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Asymmetric Distribution of Acetylcholinesterase in Gravistimulated Maize Seedlings[1]

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Acetylcholinesterase (AChE) activity has previously been studied by this laboratory and shown to occur at the interface between the stele and cortex of the mesocotyl of maize (*Zea mays* L.) seedlings. In this work we studied the distribution of AChE activity in 5-d-old maize seedlings following a gravity stimulus. After the stimulus, we found an asymmetric distribution of the enzyme in the coleoptile, the coleoptile node, and the mesocotyl of the stimulated seedlings using both histochemical and colorimetric methods for measuring the hydrolysis of acetylthiocholine. The hydrolytic capability of the esterase was greater on the lower side of the horizontally placed seedlings. Using the histochemical method, we localized the hydrolytic capability in the cortical cells around the vascular stele of the tissues. The hydrolytic activity was inhibited 80 to 90% by neostigmine, an inhibitor of AChE. When neostigmine was applied to the corn kernel, the gravity response of the seedling was inhibited and no enzyme-positive spots appeared in the gravity-stimulated seedlings. We believe these results indicate a role for AChE in the gravity response of maize seedlings.

Asymmetric growth, resulting in the characteristic gravity response, can occur in the mesocotyl of maize seedlings within 3 min following the gravity stimulus (Bandurski et al., 1986a, 1986b), and both free and ester IAA increase in the lower half of the mesocotyl cortex in that same period (Bandurski et al., 1990b). Asymmetric calcium distribution has been shown to develop in 5 to 10 min following the gravity stimulus (Goswami and Audus, 1976; Slocum and Roux, 1987). After longer times a potassium asymmetric distribution occurs (Goswami and Audus, 1976) and even sugar becomes asymmetrically distributed (Momonoki, 1988). Thus, the negatively charged IAA, the uncharged esters of IAA, and the positively charged calcium all become asymmetrically distributed in the cortical cells surrounding the stele in these short periods. What working theory can be advanced for the rapid asymmetric distribution in the cortex of these disparate substances? Bandurski et al. (1990a, 1990b) postulated as a working hypothesis that a release of solutes occurs from the stele to the cortical tissues of the mesocotyl by a gating of solute movement through the plasmodesmata, out of the stele, and into the cortex (Epel and Bandurski, 1990). The gating was accomplished by membrane depolarization, as has been observed to occur in those same periods (Sievers and Hensel, 1982).

ACh is a chemical transmitter in animals that serves to propagate an electrical stimulus across the synaptic junction (Kelly et al., 1979; Duant et al., 1980; MacIntosh, 1981). Momonoki (1992) has proposed a similar role for ACh in plants in which the chemical transmitter would function in the depolarization of the junction between the cortex and vascular stele, thus permitting the release of solutes from the stele and into the cortical tissues. AChE would then hydrolize ACh around the stele and return the system to ground state. Thus, the plasmodesmatal channels could be controlled by the binding of ACh to its receptors, just as in the animal system. Consequently, the transport of hormones and substances could be regulated by the opening and/or closing of the conductive channels.

The objective of this research was to determine whether AChE could function as a regulator of the plasmodesmatal junction between the stele and cortex, activating gates to open on one side of the stem and to close on the other. AChE activity was detected in the selected organs of 5-d-old dark-grown maize seedlings by histochemical localization of AChE with a light microscope and with a second method by chemical determination of AThCh using a colorimetric SH reagent following a gravity stimulus to the seedlings.

MATERIALS AND METHODS

Kernels of maize (*Zea mays* L. cv Silver Honey Bantam) were soaked for 24 h in running water and germinated on moist paper towels for 5 d in the dark at 25°C.

Organ Harvesting

The regions of selected organs for measuring AChE activity and preparation of cross-sections are shown in Figure 1. For the harvesting of the coleoptile, the coleoptile node, and the mesocotyl, the upper portion of 5-d-old shoots was severed at a point 4 mm below the coleoptile node. The mesocotyl was then nicked, but not cut through, at a point about 14 mm above the junction between the shoot and the root. The cortex was then slid off the stele and the 10 mm of the cortex closest to the tip was harvested (Pengelly et al., 1982). The 10 mm of coleoptile was harvested from 2 mm above the coleoptile node. The coleoptile nodes were harvested at lengths of 1.5 to 2.0 mm.

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Abbreviations: ACh, acetylcholine; AChE, acetylcholine esterase; AThCh, acetylthiocholine.

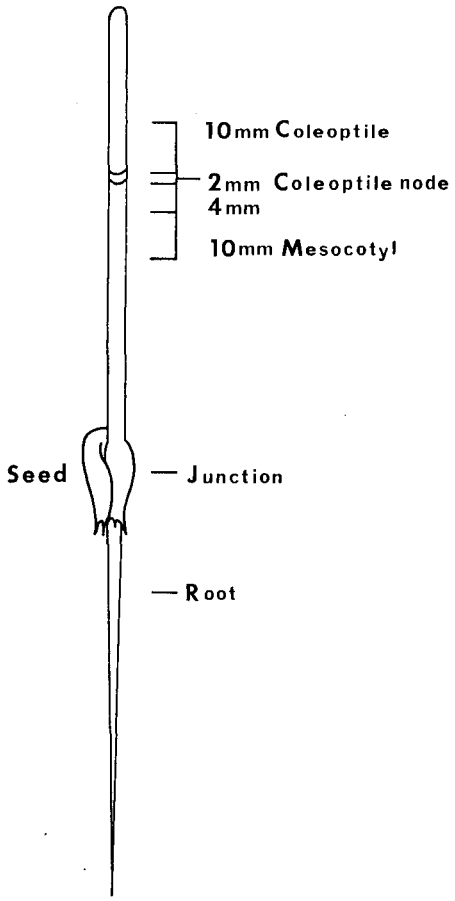

Figure 1. The regions of selected organs of the maize seedling for measuring AChE activity and preparation of cross-sections.

Analytical Materials

After the gravity stimulus was applied, the coleoptiles without leaves, the mesocotyls without steles, and the coleoptile nodes were separated into upper and lower halves or right and left halves for controls. The harvested tissues were dropped into an ice bath, and 20 plants were used for each experiment.

Gravistimulus Experiment

The gravistimulus was given by moving plants from a vertical to a horizontal position under a phototropically inactive green light (130 Erg $[cm^2]^{-1}$ s^{-1}, transmission maximum 530 nm). To obtain the maximum rate of curvature of the mesocotyl (30°), the gravistimulus was carried out following the method of Bandurski et al. (1984). About 30 kernels were pinned to a Styrofoam sheet covered with a moist paper towel and incubated for 90 min with the shoots in a vertical position. The shoots were moved to a horizontal position for 90 min for the gravity stimulus. For nongravistimulated seedlings, the shoots were held in a vertical position for 90 min and then incubated in that position. Neostigmine bromide (35 μM) was injected into kernels 90 min before incubation. Twenty plants were used for each experiment. Time-lapse photographs of a maize seedling during gravitropic curvature were made with a camera and 35-mm film attached to an intervalometer at 15-min intervals.

Assay of AChE Activity by SH Appearance

This assay was based on the colorimetric-SH reagent 5–5′-dithiobis-2-nitrobenzoic acid, as used previously by Asahi et al. (1961) and Ellman et al. (1961). Harvested organs were incubated for 30 min (Momonoki, 1992) in a vial containing 500 μL of 100 mM sodium phosphate buffer (pH 7.5) and 500 μL of AThCh chloride, and 12.5 mM in 100 mM sodium buffer, pH 7.5, as a substrate. After the organs were incubated, 200 μL of solution was transferred to a vial, and 950 μL of sodium phosphate buffer (pH 7.5) and 50 μL of 5–5′-dithiobis-2-nitrobenzoic acid (10 mM in 100 mM sodium phosphate, pH 7.0) were added. The A_{412} was read after 1 min and then the amount of SH appearing was calculated using a molar extinction coefficient of 13,600.

Table I. *ACh activity in gravistimulated maize seedlings as measured by SH appearance following hydrolysis of AThCh*

Ten-millimeter pieces of 10 coleoptiles and 10 mesocotyls without steles and 2-mm pieces of 10 coleoptile nodes from 5-d-old etiolated seedlings were used for each experiment. Neostigmine bromide (35 μM) was used as an AChE inhibitor. Values are the means ± SE of three experiments.

Plant Organ	AChE Activity			
	Gravistimulated		Nongravistimulated	
	Upper	Lower	Left	Right
	SH pmol g^{-1} fresh wt h^{-1}			
Coleoptile	164 ± 16 (158)[a]	210 ± 18 (183)	152 ± 6	157 ± 10
+ Inhibitor	32 ± 2	45 ± 3	36 ± 2	35 ± 3
Coleoptile node	569 ± 24 (571)	649 ± 20 (608)	573 ± 25	568 ± 26
+ Inhibitor	62 ± 5	86 ± 4	68 ± 4	66 ± 3
Mesocotyl	62 ± 6 (61)	99 ± 5 (79)	61 ± 5	60 ± 4
+ Inhibitor	14 ± 2	16 ± 3	12 ± 2	11 ± 3

[a] Numbers in parentheses indicate the mean of a statistical analysis of the ACh activity in the upper and lower half of the organs. The minimum difference by Tukey's procedure was 16.3 (P = 0.05).

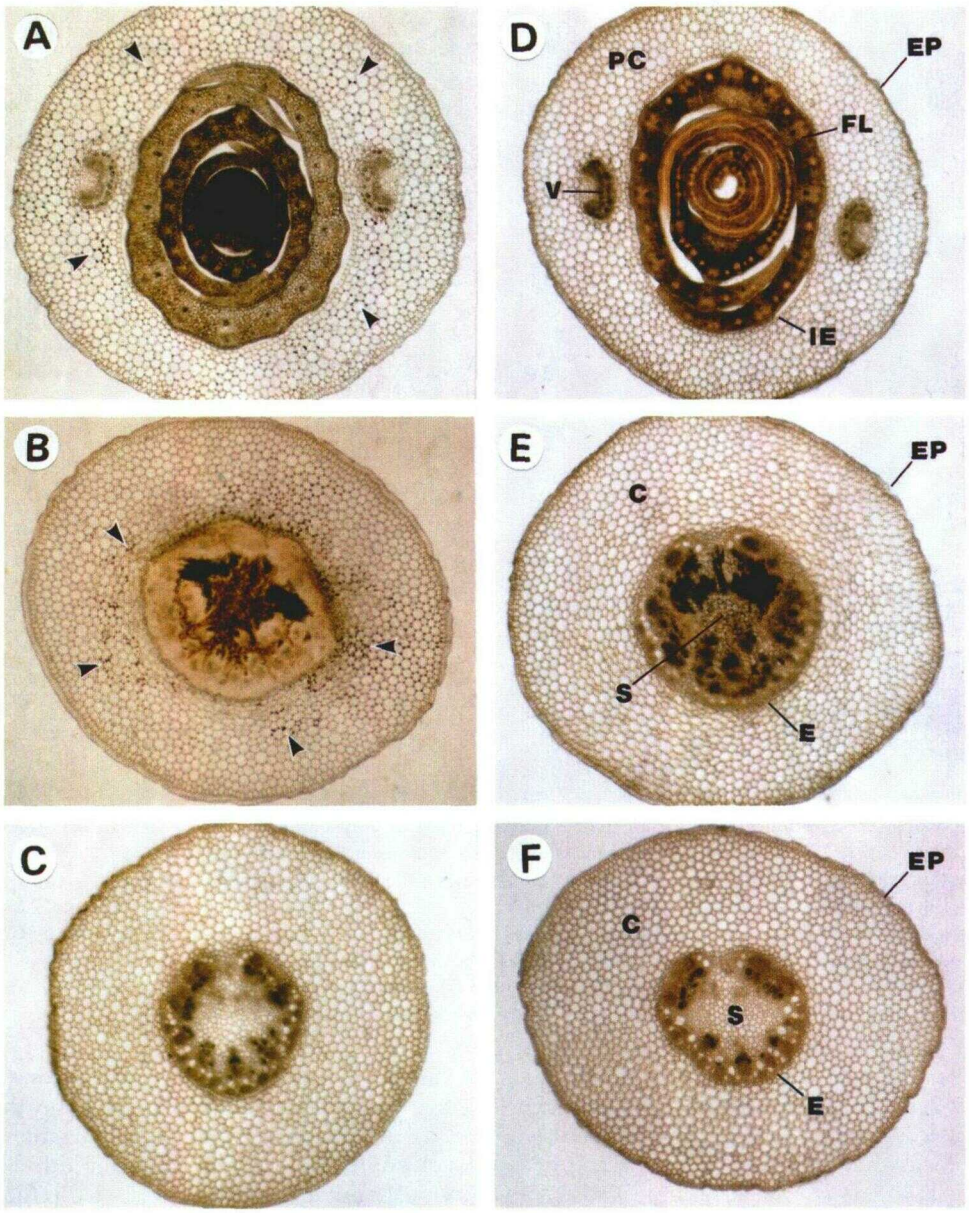

Figure 2. Histochemical evidence of AChE in selected organ tissues of nongravistimulated 5-d-old maize seedlings. A, AChE-positive spots in whole cortex cells of the coleoptile (×30); B, AChE-positive spots in whole cortex cells around the vascular system of the coleoptile node (×30); C, lack of AChE staining in the mesocotyl (×30); D, control (without a substrate), lack of AChE staining in the coleoptile (×30); E, control (without a substrate), lack of AChE staining in the coleoptile node (×30); and F, control (without a substrate), lack of AChE staining in the mesocotyl (×30). The dark residues indicated by arrowheads show AChE-positive spots of copper sulfide after a cytochemical reaction in tissues. PC, Parenchymatous cells; FL, first leaf; V, vascular bundle running into coleoptile parenchyma; IE, inner epidermis; C, cortical cells; S, stele; E, endodermis; and EP, epidermis.

Neostigmine bromide (35 μM) was used as an inhibitor of AChE. Each experiment was repeated three times.

Histochemical Detection of AChE

Cross-sections (80–100 μm) were made free-hand with a razor from selected maize organs as shown in Figure 1. Reagents for the detection of AChE were prepared by the modified methods of Koelle and Friedenwald (1949) and Koelle (1955). The preparation of the reagents and the enzyme reaction using AThCh iodide as a substrate of AChE was carried out by the same method as described in a previous paper (Momonoki and Momonoki, 1993). Diisopropylfluorophosphate was used as an inhibitor of the nonspecific esterases (Koelle and Friedenwald, 1949) because ACh is hydrolyzed by both a specific cholinesterase (AChE) and nonspecific cholinesterases. The control sections were used without diisopropylfluorophosphate for pretreatment and incubated in a medium without AThCh, to which distilled water was added instead of the substrate.

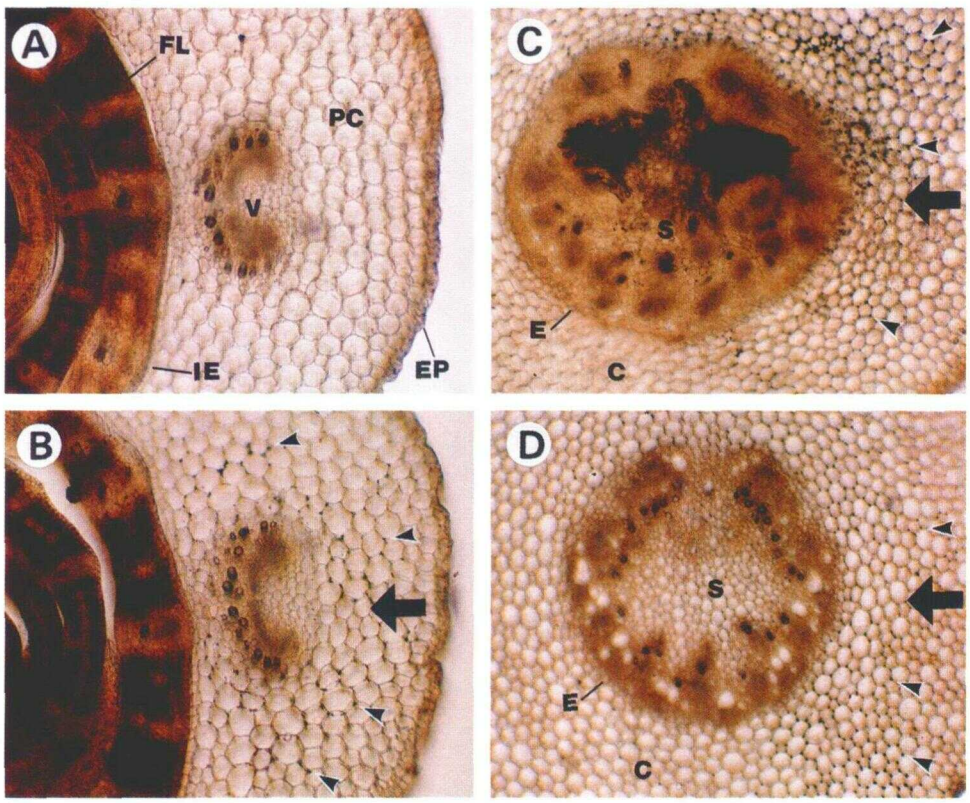

Figure 3. Asymmetric distribution of AChE in selected organ tissues of gravistimulated maize seedlings. A, Lack of AChE staining in the upper half of the cortex cells around the vascular bundles in the coleoptile (×90); B, AChE-positive spots in the lower half of cortex cells around the vascular system in the coleoptile (×90); C, asymmetric distribution of AChE-positive spots in the lower half of cortex cells around the coleoptile node (×52.5); and D, asymmetric distribution of AChE-positive spots in the lower half of cortex cells in mesocotyl (×52.5). The dark residues indicated by arrowheads show AChE-positive spots of copper sulfide after cytochemical reaction in tissues. The arrows show the direction of the lower side of the cortical cells exposed to the gravistimulus. PC, Parenchymatous cells; FL, first leaf; V, vascular bundle running into coleoptile parenchyma; IE, inner epidermis; C, cortical cells; S, stele; E, endodermis; and EP, epidermis.

AChE in plant cells was then visualized with a light microscope (Optiphot-2, photo system UFX-DX, Fujicolor film ISO400, Nikon, Tokyo, Japan). About 10 plants were used in the experiment for each organ.

RESULTS

The AChE activity in nongravistimulated selected organs is shown in Table I. The AChE activity in the coleoptile node was 4- to 10-fold higher than in the coleoptile and mesocotyl. Following the gravistimulus, ACh activity in all of the selected organs showed an asymmetric distribution. The AChE activity in the coleoptile, the coleoptile node, and the mesocotyl was greatest in the lower half of the gravistimulated seedlings. In the lower half of the gravistimulated organs, the AChE activity was 16% higher in the coleoptile, 6% higher in the coleoptile node, and 30% higher in the mesocotyl than that in the corresponding upper half of the tissues. The observed AChE activity was inhibited 80 to 90% by neostigmine bromide.

Histochemical test results for AChE in nongravity-stimulated seedlings are shown in Figure 2. The AChE activity in the coleoptile and coleoptile node was found around the vascular bundles (Fig. 2A) and around the vascular system with a strong reaction (Fig. 2B). No positive reaction for AChE was found in the mesocotyl (Fig. 2C). After a gravistimulus, localization of AChE in the coleoptile, the coleoptile node, and the mesocotyl was obviously asymmetrically distributed (Fig. 3). The asymmetric, positive AChE spots were found in the lower half of the cortex cells around the vascular bundle in the coleoptile (Fig. 3B), in the lower half of the cortex cells around the vascular system in the coleoptile node (Fig. 3C), and in the lower half of the cortex cells around the vascular system in the mesocotyl (Fig. 3D). When neostigmine bromide as an AChE inhibitor was applied to corn kernels, the seedlings did not show a gravitropical curvature (Fig. 4). The histochemical reaction was also inhibited by neostigmine bromide (Fig. 5). The positive AChE spots in the coleoptile (Fig. 3B), the coleoptile node (Fig. 3C), and the mesocotyl (Fig. 3D) were not asymmetrically distributed without a gravity stimulus.

DISCUSSION

When a plant is moved from a vertical orientation to a horizontal position, both free and ester IAA become asymmetrically distributed within 3 min following the stimulus

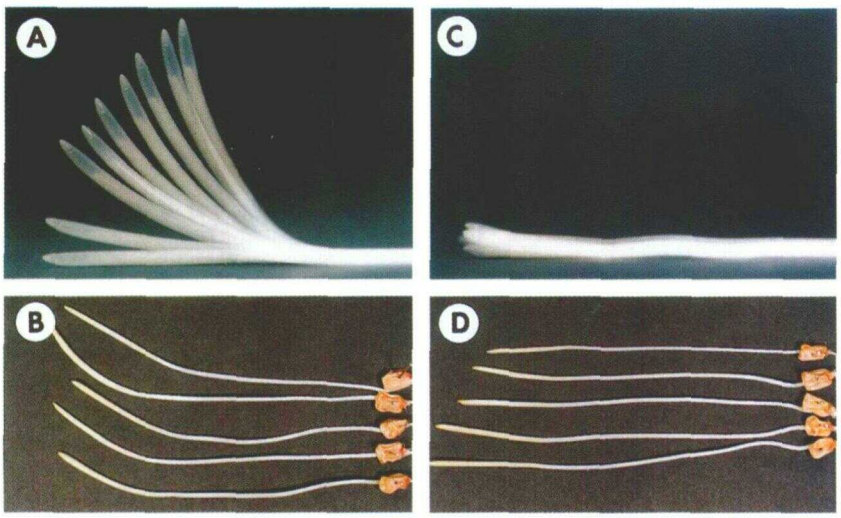

Figure 4. Effect of neostigmine bromide on gravitropic curvature in 5-d-old dark-grown maize seedlings. A, Time-lapse photograph of a maize seedling during gravitropic curvature; B, gravitropic curvature in gravistimulated seedlings; C, time-lapse photograph of inhibited gravitropic curvature of a maize seedling treated by neostigmine bromide as an inhibitor of AChE; and D, inhibited gravitropic curvature of maize seedlings by neostigmine bromide. Neostigmine bromide (35 μM) was injected into the kernels of 20 seedlings and kernels were pinned to a Styrofoam sheet covered with a moist paper towel. As a control, kernels of 20 seedlings were pinned to a Styrofoam sheet and were noninhibited and gravistimulated. All seedlings were incubated for 90 min in a vertical position, and the Styrofoam sheets were moved to a horizontal position for the gravity stimulus for 90 min. After the treatment, representative seedlings from each treatment were photographed. Successive photographs were taken at 15-min intervals. The initial photographs were taken just as the seedlings were placed horizontally.

with more of both free and ester IAA on the lower side of the gravity-stimulated shoot (Bandurski et al., 1984, 1990a). Thus, Bandurski et al. (1990a) proposed the potential-gating theory as a possible mechanism for asymmetric hormone distribution following a stimulus.

Momonoki (1992) proposed a more detailed working hypothesis in an attempt to explain the asymmetric distribution of not only IAA but also positive and neutral compounds. The AChE activity that was measured both by the appearance of SH following the hydrolysis of AThCh and by the appearance of radioactive acetate following the hydrolysis of [$^{1-14}$C]ACh occurred at the interface between the stele and the cortex. Following a gravity stimulus, the radioactive acetate derived by hydrolysis of acetate-labeled ACh was distributed asymmetrically, with about 60% of the label, in the cortex of the lower half, a horizontally placed stem, and about 40% in the upper cortical tissues (Momonoki, 1992). For these experiments the excised tissue pieces were incubated in solutions of radiolabeled ACh and, thus, it was primarily the activity of the enzyme at the tissue surface that was being measured. These results, together with the histochemical tests, suggest that the AChE activity that is present at the stele-cortex interface could play a role in the regulation of transport between the stele and the cortex. In the present work AChE activity measured by AThCh was distributed asymmetrically after a gravity stimulus. The AChE activity was 16% higher in the lower half of the coleoptile, 6% higher in the coleoptile node, and 30% higher in the mesocotyl than that of the upper side of the organs. This activity is inhibited by neostigmine bromide, an inhibitor of AChE. Using histochemical detection, we found positive AChE spots only in the lower half of tissues of the coleoptile, the coleoptile node, and the mesocotyl of gravistimulated seedlings. Furthermore, when neostigmine was applied to the corn kernels, the maize seedlings showed no curvature following a gravistimulus. We conclude from these results that AChE is involved in the tropic response of plants and could act to control the gates between the cortex and the stele.

By a further analogy to the animal ACh-ACh receptor system, there would be a release of Ca^{2+} at the site of the ACh-binding site, which, in the case of a plant, would be the plasmodesmatal cell-cell interface. In previous studies Ca^{2+} was located by means of a fluorescent indicator and confocal microscopy and was found to occur in the cortex cells surrounding the vascular system, in the epidermis, and in adhering peripheral cortical cells. There was increased AChE and Ca^{2+} in the endodermal cells between the stele and the cortex following heat stress (Momonoki et al., 1996).

A working hypothesis that ties all of these observations together (Momonoki et al., 1996) is as follows: (a) ACh occurs at the interface between the stele and the cortex in maize seedlings (Momonoki, 1992); (b) environmental stimuli cause a change in membrane potential (Schrank, 1947; Rolin, 1990); (c) the stele cell on the inside of the endodermal wall would act like a presynaptic cell and release Ca^{2+} (Momonoki et al., 1996); (d) the release of Ca^{2+} would trigger the release of ACh from the vesicles near the terminus of the presynaptic cell; (e) the released

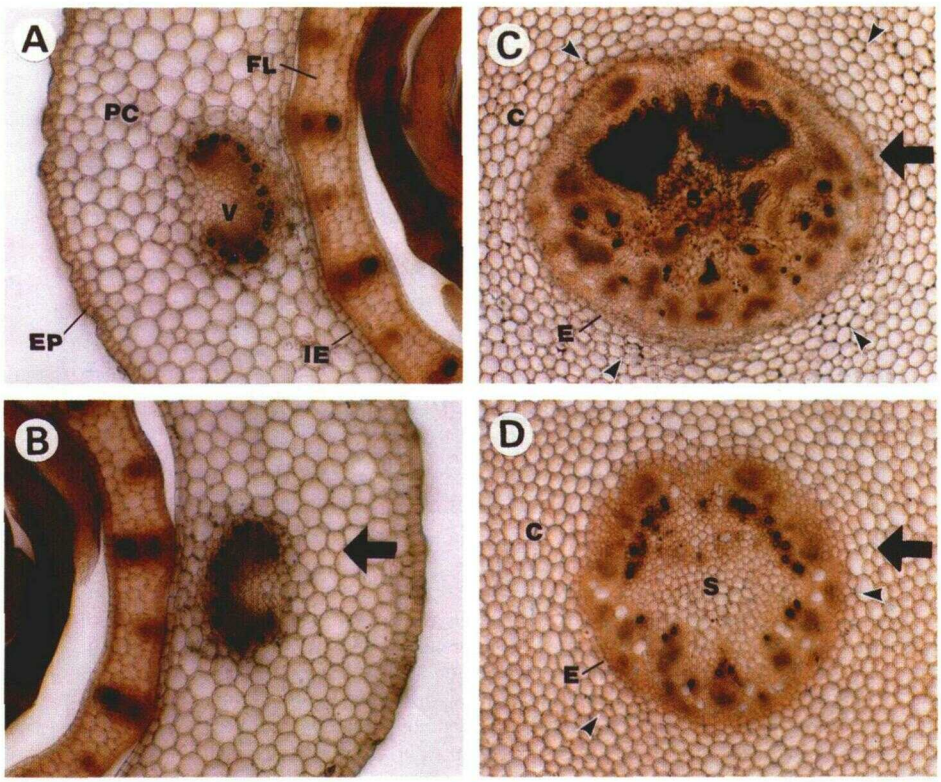

Figure 5. Effect of neostigmine bromide on AChE activity in gravistimulated maize seedlings. A, Lack of AChE staining in the upper half of the coleoptile (×90); B, lack of AChE staining inhibited by neostigmine bromide in the lower half of the coleoptile (×90); C, weak and scattered AChE-positive spots inhibited by neostigmine bromide in the coleoptile node (×52.5); and D, weak and scattered AChE-positive spots inhibited by neostigmine bromide in the mesocotyl (×52.5). The dark residues indicated by arrowheads show AChE-positive spots of copper sulfide after a cytochemical reaction in tissues. The arrows show the direction of the lower side of the cortical cells exposed to the gravistimulus. PC, Parenchymatous cells; FL, first leaf; V, vascular bundle running into coleoptile parenchyma; IE, inner epidermis; C, cortical cells; S, stele; E, endodermis; and EP, epidermis.

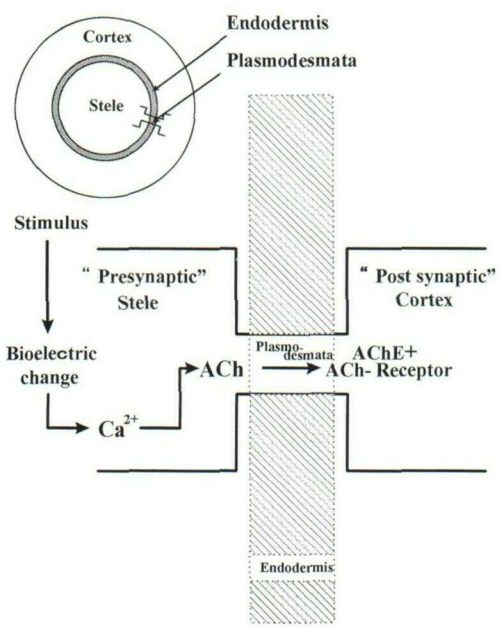

Figure 6. Diagram of a working hypothesis proposing a role for the ACh-ACh receptor system in regulating conductance between the stele and the cortex of the mesocotyl of maize seedlings.

ACh would diffuse to the junction between the stele and the cortex on the outside of the endodermis, analogous to the post-synaptic cell, and bind to an ACh receptor; (f) the propagated potential would open damper-like gates in the plasmodesmatal channel; and (g) ACh would be released from its binding site and hydrolyzed to choline plus acetate, thus bringing the system back to ground state. We have summarized our working hypothesis in Figure 6.

A working hypothesis as elaborate as ours can only serve as a guide to further work. However, the data in this paper do establish that an asymmetric distribution of AChE activity occurs in gravistimulated maize seedlings, as detected by histochemical and colorimetric test system. Thus, AChE could act as a regulator of substances moving in the plasmodesmatal channels.

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