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Calcium Levels Affect the Ability to Immunolocalize Calmodulin to Cortical Microtubules[1]

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Calcium affects the stability of cortical microtubules (MTs) in lysed protoplasts. This calmodulin (CaM)-mediated interaction may provide a mechanism that serves to integrate cellular behavior with MT function. To test the hypothesis that CaM associates with these MTs, monoclonal antibodies were produced against CaM, and one (designated mAb1D10) was selected for its suitability as an immunocytochemical reagent. It is shown that CaM associates with the cortical MTs of cultured carrot (*Daucus carota* L.) and tobacco (*Nicotiana tabacum* L.) cells. Inasmuch as CaM interacts with calcium and affects the behavior of these MTs, we hypothesized that calcium would alter this association. To test this, protoplasts containing taxol-stabilized MTs were lysed in the presence of various concentrations of calcium and examined for the association of CaM with cortical MTs. At 1 μM calcium, many protoplasts did not have CaM in association with the cortical MTs, whereas at 3.6 μM calcium, this association was completely abolished. Control experiments were performed to eliminate alternate explanations including differential antibody binding in the presence of calcium and/or taxol, detergent-induced redistribution of antigen, and epitope masking. The results are discussed in terms of a model in which CaM associates with MTs via two types of interactions, one that occurs in the presence of calcium and another that occurs only in its absence.

Higher plant intracellular Ca^{2+} concentrations fluctuate in response to a number of stimuli, and numerous correlative and experimental studies have established that this ion is an important second messenger (Marmé, 1988; Gilroy et al., 1990). However, it remains unclear how changes in Ca^{2+} levels affect relevant targets. One model for the second-messenger role of Ca^{2+} suggests that this ion interacts with Ca^{2+}-binding proteins, forming complexes that subsequently trigger an intracellular response (Dieter and Marmé, 1988).

The most widely studied Ca^{2+}-binding protein is CaM, a highly conserved, ubiquitous, eukaryotic protein that undergoes a conformational change after binding one to four molecules of Ca^{2+} (Klee, 1988). This conformational change typically alters the ability of CaM to interact with other proteins, thereby modulating their function. For example, Ca^{2+}-CaM complexes activate several enzymes in vitro (Anderson and Cormier, 1978; Dieter and Marmé, 1981; Ranjeva et al., 1983), and these biochemical activities are believed to be of regulatory importance in vivo. It has been widely reported that the binding of Ca^{2+} to CaM produces an activation complex; however, a limited number of reports have shown that CaM alone is capable of stimulating some enzymes (Greenlee et al., 1982; Kilhoffer et al., 1983; Polya, 1983). Additionally, the binding of CaM to chloroplast-membrane fractions (Roberts et al., 1983), fibroblast proteins (Van Eldik and Burgess, 1983), and neuromodulin (Alexander et al., 1988) can occur in a Ca^{2+}-independent manner. Thus, it seems that CaM can function as a positive affecter under conditions in which Ca^{2+} concentrations are either high or low.

Keith et al. (1983) demonstrated that CaM, in the presence of Ca^{2+}, could depolymerize MTs in vivo in gerbil fibroma cells. It has since been demonstrated (using indirect immunocytochemistry) that CaM associates with cytoplasmic MTs in cultured mammalian cells. This association does not require Ca^{2+} because anti-CaM antibodies stained MTs after lysis and incubation in buffers containing 10 mM EGTA (Deery et al., 1984). In plants, Ca^{2+}-CaM can affect cortical MTs in situ (Cyr, 1991), suggesting that Ca^{2+}, via CaM, may integrate MT dynamics in this array with other cellular activities. The goal of this study was to increase our understanding of the interactions among Ca^{2+}, CaM, and the cortical MT array in the cells of higher plants.

Although in situ data implicated Ca^{2+}-CaM in the regulation of the plant cortical MT array, previous immunocytochemical work failed to link CaM to this array but did localize it to the spindle apparatus, phragmoplast, and perhaps the preprophase band of plant cells (Vantard et al., 1985; Wick et al., 1985). It seemed possible that the inability to demonstrate CaM in the plant cortical MT array was due to the use of nonoptimal antibodies. To address this possibility, we produced a mAb to plant CaM for immunocytochemical studies. Immunolocalizations, utilizing protoplasts and suspension cells, have revealed that CaM is associated not only with the arrays described above but also with the cortical MT array.

Surprisingly, the absence of Ca^{2+} strongly influenced the ability to optimally demonstrate CaM in the phragmoplast, spindle apparatus, and cortical array. The possibility of two different types of interactions between CaM and MTs, one requiring Ca^{2+} and one occurring only in its absence, is discussed.

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Abbreviations: APM, amiprophos-methyl; CaM, calmodulin; mAb, monoclonal antibody; MT, microtubule; PM, Pipes and magnesium buffer; PVDF, polyvinylidene fluoride; STOP, stable tubule only protein.

MATERIALS AND METHODS

Plant Material and Protoplast Preparation

Carrot (*Daucus carota* L.) and tobacco (*Nicotiana tabacum* L. var Bright Yellow 2) suspension cells were cultured as previously described (Nagata et al., 1981; Cyr and Palevitz, 1989). Suspension-cultured cells were converted into protoplasts using standard enzymic methods (Cyr, 1991). Incubation in enzymes did not exceed 3 h. After conversion, protoplasts were filtered through cotton, collected by centrifugation at 300*g* for 5 min, and washed twice in PM buffer (50 mM Pipes [pH 6.9], 1 mM $MgSO_4$, 1 mM EGTA) with 0.4 M mannitol added as an osmoticum. PM buffer supplemented with mannitol is referred to as PMM.

In experiments in which stabilization of MTs was necessary, taxol was added to 10 μM 20 min before enzyme removal. Conversely, for the depolymerization of MTs, APM was added to 20 μM 45 min before enzyme removal.

Lysing and Fixing Protoplasts

Protoplasts, resuspended in PMM, were settled onto poly-L-Lys-coated slides (applied as a 1-mg mL^{-1} solution, M_r 300,000; Sigma Chemical Co., St. Louis, MO) for 5 min. PMM was removed by capillary action, and detergent lysis buffer was applied for 5 min. Lysis buffer consisted of 50 mM Pipes (pH 6.9), 1 mM $MgSO_4$, 1 mM EGTA, 10 mM CHAPS detergent, and 10 μg mL^{-1} each of the following protease inhibitors: antipain, aprotinin, chymostatin, pepstatin, and leupeptin. $CaCl_2$ replaced EGTA in experiments involving high levels of Ca^{2+} (above 100 μM). An EGTA/Ca^{2+} buffer system was used when the required Ca^{2+} level was less than 100 μM. When cells were osmotically lysed CHAPS and mannitol were omitted.

After lysis, the extracted cells were fixed for 20 min with 4% (w/v) formaldehyde (made fresh from paraformaldehyde), 0.1% (v/v) glutaraldehyde, 50 mM Pipes (pH 6.9), 1 mM $MgSO_4$, 5 mM EGTA. $CaCl_2$ replaced EGTA when experiments involved Ca^{2+}. The fixative was removed by capillary action, and the slides containing the lysed, fixed protoplasts were washed for 15 min with PBS.

Table I. *ELISA readings for positive hybridoma colonies*

One hundred nanograms of carrot CaM or 100 μg of BSA were absorbed to microtiter plates overnight. After the hybridoma supernatants were blocked with 0.1% BSA plus 0.05% Tween 20, they were adsorbed for 1 h and washed, and a goat anti-mouse alkaline phosphatase reporter antibody was applied for 1 h. The plates were washed and exposed to the colorimetric substrate *p*-nitrophenol phosphate at pH 9.5. The A_{405} of each well was determined with a microtiter plate reader.

Clones Colony	CaM	BSA
	A_{405}	A_{405}
1E7	1.137	0.009
5D12	0.223	0.006
4H6	0.118	0.009
10E2	2.608	0.002
1D10	1.479	0.010

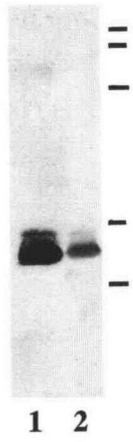

Figure 1. The antibody mAb1D10 is specific for CaM. One microgram of carrot CaM (lane 1) and 50 μg of total carrot proteins (lane 2) were separated by SDS-PAGE on a 15% gel. The polypeptides were transferred to a PVDF membrane and immunoblotted using mAb1D10. The migrated positions of reference polypeptides are indicated on the right and correspond (from top to bottom) to M_r values of 110,000, 84,000, 47,000, 20,100, and 14,200.

Fixation of Suspension-Cultured Cells

Culture medium was removed from 5-d-old cell suspension by centrifugation at 300*g* for 5 min. Fixatives identical with those described for protoplasts were applied for 1 h. Cells were rinsed once in PBS, settled onto poly-L-Lys-coated slides and then permeabilized as described by Kuss-Wymer and Cyr (1992) with minor modifications. Briefly, the permeabilization solution contained 0.5% (w/v) Cellulase YC, 0.05% (w/v) Pectolyase (Seishin Pharmaceutical Co., Ltd, Tokyo) 0.05% (v/v) Triton X-100, 0.25 M mannitol in PM buffer, and 1 μg mL^{-1} of each of the protease inhibitors mentioned above. Cells were rinsed for 10 min in PBS containing 0.05% (v/v) Tween 20 and 0.25 M mannitol.

Immunolocalization of MTs

After fixation, cells and lysed protoplasts were blocked with 3% (w/v) BSA dissolved in PBS and incubated in one of three primary antibodies (anti-carrot tubulin, designated mAb1F8; anti-soybean tubulin, Cyr et al., 1987; anti-carrot CaM, designated mAb1D10) for at least 45 min. After the slides were rinsed for 15 min in PBS, they were incubated in an appropriate secondary antibody, conjugated with fluorescein isothiocyanate for at least 45 min followed by a 15-min rinse with PBS. The slides were mounted in 4 M glycerol, 100 mM Tris (pH 9.0) containing 1 mg mL^{-1} of phenylenediamine, and 1 mg mL^{-1} of Hoechst 33258 (Calbiochem, La Jolla, CA). The slides were viewed with a Zeiss Axioskop (Carl Zeiss, Thornwood, NY) equipped with a 150-W xenon epifluorescent illuminator and ×40 and ×100 plan-Neofluar objectives. Photomicrographs were obtained using Tri-X film.

Production of mAbs

CaM was purified to apparent homogeneity from carrot suspension cells using methods described by Cyr (1991). Mice

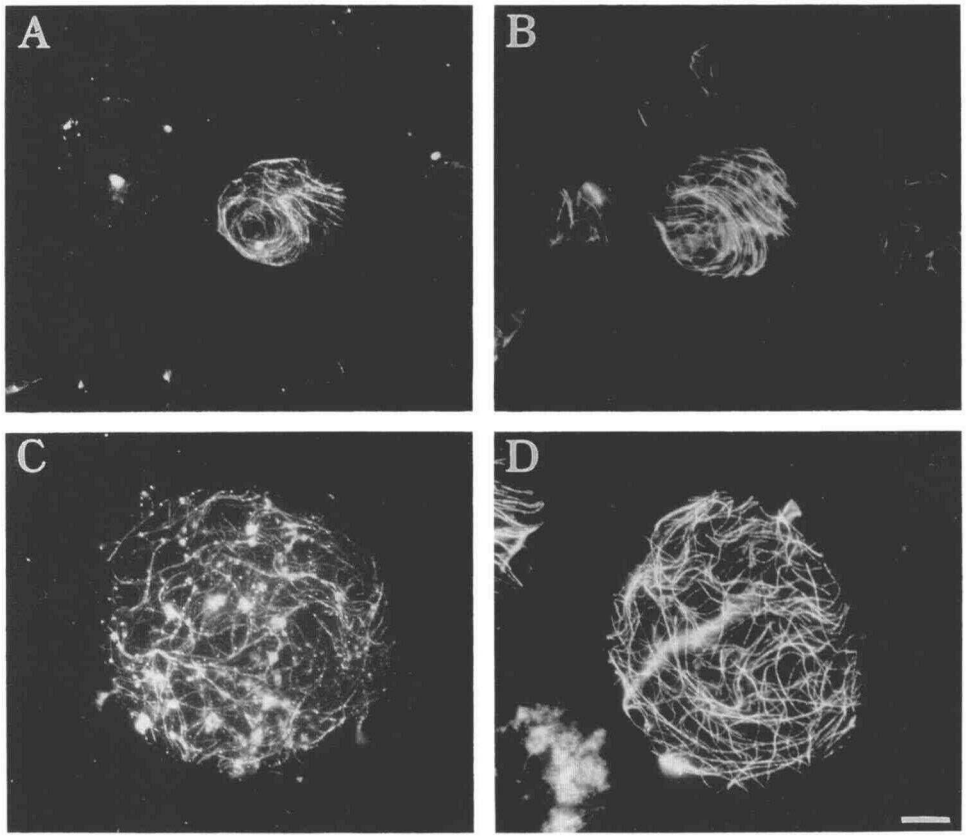

Figure 2. Immunolocalization patterns using mAb1D10 on lysed protoplasts are similar to that seen with anti-tubulin antibody. Carrot (A and B) or tobacco (C and D) protoplasts were reacted with either mAb1D10 (A and C) or an anti-tubulin antibody (B and D). These protoplasts were detergent lysed under isotonic conditions in the presence of 1 mM EGTA. Bar = 10 μm.

(BALB/c females, 6 weeks of age) were immunized on d 0 with 100 μg of CaM emulsified in complete Freund's adjuvant (Calbiochem, La Jolla, CA). On d 14 and 28 the animals were challenged with 50 μg of CaM emulsified in incomplete Freund's adjuvant. On d 40 the animals were test bled, and the one showing the highest serum titer was selected and injected intraperitoneally with 25 μg of CaM in PBS on d 3, 2, and 1 before fusion. After the mouse was killed by cervical dislocation, the spleen was aseptically removed, and the splenocytes were obtained using standard methods (Zola, 1987).

Hybridoma cells were produced by fusing splenocytes with a myeloma cell line (American Type Culture Collection CRL 1580, named X63-Ag.653; sometimes referenced as P3A63) using PEG-induced fusion according to standard procedures (Marusich, 1988), and the fusion products were screened using hypoxanthine/aminopterin/thymidine selection. Supernatants from wells harboring hybridoma colonies were assayed for CaM antibodies using ELISA.

Electrophoresis and Immunoblotting

Total proteins were obtained from suspension-cultured cells by explosive decompression using a French Press at 5000 p.s.i. as described by Cyr and Palevitz (1989). Tubulin was obtained by further processing using DEAE ion-exchange chromatography by the method of Morejohn and Fosket (1982) as modified by Cyr and Palevitz (1989). Proteins were electrophoresed by SDS-PAGE using 15% (w/v) separating gels according to the method of Laemmli (1970). For immunoblot analysis, the proteins were transferred either onto nitrocellulose membranes (Bio-Rad, Richmond, CA) using the method of Towbin et al. (1979) or onto PVDF membranes (Immobilon P; Millipore Corp., Bedford, MA) using the method of Hulen et al. (1991). After transfer, the membranes were blocked overnight with 3% (w/v) BSA in PBS and reacted for a minimum of 1 h at room temperature with the undiluted hybridoma supernatant. The blots were then washed in three changes of PBS plus 0.05% (v/v) Tween-20 and incubated with a goat anti-mouse immunoglobulin G conjugated to alkaline phosphatase, diluted 1:1000 in PBS plus 3% (w/v) BSA for a minimum of 1 h at room temperature. After they were washed as before, the membranes were briefly rinsed in substrate buffer (0.1 M Tris, pH 9.5, 1 mM $MgCl_2$) and incubated at room temperature using the method of Blake et al. (1984).

RESULTS

CaM is reported to be a poor antigen, presumably because it is small, acidic, and very highly conserved (Hulen et al.,

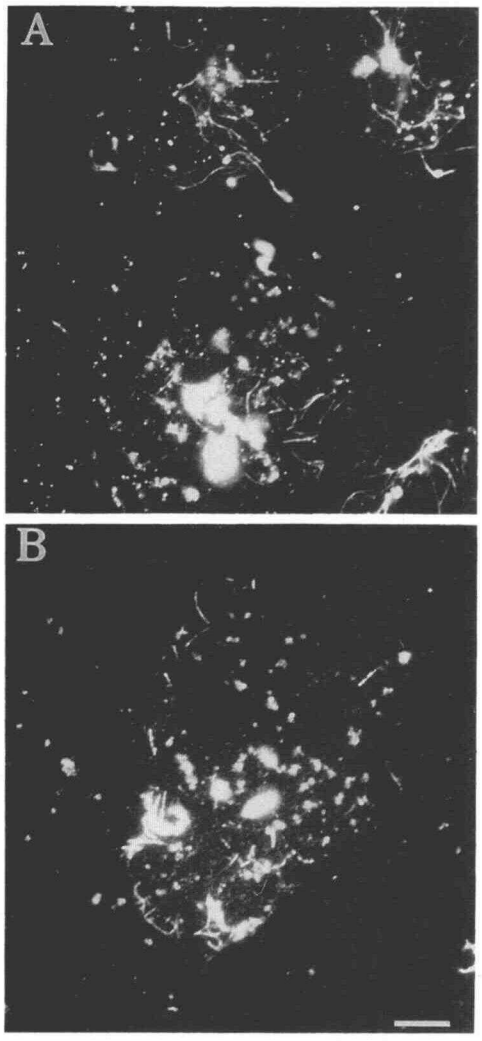

Figure 3. The filamentous staining pattern observed with mAb1D10 is dependent on intact MTs. Tobacco protoplasts were treated with the anti-MT drug APM, lysed in the presence of detergent under isotonic conditions, and reacted with either mAb1D10 (A) or an anti-tubulin antibody (B). Bar = 10 μm.

1991). Not surprisingly, we found relatively few positive hybridoma colonies that secreted antibodies that could be detected using ELISA. A total of 610 colonies (from two fusions) were screened, and 19 of these colonies gave a positive, but often weak response. ELISA data for five positive colonies that were cloned by limiting dilution are listed in Table I. The clone designated 1D10 gave the best combined signals with ELISA, immunocytochemistry, and immunoblotting and, therefore, was used in all subsequent experiments. Antibody-isotyping protocols revealed that mAb1D10 is an immunoglobulin G.

Consistent with previous reports (Van Eldik and Wolchok, 1984; Hincke, 1988; Hulen et al., 1991), it was difficult to obtain satisfactory CaM signals using standard immunoblot methods (Towbin et al., 1979), presumably due to the poor adherence of CaM to nitrocellulose membranes. However, satisfactory results were obtained utilizing a PVDF membrane and phosphate transfer buffer followed by glutaraldehyde fixation (Hulen et al., 1991). Figure 1 shows that mAb1D10 recognized two closely migrating polypeptides among total carrot proteins that co-migrated with purified carrot CaM. Similar results were observed using total tobacco proteins (data not shown). Electrophoretic variants of CaM are not unusual (Williams et al., 1984). Jablonsky et al. (1991) showed that CaM, from a variety of plant sources, often migrates as a major and minor doublet, with the slow migrating form representing a posttranslational modification in which a terminal Lys residue is removed from the carboxy terminus.

Indirect immunofluorescent staining patterns from mAb1D10 immunolocalizations on lysed carrot protoplasts revealed filamentous signals similar to those seen with anti-tubulin antibodies (Fig. 2, A and B). A fluorescent signal was not observed when culture medium was substituted for the hybridoma supernatant (data not shown). Additionally, similar distribution patterns were found in tobacco protoplasts (Fig. 2, C and D), which are larger than carrot protoplasts and consequently lend themselves better to photodocumentation. Therefore, the figures in this paper depict results obtained from tobacco cells, but in all experiments, carrot cells were examined and found to behave similarly.

One caveat of visualizing antigens in detergent-lysed cells is that artifactual rearrangements can occur during lysis (Melan and Sluder, 1992). To investigate this possibility, in relation to the filamentous pattern observed with mAb1D10, protoplasts were lysed osmotically (in the absence of detergent and mannitol) before fixation and then processed for immunolocalization. Again, a filamentous pattern was observed (data not shown), indicating that the inclusion of detergent did not cause artifactual redistribution of the antigen.

The filamentous pattern observed with mAb1D10 is similar to the MT localization seen with anti-tubulin antibodies. To verify that this staining pattern represented the localization of CaM to MTs, cells were incubated with the MT-destabilizing herbicide APM (Morejohn and Fosket, 1984) before lysis. These conditions resulted in the obliteration of MTs and consequently in a loss of fluorescent signal with both mAb1D10 and the anti-tubulin antibodies (Fig. 3, A and B). These data indicate that CaM associates with cortical MTs.

It is important to note that the above observations were made when protoplasts were lysed in the presence of EGTA. CaM typically binds to proteins in a Ca^{2+}-dependent manner; therefore, we wanted to determine what effect Ca^{2+} would have on the ability to visualize CaM associated with cortical MTs. However, because cortical MTs are sensitive to Ca^{2+} (Cyr, 1991), it was necessary to stabilize the MTs with the drug taxol before the addition of Ca^{2+}. Taxol stabilization did not interfere with the ability of CaM to bind to cortical MTs in the absence of Ca^{2+} (Fig. 4A). It is notable that a decrease in CaM-specific signal was observed at 1 μM Ca^{2+} (Fig. 4B), and at 3.6 μM Ca^{2+} the signal was no longer demonstrable on the taxol-stabilized MTs (Fig. 4C). However, MTs were clearly present as evidenced by the binding of anti-tubulin antibodies (Fig. 4D). Furthermore, higher concentrations of Ca^{2+} (up to 1 mM) gave negative immunocytochemical results with mAb1D10 (data not shown).

It is possible that the failure to immunolocalize CaM to

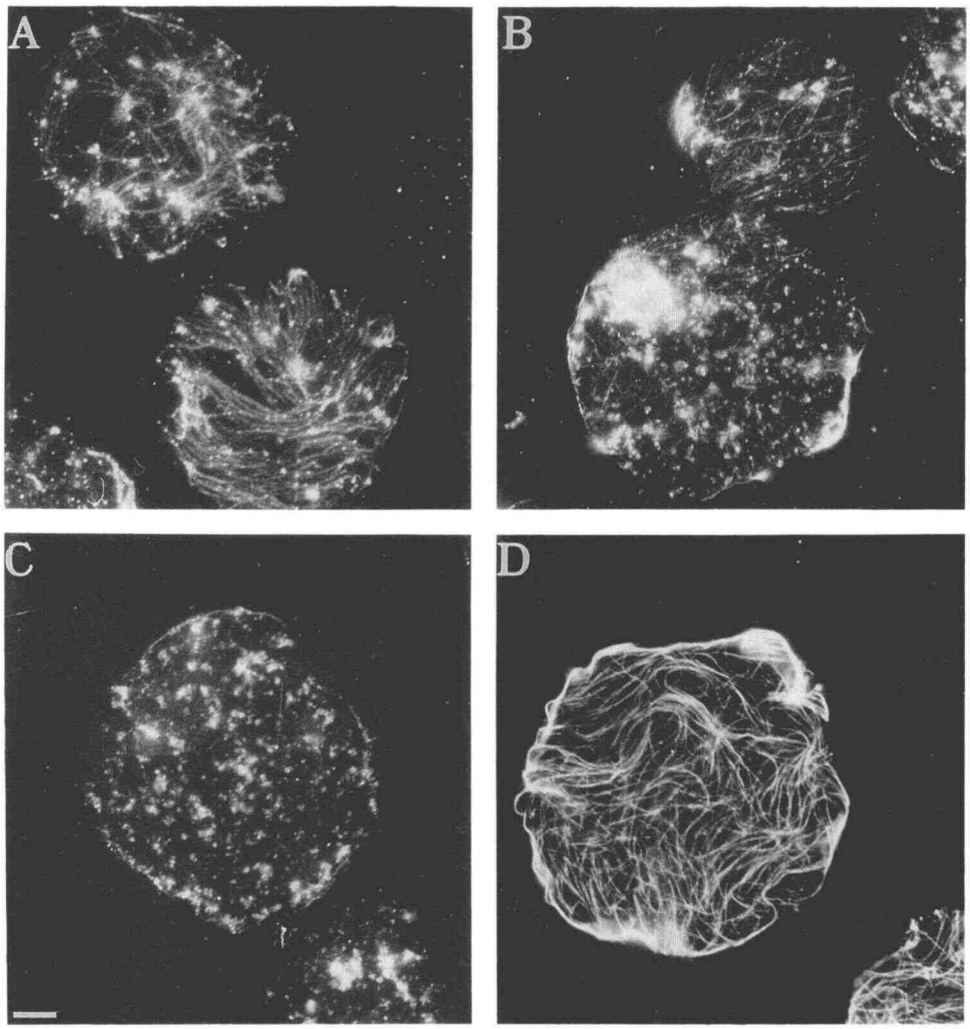

Figure 4. CaM binds to cortical MTs in the absence of Ca^{2+}. Taxol-treated tobacco protoplasts were detergent lysed in the presence of EGTA (A), 1 μM Ca^{2+} (B), 3.6 μM Ca^{2+} (C), or 500 μM Ca^{2+} (D) and immunolocalized with mAb1D10 (A, B, and C) or an anti-tubulin antibody (D). Bar = 10 μm.

cortical MTs, in the presence of Ca^{2+}, is the result of epitope masking that occurs as a result of a Ca^{2+}-dependent conformational change in CaM (Harper, 1983). To investigate whether the CaM epitope recognized by mAb1D10 behaves cryptically in the presence of Ca^{2+}, the binding characteristics for this antibody were determined in the presence and absence of Ca^{2+}. The antibody has a somewhat higher affinity for CaM in the presence of Ca^{2+} in the ELISA (Fig. 5).

Another possible explanation for the inability to immunolocalize CaM in a Ca^{2+}-dependent fashion is that a CaM-binding protein could associate with CaM and sterically interfere with the binding of mAb1D10. If this hypothesis is correct, then it should be possible to conceal the epitope in the presence of Ca^{2+} and then expose it by chelation with EGTA. Experimentally, this was done by lysing taxol-treated protoplasts in the presence of Ca^{2+} and then rinsing the extracted cells with a large excess of Ca^{2+}-free buffer (containing EGTA). The preparations were then fixed and processed for immunolocalization. These protoplasts did not have significant amounts of CaM in association with the cortical MTs (Fig. 6A), even though MTs were clearly present, as evidenced by tubulin immunolocalizations treated under identical conditions (Fig. 6B). This indicates that the failure to immunolocalize CaM in the presence of Ca^{2+} was not due to a blocking event in which a CaM-binding protein obscured the epitope recognized by mAb1D10. Rather, the inability to detect CaM after exposure to Ca^{2+} is due to its disassociation from MTs.

Lysed protoplasts were chosen as our main experimental system because the high levels of soluble cytosolic CaM made it difficult to discern CaM in association with cortical MTs in intact cells. In addition, the cell wall is capable of sequestering large amounts of Ca^{2+} (DeMarty et al., 1984); therefore, protoplasts provided a cell type in which Ca^{2+} concentrations could be known with a reasonable degree of certainty, especially when comparing treatments that included EGTA or exogenous Ca^{2+}. However, suspension-cultured cells were also examined. Although it was more difficult to discern and

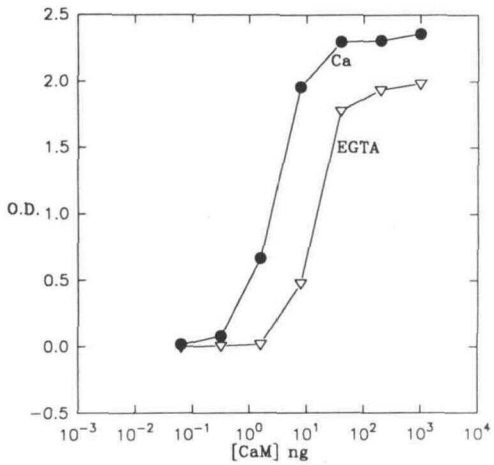

Figure 5. The mAb 1D10 binds CaM in the presence and absence of Ca^{2+}. Different amounts of carrot CaM were adsorbed overnight in microtiter plates in the presence of 1 mM Ca^{2+} (closed circles) or in its absence (1 mM EGTA; open triangles). The plates were processed for ELISA as described for Table I, except Ca^{2+} or EGTA was maintained in all solutions and Tris-buffered saline was substituted for PBS. Each point represents the mean value from three triplicate assays. Negative results were observed with bovine CaM. SD bars are not shown because they are smaller than the symbols.

document the association of CaM with cortical MTs in intact suspension-cultured cells, mAb1D10 did immunolocalize to these MTs (Fig. 7A), as well as to those in squashed apical meristematic root cells (data not shown). However, as shown with protoplasts, CaM localized to MTs only in the presence of EGTA. When cells were pretreated with 20 mM $CaCl_2$, the antibody no longer reacted with the MTs (Fig. 7B). In these experiments, high external Ca^{2+} concentrations were used because plant cells have efficient Ca^{2+} pumps, which maintain cytoplasmic concentrations at low levels (Hepler and Wayne, 1985). Although the addition of high Ca^{2+} concentrations drive cytoplasmic Ca^{2+} upward, the actual magnitude of this increase is uncertain. Immunolocalizations with anti-tubulin antibodies indicated that normal-appearing MTs were present in all cells examined.

As previously reported (Vantard et al., 1985; Wick et al., 1985), CaM associates with the mitotic apparatus; however, we found that this association was also highly influenced by the addition of Ca^{2+}. In the presence of EGTA, CaM localized to the spindle apparatus and kinetochore MTs were readily discernible (Fig. 8A), whereas in the presence of Ca^{2+}, the spindle-associated fluorescence was considerably reduced and lacked MT detail (Fig. 8C). CaM was also observed in the phragmoplast (data not shown).

A number of drugs affect the function of CaM (Allan and Hepler, 1989). Two drugs, trifluoperazine and *N*-(6-aminohexyl)-5-chloro-1-napthalenesulfonamide, were applied to protoplasts to investigate whether they altered CaM localization. Protoplasts, treated with 20 μM trifluoperazine, had a variable diminished signal in the cortical arrays when compared to untreated controls (data not shown), and therefore, their usefulness in future studies is uncertain. No inhibition of MT signal, attributable to CaM, was evident with 20 μM *N*-(6-aminohexyl)-5-chloro-1-napthalenesulfonamide.

DISCUSSION

It is possible that Ca^{2+}, in conjunction with CaM, aids in orchestrating MT function with other cellular events in higher plants. An earlier report showed that Ca^{2+}, via CaM, affects the behavior of cortical MTs in lysed carrot protoplasts (Cyr, 1991). The observation that Ca^{2+}-CaM alters the stability of cortical MTs was puzzling because previous reports in plants did not reveal immunolocalization of CaM to these MTs (although CaM is known to associate with interphase MTs in animal cells; Deery et al., 1984).

This study has shown, via immunocytochemical techniques, the localization of CaM to the plant cortical MT array. There are at least two possibilities why other immunocyto-

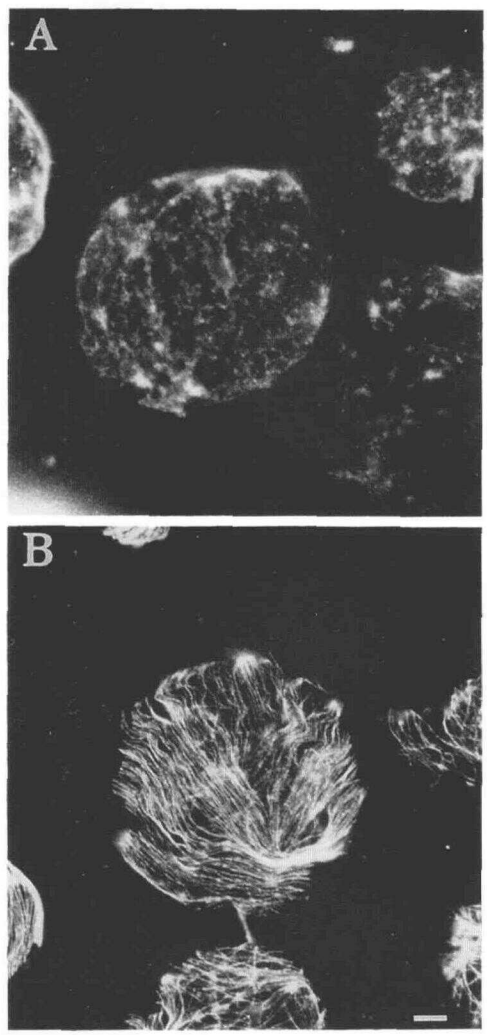

Figure 6. Ca^{2+} does not mask the epitope recognized by mAb1D10 in situ. Taxol-stabilized tobacco protoplasts were detergent lysed in the presence of 500 μM Ca^{2+}, then rinsed, and fixed with EGTA-containing buffers. The lysed protoplasts were immunolocalized with mAb1D10 (A) or anti-tubulin antibody (B). Bar = 10 μM.

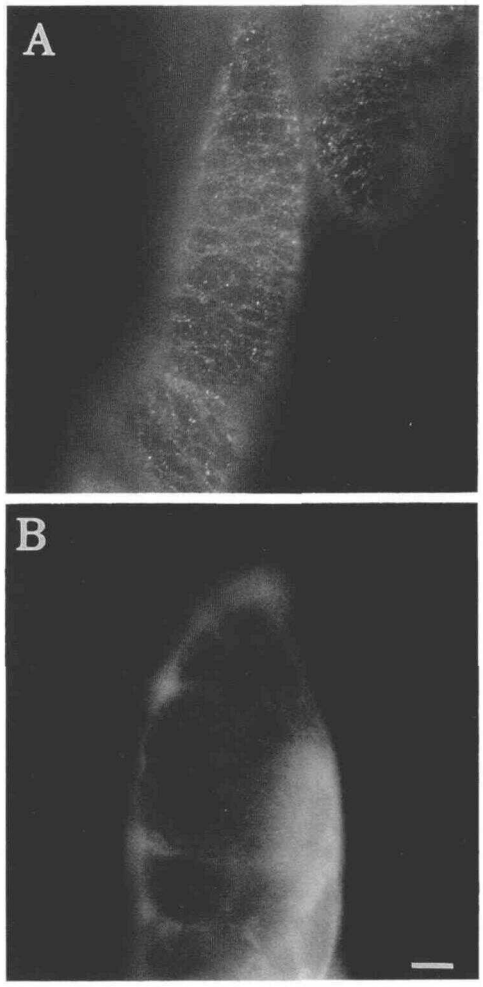

Figure 7. The association of CaM with MTs is observed in intact suspension-cultured cells. Cultured tobacco cells were pretreated without (A) or with (B) 20 mM Ca^{2+}, then fixed, and processed for immunolocalization with mAb1D10. Bar = 10 μm.

chemical studies did not detect this association in plants. First, the antibody used in this study may have a relatively high affinity for plant CaM, which permits the detection of lower amounts of antigen. This, combined with improved microscope optics, may have enhanced the visualization of this association. Second, the antibodies used in previous studies may have had a low affinity for the Ca^{2+}-free form of CaM. Either possibility (or both) would have made it difficult to detect CaM in the cortical MT array.

mAbs recognize a single epitope, which can be shared with other proteins. For example, Ca^{2+}-dependent protein kinase shares some homology with CaM and localizes to the microfilament component of the cytoskeleton (Putnam-Evans et al., 1989). However, mAb1D10 staining patterns are dependent on intact MTs, and immunoblot analysis of total proteins revealed two, closely migrating polypeptides that co-migrated with purified plant CaM (Ca^{2+}-dependent protein kinase has an M_r of about 53,000). It is most plausible that the results presented in this study represent an authentic intracellular localization of CaM and not of a protein possessing a similar epitope.

Immunolocalization studies of soluble proteins (such as CaM) have been questioned because of the possibility of artifactual rearrangements that may occur during processing. Melan and Sluder (1992) demonstrated that five fluorescently derivatized proteins could be localized within the cell after microinjection, followed by detergent extraction and aldehyde fixation. Pertinent to our study was their finding that a small number of cells (2%) had BSA weakly associated with the cytoskeleton in PtK_1 cells. This observation clearly indicates that caution must be used when interpreting immunolocalization results of soluble proteins in detergent-extracted cells, i.e. when faint cytoskeletal images are observed in a low number of cells. The results obtained in this study using mAb1D10 were observed in the majority of cells examined (>95%), and the images were easily seen using a ×40 objective. It is relevant to note also that Melan and Sluder did not notice a cytoskeletal pattern with BSA if the cells were fixed before detergent treatment; however, under similar conditions, we did observe CaM to associate with cortical MTs.

It is remarkable that the ability to localize CaM to cortical MTs is dependent on the absence of Ca^{2+} and that the signals associated with the spindle and phragmoplast were also markedly enhanced. One might ask whether the failure to immunolocalize CaM efficiently in the presence of Ca^{2+} is due to a detection artifact rather than reflective of the in vivo situation. It should be noted that mAb1D10 showed a slightly higher affinity to CaM in the presence of Ca^{2+} with the ELISA, indicating that, if any detection bias exists, it is to underestimate the amount of CaM bound in the absence of Ca^{2+}.

The data presented in this manuscript indicate that, at low Ca^{2+} concentrations, CaM associates strongly with MTs; however, when Ca^{2+} concentrations increase, the affinity of CAM for MTs decreases, and CaM disassociates more readily from MTs. The concentration at which CaM begins to lose its affinity for MTs in vivo is unknown; however, CaM associated with cortical MTs was difficult to detect in most protoplasts at 1 μM Ca^{2+}. At 3.6 μM Ca^{2+}, it was impossible to find protoplasts that showed this association. Further studies (perhaps using microinjected fluorescently derivatized CaM) are required to determine the in vivo association characteristics of CaM with MTs. We predict that at "average" or "resting" intracellular Ca^{2+} concentrations (10^{-7} M), CaM associates with MTs, and as that Ca^{2+} concentrations increase ($\geq 10^{-6}$), CaM disassociates. Regardless of the precise affinities that CaM has for MTs, it is perhaps more important to consider the relevance of the association.

It is important to note that in a lysed cell the majority of soluble CaM has been extracted. However, the data reported here show that CaM can also have a cellular location in which it is insoluble, e.g. bound to the cytoskeleton. Thus, in the cell, it appears that CaM can occur in a soluble and an insoluble state, depending on the regional Ca^{2+} concentration. This characteristic has important implications for the putative role of CaM as an MT regulator. For example, in the soluble state, CaM is free to diffuse throughout the cell (including in juxtaposition to a MT). As Ca^{2+} levels change in the vicinity of a CaM molecule, it could readily associate

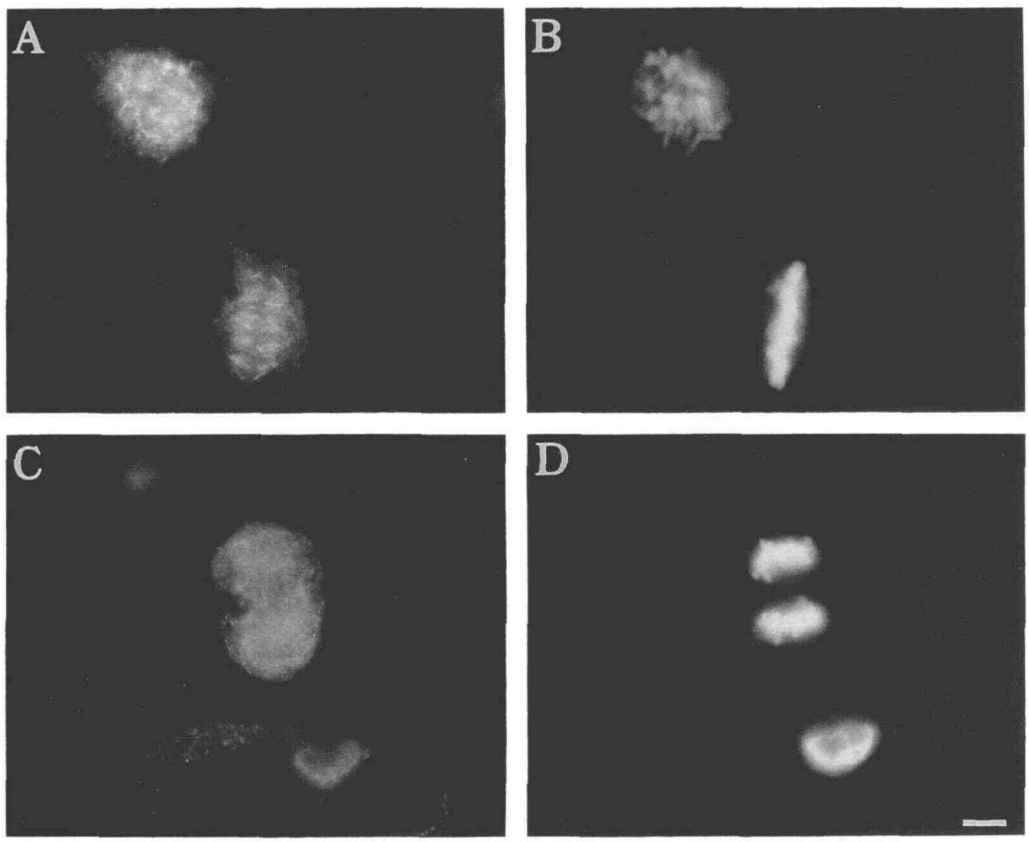

Figure 8. Ca^{2+} affects the amount of CaM that associates with spindle MTs. Taxol-stabilized protoplasts were detergent lysed in the absence of Ca^{2+} (A and B) or in its presence (500 μM; C and D). After fixation, they were immunolocalized with mAb1D10 and stained with the chromatin-binding dye, Hoechst 33258. The localization of mAb1D10 is shown in A and C, and the chromatin pattern in the identical cells is shown in B and D. Bar = 10 μm.

with a nearby MT; likewise, further change in Ca^{2+} levels would induce the disassociation of CaM from the MT. The ability to exist in both the soluble and insoluble state (depending on Ca^{2+} levels) makes CaM ideally suitable as a regulatory molecule. For CaM to participate in the regulation of MTs, its association and dissociation must alter the behavior of MTs, and it is important to consider the consequences of these events.

Cyr (1991) demonstrated that protoplasts lysed in the presence of Ca^{2+} have unstable MTs. However, protoplasts lysed in the presence of EGTA possess MTs that are insensitive to subsequent Ca^{2+} exposure; furthermore, the Ca^{2+} sensitivity of these MTs can be reconstituted with the addition of Ca^{2+} and CaM. Hence, this earlier work indicates the presence of a Ca^{2+}-dependent CaM/MT interaction.

The present immunocytochemical data showing the requirement for a lack of Ca^{2+} for the binding of CaM to MTs, together with the previous reconstitution data indicating Ca^{2+}-dependent CaM/MT interactions, suggest that cortical MTs possess two binding sites for CaM, one requiring Ca^{2+} and the other requiring its absence. This hypothesis has an underlying corollary, namely, that MTs that have CaM bound in a Ca^{2+}-free state are stable, whereas those binding CaM in a Ca^{2+}-dependent state are unstable. Although the data are consistent with the existence of two types of CaM/MT interaction, the molecular character of these two possible binding sites is unknown.

Reports indicate that CaM can bind directly to tubulin (Kumagai and Nishida, 1979). However, because of the relatively low affinity of this interaction, it is unclear whether this association occurs in vivo. It is more likely that physiologically relevant interactions occur via interactions with MT-binding proteins. Such interactions can be demonstrated biochemically, and the best characterized interaction of this type is between CaM and a protein referred to as STOP. It is interesting that, in the presence of Ca^{2+}-CaM, STOPs disassociate from MTs, and concomitantly, the tubulin polymer loses its cold-stable characteristic (Margolis et al., 1986). STOPs have not been found in plants, but homologs may exist.

MT-binding proteins that bind CaM in a Ca^{2+}-dependent fashion have been identified (Cyr, 1991); therefore, future experiments should concentrate on identifying MT-binding proteins to which CaM binds in the absence of Ca^{2+} as well. In vitro MT assembly assays should then reveal whether these two classes of proteins have opposing activities on MT assembly and/or dynamics.

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