 μg/ml cytochalasin B for 1 h at 37°C; (*b*) incubation of cells with 0.06 μg/ml colcemid for 1 h at 37°C; (*c*) incubation of cells at 0°C for 1 h; or (*d*) incubation under N_2O atmosphere at 80 lb/in² at 37°C for 16 h (17).

RESULTS

Calmodulin Immunofluorescence Controls

As shown in Figs. 1 and 2, calmodulin was found associated with the mitotic apparatus of metaphase and anaphase cells. However, fluorescence was absent from the mitotic apparatus if cells were incubated with the calmodulin-specific Ig G preincubated with fivefold excess calmodulin (Figs. 3 and 4), with pre-immune Ig G (Fig. 5), with second antibody only (Fig. 6), or with Ig G not retained on the calmodulin affinity column (Fig. 7).

Tubulin and Calmodulin Localization Through Mitosis

The distribution of calmodulin in the mitotic apparatus has been described, (13) as has that of tubulin (17). As cells enter prophase, tubulin- and calmodulin-specific fluorescence first appeared near the centrioles (Figs. 8 and 9). Through prometaphase (Figs. 10 and 11) and metaphase (Figs. 12 and 13), tubulin and calmodulin continued to show similar patterns of localization. At metaphase, however, calmodulin was primarily concentrated at the poles (Fig. 13), with strands of fluorescence projecting toward the chromosomes. As shown in the *inset* of Fig. 13, the pericentriolar region exhibited intense calmodulin fluorescence with reduced fluorescence at the centrioles (arrow). During anaphase, tubulin was present both between the poles and chromosomes (half-spindles) and in the region between the separating chromosomes (interzone) (Fig. 14). In contrast, calmodulin was found only in the half-spindles but not in the interzone (Fig. 15). Through cytokinesis, calmodulin immunofluorescence was conspicuously absent in the contractile ring of the cleavage furrow. At late anaphase or early telophase, tubulin remained present in half-spindles and interzone regions (Fig. 16). During this stage, calmodulin was first seen in the interzone region as two distinct areas on each side of the cleavage furrow (Fig. 17). Finally, in the two new daughter cells, tubulin was found throughout the midbody, except for a narrow band at the center of the structure (Fig. 18). Calmodulin was

FIGURE 14 In an anaphase cell, tubulin-specific fluorescence is found both in the greatly shortened half-spindles and in the interzone region between the separating chromosomes.

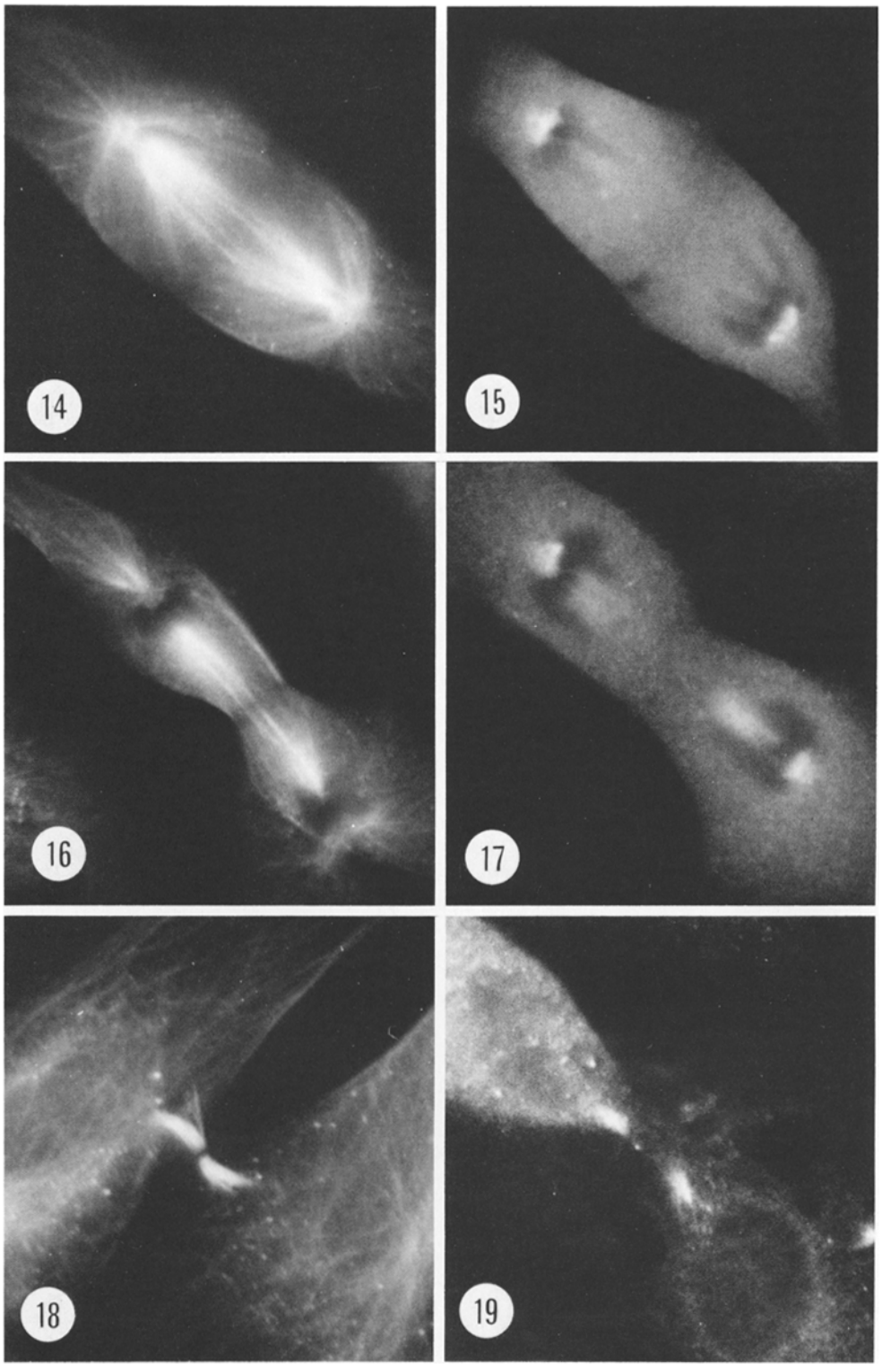
FIGURE 15 Calmodulin in an anaphase cell is localized only in the two half-spindles.

FIGURE 16 At late anaphase or teleophase, tubulin continues to be localized in the interzone region.

FIGURE 17 In late anaphase, calmodulin appears in the interzone region but is absent from the developing cleavage furrow.

FIGURE 18 In two daughter cells, tubulin is found in the midbody between the two cells.

FIGURE 19 Calmodulin in daughter cells is located at the distal end regions of the midbody.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distributed at each end of the midbody region (Fig. 19) in a pattern clearly different from that of tubulin.

Effects of Cytochalasin B and Colcemid on Tubulin and Calmodulin Localization

When CHO cells (Figs. 20 and 21) were treated with agents which disrupt microtubules, spindle structure was altered and tubulin- and calmodulin-specific fluorescence were equally affected. Colcemid treatment completely disrupted the spindle of CHO cells as reflected by tubulin immunofluorescence (Fig. 22) and equally abolished calmodulin localization in the mitotic apparatus (Fig. 23). On the other hand, treatment of cells for 1 h with cytochalasin B, a known inhibitor of cytoplasmic microfilaments (18), had no significant effect on the localization of tubulin- (Fig. 24) or calmodulin-specific (Fig. 25) fluorescence in the mitotic apparatus, although cytokinesis was prevented by the treatment.

Effects of Nitrous Oxide and Low Temperature (0°C) on Tubulin and Calmodulin Localization

Treatment of CHO cells with nitrous oxide, which causes disorganization of the spindle but does not cause disassembly of spindle microtubules (19), disrupted spindle structure as seen with tubulin-specific fluorescence. However, calmodulin was still concentrated in the cell center (Fig. 27) in the same region as the microtubules of the disorganized spindle.

When PtK_2 cells were incubated at 37°C before preparation for tubulin immunofluorescence, a large spindle with many pole-to-chromosome, pole-to-pole, and astral fibers was seen (Fig. 28). However, when cells were incubated at 0°C for 1 h before preparation for immunofluorescence, the cold-labile, pole-to-pole, and astral microtubules were disrupted leaving only the cold-stable, pole-to-chromosome tubules (Fig. 29). Finally, cold-treated cells exhibited unaltered calmodulin localization (Fig. 30).

DISCUSSION

In previous publications, we have described the characterization of specific antibodies to calmodulin (12) and, using the antibodies, the localization of calmodulin in the mitotic apparatus of dividing eukaryote cells (13). In the present paper, we compare the pattern of distribution of tubulin and that of calmodulin throughout mitosis. The localizations of calmodulin and tubulin during mitosis are distinctly different, while at the same

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FIGURES 20–27 Indirect immunofluorescence localization of tubulin or calmodulin in metaphase CHO cells. All figures × 1,200. Bar in Fig. 20, 10 μm.

FIGURE 20 Tubulin of cell subjected to no experimental treatment before processing for immunofluorescence.

FIGURE 21 Calmodulin of cell subjected to no experimental treatment.

FIGURE 22 Tubulin immunofluorescence in cell treated with 0.06 μg/ml colcemid for 1 h at 37°C just before fixing and processing for immunofluorescence. All spindle fluorescence is abolished.

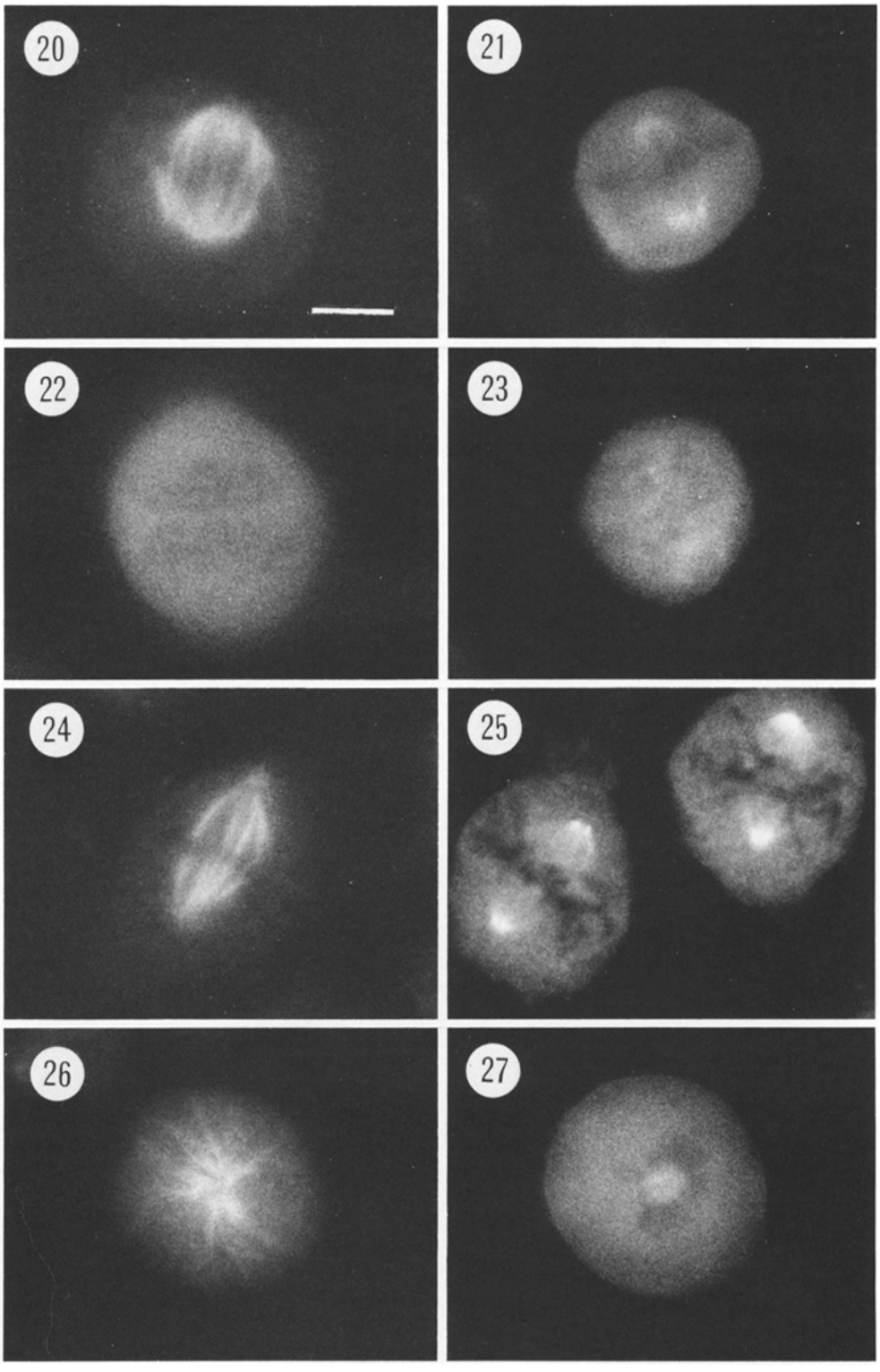
FIGURE 23 Calmodulin immunofluorescence in cell treated with colcemid. All spindle fluorescence is abolished.

FIGURE 24 Tubulin in cell treated with 10 μg/ml cytochalasin B for 1 h at 37°C just before fixing and processing for immunofluorescence. Distribution of tubulin is similar to that of untreated cells.

FIGURE 25 Calmodulin in two cells treated with cytochalasin B. Distribution of calmodulin is similar to that of untreated cells.

FIGURE 26 Tubulin in cell incubated under nitrous oxide atmosphere at 80 lb/in^2 at 37°C for 16 h just before fixing and processing for immunofluorescence. Microtubules of the spindle are disorganized but not depolymerized.

FIGURE 27 Calmodulin in cell incubated under nitrous oxide. Calmodulin is concentrated in the center of the cell, with the chromosomes arranged around the calmodulin-rich central region.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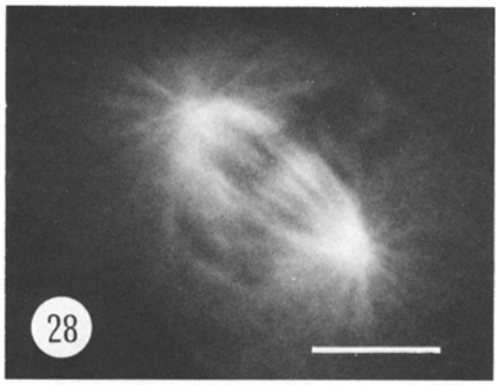

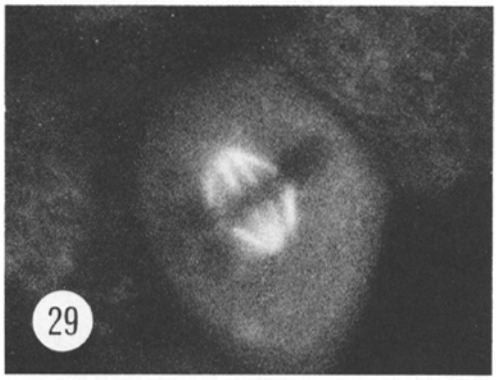

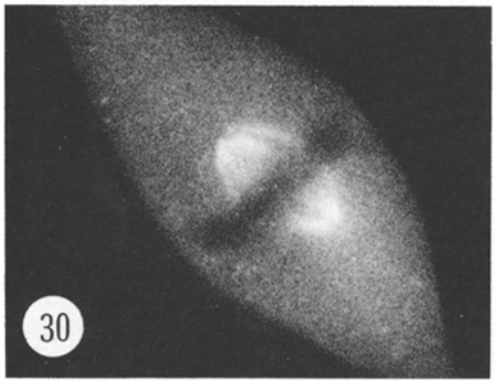

FIGURES 28–30 Indirect immunofluorescence localization of tubulin or calmodulin in metaphase PtK_2 cells. All figures × 1,660. Bar in Fig. 28, 10 μm.

FIGURE 28 Tubulin-specific fluorescence in a cell incubated at 37°C before fixing and processing for immunofluorescence. Microtubule bundles are numerous, extending from the poles to chromosomes and crossing the metaphase plate.

FIGURE 29 Tubulin-specific fluorescence in a cell incubated for 1 h at 0°C before fixing and processing for immunofluorescence. Pole-to-chromosome microtubule bundles survive cold treatment, but no microtubules which cross the metaphase plate region or asters survive.

time both proteins are components of the mitotic apparatus. The localization of calmodulin during mitosis suggests that this calcium-binding protein may be interacting with some of the microtubules or microtubule-associated proteins of the mitotic apparatus. We observed that calmodulin localization in the mitotic apparatus is always, but not exclusively, coincident with regions where disassembly of microtubules is occurring (20–22) and, therefore, we hypothesized that calmodulin might mediate calcium effects on microtubule assembly-disassembly (13). Subsequent experiments demonstrated that calmodulin would indeed prevent assembly or promote disassembly of bovine brain microtubules in vitro in a calcium-dependent manner (14). It should be pointed out that in the experiments reported by Marcum et al. (14), the ratio of calmodulin to tubulin required for maximal effect was high (4–8:1). However, additional experiments have revealed that in vitro effects can be achieved at a molar calmodulin to tubulin ratio of 2:1. These results lend support to the contention that calmodulin may play a physiologic role during chromosome movement.

Although we have substantial evidence which suggests that calmodulin mediates the calcium sensitivity of bovine brain microtubules in vitro, other functions of calmodulin in the mitotic apparatus can be postulated. Recently, calmodulin has been shown to regulate myosin-linked ATPase of muscle and nonmuscle cells by activating the myosin light chain kinase (10, 15). The localization of calmodulin coincident with the actin-containing stress fibers seen in many types of interphase cells further supported this hypothesis (12). Indeed, actin has been shown to be localized in the mitotic apparatus of lysed cells. Sanger (23) localized actin in glycerol-lysed cells by using fluorescent heavy meromyosin. Cande et al. (24) lysed cells with Triton X-100 and found actin to be similarly localized using actin antibodies. Also, using glycerol-extracted cells and fluorescein-labeled subfragment-1 of heavy meromyosin, Schloss et al. (25) found actin in the mitotic spindle. More recently, Herman and Pollard (26), again using fluorescent heavy meromyosin, found actin in the spindle of cells fixed in a manner

FIGURE 30 Calmodulin-specific fluorescence in a cell incubated for 1 h at 0°C before fixing and processing for immunofluorescence. Localization of calmodulin appears unaltered in cold-treated cells.

similar to that used in the present communication. Forer (27), using other methods of analysis, also supports the concept of actin as a component of the mitotic spindle. While some of the observations reported by these workers are not in complete agreement, in general the observations are similar.

The results of Sanger (23, 28), Schroeder (29), and Schloss et al. (25), as well as our own results (13), have shown actin concentrated in the cleavage furrow. Conversely, Cande et al. (24) and Herman and Pollard (26) failed to identify actin in the cleavage furrow. These discrepancies may result from the specific differences in techniques of cell preparation or from differences in the specific properties of antibodies. Perhaps of greater importance were the similarities of the results reported by these investigators. When actin was localized in the spindle, it was found to be concentrated in the region between the chromosomes and spindle poles. Somewhat variable is the observed presence or absence of fluorescence in the interzone region during anaphase. Overall, however, the similarities between the patterns of actin and calmodulin distribution throughout mitosis are considerable and suggest also the possibility of a calmodulin-actin interaction in the mitotic apparatus.

Our results using several treatments which disrupt specific cytoskeletal structures suggest that calmodulin may not be primarily associated with an actin-containing, microfilament-like system in the mitotic apparatus. This conclusion is derived from the results which show that neither tubulin- nor calmodulin-specific fluorescence is changed by cytochalasin B treatment. In support of this conclusion also are the results of Kiehart and Inoué (30) which provide evidence that an actomyosin system is required for cytokinesis but not for karyokinesis. However, another interpretation of the results could be that an actin-containing system is present in the mitotic apparatus but that it is different in structure and response to drugs (e.g., cytochalasin B) or antibodies from the actin-containing structures of the cleavage furrow or the microfilament bundles found in interphase cells. This could be a result of the close association of the actin with spindle microtubules as suggested by Forer (27).

The results reported herein suggest that calmodulin is associated with microtubules. Similar changes in distribution at metaphase, as reflected by specific fluorescence, occur for both tubulin and calmodulin when cells are treated with colcemid or nitrous oxide. The results further support the hypothesis that calmodulin is associated with the kinetochore-to-pole microtubules because cold treatment (31) had little effect on calmodulin localization in the spindle. Controlled disassembly of the kinetochore microtubules is necessary for chromosome separation during anaphase. Further, an analysis of counts of microtubules at various stages of mitosis (32) suggests that the microtubules of the interzone region also depolymerize during telophase, with disassembly progressing from near the reforming nucleus of each daughter cell toward the midbody. Calmodulin is found during mitosis in both regions where microtubule disassembly is known to occur, namely in the half-spindles during anaphase and the interzone during telophase.

Calmodulin was also found associated with the spindle as early as prophase of mitosis, when net assembly of microtubules is occurring. If calmodulin does indeed control microtubule assembly-disassembly in a calcium-dependent manner (14), it is possible that calmodulin may not prevent net assembly of microtubules in the early mitotic apparatus because of low calcium concentrations in the region of the forming spindle. It should be pointed out, however, that congressional movement of chromosomes is occurring during prometaphase and that considerable reduction in overall spindle length occurs between prometaphase and metaphase. Thus, even during the early phase of mitosis, calmodulin may be functionally active in the spindle.

Other roles for calmodulin, in addition to its possible effect on microtubules, may exist. As mentioned earlier, calmodulin has been shown to regulate myosin-linked ATPase of muscle and nonmuscle cells (10, 15) and may have a similar action in the mitotic spindle. Calmodulin could also be involved in the regulation of the Ca^{2+}-ATPase associated with mitosis which Mazia et al. (33), Petzelt and von Ledebur-Villiger (34), and Petzelt and Auel (35) have described. Harris (36) has proposed that Ca^{2+}-ATPase activity may be associated with the smooth endoplasmic reticulum that is in close proximity to the half-spindles of mitotic cells. Thus, calmodulin could also function as a regulator of calcium concentrations in the region of the half-spindles via a calcium-pump system similar to that found in smooth endoplasmic reticulum of muscle and nonmuscle cells. This role is suggested by the recent findings of Hinds et

al. (37). They have shown calmodulin to be a calcium-dependent regulator of the erythrocyte plasma membrane calcium pump. It is, therefore, conceivable that calmodulin may play multiple roles in the regulation and coordination of mitosis.

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