

# Lipids Trigger Changes in the Elasticity of the Cytoskeleton in Plant Cells: A Cell Optical Displacement Assay for Live Cell Measurements

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**Abstract.** An assay has been developed to quantitatively measure the tension and elasticity of the cytoskeleton in living plant cells. The cell optical displacement assay (CODA) uses a focused laser beam to optically trap and displace transvacuolar and cortical strands through a defined distance within the cell. Results from these experiments provide evidence for the classification of at least two rheologically distinct cytoskeletal assemblies, cortical and transvacuolar, that differ in their tension and response to both signaling molecules and reagents that perturb the cytoskeleton. It is further demonstrated that the tension of the transvacuolar strands can be significantly decreased by

the addition of either linoleic acid, 1,2 dioctanoyl-*sn*-glycerol, or 1,3 dioctanoylglycerol. These decreases in tension could also be induced by lowering the cytoplasmic pH. In contrast, addition of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , or the ionophore A23187 to the cells caused a considerable increase in the tension of the transvacuolar strands. The data provides evidence that: (a) linoleic acid may be a signaling molecule in plant cells; (b) diacylglycerol functions as a signaling molecule through a protein kinase C-independent pathway mediated by  $\text{PLA}_2$ ; and (c)  $\text{Ca}^{2+}$  and pH have regulatory roles for controlling cytoskeleton tension and organization.

**T**HE plant cell cytoskeleton has been demonstrated to contain at least two distinct filamentous networks that are structurally equivalent to the microfilaments and microtubules more extensively described for animal cells (Lloyd, 1989; Traas et al., 1987; Gunning and Hardham, 1982; Tiwari et al., 1984; Parthasarathy et al., 1985). Evidence for intermediate filaments has also been presented (Hargreaves et al., 1989). Actin, tubulin, myosin, and a number of other cytoskeletal associated proteins have been demonstrated in plant cells (Lloyd, 1989; Hargreaves et al., 1989; Traas et al., 1987; Gunning and Hardham, 1982; Tiwari et al., 1984; Kato and Tonomura, 1977; Jackson, 1982; Traas, 1990; Parthasarathy et al., 1985). Morphologically, the filamentous networks in plants may be further subdivided into at least four cellular domains: nuclear, transvacuolar, subcortical cytoplasm, and plasma membrane associated (Traas et al., 1987). Functionally, it appears that the control of cell division and nuclear position may involve the nuclear and transvacuolar domains (Lloyd, 1989; Traas et al., 1987; Katsuta and Shibaoka, 1988). The subcortical domain may be more involved in cytoplasmic streaming (Jackson, 1982; Traas, 1990; Kaniya, 1981), while the plasma membrane-associated network may be important for such processes as secretion, endocytosis, cell wall biosynthesis,

and initiating transmembrane signals (Traas, 1990). Although the involvement of individual cytoskeletal domains in a particular cell function may vary, e.g., cell wall deposition (Lloyd, 1989; Gunning and Hardham, 1982; Tiwari et al., 1984; Traas, 1990; Heath and Seagull, 1982), lateral diffusion of plasma membrane proteins (Metcalf et al., 1983; 1986), nuclear location (Lloyd, 1989; Traas et al., 1987; Katsuta and Shibaoka, 1988), it appears that some of the networks may be interconnected within a dynamic cytoskeletal grid composed of microfilaments and microtubules (Lloyd, 1989; Traas et al., 1987; Traas, 1990; Kobayashi et al., 1988).

Efforts to understand the relationship between organization and function of the cytoskeleton in living plant cells, in most instances, have depended on the use of agents that disrupt microfilaments or microtubules. Unlike recent work in animal cells with "suckers and pokers," mechanical devices that perturb the cytoskeleton (Elson, 1988; Evans and LaCelle, 1975; Petersen et al., 1982), direct measurements of the mechano-chemical properties of cytoskeletons, i.e., filament tensions and viscoelastic properties, have been, for the most part, technically difficult because of the presence of a rigid cell wall surrounding the cell. Past efforts to use centrifugation (Marc et al., 1989; Galatis et al., 1984; Quader et al., 1987) to distort the cytoskeleton have yielded results; however, this technique lacks both the temporal and spatial resolution that is necessary to accurately explore

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domain-specific rheological properties. Severing experiments with laser microbeams have also been useful, although invasive, to demonstrate that transvacuolar strands are maintained under tension (Goodbody et al., 1991; Hahne and Hoffmann, 1984). In pioneering work, Ashkin and Dziedzic (1989) used high-intensity laser light to immobilize or trap long, thin filaments in the cytoplasm of scallion cells. These filaments were visible using phase contrast optics because of their higher index of refraction than the surrounding cytoplasm. Displacement of the stage in  $x$ ,  $y$ , or  $z$  relative to the fixed laser beam resulted in the intracellular displacement of the filament from its normal location. The displacement distance, the laser power required for trapping, and the speed and extent of rebound were found to be dependent on the velocity of displacement. This dependency was analogous to the viscoelastic properties observed for the non-Newtonian mechanical properties of polymers and actin/actin-tubulin networks (Elson, 1988). An attractive feature of this optical-based method is that it provides a potentially nondestructive, high-resolution technique capable of repetitive measurements of the tension in filaments within specific regions of the cytoskeleton. In this manner, it can be used to examine the changes in the elasticity and tension of the individual cytoskeletal domains in response to perturbants and biosignaling molecules.

The present communication describes the *in vivo* use of optical trapping in the form of a cell optical displacement assay (CODA)<sup>1</sup>. This technique is shown to measure lipid-mediated changes in the physical properties of transvacuolar and cortical cytoskeleton networks in soybean root cells. These results suggest a heretofore unknown signaling role for such molecules in affecting important physical properties in plant cells. In particular, linoleic acid, dioctanoyl-sn-glycerol,  $Ca^{2+}$ , and cytoplasmic pH are demonstrated to modify the viscoelasticity of cytoskeletal networks in plant cells.

## Materials and Methods

### Cell Optical Displacement Assay

A typical experiment is performed in the following manner. A soybean (*Glycine max* [L.] Merr. cv. Mandarin) cell suspension (originally derived from roots) (2  $\mu$ l; 48–72 h of growth) in IB5C medium (Metcalfe et al., 1983) is placed on a slide to which a coverslip is applied and then sealed with paraffin wax. The slide, coverslip down, is placed on the computer controlled stage of an ACAS 570 Fluorescence Interactive Laser Cytometer (Meridian Instruments, Okemos, MI) (Wade et al., 1993). The cells are viewed under phase illumination with an oil immersion 100 $\times$  (1.4 NA) objective. A video camera captures the images which are then viewed on a monitor and recorded on videotape. To initiate trapping, the two-dimensional scanning stage is moved to a strand that can be identified either by its higher index of refraction or by associated vesicles. An argon ion laser beam ( $\lambda_{ex}$  = 488 nm;  $\sim$ 1- $\mu$ m diam) is focused onto the fiber or the associated vesicle. This is facilitated by the parfocality of the beam and the imaging plane. The intensity of the trapping laser beam is then monotonically increased to a level that can maintain the optical trapping of the fiber at its initial position as the stage is moved through a defined displacement at a constant velocity. For transvacuolar (also referred to as transcytoplas-

mic) strands, measurements were performed in 20 different cells. The maximum trapping intensity to achieve success in all 20 displacement attempts did not vary from day-to-day by more than 5 mW as recorded at the laser head (12% variation). The trapping beam was positioned at a point on the filament between the nucleus and the membrane/wall for each attempt in the assay. For examinations of cortical strand tension, the number of trapping measurements varied due to the difficulty in finding either defined or vesicle-associated strands from which movement could be easily monitored. To insure that the trapping intensity did not damage the fibers, each displacement at a particular power setting was performed five times and in all instances the fiber was required to rebound to the original position following termination of the trap. In some instances, an infrared laser was utilized to compare its trapping ability with that of the argon ion laser. This comparison demonstrated that at low power settings  $\leq$ 150 mW, the 488-nm line of the argon ion laser did not induce optical damage (Ashkin and Dziedzic, 1989). All trapping experiments were recorded on videotape and individual pictures of the experiments were prepared frame-by-frame from the tapes.

Ionic concentrations for the experiments described in Fig. 7 are: NaCl (100 mM), KCl (100 mM),  $MnCl_2$  (100 mM),  $CaCl_2$  (100 mM), and  $MgCl_2$  (100 mM), respectively. Cells were maintained in these solutions during the experiment. In other experiments, cells were incubated with cytochalasin D (20  $\mu$ M), amiprophosmethyl (APM) (20  $\mu$ g/ml), sodium azide (20  $\mu$ M), phalloidin (20  $\mu$ g/ml), and taxol (2  $\mu$ g/ml). The concentrations of fatty acids, phospholipids, diacylglycerol analogues, phorbol esters, and A23187 used in the experiments are listed in Table I. All effector reagents were incubated with the cells at the concentrations indicated for 30 min before measurement and were maintained with the cells throughout the CODA. Temperature-dependent treatment of cells was performed as follows: cells were incubated in ice for 30 min before measurement and maintained under these conditions throughout the measurement (an ice chamber was seated on the slide during the assay). Cells were treated at 47.5°C in a water bath for 2 min and then measured at room temperature. Cytochalasin D, phalloidin, 1,2 dioctanoyl-sn-glycerol (1,2 DiC8), 1,3 dioctanoyl-glycerol (1,3 DiC8), phorbol dibutyrate, phorbol 12,13 didecanoate, all phospholipids, and ionophore A23187 were purchased from Sigma Immunochemicals (St. Louis, MO). Unless otherwise stated, the commercially obtained phospholipids were derived from animal sources or soybeans. No difference in activity was observed when phospholipids derived from soybean were compared with the same type of phospholipid derived from either brain extracts, egg yolk, or liver. All fatty acids were from Cayman Chemical Co., Inc. (Ann Arbor, MI). APM was a gift from Dr. M. V. Parthasarathy (Section of Plant Biology, Cornell University, Ithaca, NY) and taxol was a gift from Dr. Steven Heidemann (Department of Physiology, Michigan State University, East Lansing, MI).

### Confocal Fluorescence Microscopy of F-Actin Filaments

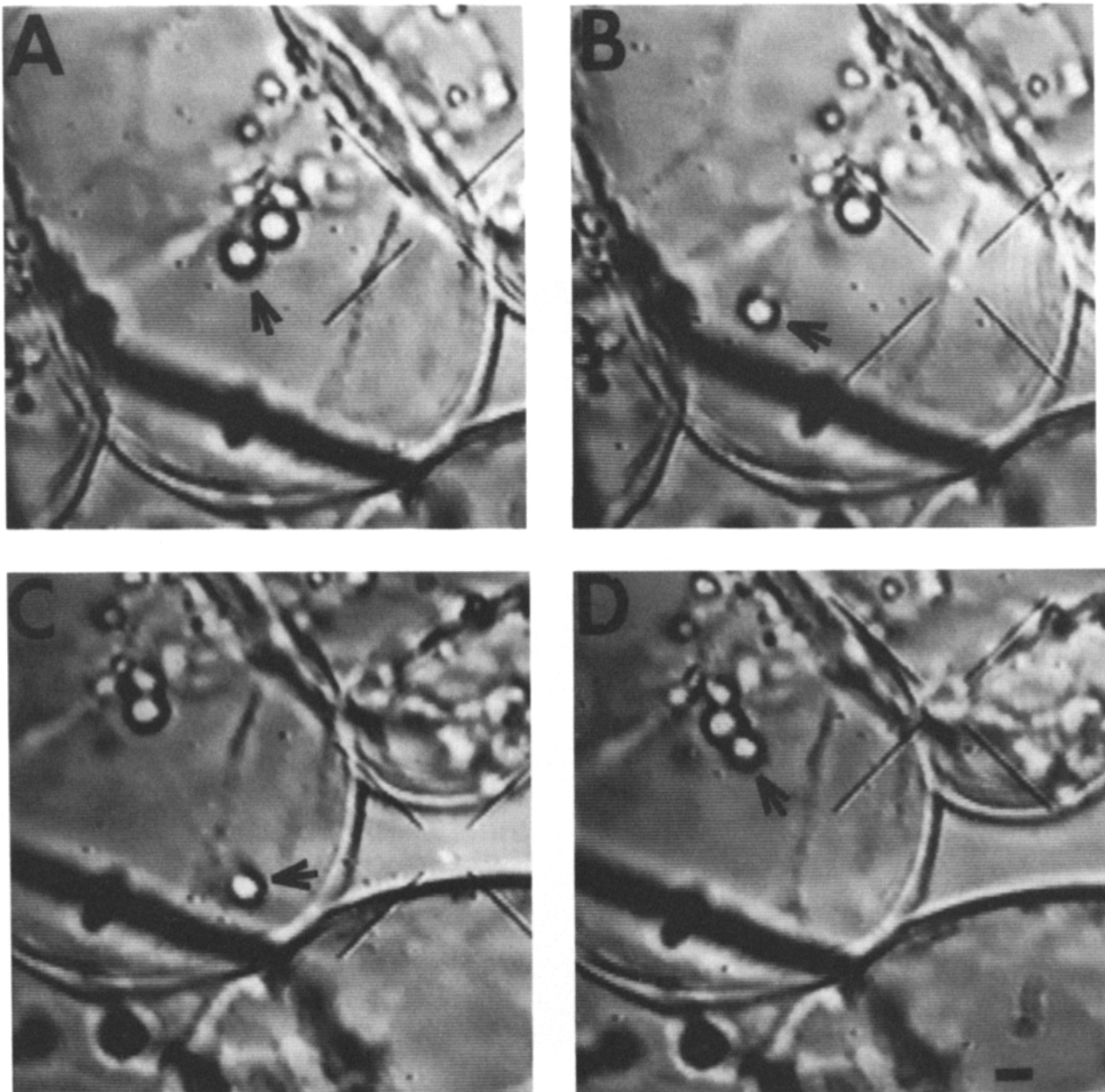
Soybean cells were fixed, permeabilized, and stained with NBD-phalloidin (Molecular Probes, Eugene, OR) as previously described for the probe rhodamineyl lysine phalloxin (Traas et al., 1987). Individual optical sections of the fluorescence distribution of the probe were acquired with an InSight Bilateral Laser Scanning Confocal microscope (Meridian Instruments, Okemos, MI) as previously described (Wade et al., 1993; Grabski et al., 1993).

## Results

### Optical Trapping of Transcytoplasmic Strands and Associated Vesicles

Ashkin and Dziedzic (1989) demonstrated that cytoplasmic filaments could be immobilized or trapped within a focused laser beam at power levels in the tens of milliwatts. In their experiments, the filament maintained its position within the stationary beam while the surrounding cell is displaced over micron distances. In Fig. 1, the trapping results of Ashkin and Dziedzic (1989) using an infrared laser (IR) (1.06  $\mu$ m line) are reproduced for a perinuclear localized vesicle (focus to the top of the vesicle) associated with a viscoelastic strand in the cytoplasm of a soybean root cell grown in sus-

1. *Abbreviations used in this paper:* APM, amiprophosmethyl; CDPK, calcium-dependent protein kinase; CODA, cell optical displacement assay; DAG, diacylglycerol; IP<sub>3</sub>, inositol 1,4,5-tris-phosphate; IR, infrared; NRK, normal rat kidney; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; 1,2 and 1,3 DiC8, 1,2 and 1,3 dioctanoyl-sn-glycerol; TPA, 12-*o*-tetradecanoylphorbol-13-acetate.

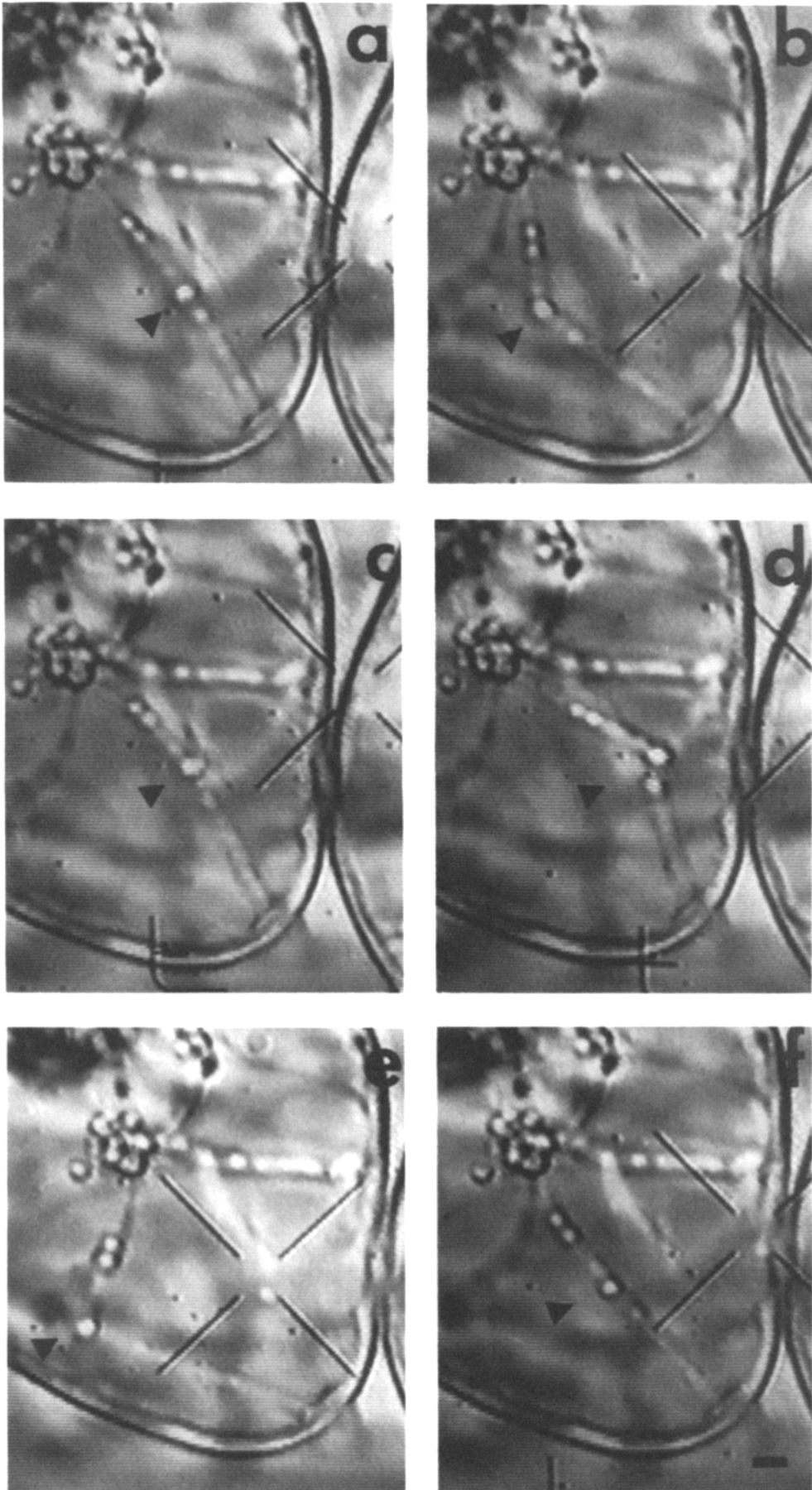


**Figure 1.** Optical displacement of a perinuclear localized vesicle in a soybean root cell using an infrared laser. Phase views of a single soybean root cell grown in suspension culture. The optical trap is focused on a vesicle (*arrow*) associated with the nucleus (*A*). The vesicle is displaced to the left (*B*) and then to the right (*C*) within the cell. Termination of the trap results in the return of the vesicle to its original location (*D*). Bar, 2.5  $\mu\text{m}$ .

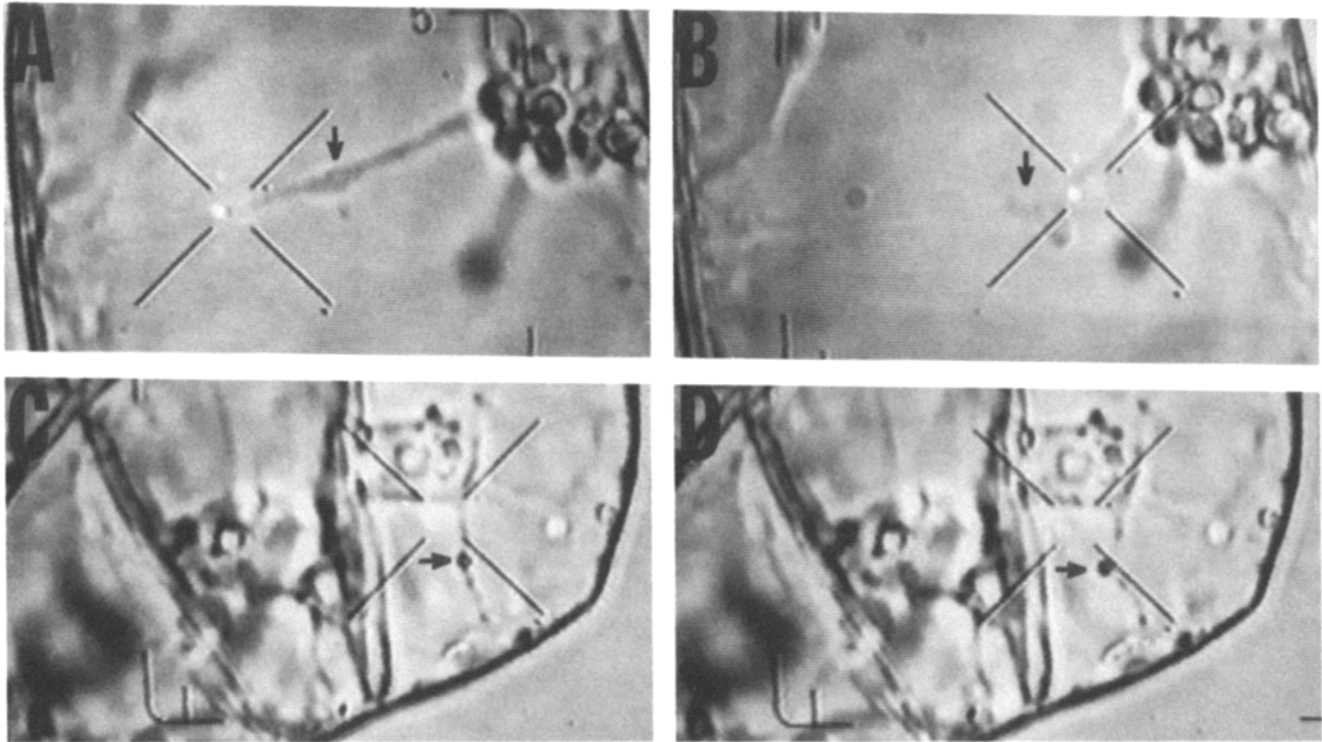
pension culture. The trapping is initiated in Fig. 1 *A* and the vesicle/strand is displaced towards the left (Fig. 1 *B*) and then right (Fig. 1 *C*) within the cytoplasm. Removal of the trap results in rebound to the original position (Fig. 1 *D*). The stretched strand that is associated with the vesicle is seen as a long defocused tail from the vesicle to the nucleus in Fig. 1 *C*. It is important to emphasize that for the optical displacements demonstrated in Fig. 1, an IR laser was used to create the optical trap. The use of an IR laser was previously suggested to minimize potential optical damage induced at energy levels that might be necessary to initiate an optical trap (Ashkin and Dziedzic, 1989). The arrow in Fig. 1 *A* indicates the target vesicle of the trapping beam. Optical trap-

ping is initiated by focusing the laser beam on the vesicle, not to the center of the cross-hairs in the image. The cross-hairs in this and all subsequent images serve as a reference to demonstrate the movement of the cell in relation to the trap. Movement of the stage results in the displacement of the vesicle and associated strand through the cell interior (Fig. 1, *B-D*). The trap is then removed and the vesicle/strand rebound to its original position (Fig. 1 *D*). If the displacement is performed too quickly, the filament breaks or can no longer contract completely (data not shown). Such velocity-dependent effects on filament integrity were also observed by Ashkin and Dziedzic (1989).

Fig. 2 provides evidence that an argon ion laser can also



*Figure 2.* Optical displacement of a vesicle associated with a transvacuolar strand using an argon ion laser. The vesicle (*arrow*) is trapped (*a*) and then displaced throughout the cell (*b-e*). Termination of the trap results in the return of the vesicle to its original position (*f*). Bar, 2.5  $\mu\text{m}$ .



**Figure 3.** Optical displacement of a transvacuolar strand. The trap is focused to a strand (arrow) (A) and the strand is displaced within the cytoplasm (B). In another sample, the trap is focused to the center of a vesicle associated with a strand (C) and is then displaced (D).

be successfully used to create a nondestructive optical trap. The trap is, again, initiated on a vesicle associated with cytoskeletal elements in the transvacuolar strand (Fig. 2 a, arrowhead). The stage is displaced and the strand can be stretched through the cytoplasm (Fig. 2, b-e) and then rebound to its original position when the trap is released (Fig. 2 f). This displacement can be performed multiple times with the same result, the strand returns to its origin. This data provides supporting evidence that at the low power levels used for the CODA, an argon ion laser can be used to produce nondestructive traps. Although cytoplasmic vesicles are a good target for the optical trap, Fig. 3 demonstrates that displacements can be performed by directly focusing on a strand that does not have an associated vesicle (note the increase in distance between the cross-hairs and the cell wall following displacement of the cell) (Fig. 3, A and B). This was also observed by Ashkin and Dziedzic (1989). Using another sample, the focus of the laser beam was shifted slightly to the focal plane of the strand (Fig. 3, C and D) rather than towards the top of vesicles associated with strands as previously observed in Figs. 1-3 (A and B). The arrows in Fig. 3 (A-D) point to the target for the laser beam, the cross-hairs serve as a reference to demonstrate the movement of the stage.

To further pursue the capability of the CODA for manipulating intracellular structures, attempts were made to trap and move the cell nucleus. Microscopic evidence has shown that the nucleus sits within an F-actin cage in the cytoplasm. This cage is continuous with actin cables/bundles that appear to anchor the nucleus to the membrane/wall surface (Traas et al., 1987). Efforts to move the nucleus were unsuccessful

at power levels that did not cause cellular damage (data not shown). Addition of cytochalasin D (20  $\mu\text{M}$ ), however, resulted in a decrease in the nuclear resistance to displacement (Fig. 4). The nucleus (Fig. 4, double arrowhead) identified by the corona of associated vesicles could be displaced from its original position (Fig. 4, A-D) within the cell and moved through the cytoplasm. This provides support for the view that the nucleus is anchored in its position by an actin network (Lloyd, 1989).

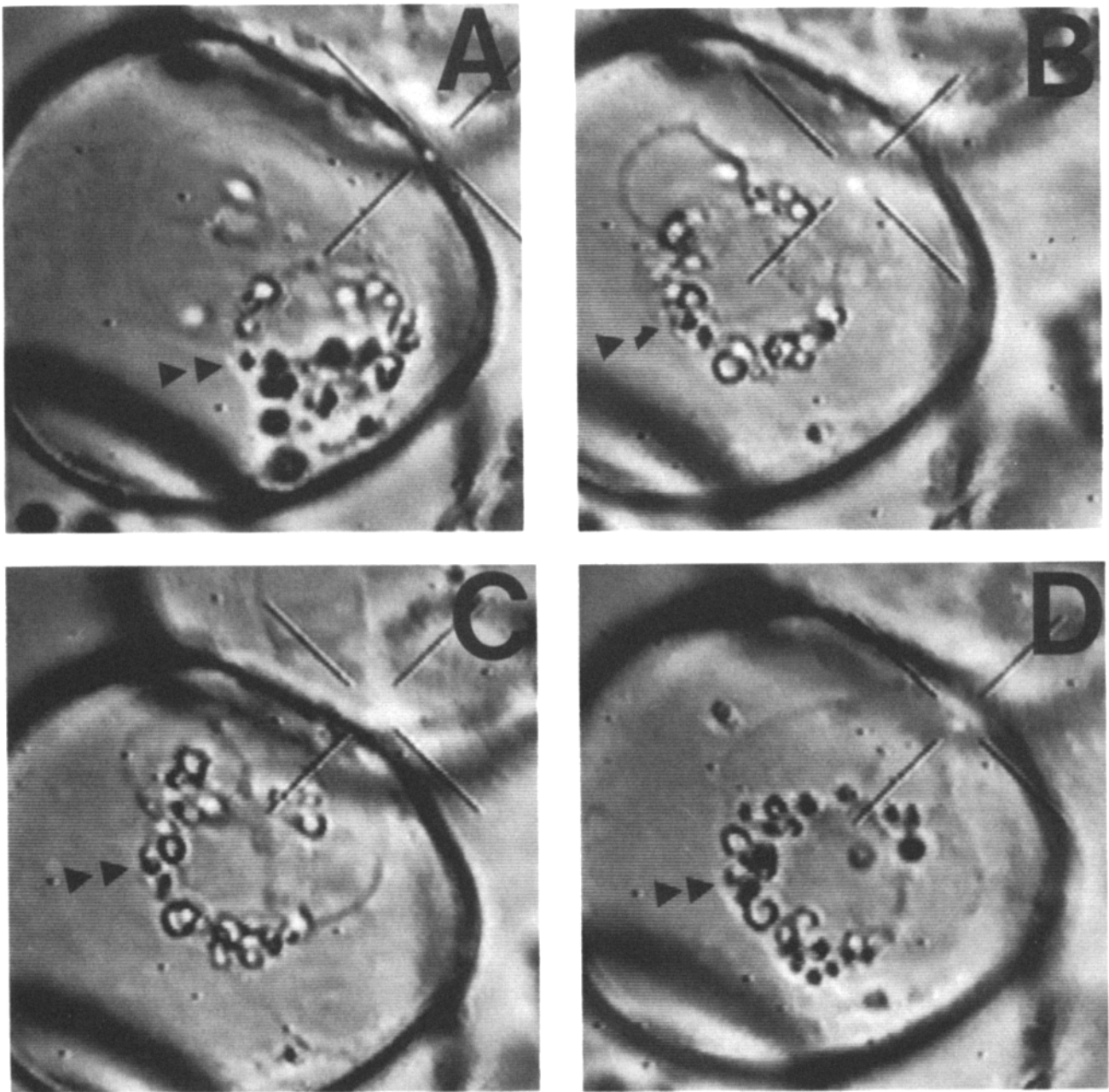
#### **Confocal Fluorescence Views of the Actin Network in Soybean Root Cells**

Fig. 5 shows confocal fluorescence views of: (a) the actin cage surrounding the nucleus; (b) the actin cables comprising the transvacuolar strands; and (c) F-actin filaments within the cytoplasm of a soybean root cell. Optical sections are displayed at 0.5  $\mu\text{m}$  slices throughout the soybean cell stained with NBD-phalloidin, a fluorescent probe for F-actin (Traas et al., 1987) (Fig. 5 A). As previously discussed by Lloyd (1989), there are a variety of filamentous structures that vary from thin, wispy filaments to heavily stained cables (Fig. 5 B). Thicker filaments (heavier staining) reminiscent of stress fibers in animal cells (Burrige, 1981) are found to comprise the transcytoplasmic strands. They appear to connect the actin cage of the nucleus to the cell periphery (Fig. 5 C).

#### **Cell Optical Displacement Assay: A Dynamic In Vivo Measurement of Cytoskeletal Tension**

The relationship between trapping power and displacement

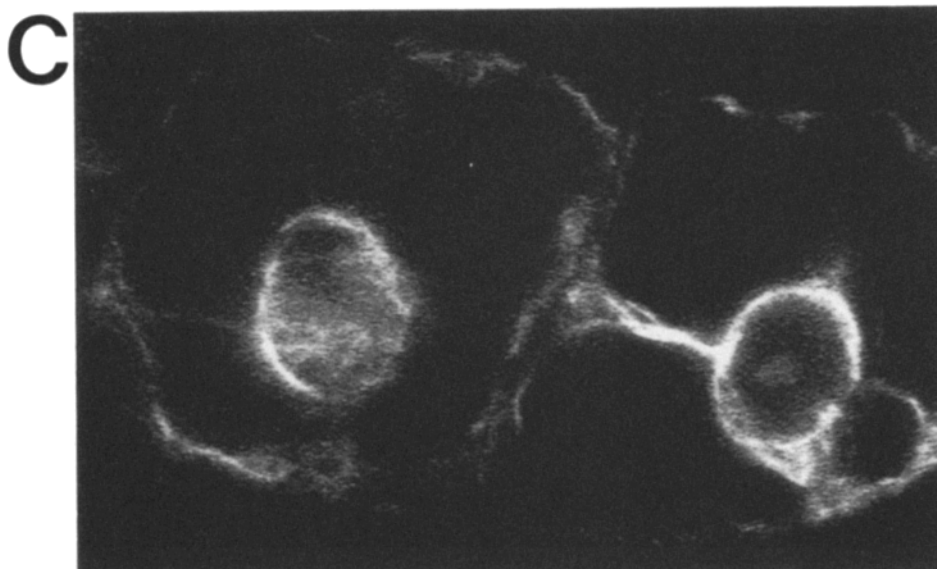
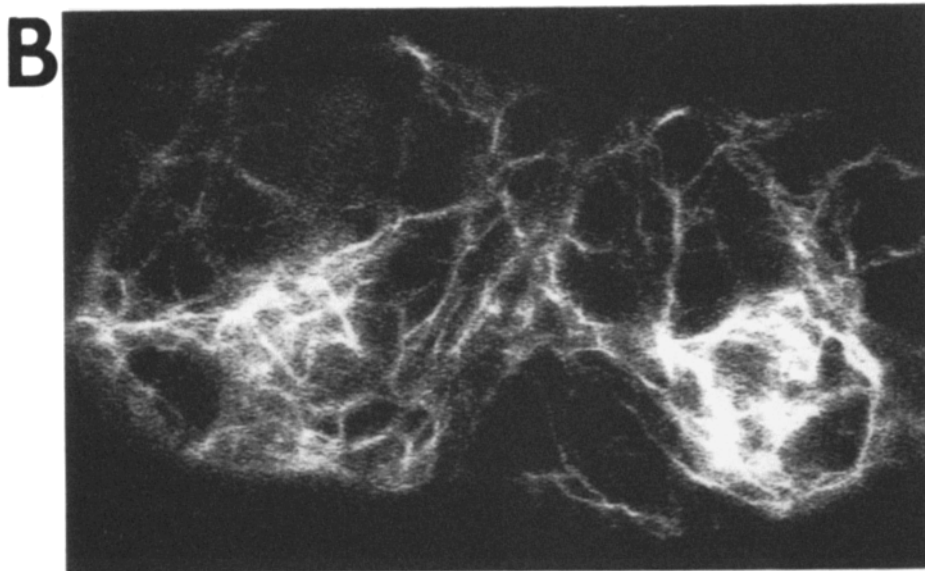
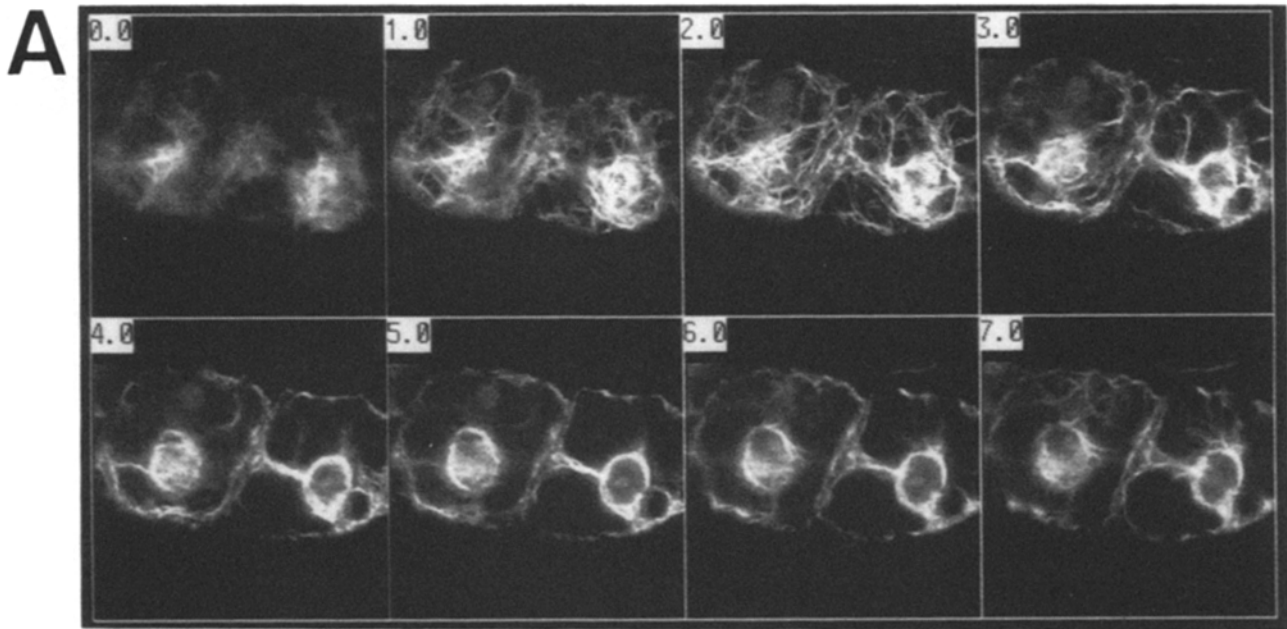




**Figure 4.** Optical displacement of the cell nucleus in soybean cells treated with cytochalasin D ( $20 \mu\text{M}$ ). The optical trap was focused to the cell nucleus (*double arrow*) (A) and the cell was then displaced, resulting in the translocation of the nucleus through the cytoplasm (B–D).

length is shown in Fig. 6 for four separate filaments measured in four different cells. To generate these curves, a filament was trapped with the focused beam of an argon ion laser and the stage was moved at constant velocity through defined distance intervals until the filament escaped the trap (maximum displacement length). A pause of 5–10 s was introduced between each displacement at each power level. Under the conditions of the experiment, each filament demonstrates a near linear relationship for a range of power settings between 5 and 40 mW. Similar dependencies were observed for extension experiments performed to determine the elasticity of the cortical cytoskeleton in erythrocytes (Evans and LaCelle, 1975). The variability observed for experiments between

filaments in different plant cells suggested that it would be useful to design an assay based on multiple measurements in a population of cells. The CODA was developed as described in Materials and Methods to compensate for cell to cell variability and provide quantitation for tension. As observed in Fig. 7, the displacement curve was created by performing 20 displacement attempts at each laser power setting. Successful displacements of transvacuolar strands through a given length at a constant velocity per 20 attempts in different cells were plotted on the ordinate for each power setting. A parameter termed the displacement threshold can be defined which is the minimum laser power necessary to produce 20 successful displacements. The displacement



*Figure 5.* Confocal fluorescence imaging of the actin network in soybean cells. Cells were permeabilized and stained with NBD-phalloidin (F-actin-specific fluorescent probe) (Molecular Probes, Eugene, OR) (Traas et al., 1987). A total of 20 sections (at  $0.5 \mu\text{m}$  intervals) were scanned and selected sections are presented in *A*. The number in the top left corner of each image refers to the distance (micron) into the cell from the bottom closest to the objective. The bottom of the cell is the first image at  $0.0 \mu\text{m}$ . A section at  $2.0 \mu\text{m}$  into the cell is enlarged to highlight fine F-actin filaments (*B*). A section at  $6.0 \mu\text{m}$  into the cell is enlarged to demonstrate the actin within a distinct transvacuolar strand (*C*).

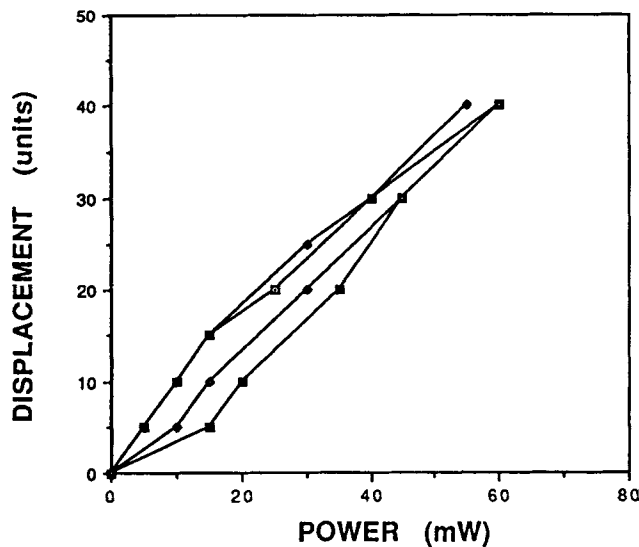


Figure 6. Relationship between laser trapping power and length of displacement. Four separate transvacuolar strands in four different cells were stretched as a function of an increasing laser power as described in Results. A unit is  $\sim 0.25 \mu\text{m}$ .

curves obtained with this method are shown in Fig. 7. The near linear response observed for each displacement experiment under the variety of incubation conditions represented by Figs. 7-11 suggested that it would be possible to utilize the 50% point (10 successful displacements out of 20 attempts) for comparisons of filament tension between different experiments. An examination of the curves in Fig. 7 shows that filaments exposed to  $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Mn}^{2+}$  demonstrated the same dependency on laser power for displacement (number of successful displacements for a set displacement length) as the control. Addition of  $\text{Mg}^{2+}$  and, to a

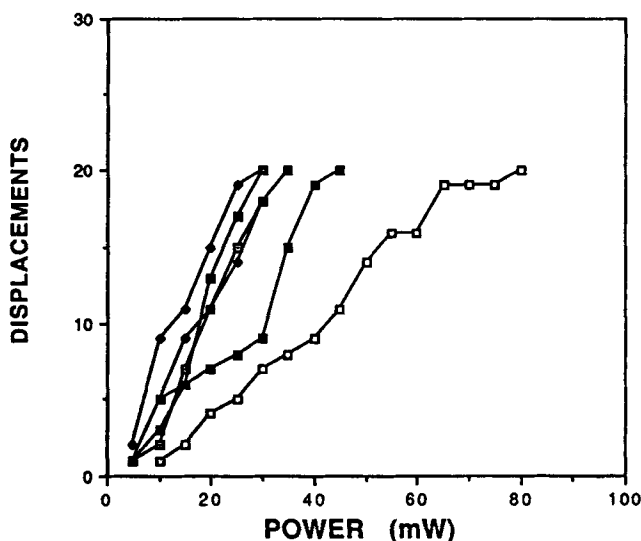


Figure 7. Influence of mono- and divalent cations on the elasticity of transvacuolar strands. CODA was performed as described in Materials and Methods. The curves represent the following incubation conditions: control ( $\square$ ),  $\text{NaCl}$  (100 mM) ( $\blacklozenge$ ),  $\text{KCl}$  (100 mM) ( $\blacksquare$ ),  $\text{MnCl}_2$  (100 mM) ( $\blacklozenge$ ),  $\text{CaCl}_2$  (100 mM) ( $\blacksquare$ ), and  $\text{MgCl}_2$  (100 mM) ( $\square$ ).

lesser extent,  $\text{Ca}^{2+}$  to cells induced a shift of the displacement threshold to higher laser intensities. Although  $\text{Ca}^{2+}$  has been previously shown to enhance tension in cytoskeleton networks (Pasternak and Elson, 1985), this appears to represent the first demonstration that  $\text{Mg}^{2+}$  can also enhance tension. This effect is reversible; the filaments returned to control values following the removal of  $\text{Mg}^{2+}$  from the incubation medium (data not shown).

To demonstrate that the observed displacements of transvacuolar strands are related to the state of the cytoskeleton, specific chemical perturbants of microfilament, and microtubule networks were used to examine their influence on strand tension. As shown in Fig. 8, cytochalasin D (Traas et al., 1987), APM (Falconer and Seagull, 1987), and incubation of cells at  $4^\circ\text{C}$  (Marc et al., 1989) all induce a decrease in tension of transvacuolar strands, while sodium azide (Pasternak and Elson, 1985), phalloidin (Andersland and Parthasarathy, 1992), and taxol (Schiff and Horwitz, 1980) cause an enhanced tension (Fig. 8). Cytochalasin D and APM, used simultaneously, appear to maximally decrease tension beyond the level of either reagent alone (Fig. 9). This enhanced effectiveness in plant cells of using cytochalasin D and APM was also observed for colchicine and cytochalasin D acting synergistically to reverse the stiffness response in lymphocytes induced by anti-IgM (Pasternak and Elson, 1985). Of particular interest is the observation that incubation of cells at  $47.5^\circ\text{C}$  for 2 min induces a significant decrease in fiber tension. Since it has been demonstrated that incubation of the internodal cell of *Nitella* at this temperature resulted in the denaturation of myosin (Chen and Kaniya, 1981), this result may be interpreted as: (a) additional evidence that the tension measured in these experiments is mediated by proteins and not phospholipids; and (b) myosin may be a component of the tension generating system in transvacuolar strands. Since cytochalasin D, APM, and heat

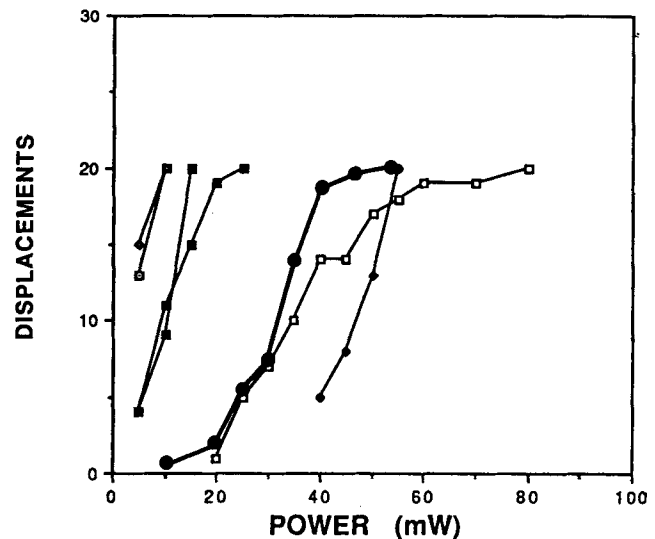


Figure 8. Effect on tension in transvacuolar strands of agents and perturbing conditions that can modify cytoskeletal organization. CODA was performed as described in Materials and Methods. The curves represent: treatment of cells at  $47.5^\circ\text{C}$  for 2' ( $\blacklozenge$ ) and  $4^\circ\text{C}$  for 30 min ( $\square$ ), incubation of cells with sodium azide ( $\blacklozenge$ ), phalloidin ( $\blacklozenge$ ), cytochalasin D ( $\blacksquare$ ), APM ( $\blacksquare$ ), and taxol ( $\square$ ), respectively.



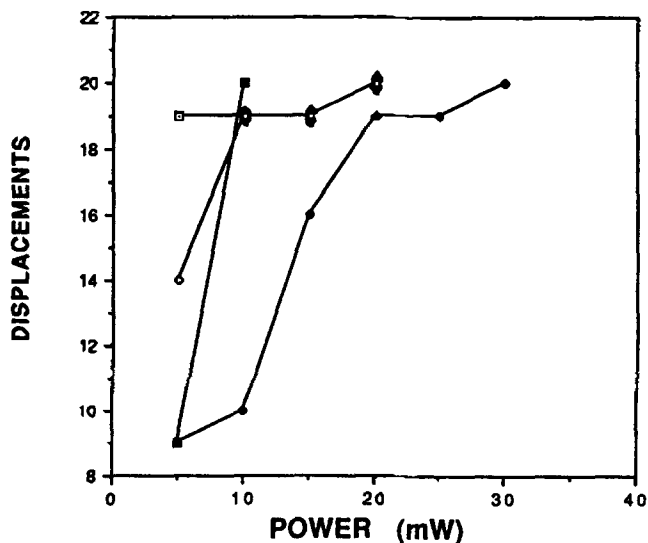


Figure 9. The role of an organized cytoskeleton in mediating the  $Mg^{2+}$ -induced increase in tension. The CODA was performed as described in Materials and Methods. The curves represent coincubation of APM (20  $\mu\text{g}/\text{ml}$ ) and cytochalasin D (20  $\mu\text{M}$ ) with cells for 30 min before measurement ( $\square$ ), treatment of cells at 47.5°C for 2 min in the presence of  $Mg^{2+}$  (100 mM) ( $\blacklozenge$ ), incubation of cells with APM (20  $\mu\text{g}/\text{ml}$ ) for 30 min before addition of  $Mg^{2+}$  (100 mM) ( $\blacksquare$ ), and incubation of cells with cytochalasin D (20  $\mu\text{M}$ ) for 30 min before addition of  $Mg^{2+}$  (100 mM) ( $\diamond$ ).

treatment can modify the organization of structural components of the cytoskeleton (Traas et al., 1987; Falconer and Seagull, 1987; Chen and Kaniya, 1981), we next examined their ability to block the tension that was induced by  $Mg^{2+}$ . As shown in Fig. 9, all these treatments inhibited the  $Mg^{2+}$ -induced increase in tension (compare Figs. 9 and 7).

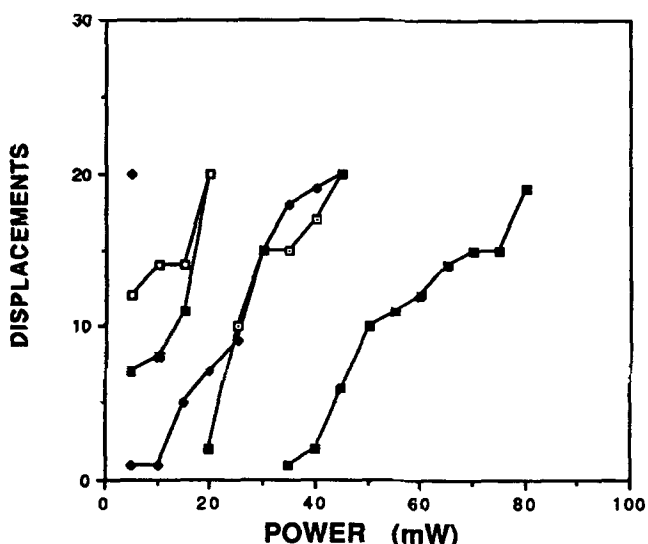


Figure 10. Influence of lipophilic signaling molecules on transvacuolar tension. CODA was performed as described in Materials and Methods. Curves represent the following conditions: incubation with phorbol dibutyrate (0.5  $\mu\text{g}/\text{ml}$ ) ( $\square$ ), phorbol 12, 13 didecanoate (0.5  $\mu\text{g}/\text{ml}$ ) ( $\blacklozenge$ ), 1,2 DiC8 ( $\blacksquare$ ), 1,3 DiC8 ( $\diamond$ ), 1,2 DiC8 +  $Mg^{2+}$  ( $\blacksquare$ ), and 1,3 DiC8 +  $Mg^{2+}$  ( $\square$ ), respectively.

## Modulation of Cytoskeletal Tension by Biosignaling Molecules

**Diacylglycerol Analogues and Phorbol Esters.** Changes in cytoskeletal organization and activity have been demonstrated to be integral responses and mitigators of transmembrane signaling events. Microfilament, microtubules, and cytoskeletal-associated control and regulatory proteins in animal cells have demonstrated changes in their organization and patterns of posttranslational modification following exposure to lipophilic biosignaling molecules (Shariff and Luna, 1992; Herman and Pledger, 1985; Luna and Hitt, 1992; Schliwa et al., 1984). These modifications appear to regulate the structural integrity of filamentous networks. In an attempt to examine the influence of lipophilic biosignaling molecules on the tension of filaments in the plant cytoskeleton, we incubated cells with the reagents shown in Fig. 10. Phorbol dibutyrate, a water-soluble congener of 12-*o*-tetradecanoylphorbol-13-acetate (TPA), an activator of protein kinase C, and phorbol 12,13 didecanoate, a nonactive (in animal cells) analogue of TPA, demonstrated little effect on the tension in transvacuolar filaments. A recent report by Shariff and Luna (1992) provided evidence for a role for diacylglycerol (DAG) as an inducer of actin nucleation at plasma membranes of *Dictyostelium discoideum* in a proposed non-pKC-mediated pathway. 1,2 DiC8, but not 1,3 DiC8 (a non-physiological diacylglycerol), was found to induce actin nucleation (Shariff and Luna, 1992). When used in the plant cells, a biological efficacy was observed that was opposite to that found in *D. discoideum*. 1,3 DiC8 caused the most pronounced decrease in tension observed for all reagents or conditions; 1,2 DiC8 was less potent. 1,3 DiC8 inhibited the tension-inducing activity of  $Mg^{2+}$  (Fig. 10) while 1,2 DiC8 initially inhibited the tension-inducing activity of  $Mg^{2+}$ , however, following a 30–40-min incubation period, the ability of  $Mg^{2+}$  to induce tension was regained (data not shown).

**Phospholipids and Fatty Acids as Modifiers of Cytoskeletal Tension.** Recently, a number of investigations have demonstrated that phospholipids and their metabolites are specific effectors for modifying the organization (Ridley and Hall, 1992; Ha and Exton, 1993) and tension (Gong et al., 1992; Kolodney and Elson, 1993) in filamentous networks comprised of F-actin and myosin. To examine for this possibility in plant cells, we added to soybean cells the fatty acids and phospholipids shown in Table I and incubated the sample as described in Materials and Methods. Since linolenic acid was previously demonstrated to be a bioactive molecule in plants as a precursor for methyl jasmonate biosynthesis (Farmer and Ryan, 1990), its effect on strand tension was examined. As shown in Table I, there was no change from control when  $\alpha$ - or  $\gamma$ -linolenic acid was added to the cells. In sharp contrast, linoleic acid, the predominant fatty acid substituent of the *sn*-2 position in plant phospholipids was found to have considerable activity in decreasing tension in transvacuolar strands at low concentrations (Table I). Arachidonic acid, the predominant fatty acid *sn*-2 substituent and bioactive molecule in animal cells also decreased the tension in transvacuolar strands (Table I). The concentrations of linoleic and arachidonic acid that were necessary to decrease tension in transvacuolar strands were within the range of arachidonate concentrations previously observed to stimu-

**Table I. Effectors of Cytoskeletal Tension**

Effectors	Displacement threshold (DT <sub>50</sub> ) <i>mW</i>
Control (1B5C media)	15
Fatty Acids (0.5 $\mu$ g/ml)	
Linoleic acid	$\leq 5$
Arachidonic acid	$\leq 5$
$\alpha$ -Linolenic acid	20
$\gamma$ -Linolenic acid	20
Diacylglycerol Analogues and Phorbol Esters (0.5 $\mu$ g/ml)	
1,2 Dioctanoyl- <i>sn</i> -glycerol	5
1,3 Dioctanoylglycerol	$\leq 5$
Phorbol dibutyrate	20
Phorbol 12,13 didecanoate	20
Phospholipids (0.5 $\mu$ g/ml)	
Lysophosphatidic acid	20
Phosphatidylcholine	15
Lysophosphatidylcholine	20
Ions (100 mM), Ca <sup>2+</sup> Ionophore A23187, and pH	
NaCl	15
KCl	15
MnCl <sub>2</sub>	15
BaCl <sub>2</sub>	15
CaCl <sub>2</sub>	30
MgCl <sub>2</sub>	45
0.2 $\mu$ M A23187	10
2.0 $\mu$ M A23187	35
5.0 $\mu$ M A23187	130
pH 4.5	$\leq 5$
pH 8.0	$\leq 5$
Lipids and Phospholipids (0.5 $\mu$ g/ml) in the presence of A23187 (0.2 $\mu$ M)	
Linoleic acid	10
$\alpha$ -Linolenic acid	15
1,2 Dioctanoyl- <i>sn</i> -glycerol	25
1,3 Dioctanoylglycerol	40

late a variety of biological activities in animal cells (Irvine, 1982). No similar decrease in tension of transvacuolar strands was observed for phosphatidylcholine, lysophosphatidylcholine, or lysophosphatidic acid (Table I). It is important to note that the inability of other phospholipids to elicit a response in the CODA may not necessarily reflect their inactivity as biological signaling molecules, but rather may represent their inability to transfer across the bilayer to the cytoplasmic leaflet of the plasma membrane to interact with the cellular-signaling enzymes (Pagano and Longmuir, 1985). This may be relevant for such phospholipids as PA or lysoPA, both of which have demonstrated an ability to alter the cytoskeleton in animal cells (Ridley and Hall, 1992; Ha and Exton, 1993).

#### **Calcium and pH as Regulators of Cytoskeletal Tension.**

Previously detailed activator/modulator roles for phospholipids and phospholipid metabolites in cell-signaling pathways have been associated with an enhanced intracellular availability of free Ca<sup>2+</sup>. In particular, phospholipase C hydrolysis of PIP<sub>2</sub> to release the calcium elicitor inositol 1,4,5-*tris*-phosphate (IP<sub>3</sub>) (Berridge and Irvine, 1989). As observed in Table I, increasing the concentration of A23187 from 0.2 to 5.0  $\mu$ M demonstrated a dramatic increase in the tension of transvacuolar strands. This increase occurs

within minutes following addition of the ionophore. Although the addition of exogenous Ca<sup>2+</sup> (100 mM) caused an increase in tension, incubation with A23187 was far more effective. This may be a result of the limited loading of the cytoplasm that occurs with exogenously added Ca<sup>2+</sup> (Bush and Jones, 1989) or may suggest a functional requirement that Ca<sup>2+</sup> must be released from a specific intracellular compartment, e.g., vacuole, endoplasmic reticulum to propagate a signal. Preincubation of cells with A23187 (0.2  $\mu$ M for 10 min) diminished and in some instances reversed the ability of fatty acids and diacylglycerol analogues to decrease the tension in transcytoplasmic strands (Table I).

Exposure of plant cells to an external pH of 4.5 (1 mM citric acid) resulted in a decrease of the cytoplasmic pH to  $\sim 6.8$  within minutes (Brummer et al., 1984). Acidification of the soybean root cells resulted in a decrease in the tension of transcytoplasmic strands (Table I). Interestingly, alkalization of the external pH to 8.0 (pH of growth media is normally 6.2), also resulted in a decrease in tension (Table I).

#### **Tension in the Cortical Cytoskeleton**

The cortical cytoskeleton appears to be comprised of at least two types of organization: membrane-associated and sub-membranous/cytoplasmic (Lloyd, 1989; Traas et al., 1987; Traas, 1990). These networks seem to be involved in the organization of the endoplasmic reticulum (Hepler et al., 1990), provide the framework for cytoplasmic streaming (Jackson, 1982; Kaniya, 1981), and apparently serve as structural cofactors in the synthesis, deposition, and incorporation of cellulose into the cell wall (Traas, 1990; Heath and Seagull, 1982). In a manner analogous to animal cells, the cortical cytoskeletal network can also influence the movement and distribution of plasma membrane glycoproteins (Metcalf et al., 1983, 1986). In an effort to examine the tension in fibers associated with the cortical cytoskeleton, filaments with associated vesicular bodies found near the cell membrane were trapped and then examined by the CODA. Since the existence of such vesicles was a rare occurrence at the cell periphery, the number of cells examined was less than that for experiments on transvacuolar strands. Examination of Fig. 11, however, clearly shows that the trapping power necessary for displacement of the maximum number of vesicle-associated strands was at least 8-10 times greater than for a similar displacement of vesicle-associated transvacuolar strands (Fig. 7). These power differences suggest a significantly greater resistance or tension for cortical strands. At present, the CODA measurements do not have the resolution to discriminate between the subassemblies comprising the cortical compartment. The two reagents that appear to have the most significant impact on the tension of these strands are cytochalasin D and APM. 1,3 DiC(8), which caused a large decrease in the tension of the transvacuolar strands, had considerably less influence on the cortical cytoskeleton.

#### **Discussion**

##### **Cell Optical Displacement Assay: A Tool for Probing the Viscoelastic Properties of Cytoskeletons in Living Cells**

Previously employed methods to directly measure rheological properties within plant cells have either lacked spatial

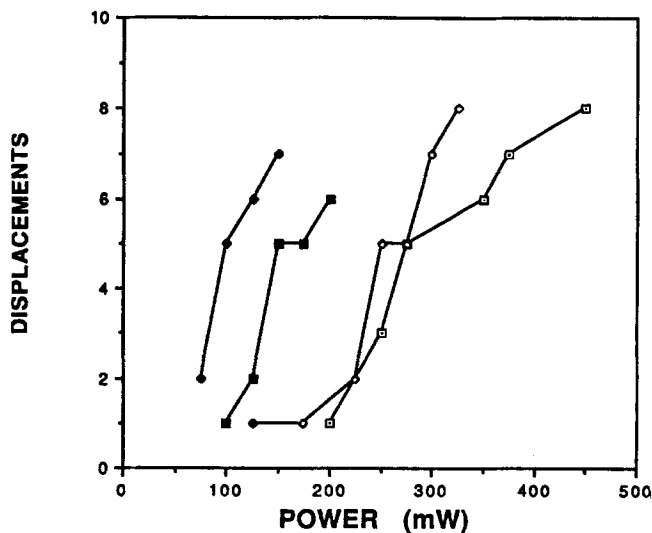


Figure 11. Tension in the cortical cytoskeleton. CODA was performed as described in Materials and Methods. The curves represent control (□), incubation with cytochalasin D (◆), APM (■), and 1,3 DiC8 (○).

resolution (Marc et al., 1989; Galatis et al., 1984; Quader et al., 1987) or have been damaging to the cell (Goodbody et al., 1991; Hahne and Hoffmann, 1984). The difficulty in pursuing these investigations in plant cells using less invasive cell "pokers" (Elson, 1988) is a consequence of the intervention of the stiff cell wall between the physical probe and the plasma membrane. Recently, the technique of optical trapping has been developed to serve as a new tool for the manipulation of submicron structures (Block, 1992) and examination of motor proteins (Kuo and Sheetz, 1993). Such measurements have been performed in reconstituted *in vitro* systems. The feasibility of this technique for *in vivo* measurements of viscoelastic properties was demonstrated by Ashkin and Dziedzic (1989). Transvacuolar strands within the cytoplasm of algae cells were displaced and then shown to rebound, demonstrating the viscoelastic properties of plastic flow, necking, stress relaxation, and set. Actin filaments have been shown to be components of transvacuolar strands and form a cytoskeletal cage around the nucleus (Fig. 5) (Traas et al., 1987). The viscoelasticity measured for transvacuolar strands therefore, should directly reflect the presence of actin filaments, actin accessory proteins, and, perhaps to a lesser extent, microtubules. This is confirmed by the CODA as a loss of tension in these structures following incubation with cytochalasin and phalloidin (Fig. 8). The pronounced influence of APM, cold, and taxol on tension in the transvacuolar strands implies that although microtubules are less abundant in the cytoplasm than at the cell periphery, they still have an influence on the tension displayed by the actin filaments that populate the strands. This could be related to a role for the cortical microtubule network in anchoring transcytoplasmic microfilaments (Lloyd, 1989) and forming other close associations with actin filaments observed throughout the cytoplasm (Traas et al., 1989).

In contrast, the elastic properties of the cortical cytoskeleton (Fig. 11) suggest a network with considerably enhanced rigidity. Both actin and tubulin networks populate this region

in both membrane-associated and -nonassociated forms. Endoplasmic reticulum is also found to align with a subpopulation of submembranous actin networks and may influence their dynamic responses (Hepler et al., 1990). A similar distinction in the physical properties of cortical and cytoplasmic filamentous actin networks was noted by Cao et al. (1992) in normal rat kidney (NRK) cells. The complexity of these structural interactions presently limit the interpretation of the data for cortical displacement measurements.

### *Phospholipids and Phospholipid Metabolites as Mediators of Cellular Signaling and Cytoskeletal Reorganization*

In attempts to assess the regulatory activity of phospholipids and their metabolic products in modulating the tension and function of the cytoskeleton in plant cells, it would be useful to frame the discussion in terms of the key physiological regulators of hormonal stimulation and growth in plants, changes in pH and intracellular calcium concentration (Gibson and Kropf, 1994; Hager and Moser, 1985; Hager et al., 1991; Rayle and Cleland, 1992; Miller et al., 1990; Pantoja et al., 1992; Putnam-Evans et al., 1990; Williams et al., 1990; Drøbak, 1992). The ensuing discussion presumes that the changes induced in the viscoelasticity of transvacuolar strands are down-stream responses that are initiated through the coupled activity of the polyphosphoinositide signaling pathway and an independent phospholipase A<sub>2</sub> (PLA<sub>2</sub>) signaling system.

**Linoleic Acid: A Second Messenger in Plant Cells?** Arachidonic acid by itself (Gong et al., 1992; Tsai et al., 1989) and as a precursor for a variety of other bioactive metabolites (Pace et al., 1993; Irvine, 1982; Hanahan, 1986) serves as a trigger for a diverse series of cellular activities, including the nonsteroidal inflammatory response (Irvine, 1982; Hanahan, 1986), bacterial invasiveness (Pace et al., 1993), and acrosomal exocytosis in sperm (Roldan and Fragio, 1994). Since exogenously supplied arachidonic acid mimics the tension-relieving activity observed for linoleic acid in the CODA, it appears likely that linoleic acid, the dominant *sn*-2 substituent in plant phospholipids, may have cellular activities in plant cells that parallel the role of arachidonic acid as a second messenger in animal cells. A recent report by Gong et al. (1992) provides evidence that arachidonate could directly inhibit myosin light chain kinase phosphatase and sensitize smooth muscle to Ca<sup>2+</sup>. This work and other observations showing that lipids may have a direct influence on modulating cytoskeletal organization, in particular actin and myosin activity (Kolodney and Elson, 1993; Ridley and Hall, 1992; Ha and Exton, 1993) support an emerging view that lipids may not only be initiators of transmembrane signaling but may also act as inhibitory or stimulatory cofactors for enzymes involved in transducing cytoskeletal reorganization.

At present, the plant cell literature provides indirect evidence for the role of linoleic acid in signal transduction. Scherer and André (1989, 1993) demonstrated that auxins rapidly stimulate PLA<sub>2</sub> in soybean cells in culture. Wheeler and Boss (1989) demonstrated that the presence of *sn*-1-palmitoyl lysophosphatidylinositol monophosphate (an indirect measurement of PLA<sub>2</sub> activity) correlates positively with the fusion capacity of protoplasts.

***Diacylglycerol: An Activator of Phospholipase A2 in Plant Cells.*** Although it now seems certain that plant cells do not contain protein kinase C homologues (Harper et al., 1991; Drøbak, 1992), plant cell transmembrane signaling appears to use all the components of the polyphosphoinositide signaling pathway (Drøbak, 1992). Recently, however, a number of PKC-independent pathways have been identified for animal cells that also use both DAG and IP<sub>3</sub> for signaling (Kramer et al., 1987; Qualliotine-Mann et al., 1993; Roldan et al., 1994; Rosenthal et al., 1993). In all instances, DAG was found to stimulate the activity of PLA<sub>2</sub> leading to the production of the pharmacologically active arachidonic acid. The introduction of the calcium ionophore A23187 (0.2–2 μM) was found to significantly enhance the resultant biological activity for DAG stimulation described above. In a similar fashion, it may be proposed that in plant cells, DAG serves to activate a calcium-dependent PLA<sub>2</sub> leading to the production of linoleic acid and the initiation of a signaling cascade. Our observation that phorbol esters (activators of PKC) have no effect on the tension in transvacuolar strands, while significant tension-relieving activity is observed for both 1,2 DiC8 and 1,3 DiC8 (diacylglycerol analogues) provides further evidence that the cytoskeletal changes must occur through a PKC-independent pathway that is mediated by DAG. The ability for both 1,2 and 1,3 DiC8 to mediate a biological phenomena has been demonstrated to be a characteristic element of pathways that use DAG to activate PLA<sub>2</sub> (Rosenthal et al., 1993; Roldan and Fragio, 1994; Qualliotine-Mann et al., 1993).

### ***Ionic Modulation of the Cytoskeleton***

***Ca<sup>2+</sup> as a Cytoskeletal Effector and PLA<sub>2</sub> Activator.*** Modification of the intracellular concentration of calcium serves as an essential trigger in the polyphosphoinositide signaling pathway. IP<sub>3</sub>-mediated changes in intracellular Ca<sup>2+</sup> serve to activate phospholipases and protein kinases (Drøbak, 1992; Berridge and Irvine, 1989). Our results clearly show that Ca<sup>2+</sup> is a significant biological effector that can enhance the tension in transvacuolar strands. It appears that in the plant cell, Ca<sup>2+</sup> has both membrane and cytoskeletal targets whose activation can lead to changes in cytoskeletal tension. DAG and Ca<sup>2+</sup> (≤1 μM) have been demonstrated to activate PLA<sub>2</sub> in a variety of in vivo and in vitro animal systems (Roldan and Fragio, 1994; Kramer et al., 1987; Leslie and Channon, 1990). The Ca<sup>2+</sup> has been suggested to recruit cytosolic PLA<sub>2</sub> to the plasma membrane as a consequence of a Ca<sup>2+</sup>-dependent phospholipid binding motif within the PLA<sub>2</sub> sequence (Clark et al., 1991).

Another target for calcium may be actomyosin that is found within the actin network of the transcytoplasmic strands. Measurement of Ca<sup>2+</sup>-induced cell stiffening has previously been reported for lymphoid cells (Pasternak and Elson, 1985). This was interpreted to be consistent with a contractile-response of the cell cytoskeleton that was presumably mediated by actomyosin. Recently, a novel calcium-dependent protein kinase (CDPK) has been isolated from soybean and demonstrated to be localized to F-actin filaments in soybean cells (Putnam-Evans et al., 1989). Because it does not apparently bind to F-actin in vitro, and antibodies to CDPK show that a CDPK-related antigen is colocalized with stress fibers in HeLa cells (Harmon et al., 1989), it

would appear reasonable to suggest that the CDPK may associate with myosin. Of particular relevance to this suggested localization is that gizzard myosin light chain was found to be a good substrate for the CDPK. Indeed, McCurdy and Harmon (1992) demonstrated that in *Chara*, a CDPK phosphorylates a putative myosin light chain. Another potential site for Ca<sup>2+</sup>-mediated signaling could involve a calcium-activated interaction of CDPK with the Ca<sup>2+</sup>-ATPase in the plasma membrane. Ca<sup>2+</sup>-ATPases have been demonstrated to contain calmodulin-binding domains (Carafoli, 1992) and, interestingly, are also stimulated by lipid-signaling molecules (Enyedi et al., 1987). Since the soybean CDPK has been demonstrated to contain a domain similar to calmodulin (Harper et al., 1991), an additional regulatory role for this CDPK could be through modifying the activity of the plant membrane Ca<sup>2+</sup>-ATPase. A membrane-bound form of CDPK has also been characterized in oat that is immunologically related to the soluble form found in oat and soybean. The membrane-bound form is activated by both Ca<sup>2+</sup> and lipids (Schaller et al., 1992). The lipid enhanced the activity of CDPK in the presence of Ca<sup>2+</sup> by 20-fold (Schaller et al., 1992). This CDPK has been suggested to phosphorylate the plasma membrane H<sup>+</sup>-ATPase (Schaller and Sussman, 1988).

The potential synergy between these calcium targets may perhaps be hinted at by results from an experiment in which cells were coincubated with A23187 and 1,2 DiC8 or 1,3 DiC8. Coincubation resulted in enhanced tension in the strands (Table I). This is distinctly different from the activity of these molecules when separately incubated with the plant cells (Table I) and may reflect the cumulative signaling response resulting from simultaneous stimulation of CDPK by IP<sub>3</sub>-mediated calcium release and DAG mediated activation of PLA<sub>2</sub>.

***The Mg<sup>2+</sup> Effect: Stimulation of CDPK?*** No measurements, to our knowledge, have examined the consequences of Mg<sup>2+</sup> in modifying the viscoelastic properties of the cytoskeleton in cells. In vitro studies have demonstrated a role for Mg<sup>2+</sup> in the assembly of actin and tubulin (Jackson, 1982), and Mg<sup>2+</sup> is necessary for the activity of the actin-activated Mg<sup>2+</sup>-ATPase associated with myosin (Jackson, 1982). Although the changes in intracellular Mg<sup>2+</sup> concentrations produced by incubation with 50–100 mM Mg<sup>2+</sup> have not been determined, it would appear that the Mg<sup>2+</sup>-induced rigidity in the transvacuolar strands is a specific effect since no similar change was observed with Mn<sup>2+</sup>, Na<sup>+</sup>, or K<sup>+</sup>. Reversibility of the Mg<sup>2+</sup> effect by reagents that disrupt microfilaments and microtubules suggest that the rigidity is not predominantly promoted by Mg<sup>2+</sup>-induced, noncovalent cross-links, but may result through an enhanced contractile response, presumably mediated by myosin (Chen and Kariya, 1981).

An alternate explanation for the Mg<sup>2+</sup> effect may relate to the observation that the activity of actin associated CDPK has a Mg<sup>2+</sup> requirement that is independent of forming a stable Mg<sup>2+</sup>-ATP complex (Putnam-Evans et al., 1990). Since intracellular concentrations of Mg<sup>2+</sup> are sufficient for enzyme function, the addition of exogenous Mg<sup>2+</sup> may lead to an increase in the intracellular concentration of Mg<sup>2+</sup> to a level that is stimulatory for the CDPK. The CDPK, demonstrating myosin light chain kinase activity (McCurdy and Harmon, 1992; Putnam-Evans et al., 1990), would then

phosphorylate myosin light chains leading to myosin contraction and enhanced rigidity in the actin network within the transvacuolar strands.

### Cross-talk between Intracellular pH and Ca<sup>2+</sup>

Auxin-induced cell elongation appears to be mediated by the acidification of the cytoplasm and the cell wall environment (Rayle and Cleland, 1992). Recently, Gibbon and Kropf (1994) have provided evidence that in the rhizoid cells of *Pelvetia* embryos, it is the formation of a longitudinal pH gradient in these cells ( $\Delta$  pH  $\sim$ 0.3–0.5 between apical and basal cytoplasm) that promotes growth. In their experiments, exposure of cells to pH 8.2 resulted in the maximal growth rate and the establishment of the largest cytoplasmic pH gradient ( $\Delta$  pH  $\sim$ 0.3). As observed in Table I, exposure of soybean cells to pH 8.0 resulted in a decrease in the tension within the transvacuolar strands. This decrease in tension was equivalent to that observed by direct acidification (Table I). These results suggest that acidification and/or the formation of spatial pH gradients within cells can result in modified cytoskeletal interactions that may be necessary for cell expansion. In attempting to define the role of pH in these processes, it is relevant to consider the biochemical coupling between pH and pCa<sup>2+</sup> within the cell. It has been shown that growing rhizoids generate cytosolic and membrane-associated Ca<sup>2+</sup> gradients that superimpose on the pH gradient (Brownlee and Wood, 1986). Changes in intracellular pH or the formation of pH gradients would impact on the activity of the plasma membrane Ca<sup>2+</sup>-ATPase which exchanges extracellular H<sup>+</sup> for Ca<sup>2+</sup> and the vacuolar Ca<sup>2+</sup>/H<sup>+</sup> antiporter (Miller et al., 1990).

### Conclusion

CODA has provided evidence for a plant cell signaling pathway that uses linoleic acid, diacylglycerol, Ca<sup>2+</sup>, and pH to modify the viscoelasticity of the plant cell cytoskeleton. It is likely that topologically defined changes in intracellular pH can lead to reduced tension in specific transvacuolar actin strands to facilitate cell expansion, while localized changes in intracellular Ca<sup>2+</sup> can activate kinases, e.g., CDPK initiates myosin light chain phosphorylation to simultaneously enhance contraction in other strands. It would appear likely that such antagonistic, but yet simultaneous, signaling events are necessary to produce the coordinated changes in tension and relaxation of actin networks that are represented by the repositioning of the nucleus and cell plate formation during mitosis in plant cells.

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