Effect of Inhibitors of Auxin Transport and of Calmodulin on a Gravisensing-Dependent Current in Maize Roots

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ABSTRACT

Some characteristics of the gravity sensing mechanism in maize root caps were investigated using a bioelectric current as an indicator of gravity sensing. This technique involves the measurement of a change in the current density which arises at the columella region coincident with the presentation time. Two inhibitors of auxin transport, triiodobenzoic acid and naphthylphthalamic acid, blocked gravitropic curvature but not the change in current density. Two inhibitors of calmodulin activity, compound 48/80 and calmidazolium, blocked both curvature and gravity-induced current. The results suggest that auxin transport is not a component of gravity sensing in the root cap. By contrast, the results suggest that calmodulin plays an intrinsic role in gravity sensing.

A method for detecting gravity sensing independently of subsequent growth components of gravitropism (1) makes it possible to probe the nature of gravity sensing through the use of metabolically inert inhibitors.

The best-known inhibitors of gravitropism in roots are known to be inhibitors of auxin transport (17, 18). Although auxin redistribution may regulate gravicurvature in coleoptiles (6, 15), auxin redistribution was not detectable in roots (15). It is not clear whether auxin transport inhibitors block gravity sensing or whether they are involved in subsequent steps in gravitropism. If the gravity-induced current shift is not inhibited by antagonists of auxin transport, it would suggest that auxin transport is not a component of gravity sensing.

There is increasing evidence that calmodulin is involved in gravitropism of roots. In maize varieties which require red light to induce root gravitropism, mRNA coding for calmodulin increases within 5 min of exposure to light (LJ Feldman, personal communication). There is a marked increase in calmodulin activity in the root cap which parallels the increase in gravitropism (23). Calmodulin is primarily localized in the sensing cells of the columella (14, 21). Calmodulin inhibitors inhibit curvature, but it has not been possible to determine whether the effect is related to gravity sensing or to subsequent growth. Many calmodulin inhibitors cause nonspecific ion leakage across the membrane making them unusable for electrophysiological measurements. Two inhibitors described recently have high activity yet do not change the membrane permeability when used at moderate concentrations. At concentrations which reversibly inhibit curvatures, these are tested here for their effect on the current associated with gravity sensing.

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MATERIALS AND METHODS

Plants. Three-old seedlings of maize (Zea mays cv 'Merit') were used when the radicles were 25 to 35 mm long.

Curvature. To measure gravitropic curvature, the germinated seeds were placed between two Plexiglas plates padded with cheesecloth with 10 mm of radicle extending beyond the edge of the Plexiglas plate. The plates were mounted in a humidified plastic box. After 15 to 30 min for equilibration, the seedlings were gravistimulated by turning the box 90°. Curvature was recorded at intervals on videotape. Curvature was subsequently measured with a protractor on the 6-fold enlarged images of the radicles displayed on the video monitor screen.

For pretreatments with inhibitors, the terminal 1.5 mm of the root tips were dipped in an inhibitor solution immediately before gravistimulation. Seedlings were dipped for 10 min for most of the inhibitors; longer times were used where noted for those inhibitors which impregnated the tissue slowly.

Curvature experiments were each repeated two to five times. Small differences in the laboratory temperature and seedling age affected the rate and extent of curvature even in the controls. Therefore, the figures are of representative experiments, rather than averages of all experiments.

Vibrating Probe. The vibrating probe apparatus was assembled as described by Björkman and Leopold (1). In principle, it is a spherical electrode of 25 μm diameter vibrating along its axis with a total displacement of 30 μm. This makes it a very rapid and extremely sensitive detector of electric potential gradients (which reflect currents) in aqueous media. The probe tip was held 100 μm from the surface of the root 500 μm back along the root from the tip of the cap, so that it was adjacent to the columella.

Gravistimulation was effected by rotating the entire apparatus 90°; the current was continuously monitored. The suspending buffer around the roots was 0.1 mM CaCl₂, 0.1 mM MgCl₂, 0.1 mM KCl, and 1 mM morpholinoethanesulfonic acid adjusted to pH 6.0 with KOH.

Inhibitors. TIBA is a competitive inhibitor of auxin transport (18), and of calcium transport (12). TIBA at 10 μM slows auxin transport in maize coleoptiles by 90% (3). Gravitropic curvature in pea roots (7) and in maize roots (12) is completely inhibited by 10 μM TIBA. TIBA (Sigma) was recrystallized from ethanol with water. For use it was dissolved in ethanol, then diluted with water so that the final ethanol concentration was less than 100 parts per million.

The auxin transport inhibitor NPA (Pfaltz & Brauer) inhibits gravitropic curvature in maize roots. Curvature is inhibited 50% in 4 μM NPA (2) and 70% after 90 min in 10 μM NPA (12). In maize coleoptiles, 100 μM NPA completely eliminated gravi-
tropic curvature as well as gravity-induced calcium redistribution (10).

Compound 48/80 (Sigma) is the most specific inhibitor of calmodulin-dependent events available (9). It is a mixture of oligomers resulting from a condensation of N-methyl-p-methoxyphenethylamine with formaldehyde. Compound 48/80 probably inhibits by binding to calmodulin (20) and has a Ki for calmodulin of 30 μg ml⁻¹ (22). Quite soluble in water, it is the most hydrophilic calmodulin inhibitor available. Some 48–80 becomes membrane bound in vitro, though that is probably bound to proteins (22). In sarcoplasmic-reticulum vesicle preparations, 50% inhibition of calmodulin-dependent calcium uptake is achieved with 10 μg ml⁻¹ compound 48/80 (24). Erythrocyte calmodulin-activated (Ca²⁺ + Mg²⁺)-ATPase is 90% inhibited at 20 μg ml⁻¹ (20). A 10-min exposure of the root tips to 100 μg ml⁻¹ compound 48/80 was sufficient to produce maximum inhibition.

CMZ (1-[bis(p-chlorophenyl)methyl]-3-[2,4-dichloro-β-(2,4-dichlorobenzoyloxy)phenoxy]imidazolium chloride) is more hydrophobic than compound 48/80 and was stored as a 4 mM ethanolic stock solution. The final ethanol concentration in the solutions applied to roots was 5% in suspending buffer; a 10-min exposure to 5% ethanol alone inhibited neither curvature nor growth. CMZ is believed to act by binding calmodulin (24). In sarcoplasmic reticulum vesicle preparations, 100 μM CMZ inhibits calmodulin-dependent calcium uptake by 90% (24). Calmodulin stimulation of erythrocyte calcium transport is 90% inhibited by 50 μM CMZ (8). In contrast, only 1 nm CMZ caused 68% inhibition of calmodulin-activated NAD kinase activity in extracts of ‘Merit’ maize roots (23).

The cells of the central columella are exposed to lower concentrations of inhibitor than that of the bulk solution because diffusion will be incomplete in the 10-min dipping time. Therefore it was considered appropriate to expose roots to concentrations as much as 10 times higher than those effective on isolated cells if necessary. During the measurements with the vibrating probe, a low concentration of inhibitor was maintained in the suspending medium to slow the washing out of inhibitor from the tissue.

RESULTS

To investigate the involvement of auxin transport and calmodulin activity in gravity sensing, the presence of the current shift which indicates the occurrence of gravity sensing (1) was determined in roots treated with inhibitors of these processes. To limit nonspecific effects of the inhibitors, the lowest dose which blocked gravitropic curvature was first determined. The initial current pattern around the roots treated with inhibitor was not noticeably different from that in controls (cf. 1).

Auxin Transport Inhibitors. To determine whether gravity sensing involves auxin transport, two agents which block auxin transport were applied. Gravitropic curvature was measured in roots after tip pretreatments with 0, 1, 10, and 100 μM TIBA in suspending buffer for 15 min. Curvature was inhibited for 1 h by the 10 μM treatment, and was completely inhibited by 100 μM TIBA (Fig. 1). In roots gravistimulated in suspending buffer supplemented with 10 μM TIBA, an outward current developed adjacent to the upper side of the columella (Fig. 2).

Pretreating root tips with 1 μM NPA for 15 min caused a 1 h delay in the onset of curvature, while 10 μM completely inhibited it (Fig. 3). These treatments did not affect the growth rate, though 1 μM did slow growth by about 70%. After treatment with 1 μM NPA, an outward current appeared on the upper side of the columella region upon gravistimulation (Fig. 4). Three parameters characteristic of the electrical response were measured for individual roots and the means of each parameter are compared in Table 1. The only significant difference from the response in
control roots was that the response in the NPA treated roots was delayed.

**Calmodulin Inhibitors.** To test the possible role of calmodulin in gravity sensing, calmodulin inhibitors were applied to root tips to test whether the sensing-induced current shift was inhibited. Roots that were pretreated with 100 µg ml⁻¹ of compound 48/80 in suspending buffer showed a complete inhibition of curvature for 1 h of gravistimulation, but by 2 h the roots had begun to curve in a normal manner (Fig. 5). Measurement of currents adjacent to the columella in roots pretreated with 100 µg ml⁻¹ 48/80 showed no change following gravistimulation (Fig. 6).

Similar experiments were carried out with roots which had been pretreated for 10 min in 0, 100, and 425 µM calmidazolium in suspension buffer with 5% ethanol. Curvature was delayed 1 h in 425 µM CMZ, but a normal rate of curvature resumed after 2 h (Fig. 7). In root tips pretreated with 425 µM CMZ for 10 min the current density adjacent to the columella did not change following gravistimulation (Fig. 8). The normal electrical response to gravistimulation was eliminated by both calmodulin inhibitors.

**DISCUSSION**

Measurement of electric current densities around maize root caps during gravistimulation allows the discrimination between specific events involved in gravity sensing as contrasted with differential growth.

The failure of auxin transport inhibitors to inhibit the sensing-induced current shift implies that auxin transport is not part of the sensing process. That is, the initial asymmetry established in the columella cells is not auxin. Microsurgical experiments (19) suggest that lateral distribution of a growth inhibitor occurs in root tissue proximal to the root cap, in response to a signal originating in the root cap.

**Table 1. Characteristics of Current Response in the Presence of Auxin Transport Inhibitors**

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a Time from gravistimulation until the current density began to increase. b Time from the beginning of a change in the current density until it reached a new steady level. c Change in current density during rise time. d Probability that difference between treatment and control is due to chance using Student's t test.
The role of calcium in root gravitropism is complex. Moore and Evans (16) point out the difficulty in reconciling the downward movement of calcium in maize root tips (12) with a presumed increase in auxin activity (as a growth inhibitor) on the lower side, as expected from the Cholodny-Went hypothesis, because the concurrent transport of these substances is in opposite directions (4). These contradictory observations might be reconciled if calcium transport processes occur independently in the root cap and in the meristematic region immediately proximal to the root cap (11). Furthermore, Mertens and Weiler (15) were unable to find any auxin redistribution in maize roots.

There are several reasons to expect calmodulin to play a role in root gravitropism. The concentration of calmodulin in the columella (14, 21), the parallel increase in calmodulin activity and gravitropisensitivity following red-light induction (23), and a 4-fold increase in calmodulin mRNA in the root cap after red light exposure (LJ Feldman, personal communication) all support a role in the sensing mechanism. The observation that calmodulin inhibitors block gravitropic curvature supports that possibility. Because calmodulin is important in metabolism (5), such inhibitors may act at steps other than sensing. But the inhibitions of the gravity sensing-induced current shift by the calmodulin inhibitors compound 48/80 and calmidazolium provide strong evidence that calmodulin is required for gravity sensing.

By using the characteristic shift in bioelectric current to detect gravity sensing, we have provided evidence that gravity sensing does not involve auxin transport, but that sensing does involve calmodulin. This technique makes it possible, for the first time, to discriminate between inhibitions of the sequential steps of gravity sensing and subsequent gravitropic curvature.

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LITERATURE CITED
20. ROSSI IPPC, AF REGA, PJ GARAHAN 1985 Compound 48/80 and calmodulin modify the interaction of ATP with the (Ca²⁺-Mg²⁺)-ATPase of red cell membranes. Biochem Biophys Acta 816: 379–386

FIG. 7. Time course of curvature in roots pretreated with CMZ. Root tips were exposed to the noted concentrations of CMZ in suspending buffer for 10 min before gravitimulation. Each point is the mean of ≥10 roots; bars indicate the standard error of the mean.

FIG. 8. Current density of roots pretreated with CMZ. Time course was measured adjacent to the columella on the upper side of the horizontal root. The roots were exposed to 425 μM CMZ for 10 min before gravitimulation and measurements made in 100 μM CMZ. Bars indicate the standard deviation of three replicate runs.