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Actin–microtubule interactions in the alga *Nitella*: analysis of the mechanism by which microtubule depolymerization potentiates cytochalasin's effects on streaming

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Summary. In the characean alga *Nitella*, depolymerization of microtubules potentiates the inhibitory effects of cytochalasins on cytoplasmic streaming. Microtubule depolymerization lowers the cytochalasin B and D concentrations required to inhibit streaming, accelerates inhibition and delays streaming recovery. Because microtubule depolymerization does not significantly alter ^{3}H-cytochalasin B uptake and release, elevated intracellular cytochalasin concentrations are not the basis for potentiation. Instead, microtubule depolymerization causes actin to become more sensitive to cytochalasin. This increased sensitivity of actin is unlikely to be due to direct stabilization of actin by microtubules, however, because very few microtubules colocalize with the subcortical actin bundles that generate streaming. Furthermore, microtubule reassembly, but not recovery of former transverse alignment, is sufficient for restoring the normal cellular responses to cytochalasin D. We hypothesize that either tubulin or microtubule-associated proteins, released when microtubules depolymerize, interact with the actin cytoskeleton and sensitize it to cytochalasin.

Keywords: Actin; Cytoplasmic streaming; Cytochalasin; Microtubule depolymerization; *Nitella*; Oryzalin.

Abbreviations: APW artificial pond water; Ca_c cytoplasmic free calcium concentration; DMSO dimethyl sulfoxide; MT– microtubule-minus; MT+ microtubule-plus.

Introduction

Actin and microtubules colocalize in many plant cells but demonstrations of either a physical or functional interaction between them have been rare. Therefore, pharmacological approaches are often useful for determining if one cytoskeletal element, inhibitable by a certain drug, has any control over the organization of the other element. For example, members of the group of actin-binding drugs known collectively as cytochalasin, also disrupt microtubules in *Gossypium* (Seagull 1990), *Adiantum* (Kodata and Wada 1992), *Lilium* (Tanaka and Wakabayashi 1992), *Chlamydomonas* (Dentler and Adams 1992), and *Allium* (Eleftheriou and Palevitz 1992). Conversely, microtubule depolymerization modifies actin in the green alga *Bryopsis* (Menzel and Schliwa 1986). In rye root tip cells, microtubule depolymerization destabilizes actin microfilaments and microtubule stabilization with taxol increases microfilament stability (Chu et al. 1993). Results like these indicate potential actin–microtubule interactions at a structural and/or functional level.

Several factors suggest the potential for actin–microtubule interaction in characean algae including the presence in the cortex of actin filaments (Collings et al. 1995, Wasteneys et al. 1996) and cortical microtubules (Wasteneys and Williamson 1987), and also the presence of microtubules alongside the subcortical actin bundles (Wasteneys and Williamson 1991). The latter finding prompted Wasteneys and Williamson (1991) to reinvestigate whether microtubule depolymerization affects cytochalasin's inhibition of the actin-based streaming. Whereas Bradley (1973) found that colchicine had no effect on cytochalasin B-induced streaming cessation, Wasteneys and Williamson (1991) found more rapid cytochalasin D-induced cessation and much slower recovery in cells treated

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with either colchicine or oryzalin. Such potentiation of cytochalasin's effects provided the first evidence that the actin and microtubule cytoskeletons in characean algae interact in some way.

To investigate the nature of actin–microtubule interactions and the mechanism by which they potentiate cytochalasin's effects, we have extended the studies of Wasteneys and Williamson (1991). We show that microtubule depolymerization is critical for potentiation, that streaming stops when cells with depolymerized microtubules are exposed to concentrations of two different cytochalasins that otherwise have no inhibitory effect, that potentiation does not result from elevated intracellular cytochalasin B concentrations, and that while the majority of cytochalasin B can be rapidly removed from cells by washing, substantial amounts exchange slowly over many hours. From these data, we argue that microtubule depolymerization sensitizes actin to cytochalasin so that lower cytochalasin concentrations are effective in streaming inhibition, and, that streaming remains inhibited after removing external cytochalasin because the slowly exchanging cytochalasin maintains a cytochalasin concentration sufficient to impair the sensitized actin. We hypothesize that sensitization to cytochalasin occurs as microtubule proteins, released on microtubule depolymerization, interact with the actin cytoskeleton.

Material and methods

Plant material

Elongating internodes of *Nitella pseudoflabellata* (and other species of characean algae tested) were grown, harvested, and pretreated as previously described (Collings et al. 1995). *Spirogyra* sp. was isolated as a contaminant of *Nitella* cultures. Higher plant material tested included *Tradescantia virginiana* and *Allium cepa* epidermal cells and *Vallisneria* mesophyll cells. *Vallisneria* was collected from Lake Burley Griffin, Canberra, ACT. *Tradescantia virginiana* was cultivated in a glasshouse and epidermal peels were taken from young leaves. Onion bulbs were purchased from a local market.

Chemicals

Stock solutions of cytochalasins B and D (Sigma, St. Louis, MO, U.S.A.) were made to 150 and 40 mM, respectively, in DMSO and stored at –20 °C. Stock solutions (10 mM in DMSO) of cremart [O-ethyl-O-(3-methyl-6-nitrophenyl) N-sec-butyl-phosphorothio-imidate] (Sumitomo Chemical Co., Hyogo, Japan) and oryzalin [4-dipropylamino-3,5-dinitrobenzene sulphamide] (Lilly Research Laboratories, Greenfield, IN, U.S.A.) were prepared just prior to experiments. Stock solutions were diluted in artificial pond water (APW; KCl 0.1 mM, NaCl 1.0 mM, $CaCl_2$ 0.1 mM, N-tris[hydroxymethyl]methyl-2-aminoethane sulfonic acid (TES) 2.0 mM, pH 7.2 Na^+). Colchicine (Sigma) was dissolved directly into APW prior to experiments. The DMSO concentration of APW was adjusted to 0.2% (v/v) in all experiments.

Inhibitor studies

The velocity of cytoplasmic streaming for the fastest visible organelles was measured with a stopwatch using a dissection microscope fitted with an ocular micrometer, usually over a distance of 625 μm. Measurements from numerous cells were averaged. For single inhibitor experiments, cells remained in APW until transferred to APW containing cytochalasin D (0.5 to 40 μM). For double inhibitor experiments, microtubules were depolymerized with oryzalin (1.0 to 10 μM for 3 to 4 h) prior to transfer to cytochalasin D solutions that maintained the concentration of oryzalin used in the pretreatments. After 90 min cytochalasin treatments, cells were briefly rinsed and left to recover in fresh samples of their original oryzalin-containing solutions. Cremart (1.0 to 10 μM) and colchicine (1.0 to 10 mM) were also used to depolymerize microtubules. Any variations on this protocol are noted in Results.

Immunofluorescence microscopy

Nitella internodes, greater than 1 cm long but still elongating, were fixed and immunolabelled by the perfusion method (Wasteneys et al. 1989, Wasteneys and Williamson 1991) using the modifications of Collings et al. (1995), with the exception that the actin stabilization agent MBS was not used. Primary antibodies were monoclonal anti-actin (clone C4 raised against chicken gizzard actin; ICN Biomedicals, Costa Mesa, CA, U.S.A.; Lessard 1989) and monoclonal anti α-tubulin (clone YL 1/2 raised against yeast tubulin; Serotec, Kidlington, U.K.; Kilmartin et al. 1982). Secondary antibodies were fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse Ig and FITC-conjugated sheep anti-rat Ig (Silenus, Hawthorn, Victoria, Australia).

^{3}H-cytochalasin B

Solvent was evaporated from a solution of ^{3}H-cytochalasin B (30.1 μM in ethanol (500 μl); 18.5 MBq/ml; Amersham, U.K.) and the ^{3}H-cytochalasin B redissolved in DMSO (40 μl) containing 150 mM cytochalasin B. Dilution in APW gave a 150 μM solution containing 0.1% DMSO with an activity of 14 200 ± 150 dpm/μl. Samples were adjusted to 0.2% DMSO with either 10 mM oryzalin in DMSO (giving 10 μM oryzalin) or with DMSO. Cell surface areas and volumes were determined from photographs taken prior to experiments. Uptake was measured in cells (n = 5 for each time point, with or without oryzalin) incubated in ^{3}H-cytochalasin for between 5 and 240 min. Cells were blotted dry (1–2 s) and uptake determined by summing the efflux from individual cells collected in 3 successive APW washes (1.5 ml with or without oryzalin) of 0.5, 29.5, and 150 min. Individual cells were then broken open and digested in 1.5 ml of APW containing 1% Triton X-100 (80 °C, 10 min). All 4 samples per cell were counted separately in 15 ml of Emulsifier Safe (Packard, Downers Grove, IL, U.S.A.).

Efflux was measured in cells (n = 9, with or without oryzalin) loaded for 90 min with ^{3}H-cytochalasin B. Blotted cells were moved through 18 washes (1.5 ml with or without oryzalin) before, at 240 min, being digested as in the influx study.

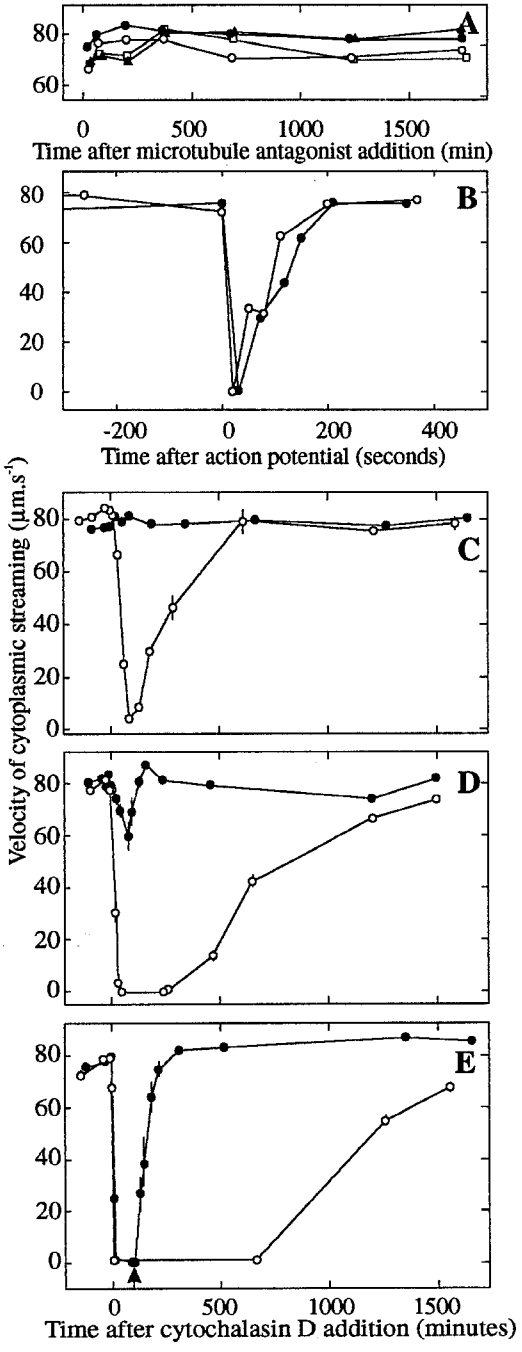
Results

In this study, we compared the sensitivity to cytochalasins of *Nitella* internodal cells, whose cortical microtubule arrays were intact (MT+), with cells

whose microtubules had been depolymerized by application of tubulin-specific drugs (MT–).

Microtubule antagonists alone do not alter streaming velocity

Microtubule depolymerization, whether by 10 μM oryzalin, 10 μM cremart, or 10 mM colchicine, did not affect cytoplasmic streaming (Fig. 1 A), and 10 μM oryzalin did not change streaming velocity, even over 72 h (data not shown). This indicates that it is unlikely that cytoplasmic free Ca^+ concentration (Ca_c) is raised by microtubule depolymerizing agents (see Hertel and Marmé 1983).



Oryzalin treatment does not potentiate action potential-induced streaming arrest

To see if the microtubule disruptor oryzalin had any effect on the cell's ability to sequester Ca^{2+} (as reported by Hertel and Marmé 1983), we compared recovery from action potential-induced streaming arrest in MT+ and MT– cells. Action potentials were generated by wounding cells adjacent to the internode in which streaming was measured, a procedure that reliably causes temporary streaming arrest because of an influx of Ca^{2+} (Williamson and Ashley 1982). We found that the recovery of streaming after the induction of an action potential was unaffected by the presence of 10 μM oryzalin (Fig. 1 B) and conclude that treatment with oryzalin does not appreciably interfere with the cell's ability to regulate Ca^{2+} levels.

Microtubule disassembly by oryzalin potentiates cytochalasin D's effects on cytoplasmic streaming

In contrast to this lack of effect on action potential-induced streaming cessation, cessation of streaming caused by cytochalasin treatments was greatly affected by the disassembly of microtubules. Microtubule depolymerization with oryzalin potentiated the effects of cytochalasin D in four ways (Fig. 1 C–E). First, inhibition of streaming by cytochalasin D occurred more rapidly (Fig. 1 D) (Wasteneys and Williamson 1991) even though oryzalin alone did not alter streaming velocity (Fig. 1 A). Second, the time taken for streaming to fully recover after cytochalasin D removal increased from a few minutes to several

Fig. 1. **A** Microtubule depolymerization did not affect streaming over the course of 1 day or longer: ● DMSO control, ○ oryzalin (10 μM), □ cremart (10 μM), ▲ colchicine (10 mM). Data are means, n = 2. **B** Microtubule depolymerization did not affect the recovery of cytoplasmic streaming after the induction of an action potential caused by wounding the adjacent internode. Representative single cells are shown: ● DMSO control, ○ oryzalin (10 μM). **C–E** Streaming was inhibited in microtubule-free cells (10 μM oryzalin) by cytochalasin D concentrations that are otherwise ineffective, and recovery after cytochalasin D removal at t = 90 min (arrow in **E**) was delayed in a dose-dependent fashion. ● Cells in the absence of oryzalin (replotted from Collings et al. 1995). ○ Cells pretreated with oryzalin (10 μM). **C** 1.0 μM cytochalasin D, **D** 2.5 μM cytochalasin D, **E** 40 μM cytochalasin D. Data are means ± SEM, n = 4; as in all figures presented, all experiments grouped together were run concurrently

hours after 1.0 μM cytochalasin D treatment and to 24 h after 40 μM cytochalasin D treatment (Fig. 1 C–E) (Wasteneys and Williamson 1991). Third, the minimal cytochalasin D concentration required to stop streaming decreased from 10 μM (Collings et al. 1995) to 1 μM (Fig. 1 C). Fourth, the short rods of cortical actin that form during cytochalasin D treatments (Collings et al. 1995) disappeared far more slowly after cytochalasin D removal; their disappearance coincided with the eventual recovery of cytoplasmic streaming (Figs. 2–7).

Potentiation was not confined to cytochalasin D but occurred similarly in cytochalasin B treatments (Fig. 30).

Potentiation of cytochalasin's effects in other Characeae and Spirogyra

After microtubule depolymerization (10 μM oryzalin), delays of 10–15 h in the recovery of streaming after cytochalasin D (40 μM) removal were recorded in 4 species of characean algae from 3 different genera tested (*Nitella pseudoflabellata, N. cristata, Nitellopsis obtusa* and *Chara corallina*). In the green alga *Spirogyra*, similar potentiation occurred. In MT+ cells, 20 and 40 μM cytochalasin D reduced but did not arrest streaming within 15 min and streaming velocity was fully restored within 10 to 20 min of washing out the cytochalasin. Lower concentrations of cytochalasin D had no effect. By contrast, MT– cells of *Spirogyra* (10 μM oryzalin) were sensitive to 5 μM cytochalasin D (streaming was reduced within 15 min and recovery took approx. 1 h) and streaming could be arrested with 10 μM cytochalasin D. Measurements of cytoplasmic streaming in epidermal cells of *Tradescantia virginiana* and *Allium cepa* and light-dependent cyclosis of chloroplasts in mesophyll cells of *Vallisneria gigantea*, however, gave no indication that the effects of cytochalasin D could be potentiated in cells pretreated with 10 μM oryzalin (Collings 1994). Notably, streaming in the higher plant material was more sensitive to cytochalasin D than in the algal cells. Cytochalasin D concentrations that were effective in the higher plant material (0.25 to 5.0 μM) were only effective in algal cells that lacked intact microtubules.

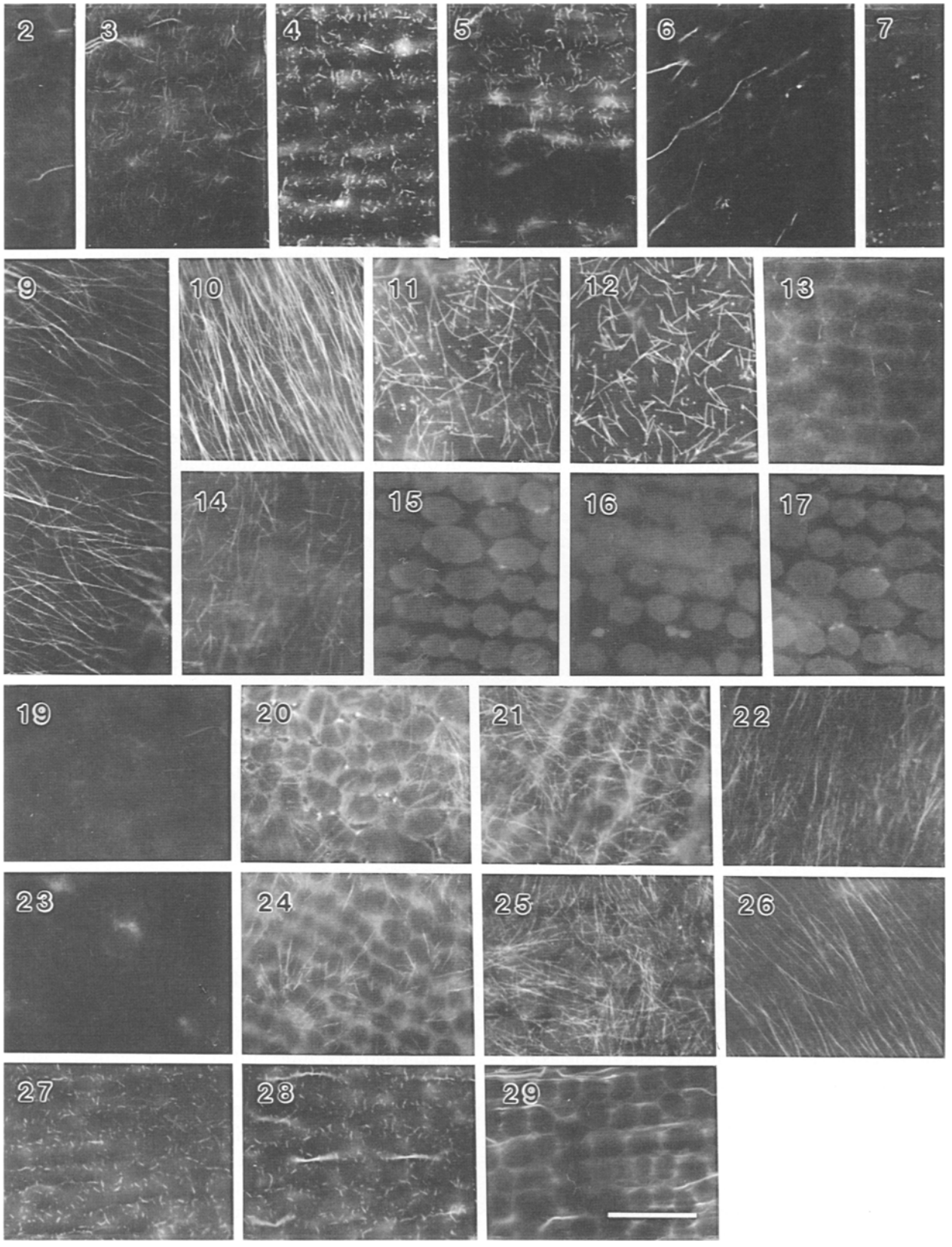
To exclude the possibility that the potentiation of cytochalasin is specific to one microtubule inhibitor, we examined the effects of three chemically distinct microtubule inhibitors, oryzalin, cremart, and colchicine, in *N. pseudoflabellata*. None of these drugs affected cytoplasmic streaming on its own (Fig. 1 A) but all showed concentration-dependent potentiation of cytochalasin treatments (Fig. 8; data for cremart were similar to those for oryzalin and not shown).

Microtubule assembly state but not orientation is critical for potentiation of cytochalasin

To clarify the relationship between potentiation and microtubule assembly states, we compared the extent of microtubule disassembly to the extent of potentiation for the 3 microtubule drugs. We also determined how rapidly potentiation follows microtubule disassembly and examined the relationship between streaming recovery and microtubule reassembly and realignment.

Immunofluorescence observations of microtubules in cells pretreated for 3 h with different concentrations of oryzalin, cremart, or colchicine were related to the recovery of cytoplasmic streaming in similarly treated cells after the removal of cytochalasin D (40 μM, 90 min) already described in Fig. 8. 1 mM colchicine caused no observable loss of microtubules (Fig. 10; compare with control in Fig. 9) and did not delay streaming recovery after cytochalasin D removal (Fig. 8 A). Higher colchicine concentrations (2.5, 5, and 10 mM) delayed streaming recovery after cytochalasin removal (Fig. 8 A) broadly in line with microtubule loss (Figs. 11–13). 1 μM oryzalin disassembled microtubules to about the same extent as 5 to 10 mM colchicine (Fig. 14) and delayed streaming recovery by approximately the same time as 5 to 10 mM colchicine (Fig. 8 B). Oryzalin (Figs. 8 B and 14–17) and cremart (data not shown), at concentrations of 2.5 μM or greater, depolymerized microtubules completely and this caused a more substantial delay in streaming recovery (Fig. 8 B). Although the threshold for complete microtubule depolymerization appeared to be at 2.5 μM, higher concentrations of oryzalin (5 and 10 μM) caused substantially longer streaming recovery times (Fig. 8 B). There was, however, relatively little difference in recovery times between cells treated with 5 and 10 μM oryzalin.

We next looked at the relationship between potentiation and the timing of microtubule disassembly and reassembly. Cells pretreated with 2.5 μM cytochalasin D continued streaming for 12 h (Fig. 18 A) but showed severe, albeit reversible, inhibition upon application of oryzalin. The time required for streaming to cease was approximately equal to the time required for oryzalin to depolymerize microtubules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(Wasteneys and Williamson 1989, Wasteneys et al. 1993) showing the close coupling between microtubule depolymerization and streaming cessation.

Microtubule disassembly did not have to be concurrent with cytochalasin treatments to cause potentiation. If cells were removed from cytochalasin D (40 μM, 90 min) and moved to oryzalin (10 μM), the recovery of streaming was greatly inhibited, although less so than if cells had been in oryzalin during the cytochalasin D treatment (Fig. 18 B). This suggested to us that cytochalasin D efflux from cells takes place over a relatively long period of time, an idea we will develop later.

To investigate the effect of microtubule reassembly on streaming recovery in potentiated cells, cells were washed free of both cytochalasin D (40 μM) and oryzalin at the same time (Fig. 18 B). Streaming recovery was still delayed for several hours compared to cells treated with only cytochalasin D but the delay was much shorter than for cells left in oryzalin after cytochalasin D removal. Once it began, streaming recovery proceeded very rapidly (Fig. 18 B). In similarly treated cells that were processed for immunofluorescence, microtubule reassembly after oryzalin removal followed the sequence of branching clusters, random microtubules and, after several hours, transversely aligned microtubules (Figs. 19–22) described by Wasteneys and Williamson (1989). Exposure to cytochalasin D (40 μM) did not alter microtubule reassembly patterns (compare Figs. 23–26 with Figs. 19–22). By fixing cells in which streaming had just restarted, we found that the recovery of cytoplasmic streaming began when microtubules were still only in the branching cluster stage of recovery (Fig. 24). Earlier studies (Wasteneys and Williamson 1989), however, indicate that maximum microtubule polymerization is reached long before microtubules are consolidated into transverse arrays. Thus, reassembly but not realignment of cortical microtubules is critical for loss of potentiation.

Potentiation of cytochalasin's effect on the structure of cortical actin

We have previously shown that cytochalasin D modifies the cortical actin array to produce short, stable

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Figs. 2–7. Recovery of cytoplasmic streaming in MT– cells after removal of cytochalasin D (40 μM, 90 min) coincided with the recovery of the cortical actin cytoskeleton visualized by anti-actin immunofluorescence. Bar in Fig. 29, for all figures: 20 μm

Fig. 2. Control cells, fixed prior to the addition of cytochalasin D, showed no short stable actin rods in the cortex

Figs. 3–5. At t = 90 min when cytochalasin D was removed (Fig. 3), cortical rods of actin were present as they were at 3.5 h (Fig. 4) and 18 h (Fig. 5)

Fig. 6. By 27 h, streaming had recovered and the cortical actin rods had disappeared. Only the longitudinally oriented cortical bundles were visible on the plasma membrane side of the chloroplasts

Fig. 7. Anti-tubulin immunofluorescence showed that microtubules did not recover after 27 h in oryzalin. Streaming rates of 0, 0, 0, 58.1, and 65.6 μm/s were recorded immediately prior to perfusions in the cells shown in Figs. 3–7

Figs. 9–17. Microtubules visualized by anti-tubulin immunofluorescence in elongating *Nitella* internodes showed a concentration-dependent response to different microtubule antagonists. Cells were pretreated for 3 to 4 h prior to perfusion

Fig. 9. APW control

Figs. 10–13. Colchicine treatment of 1.0 (Fig. 10), 2.5 (Fig. 11), 5.0 (Fig. 12), and 10 mM (Fig. 13)

Figs. 14–17. Oryzalin treatments of 1.0 (Fig. 14), 2.5 (Fig. 15), 5.0 (Fig. 16), and 10 μM (Fig. 17)

Figs. 19–29. The pattern and timing of microtubule (Figs. 19–26) and F-actin (Figs. 27–29) recovery viewed by immunofluorescence in cells following treatment with 10 μM oryzalin only (Figs. 19–22) and after co-treatment with oryzalin (10 μM) and cytochalasin D (40 μM) (Figs. 23–26). Approximate times after oryzalin removal were 90 (Figs. 19, 23, and 27), 120 (Figs. 20, 24, and 28), 150 (Figs. 21, 25, and 29), and 320 min (Figs. 22 and 26)

Figs. 19–26. Microtubule recovery proceeded from a total absence of microtubules (Figs. 19 and 23) through radiating clusters (Figs. 20 and 24) via random (Figs. 21 and 25) to transversely aligned microtubules (Figs. 22 and 26). Cytoplasmic streaming in oryzalin treated cells was unaffected; in cytochalasin D/oryzalin treated cells (Figs. 23–26), streaming rates of 0, 51.7, 32.9, and 70.8 μm/s were recorded

Figs. 27–29. In cells removed from both oryzalin (10 μM) and cytochalasin D (40 μM) cytochalasin-stabilized cortical actin rods were present at times when microtubules were still absent (Fig. 27). As microtubules repolymerized into radiating clusters, the cytochalasin D-stabilized actin remained (Fig. 28). The disappearance of cytochalasin D-stabilized actin corresponded to recovery of cytoplasmic streaming and to the establishment of a random microtubule pattern (Fig. 29). Cytoplasmic streaming rates of 0, 52.0, and 62.1 μm/s, respectively, were recorded for these cells

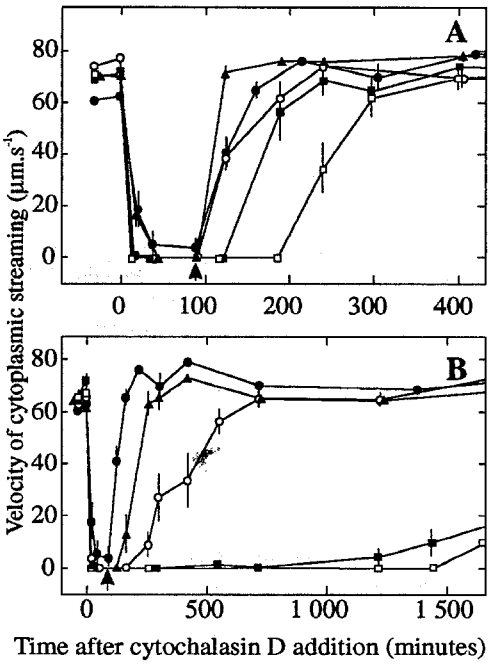

Fig. 8 A, B. The potentiation of cytochalasin D's action on cytoplasmic streaming depended on the concentration of microtubule antagonist used. Cytochalasin D treatments (40 μM, 90 min) were from t = 0 until removal (arrow). ● Cytochalasin D control. Cells were pretreated with either **A** 1 mM colchicine (▲), 2.5 mM colchicine (○), 5.0 mM colchicine (■), or 10 mM colchicine (□) or **B** 1 μM oryzalin (▲), 2.5 mM oryzalin (○), 5.0 mM oryzalin (■), or 10 μM oryzalin (□). Data are means ± SEM, n = 8. Note the different scales in **A** and **B**

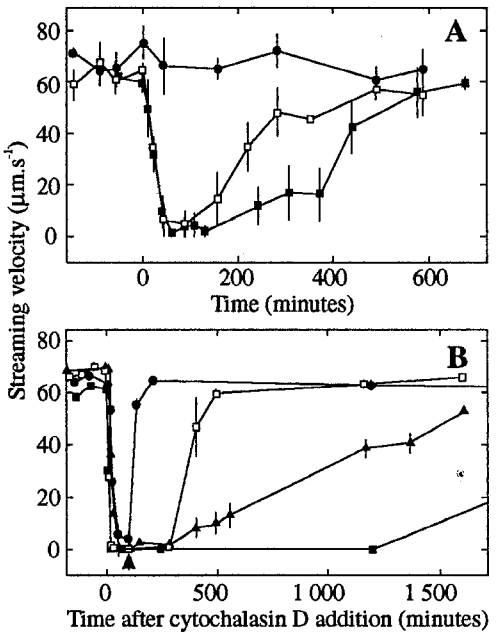

Fig. 18. **A** Cytoplasmic streaming in cells treated with 2.5 μM cytochalasin D (a concentration that did not reduce streaming velocity in this experiment; cf. the slightly different result in Fig. 1 where a different batch of cells and another lot of cytochalasin D were used) was reversibly inhibited by oryzalin (10 μM). Three separate treatments are shown. Cytochalasin D control from t = –90 min did not significantly inhibit streaming over 11 h (●). A cytochalasin D treatment from t = –90 min continuously for 11 h, with a pulse of oryzalin added at t = 0 for 90 min, caused rapid but reversible streaming inhibition after microtubule depolymerization (□). Oryzalin pretreatment (from –150 min, with cytochalasin D (90 min) from t = 0) similarly inhibited streaming (■). Data are means ± SEM, n = 4. **B** The long delay in the recovery of streaming in MT– cells after the removal (arrow) of cytochalasin D (40 μM, 90 min) was greatly reduced by the simultaneous removal of oryzalin. ● Cytochalasin D control; ■ cells pretreated with oryzalin (10 μM, 3–4 h) and remaining in oryzalin after cytochalasin D removal; □ cells pretreated with oryzalin (3–4 h) but removed from both cytochalasin D and oryzalin at 90 min; ▲ MT+ cells treated with cytochalasin D (90 min) and then allowed to recover cytoplasmic streaming in a solution containing oryzalin (10 μM). Data are means ± SEM, n = 6

rods and that, when cytochalasin D is removed, both streaming and cortical actin structures recover at similar times (Collings et al. 1995). This also happened in MT– cells when only cytochalasin D was removed (Figs. 2–7), and when both cytochalasin D and oryzalin were removed at the same time (Figs. 27–29). In the latter case, streaming recommenced (Fig. 28) and the cortical rods disappeared (Fig. 29) when cortical microtubules had reassembled but were not yet consolidated into a transverse array.

Uptake and efflux of H^3-cytochalasin B

To determine if microtubule disassembly potentiates cytochalasin's action by increasing the cellular concentration of cytochalasin, we estimated uptake and efflux of radiolabelled cytochalasin. Because cytochalasin B is the only cytochalasin commercially available with ^{3}H-labelling, we first verified that it too would be potentiated by microtubule disassembly. After a 90 min treatment with 150 μM cytochalasin B, streaming recovery took 4 h in the presence of oryzalin instead of less than 20 min in oryzalin's absence (Fig. 30) clearly demonstrating that cytochalasin B is also potentiated by microtubule disruption. That a higher concentration of cytochalasin B than cytochalasin D is required to inhibit streaming reflects a general variability between the effectiveness of different cytochalasins in both *Nitella* and higher plants (Wasteneys unpubl. obs.).

The total uptake of tritiated cytochalasin B in MT+ cells or MT– cells was not significantly different (Fig. 31 A). By 90 min, the total loading was approximately 40 000 dpm/μl, about three times the activity

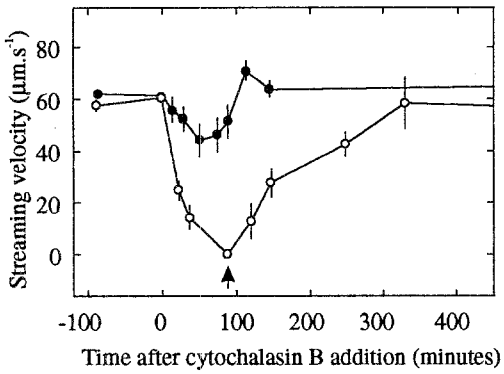

Fig. 30. Microtubule depolymerization potentiated the action of cytochalasin B. Cytochalasin B treatments (150 μM), in the absence (●) and presence (○) of oryzalin (10 μM), were for 90 min from t = 0, with removal indicated by arrow. Data are means ± SEM, n = 6

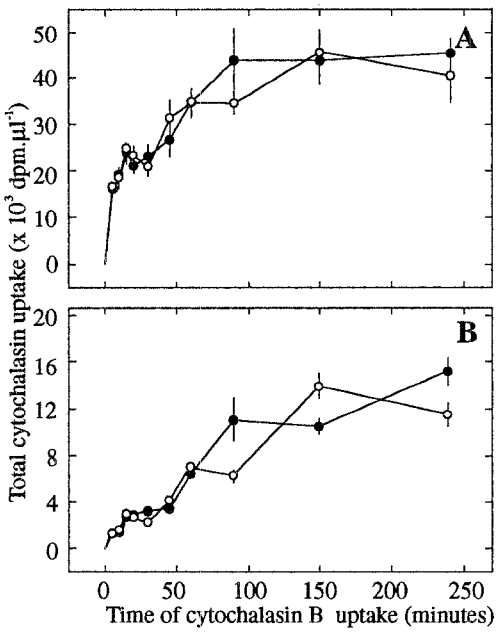

Fig. 31 A, B. The uptake of ^{3}H-cytochalasin B (150 μM) was similar in the presence (○) and absence (●) of oryzalin. Data are means ± SEM, n = 5 cells for each time point. **A** Total cytochalasin B uptake. **B** Uptake as measured by the amount of cytochalasin B remaining in the cell after 180 min of washing

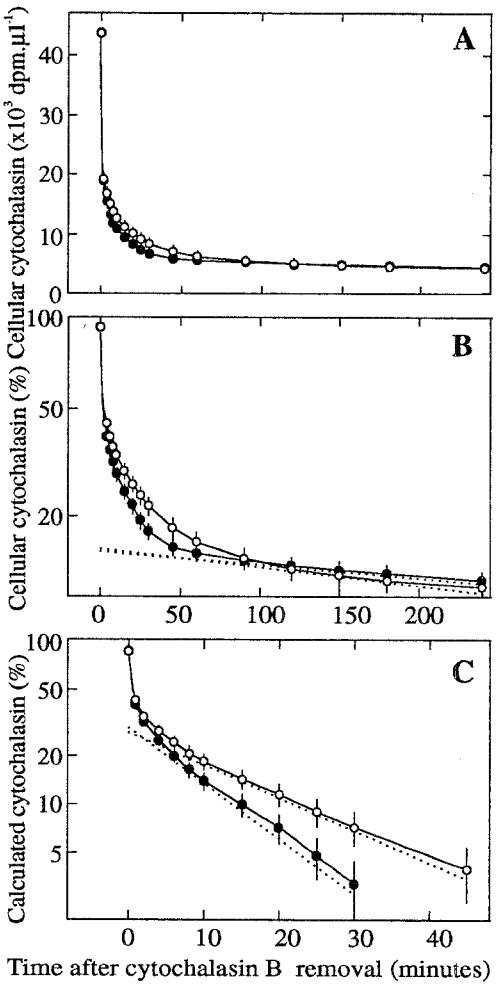

Fig. 32 A–C. The efflux of cytochalasin B (150 μM) after 90 min loading was similar in the absence (●) and presence (○) of oryzalin. Data are means ± SEM, n = 9 cells. **A** Total efflux. **B** Efflux plotted on a semi-logarithmic scale as a percentage of total uptake; there was little difference between the two treatments except in the first hour. After this time, the straight line indicates an exponential-type efflux. Lines of best fit are plotted for the data after t = 60 min and correspond to the slow phase of cytochalasin B efflux. **C** Using the method of MacRobbie and Dainty (1958), the slow phase of total efflux was subtracted from the data and the remaining radioactivity plotted on a semi-logarithmic plot. Once again, efflux approximated an exponential curve, as shown by the straight line of best fit after an initial rapid efflux

of the bathing medium. Whereas total uptake was very rapid at first and showed clear signs of levelling off, the amount of cytochalasin B that remained in the cells after 180 min of washing, that is the amount found in the cell digestion, continued to rise in an approximately linear manner in both MT+ and MT– cells, especially over the first several hours (Fig. 31 B).

The efflux of tritiated cytochalasin B was similar for MT+ and MT– cells (Fig. 31 A). Efflux resolved into three linear phases on semi-logarithmic plots using the method of MacRobbie and Dainty (1958) (Fig. 32 B, C), consistent with the existence of three compartments that empty in series with half-times of about 1, 10–15, and 400–600 min. There were no marked differences in efflux between MT+ and MT–

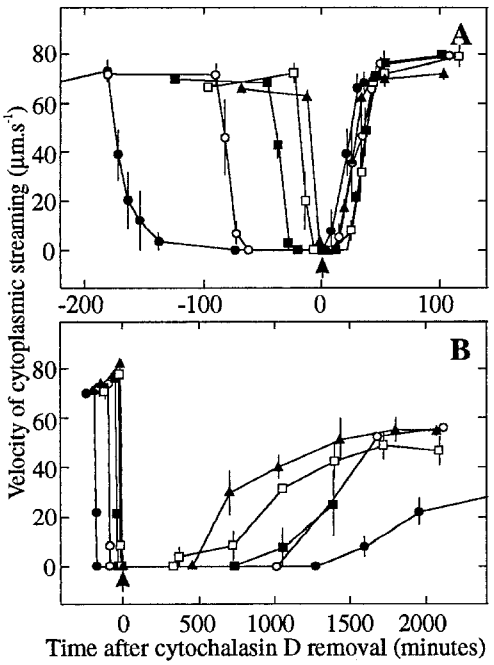

Fig. 33 A, B. The dependence of the delay in cytoplasmic streaming recovery on the length of cytochalasin D (40 μM) exposure. There was no significant delay in streaming recovery when intact microtubules were present (**A**) but increasing the exposure to cytochalasin D delayed eventual streaming recovery if microtubules were depolymerized with oryzalin (**B**). Serially-doubled cytochalasin D exposure times of 11.25 (▲), 22.5 (□), 45 (■), 90 (○), and 180 min (●) are shown for cells with intact microtubules (**A**) and for cells treated with oryzalin (10 μM) (**B**). Results are presented so that the removal of cytochalasin D (arrow) is at t = 0 min in all cases. Data are means ± SEM, n = 5

cells, except for a period in the first hour when the second efflux phase was somewhat slower from MT– cells than MT+ cells (half-times of 15 and 9 min, respectively) (Fig. 32 C). This small difference looks unpromising for understanding the timing of streaming recovery because MT+ and MT– cells showed similar cytochalasin B contents after this period even though streaming remained totally inhibited in MT– cells for over 4 h while it recovered in the MT+ cells within 20 min of the start of the efflux. This finding, together with similarities in the uptake studies, leads us to conclude that rather than being exposed to higher intracellular concentrations, the actin in MT– cells responds more strongly to cytochalasin B. How, in such a scheme, might the major delay in streaming recovery be explained?

Under conditions of microtubule polymerization and depolymerization, the efflux of cytochalasin B was rapid: approximately 80% was lost within the first 30 min. However, considerable cytochalasin B remained in the cell for at least 4 h after the removal of cytochalasin B from the external medium. This low phase efflux followed an exponential decay with a half-time of around 400–600 min, as demonstrated by the occurrence of a straight line efflux in the semi-logarithmic plot (Fig. 32 B). This suggests to us that cytochalasin (either B or D) entering the cytoplasm from the slowly exchanging compartment inhibits streaming in MT– cells even though a similar cytochalasin B (or D) concentration in MT+ cells is insufficient to inhibit streaming. To determine the importance of this slowly exchanging cytochalasin B/D, we investigated how varying the loading time affected the recovery of streaming in MT– and MT+ cells. The rationale for these experiments is that, as shown in Fig. 31 B, increasing the loading time increased the amount of cytochalasin B that remained in the cell 180 min after efflux starts when the cell is in the slow phase of cytochalasin efflux. Such variation in cytochalasin D (40 μM) treatment times from 11.25 to 180 min made no difference to streaming cessation or recovery in MT+ cells (Fig. 33 A). In MT– cells, however, longer cytochalasin D loading times did delay the recovery of cytoplasmic streaming after cytochalasin D removal in an approximately linear manner (Fig. 33 B); a delay of approximately 300 min was estimated for each doubling of loading time.

Discussion

In the giant internodal cells of characean algae, oryzalin and other microtubule antagonists affect neither the structure of the actin cytoskeleton nor cytoplasmic streaming velocity when used alone. However, when applied with cytochalasin (this term is used "generically" to refer to cytochalasin B and D), they potentiate cytochalasin's action in four ways. Compared to MT+ cells, the inhibition of cytoplasmic streaming in MT– cells is faster and occurs at lower cytochalasin concentrations and the recovery of streaming is delayed for several hours after cytochalasin's removal. Short cortical rods of actin can also be induced at lower than normal cytochalasin concentrations and, although these actin rods are similar to those occurring in cells treated with cytochalasin alone (Collings et al. 1995), their loss is also delayed and matches the recovery of cytoplasmic streaming after cytochalasin's removal.

Hypotheses for potentiation not involving microtubule depolymerization

We do not believe that our full data set can be plausibly explained by any of several hypotheses that do not invoke microtubule depolymerization as the primary mechanism.

1. Direct action of anti-microtubule agents on actin. This is implausible because binding sites for 3 chemically distinct anti-microtubule agents must all exist on actin and have binding constants that mirror those for the same compound on tubulin.
2. Direct interaction of the anti-microtubule drugs with cytochalasin. This is implausible because of the 3 chemically distinct microtubule agents used, the wide range of molar ratios involved (ca. 1 : 10 oryzalin : cytochalasin D, ca. 100 : 1 colchicine : cytochalasin D) and the need for all resultant dissimilar complexes to be more effective than the parent compounds.
3. An additive mode of action for the anti-microtubule agents and cytochalasin such as raising Ca_c. This is unlikely because there is little evidence in plants that either cytochalasins or microtubule agents raise Ca_c. Although one of the microtubule antagonists, oryzalin, was reported to reduce Ca^{2+} accumulation into isolated mitochondria (Hertel and Marmé 1983), subsequent direct measurements of Ca_c levels in *Tradescantia* have shown no increase upon oryzalin treatment (Keifer et al. 1992). Moreover, such an effect on Ca_c in characean algae is unlikely because we have shown that cytoplasmic streaming, a process which is very sensitive to even small changes in Ca_c (Williamson and Ashley 1982, Plieth and Hansen 1992), is unaffected by oryzalin, cremart or colchicine and that streaming recovery after action potentials is identical in MT+ and MT– cells. Moreover, although cytochalasin has been reported to increase Ca_c in leukocytes (Treves et al. 1987), this effect was also not demonstrable in *Tradescantia* (Keifer et al. 1992). It is thus very unlikely that potentiation involves the summation of the effects of both classes of inhibitors on Ca_c.

Potentiation of cytochalasin's action by microtubule depolymerization

Two arguments demonstrate that the potentiation of cytochalasin's effects in characean internodal cells is directly related to microtubule depolymerization. First, the three chemically dissimilar anti-microtubule agents (colchicine, cremart, and oryzalin) all potentiate the action of cytochalasin. Second, the degree of potentiation for all 3 agents directly correlates with their effectiveness at depolymerizing microtubules. Concentrations of microtubule antagonists that do not disrupt microtubules (1 mM colchicine) do not cause potentiation. Concentrations that cause only partial depolymerization of microtubules (2–10 mM colchicine, 1 μM oryzalin, and 1 μM cremart) result in intermediate potentiation. Concentrations of microtubule antagonists causing full depolymerization (which alone have no observable effect on streaming velocity or on the health of cells in general) result in the longest delays in streaming recovery and, because minimal increase in the delay occurs from 5 to 10 μM oryzalin, potentiation seems saturable. It is notable, however, that the apparently complete microtubule depolymerization by 2.5 μM oryzalin does not cause maximum potentiation of cytochalasin's effects on streaming which, instead, occur at 5 to 10 μM oryzalin. We suggest that this may be because microtubule depolymerization can be induced at substoichiometric oryzalin concentrations. Thus, the extent to which tubulin-oryzalin complexes form may be an important factor in the potentiation mechanism.

Hypotheses to explain potentiation

We believe that the arguments we have advanced provide a strong case for relating potentiation of cytochalasin-induced streaming inhibition to microtubule depolymerization rather than to some side effect common to all the inhibitors. Two hypotheses can be envisaged to account for potentiation: (1) microtubule depolymerization may either increase the concentration of intracellular cytochalasin to which *Nitella* actin is exposed, or, (2) microtubule depolymerization may sensitize actin to cytochalasin so that it responds more strongly to a given level of intracellular cytochalasin.

The first hypothesis is discounted by the lack of major differences in the uptake and efflux of ^{3}H-cytochalasin B from MT– and MT+ cells. Microtubule depolymerization does not affect uptake and the slightly slower efflux from MT– cells during the first hour is insufficient to explain the prolonged delay in streaming recovery. The slower efflux might result from the absence of mixing provided by cytoplasmic streaming. The efflux data fit a three-compartment model (Fig. 31) with time constants comparable to those for ions effluxing from the free space, cytoplasm and vacuole (MacRobbie and Dainty 1958,

Hope 1971). However, cytochalasin B is lipophilic like other cytochalasins (Bech-Hansen et al. 1976) and is unlikely to partition like an ion and we have not unequivocally confirmed the physical identities of the three compartments (Collings 1994). Nevertheless, whatever the physical location of each phase, there are no grounds to believe that microtubule depolymerization redistributes cytochalasin B between them. With no evidence of significant differences in uptake, partitioning, or efflux, we conclude that potentiation occurs because actin is sensitized to respond more strongly to intracellular cytochalasin.

Sensitization, by increasing the degree of inhibition resulting from any given concentration of cytochalasin in the cytoplasm, can qualitatively explain several facets of potentiation in MT– cells including why lower external cytochalasin concentrations can inhibit streaming, why streaming inhibition is more rapid, and why streaming remains inhibited longer after the external cytochalasin is removed. In the latter case, some 80% of the cytochalasin B loaded over 90 min is lost within 30 min of cells being moved to a cytochalasin B-free medium as the rapid and intermediate compartments (half-times of less than 15 min) are extensively depleted (Fig. 32) and the cytoplasmic cytochalasin B concentration falls. However, if we assume the cytoplasm represents the intermediate phase and that the slow phase cytochalasin B reaches the external medium via the cytoplasm, further falls in the cytoplasmic concentration of cytochalasin B occur only slowly because they depend not on the cytoplasm's own efflux characteristics but on those of the slow compartment (half-time 400–600 min).

Because the potentiation response is similar for both cytochalasins B and D, we assume that cytochalasin D demonstrates similar efflux kinetics to those of cytochalasin B. We hypothesize that the initial rapid cytochalasin efflux reduces the cytoplasmic cytochalasin concentration enough to allow streaming to recover in MT+ cells. In MT– cells, however, actin is more sensitive to cytochalasin so the cytochalasin concentration that allows streaming to restart in these cells is only reached much later, at some time during the slow phase of cytochalasin efflux. A key feature of this model is that the reservoir of slowly exchanging cytochalasin exists in both MT+ and MT– cells but that only the actin in MT– cells is sufficiently sensitized for the slowly exchanging cytochalasin to determine the time required for streaming to recover.

We tested this hypothesis by extending the loading time for cytochalasin D and observing the effect on streaming recovery in MT– and MT+ cells. Uptake of ^{3}H-cytochalasin B (and hence we assume of cytochalasin D), into the slow compartment is roughly linear with time (Fig. 31 B) so that doubling the loading time approximately doubles the slow phase content. However, the time required for the efflux of this doubled amount of cytochalasin is not doubled, but is increased by the efflux half-time for the slow compartment. Consistent with our model, recovery times in MT-cells, which are primarily dependent on the size of the slow phase reservoir of cytochalasin, are markedly affected whereas recovery in MT+ cells, which are primarily dependent on the efflux kinetics of the faster compartments, remain unaffected over the times studied. Furthermore, there is reasonable quantitative agreement with the model: each doubling of the cytochalasin D uptake period delays streaming recovery by ca. 300 min, a value in reasonable agreement with the 400–600 min half-time estimated for the slow phase of ^{3}H-cytochalasin B efflux (Fig. 32 B).

How does microtubule depolymerization affect actin?

If microtuble depolymerization sensitizes actin to cytochalasin, how do the two cytoskeletal systems interact? That microtuble depolymerization can have far reaching effects on actin is seen in fibroblasts where actin-based cell contractility is rapidly strengthened (Danowski 1989), F-actin reorganized, and the cell's F-actin content transiently increased (Kajstura and Bereiter-Hahn 1993). For *Nitella*, the simplest hypothesis would be that microtubules, some of which are close to both cortical and subcortical actin, directly stabilize the actin so that microtubule depolymerization makes the actin more susceptible to cytochalasin. We consider this implausible: there is similar potentiation of cytochalasin's effects on both the cortical actin strands which are located near to the cortical microtubules (Wasteneys et al. 1996, Collings et al. 1995) and also on the subcortical actin bundles which are, in molecular terms, distant from the vast majority of microtubules. Relatively few microtubules associate with the subcortical actin bundles (Wasteneys and Williamson 1991, Wasteneys et al. 1993, Collings 1994) where streaming is generated.

A more plausible hypothesis is that the actin filaments are sensitized to cytochalasin by either tubulin dimers or microtubule-associated proteins, large amounts of which are released when microtubules depolymerize. Microtubule components, as opposed to assembled

microtubules, could act on F-actin filaments at any point in the cell to which diffusion takes them. There is no direct evidence that either tubulin or microtubule-associated proteins sensitize actin to cytochalasin but some actin-binding proteins do modify actin's response to cytochalasin B (Suzuki and Mihashi 1991) and tubulin/microtubules bind numerous proteins of the actin cytoskeleton including actin itself (Verkhovsky et al. 1981), spectrin (Ishikawa et al. 1983), synapsin (Baines and Bennett 1986), caldesmon (Ishikawa et al. 1992a, b) and EF-1α (Marchesi and Ngo 1993). Notably, a homologue of EF1α is a component of the subcortical actin bundles of *Nitella* (Colling et al. 1994). The raised free tubulin levels that will follow microtubule depolymerization could, by increasing the quantities of actin-binding proteins complexed to tubulin, reduce the protection that actin normally receives from its associated proteins. Alternatively, numerous microtubule-associated proteins of animal cells, including kinesin (Okuhara et al. 1989), MAP1 (Asai et al. 1985), MAP2 and tau (Cross et al. 1993), bind actin in vitro and, furthermore, kinesin relocates to actin-containing stress fibres when microtubules depolymerize in bovine fibroblast cells (Okuhara et al. 1989). What effect these proteins have on actin's sensitivity to cytochalasin remains unknown. However, a family of kinesin-related proteins exists in *Arabidopsis* (Mitsui et al. 1993, 1994) and plants contain tau- and kinesin-cross reactive proteins (Vantard et al. 1991, Tiezzi et al. 1992), and probably many other microtubule-associated proteins (Schellenbaum et al. 1992). The locations of these proteins in microtubule-free cells have not yet been determined.

In conclusion, microtubule depolymerization potentiates the actions of cytochalasin on cytoplasmic streaming in *Nitella* and other characean algae so that cells respond more rapidly to cytochalasin, they respond to otherwise non-inhibitory concentrations, and they markedly delay their streaming recovery when restored to cytochalasin-free medium. Sensitization of actin to cytochalasin can explain all three facets of the response, including the long delayed recovery, if this depends on the large quantities of cytochalasin that exit only slowly from cells restored to cytochalasin-free medium. The concentrations of free tubulin and free microtubule-associated proteins will rise on microtubule depolymerization and these proteins, by interaction with actin-binding proteins and/or actin, may provide the implied linkage between the two cytoskeletal systems.

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