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Auxins and Cytokinins as Antipodal Modulators of Elasticity within the Actin Network of Plant Cells

Sharon Grabski and Melvin Schindler*

Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824

The cytoskeleton of plant and animal cells serves as a transmitter, transducer, and effector of cell signaling mechanisms. In plants, pathways for proliferation, differentiation, intracellular vesicular transport, cell-wall biosynthesis, symbiosis, secretion, and membrane recycling depend on the organization and dynamic properties of actin- and tubulin-based structures that are either associated with the plasma membrane or traverse the cytoplasm. Recently, a new in vivo cytoskeletal assay (cell optical displacement assay) was introduced to measure the tension within subdomains (cortical, transvacuolar, and perinuclear) of the actin network in living plant cells. Cell optical displacement assay measurements within soybean (*Glycine max* [L.]) root cells previously demonstrated that lipophilic signals, e.g. linoleic acid and arachidonic acid or changes in cytoplasmic pH gradients, could induce significant reductions in the tension within the actin network of transvacuolar strands. In contrast, enhancement of cytoplasmic free Ca^{2+} resulted in an increase in tension. In the present communication we have used these measurements to show that a similar antipodal pattern of activity exists for auxins and cytokinins (in their ability to modify the tension within the actin network of plant cells). It is suggested that these growth substances exert their effect on the cytoskeleton through the activation of signaling cascades, which result in the production of lipophilic and ionic second messengers, both of which have been demonstrated to directly effect the tension within the actin network of soybean root cells.

Differentiation, polarity, proliferation, mitosis, secretion, organelle motility, and migration all depend on signal-mediated rearrangements of the cell cytoskeleton (Pfeuty-David and Singer, 1980; Schliwa et al., 1984; Herman and Pledger, 1985; Luna and Hitt, 1992; Ridley and Hall, 1992; Shariff and Luna, 1992; Williamson, 1993). Microfilaments (actin) and microtubules (tubulin) form the most dynamic structural elements of the cytoskeleton. Changes in the organization of these structures have been demonstrated to occur as a consequence of signal-initated alterations in subunit interactions, e.g. actin monomer-polymer equilibria, modifications in the pattern and extent of association with the family of actin and/or tubulin-binding proteins, e.g. profilin, myosin, and microtubule-associated proteins, and differences in the degree of interaction with the plasma membrane components, e.g. polyphosphoinositides, transmembrane proteins, and G proteins (Edelman, 1976; Schliwa et al., 1984; Landreth et al., 1985; Adams and Pollard, 1989; Goldschmidt-Clermont et al., 1990; Luna and Hitt, 1992; Ridley and Hall, 1992; Shariff and Luna, 1992; Williamson, 1993).

In plant cells, dynamic microfilament and microtubule networks are implicated as mediators of nuclear migration, cytoplasmic streaming, secretion, karyokinesis, cell-wall biosynthesis, and transmembrane signaling (Williamson, 1993). A recent study by us demonstrated that the cytoskeleton may also be an important target of aluminum toxicity in plant cells (Grabski and Schindler, 1995). Studies of polarity in *Fucus* embryogenesis have suggested that plant signaling establishes both calcium and proton gradients that apparently mediate polar growth and patterns of cell differentiation (Gibbon and Kropf, 1994). Results of these and other studies utilizing agents that disrupt actin networks (Kropf et al., 1988, 1989) have been interpreted to suggest that cortical actin networks may be essential anchoring elements for maintaining topologically sequestered regions of membrane receptors and ion channels within the plasma membrane. The formation and maintenance of such spatial gradients of ion channels within the plasma membrane could result in the formation of a parallel pattern of ionic and chemical gradients within the cytoplasm. Studies of pollen tubes have also demonstrated the importance of microfilaments in forming vectoral tracks for the migration of secretory vesicles to the pollen tip for tip growth (Picton and Steer, 1983). Measurements in soybean (*Glycine max* [L.]) root protoplasts have shown that the binding of an exogenous signaling molecule (soybean agglutinin) to plasma membrane receptors can trigger large changes in the diffusional properties of unbound membrane proteins (Metcalf et al., 1983). Evidence was provided that this type of dynamic modulation of the diffusion and anchorage of membrane proteins was determined by the interaction of these membrane proteins with cytoskeletal elements, particularly cortical actin networks (Metcalf et al., 1986).

Most attempts to examine the activity and organization of the cell cytoskeleton have been directed toward cataloging changes in the pattern of organization of microfilament and microtubule networks within the cytoplasm and in characterizing the proteins involved in forming these structures. Although important in detailing structure, such studies cannot address an essential question of cytoskeletal activity, namely what is the relationship between these altered patterns of organization (form) and the mechano-

* Corresponding author; e-mail schindl1@pilot.msu.edu; fax 1–517–353–9334.

Abbreviations: CODA, cell optical displacement assay; NAA, naphthalene acetic acid.

chemical/viscoelastic properties (function) of these networks. Recently, a technique was developed by us that enabled site-specific rheological investigations for the purpose of determining the viscoelastic properties of actin-based filamentous networks within living plant cells (Grabski et al., 1994; Grabski and Schindler, 1995; Schindler, 1995). This technique has provided the first opportunity to examine the tension within three topologically and functionally distinct actin networks in plant cells: the cortical, transvacuolar, and nuclear (Grabski et al., 1994). In previous measurements, we demonstrated that a number of bioactive lipid molecules and changes in intracellular pH (increase and decrease) and pCa^{2+} could modify tension within the cortical and transvacuolar microfilament networks (Grabski et al., 1994). Lipophilic effectors or changes in pH decreased tension within the actin network, whereas changes in cytoplasmic calcium caused a considerable increase in tension within the actin network. pH and Ca^{2+} appear to function as antipodal effectors of the elasticity and integrity of the actin network.

A number of investigations have shown that the addition of auxins to plant cells results in the acidification of the cell cytoplasm/cell wall and the formation of cytosolic pH gradients (Hager and Moser, 1985; Rayle and Cleland, 1992). Other studies have shown that auxins can activate phospholipase A_2 (Scherer and André, 1989, 1993) and the polyphosphoinositide signaling pathway (Ettlinger and Lehle, 1988; Morré et al., 1984). The activity of cytokinins appears to be mediated by cytokinin-induced increases in the intracellular concentration of free Ca^{2+} (Saunders and Hepler, 1982, 1983; Hahm and Saunders, 1991; Schumaker and Gizinski, 1993). It was predicted, based on our previous work, that auxins might decrease the tension within the actin network of transvacuolar strands through their modification of intracellular pH gradients (Hager and Moser, 1985; Rayle and Cleland, 1992) and the production of lipophilic signaling molecules (Morré et al., 1984; Ettlinger and Lehle, 1988; Scherer and André, 1989, 1993). Cytokinins, in contrast, would cause an increase in tension as a result of an increase in intracellular Ca^{2+}. This increase in tension would mimic the effect previously observed following the exogenous addition of calcium or the ionophore A23187 to soybean root cells (Grabski et al., 1994).

The present report shows that the addition of auxins to soybean root cells results in a decrease in the tension within the actin network of transvacuolar strands of soybean root cells. In contrast, the addition of cytokinins, which are somewhat more variable in their effects, leads to a bimodal response with either an increase in tension (2 μM) or no change (20 μM). Although transvacuolar strands are complex multicomponent structures, measurements of tension and elasticity within the transvacuolar strands reflect the viscoelastic properties of the actin network. This was previously demonstrated by an analysis of the mechanical properties of actin, tubulin, and vimentin in model systems and animal cells (Janmey et al., 1991; Adams, 1992). The data presented in this communication may be incorporated into a model for spatially distinct control of cytoskeletal tension and organization that depends on coupling and cross-talk between the formation and dissipation of temporal and spatial gradients of free Ca^{2+} and pH initiated by biosignaling molecules.

MATERIALS AND METHODS

Reagents

2,4-D and 2,3-dichlorophenoxyacetic acid and α- and β-NAA were obtained from Aldrich. Cytokinins and analogs, GA_3, ABA, and caffeine were from Sigma.

CODA

CODA experiments were performed as described in the text below and by Grabski et al. (1994) and Schindler (1995). In a typical experiment, a suspension of soybean (*Glycine max* [L.] Merr. cv Mandarin) root cells (maintained in suspension culture and originally derived from roots) (2 μL; 48–72 h of growth) in 1B5C medium (Metcalf et al., 1983) were placed on a slide and sealed under a coverslip with melted paraffin. Incubations with auxins and cytokinins were performed as previously detailed for other bioactive molecules (Grabski et al., 1994; Grabski and Schindler, 1995; Schindler, 1995). All effector reagents were incubated with the cells, at the indicated concentrations, for 30 min prior to the CODA measurement and were maintained in the incubation mixture throughout the experiment. The cells were then placed on an ACAS 570 fluorescence laser cytometer (Meridian Instruments, Okemos, MI) (Wade et al., 1993) and viewed under phase illumination with an oil immersion ×100 (1.4 numerical aperture) objective. All trapping experiments were recorded on videotape, and individual pictures of the experiments were prepared frame by frame from the tapes.

RESULTS

Measurements of Tension within the Actin Network of Transvacuolar Strands in Soybean Root Cells

In vivo measurements of tension within the actin network of transvacuolar strands in soybean root cells were performed using the CODA previously described (Grabski et al., 1994; Grabski and Schindler, 1995; Schindler, 1995). This assay exploits the ability to trap and hold micrometer-sized structures at the focal plane of a focused beam of laser light. A laser trap (Ashkin and Dziedzic, 1989; Grabski et al., 1994; Grabski and Schindler, 1995; Schindler, 1995) was initiated with an argon ion laser beam (excitation wavelength = 488 nm; at 1 μm in diameter) focused onto a vesicle associated with a transvacuolar strand (Fig. 1). The laser beam intensity is monotonically increased until the vesicle is trapped (Fig. 1A). The stage is then moved through a defined distance at a constant velocity, which results in the displacement of the vesicle and associated actin strand through the cytoplasm (Fig. 1B). Attenuation of the trapping beam intensity results in the rebound of the actin strand to its original position (Fig. 1C). The CODA quantifies these displacements in the following manner. Displacement curves for all incubation conditions are gen-

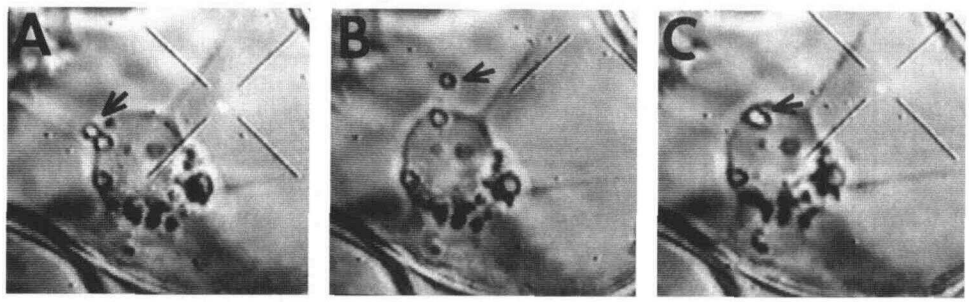

Figure 1. Optical displacement of a vesicle associated with a transvacuolar strand utilizing an optical trap. Optical displacement was performed as described in the text. As the cell is translated by means of controlled displacement of the stage (stage is moved to the lower left of the image), the vesicle (arrow) that is trapped in the laser beam (A) is moved to a position in the cell (B). When the trapping beam is terminated, the vesicle is released from the trap and rebounds to its original position (C). The beam is focused on the vesicle, not to the center of the cross-hairs in the image. The cross-hairs serve as a reference to demonstrate the movement of the cell in relation to the optical trap. Magnification ×1500.

erated by performing 20 displacement attempts at each laser power setting (Fig. 2). To ensure that the trapping intensity does not result in damage to the fibers, each displacement at a particular power setting is performed five times, and in all instances the fiber is required to rebound to the original position following termination of the trap. This provides a control for optical damage and demonstrates that manipulation of the fiber does not modify the microenvironment, resulting in changes for subsequent measurements. Previous measurements, performed on individual strands and with which the relationship between laser intensity and strand displacement was examined, demonstrated a linear response within the range of laser intensities used in these assays (Grabski et al., 1994). Such measurements provide evidence that the changes in tension that are measured in the CODA are isotropic and do not represent disruptions induced, for example, by breaking of microtubules or other more rigid structures on the initial or subsequent displacements. Successful displacements of transvacuolar strands through a given distance per 20 attempts at a constant velocity in different cells are plotted on the ordinate for each power setting. A parameter termed the displacement threshold is defined as the minimum laser power necessary to produce 20 successful displacements. As demonstrated previously by performing multiple measurements on individual strands, the maximum 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) (Grabski et al., 1994). The near-linear response observed for displacement experiments (Grabski et al., 1994) suggested the possibility of utilizing the 50% point of successful displacements as a comparator of filament tension between different experiments. As shown in Figure 2, the addition of the auxin α-NAA induced a decrease in tension (shift of curve to left of control), whereas 6-benzylaminopurine promoted a rigidity within the actin network. β-NAA, an inactive auxin analog, produced no change.

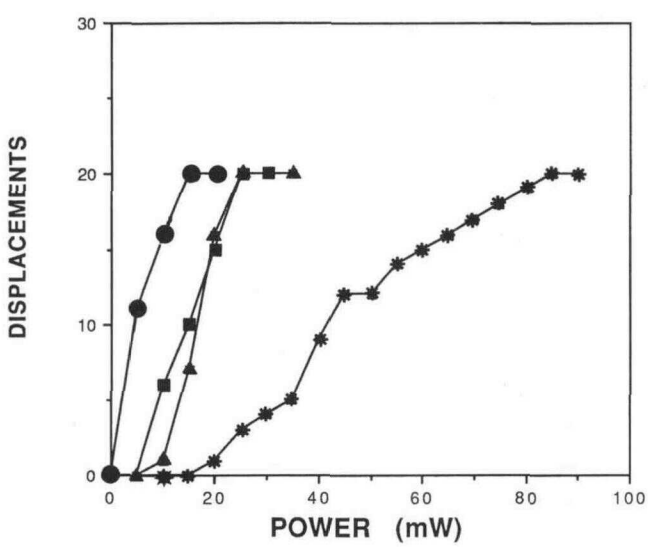

Figure 2. CODA for measuring tension in the actin network of soybean root cells. The CODA quantifies displacements as described in the text. The curves represent the following incubation conditions: ▲, control; ●, α-NAA (7.5 μM); ■, β-NAA (7.5 μM); and ✱, 6-benzylaminopurine (2 μM). Cell measurements were performed during a 60-min period following addition of the effector molecules. Unless noted, changes in tension stayed constant during this time.

Auxin- and Cytokinin-Induced Changes in Tension within the Actin Network of Transvacuolar Strands

Table I is a compilation of the CODA results for a variety of auxins and cytokinins. Only biologically active auxins, 2,4-D, IAA, and α-NAA, demonstrated an effect on the tension within the actin network of transvacuolar strands. The inactive auxin analogs 2,3-dichlorophenoxyacetic acid and β-NAA showed no effect. In all instances, this effect was manifested as a decrease in the tension of the network. In contrast, addition of the cytokinin BA, but not zeatin, induced an increase in the tension of the actin network. Similar increases in tension were seen with adenosine but not adenine. The observed bimodal responses in tension-inducing activity for BA and adenosine suggest the possibility that the response can be stimulatory (lead to increased tension) or inhibitory (lead to no increase or a mild decrease). Such bimodal activity for bioactive ligands was previously reported for the interaction of adenosine with

Table I. *Plant cell growth factor effectors of cytoskeletal tension*

Effector	Concentration	Displacement Threshold
	μM	mW
Control (1B5C medium)		15–20
Auxins		
Active		
2,4-D	2	<5
2,4-D	20	<5
IAA	2	5–10
IAA	20	5–10
α-NAA	7.5	5
Inactive		
2,3-Dichlorophenoxyacetic acid	2	15
2,3-Dichlorophenoxyacetic acid	20	15
β-NAA	7.5	15
Cytokinins and analogs		
6-Benzylaminopurine (benzyladenine)	2	40–45
6-Benzylaminopurine (benzyladenine)	20	15
6-(4-Hydroxy-3-methyl-*trans*-2-butenylamino)purine (zeatin)	2	10
6-(4-Hydroxy-3-methyl-*trans*-2-butenylamino)purine (zeatin)	20	10
Adenosine	2	40–45
Adenosine	20	15
Adenine	2 or 20	15
GAs		
GA_3	2	20
GA_3	20	20
ABA	2	15
ABA	20	15
Caffeine	2	45–50

[a] Concentration demonstrated to show maximal difference in activity between β-NAA and α-NAA (Hager and Moser, 1985).

purinoceptors in animal cells (Fredholm et al., 1994). It also appears that the enhanced tension observed with BA can increase with time to a maximum following 90 min of incubation (data not shown). The difference in tension-inducing activity for BA and zeatin may reflect a variability in plant cell response to cytokinins, which has been previously observed in bioassays and may be related to their biological efficacy and the metabolic interconversions demonstrated for cytokinins and their analogs following incubation with plant cells (Chen, 1981; Laloue et al., 1981). In related experiments, caffeine (2 mM) was incubated with cells. Caffeine has been demonstrated to trigger a release of Ca^{2+} from intracellular storage sites, leading to an increase in cytoplasmic Ca^{2+} in animal cells and a green alga (Förster et al., 1989; Fredholm et al., 1994). It has also been demonstrated that caffeine induces cell plate breakdown in *Tradescantia* (Gunning, 1982) and affects germination and differentiation in spores of the fern *Onoclea sensibilis* (Miller and Bassel, 1980). Addition of caffeine also led to an increase in tension similar to that observed for 6-benzylaminopurine (Table I). Experiments with caffeine at a concentration of 20 μM were less conclusive but appeared to demonstrate a decreased effect on tension as was observed for BA (20 μM) (data not shown). In distinction, GA_3 and ABA showed no activity in modifying the tension within the actin network at either low or high concentrations (Table I).

DISCUSSION

The cytoskeletal network in plant cells is a dynamic assemblage of structural and regulatory proteins. Changes in the polymeric organization and state of microfilaments and microtubules have been demonstrated to occur as a result of changes in actin and tubulin interactions that are mediated by actin-binding proteins, microtubule-associated proteins, motor proteins, e.g. kinesin, and G proteins (Ridley and Hall, 1992; Williamson, 1993). Typically, the cell cytoskeleton has been shown to respond to a variety of proliferative and differentiating factors (Schliwa et al., 1984; Herman and Pledger, 1985; Shariff and Luna, 1992; Janmey, 1994) that are capable of triggering changes in the ionic equilibria of the cytoplasm, e.g. pCa^{2+}, pH, and pK^{+}. These changes have been shown to lead to the production of second messengers and modifications in the activities of regulatory proteins, e.g. protein kinases and phosphatases (Harmon, 1990).

Our previous work showed that variations in the ionic environment of soybean root cells results in diverse changes in tension within the actin network (Grabski et al., 1994). Artificially induced changes in the intracellular pH (increase or decrease) of soybean root cells result in a decrease in the tension, whereas changes in pCa^{2+} (increase in free calcium) cause an increase in the tension within the actin network of transvacuolar strands. The observed tension-relieving property of auxins may therefore, in part, be related to the ability of auxins to decrease the intracellular pH (Hager et al., 1991; Rayle and Cleland, 1992). In contrast, the increases in tension induced with cytokinins were similar to those previously observed following the addition of the Ca^{2+} ionophore A23187 to soybean root cells (Grabski et al., 1994). A relationship between cytokinin activity and Ca^{2+} mobilization was demonstrated by Saunders and Hepler (1982), who showed that the activity of the cytokinin BA (1 μM) in stimulating asymmetrical division and bud formation in target cells of the moss *Funaria* could be mimicked with the Ca^{2+} ionophore A23187 in the absence of the cytokinin. A similar capacity of A23187 (2–5 μM) (Grabski et al., 1994) to mimic the activity of BA (2 μM) (Table I) was observed in the CODA. Calcium antagonists and inhibitors of calmodulin were shown to block the cytokinin-induced bud formation in *Funaria* (Saunders and Hepler, 1983). In other studies, Schumaker and Gizinski (1993) showed that cytokinin also stimulated dihydropyridine-sensitive calcium uptake in moss protoplasts. The results with caffeine are also pertinent in that, in animal cells, caffeine-induced release of intracellular Ca^{2+} was shown to correlate with the contraction of intact skeletal muscle fibers (Smith et al., 1988). As shown in Table I, addition of caffeine led to a significant enhancement of the tension within the actin network. This enhanced tension was again similar in magnitude to that previously observed following addition of the ionophore A23187 (Grabski et al., 1994) or 6-benzylaminopurine (Table I), which are both agents capable of modifying the

intracellular concentration and distribution of free Ca^{2+} (Saunders and Hepler, 1982, 1983; Hahm and Saunders, 1991; Schumaker and Gizinski, 1993). These data may tentatively be interpreted to suggest that cytokinin-induced mobilization of Ca^{2+} from intracellular storage sites can result in rigidification of the actin network. The similarity between adenosine and BA in promoting enhanced rigor within the actin network (Table I) may hint that a functional analogy exists between the cytokinin signaling pathway in plant cells and the adenosine signaling pathway in animal cells (Fredholm et al., 1994). The bimodal activity of cytokinins may, in analogy with the adenosine signaling mechanism in animal cells, reflect the existence of both stimulatory and inhibitory cytokinin receptors.

On the whole, these results support a regulatory mechanism by which auxins and cytokinins can trigger synergistic or antipodal changes within the actin network of transvacuolar strands by promoting changes in intracellular pH and pCa^{2+}, presumably through the activation of ion channels and the production of second messengers.

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