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Evidence for Opposing Effects of Calmodulin on Cortical Microtubules[1]

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Microtubule integrity within the cortical array was visualized in detergent-lysed carrot (*Daucus carota* L.) protoplasts that were exposed to various exogenous levels of Ca^{2+} and calmodulin (CaM). CaM appears to help stabilize cortical microtubules against the destabilizing action of Ca^{2+}/CaM complexes at low Ca^{2+} concentrations, but not at higher Ca^{2+} concentrations. The hypothesis that CaM interacts with microtubules at two different sites, determined by the concentration of Ca^{2+}, is supported by the effects of the CaM antagonists *N*-(6-aminohexyl)-1-naphthalene-sulfonamide and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfanamide (20 μM) and by affinity chromatography. Two classes of proteins were identified that interact with tubulin and bind to CaM. One class required Ca^{2+} for CaM binding, whereas the second class bound only when Ca^{2+} concentrations were low (<320 nM). Thus, CaM's ability to have two opposing effects upon microtubules may be regulated by the concentration of intracellular Ca^{2+} and its differential interactions with microtubule-associated proteins. Experimental manipulation of intracellular Ca^{2+} concentrations, as monitored by Indo-1, revealed that the effect of Ca^{2+} is specific to the cortical microtubules and does not affect actin microfilaments in these cells.

Cellular elements such as microtubules must operate harmoniously within the cell under a variety of conditions. To understand how the cell orchestrates the behavior of microtubules, the molecular conductors that direct intracellular signals must be identified and their effects on microtubules characterized. Ca^{2+} is thought to be one of these regulators.

Ca^{2+}, a well-known signaling molecule (Gilroy et al., 1991; Bush, 1993, 1995), binds to a number of effector proteins, including CaM (Allan and Hepler, 1989; Roberts and Harmon, 1992), which, in turn, can interact with myriad intracellular targets, including microtubules (Keith et al., 1983; Cyr, 1991a). Ca^{2+} binds to CaM via four EF-hands, each of which possesses a characteristic helix-loop-helix motif. Upon binding to Ca^{2+}, CaM changes from an extended configuration (with two EF-hands on each arm) to one that is more compact (Klee, 1988; Török and Whitaker, 1994). The configuration of the compact form is variable due to a coiled linker region termed the variable expansion joint (Török and Whitaker, 1994). This flexibility allows CaM to interact with a variety of different CaM-binding proteins (Klee, 1988; Török and Whitaker, 1994).

Microtubules are prominent components of the cytoskeleton and are used by the cell in a number of diverse processes such as the orientation of cellulose microfibrils, nuclear positioning, chromosome segregation, and the assembly of new cell plates (Goddard et al., 1994). These dynamic polymers of tubulin interact with MAPs, thereby modulating microtubule behavior (Cyr, 1991b; Schellenbaum et al., 1992). MAPs also interact with a number of other cellular components, including CaM. Microtubule-associated proteins that interact with CaM include the stable, tubule-only proteins (Margolis et al., 1986; Pirollet et al., 1992) MAP2 and tau (Kotani et al., 1985) and a homolog to EF-1α (Durso and Cyr, 1994b). These interactions with CaM have important functional repercussions: stable, tubule-only proteins lose their ability to stabilize microtubules (Pirollet et al., 1992), tau and MAP2 lose their ability to interact with actin (Kotani et al., 1985), and EF-1α loses its ability to bundle microtubules (Durso and Cyr, 1994b). The physiological significance of these interactions is demonstrated by the observation that the in situ destabilizing effect of Ca^{2+} on microtubules requires the participation of CaM (Keith et al., 1983; Cyr, 1991a).

Some of CaM's more subtle features have a profound influence on its molecular character and its role as a regulator. For example, the four Ca^{2+} molecules that bind to CaM do not associate simultaneously, but rather sequentially. This sequential binding is due to the unique arrangement of the EF-hands, which permits strong cooperation between the four Ca^{2+}-binding sites on each molecule, resulting in two high-affinity sites (K_d values about 10^{-6} M) and two low-affinity sites (K_d values about 10^{-5} M; Klee, 1988). Target proteins can also exert a strong influence on the Ca^{2+}-binding capabilities of the protein. For example, in the presence of the target protein phosphodiesterase (Gregori et al., 1985), CaM's affinity for Ca^{2+} is enhanced 2- to 10-fold at the high-affinity Ca^{2+} binding sites, and by two orders of magnitude at the low-affinity sites. As CaM binds to Ca^{2+}, its affinity for phosphodiesterase likewise increases from 10^{-3} to 10^{-10} M. Thus, there is strong cooperation between the binding of Ca^{2+} to CaM, the subse-

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Abbreviations: CaM, calmodulin; EF-1α, elongation factor 1α; MAP(s), microtubule-associated protein(s); W5, *N*-(6-aminohexyl)-1-napthalene-sulfonamide; W7, *N*-(6-aminohexyl)-5-chloro-1-napthalenesulfanamide.

quent interaction of CaM with its target proteins, and a feedback enhancement in the ability of CaM to bind progressively more Ca^{2+}. This interplay in cooperative binding allows CaM to form loose associations with target proteins, which then typically become progressively tighter and more active as Ca^{2+} levels rise (Klee, 1988). Although the binding of CaM to its target enzyme is typically enhanced as CaM binds additional Ca^{2+}, decreases have been reported (Cimler et al., 1985; Baudier et al., 1991). Relevant to the present study is the observation that CaM's affinity for microtubules can decrease at higher concentrations of Ca^{2+}.

Fisher and Cyr (1993) raised an antibody against carrot (*Daucus carota* L.) CaM, and immunocytochemistry revealed that CaM associates with the cortical microtubules; however, this localization was sensitive to Ca^{2+} and occurred only when EGTA was included in the preparations (although microtubules were clearly present under the experimental conditions). This observation is paradoxical because cortical microtubules are destabilized by Ca^{2+}/CaM (Cyr, 1991a). How can CaM simultaneously lose its affinity for microtubules, yet still participate actively in their destabilization? One possible explanation is that CaM binds to microtubules at low [Ca^{2+}], but in the presence of elevated [Ca^{2+}] disassociates and causes the passive destabilization of microtubules. However, this is unlikely because stripping CaM from microtubules via Ca^{2+} does not lead to destabilization (Cyr, 1991a; see also Fig. 1 herein). An alternative hypothesis is that CaM can associate with microtubules at two different sites: one site is Ca^{2+}-sensitive (i.e. it is disfavored by Ca^{2+}) and the other is Ca^{2+}-dependent (i.e. it requires Ca^{2+}). The data presented in this study are consistent with this alternative hypothesis and, moreover, suggest that the Ca^{2+}-sensitive site is involved in protecting microtubules against the destabilizing action by Ca^{2+}/CaM at the second site.

MATERIALS AND METHODS

Cell Culture and Protoplast Preparation

Carrot (*Daucus carota* L.) suspension cultures were maintained as previously described (Cyr and Palevitz, 1989). Protoplasts were produced using 1% Cellulase YC and 0.1% Pectolyase Y-23 (Seishin Pharmaceutical, Tokyo, Japan) with 0.35 M mannitol added as an osmoticum. Incubation in enzymes did not exceed 1.5 h. After conversion, the protoplasts were filtered through cotton, collected by centrifugation at 300*g* for 5 min, and washed twice with PMM buffer (50 mM Pipes, pH 6.9, 1 mM $MgSO_4$, 1 mM EGTA, and 0.35 M mannitol).

Isolation of CaM and Estimation of CaM Levels in Suspension Cells

CaM is a highly conserved protein (Wylie and Vanaman, 1988), and from both carrot and mammalian sources it is equally effective at destabilizing cortical microtubules in lysed carrot protoplasts (Cyr, 1991a). Experimental and phylogenetic data indicate that the source of CaM is not an experimental variable for destabilizing cortical microtubules; therefore, we used a well-characterized source material (bovine brain) from which high yields of CaM were obtained. CaM was isolated using a combination of isoelectric precipitation, heat treatment, and hydrophobic chromatography (Gopalakrishna and Anderson, 1982). The final purity was 99.9% as judged by SDS-PAGE. To estimate the amount of CaM in the suspension cells used in this study a similar purification scheme was used, and the final yield of CaM was divided by the total amount of crude starting proteins.

Ca^{2+} Measurements

An EGTA/Ca^{2+} buffer system was typically used in experiments that required a free-Ca^{2+} concentration below 100 μM. To verify the actual free-Ca^{2+} concentration, a Ca^{2+} electrode (Orion, Boston, MA) was used that had been calibrated with commercial Ca^{2+} standards from two sources (World Precision Instruments, New Haven, CT, and Molecular Probes, Eugene, OR). This electrode responded linearly down to 100 nM and enabled an accurate titration of the Ca^{2+}-EGTA (1 mM EGTA) buffer system to achieve the desired concentrations of Ca^{2+}.

To estimate the intracellular free-Ca^{2+} levels, the pentapotassium salt of Indo-1 (Molecular Probes) was loaded into cells under acidic conditions. The protoplasts were suspended in a solution containing 20 mM dimethylglutaric acid, pH 4.5, 0.35 M mannitol, and 20 μM Indo-1 for 30 min. The protoplasts, now loaded with Indo-1, were collected by centrifugation and washed several times with PMM buffer. The cells were then placed into medium containing either high or low concentrations of Ca^{2+} (100 or 1 mM, respectively). The cells were imaged within 15 min on a laser scanning confocal microscope (model LSM 410, Zeiss) equipped with a UV laser (364 nm, Coherent Ltd., Auburn, CA) and an 80/20 beam splitter (Zeiss). Emitted light was detected simultaneously at 400 to 435 nm, and wavelengths greater than 460 nm were detected using an interference filter (Zeiss) at each of the two photomultiplier detectors. A ratio of the two resulting images was obtained, and the average pixel values within the cells were determined using the morphometric software supplied by Zeiss. To obtain a standard curve, reference Ca^{2+} solutions (Molecular Probes) were mixed with 1 μM Indo-1 and a suspension of 5-μm latex beads was made. The beads facilitated focusing on the same focal plane to ensure high-fidelity measurements of each calibration solution. The average pixel value, as a function of [Ca^{2+}], was plotted.

Protoplast Lysis and Treatments

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) for 5 min. Excess PMM was removed by wicking with blotting paper, 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 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, and 10 μg mL^{-1} each of the following protease inhibitors: antipain, aprotinin, chymostatin, pepstatin, and leupeptin. The

extracted protoplasts were rinsed briefly with 50 mM Pipes, pH 6.9, and 1 mM $MgSO_4$, and exposed to the various concentrations of Ca^{2+} and CaM for 15 min at room temperature. To remove endogenous CaM that resided on the microtubules prior to Ca^{2+} treatments, the lysed protoplasts were exposed to 200 μM Ca^{2+} for 1 min and were immediately and thoroughly rinsed with PM (PMM without mannitol). This treatment effectively removes CaM from the microtubules without causing their destabilization (see Fig. 1).

Fixation and Immunolocalization of Microtubules in Lysed Cells

After treatments the protoplasts 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 $MgSO_4$, and 5 mM EGTA. The fixative was removed by wicking with blotting paper, and the slides were washed for 5 min with PBS.

After fixation the protoplasts were blocked for 5 min with 3% (w/v) BSA, dissolved in PBS, and incubated with a monoclonal antibody raised against carrot tubulin (designated mAb1F8) or carrot CaM (designated mAb1D10; Fisher and Cyr, 1993) for at least 45 min. After the slides were rinsed for 15 min in PBS, they were incubated in a goat anti-mouse fluorescein isothiocyanate-conjugated antibody (Sigma) 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} phenylenediamine (to prevent fluorescent fading) and 1 mg mL^{-1} Hoescht 33258 (Calbiochem) to visualize the nuclei. The slides were viewed with a Zeiss Axioskop equipped with a 150-W xenon epifluorescent illuminator and X40 and X100 plan-Neofluar objectives. Photomicrographs were obtained using Tri-X Pan film (Kodak), which was exposed and developed normally.

Fixation and Immunolocalization of Microtubules and Actin Filaments in Intact Protoplasts

Protoplasts were produced as described above. Half were exposed to 100 mM Ca^{2+} in carrot medium (containing mannitol as an osmoticum) for 15 min, and the rest were kept in medium only (containing 3 mM Ca^{2+}), centrifuged at 300*g* for 3 min, and the supernatant was discarded. The protoplasts were fixed and dehydrated as described by Andersland et al. (1994). Briefly, the fixative was the same as described above, but the gluteraldehyde was reduced to 0.05% (w/v) and the protoplasts were settled on the slides during the last 2 min of fixation. For dehydration, the slides were placed in methanol at −20°C for 10 min, and rinsed with PBS. The protoplasts were exposed to either an anti-tubulin soy antibody raised in rabbit or a monoclonal antibody raised in mouse against phalloidin-stabilized pea root actin (designated mAb3 H11) for 1 h. After rinsing in separate beakers of PBS plus 0.05% Tween 20 for 15 min, they were exposed to either a goat anti-rabbit or a goat anti-mouse secondary antibody (both fluorescein isothiocyanate-conjugated, Sigma) for 1 h and rinsed as described above. The slides were mounted as described above and viewed on the confocal microscope using the 488-nm line of the argon-ion laser for excitation, a 488 dichroic mirror (Zeiss), and 510- to 540-nm emission filters (Zeiss).

Image and Data Analysis

Fluorescent images of cortical microtubules from lysed protoplasts were captured using a silicon-intensified tube camera (Hamamatsu Phototronics, Hamamatsu City, Japan) and digitized using a video board (PC-Vision Plus, Imaging Technology, Woburn, MA) mounted in a microcomputer (model 286–12, CompuAdd, Austin, TX). The images were displayed on a monitor (model PVM 1344Q, Sony, Denver, CO) and analyzed with the aid of Canopy software (Los Alamos National Laboratory, Los Alamos, NM) (Rich, 1990). The area that was occupied by an individual protoplast was outlined, and a threshold feature was used to display microtubules as white pixels. Total and white pixels in the outlined region were calculated and used to compute microtubule frequency. Microtubule frequencies in at least 25 protoplasts per treatment were determined, and the average values were divided by the control values that were obtained from untreated preparations produced and processed on the same day. The data reported here represent the pooled data obtained from triplicate experiments, each performed on separate days. The data were analyzed for statistical significance using the general linear model (Minitab, State College, PA), in which the microtubule response was compared with a model consisting of: three Ca^{2+} ranges (0–10, 11–50, and >50 μM); the presence or absence of resident CaM on microtubules; and the interaction between the Ca^{2+} ranges and the presence or absence of resident CaM on microtubules.

CaM Affinity Chromatography

Total soluble tubulin-binding proteins were collected by tubulin-affinity chromatography as described by Durso and Cyr (1994b), but without a 25 mM NaCl wash step. The proteins were passed over a CaM-affinity column in the presence of 1 mM EGTA. The CaM column was loaded and washed under lower ionic conditions to avoid biasing the chromatographic isolation for ionic or hydrophobic interactions. After washing the column with PM buffer until no further proteins were detectable in the flow-through by A_{280}, PM plus 3 mM $CaCl_2$ was applied to the column and a small peak was observed by A_{280}. This peak, containing Ca^{2+}-sensitive CaM-binding proteins, was analyzed by SDS-PAGE.

CaM Inhibitors

Twenty micromolar W5, W7, and trifluoperizine was used in several experiments. Higher concentrations were not used because these antagonists reportedly lose their selectivity at elevated concentrations and inhibit other Ca^{2+}-binding proteins such as Ca^{2+}-dependent protein kinase (Harmon et al., 1987). Protoplasts were treated

for various times (30, 45, and 60 min) with these inhibitors after removal of the wall-degrading enzymes. The treated protoplasts were lysed as described above, and the inhibitors were included in the lysis solutions. The slides were fixed immediately after lysis. One set of experiments included 20 μM Ca^{2+} and 5 μM CaM in the lysis buffer, and in another set of experiments, 200 μM Ca^{2+} was applied briefly to the protoplasts after lysis to remove resident CaM from the microtubules, but before treating with inhibitors. Antibodies against tubulin and CaM were employed for immunocytochemical localization. Appropriate controls, with and without Ca^{2+}, CaM, and CaM inhibitors, were rigorously included.

RESULTS

Concentration Dependence of CaM at Destabilizing Cortical Microtubules

Data were obtained that revealed the nature of the functional interdependence between Ca^{2+}, CaM, and microtubules. In the first set of experiments, a basal dose-response curve for CaM's destabilization of microtubules was established. Protoplasts that are detergent-lysed in the presence of EGTA possess CaM on their microtubules (Fig. 1A; see also Fisher and Cyr, 1993). This microtubule-bound CaM complicated the interpretation of the dose-response curves; therefore, resident CaM was removed from the microtubules of EGTA-lysed protoplasts by a brief treatment with 200 μM Ca^{2+} (Fig. 1B). This treatment, although effective at removing demonstrable microtubule-bound CaM (as shown with the CaM monoclonal antibody mAb1D10), does not appreciably affect the appearance of cortical microtubules (as judged by immunocytochemistry using an anti-tubulin antibody) (Fig. 1C; also see Cyr, 1991a).

These CaM-free cortical microtubules were sensitive to the action of Ca^{2+}/CaM; the destabilization of these microtubules by exogenous CaM in the presence of 100 μM Ca^{2+} was found to be concentration-dependent, with a one-half-maximal effect observed at 1 μM CaM (Fig. 2). Although levels of cellular CaM can fluctuate (Braam and Davis, 1990; Braam, 1992), documented values average 1 μM. The carrot cell line has a CaM concentration of approximately 4 μM, as determined from final yields after biochemical purification. In the next set of experiments 5 μM CaM was used, because this physiologically relevant concentration, in the presence of Ca^{2+}, effectively destabilizes cortical microtubules.

Concentration Dependence of Ca^{2+} at Destabilizing Cortical Microtubules

In the second set of experiments dose-response curves for Ca^{2+} were established in the presence of 5 μM exogenous CaM. These experiments were carried out on lysed protoplasts with and without CaM residing on their microtubules. We reasoned that cortical microtubules with resident CaM might respond differently to an exogenous application of Ca^{2+}/CaM. First, when protoplasts that possessed resident CaM were lysed in the presence of EGTA and exposed to increasing concentrations of free

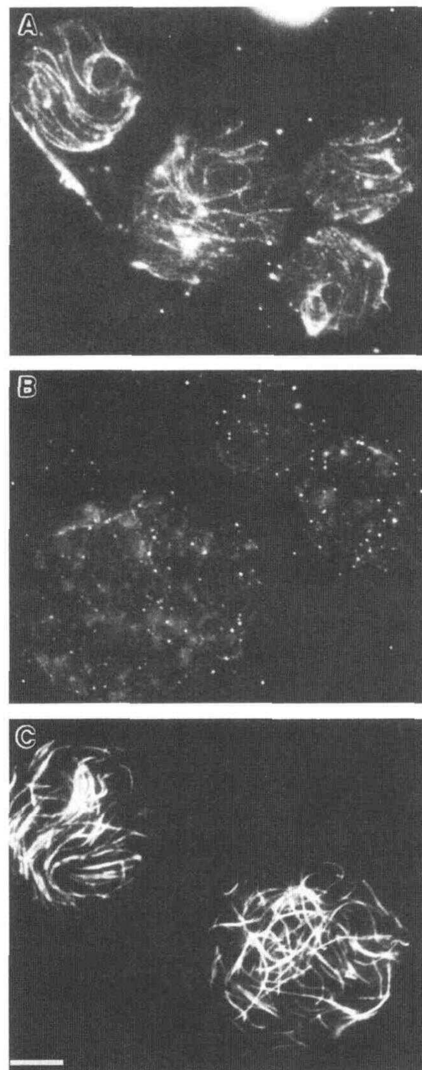

Figure 1. CaM can be removed from the cortical microtubules of lysed carrot protoplasts. Protoplasts, lysed in the presence of 1 mM EGTA, have CaM on their cortical microtubules that is demonstrable with a plant anti-CaM antibody (A). Protoplasts, lysed in the presence of 1 mM EGTA and treated with 200 μM Ca^{2+}, no longer have demonstrable CaM on their microtubules that is recognized by an anti-CaM antibody (B), although anti-tubulin antibodies clearly show the presence of an intact microtubular array (C). Bar = 2.5 μm.

Ca^{2+} (without additional exogenous CaM), their cortical microtubules appeared relatively stable (Fig. 3a). Next, protoplasts were lysed in the absence of Ca^{2+}; half were treated with 200 μM Ca^{2+} to remove resident CaM, and the other half remained untreated (microtubules in possession of resident CaM). Both preparations were then exposed to 5 μM CaM and a range of free Ca^{2+} concentrations.

Destabilization of cortical microtubules in both preparations was found to be dependent on the concentration of free Ca^{2+} (Fig. 3b). However, protoplasts with CaM residing on their cortical microtubules were significantly more resistant to destabilization by Ca^{2+} ($P < 0.05\%$). The difference in the response to Ca^{2+} was more pronounced at lower Ca^{2+} concentrations (10^{-6} to 10^{-5} M), which is sug-

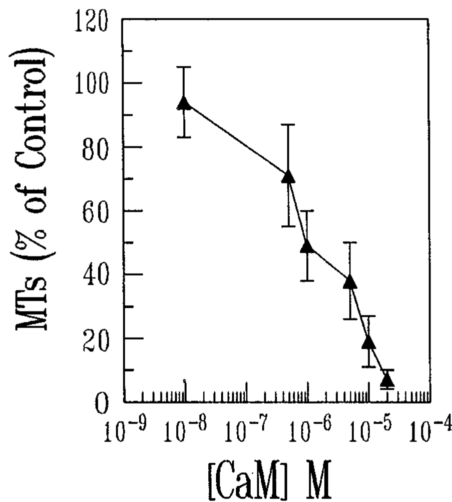

Figure 2. The destabilizing effect of exogenous CaM, in the presence of 100 μM Ca^{2+}, is concentration-dependent. Lysed and washed carrot protoplasts were exposed to 100 μM Ca^{2+} in the presence of increasing concentrations of CaM. The frequency of immunolabeled cortical microtubules was then quantified, and the data were normalized with those obtained from untreated, control cells. The data points represent the mean ± SD of pooled data obtained from three experiments; $n = 60$.

gestive of an interaction between Ca^{2+}/CaM and CaM in residence on microtubules, and a significant interaction ($P < 0.05\%$) was verified in support of this hypothesis. When the same experiment was performed with 10 μM exogenous CaM, the cortical microtubules possessing resident CaM were relatively more resistant to destabilization (Fig. 3c), again with the differences occurring between 10^{-6} and 10^{-5} M Ca^{2+}.

Effect of CaM Inhibitors on Ca^{2+}/CaM-Induced Microtubule Destabilization

We reasoned that if CaM interacts with two opposing effects on cortical microtubles, then CaM inhibitors such as W5 and W7 might perturb these responses. These related compounds are useful because W7 is more effective at inhibiting CaM interactions than is W5, and provides an important control for inferring specific inhibition of CaM (i.e. nonspecific effects were expected to be identical between the inhibitors). Lysed protoplasts, treated with Ca^{2+}/CaM, had disrupted cortical microtubules (Fig. 3b). The addition of 20 μM W5, W7, or trifluoperazine did not prevent this CaM-dependent microtubule destabilization (data not shown), and higher concentrations of these inhibitors were not used because this reportedly leads to nonspecific effects (Harmon et al., 1987).

The inability of the inhibitors to affect the microtubule destabilization action of CaM suggests the interesting possibility that this structural target of Ca^{2+}/CaM represents a novel form of interaction, differing significantly from the traditionally studied enzymatic regulation by this complex. However, when protoplasts containing CaM in residence on the cortical microtubules were treated with Ca^{2+}/CaM and W7, the microtubules were disrupted to a greater extent, compared with those treated with Ca^{2+}/CaM and W5 (Table I), and this destabilization was more pronounced as the concentration of W7 was increased (Table I). These results indicate that W7 can inhibit the microtubule-stabilizing effect of CaM, leaving the microtubules hypersensitive to the destabilizing action of Ca^{2+}/CaM, which is insensitive to the inhibitors. Immunocytochemistry, using an anti-CaM antibody, was performed on proto-

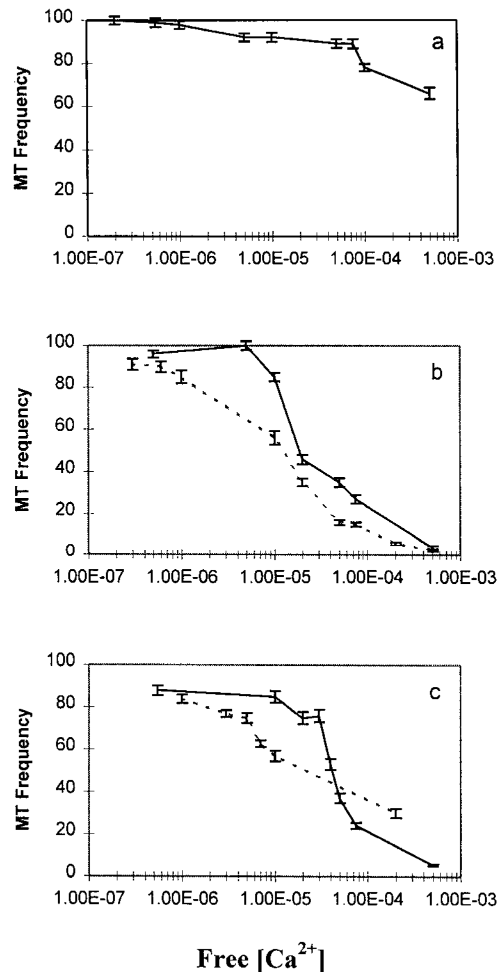

Figure 3. The destabilizing effect of exogenous CaM is dependent on the presence of Ca^{2+} and differs when resident CaM is present on microtubules. Protoplasts, lysed in the presence of 1 mM EGTA and treated with increasing concentrations of Ca^{2+}, have relatively stable cortical microtubules (A). However, protoplasts lysed in the presence of 1 mM EGTA and treated with increasing concentrations of Ca^{2+} in the presence of 5 μM (B) or 10 μM (C) CaM, show decreasing frequencies of cortical microtubules as Ca^{2+} concentrations increase (solid lines). If the protoplasts were lysed in the presence of 1 mM EGTA and treated with 200 μM Ca^{2+} (to remove resident CaM) prior to being exposed to the same concentrations of Ca^{2+} and CaM, their cortical microtubules appeared relatively more sensitive to Ca^{2+} (dashed lines). The relative percentage of the microtubules was obtained by comparing matched control preparations with the experimental treatment, i.e. in A the control preparations were treated with 1 mM EGTA only, and in B and C they were treated with Ca^{2+} (but not exogenous CaM) in either the presence or absence of resident CaM. Error bars show SE; $n = 20$.

Table I. *Effect of CaM antagonists*

Intact protoplasts were pretreated for 30 min and detergent-lysed for 5 min in the presence of the inhibitors W5 and W7. The lysed protoplasts were then treated with 20 μM Ca^{2+} and 5 μM CaM in the inhibitor's presence.

Inhibitor Concentration	Percent of Microtubules[a]
5 μM	88 ± 4
10 μM	57 ± 2.6
20 μM	47 ± 2.6

[a] The frequency of microtubules in W5-treated protoplasts was used as the control value, i.e. percent of microtubules = (frequency with W7/frequency with W5) × 100.

plasts treated with W5 and W7 to see if these inhibitors affected the ability of CaM to bind to microtubules. These studies showed that some CaM was still bound to microtubules in the presence of W5 and W7 (data not shown); thus, the inhibitors appear to block the ability of CaM to stabilize microtubules against Ca^{2+}/CaM-induced destabilization, but leave their microtubule-binding capability relatively intact.

A Small Subset of Tubulin-Binding Proteins Bind CaM in a Ca^{2+}-Sensitive Manner

It has been shown that one plant MAP, EF-1α (Durso et al., 1996), binds to tubulin affinity columns and also binds to CaM affinity columns in a Ca^{2+}-dependent fashion (Durso and Cyr, 1994). Moreover, the bundling of microtubules mediated by this MAP is abolished in vitro by Ca^{2+}/CaM. Therefore, this MAP is a candidate for the Ca^{2+}-dependent CaM-binding site (i.e. the microtubule-destabilizing site).

CaM affinity chromatography was employed to determine if a second, Ca^{2+}-sensitive CaM-binding site (i.e. the microtubule-stabilizing site) could be found on microtubules. We predicted that these Ca^{2+}-sensitive proteins would bind to CaM in the absence of Ca^{2+} but would be eluted when Ca^{2+} levels were raised. Proteins generated from tubulin affinity chromatography, followed by Ca^{2+}-sensitive CaM affinity chromatography, yielded proteins within a small elution peak. Analysis of the proteins by SDS-PAGE revealed a number of polypeptides. Prominent bands at 80, 78, and 75 kD, along with minor bands of lesser molecular mass, were observed (Fig. 4). These data show the existence of proteins that can bind tubulin in vitro and that can interact with CaM in a Ca^{2+}-sensitive manner. These proteins are candidates for mediating the Ca^{2+}-sensitive binding of CaM to microtubules that are detected by immunofluorescence.

Effect of Elevated Cytoplasmic Ca^{2+} Levels on Cortical Microtubule Destabilization in Vivo

Lysed cells are useful for studying the in situ effect of Ca^{2+}/CaM on cortical microtubules. However, because this approach involves a major perturbation of the cell, corroborative, in vivo evidence for this effect was also sought. If Ca^{2+}/CaM acts in vivo to destabilize cortical microtubules, then the elevation of cytoplasmic Ca^{2+} should have an effect on cortical microtubules. Experimentally, this was done by exposing the protoplasts to high levels of external Ca^{2+}, a treatment shown to cause transient increases in cytoplasmic Ca^{2+} levels (Gilroy et al., 1987). Using the ratiometric Ca^{2+}-binding dye Indo-1, we verified that the free cytoplasmic Ca^{2+} level increased from a basal level of approximately 90 nM to a level greater than 600 nm upon transferring the protoplasts from solutions with low levels of Ca^{2+} (3 mM or less) to solutions with high levels of Ca^{2+} (100 mM) for 15 min. Protoplasts that were treated in this manner and prepared for immunolocalization of cytoskeletal elements had full, intact, cortical microtubule arrays under conditions in which the cytoplasmic Ca^{2+} concentrations were low (Fig. 5A), but had severely disrupted arrays under conditions in which the cytoplasmic Ca^{2+} levels were high (Fig. 5B). These data are consistent with a role for Ca^{2+} in affecting the cortical microtubules in vivo. Remarkably, under these same conditions, actin filaments appeared unaffected (Fig. 5C).

DISCUSSION

CaM interacts with microtubules in plants (Vantard et al., 1985; Wick et al., 1985; Fisher and Cyr, 1993) and in animals (Welsh et al., 1979). The significance of this interaction is suggested by the observation that Ca^{2+}/CaM can destabilize microtubules in vitro (Marcum et al., 1978; Job et al., 1981) and in situ (Keith et al., 1983). Data presented here as well as in previous reports (Cyr, 1991a) show that the cortical microtubule array in plants is sensitive to Ca^{2+}/CaM in situ. Here we have shown that the concen-

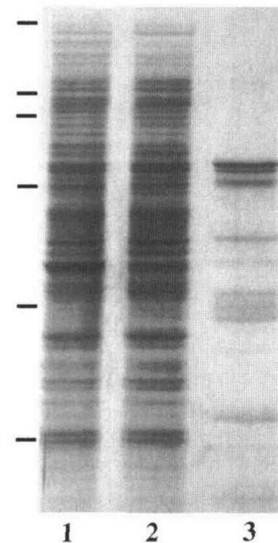

Figure 4. A subset of tubulin-binding proteins bind CaM in a Ca^{2+}-independent manner. Total tubulin-binding proteins (lane 1) were applied to a CaM affinity column in the absence of Ca^{2+} (1 mM EGTA present), and the unbound fractions were collected (lane 2). After extensive washing, Ca^{2+} was applied and a subset of proteins was released (lane 3). The positions of molecular weight standards (from the top, M_r = 205,000, 116,000, 97,500, 66,000, 45,000, and 29,000) are noted on the left.

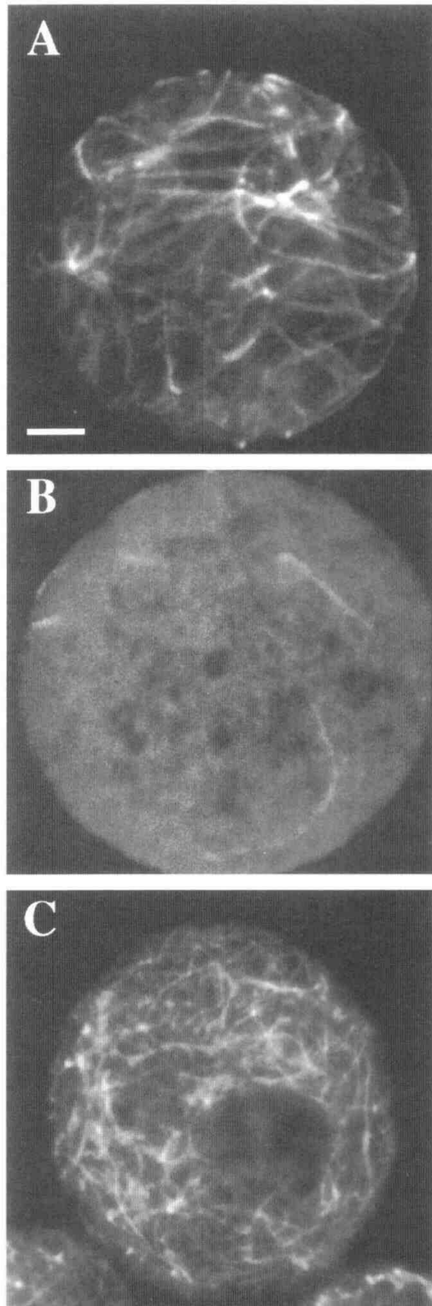

Figure 5. Protoplasts with high intracellular Ca^{2+} levels have disrupted microtubules but intact actin filaments. Intact protoplasts, suspended in medium containing relatively low amounts of Ca^{2+} (3 mM), have intracellular Ca^{2+} levels of <90 nM and possess intact microtubules (A). However, if the protoplasts are resuspended in medium containing high levels of Ca^{2+} (100 mM), the intracellular Ca^{2+} levels rise to >600 nM, and intact microtubular arrays are no longer demonstrable (B). Under the same conditions of elevated intracellular Ca^{2+}, the actin filaments appear intact (C). All three images represent immunofluorescent depictions of fixed protoplasts using anti-tubulin antibodies (A and B) or an anti-actin antibody (C) imaged with a laser confocal microscope. In all cases the protoplasts were optically sectioned (four sections, 1 μm apart), and the resulting digital images were added and interpolated, to provide an extended-focus image giving a full depiction of the cytoskeletal elements within the cells. Scale bar = 2.5 μm.

trations of Ca^{2+} and CaM that are required to observe this destabilization event are physiologically relevant.

The potential importance of Ca^{2+} as a microtubule regulator in vivo is supported in these experiments by showing that an increase in free intracellular Ca^{2+} levels, induced by increases in extracellular Ca^{2+}, does destabilize cortical microtubules. Although these data do not demonstrate a direct participation of CaM in the Ca^{2+}-mediated microtubule destabilization in vivo, it does show that physiologically relevant increases in Ca^{2+} do affect cortical microtubules. Moreover, the lysed cell data (e.g. Fig. 1) indicate that CaM is required for Ca^{2+} to destabilize cortical microtubules. Therefore, the in situ and in vivo data are consistent with the hypothesis that Ca^{2+}/CaM acts to affect the assembly state of tubulin polymers in the cell cortex. This hypothesis is not exclusive; other Ca^{2+}-dependent processes may also affect the cytoskeleton, e.g. Ca^{2+}-dependent protein kinase (Putnam-Evans et al., 1989) could also affect the appearance and/or behavior of cytoskeletal elements.

We have found evidence for two opposing modes of action for CaM on cortical microtubules. A Ca^{2+}-dependent, CaM-regulated event leads to microtubule destabilization, whereas a Ca^{2+}-sensitive (Ca^{2+}-inhibited) event stabilizes the microtubules. Because CaM probably does not bind directly to tubulin (Lee and Wolff, 1984), it is likely that Ca^{2+}/CaM is activating nontubulin, microtubule proteins, which consequently cause cortical microtubules to be stabilized or depolymerized. We propose several mechanisms that may operate alone, or in concert, to mediate the Ca^{2+}/CaM-induced depolymerization of cortical microtubules. First, EF-1α binds microtubules in vitro (Durso and Cyr, 1994b) and associates with cortical microtubules in situ (Durso et al., 1996). It also binds to CaM in a Ca^{2+}-dependent fashion. Functionally, this MAP bundles microtubules in vitro, and this activity is inhibited by Ca^{2+}/CaM. Because cortical microtubules are bundled to varying degrees (Cyr and Palevitz, 1995), it is possible that Ca^{2+}/CaM can actively unbundle these microtubules by inhibiting the action of EF-1α. Once unbundled, the microtubules may be inherently unstable and depolymerize in a zero-order reaction without the assistance of additional factors.

Alternatively, in the unbundled state microtubules may become susceptible to the action of a second Ca^{2+}/CaM-mediated process, which acts to destabilize individual microtubules. This second process may also involve EF-1α. Shiina et al. (1994) reported that EF-1α is a microtubule-severing protein. Although we have not been able to demonstrate this capability for EF-1α under the reported conditions (N.A. Durso and R.J. Cyr, unpublished observations), the possibility that the severing activity might be mediated by Ca^{2+}/CaM, leading to microtubule destabilization, is intriguing. When taxol-stabilized, carrot microtubules are allowed to bind to EF-1α in vitro, only large bundles are observed. However, in the presence of Ca^{2+}/CaM these bundles dissipate, and free microtubules are not observed (Durso and Cyr, 1994a). Currently, we are investigating the possibility that in the absence of Ca^{2+}/

CaM, EF-1α induces bundling, and that in its presence, the bundling activity is lost and the severing activity is gained, thereby inducing microtubule depolymerization. Alternatively, EF-1α may, in some other manner, participate in the depolymerization of microtubules in the presence of Ca^{2+}/CaM.

A third possible mechanism by which Ca^{2+}/CaM may affect cortical microtubules is to interfere with their attachment to the plasma membrane. Although the nature of the linking protein is unknown, its presence has been inferred from both direct and indirect evidence (Shibaoka, 1993). Shibaoka proposed that hormones may affect the interaction between microtubules and the plasma membrane. In many cells changes in Ca^{2+} concentrations accompany hormonal treatment (Bush, 1993, 1995), and it is reasonable to propose that Ca^{2+} may be involved in hormone-induced changes in the cortical array. Once the elusive plasma membrane/microtubule-linking protein has been characterized, it will be exciting to see if Ca^{2+}/CaM can modulate the affinity of microtubules for this protein.

CaM also interacts with microtubles in a Ca^{2+}-sensitive (Ca^{2+}-inhibited) fashion, both in animals (Sweet et al., 1988) and in plants (Fig. 1) (Fisher and Cyr, 1993). Sweet and Welsh (1988) proposed that CaM stabilizes microtubules in a Ca^{2+}-sensitive manner. This interpretation was based on their observation that CaM-containing microtubules were the most stable microtubules in the cell, that microinjected fluorescent CaM was found to associate with drug-resistant microtubules (Sweet et al., 1989), and that CaM, modified to disrupt its ability to bind Ca^{2+}, could still bind and stabilize microtubules in vivo (Sweet et al., 1988). Two lines of functional data presented here support a role for a Ca^{2+}-sensitive CaM interaction with microtubules that results in the stabilization of cortical microtubules. First, when CaM is removed from the Ca^{2+}-sensitive binding site of cortical microtubules by a high-Ca^{2+} wash, they become more susceptible to the destabilizing action of Ca^{2+}/CaM. Second, in the presence of CaM antagonists cortical microtubules are more sensitive to the destabilization action of exogenous Ca^{2+}/CaM. W7 does not cause CaM to disassociate from cortical microtubules; therefore, it appears that this compound interferes with CaM's ability to interactively stabilize microtubules. How this occurs is unknown; however, Figure 4 shows a set of tubulin-binding proteins that bind to CaM affinity columns in a Ca^{2+}-sensitive fashion. These proteins were chromatographed in a low-ionic environment, and it is not likely that their interaction was due to nonspecific ionic interactions because 3 mM $CaCl_2$ affected their elution. One or more of these proteins are candidates for a MAP the microtubule-stabilizing activity of which can be modulated by CaM in a Ca^{2+}-sensitive manner.

Our data are consistent with the hypothesis that CaM has two opposing effects on cortical microtubules: one that stabilizes and the other that destabilizes. Together, these opposing effects may provide a mechanism with which to "fine tune" the responsiveness of the cortical cytoskeleton to free cytoplasmic Ca^{2+} levels, which reportedly can have an oscillatory character (Tsien and Tsien, 1990; Bush, 1995). In the scheme outlined above cortical microtubules are buffered from the action of small Ca^{2+} transients, and only when larger increases occur would the cortical microtubules become destabilized in a very rapid manner. This mechanism is analogous to the proposed interaction of CaM with neuromodulin and neuroginin in developing axons, where a Ca^{2+}-sensitive interaction with CaM is hypothesized to function as a biochemical capacitor that serves to modulate the availability of Ca^{2+}/CaM (Skene, 1989; Gerendasy et al., 1994).

An unanticipated in vivo observation was that F-actin was relatively unaffected under conditions that caused the massive disruption of cortical microtubules. Ca^{2+}-activated F-actin-severing proteins have been described, and there is evidence that these proteins exist in plants; for example, actin filaments in lily pollen are Ca^{2+}-sensitive (Kohno and Shimmen, 1987). Our data indicate that the expression of a Ca^{2+}-based F-actin-disrupting system may not be expressed (or active) in all cell types, or is highly regulated.

Traditionally, the activity of CaM has been described in terms of a freely soluble molecule that interacts with its target upon binding with Ca^{2+}. Our previous immunolocalization data (Fisher and Cyr, 1993), however, indicate that CaM binds to microtubules in a low-Ca^{2+} state, and additional data show that CaM can also interact with microtubules in a high-Ca^{2+} state (Keith et al., 1983; this study). Thus, in both cases the time that CaM spends as a freely diffusing molecule is limited. Presumably, the selective advantage of such an arrangement is that CaM is always poised on, or near, a cortical microtubule. Under conditions in which the amplitude of Ca^{2+} levels is low, CaM can both locally scavenge Ca^{2+} as well as prevent a Ca^{2+}/CaM from prematurely acting to destabilize the microtubule. Under conditions in which the Ca^{2+} levels rise above a threshold level (the magnitude of which could be developmentally regulated in different cells), CaM disassociates in the form of a Ca^{2+}/CaM and then is immediately poised in the vicinity to interact with the microtubule at a close, but distinct, site on the microtubule to affect its destabilization. In this manner cortical microtubules create their own "microdomains" for monitoring and responding to fluctuations in the concentration of this important secondary messenger.

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LITERATURE CITED

Allan E, Hepler PK (1989) Calmodulin and calcium-binding proteins. *In* PK Stumpf, ed, The Biochemistry of Plants. Academic Press, New York, pp 455–484

Andersland JM, Fisher DD, Wymer CL, Cyr RJ, Parthasarathy MV (1994) Characterization of a monoclonal antibody prepared against plant actin. Cell Motil Cytoskeleton **29:** 339–344

Baudier J, Deloulme JC, Van Dorsselaer A, Black D, Matthes HWD (1991) Purification and characterization of a brain-specific protein kinase C substrate, neurogranin (p17). J Biol Chem **266:** 229–237

Braam J (1992) Regulation of expression of calmodulin and calmodulin-related genes by environmental stimuli in plants. Cell Calcium **13:** 457–463

Braam J, Davis RW (1990) Rain-, wind-, and touch-induced expression of calmodulin and calmodulin-related genes in *Arabidopsis*. Cell **60:** 357–364

Bush DS (1993) Regulation of cytoplasmic calcium in plants. Plant Physiol **103:** 7–13

Bush DS (1995) Calcium regulation in plant cells and its role in signaling. Annu Rev Plant Physiol Plant Mol Biol **46:** 95–122

Cimler BM, Andreasen TJ, Andreasen KI, Storm DR (1985) P-57 is a neural specific calmodulin-binding protein. J Biol Chem **260:** 10784–10788

Cyr RJ (1991a) Calcium/calmodulin affects microtubule stability in lysed protoplasts. J Cell Sci **100:** 311–317

Cyr RJ (1991b) Microtubule-associated proteins in higher plants. *In* CW Lloyd, ed, The Cytoskeletal Basis of Plant Growth and Form. Academic Press, San Diego, CA, pp 57–67

Cyr RJ, Palevitz BA (1989) Microtubule-binding proteins from carrot. I. Initial characterization and microtubule bundling. Planta **177:** 245–260

Cyr RJ, Palevitz BA (1995) Organization of cortical microtubules in plant cells. Curr Opin Cell Biol **7:** 65–71

Durso NA, Cyr RJ (1994a) Beyond translation: elongation factor-1α and the cytoskeleton. Protoplasma **180:** 99–105

Durso NA, Cyr RJ (1994b) A calmodulin-sensitive interaction between microtubules and a higher plant homolog of elongation factor-1α. Plant Cell **6:** 893–905

Durso NA, Leslie JD, Cyr RJ (1996) *In situ* immunocytochemical evidence that a homolog of protein translation elongation factor EF-1α is associated with microtubules in carrot cells. Protoplasma **190:** 141–150

Fisher D, Cyr R (1993) Calcium levels affect the ability to immunolocalize calmodulin to cortical microtubules. Plant Physiol **103:** 543–551

Gerendasy DD, Herron SR, Watson JB, Sutcliffe JG (1994) Mutational and biophysical studies suggest RC3/neurogranin regulates calmodulin availability. J Biol Chem **269:** 22420–22426

Gilroy S, Fricker MD, Read ND, Trewavas AJ (1991) Role of calcium in signal transduction in *Commelina* guard cells. Plant Cell **3:** 333–344

Gilroy S, Hughes WA, Trewavas AJ (1987) The measurements of intracellular calcium levels in protoplasts from higher plant cells. FEBS Lett **212:** 133–137

Goddard RH, Wick SW, Silflow CD, Snustad DP (1994) Microtubule components of the plant cell cytoskeleton. Plant Physiol **104:** 1–6

Gopalakrishna R, Anderson WB (1982) Ca^{2+}-induced hydrophobic site on calmodulin: application for purification of calmodulin by phenyl-Sepharose affinity chromatography. Biochem Biophys Res Commun **104:** 830–836

Gregori L, Gillevet PM, Doan P, Chau V (1985) Mechanism of enzyme regulation by calmodulin and Ca^{2+}. Curr Top Cell Regul **27:** 447–454

Harmon AC, Putnam-Evans C, Cormier MS (1987) A calcium-dependent but calmodulin-independent protein kinase from soybean. Plant Physiol **83:** 830–837

Job D, Fischer EH, Margolis RL (1981) Rapid disassembly of cold-stable microtubules by calmodulin. Proc Natl Acad Sci USA **78:** 4679–4682

Keith C, DiPaola M, Maxfield FR, Shelanski ML (1983) Microinjection of Ca^{++}-calmodulin causes a localized depolymerization of microtubules. J Cell Biol **97:** 1918–1924

Klee CB (1988) Interaction of calmodulin with Ca^{2+} and target proteins. *In* P Cohen, CB Klee, eds, Calmodulin, Vol 5: Molecular Aspects of Cellular Regulation. Elsevier, Amsterdam, pp 35–56

Kohno T, Shimmen T (1987) Ca^{2+}-induced fragmentation of actin filaments in pollen tubes. Protoplasma **141:** 177–179

Kotani S, Nishida E, Kumagai H, Sakai H (1985) Calmodulin inhibits interaction of actin with MAP2 and tau, two major microtubule-associated proteins. J Biol Chem **260:** 10779–10783

Lee Y, Wolff J (1984) Calmodulin binds to both microtubule-associated protein 2 and τ proteins. J Biol Chem **259:** 1226–1230

Marcum JM, Dedman JR, Brinkley BR, Means AR (1978) Control of microtubule assembly-disassembly by calcium-dependent regulator protein. Proc Natl Acad Sci USA **75:** 3771–3775

Margolis RL, Rauch CT, Job D (1986) Purification and assay of a 145-kDa protein (STOP145) with microtubule-stabilizing and motility behavior. Proc Natl Acad Sci USA **83:** 639–643

Pirollet F, Margolis RL, Job D (1992) Ca^{2+}-calmodulin regulated effectors of microtubule stability in neuronal tissues. Biochim Biophys Acta **1160:** 113–119

Putnam-Evans C, Harmon AC, Palevitz BA, Fechheimer M, Cormier MJ (1989) Calcium-dependent protein kinase is localized with F-actin in plant cells. Cell Motil Cytoskeleton **12:** 12–22

Rich PM (1990) A Manual for Analysis of Hemispherical Canopy Photography. Los Alamos National Laboratory, Los Alamos, NM

Roberts DM, Harmon AC (1992) Calcium-modulated proteins: targets of intracellular calcium signals in higher plants. Annu Rev Plant Physiol Plant Mol Biol **43:** 375–414

Schellenbaum P, Vantard M, Lambert A (1992) Higher plant microtubule-associated proteins (MAPs): a survey. Biol Cell **76:** 359–364

Shibaoka H (1993) Regulation by gibberellins of the orientation of cortical microtubules in plant cells. Aust J Plant Physiol **20:** 461–470

Shiina N, Gotoh Y, Kubomura N, Iwamatsu A, Nishida E (1994) Microtubule severing by elongation factor 1α. Science **266:** 282–285

Skene JHP (1989) Axonal growth-associated proteins. Annu Rev Neurosci **12:** 127–156

Sweet SC, Rogers CM, Welsh MJ (1988) Calmodulin stabilization of kinetochore microtubule structure to the effect of nocodazole. J Cell Biol **107:** 2243–2251

Sweet SC, Rogers CM, Welsh MJ (1989) Calmodulin is associated with microtubules forming in PTK_1 cells upon release from nocodazole treatment. Cell Motil Cytoskeleton **12:** 113–122

Sweet SC, Welsh MJ (1988) Calmodulin colocalization with cold-stable and nocodazole-stable microtubules in living PtK_1 cells. Eur J Cell Biol **47:** 88–93

Török K, Whitaker M (1994) Taking a long, hard look at calmodulin's warm embrace. Bioessays **16:** 221–224

Tsien RW, Tsien RY (1990) Calcium channels, stores, and oscillations. Annu Rev Cell Biol **6:** 715–760

Vantard M, Lambert A-M, De Mey J, Picquot P, Van Eldik LJ (1985) Characterization and immunocytochemical distribution of calmodulin in higher plant endosperm cells: localization in the mitotic apparatus. J Cell Biol **101:** 488–499

Welsh MJ, Dedman JR, Brinkley BR, Means AR (1979) Tubulin and calmodulin: effects of microtubule and microfilament inhibitors on localization in the mitotic apparatus. J Cell Biol **81:** 624–634

Wick SM, Muto S, Duniec J (1985) Double immunofluorescence labeling of calmodulin and tubulin in dividing plant cells. Protoplasma **126:** 198–206

Wylie DC, Vanaman TC (1988) Structure and evolution of the calmodulin family of calcium regulatory proteins. *In* P Cohen, CB Klee, eds, Calmodulin, Vol 5: Molecular Aspects of Cellular Regulation. Elsevier, Amsterdam, pp 1–15