Plant Physiol. (1972) 50, 523-525

Short Communication

Phytochrome-controlled Nyctinasty in *Albizzia julibrissin*

V. EVIDENCE AGAINST ACETYLCHOLINE PARTICIPATION[1]

Received for publication March 10, 1972

RUTH L. SATTER, PHILIP B. APPLEWHITE, AND ARTHUR W. GALSTON
Department of Biology, Yale University, New Haven, Connecticut 06520

We have reported that phytochrome-controlled nyctinastic leaflet movement in *Albizzia julibrissin* is accompanied and probably caused by K flux in pulvinule motor cells (16, 18); however, the metabolic events linking photon absorption by phytochrome to K flux are still unknown. Jaffe (11) reported that R[2] irradiation increased and subsequent FR decreased the titer of ACh in etiolated mung bean root tips, and proposed a general theory of phytochrome action based on ACh as a regulator of bioelectric potential and ion flow in plants. He suggested that changes in the ACh titer, following soon after photon absorption by phytochrome, couple it to both rapid behavioral changes and photomorphogenesis (12). Hartmann (10) has also found ACh levels to be mediated by phytochrome in moss callus. ACh is photomimetic in inducing sporulation in the fungus *Trichoderma* (8) but not in promoting anthocyanin synthesis in mustard seedlings (13).

These reports prompted us to investigate a possible role for ACh, not only in phytochrome-controlled nyctinastic leaflet movement in *Albizzia* (16, 18) but also in rhythmic movements, since they are also accompanied by K flux (16, 17), and rhythmic changes in ACh have been reported in animals (9). In addition, we assayed ACh in several other plants with moving leaves or with dramatic photomorphogenic behavior.

MATERIALS AND METHODS

Six- to 9-month-old *Albizzia julibrissin, Samanea saman,* and *Mimosa pudica* were grown from seed under conditions previously described (18). Beans (*Phaseolus multiflorus* and *Phaseolus vulgaris* var. Astro, Black Valentine, and Tenderhook) and light-grown peas (*Pisum sativum,* var. Alaska) were grown from seed in vermiculite in growth chambers (18) and were 2 to 3 weeks old when assayed for ACh. Peas were also grown in the dark at 27 C, 80% relative humidity. Light sources for R (4 min) and FR (1.5 min) irradiation were as previously described (18).

Extracts for ACh assay were prepared as follows. Tissue was excised and quickly frozen in ground glass homogenizing tubes kept in an acetone Dry Ice bath. Five min to 3 hr later, 1 to 5 ml of boiling sea water (pH 5.5) was added to the sample tubes, which were then immersed in a boiling bath for 10 min. The boiled tissue was homogenized in a Potter-Elvehjem tissue grinder, then centrifuged at 1200*g* for 10 min. The soluble fraction was refrigerated if assayed within a few hours or frozen if overnight storage was necessary.

ACh was assayed by the method of Florey (4), also used by Jaffe (11). The excised heart of a clam, *Mercenaria mercenaria,* was placed in a perfusion chamber containing 5 ml of aerated sea water, with one auricle tied to a stationary post and the other to a strain gauge electronically coupled to the writing lever of a chart recorder. We monitored the amplitude and frequency of the contractions, which were constant in the absence of addendum but were reduced upon addition of ACh, and compared these changes with those produced by a known quantity of standard. Differences in comparative treatments were not considered significant unless they exceeded 20%, since the sensitivity of the heart changed this much in some experiments. Acetyl cholinesterase used in pharmacological tests was type I, prepared from bovine erythrocytes (Sigma).

RESULTS

Specificity of the Bioassay for ACh in *Albizzia*. Addition of the extract from 5 mg of pinna tissue to the sea water in the perfusion chamber caused the same reduction in amplitude as did 1.5 ng standard ACh. Thus an *Albizzia* pinna contains about 300 ng ACh/g fresh wt, more than twice the concentration reported (11) for etiolated mung bean roots.

Since several other compounds in addition to ACh can inhibit the clam heart beat, we performed a series of pharmacological tests (4) each done in triplicate to determine whether ACh was the active substance in our extracts. (*a*) Pinna extracts with or without acetyl cholinesterase (15 μg/ml) were incubated at 37 C for 30 min (4). The enzyme, which hydrolyzes ACh, reduced the activity of the extract by 80% $\pm$ 3% SD. When standard ACh was similarly incubated with AChase, its activity was reduced 85% $\pm$ 2%. (*b*) The stability of ACh decreases as temperature and pH are increased (2); thus we raised the pH of a pinna extract from 5.5 to 9.2, boiled it and a control for 10 min, then reduced the pH of the test extract back to 5.5. Such treatment reduced ACh activity by 68% $\pm$ 3%. (*c*) Eserine can sensitize the clam heart to ACh by inhibiting native cholinesterase (4). Fifty μg of eserine, added to the bathing solution for 20 min, increased the activity of our extract by 84% $\pm$ 2%. (*d*) Benzoquinonium chloride interferes with the action of ACh (4). Addition of 5 μg to the bathing solution for 20 min reduced the activity of our extracts by 93% $\pm$ 1%. The results of these several tests suggest that most of the activity in our extracts was due to ACh-like compounds.

Next, we separated a pinna into its constituent parts (19) and measured the ACh activity of each organ. The secondary and tertiary pulvinules and the lamina had approximately the same

[1] This investigation was supported by grants to A.W.G. from the Herman Frasch Foundation and the National Science Foundation.

[2] Abbreviations: R: red; FR; far red; ACh: acetylcholine; AChase: acetylcholine esterase; ng: nanogram.

Table I. *Effect of R Irradiation on Leaflet Movement and ACh in the Pulvinule of Darkened Albizzia Leaflets Previously Irradiated with FR*

See text for details.

Experiment	Closure			ACh		
	R	FR	R/FR	R	FR	R/FR
	degree[1]			ng/g fresh wt		
1	65	5	13	310	288	1.1
2	90	40	2.3	—[2]	—[2]	0.8
3	80	20	4	350	330	1.1

[1] The angles of leaflets from each pinna were measured 2 hr after R irradiation. Values are averages for six pinnae.

[2] Absolute calibration was not made in this experiment; therefor only comparative data are presented.

ACh titer (325 ng/g fresh weight), while the rachilla had considerably less (100 ng/g fresh weight). Seed imbibed in the light for 3 hr contained >1 μg/g dry wt.

Does Phytochrome Control the ACh Titer in *Albizzia*? Open leaflet pairs excised from an illuminated plant will close completely upon darkening if preirradiated with R, but only partially if preirradiated with FR. K moves into dorsal and out of ventral motor cells during closure (18). K flux into the dorsal cells is 80% complete after 30 min of darkness and is independent of Pfr during this interval (17); by contrast, there is an absolute requirement for R preirradiation for K efflux from ventral motor cells.

One would expect maximum ACh changes in the pulvinus if such changes mediate K flux and leaflet movement, since the phytochrome that influences nyctinastic movement (14), the oscillator controlling endogenously rhythmic movement (17), and the motor cells (19) are all localized in this organ. Thus our most important experiments were with pulvinar tissue, but for the sake of completeness we also included experiments with rachillar and laminar tissue.

Six pairs of open pinnae excised from a plant in the light were placed with their cut ends in water; they were then irradiated with FR and transferred to darkness. Half of the pinnae received no further irradiation and served as controls. After 30 min of darkness, leaflets on all pinnae had assumed angles of 120° and had ceased their movement, indicating completion of phytochrome-independent K flux into the dorsal motor cells. At this time, we irradiated one pinna with R, and then systematically excised its pulvinules simultaneously with those of its paired FR control; the terminal three leaflet pairs were left on the rachilla for leaflet movement data. Dissections were performed under microscopes illuminated with a green safelight. As soon as one pair of pinnae was completed another was irradiated and dissected until we had collected 200 to 240 pulvinules/sample in about 100 min. Excisions were made 0 to 15 min after R irradiation, and pulvinules were frozen within 5 min of excision.

We performed this experiment three times and found no evidence of phytochrome control of ACh in the pulvinule, despite excellent phytochrome control of leaflet movement (Table I). We also performed similar experiments with rachillar and laminar tissue; they too showed no correlation between the state of phytochrome and the ACh titer.

We also tested the effect of exogenous ACh on leaflet movement. Open leaflet pairs taken from a plant in the light were floated on H_2O or ACh (iodide or chloride salts, 0.01 to 1.0 mM), irradiated with R or FR, then darkened. We repeated this experiment five times; in a typical experiment, R treated leaflets closed 78° and FR treated ones 24° during 45 min, but ACh had no effect on the closure of either group.

Tests for Light-Dark and Rhythmic Changes in ACh in *Albizzia*. We performed several other experiments to determine whether there were changes in ACh during the 30-min period following the transition from light to darkness, or during endogenously rhythmic leaflet movement. The light-dark experiments were conducted early in the photoperiod; in the rhythm experiments, *Albizzia* plants were kept in darkness for 12 hr to permit endogenously rhythmic leaflet opening (16, 17), and tissue samples were taken when leaflets were closed (hr 7–8) and during opening (hr 11–12). We performed each experiment two to three times with each tissue (pulvinule, rachilla or lamina), but did not find any significant differences in ACh associated with light-dark transition or rhythmic movement. Ratios of ACh in tertiary pulvini under these comparative conditions were: light/dark, 1.0 ± 0.3; dark closed (0°)/open (120°), 0.8 ± 0.1. Neither did we find changes in the ACh content of the secondary pulvinus when tissue was taken before and after rhythmic pinna movement (Table II).

Other Species. We also assayed ACh in other plants with moving leaves, leaflets, or tendrils. We prepared extracts of each of the following, each taken from plants in the light and in the dark: *Samanea saman* (separate samples of leaflets, secondary, and tertiary pulvini), *Mimosa pudica* (separate samples of pinnae, primary, and tertiary pulvini), four varieties of beans (pulvini from the bases of the lamina and the petiole were pooled), and pea tendrils. We were unable to find ACh activity in any of these extracts using the clam heart assay. The lower limit of detectability in these tests was 5 ng ACh/g fresh weight.

Since our extracts might have contained stimulatory substances that would mask the inhibitory action of ACh, we also performed ACh assays by a fluorometric method (3). Values

Table II. *ACh in the Secondary Pulvinus before and after Rhythmic Pinna Movement*

Experiment	Hr of Darkness	Angle between Pinnae	ACh
		degree	ng/g fresh wt
1	8	80	400
	12	115	380
2	8	65	345
	12	110	370
3	8	90	305
	12	120	355

Table III. *Effect of R Irradiation on Photomorphogenesis and ACh in Etiolated Seedlings of Pisum sativum var. Alaska*

Light Treatment	Terminal Bud		Hook		Internode	
	Gain in fresh wt[1]	ACh[2]	Opening[1]	ACh[2]	Elongation[1]	ACh[2]
	mg	ng/g fresh wt	degree	ng/g fresh wt	mm	ng/g fresh wt
R	13.2	15	135	14	16	3.2
R,FR	9.7		80		35	
Dark	7.1	16	59	18	37	2.8
R/Dark	1.8	0.9 ± 0.2	2.3	0.8 ± 0.2	0.43	1.1 ± 0.2

[1] Measured 27 hr after irradiation. Average of 20 seedlings.

[2] Tissue excised and frozen 0 to 30 min after irradiation. Average ± SD of three experiments, each with tissue from 50 seedlings.

for ACh in *Albizzia* were of the same order of magnitude as obtained with the bioassay, and we did not find any ACh in *Mimosa*.

Test for Possible ACh Changes in Etiolated Peas following R Irradiation. Our failure to find phytochrome-controlled changes in ACh in *Albizzia* or other green tissue led us to test for such changes in etiolated shoots. We used the Alaska pea for these experiments, since its phytochrome-controlled morphogenic changes are dramatic and have been carefully studied and documented (6, 7, 15). The greater succulence of etiolated compared to green tissue enabled us to prepare more concentrated homogenates and extend measurement to 1 ng ACh/g fresh weight.

Brief R irradiation of 6-day-old dark-grown peas promotes bud growth, hook opening, and inhibition of elongation of the third internode; changes potentiated by R are prevented by subsequent FR (6, 7, 15). We repeated these classic photomorphogenic experiments and prepared tissue extracts of replicate seedlings for ACh assay. Thus we irradiated a flat of seedlings with R, selected 50 uniform seedlings with recurved hooks and third internode lengths of 2.5 to 3.5 cm, separated bud, hook, and internode sections, then weighed and froze each group. Dark controls were similarly treated. Excisions were started immediately after R irradiation and were completed 30 min later. The ACh bioassay revealed no significant differences in the ACh content of R irradiated tissue and dark controls, despite the usual photomorphogenic differences in seedlings from these two groups (Table III). We obtained similar results when we reduced the harvest time to 3 min by irradiating only five seedlings at a time, or when we excised buds prior to the R light treatment and froze then immediately after irradiation.

Pharmacological tests identical to those used for *Albizzia* were also used on the pea shoots. Similar conclusions obtained: namely, ACh-like compounds are the main active substances.

DISCUSSION

The following evidence suggests that changes in ACh are not involved in phytochrome-controlled or rhythmic leaflet movement in *Albizzia* or other species tested: (*a*) The ACh content of the pulvinule is the same in the light or darkness, before or after R irradiation, and before or after rhythmic leaflet movement. By contrast, Jaffe (11) reported that R-irradiated mung bean root tips had five times as much ACh as those irradiated with FR. Although our experiments do not indicate whether ACh is redistributed within the pulvinule (*e.g.*, whether its titer increases in motor cells on one side of the pulvinule but decreases in corresponding cells on the other side), such redistribution would appear unlikely on the basis of studies of nerve tissue where ACh is not transported from one cell to another (1). (*b*) Exogenous ACh at 400 times the endogenous concentration has no effect on leaflet movement. Although we do not know whether the ACh we supplied was identical to native compounds or whether it was taken up by *Albizzia*, experiments by others (8, 11) indicate uptake and activity with other plant tissues. (*c*) The ACh titer of the pulvinule does not exceed that of the lamina. By contrast, [K] of the pulvinule is 3.5 times that of the lamina (18). (*d*) We failed to detect ACh in nastic organs of other plants. Leaflet movements in *Samanea* and *Mimosa* resemble those in *Albizzia*, since they are controlled by phytochrome and by an endogenous rhythm (5, 20), and since movement in *Mimosa* is accompanied by K flux (21). Our data argue against Toriyama and Jaffe's (22) recent suggestion that ACh might mediate movement in *Mimosa*.

Finally, our experiments with etiolated peas also weigh against the hypothesis that ACh is a general and rapid mediator in phytochrome-controlled systems, although they do not preclude possible ACh changes several hours after R irradiation when photomorphogenic changes are apparent (7).

Acknowledgment—We thank Dr. Malcolm Bowers for his generous help and use of his equipment for the clam heart bioassay.

LITERATURE CITED

1. Cooper, J. R., F. E. Bloom, and R. H. Roth. 1970. The Biochemical Basis of Neuropharmacology. Oxford University Press, New York.
2. Dawson, R. M. C., D. C. Elliott, W. H. Elliott, and K. M. Jones. 1969. Data for Biochemical Research. Oxford Press, London.
3. Fellman, J. H. 1969. A chemical method for the determination of acetylcholine: its application in a study of presynaptic release and a choline acetyltransferase assay. J. Neurochem. 16: 135–143.
4. Florey, E. 1967. The clam-heart bioassay for acetylcholine. Comp. Biochem. Physiol. 20: 365–377.
5. Fondéville, J. C., H. A. Borthwick, and S. B. Hendricks. 1966. Leaflet movements of *Mimosa pudica* L. indicative of phytochrome action. Planta 69: 357–364.
6. Furuya, M. and R. G. Thomas. 1964. Flavonoid complexes in *Pisum sativum*. II. Effects of red and far-red light on biosynthesis of kaempferol complexes and on growth of etiolated plumules. Plant Physiol. 39: 634–642.
7. Galston, A. W., A. A. Tuttle, and P. J. Penny. 1964. A kinetic study of growth movements and photomorphogenesis in etiolated pea seedlings. Amer. J. Bot. 51: 853–858.
8. Gressel, J., E. Galun, and L. Strausbauch. 1971. Photomimetic effect of acetylcholine on morphogenesis in *Trichoderma*. Nature 232: 648–649.
9. Hanin, I., R. Massarelli, and E. Costa. 1970. Acetylcholine concentrations in rat brain: diurnal oscillation. Science 170: 341–342.
10. Hartmann, E. 1971. Über den Nachweis eines Neurohormones bein Laubmooscallus und seine Beeinflussung durch das Phytochrom. Planta 101: 159–165.
11. Jaffe, M. J. 1970. Evidence for the regulation of phytochrome-mediated processes in bean roots by the neurohumor, acetylcholine. Plant Physiol. 46: 768–777.
12. Jaffe, M. J. 1972. Acetylcholine as a native metabolic regulator of phytochrome-mediated process in bean roots. *In:* V. C. Runeckles and T. C. Tso, eds., Structural and Functional Aspects of Phytochemistry, Vol. V, Chap. 4. Academic Press, Inc., New York pp. 80–104.
13. Kasemir, H. and H. Mohr. 1972. Involvement of acetylcholine in phytochrome-mediated processes. Plant Physiol. 49: 453–454.
14. Koukkari, W. L. and W. S. Hillman. 1968. Pulvini as the photoreceptors in the phytochrome effect on nyctinasty in *Albizzia julibrissin*. Plant Physiol. 43: 698–704.
15. Russell, D. W. and A. W. Galston. 1968. Comparative analysis of phytochrome-mediated growth responses in internodes of dwarf and tall pea plants. Planta 78: 1–10.
16. Satter, R. L. and A. W. Galston. 1971a. Potassium flux: a common feature *Albizzia* leaflet movement controlled by phytochrome or endogenous rhythm. Science 174: 518–520.
17. Satter, R. L. and A. W. Galston. 1971b. Photochrome-controlled nyctinasty in *Albizzia julibrissin*. III. Interactions between an endogenous rhythm and phytochrome in control of potassium flux and leaflet movement. Plant Physiol. 48: 740–746.
18. Satter, R. L., P. Marinoff, and A. W. Galston. 1970. Phytochrome controlled nyctinasty in *Albizzia julibrissin*. II. Potassium flux as a basis for leaflet movement. Amer. J. Bot. 57: 916–926.
19. Satter, R. L., D. D. Sabnis, and A. W. Galston. 1970. Phytochrome controlled nyctinasty in *Albizzia julibrissin*. I. Anatomy and fine structure of the pulvinule. Amer. J. Bot. 57: 374–381.
20. Sweet, H. G. and W. S. Hillman. 1969. Phytochrome control of nyctinasty in *Samanea* as modified by oxygen, submergence, and chemicals. Physiol. Plant. 22: 776–786.
21. Toriyama, H. 1955. Observational and experimental studies of sensitive plants. VI. The migration of potassium in the primary pulvinus. Cytologia 20: 367–377.
22. Toriyama, H. and M. J. Jaffe. 1972. Migration of calcium and its role in the regulation of seismonasty in the motor cell of *Mimosa pudica*. Plant Physiol. 49: 72–81.