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Calcium in the Regulation of Gravitropism by Light[1]

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ABSTRACT

The red light requirement for positive gravitropism in roots of corn (*Zea mays* cv "Merit") provides an entry for examining the participation of calcium in gravitropism. Applications of calcium chelators inhibit the light response. Calcium channel blockers (verapamil, lanthanum) can also inhibit the light response, and a calcium ionophore, A23187, can substitute for light. One can substitute for red light by treatments which have elsewhere been shown to trigger Ca^{2+} influx into the cytosol, *e.g.* heat or cold shock. Agents which are known to be agonists of the phosphatidylinositol second messenger system (serotonin, 2,4-dichlorophenoxyacetic acid, deoxycholate) can each partially substitute for the red light, and Li^{+} can inhibit the light effect. These experiments suggest that the induction of positive gravitropism by red light involves a rise in cytoplasmic Ca^{2+} concentration, and that a contribution to this end may be made by the phosphatidylinositol second messenger system.

The hybrid corn variety "Merit" requires a brief red light treatment of the root cap in order to exhibit positive root gravitropism, also called orthogravitropism (9, 18, 27). This red light induction of positive gravitropism is a phytochrome-mediated response that saturates at very low fluences (10). In the absence of red light treatment, roots of Merit corn will show diageotropic curvature (17, 18).

Two lines of evidence indicate that a study of the role of Ca^{2+} in red light induction of orthogravitropism might yield useful information. First, there are numerous data showing that calcium movement within the root tip is associated with corn root gravitropism (14–16, 27). For example, the variety Golden Cross Bantam × 70, which, like Merit, requires red light for positive gravitropism, also requires red light for the characteristic movement of Ca^{2+} across the root cap after gravistimulation (19). Second, at the cellular level Ca^{2+} appears to be an essential second messenger for stimulus-response coupling in many phytochrome-mediated phenomena (12, 24, 27). The work described here addresses not only the question of Ca^{2+} involvement in gravitropic behavior, but also the potential role of Ca^{2+} as a second messenger in the photocontrol of gravitropism.

MATERIALS AND METHODS

Zea mays cv Merit seeds (Asgrow Seeds, Kalamazoo MI) were imbibed in distilled water at 4°C for 2 h, and then placed on wet germination paper with the radicle end pointing downward. Rolls of germination paper containing the seeds were placed in beakers of water and grown in the dark at 25°C for 44 to 48 h.

Seedlings were selected for straightness and uniform root lengths between 10 and 25 mm. Using a dim green safelight, seedlings were mounted in holders consisting of a triple layer of wet cheesecloth between two plexiglass strips, with roots extending 10 mm beyond the bottom edge of the holders. Holders with 10 seedlings each were magnetically attached to plastic boxes lined with wet germination paper. The downward-pointing roots were allowed to equilibrate for 1 h before chemical or physical treatments.

Roots were treated by placing the holders on plexiglass stands with wells containing the appropriate reagent solutions, with the terminal mm of root in the solution. Unless otherwise noted all solutions were adjusted to a pH of 6.0. Control seedlings were always treated with the control solution (buffer) used in the rest of the treatments of the experiment (see "Results"). EDTA, ethylene glycol bis (β-aminoethyl ether), *N,N,N'*-tetraacetic acid (EGTA), $LaCl_3$, verapamil-HCl, Na-deoxycholate, 2,4-D, serotonin-HCl (5-hydroxytryptamine), and LiCl were purchased from Sigma. Compound A23187 was obtained from Boehringer Mannheim.

Red light was supplied by two red fluorescent tubes (G.E. F 40R). Exposures were 5 to 30 min depending on the experiment. 'Dark control' treatments were exposed only to the green safelight.

After chemical or light treatments, the holders were returned to the boxes and given gravistimulation by turning the boxes 90°. Seedlings remained in this horizontal position in the dark for 4 to 5 h, after which time they were taken into the light, where root length and curvature were recorded on videotape. Seedlings were maintained in humid boxes in the light for an additional 24 h in order to assess recovery of growth and light-induced gravicurvature. Any treatments which did not exhibit adequate recovery were not accepted.

Each treatment included 10 to 20 seedlings and all experiments were repeated at least three times. Data are presented as the results of a single experiment typical of the trend seen in the repeated experiments. In Table I, means are compared by calculating the LSD of the entire experiment; in all other tables and graphs, values are expressed as an average ± SE.

RESULTS

Calcium Requirements. In view of the implication of calcium in the regulation of root growth and root gravitropism, experiments were performed to deplete Merit roots of available Ca^{2+} by application of the chelators EDTA and EGTA prior to red light treatment. Results in Figures 1 and 2 indicate that at mM concentrations, both chelators yielded about a 50% inhibition of gravitropism following red light treatment. At these concentrations there were essentially no effects on growth of roots.

The effectiveness of calcium in restoring gravitropic responsiveness after chelator treatment was tested as shown in Table I. Using 1 mM of the chelators as the standard inhibitor, full gravitropic responsiveness was restored in each case by 1 mM $CaCl_2$. These effects were obtained without detectable effects on the growth rate of the roots.

The specificity of the chelator reversal effect for calcium was

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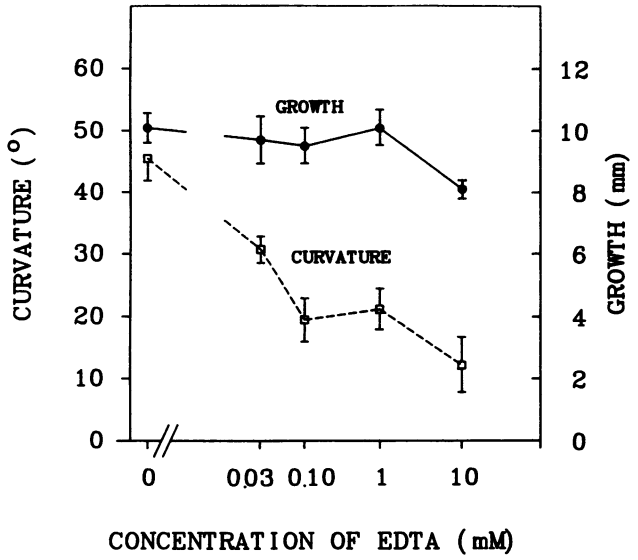

FIG. 1. Effect of EDTA on root growth and curvature after red light treatment. Roots were treated with EDTA for 60 min in the dark and 30 min in red light. Seedlings were returned to the dark and rotated 90°; root curvatures and growth were determined 5 h later.

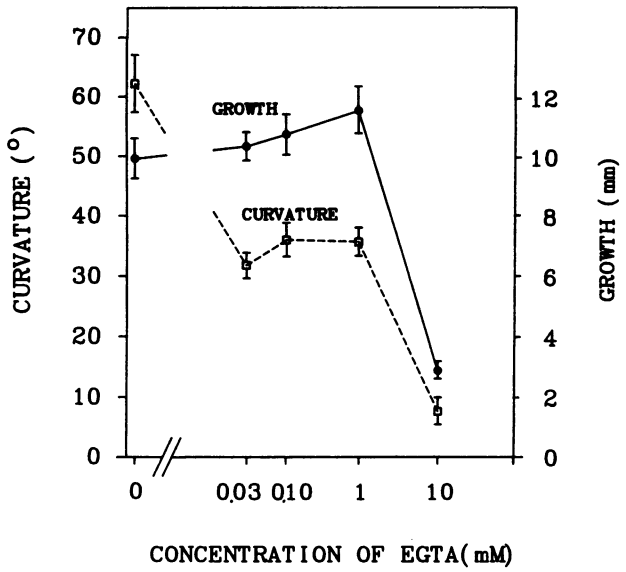

FIG. 2. Effect of EGTA on root growth and curvature after red light treatment. Roots were treated with EGTA for 90 min in the dark and 30 min in red light. Seedlings were returned to the dark and rotated 90°; root curvatures and growth were determined 5 h later.

Table I. *Effect of Divalent Cations on Recovery of Light-Induced Root Curvature after EDTA or EGTA Treamtent*

Roots were treated for 90 min in the dark with 1 mM EDTA or 1 mM EGTA. Roots were rinsed with HEPES and treated for 30 min in red light with $CaCl_2$, $MnCl_2$, or $MgCl_2$. Following treatment, seedlings were rotated 90°; root curvatures and growth were determined 5 h later.

Treatment		Curvature	Growth
First (90 min)	Second (30 min)		
		degrees	(*mm*)
Buffer 1 mM	Buffer 1 mM	58.1	12.6
EDTA 1 mM	Buffer 1 mM	19.8	10.2
1 mM	$CaCl_2$ 1 mM	74.6	12.2
1 mM	$MnCl_2$ 1 mM	47.1	12.5
1 mM	$MgCl_2$ 1 mM	24.0	9.9
LSD		12.6	2.3
Buffer 1 mM	Buffer 1 mM	55.8	13.0
EGTA 1 mM	Buffer 1 mM	36.6	14.0
1 mM	$CaCl_2$ 1 mM	59.8	12.0
1 mM	$MnCl_2$ 1 mM	37.1	12.8
1 mM	$MgCl_2$ 1 mM	37.8	14.1
LSD		6.8	1.8

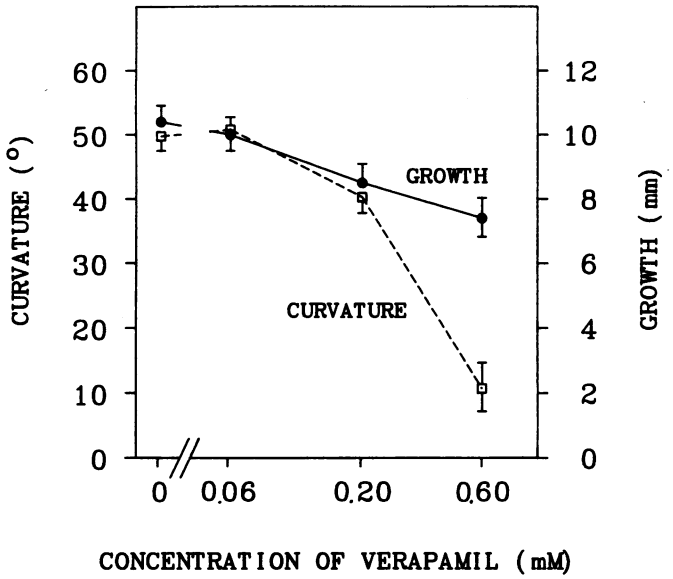

FIG. 3. Effect of verapamil on red-light-induced root curvature. Seedlings were treated for 30 min in the dark and 30 min in red light with verapamil. Following treatment, seedlings were rotated 90°; root curvatures and growth were determined after 4 h.

tested by comparisons of calcium with other divalent cations: manganese and magnesium chlorides as shown in Table I. After either of the chelator treatments, only $CaCl_2$ completely restored gravitropic responsiveness; in the case of EDTA, $MnCl_2$ gave a small amount of relief of the chelator inhibition, but after EGTA treatment neither Mn nor Mg provided relief.

Use of Ca^{2+} Channel Blockers. Experiments were carried out with putative Ca^{2+} channel blockers to further characterize the role of Ca^{2+} in light-induced positive gravitropism. Verapamil, a calcium channel blocker, or $LaCl_3$, a calcium antagonist, was applied to corn roots prior to the red light sensitization for gravitropism. The results in Figure 3 indicate that verapamil at 0.6 mM caused a 79% inhibition of curvature, while growth was inhibited only 29%. In subsequent experiments, verapamil at 0.5 mM consistently inhibited curvature while having no significant effect on root growth—for example, in a typical experiment curvature was reduced by 63% while root growth was reduced by 2% (DO Perdue, AC Leopold, unpublished data). $LaCl_3$ also produced an inhibition of gravitropic curvature, reaching a 47% inhibition at a concentration of 1 mM, while root growth was not significantly different from that of the control (Fig. 4).

Mobilization of Calcium. Many researchers report that one immediate consequence of red-light treatment of sensitive cells is a massive influx of Ca^{2+} into the cytosol (12, 27). Application of various known Ca^{2+}-mobilizing treatments was found to substitute for red light to reduce positive gravitropism in Merit roots as shown in Table II.

The tips of corn roots deprived of red light were pretreated with the Ca^{2+} ionophore A23187, with or without added Ca^{2+}

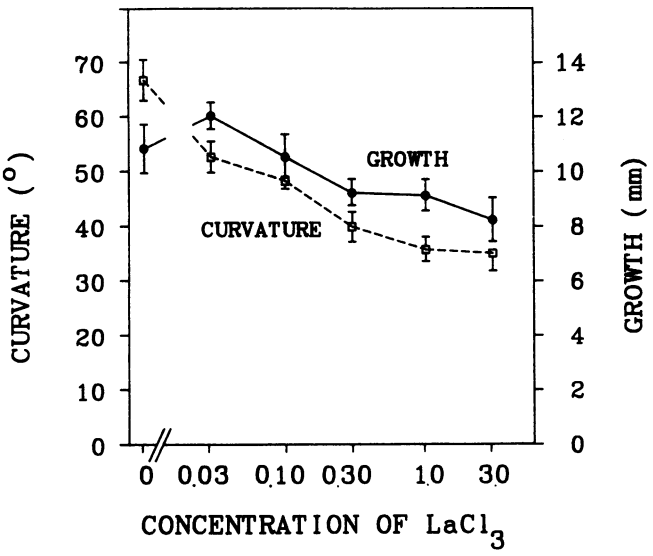

FIG. 4. Effect of $LaCl_3$ on red-light-induced root curvature. Seedlings were treated for 30 min in the dark and 15 min in red light with $LaCl_3$. Following treatment, seedlings were rotated 90°; root curvatures and growth were determined after 4 h.

Table II. *Ca^{2+}-Mobilizing Treatments Induce Root Curvature in the Dark*

Seedling root tips were treated in the dark with the following solutions for the times indicated. Curvature and growth were measured following gravistimulation in the dark. Control solutions for each treatment are described in "Results."

Treatment	Curvature	Growth
	degrees	*mm*
A23187, 5 ppm 30 min	38.2 ± 2.7	5.0 ± 0.4
Control (buffer), 30 min	7.4 ± 1.4	9.5 ± 0.8
Cold shock, 4°C, 30 s	24.8 ± 7.3	9.4 ± 0.5
Control, 25°C, 30 s	5.0 ± 2.4	9.3 ± 0.5
Heat shock, 40°C, 5 min	66.7 ± 2.4	8.8 ± 0.4
Control, 25°C, 5 min	6.3 ± 2.4	9.3 ± 0.4

in the medium prior to gravistimulation. Roots treated with A23187 for 30 min produced a 38° response to gravistimulation in the dark (Table II); this was approximately two-thirds as effective as a 5 min red light treatment. $CaCl_2$ application alone did not have any sensitizing effect; including $CaCl_2$ with the A23187 did not notably affect the result (data not shown).

Following the evidence of Rincon and Hanson (25), we examined the effects of cold and heat shock as putative mobilizers of cytoplasmic Ca^{2+}. Exposure of root tips to buffer (1 mM MES-KOH [pH 6]) chilled to 4°C, or heated to 40 to 45°C, induced positive gravitropism in darkness (Table II). Neither treatment inhibited growth.

Treatments which Affect Phosphatidylinositol Turnover. If red-light treatment of plant tissues causes an elevation of cytosolic Ca^{2+}, then it seems possible that a Ca^{2+} second-messenger system may be involved. In many animal systems, the phosphoinositides serve as agents triggering a second-messenger system for cellular reactions involving Ca^{2+} mobilization (2, 22, 28). Accordingly, we tested several agents which are known to serve as agonists for the PI[2] second messenger system for their effects on red light mediated gravitropism.

Roots of dark-grown plants were treated with the PI agonist serotonin (5-hydroxytryptamine); this resulted in substantial curvature after gravistimulation (Table III). Although serotonin is capable of promoting positive gravitropic behavior, at concentrations above 1 mM it also strongly inhibits growth (23). We presume that serotonin concentrations above 1 mM sometimes appear less effective due to those growth effects.

Exposure of dark-grown roots to solutions of deoxycholate in standard buffer (1 mM MES-KOH [pH 6.0]) induced strong graviresponsiveness in a concentration-dependent manner (Table III) Deoxycholate has been shown to promote phosphoinositide turnover in both animal and plant systems, presumably due to its stimulatory effects on the phospholipase involved in cleavage of membrane PI (4, 20).

Roots treated in the dark with the synthetic auxin 2,4-D showed strong curvature in spite of severe growth inhibition (Table III). Treatment with 2,4-D, as well as with IAA, has been reported by Morre *et al.* (21) to stimulate PI hydrolysis and turnover in isolated soybean membranes. Similar effects on stimulating gravitropic curvature have been obtained with another synthetic auxin, naphthaleneacetic acid, and with the natural auxin, IAA (AK LaFavre, AC Leopold, unpublished data).

Lithium, an inhibitor of the PI pathway, provides another probe for determining whether that pathway functions in the red light sensitization of root gravitropism. Figure 5 shows typical results of a LiCl experiment. In this experiment, we found that a 30 min treatment with 25 mM LiCl prior to red light treatment caused an 85% inhibition of gravicurvature while causing a 50% inhibition of root growth. The promotion of the red light effect on curvature at lower LiCl concentrations (2.5–5 mM) is a repeatable and expected phenomenon: lithium is known to block recycling of PI metabolites and thus results in an enhanced accumulation of soluble intermediates, including IP_3, which triggers Ca^{2+} release from internal stores (2, 28). Added inositol, at concentrations up to 0.5 M, did not relieve lithium inhibition of the light effect; inositol alone had neither an inhibitory nor a synergistic effect on light-induced gravitropic behavior (data not shown).

DISCUSSION

In this paper, we have presented indirect evidence that elevation of cytosolic Ca^{2+} levels may be an intrinsic part of the red light induction of positive graviresponsiveness in Merit corn roots, and that the phosphatidylinositol second messenger system may serve to bring about such a Ca^{2+} mobilization. We propose

Table III. *Agonists of the Phosphoinositide Pathway Induce Root Curvature in the Dark*

Seedling root tips were treated in the dark with the following solutions for the times indicated. Curvature and growth were measured following gravistimulation in the dark. Control solutions for each treatment are described in "Materials and Methods."

Treatment	Curvature	Growth
	degrees	*mm*
Serotonin (5-hydroxytryptamine)		
1 mM, 30 min	24.7 ± 2.6	5.7 ± 0.3
5 mM, 30 min	23.0 ± 3.2	3.9 ± 0.4
Control (buffer)	5.5 ± 2.7	4.4 ± 0.3
Deoxycholate		
0.1 mM, 1 h	18.5 ± 2.6	4.0 ± 0.2
0.25 mM, 1 h	44.7 ± 4.3	3.1 ± 0.2
Control (buffer)	2.3 ± 0.9	7.3 ± 0.3
2,4-D		
1 μM, 30 min	19.0 ± 4.1	11.8 ± 0.5
2.5 μM, 30 min	37.0 ± 5.1	9.8 ± 0.4
Control (buffer)	7.2 ± 4.1	14.1 ± 0.3

[2] Abbreviations: PI, phosphatidylinositol; IP_3, inositol trisphosphate.

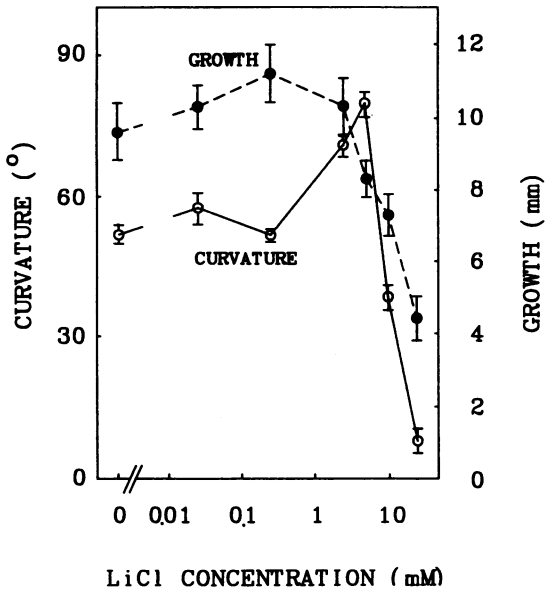

FIG. 5. Effect of LiCl on red-light-induced root curvature. Seedlings were treated for 30 min in the dark and 30 min in red light with LiCl. Following treatment, seedlings were rotated 90°; root curvatures and growth were determined after 4 h.

that red light causes a stimulation of the PI pathway, resulting in the induction of the ability of dark-grown roots to respond positively to a gravitational stimulus. We believe that this is the first report of an involvement of the PI pathway in the phenomenon of gravitropism.

A Ca^{2+} requirement for positive gravitropic behavior has been demonstrated for Merit (17) and other corn varieties (15, 16). Unfortunately, chelator treatments as utilized do not permit discrimination between possible calcium roles in the gravity sensing, transduction, and response systems: chelator treatment of Merit root tips results in inhibition of subsequent gravitropic behavior, regardless of whether treatments are applied before, during, or after red light treatment (DO Perdue, AC Leopold, unpublished results). Thus, this type of experiment is not sufficient to identify the Ca^{2+} effect on component processes such as sensing, transduction, or response.

Some workers have reported inhibitions of phytochrome-mediated phenomena by application of La^{3+}, verapamil, nifedipine, and other Ca^{2+} channel blockers (12, 24). In the intact corn root system used in our work, treatment with La^{3+} or verapamil before, during, or after red-light exposure of roots had similar inhibitory effects (DO Perdue, AC Leopold, unpublished results). Inhibition of curvature by putative calcium channel blockers does not necessarily establish a specific or unique role for calcium. For example, although La^{3+} can inhibit the red light effect in Merit roots, and we interpret this effect as being related to the La^{3+} inhibition of Ca^{2+} channels, the effect may not be related only to Ca^{2+} mobilization. La^{3+} appears to act as a competitive inhibitor of Ca^{2+} uptake and calcium-dependent processes (31): thus, La^{3+} may interfere with Ca^{2+} action following light-induced mobilization. Another type of evidence does, however, permit us to claim that the effects of Ca^{2+} described here are specific to light-induced positive gravitropism (AC Leopold, and SH Wettlauffer, unpublished data). Etiolated roots of Merit corn are strongly diageotropic before light exposure, and treatment with EDTA, EGTA, verapamil, or La^{3+} does not interfere with diageotropic curvature in the dark (DO Perdue, AC Leopold, unpublished data).

Calcium-Mobilizing Treatments. Application of A23187 can substitute for red light in certain other phytochrome-mediated phenomena (12, 24, 29). Rincon and Hanson (25) list a number of treatments which cause a substantial influx of $^{45}Ca^{2+}$ into corn roots; the most effective treatments include application of A23187, heat shock (40°C), and cold shock (4°C). The ineffectiveness of Ca^{2+} alone to promote gravitropism indicates that the positive results reported here do not reflect a mere tropistic curvature towards a region of higher Ca^{2+} concentration (15). Whether the diverse Ca^{2+} mobilizing treatments share any of the physiological and biochemical processes involved in gravitational stimulus transduction and response is not known. Collectively, however, these treatments provide substantial indirect evidence that an increase in cytosolic Ca^{2+} levels is required for dark-grown roots of Merit to exhibit positive gravitropism.

PI Role in Gravitropic Transduction. Cleavage and phosphorylation of membrane phosphatidylinositol is known to result in a transient rise in cytosolic Ca^{2+} levels, as well as activation of protein kinase C (13, 24). PI turnover has been shown to be the second messenger in stimulus-response coupling in numerous physiological phenomena: visual transduction in both vertebrates and invertebrates; excitation-contraction in muscle; stimulation of cellular processes by α-andrenergic agonists; effects of insulin and glucose on metabolism (2). The components of the PI pathway of signal transduction have been demonstrated in plants (3, 7, 11, 22, 26). We have presented evidence, both in this paper and previously (23) that known agonists of the PI pathway suffice to induce the capacity for positive gravitropism in the absence of light. The fact that PI agonists can substitute for red light activation of phytochrome is unlikely to be coincidental: the evidence presented here suggests that the PI pathway is likely to be an important second messenger involved in coupling phytochrome activation to gravitropic responsiveness.

Interpretation of the effects of LiCl is somewhat problematic. The concentrations required for inhibition of light-induced curvature also inhibit root elongation. The fact that Li^+ inhibition of growth and light-induced curvature cannot be relieved by addition of exogenous inositol indicates that Li^+ does not serve as a specific inhibitor of the PI pathway.

Influences on the PI Pathway. A23187 has been shown to stimulate PI turnover in erythrocyte ghosts of human, rabbit, and rat (6), in human platelets (1), and in pancreas (8). Thus it appears that the ionophore itself, or its Ca^{2+} mobilizing action, may directly stimulate the PI pathway. The results reported in Table II permit speculation that A23187 may, indeed, be stimulating a second messenger system involved in capacitating the positive gravitropic response of the root. Stevenson *et al.* (30) have reported that heat-shocking fibroblasts induces rapid increases in IP_3 and intracellular Ca^{2+}; this report suggests that heat shock may suffice to activate the PI pathway. Finally, the PI agonist serotonin, shown in Table III to stimulate gravitropic curvature, has previously been shown to substitute for red light to induce Ca^{2+} influx into maize leaf protoplasts (5).

The role of the PI second-messenger system in positive gravitropism will not be firmly established until the release of soluble inositol phosphates has been shown to occur after red light treatment of Merit roots, as has been demonstrated by Morse *et al.* (22) for red light regulation of pulvinar movement in *Samanea saman*. The present evidence does suggest, however, that the phytochrome regulation of positive gravitropism may involve activation of the PI pathway in stimulus-response coupling of corn root gravitropism.

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