Plant Physiol. (1992) 99, 130–133
0032-0889/92/99/0130/04/$01.00/0

Received for publication September 19, 1991
Accepted December 9, 1991

Occurrence of Acetylcholine-Hydrolyzing Activity at the Stele-Cortex Interface[1]

Yoshie S. Momonoki[2]

Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan 48824–1312

ABSTRACT

Acetylcholine (ACh) is a chemical transmitter serving to propagate an electrical perturbation across the synaptic junctions of animals. ACh and AChE have previously been demonstrated to occur in plants. In this work, we detected AChE at the interface between stele and cortex of the mesocotyl of *Zea mays* by measuring hydrolysis of acetylthiocholine and by liberation of labeled acetate from [1-^{14}C]ACh. AChE activity was also detected in a crude membrane fraction. The hydrolytic activity is inhibited by neostigmine. Hydrolysis of ACh was also measured after injection of [1-^{14}C]ACh into kernels of *Zea mays* and the radioactivity transported into the mesocotyl cortex. A gravity stimulus was then given by placing the plants in a horizontal position. Significantly more radioactivity was found in the lower cortex of horizontally placed seedlings. A working hypothesis is presented for the involvement of ACh and AChE in the tropic response of *Z. mays* seedlings.

The sequence of events in the response of a plant seedling to an environmental stimulus, such as light or gravity, is: (a) membrane depolarization that occurs in as short a time as 8 s following the stimulus (6, 16, 24, 26, 27); (b) an asymmetric distribution of IAA occurring in <3 min (4, 5); (c) an asymmetric distribution of endogenous calcium in 5 to 10 min (13, 25); followed, (d) within an 1 h or less by asymmetric distribution of ^{42}K, ^{32}P (13), and [^{14}C]glucose (21). We wish to determine how membrane depolarization can lead to asymmetric distribution of a chemical in a plant stem. For a dark-grown seedling grown in water, all solutes, salts, sugars, and hormones included, originate in the seed and move from seed to shoot through the vascular stele (9). Thus, to obtain asymmetric distribution of a substance in the cortical tissues, a selective release of solute from stele to cortex must occur. Bandurski *et al.* (2, 3) postulated a selective release of solutes from stele to cortex by gating of solute movement out of the stele and into the cortex. We rephrase the question of how membrane depolarization causes a chemical asymmetry to how is gating of solute movement from stele to cortex accomplished?

A possibly analogous mechanism may occur in the animal neural axon, where a voltage change induces ligand release. The ligand, in turn, causes a flow of ions such as Ca^{2+}, K^+, and Na^+. The ACh[3]-receptor-esterase system serves to bridge the 15- to 100-nm cleft between the afferent (pre) and efferent (post) synaptic junction of the neural system. Plants, although lacking a nervous system, may require a mechanism for transmission of an electrical stimulus from cell to cell through a cleft in the plasmodesmatal junction. The ACh-receptor-esterase system could function to chemically transmit a stimulus across the plasmodesmatal junctions between plant cells and so propagate membrane depolarization. To function in this manner, the system would require the presence of AChE at the plasmodesmatal cell-cell interface.

Both ACh and AChE have been demonstrated to be widely distributed in plants (10–12, 14, 18, 22, 28).

The objective of this research was to determine whether AChE could function as a regulator of the plasmodesmatal junction between stele and cortex, activating gates to open on one side of the stem and close on the other. To test this possibility, we searched for, and found, AChE at the interface between stele and cortex. This finding, together with the earlier works of Fluck and Jaffe (12) concerning the localization of AChE in the area between the cell wall and cell membrane, makes the AChE system a candidate for a regulator of stele to cortex transport. We assayed AChE by means of two substrates and determined the sensitivity of the activity to known inhibitors of AChE. Finally, we discuss how this enzyme could function in the gravity response of *Zea mays*.

MATERIALS AND METHODS

Plant Material

Kernels of maize (*Zea mays* L. cv Stowell's Silver Queen sweet corn, W.A. Burpee Co., Clinton, IA) were soaked for 24 h in running water and germinated in rolled moist paper towels for 4 d in a dark room at 25°C. For extraction of AChE from membranes, 3-d-old corn seedlings were used.

Organ Harvesting

For harvesting of the cortex and stele, the upper portion of 4-d-old shoots was severed at a point 4 mm below the coleoptilar node, and that portion was discarded. The mesocotyl was then nicked, but not cut through, at a point about 14 mm above the junction between shoot and root. The cortex was then slid off the stele, and the 10 mm of cortex and stele

[1] Supported by grant DCB 8805148 from the Cellular Biochemistry program of the National Science Foundation to Robert S. Bandurski.

[2] On leave from Faculty of Bioindustry, Tokyo University of Agriculture, Hokkaido, Japan.

[3] Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; SH, sulfhydryl.

closest to the tip was harvested (23). For the gravistimulus experiment, the 10 mm of 30 cortices were separated into upper and lower halves. The harvested organs were dropped into beakers in an ice bath. Ten to 30 plants were used for most experiments, and about 50 g of shoots was used for extraction of AChE from cell membranes.

Assay of AChE Activity by SH Appearance

This assay was based on the colorimetric SH reagent, 5,5′-dithiobis-2-nitrobenzoic acid, as used by Asashi *et al.* (1) and Ellman *et al.* (8). Harvested cortex and stele were incubated for 0, 15, 30, and 60 min in a vial containing 500 μL of sodium phosphate buffer (pH 7.5) and 500 μL of acetylthiocholine chloride (10 mM in buffer) as substrate. After incubation, 200 μL of solution was transferred to a vial, and 950 μL of phosphate buffer and 50 μM 5,5′-dithiobis(2-nitrobenzoic acid) were added. The resultant yellow color was read at A_{412nm} after 1 min using a molar extinction coefficient of 13,600 and an identical tube without organs as a control.

Determination of Radioactivity Derived from [1-^{14}C]ACh by Enzymic Hydrolysis

This assay for enzyme activity was based on the separation on a DEAE-Sephadex (acetate) column of the negatively charged labeled acetic acid from the starting substrate [1-^{14}C] ACh chloride using the general procedure of Leznicki and Bandurski (19). The reaction mixture included 10 mm of each of 20 steles, 1 mL of phosphate buffer (pH 7.3), 1 μL of 9000 dpm [1-^{14}C]ACh in 50% 2-propanol (specific activity, 55.5 mCi mmol^{-1}) and with or without neostigmine as an inhibitor of AChE. Incubation was for 0.5, 2, 4 and 6 h at room temperature. To stop the reaction, 3 mL of 2-propanol was added to the reaction mixture. Next, the organs were ground with liquid N_2, and the mixture was centrifuged for 5 min at 1300*g*. The supernatant fluid was chromatographed on DEAE-Sephadex (acetate). The column was washed with 10 mL of 50% ethanol, and the radioactive acetate was eluted with 5 mL of 5% acetic acid in 50% ethanol. The radioactive solution (1 mL) was counted. Neostigmine bromide (25 and 50 μM) was used as an inhibitor of AChE following the method of Fluck and Jaffe (11). A reaction mixture without steles was used for a control.

Extraction of AChE from Cell Membranes of Shoot Organs

Fifty grams of 3-d-old seedlings were made turgid by incubation for 1 h in 150 mL of ice-cold 0.01 M phosphate buffer. The tissues were then homogenized in a Waring blender with 25 g of glass beads (66–160 μm). The homogenate was filtered through two layers of cheesecloth, and the filtrate was filtered through Miracloth. The residue, containing cell walls together with adhering membranes, was suspended in 0.1% SDS for 1 h for dissociation of membrane from cell wall. Enzyme denaturation may have occurred, but SDS was believed to be helpful in dissociating membranes from the cell wall. The supernatant suspension was used for determination of AChE activity by the methods described above. Neostigmine bromide (35 μM) was again used as an inhibitor of AChE.

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}). [1-^{14}C]ACh (27,000 dpm; 3 μL) was injected into each of 30 corn kernels. The kernels were next pinned to a moistened paper-covered Styrofoam sheet and incubated with the shoots in a vertical position for 2 h. Next, the Styrofoam sheet was rotated so as to move the shoots to a horizontal position for a 90-min gravistimulus. After the gravistimulus, 1 cm of cortices were sectioned into upper and lower halves and harvested. The enzyme assay using radiolabeled ACh was used as described above.

RESULTS

AChE activity on the surface of the dissected cortex and stele of seedlings is illustrated by the data in Table I. AChE activity in the cortex increased as a sigmoidal function of time. The maximum increase of AChE activity of 47 pmol SH mL^{-1} of tissue was observed at 15 and 30 min incubation time. AChE activity in the stele also increased as a function of incubation time. A maximum increase of AChE activity of 38 pmol SH mL^{-1} of organ extract occurred between 0 and 15 min following the stimulus.

The results of a subsequent study using labeled ACh as substrate and neostigmine as an inhibitor of the activity in steles is shown in Figure 1. The radioactivity derived from hydrolysis of [1-^{14}C]ACh was about 2000 dpm h^{-1} in stele tissues. The hydrolysis was inhibited 80 to 90% by 25 and 50 μM neostigmine bromide. Table II demonstrates AChE activity in cell membranes prepared from shoot tissues. The reaction was a nearly linear function of time, and the activity was inhibited 77 to 82% by 35 μM neostigmine bromide.

The distribution of hydrolytic activity in cortex and stele after a gravistimulus is shown in Table III. The radioactivity derived from hydrolysis of [1-^{14}C]ACh was found in both cortex and stele but was distributed asymmetrically in the cortex with 58% of the activity found on the lower side and 42% on the upper side after gravistimulus.

Table I. *AChE Activity in Cortex and Stele of Z. mays Seedlings as Measured by SH Appearance following Hydrolysis of Acetylthiocholine*

Mean of three experiments ± SE. Ten-millimeter pieces of 10 corticies or 20 steles of 4-d-old etiolated seedlings were used for each experiment. Control, Reaction mixture including buffer but lacking tissue.

Treatment	Incubation Time (min)			
	0	15	30	60
	pmol SH·ml^{-1} of tissue extract			
Cortex	70 ± 4	97 ± 10	149 ± 2	154 ± 3
Control	72 ± 5.5	79 ± 1	84 ± 1.5	101 ± 1
Stele	75 ± 2	116 ± 6	143 ± 2	155 ± 4
Control	75 ± 3	78 ± 1	82 ± 1	97 ± 1

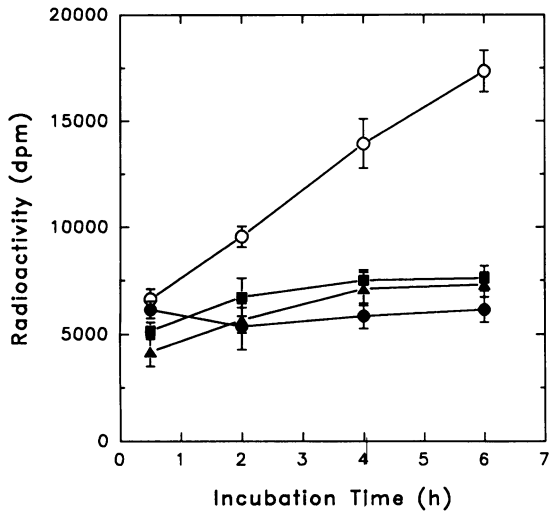

Figure 1. The radioactivity derived from [1-^{14}C]ACh by enzymic hydrolysis and effect of specific inhibitor of AChE on AChE activity in the steles of *Z. mays*. ○, With steles; ●, control (without stele); △, steles in 25 μM neostigminebromide; ■, steles in 50 μM neostigmine bromide. Each point is the mean of three experiments (vertical bars, ± SE).

DISCUSSION

ACh is a well-studied chemical transmitter in the synaptic junction causing opening of cation-selective channels in nerve synapses of animals (7, 15, 17, 20). ACh has been detected in a number of higher plants (10, 14, 18, 22, 28). Fluck and Jaffe (11) demonstrated AChE in 23 species from the following five families: *Characeae*, *Cruciferae*, *Graminae*, *Leguminosae*, and *Solanaceae*. They also demonstrated that the hydrolytic activity was inhibited by neostigmine, a recognized inhibitor of AChE.

The present data are insufficient to conclude that ACh and AChE are, or are not, involved in the tropic response of plants. Nonetheless, the occurrence of ACh and AChE in plants together with its localization at the cell wall-membrane interface (12) is suggestive of some function. In the present work, we demonstrated that AChE activity occurs at the interface between stele and cortex. AChE activity was measured both by SH appearance following the hydrolysis of acetylthiocholine and by the appearance of radioactive acetate following the hydrolysis of [1-^{14}C)ACh. These results indicate that the hydrolytic ability occurs at the interface between stele and cortex and that this activity is inhibited 80 to 90% by neostigmine bromide. We conclude that AChE activity is present at the stele cortex interface and could, therefore, play a role in the regulation of transport between stele and cortex.

Additionally, the AChE activity could be demonstrated in crude membrane preparations from whole tissue, and this activity was also inhibited by neostigmine bromide. Thus, AChE activity can be demonstrated both at the stele-cortex interface and in membranes prepared from whole tissue.

In a further attempt to test for a possible role of AChE in transport regulation following a stimulus, radioactive ACh was injected into kernels of corn while the seedling was held in a vertical position. A gravity stimulus was then given, and the distribution of radioactive acetate derived by hydrolysis of acetate-labeled ACh was determined. The radioactivity was found to be asymmetrically distributed with 58% of the label

Table II. *AChE Activity Measured by SH Appearance from Acetylthiocholine Hydrolysis in Cell Membranes Extracted from Shoots of Young Z. mays Seedlings*

Mean for three experiments ± SE. The cell membrane was extracted from 50 g of whole shoots of 3-d-old *Z. mays* seedlings. Control, Reaction mixture including buffer lacking membrane preparation. Inhibitor is 35 μM neostigmine.

Treatment	Incubation Time (min)				
	0	5	10	15	20
	pmol SH·g^{-1} fresh wt of tissue				
Control	122 ± 2	126 ± 2	129 ± 2	134 ± 2	139 ± 3
Membrane extract	136 ± 4	144 ± 3	159 ± 1	169 ± 3	183 ± 4
Inhibitor	127 ± 3	130 ± 2	136 ± 3	142 ± 3	147 ± 2.5

Table III. *Radioactivity Derived from Enzymatic Hydrolysis of [1-^{14}C]ACh at the Surface of the Cortex and Stele of Z. mays Seedlings after Gravistimulus*

	Radioactivity[a]	Ratio of Radioactivity
	dpm·30 half corticies^{-1}	%
Upper cortex	2143 ± 11	42 ± 0.5
Lower cortex	2907 ± 44[b]	58 ± 0.5
	dpm·30 steles^{-1}	
Stele	1340 ± 95	

[a] Mean for three experiments ± SE. The 4-d-old etiolated seedlings were held vertically for 2 h after injection of [1-^{14}C]ACh into the kernels and placed horizontally for 90 min for gravestimulus. Then, the 30 corticies were separated into upper and lower halves and harvested.

[b] $t = 16.8$ for 4 degrees of freedom. $P < 0.001$.

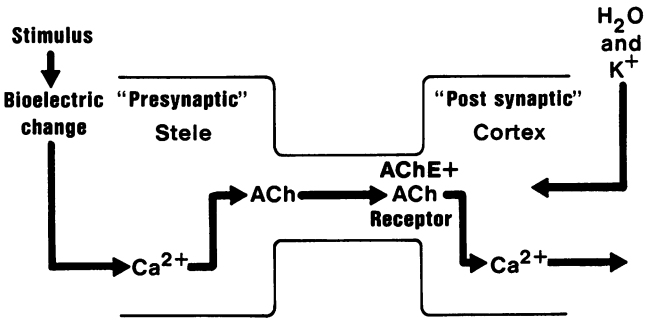

Figure 2. Diagram of a working hypothesis for a role of the ACh system at the interface between stele and cortex of *Z. mays* seedlings. The sequence of events would be exactly as is known for the propagation of an electrical system across the neural cleft of an animal neural synapse. Histochemical localization of AChE should determine whether the enzyme is intra- or intercellular.

found in the cortex of the lower half of a horizontally placed stem and 42% in the upper cortical tissues. Because AChE cannot move, an altered transport of ACh or altered AChE activity must have occurred.

An expanded working hypothesis, providing a more detailed mechanism for the voltage-gating theory, would be as follows: ACh occurs at the interface between stele and cortex in *Z. mays* seedlings. Environmental stimuli cause a bioelectric change, *i.e.* an action potential at the terminus of the stele cell. The stele cell on the inside of the endodermal wall would act like a presynaptic cell and release Ca^{2+}. The released Ca^{2+} would trigger release of ACh from vesicles near the terminus of the presynaptic cell. The released ACh would diffuse to the junction between stele and cortex on the outside of the endodermis, the postsynaptic cell, and there bind to an ACh receptor. The propagated action potential would open damper-like gates in the plasmodesmatal channel. Finally, the ACh would be released from its binding site and hydrolyzed to choline plus acetate and the system thus brought back to ground state. We have summarized our working hypothesis in Figure 2.

LITERATURE CITED

1. **Asashi T, Bandurski RS, Wilson LG** (1961) Yeast sulfate-reducing system. II. Enzymatic reduction of protein disufide. J Biol Chem **236:** 1830–1835
2. **Bandurski RS, Schulze A, Desroisiers M, Jensen P, Epel B, Reinecke D** (1990) Relationship between stimuli, IAA and growth. *In* R Pharis, S Rood (eds). Plant Growth Substances, Springer-Verlag, Heidelberg, Germany
3. **Bandurski RS, Schulze A, Desroisiers M, Jensen P, Reinecke D, Epel E** (1990) Voltage-gated channels as transducers of environmental stimuli. *In* DJ Morre, WF Boss, FA Loewus, eds, Inositol Metabolism in Plants. Wiley-Liss, Inc, New York, pp 289–300
4. **Bandurski RS, Schulze A, Domagalski W** (1986) Possible effects of organelle charge and density on cell metabolism. Adv Space Res **6:** 47–54
5. **Bandurski RS, Schulze A, Reinecke DM** (1986) Biosynthetic and metabolic aspects of auxins. *In* M Bopp, ed, Plant Growth Substances, 1985. Spring-Verlag, Heidelberg, Germany, pp 83–91
6. **Behrens HM, Gradmann D, Sievers A** (1985) Membrane potential responses following gravistimulation in roots of *Lepidium sativum* L. Planta **163:** 463–472
7. **Dunant Y, Babel-Guerin E, Droz D** (1980) Calcium metabolism and acetylcholine release at the nerve-electroplaque junction. J Physiol **76:** 471–478
8. **Ellman GL, Courtney KD, Andres V Jr, Featherstone RM** (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol **7:** 88–95
9. **Epel BL, Bandurski RS** (1990) Tissue to tissue symplastic communication in the shoots of etiolated corn seedlings. Physiol Plant **79:** 604–609
10. **Evans ML** (1972) Promotion of cell elongation in *Avena* coleoptiles by acetylcholine. Plant Physiol **50:** 414–416
11. **Fluck RA, Jaffe MJ** (1974) The distribution of cholinesterases in plant species. Phytochemistry **13:** 2475–2480
12. **Fluck RA, Jaffe MJ** (1974) Cholinesterase from plant tissues. III. Distribution and subcellular localization in *Phaseolus aureus* Roxb. Plant Physiol **53:** 752–758
13. **Goswami KKA, Audus LJ** (1976) Distribution of calcium, potassium and phosphorus in *Helianthus annus* hypocotyls and *Zea mays* coleoptiles in relation to tropic stimuli and curvatures. Ann Bot **40:** 49–64
14. **Hartmann E, Kilbinger H** (1974) Occurrence of light-dependent acetylcholine concentration in higher plants. Experientia **30:** 1387–1388
15. **Jacobson GR** (1983) Neurotransmitter. *In* G Zubay, ed, Biochemistry. Addison-Wesley Publishing Co Inc, MA, pp 1147–1167
16. **Jaffe MJ** (1968) Phytochrome-mediated bioelectric potentials in mung bean seedlings. Science **162:** 1016–1017
17. **Kelly RB, Deutsch JW, Carlson SS, Wagner JA** (1979) Biochemistry of neurotransmitter release. Annu Rev Neurosci **2:** 399–466
18. **Lees GL, Lahue R, Thompson JE** (1978) Changes in the acetylcholine titre of senescing cotyledons. J Exp Bot **29:** 1117–1124
19. **Leznicki AJ, Bandurski RS** (1988) Enzymic synthesis of indole-3-acetyl-*O*-β-D-glucose. I. Partial purification and characterization of the enzyme from *Zea mays*. Plant Physiol **88:** 1474–1480
20. **MacIntosh FC** (1981) Acetylcholine. *In* GJ Siegel, RW Albers, BW Agranoff, R Katzman, eds, Basic Neurochemistry. Little Brown and Co, Boston, MA, pp 183–204
21. **Momonoki YS** (1988) Asymmetric distribution of glucose and indole-3-*myo*-inositol in geostimulated *Zea mays* seedlings. Plant Physiol **87:** 751–756
22. **Momonoki YS, Momonoki T** (1991) Changes in acetylcholine levels following leaf wilting and leaf recovery by heat stress in plant cultivars. Jpn J Crop Sci **60:** 283–290
23. **Pengelly WL, Hall PJ, Schulze A, Bandurski RS** (1982) Distribution of free and ester indole-3-acetic acid in the cortex and stele of the *Zea mays* mesocotyl. Plant Physiol **69:** 1304–1307
24. **Schrank AR** (1947) Analysis of the effects of gravity on the electric correlation field in the coleoptile of *Avena sativa*. *In* EJ Lund, HF Rosane, eds, Bioelectric Fields and Growth. University of Texas Press, Austin, TX, pp 75–121
25. **Slocum RD, Roux SJ** (1987) Cellular and subcellular localization of calcium in gravistimulated oat coleoptiles and its possible significance in the establishiment of tropic curvatures. Planta **157:** 481–492
26. **Tanada T** (1983) Photogeneration of a bioelectric field by red and far-red irradiation in soybean hypocotyls. Plant Cell Environ **6:** 69–72
27. **Tanada T, Vinten-Johansen C** (1980) Gravity induces fast electrical field changes in soybean hypocotyls. Plant Cell Environ **3:** 127–130
28. **Verbeek M, Vendrig JC** (1977) Are acetylcholine-like cotyledon-factors involved in the growth of the cucumber hypocotyl? Z Pflanzenphysiol **83:** 335–340